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ISOLATION, EXPRESSION AND FUNCTIONAL ANALYSIS OF A cDNA ENCODING PHYTOENE DESATURASE, A CAROTENOID BIOSYNTHETIC ENZYME FROM RICE, *Oryza sativa* L.

by

Arulmolee Yoganathan

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirement for the degree of Doctor of Philosophy, The City University of New York

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

ISOLATION, EXPRESSION AND FUNCTIONAL ANALYSIS OF A cDNA ENCODING PHYTOENE DESATURASE, A CAROTENOID BIOSYNTHETIC ENZYME FROM RICE, *Oryza sativa* L.

by

Arulmolee Yoganathan

Adviser: Professor Eleanore T. Wurtzel

The mechanisms that underlie the lack of carotenoid accumulation in rice endosperm were investigated, en route to engineering carotenoid biosynthesis in rice. The genetic capacity to synthesize and accumulate carotenoids in photosynthetic tissues and not in endosperm of rice implies a tissue specific regulation. One of the possible causes of the absence of colored carotenoid accumulation in rice endosperm may be the lack of expression of one or more carotenoid biosynthetic genes. Furthermore, the presence of more than one gene involved in the tissue specific expression of carotenoid biosynthetic enzymes is a possibility too. To investigate the tissue specific regulation, the expression of the first two carotenoid biosynthetic genes, *Psy* (phytoene synthase) and *Pds* (phytoene desaturase), in rice was studied.

Results clearly shows that the carotenoid biosynthetic enzymes PSY and PDS were encoded by single copy genes mapped to chromosome # 12 and # 3, respectively. Both *Psy* and *Pds* were expressed not only in leaf but also in endosperm. *Psy* was constitutively expressed during endosperm development, whereas *Pds* showed a temporal regulation at the transcriptional level. The 2027 bp rice *Pds* cDNA isolated shared 80% and 89% identities with maize *Pds*, at the nucleotide and amino acid sequence levels, respectively. The open reading frame of 566 amino acid residues with predicted molecular weight of 63.5 kDa contained a 10.3 kDa putative transit peptide region consisting of 92 amino acid residues and a dinucleotide binding domain necessary for the enzyme activity. The predicted amino acid sequence of the mature rice PDS shared 96% identity with maize PDS. When complemented with *Erwinia uredovora* genes encoding GGPPS and PSY (for the synthesis of phytoene), the protein encoded by the rice *Pds* catalyzed a two step desaturation of phytoene resulting in the synthesis of ζ-carotene.

This dissertation is dedicated to

my parents, sister

and

husband

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LIST OF ABBREVIATIONS

aa	amino acid
ABA	abscisic acid
bp	base pair
CoA	co-enzyme A
CRTB	bacterial phytoene synthase
CRTE	bacterial geranylgeranyl pyrophosphate synthase
CRTI	bacterial phytoene desaturase
DAF	days after flowering
DMAPP	dimethylallyl pyrophosphate
EST	expressed sequence tag
FAD	flavin adenine dinucleotide
FPP	farnesyl pyrophosphate
GGPP	geranyl geranyl pyrophosphate
GGPPS	geranyl geranyl pyrophosphate synthase
GPP	geranyl pyrophosphate
HMGCoAR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HYD	carotene hydroxylase
IPP	isopentenyl pyrophosphate
kb	kilobase
kDa	kilodalton

LCY	lycopene cyclase
MVA	mevalonic acid
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
PCR	polymerase chain reaction
Pds	gene encoding phytoene desaturase
PDS	phytoene desaturase
Psy	gene encoding phytoene synthase
PSY	phytoene synthase
RACE	rapid amplification of cDNA ends
RFLP	restriction fragment length polymorphism
RT-PCR	reverse transcriptase-polymerase chain reaction
Sh	Shrunken, gene encoding sucrose synthetase
ZDS	ζ-carotene desaturase

CHAPTER 1

INTRODUCTION

Carotenoids are one of the naturally occurring group of pigments, which are widely distributed among bacteria, fungi, algae, higher plants, animals and human beings. Because of their diverse functions in these organisms, they claim the great interest of many scientists. Although the first crystalline yellow pigment carotene was isolated in 1831(Britton *et al.*, 1995), it was not until the discovery of chromatographic and spectroscopic techniques, that most of the important progress in carotenoid research was achieved. In spite of the tremendous advancement in carotenoid chemistry, the complete biological, biochemical and molecular aspects of carotenoid biosynthesis did not emerge quickly. These aspects were important because of the functions of carotenoids in biological systems.

Carotenoids play varied roles in both plants and animals. In plants they function as accessory light harvesting pigments in photosynthesis; as photo protectors; and as precursors to the phytohormone abscisic acid (ABA). In animals, carotenoids are essential precursors of vitamin A, a component of vision and to related compounds like retinoids which are important in cell differentiation, growth and reproduction (Bramley and Mackenzie, 1988).

In spite of (1) the vital physiological, medical and morphogenetic importance of carotenoids among organisms, (2) the wealth of chemical and biochemical information about carotenoids and (3) the availability of many carotenoid mutants, especially in higher plants,

the regulation of carotenoid biosynthesis at molecular level is poorly understood. The main reason for this is the difficulty in the isolation and purification of active carotenoid biosynthetic enzymes. This causes a problem in the biochemical characterization of enzymes, especially the membrane bound enzymes. Recently, to overcome this problem, molecular approaches have been carried out to characterize the regulation of carotenoid biosynthesis.

In this chapter I will discuss the structure, important biological functions of carotenoids, the importance of carotenoid biosynthesis in plastid differentiation, the carotenoid biosynthetic pathway and related enzymes, and the regulation of carotenoid biosynthesis in higher plants. Finally, I will discuss the objective of my research towards engineering carotenoid biosynthesis in rice endosperm.

1.1: Structure of Carotenoids

Carotenoids are formed by the condensation of eight C_5 isoprenoid units according to the classical 'isoprene rule', where linkage occurs in a head to tail manner (Spurgeon and Porter, 1983). At the center of the molecule the linkage order is reversed, so that the C_{40} isoprenoid skeleton is symmetrical (Spurgeon and Porter, 1983). More than 600 naturally occurring carotenoids are derived from this C_{40} isoprenoid skeleton by modifications such as cyclization, substitution, elimination, addition and rearrangement of the basic structure shown by lycopene (Figure 1.1) (Britton *et al.*, 1995). The great structural

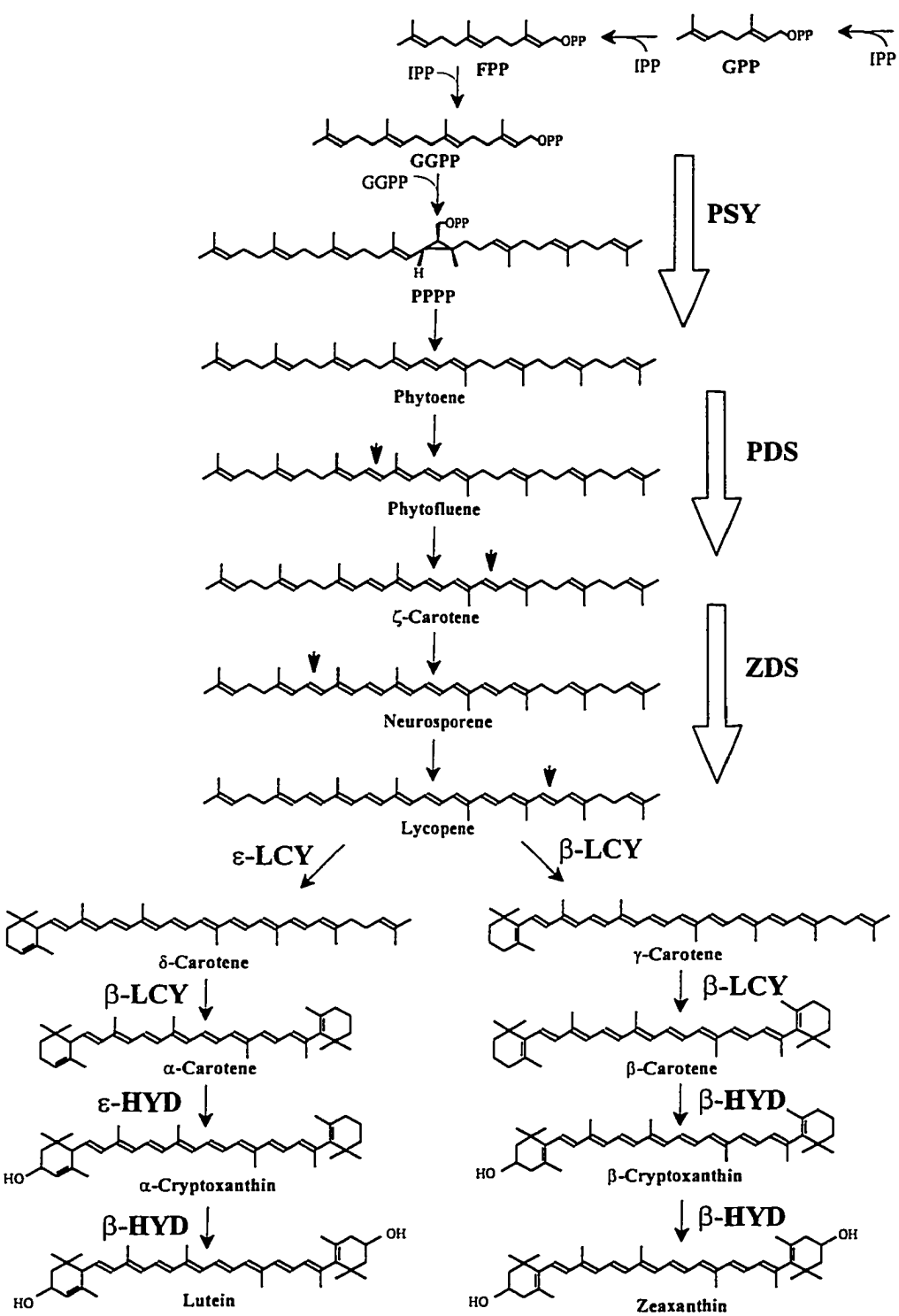

Figure 1.1: Carotenoid Biosynthetic Pathway in Higher Plants

diversity of carotenoids is also due to the existence of structurally different spatial configurations, the stereo isomers such as *cis* or *trans* forms (*Z* or *E* forms) and the optical isomers such as *d* or *l* forms (Eugster, 1995). While C_{40} carotenoids are more common in nature, additional skeletal modifications involving chain elongation result in the formation of C_{45} or C_{50} carotenoids. Although C_{30} carotenoids occur as the result of the degradation of the polyene chain of C_{40}, some C_{30} carotenoids are formed biogenetically (Bramley, 1985). The distinctive light absorbing feature of carotenoids is due to the number of conjugated double bonds resulting in a chromophore. C_{40} carotenoids can have up to 15 conjugated double bonds in the chromophore, but 7-11 double bonds are more common (Britton *et al.*, 1995). It is a common feature among carotenoids to contain oxygen and those are referred to as xanthophylls.

1.2: Biological Functions of Carotenoids

Carotenoids are synthesized *de novo* in all photosynthetic organisms and some non-photosynthetic organisms such as bacteria and fungi. In photosynthetic organisms "there would be no photosynthesis as we now recognize it were it not for the presence of carotenoids!" (Cogdell and Frank, 1987). In photosynthetic tissues of plants carotenoids function as energy transfer pigments (Mathis and Schenck, 1982). For this energy transfer carotenoids must be in close proximity to chlorophyll as they are in the pigment - protein complexes of thylakoids (Britton, 1993). The absorption of light by a carotenoid molecule generates an excited singlet state, the energy of which is higher than that of singlet excited

chlorophyll. Therefore, excitation energy can be transferred from carotenoid to chlorophyll (Britton, 1993).

Carotenoids are also vital in protection against photosensitization in (1) algae, plants, animals and humans [reviewed in (Krinsky, 1991; Bramley and Mackenzie, 1988; Goodwin, 1986)], and (2) non-photosynthetic bacteria and fungi inhabiting high light aerobic environments (Goodwin, 1980). In plants and algae, the most important function of carotenoids with a chromophore of at least seven conjugated double bonds is the protection against singlet oxygen by quenching the excitation energy of the chlorophyll triplet state or singlet oxygen (Britton, 1993). Under saturating light intensities, carotenoids protect the tissues against photo-oxidative damage of chlorophyll and destruction of cellular components by free oxygen radicals (Anderson and Robertson, 1960). Photo-inhibition, the inhibition of photosynthetic activity, occurs when the accumulation of excitation energy exceeds the rate of photochemical reaction. Photo-inhibition does not cause chlorophyll bleaching or tissue damage directly but, if the conditions are prolonged, bleaching or photo destruction of chloroplast pigments and structural damage to chloroplasts and tissues will follow (Britton, 1993). Xanthophylls, especially zeaxanthin and violaxanthin, play the protective role by preventing photo-inhibition from leading

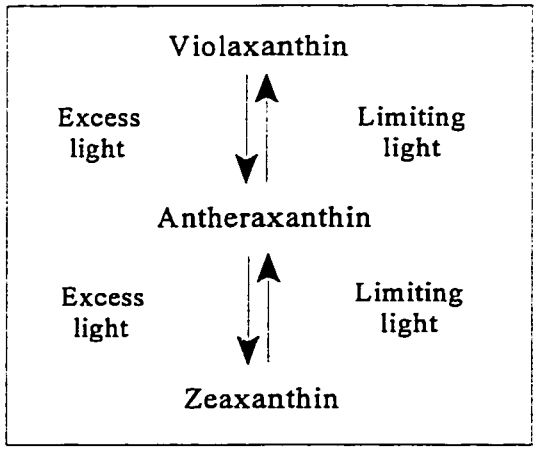

Figure 1.2: Xanthophyll Cycle

inevitably to photo-oxidative damage (Britton, 1993; Demmig-Adams, 1990). During this xanthophyll cycle (Figure 1.2), violaxanthin undergoes rapid enzymatic de-epoxidation via antheraxanthin to zeaxanthin under high light intensities and the reverse process, the re-epoxidation, takes place in the dark (Demmig-Adams, 1990). It has also been suggested that zeaxanthin acts as a competitor for excitation energy, because the thermal dissipation of excitation energy is correlated with the rapid production of zeaxanthin via the xanthophyll cycle or by *de novo* synthesis (Britton, 1993; Demmig-Adams, 1990).

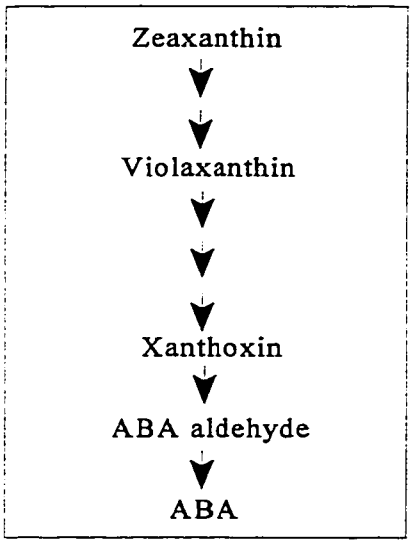

Figure 1.3: ABA Biosynthetic Pathway

Carotenoids are also the metabolic precursors of the phytohormone abscisic acid (ABA). ABA participates in the regulation of water relations, growth and seed dormancy in plants. A breakdown product of violaxanthin, derived from zeaxanthin, is the precursor for ABA biosynthesis (Koornneef, 1986; Creelman and Zeevaart, 1984; Robichaud *et al.*, 1980) (Figure 1.3). Biochemical studies, using zeaxanthin as a precursor in "feeding experiments" and work carried out using maize *viviparous* mutants (Robertson, 1975) support this indirect pathway for ABA biosynthesis. The localization of the enzymes of the ABA biosynthetic

pathway is unknown. Although the xanthophylls are localized to the plastid membranes, xanthoxin conversion to ABA appears to be cytosolic (Walton and Li, 1995). The direct pathway, where ABA can be synthesized from the C_{15} precursor FPP, exists in the fungus *Cercospora* (Walton and Li, 1995).

Dietary carotenoids, especially β-carotene, are essential metabolic precursors to Vitamin A, an essential component of vision (Morton, 1970) and to related compounds, like retinoids which are important in morphogenesis (Chytil and ul-Haq, 1990; Bendich and Olson, 1989) (Figure 1.4). Oxidative cleavage of the central double bond of β-carotene results in formation of retinal which is subsequently reduced to retinol (vitamin A) in the intestine (Krinsky *et al.*, 1993).

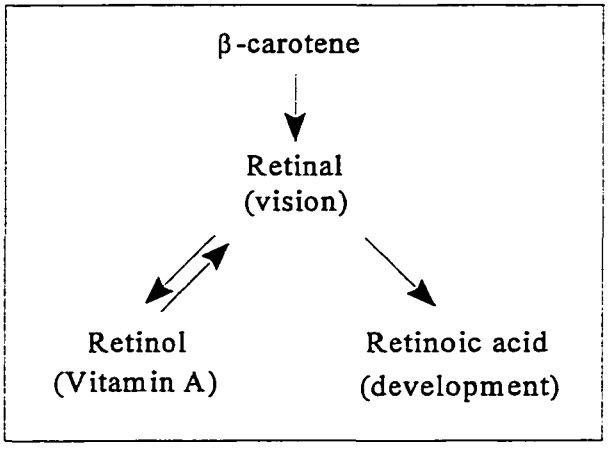

Figure 1.4: Conversion of β-carotene into Vitamin A

There are several biological functions of carotenoids related to human health and some of them are indicated below. Anti-cancer activity of carotenoids has been demonstrated (Mathews-Roth, 1986; Mathews-Roth, 1982a; Murakoshi *et al.*, 1989). Carotenoids also function as anti-oxidants (Krinsky, 1991). Effective use of carotenoids against human photosensitivity disease has also been suggested (Mathews-Roth, 1982b).

Lutein and zeaxanthin found in the eye can quench singlet oxygen and may reduce the oxidative stress on the lens proteins, preventing cataract formation (Bendich, 1993). β-carotene also provides protection against UV-induced immunosuppression (Bendich, 1993). β-carotene and canthaxanthin are involved in the activation of genes encoding connexin 43, which is an integral component of gap junctions required for cell-cell communication (Bendich, 1993).

1.3: Carotenoid Biosynthesis and Plastid Differentiation

Carotenoid biosynthetic enzymes are nuclear encoded and synthesized in the cytosol. The biosynthesis and accumulation of carotenoids in various types of plastids are important features of plastid differentiation and function. Proplastids, small undifferentiated plastids, act as the precursors of the more differentiated type of plastids, such as the chloroplasts or amyloplasts (Kirk and Tilney-Bassett, 1978). Differentiation of chromoplasts from chloroplasts occurs during flower development and fruit ripening. The different kinds of carotenoid pigments synthesized in various types of plastids are discussed below.

Chloroplast: Carotenoid biosynthesis is an essential part of chloroplast differentiation and the assembly of the functional pigment-protein complexes (Britton, 1993). Carotenoids are complexed in Photosystem I and II with chlorophyll and chlorophyll binding proteins in chloroplast thylakoid membranes. Photosystem I (PSI) contain mostly β-carotene, 25%-30% of total carotenoids, whereas photosystem II (PSII) contains xanthophylls

as the predominant carotenoids, especially lutein (45%), violaxanthin (10%-15%), and neoxanthin (10%-15%) and some β-carotene [reviewed in (Britton, 1993; Goodwin, 1980)]. It has been shown that carotenoids are required for the assembly and activity of PSII, mainly lutein, the major carotenoid of the antenna of PSII, but not for PSI (Markgraf and Oelmüller, 1991). Carotenoids are not only present in thylakoid membranes, but also in the envelope membranes. The predominant carotenoid in the envelope membrane is violaxanthin (Kirk and Tilney-Bassett, 1978).

Chromoplasts: Chromoplasts are carotenoid containing plastids responsible for some of the red, yellow, and orange colors of flowers, fruits, some roots and even the leaves of deciduous trees during the Autumn season. Chromoplasts are usually derived from chloroplasts (Kirk and Tilney-Bassett, 1978). The composition of carotenoids accumulated in various plant tissues varies depending on the type of plants and tissues. For example, chromoplasts from ripened tomato fruit contain 87% lycopene, 7% β-carotene, 3% phytofluene and traces of ζ-carotene, whereas chromoplasts from ripened red pepper fruits contain 31% capsanthin, 12% β-carotene and 7% capsorubin (Kirk and Tilney-Bassett, 1978). During the differentiation of chloroplast into chromoplast, carotenoid biosynthesis and accumulation takes place either in oil droplets, or in structures like globular, membranous, fibrillar, crystalline, tubular, or even as microcrystalline aggregates (Sitte *et al.*, 1980).

Amyloplasts: An amyloplast, a predominantly starch accumulating plastid, is differentiated from a proplastid. Amyloplasts are found in differentiated cells of roots, cotyledons, endosperms and tubers. There may be just one starch grain per amyloplast or a few (Kirk and Tilney-Bassett, 1978). The amyloplast is bound by a double membrane and unlike the chloroplast or chromoplast, it has a structureless stroma (Kirk and Tilney-Bassett, 1978). The function of amyloplast in storage tissue is presumably to synthesize starch as a reserve when carbohydrates are available in excess. It has also been suggested that amyloplasts in the root cap play a role in gravitropism of roots (Kirk and Tilney-Bassett, 1978). Unlike the differentiation of chloroplasts, carotenoid biosynthesis and accumulation are not essential for differentiation of amyloplasts. But if carotenoids are accumulated in endosperm tissues, like in maize, they function as a nutritional source for humans and animals (Lee *et al.*, 1981). Starch granules in rice are of the compound type containing up to 100 granula, in contrast to barley and wheat, in which an amyloplast contains a single starch granulum (Buttrose, 1962). Because amyloplasts are easily damaged by the enclosed starch granules, they are difficult to isolate intact. Difficulty in isolating intact amyloplasts is a reason for lack of knowledge about the amyloplast localized biochemical pathways particularly in the area of compartmentalization of substrates and enzymes and the mechanisms governing the substrate transport into the amyloplast (Lopes and Larkins, 1993).

1.4: Carotenoid Biosynthetic Pathway and Related Enzymes

Carotenoid biosynthesis is localized to plastids, but, all the carotenoid biosynthetic enzymes are nuclear encoded and synthesized as large precursor proteins in the cytosol. N-terminal extensions of these precursor proteins are called transit peptides and are necessary for post translational import into the plastid membranes (Keegstra, 1989).

1.4.1: Biosynthesis of Isoprenoid Precursors of Carotenoids

The carotenoid biosynthetic pathway is part of the general isoprenoid metabolism derived from isopentenyl pyrophosphate (IPP). The source of IPP in plastids was recently shown to be 1-deoxyxylulose-5-phosphate derived from the addition of pyruvate derived hydroxyethyl thiamine and glyceraldehyde 3-phosphate (Lichtenthaler *et al.*, 1997a) as compared to the cytosolic IPP derived from mevalonic acid (MVA). The alternate pathway for IPP synthesis in plastids was first discovered in eubacteria (Rohmer *et al.*, 1993). This alternate pathway is also supported by earlier studies with isolated spinach chloroplasts where it was shown that photosynthetically fixed carbon dioxide was used as the carbon source for the synthesis of IPP (Schulze-Siebert and Schultz, 1987). Recently, it has been shown that the plastidic isoprenoids β-carotene, lutein, and the phytol side chain of chlorophyll in *Hordeum vulgare* L., *Daucus carota* L. and *Lemna gibba* L. are synthesized via this novel mevalonate-independent pathway (Lichtenthaler *et al.*, 1997b).

Some of the IPP is isomerized into dimethylallyl pyrophosphate (DMAPP), by the enzyme isopentenyl diphosphate isomerase. IPP and DMAPP are used to build the required chain length of prenyl diphosphate intermediates by means of prenyltransferase enzymes. These prenyl diphosphate intermediates are the precursors for the biosynthesis of all classes of terpenoids, such as monoterpenes, diterpenes, tetraterpenes and sesquiterpenes (Figure 1.5). In plants, the isoprenoid pathway is topologically divided into two branches. Whereas triterpenoids and sesquiterpenoids are synthesized in the cytosol, monoterpenoids, diterpenoids and tetraterpenoids are synthesized in plastids (McGarvey and Croteau, 1995; Kleinig, 1989).

1.4.2: Biosynthesis of Geranylgeranyl Pyrophosphate

One important prenyl diphosphate intermediate is the C_{20} geranylgeranyl pyrophosphate (GGPP). GGPP is a precursor not only for carotenoids, but also for tocopherols, gibberellins, quinones, diterpenoid phytoalexins and for the phytol chain of chlorophyll (Figure 1.5). In higher plants GGPP synthase (GGPPS) catalyzes the synthesis of GGPP from IPP (Camara *et al.*, 1989). Several genes encoding GGPPS have been cloned and characterized from bacteria (To *et al.*, 1994; Math *et al.*, 1992; Armstrong *et al.*, 1990a; Misawa *et al.*, 1990), fungi (Carattoli *et al.*, 1991) and higher plants (Zhu *et al.*, 1997; Kuntz *et al.*, 1992; Scolnik and Bartley, 1994). GGPPS proteins have been shown to belong to a family of enzymes with different substrates and chain length specificity (Sandmann, 1994).

Several types of this enzyme have been isolated from bacteria, fungi, plants and animal tissues [reviewed in (Sandmann, 1994)]. The GGPPS has been purified to homogeneity from *Capsicum* chromoplasts (Dogbo and Camara, 1987) and shown to catalyze prenyl transfer reaction using IPP, geranyl pyrophosphate (GPP) or farnesyl pyrophosphate (FPP) as substrates (Dogbo and Camara, 1987). GGPP synthase activity was shown to be in the stromal fraction of isolated daffodil chromoplasts, but at a low level in the absence of membraneous structures (Kreuz *et al.*, 1982). Based on this result it has been suggested that GGPP synthase is a peripheral membrane protein. Import studies using isolated pea

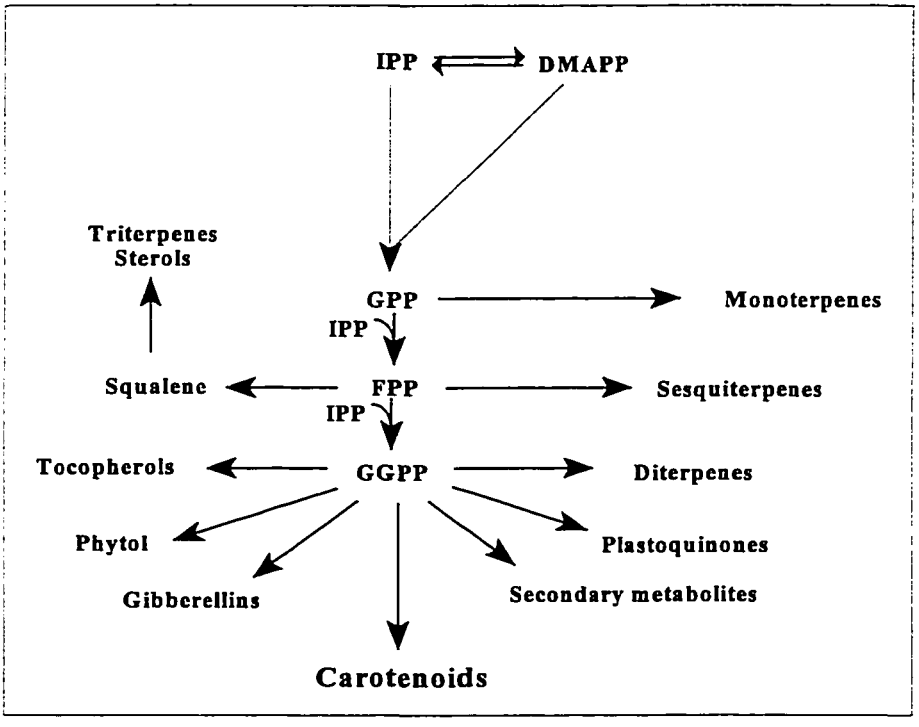

Figure: 1.5: Outline for the Major Branches in Terpenoid Metabolism

chloroplasts has demonstrated that GGPPS remains in the stroma as a soluble form free from all other carotenoid biosynthetic enzymes (Bonk *et al.*, 1997).

1.4.3: Biosynthesis of Phytoene

GGPP is considered to be the specific precursor for biosynthesis of carotenoids. Phytoene, the first intermediate in the carotenoid biosynthetic pathway, is produced from the condensation of two molecules of GGPP via a C_{40} intermediate prephytoene diphosphate. These two steps are catalyzed by the bifunctional enzyme, phytoene synthase (PSY), which has been purified to homogeneity from *Capsicum* and characterized (Dogbo *et al.*, 1988). *In vitro* reconstitution studies using daffodil chromoplasts (Kreuz *et al.*, 1982) and pepper chromoplasts (Camara *et al.*, 1982) have demonstrated that phytoene synthase activity occurs in the stroma with marked increase in the presence of membrane structures. In contrast to this phytoene synthase activity is strongly associated with chloroplast envelope membranes (Mayfield *et al.*, 1986; Lütke-Brinkhaus *et al.*, 1982). Based on these observations it has been suggested that phytoene synthase is tightly bound to the chloroplast envelope membranes (Lütke-Brinkhaus *et al.*, 1982) and in chromoplasts it is characterized as a peripheral membrane protein which can be easily removed from chromoplast membrane (Kreuz *et al.*, 1982). *In vitro* import studies using isolated daffodil chromoplasts have shown that PSY is present in both stromal fraction and membrane fraction of chromoplasts (Schledz *et al.*, 1996). In the presence of liposomes the membrane bound form is active and capable of forming phytoene solely from GGPP (Schledz *et al.*, 1996). The soluble stromal form

exists as a constituent of a chaperonin Hsp70 containing complex, which needs to be associated with membranes to be activated (Bonk *et al.*, 1997; Al-Babili *et al.*, 1996; Bonk *et al.*, 1996; Schledz *et al.*, 1996).

Genes encoding PSY have been cloned from several higher plants, cyanobacteria, fungi, and both photosynthetic bacteria and non-photosynthetic bacteria. Except in tomato, PSY is encoded by a single copy gene in all higher plants, such as *Capsicum annuum* (Römer *et al.*, 1993), *Arabidopsis thaliana* (Scolnik and Bartley, 1993a), *Cucumis melo* (Karvouni *et al.*, 1995), *Narcissus pseudonarcissus* (Schledz *et al.*, 1996), *Zea mays* (Buckner *et al.*, 1996); (Yu and Wurtzel, unpublished), and cyanobacteria *Synechocystis* (Martínez-Férez *et al.*, 1994) and *Synechococcus* PCC7942 (Chamovitz *et al.*, 1992). In tomato, chromoplast-specific and chloroplast-specific PSY proteins are encoded by two different genes, namely p*Tom5* or *Psy1* (Bartley *et al.*, 1992; Bird *et al.*, 1991; Ray *et al.*, 1987) and *Psy2* (Bartley and Scolnik, 1993), respectively.

1.4.4: Desaturation of Phytoene into Lycopene

As shown in Figure 1.1, four consecutive didehydrogenation/desaturation reactions occurring alternatively at either side of the C_{40} skeleton of phytoene introduce four conjugated double bonds (indicated in small arrows). This results in a maximally desaturated red color compound, lycopene. The intermediates are phytofluene, ζ-carotene, and neurosporene. Desaturases catalyze these desaturation reactions. There is a functional

diversity among desaturases, because of the number of desaturation steps catalyzed in different organisms. In oxygenic photosynthetic organisms (higher plants and cyanobacteria) two desaturases catalyze four desaturation steps. The first one, phytoene desaturase (PDS) uses phytoene as the substrate, catalyzes two desaturation reactions resulting in ζ-carotene via phytofluene (Li *et al.*, 1996; Pecker *et al.*, 1992; Bartley *et al.*, 1991; Linden *et al.*, 1991). The second one, ζ-carotene desaturase (ZDS), uses ζ-carotene as the substrate and catalyzes two additional desaturation reactions, thus producing lycopene via neurosporene (Albrecht *et al.*, 1996; Albrecht *et al.*, 1995; Linden *et al.*, 1994; Linden *et al.*, 1993a).

In non-oxygenic photosynthetic bacteria like *Rhodobacter*, a single phytoene desaturase CRTI catalyzes three desaturation reactions resulting in the formation of neurosporene (Linden *et al.*, 1991). In non-photosynthetic bacteria *Erwinia uredovora* and *Erwinia herbicola* (Linden *et al.*, 1991; Misawa *et al.*, 1990), and the fungus *Neurospora crassa* (Bartley *et al.*, 1990; Goldie and Subden, 1973) a single phytoene desaturase, CRTI, catalyzes all four desaturation steps resulting in the formation of lycopene.

In higher plants, PDS is an intrinsic membrane protein both in chloroplasts and chromoplasts (Hugueney *et al.*, 1992; Bartley *et al.*, 1991). Immunogold localization of PDS in higher plant chloroplasts has demonstrated that most of the PDS is primarily localized in thylakoid membranes (Linden *et al.*, 1993b). Like PSY, PDS also exists as an inactive form

associated with chaperonins Hsp70 and Cpn60 in the stroma of daffodil chromoplasts (Bonk *et al.*, 1997; Bonk *et al.*, 1996). This inactive PDS has the capacity for enzyme activation and membrane association (Al-Babili *et al.*, 1996).

The higher plant PDS is encoded by a single copy gene and has been cloned from several organisms, such as, *Arabidopsis* (Scolnik and Bartley, 1993b), tomato (Pecker *et al.*, 1992), pepper (Hugueney *et al.*, 1992), soybean (Bartley *et al.*, 1991), daffodil (Al-Babili *et al.*, 1996), maize (Li *et al.*, 1996; Hable and Oishi, 1995), *Synechococcus* (Chamovitz *et al.*, 1991), and *Synechocystis* (Martínez-Férez and Vioque, 1992). A photosynthetic gene cluster containing carotenoid biosynthetic genes has been cloned from the purple photosynthetic bacterium *Rhodobacter capsulatus* (Armstrong *et al.*, 1989) and the non-photosynthetic bacteria *Erwinia uredovora* and *Erwinia herbicola* (Armstrong *et al.*, 1990a; Misawa *et al.*, 1990). The entire gene cluster has been sequenced and well characterized by functional analysis (Math *et al.*, 1992; Armstrong *et al.*, 1990b; Misawa *et al.*, 1990; Armstrong *et al.*, 1989).

Phytoene desaturase proteins from bacteria, fungi and plants contain a dinucleotide binding domain which is usually associated with the binding of co-factors FAD or NAD(P). Co-factor FAD has been found to bind to purified pepper PDS (Hugueney *et al.*, 1992) and studies with purified PDS proteins have demonstrated that either NAD(P) or FAD is sufficient to catalyze the desaturation reactions, depending on the species (Fraser *et al.*, 1993; Fraser *et al.*, 1992; Armstrong *et al.*, 1990a; Bartley *et al.*, 1990). The desaturation reactions

also require other ancillary redox proteins like quinone oxidoreductases (Norris *et al.*, 1995; Mayer *et al.*, 1992). In photosynthetically inactive daffodil chromoplasts, oxygen is the final acceptor for electrons derived from the desaturation steps (Beyer *et al.*, 1989).

1.4.5: Biosynthesis of Cyclic Carotenoids

Cyclic carotenoids are essential components of the photosynthetic membranes and the existence of two different kinds of lycopene cyclases may determine the formation of appropriate cyclic carotenoids in photosynthetic organisms. Lycopene, the product of the sequential desaturations of phytoene, is the acyclic carotenoid precursor for monocyclic carotenes, γ-carotene and δ-carotene and bicyclic carotenoids, α-carotene and β-carotene. Genetic control of the formation of different types of cyclic end groups, the β and ε-ionone rings has been demonstrated in tomato (Kirk and Tilney-Bassett, 1978). Cunningham Jr. *et al.* have cloned two different types of cDNAs encoding β and ε-cyclases from *Arabidopsis* (Cunningham Jr. *et al.*, 1996). These two are encoded by single copy genes in *Arabidopsis*. The ε-cyclase adds single ε-ionone ring producing δ-carotene, whereas the β-cyclase introduces β-ionone rings at both ends of the lycopene to form β-carotene. Both cyclases together can add one ε-ionone ring and one β-ionone ring to form α-carotene. In a daffodil cell-free system, when poly-*cis* prolycopene was used as the substrate for the β-LCY, it was demonstrated that β-LCY activity is only possible under anaerobic conditions and β-carotene formed is in an all-*trans* configuration. Cyanobacterial β-cyclase has been cloned from *Synechococcus* sp. PCC 7942 (Cunningham Jr. *et al.*, 1993).

1.4.6: Biosynthesis of Xanthophylls

Xanthophylls are enzymatically formed hydroxy, epoxy, furanoxy and oxy derivatives of cyclic carotenoids mainly, α-carotene and β-carotene. β-carotene hydroxylase catalyzing the conversion of β-carotene to zeaxanthin has been cloned by functional complementation in *E. coli* (Sun *et al.*, 1996). The enzyme capsanthin capsorubin synthase, catalyzing the conversion of the 5,6-epoxycarotenoids antheraxanthin and violaxanthin into capsanthin and capsorubin has been purified to homogeneity from *Capsicum annuum*. The gene encoding this enzyme has been found to be up-regulated during chloroplast to chromoplast differentiation (Bouvier *et al.*, 1994). Little is known about the regulation of xanthophyll biosynthesis in higher plants.

1.5: Regulation of Carotenoid Biosynthesis in Higher Plants

Regulation of carotenoid biosynthesis is a complex process in higher plants. Work is still in progress by several groups of investigators to reveal the details of the regulation of carotenoid biosynthesis. As described earlier, carotenoid biosynthesis has a close association with plastid development, especially chloroplast and chromoplast development. Very little is known about the association between carotenoid biosynthesis and differentiation of the amyloplast and whatever information available has come from work done in maize (Li *et al.*, 1996; Wurtzel *et al.*, unpublished). Obviously more work is needed to understand the

regulation of carotenoid biosynthesis, particularly in relation to amyloplast differentiation. Maize provides an excellent model system with its well characterized carotenoid accumulating mutants (Robertson, 1975).

The reasons for complex regulatory mechanism of carotenoid biosynthesis in higher plants: (1) carotenoid biosynthesis is localized in plastids. This means nuclear encoded carotenoid biosynthetic enzymes have to be targeted to the plastids, and properly routed to various types of membranes like thylakoids and inner chromoplast membranes, (2) targeting enzymes to the membranes requires not only a transit peptide, but also some other ancillary factors, such as chaperonins, quinine oxido-reductases, co-factors and plastidic factors, some of these factors are well understood and some which are not, (3) origin of plastids and their specific functions in different tissues in higher plants, and (4) GGPP, the specific precursor for carotenoid biosynthesis, is also a common precursor for other compounds (Figure 1.5), therefore, the regulation might be tightly linked with the regulation of other biosynthetic pathways which use this common precursor. Because of the lack of complete knowledge in certain areas mentioned above the regulation of carotenoid biosynthesis is not yet fully understood. For the convenience of describing the information available on the regulation of carotenoid biosynthesis, I will discuss this in two main categories: (1) regulation upstream of the pathway, and (2) regulation within the pathway.

1.5.1: Regulation Upstream of The Carotenoid Biosynthetic Pathway

Competition for Precursors:- Carotenoid biosynthesis and its accumulation in any tissue is in competition with other pathways utilizing GGPP as a common terpenoid precursors. This may raise a question whether there is a competition for IPP, which is a common precursor for isoprenoid biosynthesis. Labeling experiment using [1-^{13}C] glucose in green alga *Scenedesmus obliquus* has revealed an alternate pathway, where phytol from the chlorophyll side chain, the polyprenyl side chain of plastoquinone-9, the carotenoids β-carotene and lutein and sterols were not produced from the classical acetate/mevalonate pathway, but via the novel glyceraldehyde 3-phosphate/pyruvate route (Schwender *et al.*, 1996) which was first discovered in eubacteria (Rohmer *et al.*, 1993). The experiments done with the antibiotic mevinolin also supports the alternate pathway. When mevinolin, a specific inhibitor of HMGCoA reductase, is used, cytosolic sterol biosynthesis in higher plants is efficiently blocked. However, the formation of chlorophylls and carotenoids in plastids [(Schwender *et al.*, 1996) and the references therein] is unaffected. Therefore, IPP formation in plastids is independent of IPP formation via mevalonate in cytosol.

The possibility for the channeling of IPP between plastids and the cytosol came from experiment carried out in pepper fruits (Hugueney *et al.*, 1996). In this experiment the induction of the cytosolic synthesis of the phytoalexin capsidiol from cytosolic FPP, results in the suppression of carotenoid biosynthesis in plastids without any effect on the activities of carotenoid biosynthetic enzymes. This suggests that the effect on carotenoid biosynthesis

is mediated through the depletion of precursors. This observation contradicts the existence of the alternate pathway for the synthesis of IPP in plastids which does not share any common precursors with cytosolic terpenoid biosynthesis. If IPP is synthesized via this alternate pathway in plastids then suppression of plastid localized carotenoid biosynthesis should not occur unless there is a channeling of IPP between cytosol and plastid across the plastid membrane. However there are situations where both plastidic and cytosolic isoprenoid pathways can be entirely independent as seen in tobacco leaves where manipulation of HMGCoAR expression affects cytosolic sterol synthesis but not plastid carotenoid synthesis (Chappell *et al.*, 1995). The likely reason for this is the presence of an alternate pathway for the synthesis of plastid isoprenoid precursors.

Effect of light on carotenoid biosynthesis during the differentiation of chloroplasts from etioplasts:- Light stimulated conversion of an etioplast to a chloroplast is accompanied by the increase in the carotenoid accumulation in maize seedlings (Albrecht and Sandmann, 1994). This 2.8 fold increase in carotenoid level is due only to an up-regulation of IPP isomerase activity under the condition where phytoene desaturation is inhibited by the herbicide Norflurazon (Sandmann and Böger, 1989). From this experiment it has been suggested that the light regulated steps are located in the pathway leading to the synthesis of phytoene (Albrecht and Sandmann, 1994). Contrarily, in seedlings of white mustard, *Sinapis alba*, light stimulated conversion of etioplast to chloroplast is accompanied by the up-regulation of PSY expression at the mRNA level, with the mRNA levels of GGPPS and PDS remaining constant (von Lintig *et al.*, 1997). This up-regulation is only

observed under light conditions suitable for chlorophyll biosynthesis which is followed by the significant increase in carotenoid content (von Lintig *et al.*, 1997). A possible explanation for this conflicting results may be that in photosynthetically inactive, herbicide treated maize seedlings, the chloroplast differentiation might have been affected. This could affect the chlorophyll biosynthesis. If chlorophyll biosynthesis and carotenoid biosynthesis are tightly co-regulated, at least in green tissues, then in photosynthetically inactive maize seedlings, the activities of carotenoid biosynthetic pathway specific enzymes might not be induced. This may explain why light treatment did not lead to induction of PSY activity in bleached maize seedlings, but did result in increased levels of PSY transcript in normal mustard seedlings.

Regulation upstream of carotenoid biosynthesis during the differentiation of chromoplast from chloroplast:- In ripening fruits of pepper, an increase in GGPPS mRNA levels has been observed just prior to the ripening stage where fruit color changes from green to red (Römer *et al.*, 1993) because of the accumulation of the xanthophyll capsanthin. Increase in GGPPS activity has also been noted during the same time (Kuntz *et al.*, 1992).

1.5.2: Regulation within The Carotenoid Biosynthetic Pathway

Regulation within the carotenoid biosynthetic pathway is quite well documented for the chloroplast to chromoplast transition during the development of fruits and flowers rather than the regulation within the carotenoid biosynthetic pathway for chloroplast differentiation.

Regulation during differentiation of chloroplast:- In tomato, as mentioned before, there are two genes encoding PSY, *Psy1* and *Psy2*. Tissue specific gene expression is observed for *Psy1* and *Psy2* (Bartley and Scolnik, 1993). Both *Pys1* and *Psy2* are expressed in mature leaves. *Pys1* is predominantly expressed in seedlings while *Psy2* transcripts are more abundant in mature leaves (Bartley and Scolnik, 1993). During photo-morphogenesis (etioplast to chloroplast transition) expression of both *Psy* and *Pds* is constitutive, but upon dark adaptation of light grown seedlings, *Psy* mRNA level decreases 7 fold without any significant change in *Pds* mRNA level (Giuliano *et al.*, 1993). This suggests that after the completion of photo-morphogenesis, light is required only for the expression of *Psy* (Giuliano *et al.*, 1993).

Regulation during fruit ripening:- In tomato, during fruit ripening the increase in total carotenoids is concomitant with the decrease in chlorophyll content. The highest carotenoid content (mainly β-carotene and lycopene) is found in ripe fruit with the greatest carotenogenic enzymatic activities found in green fruit (Fraser *et al.*, 1994). More than a 25 fold increase in *Psy* expression and only a 3 fold increase in *Pds* expression is observed for orange fruits compared to immature green fruits (Giuliano *et al.*, 1993; Hugueney *et al.*, 1992). Both *Psy1* and *Psy2* are expressed in fruits at various stages of fruit ripening, but *Psy1* is predominantly expressed at very late stages of fruit ripening (Bartley and Scolnik, 1993). An increase in *Psy* mRNA level is also observed during early stages of fruit ripening in melon (Karvouni *et al.*, 1995). A 2.2 and 1.8 kb *Psy* mRNA have been detected in pepper fruits during ripening (highest level in ripe fruit) and in leaves (lower level compared to

fruits), and in contrast to this, there is a single *Psy* transcript present in tomato fruit (Römer *et al.*, 1993). The origin of these two transcripts in pepper is not yet clear, although a post-transcriptional modification has been suggested (Römer *et al.*, 1993).

In contrast to the up-regulation of early carotenoid biosynthetic genes, *Psy* and *Pds*, the gene encoding β-LCY is down regulated at the breaker stage, where fruit color changes from green to orange (Pecker *et al.*, 1996). The significance of this down regulation is the accumulation of lycopene, the red color pigment in ripe tomato fruits. In contrast, the constant levels of LCY transcripts (Hugueney *et al.*, 1995) and strong induction of capsanthin capsorubin synthase protein (Bouvier *et al.*, 1994) is observed during fruit ripening in pepper. This results in accumulation of capsanthin, the major carotenoid pigment, in pepper fruits (Goodwin, 1986).

Regulation during flower development:- Chloroplast to chromoplast transition also occurs during flower development. In the petals of mature tomato flowers, which contain only chromoplasts, greater than seven fold decrease in chlorophyll content and greater than eight fold increase in carotenoid content has been observed in comparison with the very early green stage. This increase in carotenoid content may be due to an approximately 10 fold increase in the expression of both *Psy* and *Pds* prior to anthesis (Giuliano *et al.*, 1993).

In fully developed daffodil flowers, the carotene content is eight times more than that present in green leaves and six times more than that of young flowers at the green bulb stage (Schledz *et al.*, 1996). This increased carotene content is accompanied by a very low, but steady state *Psy* transcript levels at all four flower development stages tested (Al-Babili *et al.*, 1996) with no detectable signals in young leaves. In contrast to this, although there is no detectable PSY protein in leaves, flower stages 1 and 2 show increase in PSY protein levels. But this increase cannot account for the increase in carotene content because expressed PSY includes both the active membrane associated form and inactive soluble form (Schledz *et al.*, 1996). Al-Babili *et al.* have shown that the steady state *Pds* transcript level to be low during flower development though it is upregulated relative to green leaves (Al-Babili *et al.*, 1996). Western analysis using antibodies for daffodil PDS has indicated that PDS protein level also increases throughout the flower developmental stages tested and that the level is substantially stronger relative to the expression in green leaves (Al-Babili *et al.*, 1996).

Requirement of chaperonins for PSY localization:- Work done with isolated daffodil chromoplasts has indicated the presence of two forms of PSY, an active membrane bound form and an inactive soluble stromal form. This inactive soluble form is a constituent of a chaperonin Hsp70 containing complex. It was also shown in the same system that chaperonins are needed for the membrane association of the inactive form soluble form of PSY protein for activation (Bonk *et al.*, 1997; Al-Babili *et al.*, 1996; Bonk *et al.*, 1996; Schledz *et al.*, 1996).

1.5.3: Regulation of Carotenoid Biosynthesis in Transgenic Plants

When the carotenoid and gibberellin levels were compared between wild type tomato and a transgenic tomato expressing antisense RNA to *Psy1* (Bird *et al.*, 1991), less than 10% of the carotenoids of the wild type were found in the transgenic tomato. Gibberellin levels were also elevated in this transgenic tomato and this increase was particularly evident in immature fruits. This may be due to the fact that GGPP, the substrate for *Psy1*, is utilized for the synthesis of gibberellins (Fraser *et al.*, 1995) in fruits.

Transgenic tomatoes overexpressing *Psy1* showed a dwarf phenotype with reduced chlorophyll content, probably because the intermediate GGPP were diverted towards carotenoid biosynthesis from gibberellin and phytol biosynthesis (Fray *et al.*, 1995). However, overexpression of daffodil PSY in rice endosperm did not result in accumulation of colored carotenoid products, but only in accumulation of phytoene (Burkhardt *et al.*, 1997). This could be a due to a block further downstream of the pathway. The possibility for this has yet to be tested.

In plants transformed with *Pds* promotor/GUS fusions, the expression of GUS was very low in flower petals when compared to anthers of transgenic tobacco (Corona *et al.*, 1996). In comparison, in transgenic tomatoes, mature flower petals showed very high levels of GUS expression. This discrepancy was attributed to the lack of chromoplasts in tobacco flowers (Corona *et al.*, 1996).

Based on all the information available so far, it is probable that the regulation of carotenoid biosynthesis in higher plants may occur at different steps. In some instances it appears that the regulation occurs upstream of the pathway while it is within the pathway in other instances. Since tissue specific regulation exists in plants, it is obvious that nuclear factors are also involved in the regulation of carotenoid biosynthesis. All the information available so far indicates that regulation of carotenoid biosynthesis does not occur at a specific step. Several factors are influencing the overall regulation of carotenoid biosynthesis. This means more work is needed to be done to completely understand the regulation of carotenoid biosynthesis at molecular level.

1.6: Perspectives

1.6.1: Significance

Rice, which is the major food staple in Southeast Asia and other developing Third World Nations, lacks carotenoids in its endosperms. Therefore, it is a poor nutritional source of proVitamin A and other nutritionally important carotenoids. It has been estimated that over 250 million children worldwide suffer because of the lack of Vitamin A and providing Vitamin A to young children at risk of deficiency can reduce child mortality by an average of 30 percent (Underwood and Arthur, 1996; Beaton *et al.*, 1993; Humphrey *et al.*, 1992). Providing proVitamin A containing rice would be a convenient way of improving the daily uptake of Vitamin A for those people who rely on rice as their primary food staple.

Engineering carotenoid biosynthesis and accumulation in rice endosperm necessitates a thorough understanding of the regulation of carotenoid biosynthesis in higher plants. Regulation of carotenoid biosynthesis in chloroplasts and chromoplasts is quite well studied in higher plants. Understanding the regulation of carotenoid biosynthesis in endosperm amyloplasts is crucial to develop strategies towards engineering carotenoid biosynthesis in endosperm.

The capacity to synthesize and accumulate carotenoids in photosynthetic tissues of rice implies the presence of the necessary structural genes required for carotenoid biosynthesis. The absence of endosperm carotenoids may be due to some tissue specific block in the pathway. For example, enzymes of the pathway may be expressed only in photosynthetic tissues and not in the non-photosynthetic endosperm. If the enzymes are expressed in endosperm, they may not be properly imported into rice amyloplasts. Another possibility may be a limitation in the isoprenoid precursors or enzymes catalyzing synthesis of the isoprenoid precursors. This last possibility is unlikely as suggested by several *in vitro* labeling studies. When extracts of immature rice endosperm were treated with [1-^{14}C]IPP, ^{14}C was incorporated into GGPP detected as its corresponding alcohol (geranyl geraniol) and into other isoprenoid intermediates like farnesol and squalene, but not into phytoene (Burkhardt *et al.*, 1997). When [^{14}C] GGPP and [^{14}C] phytoene were used, no radioactivity was incorporated into any carotenoids (Burkhardt *et al.*, 1997). This indicates, that in immature endosperm the enzymes catalyzing synthesis of GGPP, are present. These experiments disproved the hypothesis that the absence of colored carotenoids in rice

endosperm is due to a block upstream of the pathway. Therefore, I have decided to test the expression in rice endosperm, of genes encoding the early carotenoid biosynthetic enzymes, especially the first two enzymes PSY and PDS.

1.6.2: Specific Aims

The main objective is to find out (1) whether the genes encoding PSY and PDS are expressed in rice endosperm, (2) is there more than one gene involved in the expression of PSY and PDS in photosynthetic vs. non-photosynthetic tissues?, and (3) isolation and functional analysis of *Pds* cDNA from leaf.

In chapter 2, I report the temporal and spatial expression patterns of genes encoding PSY and PDS and how many genes encode these two early carotenoid biosynthetic enzymes. In chapter 3, the details of the isolation of rice *Pds* cDNA by the Random Amplification of cDNA Ends and its functional complementation in *E. coli* using carotenoid biosynthetic genes from non-photosynthetic bacterium *Erwinia uredovora* are presented. Finally in chapter 4, the results obtained from my study carried out on the rice *Pds* cDNA are summarized. How this research will lead to the future studies related to the understanding the regulation of carotenoid biosynthesis in higher plants is also discussed.

CHAPTER 2

WHY DON'T CAROTENOIDS ACCUMULATE IN RICE ENDOSPERM? ANALYSIS OF GENE EXPRESSION FOR THE FIRST TWO BIOSYNTHETIC ENZYMES, PHYTOENE SYNTHASE AND PHYTOENE DESATURASE

2.1: Abstract

The absence of colored carotenoids in rice endosperm amyloplasts is linked to vitamin A deficiency in developing countries where rice (*Oryza sativa* L.) is a main food staple. Prospects for metabolic engineering of carotenoid accumulation in rice endosperm require an understanding of how this plastid-localized, nuclear-encoded pathway, is regulated for tissue specificity; carotenoids accumulate in photosynthetic plastid, but not in endosperm plastids of rice. The absence of endosperm carotenoids may be due to lack of expression of genes encoding enzymes of the carotenoid biosynthetic pathway. To address this possibility, I investigated expression of genes encoding the first two enzymes of the pathway, PSY and PDS. Based on genomic DNA hybridization, *Psy* and *Pds* appeared to be single copy genes. Despite the absence of endosperm carotenoids, transcripts for both *Psy* and *Pds* were detected by RT-PCR.

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2.2: Introduction

The biosynthesis of carotenoids is essential for plant growth and development; carotenoids function as accessory pigments in photosynthesis, as photoprotectors preventing photooxidative damage, and as precursors to ABA (Bartley and Scolnik, 1995). The presence of carotenoids in endosperm tissue also adds nutritional value; in humans and animals, dietary carotenoids are essential precursors to Vitamin A and to retinoid compounds needed in animal morphogenesis (Lee *et al.*, 1981;(Bendich and Olson, 1989). Rice, unlike maize (*Zea mays*), does not accumulate endosperm carotenoids. Therefore, Vitamin A deficiency is linked with diets based on rice endosperm, an important food staple worldwide (Humphrey *et al.*, 1992). With the availability of genes encoding biosynthetic enzymes, metabolic engineering of carotenoid accumulation in rice endosperm amyloplasts could help to solve this widespread nutritional problem. However, central to the design of such strategies is a need for understanding all steps in the regulation of carotenoid biosynthesis in the endosperm.

Carotenoids are a large class of yellow, red, and orange pigments derived from isoprenoids and synthesized in plastids by nuclear-encoded enzymes [reviewed in (Sandmann, 1994; Bartley and Scolnik, 1995)]. The C_{20} isoprenoid GGPP is the first precursor to carotenoids, and to a variety of other isoprenoid-derived pathways, including gibberellins, chlorophyll (phytol chain), prenylquinones, and many secondary metabolites (Chappell, 1995). Activation of carotenoid biosynthesis is usually accompanied by induction

of carotenoid biosynthetic enzymes (Bartley and Scolnik, 1995), although induction of "upstream" steps has also been observed (Kuntz *et al.*, 1992; Albrecht and Sandmann, 1994). Induction of carotenoid accumulation is in competition with other pathways utilizing the common GGPP precursor, suggesting that a delicate balance of substrate flow must be achieved in promoting carotenoid accumulation in different tissues and at different developmental stages.

Rice endosperm lacks both colored carotenoids and biosynthetic intermediates. The carotenoid precursor, GGPP, has been detected in extracts of rice endosperm (G. Britton, personal communication). Furthermore, incubation studies of immature rice endosperm with [1-^{14}C] IPP have revealed the presence of GGPP detected as its corresponding alcohol geranyl geraniol, and other isoprenoid intermediates like farnesol and squalene, but not phytoene (Burkhardt *et al.*, 1997). The presence of carotenoids in photosynthetic tissue implies that rice has the necessary structural genes, but absence of endosperm carotenoids is due to some tissue-specific block in the pathway. In the present investigation, I explored whether the absence of endosperm carotenoids was due to lack of endosperm-specific expression of genes encoding the carotenoid biosynthetic enzymes. That is, carotenoid accumulation in endosperm might require switching on of the entire pathway in endosperm amyloplasts, where carotenoids have no apparent essential role. I examined expression of genes encoding the first two biosynthetic enzymes. The first, PSY, catalyzes the condensation of two molecules of GGPP to form phytoene, the C_{40} backbone for all carotenoids. The second, PDS, catalyzes two of four desaturation reactions (Bartley and

Scolnik, 1995). I found that surprisingly both genes were expressed in developing rice endosperm. Therefore, at least part of the pathway is expressed in rice endosperm, despite the absence of detectable carotenoid products and intermediates.

2.3: Materials and Methods

2.3.1: Plant Materials

IR36 cultivar of *Oryza sativa* L. (obtained from S. McCouch, Cornell University) was grown in the greenhouse with supplemental lighting at Lehman College, CUNY (appendix A). The rice variety Cypress was grown at the Louisiana Rice Research Station (obtained from Dr. Steve Linscombe, Crowley, LA). To obtain staged developmental samples of IR36, controlled pollinations were carried out as follows: When panicles began emerging from the leaf sheath, florets from the top of the panicles were removed before anthesis. This was carefully done for the top one third of the panicle for 1-3 days. When anthesis had occurred in most of the florets in the panicle, the remaining florets were removed and the entire panicle covered with Lawson 117 glycine bags (Lawson, Northfield, IL) and the date recorded. Endosperm samples were collected at various DAF and seed coat and embryo removed prior to freezing and storage at -80^{0}C. The seed coat could not be removed entirely from mature endosperm (40 DAF), and therefore this stage may have included some maternal tissue.

2.3.2: RT-PCR

cDNA synthesis- Total RNA was extracted from frozen leaves of 3 week old seedlings (2-3 leaf stage) and endosperm samples of different developmental stages (Logemann *et al.*, 1987). RNA was treated with RNase free amplification grade DNase I (GibcoBRL, Gaithersburg, MD) and used for first strand cDNA synthesis (SuperScript™ Preamplification System for First Strand cDNA Synthesis, GibcoBRL, Gaithersburg, MD).

PCR amplification- Primers used in RT-PCR analysis are listed in Appendix B. The amount of total RNA used for RT-PCR was first determined by testing for a linear response between initial RNA amount and the amplified PCR product. Leaf RNA of varying amount ranging from 10 ng to 1000 ng were used for cDNA synthesis in a 20 μL final reaction volume. One fourth of this reaction mixture (5 μL) was used to amplify leaf *Psy* and *Pds* transcripts by using the rice specific primers and the amplification conditions described below. Amplification products were electrophoresed as mentioned below and quantified using the volume analysis method in Molecular Dynamics Fragment Analysis Program (Molecular Dynamics, Sunnyvale, CA). For the amplification of leaf *Sh* transcript, cDNA synthesis was carried out using the initial RNA amount from 50 ng to 800 ng in a 20 μL reaction volume. Amplified products were also analyzed the same way as those of *Psy* and *Pds*. From this determination, 100 ng total RNA was reverse transcribed to cDNA in a reaction volume of 20 μL, yielding approximately 40 ng cDNA, in a volume of 20 μL. One fourth of this reaction (5 μL) was used as a template for PCR in a final volume of 25 μL. All

amplification products were confirmed by sequencing. Specific primers and amplification conditions are as follows:

Primers for amplification of *Psy* cDNA designed from EST sequences (GenBank accession No: D48251; D48697) were # 145: 5'-CCTGAAAGGCGCAAAGTGTCTGG-3' (forward) and # 146: 5'-CGATAGCATCAAGGATCTGCCGG-3' (reverse). For the description of EST clone see the list of plasmids used in appendix C. Five μL of cDNA (10 ng) was used as template in a 25 μL PCR reaction in 20 mM Tris-HCl pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$; 200 μM each dNTP (United States Biochemicals, Cleveland, OH); 2 μM each primer; 0.06 U/μL *Taq* DNA polymerase (GibcoBRL, Gaithersburg, MD). The reaction was incubated for 1 cycle at 94°C (3 min), followed by 30 cycles at 94°C (30 sec), 65°C (30 sec), 72°C (2 min) and 1 cycle of 72°C (10 min).

For amplification of rice *Pds*, degenerate oligonucleotide primers were designed by alignment of dicot *Pds* sequences to represent a region of high homology, corresponding to nt. 1276-2099 of tomato *Pds* (GenBank accession # X59948). Forward primer # 92: 5'-GAC(T)GAGGTGTTC(T)ATA(T)GCC(T)ATGTC-3' and reverse primer # 95: 5'-ACAGCA(G)CCTTCCATG(T)GAAGCC(T)AA-3' were used to amplify rice *Pds* from leaf cDNA. The PCR protocol was the same as above except for 2.5 mM $MgCl_2$, 56°C annealing temperature and 40 cycles. A partial sequence of the 825 bp *Pds* PCR product, which showed approximately 85% homology to maize *Pds* (GenBank accession No: U37285), was used to design a rice-specific *Pds* forward primer # 151: 5'-

GACCATGTTCGCTCTTTGGGTGG-3', and reverse primer # 152: 5'-CGATGATTTCAGTGTCACTCCGTCC-3', for RT-PCR experiments. With this rice-specific primer pair and an annealing step of 62^{0}C and 40 cycles, a 430 bp rice *Pds* product was amplified from leaf and endosperm cDNA.

For amplification of rice *Sh* cDNA, primers were designed from rice cDNA sequence (GenBank accession No: Z15028). The forward primer # 139: 5'-AATTCAGAGCCTTCGTGGGC-3', and reverse primer # 140: 5'-TTTGTGAGCCAGCAGAGTGG-3' were used to amplify the *Sh* transcripts from leaf and endosperm cDNA. The PCR protocol was the same as above except for using 2 mM $MgCl_2$, 65^{0}C annealing temperature and 40 cycle incubation.

Gel electrophoresis-10 μL of each PCR reaction was electrophoresed on a 1% (w/v) agarose gel in 0.5X TBE, pH 8.3 (Sambrook *et al.*, 1989) with ethidium bromide (0.5 μg/mL).

2.3.3: DNA Sequencing

PCR amplified products, obtained directly from agarose gel pieces, were partially sequenced according to Trewick and Dearden (1994) using the Sequanase Version 2.0 DNA Sequencing Kit (United States Biochemicals, Cleveland, OH). Sequence homology analysis was carried out using PCGene software (Intelligenetics, Mountain View, CA).

2.3.4: Plant DNA Extraction and Southern Analysis

Rice leaves were collected from 3 week old seedlings and DNA was extracted (Burr and Burr, 1981). DNA (3 μg) was digested with restriction enzymes, *Eco*R I or *Hin*d III (GibcoBRL, Gaithersburg, MD) according to the vendor's recommendations except for the addition of spermidine HCl at a final concentration of 4 mM. Gel electrophoresis and Southern analysis was carried out as in Wurtzel *et al.*, (1987) using random primed [^{32}P]-labeled PCR amplified fragments of rice *Psy* and *Pds* cDNA as probes (Feinberg and Vogelstein, 1984). Southern analysis at low stringency was also carried out as mentioned above except for the use of 35% formamide in the hybridization buffer and 37^{0}C hybridization tempreature instead of 50% formamide and 42^{0}C hybridization temperature.

2.4: Results

2.4.1: Amplification of *Psy* and *Pds* Sequences.

To determine transcript accumulation for *Psy* and *Pds*, gene-specific probes and primers were developed as described in Materials and Methods. For *Psy*, search within the EST database revealed two *Psy*-homologous sequences from rice. EST clone S14375 (GenBank accession # D48251), designated as EST clone 1, sharing 75% identity for nucleotide sequences and 81% identity for amino acid residues with the maize *Y1* gene encoding PSY (GenBank accession # U32636), was used to design the rice-specific *Psy*

forward primer. EST clone S15075 (GenBank accession # D48697), designated as EST clone 2 sharing 76% identity for nucleotide sequences and 77% identity for amino acid residues with maize *Y1*, was used to design the rice-specific *Psy* reverse primer. The 680 bp cDNA amplified from rice leaf mRNA, which spanned both EST clones, showed the predicted sequence based on that for the EST clones (Matthews and Wurtzel, unpublished). This 680 bp cDNA also shared a similar pattern of hybridization to rice genomic DNA as obtained by probing with the EST clones (Figure 2.1).

For amplification of rice *Pds*, I used an approach similar to amplification of maize *Pds* (Li *et al.*, 1996). Degenerate oligonucleotide primers, designed from conserved sequences seen in alignment of several dicot *Pds* genes, were used to amplify an 825 bp product from rice cDNA. The sequence of the rice *Pds* amplification product showed 85 % homology with that of maize (Figure 2.2) and was used to design rice-specific primers for transcript analysis described below. Identity of 825 bp RT- PCR amplified cDNA was confirmed by sequence analysis. As shown in Figure 2.3, the rice *Psy* and *Pds* amplification products appeared to hybridize to single copy genes in rice. Even at lower hybridization stringencies, a similar pattern was obtained (Figure 2.4) when rice *Psy* was used as the probe. For rice *Pds,* weakly hybridizing 1.2 kb *Eco*R I fragment and 0.8 kb *Hin*d III frgment at high stringent condition, were not observed for lower hybridization stringencies (Figure 2.4). This could be due to the fact that the temperature (55^0C) at which washing was done to remove the unbound probes from the filter was too high. At this temperature some of bound probes could also have been removed. Using RFLP analysis, *Psy* and *Pds* were mapped to single

loci, in chromosome 12 distal to the marker RG181 and to chromosome 3 between markers RG191 and RZ993X, respectively (Wurtzel, Yoganathan and McCouch, unpublished).

2.4.2: Transcript analysis.

To examine expression of *Psy* and *Pds*, rice gene-specific primers were used to amplify transcripts by RT-PCR. The more sensitive RT-PCR method was chosen over Northern analysis because of the low abundance of transcripts from genes encoding carotenoid biosynthetic enzymes (Giuliano *et al.*, 1993; Li *et al.*, 1996). For comparison to a known endosperm expressed gene, primers were used to amplify transcripts of the rice homolog of maize *Sh1*. *Sh1* is a nuclear gene encoding sucrose synthetase, a cytosolic enzyme involved in starch biosynthesis and expressed in endosperm. I expected that even if *Psy* and *Pds* were not expressed in the endosperm, *Sh* trnascripts would be detected in this starch accumulating tissue.

For transcript analysis by RT-PCR, gene-specific primer sets were designed to flank intronic sequences to ensure that PCR products were derived from cDNA, since genomic DNA would give rise to higher molecular weight products than cDNA. PCR products for *Psy*, *Pds* and *Sh* amplified from cDNA were 680, 430, and 787 bp (Figure 2.5 lane 1) and approximately 1300bp, 1200bp, and 1100bp when amplified from genomic DNA (Figure 2.5 lane 3). Furthermore, cDNA synthesis reactions lacking reverse transcriptase did not give PCR products (Figure 2.5 lane 2) indicating that PCR products were specifically derived

from cDNA and not from genomic DNA. Amplification carried out with either forward or reverse primer alone did not give PCR products (Figure 2.5 lanes 4 and 5).

Prior to using RT-PCR to analyze transcript levels of *Psy*, *Pds* and *Sh*, the initial amount of total RNA used for cDNA synthesis was optimized as follows. For each primer pair, the amount of total leaf RNA used to synthesize the first strand of cDNA was varied from 10 ng - 1000 ng and the PCR amplified products were quantified using the volume analysis method in the Molecular Dynamics Fragment Analysis Program (Molecular Dynamics Corporation, Sunnyvale, CA). As shown in Figure 2.6 a linear response was obtained in the range of 50 - 500 ng starting RNA for the amplification of *Psy* and *Pds* transcripts. For the amplification of *Sh* transcripts, a linear reponse was obtained in the range of 50 - 400 ng starting RNA (Figure 2.6). Therefore, 100 ng of total RNA was used for synthesis of cDNA template used for PCR.

To find out whether the genes encoding PSY and PDS are expressed in the endosperm, the *Psy*, *Pds* transcripts were amplified from 20 DAF endosperm RNA. Figure 2.7 shows that both *Psy* and *Pds* were expressed not only in leaves, but surprisingly also in the rice endosperm. The *Sh* transcript was also detected in both tissues (Figure 2.7).

Since the endosperm used reflected only one developmental stage, which might not overlap with the window for potential carotenoid accumulation, I decided to test whether transcripts were present at other, earlier developmental stages. An example of

developmental stages used for the RNA extraction are shown in Figure 2.8. Total RNA extracted from endosperm dissected at 10, 15, and 20 DAF, and from mature (more than 40 DAF) endosperm, was used for RT-PCR. The results of RT-PCR analysis is shown in Figure 2.9. For each gene tested, transcripts were found throughout endosperm development, although temporal pattern differed. The *Psy* transcript level was constant, whereas the *Pds* transcript level was approximately two fold less at 10 DAF when compared with the level at the mature stage. The transcript levels of *Sh* was maximal at 10 DAF and steadily decreased as the endosperm matured. The transcript level of *Sh* was approximately two fold higher at 10 DAF when compared with the level at the mature stage. At all endosperm developmental stages tested, transcript levels for both *Psy* and *Pds* were lower as compared to the levels in leaves, when equal amounts of RNA were used as template for RT-PCR.

2.5: Discussion

To investigate why carotenoid accumulation is absent in rice endosperm plastids (amyloplasts), I analyzed expression of genes encoding the first two enzymes of the pathway, PSY and PDS. By hybridization to genomic DNA, I determined that rice *Psy* and *Pds* appeared to be single copy genes, supporting the possibility that tissue specificity of gene expression might play a role in modulating differential activity of this pathway. This is unlike the case of tomato (*Lycopersicon esculentum*) where *Psy* is encoded by duplicate genes varying in tissue-specific expression (Bartley and Scolnik, 1995).

I was surprised to find that both *Psy* and *Pds* were expressed during endosperm development, despite the lack of detectable carotenoids. Therefore, I could rule out the hypothesis that activation of the carotenoid biosynthetic pathway in rice endosperm amyloplasts requires a "master switch"; at least some genes are already expressed.

Since the rice endosperm does not appear to accumulate carotenoids, it was surprising to observe transcripts for two biosynthetic enzymes, representing the first two steps of the carotenoid biosynthetic pathway. The *Psy* transcript was also translated as indicated by Western analysis (Yu and Wurtzel, in preparation). The activity and sub-cellular localization of the PSY protein were not tested in this work. It is unclear whether *Pds* transcripts are translated, since a monocot PDS specific antibody is not available. Therefore, I do not know whether the rice *Psy* and *Pds* encode functional, properly localized proteins.

At this point, I only know that *Psy* and *Pds* were the only carotenoid biosynthetic genes expressed at the transcriptional level and the pattern of this expression was different in the various developmental stages of the endosperm tested. The youngest stage tested was endosperm from 10 DAF seeds. An approximate two fold increase in *Pds* transcript level was observed between 10 DAF and the mature stage. I do not have any data for the for the expression of *Psy* and *Pds* transcripts at younger developmental stages. If it is crucial for the carotenoid biosynthetic genes to be expressed at the proper endosperm developmental stages

for the pathway to be activated, extreme low level or lack of expression of carotenoid biosynthetic genes during very early stages of the endosperm development might be a reason for the lack of colored carotenoid accumulation.

The lack of phytoene accumulation in immature endosperm, as shown by the incubation studies with [1-^{14}C] IPP have revealed the presence of GGPP and other isoprenoid intermediates, but not phytoene (Burkhardt *et al.*, 1997). The over-expression of daffodil PSY in rice endosperm does not result in the accumulation of colored carotenoid products, but only in the accumulation of phytoene (Burkhardt *et al.*, 1997). These observations together with the presence of rice *Psy* transcripts and translated products in developing rice endosperm suggests that in rice endosperm the endogenous PSY might not be expressed at sufficient levels. Alternatively, the endosperm PSY protein detected might be inactive due to absence of some ancillary factors. For example, chaperonins have been found to play a role in activation of carotenoid biosynthetic enzymes in chromoplasts that develop from chloroplasts. Using daffodil (*Narcissus pseudonarcissus*) chromoplasts, where active enzymes of the pathway are membrane associated, Bonk *et al.* have shown that inactive forms of PDS and PSY exist in stromal complexes associated with chaperonins Hsp70 and Cpn60 (Bonk *et al.*, 1996) inactive PDS in these complexes was found competent for enzyme activation and membrane association (Al-Babili *et al.*, 1996).

Amyloplast carotenoid biosynthesis might also be suppressed by other pathways if branch-point enzymes compete for common precursors or interaction with biosynthetic

enzymes providing those precursors. In dicots, evidence for plastid competition for GGPP, or earlier isoprenoid precursors, was seen in transgenic tomato plants overexpressing PSY, where increased carotenoid synthesis was accompanied by reductions in gibberellins and chlorophyll (Fray *et al.*, 1995). The converse was observed when inhibition of PSY in tomato fruit resulted in increased gibberellins and cytosolic sterol (Fraser *et al.*, 1995). In maize, a monocot, increased chlorophyll content was related to decreased carotenoid content, when measured under low light, suggesting that carotenoid and chlorophyll (phytol) pathways compete for common isoprenoid precursors; a block (*cl1*) in the carotenoid pathway allowed for increased phytol synthesis, whereas suppression of the carotenoid mutation, restored the carotenoid pathway and competition for isoprenoid precursors (Robertson *et al.*, 1966). Though similar studies of pathway competition have not been carried out for the amyloplast, the examples given for the chloroplast and chromoplast suggest that competition in the amyloplast should be anticipated.

Although the plastid specific pathways can compete for common precursors, biosynthesis of isoprenoid precursors takes place via two independent pathways in plastids and cytosol. Evidence for this has been shown where manipulation of HMGCoAR expression, affects cytosolic sterol biosynthesis but not plastid carotenoid synthesis in tobacco leaves and other tissues (Chappell *et al.*, 1995) since cytosolic IPP is derived from mevalonate. Plastidic IPP is derived from 1-deoxyxylulose-5-phosphate, a condensation product derived from glyceraldehyde 3-phosphate and pyruvate derived hydroxyethyl thiamine (Lichtenthaler *et al.*, 1997b). This alternate pathway was first discovered in

eubacteria (Rohmer *et al.*, 1993). However, in other situations modulation of cytosolic enzyme activity can affect both cytosolic and plastid isoprenoid-derived pathways. For example, in pepper fruit, elicitor induction of cytosolic synthesis of a phytoalexin (capsidiol) from cytosolic FPP results in the suppression of the plastid-localized carotenoid biosynthetic pathway. Although physically separated suppression of carotenoid biosynthesis may be mediated by a shift in IPP distribution in plastid and cytosol (Hugueney *et al.*, 1996).

Concerning activation of the carotenoid biosynthetic pathway, there is obviously more work to be done. Besides possible limitations in precursors, other problems may need to be overcome. Although the relationship between *Pds* transcript level and PDS protein is unclear, induction of the pathway in rice endosperm might require higher levels of PDS, and induction of PSY activity, and perhaps other of the carotenoid biosynthetic enzymes not assessed in this study. In developing maize endosperm PSY is induced (Yu and Wurtzel, unpublished) and *Pds* transcript level is constant (Li *et al.*, 1996), in contrast with the constant level of PSY protein and the increasing level of *Pds* transcripts in developing rice endosperm.

Why would components of the rice endosperm carotenoid pathway be expressed without apparent accumulation of product? The answer is unclear. Perhaps a better understanding of endosperm development with regard to nuclear-cytoplasmic communication might explain why this pathway is not completely suppressed. Further study of the expression of all genes encoding carotenoid biosynthetic enzymes, at transcriptional and

translational levels and also the post-translational modifications of gene products, of rice will provide a better understanding of mechanisms controlling tissue-specific accumulation in this important food staple. This and future research will provide the foundation for activation of the endosperm carotenoid pathway, a solution to a widespread nutritional problem associated with absence of rice endosperm carotenoids.

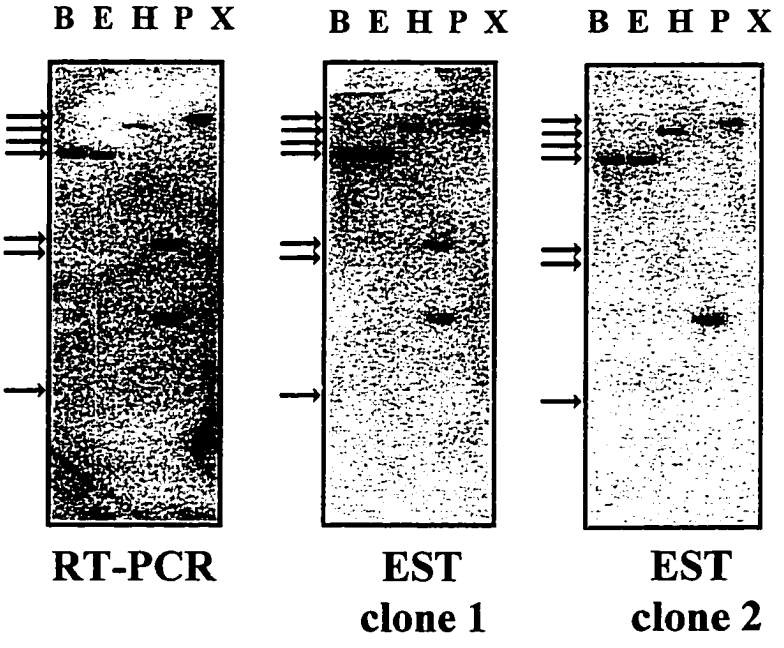


Figure 2.1: Southern analysis of rice *Psy*.

Rice genomic DNA digested with *Bam*H I (B), *Eco*R I (E), *Hin*d III (H), *Pvu* II (P) and *Xho* I (X) were subjected to Southern analysis and probed with RT-PCR amplified *Psy* cDNA fragment and two EST clones (shown below each panel in bold letters). Sizes of the probes are as follows: RT-PCR fragment-680 bp; EST clone 1(GenBank accession number D48257)-1.3kb; EST clone 2 (GenBank accession number D48697)-0.9 kb. Arrows to the left of the panel indicate the positions of molecular markers. Sizes of molecular weight markers from top are 23.1; 9.4; 6.6; 4.4; 2.3; 2.0 and 0.56 kb.

Figure 2.2: Comparison of partial sequence of 825 bp RT-PCR amplified rice *Pds* cDNA fragment with the corresponding region of the maize *Pds* cDNA sequence. Degenerate primers # 92 (forward) and # 95 (reverse) were used for amplification and sequencing. Rice specific *Pds* primers used in transcript analysis and RACE (see chapter 3) are underlined. Primers #152 and #179 are based on the sequence of the complementary strand. Nucleotide positions indicated are from (OS) rice (GenBank Accession # AF049356) and (ZM) maize (GenBank Accession # U37285) *Pds* sequences.

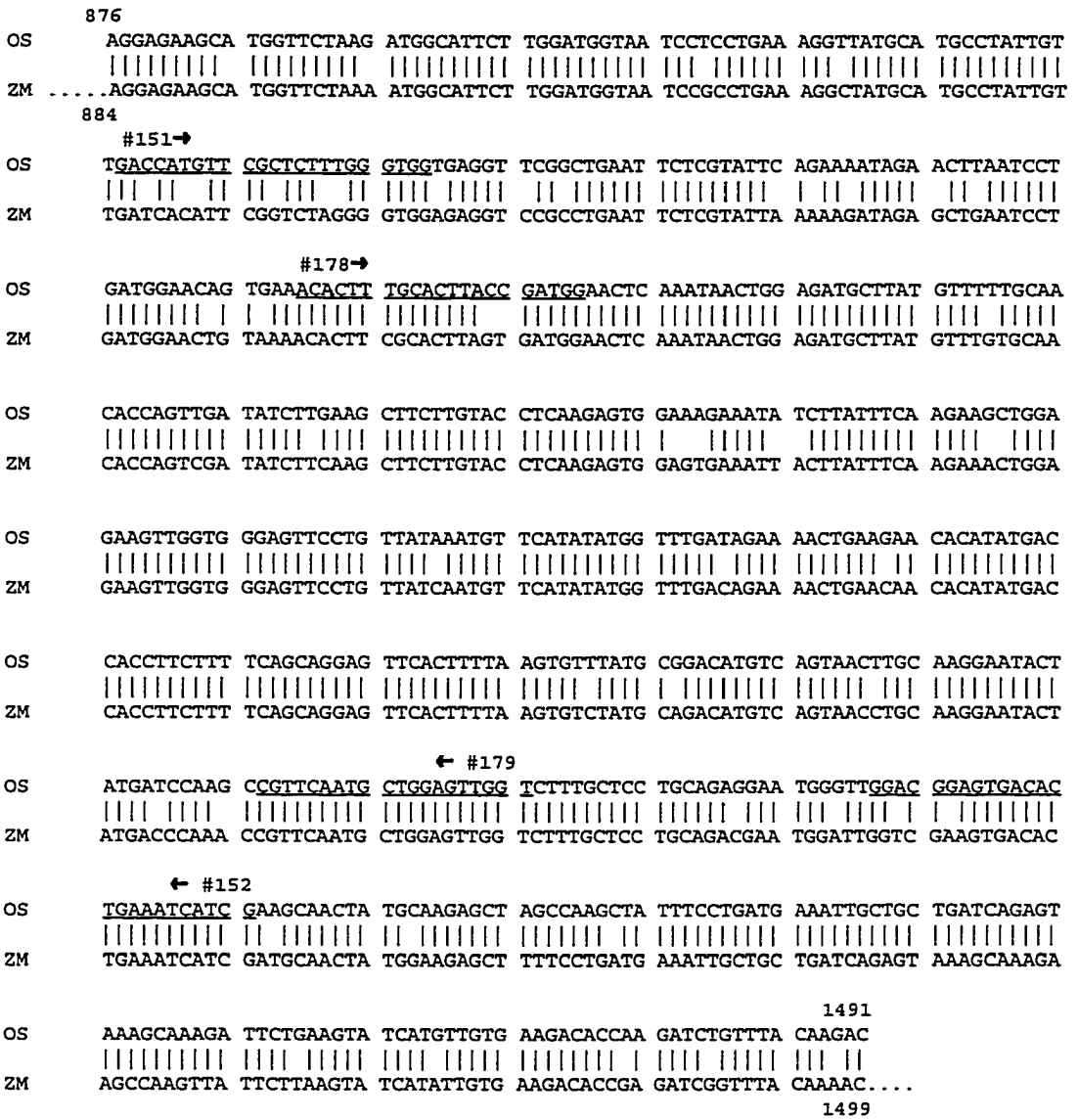


Figure 2.2: Comparison of partial sequence of 825 bp RT-PCR amplified rice *Pds* cDNA fragment with the corresponding region of the maize *Pds* cDNA sequence.

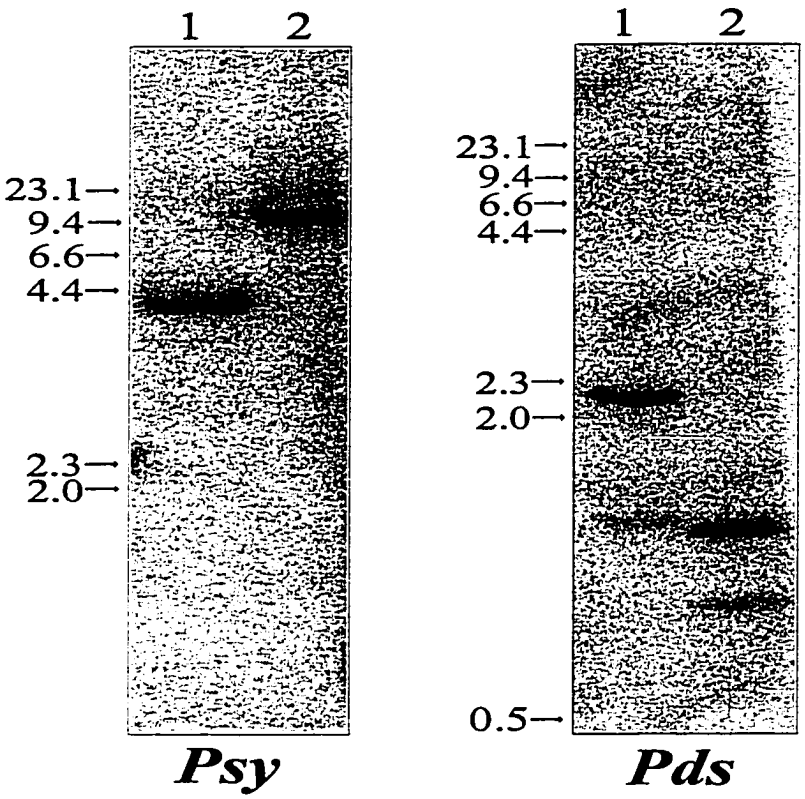


Figure 2.3: Genomic DNA hybridization.

Rice cultivar IR36 genomic DNA was digested with *Eco*R I (1) or *Hin*d III (2) and subjected to Southern analysis using either *Psy* or *Pds* RT-PCR amplification products as probes. Molecular weight markers, in kb, are shown at left of panels.

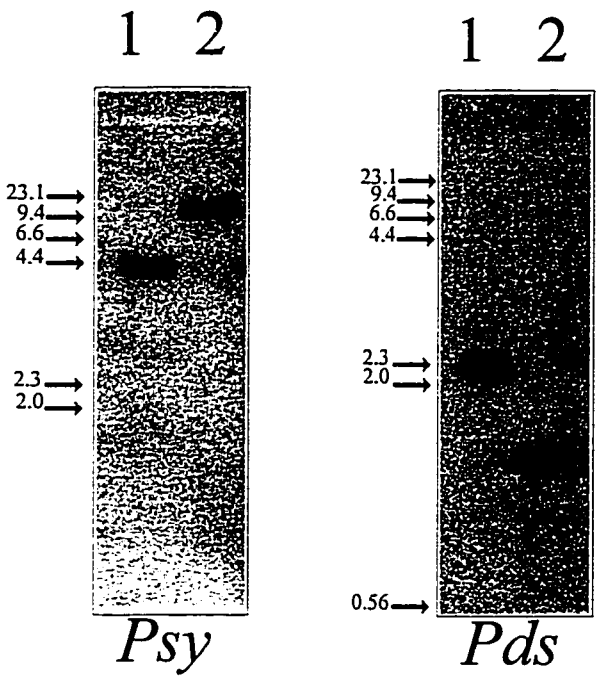


Figure 2.4: Genomic DNA hybridization at low stringency.

Rice cultivar IR36 genomic DNA was digested with *Eco*R I (1) or *Hin*d III (2) and subjected to Southern analysis using either *Psy* or *Pds* RT-PCR amplification products as probes under low stringent conditions. Molecular weight markers, in kb, are shown at left of panels.

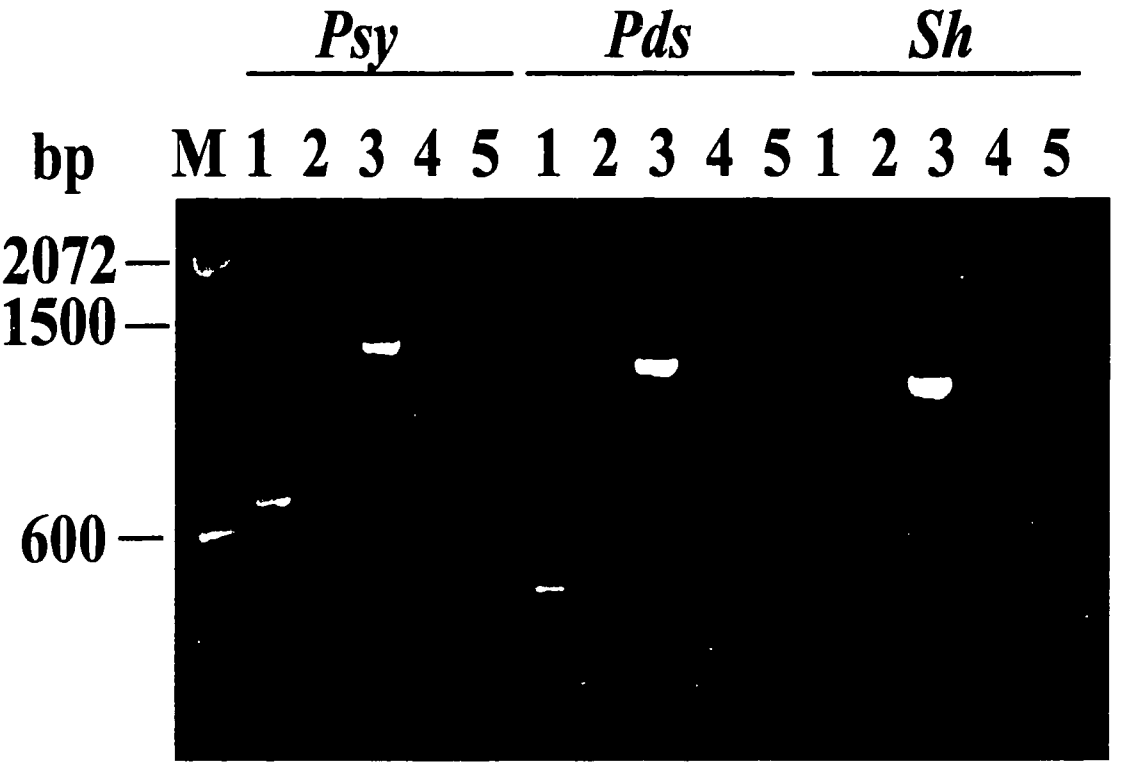


Figure 2.5: RT-PCR controls.

Lane M: 100 bp DNA ladder used as molecular weight marker

Lane 1: Amplification with reverse transcriptase in cDNA synthesis

Lane 2: Amplification without reverse transcriptase in cDNA synthesis

Lane 3: Amplification from genomic DNA

Lane 4: Amplification from cDNA with forward primer only

Lane 5: Amplification from cDNA with reverse primer only

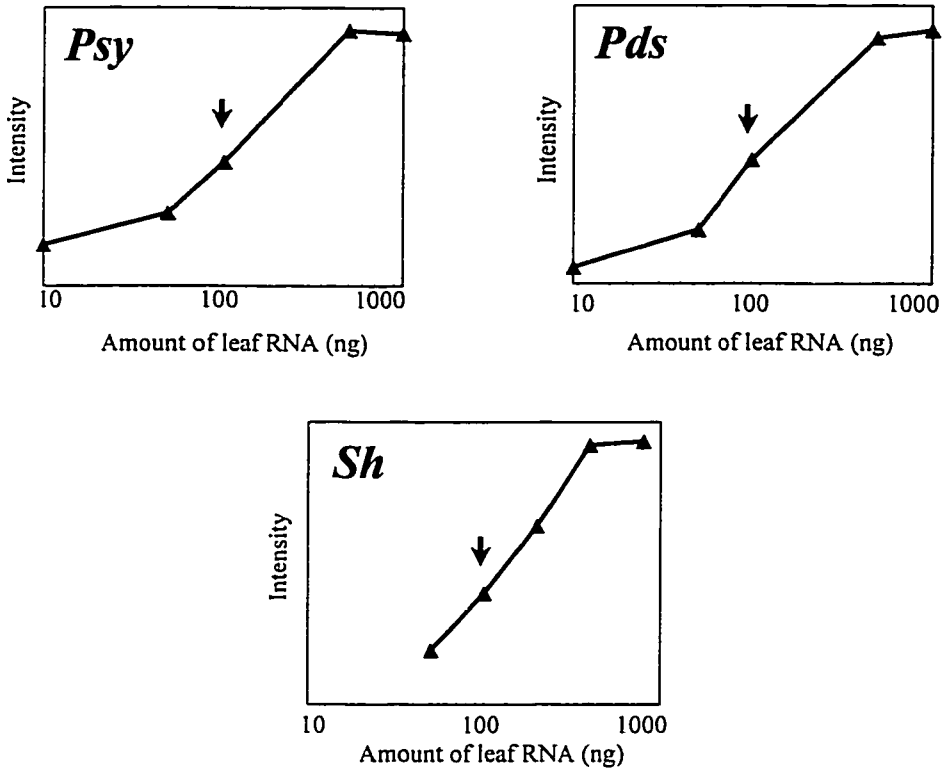


Figure: 2.6: Varying RNA template used for RT-PCR quantitation.

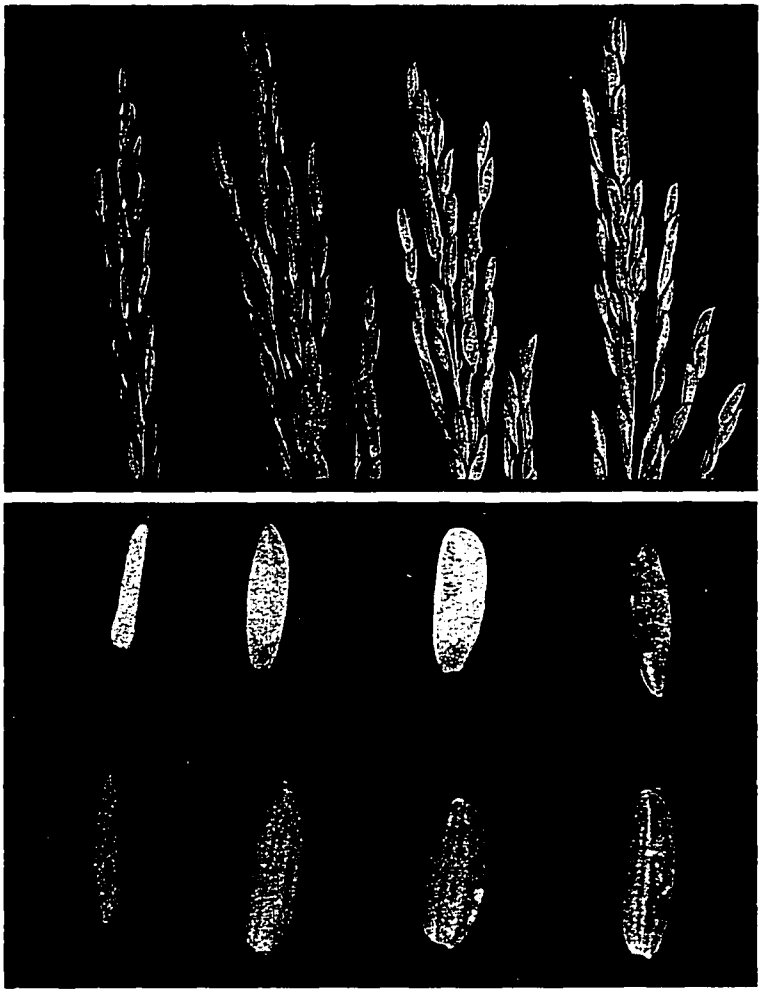
The X axis, in log scale, represents the amount of total leaf RNA used for cDNA synthesis. The Y axis, in linear scale, represents the quantity of RT-PCR amplified products as described in methods. Each point is the average of three assays. The arrow points to the initial amount of RNA used in the subsequent RT-PCR experiments.

Figure 2.7: RT-PCR analysis in rice endosperm and leaf.

Total RNA extracted from 20 DAF endosperm (E) or young leaves (L) from rice IR36 was used for cDNA template synthesis to amplify transcripts encoding proteins shown at left of panel.

Figure 2.8: Developing rice panicles and seeds.

From left to right, developmental stages of rice variety Cypress are 5, 10, 15 and 20 DAF. Developing panicles (upper panel) contain seeds shown in lower panel; lower panel shows developing seeds with seed coat removed (top) or with seed coat intact (bottom).



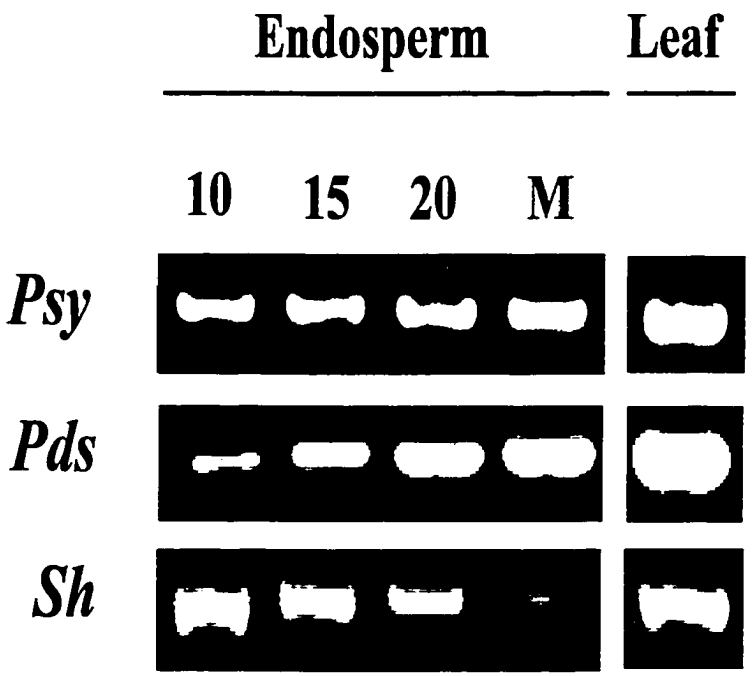


Figure 2.9: RT-PCR analysis in developing endosperm and leaf.

RT-PCR amplified transcripts (shown at left of panel), from total RNA extracted from rice IR36 endosperm at different developmental stages (DAF) (indicated above panel) or from young leaves.

CHAPTER 3

MOLECULAR CLONING AND FUNCTIONAL ANALYSIS OF A RICE cDNA ENCODING PHYTOENE DESATURASE

3.1: Abstract

A rice cDNA encoding the carotenoid biosynthetic enzyme phytoene desaturase has been isolated by Rapid Amplification of cDNA Ends (RACE). 5' and 3' RACE Amplified cDNA fragments were subcloned into pBluescript II SK(-) vector and using the restriction sites shared by these cDNA fragments, a complete 2027 bp rice *Pds* cDNA was constructed. This clone was designated as pOSPDS. Both strands of this clone were fully sequenced. The nucleotide sequence of the reconstructed cDNA showed 80% and 69% identity to maize and daffodil *Pds* sequences, respectively. The predicted amino acid sequence of rice PDS shared 89% and 79% identity to the predicted amino acid sequences of maize and daffodil PDS. The rice *Pds* cDNA encodes a 63.5 kDa protein consisting of 566 amino acid residues. Based on the comparison to the predicted amino acid sequence of cyanobacterial PDS, which does not have a transit peptide region, the amino acid sequence of the mature rice PDS and the amino acid sequence of putative transit peptide region were predicted. The putative transit peptide region contains 92 amino acid residues, predicted to be 10.3 kDa. The predicted size of the mature rice PDS protein is 53.2 kDa. A dinucleotide binding domain necessary for enzyme activity was also identified in the region of amino acid residues 100 and 130 (nucleotides 299-391). When complemented with genes encoding *Erwinia*

uredovora CRTE and CRTB, rice PDS catalyzed a two step desaturation of phytoene resulting in the synthesis of ζ-carotene in *Escherichia coli*.

3.2: Introduction

Carotenoids are important in photosynthesis, in photo-protection and they are also the precursors of the phytohormone abscisic acid (ABA) and vitamin A (Bartley and Scolnik, 1995). Carotenoid biosynthetic enzymes are encoded in the nucleus and synthesized in the cytosol. Carotenoid biosynthesis and accumulation take place in plastids. Therefore, the carotenoid biosynthetic enzymes are imported into the plastids and routed properly to the plastid membranes. Carotenoid accumulation takes place in rice photosynthetic tissues but not in non-photosynthetic tissue like endosperm. It was shown previously that the genes encoding the first two carotenoid biosynthetic enzymes PSY and PDS were expressed in the developing endosperm (chapter 2). Using RFLP analysis, rice *Pds* mapped to chromosome # 3 between the markers RG191 and RZ993X (Wurtzel, Yoganathan and McCouch, unpublished).

Enzyme desaturases introduce double bonds into the isoprenoid chain of phytoene (Britton *et al.*, 1995). In higher plants two desaturases catalyze four desaturation reactions. Phytoene desaturase (PDS) uses phytoene as the substrate and introduces two double bonds to synthesize ζ-carotene via phytofluene (Li *et al.*, 1996; Pecker *et al.*, 1992), and then ζ-carotene desaturase (ZDS) introduce two more double bonds to ζ-carotene to produce

lycopene via the intermediate neurosporene (Albrecht *et al.*, 1995). In contrast to this, the number of desaturation steps catalyzed by a single enzyme varies in photosynthetic bacteria, non-photosynthetic bacteria and fungi [reviewed in (Sandmann, 1994)]. In non-oxygenic photosynthetic bacterium like *Rhodobacter*, a single phytoene desaturase, CRTI, catalyzes three desaturation reactions resulting in the formation of neurosporene (Linden *et al.*, 1991). In non-photosynthetic bacteria *Erwinia uredovora* and *Erwinia herbicola* (Linden *et al.*, 1991; Misawa *et al.*, 1990), and the fungus *Neurospora crassa* (Bartley *et al.*, 1990; Goldie and Subden, 1973) a single phytoene desaturase, CRTI catalyzes all four desaturation steps resulting in the formation of lycopene.

Immunogold localization of PDS in higher plant chloroplasts has demonstrated that most of the PDS is localized in thylakoid membranes of chloroplasts (Linden *et al.*, 1993b). Work done with isolated daffodil chromoplasts demonstrated that PDS also exists as an inactive form associated with chaperonins Hsp70 and Cpn60 in the stroma (Bonk *et al.*, 1997; Bonk *et al.*, 1996). This inactive form of PDS has the capacity for enzyme activation and membrane association (Al-Babili *et al.*, 1996). It has also been demonstrated that PDS requires a co-factor like FAD or NAD for its catalytic function (Fraser *et al.*, 1993) and (Armstrong *et al.*, 1990a). The desaturation reactions also require other ancillary redox proteins like quinone oxidoreductases (Norris *et al.*, 1995; Mayer *et al.*, 1992).

Genes encoding PDS have been cloned from several higher plants, for example daffodil (Al-Babili *et al.*, 1996); maize (Li *et al.*, 1996); tomato (Pecker *et al.*, 1992) and

pepper (Hugueney *et al.*, 1992). In this work I will discuss the cloning and characterization of a rice cDNA encoding PDS by Rapid Amplification of cDNA Ends (RACE) and demonstration of its function in bacteria.

3.3: Materials and Methods

3.3.1: Plant RNA Extraction

Total RNA was extracted from frozen leaves of 3 weeks old seedlings (2-3 leaf stage) grown in the greenhouse of Lehman College, CUNY (Logemann *et al.*, 1987) (see appendix A for growing rice in greenhouse). RNA was treated with RNase free amplification grade DNase I (GibcoBRL, Gaithersburg, MD) and used in first strand cDNA synthesis for 5' RACE (5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0, GibcoBRL, Gaithersburg, MD), and 3' RACE (3' RACE System for Rapid Amplification of cDNA Ends, GibcoBRL, Gaithersburg, MD).

3.3.2: RT-PCR Amplification of Rice *Pds*

One hundred ng of total RNA (DNA free) was used for first strand cDNA synthesis (SuperScript™ Preamplification System for First Strand cDNA Synthesis, GibcoBRL, Gaithersburg, MD) according to the manufacturer's instructions. Primers used in the following amplifications are listed in appendix B. For amplification of rice *Pds*, degenerate

oligonucleotide primers were designed by alignment of dicot *Pds* sequences to represent a region of high homology, corresponding to nt. 1276-2099 of tomato *Pds* (GenBank accession # X59948). Forward primer # 92: 5'-GAC(T)GAGGTGTTC(T)ATA(T)GCC(T)ATGTC-3' and reverse primer # 95: 5'-ACAGCA(G)CCTTCCATG(T)GAAGCC(T)AA-3' were used to amplify rice *Pds* from leaf cDNA. 5 μL of cDNA from a 20 μL cDNA reaction was used as template in a 25 μL PCR reaction containing 20 mM Tris-HCl pH 8.4; 50 mM KCl; 2.5 mM $MgCl_2$; 200 μM each dNTP (United States Biochemicals, Cleveland, OH); 2 μM each primer; 0.06 U/μL *Taq* DNA polymerase (GibcoBRL, Gaithersburg, MD). The reaction was incubated for 1 cycle at 94°C (3 min), followed by 40 cycles at 94°C (30 sec), 56°C (30 sec), 72°C (2 min) and 1 cycle of 72°C (10 min). Sequence of the 825 bp *Pds* PCR product, which showed approximately 85% identity to maize *Pds* (GenBank accession No: U37285), was used to design a rice-specific *Pds* forward primer # 151: 5'-GACCATGTTCGCTCTTTGGGTGG-3' (nt 947-959), and reverse primer # 152: 5'-CGATGATTTCAGTGTCACTCCGTCC-3' (nt 1352-1376), for RT-PCR experiments. With this rice-specific primer pair and an annealing step of 62°C and 40 cycles, a 430 bp rice *Pds* product was amplified from leaf and endosperm cDNA. Sequence of the 825 bp *Pds* PCR product was also used to design another set of rice *Pds*-specific primers # 178 5'-ACACTTTGCACTTACCGATGG-3' (nested to #151) and # 179 5'-ACCAACTCCAGCATTGAACG-3'(nested to # 152). With this primer pair # 178 (forward) and # 179 (reverse) a 297 bp rice *Pds* cDNA fragment was amplified using the protocol used for amplification of the 430 bp cDNA fragment. These rice *Pds* specific primers were used in the 5' and 3' RACE methods described below.

3.3.3: Amplification of Rice *Pds* using 5' RACE and 3' RACE Methods

All cDNA synthesis and PCR amplification were carried out according to the vendor's recommendations except where otherwise mentioned.

5' Race Amplification of Rice *Pds* cDNA fragment 512: Five hundred ng of total RNA (DNA free) was used for first strand cDNA synthesis in 5' RACE (5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0, GibcoBRL, Gaithersburg, MD) with rice-specific *Pds* primer # 152: 5'-CGATGATTTCAGTGTCACTCCGTCC-3' (Figure 3.1). After removing excess nucleotides and gene specific primers by using vendor provided GlassMax DNA Isolation Spin Cartridges, first strand cDNA was dC tailed according to the vendor. Five μL of dC tailed cDNA was amplified using primer # 183: 5'-GGCCACGCGTCGACTAGTACGGGGGGGGGGGGGGG-3' (forward) and rice specific *Pds* primer # 179 (nested to primer # 152): 5'-ACCAACTCCAGCATTGAACG-3' (reverse) (nt 1307-1326). Primer # 183 was used instead of vendor provided Anchor Primer, because the presence of deoxyinosine residues in the 3' region of vendor provided anchor primer lowered the specificity of the gene specific amplification (data not shown). 50 μL PCR reaction containing 20 mM Tris-HCL pH 8.4; 50 mM KCl; 2.5 mM $MgCl_2$; 200 μM each dNTP (United States Biochemicals, Cleveland, OH); 2 μM each primer; 0.06 U/μL *Taq* DNA polymerase (GibcoBRL, Gaithersburg, MD) was incubated for 1 cycle at 94^{0}C (3 min), followed by 40 cycles at 94^{0}C (30 sec), 52^{0}C (30 sec), 72^{0}C (2 min) and 1 cycle at 72^{0}C (10 min). After incubation, 2 μL of amplified product was re-amplified using primer # 210; 5'-

GGCCACGCGTCGACTAGTAC-3' (forward) and primer # 179 (reverse) in a 25 μL reaction volume using reaction conditions as mentioned above. The amplified 1166 bp PCR product 512, was confirmed by partial sequencing (data not shown) and by Southern analysis (Figure 3.1).

3' RACE Amplification of Rice *Pds* cDNA fragment 31: One μg of DNA free total RNA was used for the synthesis of first strand cDNA in 3' RACE using an adapter primer (AP): 5'-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3' in a 20 μL reaction volume. Target *Pds* cDNA was amplified using 2 μL of first strand cDNA as template with rice specific *Pds* primer # 151: 5' GACCATGTTCGCTCTTTGGGTGG 3' (forward) and primer # 210 (reverse) in a 25 μL PCR reaction. The PCR protocol was the same as for amplification of cDNA fragment 512. Following PCR, 2 μL was used as template for re-amplification with primer # 178: 5'-ACACTTTGCACTTACCGATGG-3' (forward) (nt 1030-1050) and # 210 (reverse) using the same protocol. The PCR amplified 998 bp cDNA fragment 31 was confirmed for its identity by partial sequencing.

5' RACE Amplification of Rice *Pds* cDNA fragment 132: The protocol for 5' RACE amplification of fragment 132 was the same as for amplification of fragment 512 except for use of rice *Pds* specific primer # 274: 5'-GTTTATGACCAGCATCTGCCAGATTTTTTG-3' in the first strand cDNA synthesis. Primer # 183 (forward) and # 274 (reverse) were used for first round of amplification, and primer # 210 (forward) and # 274 (reverse) for re-amplification of a 356 bp rice *Pds* cDNA

fragment 132. Identity of this PCR product was confirmed by partial sequencing and Southern analysis (Figure 3.1).

3.3.4: Cloning of RACE Products and Reconstruction of Complete *Pds* cDNA

(a) Cloning of RACE products:- All restriction endonucleases and DNA modifying enzymes were purchased from GibcoBRL, Gaithersburg, MD, except where indicated otherwise. PCR amplified products and DNA restriction fragments were purified by adsorption to Glass-Milk (GeneClean II kit, BIO 101, Vista, CA) following the manufacturer's direction. Purified PCR amplified products were treated with 1 U Klenow fragment of DNA polymerase and 10 mM dNTP (GibcoBRL, Gaithersburg, MD) at 37^{0}C to create blunt ends and then ligated to *Sma* I linearized vector, pBluescript II SK(-) (Stratagene, La Jolla, CA) according to Sambrook *et al.* (Sambrook *et al.*, 1989). Plasmids containing cDNA fragments 512, 31 or 132 were designated p512, p31, and p132, respectively. The rice *Pds* insert in p512 was found to be in the sense orientation with respect to *lacZ*, on the basis of sequencing and restriction mapping, whereas p31 ans p132 were not. See appendix C for the list of plasmids used in amplification and functional analysis.

(b) Reconstruction of complete *Pds* cDNA:- Details of the plasmid construction strategy are shown in Figure 3.2. p512 was digested with *Pst* I to cut at the multiple cloning site of the vector and treated with Klenow fragment to create blunt ends. Linearized p512 was again digested with *Nde* I to cut within the rice *Pds* insert. A 4.0 kb restriction fragment

containing the rice *Pds* insert and vector sequence was gel purified using the GeneClean II kit. p31 was digested with *Bam*H I to cut at the multiple cloning site of the vector and treated with Klenow fragment to create blunt ends, followed by digestion with *Nde* I. An *Nde* I-blunt end 808 bp restriction fragment from p31 was gel purified and ligated to the *Nde* I-blunt end 4.0 kb fragment of p512. The resulting 4.8 kb plasmid was designated as p5302 and contained a partial *Pds* cDNA insert of 1867bp.

p5302 was next digested with *Not* I to generate a cut at the multiple cloning site of the vector and treated with Klenow fragment to create blunt ends. The linearized p5302 was partially digested with *Alw*N I (New England Biolabs, Beverly, MA) and the desired 4.6 kb fragment was gel purified using the GeneClean II kit. p132 was digested with *Kpn* I at the multiple cloning site of the vector and Klenow treated to generate blunt ends. The linearized p132 was partially digested with *Alw*N I and a 400 bp fragment containing the 5' end of the rice *Pds* was gel purified. This 400 bp *Alw*N I - blunt end fragment from p132 was ligated to the 4.6 kb *Alw*N I- blunt end fragment of p5302. The resulting 4988 bp plasmid was designated as pOSPDS, and contained a reconstructed *Pds* cDNA of 2027 bp.

3.3.5: DNA Sequence Analysis

PCR amplified products, obtained directly from low melting point agarose gel pieces, were partially sequenced according to Trewick and Dearden (1994) using Sequenase™ Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH). Double

stranded plasmid DNA isolated using Wizard *Plus* Miniprep DNA Purification System (Promega, Madison, WI) was used as template for the automated sequencing and to prepare a deletion series used for sequence template and in functional complementation (described below). One strand of plasmids p512, p31 and p132 and after the reconstruction of pOSPDS, the opposite strand of pOSPDS were sequenced using the deletion series. Alternatively the primer walking method was used to obtain complete sequence information. Sequence comparisons and homology analyses were done using PCGene software (Intelligenetics, Mountain View, CA). Multiple alignment of more than one sequence was carried out with the CLUSTAL program (Higgins and Sharp, 1988) of PCGene software. *Pds* sequences (followed by GenBank accession numbers) used for the comparisons and alignments with rice *Pds Oryza sativa* (AF049356) are *Zea mays* (U37285); *Narcissus pseudonarcissus* (X78815); *Lycopersicon esculentum* (X59948); *Capsicum annuum* (X68058); *Glycine max* (M64704); *Arabidopsis thaliana* (L16237); *Synechococcus* (X55289); *Synechocystis* (X62574).

3.3.6: Plant DNA Extraction and Southern Analysis

Three μg of genomic DNA extracted from rice cultivar IR36 leaves (Burr and Burr, 1981) were digested with *Eco*R I or *Hin*d III (GibcoBRL, Gaithersburg, MD) according to the vendor's recommendations except for the addition of spermidine HCl at a final concentration of 4 mM. Digested genomic DNA was separated by electrophoresis in a 0.6% agarose (GibcoBRL, Gaithersburg, MD) gel. Southern analysis was carried out as in Wurtzel

et al., (1987) using Rapid primed [^{32}P]-labeled plasmids and PCR fragments mentioned above as probes (Feinberg and Vogelstein, 1984).

3.3.7: Functional Complementation of Rice *Pds*

To create an in-frame fusion with *lacZ*, the plasmid p5302 was linearized with *Not* I and *Sst* I, and subjected to progressive 5'-end deletions using the Exo III/MungBean Nuclease Deletion Kit (Stratagene, La Jolla, CA). Religated plasmids were transformed into *Escherichia coli* strain XL1-Blue containing the plasmid pACCRT-EB encoding GGPPS and PSY from *Erwinia uredovora* (Linden *et al.*, 1991) (see appendix B for details). Transformants containing both pACCRT-EB and deleted p5302 were selected by their resistance to ampicillin (50 μg/mL) and chloramphenicol (107 μg/mL). One deletion plasmid, designated as pOSPDSdel112, was used for pigment extraction and HPLC analysis.

3.3.8: Pigment Extraction and HPLC Analysis

Fifty ml of each saturated *E. coli* culture was used to extract pigments according to a modified method of Pecker *et al.* (Pecker *et al.*, 1996). Saturated cultures were harvested by centrifugation and pellets resuspended in 5 mL acetone, incubated at 65^{0}C for 30 minutes and centrifuged at 13,000 rpm. The supernatant was evaporated under nitrogen and pigments dissolved in 1 mL of acetone and stored in amber vials at -20^{0}C. A Waters HPLC system

with 2690 separation module, and 996 photodiode array detector was used to separate the samples by reverse phase chromatography on a Sphereclone-ODS 1 (5μ, 250x4.6mm) column. The mobile phase consisted of acetonitrile:H_2O (9:1) (solvent A), 100% ethyl acetate (solvent B) and 1% trifluoroacetic acid in acetonitrile (solvent C). The pigments were separated by a linear gradient between solvent A and solvent B, starting with 100% solvent A, for 30 minutes at a flow rate of 1mL/min. Solvent C was used to wash the column between each injection. Peaks were identified by spectrophotometric profiles and retention times and comparison with pigments extracted from *E. coli* with the plasmid pACCRT-EBP (see appendix B for details), containing genes encoding *Erwinia uredovora* GGPPS and PSY (Misawa *et al.*, 1990) and maize PDS (Li *et al.*, 1996), which together confer ζ-carotene accumulation.

3.4: Results and Discussion

In chapter 2, I demonstrated, by amplification of part of the corresponding transcripts, that genes encoding PSY and PDS were expressed in rice endosperm. Here, I have further characterized the *Pds* transcript by reconstructing an almost full length cDNA. The 2027 bp *Pds* cDNA obtained from rice cultivar IR36 leaves was cloned using the Rapid Amplification of cDNA End (RACE) technique instead of the more typical cDNA library screening.

3.4.1: Isolation of Rice *Pds* cDNA by Rapid Amplification of cDNA Ends (RACE) and Construction of Rice *Pds* cDNA clone pOSPDS

Designing primers for 5' and 3' RACE method: As described in chapter 2 (section 2.3.2) and section 3.3.2 of this chapter, rice *Pds* cDNA fragment of 825 bp was obtained using degenerate oligonucleotide primers # 92 (forward) and # 95 (reverse). The nucleotide sequence of this 825 bp fragment showed 85% identity to the maize *Pds* (Li *et al.*, 1996) and used to design rice-specific *Pds* primers. These rice specific *Pds* primer pair # 151(forward) and # 152 (reverse), and # 178 (forward) and # 179 (reverse) were used in the amplification of 430 bp and 297 bp rice *Pds* cDNA fragments by RT-PCR. Identity of these PCR amplified products were confirmed by their 100% sequence homology to the 825 bp PCR amplified fragment of rice cDNA with degenerate primers # 92 (forward) and # 95 (reverse) (see Figure 2.2). Primers # 151 and 179 were used in the amplification of partial *Pds* cDNA fragment 512 by 5' RACE, and primers # 152 and 178 were used for amplification of partial *Pds* cDNA fragment 31 by 3' RACE. These fragments were used in the reconstruction of clone pOSPDS.

5' and 3' RACE: Instead of screening a cDNA library to isolate a rice *Pds* cDNA, I chose to use the Rapid Amplification of cDNA Ends (RACE) method. Initially the first strand of rice *Pds* cDNA was synthesized from leaf using a rice specific primer # 152 (Figure 3.1) in 5' RACE. This cDNA was used as the template to amplify an approximately 1166 bp *Pds* fragment designated as 512. Identity of this amplified product 512 was

confirmed by partial sequence analysis (data not shown). In addition genomic DNA hybridization was used to demonstrate that 512 hybridized to a common set of bands as obtained using the 430 bp RT-PCR product. As shown in Figure 3.1, both fragment 512 and the 430 bp RT-PCR product hybridized to 2.2 kb and 1.2 kb *Eco*R I rice genomic DNA fragments. Both probes also detected a common 830 bp *Hin*d III fragment. In addition, fragment 512 detected a 7.4 kb fragment and the 430 bp RT-PCR product detected a 1.15 kb band.

Following this confirmation, the 1166 bp fragment 512 was inserted into *Sma* I linearized pBluescript II SK(-) vector as described in section 3.3.4 and designated as p512. Plasmid p512 contains nucleotides 161- 1326 of 2027 bp rice *Pds* cDNA described below. To obtain sequence downstream of fragment 512, 3' RACE was performed using oligo dT in combination with primer # 178, as described in methods. A 998 bp *Pds* cDNA fragment was obtained and confirmed by partial sequencing. Plasmid p31 was constructed by blunt end ligation of this 998 bp fragment into the *Sma* I site of the linearized pBluescript II SK(-) vector. Plasmid p31 contains nucleotides 1030 - 2027 of the 2027 bp rice *Pds* cDNA.

Plasmids p512 and p31 were completely sequenced and found to be missing sequence encoding the transit peptide region. Therefore, another 5' RACE was carried out to amplify from leaf mRNA the missing 5' region of the rice *Pds* cDNA. Oligonucleotide primers for first strand of cDNA synthesis and subsequent PCR amplification were designed based on the sequence data obtained from plasmid p512. Primer # 274 was used for synthesis of the

first cDNA strand and then as a reverse primer with primer # 210 (forward primer) in the PCR amplification. An approximately 356 bp cDNA fragment 132 was obtained. Identity of this fragment was also confirmed by sequence analysis and genomic DNA hybridization (Figure 3.1). The 356 bp fragment 132 hybridized to a 2.2 kb *Eco*R I fragment also detected by the 430 bp RT-PCR product. Fragment 132 also hybridized to a 12 kb *Eco*R I fragment of rice genomic DNA. This 356 bp cDNA fragment was subcloned into pBluescript II SK (-) and designated as p132. Clone p132 contained nucleotides 1 - 356 of the reconstructed 2027 bp rice *Pds* cDNA described below. Complete sequencing of the coding strand of the insert of p132 revealed that it consisted of a putative start codon ATG and DNA sequence encoding a transit peptide region of the rice *Pds*.

As shown in Figure 3.2(a), unique restriction sites in the shared regions of the inserts in plasmids p512, p31 and p132 were used to reconstruct the *Pds* cDNA clone. Figure 3.2(b) shows the strategy used for the construction of clone p5302 containing a partial *Pds* insert of 1867 bp obtained by combining portions of clone p512, having a 1166 bp insert and clone p31, containing 998 bp of *Pds*. As shown in Figure 3.2(c), clone p5302 and p132, containing a partial *Pds* insert of 356 bp, were used in reconstruction of the final 2027 bp rice *Pds* cDNA. The resulting 4988 bp clone was designated as pOSPDS and contained a 2027 bp rice *Pds* cDNA insert.

3.4.2: Sequence Analysis of Rice *Pds* and Comparison with other *Pds* Genes

The complete nucleotide and predicted amino acid sequence of the rice *Pds* cDNA is shown in Figure 3.3. Rice *Pds* nucleotide sequence shows 80.3% identity to maize *Pds* cDNA (Li *et al.*, 1996) and 68.7% identity to daffodil *Pds* (Al-Babili *et al.*, 1996). The comparison of maize to daffodil also showed 70% identity at nucleotide level. Since the ATG start codon is at the 5th nucleotide position of the cDNA cloned, and there is no upstream stop codon, I am not sure whether this is the first start codon or whether there is one further upstream. The rice *Pds* cDNA also contained sequences between nucleotides 299-391 that encoded a putative dinucleotide binding domain.

Based on the deduced amino acid sequence, which included a putative transit peptide region, rice *Pds* encoded a 63.5 kDa protein, consisting of 566 amino acid residues, similar to maize (64.1 kDa; 571 aa) (Li *et al.*, 1996) and daffodil (63.8 kDa; 570 aa) (Al-Babili *et al.*, 1996). Comparison of the predicted amino acid sequence of rice *Pds* showed 89% identity with maize and 79 % identity with daffodil.

In higher plants, N-terminal extensions called transit peptides are essential for the PDS protein to be targeted to membranes of plastids. By comparison with the predicted amino acid sequence of cyanobacterial PDS, which lacks a transit peptide region, the amino acid sequence of the mature rice PDS protein and the amino acid sequence of the putative transit peptide region were predicted (Figure 3.4). The putative transit peptide region

contains 92 amino acid reidues, predicted to be 10.3 kDa. Therefore, the mature rice PDS, after import into plastids is predicted to be 53.2 kDa similar to the maize and daffodil mature PDS predicted to be 53.4 kDa and 53.3 kDa respectively. By comparison to maize, the rice PDS protein transit sequence may be missing an approximately 11 amino acid residues (33 nucleotides).

When N-terminal amino acid residues of all available monocot, most of the dicot and cyanobacterial PDS proteins were compared, the highest homology in the transit peptide region was found between amino acid residues 55-92 of rice PDS (Figure 3.5). For unknown reasons, further upstream of this region sequence homology is very low except between tomato and pepper. But within this region there is high homology between rice and maize and this is not surprising because of their close phylogenetic relationship. Overall amino acid homology based on perfectly conserved and well conserved residues between rice and other monocot *Pds* genes ranges from 78.62-88.52%, whereas between rice and other dicot *Pds* genes it is 75.97-77.74%, and between rice and cyanobacterial *Pds* genes it is 76%. The phylogenetic tree shown in Figure 3.6 is based on the alignment of amino acid sequence of most available PDS proteins from monocots, dicots and cyanobacteria, using the CLUSTAL program (Higgins and Sharp, 1988) of PCGene software.

3.4.3: Southern Analysis and Restriction Mapping

Based on hybridization results shown in Figure 3.1, when PCR amplified fragments were used as probes, a restriction map was created for *Eco*R I (Figure 3.7a) and *Hin*d III (Figure 3.7b). The 430 bp RT-PCR amplified and 1166 bp 5' RACE amplified 512 cDNA fragments hybridized to 2.2 and 1.2 kb *Eco*R I fragments. The 356 bp cDNA fragment 132 hybridized to 2.2 kb and in addition to a 12 kb *Eco*R I fragment. Based on the nucleotide sequence of rice *Pds* cDNA, fragment 132 does not have an *Eco*R I restriction site. This means the hybridizing 12 kb genomic DNA fragment must contain an intron. The *Eco*R I restriction map suggest that the rice *Pds* must be at least 2.2 kb. The 430 bp RT-PCR amplified *Pds* cDNA and 512 cDNA fragments hybridized to a 0.83 kb *Hin*d III fragment. Fragment 512 hybridized strongly to a 7.4 kb *Hin*d III genomic DNA fragment. Hybridization was also observed for this 7.4 kb fragment with cDNA fragment 132, which contained the 5' end of the rice *Pds*. This probe also hybridized strongly to a 24 kb *Hin*d III genomic DNA fragment and weekly to a 40 kb fragment. Since the *Pds* cDNA lacks a *Hin*d III site, the 24 kb *Hin*d III genomic DNA fragment contains an intron. The origin of the 40 kb faint hybridizing *Hin*d III genomic DNA fragment is not clear. It may be due to a partial digestor to presence of a pseudogene. Based on the *Hin*d III restriction map, rice *Pds* seems to be at least 7.4 kb. Tomato *Pds* genomic DNA is reported to be of 8.0 kb (Mann *et al.*, 1994).

3.4.4: Functional Analysis of Rice *Pds* by Heterologous Complementation.

To test the function of the gene product encoded by the cloned rice *Pds*, plasmid p5302 was subjected to progressive 5' end deletions to remove the sequences encoding the putative transit peptide region and to obtain an in-frame fusion. One of the deletion plasmids in which the rice *Pds* insert was in-frame with *lacZ* gene was designated pOSPDSdel112. Based on sequence analysis, pOSPDSdel112 lacked 287 nucleotides (encoding 95 aa) from the 5' end of the cDNA which included the entire putative transit peptide region. Plasmid pOSPDSdel112 was transformed into *E. coli* containing the plasmid pACCRT-EB encoding GGPPS and PSY from *Erwinia uredovora* (Linden *et al.*, 1991). GGPPS and PSY can catalyze the synthesis of phytoene in *E. coli* which is the substrate for the gene product of *Pds*. If the cloned rice *Pds* encodes a functional PDS enzyme, then this enzyme will catalyze two desaturation steps converting phytoene to ζ-carotene as for other higher plant PDS enzymes (Li *et al.*, 1996; Pecker *et al.*, 1992). *E. coli* colonies containing both plasmids, pOSPDSdel112 and pACCRT-EB, showed a light yellow color, indicative of ζ-carotene, in contrast to *E. coli* containing the plasmid pACCRT-EB only, which were cream colored (data not shown).

To test for the presence of ζ-carotene, pigments were extracted and analyzed by reversed-phase HPLC. Figure 3.8 shows the results of HPLC analysis. Pigments extracted from *E. coli* cells containing pACCRT-EB accumulated phytoene, eluting at 15.18 minutes,

detected at 280 nm, and showing the characteristic spectral profile as shown in Figure: 3.8, panel G. Plasmid pACCRT-EBP has genes encoding *Erwinia uredovora* GGPPS and PSY, and maize PDS (Luo and Wurtzel, unpublished). Since maize PDS also catalyzes a two step desaturation (Li *et al.*, 1996) cells having pACCRT-EBP can produce ζ-carotene. As a positive control, cells containing plasmid pACCRT-EBP accumulated ζ-carotene which was found to elute at 13.19 minutes and detected at 400 nm (Figure 3.8, panel E). The characteristic spectral profile of peak 2 (ζ-carotene) is shown in Figure 3.5, panel H. Cells carrying both pACCRT-EB and pOSPDSdel112 produced a pigment eluting at 13.2 minutes as detected at 400 nm (Figure 3.8, panel F). The spectral profile obtained for this peak 2 was similar to that obtained for pigments extracted from cells containing plasmid pACCRT-EBP. Based on the similar retention time the characteristic spectral profile, and comparison with authentic standards, it was demonstrated that cells carrying pACCRT-EB and pOSPDSdel112 produce ζ-carotene. Although in nature carotenoids exist as *trans* isomers, upon extraction into solutions *cis* isomers will also occur (Britton, 1995). I did not observe any isomers for ζ-carotene. This may be due to the fact that the isomers could not be separated in the system used.

Although it is now known that rice PDS is functional, and probably encoded by a single copy gene, it is still not clear whether the rice PDS protein is expressed and also properly localized to the membranes of amyloplasts in rice endosperm. Even if rice PDS is imported into the amyloplasts it could still exist as an inactive form in the stroma. It has been shown in daffodil chromoplasts that PDS exists as inactive forms associated with

chaperonins Hsp70 and Cpn60 in stroma (Bonk *et al.*, 1997; Bonk *et al.*, 1996). Availability of a functional rice *Pds* will be useful in the future studies of the expression of carotenoid biosynthetic genes in rice or other cereal endosperms.

Figure 3.1: Genomic DNA hybridizations of RACE and RT-PCR amplified products.

(A) The schematic representation of the reconstructed rice *Pds* cDNA (thick black line) in relation to RACE and RT-PCR amplified products. Numbers below indicate the nucleotide positions of amplification products. Numbers above are primers used for amplification of 5' RACE (512 and 132), 3' RACE (31) amplified cDNA fragments and 430 bp RT-PCR amplified fragment.

(B) Rice (IR36) genomic DNA digested by *Eco*R I (1) or *Hin*d III (2) and probed as indicated below each panels. Sizes of the probes: RT-PCR; 430 bp, 512; 1166 bp, 132; 356 bp. Positions of molecular weight markers shown to the left of each panel and the sizes from top are 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kb.

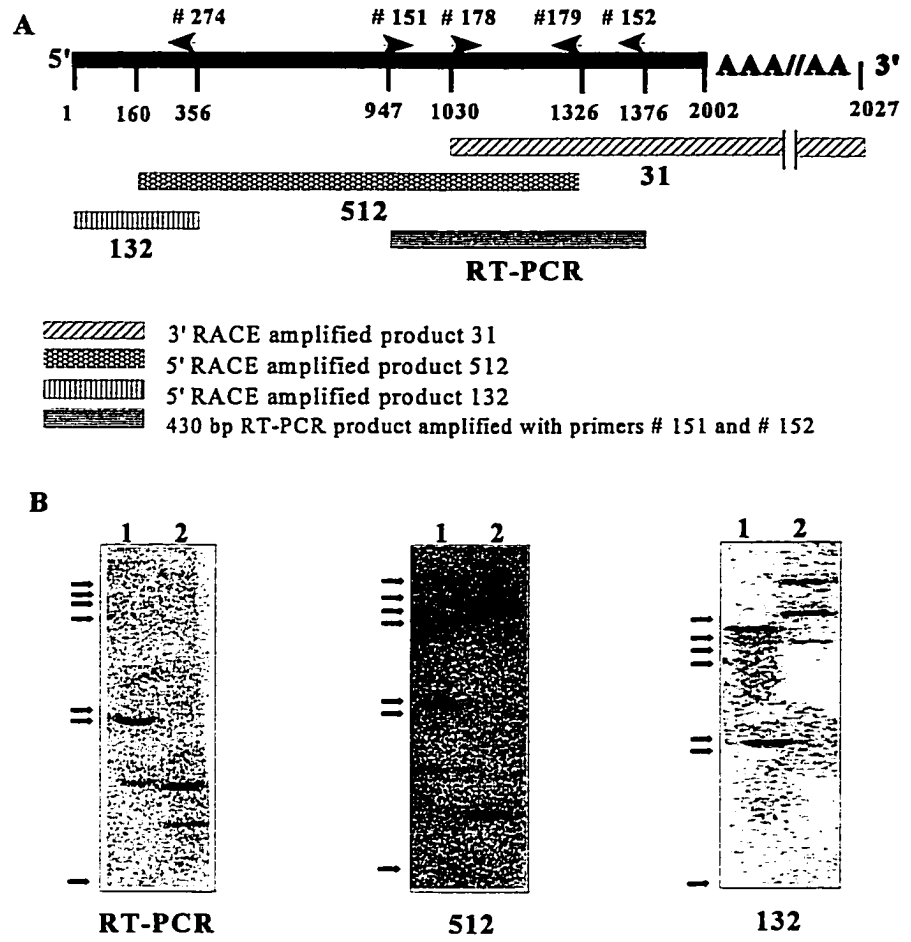


Figure 3.1: Genomic DNA hybridizations of RACE and RT-PCR amplified products.

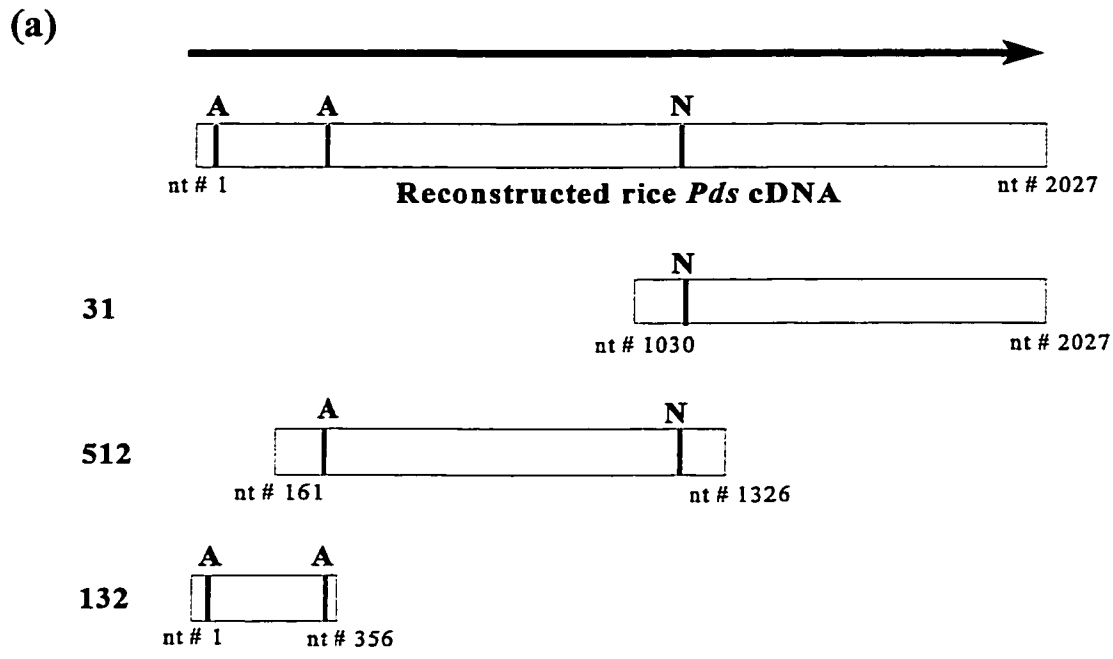


Figure 3.2(a): Schematic representation of RACE amplified products used in Reconstruction of rice *Pds* cDNA (not drawn to scale).

Arrow indicates direction of transcription.

31:- 3' RACE amplified *Pds* cDNA

512:- 5' RACE amplified *Pds* cDNA

132:- 5' RACE amplified *Pds* cDNA

Nde I (N); *Alw*N I (A).

nt #:- the first and the last nucleotide numbers of 2027 bp reconstructed rice *Pds* cDNA included on each cDNA fragment

Figure 3.2(b): Construction of plasmid p5302.

Plasmid maps are shown only as the relevant regions used for subcloning. Boxes indicate RACE amplified inserts cloned into the *Sma* I site, which is now lost. Sizes of rice *Pds* insert in the plasmids and restriction fragments of interest are indicated below the boxes. Horizontal arrows indicate direction of transcription. Thick lines represent the vector region included in the clone pOSPDS. *Pvu* II (Pv), *Sst* I (S), *Not* I (N), *Bam*H I (B), *Alw*N I (A), *Nde* I (Nd), *Pst* I (P), *Xho* I (X) and *Kpn* I (K). Restriction sites indicated as underlined letters represent the lost restriction sites because of the treatment with Klenow fragment of DNA polymerase.

(b)

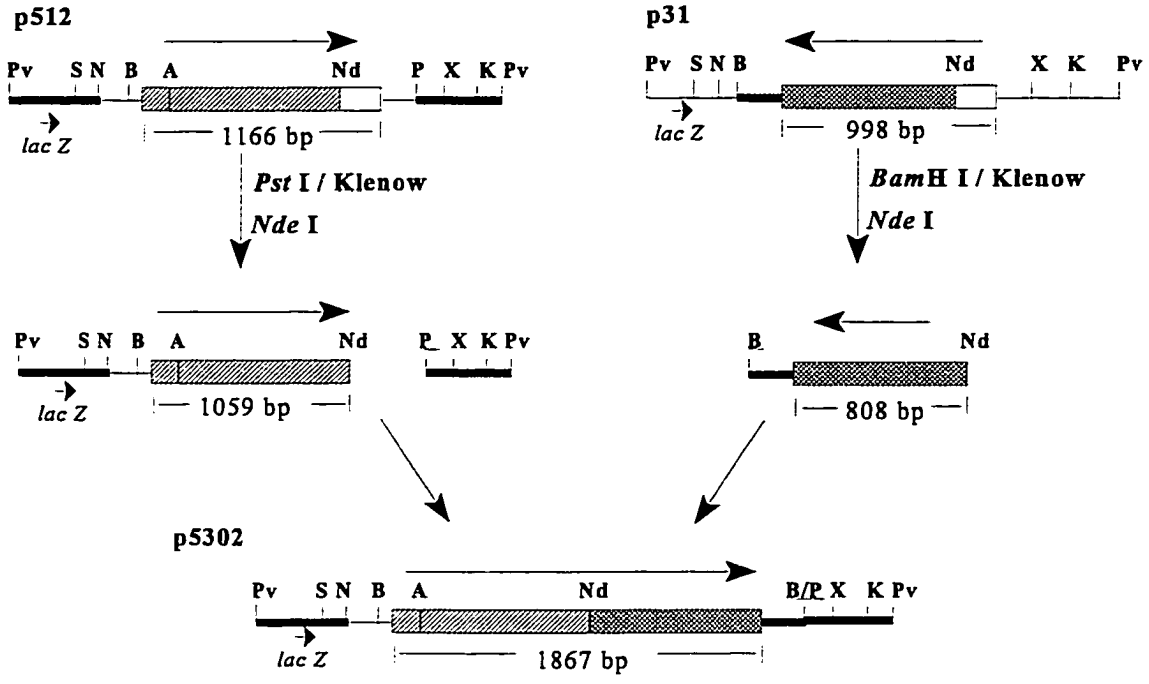


Figure 3.2(b): Construction of plasmid p5302.

(not drawn to scale)

Figure 3.2(c): Construction of plasmid pOSPDS.

Plasmid maps are shown only as the relevant regions used for subcloning. Boxes indicate RACE amplified inserts cloned into the *Sma* I site, which is now lost. Sizes of rice *Pds* insert in the plasmids and restriction fragments of interest are indicated below the boxes. Horizontal arrows indicate direction of transcription. Thick lines represent the vector region included in the clone pOSPDS. *Pvu* II (Pv), *Sst* I (S), *Not* I (N), *Bam*H I (B), *Alw*N I (A), *Nde* I (Nd), *Pst* I (P), *Xho* I (X) and *Kpn* I (K). Restriction sites indicated as underlined letters represent the lost restriction sites because of the treatment with Klenow fragment of DNA polymerase.

(C)

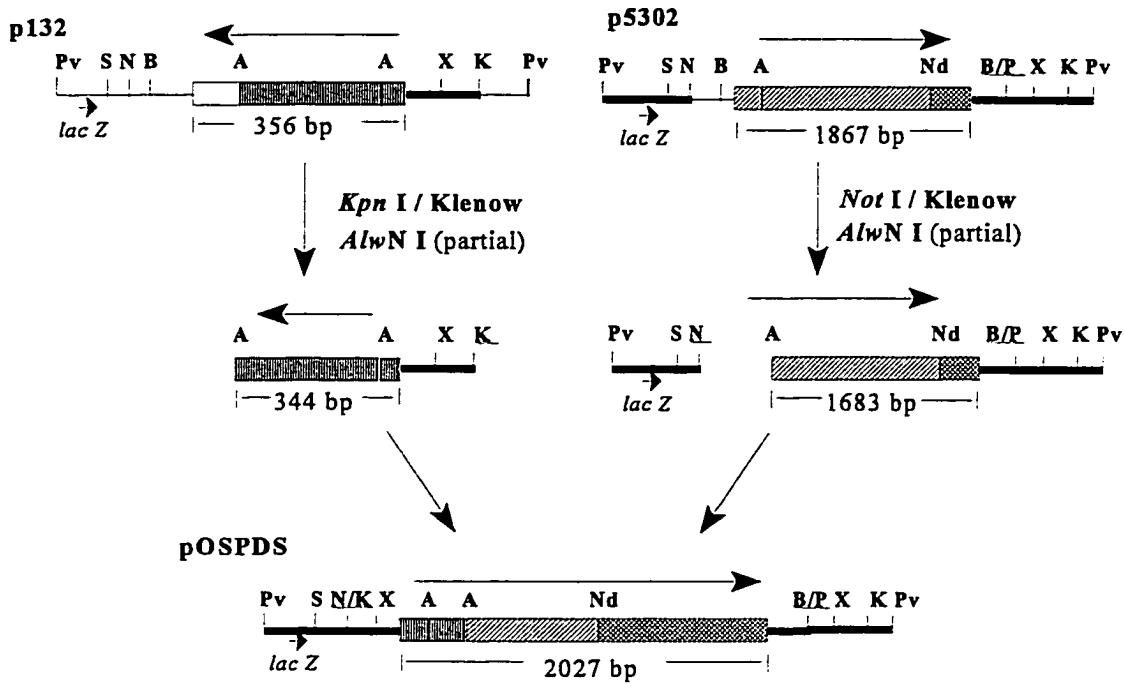


Figure 3.2(c): Construction of plasmid pOSPDS.

(not drawn to scale)

Figure 3.3: Nucleotide and amino acid sequence of the IR36 rice *Pds* cDNA.

The deduced amino acid sequence is shown as single letters below nucleotide sequence. The coding region is between nucleotides 5 - 1702. Bold letters indicate the putative transit peptide region. Letters in underlined italics indicate putative dinucleotide binding domain. This sequence from plasmid pOSPDS is deposited under GenBank Accession # AF049356.

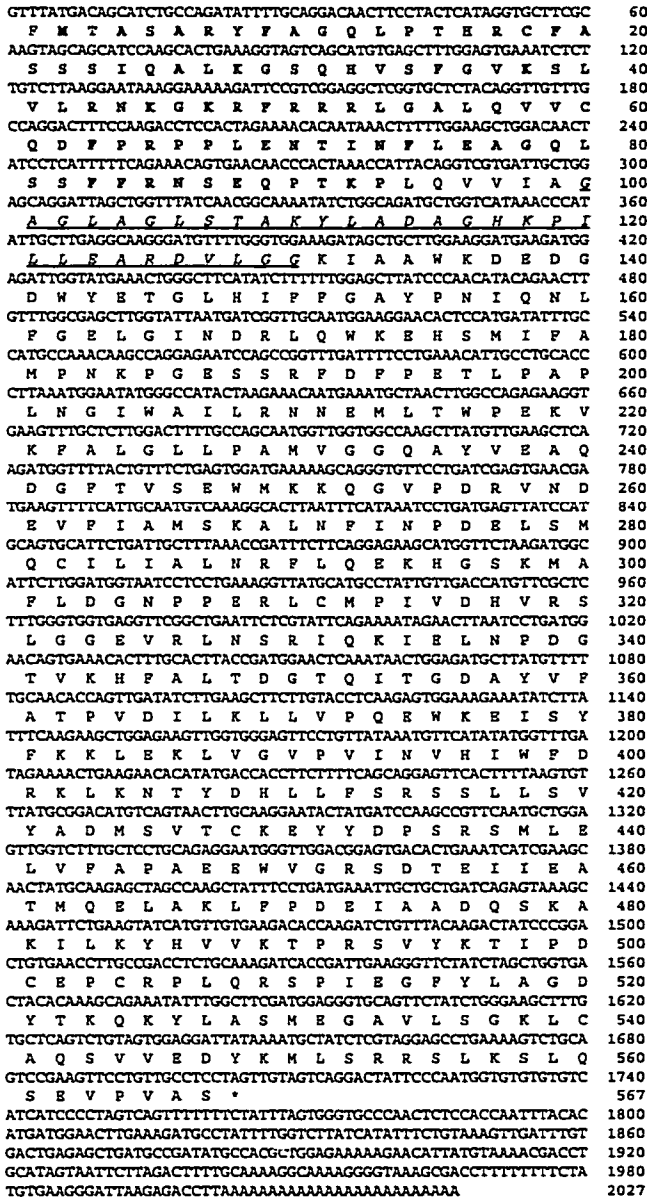


Figure 3.3: Nucleotide and amino acid sequence of the IR36 rice *Pds* cDNA.

Figure 3.4: Monocot and cyanobacterial PDS amino acid sequence alignment.

Letters in underlined italics indicate dinucleotide binding domain. Bold letters indicate putative transit peptide region. Asterisks indicate perfectly conserved residues and dots indicate well conserved residues. ***OS***: Rice; ***ZM***: Maize; ***NP***: Daffodil; ***SCO***: *Synechococcus*; ***SCY***: *Synechocystis*

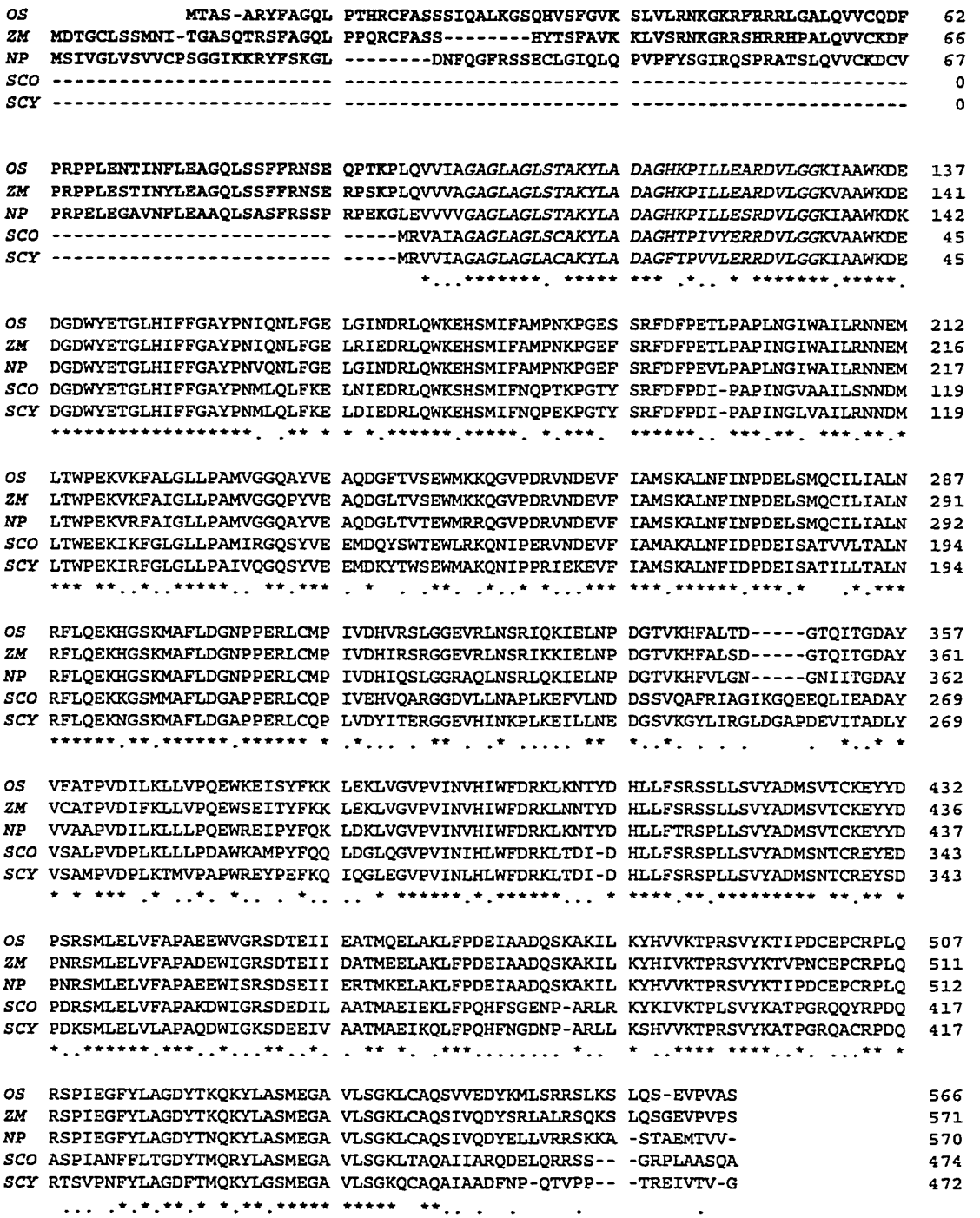


Figure 3.4: Monocot and cyanobacterial PDS amino acid sequence alignment.

```
OS                MTAS-ARYFAGQLPT-------HRCFASSSIQALKGSQ   30
ZM   MDTGCLSSMNI-TGASQTRSFAGQLPP-------QRCFASS--------H   34
NP   MSIVGLVSVVCPSGGIKKRYFSKGL---------------DNFQGFRSSE   35
LE   MPQIGLVSAVNLRVQGSSAYLWSSRSSSLGTESRDGCLQRNSL-CFAGSE   49
CA   MPQIGLVSAVNLRVQGNSAYLWSSRSS-LGTDSQDGCSQRNSL-CFGGSD   48
GM   MAACGYISAANF------NYLVGARNISKFASSDAT----ISF-SFGGSD   39
AT   MVVFGNVSAANL------PYQNG------FLEA---------L-SSGGCE   28
                                                        .

OS   HVSFGVKSLVLRNKGKRFRRRLG--ALQVVCQDFPRPPLENTINFLEAGQ   78
ZM   YTSFAVKKLVSRNKGRRSHRRHP--ALQVVCKDFPRPPLESTINYLEAGQ   82
NP   CLGIQLQVPVPFYSGIRQSPRAT--SLQVVCKDCPRPELEGAVNFLEAAQ   83
LE   SMGHKLKIRTPHATTRRLVK--DLGPLKVVCIDYPRPELDNTVNYLEAAF   97
CA   SMSHRLKIRNPHSITRRLAK--DFRPLKVVCIDYPRPELDNTVNYLEAAF   96
GM   SMGLTLRPAP-----IRAPKRNHFSPLRVVCVDYPRPELENTVNFVEAAY   84
AT   LMGHSFRVPTSQALKTRTRRRSTAGPLQVVCVDIPRPELENTVNFLEAAS   78
       .   .          *        .*.*** * *** *....*..**.

OS   LSSFFRNSEQPTKP                                       92
ZM   LSSFFRNSERPSKP                                       96
NP   LSASFRSSPRPEKG                                       97
LE   LSSTFRASPRPTKP                                      111
CA   LSSSFRSSPRPTKP                                      110
GM   LSSTFRASPRPLKP                                       98
AT   LSASFRSAPRPAKP                                       92
     **. **.. .* *
```

Figure 3.5: Comparison of amino acid sequences of putative transit peptide regions of monocot and dicot PDS.

Asterisks indicate perfectly conserved residues and dots indicate well conserved residues. ***OS***: Rice; ***ZM***: Maize; ***NP***: Daffodil; ***LE***: Tomato; ***CA***: Pepper; ***GM***: Soybean; ***AT***: *Arabidopsis*.

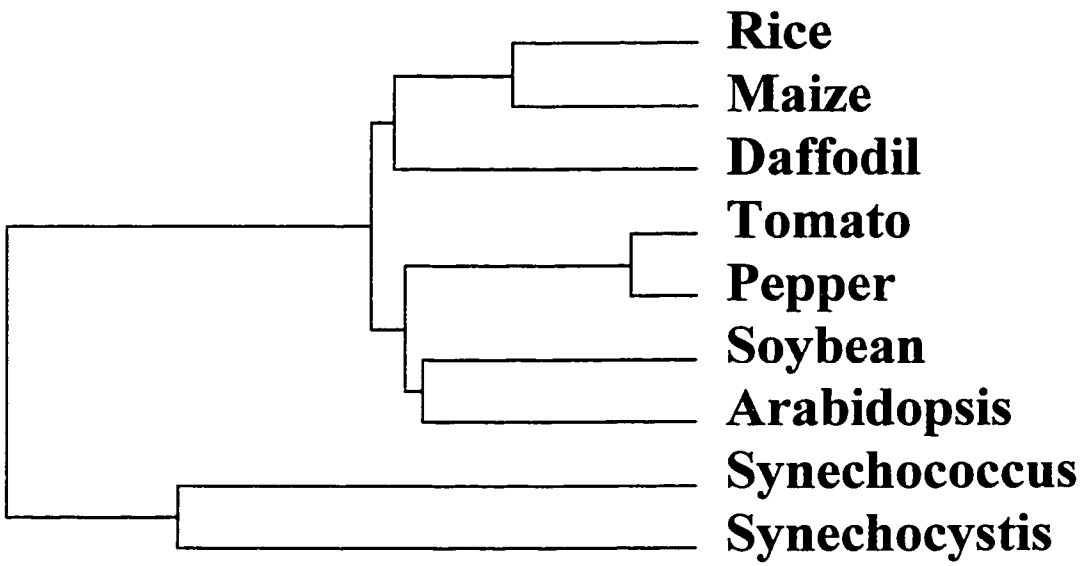


Figure 3.6: Phylogenetic tree based on alignment of PDS amino acid sequences.

The program used to generate this dendrogram is CLUSTAL, developed by Higgins and Sharp (1988), of PCGene software (Intelligenetics, Mountain View, CA)

(a)

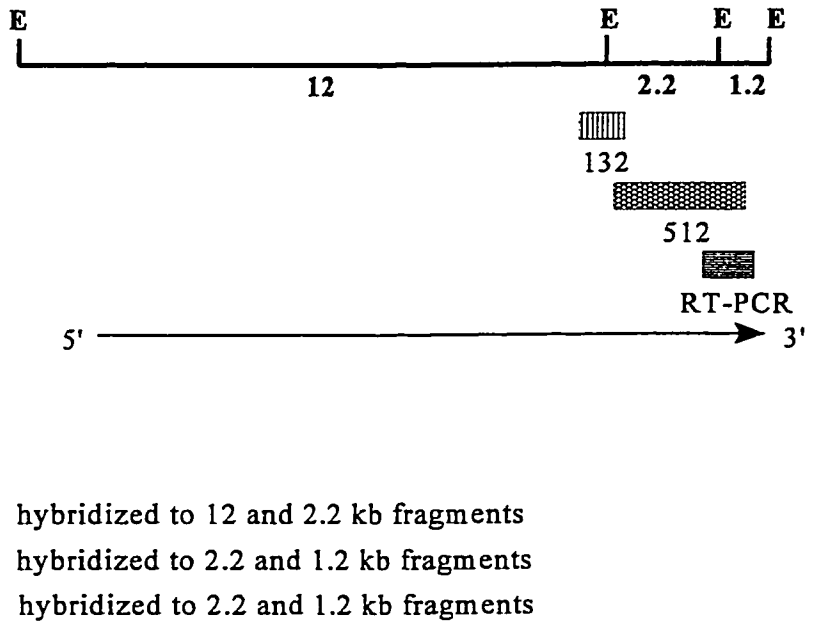
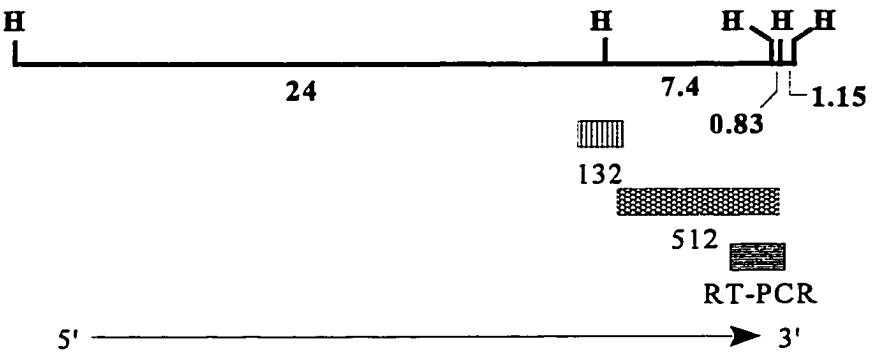


Figure 3.7(a): *Eco*R I restriction map of rice *Pds*.

Map indicated by solid thick line is drawn to scale. Numbers in bold indicate the fragment sizes in kb. Arrow indicates the direction of transcription. Sizes of the probes used:- fragment 132 is 356 bp; fragment 512 is 1166 bp; RT-PCR fragment is 430 bp.

(b)



hybridized to 24 and 7.4 kb fragments
hybridized to 7.4, 1.15 and 0.83 kb fragments
hybridized to 1.15 and 0.83 kb fragments

Figure 3.7(b): *Hind* III Restriction Map of Rice *Pds*.

Map indicated by solid thick line is drawn to scale. Numbers in bold indicate the fragment sizes in kb. Arrow indicates the direction of transcription. Sizes of the probes used:- fragment 132 is 356 bp; fragment 512 is 1.1 kb and RT-PCR fragment is 430 bp. The relative orientation of 1.15 and 0.83 kb fragments is not known.

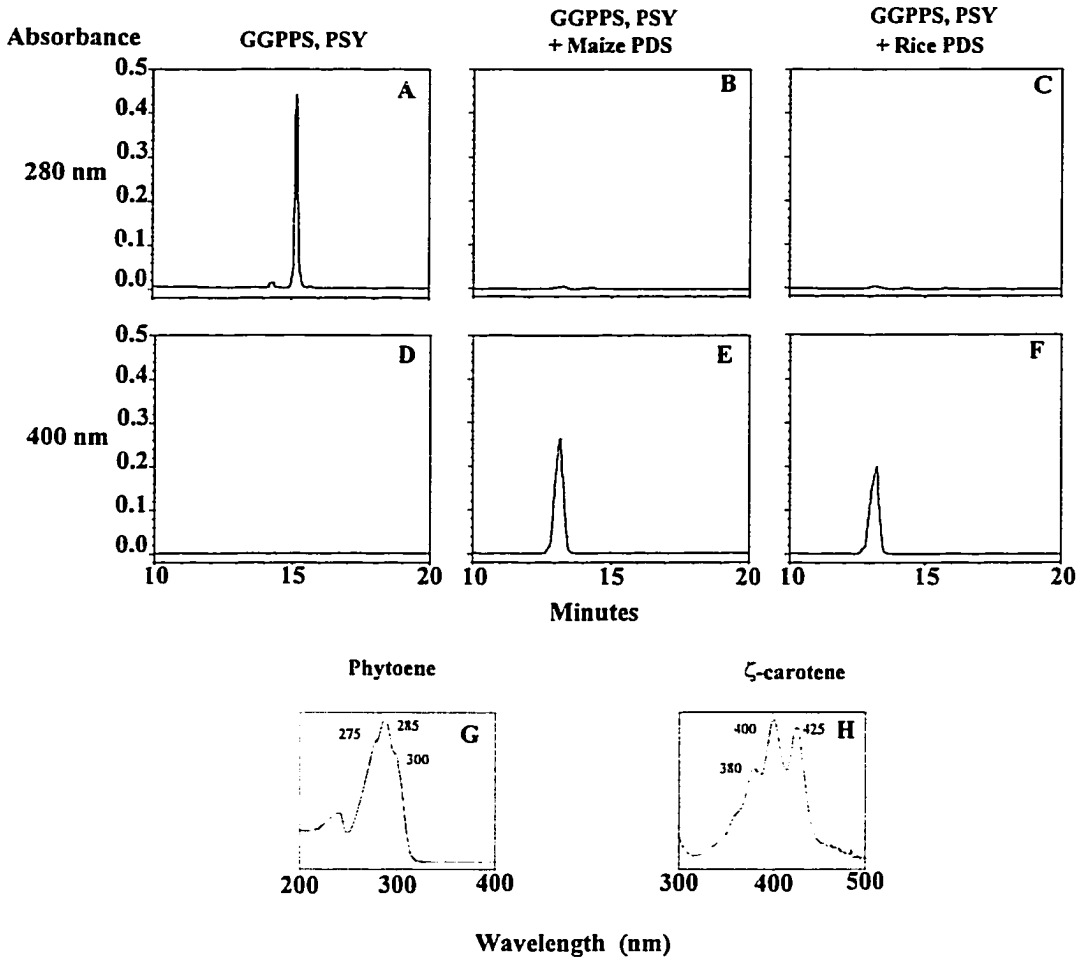


Figure 3.8: HPLC analysis of bacterial pigments.

E. coli cells transformed with pACCRT-EB only (A, D), pACCRT-EBP only (B, E) and pACCRT-EB + pOSPDSdel112 (C, F). Eluted peaks detected at 280 nm (A-C) and 400 nm (D-F). (G), Spectrophotometric profile for major peak in panel A and (H), spectrophotometric profile for major peak in panels E and F with peaks maxima as indicated.

CHAPTER 4

SUMMARY

The major findings of this study towards engineering carotenoid biosynthesis and accumulation in rice endosperm were the following: (1) *Psy* and *Pds*, the first two carotenoid biosynthetic genes, were expressed in rice endosperm at the transcriptional level, (2) PSY and PDS proteins were encoded by single copy genes mapped to rice chromosomes # 12 (distal to the marker RG181) and to chromosome # 3 (between markers RG191 and RZ993X), respectively, and (3) a 2027 bp rice *Pds* cDNA was isolated from leaf and shown to encode a functional PDS protein capable of catalyzing a two step desaturation of phytoene resulting in the synthesis of ζ-carotene.

Transcripts of *Psy* and *Pds* were not only detected in leaf, as expected, but also in rice endosperm. This observation implied that the absence of carotenoid accumulation in rice endosperm was not due to the lack of *Psy* and *Pds* expression at the transcriptional level. Based on my studies, *Psy* and *Pds* were the only carotenoid biosynthetic genes to be expressed at the transcriptional level in rice endosperm. Information is not yet available for the expression of other genes involved in the carotenoid biosynthetic pathway. The pattern of the expression of *Psy* and *Pds* transcripts varied in various endosperm developmental stages. The levels of *Psy* transcripts were constant, whereas the levels of *Pds* transcripts increased as the endosperm matured. The levels of *Pds* transcripts increased two fold from the endosperm of 10 DAF (the youngest developmental stage tested) to the mature

endosperm (more than 40 DAF). This study did not address the level of *Psy* and *Pds* transcripts at stages younger than 10 DAF, because of difficulty with RNA isolation at the very early stages of endosperm development. If it is crucial for the carotenoid biosynthetic genes to be expressed at very early endosperm developmental stages for the pathway to be activated, extreme low level or lack of expression of carotenoid biosynthetic genes during early stages of endosperm development might be a reason for the lack of colored carotenoid accumulation. The constitutive expression of *Psy* transcripts and the temporal control of *Pds* transcript accumulation may also indicate an unsynchronized expression of carotenoid biosynthetic genes during endosperm development.

Similar to *Psy* transcripts, the PSY protein was also determined to be expressed during the development of rice endosperm (Yu and Wurtzel, unpublished). Even though *Psy* transcripts and PSY proteins were present in the developing rice endosperm, phytoene did not accumulate in rice endosperm except when daffodil PSY was over expressed in young rice endosperm. However, even when PSY was over expressed, colored carotenoids did not accumulate (Burkhardt *et al.*, 1997). This observation together with my results indicate that, even though rice *Psy* was expressed throughout rice endosperm development, the level of expression might be inadequate during the very early stages of endosperm development to result in detectable levels of phytoene. Evidence that PSY is rate limiting for endosperm carotenoid accumulation is suggested from work in maize (Yu and Wurtzel, unpublished). There is also a possibility that the PSY antigen detected in rice endosperm might represent

an inactive stromal form as observed in daffodil (Bonk *et al.*, 1997; 1996). Alternatively, the PSY protein detected in the endosperm may be inactive or improperly imported into amyloplasts thereby preventing carotenoid biosynthesis.

Although *Pds* is transcribed, there may be inadequate amount of active, properly localized PDS protein, since over-expression of the daffodil PSY protein in rice endosperm did not lead to biosynthesis of compounds later than phytoene. The availability of the rice *Pds* cDNA will facilitate generation of rice specific PDS antibodies to test for expression and localization of PDS in developing rice endosperm. Although this will confirm whether PDS is imported properly into the amyloplast membrane, the activity of PDS protein in endosperm amyloplast should still be tested. The rice *Pds* cDNA can also be used to isolate the *Pds* genomic DNA to compare regulation of the *Pds* promoter in photosynthetic vs. non-photosynthetic tissues, like endosperm.

To activate biosynthesis and accumulation of colored carotenoids in rice endosperm, the isolated rice *Pds* cDNA can be used to over express PDS in rice endosperm. However, engineering carotenoid biosynthesis and accumulation of colored carotenoids in rice endosperm by over-expression of genes encoding carotenoid biosynthetic enzymes might also affect the overall morphology of the plant itself. As was discussed previously, carotenoid biosynthesis is part of the terpenoid metabolism. Several isoprenoid precursors are shared for the biosynthesis of a variety of terpenoid compounds. Although the biosynthesis of IPP takes place via two different pathways in plastids and cytosol

(Lichtenthaler *et al.*, 1997a), there is a possibility for the shift of common precursors across the plastid membranes (Hugueney *et al.*, 1996). Even within subcellular compartments like plastids, several terpenoid biosynthetic pathways compete for common precursors. For example GGPP is a common precursor for the biosynthesis of the phytol chain of chlorophyll, carotenoids, gibberellins etc. Over-expression of the phytoene synthase gene in transgenic tomatoes caused dwarf tomato plants with pale pink young leaves, because of the redirection of metabolites from gibberellin and phytol biosynthetic pathways (Fray *et al.*, 1995). Likewise, engineering carotenoid biosynthesis in rice may also redirect the metabolites from other pathways sharing common precursors. This might result in rice plants with altered morphology, and also with reduced tolerance to pathogens (because of the effect on phytoalexin biosynthesis). To reduce the disruption of the delicate balance between the various biosynthetic pathways, it is preferable to over-express PSY and PDS together under the control of an endosperm specific promoter. This might reduce the chances of producing an abnormal rice plant, when attempts are made to engineer carotenoid biosynthesis in rice endosperm.

Hopefully, the results obtained here in combination with future studies in the area of the regulation of carotenoid biosynthesis in higher plants, especially in endosperm, will lead to engineering colored carotenoid accumulation in rice endosperm, a nutritionally valuable tissue.

APPENDIX A

GROWING RICE IN GREENHOUSE

To study the regulation of carotenoid biosynthesis in rice endosperm, plants were grown in the greenhouse under a controlled environment. To harvest large amounts of leaf and endosperm material, high yielding rice cultivar IR 36, bred by The International Rice Research Institute was used. Cultivar IR 36 is an indica variety of rice. Seed material was obtained from Dr. Susan McCouch (Cornell University, Ithaca, NY) and Dr. Steve Linscombe (Louisiana Rice Research Station, Crowley, LA).

Materials Required

- Rice seeds (amount depends on need)
- Water garden soil/clay loam (Earthgro®, Lebanon, CT)
- Standard clay pots (diameter 10"-12"; height 12")
- DYNA flats (20" x 14" x 2 ¾") (Kaden Corporation, Cincinnati, OH) or any flat trays with draining holes
-Modular plastic box (17¾" x 16⅜" x 7") (Global Industrial Equipment, Port Washington, New York) or Large plastic containers
- 1000 watt Metal Halide fixture (Philips Lighting Company, Somerset, NJ)
- 1000 watt High Pressure Sodium fixture (General Electric Company, Cleveland, OH)

- Soluble fertilizer containing N:P:K at 20:20:20 (Peters Water Soluble Fertilizer, Scotts, Marysville, OH)
- Granular fertilizer containing Urea (46%) (Cadwell and Jones Inc., Manchester, CT).
- Spreader sticker

Germination of Seeds

Seeds were soaked in water in a glass or plastic container (level of water should be at least 2" above the seeds in the container) at room temperature for 5-7 days or until the emergence of radicle (one fourth of an inch) through the coleorhiza. Water was replaced every day.

Preparation of soil in flat trays/clay pots and transplantation of seedlings

Before filling the tray with soil within ½" from the top surface, it was covered with newspaper. This is to keep the soil in the tray while allowing the water to drain from the seed flat. Water was added to keep the seed flat wet but not flooded. Germinated seeds were spread (care should be taken not to damage the radicle or coleoptile) on a soil flat and a thin layer of soil was laid on top to protect germinating seeds from drying. Flat trays with young seedlings were kept in a mist room to keep it moist but not soaked with water. A 14 hour day-length was maintained from 6 am - 8 pm by using a 1000 watt High Pressure Sodium

Fixture eight feet above the floor. Day time temperature was maintain at 80^0 F-90^0 F and night time temperature was maintained at 70^0 F- 75^0 F.

Clay pots were filled with soil within 1" from the top of the pot and placed in a large plastic container. About 2" of water was added to the plastic container to keep the soil in the clay pot moist by capillary action but not saturated with water. Soluble fertilizer, containing N:P:K at 20:20:20, was added according to manufacturer's instructions, while the clay pots were prepared. Two-three weeks old seedlings (2-3 leaf stage) were uprooted from the flat trays, without damaging the roots and transplanted into the soil in the clay pots. Alternatively, germinated seeds were directly spread on the surface of the soil in clay pots and covered with soil. The soil was kept moist but not saturated with water.

Growing plants

Using the clay pots above, mature plants were grown in a 10' x 10' growth room in the greenhouse with supplemental lightening. In the growth room a 14 hour day-length was maintained from 6 am - 8 pm by using three 1000 watt Metal Halide Fixture eight feet above the floor. Day time temperature was maintained at 80^0 F-90^0 F and night time temperature was maintained at 70^0 F-75^0 F. Once the seedlings were established, soil was allowed to dry for a few days. A hole was made on the soil surface by pushing a pencil into the soil about 3 - 4 inches deep and granular fertilizer containing Urea 46% (½ teaspoon per 12" clay pot) added. Pots were flooded with 1"-2" of water above the soil surface. The ideal number of

seedlings per pot is one or two. The rest of the seedlings were removed from the pot. The water level was maintained until 4 weeks after flowering. After flooding, soluble fertilizer containing N:P:K at 20:20:20 was added once a week (4.2 lbs/5 gal). During the maturation of seeds, approximately 30-35 days after flowering (DAF), watering was stopped and the water level was reduced in the larger tub by half and maintained at this level for about one month.

Pest control

To control spider mites Avid™ (Merck inc. AgVet division, Rahway, NJ) and for mealy bugs Decathlon™ (Olympic Horticultural Products, Mainland, PA) with spreader sticker (Olympic®, Mainland, PA) as surfactant were used according to manufacturer's instructions. Insecticidal soap was avoided as this could damage the plant.

APPENDIX B

List of oligonucleotide primers used in this study.

Primer Number	Sequence 5' to 3'	Description	GenBank Accession Number
# 92	GAC(T)GAGGTGTTC(T)ATA(T)GCC(T)ATGTC	*Pds* forward degenerate primer	
# 95	ACAGCA(G)CCTTCCATG(T)GAAGCC(T)AA	*Pds* reverse degenerate primer	
# 139	AATTCAGAGCCTTCGTGGGC	Rice *Sh* forward	Z15028
# 140	TTTGTGAGCCAGCAGAGTGG	Rice *Sh* reverse	Z15028
# 145	CCTGAAAGGCGCAAAGTGTCTGG	Rice *Psy* forward	EST clone D48251
# 146	CGATAGCATCAAGGATCTGCCGG	Rice *Psy* reverse	EST clone D48697
# 151	GACCATGTTCGCTCTTTGGGTGG	Rice *Pds* forward	AF049356
# 152	CGATGATTTCAGTGTCACTCCGTCC	Rice *Pds* reverse	AF049356
# 178	ACACTTTGCACTTACCGATGG	Rice *Pds* forward	AF049356

Primer Number	Sequence 5' to 3'	Description	GenBank Accession Number
# 179	ACCAACTCCAGCATTGAACG	Rice *Pds* reverse	AF049356
# 183	GGCCACGCGTCGACTAGTACGGGGGG GGGGGGGGGG	5' RACE anchor primer	
# 210	GGCCACGCGTCGACTAGTAC	AUAP (GibcoBRL)	
# 274	GTTTATGACCAGCATCTGCCGGATATT TTG	Rice *Pds* reverse	AF049356

APPENDIX C

List of all clones used or constructed in this study.

Clone	Description	Vector	Insert Size
S14375	Rice *Psy* EST cDNA GenBank # D48251	pBluescript II SK(+)	1.3 kb
S15075	Rice *Psy* EST cDNA GenBank # D48697	pBluescript II SK(+)	0.90 kb
p512	5' RACE amplified rice *Pds* cDNA fragment 512 inserted at *Sma* I site	pBluescript II SK(-)	1166 bp
p31	3' RACE amplified rice *Pds* cDNA fragment 31 inserted at *Sma* I site	pBluescript II SK(-)	998 bp

Clone	Description	Vector	Insert Size
p132	5' RACE amplified rice *Pds* cDNA fragment 132 inserted at *Sma* I site	pBluescript II SK(-)	356 bp
p5302	*Nde* I - *Bam*H I fragment from p31 containing 808 bp rice *Pds* cDNA inserted at *Nde* I - *Pst* I site of p512	p512	1867 bp rice *Pds* cDNA fragment
pOSPDS	*Alw*N I - *Kpn* I fragment from p132 containing 344 bp rice *Pds* cDNA inserted at *Alw*N I - *Not* I site of p5302 (GenBank Accession number AF049356)	p5302	2027 bp rice *Pds* cDNA
pOSPDSdel112	deletion construct from p5302. Lacks 287 nucleotides from 5' end of the clone pOSPDS	pBluescript II SK(-)	1740 bp rice *Pds* cDNA fragment

Clone	Description	Vector	Insert Size
pACCRT-EB	contain *CrtB* and *CrtE* from *Erwinia uredovora.* Provided by Dr. N. Misawa (Linden *et al.*, 1991)	pACYC184	3.75 kb
pACCRT-EBP	*Pvu* II fragment containing maize *Pds* inserted at *Bam*H I site (Luo and Wurtzel, Unpublished; Li *et al.*, 1996)	pACCRT-EB	5.96 kb

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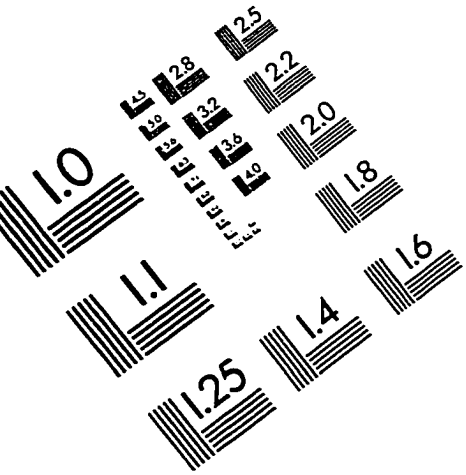
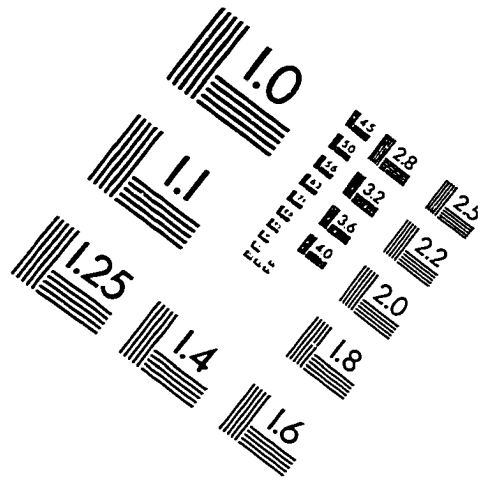
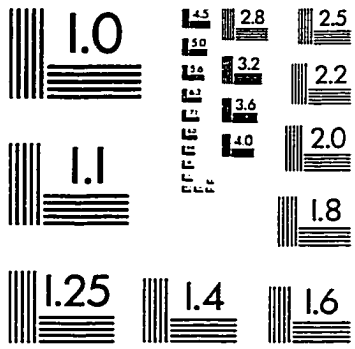
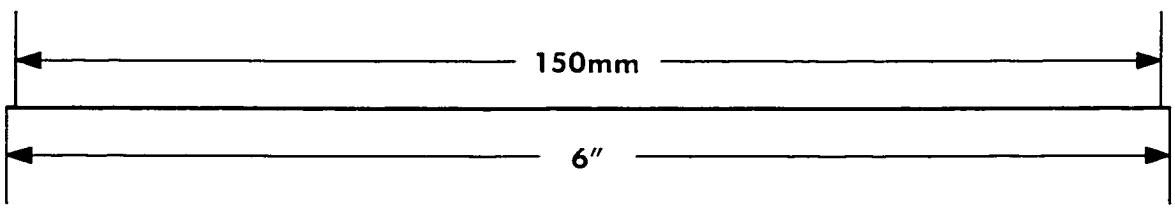
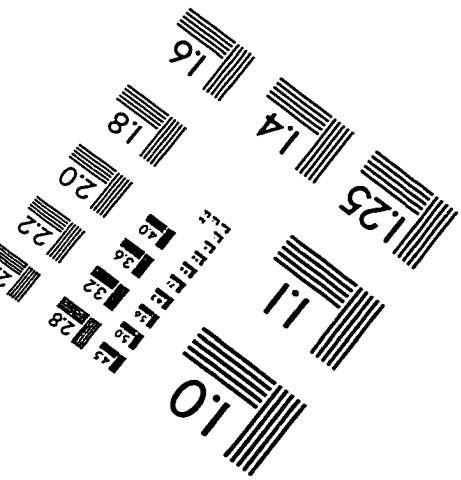
IMAGE EVALUATION TEST TARGET (QA-3)

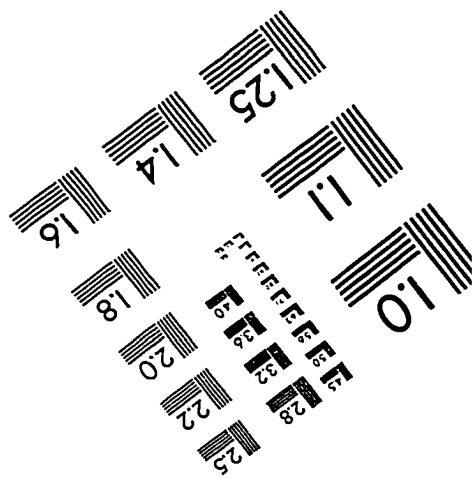












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