Carotenoid Biosynthesis in Maize: Characterization of the *PSY* Gene Family and Genetic Definition of a New Biosynthetic Step in Higher Plants

BY

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ABSTRACT

Carotenoid Biosynthesis in Maize: Characterization of the *PSY* Gene Family and Genetic Definition of a New Biosynthetic Step in Higher Plants

By FAQIANG LI

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To date, vitamin A deficiency remains a significant global health problem. Improving the carotenoid content in food crops to provide provitamin A could alleviate this nutritional problem. To achieve this goal, a complete carotenoid biosynthetic pathway and well-characterized regulatory mechanism (s) are required. The carotenoid biosynthetic pathway remains uncertain at its isomerization steps although most parts are well established. Despite ongoing regulation studies of carotenoid biosynthesis in several model organisms, few of these studies involve members of the Grass family (Poaceae), which contains most of the agronomically important crops. Thus, I chose maize as a model for the Grass family to study the regulation of the carotenoid biosynthetic pathway.

The first committed step of the carotenoid biosynthetic pathway is catalyzed by phytoene synthase or PSY, which is thought to be a rate-controlling enzyme. Therefore, genes encoding PSY were selected to study the regulation of carotenoid biosynthesis in maize. My data showed that the maize genome possesses three gene copies encoding functional PSY enzymes. Transcript assays revealed that *PSY1* but not *PSY2* or *PSY3*

mRNA levels were correlated with carotenoid accumulation in endosperm. On the other hand, the up-regulation of *PSY2* transcripts was associated with leaf carotenogenesis during greening. Maize *PSY3* expresses predominately in root and embryo tissues, where carotenoids accumulate at negligible levels. *PSY3* transcript levels in roots are induced in response to various abiotic stresses and are associated with accumulation of abscisic acid (ABA).

The carotenoid composition analysis of maize kernel mutants also led to the genetic definition of a new isomerization step required for carotenoid biosynthesis. HPLC analysis revealed that dark-grown tissues, such as roots and etiolated leaves of the maize *y9* mutant condition accumulation of the product of phytoene desaturase (PDS), 9,15,9′-tri-*cis*-ζ-carotene. This finding suggests that maize *Y9* encodes a factor required for an isomerase activity which is necessary for converting the product of PDS (9,15,9′-tri-*cis*-ζ-carotene) into the suitable ZDS substrate (9,9′-di-*cis*-ζ-carotene); this enzyme activity I named Z-ISO (15-cis zeta-carotene isomerase). In the future, cloning of the maize y9 locus will be critical for elucidation of the isomerization reactions in the carotenoid biosynthetic pathway of plants.

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LIST OF ABBREVIATIONS

aa	amino acid
ABA	abscisic acid
ABA2	short chain alcohol dehydrogenase
AAO3	abscisic acid oxidase
bp	base pair
BSA	bovine serum albumin
CCD	carotenoid cleavage dioxygenase
cDNA	complementary DNA
crtB	carotenoid gene B
crtD	carotenoid gene D
crtI	carotenoid gene I
CRTISO	carotenoid isomerase
cry-2	cryptochrome 2
dad1	*decreased apical dominance* mutant
DAP	days after pollination
DET1	DEETIOLATED1
DXS	deoxyxylulose 5-phosphate synthase
E. coli	*Escherichia coli*
elm1	*elongated mesocotyl1* mutant
FAD	flavin adenine dinucleotide
GGPP	geranylgeranyl pyrophosphate
GGPPS	geranylgeranyl pyrophosphate synthase
GUS	β-glucuronidase
HDR	hydroxymethylbutenyl 4-diphosphate reductase
HPLC	High Performance Liquid Chromatography
HYDB	*β*-hydroxylase
HYDE	*ε*-hydroxylase
IPI	isopentenyl pyrophosphate isomerase
IPP	isopentenyl pyrophosphate
IPTG	isopropylthio-β-D-galactoside
kb	kilo base pair
kDa	kilo Dalton
LB	Luria-Bertani
LCYB	lycopene beta cyclase
LCYE	lycopene epsilon cyclase
max	*more axillary growth* mutants
MEP	methylerythritol 4-phosphate
NCED	9-*cis*-epoxycarotenoid dioxygenase
ORF	open reading frame
PCR	polymerase chain reaction

PDS	phytoene desaturase
PLB	prolamellar body
PSY	phytoene synthase
rms1	*ramosus1* mutant
RT-PCR	reverse transcriptase polymerase chain reaction
Sh	Shrunken
VDE	violaxanthin de-epoxidase
vp	viviparous
w	white
y	yellow
ZDS	zeta-carotene desaturase
ZEP	zeaxanthin epoxidase
Z-ISO	zeta-carotene isomerase

CHAPTER 1

BACKGROUND AND INTRODUCTION

1.1 The general roles of carotenoids in plants and human beings

Carotenoids are a diverse group of pigments found in plants, fungi and bacteria. To date more than 750 carotenoids have been identified (Britton et al., 2004). *β*-carotene, which furnishes carrots their orange color, and lycopene, which is responsible for the red pigment found in tomato are two typical carotenoids found in plants. All carotenoids are C_{40} tetraterpenoids derived from isoprene and their polyene backbones are either linear or possess one or more cyclic *β*-ionone or *ε*-ionone rings at either end. Non-oxygenated carotenoids are referred to as carotenes, such as *β*-carotene. When they are oxygenated, they are designated as xanthophylls, such as lutein (Matthews and Wurtzel, 2007).

In higher plants, carotenoids act as accessory pigments in the photosynthetic apparatus to assist in light harvesting. Chlorophylls absorb red and blue light, while carotenoids absorb blue-green and blue light, and subsequently transfer the captured energy to chlorophylls (Taiz and Zeiger, 2006). Carotenoids also function as photoprotectants to prevent photo-oxidative damage. In intense light, excited triplet state chlorophylls are formed and interact with oxygen molecules to produce reactive and damaging singlet oxygen molecules. Carotenoids can quench these triplet chlorophylls and singlet oxygen molecules by dissipating excess excitation energy in the form of heat

(Niyogi, 1999, 2000). The major carotenoids in the plant photosynthetic apparatus are lutein, *β*-carotene, and zeaxanthin; other carotenoids found in green tissue include neoxanthin, antheraxanthin and violaxanthin.

In higher plants, some carotenoids also serve as precursors for the synthesis of the hormone abscisic acid (ABA) (Nambara and Marion-Poll, 2005). ABA is a carotenoid cleavage product and plays important roles in regulating plant stress responses, differential growth, and embryo dormancy. In addition, carotenoids also color flowers and fruits in plants to attract pollinators and seed dispersal agents.

Carotenoids not only decorate this world with their beautiful color, but also enable us to see this colorful world. In humans and animals, carotenoids are essential precursors for the synthesis of vitamin A (retinaldehyde), which can act as a chromophore in vision (retinol). Vitamin A deficiency can lead to night blindness or even irreversible blindness, especially in children. Global vitamin A deficiency affects 140 million children in 118 countries and results in increased mortality (WHO, http://www.who.int/vmnis/vitamina/en/). Vitamin A is a C_{20} compound and can be synthesized by cleaving any carotenoid containing an unmodified *β*-ionone ring, such as *β*-carotene. In flies and chickens, the enzymes that catalyze the cleavage reaction have been identified and designated as *β*-carotene 15, 15'-dioxygenase (Wyss et al., 2000; von Lintig and Wyss, 2001).

Apart from being precursors for the synthesis of vitamin A, nonprovitamin A carotenoids as antioxidants have been suggested to play beneficial roles in human health (reviewed in Rao and Rao, 2007) . For example, lutein and zeaxanthin have been associated with prevention of macular degeneration (Krinsky et al., 2003); lycopene has been associated with prostate cancer prevention (Giovannucci et al., 2002). In addition, astaxanthin has also been implicated in reducing the risk of cancer and diabetes (Hussein et al., 2006).

1.2 The carotenoid biosynthetic pathway in plants

Over the past half century, the carotenoid biosynthetic pathways of plants, fungi and bacteria have been extensively studied; nearly all the carotenogenic genes have been identified and their products characterized. All plant carotenogenic genes are located in the nucleus but their encoded enzymes must be transported into a variety of plastids where carotenoids are synthesized (Matthews and Wurtzel, 2007).

Carotenoid biosynthesis starts with the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to form a molecule of 15-*cis* phytoene (**Fig. 1-1**). This reaction is catalyzed by phytoene synthase or PSY, which is regarded as a rate-controlling step in the carotenoid biosynthetic pathway (Armstrong, 1994). Partial purification of PSY from tomato (*Lycopersicon esculentum*) chloroplasts revealed that it forms a protein complex together with the isoprenoid biosynthesis enzyme isopentenyl

diphosphate isomerase (IPI) and GGPP synthase (GGPPS)(Fraser et al., 2000).

PSY is a single copy gene in some plants, such as *A. thaliana*, however multiple copies are also found in other plants, such as tomato, tobacco and most species in the Grass family (Poaceae). *PSY1* in tomato is a fruit- and flower-specific gene, whereas *PSY2* is the predominant one expressed in green tissue (Bartley and Scolnik, 1993). Maize (*Zea mays*) *PSY1* was the first *PSY* gene identified in the Grass family (Poaceae), which was isolated through characterization of the endosperm carotenoid-free *y1* mutant (Buckner et al., 1996). Soon after a second copy was also identified in the maize and rice genomes based on the sequence homology to maize *PSY1* (Palaisa et al., 2003; Gallagher et al., 2004). PCR with ortholog-specific universal primers determined that duplicated PSY genes are present in at least eight subfamilies of the Poaceae, which suggests that this duplication may have occurred prior to the evolution of the Poaceae (Gallagher et al., 2004).

The PSY product, 15-*cis* phytoene rarely accumulates in plants and is subsequently converted to all-*trans* lycopene. In plants, this process requires two desaturases (phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS) and some supplementary isomerization reactions (Isaacson et al., 2002; Park et al., 2002; Li et al., 2007), whereas only one enzyme, CRTI, is required to carry out this entire process in bacteria (Linden et al., 1991).

The PDS and ZDS enzymes share similar structure and sequence and are unrelated to the bacterial CRTI. PDS and ZDS, together introduce four double bonds into the phytoene molecule; first at the C11, 12 and C11', 12' positions by PDS and then at the C7, 8 and C7', 8' positions by ZDS (**Fig. 1-1**). These dehydrogenation reactions require plastoquinone (Mayer et al., 1992; Norris et al., 1995) or plastid terminal oxidase (Carol and Kuntz, 2001) as an intermediate, and oxygen as the final electron acceptor (Al-Babili et al., 1996).

In plants the desaturation products of PDS (9,15, 9'-tri-*cis* ζ-carotene) and ZDS (tetra-*cis* lycopene) must be isomerized to produce acceptable geometrical isomer substrates for the following enzymes (Beyer et al., 1989). It is essential that the desaturation product of PDS, 9,15,9'-tri-*cis* ζ-carotene be isomerized to 9,9'-di-*cis* ζ-carotene in order for it to be further desaturated by ZDS (Beyer et al., 1989). Similarly, the product of ZDS, tetra-*cis* lycopene cannot be cyclized until it is converted to all-*trans* lycopene (Bartley et al., 1999) . Recently, characterization of the tomato mutant *tangerine* led to the cloning of the long-sought-after carotenoid isomerase gene, CRTISO (Isaacson et al., 2002). When CRTISO was assayed, it was revealed that CRTISO only recognized products of ZDS but not those of PDS (Isaacson et al., 2004). Although the 15-*cis* bond in the product of PDS (9,15,9'-tri-*cis* ζ-carotene) can be photoisomerized in light exposed tissues, there is still the question of whether there is a gene responsible for such activity in the dark or in dark-grown tissue (Bartley et al., 1999; Matthews et al., 2003). To identify

this missing isomerase, which we call Z-ISO (zeta-carotene isomerase), I screened carotenoid biosynthetic pathway mutants that accumulate atypical geometrical isomers. A maize pale yellow kernel mutant, *y9,* was found to accumulate the product of PDS, a 9,15,9' -tri-*cis* ζ-carotene isomer in its etiolated leaves and roots. This finding indicated that the carotenoid biosynthetic pathway is blocked at the step of converting the PDS product into the suitable ZDS substrate and that *y9* is essential for the activity of Z-ISO (Li et al., 2007). The identification process will be discussed in detail in Chapter 4.

The carotenoid biosynthetic pathway branches after the step of lycopene cyclization (**Fig. 1-1**). The *β, β* branch carotenoids (*β*-carotene and its derivatives: zeaxanthin, antheraxanthin, violaxanthin and neoxanthin) are created by adding *β*-rings onto either end of lycopene by lycopene beta cyclase (LCYB). To date, only one LCYB was identified in *A. thaliana* and two were cloned in tomato (Ronen et al., 2000). The second LCYB in tomato shows 53% identity to the first one at the amino acid level. The *β, ε* branch (*α*-carotene and its derivatives) involves the introduction of an *ε*-ring at one end of lycopene by the lycopene epsilon cyclase (LCYE) and subsequently adding a *β*-ring at the other end by LCYB (Pogson et al., 1996). Interestingly, the lettuce LCYE enzyme has the unique ability to add *ε*-rings onto both ends of lycopene to form *ε,ε*-carotene (Cunningham Jr. and Gantt, 2001).

α-carotene and *β*-carotene can be further modified to produce xanthophylls. In plant chloroplasts and chromoplasts, *α*-carotene is commonly hydroxylated at the positions

3 and 3' to form lutein. This process requires two types of hydroxylases; *β*-hydroxylases act on the *β*-ionone ring whereas the *ε*-hydroxylase hydroxylates the *ε*-ring. There are two classes of carotene β-hydroxylases: CYP97A is a cytochrome P450-type β-hydroxylase, while HYDB is a ferredoxin-dependent di-iron monooxygenase (Quinlan et al., 2007). CYP97C (carotene ε-hydroxylase) is a cytochrome P450-type monooxygenase which was identified through characterization of the *A. thaliana lut1* mutant (Tian and DellaPenna, 2004) and functionally demonstrated in *E. coli* (Quinlan et al., 2007). In the *α*-carotene branch of the pathway of plants, lutein is always the end product. The *β*-carotene branch can be hydroxylated by either HYDB or CYP97A in two steps to produce zeaxanthin via *β*-cryptoxanthin (an intermediate). Unlike lutein, zeaxanthin can be further modified by zeaxanthin epoxidase (ZEP) to produce violaxanthin via the intermediate antheraxanthin. This process can be reversed under high light stress by violaxanthin de-epoxidase (VDE); this is referred to as the xanthophyll cycle (**Fig. 1-1**). Zeaxanthin is recognized as the precursor for synthesis of ABA in plants (**Fig. 1-2**).

1.3 Carotenoid cleavage products and enzymes

Carotenoids not only serve their functions in their intact form, but can also be oxidized and broken down to form a diverse group of bioactive derivatives, called apocarotenoids. These cleavage products include vitamin A in humans and animals (Wyss et al., 2000; von Lintig and Wyss, 2001), the plant hormone ABA (Schwartz et al., 1997), aroma and flavor compounds (Bouvier et al., 2003a), root-secreted germination stimulant strigolactones

(Matusova et al., 2005) and a novel plant signal molecule involved in shoot branching inhibition (Booker et al., 2004; Akiyama et al., 2005; Matusova et al., 2005).

The cleavage enzymes are called carotenoid cleavage dioxygenases (CCD). The first gene encoding a CCD had been identified through characterization of a maize ABA-deficient *viviparous* mutant, *vp14* (Schwartz et al., 1997; Tan et al., 1997). The product of VP14 is also called 9-*cis*-epoxycarotenoid dioxygenase (NCED) because it can cleave two 9-*cis*-epoxycarotenoids (violaxanthin and neoxanthin) at their 11, 12 double bond (**Fig 1-2**). Its homologs in several other plants were also identified and characterized to recognize this enzyme's critical role in controlling ABA synthesis (Qin and Zeevaart, 1999; Iuchi et al., 2000; Thompson et al., 2000b). In most plants, NCED/CCD is encoded by a small gene family (Tan et al., 1997; Tan et al., 2003). For example, nine *CCD* genes have been identified in *A.thaliana* based on sequence homology to maize VP14 (Tan et al., 2003). Five of them (*NCED2*, *NCED3*, *NCED5*, *NCED6* and *NCED9*) are similar to *VP14* in maize; a gene that is involved in ABA synthesis. Under drought conditions, *AtNCED3* is the major stress-induced *NCED* in leaves (Iuchi et al., 2001; Tan et al., 2003). *AtNCED6* and *AtNCED9* are seed-specific and are required for ABA biosynthesis during seed development (Lefebvre et al., 2006).

The roles of *ccd7* and *ccd8* in plant development have been elucidated through characterization of a series of similar mutants, including pea *ramosus1* mutant (*rms1*), petunia *decreased apical dominance* mutant (*dad1*) and Arabidopsis *more axillary growth*

mutants (*max3* and *max4*). In these mutants, both leaves and inflorescences can emerge from typically dormant axillary buds (Sorefan et al., 2003; Booker et al., 2004). The mutated phenotype can be restored by grafting wild type root tissue with the shoot of mutants, which suggests that a mobile signaling molecule is missing in these mutants. Characterization of these mutants revealed that *ccd8* is allelic to *rms1*, *dad1* and *max4*, whereas *ccd7* is allelic to *max3* (Sorefan et al., 2003; Booker et al., 2004; Snowden et al., 2005). *In vitro*, the recombinant CCD7 protein can cleave β-carotene at the 9, 10 bond to produce 10'-apo-β-carotenal (C_{27}), which can be further broken down by CCD8 to form 13-apo-carotenone (C_{18}). Thus, these data indicated that *ccd7* and *ccd8* may team up to synthesize a novel, mobile signaling molecule involved in shoot branching inhibition, by cleaving carotenoids.

Carotenoid cleavage dioxygenases are also involved in the formation of the aroma compounds bixin in *Bixa orellana* and saffron in *Crocus sativus* respectively. The biosynthesis of bixin requires a lycopene cleavage dioxygenase (bixin aldehyde dehydrogenase), together with norbixin carboxyl methyltransferase (Bouvier et al., 2003b). Carotenoid derivatives in saffron are its major sources of color, flavor, and aroma. A crocus zeaxanthin 7,8 (7',8')-cleavage dioxygenase was identified and thought to be necessary to initiate the biosynthesis of these carotenoid derivatives (Bouvier et al., 2003a)

Strigolactones represent a group of germination stimulants, which are secreted by

the roots of host plants of the parasitic plants *Striga* and *Orobanche* (Matusova et al., 2005). They also mediate the symbiosis of arbuscular mycorrhizal fungi and their hosts by stimulating the cell proliferation of fungi (Besserer et al., 2006). Recently, Matusova *et.al* showed that strigolactones are derived from the carotenoid pathway using carotenoid biosynthetic mutants and inhibitors; the germination ratio of parasitic plants is reduced profoundly when they are treated with root exudates extracted from maize carotenoid biosynthetic mutants, such as *vp5*, *vp14*, *y9* and *y10*. Moreover, one of the carotenogenic genes, *PDS*, has been shown to be up-regulated specifically in root cells infected by mycorrhizae (Fester et al., 2002). The biosynthetic pathway of strigolactone is still unclear. However, a pathway similar to the ABA biosynthetic pathway has been proposed by Motusova *et.al* (2005) and a *ccd* member has also been suggested to be involved in strigolactone synthesis.

1.4 The regulation of carotenoid biosynthesis in higher plants

Although almost all carotenogenic genes have been successfully targeted by genetic engineering, the studies involving pathway regulation are still in the infancy stage. Carotenoids are synthesized in a variety of plant plastids and this process is usually altered significantly during plastid development, therefore most regulatory studies were carried out on four model systems involving plastid development or transition: (1) de-etiolated seedlings, (2) ripening fruits, (3) budding flowers and (4) developing seeds. In de-etiolated seedlings, etioplasts develop into chloroplasts upon exposure to light, whereas chloroplasts

transition into chromoplasts during fruit ripening and flower budding (**Fig. 1-3A, B and C**). Crop seeds usually accumulate lower amounts of carotenoid in their plastids compared with leaves, fruits and flowers. However, crop seeds still draw attention because of their economic significance. These preliminary regulatory studies have clearly demonstrated that increasing carotenoid levels is always associated with the up-regulation of key carotenogenic genes; this strongly suggests that carotenogenesis is predominantly regulated at the level of transcription (Hirschberg, 2001; Römer and Fraser, 2005).

1.4.1 Regulation in green tissue

Etiolated leaves possess plastids called etioplasts, which accumulate low levels of carotenoids in a lattice of tubular membranes known as the prolamellar body (PLB) (Park et al., 2002) (**Fig. 1-3B**). Upon exposure to light, etioplasts develop into functional chloroplasts. During this process in *A. thaliana* and *Sinapis alba*, the levels of *PSY* mRNAs are up-regulated by light, whereas the transcript levels of upstream or downstream genes, such as *GGPPS* or *PDS*, remain constant (von Lintig et al., 1997). The *cis*-elements within the *A. thaliana PSY* promoter responsible for the modulation of different light qualities were identified via promoter deletion analysis (Welsch et al., 2003). Other than transcriptional regulation, post-translational control appears to be another regulatory mechanism in controlling PSY activity in *Sinapis alba*. In etioplasts, the PSY enzyme is stored in PLBs and exhibits low enzymatic activity. Upon illumination, there is a rapid decay of the PLB and establishment of the chloroplast, accompanied by the relocalization

and activation of the PSY enzyme (Welsch et al., 2000).

In higher plants, oxycarotenoid xanthophylls (zeaxanthin, antheraxanthin and violaxanthin) function as photoprotectants for preventing photo-oxidation. Therefore, it is reasonable to envision that the carotenogenic genes related to zeaxanthin synthesis should be light-stress inducible. It is true that an increase in *HYDB* gene transcript levels have been observed in *A. thaliana*, which is correlated with higher levels of zeaxanthin under high light stress (Rossel et al., 2002). Zeaxanthin can also be synthesized from violaxanthin through de-epoxidation by VDE. However, the levels of *VDE* transcripts decrease under high light stress and post-transcriptional or translational control has been suggested to be a critical factor in the regulation of VDE (Gilmore, 2001; Rossel et al., 2002).

To date, little is known about the regulatory components or mechanisms involved in sensing light signals for up-regulation of leaf carotenogenesis. Certain photoreceptors are thought to be such regulatory components and redox control may be another factor that could have an impact on this regulation. Studies involving *A. thaliana* plants exposed to far-red light revealed that a specific phytochrome, *phyA,* may be involved in the regulation of the *PSY* gene during de-etiolation (von Lintig et al., 1997). Recently, I demonstrated that the up-regulation of maize *PSY2* is abolished in the phytochrome chromophore mutant *elm1* exposed to both red and far-red light (**Chapter 3**). Taken together, these results suggest that phytochromes are involved in the sensing of both red and far-red light signals

but not blue light. On the other hand, another photoreceptor, phototropin may be the regulatory component which mediates blue light for up-regulation of carotenogenesis; it has been reported that this photoreceptor is necessary for *PSY*, *PDS, ZDS* and *ZEP* genes induction in *Chlamydomonas reinhardtii* under blue light (Im et al., 2006). In addition, using a site-specific photosynthetic electron transport inhibitor, Woitsch *et.al* (2003) demonstrated that two genes encoding xanthophyll biosynthetic enzymes, *HYDB* and *ZEP* are redox-controlled by the plastoquinone pool.

1.4.2 Regulation in the chromoplast

Carotenogenesis during the chloroplast to chromoplast transition has been best studied in tomato fruit (**Fig.1-3C**), marigold (*Tagetes erecta*) and *Gentiana lutea* flowers. At the breaker stage of tomato ripening, lycopene starts to accumulate so that fruit color changes from green to orange. During this stage, a 10 to 20-fold increase in transcript levels has been observed for tomato *PSY1* and *PDS* (Giuliano et al., 1993), whereas the mRNA levels of both lycopene cyclases, *LCYB* and *LCYE* are not detectable (Pecker et al., 1996; Ronen et al., 1999). Such differential gene expression is thought to be the main reason for the accumulation of lycopene in ripening tomato.

Lutein and lutein fatty acid esters are major carotenoids accumulating in the petals of marigold flowers. Their amounts vary significantly from one variety to another. Transcript analysis revealed that the levels of *PSY* mRNA are positively associated with

the carotenoid levels in marigold flowers (Moehs et al., 2001). The expression of the *LCYE* also increases during flower development, which diverts lycopene to the *β, ε* branch of the carotenoid pathway and α-carotene gets hydroxylated to produce the xanthophyll lutein, which accumulates to significant amounts.

When *G. lutea* flowers are blooming, carotenoid levels in the petals start to increase while chlorophyll content significantly decreases. An increase in transcript levels of *PSY* and *ZDS* (6- to 7-fold) has been observed during the transition of the chloroplast to the chromoplast (Zhu et al., 2002). In addition, it has been shown that lycopene is diverted to the *β*, *β*- branch of the carotenoid pathway by increasing the transcript levels of *LCYB* and *HYDB* and decreasing the levels of *LCYE*. Such differential gene expression in *G. lutea* led to a shift from *β, ε* branch (ie., α-carotene) xanthophylls such as lutein to *β*- carotene derived xanthophylls (Zhu et al., 2003).

The regulation of carotenogenesis has also been studied in the flowers of daffodil, (*Narcissus pseudonarcissus*) (Schledz et al., 1996), citrus fruit (Kato et al., 2004) and pepper (*Capsicum annuum*) (Camara et al., 1995). All these studies have indicated that transcriptional regulation appeared to be the major regulatory mechanism in chromoplast carotenogenesis. However, post-transcriptional regulation has also been found to control the enzymatic activity of daffodil PSY and PDS in chromoplasts. In order to be activated both PSY and PDS of daffodil must be associated with chromoplast membranes (Al-Babili et al., 1996; Schledz et al., 1996).

Over the past several years, there has been increasing evidence which indicates that light signal transduction pathway components and photoreceptors may be involved in the regulation of fruit and leaf carotenogenesis (Liu et al., 2004; Davuluri et al., 2005; Giliberto et al., 2005). In *A. thaliana*, the blue light photoreceptor cryptochrome 2 (cry2) is an important regulatory factor in controlling flowering time and photomorphogenesis under low-light. Overexpression of a tomato *cry2* homolog under the control of the CMV35S promoter led to 2- to 3-fold increases in carotenoid levels in tomato fruit and leaf (Giliberto et al., 2005). Moreover, the nuclear protein DEETIOLATED1 (DET1) in *A. thaliana* is a negative regulator in the phytochrome signal transduction pathway (Liu et al., 2004). Fruit-specific suppression of the DET1 homolog in tomato was able to increase not only carotenoid levels but also flavonoid content significantly in fruit (Davuluri et al., 2005). Further characterization of the cross-talk between the light signal transduction pathway and the carotenoid biosynthetic pathway may enable us to identify potential regulatory genes useful in improving the carotenoid content in food.

1.4.3 Regulation and manipulation of carotenoid biosynthesis in seeds

Two kinds of plastids are present in seeds; amyloplasts are found in starchy seeds such as maize, rice and wheat for storage of starch granules (**Fig.1-3D**), whereas elaioplasts are present in oilseeds of canola and sunflower for lipid-storage (Kirk and Tilney-Bassett, 1978). In maize amyloplasts, the amount of carotenoids can be as high as

38.23 μg. g^{-1} in the maize inbred line A619 with lutein as the major component, followed by zeaxanthin (Egesel et al., 2003). However, carotenoids are present at very low levels in the amyloplasts of other grass family members, particularly in rice.

The significant color change in developing endosperm, plus a large collection of colored kernel mutants, makes maize a good candidate for seed carotenogenesis studies of cereals. As previously pointed out, the maize *PSY1* gene was identified through characterization of a colorless kernel mutant, *y1*. Moreover, the *PDS* gene was also identified through characterization of the *viviparous 5 (vp5)* mutant, which is a colorless endosperm mutant deficient in both carotenoids and ABA (Li et al., 1996; Hable et al., 1998). *ZDS* has also been associated with the ABA and carotenoid deficient mutant *viviparous 9 (vp9)* (Matthews et al., 2003).

In contrast to the seed carotenogenesis studies in maize, such studies in other cereals have received very little attention so far. But the basic biochemical and molecular knowledge of carotenogenesis obtained from maize and other plants has been used to improve the carotenoid content of rice (Ye et al., 2000; Paine et al., 2005), canola (*Brassica napus*) (Shewmaker et al., 1999) and *A. thaliana* (Lindgren et al., 2003). Rice endosperm cannot accumulate any carotenoids except the precursor GGPP (Burkhardt et al., 1997). The absence of *PSY1* transcripts in endosperm seems to account for the lack of carotenoid accumulation in rice endosperm (Burkhardt et al., 1997; Gallagher et al., 2004). Subsequently, researchers over-expressed a daffodil *PSY* and a bacterial *crtI* to create the

first generation of 'Golden rice', in which endosperm *β*-carotene, lutein and zeaxanthin accumulate at variable levels (Ye et al., 2000). In the second generation, 'Golden rice 2', maize *PSY1* was over-expressed in rice endosperm and the level of total carotenoid was increased significantly to levels that were 23-fold higher than that of the first generation of 'Golden rice' overexpressing a daffodil *PSY* (Paine et al., 2005).

PSY has also been overexpressed in the seeds of canola (Shewmaker et al., 1999) and *A. thaliana* (Lindgren et al., 2003). Wild type canola seeds accumulate negligible levels of carotenoids with lutein as the major component. Overexpression of a bacterial *PSY* gene, *crtB*, under the control of a seed-specific promoter in canola led to a 50-fold increase in total carotenoids and a 300-fold increase in *β*-carotene. The increase in carotenoid levels in this case is much higher than those observed in transgenic rice and tomato. This may be due to the changes in elaioplast morphology in transgenic canola, in which a new inclusion body surrounded by a membrane was found. Such changes in plastid structure led to the formation of a metabolic sink, which may allow for more carotenoids to be sequestered (Li and Van Eck, 2007).

1.4.4. Regulation in root tissue

In comparison to leaf, fruit and seed, root tissue contains much lower levels of carotenoids. The plastid in roots is the colorless leucoplast (**Fig 1-3E**), in which carotenoid levels are only 0.03-0.07% of the levels found in the chloroplast (Parry and

Horgan, 1992).

Rather than focusing on carotenoid content or composition manipulation, root carotenogenesis studies are concentrating on carotenoid derived signaling compounds, including the plant hormone ABA and strigolactone. As discussed above, the identification and characterization of the maize *VP14* gene has established NCED as the rate-controlling step in the ABA synthesis pathway in both leaves and roots (Schwartz et al., 1997; Tan et al., 1997). In leaves, abundant xanthophylls provide plentiful precursors so that NCED is regarded as the only regulatory step (Parry et al., 1990; Tan et al., 1997; Qin and Zeevaart, 1999; Tan et al., 2003). In contrast to leaf tissue, an increase in the mRNA levels of certain carotenogenic genes has been observed in dehydrated roots in order to supply xanthophylls for ABA synthesis (Audran et al., 1998; Thompson et al., 2000a). For example, the levels of *ZEP* mRNA increased 3- to 7-fold and 4-fold in dehydrated roots of tomato and tobacco, respectively (Audran et al., 1998; Thompson et al., 2000a). Recently, the transcript profiles of maize carotenogenic genes under drought stress were investigated and maize *PSY3*, *HYDB*, and *VP14* were found to be strongly induced by drought stress in root tissue (**Chapter 2**). This suggested that increased epoxycarotenoid supply through up-regulation of root carotenogenesis might be another bottleneck in ABA synthesis other than NCED activity.

1.5 Objectives

To date, vitamin A deficiency is still prevalent in many developing countries and areas (Sommer and Davidson, 2002). The World Health Organization reported that at least 140 million preschool children in more than 118 countries are affected by vitamin A deficiency (WHO, http://www.who.int/vmnis/vitamina/en/). To combat this problem, much attention has focused on the genetic manipulation of the carotenoid biosynthetic pathway in order to improve the nutritional content in crop plants. However, such genetic modification of carotenogenesis relies on a complete, well-studied carotenoid biosynthetic pathway and well-characterized regulatory mechanism(s) involved in carotenoid biosynthesis. As discussed above, regulation studies of carotenogenesis in various tissues have been done for several plant species. But very few of these types of studies have involved members of the Grass family (Poaceae), which contains most of the agronomically important crops. On the other hand, the carotenoid biosynthetic pathway remains uncertain at its isomerization steps although most parts are well established. Thus, our lab chose maize as a model for Grass family to study the regulation of carotenoid biosynthesis in cereal species. We also use maize kernel carotenoid mutants to elucidate the carotenoid biosynthetic pathway in higher plants. The regulation studies of carotenogenesis in maize will not only benefit carotenoid biosynthesis research in other cereal plants, but will also help researchers genetically manipulate the carotenoid pathway in order to improve the nutritional content in other crop plants.

As previously mentioned, it has been shown that duplicated *PSY* genes are present in most Grass subfamilies, including maize and rice, and the expression of rice and maize

PSY1 in endosperm is associated with endosperm carotenoid accumulation (Gallagher et al., 2004; see Appendix 1). Duplicated genes are frequently preserved by subfunctionalization, which in turn provides plants a suitable mechanism for controlling gene expression in different tissues and when subjected to various growth conditions. This is best exemplified by the subfunctionalization of tomato *PSY* genes; *PSY1* is a fruit- and flower-specific gene and *PSY2* is a leaf-specific one. Thus, I hypothesized that the duplicated *PSY* genes in the Grass family may also be subfunctionalized and study of the roles of *PSY* gene members in controlling carotenogenesis in maize became my first project.

The carotenoid biosynthetic pathway of higher plants still remains uncertain in its isomerization step as previously mentioned. The conversion from 15-*cis* phytoene to all-*trans* lycopene in plants requires two desaturases (PDS and ZDS) and some supplementary isomerization reactions, whereas only one enzyme, CRTI, is required in bacteria. Such differences in the plant and bacterial pathways are very important to be considered for metabolic engineering of plant carotenoid content and composition. Although one isomerase, CRTISO, has already been identified from *A. thaliana* and tomato (Isaacson et al., 2002; Park et al., 2002), the enzyme required to isomerize the product of PDS (9,15,9'-tri-*cis*-ζ-carotene) to produce the suitable ZDS substrate (9,9'-di-*cis*-ζ-carotene) is still a mystery, given CRTISO cannot recognize 9,15,9'-tri-*cis*-ζ-carotene as its substrate. Thus, identification of this missing enzyme (tentatively named Z-ISO) and elucidation of the isomerization steps in the carotenoid

biosynthetic pathway became my second project.

1.5.1 Specific aims

More specifically, the questions addressed here are:

(1) Enzymatic function of maize PSY3 (Chapter 2)

Very recently, a novel *PSY* gene (*PSY3*) was identified in the grass family based on sequence homology to maize *PSY1*. To verify the enzymatic function of PSY3, maize and sorghum *PSY3* full-length cDNAs were isolated and their functions were verified via a heterologous complementation assay.

(2) Tissue specificities of maize *PSY* genes (Chapter 2)

To assess the roles of maize *PSY* genes, including the newly isolated *PSY3*, their tissue specificities were investigated with quantitative RT-PCR using root, leaf, endosperm, and embryo tissues.

(3) Regulation of root carotenogenesis (Chapter 2)

To assess the roles of maize *PSY* genes in controlling root carotenogenesis, their transcript profiles were investigated using roots exposed to abiotic stresses, including drought, salt and ABA.

(4) Regulation of endosperm carotenogenesis (Chapter 3)

To evaluate the roles of the maize *PSY* genes in controlling endosperm

carotenogenesis, their expression patterns were studied with quantitative RT-PCR using developing endosperm from the B73 inbred line. Moreover, the *PSY1* endosperm mutant *y1-602C* was also used to assess the impact of *PSY2* and *PSY3* gene expression on endosperm carotenoid accumulation. The levels of maize PSY proteins in developing endosperm were also investigated with anti-PSY1 serum.

(5) Regulation of leaf carotenogenesis (Chapter 3)

To assess the roles of the maize *PSY* genes in leaf carotenogenesis, their expression patterns were analyzed using de-etiolated maize seedlings. Chromophore biosynthesis mutant *elm1* was also used to determine whether phytochromes were involved in *PSY* up-regulation by light. Finally, a maize *PSY1* null mutant, *y1-8549*, was used for leaf chlorophyll and carotenoid content analysis to assess the impact of the absence of *PSY1* on leaf carotenogenesis.

(6) ζ-carotene accumulating mutant screening (Chapter 4)

To identify Z-ISO which is responsible for the isomerization of the PDS product (9,15,9'-tri-*cis*-ζ-carotene), I screened maize mutants that accumulate ζ-carotene via HPLC and a maize pale yellow kernel mutant, *y9*, was found to accumulate the product of PDS.

To study the roles of PSY gene family in controlling carotenogenesis, quantitative RT-PCR was the main tool used to investigate transcript levels of maize PSY genes in

various tissues and in PSY1 mutants. In Chapter 2, the transcript profiles of the maize PSY genes expressed in plants exposed to various abiotic stresses were analyzed and the role of PSY3 in controlling root carotenogenesis was evaluated. In Chapter 3, the roles of PSY1 and PSY2 in controlling endosperm and leaf carotenogenesis were reported. The identification of a novel maize mutant disrupting Z-ISO activity was also reported in Chapter 4. Finally, future studies on the regulation of carotenoid biosynthesis in the grass family and the Z-ISO activity disrupting mutant are discussed in Chapter 5.

Figure 1-1. Carotenoid biosynthetic pathway in higher plants. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, zeta-carotene desaturase; Z-ISO, zeta-carotene isomerase; CRTISO, carotenoid isomerase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; HYDB, non-heme diiron carotene β-ring hydroxylase; CYP97A, P450 carotene β-ring hydroxylase;

(Acknowledgment to Rena Quinlan for drawing this pathway)

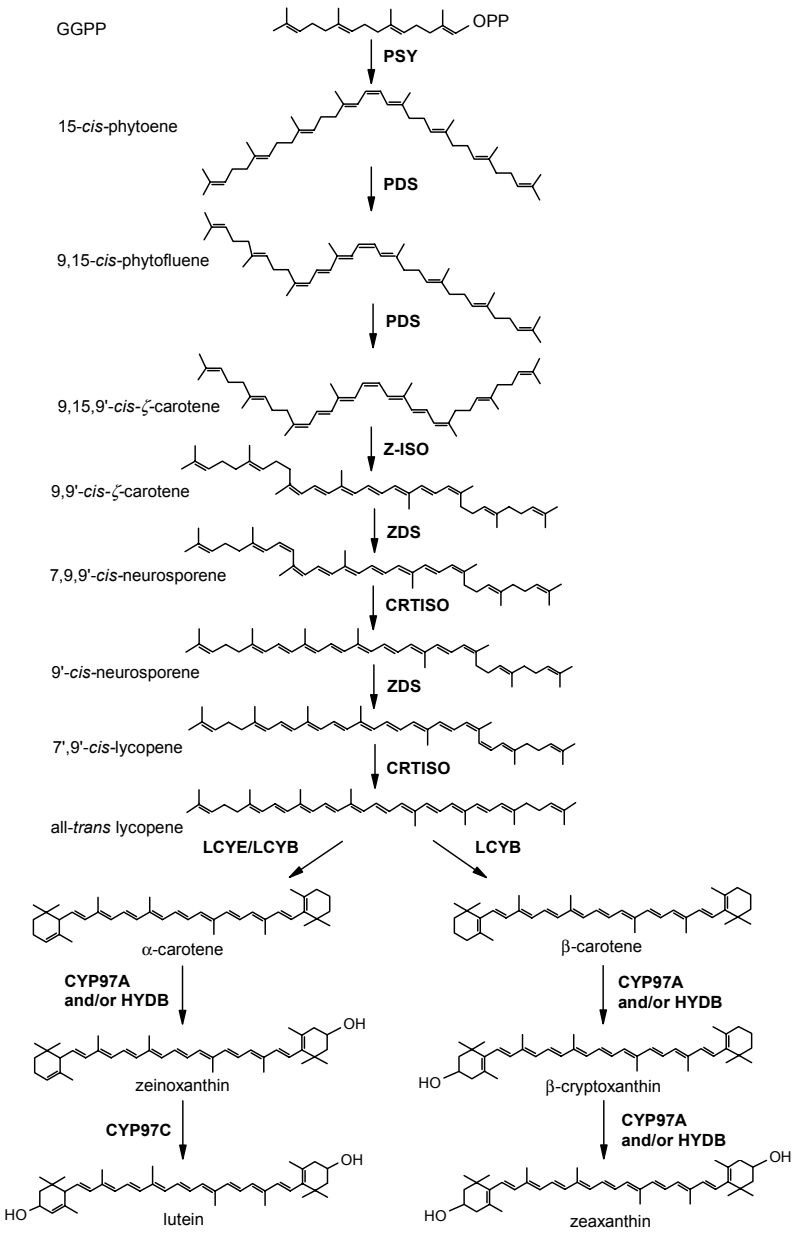
GGPP
OPP
PSY
15-*cis*-phytoene
PDS
9,15-*cis*-phytofluene
PDS
9,15,9'-*cis*-ζ-carotene
Z-ISO
9,9'-*cis*-ζ-carotene
ZDS
7,9,9'-*cis*-neurosporene
CRTISO
9'-*cis*-neurosporene
ZDS
7',9'-*cis*-lycopene
CRTISO
all-*trans* lycopene
LCYE/LCYB
LCYB
α-carotene
β-carotene
CYP97A and/or HYDB
CYP97A and/or HYDB
OH
zeinoxanthin
HO
β-cryptoxanthin
CYP97C
CYP97A and/or HYDB
OH
HO
lutein
OH
HO
zeaxanthin

Figure 1-1.

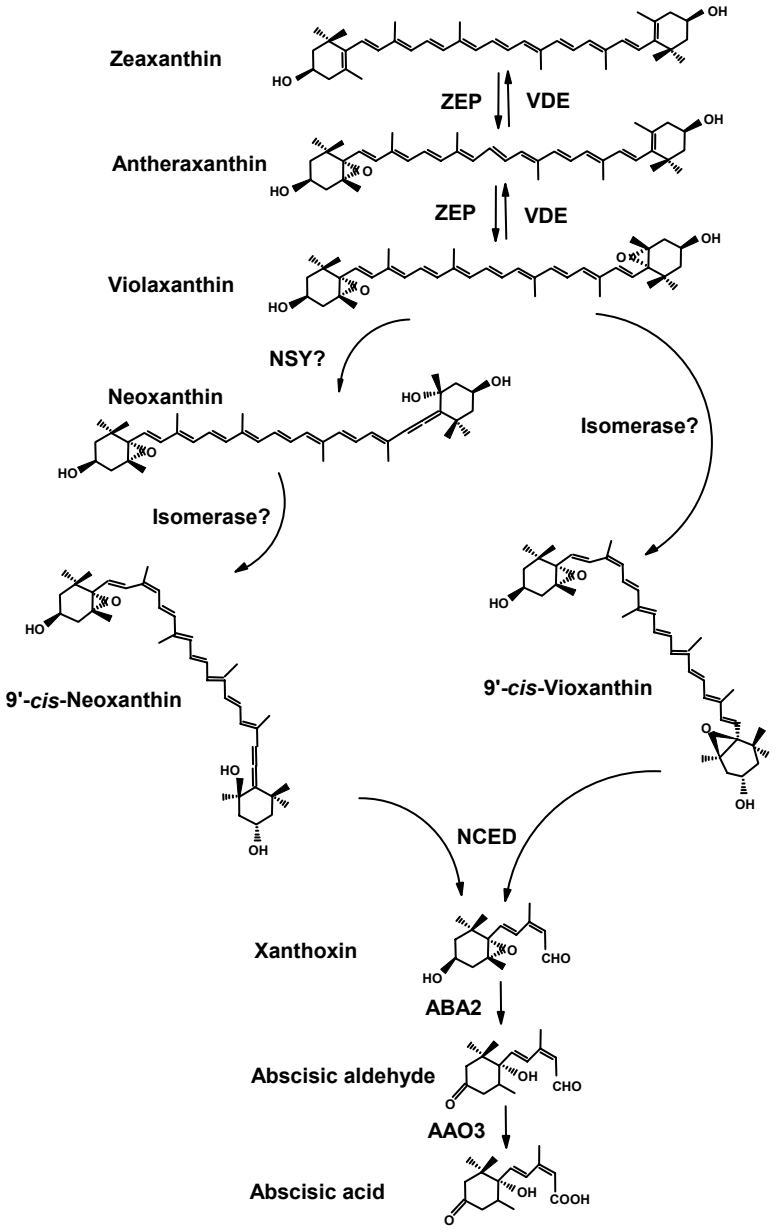

Figure 1-2. ABA biosynthetic pathway in plants (Reprinted with permission from the Annual Review of Plant Biology, Volume 56 (c) 2005 by Annual Reviews www.annualreviews.org). ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; ABA2, short chain alcohol dehydrogenase; AAO3, abscisic acid oxidase.

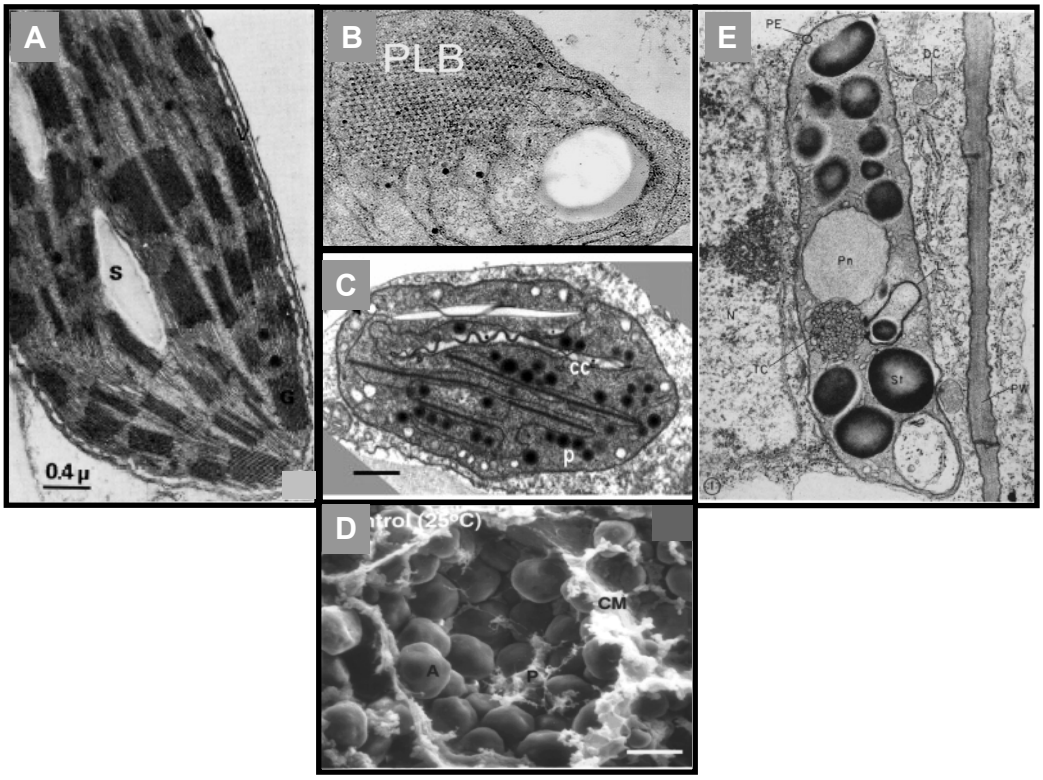

Figure 1-3. Electron micrographs of plastids

(A): Chloroplast of maize leaf. G, grana stacks; S, starch grain. (Picture reprinted from Bachmann et al., 1969 with permission)
(B): Etioplast of oat seedlings. PLB, prolamellar body. (Picture reprinted from Park et al., 2002 with permission).
(C): Chromoplast of tomato fruit. p, plastoglobules; nm, newly synthesized membranes; cc, membranes enclosing carotenoid crystals. (Picture reprinted from Simkin et al., 2007 with permission).
(D): Amyloplast of maize endosperm. A, amyloplasts; P, protein body; AM, amyloplast membrane; CM, cell membrane. (picture reprinted from Commuri and Jones, 1999 with permission).
(E): Leucoplast of bean root. DC, dilated cisterna; N, nucleus; PE, plastid envelope; Pn, protein; PW, primary wall; St, starch body; TC, tubular complex. (Newcomb, 1967 Reproduced from The Journal of Cell Biology, 1967, 33:143-163. copyright 1967 The Rockefeller University Press)

CHAPTER 2

A ROLE FOR *PSY3* IN ABIOTIC-STRESS-INDUCED ROOT CAROTENOGENESIS[1]

2.1 Abstract

Abscisic 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 and other members of the Poaceae have three paralogous genes, in contrast to only one in Arabidopsis. *PSY* gene duplication has led to subfunctionalization with each paralog exhibiting differential gene expression. I 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, I demonstrated that *PSY3* mRNA elevation correlates with carotenoid accumulation, and that blocking carotenoid biosynthesis interferes with stress-induced ABA accumulation. In parallel, I observed elevated *NCED* transcripts, and showed that, in contrast to dicots, root

1 This Chapter is a revision of Li *et al*, (2007) Plant Physiol. DOI 10.1104/pp.107.111120 with the permission from the publisher

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* mRNAs were 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 holds value in better understanding root-specific stress responses that impact plant yield.

2.2 Introduction

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 corn, four days 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 abscisic 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 derived from 9-*cis*-epoxycarotenoids, 9-*cis*-violaxanthin and 9-*cis*-neoxanthin; these C40 compounds are cleaved by 9-*cis*-epoxycarotenoid dioxygenase (NCED) to form xanthoxin, a C15 intermediate, which is subsequently converted to ABA in two steps of oxidation (see **Fig.**

2-1) (reviewed in 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 either 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. 2-1**); factors affecting flux to ABA may vary according to tissue (photosynthetic vs. nonphotosynthetic) and/or to particular species (and possibly plant families), as we demonstrate in this report for maize (*Zea mays*).

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 *Vp14*, 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 orthologous genes, *PvNCED1* of bean (*Phaseolus vulgaris*) (Qin and Zeevaart, 1999), *VuNCED1* of cowpea (*Vigna unguiculata*) (Iuchi et al., 2000), *LeNCED1* of tomato (*Lycopersicon esculentum*) (Thompson et al., 2000a) and *AtNCED3* of Arabidopsis (Iuchi et al., 2001). Stress-induced elevation of NCED transcript levels were 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 deceased 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). Though 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; the transgenic plants showed greater accumulation of ABA in leaves and only a modest increase in root ABA, suggesting that in roots there might be another step(s) 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 beta-carotene to zeaxanthin, suggests that this and possibly other components of the carotenoid biosynthetic pathway may represent putative upstream bottleneck(s) that must be released in order to elevate root ABA levels in response to abiotic stress.

To examine the nature of the root ABA bottleneck, I 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) that 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, I stumbled upon a new and unrelated *PSY* gene, *PSY3*. The sorghum *PSY3* gene led us to the rice ortholog, from which synteny with maize provided a strategy to identify a syntenic chromosome region harboring the maize *PSY3* gene.

The *PSY3* cDNAs that I 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. I 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.

2.3 Results

2.3.1 Isolation of the third *PSY* gene paralog from maize, rice and sorghum

We previously showed that phytoene synthase 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 (*Zea mays*) PSY1 and PSY2 and rice (*Oryza sativa*) PSY2. While attempting to expand *PSY* studies in sorghum (*Sorghum bicolor*), which is an important cereal crop in Africa and other parts of the world, I discovered a novel *PSY* cDNA, which I termed *PSY3*. Sorghum *PSY3* (GenBank 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, I used Blast analysis to test whether a homolog even existed in rice since earlier genome data had not revealed any additional genes; I did find a rice *PSY3* gene located on chromosome 9 (Gramene ID: LOC_Os09g38320) and 6 rice ESTs in GenBank. For maize, I identified only one genomic contig (TIGR ID: AZM4_60808), which contained an incomplete maize *PSY3* gene, but no maize ESTs were found.

To clone the maize *PSY3* gene, I exploited synteny between maize and rice and used flanking markers to identify the maize syntenic region in maize bin 7.03 near umc1865 (**Fig. 2-2**); BAC clones in this region were screened by PCR to identify one containing the maize *PSY3* gene which was sequenced by primer-walking (GenBank DQ372936) (see details in Materials and Methods). To facilitate gene annotation and later functional analyses, I used 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* (GenBankCD234165) and *PSY3* (BG46454) genes were requested from the Comparative

Grass Genomics Center (CSGR). 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. 2-3**). All *PSY* genes possess 6 exons and 5 introns, except sorghum *PSY3* whose 3th and 4th exons are fused. All of these *PSY* genes have long first exons and a very short 2nd exon. With the exception of the sorghum fused *PSY3* exons, the sizes of the 2nd, 3rd, 4th and 5th exons of all three groups of *PSY* genes in the Poaceae and Arabidopsis are identical with sizes of 51 bp, 173 bp, 236 bp and 193 bp, respectively. In addition, all *PSY1s* have a small first intron (~100 bp) and a large 2nd intron (> 600 bp) but all *PSY2s* have a large first intron (~400 bp) and small 2nd intron (~100 bp) (**Fig. 2-3**).

The deduced protein sequences for all three *PSY* genes of maize, rice and sorghum were determined and used for phylogenetic analysis (**Fig. 2-4A**). 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 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 which separates these proteins from the members of the PSY1 group with the SLRNXQ(T/K) motif and PSY2 with the ARAAVAS(S/P) motif (**Fig. 2-4B**); 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. 2-4B**).

2.3.2 *PSY3* encodes a functional phytoene synthase

I used heterologous functional complementation to verify whether the novel PSY3 proteins were functional (Gallagher et al., 2003; Matthews et al., 2003; Gallagher et al., 2004; Quinlan et al., 2007). Maize *PSY3*, and sorghum *PSY1* and *PSY3* cDNAs were subcloned into the pET23 expression vector (Novagen, Madison, WI) and transformed into *E. coli* harboring pACCAR25Δ*crtB* which carries a bacterial carotenoid gene cluster missing the bacterial *PSY* gene, *crtB* (Misawa et al., 1990). Cells produce the pathway endproduct, zeaxanthin diglucoside, only when a functional PSY enzyme is present; a peak corresponding to this endproduct is seen in an HPLC chromatogram of extracted carotenoids from positive control cells containing the entire carotenoid cluster including bacterial PSY (pACCAR25) (**Fig. 2-5A**), but not when empty vector only is co-transformed with the PSY deletion construct, pACCAR25Δ*crtB* (**Fig. 2-5B**). HPLC

analysis of carotenoid extracts from transformants containing either the maize *PSY3*, or sorghum *PSY1* and *PSY3*, showed a peak corresponding to zeaxanthin diglucoside (**Fig. 2-5C, D,** and **E**), which matches in retention time and spectrum that seen for the positive control (**Fig. 2-5A**). These results indicate that maize *PSY3*, and sorghum *PSY1* and *PSY3* all encode functional enzymes.

2.3.3 Maize *PSY3* is mainly expressed in root and embryo tissues

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. 2-6**). In leaf 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 semi-quantitative 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. 2-6**). In embryo, the 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.

2.3.4 Maize *PSY2* and *PSY3* mRNA levels up-regulated by drought

I used E-Northern from NCBI to reveal possible factors that might influence *PSY* transcript levels as indicated by source of abundant ESTs. I identified only 6 *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. The 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, I subjected maize seedlings to various abiotic stresses and measured *PSY3* transcript levels in comparison to transcript levels for *PSY1* and *PSY2*.

To test for effect of drought-stress on *PSY* transcript levels, maize seedlings were subjected to drought conditions at the 5-leaf stage. After four days of water deprivation, leaves began to wilt and ABA levels in leaf and root tissue began to increase (**Fig. 2-7A, B**); in parallel, mRNA level increases were observed for both *PSY2* and *PSY3* in leaves (**Fig. 2-7A**) and only *PSY3* in roots (**Fig. 2-7B**). Transcript levels of *PSY2* and *PSY3* reached their highest levels in seedlings subjected to continuous drought stress for 8 days, showing a 16 fold and 17 fold increase in leaves, respectively; in leaves, *PSY2* encoded the most abundant (3.4-fold compared to *PSY3*) transcript at 8 days, the time point after which water was restored. In contrast to leaves, at 4 days, roots showed a 38 fold increase of *PSY3* transcripts and by 8 days, roots showed a 50-fold induction of *PSY3* transcript levels as compared to pre-drought levels. Upon re-watering at 8 days, both ABA and mRNA levels

of *PSY2* and *PSY3* dropped to normal levels within 4 hours in both tissues. The rapid disappearance of *PSY2* and *PSY3* transcripts is suggestive of a 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.

2.3.5 Response of maize *PSY* transcript levels to NaCl and ABA treatments

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. 2-8A**) but increased in roots within 30 minutes; within 2 hours of salt stress, *PSY3* transcripts peaked six-fold, compared to untreated controls, and then dropped to lower levels by 5 hrs (**Fig. 2-8B**). In contrast, *PSY2* transcripts only slightly increased in salt-stressed root tissue. In leaves, *PSY1* transcript levels decreased rapidly within 30 minutes and then remained at a low level, whereas transcript levels of *PSY2* and *PSY3* did not show significant changes (**Fig. 2-8A**).

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 minutes,

then dropped down to normal levels within 4 hours (**Fig. 2-9A**). In contrast, *PSY1* mRNA levels steadily decreased upon ABA application while *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 compare to 1 hr for *PSY2*; *PSY3* mRNA levels increased about 7 fold in 15 minutes and *PSY2* mRNA levels increased 4.5 fold in 1 hour, and then dropped after 2 hrs (**Fig. 2-9B**).

2.3.6 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 suggested that *PSY3* plays a role in stress-induced carotenogenesis required for ABA and other apocarotenoids. Since 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 which condition accumulation of pathway intermediates (Wurtzel, 2004; Matthews and Wurtzel, 2007). I therefore chose the maize *y9* mutation which blocks carotenoid biosynthesis in nonphotosynthetic tissues through interference with isomerization of ζ-carotene by Z-ISO (Li et al., 2007); homozygous mutants accumulate the Z-ISO substrate, 9,15,9′-tri-ζ-carotene, and earlier pathway intermediates (**Fig. 3-1**) which can be easily measured by HPLC. Therefore, in this set of experiments with *y9*, I

was 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 the salt-treated *y9* roots, I observed that the total carotene content, including phytoene, phytofluene and ζ-carotene isomers, began to increase within 1 hr after salt treatment (**Fig. 2-10A**). The total carotene content peaked after 2h, to levels 1.7 fold higher than that of untreated controls. ABA levels peaked at 5 hrs 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. 2-10B**). The 2-fold increase in ABA levels in *y9* roots may be due to leaf ABA re-distribution or incomplete blocking at the Z-ISO step. In comparison to the carotenoid increase seen at 1 hr in *y9* roots, *PSY3* transcripts began elevating at 30 min and peaked by 2 hrs (**Fig. 2-10C**) as seen for *PSY3* in inbred B73 roots (**Fig. 2-8B**), while no changes were seen for *PSY1*, *PSY2* or *PDS* (**Fig. 2-10C**). 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.

2.3.7 *PSY3* is a key regulator of carotenoid biosynthesis in roots under stress

I 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, I questioned whether *PSY3* was the only gene encoding a carotenoid pathway enzyme that is abiotic-stress responsive or did other genes, including zeaxanthin epoxidase (*ZEP*) and β-carotene hydroxylase (*HYD*), contribute to enhanced carotenoids required for NCED1 activity and subsequent ABA induction. In maize, ZEP is encoded by two copies; I found that neither showed significant variation in transcript level in response to drought stress (**Fig. 2-11 A** and **B**). Maize HYD is encoded by a small gene family (Vallabhaneni and Wurtzel, unpublished), only one of which was significantly induced under drought conditions. *HYD* transcript level increases were first observed at 6d, and peaked at 8d following drought stress (**Fig. 2-11C**). In comparison, *PSY3* mRNAs increased 38-fold already at 4 d and by 8 days roots showed a 50-fold induction of *PSY3* transcript levels as compared to pre-drought levels (**Fig. 2-7B**); parallel to PSY3, *NCED1* also increased 3.7-fold at 4 d (**Fig. 2-11D**). These observations suggest that modulation of *HYD* transcript levels is delayed relative to induction of *PSY3* and *NCED* mRNA levels. I also tested *PDS*, *ZDS*, *CrtISO*, and *LCYB*, none of which showed significant changes in response to drought-stress (data not shown). Therefore, the 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 two hours of rehydration, when *PSY3* transcript levels decreased and ABA levels plummeted (**Fig. 2-7B**). 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, ABA 8' hydroxylase (ABA8ox); in Arabidopsis and *Phaseolus vulgaris*, ABA8ox genes were upregulated 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, I measured corresponding maize transcripts. I 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 (Vallabhaneni and Wurtzel, data not shown). 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. 2-11E** 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 days of drought stress compared to *ABA8ox1b* transcripts peaking at 6 days. *ABA8ox1a,* but not *ABA8ox1b*, also showed further elevation in response to rehydration within 2 hours of watering at day 8 (**Fig. 2-11E**). 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).

2.4 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, phytoene synthase, is encoded by a single copy gene, responses leading to altered flux are limited to control of that single copy gene. In contrast, I 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 available maize physical map facilitated isolation of the maize gene. I 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; while 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.

I discovered *PSY3* among sorghum EST sequences, but I 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 since 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, I examined maize for the effects of salt, drought, and ABA upon transcript levels of *PSY3* and its paralogs. I 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 *PSY*3 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, since 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 et al., 2002; Rossel et al., 2002; Woitsch and Romer, 2003; Havaux et al., 2007). Nonstressed embryo dissected at 20 days after pollination, contains little 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, I 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, I showed that the *PSY3* transcript level correlated with increased root carotenoids that are produced upstream of HYD; at this time, I am unable to evaluate the effect of elevated HYD transcripts in driving flux further to xanthophylls. I rule out later steps, such as ZEP, since I 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 IPP biosynthetic pathway for which expression of certain

enzymes has been shown to impact flux to carotenoids in maize (Vallabhaneni and Wurtzel, unpublished) and in other organisms (Matthews and Wurtzel, 2000).

2.5 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.

2.6. Materials and Methods

2.6.1 Plant materials

Maize (*Zea mays*) inbred line B73 and mutant *y9* (X07C, Maize Genetics Cooperation Stock Center, U. Illinois, Urbana/Champaign) and rice (*Oryza sativa*) indica variety 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 ^{0}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 days after pollination (DAP) from field grown plants and stored at -80 ^{0}C until analysis.

2.6.2 Cloning of maize *PSY3*

Maize *PSY1* cDNA sequence (GenBank ZMU32636) was used in Blast analysis to identify a putative homolog from sorghum (*Sorghum bicolor*) **(**GenBank 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, I used the sorghum *PSY3* cDNA (GenBank BG46454) in Blast analysis which led to 6 rice *PSY3* ESTs (CF305089, CF312554, CF312553, CF307565, AK108154 and AY078162), though none were found for maize. Among these, 5 ESTs (except AK108154) were drought inducible or associated with ABA signaling; the first 4 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 AI666177 and *rz404* on chromosome 9 (Gramene ID: LOC_Os09g38320). Next, I used synteny between rice and maize (http://www.tigr.org/tdb/synteny/) to identify the putative maize *PSY3* locus; the 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 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, Ithaca, NY) and deposited into GenBank (DQ372936).

2.6.3 Sequence analyses

The cDNA sequences and corresponding protein sequences of phytoene synthases of *A. thaliana*, *O. sativa*, *S. bicolor* and *Z. mays* were obtained from NCBI GenBank, some of which were deposited as a result of this work (DQ372936, DQ356431, AY705389, AY705390, DQ356430): *A. thaliana* (AtPSY AAA32836), *O. sativa* (OsPSY1, AAS18307; OsPSY2, AAK07735; OsPSY3, DQ356431), *S. bicolor* (SbPSY1, AY705389; SbPSY2, AW679367; SbPSY3, AY705390), *Z. mays* (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 phytoene syntheses of *A. thaliana*, *O. sativa*, *S. bicolor* and *Z. mays* were obtained from NCBI, Gramene and PlantGDB for gene structure analysis: maize *PSY1* (*ZmPSY1*; GenBank AY324431); rice *PSY1* (*OsPSY*1; GenBank AP005750); sorghum *PSY1* (*SbPSY1*; PlantGDB SbGSStuc11-12-04.5154.1); maize *PSY2* (*ZmPSY2*; GenBank AY325302); rice *PSY2* (*OsPSY2*; GenBank AL831803); sorghum *PSY2* (*SbPSY2*; PlantGDB SbGSStuc11-12-04.12062.1); maize *PSY3* (*ZmPSY3*; GenBank DQ372936) (described in this manuscript); rice *PSY3* (*OsPSY3*; Gramene LOC_Os09g38320); sorghum *PSY3* (*SbPSY3*; PlantGDB SbGSStuc11-12-04.766.1); Arabidopsis *PSY* (*AtPSY*; GenBank AB005238). The *PSY* gene structures were analyzed using Vector NTI Suite, Version 9.0 (InforMax, North Bethesda, MD).

2.6.4 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 DQ36430) 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, WI) and renamed pGEMT-RPSY3 prior to sequencing of both strands and use in phylogenetic analysis. The maize *PSY3* cDNA (DQ36430) was inserted into the pET23b (+) vector between *EcoR* I and *Xho*I, named pETb-MPSY3. Sorghum *PSY1* from CD234165 and *PSY3* from BG46454 were inserted between *EcoR* I and *Hind III* sites of the pET23a (+) vector, designated as pETa-SPSY1 and pETa-SPSY3, respectively.

2.6.5 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, *E. coli* BL21 (DE3) cells (Novagen) were transformed with combinations of pACCAR25Δ*crtB* 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).

2.6.6 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^{TM} SYBR green supermix (BioRad,

Hercules, CA) with 10 ng synthesized cDNA. For primers and PCR conditions for test genes and the internal actin control, refer to Supplementary Table I. 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 $2^{-\Delta Ct}$, 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 3 RT-PCR replicates +/- SD from five pooled plants.

2.6.7 Stress treatments

To carry out the drought stress experiment, maize B73 seedlings at the 5-leaf stage (about 3 weeks) were deprived of water for 8 days and then re-watered. Leaves began wilting 4 days after withholding of water. Therefore leaves and roots were collected at 0, 4, 6 and 8 days after water was withheld and 2, 4 and 24 h after re-watering.

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 ((±)-Abscisic acid, CAT. A1049, Sigma,

St. Louis). 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 hours of NaCl treatment, and after 0, 0.25, 0.5, 1, 2 and 4 hours of ABA treatment. All plant materials were stored at -80 ^{0}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 3 RT-PCR replicates +/- SD from five pooled plants.

2.6.8 ABA measurement

ABA extraction was carried out according to the method described by Xiong (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} $NaHCO_3$). The suspension was stirred 48 hours at 4°C for extraction and centrifuged at 3000g for 30 min. The supernatant was transferred to a new tube and dried under vacuum. Samples were dissolved in 100 µL of methanol and 900 µL of Tris-buffered saline (50 mM Tris, 0.1 mM $MgCl_2$, and 0.15M NaCl, pH 7.8) and ABA concentration determined using the Phytodetek ABA immunoassay kit (Idetek, San Bruno, CA). 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 5 samples +/- SD.

2.7 Acknowledgements

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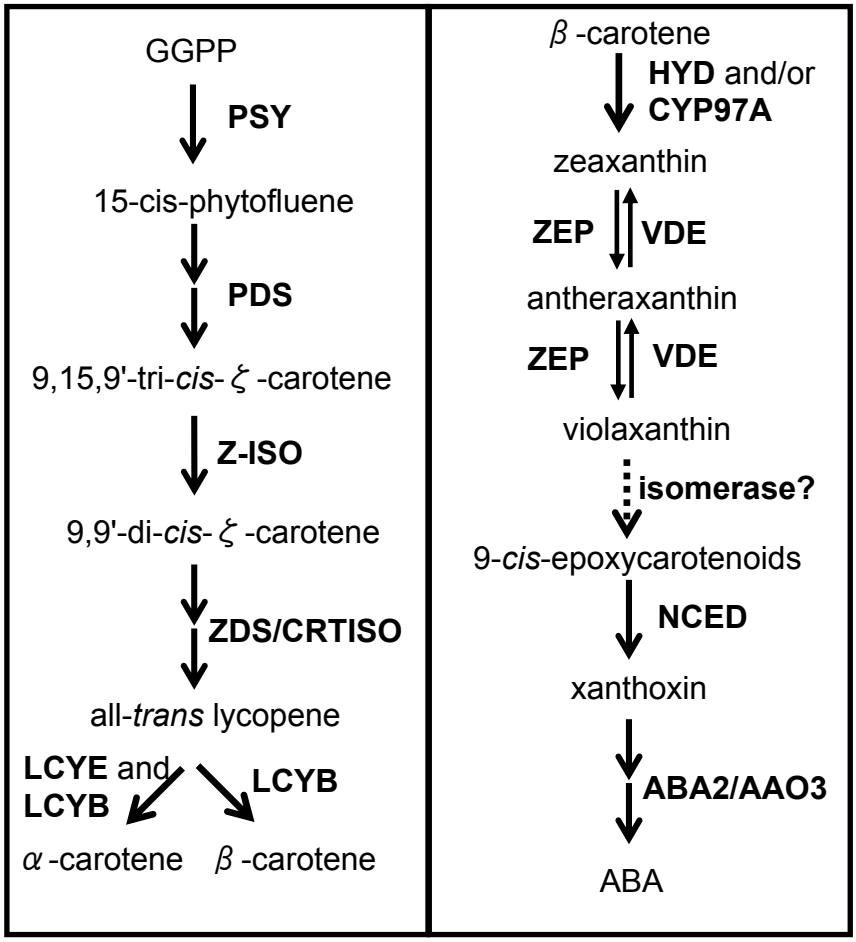


Figure 2-1. Carotenoid and ABA biosynthetic pathways in higher plants. Left panel shows carotenoid precursors of carotenes; right panel shows conversion of carotenoids to ABA. Enzymes are bold: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, zeta-carotene desaturase; Z-ISO, zeta-carotene isomerase; CRTISO, carotenoid isomerase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; HYD, non-heme diiron carotene β-ring hydroxylase; CYP97A, P450 carotene β-ring hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; ABA2, short chain alcohol dehydrogenase; AAO3, abscisic acid oxidase.

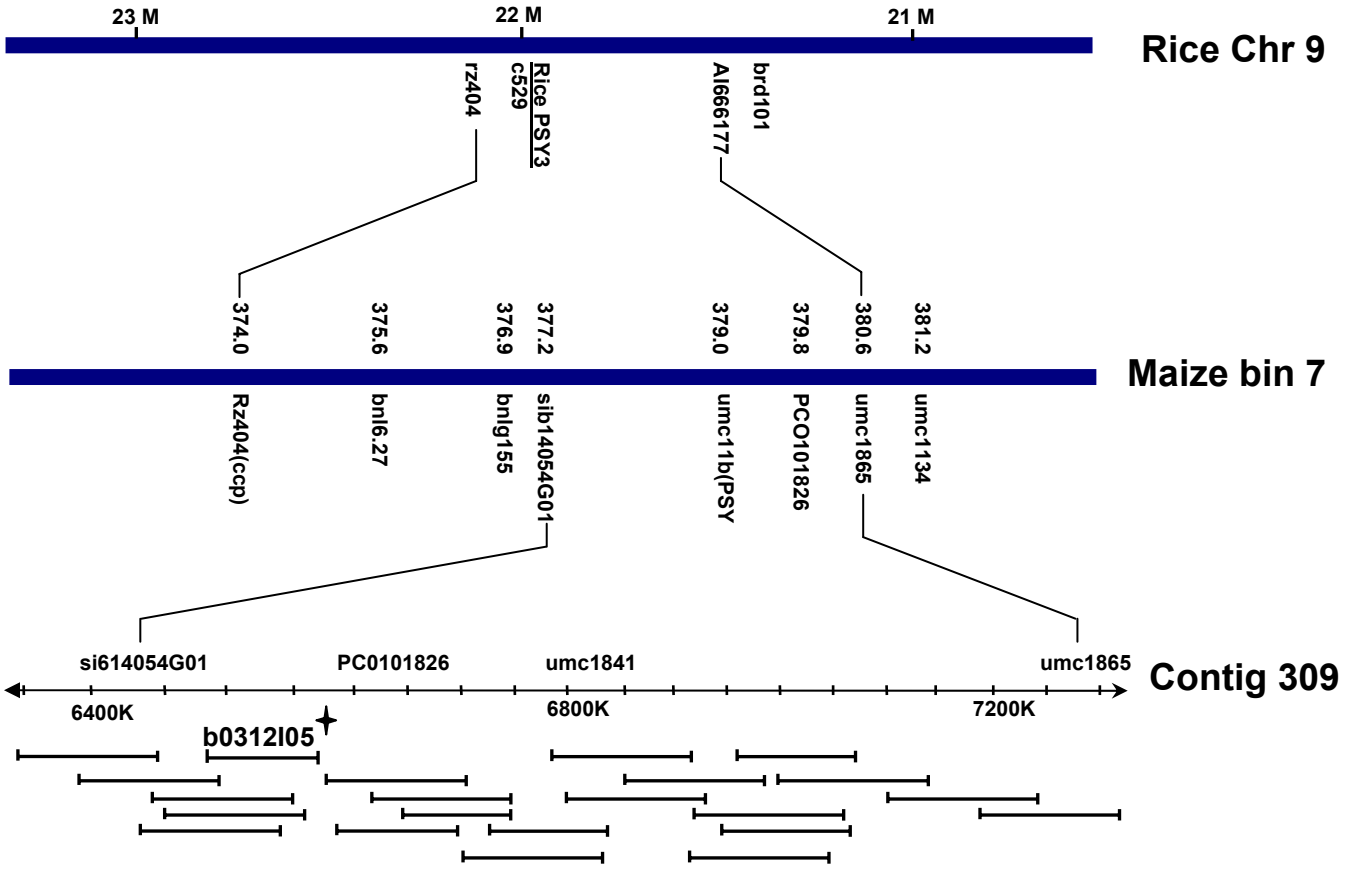


Figure 2-2. Cloning of maize PSY3 using synteny between rice and maize. Rice *PSY3* (Gramene ID: LOC_Os09g38320) was located on chromosome 9 between marker *AI666177* and *rz404* (*top*). The syntenic region on maize is bin 7 between marker *umc1865* and *Rz404 (ccp)* (*middle*). BAC clone b0312I05 containing 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.

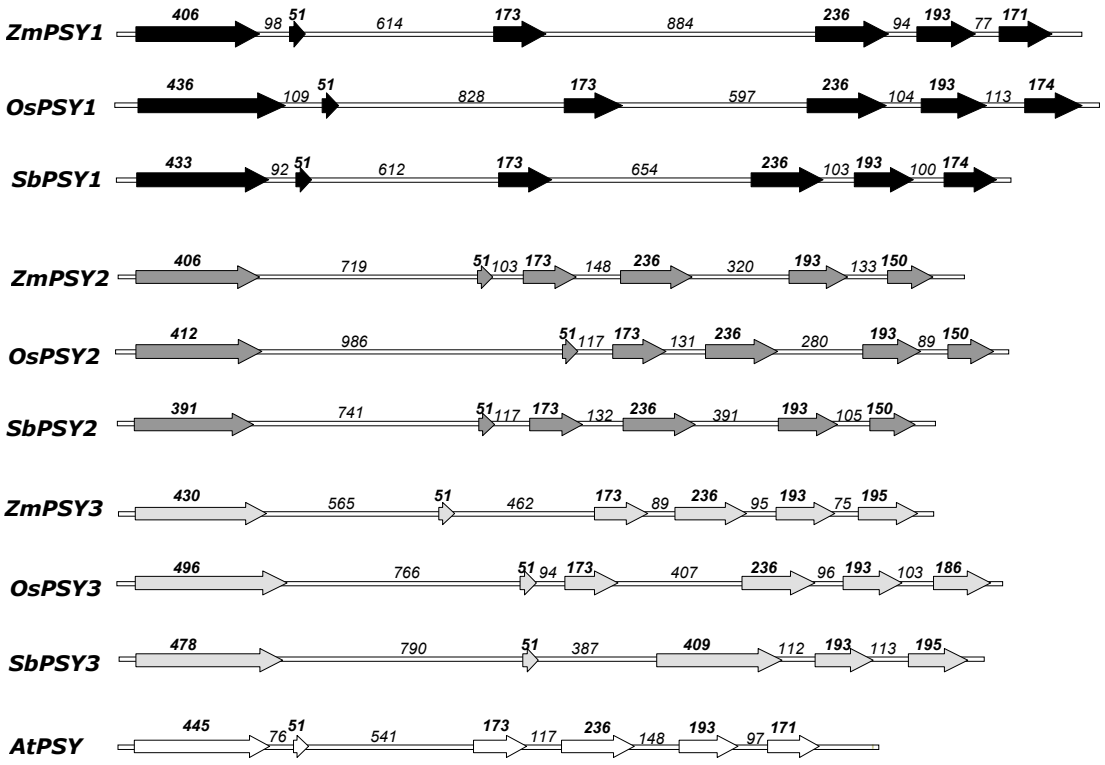
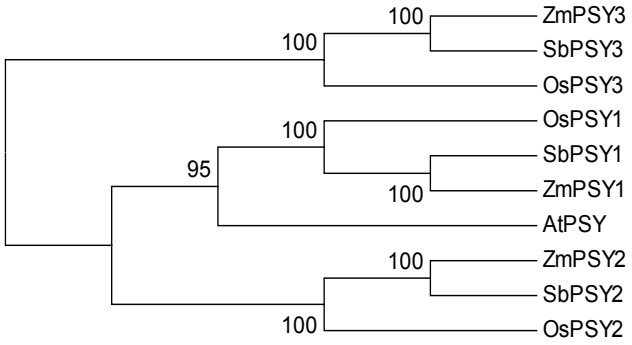


Figure 2-3. Gene structure of maize, rice, sorghum and Arabidopsis *PSY* genes. Maize *PSY1* (*ZmPSY1*; GenBank **AY324431**); rice *PSY1* (*OsPSY1*; GenBank **AP005750**); sorghum *PSY1* (*SbPSY1*; PlantGDB **SbGSStuc11-12-04.5154.1**); maize *PSY2* (*ZmPSY2*; GenBank **AY325302**); rice *PSY2* (*OsPSY2*; 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 bp.

A



B

```
OsPSY1   ----SLRNSQK
SbPSY1   ----SLRNTQT    I
ZmPSY1   ----SLRNGQT

OsPSY2   ----ARAAVAS
SbPSY2   ----ARAAVAS    II
ZmPSY2   ----ARAAVAP

OsPSY3   ----RHCSSLTSS
SbPSY3   ----RHHSSLT--  III
ZmPSY3   ----RRYSTLT--
```

Figure 2-4. 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. *A. thaliana* (AtPSY AAA32836), *O. sativa* (OsPSY1, AAS18307; OsPSY2, AAK07735; OsPSY3, DQ356431), *S. bicolor* (SbPSY1, AY705389; SbPSY2, AW679367; SbPSY3, AY705390), *Z. mays* (ZmPSY1, P49085; ZmPSY2, AAQ91837; ZmPSY3, DQ36430). The 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 highlight indicates identical residues; grey denotes similar residues.

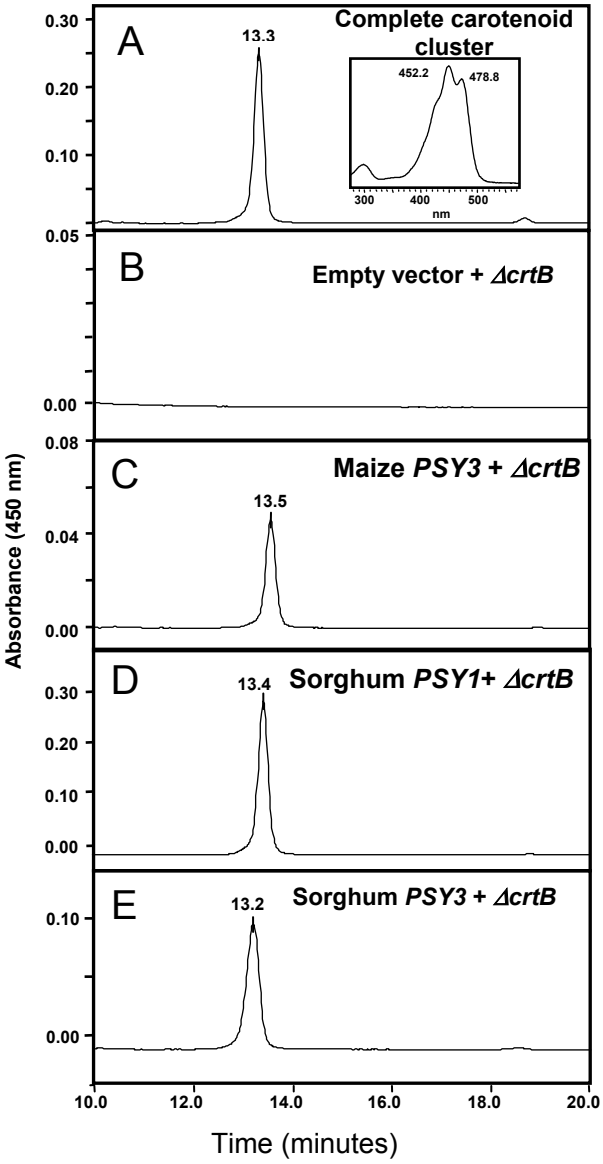


Figure 2-5. Functional complementation of PSY3 from maize and sorghum. *E. coli* cells were transformed with: (A) pACCAR25; (B) pACCAR25ΔcrtB + pET23a (empty vector); (C) pACCAR25 ΔcrtB + maize *PSY3*; (D) pACCAR25ΔcrtB + sorghum *PSY1*; (E) pACCAR25ΔcrtB + sorghum *PSY3*. Chromatograms show HPLC separation of extracted pigments; the insert in section A shows the spectral fine structure for the pathway end product, zeaxanthin diglucoside.

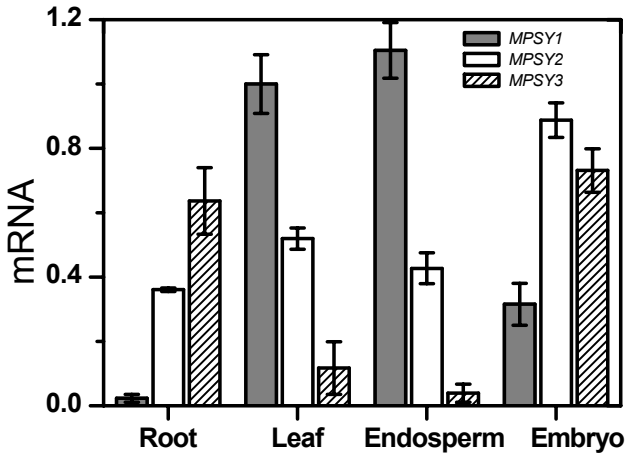


Figure 2-6. Transcript levels for *PSY* gene family members in different maize tissues. Endosperm and embryo were collected at 20 DAP from field-grown maize B73. Leaf and root samples were collected from B73 seedlings at the 5-leaf stage. Transcript levels of maize *PSY1*, *PSY2* and *PSY3* were normalized to levels of ß-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 3 RT-PCR replicates +/- SD from five pooled plants. *MPSY1*, maize *PSY1*; *MPSY2*, maize *PSY2*; *MPSY3*, maize *PSY3*

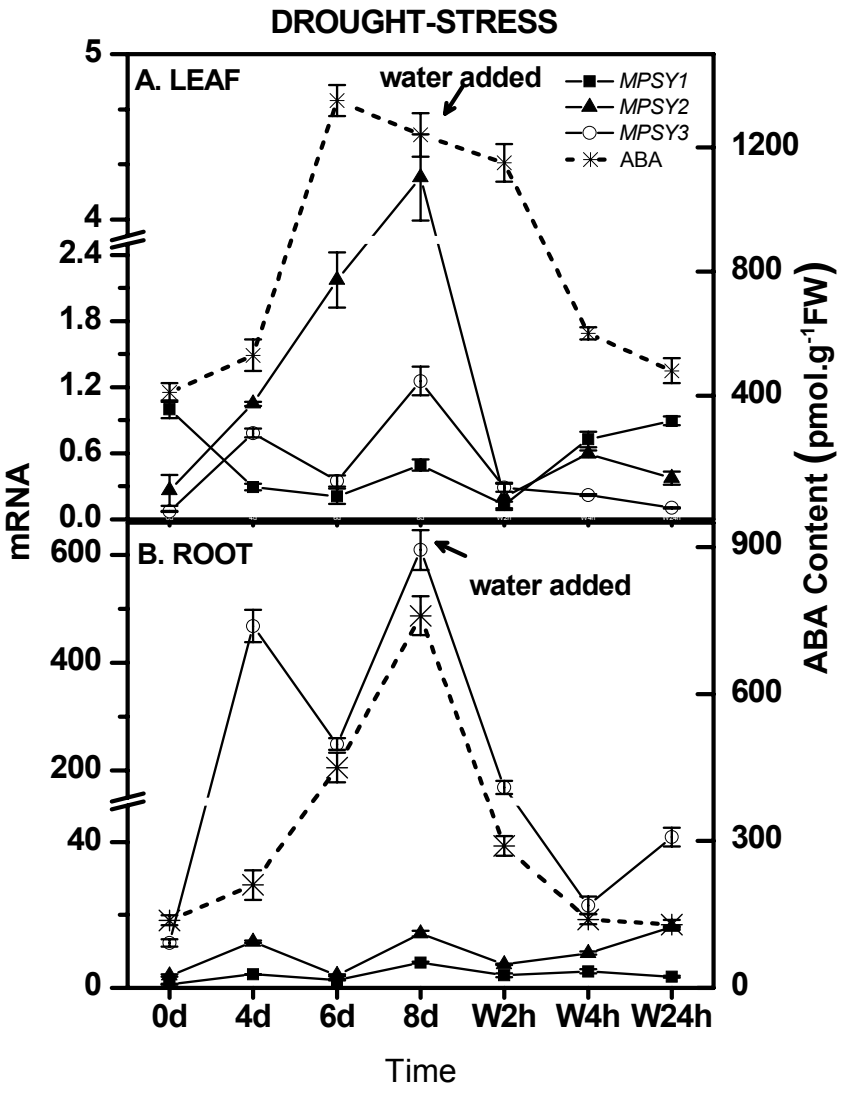


Figure 2-7. Effect of drought-stress on maize *PSY* transcript levels. Maize B73 seedlings at the 5-leaf stage were greenhouse-grown, deprived of water for 8 days followed by rewatering. Total RNA was extracted from leaves or roots at 0, 4, 6, 8 days after water was withheld and 2, 4, and 24 h after re-watering. Transcript levels of maize *PSY1*, *PSY2* and *PSY3* were normalized to levels of ß-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 3 RT-PCR replicates +/- SD from five pooled plants.
Genes are abbreviated as in Fig. 2-6.

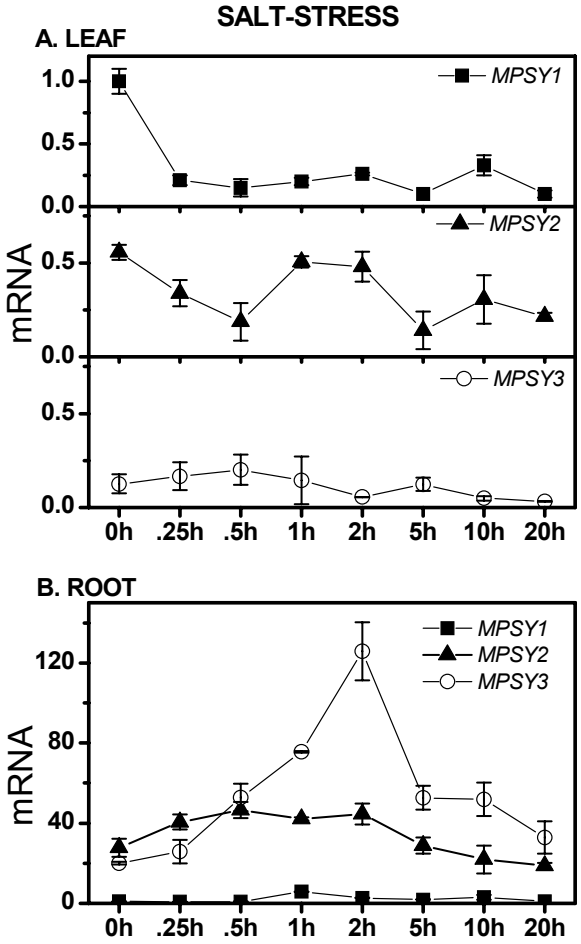


Figure 2-8. Effect of salt-stress on maize *PSY* transcript levels. Total RNA was extracted from leaves or roots at indicated hours (h) after treatment with 250 mM NaCl. Transcript levels were normalized to levels of ß-actin transcripts measured in the same sample, and are shown relative to PSY1 transcript levels in leaf (A) and root (B) at 0 h. Values represent the mean of 3 RT-PCR replicates +/- SD from five pooled plants. Genes are abbreviated as in Fig. 2-6.

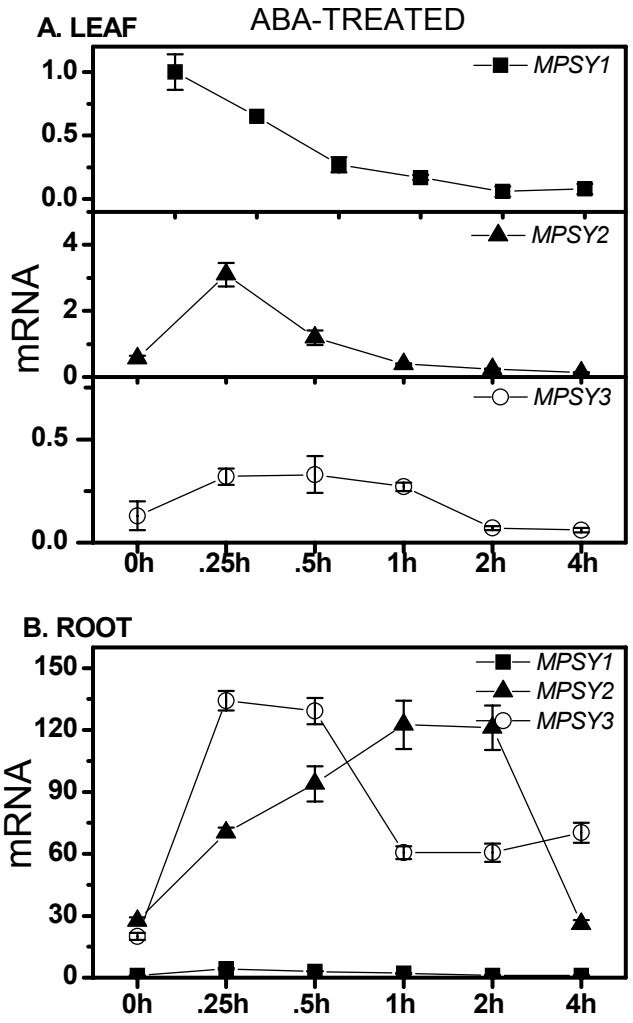


Figure 2-9. Effect of ABA on maize *PSY* transcript levels. Total RNA was extracted from leaves or roots at 0, 0.25, 0.5, 1, 2 and 4 h after ABA treatment. Transcript levels were normalized to levels of ß-actin transcripts measured in the same sample, and are shown relative to mPSY1 transcript levels in leaf (A) and root (B) at 0 h. Values represent the mean of 3 RT-PCR replicates +/- SD from five pooled plants. Genes are abbreviated as in Fig. 2-6.

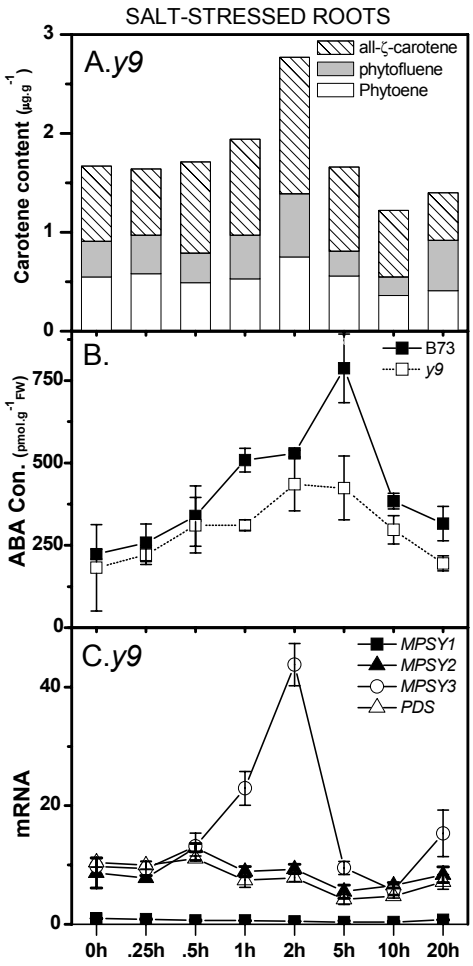


Figure 2-10. HPLC analysis of carotenoid content of maize *y9* root tissue after salt treatment. Maize *y9* mutant seedlings and normal B73 seedlings at the 5-leaf stage were salt-treated for 0, 0.25, 0.5, 1, 2, 5, 10 and 20 h. A, HPLC chromatogram (at λmax) showing carotenoid content of *y9* mutant 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 ß-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 3 RT-PCR replicates +/- SD from five pooled plants. PDS, phytoene desaturase; other genes are abbreviated as in Fig. 2-6.

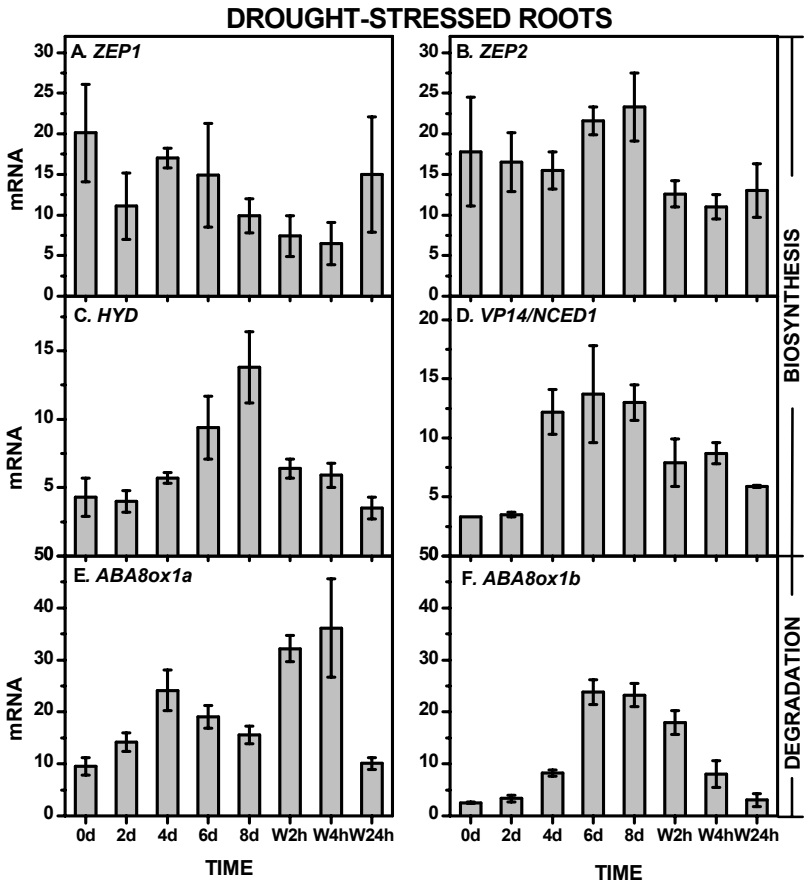


Figure 2-11. Drought-stress modulated levels of transcripts encoded by genes controlling biosynthesis and degradation of ABA in maize. The mRNA levels of (A) *zeaxanthin epoxidase 1* (*ZEP1*, *GenBank* DR820114), (B) *zeaxanthin epoxidase 2* (*ZEP2*, *GenBank* CO532283), (C) *β-carotene hydroxylase (HYD*, GenBank BQ619575), (D) *9-cis-epoxycarotenoid dioxygenase 1* (*NCED1*, *GenBank* U95953), (E) *ABA 8'hydroxylase 1a* (*ABA8ox1a*, GenBank DR806072), and (F) *ABA 8' hydroxylase 1b* (*ABA8ox1b*, GenBank EE190691), were normalized to levels of ß-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 3 RT-PCR replicates +/- SD from five pooled plants.

Table 2-1. Primers for gene isolation, BAC sequencing and quantitative real-time PCR

Genes	GenBank Acc. No.	primers	Note
Maize *PSY1*	AY324431	#505 (Forward): CATCTTCAAAGGGGTCGTCA #254 (Reverse): CAGGATCTGCCTGTACAACA	Real Time PCR
Maize *PSY2*	AY325302	#503 (Forward): TCACCCATCTCGACTCTGCTA #676 (Reverse): GATGTGATCTACGGATGGTTCAT	Real Time PCR
Maize *PSY3*	DQ372936	#996 (Forward): GCCAGAGCCTTCTTCAGGCAGG #997 (Reverse): GTCTTCGGAACGTAGGCCCTC	Real Time PCR
Maize *ZEP1*	DR820114	#1664 (Forward): TTGGTGGCAACAGCTCAAAA #1665 (Reverse): CCTTCACTTGTTGCGATGAG	Real Time PCR
Maize *ZEP2*	CO532283	#1400 (Forward): TTGGTGGCAATAGCTCAAAG #1401 (Reverse): GCTTCACTTGTTGCGAAGAG	Real Time PCR
Maize *HYD*	BQ619575	# 1419 (Forward): GAGATCAAGAAGAGGATCAGGAG #1420 (Reverse): GATCTAATGTGAGGAGAAGGTCAA	Real Time PCR
Maize *VP14/NCED1*	U95953	# 1652 (Forward): GCCCAGCTCGTAGCTTAACA #1653 (Reverse): CGAGCAAGTAACAGCAACCA	Real Time PCR
Maize *ACTIN*	J01238	#1134 (Forward): CGATTGAGCATGGCATTGTCA #1135 (Reverse): CCCACTAGCGTACAACGAA	Real Time PCR
Maize *PSY3*	DQ372936	#925 (Forward): ATGAATTCTACGAGCCGCGCGGTGAA #926 (Reverse): GAGCTCGAGACCGGTCTATGTTA	Gene isolation
Rice *PSY3*	LOC_Os09g38320*	#1155(Forward): AAGTACGTCGTCGTCGGCCATGA #1191(Reverse): CAAGCTGATCATGATGATGTTA	Gene isolation
Maize *ABA8ox1a*	DR806072	# 1774 (Forward) ATGCTCGTGCTCTTCCACCACCT #1769 (Reverse) TATACCGCCATACCATATCCATC	Real Time PCR
Maize *ABA8ox1b*	EE190691	#1774 (Forward) ATGCTCGTGCTCTTCCACCACCT #1772 (Reverse) GGAAGCGGTTTTTCGCGTTCCTGG	Real Time PCR

* Gramene ID

Table 2-1. (continued)

Real time PCR conditions for maize *PSY1*, *PSY3* and actin	Real time PCR conditions for maize *PSY2*	Real time PCR conditions for maize *ZEP1*, *ZEP2*, *HYD*, *NCED1, ABA8ox1a* and *ABA8ox1b*
Protocol Cycle 1: (1X) Step 1: 95.0ºC for 05:00 Cycle 2: (35X) Step 1: 95.0ºC for 00:30 Step 2: 56.0ºC for 00:30 Step 3: 72.0ºC for 00:45 Data collection enabled. Cycle 3: (80X) Step 1: 50.0ºC for 00:10 Repeated with temperature setpoint increased by 0.5ºC, until 90.0ºC is reached at the end (80X). Melt curve data collection and analysis enabled.	Protocol Cycle 1: (1X) Step 1: 95.0ºC for 05:00 Cycle 2: (35X) Step 1: 95.0ºC for 00:45 Step 2: 54.0ºC for 00:45 Step 3: 72.0ºC for 00:45 Data collection enabled. Cycle 3: (80X) Step 1: 50.0ºC for 00:10 Repeated with temperature setpoint increased by 0.5ºC, until 90.0ºC is reached at the end (80X). Melt curve data collection and analysis enabled.	Protocol Cycle 1: (1X) Step 1: 95.0ºC for 05:00 Cycle 2: (35X) Step 1: 95.0ºC for 00:30 Step 2: 58.0ºC for 00:30 Step 3: 72.0ºC for 00:45 Data collection enabled. Cycle 3: (80X) Step 1: 50.0ºC for 00:10 Repeated with temperature setpoint increased by 0.5ºC, until 90.0ºC is reached at the end (80X). Melt curve data collection and analysis enabled.

CHAPTER 3

THE MAIZE PHYTOENE SYNTHASE GENE FAMILY AND PARALOG-SPECIFIC ROLES IN ENDOSPERM AND LIGHT-REGULATED CAROTENOGENESIS

3.1 Abstract

Carotenoids are a diverse group of natural pigments derived from C_{40} phytoene. They serve essential functions for plants and play beneficial roles in human health as vitamin A precursors and as antioxidants. The enzyme phytoene synthase (PSY) catalyzes the first committed step in the carotenoid biosynthetic pathway. Over the last ten years, the gene encoding PSY represents a major target in the study of the regulation and manipulation of carotenoid biosynthesis in some model plants. However, few of these studies have involved members of the grass family (Poaceae), which contains most of the agronomically important crops. Thus, I chose maize as a model for the Grass family and studied the role of *PSY* genes in controlling carotenoid biosynthesis. Previously, I reported the discovery of the third *PSY* gene and its role in controlling abscisic acid (ABA) biosynthesis in roots. Here, further research was executed to study the subfunctionalization of these three *PSY* genes and their roles in endosperm and leaf carotenogenesis. I showed that *PSY1* but not *PSY2* or *PSY3* transcripts and protein levels in endosperm correlate with the carotenoid accumulation. Moreover, by using carotenoid-free *PSY1* endosperm mutant, *y1-602C*, I demonstrated that the contribution of *PSY2* and *PSY3* towards endosperm

carotenoid accumulation is negligible. On the other hand, I showed that *PSY2* is responsible for the leaf carotenogenesis during greening. By blocking the light signal transduction with chromophore deficit mutant *elm1*, I demonstrated that it is phytochromes mediate the red and far-red light signals for up-regulation of *PSY2* during de-etiolation. The role of PSY1 in leaf carotenogenesis was also assessed by using a *PSY1* null mutant, *y1-8549*. Pigment analysis revealed that functional *PSY1* is critical for maintaining the chloroplast carotenoid in green tissue under heat stress conditions. Studies of the duplication and subfunctionalization of maize *PSY* may benefit carotenoid biosynthesis research in other cereal plants.

3.2 Introduction

Carotenoids represent a diverse group of natural pigments found in bacteria, fungi, alga and plants. More than 750 kinds of carotenoids are identified to date and all of them are derivatives of phytoene (Britton et al., 2004). In higher plants, they serve as accessory pigments in the photosynthetic apparatus where they play roles in light harvesting and photoprotection (Niyogi, 2000). Carotenoids are also precursors for apocarotenoids that play roles in internal and external signaling (Matthews and Wurtzel, 2007) and precursors for abscisic acid (ABA), which plays various roles in regulating plant growth, embryo development, dormancy and stress responses (Nambara and Marion-Poll, 2005). Carotenoids are also essential components in human and animal diets, primarily as some are precursors of vitamin A (Fraser and Bramley, 2004).

The biosynthesis of carotenoids in plants (as recently revised, see Li et al., 2007) starts with the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to form one molecule of phytoene. This reaction is catalyzed by the enzyme phytoene synthase (PSY) (Dogbo et al., 1988; Gallagher et al., 2004), which is regarded as the first committed step in the carotenoid biosynthetic pathway. In the past twenty years, genes encoding PSY have been identified from a number of plants, and their products characterized (see review by Matthews and Wurtzel, 2007). It appears that only one *PSY* gene is present in some plants, such as Arabidopsis. But the duplication of the *PSY* gene has been discovered in tomato (Bartley et al., 1992; Bartley and Scolnik, 1993), tobacco (Busch et al., 2002) and all subfamilies in the Poaceae or Grass family (Gallagher et al., 2004).

Since phytoene formation represents a regulatory step in the carotenoid biosynthetic pathway, it is not surprising that over the last 10 years the *PSY* gene has represented a most important focus point in the research of the regulation and manipulation of the pathway. The important role of the *PSY* gene in controlling carotenoid biosynthesis has been demonstrated in ripening fruits (Giuliano et al., 1993; Kato et al., 2004), budding flowers (Moehs et al., 2001; Zhu et al., 2002) and de-etiolating seedlings (von Lintig et al., 1997; Welsch et al., 2003). For example, the expression levels of tomato *PSY1* appear to be tightly correlated with the levels of carotenoid accumulation in ripening tomato

(Giuliano et al., 1993), whereas tomato *PSY2* seems to be leaf-specific and expresses at low levels in the fruit and flower (Bartley and Scolnik, 1993).

Although the carotenoid biosynthetic pathway was already successfully targeted by genetic engineering in the grass family, (ie., Golden rice), the regulation of carotenogenesis in the grass family still remains poorly understood. As a result, the manipulation of carotenoid content and composition in cereal crops has led to unexpected results. To better understand the regulation of carotenoid biosynthesis in the Grass family, I chose maize as a model. The carotenoid accumulation that occurs during endosperm development and the availability of a large collection of colored kernel mutants makes maize good candidate for regulation studies of the Grass family. Previously , I reported that three *PSY* genes are present in the maize, rice and sorghum genomes (Li et al., in press). Maize *PSY1* was identified through the characterization of a mutator-induced *y1* mutant, which is unable to accumulate carotenoids in maize endosperm (Buckner et al., 1996). The expression of maize *PSY1* appears closely correlated with the carotenoid accumulation in endosperm (Gallagher et al., 2004), which is similar to the role of tomato *PSY1* in the fruit and flower. However, maize *PSY1* is not endosperm-specific and also expresses at high levels in leaf tissues, with transcript levels that are two fold higher than those of the second copy, *PSY2* (Gallagher et al., 2004; Li et al., in press). Recently, I also showed that the expression of the third *PSY* gene, *PSY3,* is associated with ABA biosynthesis in roots (Li et al., in press) .

To better understand the roles of the three maize *PSY* paralogs in controlling endosperm and leaf carotenogenesis, their transcript profiles in developing endosperm and de-etiolated leaf tissue were investigated. I showed that the transcript and protein levels of maize *PSY1* were strongly correlated with the levels of carotenoid accumulation in endosperm. On the other hand, the levels of maize *PSY2* transcript were correlated with the carotenoid accumulation in the seedlings during greening. By using a *PSY1* null mutant, I also demonstrated that functional PSY1 is essential for maintaining chloroplast carotenoids under heat stress conditions.

3.3 Results

3.3.1 Maize *PSY1* expression levels correlate to carotenoid accumulation in endosperm.

To establish when carotenoid accumulation takes place during endosperm development, total colored carotenoids from the maize B73 inbred line were measured according to the method described by Kurilich and Juvik (1999). The accumulation of carotenoids in the endosperm appeared to start as early as 10 days after pollination (DAP). From 14 DAP to 16 DAP, the carotenoid content increased 3.5 fold, from 2.39 $\mu g.g^{-1}$ to 8.44 $\mu g.g^{-1}$, and then increased gradually from 18 DAP to 28 DAP (**Fig. 3-1A**).

To determine the roles of three maize *PSY* genes in carotenoid accumulation in endosperm tissue, their transcript profiles in the developing endosperms from the B73 inbred line were investigated using real time PCR. The transcript levels of maize *PSY1* correlated with the levels of carotenoid accumulation. Maize *PSY1* mRNA levels began to increase significantly at 14 DAP and reached their peak at 18 DAP (**Fig 3-1A**), which was about 4.3 fold higher than levels at 10 DAP. Transcript levels started to decline after 22 DAP and dropped down to significantly lower levels at 28 DAP. In contrast to maize *PSY1*, the mRNA levels of maize *PSY2* remained constant during endosperm development and increased slightly after 24 DAP. Maize *PSY3* transcripts remained at relatively low levels, which were 30 fold lower than that of maize *PSY1* at 20 DAP (**Fig. 3-1A**).

The impact of maize *PSY1* expression on carotenoid accumulation was further evaluated through characterization of a maize *PSY1* endosperm mutant *y1-602C*. The *y1-602C* allele has a normal green tissue phenotype with colorless endosperm, representing low carotenoid accumulation in the endosperm of this allele. The expression of maize *PSY1* is only affected in endosperm but not in leaf tissue (Gallagher et al., 2004). The mRNA levels of maize *PSY2* and *PSY3* in tissue from all stages of development (10-28 DAP) were very similar to transcript levels in the B73 line, whereas the transcripts of maize *PSY1* were below detectable levels (**Fig 3-2**). Taken with the data from the B73 inbred line, these results clearly demonstrated that the loss of maize *PSY1* would block the carotenoid biosynthetic pathway completely in endosperm and that the presence of low amounts of maize *PSY2* and *PSY3* transcripts were unable to restore carotenoid synthesis.

The protein levels of maize *PSY1* in developing endosperm was also examined with anti-maize PSY1 antiserum. The polyclonal antiserum against maize PSY1 was raised by using an expressed fusion protein containing 70% of the maize PSY1 protein (see materials section). To test its specificity to other PSY proteins, maize PSY2 and PSY3 were expressed as T7.tag fusion proteins by using the pET23 system (Novagen, Madison, WI). With the T7.tag monoclonal antibody, I detected a polypeptide of ~35 kDa in inclusion bodies from cells containing maize *PSY2* and a polypeptide of ~50 kDa in inclusion bodies from cells containing maize *PSY3*, respectively (**Fig 3-3A**). The expressed fusion proteins were of expected sizes as predicted by cDNA sequences. However, no polypeptides of expected size for maize *PSY2* and *PSY3* were detected by using anti-maize PSY1 antiserum. These results indicate that maize PSY2 or PSY3 proteins cannot be recognized by this anti maize PSY1 antiserum. Using this antiserum, I detected a polypeptide of ~ 38 kDa in developing endosperm tissues (**Fig. 3-1B)**. The molecular weight of the maize PSY1 precursor is 46.3 kDa, with a ~ 8 kDa transit peptide predicted by ChloroP 1.1 Server service (http://www.cbs.dtu.dk/services/ChloroP/). Therefore, the 38 kDa PSY antigen detected in maize endosperm by western analysis may represent the mature maize PSY1 found in plastids. Moreover, using this antiserum, I just barely detected PSY antigen from PSY1 endosperm mutant, *y1-602C* but a strong signal was detected in the B73 inbred line (**Fig. 3-3B**), which suggest that this antiserum is very specific for the maize PSY1 protein. In the B73 inbred line, the maize PSY1 protein started to accumulate at 12 DAP, although the transcript levels of maize *PSY1* were still low (**Fig. 3-1B**). Transcripts reached a

maximal level at 18 DAP and declined after 20 DAP. Taken with the transcript profiles of the maize *PSY* genes in developing endosperm, these results clearly demonstrate that it is maize *PSY1*, but not *PSY2* or *PSY3*, which correlates with the carotenoid accumulation in maize endosperm.

3.3.2 Maize *PSY2* but not *PSY1* is up-regulated by light in photosynthetic tissue

During de-etiolation or under high light stress, the biosynthesis of carotenoids is enhanced to assist photosynthesis or to prevent photo-oxidative damage. The transcript levels of several carotenogenic genes, 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, I quantified the transcripts of three maize *PSY* genes in de-etiolated seedlings to assess their roles in leaf carotenogenesis. Dark-grown maize seedlings were illuminated for varying amounts of time (0 to 8 hours) as described in the methods section. In etiolated seedlings, the transcript levels of maize *PSY1* were about 3 folds higher than that of maize *PSY2* (**Fig. 3-4, 0h**). Upon illumination, it was observed that the mRNA levels of maize *PSY2* were up-regulated by light. Its transcript levels started to increase after 1h of illumination and reached the highest level at 2 hours, which was 6.12 folds higher than that of the unilluminated control. Subsequently, mRNA levels began to decline, reaching approximately the same levels observed in the unilluminated control after 8 h illumination. In contrast to the mRNA levels of maize *PSY2*, the mRNA levels of maize *PSY1* in leaves were not affected by light. Maize *PSY3*

expression remained at very low levels during de-etiolation, which indicated that it is not important in carotenoid biosynthesis in leaf tissue.

3.3.3 Maize *PSY2* induction needs continuous illumination

To examine whether continuous illumination is necessary for the induction of maize *PSY2*, an experiment was designed in which four sets of maize seedlings were exposed to white light and then kept in the dark for various lengths of time; the first, second, third, and fourth groups of seedlings were exposed to light for 10, 30, 60, and 120 minutes respectively and then kept in the dark for 110, 90, 60, and 0 minutes respectively in order for each set to have equal time for maize *PSY2* transcript accumulation. These studies showed that the transcript levels of maize *PSY2* did not increase until continuous illumination for 2 hours (**Fig. 3-5**). This suggested that light exposure for a short time was not sufficient for maize *PSY2* induction.

3.3.4 Red and far-red light induced maize *PSY2* mediated by phytochrome

In the above section, it was shown that only maize *PSY2* is subjected to light regulation. I also wanted to examine which kind of light can up-regulate the expression of maize *PSY2*. Dark-grown maize seedlings were exposed to red, far-red or blue light, respectively. It was observed that the mRNA levels of maize *PSY2* can be induced by all kinds of light regimes (**Fig. 3-6A, C and E**). Its mRNA levels began to increase after 2

hours of illumination by red or far-red light, with levels peaking at 4 hours, which were 5 folds higher than that of unilluminated seedlings (**Fig. 3-6A and E**). Intriguingly, the response of maize *PSY2* to blue light was more rapid as well as more intense. Its mRNA levels peaked after only 2 hours in blue light instead of 4 hours in red or far-red illuminated seedlings, with levels that were 10.5 folds higher than that of controls not exposed to light (**Fig. 3-6C**).

To further determine what kind of photoreceptor mediates such light induction of maize *PSY2*, expression profiles of maize *PSY2* in the *elm1* mutant were characterized. *elm1* is a mutant affecting chromophore biosynthesis, which is an essential constituent of all kinds of phytochromes (Sawers et al., 2002). In this mutant, there was no increase in maize *PSY2* transcripts after illumination by red or far-red light (**Fig. 3-6B and F**). These results suggested that maize *PSY2* up-regulation by red or far-red light, which was blocked in the *elm1* mutant, was mediated by phytochromes. Blue light, which is mediated by another kind of photoreceptor known as phototrophin or cryptochrome (instead of phytochrome), can still stimulate maize *PSY2* expression in the *elm1* mutants as it did in the B73 inbred line plants**(Fig. 3-6D**). In contrast, the expression profiles of maize *PSY1* and *PSY3* in *elm1* mutants were the same as those in the B73 inbred line (**Fig 3-7 and 3-8**).

3.3.5 Maize *PSY1* is essential for carotenoid biosynthesis in green tissue

I have characterized here the role of maize *PSY2* in leaf carotenogenesis. However,

due to the high levels of transcripts present for maize *PSY1* in green tissue, the role of this gene cannot be discounted with respect to its possible impact on carotenoid accumulation in green tissue (Gallagher et al., 2004). To elucidate the role of this gene I used a maize mutant which lacks a functional *PSY1*; maize *PSY1* null mutants were screened by sequencing certain *y1* alleles (see methods section).

Two classes of *y1* alleles have been reported in maize by Robertson's earlier studies (Robertson and Anderson, 1961; 1987): 1) those with white endosperm and normal green leaves ; 2) those with white endosperm and pale green leaves. According to our understanding of the roles of *PSY* gene family members in carotenogenesis and transcript profiles of *PSY* genes, I presumed that *y1-602C* is of a unique class of alleles that only affect endosperm carotenoid accumulation, whereas another class of alleles may be null mutants. Sequencing analysis of several *y1* alleles revealed that in one of the *PSY1* mutants, *y1-8549,* a G insertion at 377 bp downstream of the initiator codon ATG caused an ORF shift and resulted in a new stop codon at 388 bp (**Fig 3-9A;** GenBank EU306868). In contrast, I cannot detect any mutation inside the maize *PSY1* ORF of the *y1-602C* allele (GenBank EU306869). However, in its 5' regulatory region, no *ins2* insertion was found, which is strongly associated with maize *PSY1* expression (Palaisa et al., 2003). I used the *y1-8549* null mutant to investigate the impact of the maize *PSY1* mutation on carotenoid accumulation in etiolated and mature leaves.

y1-8549 mutants were grown under 4 kinds of conditions: 20 ^{0}C in the dark, 37 ^{0}C

in the dark, 20 ^{0}C in the light, and 37 ^{0}C in the light. Carotenoids and chlorophylls accumulating in mature leaves or only carotenoids accumulating in etiolated seedlings of *y1-8549* mutants were quantified (**Table 3-1**). Under low temperature (20 ^{0}C) in the dark, the carotenoid levels (including carotenes and xanthophylls) of *y1-8549* seedlings were 62% of the levels found in wild type seedlings (**Fig. 3-9B**). When the temperature increased to 37 ^{0}C, the carotenoid levels of *y1-8549* mutants increased slightly, whereas they increased 2.2-fold in wild type. This increase in temperature therefore had a significant impact on carotenoid accumulation for the *y1-8549* mutant compared to wild type. The *y1-8549* mature leaf samples showed only a slight difference in carotenoid accumulation from that of wild type grown at low temperatures (20 ^{0}C), and the mutant's carotenoid and chlorophyll levels were 92% and 72 % of the levels found in wild type, respectively (**Fig 3-9B**.). At 37 ^{0}C, the carotenoid levels of wild type plants only increased 6% versus the 2.2-fold increase found in dark-grown wild type seedlings. This might be due to an increase in photo-oxidation at high temperatures for plants grown in the light, which leads to more severe carotenoid degradation while also preventing carotenoid accumulation. For the same reason, the photobleaching of *y1-8549* might be due to carotenoid degradation in leaves, which exceeds biosynthetic capacity, given that there is no functional maize *PSY1*.

Under dark growth conditions, the mRNA levels of maize *PSY1* in the *y1-8549* mutant dropped to 30 % - 40 % of the levels found in wild type, while the mRNA levels of *PSY2* increased 1.5-fold at 20 ^{0}C and 3-fold at 37 ^{0}C in mutants compared to that of wild type (**Fig. 3-9B**). This increase in the transcript levels of maize *PSY2* in the mutant might

be a result of feedback regulation, given that no functional *PSY1* is present. When the temperature increased, the transcript levels of both *PSY* genes decreased in both the wild type and *y1-8549* mutant. This decrease in *PSY* transcripts was unexpected given the concurrent increase in carotenoid accumulation at high temperatures. It appears that the transcript levels of maize *PSY* genes are higher than necessary at low temperatures, and the fact that PSY transcripts decrease at high temperatures (which maize prefers) indicates that this gene is a rate-limiting factor in carotenoid biosynthesis. Therefore, the carotenoid levels double in wild type seedlings with an increase in temperature, whereas there is only a slight increase in carotenoid accumulation for *y1-8549* plants (there is no functional *PSY1*in these mutants). Carotenoids can still be maintained at a basal level in wild type seedlings despite severe photo-oxidative stress at high temperatures under light growth conditions, whereas *y1-8549* mutants are photobleached. Therefore, these data clearly demonstrated that the function of maize *PSY1* is necessary for maintaining the carotenoid level in leaf.

3.4 Discussion

The work presented here addresses the question as to how the carotenoid biosynthetic pathway is regulated in endosperm and green tissues at the molecular level in maize. I have studied the transcript and protein profiles of three maize *PSY* genes in developing endosperms, and their transcript profiles in de-etiolated seedlings. Furthermore,

a maize mutant which lacks *PSY1* function in all tissues was also used to evaluate the role of *PSY1* in maize with regard to carotenoid synthesis in chloroplasts.

PSY represents the first enzyme in the carotenoid biosynthetic pathway and is thought to catalyze a major regulatory step. It has been shown that the transcript levels of *PSY1* correlate with carotenoid accumulation in maize and rice endosperms (Buckner et al., 1996; Burkhardt et al., 1997; Gallagher et al., 2004). Here I showed that not only the transcript levels but also the protein levels of maize PSY1 strongly correlate with carotenoid accumulation in maize developing endosperm (**Fig. 3-1**). Our data also demonstrated that the period between 14 and 20 DAP was the most important stage for carotenoid accumulation in maize endosperm.

Low levels of maize *PSY2* and *PSY3* transcripts have been detected in the endosperm tissues of both the B73 inbred line and *PSY1* mutant *y1-602C* (**Fig. 3-1 and 3-2.**). Lack of carotenoid pigment in the endosperm of the *y1-602C* mutant suggests that the presence of maize *PSY2* and *PSY3* transcripts cannot restore carotenoid biosynthesis. This is also the case with rice endosperm, which contains low levels of *PSY2* transcripts along with undetectable carotenoid levels (Gallagher et al., 2004). It has been proposed that a lack of a functional PSY2 protein in maize or rice endosperms might be due to the differences in membrane architecture between endosperm amyloplasts and leaf chloroplasts, in which PSY2 is a functional protein (Gallagher et al., 2004). The

differences in the N- and C-termini of maize PSY proteins may be critical with respect to their localization.

The presence of high mRNA levels of maize *PSY1* and *PSY2* in leaves makes it difficult to determine their respective contributions to carotenoid biosynthesis in green tissue. To solve this problem, the transcript profiles in de-etiolated seedlings were investigated, since genes involved in the regulation of carotenoid biosynthesis in green tissue are always light-regulated during de-etiolation. It was observed that only maize *PSY2* mRNA levels increased 5-10 fold under all light regimes (**Fig. 3-6**). The light-regulation patterns of maize *PSY2* are very similar to those of *Arabidopsis PSY*: 1) Both genes are up-regulated by all light regimes and give the fastest and strongest response under blue light stimulation (Welsch et al., 2003); 2) Phytochromes are essential photoreceptors which mediate the regulation of far-red light (**Fig. 3-6B and F**). Maize *PSY2* mRNA levels were not induced by continuous red or far-red light in the *elm1* mutant, which is deficient in the chromophore of phytochromes. There are six phytochrome genes in the maize genome (*PhyA1, PhyA2, PhyB1, PhyB2, PhyC1,* and *PhyC2*) (Sheehan et al., 2004). Given that *PhyA* mediates the regulation of *Arabidopsis PSY* in far-red light, another phytochrome may be essential to mediate stimulation by red light, i.e. *PhyB*. The increase in maize *PSY2* transcript levels in *elm1* mutants under continuous blue light indicates that other photoreceptors may be involved, such as phototropin or cryptochrome.

It has been established that tomato *PSY1* is flower- and fruit- specific while *PSY2* is leaf-specific (Bartley and Scolnik, 1993; Fraser et al., 1999). The transcript levels of tomato *PSY2* are also 1.5 fold higher than that of *PSY1* in mature leaf tissue. However, the loss of *PSY1* function in the tomato mutant, *r,r*, can lead to lower carotenoid accumulation not only in non-green tissue but in leaf tissue (Fraser et al., 1999). Similar to the *r,r* mutant of tomato, the maize *y1-8549* mutant, which lacks maize *PSY1* function in all tissues, also accumulated lower levels of carotenoids in green tissue when they were grown under high temperature conditions (**Table 3-1**). These data clearly show that in maize and tomato, *PSY1* is not simply a non-green tissue specific gene, but is also essential for maintaining chloroplast carotenoid under high-temperature growth conditions. This contradicts the previous conclusion regarding the tissue specificities of the *PSY* genes. However, the functional redundancy of *PSY1* and *PSY2* in green tissues of tomato and maize may have been an adaptation during evolution due to the important roles of carotenoids as accessory pigments and photoprotectors in photosynthetic tissue.

The detailed characterization of the expression profiles of maize *PSY* gene family members in developing endosperm and de-etiolated seedlings in this work demonstrated that *PSY1* but not *PSY2* and *PSY3* correlates with carotenoid accumulation in endosperm. Moreover, maize *PSY1* is not simply a non-green tissue specific gene; it also contributes to chloroplast carotenoid formation. The functional redundancy of *PSY1* and *PSY2* in etiolated and mature leaves is critical for carotenoid formation in leaf tissue. The

up-regulation of maize *PSY2* during de-etiolation suggests that it plays a specific role in carotenoid biosynthesis during photomorphogenesis.

3.5 Materials and Methods

3.5.1 Plant materials

Maize (*Zea mays*) plants used were inbred line B73, phytochromobilin synthase mutant *elm1* (Sawers et al., 2002), *PSY1* recessive mutants, *y1-602C* and *y1-8549* (602M) (Maize Genetics CoOP Stock Center, University Illinois, Champaign-Urbana). The homozygous *y1-602C* have a colorless endosperm with normal leaf phenotype and homozygous *y1-8549* is a temperature sensitive mutant with pale green leaves grown at high temperature (Robertson and Anderson, 1961).

For experiments related to endosperm, maize plants were field-grown and endosperms were dissected at various days after pollination (DAP) and stored at -80 ^{0}C until analysis.

3.5.2 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 6ml of ethanol containing 0.1%

butylated hydroxyltoulene (BHT) and were incubated in an 85 ^{0}C water bath for 6min prior to a 10 min saponification with 120 µl (1g/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 (PE): Diethyl ether (DE) (2:1 v/v), vortexed and centrifuged for 10 min at 3500 rpm. The upper layer was transferred to a fresh tube and the aqueous phase was twice subjected to centrifugation with 3 ml of PE: DE each time and combined the fractions. The combined fractions were topped to 10 ml, and 1ml of aliquot was used to measure total carotenoids at OD 450nm using a Lambda 25 UV/VIS spectrometer (PerkinElmer Life Sciences, Boston).

3.5.3 Real-time PCR

RNA isolation and cDNA synthesis were carried out as described by Gallagher (2004) . Real-time PCR was performed using iQ^{TM} SYBR green supermix (BioRad, Hercules, CA) with 10 ng cDNA. Primers and PCR conditions for maize *PSY1, PSY2, PSY3* and the internal control actin were described previously (Li et al., in press). 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

$^{-\Delta Ct}$, 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 3 RT-PCR replicates +/- SD from five pooled plants.

3.5.4 Generation of anti-maize PSY1 antibodies

A partial B73 maize Y1 cDNA (encoding 287 residues, #124-410, GenBank #U32636) was subcloned as a BglII-XhoI fragment into the BamHI-XhoI sites of the Novagen vector, pPET23c(+), and renamed pY1ex1-1. After transformation of BL21(DE3) pLysS, induction with 1 mM final concentration of IPTG (isopropyl-beta-D-thiogalactopyranoside) at early stationary phase and lysis, approximately 800 μg inclusion body fraction containing the 32.9 kD Y1 fusion protein [including 12 aa, 1.32 kD of T7-Tag encoded by the vector and 70% of the Y1 protein (aa#124--aa#410, 31.57 kD), was separated on a 12% SDS-PAGE gel (110 mm wide x 140 mm high x 1.5 mm thick) at 40 mA for 4 hours at room temperature. 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 Y1 fusion protein preparation at week one, two, three, and five (Lampire Biological Laboratory Inc., Ottsville, PA). For western analysis and localization experiments, bleed from rabbit #3445 (five weeks after immunization) was used and diluted 1:10,000 in TBST

for westerns and 1:100 for confocal experiments. Specificity of the antiserum was verified by testing for presence of the antigen in normal but not homozygous *y1* endosperm, using the *y1* Maize CoOP allele 607C and 602C, which conditions reduced endosperm carotenoids, but does not affect leaf carotenoids. For western analysis of *E. coli* protein extracts, primary and secondary antisera were preincubated for 30 minutes with E. *coli* BL21(DE3) lysate at a final concentration of 0.3 mg/ml.

3.5.5 Expression of maize PSY2 and PSY3 T7.tag fusion proteins

To test the specificity of anti-maize PSY1antiserum, 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 AY450646), encoding aa#109 to aa#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 IPTG for 4 hours when the *E. 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 1:5000 dilution

was used to test for antigenicity with maize PSY2 and PSY3 fusion proteins to test the antibody specificity.

3.5.6 Western analysis to detect mPSY1 expression level in endosperm

Maize proteins from endosperms were extracted according to the method described by Wurtzel (1987). Protein samples were separated on 12% SDS-PAGE in a Criterion^{TM} Cell system (BioRad) and transferred to nitrocellulose membrane for western blot in a Trans-Blot Cell (BioRad). The first antibody, anti-maize PSY1, and the second anibody, goat anti-rabbit IgG, were diluted at 1:10,000 for use in Western anlysis according to the manufactuere'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)

3.5.7 De-etiolation experiment

Seeds of maize (B73 and *elm1*) were surface sterilized and grown on MS agar medium (Murashige and Skoog, 1962) with 1.5 % (w/v) sucrose. B73 and homozygous *elm1* plants were grown in the dark at 28 °C for 9 days and 11 days, respectively, to let them have two leaves. For de-etiolation, seedlings were transferred to continuous white (approximately 50 $\mu mol\, m^{-2}\, s^{-1}$), red (100 $\mu mol\, m^{-2}\, s^{-1}$), far-red (100 $\mu mol\, m^{-2}\, s^{-1}$) or blue light (100 $\mu mol\, m^{-2}\, s^{-1}$) and irradiated for 1, 2, 3, 4, 6 and 8 and 24 hours at room

temperature. At the end of the illumination period, leaves from 5 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.

3.5.8 Screening maize *PSY1* null mutants by sequencing

The full-length maize *PSY1* cDNA and three genomic DNA segments covering the entire ORF and 590 bp upstream were PCR amplified from *y1* alleles requested from the Maize Genetics Coop (U. Illinois, Urbana, IL). Primers were designed from the published Y1 sequence (GenBank ZMU32636) (Buckner et al., 1996). For primer sequences and PCR conditions, refer to Table 1S in supplementary material. The maize *PSY1* cDNA was amplified from total cDNA synthesized from leaves. The PCR conditions were 94 ^{0}C 30 sec, 56 ^{0}C 30 sec, 72 ^{0}C 90 sec, 35 cycles. The PCR conditions for isolating genomic DNA segments were 94 ^{0}C 45 sec, 56 ^{0}C 45 sec, 72 ^{0}C 2 min, 40 cycles with leaf genomic DNA as template. All PCR products were purified using the MinElute PCR purification kit (Qiagen, Chatsworth, CA) and verified by sequencing.

3.5.9 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 ^{0}C) or high temperature (37 ^{0}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).

The carotenoid extract and HPLC analysis were carried out as described by Quinlan (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).

3.6 Acknowledgements

I thank Dr. Thomas P. Brutnell (Boyce Thompson Institute, Cornell University, NY) for maize *elm1* seeds. I thank Dr. Timothy W. Short (Queens College, City University of New York, NY) for technical advice on light experiments, Dr. Gabriel Aisemberg for advice on confocal microscopy, and Dr. E.R. Macagno (Columbia University) for use of their microscope. I am grateful to Rena Quinlan for critical reading of the manuscript.

Figure 3-1. Transcript levels of maize *PSY* genes and total carotenoid content in developing endosperms of B73 inbred line (A) and the corresponding protein levels of maize PSY1 (B). Endosperms were dissected at 10, 12, 14, 18, 20, 22, 24, 26 and 28 days after pollination (DAP) from field-grown B73 inbred lines. UN is unfertilized ovule used as negative control. Transcript levels of maize *PSY* genes were normalized to the expression β-actin measured in the same samples and were relevant to maize *PSY1* transcript level at 20 DAP of B73 inbred line. Values represent the mean of 3 RT-PCR replicates +/- SD from five pooled plants. The total endosperm proteins were extracted according to the method described by Wurtzel (1987) and the protein levels of maize PSY1 were detected using anti-maize PSY1 antiserum.

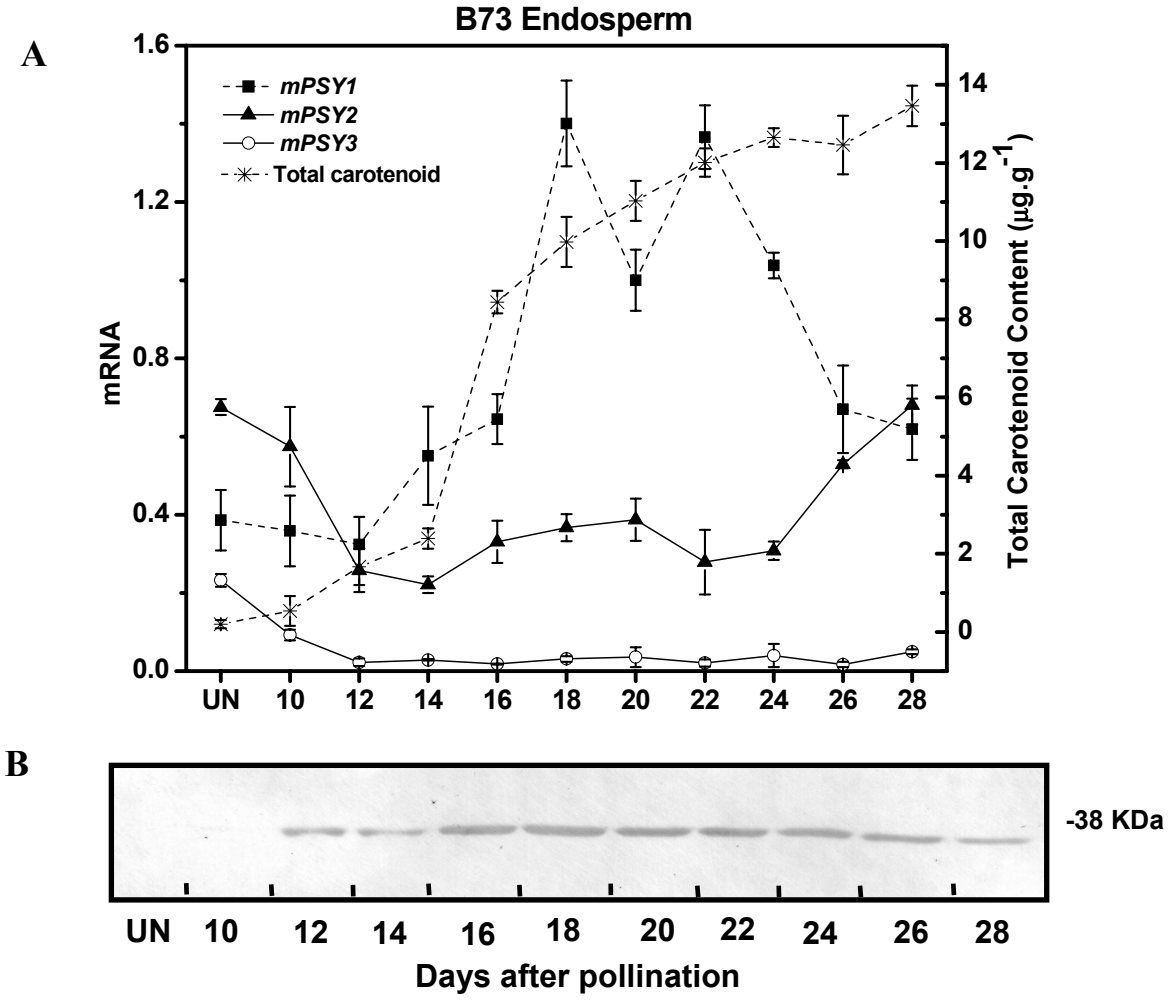

A
B73 Endosperm
mPSY1
mPSY2
mPSY3
Total carotenoid
mRNA
Total Carotenoid Content (μg.g-1)
1.6
1.2
0.8
0.4
0.0
14
12
10
8
6
4
2
0
UN
10
12
14
16
18
20
22
24
26
28
B
-38 KDa
UN
10
12
14
16
18
20
22
24
26
28
Days after pollination

Figure 3-1

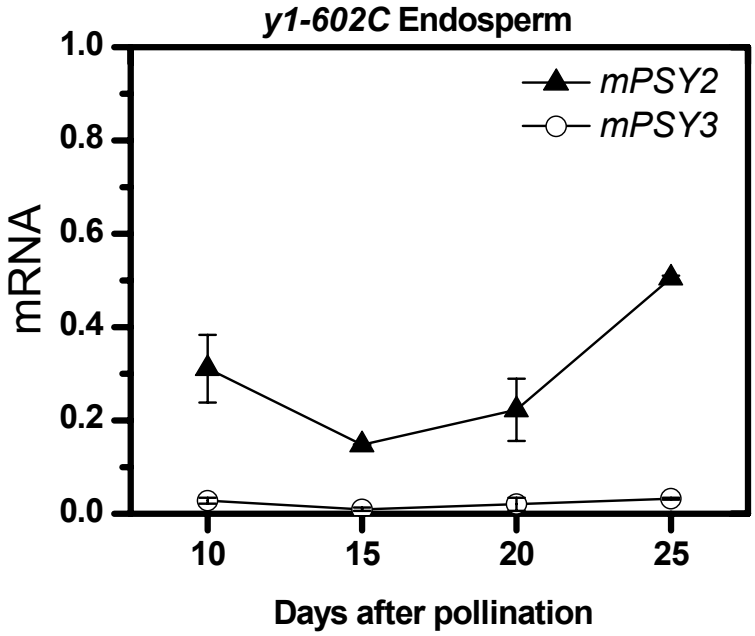


Figure 3-2. Transcript profiles of maize *PSY* genes in developing endosperms of *y1-602C* mutant. Endosperms were dissected at 10, 15, 20 and 25 DAP from field-grown *y1-602C* mutant. Transcript levels of maize *PSY2* and *PSY3* were normalized to the expression *β*-actin measured in the same samples and were relevant to maize *PSY1* transcript levels at 20 DAP of B73 inbred line. There is no detectable signal of maize *PSY1* in the endosperm of *y1-602C* allele using real time PCR. Values represent the mean of 3 RT-PCR replicates +/- SD from five pooled plants.

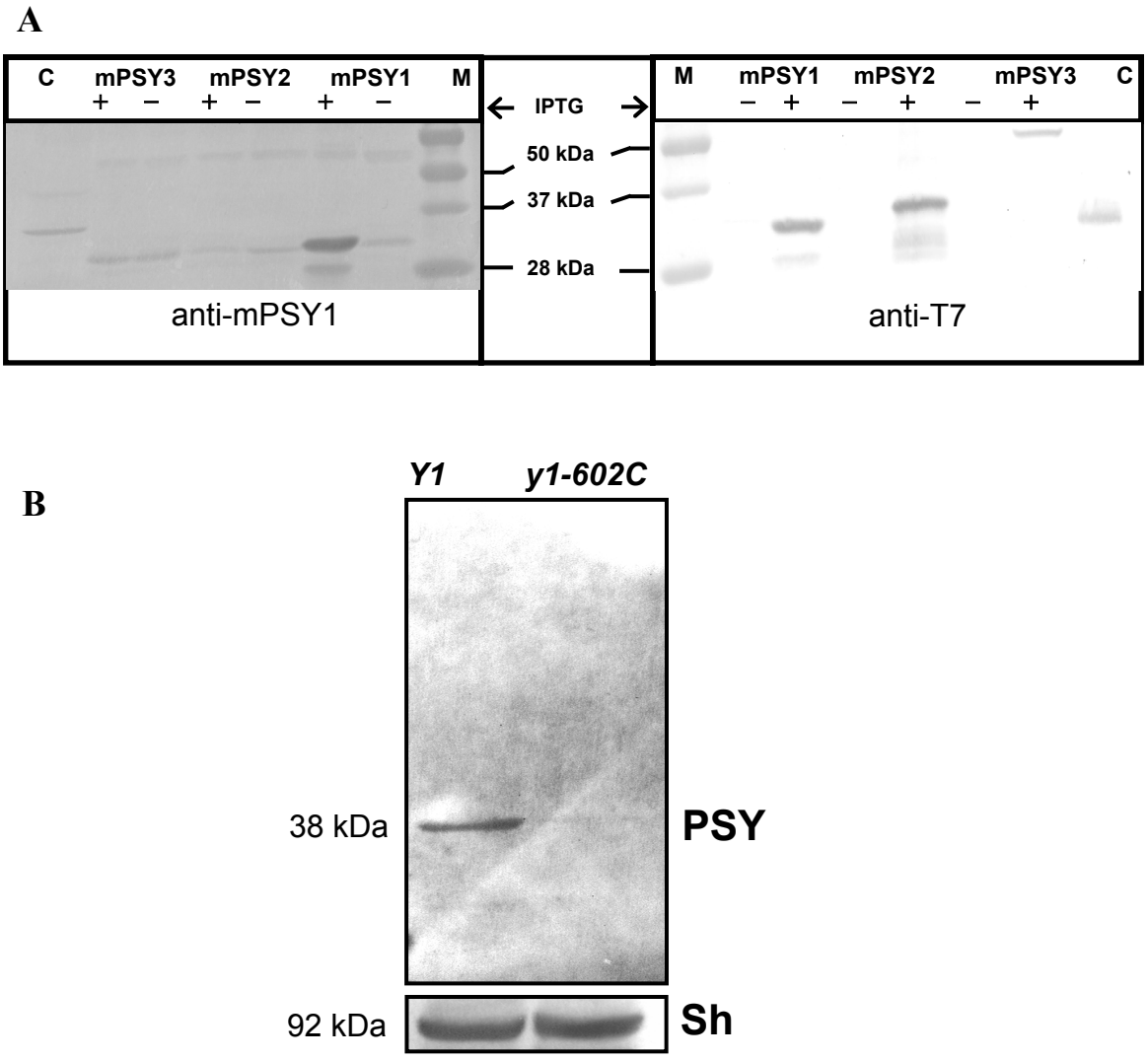


Figure 3-3. Western analysis to test the specificities of anti-maize PSY1 antiserum on maize PSY2 and PSY3 fusion proteins (A) and phytoene synthase expression in normal (Y1) and mutant (*y1*) maize endosperm (B). A. The antibodies are anti-maize PSY1 antiserum (left) and Anti-T7-Tag Antibody, AP Conjugated (right). The control "C" for anti-maize PSY1 antiserum and anti-T7 western blots are total protein extract from 20 DAP endosperm and T7.tag positive control (Novagen). B. proteins extracted from yellow endosperm (*Y1*) and white endosperm (*y1*) at 20 DAP. Immunoblots were probed by anti-PSY1 antiserum or anti-Sh antiserum.

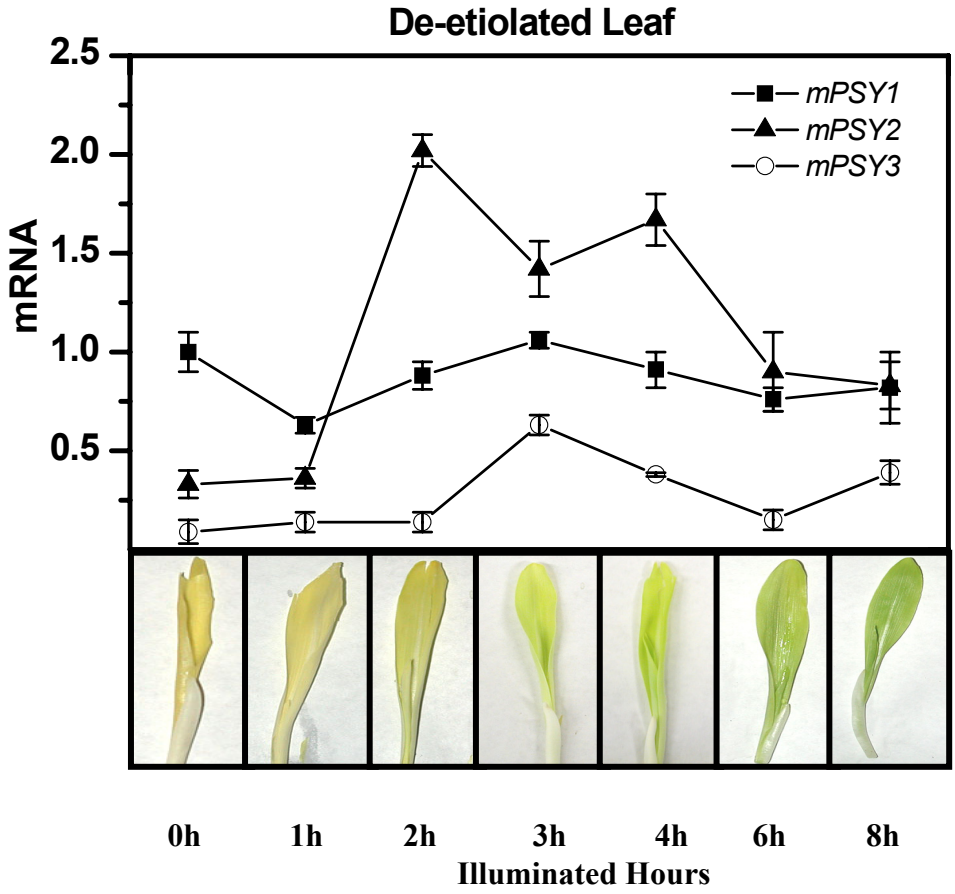


Figure 3-4. Expression profiles of maize *PSY* genes in de-etiolating seedling illuminated with white light. 9 day-old maize B73 seedlings were treated with white light (50 µmol m^{-2} s^{-1}) for 0 to 8 hours. Total cDNAs were prepared from leaves of illuminated seedlings as templates. All quantifications were normalized to *β*-actin amplified using the same conditions and were relevant to maize *PSY1* transcript levels in unlighted seedling. Values represent the mean of 3 RT-PCR replicates +/- SD from five pooled plants.

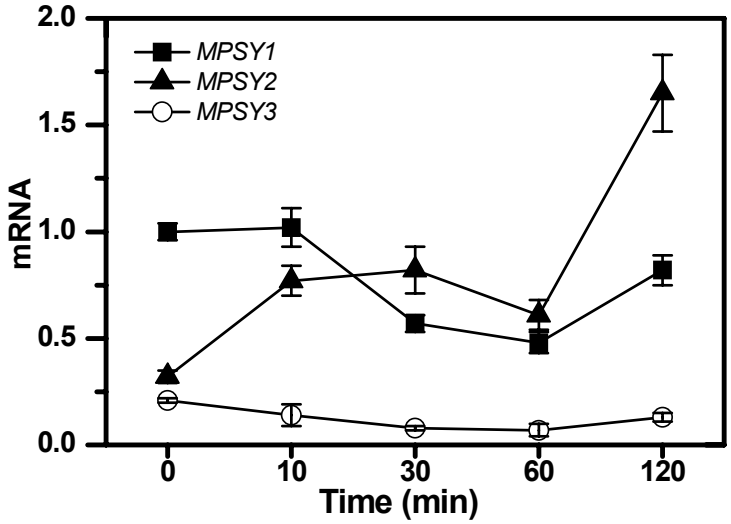


Figure 3-5. Expression profiles of maize *PSY* genes during de-etiolation. 9 day-old maize B73 seedlings were exposed to white light for 10, 30, 60, and 120 minutes respectively. Then they were kept in the dark immediately for 110, 90, 60, and 0 minutes respectively in order for each set to have equal time for transcript accumulation. Total cDNA were prepared from leaves of illuminated seedlings as templates. All quantifications were normalized to β-actin amplified using the same conditions and were relevant to maize *PSY1* transcript level at 0 h. Values represent the mean of 3 RT-PCR replicates +/- SD from five pooled plants.

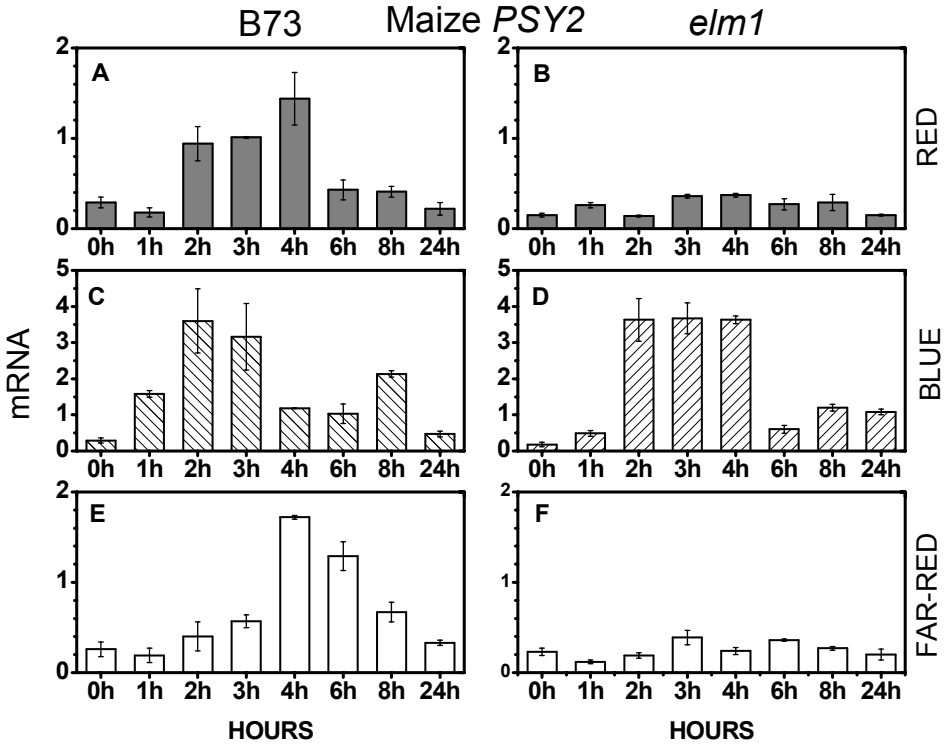


Figure 3-6. The transcript profiles of maize *PSY2* in B73 inbred line and *elm1* mutant treated with various lights during de-etiolation. Dark-grown B73 and *elm1* seedlings were illuminated with red light (100 μmol m^{-2} s^{-1}), far-red light (100 μmol m^{-2} s^{-1}) or blue lights (100 μmol m^{-2} s^{-1}) and real time PCR were used to determine the transcript levels of maize *PSY2*. All quantifications were normalized to *β*-actin and were relevant to maize *PSY1* transcript levels at 0 h using the same conditions. Values represent the mean of 3 RT-PCR replicates +/- SD from five pooled plants.

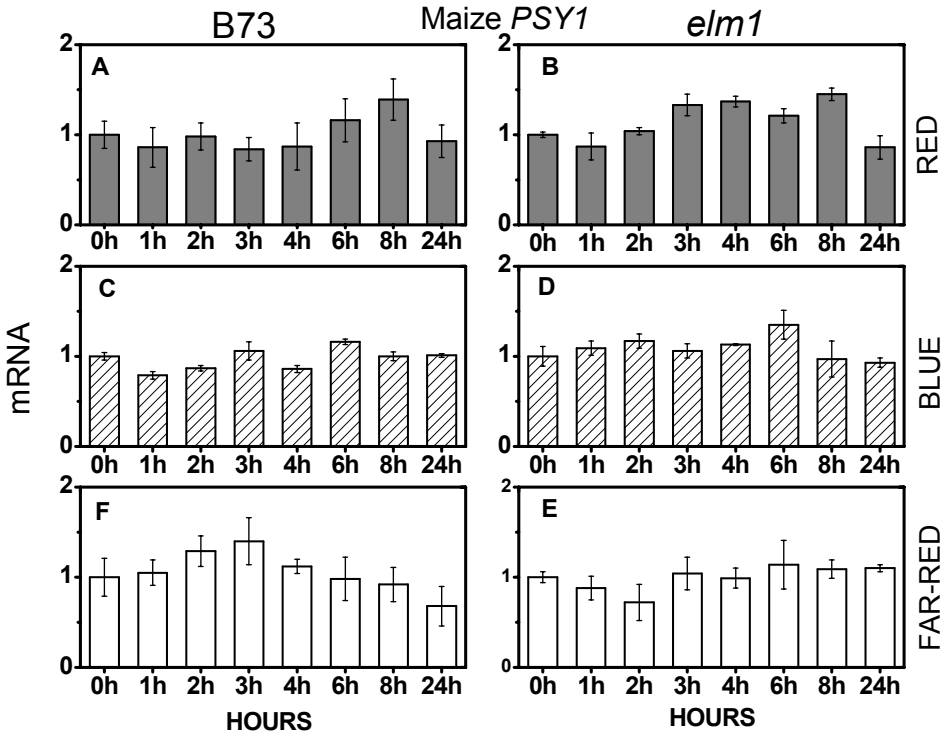


Figure 3-7. The regulation of maize *PSY1* in B73 inbred line and *elm1* mutant treated with various lights during de-etiolation. Dark-grown B73 and *elm1* seedlings were treated with red light (100 μmol m^{-2} s^{-1}), far-red light (100 μmol m^{-2} s^{-1}) and blue lights (100 μmol m^{-2} s^{-1}) and real time PCR were used to determine the transcript level of maize *PSY1*. All quantifications were normalized to β-actin and were relevant to maize *PSY1* transcript level at 0 h using the same conditions. Values represent the mean of 3 RT-PCR replicates +/- SD from five pooled plants.

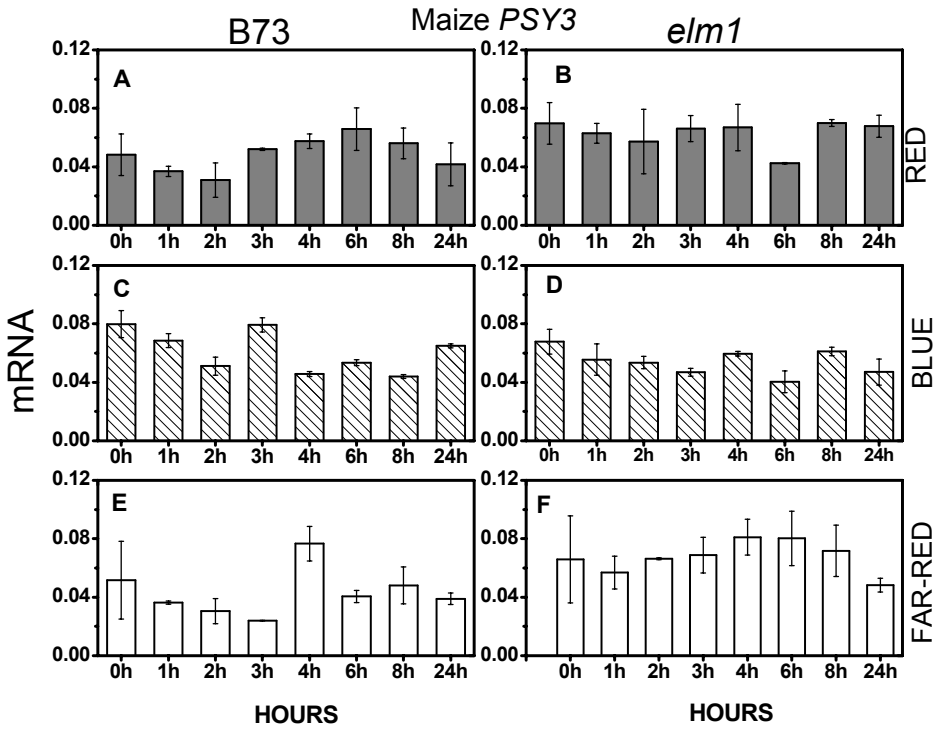


Figure 3-8. The regulation of maize *PSY3* in B73 inbred line and *elm1* mutant treated with various lights during de-etiolation. Dark-grown B73 and *elm1* seedlings were treated with red light (100 μmol m^{-2} s^{-1}), far-red light (100 μmol m^{-2} s^{-1}) and blue lights (100 μmol m^{-2} s^{-1}) and real time PCR were used to determine the transcript level of maize *PSY3*. All quantifications were normalized to β-actin and were relevant to maize *PSY1* transcript level at 0 h using the same conditions. Values represent the mean of 3 RT-PCR replicates +/- SD from five pooled plants.

Figure 3-9. Effect of PSY1 loss on maize leaf carotenogenesis. To assess the impact of functional *PSY1* loss on maize leaf carotenogenesis, *y1-8549* mutant were grown under 4 conditions (dark and 20 ^{0}C, dark and 37 ^{0}C, light and 20 ^{0}C, and light and 37 ^{0}C). The chlorophyll, carotenoid contents and transcript profiles of maize *PSY1* and *PSY2* of *y1-8549* mutant were analyzed. (**A**) Sequencing analysis of maize *PSY1* cDNA and genomic DNA prepared from leaf tissue of *y1-8549* mutant and B73 inbred line. The G insertions in the maize *PSY1* genomic DNA and cDNA prepared from *y1-8549* were highlighted. (**B**) The chlorophyll and (**C**) carotenoid contents. The mRNA levels of maize *PSY1* (**D**) and *PSY2* (**E**) in leaf tissues were normalized to levels of ß-actin transcripts measured in the same samples and are shown relative to maize *PSY1* mRNA levels from seedlings grown in the dark at 20 ^{0}C. Values represent the mean of 3 RT-PCR replicates +/- SD from five pooled plants.

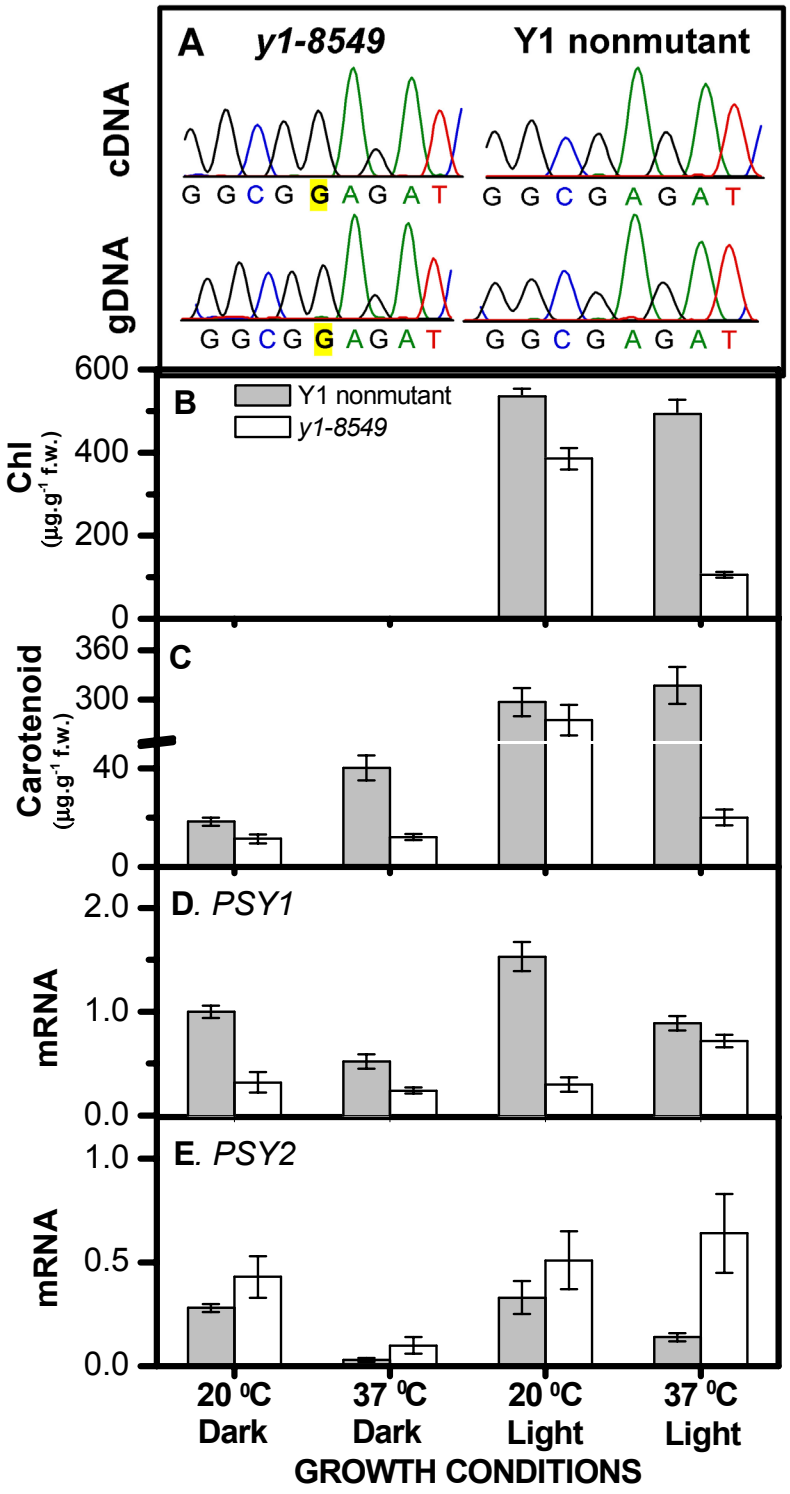

A
y1-8549
Y1 nonmutant
cDNA
gDNA
G G C G G A G A T
G G C G A G A T
B
Y1 nonmutant
y1-8549
Chl (μg.g-1 f.w.)
600
400
200
0
C
Carotenoid (μg.g-1 f.w.)
360
300
40
0
D. PSY1
mRNA
2.0
1.0
0.0
E. PSY2
mRNA
1.0
0.5
0.0
20 °C Dark
37 °C Dark
20 °C Light
37 °C Light
GROWTH CONDITIONS

Figure 3-9

Table 3-1. Chlorophyll, carotene and xanthophylls analysis of wild type and y1-8549 mutant**.**

	Wild type			*y1-8549*		
	Chlorophyll ($\mu g.g^{-1}$ f.w.)	Carotene ($\mu g.g^{-1}$ f.w.)	Xanthophylls[a] ($\mu g.g^{-1}$ f.w.)	Chlorophyll ($\mu g.g^{-1}$ f.w.)	Carotene ($\mu g.g^{-1}$ f.w.)	Xanthophylls[a] ($\mu g.g^{-1}$ f.w.)
20 ^{0}C dark	n.a.	2,10 ± 0,04	16,30 ± 1,66	n.a.	0,51 ± 0,05	10,89 ± 1,75
37 ^{0}C dark	n.a.	2,56 ± 0,17	37,74 ± 4,86	n.a.	0,61 ± 0,09	11,51 ± 1,19
20 ^{0}C light	535.31 ± 19.16	119,31 ± 9,49	177,40 ± 7,70	385,42 ± 26,11	119,24 ± 6,37	155,30 ± 12,31
37 ^{0}C light	493.24 ± 34.64	142,81 ± 11,53	174,27 ± 10,97	105.89 ± 7.25	3,72 ± 0,86	16,40 ± 2,28

a. Xanthophylls include lutein, neoxanthin, violaxanthin and antheraxanthin

Table 3-2. Primers for sequence analysis of maize *PSY1*genomic DNA and cDNA isolated from *y1-8549* and *y1-602c* mutants

Analysis of genomic DNA Segments of the maize *PSY1* of *y1-8549* and *y1-602c* mutants		
Region	Sequence Range	Primer sequences
1	1344-2472	# 217 GATGCCCAATGCCAAGAAATAACC # 344 GGCGCGGCGCCGCTCCTCTGTC
2	2201-3246	#509 GCATTGCTCAAACGCCAG #519 TCAGAGAGAGCGGCATCAAG
3	3229-5016	#257 TGATGCCGCTCTCTCTGATA #657 TCATCAAGGCCTCCTGATGTT
Analysis of cDNA Segments of the maize *PSY1* of *y1-8549* and *y1-602c* mutants		
1	Whole ORF	#627 ATGGCCATCATACTCGTACGAG #657 TCATCAAGGCCTCCTGATGTT

CHAPTER 4

MAIZE *Y9* ENCODES A PRODUCT ESSENTIAL FOR 15-*cis* ζ-CAROTENE ISOMERIZATON[2]

4.1 Abstract

Carotenoids are a diverse group of pigments found in plants, fungi, and bacteria. They serve essential functions in plants and provide health benefits for humans and animals. In plants, it was thought that conversion of the C40 carotenoid backbone, 15-*cis* phytoene, to *all-trans* lycopene, required two desaturases (PDS and ZDS) and a 'carotene isomerase'(CRTISO) plus light mediated photoisomerization of the 15-*cis* double bond; in contrast, bacteria employ one enzyme, CRTI, to provide the same geometrical isomer required by downstream enzymes. Characterization of the maize *y9* locus has brought to light a new isomerase required in plant carotenoid biosynthesis. I report that maize *Y9* encodes a factor required for isomerase activity upstream of CRTISO, which I term Z-ISO, an activity that catalyzes the *cis* to *trans* conversion of the 15-*cis* bond in 9,15,9'-tri-*cis*-ζ-carotene, the product of PDS, to form 9,9'-di-*cis*-ζ-carotene, the substrate of ZDS. I show that recessive *y9* alleles condition accumulation of 9,15,9'-tri-*cis*-ζ-carotene in dark-grown tissues, such as roots and etiolated leaves, in contrast to accumulation of 9,9'-di-*cis*-ζ-carotene in a ZDS mutant, *vp9*. I also identify a locus in Euglena which is similarly required for Z-ISO activity. These data, taken together

2 This chapter is a revision of Li *et al*, (2007) Plant Physiol. 144: 1181-1189 with the permission from the publisher.

with the geometrical isomer substrate requirement of ZDS in evolutionarily distant plants, suggest that Z-ISO activity is not unique to maize but that it will be found in all higher plants. Further analysis of this new gene-controlled step is critical to understanding the regulation of this essential biosynthetic pathway.

4.2 Introduction

Carotenoids are a diverse group of more than 750 naturally occurring pigments found in plants, fungi and bacteria (Britton et al., 2004). In higher plants, carotenoids serve as accessory pigments in photosynthesis and as photoprotectors at high light intensities. Apocarotenoids, carotenoid degradative products, are signals in plant development and in stress responses; their roles include extracellular rhizosphere signals that attract beneficial fungi and damaging parasitic plants that have opposite effects on plant yield (Milborrow, 2001; Bouvier et al., 2003a; Booker et al., 2004; Schwartz et al., 2004; Simkin et al., 2004; Castillo et al., 2005; Matusova et al., 2005; Moise et al., 2005; Nambara and Marion-Poll, 2005). Increasing interest has also been placed on carotenoid content and composition of food crops, because of the manifold importance of carotenoids in human health (Fraser and Bramley, 2004; Wurtzel, 2004; Quinlan et al., 2007).

In plant cells, carotenoids are synthesized in plastids from isoprenoid precursors through reactions catalyzed by nuclear encoded enzymes (DellaPenna and Pogson, 2006). The first committed step, mediated by phytoene synthase, is condensation of two

molecules of geranylgeranyl pyrophosphate to form 15-*cis* phytoene, containing a central 15-15' *cis* double bond (Beyer et al., 1985; Dogbo et al., 1988; Misawa et al., 1994). A four-step enzymatic desaturation of 15-*cis* phytoene to *all-trans*-lycopene also requires an electron transport chain (Mayer et al., 1990). Lycopene cyclases then catalyze ring formation at both ends of *all-trans* lycopene to form carotenes which can be further hydroxylated to produce xanthophylls (Kim and DellaPenna, 2006; Quinlan et al., 2007).

In bacteria, four desaturation steps from 15-*cis* phytoene to *all-trans* lycopene are mediated by a single enzyme, CrtI (Linden et al., 1991). In contrast, plants employ two desaturases, phytoene desaturase (PDS), which forms *trans* double bonds at 11 and 11', concomitant with *cis* bond formation at existing 9 and 9' double bonds, while ζ-carotene desaturase (ZDS) forms *cis* double bonds at 7 and 7' (Breitenbach and Sandmann, 2005) (**Fig. 4-1**). In addition, plant desaturation steps require supplementary isomerization reactions to produce acceptable geometrical isomer substrates for the desaturases and for the following lycopene cyclization steps (Beyer et al., 1989). Such differences in isomerization capacities of the plant and bacterial desaturases are important considerations for metabolic engineering of carotenoid content in food crops and in influencing biological activities of plant-derived geometrical isomers, including intestinal absorption and localization (Krinsky et al., 1990; Osterlie et al., 1999; Bjerkeng and Berge, 2000; Holloway et al., 2000; Patrick, 2000).

There has been confusion in the literature regarding the required number of carotene

isomerases needed in plant carotenoid biosynthesis, especially since reports of cloning of "the carotene isomerase gene" encoding "CRTISO", from cyanobacteria and plants (Breitenbach et al., 2001; Masamoto et al., 2001; Isaacson et al., 2002; Park et al., 2002). Early *in vitro* studies using daffodil chromoplasts (Beyer et al., 1989) suggested that further progression in the plant carotenoid biosynthetic pathway beyond ζ-carotene (e.g. ζ-carotene desaturation and onwards), required an isomerase activity and only the 15-*cis* position was recognized as an isomerase target. Expression of Arabidopsis PDS and ZDS in *E. coli* also revealed a missing plant factor required for ζ-carotene desaturation that could be replaced by photoisomerization; ζ-carotene accumulated instead of the predicted prolycopene, unless cells were exposed to light (Bartley et al., 1999). In studies of the coupled maize desaturases, it was proposed that there was a need for either two companion isomerases or a single isomerase having multiple substrates (Matthews et al., 2003). In an effort to identify the "missing isomerase gene," research groups looked at a number of pathway mutants that exhibited accumulation of atypical geometrical isomers. The tomato *Tangerine* locus, for which recessive alleles condition accumulation of prolycopene (poly *cis* lycopene), led to cloning of the corresponding gene that encoded what was thought to be the long sought-after "Carotene Isomerase," and it was so named as CRTISO (Isaacson et al., 2002). Homologs were also found in Arabidopsis and cyanobacteria (Breitenbach et al., 2001; Masamoto et al., 2001; Park et al., 2002).

When CRTISO (Isaacson et al., 2004) was assayed, it was found that isomerase activity

followed, rather than preceded, ζ-carotene desaturation which was inconsistent with earlier biochemical data indicating isomerization was needed prior to ζ-carotene desaturation; CRTISO was shown to be specific for adjacent double bonds at 7, 9 and 7',9' positions needed to convert prolycopene to *all-trans* lycopene, but it did not isomerize the single 15-15' *cis* double bond that was required for producing the proper substrate for ZDS (Beyer et al., 1989; Bartley et al., 1999; Matthews et al., 2003). Later, it was unequivocally demonstrated that in biosynthesis of prolycopene from phytoene, the product of PDS was 9,15,9'-tri-*cis*-ζ-carotene, whereas the substrate for the following desaturase, ZDS, was 9,9'-di-*cis*-ζ-carotene (Breitenbach and Sandmann, 2005). Therefore, isomerization of the 15-*cis* position of ζ-carotene was clearly required to convert the PDS product to a suitable ZDS substrate. The *in vitro* assays of CRTISO (Isaacson et al., 2004), no longer supported the possibility of a multi-substrate enzyme, but suggested that indeed a second isomerase might exist. While light might compensate for this requirement in light-exposed tissue, there was still the question of whether any genetic locus was responsible for such activity in the dark or in dark-grown tissue. In considering the phenotype associated with a lesion affecting such a second isomerase, which I will call Z-ISO (15 *cis*-ζ-carotene isomerase), it seemed that mutants would be predicted to accumulate the PDS product (and Z-ISO substrate), 9,15,9'-tri-*cis*-ζ-carotene, which could be shown to photoisomerize to 9,9'-di-*cis*-ζ-carotene. In comparison, ZDS mutants should accumulate 9,9'-di-*cis*-ζ-carotene in a genetic background conditioning functional Z-ISO. Unlike ZDS mutants that are lethal because they accumulate ζ-carotene under both light and dark

conditions, Z-ISO mutants should be nonlethal because the mutant phenotype only manifests in the dark and not in light-grown tissues. Therefore, ZDS and Z-ISO mutants should both condition accumulation of ζ-carotene in the dark but only ZDS mutants will show accumulation in light grown tissues and exhibit an albino phenotype. I therefore searched for ζ-carotene accumulating mutants in maize (Wurtzel, 2004) to test for the presence of the predicted Z-ISO geometrical isomer substrate that is distinguishable from the ZDS substrate. I identified the maize *y9* locus as a candidate and demonstrated that recessive alleles condition accumulation of 9,15,9'-tri-*cis*-ζ-carotene in "dark" tissues. This finding supports the role of an additional genetic locus in plants that controls an upstream carotene isomerase activity, Z-ISO, which is independent of CRTISO. Comparison of the *y9* phenotype with that of ζ-carotene accumulating mutants in other species shows that Z-ISO activity is not limited to maize.

4.3 Results

4.3.1 Identification of a maize candidate Z-ISO mutant

Numerous mutations affecting maize carotenoid biosynthesis, including phytoene desaturation, have been reported (Wurtzel, 2004). Endosperm phenotypes include white endosperm (compared to the yellow) and vivipary caused by an absence of abscisic acid, a carotenoid cleavage product promoting seed dormancy. Absence of leaf carotenoids cause lethality and manifest as albino tissue due to pleiotropic effects on chloroplast

biogenesis. Pathway blocks are also associated with accumulation of pathway intermediates. Accumulation of ζ-carotene was predicted for mutations in genes controlling either ZDS or a putative Z-ISO; such accumulation was found for maize *vp9* and *y9* mutant endosperms (Robertson, 1975a). These mutants had otherwise dissimilar phenotypes; *y9* homozygous mutants were nonlethal recessives affecting only endosperm and leaves remained green, while *vp9* mutants were lethal recessives affecting both endosperm and leaf tissues and plants were albino. When the maize ZDS gene was isolated (Matthews et al., 2003), it was mapped to *vp9* on chromosome *7* and not to *y9* on chromosome *10* (Robertson, 1975b); also, y9 had no affect on ZDS transcript accumulation, indicating that *y9* did not encode ZDS or control ZDS transcript levels. Given that a block in isomerase activity would be light-complemented, I predicted that a carotenoid isomerase mutant should have an almost normal leaf phenotype as seen for *y9* and not an albino phenotype as in *vp9*. Therefore, it was conceivable that *y9* might encode CRTISO, although it did not accumulate prolycopene as did other CRTISO mutants (Isaacson et al., 2002). Furthermore, mapping of *CRTISO* to chromosomes *2* and *4* (Wurtzel, 2004) (Conrad, Brutnell, and Wurtzel, unpublished) indicated that *y9* did not encode CRTISO, because *y9* mapped to chromosome *10*. While *y9* was not associated with ZDS or CRTISO, it remained a good candidate to consider for a possible genetic locus that might encode a factor necessary for putative Z-ISO activity. To validate that *y9* was necessary for Z-ISO activity (and to further prove that light was insufficient and that a genetic locus was involved), it was necessary to demonstrate that *y9* conditioned accumulation specifically of 9,15,9'-tri-*cis*-ζ-carotene in contrast to accumulation of

9,9'-di-*cis*-ζ-carotene in a ZDS mutant, such as *vp9*. Furthermore, while prior reports suggested that *y9* had no leaf phenotype (Janick-Buckner et al., 2001), I predicted that light was photoisomerizing the accumulated 9,15,9'-tri-*cis*-ζ-carotene intermediate to relieve the pathway block such that plants appeared "green." Therefore, it was necessary to demonstrate that in etiolated leaf tissue ζ-carotene accumulated because of the absence of photoisomerization. In contrast, photoisomerization would have no effect on the phenotype of a ZDS mutant.

4.3.2 HPLC assay and standards for ζ-carotene geometrical isomers

In order to distinguish the ζ-carotene isomers appearing in *y9* and *vp9* endosperm tissues, ζ-carotene isomers were extracted from *E. coli* cells containing pACCRT-EBP for use as HPLC standards (Matthews et al., 2003). It had been previously shown that expression of a plant PDS resulted in accumulation of 9,15,9'-tri-*cis*-ζ-carotene which has a characteristic spectrum including absorbance at 297 nm and which could be photoisomerized to 9,9'-di-*cis*-ζ-carotene, which elutes slower and whose spectrum lacks absorbance at 297 nm (Bartley et al., 1999; Isaacson et al., 2004; Breitenbach and Sandmann, 2005). Following HPLC conditions used earlier (Isaacson et al., 2004), in combination with photoisomerization to demonstrate isomer conversion, I was able to replicate separation and spectral identification of the tri-*cis* and di-*cis* isomers as follows. Based on spectrum and retention times, the carotenoids extracted from dark-grown *E. coli*

cells contained peaks 3a and 3b, ζ-carotene isomers, as well as a small amount of phytofluene (peak 2) and phytoene (peak 1) (**Fig. 4-2**), which was consistent with earlier reports (Bartley et al., 1999; Matthews et al., 2003; Isaacson et al., 2004; Breitenbach and Sandmann, 2005). Of the three ζ-carotene peaks, only peak 3b showed absorption at 297 nm (see spectra, **Fig. 4-2 top**), consistent with 9,15,9'-tri-*cis*-ζ-carotene (Bartley et al., 1999; Isaacson et al., 2004). When cells were exposed to light (as described in methods), the ratio of peak 3b to 3a changed from 3:1 in dark-grown cultures (**Fig. 4-2**, dashed trace) to 2:5 in light-exposed cultures (**Fig. 4-2**, solid trace), as previously seen for photoisomerization of 9,15,9'-tri-*cis*-ζ-carotene to 9,9'-di-*cis*-ζ-carotene (Bartley et al., 1999; Isaacson et al., 2004). The ζ-carotene isomer, peak 3b, was converted to the ζ-carotene isomer, peak 3a, due to photoisomerization of the 15-*cis* double bond in tri-*cis* -ζ-carotene to form di-*cis*-ζ-carotene. In addition to matching spectra and photoisomerization-induced changes in peak heights, relative retention times also matched earlier reports using the same HPLC separation system (Isaacson et al., 2004). Based on this replication, I therefore identified peak 3a as 9,9'-di-*cis*-ζ-carotene and peak 3b as 9,15,9'-tri-*cis*-ζ-carotene. A third ζ-carotene isomer, peak 3c, was also identified in the carotenoid extract of light-exposed cultures and tentatively identified as all-trans ζ-carotene based on its retention time being 0.5 min earlier than that of 9,15,9'-tri-*cis*-ζ-carotene (Isaacson et al., 2004).

4.3.3 Carotenoid compositions in endosperm tissue of *y9* and *vp9* mutants

Bacterial standards described above were used to identify the specific ζ-carotene isomers accumulating in homozygous *y9* and *vp9* mutant endosperms (see **Fig. 4-3** for chromatograms and Table 1 for corresponding peak quantifications). I compared spectral fine structure including % III/II peak ratio, presence of a *cis* peak at 297 nm and retention time with those of the standards and prior values (Isaacson et al., 2004). In *y9* endosperm, the major carotenoids were 40.4% of 9,15,9'-tri-*cis*-ζ-carotene as compared to 14.1% of 9,9'-di-*cis*-ζ-carotene, and 15% phytoene, 13.2% lutein, 11.8% phytofluene, and 3.0% zeaxanthin. In comparison, endosperms of the *Zds* mutant, *vp9*, showed the reverse geometrical isomer composition with 55.7% of 9,9'-di-*cis*-ζ-carotene and 17.6% of 9,15,9'-tri-*cis*-ζ-carotene. Taken together, these data show that *y9* and *vp9* accumulate different geometrical isomers of ζ-carotene. The carotenoid biosynthetic pathway in *y9* is blocked in the *cis* to *trans* conversion of the 15-*cis* bond in 9,15,9'-tri-*cis*-ζ-carotene and therefore accumulates 9,15,9'-tri-*cis*-ζ-carotene. In contrast, *vp9* tissue has isomerase activity needed to convert 9,15,9'-tri-*cis*-ζ-carotene to 9,9'-di-*cis*-ζ-carotene, but accumulates 9,9'-di-*cis*-ζ-carotene which indicates it is blocked in desaturation of this compound by ZDS. The accumulation of lutein and other downstream carotenoids in *y9* endosperms might be due to photoconversion of 9,15,9'-tri-*cis*-ζ-carotene in endosperms of field-grown plants. In contrast to *y9*, *vp9* endosperms did not accumulate any detectable xanthophylls as expected for a mutation in a desaturase as compared to an isomerase (**Fig.**

4-3).

4.3.4 ζ-carotene isomers in etiolated leaf and root tissues of *y9* and *vp9* mutants

If the *y9* locus indeed controls expression of a new isomerase, then a lesion in the gene should manifest as 9,15,9'-tri-*cis*-ζ-carotene accumulation in dark grown plants, when light is not available to compensate for absence of the *cis-trans* conversion; *y9* leaves of light-grown plants have been reported not to accumulate ζ-carotene (Janick-Buckner et al., 2001). Therefore, I germinated plants in the dark and collected etiolated leaves and roots, extracted carotenoids and analyzed by HPLC (**Fig. 4-3, Table 4-1**). In both etiolated leaves and roots of homozygous *y9* plants, 9,15,9'-tri-*cis*-ζ-carotene accumulated; no 9,9'-di-*cis*-ζ-carotene was detected nor were there any other downstream xanthophylls. This suggests that the carotenoid biosynthetic pathway in dark-grown *y9* is completely blocked at this step, thereby preventing downstream synthesis leading to xanthophylls typically found in either light-grown *y9* or in dark-grown maize seedlings carrying the dominant *Y9* allele (data not shown). In comparison, homozygous *vp9* roots and etiolated leaves accumulated mainly 9,9'-di-*cis*-ζ-carotene. No xanthophylls were observed in carotenoid extracts from etiolated leaves or roots of either mutant as compared to the profile in *y9* endosperm. The absence of downstream carotenoids in dark-grown tissues further supports the hypothesis that the xanthophylls detected in *y9* endosperm were due to some photoconversion in the field-grown plants. As predicted for a Z-ISO lesion,

9,15,9'-tri-*cis*-ζ-carotene was found to accumulate in dark-grown tissues of *y9* plants. These results establish that *y9* does interfere with carotenoid biosynthesis in the absence of light in leaves and roots; the only reason that *y9* plants are normally green is that light compensates for the lesion.

4.3.5 *In vitro* photoisomerization of *y9* carotenoids

To further confirm identification of the ζ-carotene isomers accumulating in dark grown *y9* roots, *in vitro* photoisomerization was used to demonstrate conversion of 9, 15, 9'-tri-*cis*-ζ-carotene to 9,9'-di-*cis*-ζ-carotene using carotenoid extracts from underground roots of field-grown homozygous *y9* plants. Carotenoid extracts were illuminated for 2 hours with white light (50 μmol $m^{-2}s^{-1}$ photon flux) and the carotenoid composition before and after illumination was examined by HPLC (**Fig. 4-4**). Before illumination, the ratio of 9, 15, 9'-tri-*cis*-ζ-carotene (peak 3b) to 9,9'-di-*cis*-ζ-carotene (peak 3a) was 3:2. (**Fig. 4-4**, lower chromatogram trace). After 2 hours of illumination, di-*cis*-ζ-carotene increased to 72% of the total ζ-carotene isomers (**Fig. 4-4**, upper chromatogram trace). The photoconversion of peak 3b to 3a is consistent with identification of peak 3b as 9,15, 9'-tri-*cis*-ζ-carotene and peak 3a as 9,9'-di-*cis*-ζ-carotene. In summary, I demonstrated that roots accumulate 9, 15, 9'-tri-*cis*-ζ-carotene which was shown to be photoisomerizable to the 9,9'-di-*cis*-ζ-carotene isomer.

4.3.6 Identification of other putative genetic loci needed for Z-ISO activity

It is unlikely that Z-ISO activity is only required for maize carotenoid biosynthesis, but it is likely ubiquitous in all plants and cyanobacteria, given that both dicot and monocot as well as cyanobacterial PDS enzymes produce 9,15,9'-tri-*cis*-ζ-carotene, which is not the isomeric substrate of ZDS, as shown here for maize and previously reported for Arabidopsis, Capsicum, and Synechococcus (Bartley et al., 1999; Isaacson et al., 2004; Breitenbach and Sandmann, 2005). To identify genetic loci in other photosynthetic organisms that may be similarly required for Z-ISO activity, I re-examined earlier studies of mutants that exhibited blocks in ζ-carotene desaturation as such blocks might alternatively represent lesions in ZDS or Z-ISO (Table 2). Specific details on how Table 2 was developed are provided (see Methods). Nine mutants were examined, all of which exhibit significant accumulation of ζ-carotene (see % ζ-carotene isomers), and some of which have already been associated with a structural locus for ZDS or CRTISO. To distinguish between ZDS and isomerase mutants, I grouped the mutants into two classes, *light non-responsive* (ZDS mutants), where light has no effect on the phenotype, or *light-responsive* (isomerase mutants), where light can reverse the phenotype by compensation for a genetic lesion.

The first class included maize *vp9* and sunflower *nondormant-1* (*nd-1)*; mutants in this class have been shown to be light-nonresponders and therefore albino, which is typical for ZDS lesions based on phenotype and in these cases, supported by gene analysis

(Matthews et al., 2003; Conti et al., 2004). Members of this group show a low ratio of tri-*cis* /di-*cis* ζ-carotene isomers and accumulate 9,9'-di-*cis*-ζ-carotene, the ZDS substrate.

The second class of mutants have been shown to be light-responders; photoisomerization releases a pathway block and hence normal carotenoid accumulation in the light can be observed (e.g. in the case of plants, they are green and viable). This class could alternatively represent either CRTISO of Z-ISO isomerase mutants. To distinguish between the two isomerase mutant types, I predicted that dark-grown CRTISO mutants should accumulate prolycopene or proneurosporene because there is no block in tri-*cis* to di-*cis* isomerization, as evidenced by a lower tri-*cis* to di-*cis* ζ-carotene isomer ratio. On the other hand, dark-grown Z-ISO mutants will not accumulate prolycopene (or proneurosporene), but instead accumulate a high ratio of tri-*cis* to di-*cis* ζ-carotene isomers due to the block in the *cis* to *trans* conversion which is otherwise released by photoisomerization.

The CRTISO class included Arabidopsis *ccr2* (Park et al., 2002), tomato *tangerin*e (Isaacson et al., 2002), *Synechocystis* sp. PCC6803 *sll003* (Breitenbach et al., 2001; Masamoto et al., 2001), *Scenedesmus* C-6D (Ernst and Sandmann, 1988) and *Scenedesmus* PG1 (Britton and Powls, 1977; Britton et al., 1977). Among them, *ccr2*, *tangerine* and *sll003*, have all been shown to represent mutations in *CRTISO* or *crtH* genes. With the exception of PG1, these mutants all accumulated prolycopene and proneurosporene, which is expected for blocks in CRTISO activity as reported (Isaacson et al., 2002). These

mutants, including PG1, all share a low tri-*cis* to di-*cis* ζ-carotene isomer ratio as predicted for a CRTISO mutant that has functional Z-ISO activity. PG1 is included in this class because it has a close to normal pigment composition in light-grown cells, indicating it carries a mutation in an isomerase because it can be light complemented; were it a lesion in ZDS, it would exhibit ζ-carotene accumulation even in light-grown cells. The low tri-*cis* to di-*cis* ζ-carotene isomer ratio found in dark-grown PG1 cells also suggests that the mutation blocks CRTISO and not Z-ISO activity, which would otherwise have given a high ratio.

The Z-ISO class of light responders included the *Euglena gracillis* mutant W_3BUL (Cunningham and Schiff, 1985) and maize *y9*. As expected for a block in Z-ISO activity, neither mutant accumulated prolycopene nor proneurosporene; both mutants exhibited a high ratio of tri-*cis* to di-*cis* ζ-carotene isomers, for which the tri-*cis* isomer was found to be photoconvertible to the di-*cis* isomer. This biochemical evidence suggests that both W_3BUL and *y9* loci affect Z-ISO and not CRTISO activity, and that Z-ISO activity is not limited to plants but is also present in a photosynthetic protist.

4.4 Discussion

I demonstrated that isomerization of 9, 15, 9'-tri-*cis*-ζ-carotene to 9,9'-di-*cis*-ζ-carotene is not simply light-mediated, but that it requires the product of a

nuclear-encoded gene. I showed that in the absence of light, the 9, 15, 9'-tri-*cis*-ζ-carotene isomer accumulates in etiolated leaves and roots of maize plants carrying the recessive *y9* allele, in comparison to a normal carotenoid composition in light-exposed *y9* leaves (Janick-Buckner et al., 2001). Therefore, an activity, which I termed Z-ISO, is essential in dark-exposed tissues. However, even in light-exposed *y9* tissues, there is evidence that Z-ISO activity is needed. Some striping seen in light-exposed *y9* plants did lead to reduced carotenoids in pale-green regions (Janick-Buckner et al., 2001), a phenotype exaggerated by cold treatment (Robertson, 1975b) or temperature fluctuations (Janick-Buckner et al., 2001). This suggests that photoisomerization is not entirely efficient in overcoming the pathway lesion associated with the recessive *y9* allele and that Z-ISO activity may be required in photosynthetic tissues when plants are subjected to abiotic stress.

The Z-ISO activity, which I demonstrated to be genetically controlled in maize, is not unique to this species, as I deduced by examining a collection of ζ-carotene accumulating mutants in multiple species. By applying a standard convention for naming the ζ-carotene isomers, I showed that the mutants fell into three classes of ζ-carotene accumulating mutants: ZDS, CRTISO and Z-ISO. The ZDS class contains two plant genes, the sunflower *nd-1* and maize *vp9* loci; CRTISO includes mutants from plants, a cyanobacterium and a green alga; the Z-ISO mutants include those in maize (*y9*) and Euglena. Therefore, unlike bacteria that encode one four-step desaturase, plants and other photosynthetic organisms possess two desaturases (PDS and ZDS) and two isomerases (Z-ISO and CRTISO) which have alternating functions in the biosynthetic pathway.

Z-ISO functions upstream of CRTISO and I ruled out *y9* as encoding CRTISO, although I don't as yet know whether *y9* encodes Z-ISO or regulates its expression. It is unlikely that *y9* encodes a factor that alters CRTISO activity, because if it did, then CRTISO mutants would not accumulate prolycopene, but instead would accumulate 9, 15, 9'-tri-*cis*-ζ-carotene.

I named the isomerase activity that is upstream of CRTISO, Z-ISO, to reflect isomerization of the 15-*cis* double bond in ζ-carotene. I don't know if Z-ISO will act on 15-*cis* bonds only in ζ-carotene, or also on 15-*cis* phytoene, or the intermediate 9,15-*cis* phytofluene. It is unlikely that 15-*cis* phytoene is a substrate, as the primary phytoene isomer that was detected in plants is the 15-*cis* isomer and not *trans* (Jungalwalab and Porter, 1965). The *y9* mutant phenotype also does not support 15-*cis* phytoene as being a substrate as it is the tri-*cis*-ζ-carotene isomer that predominates in accumulation and not 15-*cis* phytoene that would accumulate were it the preferred substrate. Therefore, perhaps Z-ISO activity requires the unique conformation of the 15-*cis* double bond in ζ-carotene which also has the 9,9' *cis* double bonds, uniquely produced during PDS desaturation. If one looks at the structure of 9,15, 9' tri-*cis* ζ-carotene, its conformation is quite distinct from that of 15-*cis* phytoene, which may be significant in substrate binding and recognition of the 15-*cis* double bond.

The naming of CRTISO, was optimistic at the time of its discovery and suggested that it catalyzed all carotene isomerizations, which I know now not to be the case. If one looks further at the isomerase substrates it becomes clear why CRTISO was not the sole carotene isomerase. CRTISO isomerizes adjacent *cis* double bonds (Isaacson et al., 2004), whereas the Z-ISO substrate is a single *cis* double bond, making for a very different substrate structure. Therefore it is likely that Z-ISO is different from CRT-ISO in terms of protein structure which may explain why there has been no success in showing Z-ISO activity for any of the plant CRTISO homologs.

Characterization of the maize *y9* locus has brought to light a new factor required in plant carotenoid biosynthesis. Future characterization of this locus will lead to isolation of all of the components needed for plant desaturation/isomerization. Biogenesis and regulation of the complex plant desaturase/isomerase metabolon is an important and future problem to address; elucidation will have direct impact on metabolic engineering of this pathway in plants.

4.5 Materials and Methods

4.5.1 Plant and bacterial materials

Maize ζ-carotene desaturase mutant *vp9-Bot100* (Maize Co-op 706B) and mutant *y9* (Maize Co-op X07C) were used in this study. For etiolated leaves and roots, seeds were

geminated and grown in the dark for two weeks at 25 ^{0}C. Endosperms were collected at 20 days after pollination from field grown plants as described (Gallagher et al., 2004). The plasmid pACCRT-EBP, carrying bacterial *crtE*, *crtB* and maize *Pds* genes confers accumulation of ζ-carotene isomers in *E. coli* and was used to produce ζ-carotene standards for HPLC (Matthews et al., 2003); transformed cells were incubated for 48 hours at 37 ^{0}C under darkness or in the light (50 μmol m^{-2} s^{-1} photon flux) prior to pigment extraction as described below.

4.5.2 Carotenoid extraction

Carotenoids were extracted from approximately 100 mL bacterial culture or 1 g endosperm, etiolated leaf or root tissues taken from dark grown plants as described (Kurilich and Juvik, 1999), dissolved in 1 mL methanol, and 50 μl injected for HPLC analysis.

4.5.3 Photoisomerization of 9,15,9'-tri-*cis*-ζ-carotene to 9,9'-cis ζ-carotene

Carotenoids were extracted (Kurilich and Juvik, 1999) from ~ 6g underground roots taken from field-grown plants and dissolved in 1mL methanol. For *in vitro* photoisomerization of ζ-carotene isomers, samples were placed in a Petri dish and exposed for 2 hours to light (50 μmol $m^{-2}s^{-1}$ photon flux) prior to HPLC analysis.

4.5.4 Carotenoid analysis by HPLC

HPLC separation was carried out using a Waters (Millipore, Franklin, MA) HPLC system with a 2695 separation module, Empower 1 software (Waters), 996 photodiode array detector (Waters), and 717 autosampler. Gradient separation was conducted as described with slight modification for improved peak resolution using a Waters Spherisorb 5µm OSD1 analytical Column (5µ, 4.6 x 250 mm, Waters, MA) and Nucleosil C18 (5µ, 4 x 3.0 mm) guard column (Phenomenex, CA) (Isaacson et al., 2004). The mobile phase consisted of acetonitrile:water (9:1; A) and ethylacetate (B), at a constant flow rate of 1.6 mL/min with the following gradient: 100% to 80% A for 8 min; 80% to 65% A for 4 min, 65% to 45% A for 28 min and a final segment at 100% B. Identification of ζ-carotene isomers was based on comparison with spectra and elution time of authentic ζ-carotene isomers produced with expression of pACCRT-EBP and from published data (Beyer et al., 1989; Sandmann, 1991; Isaacson et al., 2004). Lutein and zeaxanthin were identified based on comparison with standards purchased from Sigma (Sigma, MO). Quantification was performed by integrating the peak areas using the Empower I software (Waters) with β-carotene as an internal control. All chromatograms are shown with each peak at its λ_{max} (Maxplot).

4.5.5 Geometrical isomer profiles of ζ-carotene accumulating mutants

To compare the various ζ-carotene mutants in the literature, I converted the previously reported data to a standard format. Earlier reports named the same isomers by different naming conventions. Therefore I used several criteria to identify 9,15,9'-tri-*cis*-ζ-carotene and 9,9'-di-*cis*-ζ-carotene based on the following properties: spectral fine structure including presence or absence of the 297 nm *cis* peak characteristic of 9,15,9'-tri-*cis*-ζ-carotene, potential for photoisomerization by light to the alternate isomer, and relative retention time. Table 2 contains data derived from this study for *vp9* and *y9* plus the following mutants:

Sunflower *nondormant1* (*nd-1)* (Conti et al., 2004): Like maize *vp9* (Matthews et al., 2003), *nd-1* is a mutant with a known lesion in the *Zds* gene, both of which exhibit an albino phenotype regardless of light. For all of the other mutants, light reverses the mutant phenotype. The percentage of carotenoids in *nd-1* were calculated based on data from low light treated plants (1 μmole m^{-2} s^{-1}) as provided in Table 1 (Conti et al., 2004).

Arabidopsis thaliana ccr2 (Park et al., 2002): This mutation was demonstrated to be in the CRTISO gene. The carotenoid composition data of etiolated *ccr2* leaf was taken directly from the paper. Included are "pro-ζ-carotene" which elutes slower than "*cis*-ζ-carotene," which they describe as having a spectrum appearing to be 15*Z* (*cis*),

indicating that these compounds represent 9, 9'- di-*cis* ζ-carotene and 9, 15, 9'- tri-*cis* ζ-carotene, respectively.

Tomato *(Lycopersicon esculentum) tangerine*: this mutation was demonstrated to be in the CRTISO gene; I used the carotenoid composition from dark-grown seedlings as presented in Table 2 (Isaacson et al., 2002).

Synechocystis sll0033: this mutation was shown to be in *crtH*, which is a homolog of the plant *CRTISO* gene (Breitenbach et al., 2001; Masamoto et al., 2001); I used the carotenoid composition from Table 1 (Masamoto et al., 2001). By identifying the photoisomerizable compounds described in the paper, I interpreted "*cis*-ζ-carotene" (which contained a 15-*cis* peak at 297 nm) to be 9, 15, 9'- tri-*cis* ζ-carotene and "all-*trans* ζ-carotene" (which lacked the 15-*cis* peak) to be 9, 9'- di-*cis* ζ-carotene.

Scenedesmus obliquus C-6D (Ernst and Sandmann, 1988): The percentage of each carotenoid in *Scenedesmus* mutant C-6D was calculated based on data presented in Table I (Ernst and Sandmann, 1988). The isomer 9, 9'-di-*cis* ζ-carotene is alternatively named "pro-ζ-carotene"; the isomer "*cis*-ζ-carotene II (?Z)" was later identified as 9, 15, 9'-tri-*cis* ζ-carotene (Breitenbach and Sandmann, 2005).

Scenedesmus obliquus PG1 (Britton and Powls, 1977; Britton et al., 1977): The

percentage of each carotenoid in PG1 is calculated based on column A of Table I, which lists the total amount of ζ-carotene without specific isomers quantified (Britton et al., 1977). In Table 1 of a second paper from the same laboratory, the isomers are quantified (Britton and Powls, 1977); ζ-carotene II (15-*cis*-ζ-carotene) and ζ-carotene-III (*all-trans*) (lacking a *cis* peak and most prevalent of the ζ-carotene isomer) represent 9, 15, 9'- tri-*cis* ζ-carotene and 9, 9'- di-*cis* ζ-carotene, respectively.

Euglena gracilis var. *bacillaris* W_3BUL (Cunningham and Schiff, 1985): The carotenoid composition of W_3BUL was obtained from Table I (Cunningham and Schiff, 1985). Light was shown to convert "*cis*-ζ-carotene I" (which contains a 15-*cis* bond) to "*trans*-ζ-carotene" (which lacks the *cis* spectral peak), indicating that these compounds represent 9, 15, 9'- tri-*cis* ζ-carotene and 9, 9'- di-*cis* ζ-carotene, respectively.

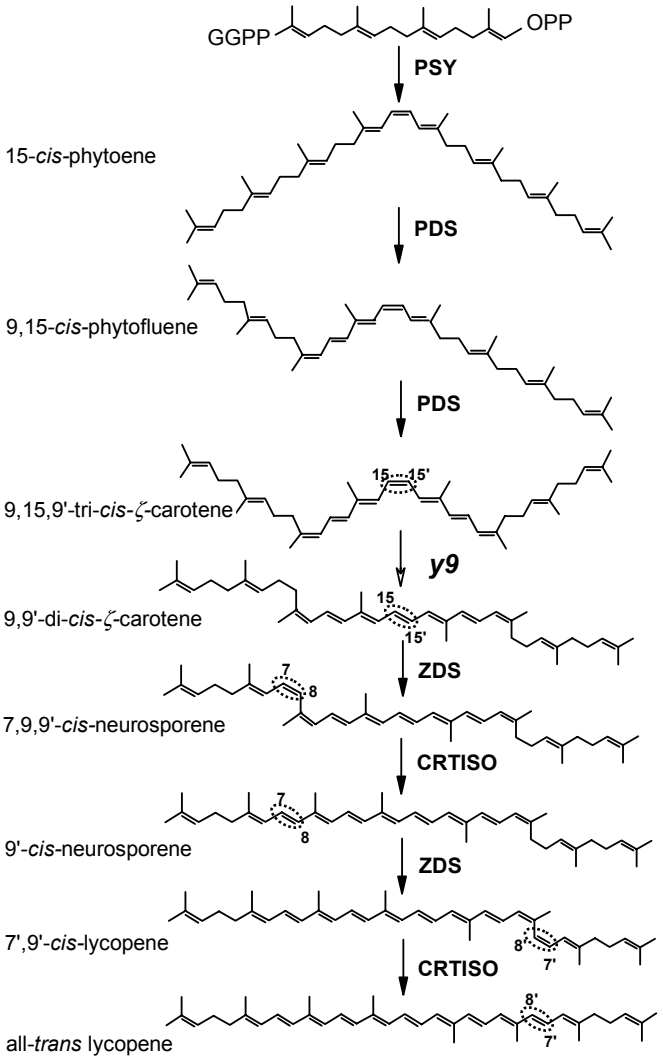


Figure 4-1. Proposed pathway of carotenoid biosynthesis in plants. CRTISO, Carotene isomerase; PDS, phytoene desaturase; PSY, Phytoene synthase; ZDS, ζ-carotene desaturase. Bonds isomerized by CRTISO and Z-ISO are circled. In maize *y9* mutant tissues, the step from tri-*cis*-ζ-carotene to di-*cis*-ζ-carotene is blocked.

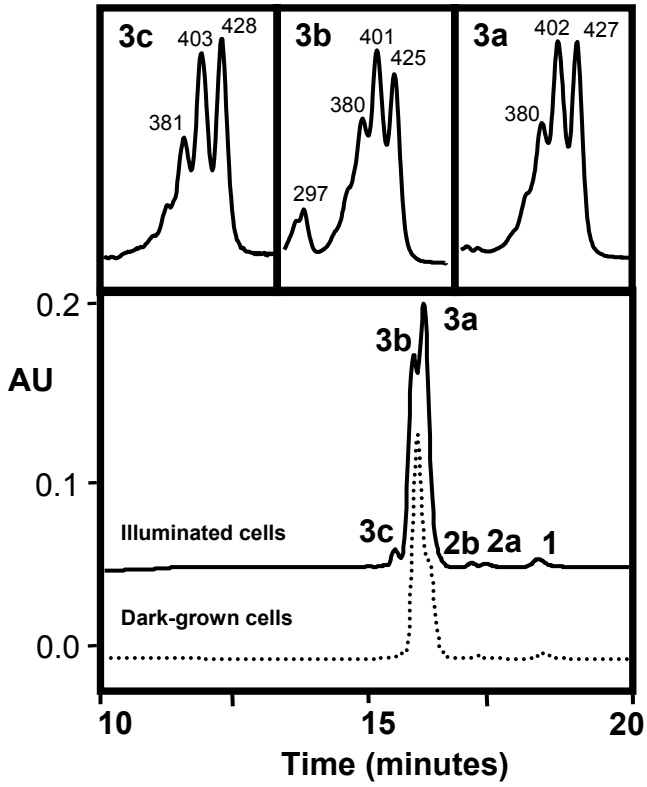


Figure 4-2. HPLC analysis of PDS enzymatic product, 9,15,9'-tri-*cis*-ζ-carotene, which is photoisomerized to 9,9'-di-*cis*-ζ-carotene. Top panel: Spectra of peaks identified in chromatogram shown in bottom panel. Bottom panel: HPLC chromatograms of carotenoids extracted from dark-grown (lower, dashed trace) or illuminated *E. coli* cells containing pACCRT-EBP plasmid (upper, solid trace). With light exposure, 9,15,9'-tri-*cis*-ζ-carotene (3b) was converted into 9,9'-di-*cis*-ζ-carotene (3a), as well as trace amounts of all-*trans*-ξ-carotene (3c). Peak1, phytoene; peak 2a and 2b, phytofluene isomers; peak 3a, 9,9'-di-*cis*-ζ-carotene; peak 3b, 9,15,9'-tri-*cis*-ζ-carotene; peak 3c, all-*trans*-ζ-carotene.

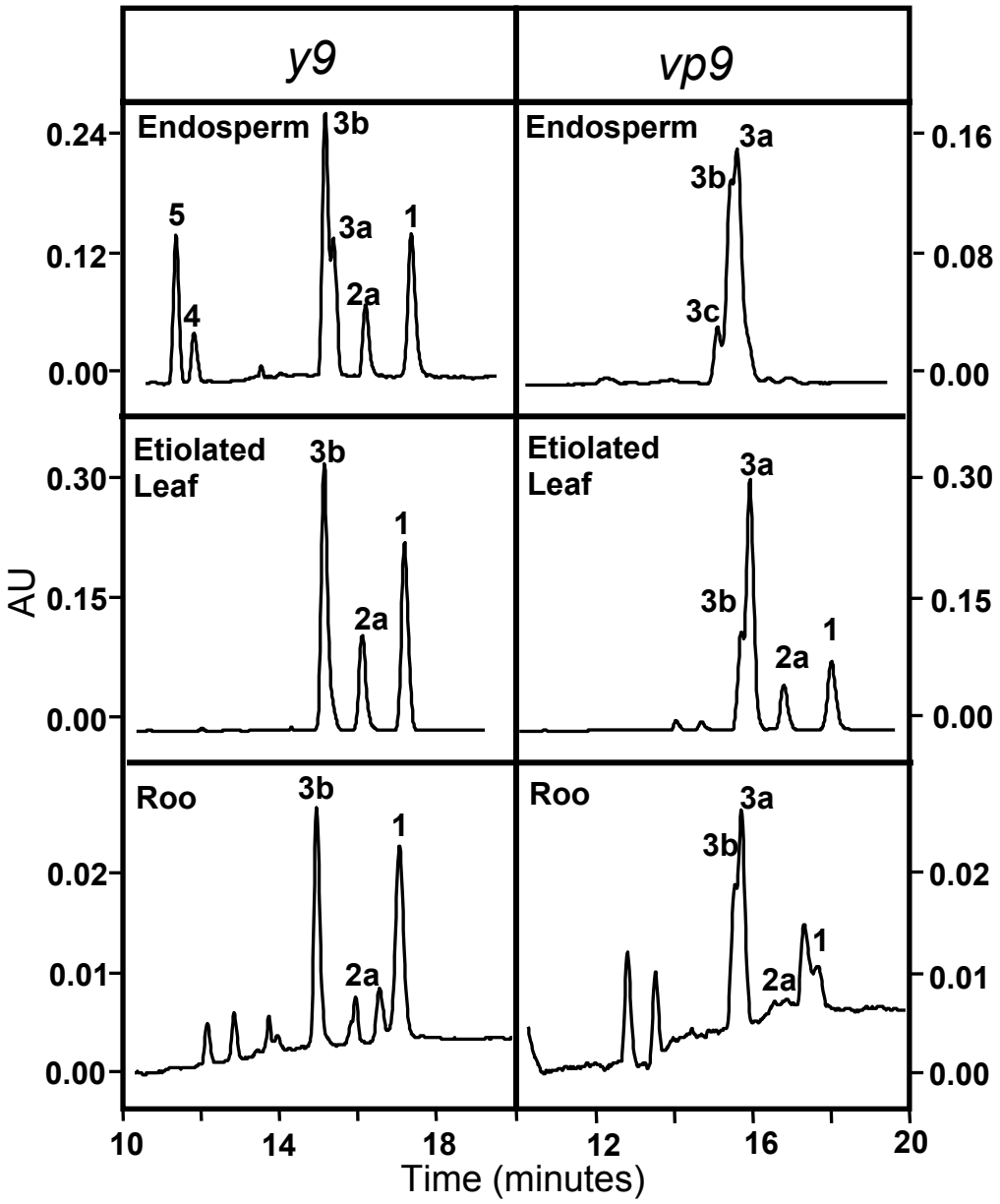


Figure 4-3. HPLC analysis of carotenoids extracted from endosperms, etiolated leaves or roots of *y9* or *vp9* mutants shows mutant-specific geometrical isomers. Etiolated leaves and roots were samples from dark-grown plants. Peak1, phytoene; peak 2a, phytofluene isomer; peak 3a, 9,9'-di-*cis*-ζ-carotene; peak 3b, 9,15,9'-tri-*cis*-ζ-carotene; peak 3c, all-*trans*-ζ-carotene; peak 4, zeaxanthin; peak 5, Lutein.

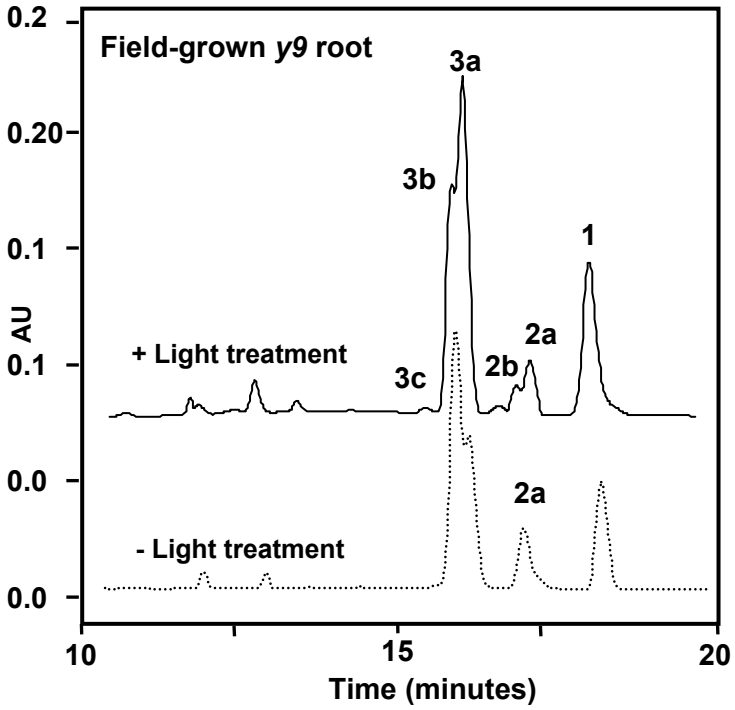


Figure 4-4. Carotenoid extracts from field-grown underground roots of *y9* plants before (lower, dashed trace) and after illumination (upper, solid trace). Light-exposure converted 9,15,9'-tri-*cis*-ζ-carotene (3b) into 9, 9'-di-*cis*-ζ-carotene (3a), as well as trace amount of all-*trans*-ζ-carotene (3c). Peaks are numbered as in figure 4-2.

Table 4-1. Carotenoid composition of y9 and vp9 mutant tissues (μg g^{-1})

	y9			*vp9*				
	Endosperm	Etiolated leaf	Root	Endosperm	Etiolated leaf	Root	Wavelength[a]	% III/II[b]
Phytoene	2.27 ± 0.14	13.0 ± 0.52	0.28 ± 0.01	-	4.06 ± 0.19	0.04 ± 0.00	276 <u>286</u> 298	0
Phytofluene	1.78 ± 0.07	5.54 ± 0.14	0.13 ± 0.01	0.06 ± 0.01	2.22 ± 0.05	0.04 ± 0.00	333 <u>349</u> 369	58
Tri-*cis*-ζ-carotene	6.07 ± 0.24	15.98 ± 0.38	0.30 ± 0.04	2.44 ± 0.06	4.17 ± 0.27	0.12 ± 0.01	(297) 380 <u>401</u> 425	75
Di-*cis*-ζ-carotene	2.12 ± 0.18	-	-	4.09 ± 0.13	13.18 ± 0.51	0.17 ± 0.01	380 <u>402</u> 427	98
all-trans ζ-carotene	-	-	-	0.61 ± 0.01	-	-	381 <u>403</u> 428	112
Lutein	1.98 ± 0.13	-	-	-	-	-	420 <u>446</u> 474	56
Zeaxanthin	0.45 ± 0.06	-	-	-	-	-	427 <u>452</u> 479	15

[a]Online absorbance spectrum taken during HPLC separation; underlined peak is highest peak (II); parentheses indicates additional *cis* peak. *in the case of phytoene, "peaks" I and III are actually shoulders.
[b]Fine structure of absorbance spectra expressed as relative height of longest wavelength peak (III) compared to middle peak (II). Value of zero indicates absence of peak III.
Values shown are averages and standard deviation for 3 samples.

Table 4-2. Geometrical isomers accumulating in dark-grown tissues of ζ-carotene accumulation mutants (% total carotenoids)

	Organism	mutation	tissue	Phytoene	Phytofluene	Tri-*cis*-ζ-carotene	Di-*cis*-ζ-carotene	All-cis ζ-carotene isomers	Proneurosporene	Pro-lycopene	Mutated gene	**% ζ-carotene isomers**	**LIGHT responsive**	**RATIO** tri-*cis*/di-*cis* ζ-carotene isomers
ZDS	Sunflower	*nd-1*[a]	c	4.8	6.7	n.a.	n.a.	88.6	-	-	*Zds*	88.6	NO	n.a.
	Corn	*vp9*	b	17.2	9.4	17.6	55.8	-	-	-	*Zds*	73.4	NO	0.3
CRTISO	Arabidopsis	*ccr2*	b	-	-	5.5	14.4	-	14.3	54.3	*CRTISO*	19.9	**YES**	0.4
	Tomato	*tangerine*	b	17.8	7.7	n.a.	n.a	24.2	15.1	33.6	*CRTISO*	24.2	**YES**	n.a.
	Synechocystis	*sll0033*	c	2	2	<1	10	-	15	60	*crtH*	10.0	**YES**	<0.1
	Scenedesmus	C-6D	c	14	8	12	44	-	12	10	unknown	56.0	**YES**	0.3
	Scenedesmus	PG1 [a]	c	20.9	3.2	4.3	62.4	-	-	-	unknown	66.7	**YES**	0.1
Z-ISO	Euglena	W_3BUL	c	-	7.6	24.7	9.8	-	-	-	unknown	34.5	**YES**	3.0
	Corn	*y9*	b	37.6	16.0	46.3	-	-	-	-	unknown	46.3	**YES**	>50

a. Percentage of total carotenoids calculated based on data provided
b. Etiolated leaf
c. Dark-grown cells
d. Leaf tissue from low-light grown plants

n.a.= not available

CHAPTER 5

SUMMARY AND FUTURE PERSPECTIVES

5.1 Summary

In this research, the regulation of carotenoid biosynthesis in maize was studied through investigating the duplication and subfunctionalization of maize PSY genes. Previously we reported that two duplicated PSY genes are present in the maize genome (Gallagher et al., 2004). Here, a novel *PSY* gene (*PSY3*) was recently identified in maize and two other members of the grass family based on sequence homology to maize *PSY1*. The enzymatic activities of maize and sorghum *PSY3* were subsequently verified using heterologous complementation tests. Tissue specificity analysis revealed that maize *PSY3* expresses predominately in root and embryo tissues, where carotenoids are barely detectable. In contrast, maize *PSY1* is the major gene expressed in leaf and endosperm tissues, where carotenoids generally accumulate to significant levels. The levels of *PSY2* mRNA in leaf and endosperm were about half of the levels observed for *PSY1* expressed in the same tissues. To assess the roles of maize *PSY* paralogs in controlling carotenogenesis in root, endosperm and leaf tissues, their transcript profiles were investigated using quantitative RT-PCR with abiotic stress-treated root, developing endosperm, and de-etiolated leaf tissues.

In roots, the levels of maize *PSY3* mRNA can be induced by various abiotic stresses, such as drought, salt and exogenous ABA. The elevation in *PSY3* transcripts was

accompanied by induced levels of carotenoid intermediates, elevation of other downstream carotenogenic genes, and followed by accumulation of ABA (**Chapter 2**). The levels of *PSY2* mRNA were also affected by abiotic stress in leaf and *PSY1* mRNAs were not elevated in any tissues tested.

In developing endosperm, the transcript and protein levels of *PSY1* appear to be correlated to carotenoid accumulation, which suggests that PSY1 plays a critical role in maize endosperm carotenogenesis. Low levels of *PSY2* and *PSY3* transcripts were also detected in the endosperm. However, the presence of *PSY2* and *PSY3* transcripts cannot compensate for carotenoid biosynthesis in the carotenoid-free *PSY1* endosperm mutant, *y1-602C*; this indicates that the contribution of PSY2 and PSY3 toward endosperm carotenoid accumulation is negligible.

In de-etiolated seedlings, the levels of *PSY2* mRNA were up-regulated by various wavelengths of light, whereas the levels of *PSY1* and *PSY3* mRNAs were not altered. These results suggested that *PSY2* plays an important role in controlling leaf carotenogenesis during de-etiolation. The sensing of red and far-red light signals for up-regulation of *PSY2* is blocked in chromophore deficit mutant *elm1*, which indicates that the red and far-red light signals are mediated by photoreceptor phytochromes while the blue light signal is not. In addition, the role of maize *PSY1* in leaf carotenogenesis was addressed using a *PSY1* null mutant. Chlorophyll and carotenoid content analysis revealed that loss of a functional *PSY1* can severely impact leaf carotenogenesis under

high-temperature growth conditions.

To elucidate the isomerization steps in the carotenoid biosynthetic pathway, I searched for maize mutants that accumulate atypical geometrical isomers. Using HPLC analysis, I showed that the isomerization of the PDS product (9,15,9′-tri-*cis*-ζ-carotene) to the ZDS substrate (9,9′-di-cis-ζ-carotene) was blocked in the dark-grown maize *y9* receive mutant (**Chapter 4**). This discovery genetically defined a new isomerization step mediated by Z-ISO (15-cis zeta carotene isomerase) in the carotenoid biosynthetic pathway and demonstrated that *Y9* encodes a product essential for the isomerase activity required for this step.

The studies described above provide some useful data on the regulatory mechanism involved in carotenoid biosynthesis in maize as well as a much clearer picture of the biosynthetic pathway. The duplicated *PSY* genes in maize vary in tissue specificity of expression and in response to environmental stresses, such as light, drought and salinity. The gene duplication and subfunctionalization provide plants a suitable mechanism for controlling carotenogenesis in different tissues and when subjected to various growth conditions. However, my studies only provide some preliminary information. More research must be performed in both fundamental and applied directions to gain a better understanding in the regulation of carotenoid biosynthesis. This will be discussed in the following sections.

5.2 Future perspectives

5.2.1 *PSY1* promoter analysis

As shown in Chapter 3, both the transcript and protein levels of *PSY1* are tightly correlated with carotenoid accumulation in endosperm. Absence of *PSY1* expression in endosperm is thought to be the major reason for low carotenoid accumulation in the endosperms of rice, white maize lines and the maize progenitor, teosinte (Burkhardt et al., 1997; Gallagher et al., 2004). How the maize *PSY1* gene gained its function in endosperm is still unclear. Moreover, we showed that the maize *PSY1* expression is temporally regulated; the levels of maize *PSY1* transcript and protein levels in B73 are higher between 14 and 20 DAP, and then decrease throughout endosperm development. However, the regulatory mechanism that controls this temporal expression is still a mystery. Analysis of *PSY1* genomic DNA sequences from a large collection of maize white and yellow lines showed that a transposon (*ins2*) is present in the promoter regions of all but one of the yellow lines, but is absent from all white lines and other *zea* species (Palaisa et al., 2003). These data indicated that the presence of the *ins2* element might be responsible for the increase in *PSY1* expression in endosperm, which leads to carotenoid accumulation. Up-regulation of gene expression or a change in tissue specificity due to transposon insertion has also been reported in *Drosophila* (Daborn et al., 2002; Schlenke and Begun, 2004). Recently, promoter analysis of the *Arabidopsis PSY* gene allowed the identification of *cis*-elements responsible for the modulation of different light qualities (Welsch et al.,

2003). Likewise, further analysis of the maize *PSY1* promoter, particularly the region including the *ins2* element, may enable us to determine how the maize *PSY1* gained its function in endosperm and lead to elucidation of the regulatory components involved in the regulation.

5.2.2 Role of *PSY3* in producing root-secreted apocarotenoids

Compared with leaf, root only accumulates trace amount of carotenoids. However, carotenoid accumulation in roots can be observed in mutations affecting carotenoid biosynthetic enzymes, suggesting that carotenoids are cleaved once synthesized. The carotenoid cleavage products, apocarotenoids, are a group of highly diverse bioactive compounds essential for plant development. ABA and strigolactone are two kinds of well-known carotenoid derived signal molecules. In chapter two, we showed that the up-regulation of *PSY3* in roots by drought was accompanied by induced levels of carotenoid intermediates, and followed by accumulation of ABA. This indicated that *PSY3* plays a critical role in controlling root carotenogenesis in response to abiotic stresses. Strigolactones are a collection of root-secreted apocarotenoids involved in the interaction between parasitic plants or mycorrhizal fungi and their hosts. A pathway similar to the ABA biosynthetic pathway has been proposed for strigolactone biosynthesis (Matusova et al., 2005). Thus, it is reasonable to hypothesize that up-regulation of *PSY3* might also be essential for strigolactone production and further experiments must be conducted to test the hypothesis.

5.2.3 Other rate-limiting steps in the carotenoid biosynthetic pathway

In Chapters 2 and 3, the important roles of the *PSY* genes in controlling maize carotenogenesis in different tissues were demonstrated. However, the existence of other rate-limiting steps besides PSY is also possible. This is best exemplified by the coordinated gene expression of certain pathway genes during tomato ripening. Large quantities of lycopene accumulate in ripe tomato by increasing the expression levels of tomato *PSY1* and *PDS*, and concomitantly decreasing the transcript levels of *LCYB* and *LCYE* (Giuliano et al., 1993; Pecker et al., 1996; Ronen et al., 1999). Gene expression transcriptome analysis and mapping analysis of quantitative trait loci (QTL) can provide valuable information for identifying other potential rate-limiting steps of the pathway (Santos and Simon, 2002; Fei et al., 2004) . For instance, Chander *et al* (2008) detected 31 putative QTL in the maize genome through QTL association analysis. Besides the well-known *PSY1* gene, his data also revealed that the *y9* locus may be another important candidate gene for controlling the levels of β-carotene, α-carotene, β-cryptoxanthin, zeaxanthin and total carotenoids in maize endosperm.

5.2.4 Regulatory components or transcription factors involved in carotenoid biosynthesis

In my study, the carotenoid biosynthesis in maize has been explored through investigating the role of the *PSY* genes. This typical approach to study a biosynthetic

pathway usually focuses on one or two rate-limiting steps, either at the gene or enzyme levels. Such a method remains valuable, but is relatively ineffective. Thus, multi-gene manipulation or the use of transcription factors to coordinately induce gene expression of the pathway has been suggested (Halpin, 2005). As transformation technology improves, multi-gene manipulation could be achieved by placing several carotenogenic genes in a plastidic polycistron and with coordinated expression under the same regulatory elements (Matthews and Wurtzel, 2007). To date, no transcription factors involved in carotenogenesis have been isolated, but some tomato fruit mutants with altered carotenoid profiles might prove useful in identifying them. Recently, studies on the *high-pigment* mutants *hp1* and *hp2* revealed the cross-talk between the light signaling pathway and the carotenoid biosynthetic pathway (Mustilli et al., 1999; Liu et al., 2004). Suppression of the photomorphogenesis regulatory gene, *DET1*, or overexpression of the photoreceptor *cry-2* has successfully enhanced carotenoid content in tomato fruits (Davuluri et al., 2005; Giliberto et al., 2005). Further characterization of these mutants and the cross-talk between these two pathways may provide more insight into the regulation of carotenoid biosynthesis.

In addition, comparative bioinformatics might be another potential tool for identifying common binding motifs, eventually the transcription factors involved in the regulation of carotenoid biosynthesis. Using this approach, the promoter regions of all carotenogenic genes from the rice and maize genomes could be studied systematically to search for potential transcription factor binding motifs.

5.2.5 Other factors to be considered with regard to carotenoid accumulation

Besides altering the expression of genes encoding carotenoid biosynthetic enzymes to manipulate carotenoid content, precursor pool enhancement, minimization of carotenoid degradation and formation of deposition sink are other factors that should be considered in efforts to attain higher carotenoid levels. The isoprenoid precursors for carotenoid biosynthesis in plants are provided through the methylerythritol 4-phosphate (MEP) pathway. Deoxyxylulose 5-phosphate synthase (DXS) and hydroxymethylbutenyl 4-diphosphate reductase (HDR) are two rate-limiting enzymes in the MEP pathway (Botella-Pavia et al., 2004; Enfissi et al., 2005). Consistent with their rate-controlling role in carotenoid precursor biosynthesis, higher levels of lycopene and β-carotene have been achieved by fruit-specific overexpression of a bacterial DXS gene in tomato (Enfissi et al., 2005). Likewise, higher carotenoid levels also have been accomplished in Arabidopsis by altering HDR levels (Botella-Pavia et al., 2004). These results highlight the importance of enhancing precursor pools to achieve elevated carotenoid content and suggest that it will be a novel alternative approach to manipulate carotenoid levels in crops. Similar to this strategy, carotenoids should also be protected from enzymatic cleavage and non-enzymatic oxidation to maintain high carotenoid concentrations.

Plant carotenoids are synthesized in nearly all types of plastids, but only chloroplasts and chromoplasts are able to store massive amounts of carotenoids. In

chloroplasts, carotenoids bind to the light-harvesting protein complexes, whereas they are deposited into so-called carotenoid sequestering structures in chromoplasts (Bartley and Scolnik, 1995; Vishnevetsky et al., 1999). Recently, a novel gene controlling chromoplast differentiation was isolated by characterization of a gain-of-function cauliflower mutant (Lu et al., 2006). This *Or* gene encodes a DnaJ cysteine-rich domain-containing protein and could turn normally carotenoid-free curd tissue orange by accumulation of *β*-carotene. When the *Or* gene was introduced into the potato, a major staple crop, it gave transgenic lines orange-yellow tubers with up to six fold higher carotenoid levels. Further examination of these transgenic tubers revealed that carotenoid sequestering structures were formed inside; thereby provided a metabolic sink for carotenoid accumulation. This result demonstrated that regulating the formation of metabolic sinks to effectively sequester carotenoids may prove to be an effective strategy for carotenoid engineering, if combined with those approaches discussed above.

5.2.6 Gene encoding Z-ISO

By analyzing the accumulated carotenoid intermediates in dark-grown *y9* seedlings, I showed that another carotenoid isomerase was necessary for carotenoid biosynthesis in higher plants **(Chapter 4)**. Further gene cloning and product characterization will be required to fill in the last gap of the carotenoid pathway of higher plants. In future, cloning and characterization of the y9 locus in maize will help to better understand plant carotenoid biosynthesis.

Appendix 1 A co-authored publication related to this dissertation[3]

Gene Duplication in the Carotenoid Biosynthetic Pathway Preceded Evolution of the Grasses[1]

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Despite ongoing research on carotenoid biosynthesis in model organisms, there is a paucity of information on pathway regulation operating in the grasses (Poaceae), which include plants of world-wide agronomic importance. As a result, efforts to either breed for or metabolically engineer improvements in carotenoid content or composition in cereal crops have led to unexpected results. In comparison to maize (*Zea mays*), rice (*Oryza sativa*) accumulates no endosperm carotenoids, despite having a functional pathway in chloroplasts. To better understand why these two related grasses differ in endosperm carotenoid content, we began to characterize genes encoding phytoene synthase (PSY), since this nuclear-encoded enzyme appeared to catalyze a rate-controlling step in the plastid-localized biosynthetic pathway. The enzyme had been previously associated with the maize *Y1* locus thought to be the only functional gene controlling PSY accumulation, though function of the *Y1* gene product had never been demonstrated. We show that both maize and rice possess and express products from duplicate PSY genes, *PSY1* (*Y1*) and *PSY2*; *PSY1* transcript accumulation correlates with carotenoid-containing endosperm. Using a heterologous bacterial system, we demonstrate enzyme function of PSY1 and PSY2 that are largely conserved in sequence except for N- and C-terminal domains. By database mining and use of ortholog-specific universal PCR primers, we found that the *PSY* duplication is prevalent in at least eight subfamilies of the Poaceae, suggesting that this duplication event preceded evolution of the Poaceae. These findings will impact study of grass phylogeny and breeding of enhanced carotenoid content in an entire taxonomic group of plant crops critical for global food security.

Carotenoids, a class of over 600 structures derived from isoprenoids, are synthesized by all photosynthetic organisms, some bacteria, and fungi. In plants, carotenoids are essential for plant growth and development; mutations blocking carotenoid accumulation have pleiotropic effects on chloroplast biogenesis and seed development (Robertson et al., 1978; Wurtzel, 1992). Carotenoids function as accessory pigments in photosynthesis, as photoprotectors preventing photooxidative damage, and as precursors to the plant hormone, abscisic acid (Hirschberg, 2001). The presence of carotenoids in plant endosperm tissue adds nutritional value; in humans and animals, dietary carotenoids are essential precursors to vitamin A and to retinoid compounds needed in development (Lee et al., 1981; Bendich and Olson, 1989). Nonprovitamin A carotenoids, such as lycopene, lutein, zeaxanthin, and others, also play beneficial roles in human health (Giovannucci et al., 1995; Kohlmeier et al., 1997; Sommerburg et al., 1998; Krinsky et al., 2003). The various roles of carotenoids affecting plant yield and nutritional potential has made them targets for breeding and metabolic engineering (Shewmaker et al., 1999; Matthews and Wurtzel, 2000; Ye et al., 2000; Davison, 2002; Blott et al., 2003; Gallagher et al., 2003).

In plants, the biosynthesis of carotenoids occurs on membranes of chloroplasts, chromoplasts, and amyloplasts, genetically identical plastids of very different internal membrane architecture. The plant enzymes, which are for the most part well established, are encoded in the nucleus and targeted to the plastids. Despite ongoing research on carotenoid biosynthesis in model organisms or carotenoid accumulating flowers, there is a paucity of information on pathway regulation operating in plants of world-wide agronomic importance, most specifically in the grasses (Poaceae). As a result, efforts to either breed for or metabolically engineer improvements in carotenoid content or composition in cereal crops have led to unexpected results because of the insufficient understanding of how metabolon assembly and function are controlled in plastids of different membrane architectures (Ye et al., 2000).

The biosynthesis of all carotenoids begins with the formation of the 40-carbon backbone, phytoene, a step mediated by phytoene synthase (PSY; Cunningham and Gantt, 1998; Hirschberg, 2001). In maize (*Zea mays*) endosperm, carotenoid content positively correlates with the dosage of the PSY structural gene, *Y1* (Randolph and Hand, 1940; Buckner et al., 1996;

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.039818.

3 This appendix is a publication of Gallgher et al (2004) Plant Physiol. 135: 1776-1783 with the permission from the publisher

Palaisa et al., 2003). In comparison to maize, rice (*Oryza sativa*) accumulates no endosperm carotenoids, despite having a functional PSY in green tissue. To better understand why these two grasses, representatives of two different subfamilies of the Poaceae, differ in endosperm carotenoid content, we began to characterize the genes encoding PSY, since this enzyme appeared to catalyze a rate-controlling step in the pathway (Bird et al., 1991; Bramley et al., 1992; Fray and Grierson, 1993; Giuliano et al., 1993; Kumagai et al., 1995; von Lintig et al., 1997). Previous cloning of the maize *Y1* locus, established this gene to encode PSY on the basis of sequence homology with other known phytoene synthase genes, though function of the gene product had never been demonstrated (Buckner and Robertson, 1993). Most plants have single genes encoding PSY and this was long thought to be true for maize and as a corollary, true for rice. We present evidence that both maize and rice possess duplicate *PSY* genes encoding structurally unique enzymes that function when tested in a heterologous bacterial system. Furthermore, the *PSY* duplication is prevalent throughout the grasses (Poaceae), suggesting that this genetic event preceded the evolution of the Poaceae.

RESULTS AND DISCUSSION

Isolation and Characterization of the Duplicate PSY Genes in Maize and Rice

In an effort to isolate the rice ortholog of maize *Y1*, a homologous rice EST (AY024350) was identified and used as a hybridization probe to isolate several rice genomic DNA bacterial artificial chromosome (BAC) clones. In Southern blots against rice genomic DNA, all of these clones shared the same pattern of hybridizing fragments, indicating they were the same gene. One genomic clone was sequenced and deposited as GenBank AY024351. Phylogenetic comparison of the deduced rice PSY against deduced peptides from all available PSY expressed sequence tags (ESTs), indicated that the rice PSY did not cluster with the deduced maize Y1 product, while another rice EST, AU082986, was the closest relative of maize *Y1* (Matthews, 2001). Although the novel rice gene encoded an apparently complete PSY protein and had an exon structure identical to maize *Y1*, cluster analyses together with genomic DNA hybridization patterns showed that the novel rice *PSY* was not the *Y1* ortholog. Either the rice gene shared sequence homology but did not encode a functional PSY, or as confirmed below, this gene represented a second but different, functional rice *PSY* gene, *PSY2*. With availability of the published rice genomic DNA sequence (http://portal.tmri.org/rice/), we identified the two different *PSY* genes, designated *PSY1* and *PSY2* on chromosomes 6 and 12, respectively, and through comparison to available cDNAs, annotated their exon/intron structures, as shown in Figure 1.

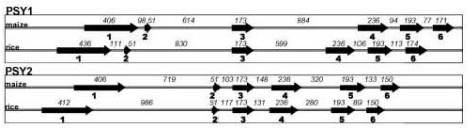


Figure 1. Gene structures of maize and rice *PSY1* and *PSY2*. Maize *PSY1* (GenBank AY324431); rice *PSY1* (GenBank AP005750); maize *PSY2* (GenBank AY325302); rice *PSY2* (GenBank AL831803). Arrows and thin bars indicate exons (bold numbers) and introns, respectively, sizes for which are in bp.

Was the PSY Gene Duplication Unique to Rice or Was It Also Present in Maize?

Prior maize mapping results identified both the *Y1* locus on chromosome 6 L (6.01) and a second locus, termed *psy2*, on chromosome 8 L (8.07; http://www.maizegdb.org/), suggesting that maize also contained duplicate *PSY* genes. Though there was no evidence that the second locus encoded a functional PSY enzyme, evidence for transcripts originating from two loci was obtained when we found maize ESTs in GenBank that showed homology to either rice *PSY1* or *PSY2*. We then screened a maize B73 genomic DNA BAC library and identified and sequenced both a *Y1* ortholog (denoted *PSY1*) and a maize ortholog for rice *PSY2*, for which gene structures are shown in Figure 1. Gene structures showed conservation across species; maize *PSY1* was more similar to rice *PSY1* than maize *PSY1* was to maize *PSY2* and the same relationship was seen for the rice genes. All four genes contain six exons and five introns. While the size of each of the six exons is conserved across all four genes, intron size is conserved only between orthologous pairs; *PSY1* genes have small first introns (approximately 100 bp), longer second and third introns (greater than 600 bp), while *PSY2* genes have long first introns (greater than 700 bp) and short second and third introns (approximately 100 bp). The possibility of the second gene, *PSY2*, being a pseudogene seemed less likely as suggested by the extensive gene structure conservation spanning two different Poaceae subfamilies. Moreover, there was evidence that all four genes were transcribed as indicated by the presence of ESTs in GenBank. At this point, we could not rule out the possibility that the transcripts might not all encode functional products.

Comparison of the Deduced Sequences for the PSY1 and PSY2 Proteins

The deduced protein sequences for PSY1 and PSY2 of maize and rice were determined and aligned (data not shown). Rice and maize PSY1 proteins shared 84.3% similar residues compared with 71.4% similar residues shared between maize PSY1 and maize PSY2. What most distinguished PSY1 from PSY2 were the distinct domains found at the carboxy termini; PSY1 proteins had a 15-residue domain distinct from the

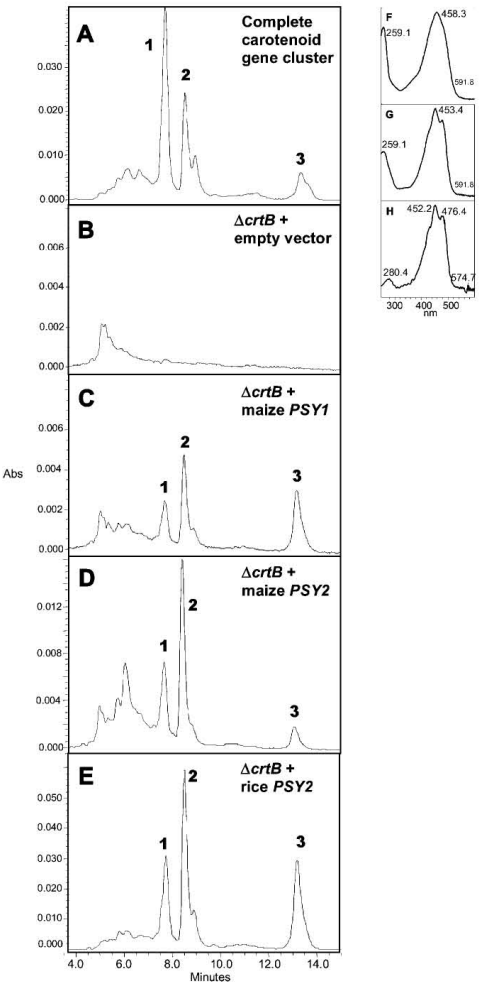


Figure 2. Functional complementation of PSY1 and PSY2. *E. coli* cells were transformed with: (A) pACCAR25; (B) pACCAR25ΔcrtB + pET23a (empty vector); (C) pACCAR25ΔcrtB + pEMPSY1-1; (D) pACCAR25ΔcrtB + pEMPSY2-1; and (E) pACCAR25ΔcrtB + pERPSY2-1 and extracted pigments analyzed by HPLC chromatograms shown at 450 nm. The spectral fine spectrum for the pathway end products, zeaxanthin β-D diglucoside (peak 1), zeaxanthin mono glucoside (peak 2), and zeaxanthin (peak 3) are shown in sections F, G, and H with retention times of 7.2 min, 8.1 min, and 13.1 min, respectively.

7-residue C-terminal domain in PSY2. Using the ChoroP Transit Peptide Predictor, all but rice PSY1 were predicted to have transit peptides (Emanuelsson et al., 1999). Maize PSY1 was predicted to be 46.5 kD (420 residues) having a 66-residue transit peptide and processed to a 39.8-kD (348 residues) mature plastid protein; rice PSY1 was predicted as 47.6 kD. Maize PSY2 was predicted as 45.2 kD (403 residues) with a 54-residue transit peptide and processed to a 39.5-kD (349 residues) mature protein; rice PSY2 was predicted as 44.7 kD (398 residues) with an 80-residue transit peptide and processed to a 36.2-kD (318 residues) mature protein. The N-terminal sequences, though not highly conserved, were more similar among the orthologs than between the paralogs.

Functional Testing of PSY1 and PSY2

To test whether the duplicated genes encoded potentially functional enzymes, we used a common tool for testing functionality of carotenoid biosynthetic enzymes (Matthews et al., 2003). Expression constructs were produced and cDNA gene products were transformed into *Escherichia coli* cells carrying a bacterial gene cluster for the entire pathway except for the gene encoding the bacterial counterpart of PSY (CrtB). Such cells produce pathway end products, zeaxanthin and its glycosylated derivates (Fig. 2, section A: peaks 3, 1, and 2, respectively), only when a functional PSY enzyme is present; these peaks are absent in the PSY deletion strain transformed with empty vector (section B) or with a truncated PSY2 construct (data not shown). When cDNAs encoding either maize PSY1 (section C), maize PSY2 (section D), or rice PSY2 (section E) were cotransformed along with the *crtB* deletion gene cluster, the expected products (peaks 1, 2, and 3) and matching spectra (sections F, G, and H) and retention times were observed, indicating that the *PSY1* and *PSY2* cDNAs tested encoded enzymes that were functional in the bacterial system.

Reverse Transcription-PCR to Test Expression of the PSY1 and PSY2 Transcripts in Maize and Rice Tissues

If either *PSY1* or *PSY2* transcript accumulation correlates with carotenoid accumulation, we would expect that maize and rice would vary specifically in endosperm transcript levels for one or both genes, given that maize endosperm accumulates carotenoids and rice endosperm does not. To test this possibility, RNA was extracted from young seedlings or endosperm from carotenoid-containing yellow maize, carotenoid-deficient white maize, and rice. Gene-specific primers, pretested on cDNAs to confirm specificity (Fig. 3A), were used to amplify the transcripts corresponding to the two PSY genes. As seen in Figure 3B, transcripts for both PSY genes were present in RNA extracted from either tissue only in the yellow maize endosperm line; for the white endosperm line,

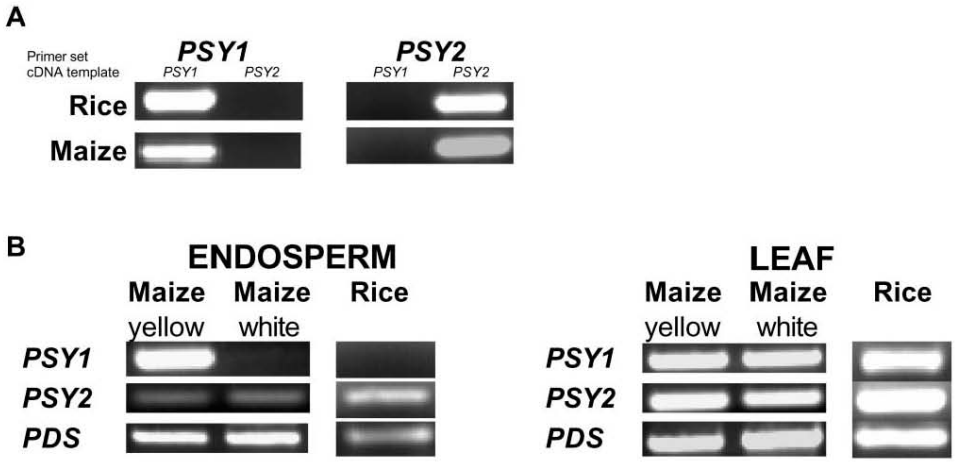


Figure 3. Leaf and endosperm transcript profiles for *PSY1* and *PSY2* in maize and rice tested by RT-PCR. A, Specificity of species- and gene-specific primers tested for both maize and rice. Left column, *PSY1* primers amplify only *PSY1* template and not *PSY2* template; right column, *PSY2* primers amplify only PSY2 template and not *PSY1* template. B, Specific transcripts for genes indicated were amplified by RT-PCR from leaf or endosperm mRNA of maize or rice. Yellow and white correspond to the endosperm phenotype of maize B73 and the maize *y1* mutant, respectively.

PSY1 transcripts were absent in endosperm, but both *PSY1* and *PSY2* transcripts were found in leaves. Similar to the white maize, *PSY1* transcripts were only present in rice leaves, but not in endosperm, while PSY2 transcripts were present in both tissues. In comparison, PDS transcripts were amplified and detected in both tissues for both plants regardless of endosperm phenotype (Li et al., 1996). These results indicate that carotenoid accumulation in endosperm correlates with expression of *PSY1* but not *PSY2* transcripts; expression of *PSY2* and *PDS* transcripts in rice endosperm is insufficient for carotenoid accumulation.

Duplication of the *PSY* Genes Preceded Evolution of the Poaceae

Since maize and rice belong to different subfamilies of the Poaceae, Panicoideae and Ehrhartoideae, respectively, it was likely that the gene duplication was more widespread in the Poaceae, a phenomenon proven correct by further GenBank database searching. *PSY* ESTs were identified for Triticum and Hordeum, species in another Poaceae subfamily, Pooideae. Together with the deduced protein sequences for the maize and rice PSY proteins, these additional sequences were compared with deduced PSY proteins of representative dicots and another monocot, Narcissus. The resulting phylogenetic tree seen in Figure 4 shows that for each of the grasses, sequences either cluster into PSY1-like or PSY2-like groups; the grass duplication appeared to have evolved from a common ancestor prior to the evolution of the grasses. In contrast, the dicot duplication seen for the tomato (Lycopersicon) PSY gene is not found in Arabidopsis, which has been fully sequenced, and generally not the rule for dicot taxa, the only other known exception being tobacco (*Nicotiana tabacum*; Bartley and Scolnik, 1993; Busch et al., 2002).

How Widespread within the Poaceae Is the Gene Duplication of *PSY*?

To test further for the distribution of the duplicated *PSY* genes among other Poaceae subfamilies, ortholog-specific universal primers were designed and tested for specificity. As seen in Figure 5A, we observed the expected *PSY1* products of 1,123 bp (maize), and not 387 bp that would be obtained if the corresponding region of *PSY2* was nonspecifically amplified; and 838 bp (rice), and not a nonspecific *PSY2* product of 370 bp. Similarly for *PSY2* universal primers, the expected products for maize and rice, 434 bp and 394 bp, respectively, were observed, and not the nonspecific amplification of *PSY1*, predicted to be 208 bp and 127 bp, respectively. These universal primers were then used to amplify DNA from representative taxa of the Poaceae. DNA sequences of the PCR amplification products, which have been deposited into GenBank, revealed that some amplified genes contained introns, while others did not. Therefore, we used only the exonic regions in a cluster analysis along with corresponding regions of either *PSY1* or *PSY2* of maize and rice to confirm that the amplified sequence clustered

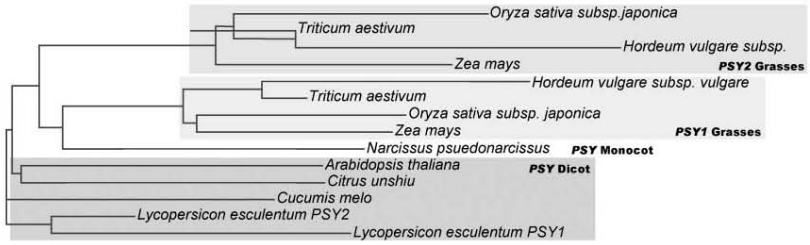
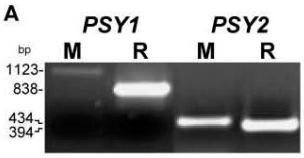


Figure 4. Phylogenic analysis of PSY amino acid sequences. SwissProt numbers, in bold, and GenBank accessions are in parentheses. *Lycopersicon esculentum* (**AAA34187**, L23424.1; J. Hirschberg, unpublished data), *L. esculentum* (**AAA34153.1**, M84744), *Cucumis melo* (**CAA85775.1**, Z37543), Arabidopsis (**AAAN17427**, BT000450.1), *Citrus unshiu* (**BAB18514**, AB037975.1), *Hordeum vulgare* subsp. *vulgare* (BI955682), *Triticum aestivum* (BM137086), *Z. mays* (AY325302), *O. sativa* (japonica, AL831803), *H. vulgare* (BE421261), *O. sativa* (japonica, AP005750), *Z. mays* (AY324431), *T. aestivum* (CD862515), and *Narcissus pseudonarcissus* (**CAA55391**, X78814).

with either *PSY1* or *PSY2* (data not shown). Using this simple PCR assay, we detected duplicate genes in 12 taxa representing 8 subfamilies in the Poaceae, as shown in Figure 5B. The use of universal *PSY* PCR primers will be valuable in assessing the distribution of the PSY duplication among the monocots. These tools will also be useful in phylogenetic analyses within the Poaceae subfamilies to offer improved resolution of evolutionary relationships.



B

Poaceae (Grass Family)			
Subfamily	**Tribe**	**Subtribe**	**Species**
Pharoideae	Phareae		*Pharus lappulaceus*
Bambusoideae	Bambuseae	Bambusinae	*Bambusa vulgaris*
Ehrhartoideae	Oryzeae	Zizaniinae	*Oryza sativa*
Pooideae	Triticeae	Hordeinae	*Hordeum muticum*
		Triticinae	*Secale cereale*
Aristidoideae	Aristideae		*Aristida adscensionis*
Arundinoideae	Arundineae		*Phragmites australis*
Centothecoideae	Centotheceae		*Zeugites pittieri*
Panicoideae	Paniceae	Cenchrinae	*Pennisetum tristachyum*
	Andropogoneae	Sorghinae	*Sorghastrum nutans*
		Tripsacinae	*Tripsacum zopilotense*
			Zea mays

Figure 5. Poaceae subfamily genomes tested and found to possess the PSY gene duplication. A, Testing of the ortholog-specific universal primers indicated at the top using as template, M (maize B73) or R (rice IR36) genomic DNA. B, Species representing 8 of the 12 subfamilies of the Poaceae family found to have both the *PSY1* and *PSY2* genes based on amplification using the ortholog-specific universal primers tested in A and further confirmed by alignment of the amplified exonic DNA sequences.

CONCLUSION

We have found that throughout the Poaceae, the gene for PSY is duplicated, suggesting that this duplication occurred prior to evolution of the grasses. Without evidence of gene product function, Palaisa et al. (2003) previously used associative genetics to correlate endosperm carotenoids with allelic states of maize *PSY1* (*Y1*) but not *PSY2* loci. Our data support that study and show that *PSY1* but not *PSY2* transcripts in endosperm correlate with endosperm carotenoid accumulation. Whereas prior studies did not address whether the genes encoded functional enzymes, we demonstrated that for maize, both *PSY1* and *PSY2* encode functional enzymes as tested in a bacterial system; rice PSY2 is also functional using this heterologous platform. However, in planta, function requires not only the potential for enzyme activity as demonstrated in the bacterial milieu, but also that the enzyme must localize to a plastid membrane where it gains access to substrate produced by an upstream enzyme. Similarly in Narcissus, where only one PSY gene has been described, PSY was found as an inactive soluble plastid stromal form and as an active plastid membrane-bound enzyme (Schledz et al., 1996). We provide data that suggest that only PSY1 seems to have both demonstrated activity in *E. coli* and to function in endosperm. While rice PSY2 is functional in *E. coli*, it is apparently not functional in rice endosperm. The transcript is translated in rice endosperm (data not shown) but the enzyme is not functional given the absence of carotenoids or carotenoid intermediates in rice endosperm (Burkhardt et al., 1997). Therefore, our data suggest that PSY1 and PSY2 are not functionally equivalent in planta and that endosperm carotenoid accumulation requires expression of *PSY1*. The duplicate grass genes are predicted to encode enzymes with variant N and C termini, suggesting that the grass PSYs may target to different plastid membranes. The difference in membrane architecture between endosperm amyloplasts and leaf chloroplasts may offer a possible explanation of why

different PSY isoforms may be associated with presence or absence of endosperm carotenoid accumulation. Further characterization of the two grass enzymes will be needed to define their roles in carotenogenesis in the variety of plastid architectures found in cereals and provide insight into the potential for endosperm carotenoid accumulation throughout the grasses.

The *PSY* duplication in the grasses predisposed evolution of tissue-specific pathway control, providing a mechanism to modify gene expression in the seed without deleterious effects on photosynthetic organs. Had there been only a single *PSY* gene, its overexpression would have interfered with the photosynthetic complex, most likely causing photosensitivity in the plant (Busch et al., 2002). The occurrence and persistence of PSY duplications suggests that recruitment of primary carotenoids as secondary metabolites has been adaptive in many species. The existence of parallel (convergent) PSY duplications among the monocots and the dicots, taken together with evidence of altered spatial expression of the gene product of one locus among photosynthetic and nonphotosynthetic organs, supports this supposition. While rice does not accumulate endosperm carotenoids, there are other grasses besides maize that do accumulate seed carotenoids, including sorghum, millet, and wheat (FAO, 1995). The existence of duplicate PSY factors in the grasses offers novel opportunities to use conventional breeding or biotechnology to select for enhanced endosperm carotenoids in grass species that are of agronomic importance.

MATERIALS AND METHODS

Plant Materials

Maize (*Zea mays*; Maize Genetic Stock Center, University of Illinois) was field-grown in Bronx, NY; rice (*Oryza sativa* indica variety) IR36 was greenhouse-grown with supplemental lighting. Maize endosperm dissected at 20 d after pollination, dissected mature rice endosperm and leaf samples from maize and rice were frozen in liquid nitrogen and stored at −80°C prior to use. For Poaceae subfamily *PSY* gene amplifications, DNA (Dr. Lynn Clark, Iowa State University) was obtained for *Bambusa vulgaris*, *Pharus lappulaceus*, and *Zeugites pittieri*, or prepared from dried leaves (Dr. Paul Peterson, Smithsonian Institution) for *Hordeum muticum* J. Presl. collected in Ayacucho, Peru, sample identification (ID): Peterson, P. M., Refulio-Rodriguez, N. 16440, *Secale cereale* collected in Maryland, ID: Pennington, S. J. 1200, *Aristida adscensionis* collected in Cajamarca. Peru. ID: Peterson. P.M.. Refulio-Rodriguez. N. 15059, *Phragmites australis* (Cav.) Trin. ex Steud., ID: Peterson, P.M. 17519, *Pennisetum tristachyum* collected in Cajamarca, Peru, ID: Peterson, P.M. Refulio-Rodriguez, N. 15031, *Sorghastrum nutans* (L.) Nash collected in Mexico, ID: Peterson, P.M., Gonzalez-Elizondo, S., Brothers, L. E. 16684 and *Tripsacum zopilotense* Hernández & Randolph collected in Tamaulipas, Mexico, ID: Peterson, P. M., Valdes-Reyna, J. 15903.

Genomic DNA Isolation and Sequence Analysis

A maize B73 genomic BAC library containing 92,160 clones in pECBAC1 and representing 5.2× genome equivalents (Dr. H. Zhang, Texas A & M University) was probed with PSY cDNAs (maize *PSY1*; GenBank ZMU32636); rice *PSY2*, GenBank AY024350; Zhang et al., 1996). Five *PSY1* and three *PSY2* BAC clones were obtained and representatives chosen for further sequencing of both strands by primer walking (DNA Sequencing Facility, University of Chicago Research Center). Sequence assembly and analysis of these and all other DNA samples were performed using Vector NTI Suite, Version 7.0 (InforMax, North Bethesda, MD), and BLAST 2.1 (Altschul et al., 1997). Maize *PSY1* and *PSY2* genomic sequences were deposited as GenBank AY324431 and AY325302, respectively. For comparison, rice (japonica) *PSY1* and *PSY2* used in Figure 1 were AP005750 and AL831803, respectively.

Plasmids and Functional Complementation

Plasmid pACCAR25 (Misawa et al., 1990) contains the *Erwinia uredovora* gene cluster conferring accumulation of glycosylated zeaxanthin when transformed into *Escherichia coli* and was used as a positive control; pACCAR25Δ*crtB* (Chamovitz et al., 1992), containing a frame-shift mutation in *crtB* (bacterial PSY), was used for heterologous complementation to test function of *PSY1* and *PSY2* cDNAs subcloned as in-frame translational fusions as follows. A maize *PSY2* cDNA (nt no. 3 to nt no. 1,348) was amplified from pAY450646 (GenBank no. AY450646) using forward primer (no. 622) 5′-AAGAGATC**GAATTC**GGCACGG-3′ with an *Eco*RI site (bolded) and reverse primer (no. 635) 5′-TCCTTGTAA**CTCGAG**CTGATTGAG-3′, with an *Xho*I site (bolded), digested with *Eco*RI and *Xho*I, subcloned into the corresponding sites of pET23a (Novagen, Madison, WI), and renamed pEMPSY2-1. The rice *PSY2* cDNA pAY452768 (GenBank no. AY452768) was inserted as an *Eco*RI/*Xho*I fragment into corresponding sites of pET23b (Novagen, Madison, WI) and renamed pERPSY2-1. A maize PSY1 cDNA was cloned in-frame in pBluescript, p12A33A, (GenBank no. ZMU32636; Dr. Brent Buckner, Truman State University). *E. coli* BL21 (DE3) cells (Novagen) were transformed (Sambrook et al., 1989) with combinations of pACCAR25Δ*crtB* and the expression constructs p12A33A, pEMPSY2-1, or pERPSY2-1 or with pACCAR25 and pET23b. Transformants were grown in liquid Luria-Bertani medium with appropriate antibiotics overnight at 37°C with aeration and held at room temperature for 2 d in the dark, centrifuged at 2,000*g* for 30 min, and pellets extracted twice with acetone. Combined extracts were dried over Na_2SO_4, concentrated to dryness under a stream of nitrogen, resuspended in injection solvent (acetonitrile, 85%; methanol, 10%; dichloromethane, 2.5%; and hexane, 2.5%), and filtered through a 0.45-μ nylon filter (Phenomenex, Torrance, CA) into a 300-μL glass insert in a 2-dram amber vial and subjected to HPLC analysis.

HPLC Analysis

Carotenoids were separated on a Waters (Millipore, Franklin, MA) HPLC system with 2690 separation module, Millenium version 2.0 software (Waters, Franklin, MA), 996 photodiode array detector (Waters), 717 autosampler, using a Nucleosil 5 C_{18} (5μ, 250 × 4.6 mm) column (Phenomenex, Torrance, CA) with a Nucleosil C_{18} (5μ, 4 × 3.0 mm) guard column (Phenomenex). Solvent mixtures used for mobile phases were A, acetonitrile:methanol (9:1, v/v) and B, hexanes:methylene chloride:methanol (4.5:4.5:1, v/v/v; Khachik et al., 1999). Sample injection was followed by 10 min of isocratic conditions using 95% A:5% B, followed by a linear gradient to 45% A:55% B over 30 min. Between samples, columns were reequilibrated for 10 min using 95% A:5% B. All solvent flow rates were 0.7 mL/min. Carotenoids were identified by comparison of retention times and absorption pattern spectra with those of authentic standards.

Isolation of RNA and RT-PCR

Total RNA was isolated from maize leaves and 20 d after pollination endosperm using B73 (a yellow endosperm line) or *y1* (a white endosperm line), and from rice IR36 leaves and mature endosperm (RNeasy Plant Mini kit for total RNA isolation, Qiagen, Valencia, CA), concentrations measured spectrophotometrically, and 100 ng of RNA used as template for first strand cDNA synthesis (SuperScript First-Strand Synthesis system for RT-PCR, Invitrogen, Carlsbad, CA). A 2-μL aliquot of the first-strand reaction, 1 to 5 ng of cDNA, was used for PCR amplification under conditions pretested for linearity. Gene-specific primers were designed to flank introns and were tested for specificity using maize *PSY1* (GenBank ZMU32636), maize *PSY2* (GenBank AY450646), rice *PSY1* (pTRPSY1-1, GenBank AY445521), and rice *PSY2* (GenBank AY024350). PCR reactions contained 20 mM Tris-HCl pH 8.4, 50 mM KCl, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.4 μM each primer, 0.025 units/μL *Taq* DNA Polymerase (Invitrogen) and were carried out for one cycle of 3 min at 94°C; followed by 35 cycles of (30 s at 94°C; 30 s at annealing temperature, 45 s at 72°C); and one cycle of 10 min at 72°C. Gene-specific

primers, annealing temperatures, and expected products were: maize *PSY1*, forward (no. 509) 5′-GCATTGCTCAAACGCCAG-3′; reverse (no. 519) 5′-CAGAGAGAGCGGCATCAAG-3′, 54°C, 300 bp; maize *PSY2*, forward (no. 532) 5′-GCGGCAAGTTCCACCACCTGT-3′; reverse (no. 529) 5′-CGAGGTCTGCGCCGAGTA-3′, 58°C, 150 bp; rice *PSY2*, forward (no. 145) 5′-CCTGAAAGGCGCAAAGCTG-3′; reverse (no. 146) 5′-CGATAG-CATCAAGGATCTGCC-3′, 65°C, 682 bp; rice *PDS*, forward (no. 151) 5′-GACCATGTTCGCTCTTTGGGTGG-3′; reverse (no. 152) 5′-CGATGATTT-CAGTGTCACTCCGTCC-3′, 61°C, 430 bp; and maize PDS, as described previously (Matthews et al., 2003).

DNA Extraction and PCR Amplification of *PSY1* and *PSY2* from Poaceae Subfamilies

For PCR, genomic DNA from dried grass samples was extracted (REDExtract-N-Amp Plant PCR kit, Sigma, St. Louis) and 4 μL added to 10 μL of REDExtract-N-Amp PCR Ready Mix and 0.5-μM final concentration of each primer in a 20-μL reaction. Universal gene-specific were designed based on conserved sequences between maize and rice: *PSY1*, forward (no. 530) 5′-TTTGGACCGGTGGGAGAA-3′ and reverse (no. 520) 5′-GCCCAT-CACAGGTACGCTCATT-3′ (annealing temperature, 54°C); *PSY2*, forward (no. 672) 5′-GACGAATATTCTCAGAGACG-3′ and reverse (no. 673) 5′-ACTTTCCCTCTGAATATGTC-3′ (annealing temperature, 50°C). All reactions were as follows: one cycle of 3 min at 94°C; followed by 40 cycles of (30 s at 94°C; 30 s at annealing temperature, 1 min at 72°C); and one cycle of 10 min at 72°C and products purified using the Qiagen MinElute PCR Purification kit (Qiagen) prior to sequencing. The PSY1 GenBank accession numbers were CG892534 (*Phragmites australis*), CG892535 (*Aristida adscensionis*), CG892537 (*Hordeum muticum*), CG892538 (*Pennisetum tristachyum*), CG892539 (*Secale cereale*), CG892540 (*Sorghastrum nutans*), CG892541 (*Tripsacum zopilotense*), CG892543 (*Pharus lappulaceus*), CG892544 (*Zeugites pittieri*) and CG892545 (*Bambusa vulgaris*). The PSY2 GenBank accession numbers were CG892547 (*Phragmites australis*), CG892548 (*Aristida adscensionis*), CG892549 (*Hordeum muticum*), CG892550 (*Secale cereale*), CG892551 (*Sorghastrum nutans*), CG892552 (*Tripsacum zopilotense*), CG892553 (*Bambusa vulgaris*), CG892554 (*Pharus lappulaceus*), CG892555 (*Zeugites pittieri*), and CG892559 (*Pennisetum tristachyum*).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY024351, ZMU32636, AY024350, AY324431, AY325302, AP005750, AL831803, AY450646, AY452768, ZMU32636, AY450646, AY445521, AY024350, CG892534, CG892535, CG892537, CG892538, CG892539, CG892540, CG892541, CG892543, CG892544, CG892545, CG892547, CG892548, CG892549, CG892550, CG892551, CG892552, CG892553, CG892554, CG892555, CG892559, L23424.1, M84744, Z37543, BT000450.1, AB0379751, BI955682, BM137086, AY325302, AL831803, BE421261, CD862515, AU082986, and X78814.

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Appendix 2 Sequences used in this study

Genomic DNA sequences	Accession No. / ID	Source	Note
Maize *PSY1*[b]	**AY324431**	GenBank	
Rice *PSY1*	**AP005750**	GenBank	
Sorghum *PSY1*	**SbGSStuc11-12-04.5154.1**	PlantGDB	
Maize *PSY2*[b]	**AY325302**	GenBank	
Rice *PSY2*	**AL831803**	GenBank	
Sorghum *PSY2*	**SbGSStuc11-12-04.12062.1**	PlantGDB	
maize *PSY3*[a]	DQ372936	GenBank	
rice *PSY3*	**LOC_Os09g38320**	Gramene	
sorghum *PSY3*	**SbGSStuc11-12-04.766.1**	PlantGDB	
Arabidopsis *PSY*	**AB005238**	GenBank	
Note: For *PSY* genomic DNA structure analysis in Chapter 2			
Protein/cDNA sequences	Accession No. / ID	Source	Note
AtPSY	AAA32836 (protein)	GenBank	*Arabidopsis* PSY
OsPSY1	AAS18307 (protein)	GenBank	Rice PSY1
OsPSY2	AAK07735 (protein)	GenBank	Rice PSY2
OsPSY3[a]	DQ356431 (cDNA)	GenBank	Rice PSY3
SbPSY1[a]	AY705389 (cDNA)	GenBank	Sorghum PSY1;
SbPSY2	AW679367 (EST)	GenBank	Sorghum PSY2
SbPSY3[a]	AY705390 (cDNA)	GenBank	Sorghum PSY3;
ZmPSY1	P49085 (protein)	GenBank	Maize PSY1
ZmPSY2[b]	AAQ91837 (protein)	GenBank	Maize PSY2
ZmPSY3[a]	DQ356430 (cDNA)	GenBank	Maize PSY3
For *PSY* phylogenetic tree analysis in Chapter 2			
Other sequences	Accession No. / ID	Source	
Sorghum *PSY1* EST	CD234165	GenBank	
Sorghum *PSY3* EST	BG46454	GenBank	
Maize *PSY3* contig	AZM4_60808	TIGR	
Rice *PSY3* EST	CF305089	GenBank	
Rice *PSY3* EST	CF312554	GenBank	
Rice *PSY3* EST	CF312553	GenBank	
Rice *PSY3* EST	CF307565	GenBank	
Rice *PSY3* EST	AK108154	GenBank	
Rice *PSY3* EST	AY078162	GenBank	
Maize *PSY1* gene sequence in *y1-602C*	EU306869	GenBank	
Maize *PSY1* gene sequence in *y1-602M*	EU306868	GenBank	

a. Sequences deposited by Li F.
b. Sequences deposited by Dr. Gallagher

Appendix 3 Plasmids used in this study and laboratory clone records

Clone Name	Brief Description	Insert Size (kb)	GenBank	Chapter
pACCAR25ΔcrtB	crtE, crtI, ΔcrtB, crtX, crtY	6.5 kb	D90087	2
BAC b031205	Maize *PSY3* containing BAC clone	137 kb	DQ372936	2
pET23b-MPSY3	Maize *PSY3* expression construct	1.4 kb	DQ356430	2
pGEMT-RPSY3	Rice *PSY3* non-expression construct	1.4 kb	DQ356431	2
pETaSPSY1	Sorghum *PSY1* expression construct	1.3 kb	CD234165	2
pETaSPSY3	Sorghum *PSY3* expression construct	1.3 kb	BG464544	2
CD230490	Sorghum *PSY1* EST	~2 kb	CD230490	2
CD234165	Sorghum *PSY1* EST	~2 kb	CD234165	2
AW679367	Sorghum *PSY2* EST	0.5 kb	AW679367	2
BG464544	Sorghum *PSY3* EST	1.6 kb	BG464544	2
pET23b(+)	Expression vector	0 kb	N/A	2
pET23a(+)	Expression vector	0 kb	N/A	2

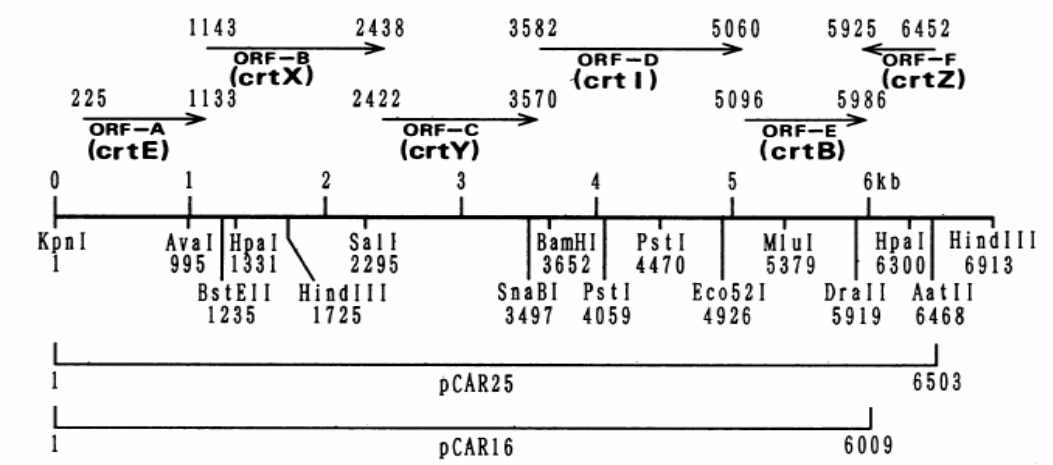
WURTZEL LAB
CLONE INFORMATION

Date Today: 08/27/07 Entered into database #403

CLONE NAME: pACCAR25ΔcrtB

Lab Clone Number/Name: pACCAR25ΔcrtB (Expression)

Clone Description: Frameshift in MluI(5379) in crtB.
Genes: crtE, crtI, ΔcrtB, crtX, crtY



Constructed by: Misawa
Purified by: Faqiang Li
DNA Location (-20°C) Box Number: 9 Position: A9 Conc. >1 µg/µl
Tube labeled as: pACCAR25ΔcrtB
Strain Location (-80°C) Box Number: 14 Position F2
Tube labeled as: pACCAR25ΔB Strain: BL21(DE3)

Cited in journal:
1. N. Misawa *et al.*, *J Bacteriol* **172**, 6704 (1990).
2. H. Linden *et al.*, *Z. Naturforsch.* **46c**, 1045 (1991).

Lab Notebook to reference: Li book 1page 127 Date: 5/28/02

Organism source of gene: *Erwinia uredovora*

Cloning vector used: pAC184 Vector size: 4.26 kb Insert size: 6.5 kb

Antibiotic markers: chloramphenicol;

Restriction enzyme(s) to release insert: N/A

WURTZEL LAB
CLONE INFORMATION

Date Today: 12/01/05 Entered into database #357

CLONE NAME: BAC b031205

Lab Clone Number/Name: BAC b031205
Clone type: Genomic

Original GenBank Accession: N/A

Clone Description:
BAC clone requested from Arizona Genomic Institute. It contains maize *PSY3* gene.
Order homepage : http://www.genome.arizona.edu/orders/
Contact info: Dave Kudrna, Plant Sciences Department,303 Forbes Building, Tucson, AZ 85721-0036,USA.(520)-626-9596 (phone) (520)-621-1259 (fax)

Constructed by: Arizona Genomic Institute Date rec'd: Apr.13.2005
Purified by: Faqiang Li
DNA Location (-20°C) Box Number: #9 Position: A7 Conc. 0.1µg/µl
Tube labeled as: Maize PSY3 genomic DNA BAC b031205 Apr.13.2005
Strain Location (-80°C) Box Number: 14 Position F5
Tube labeled as: Maize PSY3 genomic DNA BAC b031205 Strain: Top10F'

Cited in journal: Li, F., Vallabhaneni, R., and Wurtzel, E.T. (2007) PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and a key regulator of abiotic-stress-induced root carotenogenesis. Plant Physiology. First published on December 27, 2007; 10.1104/pp.107.111120

Lab Notebook to reference: Li book 4 page 42-52 Date: April 2005

Organism source of gene: Maize B73

Cloning vector used: pIndigoBac536 Vector size: N/A Insert size: ~137 kb

Antibiotic markers: Chloramphenicol

Restriction enzyme(s) to release insert: N/A

Sequence verified: yes Junction verified: no

TTGGGGAACAAGCAAGAAATGAACGGAGGAAGCGATCAGGCTCTCCTCTCTCCNCACCACACCGGCCGGGNNGCAGGCAGGAGGCCCCNCCGGGTGCTCCTCCTGTCGCTCCCACANGNNGAACAGGCCGGGGGCGAGGC
CTGCCACGATCGCGCGCCCCACCANGTGTCCCNTCCGGCGNGCNGCCNACNTGCCTGCNNTGGGTACCGGTACCGGTCTCGTCTCTGCGTGACCTGATGGCCATGTGCGTGCGGGCGGCGGATGCGGACCCGCCCCTTGG
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AAGTCTACATACAAGTTTGGAGAGGAAATCCAAATGTCTCCGTAATGATAATAGATTACGATCCAAATATATCTTCACAATTTATTTAGTCTATTTATTAACTTAATTTAAAAAAATAGATAGAATAGAGTTAAATCCTA
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TGATGGCTGCTAGCGCGGAGCGAAATGCCTCACCTGCCCCTTTCACGAACCACGCAGAGCGCCTCTCCTCCTGGACAGGAGCACCCCCTGCCAACGCCCGCCACCTGGCCTCCTCCCGGCTCCGCAATCCCGCGCATTAC
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TCCCCTCGTCGTCTCCTTCCACGCGCGGCCGACACGGGTGCCGCCCACGAGCGAGTCGGGCTCTGGCAACACCGCGCCCGCCGCGTGGGTTGGGTTGGGTTGGGACTTGGGACTCGCGGTTTGGAACCCGAACACCAGTGGC
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TGCACGAGGTGGTGCTCAGGCAGGCGGCGCTCGCGGCCGCGGGCGCCGAGGACGGCGCGGATCGAGCCGGTGCCCCTGGACGGCGGGCTGAAGGCGGCCTTCCACCGCTGCGGCGAGG
TCTGCAGGGAGTACGCCAAGACATTCTACCTTGGTAAGCGAGATCGAAGTCCCCGCTGCTCTTCTTGTTCATCTGAATACGGTCAGTTTAATTACTTGGCACACACAAAGGTTTTGCCGTGATCGAGGCACATGCATGTG
TCACAGCTGCTAGTCAGCTAGTGTGTGTTTTTCTGGCAAACTGTTTAAACAAAGTAAAAAAAAGAAACTTTTTTTTGTCCTGAAAAGGACGATAGGCAAAGGATGGAAATCTCAGTCGTACGTATCCTTGGTAGAAGCCT
GAACCGGGGGGTGTCCAAGTCCAACACCTGCCTTTTCCAATTTGTTTTCTAAATCCACTAGCGTATTTGATTAATTCTGACAATGATTGTAAAAAATAACTTATAAATTATGGACTGAGAGAATACGTAGCGTGCATTGA
GATGTTAATCATGCACACTACTAGTATTTTGCCAGTAGTTGCAGCTGTGGCCACCGTATCACAACATACCGAATACCCGGCCCAGTCAGCTTGGACAATCAGCGCGCGCATTCCACCGCACATGCATGTGTCTCTCTCAC
CTCTGATCTGATGAGATGCCGCTGCTTTTCTGTTTCAGC
GACGCAGCTGATGACGCCCGAGAGGAGGATGGCGATCTGGGCAATATACGGT
AAGCAGAGCAAGCCCCACATGTCCCTGTTTCTTGTACCGCCTGCTGTTGGCGCACTGCAGGTTCCTTTCGTTTTCTCTGAATGGTATAAAATCGTAATCCGGCTTGTGAGCTAGCTAGAGTAGTTTATGCACCGGCACGA
TGGTTTTTTTAAGGGCAGTTTGGAAGGAAAATGAGCTAATTTTCTACTCAATCTCTGAGAATACCAGAGCGGATTTAAGTTTCTAAACTAACGCTAGGACGGTGAAAGGGGGGATACAGTTTGTATGGCTTGACGGTTGA
CGATAATGACGCTCTGAGAATACCAGAGCGGATTTAAGTTTCTAAACTAACGCTAGGACGGTGAAAGTGGGGATACAGTTTGTATGGCTTGACGGTTGACGATAATGACGAGGGAAGGGATGACACTGATTGATCGCTGA
CGTGGGTGTTCTATCTCCGCGCACGCGCGCTCCTGTTCA
GTGTGGTGCAGGAGAACGGACGAGCTCGTGGACGGCCCCAACGCGTCCCACATCTCGGCGCTGGCGCTGGACCGGTGGGAGTCGCGGCTGGAGGACATCTTCGCCGGCCGGCCGTACGACATGCTCGACGCCGCCCTGTC
CGACACCGTCGCCAGGTTCCCCGTCGACATCCAG
GCACGCACGCGCGCGATCGATCCATGGATACATCTCCTCCCTCCATATATTGAAGAACATTCCCATCATATCGTGTCTCCGTGCTGCAG
CCGTTCAGGGACATGATCGAGGGGATGCGCATGGACCTGAAGAAGTCCCGGTACAGGAGCTTCGACGAGCTGTACCTCTACTGCTACTACGTGGCCGGCACCGTGGGGCTGATGAGCGTCCCGGTGATGGGCATCTCGCC
GGCGTCCAGGGCGGCCACCGAGACGGTGTACAAGGGGGCGCTGGCGCTGGGCCTGGCGAACCAGCTCACCAACATCCTCAGGGACGTCGGCGAGG
AGTACGTCGTCAACGCCACTCATCATGATCGTAGCCGGCCCATGTTGCATACAGCATACTGACACGCGCGCTTCACGTGCCTGACAAATAATAACAG
CGCCAGGAGGGGACGGATCTACCTCCCGCAGGACGAGCTGGAGATGGCGGGGCTCTCCGACGCCGACGTCCTGGACGGCCGCGTCACCGACGAGTGGAGGGGCTTCATGAGGGGCCAGATCGCGAGGGCCAGAGCCTTCT
TCAGGCAGGCGGAGGAAGGCGCCACCGAGCTCAACCAGGAGAGCCGATGGCCGGTG
AGACTCACAACGCTAGCCGTTTTCAGAACCCGATCAGATGAGTGACTGAACAGCTGACTGCTGCTGTTTCAGGTG
TGGTCTTCTCTGCTCCTGTACCGGCAGATCCTCGACGAGATCGAGGCCAACGACTACGACAACTTCACCCGGAGGGCCTACGTTCCGAAGACGAAGAAGCTGATGGCGCTGCCCAAGGCGTACCTGAGATCACTGGTGGT
GCCCTCCTCCTCTTCTCAGGCTGAGAGCCGGAGACGCTATTCCACCCTAACATAG
ACCGGTCTCTCTCTCACTGTCGGTACATAGCAGCAGCAGCAGTGAATGATCGATATGATGTACAAATTTCTGGCCAGGCCACATGCATGAGTAGTACGTACATGGTTGGCAATTGGCATGTGGTGGTGGTCGATCGGTGT
TACATACTTGTTGTACTCCTCTGTACTATACAAAACATGCCGGAGACTCGCGCGTAAGTGAGAAGCGATAAATGGAGATAGATGTTTGTCTCATAAAATGAACTAATTTACGTTAAGGTGCCGTTTGGATCCCTCTATTG
GAAGCAACTGGAATTTACTTAATAAAGGTTATTGGT

WURTZEL LAB
CLONE INFORMATION

Date Today: 12/01/05 Entered into database #358

CLONE NAME: pET23b-MPSY3

Lab Clone Number/Name: pET23b-mPSY3-10
Clone type: Expression cDNA clone

GenBank Accession: DQ356430

Clone Description:
Maize *PSY3* full-length cDNA was amplified with primers #925 and #926 and cloned into pGEMT vector. Then *PSY3* cDNA was released from pGEMT vector by complete digestion with *Eco*RI and partially with X*ho*I. Insert was placed into expressive pET23b (+) vector between *Eco*RI and X*ho*I sites to test the enzymatic function by complementary test.

Constructed by: Faqiang Li Date SEP.28.2005
Purified by: Faqiang Li
DNA Location (-20°C) Box Number: #9 Position: A6 Conc. 1µg/µl
Tube labeled as: Maize PSY3 genomic DNA pET23b-MPSY3-10 SEP.28.2005
Strain Location (-80°C) Box Number: #14 Position E2
Tube labeled as: pET23b-MPSY3-10 Strain: BL21 (DE3)

Cited in journal:
Li, F., Vallabhaneni, R., and Wurtzel, E.T. (2007) PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and a key regulator of abiotic-stress-induced root carotenogenesis. Plant Physiology. First published on December 27, 2007; 10.1104/pp.107.111120

Lab Notebook to reference: Li book 2 page 68 Date: SEP.28.2005

Organism source of gene: maize B73

Cloning vector used: pET23b (+) Vector size: 3.6 kb Insert size: ~1.4 kb

Antibiotic markers: Amp

Restriction enzyme(s) to release insert: N/A

Sequence verified: yes Junction verified: yes

> Maize PSY3 cDNA sequence

```
atgatgtctacgagccgcgcggtgaagtcgccggcgtgcgcggctcggcggcggcagtggtccgcggacgcgccgaaccgcacggcgacgttcctggcctgcaggcacgg
gaggcggctcggcggcggcggcggggcgccgtgctccgtgcgcgccgagggctccaacaccattgtctgcctcgaggccgaggcgtggggcggcgccgccggcgctgccgg
gcctccgcgtggcggcgccgtcgcccggggacgcgttcgtcgtgccgtccgagcagagggtgcacgaggtggtgctcaggcaggcggcgctcgcggcagcggcgccgagg
acggcgcggatcgagccggtgcccctggacggcgggctgaaggcggccttccaccgctgcggcgaggtctgcagggagtacgccaagacattctaccttgcgacgcagct
gatgacgcccgagaggaggatagcgatctgggcaatatacgtgtggtgcaggagaacggacgagctcgtggacggccccaacgcgtcccacatctcggcgctggcgctgg
accggtgggagtcgcggctggaggacatcttcgccggccggccgtacgacatgctcgacgccgccctgtccgacaccgtcgccaggttccccgtcgacatccagccgttc
agggacatgatcgaggggatgcgcatggacctgaagaagtcccggtacaggagcttcgacgagctgtacctctactgctactacgtggccggcaccgtggggctgatgag
cgtcccggtgatgggcatctcgccggcgtccagggcggccaccgagacggtgtacaaggggcgctggcgctgggcctggcgaaccagctcaccaacatcctcagggacg
tcggcgaggacgccaggaggggacggatctacctcccgcaggacgagctggagatggcggggctctccgacgccgacgtcctggacggccgcgtcaccgacgagtggagg
ggcttcatgaggggccagatcgcgagggccagagccttcttcaggcaggcggaggaaggcgccaccgagctcaaccaggagagccgatggccggtgtggtcttctctgct
cctgtaccggcagatcctcgacgagatcgaggccaacgactacgacaacttcacccggagggcctacgttccgaagacgaagaagctgatggcgctgcccaaggcgtacc
tgagatcactggtggtgccctcctcctcttctcaggctgagagccggagacgctattccaccctaacatag
```

>Maize PSY3 protein sequence

```
MMSTSRAVKSPACAARRRQWSADAPNRTATFLACRHGRRLGGGGGAPCSVRAEGSNTIVCLEAEAWGGAPALPGLRVAAPSPGDAFVVPSEQRVHEVVLRQAALAAAAPR
TARIEPVPLDGGLKAAFHRCGEVCREYAKTFYLATQLMTPERRIAIWAIYVWCRRTDELVDGPNASHISALALDRWESRLEDIFAGRPYDMLDAALSDTVARFPVDIQPF
RDMIEGMRMDLKKSRYRSFDELYLYCYYVAGTVGLMSVPVMGISPASRAATETVYKGALALGLANQLTNILRDVGEDARRGRIYLPQDELEMAGLSDADVLDGRVTDEWR
GFMRGQIARARAFFRQAEEGATELNQESRWPVWSSLLLYRQILDEIEANDYDNFTRRAYVPKTKKLMALPKAYLRSLVVPSSSSQAESRRRYSTLT*
```

WURTZEL LAB
CLONE INFORMATION

Date Today: 12/01/05 Entered into database #364

CLONE NAME: pGEMT-RPSY3

Lab Clone Number/Name: pGEMT-RPSY3-1
Clone type: cDNA: Non-expression

New GenBank Accession: DQ356431 Original GenBank Accession: AK108154

Clone Description:
Rice *PSY3* full-length cDNA was amplified with primers #1155 and #1191 by using rice leaf cDNA as temperate. Then PCR product was placed into pGEMT-easy vector (Promega) and verified by sequencing.

Constructed by: Faqiang Li Date Nov. 4. 2005
Purified by: Faqiang Li
DNA Location (-20°C) Box Number: #9 Position: A1 Conc. >0.5µg/µl
Tube labeled as: Rice PSY3 pGEMT-RPSY3-1 NOV.4.2005
Strain Location (-80°C) Box Number: 14 Position F2
Tube labeled as:pGEM-T-RPSY3 Strain: Top 10 F'

Cited in journal:
Li, F., Vallabhaneni, R., and Wurtzel, E.T. (2007) PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and a key regulator of abiotic-stress-induced root carotenogenesis. Plant Physiology. First published on December 27, 2007; 10.1104/pp.107.111120

Lab Notebook to reference: Li book 4 page19 Date: Nov. 4. 2005

Organism source of gene: Rice (variety indica IR36)

Cloning vector used: pGEM-easy-T vector Vector size: 3kb Insert size: 1.4 kb

Antibiotic markers: Amp

Restriction enzyme(s) to release insert: *Eco*RI

Sequence verified: yes Junction verified: no

>rice psy3 cDNA sequence edited form pGEMT-rpsy3 sequences

GTCGACCTGCAGGCGGCCGCGAATTCACTAGATGATTAAGTACGTCGTCGTCGGCC*ATGATGTCCACCACCACCACCAGCAGCGCGGCGGGGTCGCCGGTGTGCGCTCGT*
CGGCGGCAACGCGTGTTCGTCGACGTGCCGAGGCGCCGGGCGACGTCGTTGGCCAGGGTCGAGTACGCGAAGATGGCGCCGCCGCCGCCGCCGCCGTGCTCCGTGCGCGC
CGCGGGCTCCAATCCAATTGGCTGCCTCGAGGTAGCAGAGCCGTGGAGCGGCGCCGCGCCGCCCCCGCTGCCGCCGCTGCCGGGCCATCTGCATGTCGCGGCGCCGGCGG
CGGAGGACGACGACGACGCGCTCGCCGCCGCCGCCGCCGCCGTGCCGTCCGAGCAGAGGGTGCACGACGTCGTTCTGAAGCAAGCGGCGCTCGCCGCGGCAGCGCCCGAG
ATGCGGAGGCCGGCGCAGTTGGCCGAGAGGGAGCGCGTCGCCGGCGGCCTGAACGCCGCCTTCGATCGCTGCGGCGAGGTCTGCAAGGAGTACGCCAAGACATTCTACCT
CGCGACGCAGCTGATGACGCCGGAGAGGAGGAGGGCAATCTGGGCGATATACGTGTGGTGCAGGCGAACGGACGAGCTGGTGGATGGGCCCAACGCGTCGCACATGTCGG
CGCTGGCTCTCGACCGGTGGGAGTCGCGGCTGGACGACATCTTCGCCGGCCGCCCCTACGACATGCTCGACGCCGCCCTCTCCCACACCGTCGCCACCTTCCCCGTCGAC
ATCCAGCCGTTCAGGGACATGATCGAGGGGATGCGATTGGACCTAACGAAGTCGAGGTACAGGAGCTTCGACGAGCTCTACCTCTACTGCTACTACGTCGCCGGAACGGT
CGGGCTCATGACGGTGCCGGTCATGGGCATCTCGCCGGACTCGAGGGCCAACACCGAGACCGTCTACAAGGGGGCACTGGCTCTCGGCCTCGCCAACCAGCTCACCAACA
TTCTCAGGGACGTCGGCGAGGATGCAAGAAGAGGCAGGATCTATCTCCCAATGGACGAGCTCGAGATGGCCGGACTCTCTGAAGATGACATCTTCGACGGCCGCGTCACG
GACAGATGGAGATGTTTTATGAGGGATCAGATCACGAGAGCGAGGGCGTTTTTCAGACAGGCCGAAGAAGGCGCCAGCGAGCTCAATCAGGAGAGCCGATGGCCGGTTTG
GGCTTCTCTACTTCTGTACCGACAAATCCTTGACGAGATCGAGGCCAACGATTACAACAACTTCACCAAGAGGGCCTACGTCCCAAAGGCAAAGAAGATTGTGGCTCTGC
*CTAAAGCTTACTACAGATCACTCATGCTCCCCTCTTCAGTGAGGCACTGCTCTAGCCTAACATCATCATGA*TCAGCTTGTGGTATTGTTGTTACAATTTATCTTGTATTC
AAGTCTTGCAATGTACAAAGTATTGCAGTATCTGCAAATATATGTACACTTTAACATTGTGTATATATAGGGAAGTTTCTATGAAACAGAGTAGTGTATATATGACCATA
TTTCATAGGTCATACTACTCTTGCATAATGCTGTCAGCTTATCACTCATTGCTTCAGTCAATCAGCTAGGTTGTGAAAGTACCTAACTATCTATATGAAATATTAGTCTT
ATCAGCATGG

> Rice PSY3 protein sequence (444 aa)

MMSTTTTSSAAGSPVCARRRQRVFVDVPRRRATSLARVEYAKMAPPPPPPCSVRAAGSNPIGCLEVAEPWSGAAPPPLPPLPGHLHVA
APAAEDDDDALAAAAAAVPSEQRVHDVVLKQAALAAAAPEMRRPAQLAERERVAGGLNAAFDRCGEVCKEYAKTFYLATQLMTPERRR
AIWAIYVWCRRTDELVDGPNASHMSALALDRWESRLDDIFAGRPYDMLDAALSHTVATFPVDIQPFRDMIEGMRLDLTKSRYRSFDEL
YLYCYYVAGTVGLMTVPVMGISPDSRANTETVYKGALALGLANQLTNILRDVGEDARRGRIYLPMDELEMAGLSEDDIFDGRVTDRWR
CFMRDQITRARAFFRQAEEGASELNQESRWPVWASLLLYRQILDEIEANDYNNFTKRAYVPKAKKIVALPKAYYRSLMLPSSVRHCSS
LTSS

WURTZEL LAB
CLONE INFORMATION

Date Today: 01/06/04 Entered into database #404

CLONE NAME: pETa-SPSY1

Lab Clone Number/Name: pETa-SPSY1 alternative name: pETaSPSY1_2
Clone type: expression cDNA clone

Original GenBank Accession: CD234165

Clone Description:
Sorghum *PSY1* expression vector. Sorghum *PSY1* Full length cDNA was amplified from clone CD234165 with primers 820 (*EcoR*I) and 839 (*Hind*III). Then the fragment was placed into pET23a vector between *EcoRI* and *Hind*III sites. Its function was verified by complementation test.

Constructed by: Faqiang Li
Purified by: Faqiang Li
DNA Location (-20°C) Box Number: #9 Position: B7 Conc. >1µg/µl
Tube labeled as: pETaSPSY1_2
Strain Location (-80°C) Box Number: 14 Position C10
Tube labeled as: pETaSPSY1_2 Strain: BL21 (DE3)

Cited in journal:
Li, F., Vallabhaneni, R., and Wurtzel, E.T. (2007) PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and a key regulator of abiotic-stress-induced root carotenogenesis. Plant Physiology. First published on December 27, 2007; 10.1104/pp.107.111120

Lab Notebook to reference: Li book 3 page 19 and 35 Date: 5/17/04

Organism source of gene: Sorghum bicolor

Cloning vector used: pET23a (+) Vector size: 3.7 kb Insert size: 1.3 kb

Antibiotic markers: amp

Restriction enzyme(s) to release insert: *EcoR*I and *Hind*III

Sequence verified: no Junction verified: yes

WURTZEL LAB
CLONE INFORMATION

Date Today: 01/06/04 Entered into database #405

CLONE NAME: pETa-SPSY3

Lab Clone Number/Name: pETa-SPSY3 Alternative name: pETa-SPSY3_8
Clone type: Expressive cDNA clone

Original GenBank Accession: BG464544
New GenBank Accession: AY705390

Clone Description:
Sorghum *PSY3* expression vector. Full length cDNA was amplified from clone BG464544 with primers 840 (*EcoR*I) and 841 (*Hind*III). Then the fragment was placed into pET23a (+) vector between *EcoRI* and *Hind*III sites. Its function was verified by complementation test.

Constructed by: Faqiang Li
Purified by: Faqiang Li
DNA Location (-20°C) Box Number: #9 Position: B8 Conc. >1 µg/µl
Tube labeled as: pETa-SPSY3-8
Strain Location (-80°C) Box Number: #14 Position E3
Tube labeled as: pETa-SPSY3_8 Strain: BL21 (DE3)

Cited in journal:
Li, F., Vallabhaneni, R., and Wurtzel, E.T. (2007) PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and a key regulator of abiotic-stress-induced root carotenogenesis. Plant Physiology. First published on December 27, 2007; 10.1104/pp.107.111120

Lab Notebook to reference: Li book 3 page 35 – 46 Date: 05/20/04-09/08/04

Organism source of gene: Sorghum bicolor

Cloning vector used: pET23a (+) Vector size: 3.6 kb Insert size: 1.3 kb

Antibiotic markers: Amp

Restriction enzyme(s) to release insert: *EcoR*I and *Hind*III

Sequence verified: no Junction verified: yes

WURTZEL LAB
CLONE INFORMATION

Date Today: 01/06/04 Entered into database #406

CLONE NAME: CD230490

Lab Clone Number/Name: CD230490 Alternate name(s): SS1_44_D07
Clone type: EST

Original GenBank Accession: CD230490

Clone Description:
Sorghum EST containing *PSY1* full-length cDNA

Constructed by: Dr. Yutaka Suzuki and Dr. Sumio Ugano Date rec'd 4/2/04
Purified by: Faqiang Li
DNA Location (-20°C) Box Number: #9 Position: E2 Conc. >1µg/µl
Tube labeled as: SS1_44_D07
Strain Location (-80°C) Box Number: 14 Position B1
Tube labeled as: SS1_44_D07 Strain: Top 10F'

Cited in journal:
Li, F., Vallabhaneni, R., and Wurtzel, E.T. (2007) PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and a key regulator of abiotic-stress-induced root carotenogenesis. Plant Physiology. First published on December 27, 2007; 10.1104/pp.107.111120

Lab Notebook to reference: Li book 3 page 5 Date: 4/2/04

Organism source of gene: Sorghum bicolor Variety: IS3620C

Cloning vector used: pME18s-FL3 Vector size: 3392 bp Insert size: ~2kb

Antibiotic markers: amp

Restriction enzyme(s) to release insert: *Xho*I

Sequence verified: no Junction verified: yes

WURTZEL LAB
CLONE INFORMATION

Date Today: 01/06/04 Entered into database #407

CLONE NAME: CD234165

Lab Clone Number/Name: CD234165 Alternate name(s): SS1-6-D10
Clone type: EST

Original GenBank Accession: CD234165

Clone Description:
Sorghum EST containing *PSY1* full-length cDNA

Constructed by: Dr. Yutaka Suzuki and Dr. Sumio ugano Date rec'd 4/2/04
Purified by: Faqiang Li
DNA Location (-20°C) Box Number: #9 Position: E3 Conc. >1 µg/µl
Tube labeled as: SS1-6-D10
Strain Location (-80°C) Box Number: #14 Position B2
Tube labeled as: SS1-6-D10 Strain: Top10F'

Cited in journal:
Li, F., Vallabhaneni, R., and Wurtzel, E.T. (2007) PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and a key regulator of abiotic-stress-induced root carotenogenesis. Plant Physiology. First published on December 27, 2007; 10.1104/pp.107.111120

Lab Notebook to reference: Li book 3 page 5 Date: 4/2/04

Organism source of gene: Sorghum bicolor Variety: IS3620C

Cloning vector used: pME18s-FL3 Vector size: 3392 bp_ Insert size: ~2kb

Antibiotic markers: amp

Restriction enzyme(s) to release insert: *Xho*I

Sequence verified: yes Junction verified: yes

> CD234165

GCACGAGGGTTGCTCCTCGCTCTTCTGCTGATTGGTCTCCTCTCTCATCCCATCATCGTCTTCTTCGAGAGATAAGAATAAATAATACTATATGGCCATC
ATACTCGTACGAGCAGCGTCGCCGGCGGGGCTCTCCGACGCCGCCGGCATCATCAGCCACCATGGGAGTCTCCAGTGCTCCTCTCTGCCTCTGCTCAACA
AGAGGCCGGCCCCGGCCCGGCGGTGGATGCTCTGCTCGCTCAGGTACGGGTGCCTTGGCCTCGACCCCGGCCGCCCCTCCCCGCCCGCCGTCTACTCCAG
CCTCGCCGTCAACCCGGCGGGAGAGGCCGTCGTCTCGTCCGAGCAGAAGGTCTACGACGTCGTGCTCAAGCAGTCGGCATTGCTCAAACGCCAGCTGCGC
AAGCCGGTCCTCGACGTCGTCAGGCCCCAGGAGGACCTGGCGGAGATGCCACGCAACGGCCTCAACCAAGCCTACGACCGCTGCGGCGAGATCTGTGAGG
AGTATGCCAAGACGTTTTACCTCGGAACTATGTTGATGACAGAGGAGCGGCGCCGCGCCATATGGGCCATCTATGTGTGGTGTAGGAGGACAGATGAGCT
TGTGGATGGGCCAAACGCCAACTACATTACACCAACAGCTTTGGACCGGTGGGAGAAGAGACTTGAGGATCTGTTCGCGGGACGTCCTTACGATATGCTC
GATGCTGCTCTCTCTGATACCATCTCAAGGTTCCCCATAGACATTCAGCCATTCAGGGACATGATTGAAGGGATGAGGAGTGACCTTAGGAAGACAAGGT
ATAACAACTTTGACGAGCTCTACATGTACTGCTACTATGTTGCTGGAACTGTCGGGTTAATGAGCGTACCTGTGATGGGCATCGCACCCGAGTCTAAGGC
AACAACTGAAAGTGTGTATAGTGCTGCCTTGGCTCTCGGAATTGCTAACCAACTCACGAACATACTCCGGGATGTTGGAGAGGATGCTACAAGAGGGAGG
ATATATTTACCACAAGATGAGCTCGCACAGGCAGGGCTCTCTGATGAGGACATCTTCAAGGGGGTCGTCACAAACCGATGGAGAAACTTCATGAAGAGGC
AGATCAAGAGGGCGAGGATGTTTTTTGAGGAGGCAGAGAGAGGGGTAACTGAGCTCTCACAGGCTAGCAGATGGCCAGTATGGGCTTCCCTGTTGCTGTA
CAGGCAAATCCTGGATGAGATTGAAGCGAACGACTACAACAACTTCACAAAGAGGGCCTATGTTGGTAAAGGGAAAAAATTGCTGGCACTTCCTGTGGCA
TATGGGAAATCGCTACTGCTACCGTGTTCATTGAGAAATACCCAAACCTAGCCGCCAGAGAAGCTGCAAAGAAAGGGGTTCAGGTTAGGCTAGATAGAAA
TTAAATAGGGCAATGTCATCACATCAGACCTGATGAAAATAGACAACCTGGTGAATTGGTGGGGTATCATACGAATTGTTGGGATCAGGTACAGCACAGA
TTAAAGCCAACCTAGATGGATGTGGAACA

WURTZEL LAB
CLONE INFORMATION

Date Today: 01/06/04 Entered into database #408

CLONE NAME: AW679367

Lab Clone Number/Name: AW679367 Alternate name(s): WS1_24_E02
Clone type: EST

Original GenBank Accession: AW679367

Clone Description:
Sorghum EST containing partial *PSY2* cDNA

Constructed by: Dr. Cordonnier-Pratt MM Date rec'd 4/2/04
Purified by: Faqiang Li
DNA Location (-20°C) Box Number: #9 Position: E5 Conc. >1µg/µl
Tube labeled as: WS1_24_E02
Strain Location (-80°C) Box Number: #14 Position B4
Tube labeled as: WS1_24_E02 Strain: Top10F'

Cited in journal:

Lab Notebook to reference: Li book 3 page 5 Date: 4/2/04

Organism source of gene: Sorghum bicolor

Cloning vector used: pBluescript II SK (+) Vector size: 3kb Insert size: ~500 bp

Antibiotic markers: amp

Restriction enzyme(s) to release insert: *Eco*RI + *Xho*I

Sequence verified: yes Junction verified: yes

> AW679367
GCACGAGGGCCAGATTCAGCGTGCCAGATTGTTCTTTGATGAGGCTGAGAAGGGCGTCGCCCATCTAGACTCTGCTAGCAGATGGCC
GGTTCTTGCGTCTCTTTGGCTGTACAGGCAGATCCTTGATGCCATTGAGGCAAACGACTACAACAACTTCACCAAGCGTGCGTACGT
*CGGCAAGGCCAAGAAGCTGCTGTCATTACCGGTTGCATATGCAAGGGCTGCTGTTGCATCATGA*ACCATCTGTAGATCAGATGTTTT
TGTTTTGTTTTTTTCTTTTTCTTTTCCAAACCCAGTTTGTTACCCCCTCACCTCCCATTTTTTTGCTTGGTTTCTTTGCTCTTGTAT
ATAATCAGCTTCAGCTGCCTGCATGGCATAATCCTTGCCTGTCACATACAGTTCTTAGTTCAGAGGGGACTGATTCCATGTCCCTCA
ATACTCGGCTCTTGTTACCAGAAGGAATGAAGAATTAGAATTCGAGAAGCAAAAAAAAAAAAAAAAAAAACTCGAGGGGGCCCGC

WURTZEL LAB
CLONE INFORMATION

Date Today: 01/06/04 Entered into database #409

CLONE NAME: BG464544

Lab Clone Number/Name: BG464544 Alternate name(s): EM1_71_A11
Clone type: EST

Original GenBank Accession: BG464544

Clone Description:
Sorghum EST containing *PSY3* full-length cDNA

Constructed by: Dr. Cordonnier-Pratt MM Date rec'd 4/2/04
Purified by: Faqiang Li
DNA Location (-20°C) Box Number: #9 Position: E4 Conc. > 1 µg/µl
Tube labeled as: EM1_71_A11
Strain Location (-80°C) Box Number: #14 Position B3
Tube labeled as: EM1_71_A11 Strain: Top10F'

Cited in journal:
Li, F., Vallabhaneni, R., and Wurtzel, E.T. (2007) PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and a key regulator of abiotic-stress-induced root carotenogenesis. Plant Physiology. First published on December 27, 2007; 10.1104/pp.107.111120

Lab Notebook to reference: Li book 3 page 5 Date: 4/2/04
Original clone name (if different): EM1_71_A11

Organism source of gene: Sorghum bicolor

Cloning vector used: pBluescript II SK (+) Vector size: 3 kb Insert size: 1.6 kb

Antibiotic markers: amp

Restriction enzyme(s) to release insert: *Eco*RI + *Xho*I

Sequence verified: yes Junction verified: yes

> BG464544

GGCACGAGGGTCACCTGCCACAGGAGAGAGGGCTAGGTTTTGTTTTTTNTAGTCCTCGGCCAGTGTCCTGCAGGGGCAACGGACAAAACTAACGCGAGACTTGTCGCCTGCTGGCCT
CACTCTGGTCTGTCACCTCTCACCTCCAGCCCCCGCCCACCCTCTGCTCTCCCGATCCCTCTCTATATAGCTCGGTCTGGCCACCAGCTTCCGATCCATATATCCCCCATCTGCGT
CTCTCCACTGCGACTGCGACCGAGCACGTACGCCGTCGCCGCGTGTCAGCCAACC*ATGATGTCCACCAGCCATGCGGTGAAGCAGTCGCCGGCGTGCGCGGCTCGTTGGCGGCGGC*
GGCAGCACGGCCAGCGGTCCGCGGACGCGCCGGCCCGCACGGCGACGTTCTCGGCTAGGCACGGGAGGCAGCGGCGAGGAGGCCGCGGCGTGGCGCCGTGCTCCGTGCGCGCCGCG
GGCTCCAACACCATTGGCTGCCTCGAGGCCGAGGCGTGGGGCGGCGCCGCGCACGCCCCGGCTCCGCCCCTTCGCTGCTGCTGCCGAGCTTCCATGTGGAGGCGCCGGCGCCCGG
GGGCGACGCGCTCGCCGTGCCGTCCGAGCAGAGGGTGCAGGAGGTCGTGCTCAAGCAGGCGGCGCTCGCGGCCGCGGCGCCGAGGACGGCGCGGATCGAGCCCGTGCCCATGGACG
GCGGGCTCAAGGCGGCCTTCCACCGCTGCGGGGAGGTCTGCCAGGAGTACGCCAAGACATTCTACTTGGCGACGCAGCTGATGACACCGGAGAGGAGGAGGGCAATCTGGGCAATA
TACGTGTGGTGCAGGAGAACGGACGAGCTGGTCGACGGGCCCAACGCGTCCCACATCTCGGCGGTGGCGCTGGACCGGTGGGAGTCGCGGCTGGAGGACATCTTCGCCGGCCGGCC
GTACGACATGCTCGACGCCGCCCTGTCCGACACCGTCGCCAACTTCCCCGTCGACATACAGCCGTTCAGGGACATGATCGAGGGGATGCGCATGGACCTGAAGAAGTCCCGGTACC
GGAGCTTCGACGAGCTGTACCTCTACTGCTACTACGTGGCCGGCACCGTGGGGCTGATGAGCGTCCCGGTGATGGGCATCTCGCCGGACTCCAGGGCGGCCACCGAGACGGTCTAC
AAGGGGGCGCTGGCGCTGGGCCTGGCCAACCAGCTCACCAACATCCTCAGGGACGTCGGCGAGGACGCCAGAAGGGGAAGGATCTATCTCCCACAGGACGAGCTGGAGATGGCGGG
GCTCTCCGACGCCGACATCTTGGACGGCCGGGTCACCGACGAGTGGAGGAGCTTCATGAGGGGCCAGATCACGAGGGCCAGAGCCTTCTTCAGGCAGGCCGAGGAAGGCGCCACCG
AGCTCAATCAGGAGAGCCGATGGCCGGTGTGGGCTTCTCTGCTCCTGTACCGGCAGATCCTGGACGAGATCGAGGCCAACGACTACAACAACTTCACCAGGAGGGCCTACGTTCCA
*AAGACGAAGAAGCTGATGGCGCTGCCCAAGGCGTACCTGAGATCACTGGTTCCCCCCTCTTCTTCTCAGGCTGAGAGACAGAGACACCACTCCAGCCTAACATAG*AACGGTCTCTG
TCGCGTTCGGTACATAGCAGTGAATGATGTACAAATTTCTGGTCAGGCTACATACATCAATAGTAGTACATGGTTGGCATGTAGTGGTGGTGGTNGGTGGTGGTGGCGCAGATTTC
TTGTACTCGGAGTTTTGCCATGTATTACAAAAAGCACTGGATATATCACATAGATATGTACACTAA

WURTZEL LAB
CLONE INFORMATION

Date Today: 07/05/1997 Entered into database #72

CLONE NAME: pET23a
Lab Clone Number/Name: pET23a Clone type: expression vector
Clone Description: Expression vector, see Novagen catalog
Purified by: RuiBai DNA Location (-20°C) Box Number: #3 Position: 9B, 9C Tube labeled as: pET23a Strain Location (-80°C) Box Number: #7 Position: 5H Tube labeled as: pET23a Strain: Noveblue

Vector size: 3.66 kb
Antibiotic markers: amp

WURTZEL LAB
CLONE INFORMATION

Date Today: 07/05/1997 Entered into database #73

CLONE NAME: pET23b
Lab Clone Number/Name: pET23b Clone type: expression vector
Clone Description: Expression vector, see Novagen catalog
Purified by: RuiBai DNA Location (-20°C) Box Number: #3 Position: 6D Tube labeled as: pET23b Strain Location (-80°C) Box Number: #7 Position: 5I Tube labeled as: pET23b Strain: Noveblue

Vector size: 3.66 kb
Antibiotic markers: amp

Appendix 4 List of PCR primers used in this study

Appendix 4.1 List of maize *PSY1* primers

Oligo #	Sequence (5'-3')		Purpose
109<	CAGGTACGCTCATTAACCCG	nt 4267-4286[a]	Genomic DNA sequencing
217>	GATGCCCAATGCCAAGAAATAACC	nt 1344- 1367[a]	
257>	TGATGCCGCTCTCTCTGATA	nt 3229-3248[a]	
344<	GGCGCGGCGCCGCTCCTCTGTC	nt 2451-2472[a]	
509>	GCATTGCTCAAACGCCAG	nt 2201- 2218[a]	
519<	TCAGAGAGAGCGGCATCAAG	nt 3227-3246[a]	
254<	CAGGATCTGCCTGTACAACA	nt 4798-4779[a]	Real time PCR primer
505>	CATCTTCAAAGGGGTCGTCA	nt 4559-4578[a]	
627>	ATGGCCATCATACTCGTACGAGCAGCGTC	nt 1-29[b]	cDNA sequencing
657<	TCATCA AGGCCTCCTGA GTT	nt 1293 - 1313[b]	
701<	ACCTTGCATTGAAGCTTCTCTG		Full-length cDNA isolation
707>	CCGCGGGATCCTCTAGAATGGCCATC		See clone sheet: pTMPSY1f

Note: a, the GenBank Acce No. is U32636
b, The GenBank Acce No. is AY324431

Appendix 4.2 List of maize *PSY2* primers

oligo#	Sequence (5'-3')		Purpose
503>	TCACCCATCTCGACTCTGCTA	nt 1005-10025[a]	Real time PCR primers
676<	GATGTGATCTACGGATGGTTCAT	nt 1183-1205[a]	
622>	AAGGATCgaattcGGCACGG		Expression vector construction; See clone sheet pEMPSY2-1
635<	TCCTTGTAACTCGAGCTGATTGAG		

Note: a, the GenBank Accession No. is AY450646

Appendix 4.3 List of maize *PSY3* primers

oligo#	Sequence (5'-3')		Purpose
950>	ACGCTAGG ACGGTGAAAG TGGGGATACA	nt 2926- 2953[a]	Genomic DNA sequencing
960>	CAGTCGTACGTATCCTTGGTAGAAGCCTG	nt 2198- 2226[a]	
1143>	TGCTCCTCCTGTCGCTCCCA	nt 95-114[a]	
1202>	TCAGAATTAATCAAATACGCTAGTGGA	nt 2279-2305[a]	
1203<	TCAGAATTAATCAAATACGCTAGTGGA	nt 2279-2305[a]	
1121<	TGTGGCAGGCGACCGGCCACTGGTGTTCG	nt 1245-1273[a]	
1127<	TAGCAGCCATCACCATCAGCCATGG	nt 826-850[a]	
1128<	GGTTCAGACGTTGTCCTGGTGGAGA	nt 741- 765[a]	
925>	ATGAATTCTAC GAG CCG CGC GGT GAA	nt 1-26[b]	Full length cDNA isolation;
926<	GAGCTCGAGACCGGTCTATGTTA	nt 1251-1272 [b]	See clone sheet pET23b-mPSY3;
996>	GCCAGAGCCTTCTTCAGGCAGG	nt 995-1016 [b]	Real time PCR primers
997<	GTCTTCGGAACGTAGGCCCTCCG	nt 1133-1155 [b]	

Note: a, the GenBank Accession No. is DQ372936
b, the GenBank Accession No. is DQ356430

Appendix 4.4 List of rice and sorghum *PSY* primers

oligo#	Sequence (5'-3')	Purpose
569>	ACTCGAGATGGCGGCCATCACGCTCCTAC	Rice *PSY1* full-length cDNA cloning; See clone sheet: pT-RPSY1-1
570<	AGGTACCGAGGCCGTTTCTTGGCAGCTC	
597>	GAAGGTGTACGACGTCGTCCTCAAG	Rice *PSY1* full-length cDNA cloning; See clone sheet: pT-RPSY1-1;
598<	TCCTACTTCTGGCTATTTCTCAGTG	
820>	TAAAGAATTCTATATGGCCATCATA	Sorghum *PSY1* expression vector construct; See clone sheet pETaSPSY1-2;
839<	TGAAGCTTCTCTGGCGGCTAGGT	
822<	TGTACAGCCAAAGAGACGCAAGATC	sorghum psy2 reverse primer
840>	TGTCAGGAATCCATGATGTCCACCAGC	Sorghum *PSY3* expression vector construct; See clone sheet pETa-SPSY3-11;
841<	CGCGACAGAG AAGCTTCTATGTT	

Appendix 5 List of mutants used in this research

	Maize Genetics COOP stock ID	phenotype	Mutated Gene	Chapter
y1-602c	602C	white endosperm	Maize *PSY1*	3
y1-8549	602M	white endosperm zebra leaf	Maize *PSY1*	3
vp9-Bot100	706B	albino seedling white endosperm	Maize *ZDS*	4
y9	X07C	pale yellow endosperm	Unknown	2, 4
elm1	804F	pale green seedling long mesocotyl	Maize phytochromobilin synthase	3

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