

**Carotenogenesis in Maize Endosperm: Natural Genetic Variation
as a Tool for Predictive Metabolic Engineering**

BY

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ABSTRACT

**Carotenogenesis in Maize Endosperm: Natural Genetic Variation
as a Tool for Predictive Metabolic Engineering Strategies**

BY

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Adviser: Professor Eleanore T. Wurtzel

Vitamin A deficiency is a global health burden. This deficiency can be alleviated through provitamin A carotenoid biofortification of maize and other Poaceae crops. However, the predictive metabolic engineering or breeding is limited by the incomplete understanding of endogenous pathway regulation. If the pathway regulation was better understood, enhancement of carotenoid biosynthesis could be controlled by limiting rate-controlling steps and timing of expression in carotenogenic tissues.

Maize carotenogenesis was investigated using a novel approach to discover genes encoding limiting biosynthetic steps in the nutritionally targeted seed endosperm. A combination of bioinformatics and transcript profiling were first used to identify, map and study expression analysis of gene families encoding enzymes in maize and other grasses. These enzymes include the methylerythritol 4-phosphate MEP pathway for

synthesis of IPP and GGPP, the downstream carotenoid biosynthetic pathway and as well as those involved in degradation of carotenoids.

A novel approach was used to capture the genetic and biochemical diversity of a large germplasm collection, representing 80% of maize genetic diversity, without having to sample the entire collection. This core collection was used for statistical testing of correlation between carotenoid content and candidate gene transcript levels. Multiple pathway bottlenecks for isoprenoid biosynthesis and carotenoid biosynthesis controlling both total carotenoid content and individual carotenoids were discovered in specific temporal windows of endosperm development.

Transcript levels of paralogs encoding isoprenoid IPP and GGPP-producing enzymes, *DXS3*, *DXR*, *HDR*, and *GGPPS1*, were found to positively correlate with endosperm carotenoid content. For carotenoid pathway enzymes, transcript levels for *CrtISO* inversely correlated with seed carotenoid content, as compared to positive correlation of *PSY1* transcripts. Among genes encoding enzymes controlling individual carotenoids, *LCYE* was shown to control the ratio of two branches of the pathway, and *HYD3* was shown to be associated in converting provitamin A compounds to non-provitmain A compounds. Three natural alleles of *HYD3* in 51 maize lines explained 78% of variation and ~11-fold difference in beta-carotene relative to beta-cryptoxanthin and 36% of the variation and 4-fold difference in absolute levels of beta-carotene.

Further downstream, since ZEP depletes the carotenoid pool in subsequent conversion to ABA, *ZEP* transcripts were examined. Carotenoid accumulation was found to be inversely associated with *ZEP1* and *ZEP2* transcript levels. Additionally, degradation of carotenoids is another mechanism to reduce the level of carotenoids in any tissue. Degradation of carotenoids is controlled by a small gene family of eleven carotenoid cleavage genes. Although there is no direct evidence between mRNA levels of these genes and total carotenoid content, preliminary analysis suggested that copy number may be associated with reduced endosperm carotenoids.

Degradation of ABA by the action of ABA hydroxylases was also studied. The enzymatic activity of ABA 8'-hydroxylase is considered as one of the key steps in maintaining ABA levels necessary for physiological processes. *ABA8Ox* genes were identified in maize and respective gene expression was found in all tissues, with a high degree of specificity to each tissue and some degree of overlap. *ZmABA8Ox1a* and *ZmABA8Ox1b* were shown to be the major transcript components for regulating ABA catabolism in drought-stressed roots.

Extension of the maize results using phylogenetic analysis identified orthologs in other Grass species that may serve as potential metabolic engineering targets.

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To those who believe in
“Plant biology at the forefront to build a sustainable future”

TABLE OF CONTENTS

ABSTRACT	IV
ACKNOWLEDGMENTS.....	VII
TABLE OF CONTENTS	X
LIST OF TABLES	XIV
LIST OF FIGURES	XV
LIST OF ABBREVIATIONS.....	XVII
CHAPTER 1: INTRODUCTION	1
1.1 Carotenoids	2
1.2 Benefits of carotenoids to humans.....	4
1.3 Role of carotenoids in plants.....	7
1.4 Biosynthesis of carotenoids	8
1.4.1 Biosynthesis of precursors for carotenoids and other plastid isoprenoids.....	10
1.4.1.1 Isoprenoid precursor pool: MEP pathway to IPP and DMAPP	10
1.4.1.2 GGPP synthesis: Polymers from prenylation	13
1.4.2 Genes and enzymes of the carotenoid biosynthetic pathway.....	14
1.4.2.1 The first committed step: biosynthesis of phytoene	14
1.4.2.2 Desaturation and Isomerization: Biosynthesis of lycopene	15
1.4.2.3 Lycopene cyclization: biosynthesis of carotenes.....	17
1.4.2.4 Carotene hydroxylation: biosynthesis of xanthophylls.....	17
1.4.2.5 Epoxidation and de-epoxidation: Xanthophyll cycle.....	19
1.4.3 Degradation: carotenoid cleavage dioxygenases and apocarotenoids	19
1.5 Regulation of carotenoid metabolism in plants	21
1.5.1 Light and its components	22
1.5.2 Plastid biogenesis and carotenoid associated proteins.....	23
1.5.3 Role of transcription factors.....	25
1.6 Engineering of carotenoid metabolism in plants	25
1.7 Carotenogenesis in Grasses: Maize as a model species.....	28
1.7.1 Comparative genomics of Grasses.....	29
1.7.2 Maize genome evolution and diversity	31
1.7.3 Comparative genetic mapping	32
1.8 Natural genetic variation – diverse germplasm	34
1.9 Rationale and Specific Aims.....	35
1.9.1 Hypothesis.....	35
1.9.2 Specific Aims.....	36

CHAPTER 2: TIMING AND BIOSYNTHETIC POTENTIAL FOR CAROTENOID ACCUMULATION IN GENETICALLY DIVERSE GERMPLASM OF MAIZE	45
2.1 Abstract	45
2.2 Introduction	46
2.3 Results	48
2.3.1 Pathway gene families and chromosome mapping	48
2.3.2 Transcript profiling of gene family members and correlation with carotenoid content	50
2.3.3 Role of zeaxanthin epoxidase in depleting endosperm carotenoids	52
2.4 Discussion	53
2.4.1 Upstream isoprenoid biosynthesis controls downstream endosperm carotenoid content	54
2.4.2 Isomerase expression negatively affects endosperm carotenoid content	56
2.4.3 Temporal windows of gene expression that influence endosperm carotenoid content	56
2.4.4 Using maize to identify putative targets in other Grass species for improvement of seed carotenoids	57
2.5 Conclusion	57
2.6 Materials and Methods	59
2.6.1 Sequence analysis and chromosome mapping	59
2.6.2 Quantitative RT-PCR and carotenoid measurements	59
2.6.3 Plasmids and Functional Complementation	60
2.6.4 Statistical analyses	60
CHAPTER 3: METABOLITE SORTING OF A GERMPLASM COLLECTION REVEALS THE HYDROXYLASE 3 LOCUS AS A NEW TARGET FOR MAIZE PROVITAMIN A BIOFORTIFICATION	81
3.1 Abstract	81
3.2 Introduction	82
3.3 Results and Discussion	84
3.4 Materials and Methods	92
3.4.1 Plant materials	92
3.4.2 Sequence analysis and chromosome mapping	92
3.4.3 Transcript and total carotenoid analysis	94
3.4.4 Plasmids and Functional Complementation	94
3.4.5 Total carotenoid content measurement	94
3.4.6 Measurement of β -carotene in developing endosperm	95
3.4.7 Statistical analyses	96
3.4.8 Multiplex PCR assay for tracking <i>HYD3</i> alleles	96
CHAPTER 4: IDENTIFICATION AND CHARACTERIZATION OF CAROTENOID DIOXYGENASE GENE FAMILY IN THE POACEAE	119

4.1 Abstract	119
4.2 Introduction.....	120
4.3 Materials and Methods.....	122
4.3.1 Sequence analysis and chromosome mapping.....	122
4.3.2 Quantitative RT-PCR and carotenoid measurements	123
4.3.3 Relative quantification of gene-copy number.....	123
4.3.4 Statistical analyses	123
4.4 Results and Discussion	124
4.4.1 Carotenoid dioxygenase gene family in grasses	124
4.4.2 Tissue specific distribution of dioxygenase gene transcripts.....	127
4.4.3 Conclusion	131
CHAPTER 5: ABA 8' -HYDROXYLASES IN MAIZE ROOTS AFFECTED BY DROUGHT	146
5.1 Abstract	146
5.2 Introduction.....	146
5.3 Materials and Methods.....	148
5.3.1 Plant Materials and Stress treatments	148
5.3.2 Sequence analyses.....	148
5.3.3 Quantitative real-time PCR.....	149
5.4 Results and Discussion	149
5.4.1 Identification and characterization of ABA 8'-hydroxylases in maize	149
5.4.2 Spatial analysis of ABA 8' - hydroxylases	151
5.4.3 Modulation and contribution of ABA 8'-hydroxylases during water deficit in roots	152
5.5 Conclusion	152
CHAPTER 6: SUMMARY AND FUTURE WORK.....	162
6.1 Carotenoids	162
6.2 Regulation and Biosynthesis of carotenoids.....	163
6.3 Carotenogenesis in grasses: gene families and phylogenetic studies	164
6.4 Maize as a model species: Natural genetic variation as a tool.....	166
6.4.1 The biosynthetic potential and timing of gene expression.....	166
6.5 Conclusions and Future work	167
APPENDIX 1: PUBLICATIONS RELATED TO THIS DISSERTATION	177
1.1 Natural Genetic Variation in Lycopene Epsilon Cyclase Tapped for Maize Biofortification.....	177
1.2 PSY3, a New Member of the Phytoene Synthase Gene Family Conserved in the Poaceae and Regulator of Abiotic Stress-Induced Root Carotenogenesis.....	181
1.3 The Maize Phytoene Synthase Gene Family: Overlapping Roles for Carotenogenesis in Endosperm, Photomorphogenesis, and Thermal Stress Tolerance.....	194

APPENDIX 2: PLASMIDS GENERATED DURING THIS STUDY	207
APPENDIX 3: GENBANK SUBMISSIONS.....	228
APPENDIX 4: SEQUENCES USED IN THE STUDY	245
APPENDIX 5: INBREDS USED IN THE STUDY	247
APPENDIX 6: PRIMERS USED IN THIS STUDY.....	249
APPENDIX 7: RAW DATA.....	251
BIBLIOGRAPHY	293

LIST OF TABLES

Table 1-1: Enzyme Abbreviations and substrates.....	38
Table 2-1: Correlation analysis between transcripts and carotenoid content of mature seed.....	61
Table 2-2: Gene family sizes in three species of the Poaceae.	62
Table 2-3: Primers used in the study.....	63
Table 2-4: Transcript from levels in developing endosperm from diverse maize lines...	64
Table 2-5: Transcript levels of <i>ZEP</i> genes in developing endosperm from diverse maize lines.	65
Table 3-1: Primer and gene accession numbers.....	98
Table 3-2: Predicted structural characteristics of HYD proteins.	99
Table 3-3: Transcript profiling of <i>HYD3</i> in maize diversity lines.	100
Table 3-4: Genotyping of <i>HYD3</i> alleles and comparison with carotenoid composition of diverse maize germplasm accessions.	101
Table 4-1: Summary of carotenoid cleavage dioxygenase gene family	133
Table 4-2: Other carotenoid cleavage dioxygenase genes identified in this study.	134
Table 4-3: Primers used in the study.....	135
Table 5-1: Summary of ABA 8'-hydroxylases in grasses.....	154
Table 5-2: Primers used in this study.....	155
Table 6-1: Mapping of carotenoid candidate genes, quantitative trait loci, and phenotypic loci in maize.	171
Table 6-2: Chromosomal mapping of candidate genes encoding isoprenoid biosynthetic and degradative enzymes in <i>Zea mays</i> , <i>Oryza sativa</i> , <i>Triticum aestivum</i> and <i>Sorghum bicolor</i>	173

LIST OF FIGURES

Figure 1-1: Conversion of β -carotene to vitamin A derivatives	39
Figure 1-2: MEP pathway in plants	41
Figure 1-3: GGPP synthesis in plants	42
Figure 1-4: Carotenoid biosynthesis in plants	43
Figure 1-5: Xanthophyll Biosynthesis in plants	44
Figure 2-1: Simplified carotenoid biosynthetic pathway derived from isoprenoid precursors	67
Figure 2-2: Gene family structure of DXS genes from the <i>Poaceae</i>	68
Figure 2-3: Phylogenetic analysis based on translation products.	68
Figure 2-4: Maize B73 inbred transcript profiles of genes encoding enzymes for isoprenoid and carotenoid biosynthesis.....	69
Figure 2-5: Maize B73 inbred developing endosperm transcript profiles of genes encoding enzymes for isoprenoid and carotenoid biosynthesis. Mean of three replicates +/- standard deviation.....	72
Figure 2-6: Correlation analysis showing endosperm carotenoid content positively or negatively correlates with mRNA levels of specific pathway enzymes.	73
Figure 2-7: Transcript profiling of zeaxanthin epoxidase gene family members.....	74
Figure 2-8: Multiple-alignment of DXS proteins.	76
Figure 2-9: GGPPS proteins of maize.	77
Figure 2-10: Functional complementation of GGPPS1 and GGPPS2 from maize.	78
Figure 2-11: Multiple-alignment of CrtISO protein sequences.	79
Figure 2-12: ZEP gene family structure.	80
Figure 3-1: Conversion of provitamin A carotenes to nonprovitamin xanthophylls.....	102
Figure 3-2: HYD gene family structure and phylogeny.	103
Figure 3-3: Synteny between maize and rice to map maize <i>HYD</i> orthologs	105
Figure 3-4: Transcript profiling of hydroxylase gene family members in maize standard, B73 inbred.	106
Figure 3-5: Correlation analysis showing <i>HYD3</i> mRNA levels correlate with endosperm provitamin A content.	107
Figure 3-6: Alignment of maize sister paralogs <i>HYD3</i> and <i>HYD4</i> plus their rice synteny partner <i>HYD1</i>	109
Figure 3-7: Multiplex PCR diagnosis of <i>HYD3</i> alleles in high and low β -carotene lines.	110
Figure 3-8: One-way ANOVA between <i>HYD3</i> alleles (A, B, C) and the ratio of β -carotene/ β -cryptoxanthin in 51 genetically diverse maize lines.	112
Figure 3-9: Relative amount of betacarotene increases and <i>HYD3</i> transcript levels decrease during development of maize endosperm in lines carrying a C allele of <i>HYD3</i>	113

Figure 3-10: Conserved motifs in HYD proteins of maize and rice.	114
Figure 3-11: Functional complementation of <i>HYD</i> genes.	115
Figure 3-12: Alignment of maize <i>HYD3</i> in 51 lines.	117
Figure 4-1: Carotenoid cleavage dioxygenase activities in various species.	136
Figure 4-2: Phylogenetic analysis of carotenoid cleavage dioxygenases in the Grasses.	137
Figure 4-3: Phylogenetic analysis of carotenoid cleavage dioxygenases from diverse taxa.	138
Figure 4-4: Maize B73 inbred transcript profiles of genes encoding (A) carotenoid cleavage dioxygenases (CCD) (B) 9-cis-epoxy carotenoid dioxygenases (NCED).	140
Figure 4-5: Transcript profiles of genes showing detectable levels of expression in a core subset of maize inbreds	140
Figure 4-6: ZmCCD1 expression and copy number among maize inbreds.	140
Figure 4-7: Aligned <i>Zea mays</i> cleavage dioxygenase sequences	140
Figure 4-8: Protein sequence alignment of CCD8a carotenoid cleavage dioxygenase sub-family.	140
Figure 4-9: Protein sequence alignment of CCD8b carotenoid cleavage dioxygenase sub-family.	140
Figure 5-1: ABA synthesis and oxidation pathways in higher plants.	156
Figure 5-2: ABA8ox gene family structure.	157
Figure 5-3: Phylogenetic analysis of proteins encoded by members of the <i>CYP707A</i> gene subfamily for representative monocots and dicots.	158
Figure 5-4 : Transcript profiles of maize inbred line B73 genes encoding ABA 8'-hydroxylases.	159
Figure 5-5 : Drought stress-modulated levels of transcripts encoded by genes controlling inactivation of ABA in roots of <i>Zea mays</i>	160
Figure 5-6: Multiple-alignment of proteins encoded by genes encoding ABA 8'-hydroxylases of maize and orthologs from <i>Oryza sativa</i> (<i>Os</i>), <i>Sorghum bicolor</i> (<i>Sb</i>) and <i>Barley</i> (<i>Hv</i>). See table 6-1 for accession numbers	161
Figure 6-1: Simplified carotenoid biosynthetic pathway derived from isoprenoid precursors.	176

LIST OF ABBREVIATIONS

aa	amino acid
ABA	abscisic acid
AAO3	abscisic acid oxidase
AFLP	amplified fragment length polymorphism
bp	base pair
BAC	bacterial artificial chromosome
CCD	carotenoid cleavage dioxygenase
cDNA	complementary DNA
CDP-ME	4-diphosphocytidyl-2-C-methyl-D-erythritol
CHR	chromatin remodeling
CMK	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
<i>crtI</i>	carotenoid gene I
CrtO	β -carotene ketolase
CrtISO	carotenoid isomerase
<i>cry-2</i>	cryptochrome 2
CVD	cardiovascular diseases
CYP	cytochrome p450
DAP	days after pollination
DDB	damaged DNA-binding protein
DET1	DEETIOLATED1
DMAPP	dimethylallyl diphosphate
DXP	1-deoxy-D-xylulose 5-phosphate
DXS	deoxyxylulose 5-phosphate synthase
DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase
<i>E. coli</i>	<i>Escherichia coli</i>
eQTL	expression QTL

EST	expressed sequence tag
FPP	farnesyl diphosphate
FPPS	farnesyl diphosphate synthase
GPP	geranyl diphosphate
GPPS	geranyl diphosphate synthase
GGPP	geranylgeranyl pyrophosphate
GGPPS	geranylgeranyl pyrophosphate synthase
GR	Golden Rice
GSS	genome survey sequence
HDS	1-hydroxy-2-methyl-2-(E)-butenyl 4- diphosphate synthase
HDR	hydroxymethylbutenyl 4-diphosphate reductase
HMBPP	hydroxymethylbutenyl 4-diphosphate
HPLC	High Performance Liquid Chromatography
<i>hp</i>	high pigment
HSP	heat shock protein
HYDB	β -hydroxylase
HYDE	ϵ -hydroxylase
IPPI	isopentenyl diphosphate isomerase
IPP	isopentenyl pyrophosphate
IPTG	isopropylthio- β -D-galactoside
kb	kilobase pair
kDa	kilodalton
LB	Luria-Bertani
LCYB	lycopene beta cyclase
LCYE	lycopene epsilon cyclase
LD	linkage disequilibrium
LTR	long terminal repeat
<i>max</i>	<i>more axillary growth</i> mutants

MAS	marker-assisted selection
<i>MCT</i>	2-C-methyl-D-erythritol 4-phosphate cytidyltransferase
<i>MDS</i>	2-C-methyl-D-erythritol 2, 4-cyclodiphosphate synthase
MEP	methylerythritol 4-phosphate
ME-cPP	2-C-methyl-D-erythritol 2,4-cyclodiphosphate
MVA	mevalonic acid
NCED	9- <i>cis</i> -epoxycarotenoid dioxygenase
NXS	neoxanthin synthase
ORF	open reading frame
Or	orange
PCR	polymerase chain reaction
PDS	phytoene desaturase
PLB	prolamellar body
PSY	phytoene synthase
QTL	quantitative trait locus
<i>rms1</i>	<i>ramosus1</i>
RAPD	random amplification of polymorphic DNA
RFLP	restriction fragment length polymorphism
RIL	recombinant inbred line
RT-PCR	reverse transcriptase polymerase chain reaction
SDR	short chain alcohol dehydrogenase
SSR	simple sequence repeats
VDE	violaxanthin de-epoxidase
<i>vp</i>	viviparous
<i>y</i>	yellow
ZDS	zeta-carotene desaturase
ZEP	zeaxanthin epoxidase
Z-ISO	zeta-carotene isomerase

Chapter 1

Introduction

Unlike animals, plants accumulate thousands of small chemical molecules with specialized functions as a result of their sessile life style and constant interaction with pathogenic and beneficial organisms (Grotewold, 2005). These molecules are required by animals and are necessary for good health. Thus, plants are an important source of these chemicals, which are crucial in increasing the longevity of animal life. The link between diet and health is well established and there is overwhelming evidence that diet is a key environmental factor affecting the incidence of many chronic diseases (Ferguson and Philpott, 2008). However, human consumption of these phytochemicals is not adequate to combat existing diseases, prevention and reoccurrence.

The nature of the environment (biotic and abiotic interactions) in which phytochemicals participate necessitates a profound variation in their distribution, among plant sources (Wink, 2003). This natural variation represents a disparity among staple food crops which poses a particular problem in places with monotonous consumption of cereal staples, roots and pulses, leading to deficiencies of essential phytochemicals. On the other hand in developed countries nutritional diseases are most often associated with excessive consumption rather than monotonous consumption.

Today, over three billion people and with nearly two-thirds of infant deaths are associated with micronutrient nutritional deficiencies (Caballero, 2002). Major food staples contain insufficient concentrations of many phytochemicals to meet the recommended dietary allowance. In order to obtain such therapeutic levels of these

nutrients in the diet, additional fortification of the food supply as well as modification of dietary preferences, and direct modification of micronutrient levels in food crops is required (Welch and Graham, 2004). On the other hand, the interest in plant breeding for agricultural crops is growing as a result of increasing demands for food production coupled with increasing population growth and increasing environmental degradation (Wilson et al., 2004).

The fight against world hunger should take place through the appropriate use of the manipulation of plant nutrients and mobilization of the potential that is available in the plant kingdom. Crop biotechnology is being used to improve global food security by making more food available, and by enhancing the nutritional composition of foods. Developing micronutrient-enriched staple plant foods, either through traditional breeding methods or via molecular biology techniques can be a powerful intervention tool that targets the most vulnerable people (Welch and Graham, 2004). Clearly there is the potential for immense socioeconomic benefit through successful characterization and exploitation of health promoting factors in plant foods. Within this idea is a radical change towards identifying and genetically manipulating complex biosynthetic pathways in plants. For example vitamin A deficiency is a widespread problem in the developing world, causing blindness and increased childhood mortality, which has driven research into finding ways of introducing provitamin A carotenoids into staple crops.

1.1 Carotenoids

Carotenoids are a large class and the most widespread group of isoprenoid pigments in nature, synthesized *de novo* by all photosynthetic plants, protists, as well as some

heterotrophic bacteria and some fungi (Botella-Pavia and Rodriguez-Concepcion, 2006). In plants, carotenoids aid in attracting insects and other animals that act as pollinators and vehicles of seed dispersion by furnishing distinctive yellow, orange and red colors to flowers and fruits (Bartley and Scolnik, 1995; Howitt, 2006). They also play an indispensable role in light harvesting, photo-protection (Niyogi, 1999), as precursors for ABA (Schwartz et al., 2003) and in environmental adaptations (Demmig-Adams and Adams, 2002).

There are more than 750 carotenoids identified in nature so far (Britton et al., 2004). These are extremely lipophilic compounds that are almost insoluble in water. The most obvious feature of the carotenoid molecule is the long polyene chain extending between 3-15 conjugated double bonds, which influence their physical and biochemical properties (Britton, 1995). Depending on the arrangement of substituents, carbon-carbon double bonds can exist in two configurations, assigned as E/Z or *cis/trans* isomers (Britton et al., 1995; Stahl W and H, 2005). The basic carotenoid structure is a symmetrical tetraterpene (C₄₀) skeleton formed by the conjugation of two C₂₀ units. Carotenoids are sub-divided into two groups. The non-oxygenated derivatives are referred to as carotenes with one or more β -cyclic or ϵ -rings such as β -carotene and α -carotene. The other oxygenated derivatives of carotenes are designated as xanthophylls, which can contain keto, hydroxyl, or epoxy groups (Olson and Krinsky, 1995; Stahl and Sies, 2005). There are also carotenoids with fewer than 40 carbon atoms, derived by cleavage from one or both ends of the molecule and are designated as apo-carotenoids.

The properties of carotenoids have been industrially exploited for many years. They are being used as natural colorants in both animal and human food stuffs and also as nutritional supplements. Commonly used carotenoids such as astaxanthin and canthaxanthin are added to the feed of farm raised fish to produce the characteristic pink color of salmon; lutein and zeaxanthin are used to enhance the yellow pigmentation of eggs and poultry. Among apocarotenoids, crocin mimics the color of the intense red color of saffron whereas bixin and β -ionone have been widely used as a color additive in cosmetics, foods and flavors (Giuliano et al., 2003). Besides all of the above, the health-related antioxidant properties of carotenoids and roles as nutraceuticals have immense value and unprecedented use in industry.

1.2 Benefits of carotenoids to humans

Humans do not synthesize carotenoids and they rely upon plants as a dietary source of these compounds. Dietary carotenoids are mostly derived from crop plants and to a lesser extent from poultry and fish. The carotenoids in plants are usually located in roots, leaves, shoots, seeds, fruits and flowers. There are about 60 different carotenoids found in common fruits and vegetables (Fraser and Bramley, 2004), usually in deeply pigmented fruits, juices and vegetables. For example, yellow-orange vegetables and fruits provide most of the β -carotene & α -carotene, orange fruits provide α -cryptoxanthin, dark green vegetables provide lutein (Mangels et al., 1993). Tomatoes, watermelons, pink grapefruits, apricots and pink guavas provide lycopene, however 85% of dietary lycopene is obtained from tomatoes and tomato based products alone (Nguyen and Schwartz, 1999).

Diets rich in carotenoids can prevent the onset of some chronic disease states such as cardiovascular diseases (CVD) and cancer (Mares-Perlman et al., 2002). For example, β -carotene and lycopene have been shown to be inversely related to the risk of CVD (Kohlmeier and Hastings, 1995). Two of the three studies in which blood lycopene levels were measured reported an association between higher lycopene and reduction in prostate cancer risk (as referred in Giovannucci et al., 2002). Lutein and zeaxanthin are the dominating pigments present in macula lutea (Landrum et al., 1999; Khachik et al., 2002) and their presence is strongly associated with decreasing the effects of age-related macular degeneration (Schalch, 1992; Bone et al., 2001).

Consumption of a wide variety of vegetable, has a greater bearing on lung cancer than specific intake of individual carotenoid supplements (Wright et al., 2003) suggesting the synergistic effect with combination of other carotenoids and other antioxidants from fruits and vegetables to be more effective (Fraser and Bramley, 2004). Much of the research to date has been focused on the potential role of the carotenoids as dietary antioxidants, since oxidative stress is an important contributor for the occurrence of chronic diseases (Evans and Halliwell, 2001). The antioxidant properties of carotenoids have been suggested as the main mechanism by which they afford their beneficial effects.

Other mechanisms of action that are independent of their antioxidant properties are also important. In animals, some carotenoids are precursors of vitamin A, especially carotenes showing highest provitamin A activity, which is the best-established function of carotenoids. Vitamin A is a C_{20} carotenoid, where β -carotene is enzymatically cleaved

at the central 15, 15' carbon double bond in the presence of β , β -carotene 15, 15' oxygenase yielding two molecules of vitamin A aldehyde (**Fig. 1-1**). These cleavage products are essential for animal survival as both a chromophore in vision (retinals and retinols) and as a hormone (retinoic acids) (von Lintig and Vogt, 2004). Vitamin A deficiency leads to xerophthalmia and early mortality especially in children under age five, particularly in countries where rice is a main staple, essentially milled rice contains no carotenoids (West Jr et al., 1989; Grant, 1991; West et al., 2002) and also in Africa where maize is a main crop staple and has low levels of β -carotene.

The recommended dietary allowance of vitamin A is 250 μ g retinol activity equivalents (RAE)/day and 500 μ g RAE/day for children and all other age groups respectively (Nestel et al., 2006; Ortiz-Monasterio et al., 2007). The typical values of provitamin A content in traditional yellow maize variteies range between 0.2 μ g -2.5 μ g/g dry weight of kernel (Egesel et al., 2003). Assuming that 50% of carotenoids are lost during cooking and processing, and 12:1 of β -carotene to retinol bioconversion rate, approximately 200 and 400g of daily maize consumption is needed to meet the recommended dietary allowance (Ortiz-Monasterio et al., 2007). The low β -carotene amount in maize prompted a series of efforts to engineer, and reach upto a target of upto 15 μ g/g provitamin A in a dry weight kernel of maize (Ortiz-Monasterio et al., 2007). So, it is also obvious the health promoting properties of carotenoids have intensified interest in carotenoid biosynthesis in plants and its commercial exploitation (Fraser and Bramley, 2004; Fraser et al., 2008; Harjes et al., 2008; Millis et al., 2008).

1.3 Role of carotenoids in plants

In photosynthetic tissues, the color of the carotenoid is masked by chlorophyll. But during its absence, the intense color of carotenoids provides distinct pigmentation. The most abundant carotenoids in green tissues are xanthophylls, which are lutein, zeaxanthin, violaxanthin and neoxanthin. All photosynthetic tissues maintain similar carotenoid composition, with lutein 45%, β -carotene 25-30%, violaxanthin 10-15% and neoxanthin 10-15% as most abundant carotenoids (Britton, 1993). These carotenoids play an indispensable role as essential components of photosynthetic membranes acting as membrane stabilizers, consequentially they become integral to plant viability contributing to numerous processes. These act as accessory pigments absorbing light energy in the range of 450-570 nm transferring the energy to chlorophyll, channeling the excess energy away from chlorophyll to protect chlorophyll against photooxidative damage and preventing the generation of photooxidative species such as singlet oxygen molecules causing irreparable damage (Demmig-Adams et al., 1996). In addition, these also play a crucial role in the safe dissipation of excess excitation energy of the antenna chlorophyll in a process known as non-photochemical quenching (Muller et al., 2001). In higher plants, the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin is required for the pH-dependent dissipation of excess light energy as heat thereby protecting against photooxidative damage (Baroli and Niyogi, 2000).

Carotenoids accumulate in nearly all types of plastids and are thus found in most plant organs and tissues such as fruits, flowers, roots and seeds. In general, lutein is the major carotenoid present in chloroplasts of leaves followed by seeds either in amyloplasts

(starch storing plastids) or elaioplasts (lipid-storing plastids). In amyloplasts of maize, lutein and zeaxanthin are primary carotenoids followed by a trace amounts of β -carotene (Kurilich and Juvik, 1999). Mature fruit, vegetable and flower tissues are characterized by chromoplasts, where carotenoids are associated with polar lipids and carotenoid associated proteins. These carotenoid associated lipids and proteins assist in effective sequestration and retain a large quantity of carotenoids (Vishnevetsky et al., 1999; Vishnevetsky et al., 1999a).

The oxidative cleavage of carotenoids leads to the production of apocarotenoids, which mediate the interaction of plants with their biological environment such as mycorrhizal associations. Thus these apocarotenoids serve as precursors for biosynthesis of plant-growth regulator abscisic acid (ABA) and other carotenoid cleavage products (Giuliano et al., 2003; Botella-Pavia and Rodriguez-Concepcion, 2006).

1.4 Biosynthesis of carotenoids

The biochemical steps with their enzymes and substrates of the MEP pathway, GGPP synthesis and carotenoid biosynthesis are defined in Table 1-1 and Figs. 1-2 to 1-5. Biosynthesis of carotenoids is initiated by the condensation of a isopentenyl diphosphate, a 5-carbon building block (C_5) and its allylic isomer dimethylallyl diphosphate (DMAPP) into a longer carbon chain diphosphate ester leading to the C_{40} backbone of carotenoids. In plant cells, two distinct pathways have evolved for the synthesis of the universal precursors of all isoprenoid products, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The well known mevalonic acid (MVA) pathway produces cytosolic IPP, whereas the fairly recently discovered 2-C-methyl-d-erythritol 4-phosphate

(MEP) pathway synthesizes plastidial IPP and DMAPP (Rodriguez-Concepcion and Boronat, 2002; Eisenreich et al., 2004). The MEP pathway has become a promising target for the development of anti-malarial and anti-microbial drugs because this MEP pathway exists only in protozoa, malarial parasites, most eubacteria and plants but not in animals (Rodriguez-Concepcion, 2004; Rohdich et al., 2005).

Since the MEP pathway was discovered, it has been shown to provide precursors to many isoprenoids, such as gibberelins, quinones and carotenoids (Phillips et al., 2008). Isotope labeling and functional genomics have demonstrated that the prenyl diphosphates utilized in the formation of carotenoids are derived primarily from the plastid localized MEP pathway and not from the cytoplasmic mevalonate pathway (Rodriguez-Concepcion and Boronat, 2002). However, some studies indicate the exchange of prenyl diphosphates between cytoplasm and plastid (Kasahara H., 2002). Moreover, it was also suggested that environmental light and signal transduction pathways are some of the components involved in coordinated regulation of isoprenoid biosynthesis and exchange of common precursors at least during early stages of seedling development (Rodriguez-Concepcion et al., 2004).

The MEP pathway uses glyceraldehyde 3-phosphate and pyruvate as initial substrates to generate IPP and DMAPP (**Fig. 1-2**). These isoprene units are condensed in the presence of geranyl geranyl diphosphate synthase (GGPPS) to form GGPP, the precursor for the biosynthesis of carotenoids and several other groups of plastidic isoprenoids, such as phytol, quinones and diterpenes (**Fig. 1-3**). Two GGPPS are

condensed to produce the extended C₄₀ polyene carbon chain that serves as the backbone of all carotenoids. The C₄₀ polyene backbone thus formed is subject to desaturations and isomerizations to yield the colored polyene (lycopene), which is cyclized to yield carotenes (**Fig. 1-4**). Carotenes thus formed, are then hydroxylated to yield xanthophylls (lutein and zeaxanthin). Zeaxanthin alone participates in the xanthophyll cycle, where epoxidases and depoxidases involve in the interconversion of zeaxanthin, violaxanthin, antheraxanthin (**Fig. 1-5**). In similar fashion, lutein cycle operates in some higher plant species between lutein and its monoepoxide (Matsubara et al., 2003). Xanthophylls are also oxygenated to form ketocarotenoids such as capsanthin, capsorubin and astaxanthin. These carotenoids are further cleaved into smaller molecules known as apocarotenoids, essential during plant growth and development. In the past few years, several important new genes required for isoprenoid biosynthesis have been characterized and cloned in a variety of plant species and great progress has been made in the genetic manipulation of this biosynthetic pathway in plants (Fraser and Bramley, 2004; Rodriguez-Concepcion, 2006; Sandmann et al., 2006; Matthews and Wurtzel, 2007; Fraser et al., 2008; Giuliano et al., 2008).

1.4.1 Biosynthesis of precursors for carotenoids and other plastid isoprenoids

1.4.1.1 Isoprenoid precursor pool: MEP pathway to IPP and DMAPP

During the initial steps of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate (DXP) is formed by condensation of pyruvate and glyceraldehyde 3-phosphate, a reaction catalyzed by DXS (Lois et al., 1998). DXP is also the precursor for other pathways leading to pyridoxal and thiamine. 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) simultaneously catalyzes the intramolecular rearrangement and reduction of DXP

to form 2-C-methyl-D-erythritol 4-phosphate (MEP). MEP is the first committed intermediate of the pathway and the pathway is named “MEP” accordingly (**Fig. 1-2**).

In *Arabidopsis*, DXS is encoded by three homologues, however only one has been shown to be functional to date (Rodriguez-Concepcion and Boronat, 2002; Fraser and Bramley, 2004; Phillips et al., 2008). In most other plants, DXS has been shown to be encoded by two (I and II) classes (Walter et al., 2002; Kim et al., 2005; Phillips et al., 2007). Recently, a third class of DXS (DXS3) was identified in grasses, and transcripts were shown to positively correlate with total seed carotenoid content of maize (Vallabhaneni and Wurtzel, 2009; see Chapter 2). DXS expression patterns during accumulation of different plastidial isoprenoid end products suggested that increased DXS levels might be required to supply their precursors and was tentatively proposed as a potential control point (Estevez et al., 2000; Lois et al., 2000; Walter et al., 2000; Walter et al., 2002; Botella-Pavia et al., 2004; Burlat et al., 2004). Overexpression of DXS in combination with a carotenoid cluster significantly enhanced carotenoids in a bacterial platform (Matthews and Wurtzel, 2000; Kim and Keasling, 2001). Transgenic *Arabidopsis* and tomato plants in which DXS activity had been altered confirmed the regulatory role of DXS in controlling flux through the MEP pathway (Estevez et al., 2001; Enfissi et al., 2005; Morris et al., 2006; Munoz-Bertomeu et al., 2006). DXR is encoded by a single copy in all species to date. A positive correlation between enhanced plastid isoprenoid biosynthesis and transcript accumulation has been observed in some plants (Mahmoud and Croteau, 2001; Carretero-Paulet et al., 2002; Hans et al., 2004; Mayrhofer et al., 2005; Bede et al., 2006; Carretero-Paulet et al., 2006; Vallabhaneni and

Wurtzel, 2009) but not in others (Rodriguez-Concepcion et al., 2001; Dudareva et al., 2005).

In the following reactions of the MEP pathway, the enzymes encoded by the genes MCT, CMK, MDS mediate the sequential formation of 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME), CDP-ME 2-phosphate and 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate (ME-cPP), respectively (Eisenreich et al., 2001; Rodriguez-Concepcion and Boronat, 2002; Eisenreich et al., 2004; Hunter, 2007; Phillips et al., 2008). All three genes were present in organisms having *DXS* and *DXR*, indicating their role in the terpenoid pathway (Eisenreich et al., 2001). Recent evidence has shown that MCT is essential for IPP biosynthesis in *E. coli* (Kuzuyama et al., 2000) and *A. thaliana* (Rohdich et al., 2000), however the contribution of these enzymes to the regulation of the supply of intermediates for plastid isoprenoid biosynthesis is less clear.

Subsequent studies revealed that the HDS gene product catalyzes the formation of hydroxymethylbutenyl 4-diphosphate (HMBPP) from 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate (ME-cPP) (Hecht S et al., 2001; Wolff et al., 2002). The ubiquitous distribution of HDS expressed sequence tags (EST) in the tomato EST collections suggest that the corresponding gene is likely to be constitutively expressed through out the plant (Rodriguez-Concepcion et al., 2003). However, it is recently found that *HDS* has no effect towards control of isoprenoid accumulation (Flores-Perez et al., 2008; Vallabhaneni and Wurtzel, 2009). In the next step, the *HDR* gene encodes an enzyme that participates in conversion of hydroxymethylbutenyl 4- diphosphate into a 5:1 mixture of

IPP and DMAPP (Rohdich F et al., 2002). The expression of HDR is strongly upregulated with correlation to carotenoid production during both tomato fruit ripening and *Arabidopsis thaliana* seedling de-etiolation (Botella-Pavia et al., 2004); its role as a rate-controlling step confirms earlier studies in bacteria (Cunningham et al., 2000). Additionally, genes encoding enzymes of the MEP pathway have been shown to be under post transcriptional regulation (Laule et al., 2003; Guevara-Garcia et al., 2005; Sauret-Gueto et al., 2006) and strongly associated with the expression of plastome-encoded proteins (Flores-Perez et al., 2008).

IPP isomerase catalyzes the isomerization of IPP to DMAPP (Campos et al., 2001). Both IPP and DMAPP serve as starter molecules for subsequent condensation reactions with IPP to form GGPP in the presence of GGPPS. IPP isomerase have been found localized in both cytosol and plastids (Nakamura et al., 2001). However in *Arabidopsis*, IPPI is encoded by two copies and the encoded enzymes are found in multiple overlapping sub-cellular locations while each can compensate for the loss of the other (Okada et al., 2008; Philips et al., 2008). In another study, IPPI was also shown to be localized in peroxisomes (Sapir-Mir et al., 2008).

1.4.1.2 GGPP synthesis: Polymers from prenylation

GGPP is the head-to-tail condensation product of dimethylallyl diphosphate (DMAPP) with sequential addition of three isopentenyl diphosphate (IPP) molecules (**Fig. 1-3**). DMAPP could be produced either by HDR or IPPI, once produced DMAPP is condensed head to tail with IPP by geranyl diphosphate synthase (GPPS) to produce GPP (C₁₀).

Further condensations of IPP onto the growing polyene by GGPPS produce in sequence farnesyl diphosphate (FPP, C₁₅) and geranylgeranyl diphosphate (GGPP, C₂₀). It should be pointed out, that prenyl diphosphates and especially GGPP are not only the precursor for carotenoids but also participate in the synthesis of many other terpenoid compounds. Thus, the pool of GGPP is utilized for biosynthesis of carotenoids and other terpenoids and is responsible for inter-pathway competition for GGPP. Prenyl synthases are substrate-selective (Lee et al., 2005), however substrate-promiscuity with FPS, FPPS, and GGPPS activities may occur, which has been shown recently in maize, for which a GGPPS gene isolated by functional complementation is a bonafide FPPS (Cervantes-Cervantes et al., 2006). In many plant species, GGPPS is encoded by a multi-gene family and located in various cellular tissues. In Arabidopsis, GGPPS is encoded by 12 isoforms (Lange and Ghassemian, 2003), however functional data were obtained for only 5 of these family members (Okada et al., 2000). The Arabidopsis GGPPS proteins were shown to target to specific sub-cellular compartments. It is not known how many GGPPS genes are specifically linked to carotenoid biosynthesis.

1.4.2 Genes and enzymes of the carotenoid biosynthetic pathway

1.4.2.1 The first committed step: biosynthesis of phytoene

The first committed step in the carotenoid biosynthetic pathway is the head-to-head condensation of two GGPP molecules to produce 15-*cis* phytoene, a colorless carotenoid (**Fig. 1-4**). This reaction is mediated by a membrane-associated enzyme, phytoene synthase (PSY) (Cunningham and Gantt, 1998), which shares homology with both GGPPS and squalene synthases (sterol biosynthesis). PSY is encoded by a single copy gene in some species, however gene duplications have occurred both in eudicots as well

as monocots (Bramley, 2002; Palaisa et al., 2003; Gallagher et al., 2004; Giorio et al., 2008). For example, PSY in tomato is encoded by two genes; *PSY1*, which encodes a fruit- and flower specific isoform, and *PSY2*, which encodes an isoform that predominates in green tissues (Bartley and Scolnik, 1993; Fraser et al., 1999; Giorio et al., 2008). Genes encoding PSY have been isolated from many other plants and has been shown to be a rate-controlling for flux into carotene synthesis (Lu and Li, 2008). In grasses, three PSY genes were identified and functionally characterized (Gallagher et al., 2004; Li et al., 2008a, see appendix 1.2; Li et al., 2008b, see appendix 1.3). The gene duplications of PSY led to specific temporal and spatial specific expression in leaves and roots associated with biosynthesis of carotenoids conditioning for environmental adaptation. PSY1 was shown to be a rate-limiting enzyme of carotenoid biosynthesis in maize endosperm and PSY2 to have a role in plant development (Gallagher et al., 2004; Li et al., 2008b). Transcripts for PSY3 were found to be highly expressed in root, and the gene was shown to be involved in drought induced stress regulation in both rice and maize (Welsch et al., 2008; Li et al., 2008a).

1.4.2.2 Desaturation and Isomerization: Biosynthesis of lycopene

Production of lycopene requires four desaturations and several isomerizations of double bonds in the polyene chain (**Fig. 1-4**). Two structurally and functionally similar enzymes, PDS and ZDS, convert phytoene introducing conjugated *cis*-double bonds to generate a red chromophore (Isaacson et al., 2004). These enzymes catalyze two symmetric dihydrogenation eliminations occurring at the 11 and 11' positions, where PDS introduces *trans*-configured double bonds. Simultaneously, ZDS introduces *cis*-configured double bonds at 7 and 7' positions (Isaacson et al., 2004).. These serial desaturations occur in

step wise symmetry in bacteria and in some fungi, where it is carried out by a single enzyme, the CRTI-type enzyme (Hausmann and Sandmann, 2000; Isaacson et al., 2004). During the conversion of phytoene to lycopene, the CRTI-type enzyme not only introduces four double bonds, it produces different geometric isomers compared to the PDS/ZDS pair. Paired activities of PDS and ZDS in a heterologous complementation system of mixed *Synechocystis*-PDS/tomato ZDS (Bartley et al., 1999; Isaacson et al., 2004), as well as *in vitro* studies with daffodil extracts (Beyer et al., 1989), *in vivo* studies from *Arabidopsis* (Bartley et al., 1999) and maize (Matthews et al., 2003) detected the formation of prolycopene in the absence of light. However, lycopene cyclases are stereo selective for all-*trans* isomers in most systems. This observation led to identification of carotene isomerase (CrtISO) in plants (Masamoto et al., 2001; Isaacson et al., 2002).

CrtISO functions in parallel with ζ -carotene desaturation (ZDS) in plants by converting 7,9,9'-tri-*cis*-neurosporene to 9'-*cis*-neurosporene and 7,9,7'9'-tetra-*cis*-lycopene into all-*trans*-lycopene and is not involved in the isomerization of the 15-15'-*cis* double bond to the *trans* conformation (Isaacson et al., 2004). A recent characterization of the maize *y9* locus has brought to light a new isomerase required in plant carotenoid biosynthesis (Li et al., 2007). Maize *Y9* encodes a factor required for isomerase activity upstream of CrtISO, which has been termed Z-ISO, an activity that catalyzes the *cis* to *trans* conversion of the 15-*cis* bond in 9,15,9-tri-*cis*-zeta-carotene, the product of PDS, to 9,9'-di-*cis*-zeta-carotene, the substrate for ZDS. Therefore, biosynthesis of lycopene involves a two-step desaturation by PDS, followed by 15-*cis* isomerization by ζ -carotene

isomerase (Z-ISO), and followed by CrtISO mediated isomerization of each pair of double bonds introduced by ZDS. During this process, light may photoisomerize *cis*-configured carotenoids to the *trans* configuration, especially the central 15-15' *cis* double bond of phytoene, phytofluene, or ζ -carotene; thus in the absence of isomerase, poly-*cis* lycopene accumulates (Bartley et al., 1999). This was clearly seen in *Arabidopsis* null mutants with the accumulation of poly-*cis* lycopene (Park et al., 2002) and maize y9 mutants with 9,15,9'-tri-*cis*- ζ -carotene (Li et al., 2007). However, light appears to only partially compensate for the absence of Z-ISO enzyme activity.

1.4.2.3 Lycopene cyclization: biosynthesis of carotenes

The differential cyclization of the termini of the linear lycopene molecule and pathway bifurcation (**Fig. 1-4**) occurs in the presence of lycopene- β -cyclase (LCYB) and lycopene- ϵ -cyclase (LCYE). One branch leads to β -carotene with introduction of two β rings in the presence of LCYB, whereas the other leads to α -carotene in the presence of LCYB and LCYE (Cunningham and Gantt, 2001). It was also proposed that stoichiometry of these enzymes could lead to producing different composition of pathway endproducts (Cunningham Jr. et al., 1996; Pecker et al., 1996).

1.4.2.4 Carotene hydroxylation: biosynthesis of xanthophylls

Lutein and zeaxanthin are formed from α - and β -carotene, respectively by replacing the hydrogen with hydroxyl group at the 3 or 3' position of the carotene ring (**Fig. 1-5**).

Hydroxylation of one β -ring produces β -cryptoxanthin, followed by another hydroxylation leading to zeaxanthin. Hydroxylation of the β -ring and ϵ -ring or both rings of α -carotene produces zeionoxanthin, α -cryptoxanthin, and lutein respectively. Although

all β -hydroxylases carry out similar reactions, they belong to two structural classes. One class composes the non-heme di-iron monooxygenases, homologous to HYDB present in bacteria (Sun et al., 1996). The P450 type CYP97C ϵ -hydroxylase and CYP97A β -carotene hydroxylase (Tian et al., 2003; Tian et al., 2004; Quinlan et al., 2007) belong to another class.

The di-iron β -hydroxylases proteins contain conserved histidine regions originally identified in membrane fatty acid desaturases (Shanklin et al., 1994) and oxygen-dependent di-iron containing oxygenases (Fraser et al., 1997) and which are signature-motifs for non-heme di-iron monooxygenases. In *Arabidopsis* the di-iron enzymes are encoded by two paralogs (Tian and DellaPenna, 2004). However, in many other species, including both monocots and eudicots there are multiple genes encoding HYDB whose products may be expressed in different tissues or in response to different environmental cues (Wurtzel, 2004; Castillo et al., 2005; Vallabhaneni et al., 2009 In Press, see Chapter 3).

The p450 type CYP97C ϵ -hydroxylase and CYP97A β -carotene hydroxylase (Tian et al., 2003; Tian et al., 2004) are encoded by a single genes in *Arabidopsis* (Tian and DellaPenna, 2004) as well as in many other species. The function of those CYP97 enzymes and their role in β -carotene hydroxylation was demonstrated using several *Arabidopsis* mutants (Fiore et al., 2006; Kim and DellaPenna, 2006). It was further demonstrated using a heterologous complementation system that CYP97C was specific for ϵ -rings and CYP97A towards β -ring substrate with slight activity towards ϵ -ring

(Quinlan et al., 2007). However, further evidence is required to demonstrate whether or not two structurally distinct β -ring hydroxylases are specific to each branch or can compensate for each other in the absence of other.

1.4.2.5 Epoxidation and de-epoxidation: Xanthophyll cycle

Biosynthesis of antheraxanthin and violaxanthin occurs by further oxygenation of the 3-hydroxyl β -rings of zeaxanthin by the introduction of 5, 6-epoxy moieties by zeaxanthin epoxidase (**Fig. 1-5**). This reaction sequence is reversible and occurs during high light stress, where violaxanthin gets de-epoxidated in the presence of violaxanthin de-epoxidase (VDE) to the more photoprotective zeaxanthin; collectively known as the xanthophyll cycle (**Fig. 1-5**); Neoxanthin, an enzymatically (NXS) transformed isomer together with violaxanthin and antheraxanthin lead to biosynthesis of ABA.

Antheraxanthin and violaxanthin are also precursors to the red pigments, capsanthin and capsorubin, a class of keto-carotenoids in pepper fruits (Bouvier et al., 1994).

1.4.3 Degradation: carotenoid cleavage dioxygenases and apocarotenoids

Carotenoids thus synthesized are indeed controlled by a process of degradation. This process in turn leads to a specific group of compounds called apocarotenoids.

Apocarotenoids are the carotenoid cleavage products in the presence of carotenoid cleavage enzymes, which are referred to as carotenoid cleavage dioxygenases (CCDs) or 9-cis-epoxy carotenoid dioxygenases (NCEDs) (Bouvier et al., 2005; Auldrige et al., 2006). These apocarotenoids are also produced in animals and include retinoids: retinal (vitamin A), retinol (vision) and retinoic acid (Wyss, 2004). Carotenoid cleavage

dioxygenases are encoded by a small gene family, cleave at different sites, and exhibit substrate promiscuity as well as stereo-specificity (Schwartz et al., 2003). *Arabidopsis* contains nine CCD genes, and five out of nine were studied for their expression and localization (Tan et al., 2003). 9-cis epoxycarotenoids are the substrates for NCEDs that produce xanthoxin, the precursor of ABA. In leaves, NCED represents a rate-limiting step in the pathway leading to ABA (Nambara and Marion-Poll, 2005). Further more, the enzymatic activity of ABA 8'-hydroxylase (ABA8Ox) is a key step in controlling ABA levels necessary for physiological processes (Kushiro et al., 2004).

The second class of CCDs cleave the polyene backbone of linear and cyclic carotenoids at various double bonds and some are promiscuous in substrate choice (Auldridge et al., 2006). These enzymes are involved in generation of numerous apocarotenoids including β -ionone, geraniol, and β -damascenone (fruit and flower flavor), stringolactone (mycorrhizal fungal and parasitic weed growth stimulant), mycorradicin and blumenin (phytoalexin antifungals) and trisporic acid (suspected pheromone) and gained a lot of focus in a short period with their myriad functions intercepting both plant and its environment (Walter et al., 2000; Matusova et al., 2005). These apocarotenoids are important in the food, fragrance and cosmetic industries. Bixin is a red colored di-carboxylic monomethyl ester apocarotenoid, which is derived from the plant *Bixa orellana* in the presence of lycopene cleavage dioxygenase, through the action of bixin aldehyde dehydrogenase and norbixin carboxyl methyl transferase (Bouvier et al., 2003). A zeaxanthin cleavage dioxygenase is involved in the production of saffron, another commercially important colored compound from *Crocus sativus* (Bouvier et al., 2003).

The degradative mechanism and reduction in carotenoid content has been clearly shown in petals of chrysanthemum, where the expression of carotenoid cleavage dioxygenase CmCCD4a correlates inversely with the accumulation of carotenoids (Ohmiya et al., 2006). In another study, CCD1 knockouts led to 40% increase in total carotenoid, with some individual carotenoids reduced 3-5 folds in seed (Auldrige et al., 2006).

1.5 Regulation of carotenoid metabolism in plants

An array of genetic tools from both pro- and eukaryotic organisms is available for genetic engineering of individual reactions in the pathway and also to establish new branches for the pathway (Shewmaker et al., 1999; Mann et al., 2000; Zhu et al., 2008). However, the complex regulation of endogenous gene expression remains poorly understood.

Because the pathway genes are nuclear encoded and plastid localized, both environmental and endogenous signals such as plastid biogenesis, light signal transduction, developmental patterns during flower and fruit development could in turn regulate carotenoid biosynthesis (von Lintig et al., 1997; Welsch et al., 2000; Woitsch and Romer, 2003; Fraser and Bramley, 2004). Furthermore, as these carotenoids are precursors to the production of phytohormones such as ABA, qualitative changes in carotenoid content might bring physiological or biochemical changes in plants. In addition, these studies have demonstrated that carotenoid accumulation is mainly controlled by the transcriptional regulation of genes encoding carotenoid biosynthetic enzymes. Increases in carotenoid accumulation in fruits and flowers appear to correlate

with increased transcript abundance of carotenoid biosynthetic genes (Ikoma et al., 2001; Moehs et al., 2001; Bramley, 2002; Zhu et al., 2003). A deeper understanding of how the carotenogenic pathway is regulated may lead to new strategies to engineer enhanced carotenoid production apart from over expression of limiting enzymes.

1.5.1 Light and its components

Light is a signal that controls carotenoid biosynthesis, which can get stalled under prolonged darkness (Simkin et al., 2003). Phytoene synthase gene expression in *Arabidopsis* was shown to be under phytochrome control. It influences prolamellar body formation (Welsch et al., 2000) and controls lycopene accumulation in tomato fruits (Alba et al., 2000). It has also been shown by examining the *Arabidopsis* PSY promoter (Welsch et al., 2003) and exposing maize seedlings to different light qualities (Li et al., 2008a), that PSY is under phytochrome control and is modulated under different light qualities. In a similar study, by exposing tobacco seedlings, β -carotene hydroxylase and other xanthophyll biosynthetic genes were also found to be modulated under different light qualities (Woitsch and Romer, 2003). Furthermore, a co-ordinated upregulation between DXS, HDR, and PSY was observed during carotenoid accumulation at the early stages of *Arabidopsis* seedling de-etiolation suggests the influence of biosynthetic networks (Botella-Pavia et al., 2004).

Thus, manipulation of light signal transduction pathway components and photoreceptors has become a remarkable strategy for enhancement of individual reactions in the pathway in addition to the expression of structural genes in the pathway. For example, overexpression of the blue light photoreceptor, cryptochrome2 (CRY2),

increased the carotenoid and flavonoid levels in tomato fruits and leaves (Giliberto et al., 2005). In another study, fruit specific silencing of *DE-ETIOLATED1 (DET-1)* led to elevated levels of carotenoids and flavonoids in tomato fruits (Davuluri et al., 2005). The mutations in two regulatory genes of *UV-DAMAGED DNA-BINDINGPROTEIN1 (DDB1)* and *DET-1* of high pigment mutants in tomato were also shown to be responsible for increased phenolics and flavonoids (Liu et al., 2004). Increase in carotenoid accumulation in these mutants was found to be linked to an increase in plastid number and size, providing a large compartment for carotenoid biosynthesis and deposition (Cookson et al., 2003; Liu et al., 2004; Kolotilin et al., 2007).

1.5.2 Plastid biogenesis and carotenoid associated proteins

As genes encoding carotenoid biosynthetic enzymes are nuclear encoded and plastid localized, carotenoid pigment accumulation is under a variety of organellar pressures specific to different plastids. For example, in a chromoplast containing nectary tissue, overexpression of a bacterial β -carotene ketolase (CrtO) gene under the control of a tomato PDS promoter resulted in an increase of 170% in total carotenoids, however the same overexpression performed in chloroplast containing green tissue resulted in only trace amounts of astaxanthin accumulation (Mann et al., 2000).

The *Orange (Or)* gene mutation, which is caused by the insertion of a copia-type long terminal repeat (LTR) retrotransposon confer high levels of β -carotene accumulation in the cauliflower orange curd mutant and thus represents a novel regulatory gene in mediating carotenoid accumulation (Lu et al., 2006). The *Or* gene mutation in cauliflower exerts its functional role by inducing the differentiation of proplastids or

other non-colored plastids into chromoplasts, which act as a metabolic sink for carotenoid biosynthesis and accumulation (Lu et al., 2006; Li and Van Eck, 2007). Likewise, large amounts of carotenoid sequestering structures associated with high levels of carotenoid accumulation were present in cauliflower *Or* mutant similar to carrot (Li et al., 2001; Lopez et al., 2008).

In another example, carotenoid-associated proteins, such as fibrillin, CHRD and CHRC increased the metabolic sink and associated chromoplast development and carotenoid sequestration (Leitner-Dagon et al., 2006; Simkin et al., 2007). Similar to the cauliflower *Or* protein, low molecular weight molecular chaperones are also known to be involved in regulation of plastid development. In a classic study in tomato, Heat Shock Protein 21 (HSP21) was shown to protect the photosystem II components of oxidative stress, while promoting conversion of chloroplasts to chromoplasts and leading to carotenoid accumulation (Neta-Sharir et al., 2005).

The *high pigment 3 (hp3)* mutant in tomato, which is caused by a mutation in the ZEP gene, confers enhanced levels of carotenoid accumulation by increasing the size of the cellular plastid compartment to enable greater biosynthesis storage capacity (Galpaz et al., 2008). In this mutant, as well as in other ABA deficient mutants of tomato (*flacca* and *sitiens*), there is an increase in plastid number as well as an increase in lycopene levels in fruits (Galpaz et al., 2008). In another report, it was also shown that breakdown of starch led to oxidative stress which triggered production of β -carotene (Horner et al., 2007).

1.5.3 Role of transcription factors

Until now, few studies reported about regulatory factors that control carotenoid metabolism in plants. The ATCTA element was described previously in the PSY promoter in *Arabidopsis* (Welsch et al., 2003) and interestingly the same motif has also been found in the promoters of DXS and HDR (Botella-Pavia et al., 2004). Recent work described a transcriptional regulator, AtRAP2.2, a member of the *APETALA2* (*AP2*)/ethylene-responsive element binding protein transcription factor family, which binds to this ATCTA element. However, this transcription factor has been shown to modestly regulate the transcript levels of PSY and PDS as well as carotenoid content in *Arabidopsis thaliana* (Welsch et al., 2007). Unfortunately, the overexpression of AtRAP2.2 in calli from *Arabidopsis thaliana* led to decreased PSY and PDS transcription and decreased carotenoid levels.

1.6 Engineering of carotenoid metabolism in plants

Higher plants are a cost-effective source for producing secondary products. Metabolic engineering of carotenoid biosynthesis has potential interest for generating plants resistant to environmental stresses and with improved nutritional content (Wurtzel and Grotewold, 2006). Furthermore, production of nutrient rich crops represents a simple and self-sustaining solution to alleviate carotenoid deficiency in developing countries with malnourished populations. Conversely, the synergistic effect offered by multiple nutrients available through food is advantageous compared to supplementing the diet with single components (Fraser and Bramley, 2004).

The elucidation of the carotenoid biosynthetic pathway and gene cloning provided necessary tools for the metabolic engineering. For example, the *Erwinia uredovora* gene cluster has been used extensively for engineering in heterologous species (Sandmann et al., 2006). However, detrimental effects resulted from perturbations in related biochemical pathways, which were overcome by using tissue specific promoters (Giuliano et al., 2008).

“Golden Rice” is the most publicized example of a staple crop plant engineered to produce carotenoids. This is because of its potential humanitarian benefits in combating vitamin A deficiency in the developing world. Rice endosperm lacks provitamin A, and was engineered by overexpression of daffodil *PSY*, which led to accumulation of phytoene (Burkhardt et al., 1997). Later, enhanced accumulation of lutein, β -carotene and zeaxanthin were achieved by overexpression of bacterial *crtI* and daffodil *LCYB* (Ye et al., 2000). However, the utility of *crtI* and *LCYB* were found to be indispensable and led to conclusions that *PSY* plays a limiting role in the production of carotenoids (Schaub et al., 2005; Al-Babili et al., 2006). Furthermore, overexpression of *PSY* from maize alone led to the production of Golden Rice 2 with an increase of 37 μ g/g of total carotenoids of which 31 μ g/g is β -carotene. The 23 fold increase of β -carotene in GR2 compared to GR1 is promised to cover 50% of childrens RDA, which is likely to be delivered by consuming 72g of GR2 (Paine et al., 2005).

Besides rice, there is significant progress in numerous other crop plants such as potato, canola, carrot and tomato that have been engineered to enhance carotenoid

content and composition either qualitatively or quantitatively (Giuliano et al., 2008). All these efforts were successful either by expressing a rate-limiting enzyme or several enzymes in the pathway in a tissue-specific manner in seed, fruit or other storage organ. These approaches were extensively reviewed, while discussing the transgenic strategies in improving the nutritional content of crops (Fraser and Bramley, 2004; Taylor and Ramsay, 2005; Botella-Pavia and Rodriguez-Concepcion, 2006; Sandmann et al., 2006; Matthews and Wurtzel, 2007; Zhu et al., 2007; Giuliano et al., 2008).

Recent progress in understanding the regulation of carotenoid biosynthesis brought to light many different components, and led to development of a second wave in technologies related to plant metabolic engineering. The *Or* protein induced differentiation of β -carotene containing chromoplasts in the curd tissue of cauliflower has brought a new endeavor leading to high levels of β -carotene by alteration of sink strength (Lu et al., 2006; Lopez et al., 2008). Similarly, transformation of the *Or* gene into tubers produced a deep orange yellow color with 31 μ g/g of total carotenoids with an over six fold increase compared to total carotenoid content in controls (Lopez et al., 2008). Over-expression of the pepper carotenoid-binding protein, fibrillin, in tomato fruits led to an increase in the levels of carotenoids and carotenoid derived flavour volatiles (Simkin et al., 2007). Silencing of the expression of CHRD, a plastid lipid-associated protein of tomato, suppressed the quantum yield of photosynthetic electron flow in leaves as well as carotenoid accumulation in flowers (Leitner-Dagon et al., 2006). Overexpression of PSY1 in tomato also affects other isoprenoid metabolism and causes changes in plastid type to form chromoplasts-like structures prematurely during fruit development (Fraser et

al., 2007). Plastid transformation is another novel approach to metabolically engineer the pathway. Transformation of plastid with LCYB has shown four fold increase in β -carotene content of tomato fruits (Wurbs et al., 2007).

1.7 Carotenogenesis in Grasses: Maize as a model species

Maize is an important staple crop, cultivated worldwide with an average production of 779.6 million metric tons (FAO, 2008). The health benefits of yellow maize have long been recognized from specialized food in developed countries to a staple food in undeveloped countries and maize has been a target of breeding strategies for nearly a century (Gibson and Hotz, 2001; Poneleit, 2001). The primary compounds that accumulate in maize endosperm are lutein and zeaxanthin accompanied by smaller amounts of pro-vitamin A compounds at 0.25 to 2.5 μ g/g of dry weight of kernel (Kurilich and Juvik, 1999). This low composition contributes to 25-35% of child and maternal vitamin A deficiency (West, 2002). The recommended dietary allowance of vitamin A is 250 μ g retinol activity equivalents (RAE)/day and 500 μ g RAE/day for children and all other age groups respectively. Thus improving maize would have a significant impact on the target population.

To develop a comprehensive understanding of how carotenoid accumulation is regulated in cereal endosperm, four model species were being used: rice, sorghum, wheat and maize. The best feature of rice is its completely sequenced genome (Yu et al., 2002) as compared to maize. Despite this asset, research on rice is unlikely to develop as a model for understanding mechanisms controlling endosperm carotenogenesis for many reasons. The first and foremost reason is that rice does not naturally express the rate-

limiting enzyme in endosperm. Maize and sorghum belong to the same subfamily of the PACCAD clade (Lawrence and Walbot, 2007) and information derived from these model species likely will translate more readily for developing carotenoid rich grasses. In particular maize holds huge body of genetic and biochemical information and the large community of researchers to transfer the laboratory findings into valuable genetic applications.

1.7.1 Comparative genomics of Grasses

Comparative genomics is the analysis and comparison of genomes from different species. *Poaceae* is an attractive group for comparative genomics because of their evolution, speciation, domestication and their diverse native distributions (Paterson et al., 2005; Salse et al., 2008). There are about 10,000 species and 700 genera in the grass family, covering about 20% of the land surface that exceeds all other families in ecological dominance (Kellogg, 2001; Gaut, 2002). The grass family includes many important cereal crops providing about half of the calories consumed by humans. They collectively contribute ~60% of the world's food production (Keller and Feuillet, 2000) and a growing share of biofuels (Paterson et al., 2005). Thus, it is not surprising that grasses play a central role in nature by becoming a major resource for humans.

Polyploidy has played a major role in the evolution and modeling of grass genomes (Levy and Feldman, 2002). A 35-f old variation in genome size is found, ranging from 415 Mb in diploid rice to 15,000 Mb in hexaploid wheat. Repetitive DNA has been found to be one factor responsible for the range in genome size (Gaut et al., 2000; Paterson et al., 2005). For example, a four-fold difference in DNA amount between

sorghum and maize appeared to be due to retrotransposons (SanMiguel et al., 1998).

Fossil data and phylogenetic studies estimate that the different grass families diverged from a common ancestor 50 to 70 million years ago (Kellogg, 2001; Gaut, 2002). Rice is distantly related to both maize and sorghum having been separated from common progenitors around 50 million years ago (Brendel et al., 2002). Rice is considered a model cereal crop because it has a relatively small genome size with a vast germplasm collection, an efficient transformation system with an enormous repertoire of molecular and genetic resources (Paterson et al., 2005). However, as a model for tropical grasses, sorghum is a logical complement to rice, because sorghum and maize shared a common ancestor about 12 million years ago (Gaut and Doebley, 1997) and the sorghum genome is smaller (~736Mbp) in size compared to maize.

Some of the salient features in the context of comparative genomics are excellent classical genetics, rich genetic maps with thousands of markers to compare, extensive sequenced gene content and conserved gene order along the chromosomes (Moore et al., 1995). The main purpose of this research was to gain greater insight into the function of genes controlling a pro-vitamin A content and composition in maize and apply to closely related species (i.e. grasses). This study also involves the use of computer programs that can align multiple genes and look for regions of similarity among them; this way the knowledge gained studying one species can be extended or applied to others depending on their evolutionary relationships (Soltis and Soltis, 2003).

1.7.2 Maize genome evolution and diversity

Zea is the genus for maize (*Zea mays ssp. mays*) and its wild relative, teosinte (Doebley, 1990). Domestication of maize occurred about 9,000 years ago in southern Mexico and *Zea mays ssp. parviglumis* is thought to be the progenitor of cultivated maize (Wang et al., 1999; Piperno and Flannery, 2001; Matsuoka et al., 2002). Sequencing of the *adh1* locus in *Z. mays ssp. parviglumis* (the maize progenitor) and *Zea luxurians* (a distant maize relative) revealed that maize retained 77% of genetic diversity of *Z. mays ssp. parviglumis* and has greater diversity than *Z. luxurians* (Eyre-Walker et al., 1998)

Maize is diploid ($2n=20$) with chromosomes containing 2.5 billion base pairs. This genome size lies somewhere in the middle of all grass genomes size and complexity (Gaut et al., 2000), similar to the size of the human genome. The maize genome contains extensive chromosomal duplication with nearly a third of the maize genome having multiple copies (Gaut, 2001), transposons insertions and repetitive DNA (SanMiguel et al., 1998; Gaut et al., 2000; Bennetzen and Ramakrishna, 2002) lead to increase the size of the maize genome.

Maize has become an important crop for genetic analysis because of its tremendous phenotypic and molecular diversity (Whitt et al., 2002; Buckler et al., 2006), roughly about 2-5 fold higher than that of other domesticated grass crops (Buckler et al., 2001). Despite its selection history, most maize genes retain a high level of nucleotide diversity. Nucleotide diversity is measured as the average sequence divergence between any two individuals for a given locus (Buckler and Thornsberry, 2002). Cultivated maize

still retains 78% of the silent nucleotide diversity observed in its wild ancestor, and 88% of the wild microsatellite diversity (Rafalski and Morgante, 2004).

Maize carries about 30% of non-homologous gene fragments and 90% of these non-homologous regions are pseudogenes (Brunner et al., 2005; Buckler et al., 2006). In comparison of a 150kb region in two maize inbred strains, B73 and McC, sequences contain different sets of transposons (Fu and Dooner, 2002). When considering nucleotide polymorphism in genes, two maize lines are on average as divergent from one another as humans are from chimpanzees. Thus it is necessary to sequence more than one line because sequencing one line will only identify 85% of functional genes (Buckler et al., 2006).

Nucleotide variation could be due to several factors. Cultural diversity, environmental influence, domestication and various production systems facilitated development of different maize types around the world (Bellon and Berthaud, 2004). Maize is an out- crossing species that exhibits a high level of recombination (Fu et al., 2002; Vigoroux et al., 2002). This out-crossing favors continuous gene exchange between neighboring plants and in some cases with their wild relatives (Bennetzen and Devos, 2002). As a result, modern maize hybrids have higher productivity and are more stress-tolerant than their progenitors (Bruce et al., 2002)

1.7.3 Comparative genetic mapping

Gene mapping is essential for effective manipulation of important genes, QTL detection, comparative mapping, detection of chromosome duplications and marker assisted

selection. Development of genetic markers has contributed greatly to expanding the field of quantitative genetics because of use in mapping chromosomal segments or individual genes underlying a quantitative trait (Bouchez et al., 2002). An extensive genetic linkage map for maize using RFLP (Helentjaris et al., 1988; Lee et al., 2002), AFLP (Vuylsteke et al., 1999; Vuylsteke et al., 2000) and SSRs (Sharopova N, 2002) has been constructed.

The genomes of closely related species are conserved given that they are derived from a common ancestor. The conservation of gene content and order between two or more species is often referred to as genomic colinearity, while synteny is defined as the conservation of linkage on chromosomes, in the absence of a defined order (Bennetzen and Devos, 2002). Because of their conserved genetic nature, species in the grass family such as maize, sorghum, rice, oat and wheat share conserved gene collections. For example, only one of 150 maize RFLP markers failed to hybridize to sorghum DNA (Hulbert et al., 1990) and in another about 85% of rice, oat and barley cDNA clones hybridized to maize DNA (Ahn and Tanksley, 1993). Thus comparing linkage maps and determination of the order of markers along the linkage groups across species will become easier. However, precise conservations of genome colinearity and synteny can only be accomplished by high throughput sequencing and physical mapping; colinearity at the level of sequence is termed as microcolinearity.

These comparative genomic studies make it possible to use the information from several closely related species to enhance our understanding of mechanisms controlling carotenoid accumulation in grasses. Isolation and study of related genes from these

species will allow us to determine the nature of gene family variations that make each species unique (Bennetzen and Freeling, 1997). Furthermore it will allow us to identify the “best” genes for crop improvement (i.e. provitamin-A boost in maize endosperm).

1.8 Natural genetic variation – diverse germplasm

Natural genetic variation in organisms is mostly represented by simple base changes or small insertions/deletions. Humans have applied natural genetic variation for domestication and genetic improvement of more than 100 plant species. Although plant evolution under domestication has led to increased productivity, domestication reduced genetic basis of crop species (Gur and Zamir, 2004), which is due to the selection of specific gene alleles that control key morphological and agronomic traits. This resulted in reduced genetic diversity relative to unselected genes (Yamasaki et al., 2005). Despite this selection, inbred maize lines exhibit high levels of nucleotide diversity, retaining up to 77% of the diversity of the land races (Tenaillon et al., 2001).

Natural genetic variation is still an underexploited resource. This variation could occur in both structural and regulatory genes and become the key drivers in shaping the biochemical, metabolic, physiological and phenotypic variation and collectively account for many of the variety differences in crop germplasm (Kresovich et al., 2002; Alonso-Blanco et al., 2005; Tonsor et al., 2005). Exploiting natural variation is very important, as it allows crops to adapt to new biotic and abiotic stresses. Many landraces and wild relatives of crop plants contain “superior alleles” that confer resistance to pests, diseases, and quality traits (Tanksley and McCouch, 1997; Zamir, 2001). Approximately, 40-80% of the yield gain in maize, wheat and barley has been obtained through exploring the

genetic basis of these crops (Evans and Fischer, 1999). Consequently, large numbers of varieties are being collected around the world in an effort to conserve genetic variation and provide access to valuable material for plant breeders. Maize diversity germplasm with 260 inbred lines covering essentially all public lines is an important resource for studies in genetics and breeding. These lines were assayed for polymorphism at 94 microsatellite loci (Liu et al., 2003) and a model based clustering analysis placed the inbred lines in five clusters on a phylogenetic tree corresponding to major breeding groups, plus a set of lines showing evidence of mixed origins. These clusters captured 80% of the allelic diversity in the land races, suggesting that land races can provide additional genetic diversity for maize breeding (Liu et al., 2003).

1.9 Rationale and Specific Aims

With the effort of several groups studying plant isoprenoid biosynthesis, the substrates and sequential action of enzymes are already known. Modification and modulation of total carotenoid content and composition in various tissues of numerous plant species have been achieved by identifying the relevant genes and development of efficient protocols. However, the majority of manipulations have targeted single steps in the pathway with the objective to elevate either individual carotenoids or total content of carotenoids.

1.9.1 Hypothesis

Maize seed carotenoid content and carotenoid composition are controlled by the timing of endosperm transcript levels of specific gene family members encoding enzymes for carotenoid biosynthesis and degradation.

The ambitious goal behind this thesis was to identify and characterize all the genes available both in the carotenoid pathway as well as in the upstream isoprenoid biosynthetic pathway and downstream degradative pathways using natural genetic variation as a tool, a complementary approach to transgenics. My findings will lead to future in pyramiding of optimal alleles of rate-controlling steps into a single trait improving pro-vitamin A levels in maize.

1.9.2 Specific Aims

Mutational studies and genetics have already shown that expression of PSY has a major effect on the amount of total kernel carotenoids. Maize inbreds were selected showing variation in carotenoids and likely contain the most favourable and informative alleles for breeding carotenoid content and composition. Major genes controlling kernel carotenoids in maize will be identified by correlating gene expression at various time points during endosperm development to carotenoid content and composition of the mature maize seed. The proposed research is divided into several specific aims, which are as follows:

1. Identify genes encoding carotenoid biosynthetic, degradative enzymes as well as enzymes supplying isoprenoid precursors in maize
2. Locate chromosomal positions of identified genes to anchor to any known QTLs
3. Identify orthologous genes using phylogenetic analysis to extend the knowledge obtained in maize to other grass species such as sorghum, rice, wheat and barley.
4. Evaluate transcript levels in leaves, root, unfertilized ovules, developing endosperm and embryo at 20 DAP.
5. Measure transcripts from developing endosperm at 10, 15, 20 and 25 days after

pollination in 10 genetically diverse maize inbreds.

6. Test for correlation between transcript levels and carotenoid content and composition to identify rate-controlling steps and timing of gene expression.
7. Develop breeding assays for tracking optimal alleles.

Table 1-1. Enzyme Abbreviations and substrates

	Enzyme	Name	Substrate
MEP PATHWAY	DXS	1-deoxy-D-xylulose 5-phosphate synthase	D-glyceraldehyde-3-phosphate, pyruvate
	DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase	1-deoxy-D-xylulose-5-phosphate
	MCT	2-C-methyl-D-erythritol 4-phosphate cytidyltransferase	2C-methyl-D-erythritol-4-phosphate
	MCK	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	4-diphospho-2C-methyl-D-erythritol
	MCS	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate
	HDS	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	2C-methyl-D-erythritol 2,4-cyclodiphosphate
	HDR	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate
	IPPI	isopentenyl diphosphate isomerase	IPP, Dimethylallyl diphosphate
GGPP SYNTHESIS	GGPPS	Geranylgeranyl pyrophosphate synthase	Isopentenyl pyrophosphate (IPP), DMAPP
CAROTENOID BIOSYNTHESIS	PSY	phytoene synthase	geranylgeranyl pyrophosphate
	PDS	phytoene desaturase	15- <i>cis</i> -phytoene, 9,15- <i>cis</i> -phytofluene
	Z-ISO	15- <i>cis</i> - ζ -carotene isomerase	9, 15, 9'- <i>cis</i> - ζ -carotene
	ZDS	ζ -carotene desaturase	9,9'- <i>cis</i> - ζ -carotene,9'- <i>cis</i> -neurosporene
	CrtISO	carotene isomerase	7,9,9'- <i>cis</i> -neurosporene, 7'9'- <i>cis</i> -lycopene
	LCYB	lycopene β -cyclase	All-trans lycopene
	LCYE	lycopene ϵ -cyclase	All-trans lycopene
	HYDB	β -carotene hydroxylase (diiron type)	β -carotene, β -cryptoxanthin, α -carotene
	CYP97A	β -carotene hydroxylase (P450-type)	β -carotene, β -cryptoxanthin, α -carotene
	CYP97C	ϵ -carotene hydroxylase (P450-type)	α -carotene, zeinoxanthin
	ZEP	zeaxanthin epoxidase	zeaxanthin, atheraxanthin
CAROTENOID DEGRADATION	VDE	violaxanthin de epoxidase	violaxanthin, Antheraxanthin
	NXS	neoxanthin synthase	violaxanthin
	NCED	9- <i>cis</i> -epoxy carotenoid dioxygenase	various carotenoid substrates (11,12)
	CCD	carotenoid cleavage dioxygenase	various carotenoid substrates (9, 10)

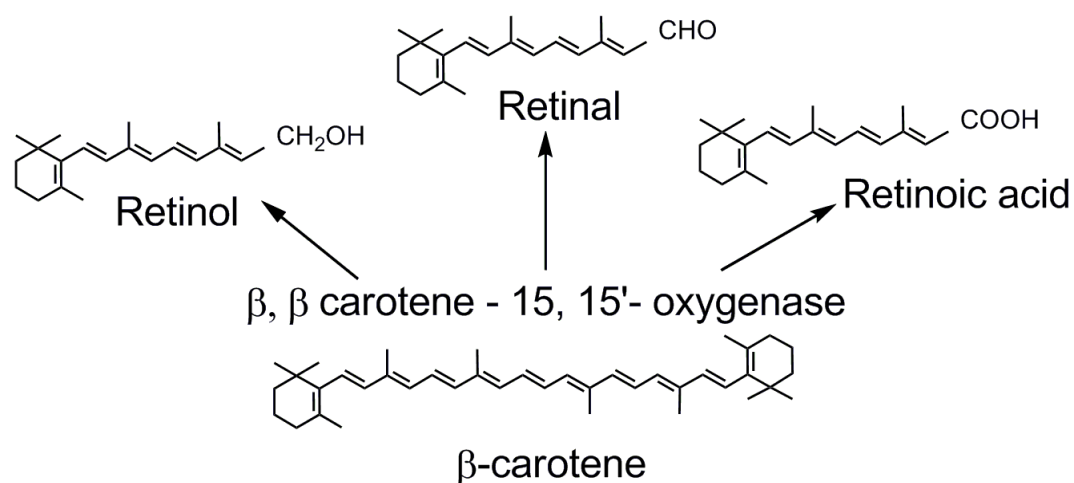


Figure 1-1. Conversion of β -carotene to Vitamin A derivatives.

Vitamin A derivatives (Retinol, Retinal and Retinoic acid) produced in mammals during the symmetric oxidative cleavage at central 15, 15' double bond in the presence of β, β carotene - 15, 15'- oxygenase.

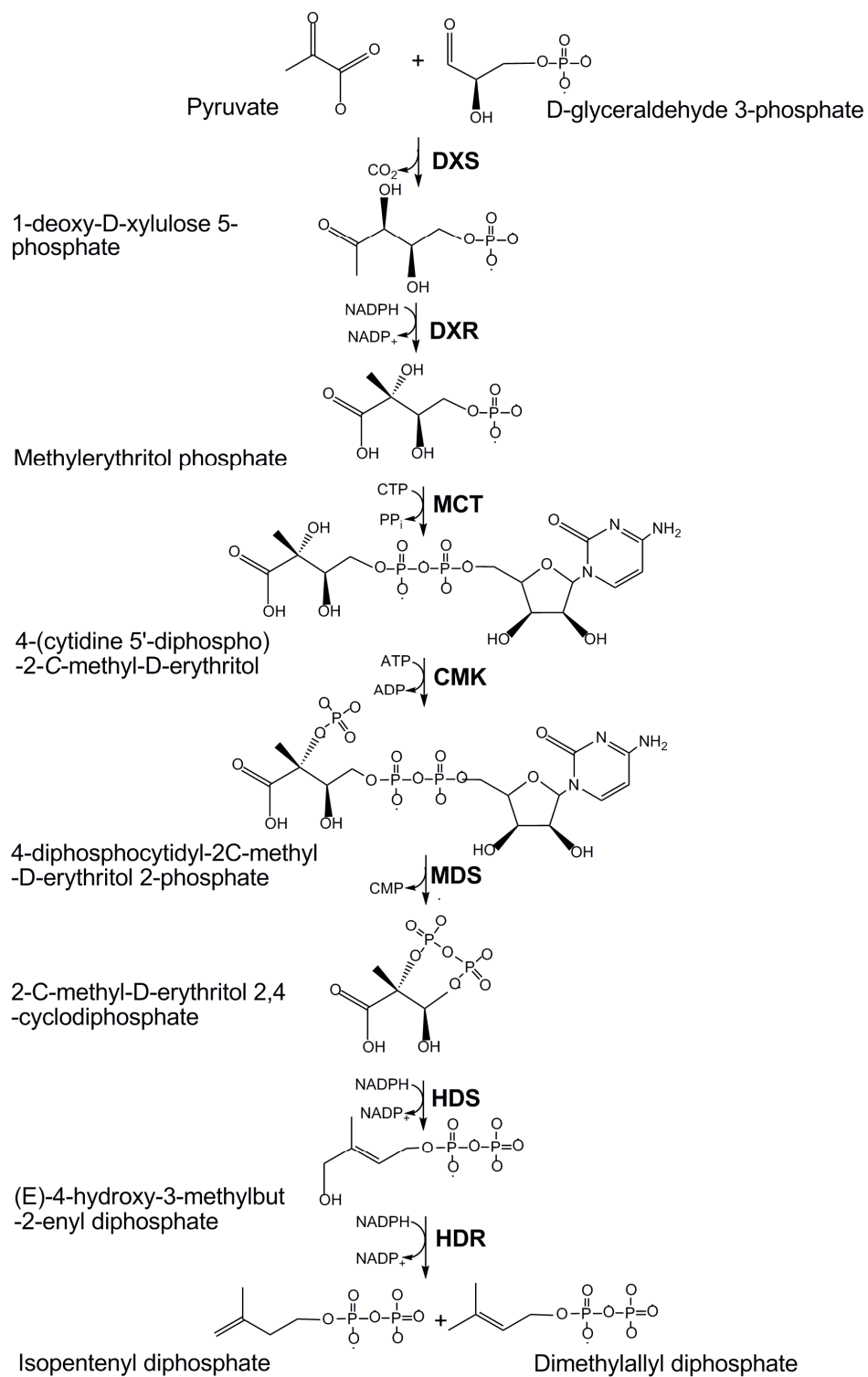


Figure 1-2. Methylerythritol phosphate (MEP) pathway in plants.

DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-Phosphate reductoisomerase; MCT, 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase ; CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase ; HDS, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase. See **Table 1-1** for list of enzymes, substrates and products.

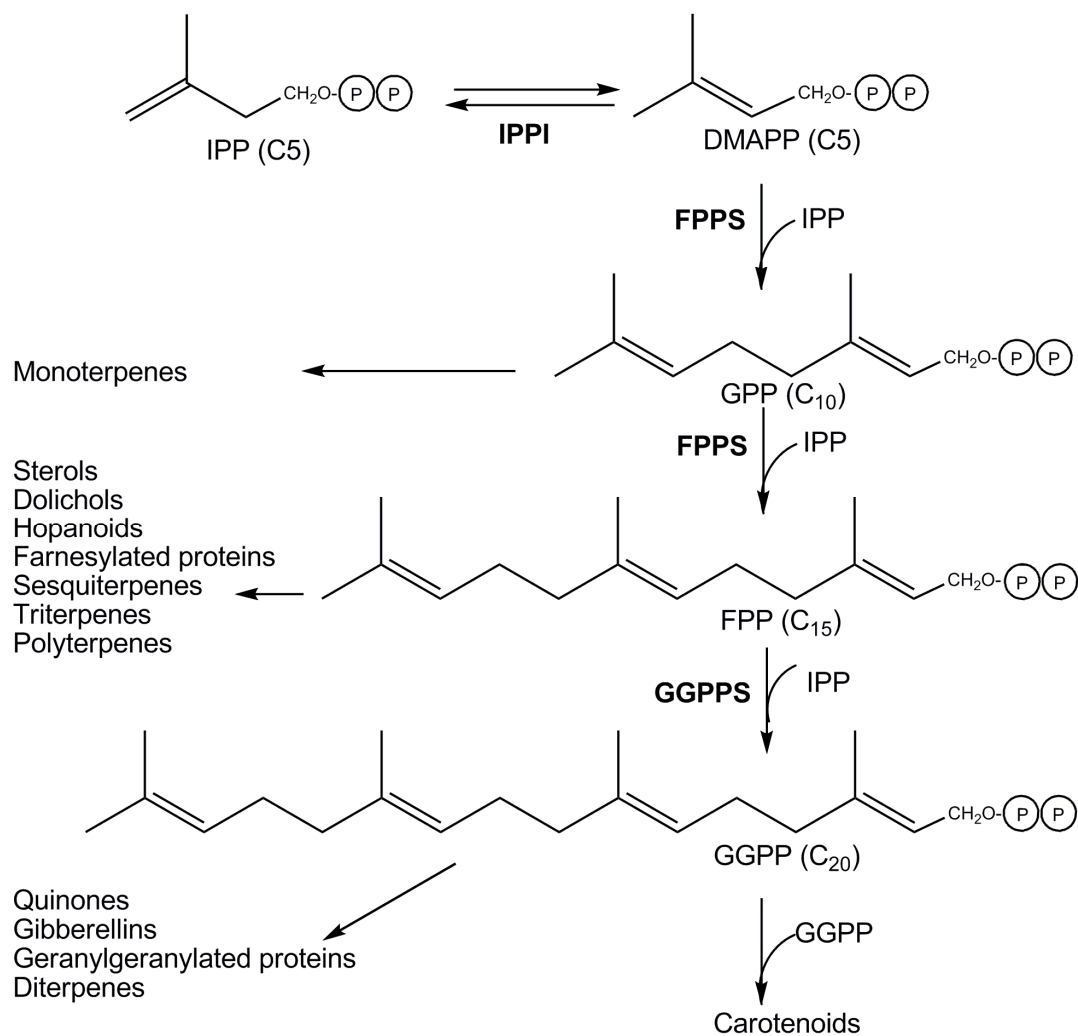


Figure 1-3. GGPP synthesis in plants.

IPPI, Isopentenyl diphosphate isomerase; FPPS, Farnesyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate.

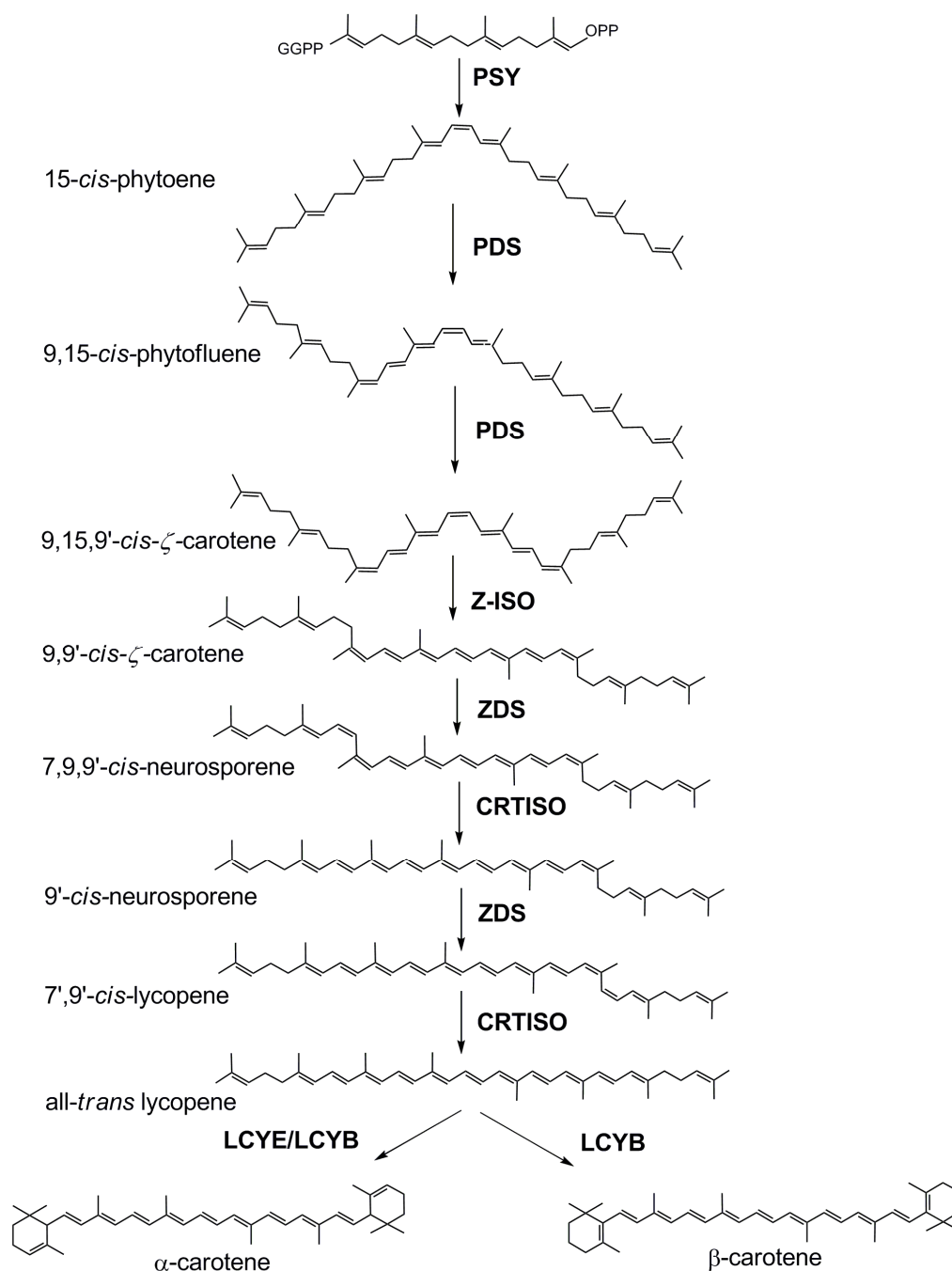


Figure 1-4. Carotenoid biosynthesis in plants.

PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, zetacarotene isomerase; ZDS, zetacarotene desaturase; CrtISO, carotene isomerase; LCYB, lycopene beta cyclase; LCYE, lycopene epsilon cyclase; GGPP, geranylgeranyl diphosphate.

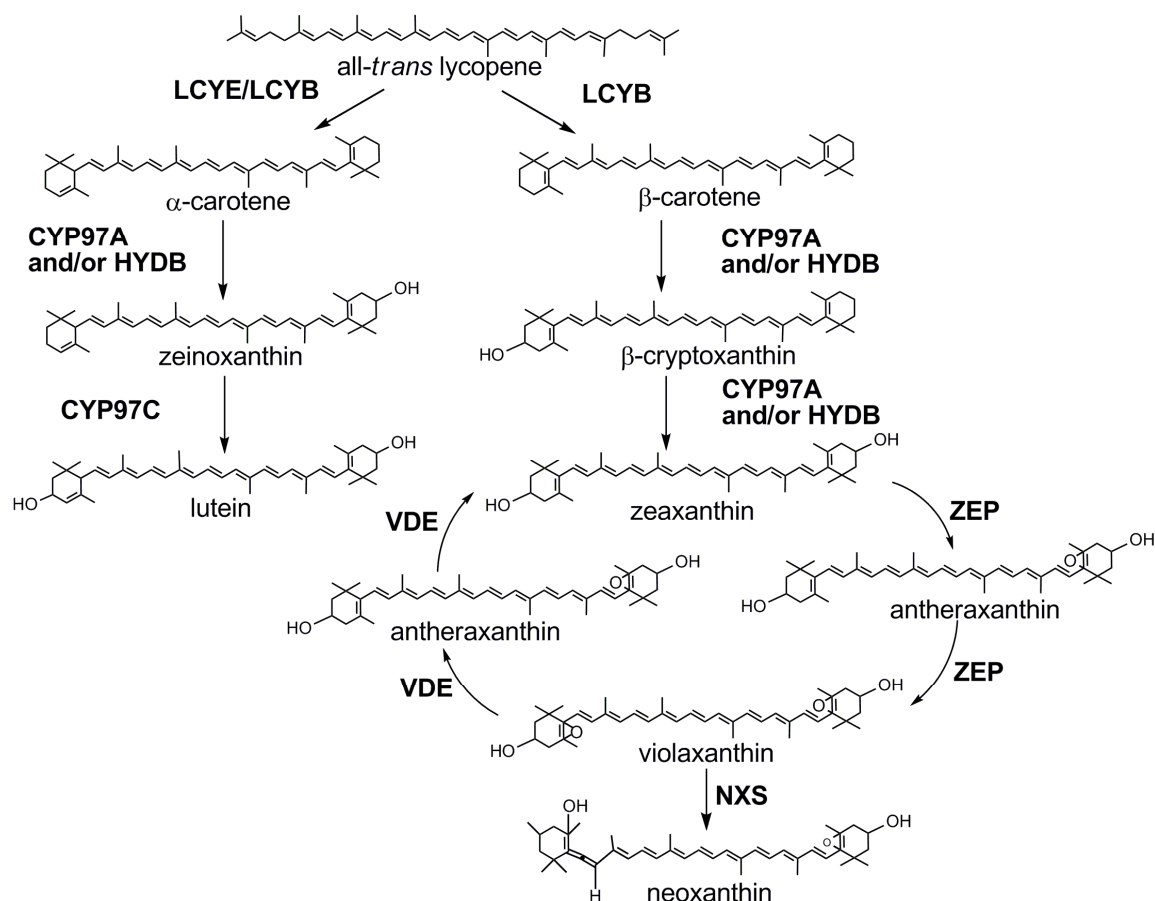


Figure 1-5. Xanthophyll biosynthesis in plants.

LCYB, lycopene beta cyclase; LCYE, lycopene epsilon cyclase; CYP97A, P450 type beta-carotene hydroxylase; CYP97C, P450 type epsilon-carotene hydroxylase; HYDB, beta-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violoxanthin de-epoxidase; NXS, neoxanthin synthase.

Chapter 2

Timing and biosynthetic potential for carotenoid accumulation in genetically diverse germplasm of maize¹

2.1 Abstract

Enhancement of the carotenoid biosynthetic pathway in food crops benefits human health and adds commercial value of natural food colorants. However, predictable metabolic engineering or breeding is limited by the incomplete understanding of endogenous pathway regulation, including rate-controlling steps and timing of expression in carotenogenic tissues. The Grass family (Poaceae) contains the major crop staples, including maize, wheat, rice, sorghum, and millets. Maize carotenogenesis was investigated using a novel approach to discover genes encoding limiting biosynthetic steps in the nutritionally targeted seed endosperm. A combination of bioinformatics and cloning were first used to identify and map gene families encoding enzymes in maize and other grasses. These enzymes represented upstream pathways for IPP and GGPP synthesis and the downstream carotenoid biosynthetic pathway, including conversion to ABA. A maize germplasm collection was used for statistical testing of correlation between carotenoid content and candidate gene transcript levels. Multiple pathway bottlenecks for isoprenoid biosynthesis and carotenoid biosynthesis were discovered in specific temporal windows of endosperm development. Transcript levels of paralogs encoding isoprenoid IPP and GGPP-producing enzymes, *DXS3*, *DXR*, *HDR*, and

¹ This chapter is a revision of Ratnakar Vallabhaneni and Eleanore T. Wurtzel, *Plant Physiology* 150:562-572 (2009) with the permission from the publisher

GGPPS1, were found to positively correlate with endosperm carotenoid content. For carotenoid pathway enzymes, transcript levels for *CrtISO* inversely correlated with seed carotenoid content, as compared to positive correlation of *PSY1* transcripts. Since ZEP depletes the carotenoid pool in subsequent conversion to ABA, *ZEP* transcripts were examined. Carotenoid accumulation was found to be inversely associated with *ZEP1* and *ZEP2* transcript levels. Extension of the maize results using phylogenetic analysis identified orthologs in other Grass species that may serve as potential metabolic engineering targets.

2.2 Introduction

Carotenoids are a complex class of isoprenoid pigments providing nutritional value as provitamin A and nonprovitamin A compounds; their varied colors provide added commercial value as colorants in foods (reviewed in Matthews and Wurtzel, 2007). Metabolic engineering of the biosynthetic pathway has been of interest to specifically address vitamin A deficiency in millions of children worldwide (reviewed in Giuliano et al., 2008); this serious global health problem could be alleviated by breeding cereal crop staples in the Poaceae (Grass family) for elevated levels of provitamin A carotenoids. Engineering high levels of specific carotenoid structures requires controlled enhancement of total carotenoid levels (enhancing pathway flux, minimizing degradation and optimizing sequestration) plus controlled composition for specific pathway endproducts. While most of the nuclear genes for the plastid-localized pathway are available (Li et al., 2007) and/or can be identified, there remain open questions about the rate-controlling steps that limit predictability of metabolic engineering in plants.

The most important cereal crop staples worldwide, such as maize, sorghum,

wheat, and rice, are all contained within the Grass family. We have taken advantage of the resources available for maize, an important crop staple in sub-Saharan Africa and Latin America, to explore those factors that control endosperm carotenogenesis. Ortholog targets in related Grass species can be identified using comparative genomics, and thereby potentially extend maize studies for predictive metabolic engineering throughout the cereal crops.

Predictable manipulation of the seed carotenoid biosynthetic pathway in diverse maize genotypes necessitates elucidation of biosynthetic step(s) that control carotenoid accumulation in endosperm tissue (**Fig. 2-1**). Extensive studies have implicated phytoene synthase (PSY), the first committed enzyme, as rate-controlling for endosperm carotenoids (Randolph and Hand, 1940; Palaisa et al., 2003; Gallagher et al., 2004; Wong et al., 2004; Pozniak et al., 2007; Li et al., 2008; Li et al., 2008a). However, upstream precursor pathways may also positively influence carotenoid accumulation (Matthews and Wurtzel, 2000; Mahmoud and Croteau, 2001) while downstream degradative pathways may deplete the carotenoid pool (Galpaz et al., 2008). We decided to utilize natural genetic and biochemical variation in a maize germplasm collection to identify potential transcriptional control points affecting endosperm carotenoid accumulation, since transcriptional control plays a large role in carotenogenesis (Giuliano et al., 2008). An alternative approach to probe the pathway with transgenes was considered less desirable as the resulting changes would only reflect and be predictive for the particular transformed genotype; and an ultimate goal is to derive regulatory/temporal information applicable to metabolic engineering/breeding of a broader range of genotypes whether by nontransgenic (Harjes et al., 2008) and/or transgenic means (Zhu et al., 2008).

Association of candidate gene *cis*-located DNA variation with kernel carotenoid levels in a germplasm collection has been effective in identifying metabolic breeding tools for the carotenoid pathway (Harjes et al., 2008). However, association analysis may overlook a rate-controlling biosynthetic step if gene expression is regulated by another locus. Moreover, optimal timing of gene expression is not revealed. Therefore, to identify rate-controlling steps, we focused on transcript level variation as manifestation of DNA polymorphisms at the candidate gene or regulation by an unlinked locus. RNA profiling would also facilitate optimization of temporal expression for transgene expression efforts.

2.3 Results

2.3.1 Pathway gene families and chromosome mapping

To be able to examine transcriptional control of pathway flux, we assembled a collection of genes from maize and other grass species to represent upstream pathways for IPP and GGPP synthesis and the downstream carotenoid biosynthetic pathway, including conversion to ABA. We used a combination of bioinformatics and cloning to identify gene families for enzymes shown in Figure 2-1. Genes were mapped in maize to chromosome position with no apparent linkage seen among the pathway genes (**Tables 2-1 and 2-2**).

MEP pathway enzymes DXR, HDS, and HDR were found to be encoded by single copy genes in the maize genome. Three paralogs for maize DXS were identified (**Figs. 2-2 and 2-3A**), (Walter et al., 2002); *DXS3* is a newly discovered paralog in maize, also present in sorghum and rice, species spanning two subfamilies of the Poaceae (**Fig. 2-2, Fig. 2-3A; Table 2-2**) (Kim et al., 2005). The ChloroP program (Emanuelsson et al., 1999) predicted a plastid localization signal in the first 51, 31 and 50 amino acids of

ZmDXS1, *ZmDXS2*, and *ZmDXS3*, yielding plastid localized proteins of 71.7, 73.5, 73.1 kD respectively. The three maize enzymes are similar in size, yet share only 52.5 % identity/65.8% similarity between DXS1 and DXS3 and 47.6 identity/62.4% similarity between DXS2 and DXS3 as compared to 69.7% identity/79.1% similarity between the more closely related DXS1 and DXS2 (**Fig. 2-8**).

The IPPI gene family contained three genes (**Table 2-2**). GGPPS was also encoded by a small gene family of three paralogs. The encoded proteins have the requisite GGPPS structural domains and chloroplast targeting signals of 52, 51 and 44 amino acids for GGPPS1, GGPPS2, and GGPPS3, yielding plastid localized forms of 33.5, 32.9, and 32.7 kD respectively (**Fig. 2-9, Table 2-2**) (Okada et al., 2000). *GGPPS3* was the only intronless gene and its predicted peptide sequence had one variant region immediately downstream of domain III. Phylogenetic analysis showed that the intron-containing *GGPPS* genes and the intron-less genes formed two distinct clades within the Angiosperms (**Fig. 2-3B**). Maize *GGPPS1* and *GGPPS2* appear to be recent duplications arising after speciation. Function of the three GGPPS genes was tested by heterologous complementation in *E. coli* (**Fig. 2-10**) using methods previously described (Cervantes-Cervantes et al., 2006); GGPPS1 and GGPPS2 proved functional but GGPPS3 did not (data not shown). *GGPPS3* transcripts were barely detectable as evidenced by RT-PCR and as reflected by low EST prevalence in maize and rice. Therefore, our studies limited evaluation to the two functional GGPPS genes.

For the carotenoid biosynthetic pathway, we identified two *CrtISO* genes in maize on chromosomes 2 and 4, adding to previously isolated pathway genes for PDS, ZDS, LCYE, and LCYB all of which are single copy in maize and other grasses (Buckner et

al., 1990; Li et al., 1996; Matthews et al., 2003; Singh et al., 2003; Harjes et al., 2008; Li et al., 2008; Li et al., 2008a) (**Table 2-2**). In contrast to two maize *CrtISO* genes, only one was found in rice, sorghum and Arabidopsis (**Table 2-2, Fig 2-11**). However, maize *CrtISO2* proved to be a pseudogene; alignment of maize *CrtISO* sequences (**Fig. 2-11**) (verified from several DNA sources; see Materials and Methods) revealed a stop codon in exon 1 of maize *CrtISO2* which is predicted to interrupt translation. The functional *CrtISO1* encodes an enzyme of 63.6 kD with a predicted plastid targeting sequence of 43 residues, yielding a plastid-localized form of 59.3 kD. Maize *CrtISO1* is located on chromosome 4 in a region syntenous with rice *CrtISO* and linked to marker *csu704* on rice chromosome 11.

For examination of carotenoid metabolism to ABA, we identified genes for zeaxanthin epoxidase (ZEP), an enzyme catalyzing the conversion of zeaxanthin to violaxanthin, a precursor of ABA in endosperm (**Fig. 2-1**). Two ZEP genes were found in maize compared to only one in rice and sorghum (**Fig. 2-12, Table 2-2**); all showed gene and protein structural similarities, including chloroplast targeting signals. Maize ZEP1 and ZEP2 have predicted transit peptides of 68 and 60 residues yielding 66.6 kD plastid-localized enzymes.

2.3.2 Transcript profiling of gene family members and correlation with carotenoid content

Predictive metabolic engineering/breeding of kernel carotenoid content is predicated on identifying genes and their temporal expression in developing endosperm that correlates with carotenoid content. Using the standard inbred B73 (Li et al., 2008), transcript levels were quantified across a panel of tissues and during endosperm development for all

functional genes that were required for carotenoid accumulation (**Fig. 2-4; Fig. 2-5**).

Paralog-specific variation in transcript levels was observed in B73 maize. However, it is unknown how transcript levels correlate with accumulated endosperm carotenoids in genetically diverse inbreds varying in carotenoid content and composition (Islam, 2004; Harjes et al., 2008). It is also unknown how timing of gene expression during endosperm development is related to net accumulation of carotenoids in mature kernels. To investigate timing and correlation between gene expression and carotenoid content, transcript levels at specific endosperm developmental stages were compared with carotenoid content of mature kernels that varied in a genetically diverse germplasm collection. Quantitative transcript profiling was conducted in a core subset of maize lines chosen from a collection varying in kernel carotenoid content and spanning 80% of maize genetic diversity (Liu et al., 2003; Islam, 2004; Harjes et al., 2008). The goal was to identify genes/paralogs with a statistically significant correlation between transcript levels at certain endosperm developmental stages and carotenoid content in the mature kernel. From a collection of 148 maize lines analyzed for carotenoid content (Islam, 2004), ten lines were chosen that represented extremes of carotenoid content and composition (e.g. highest levels of zeaxanthin, lutein etc.). These core lines spanned four major genetic diversity groups and eight subgroups (Liu et al., 2003). To validate the utility of the core subset to yield predictive results, we used the collection to test the *PSY* gene family which contains one member, *PSY1*, known to control flux to endosperm carotenoids. Only *PSY1*, of three paralogs, showed the expected correlation of transcripts and carotenoid content in endosperm (Li et al., 2008a). The validated germplasm subset was then used to assess the influence of other genes in the pathway collection described

above using paralog-specific primers (**Table 2-3**). Transcript levels were quantified in developing endosperm 10-25 days after pollination (DAP) (**Table 2-4**), stages when carotenoid accumulates (Li et al., 2008a). Transcript levels at each developmental stage were then statistically compared with carotenoid content of the mature kernel. Pearson correlation analysis (see **Table 2-1A** for r and p values) revealed that in addition to *PSY1*, several other genes showed a statistically significant correlation of transcripts with total carotenoid content (**Fig. 2-6**). Positively correlating genes upstream of the carotenoid pathway included *DXS3*, *DXR*, *HDR*, and *GGPPS1*, all at 25 days after pollination (DAP). Within the carotenoid pathway itself, *CrtISO1* transcripts negatively correlated with carotenoid content ($r = -0.76$; $p = 0.001$) as compared to the positive correlation seen with transcripts of the carotenoid pathway gene, *PSY1* ($r = 0.96$; $p = 0.001$), both at 20 DAP. The difference in r values for *CrtISO1* and *PSY1* is likely due to *PSY1* having been under strong selection (Palaisa et al., 2003), whereas *CrtISO1* has not been under any selection because it was unknown. A significant lesson drawn from these experiments is that trends in transcript accumulation patterns (**Fig. 2-5**) were not necessarily predictive of carotenoid accumulation. For example, transcripts of *DXS3* and *DXS1* both showed temporal increases during endosperm development, but only *DXS3* showed correlative expression with carotenoids. Similarly, transcript levels of *IPPI* gene family members showed temporal increases during endosperm development but were not limiting as judged by the correlation analysis.

2.3.3 Role of zeaxanthin epoxidase in depleting endosperm carotenoids

Conversion of carotenoids to ABA requires the activity of ZEP, an enzyme upstream of the rate controlling cleavage step mediated by 9-*cis*-epoxycarotenoid dioxygenase (**Fig.**

2-1). To test whether ZEP expression was associated with reduced kernel carotenoids, gene specific primers were developed for quantitative RT-PCR (**Table 2-3**). In maize B73, *ZEP* genes showed similar tissue-specific transcript levels (**Fig. 2-7A**), but differed during endosperm development. *ZEP* transcript levels were next evaluated in developing endosperm of the ten-inbred line core subset (**Table 2-5**) and tested for correlation with seed carotenoid composition (**Table 2-1B**). Both *ZEP1* and *ZEP2* transcript levels at 20 DAP showed statistically significant inverse correlation with seed zeaxanthin levels (**Table 2-7B; Table 2-5**) (*ZEP1*, $r = -0.77$; $p = 0.008$ and *ZEP2*, $r = -0.86$; $p = 0.001$). This correlation matched the temporal window (20 DAP) of the earlier carotenoid pathway genes (encoding PSY and CrtISO). In contrast, no correlation was seen between *ZEP* transcripts and lutein levels, as predicted since lutein is not a substrate of *ZEP* (**Table 2-1B**). The finding that *ZEP* expression negatively affects maize endosperm carotenoid accumulation is consistent with analysis of the tomato *ZEP* mutant (*hp3*) that showed a 30% increase in carotenoids compared to nonmutant fruits (Galpaz et al., 2008).

2.4 Discussion

Using bioinformatics and genome analysis, we produced a comprehensive identification of key genes and gene family members involved in the biosynthesis of carotenoids in maize and related Grass species. Study of a genetically diverse maize germplasm helped elucidate the rate-controlling steps in seed carotenoid biosynthesis. We distinguished gene family members for which transcript levels statistically correlated with seed carotenoid content. We also provided temporal information on gene expression that will guide future breeding efforts whether transgenes or natural alleles are utilized. These data

will contribute to predictive strategies to engineer the maize endosperm carotenoid pathway, to stack traits that affect multiple biosynthetic pathways competing for isoprenoid precursors, or to divert isoprenoid precursors to alternate pathways. Lastly, phylogenetic analysis was used to identify putative targets in related grasses, extending these studies to other species that may lack the resources of maize.

2.4.1 Upstream isoprenoid biosynthesis controls downstream endosperm carotenoid content

The observation that several steps, including upstream isoprenoid biosynthesis pathways, influence flux to carotenoids indicates that PSY is not the only rate-controlling step in controlling maize endosperm carotenoid accumulation. The identification of genes encoding the isoprenoid IPP and GGPP-producing enzymes *DXS3*, *DXR*, *HDR*, and *GGPPS1* provide new targets for metabolic engineering of carotenoids in maize, across genetically diverse germplasm. DXS and GGPPS both function at branch points of metabolism, whereby several pathways must compete for enzyme products. HDR produces a 5:1 mixture of IPP and DMAPP isomers which must be combined in specific ratios for downstream prenyl transfer reactions of multiple pathways including that for carotenoid biosynthesis (Rodriguez-Concepcion and Boronat, 2002). It is not surprising to have found these particular enzymes to be influential, given evidence in other species that the enzymes may be rate-controlling.

Upregulation of the upstream isoprenoid and downstream carotenoid pathways are needed to force flux to carotenoids. For example, overexpression of *DXS* or *GGPPS* in combination with a carotenoid cluster significantly enhanced carotenoids produced in an *Escherichia coli* bacterial platform (Wang et al., 1999; Matthews and Wurtzel, 2000).

Similar examples in other plants have shown that overexpression of genes for certain upstream precursor pathway enzymes (DXS, DXR, or HDR) plus a downstream pathway enzyme forced pathway flux to the downstream pathway (Botella-Pavia et al., 2004; Carretero-Paulet et al., 2006; Munoz-Bertomeu et al., 2006). In contrast, overexpression of *HDS* had no effect on enhancing carotenoid accumulation in *E. coli* or *Arabidopsis* (Flores-Perez et al., 2008), consistent with the results shown here for maize.

Manipulation of a downstream pathway, in the absence of elevating the isoprenoid pool, may have negative consequences. For example, early attempts to increase tomato fruit carotenoids by *PSY* overexpression caused a dwarf phenotype due to redirection of GGPP away from gibberellins (Fray et al., 1995). Similar problems in improvement of tomato volatiles by overexpression of geraniol synthase to divert isoprenoids, caused reduced carotenoid accumulation (Davidovich-Rikanati et al., 2007); and overexpression of taxadiene synthase redirected GGPP from GGPPS to taxadiene, but also depleted the carotenoid pool (Besumbes et al., 2004).

Given that certain isoprenoid biosynthetic enzymes play such a critical role in controlling endosperm carotenoids, breeders might consider new approaches to breeding that incorporate surveys for “biosynthetic potential.” That is, maize carrying a mutation in *PSY1* is blocked in the downstream carotenoid pathway and appears as “white maize.” Such an ear would not be considered suitable for breeding for high (yellow) carotenoid content. However, this line may possess high levels of transcripts for one of the “rate-controlling” isoprenoid enzymes and hence has “biosynthetic potential,” once the carotenoid block is released. Conversely, ears that have high levels of *ZEP* transcripts may appear light yellow, masking optimal expression of other required biosynthetic

steps.

2.4.2 Isomerase expression negatively affects endosperm carotenoid content

The negative correlation seen for *CrtISO* was surprising given its placement in the pathway. *PDS* and *ZDS* were not correlative and the *ZISO* gene is yet to be cloned and therefore could not be tested. The isomerase *CrtISO* is one of several enzymes, including the desaturases *PDS* and *ZDS*, and a second isomerase, *ZISO*, unique to plant carotenoid biosynthesis and required to mediate desaturation of 15-*cis* phytoene to all-*trans* lycopene (Li et al., 2007). In contrast, bacterial pathways carry out these conversions with only a single enzyme, *CrtI*, which is why bacterial *CrtI* has been used as a simple tool in plant carotenoid metabolic engineering (Matthews and Wurtzel, 2007; Giuliano et al., 2008). At this point it is a mystery as to why the *CrtISO* negative regulation is operative in this part of the pathway. Given that *CrtISO* expression controls pathway flux in endosperm, one should question whether engineering with a bacterial gene interferes with endogenous regulatory mechanisms of isomerization as hinted by recent evidence of epigenetic control of *CrtISO* in *Arabidopsis* (Cazzonelli et al., 2009). Evidence that an isomerase controls flux partitioning in photosynthetic tissue is suggested by reduced lutein observed in *CrtISO* mutants and in plants overexpressing bacterial *CrtI* (Misawa et al., 1994; Park et al., 2002).

2.4.3 Temporal windows of gene expression that influence endosperm carotenoid content

Predictive metabolic breeding also requires an understanding of the timing of expression for pathway-controlling genes. Carotenoid accumulation in maize endosperm occurs

continuously from 10 DAP onward as the seed matures (Li et al., 2008a). The correlations observed for upstream and downstream steps fell into two temporal categories; all genes controlling steps upstream of the carotenoid pathway showed correlation at 25 DAP, whereas the carotenoid pathway genes (*PSY1* and *CrtISO*, *ZEP1*, and *ZEP2*) showed correlation at 20 DAP. One possible explanation for the temporal difference is that several pathways, including the carotenoid pathway, compete for isoprenoid precursors during the later phases of endosperm development.

2.4.4 Using maize to identify putative targets in other Grass species for improvement of seed carotenoids

Extension of the maize results using phylogenetic analysis identified orthologs in other Grass species that may serve as potential engineering targets. The *DXS* phylogenetic tree (Fig. 3.3A) showed that the *DXS* triplication occurred prior to evolution of the grasses, suggesting that Grass orthologs may have similar subfunctionalized roles as shown for the *PSY* gene family (Li et al., 2008; Welsch et al., 2008; Li et al., 2009). Therefore *DXS3* orthologs in other grass species would be an attractive target for initiating investigations of rate controlling steps for endosperm isoprenoid-derived metabolites. Similarly, the correlative single copy genes (*DXR*, *HDR*, *CrtISO*) are potential endosperm targets for other Grass species having single copy genes.

2.5 Conclusion

In summary, transcript levels of 7 genes were found to positively or negatively correlate with endosperm carotenoid content in genetically diverse germplasm; the critical timing of expression of these 7 genes fell into two temporal windows of endosperm

development. Manipulation of the pathway across the diversity of maize, in cultivars worldwide, will therefore be somewhat complicated with timing adding another dimension to this complexity. Incorporating a *DXS* transgene will not overcome suboptimal alleles that limit pathway flux when the trait is introgressed into various genotypes. However, these data now allow for development of additional tools to canvass suitable alleles for the downstream steps, or to use other transgenes as necessary.

In this study, the influence of candidate gene expression on endosperm carotenoid content was examined from the perspective of synthesis. This approach can also be used to dissect those factors that control endosperm composition for specific carotenoids (e.g. provitamin A carotenoids). Future study of the degradation genes/enzymes is also needed since carotenoid degradation (Galpaz et al., 2008; Vogel et al., 2008) may potentially reduce carotenoid pools achieved by targeting the genes reported here. Beyond candidate genes, there are likely other factors as evidenced from genetic (mutant and QTL) studies in maize and other plants (Liu et al., 2003; Wurtzel, 2004).

The utilization of a genetically and biochemically diverse maize germplasm collection to infer pathway regulation is an important resource in identifying potential gene targets for controlling endosperm carotenoid content. Germplasm collections in many plant species hold untapped potential in elucidating underlying regulatory mechanisms that will guide breeding and facilitate predictive metabolic engineering. Future investigations of pathway regulation, including timing of gene expression, that underlies the global network of plant metabolism, could be accomplished by deep transcript profiling of tissues from core collections chosen by metabolite sub-sorting and correlations made between mRNA levels and metabolite profiles.

2.6 Materials and Methods

2.6.1 Sequence analysis and chromosome mapping

Rice genes (www.gramene.org) were used as a query to identify orthologs from *Zea mays* (www.tigr.org; www.plantgdb.org) and to decipher gene families. Sequence analysis was performed using Vector NTI Suite 9.0 (Invitrogen, Carlsbad, CA). Expressed sequence tags (ESTs) and conceptual translation was used to distinguish gene paralogs.

Chromosomal positions of maize genes in the *Zea mays* B73 inbred were mapped either by utilizing tools available at WebAGCoL package (Pampanwar et al., 2005) or Maize GDB. Prediction of chloroplast targeting site was made using the ChloroP software (Emanuelsson et al., 1999).

The presence of a stop codon in *CrtISO2* was confirmed from a BAC clone (98301-103300; AC183901); EST (EE045563); genomic sequence from methyl filtered library (AZM4_69491); and GSS contig sequence (ZmGSSTUC11-12-04.150.3) from PlantGDB.

2.6.2 Quantitative RT-PCR and carotenoid measurements

Plants and tissues were collected as described (Li et al., 2008a). RNA extraction and quantitative RT-PCR were performed using gene specific primers (Table 2-3) and normalized to actin, as previously described (Li et al., 2008a). Values are expressed as the mean of three RT-PCR replicates $\pm$ standard deviation. The carotenoid extraction procedure was based on Kurilich and Juvik (1999) and as previously described (Li et al., 2008a). Values are expressed as the mean of three extraction replicates $\pm$ standard deviation.

2.6.3 Plasmids and Functional Complementation

Full-length cDNAs encoding *ggpps1* and *ggpps2* were amplified using approximately 100ng of cDNA prepared from frozen maize endosperm (20 DAP), whereas *ggpps3* was amplified using 0.5µg of maize B73 genomic DNA by PCR. Primers were designed on available genomic sequence; adapted to pET23 series of vectors (Novagen, Madison, WI) with *EcoRI* and *HindIII* restriction sites. The resultant PCR products were cloned in the respective sites and designated as pET23aZmGGPPS1, pET23aZmGGPPS2 and pET23cZmGGPPS3 and used to test the function. These plasmids together with empty pET23a vector were transformed into *E. coli* BL21 (DE3) cells containing pACCAR25ΔcrtE. Double-transformants were grown in LB medium containing ampicillin and chloramphenicol and selected for their capacity to accumulate the yellow pigment zeaxanthin-β-D-glucoside. Pigments were extracted and analyzed on HPLC (Gallagher et al., 2004). The sense and anti-sense (5'- 3') primers used to construct the respective plasmids are as follows: GGPPS1-GAATTCGATAAGGAGGCCGTAACCTGTC (1000), AAGCTTGCCTGACAAAGTCACAACAGG (1001); GGPPS2 GAATTCATGGCGTTTCACTTCCACCCGCTGGTC (1005), AAGCTTAGTGAGCAAGAACAGAATAAG (1004); GGPPS3 – GAATTCCCATGAACAAGTTGGCATCCTGCT (1565), AAGCTTTCAATGCTGTCTGTGCGCCATGA (1474).

2.6.4 Statistical analyses

Pearson correlation analysis of transcript and carotenoid composition from different maize inbreds was performed using JMP v. 5.1.2 (SAS Institute, Cary, NC) to test the statistical significance ($p \leq 0.05$) of the relationship.

Table 2-1. Correlation analysis between transcripts and carotenoid content of mature seed. Pearson correlation (r) and statistical significance (p), for comparison of transcript levels and kernel carotenoid content in maize inbred lines performed using JMP v. 5.1.2 (SAS Institute, Cary, NC) to test the statistical significance ($p \leq 0.05$) of the relationship. The black letters have no effect, white letters on blue and red background showed positive correlation and negative correlation respectively. Inbred lines are as listed in Fig.6. L, lutein; Z, zeaxanthin (a=Vallabhaneni and Wurtzel, this paper; b= MaizeDatabase, www.maizegdb.org; c= (Singh et al., 2003))

A

Correlation of mRNA levels to total carotenoid content			
GENE	Chromosome Bin	CONTENT r (p) values	
		20 DAP	25 DAP
<i>DXS1</i>	6.05 ^b	-0.2 (0.5)	0.07 (0.84)
<i>DXS2</i>	9.03 ^b	0.45 (0.21)	0.34 (0.36)
<i>DXS3</i>	4.06 ^a	0.20 (0.5)	0.75 (0.01)
<i>DXR</i>	3.04 ^b	0.41 (0.23)	0.61 (0.05)
<i>HDS</i>	5.03 ^a	-0.43 (0.36)	-0.12 (0.73)
<i>HDR</i>	1.09 ^a	-0.17 (0.62)	0.64 (0.04)
<i>IPPI1</i>	7.03 ^a	-0.08 (0.81)	0.27 (0.43)
<i>IPPI2</i>	8.03 ^a	0.21 (0.54)	0.16 (0.66)
<i>IPPI3</i>	6.05 ^a	-0.12 (0.73)	-0.2 (0.49)
<i>GGPPS1</i>	2.08 ^a	0.52 (0.11)	0.69 (0.02)
<i>GGPPS2</i>	7.04 ^a	-0.27 (0.47)	-0.10 (0.76)
<i>GGPPS3</i>	8.01 ^a	NA	NA
<i>PSY1</i>	6.01 ^b	0.96 (0.001)	0.37 (0.28)
<i>PSY2</i>	8.07 ^b	-0.04 (0.89)	0.50 (0.13)
<i>PSY3</i>	7.03 ^b	0.03 (0.93)	0.59 (0.07)
<i>PDS</i>	1.02 ^b	0.12 (0.73)	-0.22 (0.53)
<i>ZDS</i>	7.02 ^b	-0.01 (0.95)	-0.103 (0.77)
<i>CrtISO1</i>	4.08 ^a	-0.76 (0.001)	-0.29 (0.57)
<i>CrtISO2</i> (pseudogene)	2.09 ^a	NA	NA
<i>LCYB</i>	5.04 ^c	0.19 (0.58)	0.60 (0.06)
<i>LCYE</i>	8.05 ^b	0.18 (0.61)	-0.31 (0.37)

B

Correlation of mRNA levels to carotenoid composition					
GENE	Chromosome Bin	20 DAP		25 DAP	
		L	Z	L	Z
<i>ZEP1</i>	2.04 ^a	0.06 (0.84)	-0.77 (0.008)	-0.19 (0.59)	-0.35 (0.31)
<i>ZEP2</i>	10.04 ^a	-0.14 (0.68)	-0.86 (0.001)	-0.33 (0.35)	-0.42 (0.22)

Table 2-2. Gene family sizes in three species of the Poaceae.

Enzymes highlighted in blue or red are encoded by a maize gene family member showing positive or negative correlation, respectively, between transcripts and total carotenoid content in maize endosperm. Accession numbers are obtained as stated: *Zea mays*, GenBank; *Oryza sativa*, Gramene; *Sorghum bicolor*, Phytozome

Enzyme	Gene family size		
	<i>Zea mays</i> GenBank ID	<i>Oryza sativa</i> Gramene ID	<i>Sorghum bicolor</i> Phytozome ID
DXS1	AY946271	LOC_Os05g33840	Sb09g020140
DXS2	AY946270	LOC_Os07g09190	Sb02g005380
DXS3	DR967452	LOC_Os06g05100	Sb10g002960
DXR	AJ297566	LOC_Os01g01710	Sb03g008650
HDS	AY562489	LOC_Os02g39160	Sb04g025290
HDR	DR789385	LOC_OS03g52170	Sb01g009140
IPPI1	AF330034	LOC_Os07g36190	Sb02g035700
IPPI2	DN215017	LOC_Os05g34180	Sb09g020370
IPPI3	CO530856	NA	NA
GGPPS1	EF417573	LOC_Os07g39270	Sb02g037510
GGPPS2	EF417574	NA	NA
GGPPS3	EF417575	LOC_Os01g14630	Sb03g009380
PSY1	AY324431	LOC_Os06g51290	Sb10g031020
PSY2	AY325302	LOC_Os12g43130	Sb08g022310
PSY3	DQ372936	LOC_Os09g38320	Sb02g032370
PDS	L39266	LOC_Os03g08570	Sb06g030030
ZDS	AF047490	LOC_Os07g10490	Sb02g006100
CrtISO1	DR812825	LOC_Os11g36440	Sb05g022240
CrtISO2	AC183901	NA	NA
LCYB	AY206862	LOC_Os02g09750	Sb04g006120
LCYE	EB674039	LOC_Os01g39960	Sb03g026020
ZEP1	DR820114	LOC_Os04g37619	Sb06g018220
ZEP2	CO532283	NA	NA

Table 2-3. Primers used in the study

Gene	Primer No.	Sequence	Gen Bank Acc #
DXS1	1206	CAGAACAGGGAGGCTCTTG	AY946271
	1207	GCGACACTTTCATCCAAAGA	
DXS2	1212	GCTGAACTACTTCCAGAAGCG	AY946270
	1213	CTGCAGGAACGACGAGTAGA	
DXS3	1194	CTGTGCCAGCAGCATAGTTT	DR967452
	1195	TTCGACCGTCAAGTAGACCA	
DXR	1164	TCCAATGTCACGCTTCTAGC	AJ297566
	1165	TGGCGAGCAACTTCTATGAC	
HDS	1176	CAGTCCAGGGTAGGAAGGAA	AY562489
	1177	TAGGGCTACCGTAGGAGCAA	
HDR	1218	GCCCAACTCGTTACATCCTT	DR789385
	1219	TGAGATCAAGCGTCAGGAAA	
IPPI1	1159A	TGAGCTTATCCAGGAGAACTA	AF330034
	1163A	ATGGTGCCTTGTAAGCATT	
IPPI2	1333	TCAGGCTGGTGGTGGACAACCTT	DN215017
	1335	TGATGGCTAAAACAAAGGCA	
IPPI3	1333	TCAGGCTGGTGGTGGACAACCTT	CO530856
	1334	AGCACCAAACAGCAGGACAA	
GGPPS1	1475	CGGAGGAGTTGCTCTCTGAT	EF417573
	1476	AGTCACAACAGGTCCGACAA	
GGPPS2	1477	CAGAGAAGTTGGTCTCTGAC	EF417574
	1478	AGATCGACAGTGAGCAAGAA	
GGPPS3	1426	ATCCATGTGCACAAGACTGC	EF417575
	1427	CACGTCCAGAACGTCGTC	
PSY1	505	CATCTTCAAAGGGGTCGTCA	AY324431
	254	CAGGATCTGCCTGTACAACA	
PSY2	503	TCACCCATCTCGACTCTGCTA	AY325302
	676	GATGTGATCTACGGATGGTTCAT	
PSY3	996	GCCAGAGCCTTCTTCAGGCAGG	DQ372936
	997	GTCTTCGGAACGTAGGCCCTC	
PDS	933	GAAATCATCGATGCAACTATGGAA	L39266
	934	CTTCGATAGGTGACCTTTGGA	
ZDS	281	GTGTGGTAAAGATCGGACAA	AF047490
	917	AGAGAGTTGCTCCTTCCAT	
CrtISO1	1325	GGTTGTCAAATGGGAAGGAG	DR812825
	1326	GAATGATGGTGCCTTGACAT	
LCYB	1095	CATCGTAAGGTTCTCGACA	AY206862
	1096	ATGCCGAAGCAGAAGAATC	
LCYE	1037	TTT ACG TGC AAA TGC AGT CAA	EB674039
	1038	TGA CTC TGA AGC TAG AGA AAG	
ZEP1	1664	TTGGTGGCAACAGCTCAAAA	DR820114
	1665	CCTTCACTTGTTGCGATGAG	
ZEP2	1400	TTGGTGGCAATAGCTCAAAG	CO532283
	1401	GCTTCACTTGTTGCGAAGAG	
Actin	1134	CGATTGAGCATGGCATTGTCA	J01238
	1135	CCCACTAGCGTACAACGAA	

Table 2-4. Transcript from levels in developing endosperm from diverse maize lines.

Transcripts were measured quantitatively endosperm collected at the indicated DAP.

Parentheses show SD, standard deviation. Quantitative RT-PCR was performed using gene specific primers (**Table 2-3**) and normalized to actin, as previously described (Li et al., 2008). Values are expressed as the mean of three RT-PCR replicates +/- standard deviation.

Relative transcript levels of DXS3, DXR, HDR, GGPPS1, PSY1, CrtISO1 in diverse maize lines						
20 DAP						
inbred	DXS3	DXR	HDR	GGPPS1	PSY1	CrtISO1
A619	1.37 (0.12)	7.02 (0.97)	2.89 (0.42)	6.52 (1.26)	2.69 (0.52)	0.86 (0.01)
B73	2.03 (0.21)	11.14 (3.86)	4.89 (0.64)	2.73 (0.83)	1.53 (0.05)	1.88 (0.08)
B37	3.59 (0.44)	7.47 (0.85)	3.17 (0.86)	12.05 (1.05)	3.74 (0.52)	0.7 (0.03)
CI7	1.80 (0.60)	3.32 (0.93)	3.58 (0.37)	6.98 (2.00)	1.71 (0.11)	0.41 (0.08)
C131A	5.14 (0.34)	3.21 (0.35)	2.47 (0.69)	0.81 (0.12)	2.15 (0.49)	2.8 (0.35)
DE3	4.16 (0.19)	8.23 (0.46)	4.26 (0.65)	2.18 (0.48)	3.04 (0.06)	0.42 (0.04)
KUI2007	4.80 (0.14)	20.93 (4.59)	3.39 (0.69)	4.86 (0.43)	3.65 (0.17)	0.21 (0.02)
NC300	2.55 (0.53)	4.26 (0.59)	2.9 (0.94)	2.51 (0.52)	1.01 (0.31)	2.08 (0.54)
SD44	6.43 (0.38)	10.96 (2.53)	4.36 (0.63)	5.15 (0.40)	1.98 (0.35)	1.77 (0.30)
TZI18	4.51 (0.18)	3.56 (0.37)	2.84 (0.43)	4.38 (0.52)	4.47 (0.96)	0.25 (0.05)
25 DAP						
inbred	DXS3	DXR	HDR	GGPPS1	PSY1	CrtISO1
A619	6.56 (0.21)	19.43 (0.95)	4.42 (0.72)	2.69 (0.46)	9.88 (1.76)	1.02 (0.34)
B73	4.79 (0.19)	14.87 (0.88)	6.84 (0.19)	5.76 (0.41)	1.15 (0.25)	3.87 (0.88)
B37	5.43 (0.66)	7.57 (1.33)	13.11 (1.81)	7.55 (1.08)	16.21 (0.29)	1.01 (0.29)
CI7	2.73 (0.21)	4.58 (0.46)	10.62 (1.88)	6.81 (1.40)	1.52 (0.42)	0.93 (0.27)
C131A	4.51 (0.28)	3.49 (0.11)	4.52 (0.14)	1.32 (0.35)	0.74 (0.23)	5.26 (0.06)
DE3	4.80 (0.19)	16.84 (0.88)	5.81 (1.29)	6.86 (0.97)	2.03 (0.74)	1.55 (0.01)
KUI2007	7.80 (0.11)	27.68 (2.66)	10.16 (1.48)	11.48 (0.40)	1.20 (0.24)	3.75 (0.29)
NC300	2.60 (0.50)	5.69 (0.38)	4.7 (0.02)	2.42 (0.40)	0.94 (0.19)	2.17 (0.12)
SD44	2.16 (0.11)	7.30 (0.11)	6.52 (1.18)	3.37 (0.50)	2.37 (0.46)	1.46 (0.12)
TZI18	11.72 (0.48)	24.43 (3.30)	8.11 (1.13)	5.69 (0.10)	3.98 (0.40)	0.59 (0.13)

Table 2-5. Transcript levels of *ZEP* genes in developing endosperm from diverse maize lines.

Transcripts were measured quantitatively from endosperm collected at the indicated DAP. Parentheses show SD, standard deviation. Quantitative RT-PCR was performed using gene specific primers (**Table 2-3**) and normalized to actin, as previously described (Li et al., 2008). Values are expressed as the mean of three RT-PCR replicates +/- standard deviation

	Relative transcript levels of <i>ZEP1</i> and <i>ZEP2</i> (SD)			
	20 DAP		25 DAP	
	<i>ZEP 1</i>	<i>ZEP2</i>	<i>ZEP1</i>	<i>ZEP2</i>
A619	0.67 (0.10)	1.30 (0.29)	0.32 (0.01)	0.53 (0.07)
B73	0.40 (0.03)	1.45 (0.24)	0.09 (0.03)	0.94 (0.22)
B37	0.51 (0.21)	0.88 (0.22)	0.22 (0.03)	0.27 (0.01)
CI7	0.75 (0.00)	1.12 (0.01)	0.09 (0.02)	0.18 (0.05)
C131A	0.94 (0.37)	1.06 (0.37)	0.46 (0.05)	0.89 (0.09)
DE3	0.40 (0.06)	0.96 (0.17)	0.02 (0.01)	0.62 (0.00)
KUI2007	0.04 (0.02)	0.05 (0.00)	0.14 (0.00)	0.56 (0.04)
NC300	0.66 (0.26)	1.41 (0.21)	0.84 (0.05)	1.11 (0.07)
SD44	0.63 (0.16)	1.15 (0.14)	0.79 (0.19)	0.97 (0.25)
TZI18	0.34 (0.08)	0.41(0.13)	0.07 (0.01)	0.16 (0.02)

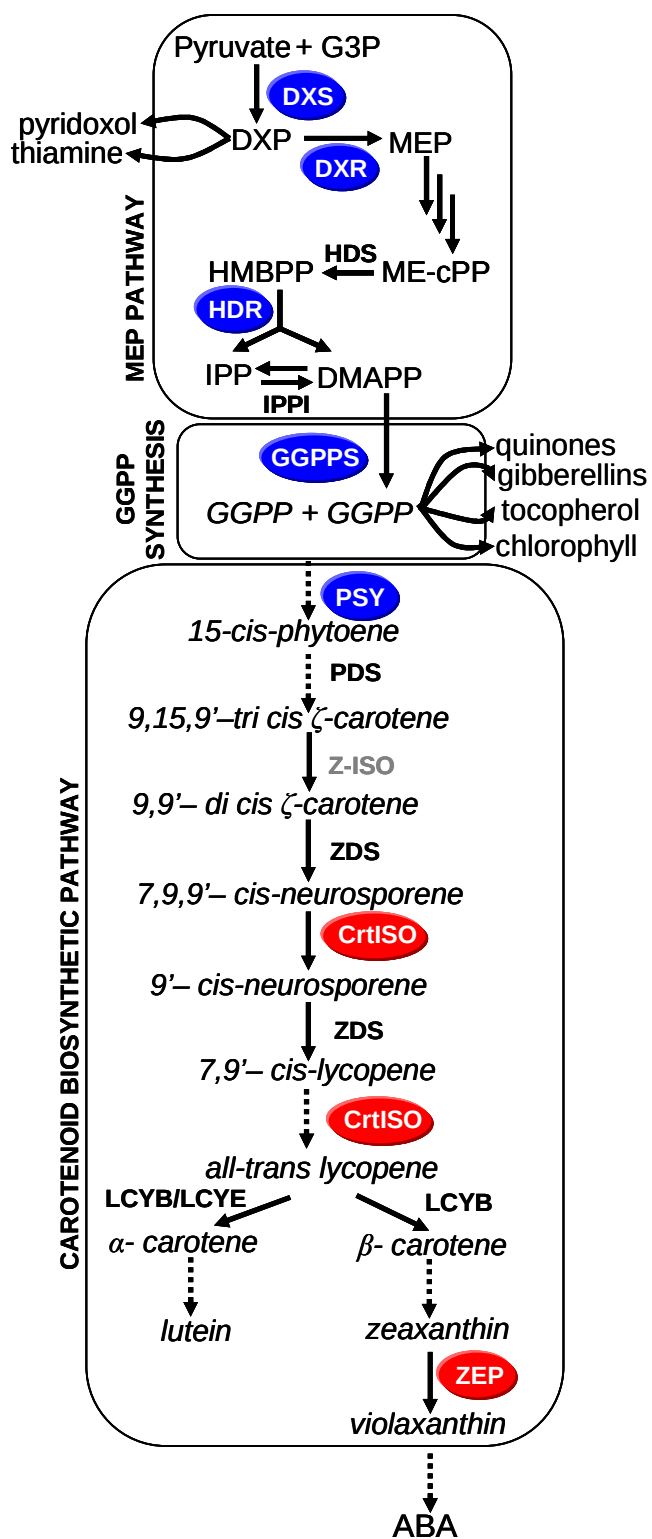


Figure 2-1. Simplified carotenoid biosynthetic pathway derived from isoprenoid precursors

Enzymatic reactions are represented by arrows. Genes encoding all enzymes shown, except ZISO, were tested for correlation with total accumulated carotenoids. Transcripts for enzymes in black letters had no effect; white letters on blue bubble, white letters on red bubble showed positive correlation and negative correlation respectively. DXS, D-1-deoxyxylulose 5-phosphate synthase; DXR, DXP reductoisomerase; HDS, 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; IPPI, Isopentenyl pyrophosphate isomerase; GGPPS, Geranylgeranyl pyrophosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, 15-*cis* zetacarotene isomerase; ZDS, zetacarotene desaturase; CrtISO, carotene isomerase; LCYB, lycopene beta cyclase; LCYE, lycopene epsilon cyclase; ZEP, zeaxanthin epoxidase; ABA, Absciscic acid.

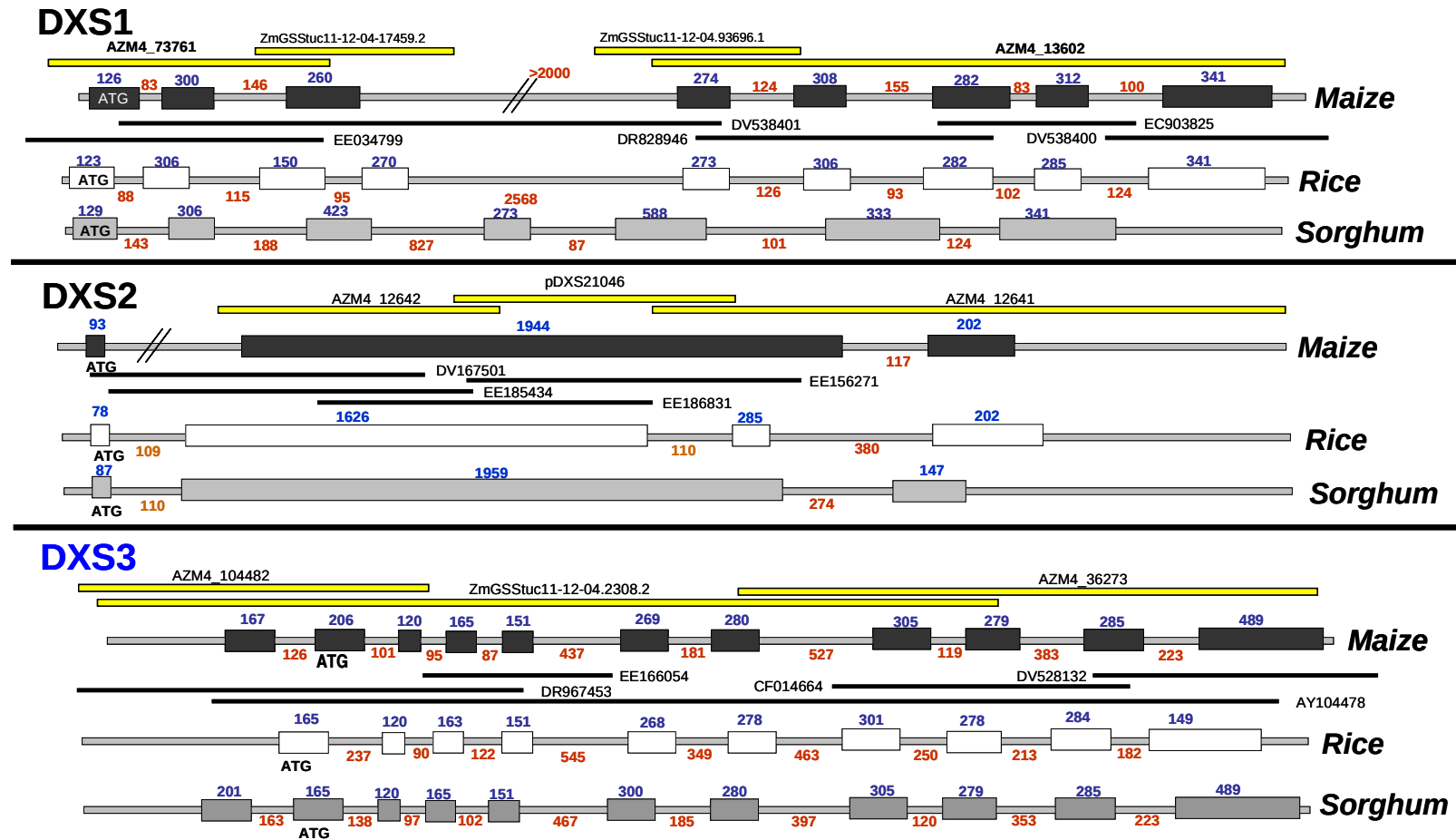
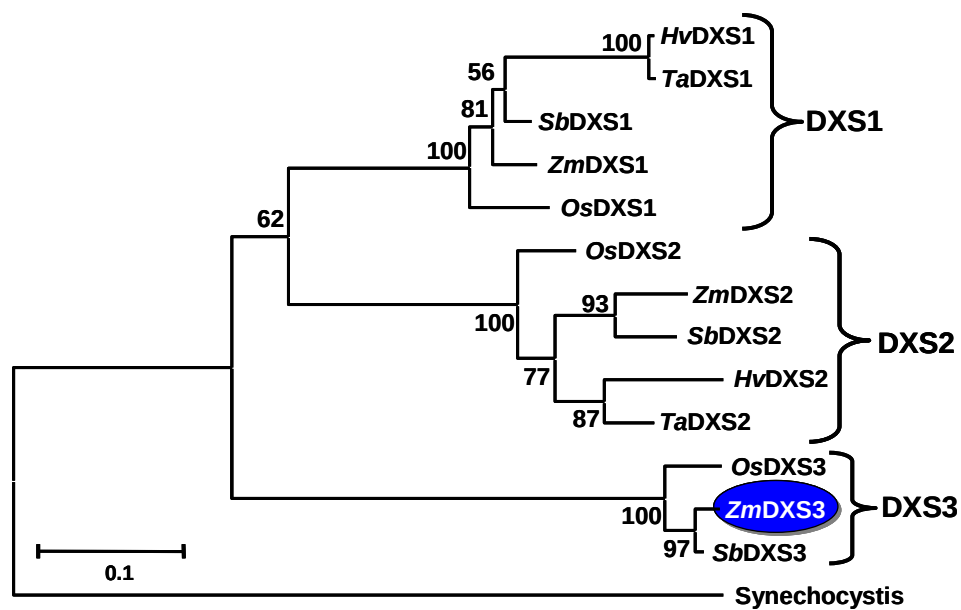


Figure 2-2. Gene family structure of DXS genes from the *Poaceae*.

Zea mays (Zm); *Oryza sativa* (Os); *Sorghum bicolor* (Sb) Boxes and lines indicate exons and introns, respectively; sizes are in base pairs. Bars above maize gene structures represent the genomic counterparts used to build the gene structure; dark bars below maize genes structure represent ESTs covering the entire coding region.

A



B

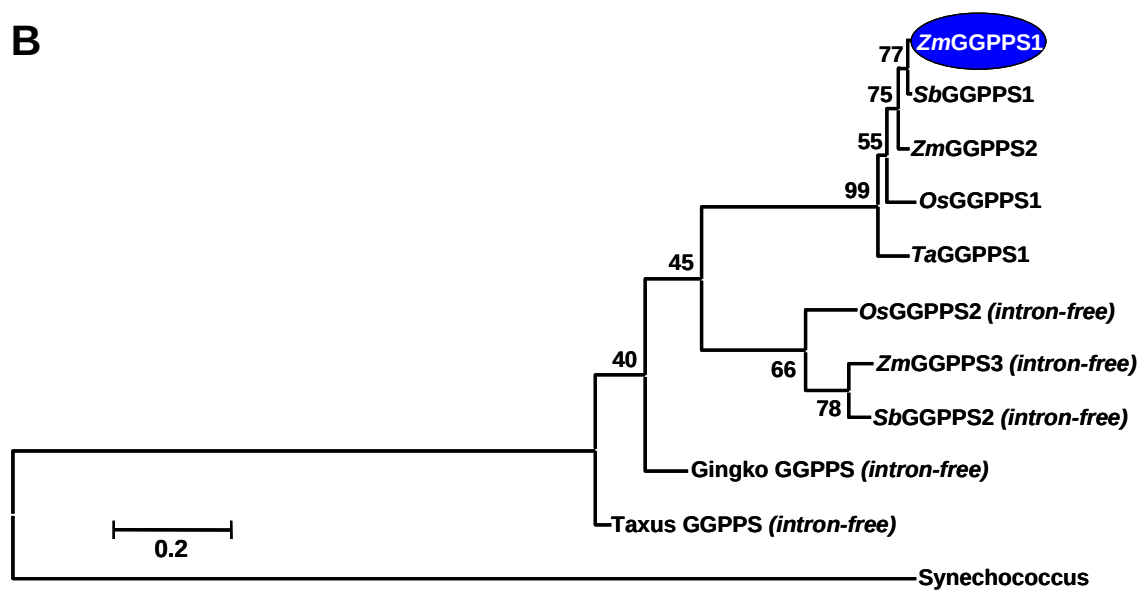


Figure 2-3. Phylogenetic analysis based on translation products.

(A) DXS gene family. GenBank accessions are in parentheses: *Synechocystis* (sll1945 BAA17089.1), *Zea mays* (ZmDXS1, TC312721; ZmDXS2, TC293862; ZmDXS3, TC307233), *Oryza sativa* (OsDXS1, LOC_Os05g33840; OsDXS2, LOC_Os07g09190; OsDXS3, LOC_Os06g05100), *Sorghum bicolor* (SbDXS1, Sb09g020140; SbDXS2, Sb02g005380; SbDXS3, Sb10g002960); *Hordeum vulgare* (HvDXS1, TC149768; HvDXS2, TC152494); *Triticum aestivum* (TaDXS1, TC238788; TaDXS2, TC258088)

(B) GGPPS gene family.. GenBank accessions are in parenthesis: *Synechococcus* (crtE, ABI45773); *Zea mays* (ZmGGPPS1, EF417573; ZmGGPPS2, EF417574; ZmGGPPS3, EF417575); *Oryza sativa* (OsGGPPS1, LOC_Os07g39270; OsGGPPS2, LOC_Os01g14630); *Sorghum bicolor* (SbGGPPS1, Sb02g037510, SbGGPPS2, Sb03g009380); *Ginkgo biloba* (AY371321); *Taxus media* (AY566309). Maize enzymes are highlighted in blue if transcripts correlated with carotenoid content.

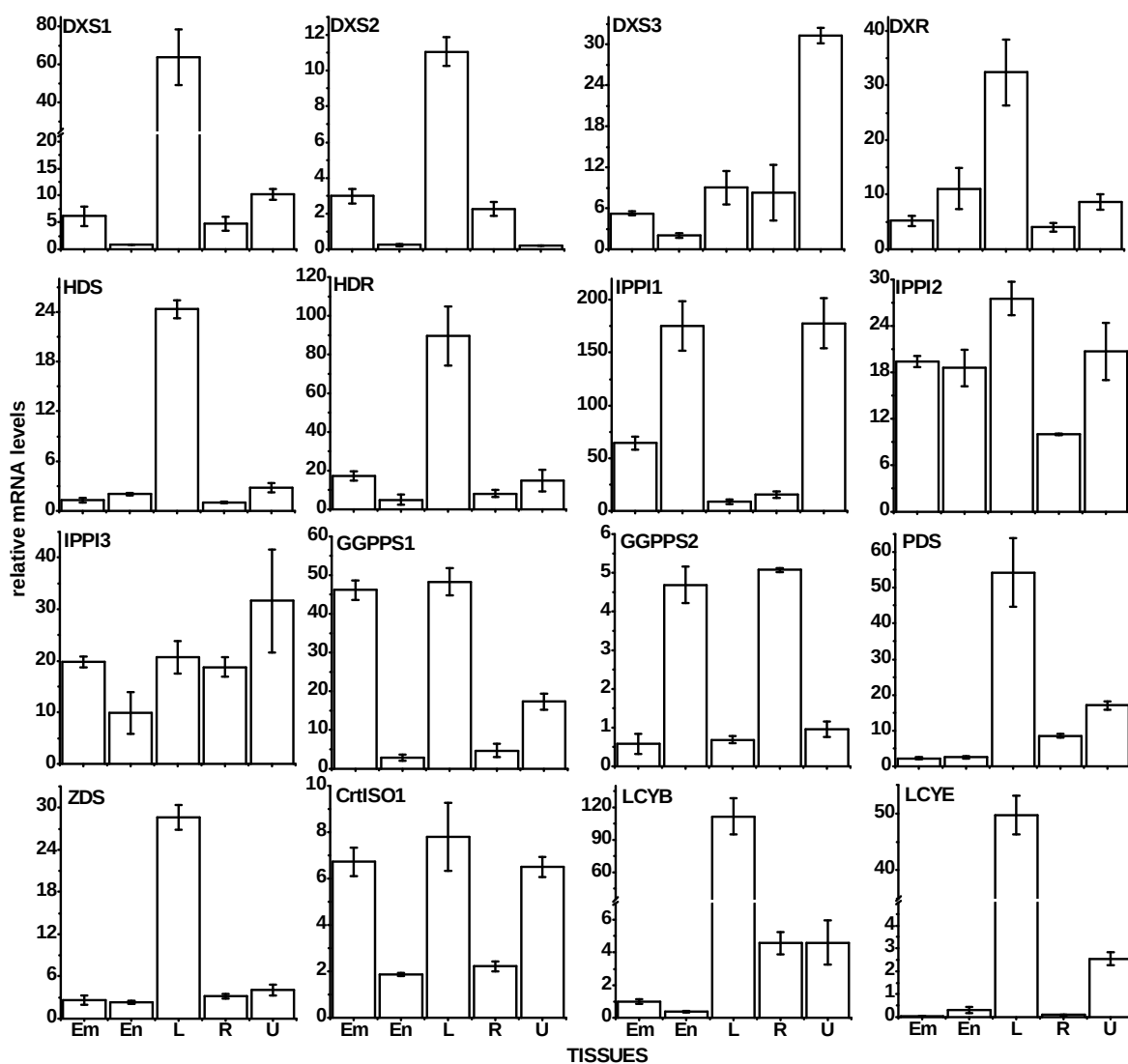


Figure 2-4. Maize B73 inbred transcript profiles of genes encoding enzymes for isoprenoid and carotenoid biosynthesis.

Transcript levels were expressed relative to endosperm levels at 20 DAP. Mean of three replicates $\pm$ standard deviation.. Embryo (Em) and endosperm (En) collected at 20 DAP from field grown plants; leaf (L) and root (R) samples collected from seedlings at the six-leaf stage and unfertilized ovule (U).

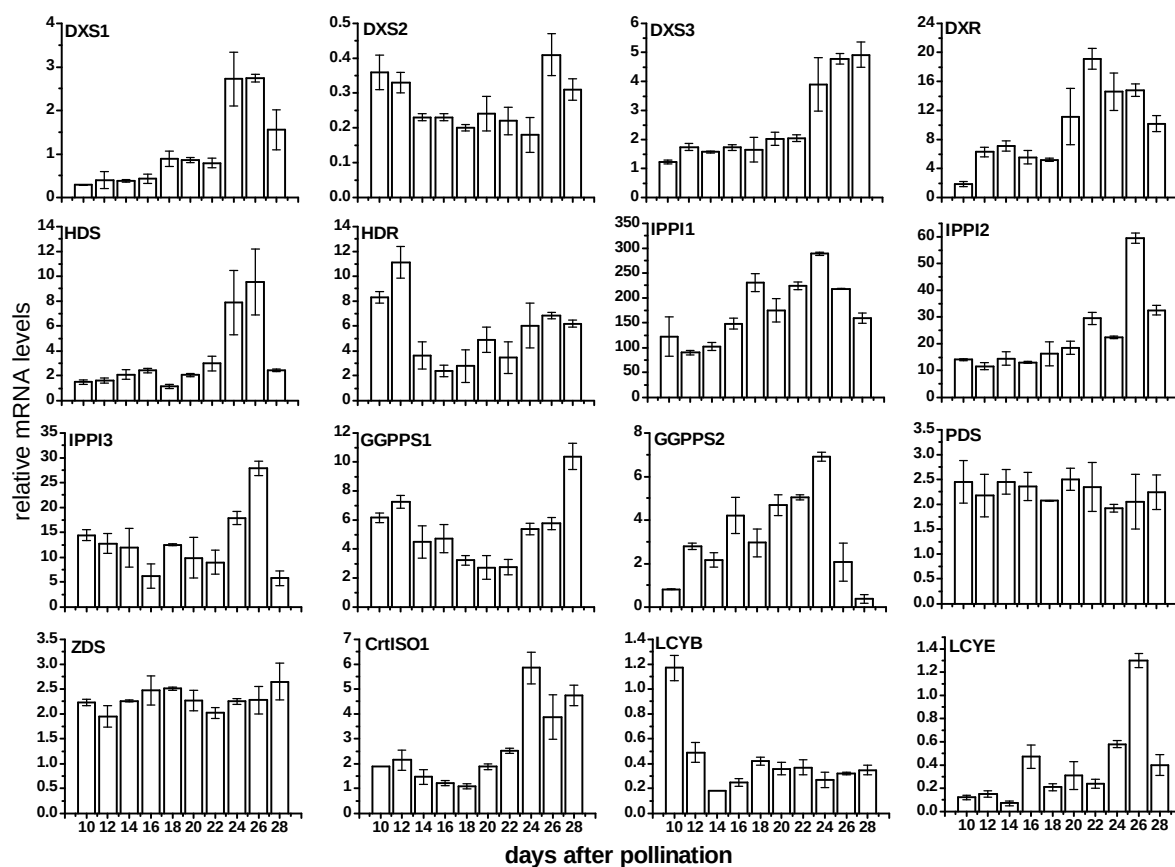


Figure 2-5. Maize B73 inbred developing endosperm transcript profiles of genes encoding enzymes for isoprenoid and carotenoid biosynthesis. Mean of three replicates +/- standard deviation.

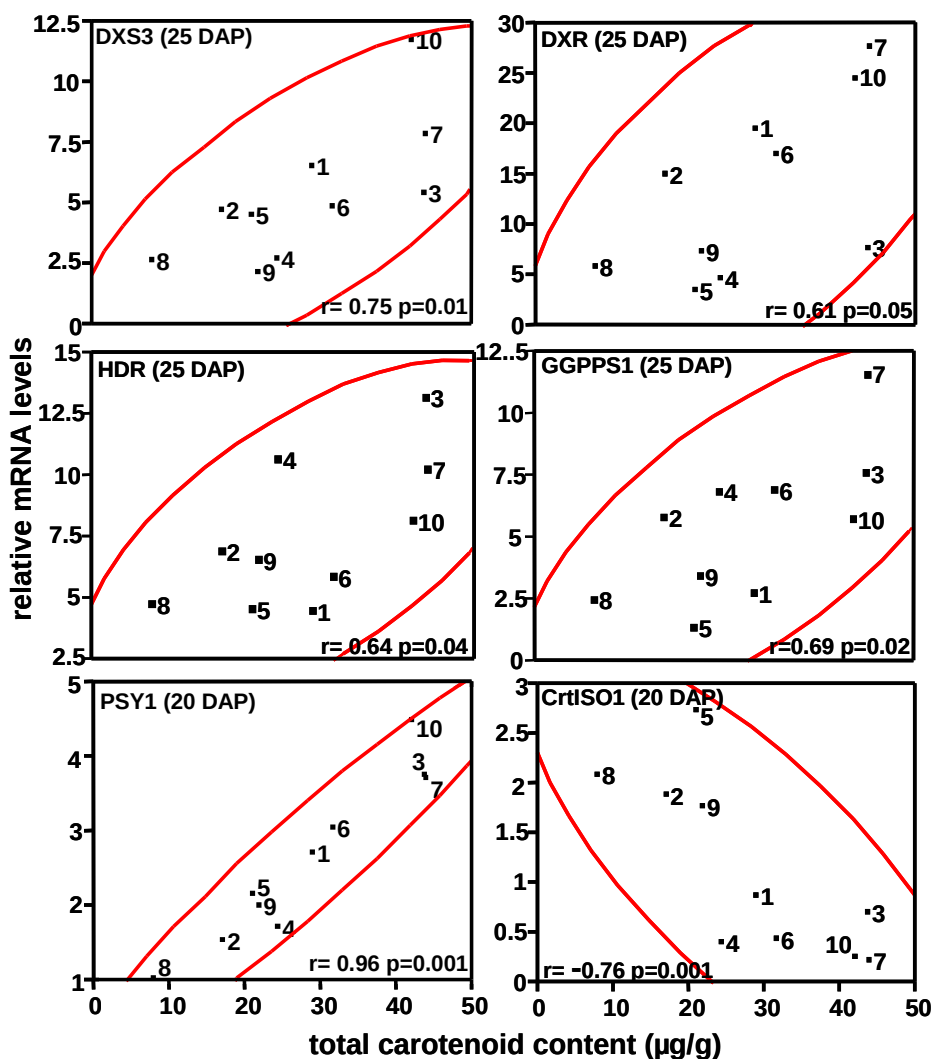


Figure 2-6. Correlation analysis showing endosperm carotenoid content positively or negatively correlates with mRNA levels of specific pathway enzymes.

Pearson correlation (r) and statistical significance (p) for comparison of transcript levels and kernel carotenoid content in maize inbred lines was performed using JMP v. 5.1.2 (SAS Institute, Cary, NC) to test the statistical significance ($p \leq 0.05$) of the relationship at a 95% confidence interval (red line) of the bivariate normal distribution. Inbred lines are: 1, A619; 2, B73; 3, B37; 4, CI.7; 5, C131A; 6, DE3; 7, KUI2007; 8, NC300; 9, SD44; 10, TZI18. PSY1 data were taken from (Li et al., 2008a).

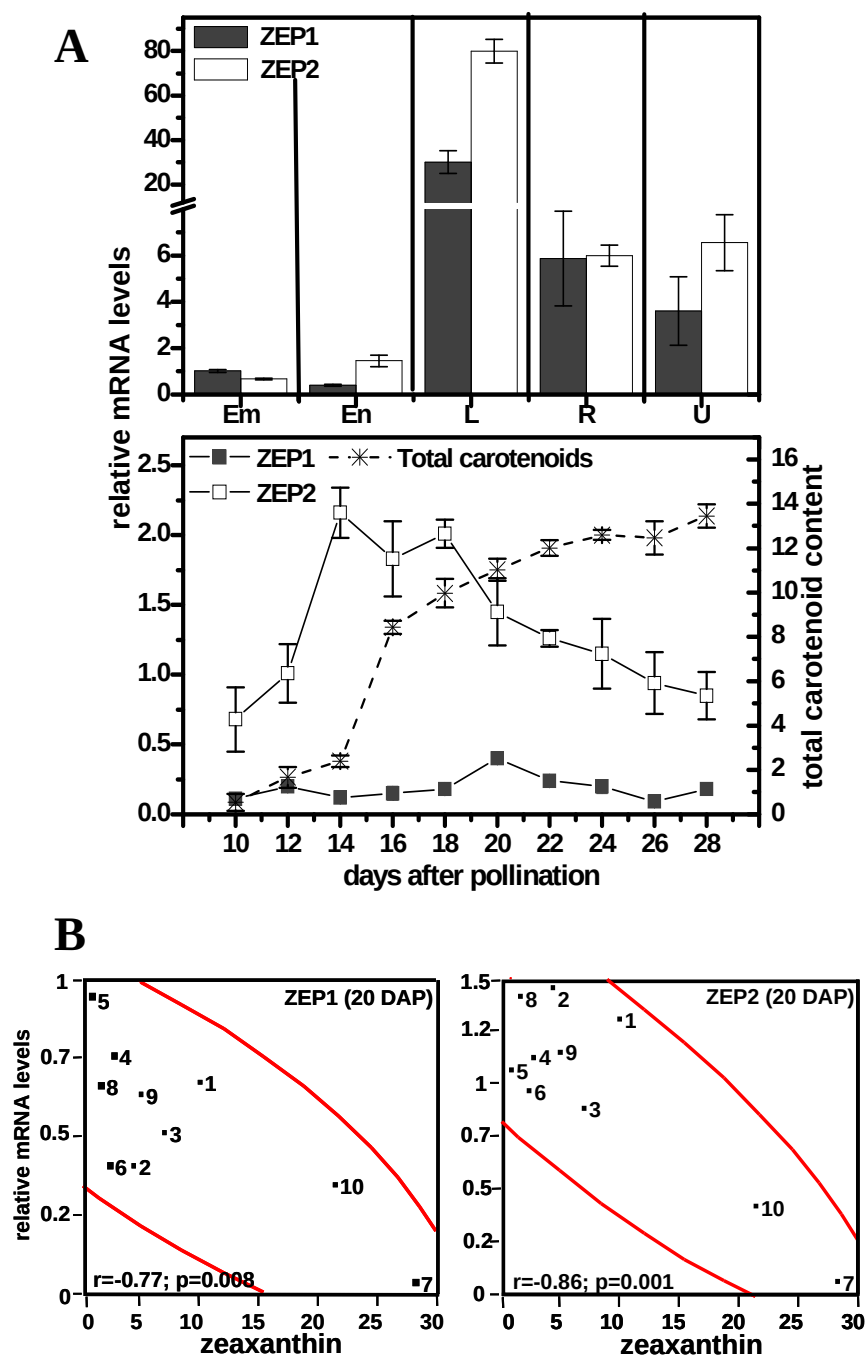


Figure 2-7. Transcript profiling of zeaxanthin epoxidase gene family members.

A) top, tissue panel (see Fig. 4); bottom, developing endosperm (see Fig. 5). Transcript levels were expressed relative to endosperm levels at 20 DAP B) Pearson correlation (r) and statistical significance (p) for comparison of transcript levels and kernel carotenoid content in maize inbred lines (see Fig. 6). Mean of three replicates $\pm$ standard deviation.



```
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SbDXS1 : DRPSCFRYPKRGIGVPLPPNYKGTPLLEVKGGRVLLGG-DRVALLGYGSVAVQCLAAASLVERHGLKVTYADARFCKPLDQTLIRRLASSHEVLLTVEEGSIGGFGSHVAQFMAALDGLLDGKIKWRPVLVLDPRYIDIGSP
ZmDXS1 : DRPSCFRYPKRGIGVPLPPNYKGTPLLEVKGGRVLLGG-DRVALLGYGSVAVQCLTAAASLVERHGLKVTYADARFCKPLDHALIRSLAKSHEVLLTVEEGSIGGFGSHVAQFMAALDGLLDGKIKWRPVLVLDPRYIDIGSP

0sDXS3 : TEQLDMDAGLTAHIAAATALLGRHRDALLMK----(722)
SbDXS3 : AEQLDLAAGLTAHIAAATALLGRHRDALLMK----(722)
ZmDXS3 : AEQLDLAAGLTAHIAAATALLGRHRDALLMK----(722)
0sDXS2 : VDQLEEAAGLTPRHIAATVLSLLGRPLEAQLS----(713)
SbDXS2 : QDQMEEAAGLTPRHIAATVLSLLGRPMEAAQLK----(727)
ZmDXS2 : QDQIEEAAGLTPRHIAATVLSLLGRPLEAQLK----(723)
0sDXS1 : ADQLAEAGLTPSHIAATVFNVLGQAREALAIMTPVNA(720)
SbDXS1 : ADQLAEAGLTPSHIAATVFNVLGQAREALAIMVAVNA(722)
ZmDXS1 : ADQLAEAGLTPSHIAATVFNVLGQAREALAIMVAVNA(719)
```

Figure 2-8. Multiple-alignment of DXS proteins.

Amino acid sequences across available grass species (*Zea mays*, *Zm*; *Oryza sativa*, *Os*; *Sorghum bicolor*, *Sb*) were used to show the sequence signatures of transketolase and DXS enzymes (Rodriguez-Concepcion et al., 2003). These include thiamine diphosphate binding domain (underlined red) and two transketolase motifs (underlined blue), with HIS residue for activity (blue triangle). Total number of aminoacids were marked at the end of each sequence in blue. The variant N-terminal amino acid sequence includes the transit peptide region. For sequence accessions, see **Table 2-2**.

```

ZmGGPPS1 : ...MAMSFHFHPLAASRVHFSPLPFAAAAGAPSPSSVAIAAHQHG.RRRRESAIVATAAAASAATTEFDFKAYMGERAV
ZmGGPPS2 : .....MAFHFHPLVASRVHFSPLPFAAATGAPSPSSVAIAAHYHHGRRRESAIVATAT.....TTEFNFKAYMEERAV
ZmGGPPS3 : MNKLASCFLOHGAPHTQIFKSYHVQRSPSLQLLENRSVSMTRHRAADRAARGTITVDVAVDSG....TSFDFESYLSAKAR

ZmGGPPS1 : AVNRALDAAVPAGEPPAALHDMARYALLAGGKVRPALCLAACAVVGGPEPWAMPAAAAVEMVHTMSLVHDDLPCMDDDD
ZmGGPPS2 : AVNRALDAATPAGGLPAALHDMARYALLAGGKVRPALCLAACAVVGGPESWAMPAAAAVEMVHTMSLVHDDLPCMDDDD
ZmGGPPS3 : AVHDALDLTLQGLRCPVLSSEMRYSVLAGGKRLRPLAIAACELVGGTAAAAPVACAVEMIHTASLIHDDMPCMDDDA

ZmGGPPS1 : LRRGKPTCHVYGEPIAVLAGDALLSLSFHHMASVGSYPDVPDEKHPARVVRIGELARCIGSEGLVAGQVVDLEMTGT
ZmGGPPS2 : LRRGKPTCHVYGEPIAVLAGDALLSLSFHHMASVGSYPDVPDEKHPARVVRIGELARCIGSEGLVAGQVVDLEMTGT
ZmGGPPS3 : LRRGRPSNHVAFGEPTALLAGDALLALAFEHVARG.....SAGAGVPADRRLRAVVELGSVAGVGGIAAGQVADWASEGA

ZmGGPPS1 : .SETVPLERLEYIHLHKTAAALLEASVVTGAIIGGGTDEQIERLRKYARSIGLLFQVDDILDVTKSSEELGKTAGKDLAS
ZmGGPPS2 : .SQTVPLELLEYIHLHKTAAALLEASVVTGAIIGGGTDEQIEALRKYARSIGLLFQVDDILDVTKSSEELGKTAGKDLAS
ZmGGPPS3 : PSGSVSLGLEYIHVHKTARLVEAAAVSGAVVGGGGDGEVERVRRYAHFLGLLGQVDDVDLDVTGTSEQLGKTAGKDVAA

ZmGGPPS1 : DKTTYPKLLGLLEKSREFAEELLSDAVEQLACFDKEKAAPLHLANYIAHRON
ZmGGPPS2 : DKTTYPKLLGLLAKSREFAEKLVSDATEQLACFDEEKAAPLHLANYIAHRON
ZmGGPPS3 : GKATYPRLMGLKGARAYMGELLAKAEALDGLDAARTAPLRHLARFMAHRQH

```

(I) (II) (III) (IV) (V) (VI) (VII)

Figure 2-9. GGPPS proteins of maize.

Blue lines indicate seven conserved domains among GGPPS gene family proteins that are highly conserved among prenyl diphosphate synthases with two Asp-rich motifs for substrate binding (domains II and VI) (Okada et al., 2000). Accession numbers: ZmGGPPS1, EF417573; ZmGGPPS2, EF417574; ZmGGPPS3, EF417575.

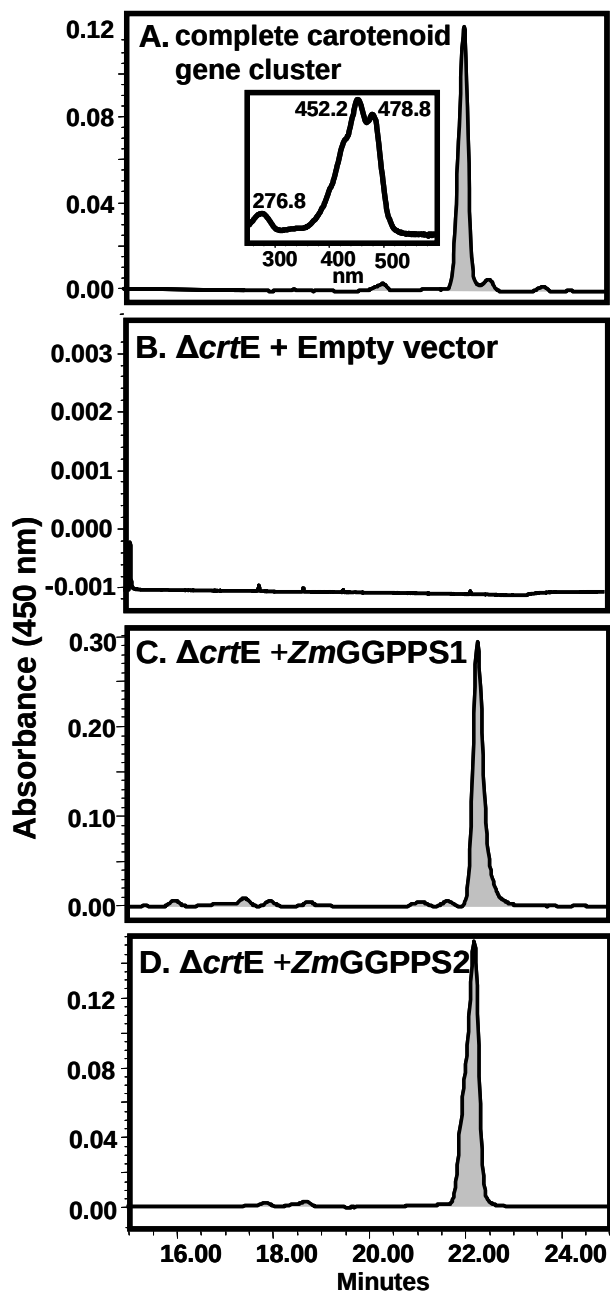


Figure 2-10. Functional complementation of GGPPS1 and GGPPS2 from maize.

E. coli cells were transformed with A, pACCAR25 (for zeaxanthin accumulation); B, pACCAR25 $\Delta crtE$ (missing bacterial GGPPS)+ pET23a (empty vector); C, pACCAR25 $\Delta crtE$ + maize GGPPS1; D, pACCAR25 $\Delta crtE$ + maize GGPPS2. Chromatograms show HPLC separation of extracted pigments; inset in A shows spectral fine structure for the pathway end product, zeaxanthin diglucoside.

```

AtCrtISO : MDLCFQNPVKCGDRLFSALNTSTYYKLGTSNLFNGPVLNRRKKKKKLPIMVTYKSVSSSVASTVQGTQRDGG---ESL
OsCrtISO : ----MPLLLAAARPCAPLLAPSAAGAVVGRSTIARPLGRSTRGAQAAAAAVAAEKTVMKAEEEEGG-----GE
SbCrtISO : -----MPPLAARVHAPILLAP---VAP-HPATIRLCASSCPRGVCGFRRGALASEKPPVAVAAEKAGGEVGGGEGGP
ZmCrtISO1 : -----MPPLAARLHAPILLVP---AASPRPATIRLCAASCPRG-VCGFRRGALASEKPPVAVAAEKAGGEGGGQGEPP
ZmCrtISO2 : -----MPPLAARLYAPLLNLAAPVPAASLRPATIRLCAASCPRN-VCGFRRGALA*EKAAAVAAEKAGGEGGGGEDEGP

AtCrtISO : YDAIVIGSGIGGLVAATQLAKKEARVLVLEKYLIPGGSSGFYERDGYTFDVGSSVMFGFSDKGNLNLITQALKAVGKKME
OsCrtISO : YDAIVVSGIGGMVAATQLAAKGARVLVLEKYVIPGGSSGYRRDGFDFDVGSSVMFGFSDKGNLNLITQALEAVGKKME
SbCrtISO : YDAIVIGSGIGGLVAATQLAAKGARVLVLEKYLIPGGSSGYRRDGFDFDVGSSVMFGFSDKGNLNLITQALEAVGKKME
ZmCrtISO1 : YDAIVIGSGIGGLVAATQLAAKGARVLVLEKYLIPGGSSGYRRDGFDFDVGSSVMFGFSDKGNLNLITQALEAVGKKME
ZmCrtISO2 : YDAIVIGSGIGGLVAATQLAAKGARVLVLEKYLIPGGSSGYRRDGFDFDVGSSVMFGFSDKGNLNLITQALEAVGKKMR

AtCrtISO : VIPDPTTVHFHLPNNLSVRIHREYDDFTIELTSKFPHEKEGILGFYGDCKWIFNSLNSLELKSLEEPIYLFQGFFQKPLE
OsCrtISO : VIPDPSTVHFHLPDLSVLVHREYDDFVTELVNKKFPHEKEGILKFYGTCKWIFNSLNSLELKSLEEPLYLFGQFFQKPLE
SbCrtISO : VLPDPSTVHFHLPDLSVLVHRKYEDFINELISKFPHEKEGILKFYDTCWKIFNSLNSLELKSLEEPLYLFGQFFQKPLE
ZmCrtISO1 : VLPDPSTVHFHLPDLSVLVHRKYEDFINELISKFPHEKEGILKFYGTCKWIFNSLNSLELKSLEEPLYLFGQFFQKPLE
ZmCrtISO2 : VLPDPSTVHFHLPDLSVLVHRKYEDFINELVSKFPHEKEGILKFYDTCWIFNSLNSLELKSLEEPLYLFRQFFQKPRE

AtCrtISO : CLTLAYYLPQNAGDIARKFIKDDQLLSFIDAECFIVSTVNALQTPMINASMVLCDRHFGGINYPVGGVGGIATSLADGLV
OsCrtISO : CLTLAYYLPQNAGDIARKFIKDDQLLSFIDAECFIVSTVNALQTPMINASMVLCDRHFGGINYPVGGVGGIATSLADGLV
SbCrtISO : CLTLAYYLPQNAGDIARKFIKDDQLLSFIDAECFIVSTVNALQTPMINASMVLCDRHFGGINYPVGGVGGIATSLADGLV
ZmCrtISO1 : CLTLAYYLPQNAGDIARKFIKDDQLLSFIDAECFIVSTVNALQTPMINASMVLCDRHFGGINYPVGGVGGIATSLADGLV
ZmCrtISO2 : CLTLAYYLPQNAGDIARKFIKDDQLLSFIDAECFIVSTVNALQTPMINASMVLCDRHFGGINYPVGGVGGIATSLADGLV

AtCrtISO : DKGSEIRYKANVKSITLDHGKAVGVRLADGREFFAKTIISNATRWDTFGKLLKEKLPKEEFNFQKNYVKAPSFLSIHMG
OsCrtISO : DKGSEIRYKANVTNVILENGKAVGVRLSNGKEFFAKTVISNATRWDTFGKLLKEELPEEEKNFQKNYVKAPSFLSIHMG
SbCrtISO : EKGSEIRYKANVTNVILENGKAVGVRLSNGKEFFAKTVISNATRWDTFGKLLKEKLPKEEFNFQKNYVKAPSFLSIHMG
ZmCrtISO1 : EKGSEIRYKANVTNVILENGKAVGVRLSNGKEFFAKTVISNATRWDTFGKLLKEKLPKEEFNFQKNYVKAPSFLSIHMG
ZmCrtISO2 : EKGSEIRYKANVTNVILENGKAVGVRLSNGKEFFAKTVISNATRWDTFGKLVK-EELPEEEKNFQKNYVKAPSFLSIHMG

AtCrtISO : VKASVLPADTDCHHFVLEDDWNNLEKPYGSIFLSIPTVLDPSLAPEGHHILHIFTTASIEDWGLSRKDYEEKKELVATE
OsCrtISO : VKASVLPADTDCHHFVLEDDWNNLEKPYGSIFLSIPTVLDPSLAPEGHHILHIFTTASIEDWGLSRKDYEEKKELVATE
SbCrtISO : VKASVLPAGTDCHHFVLEDNWNLEKPYGSIFLSIPTVLDPSLAPEGHHILHIFTTASIEDWGLSRKEYEEKKEEVANE
ZmCrtISO1 : VKASVLPAGTDCHHFVLEDNWNLEKPYGSIFLSIPTVLDPSLAPEGHHILHIFTTASIEDWGLSRKEYEEKKEEVANE
ZmCrtISO2 : VKASVLPAGTDCHHFVLEDNWNLEKPYGSIFLSIPTVLDPSLAPEGHHILHIFTTASIEDWGLSRKEYEEKKEEVANE

AtCrtISO : IIRLEKKLFPGLQDSIVLKEVGSPKTHRRFLARNDGTGPMPRGPKPGLLAMPFNNTSIDGLYCVGDSFCFPGQGVIAVA
OsCrtISO : IIRLEKKLFPGLQDSIVLKEVGSPKTHRRFLARNDGTGPMPRGPKPGLLAMPFNNTSIDGLYCVGDSFCFPGQGVIAVA
SbCrtISO : IIRLEKKLFPGLQDSIVLKEVGSPKTHRRFLARNDGTGPMPRGPKPGLLAMPFNNTSIDGLYCVGDSFCFPGQGVIAVA
ZmCrtISO1 : IIRLEKKLFPGLQDSIVLKEVGSPKTHRRFLARNDGTGPMPRGPKPGLLAMPFNNTSIDGLYCVGDSFCFPGQGVIAVA
ZmCrtISO2 : IIRLEKKLFPGLQDSIVLKEVGSPKTHRRFLARNDGTGPMPRGPKPGLLAMPFNNTSIDGLYCVGDSFCFPGQGVIAVA

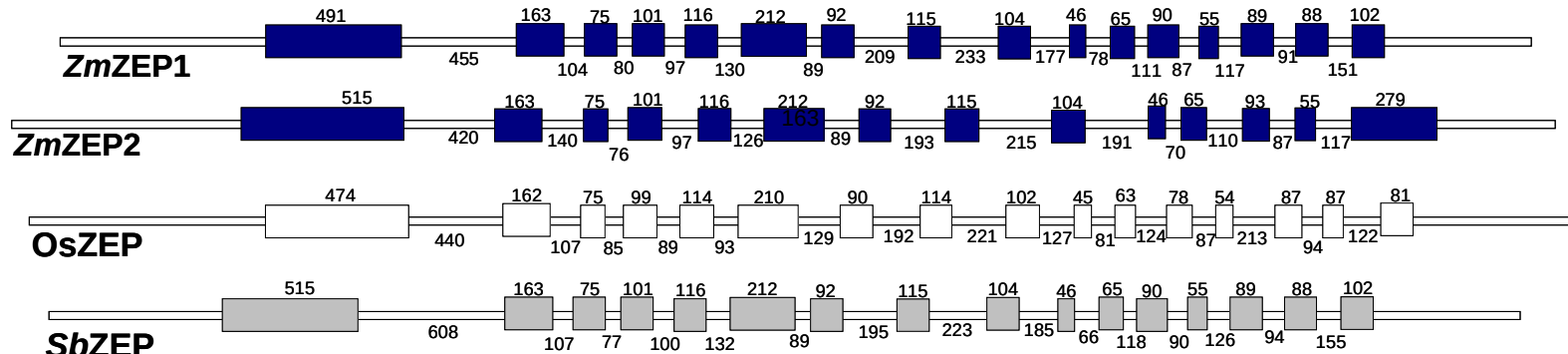
AtCrtISO : FSGVMCAHRVAADIGLEKKSRLVDVGLLGLLRLWRTLA(595)
OsCrtISO : FSGIMCAHRVAADIGLEQKSPVLDAGLLGLLRLWRTLA(586)
SbCrtISO : FSGIMCAHRVAADIGLEQKSPVLDAGLLGLLRLWRTLA(588)
ZmCrtISO1 : FSGIMCAHRVAADIGLEQKSPALDAGLLGLLRLWRTLA(587)
ZmCrtISO2 : FSGIMCAHRVAADIGLEQKSPVLDAGLLGLLRLWRTLA(588)

```

Figure 2-11. Multiple-alignment of CrtISO protein sequences.

Amino acid sequences across available grass species (*Zea mays*, *Zm*; *Oryza sativa*, *Os*; *Sorghum bicolor*, *Sb*) and *Arabidopsis thaliana* (*At*) were used. Total number of amino acids were marked at the end of each sequence in blue. Accession numbers: **AtCrtISO**, At1g06820; **OsCrtISO**, LOC_Os11g36440; **SbCrtISO**, Sb05g022240; **ZmCrtISO1**, AC218991; **ZmCrtISO2**, AC183901.

A



B

```

OsZEP : -----MAPLPSSSS-TRATSLVATPGASCPHGQAQRLLAFR---PPRRGSQWRGLCVSRGHEATVAMAMPAAQAAG-RRARVLVAGGGIGGLVFALAARRKGFVVVLERDMSAVRGEGRYRGPQLQSN
ZmZEP1 : MPTVLSTRRAASPETISLPSSRS-SHSRTASPLHPVSRGN-RALRLLAALRSPSAPRPLRRRPGLPPARAGLAAAAAMPAPET----KARILVAGGGIGGLVFALAARRKGFVVLERDMSAVRGEGRYRGPQLQSN
SbZEP : MPATLSTTRAP-IRATSLPSSR---ASQTAILRHPVSRRN-RALRLALP-SPSAPRRRPPGLPPARAGLVATAAMPAPAEPEPKARVLVAGGGIGGLVFALAARRKGFVVLERDMSAVRGEGRYRGPQLQSN
ZmZEP2 : ---MLSTTWAS-KATPIPSPP---ASQTATRLHPASSN-RTLRLVALP-SPSAPRRR-RPGLPPPARAGLVATAAMPAPAEPE---KARVLVAGGGIGGLVFALAARRKGFVVLERDMSAVRGEGRYRGPQLQSN

OsZEP : ALAALAEAVDAAADQVMDAGCITGNRVNGIVDGVSGSWYIKFDTFTPAAEERGLPVTRVISRMTLQQILARAVGDDAILNGSHVVDFTDDGSKVTAILEDGRKFEGDLLVGADGIWSKVRKTLFGHSDATYSEYTCYTGIA
ZmZEP1 : ALAALAEAVDAAADQVMDAGCITGNRVNGIVDGVSGSWYIKFDTFTPAAEERGLPVTRVISRMTLQQILARAVGDDAILNGSHVVDFTDDGSKVTAILEDGRKFEGDLLVGADGIWSKVRKTLFGHSDATYSEYTCYTGIA
SbZEP : ALAALAEAVDAAADQVMDAGCITGNRVNGIVDGVSGSWYIKFDTFTPAAEERGLPVTRVISRMTLQQILARAVGDDAILNGSHVVDFTDDGSKVTAILEDGRKFEGDLLVGADGIWSKVRKTLFGHSDATYSEYTCYTGIA
ZmZEP2 : ALAALAEAVDAAADQVMDAGCITGNRVNGIVDGVSGSWYIKFDTFTPAAEERGLPVTRVISRMTLQQILARAVGDDAILNGSHVVDFTDDGSKVTAILEDGRKFEGDLLVGADGIWSKVRKTLFGHSDATYSEYTCYTGIA

OsZEP : DFVPPDIDTVGYRVFLGHKQYFVSSDVGAGKMQWYAFHKEAGGTPENGKKNRLLEIFDNGWCDNVVDLINATDEEAILRRDIYDRPPTINWGKGRVTLTGDSVHAMQPNLGQGGCMAIEDGYQLAVELEKSWQESAKSG
ZmZEP1 : DFVPPDIDTVGYRVFLGHKQYFVSSDVGAGKMQWYAFHKEAGGTPENGKKNRLLEIFDNGWCDNVVDLINATDEEAILRRDIYDRPPTINWGKGRVTLTGDSVHAMQPNLGQGGCMAIEDGYQLAVELEKSWQESAKSG
SbZEP : DFVPPDIDTVGYRVFLGHKQYFVSSDVGAGKMQWYAFHNEAGGTPENGKKNRLLEIFDNGWCDNVVDLINATDEEAVLRDIYDRPPTINWGKGRVTLTGDSVHAMQPNLGQGGCMAIEDGYQLAVELEKSWQESAKSG
ZmZEP2 : DFVPPDIDTVGYRVFLGHKQYFVSSDVGAGKMQWYAFHNEAGGTPENGKKNRLLEIFDNGWCDNVVDLINATDEEAVLRDIYDRPPTINWGKGRVTLTGDSVHAMQPNLGQGGCMAIEDGYQLAVELEKSWQESAKSG

OsZEP : TPMDIVSSLRRYEKERRLRVSVIHGLARMAAIMATTYRYPYLGVLGPLSFLTKLRIPHPGRVGGRRFFIKYGMPTMLSWLGGNSTKLEGRPLSCRLSDKANDOLRRWFEDDDALEQAMGGEWYLLPTSSGDSQ----PTR
ZmZEP1 : TPMDIVSSLRRYEKERRLRVSVIHGLARMAAIMATTYRYPYLGVLGPLSFLTKLRIPHPGRVGGRRFFIKYGMPTMLSWLGGNSTKLEGRPLSCRLSDKAHDELYRWFDDDDALEQAMGGEWYLLPTSEANNNSLQPIR
SbZEP : TPMDIVSSLRRYEKERRLRVSVIHGLARMAAIMATTYRYPYLGVLGPLSFLTKLRIPHPGRVGGRRFFIKYGMPTMLSWLGGNSTKLEGRPLSCRLSDKANDOLYRWFEEDDDALEQAMGGEWYLLPTSEGNNS-SFQPIR
ZmZEP2 : TPMDIVSSLRRYEKERRLRVSVIHGLARMAAIMATTYRYPYLGVLGPLSFLTKLRIPHPGRVGGRRFFIKYGMPTMLSWLGGNSTKLEGRPLSCRLSDKANDOLYRWFEEDDDALEQAMGGEWYLLPTSEGNNS-SLQPIR

OsZEP : LIRDEKKSLSIGSRSDPSNSTASLALPLPOISERHATITCKNAFYLTDLGSEHGWTIDNEGRRYRVPPNFPVRFHPSDVEIEFGSDKKAMFRVKVNLTPYESARSGKQOGLQOEQVLQAA
ZmZEP1 : LIRDEKKSLSIGSRSDPSNSTASLALPLPOISERHATITCKNAFYLTDLGSEHGWTIDNEGRRYRVPPNFPVRFHPSDVEIEFGSDKKAMFRVKVNLTPYESARSGKQOGLQOEQVLQAA
SbZEP : LIRDEKKSLSIGSRSDPSNSTASLALPLPOISERHATITCKNAFYLTDLGSEHGWTIDNEGRRYRVPPNFPVRFHPSDVEIEFGSDKKAMFRVKVNLTPYESARSGKQOGLQOEQVLQAA
ZmZEP2 : LIRDEKKSLSIGSRSDPSNSTASLALPLPOISERHATITCKNAFYLTDLGSEHGWTIDNEGRRYRVPPNFPVRFHPSDVEIEFGSDKKAMFRVKVNLTPYESARSGKQOGLQOEQVLQAA

```

Figure 2-12. ZEP gene family structure.

(A) Gene organizational structure showing paralogs and orthologs of the ZEP gene family in *Zea mays* (*ZmZEP1*, AZM5_13315; *ZmZEP2*, AZM5_13312); *Oryza sativa* (*OsZEP*, LOC_Os04g37619) and *Sorghum bicolor* (*SbZEP*, Sb06g018220) species representing two subfamilies of the Poaceae (grasses). Boxes and lines indicate exons and introns respectively, sizes that are in base pairs **(B)** Multiple-alignment of ZEP paralogs in maize and orthologs from *Oryza sativa* (*Os*)

Chapter 3

Metabolite sorting of a germplasm collection reveals the Hydroxylase3 locus as a new target for maize provitamin A biofortification²

3.1 Abstract

Vitamin A deficiency, a global health burden, can be alleviated through provitamin A carotenoid biofortification of major crop staples such as maize and other grasses in the Poaceae. If regulation of carotenoid biosynthesis was better understood, enhancement could be controlled by limiting beta-carotene hydroxylation to compounds with lower or no nonprovitamin A activity. Natural maize genetic diversity enabled identification of hydroxylation genes associated with reduced endosperm provitamin A content. A novel approach was used to capture the genetic and biochemical diversity of a large germplasm collection, representing 80% of maize genetic diversity, without having to sample the entire collection. Metabolite data-sorting was applied to select a 10 line genetically diverse subset representing biochemical extremes for maize kernel carotenoids. Transcript profiling led to discovery of the *Hydroxylase3* locus that coincidentally mapped to a carotene QTL, thereby prompting investigation of allelic variation in a broader collection. Three natural alleles in 51 maize lines explained 78% of variation and ~11-fold difference in beta-carotene relative to beta-cryptoxanthin and 36% of the variation and 4-fold difference in absolute levels of beta-carotene. A simple PCR assay to track and identify *HYD3* alleles will be valuable for predicting nutritional content in genetically diverse cultivars found world-wide.

² This Chapter is a revision of Vallabhaneni *et al*, (2009), Plant Physiology, In Press

3.2 Introduction

Vitamin A deficiency is a global health problem affecting 140-250 million children and accounts for increased childhood mortality and disease (World Health Organization (Geneva), 1995; Underwood, 2004; Black et al., 2008). Humans and animals are unable to synthesize their own vitamin A and rely on dietary provitamin A carotenoid pigments; plant-derived carotenes are metabolized to produce vitamin A, two molecules from β -carotene and only one from α -carotene or β -cryptoxanthin. Seed endosperm tissue of maize and other grasses (Poaceae) represents 70% of worldwide food production (Chandler and Brendel, 2002), but has limited provitamin A value. Improvement of provitamin A content in staple crops is therefore a critical step towards alleviating vitamin A deficiency worldwide. Maize, a major food staple in vitamin A deficient sub-Saharan Africa and Latin America, exhibits a wide range of carotenoid content and composition. The underlying basis for this variation has been the subject of Quantitative Trait Locus (QTL) and association studies (Wong et al., 2002; Palaisa et al., 2003; Chander et al., 2007; Harjes et al., 2008). Given the genetic and biochemical diversity of maize, opportunity exists for developing breeding markers, based on candidate genes of the carotenoid biosynthetic pathway, to select for predicted nutritional content.

The carotenes are intermediates in the (recently revised) carotenoid biosynthetic pathway (Li et al., 2007; Matthews and Wurtzel, 2007) leading to the predominant nonprovitamin A xanthophylls in maize kernels (**Fig. 3-1**), which is why maize kernels generally have limited provitamin A content despite being rich in carotenoids (unlike rice

which does not naturally accumulate endosperm carotenoids) (Islam, 2004). Lycopene, the immediate precursor of provitamin A carotenes, represents a branch point in the carotenoid biosynthetic pathway. The linear lycopene molecule is further modified by LCYB and LCYE enzymes that catalyze formation of terminal β -ionone rings and ϵ -rings, respectively. Depending on relative activity levels of the cyclases, the products are β -carotene with two β -ionone rings, the most potent dietary source of vitamin A, and α -carotene, with only one β -ionone ring and therefore half the provitamin A potential compared to β -carotene; the hydroxylation intermediate β -cryptoxanthin, with only one β -ionone ring, has half the provitamin A activity compared to β -carotene. The pathway continues with hydroxylation of the carotenes which depletes the provitamin A pool by converting provitamin A compounds to nonprovitamin A xanthophylls (Matthews and Wurtzel, 2007). Therefore, pathway branching and hydroxylation are key determinants in controlling provitamin A levels. Both of these aspects have been targets for metabolic engineering of endosperm carotenoids in other species (Diretto et al., 2006; Diretto et al., 2007). However, transgenic solutions to manipulate the carotenoid biosynthetic pathway, as achieved in Golden Rice (Ye et al., 2000) and other plants (Giuliano et al., 2008), are not always acceptable as observed by the overwhelming public resistance to GMO food crops. Moreover, transgene incorporation into one genetic variety used for laboratory transformation produces variable carotenoid phenotypes (Aluru et al., 2008); and the desired transgene-produced trait is not predictably transferred to other genetic backgrounds of cultivars worldwide. This lack of predictability could be overcome given a better understanding of the rate-controlling steps and tools to genotype and/or phenotype varieties for predicting the outcome of transgene introduction as we report

here. Given the extensive natural diversity inherent in maize, it is conceivable to predictably breed high provitamin A maize in a wide range of genotypes given a thorough understanding of pathway bottlenecks and development of corresponding breeding alleles.

Our collaborative effort in the recent development of *LCYE*-based breeding markers for maize demonstrated feasibility of a nontransgenic, traditional breeding approach to control the pathway branching step and force pathway flux towards β -carotene and its nonprovitamin A derivatives (the β -branch) (Harjes et al., 2008). However, unless hydroxylation is also controlled, nonprovitamin A xanthophyll compounds will predominate. Given that lycopene cyclization can be forced towards the β -branch, the next challenge in breeding high β -carotene in maize endosperm is to block β -carotene hydroxylation to increase levels of β -carotene relative to β -cryptoxanthin and downstream xanthophylls. Therefore, we embarked on a study capitalizing on maize germplasm resources to characterize the maize β -carotene hydroxylase genes and to discover breeding markers for enhancing the relative levels of seed β -carotene.

3.3 Results and Discussion

Two structurally distinct classes of carotene hydroxylases are known: the P450 heme-thiolate CYP97A and CYP97C enzymes and the nonheme diiron monooxygenases [reviewed in (Matthews and Wurtzel, 2007; Giuliano et al., 2008)]. As found in rice (Quinlan et al., 2007), maize contained one gene each for CYP97A and CYP97C, respectively. A total of six unlinked maize paralogs encoding nonheme diiron β -carotene

hydroxylases (*HYD*) were identified in contrast to three rice genes (**Fig. 3-2; Tables 3-1 and 3-2**); phylogenetic analysis suggested that the gene duplications found in the grasses occurred after the monocot and dicot evolutionary split. Maize *HYD1* and *HYD2* are pseudogenes while *HYD3-6* encode enzymes with characteristic hydroxylase domains and plastid targeting signals (Sun et al., 1996) (**Fig. 3-2**). *HYD3* and *HYD4*, are syntenous with rice *HYD1* (**Fig. 3-3**), and are predicted to encode proteins with markedly different isoelectric points, but functional testing confirmed that both encode carotene β -ring hydroxylases (**Fig. 3-11**). The *CYP97A* and *CYP97C* genes have been functionally tested in rice (Quinlan et al., 2007); phylogenetic analysis of this ancient evolutionary clade would suggest that the maize encoded enzymes behave similarly.

In the maize inbred B73 which has been used extensively for investigating regulation of endosperm carotenogenesis (Li et al., 1996; Matthews et al., 2003; Li et al., 2008; Li et al., 2008a; Li et al., 2009; Vallabhaneni and Wurtzel, 2009), mRNA abundance for the six functional carotene hydroxylases genes varied among tissues and during endosperm development, where comparison was also made with carotenoid accumulation (**Fig. 3-4**). To test which, if any, carotene hydroxylase gene showed a statistical correlation between mRNA levels and endosperm provitamin A β -carotene content, transcript abundance was quantified in a genetically diverse maize germplasm collection (Liu et al., 2003; Islam, 2004; Harjes et al., 2008). However, it was impractical to screen endosperm developmental samples in staged, hand-pollinated plants for the entire collection representing 80% of maize genetic diversity. Therefore, a uniquely selected subset of lines for testing was chosen from 148 lines with known carotenoid

content and composition (Islam, 2004). From existing metabolite data, we sorted lines for highest total carotenoid content, highest ratio of β -carotene to β -cryptoxanthin, highest ratio of β -cryptoxanthin to zeaxanthin, highest ratio of β -carotene to zeaxanthin, highest ratio of α -carotene to lutein, and highest ratio of lutein to zeaxanthin. The resulting ten-line subset (**Table 3-3; Table 3-4**) exhibited the most extreme carotenoid biochemical phenotypes and likely contained the most favorable and informative alleles for breeding carotenoid content and composition. For example, a block in carotene hydroxylase activity would be predicted to manifest as a high ratio of β -carotene to β -cryptoxanthin, while factors that controlled flux would be predicted to influence levels of total carotenoids. The lines are genetically diverse, spanning four major genetic diversity groups and eight subgroups (**Table 3-4**) among the 260 Goodman Diversity lines (Liu et al., 2003). The 10 lines were then used for quantitative transcript profiling of endosperm samples at different developmental stages (days after pollination, DAP). To demonstrate adequacy of sample size and to validate use of this biochemically diverse subset to infer pathway regulation, we applied Pearson correlation analysis to show that *PSY1* but not *PSY2* or *PSY3* transcript levels showed statistically significant correlation with seed carotenoid content (Li et al., 2008a; Vallabhaneni and Wurtzel, 2009). The findings were consistent with molecular and genetic studies associating *PSY1* and control of pathway flux in endosperm (Randolph and Hand, 1940; Palaisa et al., 2003; Wong et al., 2004; Pozniak et al., 2007; Li et al., 2008a). The subset has also been recently tested to examine additional genes that encode enzymes for steps that represent carotenoid biosynthetic pathway bottlenecks and those steps that do not (Vallabhaneni and Wurtzel, 2009). For example, the finding of carotenoid bottlenecks in the upstream isoprenoid biosynthetic

pathway is consistent with other studies in the literature. The validated germplasm subset was then used to assess the possible role of carotene hydroxylase gene expression in controlling seed β -carotene composition. Transcript abundance was quantitatively measured, over a range of endosperm developmental stages, for the five carotene β -ring hydroxylase genes, *HYD3*, *HYD4*, *HYD5*, *HYD6*, and *CYP97A* and the one ϵ -ring hydroxylase gene, *CYP97C*. Time points chosen were based on earlier studies that investigated carotenoid accumulation during endosperm development (Li et al., 2008a). We show the transcript data for *HYD3* only, as it was the only gene for which transcripts were found to correlate with carotenoids (**Table 3-3**). Interestingly, some of the lines showed a steep reduction in *HYD3* transcripts between 15 and 25 DAP ranging from 0.7 to >5000-fold. A Pearson correlation analysis comparing β -carotene content and transcript levels for all carotene hydroxylase genes was conducted and *HYD3* was the only gene for which there was a statistically significant (p value ≤ 0.05) correlation between transcript levels and provitamin A β -carotene content (**Fig. 3-5**). In the correlation plots shown, where the colored lines denote a 95% confidence interval, the “fold reduction” of *HYD3* transcripts over endosperm development (15 to 25 DAP) positively correlated with seed β -carotene ($\mu\text{g/g}$) levels ($r=0.79$, $p=0.005$) (**Fig. 3-5A**). As would be predicted for a critical endosperm hydroxylase gene, *HYD3* transcripts at 25 DAP also positively correlated with zeaxanthin ($\mu\text{g/g}$) levels ($r=0.78$, $p=0.007$) (**Fig. 3-5B**). That is, the greater the reduction in transcripts over development, the higher the β -carotene content, as would be predicted from a correspondingly lower level of hydroxylase activity; conversely, the higher the transcripts at 25 DAP, the greater the level of zeaxanthin, as would be predicted from increased hydroxylase activity.

We noted the presence of a QTL for endosperm β -carotene content (Chander et al., 2007) that maps together with *HYD3*, between markers *umc1506* and *bnlg1028* on maize chromosome 10.05 (**Fig. 3-3**). Therefore, we sequenced all *HYD3* alleles in the 10 line subset. Variation consistently seen in the high β -carotene lines was found in a ~ 40 bp region adjacent to the transcript start site (**Fig. 3-6, blue box**). A conserved transcript start site and first ATG were mapped by aligning available paralog-specific ESTs and genomic DNA for maize B73 *HYD3* and sister paralog *HYD4*, and rice synteny partner *HYD1* (**Fig. 3-6**). B73 and the other six low β -carotene lines had identical sequence in this region (variant “A”); A619 had sequence variant “B” while CI.7 and DE3 shared variation “C”. “B” and “C” in the high β -carotene lines appear to contain a duplicated sequence from a downstream region that replaced the progenitor sequence seen in “A”, although allele C shows a more complete match (14/17 nt) as compared to allele B (10/17 nt). Diversity analysis (Liu et al., 2003) of the 260 Goodman lines placed A619 and CI.7, which carried different *HYD3* variations, in two genetically distinct subgroups of the maize non-Stiff stalk lines; such diversity grouping is consistent with finding that they do not share the same polymorphism. The identical “C” polymorphism seen in DE3 and CI.7 may be a result of a possible overlapping pedigree since DE3 was placed in a “mixed” group as an indication of genetic structure shared with more than one maize diversity group. However, further sequencing of the entire *HYD3* gene of DE3 and CI.7 revealed some minor sequence differences between *HYD3* alleles in these two inbreds (**Fig 3-12**).

To test whether *HYD3* allelic variation could explain β -carotene variation, a PCR assay was developed to rapidly genotype a broader maize collection. The assay distinguished between **A** and **C** or between **A** and **B** alleles and generated a common *HYD3* paralog-specific product which could be sequenced to reveal new alleles; the assay correctly genotyped all 10 lines for their *HYD3* allele (**Fig. 3-7**). We then selected an additional 41 lines spanning maize genetic diversity and range of carotenoid content and composition (**Table 3-4**). The combined group of 51 lines included 9 new lines identified by “metabolite-sorting” to exhibit high ratios of β -carotene to β -cryptoxanthin, as possible indicator of a block in β -carotene hydroxylation. The common *HYD3* paralog-specific product was amplified and sequenced to characterize *HYD3* alleles in this broader maize collection (**Fig. 3-7**). In the combined 51 lines, we identified fourteen “high carotene” alleles: 7 **C** alleles, including one new **C**-like allele, and 7 **B** alleles. The fourteen **B** and **C** alleles were widely distributed among genetic diversity groups, as were the 37 **A** alleles (**Table 3-4**). One-way ANOVA showed that the mean ratios of β -carotene/ β -cryptoxanthin exhibited a statistically significant difference among the three *HYD3* alleles ($F_{2,48} = 89.3$, $p < 0.0001$) and that the *HYD3* allele explained 78% of the variation (R^2) in the β -carotene/ β -cryptoxanthin ratio (**Fig.3-8**). The absolute amount of β -carotene (ug/gm) also showed a statistically significant difference among the three alleles (A, B, C) in one-way ANOVA ($F_{2,48} = 13.7$, $p < 0.0001$) and *HYD3* allele explained 36% of total variation (R^2) in level of β -carotene (ug/gm). It is reasonable that *HYD3* allele will explain less of the variation in absolute levels of β -carotene compared to ratio of β -carotene/ β -cryptoxanthin. Absolute levels of β -carotene will be influenced not only by LCYE and *HYD3* activities, but also by multiple factors controlling pathway flux

(Vallabhaneni and Wurtzel, 2009). In comparison, β -carotene/ β -cryptoxanthin ratio will be influenced most directly by the *HYD3* enzyme activity.

We next compared the means of the three alleles using the Tukey-Kramer HSD test at a 0.05 level. We observed a significant difference ($p < .0001$) between alleles **C** and **B**, and **C** and **A** for the ratio of β -carotene and β -cryptoxanthin and β -carotene alone. No significant difference was observed between alleles **B** and **A** for both the ratio of β -carotene and β -cryptoxanthin and β -carotene alone.

The most favorable *HYD3* **C** allele was associated with ~11-fold higher levels of β -carotene relative to β -cryptoxanthin and 4-fold higher levels of β -carotene (ug/gm). The most likely explanation is that during endosperm development a reduction in *HYD3* transcripts leads to reduced conversion of β -carotene to downstream xanthophylls causing β -carotene to accumulate. To test this possibility, we selected CI.7 and DE3, lines carrying the **C** allele, and measured β -carotene at each of three developmental stages and compared to *HYD3* transcript level for triplicate replicates of endosperms collected at 15, 20 and 25 DAP. As shown in Fig. 3-9, levels of β -carotene increased during endosperm development (for equal amounts of extracted carotenoids), as *HYD3* mRNA levels decreased in the same endosperm developmental samples. Therefore, there is a direct link between *HYD3* transcript level and accumulated β -carotene.

Enhancement of provitamin A levels necessitates control of both pathway branching and carotene hydroxylation in addition to elevating pathway flux and

minimizing product degradation. Previous development of *LCYE* markers (Harjes et al., 2008) provided the first step in tracking alleles to control pathway branching through traditional nontransgenic breeding selection. However, the *LCYE* markers were insufficient. Even if pathway branching could be controlled through selection of optimal *LCYE* alleles, there remained the problem that the provitamin A carotenes produced in the pathway would automatically be hydroxylated to nonprovitamin A compounds, as typically seen for maize endosperm composition of zeaxanthin or lutein (Kurilich and Juvik, 1999). Other upstream steps may influence carotenoid content (Vallabhaneni and Wurtzel, 2009), but enhancement of provitamin A composition will specifically require the combined use of tools that target both *LCYE* and *HYD3*. The presence of the **C** allele can be monitored by the simple PCR assay and its presence in genetically diverse lines will facilitate further use in different geographical regions. This simple PCR assay may now be used to further test predictability of the *HYD3* allele in controlling β -carotene content in diverse cultivars.

Many ongoing studies have investigated pathway regulation through transgene expression in one cultivar (Giuliano et al., 2008); the resulting phenotype is dependent on the genotype used and the resulting data are not predictably transferred to other genotypes, perhaps because of limited understanding of pathway regulation. Here we took advantage of natural variation inherent in a large germplasm collection; by judicious sampling of the collection, we were able to pinpoint a specific gene family member for which favorable alleles were discovered for predicting enhanced β -carotene composition. Metabolite-based sorting of a germplasm collection provided accessibility to a valuable

resource that yielded critical knowledge and tools that will be needed for breeding high provitamin A in maize, an important food staple in vitamin A-deficient sub-Saharan Africa and Latin America, where vitamin A deficiency is a serious health problem. This approach can be easily adapted to other metabolite targets in other species to rapidly discover pathway regulation and to develop tools for predictive breeding.

3.4 Materials and Methods

3.4.1 Plant materials

Maize inbred lines (A619, B73, B37, CI.7, C131A, DE3, KUI2007, NC300, SD44 and TZI18) (Harjes et al., 2008) were field grown in Bronx, NY and sibling pollinated. Unfertilized ear, embryo (20 days after pollination, DAP) and endosperm (10 -30 DAP) were collected from field-grown plants, whereas leaf and root samples were collected from 6-leaf-stage seedlings grown in a green house (16 h day at 25°C). Samples were stored at -80°C prior to use.

3.4.2 Sequence analysis and chromosome mapping

Cloning and DNA sequence analysis: A maize B73 genomic BAC library (Gallagher et al., 2004) was probed with the maize *HYD4* cDNA, GenBank AY844956. Eight BAC clones representing three groups were identified and a representative of each group was sequenced by primer walking (DNA Sequencing Facility, University of Chicago Research Center) and the data deposited in GenBank (*HYD1*, EU638325; *HYD2*, EU638326; *HYD3* AY844957). The *HYD3* cDNA sequence (Genbank# AY844958) was

used as a query to identify additional *Zea mays* paralogs and orthologs from *Oryza sativa* (www.gramene.org) and *Sorghum bicolor* (www.phytozome.org). Maize methyl filtered contig sequences, and corresponding BAC clones harboring carotene hydroxylase genes (diiron type) were identified (**Table 3-1**). Gene models were drawn using Genscan (www.genscan.com), Softberry (www.softberry.com) and Vector NTI Suite 9.0 (Invitrogen, Carlsbad, CA). Transcription start sites were estimated by EST and promoter analysis (www.softberry.com). Maize ESTs corresponding to CYP97A (DV169913) and CYP97C (BE552887) were found by searching plantGDB maize database using orthologous gene sequences of CYP97A (LOC_Os02g57290) and CYP97C (LOC_Os10g39930) from *Oryza sativa* (Quinlan et al., 2007). As found for rice and sorghum, only one maize ortholog was found for each gene encoding these two CYP97 enzymes (**Table 3-1**).

Mapping and Synteny: Chromosomal positions of maize genes in the *Zea mays* B73 inbred were mapped either by utilizing tools available at WebAGCoL package (Pampanwar et al., 2005) or Maize GDB. Orthologous genes from *Oryza sativa* ssp. Japonica were identified through synteny comparisons with maize (www.tigr.org/tdb/synteny/).

Phenetic and Protein Analysis: HYD amino acid sequences were aligned using ClustalW and a neighbor-joining tree was constructed with bacterial *crtZ* from *Erwinia herbicola* (Genbank # AAA64983) as an out group with 500 bootstrap replication support using MEGA3 software (Kumar et al., 2001). Chloroplast transit peptide signal and transmembrane analysis were predicted using ChloroP 1.1 (Emanuelsson et al., 1999) and TMHMM 2.0 (Krogh A et al., 2001) respectively.

3.4.3 Transcript and total carotenoid analysis

RNA extraction and quantitative RT-PCR were performed using gene specific primers (**Table 3-1**) and normalized to actin, as described (Li et al., 2008). Values are expressed as the mean of three RT-PCR replicates +/- standard deviation. Total carotenoids were extracted as previously described (Li et al., 2008a) and the concentration (shown in **Fig. 3-4**) was calculated using the Lambert-Beer equation (Schiedt and Liaaen-Jensen, 1995).

3.4.4 Plasmids and Functional Complementation

E. coli BL21 (DE3) cells (Novagen, Madison, WI) containing pAC-BETA-04 accumulate β -carotene and were used to test hydroxylase function (Sun et al., 1996). BM382572 (*HYD3*) and BG320875 (*HYD4*) cDNAs were sub-cloned for functional analysis into the *EcoRI* / *NotI* and *EcoRI* / *XhoI* sites of the pET23c expression vector (Novagen, Madison, WI), and renamed as pTHYD3 and pTHYD4 respectively. Transformants carrying the test plasmids or empty vector were grown on selective medium and pigments extracted and analyzed by HPLC as described (Gallagher et al., 2004).

3.4.5 Total carotenoid content measurement

The carotenoid extraction procedure was based on Kurilich and Juvik (Kurilich and Juvik, 1999). Five hundred mg maize endosperm was ground in ethanol and incubated for 6 min (6 ml ethanol, 0.1% butylated hydroxytoluene) at 85⁰C, followed by 10 min saponification with 120 μ l (1g/ml) KOH. Samples were vortexed, placed on ice and 4 ml cold dH₂O added. Three ml of 2:1 PE:DE (v/v) were added to each sample, vortexed and centrifuged for 10 min at 3500 rpm. The upper layer was retrieved and the separation was

repeated twice with 3 ml of 2:1 PE:DE (v/v). The combined fractions were made up to 10 ml, and carotenoids were measured spectrophotometrically at OD 450nm using a Lambda UV/VIS spectrophotometer (Perkin Elmer Life Sciences, Boston), and the concentration of carotenoids were calculated using the Lambert-Beer equation (Schiedt and Liaaen-Jensen, 1995).

3.4.6 Measurement of β -carotene in developing endosperm

The carotenoid extraction procedure was based on Kurilich and Juvik (Kurilich and Juvik, 1999). Five hundred mg maize endosperm was ground in ethanol and incubated for 6 min (6 ml ethanol, 0.1% butylated hydroxytoluene) at 85⁰C, followed by 10 min saponification with 120 μ l (1g/ml) KOH. Samples were vortexed, placed on ice and 4 ml cold dH₂O added. Three ml of 2:1 PE:DE (v/v) were added to each sample, vortexed and centrifuged for 10 min at 3500 rpm. The upper layer was retrieved and transferred to a tube with a known weight. The separation was repeated twice with 3 ml of 2:1 PE:DE (v/v) and upper layers combined and dried under nitrogen gas. Total weight of the tube was measured to obtain the net weight of dried extract in each tube. All extractions and measurements were performed in triplicates, and finally dried extract was redissolved appropriately in HPLC grade isopropanol to maintain equal concentration (100 μ g/ μ l) in all samples. 4 μ l of each sample was injected onto a Acquity UPLC BEH C18 1.7 μ m 2.1x100mm (part # 186002352) column kept at 65 deg C and attached to a Waters Acquity Ultra Performance LC (UPLC) with a photodiode array detector. Separation was carried out using a linear gradient from 100% acetonitrile to 80% acetonitrile/20% isopropanol over 6 min, followed by 100% acetonitrile at 6.5 min. The solvent flow rate

was 0.3 mL/min. Data were collected for 7 min total and analyzed with Empower Pro software to determine β -carotene peak area for each sample. All samples were run in triplicates and standard deviation calculated.

3.4.7 Statistical analyses

Pearson correlation analysis of transcript and carotenoid composition from 10-line subset of maize inbreds was performed using JMP v. 5.1.2 (SAS Institute, Cary, NC) to test the statistical significance ($p \leq 0.05$) of the relationship. One-way ANOVA and Tukey-Kramer HSD test for comparisons between the alleles (A, B, C) and ratio of β -carotene/ β -cryptoxanthin or absolute levels of β -carotene in 51 lines was performed using JMP 5.1 (SAS institute Inc.).

3.4.8 Multiplex PCR assay for tracking *HYD3* alleles

A multiplex PCR assay based on *HYD3* promoter variation (**A**, **B**, or **C**) was developed to distinguish between *HYD3* allele **A** (in the low carotene B73 inbred) and the *HYD3* alleles **B** and **C** found in the high carotene inbreds A619 (allele **B**), CI.7 (allele **C**) and DE3 (allele **C**). The assay involved amplification of one *HYD3* paralog-specific “control” PCR product produced by all alleles and a second product that distinguished allele **A** from **C** or allele **A** from **B**, in both homozygous and heterozygous samples. The PCR assay utilized four primers: (a) two external primers **P1** (#1595, GACTTGTGAGCAAGGGGAAG) and **P2** (#1592, GACGTGACTCCGAGGCTAGA) for amplification of a “control” product that was conserved in all alleles; and (b) two internal primers added for amplification of the *HYD3* specific alleles. To track alleles **C** and **A**, the specific allele forward primer was **C**, (#2111,

AACACTCCCGCTCCCGCGGCTCG, allele **C**), and the reverse primer was **A** (#2116, TTATATGGATAGTTCACATACCTC, allele **A**). Alternatively, to distinguish allele **B** from **A**, the forward primer used was primer **B** (#2109, AACACTCACGCTCCCGCG, allele **B**) together with reverse primer **A**. The 50 µl PCR reaction contained: 1X PCRx Amplification Buffer (Invitrogen, Carlsbad, CA); 0.2 mM dNTPs (Invitrogen, Carlsbad, CA), 1.5 mM MgSO₄ (Invitrogen, Carlsbad, CA), 2.5 units iTaqTM DNA polymerase (BIO-RAD, La Jolla, CA); 0.6 µm each of two external primers; 0.4 µm each of two internal primers; 0.5 µg genomic DNA template, 3X final concentration of PCRx Enhancer Solution (Invitrogen, Carlsbad, CA). Thermal cycling conditions were: one cycle (94°C for 3min) followed by 40 cycles (94°C for 45sec, 54°C for 45 sec and 72°C for 1min) and one cycle (72°C for 10min). Amplified products were separated on 1% agarose gels and validated by sequencing. Maize inbred sequences used for developing the multiplex primers were: FJ228406, FJ228407, FJ228408, FJ228409, FJ228410, FJ228411, FJ228412, FJ228413, FJ228414, FJ228415.

Table 3-1. Primer and gene accession numbers

Maize Gene	Primer No	Sequence	ACCESSION NO.				
			Maize			Rice	Sorghum
			MAP	GENOMIC	cDNA		
HYD3	1409	TGGCCTGGACGAGCTTGTTAG	10.05	AZM5_1855 ^a	BM382572 ^e /	NA	NA
	995	CACACTCATTGCCCCACTCGC		AY844957 ^e	AY844958 ^e		
HYD4	1408	TGGCCTGGACGAGCTGGAAAA	2.03	AZM5_1857 ^a	BG320875 ^e /	LOC_Os04g48880 ^c (HYD1)	Sb06g026190 ^d (HYD1)
	1011	GGCTCGCGCATCCAAACC			AY844956 ^e		
HYD5	1513	GCAGCCCATCAGATACATCA	9.07	AZM5_23363 ^a	DR817188 ^e	LOC_Os03g03370 ^c (HYD2)	NA
	1514	GCAAGCAGGTCTTATTGGGT					
HYD6	1419	GAGATCAAGAAGAGGATCAGGAG	1.01	AZM5_7254 ^a	BQ619575 ^e	LOC_Os10g38940 ^c (HYD3)	Sb01g048860 ^d (HYD2)
	1420	GATCTAATGTGAGGAGAAGGTCAA					
CYP97A	1344	CTGGAGCCATCTGAAAGTCA	5	ZmGSStuc11- 12-04.50592.1 ^b	DV169913 ^e	LOC_Os02g57290 ^c	Sb04g037300 ^d
	1345	GGACCAAATCCAAACGAGAT					
CYP97C	1348	GTTGACATTGGATGTGATTGG	3.05	NA	BE552887 ^e	LOC_Os10g39930 ^c	Sb01g030050 ^d
	1349	AACCAACCTTCCAGTATGGC					

^a tigr.org; ^b plantgdb.org; ^c gramene.org; ^d phytozome.org; ^e NCBI

Table 3-2. Predicted structural characteristics of HYD proteins.

Gene	Accession #		Map Position	Transit peptide		Mature peptide		Isoelectric point (pI)		# Transmembrane domains (residues)
	Genomic	cDNA		Residues	Mass (kD)	Residues	Mass (kD)	Mature protein	Un-processed	
HYD3	AZM5_1855/ ^a AY844957 ^b	BM382572 ^b / AY844958 ^b	10.05	69	7.1	250	27.5	6.32	8.81	4 (22-41; 53-73; 107-124; 129-151)
HYD4	AZM5_1857 ^a	BG320875 ^b / AY844956 ^b	2.03	68	7.0	241	26.5	9.51	9.99	4 (42-61; 73-94; 127-144; 149-171)
HYD5	AZM5_23363 ^a	DR817188 ^b	9.07	73	7.1	220	24.5	8.11	10.28	4 (12-31; 44-64; 99-116; 121-143)
HYD6	AZM5_7254 ^a	BQ619575 ^b	1.01	56	5.8	243	26.6	9.62	9.88	4 (36-55; 67-83; 122-139; 144-166)

^a tigr.org; ^b NCBI

Table 3-3. Transcript profiling of *HYD3* in maize diversity lines.

Transcripts were measured quantitatively from endosperm collected at the indicated

DAP. Parentheses show SD, standard deviation. The fold reduction in transcripts (15/25

DAP) was calculated by dividing the 15 DAP value by the 25 DAP value. Quantitative

RT-PCR was performed using gene specific primers (**Table 3-1**) and normalized to actin,

as previously described (Li et al., 2008). Values are expressed as the mean of three RT-

PCR replicates +/- standard deviation. β -carotene levels in mature kernels are from

published values (Harjes et al., 2008).

	β -carotene	Relative transcript levels of <i>HYD3</i> (SD)			Fold reduction
Inbred	$\mu\text{g/g}$	15 DAP	20 DAP	25 DAP	15/25 DAP
A619	6.11	408.4 (14.01)	0.23 (0.13)	0.08 (0.02)	5105
B73	0.57	163.96 (19.04)	135.97 (49)	90.23 (5.07)	1.8
B37	1.15	482.32 (111.6)	246.51(67.39)	100.46 (8.07)	4.8
CI.7	13.63	515.15 (98.79)	26.40 (4.87)	0.12 (0.01)	4293
CI31A	0.82	791.72 (73.78)	164.71 (30.5)	88.47 (14.8)	9.0
DE3	13.34	147.93 (14.36)	2.70 (0.48)	0.05 (0)	2959
KUI2007	3.15	240.32 (13.9)	317.84 (47.2)	352.26 (64.5)	0.7
NC300	1.90	363 (21.3)	164.52 (62.5)	99.53 (21.31)	3.7
SD44	1.95	350.64 (65.4)	5.79 (1.76)	4.45 (0.19)	64.9
TZI18	2.36	375.8 (7.36)	159.10 (8.2)	130.43 (15.4)	2.9

Table 3-4. Genotyping of *HYD3* alleles and comparison with carotenoid composition of diverse maize germplasm accessions.

The *HYD3*-specific product was amplified as described in Figure 7 and DNA sequence alignments with lines evaluated in Fig. 6 were used to identify the *HYD3* allele in the larger group of 51 maize lines.

Genotyping of <i>HYD3</i> alleles and comparison with carotenoid composition of diverse maize germplasm accessions															
Accession*	GENETIC DIVERSITY GROUP**	GENETIC DIVERSITY SUBGROUP**	MAIZE VARIETY	<i>HYD3</i> Allele***	CAROTENOID CONTENT AND COMPOSITION****										
					Lutein (L)	Zeaxanthin (Z)	β-cryptoxanthin (BC)	α-carotene (A)	β-carotene (B)	Total	Z/L	L/Z	B/Z	B/A	A/L
Ames 19000	unknown		YanV6	C	7.17	1.48	0.25	0.14	1.83	10.9	0.31	4.84	1.24	13.07	0.02
NSL 30339	unknown		H4	A	7.97	3.5	1.9	0.22	5.29	18.68	0.44	2.25	1.91	24.05	0.03
Ames 26290	unknown		C49	A	17.16	1.41	1.06	2.03	1.91	23.17	0.08	12.17	1.07	0.74	0.12
NSL 85965	unknown		B10	A	27	842	1.36	0.79	1.87	39.44	0.31	3.21	0.22	2.37	0.03
Ames 27171	TS	NC	NC350	A	2.6	1.4	2.35	0.44	2.46	9.39	0.54	1.96	1.76	5.99	0.17
Ames 27261	TS	NC	NC298	A	18.1	18.32	2.83	0.22	2.76	42.23	1.01	0.99	0.15	12.55	0.01
Ames 27088	TS	Swan	CML 228	A	7.98	18.24	4.52	0.21	1.87	32.22	2.47	0.40	0.10	8.90	0.03
Ames 17174	TS	TS-mixed	NC300	A	0.7	1.04	3.08	0.23	1.90	6.95	1.49	0.67	1.83	8.26	0.33
Ames 17174	TS	TS-mixed	NC356	A	8.36	31.93	4.12	0.3	2.5	47.28	3.82	0.26	0.08	8.33	0.04
Ames 17174	TS	TS	TZ1	A	14.40	21.60	3.47	0.36	2.36	42.18	1.50	0.67	0.11	6.60	0.02
Ames 27062	TS	TZ1	A6	A	7.59	22.94	5.5	0.07	2.99	39.09	3.02	0.33	0.13	42.71	0.01
Ames 27175	TS	TZ1	NC358	A	17.17	8.3	1.9	1.09	0.78	29.2	0.48	2.07	0.09	0.72	0.06
Ames 26186	Sweet corn	P39		A	8.81	8.92	1.12	0.4	0.78	20.12	1.01	0.99	0.09	1.95	0.05
Ames 27114	SS	B14A	H91	A	7.21	3.33	0.18	0.06	0.18	10.96	0.46	2.17	0.05	3.00	0.01
PI 408112	SS	B14A	A214N	A	13.74	8.43	0.71	0.25	0.5	23.63	0.61	1.63	0.06	2.00	0.02
PI 587126	SS	B37	H84	C	8.44	2.38	0.31	0.31	2.72	14.16	0.28	3.55	1.14	8.77	0.04
PI 550555	SS	B37	NC250	B	9.98	3.17	0.33	0.08	0.37	13.93	0.32	3.15	0.12	4.63	0.01
PI 550555	SS	B37		A	17.07	4.57	0.90	0.57	1.15	24.26	0.27	3.74	0.25	2.02	0.03
PI 550483	SS	B37	B76	A	31.28	5.72	1.18	1.11	1.28	40.57	0.18	5.47	0.22	1.15	0.04
PI 587139	SS	B73	A679	B	9.11	2.51	0.58	0.8	0.57	13.57	0.28	3.63	0.23	0.71	0.09
PI 608767	SS	B73	B84	A	12.15	4.18	0.33	0.22	0.49	17.37	0.34	2.91	0.12	2.23	0.02
Ames 27138	SS	B73	B73	A	11.75	4.79	1.27	0.91	0.57	19.29	0.41	2.45	0.12	0.63	0.08
Ames 27138	SS	N28	N29Ht	A	22.9	9.17	0.9	0.31	0.58	33.66	0.40	2.50	0.06	1.87	0.01
Ames 27143	SS	SS-mixed	NC324	B	17.62	4.86	0.95	0.99	3.9	29.31	0.39	3.63	0.46	3.98	0.06
Ames 27148	SS	SS-mixed	H100	A	11.12	8.17	0.88	0.14	0.48	20.79	0.73	1.36	0.06	3.43	0.01
Ames 19311	SS	SS-mixed	A641	A	8.29	5.97	3.57	0.34	1.1	19.27	0.72	1.39	0.18	3.24	0.04
PI 587131	Popcorn	HP901		A	8.25	12.76	3.03	0.36	2.1	26.79	1.55	0.65	0.16	5.83	0.04
PI 531085	NSS	C103	NC262	A	4.89	13.66	1.4	0.09	1.46	21.5	2.79	0.36	0.11	16.22	0.02
PI 550472	NSS	C103	B57	A	21.49	2.45	1.12	1.39	0.74	27.19	0.11	8.77	0.30	0.53	0.06
Ames 23450	NSS	CO109 Mo17	A556	B	14.5	4.26	0.76	0.79	1.75	22.06	0.29	3.40	0.41	2.22	0.05
PI 558532	NSS	CO109 Mo17	Mo17	A	17.24	14.25	1.18	0.14	1.1	38.91	0.83	1.21	0.08	7.86	0.01
NSL 30904	NSS	GaSC	4226	A	16.9	8.01	0.68	0.15	0.34	26.08	0.47	2.11	0.04	2.27	0.01
Ames 19929	NSS	Hy.T8 Wf9	H49	A	22.01	10.94	0.47	0.24	1.3	34.96	0.50	2.01	0.12	5.42	0.01
Ames 28367	NSS	K54W	C17	C	6.36	2.77	0.64	0.87	0.63	24.47	0.44	2.30	4.92	15.67	0.14
Ames 28367	NSS	K54W	C131A	A	18.36	0.89	0.56	0.46	0.92	21.08	0.05	20.63	0.92	1.79	0.03
Ames 19288	NSS	M14.Dn43	Dn43	B	24.9	7	0.78	0.35	2.25	35.28	0.28	3.56	0.32	0.82	0.01
PI 587139	NSS	M14.Dn43	A619	B	10.03	10.05	2.46	0.41	6.11	29.06	1.00	1.00	0.61	14.90	0.04
NSL 30967	NSS	M14.Dn43	M14	A	31.33	8.79	1.06	0.68	1.8	43.66	0.28	3.56	0.20	2.65	0.02
Ames 27190	NSS	NSS-mixed	T234	C-like	7.004	0.479	0.096	0.12	0.808	8.5	0.07	14.62	1.69	6.73	0.02
PI 608765	NSS	NSS-mixed	B77	C	8.19	5.95	1.23	0.61	8.46	24.44	0.73	1.38	1.42	13.87	0.07
Ames 27129	NSS	NSS-mixed	SD44	A	13.92	5.12	0.66	0.22	1.95	21.88	0.37	2.72	0.38	8.72	0.02
Ames 26604	NSS	NSS-X	3811	A	25.39	8.52	0.6	1.42	1.69	37.62	0.34	2.98	0.20	1.19	0.06
Ames 23405	NSS	NSS-X	A239	A	13.28	18.14	2.18	0.07	2.71	36.38	1.37	0.73	0.15	38.71	0.01
Ames 23405	NSS	NSS-X	A239	A	13.93	13.26	2.92	0.44	2.39	32.94	0.95	1.05	0.18	5.43	0.03
Ames 27189	Mixed	DE3		C	13.95	2.40	0.70	1.52	19.34	31.82	0.17	5.77	5.56	8.76	0.11
Ames 27189	Mixed	SC26		C	2.08	0.14	0.801	0.97	7.2	10.7	0.07	14.96	9.28	0.42	11.98
Ames 27066	Mixed	B104		B	11.54	5.53	0.93	0.11	0.91	18.62	0.47	2.14	0.15	7.36	0.01
Ames 27098	Mixed	CML 328		A	3.95	23.56	10.84	0.51	7.7	45.96	7.03	0.14	0.33	15.10	0.15
Ames 26366	Mixed	N7A		A	24.48	6.18	1.48	1.34	0.64	34.12	0.25	3.96	0.10	0.48	0.05
Ames 27067	Mixed	C49A		A	6.95	9.19	1.33	0.1	0.53	18.1	1.32	0.76	0.06	5.30	0.01
					13.05	9.00	1.72	0.51	2.50	26.56	0.94	3.32	1.60	8.14	0.05
					average										

* Accessions: Maize lines were obtained from the National Germplasm Collection, except for the initial 10 line subset described in the manuscript.

** Genetic diversity group/subgroup: Taken from Liu, K. et al. Genetic structure and diversity among maize Inbred lines as inferred from DNA microsatellites. Genetics 165, 2117-2128 (2003). The authors state in that paper: "The 260 lines ...are listed with the groups and subgroups from the STRUCTURE analysis. Lines in the mixed group show 80% membership for any group. Seven popcorn lines and five sweet corn lines were assigned into predefined popcorn and sweet corn groups. Within TS, SS, and NSS groups, lines are organized into several distinct subgroups and one mixed subgroup on the basis of analyses with the program STRUCTURE. The subgroups are named after a defining inbred line(s), principal source (e.g., NC for North Carolina), maturity (early vs. late), or mixed for lines that showed 80% membership for any subgroup."

*** *HYD3* Allele was determined by PCR genotyping and DNA sequence analysis.

****Carotenoid composition and content is based on data provided in Harjes et al., 2008 Science 319, 330-3 (2008) except for CML228, P39, HP301 which were described in Islam, S. N., Masters Thesis in Crop Sciences, University of Illinois at Urbana-Champaign 93 (University of Illinois at Urbana-Champaign, Urbana-Champaign, 2004).

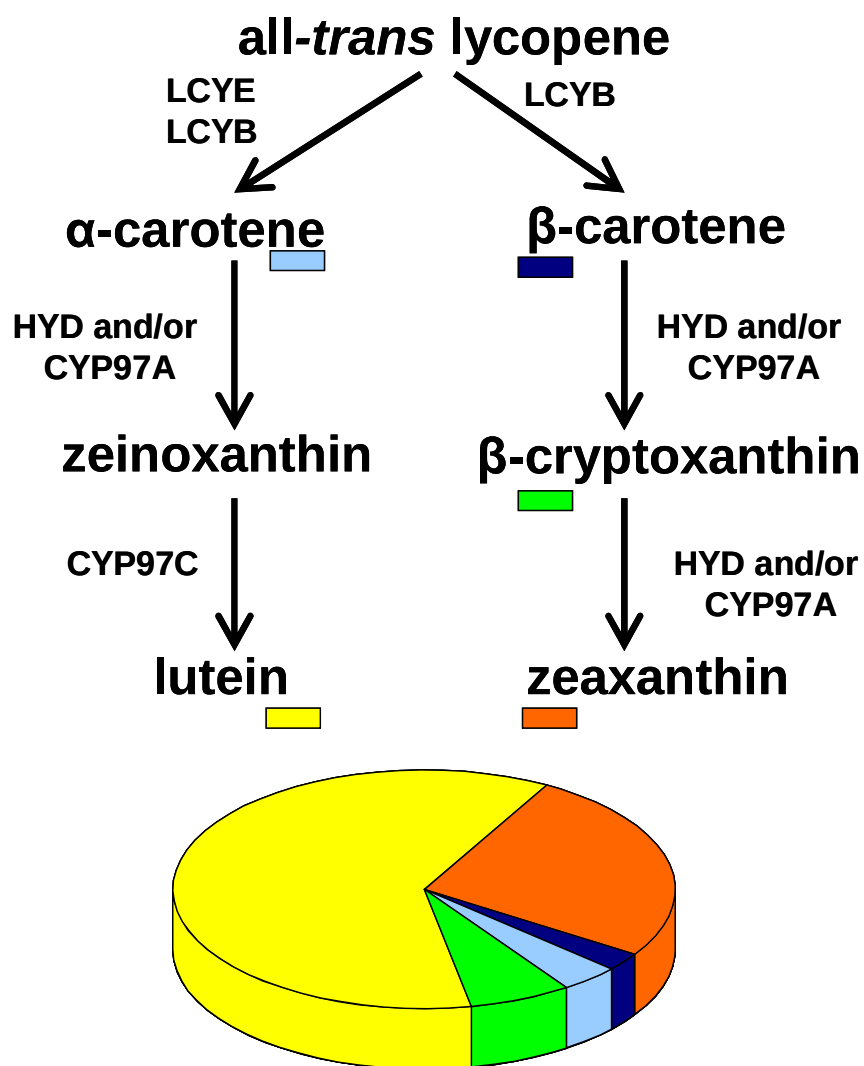
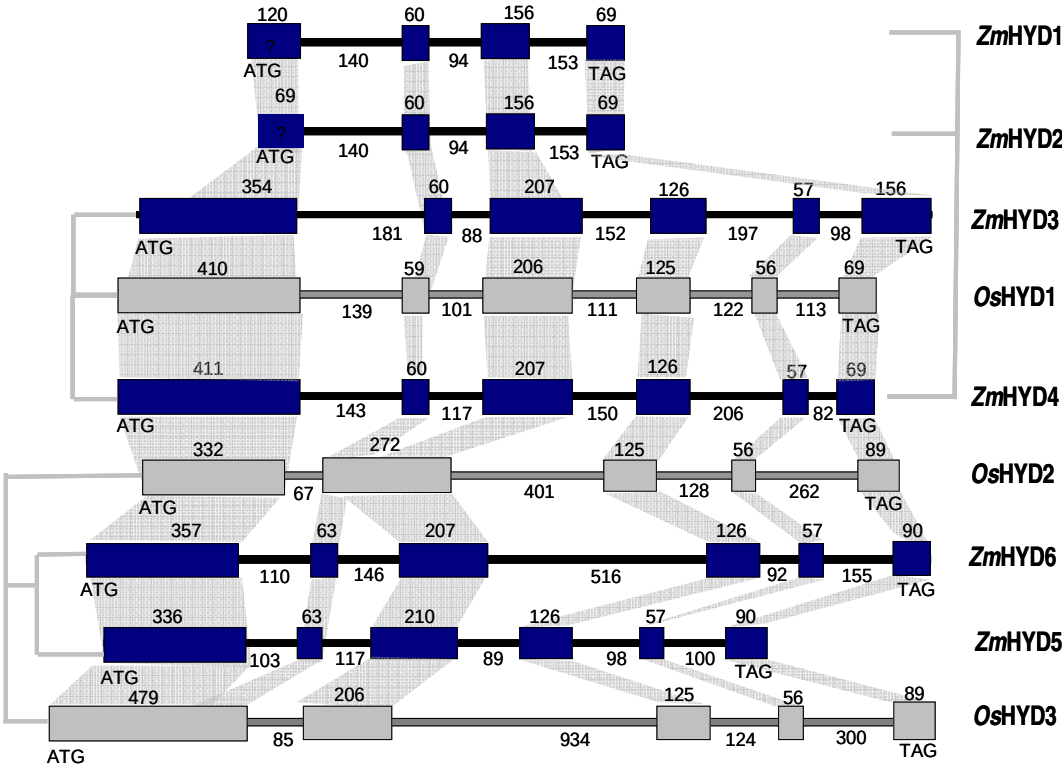


Figure 3-1. Conversion of provitamin A carotenenes to nonprovitamin xanthophylls. Relative abundance of mature kernel carotenoids in maize inbred B73. LCYB, lycopene β -cyclase; LCYE, lycopene ϵ -cyclase; HYD, β -carotene hydroxylase (diiron); CYP97A, β -carotene hydroxylase (P450); CYP97C, ϵ -carotene hydroxylase (P450).

A



B

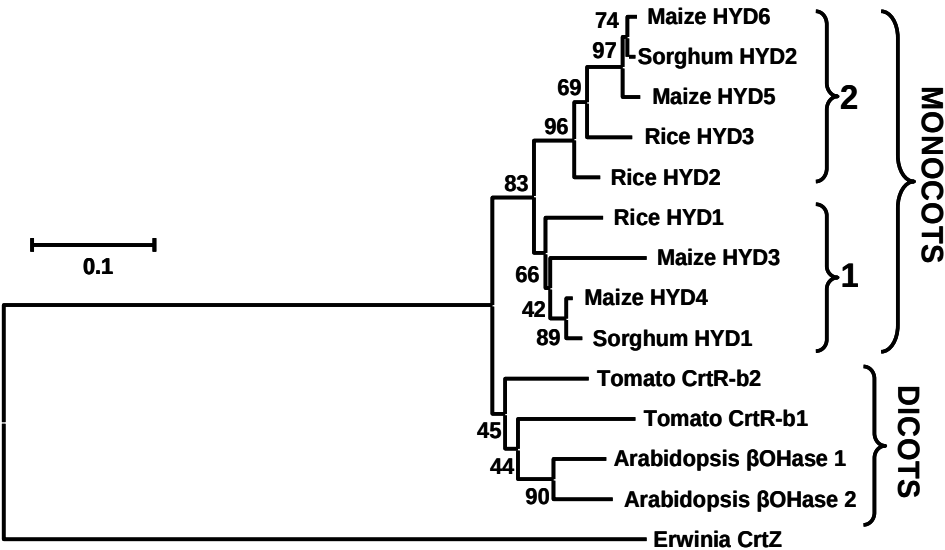


Figure 3-2. HYD gene family structure and phylogeny.

(A) Gene organizational structure showing paralogs and orthologs of the *HYD* gene family in maize (Zm) and rice (Os) species representing two subfamilies of the Poaceae (grasses). Boxes and lines indicate exons and introns respectively, sizes for which are in base pairs. Vertical lines connecting paralogs represent a sub-family. **(B)** Phenetic analysis showing the evolution and duplication of carotenoid hydroxylase amino acid sequences. Monocot sequences fall into two major groups labeled 1 and 2. GenBank accessions are in parentheses: *Erwinia* (*crtZ* AAA64983), *Arabidopsis* (β OHase1, AT4G25700; β OHase2, AT5G52570), tomato (*crtR*-b1, Y14809; *crtR*-b2, Y14810); See Table 3-1 for list of monocot accession numbers.

Synteny between maize and rice to locate HYD orthologs

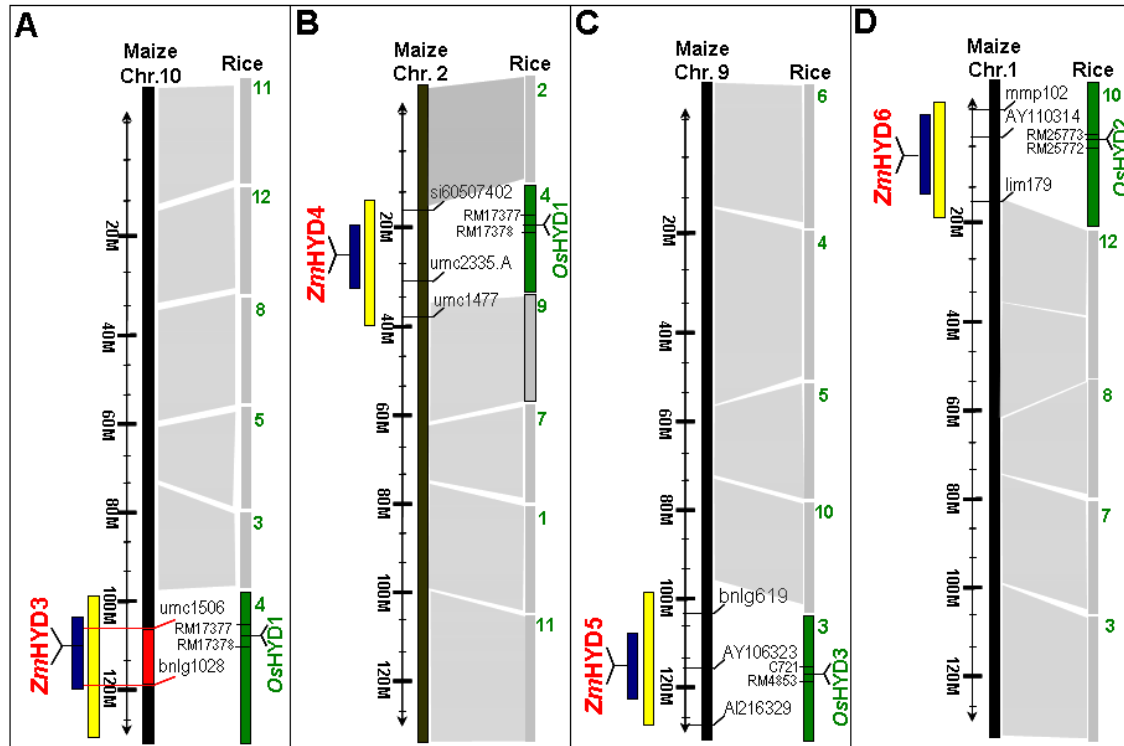


Figure 3-3. Synteny between maize and rice to map maize *HYD* orthologs

Orthologous genes encoding maize and rice nonheme diiron β -carotene hydroxylases were compared by synteny, where each chromosome of maize is syntenous to multiple rice chromosome regions numbered in green. Each maize chromosome is marked by bin (yellow box), contig (dark blue box), with its respective maize gene and linked genetic markers. Maize *HYD3* (*ZmHYD3*) maps to chromosome 10 between DNA markers *umc1506* and *bnlg1028* which flank a β -carotene QTL (Chander et al., 2007) colored in red. A. *ZmHYD3* (BAC clone # c0156B23; contig# 415; chr 10.05); B. *ZmHYD4* (BAC clone # c0112K16; contig #73; chr 2.03); C. *ZmHYD5* (BAC clone # b0589L12; contig # 391; chr 9.07); D. *ZmHYD6* (BAC clone # b0072L05; contig #03; chr 1.01).

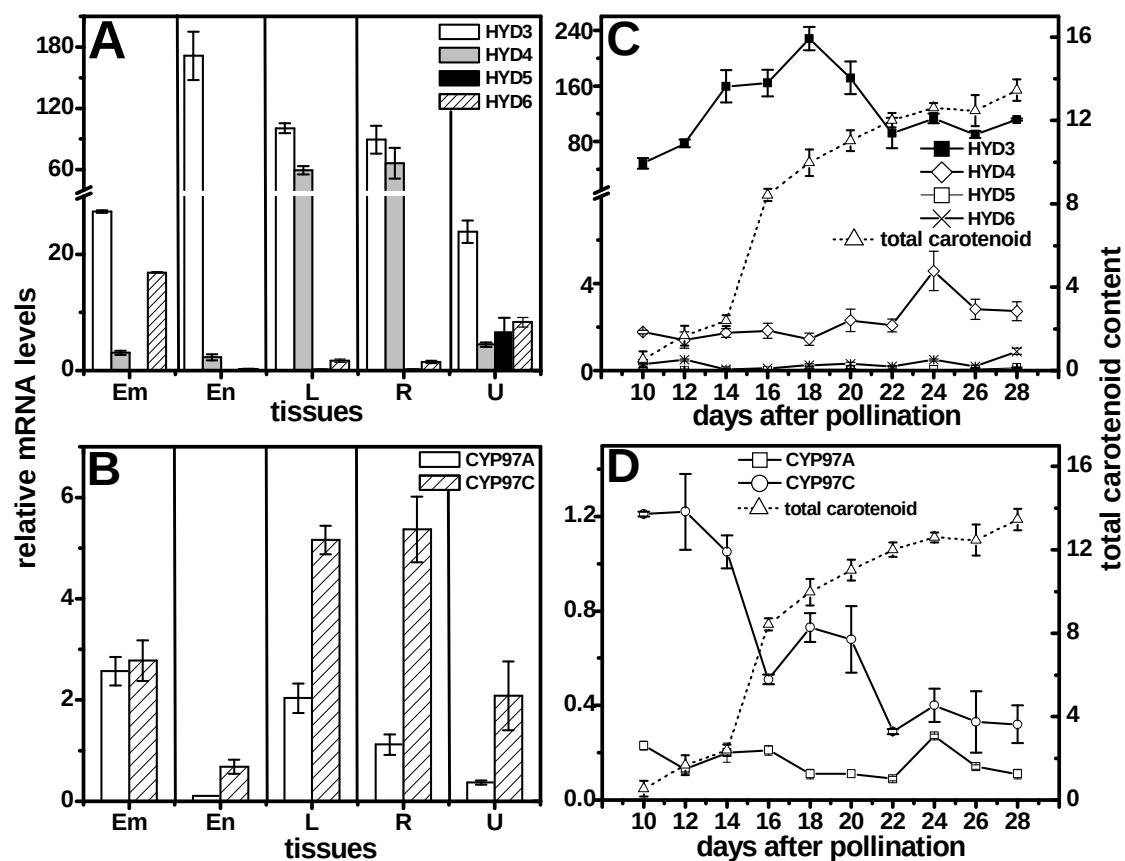


Figure 3-4. Transcript profiling of hydroxylase gene family members in maize standard, B73 inbred.

Transcript levels of *HYD* (A) and P450-type (B) hydroxylase genes in different maize tissues: embryo (Em) and endosperm (En) collected at 20 DAP from field grown plants; leaf (L) and root (R) samples collected from seedlings at the six-leaf stage; unfertilized ovule (U). Transcript levels were expressed relative to endosperm levels at 20 DAP. Transcript abundance in developing endosperm is compared to accumulation of total carotenoids (C and D). Mean of three replicates $\pm$ standard deviation.

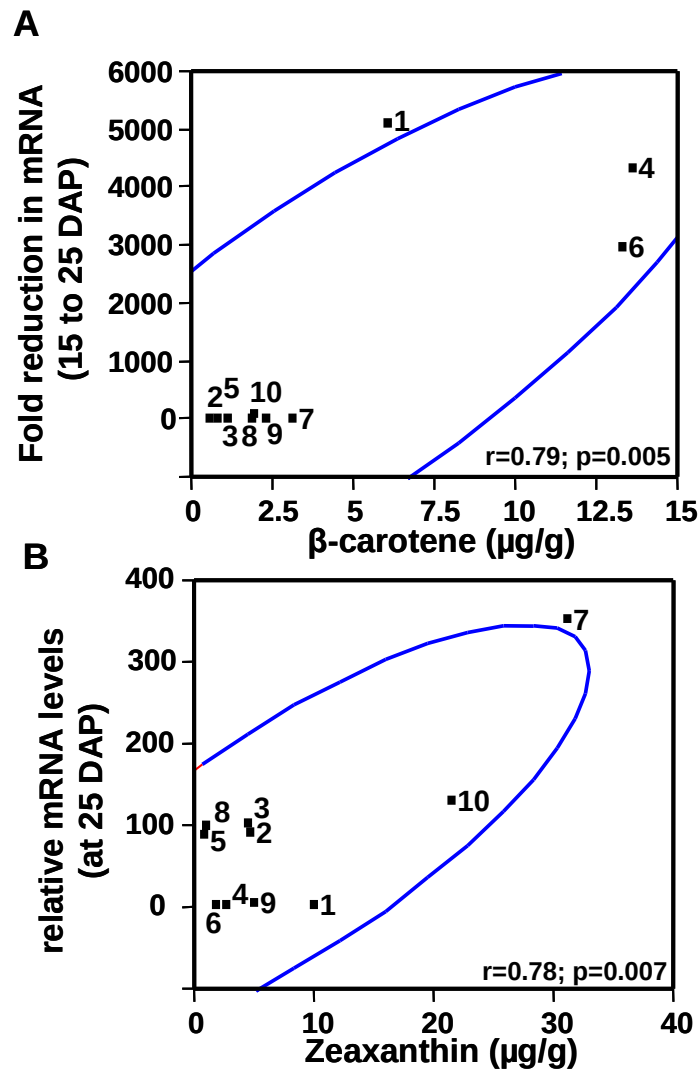


Figure 3-5. Correlation analysis showing *HYD3* mRNA levels correlate with endosperm provitamin A content.

A) Provitamin A β -carotene levels correlate with the fold reduction in *HYD3* transcripts between 15 and 25 DAP. B) Zeaxanthin levels correlate with *HYD3* transcripts at 25 DAP. Pearson correlation (r) and statistical significance (p), for comparison of *HYD3* transcript levels and kernel carotenoid composition (taken from Table 3-4) in maize inbred lines (Harjes et al., 2008) performed using JMP v. 5.1.2 (SAS Institute, Cary, NC) to test the statistical significance ($p \leq 0.05$) of the relationship at a 95% confidence interval (blue line) of the bivariate normal distribution. Inbred lines are: 1, A619; 2, B73; 3, B37; 4, CI.7; 5, C131A; 6, DE3; 7, KUI2007; 8, NC300; 9, SD44; 10, TZI18.

. OsHYD1 : GCCCTTGACGCGTGTGGATC AATCGACCGG AACCAGGGC ATC. C TTC GCTGACAT CGTCT
 . ZmHYD4 B73 : CTGCGTACTACAGTAAATGCA GAAACATGGACCGTTGCTTTTGA GAAAC...TG
 A. ZmHYD3 B73 : CATTCCAATTCCGCCACCGCA TTCTTTCTTTCCGCGGACGCGTTC GAAAC...AG
 A. ZmHYD3 B37 : CATTCCAATTCCGCCACCGCA TTCTTTCTTTCCGCGGACGCGTTC GAAAC...AG
 A. ZmHYD3 C131A : CATTCCAATTCCGCCACCGCA TTCTTTCTTTCCGCGGACGCGTTC GAAAC...AG
 A. ZmHYD3 KUI2007 : CATTCCAATTCCGCCACCGCA TTCTTTCTTTCCGCGGACGCGTTC GAAAC...AG
 A. ZmHYD3 NC300 : CATTCCAATTCCGCCACCGCA TTCTTTCTTTCCGCGGACGCGTTC GAAAC...AG
 A. ZmHYD3 SD44 : CATTCCAATTCCGCCACCGCA TTCTTTCTTTCCGCGGACGCGTTC GAAAC...AG
 A. ZmHYD3 TZI18 : CATTCCAATTCCGCCACCGCA TTCTTTCTTTCCGCGGACGCGTTC GAAAC...AG
 B. ZmHYD3 A619 : CATTCCAATTCCGCCACCGCA TTCTTTCTTTCCGCGGACGCGTTC GAAAC...AG
 C. ZmHYD3 CI.7 : CATTCCAATTCCGCCACCGCA TTCTTTCTTTCCGCGGACGCGTTC GAAAC...AG
 C. ZmHYD3 DE3 : CATTCCAATTCCGCCACCGCA TTCTTTCTTTCCGCGGACGCGTTC GAAAC...AG

. OsHYD1 : TGAGCCAACAGCAATACAGTCAATTTCCCGCTTCGTCGTCGCCAGATGACACAGGCTTCCACCTAAGCC
 . ZmHYD4 B73 : AGAACCAATTTCGATTTTCTCATTCCGCGACGCGCACGCTTTTC TTCTCTCCGCG CAAAGCCAGCC
 A. ZmHYD3 B73 : GGAGCCTCTGGGGAGACTCGAG GGCACCTCTGCTCTTCCCG TTCTATCTCTG TGAGCTGTAAAC
 A. ZmHYD3 B37 : GGAGCCTCTGGGGAGACTCGAG GGCACCTCTGCTCTTCCCG TTCTATCTCTG TGAGCTGTAAAC
 A. ZmHYD3 C131A : GGAGCCTCTGGGGAGACTCGAG GGCACCTCTGCTCTTCCCG TTCTATCTCTG TGAGCTGTAAAC
 A. ZmHYD3 KUI2007 : GGAGCCTCTGGGGAGACTCGAG GGCACCTCTGCTCTTCCCG TTCTATCTCTG TGAGCTGTAAAC
 A. ZmHYD3 NC300 : GGAGCCTCTGGGGAGACTCGAG GGCACCTCTGCTCTTCCCG TTCTATCTCTG TGAGCTGTAAAC
 A. ZmHYD3 SD44 : GGAGCCTCTGGGGAGACTCGAG GGCACCTCTGCTCTTCCCG TTCTATCTCTG TGAGCTGTAAAC
 A. ZmHYD3 TZI18 : GGAGCCTCTGGGGAGACTCGAG GGCACCTCTGCTCTTCCCG TTCTATCTCTG TGAGCTGTAAAC
 B. ZmHYD3 A619 : GGAGCCTCTGGGGAGACTCGAG GGCACCTCTGCTCTTCCCG TTCTATCTCTG TGAGCTGTAAAC
 C. ZmHYD3 CI.7 : GGAGCCTCTGGGGAGACTCGAG GGCACCTCTGCTCTTCCCG TTCTATCTCTG TGAGCTGTAAAC
 C. ZmHYD3 DE3 : GGAGCCTCTGGGGAGACTCGAG GGCACCTCTGCTCTTCCCG TTCTATCTCTG TGAGCTGTAAAC

. OsHYD1 : ACCGTCGAGACTCGAGCTCGAGCTC.TCTTTAAGTAGTAGTACTACGACGAGAGCGCGGGGTCTCTCGATTAC
 . ZmHYD4 B73 : TCTGTCGAGACTCGAGCTCT.TAAATAGT.GTGACCGCTGTCTCTTTCTTCTGCTCTGTCTTCTGCTCAT...C
 A. ZmHYD3 B73 : GCTTGGAGGTATGTCAACTATCCATATAAGTTCTGTGAGCTG.TTTCGCGCTCACTCGGTCCCTGTCCAT...C
 A. ZmHYD3 B37 : GCTTGGAGGTATGTCAACTATCCATATAAGTTCTGTGAGCTG.TTTCGCGCTCACTCGGTCCCTGTCCAT...C
 A. ZmHYD3 C131A : GCTTGGAGGTATGTCAACTATCCATATAAGTTCTGTGAGCTG.TTTCGCGCTCACTCGGTCCCTGTCCAT...C
 A. ZmHYD3 KUI2007 : GCTTGGAGGTATGTCAACTATCCATATAAGTTCTGTGAGCTG.TTTCGCGCTCACTCGGTCCCTGTCCAT...C
 A. ZmHYD3 NC300 : GCTTGGAGGTATGTCAACTATCCATATAAGTTCTGTGAGCTG.TTTCGCGCTCACTCGGTCCCTGTCCAT...C
 A. ZmHYD3 SD44 : GCTTGGAGGTATGTCAACTATCCATATAAGTTCTGTGAGCTG.TTTCGCGCTCACTCGGTCCCTGTCCAT...C
 A. ZmHYD3 TZI18 : GCTTGGAGGTATGTCAACTATCCATATAAGTTCTGTGAGCTG.TTTCGCGCTCACTCGGTCCCTGTCCAT...C
 B. ZmHYD3 A619 : GCTTGGAGGTATGTCAACTATCCATATAAGTTCTGTGAGCTG.TTTCGCGCTCACTCGGTCCCTGTCCAT...C
 C. ZmHYD3 CI.7 : ACTTGGACCTCCGCTCC.C.....GGGCTGG.....TTCCGCGCTCACTCGGTCCCTGTCCAT...C
 C. ZmHYD3 DE3 : ACTTGGACCTCCGCTCC.C.....GGGCTGG.....TTCCGCGCTCACTCGGTCCCTGTCCAT...C

. OsHYD1 : ACTCCCGCTCACACTCTC...CGCTCCATCCCTGTCCACGAAAGGCGCGCG...CGTAAGATACGAGGCCGG.
 . ZmHYD4 B73 : TCTACCG...TTCCATTCCTA...ATTCCATCCG...CGGAAAGGCGAGGCC...CGGGGAGAGCGGCCCGG.
 A. ZmHYD3 B73 : CTTAGCG...TTCCATTCCTA...ATTCCATCCG...CGGAAAGGCGAGGCC...CGGGGAGAGCGGCCCGG.
 A. ZmHYD3 B37 : CTTAGCG...TTCCATTCCTA...ATTCCATCCG...CGGAAAGGCGAGGCC...CGGGGAGAGCGGCCCGG.
 A. ZmHYD3 C131A : CTTAGCG...TTCCATTCCTA...ATTCCATCCG...CGGAAAGGCGAGGCC...CGGGGAGAGCGGCCCGG.
 A. ZmHYD3 KUI2007 : CTTAGCG...TTCCATTCCTA...ATTCCATCCG...CGGAAAGGCGAGGCC...CGGGGAGAGCGGCCCGG.
 A. ZmHYD3 NC300 : CTTAGCG...TTCCATTCCTA...ATTCCATCCG...CGGAAAGGCGAGGCC...CGGGGAGAGCGGCCCGG.
 A. ZmHYD3 SD44 : CTTAGCG...TTCCATTCCTA...ATTCCATCCG...CGGAAAGGCGAGGCC...CGGGGAGAGCGGCCCGG.
 A. ZmHYD3 TZI18 : CTTAGCG...TTCCATTCCTA...ATTCCATCCG...CGGAAAGGCGAGGCC...CGGGGAGAGCGGCCCGG.
 B. ZmHYD3 A619 : CTTAGCG...TTCCATTCCTA...ATTCCATCCG...CGGAAAGGCGAGGCC...CGGGGAGAGCGGCCCGG.
 C. ZmHYD3 CI.7 : CTTAGCG...TTCCATTCCTA...ATTCCATCCG...CGGAAAGGCGAGGCC...CGGGGAGAGCGGCCCGG.
 C. ZmHYD3 DE3 : CTTAGCG...TTCCATTCCTA...ATTCCATCCG...CGGAAAGGCGAGGCC...CGGGGAGAGCGGCCCGG.

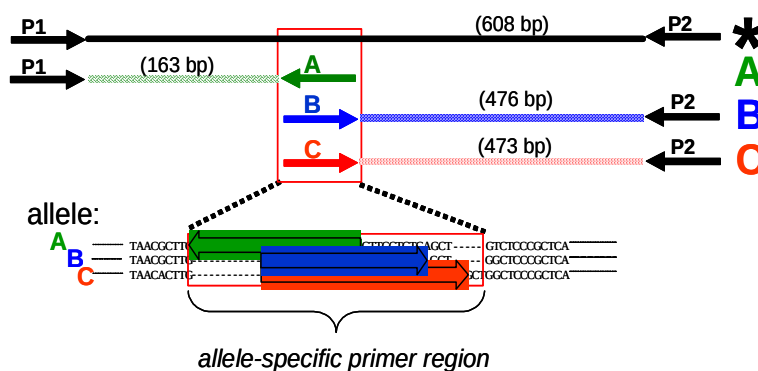
. OsHYD1 : GAGGCAAGAGGACCAAGGATGCGCCACCGCTCTCTCCGCGG.....CGCTATGACACAGCTTCGC
 . ZmHYD4 B73 : GTAGCTACAGGAGGACCGCAGGCCAGGGGCGATGCCCCGCTGTCTCGGCGCCCGATGACACAGCTTCGT
 A. ZmHYD3 B73 : G...CGT...CACAAATACACAGGCCAGGGGCGGATGCCCCG.....CGATGACACAGCTTCGT
 A. ZmHYD3 B37 : G...CGT...CACAAATACACAGGCCAGGGGCGGATGCCCCG.....CGATGACACAGCTTCGT
 A. ZmHYD3 C131A : G...CGT...CACAAATACACAGGCCAGGGGCGGATGCCCCG.....CGATGACACAGCTTCGT
 A. ZmHYD3 KUI2007 : G...CGT...CACAAATACACAGGCCAGGGGCGGATGCCCCG.....CGATGACACAGCTTCGT
 A. ZmHYD3 NC300 : G...CGT...CACAAATACACAGGCCAGGGGCGGATGCCCCG.....CGATGACACAGCTTCGT
 A. ZmHYD3 SD44 : G...CGT...CACAAATACACAGGCCAGGGGCGGATGCCCCG.....CGATGACACAGCTTCGT
 A. ZmHYD3 TZI18 : G...CGT...CACAAATACACAGGCCAGGGGCGGATGCCCCG.....CGATGACACAGCTTCGT
 B. ZmHYD3 A619 : G...CGT...CACAAATACACAGGCCAGGGGCGGATGCCCCG.....CGATGACACAGCTTCGT
 C. ZmHYD3 CI.7 : G...CGT...CACAAATACAGTTGCCAGGGGCGGATGCCCCG.....CGATGACACAGCTTCGT
 C. ZmHYD3 DE3 : G...CGT...CACAAATACAGTTGCCAGGGGCGGATGCCCCG.....CGATGACACAGCTTCGT

. OsHYD1 : CGTCAAGAAACCGCTCTCTCGCGGCGCGGCGCGCGCGCGCTGCGCCCGCGTCTCGGCGCGCGCTGCGCGT
 . ZmHYD4 B73 : CGCCAAGAAACCGCTCTCTCGCGGCGCGGCGCGCGCGCGCGCTGCGCCCGCGTCTCGGCGCGCGCTGCGCGT
 A. ZmHYD3 B73 : CGCCAAGAAACCGCTCTCTCGCGGCGCGGCGCGCGCGCGCGCG...CTCCCTCTCGCGGCGCGCGCTGCGCGT
 A. ZmHYD3 B37 : CGCCAAGAAACCGCTCTCTCGCGGCGCGGCGCGCGCGCGCGCG...CTCCCTCTCGCGGCGCGCGCTGCGCGT
 A. ZmHYD3 C131A : CGCCAAGAAACCGCTCTCTCGCGGCGCGGCGCGCGCGCGCGCG...CTCCCTCTCGCGGCGCGCGCTGCGCGT
 A. ZmHYD3 KUI2007 : CGCCAAGAAACCGCTCTCTCGCGGCGCGGCGCGCGCGCGCGCG...CTCCCTCTCGCGGCGCGCGCTGCGCGT
 A. ZmHYD3 NC300 : CGCCAAGAAACCGCTCTCTCGCGGCGCGGCGCGCGCGCGCGCG...CTCCCTCTCGCGGCGCGCGCTGCGCGT
 A. ZmHYD3 SD44 : CGCCAAGAAACCGCTCTCTCGCGGCGCGGCGCGCGCGCGCGCG...CTCCCTCTCGCGGCGCGCGCTGCGCGT
 A. ZmHYD3 TZI18 : CGCCAAGAAACCGCTCTCTCGCGGCGCGGCGCGCGCGCGCGCG...CTCCCTCTCGCGGCGCGCGCTGCGCGT
 B. ZmHYD3 A619 : CGCCAAGAAACCGCTCTCTCGCGGCGCGGCGCGCGCGCGCGCG...CTCCCTCTCGCGGCGCGCGCTGCGCGT
 C. ZmHYD3 CI.7 : CGCCAAGAAACCGCTCTCTCGCGGCGCGGCGCGCGCGCGCGCG...CTCCCTCTCGCGGCGCGCGCTGCGCGT
 C. ZmHYD3 DE3 : CGCCAAGAAACCGCTCTCTCGCGGCGCGGCGCGCGCGCGCGCG...CTCCCTCTCGCGGCGCGCGCTGCGCGT

Figure 3-6. Alignment of maize sister paralogs *HYD3* and *HYD4* plus their rice syntenic partner *HYD1*.

DNA sequences were aligned for *HYD3* and *HYD4*, their rice syntenic partner *HYD1*, and the *HYD3* sequences of all of the maize inbreds used in this study. Transcription start sites (colored nucleotides) for B73 *HYD3*, B73 *HYD4* and rice *HYD1* were estimated by aligning ESTs. The first ATG for *HYD1* is denoted by a blue overline and contained within a red box for the remaining *HYD3* and *HYD4* sequences. A blue box encompasses the variant region that distinguishes the high and low β -carotene lines. Black, dark grey, and light grey shading indicate degree of conserved nucleotides where black is the highest (100%) match.

A. Allele-specific primer design



B. Allele screen in high and low β -carotene lines

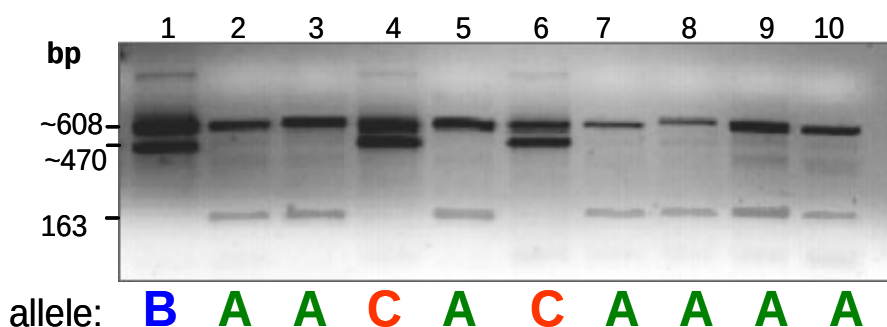


Figure 3-7. Multiplex PCR diagnosis of *HYD3* alleles in high and low β -carotene lines.

The sequence variation in *HYD3* (Fig. 3-6, blue box) was used to develop a multiplex PCR assay to track *HYD3* alleles (**B** and **C**) in the “high β -carotene” lines and to distinguish these from the **A** allele in the “low β -carotene” line. With this simple PCR assay, natural alleles can be tracked using traditional, nontransgenic breeding. The assay contained four primers to (a) amplify a control, *HYD3*-specific product (*), which was conserved in all alleles and encompassed the allele-specific region (Fig. 3-12; Fig. 3-6, blue box), and (b) to amplify and distinguish specific alleles **A** from **C** or **A** from **B**. The multiplex PCR assay was next used to genotype all of the 10 inbred lines used in the correlation study and as predicted, all lines having **B** or **C** alleles, also had a high β -carotene content (21-55% of total carotenoids) as compared to maize B73 (**A** allele) where kernels have only 3% β -carotene. A) Allele specific primer design. Top, *HYD3* primers (P1 and P2) amplify all alleles, as product (*), the size of which is shown based on the A allele (B and C alleles generate a * product that differs by a few bp); allele-

specific primers (A, B, or C) amplify maize allele A from low β -carotene line B73, allele B from high β -carotene line A619 or allele C from high β -carotene lines CI.7 and DE3, respectively. **Bottom**, The boxed region used to design the allele-specific primers is shown in more detail. Sequences and amplification direction are shown in corresponding colors for the three alleles. PCR product sizes are based on the **A** allele; products from the other alleles differ slightly. *B) Screening of alleles in the maize diversity lines.* Primers used were **P1/P2/A** plus either **B** (lane 1) or **C** (lanes 2-10). Inbred lines are: 1, A619; 2, B73; 3, B37; 4, CI.7; 5, C131A; 6, DE3; 7, KUI2007; 8, NC300; 9, SD44; 10, TZI18.

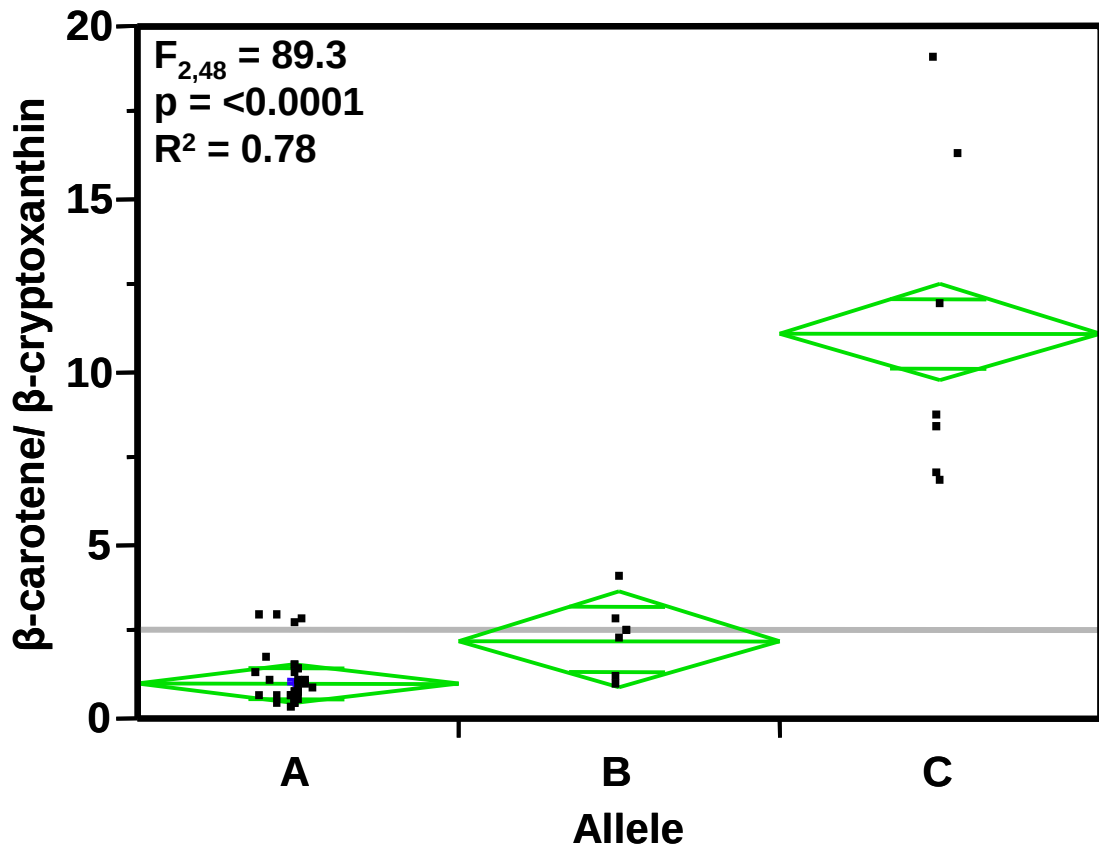
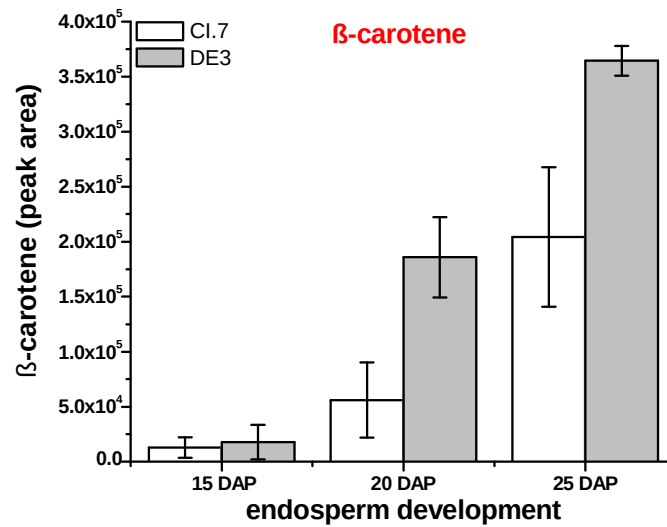


Figure 3-8. One-way ANOVA between *HYD3* alleles (A, B, C) and the ratio of β -carotene/ β -cryptoxanthin in 51 genetically diverse maize lines.

The mean ratios of β -carotene/ β -cryptoxanthin showed a statistically significant difference among the three *HYD3* alleles in One-way ANOVA ($F_{2,48} = 89.3$, $p < 0.0001$) and the *HYD3* allele explained 78% of the variation (R^2) in the β -carotene/ β -cryptoxanthin ratio. Three different alleles denoted as A, B, C are plotted on the X-axis and ratios of β -carotene (ug) / β -cryptoxanthin (ug) are plotted on the Y-axis. Gray line indicates the grand mean of the entire sample. The green diamonds indicate 95% confidence interval of the mean, and the dots represent each inbred line in study.

A



B

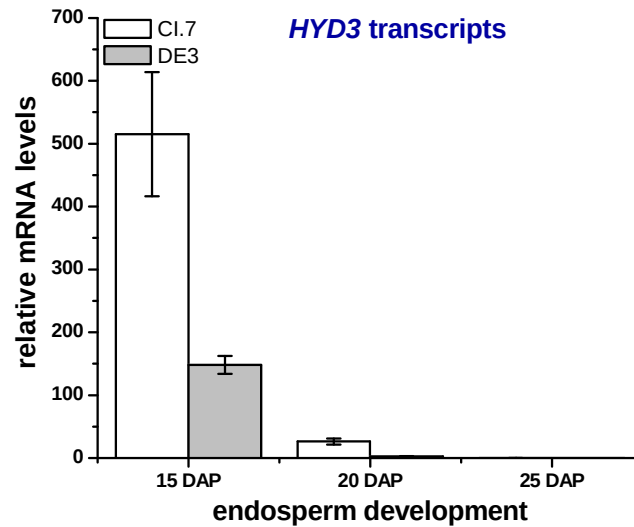


Figure 3-9. Relative amount of β -carotene increases and *HYD3* transcript levels decrease during development of maize endosperm in lines carrying a C allele of *HYD3*.

Total carotenoids were extracted and equal amounts of total carotenoids subjected to UPLC separation and quantification of betacarotene in triplicate. Samples from the same endosperm samples were subjected to quantitative RT-PCR. Means with standard deviation were plotted.

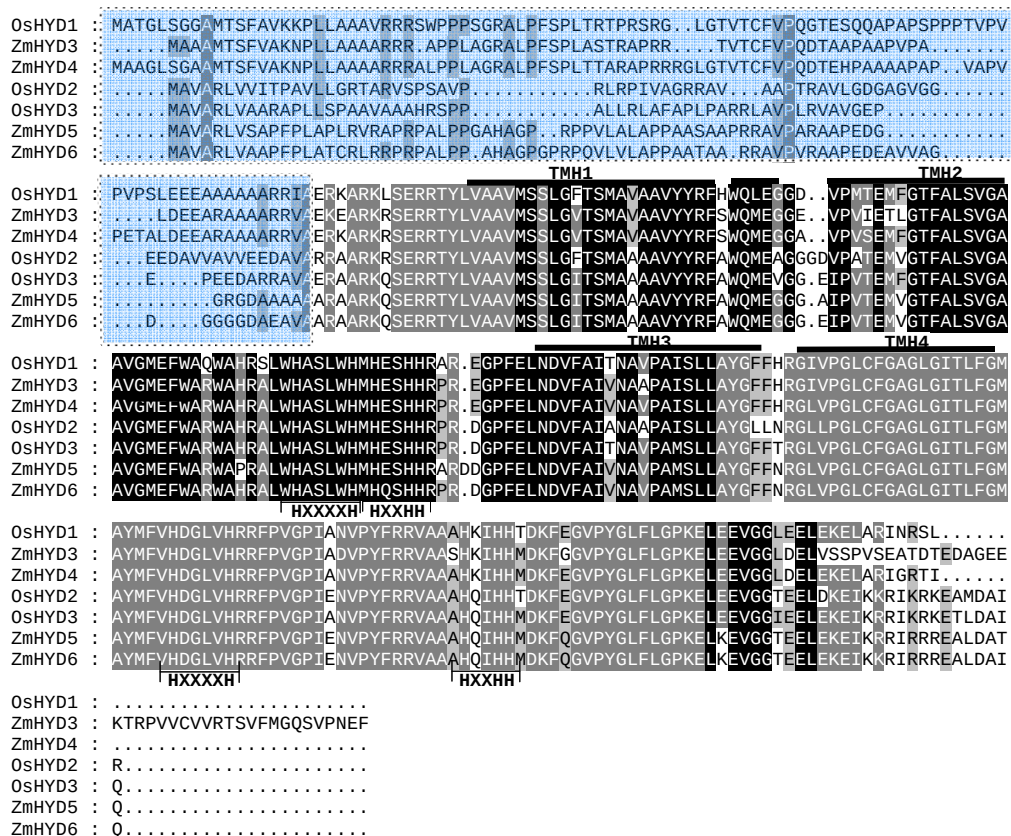


Figure 3-10. Conserved motifs in HYD proteins of maize and rice.

Multiple sequence alignment of HYD protein sequences from maize (*Zm*; *Zea mays*) and rice (*Os*; *Oryza sativa*) indicates variable N-termini harboring the chloroplast transit peptide (light blue shaded box), four transmembrane helices (TMH1-4) and four histidine motifs necessary for enzymatic function (consensus sequences indicated by a thin overline).

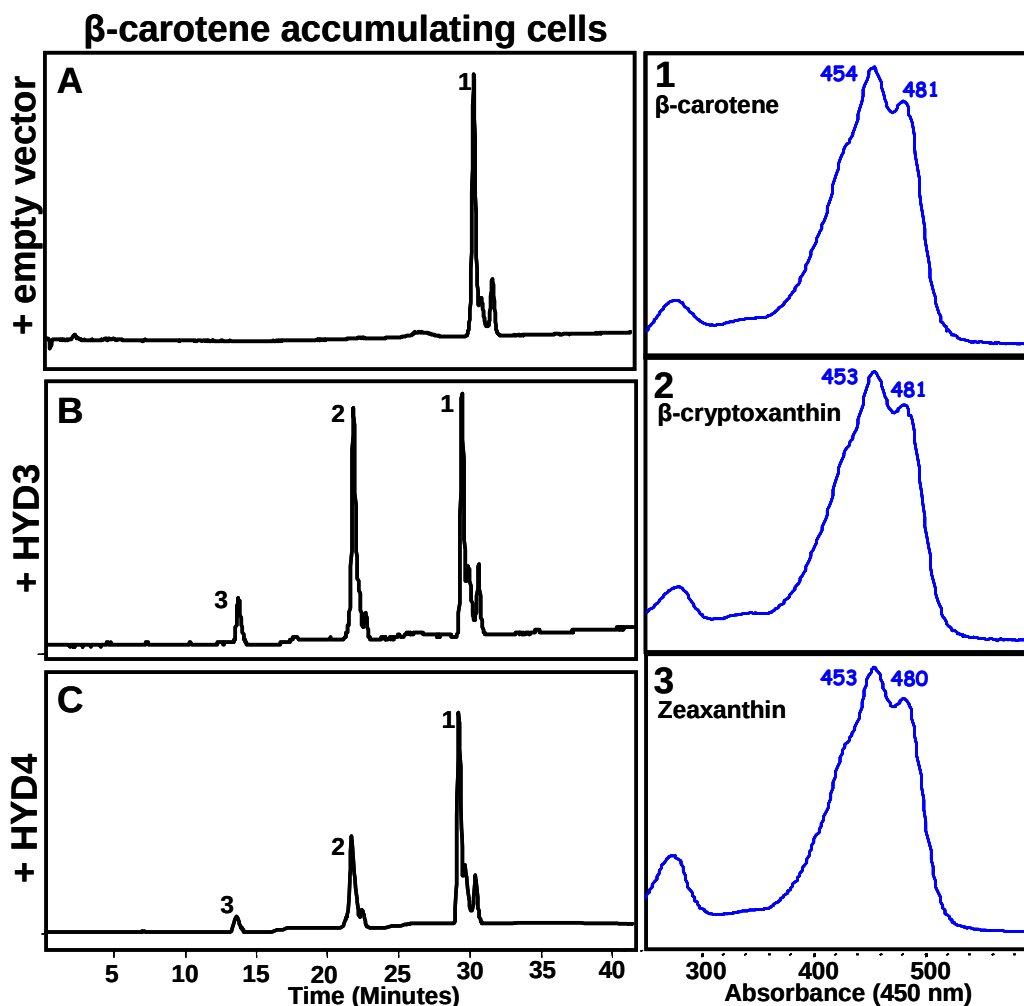


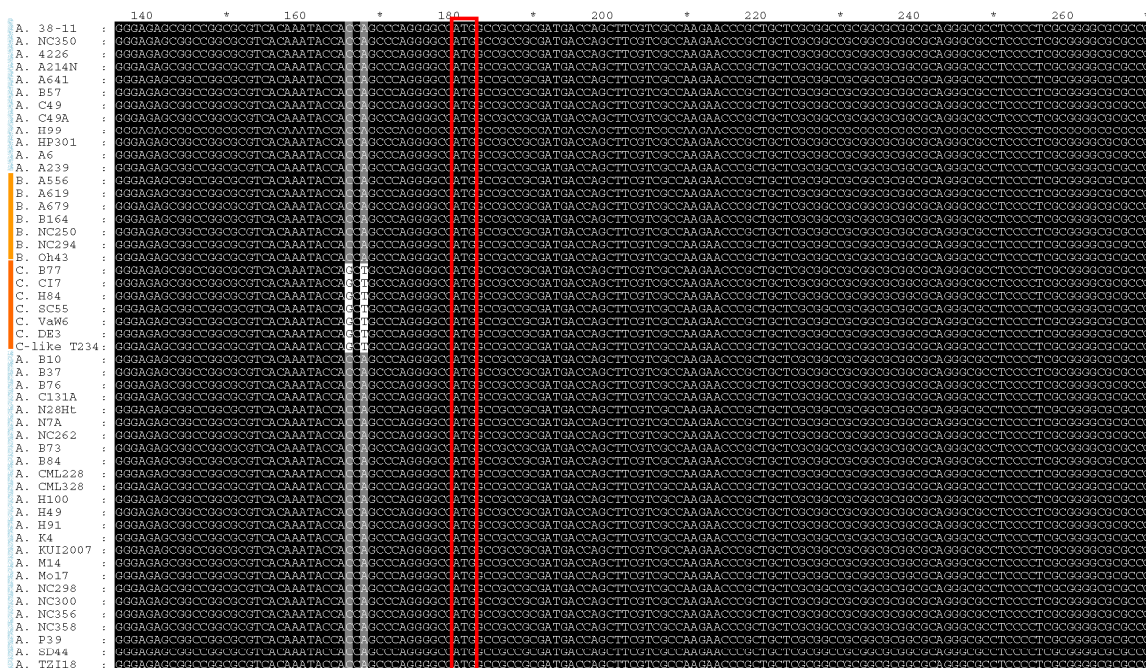
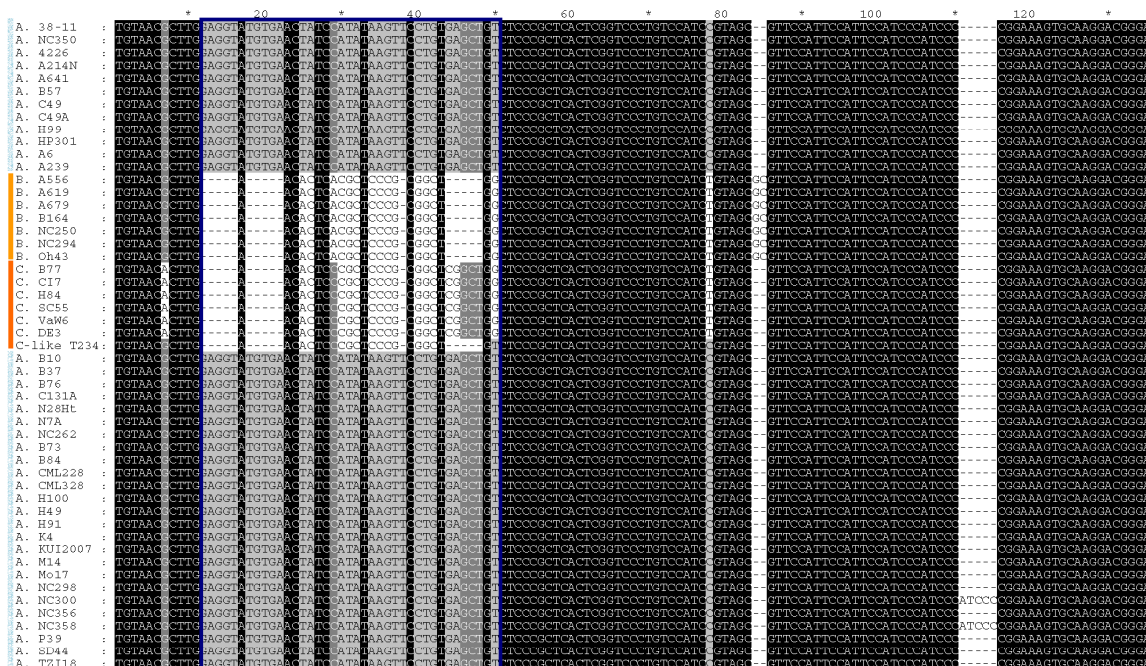
Figure 3-11. Functional complementation of *HYD* genes.

Control cells carrying pAC-BETA-04 which contains genes encoding enzymes needed for β -carotene production (Sun et al., 1996) and an empty vector (pET23c) accumulated the β -carotene substrate (Fig. S2A). Alternatively, when β -carotene –accumulating cells were transformed with the *HYD3* (Fig. S2B) or *HYD4* (Fig. S2C) expression constructs, additional peaks were observed that corresponded to enzyme intermediate, β -cryptoxanthin, and enzyme product, zeaxanthin. This observed hydroxylase activity was similar to that seen for the other class of β -ring hydroxylase, the P450 CYP97A enzyme, which also hydroxylates β -rings, in contrast to CYP97C which was shown to hydroxylate ϵ -rings (Quinlan et al., 2007). **Left**, HPLC chromatograms, at 450 nm of extracted pigments from *E. coli* transformed with pAC-BETA-04 and (A) pET23C (empty vector), showing accumulated β -carotene (peak 1); or (B) pTHYD3 encoding HYD3,

showing β -carotene plus β -cryptoxanthin and zeaxanthin (peaks 1-3, respectively); or (C) pTHYD4 encoding HYD4, showing β -carotene plus β -cryptoxanthin and zeaxanthin (peaks 1-3, respectively). **Right**, UV spectra of peaks 1-3 shown in the chromatograms on the left.

Figure 3-12. Alignment of maize *HYD3* in 51 lines.

Variant 5' region was amplified by PCR primers P1/P2 (Fig. 3-7). Allele and corresponding inbred are denoted on left of each sequence. Colors are as in Fig 6.



4

[illegible]

Chapter 4

Identification and characterization of carotenoid dioxygenase gene family in the Poaceae³

4.1 Abstract

Carotenoids and their apocarotenoid degradative derivatives play essential physiological and developmental roles and provide plants tolerance to a variety of stresses. Degradation of carotenoids to apocarotenoids is mediated by carotenoid cleavage dioxygenases. A better understanding of biosynthesis vs. degradation could be useful for controlling carotenoid levels leading to improved plant fitness and/or enhanced content of nutritionally valuable carotenoids. The Poaceae contains many crops of agronomic value, therefore this study focused on characterizing the carotenoid dioxygenase gene family in the grass species maize, rice, and sorghum. Corresponding sequences were also identified from recently published genomes of the lycophyte, *Selaginella moellendorffii* and the moss *Physcomitrella patens* and an algal genome of *Chlamydomonas reinhardtii*. Genes were mapped to chromosome locus in maize and encoded proteins were phylogenetically compared. CCD8b was identified as a new class of cleavage dioxygenases that may play a specialized role in apocarotenoid biogenesis; CCD4 was found only in flowering plants. The maize gene family was assessed for transcript profiles in tissues varying in accumulated carotenoid levels. *CCD1* in maize was found to vary in gene copy number and preliminary analysis suggested that copy number may be associated with reduced

³ This chapter is submitted to Journal of Experimental Botany as Vallabhaneni et al., [In review]

endosperm carotenoids. The possible role of *CCD1* in diminishing endosperm carotenoid accumulation is consistent with mapping of *CCD1* to maize chromosome 9.07, location of the *white cap1* locus for which dominant alleles condition reduced endosperm carotenoids. This finding has implications for efforts to increase provitamin A carotenoid content of maize endosperm.

4.2 Introduction

In nature, carotenoids have diverse functions in both plants and animals. In plants, algae and cyanobacteria, carotenoids serve as structural and accessory pigments within the light harvesting complex to mediate photosynthesis and for photoprotection. Carotenoids are also enzymatically cleaved to produce apocarotenoids, such as strigolactones and abscisic acid (ABA). Apocarotenoids play numerous physiological roles, including control of plant architecture, dormancy and stress responses, signals attracting beneficial mycorrhizal fungi that promote plant growth and signals attracting harmful parasitic plants (e.g. *Striga*) that compete for plant resources (reviewed in Nambara and Marion-Poll, 2005; Akiyama and Hayashi, 2008). Therefore, synthesis of carotenoids and catabolism to apocarotenoid hormones must be properly balanced for optimal plant development and function.

The primary route in the formation of biologically active apocarotenoids is oxidative cleavage of carotenoids mediated by carotenoid dioxygenases (Bouvier et al., 2005). Two types of carotenoid dioxygenases have been identified in plants, 9-cis carotenoid cleavage dioxygenases (NCEDs) and carotenoid cleavage dioxygenases (CCDs) (**Fig. 4-1**). These enzymes were identified by analyzing the *viviparous* (*vp*) mutants of maize, which led to

cloning of the maize *NCED1* (*Vp14*) (Tan et al., 1997). NCED enzymes cleave the 11,12 (11',12') double bond of 9-*cis*-violaxanthin or 9-*cis*-neoxanthin, catalyzing the first step in ABA biosynthesis (Schwartz et al., 1997; Tan et al., 1997). In contrast, CCD enzymes do not share cleavage specificity with NCED enzymes; some CCD enzymes recognize specific carotenoid or apocarotenoid substrates while others show promiscuity in choice of substrate (as reviewed in Giuliano et al., 2003). As a group, the plant CCD enzymes generate various phytohormones and aroma compounds. Related CCD enzymes in animals cleave carotenoids, such as β -carotene, at the 9,10 (9',10') double bond to form vitamin A (Schwartz et al., 2001).

Carotenoid dioxygenase genes have been identified in various plant species (Giuliano et al., 2003). In the complete genome of *Arabidopsis*, nine genes similar to *VP14* were identified and characterized with regard to their tissue specificity of expression and subcellular localization (Iuchi et al., 2001; Tan et al., 2003; Lefebvre et al., 2006). Five genes encode NCEDs involved in ABA biosynthesis, while the remaining genes encode CCDs.

Less is known about carotenoid cleavage enzymes in crops of agronomic importance. This enzyme class may potentially influence nutritional content (e.g. provitamin A carotenoids) and plant yield. The Poaceae includes the major food crops, for which carotenoid content is a target for improvement. Recent studies revealed genes for biosynthesis that influence carotenoid accumulation, especially in endosperm (Gallagher et al., 2004; Wurtzel, 2004; Li et al., 2007; Matthews and Wurtzel, 2007; Harjes et al.,

2008; Li et al., 2008; Li et al., 2008a; Li et al., 2009; Vallabhaneni and Wurtzel, 2009). However, there is limited knowledge of the carotenoid cleavage genes and their roles in various tissues of maize and other grasses. Therefore this study focused on identifying and characterizing the carotenoid dioxygenase gene family in maize, rice, and sorghum.

4.3 Materials and Methods

4.3.1 Sequence analysis and chromosome mapping

Rice genes (www.gramene.org) were used as a query to identify orthologs from *Zea mays* (www.tigr.org; www.plantgdb.org) and to decipher gene families. The genomic sequence, obtained from the respective BAC clones (**Table 4-1**), was used to deduce the full length cDNA sequence. The exon-intron boundaries were confirmed through selected ESTs, where available. Sequence analysis was performed using Vector NTI Suite 9.0 (Invitrogen, Carlsbad, CA). Conceptual translation of expressed sequence tags (ESTs) was used to distinguish gene paralogs. Chromosomal positions of maize genes in the *Zea mays* B73 inbred were mapped either by utilizing tools available at WebAGCoL package (<http://www.agcol.arizona.edu/software/webagcol/>) (Pampanwar et al., 2005) or Maize GDB. Prediction of chloroplast targeting site was made using the ChloroP software (<http://www.cbs.dtu.dk/services/ChloroP/>) (Emanuelsson et al., 1999) and pSORT (<http://wolfpsort.org/>). Phytozome (<http://www.phytozome.org>) was used to retrieve all non-seed plant sequences. Phenetic analysis was performed using MEGA2 (Kumar et al., 2001).

4.3.2 Quantitative RT-PCR and carotenoid measurements

Plants and tissues of different maize inbreds in a core subset of a maize germplasm collection were collected as described (Vallabhaneni and Wurtzel, 2009). RNA extraction and quantitative RT-PCR were performed using gene specific primers (**Table 4-3**) and normalized to actin, as previously described (Vallabhaneni and Wurtzel, 2009). Values are expressed as the mean of three RT-PCR replicates +/- standard deviation.

4.3.3 Relative quantification of gene-copy number

The genomic DNA prep was quantified using the NanoDrop 1000 (Thermo-Scientific) and diluted to 10ng/ul. Reactions for quantitative real-time PCR were prepared as follows: the 30ul reaction volume contained 15ul Sybr Green master mix (BioRad, CA), 1ul (20pmol/ul) of each primer (**Table 4-3**), 11ul of water and 2ul (10ng/ul) of template. Amplifications were performed in a Bio Rad- iCycler real-time PCR machine. Thermal cycling conditions included an initial incubation at 94°C for 10s, followed by 35 cycles of 95°C for 10s, 58°C for 35s, and 72°C 10s. Both control *phytoene desaturase* (*PDS*) and target *carotenoid cleavage1* (*CCD1*) genes were amplified from each sample to calculate ΔC_t ($\Delta C_t = \text{Control } C_t - \text{Target } C_t$) values. *PDS* is a single copy gene in the maize genome, thus it was used as to normalize values for copy number. All samples were run in triplicate and standard deviation was calculated. Relative quantification of gene copy number ($2^{\Delta C_t}$) was used to plot against total carotenoid content of each sample.

4.3.4 Statistical analyses

Pearson correlation analysis of transcript and carotenoid composition of different maize inbreds in a core subset of a maize germplasm collection (Vallabhaneni and Wurtzel,

2009) and linear regression plot for gene copy number vs. total carotenoid content was performed using JMP v. 5.1.2 (SAS Institute, Cary, NC) to test the statistical significance ($p \leq 0.05$) of the relationship.

4.4 Results and Discussion

4.4.1 Carotenoid dioxygenase gene family in grasses

Using available sequence data, genes encoding carotenoid dioxygenases were identified from the grass species maize, rice and sorghum, and from several non-seed plants and algae (**Tables- 4-1 and 4-2; Fig. 4-3**). Genes were characterized and named according to similarity with genes from *Arabidopsis* (Tan et al., 2003). For the three species spanning two subfamilies of the Poaceae, carotenoid dioxygenases were found to be encoded by a large gene family. This family was divided into seven groups encompassing 2 clades having homology to genes encoding 9-cis-epoxy carotenoid cleavage dioxygenases (NCEDs) and 5 clades having homology to carotenoid cleavage dioxygenases (CCDs).

Table 4-1 summarizes the structural properties of the genes and encoded proteins from maize, sorghum and rice. The alignment of the predicted proteins from maize is shown in Figure 4-7. The amino acid sequences of carotenoid dioxygenases reveal the presence of four conserved histidines that coordinate iron atoms and are required for catalytic activity (Schwartz et al., 1997; Kiefer et al., 2001; Marasco et al., 2006). All maize cleavage dioxygenases are predicted to be plastid-localized except for CCD1, as previously reported (Auldrige et al., 2006). Maize genes were mapped to chromosomes (**Table 4-1**) and no apparent linkage was seen among the gene family members.

Grasses. As shown in Figure 4-2, the *NCED* genes and the *CCD4*-related genes lack introns, while all other *CCD* genes contained multiple introns. There is only one reported gene structure for a *CCD4* gene from *Chrysanthemum morifolium*, containing a single 200 bp intron (Ohmiya et al., 2006); *CCD4* homologs could not be identified in non-seed plants (**Fig. 4-3**). It is known that *NCED* genes lack introns (Tan et al., 2003), but it is unusual for *CCD* genes to lack introns. Maize *CCD4* is unlikely to be an *NCED*, despite its shared intron-less gene structure and sister position to *NCED* genes in the phylogenetic tree. The reasoning for this prediction is that the gene product of a *CCD4* homolog from *Crocus sativus* has been enzymatically characterized as a CCD and not an *NCED* (Rubio et al., 2008). Unlike the substrate-specific *NCED* enzymes, *CCD4* was promiscuous in substrate choice, utilizing zeaxanthin or β -carotene (Rubio et al., 2008).

Diverse taxa. In the *NCED* clade, the phylogenetic tree shows gene duplications having arisen in the grasses after the monocot-dicot evolutionary split (**Fig. 4-3**). Based on this evidence, expression profiles in dicots would not be predictive of *NCED* profiles in the grasses, thus warranting further analysis for the grasses. *NCED* genes were also characterized from the recently published *Selaginella moellendorffii* genome; the nine identified genes (labeled as *Sm*) showed sequence placement basal to all other *NCED*s in the tree. When taking into account the relative placement of higher and lower plant *NCED*s, the *CCD4* group does not cluster within the *NCED* cluster but remains as a sister group, further supporting that it is not an *NCED*. Interestingly, *CCD4* homologs have only been found in flowering plants. Preferential expression of *CCD4* in floral organs of *Chrysanthemum* and *Crocus* (Ohmiya et al., 2006; Rubio et al., 2008) suggests that

evolution of *CCD4* genes may have facilitated adaptation for specific physiological capacities unique to flowering plants.

CCD1, is another CCD that is promiscuous in substrate choice, and produces volatiles derived from 9,10 (9'10') bonds of multiple carotenoids and 5,6 (5'6') bonds of lycopene (Sun et al., 2008; Vogel et al., 2008). *CCD1* expression causes the notable emission of diverse volatiles characteristic of tomato fruit and petunia flowers (Simkin et al., 2004; Simkin et al., 2004). Homologs of *CCD1* were identified in the grasses along with two and three previously unknown sequences in the lower plants *Selaginella* (*Sm*) and *Physcomitrella patens* (*Ppa*), respectively.

A new class of *CCD* genes, discovered in the phylogenetic analysis (**Fig. 4-3**) was the group containing maize *CCD8b*. The aligned protein sequences for *CCD8* related sequences are shown in Figure 5-8 (*CCD8a*) and Figure 4-9 (*CCD8b*). *CCD8b* is sister to a group that includes *CCD1*, *CCD4* and *NCED* and has a unique exon-intron structure distinguishable from the *CCD8a* genes (**Fig. 4-2**). *CCD8b* homologs were not found in *Arabidopsis*. The *CCD8b* group is not unique to the grasses since homologs were found for a eudicot, *Vitis vinifera* (*Vv*), and a non-seed plant, *Selaginella moellendorffii* (*Sm*). Homologs of the sister group, *CCD8a*, have been described in *Arabidopsis* and other species. Mutants in the *CCD8a* homolog of *Arabidopsis* and pea (e.g. *MAX4*, *RMS1*) are known to interfere with plant development (Sorefan et al., 2003). *CCD8a* cleaves the apocarotenoid C₂₇ 10'-apo-β-carotenal generating C₁₈ 13'-apo-β-carotenal (Schwartz et al., 2004) in formation of strigolactones, the branching inhibition hormones (Gomez-

Roldan et al., 2008; Umehara et al., 2008). The human CCD enzyme (*HsRPE65*), phylogenetic sister of both CCD8a and CCD8b enzymes, cleaves β -carotene at the 9,10 (9',10') double bond to form vitamin A (Redmond et al., 1998). The CCD8b enzymes form a separate group from CCD8a suggesting that CCD8b enzymes could potentially serve a novel role unrelated to strigolactone biosynthesis. Alternatively, *CCD8b* genes may be duplicative in terms of enzyme activity but have alternative expression profiles or cell-type specificities. The specific enzymatic role of the *CCD8b* group remains elusive.

The CCD8a substrate for biosynthesis of strigolactones is produced by CCD7, represented by the remaining CCD clade in the phylogenetic tree. Homologs were identified in maize (*Zm*), rice (*Os*), and sorghum (*Sb*), along with the lower plants, *Selaginella* (*Sm*) and *Physcomitrella patens* (*Ppa*). To date, all *CCD7* genes from lower and higher plants appear to be single copy. The significance of the single gene copy characteristic unique to the *CCD7* clade is unknown. Homologs for CCD7 and CCD8 were also found in *Chlamydomonas reinhardtii* (*Cre*); interestingly this algal species lacked homologs to all other CCD and NCED genes. The CCD7 clade remained most closely related to lipoxygenase dioxygenases that are encoded by the soil bacterium *Sphingomonas paucimobilis* (Redmond et al., 2001).

4.4.2 Tissue specific distribution of dioxygenase gene transcripts

Tissue specificity was examined in the maize inbred B73, where transcript levels for the various *NCED* and *CCD* genes were assessed in a panel of tissues including leaves, roots, endosperm and embryo, the latter two staged at 20 days after pollination (DAP) (**Fig. 4-4**

and 4-5). This particular inbred line was chosen since it has been used extensively for analysis of genes that influence carotenoid content (Gallagher et al., 2004; Li et al., 2008; Li et al., 2008a; Vallabhaneni and Wurtzel, 2009) and this inbred was the source of the first maize genome sequence.

NCED genes. NCED cleavage of 9-*cis*-epoxycarotenoids represents the rate-limiting step in *de novo* biosynthesis of ABA, especially in leaf tissue where carotenoids are abundant (Nambara and Marion-Poll, 2005). In contrast, although *NCED1* transcript accumulation is induced in roots of abiotic-stressed maize plants (Li et al., 2008), ABA formation is limited by biosynthesis of carotenoid precursors. To assess the potential contribution of *NCED* gene transcripts for carotenoid degradation in different tissues under non-stressed conditions, transcript levels were quantitatively measured for the tissue panel (**Fig. 4-4**). In most tissues including leaves, *NCED1* transcripts represented the most prevalent NCED transcript. *NCED3a* and *NCED9* transcripts were also present in leaves while roots contained equivalent levels of *NCED1* and *NCED3a* with 60% relative levels of *NCED9* transcripts. Embryos contained mostly *NCED1* and ~ 25% relative levels of *NCED9* transcripts. In endosperm, transcripts of *NCED1* and *NCED9* represented the most prevalent *NCED* classes. However, as the endosperm approached dormancy from 20 to 25 DAP, relative levels switched between the two genes.

CCD genes. CCD enzymes vary in choice of substrate and in potential apocarotenoid product (Auldrige et al., 2006). Differential expression of CCD genes combined with tissue-specific carotenoid profiles will yield a suite of apocarotenoid products depending

on combinations of genes expressed. For example, CCD7 cleaves β -carotene asymmetrically producing one β -ionone and C₂₇ 10'-apo- β -carotenal; the latter product is further cleaved by CCD8 generating C₁₈ 13'-apo- β -carotenal (Schwartz et al., 2004).

From studies in peas and rice, CCD7 and CCD8 were shown to be involved in generating the branching inhibition strigolactone hormones formed in roots. These hormones signal symbiotic mycorrhizal fungal hyphae to branch and associate with plant roots or can be transported to aerial parts of the plant and inhibit branching, an important agronomic trait in the grasses (Gomez-Roldan et al., 2008; Umehara et al., 2008; Purugganan and Fuller, 2009). Leaves showed the highest expression of *CCD* transcripts, with *CCD1* and *CCD4a* representing the most abundant transcript classes, respectively (**Fig. 4-4**); lower amounts of *CCD4b* and barely detectable levels of *CCD7* transcripts were also detected in leaves. Leaves did not contain any detectable levels of *CCD8a* or *CCD8b* transcripts. In contrast to the high levels seen in leaves, endosperm of the maize B73 inbred had *CCD1* mRNA levels that were three orders of magnitude lower. Under normal growth conditions, the prevalent CCD classes found in roots, in order of abundance, were *CCD1*, *CCD8b*, and *CCD7*. However, these levels are significantly lower than are found in leaf tissue. For example, *CCD1* was about 30-fold lower in roots as compared to leaves. *CCD1* expression in roots has been associated with formation of mycorradicin, a root apocarotenoid produced after mycorrhizal fungal colonization (Floss et al., 2008; Sun et al., 2008). Despite the low levels seen for roots, this was the only tissue where transcripts could be detected for *CCD7* and *CCD8a* and *CCD8b*. Compared to the other tissues analyzed, embryo contained very low levels of *CCD1* and *CCD8b* transcripts, and barely detectable levels of *CCD4a*, *CCD4b*, *CCD7*, and *CCD8a* transcripts. The expression of

CCD7 in roots is consistent with expression of orthologs in Arabidopsis, pea and petunia (Sorefan et al., 2003; Booker et al., 2004; Snowden et al., 2005; Auldridge et al., 2006). While maize roots preferentially accumulate *CCD8b* over *CCD8a* transcripts, the three dicot species accumulate only transcripts for a *CCD8a* homolog, given the absence of a *CCD8b* gene.

Other maize inbred lines. While the dioxygenase gene family members show specific patterns of expression in B73, there may be variation among genetically diverse maize cultivars. Therefore, transcript levels were assessed for the NCED and CCD family members in a 10-line core set of genetically diverse maize inbred lines. These particular lines have been used for identifying control points in the carotenoid biosynthetic pathway and for pinpointing specific gene family members responsible for affecting pathway flux to carotenoids (Vallabhaneni and Wurtzel, 2009). Expression in endosperm was given specific focus since genes had already been identified that promote carotenoid accumulation but it was unknown whether there might be variation in expression of carotenoid cleavage genes that could counteract efforts to maximize levels of nutritionally targeted carotenoids in endosperm. Of all the cleavage genes analyzed, *CCD1* was the only gene family member showing unusually elevated transcripts in one maize line, NC300 (**Fig. 4-5 and 4-6A**). In the other nine maize lines, *CCD1* mRNA level ranged from ~ 100-300 fold lower. Some genes lacked detectable transcripts in endosperm of the ten lines and were therefore not shown. For the five other cleavage genes shown, there was variation in expression, but not of such magnitude.

Maize *CCD1* was located on chromosome 9.07 (see mapping, **Table 4-1**), the chromosome bin containing the *white cap1* (*wc1*) locus for which dominant alleles exhibit reduced endosperm carotenoids (Maize Genetics and Genomics Database: <http://www.maizegdb.org/>). The Maize Genetics and Genomics Database provides unpublished data indicating that the *wc1* locus encoding *CCD1* is associated with variation in gene copy number (www.maizegdb.org). Therefore, correlation between gene copy number and carotenoid content of the 10 lines was tested. Quantitative real-time PCR was conducted, using genomic DNA from each line, to establish copy number at the *CCD1* locus by normalizing with *PDS* (phytoene desaturase), a single copy gene. As shown in Figure 4-6B, linear regression analysis revealed a statistically significant correlation ($R^2 = 0.54$ and $p = 0.01$) between copy number of *CCD1* and carotenoid content. Of note was that line NC300 showed ~7-8 copies of the *CCD1* gene which could explain the unusually high *CCD1* transcript level in that line compared to the other 9 lines tested.

These results suggest that *CCD1* expression may limit maize seed carotenoid content. A similar effect of variation of *CCD1* expression in seed was observed for an Arabidopsis loss-of-function *CCD1* mutant that exhibited a significant increase in carotenoid content of mature seeds (Auldridge et al., 2006). Together, these results suggest a role for *CCD1* in seed carotenoid catabolism in monocots and dicots.

4.4.3 Conclusion

The carotenoid cleavage dioxygenase gene family was defined for three grass species in the context of higher and lower plant phylogenetic analysis. Gene mapping and transcript

profiling for maize, together with identification of homologs in lower plants, provide a foundation needed for basic research and future crop improvement. Apocarotenoids contribute to valuable agronomic traits such as tillering and abiotic and biotic stress responses. From a human nutritional perspective, degradation of carotenoids to apocarotenoids limits nutritional value of endosperm tissue. A better understanding of the role of apocarotenoids and the requisite carotenoid cleavage genes is critical in achieving the right balance in engineering high carotenoid endosperm while maintaining optimal plant fitness of maize and other important cereal crops.

Table 4-1. Summary of carotenoid cleavage dioxygenase gene family.

For maize genes, information is provided for chromosome location (Map); sizes of genomic DNA, cDNA, and open reading frame (ORF) are given in base pairs. Genomic DNA sequence covers from ATG to stop codon. The predicted cDNA sequences starts from predicted transcription start site to poly A signal. For maize protein information, number of amino acid residues is given for the mature protein (M) and TP (transit peptide). Loc, predicted cellular location; P, plastid and C, cytosolic. Pre/pI, preprotein (MW in kD)/isoelectric point; M/pI, predicted mature protein (MW in kD)/isoelectric point.

	<i>Oryza sativa</i>	<i>Zea mays</i>												<i>Sorghum bicolor</i>
		ACCESSIONS		GENE					PROTEIN					
		Genomic	ESTs	Map	Introns	GENOMIC	cDNA [§]	ORF	M	TP	Loc	Pre/pI	M/pI	
NCED1		AC201886	U95953	1.08	0	1815	2498	1815	605	23	P	65/5.5	63/5.3	
NCED2	LOC_Os12g42280	AC199036	DV032362	3.04	0	NA	NA	1008 ^p	403 ^p	-	-	-	-	Sbi_0.36552 ^p
NCED3a	LOC_Os07g05940	AC212820	DY541318	NA	0	1722	2208	1722	574	46	P	65/6.1	57/5.8	Sb02g003230
NCED3b		AC205109	NA	2.09	0	1728	2843	1728	576	52	P	62/6.6	56/5.8	
NCED9	LOC_Os03g44380	AC190614	NA	5.03	0	1806	2157	1806	602	23	P	65/6.4	62/6.1	Sb01g013520
CCD1	LOC_Os12g44310	DQ100347	DQ100346	9.07	13	5414	2388	1653	551	-	C	62/5.9	55/5.8	Sb01g047540
CCD4a	LOC_Os02g47510	AC190588	EE172116	4.06	0	1917	2262	1917	639	50	P	68/6.3	63/5.7	Sb04g030640
CCD4b	LOC_Os12g24800	AC194862	NA	5.06	0	1920	2382	1920	640	52	P	69/6.8	63/5.8	
CCD7	LOC_Os04g46470	AC211432	NA	2.03	6	2607	3140	1860	620	29	P	68/8.7	65/7.8	Sb06g024560
CCD8a	LOC_Os01g54270 (CCD8a)	AC185113	CO526902	3.07	3	3007	2672	1662	554	36	P	61/6.5	57/6.0	Sb05g009950 (CCD8a)
CCD8b	LOC_Os09g15240 (CCD8b)	AC198395	DW954641	7.02	11	3771	2402	1632	544	79	P	60/5.6	51/5.5	Sb03g034400 (CCD8b)
CCD8c	LOC_Os01g38580 (CCD8c)													Sb10g004360 (CCD8c)
														Sb10g004370 (CCD8c-like)
CCD8d	LOC_Os08g28240 (CCD8d)													Sb02g021490 (CCD8d)
														Sb07g024250 (CCD8d-like)

Table 4-2. Other carotenoid cleavage dioxygenase genes identified in this study.

Vv, *Vitis vinifera*; *Sm*, *Selaginella moellendorffii*; *Ppa*, *Physcomitrella patens*; *Cre*, *Chlamydomonas reinhardtii*. Note that *Physcomitrella* and *Chlamydomonas* contain only one *CCD8* gene of the *CCD8a* type (see **Fig. 4-3**)

Species	# genes	Gene	Accession numbers
<i>Vitis vinifera</i>	9	<i>VvNCED2</i>	GSVIVT00028310001
		<i>VvNCED3</i>	GSVIVT00000988001
		<i>ViNCED6</i>	GSVIVT00020467001
		<i>VvCCD1</i>	GSVIVT00028793001
		<i>VvCCD4a</i>	GSVIVT00001163001
		<i>VvCCD4b</i>	GSVIVT00013949001
		<i>VvCCD7</i>	GSVIVT0001914600
		<i>VvCCD8a</i>	GSVIVT0003242300
		<i>VvCCD8b</i>	GSVIVT00032436001
<i>Selaginella moellendorffii</i>	15	<i>SmNCEDa</i>	JGI11287, JGI11289, JGI11292, JGI11304, , JGI79628, JGI80651, JGI91815, JGI94523
		<i>SmNCEDb</i>	JGI233638
		<i>SmNCEDc</i>	JGI75383
		<i>SmCCD1</i>	JGI165469, JGI272067
		<i>SmCCD7</i>	JGI11120
		<i>SmCCD8a</i>	JGI173016
		<i>SmCCD8b</i>	JGI132803
<i>Physcomitrella patens</i>	6	<i>PpaNCED</i>	JGI57876
		<i>PpaCCD1</i>	JGI159406, JGI158869, JGI108830
		<i>PpaCCD7</i>	JGI115720
		<i>PpaCCD8</i>	JGI115616
<i>Chlamydomonas reinhardtii</i>	2	<i>CreCCD7</i>	JGI144745
		<i>CreCCD8</i>	JGI141185

Table 4-3. Primers used in the study

Gene	No.	Sequence	Gen Bank ID.
NCED1	1652	GCCCAGCTCGTAGCTTAACA	AC201886
	1653	CGAGCAAGTAACAGCAACCA	
NCED2	2188	ACGGCTACATCCTGTCCCTC	AC199036
	1881	AGAACAACCAAGGAATCATCAGG	
NCED3a	2237	CGATCACGAACTCGACCAAC	AC212820
	2060	CCGGCGTCTGCCTCAA	
NCED3b	2055	CGATCACGAACTCGACCACC	AC205109
	2081	GCATGCAAATATACATATGTGCGG	
NCED9	2185	GAGAGATTATTAGTAGCAGTAGGGAGAG	AC190614
	2018	GCAACCGATCCTAGACACCC	
CCD1	2064	CTGGAGAAGAAGATGACGGCTAT	DQ100347
	2065	AGGCATGGAATCCATAAGGAAC	
CCD4a	1816	AAGCACCGAACACTCGTCCA	AC190588
	1817	ACTCTTTTATGGGTTCTGCTACATTT	
CCD4b	1818	TCCCCTACGGCTTCCACG	AC194862
	1819	ATCCAGCGGCGTGTTTCTC	
CCD7	1836	TGACGCCACAGACATCCATT	AC211432
	1837	TGGGACCGACGAGCAGAA	
CCD8a	1834	TGCTGCTCACATCTCCCACA	AC185113
	1835	CGTGTAATTGCTACAGCCACATT	
CCD8b	2321	GAAAGCTATGCGAGAATCGG	AC198395
	2322	GTATTATGGAGTCCGGCGAA	
PDS	933	GAAATCATCGATGCAACTATGGAA	L39266
	934	CTTCGATAGGTGACCTTTGGA	
Actin	1134	CGATTGAGCATGGCATTGTCA	J01238
	1135	CCCACTAGCGTACAACGAA	

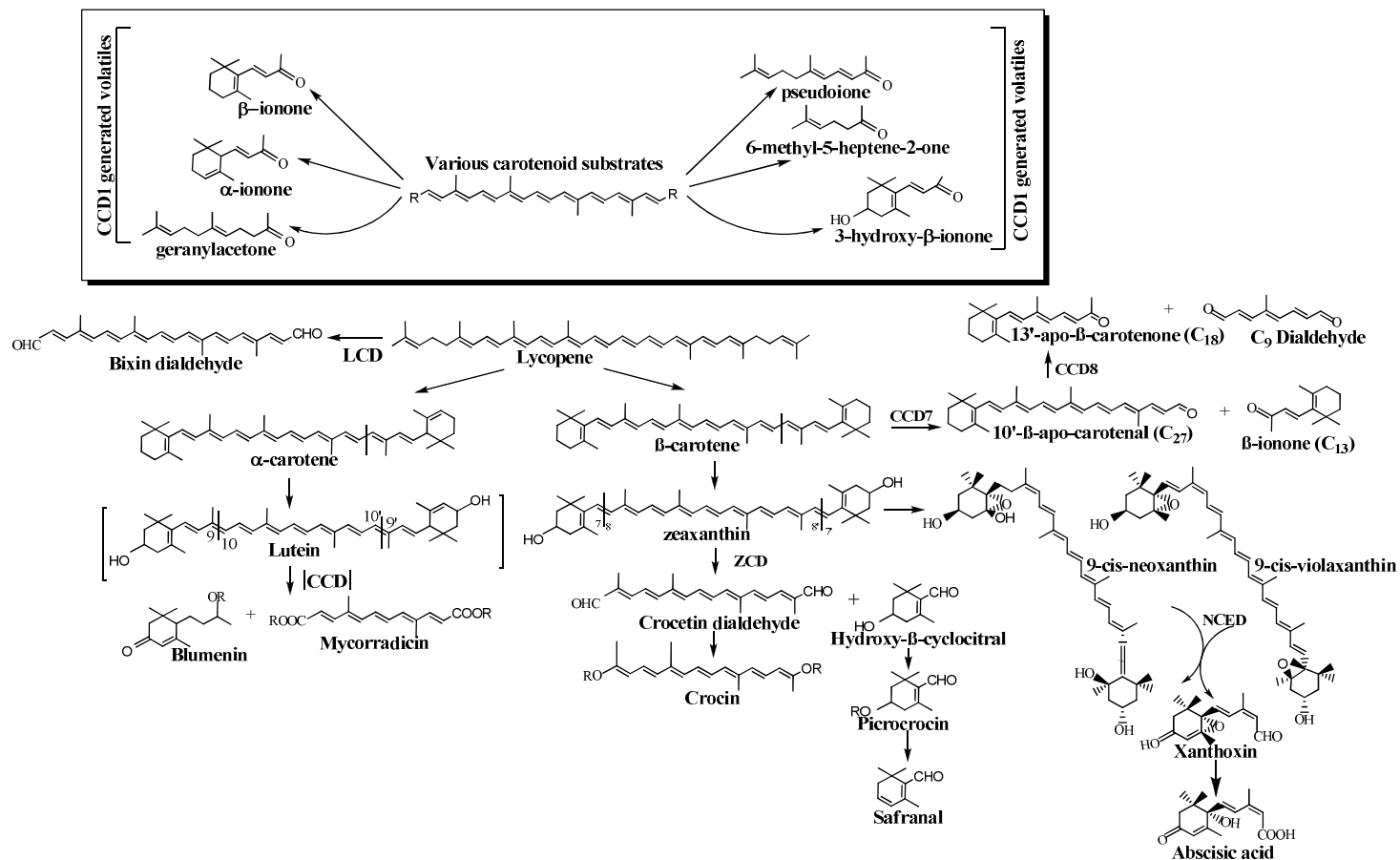


Figure 4-1. Carotenoid cleavage dioxygenase activities in various species.

CCD1 is promiscuous in the choice of substrate, thus producing an array of volatiles as shown inside the box (Auldrige et al., 2006). Zeaxanthin cleavage dioxygenase, ZCD (*Crocus sativus*) (Bouvier et al., 2003); lycopene cleavage dioxygenase, LCD (*Bixa orellana*) (Bouvier et al., 2005). Formation of abscisic acid is mediated by 9-cis-epoxy carotenoid dioxygenases (NCED) (Schwartz et al., 1997). CCD activity in mycorrhizal maize roots leads to formation of apocarotenoids mycorradicin and blumenin (Walter et al., 2000); the hypothetical lutein substrate is indicated in square parentheses. CCD7 and CCD8 participate sequentially as shown (Bouvier et al., 2005).

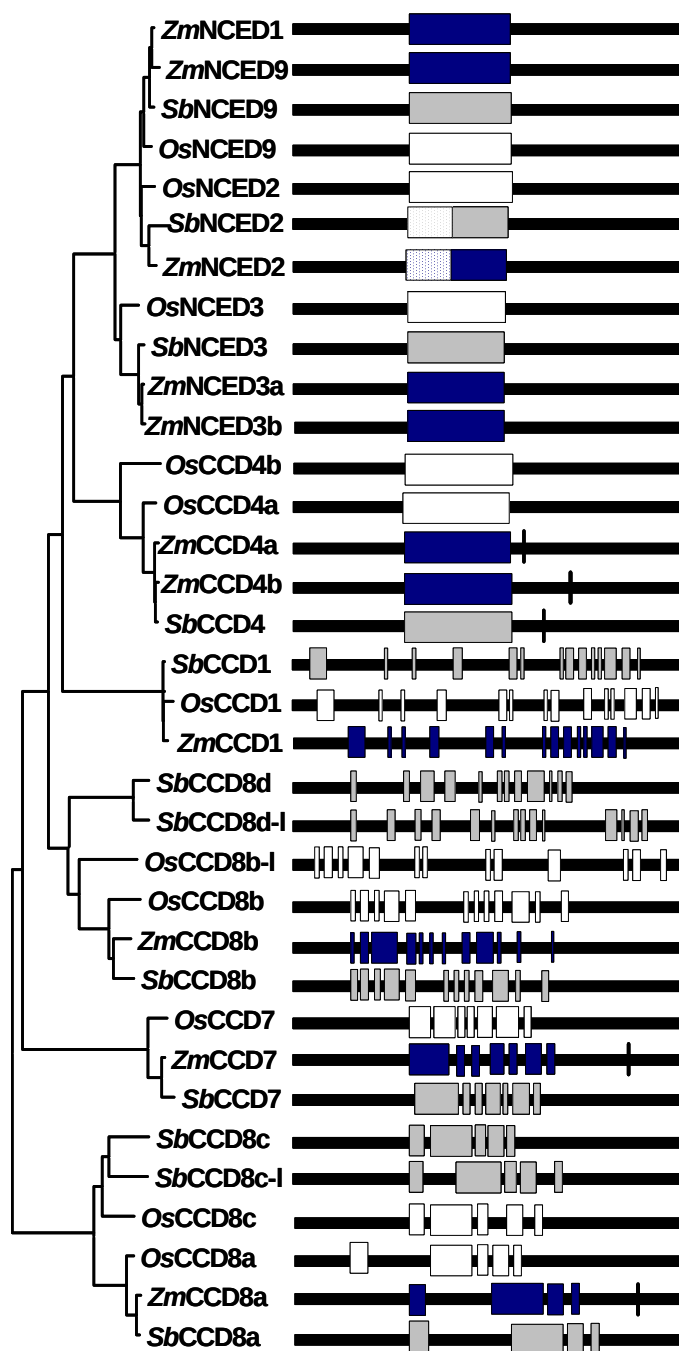


Figure 4-2. Phylogenetic analysis of carotenoid cleavage dioxygenases in the Grasses.

Un-rooted neighbour-joining tree was constructed using protein sequences shown at left. Corresponding gene structures are shown to right of each protein. *Zea mays* (Zm); *Oryza sativa* (Os); *Sorghum bicolor* (Sb). Exons for maize, sorghum and rice are indicated by boxes colored dark, grey, and white, respectively; thick horizontal lines are introns. *ZmNCED2* and *SbNCED2* are partial as indicated by dotted boxes. Exon structures starts with ATG, and vertical thin lines indicate poly-A signals.

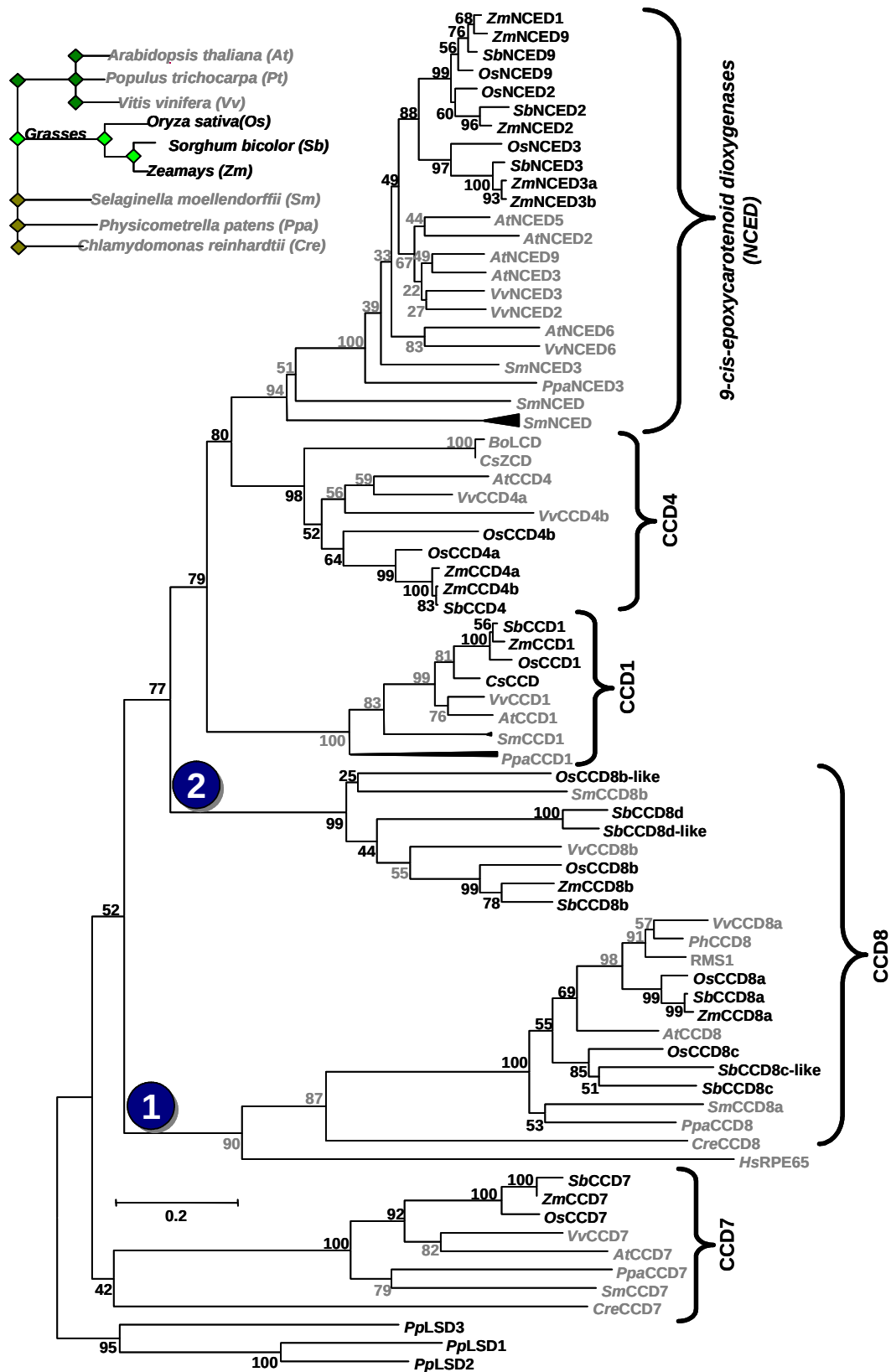


Figure 4-3. Phylogenetic analysis of carotenoid cleavage dioxygenases from diverse taxa.

Neighbour-joining tree was constructed using protein sequences. *Zea mays*, *Oryza sativa*, *Sorghum bicolor* (see Table 1 for accession numbers), *Arabidopsis thaliana* (AtNCED2, At4g18350; AtNCED3, At3g14440; AtNCED5, At1g30100; AtNCED6, At3g24220; AtNCED9, At1g78390; AtCCD1, At3g63520; AtCCD4, At4g19170; AtCCD7, At2g44990; AtCCD8, At4g32810), *Bixa Orellana* (BoLCD, AJ489277); *Crocus sativus* (CsZCD, CAD33262, CsCCD, AJ132927; CsCCD4a, EU523662; CsCCD4b, EU523663); *Sphingomonas paucimobilis* (PpLSD1, S80637; PpLSD2, S65040; PpLSD3, AB073227); *Homo sapiens* (HsRPE65, AF039855); *Phaseolus vulgaris* (RMS1, AY557341); *Petunia hybrida* (PhCCD8, AY743219). Refer to Table 2 for genes from: *Chlamydomonas reinhardtii* *Selaginella moellendorffii*, *Physcometrella patens* and *Vitis vinifera*. Numbers 1 and 2 indicate two distinct clusters of CCD8. Amino acid distances were corrected by Poisson correction. Numbers indicate bootstrap support for individual nodes. Bootstrap support higher than 40% is indicated at respective nodes (n = 500). Grass species enzymes are shown in black; except for *Pp* genes, others are grey. Black triangles represent additional gene copies shown in a compressed fashion (detailed in **Table 4-2**). The evolution of representative green plants is shown at the top left hand corner.

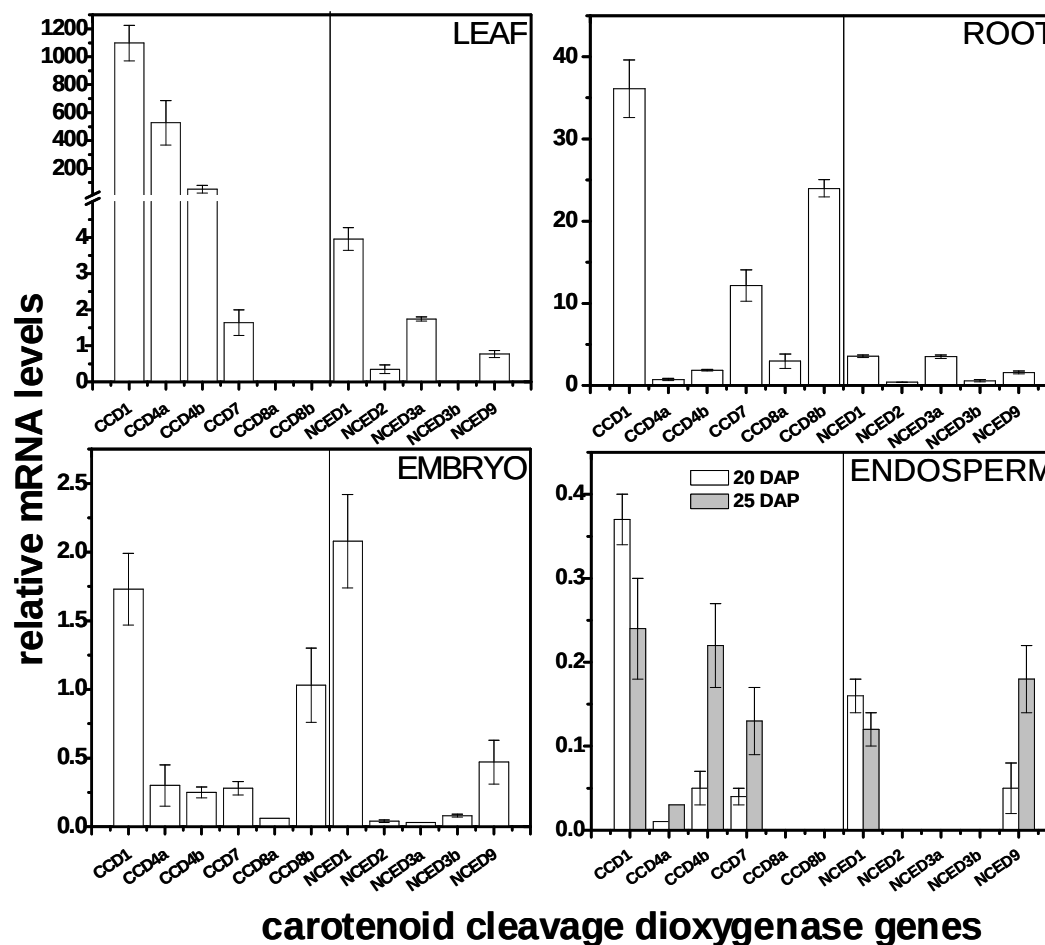


Figure 4-4. Maize B73 inbred transcript profiles of genes encoding (A) carotenoid cleavage dioxygenases (CCD) (B) 9-cis-epoxy carotenoid dioxygenases (NCED). Mean of three replicates $\pm$ standard deviation. Embryo collected at 20 DAP and endosperm collected at 20 and 25 DAP from field grown plants; leaf and root samples collected from seedlings at the six-leaf stage.

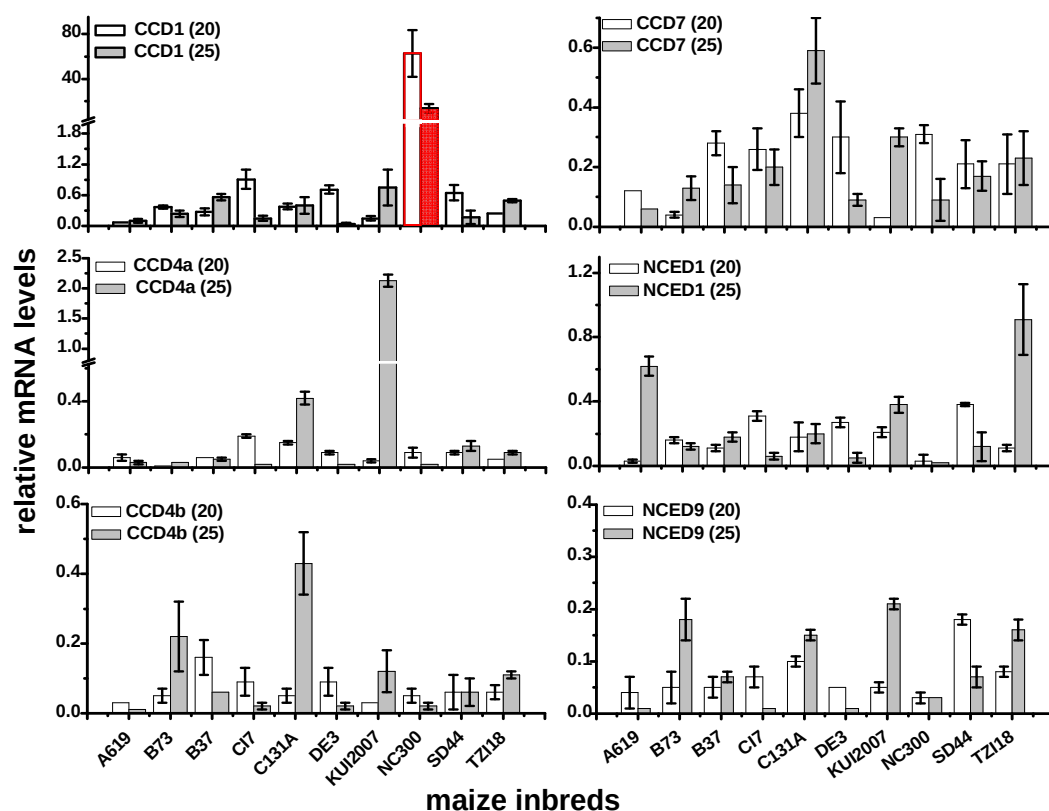


Figure 4-5. Transcript profiles of genes showing detectable levels of expression in a core subset of maize inbreds.

Transcript levels were measured at both 20 and 25 DAP during endosperm development. Mean of three replicates $\pm$ standard deviation. Inbred lines are: 1, A619; 2, B73; 3, B37; 4, C17; 5, C131A; 6, DE3; 7, KUI2007; 8, NC300; 9, SD44; 10, TZ118.

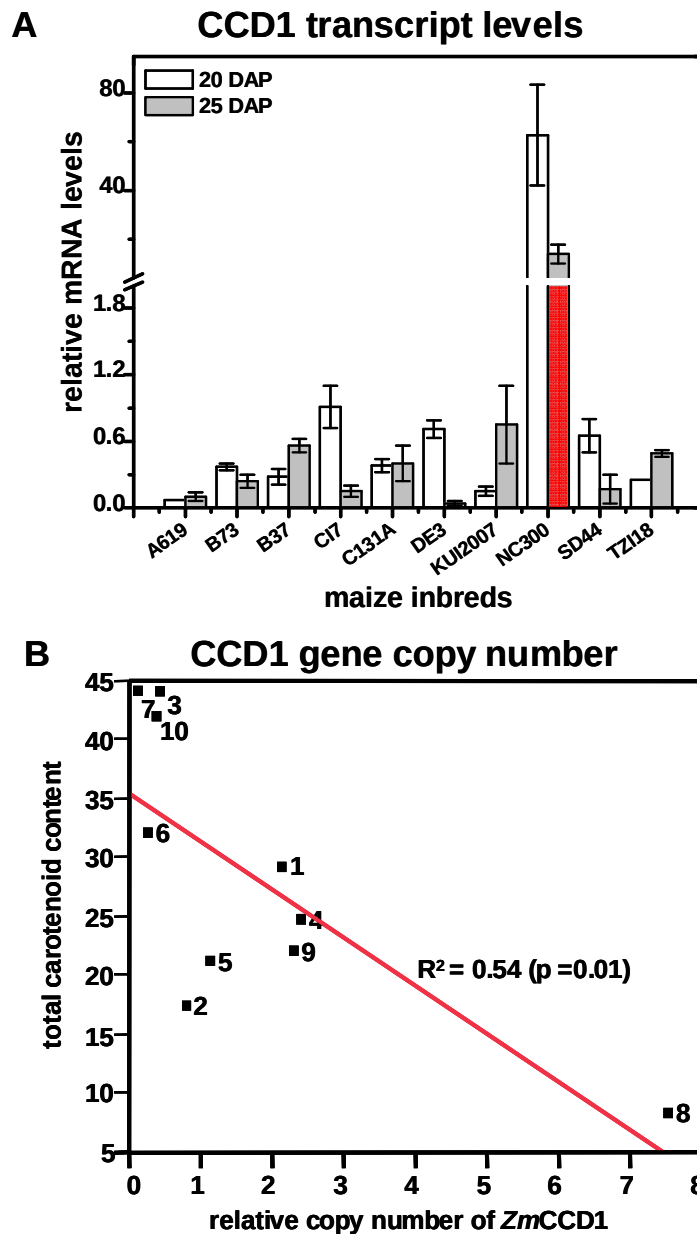


Figure 4-6. *ZmCCD1* expression and copy number among maize inbreds

(A) Transcript levels were measured at both 20 and 25 DAP during endosperm development. Mean of three replicates $\pm$ standard deviation (B) Gene-copy number of *ZmCCD1* relative to *ZmPDS*. Mean of three replicates $\pm$ standard deviation. Red line indicates linear fit line. Inbred lines are: 1, A619; 2, B73; 3, B37; 4, CI.7; 5, C131A; 6, DE3; 7, KUI2007; 8, NC300; 9, SD44; 10, TZI18.


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AtCCD8      : -----MASLITTKAMMSHHVLSSTRITTLYSN--SIGDQIKTKPQVPHRLFARRIFGVTR-----AVINSAAPSPLPEKEKVEGERR-----CHVAMTSVQENWEGELTVQGIPTWLNGLTYLRNGPGLWNIGD
CsCCD       : -----MGEVAKKEEVEERSIVAVN-----PQPSKGLVSSAVDLIEKAVYLFHDK-----S-----KPCHYLSGNFAPVVDTPPCPDLPVRCHLPECLNGEEFVRGP-NPKFMP
HsRPE65     : -----MSIQVEHPAG-----GYKKLETVEELSSPLTAHVTGRIPNLTLGSLRRCGPGLEFVGS
OsCCD8c     : ---MATSLTLIATPCTAPRSSSSSFAL-----APRLPPRCN-ATAARRRAVRATTLQS-----DQEPAGSG-----DSG--ATTTKLSASTSVRQERWEGDLPIEGCLPPNLNGTYIRNGPGMWVGE
SbCCD8c     : ---MATYSTPLGAAPKSFFLSN-----SLFNISLGT-ATPSRISSVKAAG-----ECWTKHPAMTNVRQERWEGHLAVEGHLPTNLNGTYLRNGPGLWEVD
SbCCD8c-li  : ---MAACKLPTLCTAPTPLYASR-----RSAGQTIIST-AAASACHRCRASRRR-----I-----QAA--GDCTKHSAMTSIRHERWEGDLATEGHIPANLNGTYLRNGPGVMEVD
OsCCD8a     : MSPAMLQASSLCVSAALSGAASRPG-----RLASQGHQKRAVAQPLAASAVTEAAPP-----APVVAPPARPVDAP-RRRGGGGGGGGGELVAKSVRQERWEGALEVDGELPLNLNGTYLRNGPGLWNIGD
SbCCD8a     : --MSPTMASSLCVFAAMSGATGRPSSTGGSVPPGRLSSTAQGTGKRAVVQPLAASVVTPTPTPAIAPAAPPARPVDAP-RRRGGRG-----TGEHAAMKSVRQERWEGALELEGEPLNLNGTYLRNGPGLWNIGD
ZmCCD8a     : -----MSGASGRPS-AGGSAPV-GRLSSTQGGKRAVVQPLAASVVTETP-----APAVAPARPVDAPRRRGGRG-----TVEHAAMKSVRQERWEGALELEGEPLNLNGTYLRNGPGLWNIGD

AtCCD8      : HDERHLFDGYSTLVKLQEDG--GRIFAAHRLLESAYKAAK-KHNR---LCYREFSETPKSVIINKNPFSGTGETVRLFSGESLTDNANT--GVIKLGDGRVMCLTETQK--GSILVDHETLETIGKFEYDDVLSDHM
CsCCD       : VAGYHWFDDGDMIHGIRIDG--KATYASRYVKISRLQEEYFEGP-----KFMKIGDLKGFFGLFVQVQLRAKLKVIDVSYGVGIGNTALIYHKGKLLALSEADKPYVVKVLEDGDLTLGLLDLDRKLSH--
HsRPE65     : EPFYHLFDGQALLHKFDKEG--HVTYHRRFIRTDAYVRAM-TEKR---IVITIEFGTCAFPDPCK---NIFSRFFSYFRVEVTDNALV--NLYPVGEDYYACTETN---FITKINPETLETIKQVDCNLYSV-N
OsCCD8c     : HAEHHLFDGYATLVRVSFRGGGARATGAHQIETSEAYRAAV-ARGR---PVLREFSL-CPAPAK--SLLHRFGDLVGLVTGAALTDPNS--AVLPLGDGRVMCLTETTK--SSVLIDPDTLETIGKFRYDRLG-M
SbCCD8c     : HSSHIFLDGYATLVRISFR--RGCATGAHQVESDAYKAAR-AHGR---PLHREFSLQCPSEPG--TLDRVRDVVGLASGTLTLDNANV--SVLPLGDGRVCLTEATK--GSVLIDPETLDTIGKFRYADRLWG-L
SbCCD8c-li  : HALDHVFDGYATLVRVYFQGA-RGRATGAHQIETSEAYKAAARAHGRRRLMRMREFSLQCPSEPG--TLDRLRHVGLVTCAGMSDNANT--AVLPLGDGRVCLADVTK--SSVLIDPETLETIGKFRYADRLWC-P
OsCCD8a     : YGFRHLFDGYATLVRVSFRG--GRAVGAHQIETSEAYKAAR-AHGK---VCYREFSEVPKPDNF--LSYVGLATLFSGSSLTDSNT--GVVMLGDGRVCLTETIK--GSIVDPTLDTIGKFRYADRLWG-L
SbCCD8a     : YGFRHLFDGYATLVRVSFRN--GHAVGAHQIETSEAYKAAR-ANGK---VCYREFSEVPKADSF--LSHVGLATLFSGSSLTDSNT--GVVRLGDGRVCLTETIK--GSIVDPTLDTIGKFRYADRLWG-L
ZmCCD8a     : YGFRHLFDGYATLVRVSFRD--GOAVGAHQIETSEAYKAAR-AHGK---VCYREFSEVPKAEFG--LSHVGLATLFSGSSLTDSNT--GVVRLGDGRVCLTETIK--GSIVDPTLDTIGKFRYADRLWG-L

AtCCD8      : IQSAHPITVDT-----EFWTLIPDLVPGYVVRMDAG-----SNERKQVGRVDCRGG-PAPGWHVSFVTEHYVVVPEMPLRYCAKNLLRAEPTPLYKEWHLES--GSYMHVMCKASG-KIVASVEVPPF
CsCCD       : SFTAHFKVPDPT-----DEMFTFGYAHTPPVTVRVISK-----DGMVDPVPITIPASVMMHDFAITENYSIFMDLPLYEQKEVYKGGKLIFFSDATKKAR-----EGVLPYAKDDSLIRWFELPNC
HsRPE65     : GATAHPHIENDGTVYNIIGNCFGKNSTIAYNIKIPLQADKEDP-----ISKSEIVQPCSDR-FKSYVHSFGLTPNYIVFEVTPVKNLNFKFLSSWSLWGANMDCFESETNETMGVLLHADKRRKKYLNKKYRTSPF
OsCCD8c     : IQSAHPITVDT-----EFLTLPLDLVRPGHLVVRMDAG-----SNERKQVGRVDCRGG-PSPGWLHSAFVTEKYAVVPEMPLRYSSASILLASELAIFYADVPAS--GSYMHVMCKSTG-KTVASVEVPPF
SbCCD8c     : IQSAHPITVGN-----ELLTLPLDMFRGRHVRVMDAG-----SNERKQVGRVDCRGG-QAPGWHVSFAVTEKYIVVPEMPLRYSLAGVLKSMPTWYLLDWLPES--GSYMHVMCKSTG-KTVASVEVPPF
SbCCD8c-li  : VQCTHPVVTITTTTTTTRAAAAAELMLHDPDFARRGYLVARMMAAGGTGSNDREVVGRVRCRGG-TTPAVHVSFAVTAKEYIVVPEMPLRYSVACLLMSLTPFYIMDWLPHS--GSYMHVMCKSTG-KTVASVEVPPF
OsCCD8a     : IQSAHPITVDT-----EFWTLIPDLIRPGYVVRMDAG-----SNERQVGRVDCRGG-PAPGWHVSFVTEHYVVVPEMPLRYCAKNLLRAEPTPLYKEWHLES--GSYMHVMCKASG-KIVASVEVPPF
SbCCD8a     : IQSAHPITVDT-----EFWTLIPDLIRPGYSVVRMDAG-----TNERRFVGRVDCRGG-PAPGWHVSFPTTDHYVVVPEMPLRYCARNLLRAEPTPLYKEWHLES--GSYMHVMCKASG-RVASVEVPPF
ZmCCD8a     : IQSAHPITVDT-----EFWTLIPDLIRPGYSVVRMDAG-----TNERRFVGRVDCRGG-PAPGWHVSFPTTDHYVVVPEMPLRYCARNLLRAEPTPLYKEWHLES--GSYMHVMCKASG-RVASVEVPPF

AtCCD8      : VTFHFINAYEEDKNG--DGKATVITADCEHNADTRIQLMRLDITLRS--HGHDVLPDARIG-----RFRIPLDG-----SKYCKLETAEAEKHGRAMDVCINPLYLQKYR---YVYACGAQ
CsCCD       : FIEHNANAWEEG-----DEVVLTICRLENPDLMVNGAVKEKENF--K-NELYEMRFN-----MKTGAASQKQLSVSAVDFPRINESYTTIKQR---YVYGTILD
HsRPE65     : NLHFHINTYEDNG-----FLIVDLCCWKGFEFVYNYLYANLREN--WEEVKKNARKAPQPEVRVYVPLNIDKADTGKNLVTLNPTTATATLCSDETIWLEPEVLFSQPROAFEPQINYQKCGKPYTYAYGLG
OsCCD8c     : MAHFHINAYEEG-----DEAAVVVDCCEHYGDPATITETLVLSRLRL--RGKQVLPNARVG-----RFRIPLDG-----SPFGELETAIDPEEHGRGMDVCINPARLGRKYQ---YAYACGAR
SbCCD8c     : MALHFHINAYEQ-----DAITADCEYYADPSVICALALRRLRSPGMNNDAPDVRVA-----RFSIPLDG-----TLMGELETVIDPEVHGRGVEPSINPAYQKEYR---YVYACGAR
SbCCD8c-li  : VAFHFHINAYEENGDDDDGVPRNATITADCEYYADPATIQAALHRLRSP-ETAKDFPDSRVA-----RFRIPLDG-----AAMGELETVIDPEEHGRGVELSTINPDYVGKEYR---YLYACTAR
OsCCD8a     : VTFHFHINAYEETDE--EGRVTATITADCEHNANTAILDKLRQLHNLRS--SGQDVLDPARVG-----RFRIPLDG-----SQFGELETAIDPEEHGRGMDVCINPAHVGREYR---YAYACGAR
SbCCD8a     : VTFHFHINAYEEKDE--EGRVTATITADCEHNANTITIDKLRQLNLRS--TGQDVLDPARVG-----RFRIPLDG-----SPFGELESAIDPDQHGRGMDVCINPAHVGGKYR---YAYACGAQ
ZmCCD8a     : VTFHFHINAYEKDD--EGRVTATITADCEHNANTSIDKLRQLNLRS--TGQDVLPA-----RIPLDG-----SPFGELEPALDPDQHGRGMDVCINPAHVGGKYR---YAYACGAH

AtCCD8      : RPCNFPNALSKVDIVKVKV-----WHEHGMIPSEPFVPRPGAT--HEDDGVVISIVSEENG--SFATLLDGSFEEIARAKFPYGLPYGLHGCWIPKD-----
CsCCD       : NITKVKGIKFDLHAPEAGKKKLEVGGNVQGFIDLGPGRYGEAVFVPRERGIKSEEDDGYLIFVHDENTGKSEVNVVIDAKTMSAEPVAVVVELNRPVYGFHAFVNEEQQLWQQTQDV
HsRPE65     : LNHFVPDRCLKLVKTKETW-----WQEPDSYSEPFVFSHPDAL--EEDDGVLSVSVSPGAGQKPAYLLILNAKDLSVARAEVEINIPVTFHGLFKS-----
OsCCD8c     : RPCNFPNTLTIDLVKKAAS-----WHEEGSVSEPFVPRPGAT--DEDDGVVISIVSDDGE--GYALVLDATTFEIIARVRLPYGLPYGHGCWIPATEE-----
SbCCD8c     : RPCNFPNCLTKIDLGEKEAKN-----WHELGSVSEPFVPRPGS--DEDDGVVISIVSTMEG--GYALLLDAMTFEIIARVRLPYGLPYGHGCWIPENV-----
SbCCD8c-li  : RPCNFPNALTIDKMDLVEKETTS-----WHEEGTVSEPFVPRPGAT--HEDDGVVISIVSTMDG--GYVLLLDAAFTFEIARVRLPYGLPYGHGCWIPDNN-----
OsCCD8a     : RPCNFPNTLTIDLVERTAKN-----WHEEGSVSEPFVPRPGAT--DEDDGVVISIVSDDGE--GYALVLDGKTFEIVARAKFPYGLPYGLHGCWVPRKRNSK-----
SbCCD8a     : RPCNFPNTLTIDLVERTAKN-----WHEEGSVSEPFVPRPGAV--EEDDGVVISIVSDDGE--GYALVLDGKTFEIVARAKFPYAMPYGLHGCWVPRRTSDA-----
ZmCCD8a     : RPCNFPNTLTIDLVERTAKN-----WHEEGSVSEPFVPRPGAV--EEDDGVVISIVSDDGE--GYALVLDGKTFEIVARAKFPYAMPYGLHGCWVPRRTSDA-----

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Figure 4-8. Protein sequence alignment of CCD8a carotenoid cleavage dioxygenase sub-family.

AtCCD8 : -----MASLITTKAMMSHHVLSSTRITTLTYSNIGDQQIKTKPQVPHRLFARRIFGVTRAVNSAAPSPLPEKEKVEGERRCHVAWTSVQENWEGELTVQKPTPTWLNGLTYLRNG
HsrPE65 : -----MSIQVEHPAGG---YKKLFETVEELSSPLTAHVTGRIPLWLTLG-SLLRCG
CsCCD : -----MGEVAKEEVEER-----RSIVAVNPQP-----SKGLVSSAVDLIEKAVYLFDHDKSKPCHYLSG---NFAPVDETPPCPDLP---VRGHLPECLNG-EFVRVG
OsCCD8b-li : -----MYTLQPRVCISSS-----RSSISP-----KAARLSHQKASTG--KPYFREIQVHLSKLGASNAMNSTYQQLDSFDHTFTTKCQPLRPTE---NFAPVDETEITRVE---IEGEIPADFPPEGVYIRNG
OsCCD8b : -----MMTASLHPCVCKA-----SPAFRP-----ASSLGARTQPKSTATNPKRPLFQELQRRLSFRIDEASKALETAKQGLLDALVDSTFKFSQDQMLPSEN---NFAPVNEISEAIEILQ---IEGEIPEDFPE-----G
SbCCD8b : MVLKASASFSPSLPLPLSCR-----INGGRPSMSMSAGARTAASVGTSS-QKPLLGEELLGNLSSKMDRASKALKDVPQRFLDVLVDATFKFTDEALNPSES---NFAPVDEIGEAIEIHQNQVEGAIPDDFPPEGVYIRNG
ZmCCD8b : -----M-----AVGRRP-----CPCLPAHGPGHQWAPA-LRPWFGLDLGRLSSTM DGASRALKDAPQRFLDALVDATERFTDQALNSAES---NFAPVDETGAEIEIHQSOLQGAIPDGFPEGVYIRNG
SbCCD8D : -----MFRAGLKPTTTSSSRCIHSRRAP-----DHALP-FNPPNAIKG-----NFRPVNEIDEAVLLNN---LDGVPVGDFFPEGVYIRNG
SbCCD8d-li : -----MFRDGLKPTNSCR-CRCIRSHRAPA-----DHALPCFTPPNAIKVPPSPGLKKQALILQGISQALKSVSSNLLERFDRAVRFSEQPSL-NEG---NFRPVNEIDEAVLLNS---LDGVPVGDFFPEGVYIRNG

AtCCD8 : PGLWNIGDHD-----FRHLFDGYSTLVKIQDGGRIFAAHLLESAYKAAKHNRLCYREFSETPKSVIIN--KNPFSGIGEIVRLFS---GESLTDNANTGVIKLGDGRVMCLTETQKGSITLYDHET-LETIGK
HsrPE65 : PGLFEVGSE-----FYHLFDGQALLHKFD-KEGHVYTHRRFIRTDAYVRAMT---EKRIVITEGTCAFPDPCKNIFSRFFSYFRG---VEVTDNALVNVVPVGEDYACTETNFTIKNPET---LETIKQ
CsCCD : PNPKFMPVAG-----YHFDGDMIHGRIKDGKAT-----YASRYKTSRLKQEEYFEGPKFMKIGDLKGFGLFMVQMQLLAKLKVIDSVYGVGTGNTALITYHKGKLLALSEADKPYVVKLEDGDLTLGL
OsCCD8b-li : GNPLYGGLQSVSIFGQSHNIWVEGEGMLHAYVYCKSNNS-TWSTSYNNRYVQSETFRIEKERQKPCFLPMTDGNPPAMLIASVLNTRLF-----RKVMKMSMSTSVFEHAGRVYAASEDDVPHEVDLHN---LSTLGS
OsCCD8b : SNPLFGALHSTVSIFGKSSEIWVEGEGMLHAYVYTKNSSD-TWSVSYANRYVQSETLTKETKRQKPCFLPAIMGDSAAITAAAYILNMYR-----GKVNKISINTNVFEHAGRVYAVSENHLPQEISTQN---LDTGDS
SbCCD8b : SNPLFGALHSTVSIFGQSREIWVEGEGMLHAYVYTKNTSG-SWSVSYANRYVQSETLTKETARQKPCFLPAIEGDSAAITAAAYIFNHLR-----GKVNKISINTNVFEHAGRVYAVSENHLPQEIGIDN---LDTSGT
ZmCCD8b : SNPLFGALHSTSVLQGSREIWVEGEGMLHAYVYTKSSAGHLWSVYASRYVQSETLELETARHKPCFLPAVEGDSAAITAAAYVFNHLR-----GKVSKDISINTNVFEHAGRVYAVSENHLPYEICVGS---LDTTRA
SbCCD8D : PNPLNPTQTIADSI FGSTSYMYEGHGLHAYVYDKSSLG-EWKISYRNKYNSDTFQLERKNQV-AFVPSADGQPYATLVAFVLNILR-----EKAVKDSANTNI FEHAGRAFAVTENHLPYEINISN---LNTLGP
SbCCD8d-li : PNPLNPTQTIADSI FGSTSYMYEGHGLHAYVYFNKSNLG-EWKISYRNKFG-----KAVKDSANTNIFEHAGRAFAVTENHLPYEININN---LNTLGP

AtCCD8 : FEYDDVLSDHMIQSAHPTVTEEMWTLPIDLKVP---GYRVRMEAGSNKREVVGRVRCR-----SGSWGPGWHSFAVTEN---YVVIPEMPLRYSVKNLLRAEPTPIYKFEWCPQDGAFTLVSKLTGEVVS-----
HsrPE65 : VDLCNVSVNGATAHPHIENDGTVYNIIGNCFGKNFSIAYNIIVKIPLQADKEDPLSKSEIVVQFPCSDRFKPSYVHSFGLTPN---YIVVETPVKINLKFSSWSLWGANYMDCFESNETMGVWLHIAKKRKKYLNKK
CsCCD : LDYDKRLSHS-FTAHPKVDPFIDENFTFCYAHTPPYVTVRISKDG-VIRDPVPIITIP-----ASVMHDFALITEN---YSIFMDPLIFYQPKEMKGGKLI FSFATKKAR---FGVLPRYAKDLSLIR---W
OsCCD8b-li : WHLGGEWKLP-FTAHPKVIPGSKEMVIFCINAVQPLTVGIIISEDGEKIKQKVGLKLD-----RCTYCHEIGVGTGT---YNIITID SPLTLNPTRM LRG-APVLEFEEESYSR---IGVMPHYGDADSVI---W
OsCCD8b : WDINGEWKRP-FTAHPKVAPGSGELVIFCSDAKRPFMLMGVVSADGTQKHKVDLKL-----RCILCHDIGVTVK---YNIIMDPLTIDISRLIRG-NQLTKFEKDSYAR---IGVMPRYGDAESVM---W
SbCCD8b : WDVGGEDWRPA-FTAHPKVAPGSGELVIFCMDAKRPFVLVIGVVSADGTQKHRVDLKL-----RSTLCHDIGVTLYKGHNVMIDPLTIDISRLVKG-GQLIQFEKESYAR---IGVMPRYGDAESV---W
ZmCCD8b : WDVGGEDWRP-FTAHPKVAPGSGELVIFCTDAKPFVLVGVVSADGTQKHRADLKL-----RCTLCHDIGVTPK---HNVMIDPLTMDVGRIVKG-GQLIQFEKESYAR---IGVMPRYGDAESV---W
SbCCD8D : YNNGAWNQP-FTSHPKKIHESGELVIMMCTNPEKPHYVLGVISPDGERVHKADLKE-----EGKLTHDIGVTKR---YNIIMDPLRFGISRTFLR-KPFIENDMNGKSR---IGVMPRFDAESII---W
SbCCD8d-li : YSNGAWNQP-FTSHPKKIHESGELVIMMCTNTEKPYVVLGVISDGSCEVHKVDLKEFG-----DGRLTHDIGVTKR---YNIIMDPLRFGISRTFLQ-KPFIENDLNGKSR---IGVMPRFDAESII---W

AtCCD8 : VEVPAYVTFFHTNAYEDKNGDGKATVIADCEHN-----ADTRIDLMLRLDTRLSSGHGDVLPDARIGRIPRLDGSKYGKLETAVEAEKHG-----RAMDMCSIN-PLVYLQKYR
HsrPE65 : YRTSPENLFHHINTYEDNGFLIVDLCCWKGFEFVYN-----YLYLANLRENWEEVKKARKAPQEVRRVYLP LNIDKADTGKNLVTLPNTTATAILCSDETZWLEPEVLFSGPRQAFEPQINQKYCKPYT
CsCCD : FEIPNCFIHNNANAWEEGDVVLITCRLENPDVMN-----GAVKEKLEN-----FKNELYEMRFNKTGAASQKQLS-----VSAVDFPRIN-ESYTRKQR
OsCCD8b-li : FVVEPFCFHLVNCFEEGHEVVVRGFHVPSSAIMGPR---R-----QKNMVMDTSSQEPN-----EENFSRLYEWRLNLTTRTVAGKYLTSL-----DVALEFPVIN-DKFSCLRHS
OsCCD8b : FVVEPFCFHLVNCFEEGHEVVIRGFRAADSIIPGPRISLNKN-----DLLSDPSKCSVKQG---INEEFFSRLYQWRNLNKTAKSVGQYLSGT-----EFSMEFPVIN-DHYTGLHQS
SbCCD8b : FVVEPFCFHLVNCFEEGDEG---LRSPDSIIPGRLAPNKC-----DSKMSELTEDDKPN-GTTKEFFRRLYQWRNLNKTAKSVGSEYLTGT-----EFSFEPIIN-NQYTGLQHS
ZmCCD8b : FVVEPFCFHLVNCFEEGHEVVVQALRSPDSIIPGSTIALDKL-----DSEMPEVAGDDKPAKRPTAEFFFRLYQWRNLNRTSVSGEYLSGT-----DYSLEFPVINS-SQYTGLQHR
SbCCD8D : FVVENHCSYHLNCFEDENEVVIRGCRLLGSIIPSGRHRVDKSKWYGRAFLQPKDSDSEDFPS---LDGTLFSRPYEWRLNENGSVHEGYITSE-----KVAMDFPVINS-DKFCVQVQNK
SbCCD8d-li : FVKNHCSYHLNCFEDGNEVVIRGCRVLASLIPSG-----HEDSEDFPS---LDGTLFSRPYEWRLNENSIVCEDYITSE-----KIAMDFPVIN-DKFIQIQNK

AtCCD8 : VYVACGAQRPCNFPNALSVDIVEKKVKNWHEHGMP-----SEPFVPRPGATHEDDGVVISIYSENGG---SFATLLDGSSFEETARAKFYGLPYGLHGCMIPKD-----
HsrPE65 : YAYGLGLNHFVPDRCLKLVKTKETWVQEPDSDYP-----SEPIFVSHPDAL EEDDGVVLSVVSPGAG-QKPAYLLILNAKDLSEVARAEVEINIPVTFHGLFKKS-----
CsCCD : VYVGTILDNITKVKGIIKFDLHAEPEAGKKLEVGNVQIGFDLGPGRYGSEAVFVPRERGIKSEEDDGYLIFFVHDENTG-KSEVNVIDAKTMSAEPVAVVLPNRPVPGFHFVNEEQLQWQQTDV
OsCCD8b-li : LNL AARPKEIGFAKLCEEKQNIATKIDREDLIK-----VEYHQLAKNQFCSGVTFVPKAAGAHEDDGVVISVYHDETN-ISKYHIIDARNFESEPIAKIILPQRVPYGLHGAFTIKRT-----
OsCCD8b : YAYAQVADSLESSYGVNE-----KVILKYGLAKLCLEADNVIAEHIIIDAQTFEGAPVAKITLPRVPYGFHGNFFYTSNQR-----
SbCCD8b : YAYAQIVDSCENCG-----KVNPKYGGFAKFYLDERN---TEVHTVDAKRFEDAPVAKITLPRVPYGFHGNFFYTSNQR-----
ZmCCD8b : YAYAQVVDSCGNG-----KVNPKYGGFAKFYLDERN---AEVHTVDAKRFEDAPVAKITLPRVPYGFHGNFFYTSNQR-----
SbCCD8D : YGVAQVADSLATSKTGLFKFKMIAKLHFNMPDK-----KNGIDEDDGVVITYVHDEGTN-SSQYIIDA KRFSEEPVAKITLPRVPYGFHGNFFYTSNQR-----
SbCCD8d-li : YGVAQVADSLATSKTGLFKFKMIAKLHFNMPDKENKELISVEYHTLKEKQFCSGVQFVAKKNGIDEDDGVVITYVHDEGTN-VSQYIIDA KRFSEEPVAKITLPRVPYGFHGNFFYTSNQR-----

Figure 4-9. Protein sequence alignment of CCD8b carotenoid cleavage dioxygenase sub-family.

Chapter 5

ABA 8'-hydroxylases in maize roots affected by drought⁴

5.1 Abstract

The ability of plants to withstand drought, a potentially major constraint to yield and production, is influenced by abscisic acid (ABA). ABA is synthesized in the cytosol from plastid carotenoid-pathway precursors, and later degraded by the action of ABA hydroxylases. Endogenous accumulation of ABA is controlled by both its synthesis and breakdown. Thus the enzymatic activity of ABA 8'-hydroxylase (ABA8Ox) may be considered one of the key steps in maintaining ABA levels for necessary physiological processes. To investigate the role of this enzyme, maize *ABA8Ox* gene family members were identified. *ABA8Ox* gene expression was then analyzed in different tissues and roots during the drought-stress response in maize. These genes were found to be expressed in all tissues, with a high degree of specificity to each tissue and some degree of overlap. *ZmABA8Ox1a* and *ZmABA8Ox1b* were shown to be the major transcript components for regulating ABA catabolism in drought-stressed roots. Phylogenetic and gene-structure analyses were performed to extend the implications and infer the cause of ABA breakdown in other cereal crops.

5.2 Introduction

Human survival depends on food production, and drought is a potentially major

⁴ This chapter is submitted to Plant Molecular Biology as Vallabhaneni et al., [In review]

constraint to yield and production of food crops. In maize, for example, drought accounts for at least 15% of the crop loss in sub-Saharan Africa (Badu-Apraku *et al.*, 2009).

Absciscic acid (ABA) is a plant hormone that influences the plant's ability to withstand abiotic stresses such as drought, cold, salt and wounding (Finkelstein *et al.*, 2002).

Transgenic plants overexpressing ABA biosynthetic genes show improved drought tolerance (Iuchi *et al.*, 2001), while overexpression of ABA 8' hydroxylases (ABA8Ox) reduces ABA levels and produces ABA-deficient phenotypes (Nitsch *et al.*, 2009). Thus, the amount of ABA is determined by the balance between biosynthesis and catabolism (Kushiro *et al.*, 2004), and one strategy for achieving maximum drought tolerance in crop plants, and thereby avoiding losses, is to increase the synthesis of endogenous ABA while controlling catabolism.

The first committed step in ABA biosynthesis is the oxidative cleavage of a 9-cis-epoxycarotenoid (C₄₀) to form xanthoxin (C₁₅). Xanthoxin is oxidized to form abscisic aldehyde, and then further oxidized to ABA (Schwartz *et al.*, 2003; Nambara and Marion-Poll, 2005). Hydroxylation of ABA to yield 8'-hydroxy ABA (8'-OH-ABA), catalyzed by ABA 8'-hydroxylase (CYP707A or ABA8Ox), is a committed step in ABA inactivation (**Fig. 5-1**).

ABA-biosynthesis-related genes (Schwartz *et al.*, 2003; Li *et al.*, 2008) and their regulatory mechanisms (Seo and Koshiba, 2002; Xiong and Zhu, 2003) have been identified in a wide range of plants. Extensive studies have been conducted on genes encoding enzymes with ABA catabolic functions in *Arabidopsis* (CYP707A1-CYP707A4) (Kushiro *et al.*, 2004; Saito *et al.*, 2004) and barley (*HvABA8Ox1* and *HvABA8Ox2*) (Millar *et al.*, 2006). Their expression patterns have indicated that ABA catabolism under

stress and recovery is mainly regulated at the transcriptional level. Furthermore, each CYP707A gene has a distinctive role in plants with some degree of tissue-specific expression (Yang and Zeevaart, 2006).

Although there is a wealth of knowledge on ABA catabolism in model species, ABA catabolism in staple food crops is not as well understood. Cereal crops of global importance, such as maize, sorghum, wheat and rice, are evolutionarily related and contained within the family Poaceae (grasses). The aim of this study was to characterize the ABA 8' hydroxylase (ABA8Ox) gene family and elucidate the expression of ABA-breakdown-related genes in unstressed maize tissues and in maize roots affected by drought stress. Using comparative genomics, orthologs were also identified in other grass genomes opening up the possibility of breeding these plants for drought tolerance.

5.3 Materials and Methods

5.3.1 Plant Materials and Stress treatments

Maize (*Zea mays* L.) inbred line B73 plants were field grown in the Bronx, New York, sibling-pollinated, and endosperm and embryo tissues were dissected 20 days after pollination (DAP). For drought experiments and collection of leaf and root samples at the six-leaf stage, B73 was grown in a greenhouse as described previously (Li et al., 2008). Dissected tissues were stored at -80°C until analysis.

5.3.2 Sequence analyses

The genomic DNA sequences of ABA hydroxylases of *Arabidopsis thaliana*, *Oryza*

sativa, *Sorghum bicolor* and *Z. mays* were obtained from NCBI GenBank, Gramene, Phytozome and PlantGDB, respectively (**Table 5-1**). The sequences were analyzed using Vector NTI Suite, Version 9.0 (InforMax, North Bethesda, MD), and processed for gene-structure analysis, while deducing cDNA and protein sequences. Amino acid sequences were aligned using ClustalW and a neighbor-joining tree was constructed with 500 bootstrap replication support using MEGA3 software (Kumar et al., 2001). Chromosomal mapping was performed using the WebAGCoL package (Pampanwar et al., 2005) or MaizeGDB.

5.3.3 Quantitative real-time PCR

RNA extraction and quantitative RT(real-time)-PCR were performed using gene-specific primers (**Table 5-2**) and normalized to actin, as previously described (Vallabhaneni and Wurtzel, 2009).

5.4 Results and Discussion

5.4.1 Identification and characterization of ABA 8'-hydroxylases in maize

Five putative *ABA8Ox* genes were found in maize (*ZmABA8Ox1a*, *ZmABA8Ox1b*, *ZmABA8Ox2*, *ZmABA8Ox3a*, *ZmABA8Ox3b*). These genes are orthologous to *Arabidopsis* (Kushiro et al., 2004; Saito et al., 2004) and rice (Saika et al., 2007) genes, and their presence is conserved across the grasses (Poaceae). The predicted maize proteins are implicated in ABA inactivation based on sequence homology with *CYP707* genes in *Arabidopsis*. The predicted maize proteins share 79.7% similarity and 40.2%

sequence identity (**Fig. 5-6**) and are predicted to localize to the cytosol, the predominant site for ABA synthesis and catabolism to phaseic acid and dihydrophaseic acid (**Fig. 5-1**). Table 1 summarizes the putative genes encoding ABA8Ox enzymes and their chromosomal positions in maize along with orthologs in Arabidopsis, rice, and sorghum.

The exon-intron boundaries were deduced and confirmed through selected expressed sequence tags (ESTs) where available (**Table 5-1**). All members of the *ABA8Ox* gene family from all three grass species carried a typical eukaryotic gene structure with multiple introns (**Fig. 5-2**). Structures of genes are shown with orthologs grouped into one of three clades, *Ox1*, *Ox2*, and *Ox3*. Orthologs in the three species shared exon-intron organization that distinguished each paralog. The *Ox1* clade had the fewest number of exons (five) whereas the *Ox2* clade had the most exons (nine) (**Fig. 5-2**). In addition, the classification of clades based on gene structure was similar to the protein phylogenetic organization.

Phylogenetic analysis of ABA8Ox protein sequences from the grasses and select dicots showed three distinct clades (**Fig. 5-3**), designated *Ox1*, *Ox2* and *Ox3*. The formation of three clades is supported by the presence of orthologous genes in rice, designated *OsABA8Ox1* to *OsABA8Ox3* (Saika et al., 2007). Both *Ox2* and *Ox3* appear to have diverged after the division of monocots and dicots. During the speciation events among monocots, it appears that some grasses lost some of their genes or acquired specialized functions during duplication leading to *Ox2* and *Ox3*. This assumption is partially supported by the absence of *Ox2* in barley (data not shown). The *Ox2* (labeled as such) from barley is actually a true *Ox3* based on its sequence similarities (**Fig. 5-6**). The absence of *Ox2* in barley could be due to either incomplete genome sequencing or, at

least in part, evolutionary mechanisms.

5.4.2 Spatial analysis of ABA 8'-hydroxylases

Among the sampled tissues, maize paralogs of *ABA8Ox* varied in relative expression, suggesting that in each tissue, different combinations of *ABA8Ox* are responsible for ABA catabolism. *Ox3a* was found to be predominantly expressed in leaves, *Ox1a* in roots, *Ox2* and *Ox1a* in the endosperm, and *Ox2*, *Ox1a* and *Ox1b* in the embryo (**Fig. 5-4**). In contrast, *Arabidopsis* CYP707A3 (an *Ox1* homolog based on Fig. 5-3) plays a prominent role in ABA catabolism in the vegetative tissues of *Arabidopsis* (Kushiro et al., 2004; Umezawa et al., 2006). The abundance of maize *ABA8Ox1a* in the roots suggests a prominent role in root ABA catabolism. The putative role of *ABA8Ox2* in endosperm and embryo seed tissues is consistent with previous studies in *Arabidopsis*, where CYP707A2 was found to play a significant role in seed dormancy (Okamoto et al., 2006), while CYP707A3 (an *Ox1* homolog based on Fig. 5-3) did not affect it. In this study, *Ox3a* exhibited over 1000-fold higher expression in leaves compared to other paralogs, a much higher difference than that exhibited by paralogs expressed in other sampled tissues. In a similar study with *Phaseolus vulgaris*, high ABA8Ox activity in leaves during dehydration was found to be due solely to an increase in CYP707A3; no change was observed in CYP707A1 or A2 with dehydration (Yang and Zeevaart, 2006). Therefore, *ABA8Ox3* could be an important target in the control of maize leaf ABA catabolism, given that ABA can be mobilized from any tissue.

5.4.3 Modulation and contribution of ABA 8'-hydroxylases during water deficit in roots

Because drought stress is first detected by the roots, it is likely that this organ can sense and respond to abiotic stress, leading to the mechanics of ABA accumulation. This suggests that ABA8Ox modulation in roots can determine drought tolerance in the entire plant. Data presented in Fig. 5-5 suggest that all five *ZmABA8Ox* genes are modulated by drought stress: their relative expressions paralleled dehydration and rehydration (**Fig. 5-5**) and modulation of ABA levels (Li et al., 2008). In roots, expression of *ZmABA8Ox1a*, followed temporally by that of *ZmABA8Ox1b*, suggests an important role for these two genes in modulation of root ABA levels. *ZmABA8Ox1a* levels peaked at 4 days of drought stress, whereas *ZmABA8Ox1b* transcript peaked at 6 days. *ZmABA8Ox1a*, but not *ZmABA8Ox1b*, also showed further elevation in response to rehydration within 2 h of watering on day 8 (**Fig. 5-5**). These data also suggest that since all *ZmABA8Ox* genes are expressed in roots, it is reasonable to assume that each of them contributes to the modulation of ABA concentration in this organ. Pooled transcript levels suggested temporal, compensatory variations between expression of each of the paralogous genes, particularly between *Ox1a* and *Ox1b*. *ZmABA8Ox1a* was found to contribute the most to root ABA8Ox transcript levels in early responses to drought stress, followed by *ZmABA8Ox1b*, *ZmABA8Ox3b*, *ZmABA8Ox3a* and *ZmABA8Ox2* (**Fig. 5-5**).

5.5 Conclusion

Many processes coexist, involving a cascade of genes contributing to whole-plant adaptation and tolerance to drought stress. This hypothesis is consistent with the existence of various quantitative trait loci reported in previous studies on drought stress

(Sari-Gorla et al., 1999; Tuberosa et al., 2002). Here, evidence is provided for control of ABA levels in roots mediated by ZmABA8Ox1a as the main early response degradative enzyme followed temporally by ZmABA8Ox1b. Drawing from the phylogenetic analysis, Ox1 orthologs can be considered as potential targets for engineering drought-tolerance across the grass family.

Table 5-1. Summary of ABA 8'-hydroxylases in grasses

Enzyme	<i>Arabidopsis thaliana</i>	<i>Oryza sativa</i>	<i>Zea mays</i>		<i>Sorghum bicolor</i>
		Gramene	BAC clones / ESTs	MAP	Phytozome
ABA8ox1 (cytochrome P450 707A5)	At4g19230 (CYP707A1) At5g45340 (CYP707A3)	LOC_Os02g47470	AC194862 (ZmABA8Ox1a) DR806072; EC902187; DY688015; CO520018; EB706067; DR803102; EE157729	5.06	Sb04g030660
			AC182107 (ZmABA8Ox1b) CD433445; EE190691; EE169703; EE036846; EE023371; EC902781; EE036847; EC894886; EE169704; EC884504; EC898571; EC884505	4.06	
ABA8ox2 (cytochrome P450 707A6).	AT2G29090 (CYP707A2)	LOC_Os08g36860	AC212409 (ZmABA8Ox2) CO460095; CO456959 ; CO459318; DV494257; EB701816; AI670285; EE040561; CO456726 ; DV529214; CA398898 ; DY623142; DY239249; EB701815	4.04	Sb07g022990
ABA8ox3 (cytochrome P450 707A7)	At3g19270 (CYP707A4)	LOC_Os09g28390	AC190490 (ZmABA8Ox3a) CD941324; CD941122; CD955590	2.06	Sb02g026600
			AC195926 (ZmABA8Ox3b) EC904849; DV527897; EE043845; DR785156; EE175998; EE036989 DV514713; DR962771; DV519081	7.02	

Table 5-2. Primers used in this study

Gene	No.	Primer Sequence	Orientation	Accession No.
ZmABA8Ox1a	1774	ATGCTCGTGCTCTTCCACCACCT	Forward	AC194862
	1769	TATACCGCCATACCATATCCATCCGCC	Reverse	
ZmABA8Ox1b	1774	ATGCTCGTGCTCTTCCACCACCT	Forward	AC182107
	1772	GGAAGCGGTTTTTCGCGTTCCTGG	Reverse	
ZmABA8Ox2	1785	AGCCTACGAGGAGAACGATG	Forward	AC212409
	1786	TCAGGAACCCTTGGTAGTGC	Reverse	
ZmABA8Ox3a	1783	ACAGAAAGGGGCGTGAGACCGA	Forward	AC190490
	1778	AGGCGAGCAAAGAAGAATTTCAA	Reverse	
ZmABA8Ox3b	1784	ACAGAAATGGCCGCCATGAGACCGA	Forward	AC195926
	1781	ATTTCTTCTCCCCCTCAAGGTAAT	Reverse	
Actin	1134	CGATTGAGCATGGCATTGTCA	Forward	J01238
	1135	CCCCTAGCGTACAACGAA	Reverse	

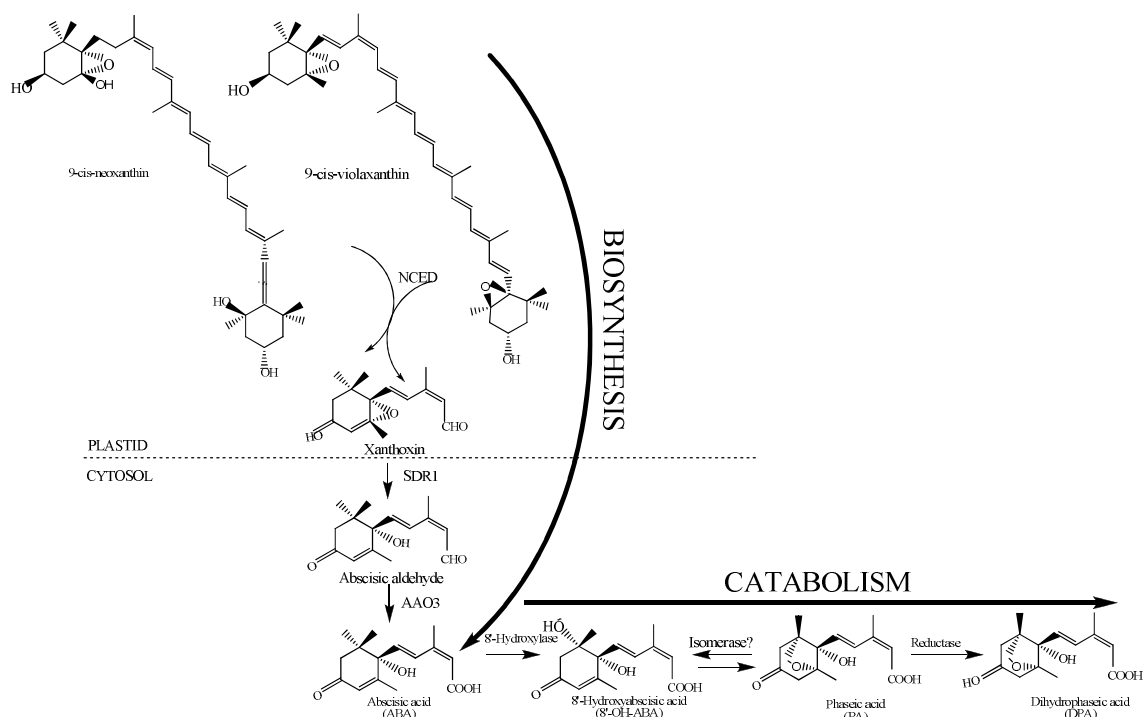


Figure 5-1. ABA synthesis and oxidation pathways in higher plants.

Carotenoid precursors of ABA are synthesized in the plastid. The cis-xanthophylls are cleaved by a family of 9-cis-epoxycarotenoid dioxygenases (NCED) to form the first 15-carbon precursor, xanthoxin. Xanthoxin moves to the cytosol and is converted to absciscic aldehyde by a short-chain dehydrogenase reductase (SDR1), which is then oxidized to ABA by an absciscic aldehyde oxidase (AAO3). ABA is hydroxylated to 8'-hydroxy ABA in the presence of 8' hydroxylase, and subsequently converted to phaseic acid and dihydrophaseic acid. The genes involved in these downstream steps are still unknown.

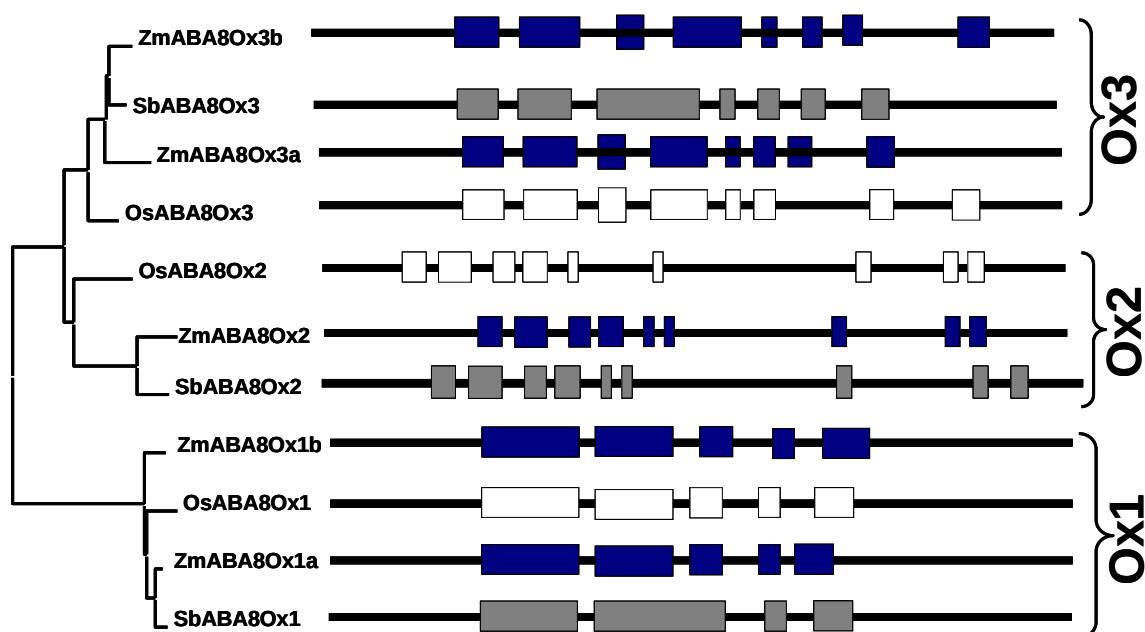


Figure 5-2. ABA8ox gene family structure.

ABA8Ox gene family. Gene structures (with boxes indicating exons and thin lines representing introns) for paralogs and orthologs of the ABA8Ox gene family in *Zea mays*, *Oryza sativa* and *Sorghum bicolor* (accession numbers are presented in Table 5-1). Gene structures are compared to protein phylogenetic tree on left which was produced based on sequences shown in Fig. 5-6.

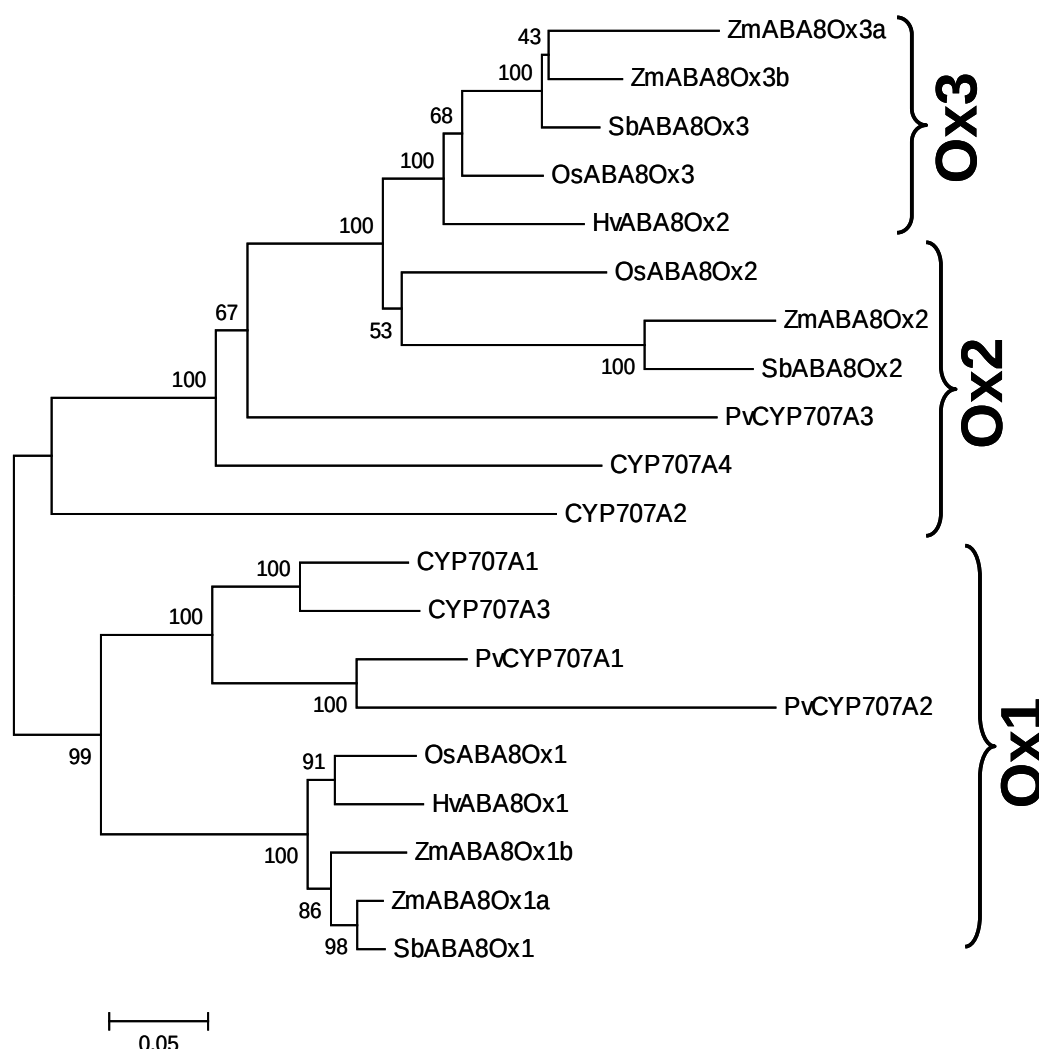


Figure 5-3. Phylogenetic analysis of proteins encoded by members of the *CYP707A* gene subfamily for representative monocots and dicots.

A neighbor-joining tree was constructed using the following protein sequences (given as accession numbers): ***Zea mays*** (ZmABA8Ox1a, DR806072; ZmABA8Ox1b, CD433445; ZmABA8Ox2, CO460095; ZmABA8Ox3a, EC904849; ZmABA8Ox3b, [CD941324](#)), ***Oryza sativa*** (OsABA8Ox1, LOC_Os02g47470; OsABA8Ox2, LOC_Os08g36860; OsABA8Ox3, LOC_Os09g28390), ***Sorghum bicolor*** (SbABA8Ox1, Sb04g030660; SbABA8Ox2, Sb07g022990; SbABA8Ox3, Sb02g026600), ***Hordeum vulgare*** (HvABA8Ox1, DQ145930; HvABA8Ox2, DQ145931), ***Arabidopsis thaliana*** (CYP707A1, At4g19230; CYP707A2, At2g29090; CYP707A3, At5g45340; CYP707A4, At3g19270), ***Phaseolus vulgaris*** (PvCYP707A1, DQ352541; PvCYP707A2, DQ352542; PvCYP707A3, DQ352543). Amino acid distances were subjected to Poisson correction. Numbers indicate bootstrap support for individual nodes. Bootstrap support higher than 40% is indicated at respective nodes ($n = 500$).

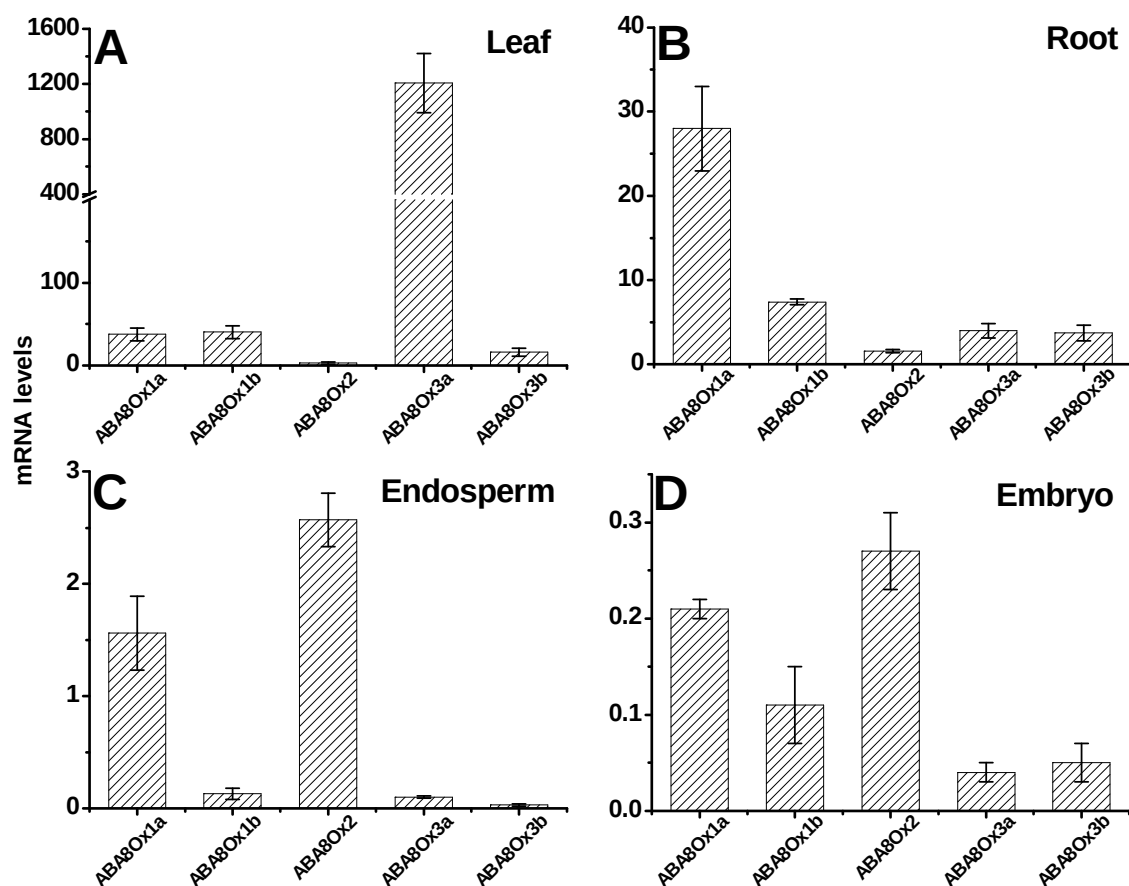


Figure 5-4. Transcript profiles of maize inbred line B73 genes encoding ABA 8'-hydroxylases.

Means of three replicates + SD are presented. Embryo and endosperm were collected at 20 DAP from field-grown plants; leaf and root samples were collected from seedlings at the six-leaf stage

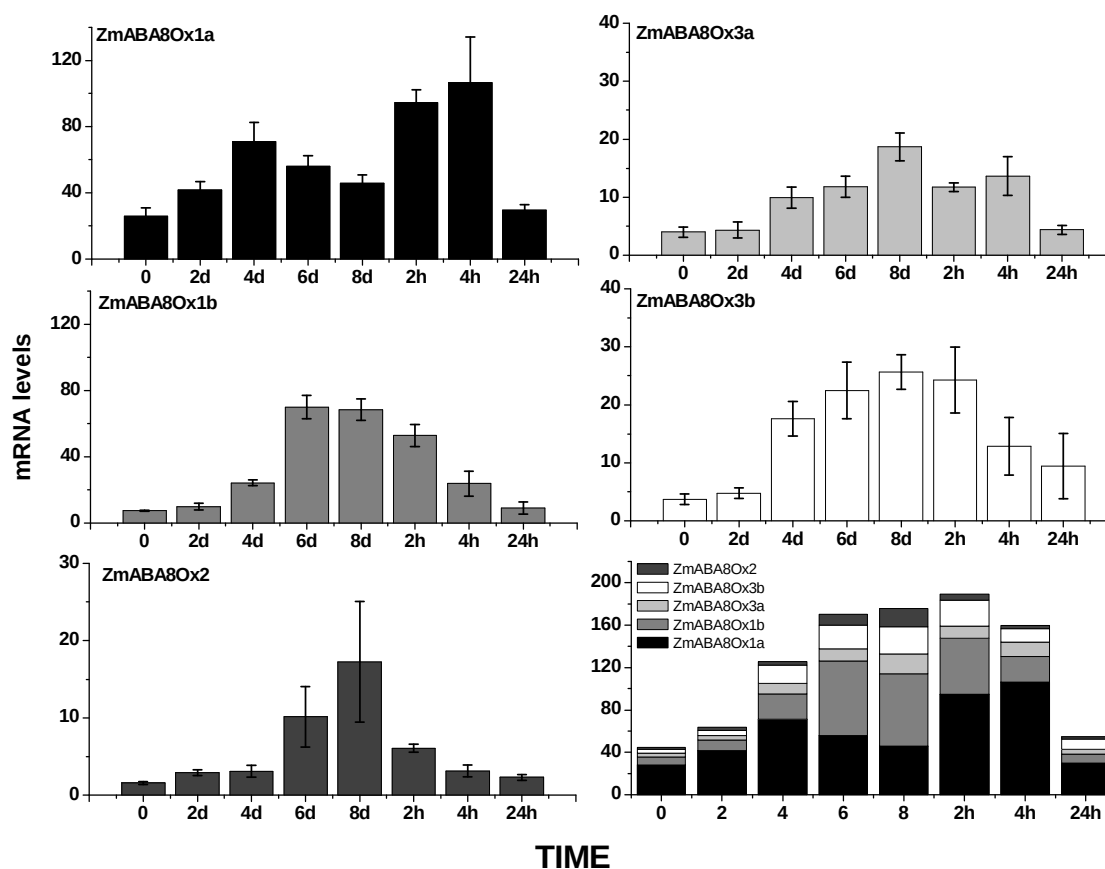


Figure 5-5. Drought stress-modulated levels of transcripts encoded by genes controlling inactivation of ABA in roots of *Zea mays*.

Means of three replicates $\pm$ SD are presented. See Table 1 for GenBank accession numbers. Bottom right panel shows the sum (stacked) of all transcripts controlling ABA catabolism.

HvABA80x1 : ---GAFILLLLVPLVLCVAVR--ARKGAGGR-----SSSGGKKKGLP---PGSMGWPYVGETTOLYSSKNPVVFARKNRYGPIFKTHILGCPVMVSSPEAAKFLVLTQAHFKPTFPASKERMLGRQAI
 HvABA80x2 : ---MAFFLLLLILVSVAVSYAHHAIRRRRGQ---C-AHGRHEQAALKP---PGSMGLPYVGETTOLYS-QDPSVFLSSQKRYGPIFKTHILGCPVMLASPEAAKFLVLSRAHLFKPTYPASKERLIGSAL
 OsABA80x1 : ---GAFLLFVCLVAPFLVLCVAVR--GRRRQAG-----SSEAAACGLP---PGSMGWPYVGETTOLYSSKNPVVFARKNRYGPIFKTHILGCPVMVSSPEAAKFLVLTQAHFKPTFPASKERMLGRQAI
 OsABA80x2 : MAFLFFVFTAAVLFCVVPFALLLCTSVQRRD---VGQGGGRDWQKKKRLP---PGSMGWPYVGETTOLYS-QDPIVFFASQKRYGPIFKTHILGCPVMLASPEAAKFLVLSRAHLFKPTYPASKERMIGSAL
 OsABA80x3 : ---MAASFVIVIVISFFISLAFMCYVHYTSRQRRK-----LHGVGHEKAVRLP---PGSMGWPYVGETTOLYS-QDPIVFFASQKRYGPIFKTHILGCPVMLASPEAAKFLVLTQAHFKPTYPASKERMIGSAL
 SbABA80x1 : ---GALLLFVCLLAPIVLLCAAVRGRSRKRSAS-----PASSCGKALP---PGSMGWPYVGETTOLYSSKNPVVFARKNRYGPIFKTHILGCPVMVSSPEAAKFLVLTQAHFKPTFPASKERMLGRQAI
 SbABA80x2 : ---MAFLLAVALIYIVFSLLTLLVLLVVSRRRTTAVTAGTGGATTEQDHNKLP---PGSLGLPIIGETTOLYLT-QNPDKFFASRLKRYGVFKTHVLGCPVILASPEAAKFLVLSRAHLFKPTYPASKERMIGQAAL
 SbABA80x3 : ---MAFFFLAATLLIFLAAYFYQYARWQGRG-----RS-GGGHEKQASSLSKLP---PGSMGWPYLGETTOLYS-QDPIVFFASQKRYGPIFKTHILGCPVMVSSPEAAKFLVLTQAHFKPTYPASKERMIGSAL
 ZmABA80x1a : ---GAEFLFVCLLAPVVLACAVRG-RKGR-AS-----SAAAGGKALP---PGSMGWPYVGETTOLYSSKNPVVFARKNRYGPIFKTHILGCPVMVSSPEAAKFLVLTQAHFKPTFPASKERMLGRQAI
 ZmABA80x1b : ---MAFFLLFVCLLAPVVLACAVRG-RKGR-AS-----SAAAGGKALP---PGSMGWPYVGETTOLYSSKNPVVFARKNRYGPIFKTHILGCPVMVSSPEAAKFLVLTQAHFKPTFPASKERMLGRQAI
 ZmABA80x2 : ---MAFFLAVALIYIVS-LLTLLVLLDASRR-RTTG-----GGSSSTECHKLP---PGSLGLPIIGETTOLYS-QNPDKFFASRLKRYGVFKTHVLGCPVILASPEAAKFLVLSRAHLFKPTYPASKERMIGQAAL
 ZmABA80x3a : ---MAFFLALVCLLILLAASYVQYTRWQGGK-----R-FGGHGRSAPLKP---PGSMGWPYLGETTOLYS-QDPSFFASQKRYGPIFKTHILGCPVMVSSPEAAKFLVLTQAHFKPTYPASKERMIGSAL
 ZmABA80x3b : ---MAFFFLAATLLIFLAAYFYQYARWQGGK-----R-LGGHEKASSSSSK-LP---PGSMGWPYLGETTOLYS-QDPIVFFASQKRYGPIFKTHILGCPVMVSSPEAAKFLVLTQAHFKPTYPASKERMIGSAL

HvABA80x1 : FFQGGDYHLLRLRLVSRFSPFAIRGSVSSIEAIALRSLGSMWG-HEVNTFQEMKTYALNVALLSIFGE--EEMQYIEELKQCYLTLEKGYNSMPVNLPGTLFHKAMKARKRLGATVAHIISARR-----ERERG---
 HvABA80x2 : FFHQGDYHLLRLRLVQGPLGPEALRKLVPDIEAAVRSSTLAAMADGDVASTFHAMKRLSFDVGIVTIFCG-RLDERRKEELRRNYAVVEKGYNSFPNSFPGLTYKATQARRRLNGVLSDDVHERR-----ERGEH---
 OsABA80x1 : FFQGGDYHLLRLRLVSRFSPFAIRGSVSSIEAIALRSLGSMWG-QFVNITFQEMKTYALNVALLSIFGE--EEMRYIEELKQCYLTLEKGYNSMPVNLPGTLFHKAMKARKRLGATVAHIISARR-----ERERG---
 OsABA80x2 : FFHQGDYHLLRLRLVQGPLGPEALRKLVPDIEAAVRSSTLAAMADGDVASTFHAMKRLSFDVGIVTIFCG-RLDERRKEELRRNYAVVEKGYNSFPNSFPGLTYKATQARRRLNGVLSDDVHERR-----ERGEH---
 OsABA80x3 : FFHQGDYHLLRLRLVQGPLGPEALRKLVPDIEAAVRSSTLAAMADGDVASTFHAMKRLSFDVGIVTIFCG-RLDERRKEELRRNYAVVEKGYNSFPNSFPGLTYKATQARRRLNGVLSDDVHERR-----ERGEH---
 SbABA80x1 : FFQGGDYHLLRLRLVSRFSPFAIRGSVSSIEAIALRSLGSMWG-QFVNITFQEMKTYALNVALLSIFGE--EEMRYIEELKQCYLTLEKGYNSMPVNLPGTLFHKAMKARKRLGATVAHIISARR-----ERERG---
 SbABA80x2 : FFHQGDYHLLRLRLVQGPLGPEALRKLVPDIEAAVRSSTLAAMADGDVASTFHAMKRLSFDVGIVTIFCG-RLDERRKEELRRNYAVVEKGYNSFPNSFPGLTYKATQARRRLNGVLSDDVHERR-----ERGEH---
 SbABA80x3 : FFHQGDYHLLRLRLVQGPLGPEALRKLVPDIEAAVRSSTLAAMADGDVASTFHAMKRLSFDVGIVTIFCG-RLDERRKEELRRNYAVVEKGYNSFPNSFPGLTYKATQARRRLNGVLSDDVHERR-----ERGEH---
 ZmABA80x1a : FFQGGDYHLLRLRLVSRFSPFAIRGSVSSIEAIALRSLGSMWG-QFVNITFQEMKTYALNVALLSIFGE--EEMRYIEELKQCYLTLEKGYNSMPVNLPGTLFHKAMKARKRLGATVAHIISARR-----ERERG---
 ZmABA80x1b : FFQGGDYHLLRLRLVSRFSPFAIRGSVSSIEAIALRSLGSMWG-QFVNITFQEMKTYALNVALLSIFGE--EEMRYIEELKQCYLTLEKGYNSMPVNLPGTLFHKAMKARKRLGATVAHIISARR-----ERERG---
 ZmABA80x2 : FFHQGDYHLLRLRLVQGPLGPEALRKLVPDIEAAVRSSTLAAMADGDVASTFHAMKRLSFDVGIVTIFCG-RLDERRKEELRRNYAVVEKGYNSFPNSFPGLTYKATQARRRLNGVLSDDVHERR-----ERGEH---
 ZmABA80x3a : FFHQGDYHLLRLRLVQGPLGPEALRKLVPDIEAAVRSSTLAAMADGDVASTFHAMKRLSFDVGIVTIFCG-RLDERRKEELRRNYAVVEKGYNSFPNSFPGLTYKATQARRRLNGVLSDDVHERR-----ERGEH---
 ZmABA80x3b : FFHQGDYHLLRLRLVQGPLGPEALRKLVPDIEAAVRSSTLAAMADGDVASTFHAMKRLSFDVGIVTIFCG-RLDERRKEELRRNYAVVEKGYNSFPNSFPGLTYKATQARRRLNGVLSDDVHERR-----ERGEH---

HvABA80x1 : SLLGLMRSRAGDDADDEGALLTDEQVADNIGVLFAAQDTTASVLTWVKYLDHDPKLLAEVRAEQAAAREATDGG-RLPLTWAOTRRMPTITHLVILESLRMAIIISFTFREAVADVEYQGYLIPKGWKVPLFRNI
 HvABA80x2 : SLLGLMRSRAGDDADDEGALLTDEQVADNIGVLFAAQDTTASVLTWVKYLDHDPKLLAEVRAEQAAAREATDGG-RLPLTWAOTRRMPTITHLVILESLRMAIIISFTFREAVADVEYQGYLIPKGWKVPLFRNI
 OsABA80x1 : SLLGLMRSRAGDDADDEGALLTDEQVADNIGVLFAAQDTTASVLTWVKYLDHDPKLLAEVRAEQAAAREATDGG-RLPLTWAOTRRMPTITHLVILESLRMAIIISFTFREAVADVEYQGYLIPKGWKVPLFRNI
 OsABA80x2 : SLLGLMRSRAGDDADDEGALLTDEQVADNIGVLFAAQDTTASVLTWVKYLDHDPKLLAEVRAEQAAAREATDGG-RLPLTWAOTRRMPTITHLVILESLRMAIIISFTFREAVADVEYQGYLIPKGWKVPLFRNI
 OsABA80x3 : SLLGLMRSRAGDDADDEGALLTDEQVADNIGVLFAAQDTTASVLTWVKYLDHDPKLLAEVRAEQAAAREATDGG-RLPLTWAOTRRMPTITHLVILESLRMAIIISFTFREAVADVEYQGYLIPKGWKVPLFRNI
 SbABA80x1 : SLLGLMRSRAGDDADDEGALLTDEQVADNIGVLFAAQDTTASVLTWVKYLDHDPKLLAEVRAEQAAAREATDGG-RLPLTWAOTRRMPTITHLVILESLRMAIIISFTFREAVADVEYQGYLIPKGWKVPLFRNI
 SbABA80x2 : SLLGLMRSRAGDDADDEGALLTDEQVADNIGVLFAAQDTTASVLTWVKYLDHDPKLLAEVRAEQAAAREATDGG-RLPLTWAOTRRMPTITHLVILESLRMAIIISFTFREAVADVEYQGYLIPKGWKVPLFRNI
 SbABA80x3 : SLLGLMRSRAGDDADDEGALLTDEQVADNIGVLFAAQDTTASVLTWVKYLDHDPKLLAEVRAEQAAAREATDGG-RLPLTWAOTRRMPTITHLVILESLRMAIIISFTFREAVADVEYQGYLIPKGWKVPLFRNI
 ZmABA80x1a : SLLGLMRSRAGDDADDEGALLTDEQVADNIGVLFAAQDTTASVLTWVKYLDHDPKLLAEVRAEQAAAREATDGG-RLPLTWAOTRRMPTITHLVILESLRMAIIISFTFREAVADVEYQGYLIPKGWKVPLFRNI
 ZmABA80x1b : SLLGLMRSRAGDDADDEGALLTDEQVADNIGVLFAAQDTTASVLTWVKYLDHDPKLLAEVRAEQAAAREATDGG-RLPLTWAOTRRMPTITHLVILESLRMAIIISFTFREAVADVEYQGYLIPKGWKVPLFRNI
 ZmABA80x2 : SLLGLMRSRAGDDADDEGALLTDEQVADNIGVLFAAQDTTASVLTWVKYLDHDPKLLAEVRAEQAAAREATDGG-RLPLTWAOTRRMPTITHLVILESLRMAIIISFTFREAVADVEYQGYLIPKGWKVPLFRNI
 ZmABA80x3a : SLLGLMRSRAGDDADDEGALLTDEQVADNIGVLFAAQDTTASVLTWVKYLDHDPKLLAEVRAEQAAAREATDGG-RLPLTWAOTRRMPTITHLVILESLRMAIIISFTFREAVADVEYQGYLIPKGWKVPLFRNI
 ZmABA80x3b : SLLGLMRSRAGDDADDEGALLTDEQVADNIGVLFAAQDTTASVLTWVKYLDHDPKLLAEVRAEQAAAREATDGG-RLPLTWAOTRRMPTITHLVILESLRMAIIISFTFREAVADVEYQGYLIPKGWKVPLFRNI

HvABA80x1 : HHNPDHFPCEKFDPSRFVAPKPNITFPPFGNGTHSCPGNELAKLEMLVLHLLVITGYRMEVVGSDDEVEYSPFPVRKGLLARVRDDGVCAGRKGCTDEDDNYDDDEVIV
 HvABA80x2 : HHNPDHFPCEKFDPSRFVAPKPNITFPPFGNGTHSCPGNELAKLEMLVLHLLVITGYRMEVVGSDDEVEYSPFPVRKGLLARVRDDGVCAGRKGCTDEDDNYDDDEVIV
 OsABA80x1 : HHNPDHFPCEKFDPSRFVAPKPNITFPPFGNGTHSCPGNELAKLEMLVLHLLVITGYRMEVVGSDDEVEYSPFPVRKGLLARVRDDGVCAGRKGCTDEDDNYDDDEVIV
 OsABA80x2 : HHNPDHFPCEKFDPSRFVAPKPNITFPPFGNGTHSCPGNELAKLEMLVLHLLVITGYRMEVVGSDDEVEYSPFPVRKGLLARVRDDGVCAGRKGCTDEDDNYDDDEVIV
 OsABA80x3 : HHNPDHFPCEKFDPSRFVAPKPNITFPPFGNGTHSCPGNELAKLEMLVLHLLVITGYRMEVVGSDDEVEYSPFPVRKGLLARVRDDGVCAGRKGCTDEDDNYDDDEVIV
 SbABA80x1 : HHNPDHFPCEKFDPSRFVAPKPNITFPPFGNGTHSCPGNELAKLEMLVLHLLVITGYRMEVVGSDDEVEYSPFPVRKGLLARVRDDGVCAGRKGCTDEDDNYDDDEVIV
 SbABA80x2 : HHNPDHFPCEKFDPSRFVAPKPNITFPPFGNGTHSCPGNELAKLEMLVLHLLVITGYRMEVVGSDDEVEYSPFPVRKGLLARVRDDGVCAGRKGCTDEDDNYDDDEVIV
 SbABA80x3 : HHNPDHFPCEKFDPSRFVAPKPNITFPPFGNGTHSCPGNELAKLEMLVLHLLVITGYRMEVVGSDDEVEYSPFPVRKGLLARVRDDGVCAGRKGCTDEDDNYDDDEVIV
 ZmABA80x1a : HHNPDHFPCEKFDPSRFVAPKPNITFPPFGNGTHSCPGNELAKLEMLVLHLLVITGYRMEVVGSDDEVEYSPFPVRKGLLARVRDDGVCAGRKGCTDEDDNYDDDEVIV
 ZmABA80x1b : HHNPDHFPCEKFDPSRFVAPKPNITFPPFGNGTHSCPGNELAKLEMLVLHLLVITGYRMEVVGSDDEVEYSPFPVRKGLLARVRDDGVCAGRKGCTDEDDNYDDDEVIV
 ZmABA80x2 : HHNPDHFPCEKFDPSRFVAPKPNITFPPFGNGTHSCPGNELAKLEMLVLHLLVITGYRMEVVGSDDEVEYSPFPVRKGLLARVRDDGVCAGRKGCTDEDDNYDDDEVIV
 ZmABA80x3a : HHNPDHFPCEKFDPSRFVAPKPNITFPPFGNGTHSCPGNELAKLEMLVLHLLVITGYRMEVVGSDDEVEYSPFPVRKGLLARVRDDGVCAGRKGCTDEDDNYDDDEVIV
 ZmABA80x3b : HHNPDHFPCEKFDPSRFVAPKPNITFPPFGNGTHSCPGNELAKLEMLVLHLLVITGYRMEVVGSDDEVEYSPFPVRKGLLARVRDDGVCAGRKGCTDEDDNYDDDEVIV

Figure 5-6. Multiple-alignment of proteins encoded by genes encoding ABA 8'-hydroxylases of maize and orthologs from *Oryza sativa* (Os), *Sorghum bicolor* (Sb) and *Barley* (Hv). See table 5-1 for accession numbers

Chapter 6

Summary and Future Work

Carotenogenesis in Maize Endosperm and Natural Genetic Variation as a Tool for Predictive Metabolic Engineering Strategies⁵

6.1. Carotenoids

Carotenoids are a large class of isoprenoid pigments synthesized *de novo* by all photosynthetic plants, protists, bacteria and some fungi (Botella-Pavia and Rodriguez-Concepcion, 2006). In plants, carotenoids play a major role in many fundamental processes such as light harvesting, photo-protection and carotenoids aid in attracting pollinators. There are more than 750 carotenoids identified in nature so far (Britton et al., 2004). Carotenoids contain a system of conjugated double bonds that influence their physical and biochemical properties. These carotenoids are sub-divided into two groups. The first class are the non-oxygenated derivatives, which contain only carbon and hydrogen atoms with one or more β -cyclic or ϵ -rings such as β -carotene and α -carotene are referred to as carotenes. The second class are oxygenated derivatives of carotenes, designated as xanthophylls, which can contain keto, hydroxyl, or epoxy groups (Olson and Krinsky, 1995; Stahl and Sies, 2005). Carotenoid derivatives, as apocarotenoids are generated by cleavage from one or both ends of the carotenoid molecule (Giuliano et al., 2003).

⁵ Vallabhaneni and Wurtzel, review in preparation

In animals, some carotenoids are precursors of vitamin A. Vitamin A deficiency leads to xerophthalmia and early mortality, particularly in countries where rice, which is low in vitamin A, is the main staple food in the diet (West Jr et al., 1989; Grant, 1991; West et al., 2002). In addition, carotenoids prevent the onset of some chronic diseases states such as cardiovascular diseases and cancer (Mares-Perlman et al., 2002).

Humans rely upon plants as a dietary source of carotenoids as they do not synthesize carotenoids. The need to increase carotenoids in foods promoted a series of efforts to engineer high carotenoid levels into staple crops and the health promoting properties of carotenoids have intensified interest in carotenoid biosynthesis in plants and its commercial exploitation led to an unprecedented value in industry (Fraser and Bramley, 2004; Fraser et al., 2008; Harjes et al., 2008; Millis et al., 2008).

6.2 Regulation and Biosynthesis of carotenoids

Biosynthesis of carotenoids which take place in plastids is initiated by condensation of isopentenyl diphosphate, a 5-carbon (C_5) building block (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP) into a longer carbon chain diphosphate ester leading to the C_{40} backbone of carotenoids. The substrates and sequential action of enzymes in producing precursors for synthesis of carotenoids and later degradative pathway is described in detail elsewhere (see Chapter 1).

Previous studies demonstrated that carotenoid accumulation is mainly under control of transcriptional regulation. Precursor supply, developmental cues, environmental adaptations and effect of light over structural genes plays a major role in conferring

metabolic turnover of carotenoids [reviewed in (Sandmann et al., 2006; Giuliano et al., 2008)]. Regulation of carotenoid biosynthesis and current strategies to enhance carotenoid accumulation is described in detail elsewhere (see Chapter 1). Further development in this area depends on identifying specific alleles and understanding the relation between carotenoid accumulation and endogenous expression of carotenoid biosynthetic genes.

6.3 Carotenogenesis in grasses: gene families and phylogenetic studies

In grasses, most enzymes are encoded by single to multi gene families. The composition of gene family and its characterization with regard to endosperm carotenogenesis is described in chapters 2-4. Briefly, among genes encoding enzymes of the MEP pathway, DXS is encoded by three paralogs. *DXS3* is newly discovered paralog in maize (Vallabhaneni and Wurtzel, 2009), also present in sorghum and rice, spanning two subfamilies of the Poaceae. Other MEP pathway enzymes DXR, HDS and HDR were found to be encoded by single copy genes. IPPI is encoded by three genes and GGPPS is also encoded by three genes.

Within the carotenoid pathway, PSY is encoded by three genes throughout the poaceae (Gallagher et al., 2004; Li et al., 2008a; Li et al., 2008b, see appendix) and PDS, ZDS, LCYE, and LCYB all of which are encoded by single copy genes in maize and other grasses (Buckner et al., 1990; Li et al., 1996; Matthews et al., 2003; Singh et al., 2003; Harjes et al., 2008). *CrtISO* was found to be encoded by two genes in maize on chromosomes 2 and 4. However, *CrtISO2* proved to be a pseudogene in maize B73

(Vallabhaneni and Wurtzel, 2009). In contrast to maize, *CrtISO* was found to be encoded by only a single copy gene in rice, sorghum and Arabidopsis.

Carotenes thus formed are hydroxylated in the presence of two structurally distinct classes of carotene hydroxylases: the P450 heme-thiolate CYP97A and CYP97C enzymes and the nonheme diiron monooxygenases [reviewed in (Matthews and Wurtzel, 2007; Giuliano et al., 2008)]. As found in rice, maize contained one gene each for CYP97A and CYP97C, respectively. A total of six unlinked maize paralogs encoding nonheme diiron β -carotene hydroxylases (*HYD*) were identified in contrast to three rice genes with maize *HYD3* and maize *HYD4* syntenous to rice *HYD1* (Vallabhaneni et al 2009 in press; see Chapter 3).

Hydroxylated carotenoids are depleted by the action of Zeaxanthin epoxidase (ZEP) catalyzing the conversion of zeaxanthin to violaxanthin, a precursor of ABA in endosperm. I extended gene family identification and characterization and found that ZEP is encoded by two gene copies compared to only one in rice and sorghum (Quinlan et al., 2007). The ABA thus formed is catabolized by hydroxylation in the presence of ABA hydroxylases, which are also found to be encoded by a small gene family with tissue specific expression (see Chapter 5). Simultaneously, carotenoids are degraded by the action of carotenoid dioxygenase enzymes, which are also encoded by a large multi-gene family in the Poaceae (see Chapter 4). This gene family is divided into two subfamilies known as 9-*cis*-epoxy carotenoid cleavage dioxygenase (NCEDs) and carotenoid cleavage dioxygenases (CCDs), similar to other plant families (see Chapter 4).

All of the genes for carotenoid synthesis and degradation as described in chapters 2-5 were mapped to chromosome and compared to known QTL and carotenoid mutant loci (Tables 6.1 and 6.2).

6. 4 Maize as a model species: Natural genetic variation as a tool

It is known that carotenoid accumulation is mainly under control of transcriptional regulation. To be able to examine transcriptional control of pathway flux, I assembled a collection of genes from maize to represent upstream pathways for IPP and GGPP synthesis and the downstream carotenoid biosynthetic pathway (see Chapter 2), genes controlling individual carotenoid composition (see Chapter 3) including conversion to ABA (see Chapter 2), ABA hydroxylation (see Chapter 5) and also genes involved in degradation of carotenoids (see Chapter 4). Transcript levels were quantified for all functional genes in various tissues and across endosperm development for standard inbred B73; and a core subset comprising of 10 genetically diverse inbreds (see chapters 2-4). Thus, predictive metabolic engineering/breeding of kernel carotenoid content is performed by identifying genes and the temporal expression in developing endosperm that correlates with carotenoid content and composition.

6.4.1 The biosynthetic potential and timing of gene expression

To validate the utility of the core subset to yield predictive results, I used the collection to test the *PSY* gene family which contains one member, *PSY1*, known to control flux to endosperm carotenoids. Only *PSY1*, of three paralogs, showed the expected correlation of transcripts and carotenoid content in endosperm (Vallabhaneni and Wurtzel, 2009; see Chapter 2). The validated germplasm subset was then processed for other gene family

members both upstream, downstream, genes involved in depletion of carotenoids and degradation of carotenoids at different stages of development. In addition to *PSY1*, several other genes showed a statistically significant correlation of transcripts with total carotenoid content. Positively correlating genes upstream of the carotenoid pathway included *DXS3*, *DXR*, *HDR*, and *GGPPS1*, all at 25 days after pollination (DAP). Within the carotenoid pathway itself, *CrtISO1* transcripts negatively correlated with carotenoid content as compared to the positive correlation seen with transcripts of the carotenoid pathway gene, but interestingly, *PSY1* at 20 DAP. These experiments revealed a paralog-specific variation in transcript levels; paralog specific contribution to carotenoid accumulation in a specific tissue, timing of gene expression where transcript levels at specific endosperm developmental stages correlated to net accumulation of carotenoid content in mature kernel.

6.5 Conclusions and Future work

I showed that there exist multiple control points among the pathway genes controlling the accumulation of total carotenoid content and found optimal alleles for one of the key genes controlling β -carotene in maize seed. To best use this huge body of knowledge, pyramiding the optimal alleles for multiple control points into a single genotype is a best strategy to greatly improve total carotenoid and composition in maize endosperm (**Fig. 6.1**). However there were still open questions to fill in the gaps towards understanding how carotenogenesis is regulated in maize seed. In this regard, subsequent studies must focus towards post-transcriptional, sequestration, metabolon assembly, transcription networks, promoter content analysis and trans-acting factors, each regulating the

structural genes towards enhancing pigment accumulation.

6.5.1 Develop homozygous lines with LCYE and HYD3 optimal alleles

This can readily be achieved by crossing inbred lines harbouring LCYE (Harjes et al., 2008) and HYD3 (see chapter 3) optimal alleles. Using marker assisted selection, select the progeny carrying both LCYE and HYD3 optimal alleles. Understand the possible outcome of increasing β -carotene in endosperm by performing gene expression and chemical carotenoid analysis.

6.5.2 Sequence all known multiple control points to identify newer optimal alleles

To this date, I found DXS3, DXR, HDR, GGPPS1, PSY1, CrtISO1, ZEP1 and ZEP2 as multiple control points (Vallabhaneni and Wurtzel, 2009). . New optimal alleles can be found by sequencing the entire gene on all available inbred lines and perform statistical analysis correlating the sequence polymorphisms to metabolite content. For example, one can start with HDR, as it anchors one of the available QTLs.

6.5.3 Identify putative candidate genes that anchor available QTLs

Chander et al., (2007) identified many QTLs controlling both carotenoid content and composition in maize kernel. However, only few QTLs were linked to candidate genes encoding carotenoid biosynthetic genes (Table 6.1 and Table 6.2). It would not be surprising if some of these were linked to genes encoding transcription factors. As the maize genome approaches completion, it will be easier to identify the candidate genes and perform functional testing in relation to metabolite content in maize seed.

6.5.4 Systems approach to identify transcriptional networks

The availability of large-scale transcriptome, proteome, and metabolome datasets and new high-throughput methods for systems level analysis will enable us to use the information to integrate and comprehend the interactions of multiple components. This kind of analysis will let us elucidate the co-ordinated regulation between carotenoid metabolism and other metabolic pathways. For example co-expression analysis and arrays available for Arabidopsis can provide a wealth of information to hypothesize regulatory mechanisms controlling seed metabolite content and composition in maize.

6.5.5 Promoter content analysis and identification of master transcription factors

The ultimate increase in carotenoid content requires understanding of how pathway genes (structural enzymes) are activated. As genome sequence data increases, promoter sequences of each carotenoid gene and their network partners can be extracted from both Arabidopsis and rice, to analyze promoter regulatory sequences systematically. Such data will lead us to identify putative transcription factor binding sites.

Functional testing of each transcription factor should ultimately lead us to identify a global transcription factor or rather master transcription factor that can control the full metabolic pathway e.g. maize LC/C1 transcription factor affecting the flavonol content in tomato (Bovy et al., 2002). Such a transcription factor would enable single step metabolic engineering.

6.5.6. Post-transcriptional regulation studies

Transcriptional regulation was shown to be an important mechanism in controlling carotenoid accumulation. However, there is evidence that carotenoid biosynthetic

enzymes as well as MEP pathway enzymes (Guevera-garcia et al., 2005) supplying precursors are under post-transcriptional regulation. Post-transcriptional regulation is a key point to address in examining how carotenoid accumulation shifts under different post-transcriptional regimes.

6.5.7 Role of remaining MEP pathway genes in total carotenoid accumulation

Although this study generated extensive data that pinpoint multiple pathway control points (Fig. 6.1) that influence carotenoid content and composition, there still remain three genes in the MEP pathway to be studied (MCT, MCK, MCS).

Table 6-1. Mapping of carotenoid candidate genes, quantitative trait loci, and phenotypic loci in maize.

Chromosome bins (column 3, 7) are indicated for each carotenoid locus representing a structural gene, quantitative trait locus (*qtl*) or known genetic (phenotypic) locus. Where the structural gene and gene product are known for a genetic locus, the enzyme product is listed (column 1, 5) on the same line as the genetic locus (column 2, 6). In cases where an enzyme is encoded by a gene family, isozymes are followed by a number (eg. DXS1, DXS2, DXS3). For genetic loci for which there is only a carotenoid phenotype or *qtl*, but the product is unknown, no corresponding enzyme (column 1, 5) are indicated.

References for mapping data provided in columns 1-3 and 5-7 are respectively in columns 4 and 8. *qtl*, quantitative trait locus for enhanced: a, α -carotene b, β -carotene; c, β -cryptoxanthin; z, zeaxanthin; l, lutein; or t, total carotenoids in the seed endosperm.

References cited are a=(Vallabhaneni and Wurtzel, 2009); b=(Wong et al., 2004); c=Maize Database; d=(Buckner et al., 1990); e=(Singh et al., 2003); f=(Chander S et al., 2007); g= (Matthews et al., 2003); h=Brutnell et al, unpublished.

Enzyme	Genetic Locus	C. Map	Ref.	Enzyme	Genetic Locus	C. Map	Ref.
HYD6		1.01	a		<i>qtl^{bczlt}</i>	6.01	b
	<i>qtl^d bct</i>	1.01-.03	b	PSY1	<i>y1</i>	6.01	c,d
PDS	<i>vp5</i>	1.02	c,d		<i>qtl^{a,b,z,t} (Y1ssr –umc1595)</i>	6.01-6.03	f
	<i>qtl^{lt} (umc1403-fad83)</i>	1.03-	e		<i>qtl^{lct} (umc2313-Y1ssr)</i>	6.01-6.01	f
	<i>qtl^f (bnlg2086-umc1988)</i>	1.04-1.06	f	DXS1		6.05	a
	<i>wlu7</i>	1.05	c		<i>qtl^{bzl}</i>	6.05	b
NCED1		1.08	a	IPPI3		6.05	a
	<i>qtl^{bczlt}</i>	1.05-.09	b		<i>qtl^q (umc2162 – phi29985)</i>	6.06-6.07	f
	<i>wlu5</i>	1.07	c		<i>y8 (lty2)</i>	7.01	c
	<i>lw1</i>	1.10	c		<i>qtl^{bczlt}</i>	7.02	b
HDR	<i>umc1082</i>	1.09	a	GGPPS2		7.04	a
	<i>qtl^f (umc2047 – phi30870)</i>	1.09-1.10	f				
	<i>qtl^f (umc2047-ols1)</i>	1.09	a				
	<i>qtl^z (phi30870 – umc1553)</i>	1.10-1.11	e				
	<i>Y3-al1</i>	2.00-.01	c	ZDS	<i>vp9</i>	7.02	c,g
HYD4		2.03	a		<i>qtl^{bz} (bnlg1792-atf2)</i>	7.02-7.03	f
CCD7		2.03	a		<i>qtl^f (phi091-atf2)</i>	7.03	f
	<i>se1</i>	2.01	c		<i>qtl^l (atf2-umc2332)</i>	7.03-7.04	f
ZEP1	<i>gpm594</i>	2.04	a				
	<i>w3 (y11)</i>	2.06	c		<i>wlu2</i>	7.02-.06	b
GGPPS1		2.08	a	PSY3		7.03	c
CrtISO2		2.09	a				
NCED3b		2.09	a	IPPI1		7.04	h
	<i>qtl^{bclt}</i>	3.01	b		<i>Clm1</i>	8.00-.09	c
	<i>qtl^{bczlt}</i>	3.03-.05	b	GGPPS3		8.01	a
	<i>cl1</i>	3.04	c	IPPI2		8.03	a
DXR		3.04	c		<i>qtl^{bclt}</i>	8.02-03	b
NCED2		3.04	a		<i>qtl^z (umc1562-umc1141)</i>	8.05 -8.06	f
CYP97C	343.75cM	3.05	a				
	<i>qtl^a (umc2408-bnlq197)</i>	3.06	f				
	<i>qtl^q (umc1399-phi046)</i>	3.07-3.08	f		<i>qtl^z (phi115-umc1735)</i>	8.03	f
	<i>y10</i>	3.07	c		<i>wlu3</i>	8.04-.09	c
CCD8		3.07	a	LCYE		8.05	h
	<i>wlu1</i>	3.07-.08	c		<i>qtl^{bczlt}</i>	8.05-.06	b
	<i>qtl^{zlt}</i>	4.02-.05	b				
CYP97A	392.43cM	4	a	PSY2		8.07	i
	<i>lw4</i>	4.06	a		<i>qtl^{czlt}</i>	9.02-.04	b
DXS3		4.06	c	HYD5		9.07	a
CCD4a		4.06	a		<i>wlu4</i>	9.03-.08	c
	<i>qtl^{clt}</i>	4.07-.09	b	DXS2		9.03	c
CrtISO1	<i>csu704</i>	4.08	a		<i>qtl^{bczlt}</i>	9.06-.07	b
	<i>qtl^{bczlt}</i>	5.01-.04	b	CCD1	<i>Wc1</i>	9.07-.08	c
HDS	<i>umc1048</i>	5.03	a		<i>qtl^b</i>	10.01-.02	b
NCED9		5.03	a		<i>qtl^q (umc2016 –bnlg1655)</i>	10.03	f
	<i>qtl^{bz} (umc1692 – umc2373)</i>	5.03-5.04	f		<i>qtl^f (phi050 -umc1367-umc2016)</i>	10.03	f
	<i>qtl^f (umc1447-umc1692-umc2373)</i>	5.03-5.04	f		<i>y9 (y12)</i>	10.03- .04 (10.02?)	c
	<i>qtl^f (umc2115 – umc1447)</i>	5.02-5.03	f		<i>qtl^{cl}</i>	10.04-.05	b
				ZEP2		10.04	a
	<i>vp2</i>	5.04	c	HYD3		10.05	a
LCYB	<i>vp7</i>	5.04	e		<i>qtl^{b,z,t} (umc1506-bnlq1028)</i>	10.04-10.06	f
	<i>lw2/vp12</i>	5.05	c				
	<i>lw3</i>	5.06	c				
CCD4b		5.06	a				
	<i>qtl^{bczlt}</i>	5.06-.07	b	HYD1, 2			

Table 6-2. Chromosomal mapping of candidate genes encoding isoprenoid biosynthetic and degradative enzymes in *Zea mays*, *Oryza sativa*, *Triticum aestivum* and *Sorghum bicolor*. Pseudo indicates the gene is non functional. P value indicates the strength of association between qtl and the metabolite. The bin positions and qtl data for sorghum are collected from Fernandez et al (*Fernandez et al.*, 2008)

Enzyme	Maize (<i>Zea mays</i>)		Rice (<i>Oryza sativa</i>)		Wheat (<i>Triticum aestivum</i>)		Sorghum (<i>Sorghum bicolor</i>)	
	Chr. bin	Reference/ GenBank	Chr. bin	Reference GenBank	Chr. bin	Reference/ GenBank	Chr. Bin and (qtl)	Reference/ Phytozome
DXS1	6.05	(2008)AY110050	5	AK064944			9	Sb09g020140
DXS2	9.03	AY946270	7	AK100909			2	Sb02g005380
DXS3	4.06	AY104478	6	AK121920			10	Sb10g002960
DXR	3.04	BG840684	1	AK099702			3	Sb03g008650
MCT		DR818082	1	AP003379			3	Sb03g042160
CMK	3.06	BG354410	1	AK067589			3	Sb03g037310
MDS		CD974184	2	AK060238			4	Sb04g031830
HDS	5.03	AC198323	2	AK120769	6D	BE585702	4	Sb04g025290
HDR	1.09	DR789385	3	AK062113			1	Sb01g009140
IPI1	7.04	AF330034	7	AK065871	1A	CD452500	2	Sb02g035700
IPI2	8.03	AC203067	5	AK060336			9	Sb09g020370
IPI3	6.05	AC206783						
GGPPS1	2.08	EE417573	7	AK121529			2	Sb02g037510
GGPPS2	7.04	EF417574	1	AP002865				
GGPPS3	8.01	AC211188					3	Sb03g009380
PSY1	6.01	AY324431	6	AY445521	7AL, 7BL, 7A	Yellow pigment QTL	10b, 0.0cM qtl (color; p≤ 0.0001	Sb10g031020
PSY2	8.07	AY325302	12	AY773943	5A, 5B	either PSY2 or PSY3	8	Sb08g022310
PSY3	7.03	DQ372936	9	FJ214953			2, 165.9cM	Sb02g032370
PDS	1.02	L39266	3	AF049356	4A, 4B		6, 16.9cM	Sb06g030030
ZDS	7.02	AF047490	7	AK065213	2A, 2B		2, 37.1cM	Sb02g006100
CrtISO1	4.08	CC749876 AC218991	11	AC108871			5	Sb05g022240
CrtISO2 pseudo	2.09	EE045563/ AC183901						
LCYB	5.04	AY206862		AP008208			4	Sb04g006120
LCYE	8.05	BV727273		AK072015			3, 123.1cM	Sb03g026020
HYD1 pseudo		EU638325						
HYD2 pseudo		EU638326						
HYD3	10.05	AY844958						
HYD4	2.03	AC196442	4	AL606624			6	Sb06g026190
HYD5	9.07	AC149033	3	AC119747			1	Sb01g048860
HYD6	1.01	AC155364	10	AE016959				
CYP97C2	3.05	BE552887	10	AK065689	6AL, 6BL, 6DL	USDA	1, 146.3cM	Sb01g030050
CYP97A4	4	AY112171	2	AK068163			1, 38.9cM 1 or4?	Sb04g037300
ZEP1	2.04	AC194845	4	AL606448			6	Sb06g018220
ZEP2	10.04	AC206194	NA	NA				
VDE								
NXS								
CCD1	9.07	AY106323	12	Os12g44310			1, 48.5cM qtl (color; p≤ 0.05)	Sb01g047540
NCED1	1.08	AC201886					1, 210.3cM	
NCED2	3.04	AC199036	12	Os12g42280			1	Sbi_0.36552
NCED3a		AC212820	7	Os07g05940			2	Sb02g003230
NCED3b	2.09	AC205109						
CCD4a	4.06	AC190588	2	Os02g47510			4	Sb04g030640
CCD4b	5.06	AC194862	12	Os12g24800				
CCD7	2.03	AC211432	4	Os04g46470			6	Sb06g024560
CCD8a	3.07	AC185113	1	Os01g54270			5	Sb05g009950
CCD8b	7.02	AC198395	9	Os09g15240			2	Sb02g021490
NCED9	5.03	AC190614	3	Os03g44380			1	Sb01g013520

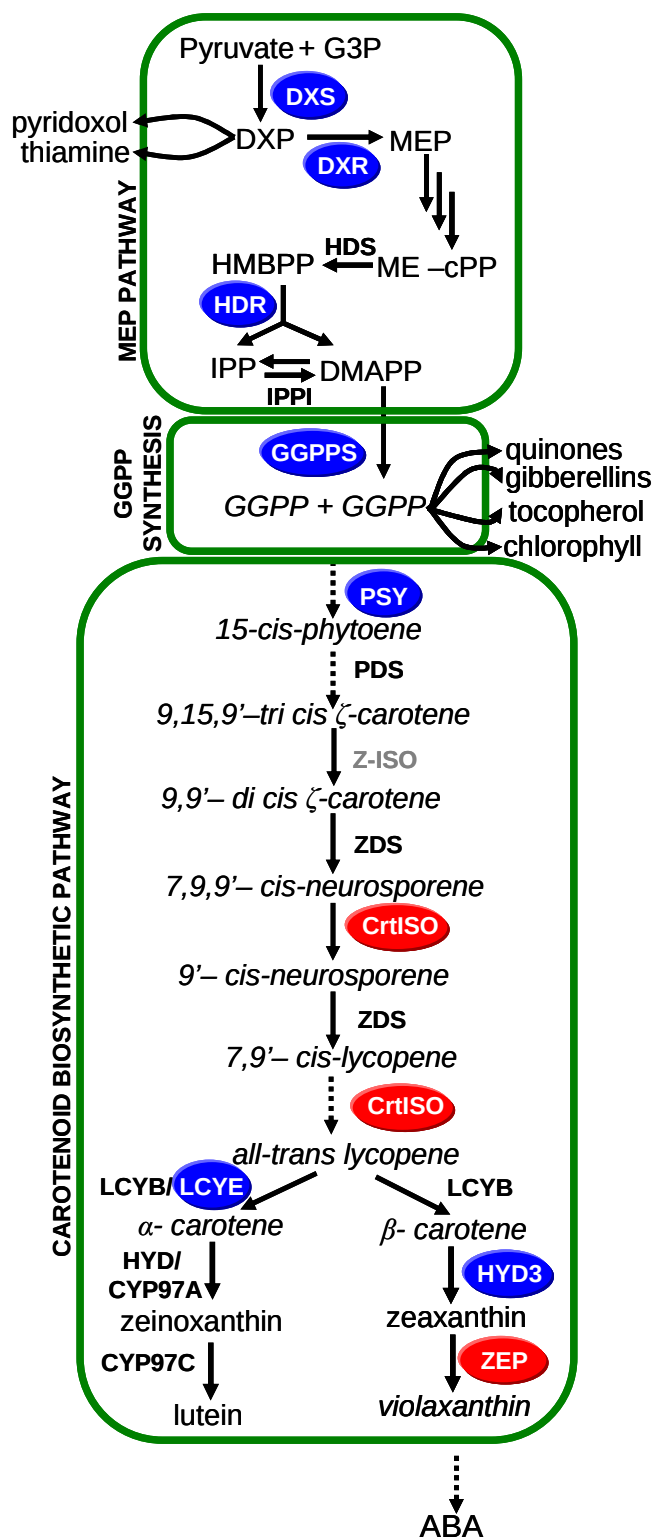


Figure 6-1. Simplified carotenoid biosynthetic pathway derived from isoprenoid precursors.

Enzymatic reactions are represented by arrows. Genes encoding all enzymes shown, except ZISO, were tested for correlation with total accumulated carotenoids. Transcripts for enzymes in black letters had no effect; white letters on blue bubble, white letters on red bubble showed positive correlation and negative correlation respectively. DXS, D-1-deoxyxylulose 5-phosphate synthase; DXR, DXP reductoisomerase; HDS, 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; IPPI, Isopentenyl pyrophosphate isomerase; GGPPS, Geranylgeranyl pyrophosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, 15-*cis* zetacarotene isomerase; ZDS, zetacarotene desaturase; CrtISO, carotene isomerase; LCYB, lycopene beta cyclase; LCYE, lycopene epsilon cyclase; HYD, β -carotene hydroxylase; CYP97A, p450 type β -carotene hydroxylase; CYP97C, p450 type ϵ -carotene hydroxylase; ZEP, zeaxanthin epoxidase; ABA, Absciscic acid

Appendix 1: Publications related to this dissertation

1.1 Natural Genetic Variation in Lycopene Epsilon Cyclase Tapped for Maize Biofortification⁶

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Materials and Methods
Figs. S1 to S4
References

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Natural Genetic Variation in Lycopene Epsilon Cyclase Tapped for Maize Biofortification

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Dietary vitamin A deficiency causes eye disease in 40 million children each year and places 140 to 250 million at risk for health disorders. Many children in sub-Saharan Africa subsist on maize-based diets. Maize displays considerable natural variation for carotenoid composition, including vitamin A precursors α -carotene, β -carotene, and β -cryptoxanthin. Through association analysis, linkage mapping, expression analysis, and mutagenesis, we show that variation at the *lycopen epsilon cyclase* (*lycE*) locus alters flux down α -carotene versus β -carotene branches of the carotenoid pathway. Four natural *lycE* polymorphisms explained 58% of the variation in these two branches and a threefold difference in provitamin A compounds. Selection of favorable *lycE* alleles with inexpensive molecular markers will now enable developing-country breeders to more effectively produce maize grain with higher provitamin A levels.

Maize is the dominant subsistence crop in much of sub-Saharan Africa and the Americas, where between 17 and 30% of children under age of 5 are vitamin A-deficient. This results in xerophthalmia (progressive blindness), increased infant morbidity and mortality, and depressed immunological responses (1). Vitamin A deficiency starts with inadequate provitamin A or vitamin A content or bioavailability

in foods and is exacerbated by disease-induced malabsorption.

Diet diversification, food fortification, and supplementation (2–4) have all been used to combat dietary micronutrient deficiencies. Ideally, all children would have access to a varied diet rich in fruits and vegetables, but diet diversification is often limited by crop seasonality, expense, and low bioavailability of green leafy plant carotenoids (5, 6). Poor infrastructure in developing countries has limited widespread use of direct vitamin supplementation. Perhaps the most feasible approach to eradicating death and disease caused by dietary deficiencies is biofortification, a process by which staple crops are purposefully bred for higher nutritional density (7, 8). Although biofortified foods can potentially be an inexpensive, locally adaptable, and long-term solution to diet deficiencies, cultural preferences may limit their acceptance. This may be particularly true for those crops where transgenics are the only alternative to boost provitamin A content, given limited acceptance of genetically modified organisms in developing countries.

Carotenoids are derived from the isoprenoid biosynthetic pathway and are precursors of the plant hormone abscisic acid and of other apo-

carotenoids (9). The first committed step of this pathway [as recently revised (10)] is formation of phytoene from geranylgeranyl diphosphate by phytoene synthase (*psy*) (Fig. 1) (11). Recent studies in maize suggest that the *psy* locus has been the target of a selective sweep following selection for endosperm-accumulating carotenoids and shift from white to yellow kernels (12). The first branch point of this pathway (Fig. 1) occurs at cyclization of lycopene where action of lycopene beta cyclase (LCYB) at both ends of linear lycopene produces a molecule with two β rings. Alternatively, the coaction of LCYB and lycopene epsilon cyclase (LCYE) generates a β , ϵ -carotene that is a precursor to lutein (13). Relative activities of LCYB and LCYE are hypothesized to regulate the proportion of carotenes directed to each branch of this pathway (13–15). Indeed, transgenic manipulations of LCYE expression in *Arabidopsis*, potato, and *Brassica* increase the pool of β ring-containing carotenes and xanthophylls (13, 16–18).

Maize exhibits considerable natural variation for kernel carotenoids, with some lines accumulating as much as 66 μ g/g. The pre-

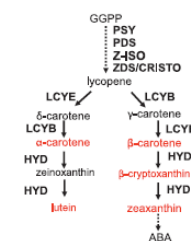


Fig. 1. Simplified carotenoid biosynthetic pathway in plants (29). Enzymatic reactions are represented by arrows, dashed lines represent multiple enzymatic steps. Substrates in red were evaluated in this study. Compounds: GGPP, geranylgeranyl diphosphate; ABA, abscisic acid. Enzymes: PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, 15-*cis* zetacarotene isomerase; ZDS, zetacarotene desaturase; CRISO, carotene isomerase; HYD, carotene hydroxylase enzymes, which include ϵ - and β -ring hydroxylases.

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dominant carotenoids in maize kernels, in decreasing order of concentration, are lutein, zeaxanthin, β -carotene, β -cryptoxanthin, and α -carotene. β -Carotene contains two provitamin A structures (two nonhydroxylated β -ionone rings) and β -cryptoxanthin and α -carotene one each (single nonhydroxylated β -ionone ring). Among lines included in our diverse maize panel, β -carotene levels reached 13.6 $\mu\text{g/g}$. However, most yellow maize grown and consumed throughout the world has only 0.5 to 1.5 $\mu\text{g/g}$ β -carotene. Comparisons between β -carotene and total carotenoids with grain color (scaled according to shade of yellow) revealed poor correlations with low R^2 values (Fig. 2), which

indicated that marker-assisted selection (MAS) may prove much more efficient than selection based on color alone.

To dissect the phenotypic diversity, we used an association-mapping approach that exploits the genetic diversity of maize to provide resolution within 2000 base pairs (bp) (19–21). In the context of plant breeding, this has the added advantage of identifying the most favorable allele within a diverse genetic background, which provides the necessary genotypic information to facilitate the design of efficient maize introgression and selection schemes throughout the world. We complemented the association mapping with linkage mapping to evaluate the

effects in a genetically less complex background and with a mutagenesis program to isolate novel allelic variation within an elite near-isogenic background.

To evaluate functional diversity (Fig. 1), eight candidate genes representing select members of gene families encoding biosynthetic enzymes of the carotenoid pathway were sampled across a diverse panel of 288 maize lines, of which 204 were yellow. Subsets of yellow lines were grown in four different years and surveyed for whole-kernel carotenoids by high-performance liquid chromatography (HPLC). The yellow lines averaged 23 $\mu\text{g/g}$ for total carotenoids (range 5.5 to 66.0 $\mu\text{g/g}$) and 1.7 $\mu\text{g/g}$ for β -carotene (range 0.06 to 13.6 $\mu\text{g/g}$).

For association analysis, we used a mixed-model approach that controlled for complex population and pedigree relationships (22). Among our current sampling of candidate genes, *lycopen epsilon cyclase* (*lcyE*) (14) had the largest effect on partitioning the two branches of carotenoids and, consequently, on β -carotene and β -cryptoxanthin content. In maize, the single-copy *lcyE* gene consists of 10 exons spanning 3640 bp (Fig. 3). After initial association and screening for polymorphisms in key haplotypes, four regions were selected and scored across the entire panel. On the basis of the position of LCYE in the biochemical pathway, we predicted that the ratio of the sum of kernel carotenoids from each pathway branch would form the strongest association. Indeed, this was confirmed (Table 1), with the strength of the association confirming that *lcyE* plays a key role in controlling this ratio. Correspondingly, levels of predominant provitamin A compounds β -carotene and β -cryptoxanthin were also highly associated with *lcyE*.

Subsequent haplotype analysis revealed several probable causative polymorphisms for the ratio of α - and β -carotene branches for the 2003 field season (table S1). A large promoter indel and an amino acid substitution in exon 1 explain most of the variation ($R^2 = 36\%$; $n = 135$; $P = 1.27 \times 10^{-12}$) with a 5.2-fold effect. A second indel in the 3' UTR also has a significant 3.3-fold effect and contributes to variation not explained by the promoter polymorphism (type III SS; $P = 1.9 \times 10^{-4}$). The fourth significant polymorphism at position 2238 in intron 4 was associated with a 2.5-fold effect (type III SS; $P = 0.0003$). The overall, four-term model explains 58% of the variation ($P = 9.2 \times 10^{-17}$). These significant polymorphisms exhibit some linkage disequilibrium (LD), and only nine haplotypic classes exist in our sample, which limits full differentiation of the effects of each polymorphism. Overall, there is a ninefold difference between two of the more differentiated haplotype classes, and sixfold between two more common haplotypes (table S2). There was a threefold increase in the proportion of β -carotene and β -cryptoxanthin between the common haplotypes. Verification of these results was provided by

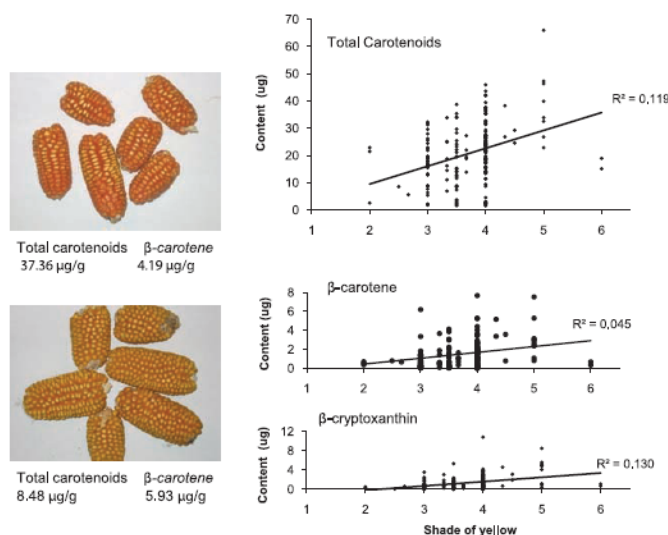


Fig. 2. Grain color and carotenoid content. The graphs depict the low correlation between visual grain color and total carotenoids, β -carotene, and β -cryptoxanthin in diverse inbreds. In these kernels, the shade of yellow ranges from white (score of 1) to dark orange (score of 6). White kernels were excluded from the analysis. The difficulty in visual selection for β -carotene content is further exemplified by the images on the left, where the yellow maize below has higher β -carotene than the orange variety above. These correlations are across the diverse panel of 228 maize inbreds; correlations for grain color and total carotenoids are higher when scored across segregating populations and narrow ranges of germ plasm, but correlations for β -carotene and β -cryptoxanthin remain low.

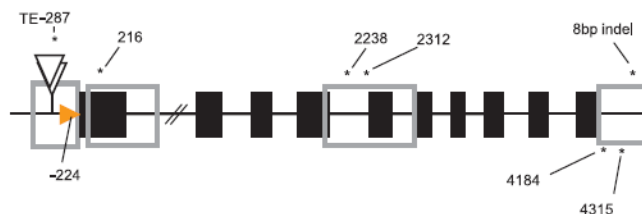


Fig. 3. Schematic diagram of the maize *lcyE*. Putative transcription start sites are depicted with orange arrow, translated exons as black squares, and the sampled regions as gray boxes. Polymorphisms that significantly associated with changes in flux between the lutein and zeaxanthin branches of the pathway are labeled with asterisks. The 5' transposable element insertion(s) are represented by the white triangles. Positions relative to the sequence alignment are indicated numerically above the polymorphisms.

REPORTS

significant associations in subsequent field seasons (Table 1).

Expression analysis indicated that *lcyE* is preferentially expressed in the endosperm relative to the embryo (fig. S1). Expression profiling of kernels at 15 and 20 days after pollination (DAP) indicated expression levels correlated well with the ratio of carotenoids from each pathway branch, explaining 70 to 76% of the variance. Lines with transposon insertions near the start site had much lower expression levels [in 15 DAP and 20 DAP lower by a factor of 3.7 and 13, respectively (fig. S2)]. The 3' indel may also have expression effects, but our statistical tests lacked the power to confirm this hypothesis. A quantitative trait locus (QTL) experiment that examined segregation of B73-Mo17 alleles in leaves found significant variation in the cis-regulation of *lcyE* expression, along with several other regions that also contribute to expression level control of *lcyE* (fig. S3).

In a previous study, three major QTL were identified for accumulation of carotenoids in maize (23). Two of these QTL colocalized with *yl* and *zeta carotene desaturase* (*zds*); the third QTL mapped to a region without a candidate gene. We mapped *lcyE* to chromosome 8 bin 5, near marker bnl1599, and it colocalized with this previously undetermined QTL. This QTL showed significant effects for modification of the ratio of α to β branch carotenoids [logarithm of the odds ratio for linkage (or lod) score of 34.05; R^2 54.4%] and explained 31.7% of the variation for lutein (lod 16.5). The magnitude of effects was not as large as in association or mutagenesis analysis. However, this biparental QTL population only segregated for the amino substitution (at codon 216) and a modest promoter polymorphism and does not segregate for the 3' polymorphism. Notably, this QTL was not significant for total carotenoids, which further supports the conclusion that variation within *lcyE* gene underlies this QTL for carotenoid composition.

To confirm association and QTL results, mutagenesis induced by ethane methyl sulfonate (EMS) was conducted to isolate additional alleles of *lcyE*. Two M_2 ears of inbred Qx47 segregated for a distinct change in endosperm color from yellow to orange, with orange recessive to yellow (these color changes were apparent in the inbred isogenic background, but not in diverse breeding materials). HPLC analysis of orange and yellow kernels confirmed a shift in the zeaxanthin:lutein ratio in the direction of zeaxanthin. This orange endosperm mutation was backcrossed into the standard genetic inbred line B73, and *lcyE* was tested as a candidate gene, which revealed that the Qx47 *lcyE* haplotype cosegregates with orange endosperm and ratio of α -carotene versus β -carotene branch carotenoids (fig. S4).

The most favorable haplotype for higher β -carotene branch carotenoids included both the large promoter insertion and 3' 8-bp insertion. In the diverse panel we tested, this haplotype occurs in 5% of temperate inbreds and 16% of tropical inbreds. MAS at this locus should be effective for several reasons: (i) The most favorable haplotype is found with at least modest frequency in different germ plasm sources and thus breeders can select donors from their relatively more adapted sources. (ii) The favorable haplotype has a large effect. (iii) Visual selection is ineffective for differentiating carotenoid composition and selecting provitamin A compounds. (iv) In comparison with HPLC analysis of carotenoids, polymerase chain reaction (PCR) scoring of the *lcyE* locus is much less expensive (costing perhaps 1/1000th that of HPLC) and more accessible to developing countries with greatest need for provitamin A.

An approach that empowers local breeder involvement through inexpensive visual selection for darker yellow to orange kernels to enhance flux into carotenoid pathway, and also incorporates MAS for *lcyE*, should result in

increased levels of provitamin A compounds. To expedite creation of improved germ plasm globally, we provide information on PCR-based markers (fig. S5). Donor inbreds and improved breeding lines derived at the International Maize and Wheat Improvement Center (CIMMYT) from synthetics of diverse panel inbreds with higher β -carotene are available by contacting T. R.R. This will facilitate selection worldwide of the most favorable *lcyE* alleles, which we have begun in our program. We are screening tropical breeding germ plasm collections in collaboration with CIMMYT.

To date, MAS for natural variation has been limited by resolution and scope (germ plasm diversity). Alleles have generally been characterized in the limited genetic background and resolution of biparental QTL studies, leaving in question their relevance to broader germ plasm (24), particularly for germ plasm outside of the temperate United States. As a result, the primary use for MAS is backcross breeding of transgenic traits. In contrast, the association mapping approach used here allows for rapid generation of selectable markers based on performance of diverse germ plasm. This provides markers more relevant in a broad genetic background, and that enables breeders to search for favorable alleles in their locally adapted germ plasm sources.

In ongoing studies, we are attempting to identify alleles for other genes in the pathway that increase total carotenoids and that slow the conversion of β -carotene to β -cryptoxanthin and zeaxanthin, to exploit more fully the natural genetic variation potential in provitamin A biofortification of maize. These results will then be further incorporated in breeding efforts to create a healthier maize crop for the world's poorest people.

Although the genetic results and strategy presented here are encouraging, they need to be placed in context as part of an overall biofortification effort encompassing breeding infrastructure, seed distribution, societal acceptance,

Table 1. *lcyE* associations across seasons. Association results for significant polymorphisms identified in the four regions sampled along the *lcyE* gene. Each polymorphism is labeled numerically by its position on the alignment relative to the exon 1 start codon. Followed by the favorable allele (bold)/unfavorable allele at the site. An initial scan for association using both β -carotene and the ratio of the two pathway branches was

conducted using the mixed model incorporating population structure and kinship. Subsequently a simpler general linear model (GLM) was used to evaluate data sets from additional years, including population structure (Q), given the oligogenic behavior of the trait the change in flux estimates for 2003 do not include Q. Avg., average; n.c., nonconvergence; n.s., not significant.

	<i>lcyE</i> association (P), mixed model of			<i>lcyE</i> association as a ratio across environments (GLM) (P)			Fold change in flux
	β -Carotene/all	Ratio of branches		(2003)	(2004)	(2005)	
Environment (year)	(2003)	(2003)	(2002)	(2003)	(2004)	(2005)	(2003)
Avg. observation no.	(157)	(154)	(44)	(156)	(154)	(156)	
Polymorphic site							
5' TE 1+4/2/3	5.42×10^{-4}	3.96×10^{-11}	0.024	8.05×10^{-11}	0.008	8.61×10^{-9}	6.5
216 G / T	n.c.	1.35×10^{-10}	0.059	1.24×10^{-10}	0.003	2.93×10^{-10}	2.8
2238 G / T	1.22×10^{-4}	1.69×10^{-9}	0.008	2.12×10^{-10}	0.023	1.08×10^{-9}	2.7
2312 A / T	1.70×10^{-3}	n.c.	n.s.	6.84×10^{-4}	0.026	0.005	2.9
4184 G / A	3.06×10^{-4}	n.c.	8.87×10^{-4}	2.23×10^{-10}	0.019	1.13×10^{-8}	2.6
4315 C / G	1.84×10^{-4}	7.01×10^{-10}	0.012	3.07×10^{-9}	5.75×10^{-4}	6.79×10^{-7}	2.6
3'Indel 8/0	4.80×10^{-3}	2.75×10^{-9}	n.s.	1.46×10^{-8}	8.97×10^{-4}	4.13×10^{-6}	3.5

dietary habits, and nutritional impact. Information now available on some of these issues is encouraging. Results from an animal model for human vitamin A metabolism indicated vitamin A activity of provitamin A in orange maize was greater than assumed by a factor of about four (25). A successful intervention to introduce β -carotene-rich, orange sweet potato in Mozambique, where only white sweet potato was previously cultivated, suggests that orange-colored staple foods can be acceptable, and their regular consumption results in improved vitamin A status (26). Related follow-up acceptance studies of yellow and orange maize in Mozambique and Zimbabwe are in progress with initial results encouraging (27). The dietary habits of many Africans, in which maize is consumed for all three meals a day, indicates that maize is a good target for biofortification (28). The recent positive nutritional and acceptance results will need to be coordinated with comprehensive breeding and seed distribution efforts to realize the potential of provitamin A-biofortified maize, as, for example, is coordinated by the HarvestPlus Global Challenge Program.

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Supporting Online Material

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Materials and Methods

Figs. S1 to S9

Tables S1 to S5

References

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1.2 PSY3, a New Member of the Phytoene Synthase Gene Family Conserved in the Poaceae and Regulator of Abiotic Stress-Induced Root Carotenogenesis⁷

PSY3, a New Member of the Phytoene Synthase Gene Family Conserved in the Poaceae and Regulator of Abiotic Stress-Induced Root Carotenogenesis^{1[W][OA]}

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Abscissic acid (ABA) plays a vital role in mediating abiotic stress responses in plants. De novo ABA biosynthesis involves cleavage of carotenoid precursors by 9-cis-epoxycarotenoid dioxygenase (NCED), which is rate controlling in leaves and roots; however, additional bottlenecks in roots must be overcome, such as biosynthesis of upstream carotenoid precursors. Phytoene synthase (PSY) mediates the first committed step in carotenoid biosynthesis; with *PSY3* described here, maize (*Zea mays*) and other members of the Poaceae have three paralogous genes, in contrast to only one in *Arabidopsis thaliana*. *PSY* gene duplication has led to subfunctionalization, with each paralog exhibiting differential gene expression. We showed that *PSY3* encodes a functional enzyme for which maize transcript levels are regulated in response to abiotic stresses, drought, salt, and ABA. Drought-stressed roots showed elevated *PSY3* transcripts and ABA, responses reversed by rehydration. By blocking root carotenoid biosynthesis with the maize *y9* mutation, we demonstrated that *PSY3* mRNA elevation correlates with carotenoid accumulation and that blocking carotenoid biosynthesis interferes with stress-induced ABA accumulation. In parallel, we observed elevated *NCED* transcripts and showed that, in contrast to dicots, root zeaxanthin epoxidase transcripts were unchanged. *PSY3* was the only paralog for which transcripts were induced in roots and abiotic stress also affected leaf *PSY2* transcript levels; *PSY1* mRNA was not elevated in any tissues tested. Our results suggest that *PSY3* expression influences root carotenogenesis and defines a potential bottleneck upstream of *NCED*; further examination of *PSY3* in the grasses is of value for better understanding root-specific stress responses that impact plant yield.

Abiotic stresses, such as water deficit, salinity, and high or low temperatures, have profound negative effects on plant growth; such stresses are the primary causes of crop productivity losses (Bray et al., 2000). For example, during the flowering and silking stages of maize (*Zea mays*), 4 d of mild drought stress can cause up to a 50% decrease in yield productivity (Classen and Shaw, 1970). Plants have evolved various levels of adaptation in response to stress conditions, and best understood is mediation by the hormone abscissic acid (ABA; Xiong et al., 2002).

Given its important role in plant stress tolerance, regulation of ABA biosynthesis and accumulation is a focal point of research. In higher plants, ABA is de-

rived from the 9-cis-epoxycarotenoids 9-cis-violaxanthin and 9-cis-neoxanthin; these C_{40} compounds are cleaved by 9-cis-epoxycarotenoid dioxygenase (NCED) to form xanthoxin, a C_{15} intermediate, which is subsequently converted to ABA in two steps of oxidation (see Fig. 1; for review, see Nambara and Marion-Poll, 2005). ABA accumulation is a balance of biosynthesis and catabolism, the latter process primarily regulated by ABA 8' hydroxylase (ABA8ox; Kushiro et al., 2004; Saito et al., 2004). ABA biosynthesis, as derived from de novo biosynthesis in a given tissue, may be potentially limited by the plastid enzymes involved in producing the carotenoid (epoxycarotenoid) precursors (Li et al., 2007; Matthews and Wurtzel, 2007; Quinlan et al., 2007) and/or the enzymes involved in conversion of specific epoxycarotenoids to ABA (Taylor et al., 2005; Fig. 1); factors affecting flux to ABA may vary according to tissue (photosynthetic versus nonphotosynthetic) and/or to particular species (and possibly plant families), as we demonstrate in this article for maize.

In leaves of dicot and monocot plants, including maize, de novo biosynthesis of ABA is induced by water stress (Sindhu and Walton, 1987). The cloning and characterization of maize *Viviparous14*, which encodes NCED1, led to identification of NCED as a rate-controlling enzyme in stress-induced de novo biosynthesis of ABA, particularly in leaf tissue (Schwartz et al., 1997; Tan et al., 1997). Similar results were obtained from characterization of other ortholo-

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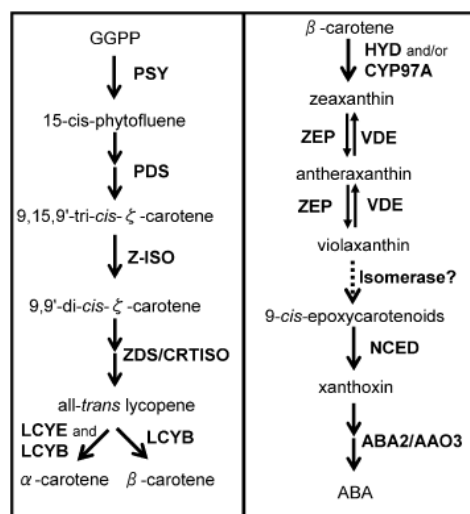


Figure 1. Carotenoid and ABA biosynthetic pathways in higher plants. Left, Carotenoid precursors of carotenes. Right, Conversion of carotenes to ABA. Enzymes are bold: LCYE, lycopene ϵ -cyclase; CYP97A, P450 carotene β -ring hydroxylase; VDE, violaxanthin de-epoxidase; ABA2, short-chain alcohol dehydrogenase; AAO3, ABA oxidase.

gous genes, *PvNCED1* of bean (*Phaseolus vulgaris*; Qin and Zeevaart, 1999), *VuNCED1* of cowpea (*Vigna unguiculata*; Iuchi et al., 2000), *LeNCED1* of tomato (*Solanum lycopersicum*; Thompson et al., 2000a), and *AtNCED3* of Arabidopsis (*Arabidopsis thaliana*; Iuchi et al., 2001). Stress-induced elevation of NCED transcript levels was observed to precede ABA accumulation (Qin and Zeevaart, 1999), and up- or down-regulation of the gene affected both ABA levels and drought sensitivity (Thompson et al., 2000b; Iuchi et al., 2001). Conversion of the downstream xanthoxin to ABA is not limiting as evidenced by the invariant levels of a requisite cytosolic enzyme activity in comparing normal and water-stressed leaves (Sindhu and Walton, 1987). Similarly, the upstream carotenoid levels are not thought to limit flux to ABA in leaves; leaf epoxycarotenoids are abundant (Parry et al., 1990), and transcripts for zeaxanthin epoxidase (ZEP), which converts zeaxanthin to violaxanthin, the precursor for epoxycarotenoids, were shown to be constant or decreased in leaves under drought stress (Audran et al., 1998; Iuchi et al., 2000; Thompson et al., 2000a).

Roots of maize and other plants also respond to osmotic or water stress through elevation of ABA, only some of which is due to increased translocation from other tissues (Rivier et al., 1983; Cornish and Zeevaart, 1985). However, the mechanism for increasing flux to ABA in nonphotosynthetic tissues, such as roots, contrasts with that operating in leaves. ABA epoxycarotenoid precursors are lower in roots as compared with leaves (Parry and Horgan, 1992). Although NCED is a

limiting enzyme for ABA biosynthesis in roots, just as it is in leaves (Qin and Zeevaart, 1999; Thompson et al., 2000a), increased rates of carotenoid synthesis in roots may also be necessary for elevating flux to root ABA. Unlike the constant levels seen in leaves, drought stress-induced elevation of root ABA was associated with ZEP transcript level increases of 3- to 7-fold and 4-fold in roots of tobacco (*Nicotiana plumbaginifolia*) and tomato, respectively (Audran et al., 1998; Thompson et al., 2000a). Another indication that there is an additional rate-controlling step upstream of NCED that limits flux to ABA in roots is suggested by experiments where transgenic tomatoes were modified for constitutive overexpression of NCED1; transgenic plants showed greater accumulation of ABA in leaves and only a modest increase in root ABA, suggesting that in roots there might be other steps upstream of NCED that limited flux to ABA (Thompson et al., 2007). Therefore, induction of elevated root ABA must also require enhanced levels of NCED precursors to accommodate elevated NCED levels induced under drought stress, but absent in NCED-overexpressing transgenic plants. The observation that stress-induced accumulation of ABA was also associated with elevated transcripts for the nonheme diiron β -carotene hydroxylase (HYD), an enzyme catalyzing hydroxylation of β -carotene to zeaxanthin, suggests that this and possibly other components of the carotenoid biosynthetic pathway may represent putative upstream bottlenecks that must be released to elevate root ABA levels in response to abiotic stress.

To examine the nature of the root ABA bottleneck, we decided to look at maize, an important food crop worldwide, and model for translational genomics in the grass family (Poaceae; Lawrence and Walbot, 2007); insight from study of maize paralogs will be useful in predicting ortholog targets in related grasses, many of which also serve important agronomic roles worldwide. The carotenoid biosynthetic pathway in maize and other grasses is complex compared to Arabidopsis and other dicots; many of the enzymes in the grasses are encoded by small gene families for which much ongoing investigation is focused on elucidating specific roles in carotenogenesis (Wurtzel, 2004; Li et al., 2007; Matthews and Wurtzel, 2007).

The first enzyme in the plastid-localized carotenoid biosynthetic pathway, phytoene synthase (PSY), which is known to control flux to carotenoids in the seed (Gallagher et al., 2004), is nuclear encoded by a small gene family consisting of PSY1 and PSY2, which we had previously shown to exist throughout the Grasses (Gallagher et al., 2004). In the process of searching for orthologs of PSY1 and PSY2 in sorghum (*Sorghum bicolor*), a cultivated species in drought- and salt-stressed environments, we stumbled upon a new and unrelated PSY gene, PSY3. The sorghum PSY3 gene led us to the rice (*Oryza sativa*) ortholog, from which synteny with maize provided a strategy to identify a syntenic chromosome region harboring the maize PSY3 gene.

PSY3 cDNAs that we identified in the public databases were primarily associated with abiotic stress, suggesting that the *PSY3* gene might play a role in regulating carotenoid flux in response to stress. We therefore tested this possibility and showed that, indeed, *PSY3* gene expression represented at least one bottleneck in controlling flux to carotenoid precursors that are required for elevating ABA in maize roots.

RESULTS

Isolation of the Third *PSY* Gene Paralog from Maize, Rice, and Sorghum

We previously showed that *PSY* was encoded by two paralogs, *PSY1* and *PSY2*, in 12 species across eight subfamilies of the grasses (Poaceae; Gallagher et al., 2004) in comparison to a single *PSY* gene in Arabidopsis; we also demonstrated enzymatic functions for maize *PSY1* and *PSY2* and rice *PSY2*. While attempting to expand *PSY* studies in sorghum, which is an important cereal crop in Africa and other parts of the world, we discovered a novel *PSY* cDNA, which we termed *PSY3*. Sorghum *PSY3* (GenBank accession no. BG464544) has a unique 3' end distinguishing it from sorghum *PSY1* and *PSY2* homologs. To rule out the possibility that sorghum was unusual among the grasses in having a third gene, we used BLAST analysis to test whether a homolog even existed in rice because earlier genome data had not revealed any additional genes; we did find a rice *PSY3* gene located on chromosome 9 (Gramene ID LOC_Os09g38320) and six rice ESTs in GenBank. For maize, we identified only one genomic contig (The Institute for Genomic Research ID AZM4_60808), which contained an incomplete maize *PSY3* gene, but no maize ESTs were found.

To clone the maize *PSY3* gene, we exploited synteny between maize and rice and used flanking markers to identify the maize syntenic region in maize bin 7.03 near *umc1865* (Fig. 2); bacterial artificial chromosome (BAC) clones in this region were screened by PCR to identify one containing the maize *PSY3* gene, which was sequenced by primer walking (GenBank accession no. DQ372936; see details in "Materials and Methods"). To facilitate gene annotation and later functional analyses, we used reverse transcription (RT)-PCR to clone full-length *PSY3* cDNAs for maize and rice (as described in "Materials and Methods"). The cDNA clones containing the sorghum *PSY1* (GenBank accession no. CD234165) and *PSY3* (GenBank accession no. BG46454) genes were requested from the Comparative Grass Genomics Center. Analysis of genomic and cDNA sequences revealed conserved gene structure between *PSY3* as compared to *PSY1* and *PSY2* in the grass family, as well as with Arabidopsis *PSY* (Fig. 3). All *PSY* genes possess six exons and five introns, except sorghum *PSY3*, whose third and fourth exons are fused. All of these *PSY* genes have long first exons and a very short second exon. With the exception of the sorghum-fused *PSY3* exons, the sizes of the second, third, fourth, and fifth exons of all three groups of *PSY* genes in the Poaceae and Arabidopsis are identical with sizes of 51, 173, 236, and 193 bp, respectively. In addition, all *PSY1*s have a small first intron (approximately 100 bp) and a large second intron (>600 bp), but all *PSY2*s have a large first intron (approximately 400 bp) and a small second intron (approximately 100 bp; Fig. 3).

The deduced protein sequences for all three *PSY* genes of maize, rice, and sorghum were determined and used for phylogenetic analysis (Fig. 4A). This analysis showed that all *PSY3* proteins belong to a novel group, whereas the *PSY1* group in monocot species in the grass family is most closely related to the

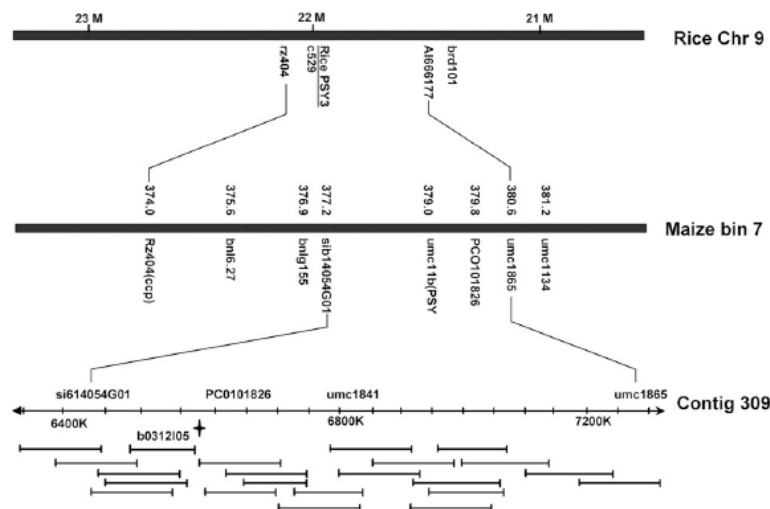


Figure 2. Cloning of maize *PSY3* using synteny between rice and maize. Rice *PSY3* (Gramene ID LOC_Os09g38320) was located on chromosome 9 between markers *Al666177* and *rz404* (top). The syntenic region on maize is bin 7 between markers *umc1865* and *Rz404* (*ccp*; middle). BAC clone *b0312105* containing maize *PSY3* was identified by PCR from contig 309 BACs that covered this region (bottom). The maize bin 7 map was adapted from IBM2 2004 neighbors 7 of MaizeGDB. Contig 309 of the Maize Agarose FPC Map was developed by the Arizona Genomic Institute.

Li et al.

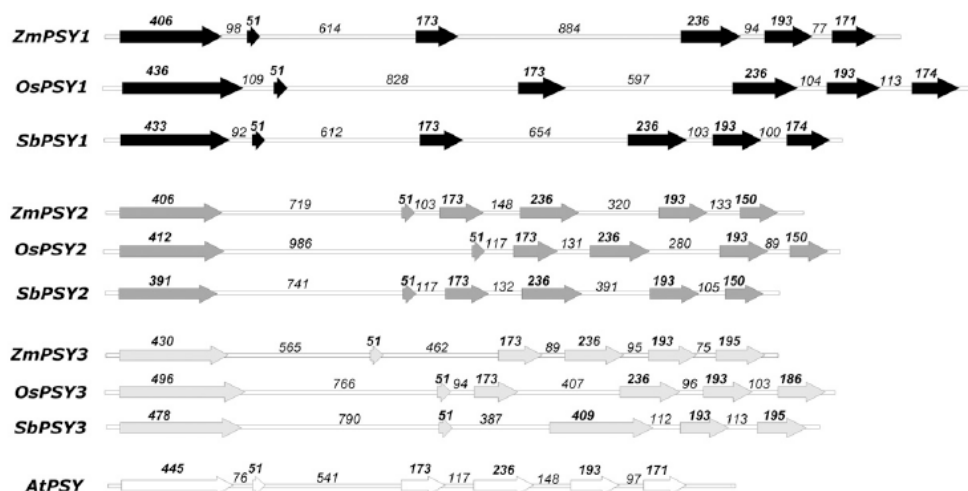


Figure 3. Gene structure of maize, rice, sorghum, and Arabidopsis PSY genes. Maize *PSY1* (ZmPSY1; GenBank AY324431); rice *PSY1* (OsPSY1; GenBank AP005750); sorghum *PSY1* (SbPSY1; PlantGDB SbGSStuc11-12-04.51.54.1); maize *PSY2* (ZmPSY2; GenBank AY325302); rice *PSY2* (OsPSY2; GenBank AL831803); sorghum *PSY2* (SbPSY2; PlantGDB SbGSStuc11-12-04.12062.1); maize *PSY3* (ZmPSY3; GenBank DQ372936); rice *PSY3* (OsPSY3; Gramene LOC_Os09g38320); sorghum *PSY3* (SbPSY3; PlantGDB SbGSStuc11-12-04.766.1); Arabidopsis *PSY* (AtPSY; GenBank AB005238). Arrows and thin bars indicate exons (bold numbers) and introns, respectively, sizes for which are in base pairs.

PSY in the dicot plant Arabidopsis. PSY3 proteins possess a distinct domain found at the carboxyl terminal, R(H/R)XS(S/T)LT, a motif that separates these proteins from the members of the PSY1 group with the SLRNXQ(T/K) motif and PSY2 with the ARAVAS(S/P) motif (Fig. 4B); H/R are charged amino acids, and S/T are polar but uncharged amino acids. All PSY3 proteins possess transit peptides for chloroplast targeting as predicted by the ChloroP 1.1 server (Emanuelsson et al., 1999). Maize PSY3 was predicted to be 47.3 kD (426 residues), having a 51-residue transit peptide and processed to a 41.9-kD (375 residues) mature plastid protein; rice PSY3 was predicted as 49.3 kD (444 residues) and having a 54-residue transit peptide and processed to a 43.5-kD (390 residues) mature plastid protein. Sorghum PSY3 was predicted as 48.9 kD (441 residues) with a 57-residue transit peptide and processed to a 42.7-kD (384 residues) mature protein. When comparing the processed and plastid-localized PSY proteins, the 41.9-kD maize PSY3 is expected to be larger by 2 kD at the N terminus as compared to maize PSY1 (39.8 kD) and PSY2 (39.4 kD). The unconserved first exon of the PSY genes encodes the PSY transit peptide and the N-terminal 2-kD extension in the case of PSY3 proteins; the last gene exon, which also lacks conservation among PSY genes, encodes the distinguishing C-terminal PSY motif (Fig. 4B).

PSY3 Encodes a Functional PSY

We used heterologous functional complementation to verify whether the novel PSY3 proteins were func-

tional (Gallagher et al., 2003, 2004; Matthews et al., 2003; Quinlan et al., 2007). Maize *PSY3* and sorghum *PSY1* and *PSY3* cDNAs were subcloned into the pET23 expression vector (Novagen) and transformed into *Escherichia coli* harboring pACCAR25 Δ crtB, which carries a bacterial carotenoid gene cluster missing the bacterial PSY gene *crtB* (Misawa et al., 1990). Cells produce the pathway end product, zeaxanthin diglucoside, only when a functional PSY enzyme is present; a peak corresponding to this end product is seen in an HPLC chromatogram of extracted carotenoids from positive control cells containing the entire carotenoid cluster, including bacterial PSY (pACCAR25; Fig. 5A), but not when the empty vector only is cotransformed with the PSY deletion construct, pACCAR25 Δ crtB (Fig. 5B). HPLC analysis of carotenoid extracts from transformants containing either maize *PSY3* or sorghum *PSY1* and *PSY3* showed a peak corresponding to zeaxanthin diglucoside (Fig. 5, C–E), which matches in retention time and spectrum that seen for the positive control (Fig. 5A). These results indicate that maize *PSY3* and sorghum *PSY1* and *PSY3* all encode functional enzymes.

Maize PSY3 Is Mainly Expressed in Root and Embryo Tissue

To assess the role of maize *PSY3*, its tissue specificity was investigated with quantitative RT-PCR using endosperm and leaf tissues, where carotenoids generally accumulate to visible levels, and root and embryos, where carotenoids are barely detectable (Fig. 6). In leaf

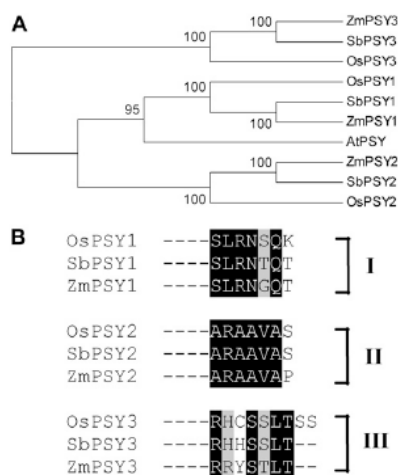


Figure 4. PSY1, PSY2, and PSY3 protein sequence comparisons. A, Phylogenetic analysis of amino acid sequences. B, Conserved carboxyl termini of PSY proteins. GenBank accessions are in parentheses. Arabidopsis (*AtPSY* AAA32836), rice (*OsPSY1*, AAS18307; *OsPSY2*, AAK07735; *OsPSY3*, DQ356431), sorghum (*SbPSY1*, AY705389; *SbPSY2*, AW679367; *SbPSY3*, AY705390), maize (*ZmPSY1*, P49085; *ZmPSY2*, AAQ91837; *ZmPSY3*, DQ356430). Amino acid sequences were aligned using ClustalW and a neighbor-joining tree was constructed with a 500-bootstrap replication support using MEGA3 software (Kumar et al., 2001). The carboxyl-terminal conserved domain of each PSY group was identified manually after alignment. Black highlighting indicates identical residues; gray denotes similar residues.

and endosperm tissue, maize *PSY3* mRNA levels were 4- to 5-fold lower than those of *PSY2* and 10- to 15-fold lower than those of *PSY1*, which is consistent with the semiquantitative data we previously reported (Gallagher et al., 2004). In contrast, maize *PSY3* mRNAs represented the most prevalent *PSY* transcript in roots, being about 4-fold higher than that of *PSY2* and 10-fold higher than that of *PSY1* (Fig. 6). In embryo, transcript levels of maize *PSY2* and *PSY3* were 3- and 2-fold higher than that of maize *PSY1*, respectively. The observed abundance of *PSY3* transcripts in tissues that accumulate little colored carotenoids suggests that *PSY3* may have a unique role in maize instead of merely being a redundant copy.

Maize *PSY2* and *PSY3* mRNA Levels Up-Regulated by Drought

We used E-northern from the National Center for Biotechnology Information (NCBI) to reveal possible factors that might influence *PSY* transcript levels as indicated by the source of abundant ESTs. We identified only six *PSY3* ESTs from rice, but none from maize; as described in "Materials and Methods," five were related to the ABA pathway or associated with plants subjected to drought conditions. E-northern

data suggested that *PSY3* in the grass family may be involved in drought stress or in regulation of ABA biosynthesis under abiotic stresses. To test these hypotheses, we subjected maize seedlings to various abiotic stresses and measured *PSY3* transcript levels in comparison to transcript levels for *PSY1* and *PSY2*.

To test for the effect of drought stress on *PSY* transcript levels, maize seedlings were subjected to drought conditions at the five-leaf stage. After 4 d of

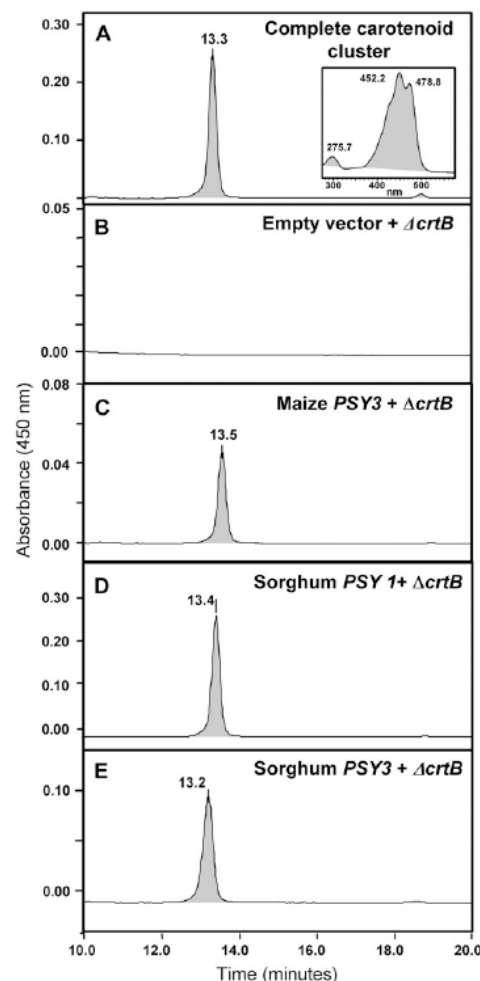


Figure 5. Functional complementation of *PSY3* from maize and sorghum. *E. coli* cells were transformed with pACCAR25 (A); pACCAR25 $\Delta crtB$ + pET23a (empty vector; B); pACCAR25 $\Delta crtB$ + maize *PSY3* (C); pACCAR25 $\Delta crtB$ + sorghum *PSY1* (D); and pACCAR25 $\Delta crtB$ + sorghum *PSY3* (E). Chromatograms show HPLC separation of extracted pigments; inset in A shows the spectral fine structure for the pathway end product, zeaxanthin diglucoside.

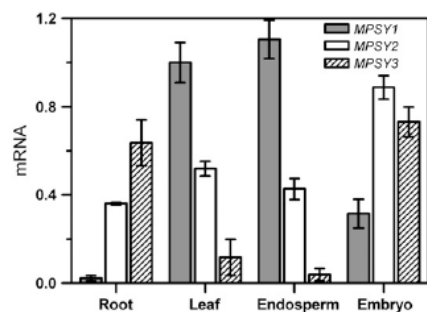


Figure 6. Transcript levels for PSY gene family members in different maize tissues. Endosperm and embryo were collected at 20 DAP from field-grown maize B73. Leaf and root samples were collected from B73 seedlings at the five-leaf stage. Transcript levels of maize *PSY1*, *PSY2*, and *PSY3* were normalized to levels of β -actin transcripts measured in the same samples and are shown relative to *PSY1* transcript levels in leaves (the expression level of maize *PSY1* in leaf = 1). Values represent the mean of three RT-PCR replicates $\pm$ SD from five pooled plants. MPSY1, maize *PSY1*; MPSY2, maize *PSY2*; MPSY3, maize *PSY3*.

water deprivation, leaves began to wilt and ABA levels in leaf and root tissue began to increase (Fig. 7, A and B); in parallel, mRNA level increases were observed for both *PSY2* and *PSY3* in leaves (Fig. 7A) and only *PSY3* in roots (Fig. 7B). Transcript levels of *PSY2* and *PSY3* reached their highest levels in seedlings subjected to continuous drought stress for 8 d, showing a 16- and 17-fold increase in leaves, respectively; in leaves, *PSY2* encoded the most abundant (3.4-fold compared to *PSY3*) transcript at 8 d, the time point after which water was restored. In contrast to leaves, at 4 d, roots showed a 38-fold increase of *PSY3* transcripts and, by 8 d, roots showed 50-fold induction of *PSY3* transcript levels as compared to predrought levels. Upon rewatering at 8 d, both ABA and mRNA levels of *PSY2* and *PSY3* dropped to normal levels within 4 h in both tissues. The rapid disappearance of *PSY2* and *PSY3* transcripts is suggestive of tight control of mRNA stability and/or gene transcription rate. In contrast, maize *PSY1* mRNA levels were only slightly altered by drought stress in both tissues.

Response of Maize PSY Transcript Levels to Salt and ABA Treatment

For plants, the responses to drought and salt are closely related and their mechanisms overlap (Zhu, 2002). Moreover, in *Arabidopsis*, many drought-inducible genes can also be induced by exogenous application of ABA (Seki et al., 2002). Therefore, salt and exogenous ABA treatments were also used to study *PSY* responses.

When maize inbred B73 seedlings were subjected to salt stress, *PSY3* transcript levels were barely altered in leaves (Fig. 8A), but increased in roots within 30 min; within 2 h of salt stress, *PSY3* transcripts peaked

6-fold, compared to untreated controls, and then dropped to lower levels by 5 h (Fig. 8B). In contrast, *PSY2* transcripts only slightly increased in salt-stressed root tissue. In leaves, *PSY1* transcript levels decreased rapidly within 30 min and then remained at a low level, whereas transcript levels of *PSY2* and *PSY3* did not show significant changes (Fig. 8A).

To test *PSY* responses to ABA treatment, maize B73 seedlings were subjected to 100 mM ABA. In leaves, *PSY2* transcript levels increased about 8-fold within 15 min, then dropped down to normal levels within 4 h (Fig. 9A). In contrast, *PSY1* mRNA levels steadily decreased upon ABA application, whereas *PSY3* transcripts remained at a low level for the entire 4-h period. In roots, within 15 min of treatment, both *PSY2* and *PSY3* mRNA levels increased in response to ABA, with distinguishable temporal patterns; the *PSY3* response was higher and peaked earlier at 15 min compared to 1 h for *PSY2*; *PSY3* mRNA levels increased about 7-fold in 15 min and *PSY2* mRNA levels increased 4.5-fold in 1 h, and then dropped after 2 h (Fig. 9B).

Up-Regulation of PSY3 Expression Correlates with the Increase of Carotenoid Flux under Salt Treatment

The up-regulation of maize *PSY3* in roots in response to drought, salt, and exogenous ABA strongly

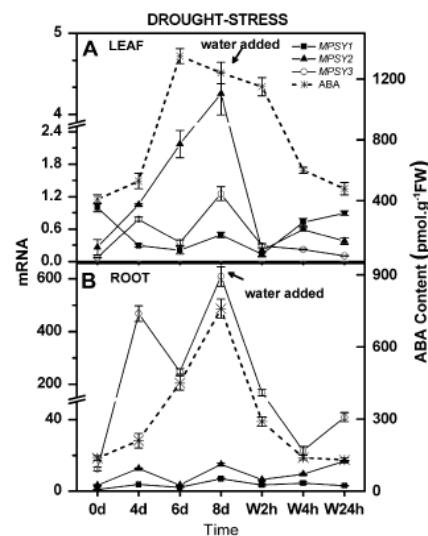


Figure 7. Effect of drought stress on maize *PSY* transcript levels. Maize B73 seedlings at the five-leaf stage were greenhouse grown, deprived of water for 8 d, followed by rewatering. Total RNA was extracted from leaves or roots at 0, 4, 6, and 8 d after water was withheld and 2, 4, and 24 h after rewatering. Transcript levels of maize *PSY1*, *PSY2*, and *PSY3* were normalized to levels of β -actin transcripts measured in the same sample, and are shown relative to *PSY1* transcript levels in leaf (A) and root (B) at 0 d. Values represent the mean of three RT-PCR replicates $\pm$ SD from five pooled plants. Genes are abbreviated as in Figure 6.

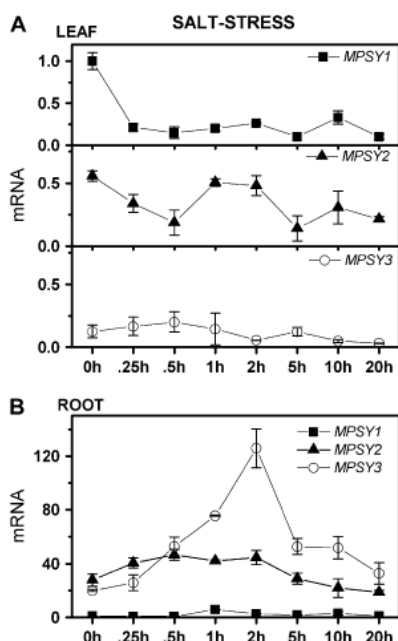


Figure 8. Effect of salt stress on maize *PSY* transcript levels. Total RNA was extracted from leaves or roots at indicated hours (h) after treatment with 250 mM NaCl. Transcript levels were normalized to levels of β -actin transcripts measured in the same sample, and are shown relative to *PSY1* transcript levels in leaf (A) and root (B) at 0 h. Values represent the mean of three RT-PCR replicates $\pm$ SD from five pooled plants. Genes are abbreviated as in Figure 6.

suggested that *PSY3* plays a role in stress-induced carotenogenesis required for ABA and other apocarotenoids. Because roots do not ordinarily accumulate carotenoids, it is difficult to assess the correlation between increased *PSY3* transcripts and increased carotenoid accumulation. However, it is possible to block the pathway using mutants that condition accumulation of pathway intermediates (Wurtzel, 2004; Matthews and Wurtzel, 2007). We therefore chose the maize *y9* mutation, which blocks carotenoid biosynthesis in nonphotosynthetic tissues through interference with isomerization of ζ -carotene by ζ -carotene isomerase (Z-ISO; Li et al., 2007); homozygous mutants accumulate the Z-ISO substrate 9,15, η^7 ζ -carotene and earlier pathway intermediates (Fig. 1), which can be easily measured by HPLC. Therefore, in this set of experiments with *y9*, we were able to compare changes in gene expression for carotenoid enzymes with changes in carotenoid content and ABA. Homozygous maize *y9* seedlings were salt treated, and carotenoid content, ABA, and *PSY* transcript levels in roots were measured and compared with ABA levels in normal B73 roots subjected to salt stress. In salt-treated *y9* roots, we observed that the total carotene content,

including phytoene, phytofluene, and ζ -carotene isomers, began to increase within 1 h after salt treatment (Fig. 10A). The total carotene content peaked after 2 h to levels 1.7-fold higher than that of untreated controls. ABA levels peaked at 5 h both in B73 and in *y9* roots; due to the block at Z-ISO, the increase in ABA concentration in the root tissue of the *y9* mutant was 46% less than that of the normal B73 control (Fig. 10B). The 2-fold increase in ABA levels in *y9* roots may be due to leaf ABA redistribution or incomplete blocking at the Z-ISO step. In comparison to the carotenoid increase seen at 1 h in *y9* roots, *PSY3* transcripts began elevating at 30 min and peaked by 2 h (Fig. 10C) as seen for *PSY3* in inbred B73 roots (Fig. 8B), whereas no changes were seen for *PSY1*, *PSY2*, or *PDS* (phytoene desaturase; Fig. 10C). Therefore, the elevated carotenoid content appears to be preceded by elevation in *PSY3* transcripts. A block in the pathway leads to reduced ABA, implying that elevated carotenoids are needed for stress-induced elevated ABA. These results demonstrate that salt-induced elevation of *PSY3* mRNA level correlates with increased carotenoid flux in roots, which contributes precursors needed for conversion to ABA.

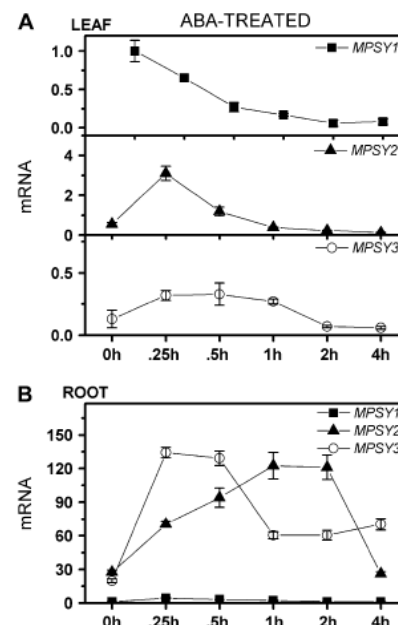


Figure 9. Effect of ABA on maize *PSY* transcript levels. Total RNA was extracted from leaves or roots at 0, 0.25, 0.5, 1, 2, and 4 h after ABA treatment. Transcript levels were normalized to levels of β -actin transcripts measured in the same sample and are shown relative to *mPSY1* transcript levels in leaf (A) and root (B) at 0 h. Values represent the mean of three RT-PCR replicates $\pm$ SD from five pooled plants. Genes are abbreviated as in Figure 6.

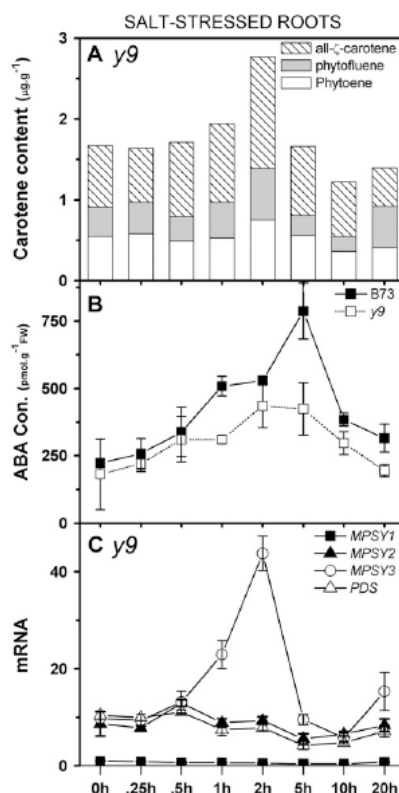


Figure 10. HPLC analysis of carotenoid content of maize *y9* root tissue after salt treatment. Maize *y9* mutant seedlings and normal B73 seedlings at the five-leaf stage were salt treated for 0, 0.25, 0.5, 1, 2, 5, 10, and 20 h. A, HPLC chromatogram (at λ_{max}) showing carotenoid content of *y9* mutant seedling roots (the mean of $N = 5$) subjected to salt stress for hours indicated. B, ABA content of roots from maize *y9* mutant seedlings and normal B73 seedlings subjected to salt stress for hours indicated. C, Quantitative RT-PCR of salt-treated roots for analysis of genes listed. Transcript levels were normalized to levels of β -actin transcripts measured in the same sample and are shown relative to *PSY1* transcript levels in root at 0 h. Values represent the mean of three RT-PCR replicates $\pm$ SD from five pooled plants. Other genes are abbreviated as in Figure 6.

PSY3 Is a Key Regulator of Carotenoid Biosynthesis in Roots under Stress

We showed that *PSY3* transcript levels are increased in response to drought, salt, and ABA treatment; elevated *PSY3* transcripts were also shown to precede carotenoid and ABA accumulation in *y9* roots. It has been previously shown in dicots that *ZEP* and *HYD* transcripts are elevated in roots subjected to drought stress (Audran et al., 1998; Thompson et al., 2007). Therefore, we questioned whether *PSY3* was the only gene encoding a carotenoid pathway enzyme that is abiotic stress responsive or whether other genes, in-

cluding *ZEP* and *HYD*, contribute to enhanced carotenoids required for *NCED1* activity and subsequent ABA induction. In maize, *ZEP* is encoded by two copies; we found that neither showed significant variation in transcript level in response to drought stress (Fig. 11, A and B). Maize *HYD* is encoded by a small gene family (R. Vallabhaneni and E.T. Wurtzel, unpublished data), only one of which was significantly induced under drought conditions. *HYD* transcript level increases were first observed at 6 d and peaked at 8 d following drought stress (Fig. 11). In comparison, *PSY3* mRNAs increased 38-fold already at 4 d and by 8 d roots showed 50-fold induction of *PSY3* transcript levels as compared to predrought levels (Fig. 7B); parallel to *PSY3*, *NCED1* also increased 3.7-fold at 4 d (Fig. 11D). These observations suggest that modulation of *HYD* transcript levels is delayed relative to induction of *PSY3* and *NCED1* transcript levels. We also tested *PDS*, *ZDS* (ζ -carotene desaturase), *CrtISO* (carotenoid isomerase), and *LCYB* (lycopene β -cyclase), none of which showed significant changes in response to drought stress (data not shown). Therefore, temporal control of stress-induced root carotenogenesis and ABA biosynthesis appears to be regulated first through induction of *PSY3* and *NCED1* transcript levels, followed by induction of *HYD* transcript levels, but not induction of *ZEP* as seen in dicots.

Reversal of drought stress in roots was observed within 2 h of rehydration, when *PSY3* transcript levels decreased and ABA levels plummeted (Fig. 7B). One explanation for the rapid loss of ABA, besides loss of *PSY3* transcripts, is that rehydration likely induced transcript levels of the ABA degradative enzyme *ABA8ox*; in *Arabidopsis* and bean, *ABA8ox* genes were up-regulated in response to drought stress and rehydration caused additional increases in transcript levels associated with reduced ABA (Kushiro et al., 2004; Yang and Zeevaart, 2006). To test whether reduced ABA was due to induction of the degradative enzyme *ABA8ox*, we measured corresponding maize transcripts. We identified maize orthologs of the recently identified rice gene (Saika et al., 2007) and examined two maize orthologs (*ABA8ox1a* and *ABA8ox1b*) that were most abundantly expressed in maize roots (R. Vallabhaneni and E.T. Wurtzel, unpublished data). Transcript levels of these root-abundant paralogs were measured in response to drought stress and rehydration; both genes showed increased transcript levels upon drought stress (Fig. 11, E and F). *ABA8ox1a*, having the most abundant root transcripts, showed a more rapid response to drought stress in comparison with *ABA8ox1b*; *ABA8ox1a* levels peaked at 4 d of drought stress compared to *ABA8ox1b* transcripts peaking at 6 d. *ABA8ox1a*, but not *ABA8ox1b*, also showed further elevation in response to rehydration within 2 h of watering at day 8 (Fig. 11E). These results explain the rapid loss of ABA upon watering and are consistent with previous reports of *ABA8ox* gene responses to drought stress in other plants (Kushiro et al., 2004; Yang and Zeevaart, 2006).

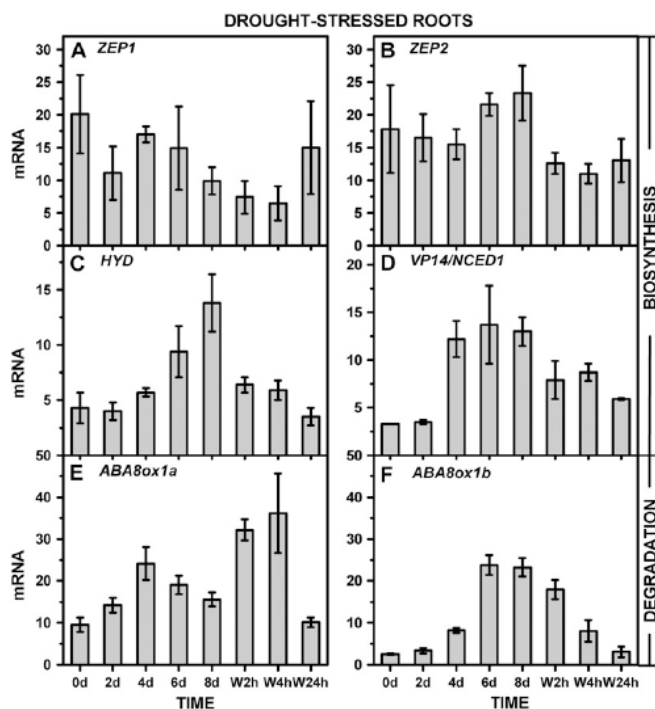


Figure 11. Drought stress-modulated levels of transcripts encoded by genes controlling biosynthesis and degradation of ABA in maize. The mRNA levels of *ZEP1* (A), GenBank DR820114, *ZEP2* (B), GenBank CO532283, *HYD* (C), GenBank BQ619575, *NCED1* (D), GenBank U95953, *ABA8ox1a* (E), GenBank DR806072, and *ABA8ox1b* (F), GenBank EE190691, were normalized to levels of β -actin transcripts measured in the same samples and are shown relative to maize *PSY1* mRNA levels in roots at 0 h. Values represent the mean of three RT-PCR replicates $\pm$ SD from five pooled plants.

DISCUSSION

The role of carotenogenesis in plants is multifaceted, including functions in photosynthesis and photoprotection, precursors to ABA and to other apocarotenoids that function as signals in development, and in communication between plants and their biotic environment. Yet, in a species such as *Arabidopsis*, where a rate-controlling carotenoid biosynthetic enzyme, *PSY*, is encoded by a single-copy gene, responses leading to altered flux are limited to control of that single-copy gene. In contrast, we showed that maize and other members of the Poaceae have three paralogs; gene duplication has provided an opportunity for subfunctionalization, whereby gene family members vary in tissue specificity of expression and in responses to abiotic stress.

Database searching led to the fortuitous discovery of *PSY3* in the grasses; syntenic comparisons between the published rice genome and the available maize physical map facilitated isolation of the maize gene. We showed that *PSY3* is present in maize, sorghum, and rice, three species that span two subfamilies in the Poaceae. Enzymes encoded by each of the three paralogs were shown to be functional, as demonstrated in a commonly used *E. coli* platform. Each enzyme is also predicted to be chloroplast-localized and marked by a paralog-specific C-terminal domain, whereas *PSY3* is

predicted to have an extended N terminus as compared to *PSY1* and *PSY2*. The fact that *PSY3* sequences across three species are more closely related than they are to *PSY1* and *PSY2* within a species suggests that gene family members may share paralog-specific roles in the plant either in terms of gene regulation or with regard to metabolon assembly and/or membrane-specific localization within different plastids.

We discovered *PSY3* among sorghum EST sequences, but we could not find any evidence that the gene was expressed in maize, suggesting that specific tissues or conditions were needed to elicit expression, which later proved to be true. In maize endosperm and leaves, tissues high in carotenoids, low levels of *PSY3* transcripts were observed. In contrast, the gene is expressed in minimally carotenogenic tissues; further analysis of roots revealed that the absence of accumulated carotenoids is likely due to carotenoid cleavage because carotenoid accumulation can be observed if further conversions are blocked by mutations affecting carotenoid biosynthetic enzymes.

The rationale for investigating stress-induced regulation was based on prevalence of rice *PSY3* ESTs associated with abiotic stress. Plant responses to drought and salt show overlapping mechanisms (Zhu, 2002) and many drought-inducible genes can also be induced by exogenous application of ABA (Seki et al.,

2002). Therefore, we examined maize for the effects of salt, drought, and ABA upon transcript levels of *PSY3* and its paralogs. We discovered that drought-induced elevation of ABA in leaves and roots was accompanied by elevated leaf mRNA levels for *PSY2* and, to a lesser extent, *PSY3*, in contrast to roots where only *PSY3* transcript levels increased; salt had a similar effect on induction of *PSY3* mRNA levels in roots, but not on *PSY2* transcripts in leaves. Rehydration reversed induction of *PSY3* transcript levels in roots, stimulated induction of transcripts for the ABA degradation enzyme, ABA8ox, and led to diminution of ABA. *PSY1* mRNA levels never showed enhancement by any of the abiotic stresses tested. Direct treatment with ABA induced elevation of both *PSY2* and *PSY3* transcripts in roots, although the *PSY3* response was earlier. The effect of abiotic stress on *PSY3* transcripts in maize was consistent with the observed pattern of rice ESTs associated with plant stress, suggesting that *PSY3* responses seen in maize are not unique to this species.

In nonstressed leaves, the mRNA levels of maize *PSY2* were 3.4-fold higher than that of *PSY3*, suggesting that *PSY2* is the primary gene responding to drought stress in leaves. However, the response mechanism may not involve increased flux to ABA because leaf ABA biosynthesis is limited not by the carotenoid precursor pool, but by NCED-mediated conversion of the xanthophyll precursors to ABA (Parry et al., 1990; Marin et al., 1996; Audran et al., 1998; Thompson et al., 2000a). Instead, increased carotenoid flux in leaves may relate to other processes, such as photosynthesis, temperature stress tolerance, and photoprotection (Davison, 2002; Rossel et al., 2002; Woitsch and Romer, 2003; Havaux et al., 2007). Nonstressed embryo, dissected at 20 d after pollination (DAP), contains few accumulated carotenoids, and the most prevalent *PSY* transcripts were those of *PSY2* followed by *PSY3*; perhaps, in this tissue, which is lacking in photosynthetic plastids, *PSY* expression could possibly relate to plant development (Sorefan et al., 2003; Booker et al., 2004) and/or apocarotenoid biosynthesis (Matusova et al., 2005).

In roots, transgenic overexpression of NCED previously suggested that there were other factors that were bottlenecks to ABA (Thompson et al., 2007). The results shown here suggest that overexpression of *PSY3* in combination with NCED may overcome the bottleneck that was observed when NCED was overexpressed alone. By using the maize *y9* mutation to block root carotenoid biosynthesis, we observed elevated root *PSY3* mRNAs in parallel with carotenoid accumulation; the block in carotenoids also led to reduction in accumulated ABA. *PSY3* and NCED shared a similar temporal response to drought stress and were followed by elevation of ABA. *PSY3* was the only gene in the carotenoid pathway that showed significant changes in transcript levels needed to control flux to carotenoids in roots; induction of elevated *PSY3* transcripts was followed by moderate induction of *HYD* transcript levels, which might be interpreted as a

response to the increased carotenoid flux mediated by elevation of *PSY3* transcripts. By using the *y9* mutant to block Z-ISO in the carotenoid pathway, we showed that the *PSY3* transcript level correlated with increased root carotenoids that are produced upstream of *HYD*; at this time, we are unable to evaluate the effect of elevated *HYD* transcripts in driving flux further to xanthophylls. We rule out later steps, such as *ZEP*, because we did not observe any significant change in *ZEP* transcripts in roots when plants were subjected to drought stress. The lack of changes in maize root *ZEP* transcripts is in contrast to dicots, where stress did cause changes in *ZEP* mRNA levels in roots, but not in leaves (Audran et al., 1998; Thompson et al., 2000a). Other factors that might additionally affect flux in roots may be attributed to the upstream nonmevalonate isopentenyl diphosphate biosynthetic pathway for which expression of certain enzymes has been shown to impact flux to carotenoids in maize (R. Vallabhaneni and E.T. Wurtzel, unpublished data) and in other organisms (Matthews and Wurtzel, 2000).

CONCLUSION

In summary, *PSY3* expression plays a role in controlling flux to carotenoids in roots in response to drought stress; changes in *PSY3* transcripts were accompanied by induced levels of carotenoid intermediates, elevation of *HYD* and NCED transcripts, and followed by accumulation of ABA. *PSY3*, which exists in multiple species within two subfamilies of the Poaceae, is a new target to consider for enhancing tolerance to drought and salt stress. Stress tolerance is an important factor affecting plant yield that could contribute to increasing the food supply or to improved biofuel production from grass species of the Poaceae.

MATERIALS AND METHODS

Plant Materials

Maize (*Zea mays*) inbred line B73 and mutant *y9* (X07C; Maize Genetics Cooperation Stock Center, University of Illinois) and rice (*Oryza sativa*) 'indica' var. IR36 were propagated as follows. Maize B73 and *y9* mutant were grown in a greenhouse with a photoperiod of 16 h supplemented with artificial lighting at 25°C with appropriate watering prior to drought, salt, or ABA treatment. Rice was grown under the same conditions and leaves were collected for cDNA isolation and gene cloning. The endosperm and embryo tissues of maize B73 were dissected at 20 DAP from field-grown plants and stored at -80°C until analysis.

Cloning of Maize *PSY3*

Maize *PSY1* cDNA sequence (GenBank accession no. ZMU32636) was used in BLAST analysis to identify a putative homolog from sorghum (*Sorghum bicolor*; GenBank accession no. BG46454; Altschul et al., 1997). Further sequence comparisons revealed that this sorghum EST had a unique 3' end, which distinguished it from *PSY1* and *PSY2*, and it was therefore named *PSY3*. To search for *PSY3* homologs in other grass species, we used the sorghum *PSY3* cDNA (GenBank accession no. BG46454) in BLAST analysis, which led to six rice *PSY3* ESTs (CF305089, CF312554, CF312553, CF307565,

AK108154, and AY078162), although none was found for maize. Among these, five ESTs (except AK108154) were drought inducible or associated with ABA signaling; the first four ESTs originated from a cDNA library prepared from transgenic rice modified for overexpression of ABF3, an ABA-responsive element-binding factor, which belongs to a distinct subfamily of bZIP proteins (Choi et al., 2000). BLAST analysis (Altschul et al., 1997) with the sorghum *PSY3* cDNA (BG46454) was then used to identify the rice *PSY3* gene between loci A1666177 and rz404 on chromosome 9 (Gramene ID LOC_Os09g38320). Next, we used synteny between rice and maize (<http://www.tigr.org/tdb/synteny>) to identify the putative maize *PSY3* locus; rice *PSY3* flanking markers were used to mark the syntenic region, which putatively encompassed maize *PSY3*, in bin 7.03 between loci rz404 (ccp) and umc1865. This maize region was covered by BAC contig 309 developed by the Maize Agarose FPC Map project (<http://www.genome.arizona.edu/fpc/maize>). Maize B73 BAC clones within this contig were requested and screened via PCR; PCR primers were designed from maize genomic contig AZM4_60808, which contained a partial maize *PSY3* genomic DNA sequence deduced by alignment with sorghum and rice *PSY3* cDNAs. Maize *PSY3* containing BAC clone b031205 was identified and both strands were sequenced by primer walking (DNA Sequencing Facility, Biotechnology Resource Center, Cornell University) and deposited into GenBank (DQ372936).

Sequence Analyses

cDNA sequences and corresponding protein sequences of PSYs of Arabidopsis (*Arabidopsis thaliana*), rice, sorghum, and maize were obtained from NCBI GenBank, some of which were deposited as a result of this work (DQ372936, DQ356431, AY705389, AY705390, DQ356430): Arabidopsis (AtPSY AAA32836), rice (OsPSY1, AAS18307; OsPSY2, AAK07735; OsPSY3, DQ356431), sorghum (SbPSY1, AY705389; SbPSY2, AW679367; SbPSY3, AY705390), maize (ZmPSY1, P49085; ZmPSY2, AAQ91837; ZmPSY3, DQ356430). Amino acid sequences were aligned using ClustalW and a neighbor-joining tree was constructed with 500 bootstrap replication support using MEGA3 software (Kumar et al., 2001). The carboxyl-terminal conserved domain of each PSY group was identified manually after alignment.

The genomic DNA sequences of PSYs of Arabidopsis, rice, sorghum, and maize were obtained from NCBI, Gramene, and PlantGDB for gene structure analysis: maize *PSY1* (ZmPSY1; GenBank AY324431); rice *PSY1* (OsPSY1; GenBank AP005750); sorghum *PSY1* (SbPSY1; PlantGDB SbGSSstuc11-12-04.5154.1); maize *PSY2* (ZmPSY2; GenBank AY325302); rice *PSY2* (OsPSY2; GenBank AL831803); sorghum *PSY2* (SbPSY2; PlantGDB SbGSSstuc11-12-04.12062.1); maize *PSY3* (ZmPSY3; GenBank DQ372936; described in this article); rice *PSY3* (OsPSY3; Gramene LOC_Os09g38320); sorghum *PSY3* (SbPSY3; PlantGDB SbGSSstuc11-12-04.766.1); Arabidopsis *PSY* (AtPSY; GenBank AB005238). The *PSY* gene structures were analyzed using Vector NTI Suite Version 9.0 (InforMax).

Plasmids

Sorghum *PSY*-containing ESTs, CD234165, AW679367, and BG46454, were requested and verified by further sequencing. Both CD234165 and BG46454 contained full-length sorghum *PSY1* and *PSY3* cDNAs, respectively. The maize *PSY3* (sequence deposited as DQ356430) and rice *PSY3* (sequence deposited as DQ356431) full-length cDNAs were amplified from cDNAs prepared from leaf tissue of the corresponding plant species using RT-PCR primers designed based on genomic DNA sequences of maize BAC clone b031205 and rice LOC_Os09g38320, respectively. The rice *PSY3* (DQ356431) was subcloned into the pGEMT-vector (Promega) and renamed pGEMT-RPSY3 prior to sequencing of both strands and use in phylogenetic analysis. The maize *PSY3* cDNA (DQ356430) was inserted into the pET23b (+) vector between *EcoRI* and *XhoI*, named pETb-MPSY3. Sorghum *PSY1* from CD234165 and *PSY3* from BG46454 were inserted between the *EcoRI* and *HindIII* sites of the pET23a (+) vector, designated as pETa-SPSY1 and pETa-SPSY3, respectively.

Functional Complementation

To test the function of *PSY* gene products, a heterozygous complementation assay was carried out as previously described (Gallagher et al., 2004). Briefly, *Escherichia coli* BL21 (DE3) cells (Novagen) were transformed with combinations of pACCAR25*Δcrb* and the expression constructs pETb-MPSY3, pETa-SPSY1, pETa-SPSY3, or empty vector pET23b (+). Carotenoids

were extracted (Gallagher et al., 2004), resuspended in methanol, and subjected to HPLC analysis (Quinlan et al., 2007); zeaxanthin diglucoside was identified as before (Gallagher et al., 2004) and in comparison with literature data (Misawa et al., 1990).

Quantitative Real-Time PCR

RNA isolation and cDNA synthesis were carried out as described (Gallagher et al., 2004). Real-time PCR was performed using iQ SYBR green supermix (Bio-Rad) with 10 ng synthesized cDNA. For primers and PCR conditions for test genes and the internal actin control, refer to Supplemental Table S1. Specificity of amplification was confirmed via melt curve analysis of final PCR products by ramping the temperature from 50°C to 90°C with fluorescence acquired after every 0.5°C increase. All quantifications were normalized to the signal of actin cDNA for the same sample. The fold change of transcript abundance of target genes was first calculated as $2^{-\Delta C_T}$, where ΔC_T is the number of PCR cycles required to reach the log phase of amplification for the target gene minus the same measure for actin. Transcript abundance of maize *PSY1* was then adjusted to 100% and fold changes of transcripts from other genes from the same tissue were normalized via comparison with that of maize *PSY1*. Values represent the mean of three RT-PCR replicates $\pm$ SD from five pooled plants.

Stress Treatments

To carry out the drought stress experiment, maize B73 seedlings at the five-leaf stage (about 3 weeks) were deprived of water for 8 d and then rewatered. Leaves began wilting 4 d after withholding water. Therefore, leaves and roots were collected at 0, 4, 6, and 8 d after water was withheld and 2, 4, and 24 h after rewatering.

For high salt and ABA treatments, maize B73 seedlings were carefully removed from soil to avoid injury, rinsed with water, and then hydroponically grown in solutions that contained either 250 mM NaCl or 100 mM ABA [(±)-ABA; catalog A1049; Sigma]. For an effective concentration of 100 mM ABA, 200 mM of the enantiomeric mixture was used. Leaves and roots were collected after 0, 0.25, 0.5, 1, 2, 4, 5, 10, and 20 h of salt treatment, and after 0, 0.25, 0.5, 1, 2, and 4 h of ABA treatment. All plant materials were stored at -80°C until analysis. For the salt treatment of *y9* mutants, *y9* seedlings were treated with 250 mM NaCl and roots were collected for HPLC analysis to measure carotenoid content, which was performed and quantified as previously described (Li et al., 2007). Values represent the mean of three RT-PCR replicates $\pm$ SD from five pooled plants.

ABA Measurement

ABA extraction was carried out according to the method described by Xiong et al. (2001). Briefly, 1 g of frozen tissue was ground with liquid nitrogen and suspended in 5 mL of extraction solution (80% methanol, 100 mg L⁻¹ butylated hydroxytoluene, and 1.7 g L⁻¹ NaHCO₃). The suspension was stirred 48 h at 4°C for extraction and centrifuged at 3,000g for 30 min. The supernatant was transferred to a new tube and dried under vacuum. Samples were dissolved in 100 μ L of methanol and 900 μ L of Tris-buffered saline (50 mM Tris, 0.1 mM MgCl₂, and 0.15 M NaCl, pH 7.8) and ABA concentration determined using the Phytodetek ABA immunoassay kit (Idetek). Note that the monoclonal antibody is specific for ABA and does not bind to ABA-GE [2-*cis*-(s)-ABA-B-D-glucopyranosyl ester]. Values are reported as the mean of five samples $\pm$ SD.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY705389, AY705390, DQ356430, DQ356431, and DQ372936.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Primers for gene isolation, BAC sequencing, and quantitative real-time PCR.

ACKNOWLEDGMENTS

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1.3 The Maize Phytoene Synthase Gene Family: Overlapping Roles for Carotenogenesis in Endosperm, Photomorphogenesis, and Thermal Stress Tolerance⁸

The Maize Phytoene Synthase Gene Family: Overlapping Roles for Carotenogenesis in Endosperm, Photomorphogenesis, and Thermal Stress Tolerance^{1[C][W][OA]}

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Carotenoids are essential for photosynthesis and photoprotection; they also serve as precursors to signaling molecules that influence plant development and biotic/abiotic stress responses. With potential to improve plant yield and nutritional quality, carotenoids are targets for metabolic breeding/engineering, particularly in the Poaceae (grass family), which includes the major food crops. Depending on genetic background, maize (*Zea mays*) endosperm carotenoid content varies, and therefore breeding-enhanced carotenoid levels have been of ongoing interest. The first committed step in the plastid-localized biosynthetic pathway is mediated by the nuclear-encoded phytoene synthase (PSY). The gene family in maize and other grasses contains three paralogs with specialized roles that are not well understood. Maize endosperm carotenoid accumulation requires *PSY1* expression. A maize antibody was used to localize *PSY1* to amyloplast envelope membranes and to determine *PSY1* accumulation in relation to carotenoid accumulation in developing endosperm. To test when and if *PSY* transcript levels correlated with carotenoid content, advantage was taken of a maize germplasm diversity collection that exhibits genetic and chemical diversity. Total carotenoid content showed statistically significant correlation with endosperm transcript levels at 20 d after pollination for *PSY1* but not *PSY2* or *PSY3*. Timing of *PSY1* transcript abundance, previously unknown, provides critical information for choosing breeding alleles or properly controlling introduced transgenes. *PSY1* was unexpectedly found to have an additional role in photosynthetic tissue, where it was required for carotenogenesis in the dark and for heat stress tolerance. Leaf carotenogenesis was shown to require phytochrome-dependent and phytochrome-independent photoregulation of *PSY2* plus nonphotoregulated *PSY1* expression.

Carotenoids represent a diverse group of more than 750 structures found in bacteria, fungi, algae, and plants (Britton et al., 2004). In higher plants, these pigments have numerous biological roles. For example, carotenoids serve as accessory pigments in the photosynthetic apparatus where they function in light harvesting and photoprotection (Niyogi, 2000). Heat and light stress tolerance is mediated by carotenoid

antioxidants that protect membranes from lipid peroxidation (Davison, 2002; Havaux et al., 2007; Johnson et al., 2007). Carotenoids are also precursors to cleavage products, such as the apocarotenoid abscisic acid (ABA), which regulates plant growth, embryo development, dormancy, and stress responses (Nambara and Marion-Poll, 2005); additional apocarotenoid products, such as strigolactone and others, have less defined roles in internal and external signaling but are essential elements that may affect plant yield (Booker et al., 2004; Akiyama and Hayashi, 2006). As plant dietary sources, carotenoids contribute to human and animal health, such as the provitamin A compound β -carotene (Fraser and Bramley, 2004). Accordingly, regulatory mechanisms for pathway induction are complex and not well understood, especially in plants of agronomic value found throughout the Poaceae (grass family).

The first committed and rate-controlling step in the plastid-localized carotenoid biosynthetic pathway (as recently revised; see Li et al., 2007) is mediated by the nuclear-encoded phytoene synthase (PSY); PSY catalyzes formation of 15-*cis* phytoene from two molecules of geranylgeranyl pyrophosphate (for review, see Matthews and Wurtzel, 2007). It is well known for many species that the PSY step affects flux to carot-

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enoids and much, though not all of the regulation is at the transcriptional level. In the grass family, which includes maize (*Zea mays*), sorghum (*Sorghum bicolor*), and rice (*Oryza sativa*), there are three paralogous *PSY* genes (Gallagher et al., 2004; Li et al., 2008). It is unknown whether the three *PSY* genes in the grasses have overlapping functions in modulation of carotenogenesis in different tissues and in response to multiple developmental and/or stress signals. For efforts to metabolically engineer enhanced levels of endosperm carotenoids, it would be helpful to elucidate the timing of gene expression for pathway enzymes to determine when and if transcript levels in developing endosperm correlate with carotenoid accumulation. In the absence of such data, use of a generic endosperm-specific promoter to drive transgene expression may be less effective in providing adequate levels of enzyme at the optimal time (Ye et al., 2000). As a result, a generic endosperm promoter may be a limiting factor in metabolic engineering of enhanced carotenoid content. *PSY* gene duplications in other species such as tomato (*Solanum lycopersicum*; Bartley and Scolnik, 1993; Giorio et al., 2008) and tobacco (*Nicotiana tabacum*; Busch et al., 2002) are examples of convergent evolution that have limited value in predicting roles and regulation for members of the grass *PSY* gene family. Pathway localization is another factor adding to complexity of carotenogenesis; pathway biogenesis may be specific to plastid types (e.g. amyloplasts, chromoplasts, chloroplasts) that differ in membrane architecture, physiological, and/or developmental state. Further examination of the grass *PSY* gene family will contribute data that will facilitate predictable outcomes in metabolic engineering ("predictive metabolic engineering"; Wurtzel and Grotewold, 2006) in improvement of plant yield and/or enhancement of nutritional content in maize and other related crops (Kean et al., 2007; Pozniak et al., 2007; Giuliano et al., 2008; Harjes et al., 2008).

Maize, a key food crop and target for improvement of endosperm carotenoids, is an attractive grass model having useful resources for investigating roles of the *PSY* gene family members in mediating carotenogenesis. Resources include pathway mutants (Wurtzel, 2004) and a genetically and chemically diverse germplasm collection (Harjes et al., 2008). The germplasm collection could be used to investigate the timing and abundance of transcripts that might correlate with carotenoid content in endosperm. For grasses lacking these tools, it may be possible to employ phylogenetic and syntenic genome analyses to identify orthologous sequences having potentially similar roles (Li et al., 2008). The three paralogs in the maize *PSY* gene family all encode functional enzymes. *PSY3* is associated with root carotenogenesis needed for drought and salt stress-induced production of ABA (Li et al., 2008), and *PSY1*, encoded by the *Yellow1* (*Y1*) locus, is required for endosperm carotenoid accumulation (Randolph and Hand, 1940; Buckner et al., 1996; Palaisa et al., 2003; Gallagher et al., 2004). The roles of the three *PSY*

gene family members were further investigated utilizing mutant and diversity collections. Data obtained on timing of expression and correlation with carotenoid content, enzyme localization, and overlapping roles in carotenogenesis will be useful in developing strategies to provide predictable outcomes in metabolic engineering and/or breeding of enhanced carotenoid content.

RESULTS

Immunolocalization of *PSY1* in Endosperm Amyloplasts

Having previously established that accumulation of *PSY1* transcripts correlated with presence of endosperm carotenoids in maize and absence in rice (Buckner et al., 1996; Gallagher et al., 2004), an antiserum was developed (as described in "Materials and Methods") to further examine *PSY* enzyme accumulation and localization in maize endosperm. *PSY1*, encoded by maize *Y1*, was overexpressed and purified from *Escherichia coli* to raise a polyclonal antiserum. To test specificity of the antiserum, protein extracted from normal *Y1* and mutant *y1-602C* endosperm was subjected to western analysis, as shown in Figure 1. This

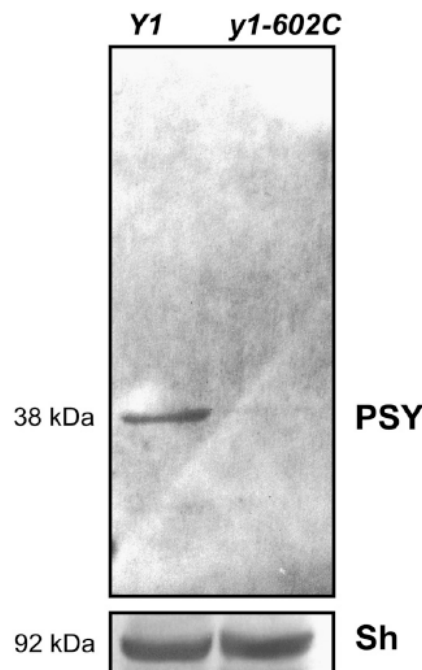


Figure 1. Western analysis demonstrating specificity of anti-*PSY1* antiserum for *PSY* in maize endosperm. Proteins were extracted from yellow (*Y1*) and white (*y1*) endosperm at 20 DAP. Immunoblots were probed by anti-*PSY1* antiserum (top) or anti-Shrunken1 (*Sh*) antiserum (bottom).

particular *y1* allele blocks endosperm carotenogenesis but does not interfere with leaf carotenogenesis; *PSY1* transcript accumulation is only affected in endosperm but not in leaf tissue (Gallagher et al., 2004). Sequence analysis of the *y1-602C* allele (GenBank accession no. EU306869) revealed no mutations in the open reading frame, but the 5' regulatory region was missing an *ins2* insertion, the presence of which has been strongly associated with *PSY1* expression in endosperm (Palaisa et al., 2003). The antiserum recognized an approximately 38-kD antigen, as expected for the processed, plastid-localized protein (Gallagher et al., 2004), and detected this antigen only in protein extracted from normal *Y1* but not from the mutant *y1* endosperm. Antiserum specificity was tested against *PSY2* and *PSY3*. The antiserum proved to recognize only *PSY1*, but not *PSY2* or *PSY3*, when all three proteins were individually expressed in *E. coli* by introduction of the corresponding cDNAs carrying a T7 Tag at the N terminus; as a control, all *E. coli*-expressed proteins were detectable with the T7 Tag monoclonal antibody (see Supplemental Fig. S1). To demonstrate localization of *PSY1* within endosperm amyloplasts, endosperm thin sections were examined using confocal microscopy for immunolocalization of *PSY1*. As shown in Figure 2A, the antiserum localizes the protein to the amyloplast envelope; no plastid signal was obtained in

the absence of the primary antibody (Fig. 2B). The antiserum also detected *PSY* antigen in isolated maize amyloplasts but not in isolated amyloplasts of rice endosperm that does not accumulate *PSY1* transcripts (E.T. Wurtzel and J. Yu, unpublished data).

Timing of *PSY* Expression and Carotenoid Accumulation in Endosperm

To better understand the relationship between temporal regulation of *PSY* transcript accumulation as compared to carotenoid accumulation during endosperm development, quantitative reverse transcription (RT)-PCR was used to monitor transcript levels for *PSY1*, *PSY2*, and *PSY3* in combination with spectrophotometric analysis (Kurilich and Juvik, 1999) of total carotenoid content for developing endosperms of maize inbred B73, a line that carries the dominant *Y1* allele. As shown in Figure 3A, carotenoid content increased 26.4-fold between 10 and 28 d after pollination (DAP). At 10 DAP, the levels were not statistically different from those of the maternal ovule tissue collected from unfertilized ear. The period of 10 to 16 DAP represented the most significant increase in content, 15.6-fold, though carotenoid accumulation continued throughout development (Fig. 3A).

While *PSY2* levels were highest in the unfertilized ovule, compared to *PSY1* and *PSY3*, only *PSY1* transcript levels increased significantly during endosperm carotenogenesis, starting after 12 DAP. In the B73 inbred, *PSY1* mRNA levels declined after 22 DAP and dropped down to significantly lower levels at 28 DAP. In contrast to maize *PSY1*, *PSY2* mRNA levels were constant during endosperm development, except between 10 and 14 DAP, when *PSY1* transcripts increased and *PSY2* transcripts decreased; when *PSY1* transcript levels decreased late in endosperm development at 24 DAP, *PSY2* transcript levels increased. Maize *PSY3* transcripts remained at relatively low levels, which were 30-fold lower than that of maize *PSY1* at 20 DAP (Fig. 3A); a slight reduction in *PSY3* transcripts was also observed from 10 to 12 DAP, as seen for *PSY2*.

The *PSY1* antiserum was used to examine temporal accumulation of *PSY1* for comparison with transcript accumulation in developing endosperm (Fig. 3B). Early in endosperm development (10 DAP) and in unfertilized ovule tissue, transcripts were present, but no *PSY1* antigen was detected. Even though transcript levels were about the same between 10 and 12 DAP, *PSY* protein was first detected at 12 DAP, suggesting that *PSY* accumulation might be linked to endosperm development and plastid biogenesis, as previously observed for posttranscriptional regulation of *PSY* destined for chloroplasts (Welsch et al., 2000). The period of 14 to 16 DAP also showed the steepest rise in accumulated carotenoids for a given time frame, following induction of *PSY* protein seen between 10 and 12 DAP. After 22 DAP when carotenoid accumulation was leveling off, protein levels started to be reduced, in parallel to the reduced *PSY1* transcript levels.

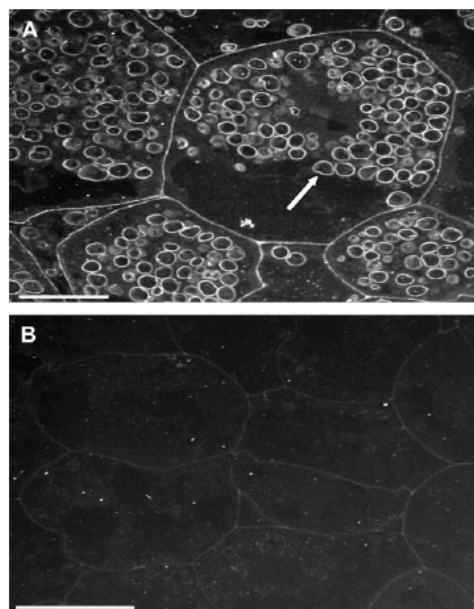


Figure 2. Localization of maize *PSY1* in maize B73 endosperm. Endosperm dissected at 17 DAP was probed with: A, anti-maize *PSY1* antiserum and anti-rabbit IgG FITC conjugate; or B, anti-rabbit IgG FITC conjugate only. Bar = 50 μ m (A) and 100 μ m (B). Arrow points to amyloplast within an endosperm cell.

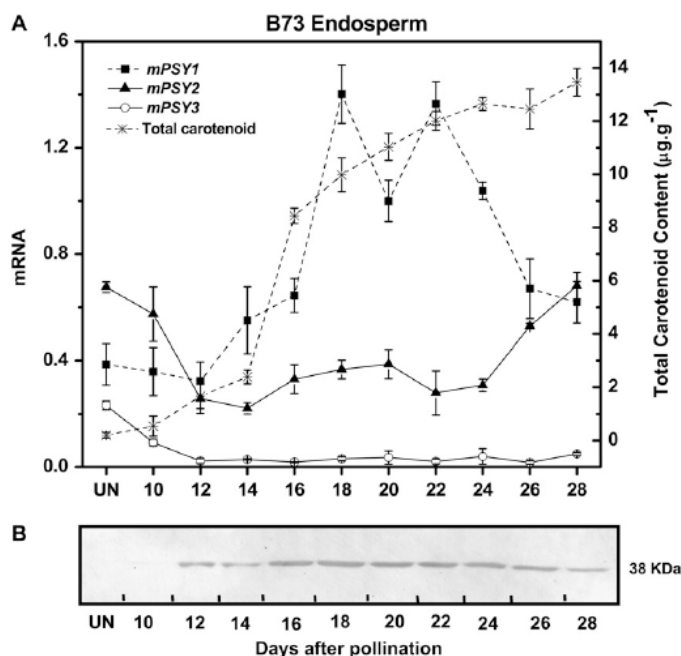


Figure 3. Temporal control of *PSY* transcripts and carotenoid accumulation in developing maize endosperm. Maize B73 inbred was pollinated and endosperms or unfertilized ovules (UN) dissected at indicated days after pollination. A, Quantitative RT-PCR amplification of transcripts for *PSY* gene family members compared with mature kernel carotenoid content; B, *PSY1* protein levels detected with anti-*PSY1* antiserum. Transcript levels of maize *PSY* genes were normalized to levels of β -actin transcripts measured in the same samples and made relative to the maize *PSY1* transcript level at 20 DAP. Values represent the mean of three RT-PCR replicates $\pm$ sd.

PSY gene family transcript accumulation was also examined in maize carrying the *PSY1* mutant allele, *y1-602C*. *PSY2* transcripts increased about 3-fold between 15 and 25 DAP (Fig. 4) in comparison to more constant levels observed for *PSY2* in B73, which carries the *Y1* allele (Fig. 3A); *PSY3* transcripts in *y1-602C* were at a constant and low level throughout development, as seen in B73. These results suggest that under nonmutant conditions of the *Y1* allelic background, *PSY1* expression (or some carotenoid intermediate/product) may have a negative feedback effect on transcript accumulation for *PSY2* but not *PSY3*. The inverse temporal accumulation of *PSY1* relative to *PSY2* and *PSY3* in the *Y1* endosperm of B73 (Fig. 3A) is consistent with this interpretation, for which further analysis is needed to better understand the underlying mechanisms. Endosperm transcript accumulation for *PSY2* in the *y1* background was later (20–25 DAP) than seen for *PSY1* in the *Y1* background (12–18 DAP), which might suggest that endosperm carotenogenesis is linked with a specific window of gene expression that is associated with the *PSY1* (*Y1*) gene but not with *PSY2*.

Correlating Timing of *PSY1* Transcript Levels with Endosperm Carotenogenesis

Although *PSY1* was recognized for its importance in endosperm carotenogenesis, it was unknown how timing of expression might influence carotenoid con-

tent. Also, maize *PSY3* was a new paralog, not yet evaluated in terms of endosperm carotenogenesis (Li et al., 2008). If it were known when and if *PSY* transcript levels correlated with carotenoid accumulation, then timing could be applied as a factor in breeding elevated carotenoid levels; timing of expres-

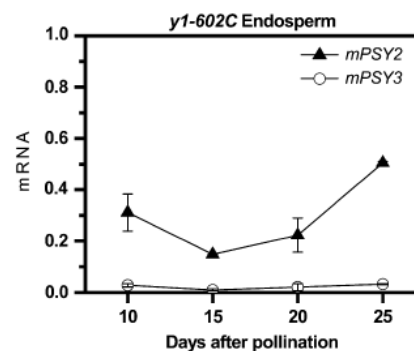


Figure 4. *PSY* transcripts in developing endosperms of *y1-602C* plants. Endosperms were dissected at indicated days after pollination from field-grown *y1-602C* plants. Transcript levels were measured by quantitative RT-PCR and normalized to transcript levels of β -actin measured in the same samples and made relative to maize *PSY1* transcript levels at 20 DAP in maize B73. There was no detectable signal for *PSY1* transcripts in the endosperm of *y1-602C* allele using real-time PCR. Values represent the mean of three RT-PCR replicates $\pm$ sd.

sion could be used in screening for optimal alleles (e.g. assessing mRNA levels at specific endosperm developmental time points) or controlling temporal expression of *PSY* transgenes (e.g. by choice of promoter that provides transcription in a given temporal window during endosperm development). To assess whether timing of *PSY* transcript level correlated with carotenoid content, advantage was taken of the genetic and chemical diversity available in maize. A subset of 10 inbred lines was chosen that exhibited a range in carotenoid content and composition (Harjes et al., 2008). Plants were control pollinated, endosperms dissected at 15, 20, and 25 DAP, and then *PSY* transcripts and carotenoid content quantified as described in "Materials and Methods." Pearson correlation analysis revealed that *PSY1* was the only gene whose transcripts showed significant correlation with total carotenoids (Fig. 5, A and B); this statistically significant correlation was specifically seen at 20 DAP (96% correlation [r] and P value of 0.001). Therefore, *PSY1* transcript level at 20 DAP is a good predictor of carotenoid content influenced by *PSY1* expression and therefore may be used in selecting optimal *PSY1*

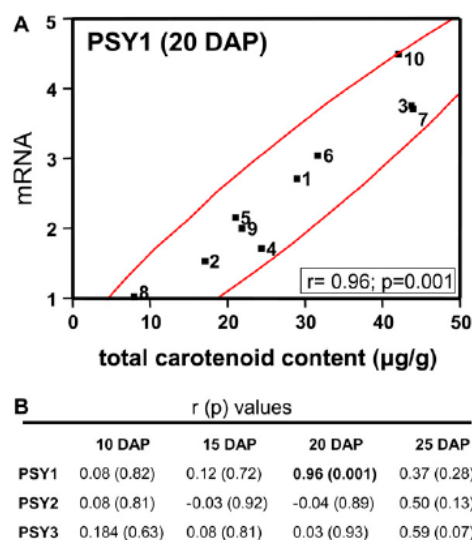


Figure 5. Timing *PSY* expression in a diverse maize germplasm collection. **A**, Correlation analysis of *PSY1* transcript levels at 20 DAP compared to carotenoid content of mature kernels in 10 chemically and genetically diverse lines (Harjes et al., 2008). Inbred lines are: 1, A619; 2, B73; 3, B37; 4, C17; 5, C131A; 6, DE3; 7, KUI2007; 8, NC300; 9, SD44; 10, TZ118. **B**, Pearson correlation (r) and statistical significance (P) for correlations made between *PSY* transcript levels at various endosperm developmental time points and carotenoid content for *PSY1*, *PSY2*, and *PSY3*. Correlation is significant for $P < 0.05$. Only *PSY1* transcript levels at 20 DAP showed significant correlation with carotenoid content. [See online article for color version of this figure.]

alleles for breeding or designing optimal timing of transgenes.

PSY2, the Only Paralog That Is Up-Regulated by Light in Photosynthetic Tissue

During de-etiolation or under high-light stress, carotenoid biosynthesis is induced to assist photosynthesis and to protect against photooxidative damage. Transcript levels of several genes encoding carotenogenic enzymes, especially *PSY*, have been shown to be up-regulated by light, which leads to carotenoid accumulation (Bartley and Scolnik, 1993; von Lintig et al., 1997; Simkin et al., 2003). Therefore, maize *PSY* gene family transcripts were quantified during de-etiolation to assess paralog-specific roles in leaf carotenogenesis. Dark-grown maize B73 inbred (Y1) seedlings were illuminated with white light (approximately $50 \mu\text{mol m}^{-2} \text{s}^{-1}$) for varying amounts of time (0–8 h) as described in the "Materials and Methods" section. In etiolated seedlings, the transcript level of *PSY1* was about 3-fold higher than that of *PSY2* (Fig. 6, 0 h). Upon illumination, it was observed that the *PSY2* mRNA levels were up-regulated by light. *PSY2* transcript levels started to increase after 1 h of illumination and reached the highest level at 2 h, being 6-fold higher than that of the unilluminated control. After 2 h, *PSY2* transcript levels began to decline, reaching approximately the same levels observed in the unilluminated control measured after 8 h illumination. In contrast to *PSY2*, which exhibited light modulation of transcript levels, *PSY1* transcript levels in leaves were not affected by light. Maize *PSY3* transcript levels remained at very low levels during de-etiolation, suggesting that *PSY3* is not involved in light-induced leaf carotenogenesis.

Role of Phytochrome in Light-Mediated Induction of *PSY2* Transcript Levels

To determine the light quality required for photoinduction of *PSY2* transcript levels, dark-grown maize B73 seedlings were exposed to $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red, far-red, or blue light and transcripts measured by quantitative RT-PCR. All of the light regimes induced transcript elevation, although they had specific temporal signatures and effects (Fig. 7, A, C, and E). In red and far-red light (Fig. 7, A and E), *PSY2* mRNA levels began increasing after 2 h and peaked at 4 h, with a 5-fold increase in *PSY2* mRNAs as compared to the unilluminated control; the far-red light response showed slower kinetics compared to the red-light response. In comparison, blue light (Fig. 7C) caused a more rapid and elevated accumulation of *PSY2* mRNA levels; levels increased at 1 h and peaked after only 2 h in blue light, causing a 10.5-fold increase in *PSY2* transcripts as compared to the unilluminated control. These light regimes were also tested with *PSY1* and *PSY3*, and as predicted from the white light experiments (Fig. 6), photoinduction was not observed (Supplemental Figs. S2 and S3).

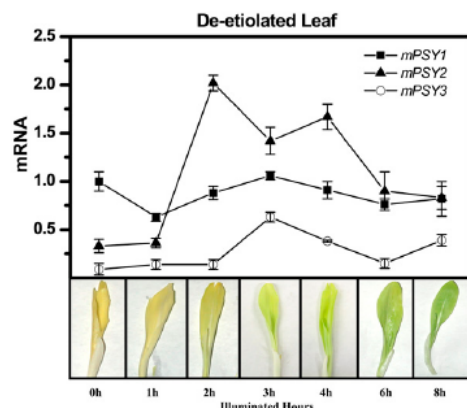


Figure 6. *PSY* transcript profiles during white light de-etiolation of seedlings. Nine-day-old maize B73 seedlings were treated with white light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 0 to 8 h. Total cDNAs were prepared from leaves of illuminated seedlings for use as templates for quantitative RT-PCR. All quantifications were normalized to β -actin amplified using the same conditions and were made relative to maize *PSY1* transcript levels in unilluminated seedlings. Values represent the mean of three RT-PCR replicates $\pm$ SD from five pooled plants. [See online article for color version of this figure.]

The observed induction by red and far-red light suggested that phytochrome was involved in mediating *PSY2* photoinduction. To verify this, loss of the red and far-red light photoinduction was tested by blocking phytochrome activity. This was accomplished by using maize seedlings that were homozygous for the recessive *elongated mesocotyl1 (elm1)* allele that interferes with biosynthesis of the phytochrome chromophore, thus preventing assembly of all phytochrome types (Sawers et al., 2002). Dark-grown *elm1* (*Y1*) seedlings were subjected to the three light regimes. No increase in *PSY2* transcripts was observed after illumination by red or far-red light (Fig. 7, B and F). These results were consistent with the role of phytochrome in mediating *PSY2* up-regulation by red or far-red light. When *elm1* plants were subjected to blue light (Fig. 7D), photoinduction was still observed and *PSY2* transcript levels peaked at 2 h as seen for B73 (which carries the dominant *Elm1* allele), although the down-regulation at 4 h in B73 was delayed to 6 h in *elm1* plants. The blue light result in *elm1* plants was also as expected, because blue light photoinduction is not phytochrome mediated but instead mediated by other photoreceptors such as phototropins or cryptochromes (Briggs and Olney, 2001; Im et al., 2006). The slight temporal change in the blue light induction may be due to some cross talk between the red and blue light signaling. Again, *PSY1* and *PSY3* transcript levels were tested in the *elm1* plants, and, as expected, no change was observed as compared to B73 seedlings treated with the three light regimes (Supplemental Figs. S2 and S3). In summary, carotenogenesis that

accompanies photomorphogenesis involves the photoinduction of *PSY2*, which is mediated both by phytochrome and nonphytochrome receptors.

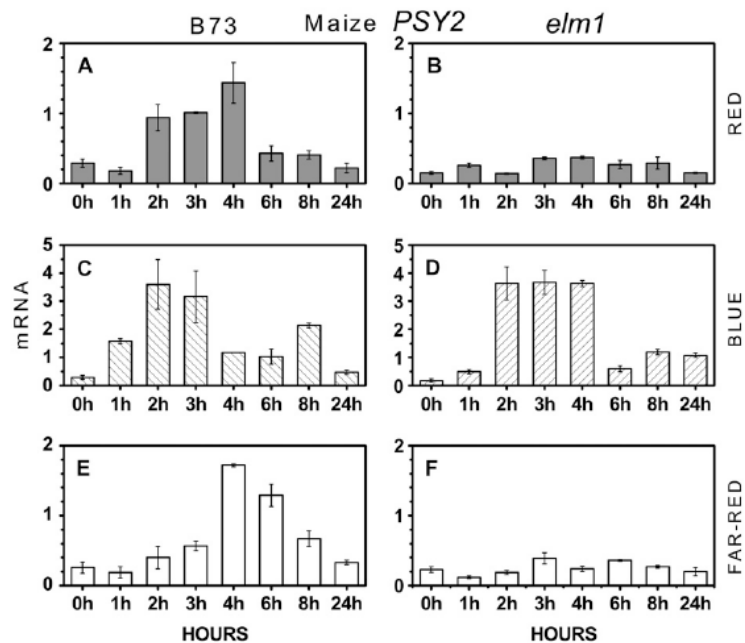
PSY1 Is Essential for Heat Stress-Induced Carotenogenesis in Photosynthetic Tissue

As shown above, photoinduction of *PSY2* transcript levels was shown to be linked with carotenogenesis in tissue undergoing photomorphogenesis; *PSY1* and *PSY3* were clearly found to be unresponsive to light. Assuming that *PSY1* plays no role in leaf carotenogenesis, it was puzzling to note that in etiolated seedlings that harbor proplastids and low carotenoid levels relative to light-grown seedlings (Table I), *PSY1* transcripts were more abundant than those for *PSY2* (Fig. 6). Moreover, in fully green, de-etiolated seedlings (Fig. 6) or in light-grown seedlings (Gallagher et al., 2004), transcript levels for *PSY1* and *PSY2* were equivalent; in mature leaves, the level of *PSY2* transcripts actually exceeded those for *PSY1* (Li et al., 2008). Although the role of *PSY1* in endosperm carotenogenesis is well established, this unexpected abundance of *PSY1* transcripts in green and etiolated leaves suggested that perhaps *PSY1* might also have a role in green tissue carotenogenesis. To test this possibility, a *PSY1* null allele of *y1* was needed. Two classes of *y1* alleles have been reported in maize by Robertson's earlier studies (Robertson and Anderson, 1961; Robertson, 1987): those with white endosperm and normal green leaves, and those with white endosperm and pale-green leaves. The *y1-602C* allele used in earlier experiments (Fig. 1) is representative of Robertson's class 1, which affects carotenogenesis in endosperm but not leaves, because the mutation affects only the promoter. Therefore, plants carrying *y1* alleles (obtained from the Maize Genetics Stock Center, University of Illinois, Champaign-Urbana, IL) were screened to identify one carrying a null allele, which would likely fall into Robertson's class 2 mutants. Sequence analysis revealed that in one of the *PSY1* mutants, *y1-8549*, a G was inserted 377 bp downstream of the ATG initiator codon, which caused a frame shift and resulted in a new stop codon at 388 bp (Fig. 8A). Previous reports of this allele (Robertson and Anderson, 1961) indicated that higher temperatures exacerbated the mutant phenotype and homozygous plants were extremely weak and generally did not survive to maturity under their field conditions, although the cause was unknown at the time. Therefore, the *y1-8549* null allele was used to investigate the impact of blocking *PSY1* activity on carotenoid accumulation in etiolated and mature leaves.

The *Y1* nonmutant and *y1-8549* mutant seedlings were grown under four conditions varying for light and heat stress, where 37°C is considered heat stress and 20°C is within the optimal temperature range (Wilhelm et al., 1999). Under low temperature (20°C) in the dark, the total carotenoid levels (including both carotenes and xanthophylls) of *y1-8549* seedlings were

Li et al.

Figure 7. *PSY2* transcript levels in de-etiolating maize B73 (*Elm1*) and *elm1* mutant seedlings treated with light of various spectral qualities. Dark-grown B73 (A, C, and E, left) and *elm1* (B, D, and F, right) seedlings were illuminated with red light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$; A and B), blue light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$; C and D), or far-red light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$; E and F) and quantitative real-time PCR was used to determine transcript levels for maize *PSY2*. All quantifications were normalized to β -actin and shown relative to maize *PSY1* transcript levels at 0 h using the same conditions. Values represent the mean of three RT-PCR replicates $\pm$ SD from five pooled plants.



62% of the levels found in nonmutant *Y1* seedlings (Table I, values); carotenes alone were only 24% of the normal (Table I, ratios). When the temperature was increased to 37°C, *Y1* seedlings grown in the dark responded with a 2.2-fold higher level of carotenoids, whereas the carotenoid level in *y1-8549* seedlings was unchanged and therefore exhibited only 30% of normal levels, suggesting that the *PSY1* null allele was interfering with the response to elevated temperature under dark growth conditions.

Under low temperature (20°C) in the light, *y1-8549* seedlings showed only a slight difference compared to *Y1* seedlings; carotenoid and chlorophyll levels in *y1-8549* were 92% and 72% of the levels found in *Y1* seedlings, respectively (Fig. 8, B and C; Table I). However, when grown at 37°C in the light, the *PSY1* null mutant was photobleached and showed only 3% carotenes, 9% xanthophylls, and 21% chlorophyll, as compared to plants carrying the normal allele (Table I,

ratios). Carotenoid levels in leaves of *Y1* plants grown at 37°C in the light were approximately 6% higher than found in plants grown at 20°C in the light, as compared to a 2.2-fold elevation in carotenoids when plants were grown in the dark at 37°C. One explanation for the limited heat-stress response in the light may be due to photooxidation of pigments, which takes place at high temperatures for plants grown in the light and leads to carotenoid degradation, thus limiting carotenoid accumulation (Britton, 1995). If the role of *PSY1* is to contribute to heat-stress activation of carotenoid biosynthesis, then the observed photobleaching seen in *y1-8549* plants that was exacerbated by high temperatures (Robertson and Anderson, 1961) might be due to rapid degradation of carotenoids, which are insufficiently replenished due to limited biosynthetic capacity in the absence of a functional maize *PSY1*.

Under dark growth conditions, the mRNA levels of *PSY1* in the *y1-8549* mutant were 30% and 40% of the

Table I. Chlorophyll, carotene, and xanthophyll analysis of *Y1* and *y1-8549* mutant seedlings
f.w., Fresh weight; n.a., not applicable.

Growth Condition	Y1 Normal Values			y1-8549 Mutant Values			Mutant to Normal Ratio		
	Chlorophyll	Carotene	Xanthophylls ^a	Chlorophyll	Carotene	Xanthophylls ^a	Chlorophyll	Carotene	Xanthophylls ^a
	$\mu\text{g g}^{-1} \text{f.w.}$								
20°C dark	n.a.	2.10 $\pm$ 0.04	16.30 $\pm$ 1.66	n.a.	0.51 $\pm$ 0.05	10.89 $\pm$ 1.75	n.a.	0.24	0.67
37°C dark	n.a.	2.56 $\pm$ 0.17	37.74 $\pm$ 4.86	n.a.	0.61 $\pm$ 0.09	11.51 $\pm$ 1.19	n.a.	0.24	0.30
20°C light	535.31 $\pm$ 19.16	119.31 $\pm$ 9.49	177.40 $\pm$ 7.70	385.42 $\pm$ 26.11	119.24 $\pm$ 6.37	155.30 $\pm$ 12.31	0.72	1.00	0.88
37°C light	493.24 $\pm$ 34.64	142.81 $\pm$ 11.53	174.27 $\pm$ 10.97	105.89 $\pm$ 7.25	3.72 $\pm$ 0.86	16.40 $\pm$ 2.28	0.21	0.03	0.09

^aXanthophylls include lutein, neoxanthin, violaxanthin, and antheraxanthin.

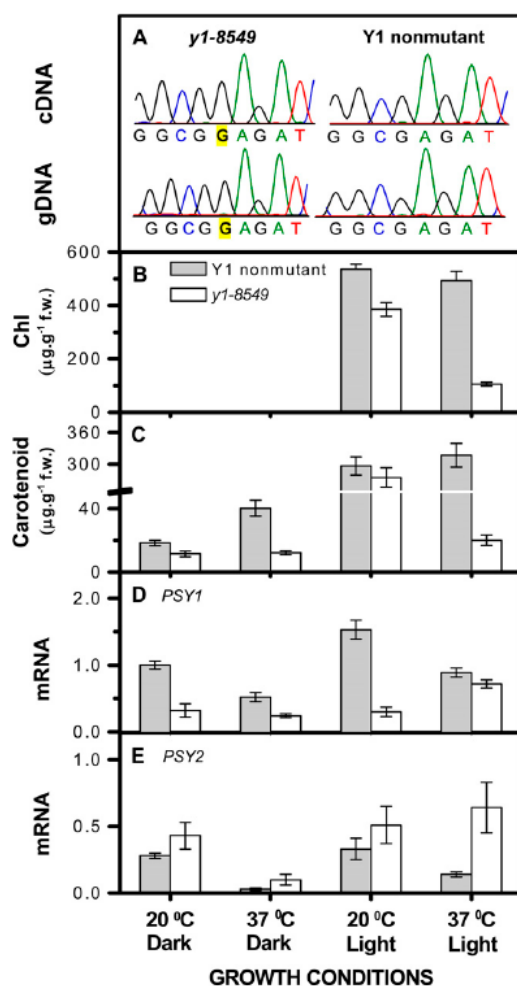


Figure 8. Effect of *PSY1* loss on maize leaf carotenogenesis. To assess the impact of functional *PSY1* loss on maize leaf carotenogenesis, the *y1-8549* mutant was grown under four conditions (dark and 20°C, dark and 37°C, light and 20°C, and light and 37°C). Chlorophyll and carotenoid content and transcript profiles of maize *PSY1* and *PSY2* of *y1-8549* mutant were analyzed. A, Sequence analysis of maize *PSY1* cDNA and genomic DNA prepared from leaf tissue of *y1-8549* mutant and B73 inbred line. The G insertion in the *PSY1* genomic DNA and cDNA prepared from *y1-8549* is bold/highlighted. B, Chlorophyll content; C, carotenoid content. D and E, The mRNA levels of maize *PSY1* (D) and *PSY2* (E) in leaf tissue were normalized to levels of β -actin transcripts measured in the same samples and are shown relative to maize *PSY1* mRNA levels from seedlings grown in the dark at 20°C. Values represent the mean of three RT-PCR replicates $\pm$ sd from five pooled plants. [See online article for color version of this figure.]

levels found in Y1 at 20°C and 37°C, respectively, while the mRNA levels of *PSY2* were 1.5-fold greater at 20°C and 3-fold greater at 37°C in the mutant

compared to Y1 (Fig. 8B). The higher accumulation of *PSY2* transcripts seen in the mutant might be a result of feedback regulation of *PSY1* on *PSY2* expression; in the Y1 background, expression of the functional Y1 product and/or its ensuing activity somehow represses *PSY2* mRNA accumulation, and in the mutant, this repression is absent such that *PSY2* expression partially compensates for the *PSY1* deficiency. This observation is reminiscent of the case seen in endosperm when either *PSY1* transcripts were temporally reduced or absent, in the case of the *y1-602C* endosperm mutant (Fig. 4). When the seedling growth temperature was raised, the transcript levels of both *PSY* genes decreased in Y1 and *y1-8549* seedlings. This decrease in *PSY* transcripts in etiolated Y1 plants was unexpected given the concurrent increase in carotenoid accumulation at high temperatures.

In summary, carotenoid levels doubled in etiolated Y1 seedlings subjected to heat stress as compared to normal temperature, whereas *y1-8549* plants responded with only a slight increase in carotenoid accumulation; in the light, *y1* mutants actually showed a severe decrease in carotenoids under heat stress conditions and appeared photobleached. The simplest explanation is that in the light, heat-stressed plants are subjected to photo-oxidative damage that can be relieved by increased carotenoid synthesis that is mediated by *PSY1*, a protective mechanism not available in the *PSY1* null mutant seedlings. Therefore, these data support a role for *PSY1* in leaf carotenogenesis, especially under heat stress conditions.

DISCUSSION

We recently established that three maize *PSY* paralogs arose prior to evolution of the grasses (Gallagher et al., 2004; Li et al., 2008). There remain open questions about the specific roles of these three genes that encode a key enzyme operating at the pathway entry point; the six downstream steps all appear to involve single-copy genes in maize (Li et al., 1996; Matthews et al., 2003; Li et al., 2007; Harjes et al., 2008). Whereas some species contain a single *PSY* gene, the *PSY* gene duplication in the grasses could potentially provide grasses with a fine tune control of carotenoid biosynthesis for different regulatory scenarios.

PSY1 Timing in Endosperm Carotenogenesis

From early genetic analysis to more recent association studies, the *PSY1* locus has been associated with maize endosperm carotenoids (Randolph and Hand, 1940; Palaisa et al., 2003). Additionally, in maize and wheat, a major quantitative trait locus for endosperm carotenoids maps to the *PSY1* locus (Wong et al., 2004; Pozniak et al., 2007). However, it was unclear what was the temporal relationship between gene expression during endosperm development and kernel carotenoid content. Efforts to develop strategies that provide pre-

dictable changes in plant chemistry, or “predictive metabolic engineering,” are dependent on availability of such data. In the absence of such information, use of a tissue-specific promoter provides tissue specificity but not necessarily the optimal window of expression for maximizing pathway end-product accumulation. Therefore, endosperm carotenoid accumulation was determined for developing kernels, together with *PSY* transcripts and protein. Carotenoid accumulation was observed from 10 DAP to maturity. This pattern of continuous accumulation is in contrast to that seen in sorghum varieties, which exhibited a peak of accumulation mid-development followed by a steep drop (Kean et al., 2007). Next, temporal expression of *PSY1* was quantified in maize B73 and nine other maize inbred lines that spanned carotenoid content and composition in the maize diversity panel (Harjes et al., 2008). By combining both transcript and carotenoid data, correlation was tested for carotenoid content and transcript levels of *PSY* gene family members. *PSY1* transcript level at 20 DAP was found to be a good predictor of carotenoid accumulation and therefore may be useful in selecting optimal *PSY1* alleles for breeding or designing optimal timing of transgenes.

The maize diversity lines are a powerful resource for gaining insight into the regulation of plant chemistry across genetically diverse germplasm. When used in association analysis, the diversity lines may provide information on polymorphisms that can be used for breeding, as in the case of improving provitamin A carotenoid content (Harjes et al., 2008). As applied here, these lines provided insight into the timing of candidate gene expression. There will likely be other steps and/or regulatory factors that may affect carotenoid accumulation and for which expression can be examined using these diversity lines. Germplasm diversity collections of other species, for which metabolic breeding holds interest, represent untapped value to examine candidate gene expression in relation to metabolite composition.

Amyloplast Envelope Membrane Localization of *PSY1*

Maize *PSY1* was shown to be resident on endosperm amyloplast envelope membranes, suggesting that the envelope membrane is the site of the carotenoid biosynthetic metabolon in amyloplasts. An envelope location for the pathway may not be limited to amyloplasts. Early studies provided evidence that an envelope-localized carotenoid biosynthetic pathway might also exist in chloroplasts. Chloroplast envelope membranes were shown to contain a unique carotenoid profile compared to thylakoid membranes (Jeffrey et al., 1974; Siefermann-Harms et al., 1978); chloroplast envelope membrane extracts also exhibited enzyme activity for conversion of zeaxanthin to violaxanthin (Costes et al., 1979). More recent reports have localized *PSY* and other pathway enzymes to thylakoid membranes in chloroplasts (Linden et al., 1993; Bonk et al., 1997; Welsch et al., 2000). Therefore, the carotenoid pathway may

actually be two pathways from a topological standpoint, potentially existing on envelope and thylakoid membranes. In amyloplasts, the pathway is likely associated with the envelope membrane while in chloroplasts, the pathway may be associated with both envelope and thylakoid membranes. In assembly of the biosynthetic metabolons, the nuclear-encoded pathway enzymes are recruited to membrane locations that may differ from one plastid type to another. Because many of the pathway enzymes are encoded by single copy genes (Li et al., 1996; Matthews et al., 2003), specificity of targeting must be controlled by factors beyond the transit sequence. Optimization of metabolic engineering/breeding of carotenoids is predicated on elucidating the mechanisms that control recruitment of pathway enzymes to metabolons on different plastid membranes in various plastid types.

PSY2 Is Associated with Photomorphogenesis

Given the involvement of carotenoids in photosynthetic tissue, it was expected that the *PSY* genes were photoregulated. For example, the single *PSY* gene in Arabidopsis was shown to be regulated by continuous red, far-red, and blue light regimes (von Lintig et al., 1997). However, in a *Y1* genetic background, only *PSY2* was photoregulated by light, mediated by phytochrome-dependent and phytochrome-independent mechanisms that involved blue light signals. Maize *PSY1* and *PSY3* did not respond to light of any spectral type. It was previously demonstrated that *PSY3* was associated with carotenogenesis leading to drought and salt-induced ABA biosynthesis in roots (Li et al., 2008); recent work on rice *PSY3* showed the rice homolog to have a similar role in rice roots (Welsch et al., 2008). Unlike maize, rice *PSY1* and *PSY2* are both photoregulated, although blue light was not tested (Welsch et al., 2008), and rice *PSY1* is not expressed in endosperm (Gallagher et al., 2004). Therefore, the ancestral Poaceae *PSY* was a photoregulated gene; the duplications analyzed in *Y1* maize represent losses in the photoregulatory mechanisms and evolution of other features, including the gain-of-function endosperm-specific expression seen for *PSY1* in *Y1* maize, but absent in its evolutionary ancestor teosinte and other subfamilies of the grasses, including species such as rice.

A Second Role for *PSY1* in Photosynthetic Tissue and Thermal Tolerance

Examination of leaf carotenogenesis revealed involvement for both maize *PSY1* and *PSY2*, indicating that *PSY1* is not exclusively associated with endosperm carotenogenesis. In addition to endosperm function, *PSY1* was shown to be required for leaf carotenogenesis, in the dark and under conditions of heat stress. When *PSY1* expression was eliminated in the maize *y1-8549* *PSY1* null mutant, the plant had reduced carotenoids in the dark and suffered severe

photobleaching at elevated temperatures in the light. Dual roles for a supposedly "nonphotosynthetic tissue" *PSY* paralog are not unique to maize. The loss of the "fruit-specific" *PSY1* in the tomato *r,r* mutant showed reduced carotenoid accumulation not only in fruit but also in leaf tissue (Fraser et al., 1999). In that case, it is not known whether the tomato *PSY1* plays a role in stress resistance. While *PSY1* (*y1*) has been labeled as the gene needed for endosperm carotenogenesis, the data presented indicate that *PSY1* is essential in controlling carotenogenesis in photosynthetic tissue. Its absence has severe effects on plant vigor that are exacerbated by high temperature. It is unclear at present how *PSY1*-controlled carotenogenesis mediates stress resistance in leaf tissue. *PSY1* is not impacting ABA biosynthesis, as carotenoids are not limiting in photosynthetic tissue as they are in roots (Li et al., 2008). Because carotenoids are known to protect against heat stress-induced lipid peroxidation, it appears that *PSY1* is the maize paralog that is essential for heat stress-induced biosynthesis of carotenoid antioxidants that protect plastid membranes (Davison, 2002; Havaux et al., 2007). The implications of these findings are that efforts to manipulate *PSY1* expression in endosperm may potentially cause changes in *PSY1* expression in photosynthetic tissue if *PSY1* alleles are chosen for use in breeding programs to enhance endosperm carotenoids without considering the dual role of *PSY1* in endosperm and leaf stress responses. On the other hand, this aspect of *PSY1* related to thermotolerance may open new avenues for breeding more stress-tolerant lines.

Posttranscriptional Regulation

Examination of gene expression in some of the mutants suggested that the various *PSY* genes may be subject to feedback regulation. In different tissues, absence of *PSY1* transcripts was associated with elevation of *PSY2* transcripts. During endosperm development, there was an inverse relationship seen between transcripts for *PSY1* relative to transcripts for *PSY2* or *PSY3*. In leaf tissue, where *PSY1* was absent due to a null mutation in *y1*, there appeared a concomitant increase in transcripts for *PSY2* under heat stress conditions. *PSY1* may also be subject to posttranscriptional regulation as suggested by the absence of endosperm *PSY1* protein at early developmental time points where transcripts were detected; perhaps translation is linked to amyloplast biogenesis in a similar fashion, whereby there appears to be a link between chloroplast biogenesis and translation of *PSY* mRNAs in Arabidopsis (von Lintig et al., 1997).

Each of the maize *PSY* paralogs evolved unique roles to control carotenogenesis in different tissues and in response to stress. The ongoing maize studies are greatly facilitated by the mutant and germplasm diversity collections, which generally are unavailable tools for research on other agronomically important grasses. However, phylogenetic identification of or-

thologous genes may suggest potential targets for plant improvement whether it is to enhance plant yield through stress resistance or in nutritional improvement.

MATERIALS AND METHODS

Plant Materials

Maize (*Zea mays*) plants used were phytochromobilin synthase mutant *dm1* (Sawers et al., 2002), inbred line B73 (which carries dominant alleles for *Y1* and *Elm1*), *PSY1* (*Y1*) recessive mutants, *y1-602C* and *y1-8549* (602M), also known as pastel-8549 (Robertson and Anderson, 1961), and maize germplasm diversity lines (T. Rocheford, University of Illinois, Champaign-Urbana, IL). Plants homozygous for *y1-602C* have a colorless endosperm with normal leaf phenotype, and plants homozygous for *y1-8549* have colorless endosperm and are temperature sensitive with pale-green leaves when grown at high temperature (Robertson and Anderson, 1961). For experiments related to endosperm, maize plants were field grown, and endosperms were dissected at various DAP and stored at -80°C until analysis.

Total Carotenoid Content Measurement

The carotenoid extraction procedure used was slightly modified from Kurilich and Juvik (1999). Tissue samples of 500 mg were ground in 6 mL of ethanol containing 0.1% butylated hydroxytoluene and were incubated in an 85°C water bath for 6 min prior to a 10-min saponification with 120 μL (1 g/mL) of KOH. All samples were vortexed once during saponification, after which they were immediately placed in ice to which 4 mL of cold deionized distilled water was added to each sample, followed by 3 mL of petroleum ether:diethyl ether (2:1, v/v), vortexed, and centrifuged for 10 min at 3,500 rpm. The upper layer was transferred to a fresh tube, and the aqueous phase was twice subjected to centrifugation with 3 mL of petroleum ether:diethyl ether each time and combined the fractions. The combined fractions were topped to 10 mL, and 1 mL of aliquot was used to measure total carotenoids at optical density of 450 nm using a Lambda 25 UV/VIS spectrometer (PerkinElmer Life Sciences).

Real-Time PCR

RNA isolation and cDNA synthesis were carried out as described by Gallagher et al. (2004). Real-time PCR was performed using iQ SYBR green Supermix (Bio-Rad) with 10 ng of cDNA. Primers and PCR conditions for maize *PSY1*, *PSY2*, *PSY3*, and the internal control actin were described previously (Li et al., 2008). Specificity of amplification was confirmed via melt curve analysis of final PCR products by ramping the temperature from 50°C to 90°C with fluorescence acquired after every 0.5°C increase.

The fold change of transcript abundance of target genes was first calculated as $2^{-\Delta\text{Ct}}$, where ΔCt is the number of PCR cycles required to reach the log phase of amplification for the target gene minus the same measure for actin. Transcript abundance of maize *PSY1* was then adjusted to 100% and fold changes of transcripts from other genes from the same tissue were normalized via comparison with that of maize *PSY1*. Values represent the mean of three RT-PCR replicates $\pm$ SD.

Generation of Anti-Maize *PSY1* Antibodies

A partial B73 maize *Y1* cDNA (clone 12A33A, GenBank no. NM_001114652), provided by Dr. Brent Buckner (Truman State University, MO), was subcloned as a *Bgl*II-*Xho*I fragment into the *Bam*HI-*Xho*I sites of the Novagen vector pET23c(+) and renamed pY1ex1-1. After transformation of BL21(DE3) pLysS, induction with 1 mM final concentration of isopropyl- β -D-thiogalactopyranoside at early stationary phase, and lysis, approximately 800 μg of inclusion body fraction containing the 32.9-kD *Y1* fusion protein (including 12 amino acids, 1.32 kD of T7 Tag encoded by the vector, and *Y1* protein [amino acid nos. 124–410, 31.57 kD]) was separated by 12% SDS-PAGE and the fusion protein band (about 50% yield) was excised and immersed in 5 mL of 125 mM NaCl. Rabbits, chosen for absence of preexisting immune response to maize proteins, were injected with 100 μg of *Y1* fusion protein preparation at weeks 1, 2, 3, and 5 (Lampire Biological Laboratory). For western analysis and localization experiments, blood from rabbit number 3445 (5 weeks after immuniza-

Li et al.

tion) was used and diluted 1:10,000 in Tris-buffered saline plus Tween 20 for westerns and 1:25 for confocal experiments. Specificity of the antiserum was verified by testing for presence of the antigen in normal, but not in homozygous *y1* endosperm, using the *y1* maize COOP alleles 602C (shown in Fig. 1) and 607C (data not shown), which reduced endosperm carotenoids but did not affect leaf carotenoids.

Expression of Maize PSY2 and PSY3 T7 Tag Fusion Proteins

To test the specificity of anti-maize PSY1 antiserum, maize PSY2 and maize PSY3 T7 Tag fusion proteins were also expressed using the pET23 vector (Novagen). pEMPSY2-1 contained a partial B73 maize PSY2 cDNA (GenBank accession no. AY450646), encoding amino acids 109 to 402 (33.46 kD) of PSY2 (Gallagher et al., 2004) fused to 16 residues (1.67 kD) of the T7 Tag, resulting in a PSY2 fusion product of 35.13 kD. pEMPSY3 was constructed by inserting a full-length maize PSY3 cDNA (encoding residues 1–426, 47.32 kD, GenBank DQ36430) into the pET23c(+) vector (in-frame fusion with 16 residues of a T7 Tag, encoding 1.67 kD) to produce a PSY3 fusion protein of 48.99 kD. Expression of the fusion proteins was induced by addition of 0.5 mM isopropyl- β -D-thiogalactopyranoside for 4 h when the *Escherichia coli* cells reached an A_{600} of 0.6 at 37°C. To detect the fusion protein by immunoblot analysis, the inclusion bodies were isolated and fractionated on a 12% SDS-polyacrylamide gel. Anti-T7 Tag antibody, AP conjugated (Novagen), was used at 1:10,000 dilution to detect the induced T7 Tag fusion proteins. Then anti-maize PSY1 antiserum at 1:5,000 dilution was used to test for antigenicity with maize PSY2 and PSY3 fusion proteins to test the antibody specificity.

Western Analysis to Detect mPSY1 Expression Level in Endosperm

Maize proteins from endosperms were extracted according to the method described by Wurtzel et al. (1987). Protein samples were separated on 12% SDS-PAGE in a Criterion Cell system (Bio-Rad) and transferred to nitrocellulose membrane for western blot in a Trans-Blot Cell (Bio-Rad). The first antibody, anti-maize PSY1, and the second antibody, goat anti-rabbit IgG, were diluted at 1:10,000 for use in western analysis according to the manufacturer's protocol (Novagen). To control for protein loading, a duplicate western was probed with a polyclonal antiserum raised against maize Shrunken1 protein (Wurtzel et al., 1987).

Immunolocalization of PSY in Maize Endosperm

Maize B73 endosperms at 17 DAP were dissected and immersed in 50 mM sodium phosphate buffer, pH 7.2, and fixed according to Zhang and Boston (1992) with modifications. Several slices ($2 \times 4 \times 1$ mm) for each tissue were fixed in 3% glutaraldehyde in sodium phosphate buffer either for 2 h at room temperature or overnight at 4°C. Fixed slices were washed with sodium phosphate buffer at room temperature three times for 15 min each, dehydrated at room temperature through a series of ethyl alcohol 10%, 25%, 50%, 70%, and 95% (v/v) for 10 min each, then twice in 100% ethyl alcohol for 10 min each time. Slices were infiltrated in 25%, 50%, and 75% (v/v) LR white (Polysciences) in ethyl alcohol at room temperature for 2 h each and then in 100% (v/v) LR white at room temperature overnight. Slices were transferred to 100% LR white in beam capsules (Polysciences) and polymerized at 65°C for 24 h. One μ m semi-thin sections were prepared using glass knives on an Ultra Microtome (Leica). Sections were collected onto precoated glass slides (Polysciences) and air dried. All steps of immunofluorescent labeling were carried out at room temperature. Slides were rehydrated in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4) for 15 min, blocked in 0.5% (w/v) bovine serum albumin (BSA) in PBS for 30 min, incubated with primary antiserum (anti-maize PSY1) diluted at 1:25 in BSA/PBS overnight in a moist chamber, washed with PBS three times (5 min each), labeled with the goat anti-rabbit IgG-FITC conjugate (Sigma Diagnostics) for 2 h, and washed with PBS three times at 5 min each. Slides were air dried and mounted with coverslips using Aqua Poly/Mount as an anti-fading agent (Polysciences). For negative controls, primary antisera were replaced with 0.5% (w/v) BSA in PBS. Slides were observed using a fluorescent microscope with a blue filter, excitable at 488 nm. Photographs were taken under a MRC-600 Laser Scanning Confocal Imaging system (Bio-

Rad) at Columbia University. Images were processed using Adobe Photoshop software (Adobe Systems).

Correlation Analysis between PSY Transcript Levels and Carotenoid Content of Maize Diversity Lines

For each of 10 diverse lines, PSY transcript levels in developing endosperms at specified DAP were measured by quantitative RT-PCR as described; total kernel carotenoid content was described (Islam, 2004; Harjes et al., 2008). Correlation between transcript levels and carotenoid content was evaluated using JMP (version 5.1; SAS Institute) to perform the Pearson correlation analysis (r) and to test for statistical significance (P) of the correlation between transcript levels and carotenoid content. Statistically significant correlation was based on a P value ≤ 0.05 .

De-Etiolation Experiment

Seeds of maize (B73 and *elm1*) were surface sterilized and grown on Murashige and Skoog agar medium (Murashige and Skoog, 1962) with 1.5% (w/v) Suc. B73 and homozygous *elm1* plants were grown, until the two-leaf stage, in the dark at 28°C for 9 d and 11 d, respectively. For de-etiolation, seedlings were transferred to continuous white (approximately $50 \mu\text{mol m}^{-2} \text{s}^{-1}$), red ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$), far-red ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$), or blue light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) and irradiated for 1, 2, 3, 4, 6, and 8 and 24 h at room temperature. At the end of the illumination period, leaves from five plants were randomly collected and immediately frozen for RNA extraction. Etiolated seedlings that were kept in the dark for the same time periods served as controls. Far-red light was supplied at a fluence rate of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ by Q-beam FR LED light sources (Quantum Devices) with λ_{max} at 735 nm and filtered through far-red plastic resin (FRF700; Westlake Plastics) to yield a half-maximum bandwidth of <25 nm with no detectable emissions below 690 nm. Red light was obtained from 667-nm Q-beam LED sources (Quantum Devices) at a fluence rate of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ half bandwidth <25 nm, and blue light was supplied from 464-nm LED sources in a Percival E30-LED incubator with fluence rate of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, half bandwidth <30 nm. White light was obtained from cool-white fluorescent bulbs. Light intensity and spectral output were measured with a LI-COR Li-1800 spectroradiometer.

Screening Maize PSY1 Null Mutants by Sequencing

The full-length maize PSY1 cDNA and three genomic DNA segments covering the entire open reading frame and 590 bp upstream were PCR amplified from *y1* alleles requested from the Maize Genetics Cooperation Stock Center (University of Illinois, Urbana, IL). Primers were designed from the published Y1 sequence (GenBank accession no. ZMU32636; Buckner et al., 1996). For primer sequences and PCR conditions, refer to Supplemental Table S1. The maize PSY1 cDNA was amplified from total cDNA synthesized from leaves. The PCR conditions were 94°C for 30 s, 56°C for 30 s, 72°C for 90 s, 35 cycles. The PCR conditions for isolating genomic DNA segments were 94°C for 45 s, 56°C for 45 s, 72°C for 2 min, 40 cycles with leaf genomic DNA as template. All PCR products were purified using the MinElute PCR purification kit (Qiagen) and verified by sequencing.

Analysis of Carotenoid Composition of *y1-8549* Mutant

Homozygous *y1-8549* mutants were grown in the dark or in the light under either low temperature (20°C) or high temperature (37°C) conditions. The etiolated leaves (two) of dark-grown plants and the third leaves of light-grown plants were collected for chlorophyll and carotenoid analysis using HPLC. Chla and Chlb were extracted into 80% (v/v) acetone and quantified as described (Lichtenthaler, 1987). Carotenoid extraction and HPLC analysis were carried out as described by (Quinlan et al., 2007). Lutein and β -carotene were identified by comparison with commercial standards. Neoxanthin and violaxanthin standards were isolated by thin-layer chromatography of carotenoids (Cammarata and Schmidt, 1992).

Accession Numbers

Sequence data from this article can be found in the GenBank under accession numbers EU306868 (*y1-8549*) and EU306869 (*y1-602C*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Western analysis to test specificity of the anti-maize *PSY1* antiserum.

Supplemental Figure S2. *PSY1* mRNA levels in maize treated with light of various spectral qualities.

Supplemental Figure S3. *PSY3* mRNA levels in maize treated with light of various spectral qualities.

Supplemental Table S1. Primers for analyzing *PSY1* mutant alleles.

ACKNOWLEDGMENTS

We thank Dr. Brent Buckner (Truman State University) for providing the Y1 cDNA, which allowed us to prepare a *PSY1* antiserum, Dr. Thomas P. Brutnell (Boyce Thompson Institute, Cornell University) for maize *dm1* seeds, the Maize Genetics Cooperation Stock Center (University of Illinois, Champaign-Urbana) for *y1* alleles, Dr. Timothy W. Short (Queens College) for technical advice and provision of growth chambers used for light experiments, Dr. Gabriel Aisemberg (Lehman College) for advice on confocal microscopy, Dr. Dwight Kincaid (Lehman College) for advice on statistical analysis, and Dr. E.R. Macagno (formerly at Columbia University) for use of their confocal microscope. We are grateful to Rena Quinlan for critical reading of the manuscript.

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Li et al.

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Appendix 2: Plasmids generated during this study

Clone Name	Brief Description	Insert Size (kb)	Cha. No.	GenBank Number
pET23aZmGGPPS1	Maize GGPPS1	1.1	3	EF417573
pET23aZmGGPPS2	Maize GGPPS2	1.1	3	EF417574
pET23cZmGGPPS3	Maize GGPPS3	1.0	3	EF417575
HYD-B_14	TAMU BAC Clone	40-150	4	EU638325
HYD-B_41	TAMU BAC Clone	40-150	4	EU638326
HYD-B_80	TAMU BAC Clone	40-150	4	AY844957
BM382572	Maize HYD3 containing EST	1.2	4	BM382572
BG320875	Maize HYD4 containing EST	1.4	4	BG320875
pTHYD4	Maize HYD4 cDNA in pET23C	1.4	4	AY844956
pTHYD3	Maize HYD3 cDNA in pET23C	1.2	4	AY844958
pAC-BETA-04	Accumulates beta-carotene; has ipp isomerase to give deeper color.		4	
pGEMTZmDXS1	Maize DXS1	2.1	3	AY946271
pBSZmDXS1	Maize DXS1	2.1	3	AY946271
pGEMTOsDXS1	Rice DXS1	2.1	3	LOC_Os05g33840
pBSOSDXS1	Rice DXS1	2.1	3	LOC_Os05g33840
pGEMTOsDXS2	Rice DXS2	2.1	3	LOC_Os07g09190
pBSOsDXS2	Rice DXS2	2.1	3	LOC_Os07g09190
pGEMTOsDXS3	Rice DXS3	2.1	3	LOC_Os06g05100
pBSOsDXS3	Rice DXS3	2.1	3	LOC_Os06g05100
pGEMTZmDXS3	Maize DXS3	2.1	3	DR967452

WURTZEL LAB CLONE INFORMATION

Date Today: 07 /02/09

Entered into database 427

CLONE NAME: pET23aZmGGPPS1	
Lab Clone Number/Name: pET23aZmGGPPS1 Clone type: cDNA expression construct	Alternate name(s): GenBank Acc.No. EE417573
Clone Description: The maize GGPPS1 cDNA, was cloned in EcoRI and HindIII sites and cloned into the corresponding sites of the pET23a. This construct complements the pACCAR25ΔcrtE successfully.	
Constructed by: Ratnakar Vallabhaneni Purified by: Ratnakar Vallabhaneni DNA Location (-20°C) Box Number: 6 Position: H3 Conc. >0.5 μg/μl Tube labeled as: pET23aZmGGPPS1 Strain Location (-80°C) Box Number: 18 Position B9 Tube labeled as: pET23aZmGGPPS1 Strain: BL21 (DE3)	

Cited in journal:

Lab Notebook to reference: Ratnakar Vallabhaneni, Note book 2 Page 77
Organism source of gene: maize B73 inbred line Endosperm
Cloning vector used: pET23a Vector size: 3.6 kb Insert size: 1.1 kb
Antibiotic markers: Amp
Restriction enzyme(s) to release insert: <i>EcoRI</i> & <i>HindIII</i>
Sequence verified: Yes Junction verified: Yes

WURTZEL LAB
CLONE INFORMATION

Date Today: 07 /02/09

Entered into database 428

CLONE NAME: pET23aZmGGPPS2	
Lab Clone Number/Name: pET23aZmGGPPS2 Clone type: cDNA expression construct	Alternate name(s): GenBank Acc.No. EE417574
Clone Description: The maize GGPPS2 cDNA, was cloned in EcoRI and HindIII sites and cloned into the corresponding sites of the pET23a. This construct complements the pACCAR25ΔcrtE successfully.	
Constructed by: Ratnakar Vallabhaneni Purified by: Ratnakar Vallabhaneni DNA Location (-20°C) Box Number: 6 Position: H4 Conc. >0.5 μg/μl Tube labeled as: pET23aZmGGPPS2 Strain Location (-80°C) Box Number: 18 Position B10 Tube labeled as: pET23aZmGGPPS2 Strain: BL21 (DE3)	
Cited in journal:	
Lab Notebook to reference: Ratnakar Vallabhaneni, Note book 7 Page 10	
Organism source of gene: maize B73 inbred line Endosperm	
Cloning vector used: pET23a Vector size: 3.6 kb Insert size: 1.1 kb	
Antibiotic markers: Amp	
Restriction enzyme(s) to release insert: <i>EcoRI</i> & <i>HindIII</i>	
Sequence verified: Yes Junction verified: Yes	

WURTZEL LAB
CLONE INFORMATION

Date Today: 07 /02/09

Entered into database 429

CLONE NAME: pET23cZmGGPPS3	
Lab Clone Number/Name: pET23cZmGGPPS3 Clone type: cDNA expression construct	Alternate name(s): GenBank Acc.No. EE417575
Clone Description: The maize GGPPS3 cDNA, was cloned in EcoRI and HindIII sites and cloned into the corresponding sites of the pET23c. This construct does not complement pACCAR25ΔcrtE, thus it is not functional.	
Constructed by: Ratnakar Vallabhaneni Purified by: Ratnakar Vallabhaneni DNA Location (-20°C) Box Number: 6 Position: H5 Conc. >0.5 µg/µl Tube labeled as: pET23cZmGGPPS3 Strain Location (-80°C) Box Number: 18 Position C1 Tube labeled as: pET23cZmGGPPS3 Strain: BL21 (DE3)	

Cited in journal:

Lab Notebook to reference: Ratnakar Vallabhaneni, Note book 7 Page 66
Organism source of gene: maize B73 inbred line Genomic DNA
Cloning vector used: pET23c Vector size: 3.6 kb Insert size: 1.0 kb
Antibiotic markers: Amp
Restriction enzyme(s) to release insert: <i>EcoRI</i> & <i>HindIII</i>
Sequence verified: Yes Junction verified: Yes

WURTZEL LAB CLONE INFORMATION

Date Today: 05 /07/08

Entered into database 127

CLONE NAME: HYD-B_14 harbouring carotene hydroxylase HYD1
Lab Clone Number/Name: <u>HYD-B 14</u> Alternate name(s): Carotene Hydroxylase 1 Clone type: Genomic: <u>X</u> cDNA: Expression <u> </u> Non-expression <u>X</u>
New GenBank Accession: <u>EU638325</u> Original GenBank Accession: <u> </u>
Clone Description: The 1.3kb insert from the β -carotene hydroxylase EST (Genbank BG320875) as hybridized to the maize B73 genomic BAC library (92,160 clones; 5.2X pECBAC1 015-B73-RI) representing 5.2X genomic equivalents
Constructed by: Texas A & M University Purified by: C.E.Gallagher DNA Location (-20°C) Box Number: 6 Position: B3 Conc. >0.5 μ g/ μ l Tube labeled as: HYD-B_14 Strain Location (-80°C) Box Number: 12 Position D7 Tube labeled as: HYD-B_14 Strain: NA

Cited in journal:

Lab Notebook to reference: C.E.Gallagher, Note book 2 Page 31
Organism source of gene: maize B73 inbred line
Cloning vector used: pECBAC1 Vector size: 7.5 kb Insert size: 40-150 kb
Antibiotic markers: Chloroamphenicol
Restriction enzyme(s) to release insert: <i>EcoRI</i>
Sequence verified: yes Junction verified: no
GenBank sheet attached: Yes <u> </u> No <u>x</u> Hybridization results attached: Yes <u> </u> No <u>x</u> Other: (attach additional information here i.e. cartoon, VectorNTI details, sequence)

WURTZEL LAB
CLONE INFORMATION

Date Today: 05/07/2008

Entered into database 127

CLONE NAME: HYD-B_41, a BAC clone harbouring HYD2		
Lab Clone Number/Name: <u>HYD-B_41</u> Alternate name(s): Carotene Hydroxylase 2		
Clone type: Genomic: <u>X</u>		
cDNA: Expression <u> </u> Non-expression <u>X</u>		
New GenBank Accession: <u>EU638326</u>		Original GenBank Accession: <u> </u>
Clone Description: The 1.3kb insert from the β -carotene hydroxylase EST (Genbank BG320875) as hybridized to the maize B73 genomic BAC library (92,160 clones; 5.2X pECBAC1 015-B73-RI) representing 5.2X genomic equivalents		
Constructed by: Texas A & M University Purified by: C.E.Gallagher DNA Location (-20°C) Box Number: 6 Position: B4 Conc. >0.2 μ g/ μ l Tube labeled as: HYD-B_41 Strain Location (-80°C) Box Number: 12 Position: D10 Tube labeled as: HYD-B_41 Strain: NA		

Cited in journal:

Lab Notebook to reference: C.E.Gallagher, Note book 2 Page 31
Original clone name (if different):
Organism source of gene: Maize B73
Cloning vector used: pECBAC1vector Vector size: 7.5Kb Insert size: 40-150kb
Antibiotic markers: Chloramphenicol
Restriction enzyme(s) to release insert: <i>EcoRI</i>
Sequence verified: yes Junction verified: no
GenBank sheet attached: Yes <u> </u> No <u>x</u> Hybridization results attached: Yes <u> </u> No <u>x</u> Other: (attach additional information here i.e. cartoon, VectorNTI details, sequence)

WURTZEL LAB CLONE INFORMATION

Date Today: 05/07/08

Entered into database 127

Clone Number/Name: HYD-B_80, a BAC clone harbouring HYD3	
Lab Clone Number/Name: <u>HYD-B_80</u> Alternate name(s): Carotene Hydroxylase 3	
Clone type: Genomic: <u>X</u> cDNA: Expression <u> </u> Non-expression <u>X</u>	
New GenBank Accession: <u>AY844957</u> Original GenBank Accession: <u>BM382572</u>	
Clone Description: The 1.3kb insert from the β -carotene hydroxylase EST (Genbank BG320875) as hybridized to the maize B73 genomic BAC library (92,160 clones; 5.2X pECBAC1 015-B73-RI) representing 5.2X genomic equivalents	
Constructed by: Texas A&M University Purified by: C.E.Gallagher Storage Location (-20°C) Box Number: 6 Position: E6 Conc. 0.2 ug/ul Tube labeled as: HYD-B_80 Storage Location (-80°C) Box Number: 12 Position: E6 Tube labeled as: HYD-B_80 strain: NA	

Cited in journal:

Lab Notebook to reference: C.E.Gallagher, Note book 2 Page 31
Original clone name (if different):
Organism source of gene: Maize B73
Cloning vector used: pECBAC1 Vector size: 7.5kb Insert size: 40-150 kb
Antibiotic markers: Chloramphenicol
Restriction enzyme(s) to release insert: <i>EcoRI</i>
Strain Transformed into:
Sequence verified: yes Junction verified: no
GenBank sheet attached: Yes <u> </u> No <u>x</u>
Hybridization results attached: Yes <u> </u> No <u>x</u>
Other: (attach additional information here i.e. cartoon, VectorNTI details, sequence)

WURTZEL LAB
CLONE INFORMATION

Date Today: 05/08/2008

Entered into database 202

CLONE NAME: BM382572 is an EST harbouring carotene hydroxylase 3	
Lab Clone Number/Name: <u>BM382572</u> Alternate name(s): Carotene Hydroxylase 3 Clone type: Genomic: _____ cDNA: Expression _____ Non-expression <u>X</u> _____	
New GenBank Accession: <u>AY844958</u> Original GenBank Accession: <u>BM382572</u>	
Clone Description: This clone was identified by sequence homology to BAC clone HYD_80 and other beta-carotene hydroxylases	
Constructed by: Schnable Lab Purified by: C.E.Gallagher DNA Location (-20°C) Box Number: 4 Position: 10C Conc. 0.5 µg/µl Tube labeled as: BM382572 Strain Location (-80°C) Box Number: 13 Position: E7 Tube labeled as: BM382572 Strain: Top10 F'	
Cited in journal:	
Lab Notebook to reference: C.E.Gallagher, Note book 2 Page 137	
Organism source of gene: Maize B73	
Cloning vector used: pSlip7 Vector size: 4.4kb Insert size: 1.2 kb	
Antibiotic markers: Ampicilin	
Restriction enzyme(s) to release insert: EcoRI/Not1	
Sequence verified: yes Junction verified: no	
GenBank sheet attached: Yes _____ No <u>x</u> _____ Hybridization results attached: Yes _____ No <u>x</u> _____ Other: (attach additional information here i.e. cartoon, VectorNTI details, sequence)	

WURTZEL LAB
CLONE INFORMATION

Date Today: 05/08/2008 Entered into database 196

CLONE NAME: BG320875 is an EST harbouring carotene hydroxylase 4	
Lab Clone Number/Name: <u> BG320875 </u> Alternate name(s): Carotene Hydroxylase 4	
Clone type: Genomic: _____ cDNA: Expression _____ Non-expression <u> X </u>	
New GenBank Accession: <u> AY844956 </u> Original GenBank Accession: <u> BG320875 </u>	
Clone Description: Maize EST identified as beta-carotene hydroxylase by sequence homology. Zm_04_09b07_RZm_04AAFC_ECORC_cold stressed maize seedlings	
Constructed by: J.A.Singh Purified by: C.E.Gallagher DNA Location (-20°C) Box Number: 4 Position: 8C Conc. >0.5 µg/µl Tube labeled as: BG320875 Strain Location (-80°C) Box Number: 11 Position I10 Tube labeled as: BG320875 Strain: TOP10F`	

Cited in journal:

Lab Notebook to reference: C.E.Gallagher, Note book 2 Page 25
Organism source of gene: Maize CO328
Cloning vector used: pBluescript SK- Vector size: 2.9kb Insert size: 1.4 kb
Antibiotic markers: Ampicilin
Restriction enzyme(s) to release insert: EcoRI/Xho1
Sequence verified: yes Junction verified: no
GenBank sheet attached: Yes _____ No <u> x </u> Hybridization results attached: Yes _____ No <u> x </u> Other: (attach additional information here i.e. cartoon, VectorNTI details, sequence)

WURTZEL LAB
CLONE INFORMATION

Date Today: 05/08/2008

Entered into database 299

Clone Number/Name: pTHYD4		
Lab Clone Number/Name: <u>pTHYD4</u> Alternate name(s): Carotene Hydroxylase 4		
Clone type: Genomic: _____ cDNA: Expression <u>x</u> Non-expression _____		
New GenBank Accession: <u>AY844956</u> Original GenBank Accession: <u>BG320875</u>		
Clone Description: The maize HYD4 cDNA, derived from the BG320875 plasmid, was excised by restriction digestion with EcoRI and XhoI and cloned into the corresponding sites of the pET23c. This construct complements the pAC-Beta-04 successfully.		
Constructed by: Nicholas Licciardello Purified by: Nicholas Licciardello Storage Location (-20°C) Box Number: 6 Position: A1 Conc. 1.0_ug/ul Tube labeled as: pTHYD4 Storage Location (-80°C) Box Number: 14 Position: A1 Tube labeled as: pTHYD4 Strain: E.coli Nova Blue		

Cited in journal:

Lab Notebook to reference: Nicholas Licciardello note book#3 June 4, 2004
Original clone name (if different): _____
Organism source of gene: Maize CO328
Cloning vector used: pET23C Vector size: 3.6 kb Insert size: 1.38 kb
Antibiotic markers: amp
Restriction enzyme(s) to release insert: <i>EcoRI/XhoI</i>
Sequence verified: yes Junction verified: yes
GenBank sheet attached: Yes____No__x____ Hybridization results attached: Yes____No__x____ Other: (attach additional information here i.e. cartoon, VectorNTI details, sequence)

WURTZEL LAB
CLONE INFORMATION

Date Today: 05/08/2008

Entered into database : 203

Clone Number/Name: pTHYD3		
Lab Clone Number/Name: <u>pTHYD3</u> Alternate name(s): Carotene Hydroxylase 3		
Clone type: Genomic: _____ cDNA: Expression <u>x</u> Non-expression _____		
New GenBank Accession: <u>AY844958</u> Original GenBank Accession: <u>BM382575</u>		
Clone Description: The maize HYD3 cDNA, derived from the BM382575 plasmid, was excised by restriction digestion with <i>EcoRI</i> and <i>NotI</i> and cloned into the corresponding sites of the pET23c. This construct complements the pAC-Beta-04 successfully.		
Constructed by: C.E.Gallagher Purified by: C.E.Gallagher Storage Location (-20°C) Box Number: 6 Position: A2 Conc. 1.0_ug/ul Tube labeled as: pTHYD3 Storage Location (-80°C) Box Number: 14 Position: A2 Tube labeled as: pTHYD3 Strain: <i>E.coli</i> Nova Blue		

Cited in journal:

Lab Notebook to reference: C.E.Gallagher, Note book 2 Page 140
Original clone name (if different): _____
Organism source of gene: Maize B73
Cloning vector used: pET23C Vector size: 3.6 kb Insert size: 1.2 kb
Antibiotic markers: amp
Restriction enzyme(s) to release insert: <i>EcoRI/NotI</i>
Sequence verified: yes Junction verified: yes
GenBank sheet attached: Yes____No__x__ Hybridization results attached: Yes____ No__x__ Other: (attach additional information here i.e. cartoon, VectorNTI details, sequence)

WURTZEL LAB

CLONE INFORMATION

Date Today: 08 / 21 /97Entered into database yes 17 / no

CLONE NAME: pAC-BETA-04
Lab Clone Number/Name: <u>pAC-BETA-04</u> Alternate name(s): _____ Clone type: Genomic: _____ cDNA: Expression <u> x </u> Non-expression _____
New GenBank Accession: _____ Original GenBank Accession: _____
Clone Description: Accumulates beta-carotene; has ipp isomerase to give deeper color.
Constructed by: <u>Dr. Cunningham</u> Date rec'd _____ Purified by: _____ DNA Location (-20°C) Box Number: <u>6</u> Position: <u>A9</u> Conc. <u>32 ng/μl</u> Tube labeled as: <u>pAC-BETA-04</u> Strain Location (-80°C) Box Number: <u>15</u> Position <u>A4</u> Tube labeled as: <u>pAC-BETA-04</u> Strain: <u>BL21 (DE3)</u>
Cited in journal: J. Biol. Chem 271: 24349-24352, 1996
Lab Notebook to reference: _____ Date: _____ Original clone name (if different): _____
Organism source of gene: Erwinia uredovora and H. pluvialis (IPP) Variety <u> </u>
Cloning vector used: <u>pACYC 184</u> Vector size: <u>4.2 Kb</u> Insert size: _____
Antibiotic markers: _____ amp; _____ tet; <u> x </u> chloramphenicol; _____ other (_____)
Restriction enzyme(s) to release insert: <u>Hind III</u>
Sequence verified: yes _____ no _____ Junction verified: yes _____ no _____
GenBank sheet attached: Yes _____ No <u> x </u> Hybridization results attached: Yes _____ No <u> x </u> Other: A cDNA encoding an isopentenyl pyrophosphate (IPP) isomerase in the green alga Haematococcus pluvialis was excised from the library cloning vector (pBluescript SK +) with BamHI and KpnI sites of the pTrcHis A (invitrogen). Digestion of the resulting plasmid with EcoRV and KpnI produces a fragment of 2.0 Kb containing the H. pluvialis IPP isomerase downstream of the strong bacterial trc promoter. This fragment was cloned in the blunted HindIII site of pAC-BETA to produce pAC-BETA-04.

WURTZEL LAB
CLONE INFORMATION

Date Today: 05 /26/09

Entered into database 439

CLONE NAME: pGEMTZmDXS1	
Lab Clone Number/Name: pGEMTZmDXS1 Clone type: cDNA non-expression construct	Alternate name(s): GenBank Acc.No.
Clone Description: The maize DXS1 cDNA, was PCR amplified using primers listed with EcoRI and HindIII sites and cloned into pGEMT, and later sub-cloned into pBS 1666: A G AAT TCA ATGGCTCTGTCGACGT 1307: AAGCTTCTACGCGTTTGCACT	
Constructed by: Ratnakar Vallabhaneni Purified by: Ratnakar Vallabhaneni DNA Location (-20°C) Box Number: 9 Position: E7 Conc. >0.5 µg/µl Tube labeled as: pGEMTZmDXS1 Strain Location (-80°C) Box Number: 19 Position H3 Tube labeled as pGEMTZmDXS1 Strain: TOP10F'	

Cited in journal: Un Published

Lab Notebook to reference: Ratnakar Vallabhaneni, Note book 9 Page 11
Organism source of gene: maize B73 inbred line Endosperm
Cloning vector used: pGEMT Vector size: 3.0 kb Insert size: 2.1 kb
Antibiotic markers: Amp
Restriction enzyme(s) to release insert: EcoRI & HindIII
Sequence verified: Yes Junction verified: NO

WURTZEL LAB
CLONE INFORMATION

Date Today: 05 /26/09

Entered into database 440

CLONE NAME: pBSZmDXS1	
Lab Clone Number/Name: pBSZmDXS1 Clone type: cDNA expression construct	Alternate name(s): GenBank Acc.No.
Clone Description: The maize DXS1 cDNA, was PCR amplified using primers listed with EcoRI and HindIII sites and cloned into pGEMT, and later sub-cloned into pBS into their corresponding sites and named it as pBSZmDXS1 1666: A G AAT TCA ATGGCTCTGTCGACGT 1307: AAGCTTCTACGCGTTTGCACT	
Constructed by: Ratnakar Vallabhaneni Purified by: Ratnakar Vallabhaneni DNA Location (-20°C) Box Number: 9 Position: E8 Conc. >0.5 µg/µl Tube labeled as: pBSZmDXS1 Strain Location (-80°C) Box Number: 19 Position H4 Tube labeled as: pBSZmDXS1 Strain: TOP10F'	

Cited in journal: Un published

Lab Notebook to reference: Ratnakar Vallabhaneni, Note book 9 Page 12
Organism source of gene: maize B73 inbred line Endosperm
Cloning vector used: pBSK II (-) Vector size: 3.6 kb Insert size: 2.1 kb
Antibiotic markers: Amp
Restriction enzyme(s) to release insert: EcoRI & HindIII
Sequence verified: Yes Junction verified: Yes

WURTZEL LAB CLONE INFORMATION

Date Today: 05 /26/09

Entered into database 441

CLONE NAME: pGEMTOsDXS1	
Lab Clone Number/Name: pGEMTOsDXS1 Clone type: cDNA expression construct	Alternate name(s): GenBank Acc.No.
Clone Description: The Rice DXS1 cDNA, was PCR amplified using primers listed with EcoRI and HindIII sites and cloned into pGEMT, and later sub-cloned into pBS. 1667: A G AAT TCA ATG GCG CTC ACG ACG TTC TC 1668: AAG CTT CTA CGC GTT GGG CAC CGT CAT GA	
Constructed by: Ratnakar Vallabhaneni Purified by: Ratnakar Vallabhaneni DNA Location (-20°C) Box Number: 9 Position: H5 Conc. >0.5 µg/µl Tube labeled as: pGEMTOsDXS1 Strain Location (-80°C) Box Number: 19 Position E9 Tube labeled as: pGEMTOsDXS1 Strain: TOP10F'	

Cited in journal: Un Published

Lab Notebook to reference: Ratnakar Vallabhaneni, Note book 9 Page 12
Organism source of gene: Rice Nipponbare leaf
Cloning vector used: pGEMT Vector size: 3.0 kb Insert size: 2.1 kb
Antibiotic markers: Amp
Restriction enzyme(s) to release insert: EcoRI & HindIII
Sequence verified: Yes Junction verified: NO

WURTZEL LAB
CLONE INFORMATION

Date Today: 05 /26/09

Entered into database 442

CLONE NAME: pBSOsDXS1	
Lab Clone Number/Name: pBSOsDXS1 Clone type: cDNA expression construct	Alternate name(s): GenBank Acc.No.
Clone Description: The Rice DXS1 cDNA, was PCR amplified using primers listed with EcoRI and HindIII sites and cloned into pGEMT, and later sub-cloned into pBS in to their corresponding sites and named it as pBSOsDXS1. 1667: A G AAT TCA ATG GCG CTC ACG ACG TTC TC 1668: AAG CTT CTA CGC GTT GGG CAC CGT CAT GA	
Constructed by: Ratnakar Vallabhaneni Purified by: Ratnakar Vallabhaneni DNA Location (-20°C) Box Number: 9 Position: H6 Conc. >0.5 µg/µl Tube labeled as pBSOsDXS1 Strain Location (-80°C) Box Number: 19 Position E10 Tube labeled as: pBSOsDXS1 Strain: TOP10F'	

Cited in journal: Un published

Lab Notebook to reference: Ratnakar Vallabhaneni, Note book 9 Page 11
Organism source of gene: Rice Nipponabre leaf
Cloning vector used: pBSSKII(-) Vector size: 3.6 kb Insert size: 2.1 kb
Antibiotic markers: Amp
Restriction enzyme(s) to release insert: EcoRI & HindIII
Sequence verified: Yes Junction verified: Yes

WURTZEL LAB
CLONE INFORMATION

Date Today: 05 /26/09

Entered into database 443

CLONE NAME pGEMTOsDXS2			
Lab Clone Number/Name: pGEMTOsDXS2		Alternate name(s): GenBank Acc.No.	
Clone type: cDNA non-expression construct			
Clone Description: The Rice DXS2 cDNA, was PCR amplified using primers listed with EcoRI and HindIII sites and cloned into pGEMT, and later sub-cloned into pBS. 1682: AGAATTCAATGGCGCTCCAGGCATCGTCGTCGC 1683: AAG CTT T CAG CTG AGC TGA AGT GCC TCC AAT			
Constructed by: Ratnakar Vallabhaneni Purified by: Ratnakar Vallabhaneni DNA Location (-20°C) Box Number: 9 Position: H7 Conc. >0.5 µg/µl Tube labeled as: pGEMTOsDXS2 Strain Location (-80°C) Box Number: 18 Position B9 Tube labeled as: pGEMTOsDXS2 Strain: TOP10F'			

Cited in journal:

Lab Notebook to reference: Ratnakar Vallabhaneni, Note book 9 Page 11
Organism source of gene: Rice nipponbare leaf
Cloning vector used: pGEMT Vector size: 3.0 kb Insert size: 2.1 kb
Antibiotic markers: Amp
Restriction enzyme(s) to release insert: EcoRI & HindIII
Sequence verified: Yes Junction verified: No

WURTZEL LAB
CLONE INFORMATION

Date Today: 05 /26/09

Entered into database 444

CLONE NAME: pBSOsDXS2	
Lab Clone Number/Name: pBSOsDXS2 Clone type: cDNA expression construct	Alternate name(s): GenBank Acc.No.
Clone Description: The Rice DXS2 cDNA, was PCR amplified using primers listed with EcoRI and HindIII sites and cloned into pGEMT, and later sub-cloned into pBS into their corresponding sites and renamed it as pBSOsDXS2 1682: AGAATTC AATGGCGCTCCAGGCATCGTCGTCGC 1683: AAG CTT T CAG CTG AGC TGA AGT GCC TCC AAT	
Constructed by: Ratnakar Vallabhaneni Purified by: Ratnakar Vallabhaneni DNA Location (-20°C) Box Number: 9 Position: H8 Conc. >0.5 µg/µl Tube labeled as: pBSOsDXS2 Strain Location (-80°C) Box Number: 19 Position F2 Tube labeled as: pBSOsDXS2 Strain: TOP10F'	

Cited in journal: UnPublished

Lab Notebook to reference: Ratnakar Vallabhaneni, Note book 9 Page 12
Organism source of gene: Rice Nipponbare leaf
Cloning vector used: pBSIISK (-) Vector size: 3.6 kb Insert size: 2.1 kb
Antibiotic markers: Amp
Restriction enzyme(s) to release insert: EcoRI & HindIII
Sequence verified: Yes Junction verified: Yes

WURTZEL LAB
CLONE INFORMATION

Date Today: 05 /26/09

Entered into database 445

CLONE NAME: pGEMTOsDXS3	
Lab Clone Number/Name: pGEMTOsDXS3 Clone type: cDNA non-expression construct	Alternate name(s): GenBank Acc.No.
Clone Description: The Rice DXS3 cDNA, was PCR amplified using primers listed with EcoRI and HindIII sites and cloned into pGEMT, and later sub-cloned into pBS. 1674: A G AAT TCG ATG GGT GGG AGA TAT CTG CA 1675: AAG CTT TTA CTT CAT CAA CAA AAG TGC GTC T	
Constructed by: Ratnakar Vallabhaneni Purified by: Ratnakar Vallabhaneni DNA Location (-20°C) Box Number: 9 Position: H9 Conc. >0.5 µg/µl Tube labeled as: pGEMTOsDXS3 Strain Location (-80°C) Box Number: 19 Position F3 Tube labeled as: pGEMTOsDXS3 Strain: TOP10F'	

Cited in journal: Unpublished

Lab Notebook to reference: Ratnakar Vallabhaneni, Note book 9 Page 12
Organism source of gene: Rice nipponabare leaf
Cloning vector used: pGEMT Vector size: 3.0 kb Insert size: 2.1 kb
Antibiotic markers: Amp
Restriction enzyme(s) to release insert: EcoRI & HindIII
Sequence verified: Yes Junction verified: No

WURTZEL LAB
CLONE INFORMATION

Date Today: 05 /26/09

Entered into database 446

CLONE NAME: pBSOSDXS3	
Lab Clone Number/Name: pBSOSDXS3 Clone type: cDNA expression construct	Alternate name(s): GenBank Acc.No.
Clone Description: The Rice DXS3 cDNA, was PCR amplified using primers listed with EcoRI and HindIII sites and cloned into pGEMT, and later sub-cloned into pBS into their corresponding sites and renamed it as pBSOs DXS3 1674: A G AAT TCG ATG GGT GGG AGA TAT CTG CA 1675: AAG CTT TTA CTT CAT CAA CAA AAG TGC GTC T	
Constructed by: Ratnakar Vallabhaneni Purified by: Ratnakar Vallabhaneni DNA Location (-20°C) Box Number: 9 Position: H10 Conc. >0.5 µg/µl Tube labeled as: pBSOSDXS3 Strain Location (-80°C) Box Number: 19 Position F4 Tube labeled as: pBSOSDXS3 Strain: TOP10F'	

Cited in journal: Unpublished

Lab Notebook to reference: Ratnakar Vallabhaneni, Note book 9 Page 12
Organism source of gene: Rice nipponbare leaf
Cloning vector used: pBSSKII (-) Vector size: 3.6 kb Insert size: 2.1 kb
Antibiotic markers: Amp
Restriction enzyme(s) to release insert: EcoRI & HindIII
Sequence verified: Yes Junction verified: Yes

WURTZEL LAB
CLONE INFORMATION

Date Today: 05 /26/09

Entered into database 447

CLONE NAME: pGEMTZmDXS3	
Lab Clone Number/Name: pGEMTZmDXS3 Clone type: cDNA non-expression construct	Alternate name(s): GenBank Acc.No.
Clone Description: The Maize DXS3 cDNA, was PCR amplified using primers listed with PstI and HindIII sites and cloned into pGEMT 1641: A C TGC AGC ATG GAC ACG GCG TTT CTG AGT C 1642: AAAGCTTCTACTTCATCAACAGAAGGGGCATCACG	
Constructed by: Ratnakar Vallabhaneni Purified by: Ratnakar Vallabhaneni DNA Location (-20°C) Box Number: 9 Position: F5 Conc. >0.5 µg/µl Tube labeled as: pGEMTZmDXS3 Strain Location (-80°C) Box Number: 19 Position I1 Tube labeled as: pGEMTZmDXS3 Strain: TOP10F'	

Cited in journal: Unpublished

Lab Notebook to reference: Ratnakar Vallabhaneni, Note book 9 Page 11
Organism source of gene: maize B73 inbred line Endosperm
Cloning vector used: pGEMT Vector size: 3.0 kb Insert size: 2.1 kb
Antibiotic markers: Amp
Restriction enzyme(s) to release insert: PstI and HindIII
Sequence verified: YES Junction verified: NO

Appendix 3: GenBank Submissions

No	Gene name	Cultivar	Accession name	Sequence Type	Source	Chapter
1	ZmGGPPS1	B73	EF417573	cDNA	RNA	2
2	ZmGGPPS2	B73	EF417574	cDNA	RNA	2
3	ZmGGPPS3	B73	EF417575	cDNA	RNA	2
4	ZmGGPPS4	B73	EF417576	cDNA	RNA	2
5	ZmHYD1	B73	EU638325	Genomic DNA	BAC clone	3
6	ZmHYD2	B73	EU638326	Genomic DNA	BAC clone	3
7	ZmHYD3	A619	FJ228406	Genomic DNA	Genomic DNA	3
8	ZmHYD3	B73	FJ228407	Genomic DNA	Genomic DNA	3
9	ZmHYD3	B37	FJ228408	Genomic DNA	Genomic DNA	3
10	ZmHYD3	CI7	FJ228409	Genomic DNA	Genomic DNA	3
11	ZmHYD3	C131A	FJ228410	Genomic DNA	Genomic DNA	3
12	ZmHYD3	DE3	FJ228411	Genomic DNA	Genomic DNA	3
13	ZmHYD3	KUI2007	FJ228412	Genomic DNA	Genomic DNA	3
14	ZmHYD3	NC300	FJ228413	Genomic DNA	Genomic DNA	3
15	ZmHYD3	SD44	FJ228414	Genomic DNA	Genomic DNA	3
16	ZmHYD3	TZI18	FJ228415	Genomic DNA	Genomic DNA	3

1. EF417573

LOCUS bankit880503 1104 bp mRNA circular PLN 31-JAN-2007
 DEFINITION Zea mays Geranylgeranyl pyrophosphate synthase (GGPPS1) mRNA,
 complete cds.
 ACCESSION 880503
 VERSION
 KEYWORDS .
 SOURCE Zea mays
 ORGANISM Zea mays
 Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
 Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD
 clade; Panicoideae; Andropogoneae; Zea.
 REFERENCE 1 (bases 1 to 1104)
 AUTHORS Vallabhaneni, R. and Wurtzel, E.T.
 TITLE Cloning and Characterization of GGPPS gene family in Zea mays
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 1104)
 AUTHORS Vallabhaneni, R. and Wurtzel, E.T.
 TITLE Direct Submission
 JOURNAL Submitted (31-JAN-2007) Biological Sciences, Lehman College, CUNY,
 250 Bedford park Blvd (W), Bronx, Bronx, NY 10468, USA
 FEATURES Location/Qualifiers
 source 1..1104
 /organism="Zea mays"
 /mol_type="mRNA"
 /cultivar="B73"
 /db_xref="taxon:4577"
 CDS 1..1104
 /codon_start=1
 /protein_id="PR0T_1_bankit880503"
 /translation="MAMSFHFHPLAASRVHFSPLPFAAAAGAPSPSSSSVAIAAHQHGG
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 LLAGGKRVRPALCLAAACAVVGGPEPWAMPAAAAVEMVHTMSLVHDDLPCDDDLRRG
 KPTCHVVYGEPIAVLAGDALLSLSFHHMASVGSYPDPVDPEKHPARVVRAIGELARCI
 GSEGLVAGQVVDLEMTGTSETVPLERLEYIHLHKTAALLEASVIGAIIGGGTDEQIE
 RLRKYARSIGLLFQVDDILDVTKSSEELGKTAGKDLASDKTTPKLLGLEKSREFAE
 ELLSDAVEQLACFDKEKAAPLLHLANYIAHRQN"
 BASE COUNT 170 a 398 c 368 g 168 t
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 1 atggccatgt cgttccactt ccacccgctg gccgcctcgc gcgtccactt ctccccactc
 61 ccgttcgccg ccgccgcggg ggcgccctcc ccctcctcct ccgtcgccat cgcggcgccac
 121 caccagcacg gtcgccgcg cttctccgcc atcgtggcca cggcggccgc cgcgtcgccg
 181 gcgaccacg agttcgactt caaggcgtag atgggggagc gcgcggtcgc ggtgaaccgg
 241 gccctggacg cggccgtccc cgccggggag ccccccgcgc gcgtgcacga cgcgatgcgt
 301 tacgcgctgc tggcggggcg gaagcgcgtg cgccccgcgc tgtgcctggc cgcgtgcgcc
 361 gtggtgggcg ggccggagcc ctgggccatg ccggccgcgc cggccgtcga gatggtgcac
 421 accatgtcgc tcgtccacga cgacctcccc tgcattggac acgacgacct ccgccgcggc
 481 aagcccacct gccacgtcgt ctacggcgag cccatcgccg tcctcgccgg ggacgcgctg
 541 ctctcgctct cctccacca catggccagc gtcgggtcct accctccgga cgtggaccgg
 601 gagaagcacc ccgccgcgct cgtccgagcc attggggagc tcgcgcgctg catcggatcc
 661 gagggactcg tcgccggcca ggttgctcat ctcgagatga cgggcacatc agagacggtg
 721 cccctcgaac gcctcgagta catccacctc cacaagaccg ccgcattgct cgaggcctcg
 781 ttggtgattg gggcgatcat cggaggcgcc acggacgagc agatcgagag gctgcggaag
 841 tacgcgaggt cgatcgggct gctgttccag gtggtcgacg acatactcga tgtcaccaag
 901 tcgtcagagg agctcgcaa gacggcgggg aaggacctgg caagcgacaa aacgacgtac
 961 ccgaagctgc tggggctaga aaagtcgcgg gagttcgcgg aggagttgct ctctgatgcc
 1021 gtagagcagc ttgcttgctt cgacaaggag aaggcagcgc ctctgttgca tctggccaac
 1081 tatatcgccc ataggcagaa ctga

2. EF417574

LOCUS bankit880505 1086 bp mRNA circular PLN 31-JAN-2007
 DEFINITION Putative Zea mays Geranylgeranyl pyrophosphate synthase (GGPPS2)
 mRNA, complete CDS.
 ACCESSION 880505
 VERSION
 KEYWORDS
 SOURCE Zea mays
 ORGANISM Zea mays
 Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
 Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD
 clade; Panicoideae; Andropogoneae; Zea.
 REFERENCE 1 (bases 1 to 1086)
 AUTHORS Vallabhaneni, R. and Wurtzel, E.T.
 TITLE Cloning and Characterization of GGPPS gene family in Zea mays
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 1086)
 AUTHORS Vallabhaneni, R. and Wurtzel, E.T.
 TITLE Direct Submission
 JOURNAL Submitted (31-JAN-2007) Biological Sciences, Lehman College, CUNY,
 250 Bedford park Blvd (W), Bronx, Bronx, NY 10468, USA
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 VYGEPIAVLAGDALLSLSFHHMASVGSYPYPAVDPEKHPARVVRAIGELARCIGSEGLV
 AGQVVDLEMTGTSQTVPLELLEYIHLHKTAALLEASVVIGAIIGGGTDEQIEALRKYA
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 BASE COUNT 180 a 386 c 344 g 176 t
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 121 caccacggcc gccgccgctt ctccgccatc gtggccacgg ccaccaccac ggagttcaac
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 241 cccgccgggg gcctccccgc ggcgctgcac gacgcgatgc ggtacgcgct gctggcgggc
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 541 cacatggcca gcgtcggatc ctaccctccg gccgttgacc cggagaagca ccccgcccgc
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 1081 aactga

3. EF417575

LOCUS bankit880507 1092 bp mRNA circular PLN 31-JAN-2007
 DEFINITION Putative Zea mays Geranylgeranyl pyrophosphate synthase
 (GGPPS3)mRNA, complete CDS.
 ACCESSION 880507
 VERSION
 KEYWORDS .
 SOURCE Zea mays
 ORGANISM Zea mays
 Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
 Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD
 clade; Panicoideae; Andropogoneae; Zea.
 REFERENCE 1 (bases 1 to 1092)
 AUTHORS Vallabhaneni, R. and Wurtzel, E.T.
 TITLE Cloning and Characterization of GGPPS gene family in Zea mays
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 1092)
 AUTHORS Vallabhaneni, R. and Wurtzel, E.T.
 TITLE Direct Submission
 JOURNAL Submitted (31-JAN-2007) Biological Sciences, Lehman College, CUNY,
 250 Bedford park Blvd (W), Bronx, Bronx, NY 10468, USA
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 YAHFLGLLGQVDDVLDVTGTSEQLGKTAGKDVAAGKATYPRLMGLKGARAYMGELLA
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BASE COUNT 156 a 387 c 389 g 160 t
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 121 acccggcacc gcgccgcgga ccgtgctgcc agaggcacca tcgtcgacgt cgccgtcgac
 181 agtggcacca gcttcgactt cgagagctac ctgtcggcca aggccagggc cgtgcacgac
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 301 tactccggtt tcgcggggcg caagcgcctc cgccccgtgc tggccatcgc cgcggtcgag
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4. EF417576

LOCUS bankit884020 1032 bp mRNA circular PLN 31-JAN-2007
 DEFINITION Putative Zea mays Geranylgeranyl pyrophosphate synthase(GGPPS4)mRNA,complete CDS.
 ACCESSION 884020
 VERSION
 KEYWORDS .
 SOURCE Zea mays
 ORGANISM Zea mays
 Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD clade; Panicoideae; Andropogoneae; Zea.
 REFERENCE 1 (bases 1 to 1032)
 AUTHORS Vallabhaneni,R. and Wurtzel,E.T.
 TITLE Cloning and Characterization of GGPPS gene family in Zea mays
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 1032)
 AUTHORS Vallabhaneni,R. and Wurtzel,E.T.
 TITLE Direct Submission
 JOURNAL Submitted (31-JAN-2007) Biological Sciences, Lehman College, CUNY, 250 Bedford park Blvd (W), Bronx, Bronx, NY 10468, USA
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 source Location/Qualifiers
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 LFPLAYTHVISHTPSPDPVPHAVLLRVLAELARAVGSTGMAAGQFLDLGATALGEAE
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 BASE COUNT 156 a 390 c 319 g 167 t
 ORIGIN
 1 atggctctct cctccctctt catcaccctc cccatcccca atgtcccttc ccccaaacc
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 1021 gcaactccat ag

5. EU638325

LOCUS EU638325 3615 bp DNA linear PLN 01-JUL-2008
 DEFINITION Zea mays diiron type beta-carotene hydroxylase (HYD1) genomic sequence.
 ACCESSION EU638325
 VERSION EU638325
 KEYWORDS .
 SOURCE Zea mays
 ORGANISM Zea mays, Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD clade; Panicoideae; Andropogoneae; Zea.
 REFERENCE 1 (bases 1 to 3615)
 AUTHORS Vallabhaneni, R., Gallagher, C.E. and Wurtzel, E.T.
 TITLE Characterization of beta-carotene hydroxylase gene family in maize
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 3615)
 AUTHORS Vallabhaneni, R., Gallagher, C.E. and Wurtzel, E.T.
 TITLE Direct Submission
 JOURNAL Submitted (04-APR-2008) Biological Sciences, Lehman College, CUNY, 250 Bedford Park Blvd (w), Bronx, New York, NY 10468, USA
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 CDS join(1970..2089,2231..2290,2386..2542,2694..2762)
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 /note="diiron type beta-carotene hydroxylase"
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LOCUS          EU638326                3484 bp    DNA        linear     PLN 01-JUL-2008
DEFINITION     Zea mays diiron type beta-carotene hydroxylase(HYD2-Pseudo)genomic
sequence.
ACCESSION      EU638326
VERSION        EU638326
KEYWORDS       .
SOURCE         Zea mays
ORGANISM       Zea mays; Eukaryota; Viridiplantae; Streptophyta; Embryophyta;
Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae;
PACCAD clade; Panicoideae; Andropogoneae; Zea.
REFERENCE      1 (bases 1 to 3484)
AUTHORS        Vallabhaneni,R., Gallagher,C.E. and Wurtzel,E.T.
TITLE          Characterization of beta-carotene hydroxylase (HYD2;
pseudogene)genomic sequence
JOURNAL        Unpublished
REFERENCE      2 (bases 1 to 3484)
AUTHORS        Vallabhaneni,R., Gallagher,C.E. and Wurtzel,E.T.
TITLE          Direct Submission
JOURNAL        Submitted (04-APR-2008) Biological Sciences, Lehman College,
CUNY, 250 Bedford Park Blvd (w), Bronx, New York, NY 10468, USA
FEATURES             Location/Qualifiers
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7. FJ228406

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LOCUS      bankit1135531          560 bp    DNA        linear    PLN 22-SEP-2008
DEFINITION Zea mays diiron type beta-carotene hydroxylase (HYD3), genomic~~
            sequence includes exon 1 and partial promoter.
ACCESSION  1135531
VERSION
KEYWORDS   .
SOURCE     Zea mays
  ORGANISM Zea mays
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD
            clade; Panicoideae; Andropogoneae; Zea.
REFERENCE  1 (bases 1 to 560)
  AUTHORS  Vallabhaneni,R. and Wurtzel,E.T.
  TITLE    Characterization of beta-carotene hydroxylase gene family in maize
  JOURNAL  Unpublished
REFERENCE  2 (bases 1 to 560)
  AUTHORS  Vallabhaneni,R. and Wurtzel,E.T.
  TITLE    Direct Submission
  JOURNAL  Submitted (22-SEP-2008) Biological Sciences, Lehman college of
            CUNY, 250 Bedford park blvd (w), Bronx, NY 10468, USA
FEATURES   Location/Qualifiers
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   121 ctcccgtcga ctcggtccct gtccatctgt agcgcgttcc attccattcc atcccatccc
   181 cgaaaagtgc aaggacggga gggagagcgg ccggcgcgctc acaaatacca ccagcccagg
   241 ggccatggcc gccgcgatga ccagcttcgt cgccaagaac ccgctgctcg cggccgcggc
   301 gcggcgcagg gcgcctcccc tcgcggggcg cgccctgccg ttctctccgc tcgccagcac
   361 caggggcccc cgccgcaggg ggctcaggct gaggaccgtc acgtgcttcg tgccgcagga
   421 cacggcggcc ccggccgctc ccgtgccggc gctggacgag gaggccaggg ccgcggcggc
   481 gcggcgcgctc gcggagaagg aggcgcggaa gcggtccgag cggcggacgt acctggtggc
   541 cgccgtgatg tctagcctcg

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8. FJ228407

LOCUS bankit1135538 557 bp DNA linear PLN 22-SEP-2008
 DEFINITION Zea mays diiron type beta-carotene hydroxylase (HYD3), genomic~~
 sequence includes exon 1 and partial promoter.
 ACCESSION 1135538
 VERSION
 KEYWORDS .
 SOURCE Zea mays
 ORGANISM Zea mays
 Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
 Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD
 clade; Panicoideae; Andropogoneae; Zea.
 REFERENCE 1 (bases 1 to 557)
 AUTHORS Vallabhaneni, R. and Wurtzel, E.T.
 TITLE Characterization of beta-carotene hydroxylase gene family in maize
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 557)
 AUTHORS Vallabhaneni, R. and Wurtzel, E.T.
 TITLE Direct Submission
 JOURNAL Submitted (22-SEP-2008) Biological Sciences, Lehman college of
 CUNY, 250 Bedford park blvd (w), Bronx, NY 10468, USA
 FEATURES
 source Location/Qualifiers
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 61 tctgccttcc cctcctatcc tgtgagctgt aacgccttga ggtatgtgaa ctatccatat
 121 aagttcctgt gagctgtctc ccgctcactc ggtccctgtc catccgtagc gttccattcc
 181 attccatccc atccccggaa agtgcaagga cgggagggag agcggccggc gcgtcacaaa
 241 taccaccagc ccaggggcca tggccgcgcg gatgaccagc ttcgtcgcca agaaccgcgt
 301 gctcgcggcc gcggcgcgcc gcagggcgcc tcccctcgcg gggcgcgccc tgccgttctc
 361 tccgctcgcc agcaccaggg ccccgcgccg caccgtcacg tgcttcgtgc cgcaggacac
 421 ggcggccccc gccgctcccg tgcggcgct ggacgaggag gccagggccg cggcggcgcg
 481 gcgcgtcgcg gagaaggagg cgcggaagcg gtccgagcgg cggacgtacc tgggtggccg
 541 cgtgatgtct agcctcg

9. FJ228408

LOCUS bankit1135545 574 bp DNA linear PLN 22-SEP-2008
 DEFINITION Zea mays diiron type beta-carotene hydroxylase (HYD3), genomic~~
 sequence includes exon 1 and partial promoter.
 ACCESSION 1135545
 VERSION
 KEYWORDS .
 SOURCE Zea mays
 ORGANISM Zea mays
 Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
 Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD

clade; Panicoideae; Andropogoneae; Zea.

REFERENCE 1 (bases 1 to 574)
 AUTHORS Vallabhaneni,R. and Wurtzel,E.T.
 TITLE Characterization of beta-carotene hydroxylase gene family in maize
 JOURNAL Unpublished

REFERENCE 2 (bases 1 to 574)
 AUTHORS Vallabhaneni,R. and Wurtzel,E.T.
 TITLE Direct Submission
 JOURNAL Submitted (22-SEP-2008) Biological Sciences, Lehman college of CUNY, 250 Bedford park blvd (w), Bronx, NY 10468, USA

FEATURES
 source Location/Qualifiers
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 SSL"

BASE COUNT 84 a 208 c 191 g 91 t

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 121 agttcctgtg agctgtctcc cgctcactcg gtccctgtcc atccgtagcg ttccattcca
 181 ttccatccca tccccgga aa gtgcaaggac gggagggaga gcggcgggcg cgtcacaaat
 241 accaccagcc caggggccat ggccgcgcgc atgaccagct tcgtcgccaa gaaccgcgtg
 301 ctgcgcggcg cggcgcggcg cagggcgctt cccctcgcg ggcgcgccct gccgttctct
 361 ccgctcgcca gcaccagggc cccgcgcgcg aggggggtca ggctgaggac cgtcacgtgc
 421 ttcgtgccgc aggacacggc ggccccggcc gctcccgtgc cggcgctgga cgaggaggcc
 481 agggccgcgg cggcgcggcg cgtcgcggag aaggaggcgc ggaagcggtc cgagcggcgg
 541 acgtacctgg tggccgccgt gatgtctagc ctcg

10. FJ228409

LOCUS bankit1135562 568 bp DNA linear PLN 22-SEP-2008

DEFINITION Zea mays diiron type beta-carotene hydroxylase (HYD3), genomic--
 sequence includes exon 1 and partial promoter.

ACCESSION 1135562

VERSION

KEYWORDS .

SOURCE Zea mays

ORGANISM Zea mays
 Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
 Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD
 clade; Panicoideae; Andropogoneae; Zea.

REFERENCE 1 (bases 1 to 568)
 AUTHORS Vallabhaneni,R. and Wurtzel,E.T.
 TITLE Characterization of beta-carotene hydroxylase gene family in maize
 JOURNAL Unpublished

REFERENCE 2 (bases 1 to 568)
 AUTHORS Vallabhaneni,R. and Wurtzel,E.T.
 TITLE Direct Submission
 JOURNAL Submitted (22-SEP-2008) Biological Sciences, Lehman college of CUNY, 250 Bedford park blvd (w), Bronx, NY 10468, USA

FEATURES
 source Location/Qualifiers
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AAVLSSL"
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   61 cttctcctat cctgtgagct gtaacacttg aacactcccg ctcccgcggc tcggctggct
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  181 aagtgcaagg acgggagggg gagcggccgg cgcgtcacaa ataccagctg cccagggggc
  241 atggccgccg cgatgaccag cttcgtcgcc aagaaccgcg tgctcgcggc cgcggcgcg
  301 cgcagggcgc ctcccctcgc gggcgcgccc ctgccgttct ctccgctcgc cagcaccagg
  361 gccccgcgcc gcagggggct caggctgagg accgtcacgt gcttcgtgcc gcaggaccgc
  421 gcggaccgcg ccgtcccgtg gccggcgccg gagacggcgc tggacgagga ggccagggcc
  481 gcggcggcgc ggcgcgtcgc ggagaaggag gcgcggaagc ggtccgagcg gcggacgtac
  541 ctggtggccg ccgtgtgtgc tagcctcg

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11. FJ228410

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LOCUS      bankit1135566          574 bp    DNA        linear    PLN 22-SEP-2008
DEFINITION Zea mays diiron type beta-carotene hydroxylase (HYD3), genomic~~
sequence includes exon 1 and partial promoter.
ACCESSION  1135566
VERSION
KEYWORDS   .
SOURCE     Zea mays
  ORGANISM Zea mays
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD
            clade; Panicoideae; Andropogoneae; Zea.
REFERENCE  1 (bases 1 to 574)
  AUTHORS  Vallabhaneni,R. and Wurtzel,E.T.
  TITLE    Characterization of beta-carotene hydroxylase gene family in maize
  JOURNAL  Unpublished
REFERENCE  2 (bases 1 to 574)
  AUTHORS  Vallabhaneni,R. and Wurtzel,E.T.
  TITLE    Direct Submission
  JOURNAL  Submitted (22-SEP-2008) Biological Sciences, Lehman college of
            CUNY, 250 Bedford park blvd (w), Bronx, NY 10468, USA
FEATURES   Location/Qualifiers
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            /translation="MAAAMTSFVAKNPLAAAAARRRAPLAGRALPFSPLASTRAPRR
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BASE COUNT  84 a      208 c      191 g      91 t

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181 ttccatccca tcccggaaa gtgcaaggac gggagggaga gcggccggcg cgtcacaat
241 accaccagcc caggggccat ggccgcgcg atgaccagct tcgtcgccaa gaaccgcgtg
301 ctgcgcggcg cggcgcggcg caggggcgct cccctcgcgg ggccgcgcct gccgttctct
361 ccgctcgcca gcaccagggc cccgcgcgc aggggggtca ggctgaggac cgtcacgtgc
421 ttctgtccgc aggacacggc ggccccggc gctcccgtgc cggcgctgga cgaggaggcc
481 agggccgcgg cggcgcggcg cgtcgcggag aaggaggcgc ggaagcggtc cgagcggcgg
541 acgtacctgg tggccgccgt gatgtctagc ctgc

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12. FJ228411

LOCUS bankit1135573 556 bp DNA linear PLN 22-SEP-2008

DEFINITION Zea mays diiron type beta-carotene hydroxylase (HYD3), genomic-- sequence includes exon 1 and partial promoter.

ACCESSION 1135573

VERSION

KEYWORDS .

SOURCE Zea mays

ORGANISM Zea mays

Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD clade; Panicoideae; Andropogoneae; Zea.

REFERENCE 1 (bases 1 to 556)

AUTHORS Vallabhaneni, R. and Wurtzel, E.T.

TITLE Characterization of beta-carotene hydroxylase gene family in maize

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 556)

AUTHORS Vallabhaneni, R. and Wurtzel, E.T.

TITLE Direct Submission

JOURNAL Submitted (22-SEP-2008) Biological Sciences, Lehman college of CUNY, 250 Bedford park blvd (w), Bronx, NY 10468, USA

FEATURES

Location/Qualifiers

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/organism="Zea mays"

/mol_type="genomic DNA"

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/db_xref="taxon:4577"

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/codon_start=1

/product="HYD3"

/translation="MAAAMTSFVAKNPLAAAAARRRAPLAGRALPFSPLASTRAPRR RGLRLRTVTCTFVPQDTAAPAPVPALDEEARAAAAARRVAEKEARKRSERTYLVAAMV SSL"

BASE COUNT 77 a 209 c 189 g 81 t

ORIGIN

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121 cccgctcact cggtcctgt ccactctgtag cgttccattc cattccatcc catccccgga
181 aagtgcagg acgggagggg gagcggcgg cgctcacaa ataccagctg cccagggggc
241 atggccgccc cgatgaccag cttcgtcgcc aagaaccgc tgcctcgccc cgcggcgcgg
301 cgcaggggcg ctcctctgc gggcgcgccc ctgccgttct ctccgtcgc cagcaccagg
361 gccccgcgcc gcagggggct caggctgagg accgtcacgt gcttcgtgcc gcaggacacg
421 gcggccccgg ccgtcccgt gccggcgctg gacgaggagg ccagggccgc ggcggcgcgg
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541 gtgatgtcta gcctcg

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13. FJ228412

LOCUS bankit1135582 556 bp DNA linear PLN 22-SEP-2008
 DEFINITION Zea mays diiron type beta-carotene hydroxylase (HYD3), genomic~~
 sequence includes exon 1 and partial promoter.
 ACCESSION 1135582
 VERSION
 KEYWORDS .
 SOURCE Zea mays
 ORGANISM Zea mays
 Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
 Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD
 clade; Panicoideae; Andropogoneae; Zea.
 REFERENCE 1 (bases 1 to 556)
 AUTHORS Vallabhaneni,R. and Wurtzel,E.T.
 TITLE Characterization of beta-carotene hydroxylase gene family in maize
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 556)
 AUTHORS Vallabhaneni,R. and Wurtzel,E.T.
 TITLE Direct Submission
 JOURNAL Submitted (22-SEP-2008) Biological Sciences, Lehman college of
 CUNY, 250 Bedford park blvd (w), Bronx, NY 10468, USA
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 121 agttcctgtg agctgtctcc cgctcactcg gtccctgtcc atccgtagcg ttccattcca
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 241 accaccagcc caggggccat ggccgcccgc atgaccagct tcgtcgccaa gaaccgctg
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 541 gtgatgtcta gcctcg

14. FJ228413

LOCUS bankit1135584 578 bp DNA linear PLN 22-SEP-2008
 DEFINITION Zea mays diiron type beta-carotene hydroxylase (HYD3), genomic~~
 sequence includes exon 1 and partial promoter.
 ACCESSION 1135584
 VERSION
 KEYWORDS .
 SOURCE Zea mays
 ORGANISM Zea mays
 Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;

Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD clade; Panicoideae; Andropogoneae; Zea.

REFERENCE 1 (bases 1 to 578)
 AUTHORS Vallabhaneni,R. and Wurtzel,E.T.
 TITLE Characterization of beta-carotene hydroxylase gene family in maize
 JOURNAL Unpublished

REFERENCE 2 (bases 1 to 578)
 AUTHORS Vallabhaneni,R. and Wurtzel,E.T.
 TITLE Direct Submission
 JOURNAL Submitted (22-SEP-2008) Biological Sciences, Lehman college of CUNY, 250 Bedford park blvd (w), Bronx, NY 10468, USA

FEATURES
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 SSL"

BASE COUNT 85 a 211 c 190 g 92 t

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 121 agttcctgtg agctgtctcc cgctcactcg gtccctgtcc atccgtagcg ttccattcca
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 241 caaataccac cagcccaggg gccatggccg ccgcgatgac cagcttcgtc gccaagaacc
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 541 ggcgggacgta cctggtggcc gccgtgatgt ctagcctc

15. FJ228414

LOCUS bankit1135587 556 bp DNA linear PLN 22-SEP-2008

DEFINITION Zea mays diiron type beta-carotene hydroxylase (HYD3), genomic~~
 sequence includes exon 1 and partial promoter.

ACCESSION 1135587

VERSION

KEYWORDS .

SOURCE Zea mays
 ORGANISM Zea mays
 Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
 Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD
 clade; Panicoideae; Andropogoneae; Zea.

REFERENCE 1 (bases 1 to 556)
 AUTHORS Vallabhaneni,R. and Wurtzel,E.T.
 TITLE Characterization of beta-carotene hydroxylase gene family in maize
 JOURNAL Unpublished

REFERENCE 2 (bases 1 to 556)
 AUTHORS Vallabhaneni,R. and Wurtzel,E.T.
 TITLE Direct Submission
 JOURNAL Submitted (22-SEP-2008) Biological Sciences, Lehman college of CUNY, 250 Bedford park blvd (w), Bronx, NY 10468, USA

FEATURES Location/Qualifiers

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source      1..556
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  181 ttccatccca tccccgaaa gtgcaaggac gggagggaga gcggccggcg cgtcacaaat
  241 accaccagcc caggggccat ggccgccgag atgaccagct tcgtcgccaa gaaccgctg
  301 ctgcgcggcg cgcgcgggcg cagggcgccct cccctcgcgg ggcgcgccct gccgttctct
  361 ccgctcgcca gcaccagggc cccgcgcgcg accgtcacgt gcttcgtgcc gcaggacacg
  421 gcggccccgg ccgtcccgt gccggcgctg gacgaggagg ccagggccgc ggcggcgcgg
  481 cgcgtcgcgg agaaggaggc gcggaagcgg tccgagcggc ggacgtacct ggtggccgcc
  541 gtgatgtcta gcctcg

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16. FJ228415

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LOCUS      bankit1135588          541 bp    DNA        linear    PLN 22-SEP-2008
DEFINITION Zea mays diiron type beta-carotene hydroxylase (HYD3), genomic~~
sequence includes exon 1 and partial promoter.
ACCESSION  1135588
VERSION
KEYWORDS   .
SOURCE     Zea mays
  ORGANISM Zea mays
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD
            clade; Panicoideae; Andropogoneae; Zea.
REFERENCE  1 (bases 1 to 541)
  AUTHORS  Vallabhaneni,R. and Wurtzel,E.T.
  TITLE    Characterization of beta-carotene hydroxylase gene family in maize
  JOURNAL  Unpublished
REFERENCE  2 (bases 1 to 541)
  AUTHORS  Vallabhaneni,R. and Wurtzel,E.T.
  TITLE    Direct Submission
  JOURNAL  Submitted (22-SEP-2008) Biological Sciences, Lehman college of
            CUNY, 250 Bedford park blvd (w), Bronx, NY 10468, USA
FEATURES   Location/Qualifiers
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BASE COUNT      80 a      193 c      178 g      90 t
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121 agttcctgtg agctgtctcc cgctcactcg gtccctgtcc atccgtagcg ttccattcca
181 ttccatccca tccccgaaa gtgcaaggac gggagggaga gcggccggcg cgtcaciaat
241 accaccagcc caggggcat ggccgccgag atgaccagct tcgtcgccaa gaacccgctg
301 ctgcgcggcg cggcgccgag cagggcgccg cccctcgagg ggcgcgccct gccgttctct
361 ccgctcaggc tgaggaccgt cacgtgcttc gtgccgcagg acacggcggc cccggccgct
421 cccgtgccgg cgctggacga ggaggccagg gccgcggcgg cgcggcgcggt cgcggagaag
481 gaggcgcgga agcggtcgga gcggcgagag tacctgggtg ccgccgtgat gtctagcctc
541 g
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Appendix 4: Sequences used in the Study

Gene	<i>Zea Mays</i>		<i>Oryza sativa</i>	<i>Sorghum bicolor</i>
	ESTs/cDNA	BAC/Genomic		
DXS1	AY946271	AC206058	LOC_Os05g33840	Sb09g020140
DXS2	AY946270	AC208336	LOC_Os07g09190	Sb02g005380
DXS3	DR967452	AC204410	LOC_Os06g05100	Sb10g002960
DXR	AJ297566	AC203861	LOC_Os01g01710	Sb03g008650
HDS	AY562489	AC198323	LOC_Os02g39160	Sb04g025290
HDR	DR789385	AC209801	LOC_Os03g52170	Sb01g009140
IPPI1	AF330034	AC213618	LOC_Os07g36190	Sb02g035700
IPPI2	DN215017	AC203067	LOC_Os05g34180	Sb09g020370
IPPI3	CO530856	AC206783	NA	NA
GGPPS1	EF417573	AC194970	LOC_Os07g39270	Sb02g037510
GGPPS2	EF417574	AC196175	NA	NA
GGPPS3	EF417575	AC211188	LOC_Os01g14630	Sb03g009380
PSY1	AY324431		LOC_Os06g51290	Sb10g031020
PSY2	AY325302		LOC_Os12g43130	Sb08g022310
PSY3	DQ372936		LOC_Os09g38320	Sb02g032370
PDS	L39266	AC205461	LOC_Os03g08570	Sb06g030030
ZDS	AF047490	AC197604	LOC_Os07g10490	Sb02g006100
CrtISO1	DR812825	AC218991	LOC_Os11g36440	Sb05g022240
CrtISO2	EE045563	AC183901	NA	NA
LCYB	AY206862	AC205536	LOC_Os02g09750	Sb04g006120
LCYE	EB674039	AC196426	LOC_Os01g39960	Sb03g026020
HYD1	NA	EU638325	NA	NA
HYD2	NA	EU638326	NA	NA
HYD3	BM382572	AC194430	NA	NA
HYD4	BG320875	AC196442	LOC_Os04g48880	Sb06g026190
HYD5	DR817188	AC149033	LOC_Os03g03370	Sb01g048860
HYD6	BQ619575	AC155364	LOC_Os10g38940	NA
CYP97A	DV169913	AC197544	LOC_Os02g57290	Sb04g037300
CYP97C	BE552887	AC177851	LOC_Os10g39930	Sb01g030050
ZEP1	DR820114	AC194845	LOC_Os04g37619	Sb06g018220

ZEP2	CO532283	AC206194	NA	NA
NCED1	U95953	AC201886	NA	NA
NCED2	DV032362	AC199036	LOC_Os12g42280	Sbi_0.36552
NCED3a	DY541318	AC212820	LOC_Os07g05940	Sb02g003230
NCED3b	NA	AC205109	NA	NA
NCED9	NA	AC190614	LOC_Os03g44380	Sb01g013520
CCD1	DQ100346	DQ100347	LOC_Os12g44310	Sb01g047540
CCD4a	EE172116	AC190588	LOC_Os02g47510	Sb04g030640
CCD4b	NA	AC194862	LOC_Os12g24800	NA
CCD7	NA	AC211432	LOC_Os04g46470	Sb06g024560
CCD8a	CO526902	AC185113	LOC_Os01g38580	Sb05g009950
CCD8b	DW954641	AC198395	LOC_Os09g15240	Sb03g034400
CCD8c	NA	NA	LOC_Os01g54270	Sb10g004360 ; Sb10g004370 (8c- like)
CCD8d	NA	NA	LOC_Os08g28240	Sb02g021490 ; Sb07g024250 (8d- like)
ABA8Ox1a	DR806072	AC194862	LOC_Os02g47470	Sb04g030660
ABA8Ox1b	CD433445	AC182107	NA	NA
ABA8Ox2	CO460095	AC212409	LOC_Os08g36860	Sb07g022990
ABA8Ox3a	CD941324	AC190490	LOC_Os09g28390	Sb02g026600
ABA8Ox3b	EC904849	AC195926	NA	NA
Actin	J01238			

Appendix 5: Inbreds Used in the Study

Accession*	GENETIC DIVERSITY GROUP**	GENETIC DIVERSITY SUBGROUP**	MAIZE VARIETY
Ames 19000	unknown		VaW6
NSL 30338	unknown		K4
Ames 28290	unknown		C49
NSL 65865	unknown		B10
Ames 27171	TS	NC	NC350
Ames 27261	TS	NC	NC298
	TS	Suwan	KUI2007
Ames 27088	TS	Suwan	CML 228
	TS	TS-mixed	NC300
Ames 17174	TS	TS-mixed	NC356
	TS	TZI	TZI18
Ames 27062	TS	TZI	A6
Ames 27175	TS	TZI	NC358
Ames 28186	Sweet corn		P39
Ames 27114	SS	B14A	H91
PI 406112	SS	B14A	A214N
PI 587128	SS	B37	H84
PI 550555	SS	B37	NC250
	SS	B37	B37
PI 550483	SS	B37	B76
PI 587139	SS	B73	A679
PI 608767	SS	B73	B84
	SS	B73	B73
Ames 27138	SS	N28	N28Ht
Ames 27143	SS	SS-mixed	NC294
Ames 22748	SS	SS-mixed	H100
Ames 19311	SS	SS-mixed	A641
PI 587131	Popcorn		HP301
PI 531085	NSS	C103	NC262
PI 550472	NSS	C103	B57
Ames 23450	NSS	CO109:Mo17	A556
PI 558532	NSS	CO109:Mo17	Mo17
NSL 30904	NSS	Ga:SC	4226
Ames 15929	NSS	Hy:T8:Wf9	H49
Ames 28367	NSS	K64W	C17
	NSS	K64W	C131A
Ames 19288	NSS	M14:Oh43	Oh43
PI 587138	NSS	M14:Oh43	A619

NSL 30867	NSS	M14:Oh43	M14
Ames 27190	NSS	NSS-mixed	T234
PI 608765	NSS	NSS-mixed	B77
	NSS	NSS-mixed	SD44
PI 587129	NSS	NSS-mixed	H99
Ames 26604	NSS	NSS-X	38-11
Ames 23405	NSS	NSS-X	A239
	Mixed		DE3
Ames 27189	Mixed		SC55
Ames 27066	Mixed		B164
Ames 27098	Mixed		CML 328
Ames 28366	Mixed		N7A
Ames 27067	Mixed		C49A

* Accessions: Maize lines were obtained from the National Germplasm Collection (<http://www.ars-grin.gov/npgs/holdings.html>), except for the initial 10 line subset described in the manuscript.

** Genetic diversity group/subgroup: Taken from Liu, K. et al. Genetic structure and diversity among maize Inbred lines as inferred from DNA microsatellites. Genetics 165, 2117-2128 (2003). The authors state in that paper: "The 260 lines ...are listed with the groups and subgroups from the STRUCTURE analysis. Lines in the mixed group show 80% membership for any group. Seven popcorn lines and five sweet corn lines were assigned into predefined popcorn and sweet corn groups. Within TS, SS, and NSS groups, lines are organized into several distinct subgroups and one mixed subgroup on the basis of analyses with the program STRUCTURE. The subgroups are named after a defining inbred line(s), principal source (e.g., NC for North Carolina), maturity (early vs. late), or mixed for lines that showed 80% membership for any subgroup.

Appendix 6: Primers used in this study

No.	Sequence	Gene	Accession number	Chapter
1206	CAGAACAGGGAGGCTCTTG	DXS1	AY946271	2
1207	GCGACACTTTCATCCAAAGA			
1212	GCTGAACTACTTCCAGAAGCG	DXS2	AY946270	2
1213	CTGCAGGAACGACGAGTAGA			
1194	CTGTGCCAGCAGCATAGTTT	DXS3	DR967452	2
1195	TTCGACCGTCAAGTAGACCA			
1164	TCCAATGTCACGCTTCTAGC	DXR	AJ297566	2
1165	TGGCGAGCAACTTCTATGAC			
1176	CAGTCCAGGGTAGGAAGGAA	HDS	AY562489	2
1177	TAGGGCTACCGTAGGAGCAA			
1218	GCCCCAACTCGTTACATCCTT	HDR	DR789385	2
1219	TGAGATCAAGCGTCAGGAAA			
1159A	TGAGCTTATCCAGGAGAACTA	IPPI1	AF330034	2
1163A	ATGGTGCCTTGTAAGCATT			
1333	TCAGGCTGGTGGTGGACAACCTT	IPPI2	DN215017	2
1335	TGATGGCTAAAACAAAGGCA			
1333	TCAGGCTGGTGGTGGACAACCTT	IPPI3	CO530856	2
1334	AGCACCAAACAGCAGGACAA			
1475	CGGAGGAGTTGCTCTCTGAT	GGPPS1	EF417573	2
1476	AGTCACAACAGGTCCGACAA			
1477	CAGAGAAGTTGGTCTCTGAC	GGPPS2	EF417574	2
1478	AGATCGACAGTGAGCAAGAA			
1426	ATCCATGTGCACAAGACTGC	GGPPS3	EF417575	2
1427	CACGTCCAGAACGTCGTC			
505	CATCTTCAAAGGGGTCTGCA	PSY1	AY324431	2
254	CAGGATCTGCCTGTACAACA			
503	TCACCCATCTCGACTCTGCTA	PSY2	AY325302	2
676	GATGTGATCTACGGATGGTTCAT			
996	GCCAGAGCCTTCTTCAGGCAGG	PSY3	DQ372936	2
997	GTCTTCGGAACGTAGGCCCTC			
933	GAAATCATCGATGCAACTATGGAA	PDS	L39266	2
934	CTTCGATAGGTGACCTTTGGA			
281	GTGTGGTAAAGATCGGACAA	ZDS	AF047490	2
917	AGAGAGTTGCTCCTTCCAT			
1325	GGTTGTCAAATGGGAAGGAG	CrtISO1	DR812825	2
1326	GAATGATGGTGCCTTGACAT			
1095	CATCGTAAGGTTCCCTCGACA	LCYB	AY206862	A
1096	ATGCCGAAGCAGAAGAACTC			
1037	TTT ACG TGC AAA TGC AGT CAA	LCYE	EB674039	A
1038	TGA CTC TGA AGC TAG AGA AAG			
1664	TTGGTGGCAACAGCTCAAAA	ZEP1	DR820114	2
1665	CCTTCACTTGTTGCGATGAG			
1400	TTGGTGGCAATAGCTCAAAG	ZEP2	CO532283	2
1401	GCTTCACTTGTTGCGAAGAG			
1134	CGATTGAGCATGGCATTGTCA	Actin	J01238	C
1135	CCCCTAGCGTACAACGAA			
1409	TGGCCTGGACGAGCTTGTTAG	HYD3	BM382572	3
995	CACACTCATTTGGCCCACTCGC			
1408	TGGCCTGGACGAGCTGGAAAA	HYD4	BG320875	3
1011	GGCTCGCGCATCCAAACC			
1513	GCAGCCCATCAGATACATCA	HYD5	DR817188	3
1514	GCAAGCAGGTCTTATTGGGT			

1419	GAGATCAAGAAGAGGATCAGGAG	HYD6	BQ619575	3
1420	GATCTAATGTGAGGAGAAGGTCAA			
1344	CTGGAGCCATCTGAAAGTCA	CYP97A	DV169913	3
1345	GGACCAAATCCAAACGAGAT			
1348	GTTGACATTGGATGTGATTGG	CYP97C	BE552887	3
1349	AACCAACCTTCCAGTATGGC			
1652	GCCCAGCTCGTAGCTTAACA	NCED1	AC201886	4
1653	CGAGCAAGTAACAGCAACCA			
2188	ACGGCTACATCCTGTCCCTC	NCED2	AC199036	4
1881	AGAACAACCAAGGAATCATCAGG			
2237	CGATCACGAACCTCGACCAAC	NCED3a	AC212820	4
2060	CCGGCGTCTGCCTCAAA			
2055	CGATCACGAACCTCGACCACC	NCED3b	AC205109	4
2081	GCATGCAAATATACATATGTGCGG			
2185	GAGAGATTATTAGTAGCAGTAGGGAGAG	NCED9	AC190614	4
2018	GCAACCGATCCTAGACACCC			
2064	CTGGAGAAGAAGATGACGGCTAT	CCD1	DQ100347	4
2065	AGGCATGGAATCCATAAGGAAC			
1816	AAGCACCGAACAACCTCGTCCA	CCD4a	AC190588	4
1817	ACTCTTTTATGGGTTCTGCTACATTT			
1818	TCCCCTACGGCTTCCACG	CCD4b	AC194862	4
1819	ATCCAGCGGCGTGTTTCTC			
1836	TGACGCCACAGACATCCATT	CCD7	AC211432	4
1837	TGGGACCGACGAGCAGAA			
1834	TGCTGCTCACATCTCCCACA	CCD8a	AC185113	4
1835	CGTGTAATTGCTACAGCCACATT			
2321	GAAAGCTATGCGAGAATCGG	CCD8b	AC198395	4
2322	GTATTATGGAGTCCGGCGAA			
1774	ATGCTCGTGCTCTTCCACCACCT	ABA8Ox1a	AC194862	5
1769	TATACCGCCATACCATATCCATCCGCC			
1774	ATGCTCGTGCTCTTCCACCACCT	ABA8Ox1b	AC182107	5
1772	GGAAGCGGTTTTTCGCGTTCCTGG			
1785	AGCCTACGAGGAGAACGATG	ABA8Ox2	AC212409	5
1786	TCAGGAACCCTTGGTAGTGC			
1783	ACAGAAAGGGGCGTGAGACCGA	ABA8Ox3a	AC190490	5
1778	AGGCGAGCAAAGAAGAATTTCAA			
1784	ACAGAAATGGCCGCCATGAGACCGA	ABA8Ox3b	AC195926	5
1781	ATTTCTTCTCCCCCTCAAGGTAAT			

A= (Harjes et al., 2008); C= common primer for all chapters

Appendix 7: Raw data

Chapter	Type of DATA	Figures	Citation
2	Temporal and Spatial profiles	2-4 & 2-5	Vallabhaneni et al., 2009
2	Correlation analysis	2-6	Vallabhaneni et al., 2009
3	Temporal and Spatial analysis	3-4	Vallabhaneni et al., 2009 (Plant Physiology, In Press)
3	Correlation analysis	3-5	Vallabhaneni et al., 2009 (Plant Physiology, In Press)
4	Spatial analysis	4-4	Vallabhaneni et al., 2009 (Submitted to JXB)
4	Correlation analysis	4-6	Vallabhaneni et al., 2009 (Submitted to JXB)
4	Gene-copy number	4-5	Vallabhaneni et al., 2009 (Submitted to JXB)
5	Spatial analysis	5-4	Vallabhaneni et al., 2009 (Submitted to PMB)
5	Drought treatment	5-5	Vallabhaneni et al., 2009 (Submitted to PMB)

Chapter 2: Temporal and Spatial profiles (B73)

Endosperm Developmental profiles of PDS											
	0	10	12	14	16	18	20	22	24	26	28
MPDS	24.90	26.70	26.51	25.31	25.79	26.80	27.79	27.19	28.24	28.89	27.63
MPDS	25.09	26.25	26.33	25.59	25.44	26.79	27.92	27.24	28.15	28.59	27.63
MPDS	25.08	26.23	25.97	25.38	25.62	26.80	27.66	27.77	28.26	29.37	27.25
AVG	25.02	26.39	26.27	25.43	25.62	26.80	27.79	27.40	28.22	28.95	27.50
StDEV	0.11	0.27	0.27	0.15	0.18	0.01	0.13	0.32	0.06	0.39	0.22
ACTIN	23.70	21.09	20.80	20.44	20.05	21.14	21.97	21.05	22.35	22.40	22.02
ACTIN	23.35	20.87	20.71	20.01	20.49	21.21	22.76	22.18	22.49	23.57	22.02
ACTIN	23.61	21.17	20.75	19.77	20.10	21.25	22.68	22.73	22.71	24.05	22.02
AVG	23.55	21.04	20.75	20.07	20.21	21.20	22.47	21.99	22.52	23.34	22.02
StDEV	0.18	0.16	0.05	0.34	0.24	0.06	0.43	0.86	0.18	0.85	0.00
Act MPDS	-1.47	-5.35	-5.52	-5.35	-5.40	-5.60	-5.32	-5.41	-5.70	-5.61	-5.48
PDS	36.10	2.45	2.18	2.45	2.36	2.07	2.50	2.35	1.92	2.05	2.24
sPDS	39.32	1.98	1.85	2.65	2.10	2.06	2.50	2.71	1.89	2.13	2.05
sPDS	34.47	2.71	2.10	2.18	2.67	2.08	2.29	2.62	2.01	2.63	2.05
sPDS	34.71	2.75	2.69	2.53	2.36	2.06	2.74	1.82	1.87	1.53	2.66
stDEV	3.66	0.43	0.43	0.24	0.29	0.01	0.23	0.49	0.08	0.55	0.36

Endosperm Developmental profiles of ZDS											
	0	10	12	14	16	18	20	22	24	26	28
MZDS	24.59	25.66	25.32	25.32	25.75	25.68	25.56	25.80	25.38	25.59	26.22
MZDS	25.14	25.63	25.49	25.30	26.08	25.68	25.82	25.65	25.48	25.95	26.62
MZDS	24.82	25.57	25.63	25.32	25.93	25.65	25.70	25.74	25.40	25.75	26.43
AVG	24.85	25.62	25.48	25.31	25.92	25.67	25.69	25.73	25.42	25.76	26.42
STDEV	0.28	0.05	0.16	0.01	0.17	0.02	0.13	0.08	0.05	0.18	0.20
ACTIN	23.9	19.92	19.77	20.51	20.56	20.52	20.25	20.07	19.73	20.46	21.00
ACTIN	24.23	20.34	19.81	19.24	20.62	20.18	20.27	20.10	20.14	20.18	21.37
ACTIN	24.08	20.10	19.82	19.80	20.57	20.36	20.20	20.14	19.98	20.30	21.20
AVG	24.07	20.12	19.80	19.85	20.58	20.35	20.24	20.10	19.95	20.31	21.19
STDEV	0.17	0.21	0.03	0.64	0.03	0.17	0.04	0.04	0.21	0.14	0.19
Act ZDS	-0.79	-5.50	-5.68	-5.46	-5.34	-5.32	-5.45	-5.63	-5.47	-5.45	-5.23
ZDS	57.83	2.21	1.95	2.27	2.47	2.51	2.28	2.02	2.26	2.29	2.66
SZDS	5.67	2.15	2.18	2.26	2.78	2.49	2.50	1.93	2.32	2.58	3.06
SZDS	4.91	2.19	1.94	2.29	2.21	2.49	2.09	2.14	2.16	2.01	2.32
SZDS	4.95	2.29	1.76	2.26	2.46	2.54	2.27	2.01	2.29	2.31	2.65
stDEV	0.78	0.07	0.21	0.02	0.29	0.03	0.21	0.11	0.08	0.28	0.37

Spatial expression profiles of PDS						Relative to Endosperm					
	Em	En(20)	L	R	O		Em	En(20)	L	R	O
MPDS	28.07	27.79	23.08	26.09	24.90	NPDS	0.23	0.00	1.59	1.51	-1.08
MPDS	27.72	27.92	23.56	26.18	25.09	NPDS+Act	-5.50	-5.32	-0.88	-3.62	-2.55
MPDS	28.11	27.66	23.42	26.00	25.08	Absolute	2.21	2.50	54.21	8.13	17.04
Average	27.97	27.79	23.35	26.09	25.02	StDEV1	2.06	2.50	65.52	8.13	18.56
StDEV	0.21	0.13	0.25	0.09	0.11	StDEV2	2.63	2.29	46.98	7.64	16.27
Actin	22.11	21.97	21.24	20.99	23.70	StDEV3	2.01	2.74	51.76	8.66	16.38
Actin	22.33	22.76	20.45	20.93	23.35	StDEV	0.34	0.23	9.63	0.51	1.29
Actin	22.28	22.68	20.96	20.95	23.61						
Average	22.24	22.47	20.88	20.96	23.55						
StDEV	0.12	0.43	0.40	0.03	0.18						
Act PDS	-5.73	-5.32	-2.47	-5.13	-1.47						
NLPDS	1.89	2.50	18.05	2.85	36.10						
StDEV1	1.8	2.5	21.8	2.8	39.3						
StDEV2	2.2	2.3	15.6	2.7	34.5						
StDEV3	1.7	2.7	17.2	3.0	34.7						
StDEV	0.3	0.2	3.2	0.2	2.7						

Spatial expression profiles of ZDS						Relative to Endosperm					
	Em	En(20)	L	R	O		Em	En(20)	L	R	O
MZDS	25.77	25.56	22.13	25.06	24.59	NZDS	-2.00	0.00	-0.64	-0.72	-3.83
MZDS	25.12	25.82	21.95	25.33	25.14	NZDS+Act	-5.25	-5.45	-1.80	-4.96	-4.61
MZDS	25.57	25.70	22.04	25.20	24.82	Absolute	2.63	2.28	28.72	3.22	4.09
Average	25.49	25.69	22.04	25.20	24.85	StDEV1	2.16	2.50	26.98	3.54	4.90
StDEV	0.33	0.13	0.09	0.14	0.28	StDEV2	3.40	2.09	30.57	2.94	3.35
Actin	22.11	20.25	21.24	20.99	23.9	StDEV3	2.49	2.27	28.72	3.21	4.18
Actin	22.33	20.27	20.45	20.93	24.23	StDEV	0.64	0.21	1.79	0.30	0.78
Actin	22.28	20.20	20.96	20.95	24.08						
Average	22.24	20.24	20.88	20.96	24.07						
StDEV	0.12	0.04	0.40	0.03	0.17						
Act ZDS	-3.25	-5.45	-1.16	-4.24	-0.78						
NLZDS	10.54	2.28	44.85	5.29	58.24						
StDEV1	8.7	2.5	42.1	5.8	69.7						
StDEV2	13.6	2.1	47.7	4.8	47.6						
StDEV3	9.9	2.3	44.9	5.3	59.5						
StDEV	2.6	0.2	2.8	0.5	11.1						

Endosperm developmental profiles of crtISO1											
	0	10	12	14	16	18	20	22	24	26	28
MISO	25.98	26.29	26.26	26.13	26.94	27.75	27.74	27.64	26.83	27.75	27.15
MISO	25.81	26.31	25.71	26.72	26.79	27.83	27.63	27.70	26.55	27.13	26.97
MISO	25.87	26.27	25.90	26.40	26.82	27.70	27.66	27.70	26.68	27.40	27.00
AVG	25.89	26.29	25.96	26.42	26.85	27.76	27.68	27.68	26.69	27.43	27.04
STDEV	0.09	0.02	0.28	0.30	0.08	0.07	0.06	0.03	0.14	0.31	0.10
ACTIN	24.28	20.39	20.27	19.82	20.01	21.01	22.15	20.95	21.66	22.79	22.38
ACTIN	25.10	20.75	20.56	20.80	20.99	21.43	21.70	23.86	23.56	22.72	22.90
ACTIN	24.60	20.54	20.40	20.36	20.49	21.25	21.97	22.30	22.55	22.70	22.64
AVG	24.66	20.56	20.41	20.33	20.50	21.23	21.94	22.37	22.59	22.74	22.64
STDEV	0.41	0.18	0.15	0.49	0.49	0.21	0.23	1.46	0.95	0.05	0.26
Act ISO	-1.23	-5.73	-5.55	-6.09	-6.35	-6.53	-5.74	-5.31	-4.10	-4.69	-4.40
NLISO	42.73	1.88	2.14	1.47	1.22	1.08	1.88	2.52	5.84	3.87	4.74
STDEV1	40.1	1.9	1.7	1.8	1.1	1.1	1.8	2.6	5.3	3.1	4.4
STDEV2	45.1	1.9	2.5	1.2	1.3	1.0	1.9	2.5	6.4	4.8	5.0
STDEV3	43.2	1.9	2.2	1.5	1.2	1.1	1.9	2.5	5.9	3.9	4.9
SIDEV	2.5	0.0	0.4	0.3	0.1	0.0	0.1	0.1	0.6	0.8	0.3

Endosperm development profiles of MGGPPS1											
	0	10	12	14	16	18	20	22	24	26	28
MGGPPS1	24.81	24.69	24.07	24.62	25.64	26.65	27.79	26.71	26.61	26.09	25.52
MGGPPS1	24.97	24.67	23.90	24.75	25.04	26.94	27.01	27.16	26.59	26.10	25.68
MGGPPS1	24.62	24.82	24.04	24.12	25.27	26.85	27.60	26.60	26.79	25.92	25.57
AVG	24.80	24.73	24.00	24.50	25.32	26.81	27.47	26.82	26.66	26.04	25.59
SIDEV	0.18	0.08	0.09	0.33	0.30	0.15	0.41	0.30	0.11	0.10	0.08
ACTIN	25.78	20.70	20.18	20.04	20.87	21.67	22.15	21.47	22.36	21.91	22.26
ACTIN	26.39	20.73	20.20	19.91	20.97	22.08	22.40	21.82	22.58	21.95	22.40
ACTIN	26.07	20.69	20.27	20.13	20.89	21.83	22.26	21.62	22.40	21.90	22.30
AVG	26.08	20.71	20.22	20.03	20.91	21.86	22.27	21.64	22.45	21.92	22.32
SIDEV	0.31	0.02	0.05	0.11	0.05	0.21	0.13	0.18	0.12	0.03	0.07
Act MGGPPS	1.28	-4.02	-3.79	-4.47	-4.41	-4.95	-5.20	-5.19	-4.22	-4.12	-3.27
MGGPPS1	242.84	6.16	7.25	4.51	4.71	3.23	2.73	2.75	5.38	5.76	10.37
SGGPPS1	241.16	6.32	6.92	4.14	3.77	3.61	2.18	2.97	5.58	5.56	10.88
SGGPPS1	215.85	6.41	7.78	3.79	5.71	2.96	3.74	2.17	5.66	5.52	9.74
SGGPPS1	275.11	5.78	7.06	5.86	4.87	3.15	2.49	3.21	4.93	6.25	10.51
SIDEV	29.74	0.34	0.46	1.11	0.97	0.34	0.83	0.54	0.40	0.41	0.81

Spatial expression profiles of crtISO1						Relative to Endosperm					
	Em	En(20)	L	R	O		Em	En (20)	L	R	O
MISO	25.7	27.74	25.89	27.28	25.98	NISO	-0.35	0.00	-1.30	-1.86	-2.71
MISO	25.97	27.63	25.35	27.58	25.79	NISO+Act	-3.90	-5.74	-3.68	-5.49	-3.95
MISO	25.84	27.66	25.62	27.43	25.89	Abs.	6.71	1.88	7.80	2.23	6.49
Average	25.84	27.68	25.62	27.43	25.89	SIDEV1	7.38	1.79	6.47	2.47	6.08
SIDEV	0.14	0.06	0.27	0.15	0.10	SIDEV2	6.12	1.94	9.41	2.01	6.93
Actin	21.55	22.15	23.12	22.99	24.28	SIDEV3	6.70	1.90	7.80	2.23	6.47
Actin	23.03	21.70	23.4	24.63	25.06	SIDEV	0.63	0.07	1.47	0.23	0.43
Actin	22.29	21.97	23.2	23.78	24.62						
Average	22.29	21.94	23.24	23.80	24.65						
SIDEV	0.74	0.23	0.14	0.82	0.39						
Act I PPI	-3.55	-5.74	-2.38	-3.63	-1.23						
NISO	8.56	1.88	19.21	8.08	42.53						
SIDEV1	0.1	1.8	0.2	0.1	39.9						
SIDEV2	0.1	1.9	0.2	0.1	45.5						
SIDEV3	0.1	1.9	0.2	0.1	42.4						
SIDEV	0.0	0.1	0.0	0.0	2.8						

Spatial expression profiles of I-GGPPS1						Relative to Endosperm					
	Em	En(20)	L	R	O		Em	En (20)	L	R	O
MGGPPS1	22.39	27.79	23.20	26.19	24.81	NGGPPS1	-0.73	0.00	-2.02	-2.95	-3.81
MGGPPS1	23.30	27.01	23.41	27.21	24.97	NGGPPS1+2	-0.78	-5.20	-0.72	-4.42	-2.53
MGGPPS1	23.46	27.60	22.34	26.67	24.62	Absolute	58.10	2.73	60.85	4.67	17.31
Average	23.05	27.47	22.98	26.69	24.80	SIDEV1	90.13	2.13	51.41	6.47	16.84
SIDEV	0.58	0.41	0.57	0.51	0.18	SIDEV2	47.96	3.67	44.44	3.19	15.07
Actin	22.88	22.15	24.02	24.94	25.78	SIDEV3	42.93	2.43	93.30	4.64	19.21
Actin	23.02	22.40	24.38	25.38	26.39	SIDEV	25.92	0.81	26.43	1.64	2.08
Actin	23.09	22.26	24.46	25.34	26.07						
Average	23.00	22.27	24.29	25.22	26.08						
SIDEV	0.11	0.13	0.23	0.24	0.31						
Act GGPPS	-0.05	-5.20	1.30	-1.47	1.28						
NLMGGPPS	96.37	2.73	246.80	36.10	242.84						
SIDEV1	152.3	2.2	212.4	51.1	241.2						
SIDEV2	81.0	3.7	183.6	25.2	215.8						
SIDEV3	72.5	2.5	385.5	36.6	275.1						
SIDEV	43.8	0.8	109.2	13.0	29.7						

Endosperm development profiles of cyclases											
	0	10	12	14	16	18	20	22	24	26	28
MLCYE	27.46	30.58	29.67	29.98	28.75	30.80	30.67	30.52	28.59	28.26	30.25
MLCYE	27.18	30.10	29.11	29.91	28.86	30.80	30.66	30.54	28.78	28.13	29.70
MLCYE	27.48	30.16	29.21	29.21	28.28	30.43	29.79	30.14	28.67	28.15	29.89
AVG	27.37	30.28	29.33	29.70	28.63	30.68	30.37	30.40	28.68	28.18	29.95
StDEV	0.17	0.26	0.30	0.43	0.31	0.21	0.51	0.23	0.10	0.07	0.28
MLCYB	26.04	26.93	27.83	28.49	29.59	29.72	30.06	29.82	30.17	30.22	30.44
MLCYB	26.71	27.25	27.76	28.38	29.32	29.79	30.00	29.56	29.49	30.22	30.17
MLCYB	26.82	26.98	27.41	28.46	29.63	29.59	30.45	30.05	29.57	30.31	30.45
AVG	26.52	27.05	27.67	28.44	29.51	29.70	30.17	29.81	29.74	30.25	30.35
StDEV	0.47	0.23	0.05	0.08	0.19	0.05	0.04	0.18	0.48	0.00	0.19
ACTIN	22.03	20.73	20.03	19.35	20.66	21.70	22.09	21.63	20.70	21.72	22.59
ACTIN	22.34	20.60	19.94	19.38	21.03	21.96	22.06	21.79	21.94	22.09	21.80
ACTIN	22.19	20.60	20.08	19.46	21.03	21.84	22.10	21.84	21.12	22.08	22.31
AVG	22.19	20.64	20.02	19.40	20.91	21.83	22.08	21.75	21.25	21.96	22.23
StDEV	0.16	0.08	0.07	0.06	0.21	0.13	0.02	0.11	0.63	0.21	0.40
ΔCt MLCYE	-5.19	-9.64	-9.31	-10.30	-7.72	-8.84	-8.29	-8.65	-7.43	-6.22	-7.71
ΔCt MLCYB	-4.34	-6.41	-7.65	-9.05	-8.61	-7.87	-8.09	-8.06	-8.49	-8.29	-8.12
MLCYE	2.75	0.13	0.16	0.08	0.47	0.22	0.32	0.25	0.58	1.34	0.48
MLCYB	4.95	1.18	0.50	0.19	0.26	0.43	0.37	0.38	0.28	0.32	0.36
SLCYE	2.59	0.10	0.12	0.07	0.44	0.20	0.26	0.23	0.62	1.27	0.39
SLCYE	3.14	0.14	0.18	0.07	0.40	0.20	0.26	0.23	0.54	1.39	0.57
SLCYE	2.55	0.14	0.17	0.11	0.60	0.26	0.48	0.30	0.59	1.37	0.50
StDEV	0.33	0.02	0.03	0.03	0.11	0.03	0.13	0.04	0.04	0.06	0.09
SLCYB	6.92	1.28	0.44	0.18	0.24	0.42	0.40	0.37	0.21	0.33	0.34
SLCYB	4.35	1.03	0.47	0.20	0.29	0.40	0.41	0.45	0.33	0.33	0.41
SLCYB	4.03	1.24	0.59	0.19	0.24	0.46	0.30	0.32	0.31	0.31	0.34
StDEV	1.58	0.14	0.08	0.01	0.03	0.03	0.06	0.06	0.07	0.01	0.04

Endosperm development profiles of MGGPPS2											
	0	10	12	14	16	18	20	22	24	26	28
MGGPPS2	29.17	27.63	25.44	25.70	25.68	26.72	26.58	25.93	26.33	26.13	30.52
MGGPPS2	28.76	27.72	25.35	25.35	25.29	27.20	26.80	26.00	26.25	25.56	30.56
MGGPPS2	28.94	27.61	25.37	25.60	25.47	26.92	26.68	25.94	26.32	27.30	30.18
AVG	28.96	27.65	25.39	25.55	25.48	26.95	26.69	25.96	26.30	26.33	30.42
StDEV	0.21	0.06	0.05	0.18	0.20	0.24	0.11	0.04	0.04	0.89	0.21
ACTIN	25.78	20.70	20.18	20.07	20.87	21.67	22.15	21.47	22.38	20.43	22.84
ACTIN	26.40	20.72	20.27	20.01	20.96	22.01	22.40	21.87	22.53	20.74	22.02
ACTIN	26.06	20.70	20.20	20.00	20.90	21.90	22.26	21.60	22.43	21.05	22.40
AVG	26.08	20.71	20.22	20.03	20.91	21.86	22.27	21.65	22.45	20.74	22.42
StDEV	0.31	0.01	0.05	0.04	0.05	0.17	0.13	0.20	0.08	0.31	0.41
ΔCt MGGPPS2	-2.88	-6.95	-5.17	-5.52	-4.57	-5.09	-4.42	-4.31	-3.85	-5.59	-8.00
MGGPPS2	13.62	0.81	2.78	2.17	4.21	2.94	4.68	5.04	6.92	2.08	0.39
SGGPPS2	11.74	0.82	2.68	1.96	3.67	3.44	5.04	5.14	6.78	2.38	0.36
SGGPPS2	15.60	0.77	2.85	2.50	4.80	2.47	4.33	4.89	7.16	3.54	0.35
SGGPPS2	13.77	0.84	2.81	2.10	4.24	3.00	4.70	5.10	6.82	1.06	0.46
StDEV	2.73	0.04	0.12	0.38	0.80	0.69	0.50	0.17	0.27	0.82	0.01

Spatial Expression profiles of cyclases						Relative to Endosperm					
	Em	En(20)	L	R	O		Em	En(20)	L	R	O
MLCYB	28.53	30.06	21.74	26.43	26.04	NLCYB	0.51	0.00	1.20	1.13	-0.10
MLCYB	28.77	30.00	22.07	26.59	26.71	NLCYB+ΔCt	-6.65	-8.09	0.16	-4.45	-4.44
MLCYB	28.91	30.45	21.96	26.58	26.82	Absolute	0.99	0.37	111.73	4.58	4.61
AVG	28.74	30.17	21.92	26.53	26.52	StDEV1	1.14	0.40	126.58	4.90	6.43
StDEV	0.19	0.24	0.17	0.09	0.47	StDEV2	1.15	0.40	142.41	5.79	4.90
MLCYE	33.50	30.67	23.07	31.93	27.46	StDEV3	0.88	0.30	108.67	4.42	3.74
MLCYE	33.37	30.66	23.20	32.34	27.18	StDEV	0.16	0.05	16.88	0.70	1.35
MLCYE	33.44	29.79	23.00	32.14	27.48	Relative to Endosperm					
AVG	33.44	30.37	23.09	32.14	27.37		Em	En (20)	L	R	O
StDEV	0.07	0.51	0.10	0.21	0.17	NLCYE	0.51	0.00	1.20	1.13	-0.10
ACTIN	21.02	22.09	21.24	20.99	22.03	NLCYE+ΔCt	-11.36	-8.29	-1.01	-10.05	-5.29
ACTIN	21.92	22.06	20.45	20.93	22.34	Absolute	0.04	0.32	49.77	0.09	2.56
ACTIN	21.77	22.10	20.96	20.95	22.19	StDEV1	0.04	0.26	50.35	0.11	2.40
AVG	21.57	22.08	20.88	20.96	22.19	StDEV2	0.04	0.26	46.01	0.08	2.92
StDEV	0.48	0.02	0.56	0.04	0.22	StDEV3	0.04	0.48	52.85	0.09	2.37
ΔCt LCYB	-7.17	-8.09	-1.04	-5.58	-4.34	StDEV	0.00	0.13	3.46	0.01	0.31
ΔCt LCYE	-11.87	-8.29	-2.21	-11.18	-5.19						
LCYB	0.70	0.37	48.63	2.10	4.94						
LCYE	0.03	0.32	21.66	0.04	2.74						
StDEVb	1.14	0.40	126.58	4.90	6.43						
StDEVb	0.97	0.41	100.70	4.39	4.04						
StDEVb	0.88	0.30	108.67	4.42	3.74						
StDEV	0.13	0.06	13.25	0.29	1.47						
StDEVE	0.04	0.26	50.35	0.11	2.40						
StDEVE	0.04	0.26	46.01	0.08	2.92						
StDEVE	0.04	0.48	52.85	0.09	2.37						
StDEV	0.00	0.13	3.46	0.01	0.31						

Spatial expression profiles of t-GGPPS2						Relative to Endosperm					
	Em	En(20)	L	R	O		Em	En (20)	L	R	O
MGGPPS2	29.24	26.58	29.37	26.56	29.19	NGGPPS1	-0.73	0.00	-2.02	-2.95	-3.81
MGGPPS2	30.17	26.80	29.58	26.60	28.74	NGGPPS1+ΔCt	-7.43	-4.42	-7.18	-4.30	-6.69
MGGPPS2	29.68	26.68	29.40	26.55	28.94	Absolute	0.58	4.68	0.69	5.08	0.97
Average	29.70	26.69	29.45	26.57	28.96	StDEV1	0.78	4.94	0.71	5.01	0.81
StDEV	0.47	0.11	0.11	0.03	0.23	StDEV2	0.41	4.24	0.62	4.87	1.10
Actin	22.88	22.15	24.02	24.94	25.78	StDEV3	0.58	4.61	0.16	0.08	0.96
Actin	23.02	22.40	24.38	25.38	26.40	StDEV	0.26	0.49	0.07	0.10	0.21
Actin	23.09	22.26	24.46	25.34	26.06						
Average	23.00	22.27	24.29	25.22	26.08						
StDEV	0.11	0.13	0.23	0.24	0.31						
ΔCt GGPPS2	-6.70	-4.42	-5.16	-1.35	-2.88						
NLMGGPPS2	0.96	4.68	2.79	39.23	13.62						
StDEV1	1.3	5.0	2.9	39.5	11.6						
StDEV2	0.7	4.3	2.6	38.4	15.8						
StDEV3	1.0	4.7	2.9	39.8	13.8						
StDEV	0.3	0.4	0.2	0.7	2.1						

Endosperm developmental profiles of HDR											
	0	10	12	14	16	18	20	22	24	26	28
MHDR	24.91	23.70	23.35	24.29	25.33	28.68	26.59	26.54	26.67	25.74	25.38
MHDR	24.17	23.86	23.32	24.41	25.73	27.45	26.04	26.79	26.86	25.66	25.51
MHDR	23.78	23.78	23.06	25.15	25.15	27.30	26.03	25.86	26.09	25.70	25.46
AVG	24.29	23.78	23.24	24.62	25.40	27.81	26.22	26.40	26.54	25.70	25.45
STDEV	0.57	0.08	0.16	0.47	0.30	0.76	0.32	0.48	0.40	0.04	0.07
ACTIN	25.78	19.83	19.69	20.29	19.77	22.45	21.59	20.98	22.81	21.78	21.65
ACTIN	25.70	19.84	20.18	19.55	19.76	22.57	21.99	22.12	22.21	21.90	21.24
ACTIN	26.38	20.89	20.35	19.66	20.48	22.91	21.99	21.53	22.45	21.81	21.42
AVG	25.95	20.19	20.07	19.83	20.00	22.64	21.86	21.54	22.49	21.83	21.44
STDEV	0.37	0.61	0.34	0.40	0.41	0.24	0.23	0.57	0.30	0.06	0.21
ΔCt HDR	1.67	-3.59	-3.17	-4.78	-5.40	-5.17	-4.36	-4.85	-4.05	-3.87	-4.01
NLHDR	317.48	8.29	11.11	3.63	2.37	2.78	4.86	3.46	6.04	6.84	6.19
SIDEV1	206.10	8.76	10.32	4.55	2.49	1.52	3.76	3.13	5.52	6.65	6.50
SIDEV2	344.22	7.84	10.54	4.19	1.89	3.57	5.50	2.63	4.84	7.03	5.94
SIDEV3	451.06	8.29	12.62	2.51	2.82	3.96	5.54	5.02	8.25	6.84	6.15
SIDEV	122.82	0.46	1.27	1.09	0.47	1.31	1.02	1.26	1.81	0.27	0.28

Endosperm developmental profiles of DXR											
	0	10	12	14	16	18	20	22	24	26	28
MDXR	23.85	25.67	23.47	22.65	23.67	23.79	24.18	25.27	25.53	25.62	25.92
MDXR	24.32	25.39	23.55	22.67	23.65	23.92	23.68	25.11	25.03	25.45	25.65
MDXR	23.96	25.11	23.25	22.41	23.25	23.87	23.20	25.31	25.32	25.53	25.63
AVG	24.04	25.39	23.42	22.58	23.52	23.86	23.69	25.23	25.29	25.53	25.73
STDEV	0.25	0.28	0.16	0.14	0.24	0.07	0.49	0.11	0.25	0.09	0.16
ACTIN	22.47	19.65	19.21	18.80	19.11	19.31	20.19	21.79	22.24	22.40	22.09
ACTIN	22.37	19.59	19.26	18.73	19.12	19.56	20.82	24.91	22.74	23.55	23.13
ACTIN	22.38	19.66	19.82	18.77	19.82	19.91	20.55	21.83	22.56	22.40	22.09
AVG	22.41	19.63	19.43	18.77	19.35	19.59	20.52	22.84	22.51	22.78	22.44
STDEV	0.06	0.04	0.34	0.04	0.41	0.30	0.32	1.79	0.25	0.66	0.60
ΔCt DXR	-1.64	-5.76	-3.99	-3.81	-4.17	-4.27	-3.17	-2.39	-2.78	-2.75	-3.30
NL DXR	32.16	1.85	6.28	7.13	5.54	5.20	11.14	19.12	14.56	14.87	10.18
SIDEV1	36.77	1.52	6.08	6.78	5.01	5.45	7.91	18.60	12.36	14.00	8.94
SIDEV2	26.55	1.85	5.75	6.68	5.08	4.98	11.19	20.78	17.47	15.75	10.78
SIDEV3	34.07	2.25	7.08	8.00	6.70	5.16	15.60	18.09	14.29	14.90	10.93
SIDEV	5.30	0.36	0.69	0.74	0.96	0.24	3.86	1.43	2.58	0.88	1.11

Spatial expression profiles of I HDR						Relative to Endosperm					
	Em	En (20)	L	R	O		Em	En(20)	L	R	O
MHDR	24.66	26.59	22.15	25.35	24.91	NHDR	-0.80	0.00	-2.43	-3.36	-4.10
MHDR	24.98	26.04	21.85	25.27	24.17	NHDR+ΔCt	-2.54	-4.36	-0.16	-3.61	-2.76
MHDR	24.57	26.03	22.04	25.78	24.78	Absolute	17.19	4.86	89.71	8.19	14.73
Average	24.74	26.22	22.01	25.47	24.62	SIDEV1	18.17	3.77	81.79	11.27	15.28
SIDEV	0.22	0.32	0.15	0.27	0.40	SIDEV2	14.56	5.52	100.70	11.91	25.53
Actin	22.88	21.59	24.02	24.94	25.78	SIDEV3	19.34	5.56	111.73	8.36	16.72
Actin	23.02	21.99	24.38	25.38	25.70	SIDEV	2.49	1.02	15.14	1.89	5.54
Actin	23.09	21.99	24.46	25.34	26.38						
Average	23.00	21.86	24.29	25.22	25.95						
SIDEV	0.11	0.23	0.23	0.24	0.37						
ΔCt HDR	-1.74	-4.36	2.27	-0.25	1.33						
NLHDR	29.94	4.86	483.44	84.28	251.98						
SIDEV1	31.6	3.8	439.7	91.4	206.1						
SIDEV2	25.3	5.5	541.4	96.6	344.2						
SIDEV3	33.6	5.5	474.6	67.8	225.5						
SIDEV	4.3	1.0	51.7	15.3	74.8						

Spatial expression profiles of II DXR						Normalizing to Endosperm					
	Em	En(20)	L	R	O		Em	En(20)	L	R	O
MDXR	24.58	24.18	22.16	25.45	23.85	NDXR	-1.72	0.00	-4.65	-2.73	-1.89
MDXR	24.68	23.68	22.40	25.09	24.32	NDXR+ΔCt	-4.27	-3.17	-1.62	-4.62	-3.52
MDXR	25.11	23.20	21.87	24.88	23.96	Absolute	5.18	11.13	32.45	4.06	8.69
Average	24.79	23.69	22.14	25.14	24.04	SIDEV1	5.99	7.91	32.08	3.28	9.94
SIDEV	0.28	0.49	0.27	0.29	0.25	SIDEV2	5.59	11.18	27.16	4.21	7.17
Actin	22.11	20.19	25.25	23.67	22.47	SIDEV3	4.15	15.60	39.20	4.86	9.21
Actin	22.33	20.82	25.72	22.94	22.37	SIDEV	0.96	3.80	6.06	0.79	1.43
Actin	22.28	20.55	24.54	23.14	22.38						
Average	22.24	20.52	25.17	23.25	22.41						
SIDEV	0.12	0.32	0.59	0.38	0.06						
ΔCt DXR	-2.55	-3.17	3.03	-1.89	-1.64						
NL DXR	17.08	11.14	814.92	26.98	32.16						
SIDEV1	0.2	7.9	8.1	0.2	36.8						
SIDEV2	0.2	11.2	6.8	0.3	26.5						
SIDEV3	0.1	15.6	9.8	0.3	34.1						
SIDEV	0.0	3.9	1.5	0.1	5.3						

Endosperm developmental profiles of DXS gene family												
	0	10	12	14	16	18	20	22	24	26	28	
MDXS1	25.11	29.20	28.70	27.83	29.12	28.30	28.73	28.19	28.14	28.20	28.35	
MDXS1	25.14	29.23	27.70	27.72	28.67	28.00	28.60	28.51	27.48	28.11	28.93	
MDXS1	25.11	29.18	28.27	27.76	28.80	28.01	28.66	28.30	27.75	28.23	28.65	
AVG	25.12	29.20	28.22	27.77	28.86	28.10	28.66	28.33	27.79	28.18	28.64	
SiDEV	0.02	0.03	0.50	0.06	0.23	0.17	0.07	0.16	0.33	0.06	0.29	
MDXS3	23.53	27.08	26.20	25.80	26.96	27.57	27.29	27.05	27.63	27.34	27.12	
MDXS3	23.44	27.24	26.03	25.76	26.80	26.85	27.64	26.89	26.84	27.44	26.85	
MDXS3	23.51	27.14	26.08	25.77	26.87	27.25	27.40	26.95	27.20	27.35	27.00	
AVG	23.49	27.15	26.10	25.78	26.88	27.22	27.44	26.96	27.22	27.38	26.99	
SiDEV	0.05	0.08	0.09	0.02	0.08	0.36	0.18	0.08	0.40	0.06	0.14	
ACTIN	22.52	20.66	19.85	19.60	21.20	21.02	21.61	20.97	22.41	22.85	22.24	
ACTIN	22.65	20.90	20.69	19.96	20.90	21.53	22.05	21.73	22.79	23.13	23.09	
ACTIN	22.57	20.85	20.25	19.82	21.00	21.35	21.80	21.36	22.57	23.00	22.60	
AVG	22.58	20.80	20.26	19.79	21.03	21.30	21.82	21.35	22.59	22.99	22.64	
SiDEV	0.07	0.13	0.42	0.18	0.15	0.26	0.22	0.38	0.19	0.14	0.43	
ΔCt MDXS1	-2.54	-8.40	-7.96	-7.98	-7.83	-6.80	-6.84	-6.98	-5.20	-5.19	-6.00	
ΔCt MDXS3	-0.91	-6.35	-5.84	-5.98	-5.84	-5.92	-5.62	-5.61	-4.63	-4.38	-4.35	
MDXS1	17.27	0.30	0.40	0.40	0.44	0.90	0.87	0.79	2.73	2.75	1.56	
MDXS3	53.10	1.23	1.75	1.58	1.74	1.65	2.03	2.05	3.94	4.79	4.92	
SDXS1	0.17	0.29	0.29	0.38	0.37	0.77	0.01	0.88	2.15	2.68	1.92	
SDXS1	0.17	0.30	0.56	0.42	0.52	1.04	0.01	0.71	3.47	2.82	1.27	
SDXS1	0.17	0.30	0.39	0.40	0.46	0.96	0.87	0.81	2.80	2.65	1.56	
SiDEV	0.00	0.00	0.19	0.03	0.11	0.18	0.00	0.12	0.62	0.09	0.46	
SDXS3	0.51	1.30	1.63	1.54	1.64	1.28	0.02	1.94	3.08	4.97	4.51	
SDXS3	0.55	1.16	1.87	1.62	1.85	2.11	0.02	2.16	5.29	4.61	5.37	
SDXS3	0.52	1.24	1.77	1.59	1.75	1.62	2.09	2.07	4.09	4.88	4.90	
SiDEV	0.03	0.10	0.17	0.05	0.15	0.59	0.00	0.16	0.93	0.26	0.60	
MDXS2	30.91	28.65	27.72	28.13	29.60	30.64	30.60	30.27	30.15	29.37	30.36	
MDXS2	30.56	28.96	28.86	28.22	29.78	30.91	30.06	30.73	30.83	29.79	30.55	
MDXS2	30.12	28.54	28.00	28.05	29.56	30.76	30.50	30.08	30.00	29.69	30.24	
AVG	30.53	28.72	28.19	28.13	29.65	30.77	30.39	30.36	30.33	29.62	30.38	
SiDEV	0.40	0.22	0.59	0.09	0.12	0.14	0.29	0.33	0.44	0.22	0.16	
ACTIN	22.03	20.73	20.03	19.35	20.66	21.70	21.70	21.48	20.70	21.76	22.23	
ACTIN	22.34	20.60	19.94	19.38	21.03	21.96	21.70	21.79	21.94	21.83	21.97	
ACTIN	22.56	20.47	20.00	19.46	21.00	21.96	21.72	21.36	21.00	21.50	22.00	
AVG	22.31	20.60	19.99	19.40	20.90	21.87	21.71	21.54	21.21	21.70	22.07	
SiDEV	0.27	0.13	0.05	0.06	0.21	0.15	0.01	0.22	0.65	0.17	0.14	
ΔCt MDXS2	-8.22	-8.12	-8.20	-8.74	-8.75	-8.90	-8.68	-8.82	-9.11	-7.92	-8.32	
MDXS2	0.34	0.36	0.34	0.23	0.23	0.21	0.24	0.22	0.18	0.41	0.31	
SDXS2	0.00	0.38	0.47	0.23	0.24	0.23	0.21	0.24	0.20	0.49	0.32	
SDXS2	0.00	0.30	0.21	0.22	0.21	0.19	0.31	0.17	0.13	0.37	0.28	
SDXS2	0.00	0.41	0.39	0.25	0.25	0.21	0.23	0.27	0.23	0.39	0.35	
SiDEV	0.00	0.05	0.13	0.01	0.02	0.02	0.05	0.05	0.05	0.07	0.03	

Spatial expression profiles of DXS						Normalizing to Endosperm					
	Em	En(20)	L	R	O		Em	En(20)	L	R	O
MDXS1	26.16	28.73	22.82	26.70	25.11	NDXS1	1.00	0.00	-3.04	-1.24	-0.76
MDXS1	25.41	28.60	22.16	26.01	25.12	NDXS1+ΔCt	-4.03	-6.84	-0.65	-4.39	-3.30
MDXS1	26.00	28.66	22.44	25.93	25.13	Absolute	6.12	0.87	63.88	4.78	10.15
Average	25.86	28.66	22.47	26.21	25.12	SiDEV1	4.97	0.84	50.35	3.42	10.29
SiDEV	0.40	0.07	0.33	0.42	0.01	SiDEV2	8.36	0.92	79.55	5.52	10.22
MDXS3	26.17	27.29	25.77	25.29	23.53	SiDEV3	5.56	0.88	65.52	5.83	10.15
MDXS3	26.05	27.64	25.08	24.77	23.45	SiDEV	1.81	0.04	14.61	1.31	0.07
MDXS3	26.02	27.40	25.02	26.20	23.50	NDXS3	1.00	0.00	-3.04	-1.24	-0.76
Average	26.08	27.44	25.29	25.42	23.49	NDXS3+ΔCt	-4.25	-5.62	-3.46	-3.59	-1.67
SiDev	0.08	0.18	0.42	0.72	0.04	Absolute	5.24	2.03	9.07	8.29	31.35
Actin	20.97	21.61	24.72	22.80	22.50	SiDEV1	4.94	2.27	6.52	9.09	30.78
Actin	20.68	22.05	24.83	23.09	22.65	SiDEV2	5.37	1.78	10.51	13.03	32.53
Actin	20.83	21.80	25.05	23.31	22.59	SiDEV3	5.48	2.11	10.96	4.84	31.43
Average	20.83	21.82	24.87	23.07	22.58	SiDEV	0.29	0.25	2.45	4.10	0.89
SiDEV	0.15	0.22	0.17	0.26	0.08	NDXS2	0.88	0.00	-3.16	-1.36	-0.60
ΔCt DXS1	-5.03	-6.84	2.39	-3.15	-2.54	NDXS2+ΔCt	-5.07	-8.68	-3.18	-5.48	-8.82
NLXS1	3.06	0.87	525.37	11.29	17.19	Absolute	2.97	0.24	11.06	2.25	0.22
SiDEV1	2.5	0.8	413.2	8.1	17.3	SiDEV1	3.00	0.21	11.83	1.88	0.17
SiDEV2	4.2	0.9	652.8	13.0	17.2	SiDEV2	2.57	0.31	11.27	2.65	0.22
SiDEV3	2.8	0.9	537.6	13.7	17.1	SiDEV3	3.42	0.23	10.22	2.29	0.29
SiDEV	0.9	0.0	119.9	3.1	0.1	SiDEV	0.42	0.05	0.81	0.38	0.06
ΔCt DXS3	-5.25	-5.62	-0.42	-2.35	-0.91						
NLXS3	2.62	2.03	74.57	19.57	53.10						
SiDEV1	2.5	2.3	53.5	21.4	51.8						
SiDEV2	2.7	1.8	86.3	30.7	54.7						
SiDEV3	2.7	2.1	89.9	11.4	52.9						
SiDEV	0.1	0.2	20.1	9.7	1.5						
MDXS2	26.77	30.60	24.79	27.44	30.91						
MDXS2	26.99	30.06	24.86	26.95	30.56						
MDXS2	26.58	30.50	25.00	27.16	30.12						
Average	26.78	30.39	24.88	27.183	30.53						
SiDEV	0.21	0.29	0.11	0.25	0.40						
Actin	20.97	21.70	24.72	22.80	22.03						
Actin	20.68	21.70	24.83	23.09	22.34						
Actin	20.83	21.72	25.05	23.31	22.56						
Average	20.83	21.71	24.87	23.07	22.31						
SiDEV	0.15	0.01	0.17	0.26	0.27						
ΔCt DXS2	-5.95	-8.68	-0.02	-4.12	-8.22						
NLXS2	1.61	0.24	98.85	5.76	0.34						
SiDEV1	1.6	0.2	105.5	4.8	0.3						
SiDEV2	1.4	0.3	100.5	6.8	0.3						
SiDEV3	1.9	0.2	91.2	5.9	0.4						
SiDEV	0.2	0.1	7.2	1.0	0.1						

Endosperm development profiles of Zeaxanthin epoxidase 1											
	0	10	12	14	16	18	20	22	24	26	28
MZEP1	26.34	29.58	28.54	29.71	30.38	31.04	29.64	29.19	29.76	29.8	29.89
MZEP1	25.73	29.67	28.53	29.8	29.57	30.99	29.39	28.88	30.46	29.86	29.94
MZEP1	26.86	29.63	28.8	30.46	30.12	30.77	29.41	29.04	29.86	30.79	29.39
AVG	26.31	29.63	28.62	29.99	30.02	30.93	29.48	29.04	30.03	30.15	29.74
SiDEV	0.57	0.06	0.15	0.41	0.41	0.14	0.14	0.16	0.38	0.56	0.30
ACTIN	25.39	19.88	19.96	20.16	20.58	22.94	21.82	20.24	20.94	20.15	20.52
ACTIN	24.98	19.80	19.70	20.89	21.30	21.40	21.82	20.49	21.17	20.06	20.67
ACTIN	25.70	19.71	19.29	19.79	20.05	21.13	21.22	20.32	21.00	20.05	20.58
AVG	25.36	19.80	19.65	20.28	20.64	21.82	21.62	20.35	21.04	20.09	20.59
SiDEV	0.36	0.09	0.34	0.56	0.63	0.98	0.35	0.13	0.12	0.06	0.08
ΔCt MZEP1	-0.95	-9.83	-8.97	-9.71	-9.38	-9.11	-7.86	-8.69	-8.99	-10.06	-9.15
MZEP1	51.64	0.11	0.20	0.12	0.15	0.18	0.43	0.24	0.20	0.09	0.18
SZEP1	50.58	0.11	0.21	0.14	0.12	0.17	0.39	0.22	0.24	0.12	0.16
SZEP1	77.20	0.11	0.21	0.14	0.21	0.17	0.46	0.27	0.15	0.11	0.15
SZEP1	35.27	0.11	0.18	0.09	0.14	0.20	0.45	0.24	0.22	0.06	0.22
SiDEV	21.22	0.00	0.02	0.03	0.05	0.02	0.04	0.04	0.05	0.03	0.00

Endosperm development profiles of Zeaxanthin epoxidase 2											
	0	10	12	14	16	18	20	22	24	26	28
MZEP2	25.17	26.69	26	25.68	26.5	27.39	27.4	26.73	27.25	26.46	27.26
MZEP2	25.51	26.68	26.59	25.87	26.57	27.45	27.88	26.59	27.31	26.88	27.66
MZEP2	25.67	27.6	26.26	25.89	26.18	27.54	27.59	26.65	27.86	27.1	27.46
AVG	25.45	26.99	26.28	25.81	26.42	27.46	27.62	26.66	27.47	26.81	27.46
SiDEV	0.26	0.53	0.30	0.12	0.21	0.08	0.24	0.07	0.34	0.33	0.20
ACTIN	25.39	19.88	19.96	20.16	20.58	22.94	21.82	20.24	20.94	20.15	20.52
ACTIN	24.98	19.80	19.70	20.89	21.30	21.40	21.82	20.49	21.17	20.06	20.67
ACTIN	25.70	19.71	19.29	19.79	20.05	21.13	21.22	20.32	21.00	20.05	20.58
AVG	25.36	19.80	19.65	20.28	20.64	21.82	21.62	20.35	21.04	20.09	20.59
SiDEV	0.36	0.09	0.34	0.56	0.63	0.98	0.35	0.13	0.12	0.06	0.08
ΔCt MZEP2	-0.09	-7.19	-6.63	-5.53	-5.77	-5.64	-6.00	-6.31	-6.44	-6.73	-6.87
MZEP2	93.74	0.68	1.01	2.16	1.83	2.01	1.56	1.26	1.15	0.94	0.85
SZEP1	113.81	0.84	1.23	2.37	1.73	2.11	1.82	1.20	1.35	1.21	0.98
SZEP1	89.92	0.85	0.81	2.08	1.64	2.02	1.30	1.32	1.29	0.90	0.74
SZEP1	80.48	0.45	1.02	2.05	2.15	1.90	1.60	1.27	0.88	0.77	0.85
SiDEV	17.18	0.23	0.21	0.18	0.27	0.10	0.26	0.06	0.25	0.22	0.17

Spatial expression profiles of ZEP1						Relative to Endosperm					
	Em	En (20)	L	R	O		Em	En (20)	L	R	O
MZEP1	28.07	29.64	23.55	25.61	26.34	NZEP1	-1.93	0.00	-3.44	-1.90	-3.84
MZEP1	28.23	29.39	23.10	26.10	25.73	NZEP1+ΔCt	-6.62	-7.96	-1.73	-4.09	-4.79
MZEP1	28.13	29.41	23.11	25.12	26.86	Absolute	1.01	0.40	30.08	5.87	3.61
Average	28.14	29.48	23.25	25.61	26.31	SiDEV1	1.07	0.36	24.49	5.87	3.54
SiDEV	0.08	0.14	0.26	0.49	0.57	SiDEV2	0.96	0.43	33.45	4.18	5.40
Actin	23.32	21.52	25.01	23.64	25.39	SiDEV3	1.02	0.42	33.22	8.25	2.47
Actin	23.52	21.82	25.02	23.24	24.98	SiDEV	0.06	0.04	5.11	2.04	1.48
Actin	23.50	21.22	24.86	23.38	25.70						
Average	23.45	21.52	24.96	23.42	25.36						
SiDEV	0.11	0.30	0.09	0.20	0.36						
ΔCt ZEP1	-4.70	-7.96	1.71	-2.19	-0.95						
NLZEP1	3.86	0.40	327.16	21.92	51.64						
SiDEV1	4.1	0.36	266.4	21.9	50.58						
SiDEV2	3.6	0.43	363.8	15.6	77.20						
SiDEV3	3.9	0.42	361.3	30.8	35.27						
SiDEV	0.2	0.04	55.6	7.6	21.22						

Spatial expression profiles of ZEP2						Relative to Endosperm					
	Em	En (20)	L	R	O		Em	En (20)	L	R	O
MZEP2	28.65	27.4	21.94	25.53	25.17	NZEP2	-1.93	0.00	-3.44	-1.90	-3.84
MZEP2	28.79	27.88	21.75	25.50	25.51	NZEP2+ΔCt	-7.23	-6.10	-0.32	-4.06	-3.93
MZEP2	28.81	27.59	21.84	25.71	25.67	Absolute	0.67	1.45	79.92	6.00	6.56
Average	28.75	27.62	21.84	25.58	25.45	SiDEV1	0.71	1.70	74.74	6.21	7.97
SiDEV	0.09	0.24	0.10	0.11	0.26	SiDEV2	0.65	1.22	85.26	6.34	6.29
Actin	23.32	21.52	25.01	23.64	25.39	SiDEV3	0.64	1.49	80.11	5.48	5.63
Actin	23.52	21.82	25.02	23.24	24.98	SiDEV	0.04	0.24	5.26	0.46	1.20
Actin	23.50	21.22	24.86	23.38	25.70						
Average	23.45	21.52	24.96	23.42	25.36						
SiDEV	0.11	0.30	0.09	0.20	0.36						
ΔCt ZEP2	-5.30	-6.10	3.12	-2.16	-0.09						
NLZEP2	2.53	1.45	869.39	22.38	93.74						
SiDEV1	2.7	1.70	813.0	23.2	113.81						
SiDEV2	2.5	1.22	927.5	23.7	89.92						
SiDEV3	2.4	1.49	871.4	20.4	80.48						
SiDEV	0.2	0.24	57.2	1.7	17.18						

Endosperm developmental profiles of IPPI1											
	0	10	12	14	16	18	20	22	24	26	28
MIPPI	19.98	19.71	19.01	19.01	19.20	18.86	20.41	20.74	20.97	21.65	21.66
MIPPI	20.34	18.94	18.86	18.80	19.41	18.76	20.19	20.66	21.00	21.66	21.82
MIPPI	20.24	18.78	18.95	18.95	19.29	18.98	20.02	20.64	20.98	21.66	21.81
AVG	20.19	19.14	18.94	18.92	19.30	18.87	20.21	20.68	20.98	21.66	21.76
STDEV	0.19	0.50	0.08	0.11	0.11	0.11	0.20	0.05	0.02	0.01	0.09
ACTIN	22.47	19.51	18.56	19.03	19.69	20.04	21.04	21.79	22.24	22.40	22.09
ACTIN	22.37	19.20	18.70	18.71	19.71	19.97	20.92	21.91	22.74	23.55	23.13
ACTIN	22.38	19.59	19.11	19.12	20.20	20.21	21.09	21.83	22.56	22.40	22.09
AVG	22.41	19.43	18.79	18.95	19.87	20.07	21.02	21.84	22.51	22.78	22.44
STDEV	0.06	0.21	0.29	0.22	0.29	0.12	0.09	0.06	0.25	0.66	0.60
ΔCt IPPI	2.22	0.29	-0.15	0.03	0.57	1.21	0.81	1.16	1.53	1.13	0.67
NLIPPI	465.89	122.26	90.13	102.34	148.11	230.80	175.32	223.97	288.79	218.35	159.48
STDEV1	537.6	82.5	85.9	96.1	158.7	231.9	152.3	214.9	291.5	219.4	171.3
STDEV2	418.9	140.8	95.3	111.2	137.2	248.5	177.4	227.1	285.5	217.8	153.3
STDEV3	449.0	157.3	89.5	100.2	149.1	213.4	199.5	230.3	289.5	217.8	154.4
SIDEV	61.7	39.3	4.7	7.8	10.8	17.6	23.6	8.1	3.1	0.9	10.1

Spatial expression profiles of IPPI1						Normalizing to Endosperm					
	Em	En (20)	L	R	O		Em	En(20)	L	R	O
MIPPI	21.52	20.41	24.89	23.90	19.98	NIPPI	-1.36	0.00	-4.15	-2.23	-1.39
MIPPI	21.65	20.19	24.22	23.88	20.34	NIPPI+ΔCt	-0.64	0.81	-3.48	-2.72	0.83
MIPPI	21.80	20.02	24.37	23.44	20.24	Absolute	64.17	175.32	8.98	15.14	177.77
Average	21.66	20.21	24.49	23.74	20.19	SIDEV1	70.71	152.62	6.83	13.50	205.62
STDEV	0.14	0.20	0.35	0.26	0.19	SIDEV2	64.61	177.77	10.80	13.70	160.21
Actin	22.34	21.04	25.25	23.67	22.47	SIDEV3	58.20	200.00	9.80	18.60	171.71
Actin	22.31	20.92	25.72	22.94	22.37	SIDEV	6.23	23.70	2.09	2.89	23.60
Actin	22.47	21.09	24.54	23.14	22.38						
Average	22.37	21.02	25.17	23.25	22.41						
STDEV	0.09	0.09	0.59	0.38	0.06						
ΔCt IPPI	0.72	0.81	0.68	-0.49	2.22						
NLIPPI	164.34	175.32	159.84	71.20	465.89						
STDEV1	1.8	152.3	1.2	0.6	537.6						
STDEV2	1.7	177.4	1.9	0.6	418.9						
STDEV3	1.5	199.5	1.7	0.9	449.0						
SIDEV	0.2	23.6	0.4	0.1	61.7						

Endosperm developmental profiles of IPPI gene family											
	0	10	12	14	16	18	20	22	24	26	28
MIPPI2	21.67	22.18	21.62	22.68	22.80	23.87	23.60	23.78	24.74	23.13	23.81
MIPPI2	22.06	22.18	21.86	20.62	22.47	21.38	23.22	23.44	24.56	24.32	24.65
MIPPI2	21.83	22.42	22.22	21.90	23.14	22.83	23.52	23.59	24.72	23.15	23.71
AVG	21.85	22.26	21.90	21.73	22.80	22.69	23.45	23.60	24.67	23.53	24.06
STDEV	0.20	0.14	0.30	1.04	0.33	1.25	0.20	0.17	0.10	0.68	0.52
MIPPI3	21.56	21.60	21.01	21.86	22.89	23.38	23.96	24.78	24.92	24.56	27.08
MIPPI3	20.95	22.70	22.19	22.02	24.51	22.63	24.67	25.87	25.02	25.08	26.37
MIPPI3	21.21	22.39	22.08	22.19	24.20	23.21	24.43	25.30	25.04	24.24	26.20
AVG	21.24	22.23	21.76	22.02	23.87	23.07	24.35	25.32	24.99	24.63	26.55
STDEV	0.31	0.57	0.65	0.17	0.86	0.39	0.36	0.55	0.06	0.42	0.47
ACTIN	22.47	19.51	18.56	19.03	19.69	20.04	21.04	21.79	22.24	22.40	22.09
ACTIN	22.37	19.20	18.70	18.71	19.71	19.97	20.92	21.91	22.74	23.55	23.13
ACTIN	22.38	19.59	19.11	19.12	20.20	20.21	21.09	21.83	22.56	22.40	22.09
AVG	22.41	19.43	18.79	18.95	19.87	20.07	21.02	21.84	22.51	22.78	22.44
STDEV	0.06	0.21	0.29	0.22	0.29	0.12	0.09	0.06	0.25	0.66	0.60
ΔCt MIPPI2	0.55	-2.83	-3.11	-2.78	-2.94	-2.62	-2.43	-1.76	-2.16	-0.75	-1.62
ΔCt MIPPI3	1.17	-2.80	-2.97	-3.07	-4.00	-3.00	-3.34	-3.47	-2.48	-1.84	-4.11
MIPPI2	146.92	14.11	11.58	14.56	13.08	16.27	18.56	29.52	22.38	59.46	32.53
MIPPI3	224.49	14.39	12.76	11.91	6.25	12.50	9.90	9.00	17.92	27.87	5.78
SIPPI2	0.17	0.29	0.29	0.38	0.37	0.77	0.01	0.88	2.15	2.68	1.92
SIPPI2	0.17	0.30	0.56	0.42	0.52	1.04	0.01	0.71	3.47	2.82	1.27
SIPPI2	1.50	12.66	9.28	12.97	10.38	14.80	17.64	29.80	21.66	77.56	41.37
SIDEV	0.00	0.00	0.19	0.03	0.11	0.18	0.00	0.12	0.62	0.09	0.46
SIPPI3	0.51	1.30	1.63	1.54	1.64	1.28	0.02	1.94	3.08	4.97	4.51
SIPPI3	0.55	1.16	1.87	1.62	1.85	2.11	0.02	2.16	5.29	4.61	5.37
SIPPI3	2.29	12.88	10.22	10.61	4.96	11.37	9.39	9.11	17.35	36.43	7.36
SIDEV	0.03	0.10	0.17	0.05	0.15	0.59	0.00	0.16	0.93	0.26	0.60

Spatial Expression profiles of IPPI						Relative to Endosperm					
	Em	En(20)	L	R	O		Em	En(20)	L	R	O
MIPPI2	23.76	23.60	23.14	24.67	23.45	NIPPI2	-0.32	0.00	-2.54	-2.91	-2.83
MIPPI2	23.69	23.22	23.3	24.69	23.81	NIPPI2+ΔCt	-2.37	-2.43	-1.86	-3.32	-2.27
MIPPI2	23.725	23.52	23.22	24.68	23.63	Absolute	19.41	18.56	27.55	10.01	20.73
AVG	23.73	23.45	23.22	24.68	23.63	SIDEV1	31.21	34.87	47.96	16.61	38.69
STDEV	0.04	0.20	0.08	0.01	0.18	SIDEV2	41.90	48.30	59.46	21.61	44.75
MIPPI3	23.64	23.96	23.79	23.67	23.34	SIDEV3	31.97	36.86	45.38	16.49	34.15
MIPPI3	23.75	24.67	23.48	23.87	22.7	SIDEV	5.96	7.25	7.50	2.92	5.32
MIPPI3	23.70	24.43	23.64	23.77	23.02						
AVG	23.70	24.35	23.64	23.77	23.02	Relative to Endosperm					
STDEV	0.05	0.36	0.15	0.10	0.32						
ACTIN	21.01	21.04	23.10	23.66	24.25	NIPPI3	-0.32	0.00	-2.54	-2.91	-2.83
ACTIN	22.36	20.92	24.71	24.89	24.14	NIPPI3+ΔCt	-2.34	-3.34	-2.28	-2.41	-1.66
ACTIN	21.69	21.09	23.88	24.25	24.17	Absolute	19.82	9.90	20.66	18.82	31.64
AVG	21.69	21.02	23.90	24.27	24.19	SIDEV1	33.92	27.17	30.57	33.22	41.75
STDEV	0.09	0.09	0.59	0.38	0.06	SIDEV2	31.43	16.61	37.89	28.92	65.07
ΔCt IPPI2	-2.04	-2.43	0.68	-0.41	0.56	SIDEV3	32.65	19.61	34.03	30.99	52.12
ΔCt IPPI3	-2.01	-3.34	0.26	0.50	1.17	SIDEV	1.24	5.44	3.67	2.15	11.68
IPPI2	24.32	18.56	159.84	75.09	147.09						
IPPI3	24.83	9.90	119.89	141.09	224.49						
STDEV2	23.73	16.69	168.96	75.61	166.63						
STDEV2	24.91	21.71	151.22	74.57	129.83						
STDEV2	24.32	17.64	159.84	75.09	147.09						
STDEV	0.59	2.67	8.87	0.52	18.41						
STDEV3	25.79	13.00	107.67	151.22	179.83						
STDEV3	23.90	7.95	133.48	131.65	280.24						
STDEV3	24.83	9.39	119.89	141.09	224.49						
STDEV	0.95	2.60	12.91	9.79	50.31						

Endosperm developmental profiles of HDS											
	0	10	12	14	16	18	20	22	24	26	28
MHDS	26.73	25.75	25.57	26.17	25.99	28.31	27.01	27.31	26.03	25.48	28.57
MHDS	26.30	25.76	25.80	25.66	26.12	28.07	27.18	27.17	26.70	25.41	28.54
MHDS	26.83	26.08	25.42	25.74	25.92	28.51	27.00	27.76	26.84	24.81	28.65
AVG	26.62	25.86	25.60	25.86	26.01	28.30	27.06	27.41	26.52	25.23	28.59
STDEV	0.28	0.19	0.19	0.27	0.10	0.22	0.10	0.31	0.43	0.37	0.06
ACTIN	25.39	19.88	19.96	20.16	20.58	22.94	21.32	22.55	23.03	22.23	22.89
ACTIN	24.98	19.80	19.70	20.89	21.30	21.40	21.82	22.98	22.68	21.94	23.57
ACTIN	25.70	19.71	19.29	19.79	20.05	21.13	21.22	21.52	22.86	21.37	23.23
AVG	25.36	19.80	19.65	20.28	20.64	21.82	21.45	22.35	22.86	21.85	23.23
STDEV	0.36	0.09	0.34	0.56	0.63	0.98	0.32	0.75	0.18	0.44	0.34
ΔCt HDS	-1.26	-6.07	-5.95	-5.58	-5.37	-6.47	-5.61	-5.06	-3.67	-3.39	-5.36
NLHDS	41.66	1.49	1.62	2.10	2.42	1.13	2.05	2.99	7.87	9.56	2.44
SIDEV1	38.60	1.61	1.65	1.69	2.46	1.12	2.12	3.21	11.08	8.06	2.47
SIDEV2	52.00	1.60	1.41	2.40	2.25	1.32	1.89	3.54	6.97	8.46	2.52
SIDEV3	36.01	1.28	1.83	2.27	2.58	0.97	2.14	2.35	6.32	12.82	2.34
SIDEV	8.58	0.19	0.21	0.38	0.17	0.17	0.14	0.61	2.58	2.64	0.10

Spatial expression profiles of HDS						Normalizing to Endosperm					
	Em	En(20)	L	R	O		Em	En(20)	L	R	O
MHDS	27.82	27.01	23.55	28.25	26.73	NHDS	-1.55	0.00	-2.84	-3.77	-3.91
MHDS	27.94	27.18	23.42	27.93	26.30	NHDS+ΔCt	-6.26	-5.61	-2.04	-6.61	-5.17
MHDS	27.36	27.00	23.49	28.00	26.83	Absolute	1.30	2.05	24.32	1.02	2.77
Average	27.71	27.06	23.49	28.06	26.62	SIDEV1	1.21	2.12	23.33	0.90	2.57
SIDEV	0.31	0.10	0.07	0.17	0.28	SIDEV2	1.11	1.88	25.53	1.12	3.47
Actin	22.88	21.32	24.02	24.94	25.39	SIDEV3	1.66	2.13	24.32	1.07	2.40
Actin	23.02	21.82	24.38	25.38	24.98	SIDEV	0.29	0.14	1.10	0.12	0.57
Actin	23.09	21.22	24.46	25.34	25.70						
Average	23.00	21.45	24.29	25.22	25.36						
SiDEV	0.11	0.32	0.23	0.24	0.36						
ΔCt HDS	-4.71	-5.61	0.80	-2.84	-1.26						
NLHDS	3.82	2.05	174.11	13.97	41.66						
SIDEV1	3.5	2.1	166.6	12.2	38.6						
SIDEV2	3.3	1.9	182.3	15.3	52.0						
SIDEV3	4.9	2.1	173.7	14.6	36.0						
SIDEV	0.9	0.1	7.9	1.6	8.6						

Chapter 2: Correlation analysis (Inbreds)

20 DAP	C131A	B37	A619	KUI2007	TZ118	DE3	C17	NC300	SD44	B73
MDXS1	27.88	27.83	28.79	29.13	32.14	30.79	30.77	26.29	29.21	28.73
MDXS1	27.47	27.32	28.73	30.52	32.78	30.77	29.24	26.66	28.91	28.59
MDXS1	27.66	27.56	28.74	29.81	32.47	30.79	30.02	26.49	29.07	28.67
AVG	27.67	27.57	28.75	29.82	32.46	30.78	30.01	26.48	29.06	28.66
SiDEV	0.21	0.26	0.03	0.70	0.32	0.01	0.77	0.19	0.15	0.07
ACTIN	19.63	19.71	21.17	20.34	20.78	19.66	19.70	18.01	20.60	21.61
ACTIN	20.01	20.68	21.07	20.99	21.67	21.31	21.14	18.56	20.39	22.02
ACTIN	19.80	20.21	21.10	20.65	21.24	20.50	20.43	18.30	20.51	21.83
AVG	19.81	20.20	21.11	20.66	21.23	20.49	20.42	18.29	20.50	21.82
SiDEV	0.19	0.49	0.05	0.33	0.45	0.83	0.72	0.28	0.11	0.21
ΔCt MDXS1	-7.86	-7.37	-7.64	-9.16	-11.23	-10.29	-9.59	-8.19	-8.56	-6.84
DXS1	0.43	0.60	0.50	0.17	0.04	0.08	0.13	0.34	0.26	0.87
SiDEV1	0.37	0.50	0.49	0.28	0.05	0.08	0.08	0.39	0.24	0.83
SiDev2	0.50	0.72	0.51	0.11	0.03	0.08	0.22	0.30	0.29	0.92
SiDEV3	0.43	0.61	0.51	0.18	0.04	0.08	0.13	0.34	0.26	0.87
SiDEV	0.06	0.11	0.01	0.09	0.01	0.00	0.07	0.04	0.03	0.04
15 DAP	TZ118	KUI2007	B37	C17	NC300	SD44	DE3	A619	C131A	B73
MDXS1	33.21	29.07	28.34	30.07	27.68	32.30	29.17	29.53	32.93	29.12
MDXS1	33.30	28.73	28.77	30.13	27.47	31.77	27.58	29.51	28.85	28.62
MDXS1	33.26	28.96	28.68	30.12	27.56	31.96	28.36	29.53	30.9	28.85
AVG	33.26	28.92	28.60	30.11	27.57	32.01	28.37	29.52	30.89	28.86
SiDEV	0.04	0.14	0.19	0.03	0.09	0.22	0.65	0.01	1.67	0.25
ACTIN	18.12	19.44	18.91	19.27	18.70	21.39	18.88	19.80	20.52	21.20
ACTIN	19.05	19.56	20.21	19.92	18.70	22.55	21.12	20.41	23.44	20.86
ACTIN	19.07	19.00	20.01	19.54	18.97	22.42	20.01	20.12	21.96	21.04
AVG	18.75	19.33	19.71	19.58	18.79	22.12	20.00	20.11	21.97	21.03
SiDEV	0.54	0.29	0.70	0.33	0.16	0.64	1.12	0.31	1.46	0.17
ΔCt MDXS1	-14.51	-9.59	-8.89	-10.53	-8.78	-9.89	-8.37	-9.41	-8.92	-7.83
DXS1	0.00	0.13	0.21	0.07	0.23	0.11	0.30	0.15	0.21	0.44
SiDEV1	0.00	0.12	0.25	0.07	0.21	0.09	0.17	0.15	0.05	0.37
SiDev2	0.00	0.15	0.19	0.07	0.24	0.12	0.52	0.15	0.85	0.52
SiDEV3	0.00	0.13	0.20	0.07	0.23	0.11	0.31	0.15	0.21	0.44
SiDEV	0.00	0.02	0.03	0.00	0.02	0.02	0.18	0.00	0.42	0.08
25 DAP	DE3	C17	NC300	SD44	C131A	B37	TZ118	KUI2007	A619	B73
MDXS1	30.16	29.59	27.15	29.67	26.93	29.20	32.45	30.65	28.39	28.20
MDXS1	31.21	32.14	28.22	30.95	27.35	29.57	31.79	29.95	27.74	28.15
MDXS1	30.67	30.88	27.69	30.29	27.12	29.40	32.13	30.31	28.08	28.19
AVG	30.68	30.87	27.69	30.30	27.13	29.39	32.12	30.30	28.07	28.18
SiDEV	0.53	1.28	0.54	0.64	0.21	0.19	0.33	0.35	0.33	0.03
ACTIN	19.62	20.13	20.00	20.71	20.47	23.59	22.19	23.61	23.97	22.85
ACTIN	20.57	21.55	20.56	20.88	20.75	23.74	22.50	23.83	22.74	23.13
ACTIN	20.11	20.82	20.29	20.78	20.62	23.65	22.36	23.73	23.37	23.00
AVG	20.10	20.83	20.28	20.79	20.61	23.66	22.35	23.72	23.36	22.99
SiDEV	0.48	0.71	0.28	0.09	0.14	0.08	0.16	0.11	0.62	0.14
ΔCt MDXS1	-10.58	-10.04	-7.40	-9.51	-6.52	-5.73	-9.77	-6.58	-4.71	-5.19
DXS1	0.07	0.10	0.59	0.14	1.09	1.88	0.11	1.05	3.82	2.75
SiDEV1	0.09	0.23	0.86	0.21	1.25	2.15	0.09	0.82	3.06	2.71
SiDev2	0.05	0.04	0.41	0.09	0.94	1.66	0.14	1.34	4.80	2.80
SiDEV3	0.07	0.09	0.59	0.14	1.10	1.87	0.11	1.04	3.79	2.73
SiDEV	0.02	0.10	0.23	0.06	0.16	0.24	0.03	0.26	0.87	0.05
10 DAP	DE3	TZ118	C17	SD44	KUI2007	A619	B37	C131A	NC300	B73
MDXS1	31.77	30.29	32.38	31.43	29.22	30.21	31.40	30.02		29.20
MDXS1	31.75	30.25	30.39	29.73	27.70	29.16	29.65	30.37		29.20
MDXS1	31.77	30.28	31.37	30.56	28.47	29.70	30.54	30.21		29.21
AVG	31.76	30.27	31.38	30.57	28.46	29.69	30.53	30.20		29.20
SiDEV	0.01	0.02	1.00	0.85	0.76	0.53	0.88	0.18		0.01
ACTIN	20.13	19.62	20.01	19.92	20.59	20.64	19.10	20.82		20.66
ACTIN	21.23	20.00	20.95	19.85	20.81	20.14	20.48	21.77		20.94
ACTIN	21.25	19.82	20.49	19.90	20.71	20.40	19.80	21.31		20.81
AVG	20.87	19.81	20.48	19.89	20.70	20.39	19.79	21.30		20.80
SiDEV	0.64	0.19	0.47	0.04	0.11	0.25	0.69	0.48		0.14
ΔCt MDXS1	-10.89	-10.46	-10.90	-10.68	-7.76	-9.30	-10.74	-8.90		-8.40
DXS1	0.05	0.07	0.05	0.06	0.46	0.16	0.06	0.21		0.30
SiDEV1	0.05	0.07	0.03	0.03	0.27	0.11	0.03	0.24		0.30
SiDev2	0.05	0.07	0.10	0.11	0.78	0.23	0.11	0.19		0.30
SiDEV3	0.05	0.07	0.05	0.06	0.46	0.16	0.06	0.21		0.29
SiDEV	0.00	0.00	0.04	0.04	0.26	0.06	0.04	0.03		0.00

20 DAP	A619	B37	C131A	C17	DE3	KUI2007	NC300	SD44	TZ118	B73
MDXS2	29.80	29.76	29.65	30.22	26.15	30.05	31.51	31.75	28.88	30.58
MDXS2	29.78	32.29	30.77	31.34	27.58	32.96	31.51	29.21	28.38	30.06
MDXS2	29.80	31.04	30.21	30.76	26.85	31.51	31.49	30.49	28.64	30.50
AVG	29.79	31.03	30.21	30.78	26.87	31.51	31.51	30.48	28.63	30.38
StDEV	0.01	1.79	0.79	0.79	1.01	2.06	0.00	1.80	0.35	0.28
ACTIN	21.67	20.51	20.79	20.47	19.40	20.98	19.26	21.61	22.32	21.68
ACTIN	22.90	21.05	20.91	22.16	21.00	22.51	19.45	22.73	24.74	21.70
ACTIN	22.30	20.76	20.85	21.30	20.18	21.75	19.36	22.18	23.51	21.72
AVG	22.29	20.77	20.85	21.31	20.19	21.75	19.36	22.17	23.53	21.70
StDEV	0.62	0.27	0.06	0.85	0.80	0.77	0.13	0.79	1.71	0.02
ΔCt MDXS2	-7.50	-10.25	-9.36	-9.47	-6.67	-9.76	-12.16	-8.31	-5.10	-8.68
DXS2	0.55	0.08	0.15	0.14	0.98	0.12	0.02	0.32	2.92	0.24
StDEV1	0.55	0.20	0.22	0.21	1.61	0.32	0.02	0.13	2.45	0.21
StDev2	0.56	0.03	0.10	0.10	0.60	0.04	0.02	0.76	3.47	0.30
StDEV3	0.55	0.08	0.15	0.14	0.99	0.12	0.02	0.31	2.90	0.22
StDEV	0.00	0.08	0.06	0.06	0.51	0.14	0.00	0.32	0.51	0.05
25 DAP	A619	B37	C131A	C17	DE3	KUI2007	NC300	SD44	TZ118	B73
MDXS2	27.16	30.63	27.63	25.92	25.22	27.51	26.74	27.41	28.81	29.37
MDXS2	27.78	30.56	27.48	26.40	24.81	28.22	28.76	29.78	29.28	29.77
MDXS2	27.48	30.61	27.57	26.17	25.02	27.88	27.76	28.61	29.06	29.69
AVG	27.47	30.60	27.56	26.16	25.02	27.87	27.75	28.60	29.05	29.61
StDEV	0.31	0.04	0.08	0.24	0.21	0.36	1.01	1.19	0.24	0.21
ACTIN	23.08	28.22	22.29	20.74	19.84	22.51	18.99	20.97	24.31	21.76
ACTIN	25.64	28.53	22.30	21.88	21.78	22.32	21.85	21.83	23.94	21.81
ACTIN	24.37	28.38	22.31	21.32	20.79	22.43	20.4	21.41	24.14	21.50
AVG	24.36	28.38	22.30	21.31	20.80	22.42	20.41	21.40	24.13	21.69
StDEV	1.28	0.16	0.01	0.57	0.97	0.10	1.43	0.61	0.19	0.17
ΔCt MDXS2	-3.11	-2.22	-5.26	-4.85	-4.21	-5.45	-7.34	-7.20	-4.92	-7.92
DXS2	11.58	21.41	2.61	3.48	5.40	2.30	0.62	0.68	3.31	0.41
StDEV1	14.39	20.97	2.49	4.10	4.68	2.94	1.25	1.55	3.90	0.49
StDev2	9.36	22.02	2.76	2.94	6.22	1.79	0.31	0.30	2.82	0.37
StDEV3	11.53	21.27	2.59	3.45	5.38	2.27	0.61	0.68	3.28	0.39
StDEV	2.52	0.54	0.14	0.58	0.77	0.57	0.48	0.64	0.54	0.06
15 DAP	A619	B37	C131A	C17	DE3	KUI2007	NC300	SD44	TZ118	B73
MDXS2	29.13	30.05	30.61	32.20	27.21	30.62	31.93	29.09	29.88	29.60
MDXS2	28.83	29.73	30.08	30.62	27.21	31.22	31.93	27.73	32.97	29.76
MDXS2	28.96	29.90	30.36	31.39	27.22	30.90	31.91	28.42	31.41	29.56
AVG	28.98	29.89	30.35	31.41	27.21	30.92	31.93	28.41	31.43	29.64
StDEV	0.21	0.23	0.37	1.12	0.00	0.42	0.00	0.96	2.18	0.11
ACTIN	21.28	20.79	21.63	20.47	19.40	20.15	19.76	22.66	20.04	20.64
ACTIN	20.61	21.21	21.05	21.04	19.15	20.47	19.76	22.80	20.02	21.03
ACTIN	20.96	21.01	21.35	20.77	19.29	20.32	19.77	22.74	20.04	21.00
AVG	20.95	21.00	21.34	20.76	19.28	20.31	19.76	22.73	20.03	20.89
StDEV	0.34	0.21	0.29	0.29	0.13	0.16	0.00	0.10	0.01	0.22
ΔCt MDXS2	-8.03	-8.89	-9.00	-10.65	-7.93	-10.61	-12.17	-5.68	-11.40	-8.75
DXS2	0.38	0.21	0.20	0.06	0.41	0.06	0.02	1.95	0.04	0.23
StDEV1	0.34	0.19	0.16	0.04	0.41	0.08	0.02	1.22	0.11	0.24
StDev2	0.42	0.24	0.23	0.11	0.41	0.05	0.02	3.13	0.01	0.21
StDEV3	0.39	0.21	0.19	0.06	0.41	0.07	0.02	1.94	0.04	0.25
StDEV	0.04	0.02	0.04	0.04	0.00	0.01	0.00	0.96	0.05	0.02
10 DAP	A619	B37	C131A	C17	DE3	KUI2007	NC300	SD44	TZ118	B73
MDXS2	29.38	29.15	31.83	32.77	30.16	32.18		27.97	29.87	28.65
MDXS2	30.61	29.12	31.40	26.37	28.05	31.02		27.96	30.62	28.94
MDXS2	30.01	29.15	31.63	29.58	29.12	31.61		27.98	30.26	28.54
AVG	30.00	29.14	31.62	29.57	29.11	31.60		27.97	30.25	28.71
StDEV	0.62	0.02	0.22	3.20	1.06	0.58		0.01	0.38	0.21
ACTIN	20.30	19.70	20.35	20.72	20.18	21.01		19.54	20.55	20.71
ACTIN	20.43	19.74	20.94	21.94	21.11	21.82		20.76	21.25	20.60
ACTIN	20.38	19.70	20.66	21.34	20.63	21.43		20.16	20.91	20.47
AVG	20.37	19.71	20.65	21.33	20.64	21.42		20.15	20.90	20.59
StDEV	0.07	0.02	0.30	0.61	0.47	0.41		0.86	0.49	0.12
ΔCt MDXS2	-9.63	-9.43	-10.97	-8.24	-8.47	-10.18		-7.82	-9.35	-8.12
DXS2	0.13	0.15	0.05	0.33	0.28	0.09		0.44	0.15	0.36
StDEV1	0.19	0.14	0.04	0.04	0.14	0.06		0.44	0.20	0.38
StDev2	0.08	0.15	0.06	3.05	0.59	0.13		0.45	0.12	0.31
StDEV3	0.13	0.14	0.05	0.33	0.28	0.09		0.44	0.15	0.41
StDEV	0.06	0.00	0.01	1.66	0.23	0.04		0.00	0.04	0.05

20 DAP	C131A	B37	A619	KUI2007	TZI18	DE3	C17	NC300	SD44	B73
MDXS3	24.06	25.14	27.30	25.21	25.04	23.75	25.75	23.29	24.38	27.29
MDXS3	23.87	25.05	27.04	25.13	25.15	23.88	26.68	23.89	24.55	27.59
MDXS3	23.98	24.81	27.18	25.18	25.11	23.83	26.23	23.57	24.48	27.45
AVG	23.97	25.00	27.17	25.17	25.10	23.82	26.22	23.58	24.47	27.44
SiDEV	0.10	0.17	0.13	0.04	0.06	0.07	0.47	0.30	0.09	0.15
ACTIN	19.79	19.69	21.02	20.58	20.61	18.95	19.70	18.01	20.37	21.61
ACTIN	19.58	20.70	20.93	21.00	20.64	19.51	21.14	18.56	20.64	22.02
ACTIN	19.69	20.21	20.99	20.80	20.64	19.24	20.43	18.30	20.52	21.83
AVG	19.69	20.20	20.98	20.79	20.63	19.23	20.42	18.29	20.51	21.82
SiDEV	0.11	0.51	0.05	0.21	0.02	0.28	0.72	0.28	0.14	0.21
ΔCt MDXS3	-4.28	-4.80	-6.19	-4.38	-4.47	-4.59	-5.80	-5.29	-3.96	-5.62
DXS3	5.14	3.59	1.37	4.80	4.51	4.16	1.80	2.55	6.43	2.03
SiDEV1	4.82	3.26	1.25	4.68	4.70	4.37	2.49	3.13	6.84	2.26
SiDev2	5.50	3.47	1.50	4.95	4.36	3.99	1.31	2.06	6.08	1.83
SiDEV3	5.10	4.09	1.36	4.78	4.48	4.13	1.79	2.57	6.38	2.02
SiDEV	0.34	0.44	0.12	0.14	0.18	0.19	0.60	0.53	0.38	0.21
15 DAP	TZI18	KUI2007	B37	C17	NC300	SD44	DE3	A619	C131A	B73
MDXS3	25.17	25.71	24.91	26.00	25.14	26.30	24.93	23.79	24.39	26.95
MDXS3	25.08	25.51	24.74	26.08	25.13	26.40	24.48	24.27	24.84	26.80
MDXS3	25.14	25.62	24.74	26.03	25.11	26.25	24.71	24.03	24.63	26.89
AVG	25.13	25.61	24.80	26.04	25.13	26.32	24.71	24.03	24.62	26.88
SiDEV	0.05	0.10	0.10	0.04	0.02	0.08	0.23	0.24	0.23	0.08
ACTIN	19.02	19.96	18.91	19.27	18.70	21.39	18.88	19.80	19.99	21.20
ACTIN	19.60	20.14	20.21	19.92	18.70	22.55	21.14	20.43	20.29	20.86
ACTIN	19.32	20.06	20.01	19.54	18.97	22.42	20.01	20.12	20.15	21.04
AVG	19.31	20.05	19.71	19.58	18.79	22.12	20.01	20.12	20.14	21.03
SiDEV	0.29	0.09	0.70	0.33	0.16	0.64	1.13	0.32	0.15	0.17
ΔCt MDXS3	-5.82	-5.56	-5.09	-6.46	-6.34	-4.20	-4.70	-3.91	-4.48	-5.85
DXS3	1.77	2.12	2.94	1.14	1.24	5.45	3.86	6.64	4.49	1.74
SiDEV1	1.73	1.98	2.72	1.17	1.23	5.52	3.30	7.84	5.27	1.66
SiDEV2	1.84	2.28	3.06	1.10	1.23	5.15	4.51	5.62	3.86	1.84
SiDEV3	1.76	2.11	3.06	1.14	1.25	5.71	3.85	6.64	4.46	1.73
SiDEV	0.06	0.15	0.20	0.03	0.01	0.29	0.61	1.11	0.71	0.09
25 DAP	DE3	C17	NC300	SD44	C131A	B37	TZI18	KUI2007	A619	B73
MDXS3	24.53	26.17	25.82	25.82	24.99	27.78	26.42	27.42	26.27	27.33
MDXS3	24.42	26.34	25.27	25.96	25.17	27.74	26.51	27.38	26.18	27.44
MDXS3	24.49	26.37	25.56	25.90	25.09	28.07	26.53	27.41	26.24	27.36
AVG	24.48	26.29	25.55	25.89	25.08	27.86	26.49	27.40	26.23	27.38
SiDEV	0.06	0.11	0.28	0.07	0.09	0.18	0.06	0.02	0.05	0.06
ACTIN	19.92	20.93	20.00	20.53	20.47	23.59	23.46	23.61	22.44	22.85
ACTIN	20.37	21.53	20.56	20.18	20.77	23.72	23.33	23.83	22.15	23.13
ACTIN	20.01	20.84	20.29	20.37	20.60	23.67	23.40	23.73	22.31	23.00
AVG	20.10	21.10	20.28	20.36	20.61	23.66	23.40	23.72	22.30	22.99
SiDEV	0.24	0.38	0.28	0.18	0.15	0.07	0.07	0.11	0.15	0.14
ΔCt MDXS3	-4.38	-5.19	-5.27	-5.53	-4.47	-4.20	-3.09	-3.68	-3.93	-4.38
DXS3	4.80	2.73	2.60	2.16	4.51	5.43	11.74	7.80	6.56	4.79
SiDEV1	4.64	2.98	2.15	2.27	4.81	5.75	12.30	7.71	6.38	4.95
SiDEV2	5.01	2.65	3.15	2.06	4.25	5.91	11.56	7.93	6.79	4.59
SiDEV3	4.77	2.59	2.58	2.15	4.49	4.70	11.40	7.77	6.52	4.85
SiDEV	0.19	0.21	0.50	0.11	0.28	0.66	0.48	0.11	0.21	0.19
10 DAP	DE3	TZI18	C17	SD44	KUI2007	A619	B37	C131A	NC300	B73
MDXS3	27.79	25.68	27.19	24.01	24.97	25.16	25.31	26.33		27.08
MDXS3	26.30	25.46	26.94	25.10	25.26	25.03	25.41	25.77		27.22
MDXS3	27.06	25.58	27.08	24.54	25.13	25.11	25.37	26.06		27.16
AVG	27.05	25.57	27.07	24.55	25.12	25.10	25.36	26.05		27.15
SiDEV	0.75	0.11	0.13	0.55	0.15	0.07	0.05	0.28		0.07
ACTIN	20.13	19.62	20.01	19.94	20.59	20.64	19.10	20.82		20.66
ACTIN	21.23	20.02	20.94	19.85	20.81	20.14	20.50	21.77		20.94
ACTIN	21.25	19.80	20.49	19.88	20.71	20.40	19.78	21.31		20.81
AVG	20.87	19.81	20.48	19.89	20.70	20.39	19.79	21.30		20.80
SiDEV	0.64	0.20	0.47	0.05	0.11	0.25	0.70	0.48		0.14
ΔCt MDXS3	-6.18	-5.76	-6.59	-4.66	-4.42	-4.71	-5.57	-4.75		-6.35
DXS3	1.38	1.85	1.04	3.96	4.68	3.83	2.11	3.71		1.23
SiDEV1	0.83	1.71	0.96	5.75	5.20	3.67	2.18	3.06		1.29
SiDEV2	2.32	2.00	1.14	2.70	4.25	4.02	2.04	4.51		1.17
SiDEV3	1.37	1.84	1.03	3.98	4.65	3.80	2.10	3.69		1.22
SiDEV	0.76	0.14	0.09	1.53	0.47	0.18	0.07	0.73		0.06

20DAP	SD44	NC300	C131A	C17	B37	TZI18	KUI2007	A619	B73	DE3
MDXR	23.85	23.03	25.09	24.67	23.61	25.79	24.00	25.83	24.18	22.77
MDXR	23.19	23.02	24.77	24.76	23.34	25.49	23.37	25.43	23.68	22.61
MDXR	23.31	22.69	24.91	25.46	23.62	25.62	23.67	25.61	23.20	22.70
AVG	23.45	22.91	24.92	24.96	23.52	25.63	23.68	25.62	23.69	22.69
StDEV	0.35	0.19	0.16	0.43	0.16	0.15	0.32	0.20	0.49	0.08
ACTIN	19.94	18.26	19.66	19.61	19.16	20.65	20.93	21.30	20.19	19.01
ACTIN	20.36	18.34	20.27	19.86	20.38	21.01	21.93	22.27	20.82	19.16
ACTIN	20.48	18.48	19.95	20.68	19.80	20.81	21.41	21.80	20.55	19.10
AVG	20.26	18.36	19.96	20.05	19.78	20.82	21.42	21.79	20.52	19.09
StDEV	0.28	0.11	0.31	0.56	0.61	0.18	0.50	0.49	0.32	0.08
ΔCt MDXR	-3.19	-4.55	-4.96	-4.91	-3.74	-4.81	-2.26	-3.83	-3.17	-3.60
DXR	10.96	4.26	3.21	3.32	7.47	3.56	20.93	7.02	11.14	8.23
StDEV1	8.30	3.93	2.86	4.07	7.03	3.20	16.76	6.08	7.91	7.80
StDEV2	13.12	3.96	3.56	3.82	8.48	3.94	25.94	8.02	11.19	8.72
StDEV3	12.07	4.97	3.24	2.35	6.98	3.60	21.07	7.08	15.60	8.19
StDEV	2.53	0.59	0.35	0.93	0.85	0.37	4.59	0.97	3.86	0.46
15DAP	SD44	NC300	C131A	C17	B37	TZI18	KUI2007	A619	B73	DE3
MDXR	23.87	24.66	24.98	24.89	24.61	24.87	25.39	23.65	23.67	23.10
MDXR	24.06	24.47	24.96	24.80	24.60	24.88	25.09	23.81	23.65	23.21
MDXR	23.95	24.55	24.95	24.83	24.60	24.86	25.22	23.71	23.23	23.14
AVG	23.96	24.56	24.96	24.84	24.60	24.87	25.23	23.72	23.52	23.15
StDEV	0.10	0.10	0.02	0.05	0.01	0.01	0.15	0.08	0.25	0.06
ACTIN	21.75	19.04	19.88	20.29	20.36	19.34	20.55	19.47	19.11	19.81
ACTIN	22.45	18.87	20.03	20.09	19.78	19.13	19.59	19.92	19.12	20.24
ACTIN	22.11	18.97	19.94	20.12	20.06	19.22	20.05	19.71	19.80	20.04
AVG	22.10	18.96	19.95	20.17	20.07	19.23	20.06	19.70	19.34	20.03
StDEV	0.35	0.09	0.08	0.11	0.29	0.11	0.48	0.23	0.40	0.22
ΔCt MDXR	-1.86	-5.60	-5.01	-4.67	-4.54	-5.64	-5.17	-4.02	-4.17	-3.12
DXR	27.61	2.06	3.10	3.92	4.31	2.01	2.78	6.15	5.54	11.50
StDEV1	29.39	1.92	3.06	3.79	4.29	2.01	2.49	6.47	4.98	11.91
StDEV2	25.76	2.19	3.10	4.03	4.32	1.99	3.07	5.79	5.05	11.03
StDEV3	27.80	2.08	3.13	3.95	4.32	2.02	2.80	6.21	6.76	11.58
StDEV	1.82	0.14	0.03	0.12	0.02	0.01	0.29	0.34	1.01	0.44
10DAP	SD44	NC300	C131A	C17	B37	TZI18	KUI2007	A619	B73	DE3
MDXR	24.56		27.16	26.05	25.04	25.91	25.68	26.37	25.67	25.66
MDXR	24.01		26.93	26.09	24.97	25.16	24.94	26.43	25.37	25.31
MDXR	24.30		27.06	26.05	25.02	25.52	25.32	26.41	25.11	25.47
AVG	24.29		27.05	26.06	25.01	25.53	25.31	26.40	25.38	25.48
StDEV	0.28		0.12	0.02	0.04	0.38	0.37	0.03	0.28	0.18
ACTIN	19.37		20.82	20.67	19.10	20.44	21.68	19.91	19.65	21.77
ACTIN	20.52		21.79	20.76	20.48	20.67	21.24	20.66	19.59	20.61
ACTIN	20.48		19.77	20.70	19.80	20.56	20.76	20.44	19.64	21.20
AVG	20.12		20.79	20.71	19.79	20.56	21.23	20.34	19.63	21.19
StDEV	0.65		1.01	0.05	0.69	0.12	0.46	0.39	0.03	0.58
ΔCt MDXR	-4.17		-6.26	-5.35	-5.22	-4.97	-4.09	-6.07	-5.76	-4.29
DXR	5.57		1.31	2.45	2.69	3.18	5.89	1.49	1.85	5.12
StDEV1	4.62		1.21	2.47	2.63	2.45	4.56	1.53	1.52	4.52
StDEV2	6.76		1.42	2.40	2.76	4.11	7.62	1.46	1.87	5.76
StDEV3	5.53		1.30	2.47	2.67	3.21	5.86	1.49	2.24	5.16
StDEV	1.08		0.11	0.04	0.07	0.84	1.54	0.03	0.36	0.62
25DAP	SD44	NC300	C131A	C17	B37	TZI18	KUI2007	A619	B73	DE3
MDXR	23.91	24.13	26.24	25.29	27.37	26.40	25.59	25.53	25.62	22.49
MDXR	23.87	24.32	26.15	25.00	27.87	26.01	25.31	25.67	25.45	22.34
MDXR	23.90	24.24	26.21	25.13	27.63	26.19	25.43	25.61	25.51	22.40
AVG	23.89	24.23	26.20	25.14	27.62	26.20	25.44	25.60	25.53	22.41
StDEV	0.02	0.10	0.05	0.15	0.25	0.20	0.14	0.07	0.09	0.08
ACTIN	20.17	19.77	21.34	20.76	23.77	23.48	22.55	22.82	22.40	19.60
ACTIN	20.07	20.41	21.37	20.61	24.02	24.85	24.62	23.67	23.53	20.09
ACTIN	20.11	20.10	21.37	20.70	23.91	24.17	23.60	23.23	22.40	19.83
AVG	20.12	20.09	21.36	20.69	23.90	24.17	23.59	23.24	22.78	19.84
StDEV	0.05	0.32	0.02	0.08	0.13	0.69	1.04	0.43	0.65	0.25
ΔCt MDXR	-3.78	-4.14	-4.84	-4.45	-3.72	-2.03	-1.85	-2.36	-2.75	-2.57
DXR	7.30	5.69	3.49	4.58	7.57	24.43	27.68	19.43	14.87	16.84
StDEV1	7.21	6.09	3.40	4.12	9.02	21.27	25.00	20.45	13.93	15.93
StDEV2	7.42	5.34	3.61	5.04	6.38	27.87	30.35	18.56	15.68	17.68
StDEV3	7.26	5.65	3.47	4.61	7.54	24.60	27.93	19.34	15.04	16.96
StDEV	0.11	0.38	0.11	0.46	1.33	3.30	2.68	0.95	0.88	0.88

Transcript profiles of HDS at 20 and 25 DAP in 10 inbred lines										
25DAP	A619	B73	B37	Cl.7	C131A	DE3	KUI2007	NC300	SD44	TZI18
MHDS	28.13	25.48	29.17	28.73	28.50	26.79	26.97	28.55	26.37	28.71
MHDS	27.92	25.41	28.87	28.58	27.16	26.22	26.78	27.39	26.00	28.54
MHDS	27.39	24.81	28.56	28.96	28.49	26.67	26.59	27.83	26.19	28.63
AVG	27.81	25.23	28.87	28.76	28.05	26.56	26.78	27.92	26.19	28.63
StDEV	0.38	0.37	0.31	0.19	0.77	0.30	0.19	0.59	0.19	0.09
ACTIN	22.04	22.23	22.09	23.86	22.53	21.47	23.19	22.24	21.65	23.79
ACTIN	23.25	21.94	22.53	23.91	21.98	20.40	22.01	20.87	21.64	23.17
ACTIN	22.60	21.37	22.30	23.95	22.23	20.91	22.59	21.53	21.63	23.45
AVG	22.63	21.85	22.31	23.91	22.25	20.93	22.60	21.55	21.64	23.47
StDEV	0.61	0.44	0.22	0.05	0.28	0.54	0.59	0.69	0.01	0.31
Δ Ct HDS	-5.18	-3.39	-6.56	-4.85	-5.80	-5.63	-4.18	-6.38	-4.55	-5.16
MHDS	2.75	9.56	1.06	3.47	1.79	2.01	5.50	1.20	4.28	2.80
StDEV1	2.21	8.06	0.86	3.53	1.31	1.72	4.82	0.78	3.77	2.65
StDEV2	2.56	8.46	1.06	3.92	3.32	2.55	5.50	1.74	4.87	2.98
StDEV3	3.69	12.82	1.31	3.01	1.32	1.87	6.28	1.28	4.27	2.80
StDEV	0.77	2.64	0.23	0.46	1.16	0.44	0.73	0.48	0.55	0.17
20DAP	A619	B73	B37	Cl.7	C131A	DE3	KUI2007	NC300	SD44	TZI18
MHDS	26.26	25.91	27.14	27.91	28.34	26.03	27.45	25.61	27.37	26.14
MHDS	25.95	26.30	26.75	28.64	28.98	27.60	26.95	25.12	27.04	26.77
MHDS	26.08	26.10	26.92	28.25	28.65	26.75	27.17	25.35	27.15	26.40
AVG	26.10	26.10	26.94	28.27	28.66	26.79	27.19	25.36	27.19	26.44
StDEV	0.16	0.20	0.20	0.37	0.32	0.79	0.25	0.25	0.17	0.32
ACTIN	23.20	22.20	21.67	23.65	22.05	20.19	22.43	20.72	21.64	21.35
ACTIN	22.52	22.15	22.29	22.92	22.77	21.96	23.18	21.06	21.64	21.35
ACTIN	22.85	22.15	21.97	23.25	22.40	21.05	22.83	20.85	21.61	21.34
AVG	22.86	22.17	21.98	23.27	22.41	21.07	22.81	20.88	21.63	21.35
StDEV	0.34	0.03	0.31	0.37	0.36	0.89	0.38	0.17	0.02	0.01
Δ Ct HDS	-3.24	-3.94	-4.96	-4.99	-6.25	-5.73	-4.38	-4.48	-5.56	-5.09
MHDS	10.58	6.53	3.21	3.14	1.31	1.89	4.81	4.47	2.12	2.94
StDEV1	9.45	7.47	2.79	4.02	1.64	3.21	4.02	3.76	1.87	3.61
StDEV2	11.72	5.70	3.66	2.42	1.05	1.08	5.69	5.28	2.35	2.33
StDEV3	10.71	6.55	3.25	3.18	1.32	1.95	4.88	4.50	2.18	3.01
StDEV	1.14	0.88	0.43	0.80	0.29	1.07	0.83	0.76	0.24	0.64

Transcript profiles of HDR at 20 and 25 DAP in 10 inbred lines										
20 DAP	A619	B73	B37	CI7	C131A	DE3	KUI2007	NC300	SD44	TZI18
MHDR	26.88	26.75	26.88	28.42	25.73	26.51	26.24	25.56	26.10	26.51
MHDR	26.91	26.54	26.91	28.21	26.54	26.07	26.30	25.70	25.88	26.26
MHDR	27.27	26.37	26.27	28.49	26.05	26.23	25.79	24.90	26.29	26.69
AVG	27.02	26.55	26.69	28.37	26.11	26.27	26.11	25.39	26.09	26.49
StDEV	0.22	0.19	0.36	0.15	0.41	0.22	0.28	0.43	0.21	0.22
ACTIN	21.74	21.99	21.77	23.72	20.69	21.61	21.41	19.73	21.47	21.15
ACTIN	22.08	22.20	21.62	23.44	20.86	21.84	21.06	20.85	21.69	21.54
ACTIN	21.90	22.41	21.73	23.55	20.76	21.70	21.21	20.25	21.55	21.36
AVG	21.91	22.20	21.71	23.57	20.77	21.72	21.23	20.28	21.57	21.35
StDEV	0.17	0.21	0.08	0.14	0.09	0.12	0.18	0.56	0.11	0.01
Δ Ct MHDR	-5.11	-4.35	-4.98	-4.80	-5.34	-4.55	-4.88	-5.11	-4.52	-5.14
HDR	2.89	4.89	3.17	3.58	2.47	4.26	3.39	2.90	4.36	2.84
StDEV1	3.18	4.27	2.77	3.47	3.21	3.61	3.10	2.57	4.33	2.80
StDEV2	3.12	4.94	2.71	4.01	1.83	4.89	2.97	2.33	5.04	3.33
StDEV3	2.43	5.56	4.23	3.30	2.57	4.38	4.23	4.06	3.79	2.47
StDEV	0.42	0.64	0.86	0.37	0.69	0.65	0.69	0.94	0.63	0.43
25 DAP	A619	B73	B37	CI7	C131A	DE3	KUI2007	NC300	SD44	TZI18
MHDR	27.37	25.74	25.16	26.86	26.71	24.76	25.69	25.96	25.32	27.10
MHDR	26.90	25.66	25.08	27.27	26.67	24.93	25.88	25.96	25.84	27.29
MHDR	27.12	25.70	25.47	27.29	26.76	25.40	26.11	25.95	25.58	26.89
AVG	27.13	25.70	25.24	27.14	26.71	25.03	25.89	25.96	25.58	27.09
StDEV	0.24	0.04	0.21	0.24	0.05	0.33	0.21	0.01	0.26	0.20
ACTIN	22.06	21.74	22.09	23.87	22.53	21.48	23.16	22.24	21.64	23.78
ACTIN	23.22	21.90	22.50	23.96	21.97	20.39	22.00	20.87	21.66	23.15
ACTIN	22.61	21.85	22.33	23.89	22.24	20.92	22.63	21.53	21.62	23.48
AVG	22.63	21.83	22.31	23.91	22.25	20.93	22.60	21.55	21.64	23.47
StDEV	0.11	0.24	0.24	0.18	0.09	0.16	0.11	0.37	0.13	0.61
Δ Ct MHDR	-4.50	-3.87	-2.93	-3.24	-4.47	-4.10	-3.30	-4.41	-3.94	-3.62
HDR	4.42	6.84	13.12	10.62	4.52	5.82	10.16	4.70	6.52	8.11
StDEV1	3.74	6.65	13.84	12.90	4.53	7.02	11.70	4.69	7.80	8.08
StDEV2	5.18	7.03	14.63	9.71	4.66	6.24	10.26	4.69	5.44	7.08
StDEV3	4.45	6.84	11.16	9.57	4.37	4.51	8.75	4.73	6.52	9.34
StDEV	0.72	0.19	1.82	1.88	0.14	1.29	1.48	0.02	1.18	1.13

Transcript profiles of IPPI1 at 10, 15, 20, 25 DAP in 10 inbred lines										
20DAP	DE3	TZ18	C17	NC300	KUI2007	SD44	B37	C131A	A619	B73
MIPPI	18.43	19.78	19.15	18.00	19.91	19.69	19.06	19.71	20.80	20.41
MIPPI	18.09	19.52	18.85	17.59	19.90	19.55	19.97	19.96	20.66	20.17
MIPPI	18.24	19.66	18.98	17.78	19.92	19.63	19.52	19.82	20.73	20.02
AVG	18.25	19.65	18.99	17.79	19.91	19.62	19.52	19.83	20.73	20.20
SIDEV	0.17	0.13	0.15	0.21	0.01	0.07	0.46	0.13	0.07	0.20
ACTIN	19.06	20.43	19.46	18.23	20.30	20.21	19.93	19.78	21.98	21.04
ACTIN	19.35	20.90	19.89	18.40	20.31	20.59	19.52	20.10	21.78	20.92
ACTIN	19.19	20.65	19.66	18.30	20.32	20.41	19.73	19.90	21.88	21.07
AVG	19.20	20.66	19.67	18.31	20.31	20.40	19.73	19.93	21.88	21.01
SIDEV	0.15	0.24	0.22	0.09	0.01	0.19	0.21	0.16	0.10	0.08
ΔCt MIPPI	0.95	1.01	0.68	0.52	0.40	0.78	0.21	0.10	1.15	0.81
IPPI	192.74	200.93	159.84	143.40	131.95	171.71	115.67	106.93	221.91	175.32
SIDEV1	170.53	184.04	143.40	123.97	131.95	163.96	158.74	116.20	211.40	151.57
SIDEV2	215.85	220.38	176.54	164.72	132.87	180.67	84.48	97.72	232.95	179.01
SIDEV3	194.53	200.00	161.33	144.39	131.04	170.92	115.40	107.67	221.91	198.62
SIDEV	22.67	18.22	16.59	20.37	0.91	8.39	37.30	9.25	10.77	23.63
25DAP	DE3	TZ18	C17	NC300	KUI2007	SD44	B37	C131A	A619	B73
MIPPI	19.38	22.70	20.51	19.49	20.55	19.96	23.78	20.50	20.38	21.65
MIPPI	19.65	21.90	20.66	19.22	20.70	19.80	23.82	20.30	20.41	21.66
MIPPI	19.50	22.31	20.60	19.34	20.64	19.86	23.81	20.41	20.40	21.66
AVG	19.51	22.30	20.59	19.35	20.63	19.87	23.80	20.40	20.40	21.66
SIDEV	0.14	0.40	0.08	0.14	0.08	0.08	0.02	0.10	0.02	0.01
ACTIN	19.89	23.36	20.18	19.64	21.60	20.07	25.08	21.11	22.71	22.40
ACTIN	19.70	23.44	20.54	19.45	21.18	19.87	25.05	21.39	22.22	23.55
ACTIN	19.81	23.41	20.34	19.56	21.37	19.95	25.08	21.23	22.47	22.40
AVG	19.80	23.40	20.35	19.55	21.38	19.96	25.07	21.24	22.47	22.78
SIDEV	0.10	0.04	0.18	0.10	0.21	0.10	0.02	0.14	0.25	0.66
ΔCt MIPPI	0.29	1.10	-0.24	0.20	0.75	0.09	1.27	0.84	2.07	1.13
IPPI	122.26	21.44	8.49	11.49	16.86	10.64	24.06	17.90	41.99	21.84
SIDEV1	133.79	162.83	89.71	104.25	178.18	100.23	244.53	167.40	424.77	219.36
SIDEV2	110.96	283.50	80.85	125.70	160.58	111.99	237.84	192.30	416.02	217.85
SIDEV3	123.11	213.37	84.28	115.67	167.40	107.43	239.50	178.18	418.92	217.85
SIDEV	11.43	60.60	4.47	10.73	8.87	5.93	3.48	12.48	4.45	0.87
15 DAP	DE3	C131A	SD44	NC300	C17	B37	TZ18	KUI2007	A619	B73
MIPPI	19.03	20.78	20.77	18.62	20.06	20.24	19.06	19.18	18.98	19.20
MIPPI	18.97	21.33	21.65	18.75	20.04	20.81	19.06	19.12	20.01	19.41
MIPPI	19.01	21.04	21.21	18.67	20.03	20.53	19.07	19.15	19.50	19.27
AVG	19.00	21.05	21.21	18.68	20.04	20.53	19.06	19.15	19.50	19.29
SIDEV	0.03	0.28	0.44	0.07	0.02	0.29	0.01	0.03	0.52	0.11
ACTIN	19.20	19.69	21.76	18.72	19.76	20.23	19.16	20.00	19.46	19.67
ACTIN	19.67	22.22	22.31	20.66	21.82	20.39	21.04	20.45	20.03	19.71
ACTIN	19.44	20.94	22.04	19.70	20.80	20.31	20.11	20.23	19.75	20.20
AVG	19.44	20.95	22.04	19.69	20.79	20.31	20.10	20.23	19.75	19.86
SIDEV	0.24	1.27	0.28	0.97	1.03	0.08	0.94	0.23	0.29	0.30
ΔCt MIPPI	0.43	-0.10	0.83	1.01	0.75	-0.22	1.04	1.08	0.25	0.57
IPPI	135.03	9.33	17.74	20.19	16.82	8.61	20.56	21.09	11.89	14.81
SIDEV1	132.56	112.51	240.61	210.43	166.25	104.97	206.10	206.58	170.13	158.01
SIDEV2	138.19	76.84	130.74	192.30	168.57	70.71	206.10	215.35	83.32	136.60
SIDEV3	134.41	93.95	177.36	203.26	169.74	85.86	204.67	210.92	118.65	150.52
SIDEV	2.87	17.84	55.14	9.13	1.78	17.17	0.82	4.39	43.66	10.86
10 DAP	DE3	TZ18	C17	B37	C131A	KUI2007	SD44	A619	B73	NC300
MIPPI	21.04	20.58	21.31	20.01	21.92	21.39	20.17	20.77	19.71	
MIPPI	20.73	20.22	20.72	19.81	21.35	21.19	19.81	20.50	18.94	
MIPPI	20.90	20.41	21.03	19.92	21.62	21.27	20.00	20.62	18.78	
AVG	20.89	20.40	21.02	19.91	21.63	21.28	19.99	20.63	19.14	
SIDEV	0.16	0.18	0.30	0.10	0.29	0.10	0.18	0.14	0.50	
ACTIN	20.42	20.01	20.64	19.17	20.32	20.81	19.25	20.80	19.51	
ACTIN	21.90	20.01	20.54	19.12	21.15	21.29	19.09	20.09	19.20	
ACTIN	21.14	20.02	20.57	19.13	20.72	21.03	19.18	20.43	19.59	
AVG	21.15	20.01	20.58	19.14	20.73	21.04	19.17	20.44	19.43	
SIDEV	0.74	0.01	0.05	0.03	0.42	0.24	0.08	0.36	0.21	
ΔCt MIPPI	0.26	-0.39	-0.44	-0.77	-0.90	-0.24	-0.82	-0.19	0.29	
IPPI	120.02	7.63	7.39	5.85	5.36	8.47	5.66	8.77	12.23	
SIDEV1	108.17	67.52	60.43	54.71	43.83	78.64	50.12	79.55	82.55	
SIDEV2	134.10	86.65	90.96	62.85	65.07	90.33	64.32	95.93	140.77	
SIDEV3	119.20	75.96	73.37	58.24	53.96	85.46	56.38	88.27	157.28	
SIDEV	13.01	9.59	15.32	4.08	10.62	5.87	7.12	8.19	39.26	

Transcript profiles of IPPI2 and IPPI3 at 10, 15, 20, 25 DAP in 10 inbred lines										
20DAP	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZ118	A619	B73
MIPPI2	24.36	25.91	24.08	24.08	25.01	24.00	25.37	24.46	24.78	23.92
MIPPI2	24.08	25.61	23.82	24.37	25.05	23.89	25.05	23.98	23.71	23.66
MIPPI2	24.22	25.76	23.95	24.23	25.03	23.95	25.21	24.22	24.25	23.79
AVG	24.22	25.76	23.95	24.23	25.03	23.95	25.21	24.22	24.25	23.79
StDEV	0.14	0.15	0.13	0.15	0.02	0.06	0.16	0.24	0.54	0.13
MIPPI3	24.62	25.84	24.09	24.01	29.16	24.71	25.35	28.84	23.95	24.28
MIPPI3	24.54	25.78	24.28	23.94	29.12	24.51	25.43	28.57	23.66	25.11
MIPPI3	24.58	25.81	24.19	23.98	29.14	24.61	25.39	28.71	23.81	24.70
AVG	24.58	25.81	24.19	23.98	29.14	24.61	25.39	28.71	23.81	24.70
StDEV	0.04	0.03	0.10	0.04	0.02	0.10	0.04	0.14	0.15	0.42
ACTIN	21.13	21.90	19.63	19.97	20.72	19.59	21.87	21.81	21.12	21.85
ACTIN	21.97	23.03	19.76	21.03	21.10	20.48	22.91	23.08	21.63	23.14
ACTIN	21.55	22.47	19.70	20.50	20.91	20.04	22.39	22.45	21.38	22.50
AVG	21.55	22.47	19.70	20.50	20.91	20.04	22.39	22.45	21.38	22.50
StDEV	0.42	0.57	0.07	0.53	0.19	0.45	0.52	0.64	0.26	0.65
ΔCt MIPPI2	-2.67	-3.29	-4.25	-3.73	-4.12	-3.91	-2.82	-1.77	-2.87	-1.29
ΔCt MIPPI3	-3.03	-3.34	-4.49	-3.48	-8.23	-4.57	-3.00	-6.26	-2.43	-2.20
IPPI2	15.71	10.20	5.24	7.55	5.75	6.65	14.16	29.25	13.68	40.80
IPPI3	12.24	9.85	4.45	8.98	0.33	4.20	12.50	1.30	18.56	21.76
StDEV1	14.26	9.19	4.79	8.36	5.83	6.41	12.67	24.77	9.45	37.28
StDEV2	17.31	11.32	5.74	6.84	5.67	6.92	15.82	34.55	19.84	44.65
StDEV3	15.71	10.20	5.24	7.54	5.75	6.64	14.16	29.25	13.65	40.80
StDEV	1.53	1.06	0.47	0.76	0.08	0.25	1.57	4.89	5.23	3.68
StDEV1	11.91	9.65	4.76	8.78	0.33	3.92	12.85	1.19	16.80	29.05
StDEV2	12.59	10.06	4.17	9.21	0.34	4.50	12.16	1.43	20.54	16.34
StDEV3	12.24	9.85	4.44	8.96	0.33	4.20	12.50	1.30	18.51	21.71
StDEV	0.34	0.20	0.29	0.22	0.00	0.29	0.35	0.12	1.87	6.38
25 DAP	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZ118	A619	B73
MIPPI2	23.80	25.11	24.52	22.98	25.85	23.33	23.19	25.22	25.21	23.48
MIPPI2	23.47	24.68	24.90	23.11	25.42	23.99	23.90	25.89	24.84	23.67
MIPPI2	23.14	24.90	24.71	23.05	25.64	23.66	23.55	25.56	25.03	23.58
AVG	23.47	24.90	24.71	23.05	25.64	23.66	23.55	25.56	25.03	23.58
StDEV	0.33	0.22	0.19	0.07	0.22	0.33	0.36	0.34	0.19	0.10
MIPPI3	23.89	25.05	24.72	23.58	28.59	24.90	24.82	28.35	24.96	24.07
MIPPI3	23.51	24.87	24.50	23.73	27.89	23.97	25.41	27.73	24.82	23.92
MIPPI3	23.20	24.96	24.61	23.66	28.24	24.44	25.12	28.04	24.89	24.00
AVG	23.53	24.96	24.61	23.66	28.24	24.44	25.12	28.04	24.89	24.00
StDEV	0.35	0.09	0.11	0.08	0.35	0.47	0.30	0.31	0.07	0.08
ACTIN	20.69	20.65	21.11	20.53	22.03	20.77	20.72	23.47	22.93	21.23
ACTIN	20.71	20.73	21.35	19.95	21.90	18.96	20.80	23.13	21.79	22.69
ACTIN	20.20	20.69	21.23	20.24	21.97	19.87	20.76	23.30	22.36	21.96
AVG	20.53	20.69	21.23	20.24	21.97	19.87	20.76	23.30	22.36	21.96
StDEV	0.29	0.04	0.12	0.29	0.07	0.91	0.04	0.17	0.57	0.73
ΔCt MIPPI2	-2.94	-4.21	-3.48	-2.81	-3.67	-3.79	-2.79	-2.26	-2.67	-1.62
ΔCt MIPPI3	-3.00	-4.27	-3.38	-3.42	-6.27	-4.57	-4.36	-4.74	-2.53	-2.04
IPPI2	13.08	5.42	8.96	14.29	7.86	7.21	14.49	20.93	15.75	32.61
IPPI3	12.50	5.18	9.61	9.36	1.29	4.21	4.88	3.74	17.31	24.37
StDEV1	10.39	4.67	10.22	14.97	6.78	9.07	18.56	26.43	13.87	34.87
StDEV2	13.06	6.29	7.86	13.68	9.13	5.74	11.34	16.61	17.92	30.57
StDEV3	16.47	5.40	8.96	14.26	7.84	7.21	14.46	20.88	15.71	32.53
StDEV	3.05	0.81	1.18	0.65	1.18	1.67	3.62	4.92	2.03	2.15
StDEV1	9.76	4.87	8.90	9.88	1.01	3.05	6.00	3.02	16.49	23.16
StDEV2	12.70	5.52	10.37	8.90	1.65	5.82	3.98	4.64	18.17	25.70
StDEV3	15.75	5.18	9.61	9.34	1.29	4.20	4.87	3.74	17.31	24.32
StDEV	2.99	0.32	0.73	0.49	0.32	1.39	1.01	0.81	0.84	1.27

15DAP	A619	B37	CI&	C131A	DE3	KUI2007	NC300	SD44	TZ118	B73
MIPPI2	23.99	23.08	24.02	23.97	22.64	23.88	23.46	25.44	22.73	22.80
MIPPI2	25.31	23.23	24.26	23.74	22.54	24.27	23.71	25.98	22.95	22.47
MIPPI2	24.15	23.16	24.14	23.86	22.59	24.08	23.59	25.71	22.84	23.14
AVG	24.65	23.16	24.14	23.86	22.59	24.08	23.59	25.71	22.84	22.80
StDEV	0.93	0.08	0.12	0.12	0.05	0.20	0.13	0.27	0.11	0.33
MIPPI3	24.73	24.16	23.95	23.44	22.79	30.68	24.73	24.94	27.83	22.89
MIPPI3	25.33	25.80	24.02	25.17	23.12	28.91	24.50	26.20	30.27	24.51
MIPPI3	24.53	24.98	23.99	24.31	22.96	29.80	24.62	25.57	29.05	24.20
AVG	24.86	24.98	23.99	24.31	22.96	29.80	24.62	25.57	29.05	23.87
StDEV	0.42	0.82	0.04	0.87	0.17	0.89	0.12	0.63	1.22	0.86
ACTIN	20.47	20.58	21.06	20.92	19.64	20.60	19.86	22.45	20.07	19.69
ACTIN	19.89	20.80	21.48	20.80	19.67	20.52	20.00	22.88	20.03	19.71
ACTIN	20.18	20.69	21.27	20.86	19.66	20.56	19.93	22.67	20.05	20.20
AVG	20.18	20.69	21.27	20.86	19.66	20.56	19.93	22.67	20.05	19.87
StDEV	0.29	0.11	0.21	0.06	0.02	0.04	0.07	0.22	0.02	0.29
ΔCt MIPPI2	-4.47	-2.47	-2.87	-3.00	-2.93	-3.52	-3.66	-3.04	-2.79	-2.94
ΔCt MIPPI3	-4.68	-4.29	-2.72	-3.45	-3.30	-9.24	-4.69	-2.90	-9.00	-4.00
IPPI2	4.51	18.09	13.68	12.53	13.09	8.74	7.93	12.13	14.46	13.08
IPPI3	3.89	5.11	15.21	9.17	10.15	0.17	3.88	13.37	0.20	6.25
StDEV1	7.13	19.08	14.87	11.58	12.65	10.01	8.66	14.63	15.60	13.09
StDEV2	2.86	17.19	12.59	13.58	13.55	7.64	7.28	10.06	13.40	16.46
StDEV3	6.38	18.05	13.68	12.50	13.09	8.72	7.91	12.13	14.46	10.38
StDEV	2.28	0.94	1.14	1.00	0.45	1.19	0.69	2.29	1.10	3.04
StDEV1	4.27	9.02	15.60	16.72	11.40	0.09	3.59	20.69	0.45	12.30
StDEV2	2.82	2.90	14.87	5.04	9.07	0.31	4.21	8.64	0.08	4.00
StDEV3	4.90	5.11	15.18	9.15	10.13	0.17	3.87	13.37	0.20	4.96
StDEV	1.07	3.10	0.37	5.93	1.17	0.11	0.31	6.07	0.19	4.54
10DAP	A619	B37	CI7	C131A	DE3	KUI2007	SD44	TZ118	B73	NC300
MIPPI2	23.39	23.21	23.34	23.17	22.76	24.28	22.97	23.83	22.18	
MIPPI2	23.12	23.01	23.04	23.77	22.93	24.21	22.89	23.46	22.18	
MIPPI2	23.26	23.11	23.19	23.47	22.85	24.25	22.93	23.65	22.42	
AVG	23.26	23.11	23.19	23.47	22.85	24.25	22.93	23.65	22.26	
StDEV	0.19	0.10	0.15	0.30	0.09	0.04	0.04	0.19	0.14	
MIPPI3	24.76	25.49	23.20	23.25	22.66	26.23	23.65	27.17	21.60	
MIPPI3	23.00	22.87	23.39	23.66	22.74	27.85	24.58	27.77	22.70	
MIPPI3	23.88	24.18	23.30	23.46	22.70	27.04	24.12	24.47	22.39	
AVG	23.88	24.18	23.30	23.46	22.70	27.04	24.12	26.47	22.23	
StDEV	0.88	1.31	0.10	0.21	0.04	0.81	0.47	1.76	0.57	
ACTIN	22.26	21.65	23.01	22.62	21.17	22.49	21.07	21.33	19.51	
ACTIN	21.29	20.59	19.73	21.57	21.07	22.70	22.04	21.74	19.20	
ACTIN	21.78	21.12	21.37	22.10	21.12	22.60	21.56	21.54	19.59	
AVG	21.78	21.12	21.37	22.10	21.12	22.60	21.56	21.54	19.43	
StDEV	0.49	0.53	1.64	0.53	0.05	0.11	0.49	0.21	0.21	
ΔCt MIPPI2	-1.48	-1.99	-1.82	-1.37	-1.73	-1.65	-1.37	-2.11	-2.83	
ΔCt MIPPI3	-2.10	-3.06	-1.93	-1.36	-1.58	-4.44	-2.56	-4.93	-2.80	
IPPI2	35.89	25.17	28.32	38.60	30.21	31.86	38.60	23.16	14.11	
IPPI3	23.27	11.99	26.30	38.96	33.45	4.60	16.96	3.27	14.39	
StDEV1	32.68	23.49	25.53	47.52	32.09	31.14	37.54	20.40	14.90	
StDEV2	39.41	26.98	31.43	31.35	28.52	32.68	39.69	26.36	14.90	
StDEV3	35.77	25.17	28.32	38.60	30.15	31.79	38.60	23.11	12.66	
StDEV	3.37	1.75	2.95	8.10	1.79	0.78	1.07	2.99	1.29	
StDEV1	12.65	4.84	28.13	44.96	34.39	8.06	23.43	2.01	22.27	
StDEV2	42.83	29.73	24.66	33.84	32.53	2.62	12.30	1.33	10.39	
StDEV3	23.27	11.99	26.24	38.87	33.45	4.60	16.92	13.09	12.88	
StDEV	15.31	12.82	1.74	5.57	0.93	2.75	5.59	6.60	6.27	

Transcript profiles of GGPPS1 at 20 and 25 DAP in 10 inbred lines										
20 DAP	A619	B73	B37	CI7	C131A	DE3	KUI2007	NC300	SD44	TZI18
MGGPPS1	22.34	27.79	24.88	27.24	27.52	27.21	25.63	25.91	25.73	25.87
MGGPPS1	22.87	27.01	24.63	27.91	27.94	26.93	25.45	25.56	25.87	25.69
MGGPPS1	22.39	27.60	24.77	27.08	27.72	27.56	25.69	25.31	25.95	26.03
AVG	22.53	27.47	24.76	27.41	27.73	27.23	25.59	25.59	25.85	25.86
StDEV	0.29	0.41	0.13	0.44	0.21	0.32	0.12	0.30	0.11	0.17
ACTIN	18.62	22.18	21.77	23.72	20.69	21.64	21.39	19.73	21.45	21.16
ACTIN	18.72	22.39	21.65	23.44	20.87	21.82	21.09	20.84	21.71	21.55
ACTIN	18.44	22.24	21.70	23.55	20.75	21.69	21.20	20.26	21.55	21.34
AVG	18.59	22.27	21.71	23.57	20.77	21.72	21.23	20.28	21.57	21.35
StDEV	0.14	0.11	0.06	0.14	0.09	0.09	0.15	0.56	0.13	0.01
Δ Ct MGGPP	-3.94	-5.20	-3.05	-3.84	-6.96	-5.52	-4.36	-5.32	-4.28	-4.51
MGGPPS1	6.52	2.73	12.05	6.98	0.81	2.18	4.86	2.51	5.15	4.38
StDEV1	7.45	2.18	11.08	7.86	0.93	2.22	4.73	2.01	5.59	4.36
StDEV2	5.16	3.74	13.18	4.94	0.69	2.70	5.35	2.57	5.08	4.94
StDEV3	7.20	2.49	11.96	8.78	0.81	1.74	4.53	3.05	4.80	3.90
StDEV	1.26	0.83	1.05	2.00	0.12	0.48	0.43	0.52	0.40	0.52
25 DAP	A619	B73	B37	CI7	C131A	DE3	KUI2007	NC300	SD44	TZI18
MGGPPS1	25.79	26.09	25.13	25.10	27.55	23.88	26.28	26.73	26.09	26.50
MGGPPS1	25.42	26.10	25.54	25.40	26.93	23.88	26.33	26.25	26.51	26.45
MGGPPS1	25.86	25.92	25.34	24.81	27.54	23.54	26.38	26.43	26.33	26.48
AVG	25.69	26.04	25.34	25.10	27.34	23.77	26.33	26.47	26.31	26.48
StDEV	0.24	0.10	0.21	0.30	0.36	0.20	0.05	0.24	0.21	0.03
ACTIN	20.29	21.91	21.51	21.19	21.72	20.07	23.21	21.33	21.80	22.31
ACTIN	20.79	21.93	21.71	21.21	21.00	19.85	23.11	20.72	21.47	22.37
ACTIN	20.34	21.92	21.61	21.28	20.57	19.78	23.30	21.26	20.99	22.34
AVG	20.47	21.92	21.61	21.23	21.10	19.90	23.21	21.10	21.42	22.34
StDEV	0.11	0.24	0.24	0.18	0.09	0.16	0.11	0.37	0.13	0.61
Δ Ct MGGPP	-5.22	-4.12	-3.73	-3.88	-6.24	-3.87	-3.12	-5.37	-4.89	-4.14
MGGPPS1	2.69	5.76	7.55	6.81	1.32	6.86	11.48	2.42	3.37	5.69
StDEV1	2.51	5.56	8.72	6.82	1.14	6.34	11.88	2.02	3.93	5.59
StDEV2	3.24	5.52	6.56	5.54	1.75	6.34	11.48	2.82	2.94	5.79
StDEV3	2.39	6.25	7.54	8.34	1.15	8.02	11.08	2.49	3.33	5.67
StDEV	0.46	0.41	1.08	1.40	0.35	0.97	0.40	0.40	0.50	0.10

Transcript profiles of GGPPS2 at 20 and 25 DAP in 10 inbred lines										
20DAP	A619	B73	B37	Cl.7	C131A	DE3	KUI2007	NC300	SD44	TZI18
MGGPPS2	27.22	26.60	26.22	27.25	24.07	30.18	29.91	29.70	26.99	29.20
MGGPPS2	27.98	26.79	26.97	27.77	23.91	30.37	30.00	30.78	27.70	28.35
MGGPPS2	27.79	26.67	26.61	27.53	24.01	30.25	29.97	30.21	27.35	28.76
AVG	27.66	26.69	26.60	27.52	24.00	30.27	29.96	30.23	27.35	28.77
StDEV	0.40	0.10	0.38	0.26	0.08	0.10	0.05	0.54	0.36	0.43
ACTIN	21.74	22.15	21.77	23.72	20.69	21.61	21.39	19.73	21.47	21.16
ACTIN	22.08	22.41	21.65	23.44	20.83	21.83	21.04	20.84	21.69	21.55
ACTIN	21.90	22.25	21.70	23.55	20.79	21.71	21.25	20.26	21.55	21.34
AVG	21.91	22.27	21.71	23.57	20.77	21.72	21.23	20.28	21.57	21.35
StDEV	0.17	0.13	0.06	0.14	0.07	0.11	0.18	0.56	0.11	0.20
ΔCt GGPPS2	-5.76	-4.42	-4.89	-3.95	-3.23	-8.55	-8.73	-9.95	-5.78	-7.42
MGGPPS2	1.85	4.68	3.36	6.49	10.68	0.27	0.23	0.10	1.82	0.58
StDEV1	2.51	4.97	4.38	7.80	10.15	0.28	0.24	0.15	2.34	0.43
StDEV2	1.49	4.36	2.60	5.44	11.34	0.25	0.23	0.07	1.43	0.78
StDEV3	1.69	4.74	3.34	6.43	10.58	0.27	0.23	0.10	1.82	0.59
StDEV	0.54	0.31	0.89	1.19	0.60	0.02	0.01	0.04	0.46	0.17
25DAP	A619	B73	B37	Cl.7	C131A	DE3	KUI2007	NC300	SD44	TZI18
MGGPPS2	29.94	25.71	26.30	27.50	25.27	29.58	27.36	28.18	25.27	27.17
MGGPPS2	30.56	25.48	26.19	27.33	25.95	29.11	29.00	30.00	25.27	27.26
MGGPPS2	30.24	25.63	26.22	27.40	25.55	29.33	28.15	29.05	25.27	27.20
AVG	30.25	25.61	26.24	27.41	25.59	29.34	28.17	29.08	25.27	27.21
StDEV	0.31	0.12	0.06	0.09	0.34	0.24	0.82	0.91	0.00	0.05
ACTIN	20.29	21.91	21.51	21.19	21.72	20.07	23.21	21.33	21.80	21.66
ACTIN	20.79	21.93	21.71	21.21	21.00	19.85	23.11	20.72	21.47	21.58
ACTIN	20.34	21.92	21.61	21.28	20.57	19.78	23.30	21.26	20.99	21.68
AVG	20.47	21.92	21.61	21.23	21.10	19.90	23.21	21.10	21.42	21.64
StDEV	0.28	0.01	0.10	0.05	0.58	0.15	0.10	0.33	0.41	0.05
ΔCt GGPPS2	-9.77	-3.69	-4.63	-6.18	-4.49	-9.44	-4.96	-7.97	-3.85	-5.57
MGGPPS2	0.11	7.77	4.05	1.38	4.44	0.14	3.21	0.40	6.93	2.11
StDEV1	0.14	7.23	3.87	1.29	5.54	0.12	5.62	0.74	6.93	2.16
StDEV2	0.09	8.48	4.18	1.45	3.46	0.17	1.80	0.21	6.93	2.03
StDEV3	0.11	7.64	4.09	1.39	4.56	0.14	3.25	0.41	6.93	2.12
StDEV	0.02	0.64	0.16	0.08	1.04	0.02	1.93	0.27	0.00	0.07

20 DAP	A619	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZI18	B73
MPSY1	26.10	24.26	25.44	25.40	23.75	26.06	24.79	25.81	27.51	27.76
MPSY1	26.66	24.66	25.27	24.75	23.70	26.19	25.67	26.34	28.12	27.69
MPSY1	26.36	24.42	25.31	25.09	23.74	26.11	25.21	26.06	27.83	27.79
AVG	26.37	24.45	25.34	25.08	23.73	26.12	25.22	26.07	27.82	27.75
SIDEV	0.28	0.20	0.09	0.33	0.03	0.07	0.44	0.27	0.31	0.05
ACTIN	21.02	19.25	19.20	19.37	18.44	21.35	18.04	19.96	23.34	21.69
ACTIN	21.31	20.19	19.76	19.72	18.93	21.35	19.13	20.85	23.32	21.70
ACTIN	21.15	19.70	19.45	19.53	18.70	21.33	18.6	20.42	23.34	21.75
AVG	21.16	19.71	19.47	19.54	18.69	21.34	18.59	20.41	23.33	21.71
SIDEV	0.15	0.47	0.28	0.18	0.25	0.01	0.55	0.45	0.01	0.01
ΔCt MPSY1	-5.21	-4.73	-5.87	-5.54	-5.04	-4.78	-6.63	-5.66	-4.49	-6.03
PSY1	2.69	3.74	1.71	2.15	3.04	3.65	1.01	1.98	4.47	1.53
SIDEV1	3.26	4.28	1.60	1.72	3.00	3.80	1.36	2.37	5.53	1.51
SIDEV2	2.21	3.24	1.79	2.70	3.10	3.48	0.74	1.64	3.62	1.59
SIDEV3	2.72	3.83	1.75	2.13	3.02	3.67	1.02	1.99	4.43	1.48
SIDEV	0.52	0.52	0.10	0.49	0.06	0.17	0.31	0.36	0.96	0.05
25 DAP	A619	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZI18	B73
MPSY1	25.23	31.05	26.70	27.32	24.54	27.80	26.23	25.12	28.02	27.23
MPSY1	25.85	31.06	26.06	26.43	24.25	27.90	26.62	25.66	27.84	27.44
MPSY1	25.61	31.01	26.69	26.84	25.72	28.45	27.03	25.47	28.19	27.15
AVG	25.56	31.01	26.48	26.88	24.40	27.85	26.13	25.39	27.73	25.39
SIDEV	0.87	1.35	0.55	0.85	0.78	0.35	0.88	0.27	0.58	0.67
ACTIN	21.81	28.55	20.61	19.67	18.84	21.58	19.47	19.73	22.99	21.37
ACTIN	21.97	28.21	20.27	19.93	18.71	21.36	19.31	20.25	23.17	21.51
ACTIN	21.87	28.36	20.42	19.78	18.76	21.45	19.37	19.91	23.08	21.24
AVG	21.89	28.38	20.44	19.80	18.78	21.47	19.39	19.99	23.08	21.94
SIDEV	0.11	0.24	0.24	0.18	0.09	0.16	0.11	0.37	0.13	0.61
ΔCt MPSY1	-3.34	-2.63	-6.04	-7.08	-5.62	-6.38	-6.74	-5.40	-4.65	-6.45
PSY1	9.88	16.21	1.52	0.74	2.03	1.20	0.94	2.37	3.98	1.15
SIDEV1	9.88	15.71	1.30	0.54	1.84	1.24	0.87	2.86	3.26	2.56
SIDEV2	6.43	15.60	2.03	1.01	2.25	1.16	0.67	1.96	3.69	2.21
SIDEV3	7.59	16.15	1.31	0.76	0.81	0.79	0.50	2.24	2.90	2.70
SIDEV	1.76	0.29	0.42	0.23	0.74	0.24	0.19	0.46	0.40	0.25
15 DAP	A619	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZI18	B73
MPSY1	24.82	24.36	25.18	25.21	23.82	24.96	25.70	26.68	26.63	25.81
MPSY1	24.91	24.21	24.93	25.16	24.24	24.78	25.07	27.14	27.08	25.13
MPSY1	24.86	24.27	25.05	25.19	24.01	24.85	25.38	26.92	26.85	24.96
AVG	24.87	24.29	25.06	25.19	24.03	24.87	25.39	26.91	26.86	25.47
SIDEV	0.06	0.11	0.18	0.04	0.30	0.13	0.45	0.33	0.32	0.48
ACTIN	20.11	20.13	20.11	20.30	18.78	19.83	19.50	22.26	19.73	21.18
ACTIN	20.39	20.41	21.32	20.94	19.73	20.38	19.69	22.59	19.62	20.33
ACTIN	20.21	20.26	20.70	20.61	19.24	20.10	19.6	22.41	19.6	20.26
AVG	20.25	20.27	20.72	20.62	19.26	20.11	19.60	22.43	19.68	20.76
SIDEV	0.20	0.20	0.86	0.45	0.67	0.39	0.13	0.23	0.08	0.60
ΔCt MPSY1	-4.62	-4.02	-4.34	-4.57	-4.78	-4.77	-5.79	-4.49	-7.18	-4.72
PSY1	4.08	6.19	4.94	4.22	3.65	3.68	1.81	4.47	0.69	3.81
SIDEV1	4.21	5.87	4.53	4.15	4.22	3.46	1.45	5.24	0.81	3.01
SIDEV2	3.96	6.52	5.38	4.30	3.16	3.91	2.25	3.81	0.59	4.82
SIDEV3	4.09	6.25	4.95	4.21	3.70	3.73	1.81	4.43	0.69	5.42
SIDEV	0.13	0.32	0.43	0.07	0.53	0.23	0.40	0.72	0.11	1.26
10 DAP	A619	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZI18	B73
MPSY1	27.48	25.25	28.80	28.52	27.42	27.02		25.47	27.61	25.68
MPSY1	26.39	25.03	27.31	27.57	26.57	26.15		25.14	27.03	26.04
MPSY1	26.92	25.11	28.05	28.03	27.00	26.55		25.31	27.32	26.40
AVG	26.94	25.14	28.06	28.05	27.00	26.59		25.31	27.32	25.86
SIDEV	0.77	0.16	1.05	0.67	0.60	0.62		0.23	0.41	0.36
ACTIN	20.76	19.73	20.99	19.94	20.52	20.73		19.48	19.82	20.47
ACTIN	21.05	19.84	20.71	20.47	20.25	21.00		19.45	19.97	20.55
ACTIN	20.91	19.80	20.86	20.22	20.38	20.87		19.45	19.95	20.52
AVG	20.91	19.79	20.85	20.21	20.38	20.87		19.46	19.91	20.51
SIDEV	0.22	0.09	0.18	0.39	0.20	0.19		0.01	0.11	0.05
ΔCt MPSY1	-6.02	-5.35	-7.20	-7.83	-6.62	-5.72		-5.84	-7.42	-5.36
PSY1	1.54	2.46	0.68	0.44	1.02	1.90		1.75	0.58	2.44
SIDEV1	1.05	2.28	0.41	0.32	0.76	1.40		1.54	0.49	2.80
SIDEV2	2.24	2.66	1.14	0.61	1.37	2.56		1.94	0.74	2.18
SIDEV3	1.55	2.51	0.68	0.44	1.02	1.94		1.72	0.60	1.70
SIDEV	0.60	0.19	0.37	0.15	0.31	0.58		0.20	0.12	0.55

20 DAP	A619	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZI18	B73
MPSY2	27.60	27.95	27.92	26.90	25.78	27.13	25.75	27.69	28.90	26.40
MPSY2	27.43	27.24	26.88	26.21	25.61	27.15	25.27	27.02	28.38	27.15
MPSY2	27.51	27.59	27.41	26.55	25.70	27.13	25.50	27.34	28.65	27.93
AVG	27.52	27.60	27.40	26.56	25.70	27.14	25.51	27.36	28.64	26.78
StDEV	0.12	0.50	0.74	0.49	0.12	0.01	0.34	0.47	0.37	0.53
ACTIN	22.75	20.31	21.20	20.68	20.42	21.82	19.91	22.49	23.46	21.69
ACTIN	24.07	22.06	21.93	21.27	20.05	22.07	19.85	21.95	23.30	21.70
ACTIN	23.42	21.19	21.56	20.96	20.23	21.96	19.87	22.20	23.35	21.75
AVG	23.41	21.19	21.57	20.98	20.24	21.95	19.88	22.22	23.38	21.70
StDEV	0.93	1.24	0.52	0.42	0.26	0.18	0.04	0.38	0.11	0.01
ΔCI MPSY	-4.11	-6.41	-5.84	-5.58	-5.46	-5.20	-5.63	-5.14	-5.26	-5.08
PSY2	5.81	1.18	1.75	2.09	2.27	2.73	2.02	2.85	2.61	2.96
StDEV1	5.48	0.92	1.22	1.65	2.14	2.75	1.71	2.26	2.18	3.83
StDEV2	6.16	1.50	2.51	2.66	2.41	2.71	2.38	3.59	3.13	2.28
StDEV3	5.83	1.18	1.74	2.10	2.26	2.75	2.03	2.88	2.59	1.33
StDEV	0.34	0.29	0.65	0.51	0.13	0.02	0.34	0.67	0.47	1.27
25 DAP	A619	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZI18	B73
MPSY2	27.16	30.65	27.65	25.94	25.23	27.51	26.74	27.41	28.81	26.39
MPSY2	27.80	30.56	27.48	26.40	24.81	28.24	28.78	29.80	29.30	26.35
MPSY2	27.47	30.62	27.56	26.15	25.03	27.85	27.77	28.60	29.04	26.65
AVG	27.48	30.61	27.57	26.17	25.02	27.88	27.76	28.61	29.06	26.37
StDEV	0.45	0.06	0.12	0.33	0.30	0.52	1.44	1.69	0.35	0.03
ACTIN	23.08	29.64	22.31	20.74	19.84	22.53	18.99	20.97	24.31	22.37
ACTIN	25.66	30.46	22.30	21.90	21.78	22.32	21.85	21.85	23.96	21.51
ACTIN	24.37	30.02	22.35	21.33	20.81	22.44	20.42	21.41	24.14	21.24
AVG	24.37	30.05	22.31	21.32	20.81	22.43	20.42	21.41	24.14	21.94
StDEV	1.82	0.58	0.01	0.82	1.37	0.15	2.02	0.62	0.25	0.61
ΔCI MPSY	-3.11	-0.55	-5.26	-4.85	-4.21	-5.45	-7.34	-7.20	-4.92	-4.43
PSY2	11.58	68.07	2.61	3.47	5.40	2.29	0.62	0.68	3.30	4.64
StDEV1	14.46	65.98	2.46	4.07	4.67	2.95	1.25	1.56	3.91	4.58
StDEV2	9.28	70.22	2.77	2.96	6.25	1.78	0.30	0.30	2.79	4.70
StDEV3	11.66	67.36	2.62	3.52	5.37	2.33	0.61	0.68	3.34	3.82
StDEV	2.59	2.17	0.15	0.56	0.79	0.59	0.48	0.65	0.56	0.48
15 DAP	A619	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZI18	B73
MPSY2	27.45	26.70	26.87	28.58	25.84	30.57	28.87	29.78	27.84	26.26
MPSY2	26.68	26.48	25.98	27.61	24.92	27.47	27.16	28.05	26.88	26.03
MPSY2	27.05	26.58	26.42	28.10	25.38	29.03	28.00	28.90	27.34	26.50
AVG	27.07	26.59	26.43	28.10	25.38	29.02	28.02	28.92	27.36	26.15
StDEV	0.54	0.16	0.63	0.69	0.65	2.19	1.21	1.22	0.68	0.16
ACTIN	20.27	20.94	20.67	20.32	19.89	20.42	21.05	21.87	19.54	21.18
ACTIN	19.74	20.70	20.63	19.63	18.33	20.19	19.16	22.10	19.87	20.33
ACTIN	20.02	20.80	20.65	19.98	19.10	20.32	20.13	21.97	19.72	20.26
AVG	20.01	20.82	20.65	19.98	19.11	20.31	20.11	21.99	19.71	20.76
StDEV	0.37	0.17	0.03	0.49	1.10	0.16	1.34	0.16	0.23	0.60
ΔCI MPSY	-7.06	-5.77	-5.78	-8.12	-6.27	-8.72	-7.91	-6.93	-7.66	-5.39
PSY2	0.75	1.83	1.83	0.36	1.30	0.24	0.42	0.82	0.50	2.38
StDEV1	0.57	1.70	1.34	0.26	0.94	0.08	0.23	0.45	0.36	2.20
StDEV2	0.98	1.98	2.49	0.50	1.78	0.70	0.75	1.49	0.69	2.58
StDEV3	0.76	1.85	1.83	0.36	1.30	0.24	0.42	0.83	0.50	1.86
StDEV	0.20	0.14	0.57	0.12	0.42	0.32	0.26	0.53	0.17	0.36
10 DAP	A619	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZI18	B73
MPSY2	25.75	24.36	25.76	25.83	25.59	25.62		26.36	25.91	25.67
MPSY2	25.75	24.76	26.02	26.37	25.83	25.93		26.11	26.55	25.21
MPSY2	25.75	24.55	25.87	26.12	25.72	25.76		26.00	26.23	25.20
AVG	25.75	24.56	25.89	26.10	25.71	25.78		26.24	26.23	25.44
StDEV	0.00	0.28	0.18	0.38	0.17	0.22		0.18	0.45	0.27
ACTIN	20.70	19.75	20.53	20.65	20.15	20.65		20.53	21.31	20.47
ACTIN	21.58	19.84	23.01	21.84	21.81	22.75		22.12	20.73	20.54
ACTIN	21.12	19.82	21.75	21.24	20.95	21.68		21.32	21.00	20.44
AVG	21.14	19.80	21.77	21.25	20.98	21.70		21.33	21.02	20.51
StDEV	0.62	0.06	1.75	0.84	1.17	1.48		1.12	0.41	0.05
ΔCI MPSY	-4.61	-4.77	-4.12	-4.86	-4.73	-4.08		-4.91	-5.21	-4.94
PSY2	4.09	3.68	5.75	3.46	3.77	5.93		3.33	2.70	3.27
StDEV1	4.09	4.22	6.29	4.17	4.09	6.61		3.05	3.37	2.79
StDEV2	4.09	3.20	5.26	2.87	3.47	5.33		3.63	2.16	3.83
StDEV3	4.09	3.70	5.83	3.41	3.74	6.00		3.91	2.70	3.86
StDEV	0.00	0.51	0.52	0.65	0.31	0.64		0.44	0.61	0.61

20 DAP	A619	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZI18	B73
MPSY3	33.38	28.78	30.89	28.43	27.65	29.74	31.12	28.14	29.78	30.75
MPSY3	33.38	29.94	31.26	29.10	27.20	29.69	31.62	28.55	32.11	31.43
MPSY3	33.38	29.34	31.06	28.74	27.42	29.70	31.35	28.33	30.92	29.62
AVG	33.38	29.36	31.08	28.77	27.43	29.72	31.37	28.35	30.95	30.61
SiDEV	0.00	0.82	0.26	0.47	0.32	0.04	0.35	0.29	1.65	0.48
ACTIN	22.70	20.71	20.71	20.39	19.54	20.98	19.20	21.75	22.07	21.69
ACTIN	22.76	20.88	20.82	20.82	19.72	21.27	19.69	21.69	22.55	21.70
ACTIN	22.72	20.83	20.78	20.60	19.67	21.11	19.46	21.75	22.33	21.75
AVG	22.73	20.80	20.77	20.61	19.63	21.12	19.45	21.72	22.31	21.70
SiDEV	0.04	0.12	0.08	0.30	0.13	0.20	0.35	0.04	0.34	0.01
ΔCt MPSY3	-10.65	-8.57	-8.57	-8.16	-7.80	-8.60	-11.93	-6.63	-8.64	-8.92
PSY3	0.06	0.26	0.08	0.35	0.45	0.26	0.03	1.01	0.25	0.21
SiDEV1	0.06	0.39	0.09	0.44	0.39	0.25	0.03	1.17	0.56	0.19
SiDEV2	0.06	0.18	0.07	0.28	0.53	0.26	0.02	0.88	0.11	0.12
SiDEV3	0.06	0.27	0.08	0.36	0.45	0.26	0.03	1.02	0.26	0.41
SiDEV	0.00	0.11	0.01	0.08	0.07	0.00	0.00	0.14	0.23	0.15
25 DAP	A619	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZI18	B73
MPSY3	30.77	31.03	31.05	29.13	26.90	29.77	29.14	30.05	30.48	32.07
MPSY3	30.86	30.71	29.32	28.34	27.94	30.76	30.87	26.86	30.27	31.05
MPSY3	30.81	30.84	30.15	28.76	27.40	30.24	30.05	28.44	30.32	30.91
AVG	30.82	30.87	30.19	28.74	27.42	30.27	30.01	28.46	30.38	31.34
SiDEV	0.06	0.23	1.22	0.56	0.74	0.70	1.22	2.26	0.15	0.63
ACTIN	22.97	30.66	20.91	20.98	19.81	22.42	20.57	20.88	23.99	22.37
ACTIN	23.32	32.08	20.67	21.48	19.75	22.48	20.27	21.08	24.30	21.51
ACTIN	23.14	31.32	20.75	21.24	19.77	22.45	20.41	20.97	24.13	21.24
AVG	23.15	31.37	20.79	21.23	19.78	22.45	20.42	20.98	24.15	21.38
SiDEV	0.25	1.00	0.17	0.35	0.04	0.04	0.21	0.14	0.22	0.61
ΔCt MPSY3	-7.67	0.50	-9.40	-7.51	-7.64	-7.82	-9.59	-7.48	-6.23	-9.96
PSY3	0.49	0.41	0.15	0.55	0.50	0.44	0.13	0.56	1.33	0.10
SiDEV1	0.51	126.58	0.08	0.42	0.72	0.63	0.24	0.19	1.24	0.06
SiDEV2	0.48	158.01	0.27	0.72	0.35	0.32	0.07	1.70	1.43	0.12
SiDEV3	0.49	144.39	0.15	0.54	0.51	0.45	0.13	0.57	1.38	0.14
SiDEV	0.02	15.76	0.10	0.15	0.19	0.16	0.08	0.79	0.10	0.04
15 DAP	A619	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZI18	B73
MPSY3	33.43	29.25	31.80	30.60	28.56	30.85	29.87	30.07	31.73	30.36
MPSY3	33.43	29.42	29.71	29.72	28.56	30.74	31.11	29.73	32.12	30.51
MPSY3	33.43	29.35	30.74	30.14	28.52	30.82	30.45	29.88	31.97	30.50
AVG	33.43	29.34	30.76	30.16	28.56	30.80	30.49	29.90	31.93	30.46
SiDEV	0.00	0.12	1.48	0.62	0.00	0.08	0.88	0.24	0.28	0.11
ACTIN	21.16	21.07	21.82	20.40	18.96	20.73	19.36	22.34	20.02	21.38
ACTIN	20.77	22.01	21.78	20.80	19.45	21.03	20.29	22.75	20.29	20.33
ACTIN	20.96	21.52	21.78	20.57	19.20	20.85	19.84	22.57	20.14	20.26
AVG	20.97	21.54	21.80	20.60	19.21	20.88	19.83	22.55	20.16	20.59
SiDEV	0.28	0.66	0.03	0.28	0.35	0.21	0.66	0.29	0.19	0.74
ΔCt MPSY3	-12.47	-7.80	-8.96	-9.56	-9.36	-9.92	-10.67	-7.36	-11.77	-9.87
PSY3	0.02	0.45	0.13	0.13	0.15	0.10	0.06	0.61	0.03	0.11
SiDEV1	0.02	0.48	0.10	0.10	0.15	0.10	0.09	0.54	0.03	0.11
SiDEV2	0.02	0.42	0.42	0.18	0.15	0.11	0.04	0.69	0.03	0.10
SiDEV3	0.02	0.45	0.20	0.13	0.16	0.10	0.06	0.62	0.03	0.10
SiDEV	0.00	0.03	0.16	0.04	0.00	0.00	0.03	0.07	0.00	0.01
10 DAP	A619	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZI18	B73
MPSY3	32.67	27.24	26.82	27.64	26.01	27.04		29.41	29.92	27.76
MPSY3	32.67	26.57	26.97	28.25	27.22	26.47		28.60	31.20	28.15
MPSY3	32.67	26.92	26.85	27.94	26.65	26.75		29.08	30.55	28.09
AVG	32.67	26.91	26.90	27.95	26.62	26.76		29.01	30.56	28.00
SiDEV	0.00	0.47	0.11	0.43	0.86	0.40		0.57	0.91	0.21
ACTIN	21.67	21.07	22.01	21.76	21.01	22.06		20.62	21.50	20.47
ACTIN	21.39	20.83	21.71	22.50	21.55	22.07		22.51	20.83	20.54
ACTIN	21.54	20.98	21.87	22.15	21.28	22.05		21.51	21.14	20.44
AVG	21.53	20.95	21.86	22.13	21.28	22.07		21.57	21.17	20.48
SiDEV	0.20	0.17	0.21	0.52	0.38	0.01		1.34	0.47	0.05
ΔCt MPSY3	-11.14	-5.96	-5.04	-5.82	-5.34	-4.69		-7.44	-9.40	-7.52
PSY3	0.04	1.61	3.05	1.78	2.48	3.87		0.58	0.15	0.54
SiDEV1	0.04	1.28	3.21	2.19	3.77	3.18		0.43	0.23	0.64
SiDEV2	0.04	2.03	2.90	1.44	1.63	4.72		0.76	0.10	0.49
SiDEV3	0.04	1.60	3.15	1.78	2.42	3.89		0.55	0.15	0.51
SiDEV	0.00	0.38	0.17	0.38	1.08	0.77		0.17	0.07	0.08

25 DAP	DE3	TZ118	C17	NC300	KUI2007	SD44	B37	C131A	A619	B73
MPDS	23.82	28.60	24.60	24.48	27.35	24.13	30.31	25.87	25.43	28.89
MPDS	23.83	28.45	24.09	24.94	27.47	23.34	28.18	25.31	25.24	28.59
MPDS	23.84	28.54	24.33	24.72	27.42	23.72	29.23	25.60	26.98	29.37
Average	23.83	28.53	24.34	24.71	27.41	23.73	29.24	25.59	25.88	28.95
StDev	0.01	0.08	0.26	0.23	0.06	0.40	1.07	0.28	0.13	0.39
MZDS	26.75	27.83	24.87	26.24	27.91	26.53	29.60	25.09	25.87	25.59
MZDS	26.20	27.76	24.71	25.77	27.94	26.66	29.50	25.35	26.19	25.95
MZDS	26.46	27.81	24.80	26.02	27.94	26.61	29.53	25.23	30.16	25.75
Avrage	26.47	27.80	24.79	26.01	27.93	26.60	29.54	25.22	27.41	25.76
StDev	0.28	0.04	0.08	0.24	0.02	0.07	0.05	0.13	2.39	0.18
Actin	19.89	22.97	19.90	20.52	22.07	21.34	26.44	23.47	22.48	20.46
Actin	20.07	23.65	20.90	20.77	22.90	20.72	27.34	21.76	22.93	20.16
Actin	19.96	23.32	20.41	20.66	22.50	21.04	26.87	22.60	20.60	20.32
Average	19.97	23.31	20.40	20.65	22.49	21.03	26.88	22.61	22.00	20.31
StDev	0.13	0.48	0.71	0.18	0.59	0.44	0.64	1.21	1.24	0.15
Act PDS	-3.86	-5.22	-3.94	-4.06	-4.92	-2.70	-2.36	-2.98	-3.88	-8.64
Act ZDS	-6.50	-4.49	-4.39	-5.36	-5.44	-5.57	-2.66	-2.61	-5.40	-5.45
PDS	6.90	2.69	6.53	5.98	3.30	15.42	19.52	12.65	6.79	2.64
ZDS	1.11	4.46	4.77	2.43	2.30	2.11	15.82	16.34	2.36	2.29
StDEV1	6.95	2.56	5.45	7.03	3.44	11.69	9.30	10.44	9.30	0.26
StDEV2	6.90	2.84	7.77	5.11	3.17	20.21	40.71	15.39	10.61	0.32
StDEV3	6.86	2.67	6.58	5.95	3.28	15.53	19.66	12.59	3.18	0.19
StDEV	0.05	0.14	1.16	0.96	0.14	4.27	16.00	2.48	3.97	0.07
StDEV1	0.91	4.37	4.52	2.08	2.34	2.21	15.21	17.92	6.86	2.58
StDEV2	1.34	4.59	5.05	2.88	2.29	2.02	16.30	14.97	5.49	2.01
StDEV3	1.12	4.43	4.75	2.42	2.29	2.10	15.97	16.27	0.35	2.31
StDEV	0.21	0.11	0.27	0.40	0.03	0.10	0.56	1.48	3.43	0.28
20DAP	TZ118	C131A	C17	SD44	B37	NC300	DE3	A619	KUI2007	B73
MZDS	28.01	23.66	24.58	26.22	25.71	24.19	26.93	25.73	26.99	25.56
MZDS	28.32	24.75	25.22	25.84	25.32	24.52	26.64	25.03	27.02	25.82
MZDS	27.71	24.08	24.62	25.77	25.40	25.16	26.79	25.38	27.01	25.70
AVG	28.01	24.16	24.81	25.94	25.48	24.62	26.79	25.38	27.01	25.69
STDEV	0.31	0.55	0.36	0.24	0.21	0.49	0.15	0.35	0.02	0.13
MPDS	27.35	23.05	24.63	23.11	24.80	24.25	24.20	23.68	25.93	27.79
MPDS	27.95	23.05	23.35	23.24	23.72	24.15	24.73	23.97	27.31	27.92
MPDS	27.66	24.50	24.73	23.78	24.76	25.77	24.47	23.83	26.62	27.66
AVG	27.65	23.53	24.24	23.38	24.43	24.72	24.47	23.83	26.62	27.79
STDEV	0.30	0.84	0.77	0.36	0.61	0.91	0.27	0.15	0.69	0.13
ACTIN	20.42	19.65	19.86	20.57	20.45	19.16	21.98	21.90	21.19	20.25
ACTIN	19.97	19.24	19.96	20.35	20.32	19.44	23.62	23.29	22.29	20.22
ACTIN	20.22	19.21	19.74	20.71	20.22	19.47	22.80	22.60	21.74	20.25
AVG	20.20	19.37	19.85	20.54	20.33	19.36	22.80	22.60	21.74	20.24
stDEV	0.23	0.25	0.11	0.18	0.12	0.17	0.82	0.70	0.55	0.02
Act ZDS	-7.81	-4.80	-4.95	-5.40	-5.15	-5.27	-3.99	-2.78	-5.27	-5.45
Act PDS	-7.45	-4.17	-4.38	-2.83	-4.10	-5.37	-1.67	-1.23	-4.88	-4.88
ZDS	0.45	3.60	3.23	2.37	2.82	2.60	6.31	14.53	2.60	2.50
PDS	0.57	5.57	4.79	14.03	5.84	2.42	31.50	42.63	3.40	2.28
StDEV1	0.45	5.10	3.78	1.96	2.40	3.51	5.71	11.40	2.63	2.50
StDEV2	0.36	2.40	2.42	2.54	3.15	2.79	6.98	18.51	2.57	2.09
StDEV3	0.55	3.81	3.67	2.67	2.98	1.79	6.29	14.53	2.59	2.27
StDEV	0.09	1.35	0.75	0.38	0.39	0.86	0.64	3.57	0.03	0.21
StDEV1	0.71	7.78	3.65	16.88	4.51	3.36	37.89	47.19	5.48	0.53
StDEV2	0.47	7.78	8.86	15.42	9.54	3.61	26.24	38.60	2.11	0.49
StDEV3	0.57	2.85	3.40	10.61	4.64	1.17	31.43	42.53	3.40	0.58
StDEV	0.12	2.85	3.08	3.28	2.87	1.34	5.84	4.30	1.70	0.05

15 DAP	DE3	TZI18	C17	NC300	KUI2007	SD44	B37	C131A	A619	B73
MPDS	22.78	25.06	23.77	23.87	26.04	25.28	24.82	25.41	24.17	25.79
MPDS	22.39	25.60	23.91	23.55	25.55	25.50	24.68	25.03	24.39	25.44
MPDS	22.57	25.31	23.82	23.72	25.81	25.37	24.73	25.20	26.96	25.62
Average	22.58	25.32	23.83	23.71	25.80	25.38	24.74	25.21	25.17	25.62
StDev	0.20	0.27	0.07	0.16	0.25	0.11	0.07	0.19	0.16	0.18
MZDS	24.48	25.92	24.85	24.73	25.65	27.87	25.33	25.20	25.92	25.75
MZDS	24.87	26.80	26.12	25.65	25.76	27.88	26.70	25.79	26.28	26.08
MZDS	24.69	26.37	25.50	25.20	25.72	27.89	26.03	25.51	30.16	25.93
Avrage	24.68	26.36	25.49	25.19	25.71	27.88	26.02	25.50	27.45	25.92
StDev	0.20	0.44	0.64	0.46	0.06	0.01	0.69	0.30	2.35	0.17
Actin	20.20	19.90	20.10	19.64	21.67	23.07	22.12	21.51	22.13	20.56
Actin	21.61	20.71	20.28	18.82	19.57	21.56	21.06	21.06	20.32	20.60
Actin	20.89	20.32	20.20	19.24	20.63	22.33	21.57	21.27	20.60	20.59
Average	20.90	20.31	20.19	19.23	20.62	22.32	21.58	21.28	21.02	20.58
StDev	1.00	0.57	0.13	0.58	1.48	1.07	0.75	0.32	0.97	0.02
ΔCt PDS	-1.68	-5.01	-3.64	-4.48	-5.18	-3.06	-3.16	-3.93	-4.16	-5.03
ΔCt ZDS	-3.78	-6.05	-5.30	-5.96	-5.09	-5.56	-4.44	-4.22	-6.44	-5.34
PDS	31.21	3.10	8.02	4.48	2.76	11.96	11.19	6.55	5.61	2.36
ZDS	7.28	1.51	2.54	1.61	2.94	2.12	4.62	5.37	1.15	2.47
StDEV1	27.17	3.72	8.38	4.02	2.34	12.85	10.61	5.71	11.24	2.71
StDEV2	35.60	2.56	7.61	5.02	3.29	11.03	11.69	7.43	9.65	3.45
StDEV3	31.43	3.13	8.10	4.46	2.75	12.07	11.29	6.61	1.63	3.05
StDEV	4.22	0.58	0.39	0.50	0.48	0.91	0.55	0.86	5.15	0.37
StDEV1	8.36	2.05	3.96	2.21	3.07	2.13	7.45	6.61	3.34	2.78
StDEV2	6.38	1.11	1.64	1.17	2.84	2.12	2.88	4.39	2.60	2.21
StDEV3	7.23	1.50	2.53	1.60	2.92	2.11	4.59	5.33	0.18	2.46
StDEV	0.99	0.47	1.17	0.52	0.11	0.01	2.31	1.11	1.66	0.29
10 DAP	DE3	TZI18	C17	KUI2007	SD44	B37	C131A	A619	NC300	B73
MPDS	25.12	25.55	24.92	26.29	24.34	26.24	25.68	25.11		26.70
MPDS	25.45	26.25	24.90	25.48	24.27	25.08	25.87	24.47		26.25
MPDS	25.30	25.91	24.92	25.87	24.32	25.64	25.79	24.77		26.23
Average	25.29	25.90	24.91	25.88	24.31	25.65	25.78	24.78		26.39
StDev	0.17	0.35	0.01	0.41	0.04	0.58	0.10	0.45		0.27
MZDS	25.94	25.10	25.70	24.74	25.16	25.50	25.60	25.52		25.66
MZDS	25.31	25.00	24.20	23.63	24.17	24.60	25.02	24.75		25.57
MZDS	25.64	25.06	24.93	24.20	24.65	25.06	25.32	25.15		25.63
Avrage	25.63	25.05	24.94	24.19	24.66	25.05	25.31	25.14		25.62
StDev	0.32	0.05	0.75	0.56	0.50	0.45	0.29	0.39		0.05
Actin	21.12	19.72	20.51	20.70	19.01	18.93	21.01	20.76		21.09
Actin	21.09	19.91	20.45	22.01	19.83	19.50	22.00	21.23		20.87
Actin	21.12	19.83	20.49	21.37	19.43	19.23	21.49	20.60		21.17
Average	21.11	19.82	20.48	21.36	19.42	19.22	21.50	20.86		21.04
StDev	0.02	0.13	0.04	0.93	0.58	0.40	0.70	0.33		0.16
ΔCt PDS	-4.18	-6.08	-4.43	-4.52	-4.89	-6.43	-4.28	-3.92		-5.35
ΔCt ZDS	-4.52	-5.23	-4.46	-2.83	-5.24	-5.83	-3.81	-4.28		-4.58
PDS	5.52	1.47	4.64	4.36	3.38	1.16	5.15	6.61		2.45
ZDS	4.36	2.66	4.54	14.06	2.65	1.75	7.11	5.16		4.19
StDEV1	6.21	1.88	4.62	3.28	3.31	0.77	5.52	5.27		1.98
StDEV2	4.94	1.16	4.68	5.75	3.48	1.72	4.84	8.21		2.71
StDEV3	5.48	1.47	4.62	4.39	3.36	1.17	5.11	6.67		2.75
StDEV	0.64	0.36	0.04	1.24	0.08	0.48	0.34	1.47		0.43
StDEV1	3.52	2.57	2.69	9.61	1.88	1.29	5.83	3.96		4.08
StDEV2	5.44	2.76	7.61	20.73	3.72	2.40	8.72	6.76		4.34
StDEV3	4.33	2.65	4.59	13.97	2.67	1.75	7.08	5.12		4.16
StDEV	0.97	0.09	2.48	5.61	0.93	0.56	1.45	1.40		0.13

20 DAP	A619	B73	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZ118
MISO1	28.03	27.74	27.95	30.11	25.03	27.97	28.93	25.42	27.94	28.87
MISO1	28.00	27.61	27.95	30.02	24.67	27.87	29.11	25.64	28.17	29.38
MISO1	28.00	27.68	28.05	29.60	24.79	27.70	29.13	24.94	27.68	29.15
AVG	28.01	27.68	27.98	29.91	24.83	27.85	29.06	25.33	27.93	29.13
SiDEV	0.02	0.07	0.06	0.27	0.18	0.14	0.11	0.36	0.25	0.26
ACTIN	20.88	22.15	20.53	21.81	19.39	19.90	20.17	19.34	21.55	21.03
ACTIN	21.47	21.73	21.33	22.20	19.95	19.97	20.15	19.88	22.65	20.51
ACTIN	21.10	21.94	20.63	21.90	19.68	19.97	20.15	20.02	22.12	19.95
AVG	21.15	21.94	20.83	21.97	19.67	19.95	20.16	19.75	22.11	20.50
SiDEV	0.30	0.21	0.44	0.20	0.28	0.04	0.01	0.36	0.55	0.01
ΔCI MISO1	-6.86	-5.74	-7.15	-7.94	-5.16	-7.90	-8.90	-5.59	-5.82	-8.64
ISO1	0.86	1.88	0.70	0.41	2.80	0.42	0.21	2.08	1.77	0.25
SiDEV1	0.85	1.79	0.72	0.35	2.44	0.38	0.23	1.96	1.75	0.30
SiDEV2	0.87	1.96	0.72	0.38	3.13	0.41	0.20	1.68	1.50	0.21
SiDEV3	0.87	1.88	0.67	0.50	2.88	0.46	0.20	2.73	2.10	0.25
SiDEV	0.01	0.08	0.03	0.08	0.35	0.04	0.02	0.54	0.30	0.05
25 DAP	A619	B73	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZ118
MISO1	28.10	27.75	27.98	27.27	26.08	26.37	28.00	26.02	27.27	29.41
MISO1	28.87	27.10	27.69	27.99	26.11	26.35	28.22	26.18	27.03	28.88
MISO1	28.85	27.43	27.19	27.88	26.10	26.36	28.12	26.10	27.15	28.77
AVG	28.61	27.43	27.62	27.71	26.10	26.36	28.11	26.10	27.15	29.02
SiDEV	0.87	1.35	0.55	0.85	0.78	0.35	0.88	0.27	0.58	0.67
ACTIN	22.27	22.75	20.88	21.32	21.57	19.96	22.94	20.86	21.19	22.07
ACTIN	21.76	22.72	20.98	20.62	22.12	20.74	23.41	20.84	20.91	21.41
ACTIN	21.95	22.74	21.12	20.97	21.85	20.35	23.78	20.02	21.05	21.34
AVG	21.99	22.74	20.99	20.97	21.85	20.35	23.38	20.57	21.05	21.61
SiDEV	0.11	0.24	0.24	0.18	0.09	0.16	0.11	0.37	0.13	0.61
ΔCI MISO1	-6.61	-4.69	-6.63	-6.74	-4.25	-6.01	-4.74	-5.53	-6.10	-7.41
ISO1	1.02	3.87	1.01	0.93	5.26	1.55	3.75	2.17	1.46	0.59
SiDEV1	1.45	3.09	0.79	1.27	5.32	1.54	4.06	2.29	1.34	0.45
SiDEV2	0.85	4.85	0.96	0.77	5.21	1.56	3.48	2.05	1.58	0.65
SiDEV3	0.86	3.87	1.36	0.83	5.24	1.55	3.73	2.17	1.46	0.70
SiDEV	0.34	0.88	0.29	0.27	0.06	0.01	0.29	0.12	0.12	0.13
15 DAP	A619	B73	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZ118
MISO1	28.17	26.91	27.13	28.93	24.30	25.93	28.45	25.60	28.79	27.78
MISO1	27.94	26.81	27.39	28.28	25.02	26.44	30.55	26.73	29.56	27.13
MISO1	24.85	24.27	25.05	25.17	24.01	24.85	25.30	26.90	26.84	25.44
AVG	24.87	24.29	25.06	25.19	24.03	24.87	25.39	26.91	26.86	25.47
SiDEV	0.06	0.11	0.18	0.04	0.30	0.13	0.45	0.33	0.32	0.48
ACTIN	21.38	20.05	22.12	22.74	22.04	20.55	21.52	21.32	23.98	21.64
ACTIN	21.79	20.90	22.69	22.90	21.78	19.86	21.26	20.25	23.74	20.84
ACTIN	20.23	20.25	20.71	20.60	19.27	20.10	19.65	22.41	19.67	20.75
AVG	20.25	20.27	20.72	20.62	19.26	20.11	19.60	22.43	19.68	20.76
SiDEV	0.20	0.20	0.86	0.45	0.67	0.39	0.13	0.23	0.08	0.60
ΔCI MISO1	-4.62	-4.02	-4.34	-4.57	-4.78	-4.77	-5.79	-4.49	-7.18	-4.72
ISO1	4.08	6.19	4.94	4.22	3.65	3.68	1.81	4.47	0.69	3.81
SiDEV1	0.41	1.00	1.17	0.32	3.03	1.76	0.22	11.07	0.18	0.77
SiDEV2	0.48	1.07	0.98	0.49	1.84	1.24	0.05	5.06	0.11	1.20
SiDEV3	4.12	6.25	4.95	4.27	3.70	3.73	1.92	4.50	0.70	3.89
SiDEV	2.12	3.01	2.24	2.23	0.94	1.31	1.03	3.64	0.32	1.69
10DAP	A619	B37	C17	C131A	DE3	KUI2007	SD44	TZ118	B73	TZ118
MISO	26.95	24.87	25.59	23.65	24.94	25.53	25.20	25.62	26.26	25.62
MISO	26.84	24.98	25.69	24.24	24.89	24.99	25.48	24.92	26.31	24.90
MISO	26.91	24.90	25.65	23.90	24.95	25.23	25.39	25.24	26.27	25.68
AVG	26.90	24.93	25.64	23.95	24.92	25.26	25.34	25.27	26.29	25.86
SiDEV	0.08	0.08	0.07	0.42	0.04	0.38	0.20	0.49	0.04	0.36
ACTIN	20.45	20.69	20.20	19.98	20.40	21.17	19.77	19.82	20.39	19.82
ACTIN	20.12	19.42	20.39	20.04	20.04	20.78	20.04	20.73	20.73	20.73
ACTIN	20.27	20.04	20.28	20.00	20.20	20.95	19.90	20.25	20.55	20.50
AVG	20.28	20.05	20.29	20.01	20.21	20.97	19.91	20.28	20.56	20.51
SiDEV	0.17	0.64	0.10	0.03	0.18	0.20	0.19	0.64	0.24	0.05
ΔCI MISO	-6.62	-4.88	-5.35	-3.94	-4.70	-4.29	-5.44	-5.00	-5.73	-5.36
MISO	1.02	3.41	2.45	6.52	3.84	5.10	2.31	3.14	1.89	2.44
SiDEV1	0.98	3.54	2.54	8.00	3.78	4.23	2.55	2.46	1.92	2.89
SiDEV2	1.06	3.28	2.37	5.32	3.91	6.15	2.10	4.00	1.86	4.75
SiDEV3	1.01	3.47	2.43	6.73	3.75	5.21	2.23	3.20	1.91	2.77
SiDEV	0.04	0.13	0.09	1.34	0.09	0.96	0.23	0.77	0.03	1.11

25DAP	TZI18	C131A	B37	A619	DE3	C17	NC300	SD44	B73	KUI2007
MLCYB	31.06	30.88	28.14	28.53	28.06	30.12	31.18	29.63	30.22	29.17
MLCYB	31.03	30.76	27.82	28.58	28.27	30.16	31.26	29.62	30.22	28.73
MLCYB	31.01	30.82	28.02	28.56	28.17	30.19	31.19	29.65	30.31	28.95
AVG	31.03	30.82	27.99	28.56	28.17	30.16	31.21	29.63	30.25	28.95
STDEV	0.03	0.06	0.16	0.03	0.11	0.04	0.04	0.02	0.00	0.22
MLCYE	34.95	30.53	29.32	33.13	31.52	32.07	31.01	28.82	28.26	32.97
MLCYE	34.48	30.87	28.62	33.16	30.32	32.50	31.01	28.97	28.13	32.96
MLCYE	35.11	30.93	29.00	33.24	30.92	32.29	31.01	28.90	28.15	32.84
AVG	34.85	30.78	28.98	33.18	30.92	32.29	31.01	28.90	28.18	32.92
STDEV	0.33	0.22	0.35	0.06	0.60	0.22	0.00	0.08	0.07	0.07
ACTIN	23.30	20.94	20.75	21.44	19.62	20.13	20.00	20.71	21.72	21.02
ACTIN	22.70	20.42	21.11	21.48	20.59	21.55	20.58	20.88	22.09	21.60
ACTIN	19.09	19.00	19.54	18.97	20.11	20.84	20.29	20.80	22.08	20.01
AVG	21.70	20.12	20.47	20.63	20.11	20.84	20.29	20.80	21.96	20.88
STDEV	0.42	0.37	0.25	0.03	0.69	1.00	0.41	0.12	0.21	0.41
ΔCtLCYB	-9.34	-10.70	-7.53	-7.93	-8.06	-9.32	-10.92	-8.84	-8.29	-8.07
ΔCtLCYE	-13.90	-10.66	-8.51	-12.55	-10.81	-11.45	-10.72	-8.10	-6.22	-12.05
LCYB	0.15	0.06	0.54	0.41	0.37	0.16	0.05	0.22	0.32	0.37
LCYE	0.01	0.06	0.27	0.02	0.06	0.04	0.06	0.36	1.34	0.02
SLCYB	0.15	0.06	0.49	0.42	0.40	0.16	0.05	0.22	0.33	0.32
SLCYB	0.16	0.06	0.61	0.40	0.35	0.16	0.05	0.22	0.33	0.43
SLCYB	0.16	0.06	0.53	0.41	0.37	0.15	0.05	0.22	0.31	0.37
Average	0.00	0.00	0.06	0.01	0.03	0.00	0.00	0.00	0.01	0.06
SLCYE	0.01	0.06	0.27	0.02	0.06	0.04	0.06	0.36	1.34	0.02
SLCYE	0.01	0.06	0.35	0.02	0.08	0.03	0.06	0.35	1.39	0.02
SLCYE	0.01	0.06	0.27	0.02	0.06	0.04	0.06	0.36	1.37	0.03
Average	0.00	0.00	0.05	0.00	0.02	0.00	0.00	0.01	0.02	0.00
20DAP	TZI18	C131A	B37	A619	DE3	C17	NC300	SD44	B73	KUI2007
MLCYB	31.78	30.69	28.00	29.77	28.18	28.85	28.57	29.24	30.06	29.86
MLCYB	31.56	30.13	27.56	29.46	27.72	28.53	28.19	28.72	30.00	29.66
MLCYB	30.78	29.54	28.16	30.37	27.95	28.07	27.70	28.41	30.45	29.88
AVG	31.37	30.12	27.91	29.87	27.95	28.48	28.15	28.79	30.17	29.80
STDEV	0.16	0.42	0.31	0.22	0.23	0.23	0.27	0.22	0.32	0.12
MLCYE	34.12	29.25	28.23	32.95	30.80	31.59	30.18	29.70	30.67	32.80
MLCYE	34.48	28.96	27.68	33.01	30.77	31.55	30.31	29.57	30.60	32.83
MLCYE	35.11	29.69	28.21	32.70	30.79	31.94	30.75	29.88	29.79	32.70
AVG	34.57	29.30	28.04	32.89	30.79	31.69	30.41	29.72	30.35	32.78
STDEV	0.25	0.21	0.31	0.16	0.02	0.03	0.09	0.16	0.05	0.07
ACTIN	20.42	19.65	20.45	20.88	19.80	19.86	19.16	20.57	22.09	20.01
ACTIN	19.97	19.24	20.32	20.33	19.88	19.96	19.44	20.35	22.06	20.45
ACTIN	20.22	19.21	20.22	20.44	19.84	19.74	19.47	20.71	22.10	20.78
AVG	20.20	19.37	20.33	20.55	19.84	19.85	19.36	20.54	22.08	20.41
STDEV	0.23	0.25	0.12	0.29	0.04	0.11	0.17	0.18	0.02	0.39
ΔCtLCYB	-11.17	-10.75	-7.58	-9.32	-8.11	-8.63	-8.80	-8.25	-8.09	-9.39
ΔCtLCYE	-14.37	-9.93	-7.71	-12.34	-10.95	-11.84	-11.06	-9.17	-8.27	-12.36
LCYB	0.04	0.06	0.52	0.16	0.36	0.25	0.22	0.33	0.37	0.15
LCYE	0.00	0.10	0.48	0.02	0.05	0.03	0.05	0.17	0.32	0.02
SLCYB	0.03	0.04	0.49	0.17	0.31	0.20	0.17	0.24	0.40	0.14
SLCYB	0.04	0.06	0.67	0.21	0.42	0.24	0.22	0.35	0.41	0.16
SLCYB	0.07	0.09	0.44	0.11	0.36	0.34	0.31	0.43	0.30	0.14
STDEV	0.02	0.02	0.12	0.05	0.06	0.07	0.07	0.09	0.06	0.01
SLCYE	0.01	0.11	0.42	0.02	0.05	0.03	0.06	0.18	0.26	0.02
SLCYE	0.01	0.13	0.61	0.02	0.05	0.03	0.05	0.19	0.27	0.02
SLCYE	0.00	0.08	0.42	0.02	0.05	0.02	0.04	0.15	0.48	0.02
STDEV	0.00	0.03	0.11	0.00	0.00	0.00	0.01	0.02	0.12	0.00

15 DAP	TZI18	C131A	B37	A619	DE3	C17	NC300	SD44	B73	KUI2007
MLCYB	29.99	29.81	28.02	28.63	28.15	28.37	30.61	30.93	29.59	28.36
MLCYB	30.46	30.08	27.81	29.73	27.08	30.01	29.45	31.72	29.32	27.73
MLCYB	30.01	31.08	27.82	29.18	27.62	29.00	29.56	31.72	29.63	27.96
AVG	30.15	30.32	27.88	29.18	27.62	29.13	29.87	31.46	29.51	28.02
STDEV	0.27	0.19	0.12	0.39	0.38	0.71	0.08	0.00	0.19	0.16
MLCYE	32.52	30.81	27.86	32.78	30.64	32.67	29.89	33.01	28.75	31.40
MLCYE	32.87	32.47	28.02	33.01	29.67	31.55	29.92	32.98	28.86	31.54
MLCYE	32.87	31.64	27.94	32.90	30.16	31.56	29.89	32.96	28.28	31.56
AVG	32.75	31.64	27.94	32.90	30.16	31.93	29.90	32.98	28.63	31.50
STDEV	0.00	0.59	0.08	0.12	0.35	0.01	0.00	0.03	0.08	0.09
ACTIN	18.12	20.42	18.91	20.82	19.49	19.27	18.70	21.39	20.66	19.44
ACTIN	19.05	20.08	20.21	22.49	20.30	19.92	18.70	22.55	21.03	19.56
ACTIN	19.09	20.25	20.01	21.66	19.90	19.54	18.97	22.42	21.03	19.00
AVG	18.75	20.25	19.71	21.66	19.90	19.58	18.79	22.12	20.91	19.33
STDEV	0.66	0.24	0.92	1.18	0.57	0.46	0.00	0.82	0.21	0.08
ΔCt LCYB	-11.40	-10.07	-8.17	-7.52	-7.72	-9.55	-11.08	-9.34	-8.61	-8.68
ΔCt LCYE	-14.00	-11.39	-8.23	-11.24	-10.26	-12.35	-11.11	-10.86	-7.72	-12.17
LCYB	0.04	0.09	0.35	0.54	0.47	0.13	0.05	0.15	0.26	0.24
LCYE	0.01	0.04	0.33	0.04	0.08	0.02	0.05	0.05	0.47	0.02
SLCYB	0.04	0.13	0.32	0.80	0.33	0.23	0.03	0.22	0.24	0.19
SLCYB	0.03	0.11	0.36	0.37	0.69	0.07	0.06	0.13	0.29	0.30
SLCYB	0.04	0.05	0.36	0.54	0.47	0.15	0.06	0.13	0.24	0.25
STDEV	0.01	0.04	0.03	0.21	0.18	0.08	0.02	0.05	0.03	0.05
SLCYE	0.01	0.07	0.35	0.04	0.06	0.01	0.05	0.05	0.44	0.02
SLCYE	0.01	0.02	0.32	0.04	0.11	0.02	0.04	0.05	0.40	0.02
SLCYE	0.01	0.04	0.33	0.04	0.08	0.02	0.05	0.05	0.60	0.02
STDEV	0.00	0.02	0.02	0.00	0.03	0.01	0.00	0.00	0.11	0.00
10DAP	TZI18	C131A	B37	A619	DE3	C17	SD44	B73	KUI2007	NC300
MLCYB	28.93	26.11	24.94	28.23	27.36	27.63	28.08	26.93	27.79	
MLCYB	28.54	26.64	25.56	28.67	27.39	27.72	28.85	27.25	27.98	
MLCYB	28.14	26.36	25.32	29.11	27.38	27.68	28.47	26.98	27.78	
Average	28.54	26.37	25.27	28.67	27.38	27.68	28.47	27.05	27.85	
StDev	0.28	0.20	0.17	0.44	0.02	0.05	0.27	0.23	0.13	
MLCYE	32.67	30.11	26.52	32.10	31.41	33.48	29.99	30.58	28.47	
MLCYE	32.62	28.99	26.99	32.20	30.57	33.48	29.23	30.10	28.41	
MLCYE	32.65	29.00	26.56	32.18	30.99	33.48	29.61	30.16	28.14	
Avrage	32.65	29.37	26.69	32.19	30.99	33.48	29.61	30.28	28.34	
StDev	0.03	0.01	0.26	0.05	0.30	0.00	0.27	0.26	0.19	
Actin	19.48	17.88	16.94	21.04	21.21	20.51	19.08	20.73	22.22	
Actin	21.13	18.87	17.61	20.94	20.67	20.97	18.85	20.60	22.65	
Actin	20.31	18.00	17.52	20.99	20.94	20.74	18.97	20.60	22.14	
Average	20.31	18.25	17.36	20.99	20.94	20.74	18.97	20.64	22.34	
StDev	1.17	0.70	0.47	0.07	0.38	0.33	0.16	0.08	0.27	
ΔCt LCYB	-8.23	-8.12	-7.92	-7.68	-6.44	-6.94	-9.50	-6.41	-5.51	
ΔCt LCYE	-12.34	-11.12	-9.33	-11.20	-10.05	-12.74	-10.64	-9.64	-6.00	
LCYB	0.33	0.36	0.41	0.49	1.15	0.82	0.14	1.18	2.19	
LCYE	0.02	0.05	0.16	0.04	0.09	0.01	0.06	0.13	1.56	
SLCYB	0.25	0.43	0.52	0.66	1.17	0.84	0.18	1.28	2.28	
SLCYB	0.33	0.30	0.34	0.49	1.14	0.79	0.11	1.03	2.00	
SLCYB	0.44	0.36	0.40	0.36	1.15	0.81	0.14	1.24	2.30	
stDEV	0.09	0.07	0.09	0.15	0.01	0.03	0.04	0.14	0.17	
SLCYE	0.02	0.03	0.17	0.05	0.07	0.01	0.05	0.10	1.42	
SLCYE	0.02	0.06	0.13	0.04	0.13	0.01	0.08	0.14	1.49	
SLCYE	0.02	0.06	0.17	0.04	0.09	0.01	0.06	0.14	1.79	
stDEV	0.00	0.02	0.03	0.00	0.03	0.00	0.02	0.02	0.20	

20DAP	A619	B73	B37	Cl.7	C131A	DE3	KUI2007	NC300	SD44	TZ118
ZEP1	29.13	29.64	28.71	30.64	27.76	29.49	32.90	26.93	28.50	29.91
ZEP1	29.36	29.39	29.40	30.61	27.80	29.93	32.71	27.72	29.21	29.55
ZEP1	28.89	29.41	29.81	30.61	26.91	29.57	31.56	27.90	28.87	29.19
AVG	29.13	29.48	29.31	30.62	27.49	29.66	32.39	27.52	28.86	29.55
SIDEV	0.33	0.14	0.56	0.02	0.50	0.23	0.73	0.52	0.36	0.36
ZEP2	28.17	27.40	28.89	30.05	27.32	28.12	32.13	26.20	27.82	29.14
ZEP2	27.84	27.88	28.18	30.02	26.82	28.59	32.20	26.64	28.18	28.86
ZEP2	28.49	27.59	28.50	30.07	27.82	28.53	32.05	26.42	28.00	29.80
AVG	28.17	27.62	28.52	30.05	27.32	28.41	32.13	26.42	28.00	29.27
SIDEV	0.46	0.24	0.36	0.03	0.50	0.26	0.08	0.22	0.18	0.48
ACTIN	21.74	21.82	21.77	23.72	20.66	21.61	21.39	19.73	21.42	21.20
ACTIN	22.08	21.24	21.65	23.44	20.87	21.82	21.09	20.81	21.70	21.55
ACTIN	21.90	21.50	21.69	23.55	20.75	21.70	21.20	20.29	21.54	21.30
AVG	21.91	21.52	21.70	23.57	20.76	21.71	21.23	20.28	21.55	21.35
SIDEV	0.17	0.29	0.06	0.14	0.11	0.11	0.15	0.54	0.14	0.18
ΔCt ZEP1	-7.22	-7.96	-7.60	-7.05	-6.73	-7.95	-11.16	-7.24	-7.31	-8.20
MZEP1	0.67	0.40	0.51	0.75	0.94	0.40	0.04	0.66	0.63	0.34
ΔCt ZEP2	-6.26	-6.10	-6.82	-6.48	-6.56	-6.70	-10.90	-6.14	-6.45	-7.92
MZEP2	1.31	1.45	0.89	1.12	1.06	0.96	0.05	1.41	1.15	0.41
ZEP1	0.67	0.36	0.78	0.74	0.78	0.45	0.03	0.99	0.81	0.26
ZEP1	0.57	0.43	0.48	0.76	0.76	0.34	0.03	0.57	0.50	0.34
ZEP1	0.79	0.42	0.36	0.76	1.41	0.43	0.08	0.51	0.63	0.44
SIDEV	0.11	0.04	0.21	0.01	0.37	0.06	0.03	0.26	0.16	0.09
ZEP2	1.30	1.70	0.69	1.12	1.06	1.18	0.05	1.65	1.30	0.45
ZEP2	1.64	1.22	1.12	1.14	1.50	0.85	0.05	1.21	1.01	0.55
ZEP2	1.04	1.49	0.90	1.10	0.75	0.89	0.06	1.41	1.15	0.29
SIDEV	0.30	0.24	0.22	0.02	0.38	0.18	0.00	0.22	0.14	0.13
25DAP	A619	B73	B37	Cl.7	C131A	DE3	KUI2007	NC300	SD44	TZ118
ZEP1	28.71	29.80	29.82	31.27	28.53	31.61	32.59	27.46	29.90	32.35
ZEP1	28.77	29.86	29.49	31.00	28.52	31.42	32.67	27.63	29.38	31.50
ZEP1	28.64	30.79	29.42	31.76	28.85	33.30	32.67	27.55	29.78	31.67
AVG	28.71	30.15	29.58	31.34	28.63	32.11	32.64	27.55	29.69	31.84
SIDEV	0.07	0.56	0.21	0.39	0.19	1.03	0.05	0.09	0.27	0.45
ZEP2	27.92	26.46	29.21	30.12	27.71	27.20	30.69	27.04	29.72	30.23
ZEP2	27.89	26.88	29.23	30.02	27.51	27.24	30.56	27.14	29.00	31.25
ZEP2	28.25	27.10	29.38	30.75	27.80	27.23	30.79	27.23	29.47	31.15
AVG	28.02	26.81	29.27	30.30	27.67	27.22	30.68	27.14	29.40	30.88
SIDEV	0.20	0.33	0.09	0.40	0.15	0.02	0.12	0.10	0.37	0.56
ACTIN	20.35	20.15	20.68	21.19	20.93	19.74	23.21	20.75	22.75	21.66
ACTIN	20.71	20.04	20.71	21.21	20.75	19.86	23.11	20.50	22.67	21.58
ACTIN	20.36	20.07	20.95	21.28	20.97	20.10	23.30	20.69	22.71	21.66
AVG	20.47	20.09	20.78	21.23	20.88	19.90	23.21	20.65	22.71	21.63
SIDEV	0.21	0.06	0.15	0.05	0.12	0.18	0.10	0.13	0.04	0.05
ΔCt ZEP1	-8.23	-10.06	-8.80	-10.12	-7.75	-12.21	-9.44	-6.90	-6.98	-10.21
MZEP1	0.33	0.09	0.22	0.09	0.46	0.02	0.14	0.84	0.79	0.08
ΔCt ZEP2	-7.55	-6.73	-8.49	-9.07	-6.79	-7.32	-7.47	-6.49	-6.69	-9.24
MZEP2	0.53	0.94	0.28	0.19	0.90	0.62	0.56	1.11	0.97	0.16
ZEP1	0.33	0.12	0.19	0.09	0.50	0.03	0.15	0.89	0.68	0.06
	0.32	0.11	0.24	0.11	0.50	0.03	0.14	0.79	0.98	0.11
	0.35	0.06	0.25	0.07	0.40	0.01	0.14	0.84	0.74	0.10
SIDEV	0.02	0.03	0.03	0.02	0.06	0.01	0.00	0.05	0.16	0.02
ZEP2	0.57	1.21	0.29	0.21	0.88	0.63	0.56	1.19	0.78	0.26
	0.59	0.90	0.29	0.23	1.01	0.62	0.61	1.11	1.28	0.13
	0.46	0.77	0.26	0.14	0.83	0.62	0.52	1.04	0.92	0.14
SIDEV	0.07	0.22	0.02	0.05	0.09	0.01	0.05	0.07	0.26	0.07

Chapter 3: Spatial and Temporal profiles (B73)

Endosperm development profiles of Carotene hydroxylase 3										
	10	12	14	16	18	20	22	24	26	28
MHYD3	21.11	20.04	19.84	19.83	20.74	20.65	20.67	20.96	20.31	20.41
MHYD3	20.72	20.12	19.57	19.83	20.53	20.60	20.10	20.82	20.15	20.44
MHYD3	20.70	19.91	19.42	20.13	20.64	20.98	20.64	20.80	20.24	20.45
AVG	20.84	20.02	19.61	19.93	20.64	20.74	20.47	20.86	20.23	20.43
StDEV	0.23	0.11	0.21	0.17	0.11	0.21	0.32	0.09	0.08	0.02
ACTIN	19.88	19.96	20.16	20.58	22.94	21.52	20.21	20.90	20.15	20.52
ACTIN	19.80	19.70	20.89	21.30	21.40	21.82	20.49	21.17	20.02	20.66
ACTIN	19.71	19.29	19.79	20.05	21.13	21.22	20.35	21.04	20.09	20.59
AVG	19.80	19.65	20.28	20.64	21.82	21.52	20.35	21.04	20.09	20.59
StDEV	0.09	0.34	0.56	0.63	0.98	0.30	0.14	0.14	0.07	0.07
ΔCt MHYD3	-1.05	-0.37	0.67	0.71	1.19	0.78	-0.12	0.18	-0.15	0.16
MHYD3	48.41	77.20	159.11	163.96	227.63	171.32	92.02	113.03	90.33	111.47
SHYD3	40.24	76.31	135.66	175.73	211.89	182.77	80.11	105.46	85.66	113.29
SHYD3	52.73	72.20	163.58	175.73	245.09	189.21	118.92	116.20	95.71	110.96
SHYD3	53.46	83.51	181.50	142.73	227.10	145.40	81.79	117.83	89.92	110.19
StDEV	7.43	5.73	23.10	19.05	16.62	23.66	21.94	6.72	5.04	1.65

Endosperm development profiles of Carotene hydroxylase 5										
	10	12	14	16	18	20	22	24	26	28
MHYD5	32.46	31.41	33.48	32.33	31.38	32.37	32.84	31.92	31.67	31.03
MHYD5	31.40	30.62	32.50	32.24	31.65	31.75	32.37	31.90	31.10	30.73
MHYD5	32.23	30.76	32.69	32.27	31.48	31.76	32.30	31.76	31.34	30.86
AVG	32.03	30.93	32.89	32.28	31.50	31.96	32.50	31.86	31.37	30.87
StDEV	0.75	0.56	0.69	0.06	0.19	0.44	0.33	0.01	0.40	0.21
ACTIN	18.93	18.65	18.92	18.87	20.59	21.72	20.27	20.65	19.95	21.65
ACTIN	18.72	18.64	18.89	18.87	20.22	20.45	20.59	20.77	20.72	21.14
ACTIN	18.83	18.65	18.91	18.87	20.41	21.09	20.43	20.71	20.34	21.40
AVG	18.83	18.65	18.91	18.87	20.41	21.09	20.43	20.71	20.34	21.40
StDEV	0.11	0.00	0.02	0.00	0.18	0.63	0.16	0.06	0.39	0.25
ΔCt MHYD5	-13.21	-12.29	-13.99	-13.41	-11.10	-10.88	-12.07	-11.15	-11.04	-9.48
MHYD5	0.01	0.02	0.01	0.01	0.05	0.05	0.02	0.04	0.05	0.14
SHYD5	0.01	0.01	0.00	0.01	0.05	0.04	0.02	0.04	0.04	0.13
SHYD5	0.02	0.02	0.01	0.01	0.04	0.06	0.03	0.04	0.06	0.15
SHYD5	0.01	0.02	0.01	0.01	0.05	0.06	0.03	0.05	0.05	0.14
StDEV	0.01	0.01	0.00	0.00	0.01	0.02	0.01	0.00	0.01	0.02

Spatial expression profiles of HYD3						Relative to Endosperm					
	Em	En(20)	L	R	U		Em	En(20)	L	R	U
MHYD3	24.13	20.65	21.47	21.82	23.46	NHYD3	0.93	0.00	-4.19	-1.22	-3.84
MHYD3	24.09	20.60	21.45	21.53	23.29	NHYD3+ΔCt	-1.87	0.78	0.01	-0.16	-1.87
MHYD3	24.12	20.98	21.61	21.70	23.41	Absolute	27.36	171.32	100.46	89.30	27.36
Average	24.11	20.74	21.51	21.68	23.39	StDEV1	16.38	182.77	103.53	81.23	26.06
StDEV	0.02	0.21	0.09	0.15	0.09	StDEV2	16.84	189.21	104.97	99.31	29.32
Actin	21.40	21.52	25.71	22.98	25.39	StDEV3	16.49	145.40	93.95	88.27	26.98
Actin	21.27	21.82	25.63	22.73	24.98	StDEV	0.24	23.66	5.99	9.12	1.68
Actin	21.27	21.22	25.78	22.51	25.70						
Average	21.31	21.52	25.71	22.74	25.36						
StDEV	0.08	0.30	0.08	0.24	0.36						
ΔCt HYD3	-2.80	0.78	4.20	1.06	1.97						
NLHYD3	14.36	171.32	1833.68	208.01	391.77						
StDEV1	14.2	182.8	1885.2	189.2	372.4						
StDEV2	14.6	189.2	1911.5	231.3	418.9						
StDEV3	14.3	145.4	1710.9	205.6	385.5						
StDEV	0.2	23.7	109.1	21.2	24.0						

Spatial expression profiles of HYD5						Relative to Endosperm					
	Em	En(20)	L	R	UN		Em	En(20)	L	R	U
MHYD5	31.45	32.37	30.50	31.65	25.19	NHYD3	-1.05	0.00	-4.06	-1.78	-3.22
MHYD5	31.12	31.75	30.37	29.9	24.84	NHYD3+ΔCt	-10.20	-10.87	-9.28	-9.37	-3.93
MHYD5	31.29	31.76	30.24	29.83	25.02	Absolute	0.09	0.05	0.16	0.15	6.56
Average	31.29	31.96	30.37	30.46	25.02	StDEV1	0.08	0.04	0.15	0.07	5.83
StDEV	0.17	0.36	0.13	1.03	0.18	StDEV2	0.10	0.06	0.16	0.22	7.43
Actin	21.85	21.72	25.15	22.76	24.64	StDEV3	0.19	0.14	0.18	0.23	14.56
Actin	21.61	20.45	25.31	22.80	23.97	StDEV	0.01	0.02	0.01	0.09	1.13
Actin	22.96	21.09	24.99	23.05	24.31						
Average	22.14	21.09	25.15	22.87	24.31						
StDEV	0.72	0.64	0.16	0.16	0.34						
ΔCt HYD3	-9.15	-10.87	-5.22	-7.59	-0.71						
NLHYD3	0.18	0.05	2.68	0.52	61.13						
StDEV1	0.2	0.0	2.5	0.2	54.2						
StDEV2	0.2	0.1	2.7	0.8	69.1						
StDEV3	0.2	0.1	2.9	0.8	61.0						
StDEV	0.0	0.0	0.2	0.3	7.5						

Endosperm development profiles of Carotene hydroxylase 4 and 6										
	10	12	14	16	18	20	22	24	26	28
MHYD4	25.71	25.56	26.23	26.32	27.76	27.09	25.79	25.70	25.64	25.92
MHYD4	25.65	25.86	26.13	26.68	27.93	26.58	26.02	25.57	25.04	25.88
MHYD4	25.61	25.98	25.94	26.23	28.11	27.17	25.98	25.18	25.00	25.56
AVG	25.66	25.80	26.10	26.41	27.93	26.95	25.93	25.48	25.23	25.79
StDEV	0.05	0.22	0.15	0.24	0.18	0.32	0.12	0.27	0.36	0.20
MHYD6	28.43	27.78	30.2	30.85	31.38	30.11	29.44	28.7	29.23	27.49
MHYD6	28.21	27.1	31.61	31.03	30.08	30.34	29.2	28.85	29.17	27.59
MHYD6	27.96	27.13	31.14	30.05	30.13	29.19	29.32	28.56	28.64	27.16
AVG	28.20	27.34	30.98	30.64	30.53	29.88	29.32	28.70	29.01	27.41
StDEV	0.16	0.08	0.83	0.00	0.41	0.06	0.17	0.11	0.13	0.09
ACTIN	19.88	19.96	20.16	20.58	22.94	21.52	20.21	20.90	20.15	20.52
ACTIN	19.80	19.70	20.89	21.30	21.40	21.82	20.49	21.17	20.02	20.66
ACTIN	19.71	19.29	19.79	20.05	21.13	21.22	20.35	21.04	20.09	20.59
AVG	19.80	19.65	20.28	20.64	21.82	21.52	20.35	21.04	20.09	20.59
StDEV	0.09	0.34	0.56	0.63	0.98	0.30	0.14	0.14	0.07	0.07
ΔCt MHYD4	-5.86	-6.15	-5.82	-5.77	-6.11	-5.43	-5.58	-4.45	-5.14	-5.20
ΔCt MLCY6	-8.40	-7.69	-10.70	-10.00	-8.71	-8.36	-8.97	-7.67	-8.93	-6.82
MHYD4	1.72	1.41	1.77	1.84	1.45	2.32	2.09	4.59	2.84	2.73
MHYD6	0.30	0.49	0.06	0.10	0.24	0.30	0.20	0.49	0.21	0.88
SHYD4	1.66	1.66	1.62	1.96	1.63	2.11	2.30	3.95	2.13	2.49
SHYD4	1.73	1.35	1.73	1.52	1.45	3.00	1.96	4.32	3.23	2.56
SHYD4	1.78	1.24	1.98	2.08	1.28	1.99	2.02	5.66	3.32	3.19
StDEV	0.06	0.22	0.18	0.29	0.18	0.55	0.18	0.90	0.66	0.39
SHYD6	0.33	0.68	0.28	0.19	0.62	0.48	0.18	0.42	0.13	0.69
SHYD6	0.38	0.73	0.12	0.19	0.42	0.45	0.22	0.38	0.11	0.76
SHYD6	0.41	0.59	0.10	0.19	0.38	0.69	0.20	0.46	0.17	0.94
StDEV	0.04	0.07	0.10	0.00	0.13	0.13	0.02	0.04	0.03	0.13

Spatial expression profiles of HYD4						Relative to Endosperm					
	Em	En(20)	L	R	U		Em	En(20)	L	R	U
MHYD4	26.77	27.09	22.19	22.49	26.14	NHYD3	-0.26	0.00	-4.00	-3.01	-4.02
MHYD4	26.45	26.58	22.23	21.84	25.95	NHYD3+ΔCt	-5.04	-5.43	-0.75	-0.59	-4.47
MHYD4	26.46	27.17	22.38	21.99	25.89	Absolute	3.04	2.32	59.60	66.59	4.50
Average	26.56	26.95	22.27	22.11	25.99	StDEV1	2.63	2.11	62.85	51.05	4.07
StDEV	0.18	0.32	0.10	0.34	0.13	StDEV2	3.28	3.00	61.13	80.11	4.64
Actin	22.05	21.52	25.42	24.74	25.45	StDEV3	3.26	1.99	55.10	72.20	4.84
Actin	21.65	21.82	25.61	24.32	25.62	StDEV	0.37	0.55	4.07	15.02	0.40
Actin	21.65	21.22	25.52	24.52	25.54						
Average	21.78	21.52	25.52	24.53	25.54						
StDEV	0.23	0.30	0.10	0.21	0.09						
ΔCt HYD4	-4.78	-5.43	3.25	2.42	-0.46						
NLHYD4	3.65	2.32	951.37	535.17	72.87						
StDEV1	3.2	2.1	1003.3	410.3	65.8						
StDEV2	3.9	3.0	975.9	643.8	75.1						
StDEV3	3.9	2.0	879.5	580.2	78.3						
StDEV	0.4	0.6	65.0	120.7	6.5						

Spatial expression profiles of HYD6						Relative tp Endosperm					
	Em	En (20)	L	R	U		Em	En (20)	L	R	U
MHYD6	24.10	30.11	27.21	27.27	25.21	NHYD3	-0.26	0.00	-4.00	-3.01	-4.02
MHYD6	24.08	30.34	27.82	27.74	24.94	NHYD3+ΔCt	-2.57	-8.36	-5.84	-6.04	-3.58
MHYD6	24.09	29.19	27.05	27.67	25.15	Absolute	16.84	0.30	1.75	1.52	8.36
Average	24.09	29.88	27.36	27.56	25.10	StDEV1	16.72	0.26	1.94	1.86	7.75
StDEV	0.01	0.63	0.41	0.25	0.14	StDEV2	16.96	0.22	1.27	1.34	9.34
Actin	22.05	21.52	25.42	24.74	25.45	StDEV3	16.84	0.49	2.16	1.41	8.08
Actin	21.65	21.82	25.61	24.32	25.62	StDEV	0.12	0.15	0.47	0.28	0.84
Actin	21.65	21.22	25.52	24.52	25.54						
Average	21.78	21.52	25.52	24.53	25.54						
StDEV	0.23	0.30	0.10	0.21	0.09						
ΔCt HYD3	-2.31	-8.36	-1.84	-3.03	0.44						
NLHYD3	20.21	0.30	27.87	12.21	135.35						
StDEV1	20.1	0.3	30.9	14.9	125.4						
StDEV2	20.4	0.2	20.3	10.8	151.2						
StDEV3	20.2	0.5	34.5	11.3	130.7						
StDEV	0.1	0.1	7.4	2.3	13.6						

Endosperm development profiles of CYP97A										
	10	12	14	16	18	20	22	24	26	28
MCYP97A	28.68	29.29	29.56	29.7	32.03	31.34	30.77	29.66	29.61	30.88
MCYP97A	28.48	29.18	29.19	29.4	31.47	31.32	30.58	29.51	29.44	30.42
MCYP97A	28.58	29.14	29.03	29.61	31.54	31.42	30.07	29.56	29.56	30.08
AVG	28.58	29.20	29.26	29.57	31.68	31.36	30.47	29.58	29.54	30.46
StDEV	0.14	0.08	0.27	0.15	0.31	0.05	0.36	0.08	0.09	0.40
ACTIN	19.88	19.96	20.16	20.58	22.94	21.52	20.21	20.90	20.15	20.52
ACTIN	19.80	19.70	20.89	21.30	21.40	21.82	20.49	21.17	20.02	20.66
ACTIN	19.71	19.29	19.79	20.05	21.13	21.22	20.35	21.04	20.09	20.59
AVG	19.80	19.65	20.28	20.64	21.82	21.52	20.35	21.04	20.09	20.59
StDEV	0.09	0.34	0.56	0.63	0.98	0.30	0.14	0.14	0.07	0.07
ΔCt MCYP97A	-8.78	-9.55	-8.98	-8.93	-9.86	-9.84	-10.12	-8.54	-9.45	-9.87
MCYP97A	0.23	0.13	0.20	0.21	0.11	0.11	0.09	0.27	0.14	0.11
SCYP97A	0.21	0.13	0.16	0.19	0.08	0.11	0.07	0.25	0.14	0.08
SCYP97A	0.24	0.14	0.21	0.23	0.12	0.11	0.08	0.28	0.15	0.11
SCYP97A	0.24	0.14	0.23	0.20	0.12	0.10	0.12	0.27	0.14	0.14
StDEV	0.02	0.01	0.04	0.02	0.02	0.00	0.01	0.01	0.01	0.02

Spatial expression profiles of CYP97A						Relative to Endosperm					
	Em	En (20)	Leaf	Root	UN		Em	En (20)	Leaf	Root	UN
MCYP97A	26.97	31.34	27.37	28.24	29.81	NHYD3	-1.93	0.00	-3.44	-1.90	-3.84
MCYP97A	26.65	31.32	26.97	28.02	29.51	NHYD3+ΔCt	-5.28	-9.84	-5.61	-6.48	-8.09
MCYP97A	26.78	31.42	27.06	27.74	29.51	Absolute	2.57	0.11	2.04	1.12	0.37
Average	26.80	31.36	27.13	28.00	29.61	StDEV1	2.29	0.11	1.73	0.95	0.32
StDEV	0.16	0.05	0.21	0.25	0.17	StDEV2	2.86	0.11	2.29	1.10	0.39
Actin	23.32	21.52	25.01	23.64	25.39	StDEV3	2.61	0.10	2.15	1.34	0.39
Actin	23.52	21.82	25.02	23.24	24.98	StDEV	0.28	0.00	0.29	0.20	0.04
Actin	23.50	21.22	24.86	23.38	25.70						
Average	23.45	21.52	24.96	23.42	25.36						
StDEV	0.11	0.30	0.09	0.20	0.36						
ΔCt HYD3	-3.35	-9.84	-2.17	-4.58	-4.25						
NLHYD3	9.78	0.11	22.22	4.18	5.24						
StDEV1	8.7	0.11	18.9	3.5	4.56						
StDEV2	10.9	0.11	24.9	4.1	5.62						
StDEV3	9.9	0.10	23.4	5.0	5.62						
StDEV	1.1	0.00	3.1	0.7	0.61						

Endosperm development profiles of CYP97C										
	10	12	14	16	18	20	22	24	26	28
MCYP97C	26.18	25.94	26.95	26.92	28.79	28.76	28.79	28.74	27.77	28.5
MCYP97C	26.16	26.23	26.76	26.87	29.03	29	28.74	29.04	28.87	28.97
MCYP97C	26.5	25.85	26.86	26.97	28.96	28.41	28.85	29.19	28.34	29.13
AVG	26.17	26.01	26.86	26.92	28.93	28.72	28.79	28.99	28.33	28.87
StDEV	0.01	0.20	0.10	0.05	0.12	0.30	0.06	0.23	0.55	0.33
ACTIN	19.88	19.96	20.16	19.58	22.94	21.52	20.21	20.90	20.15	20.52
ACTIN	19.80	19.70	20.89	19.30	21.40	21.82	20.49	21.17	20.02	20.66
ACTIN	19.71	19.29	19.79	19.05	21.13	21.22	20.35	21.04	20.09	20.59
AVG	19.80	19.65	20.28	19.31	21.82	21.52	20.35	21.04	20.09	20.59
StDEV	0.09	0.34	0.56	0.27	0.98	0.30	0.14	0.14	0.07	0.07
ΔCt MCYP97C	-6.37	-6.36	-6.58	-7.61	-7.10	-7.20	-8.44	-7.95	-8.24	-8.28
MCYP97C	1.21	1.22	1.05	0.51	0.73	0.68	0.29	0.40	0.33	0.32
SCYP97C	1.20	1.28	0.98	0.51	0.80	0.66	0.29	0.48	0.49	0.42
SCYP97C	1.21	1.05	1.12	0.53	0.68	0.56	0.30	0.39	0.23	0.30
SCYP97C	1.21	1.36	1.05	0.49	0.71	0.84	0.28	0.35	0.33	0.27
StDEV	0.01	0.16	0.07	0.02	0.06	0.14	0.01	0.07	0.13	0.08

Spatial expression profiles of CYP97C						Relative to Endosperm					
	Em	En (20)	Leaf	Root	UN		Em	En (20)	Leaf	Root	UN
MCYP97C	26.72	28.76	25.74	25.95	27.68	NHYD3	-1.93	0.00	-3.44	-1.90	-3.84
MCYP97C	26.87	29	25.89	25.63	26.92	NHYD3+ΔCt	-5.17	-7.20	-4.28	-4.22	-5.59
MCYP97C	26.47	28.41	25.76	25.64	26.72	Absolute	2.78	0.68	5.16	5.37	2.08
Average	26.69	28.72	25.80	25.74	27.11	StDEV1	2.72	0.66	5.37	4.64	1.40
StDEV	0.20	0.30	0.08	0.18	0.51	StDEV2	2.45	0.56	4.84	5.79	2.37
Actin	23.32	21.52	25.01	23.64	25.39	StDEV3	3.24	0.84	5.29	5.75	2.72
Actin	23.52	21.82	25.02	23.24	24.98	StDEV	0.40	0.14	0.29	0.65	0.68
Actin	23.50	21.22	24.86	23.38	25.70						
Average	23.45	21.52	24.96	23.42	25.36						
StDEV	0.11	0.30	0.09	0.20	0.36						
ΔCt HYD3	-3.24	-7.20	-0.83	-2.32	-1.75						
NLHYD3	10.58	0.68	56.12	20.03	29.73						
StDEV1	10.3	0.66	58.4	17.3	19.98						
StDEV2	9.3	0.56	52.6	21.6	33.84						
StDEV3	12.3	0.84	57.6	21.5	38.87						
StDEV	1.5	0.14	3.1	2.4	9.78						

Chapter 3: Correlation analysis (Inbreds)

15DAP	A619	B73	B37	Cl.7	C131A	DE3	KUI2007	NC300	SD44	TZI18
MHYD3	17.27	19.85	17.02	17.23	16.93	17.12	16.45	16.89	17.01	16.75
MHYD3	17.18	19.81	16.74	17.01	16.77	17.05	16.32	16.85	16.86	16.73
MHYD3	17.20	20.13	17.23	17.37	16.90	17.05	16.71	16.75	17.28	16.65
AVG	17.22	19.93	17.00	17.20	16.87	17.07	16.49	16.83	17.05	16.71
StDEV	0.05	0.17	0.25	0.18	0.08	0.04	0.20	0.07	0.21	0.05
ACTIN	19.11	20.58	19.15	19.41	19.80	17.43	17.70	18.39	18.89	18.87
ACTIN	19.38	21.30	19.38	19.73	19.90	17.84	17.81	18.99	18.83	18.37
ACTIN	19.25	20.05	19.27	19.57	19.85	17.64	17.76	18.69	18.86	18.62
AVG	19.25	20.64	19.27	19.57	19.85	17.64	17.76	18.69	18.86	18.62
StDEV	0.14	0.63	0.12	0.16	0.05	0.21	0.06	0.30	0.03	0.25
Δ Ct MHYD3	2.03	0.71	2.27	2.37	2.99	0.56	1.27	1.86	1.81	1.91
MHYD3	408.40	163.96	482.32	515.75	791.73	147.73	240.33	363.01	350.64	375.81
MHYD3	393.58	173.31	474.59	506.30	759.47	143.06	247.37	348.22	360.50	365.53
	418.92	178.18	576.24	589.71	845.61	150.18	271.63	358.01	400.00	370.64
	413.15	142.73	410.30	459.48	772.75	150.07	206.58	383.71	298.97	391.77
StDEV	17.91	19.21	71.88	58.98	60.91	5.03	17.16	6.92	27.93	3.61

20DAP	A619	B73	B37	Cl.7	C131A	DE3	KUI2007	NC300	SD44	TZI18
MHYD3	29.88	21.65	20.00	25.20	19.93	27.14	19.52	20.21	25.56	20.67
MHYD3	31.40	20.60	20.46	25.61	20.37	26.98	19.36	19.37	25.94	20.61
MHYD3	30.67	20.98	20.75	25.66	19.85	26.65	19.79	19.09	25.54	20.76
AVG	30.65	21.08	20.40	25.49	20.05	26.92	19.56	19.56	25.68	20.68
StDEV	0.76	0.53	0.38	0.25	0.28	0.25	0.22	0.58	0.23	0.08
ACTIN	21.74	21.52	21.77	23.72	20.69	21.61	21.39	19.73	21.45	21.15
ACTIN	22.07	21.82	21.64	23.42	20.85	21.82	21.06	20.82	21.69	21.55
ACTIN	21.91	21.22	21.71	23.57	20.77	21.72	21.23	20.28	21.57	21.35
AVG	21.91	21.52	21.71	23.57	20.77	21.72	21.23	20.28	21.57	21.35
StDEV	0.17	0.30	0.07	0.15	0.08	0.11	0.17	0.55	0.12	0.20
Δ Ct MHYD3	-8.74	0.44	1.30	-1.92	0.72	-5.21	1.67	0.72	-4.11	0.67
MHYD3	0.23	135.97	246.80	26.43	164.72	2.71	318.21	164.72	5.79	159.11
MHYD3	0.40	91.38	326.41	32.31	179.01	2.33	326.41	104.73	6.29	160.21
	0.14	189.21	237.29	24.32	131.95	2.60	364.69	187.47	4.84	167.02
	0.23	145.40	194.08	23.49	189.21	3.27	270.69	227.63	6.38	150.52
StDEV	0.13	49.00	67.48	4.87	30.54	0.48	47.27	62.67	0.87	8.29

25DAP	A619	B73	B37	Cl.7	C131A	DE3	KUI2007	NC300	SD44	TZ118
MHYD3	30.29	20.31	20.91	30.88	21.35	30.66	21.71	21.03	25.76	21.46
MHYD3	30.73	20.15	20.76	30.70	20.89	30.69	21.21	20.44	25.74	21.17
MHYD3	30.91	20.24	20.68	30.93	20.94	30.47	21.25	20.49	25.84	21.14
AVG	30.64	20.23	20.78	30.84	21.06	30.61	21.39	20.65	25.78	21.26
StDEV	0.32	0.08	0.12	0.12	0.25	0.12	0.28	0.33	0.05	0.18
ACTIN	20.29	20.15	20.68	21.19	20.93	19.74	23.21	20.75	21.45	21.66
ACTIN	20.79	20.02	20.75	21.21	20.75	19.86	23.11	20.50	21.69	21.58
ACTIN	20.34	20.09	20.94	21.28	20.97	20.10	23.30	20.69	21.57	21.68
AVG	20.47	20.09	20.79	21.23	20.88	19.90	23.21	20.65	21.57	21.64
StDEV	0.28	0.07	0.13	0.05	0.12	0.18	0.10	0.13	0.12	0.05
ΔCt MHYD3	-10.17	-0.15	0.01	-9.61	-0.18	-10.71	1.82	-0.01	-4.21	0.38
MHYD3	0.09	90.33	100.46	0.128	88.474	0.05984	352.2663	99.53897	5.40336	130.44
MHYD3	0.11	85.66	92.02	0.12	72.36	0.06	282.19	76.67	5.48	113.29
	0.08	95.71	102.10	0.14	99.54	0.06	399.08	115.40	5.56	138.51
	0.07	89.92	107.92	0.12	96.15	0.07	388.16	111.47	5.18	141.42
StDEV	0.02	5.04	8.05	0.01	14.81	0.01	64.57	21.32	0.20	15.47

20DAP	A619	B73	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZ118
CYP97A	27.96	31.34	28.38	30.16	27.66	29.33	26.60	27.73	28.83	26.70
CYP97A	27.41	31.32	28.47	30.12	28.03	29.33	26.41	27.46	28.69	26.90
CYP97A	27.35	31.42	28.12	30.13	28.03	28.87	26.78	27.14	29.83	26.65
AVG	27.57	31.36	28.32	30.14	27.91	29.18	26.60	27.44	29.12	26.75
STDEV	0.39	0.01	0.25	0.03	0.21	0.00	0.13	0.23	0.81	0.13
CYP97C	26.54	28.76	26.43	28.06	25.40	27.07	27.71	26.08	26.01	26.44
CYP97C	26.84	29	26.44	27.66	25.31	26.99	27.74	25.94	26.23	26.73
CYP97C	26.20	28.41	26.46	27.68	25.35	27.42	27.28	26.06	26.19	27.01
AVG	26.53	28.72	26.44	27.80	25.35	27.16	27.58	26.03	26.14	26.73
STDEV	0.21	0.30	0.01	0.23	0.05	0.06	0.02	0.08	0.16	0.29
ACTIN	21.74	21.82	21.22	23.72	20.69	21.61	21.39	19.73	21.45	21.15
ACTIN	22.07	21.22	21.82	23.42	20.85	21.82	21.06	20.82	21.69	21.55
ACTIN	21.91	21.52	21.52	23.57	20.77	21.72	21.23	20.28	21.57	21.35
AVG	21.91	21.52	21.52	23.57	20.77	21.72	21.23	20.28	21.57	21.35
StDEV	0.23	0.42	0.42	0.21	0.11	0.15	0.23	0.77	0.17	0.28
ΔCt CYP97A	-5.67	-9.84	-6.80	-6.57	-7.14	-7.46	-5.37	-7.17	-7.55	-5.40
ΔCt CYP97C	-4.62	-7.20	-4.92	-4.23	-4.58	-5.44	-6.35	-5.75	-4.57	-5.38
CYP97A	1.97	0.11	0.90	1.05	0.71	0.57	2.42	0.70	0.53	2.37
CYP97C	4.07	0.68	3.30	5.33	4.17	2.30	1.23	1.86	4.20	2.41
CYP97A	1.51	0.11	0.86	1.04	0.84	0.51	2.41	0.57	0.65	2.45
CYP97A	2.20	0.11	0.81	1.07	0.65	0.51	2.75	0.69	0.72	2.13
CYP97A	2.30	0.10	1.03	1.06	0.65	0.70	2.13	0.86	0.33	2.54
STDEV	0.43	0.00	0.12	0.02	0.11	0.11	0.31	0.14	0.21	0.21
CYP97C	4.03	0.66	3.33	4.45	4.04	2.45	1.12	1.79	4.61	2.94
CYP97C	3.27	0.56	3.30	5.87	4.30	2.59	1.09	1.97	3.96	2.40
CYP97C	5.10	0.84	3.26	5.79	4.18	1.92	1.51	1.82	4.07	1.98
STDEV	0.92	0.14	0.03	0.80	0.13	0.35	0.23	0.10	0.35	0.48

25DAP	A619	B73	B37	C17	C131A	DE3	KUI2007	NC300	SD44	TZ118
CYP97A	27.87	29.61	29.11	31.19	30.17	30.36	31.19	29.63	32.27	29.21
CYP97A	27.12	29.44	28.32	31.44	29.75	29.80	31.30	28.87	32.21	28.82
CYP97A	27.31	29.56	28.90	31.46	29.23	29.49	31.27	29.04	32.42	28.70
AVG	27.43	29.54	28.78	31.36	29.72	29.88	31.25	29.18	32.30	28.91
STDEV	0.53	0.12	0.41	0.18	0.30	0.44	0.08	0.54	0.04	0.08
CYP97C	26.80	27.77	26.18	27.79	26.67	25.96	28.37	28.18	29.43	27.35
CYP97C	27.08	28.87	26.87	29.52	27.04	26.15	28.11	27.65	29.33	27.50
CYP97C	26.68	28.34	26.81	27.66	26.92	26.56	28.18	28.10	29.53	27.64
AVG	26.85	28.33	26.62	28.32	26.88	26.22	28.22	27.98	29.43	27.50
STDEV	0.20	0.78	0.49	1.04	0.19	0.31	0.18	0.37	0.07	0.15
ACTIN	20.29	20.15	20.68	21.19	20.93	19.74	23.21	20.75	23.67	21.66
ACTIN	20.79	20.02	20.75	21.21	20.75	19.86	23.11	20.50	23.58	21.58
ACTIN	20.34	20.09	20.94	21.28	20.97	20.10	23.30	20.69	23.17	21.68
AVG	20.47	20.09	20.79	21.23	20.88	19.90	23.21	20.65	23.47	21.64
SiDEV	0.28	0.07	0.13	0.05	0.12	0.18	0.10	0.13	0.27	0.05
Δ Ct CYP97A	-6.96	-9.45	-7.99	-10.14	-8.83	-9.98	-8.05	-8.53	-8.83	-7.27
Δ Ct CYP97C	-6.38	-8.24	-5.83	-7.10	-5.99	-6.32	-5.01	-7.33	-5.96	-5.86
CYP97A	0.80	0.14	0.39	0.09	0.22	0.10	0.38	0.27	0.22	0.65
CYP97C	1.20	0.33	1.76	0.73	1.57	1.25	3.10	0.62	1.61	1.73
CYP97A	0.59	0.14	0.31	0.10	0.16	0.07	0.40	0.20	0.22	0.53
CYP97A	1.00	0.15	0.54	0.08	0.21	0.10	0.37	0.33	0.23	0.69
CYP97A	0.87	0.14	0.36	0.08	0.31	0.13	0.37	0.30	0.20	0.75
STDEV	0.21	0.01	0.12	0.01	0.07	0.03	0.02	0.07	0.02	0.12
CYP97C	1.25	0.49	2.38	1.06	1.81	1.50	2.79	0.54	1.61	1.91
CYP97C	1.03	0.23	1.48	0.32	1.40	1.31	3.34	0.78	1.73	1.72
CYP97C	1.35	0.33	1.54	1.16	1.52	0.99	3.18	0.57	1.50	1.56
STDEV	0.17	0.13	0.51	0.46	0.21	0.26	0.28	0.13	0.11	0.17

Chapter 4: Spatial analysis (B73)

Spatial profiles of dioxigenases in maize						Relative to Endosperm at 20 DAP					
	Em	En (20)	En (25)	L	R		Em	En (20)	En (25)	L	R
MCCD1	26.84	29.65	30.66	20.93	25.63	Normalization	0.88	0.00	-2.14	-2.77	-2.46
MCCD1	26.77	29.75	30.05	20.81	25.38	NCCD1+ΔCt	-4.97	-8.08	-8.72	0.68	-3.93
MCCD1	26.40	29.92	30.53	21.30	25.87	Absolute	3.18	0.37	0.24	160.58	6.56
Average	26.67	29.77	30.41	21.01	25.63	SiDEV1	2.83	0.40	0.20	170.53	6.56
SiDEV	0.24	0.14	0.32	0.26	0.25	SiDEV2	2.97	0.38	0.31	185.32	7.80
MCCD4a	29.20	35.01	35.07	21.95	31.08	SiDEV3	3.85	0.34	0.22	131.95	5.56
MCCD4a	29.34	35.03	35.09	22.02	31.27	SiDEV	0.55	0.03	0.06	27.55	1.13
MCCD4a	29.05	35.04	35.12	22.24	31.33	NCCD4a+ΔCt	-7.50	-13.33	-13.40	-0.37	-9.53
Average	29.20	35.03	35.09	22.07	31.23	Absolute	0.55	0.01	0.01	77.20	0.14
SiDEV	0.15	0.02	0.03	0.15	0.13	SiDEV1	0.55	0.01	0.01	84.09	0.15
MCCD4b	29.76	32.43	32.44	25.51	29.97	SiDEV2	0.50	0.01	0.01	80.11	0.13
MCCD4b	29.74	33.15	33.10	25.24	29.96	SiDEV3	0.61	0.01	0.01	68.78	0.13
MCCD4b	28.92	32.24	31.90	25.44	29.81	SiDEV	0.06	0.00	0.00	7.94	0.01
Average	29.47	32.61	32.48	25.40	29.91	NCCD4b+ΔCt	-7.78	-10.91	-10.78	-3.70	-8.22
SiDEV	0.48	0.48	0.60	0.14	0.09	Absolute	0.46	0.05	0.06	7.69	0.34
MCCD7	29.66	32.59	32.86	30.30	27.09	SiDEV1	0.37	0.06	0.06	7.13	0.32
MCCD7	29.55	33.07	33.58	30.10	27.23	SiDEV2	0.38	0.04	0.04	8.60	0.33
MCCD7	28.66	33.27	33.28	30.80	27.27	SiDEV3	0.67	0.07	0.09	7.48	0.36
Average	29.29	32.98	33.24	30.40	27.20	SiDEV	0.17	0.02	0.02	0.77	0.02
SiDEV	0.55	0.35	0.36	0.36	0.09	NCCD7+ΔCt	-7.59	-11.28	-11.54	-8.70	-5.50
MCCD8a	31.81	ND	ND	ND	28.62	Absolute	0.52	0.04	0.03	0.24	2.21
MCCD8a	31.84	ND	ND	ND	29.22	SiDEV1	0.40	0.05	0.04	0.26	2.38
MCCD8a	30.80	ND	ND	ND	30.34	SiDEV2	0.43	0.04	0.03	0.30	2.16
Average	31.48	ND	ND	ND	29.39	SiDEV3	0.80	0.03	0.03	0.18	2.11
SiDEV	0.59	ND	ND	ND	0.87	SiDEV	0.22	0.01	0.01	0.06	0.15
MCCD8b	27.28	ND	ND	ND	26.26	NCCD8a+ΔCt	-9.79	ND	ND	ND	-7.70
MCCD8b	27.83	ND	ND	ND	26.15	Absolute	0.11	ND	ND	ND	0.48
MCCD8b	27.12	ND	ND	ND	26.24	SiDEV1	0.09	ND	ND	ND	0.83
Average	27.41	ND	ND	ND	26.22	SiDEV2	0.09	ND	ND	ND	0.54
SiDEV	0.37	ND	ND	ND	0.06	SiDEV3	0.18	ND	ND	ND	0.25
MNCEd1	26.58	30.85	31.15	28.97	29.25	SiDEV	0.05	ND	ND	ND	0.29
MNCEd1	26.72	31.19	31.51	28.83	29.07	NCCD8b+ΔCt	-5.71	ND	ND	ND	-4.52
MNCEd1	26.04	30.90	31.69	29.46	28.80	Absolute	1.91	ND	ND	ND	4.36
Average	26.45	30.98	31.45	29.09	29.04	SiDEV1	2.09051	ND	ND	ND	4.23939
SiDEV	0.36	0.18	0.27	0.33	0.23	SiDEV2	1.42786	ND	ND	ND	4.57527
MNCEd2	32.60	ND	ND	32.66	31.72	SiDEV3	2.3357	ND	ND	ND	4.29857
MNCEd2	31.73	ND	ND	32.67	32.86	SiDEV	0.46964	ND	ND	ND	0.17929
MNCEd2	31.65	ND	ND	32.45	31.97	NNCEd1+ΔCt	-4.75	-9.28	-9.75	-7.39	-7.34
Average	31.99	ND	ND	32.59	32.18	Absolute	3.72	0.16	0.12	0.60	0.62
SiDEV	0.53	ND	ND	0.12	0.60	SiDEV1	3.40	0.18	0.14	0.65	0.53
MNCEd3a	32.32	ND	ND	30.31	28.74	SiDEV2	3.08	0.14	0.11	0.71	0.60
MNCEd3a	32.32	ND	ND	30.31	29.13	SiDEV3	4.94	0.17	0.10	0.46	0.73
MNCEd3a	32.31	ND	ND	30.31	29.10	SiDEV	0.99	0.02	0.02	0.13	0.10
Average	32.32	ND	ND	30.31	28.99	NNCEd2+ΔCt	-10.30	ND	ND	-10.90	-10.49
SiDEV	0.01	ND	ND	0.00	0.22	Absolute	0.08	ND	ND	0.05	0.07
MNCEd3b	31.47	ND	ND	ND	31.72	SiDEV1	0.05	ND	ND	0.05	0.10
MNCEd3b	30.72	ND	ND	ND	31.59	SiDEV2	0.10	ND	ND	0.05	0.04
MNCEd3b	30.95	ND	ND	ND	31.45	SiDEV3	0.10	ND	ND	0.06	0.08
Average	31.05	ND	ND	ND	31.59	SiDEV	0.03	ND	ND	0.00	0.03
SiDEV	0.38	ND	ND	ND	0.14	NNCEd3a+ΔCt	-10.62	ND	ND	-8.61	-7.29
MNCEd9	28.12	32.19	32.22	31.36	29.91	Absolute	0.06	ND	ND	0.26	0.64
MNCEd9	28.76	33.60	32.70	31.54	30.24	SiDEV1	0.06	ND	ND	0.26	0.76
MNCEd9	28.57	32.29	31.64	31.52	30.14	SiDEV2	0.06	ND	ND	0.26	0.58
Average	28.48	32.69	32.52	31.47	30.10	SiDEV3	0.06	ND	ND	0.26	0.59
SiDEV	0.33	0.79	0.81	0.10	0.17	SiDEV	0.00	ND	ND	0.00	0.10
Actin	20.69	21.82	23.81	24.32	23.83	NNCEd3b+ΔCt	-9.35	ND	ND	ND	-9.89
Actin	20.68	21.65	23.88	24.44	24.32	Absolute	0.15	ND	ND	ND	0.11
Actin	21.08	21.62	23.82	24.65	24.32	SiDEV1	0.11	ND	ND	ND	0.10
Average	20.82	21.70	23.84	24.47	24.16	SiDEV2	0.19	ND	ND	ND	0.11
SiDEV	0.23	0.11	0.04	0.17	0.28	SiDEV3	0.16	ND	ND	ND	0.12
ΔCt1 CCD1	-5.85	-8.08	-6.58	3.46	-1.47	SiDEV	0.04	ND	ND	ND	0.01
MCCD1	1.73	0.37	1.05	1097.89	36.10	NNCEd9+ΔCt	-6.79	-11.00	-10.82	-9.78	-8.40
ΔCt1 CCD4a	-8.38	-13.33	-11.26	2.40	-7.07	Absolute	0.91	0.05	0.06	0.11	0.30
MCCD4a	0.30	0.01	0.04	527.80	0.74	SiDEV1	1.17	0.07	0.03	0.12	0.34
ΔCt1 CCD4b	-8.66	-10.91	-8.64	-0.93	-5.76	SiDEV2	0.75	0.03	0.05	0.11	0.27
MCCD4b	0.25	0.05	0.25	52.61	1.85	SiDEV3	0.85	0.06	0.10	0.11	0.29
ΔCt1 CCD7	-8.47	-11.28	-9.40	-5.93	-3.04	SiDEV	0.22	0.02	0.04	0.01	0.04
MCCD7	0.28	0.04	0.15	1.64	12.16	ND= not detectable					
ΔCt1 CCD8a	-10.67	ND	ND	ND	-5.24						
MCCD8a	0.06	ND	ND	ND	2.65						
ΔCt1 CCD8b	-5.59	ND	ND	ND	-2.06						

Chapter 4: Correlation analysis (Inbreds)

20DAP	A619	B73	B37	Cl.7	C131A	DE3	KUI2007	NC300	SD44	TZ118
MNCD1	33.21	30.85	31.48	29.98	31.75	28.69	29.73	33.35	30.31	31.87
MNCD1	33.46	31.19	32.13	30.60	31.69	28.86	30.14	33.26	29.73	31.72
MNCD1	32.66	30.90	32.09	30.68	31.14	29.02	30.28	32.36	31.22	31.33
AVG	33.11	30.98	31.90	30.42	31.53	28.86	30.05	32.99	30.42	31.64
StDEV	0.41	0.18	0.36	0.38	0.34	0.17	0.29	0.55	0.75	0.28
MCCD1	31.93	29.65	30.33	29.00	30.27	27.68	30.41	22.75	29.90	30.47
MCCD1	31.95	29.75	30.96	28.56	30.34	27.37	30.20	21.90	29.27	30.45
MCCD1	31.97	29.92	30.21	29.09	30.72	27.44	30.93	21.81	29.72	30.47
AVG	31.95	29.77	30.50	28.88	30.44	27.50	30.51	22.15	29.63	30.46
StDEV	0.02	0.14	0.40	0.28	0.24	0.16	0.38	0.52	0.32	0.01
ACTIN	21.14	22.03	22.03	22.26	22.58	20.11	20.95	21.95	22.97	21.85
ACTIN	21.11	21.82	21.92	21.73	22.37	20.54	21.05	21.69	22.00	21.89
ACTIN	22.22	21.65	22.14	22.32	22.30	20.40	21.51	20.80	22.12	21.77
AVG	21.49	21.83	22.03	22.10	22.42	20.35	21.17	21.48	22.36	21.84
StDEV	0.63	0.19	0.11	0.32	0.15	0.22	0.30	0.60	0.53	0.06
ΔCl NCED1	-11.62	-9.15	-9.87	-8.32	-9.11	-8.51	-8.88	-11.51	-8.06	-9.80
MNCD1	0.03	0.18	0.11	0.31	0.18	0.27	0.21	0.03	0.38	0.11
ΔCl CCD1	-10.46	-7.94	-8.47	-6.78	-8.03	-7.15	-9.34	-0.67	-7.27	-8.63
MCCD1	0.07	0.41	0.28	0.91	0.38	0.71	0.15	62.71	0.65	0.25
NCED1	0.03	0.19	0.14	0.43	0.16	0.31	0.26	0.03	0.41	0.10
	0.02	0.15	0.09	0.28	0.16	0.27	0.20	0.03	0.61	0.11
	0.04	0.19	0.09	0.26	0.24	0.25	0.18	0.05	0.22	0.14
StDEV	0.01	0.02	0.03	0.09	0.05	0.03	0.04	0.01	0.20	0.02
CCD1	0.07	0.44	0.32	0.84	0.43	0.62	0.17	41.47	0.54	0.25
	0.07	0.41	0.21	1.14	0.41	0.77	0.19	74.74	0.83	0.26
	0.07	0.37	0.34	0.79	0.32	0.73	0.12	79.55	0.61	0.25
StDEV	0.00	0.04	0.07	0.19	0.06	0.08	0.04	20.74	0.15	0.00

20DAP	A619	B73	B37	Cl.7	C131A	DE3	KUI2007	NC300	SD44	TZ118
MCCD7	31.05	32.59	30.28	30.26	30.11	28.11	32.71	29.88	30.71	31.23
MCCD7	31.12	33.07	30.65	30.92	30.60	29.11	32.81	29.79	31.57	30.06
MCCD7	31.10	33.27	30.45	30.75	30.55	28.91	32.75	29.65	31.45	30.75
AVG	31.09	32.98	30.46	30.64	30.42	28.71	32.76	29.77	31.24	30.68
StDEV	0.04	0.35	0.19	0.34	0.27	0.53	0.05	0.12	0.47	0.59
ACTIN	21.14	22.03	22.03	22.26	22.58	20.11	20.95	21.95	22.97	21.85
ACTIN	21.11	21.82	21.92	21.73	22.37	20.54	21.05	21.69	22.00	21.89
ACTIN	22.22	21.65	22.14	22.32	22.30	20.40	21.51	20.80	22.12	21.77
AVG	21.49	21.83	22.03	22.10	22.42	20.35	21.17	21.48	22.36	21.84
StDEV	0.63	0.19	0.11	0.32	0.15	0.22	0.30	0.60	0.53	0.06
ΔCl CCD7	-9.60	-11.14	-8.43	-8.54	-8.00	-8.36	-11.59	-8.29	-8.88	-8.84
MCCD7	0.13	0.04	0.29	0.27	0.39	0.30	0.03	0.32	0.21	0.22
CCD7	0.13	0.06	0.33	0.35	0.48	0.46	0.03	0.30	0.31	0.15
	0.13	0.04	0.25	0.22	0.34	0.23	0.03	0.32	0.17	0.33
	0.13	0.04	0.29	0.25	0.36	0.26	0.03	0.35	0.18	0.21
StDEV	0.00	0.01	0.04	0.07	0.08	0.12	0.00	0.03	0.08	0.10

20DAP	A619	B73	B37	Cl.7	C131A	DE3	KUI2007	NC300	SD44	TZ118
MCCD4a	32.66	35.01	32.59	31.15	31.72	30.48	32.15	32.04	32.44	32.67
MCCD4a	31.65	35.03	32.57	31.21	31.83	30.53	32.39	31.13	32.31	32.75
MCCD4a	31.95	35.04	32.50	31.05	31.75	30.35	32.50	31.55	32.55	32.91
AVG	32.09	35.03	32.55	31.14	31.77	30.45	32.35	31.57	32.43	32.78
StDEV	0.52	0.02	0.05	0.08	0.06	0.09	0.18	0.46	0.12	0.12
MCCD4b	32.68	32.43	30.78	31.78	32.82	29.89	32.35	31.80	32.03	31.83
MCCD4b	33.01	33.15	31.51	32.97	33.56	30.11	32.68	32.37	33.49	32.81
MCCD4b	33.00	32.24	31.55	31.85	33.25	30.95	32.45	32.51	33.50	32.45
AVG	32.90	32.61	31.28	32.20	33.21	30.32	32.49	32.23	33.01	32.36
StDEV	0.19	0.48	0.43	0.67	0.37	0.56	0.17	0.38	0.85	0.50
ACTIN	21.14	22.03	22.03	22.26	22.58	20.11	20.95	21.95	22.97	21.85
ACTIN	21.11	21.82	21.92	21.73	22.37	20.54	21.05	21.69	22.00	21.89
ACTIN	22.22	21.65	22.14	22.32	22.30	20.40	21.51	20.80	22.12	21.77
AVG	21.49	21.83	22.03	22.10	22.42	20.35	21.17	21.48	22.36	21.84
StDEV	0.63	0.19	0.11	0.32	0.15	0.22	0.30	0.60	0.53	0.06
ΔCl CCD4a	-10.60	-13.19	-10.52	-9.03	-9.35	-10.10	-11.18	-10.09	-10.07	-10.94
MCCD4a	0.06	0.01	0.07	0.19	0.15	0.09	0.04	0.09	0.09	0.05
ΔCl CCD4b	-11.41	-10.77	-9.25	-10.10	-10.79	-9.97	-11.32	-10.75	-10.64	-10.53
MCCD4b	0.04	0.06	0.16	0.09	0.06	0.10	0.04	0.06	0.06	0.07
CCD4a	0.04	0.01	0.07	0.19	0.16	0.09	0.05	0.07	0.09	0.05
	0.09	0.01	0.07	0.18	0.15	0.09	0.04	0.12	0.10	0.05
	0.07	0.01	0.07	0.20	0.16	0.10	0.04	0.09	0.09	0.05
StDEV	0.02	0.00	0.00	0.01	0.01	0.01	0.01	0.03	0.01	0.00
CCD4b	0.04	0.06	0.23	0.12	0.07	0.13	0.04	0.08	0.12	0.10
	0.03	0.04	0.14	0.05	0.04	0.12	0.03	0.05	0.04	0.05
	0.03	0.07	0.14	0.12	0.05	0.06	0.04	0.05	0.04	0.06

25DAP	A619	B73	B37	CL7	C131A	DE3	KUI2007	NC300	SD44	TZ18
MNCED1	28.10	31.15	30.77	31.59	30.84	29.63	29.79	32.23	31.44	30.06
MNCED1	28.01	31.51	30.30	30.69	30.39	30.51	29.65	32.74	32.59	30.74
MNCED1	27.83	31.69	30.56	31.33	30.00	31.17	30.00	32.69	30.62	30.13
AVG	27.98	31.45	30.54	31.20	30.41	30.44	29.81	32.55	31.55	30.31
SIDEV	0.14	0.27	0.24	0.46	0.42	0.77	0.18	0.28	0.99	0.37
MCCD1	29.97	30.66	28.81	29.72	28.82	29.97	29.27	23.00	30.00	31.09
MCCD1	30.77	30.05	29.14	29.80	29.44	31.17	29.10	23.04	31.23	31.25
MCCD1	30.74	30.53	28.96	30.62	29.92	31.17	28.18	23.78	31.72	31.21
AVG	30.49	30.41	28.97	30.05	29.39	30.77	28.85	23.27	30.98	31.18
SIDEV	0.45	0.32	0.17	0.50	0.55	0.69	0.59	0.44	0.89	0.08
ACTIN	20.49	21.05	21.15	20.77	21.16	19.40	20.88	20.09	21.04	22.71
ACTIN	20.58	22.70	21.35	20.45	21.44	19.58	22.60	20.34	22.73	23.71
ACTIN	20.87	21.75	21.98	20.87	21.75	19.50	21.90	20.83	21.79	24.18
AVG	20.65	21.83	21.49	20.70	21.45	19.49	21.79	20.42	21.85	23.53
SIDEV	0.20	0.83	0.43	0.22	0.30	0.09	0.86	0.38	0.85	0.75
ΔCI NCED1	-7.33	-9.62	-9.05	-10.51	-8.96	-10.94	-8.02	-12.13	-9.70	-6.78
MNCED1	0.62	0.12	0.19	0.07	0.20	0.05	0.39	0.02	0.12	0.91
ΔCI CCD1	-9.85	-8.58	-7.48	-9.35	-7.94	-11.28	-7.06	-2.85	-9.13	-7.65
MCCD1	0.11	0.26	0.56	0.15	0.41	0.04	0.75	13.84	0.18	0.50
NCED1	0.57	0.16	0.16	0.05	0.15	0.09	0.39	0.03	0.13	1.08
	0.61	0.12	0.22	0.10	0.20	0.05	0.43	0.02	0.06	0.68
	0.69	0.11	0.19	0.06	0.27	0.03	0.34	0.02	0.23	1.03
SIDEV	0.06	0.03	0.03	0.02	0.06	0.03	0.05	0.00	0.09	0.22
CCD1	0.16	0.22	0.63	0.19	0.60	0.07	0.56	16.72	0.35	0.53
	0.09	0.34	0.50	0.18	0.39	0.03	0.63	16.27	0.15	0.48
	0.09	0.24	0.57	0.10	0.28	0.03	1.20	9.74	0.11	0.49
SIDEV	0.04	0.06	0.06	0.05	0.16	0.02	0.35	3.91	0.13	0.03

25DAP	A619	B73	B37	CL7	C131A	DE3	KUI2007	NC300	SD44	TZ18
MCCD7	31.31	32.86	30.44	29.88	29.15	29.70	30.34	29.51	30.58	32.94
MCCD7	31.32	33.58	31.69	29.19	28.61	29.51	30.02	31.14	31.40	32.07
MCCD7	31.31	33.28	30.79	29.74	28.74	29.22	30.14	30.75	30.95	31.85
AVG	31.31	33.24	30.97	29.60	28.83	29.48	30.17	30.47	30.98	32.29
SIDEV	0.01	0.36	0.64	0.36	0.28	0.24	0.16	0.85	0.41	0.58
ACTIN	20.49	23.45	21.15	20.77	21.16	19.40	20.88	20.09	21.04	22.71
ACTIN	20.58	23.81	21.35	20.45	21.44	19.58	22.60	20.34	22.73	23.71
ACTIN	20.87	23.88	21.98	20.87	21.75	19.50	21.90	20.83	21.79	24.18
AVG	20.65	23.71	21.49	20.70	21.45	19.49	21.79	20.42	21.85	23.53
SIDEV	0.20	0.23	0.43	0.22	0.30	0.09	0.86	0.38	0.85	0.75
ΔCI CCD7	-10.67	-9.53	-9.48	-8.91	-7.38	-9.98	-8.37	-10.05	-9.12	-8.75
MCCD7	0.06	0.14	0.14	0.21	0.60	0.10	0.30	0.09	0.18	0.23
CCD7	0.06	0.18	0.20	0.17	0.48	0.08	0.27	0.18	0.24	0.15
	0.06	0.11	0.09	0.28	0.70	0.10	0.33	0.06	0.13	0.27
	0.06	0.13	0.16	0.19	0.64	0.12	0.31	0.08	0.18	0.31
SIDEV	0.00	0.04	0.06	0.06	0.11	0.02	0.03	0.07	0.05	0.09

25DAP	A619	B73	B37	CL7	C131A	DE3	KUI2007	NC300	SD44	TZ18
MCCD4a	32.35	35.07	32.55	32.75	29.40	31.42	27.27	32.34	32.49	33.48
MCCD4a	32.00	35.09	32.21	32.47	29.18	31.39	27.40	32.14	30.73	33.79
MCCD4a	32.68	35.12	32.11	32.47	29.45	31.55	27.35	32.55	30.95	33.56
AVG	32.34	35.09	32.29	32.56	29.34	31.45	27.34	32.34	31.39	33.61
SIDEV	0.34	0.03	0.23	0.16	0.14	0.09	0.07	0.21	0.96	0.16
MCCD4b	33.45	32.44	32.26	32.06	29.07	30.94	31.59	31.88	33.45	33.16
MCCD4b	32.97	33.10	32.11	32.75	29.65	31.76	31.93	33.03	31.71	33.53
MCCD4b	33.85	31.90	32.20	33.01	29.15	31.14	30.79	32.97	31.97	33.36
AVG	33.42	32.48	32.19	32.61	29.29	31.28	31.44	32.63	32.38	33.35
SIDEV	0.44	0.60	0.08	0.49	0.31	0.43	0.59	0.65	0.94	0.19
ACTIN	20.49	23.45	21.15	20.77	21.16	19.40	20.88	20.09	21.04	22.71
ACTIN	20.58	23.81	21.35	20.45	21.44	19.58	22.60	20.34	22.73	23.71
ACTIN	20.87	23.88	21.98	20.87	21.75	19.50	21.90	20.83	21.79	24.18
AVG	20.65	23.71	21.49	20.70	21.45	19.49	21.79	20.42	21.85	23.53
SIDEV	0.20	0.23	0.43	0.22	0.30	0.09	0.86	0.38	0.85	0.75
ΔCI CCD4a	-11.70	-11.38	-10.80	-11.87	-7.89	-11.96	-5.55	-11.92	-9.54	-10.08
MCCD4a	0.03	0.04	0.06	0.03	0.42	0.03	2.14	0.03	0.13	0.09
ΔCI CCD4b	-12.78	-8.77	-10.70	-11.91	-7.84	-11.79	-9.64	-12.21	-10.52	-9.82
MCCD4b	0.01	0.23	0.06	0.03	0.44	0.03	0.13	0.02	0.07	0.11
CCD4a	0.03	0.04	0.05	0.02	0.40	0.03	2.25	0.03	0.06	0.10
	0.04	0.04	0.06	0.03	0.47	0.03	2.05	0.03	0.21	0.08
	0.02	0.04	0.06	0.03	0.39	0.02	2.12	0.02	0.18	0.10
SIDEV	0.01	0.00	0.01	0.00	0.04	0.00	0.10	0.00	0.08	0.01
CCD4b	0.01	0.24	0.06	0.04	0.51	0.04	0.11	0.04	0.03	0.13
	0.02	0.15	0.06	0.02	0.34	0.02	0.09	0.02	0.11	0.10
	0.01	0.34	0.06	0.02	0.48	0.03	0.20	0.02	0.09	0.11

Figure 5A: Extracted from figure S3

20DAP	A619	B73	B37	CI.7	C131A	DE3	KUI2007	NC300	SD44	TZI18
MCCD1	31.93	29.65	30.33	29.00	30.27	27.68	30.41	22.75	29.90	30.47
MCCD1	31.95	29.75	30.96	28.56	30.34	27.37	30.20	21.90	29.27	30.45
MCCD1	31.97	29.92	30.21	29.09	30.72	27.44	30.93	21.81	29.72	30.47
AVG	31.95	29.77	30.50	28.88	30.44	27.50	30.51	22.15	29.63	30.46
StDEV	0.02	0.14	0.40	0.28	0.24	0.16	0.38	0.52	0.32	0.01
ACTIN	21.14	22.03	22.03	22.26	22.58	20.11	20.95	21.95	22.97	21.85
ACTIN	21.11	21.82	21.92	21.73	22.37	20.54	21.05	21.69	22.00	21.89
ACTIN	22.22	21.65	22.14	22.32	22.30	20.40	21.51	20.80	22.12	21.77
AVG	21.49	21.83	22.03	22.10	22.42	20.35	21.17	21.48	22.36	21.84
StDEV	0.63	0.19	0.11	0.32	0.15	0.22	0.30	0.60	0.53	0.06
ΔCt CCD1	-10.46	-7.94	-8.47	-6.78	-8.03	-7.15	-9.34	-0.67	-7.27	-8.63
MCCD1	0.07	0.41	0.28	0.91	0.38	0.71	0.15	62.71	0.65	0.25
CCD1	0.07	0.44	0.32	0.84	0.43	0.62	0.17	41.47	0.54	0.25
	0.07	0.41	0.21	1.14	0.41	0.77	0.19	74.74	0.83	0.26
	0.07	0.37	0.34	0.79	0.32	0.73	0.12	79.55	0.61	0.25
StDEV	0.00	0.04	0.07	0.19	0.06	0.08	0.04	20.74	0.15	0.00

25DAP	A619	B73	B37	CI.7	C131A	DE3	KUI2007	NC300	SD44	TZI18
MCCD1	29.97	30.66	28.81	29.72	28.82	29.97	29.27	23.00	30.00	31.09
MCCD1	30.77	30.05	29.14	29.80	29.44	31.17	29.10	23.04	31.23	31.25
MCCD1	30.74	30.53	28.96	30.62	29.92	31.17	28.18	23.78	31.72	31.21
AVG	30.49	30.41	28.97	30.05	29.39	30.77	28.85	23.27	30.98	31.18
StDEV	0.45	0.32	0.17	0.50	0.55	0.69	0.59	0.44	0.89	0.08
ACTIN	20.49	21.05	21.15	20.77	21.16	19.40	20.88	20.09	21.04	22.71
ACTIN	20.58	22.70	21.35	20.45	21.44	19.58	22.60	20.34	22.73	23.71
ACTIN	20.87	21.75	21.98	20.87	21.75	19.50	21.90	20.83	21.79	24.18
AVG	20.65	21.83	21.49	20.70	21.45	19.49	21.79	20.42	21.85	23.53
StDEV	0.20	0.83	0.43	0.22	0.30	0.09	0.86	0.38	0.85	0.75
ΔCt CCD1	-9.85	-8.58	-7.48	-9.35	-7.94	-11.28	-7.06	-2.85	-9.13	-7.65
MCCD1	0.11	0.26	0.56	0.15	0.41	0.04	0.75	13.84	0.18	0.50
CCD1	0.16	0.22	0.63	0.19	0.60	0.07	0.56	16.72	0.35	0.53
	0.09	0.34	0.50	0.18	0.39	0.03	0.63	16.27	0.15	0.48
	0.09	0.24	0.57	0.10	0.28	0.03	1.20	9.74	0.11	0.49
StDEV	0.04	0.06	0.06	0.05	0.16	0.02	0.35	3.91	0.13	0.03

Figure 5B: Relative quantification of gene copy number

	A619	B73	B37	CI7	C131A	DE3	KUI2007	NC300	SD44	TZI18
CCD1	26.11	22.67	24.46	23.06	23.21	24.52	27.54	20.96	23.43	26.30
	24.70	22.35	24.34	23.61	23.42	24.12	29.18	21.09	22.74	25.83
	24.98	23.17	24.46	24.04	23.71	25.86	27.29	21.11	23.87	27.56
Average	25.26	22.73	24.42	23.57	23.45	24.83	28.00	21.05	23.35	26.56
Stdev	0.75	0.41	0.07	0.49	0.25	0.91	1.03	0.08	0.57	0.89
PDS	26.68	22.31	23.89	25.28	23.91	23.37	24.52	24.15	24.78	25.44
	26.07	21.81	22.52	24.38	23.36	22.68	24.25	23.82	24.33	24.11
	26.36	22.01	23.19	24.82	23.63	22.96	24.35	23.92	24.55	24.76
Average	26.37	22.04	23.20	24.83	23.63	23.00	24.37	23.96	24.55	24.77
Stdev	0.31	0.25	0.69	0.45	0.28	0.35	0.14	0.17	0.23	0.67
PDS-CCD1	1.11	-0.69	-1.22	1.26	0.19	-1.83	-3.63	2.91	1.21	-1.79
Fold	2.15	0.62	0.43	2.39	1.14	0.28	0.08	7.52	2.31	0.29

Chapter 5: Spatial analysis (B73)

	Leaf	Root	Endosperm	Embryo
ABA8Ox1a	27.42	24.82	26.01	28.25
ABA8Ox1a	27.05	24.57	25.99	28.35
ABA8Ox1a	27.63	25.09	26.57	28.43
AVE	27.37	24.83	26.19	28.34
Stdev	0.29	0.26	0.33	0.09
ABA8Ox1a	27.24	26.65	29.26	28.91
ABA8Ox1a	26.99	26.77	30.12	29.14
ABA8Ox1a	27.57	26.77	30.02	29.88
AVE	27.27	26.73	29.80	29.31
Stdev	0.29	0.07	0.47	0.51
ABA8Ox2	30.88	29.20	25.57	28.15
ABA8Ox2	30.48	28.87	25.31	28.11
ABA8Ox2	31.83	28.88	25.48	27.80
AVE	31.06	28.98	25.45	28.02
Stdev	0.69	0.19	0.13	0.19
ABA8Ox3a	22.14	27.84	29.99	30.23
ABA8Ox3a	22.27	27.78	29.98	30.90
ABA8Ox3a	22.66	27.30	30.25	30.82
AVE	22.36	27.64	30.07	30.65
stdev	0.27	0.30	0.15	0.37
ABA8Ox3a	28.86	27.85	32.66	30.76
ABA8Ox3a	28.84	27.37	31.86	30.44
ABA8Ox3a	28.15	28.05	31.97	29.88
AVE	28.62	27.76	32.16	30.36
Stdev	0.40	0.35	0.43	0.45
Actin	25.6	22.68	20.09	19.38
Actin	25.67	22.99	20.17	19.31
Actin	26.53	23.25	20.24	19.68
Average	25.93	22.97	20.17	19.46
Stdev	0.52	0.29	0.08	0.20
Actin-ABA8Ox1a	-1.49	-1.85	-5.84	-8.79
Actin-ABA8Ox1a	-1.12	-1.60	-5.82	-8.89
Actin-ABA8Ox1a	-1.70	-2.12	-6.40	-8.97
Average	-1.43	-1.85	-6.02	-8.89
Power	37.03	27.68	1.54	0.21
Actin-ABA8Ox1b	-1.31	-3.68	-9.09	-9.45
Actin-ABA8Ox1b	-1.06	-3.80	-9.95	-9.68
Actin-ABA8Ox1b	-1.64	-3.80	-9.85	-10.42
Average	-1.33	-3.76	-9.63	-9.85
Power	39.69	7.40	0.13	0.11
Actin-ABA8Ox2	-4.95	-6.23	-5.40	-8.69
Actin-ABA8Ox2	-4.55	-5.90	-5.14	-8.65
Actin-ABA8Ox2	-5.90	-5.91	-5.31	-8.34
Average	-5.13	-6.01	-5.29	-8.56
Power	2.86	1.55	2.56	0.26
Actin-ABA8ox3a	3.79	-4.87	-9.82	-10.77
Actin-ABA8ox3a	3.66	-4.81	-9.81	-11.44
Actin-ABA8ox3a	3.27	-4.33	-10.08	-11.36
Average	3.58	-4.67	-9.91	-11.19
Power	1193.12	3.94	0.10	0.04
Actin-ABA8ox3b	-2.93	-4.88	-12.49	-11.30
Actin-ABA8ox3b	-2.91	-4.40	-11.69	-10.98
Actin-ABA8ox3b	-2.22	-5.08	-11.80	-10.42
Average	-2.68	-4.78	-12.00	-10.90
Power	15.57	3.63	0.02	0.05
	Leaf	Root	Endo	Embryo

Chapter 5: Drought treatment (B73)

	0	2	4	6	8 2h	4h	24h	
ABA8Ox1a	24.82	24.2	23.89	24.57	24.82	24.38	23.7	25.03
ABA8Ox1a	24.57	23.98	24.3	24.24	24.79	24.16	22.98	24.81
ABA8Ox1a	25.09	24.33	24.26	24.36	24.54	24.31	23.46	24.71
AVE	24.83	24.17	24.15	24.39	24.72	24.28	23.38	24.85
Stdev	0.26	0.18	0.23	0.17	0.15	0.11	0.37	0.16
ABA8Ox1b	26.65	25.95	25.58	23.91	23.99	24.96	25.73	26.04
ABA8Ox1b	26.77	26.44	25.74	24.10	24.16	25.09	25.06	26.71
ABA8Ox1b	26.77	26.39	25.77	24.19	24.26	25.33	25.88	27.22
AVE	26.73	26.26	25.70	24.07	24.14	25.13	25.56	26.66
Stdev	0.07	0.27	0.10	0.14	0.14	0.19	0.44	0.59
ABA8Ox3a	27.84	28.00	26.93	26.66	25.84	27.28	26.37	27.33
ABA8Ox3a	27.78	27.03	26.76	26.48	26.22	27.39	26.68	27.75
ABA8Ox3a	27.30	27.42	27.30	26.77	25.98	27.21	25.97	27.80
AVE	27.64	27.48	27.00	26.64	26.01	27.29	26.34	27.63
stdev	0.30	0.49	0.28	0.15	0.19	0.09	0.36	0.26
ABA8Ox3b	27.85	27.50	26.23	25.72	25.37	26.40	26.75	25.94
ABA8Ox3b	27.37	27.42	25.90	26.04	25.60	25.90	25.86	28.09
ABA8Ox3b	28.05	27.02	26.36	25.41	25.69	26.5	26.77	26.24
AVE	27.76	27.31	26.16	25.72	25.55	26.27	26.46	26.76
Stdev	0.35	0.26	0.24	0.32	0.17	0.32	0.52	1.16
Actin	22.68	22.81	23.85	23.67	23.7	24.61	23.8	22.79
Actin	22.99	22.85	23.99	23.31	23.44	24.2	23.17	23.75
Actin	23.25	23.05	23.09	23.66	23.61	23.79	23.34	22.73
Average	22.97	22.90	23.64	23.55	23.58	24.20	23.44	23.09
Stdev	0.29	0.13	0.48	0.21	0.13	0.41	0.33	0.57
Actin-ABA8Ox1a	-1.85	-1.30	-0.25	-1.02	-1.24	-0.18	-0.26	-1.94
Actin-ABA8Ox1a	-1.60	-1.08	-0.66	-0.69	-1.21	0.04	0.46	-1.72
Actin-ABA8Ox1a	-2.12	-1.43	-0.62	-0.81	-0.96	-0.11	-0.02	-1.62
Average	-1.85	-1.27	-0.51	-0.84	-1.13	-0.08	0.06	-1.76
Power	0.28	0.41	0.84	0.49	0.42	0.88	0.83	0.26
Power	0.33	0.47	0.63	0.62	0.43	1.03	1.37	0.30
Power	0.23	0.37	0.65	0.57	0.52	0.93	0.98	0.33
Average	0.28	0.42	0.71	0.56	0.46	0.95	1.06	0.30
Stdev	0.05	0.05	0.12	0.06	0.05	0.07	0.28	0.03
Factor	27.98	41.77	70.98	55.98	45.76	94.58	106.32	29.65
Factor	5.01	5.19	11.56	6.37	5.01	7.46	27.82	3.29
Actin-ABA8Ox1b	-3.68	-3.05	-1.94	-0.36	-0.41	-0.76	-2.29	-2.95
Actin-ABA8Ox1b	-3.80	-3.54	-2.10	-0.55	-0.58	-0.89	-1.62	-3.62
Actin-ABA8Ox1b	-3.80	-3.49	-2.13	-0.64	-0.68	-1.13	-2.44	-4.13
Average	-3.76	-3.36	-2.05	-0.52	-0.55	-0.93	-2.12	-3.57
Power	0.08	0.12	0.26	0.78	0.75	0.59	0.20	0.13
Power	0.07	0.09	0.23	0.68	0.67	0.54	0.32	0.08
Power	0.07	0.09	0.23	0.64	0.63	0.46	0.18	0.06
Average	0.07	0.10	0.24	0.70	0.68	0.53	0.24	0.09
Stdev	0.00	0.02	0.02	0.07	0.07	0.07	0.08	0.04
Factor	7.40	9.88	24.13	69.97	68.35	52.90	23.75	8.93
Factor	0.36	1.93	1.74	7.04	6.54	6.74	7.61	3.68
Actin-ABA8ox3a	-4.87	-5.10	-3.29	-3.11	-2.26	-3.08	-2.93	-4.24
Actin-ABA8ox3a	-4.81	-4.13	-3.12	-2.93	-2.64	-3.19	-3.24	-4.66
Actin-ABA8ox3a	-4.33	-4.52	-3.66	-3.22	-2.40	-3.01	-2.53	-4.71
Average	-4.67	-4.58	-3.35	-3.09	-2.43	-3.09	-2.90	-4.54
Power	0.03	0.03	0.10	0.12	0.21	0.12	0.13	0.05
Power	0.04	0.06	0.12	0.13	0.16	0.11	0.11	0.04
Power	0.05	0.04	0.08	0.11	0.19	0.12	0.17	0.04
Average	0.04	0.04	0.10	0.12	0.19	0.12	0.14	0.04
Stdev	0.01	0.01	0.02	0.01	0.02	0.01	0.03	0.01
Factor	3.99	4.34	9.90	11.78	18.67	11.73	13.64	4.36
Factor	0.86	1.40	1.82	1.21	2.44	0.73	3.39	0.81
Actin-ABA8ox3b	-4.88	-4.60	-2.59	-2.17	-1.79	-2.20	-3.31	-2.85
Actin-ABA8ox3b	-4.40	-4.52	-2.26	-2.49	-2.02	-1.70	-2.42	-5.00
Actin-ABA8ox3b	-5.08	-4.12	-2.72	-1.86	-2.11	-2.30	-3.33	-3.15
Average	-4.78	-4.41	-2.52	-2.18	-1.97	-2.07	-3.02	-3.67
Power	0.03	0.04	0.17	0.22	0.29	0.22	0.10	0.14
Power	0.05	0.04	0.21	0.18	0.25	0.31	0.19	0.03
Power	0.03	0.06	0.15	0.27	0.23	0.20	0.10	0.11
Average	0.04	0.05	0.18	0.22	0.26	0.24	0.13	0.09
Stdev	0.01	0.01	0.03	0.05	0.03	0.06	0.05	0.06
Factor	3.70	4.76	17.60	22.47	25.64	24.28	12.87	9.42
Factor	0.93	0.88	2.97	4.87	2.99	5.67	5.00	5.61

ABA8Ox2	29.20	28.14	28.30	27.09	26.84	28.37	28.87	28.33
ABA8Ox2	28.87	27.82	28.98	27.33	26.27	28.12	28.15	28.53
ABA8Ox2	28.88	28.07	28.78	26.32	25.54	28.25	28.37	28.77
AVE	28.98	28.01	28.69	26.91	26.22	28.25	28.46	28.54
Stdev	0.19	0.17	0.35	0.53	0.65	0.13	0.37	0.22
Actin	22.68	22.81	23.85	23.67	23.7	24.61	23.8	22.79
Actin	22.99	22.85	23.99	23.31	23.44	24.2	23.17	23.75
Actin	23.25	23.05	23.09	23.66	23.61	23.79	23.34	22.73
Average	22.97	22.90	23.64	23.55	23.58	24.20	23.44	23.09
Stdev	0.29	0.13	0.48	0.21	0.13	0.41	0.33	0.57
Actin-ABA8Ox2	-6.23	-5.24	-4.66	-3.54	-3.26	-4.17	-5.43	-5.24
Actin-ABA8Ox2	-5.90	-4.92	-5.34	-3.78	-2.69	-3.92	-4.71	-5.44
Actin-ABA8Ox2	-5.91	-5.17	-5.14	-2.77	-1.96	-4.05	-4.93	-5.68
Average	-6.01	-5.11	-5.04	-3.37	-2.63	-4.05	-5.03	-5.45
Power	0.01	0.03	0.04	0.09	0.10	0.06	0.02	0.03
Power	0.02	0.03	0.02	0.07	0.16	0.07	0.04	0.02
Power	0.02	0.03	0.03	0.15	0.26	0.06	0.03	0.02
Average	0.02	0.03	0.03	0.10	0.17	0.06	0.03	0.02
Stdev	0.00	0.00	0.01	0.04	0.08	0.01	0.01	0.00
Factor	1.56	2.92	3.09	10.16	17.25	6.07	3.13	2.30
Factor	0.19	0.35	0.78	3.93	7.79	0.53	0.76	0.35
	0	2	4	6	8	2	4	24

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