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Carotenogenesis in maize and rice

By

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Abstract

CAROTENOGENESIS IN MAIZE AND RICE

by

Paul David Matthews

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Carotenoids have diverse roles at all levels of biological organization. These plant pigments serve as light-harvesters, photoprotectors, antioxidants, colorants, animal attractants, and as metabolic precursors to odorants and abscisic acid. In animals they are dietary precursors to retinoic acid, retinal, and retinol. Because of these health-related roles in human and animals, they impact on both natural and human ecology. Presented is the development and improvement of tools for the comparative understanding and metabolic engineering of carotenogenesis in representative cereal crops, maize (*Zea mays* L) and rice (*Oryzae sativa* L.). Improvements to an *Erwinia uredovora* heterologous genetic functional complementation system in *Escherichia coli* include (1) ranking and choice of bacterial strains (TOP10F' ranked best) for stability and degree of carotenoid accumulation and (2) recommendations for color complementation screening of cDNA libraries and (3) increased (up to10.8 times) accumulation of zeaxanthin and lycopene by metabolic engineering of carotenogensis with genetic modulation of the isoprenoid precursor pool by overexpression of deoxyxylulose phosphate synthase (DOXP). I also report functional analyses of the concerted action of maize phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS) by combined HPLC separation and PDA analyses of

geometric isomers of carotenoids accumulating in *E. coli*. Maize PDS and ZDS mediated a poly-Z-desaturation pathway to 7,9,7,9'-*Z*-lycopene (poly-*cis*-lycopene or prolycopene). The existence of a poly-Z-desaturation pathway for maize PDS and ZDS is discussed in relation to two divergent interpretations. For each interpretation implications for genetic manipulation and further study of maize carotenogenesis are given. Finally, I report isolation and characterization of a partial, rice cDNA and corresponding gene of phytoene synthase (*Psy*). I also report the chromosomal map location of rice *Psy*. I analyzed protein and nucleic acid primary sequence for chloroplast transit peptide prediction, protein domain homology, phenetic phylogenetic relationships, gene copy number and possible syntenic relationships among maize and rice. The relation of the novel rice *Psy* gene to the genetic locus, maize *Yellow1* (*Y1*), is also considered.

Dedicated

to

those who inspired me early:

~through the literature~

Armin Braun and Mary-Dell Chilton

~through the vine~

Dominick V. Basile, Mary KB. Berlyn, and Dwight T. Kincaid

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Abbreviations

%G+C	per cent guanosine plus cytosine
ABA	abscisic acid
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BLAST	basic local alignment of sequence tool
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
ChloroP	chloroplast transit peptide prediction
Cpn60	chaperonin60
CPonCD	Current Protocols on compact disc
CRTB	bacterial phytoene synthase
CTP	cytosine triphosphate
DAP	days after pollination
DEPC	diethylpyrocarbamate
DMAPP	dimethylallyl pyrophosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dpi	dots per inch
DTT	dithiothreitol
Dw	dry weight
DXP	D-1-deoxyxylulose 5-phosphate

DXR	D-1-deoxyxylulose 5-phosphate reductase
DXS	D-1-deoxyxylulose 5-phosphate synthase
E/Z	*entgeigen/zuzammen*
EDTA	ethylenediamine tetraacetate
EST	expressed sequence tag
ESTDB	expressed sequence tag database
FAD	flavoadenine dinucleotide
FDFT	farnesyl diphosphate farnesyl transferase
FPH	formamide prehybridization buffer
FPP	farnesyl pyrophosphate
FPS	farnesyl phosphate synthase
GAP	glyceraldehyde 3-phosphate
GGPP	geranylgeranyl pyrophosphate
GGPPS	geranylgeranyl pyrophosphate synthase
GPS	*geranyl phosphate synthase*
GTE	glucose tris ethylenediamine tetraactate
GTP	guanosine triphosphate
HMGCoA	hydroxymethylglutaryl-CofactorA
HPLC	high performance liquid chromatography
HYD	hydroxylase
Idi	*isopentyl diphosphate isomerase*
IOD	integrated optical density
IPP	isopentyl pyrophosphate

IPPI	isopentyl pyrophosphate isomerase
IPTG	isopropyl thioglactoside
Kb	kilobase
LB	Luria-Bertani
LCY	lycopene cyclase
MaizeDB	maize database
MRNA	messenger ribonucleic acid
NAD	nicotinamide adenine diphosphate
NADP	nicotinamide adenine diphosphate phosphatase
NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction
PDA	photodiode array
PDS	phytoene desaturase
Pfu	plaque forming units
pH	partial hydrogen ion concentration
pI	isoelectric point
Prodom	protein domain
PSY	phytoene synthase
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	retention time
RT-PCR	reverse transcriptase-polymerase chain reaction
SAPS	statistical analysis of protein sequence

SDS	sodium dodecyl sulfate
Sh	*shrunken*
Sh1	*shrunken1*
SQS	squalene synthase
SSC	sodium chloride sodium citrate
STET	sodium chloride tris ethylenediamine tetraacetate triton
Swissprot	Swiss protein database
Taq	*Thermophilus aquaticus* DNA-dependent DNA polymerase
TBE	tris borate ethylenediamine tetraacetate
TE	tris ethylenediamine tetraacetate
Tris	[tris(hydroxymethyl)aminomethane]
Tris-HCL	Tris-hydrochloride
tRNA	transfer ribonucleic acid
UCD	University of California at Davis
UTP	uracil triphosphate
UV	ultraviolet
vp2	*viviparous2*
vp5	*viviparous5*
vp9	*viviparous9*
Y1	*Yellow1*
ZDS	zetacarotene desaturase
Z-GGPP	*zuzammen*-geranylgeranyl pyrophosphate

Chapter 1. Introduction

1.1 Roles and distribution of carotenoids

Carotenoids are polyene pigments that are widely distributed at the ecological, organismal, tissue and cellular levels of biological organization (Pfander, 1992). Although some are colorless, carotenoids generally range in color from light yellow to deep orange and pink. Because of their diverse functions in various organisms, from bacteria, fungi, and algae, to higher plants, animals, and humans, carotenoids have continued to remain of great scientific and popular interest (Wurtzel, 2001; Ye et al., 2000). In plants, carotenoids serve as light harvesters in photosynthesis, photo-oxidative stress protectors, animal attractors, and precursors of abscisic acid (Goodwin, 1980b). Because of their role in the synthesis of Vitamin A and retinoids in humans and animals (Britton et al., 1995), they have obvious relations to human-health issues [reviewed in (Bendich and Olson, 1989; Bertram, 1993; Bhaskaram, 1995; Fisher et al., 1970; Goodwin, 1986; Nishino, 1998; Simpson and Chichester, 1981; Sommer, 1997).

1.2 Structure of carotenoids

Carotenoids are C_{40} polyenes formed from the condensation of the C_5 phosphate-activated isoprenes (reviewed in (Pfander, 1992)). Thus, they are a polymer of eight isoprenes. An example of a carotenoid, lycopene, is show in **Figure 1.1** along with carbon numbering conventions.

The polyene is the result of the head to head condensation of two C_{20} polyenes that have

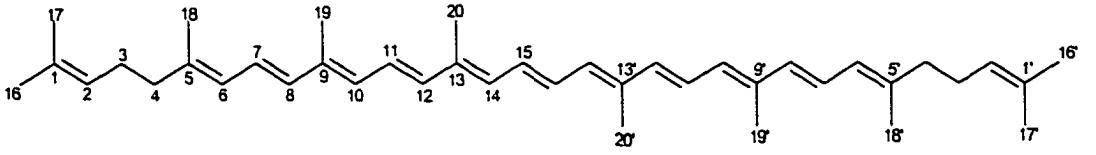

Figure 1.1 Numbering of carbon atoms in lycopene, the core of plant carotenoids

condensed in a head to tail fashion. An illustrative notation where ip indicates an isoprenoid is: ipipipippipipipi. The hydrocarbon chain is symmetrical around the 15-15' bond. Modifications of end-structures, such as cyclization and subsequent oxygenation, may preserve the symmetry if the end-groups are identical or destroy it if they are non-identical.

Figure 1.2 shows β-carotene with its identical end-structures.

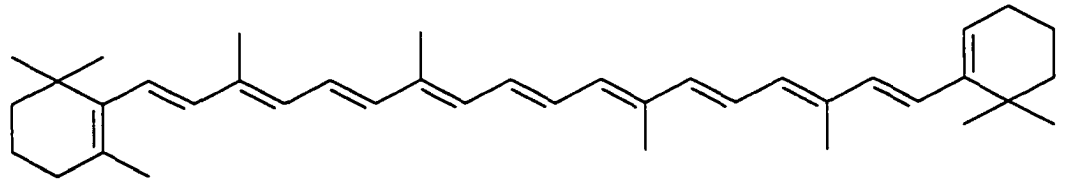

Figure1.2 End structures of the symmetric β-carotene.

A functionally distinguishing feature of carotenoids is a chromophoric system of conjugated double bonds along the extended polyene chain. In plants modifications of the core lycopene chain by hydrogenation, dehydrogenation, cyclization, and oxidation occur primarily at both ends of the polyene (Weedon and Moss, 1995), affecting color,

solubility and antioxidative potential. While variable desaturation and increase in the extent of the double bond system changes the color from colorless to reds to oranges, geometric isomerization of the double bonds favoring *Z*-configurations (*cis*-configurations) decreases the coloration. Carbon chirality also gives carotenoids optical isomeric forms. Each small variation in form may correlate with variation in biological activity.

1.3 Biological functions of carotenoids

Discussion of biological function must be prefaced with a confession of philosophical prejudice (Kuhn, 1962). In biology, functional classifications are ideally framed within phyletic hypothesis (Dobzhansky, 1970). A phyletic hypothesis may be gleaned from relationships among the genes, which code for the carotenoid biosynthetic complexes, and an understanding of the functional mechanics of the complexes themselves. Only a thorough understanding of the comparative mechanics of carotenoid functions among plastid-types, tissue-types and species will allow such understanding. A description of the evolutionary relationships among carotenoid functions is a superior classification system, but such a description is currently embryonic. Complete sets of genes and enzyme complexes need to be isolated and probed for function. Furthermore, **Chapters 5** and **7** will present and discuss evidence for the shortcomings of the reductionistic approach employed here. An information component of a complex, interacting system will be inferred in interpretation of the results in **Chapter 5** and a systems approach (Wiener, 1948) suggested as an anticipated solution.

Meanwhile, I believe a multiplicity of artificial, functional classifications—despite their inconsistencies—may serve *en route* as heuristic hypotheses to guide the implementation of reductionistic tools. Two such flawed *facilie au tempaire* are presented below as examples of paradigms (Kuhn, 1962) that invade my later discussions.

Carotenoids function in three general functional modes (1) absorption of light (2) metabolic precursor and (3) mitigation of oxidative stress. Specific examples of each role are given in subsections dedicated to each functional mode.

1.3.1 Functional mode 1: Properties as pigments

The color of carotenoids is due to the extended system of double-conjugated bonds within the polyene chain. The electrons in this extended energy-probability space interact with photons in a variety of ways to realize the multifarious functions. By virtue of their absorption and transfer of photonic energy, carotenoids serve in:

(1) Light absorption as photosynthetic accessory pigments. In leaves of plants the carotenoids, β-carotene, lutein, neoxanthin, antheraxanthin, zeaxanthin, and violaxanthin are found associated with proteins of photosystem I and II, where they harness energy from 400-500 nm light (Mathis and Schenck, 1982; Young, 1991). In chloroplasts, the carotenoids are in close association with the chlorophyll in pigment-protein complexes of the thylakoid membranes (Britton, 1993). In brown algae, fucoxanthin, the most abundant carotenoid in Nature, harnesses energy from light in a photosynthetic membrane complex (Siefermann-Harms et al., 1985). The energy absorbed by carotenoids is transferred to chlorophyll or bacteriochlorophyll by singlet-singlet excitation between the pigment

molecules in a subsequent and distinct process (Cogdell and Frank, 1987; Young and Frank, 1996).

(2) Photoprotection. Because of their ability to absorb light energy and release the energy as heat or fluorescence (Demming et al., 1989; Phillip et al., 1996), carotenoids act to block penetration of light into tissues (Bartley and Scolnik, 1995; Fan et al., 1998; Tuveson and Sandmann, 1993) and protect against photosensitization (Sandmann et al., 1998) (reviewed in (Bramley and Mackenzie, 1988; Goodwin, 1980b; Krinsky, 1991)). Protection against light induced photo-oxidative stress also occurs as an inducible response system.

Xanthophylls, which are oxygenated carotenoids, such as zeaxanthin and violaxanthin, participate in a non-radiative dissipation of excess chlorophyll excitation energy in response to photo-oxidative stress (Demmig-Adams, 1990; Demmig-Adams and Adams, 1993; Young, 1991). Zeaxanthin competes for excitation energy with chlorophyll, but releases it harmlessly as non-radiative energy (heat). The induction occurs when violaxanthin, which accumulates under low-light conditions, undergoes rapid recursive de-expoxidation to zeaxanthin via antheraxanthin at high light intensities. The reverse process (expoxidation) restores the responsive potential in the dark (Demmig-Adams, 1990; Demmig-Adams and Adams, 1993; Young, 1991).

(3) Ornamentation and animal attractant. Carotenoids function as ornamentation in flowers and fruits. They attract animal vectors, namely: pollinators and seed dispersers.

Additionally, the oxidative breakdown products of carotenoids are sweetly fragrant and impart attractive odors to flower and fruit (Govindarajan, 1986). Carotenoids, produced by plants and ingested by animals, also serve as colorants and photoprotectants in the skins of animals and the feathers of birds. Humans are also attracted to the color of animal flesh that has accumulated carotenoids, favoring the yellow skins of chickens (Bauernfeind, 1972; Brush, 1990) and the pinkness of salmon (Andersen et al., 1990; Khare et al., 1973). Carotenoids have been proposed as indicators of parasite-load in sexual selection systems. For example, the cockerel's bright red wattle and comb are due to accumulation of dietary carotenoids and this accumulation is negatively affected by intestinal parasites (Zuk, 1992). Using the pigment-filled organs as visual cues of resistance to parasite infection, hens select healthy males with the reddest wattles and combs for mating (Zuk, 1992; Zuk et al., 1990). Hence, carotenoids may have been enlisted in an evolutionary arms race between parasite and host as a visual cue operating in the host's sexual selection system (Hamilton et al., 1990; Ridley, 1993).

1.3.2 Functional mode 2: Properties as metabolic precursors.

(1) Precursor to abscisic acid (ABA). The oxidative cleavage of the carotenoid zeaxanthin produces xanthoxin, the precursor to ABA. ABA regulates plant growth and development, leaf and petal abscission, embryo development (Fong et al., 1983), dormancy (Fong et al., 1983), and responses to water stress (Parry and Hogan, 1991; Zeevaart and Creelman, 1988).

(2) Precursor to Vitamin A and retinoids. In animals, some dietary carotenoids (mostly β-carotene) serve as precursors for retinal (vision), retinoic acid (morphogen) and retinol (vitamin A). These compounds have well known roles in health for limb development (Jiang et al., 1995), vision (Sommer, 1988), immune function (Ross, 1992; Ross, 1996; Sommer, 1988), as well as mucosal membrane integrity and related disease susceptibility (Berman, 1991; Bhaskaram, 1995; Tomkins, 1991).

1.3.3 Functional mode 3. Properties in mitigation of oxidative stress

(1) Scavenging of singlet oxygen or preventing its formation. In high light environments, excess excitation energy can produce singlet chlorophyll and singlet oxygen, which causes photo-oxidative damage (Anderson and Robertson, 1960; Young, 1991). Photo-inhibition of photosyntheic activity occurs when the excitation energy exceeds the rate of energy transfer in photosynthesis. Prolonged states of photo-inhibition eventually lead to photo-destruction of chlorophyll and the chloroplast (Britton, 1993).

(2) Free radical scavenging. Carotenoids, serving as antioxidants and free radical scavengers, have been shown to promote prostate health (lycopene) (Dahiya et al., 1994; Levy et al., 1995; Thompson et al., 1995) and prevent macular degeneration (lutein) (Eldred, 1989) and cancer (carotenes) (Acevedo and Pooh, 1995; Dahiya et al., 1994; Nishino, 1998).

Another classification system separates carotenoids into two classes, primary carotenoids (PCs) and secondary carotenoids (SCs). PCs are essential metabolites involved in

photosynthesis and photoprotection as well as adaptation to oxidative-stress. SCs are secondary metabolites involved in fruit and flower coloration and precursors to odorants and other catabolites, such as apocarotenoids. SCs are not constitutively present or necessary for development. This dichotomy sometimes falls naturally over plastid types, such as chloroplasts (PCs) and chromoplasts (SCs). For example, the wild relatives of tomato and maize do not have carotenoids in their petals and endosperms (personal observation), respectively; therefore, these petal and endosperm carotenoids are SCs in the domesticated plants. Although they are not likely to be essential to ontologic development in these organs, SCs may have functions on phyletic time scales and in the evolutionary ecology of the species. For example, carotenoids associated with the pollen spore coat (*pollenkitt*) may protect the germline from photo-oxidative stress, a function that may operate more importantly in phyletic than ontologic time.

1.4 Carotenoid Biosynthesis

The biosynthetic pathway and the structures of the common plant carotenoids are depicted in **Figure 1.3** and reviewed in (Britton and Goodwin, 1982; Cunningham and Gantt, 1998a; Goodwin, 1980b; Spurgeon and Porter, 1980). The biosynthesis begins with condensations of eight phosphate-activated isoprene units, IPP. In plastids, the C_{20} isoprenoid GGPP (geranylgeranyl pyrophosphate) is produced by GGPP synthase (GGPPS). GGPP is the precursor to many other secondary metabolites (Chappell 1995). GGPP is formed from four units of IPP, one of which is an isomer (DMAPP). IPP isomerase converts IPP to DMAPP; DMAPP is condensed with consecutive molecules of IPP to form FPP (farnesyl pyrophosphate), GPP (geranyl pyrophosphate), and then

GGPP. In plastids, GGPPS carries-out each condensation in a head to tail fashion. The addition of IPP to DMAPP is catalyzed by GGPPS. The addition of IPP to FPP is catalyzed by GPPPS. And the addition of IPP to GPP is also catalyzed by GGPPS to form GGPP (see **Figure 1.3**). FPS, which catalyzes the addition of DMAPP to IPP, and GPS, which catalyzes the addition of IPP to GPS, exist and are active in the cytosol of plants and in various bacteria, fungi, and animals. The substrate chain length specificity of GGPPS among various species is reviewed in (Sandmann, 1994)

The bi-functional enzyme, phytoene synthase (PSY), continues the condensation of two GGPP molecules in a head to head fashion to produce prephytoene pyrophosphate (PPPP), which is further converted by PSY to phytoene. Colorless phytoene is the first C_{40} carotenoid of the pathway. A series of four desaturations then occurs, extending the conjugated double bond system that forms a chromophore (Goodwin, 1980a).

The desaturations in the pathway have been ordered through analysis of products accumulated by mutants blocked in progression of the pathway (Robertson, 1975), and by dissection of the pathway in bacteria (Armstrong et al., 1990; McDermott et al., 1973; Misawa et al., 1990; Schnurr et al., 1991) and algae (Hirschberg et al., 1997; Powls and Britton, 1977).

Desaturations occur at the 11 and 11' positions first, then, at the 7 and 7' positions (Goodwin, 1980a; Porter, 1969). The double bond resides between carbons 7 and 8 or between carbons 11 and 12, or 7'and 8' or 11' and 12' (see **Figure 1.1** for naming

conventions and **Figure 1.3** for position of desaturations indicated on structures). Phytoene desaturase (PDS) catalyzes the dehydrogenations at 11 and 11'. The intermediate produced after one desaturation at the 11 position is called phytofluene. PDS produces ξ-carotene, after two desaturations. ξ-carotene desaturase (ZDS) catalyses the dehydrogenations at 7 and 7'. The intermediate produced after one desaturation at the 7 position is called neurosporene. ZDS produces lycopene after two desaturations. The order of paired catalyses (11,11' then 7,7') may be inferred from our failure to detect any intermediates having 7-double-bonds that do not have both 11- and 11'-double-bonds. That is, PDS finishes two desaturations before ZDS acts.

Given that in some bacteria (Linden et al., 1991; Misawa et al., 1990) and some fungi (Bartley et al., 1990; Goldie and Subden, 1973; Hausmann and Sandmann, 2000) three to five desaturations of phytoene are carried-out by a single enzyme, the CRTI-type enzyme, it is possible that ZDS might conceivably use phytoene as a substrate. The lack of reported intermediates that contain 7,7'-double-bonds with no 11,11'-double-bonds, considered together with the finding that no desaturation products are detectable in a heterologous complementation system expressing phytoene and over-expressing just ZDS (Luo, 2000), indicates that phytoene does not serve as a substrate for ZDS. Similarly, the lack of detection of intermediates containing 7,7'-double-bonds and just one 11-double bond (Goodwin, 1980a) suggests that phytofluene is not a substrate for ZDS. PDS and ZDS do not share substrates. This finding is true of ZDS from other plants, for example, *Capsicum annum* (Albrecht et al., 1995a). Similarly, lycopene is not subject to further desaturations by PDS or ZDS. Consequently, other desaturation states, such as 3,3',4'4'-

lycopene, are not observed in plants and have only been produced in *E. coli* as the result of *in vitro* gene shuffling of bacterial phytoene desaturases (Schmidt-Dannert et al., 2000). Hence, 7, 11-double-bond polyenes, 7, 11,11'-double-bond polyenes, and further desaturation products of lycopene do not occur naturally and need not be considered in the analysis of maize PDS and ZDS products (**see Chapter 5**).

Further reactions in the biosynthesis of carotenes and xanthophylls will not be emphasized here, as they are beyond the scope of this dissertation, which focuses on PSY, PDS, ZDS, and an enzyme upstream of the carotenoid pathway, DXS (D-1-deoxyxylulose-5-phosphate synthase). For completeness, further conversions are described here in brief with copious literature references.

Lycopene is further modified by the action of a series of two end-cyclases to produce the carotenes (Cunningham and Gantt, 1998a; Goodwin, 1980b; Spurgeon and Porter, 1980). Here the pathway (**Figure 1.3**) splits depending on whether the ends are adorned with the same or different types of rings. The genes of the two cyclases have been cloned from Arabidopsis (Cunningham Jr. et al., 1996), a cyanobacterium (Cunningham et al., 1993), and a unicellular algae (Cunningham et al., 1994), among others. One cyclase catalyzes formation of a β-ring, the other an ϵ-ring. These rings vary in the position of a double bond. If two β-rings are added to the ends of lycopene, then β-carotene is formed. If one β-ring is added to one end of lycopene, and an ϵ-ring is added to the other, then α-carotene is formed. Apparently, some ϵ-cyclases will only introduce one ring such that monocylics with one ϵ-ring occur but bicyclics with two ϵ-rings do not occur

(Cunningham and Gantt, 1998b). Recently, however, an ϵ,ϵ-cyclase has been discovered in lettuce (Cunningham and Gantt, 1998b). The occurrence of ϵ,ϵ-cyclase among plants is thought to be rare. The control of flow through the branches is controlled by the ϵ-cyclase activity relative to that of β-cyclase (Cunningham and Gantt, 1998b; Cunningham Jr. et al., 1996) and determines the ratio of the functionally distinct xanthophylls lutein and zeaxanthin. Most plants and tissues share the pathway up to the point of the carotenes. Organism and tissue specific differences occur at the point of hydroxylase action.

Oxygen additions produce the xanthophylls, as shown in **Figure 1.3**. For example, β-carotene is converted to zeaxanthin by β-carotene hydroxylase (Bouvier et al., 1998b; Sandmann, 1994; Sun et al., 1996). Such xanthophylls may be converted into epoxy-xanthophylls, violaxanthin or neoxanthin by zeaxanthin epoxidase (Bouvier et al., 1996; Bugos et al., 1998; Marin et al., 1996). As noted previously, the expoxidations are reversed by action of de-epoxidations to constitute the xanthophyll cycle.

Xanthophyll-dioxygenase, produces C25 apo-aldehydes, such as xanthoxin, which is the precursor to ABA. In maize, the dioxygenase is stereoselective for 9-*Z*-epoxy-xanthophylls (Schwartz et al., 1997). Since plant carotenoids after phytoene are thought to be all-*E* geometric isomers, an isomerase is proposed to be associated with epoxy-xanthophyll dioxygenases oxidative cleavage (Schwartz et al., 1997). The all-*E* isomeric nature of plant carotenoids has recently been called into question (elaborated in **Chapter 5**).

Mutations blocking carotenoid biosynthesis in higher plants are inherited in a Mendelian fashion (Robertson, 1955), indicating they are nuclear coded enzymes (Kirk, 1967). Since carotenoid biosynthesis occurs in plastids (Schulze-Siebert and Schultz, 1987a; Schulze-Siebert and Schultz, 1987b), carotenoid biosynthetic enzymes must be imported and localized among compartments within the plastid (Al-Babili et al., 1996; Bonk et al., 1997; Camara et al., 1982; Heintze et al., 1990; Kreuz et al., 1982; Price et al., 1995; Schledz et al., 1996). These compartments vary greatly in hydrophilicity and hydrogen ion concentration. **Table 1.1** lists (1) the various functional conversions of selected carotenoid biosynthetic enzymes (2) the points which substrates become dedicated to a particular class of compounds, *e.g.*, isoprenoids, terpenoids, carotenes, or xanthophylls (3) the localization of the enzymes and (4) selected references for the localization studies. The localization and the molecular genetics of each of the carotenogenic enzymes studied in this dissertation are given in detail below in **Section 1.6**.

1.5 The pathway to the carotenoid precursor, IPP

Isopentenyl pyrophosphate (IPP) is the precursor to the largest class of natural products, the isoprenoids. The production of IPP varies among organisms and organelles. In animals and fungi and plants, IPP is produced from mevalonic acid in the cytoplasm, but in some bacteria, algae and plastids of plants, another pathway operates. This novel pathway (Lichtenthaler et al., 1997a), called the 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway is shown in **Figure 1.4**. The mevalonate pathway is shown in **Figure 1.5** for comparison. While the DOXP, non-mevalonate pathway leads to the plastidic

isoprenoids, such as carotenoids, phytol, plastoquinone-9, isoprene, mono- and diterpenes, the classic mevalonic pathway to IPP leads to sterols, sesquiterpenes, and triterpenoids (Lichtenthaler, 1999).

DOXP starts with glycolytic intermediates, the 3-carbon D-glyceraldehyde-3-phosphate (GA-3-P) and the 2-carbon pyruvate. The first enzyme in the pathway, and likely a rate-controlling step towards isoprenoid biosynthesis, is deoxy-D-xylulose-5-phosphate synthase (DOXP-synthase or DXS), which mediates a thiamine-dependent transketolase reaction. The intermediate, 2-C-methyl-D-erythritol-4-phosphate, is converted to IPP (Lois et al., 1998).

The genes coding for DXS and a reductase. DXR,which is the next step in te pathway, have been cloned from *E. coli* (Lois et al., 1998). *Dxs* has also been isolated from peppermint (Lange and Croteau, 1999) and (as *CLA1)* from arabidopsis (Mandel et al., 1996). Recently, *Dxs* has also been isolated from *Bacillus subtilis* and *Synechocystis*, and these genes have been expressed in an *E. coli* heterologous complementation system (Harker and Bramley, 1999), providing results similar to those presented in **Chapter 4.** Other genes and enzymes of the pathway to IPP are not yet known.

In plants the two pathways are separated by the plastid membrane (Lichtenthaler et al., 1997a), as shown in **Figure 1.6.** Presently, it is not clear whether IPP moves across the plastid membrane.

1.6 Molecular genetics of carotenoid biosynthetic enzymes PSY, PDS, and ZDS

1.6.1 Molecular genetics of PSY

1.6.1.1 Localization of PSY

PSY has been localized in the stroma of the plastid as a peripheral membrane protein (Al-Babili et al., 1996; Bartley et al., 1992; Bonk et al., 1997; Lutke-Brinkhaus et al., 1982; Misawa et al., 1994; Schledz et al., 1996; Yu, 1999), where it functions in a phytoene biosynthetic complex also containing homodimeric GGPPS (Camara et al., 1982; Dogbo and Camara, 1987). Whether PSY is a homodimer within the complex is uncertain. The pepper PSY functions as a purified soluble monomeric peptide (Camara et al., 1988). An association with the plastid membrane is expected since the product of enzyme complex, phytoene, is highly lipophilic and must be delivered to a membrane bound complex of desaturases, cyclases, and oxygenases (Cunningham and Gantt, 1998a). In daffodil, two isoforms of PSY were found (Schledz et al., 1996); one in the stroma and one associated with the plastid membranes. Association with the membrane and galactolipids was necessary for activity *in vitro* (Schledz et al., 1996). The soluble, stromal form was found to be associated in a large complex with chaperonin60 (Cpn60) (Bonk et al., 1997). Yu (1999) localized PSY to the envelope in maize endosperm amyloplasts, but to the cytoplasm in rice endosperm amyloplasts using an anti-maize PSY antiserum. The mis-localization of PSY in rice was suggested to be functionally correlated with lack of carotenoid biosynthesis, since rice amyloplasts do not make carotenoids (Yu, 1999).

1.6.1.2 Putative transit peptide and molecular mass of PSY

The molecular mass of PSY determined by Western blotting from cDNA and gene sequence predictions varies among studies and among species. For example, a pea chloroplast import study showed that tomato PSY1 is produced as a 47 kDa pre-protein and was processed to a 42 kDa mature protein upon import (Bartley et al., 1992). A Western blot of tomato stromal proteins hybridized with a monoclonal antibody raised against PSY1 demonstrated a single antigen of apparent molecular mass of 38 kDa (Misawa et al., 1994). In maize and rice, an antigen with an apparent molecular mass of 38 kDa was also found with Western blotting and immunodetection of amyloplast proteins (Yu, 1999). In Arabidopsis, the nucleotide sequence of a PSY cDNA has been used to predict a pre-protein with a molecular mass of 47.6 kDa (Cunningham and Gantt, 1998a). Bonk, et al. (1997) predict a daffodil PSY of calculated molecular mass, 47.8 kDa. After import into pea chloroplasts a 3 kDa cleavage product is found by blue native-PAGE and autoradiography of an *in vitro* translation product (Bonk et al., 1997). Other predictions of chloroplast transit cleavage sites suggest mature PSY proteins of about 38-40 kDA (see this study **Chapter 6** for arabidopsis and rice and (Karvouni et al., 1995) for musk melon). Confirmation of the transit peptide cleavage site by N-terminal microsequencing of purified, mature PSY has not been reported; therefore, predictions of transit peptide size remain conjectural. The existence of PSY isoforms coded by a small number of genes within a species may add to the variation among PSY sizes and differential expression of isoforms among tissues with plastids of different types, such as proplastids, amyloplasts, chromoplasts, chloroplasts, etioplasts, and elaioplasts.

1.6.1.3 Gene copy number and regulation of PSY expression

Tomato has two PSY isoforms coded by unique, unlinked genes designated *Psy1* and *Psy2*. *Psy1* is induced at the transcriptional level during fruit ripening and chromoplast development (Bartley and Scolnik, 1993), but is not essential in leaf chloroplasts: anti-sense *Psy1* constructs which severely attenuate carotenoid accumulation in fruits to levels below detection (Bird et al., 1991) do not perturb carotenoid levels in leaves (Bramley et al., 1992). Both *Psy1* and *Psy2* are expressed in seedlings, leaves and fruits, but only *Psy1* is induced in fruit ripening at the transcriptional level as measured by RT-PCR (Bartley and Scolnik, 1993) and at protein level as measured by immunological techniques (Fraser et al., 1999). *Psy1* transcripts are constitutively expressed in shoots showing a small increase during photomorphogenesis, but decrease approximately seven-fold during dark adaptation; thus light is necessary for continued expression of *Psy1* (Giuliano et al., 1993). Treatment with the bleaching herbicide norflourazon, which is an inhibitor of PDS enzyme activity and thus carotenoid accumulation (Bramley, 1994), induces the expression of *Psy1* transcripts by two-three-fold as well as those of *Pds* five-ten-fold (Giuliano et al., 1993). A tomato mutation, *ghost*, that exhibits white leaf sectors free of carotenoids also exhibits increased levels of *Psy1* and *Pds* transcripts (Giuliano et al., 1993). Surprisingly, this apparent desuppression of carotenoid biosynthetic gene expression in tissues blocked in the accumulation of carotenoid end-products was not observed in a white mutant of arabidopsis, *immutans*, which is similar in phenotype to *ghost* (Wetzel et al., 1994); nor in norflurazon treated seedlings (Wetzel and Rodermel, 1998).

In tomato, although *Psy1* transcript levels vary little (2-3 fold) throughout leaf development, steady-state transcript levels rise more than nine-fold in flowers, especially near the onset of anthesis, where carotenoids are present largely in petals and anthers (Bramley et al., 1992). Further studies of the localization of isoforms and enzyme activities have not been reported as PSY1 and PSY2 are not immunologically or functionally distinct. Tomato null mutants of *Psy1* have been of some use in delineating the degree of overlap of PSY functions (Fraser et al., 1999).

In other model plants there are indications of more than one PSY. In bell pepper, RT-PCR amplified two transcripts of different size in mature fruit (Romer et al., 1993). The different transcripts could potentially represent two distinct genes or variants in either transcription or processing of the hetero-nuclear transcript from a single gene. In maize, a gene for *Psy* has been sequenced and mapped to the morphogenetic locus *Y1* on chromosome 6 (Buckner et al., 1996). However, when used as a probe in maize recombinant-inbred mapping studies, tomato *Psy2* was found in association with markers on chromosome 8. Another putative *Psy* probe (umn111) maps to maize chromosome 4 (Davis et al., 1999). PSY in Arabidiopsis may be a single copy gene (Scolnik and Bartley, 1993), since only one group of highly homologous expressed sequence tags were found (TIGR Arabidopsis Gene Index, http:www.tigr.org/tdb/agi/). **Chapter 6** of this dissertation presents data suggesting the existence of at least two *Psy* genes in rice and two or more in maize.

Psy genes are induced during photomorphogenesis. While GGPPS and PDS transcript levels remain constant, PSY transcripts have been shown to increase in light treated seedlings of Arabidopsis, *Sinapis alba* (Fraser et al., 1999) and tomato (Fraser et al., 1999; Giuliano et al., 1993). The induction depends on the quality of the light. Continuous far-red and red light induce *Psy* transcript levels, as measured by RT-PCR, indicting the involvement of phytochromes. This phytochrome-mediated response is abolished in the arabidopsis *phyA*, a null mutant of phytochrome A (Fraser et al., 1999). This induction is also seen at the level of protein accumulation and enzyme activity. Interestingly, after growth under far-red light conditions, although mRNA and protein levels increase, enzyme activity does not, since PSY is localized in an inactive form within prolamellar bodies of the thylakoids. PSY is then relocated to developing thylakoids and becomes active during de-etiolation (Welsh et al., 1999).

In maize and rice, *Psy* transcript levels have been measured in endosperms of various developmental stages gauged chronologically from the time of ovule fertilization. Li (1998) demonstrated that *Psy* transcripts increased in maize endosperm just prior to and concomitant with the commencement of carotenoid accumulation as profiled by Yu (1999). In rice, *Psy* transcripts were constitutively present throughout endosperm development even though carotenoids were not produced in these amyloplasts (Yoganathan, 1998a).

1.6.1.4 Homologies among PSY proteins

The bacterial or fungal homolog of plant, algal and cyanobacterial PSY is called CRTB. PSY and CRTB proteins share a prenyl transfer domain with squalene synthase enzymes (SQS or FDFT) (Chamovitz et al., 1992; Cunningham and Gantt, 1998a; Sandmann, 1994). Squalene, the C_{30} precursor to sterols in animals and plants, is the condensation product of two molecules of FPP. This condensation resembles the condensation of two molecules of GGPP to form phytoene. PSY and CRT proteins share additional domains of homology that do not occur in SQS proteins. Furthermore, the higher plant PSY proteins have an N-terminal domain of homology that is not found among the bacterial, cyanobacterial or algal PSY proteins. Since chloroplast transit signal peptide cleavage sites are conjectural, it is not known whether this domain is part of the transit peptide or the mature protein (Cunningham and Gantt, 1998b).

1.6.2 Molecular genetics of the carotenoid desaturases, PDS and ZDS

1.6.2.1 Localization of PDS and ZDS

PDS and ZDS are thought to be closely associated with each other as well as with the plastid membranes (Al-Babili et al., 1996; Armstrong and Hearst, 1996; Bonk et al., 1997; Bramley, 1985; Cunningham and Gantt, 1998b; Grunewald et al., 2000; Linden et al., 1993a; Linden et al., 1993b; Misawa et al., 1993; Serrano et al., 1990; Yu, 1999). While immunolocalization experiments in cyanobacteria (Serrano et al., 1990) and in spinach and tobacco (Linden et al., 1993a), show PDS to be associated with a thylakoid fraction of plastids, studies with daffodil chromoplasts suggest PDS may not be an integral membrane protein (Schledz et al., 1996). Like PSY, PDS is found in a high

molecular weight complex in daffodil chromoplasts (Schledz et al., 1996). Upon import into pea chloroplasts (Bonk et al., 1997), PDS is associated with plastidal chaperonin (Al-Babili et al., 1996; Bonk et al., 1997; Bonk et al., 1996). Since ZDS has only been isolated and characterized recently from higher plants (Albrecht et al., 1995b; Bartley et al., 1999; Breitenbach et al., 1999; Luo, 2000), it has not yet been the subject of localization studies in higher plants. One may assume it is found in close association with PDS.

1.6.2.2 Regulation of PDS and ZDS expression

Induction of transcription may account for differential regulation of carotenoid accumulation in development (Corona et al., 1996) and in response to stress (Breitenbach et al., 1999). *Pds* transcript accumulation has been profiled in development in tomato (Giuliano et al., 1993), maize (Li, 1998; Li et al., 1996b), rice (Yoganathan, 1998a), and pepper fruits (Breitenbach et al., 1999; Hugueney et al., 1996). In tomato, *Pds* transcript accumulation is developmentally regulated in leaves, roots, fruits and flowers. *Pds* transcript levels are lowest in roots, intermediate in leaves and highest in petals and anthers. Immediately before anthesis during flower development, PDS is induced more than 10-fold. On the other hand, while PSY is induced more than 20-fold during fruit ripening, PDS is induced less than 3-fold (Giuliano et al., 1993).

In maize and rice, PDS transcript levels are constant or decrease in developing endosperms (Li et al., 1996b; Yoganathan, 1998). Carotenoid biosynthetic genes in bell pepper fruits, including Pds, were increased at the ripening stage by wounding

(Hugueney et al., 1996) and by chemically induced oxidative stress (Breitenbach et al., 1999).

Pds transcription levels have been measured by *Pds*-promoter-*uidA* fusions, which produce a chromogenic reporter activity, β-glucuronidase (GUS), in transgenic tomato and tobacco. GUS expression was high in tissues with chromoplasts, such as tomato anthers, petals, and fruits; and low in tissues without chromoplasts, such as tobacco petals and fruits (Corona et al., 1996). Like the higher accumulation seen for PSY and PDS transcript levels in *ghost* and bleaching herbicide treated seedlings described in **Section 1.6.1**, GUS staining was higher in bleached seedlings of tobacco (Corona et al., 1996). This suggests pathway end-product inhibition of PDS transcription. Likewise, product feedback- inhibition of transcription has been suggested for pepper structural genes of carotenogenesis (Bouvier et al., 1998a). Enzyme-level feedback inhibition has been observed for PDS (Sandmann and Böger, 1989) and tentatively for ZDS (Breitenbach et al., 1999).

Few studies have measured the expression of genes for carotenoid biosynthetic enzymes coordinately, aside from the above mentioned concomitant measurements of PSY and PDS and multiple genes from bell pepper. The noteworthy example from Hugueney et al. (1996), profiles the expression of carotenoid biosynthetic genes, *Ggps*, *Psy*, *Pds*, and *Capsorubin-capsoxanthin synthase* as measured by steady state accumulation of mRNA in pepper fruit (Hugueney et al., 1996). These genes have been shown to be synchronously induced by transient oxidative stress produced from generators of reactive

oxygen species (Bouvier et al., 1998a). Further progress in isolation of complete sets of genes from a variety of organisms will allow for comprehensive, comparative expression studies to be interpreted in a phyletically-phrased, hypothesis-driven approach.

1.6.2.3 Putative transit peptide and molecular mass and homologies among PDS and ZDS

PDS and ZDS are both flavoproteins and show limited primary sequence homology (Breitenbach et al., 1998; Cunningham and Gantt, 1998b). A cyanobacterial ZDS was found to be more similar to PDS than to CRTI (Armstrong et al., 1990), the bacterial four–step desaturase, or to All, the five-step desaturase (Hausmann and Sandmann, 2000) from *Neurospora crassa* (Breitenbach et al., 1998). Plant and bacterial phytoene desaturases are thought to have an independent evolutionary origin. In *Anabaena*, the PDS resembles the plant-type PDS, but the ZDS does not: it resembles CRTI. In *Synechocystis*, there is a PDS-type homolog, but the CRTI homolog codes for a β-carotene monoketolase while another gene codes for ZDS. This algal ZDS is homologous to the plant ZDS. Moreover the *Synechocystis* ZDS and plant ZDS are more closely related to one another than either is to PDS; therefore, the divergence of *Anabaena*-type desaturase from the plant-type desaturases predates the separation of the plant-type desaturases from each other. Consequently, Breintenbach *et al.* (1998) conclude that higher plant PDS and ZDS diverged from a duplication event occurring in the algal progenitor of chloroplasts.

Although poorly conserved, plant PDS and ZDS share a number of conserved primary structural domains. One is a putative transit peptide that is relatively well conserved (Li et al., 1996a; Luo, 2000) in comparison to that of PSY putative transit sequences (**Chapter 6**). The *Arabidopsis* PDS transit peptide cleavage site has not been determined (Cunningham and Gantt, 1998b) and currently defies neural network prediction with ChloroP (see **Chapter 6 Methods**). PDS alignment with the well-conserved (65% identity, 85% similarity) *Synechocystis* PDS, suggests a transit peptide of about 50 amino acid residues, and pre-protein and mature protein molecular masses of about 61.9 and 57.0 kDa, respectively, were predicted (Cunningham and Gantt, 1998b). In maize, a similar prediction strategy yields a conjectural transit peptide of 96 amino acid residues from a pre-protein of a calculated molecular mass of 64.1 kDa and a mature, plastid localized protein of 53.5 kDa (Li et al., 1996a). Similar predictions are available for ZDS from *Arabidopsis* (Scolnik and Bartley, 1995), bell pepper (Breitenbach et al., 1999), and maize and rice (Luo, 2000).

The second homology between PDS and ZDS is a dinucleotide-binding domain, which is shared among carotene desaturases found in phylogenetically distant organisms (see (Li et al., 1996a) for alignments). The particular dinucleotide that PDS or ZDS binds varies among organisms, for examples see (Breitenbach et al., 1998; Breitenbach et al., 1999; Hugueney et al., 1992).

1.6.2.4 Cofactor requirements for desaturases

Overall, the desaturation reactions require a dinucleotide and an electron transport chain with a plastoquinone electron carrier and oxygen as a final electron acceptor (Beyer et al., 1989). Both PDS and ZDS are thought to be flavoenzymes, since the deduced amino acid sequences have putative dinucleotide-binding domains as described above. For example, analyses of cofactor requirements for purified bell pepper systems (Armstrong et al., 1990; Bartley et al., 1990; Fraser et al., 1992) have demonstrated that either NAD(P) or FAD is sufficient to catalyze the desaturations. On the other hand, the ZDS of *Capsicum annuum* is insensitive to the presence or absence of oxygen and is not enhanced by dinucleotides FAD, NAD, or NADP (Breitenbach et al., 1999). Here, an alternative plastidal oxidase may be the terminal electron acceptor (Mayer et al., 1990; Mayer et al., 1992). Other requirements for the activity of PDS and ZDS, especially the relationship of the desaturase activities to geometric isomerism of substrates, are treated in detail in **Chapter 5,** which presents results of a study of the concerted action of maize PDS and ZDS in an *E. coli* heterologous function complementation system.

1.7 Metabolic engineering

Many genes coding plant carotenoid biosynthetic enzymes have now been isolated by molecular biological techniques, such as (1) homologous and heterologous probe hybridization (Bartley and Scolnik, 1995; Bartley et al., 1994; Bouvier et al., 1994), (2) PCR using degenerate primers (Li et al., 1996b), (3) transposon tagging (Buckner and Robertson, 1993), (4) immuno-expression and differential screening of cDNA libraries (Dogbo and Camara, 1987; Kuntz et al., 1992), (5) color complementation screening of

cDNA expression libraries against bacterial carotenogenic gene clusters expressed in *E. coli* heterologous systems (Cunningham and Gantt, 1998a; Lotan and Hirschberg, 1995; Misawa et al., 1995), and (6) utilization of discovery-driven, random, genomic approaches (Luo, 2000; Yoganathan, 1998b) (this study, **Chapter 6**). The cloning of genes coding enzymes for carotenoid biosynthesis enzymes and genes for the biosynthesis of isoprenoid precursors allows metabolic engineering of the accumulation of carotenoids. Some examples are given below as a preface to:

- the metabolic engineering described in **Chapter 4**,
- the importance of the functional complementation results given in **Chapter 5**,
- the rationale for the cloning and analysis of the rice gene for a likely rate-controlling, branch-point enzyme, PSY, described in **Chapter 6**.

The cloning of IPP biosynthetic genes opened a door for metabolic engineering of the IPP pool. Competition for DOXP might affect end-product accumulation for different isoprenoid-derived biosynthetic pathways. The enzyme responsible for DOXP synthesis, DXS, might code for a novel rate-controlling enzyme, whose expression could be modified to increase substrate availability for pathways under consideration for metabolic engineering. Carotenoids are one such pathway.

Other studies have shown rate-control or induction of transcription for the genes coding enzymes active within the isoprenoid biosynthetic pathway before PSY, such as GGPPS (Kuntz et al., 1992) and IPPI (Albrecht and Sandmann, 1994; Cunningham and Gantt, 1998a), which also correlate with accumulation of carotenoids. Plant IPP isomerase

catalyzes the reversible isomerization of IPP to DMAPP. Despite the reversibility, overproduction of IPP isomerase has been shown to increase the flow of carbon into the carotenoids by a factor of about two-fold in a heterologous *E. coli* complementation system (Cunningham and Gantt, 1998b).

In a cyanobacterium, PDS was shown to be "a rate-limiting step" (Chamovitz et al., 1993) and progression of substrates through the pathway at the step of ZDS has also been suggested as being dependent on the stereoselectivity of ZDS. Thus an obligate isomerase may limit progression through the pathway, as has been suggested for isolated daffodil chromoplasts (Beyer et al., 1989) and *Arabidopsis* ZDS assayed in a heterologous *E. coli* functional complementation system (Bartley et al., 1999).

PSY is a branch-point enzyme that controls the dedication of isoprene to the carotenoids. Thus, it is likely to be a rate-controlling step for carotenoid accumulation. Many studies have demonstrated a correlation between the apparent induction of *Psy* transcript accumulation and protein accumulation concomitant with carotenoid accumulation in fruits, flowers, and leaves (see **Section 1.6.1**). PSY competes for GGPP, which is a precursor common to the synthesis of the phytol chain of chlorophyll, gibberellins, and prenylquinones, among others. Not surprisingly, over-expression of PSY has deleterious effects on chlorophyll content and on plant growth and stature. The dwarfism is partly due to a deficiency in accumulation of gibberellic acid caused by depletion of GGPP in deference to over-accumulation of carotenoids (Fray et al., 1995). Obviously, a regulated, delicate balance in carbon flow exists and efforts to over-accumulate secondary

metabolites by genetic manipulation of substrate-flow-controlling enzyme levels may require adjustment to precursor pools, by modulation of biochemical conversions that are more closely linked to central carbon metabolism. Such substrate pools may be more resilient to artificially induced outflow. This general hypothesis is tested with a specific model case as presented in **Chapter 4**. The cloning and characterization of rice PSY is reported in **Chapter 6**. Together the results presented in this dissertation contribute to a foundation for the metabolic engineering of carotenogenesis in maize and rice.

1.8 General Aims

The object of these studies was to contribute to the development of tools for the comparative understanding of carotenogenesis in rice and maize. The long-term goal is not only to understand the biology of carotenoid biosynthesis and the function and localization of carotenoids, but also to precondition the genetic manipulation of this process in the most economically important representatives of the cereal crops (Wurtzel, 2001).

1.9 Specific Aims

Specific objectives included:

- To develop methods for isolation of genes involved in carotenogensis, such as improving color complementation screening and high-throughput screening procedures
- To improve methods of testing the functions of carotenogensis-related genes

- To test hypotheses regarding the metabolic engineering of carotenogenesis in relationship to genetic modulation of the isoprenoid precursor pool
- To characterize the specific function of maize phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) with respect to isomerism of their substrates and products
- To isolate and characterize rice *phytoene synthase* (*Psy*), a gene coding for the first step of the carotenoid biosynthetic pathway and to compare it to maize *phytoene synthase* (*Y1*)

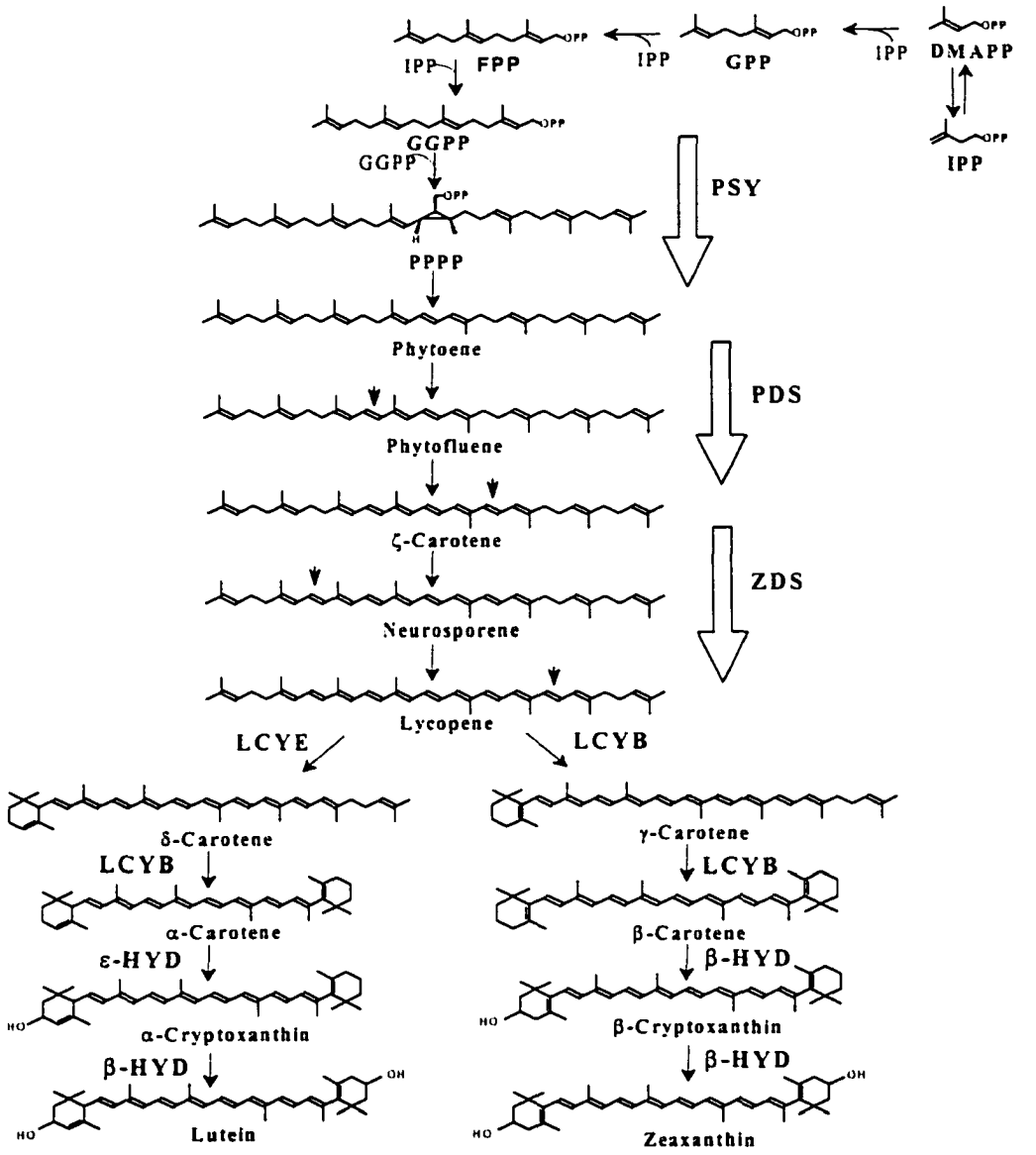

Figure 1.3 General plant carotenoid biosynthetic pathway. The pathway depiction begins with the isoprenoid precursor IPP. Phytoene is the first compound unique to carotenogenesis. Enzymatic steps within the pathway that are studied here are indicated with open arrows. Long arrows indicate other biochemical conversions, short arrows indicate positions of desaturations. Abbreviations for enzyme names shown in bold capitals are listed with details in **Table 1.1**. Redrawn after A. Yoganathan [Yoganathan, 1998 #4358].

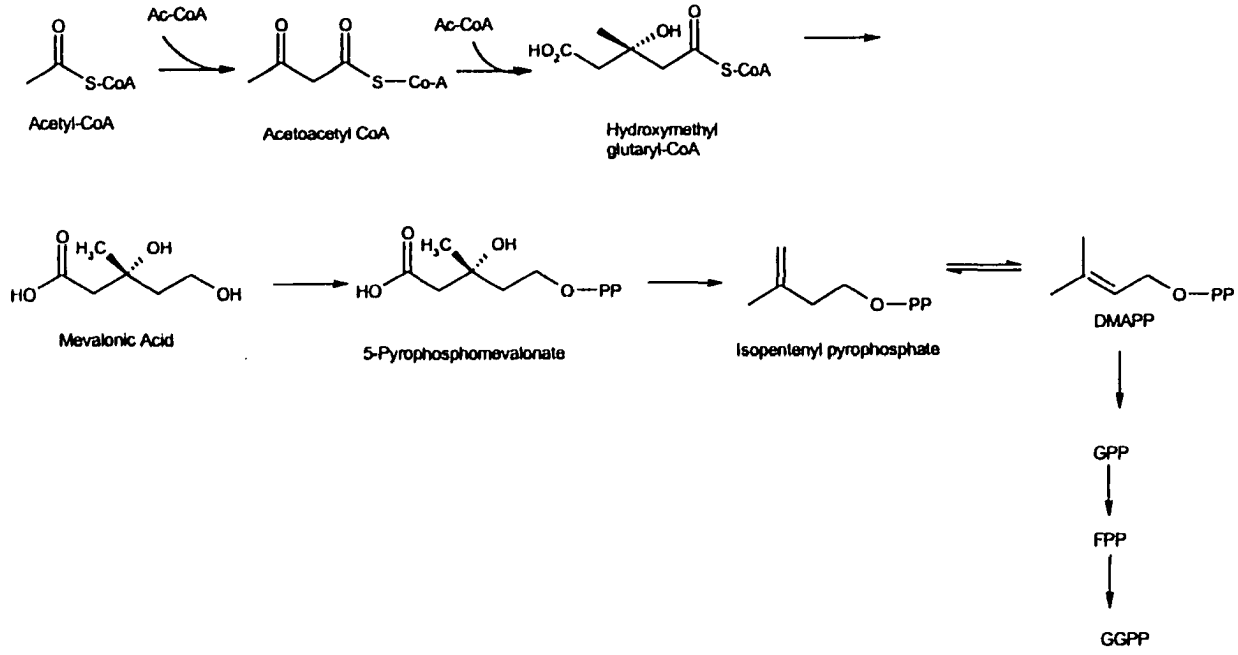

Figure 1.6 Mevalonate pathway to IPP and GGPP. Drawn by Cynthia Gallagher.

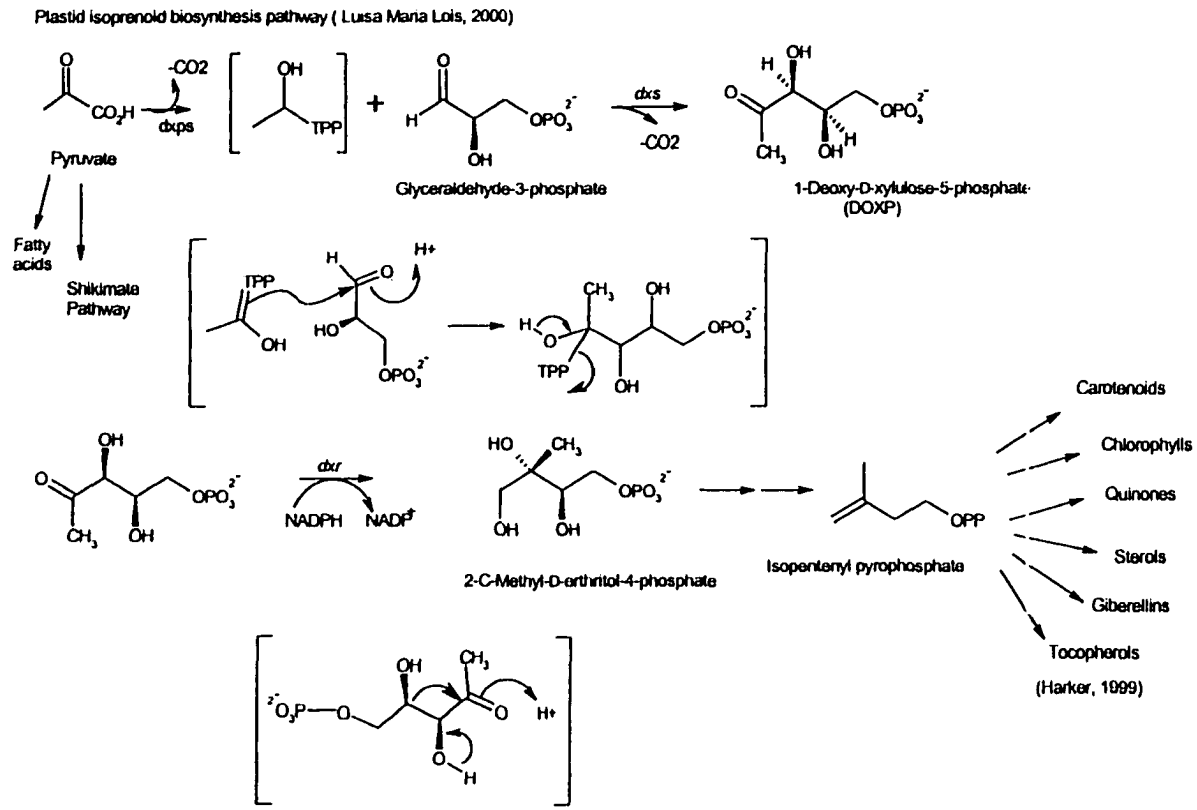

Figure 1.5. DOXP pathway to IPP formation in plant plastids. Redrawn by Cynthia Gallagher.

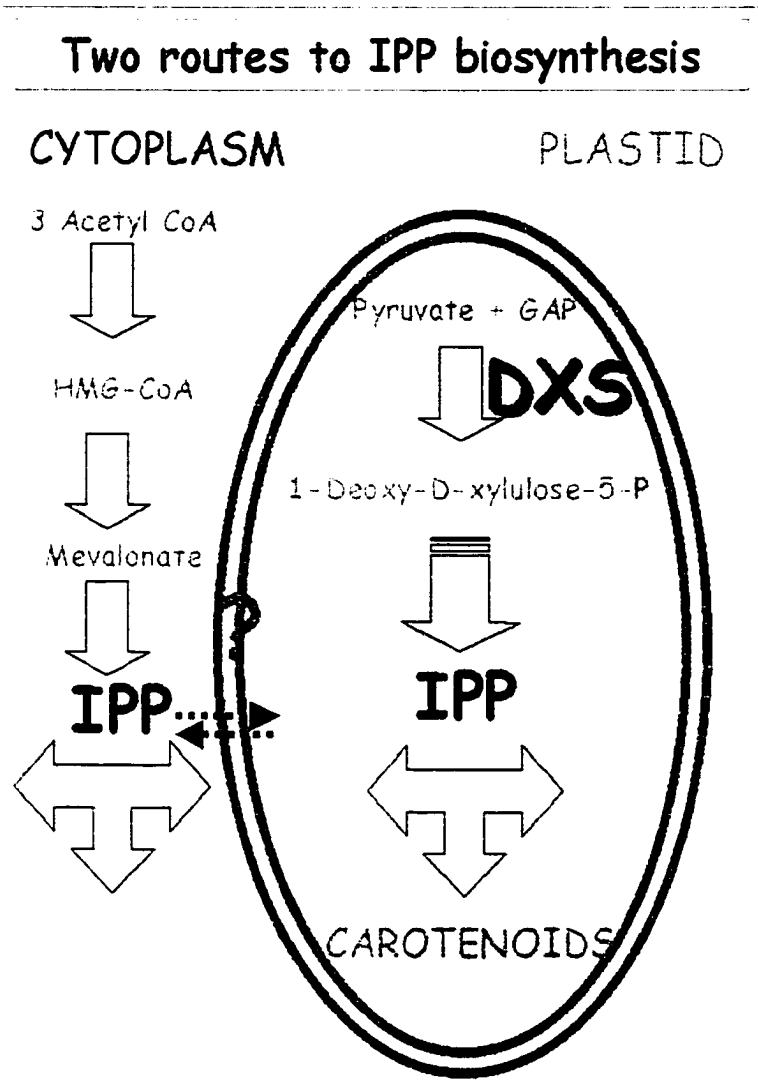

Figure 1.6 Compartmentalization of the DXOP pathway to IPP and the mevalonate pathway to IPP in a plant cell. Adapted from (Lichtenthaler, 1999) by E.T. Wurtzel.

Table 1.1 Enzymes, substrates, and localization of plastidal carotenogenesis

Table 1.1 Enzymes, substrates and localization of enzymes in plastidal carotenogenesis. Continued next page.

Desaturation	ξ-carotene desaturase I	ZDS	ξ-carotene	neurosporene	40	carotenoid	membrane	(Hugueney et al., 1992)
Desaturation	ξ-carotene desaturase II	ZDS	neurosporene	lycopene	40	carotenoid	membrane	
Cyclization	ε-cyclase	ε-LYC	lycopene	δ-carotene	40	carotene	integral membrane	(Cunningham Jr. et al., 1996)
Cyclization	β-cyclase I	β-LYC	lycopene	γ-carotene	40	carotene	integral membrane	(Cunningham Jr. et al., 1996)
Cyclization	β-cyclase II	β-LYC	γ-carotene	β-carotene	40	carotene	integral membrane	(Cunningham Jr. et al., 1996)
Cyclization	β-cyclase III	β-LYC	δ-carotene	α-carotene	40	carotene	integral membrane	(Cunningham Jr. et al., 1996)
Oygenation β-ring hydroxylation	β-carotene hydroxylase	β-HYD	β-carotene, α-carotene	zeaxanthin, lutein	40	xanthophyll	integral membrane	(Bouvier et al., 1998b; Cunningham and Gantt, 1998b)
Epoxidation	Zeaxanthin epoxidase	ZEP	zeaxanthin	antheraxanthin, violaxanthin	40	xanthophyll	stroma/ peripheral membrane	(Bugos et al., 1998)
De-epoxidation	Violaxanthin de-epoxidase	VDE	violaxanthin	antheraxanthin, zeaxanthin	40	xanthophyll	thylakoid lumen	(Bugos et al., 1998)

Table 1.1 Enzymes, substrates and localization of enzymes in plastidal carotenogenesis. Recursions of enzyme activities are listed multiple times and given Roman numerals. "Carbons" is the number of carbons in the substrate for a particular enzyme.

Table 1.1 Enzymes, substrates and localization of enzymes in plastidal carotenogenesis. Continued next page.

Desaturation	ξ-carotene desaturase I	ZDS	ξ-carotene	neurosporene	40	carotenoid	membrane	(Hugueney et al., 1992)
Desaturation	ξ-carotene desaturase II	ZDS	neurosporene	lycopene	40	carotenoid	membrane	
Cyclization	ε-cyclase	ε-LYC	lycopene	δ-carotene	40	carotene	integral membrane	(Cunningham Jr. et al., 1996)
Cyclization	β-cyclase I	β-LYC	lycopene	γ-carotene	40	carotene	integral membrane	(Cunningham Jr. et al., 1996)
Cyclization	β-cyclase II	β-LYC	γ-carotene	β-carotene	40	carotene	integral membrane	(Cunningham Jr. et al., 1996)
Cyclization	β-cyclase III	β-LYC	δ-carotene	α-carotene	40	carotene	integral membrane	(Cunningham Jr. et al., 1996)
Oygenation β-ring hydroxylation	β-carotene hydroxylase	β-HYD	β-carotene, α-carotene	zeaxanthin, lutein	40	xanthophyll	integral membrane	(Bouvier et al., 1998b; Cunningham and Gantt, 1998b)
Epoxidation	Zeaxanthin epoxidase	ZEP	zeaxanthin	antheraxanthin, violaxanthin	40	xanthophyll	stroma/ peripheral membrane	(Bugos et al., 1998)
De-epoxidation	Violaxanthin de-epoxidase	VDE	violaxanthin	antheraxanthin, zeaxanthin	40	xanthophyll	thylakoid lumen	(Bugos et al., 1998)

Table 1.1 Enzymes, substrates and localization of enzymes in plastidal carotenogenesis. Recursions of enzyme activities are listed multiple times and given Roman numerals. "Carbons" is the number of carbons in the substrate for a particular enzyme.

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[1]Chapter 2. High-throughput microplate format for producing and screening riboprobes from bacterial cells

2.1 Prologue

Early efforts aimed at isolation of higher plant genes coding for enzymes involved in carotenogensis in maize took advantage of the wealth of mutants (Neuffer et al., 1997) (Buckner and Robertson, 1993) and genetic tools (Wurtzel, 1992) available for maize. Before the advent of homologous algal genes and facile heterologous functional complementation systems in *E. coli*, I pursued risky efforts in maize transposon tagging, transposon display, differential library screening among mutant and non-mutant endosperm cDNA libraries, subtractive hybridization, and immunological screening of cDNA expression libraries. Many of these efforts were doomed to failure, because of the extraordinary low-level prevalence of transcripts for the carotenoid biosynthetic genes, a fact that only came to light later. Nevertheless, one result of these brute-force attempts at gene isolations was the development of a method for high-throughput screening of putative differentially expressed cDNA clones. Such methods have now been commercialized and have become central to molecular genetic approaches to biology.

2.2 Introduction

Differential screening and subtractive hybridization techniques may yield large numbers of clones enriched for differentially expressed sequences. The preferred method of

[1] This chapter is a revised version of P. D. Matthews and E.T. Wurtzel. 1995. High-throughput microplate format for producing and screening riboprobes from bacterial cells. BioTechniques 18(6):1000-1004.

selecting and confirming *bone fide* differentially expressed clones is to prepare each sequence as a hybridization probe and challenge Northern blots or slot blots. If the enrichment factor is modest or a low prevalence clone is sought, screening hundreds of clones becomes desirable but is prohibitively laborious in conventional format. The need to prepare a large number of nucleic acid probes occurs often in clinical, environmental, and genomic projects. Polymerase chain reaction-based labeling protocols (Henkel et al., 1993) require a microplate thermocycler and may accommodate only 96 clones simultaneously. "Checkerboard" hybridizations (Socransky et al., 1994) are limited by the size and number of non-disposable blotting manifolds. Here I present methods, which may be amenable to automation, for the growth of bacterial cultures, purification of plasmid DNA, preparation of riboprobes, and nucleic acid hybridizations all in a microplate format. The simultaneous process capacity of my method is 1152 clones (12 microplates), the limitation imposed by centrifuge capacity and vacuum filtration manifold size.

2.3 Methods

Bacterial culture growth and plasmid minipreparation by the alkaline lysis technique in microplates have been described (Ausubel et al., 1987). I prefer a boiling method (Holmes and Quigley, 1981) modified for microplate format, as the plasmid is more susceptible to restriction endonuclease digestion, and employ it as described here. Primary transformants are picked with sterile toothpicks to 200 ul LB medium supplemented with 0.1% v/v KNO_3, which allows denser growth in the absence of

agitation (Ausubel et al., 1987), to 300 ul round bottom 96 well microplates. Incubation is for 24-48 hr at 37°C. Twelve plates are centrifuged together at 2500 rev/min for 10 min in a Beckman GPR benchtop centrifuge with a GH3.7 rotor fitted with plate carriers. The supernatant is removed by inverting the plates with a whipping motion followed by blotting on absorbent paper. Cross-contamination is minimal. The bacterial pellets are washed with 200 ul T. E. and repelleted, whipped and drained as before. After addition of 100 ul freshly prepared STET (0.1 M NaCl; 10mM Tris - HCl, pH 8; 1 mM EDTA, 5% Triton X-100) with 1 mg/ml lysozyme, cells are resuspended by pipetting. Plates are lowered onto boiling water in centrifuge plate carriers for 40 sec or placed for 60 sec in a baking dish containing just-boiled water. It is important to remove any plate lids to avoid flooding by capillary action. Centrifuge the lysate at 2500 rev/min for 10 min to pellet cell *debris* and transfer 70-100 ul supernatant to a new assay plate. The plasmid is precipitated with 0.7 vols of isopropanol at room temperature and centrifuged for 30 min at 2500 rev/min. The plasmid pellet (approximately 1 ug) is washed with cold 70% EtOH and air dried. Plasmids are dissolved in 10 ul water. Alternately, plasmids may be selectively concentrated by adhesion to glass particles by addition of 200 ul of a freshly prepared slurry of 6 M NaI and 1 ul/ml GeneClean Glass Milk II (Bio101). The glass is pelleted by centrifugation for 3 min at 2500 rev/min and washed twice with 50% EtOH, 0.3 M NaOAc, pH 5.2 and once with 70% EtOH. The final glass pellet is air dried and resuspended in 10 ul water. The glass may then be left in the wells in subsequent steps and re-used for concentration of the plasmid. The glass does not interfere with subsequent restriction digests.

Riboprobe templates must be completely linearized with a restriction enzyme that does not produce 3' overhangs. 5 ul of plasmid (500ng) or 5 ul of plasmid glass slurry is mixed in a plate with 5 ul 2x restriction enzyme mix containing 1 U of enzyme and 8 mM spermidine HCl. After 2-3 hours incubation at the optimal temperature, an additional 10ul of restriction mix containing 1 U enzyme is added. This reaction is generally incubated overnight. For some restriction enzymes heat inactivation is appropriate. Linearized plasmid is concentrated and dried as above.

Riboprobes are synthesized under the following conditions described (Sambrook et al., 1989): In a final concentration of 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2mM spermidine HCl, 5 mM NaCl, 10 mM dithiothreitol, 100 ug/ml acetylated BSA, 500 uM each ATP, CTP, UTP, 1 unit/ul human placental ribonuclease inhibitor, 5 units/ul bacteriophage DNA-dependent RNA polymerase, and 1 ul/25 ul final volume 3000 Ci/mmole; 10 uCi/ ul [α-^{32}P]GTP. The first four components are pre-mixed as a 10X transcription buffer. 5 ul of transcription mix is added to the dried pellet of linearized template and mixed by pipetting. Transcription proceeds at 37°C for 1-2 hours and is terminated upon removal of the template by addition of 1 ul of 10mg/ml RNAse-free DNAse RQ1 (Promega). I do this routinely, but have not determined whether removal of template is necessary to reduce hybridization background. 100ul hybridization solution (50% formamide, 6X SSC, 0.1% SDS, 0.001% Tween 20, 1X Denhardt's solution, 100 ug/ul yeast tRNA) is then added. Removal of unincorporated radiolabel is unnecessary as

incorporation is generally high. This hybridization solution is added directly to target nucleic acids immobilized to nitrocellulose bottoms of 96 well filtration plates (Millipore).

Nitrocellulose filtration plates (Multiscreen Assay System) are pre-wetted by submersion in a tub of 3 X SSC. Target DNA or RNA is denatured by heating in 50% formamide, 6x SSC, and is applied to the plates in a maximum volume of 300 ul. A large application volume favors even binding to the membrane. Twelve filtration plates, i.e. 1152 individual hybridization reactions, may be prepared simultaneously on a 11 X 14 inch gel drier with light vacuum. It is necessary to cover the plates with the gel drier rubber sheet or plastic wrap to seal the vacuum. The filters are washed once by vacuum filtration of 300 ul 3 x SSC, dried, and baked at 80^0C for 1.5 hours.

Following overnight hybridization, fluid is removed from the wells and disposed as liquid radioactive waste and the plates are washed at 65°C by agitation in 3 changes of a large volume of 0.1 X SSC, 1% SDS. Plates are air dried and exposed to X-ray film in empty 8 X 10 photographic paper boxes. A heavy book is placed on the plates in the boxes to assure even contact of the filters with the film. One intensifying screen is generally used. Signals are analyzed for integrated optical density (IOD) with ImageQuant (Molecular Dynamics, Sunnyvale, CA).

2.4 Results

Incorporation of radionucleotide is about 50%, as determined by DE81 adsorption (Sambrook et al., 1989) of the RNA product. Hybridization signals are dose dependent on target sequence, as see in Figure 1; and sense and antisense maize *Shrunken* (Wurtzel et al., 1987) probes show the signal to noise ratio is about 4:1, see Figure 2. Background levels may be further decreased with a ribonuclease A washing step (not attempted), but the signal is sufficient for detection of moderately abundant (0.1% of mRNA) transcripts. This procedure takes advantage of the promoter specificity and relative tolerance of bacteriophage DNA dependent RNA polymerases to contaminated templates. The templates produced here are doubtfully sufficiently pure for other template applications such as DNA polymerase-based sequencing. Protocols based on magnetic microsphere technology may be superior for applications requiring high-purity plasmid.

2.5 Discussion

I describe a high throughput system, starting with bacterial clones and preceding through plasmid purification and riboprobe production, for sensitive and reliable Northern and Southern hybridizations in microplate format. This format is amenable to automation. Directional cloning of cDNA inserts is essential to efficiency for Northern hybridizations. Future improvements to this protocol may include an RNase washing step for riboprobe hybridizations and UV crosslinking of target nucleic acid to nitrocellulose filters, both of which may increase signal to noise ratios.

2.6 Acknowledgements

This work was supported by a McKnight Foundation Individual Research Award, by The American Cancer Society (grant #BC-661) and by NIH grant #5SO6GM/AGO8225-10. I also thank Bio101 for the generous gift of GeneClean II Glass Milk.

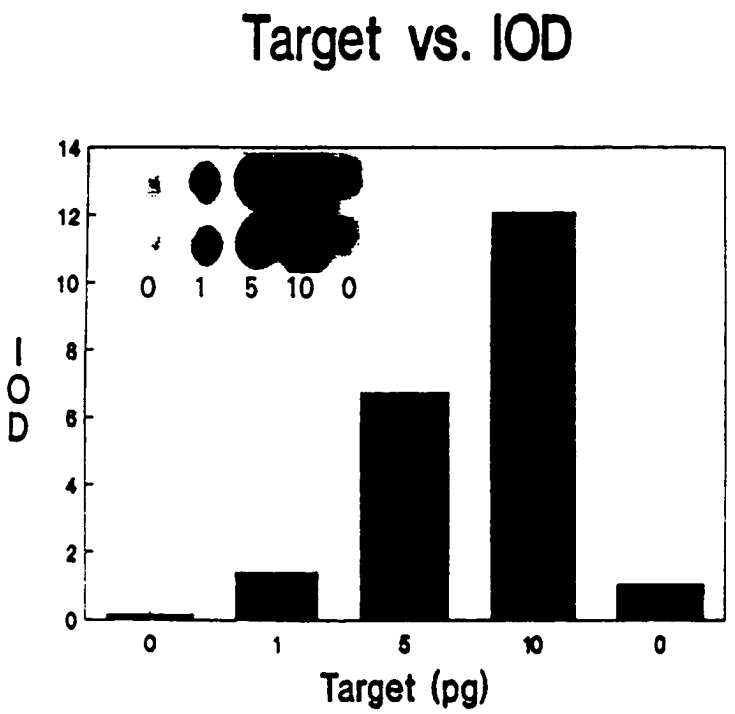

Figure 2.1. A 3.4 *Bgl*II fragment of maize sucrose synthase gene (*Shrunken*) in the transcription vector pBluescript® II (Stratagene, La Jolla, CA, USA) is labeled as a riboprobe and hybridized to various amounts of target plasmid DNA (a 3.4 genomic fragment of *Sh* in pBR322) applied to Multiscreen nitrocellulose microplates in a background of 10μg denatured salmon sperm DNA. Hybridization signals are shown in the inset in duplicate for 1, 5, and 10 pg of target and two positions of 0pg target negative controls. Average (n=2) integrated optical density (IOD), shown as bars, is dose-dependent on picograms (pg) of target DNA, and nonspecific hybridization to nontarget background DNA is low.

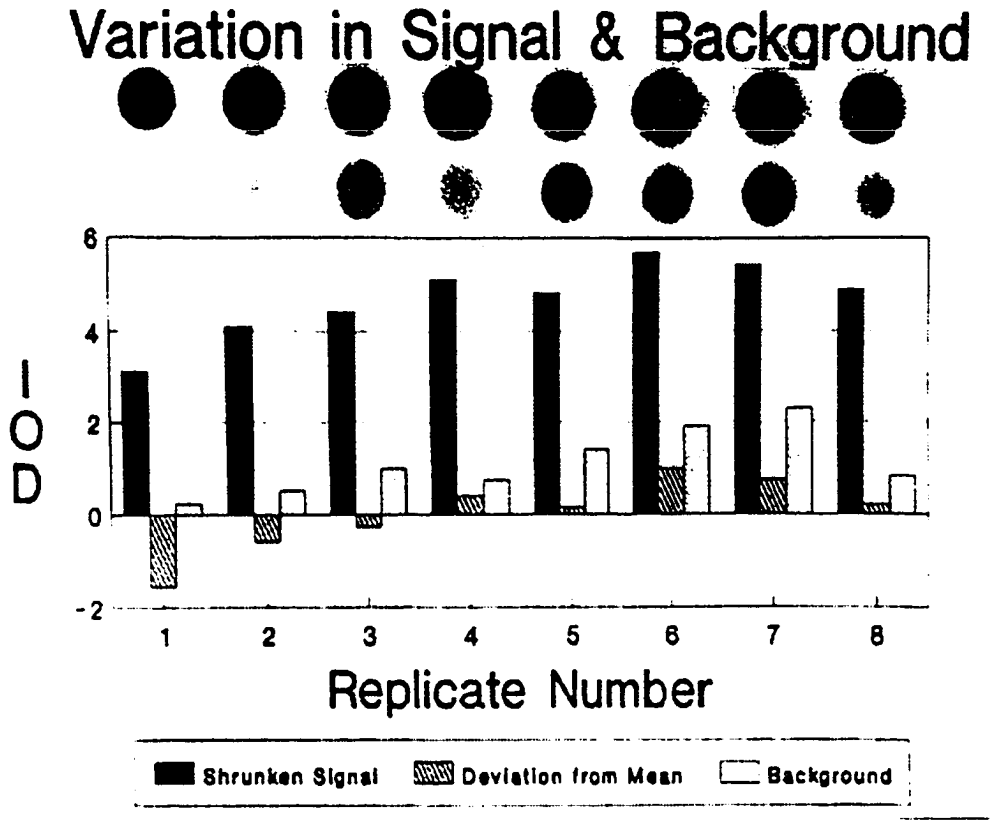

Figure 2.2. Actual signals and graphical display representing variation in signals and background. A row of microplate targets containing 10ug total maize kernel RNA (14 days after pollination) was probed with *Sh* antisense (top row, signals) and *Sh* sense (bottom row, background) probes of equal specific activity. Integrated optical density (IOD) of the signal, the deviation of the signal from the mean IOD of the eight replicates (as shown in top row), and the IOD of the background sense-probe negative control (as shown in bottom row) are shown as bars.

2.7 References

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[2]Chapter 3. Variation in Expression of Carotenoid Genes in Transformed *E. coli* Strains

3.1 Prologue

Heterologous complementation systems were crucial for the development of the molecular genetic and biochemical study of carotenogenesis. In addition to the prospect of bioengineering the accumulation of useful and unusual carotenoids, the expression of carotenogenic genes in *E. coli* represents a unique opportunity for (1) cloning of new genes by heterologous complementation (Sun et al., 1996), (2) for functional testing of gene products (Li et al., 1996b) and **Chapter 4**, (3) the molecular breeding of new carotenoid biosynthetic pathways (Albrecht et al., 2000; Schmidt-Dannert et al., 2000), and (4) for examination of flow through the pathway (Kajiwara et al. 1997; Ruther et al. 1997).

The huge diversity and distribution of natural carotenoids present a challenge for isolation and comparative understanding of a large array of genes and gene products. The utility of *E. coli* for study of carotenoid genes, depends on adequate expression of those genes giving visible changes in *E. coli* pigmentation that can also be detected by analytical methods such as spectrophotometry or HPLC (high pressure liquid chromatography). Yet, expression of carotenoid genes is not consistent among *E. coli* strains. Therefore, I compared fourteen *E. coli* strains for level and stability of expression

[2] This chapter is a revison of Wurtzel ET, Valdez G, Matthews PD (1997) Variation in expression of carotenoid genes in transformed *E. coli* strains. Bioresearch Journal 1:1-11.

of genes encoding carotenoid biosynthetic enzymes in order to improve the heterologous complementation system.

3.2 Abstract

The carotenoid biosynthetic gene cluster of *Erwinia uredovora* has been used to condition the expression of colored carotenoids in various heterologous systems including *Escherichia coli*. Heterologous expression of these genes in *E. coli* is a powerful tool for investigating carotenoid gene function and for screening for new carotenoid related genes. I have assessed fourteen *E. coli* strains for level and stability of expression of *E. uredovora* carotenoid genes. Lycopene and zeaxanthin producing strains were examined for colony pigmentation and for accumulated carotenoids extractable from liquid cultures. Strains varied independently for colony pigmentation and stability of expression. The strain differences observed did not appear to be due to the particular carotenoid accumulated. The strains TOP10 F' and AB2463 exhibited the best accumulation of carotenoids. Recommendations are made for heterologous complementation expression screening for higher plant cDNA libraries and for improvements to this system.

3.3 Introduction

Carotenoids, a large class of yellow to orange hydrophobic pigments, photoprotect nonphotosynthetic microorganisms in high light aerobic environments. In photosynthetic organisms, carotenoids have additional functions as accessory pigments in

photosynthesis. A property unique to plants, certain carotenoids are hormone precursors (Bartley and Scolnik, 1995).

In microorganisms, such as *Erwinia uredovora* or *Erwinia herbicola*, the carotenoid biosynthetic pathway is specified by a gene cluster encoding biosynthetic enzymes that function in a pathway beginning with the synthesis of geranylgeranyl pyrophosphate (GGPP) and ending in the synthesis of zeaxanthin glucosides ((Hundle et al., 1994; Misawa et al., 1990; Sandmann et al., 1990). All or part of the *E. uredovora* and *E. herbicola* carotenoid gene clusters have been introduced into *Escherichia coli*, conferring upon this otherwise nonpigmented bacterium, the ability to accumulate a range of colored carotenoids ((Misawa et al., 1990; Sandmann et al., 1990). Since carotenoids are derived from isoprenoid precursors ((Chappell, 1995; Sandmann, 1994), *E. coli* can accumulate carotenoids by coupling an endogenous isoprenoid biosynthetic pathway with enzymes encoded by transformed genes of carotenogenic organisms such as *E. uredovora*. In fact, the biosynthetic pathway can be reconstituted *in vivo* even if the enzymes are of such diverse origin as those encoded by bacteria and plants (for examples, see ((Chamovitz et al., 1992), (Li et al., 1996a; Misawa et al., 1994)).

The expression of carotenoid genes in *E. coli* has been useful for identifying function of gene products ((Chamovitz et al., 1992; Cunningham et al., 1993; Hugueney et al., 1992; Kuntz et al., 1992; Li et al., 1996a; Linden et al., 1991; Linden et al., 1994; Math et al., 1992; Pecker et al., 1992; Penfold and Pemberton, 1994), dissection of the pathway

((Hundle et al., 1991; Sandmann et al., 1990), study of transcriptional regulators of carotenogenic genes (Penfold and Pemberton, 1994), and isolation of new genes encoding enzymes of the carotenoid biosynthetic pathway (Kajiwara et al., 1997; Lotan and Hirschberg, 1995) or enzymes catalyzing the synthesis of carotenoid precursors (Ohnuma et al., 1994).

The huge diversity and distribution of natural carotenoids present a challenge for isolation and comparative understanding of a large array of genes and gene products. The utility of *E. coli* for study of carotenoid genes, depends on adequate expression of those genes giving visible changes in *E. coli* pigmentation that can also be detected by analytical methods such as spectrophotometry or HPLC (high pressure liquid chromatography). Yet, expression of carotenoid genes is not consistent among *E. coli* strains (unpublished observations). Therefore, I compared fourteen *E. coli* strains for level and stability of expression of genes encoding carotenoid biosynthetic enzymes. The strains analyzed included those with genetic markers known to minimize recombination of cloning vectors or to promote infection by phage cloning vectors. *E. coli* strains were transformed with one of two plasmids encoding portions of the *E. uredovora* biosynthetic pathway, resulting in accumulation of either lycopene or zeaxanthin. I observed that the *E. coli* strains tested varied widely in carotenoid accumulation and stability of expression.

3.4 Materials and Methods

E. coli K-12 strains (Table 1) were transformed by the $CaCl_2$ protocol (Sambrook et al., 1989) with plasmids pACCART-EIB and pACCAR25ΔcrtX carrying gene clusters from *Erwinia uredovora* for biosynthesis of lycopene and zeaxanthin, respectively (Misawa et al., 1990). Transformants selected and maintained by resistance to 170 μg/mL chloramphenicol were confirmed by visual assessment of colony color on two day old Luria-Bertani (LB) plates and by plasmid DNA extraction from liquid culture (Sambrook et al., 1989). For quantitative extraction of carotenoids, a 10 μl stationary culture from a frozen glycerol stock was inoculated into 10 mL LB with antibiotic, grown overnight at 37 degree Celsius to stationary phase and 10 μl inoculated into 100 ml LB with antibiotic in a 250 ml Erlenmeyer flask, incubated for 24 hrs at 37 degree Celsius with agitation of *c.a.* 150 rpm. Aliquots were used to determine cell density and total protein concentration (Lowry Protein Determination Kit, Sigma Chemical Co., St. Louis, MO). Wet weight of cell pellets from 40 mL cultures were determined before carotenoid extraction. Lycopene was extracted according to Linden (1991); zeaxanthin was extracted with modifications of Linden's protocol for improved yields. In reduced light, a 40 ml culture was pelleted by centrifugation, drained and resuspended in 10 ml methanol with 6% KOH. Ten ml saturated NaCl was added, and zeaxanthin partitioned into 7.5 ml petroleum ether. The partitioning was repeated with an additional 7.5 ml petroleum ether, and the combined extracts dried at room temperature in a Savant Speedvac centrifugal evaporator. Carotenoid concentrations were determined spectrophotometrically with molar extinction coefficients of 184900 for lycopene in petroleum ether at 470 nm absorbance and 133400

for zeaxanthin in petroleum ether at 449 nm absorbance (Britton, 1975). Values for extractable carotenoids showed high correlation with values normalized to protein concentration or to cell titer (data not shown).

3. 5 Results

Fourteen *E. coli* strains were chosen from various sources, including those commonly used for gene transformation. These strains were transformed with one of two plasmids that differed in the number of *E. uredovora* genes encoding carotenoid biosynthetic enzymes. Lycopene accumulated in transformants containing pACCART-EIB, which has only part of the gene cluster, *crtE*, *crtB*, and *crtI*, encoding GGPP synthase, phytoene synthase, and phytoene desaturase, respectively. Zeaxanthin accumulated in transformants containing the second plasmid, pACCAR25ΔcrtX, which encodes the entire gene cluster except *crtX* (Misawa et al., 1990).

As shown in **Table 3.1**, the fourteen strains were ranked based on carotenoid pigmentation of transformants grown on a solid medium. I observed that transformed strains varied widely for both level of pigmentation and for stability of expression. When grown on plates, TOP10 F' and AB2463 showed the highest accumulation of pigments, whereas SURE and Y1088 showed the lowest accumulation. Except strain V73, the accumulation of lycopene and zeaxanthin did not differ for a particular strain. Plating density and colony size affected pigmentation markedly: small (<1mm diameter) colonies, resulting from high density (>12 colonies/ cm^2) plating, showed little

pigmentation, regardless of plasmid present. Pigment accumulation could be enhanced by growth over several days, although differences among strains remained. At lower plating densities, pigment accumulation was improved.

In some strains, such as SK2267, V71, KL168, and SK3451, expression was unstable: colonies showed sectoring of pigmentation, and many unpigmented colonies were present among chloramphenicol resistant primary transformants.

Strains grown in liquid culture also reflected variation in accumulation, although the range was not as dramatic as when strains were grown on solid medium. When TOP10 F' and SURE cells were grown in liquid culture, the TOP10 F' culture accumulated almost twice as much lycopene (approximately 100 μg/gm wet cell weight) as compared to SURE cells.

3.6 Discussion

Carotenoid pigmentation in fourteen strains grown on solid medium varied widely. There was also no obvious correlation between genetic markers and pigmentation (**Table 3.1**). It is likely that unidentified markers affect pigment accumulation. For example, since the pathway utilizes isoprenoid precursors, expression of loci controlling isoprenoid biosynthesis or pathways competing for precursors pools (Chappell, 1995), might affect overall carotenoid accumulation from strain to strain. Examination of isoprenoid levels in these strains might reveal differences in isoprenoid pools that would support the observed

differences in pigment accumulation. Alternatively, strains may vary in level of expression of the heterologous *E. uredovora* genes that could also account for differences in carotenoid accumulation between strains.

The carotenoid pigments themselves, which are hydrophobic and may perturb the membrane through association, could also affect accumulation. But as shown in **Table 3.1**, in most cases, the accumulation and stability of expression was independent of carotenoid accumulated.

Instability in expression, such as sectored colonies and colony to colony pigment variation, was also observed. However, this instability appeared to be independent of pigmentation level. For example, both HB101 and KL168 showed medium pigmentation, although expression in KL168 was very unstable compared with HB101. Similarly, strains showing weak pigmentation, such as SURE and Y1088, were not necessarily unstable. Perhaps, these strains vary in stability of the plasmid. Both plasmids have in common *crtE*, *crtB*, and *crtI*, encoding GGPP synthase, phytoene synthase, and phytoene desaturase, respectively, but pACCAR25ΔcrtX additionally contains *crtX*, *crtY* and *crtZ* encoding zeaxanthin glucosyl transferase, lycopene cyclase, and β-carotene hydroxylase, respectively, with a frameshift mutation in *crtX*. The gene cluster of pACCART-EIB is 3.8 kb, while the cluster of pACCAR25ΔcrtX is 6.5 kb. Since some strains showing instability of carotenoid pigmentation were recombination deficient (for example, *recA*), it is unlikely that the observed instability was a result of marker loss from the plasmid.

I have identified several strains suitable for studies of carotenoid biosynthesis, such as functional analysis of cloned genes. Although *E. coli* strains have been used for complementation screening using M13-based cDNA expression libraries, I found that some stable carotenoid accumulating strains became unstable when superinfected with such M13-based cDNA expression libraries (data not shown). Therefore, some caution is needed in using even the stable and strongly pigmented strains for such studies. Since I observed that optimal colony pigmentation is obtained with low density plating, screening for higher plant genes encoding carotenoid enzymes will be more difficult as these genes are generally expressed at low levels (for example, see (Li et al., 1996b)). To screen one million clones by complementation, would require one hundred and forty 625cm^2 screening plates. Methods for selection of carotenoid producing cells in liquid culture could greatly improve the efficacy of screening. For example, fluorometric flow cytometric cell sorting devices have been used to isolate carotenoid producing yeasts (An et al., 1991). Such devices might prove useful for screening by complementation where gene expression alters carotenoid pigmentation compared to background cells. Advantage could be also be taken of the photoprotective (Tuveson and Sandmann, 1993) or singlet-oxygen toxicity-protective properties (Dahl et al., 1989) (Kagan et al., 1992) of carotenoids to select against non-pigmented cells before plating.

3. 7 Acknowledgments

I thank Dr. Norihiko Misawa for plasmids containing the *Erwinia* genes, the *E. coli* Genetic Stock Center and Dr. Mary Berlyn for advice and strains, and Dr. Vivek Upasani for technical advice. I am especially grateful to Gregorio Valdez, who did these experiments, and furnished great friendship, always. This research was supported by grants from The National Institutes of Health-Minority Biomedical Research Support Program (2S06 GM08225-11) and The Rockefeller Foundation International Rice Biotechnology Program.

Table 3.1 Strains Ranked for Carotenoid Pigmentation. Continues next page.

			argH1(del)	
13	Y1088	Stratagene	supE supF metB trpR hsdR$^-$ hsdM$^+$ tonA21 strA$^-$ lacU169 proC::Tn5 (pMC9 lacI)	low
14	SURE	Stratagene	[F' proAB lacI^{q}ZΔM15 Tn10 (tet^r)] e14-(mcrA) Δ(mcrCB-hsdSMR-mrr)171 sbcC recB recJ umuC::Tn5(kan^r) uvrC supE44 lac gyrA96 relA1 thi-1 endA1	low

[a] High, medium, and low are relative ratings of carotenoid accumulation based on visual assessment of strain pigmentation after 48 hrs of growth on a solid medium. Unless indicated otherwise, the rating is identical for lycopene (lyc)-expressing cells and zeaxanthin (zea)-expressing cells.
[b] unstable, many colonies sectored for pigmentation and marked pigment variation between colonies; very unstable, a majority of unpigmented colonies.

Table 3.1. Descending rank of *E. coli* strains transformed with *E. uredovora* carotenoid genes.

Table 3.1 Strains Ranked for Carotenoid Pigmentation. Continues next page.

			glnV44(AS) galK2 λ⁻ rac-0 uvrC34 hisG4(Oc) rfbD1 mgl-51 rpsL31(strR) kdgK51 xylA5 mtl-1 argE3(Oc) thi-1	
7	AB2480	CGSC#2480	Δ(gpt-proA)62 lacY1 tsx-33 glnV44(AS) galK2 λ⁻ recA13 rpsL31(strR) or rpsL8 xylA5 mtl-1 thi-1 uvrA6	medium
8	AB1886	CGSC#1886	F⁻ thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 qsr'-0 glnV44(AS) galK2 λ⁻ rac-0 hisG4(Oc) rfbD1 mgl-51 rpsL31(strR) kdgK51 xylA5 mtl-1 argE3(Oc) thi-1 uvrA6	medium
9	SK2267	CGSC#12959	F⁻ lacZ4 or lac-61 glnV44(AS) gal-44 λ⁻ sbcB15 recA1 endA1 rfa-([C]) thi-1 hsdR4 ton-58	medium /unstable
10	V71	CGSC#6734	F⁻ tsx-33 galK2 λ⁻ rac-0 hisG4(Oc) recC73 recC1003(χ) argA21 rpsL31(strR) kdgK51 xylA5 mtl-1 recF143 bglR17 Met-(ts)	medium /unstable
11	KL168	CGSC#4202	Hfr λ⁻ e14- relA1 recB21 thi-1 deoB13	medium/very unstable
12	SK3451	CGSC#6882	F⁻Δ(lacY-lacZ)286 tsx-3 glnV44(AS) λ⁻ lacZ95=φ80dIIlacZ9 hisG4(Oc) rpsL281(strR) xylA7 mtl-1 metE46 uvrD282::Tn5	medium/very unstable

Table 3.1 Strains Ranked for Carotenoid Pigmentation. Continues next page.

	STRAIN	SOURCE	GENOTYPIC MARKERS	RATING[a]/comment[b]
1	TOP10 F'	Invitrogen	F'{tet^r} mcrA Δ(mrr hsdRMS mcrBC) φ80Δlac-ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara, leu)7697 galU galK λ$^-$ rpsL endA1 nupG	high
2	AB2463	CGSC#2463	F$^-$ thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 qsr'-0 glnV44(AS) galK2 λ$^-$ rac-0 hisG4(Oc) rfbD1 recA13 rpsL31(strR) kdgK51 xylA5 mtl-1 argE3(Oc) thi-1	high
3	HB101	B R L	F$^-$ mcrB mrr hsdS20(r_B^-,m_B^-) recA13 supE44 ara14 galK2 lacY1 proA2 rpsL20(Str^r) xy15 λ$^-$ leu mtl1	medium
4	SOLR	Stratagene	[F' proAB lac1^q ZΔ M15] e14-(mcrA) Δ(mcrCB-hsdSMR-mrr)171 sbcC recB recJ uvrC umuC::Tn5(kan^r) lac gyrA96 relA1 thi-1 endA1 λ^R Su$^-$ (non-suppressing)	medium
5	V73	CGSC#6736	F$^-$ tsx-33 galK2 λ$^-$ rac-0 hisG4(Oc) recC73 recC1001(χ) argA21 rpsL31(strR) kdgK51 xylA5 mtl-1 recF143 bglR17 Met-(ts)	medium (lyc), high (zea)
6	AB1884	CGSC#1884	F$^-$ thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 qsr'-0	medium

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[3]Chapter 4. Metabolic engineering of carotenoid accumulation in *Escherichia coli* by modulation of the isoprenoid precursor pool with expression of deoxyxylulose phosphate synthase

4.1 Prologue

The *E. coli* heterologous complementation carotenogenic system is not only a valuable tool for the isolation and functional analyses of carotenoid biosynthetic genes and a potentially valuable source of new and unusual carotenoids but also a testing ground for hypotheses regarding relationships among metabolic pathways. One hypothesis, that carbon flow from an increased isoprenoid precursor pool could be directed into the production of useful secondary metabolites, such as carotenoids, became possible with the availability of gene expression constructs for a key, rate-controlling enzyme in a recently discover biosynthetic route to activated isoprenes. A second hypothesis, that this phenomenon is extendable to genetically engineered plants, has yet to be tested.

4.2 Abstract

The recently discovered non-mevalonate pathway to isoprenoids, which uses glycolytic intermediates, has been modulated by overexpression of *E. coli* D-1-deoxyxylulose 5-phosphate synthase (DXS) to increase deoxyxylulose 5-phosphate (DXP) and, consequently, increase the isoprenoid precursor pool in *E. coli*. Carotenoids are a large class of biologically important compounds synthesized from isoprenoid precursors and of interest for metabolic engineering. However, carotenoids are not ordinarily present in *E.*

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coli. Co-overexpression of *E. coli dxs* with *Erwinia uredovora* gene clusters encoding carotenoid biosynthetic enzymes, led to an increased accumulation of the carotenoids lycopene or zeaxanthin over controls not expressing DXS. Thus, rate-controlling enzymes encoded by the carotenogenic gene clusters are responsive to an increase in isoprenoid precursor pools. Levels of accumulated carotenoids were increased up to 10.8 times the levels of controls not overexpressing DXS. Lycopene accumulated to a level as high as 1333 μg/gdw, and zeaxanthin accumulated to a level as high as 592 μg/gdw, when pigments were extracted from colonies. Zeaxanthin-producing colonies grew about twice as fast as lycopene-producing colonies throughout a time course of 11 days. Metabolic engineering of carbon flow from simple glucose metabolites to representatives of the largest class of natural products was demonstrated in this model system.

4.3 Introduction

Isoprenoids are a diverse group of natural products found in all organisms. They are derived from the C_5 skeleton of isopentenyl pyrophosphate (IPP). IPP was thought to be strictly derived from the mevalonate pathway, and the enzyme HMGCoA reductase was considered to be a rate-limiting enzyme for this biosynthetic route (Dimster-Denk et al. 1994; Chappell 1995). Recently, a novel non-mevalonate biosynthetic pathway was discovered that operates specifically in plastids of plants, reviewed in (Lichtenthaler 1999), as well as in bacteria and in cyanobacteria; in plants the mevalonate pathway operates in the cytoplasm in parallel to the plastid-localized non-mevalonate IPP pathway

(Rohmer et al. 1993; Schwender et al. 1996). In the non-mevalonate route, IPP is derived from deoxyxylulose 5-phosphate (DXP), which in *Escherichia coli* has also been found to be a common precursor to biosynthesis of vitamins B1 (thiamin) and B6 (pyridoxal) (Meyer and Sahm 1997). The enzyme, D-1-deoxyxylulose 5-phosphate synthase (DXS), is responsible for catalyzing the synthesis of DXP from pyruvate and GAP (glyceraldehyde 3-phosphate) (Sprenger et al. 1997; Lange et al. 1998; Lois et al. 1998). In plants and bacteria, competition for DXP might affect end-product accumulation for different isoprenoid-derived biosynthetic pathways. The enzyme responsible for DXP synthesis might represent a novel rate-controlling enzyme, whose expression could be modified to increase substrate availability for pathways under consideration for metabolic engineering.

Carotenoids are one example of isoprenoids for which interest in metabolic engineering lies in their utility as coloring agents and as precursors to vitamin A and to retinoids, compounds essential in growth and development (Misawa et al. 1991; Misawa et al. 1993; Yamano et al. 1994; Burkhardt et al. 1997; Ruther et al. 1997; Wang et al. 1999). Carotenoids are synthesized in certain bacteria, fungi, and in plastids of plants, as reviewed in (Armstrong and Hearst 1996; Cunningham and Gantt 1998). Gene clusters encoding the carotenoid biosynthetic enzymes have been isolated from epiphytic bacteria such as *Erwinia uredovora*, and their introduction into *E. coli* has resulted in carotenoid accumulation (Misawa et al. 1990). In addition to the prospect of bioengineering the accumulation of useful and unusual carotenoids, the expression of carotenogenic genes in

E. coli represents a unique opportunity for cloning of new genes by heterologous complementation (Sun et al. 1996), for functional testing of gene products (Li et al. 1996), and for examination of flow through the pathway (Kajiwara et al. 1997; Ruther et al. 1997).

The C_{20} isoprenoid GGPP (geranylgeranyl pyrophosphate), produced by GGPP synthase (GGPPS), is the first precursor to carotenoids, and to a variety of other isoprenoid-derived products, including gibberellins, the phytol chain of chlorophyll, prenylquinones, tocopherols, prenylated proteins, and many secondary metabolites such as taxol and casbene (Chappell 1995). GGPP is formed from four units of IPP, one of which is an isomer (DMAPP). IPP isomerase converts IPP to DMAPP in *E. coli*; DMAPP condensed with consecutive molecules of IPP form FPP (farnesyl pyrophosphate), GPP (geranyl pyrophosphate), and then GGPP.

In *E. coli,* the presence of GGPP depends on the activity of endogenous IPP isomerase and FPP synthase, and exogenous GGPPS. Recent efforts have focused on increasing carotenoid accumulation by overexpression of these three enzymes (Wang et al. 1999), as well as overexpression of enzymes within the carotenoid pathway (Kajiwara et al. 1997; Ruther et al. 1997). Hypothetically, the accumulation of carotenoids also depends on the concentration of IPP as derived from the non-mevalonate pathway. Since the DXS enzyme functions at the junction of three pathways, manipulation of DXS enzyme levels

might be as important in enhancing endproduct accumulation as increasing levels of downstream enzymes.

To test the flux relationships between the DXS-mediated isoprenoid pathway and a carotenoid biosynthetic sink in *E. coli,* I examined the effect of overexpressing *E. coli* DXS in combination with carotenoid enzymes encoded by *E. uredovora*. For this purpose, I used an *E. coli dxs* gene construct which had previously been shown to confer over a 100-fold increase in DXS activity in *E. coli* cell-free extracts (Sprenger et al. 1997; Lois et al. 1998). The over-expression of *E. coli* DXS, in the presence of the *Erwinia* carotenoid gene cluster, resulted in strikingly rich-colored colonies. I present the time course and extent of carotenoid accumulation in these strains.

4.4 Materials and methods

4.4.1 Growth, strains, and plasmids

Lycopene (ψ,ψ-carotene) and zeaxanthin (β,β-carotene-3,3'-diol) accumulation in *E. coli* TOP10 F' (Invitrogen, Carlsbad, CA) was accomplished by expression of the *Erwinia uredorova* carotenoid gene cluster present in plasmids pACCRT-*EIB* or pACCAR25Δ*crt*X, alternatively named pCAR25del*Y* (Misawa et al. 1990), as previously described (Wurtzel et al. 1997). Plasmid pACCAR25Δ*crtB* (Misawa et al. 1990), which has a frameshift mutation in the gene encoding phytoene synthase, produces no carotenoid and was used as a negative control. These carotenoid accumulating strains were transformed by the $CaCl_2$ protocol (Sambrook et al. 1989) with an additional

plasmid, pTAC-ORF2 (*dxs*) expressing *E. coli* D-1-deoxyxylulose-5-phosphate synthase (Lois et al. 1998). Bacteria were cultivated in Luria-Bertani (LB) medium in liquid culture or agar plates (Sambrook et al. 1989) supplemented with 50 μg/ml ampicillin to select for pUC-derived plasmids carrying *dxs* and 70 μg/ml chloramphenicol to select for pACY184-derived plasmids carrying the *Erwinia* carotenogenic gene clusters. In some cases DXS expression was induced by addition of 1mM isopropyl-1-thio-β-D-galactoside (IPTG). Liquid cultures were inoculated from a glycerol stock (Sambrook et al. 1989) prepared from one primary transformant colony. Ten μl of the glycerol stock was added to 10 ml of LB with antibiotic and grown to stationary phase for 12 hrs at 37°C and shaking at 240 rpm. Fifty μl of these starter cultures were then inoculated into 50 ml of LB with antibiotics in 125 ml Erlenmeyer flasks, incubated at 37 °C with shaking for 30 hrs, then held at room temperature for an additional 30 hours. For growth of colonies on solid medium, cells from a glycerol stock of c.a. 500 primary transformant colonies scraped from a plate and mixed together, were serially diluted and plated onto LB plates with antibiotics at a density of c.a. 150 colonies per 15 X 100 mm Petri dish. These plates were incubated at 37 °C for 8 hours and then at room temperature for 2-11 days. All cultures were grown in the dark and shielded from light during all manipulations and subsequent carotenoid extractions.

4.4.2 Carotenoid analysis

Colonies lifted onto dry 5.4 cm^2 nitrocellulose membranes or single colonies lifted onto 7 mm diameter discs were placed colony-side up in a pool of 50 mM glucose/0.25 mM

Tris, pH 8.0/10mM EDTA (GTE) on a glass plate or microtiter plate lid and scanned wet on a Hewlett Packard ScanJet 6100C at settings: contrast 200, brightness 200, scaling 300%, and resolution 100 DPI, 256 greys. Colony boundaries were selected manually and pixel values determined with ImageQuaNT V4.1B (Molecular Dynamics Sunnyvale, CA). After scanning, cells were released from the filters by vortexing (in the case of single colony lifts) or massaging with GTE (in the case of entire plate lifts) and stored frozen in glycerol until extraction. Alternatively, cells from liquid cultures were collected by centrifugation and weighed wet.

Carotenoids were exhaustively extracted essentially according to the protocol of De Leehnheer (De Leehnheer and Nelis 1992), except starting cells were not freeze-dried, but rather suspended in a small volume of GTE. All samplings and extractions were performed in at least duplicate and all samples were extracted simultaneously in parallel. Carotenoids were quantified spectrophotometrically in hexanes at 479 nm and concentration of extracts was determined by interpolation of a curve ($r^2 = 0.991$) of lycopene standards (Sigma, St. Louis, MO). Intermediates in the carotenoid biosynthetic pathway do not accumulate to significant levels; hence, the quantified carotenoid represents the end product of the pathway engineered on the basis of the carotenoid gene cluster present (Ruther et al. 1997; and data not shown).

Since extracted carotenoids were highly correlated ($r = 0.998$) with the area of colonies pooled for extraction, relative comparisons were based on the concentration of the extract

normalized to colony area. Wet and dry colony mass and area relationships were determined by lifting colonies to pre-weighed membranes, scanning, weighing, then reweighing air-dried colonies until no further weight change occurred. The ratio of fresh weight colonies to dry weight was 0.24 +/- 0.04 (n=9 filters, +/-SD), and the ratio of colony area in pixels to gram dry weight was 4,130,000 +/- 430,000 (n=9 filters, +/-SD).

4.5 Results

I tested the hypothesis that the IPP pool available for carotenoid accumulation in *E. coli* is limited by expression of *dxs*, the gene encoding D-1-deoxyxylulose 5-phosphate synthase. The *dxs* gene was recently cloned from *E. coli* and expression of its gene product characterized (Sprenger et al. 1997). Lois, et al. (1998) have demonstrated high levels of DXS activity in *E. coli* cell-free extracts transformed with the plasmid pTAC-ORF2, which contains *dxs* under control of a strong promoter. I introduced this plasmid into *E. coli* harboring *E. uredovora* genes needed to confer carotenoid biosynthesis. The *E. coli* strain TOP10 F' was chosen for transformation based on an earlier study (Wurtzel, et al.; and **Chapter 3**) suggesting that, unlike other strains, carotenoid accumulation was clonally stable and fairly strong in TOP10 F' (Wurtzel et al. 1997). As shown in **Figure 4.1**, colonies accumulating the red lycopene showed a dramatic increase in pigmentation when the overexpressed DXS was present. This increase in accumulation, conferred by the overexpressed DXS, was apparent for both lycopene- and zeaxanthin-accumulating strains and was further quantified as listed in the **Table 4.1**.

Based on values from exhaustively extracted carotenoids from Top10 F' strains, I demonstrate a 4.4-10.8-fold increase in lycopene accumulation in colonies grown on agar plates containing pACCRT-*EIB* and *E. coli* D-1-deoxyxylulose-5-phosphate synthase (*EIB+dxs*) as compared to the same strain containing just pACCRT-*EIB* (*EIB*). Similarly, zeaxanthin-accumulating strains exhibited a 2.1-3.9-fold increase when cells expressed both the carotenoid gene cluster of pACCAR25Δcrt*X* and *dxs* (Δ*crtX+dxs*) as compared to the same strain containing just pACCAR25Δ*crtX* (Δ*crtX*).

Carotenoid accumulation is the product of cell growth and pigment biosynthesis, each of which might vary independently over time. Therefore, I carried out a time course of lycopene accumulation over 11 days of colony culture on plates as shown in Figure 2. Pigmentation reached maximum after 11 days of incubation at room temperature in the dark. Lycopene accumulation per gram dry cell weight was consistently two times higher than that of zeaxanthin accumulation during the entire growth period, regardless of increases due to overexpression of DXS (zeaxanthin data not shown). Over the incubation time of 11 days, lycopene levels in *EIB+dxs* increased up to 15 fold higher as compared to 1 day growth. In strains expressing *dxs*, increases in pigmentation continued only up to 4 days for zeaxanthin but up to 11 days for lycopene. In all cases, zeaxanthin-producing colonies grew faster and were nearly twice as large as lycopene producing colonies, therefore, levels of zeaxanthin production were similar to lycopene production on a per colony or per plate basis (data not shown).

The differences observed when strains were grown on solid medium were further explored by growth in liquid culture. Extracts from liquid cultures showed a similar range of pigment expression as that measured in colonies, but with much greater variation (data not shown). I saw a large variation in carefully prepared replicate cultures, as well as large variation among cultures prepared from different primary transformants. IPTG induction of DXS expression at various time points had a negative effect on carotenoid accumulation in liquid cultures as well as in colonies on plates (data not shown).

4.6 Discussion

Accumulation of both lycopene and zeaxanthin in *E. coli* was greatly enhanced by overexpression of DXS, suggesting that IPP pools may be limited by DXS expression and activity. Other studies have shown effects on the rate of carotenoid synthesis by causes downstream of the IPP pool. For example, Wang et al. (1999) have shown that co-overexpression of heterologous GGPPS (CrtE) and homologous IPP isomerase, enzymes upstream of the carotenoid biosynthetic pathway, but downstream from DXS, increased astaxanthin accumulation in *E. coli* up to a level of 234 μg/gdw. Co-overexpression of genes encoding a novel bifunctional GGPPS (*gps*) and IPP isomerase (*idi*) led to a drastic increase in astaxanthin levels up to 1419 μg/gdw, demonstrating that GGPPS and IPP isomerase are rate-controlling. By modulation of IPP precursor levels in the presence of native levels of IPP isomerase and FPP synthase, my maximum value for accumulation of lycopene was similar at 1333 μg/gdw. Together these studies clearly indicate, that flux

into the heterologous carotenoid biosynthetic pathway is influenced by rate-controlling enzyme levels which are also responsive to precursor concentrations.

Overexpression of enzymes within the carotenoid biosynthetic pathway has not led to large increases of carotenoid accumulation. For example, co-transformation of carotenogenic strains harboring pACYC-derived plasmids with an additional pUC-derived plasmid overexpressing one of enzymes of the biosynthetic gene cluster, such as phytoene synthase, phytoene desaturase, or lycopene cyclase, had a negative effect on carotenoid accumulation. Only carefully controlled (by growth-phase specific IPTG induction) overexpression of a terminal enzyme, β-carotene hydroxylase, created a metabolic sink and a 1.3 fold increase in zeaxanthin accumulation to 276 ug/gdw (Ruther et al. 1997).

On the contrary, I report a 2.2-10.8 fold increase from my average values of extracted carotenoid, when DXS is overexpressed. While my average values for accumulation in Δ*crtX* and *EIB* are similar to literature values for other carotenoid gene cluster containing *E. coli* strains (Ruther et al. 1997; Wurtzel et al. 1997; Wang et al. 1999), I measured increases up to 592 g/gdw for zeaxanthin and up to 1333 ug/gdw for lycopene with overexpression of DXS. pTAC-TAC expression vectors are IPTG inducible, but lack tight control of basal expression level from the strong P_{tac} promoter, leading to DXS expression in the absence of induction (Rohmer et al. 1993). Preliminary experiments with induction of DXS expression by inclusion of IPTG in solid medium or addition of

IPTG to liquid cultures at inoculation, at midlog, and at stationary phases of growth had a negative effect on lycopene and zeaxanthin accumulation.

Inclusion of glucose in the growth medium increases astaxanthin pigmentation in *E. coli* overexpressing GGPPS and IPP isomerase in combination with an astaxanthin biosynthetic gene cluster (Wang, et al. 1999). Co-overexpression of IPP isomerase in my DXS-overexpressing lines in the presence of glucose may further stimulate carotenoid accumulation in my strains.

Success with manipulations of these coupled pathways in bacteria may be extendable to plants, especially as the carotenoid biosynthetic pathway and the non-mevalonate route to IPP via DXS are plastid localized. Efforts are underway to extend metabolic engineering of the isoprenoid precursor pool in the presence of a carotenoid biosynthetic sink in higher plants.

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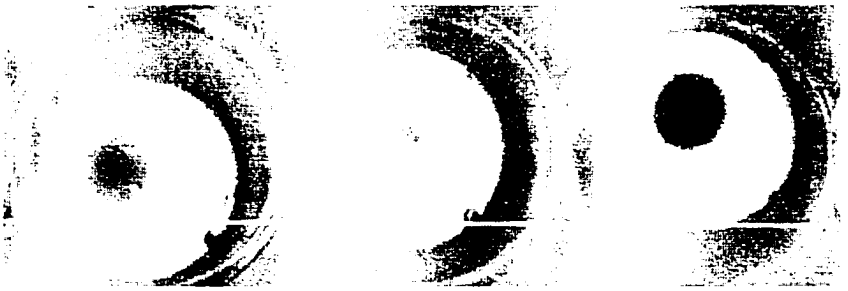

Fig. 4.1 Overexpression of D-1-deoxyxylulose 5-phosphate synthase (DXS) causes increase in lycopene accumulation in *E. coli* colonies. *E. coli* transformed with *Erwinia uredovora* gene cluster conferring lycopene biosynthesis without (left) or with (right) overexpression of *E. coli* DXS. Middle, shows unpigmented colony.

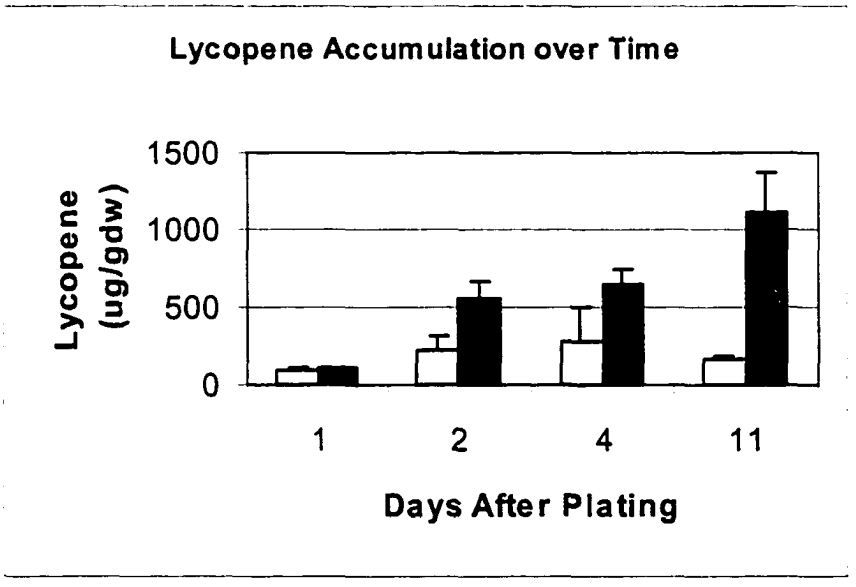

Fig. 4.2 Timecourse of carotenoid accumulation in strains containing pACCRT-*EIB* (unshaded bar) pACCRT-*EIB*+pTAC-ORF2(DXS) (shaded bar). Values are averages of 2-4 determinations and error bars are +/- SD.

Plasmids	Enzymes	Pigment	Carotenoid µg/gfw)	Carotenoid µg /gdw)
pACCRT-EIB	GGPPS, PSY, CRTI	lycopene	38 +/- 8	160 +/- 32
pACCRT-EIB pTAC-ORF2	GGPPS, PSY, CRTI DXS	lycopene	266 +/- 64	1106 +/- 270
pACCAR25ΔcrtX	GGPPS, PSY, CRTI, LCY, HYD	zeaxanthin	44 +/- 8	186 +/- 36
pACCAR25ΔcrtX pTAC-ORF2	GGPPS, PSY, CRTI, LCY, HYD DXS	zeaxanthin	126 +/- 16	526 +/- 66
pACCAR25ΔcrtB	GGPPS, CRTI, LCY, HYD	none	none	none

Table 4.1. Average carotenoid accumulation among strains carrying plasmids with or without the *E. coli dxs* gene. Enzymes encoded by the plasmids are as indicated. Carotenoid content data were taken from 4 days growth on solid medium for zeaxanthin and 11 days growth for lycopene. Gfw = grams fresh weight and gdw = grams dry weight, for which carotenoid content was normalized to colony surface area.

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Chapter 5. Determination of *E/Z* isomers states of products of a maize phytoene desaturase and zetacarotene desaturase

5.1 Prologue

"Normally carotenoids occur in Nature as the (all-*E*)-isomer; however, exceptions are known, such as the (15*Z*)-phytoene isolated from carrots, tomatoes, and other organisms. On the other hand, some carotenoids undergo isomerization very easily during workup; therefore many (*Z*)-isomers that are described in the literature as natural products are artifacts. For experimental work it must be kept in mind that (*E/Z*)-isomerization may occur when a carotenoid is kept in solution. Normally the percentage of the (*Z*)-isomers is rather low, but it is enhanced at higher temperature. Furthermore, the formation of (*Z*)-isomers is increased by exposure to light." — Hanspeter Pfander (Pfander, 1992)

5.2 Abstract

The carotenoid desaturases of maize, phytoene desaturase (PDS) and zetacarotene desaturase (ZDS), when overexpressed together in a heterologous *E. coli* carotenogenic system that supplies phytoene, catalyze the production of 7,9,7',9'-tetra-Z-lycopene (poly-*Z*-lycopene or prolycopene). By HPLC and PDA analysis of carotenoids produced in various gene expression systems, I have found that the products of PDS to be approximately a 4:1 mixture of *Z*-ξ-carotene and all-*E*-ξ-carotene. I also show ZDS to produce poly-*Z*-lycopene upon desaturation of the product produced by PDS, through poly-Z-neurosporene intermediates, including 7,9,9'-tri-Z-neurosporene. Thus, I

demonstrate a poly-Z desaturation pathway for maize PDS and ZDS. These results suggest maize ZDS is capable of introducing *Z*-double bonds. I also show that PDS and ZDS produce small amounts of all-*E*-lycopene, hence both may also catalyze the *E* - elimination routes. These results are discussed in relation to two divergent hypotheses of the range of occurrence of poly-*Z*-desaturation pathways to carotenoids in plants.

5.3 Introduction

This section recalls the details of the carotenoid biosynthetic pathway elaborated in **Chapter 1** with new emphasis on the stereochemistry of the desaturation reactions and the geometric isomer states of the carotenoids produced. Background relevant to interpretation of HPLC and PDA of carotenoid *E/Z* isomers and recent literature relevant to interpretation of results is also presented.

5.3.1 A carotenogenic metabolon

The central reactions resulting in the production of carotenoids from activated isoprene precursors can be divided into four functional classes. A series of condensations (transfers of prenyl groups) are followed by desaturations, cyclizations, and oxygenations. These covalent conversions occur in linear sequence (Goodwin, 1983). Further modifications such as hydrations, eliminations, and glycosylations provide more diversity in structure and function. **Figure 1.3** shows the substrates and the sequence of conversions for the common core of reactions.

Many of the conversions are reiterated within this linear sequence. Often, a reiterated conversion is completed by recursion of the same catalytic activity. For example, desaturations occur on a mirror-equivalent site of a bilaterally symmetrical polyene chain (see **Figure 1.1** for lycopene). Meanwhile, some recursive conversions, such as cyclization, may be completed by unique enzymes acting at opposite ends of the same molecule. For example, α-carotene results from the action of an ϵ-cyclase acting on one end of the polyene and β-cyclase on the other. The number of enzymes involved and the number of recursions of a particular catalytic activity vary among different organisms (Linden et al., 1991).

The linear partitioning of functions, some of which recur two to four times, suggests a spatial array of catalytic activities (Cunningham and Gantt, 1998a; Goodwin, 1983; Kleinig and Britton, 1982). The usual lack of detectable intermediates *in planta*, suggests that the substrates are tightly held in an enzyme complex (Beyer et al., 1985b; Cunningham and Gantt, 1998a). This assembly-line-like channeling of substrates allows for the possibility that earlier conversions may influence the nature of the catalytic properties of subsequent enzymes directly, *i.e.*, what happens at one point in the biosynthetic complex may be communicated to another part of the complex by virtue of the physical contact of the structures involved. The communication may be transmitted through the protein moiety of the enzymes or the structures of the substrates themselves, as they are constrained within the complex. The symmetry of the substrates and the symmetry of actions on the substrate also suggest that enzymatic complexes may have a

symmetrical, multimeric structure that dictates function (Cunningham and Gantt, 1998a; Goodwin, 1983). Two recursive functions may be the action of a homodimeric enzyme. For example, GGPPS exists in a phytoene synthase biosynthetic complex as a homodimer (Dogbo and Camara, 1987). A holomeric enzyme complex in which information is transmitted through the complex in a series of cause and effects on channeled substrates may be called a "metabolon."

Different carotenoid biosynthetic enzymes are localized in different plastid compartments (Al-Babili et al., 1996; Bonk et al., 1997; Camara et al., 1982; Heintze et al., 1990; Kreuz et al., 1982; Price et al., 1995; Schledz et al., 1996). These compartments vary greatly in hydrophilicity and hydrogen ion concentration. **Table 1.1** reviews the various functional conversions, where enzymes are localized, and the points which substrates become dedicated to a particular class of compounds, *e.g.*, isoprenoids, terpenoids, carotenes, or xanthophylls. The sequence of reactions will be recalled below briefly.

5.3.2 The sequence of reactions

The first class of conversions is sequential transfer of eight diphosphate-activated isoprene units to produce a growing chain of hydrocarbons with double bonds. The final product of these condensations is phytoene as shown in **Figure 5.1.** The product of the bifunctional enzyme phytoene synthase (PSY) is predominately 15-Z-phytoene (Powls and Britton, 1977a).

Phytoene is the first substrate unique to the carotenoids. The second class of conversions is the recursive desaturation of a C40 hydrocarbon to produce an extended, photoelectronically active system of double conjugated bonds. The third class of conversions is cyclizations of the hydrocarbon end structures. The fourth class is oxygenation, with mechanistic sub-classes, namely: oxygenation, hydroxylation, and epoxidation.

While the first class of conversions are stromal and peripherally associated with the plastid membranes (Al-Babili et al., 1996; Camara et al., 1982; Lutke-Brinkhaus et al., 1982; Schledz et al., 1996), the later three classes of conversions likely occur within the plastid membrane system (Cunningham and Gantt, 1998b; Grunewald et al., 2000; Lutke-Brinkhaus et al., 1982), although there are important exceptions (Grunewald, personal communication) and some debate. Variations in the number and type of these conversions generate the large diversity of carotenoids. In some organisms, hydrogenation, elimination, glycosylation and cleavage add yet further diversity to generate secondary metabolites. Additionally, catalytic activities may be tissue- and plastid-specific within an organism. The nature of this variation within and among organisms has not been examined inclusively. The molecular genetics and biochemistry of each class of relevant conversions is reviewed in detail in **Chapter 1**. This introduction focuses on the stereochemistry of the desaturations.

5.3.3 Stereochemistry of the desaturations

Imposed on this series of covalent conversions is a milieu of substrate isomerization states. Much investigation has been made about *E/Z* isomer states among extended double bond systems of carotenoid backbone structures. The desaturation reactions not only produce particular *E/Z* isomers depending on the route of hydrogen elimination, but also the enzymes involved may require a particular *E/Z* conformation of the substrate (stereoselectivity) or behave according to the *E/Z* conformation of the substrate (stereosensitivity). While the stereochemistry of the formation of all-*E* lycopene and 15-*Z*-lycopene has been fully described, few studies (Beyer et al., 1989; Breitenbach et al., 1999; Qureshi et al., 1974) have addressed the interaction between the substrate isomer state and the enzyme's catalytic properties within the desaturation series. Given the existence of isomeric states, many questions arise, such as:

- Are the desaturases stereoselective?
- Are desaturases flexible in their hydrogen elimination routes?
- Does the substrate's isomeric state influence the route of elimination of the desaturase?
- Must the paired didehydrogenations have the same stereo-elimination route?
- Are there variations in stereoselectivity or leaving-group-selection that are tissue-, organ-, plastid- or species- specific?
- Are there isomerase activities or photoisomerizations that are necessary for progression though the pathway?

- Does the activity of the enzyme *in vitro* or in a heterologous *E. coli* carotenogenic system mimic the behavior of the enzyme *in situ* with respect to isomeric states of substrates?

This chapter presents studies of the maize phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) working together in *E. coli*. These activities are expressed from cDNAs. The maize enzymes are over-expressed from plasmids in an *E. coli* heterologous complementation system (Misawa and Shimada, 1997; Sandmann, 1997) (see also **Chapters 3** and **4**). The study augments initial characterizations of the carotenoid products of PDS (Li et al., 1996b) and of ZDS (Luo, 2000) and also identifies the final product of the maize desaturases as lycopene, an issue that was not resolved by Luo. But here the focus is on the *E/Z* conformation of the intermediates and products of the desaturations. In concert, PDS and ZDS introduce four double bonds into phytoene, which already carries 9 double bonds. These existing double bonds come from the substrates of the condensations and the condensations themselves. These double bonds do not come from polyene desaturations. PDS introduces the first two double bonds into the polyene. ZDS introduces the second two double bonds into the polyene. Each enzyme acts recursively on bilaterally symmetrical substrates. Potentially, the dehydrogenations may occur by elimination of the hydrogen in the *Z*-configuration (*cis*) to generate a *Z*-double-bond or elimination in the *E*-configuration (*trans*) to yield an *E*-double-bond. Characterization of the isomeric states products of each of the enzymes reveals the routes

of elimination. Carotenoids from higher plants such as lycopene and carotenes are thought to occur in the all-*E* configuration (Bramley, 1985).

A comparison of the structures of products of the concerted action of the two enzymes to previous studies of the products of desaturases alone may help define the sequence of potentially overlapping activities and also the extent of interdependence of the activities. Here, particular attention is given to the possible dependence of the activity of ZDS on the *E/Z*-isomer-states of the substrates and products of PDS. This analysis is conducted with a cautious cognizance of the distinction between the molecular function of an isolated enzyme *in vitro* or in a heterologous *E. coli* carotenogenic system and the molecular function in the complex internal environment of the plastid.

5.3.4 Specific Background

5.3.4.1 Prerequisites to interpretation of HPLC results

An understanding of my interpretation of HPLC and spectral characterization of the desaturation products may require the review of (1) the specific enzyme activities in the desaturation pathway and evidence for substrate specificity and sequence of catalytic action, (2) the possible intermediates and products of the desaturation series, (3) cofactors and other requirements for desaturations, and (4) the interpretation of HPLC elution profiles and PDA spectral profiles for isomer identification. These will be presented here, briefly.

5.3.4.1.1 The specific desaturations in the pathway and evidence for substrate specificity and sequence of action

The desaturations mediated by PDS and ZDS are depicted in **Figure 5.2.**

The substrate for the four desaturations is phytoene, a colorless carotenoid. The order of activities in the pathway has been delineated by analysis of products that accumulate in mutants that are blocked in progression in the pathway, as for example in maize (Robertson, 1975), and by dissection of the pathway in bacteria (Armstrong et al., 1990) (Schnurr et al., 1991) (McDermott et al., 1973) (Misawa et al., 1990) and algae (Hirschberg et al., 1997) (Powls and Britton, 1977b).

Desaturations occur at the 11 and 11' positions first, then, at the 7 and 7' positions (Goodwin, 1980) (Porter, 1969). The double bond resides between carbons 7 and 8 or between carbons 11 and 12, or 7'and 8' or 11' and 12' (see **Figure 1.1** for naming conventions and **Figure 1.3** for position of desaturations indicated on structures). Phytoene desaturase (PDS) catalyzes the dehydrogenations at 11 and 11'. The intermediate produced after one desaturation at the 11 position is called phytofluene. PDS produces ξ-carotene, after two desaturations. ξ-carotene desaturase (ZDS) catalyzes the dehydrogenations at 7 and 7'. The intermediate produced after one desaturation at the 7 position is called neurosporene. ZDS produces lycopene after two desaturations. The order of paired catalyses (11,11' then 7,7') may be inferred from failure to detect any intermediates having 7-double-bonds that do not have both 11- and 11'-double-bonds. That is, PDS finishes two desaturations before ZDS acts.

Given that in some bacteria (Linden et al., 1991; Misawa et al., 1990) and some fungi (Bartley et al., 1990; Goldie and Subden, 1973; Hausmann and Sandmann, 2000) three to five desaturations of phytoene are carried-out by a single enzyme, the CRTI-type enzyme, it is possible that ZDS might conceivably use phytoene as a substrate. The lack of reported intermediates that contain 7,7'-double-bonds with no 11,11'-double-bonds, considered together with the finding that no desaturation products are detectable in a heterologous complementation system expressing phytoene and over-expressing just ZDS (Luo, 2000), indicates that phytoene does not serve as a substrate for ZDS. Similarly, the lack of detection of intermediates containing 7,7'-double-bonds and just one 11-double bond (Goodwin, 1980) suggests that phytofluene is not a substrate for ZDS. PDS and ZDS do not share substrates. This finding is true of ZDS from other plants, for example, *Capsicum annum* (Albrecht et al., 1995). Similarly, lycopene is not subject to further desaturations by PDS or ZDS. Consequently, other desaturation states, such as 3,3',4'4'-lycopene, are not observed in plants and have only been produced in *E. coli* as the result of *in vitro* gene shuffling of bacterial phytoene desaturases (Schmidt-Dannert et al., 2000). Hence, 7, 11-double-bond polyenes, 7, 11,11'-double-bond polyenes, and further desaturation products of lycopene do not occur naturally and need not be considered in the analysis of maize PDS and ZDS products.

5.3.4.1.2 The possible *E/Z* isomers of intermediates and products of the PDS and ZDS desaturation series

While there is a wealth of information on the isomer states of carotenoids (Goodwin, 1969; Goodwin, 1980; Neudert et al., 1998; Sandmann et al., 1990), few studies have addressed the stereochemical requirements of the desaturase enzymes themselves (Beyer et al., 1989; Breitenbach et al., 1999; Linden et al., 1991). Difficulty in preparing active enzymes, because of their hydrophobicity and low prevalence, have hindered *in vitro* studies of substrate usage and catalytic capabilities. Studies have been accomplished in cell-free, flower chromoplast systems from *Narcissus pseudonarcissus* (daffodil), where carotenogenesis is induced (Beyer et al., 1985a) (Beyer et al., 1989), but these results may not be extendable to other plants, tissues and plastid types. The availability of cloned genes, reviewed in (Bartley et al., 1994), has allowed the study of these enzymes by over-expression in heterologous systems. Recently, success in over-expression of PSY (Neudert et al., 1998), PDS (Li et al., 1996b; Sandmann and Kowalczyk, 1989) and ZDS (Beyer et al., 1989; Breitenbach et al., 1999; Luo, 2000) in *E. coli*, has led to *in vivo* complementation of exogenous carotenoid biosynthetic gene clusters as well as the ability to purify active enzyme for *in vitro* analyses (Breitenbach et al., 1999). These systems offer the advantage of being able to truncate the conversion series and examine the accumulating intermediates. Additionally, overproduction in *E. coli* allows that *in vitro* purified enzymes may be fed products that are extracted from a truncated

heterologous *E. coli* carotenogenic system. While each of these approaches offers specific advantages, each is also confounded by analysis of the enzyme *ex situ*.

Figure 5.3 shows some illustrative examples of E/Z isomers among the products of the carotenoid desaturases. These are isomers of (**A**) ζ-carotene, (**B**) neurosporene; and (**C**) lycopene. For example ζ-carotene accumulates where the last enzyme is PDS and may produce all-*E*- and 9,9'-Z-ζ-carotene. These isomers are thus shown in **Figure 5.3 A**. To acquaint the reader with the structure of geometric isomers along the pathway mediated by PDS and ZDS, in each figure an all-*E* isomer is compared to an isomer of the pathway leading to poly-*Z*-lycopene (7,7',9,9'-tetra-*Z*-lycopene). Experimental data in **Tables 5.1** and **5.2** and **Figures 5.6, 5.8, 5.9,** and **5.10** refer to comparisons among these structures as well as others not shown.

5.3.4.1.3 Cofactors and other requirements for desaturations

An understanding of the cofactor requirements for function of PDS and ZDS is essential to consideration of the behavior of PDS and ZDS in a heterologous complementation system and the discussion of metabolic engineering of the carotenoid biosynthetic pathway. Perturbations of required cofactors may perturb desaturase functions. For example, interruption of plastiquinone biosynthesis in the maize mutant *vp2* has pleiotropic effects on carotenoid biosynthesis and consequently ABA functions (Luo, 2000). Known factors that affect the desaturations are thus elaborated below.

Overall, the desaturation reactions require dinucleotide cofactors and an electron transport chain with a plastoquinone electron carrier and oxygen as a final electron acceptor (Beyer et al., 1989). Both PDS and ZDS are thought to be flavoenzymes, since the deduced amino acid sequences have putative dinucleotide-binding domains. For example, purified bell pepper PDS binds the cofactor FAD (Hugueney et al., 1992), while other studies in bacterial systems (Armstrong et al., 1990; Bartley et al., 1990; Fraser et al., 1992) have demonstrated that either NAD(P) or FAD is sufficient to catalyze the desaturations. On the other hand, the ZDS of *Capsicum annum* is insensitive to the presence or absence of oxygen and is not enhanced by dinucleotides FAD, NAD, or NADP (Breitenbach et al., 1999). Here, an alternative plastidal oxidase may be the terminal electron acceptor (Mayer et al., 1990; Mayer et al., 1992). In daffodil chromoplasts, a light-induced *Z* to *E* isomerization of 15-*E*-ζ-carotene is essential for activity of ZDS (Beyer et al., 1989). Thus, light is proposed as a requirement. Stereoselectivity is also a proposed influencing factor (Bartley et al., 1999) for arabidopsis ZDS. The biosynthesis of 15-*Z*-isomers of lycopene (**Figure 5.1**) and a scheme for the photoisomerization requirement is shown in **Figure 5.4.**

Other factors involved in the desaturation reactions include, for example, chaperonin for proper membrane localization (Al-Babili et al., 1996; Bonk et al., 1996) and galactolipids (Schledz et al., 1996). The membrane environment and carotenoid binding proteins (Cervantes-Cervantes et al., 1990) may also affect sequestration of carotenoid end-

products (Rabbani et al., 1998). End-product inhibition has been observed for PDS (Sandmann and Kowalczyk, 1989) and tentatively for ZDS (Breitenbach et al., 1999).

The PDS and ZDS desaturases are thought to be part of a membrane-bound complex. While a two-hybrid reporter system indicates a self-interaction for bell pepper ZDS purified from an over-expressing *E. coli* host, the *in vitro* activity was found to be independent of oligomerization (Breitenbach et al., 1999). Although suspected (Cunningham and Gantt, 1998b; Goodwin, 1983), the requirements for protein-protein interactions in the biosynthetic complex are unknown. Some plant enzymes function ineffectively (Luo, 2000), and, perhaps, aberrantly in a heterologous *E. coli* carotenogenic system, as some of the above mentioned cofactor requirements may not be met.

5.3.4.1.4 Interpretation of HPLC elution profiles and spectral profiles for isomer identification

The retention time of a cartotenoid within an HPLC system compared to an authentic standard can yield a tentative identification of the substance. Retention times may vary markedly between individual injections, so co-injection of the suspected standard with the sample is warranted. Alternatively, positive identification of carotenoids can be accomplished by analysis of spectral fine structure of high performance liquid chromatography (HPLC) purified forms. Further methods, such as nuclear magnetic resonance, mass spectroscopy and circular dichroic spectroscopy are confirmatory for

new or unusual carotenoids. Fortunately, HPLC separation and photodiode array detection (PDA) in conjunction with careful consultation of the literature is sufficient to identify *E/Z* isomers of commonly found plant carotenoids.

For carotenoids, spectral fine structure includes a number of "shoulders" of increased relative molar absorbency that result from discrete electron vibrational states. These are found within a few decades of the spectral value (nm) of the most absorbed color of light, called the "major peak" or $\lambda_{maximum}$ (λ_{max}). For carotenoids, there are usually three λ_{max}s, which are designated with Roman numerals I, II, and III from lowest to highest spectral values. For carotenoids, II is the major peak and I and III are minor peaks or "shoulders". The relative height of Peak II compared to Peak III, usually expressed as %III/II, but sometimes as II/III, is characteristic of a particular carotenoid. I will use II/III here, as it corresponds to values in recent pertinent literature (**Section 5.3.4.2**). The existence of *Z*-double-bonds within the system of conjugated double bonds can be inferred from changes in spectral fine structure in comparison to commercially available (Hoffmann LaRoche, Nutley, NJ or Sigma chemical, St. Lois, MO., see **5.6 Methods**), authentic, synthetic standards. These standards are composed predominantly (>90%) of the all-*E*-configured isomer. **Figure 5.5** shows the trends in spectral fine structure among selected isomers.

The introduction of a *Z* bond into the all *E* isomer leads to a decrease in relative molar absorbency of the carotenoid isomer. Also, each λ_{max} exhibits a bathochromic shift of a few nm. The placement of the *Z*-bond within the double conjugated bond system may also be inferred by the size and placement of an additional peak (λ_{max}) occurring

approximately 20 nm hypochromic to the major peak. This is called the "*cis*-peak" and occurs when the *Z*-double-bond is centrally located, as shown in **Figure 5.5.** Carotenoids need to be significantly pure in order to exhibit an interpretable fine structure, hence HPLC purification is necessary. Recent advances in separation technology now allow the clear separation of many carotenoids and their isomers. Separation in two solvent systems is often necessary.

5.3.4.2 Recent literature background

My results of HPLC analysis of the products of maize PDS and ZDS are perfectly congruent with results published recently for the ZDS of arabidopsis and of *Capsicum annuum*. First, the details and conclusions of these two reports will be discussed briefly, then my general and specific aims, followed by analysis of HPLC elution profiles and spectral fine structures of intermediates and products of maize PDS and ZDS produced in a heterologous *E. coli* carotenogenic system.

Prior to my study, poly-*Z* activity had only been demonstrated for enzymes from mutants of *Scenedesmus* (Sandmann, 1993), a tomato fruit mutant (Clough and Pattenden, 1983), isolated daffodil chromoplasts (Beyer et al., 1989), and *ex situ* arabidopsis (Bartley et al., 1999) and *Capsicum annuum* (Breitenbach et al., 1999). Other studies with tomato (Fraser et al., 1994) and *Capsicum annuum* (Hugueney et al., 1992) desaturases in the context of the heterologous complementation host *E. coli*, have shown an all-*E*-carotene desaturation pathway or did not discriminate between isomers (Albrecht et al., 1995).

5.3.4.2.1 Arabidopsis ZDS in a heterologous *E. coli* carotenogenic system

Bartley, *et al.* (Bartley et al., 1999) have examined the catalytic properties of PDS and ZDS from *Arabidopsis thaliana*. This dicot mustard is a close relative and good model for the agriculturally important species of *Brassica*, but, given the variation among the organisms noted above, it may be divergent with respect to carotenoid biosynthetic genes from the monot *Narcissus* or the cereals, such as maize and rice. These authors demonstrated a poly-*Z*-desaturation pathway. The activity was demonstrated by co-expression of PDS and ZDS in an *E. coli* carotenogenic system using genes from *Erwinia stewartii*. The system was completely analogous to the experimental system reported here, with the exception that the system I adopted (Luo, 2000) did not over-express isopentenyl pyrophosphate isomerase (IPPI), an enzyme upstream of the carotenoid biosynthetic pathway that increases carotenoid accumulation by only about two-fold (data not shown). *E. coli* has a native IPPI (*idi* gene), GPS and FPS and, with addition of a foreign GGPPS (*crtE*), will produce GGPP (Matthews and Wurtzel, 2000; Misawa et al., 1990) and **Chapters 2** and **3**. The substrate phytoene is then produced by the action of phytoene synthase CRTB. Arabidopsis PDS and ZDS then desaturate this phytoene.

Interestingly, the activity of arabidopsis ZDS was found to be regulated by light (Bartley et al., 1999). When bacterial cells were grown and extracted in the dark, the carotenoid ζ-carotene accumulated, but very little lycopene accumulated, as determined by HPLC and PDA. Treatment of cells with light reversed the profile of accumulation with little ζ-

carotene and poly-*Z*-lycopene as the major product via *Z*-neurosporenes, including 7,9,9'-tri-*Z*-neurosporene, the expected precursor to poly-*Z*-lycopene (7,9,7',9'-tetra-*Z*-lycopene; see **Figure 5.3 B** and **C** for structures). Extracted ζ-carotene that accumulated in dark grown cells was photo-isomerizable from the 15-*Z*- to 15-*E* configuration *in vitro* in methanol. The hypothesis that the arabidopsis ZDS is stereoselective for the 15-*E* substrate, and that blockage of the desaturation pathway is released by photoisomerization to the 15-*E* isomer, as proposed by Bartley, *et al.* (Bartley et al., 1999), is show schematically in **Figure 5.4.**

For arabidopsis PDS and ZDS in a heterologous *E. coli* carotenogenic system, the dependence on photo-isomerization of 15-*Z* ζ-carotene was not strictly dependent on light (Bartley et al., 1999). Some product did accumulate in the dark. Thus, the configuration change may yet occur in other ways. Minor products of the desaturation are many and include all-*E*- and intermediate di- and tri-*Z* neurosporenes (see **Figure 5.3** for examples of these structures). Since *E*-configuations are of lower energy due to less steric hindrance and since *Z*-configurations are photo-labile (Bartley et al., 1999), it is unclear whether the *E*-forms are true products of the enzyme action or decay products generated in a heterologous *E. coli* carotenogenic system or *in vitro*. Nevertheless, these authors conclude, that unlike bacterial desaturases, the plant desaturases, which are probably coded by single copy genes (Li, 1998; Li et al., 1996a; Luo, 2000; Yoganathan, 1998), carry out a light-dependent poly-*Z* desaturation pathway, and suggest generalizing this

result more widely (to all higher plants). These results are comparable to those obtained with *Narcissus* chromoplasts and certain algal mutants (Beyer et al., 1989).

These authors also suggest that the presence of *Z*-bonds at the 9 position seems to determine the *Z*-elimination by ZDS at the 7,7' positions. The double bonds at 9,9' positions may come from *Z*-GGPP (**see Figure 5.1**) or potentially from an isomerization associated with PDS, which desaturates the 11 and 11' positions (Bartley et al., 1999).

Since the poly-*Z*-pathway also operates in the dark *in planta,* the authors argue that there must be some other isomerase factor, which is lost in *in vitro* chromoplast experiments and in *E. coli* heterologous expression systems that may be artificially replaced by the action of light. If there is another obligate secondary factor for ZDS action in plants, that has eluded genetic dissection of the pathway, it is of immediate concern for those who wish to metabolically engineer pro-vitamin A content in various agronomic plants. Recent success (Ye et al., 2000) has, for example, only been possible with the use of the CRTI-type bacterial enzyme, which carries-out all four desaturations in a poly-*E* desaturation pathway (Giuliano et al., 1986).

5.3.4.2.2 Capsicum ZDS in a heterologous *E. coli* carotenogenic system and *in vitro*

In vitro feeding experiments with purified *Capsicum annuum* ZDS have shown that the enzyme is sensitive to the isomeric form of the substrate (Breitenbach et al., 1999). If the enzyme is fed a purified all-*E*-substrate, it produces an all-*E*-product. On the other hand,

if it is fed 7,7',9-*Z*-neurosporene, it produces 7,7',9,9'-poly-*Z*-lycopene. The enzyme is not stereoselective. The *Capsicum* ZDS produces predominately all-*E* lycopene *in situ*. No dependence on light or the presence of molecular oxygen or the presence of a dinucleotide was noted for *Capsicum* ZDS (Breitenbach et al., 1999). Breitenbach *et al.* also showed that plant PDS in *E. coli* produces mainly *Z* isomers. Again, although not stereoselective, *Capsicum* ZDS is stereosensitive to its substrate. If the substrate already contains a 9,9'-*Z* double-bond, it tends to introduce additional *Z*-double-bonds at the 7 and 7' positions. The authors extend Goodwin's hypothesis (**Figure 5** and **Section 5.3.1**) of a stereochemical mechanism that holds and strains the substrate in a channeled complex. The enzyme complex is adapted to *E*-configurations of substrates and holds and maintains the substrate and products in the *E*-configuration. If the substrate is in the *Z*-configuration then the bending of the substrate favors dual *Z*-di-dehydrogenation. If the metabolon is disrupted, as potentially occurs in *E. coli*, where a mixture of heterologous enzymes produce lycopene, information is not transmitted, and the substrates may accumulate and be released from their all-*E* fate.

5.4 General aim

Together, the availability of facile heterologous functional complementation systems and isolation of active desaturase enzymes for *in vitro* substrate feeding experiments, have led to analysis of the isomers among the products and intermediates of a few carotenoid desaturases. Interestingly, these studies have suggested that the dogma of a preponderance of all-*E* carotenoids in higher plants (see also **5.1 Prologue**) may not hold.

The occurrence of poly-Z-carotenenoids, was once thought to be a rarity, existing only perhaps in chromoplasts of some flowers, a Mendelian mutant variety of tomato (Qureshi et al., 1974), and a mutant of *Scenedesmus* C-6D (Sandmann, 1993). It was cast in doubt by the finding that the combined activities of PDS and ZDS from arabidopsis (Bartley et al., 1999) and *Capsicum annum* (Breitenbach et al., 1999) yield poly-*Z*-lycopene *in vitro* and in *E. coli* heterologous complementation systems, respectively.

These results have been variously interpreted as artifacts of an *ex situ* biochemical pathway (Breitenbach et al., 1999) or as a new twist in the tale of carotenoid substrate stereoselectivity belying the prospect of an obligate isomerase needed for progression in the pathway through a stereoselective ZDS (Bartley et al., 1999) (**Figure 5.3**). In one case, characterization of the desaturase activity of newly isolated functional cDNAs from various plants is one approach to accessing the distribution of the poly-*Z* desaturation pathway among higher plants. In particular, analysis of a maize PDS and ZDS system contributes to the clarification of the presence of the poly-*Z*-pathway among this model cereal crop. The potential existence of essential secondary factors, such as an isomerase, has important implications for bioengineering of maize and related crops, such as rice. The carotenoid desaturation reactions have been shown to require other essential factors, as outlined in **Section 5.3.4.1.3** Thus, an understanding of the enzyme requirements may have importance to the metabolic engineering of crops. For example, metabolic engineering of carotenoid containing rice endosperm failed when a daffodil PDS was used (Burkhardt et al., 1997). In the other case, the behavior of the maize coupled PDS

and ZDS desaturation system in *E. coli*, especially with regard to its behavior in response to isomeric states of intermediate substrates, formally extends Goodwin's hypothesis (**Section 5.3.1**) of a tightly orchestrated carotenoid metabolon to a representative of the cereal crops. Because of the importance of either interpretation of a poly-*Z* desaturation pathway to understanding and bioengineering of cereal carotenogenesis, an investigation of the potential for poly-*Z* activity of maize PDS and ZDS was launched.

The isolation and expression-cloning of a full-length cDNA from maize (Luo, 2000), and a maize PDS by Li *et al.* (Li, 1998; Li et al., 1996b) and subsequent incomplete characterization of the enzyme products as neurosporene or poly-*Z*-lycopene, necessitate further characterization of the isomeric state of these products. While Li *et al.* found the activity of PDS in a heterologous *E coli* carotenogenic system to be robust, as evidenced by conversion of most of the substrate to product, Luo found maize ZDS to be inefficient in a heterologous *E. coli* carotenogenic system, even after grooming for maximal enzyme activity by removal of the putative chloroplast transit sequence (Luo, 2000).

5.5 Specific aims

1. To develop culture growth and gene induction conditions necessary to increase low-level enzyme activity to allow product detection
2. To develop a carotenoid extraction method with the efficiency necessary to detect low-level product accumulation

3. To choose an HPLC method for the separation and determination of poly-*Z*-lycopene and neurosporene isomers
4. To determine the *E/Z* conformation of the products of maize PDS and ZDS in a herterologous *E. coli* carotenogenic system

5.6 Methods

5.6.1 Plasmids and host bacterial strains

To test for functional complementation of the maize *Zds* cDNA, the plasmid pACCRT-EBP, conferring ζ-carotene accumulation, was first constructed as described (Luo, 2000). The resulting plasmid pACCRT-EBP carries *Erwinia uredovora crtE*, encoding GGPP synthase, *crtB*, encoding phytoene synthase, and maize PDS. pMzds-107 (constructed by Luo, (Luo, 2000)) coding maize ZDS for which 106 bp (chloroplast localization signal) had been deleted and confirmed to be in frame with *lac*Z was also used. *E. coli* XL-1 Blue (Stratagene, La Jolla, CA) cells carrying pACCRT-EBP were transformed with pMzds-107. Ampicillin (50 μg/ml) and chloramphenicol (70 μg/ml) were used to select transformants containing both pMZDS-107 and pACCRT-EBP, since pMzds-107 carries only an Amp^R marker, and pACCRT-EBP possesses only a Chl^R marker. For production of carotenoid standards various, previously-characterized carotenogenic strains were used, namely: pAC-Neurosporene (Cunningham Jr. et al., 1996) for neurosporene production, and pACCRT-EIB/ pTAC-ORF2 for lycopene production (Matthews and Wurtzel, 2000).

5.6.2 Development of culture and gene induction conditions necessary to increase low-level enzyme activity and allow product detection

Since Luo had shown (Luo, 2000) that the above strain produced very little neurosporene or lycopene, experiments were carried-out to increase accumulation of products of maize ZDS. Inclusion of DXS, as was reported in **Chapter 3,** was not a convenient option, as the plasmid carrying *dxs* is not compatible with plasmids already present in the heterologous system. Carefully-timed promoter induction with isopropylthiogalactoside (IPTG) has been shown to be critical to carotenoid biosynthetic gene expression in *E. coli* (Kajiwara et al., 1997). Therefore, co-transformed cells were grown in 1000 ml rotary (260 rpm) shake-cultures in Luri-Betani broth pH 7.2 for 8 hours at 37°C, and induced with 10 mM IPTG at various growth phases, namely: inoculation, midlog, early stationary and late stationary. Cultures were then further incubated for 46 hours at 26°C with mild agitation (100 rpm), based on previous experience with incubation temperature and time (**Chapter 2**), and assessed visually for pigmentation. Addition of IPTG at early stationary phase (8 hours after inoculation) led to the best pigment accumulation (data not shown). Control cells with either pACCRT-EBP, producing ξ-carotene (Li, 1998); or pACCRT-EIB/ pTAC-ORF2 producing lycopene (Matthews and Wurtzel, 2000); or pAC-Neurosporene] producing neurosporene [Cunningham Jr., 1996 #47, were grown similarly with appropriate antibiotics. Pigmented cells were harvested by centrifugation.

5.6.3 Development of a carotenoid extraction method with the efficiency necessary to detect low-level product accumulation

Various extraction protocols were also tried in order to increase extracted pigment yields to a level sufficient to separate and identify products and intermediates of the carotenoid biosynthetic pathway. Although phenolization, as described in **Chapter 3,** yielded higher amounts of lycopene (data not shown), it was a long and arduous procedure. In an effort to minimize extraction time and potential isomerization and oxidation artifacts, I eventually deferred to the protocol outlined below. Five grams (fresh weight) of cell pellets were exhaustively extracted with three changes of 50 ml acetone with homogenization in a Brinkman Polytron (Kinematica, Switzerland) at 30,000 rpm. NaCl was added to 2.5 % w/v of acetone extracts, and carotenoids were partitioned into an equal volume of diethyl ether. The diethyl ether phase was dried over anhydrous $NaSO_4$, and evaporated to dryness in a SpeedVac rotary evaporator (Savant, Farmingdale, NY) under vacuum. Samples were subjected to HPLC immediately, or when necessary, stored under nitrogen atmosphere at –80 °C in amber vials. All manipulations of cells and pigments were carried out in the dark or very dim light. Great care was taken to prevent *in vitro* geometric isomerization.

5.6.4 Choice of an HPLC method for the separation and identification of poly-Z-lycopene and neurosporene isomers

A Waters HPLC system (Millipore, Franklin, MA) with a 2690 separation module, a 996 photodiode array detector, and a 717 autosampler was used to identify carotenoids by reverse phase chromatography on a Nucleosil 5 C18 (5μ, 250 x 4.6mm) column (Phenomenex, Torrance, CA). Having initially found that a mobile phase (1 ml/min) of

90:15:5 acetonitrile/methanol/isopropanol used by Luo (Luo, 2000) and Li *et al.* (Li et al., 1996b) was unsatisfactory, since it did not allow separation of isomers, I adopted the solvent systems used by Bartley *et al.* (Bartley et al., 1999) with minor modifications (as described below) to increase isomer resolution. Using this HPLC system also facilitated comparison to the data obtained by Bartley et al. and Breitenbach et al. (Breitenbach et al., 1999). Extracts were resuspended in methanol or hexane, depending on their solubility, and injected in a mobile (0.8 ml/min) phase of 100% acetonitrile (henceforth designated as HPLC **System I**). Elution profiles were monitored at 400 and 450 nm and selected peaks collected, evaporated and resuspended in methanol for reinjection into a mobile (1ml/min) phase of 100% methanol (henceforth designated as HPLC **System II**).

5.6.5 HPLC and PDA standards

Some carotenoid standards were produced and extracted from bacterial strains that had been previously characterized for product accumulation. These strains are described above in **5.6.1 Plasmids and host bacterial strains.** Carotenoid isomers in these strains were further identified by comparison of spectral profiles to published values. Other standards were purchased from Sigma Chemical Co. (St. Louis, MO) or supplied by Hoffmann LaRoche (Nutley, NJ).

5.7 Results

5.7.1 Determination of the E/Z conformation of the products of maize PDS and ZDS

5.7.1.1 Elution Profiles in System I

The identification of carotenoids was based on elution time and spectral fine structure. For some carotenoids, separation and PDA analysis in **System I** was sufficient for identification. Mixtures of major products of PDS and ZDS required further purification after isolation from **System I** by chromatography in **System II**. **Figure 5.6** shows the elution profile in **System I** of reaction products extracted from cells containing PDS and ZDS, by virtue of containing plasmids pMzds-107, coding maize ZDS and pACCRT-EBP, coding *Erwinia* GGPPS, *Erwinia* PSY and maize PDS.

The major accumulated products, seen in **Figure 5.6**, were isomers of ζ-carotene, the product of PDS. These are elution **Peaks 1A** and **1B**, **Maxplot**, **Figure 5.6**. Using ζ-carotene, ZDS produced isomers of the intermediate, neurosporene, **Peaks 2A,B** and **C**, **Maxplot**, **Figure 5.6**, and the final product, lycopene **Peak 3**, **Maxplot**, **Figure 5.6**. These tentative identifications are based on relative retention times (RT) of positively identified products from Bartley *et al.* (Bartley et al., 1999), who present an almost identical chromatogram for products of arabidopsis PDS and ZDS, but who do not report exact RTs. While a Maxplot {Millenium32 software,Waters HPLC system (Millipore, Franklin, MA} depicts each peak at its maximum absorption, thus giving a visual approximation of the relative quantities of the products, individual products may be emphasized by viewing the chromatogram at various single wavelengths near the λ_{max} of

the individual product. **Figure 5.6** also shows the products of PDS emphasized at **400nm** and the products of ZDS emphasized at **450nm. Peak 3** is prominent at **450nm.** At this wavelength, further features of the products of ZDS may be observed: (1) **Peak 3** is not well separated from neurosporene isomers (**Peaks 2A, B** and **C**) and (2) additional very small peaks are seen (labeled **Peak L**). The small peaks are potentially lycopene isomers. No meaningful relationships among the estimated quantities (raw peak heights or peak integrals or mol%) of various products were found (data not shown).

Tentative identifications based on RT above were confirmed by examination of spectral fine structure of each product, as shown in **Figures 5.7, 5.8.** and **5.9** in comparison to published values of λ_{max} s listed in **Table 5.1** (Breitenbach et al., 1999). For each product the values of RT and λ_{max}s, I, II, III, and II/III were determined and tabulated (**Table 5.1**) for comparison to values from the literature (Bartley et al., 1999; Breitenbach et al., 1999). Additionally, each comparison is made explicit below.

The accumulating products of PDS were *Z*-ζ-carotene and all-*E*-ζ-carotene. *Z*-ζ-carotene predominates over all-*E*-ζ-carotene by a ratio of about 4:1, as can be seen from inspection of relative peak height in **Figure 5.6 (400nm).** In order to detect and isolate the products of ZDS, the amount of injected total carotenoid had combined molar absorbency at 450 nm of about 4, most of which is ζ-carotene (**Figure 5.6**). The mono-di-dehydrogenation intermediate, phytofluene is not detected. Phytoene, which also accumulates in this system, does not absorb much at 450 nm.

Carotenoid		I	II	III	II/III	RT
My identified unknowns						
Peak 3	*Z*-neurosporene	416	440	469	1.28	57
or	poly-*Z*-lycopene					
Peak 2A	*Z*-neurosporene	nd	nd	nd	nd	71
Peak 2B	*Z*-neurosporene	410	434	462	1.84	63
Peak 2C	*Z*-neurosporene	411	434	462	1.84	66
Peak 1B	*Z*-ζ-carotene	379	400	424	1.08	72
Peak 1A	all-*E*-ζ-carotene	381	401	426	0.99	77
Peak L	lycopene	nd	nd	nd	nd	50
Peak L	lycopene	nd	nd	nd	nd	52
Standards (Breitenbach et al., 1999)						
Peak 2-4	*Z*-neurosporene	412	438	465	1.33	nd
Peak 2-1	*Z*-neurosporene	410	434	462	1.09	nd
Peak 2-2	*Z*-neurosporene	414	440	468	1.11	nd
Peak 2-3	all-*E* neurosporene	414	440	469	1.02	nd
Stand III	all-*E* neurosporene	412	440	468	1.08	nd
Peak 1-3	poly-*Z*-lycopene	416	440	469	1.27	nd
Stand II	poly-*Z*-lycopene	416	440	468	1.25	nd
Stand I	all-*E* lycopene	444	472	504	1.12	nd
Peak 1-1	all-*E* lycopene	446	471	503	1.13	nd
Peak 3-2	*Z*-ζ-carotene	378	400	428	1.07	nd
Peak 3- 3	*Z*-ζ-carotene	380	402	429	1.06	nd
Peak 3-1	all *E*-ζ-carotene	384	402	425	1.02	nd
Stand IV	all-*E*-ζ-carotene	378	402	425	0.99	nd
My standards						
Z-ζ-carotene (pACCRT-EPB)		379	401	425	1.10	73
Z-ζ-carotene (pACCRT-EPB)		379	400	424	1.07	18
all-*E* ζ-carotene (pACCRT-EPB)		379	401	426	0.99	20
lycopene (p ACCRT-EIB/pTAC-ORF2)		442	471	499	1.12	46
all-*E* lycopene (p ACCRT-EIB/pTAC-ORF2)		444	473	500	1.19	48

Table 5.1 Identifications of accumulated carotenoids extracted from a heterologous *E. coli* carotenogenic system containing plasmids pMzds-107 coding for maize ZDS and pACCRT-EBP coding for *Erwinia* GGPPS and *Erwinia* PSY and maize PDS and subjected to separation in HPLC System I. Peak numbering and RT corresponds to **Figure 5.6** and spectral fine structure λ_{max}s correspond to **Figures 5.7, 5.8** and **5.9**. "nd" denotes not determined due to small peak size. Standards with peak designations are from the literature (Breitenbach et al., 1999) and from control strains indicated parenthetically and described in **4.6.1 Plasmids and host bacterial strains.**

The RT of all-*E*-ζ-carotene is 77 min (**Figure 5.6, Peak 1A**). The values of I, II, III and II/III are 381, 401, 426 and 0.99. These values are similar to the *E*-ζ-carotene standard and indicate a ζ-carotene. The control *E. coli* strain pACCRT-EPB, yields values of I, II, III, and II/III of 379, 401, 426, and 0.99 for all-*E*-ζ-carotene. The spectral fine structure of the proposed all-*E*-ζ-carotene does not show a *cis*-peak (see **Figure 5.7**). Values from the literature for all-*E*-ζ-carotene for I, II, III, and II/III are 378,402,425, and 0.99 (Breitenbach et al., 1999). **Peak 1A** is all-E-ζ-carotene.

The RT of *Z*-ζ-carotene is 72 min (**Figure 5.6, Peak 1B**) and is similar to the RT of 73 min for *Z*-ζ-carotene produced in *E coli* containing pACCRT-EPB alone (chromatogram not shown), and the values of I, II, and III, and II/III are 379, 400, 424, and 1.08 for **Peak 1B** and 379, 400, 425, and 1.10 for my standard *Z*-ζ-carotene from pACCRT-EPB (see **Table 5.1**). This isomer additionally shows a *cis*-peak at about 300nm (see **Figure 5.7**). Values from the literature for *Z*-ζ-carotene for I, II, III and II/III are 378, 400, 428, and 1.07 (Breitenbach et al., 1999). **Peak 1B** is *Z*-ζ-carotene.

The mono-di-dehydrogenation intermediate product of ZDS, neurosporene, accumulates in three partially separable isomeric states (**Figure 5.6**). Only two of these, **Peak 2B,** at a RT of 66 min, and **Peak 2C,** at a RT of 63 min, accumulate to a level to allow interpretation of spectral fine structure. **Peak 2A,** at RT of 70 min, has the same relative height and relative RT (data not shown) as a product confirmed as *Z*-neurosporene (Bartley et al., 1999) (**Figure 5.8**). Both **Peaks 2B** and **C** are *Z*-neurosporene geometric

isomers: **Peak 2B** has values of I, II, III, and II/III of 411, 434, 462 and 1.84, while **Peak 2C** has values of 410, 434, 462, and 1.84. The values for neurosporene extracted from a control strain of carotenogenic *E. coli* pAC-Neurosporene (see **5.6.1 Plasmids and host bacterial strains**) are identical (data not shown). Literature values of I, II, III, and II/III for three *Z*-neurosporenes are 410, 434, 462, and 1.09; 414, 440, 468, and 1.11; and 412, 438, 465 and 1.33 (Breitenbach et al., 1999). Each isomer shows a *cis*-peak. The discrepancy in II/III values for **Peaks 2B** and **C** *vs.* literature values are due to impurity of **Peaks 2 b** and **C**. The individual positions of the *Z*-double-bond cannot be determined from the fine spectra, nor were they determined in the compared studies (Bartley et al., 1999; Breitenbach et al., 1999) An all-*E*-neurosporene was not isolated in **System I.**

5.7.1.2 Elution profiles in System II

The final accumulating products of the carotenogenic system, pMzds-107 coding for maize ZDS and pACCRT-EBP coding for *Erwinia* GGPPS and *Erwinia* PSY and maize PDS in *E. coli*, are geometric isomers of lycopene (see **Figure 5.6, Peaks 3** and **L**). The fine spectral structure of **Peaks 3** and **L** of **Figure 5.6** are shown in **Figure 5.9**. Whether **Peak 3** was poly-*Z*-lycopene or neurosporene isomers could not be determined. This major final product was not sufficiently pure for assessment of PDA fine structure. Additionally, minor **Peak L** was possibly lycopene isomers. While the quantity of **Peak L** (Figure 3.7) was too small (absorbency at 466 nm = 0.0045) to allow further study or definitive fine spectral structure (see **Figure 5.9**), the major product, **Peak 3,** was collected and subjected to **System II.**

Figure 5.10 shows the elution profile at 400 nm of **System I, Peak 3** separated in **System II** and the spectral fine structure of the ultimate products of maize PDS and ZDS. **Peak 1** is all-*E*-lycopene, **Peak 2A**, and **2B** are poly-*Z*-lycopene, and **Peaks 3** are neurosporene isomers. The major product is poly-*Z*-lycopene. **Table 5.2** presents the values of I, II, III, and II/III for the spectral fine structures of all-*E*-lycopene and poly-*Z*-lycopene and all available, pertinent standards in **System II**. The values of I, II, III, and II/III for all-*E*-lycopene are 452, 469, 500, and 1.18. The values of I, II, III, and II/III for all-*E*-lycopene authentic standard from Hoffmann-LaRoche (Nutley, NJ, USA) are 447, 469, 500, and 1.23 (see **Table 5.2**).

Carotenoid	I	II	III	II/III	*Cis* peak	RT
Identified unknowns						
Peak 2A.....poly-*Z*-lycopene	420	437	nd	nd	+	35
Peak2B.....poly-*Z*-lycopene	420	437	nd	nd	+	34
Peaks 3.....neurosporenes	nd	nd	nd	nd	nd	43-39
Peak 1.....all-*E*-lycopene	452	469	500	1.18	-	22
Literature standards (Bartley et al., 1999)						
poly-*Z*-lycopene	419	437	nd	nd	+	22
My standards						
all-*E*-lycopene (Hoffmann-La Roche)	447	469	500	1.23	-	22
15-*Z*-lycopene (Hoffmann-La Roche)	438	463	493	1.18	+	24

Table 5.2 Identifications of accumulated carotenoids extracted from a heterologous *E coli* carotenogenic system contain plasmids pMzds-107 coding maize ZDS and pACCRT-EBP coding *Erwinia* GGPPS and *Erwinia* PSY and maize PDS and subjected to separation in HPLC System II. Peak numbering, RT, and spectral fine structure λ_{max}s correspond to **Figure 5.10**; "nd" denotes not determined due to small peak size. "+" indicates the presence of a *cis*-peak, "-" indicates an absence of a *cis*-peak. Standards with peak designations are from the literature (Bartley, et al, 1999; Breitenbach et al., 1999). **Peak 3** from **System I** was injected into **System II** to produce the above peaks.

The spectral fine structure of the major product, **Peak2A,** is unmistakably that of poly-Z-lycopene. The values of I and II, are 420 and 437 (compare to literature values in **Table 5.2** and spectral fine structures in (Bartley et al., 1999; Beyer et al., 1989; Breitenbach et al., 1999; Clough and Pattenden, 1983; Sandmann, 1993).) These values perfectly match published standard values (Bartley et al., 1999). The λ_{max} III is not exhibited in **System II**.

5.8 Discussion

Since a poly-*Z*-pathway for the desaturation of phytoene to lycopene was recently reported for arabidopsis PDS and ZDS and bell pepper ZDS, I was compelled to determine the stereochemical specificity of the maize PDS and ZDS. Like the previously studied plant desaturases, the maize enzymes also catalyze a poly-*Z*-pathway in the context of an *E. coli* heterologous complementation system, which uses bacterial enzymes to supply phytoene. The major accumulation product of the four desaturations carried out by PDS and ZDS is poly-*Z*-lycopene (7,7',9,9'-tetra-Z-lycopene).

My results with maize PDS and ZDS match the results obtained with the Arabidopsis desaturases very well. The spectral fine structural properties and elution times for these Arabidopsis enzyme products and the results of my study are show in **Table 5.1** and **Table 5.2** for comparison with those of available, authentic standards.

PDS produces Z-ζ-carotenes. Since the instrumental analyses employed here and elsewhere (Bartley et al., 1999; Breitenbach et al., 1999; Linden et al., 1991), are only able to differentiate all-*E* from various *Z*- isomers, the *Z*-ζ-carotenes identified here could be either 15-*Z*-ζ-carotene; 9,9'-di-*Z*-ζ-carotene; 9,9'15-tri-*Z*-ζ-carotene; or 11,11'-di-*Z*-ζ-carotene.

The origin of 9 and 9'-*Z*-double bonds may be from *Z*-GGPP or could be introduced by an accessory isomerase activity associated with PDS itself. It is unlikely that a separate, intrinsic isomerase activity is active *in planta* at the desaturation step, since the 9,9'-*Z*-configuaration occurs in *E. coli* where an additional carotenoid isomerase is extremely unlikely. On the other hand, the 11 and 11'-*E*-activity must definitely come from PDS.

That ZDS introduces di-*Z*-didehydrogenations is evident by the unmistakable identification of 7,7'9,9'-tetra-*Z*-lycopene as the major product. That is, for ZDS, the paired didehydrogenations at the 7 and 7' positions have the same stereo-elimination route. The small number of chromatographically resolved geometric isomers of the intermediate, neurosporene, also is circumstantial evidence for paired elimination having the same stereo-elimination route. The three accumulating isomers of neurosporene, are likely to be 7,9,9'-tri-*Z*-neurosporene; 9, 9'-*Z*-neurosporene; and 15-*Z*-neurosporene.

Interpretation of the accumulation of the small amounts of all-*E*-isomers is confounded by the thermodynamically favored conversion of *Z*-isomers to the less sterically-hindered

E-isomers by heat and light. Their accumulation may not be indicative of enzyme action. For example the large accumulation of all-*E*-ζ-carotene (approximately one fourth of the product of PDS) could be variously interpreted as a significant *E*-elimination activity of PDS or the accumulation of lower-energy-state *E*-isomers. *E*-isomers have the opportunity to accumulate in *E. coli* as the products of PDS accumulate to high levels due to the relatively low activity of ZDS. Therefore, it is unclear whether these enzymes are catalyzing *E*-elimination. The general preponderance of all-*E*-carotenoids in Nature, suggests that these enzymes do show *E*-elimination activity *in planta*, or alternatively could result from the activity of isomerase(s).

On the other hand, the accumulation of poly-*Z* isomers is certainly not an artifact of experimental work-up, although *E*- to *Z*- isomerization can be induced by heat and light (see **Prologue**). *Z*-isomers are the major product, not minor, induced artifacts. The isolation and analyses of products were designed to avoid the production of such artifacts.

Since the *Z*-isomer products of PDS could possibly be 15-*Z* or 9,9'-Z forms, and thus not the product of PDS *Z*-elimination activity, I cannot conclude the PDS exhibits Z-elimination activity. Nor can I conclude that it does not, since *E*-isomers may be artifacts. Since 7,7',9'9-tetra-*Z*-lycopene is eventually produced, an isomerase activity for conversion of any 11,11'-Z-bonds would be necessary. This activity could be associated with ZDS. Such an isomerase activity seems unlikely. I can conclude that maize PDS

must be able to produce or process at least the 9,9'-*Z*-ζ-carotene, since 7,7',9'9-tetra-*Z*-lycopene is produced.

Since a large amount of both the *E*- and *Z*- isomers of ζ-carotene are available to ZDS, yet it produces mostly or all *Z*-elimination products (7,7',9'9-tetra-Z-lycopene), this enzyme appears to have some degree of stereoselectivity for the *Z*-isomers of ζ-carotene as well as the definitively demonstrated *Z*-elimination activity. For purified bell pepper ZDS *in vitro*, the distribution of geometric isomers in the products reflects the distribution of the substrates (Breitenbach et al., 1999). Examination of the ratios of products to substrate in my system showed no such relationship. Given that bell pepper ZDS is stereosensitive, but not stereoselective to substrates fed to it *in vitro*, and that the origin of *E*-isomers in my system is unclear, it is not possible to conclude whether maize ZDS has constitutive *Z*-elimination activity or is in fact stereosensitive, yet also stereoselective. Resolution of this issue requires substrate-feeding experiments or *in situ* analysis of the carotenogenic system with substrates in place.

The major product of PDS is 15-*Z*-ζ-carotene. One explanation for the low activity of ZDS is that it does not process 15-*Z*-ζ-carotene efficiently, because it is stereoselective for the 15-*E* isomer (Bartley et al., 1999; Beyer et al., 1989). Bartely et al. showed that light induces arabidopsis ZDS activity and that the 15-*Z* isomer is photoisomerizable to the 15-*E* form *in vitro* in methanol. The requirement for isomerization has also been noted for daffodil chromoplast carotenoid desaturations and cyclyzations (Beyer et al.,

1989). Neither I (data not shown) nor Breitenbach et al. (Breitenbach et al., 1999) could demonstrate a photo-induction of ZDS in an *E. coli* heterologous complementation system treated with light, for maize or bell pepper ZDS, respectively. The lack of an obligate isomerase in rice endosperm, is one (of many) potential explanations for the failure of daffodil PDS and PSY transgenes to show enzyme activity (Burkhardt et al., 1997), while a gene coding the four-step bacterial desaturase was functional (Ye et al., 2000). Since the observations of sereselectivity are disparate among experimental systems and species, the possibility of species- or plastid-type specificity exists. The potential existence of a poly-*Z*-pathway in plants has important ramifications for the need for isomerase activity within the desaturase series, but also for an isomerase proposed to be associated with epoxy-xanthophyll dioxygenases oxidative cleavage specificity for 9-*Z*-epoxy-xanthophylls in maize (Schwartz et al., 1997).

An alternative explanation for the stereochemical behavior of PDS and ZDS *ex situ*, is that the behaviors are an artifact of the disruption of a carotenoid assembly complex. Such an explanation is favored by Occam's Razor, since rather than proposal of further, yet unobserved, system components (like Ptolemy's need for epicycles to explain retrograde planetary motion), the explanation indicts communicative interactions among existing components.

Although it may be unappealing to interpret the results of this study as a likely artifact of an *ex situ* biosynthetic pathway, the existence of an altered, aberrant activity, namely the

poly-*Z*-elimination route, may lend further credence to the existence and functioning of a carotenogenic metabolon. Further studies of the accumulation products of carotenoid intermediates in, for example, bleaching-herbicide treated plants (Sandmann and Böger, 1989) or mutants blocking the pathway in maize, may help define the geometric isomeric states of accumulation products *in planta*. While studies based on dissection and aberration of the pathway *in planta* may be equally confounded, currently reconstitution or analysis *in situ* is intractable. In addition to extending the suggestion (Breitenbach et al., 1999) of what to seek in the model molecular genetic organism, maize (this chapter), my studies (other chapters herein) contribute to production of molecular tools for such analysis. The constitution and behavior of such a complex system allows subtle variation among organisms, tissues, and plastid types. Understanding of such interactions may be crucial to an understanding of the distribution of carotenoid pigments, their multiplicity of functions, and the prospect of metabolic engineering for accumulation and function.

5.9 Acknowledgement

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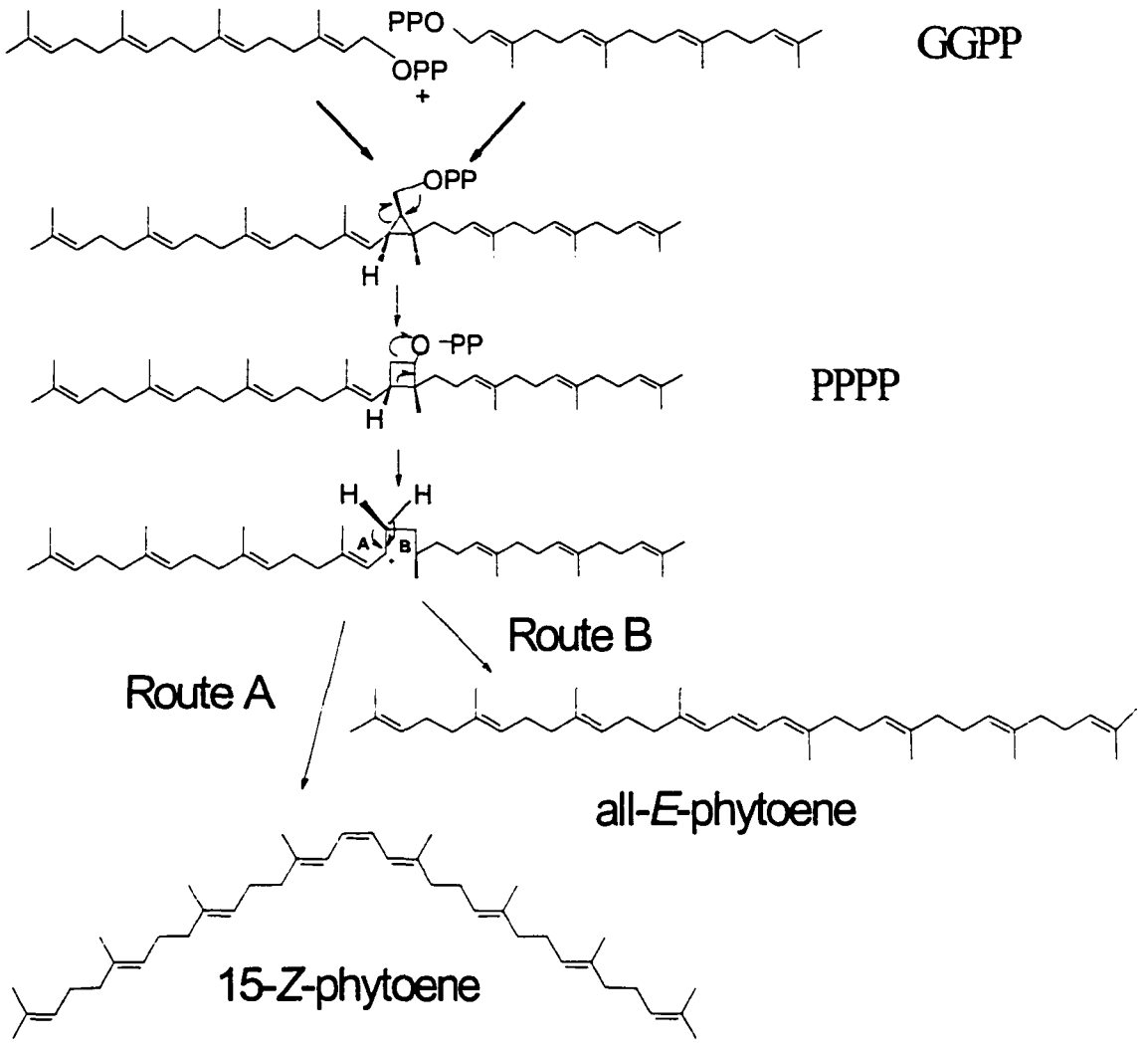


Figure 5.1 Stereochemistry of phytoene synthesis. Two molecules of GGPP are condensed to form PPPP. This unstable intermediate undergoes dehydrogenation by **Route A** to produce 15-Z-phytoene or by **Route B** to produce all-trans-phytoene. Redrawn by Cynthia Gallagher from (Pfander, 1992).

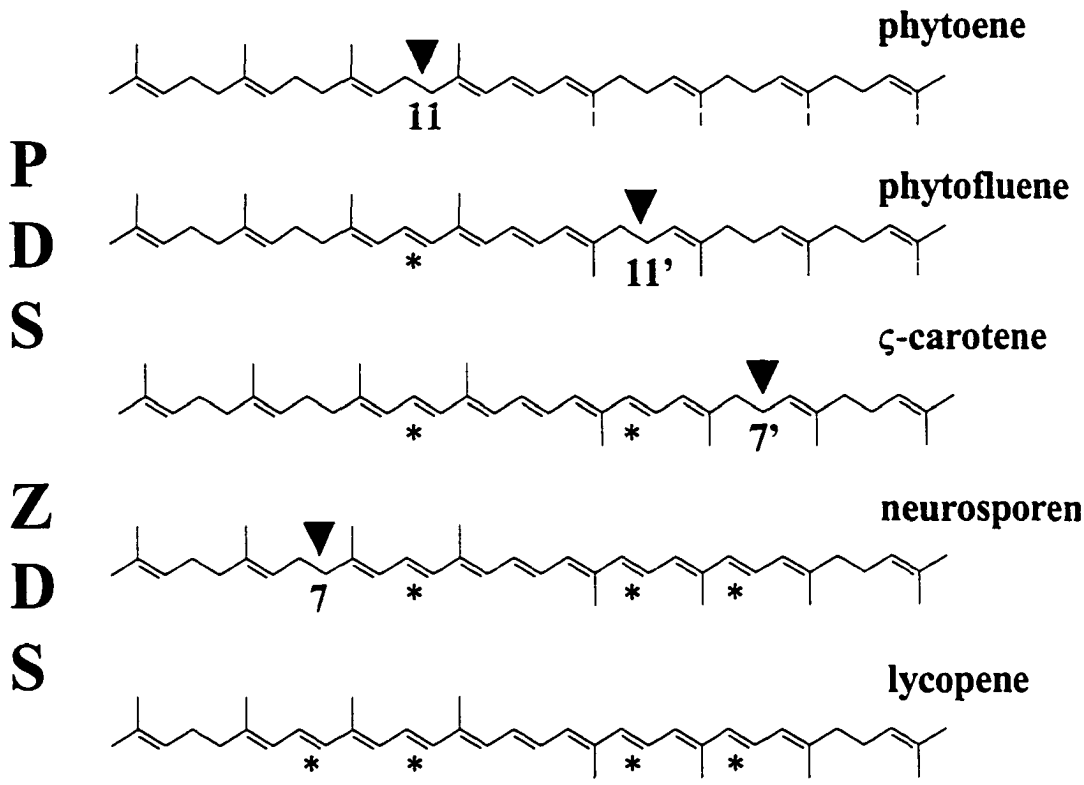


Figure 5.2 Desaturations catalyzed by PDS and ZDS. Desaturations of phytoene occur at the sites indicated by triangles to produce the double bonds shown by asterisks. The order of desaturations occurs as shown from top to bottom; all possible intermediates are shown.

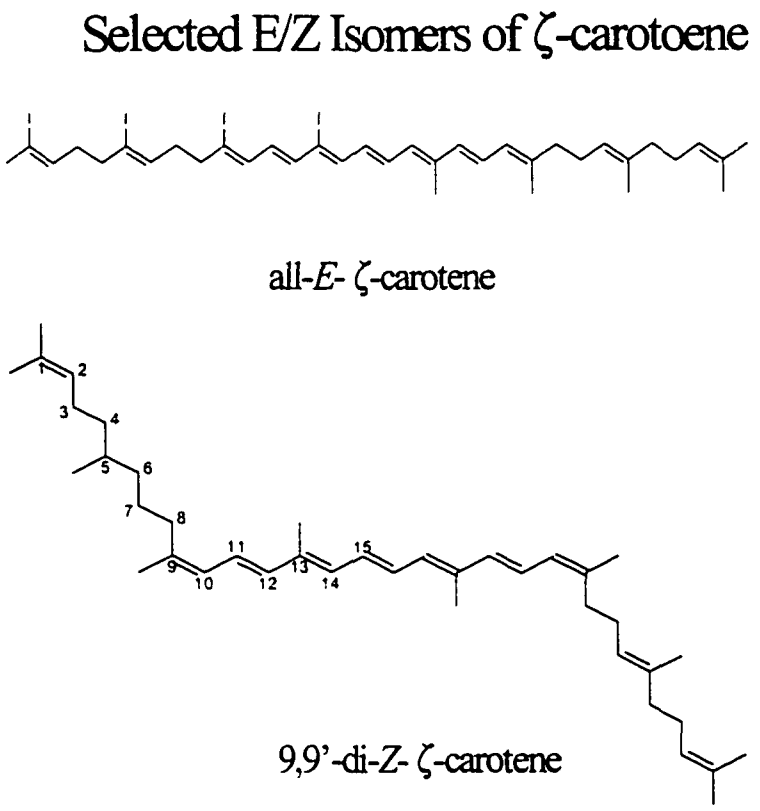


Figure 5.3 Examples of carotenoid E/Z isomers. Examples of E/Z isomers that may be produced during the desaturation of phytoene to lycopene. **Panel A**, ζ-carotene isomers. For each product, the all-*E*-isomer and a representative of the poly-*Z*-pathway are shown.

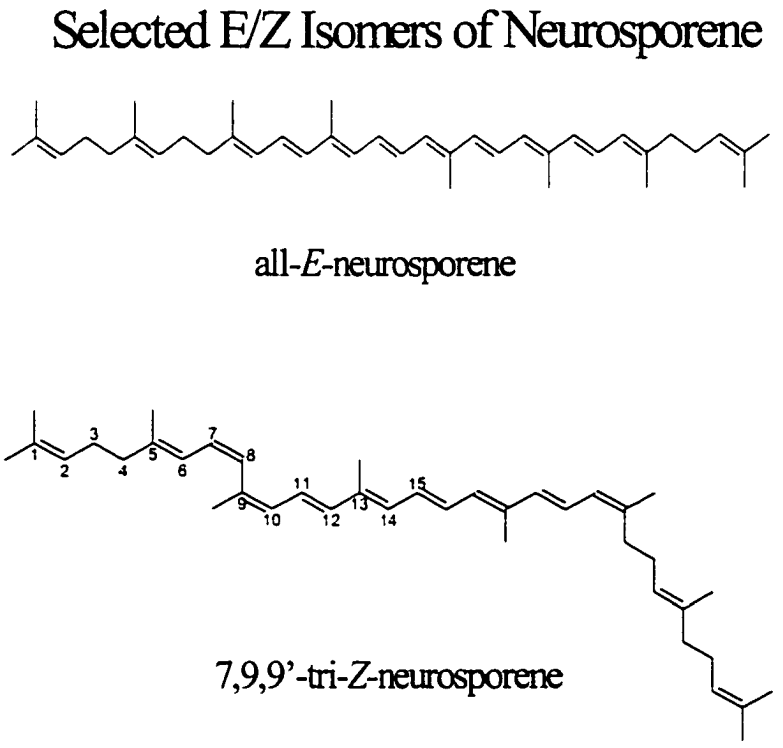


Figure 5.3 Examples of carotenoid E/Z isomers. Examples of E/Z isomers that may be produced during the desaturation of phytoene to lycopene. **Panel B**, neurosporene isomers. For each product, the all-*E*-isomer and a representative of the poly-*Z*-pathway are shown.

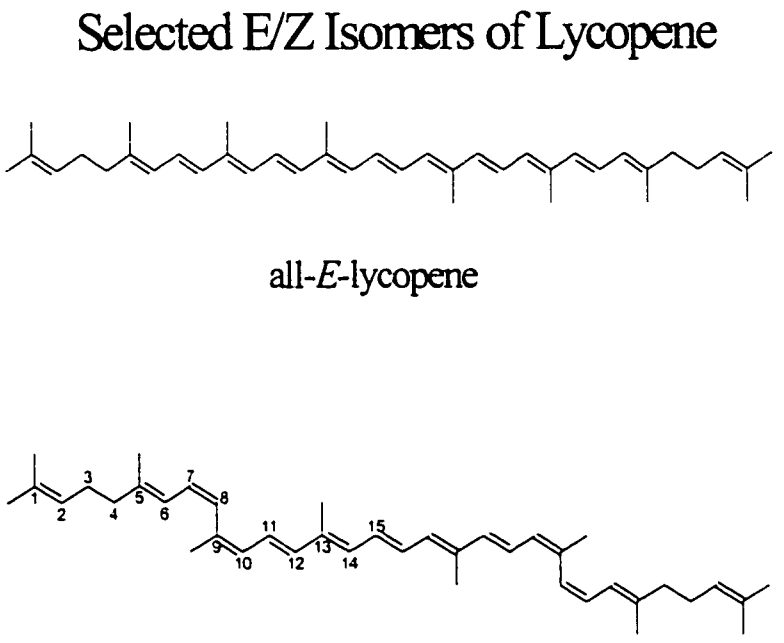


Figure 5.3 Examples of carotenoid E/Z isomers. Examples of E/Z isomers that may be produced during the desaturation of phytoene to lycopene. **Panel C**, lycopene isomers. For each product, the all-*E*-isomer and a representative of the poly-*Z*-pathway are shown.

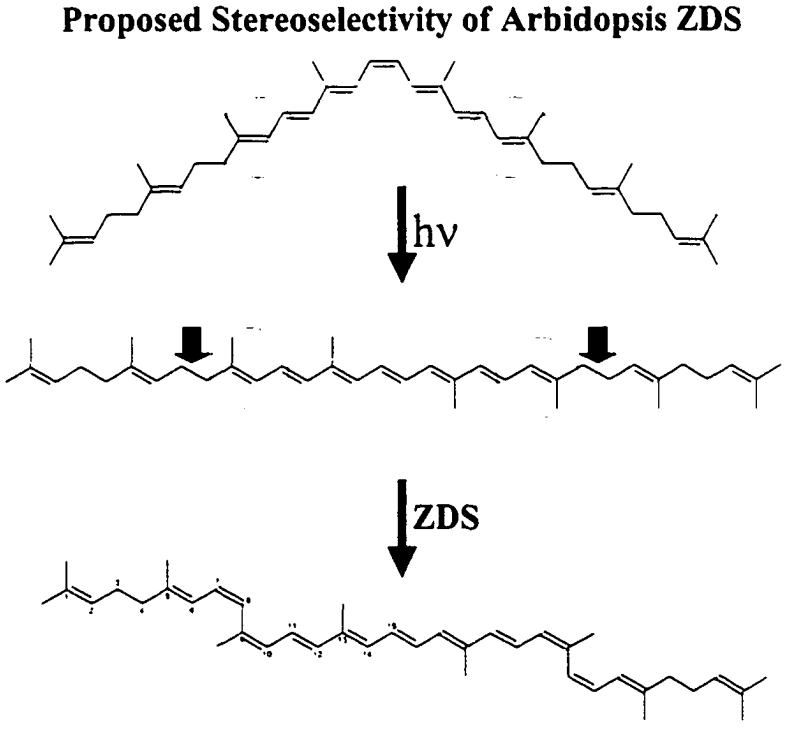


Figure 5.4 The stereochemical selectivity of ZDS. A hypothesis that ZDS is stereoselective for the 15-E isomer of ξ-carotene (center structure) has been proposed (Beyer, et al, 1989; Bartley, 1999). PSY and PDS produce the 15-*Z* isomer (top structure) in the dark and it accumulates in a heterologous *E. coli* carotenogenic system. The 15-*Z* isomer is photo-converted to the 15-*E* isomer and only then may ZDS introduce *Z* double bonds at the 7 and 7' positions (arrows). The origin of *Z* double bonds at the 9,9' positions (circles, shown in *E* configuration) of the poly-*Z*-lycopene product (bottom) is unclear, although they potentially originate from *Z*-GGPP or may come from an isomerase activity associated with PDS.

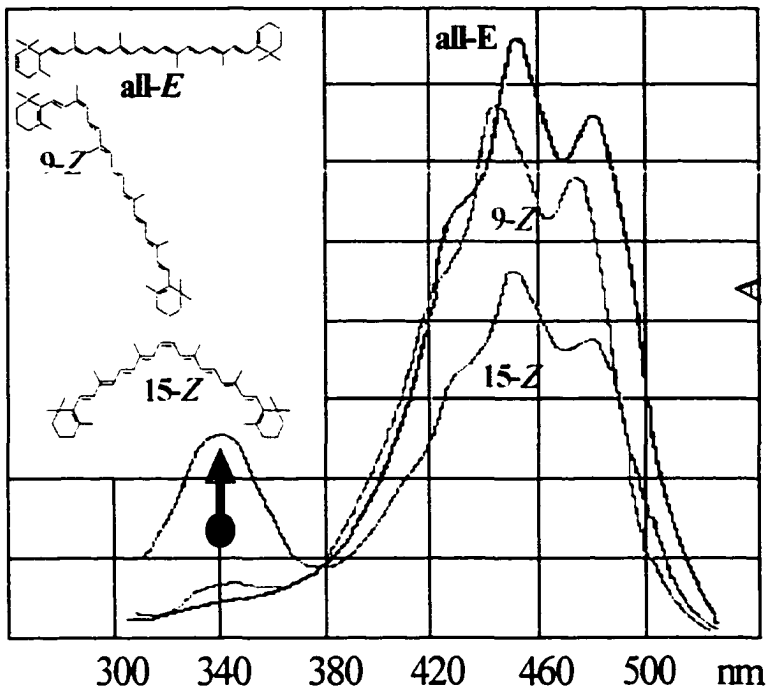


Figure 5.5 Interpretation of carotenoid spectral fine structure. Exemplar spectral fine structure of β-carotene isomers shows trends with changes in isomer states. Introduction of a lateral *Z*-double-bond (9-*Z*) decreases overall absorption and bathochromically shifts the λ_{max}s a few nanometers (seen in 9-*Z* spectrum compared to all-*E* spectrum). A central *E*-double-bond further decreases overall absorbency and exhibits an additional "*cis*-peak" at 340nm (see the15-*Z* spectrum, *cis*-peak denoted by arrow). Redrawn from (Pfander, 1992).

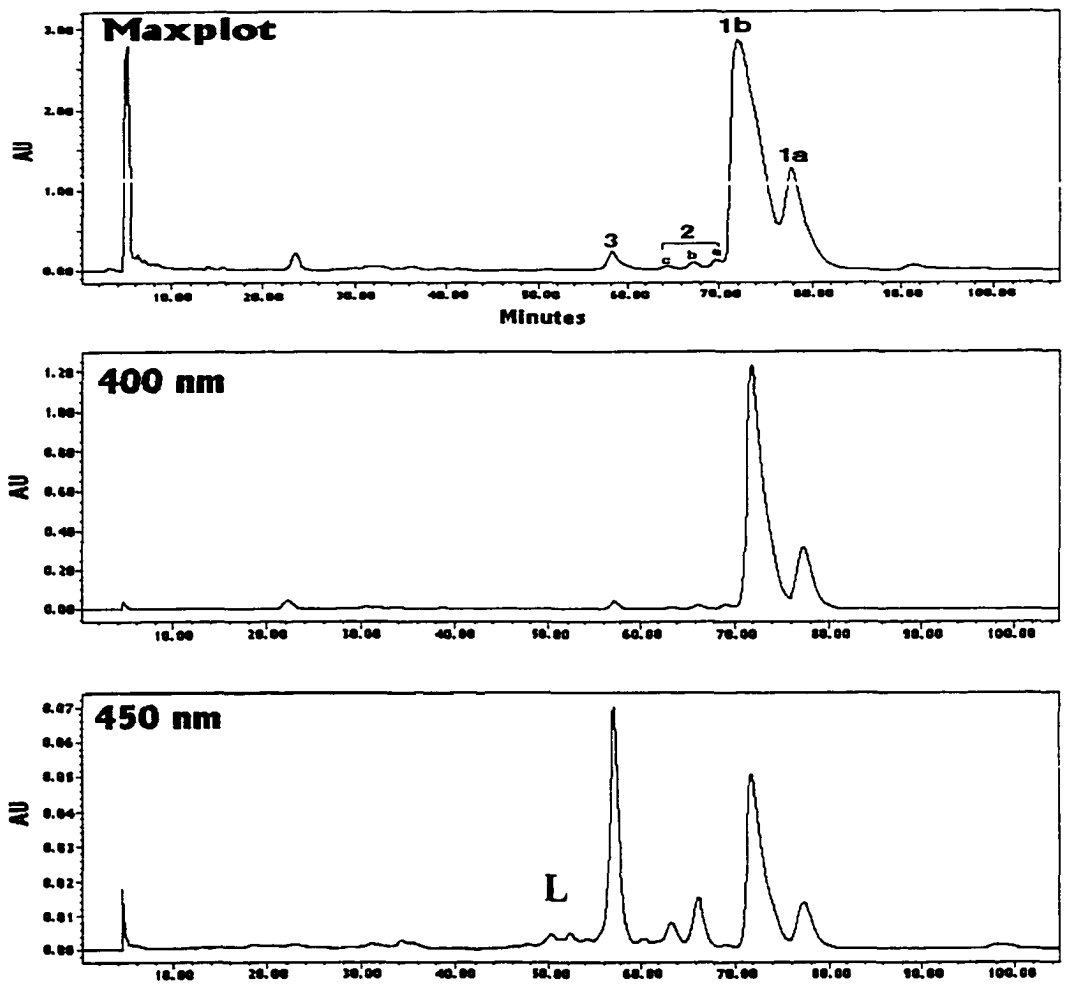
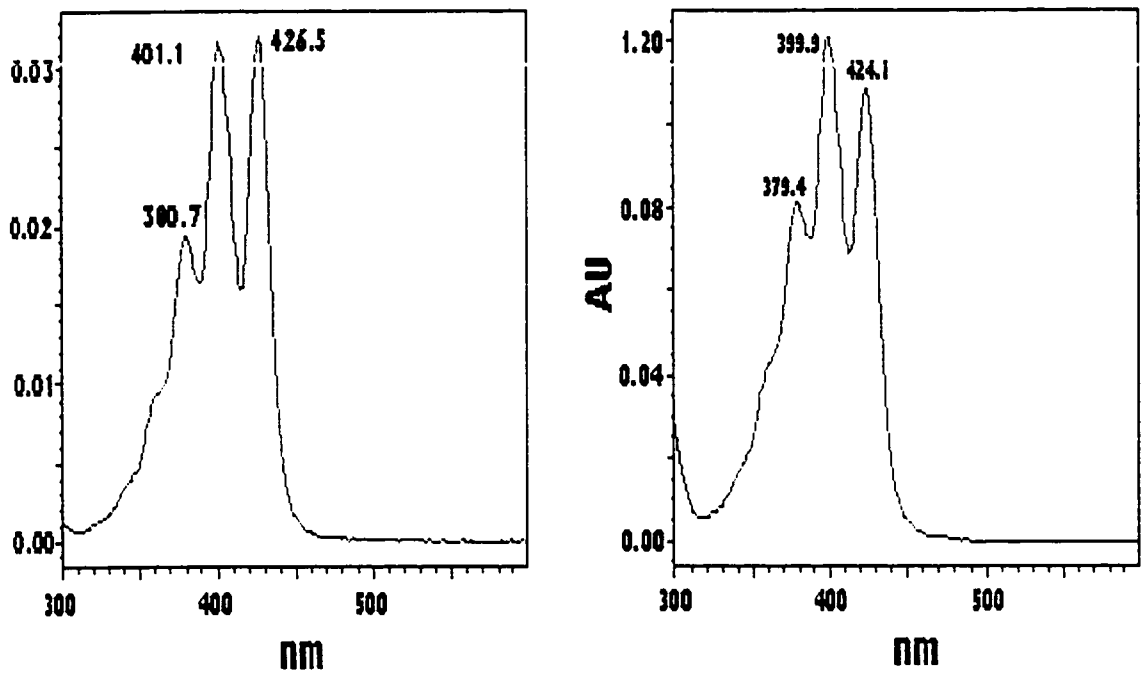


Figure 5.6 HPLC System I elution profiles of carotenoids produced in a heterologus *E. coli* carotenogenic system containing plasmids pMzds-107 coding maize ZDS and pACCRT-EBP coding *Erwinia* GGPPS and *Erwinia* PSY and maize PDS. The first panel, **Maxplot,** shows all peaks plotted at their individual λ_{max}s. The second and third panels shows all peaks plotted at 400 nm and 450 nm, respectively. The panel at 400nm emphasizes the products of PDS, which absorbs maximally at this wavelength. The panel at 450 nm emphasizes the products of ZDS, which absorb maximally at this wavelength. **Peak 1** is ζ-carotene; **Peak 2** is neurosporene; **Peak 3** is lycopene.

Products of PDS



Peak 1A, all-*E*-ζ-carotene **Peak 1B**, *Z*-ζ-carotene

Figure 5.7 Spectral fine structure of products of PDS accumulating in a heterologous *E. coli* carotenogenic system containing plasmids pMzds-107 coding for maize ZDS and pACCRT-EBP coding for *Erwinia* GGPPS and *Erwinia* PSY and maize PDS. The spectral profiles are indicative of all-*E*-ζ-carotene and a *Z*-ζ-carotene. Peak numbers correspond to **Figure 5.6**.

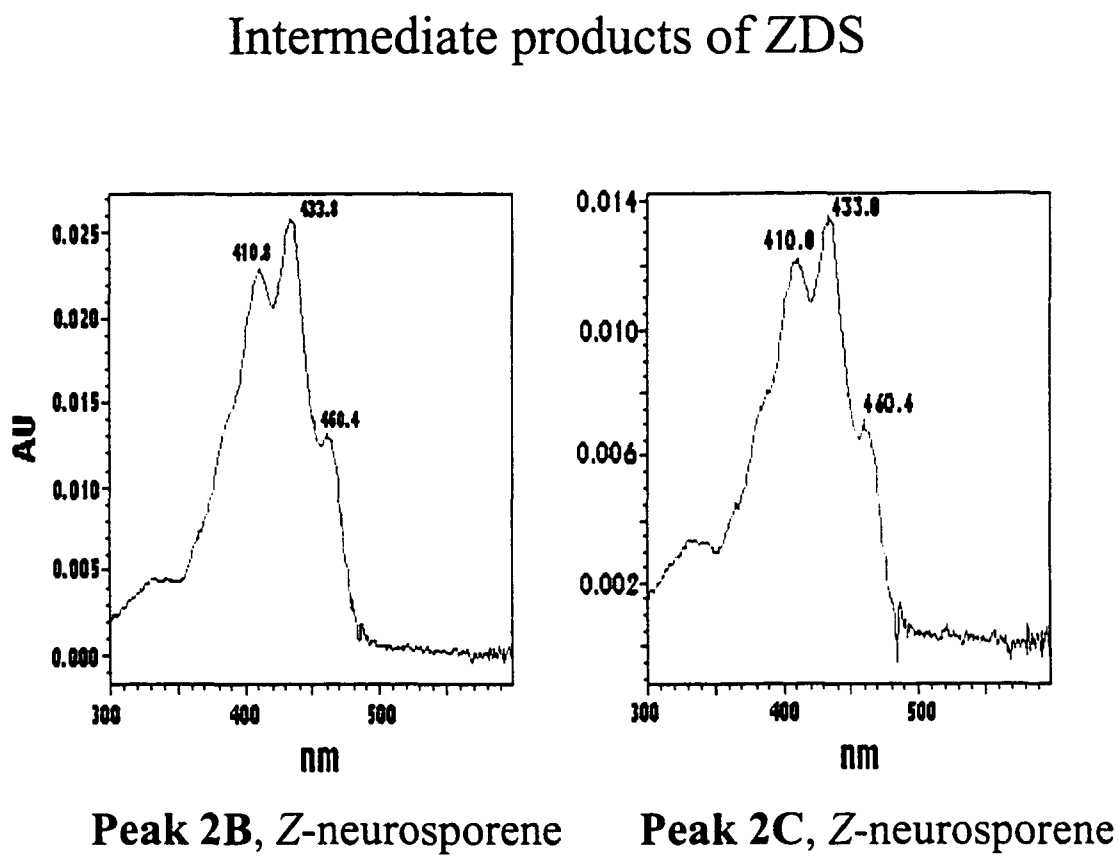
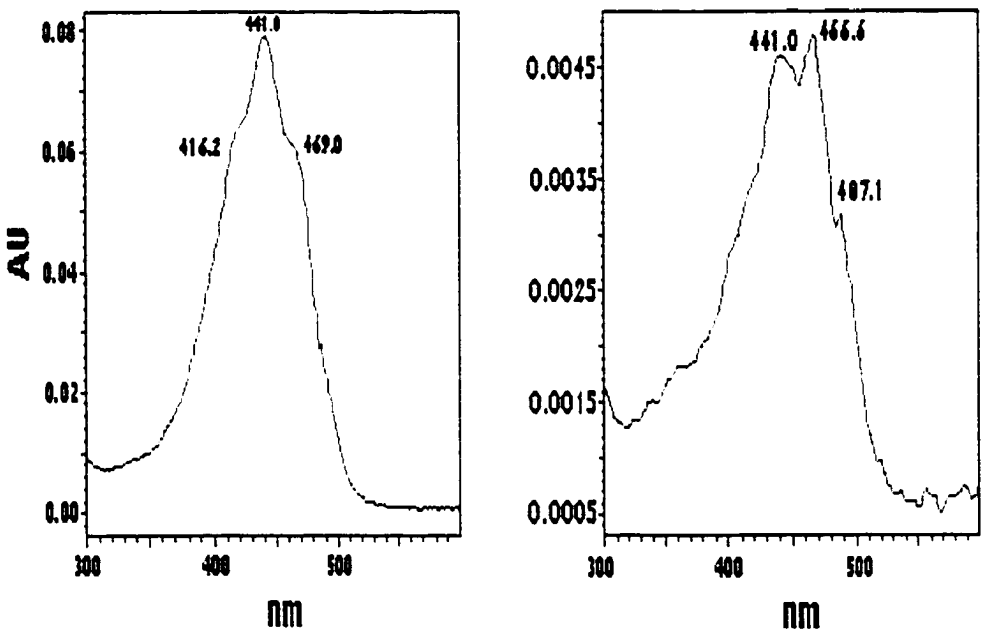


Figure 5.8 Spectral fine structure of intermediate products of ZDS accumulating in a heterologous *E. coli* carotenogenic system containing plasmids pMzds-107 coding for maize ZDS and pACCRT-EBP coding for *Erwinia* GGPPS and *Erwinia* PSY and maize PDS. The spectral profiles are indicative of *Z*-neurosporenes. Peak numbers correspond to **Figure 5.6.**

Final products of ZDS



Peak 3, poly-*Z*-lycopene **Peak L**, lycopene

Figure 5.9 Spectral fine structure of final products of ZDS accumulating in a heterologous *E. coli* carotenogenic system containing plasmids pMzds-107 coding for maize ZDS and pACCRT-EBP coding for *Erwinia* GGPPS and *Erwinia* PSY and maize PDS. The spectral profiles are indicative of poly-*Z*-lycopene and other lycopene isomers. Peak numbers correspond to **Figure 5.6**.

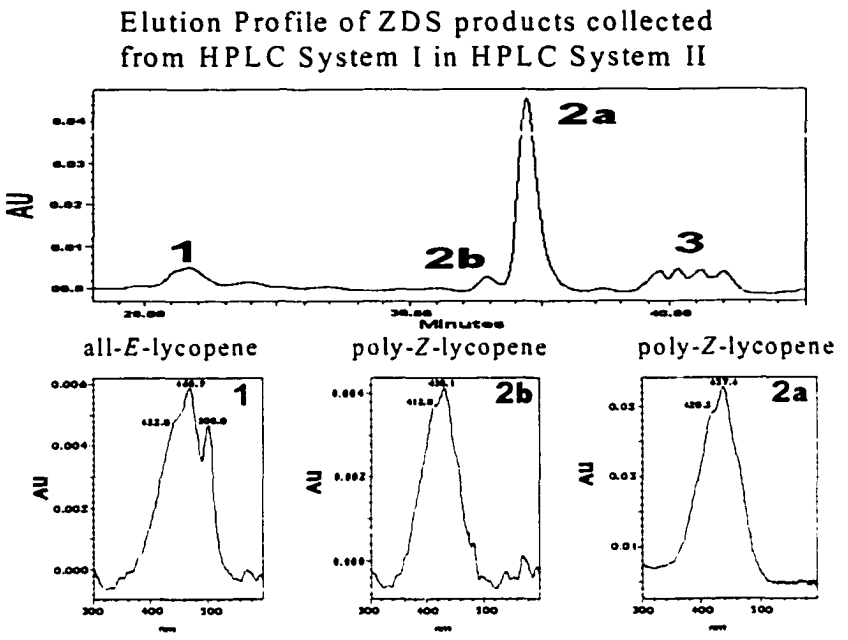


Figure 5.10 Elution profile and spectral fine structures of selected peaks from products of ZDS accumulating in a heterologous *E coli* carotenogenic system contain plasmids pMzds-107 coding for maize ZDS and pACCRT-EBP coding for *Erwinia* GGPPS and *Erwinia* PSY and maize PDS at 400 nm. The top panel is elution profile demonstrating separation of lycopene (**Peak 1**), poly-*Z*-lycopene (**Peaks 2A and 2B**), and four neurosporene isomers (**Peaks 3**). The bottom panel shows spectral fine structure for selected products. The peaks were produced by injection of **Peak 3** from **System I** into **System II.**

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Chapter 6. Cloning and characterization of cDNAs and a genomic DNA fragment coding phytoene synthase in rice.

6.1 Prologue

Maize *Psy* was cloned in 1990 by transposon tagging (Buckner et al., 1990). It is the *Y1* locus of maize. For some recessive mutant alleles of *Y1*, the phenotype is a white or light yellow kernel, but plants are green. Many other mutations in maize, which interrupt carotenoid biosynthesis (Robertson, 1955), lead to pleiotropic effects on the plant, such as precocious germination and a lethal albino seedling phenotype (Robertson, 1952). The *y1* mutation, which accumulated phytoene in the endosperm (Robertson, 1975), and thus was a block in carotenoid biosynthesis at the level of phytoene synthase, had a tissue specific expression pattern. It seemed that the *Y1* gene either behaved differently in the kernel than in the plant and that the mutation only affected the gene's function in the endosperm, or, perhaps, that *Y1* was part of a system of duplicate factors. I have cloned and sequenced a *Psy* from rice, which I expected to be the orthologous gene to maize *Y1*.

6.2 Introduction

6.2.1 Biochemistry

PSY is found in all organisms that synthesize carotenoids: plants and photosynthetic bacteria as well as some non- photosynthetic bacteria and fungi (reviewed in (Goodwin, 1980) (Armstrong and Hearst, 1996) (Sandmann, 1994)). The reaction carried out by PSY is catalyzed in two separate steps: the first is a head-to-head condensation of two molecules of GGPP to form prephytoene diphosphate; this intermediate is then

rearranged to form phytoene (Dogbo et al., 1988; Sandmann and Misawa, 1992). In bacteria, PSY is encoded by the gene *crtB*.

Phytoene synthase is the first enzyme of the carotenoid pathway, as shown in **Figure 1.3**. Its substrate GGPP has many metabolic fates. Examples of the primary and secondary anabolites of GGPP include the phytol chain of chlorophyll, the tocopherols, *ent* -kaurene and giberellic acid, as well as the diterpenes, such as casbene and taxol. Carbon in phytoene is committed to become a carotenoid or a catabolite of carotenoids. In plants, phytoene synthase usually produces 15-*Z*-phytoene. The stereochemistry of the reaction is shown in **Figure 5.1**. The enzyme functions as a component of a membrane-associated multi-component complex in chloroplasts and chromoplasts (Camara et al., 1982; Fraser et al., 1999).

6.2.2 The phytoene biosynthetic complex

The components of the phytoene synthase complex are geranyl-geranyl pyrophosphate synthase (GGPPS) and phytoene synthase (Beyer et al., 1985). IPPI is active in the stroma (Cunningham and Gantt, 1998a). The PSY-GGPPS complex can be isolated in a highly active form from *Capsicum annuum* chromoplasts (Camara et al., 1989). Purified PSY shows a strict requirement for Mn^{2+} and is inhibited by inorganic phosphate (Camara et al., 1989). PSY and GGPPS have been immunolocalized in a number of fruit chromoplast and chloroplasts.

6.2.3 Localization of PSY

PSY has been localized in the stroma of the plastid as a peripheral membrane protein (Al-Babili et al., 1996; Bartley et al., 1992; Bonk et al., 1997; Lutke-Brinkhaus et al., 1982; Misawa et al., 1994; Schledz et al., 1996; Yu, 1999), where it functions in a phytoene biosynthetic complex also containing homodimeric GGPPS (Camara et al., 1982; Dogbo and Camara, 1987). Whether PSY is a homodimer within the complex is uncertain. The pepper PSY functions as a purified soluble monomeric peptide (Dogbo, et al., 1988). An association with the plastid membrane is expected since the product of enzyme complex, phytoene, is highly lipophlic and must be delivered to a membrane bound complex of desaturases, cyclases, and oxygenases (Cunningham and Gantt, 1998a). In daffodil, two isoforms of PSY were found (Schledz et al., 1996); one in the stroma and one associated with the plastid membranes. Association with the membrane and galactolipids was necessary for activity *in vitro* (Schledz et al., 1996). The soluble, stromal form was found to be associated in a large complex with chaperonin60 (Cpn60) (Bonk et al., 1997). Yu (1999) localized PSY to the envelope in maize endosperm amyloplasts, but to the cytoplasm in rice endosperm amyloplasts using an anti-maize PSY antiserum. The mis-localization of PSY in rice was suggested to be functionally correlated with lack of carotenoid biosynthesis, since rice amyloplasts do not make carotenoids (Yu, 1999).

6.2.4 Putative transit peptide and molecular mass of PSY

The molecular mass of PSY determined by Western blotting from cDNA and gene sequence predictions varies among studies and among species. For example, a pea

chloroplast import study showed that tomato PSY1 is produced as a 47 kDa pre-protein and was processed to a 42 kDa mature protein upon import (Bartley et al., 1992). A Western blot of tomato stromal proteins hybridized with a monoclonal antibody raised against PSY1 demonstrated a single antigen of apparent molecular mass 38 kDa (Misawa et al., 1994). In maize and rice, an antigen with an apparent molecular mass of 38 kDa was also found with Western blotting and immunodetection of amyloplast proteins (Yu, 1999). In Arabidopsis, the nucleotide sequence of a PSY cDNA has been used to predict a pre-protein with a molecular mass of 47.6 kDa (Cunningham and Gantt, 1998a). Bonk, et al. (1997) predict a daffodil PSY of calculated molecular mass, 47.8 kDa. After import into pea chloroplasts a 3 kDa cleavage product is found by blue native-PAGE and autoradiography of an *in vitro* translation product (Bonk et al., 1997). Other predictions of chloroplast transit cleavage sites suggest mature PSY proteins of about 38-40 kDA (see this chapter for arabidopsis and rice and (Karvouni et al., 1995a) for musk melon). Confirmation of the transit peptide cleavage site by N-terminal microsequencing of purified, mature PSY has not been reported; therefore, predictions of transit peptide size remain conjectural.

6.2.5 Protein Homologies

Selected complete plant PSY proteins sequences and deposited to Prosite or Genbank are shown in **6.4.10 Hypotheses of the evolutionary relationships of rice PSY and maize PSY1 proteins, Table 6.3**. Psy is homologous to bacterial CRTB and a small family of polyisoprenoid synthases, including squalene synthase (SQS or also farnesyl diphosphate

farnesyltransferase, FDFT), which vary in the chain length of their substrates but have similar reaction mechanisms (Sandmann, 1994). These genes share one to three conserved, putatively functional domains (Chamovitz et al., 1992; Cunningham and Gantt, 1998a; Sandmann, 1994). These homologous domains as listed in the databases Prodom (Corpet et al., 2000), Prosite (Hofmann et al., 1999), and BLOCKS (Henikoff and Henikoff, 1994) are outlined with accession numbers below.

The plant PSY proteins share an N-terminal domain (Prodom accession 7377) that does not occur in SQSs or in the bacterial CRTBs. Since chloroplast transit signal peptide cleavage sites are conjectural, it is not known whether this domain is part of the transit peptide or the mature protein (Cunningham and Gantt, 1998b). A domain in the middle of the protein (Prodom accession 1082) is thought to be likely a pyrophosphate binding-and-removing-domain (Chamovitz et al., 1992). The middle domian also shares similarity with GGPPS and FFPS (Chamovitz et al., 1992). The plant PSYs share a domain of strong similarity at the carboxy terminal 37 amino acids (Prodom accession 9861), for which a function has not yet been proposed. The SQSs share a domain (Prodom accession 3537) that is not found among phytoene synthases. Within the middle conserved domain, Prosite lists two conserved subdomains, namely: Squalene_phytoene_synthase1 (accession 1044) having the signature:

Y-[CSAM]-x(2)-[VSG]-A-[GSA]-[LIVAT]-[IV]-G-x(2)-[LMSC]-x(2)-[LIV]

and Squalene_phytoene_synthase2 (accession1045), with the signature:

[LIVM]-G-x(3)-Q-x(2,3)-N-[IF]-x-R-D-[LIVMFY]-x(2)-[DE]-x(4,7)-R-x-[FY]- x-P.

This second domain has a small cluster of charged amino acid residues as noted by Chamovitz et al. (Chamovitz et al., 1992) for PSY proteins.

The BLOCKS database lists homologies among PSY proteins and SQS proteins (Henikoff and Henikoff, 1994). BLOCKS accession IPB002060A and BLOCKS accession IPB002060C are within Prodom accession1082 and correspond to Prosite Squaline_phytoene_synthase signatures 1 and 2, respectively. BLOCKS number IPB002060B is unique to plant PSY proteins. IBP002060 has a small cluster of charged amino acid residues, as calculated with SAPS. Prodom domains and BLOCKS are shown in a multiple alignment of plant PSY proteins in **Figure 6.13.**

The overall homology among bacterial CRTB proteins is only about 30 % identity and 50% similarity, while the conservation among plant PSYs is about 75% identity and 80 % similarity (data not shown). Specific comparisons to rice PSY will be presented in **6.4.10 Hypotheses of the evolutionary relationships of rice PSY and maize PSY1 proteins.**

6.2.6 Predicting chloroplast transit peptides

All the genes of the carotenoid biosynthetic pathway are nuclear-coded and the gene products are targeted to the chloroplast by an N-terminal transit peptide that is cleavaged from the pre-protein upon import. Additional signals for localization to thylakoid membranes may reside within the mature peptide. There is no strong consensus among chloroplast transit peptide domains. Putative prediction of chloroplast transit peptide has been approached computationally with ChloroP v1.1 (Emanuelsson et al., 1999), although many of the carotenoid deduced amino acid primary structures defy transit cleavage site prediction (personal observation). Another predictor of a chloroplast transit domain might be a change in basic properties, such as amino acid composition or hydrophobicity, as the functional constraints on catalytic domains and transit sequence domains are likely to be widely disparate. Finally *in vitro* chloroplast and chromoplast import studies combined with immunodection tools (Bonk et al., 1997; Bartley et al., 1992; Karvouni et al., 1995; Klosgen et al., 1989), facilitate approximations of the molecular mass of pre-protein *vs.* mature protein as well as suborganellar localization (see also **6.2.4 Putative transit peptide and molecular mass of PSY**). For example, an *in vitro* import study showed that tomato PSY1 is expressed as a 47 kDa pre- protein, and targeted into pea chloroplasts as a 42 kDa mature protein (Bartley et al., 1992). Therefore the transit peptide of tomato PSY in the ripe fruit is about 9 kDa, which suggests about 80 amino acid residues as a cleaved transit peptide. However, Karvouni *et al.* have proposed

that import assays with heterologous systems might not be reliable for correct processing of mature proteins (Karvouni et al., 1995a).

Another mode of inference for prediction of a transit peptide by alignment methods such as CLUSTALW is comparison of amino acid primary structure to the bacterial PSY homologous CRTB proteins and the cyanobacterial PSY proteins. Since bacteria do not have plastids, the CRTBs do not have transit peptides, but retain catalytic domain homologies due to functional constraints on protein structural evolution. Finally by similar inference, the prediction of a transit peptide for one plant PSY may be extended to prediction for another highly-homologous plant PSY.

6.2.7 Gene structure

Previous to the sequencing of rice *Psy* in this study, three other plant *Psy* genes had been sequenced. These are *Psy1* (GTOM5, LEGTOM, Genbank X60441*)* and *Psy2* (GTOMF, LEPSPS,Genbank X60440*)* of tomato (Ray et al., 1992) and several alleles of *Y1* (*Psy,* ZMU32636, Genbank U32636) from maize (Buckner et al., 1996). Both the tomato and maize *Psy1* genes share identical 6-exon structure, 5 non-homologous (personal observation) introns and high homology (85%) among exons (presented in **Figure 6.7**, below), that is typical among plant PSY genes. On the other hand, tomato *Psy2* has limited coding sequence homology to *Psy1* genes (65-85%, depending on the exon) but a different exon/intron structure with 8 exons and 7 introns. The first exon of homology to *Psy1* is split into 3 exons. Other exons retain similar exon/intron boundaries. The

promoter of tomato *Psy1* and *Psy2* and of maize *Psy1* show no obviously significant similarities at the sequence level. The phyletic relationships among these two gene-types have not been determined due to the paucity of the sequenced family members. Tomato *Psy2* has been mapped to a different chromosome in maize, specifically, linkage group 8, than that of *Y1* (Psy1) in linkage group 6 (MaizeDB, http://www.agron.missouri.edu/). Given the rapid advances in comparative genomics among the Poaceae (Gale, et al.1998; Ahn, et al., 1993), sequencing, chromosome mapping, and analyses of syntenic relationships (Feuillet, et al., 1999) for a rice *Psy* were deemed to be of merit.

6.2.8 Analyses of *Psy* gene expression during development in plants

In tomato, both *psy1* and *psy2* are expressed during tomato development. Reverse transcriptase-polymerase chain reaction (RT-PCR) measurements of steady state messengerRNA (mRNA) levels have shown that *psy1* transcripts are abundant in seedlings and ripe fruits, while *psy2* transcripts are more abundant in leaves (Bartley and Scolnik, 1993). Treatment with Norflurazon, an inhibitor blocking carotenoid synthesis at the PDS step (reviewed in (Bramley, 1994), triggered the expression of both *psy1* and *psy2* at the transcript level. Psy transcript levels were low in tomato roots where less carotenoid accumulated. The amount of *Psy1* transcript increased during flower development, while that of *Psy2* did not. During fruit ripening, carotenoid levels increase and PSY expression increases dramatically.

In maize, where PSY is coded by the *Y1* gene (Buckner et al., 1996), the dominant *Y1* transcript was highly expressed in embryo, seedling and endosperm while the mutant *y1* transcript was expressed at wild-type levels in the embryo and seedling, but was not expressed in the endosperm. Li (Li, 1998) studied *Psy1* expression at the transcript level in endosperms and embryo at various days after pollination (DAP) of the ovule in maize. In maize transcript levels are increased between 10-15 DAP and in rice endosperm transcript levels are constant. For example, a cultivar accumulating high levels carotenoids showed Psy transcript accumulation earlier in development. On the other hand, constant levels of Psy transcripts were found in rice endosperms throughout development (Yoganathan, 1998). Yu (Yu, 1999) measured PSY accumulation in a similar series of maize tissues and found that antigens of 38Kd, corresponding to the predicted size of a mature maize PSY appeared about 10 DAP and peaked at about 15-20 DAP, which corresponds to the beginning of carotenoid accumulation. These maize antigens were assocaited with amyloplasts. In rice endosperm, where carotenoids do not accumulate, a putative PSY antigen was constitutively present, though not associated with amyloplasts.

The expression of PSY has been studied in fruit chromoplasts of *Capsicum annuum* (Hugueney et al., 1996). *Psy* transcript was lower in leaves and young fruits, but higher in ripe fruits (Römer *et al.*, 1993). PSY transcripts of different sizes have been observed in pepper leaves and fruits, which suggested that the regulation of PSY expression in pepper was tissue specific. In addition, post-transcriptional modification may be involved

(Römer *et al.*, 1993). PSY expression, profiled with an anti-PSY antiserum, was developmentally regulated in fruit ripening, and was also induced after fungal elicitor treatment and wounding of fruit (Hugueney et al., 1996).

The accumulation of *Psy* was examined in various tissues in melon (Karvouni et al., 1995). Northern analysis showed that transcript levels were low in green fruits, but increased and reached the maximum in fruits during the transition from green to orange, and then decreased in late ripening stages. In leaves and roots, *Psy* transcript levels were low. Intermediate amounts of transcripts were detected in flower petals.

In daffodil (*Narcissus pseudonarcissus*) flowers, the chloroplasts are converted into chromoplasts with concomitant accumulation of carotenes (Schledz *et al.*, 1996). During the transition, PSY transcript levels were not detectable on Northern blots from green leaves, were high in the early flower stage, then, later, slightly decreased. PSY antigen levels increased in the early flower stage, then decreased (Schledz *et al.*, 1996). In addition, PSY activity required the association of the enzyme to galactolipids (Schledz *et al.*, 1996). Using an antibody against daffodil PSY, two isoforms of PSY were identified. The active PSY form was present on the chromoplast membrane, while the inactive PSY form resided in the chromoplast stroma associated with a soluble Cpn60 protein complex (Bonk *et al.*, 1997). Further background information and more example of the biochemistry, localization and molecular genetics of PSY may be found in **Chapter 1, section 1.6.1 Molecular genetics of PSY.**

While the regulation of gene expression and the localization of gene products is not the focus of this study, an overview of the complexity of expression among genes, tissues-types and plastid-types (**Chapter 1**) is germane to the proposal of a small gene family of *Psy* genes among the grasses.

6.3 Materials and Methods

6.3.1 Plasmids and BACs

Table 6.1 and **Appendix A** show genomic clones used in this study. BAC clones are from University of California at Davis (UCD) and described in (Wang et al., 1995) and from Texas A&M as described in (Zhang, et al, 1996). Clones were chosen from libraries by hybridization to a 1.3 Kb *SalI/Not*I fragment of EST D48251 (now also Genbank AY024350) coding a partial rice PSY cDNA. Only UCD #2 was used for sequencing and subcloning. Other BAC library clones that hybridized are listed but were not investigated further.

Clone Designation	Vector	Clone Source Reference	Inbred	Comment
BAC UCD 2	pBeloBACII	(**Wang et al.**, 1995)	IR-BB21 rice Nipponbare	Used to generate subclones for sequencing
BAC UCD 9	pBeloBACII	(**Wang et al.**, 1995)	IR-BB21 rice Nipponbare	Not used
BAC LM 1	pBeloBACII	[Zhang, 1996 #4398]	Lemont	Not used
BAC LM 2	pBeloBACII	[Zhang, 1996 #4398]	Lemont	Not used
pVIVYH5	pBluescript SK- *Hin*dIII site	this study, 10kb *Hin*dIII subfragment of UCD 2	IR-BB21 rice Nipponbare	Restriction map **Appendix B**
pVIVYB10	PBluescript SK- *Bam*HI	this study, 5 kb *Bam*HI subfragment of UCD 2	IR-BB21 rice Nipponbare	Restriction map **Appendex B**
pVIVYE3	pBluescript SK- *Eco*RI	this study, 2.3 kb *Eco*RI subfragment of UCD 2	IR-BB21 rice Nipponbare	Restriction map **Appendix B**
pRCPSYC6.0 (Genbank AY024351)	pBluescript II SK- *Cla*I site	this study, 6 kb *Cla*I subfragment of pVIVYH5	IR-BB21 rice Nipponbare	Sequenced, restiction map **Appendix D**

Table 6.1 Genomic clones used in this study. Also see **Appendix A** and restriction maps in appendices listed in **Comment**. All rice varieties were Japonica.

BAC clone UCD #2 was fragmented with *Bam*HI, *Hin*dIII, or *Eco*RI and combinations of each and subjected to Southern blotting. Fragments hybridizing to a 1.3 Kb *Sal*I/*Not*I fragment of EST D48251 coding a partial rice PSY, were size-scored for restriction mapping (**Appendix B**) and isolated and subcloned into pBluescript SK- to produce, that had been prepared with the appropriate enzyme, to created plasmids pVIVYH5,

pVIVYB10, and pVIVYE3 containing a 5 Kb *Hin*dIII, a 10*Bam*HI, and a 3Kb *Eco*RI restriction fragment, respectively. Library screening, restriction mapping and subcloning were performed by Vivek Upasani, MGM Science Institute, India. Clone pVIVYH5 was digested with *Cla*I to release a 6 kb fragment containing the PSY coding sequence. This fragment was subcloned into pBluescript II SK- to produce pRCPSYC6.0 which was used for sequence analysis and deposited as Genbank AY024351.

6.3.2 Expressed Sequence Tags

EST sequences were collected from Genbank and MAFF (http://www.dna.affrc.go.jp/). **Table 6.2** shows EST sequences used in this study and is referred to later in the **Results** and **Discussion**.

Accession	End/size	Tissue/strain	Homology
AU082727	3' poly A/ 489 n.t.	seedling root/Nipponbare	Group 2
AU082986	3' / 556 n.t.	>10 cm panicle/Nipponbare	Group 1
C73801	3' / 484 n.t.	> 10 cm panicle/Nipponbare	Group 1
C74590	5' / 407 n.t.	3-10 cm panicle/Nipponbare	Group 2
C98515	3' poly A/ 456 n.t.	flowering panicle/Nipponbare	Group 2
D48251* (AY02450)	5' / 402 n.t.	green shoot/Nipponbare	Group 2
D48697*	3' / 315 n.t.	8 da green shoot/Nipponbare	Group 2

Table 6.2 Genbank accession numbers of Expressed Sequence Tags used in this study. * denotes EST cDNA clones that have been completely sequenced in this study. "n.t." denotes nucleotides. Two protein primary-structure, homology groups are indicated.

6.3.3 Screening of BAC libraries.

BAC libraries were screened by DNA hybridization at high stringency according to (Wang et al., 1995).

6.3.4 DNA manipulations.

Subcloning, Southern blotting, restriction digestion, sequencing template preparation, colony screening, and growth and preparation of bacterial cultures were carried out according to standard protocols in Current Protocols (CponCD). High stringency Southern hybridization conditions were the use of formamide prehybridization (FPH) (CponCD) with a radio-labeled probe at 42 °C for 18 hours, while low stringency conditions were FPH adjusted to 35% formamide and hybridized at 37 °C for 24 hours. Washing was in two changes of 6x SSC, 0.1% SDS at 42 °C. Preparation of sequencing template cut-back series for manual sequencing of EST cDNA was preformed using an ExoIII/Mung Bean Deletion Kit from Stratagene (LaJolla , CA, USA), according to the manufacturer's instructions. Manual DNA sequencing was performed using Sequenase Version 2 sequencing kit from USB (Gainsville, FA, USA) according to the manufacturer's instructions. Automated sequencing of the genomic subcloned BAC fragment contained in pRCPSYC6.0 was performed by ABI Prism 377XL automated DNA sequencers at University of Chicago Cancer Research Center DNA Sequencing Facility (http://cancer-seqbase.uchicago.edu/) by primer walking with primers produced at BioSynthesis Inc. (Lewisville, TX, USA). Text searches of Genbank ESTDB yielded

rice EST clones D48251 (D48251 is now also AY024350) and D48697, which were sized by restriction mapping and subjected to cut-back deletions and manually sequenced.

6.3.5 Computational methods.

Sequence editing and grooming were perform initially in Chromas Version 1.56 (Technelysium Pty. Ltd., Helensvale, Queensland, Australia). Sequence assembly, primer selection, restriction site mapping, alignments (ClustalW), dendrograms, hydrophobicity plotting, and graphics were prepared in VectorNTI Version 5.1. (Informax, North Bethesda, MD, USA). Database searches were performed at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Protein similarity searching was performed at Prodom 2000.1 (http://www.toulouse.inra.fr/prodom.html), Blocks Database (http://www.blocks.fhcrc.org/), and Prosite Database (http://expasy.cbr.nrc.ca/prosite/). Transcription factor database searching was performed against TransFac Release 4.0 (http://transfac.gbf.de/TRANSFAC/index.html). Transit peptide prediction was performed with ChloroP V1.1 [Emanuelsson, 1999 #4413]. Promoter elements and polyadenylation signals were predicted by manual inspection.

6.4 Results

6.4.1 EST cDNA sequence

Clone D48697 was found to be a 940 bp-shorter-version of clone D48251, which had an insert of 1229 bp. The sequence of clone D48251 is shown in **Figure 6.1** along with a deduced open reading frame capable of encoding 71 amino acids. A feature map of the

insert in a partial vector sequence is shown in **Figure 6.2.** The clone contained a small stretch of unidentifiable DNA of high %G+C content at the 5' end as shown in the **Figure 6.2** as a thin line. Comparison of this amino acid sequence by AlignX was made to Maize PSY1 and daffodil PSY sequence from Genbank (accession numbers shown in **Table 6.3**). The sequence of EST D48251 has been submitted to Genbank under accession number **AY024350.** The Genbank flat file is presented as **Appendix D.**

Distance analysis of the alignment yielded the relationship shown in **Figure 6.3.** The deduced amino acid sequence was highly homologous to PSY proteins from monocotyledonous plants, but the deduced sequence seemed to be missing its N-terminus. The cDNA from which it was deduced was incomplete. This is a common problem with cDNAs, due to limitations in cDNA library construction methods. Since the prevalence of *Psy* transcripts was thought to be low (Li, 1998) and 6 attempts at screening various cDNA libraries had failed to produce PSY homologous clones, I decided to complete the cDNA sequence deductively by investigating the genomic DNA. I took advantage of an ongoing screening program for rice BAC library clones with homology to carotenoid genes that had been started by Vivek Upasani, in E.T. Wurtzel's laboratory.

6.4.2 Genomic Southern blot

Southern blot analysis of rice genomic DNA indicated that both sequenced rice ESTs were likely to be single copy genes, as shown in **Figure 6.4.** The small number of

hybridizing bands for a variety of restriction enzymes (1-5) that recognize 6-base-pair palandromic sequences is circumstantial evidence for a single copy gene. Blots at low stringency showed the same simple pattern, while hybridization of the rice cDNA to maize genomic DNA similarly prepared, indicated a number of minor bands among prominent bands, indicative of a small gene family in maize (data not shown).

6.4.3 BAC clone isolation

Screening the Rice BAC libraries resulted in four positive BAC clones as shown in **Figure 6.5** and listed in **Table 6.1**.

6.4.4 BAC Subclone isolation and sequencing

The BAC clone UCD #2 was selected for further analysis. This clone had an insert ranging from 120-140 Kb (data not shown) and so was subjected to restriction enzyme fragmentation to produce a template more appropriate in size for sequencing. Subfragments were cloned and are described in **Materials and Methods** and listed in **Table 6.1.** A restriction map of the region containing the gene was generated in the process and is shown for a subregion of UCD #2.

*Cla*I was used to generate three restriction fragments from pVIVYH5 which contained the *Hin*dIII fragment shown in **Figure 6.6**. The larger fragment was cloned to produce a plasmid containing a 6 kb *Cla*I fragment containing *Psy*. *Cla*I was chosen as it produced the smallest possible single fragment that hybridized to a 1.3 SalI/NotI fragment from

EST D48697, which was know to contain a *Cla*I site near its 5' truncated end. This strategy was devised to place the missing 5' end of *Psy* near (within 3Kb) a vector sequencing primer site. Chromosome walking was then pursued from vector primers and from a primer designed from sequence near the 5' end of the truncated cDNA. The primer -walking sequencing strategy is shown and the extent of sequencing coverage (3 runs each strand) is shown in **Figure 6.7** to demonstrate sequence quality. Such a standard for coverage produced 98% accuracy.

6.4.5 The sequence of rice *Psy*

The sequence of rice *Psy* is given in **Figure 6.8** and has been submitted to Genbank under accession number AY024351. The Genbank flat file is presented as **Appendix E.**

6.4.6 Features of rice *Psy*

6.4.6.1 Putative regulatory features

Rice *Psy* exhibits typical plant gene features that may be presented as hypotheses. The putative promoter element contains CAAT and TATAA boxes and a putative signal for 5' mRNA capping structure addition. These features are rather close to the putative start codon, and since transcriptional start site mapping was not performed, should be viewed as highly conjectural. Some features were recognized by TransFac, a transcription factor binding site database search engine. TransFac also finds a putative binding site for the myb-domain, transcription factor activator P. This transcription factor activates flavonoid and anthocyanin pigment biosynthesis genes in maize (Grotewold, et al, 1994). A

sequence of poly-guanine then poly-cytosine causes strong attenuation of sequencing reactions and defies passage by conventional sequencing. This unusual element is within the likely promoter. A putative poly adenylation site is also evident.

6.4.6.2 Intron/Exon structure

By comparing the sequences of the cDNAs, the coding region homologies of rice *Psy* and the maize and tomato *Psy1* genes, I was able to deduce the exon/intron boundaries of the rice *Psy* gene. This also allowed deduction of the complete protein sequence and a putative translation start site.

The intron/exon structure of rice *Psy* is shown in **Figure 6.9**. The exon structure of maize *Psy1* (Y1) and tomato Psy1 are perfectly conserved (Buckner et al., 1996), and the deduced rice *Psy* exon structure is similarly conserved.

These genes all have six exons of the same size. On the other hand, the intron size, with few exceptions, are not the same size and pairwise, DNA sequence similarity searches {BLAST2 (NCBI)and similarity plots (VectorNTI)} show no significant similarity. Rice *Psy* has a large first exon (948 nucleotides, see **Appendix G** for other intron/exon sizes) compared to the other *Psy* genes (~100 necleotides, **see Figure 6.9** for scale drawings). As noted previously, tomato *Psy2* does not share this exon/intron structure and shows limited exon homologies.

6.4.7 Deduced protein primary sequence

The primary protein sequence of rice PSY is shown is **Figure 6.10.** Further features of this sequence, such as transit peptide cleavage site, molecular mass, and domains of homology to other proteins are discussed below.

6.4.8 Prediction of the chloroplast transit sequence

In order to report deduced properties of a mature protein it is essential to predict a transit peptide cleavage site. Strategies and predictions for putative cleavage sites of other PSY proteins have been discussed in the **Introduction.**

Since transit peptides are non-homologous and PSY exhibits strong homology as a protein family, an examination of residue similarity in a pair-wise alignment of the closest know relative (likely maize) was performed. **Figure 6.11** shows a similarity plot of maize PSY against rice PSY.

Sequence similarity of rice to maize PSY only begins at about residue 122 with respect to the rice PSY N-terminus. Since PSYs have strong functionally conserved domains that reside and are active in a particular subcellular environment, and the transit peptide is not subject to such evolutionary constraints, the transit peptide may exhibit disparate general properties such as hydropathy or hydrophobicity. Such a relationship was found for hydrophobicity at low physiologic hydrogen ion concentration. A hydrophobicity plot at

pH3.4 in **Figure 6.12** shows the pre-protein to be bi-phasic. The phase change occurs at approxiamtely amino acid 122. This suggests the N-terminal end when compared to the rest of the protein may have evolved to reside in an environment of different charge-distribution.

Alignment of PSY among plants in comparison to bacterial CRTB also allows prediction of the functional domains important to enzyme function, and those that are likely to serve other functions, such as localization. Since bacteria do not have plastid transit sequences, their 5' terminus is instructive. **Figure 6.13** shows a multiple alignment (ClustalW) of plant PSYs, including rice PSY against bacterial CrtB. A bold underscore in **Figure 6.13**, indicates the limit of bacterial homology to the plant PSYs proteins This limit is also at amino acid 122.

Finally, computational means for prediction of chloroplast transit sequence cleavage sites have been developed. I compared the predictions for all the plant PSY proteins using ChloroP v1.1 (see **Materials and methods**). For most, no or very weak predictions were made for the cleavage site (data not shown). Analysis of arabidopsis PSY yielded a strong prediction of a cleavage site within the sequence at amino acid residue 70 and 71 in the sequence VVSSSLV, where the first two serines are 70-71. For rice PSY there is a strong prediction at the limit of the bacterial sequence homology depicted in **Figure 6.13**, and a weak prediction of a transit peptide cleavage site at the point that is homologous to the predicted arabidopsis cleavage site, shown by an arrow in **Figure 6.13** at amino acid

58. Homology to the limit of homology to the bacterial protein which contains strongly conserved functional domains and limit of the change in hydrophobicity coincide. This coincidence may just mark the difference between plant and bacterial proteins, and does not imply a cleavage site, but rather a potential region that could contain a transit peptide. ChloroP seeks cleavage sites within the putative transit peptide region based on experience with many proteins. Therefore, I will assume the cleavage site prediction is best, although it remains merely a conjecture.

6.4.9 Predicted Molecular Mass

Taking the predicted peptide cleavage site as the one that is homologous to the site in arabidopsis, the transit peptide is 58 amino acids, and the mature protein is 346 amino acids. The calculated molecular mass (MM) of the pre-protein is thus 44716 Daltons with a isoeletric focus point (pI) of 8.02. The MM of the mature protein is 38, 844 Daltons with pI of 8.81. This size prediction is consistent with previous immunodetection results of rice PSY antigens as being 38 kDa in apparent mass (Yu, 1999).

6.4.10 Hypotheses of the evolutionary relationships of rice PSY and maize PSY1 proteins

In order to look for features of protein primary structure that could have possible informative value about the relationship of rice PSY to other members of the gene family, especially that of maize PSY, I performed a number of alignments and examined

dendrograms for a variety of related proteins and protein domains. Selected proteins with homology are listed in **Table 6.3**, with Prosite accession numbers.

An alignment that encompasses the whole range of homologies is given in **Figure 6.14.** Homology blocks that have been identified by comparisons of sequence databases are enumerated in **6.2.5 Protein Homologies**. These blocks are readily apparent in **Figure 6.14** and have been mapped onto the plant PSY protein comparison in **Figure 6.13.** The alignment in **Figure 6.14** and a dendrogram of the alignment in **Figure 6.15**, demonstrate that as expected, the plant PSYs are more related to each and to the algal PSY proteins than to CRTBs or SQSs. Interestingly the position of the Archea, *T. thermophilus*, is also as expected.

The dendrogram of the alignment of just the plant PSY proteinss , as in **Figure 6.15**, does not give the expected sister relationship of rice PSY to maize PSY, but groups rice PSY with that of carrot, while maize is sister, as expected, to the monocotyledonous daffodil. Furthermore, tomato PSY1 and PSY2 are more closely related than to each other and bell peper than they are to rice PSY. That is, rice PSY does not have the same relationship that tomato PSY1 and PSY 2 share with maize PSY1. Removal of the transit peptides and comparison of various homology blocks gave a similar gene tree (data not shown).

Microbial phytoene synthase	Farnesyl diphosphate farnesyltransferase (Squalene synthase)	Plant phytoene synthase
CRTB_THETH *Thermus thermophilus* (P37270)	FDFT_MOUSE *Mus musculus* (P53798)	PSY_ARATH *Arabidopsis thaliana* (P37271)
CRTB_ERWUR *Pantoea ananas* (P21683)	FDFT_HUMAN *Homo sapiens* (P37268)	PSY_CAPAN *Capsicum annuum* bell pepper (P37272)
CRTY_SPIPL *Spirulina platensis* (Q07333)	FDFT_RAT *Rattus norvegicus* (Q02769)	PSY_CITUN *Citrus unshiu* Satsuma orange (AAF33237)
CRTB_MYCTU *Mycobacterium tuberculosis* (Q50728)	FDFT_CANAL *Candida albicans* (P78589)	PSY_CUCME *Cucumis melo* musk melon (P49293)
CRTB_STRGR *Streptomyces griseus* (P54977)	FDFT_SCHPO *Schizosaccharomyces pombe* (P36596)	PSY_DAUCA *Daucus carota* carrot (Q9SSU8)
CRTB_SYNY3 *Synechocystis* PCC6803 (P37294)	FDFT_YEAST *Saccharomyces cerevisiae* (P29704)	PSY_HELAN *Helianthus annuus* sunflower (CAC19567)
CRTB_RHOCA *Rhodobacter capsulatus* (P17056)	FDFT_USTMA *Ustilago maydis* (Q92459)	PSY_MAIZE *Zea mays* maize (P49085)
CRTB_RHOSH *Rhodobacter sphaeroides* (P54905)	FDFT_ARATH *Arabidopsis thaliana* Mouse-ear cress (P53799)	PSY_NARPS *Narcissus pseudonarcissus* daffodil (P53797)
CRTB_SYNP7 *Synechococcus sp.* PCC 7942 (P37269)	FDFT_NICBE *Nicotiana benthamiana* (P53800)	PSY1_GENLU *Gentiana lutea* great yellow gentian (E15680)
CRTB_AGRAU *Agrobacterium aurantiacum* (P54975)	FPP_RICE *Oryzae sativa* rice BF430519	PSY1_LYCES *Lycopersicon esculentum* tomato (P08196)
PSY_NEUCR *Neurospora crassa* (P37295)	FPPS_MAIZE *Zea mays* maize P49353	PSY_TAGER *Tagetes erecta* marigold AF251015
CRTB_ERWHE *Erwinia herbicola* (P22872)		
Y4AC_RHISN *Rhizobium sp.* NGR234 (P55350)		

Table 6.3 Selected Prosite or Genbank accessions with homology to rice PSY. Accession number in parentheses.

6.4.11 Two protein primary-strucure homology groups of rice *Psy* ESTs

Alignments and contig analysis with VectorNTI subprograms indicated that rice EST sequences from the database and the complete sequence of a hypothetical cDNA deduced from the sequence of the rice *Psy* gene fell into two homology groups. The gene sequenced in this study matches the cDNA sequenced in this study as well as other ESTs in the database (Group I), but another group (2) or ESTs did not match the gene sequenced here.This was also true at the level of deduced amino acid primary structure for the available incomplete sequences. The ESTs and the groups are listed in **Table 6.2**. EST AU082986 (Group 1) possess a 3' end that has no significant similarity to the rice *Psy*, that also was significantly longer (100nt) than that of the other EST group. EST D48251 (Group 2) matched the genomic sequence reported here, as predicted. This EST also showed significant homology, at the 5' end of the available sequence that matched the conserved carboxy-terminus of all plant PSYs. Adding the deduced amino acid sequence of this Group 1 EST, which corresponds to Prodom PD009861, to the alignment of selected PSYs, demonstrates that this Group of PSY is the sister group of maize PSY1 and the gene sequenced is an outlier. This hypothesis is presented along with aligned domain block in **Figure 6.16**.

6.4.12 Mapping of rice *Psy* to chromosome and rice maize synteny relationships at rice Psy and maize *Y1* loci

EST D48697 (Genbank AY024350) was mapped by recombinant inbred mapping by Susan McCouch of Cornell University. Rice *Psy* mapped to rice chromosome 12 near marker CDO344, which in maize corresponds to chromosome 3S in an area that is not well conserved in rice (Ahn, et al., 1993). Maize Y1 is on maize chromosome 6 in an area that is syntenic with rice chromosome 6. Tomato Psy2 maps maize chromosome 8. (MaizeDB).

6.5 Discussion

PSY is the first enzyme in an important biochemical pathway that produces essential carotenoids with a myriad of biological roles. PSY is likely a rate-controlling step for entry of GGPP in to the pathway. PSY also exhibits regulated modulation at both the transcriptional and translation levels. As part of a biosynthetic complex, its temporal expression, localization, and processing may be intricate and critical. Different isoforms of PSY may occur in different tissues and be differentially regulated as has been shown for tomato and pepper fruits. Additionally, much variation in induction and steady state levels has been observed for *Psy* transcripts among the various tissue and plastid types of pepper (Hugueney et al., 1996), daffodil (Schledz et al., 1996), tomato (Bartley and Scolnik, 1993) and melon (Karvouni et al., 1995b).

Having a specific gene allows one to probe its function. Giving the close relationship among rice and maize among the other cereal grasses, the understanding of one

predisposes comparative understanding of the other. For example, rice *Psy* may be important for understanding the a potential duplicate PSY in maize.

While the rice *Psy* gene described in detail here shows much structural homology to maize *Y1*, at the level of gene structure, it significantly differs in coding regions for highly conserved protein functional domain at the C-terminus. This domain shared more similarity to the carrot protein, for example, than do the maize protein. Because there is a class of rice EST cDNAs that show greater homology to the maize PSY than does the gene I sequenced, it may be that there is a second *Psy* gene in rice, that is the ortholog of *Y1*. Differential exon splicing is a possible but less likely phenomenon. A careful cladistic analysis of the PSY proteins deduced from these Group 1 (*Y1* homologous group) ESTs and mapping to chromosome is merited when these clones have been procured and sequenced.

The promoter of rice Psy contains some potentially very interesting elements. I present no data that these features have any functional significance. One element is a GC-rich island in the promoter region. Another is a transcription-factor binding-site for an activator of pigment biosynthesis, maize activator P. This binding site is in a putative TAATA box CAAT box region. Further work, such as transcriptional start site mapping and additional sequencing upstream of the coding region are needed. The GC-rich island has impeded sequencing efforts. Techniques to overcome attenuation of sequencing reactions should be applied.

Most importantly, analysis of the maize-rice synteny relationships and identification of two homology groups among rice ESTs add circumstantial evidence to the hypothesis that the presented rice *Psy* is novel and is not the ortholog of Y1.

6.6 Acknowlegdements

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```
   1  TGGAGGTGGACCCAACGCGTCCGACCCCAACCATTCGACCCGCGCAGCGAGCCCCAACCC
  61  CACATCGCCGCGCGCCCCCCGATGGCGTCCTCCTCGTCGGCGGCGGCGCTCTGGACGGCGG
                  K  T  F  Y  L  G  T  Q  L  M  T  P  E  R  R  K
 121  CGCCCCACCCCCAAGACCTTCTACCTAGGTACTCAGCTTATGACTCCTGAAAGGCGCAAA
       A  V  W  A  I  Y  V  W  C  R  R  T  D  E  L  V  D  G  P  N
 181  GCTGTCTGGGCAATCTATGTATGGTGCAGAAGAACTGATGAACTGGTAGATGGCCCTAAC
       S  S  Y  I  T  P  K  A  L  D  R  W  E  K  R  L  E  D  L  F
 241  TCGTCTTACATTACACCAAAGGCACTTGATCGATGGGAGAAGAGATTAGAAGATCTCTTC
       E  G  R  P  Y  D  M  Y  D  A  A  L  S  D  T  V  S  K  F  P
 301  GAAGGCAGGCCATATGATATGTATGATGCAGCCCTCTCGGACACAGTGTCAAAGTTTCCA
       V  D  I  Q  P  F  K  D  M  I  E  G  M  R  L  D  L  W  K  S
 361  GTAGATATCCAGCCATTCAAAGACATGATTGAAGGAATGAGGCTTGACCTGTGGAAATCA
       R  Y  R  S  F  D  E  L  Y  L  Y  C  Y  Y  V  A  G  T  V  G
 421  AGGTATAGGAGCTTTGATGAGCTCTACCTCTACTGCTACTACGTTGCTGGCACGGTTGGT
       L  M  T  V  P  V  M  G  I  A  P  D  S  K  A  S  T  E  S  V
 481  CTCATGACAGTACCGGTGATGGGGATTGCCCCCGACTCGAAGGCCTCAACCGAGAGCGTG
       Y  N  A  A  L  A  L  G  I  A  N  Q  L  T  N  I  L  R  D  V
 541  TACAACGCTGCGCTAGCTCTTGGGATCGCCAACCAGCTGACGAATATTCTCAGAGACGTA
       G  E  D  S  R  R  G  R  I  Y  L  P  L  D  E  L  A  E  A  G
 601  GGCGAAGACTCAAGGAGGGGAAGAATCTACCTTCCATTGGATGAATTGGCAGAGGCAGGT
       L  T  E  E  D  I  F  R  G  K  V  T  D  K  W  R  K  F  M  K
 661  CTGACAGAAGAAGACATATTCAGAGGGAAAGTGACTGATAAATGGAGGAAGTTCATGAAG
       G  Q  I  L  R  A  R  L  F  F  D  E  A  E  K  G  V  A  H  L
 721  GGACAAATTCTGCGTGCCAGGTTATTCTTTGATGAGGCGGAGAAGGGCGTTGCGCATCTA
       D  S  A  S  R  W  P  V  L  A  S  L  W  L  Y  R  Q  I  L  D
 781  GACTCTGCGAGTAGATGGCCGGTTCTGGCATCTTTGTGGTTATACCGGCAGATCCTTGAT
       A  I  E  A  N  D  Y  N  N  F  T  K  R  A  Y  V  N  K  A  K
 841  GCTATCGAAGCAAACGACTACAACAACTTCACCAAGCGCGCGTATGTAAACAAGGCAAAG
       K  L  L  S  L  P  V  A  Y  A  R  A  A  V  A
 901  AAGCTGCTGTCTTTACCGGTCGCTTATGCAAGAGCGGCAGTTGCATCATGAACAATCACT
 961  AGATCAGATGCCTTATTATTTTTTTCTTTCATTTTCTTTTCTTTTGATTTCGCACGATT
1021  TCTTGGCTGTTGTATATATTCAAGCAGCTACCTGTATGCCATAAGCCTGCCACAGTTTTT
1081  CTTTAGTTCAAGGGACTGATTTCAGGTCCCTCAATACTCAACTCTTGTTAGAAACAAATA
1141  CAGAGGGGGTAAGCCCCACAGTTCAAGAAGCATATTAAAAAAAAAAAAAAAA
```

Figure 6.1 Sequence and translation of partial cDNA from EST D48251 deposited as Genbank AY024350) The translation shows the beginning limit of homology to other PSY proteins.

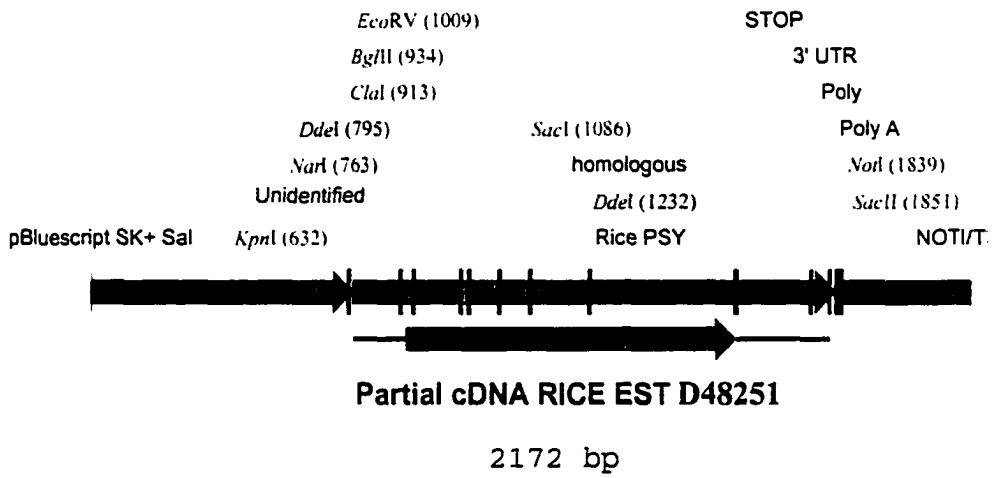

Figure 6.2 Feature map of partial cDNA of Rice EST D48251 (Genbank AY024350) Upper line drawing shows cDNA inserted in pBluescript SK+, lower shows PSY homologous coding sequence (CDS) to the putative stop codon (TGA)

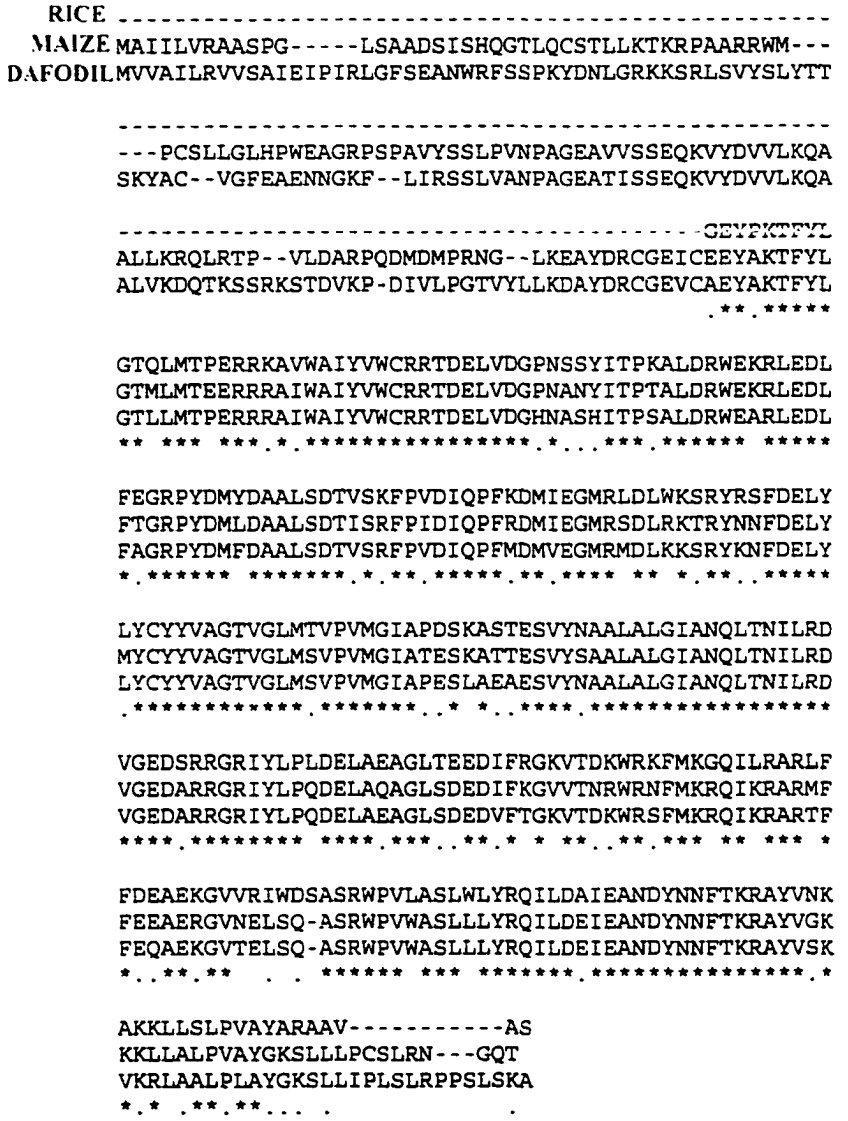

Figure 6.3 An alignment of deduced amino acid sequence from EST D48251 against maize PSY (Swissprot: P49085) and daffodil PSY (Swissprot: P53797) shows that the rice cDNA CDS is truncated at the 5' end.

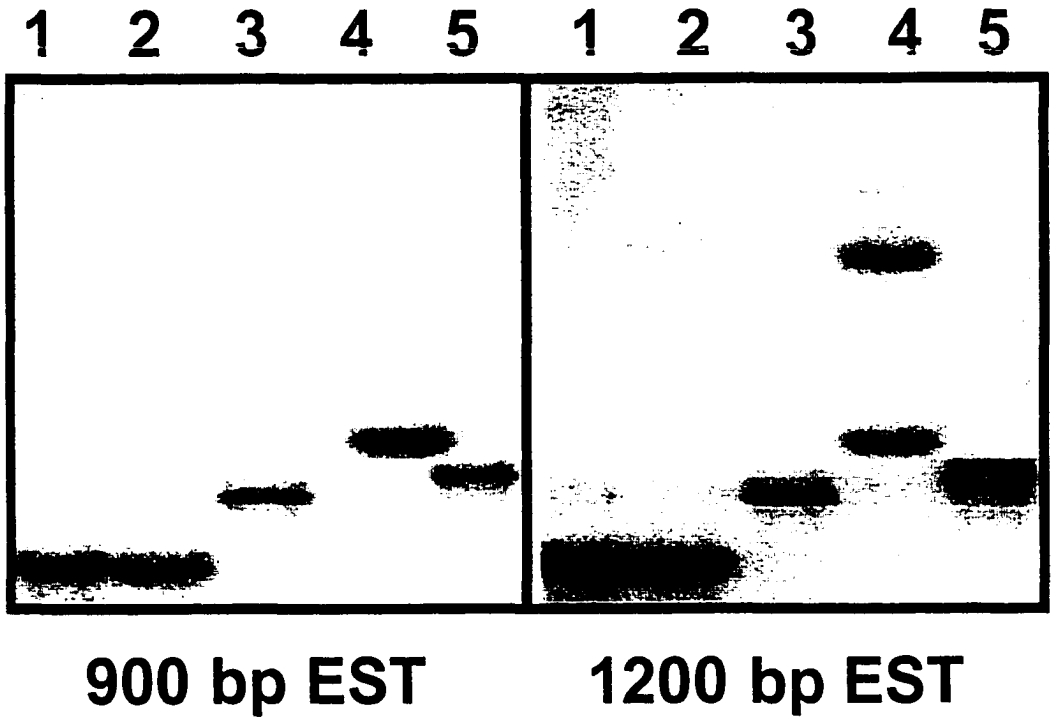

Figure 6.4 Genomic Southern Blots. Two partial rice PSY homologous cDNAs, one 940bp (D48697) and one 1229 bp (D48251, AY024350), exhibit the same pattern when hybridized to IR36 rice genomic DNA fragmented with five different restriction enzymes, labeled Lanes1-5.

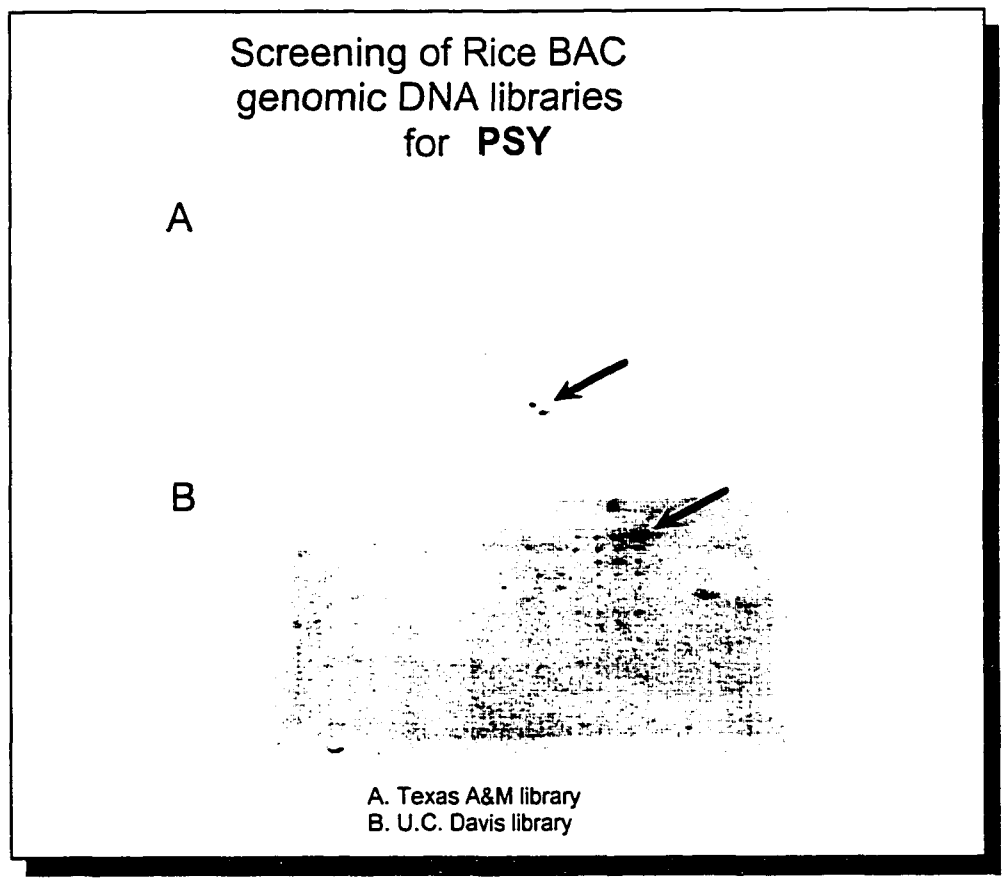

Figure 6.5 BAC clones selected by hybridization of mini-arrays to rice PSY EST D48251. Experiment and figure by Dr. Vivek Upasani.

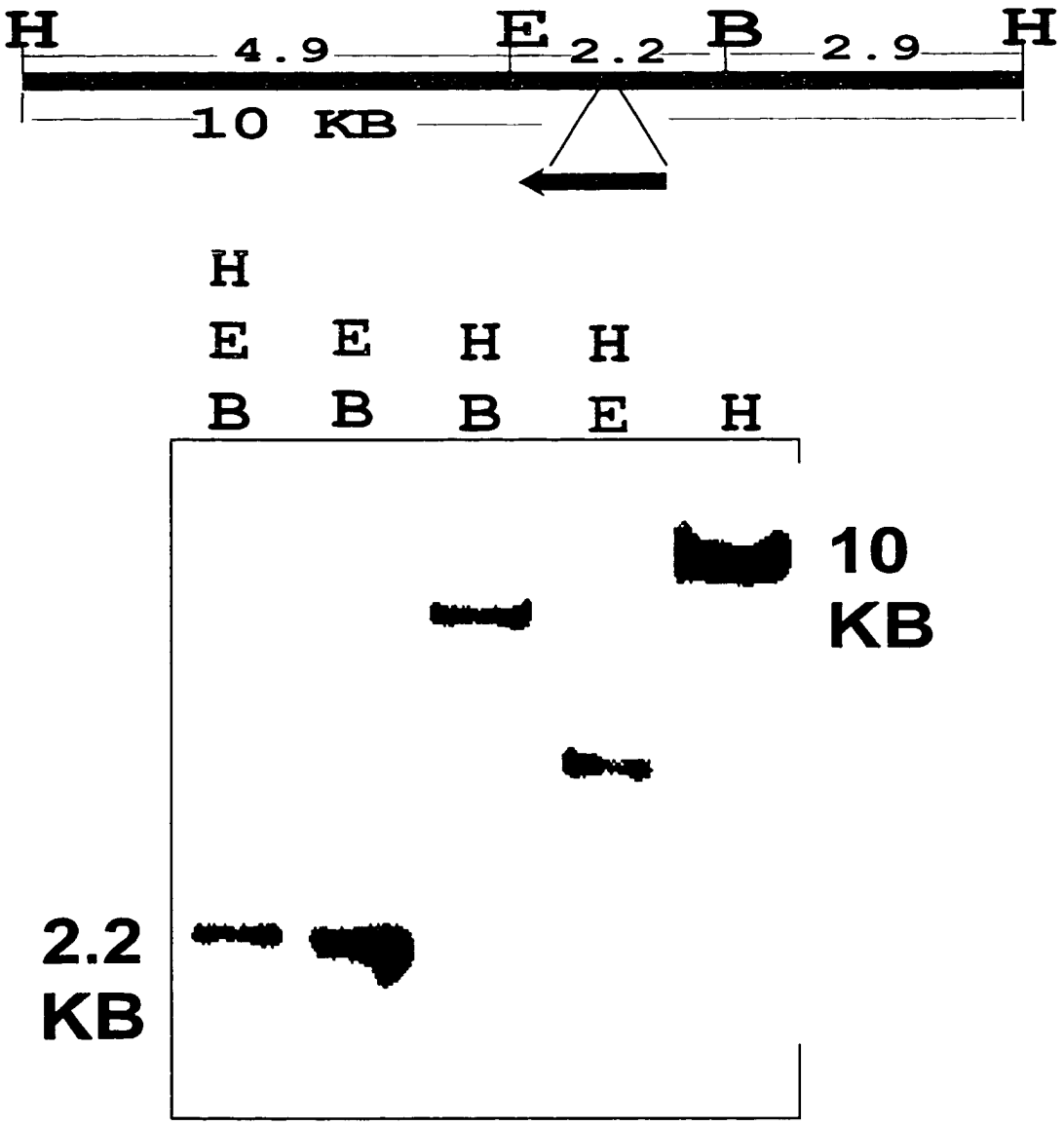

Figure 6.6 Restriction mapping of a approximately 10 kilobase cloned subfragment (pVIVYH5) of UCD BAC clone Number 2.H , *Hin*dIII, E, *Eco*RI, B, *Bam*HI, Kb, kilobase pairs. The arrow indicates possible position of cDNA fragment (EST D48251, AY024351) used as probe.

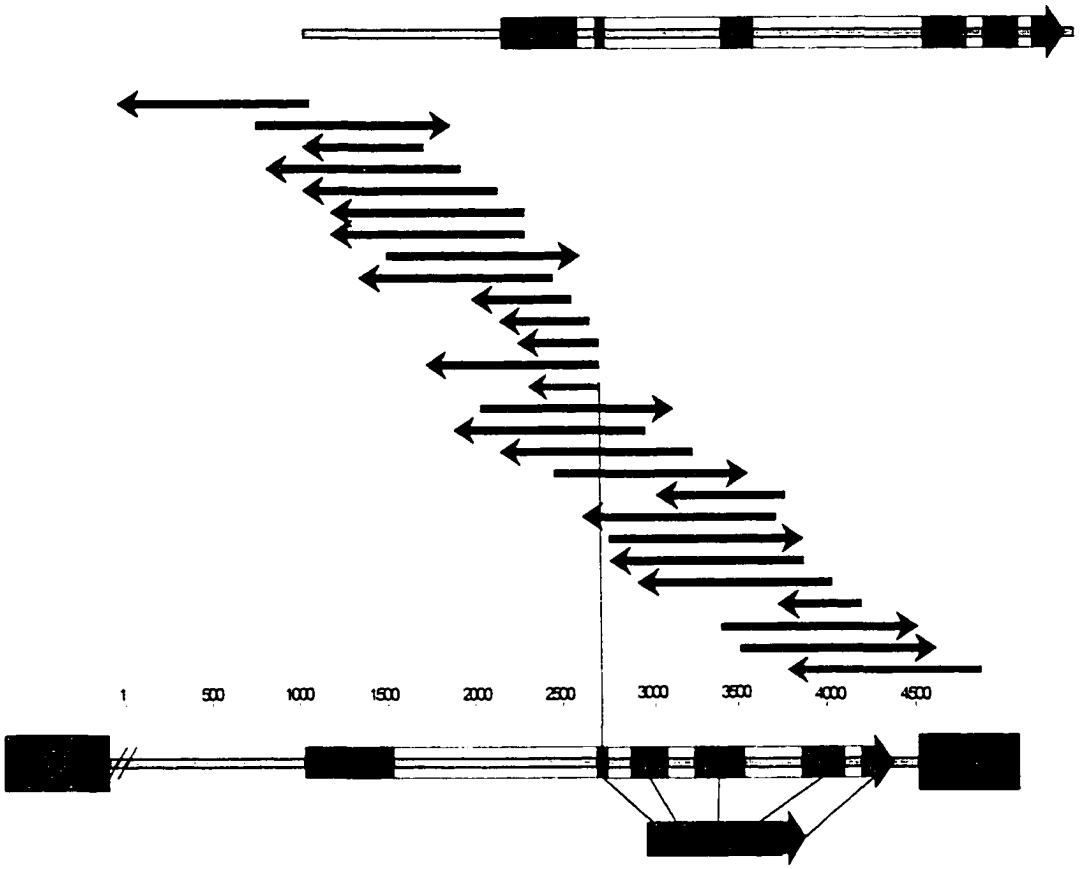

Figure 6.7 Sequencing strategy of a 6kb ClaI subfragment of clone pVIVH3, pRCPSYC6.0 (Genbank AY024351). Top line drawing is exon/intron structure of maize PSY gene (Genbank: ZMU32636) for comparison to deduce structure of the rice PSY gene (Genbank AY024351, this study). T3 and T7 indicate promoter sequences in pBluescript SK- for insert orientation. Shaded arrows indicate starting sequencing runs of primer walking. Solid arrows indicate sequencing runs. The direction of the arrow indicates which strand was sequenced.

```
   1  AANAAAAAGANATNATTNAAAAGGGAAATGGGTNATTAATAAAGGGGAAGAAATTTTANGAAGGGG
  67  AGAAATAGTTNGGGGGGGGAGGGTTTANTNTNNTTTAANGAAANAGGNAAANGGGGGTTNNATANA
 133  ATTTAATAATTTTNGNAAGGTAAAAAAGGCCGNGAAAAAAAAGGATNACAAAAATTNGGGGGGTTN
 199  ATATNNGTTGNCAAAAAAAAAAAAANGAAGCCNAATAAATANTCCCCTAAAANAGAATANTTTTTT
 265  TANGAAGGGATGGNGGNANTTTTTNGTGGGTTAAAACAGGNANNNATCNNNAGANNGAAANTAAAA
 331  GAAANGAGGNANTANAAGAAGTTTAACCAAGGGGGGGGNNANTGNAAANAAAGGGAAAANAAANAT
 397  GANTNGNTATTGGNNGGNTGGTTNAAAGAAAAGAGGGGNAGTTTGGNTTCAATGAGAAAAATAAAT
 463  NAGNANAAAAGGNGNGAAAAGANNNTNTTTTANAGTTAAATATTAGTTATACAATTAGAAATTGAC
 529  GAGAATNTTTTGTGGGNGGTNGANAAAATATTCAAAGAAGATACATTGAANGCCNAAAAAACCNCG
 595  CNACAGGNTTTTTTTTTAAGNNAAAAGGGNANNANNTNAGGTNCACTNAAAAAATNAAATTNTTTGT
 661  TAAAAAAAAAAAAAAAAGGGGGGNAAGNCNCAAAAGTTNAAAAAGGNATAATACTNTTTATTTTGTT
 727  TTCAANAAANTNAAGGTTGAAATAGAAATTNCTGNANNNNGGNGGGATCCAATAGTTANAGAAAGG
 793  NCGCCANNGNGGNGGAAGTNCAANTTTTGTTCCANTTANTGAAGGGNNAAAT GGGGGCCCCCCCCC
 859  CCCCCAGAAAAATTAATTAAAATTAATTGGGGGTAACCAATTTAAACCCACCCTCCTTTCAAAAGA
                          M  T  G  E  G  S  P  N  Q  N  C  R  G  A  P  G ·
 925  AAACAAACCCCAACCAATGACCGGGGAAGGAAGCCCAAACCAAAATTGCCGGGGGGCCCCAAGGGG
      · L  L  A  G  G  F  E  G  G  P  P  P  P  R  Q  V  E  Q  D  S  H ·
 991  TCTCTTGGCGGGGGGTTTTGAAGGTGGGCCCCCACCCCCACGGCAAGTGGAACAGGATTCAAGCCA
      · F  S  Q  G  Y  N  E  G  E  E  A  G  C  C  R  L  V  G  E  A  A ·
1057  TTTTTCACAAGGTTACAACGAAGGGGAAGAAGCCGGTTGTTGTCGCCTCGTCGGTGAGGCCGCTGC
      · G  R  E  P  G  G  G  H  G  A  G  G  R  G  V  E  E  D  G  G  E ·
1123  AGGCCGCGAGCCTGGCGGTGGCCACGGCGCCGGTGGCCGTGGCGTCGAGGAGGACGGCGGCGGAGA
      · A  S  R  G  R  L  G  T  R  C  G  R  P  H  R  A  A  R  G  R  G ·
1189  GGCGTCTAGAGGTCGGCTCGGCACGCGCTGTGGAAGGCCCCACCGCGCGGCGCGGGGGCGCCGCGG
      · G  R  G  G  R  V  D  W  G  L  L  L  G  D  A  Y  H  R  C  G  V ·
1255  TGGGCGAGGAGGACGCGTCGACTGGGGCCTCCTCCTCGGCGACGCCTACCACCGCTGCGGCGAGGT
      · C  A  E  Y  A  K  T  F  Y  L
1321  CTGCGCCGAGTACGCCAAGACCTTCTACCTAGGTCAGCCAACCCAACATTGACTAATCATTCAATT
1387  TTGTTTTTATCACCGTTATTACTTTATCATTTCACAGGTGAAAGCTAGCTACTATTCGTTTGCCAC
1453  TACTACTCCCTAGCTTAGCTAGCGTCTCTTCCTTTTCTTGATTTGTTCATAGTACTGTATATGATA
1519  ATTGTAGACCTACCCTTTCTCTATTTCTTTTTGGGTTCTTGGGTGGAATGGCTGGCTTGATAAAAT
1585  TTTGCGACCCTTTTTGTGGTTCTAGGAGCCACATCTCACTTTCACTTTCTTGTACTTGTTGGTCTA
1651  AAAAGATTAGTTAATGCTGCAATTAGTTGGCTTTTACTAGGAATTGTAAGAGGGTGGTGTTAAAAG
1717  GTGATTAAAAAAAAATCTATGAACATTCCTAGATTCCTATTACGTGTTAAGAAGATAAATATTCCT
1783  GCGTATAATTTATGGAATACTCCTTCTGATGGCAAAAATATATTAGCAGTAGCCAAACAACACCAG
1849  CCTGAGTGAGTGAGTGGCTCTCAGTGCTCTGAACAACTGTACATGTATATACAAATGTTTGCCGAC
1915  TTCAGAGTAGGTAAGATAATGCACTTGGATGATAGATGCCATCCCAAGGAAATGATCCAACAAGCA
1981  GATGCGCAGATATGGTGATCTCTCTAGTGCACCACTGGCAATTTATAGATTTCTCTTCTTAATTAG
2047  GGTCAGGGATATGCTGGTTGGTAGTGGGTTTGTGGGTGATGTGAGGACGCATGGGCTTGATTGATG
2113  AATCCAGTAACCTTCTTGTTTTTTTTAATCTTGCTTTCTGTATCACCGTGTTTCCACTTGGATCAT
2179  CCCTGACCTGGTAAGGGACTCATTTTCTATTGCTACGAGGACCTTTTGGGGACTAGCTCAAACTCT
2245  GATGTATGGTTGATACTTAGATTTTCTACGTAAAGTGGAATTTCACCTTGTGTAATTTCTCATGGT
                                T  Q  L  M  T  P  E  R  R  K  A  W ·
2311  TTTTGTTTCCATCGTTTACATTTCAGGTACTCAGCTTATGACTCCTGAAAGGCGCAAAGCTGTCTG
      · A  I
2377  GGCAATCTATGGTATGGATTAGTTAACTATTCATTGACCTACCAGTTACTCTCACTTGTGAAAATT
                                                                  V  C ·
2443  CAGATAACTTCAATTGTTTGTGGAATTTTTAATTCTTACCTTTGTTTTTGTCTTTGGCAGTATGGT
      · R  R  T  D  E  L  V  D  G  P  N  S  S  Y  I  T  P  K  A  L  R ·
2509  GCAGAAGAACTGATGAACTGGTAGATGGCCCTAACTCGTCTTACATTACACCAAAGGCACTTGATC
      · W  E  K  R  L  E  D  L  F  E  G  R  P  Y  D  M  Y  D  A  A  S ·
2575  GATGGGAGAAGAGATTAGAAGATCTCTTCGAAGGCAGGCCATATGATATGTATGATGCAGCCCTCT
      · D  T  V  S  K  F  P  V  D  I
2641  CGGACACAGTGTCAAAGTTTCCAGTAGATATCCAGGTACAGCCAACAAAAAAGCTACTGTAAAAGC
2707  AGGATATGAAGTGCTTTTAAAAGTTATTAGTACACAGTAAATTGGTGACACGCATTTGTCTGATGT
                                      P  F  K  D  M  I  E  G  M  R  D ·
2773  TTCTGCACCGTCGGGATCTCTTTTATTTACAGCCATTCAAAGACATGATTGAAGGAATGAGGCTTG
      · L  W  K  S  R  Y  R  S  F  D  E  L  Y  L  Y  C  Y  Y  V  A  T ·
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2839    ACCTGTGGAAATCAAGGTATAGGAGCTTTGATGAGCTCTACCTCTACTGCTACTACGTTGCTGGCA
        · V G L M T V P V M G I A P D S K A S T E V ·
2905    CGGTTGGTCTCATGACAGTACCGGTGATGGGGATTGCCCCCGACTCGAAGGCCTCAACCGAGAGCG
        · Y N A A L A L G I A N Q L T N I L R D V E ·
2971    TGTACAACGCTGCGCTAGCTCTTGGGATCGCCAACCAGCTGACGAATATTCTCAGAGACGTAGGCG
        · E
3037    AAGAGTAAGTACCAATGTGCTCACTACCAGCATGTTCTTTTTCTGAGTTAATAATTGCAGACTTTC
3103    ACATGATAAATATGTAGCTTGACACCCAATATGTATTGCACAAACCATTTGCGCTTTAAAGTCTAG
3169    TCCAAAATTGTGCCTAAACAGATGCCTTTCACATTAAAAATTTGAAGCGCACATAAGTTTAATTTA
3235    AGGCATATGTACCGATTTGTCGCTATTGAGATTAAGTTTTTTGGTTGATTGTGATGTAATATGTTT
                              S R R G R I Y L P L D E L A A ·
3301    GTTGGGATGGAATTTCAGCTCAAGGAGGGGAAGAATCTACCTTCCATTGGATGAATTGGCAGAGGC
        · C L T E E D I F R G K V T D K W R K F M G ·
3367    AGGTCTGACAGAAGAAGACATATTCAGAGGGAAAGTGACTGATAAATGGAGGAAGTTCATGAAGGG
        · Q I L R A R L F F D E A E K G V A H L D A ·
3433    ACAAATTCTGCGTGCCAGGTTATTCTTTGATGAGGCGGAGAAGGGCGTTGCGCATCTAGACTCTGC
        · S R W
3499    GAGTAGATGGCCGGTATGAGAGGCTACAAATTCTGCGGTTTTATGTTCCACAAATAAAATAAACCA
                                                V L A S L W L Y R I ·
3565    CTGAAATTTACCTTAATCGATTATTTTCTCGTAGGTTCTGGCATCTTTGTGGTTATACCGGCAGAT
        · L D A I E A N D Y N N F T K R A Y V N K K ·
3631    CCTTGATGCTATCGAAGCAAACGACTACAACAACTTCACCAAGCGCGCGTATGTAAACAAGGCAAA
        · K L L S L P V A Y A R A A V A S *
3697    GAAGCTGCTGTCTTTACCGGTCGCTTATGCAAGAGCGGCAGTTGCATCATGAACAATCACTAGATC
3763    AGATGCCTTATTATTTTTTTTCTTTCATTTTCTTTTCCTTTGATTTCGCACGATTTCTTGGCTGTT
3829    GTATATATTCAAGCAGCTACCTGTATGCCATAAGCCTGCCACAGTTTTTCTTTAGTTCAAGGGACT
3895    GATTTCAGGTCCCTCAATACTCAACTCTTGTTAGAAACAAATACAGAGGGGGTAAGCCCCACAGTT
3961    CAAGAAGC ATATTA CTCTTCATTTGTCTTCAAGAATTCAA
```

Figure 6.8 Nucleotide sequence of the a rice PSY gene (Genbank AY024351) showing deduced amino acid sequence and intron/exon structure. Potential promoter elements are shown: a CAAT box in bold, a TATAA box with underline within which is a binding site for maize activator P in bold underline, a cap signal with dotted underline, and a CG rich island italics, a poly(A) addition site in bold, and the stop codon with an asterisk. A pair of inverted repeats are shown in the first intron with squiggly underlines.

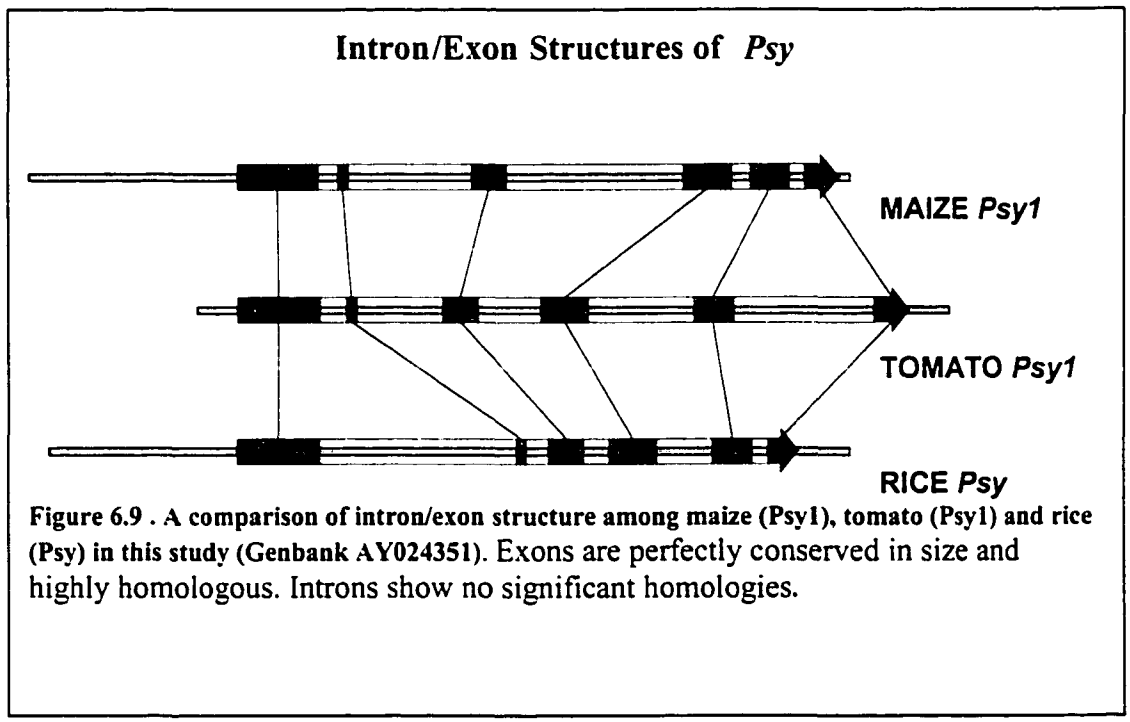

Figure 6.9 . A comparison of intron/exon structure among maize (Psy1), tomato (Psy1) and rice (Psy) in this study (Genbank AY024351). Exons are perfectly conserved in size and highly homologous. Introns show no significant homologies.

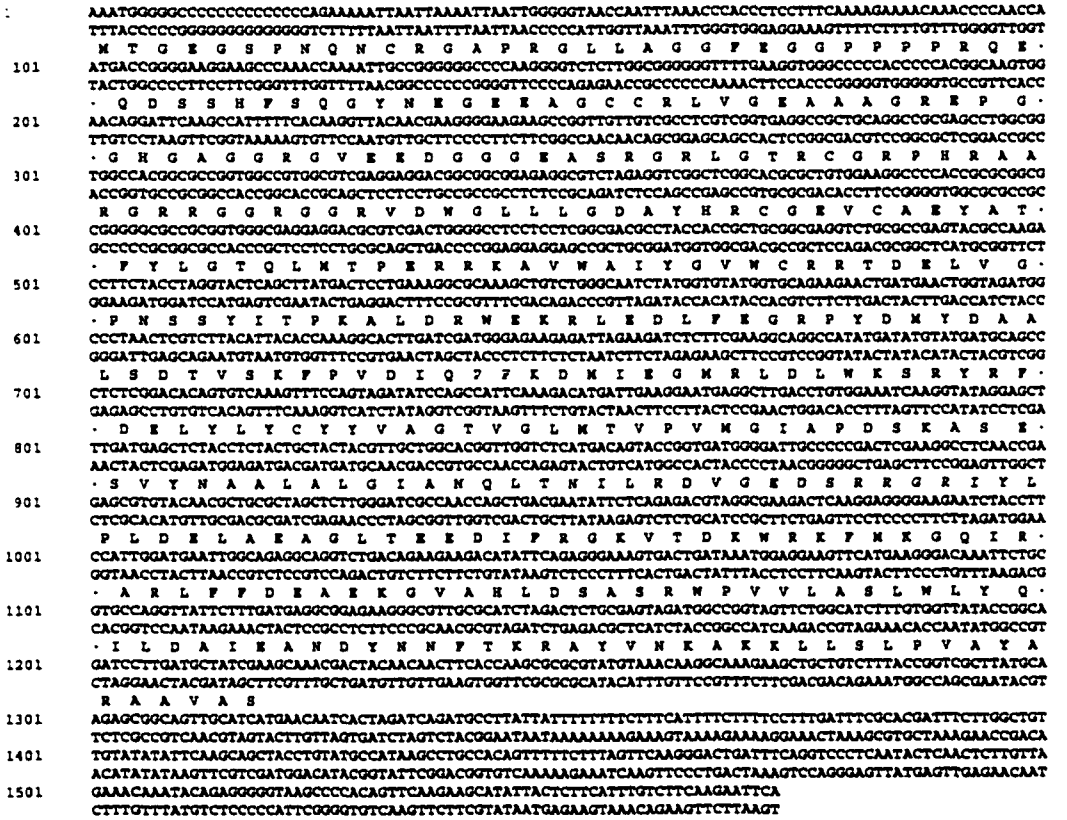

Figure 6.10 Complete cDNA and peptide sequence of rice *Psy* deduced from the gene. Deduced amino acid sequence is shown. The transcription start site has not been mapped.

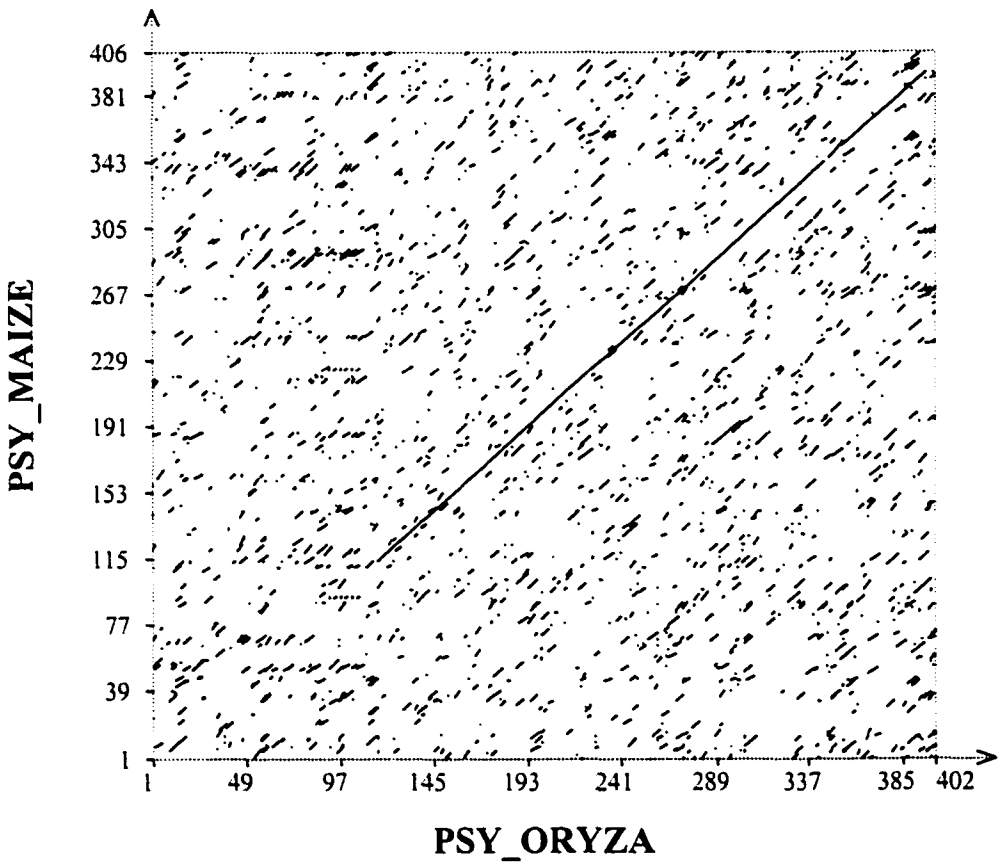

Figure 6.11 Similarity plot of PSY_MAIZE vs PSY_RICE. There is no significant similarity for the N-terminal portion of the proteins. The scales indicate amino acid residue numbered from the first methionine. See **Table 6.3** for Genbank accession numbers of proteins.

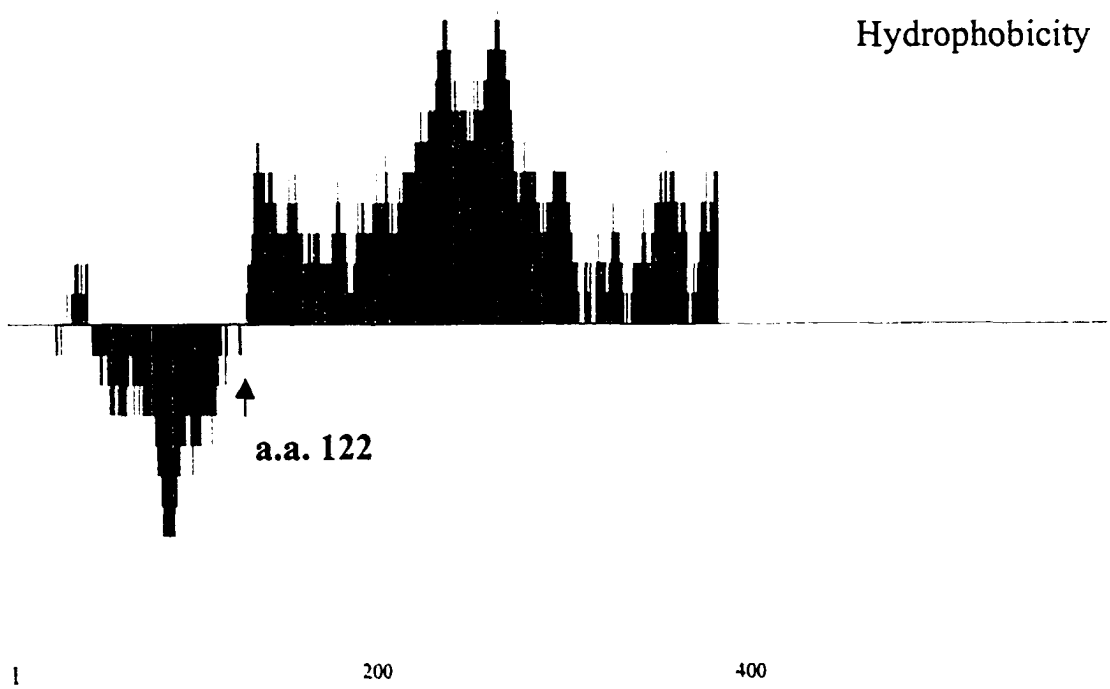

Figure 6.12 Hydrophobicity plot of rice PSY protein shows N-terminus is different from the rest of the protein. The horizontal scale indicates amino acid residue numbered from the first methionine. The scale on the vertical axis is relative hydrophobicity.

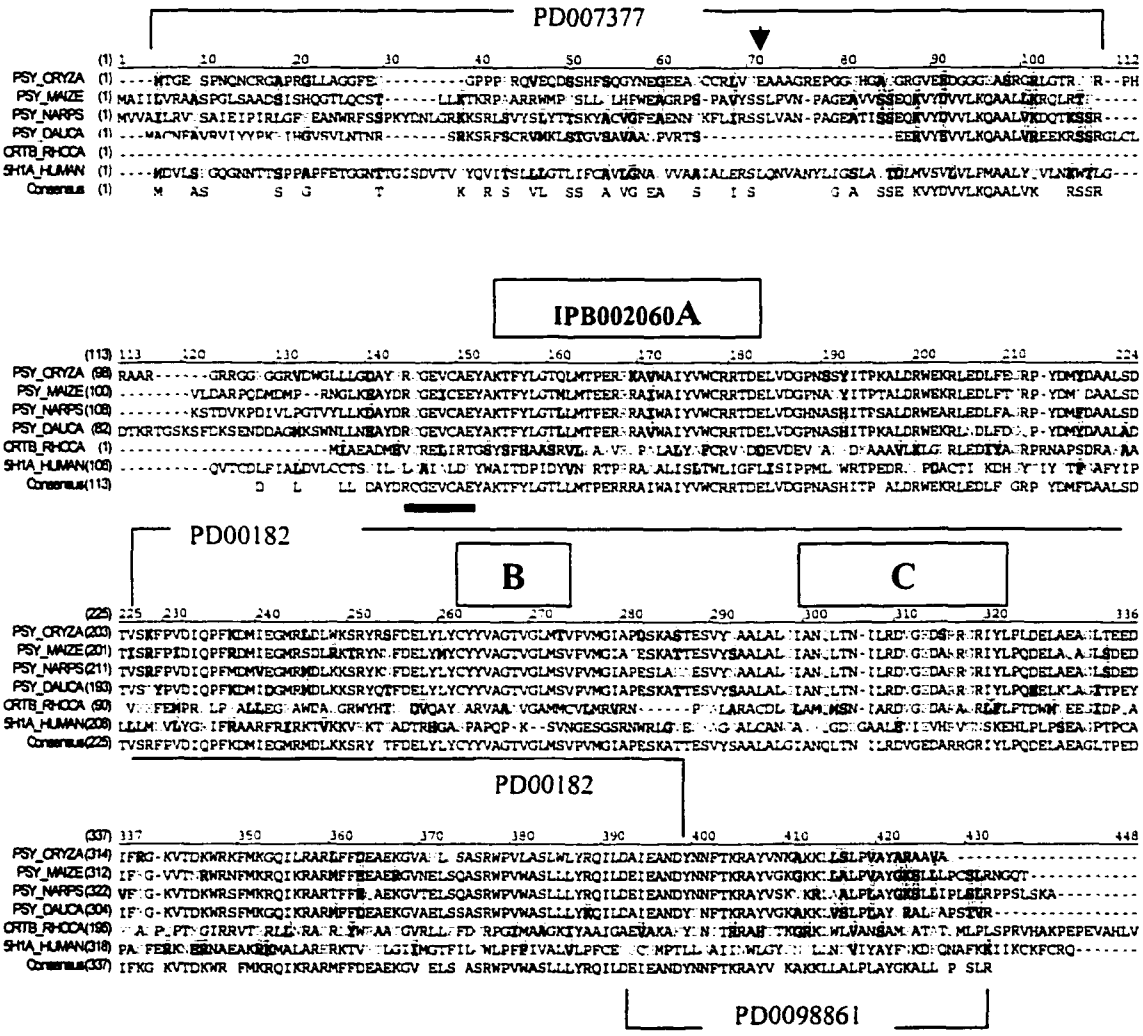

Figure 6.13. Amino acid sequence alignment of rice, maize, daffodil, and carrot PSY against a bacterial CRTB and human squalene synthase. Shaded lines designate identical amino acids, grey blocks are similar, dark grey conserved, and uncolored are non-homologous. The arrow indicates predicted cleavage site, the bold line is under the limit of N-terminal homology to bacterial phytoene synthases, and the boxes with accession numbers are above conserved domains listed in BLOCKS database. Domains in the Prodom database are shown as lines. Putative functional domains are further described in the text. A bold underscore indicates the limit of bacterial homology to the plant PSY proteins.

Figure 6.14 Pylogenetically-broad alignment of rice PSY to various poly-isoprenoid synthases.

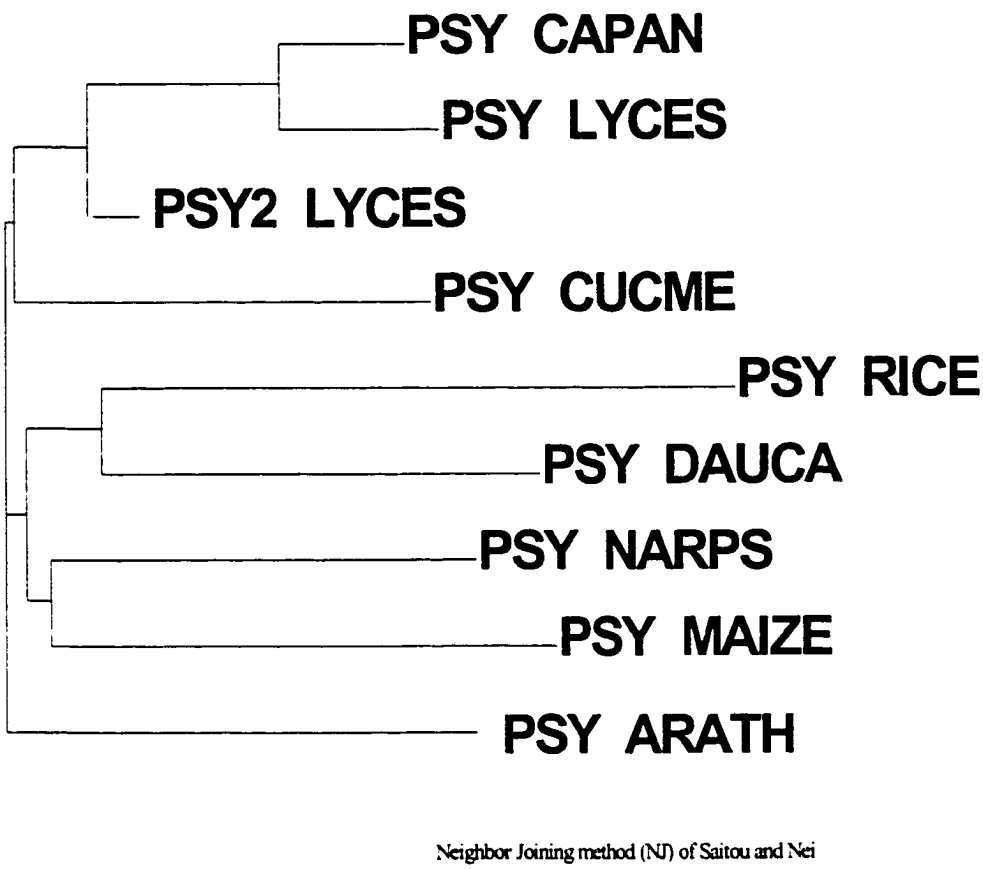

Figure 6.15 Dendrogram of alignment among plant PSY proteins. Length of branches corresponds to phenetic distance.

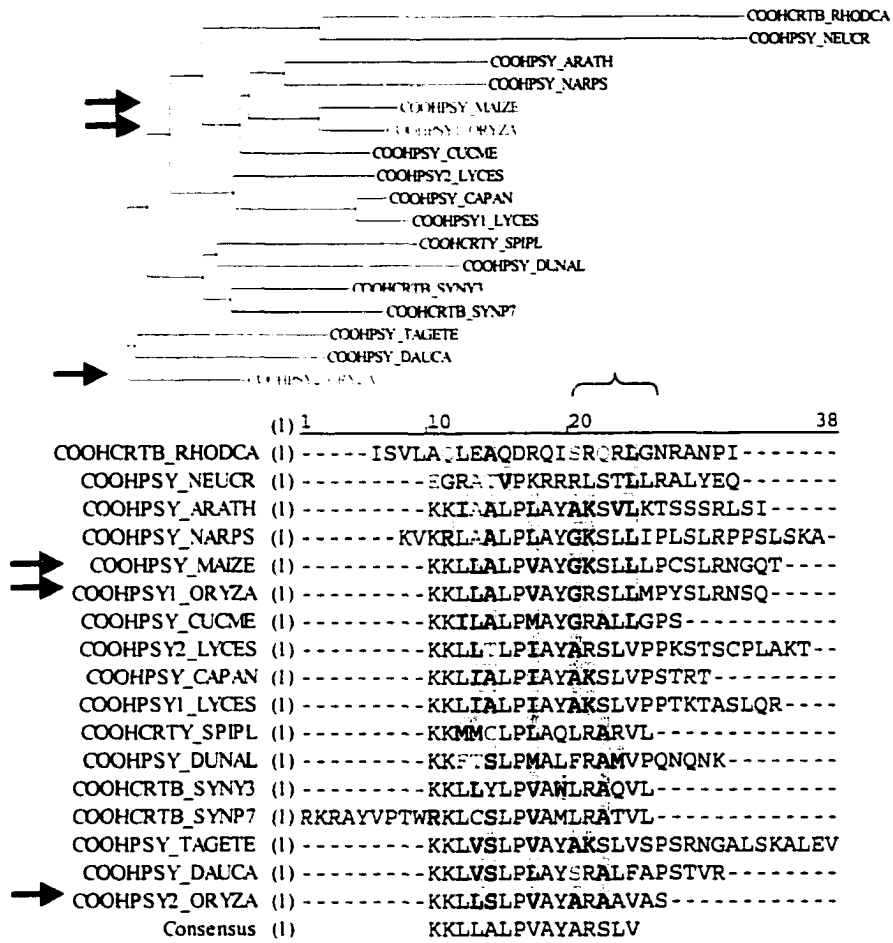

Figure 6.16 Alignment of the carboxy-terminus of selected PSY proteins, including Group 1 and Group 2 rice PSY ESTs. Light grey blocks are similar, gray conserved, and light shaded weakly similar, and uncolored are non-homologous. Arrows show sisterhood of rice Group 1 ESTs with maize PSY1 (Y1 gene product) and rice PSY as an outgroup. Species names are defined in **Table 6.3**.Comparsions are to the C-terminal 25 residues of rice PSY protein.

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Chapter 7 Summary and future perspectives

7.1 Summary of accomplishments

Carotenogenesis has received much attention from various scientific fronts. Recent advances include success with (1) isolation and manipulation of lipohilic enzymes in cell-free systems, for examples see (Bonk et al., 1997; Breitenbach et al., 1999; Schledz et al., 1996) (2) the production of enzyme activities in heterologous functional complementation systems (Bartley et al., 1999; Misawa et al., 1990; Misawa et al., 1994), and (3) genomic approaches to gene isolation and identification of gene products by color complementation screening and functional analyses (Li et al., 1996; Scolnik and Bartley, 1993; Luo, 2000; Matthews and Wurtzel, 2000; Yoganathan, 1998). The bacterial carotenogenic systems have now also become important as models of metabolic engineering (Jones et al., 2000; Matthews and Wurtzel, 2000) and potential factories for new and unusual carotenoids (Sandmann et al., 1999; Schmidt-Dannert et al., 2000; Wang et al., 1999). The advances have led to the bioenginneering of important crop species (Wurtzel, 2001; Ye et al., 2000).

The goal of this dissertation was to improve and expand on these recent developments and apply them to the understanding of carotenogenesis in maize and rice. One long-term goal, to be gleaned from a comparative understanding, is the ability to manipulate carotenoid content and quality among the cereal crops. To these ends, I improved screening procedures and improved the functional complementation system of Misawa. I first found better bacterial strains and then genetically modulated the isoprenoid

precursor pool to affect high-level lycopene or zeaxanthin accumulation in *E. coli*. In doing so, I tested a hypothesis concerning the flow or metabolites from glycolytic intermediates into secondary metabolites in a model system. Such a system may be one necessary component of plants genetically modified for high-level production of many, various isoprenoids. I also applied the functional complementation system to a study of the enzymatic activity of enzymes in the pathway to carotenogenesis in maize. Taking the lead left to me by others in Prof. E.T. Wurtzel's laboratory, I used a pre-existing system (Li et al., 1996; Luo, 2000) to demonstrated that maize PDS and ZDS acting together produce intermediates and products of a poly-*Z*-desaturation pathway to poly-*Z*-lycopene (poly-*cis*-lycopene or prolycopene). This finding opens debate on the occurrence and importance of a potential poly-*Z*-desaturation pathway in maize. Finally, I used available genomic approaches to isolate and characterize a key gene, coding for a likely rate-controlling step in carotenogenesis in rice, *phytoene synthase*. The gene itself now serves as a tool for the further study and perhaps manipulation of cereal carotenogenesis. By examining the sequence of the gene, I was able to present hypotheses of its evolutionary relationships among other plant *Psy* genes and evidence the proposition that the *Psy* gene presented herein is a novel isolate among the grasses and among monocotyledonous plants.

7.2 Future Perspectives

7.2.1 Heterologous complementation systems

Improvements to carotenoid accumulation in the *E. coli* carotenogenic systems may have met their limits in this and other studies (Harker and Hirschberg, 1997; Wang et al., 1999). Different approaches to increase accumulation have yielded similarly high levels of accumulation, on the order of 1000-1500 μg/gFW. This may be an upper limit of the sequestration of the lipopohilic carotenoid products in *E. coli*. Improvements to the system reported in **Chapter 4** could involve expression of the DXS from a low-copy-number plasmid. Such a gene expression platform has recently allowed a lower general "metabolic burden", and provides a two-fold increase in lycopene accumulation over high-copy plasmid platforms (Jones et al., 2000), as I suggested in **Chapter 3** (Wurtzel et al., 1997) and **Chapter 4** (Matthews and Wurtzel, 2000). Integration of a DXS expression cassette into the bacterial chromosome may further enhance the utility of carotenogenic strains by opening capatibility with additional plasmids, for color complementation screening of cDNA libraires, for testing ideas in metabolic engineering, and for molecular breeding systems. The results presented in **Chapter 4** may prove that high levels of pigmentation allow screening of smaller colonies plated at lower density and thus increase the throughput potential for functional genomic screening enterprises.

7.2.2 The question of a poly-*Z*-desaturation pathway posed for maize

The wealth of classical genetics (Robertson, 1975) and burgeoning comparative genomics (Gale and Katrien, 1998) may help resolve questions about the existence of a

poly-Z-desaturation pathway in maize. As discussed in **Chapter 5**, the existence of the pathway is relevant to genetic modification strategies, aimed at modifying the amount of carotenioids and the vitamin A quality of carotenoids produced in maize endosperm. Findings may be extendable to rice and other crops, such as fox millet. Mutant lines that offer promise for assessing a poly-Z-pathway in maize include those viviparous mutants that block desaturation and accumulate intermediates of the desaturation pathway. Such mutations are *vp2,* which accumulates phytoene (Luo, 2000), *vp5* which accumulates phytoene (Li, 1998), and *vp9,* which accumulates ζ-carotene (Luo, 2000), among others. Alternatively, the completed genomic sequences of rice and arabidopsis, along with functional genomics techniques, such as gene traps and insertional mutagenesis, may help identify additional, obligate isomerases associated with carotenogeneis. Because the activity of the desaturases potentially reflect the transfer of information through an intact metabolon, reductionist attempts may fail to resolve the issues. Attention perhaps should be directed to the flow of information within the intact, complex, adaptive metabolon. Subtle probes for function and distribution of carotenoid isomers and carotenogenic enzymes *in situ* may be necessary. Isolation of the genes coding for enzymes of carotenogenesis predisposes the use of transgenic plants to analyze function *in planta*, for example see (Wetzel and Rodermel, 1998). A combination of translational fusions of fluorescent proteins to carotenogenic enzymes, along with perhaps *in situ* detection of carotenoid isomer states with as yet-to-be-developed subtrate analogs incorporating molecular beacons or use of high-resolution spectrophotometry may allow imaging of metablon functioning and programing of information imput.

7.2.3 Rice *phytoene synthase* gene

Isolation of rice PSY may present a valuable tool to further understanding of gene copy and tissue specific expression of PSY in rice and maize. Given the bioengineering interest in maize and rice (Ye et al., 2000; Wurtzel, 2001a), further intense study is merited. The two different protein primary-structure homology groups among rice cDNAs should be investigated in rice and among maize and related grasses, including the landraces of each. The cDNAs represented by EST E20665 (see **Chapter 6** and **Appendix A**) should be sequenced and the amino acid sequence deduced and compared among plant PSY proteins. Such an analysis may shed more light on the phyletic relationships among maize *Y1* and the locus in maize hybridizing to tomato *Psy2* and putative *Psy*-homologous probe, umn111, and the rice gene (AY024351) presented here and that represented by EST E20665. Attempts should be made to develope and hybridize each of these gene-specific probes across the model species of grasses and generate complete chromosomal mapping data among recombinant inbred lines (Burr et al., 1988) or among oat-maize introgression lines and radiation hybrids (Riera-Lizarazu et al., 2000), where duplicate genes of maize have likely been separated from each other.

The promoter sequence of the rice *Psy* is not complete due technical problems with a sequencing read-through of a GC-rich area. Efforts underway to finish this nucleic acid sequence, in addition to transcriptional start-site mapping, will produce more confidence in several potential interesting promoter elements, such as a putative binding site for maize activator P. Having this promoter as well as, perhaps, another, the one orthologous

to the maize *Y1* (Buckner et al., 1996), will allow comparative analysis of the tissue specific expression patterns of each of these genes. This may be accomplished with transgenic plants containing promoter-reporter fusions.

Many of the genes related to carotenogenesis have now been cloned and characterized in maize and rice. These genes will serve as tools not only to genetically modify this process in cereals but also to understand the evolution and natural history of the precious, yellow endosperms of maize.

7.3 References

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Appendix

Plasmids used in this study and laboratory clone records

Name	Genbank	Chapter sections	Genes	Products/ comment	Resistence Marker	Reference
BAC LM 1		6.3.1	Rice *Psy*	Hybridizes to EST D48251	*ChlorR*	(Zhang, et al, 1996)
BAC LM 2		6.3.1	Rice *Psy*	Hybridizes to EST D48251	*ChlorR*	(Zhang, et al, 1996)
BAC UCD 2		6.3.1	Rice *Psy*	Hybridizes to EST D48251	*ChlorR*	(Wang, et al, 1995)
BAC UCD 9		6.3.1	Rice *Psy*	Hybridizes to EST D48251	*ChlorR*	(Wang, et al, 1995)
EST D48251	AY024350	6.3.1	Rice *Psy*	Sequenced ~1.3 kb, not full length	*AmpR*	Genbank/MAFF
EST D48697		6.3.1	Rice *Psy*	Sequenced, ~0.9kb, not full length	*AmpR*	Genbank/MAFF
EST E20665		6.3.1	Rice *Psy*	Different than gene sequenced in this study/ not sequenced	*AmpR*	Genbank/MAFF
pACCAR25ΔcrtX		3.4, 4.4.1	*crtE, crtI, crtB, ΔcrtX, crtY, crtZ*	GGPPS, PSY, PDS, LYC, HYD, zeaxanthin	*ChlorR*	(Misawa et al., 1990)
pACCART-EIB		3.4, 4.4.1, 5.6.1	*crtE, crtI, crtB*	GGPPS, PSY, PDS lycopene	*ChlorR*	(Misawa et al., 1990)
PACCRT-EBP		5.6.1	*crtE, crtB,* maize *Pds*	GGPPS, PSY, maizePDS	*AmpR*	(Hartmut et al., 1919)
pAC-Neurosporene		5.6.1	*crtE, crtI, crtB, crtI*	GGPPS, PSY, CRTI neurosporene	*ChlorR*	(Cunningham et al., 1994)
pBgl 3.4		2.4	Maize *shrunken1*	Sucrose synthase	*AmpR*	(Wurtzel et al., 1987)
PMzzds-107	AF047490	5.6.1	Maize *Zds*	Maize ZDS	*AmpR*	(Luo, 2000)
pRCPSYC6.0	AY024351	6.3.1	Rice *Psy*	Hybridizes to EST D48251	*AmpR*	This study
pTAC-ORF2		4.4.1	dxs	DXS	*AmpR*	(Rohmer et al., 1993)
pVIVYB10		6.3.1	Rice *Psy*	Hybridizes to EST D48251	*AmpR*	This study
pVIVYE3		6.3.1	Rice *Psy*	Hybridizes to EST D48251	*AmpR*	This study
pVIVYH5		6.3.1	Rice *Psy*	Hybridizes to EST D48251	*AmpR*	This study

References for clones

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WURTZEL LAB
CLONE INFORMATION

Date Today: 01.28.01
(MONTH) (DAY) (YEAR)

Clone Number/Name: **BAC LM 1**

Constructed by: vivek, updated by paul
Purified by:
Date constructed:
(MONTH) (DAY) (YEAR)
Storage Location- Box Number: 2 Position: A1, B1

Clone Description: Rice PSY genomic BAC clone, hybridizes to EST D48251 LEMONT(JAPONICA)
Cited in journal: **Zhang HB, Choi s, Woo SS, Li Z, Wing RA.** (1996) Construction and characterization of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. *Mol Breed* **2**, 11-24.

Cloning vector used: Vector size: pBELO BAC 11, 7.5 KB
Organism source of gene: Insert size: 45-260 KB
Restriction enzyme(s) to release insert:
Lab Notebook to reference: PAUL DISSERTATION, VIVEK 1996
Concentration:
E. coli Antibiotic markers: CHLORR
Strain Transformed into: Frozen glycerols, strain box STAB #1 , GLYCEROL #6

Cartoon of construct:

WURTZEL LAB
CLONE INFORMATION

Date Today: 01.28.01
(MONTH)/(DAY)/(YEAR)

Clone Number/Name: **BAC LM 2**

Constructed by: vivek, updated by paul
Purified by:
Date constructed:
(MONTH)/(DAY)/(YEAR)
Storage Location- Box Number: 2 Position: A1, B1

Clone Description: Rice PSY genomic BAC clone, hybridizes to EST D48251 LEMONT(JAPONICA)
Cited in journal: **Zhang HB, Choi s, Woo SS, Li Z, Wing RA.** (1996) Construction and characterization of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. *Mol Breed* **2**, 11-24.

Cloning vector used: Vector size: pBELO BAC 11, 7.5 KB
Organism source of gene: Insert size: 45-260 KB
Restriction enzyme(s) to release insert:
Lab Notebook to reference: PAUL DISSERTATION, VIVEK 1996
Concentration:
E. coli Antibiotic markers: CHLORR
Strain Transformed into: Frozen glycerols, strain box STAB #1 , GLYCEROL #6

Cartoon of construct:

WURTZEL LAB
CLONE INFORMATION

Date Today: 01.28.01
(MONTH) (DAY) (YEAR)

Clone Number/Name: **BAC UCD 2**

Constructed by: VIVEK, UPDATED BY PAUL
Purified by:
Date constructed:
(MONTH) (DAY) (YEAR)
Storage Location- Box Number: 4 Position: D3

Clone Description: RICE PSY GENOMIC, HYBRIDIZES TO EST D48251 Used for sequencing.
Cited in journal: **Wang GL, Holsten TE, Song WY, Wang HP, Ronald PC.** 1995. Construction of a rice bacterial artificial chromosome library and identification of clones linked to the Xa-21 disease Resistence locus. *Plant J* 7, 523-33.

Cloning vector used: Vector size: pBelo BACII, 7.5 kb
Organism source of gene: Insert size: 40-210 kb
Restriction enzyme(s) to release insert: 3222 bp BamHI/EcoRI fragment contain rice psy gene and 1kb promoter
Lab Notebook to reference: vivek 1996, paul thesis
Concentration: 8 ug/ul
E. coli Antibiotic markers: chlorR
Strain Transformed into: Frozen glycerols, strain box 5, positions F9, G-9

Cartoon of construct:

WURTZEL LAB
CLONE INFORMATION

Date Today: 01.28.01
(MONTH)/ (DAY)/ (YEAR)

Clone Number/Name: **BAC UCD 9**

Constructed by: VIVEK, UPDATED BY PAUL
Purified by:
Date constructed:
(MONTH)/ (DAY)/ (YEAR)
Storage Location- Box Number: 4 Position: D2

Clone Description: RICE PSY GENOMIC, HYBRIDIZES TO EST D48251 Not used for sequencing.
Cited in journal: **Wang GL, Holsten TE, Song WY, Wang HP, Ronald PC.** 1995. Construction of a rice bacterial artificial chromosome library and identification of clones linked to the Xa-21 disease Resistence locus. *Plant J* 7, 523-33.

Cloning vector used: Vector size: pBelo BACII, 7.5 kb
Organism source of gene: Insert size: 40-210 kb
Same restriction pattern as UCD 2, not seuqunced
Lab Notebook to reference: vivek 1996, paul thesis
Concentration: 8 ug/ul
E. coli Antibiotic markers: chlorR
Strain Transformed into: Frozen glycerols, strain box 5, positions E8, F8

Cartoon of construct:

WURTZEL LAB
CLONE INFORMATION

Date Today: _____
(MONTH)/(DAY)/(YEAR)

Clone Number/Name: **EST D48251** also MAFF S14375 also now GENBANK **AY024350 also RY1.3**

Constructed by: MAFF
Purified by: updated by paul, arumulee's acquisition
Date constructed:
(MONTH) (DAY) (YEAR)
Storage Location- Box Number: 4 Position: D5

Clone Description: partial rice Psy cDNA, complete sequence Genbank accession AY024350
Cited in journal:paul's thesis

Cloning vector used: SK+ Vector size: 2.9 kb
Organism source of gene: Rice, Nipponbare, 8 da shoot Insert size: 1260 bp
Restriction enzyme(s) to release insert: 5'SalI/3'NotI
Lab Notebook to reference: paul's thesis
Concentration: 10 ug/ul
E. coli Antibiotic markers: AmpR
Strain Transformed into: TOP10F' Frozen glycerols, strain box 8, RY1.3

Cartoon of construct:

WURTZEL LAB
CLONE INFORMATION

Date Today: 01.29.01
(MONTH) (DAY) (YEAR)

Clone Number/Name: **EST D48697** also MAFF S15075 Also RY.9

Constructed by:
Purified by:
Date constructed:
(MONTH) (DAY) (YEAR)
Storage Location- Box Number: Position:

Clone Description: partial cDNA sequenced, same as EST D48251, but shorter, not deposited to Genbank
Cited in journal:paul's thesis

Cloning vector used: SK+ Vector size: 2.9
Organism source of gene: Rice, Nipponbare, 8 da shoot Insert size: 900 bp
Restriction enzyme(s) to release insert: 5'Sal1/3'NotI
Lab Notebook to reference: paul's thesis
Concentration: 1 ug/ul
E. coli Antibiotic markers: AmpR
Strain Transformed into: TOP10F' Frozen glycerols, strain box 8, RY9

Cartoon of construct:

WURTZEL LAB
CLONE INFORMATION

Date Today: 01.29.01
(MONTH)/ (DAY)/ (YEAR)

Clone Number/Name: **pACCAR25ΔcrtX**

Constructed by: Misawa
Purified by:
Date constructed:
(MONTH)/ (DAY)/ (YEAR)
Storage Location- Box Number: 3 Position:

Clone Description: Produces zeaxanthin from *Erwinia* genes. Lacks zeaxanthin glucosidase. Genes: crtE, crtI, crtB,ΔcrtX, crtY, crtZ
Cited in journal:Hartmut, et al. (1990) J Bacteriol.172 :6704-12;. **Hartmut L, Misawa N, Chamowitz D, Pecker I, Hirschberg J, Sandmann G.** 1991. Functional complementation in *Eschericia coli* of different phytoene desaturase genes and analysis of accumulated carotenes. *Naturforsch* **46,** 1045-1051.

Cloning vector used: pAC184 Vector size: 4.26 kb
Organism source of gene: *Erwinia* Insert size: cluster 6.5 kb
Restriction enzyme(s) to release insert:
Lab Notebook to reference:
Concentration:
E. coli Antibiotic markers: ChlorR
Strain Transformed into: TOP10F' Frozen glycerols, strain box 8

Cartoon of construct:

WURTZEL LAB
CLONE INFORMATION

Date Today: 01.29.01
(MONTH)/(DAY)/(YEAR)

Clone Number/Name: **pACCART-EIB**

Constructed by:
Purified by:
Date constructed:
(MONTH)/(DAY)/(YEAR)
Storage Location- Box Number: 3 Position:

Clone Description: produces lycopene Genes: crtE, crtI, crtB
Cited in journal: Hartmut, et al. (1990) J Bacteriol.172 :6704-12; **Hartmut L, Misawa N, Chamowitz D, Pecker I, Hirschberg J, Sandmann G.** 1919. Functional complementation in *Eschericia coli* of different phytoene desaturase genes and analysis of accumulated carotenes. *Naturforsch* **46,** 1045-1051.

Cloning vector used: pAC184 Vector size: 4.26 kb
Organism source of gene: *Erwinia* Insert size: cluster 3.8 kb
Restriction enzyme(s) to release insert:
Lab Notebook to reference:
Concentration:
E. coli Antibiotic markers: ChlorR
Strain Transformed into: Frozen glycerols, strain box 8

Cartoon of construct:

Revised

WURTZEL LAB

CLONE INFORMATION

3. pACC-EBP

Clone Number/Name: pACCRT-EBP (CrtE, CrtB and Maize PDS)

Constructed by: RuiBai Luo
Purified by: RuiBai Luo
Date constructed: 5 (MONTH) / 11 (DAY) / 1997 (YEAR)
Storage Location-
Box Number: 3 Position: 5 J

Clone description: Maize full lengh *pds* cDNA (2.0 kb) was released from pBluescript II SK (-) by *Pvu*II from clone pMPDSd3-33, filled in by Klenow; and inserted into pACCRT-EB (7.99 kb) *Bam*HI site (which filled in by Klenow already), finally contains *CrtE*, *CrtB* and maize *pds*, it confers accumulation of ζ-carotene.
Cited in journal: **Maize Pds cDNA:** Li ZH, Matthews PD, Burr B, Wurtzel ET. Cloning and characterization of a maize cDNA encoding phytoene desaturase, an enzyme of the carotenoid biosynthetic pathway. Plant Mol Biol Jan;30(2):269-279 (1996) **pACCRT-EB:** Hartmut L, Misawa N, Chamovitz D, Pecker I, Hirschberg J, Sandmann G. Functional Complementation in *Escherichia coli* of different phytoene desaturase genes and analysis of accumulated carotenes. Naturforsch, 46c, 1045-1051 (1991). See Luo thesis Figure 2-2.
Cloning vector used: pACCRT-EB Vector size: 7.99 kb
Organism source of gene: *E.uredovara*; maize Insert size: 2.0 kb (Pds)
Restriction enzyme(s) to release insert:
Lab Notebook to reference: Ruibai #1.
Concentration: 0.01 µg/ul
E. coli Antibiotic markers: __amp; __tet; X chloramphenicol; (34 µg/ml Chl)
Strain Transformed into: XL-1 Blue Frozen glycerols, strain box # 7-6-E

WURTZEL LAB
CLONE INFORMATION

Date Today: _____
(MONTH) (DAY) (YEAR)

Clone Number/Name: **pAC-Neurosporene**

Constructed by:
Purified by:
Date constructed:
(MONTH) (DAY) (YEAR)
Storage Location- Box Number: Position:

Clone Description: produces neurosporene enzymes: GGPPS, PSY, CRTI
Cited in journal: **Cunningham, FX, Jr., Sun Z, Chamovitz D, Hirschberg J, Gantt E.** 1994. Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium Synechococcus sp strain PCC7942. *Plant Cell* **6**, 1107-1121

Cloning vector used: pAC184 Vector size: 4.26 kb
Organism source of gene: Erwinia Insert size:
Restriction enzyme(s) to release insert:
Lab Notebook to reference:
Concentration: 0.5 ug/ul
E. coli Antibiotic markers: ChlorR
Strain Transformed into: ?? Frozen glycerols, strain box 7, A5

Cartoon of construct:

WURTZEL LAB
CLONE INFORMATION

Date Today: 01.29.01
(MONTH) (DAY) (YEAR)

Clone Number/Name: **pBgl3.4**
Also pBF214.1 or.2 in notes

Constructed by: Ben Burr
Purified by:
Date constructed:
(MONTH) (DAY) (YEAR)
Storage Location- Box Number: 3 Position: 8D

Clone Description: 3.4 kb BglII fragment of maize *Shrunken1* genomic DNA

Cited in journal: **Wurtzel ET, Burr FA, Burr B.** 1987. DNase I hypersensitivity and expression of the *Shrunken-1* gene of maize. *Plant Mol Biol* **8**, 251-264

Cloning vector used: pUC9 Vector size:

Organism source of gene: maize Insert size: 3.4 kb

Restriction enzyme(s) to release insert: BglII

Lab Notebook to reference: (Wurtzel et al., 1987)

Concentration: 3.75 ug/ul

E. coli Antibiotic markers: AmpR

Strain Transformed into: Frozen glycerols, strain box

Cartoon of construct:

WURTZEL LAB

CLONE INFORMATION

2. pMzds-107

Clone Number/Name: Mzds (48, clone 10), [Renamed as pMzds-107, for Maize *Zds* expression]

Constructed by: RuiBai Luo
Purified by: RuiBai Luo
Date constructed: 7/ 3/ 1997
(MONTH)/ (DAY)/ (YEAR)
Storage Location-
Box Number: 3 Position: 6 B

Clone description: Delete Mzds48 5' 106 bp, in frame with lac Z promoter. (By double digested Mzds48 by *Not*I and filled in *Not*I, then by *Xba*I, finally perform 5' deletion).Transformed into pACCRT-EBP cells, neurosporene and lycopene can be detected by HPLC. (Junction sequence:ggtggcg|ttctcctcctcttt=vector/*zds*).
Cited in journal: Genbank access #AF047490 (Squence of pMzds48). See also Luo thesis Figure 2-1. (Restriction map), Figure 2-3 (Junction sequence)
Cloning vector used: pBluescript SK II (-) Vector size: 2.96 kb
Organism source of gene: maize Insert size: 2159 bp
Restriction enzyme(s) to release insert: *Sac*II and *Sal*I
Lab Notebook to reference: Ruibai #1, #6.
Concentration: 0.1 µg/ul
E. coli Antibiotic markers: X amp; __tet; _ chloramphenicol; (50 µg/ml Amp)
Strain Transformed into: XL-1Blue Frozen glycerols, strain box # 7-5-F

WURTZEL LAB
CLONE INFORMATION

Date Today: 01.28.01
(MONTH)/ (DAY)/ (YEAR)

Clone Number/Name: **pRCPSYC6.0**
Also IG6 and IG2, partial sequence deposited as GENBANK AY024351

Constructed by: PAUL
Purified by:
Date constructed:
(MONTH)/ (DAY)/ (YEAR)
Storage Location- Box Number: 4 Position: D8

Clone Description: RICE PSY GENOMIC, HYBRIDIZES TO EST D48251 Used for sequencing. A 6 kb ClaI fragment from pVIVYH5 was subcloned into the ClaI site of pBluescript SK-
Cited in journal:paul's thesis

Cloning vector used: SK- Vector size: 2.9 kb
Organism source of gene: Rice Insert size: 6 kb
Restriction enzyme(s) to release insert: ClaI, BamHI and EcoRI will elease 3222 bp fragment with *Psy* gene
Lab Notebook to reference: vivek 1996, paul thesis
Concentration: 3 ug/ul
E. coli Antibiotic markers: ChlorR
Strain Transformed into: Frozen glycerols, strain box 8, positions 6D

Cartoon of construct:

WURTZEL LAB
CLONE INFORMATION

Date Today: 5/20/98
(MONTH/ DAY/ YEAR)

Clone Number/Name:	*E. coli dxs* gene (deoxyxylulose phosphate synthase) (expression construct)

Constructed by: Boronat Lab, Spain*
Purified by: _____
Date constructed: ____/____/____
(MONTH/ DAY/ YEAR)

Storage Location-
Box Number: 4 Position: G-10

Clone description: pTAC-ORF2 *E. coli* dxs gene (deoxyxylulose phosphate synthase) only, under control of TACTAC promoter.
GenBank #AF035440
Cited in journal: Lois *et al.* PNAS (1998) 95: 2105-2110.
Cloning vector used: pTACTAC Vector size: _____ Reference: Browner *et al.*, (1991) Protein Engineering 4:351-7.
Organism source of gene: *E. coli* Insert size: 2 kb
Restriction enzyme(s) to release insert: *NdeI- SalI*
Induction conditions: Grow in TY + amp (see Current Protocols in Molecular Biology by Ausubel) at 37 C, to OD 600 (0.6), induce with 1 mM IPTG for 1-4 hrs.....see 65 kDa protein by SDS-PAGE.
Concentration: 75 ng/ul (?) ug/ul
E. coli Antibiotic markers: X amp (100 ug/ml); __tet; __chloramphenicol; __other (__)
Strain Transformed into: XL 10 amp kan tet Frozen glycerols, strain box # 8

Dr. Albert Boronat
Facultat Quimica 716C
Departament de Bioquímica i Biologia Molecular
Universitat de Barcelona
Marti i Franques 1
08028-Barcelona, Spain
Tel.: (93)4021194, FAX: (93)4021219
e-mail: aboronat@sun.bq.ub.es

G4, G5, G6
in XL 10
(amp, kan, tet)

File: Ecoli dxs.wpd

WURTZEL LAB
CLONE INFORMATION

Date Today: 01.29.01
(MONTH) (DAY) (YEAR)

Clone Number/Name: **pVIVYB10**
Renamed from Vivek's B8 to B11 series. It is B10.

Constructed by: Vivek
Purified by:
Date constructed: 00.07.96
(MONTH) (DAY) (YEAR)
Storage Location- Box Number: Position:

Clone Description: 5 kb BamHI fragment from UCD#2 rice BAC PSY Genomic DNA

Cited in Journal : **Zhang HB, Choi s, Woo SS, Li Z, Wing RA.** (1996) Construction and characterization of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. *Mol Breed* **2**, 11-24.

Cloning vector used: SK- Vector size: 2.9 kb

Organism source of gene: Rice Insert size: 5 kb

Restriction enzyme(s) to release insert: integrity of MCS not determined, BamHI

Lab Notebook to reference: Vivek 1996

Concentration:

E. coli Antibiotic markers: AmpR

Strain Transformed into: TOP10F' Frozen glycerols, strain box 8

Cartoon of construct:

WURTZEL LAB
CLONE INFORMATION

Date Today: 01.29.01
(MONTH) (DAY) (YEAR)

Clone Number/Name: **pVIVYE3**
Renamed from Vivek's E1 to E4 series. It is E3.

Constructed by:
Purified by:
Date constructed:
(MONTH) (DAY) (YEAR)
Storage Location- Box Number: Position:

Clone Description: 2.3 kb EcoRI fragment from UCD#2 rice BAC PSY Genomic DNA

Cited in Journal: **Zhang HB, Choi s, Woo SS, Li Z, Wing RA.** (1996) Construction and characterization of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. *Mol Breed* **2**, 11-24.

Cloning vector used: SK- Vector size: 2.9 kb

Organism source of gene: Rice Insert size: 2.3 kb

Restriction enzyme(s) to release insert: integrity of MCS not determined, EcoRI

Lab Notebook to reference: Vivek 1996

Concentration:

E. coli Antibiotic markers:

Strain Transformed into: TOP10F' Frozen glycerols, strain box 8

Cartoon of construct:

WURTZEL LAB
CLONE INFORMATION

Date Today: 01.29.01
(MONTH)/(DAY)/(YEAR)

Clone Number/Name: **pVIVYH5**
Renamed from Vivek's H1, H2, H3, H5 series. It is H5.

Constructed by: Vivek
Purified by:
Date constructed: 00.07.96
(MONTH)/ (DAY)/ (YEAR)
Storage Location- Box Number: Position:

Clone Description: 2.3 kb EcoRI fragment from UCD#2 rice BAC PSY Genomic DNA
Used for sequencing, used to generate subclone pRCPSYC6.0

Cited in Journal: **Zhang HB, Choi s, Woo SS, Li Z, Wing RA.** (1996) Construction and characterization of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. *Mol Breed* **2**, 11-24.

Cloning vector used: SK- Vector size: 2.9 kb

Organism source of gene: Rice Insert size: 5 kb

Restriction enzyme(s) to release insert: HindIII

Lab Notebook to reference: Vivek 1996

Concentration:

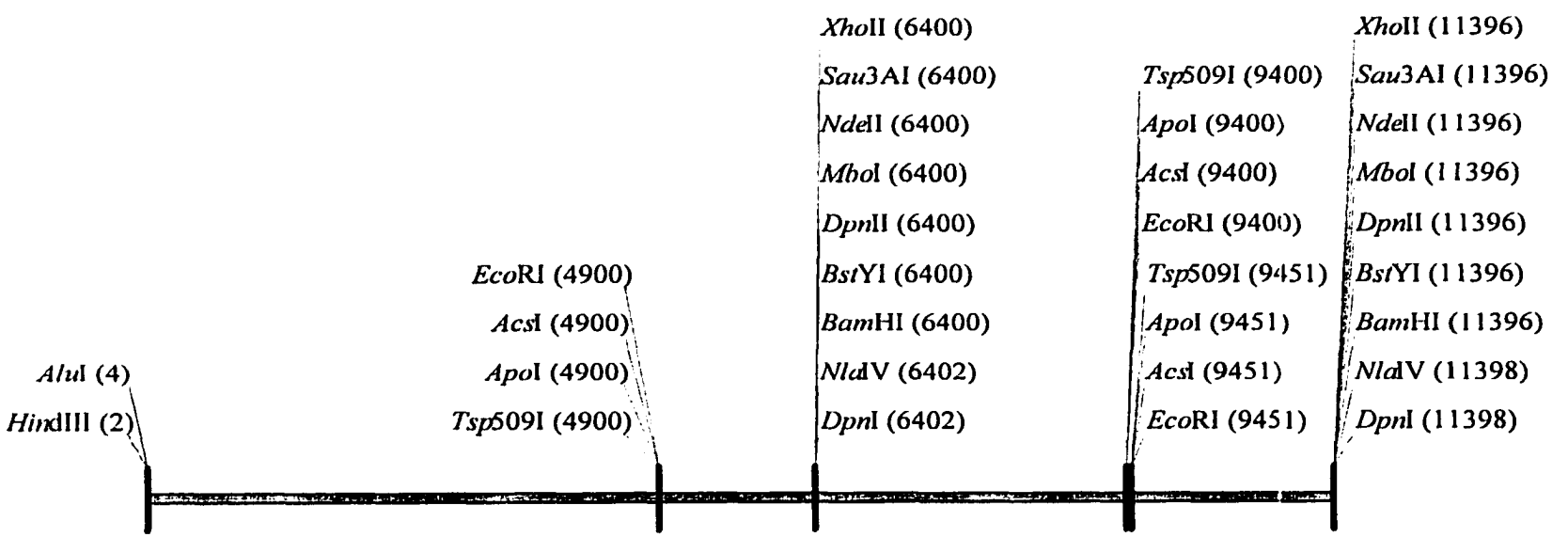
E. coli Antibiotic markers:

Strain Transformed into: TOP10F' Frozen glycerols, strain box 8

Cartoon of construct:

Appendix B

Restriction map of fragments that hybridize to a 1.3 kb SalI/NotI fragment of rice EST D48251



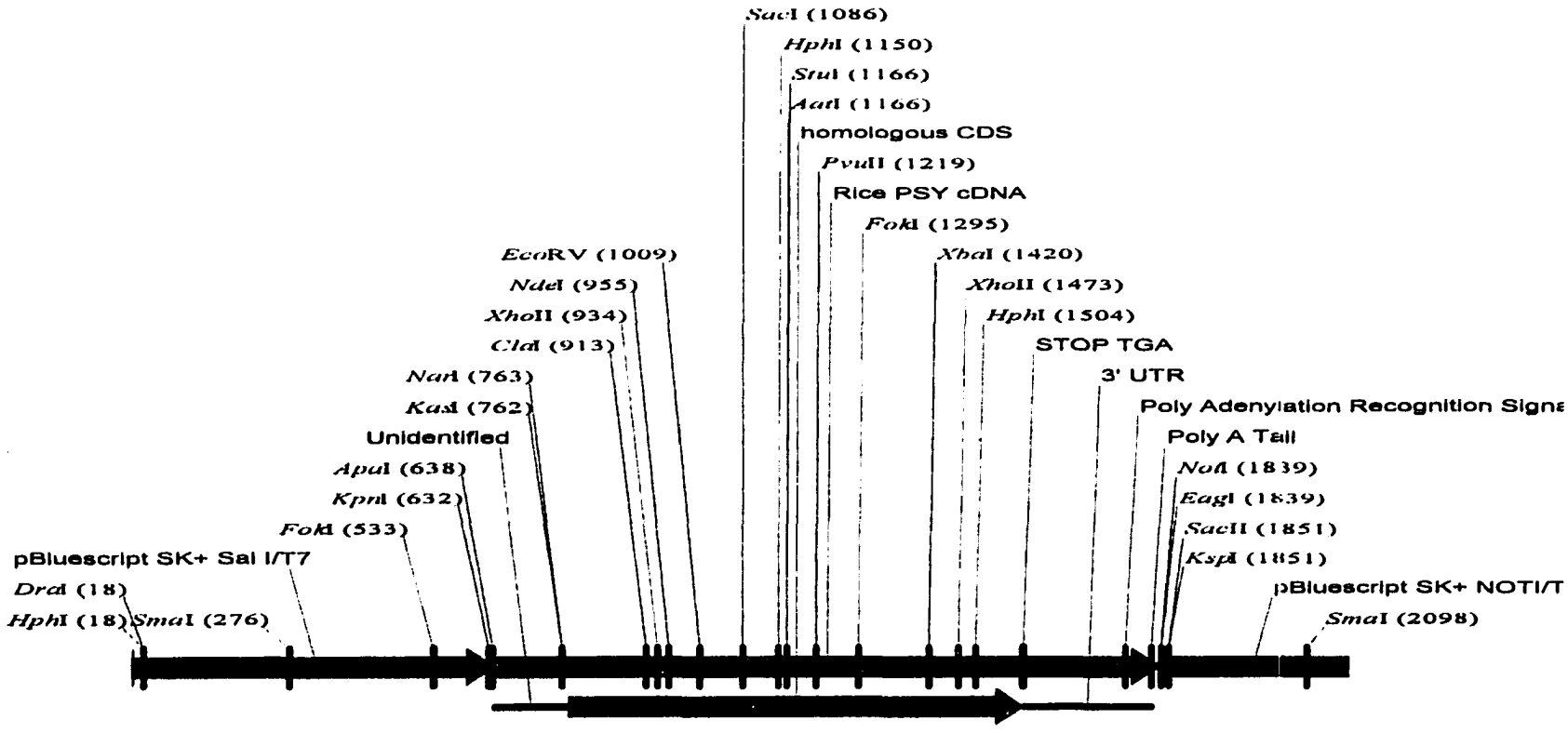
UCD #2 Map of Probed Fragments

11400 bp

Approximate nucleotide position with respect to left end shown in brackets. BamHI, EcoRI, and HindIII site were experimentally determined, other sites shown are deduced from the recognition sequences of experimentally–determined sites. These fragments were used to construct pVIVYH5 (10 kb), pVIVYE3 (2.3 kb), and pVIVYB10 (5kb). **This map is tentative and not fully resolved.**

Appendix C

Restriction map deduced from sequence shown on a graphic and on the sequence for partial rice cDNA EST D48251, Genbank AY24350, including partial vector sequence



Partial cDNA RICE EST D48251
2172 bp

EST D48251 (Genbank AY024350) restriction sites

```
          HphI        DraI
          -----       -------
   1  GTAAGGTGAT ATTTTTTAAA ATTTGGGGAA TGGGTTAAGT AGATCATTAT
      CATTCCACTA TAAAAAATTT TAAACCCCTT ACCCAATTCA TCTAGTAATA
  51  TTAACCATTA GGCGAATTGG CAAATCCTGT TTAATCAAAA GAATAGACGA
      AATTGGTAAT CCGCTTAACC GTTTAGGACA AATTAGTTTT CTTATCTGCT
 101  GATAGGGTTA GGTTGTTCAG TGGGAACAGA TTCAGTATTA AAGAAGTGGA
      CTATCCCAAT CCAACAAGTC ACCCTTGTCT AAGTCATAAT TTCTTCACCT
 151  TTCAATGTCA AAGGGCGAAA AACCGTGTAT AGGGGAGGCC CAGAGAGAAC
      AAGTTACAGT TTCCCGCTTT TTGGCACATA TCCCCTCCGG GTCTCTCTTG
 201  ATCGAACCCA TAATAAAGTT TTTGGGGTAG GAGGTGCCGT AAAGCATTAA
      TAGCTTGGGT ATTATTTCAA AAACCCCATC CTCCACGGCA TTTCGTAATT
                              SmaI
                             -------
 251  ATGGGGGAAA CCTAAAGGGG ACCCCGGGAT TTGAGAGCTG ACGGGGAAAG
      TACCCCCTTT GGATTTCCCC TGGGGCCCTA AACTCTCGAC TGCCCCTTTC
 301  CAGGGCGAAG GTGGGGAGAA AGGAAGGGAT AGAAAGCGAA AGGAGCGGGC
      GTCCCGCTTC CACCCCTCTT TCCTTCCCTA TCTTTCGCTT TCCTCGCCCG
 351  GTTAGGGACG ATGGCAAGTG TAGGGGTAAA AGCGTGGGGG TAACCACCAC
      CAATCCCTGC TACCGTTCAC ATCCCCATTT TCGCACCCCC ATTGGTGGTG
 401  ACCACGCCGA GTTTAATGAG CGCGATACAG GGGGGTCCCA TTCGCCATTC
      TGGTGCGGCT CAAATTACTC GCGCTATGTC CCCCCAGGGT AAGCGGTAAG
 451  AGGCGCGCAA CTGTTGGGAA GGGGAATCGG TGCGGGCCTC TTCGCTATTA
      TCCGCGCGTT GACAACCCTT CCCCTTAGCC ACGCCCGGAG AAGCGATAAT
                         FokI
                        ------
 501  CGCCAGTGTG CGAAAGGGGG ATGTGTGCAA GGGATTAAGT TGGGTAACGC
      GCGGTCACAC GCTTTCCCCC TACACACGTT CCCTAATTCA ACCCATTGCG
 551  CAGGGTTTTC CCAGTCATTG ACGTTGTAAA CGACGGCCAG TGAGCGCGCG
      GTCCCAAAAG GGTCAGTAAC TGCAACATTT GCTGCCGGTC ACTCGCGCGC
                                   KpnI
                                  -------
                                          ApaI
                                         -------
 601  TAATAGACTA AATAGAGGGC GAATTGGGTA CCGGGCCCCC CTTGGAGGTG
      ATTATCTGAT TTATCTCCCG CTTAACCCAT GGCCCGGGGG GAACCTCCAC
 651  GACCCAACGC GTCCGACCCC AACCATTCGA CCCGCGCAGC GAGCCCCAAC
      CTGGGTTGCG CAGGCTGGGG TTGGTAAGCT GGGCGCGTCG CTCGGGGTTG
 701  CCCACATCGC CGCGCGCCCC CGATGGCGTC CTCCTCGTCG GCGGCGGCGC
      GGGTGTAGCG GCGCGCGGGG GCTACCGCAG GAGGAGCAGC CGCCGCCGCG
                NarI
               ------
                KasI
               ------
 751  TCTGGACGGC GGCGCCCCAC CCCCAAGACC TTCTACCTAG GTACTCAGCT
      AGACCTGCCG CCGCGGGGTG GGGGTTCTGG AAGATGGATC CATGAGTCGA
 801  TATGACTCCT GAAAGGCGCA AAGCTGTCTG GGCAATCTAT GTATGGTGCA
      ATACTGAGGA CTTTCCGCGT TTCGACAGAC CCGTTAGATA CATACCACGT
 851  GAAGAACTGA TGAACTGGTA GATGGCCCTA ACTCGTCTTA CATTACACCA
      CTTCTTGACT ACTTGACCAT CTACCGGGAT TGAGCAGAAT GTAATGTGGT
                ClaI                    XhoII
               ------                   -------
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 901   AAGGCACTTG ATCGATGGGA GAAGAGATTA GAAGATCTCT TCGAAGGCAG
       TTCCGTGAAC TAGCTACCCT CTTCTCTAAT CTTCTAGAGA AGCTTCCGTC
          NdeI
         ------
 951   GCCATATGAT ATGTATGATG CAGCCCTCTC GGACACAGTG TCAAAGTTTC
       CGGTATACTA TACATACTAC GTCGGGAGAG CCTGTGTCAC AGTTTCAAAG
            EcoRV
           -------
1001   CAGTAGATAT CCAGCCATTC AAAGACATGA TTGAAGGAAT GAGGCTTGAC
       GTCATCTATA GGTCGGTAAG TTTCTGTACT AACTTCCTTA CTCCGAACTG
                                         SacI
                                        ------
1051   CTGTGGAAAT CAAGGTATAG GAGCTTTGAT GAGCTCTACC TCTACTGCTA
       GACACCTTTA GTTCCATATC CTCGAAACTA CTCGAGATGG AGATGACGAT
                                              HphI
                                             ------
1101   CTACGTTGCT GGCACGGTTG GTCTCATGAC AGTACCGGTG ATGGGGATTG
       GATGCAACGA CCGTGCCAAC CAGAGTACTG TCATGGCCAC TACCCCTAAC
                    StuI
                   ------
                    AatI
                   ------
1151   CCCCCGACTC GAAGGCCTCA ACCGAGAGCG TGTACAACGC TGCGCTAGCT
       GGGGGCTGAG CTTCCGGAGT TGGCTCTCGC ACATGTTGCG ACGCGATCGA
                      PvuII
                     -------
1201   CTTGGGATCG CCAACCAGCT GACGAATATT CTCAGAGACG TAGGCGAAGA
       GAACCCTAGC GGTTGGTCGA CTGCTTATAA GAGTCTCTGC ATCCGCTTCT
                                        FokI
                                       -----
1251   CTCAAGGAGG GGAAGAATCT ACCTTCCATT GGATGAATTG GCAGAGGCAG
       GAGTTCCTCC CCTTCTTAGA TGGAAGGTAA CCTACTTAAC CGTCTCCGTC
1301   GTCTGACAGA AGAAGACATA TTCAGAGGGA AAGTGACTGA TAAATGGAGG
       CAGACTGTCT TCTTCTGTAT AAGTCTCCCT TTCACTGACT ATTTACCTCC
1351   AAGTTCATGA AGGGACAAAT TCTGCGTGCC AGGTTATTCT TTGATGAGGC
       TTCAAGTACT TCCCTGTTTA AGACGCACGG TCCAATAAGA AACTACTCCG
                           XbaI
                          -------
1401   GGAGAAGGGC GTTGCGCATC TAGACTCTGC GAGTAGATGG CCGGTTCTGG
       CCTCTTCCCG CAACGCGTAG ATCTGAGACG CTCATCTACC GGCCAAGACC
                             XhoII
                             ------
1451   CATCTTTGTG GTTATACCGG CAGATCCTTG ATGCTATCGA AGCAAACGAC
       GTAGAAACAC CAATATGGCC GTCTAGGAAC TACGATAGCT TCGTTTGCTG
                  HphI
                 -----
1501   TACAACAACT TCACCAAGCG CGCGTATGTA AACAAGGCAA AGAAGCTGCT
       ATGTTGTTGA AGTGGTTCGC GCGCATACAT TTGTTCCGTT TCTTCGACGA
1551   GTCTTTACCG GTCGCTTATG CAAGAGCGGC AGTTGCATCA TGAACAATCA
       CAGAAATGGC CAGCGAATAC GTTCTCGCCG TCAACGTAGT ACTTGTTAGT
1601   CTAGATCAGA TGCCTTATTA TTTTTTTTCT TTCATTTTCT TTTCTTTTGA
       GATCTAGTCT ACGGAATAAT AAAAAAAAGA AAGTAAAAGA AAAGAAAACT
1651   TTTCGCACGA TTTCTTGGCT GTTGTATATA TTCAAGCAGC TACCTGTATG
       AAAGCGTGCT AAAGAACCGA CAACATATAT AAGTTCGTCG ATGGACATAC
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1701    CCATAAGCCT GCCACAGTTT TTCTTTAGTT CAAGGGACTG ATTTCAGGTC
        GGTATTCGGA CGGTGTCAAA AAGAAATCAA GTTCCCTGAC TAAAGTCCAG
1751    CCTCAATACT CAACTCTTGT TAGAAACAAA TACAGAGGGG GTAAGCCCCA
        GGAGTTATGA GTTGAGAACA ATCTTTGTTT ATGTCTCCCC CATTCGGGGT
                                                   NotI
                                                  ~~~~~~~~~
                                                   EagI
                                                  ~~~~~~
1801    CAGTTCAAGA AGCATATTAA AAAAAAAAAA AAAAGGGCGG CCGCCACCGC
        GTCAAGTTCT TCGTATAATT TTTTTTTTTT TTTTCCCGCC GGCGGTGGCG
        SacII
        ~~
        KspI
        ~~
1851    GGTGGACTCC AGCTTTTGTT CCCTTTATGA GGTTAATTGC GCGCTTGGCG
        CCACCTGAGG TCGAAAACAA GGGAAATACT CCAATTAACG CGCGAACCGC
1901    TAATCATGGT CATACTGTTT CCTGTGTGAA ATTGTTATCC GCTCACAATT
        ATTAGTACCA GTATGACAAA GGACACACTT TAACAATAGG CGAGTGTTAA
1951    CCACACAACA TACGAACCGG AAGCATAAAG TGTTAAGCCT GGGGTGCCTA
        GGTGTGTTGT ATGCTTGGCC TTCGTATTTC ACAATTCGGA CCCCACGGAT
2001    ATGAATGACT AACTCACATT AATTGCGTTG CGCTCACTGC CCGCTTTCCA
        TACTTACTGA TTGAGTGTAA TTAACGCAAC GCGAGTGACG GGCGAAAGGT
                                                        SmaI
                                                        ~~
2051    GTCGGAAACC TGTCGTGCCA CCTGCATTAA TAATCCGCCA ACCCCCCGGG
        CAGCCTTTGG ACAGCACGGT GGACGTAATT ATTAGGCGGT TGGGGGGCCC
2101    AAAAGCGGTT GCCTTTTTGG GGCTCTTCCC CTTCCTCCCT CATTAAATCC
        TTTTCGCCAA CGGAAAAACC CCGAGAAGGG GAAGGAGGGA GTAATTTAGG
2151    CTGGCCCGGT CTTTCGCTGC GG
        GACCGGGCCA GAAAGCGACG CC
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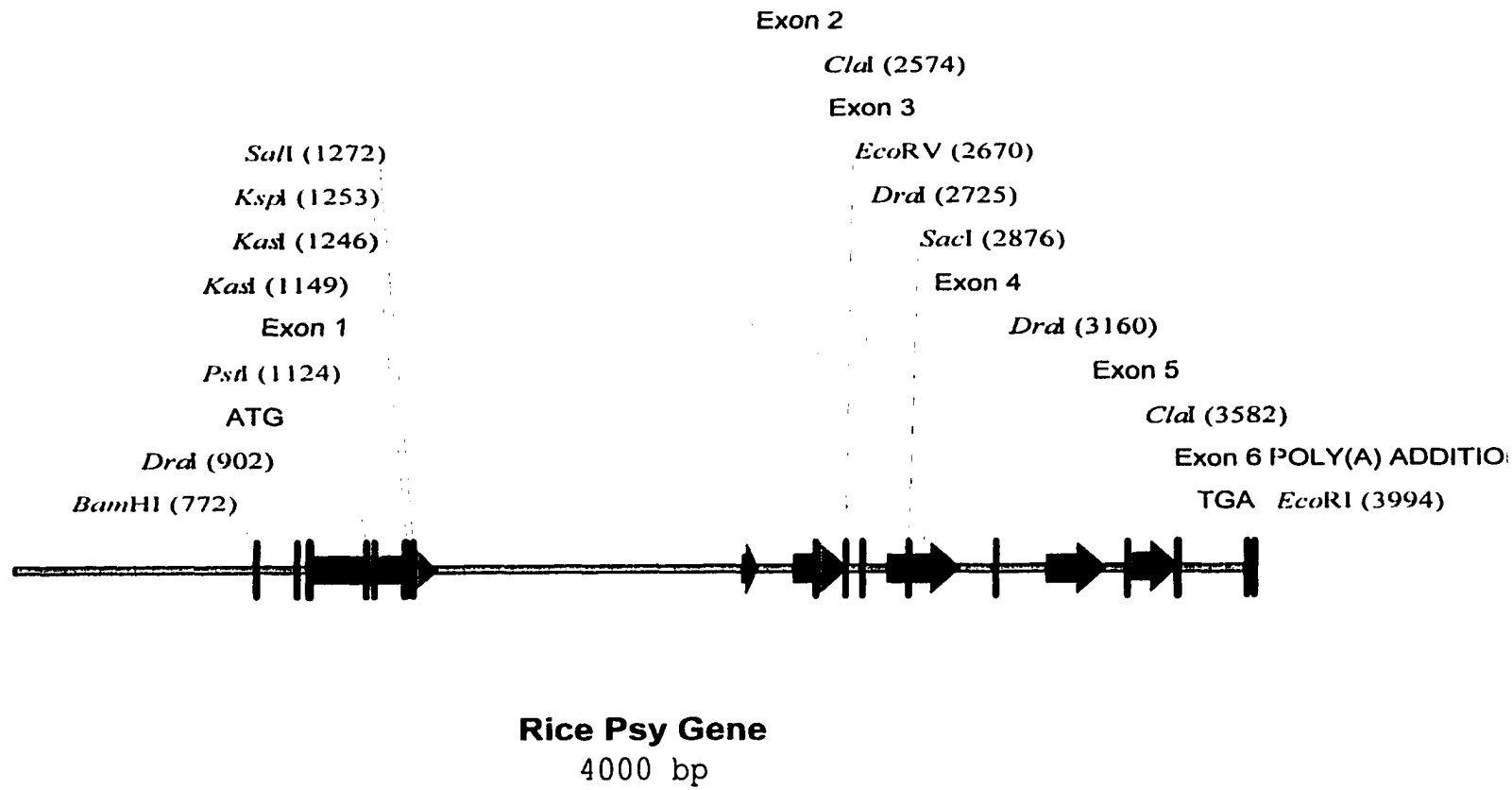
Appendix D

Restriction map deduced from sequence (Genbank AY02351) shown on a graphic and on the sequence (next page)



Restriction map on sequence of rice *Psy* gene (Genbank AY024351)

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      TTNTTTTTCT NTANTAANTT TTCCCTTTAC CCANTAATTA TTTCCCCTTC
  51  AAATTTTANG AAGGGGAGAA ATAGTTNGGG GGGGGAGGGT TTANTNTNNT
      TTTAAAATNC TTCCCCTCTT TATCAANCCC CCCCCTCCCA AATNANANNA
 101  TTAANGAAAN AGGNAAANGG GGGTTNNATA NAATTTAATA ATTTTNGNAA
      AATTNCTTTN TCCNTTTNCC CCCAANNTAT NTTAAATTAT TAAAANCNTT
 151  GGTAAAAAAG GCCGNGAAAA AAAAGGATNA CAAAAATTNG GGGGGTTNAT
      CCATTTTTTC CGGCNCTTTT TTTTCCTANT GTTTTTAANC CCCCCAANTA
 201  ATNNGTTGNC AAAAAAAAAA AAANGAAGCC NAATAAATAN TCCCCTAAAA
      TANNCAACNG TTTTTTTTTT TTTNCTTCGG NTTATTTATN AGGGGATTTT
 251  NAGAATANTT TTTTTANGAA GGGATGGNGG NANTTTTTNG TGGGTTAAAA
      NTCTTATNAA AAAAATNCTT CCCTACCNCC NTNAAAAANC ACCCAATTTT
 301  CAGGNANNNA TCNNNAGANN GAAANTAAAA GAAANGAGGN ANTANAAGAA
      GTCCNTNNNT AGNNNTCTNN CTTTNATTTT CTTTNCTCCN TNATNTTCTT
 351  GTTTAACCAA GGGGGGGGNN ANTGNAAANA AAGGGAAAAN AAANATGANT
      CAAATTGGTT CCCCCCCCNN TNACNTTTNT TTCCCTTTTN TTTNTACTNA
 401  NGNTATTGGN NGGNTGGTTN AAAGAAAAGA GGGGNAGTTT GGNTTCAATG
      NCNATAACCN NCCNACCAAN TTTCTTTTCT CCCCNTCAAA CCNAAGTTAC
 451  AGAAAAATAA ATNAGNANAA AAGGNGNGAA AAGANNNTNT TTTANAGTTA
      TCTTTTTATT TANTCNTNTT TTCCNCNCTT TTCTNNNANA AAATNTCAAT
 501  AATATTAGTT ATACAATTAG AAATTGACGA GAATNTTTTG TGGGNGGTNG
      TTATAATCAA TATGTTAATC TTTAACTGCT CTTANAAAAC ACCCNCCANC
 551  ANAAAATATT CAAAGAAGAT ACATTGAANG CCNAAAAAAC CNCGCNACAG
      TNTTTTATAA GTTTCTTCTA TGTAACTTNC GGNTTTTTTG GNGCGNTGTC
 601  GNTTTTTTTT AAGNNAAAAG GGNANNANNT NAGGTNCACT NAAAAAATNA
      CNAAAAAAAA TTCNNTTTTC CCNTNNTNNA NTCCANGTGA NTTTTTTANT
 651  AATTNTTTGT TAAAAAAAAA AAAAAAGGGG GGNAAGNCNC AAAAGTTNAA
      TTAANAAACA ATTTTTTTTT TTTTTTCCCC CCNTTCNGNG TTTTCAANTT
 701  AAAGGNATAA TACTNTTTAT TTTGTTTTCA ANAAANTNAA GGTTGAAATA
      TTTCCNTATT ATGANAAATA AAACAAAAGT TNTTTNANTT CCAACTTTAT
                           BamHI
                           ------
 751  GAAATTNCTG NANNNNGGNG GGATCCAATA GTTANAGAAA GGNCGCCANN
      CTTTAANGAC NTNNNNCCNC CCTAGGTTAT CAATNTCTTT CCNGCGGTNN
 801  GNGGNGGAAG TNCAANTTTT GTTCCANTTA NTGAAGGGNN AAATGGGGGC
      CNCCNCCTTC ANGTTNAAAA CAAGGTNAAT NACTTCCCNN TTTACCCCCG
 851  CCCCCCCCCC CCCAGAAAAA TTAATTAAAA TTAATTGGGG GTAACCAATT
      GGGGGGGGGG GGGTCTTTTT AATTAATTTT AATTAACCCC CATTGGTTAA
      DraI
      ----
 901  TAAACCCACC CTCCTTTCAA AAGAAAACAA ACCCCAACCA ATGACCGGGG
      ATTTGGGTGG GAGGAAAGTT TTCTTTTGTT TGGGGTTGGT TACTGGCCCC
 951  AAGGAAGCCC AAACCAAAAT TGCCGGGGGG CCCCAAGGGG TCTCTTGGCG
      TTCCTTCGGG TTTGGTTTTA ACGGCCCCCC GGGGTTCCCC AGAGAACCGC
1001  GGGGGTTTTG AAGGTGGGCC CCCACCCCCA CGGCAAGTGG AACAGGATTC
      CCCCCAAAAC TTCCACCCGG GGGTGGGGGT GCCGTTCACC TTGTCCTAAG
1051  AAGCCATTTT TCACAAGGTT ACAACGAAGG GGAAGAAGCC GGTTGTTGTC
      TTCGGTAAAA AGTGTTCCAA TGTTGCTTCC CCTTCTTCGG CCAACAACAG
                          PstI
                          -------
1101  GCCTCGTCGG TGAGGCCGCT GCAGGCCGCG AGCCTGGCGG TGGCCACGGC
      CGGAGCAGCC ACTCCGGCGA CGTCCGGCGC TCGGACCGCC ACCGGTGCCG
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        KasI
        ~~~
1151    GCCGGTGGCC GTGGCGTCGA GGAGGACGGC GGCGGAGAGG CGTCTAGAGG
        CGGCCACCGG CACCGCAGCT CCTCCTGCCG CCGCCTCTCC GCAGATCTCC
                                                      KasI
                                                      --
1201    TCGGCTCGGC ACGCGCTGTG GAAGGCCCCA CCGCGCGGCG CGGGGGCGCC
        AGCCGAGCCG TGCGCGACAC CTTCCGGGGT GGCGCGCCGC GCCCCCGCGG
        KspI                    SalI
        ~~~~                    ~~~~~~
1251    GCGGTGGGCG AGGAGGACGC GTCGACTGGG GCCTCCTCCT CGGCGACGCC
        CGCCACCCGC TCCTCCTGCG CAGCTGACCC CGGAGGAGGA GCCGCTGCGG
1301    TACCACCGCT GCGGCGAGGT CTGCGCCGAG TACGCCAAGA CCTTCTACCT
        ATGGTGGCGA CGCCGCTCCA GACGCGGCTC ATGCGGTTCT GGAAGATGGA
1351    AGGTCAGCCA ACCCAACATT GACTAATCAT TCAATTTTGT TTTTATCACC
        TCCAGTCGGT TGGGTTGTAA CTGATTAGTA AGTTAAAACA AAAATAGTGG
1401    GTTATTACTT TATCATTTCA CAGGTGAAAG CTAGCTACTA TTCGTTTGCC
        CAATAATGAA ATAGTAAAGT GTCCACTTTC GATCGATGAT AAGCAAACGG
1451    ACTACTACTC CCTAGCTTAG CTAGCGTCTC TTCCTTTTCT TGATTTGTTC
        TGATGATGAG GGATCGAATC GATCGCAGAG AAGGAAAAGA ACTAAACAAG
1501    ATAGTACTGT ATATGATAAT TGTAGACCTA CCCTTTCTCT ATTTCTTTTT
        TATCATGACA TATACTATTA ACATCTGGAT GGGAAAGAGA TAAAGAAAAA
1551    GGGTTCTTGG GTGGAATGGC TGGCTTGATA AAATTTTGCG ACCCTTTTTG
        CCCAAGAACC CACCTTACCG ACCGAACTAT TTTAAAACGC TGGGAAAAAC
1601    TGGTTCTAGG AGCCACATCT CACTTTCACT TTCTTGTACT TGTTGGTCTA
        ACCAAGATCC TCGGTGTAGA GTGAAAGTGA AAGAACATGA ACAACCAGAT
1651    AAAAGATTAG TTAATGCTGC AATTAGTTGG CTTTTACTAG GAATTGTAAG
        TTTTCTAATC AATTACGACG TTAATCAACC GAAAATGATC CTTAACATTC
1701    AGGGTGGTGT TAAAAGGTGA TTAAAAAAAA ATCTATGAAC ATTCCTAGAT
        TCCCACCACA ATTTTCCACT AATTTTTTTT TAGATACTTG TAAGGATCTA
1751    TCCTATTACG TGTTAAGAAG ATAAATATTC CTGCGTATAA TTTATGGAAT
        AGGATAATGC ACAATTCTTC TATTTATAAG GACGCATATT AAATACCTTA
1801    ACTCCTTCTG ATGGCAAAAA TATATTAGCA GTAGCCAAAC AACACCAGCC
        TGAGGAAGAC TACCGTTTTT ATATAATCGT CATCGGTTTG TTGTGGTCGG
1851    TGAGTGAGTG AGTGGCTCTC AGTGCTCTGA ACAACTGTAC ATGTATATAC
        ACTCACTCAC TCACCGAGAG TCACGAGACT TGTTGACATG TACATATATG
1901    AAATGTTTGC CGACTTCAGA GTAGGTAAGA TAATGCACTT GGATGATAGA
        TTTACAAACG GCTGAAGTCT CATCCATTCT ATTACGTGAA CCTACTATCT
1951    TGCCATCCCA AGGAAATGAT CCAACAAGCA GATGCGCAGA TATGGTGATC
        ACGGTAGGGT TCCTTTACTA GGTTGTTCGT CTACGCGTCT ATACCACTAG
2001    TCTCTAGTGC ACCACTGGCA ATTTATAGAT TTCTCTTCTT AATTAGGGTC
        AGAGATCACG TGGTGACCGT TAAATATCTA AAGAGAAGAA TTAATCCCAG
2051    AGGGATATGC TGGTTGGTAG TGGGTTTGTG GGTGATGTGA GGACGCATGG
        TCCCTATACG ACCAACCATC ACCCAAACAC CCACTACACT CCTGCGTACC
2101    GCTTGATTGA TGAATCCAGT AACCTTCTTG TTTTTTTTAA TCTTGCTTTC
        CGAACTAACT ACTTAGGTCA TTGGAAGAAC AAAAAAAATT AGAACGAAAG
2151    TGTATCACCG TGTTTCCACT TGGATCATCC CTGACCTGGT AAGGGACTCA
        ACATAGTGGC ACAAAGGTGA ACCTAGTAGG GACTGGACCA TTCCCTGAGT
2201    TTTTCTATTG CTACGAGGAC CTTTTGGGGA CTAGCTCAAA CTCTGATGTA
        AAAAGATAAC GATGCTCCTG GAAAACCCCT GATCGAGTTT GAGACTACAT
2251    TGGTTGATAC TTAGATTTTC TACGTAAAGT GGAATTTCAC CTTGTGTAAT
        ACCAACTATG AATCTAAAAG ATGCATTTCA CCTTAAAGTG GAACACATTA
2301    TTCTCATGGT TTTTGTTTCC ATCGTTTACA TTTCAGGTAC TCAGCTTATG
        AAGAGTACCA AAAACAAAGG TAGCAAATGT AAAGTCCATG AGTCGAATAC
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2351   ACTCCTGAAA GGCGCAAAGC TGTCTGGGCA ATCTATGGTA TGGATTAGTT
       TGAGGACTTT CCGCGTTTCG ACAGACCCGT TAGATACCAT ACCTAATCAA
2401   AACTATTCAT TGACCTACCA GTTACTCTCA CTTGTGAAAA TTCAGATAAC
       TTGATAAGTA ACTGGATGGT CAATGAGAGT GAACACTTTT AAGTCTATTG
2451   TTCAATTGTT TGTGGAATTT TTAATTCTTA CCTTTGTTTT TGTCTTTGGC
       AAGTTAACAA ACACCTTAAA AATTAAGAAT GGAAACAAAA ACAGAAACCG
2501   AGTATGGTGC AGAAGAACTG ATGAACTGGT AGATGGCCCT AACTCGTCTT
       TCATACCACG TCTTCTTGAC TACTTGACCA TCTACCGGGA TTGAGCAGAA
                              ClaI
                             ------
2551   ACATTACACC AAAGGCACTT GATCGATGGG AGAAGAGATT AGAAGATCTC
       TGTAATGTGG TTTCCGTGAA CTAGCTACCC TCTTCTCTAA TCTTCTAGAG
2601   TTCGAAGGCA GGCCATATGA TATGTATGAT GCAGCCCTCT CGGACACAGT
       AAGCTTCCGT CCGGTATACT ATACATACTA CGTCGGGAGA GCCTGTGTCA
                         EcoRV
                        -------
2651   GTCAAAGTTT CCAGTAGATA TCCAGGTACA GCCAACAAAA AAGCTACTGT
       CAGTTTCAAA GGTCATCTAT AGGTCCATGT CGGTTGTTTT TTCGATGACA
                              DraI
                             ------
2701   AAAAGCAGGA TATGAAGTGC TTTTAAAAGT TATTAGTACA CAGTAAATTG
       TTTTCGTCCT ATACTTCACG AAAATTTTCA ATAATCATGT GTCATTTAAC
2751   GTGACACGCA TTTGTCTGAT GTTTCTGCAC CGTCGGGATC TCTTTTATTT
       CACTGTGCGT AAACAGACTA CAAAGACGTG GCAGCCCTAG AGAAAATAAA
2801   ACAGCCATTC AAAGACATGA TTGAAGGAAT GAGGCTTGAC CTGTGGAAAT
       TGTCGGTAAG TTTCTGTACT AACTTCCTTA CTCCGAACTG GACACCTTTA
                              SacI
                             ------
2851   CAAGGTATAG GAGCTTTGAT GAGCTCTACC TCTACTGCTA CTACGTTGCT
       GTTCCATATC CTCGAAACTA CTCGAGATGG AGATGACGAT GATGCAACGA
2901   GGCACGGTTG GTCTCATGAC AGTACCGGTG ATGGGGATTG CCCCCGACTC
       CCGTGCCAAC CAGAGTACTG TCATGGCCAC TACCCCTAAC GGGGGCTGAG
2951   GAAGGCCTCA ACCGAGAGCG TGTACAACGC TGCGCTAGCT CTTGGGATCG
       CTTCCGGAGT TGGCTCTCGC ACATGTTGCG ACGCGATCGA GAACCCTAGC
3001   CCAACCAGCT GACGAATATT CTCAGAGACG TAGGCGAAGA GTAAGTACCA
       GGTTGGTCGA CTGCTTATAA GAGTCTCTGC ATCCGCTTCT CATTCATGGT
3051   ATGTGCTCAC TACCAGCATG TTCTTTTTCT GAGTTAATAA TTGCAGACTT
       TACACGAGTG ATGGTCGTAC AAGAAAAAGA CTCAATTATT AACGTCTGAA
3101   TCACATGATA AATATGTAGC TTGACACCCA ATATGTATTG CACAAACCAT
       AGTGTACTAT TTATACATCG AACTGTGGGT TATACATAAC GTGTTTGGTA
              DraI
             -------
3151   TTGCGCTTTA AAGTCTAGTC CAAAATTGTG CCTAAACAGA TGCCTTTCAC
       AACGCGAAAT TTCAGATCAG GTTTTAACAC GGATTTGTCT ACGGAAAGTG
3201   ATTAAAAATT TGAAGCGCAC ATAAGTTTAA TTTAAGGCAT ATGTACCGAT
       TAATTTTTAA ACTTCGCGTG TATTCAAATT AAATTCCGTA TACATGGCTA
3251   TTGTCGCTAT TGAGATTAAG TTTTTTGGTT GATTGTGATG TAATATGTTT
       AACAGCGATA ACTCTAATTC AAAAAACCAA CTAACACTAC ATTATACAAA
3301   GTTGGGATGG AATTTCAGCT CAAGGAGGGG AAGAATCTAC CTTCCATTGG
       CAACCCTACC TTAAAGTCGA GTTCCTCCCC TTCTTAGATG GAAGGTAACC
3351   ATGAATTGGC AGAGGCAGGT CTGACAGAAG AAGACATATT CAGAGGGAAA
       TACTTAACCG TCTCCGTCCA GACTGTCTTC TTCTGTATAA GTCTCCCTTT
3401   GTGACTGATA AATGGAGGAA GTTCATGAAG GGACAAATTC TGCGTGCCAG
       CACTGACTAT TTACCTCCTT CAAGTACTTC CCTGTTTAAG ACGCACGGTC
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3451   GTTATTCTTT GATGAGGCGG AGAAGGGCGT TGCGCATCTA GACTCTGCGA
       CAATAAGAAA CTACTCCGCC TCTTCCCGCA ACGCGTAGAT CTGAGACGCT
3501   GTAGATGGCC GGTATGAGAG GCTACAAATT CTGCGGTTTT ATGTTCCACA
       CATCTACCGG CCATACTCTC CGATGTTTAA GACGCCAAAA TACAAGGTGT
                                    ClaI
                                    ------
3551   AATAAAATAA ACCACTGAAA TTTACCTTAA TCGATTATTT TCTCGTAGGT
       TTATTTTATT TGGTGACTTT AAATGGAATT AGCTAATAAA AGAGCATCCA
3601   TCTGGCATCT TTGTGGTTAT ACCGGCAGAT CCTTGATGCT ATCGAAGCAA
       AGACCGTAGA AACACCAATA TGGCCGTCTA GGAACTACGA TAGCTTCGTT
3651   ACGACTACAA CAACTTCACC AAGCGCGCGT ATGTAAACAA GGCAAAGAAG
       TGCTGATGTT GTTGAAGTGG TTCGCGCGCA TACATTTGTT CCGTTTCTTC
3701   CTGCTGTCTT TACCGGTCGC TTATGCAAGA GCGGCAGTTG CATCATGAAC
       GACGACAGAA ATGGCCAGCG AATACGTTCT CGCCGTCAAC GTAGTACTTG
3751   AATCACTAGA TCAGATGCCT TATTATTTTT TTTCTTTCAT TTTCTTTTCC
       TTAGTGATCT AGTCTACGGA ATAATAAAAA AAAGAAAGTA AAAGAAAAGG
3801   TTTGATTTCG CACGATTTCT TGGCTGTTGT ATATATTCAA GCAGCTACCT
       AAACTAAAGC GTGCTAAAGA ACCGACAACA TATATAAGTT CGTCGATGGA
3851   GTATGCCATA AGCCTGCCAC AGTTTTTCTT TAGTTCAAGG GACTGATTTC
       CATACGGTAT TCGGACGGTG TCAAAAAGAA ATCAAGTTCC CTGACTAAAG
3901   AGGTCCCTCA ATACTCAACT CTTGTTAGAA ACAAATACAG AGGGGGTAAG
       TCCAGGGAGT TATGAGTTGA GAACAATCTT TGTTTATGTC TCCCCCATTC
                                                   EcoRI
                                                   ----
3951   CCCCACAGTT CAAGAAGCAT ATTACTCTTC ATTTGTCTTC AAGAATTCAA
       GGGGTGTCAA GTTCTTCGTA TAATGAGAAG TAAACAGAAG TTCTTAAGTT
```

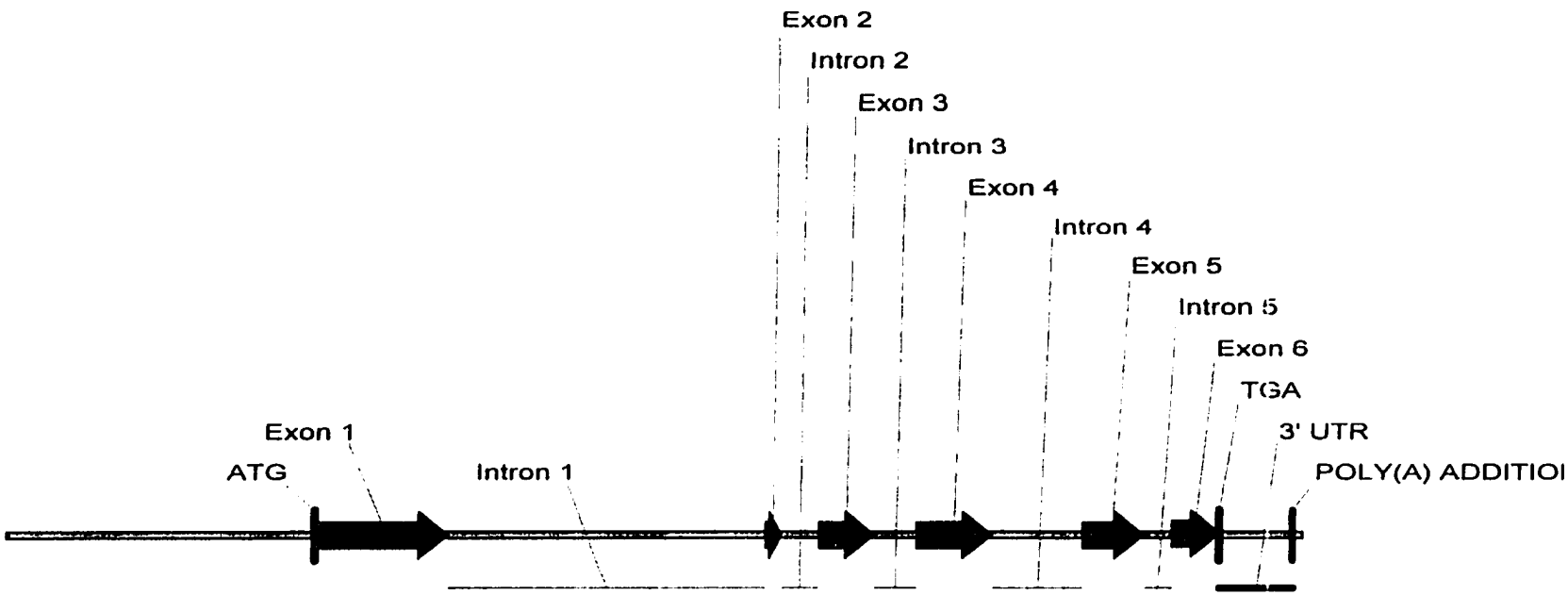
Appendix E

Rice *Psy* gene (Genbank AY024351) and maize *Psy* (ZMU32636) features, including introns and exons

Feature Map

- Intron (5 total)
  - Intron 1
    Start: 1353 End: 2337
  - Intron 2
    Start: 2387 End: 2502
  - Intron 3
    Start: 2676 End: 2805
  - Intron 4
    Start: 3041 End: 3319
  - Intron 5
    Start: 3512 End: 3599
- Misc. Feature (2 total)
  - ATG
    Start: 941 End: 943
  - TGA
    Start: 3746 End: 3748
- PolyA Site (1 total)
  - POLY(A) ADDITION
    Start: 3969 End: 3974
- 3' UTR (1 total)
  - 3' UTR
    Start: 3746 End: 3974
- Exon (6 total)
  - Exon 1
    Start: 941 End: 1352
  - Exon 2
    Start: 2337 End: 2386
  - Exon 3
    Start: 2502 End: 2675
  - Exon 4
    Start: 2805 End: 3040
  - Exon 5
    Start: 3319 End: 3511
  - Exon 6
    Start: 3599 End: 3748

Graphical feature map of Rice *Psy* (Genbank AY024351)



Rice PSY gene with introns and exons

4000 bp

ZMU32636 MAIZE PSY GENE introns and exons

Feature Map

Exon (6 total)

Exon 1
Start: 1934 End: 2344

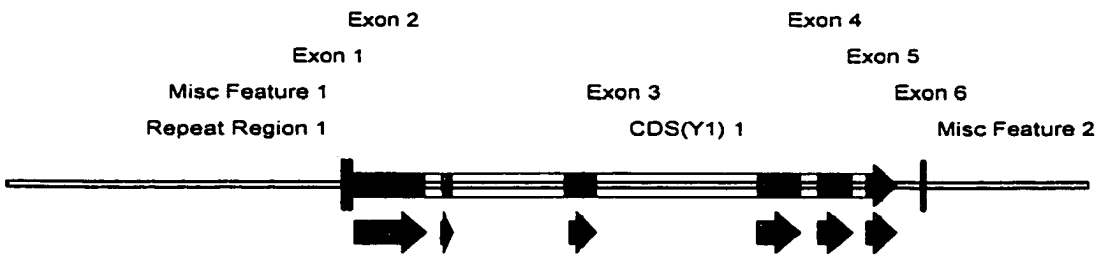
Exon 2
Start: 2428 End: 2496

Exon 3
Start: 3134 End: 3277

Exon 4
Start: 4160 End: 4398

Exon 5
Start: 4494 End: 4688

Exon 6
Start: 4763 End: 4933



ZMU32636 MAIZE PSY GENE
5995 bp

Appendix F. Rice PSY protein exon domain map

Feature Map

Domain: Misc (6 total)

Exon1
Start: 1 End: 138

Exon2
Start: 139 End: 154

Exon3
Start: 155 End: 212

Exon4
Start: 213 End: 290

Exon5
Start: 291 End: 355

Exon6
Start: 356 End: 404

Graphic of exons drawn to scale on protein:

Rice PSY Exon Domain Map
405 aa

Appendix G Intron splice junctions and intron/exon sizes of rice Psy (Genbank AY024351)

Splice junctions

Rice PSY	Start	Stop	Size	5' Splice	3' Splice
Intron 1	413	1397	984	ag/gtcagc	ttcag/gt
Intron 2	1450	1562	112	gt/atggat	tggca/gt
Intron 3	1736	1865	129	ag/gtacag	tacag/cc
Intron 4	2101	2379	278	ga/gtaagt	ttcag/ct
Intron 5	2575	2659	84	ta/tgagag	cgtag/gt

Introns and exon sizes and coding regions

Feature	Number of nucleotides	Codes for amino acid #
Exon 1	411	1-138
Intron 1	948	
Exon 2	49	139-154
Intron 2	115	
Exon 3	173	155-212
Intron 3	129	
Exon 4	235	213-290
Intron 4	278	
Exon 5	192	291-355
Intron 5	87	
Exon 6	149	356-404

Appendix H Selected oligonucleotides used for sequencing rice *Psy* (Genbank AY024351)

451

General Description
DNA 451
Entire molecule length: 22 bp

Sequence
1 aagagaggga tgaaactggg tc

450

General Description
DNA 450
Entire molecule length: 22 bp

Sequence
1 agtcccaacc ctccacctag ga

448

General Description
DNA 448
Entire molecule length: 22 bp

Sequence
1 tggttacccc caattaattt ta

447

General Description
DNA 447
Entire molecule length: 22 bp

Sequence
1 ggtcattggt tggggtttgt tt

446

General Description
DNA 446
Entire molecule length: 22 bp

Sequence
1 gtgggggccc accttcaaaa cc

445

General Description
DNA 445
Entire molecule length: 22 bp

Sequence
1 cagcggcctc accgacgagg cg

444

General Description
DNA 444
Entire molecule length: 22 bp

Sequence
1 gaggaggagg ccccagtcga cg

443

General Description
DNA 443
Entire molecule length: 22 bp

Sequence
1 ttagtcaatg ttgggttggc tg

442

General Description
DNA 442
Entire molecule length: 22 bp

Sequence
1 gaatcctgtt ccacttgccg tg

441

General Description
DNA 441
Entire molecule length: 22 bp

Sequence
1 ggggtaacca atttaaaccc ac

428

General Description
DNA 428
Entire molecule length: 22 bp

Sequence
1 cgcttggtga agttgttgta gt

427

General Description
DNA 427
Entire molecule length: 22 bp

Sequence
1 gcatacaggt agctgcttga at

426

General Description
DNA 426
Entire molecule length: 22 bp

Sequence
1 caaattctgc gtgccaggtt at

425

General Description
DNA 425
Entire molecule length: 24 bp

Sequence
1 gcaccgtcgg gatctctttt attt

424

General Description
DNA 424
Entire molecule length: 22 bp

Sequence
1 cttctaccta ggtcagccaa cc

423

General Description
DNA 423
Entire molecule length: 22 bp

Sequence
1 gtggccgtgg cgtcgaggag ac

422

General Description
DNA 422
Entire molecule length: 22 bp

Sequence
1 tctgcaggcc tcaccgaccg ag

421

General Description
DNA 421
Entire molecule length: 22 bp

Sequence
1 cctccaccag cgccgcctgc cg

417

General Description
DNA 417
Entire molecule length: 21 bp

Sequence
1 gaaatggata aaagtaataa c

416

General Description
DNA 416
Entire molecule length: 22 bp

Sequence

1 ggaatctagg aatgttcata ga

415

General Description
DNA 415
Entire molecule length: 22 bp

Sequence
1 gccatcagaa ggagtattcc at

414

General Description
DNA 414
Entire molecule length: 22 bp

Sequence
1 cagcataatc cctggaccct aa

413

General Description
DNA 413
Entire molecule length: 22 bp

Sequence
1 tttgacactg tgtccgagag gg

412

General Description
DNA 412
Entire molecule length: 22 bp

Sequence
1 cggtatgaga ggctacaaat tc

411

General Description
DNA 411
Entire molecule length: 22 bp

Sequence
1 caatcgctag atcagatgcc tt

410

General Description
DNA 410
Entire molecule length: 22 bp

Sequence
1 tttctgtcag acctgcctct gc

409

General Description
DNA 409
Entire molecule length: 22 bp

Sequence
1 tttacatacg cgcgcttggt ga

408

General Description
DNA 408
Entire molecule length: 22 bp

Sequence
1 ttcttgaact gtggaggctt ac

407

General Description
DNA 407
Entire molecule length: 22 bp

Sequence
1 tcacatcacc cacaaaccca ct

406

General Description
DNA 406
Entire molecule length: 22 bp

Sequence
1 cccagacagc tttgcgcctt tc

404

General Description
DNA 404
Entire molecule length: 22 bp

Sequence
1 aaccagcata tccctgaccc ta

403

General Description
DNA 403
Entire molecule length: 22 bp

Sequence
1 gcagaagaac tgatgaactg gt

402

General Description
DNA 402
Entire molecule length: 22 bp

Sequence
1 aacctttgg ggactagctc aa

401

General Description
DNA 401

Entire molecule length: 24 bp

Sequence

1 gccgacttca gagtaggtaa gata

400

General Description

DNA 400

Entire molecule length: 22 bp

Sequence

1 ggntnctgnc cctaattant tg

399

General Description

DNA 399

Entire molecule length: 22 bp

Sequence

1 ggntnctgnc cctaattant tg

398

General Description

DNA 398

Entire molecule length: 22 bp

Sequence

1 ataccgtgga cctcgagnaa gg

397

General Description

DNA 397

Entire molecule length: 25 bp

Sequence

1 tgtaggactt ttagcttatg tgaca

396

General Description

DNA 396

Entire molecule length: 22 bp

Sequence

1 ggntnctgnc cctaattant tg

318

General Description

DNA 318

Entire molecule length: 21 bp

Sequence

1 acaccaccct cttacaattc c

Appendix I Genbank flat files rice Psy partial cDNA AY024350 and Psy gene AY024351

```
LOCUS       AY024350   1060 bp    mRNA            PLN       20-JAN-2001
DEFINITION  Oryzae sativa, Strain: Nipponbare, green shoot (8 days old),
            phytoene synthase, cDNA, partial cds, dbEST ID 146174.
ACCESSION   ;
KEYWORDS    .
SOURCE      Oryza sativa.
  ORGANISM  Oryza sativa
            Eukaryota; Viridiplantae; Embryophyta; Tracheophyta; Spermatophyta;
            Magnoliophyta; Liliopsida; Poales; Poaceae; Ehrhartoideae; Oryzeae;
            Oryza.
REFERENCE   1  (bases 1 to 1060)
  AUTHORS   Sasaki,T., Miyao,A. and Yamamoto,K.
  TITLE     Rice cDNA from callus 1995
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1060)
  AUTHORS   Matthews,P.D. and Wurtzel,E.T.
  TITLE     Cloning and characterization of a phytoene synthase gene from rice
            (Oryzae sativa L.)
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 1060)
  AUTHORS   Matthews,P.D. and Wurtzel,E.T.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-JAN-2001) Biological Sciences, Lehman College, City
            University of New York, 250 Bedford Park Blvd. W., Bronx, NY 10468,
            USA
FEATURES             Location/Qualifiers
     source          1..1060
                     /organism="Oryza sativa"
                     /strain="Nipponbare"
                     /db_xref="taxon:4530"
                     /chromosome="12"
                     /map="above RG 181"
BASE COUNT      303 a    224 c    259 g    274 t
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       61 atctatgtat ggtgcagaag aactgatgaa ctggtagatg gccctaactc gtcttacatt
      121 acaccaaagg cacttgatcg atgggagaag agattagaag atctcttcga aggcaggcca
      181 tatgatatgt atgatgcagc cctctcggac acagtgtcaa agtttccagt agatatccag
      241 ccattcaaag acatgattga aggaatgagg cttgacctgt ggaaatcaag gtataggagc
      301 tttgatgagc tctacctcta ctgctactac gttgctggca cggttggtct catgacagta
      361 ccggtgatgg ggattgcccc cgactcgaag gcctcaaccg agagcgtgta caacgctgcg
      421 ctagctcttg ggatcgccaa ccagctgacg aatattctca gagacgtagg cgaagactca
      481 aggaggggaa gaatctacct tccattggat gaattggcag aggcaggtct gacagaagaa
      541 gacatattca gagggaaagt gactgataaa tggaggaagt tcatgaaggg acaaattctg
      601 cgtgccaggt tattctttga tgaggcggag aagggcgttg cgcatctaga ctctgcgagt
      661 agatggccgg ttctggcatc tttgtggtta taccggcaga tccttgatgc tatcgaagca
      721 aacgactaca acaacttcac caagcgcgcg tatgtaaaca aggcaaagaa gctgctgtct
      781 ttaccggtcg cttatgcaag agcggcagtt gcatcatgaa caatcactag atcagatgcc
      841 ttattatttt ttttctttca ttttcttttc ttttgatttc gcacgatttc ttggctgttg
      901 tatatattca agcagctacc tgtatgccat aagcctgcca cagtttttct ttagttcaag
      961 ggactgattt caggtccctc aatactcaac tcttgttaga aacaaataca gaggggtaa
     1021 gccccacagt tcaagaagca tattaaaaaa aaaaaaaaaa
//
```

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LOCUS       AY024351   3222 bp    DNA             PLN       20-JAN-2001
DEFINITION  Oryzae sativa, Strain: Nipponbare, phytoene synthase gene.
ACCESSION   ;
KEYWORDS    .
SOURCE      Oryza sativa.
  ORGANISM  Oryza sativa
            Eukaryota; Viridiplantae; Embryophyta; Tracheophyta; Spermatophyta;
            Magnoliophyta; Liliopsida; Poales; Poaceae; Ehrhartoideae; Oryzeae;
            Oryza.
REFERENCE   1  (bases 1 to 3222)
  AUTHORS   Matthews,P.D. and Wurtzel,E.T.
  TITLE     Cloning and characterization of a phytoene synthase from
            rice(Oryzae sativa L.)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 3222)
  AUTHORS   Matthews,P.D. and Wurtzel,E.T.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-JAN-2001) Biological Sciences, Lehman College, City
            University of New York, 250 Bedford Park Blvd. W., Bronx, NY 10468,
            USA
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                     /strain="Nipponbare"
                     /db_xref="taxon:4530"
                     /chromosome="12"
                     /map="above RG 181"
     exon            170..581
     exon            1566..1615
     exon            1731..1904
     exon            2034..2269
     exon            2548..2740
     exon            2828..2977
BASE COUNT      843 a    669 c    756 g    942 t     12 others
ORIGIN
        1 gatccaatag ttanagaaag gncgccanng nggnggaagt ncaanttttg ttccanttan
       61 tgaagggnna aatgggggcc ccccccccc ccagaaaaat taattaaaat taattggggg
      121 taaccaattt aaacccaccc tcctttcaaa agaaaacaaa ccccaaccaa tgaccgggga
      181 aggaagccca aaccaaaatt gccggggggc cccaaggggt ctcttggcgg ggggttttga
      241 aggtgggccc ccacccccac ggcaagtgga acaggattca agccattttt cacaaggtta
      301 caacgaaggg gaagaagccg gttgttgtcg cctcgtcggt gaggccgctg caggccgcga
      361 gcctggcggt ggccacggcg ccggtggccg tggcgtcgag gaggacggcg gcggagaggc
      421 gtctagaggt cggctcggca cgcgctgtgg aaggccccac cgcgcggcgc gggggcgccg
      481 cggtgggcga ggaggacgcg tcgactgggg cctcctcctc ggcgacgcct accaccgctg
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      601 actaatcatt caattttgtt tttatcaccg ttattacttt atcatttcac aggtgaaagc
      661 tagctactat tcgtttgcca ctactactcc ctagcttagc tagcgtctct tccttttctt
      721 gatttgttca tagtactgta tatgataatt gtagacctac cctttctcta tttctttttg
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      841 gccacatctc actttcactt tcttgtactt gttggtctaa aaagattagt taatgctgca
      901 attagttggc ttttactagg aattgtaaga gggtggtgtt aaaaggtgat taaaaaaaaa
      961 tctatgaaca ttcctagatt cctattacgt gttaagaaga taaatattcc tgcgtataat
     1021 ttatggaata ctccttctga tggcaaaaat atattagcag tagccaaaca acaccagcct
     1081 gagtgagtga gtggctctca gtgctctgaa caactgtaca tgtatataca aatgtttgcc
     1141 gacttcagag taggtaagat aatgcacttg gatgatagat gccatcccaa ggaaatgatc
     1201 caacaagcag atgcgcagat atggtgatct ctctagtgca ccactggcaa tttatagatt
     1261 tctcttctta attagggtca gggatatgct ggttggtagt gggtttgtgg gtgatgtgag
     1321 gacgcatggg cttgattgat gaatccagta accttcttgt ttttttaat cttgctttct
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1501 acgtaaagtg gaatttcacc ttgtgtaatt tctcatggtt tttgtttcca tcgtttacat
1561 ttcaggtact cagcttatga ctcctgaaag gcgcaaagct gtctgggcaa tctatggtat
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1801 atcgatggga gaagagatta gaagatctct tcgaaggcag gccatatgat atgtatgatg
1861 cagccctctc ggacacagtg tcaaagtttc cagtagatat ccaggtacag ccaacaaaaa
1921 agctactgta aaagcaggat atgaagtgct tttaaaagtt attagtacac agtaaattgg
1981 tgacacgcat ttgtctgatg tttctgcacc gtcgggatct cttttattta cagccattca
2041 aagacatgat tgaaggaatg aggcttgacc tgtggaaatc aaggtatagg agctttgatg
2101 agctctacct ctactgctac tacgttgcrg gcacggttgg tctcatgaca gtaccggtga
2161 tggggattgc ccccgactcg aaggcctcaa ccgagagcgt gtacaacgct gcgctagctc
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2341 atatgtagct tgacacccaa tatgtattgc acaaaccatt tgcgctttaa agtctagtcc
2401 aaaattgtgc ctaaacagat gcctttcaca ttaaaaattt gaagcgcaca taagtttaat
2461 ttaaggcata tgtaccgatt tgtcgctatt gagattaagt tttttggttg attgtgatgt
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2581 tgaattggca gaggcaggtc tgacagaaga agacatattc agagggaaag tgactgataa
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2761 tgcggtttta tgttccacaa ataaaataaa ccactgaaat ttaccttaat cgattatttt
2821 ctcgtaggtt ctggcatctt tgtggttata ccggcagatc cttgatgcta tcgaagcaaa
2881 cgactacaac aacttcacca agcgcgcgta tgtaaacaag gcaaagaagc tgctgtcttt
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3001 attatttttt ttctttcatt ttcttttcct ttgatttcgc acgatttctt ggctgttgta
3061 tatattcaag cagctacctg tatgccataa gcctgccaca gtttttcttt agttcaaggg
3121 actgatttca ggtccctcaa tactcaactc ttgttagaaa caaatacaga gggggtaagc
3181 cccacagttc aagaagcata ttactcttca tttgtcttca ag
//
```

Appendix J A co-authored publication related to this dissertation

Plant Molecular Biology 30: 269–279, 1996.

Cloning and characterization of a maize cDNA encoding phytoene desaturase, an enzyme of the carotenoid biosynthetic pathway

Zhou-Hui Li[1], Paul D. Matthews[1], Benjamin Burr[2] and Eleanore T. Wurtzel[1,*]
[1] *Department of Biological Sciences, Lehman College, The City University of New York, 250 Bedford Park Boulevard, West Bronx, NY 10468, USA (*author for correspondence); [2] Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA*

Received 3 August 1995; accepted in revised form 12 October 1995

Key words: carotenoid biosynthesis, endosperm, gene, maize, phytoene desaturase, regulation

Abstract

To study regulation of the plastid-localized maize carotenoid biosynthetic pathway, a cDNA encoding phytoene desaturase (PDS) was isolated and characterized. The DNA sequence of the maize *Pds* cDNA was determined and compared with available dicot *Pds* genes. The deduced PDS protein, estimated at 64.1 kDa (unprocessed), had a dinucleotide binding domain and conserved regions characteristic of other carotene desaturases. Alignment of available PDS sequences from distantly related organisms suggests that *Pds* has potential as a phylogenetic tool. By use of heterologous complementation in *Escherichia coli*, maize PDS was shown to catalyze two desaturation steps converting phytoene to ζ-carotene. RFLP (restriction fragment length polymorphism) mapping was used to place *Pds* on chromosome 1S near *viviparous5* (*vp5*), and RT-PCR (reverse-transcriptase polymerase chain reaction) analysis indicated reduced *Pds* transcript in *vp5* mutant relative to normal endosperm. Other phytoene-accumulating mutant endosperms, *vp2* and *white3* (*w3*), showed no difference in *Pds* transcript accumulation as compared with normal endosperm counterparts. RT-PCR analysis of *Pds* transcript accumulation in developing endosperm showed *Pds* was constitutively expressed. Therefore, endosperm carotenogenesis is not regulated by increasing the level of *Pds* transcripts.

Introduction

Carotenoids serve multiple functions in plants; as accessory pigments in photosynthesis; as photoprotectors; and as precursors to the hormone, abscisic acid (ABA). In animals, carotenoids are essential precursors to Vitamin A and related compounds (reviewed in [2]). Carotenoids are synthesized and accumulated in plastids; these plastids include chloroplasts and nonphotosynthetic plastids, such as chromoplasts of fruits, flowers, some endosperms and roots (for reviews, see [5, 12, 29]).

Because of the varied roles and location of

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Database under the accession number U37285.

carotenoids within plastids of different membrane architectures, we expect tissue-specific regulation of the pathway. Maize is an excellent model system to explore regulation of carotenoid biosynthesis because of the many mapped and biochemically characterized mutations blocking the pathway. These include recessive, dominant, and suppressor/modifier alleles [27].

The synthesis of C_{40} carotenoids begins with condensation of two molecules of geranylgeranyl pyrophosphate to produce phytoene, a step catalyzed by phytoene synthase (PSY). Phytoene synthesis occurs in plastid stroma, whereas subsequent steps leading to synthesis of colored carotenoids occur on plastid membranes [4, 17, 21, 23]. This latter phase includes four sequential desaturations of phytoene. In dicots and cyanobacteria, these steps are catalyzed by two enzymes, PDS (phytoene desaturase) and ZDS (ζ-carotene desaturase), each mediating two steps (reviewed in [3]). In other carotenogenic organisms, including fungi, nonphotosynthetic bacteria, and photosynthetic bacteria, one phytoene desaturase enzyme may catalyze up to four desaturation steps (reviewed in [2]). As shown in Fig. 1, recessive alleles of four maize loci, *vp2*, *vp5*, *w3*, and *vp9*, block desaturation both in endosperm and plant [25, 32]. It is presently unclear what functions are represented by these genes and whether these genes reflect an alternative array of overlapping or non-overlapping desaturation functions, each mediating a particular number of steps, or encode regulatory or ancillary functions such as pigment-binding proteins or oxidoreductases [22].

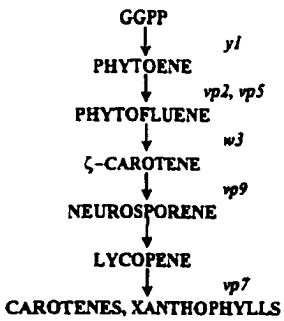

Fig. 1. Lethal recessive mutations blocking the carotenoid biosynthetic pathway of maize. *w3* results in accumulation of both phytoene and phytofluene. Many, but not all, *y1* mutations affect only endosperm and are not lethal to the plant.

With the exception of maize *Y1*, which has been shown by heterologous complementation to encode PSY [6; Yoganathan and Wurtzel, unpublished], there has been little characterization of genes encoding the biosynthetic enzymes in monocots. Genes encoding PDS have primarily been isolated from dicots (reviewed in [3]) and the temporal regulation of their expression examined in chromoplasts [10, 11, 15, 26]. Here PDS transcript abundance appears to correlate with carotenoid accumulation. Therefore, we decided to isolate the maize gene encoding PDS to characterize the temporal regulation of PDS expression in endosperm plastids (amyloplasts). Using RFLP mapping and analysis of steady-state levels of accumulated transcripts, we have associated a known maize carotenoid locus with a specific gene product, PDS. To demonstrate the function and determine the number of desaturation steps catalyzed by maize PDS, we employed a heterologous complementation system. Finally, we showed that the carotenoid biosynthetic pathway in maize endosperm was not regulated by modulation of *Pds* transcript levels.

Material and methods

Plant materials

For developmental studies, maize plants were grown under standard field conditions at the Black Rock Forest (Cornwall, NY). For other studies, plants were grown either in the field or in greenhouses at Lehman College, City University of New York. Inbred lines were obtained from Dr S. Briggs (Pioneer Hy-Bred) and mutant lines were provided by Dr D. Robertson (Iowa State University) and from the Maize Coop (University of Illinois, Urbana, IL). The *vp5* mutant used in this study was generated in a *Mutator* background, by Dr Robertson, and is therefore designated *vp5-*

Mum. By introduction of this mutation into another genetic background in which the *Mutator* element was inactive, a stable phenotype was obtained and revertant sectors no longer observed. *vp2* and *w3* are stable mutations. Developing endosperms were collected and frozen at −80 °C prior to use.

Amplification of the maize Pds *gene*

The CLUSTAL program [14] from PC/Gene software (Intelligenetics, Mountain View, CA) was used to align *Pds* sequences from tomato (GenBank accession numbers X59948, S36691), soybean (M64704), pepper (X68058) and *Arabidopsis* (L16237). A region of high homology, corresponding to nucleotides 1905–2099 of tomato *Pds*, was shared by all available dicot *Pds* genes. Degenerate oligonucleotide primers, 5′-CCTGATGAAATC(T)TCG(A,T)GCG(A,T)GAC(T)CA-3′ and 5′-ACAGCA(G)CCTTCCATG(T)GAAGCC(T)AA)-3′, were used to amplify the corresponding region of maize *Pds* from maize B73 genomic DNA as follows: DNA, 0.1 μg, in 20 μl PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1 mM $MgCl_2$, 160 μM each dNTP, 0.4 μM each primer; 1 μg/μl bovine serum albumin (BSA), 0.1 unit/μl *Taq* polymerase (Gibco-BRL, Gaithersburg, MD)) was incubated for 1 cycle at 94 °C (3 min), followed by 40 cycles at 94 °C (30 s), 48 °C (30 s), 72 °C (30 s) and one cycle of 72 °C (10 min).

Isolation of maize Pds *cDNA clones*

One to two million clones of a λgt11 cDNA library [9], prepared from RNA extracted from maize endosperm dissected at 14 days after pollination (DAP), were screened with the PCR-amplified maize *Pds* fragment [28]. Seven positive clones were isolated, phage DNA extracted according to Sambrook *et al.* [28], and inserts amplified using primers, 5′-AGGCACATGGCTGAATATCG-3′ and 5′-CGGCAGTACAATGGATTTCC-3′. Lambda DNA, 0.1 μg, in 20 μl PCR buffer (20 mM Tris pH 8.2, 10 mM KCl, 2.5 mM $MgCl_2$, 1 mM $(NH_4)_2SO_4$, 200 μM each dNTP; 1 μM each primer, 1 μg/μl BSA, 0.1% Triton X-100, 0.025 unit/μl *Pfu* DNA polymerase (Stratagene)) was incubated for one cycle at 94 °C (2.5 min), then 40 cycles at 94 °C (30 s), 55 °C (30 s), 72 °C (2 min) and one cycle of 72 °C (10 min).

DNA sequence analysis

The *fmol*™ DNA Sequencing System (Promega, Madison, WI) was used for initial sequencing of phage DNA inserts using primers described for PCR. The plasmid deletion series prepared for the complementation analysis was used for making single-stranded templates for sequence analysis of the entire gene. Complete sequencing of maize *Pds* was carried out using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH). Sequence analysis and homology comparisons were carried out using PCGene software (Intelligenetics). Alignments were carried out using the program CLUSTAL. *Pds* sequences used for comparisons shown in Figs. 3 and 4 are as follows: maize, this paper (GenBank U37285); for *Arabidopsis*, pepper, soybean, tomato, see GenBank numbers listed above; *Synechocystis*, X62574; *Synechococcus*, X55289; sequence encoding CRTI from *Erwininia herbicola*, GenBank M87280; sequence encoding CRTI and CRTD from *Rhodobacter sphaeroides*, X82458; sequence encoding ZDS from *Anabaena*, S43324. Maize and pepper *rbcL* sequences for DNA and protein comparisons described in the discussion were deposited under accession numbers Z11973 and U08610, respectively.

Subcloning, expression and functional complementation of maize Pds

Amplified λgt11 inserts were purified by adsorption to Glass-Milk (GeneClean II kit, BIO 101, Vista, CA) following the manufacturer's direc-

tions, treated at 37 °C for 1 h with Klenow fragment and 10 mM dNTP to create blunt ends, and then ligated to *Sma*I-linearized vector, pBluescript II SK(−) (Stratagene). One clone, found to be in the sense orientation with respect to *lacZ*, on the basis of sequencing and restriction mapping, was designated pMPDS3. This plasmid was purified by CsCl equilibrium density centrifugation according to Sambrook *et al.* [28]. To create an in-frame fusion with *lacZ*, the plasmid was linearized with *Not* I and *Bst* XI, and subjected to 5'-end deletions using the Exonuclease III and Mung Bean Nuclease Deletion Kit (Stratagene, La Jolla, CA). Religated plasmids were transformed into *Escherichia coli* JM101 containing plasmid pACCRT-EB, encoding GGPPS and PSY from *Erwinia uredovora* [19]. Transformants containing both the deletion derivative of pMPDS3 and pACCRT-EB were selected by resistance to ampicillin and chloramphenicol and then grown in liquid culture (LB medium) with appropriate antibiotics. Expression of the LacZ-PDS fusion proteins was induced by addition of isopropylthio-β-D-galactoside (IPTG) (1 mM final concentration) during log phase. After growth to stationary phase, pigments were extracted and analyzed by HPLC.

Pigment extraction and HPLC analysis

Fifteen ml stationary phase cultures of *E. coli* cells were pelleted and resuspended in 20 ml methanol and carotenoids extracted according to Sandmann [30], except that 15 ml petroleum ether was used in place of diethyl ether. Pigments were separated by reverse phase HPLC, using a 25 cm × 4.6 mm Spherisorb ODS-1 5u C18 column (Phenomenex, Torrance, CA), and a solvent of acetonitrile/methanol/isopropanol (85:10:5) with flow rate of 1 ml/min using a Series 410 BIO LC Pump (Perkin Elmer, Norwalk, CT). Peaks were detected using an LC-480 Auto Scan photodiode array detector (Perkin Elmer). Alternatively, a Waters HPLC system with 600 controller and pump, a 996 photodiode array detector, and WISP 717 autosampler were used. Peaks were identified on the basis of co-migration and shared spectrophotometric profiles with known standards.

RNA extraction and RT-PCR

Total RNA of maize was extracted from endosperms collected at various DAP and from leaves of young plants (2–3 leaf stage) [20]. RNA pellets were resuspended in DEPC (diethyl pyrocarbonate)-treated water, centrifuged 5 min at 14 000 rpm in an Eppendorf centrifuge, and the supernatants collected. RNA concentration was estimated spectrophotometrically and total RNA (1 μg) used as template for cDNA synthesized with the SuperScript Preamplification System (Gibco-BRL) for first-strand cDNA synthesis. One fourth (5 μl) of product, ca. 1–4 ng cDNA, was used for PCR in a final volume of 25 μl. The amount of total RNA used was first tested to ensure linearity of response in the RT-PCR reaction (data not shown). Primers used for amplification were: *Pds*, 5'-GGAACTGTGAAACACTTCGC-3' and 5'-GAAACCTTCGATAGGTGACC-3'; *Sh1*, 5'-ATCCCTGAGAAAGGCAGAGG-3' and 5'-AGTGACTCCCAACTTGTGCG-3' (GenBank accession number X02382). The conditions for PCR were: 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 100 μg/μl BSA; 2 mM DTT (dithiothreitol), 0.1 mM each dNTP, 0.4 μM each primer, 0.1 μl/μl *Taq* DNA polymerase (BRL). The protocol for PCR was: one cycle of 94 °C (2 min), followed by 40 cycles of 94 °C (30 s), 52 °C (30 s), 72 °C (30 s) and one cycle of 72 °C (10 min). 10 μl of each PCR reaction was analyzed by electrophoresis on 1.8% agarose gels in 0.5 × TBE. The sizes of the PCR products for *Sh* and for *Pds* were 673 and 528 bp, respectively.

Results

Isolation of a maize Pds *cDNA*

Since we observed that dicot *Pds* genes hybridized poorly to monocot sequences, we chose to amplify maize *Pds* from genomic DNA using de-

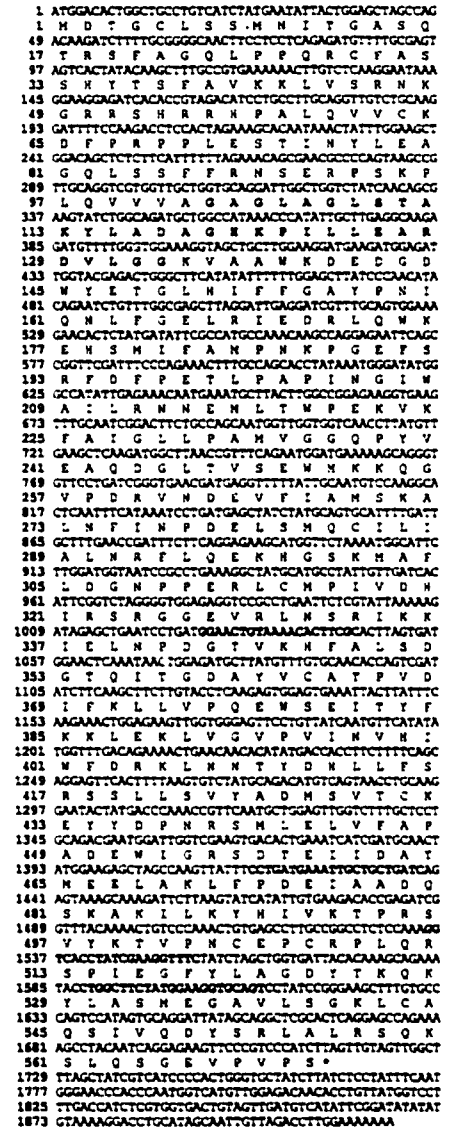

Fig. 2. Nucleotide and amino acid sequence of the maize *Pds* cDNA. Deduced amino acid sequence is shown as single letters below the nucleotide sequence. Bold letters indicate the putative dinucleotide binding domain in the protein sequence and the oligonucleotide primers used for PCR in the nucleotide sequence. The sequence as shown represents the entire sequence available from the cloned cDNA.

generate oligonucleotide primers designed by alignment of all available dicot *Pds* sequences. We sequenced the maize PCR product to verify homology to other *Pds* genes, and this PCR fragment was then used to screen a maize cDNA library of 1–2 million clones. We obtained seven clones. Based on preliminary sequence analysis and alignment with the dicot *Pds* sequences, we chose a 2.0 kb clone for further characterization.

Sequence analysis of maize Pds *and comparison with other dicot* Pds *genes*

We sequenced the maize *Pds* cDNA as shown in Fig. 2. Based on the deduced amino acid sequence, maize PDS, including its putative transit peptide, was found to be 571 amino acid residues with a mass of 64.1 kDa. The sequence determined here, is almost identical to a sequence of a *Pds* cDNA from another maize line, Funk F, except at 14 nucleotide positions, only four of which resulted in a change of amino acid sequence (residues 61–63, 68, and 555) [13]. However, at amino acid position 61–63, the maize *Pds* protein sequence reported here is identical to the dicot sequences shown in Fig. 3A, whereas the Funk F *Pds* protein sequence is different due to a shift in reading frame.

Figure 3A shows the comparison of the N-terminal sequence of available dicot and cyanobacterial PDS proteins. Based on this comparison, we estimate that the maize PDS transit peptide is approximately 96 residues or 10.6 kDa and therefore the plastid-localized PDS should be about 53.5 kDa. The highest homology found between the dicot and monocot putative transit sequences corresponds to residues 59–96 of maize PDS. The comparison of the proposed dinucleotide binding domain, shared by carotene desaturases (PDS, CRTI, CRTD, ZDS) found in phylogenetically distant carotenogenic organisms, is shown in Fig. 3B. Figure 3C shows the region

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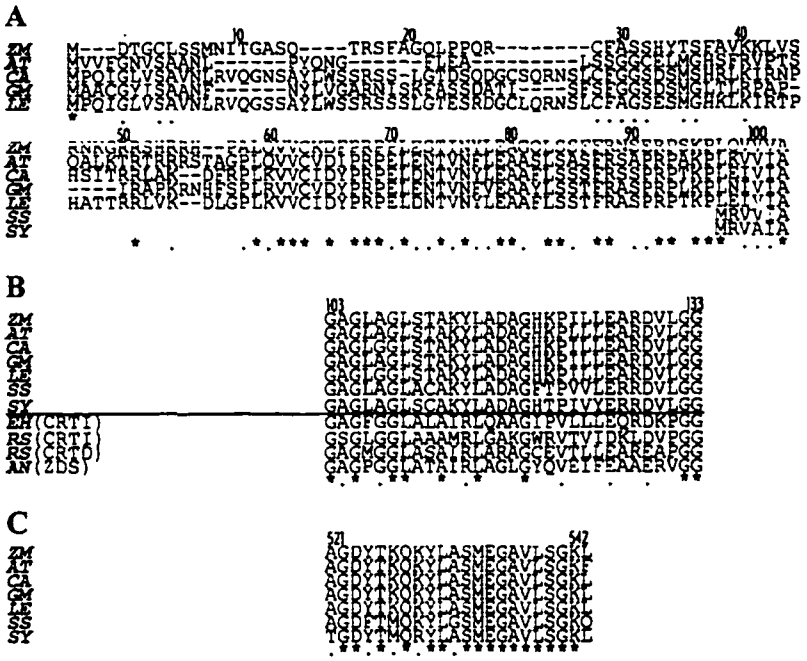

Fig. 3. PDS amino acid sequence alignments. A, N-terminus and transit sequence; B, putative dinucleotide-binding domain; C, highly conserved region used for design of degenerate oligonucleotide primers used for amplification of the maize gene. Asterisks indicate identical residues and dots indicate similar residues. ZM, maize; AT, *Arabidopsis*; CA, pepper; GM, soybean; LE, tomato; SS, *Synechocystis*; SY, *Synechococcus*; EH, *Erwinia herbicola*; RS, *Rhodobacter sphaeroides*; AN, *Anabaena*.

of high homology at the C-terminus, which was used for design of the degenerate oligonucleotide primers used initially to amplify the maize *Pds* gene. Overall nucleotide homology between the maize and other dicot *Pds* genes ranges from 70.5–72%, whereas the amino acid homology based on identical or similar residues is about 77% and 82.8–84.2%, respectively. An alignment of all available PDS protein sequences was carried out and the results are shown in Fig. 4.

Functional analysis of maize PDS by heterologous complementation in E. coli

To test the function of the maize *Pds* gene product, we subcloned the 2.0 kb insert into pBluescript II SK− and designated the clone pMPDS3. By creating progressive 5′-end deletions, we obtained

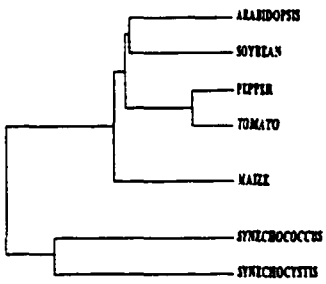

Fig. 4. Phylogenetic tree based on alignment of PDS amino acid sequences in monocots, dicots, and cyanobacteria.

between T232 and CM37 [7]. On the basis of strong hybridization to one fragment, we mapped maize *Pds* to chromosome 1S, near *vp5* (data not shown). Additional weak hybridization signals mapped to three other loci; 4L (1 map unit from *o2*); 2L (1 map unit from bnl 17.25); 1L (1 map unit from dup103).

RT-PCR analysis of Pds *in phytoene-accumulating mutant endosperms*

RT-PCR was used to examine *Pds* transcript levels in mutant endosperms accumulating phytoene. The low transcript abundance required the use of this sensitive technique over conventional northern analysis. Endosperms of the genotypes *vp5*, *vp2*, and *w3* accumulate phytoene and were tested for *Pds* transcript accumulation in comparison to normal endosperm counterparts. As shown in Fig. 6A, only *vp5* endosperms showed a visible difference in transcript accumulation as compared with transcripts accumulating in the normal endosperms. For normalization, we amplified *Sh1* (*Shrunken1*) sequences from the same cDNA; no differences in the amount of *Sh* amplification product were observed.

Determination of Pds *transcript accumulation in leaves and developing endosperms*

RT-PCR analysis was used to assess the temporal pattern of *Pds* transcript accumulation in developing endosperms. Also the level of transcript accumulation in endosperm was compared to that in leaves. In comparison, we also examined the accumulation of the maize *Sh* transcript which has been previously studied in developing endosperm and in leaves using northern analysis [31, 34]. As shown in Fig. 6B, the abundance of *Pds* transcripts in maize leaves and in endosperms were of comparable levels. Figure 6C shows that *Pds* was expressed in the unfertilized ear and transcript levels did not vary substantially (less than a 1.5-fold difference determined densitometrically) over the entire period of endosperm development, except at 40 DAP (days after pollination), when an almost three-fold reduction was observed. In contrast, accumulation of the *Sh* transcript appeared to be under temporal control; the *Sh* amplification product was first detected at 10 DAP, increased at 15 DAP and remained at a constant level between 15–30 DAP, at which point the level dropped, and no product was obtained from the 40 DAP endosperm. The temporal pattern of *Sh* transcript accumulation in developing endosperm detected by RT-PCR was consistent with previous results using northern analysis [34].

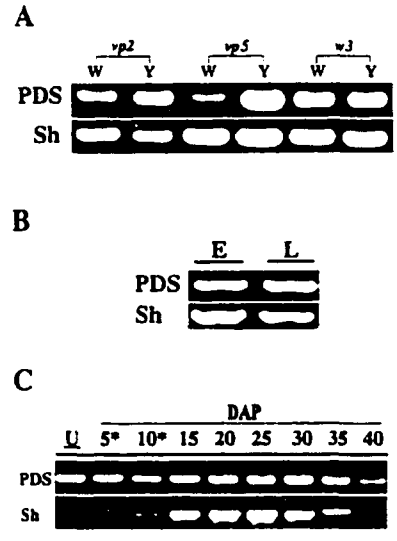

Fig. 6. RT-PCR analysis of *Pds* and *Sh* transcripts. RNA for RT-PCR was extracted from A, white carotenoid mutant (W) and normal yellow endosperms (Y) segregating on maize ears harvested at 20 DAP; B, endosperms harvested at 20 DAP and young leaves; C, developing endosperms harvested at varying DAP (as shown by corresponding numbers) and unfertilized ear (*U*). * indicates that some maternal tissue may also be present in the endosperm sample. Identity of amplification products are shown at left of bands in each panel.

Discussion

A maize *Pds* cDNA clone was isolated, and by using a heterologous complementation system in

E. coli, shown to encode a two-step desaturase, an enzyme catalyzing the desaturation of phytoene to ζ-carotene in two steps. The presence of a two-step desaturase in maize, a monocot, as well as in several dicots, suggests that all higher plants must encode such a two-step desaturase.

Recessive alleles of four unlinked loci, *vp2*, *vp5*, *w3*, and *vp9*, condition a block in the desaturation steps; the first three condition an accumulation of phytoene, the substrate of PDS. Therefore, we proposed that one of these three loci might encode PDS. RFLP mapping results showed that *Pds* mapped near *vp5* on chromosome 1S. Furthermore, transcript analysis, using RT-PCR, showed that only *vp5* endosperms had lower levels of *Pds* transcripts accumulating in comparison to normal endosperms segregating on the same ear. Taken together, these results suggest that maize PDS may be encoded by the *vp5* locus. Consistent with these results is a previous genetic experiment showing *vp5* to encode a cell-autonomous product and not some diffusible regulator [33]. Since *vp2* and *w3* endosperms also accumulate phytoene, but do not affect *Pds* transcript accumulation as detected by RT-PCR (Fig. 6A), it is unlikely that these loci encode transcriptional regulators. These other loci may encode a phylogenetically diverged *Pds*, such as found in the case of *Psy* genes of tomato [3]. However, DNA hybridization results obtained using maize *Pds* as a probe suggest *Pds* is a single-copy gene (not shown). Other weak hybridization signals did not map to loci associated with blocks in the desaturation steps. Therefore, *vp2* and *w3* are not as likely to be structural genes and might encode ancillary functions such as pigment binding proteins or oxidoreductases [22]. The *vp9* gene might encode or regulate expression of ZDS, since recessive alleles confer accumulation of ζ-carotene (see Fig. 1).

Like Rubisco (ribulose bisphosphate carboxylase), which has been widely used for plant evolutionary studies, PDS shows high homology in comparing amino acid sequences of dicots with that of maize, a monocot. For example, amino acid identity and similarity for maize and pepper PDS proteins is 77.2 and 84.2%, respectively. In contrast, Rubisco identity and similarity are 90.6 and 95%, respectively. The phylogenetic tree (Fig. 4) produced by alignment of cyanobacterial, monocot, and dicot PDS amino acid sequences is consistent with current hypotheses of plant evolutionary relationships. However, unlike the chloroplast encoded *rbcL*, which is highly conserved at the nucleotide level between monocots and dicots, the nuclear-encoded *Pds* nucleotide sequence is more variable. Nucleotide homology between maize and pepper *Pds* genes is 72%, whereas for *rbcL*, the homology is 85.4%.

The *rbcL* gene has been an important tool for plant evolutionary studies. However, its high degree of conservation limits its utility for evolutionary studies concerning lower ranked taxonomic groupings. In contrast, *Pds* is an essential, nuclear-encoded gene with greater variability, suggesting that it holds great potential for studies at lower taxonomic ranks than shown in Fig. 4.

A major question regarding control of carotenoid biosynthesis is whether the pathway is differentially regulated in various tissues, i.e. in different plastid types. During endosperm development, plastids triple in number [24] and total colored carotenoids increase dramatically (Yu and Wurtzel, unpublished) during the period of 10–20 days after pollination. Using RT-PCR, we studied the expression of *Pds* transcripts in developing maize endosperm during the period of carotenoid accumulation. No marked change in *Pds* transcript level was found between 5–35 DAP, as compared with the temporally regulated expression of *Sh*. At 40 DAP, there was a reduction in *Pds* transcripts. However, this stage is late in endosperm development and well past the greatest period of carotenoid accumulation. This constitutive expression of *Pds* during the period of carotenoid accumulation in developing maize endosperm is in contrast to the temporal control of *Psy* and *Pds* transcript accumulation in developing tomato fruit; during development of tomato chromoplasts from chloroplasts, carotenoid accumulation is accompanied by a 25-fold increase in *Psy* transcripts and a 3–10 fold increase in *Pds* transcripts [11, 26]. This difference is not unexpected, since carotenoid-containing plastids

of endosperm (amyloplasts) and fruit (chromoplasts) are the products of different developmental processes [16].

It is not surprising that maize endosperm carotenoid accumulation is not regulated by specific induction of *Pds* transcript accumulation. The induction of carotenoid accumulation in the endosperm may not necessarily be regulated at the level of transcript accumulation. Alternatively, the endosperm pathway may be regulated by transcriptional control of *Psy*. Furthermore, the endosperm pathway may not be regulated by controlling expression of the enzymes within the pathway, but alternatively by controlling the flow of substrates to the pathway. Such upstream control (above PSY, the first enzyme specific to carotenogenesis) has been previously documented. Albrecht and Sandmann [1] demonstrated by *in vitro* labeling experiments that the phytochrome-mediated accumulation of carotenoids during the course of conversion of an etioplast to a chloroplast is regulated upstream of the pathway, via activation of IPP (isopentenyl pyrophosphate) isomerase. Another example of upstream regulation of carotenogenesis, occurs in developing pepper fruits; the abundance of transcripts encoding GGPP synthase show a concomitant increase, followed by an increase in enzyme activity, that is associated with carotenoid accumulation [18]. Both IPP and GGPP are precursors to a variety of terpenoid pathways [8]. Therefore, evidence is mounting that the pathway is not only regulated with respect to tissue specificity, but is controlled both within the pathway as well as upstream of the pathway.

The genetically identified mutant alleles affecting carotenoid synthesis in maize endosperm, but not leaves, will be useful for determining how the carotenoid biosynthetic pathway is regulated in different plastid/tissue types, where the role and localization of carotenoids varies. Furthermore, an understanding of the molecular regulation of carotenoid biosynthesis in endosperm is of great value for engineering the pathway in endosperms of other cereal crops that are otherwise poor nutritional sources of carotenoids. The stable expression of *Pds* transcripts in developing endosperm may also serve as a useful internal experimental control for future studies of endosperm gene expression, including other genes involved in carotenogenesis.

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