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Molecular and genetic studies related to zeta-carotene desaturation and carotenoid biosynthesis in maize and rice

By

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Abstract

Molecular and genetic studies related to zeta-carotene desaturation and carotenoid biosynthesis in maize and rice

By

Ruibai Luo

Advisor: Dr. Eleanore Wurtzel

Carotenoids are present in all photosynthetic organisms and serve several major functions: as accessory pigments for light harvesting, as photoprotectors against photooxidative damage and as precursors of the plant hormone-abscisic acid (ABA). In this work, I have isolated and sequenced a cDNA encoding a ZDS (ζ-carotene desaturase) from maize and sequenced the corresponding gene from rice, in the carotenoid biosynthetic pathway. In both plants, the gene is likely to be single copy. The maize cDNA was functionally tested in *E. coli* and demonstrated to encode a two-step desaturase. With the ZDS encoding cDNA, combined with RFLP analysis, transcript and HPLC (High-pressure liquid chromatography) analysis of maize carotenoid mutants, the potential function of several of the maize genetic loci that are linked to the carotenoid

desaturation steps were further characterized. It was found that maize mutants *vp9* and *y9* accumulate ζ-carotene, while *y8* mutants are completely blocked in the pathway and do not accumulate any intermediates; it appears that the *vp9* locus is the likely candidate for the *zds* structural gene, while *y9* affects ζ-carotene desaturation in some unknown manner. Of the other desaturation mutants tested, *vp2* was found to be blocked in the biosynthesis of homogentisic acid (HGA), an intermediate of plastoquinone biosynthesis, while *w3* was not, despite its conditioning of phytoene accumulation.

Preliminary studies were also carried out to characterize a rice *Zds* cDNA and the corresponding rice genomic DNA. To raise a maize ZDS antibody, the expression of a fusion protein-ZDS construct, pET23a-Mzds348, in the expression vector pET23a, was also achieved.

A rice mutant 84NMEMdr2 was characterized by HPLC analysis. Pigments were extracted from endosperm, embryo, and leaves of the rice mutant. In embryo and albino seedlings, a carotenoid intermediate, phytoene, was found to accumulate. This suggests that this rice mutation is due to a block in the carotenoid biosynthetic pathway at the step mediated by the enzyme phytoene desaturase (PDS). This is the first rice mutant found to be blocked in the carotenoid biosynthetic pathway.

Abbreviations

aa	amino acid
ABA	abscisic acid
amp	ampicillin
ADP	adenosine diphosphate
BAC	bacterial artificial chromosome
bp	base pair
BSA	bovine serum albumin
CCS	capsanthin-capsorubin synthase
Css	capsanthin-capsorubin synthase gene
cDNA	complementary DNA
Chl	chloramphenicol
Cpn	chaperonin
crtB	carotenoid gene B
crtE	carotenoid gene E
crtI	carotenoid gene I
crtY	carotenoid gene Y
DAP	days after pollination
DMAPP	dimethylallyl pyrophosphate
EST	expressed sequence tag
Fps	farnesyl pyrophosphate synthase gene
FAD	flavin adenine dinucleotide
FPP	farnesyl pyrophosphate

GPP	geranylgeranyl pyrophosphate
GGPPS	geranylgeranyl pyrophosphate synthase
Ggpps	geranylgeranyl pyrophosphate synthase gene
HGA	homogentisic acid
HPLC	high-performance liquid chromatography
Hsp	heat shock protein
IPP	isopentenyl pyrophosphate
IPTG	isopropylthio-β-galactoside
kb	kilobase pair
kDa	kilodalton
LB	Luria-Bertani
LCY	lycopene cyclase
LCYB	lycopene β-cyclase
LCYE	lycopene ε-cyclase
Lcy	lycopene cyclase gene
OHPP	4-hydroxyphenylpyruvate
ori	origin
min	minutes
Mum	Mutator
MCS	multiple cloning site
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate

NMR	nuclear magnetic resonance
nt	nucleotide
PCR	polymerase chain reaction
PDS	phytoene desaturase
Pds	phytoene desaturase gene
PPPP	prephytoene diphosphate
PSY	phytoene synthase
Psy	phytoene synthase gene
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
s	seconds
TLC	thin layer chromatography
vp	viviparous
w	white
y	yellow
ZDS	ζ-carotene desaturase
Zds	ζ-carotene desaturase gene

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Chapter 1. Introduction

1.1 Carotenoids and the carotenoid biosynthetic pathway

1.1.1 Chemical structure and functions of carotenoids

Carotenoids are a well-characterized class of naturally occurring terpenoids. They are synthesized *de novo* by all photosynthetic and many non-photosynthetic organisms such as certain bacteria and fungi (Goodwin, 1980). They are red, brilliant yellow or orange lipid-soluble pigments present in microorganisms, algae, higher plants and animals, including humans. In 1813, Wackenroder first isolated carotene from carrots. In 1837, Berzelius named the yellow pigment from autumn leaves xanthophylls. These events marked the beginning of research on carotenoids (Isler, 1971). Many structures of carotenoids have been determined since the early 1900s. In the past 20 to 30 years, the advances of various techniques such as chromatography, UV/Vis spectrometry, Infrared and Raman spectroscopy, mass spectrometry and nuclear magnetic resonance (NMR) have permitted the structural determination of more than 600 carotenoids in nature, not including isomeric forms. For example, in the case of α-carotene, a total of 512 possible isomers exists, although the number of possible isomers of symmetric carotenoids such as β-carotene is smaller (Britton and Goodwin, 1982). Some carotenoid isomers, and their cleavage products, play important biological roles (Britton et al., 1995b; Goodwin, 1980; Pfander, 1992). The total production of carotenoids in nature has been estimated at about 10 tons a year (Britton et al., 1995b). Most of this production is in the form of four major

carotenoids: fucoxanthin, the characteristic pigment of many marine algae, which is the most abundant natural carotenoid; and lutein, violaxanthin, and nexoanthin, the carotenoids in green leaves (Britton et al., 1995b). Dietary intake of carotenoids by some animals such as flamingo, lobster, and sea urchin provide them with brilliant colors (Britton and Goodwin, 1982; Britton et al., 1995b; Pfander, 1992).

In plants, carotenoids have several major functions: (1) Carotenoids are light harvesting pigments in photosynthesis (Siefermann-Harms, 1987). Carotenoids, along with the chlorophylls and various pigment-binding proteins, form the photosystem complexes in the chloroplast thylakoid membranes. Photosystem I (PSI) contains mainly β-carotene, while lutein plays a key role in the light harvesting antenna in photosystem II (PSII) (Humbeck et al., 1989; Siefermann-Harms et al., 1985). (2) Carotenoids provide essential photoprotective functions against potentially harmful photooxidative damage (Demmig-Adams and Adams, 1992; Rau, 1988). (3) In certain non-photosynthetic organs of plants, such as ripening fruits and flowers, carotenoids accumulate in plastids. They are involved in the attraction of insects or other animals that help in pollination and seed dispersal. (4) Some carotenoids, such as violaxanthin, are precursors of abscisic acid (ABA), an important plant hormone which is produced in plants in response to low temperature or water stress and is vital for plant growth and development (Koornneef, 1986).

In mammals, provitamin A activity is one of the most important biological functions of carotenoids. The central process of vision involves vitamin A (retinal) as the chromophore of the visual pigments. Humans require vitamin A in their diet; 400 μg to

1300 μg of retinal is required per day, depending on age and sex (Erdman et al., 1993). However, inadequate intake of vitamin A, especially by children, is a severe health problem in developing countries. According to a report of the World Health Organization (WHO), approximately 250,000 to 500,000 children die yearly as a result of vitamin A deficiency (Humphrey et al., 1992). Only 40 types of carotenoids have provitamin A activity; β-carotene has the highest potential vitamin A activity (Simpson, 1983; Weiser and Kormann, 1993). Carotenoids are also precursors of vitamin A-related compounds, such as retinoic acids, which are essential in animal morphogenesis (Ruberte et al., 1991). Multiple biological functions, besides provitamin A activity, have also been ascribed to carotenoids. Carotenoids act as quenchers of singlet oxygen; as free-radical scavengers (Oliveros et al., 1992); and as anticarcinogenic agents (Bertram, 1993). Some evidence shows that carotenoids are involved in cell-cell communication (Bendich, 1993; Zhang et al., 1992); and also might play important roles in prevention of or protection against cancer, heart disease and supporting the human immune system (Britton, 1995a; Ross, 1992).

1.1.2 Biosynthesis of carotenoids

Carotenoids belong to the isoprenoid family which comprises the largest group of natural products. Until now, more than 22,000 types of isoprenoids have been identified in plants. Isoprenoids play many diverse functions in plants for example, as plant hormones (e.g., cytokinin, from IPP; and gibberellin, from GGPP); as electron carriers (ubiquinone and plastoquinone); as antimicrobial agents (phytoalexins); or as photosynthetic pigments

(phytol and carotenoid) (Chappell, 1995; McGarvey and Croteau, 1995). Because of the importance of their functions, carotenoids play irreplaceable roles in nature. Carotenoids, as a group of isoprenoids, are C_{40} tetraterpenoids that generally consist of eight isoprene units joined together. The general carotenoid biosynthetic pathway in higher plants is shown in Figure 1-1A, B.

The first specific precursor of all isoprenoids is the universal C_5 biological isoprene precursor, IPP. In plants, the synthesis of isoprenoids takes place at various subcellular compartments: plastids (for prenylquinone and carotenoid), mitochondria (for ubiquinone) and ER/cytosol (for sterols). Recently, it was been shown that IPP can be synthesized via two different pathways. In the cytosol, IPP is synthesized from acetyl-CoA via mevalonic acid (MVA) by the classical acetate/mevalonate pathway (McGarvey and Croteau, 1995). In plastids, it was recently found that IPP can be synthesized via pyruvate/glyceraldehyde 3-phosphate in the green alga *Scenedesmus* (Schwender et al., 1996). In higher plants, it also has been shown that the plastid-bound isoprenoids are synthesized through this newly identified pyruvate/glyceraldehyde 3-phosphate pathway (Lichtenthaler et al., 1997). The isomerization of IPP to DMAPP is followed by a series of condensation reactions, with successive addition of IPP to DMAPP, resulting in the formation of GPP (C_{10}), FPP (C_{15}) and GGPP (C_{20}). Carotenoid biosynthesis starts with the head to head condensation of two molecules of GGPP to form phytoene. Phytoene undergoes a series of four desaturation steps sequentially forming phytofluene, ζ-carotene, neurosporene and lycopene. Both ends of lycopene undergo cyclization, leading to the formation of α-carotene, carrying a β and a ε-ionone ring; or β-carotene, containing

two β-ionone rings. Finally, additions of various oxygen-containing side groups and other chemical modifications form different kinds of xanthophylls. The basic chemical feature of carotenoids is the polyene chain that may present from 3 to 15 conjugated double bonds. All the particular variations of carotenoids are derived from the basic C_{40} carbon skeleton of lycopene and β-carotene; their structures are shown in Figure 1-2.

These skeletons of carotenoids can be varied by cyclization at one or both end groups; by different cyclization reactions and by addition of various oxygen-containing functional groups. For instance, zeaxanthin, violaxanthin, and lutein have hydroxyl substituents at C-3 and C-3'. The degree of conjugation and isomerization states of the backbones determine the spectroscopic properties of particular carotenoids. Overall, the size, shape, and functional groups enable carotenoids compounds to fit into cellular and subcellular structures properly and to function efficiently. The long alternating double and single bond system of carotenoids, besides giving them characteristic light absorbing properties, also confers distinctive chemical reactivities. The specific interactions of carotenoids with other molecules, such as proteins, enhance more diverse functions of carotenoids. For instance, in the photosynthetic complexes, apoproteins can attach nearby carotenoids and chlorophylls in the right orientation to allow for efficient energy transfer. As Dr. George Britton pointed out: "Without carotenoids, photosynthesis and all life in an oxygen atmosphere would be impossible" (Britton, 1995a).

1.2 Genes encoding carotenoid biosynthetic enzymes

In higher plants, genes encoding the carotenoid biosynthetic enzyme are nuclear-encoded and the enzymes are targeted to the plastids. Many genes encoding carotenoid biosynthetic enzymes have been isolated by heterologous or homologous probe hybridization (Bartley and Scolnik, 1995; Bartley et al., 1994; Bouvier et al., 1994). Color complementation (Cunningham and Gantt, 1998; Lotan and Hirschberg, 1995; Misawa et al., 1995a) has been a useful method to screen for genes which encode carotenoid biosynthetic enzymes on the basis of color changes associated with the biosynthesis of carotenoids of different colors. Also, transposon tagging (Buckner and Robertson, 1993); differential cDNA screening or screening of expression cDNA libraries with antibodies against the purified enzyme have also been used for cloning genes that encode carotenoid biosynthetic enzymes (Dogbo and Camara, 1987; Kuntz et al., 1992).

The enzyme geranylgeranyl pyrophosphate synthase (GGPPS) catalyzes the first step of the carotenoid biosynthetic pathway. GGPPS converts three molecules of isopentenyl pyrophosphate (IPP) and one molecular of dimethylallyl pyrophosphate (DMAPP) to form geranylgeranyl pyrophosphate (GGPP). GGPPS from *Capsicum annuum* has been purified and catalyzes the *in vitro* formation of GGPP (Dogbo and Camara, 1987). Antibodies against GGPPS purified from pepper chromoplasts have been used to clone the corresponding cDNA from ripening fruit mRNA (Badillo et al., 1995; Kuntz et al., 1992). In *Arabidopsis,* six isoforms of GGPPS have been identified (Bartley et al., 1994; Scolnik and Bartley, 1994; Zhu et al., 1997); one of them, GGPPS6, was localized to mitochondria. GGPPS genes have also been characterized in *Saccharomyces cerevisiae* (Jiang et al., 1995); *Sinapis alba* (Laferriere and Beyer, 1991); and *Lupinus albus* (Aitken

et al., 1995).

Phytoene synthase (PSY) catalyzes the condensation of two molecules of GGPP to form PPPP (prephytoene diphosphate) and the conversion of PPPP to phytoene, the first specific product in the carotenoid biosynthetic pathway. Clone pTOM5, a cDNA from tomato, was found to encode PSY (Ray et al., 1987). It is now known that there are two copies of *psy* in tomato, *psy1* and *psy2*. While *psy1* transcripts encoded by pTOM5 were found mainly in seedlings and in late stages of fruit ripening, *psy2* transcripts were relatively abundant in mature leaves (Bartley and Scolnik, 1993; Romer et al., 1993); whereas other plants appear to have only one gene copy of *psy* (Kajiwara et al., 1997; Schledz et al., 1996). Until now, *Psy* cDNAs from *Arabidopsis*, maize, daffodil, pepper and muskmelon have been cloned (Buckner et al., 1996; Karvouni et al., 1995; Romer et al., 1993; Schwender et al., 1996; Scolnik and Bartley, 1993a).

The desaturation of phytoene proceeds differently in carotenoid-producing micoorganisms and photosynthetic organisms including cyanobacteria, algae and higher plants. There are two types of enzymes involving in the desaturation of phytoene: CRTI-type desaturases and phytoene desaturases (PDS). In bacteria, the CRTI-type desaturase catalyzes three to four desaturation steps by converting phytoene into neurosporene or lycopene. The CRTI-type desaturases can be classified into two classes: (1) The enzyme from *Rhodobacter* can catalyze phytoene desaturation by adding three double bonds, thus forming neurosporene (Armstrong et al., 1989; Lang et al., 1994); and (2), The enzyme from *Erwinia* (Misawa et al., 1990), where the *crtI* product can catalyze the desaturation

of phytoene by adding four double bonds to form lycopene. In higher plants, phytoene desaturase (PDS) catalyzes only two desaturation steps, converting phytoene to phytofluene and then phytofluene into ζ-carotene. Each desaturation step releases two electrons; some studies have shown that these two desaturation steps are coupled with the photosynthetic electron transport chain, where the first electron acceptor could be a membrane-bound plastoquinone, and oxygen as the final electron acceptor (Beyer et al., 1989; Mayer et al., 1992; Schulz et al., 1993). Furthermore, it has been revealed that oxidized quinones, but not reduced quinones, are able to replace oxygen as the terminal electron acceptor for the desaturation of phytoene in an anaerobic enviroment (Mayer et al., 1990). The biosynthetic pathway of plastoquinones is outlined in Figure 1-3.

Homogentisic acid (HGA), a precursor of the plastoquinone and tocopherol biosynthetic pathways, is produced from 4-hydroxyphenylpyruvate (OHPP) via the OHPP dioxygenase. Schulz *et al.* demonstrated that the bleaching herbicide SC-0051 functions as a competitive inhibitor of OHPP dioxygenase, causing accumulation of phytoene in the leaves of *Lemna gibba* L. (Schulz et al., 1993). In certain *Arabidopsis* mutants, recessive alleles of the loci *pds1* and *pds2* confer accumulation of phytoene; neither mutation maps to the gene encoding phytoene desaturase (Norris et al., 1995). Tests of *pds1* and *pds2* showed that either mutation confers abnormally low levels of plastoquinone and tocopherol. The *pds1* locus may encode OHPP dioxygenase, because white seedlings of *pds1* will turn green when grown on homogentisic acid (HGA), the product of this enzyme. When *pds1* mutant plants were grown on OHPP, the substrate of OHPP dioxygenase, the mutant plants remained albino (Norris et al., 1995). Furthermore, Norris

et al. isolated a cDNA encoding the OHPP dioxygenase from *Arabidopsis*. They demonstrated the function of this cDNA gene product by expression in *E. coli* and *pds1* transgenic plants, and found that this OHPP dioxygenase gene was linked with *pds1* by mapping and co-segregation analysis. *pds1* was further characterized; a 17-bp deletion in the *pds1* allele leads to truncation of 26 amino acids at the carboxyterminal of the OHPP dioxygenase protein (Norris et al., 1998). Additionally, Henry *et al.* demonstrated that a mutant of the green algae *Scenedesmus obliquus*, which is not able to synthesize tocopherol, has normal levels of carotenoids and plastoquinone; this illustrated that tocopherol is not required for carotenoid synthesis (Henry et al., 1986). Nievelstein *et al.* revealed that the electron passage from phytoene to membrane-bound quinone, and then oxygen, involves a link to a membrane-bound NAD(P)H-dependent respiratory path, which also utilizes quinone as the redox active component (Nievelstein et al., 1995). Overall, the redox state of quinone has a significant effect on the desaturation of phytoene.

Many *Pds* cDNAs from higher plants have been cloned. To date, in maize, a cDNA encoding PDS was isolated in our laboratory and PDS was found to be encoded by the *vp5* locus (Hable et al., 1998; Li et al., 1996). *Pds* cDNAs have also been isolated from *Arabidopsis*, daffodil, pepper, rice, soybean, and tomato (Al-Babili et al., 1996b; Bartley et al., 1991; Hugueney et al., 1992; Mann et al., 1994; Scolnik and Bartley, 1993b; Yoganathan, 1998).

In higher plants, ζ-carotene desaturase (ZDS) mediates the dehydrogenation of ζ-carotene

and neurosporene to lycopene. This enzyme catalyzes the formation of a double bond in position C7-8 of ζ-carotene to generate neurosporene, and introduces a double bond in position C7'-8' of neurosporene to form lycopene (Figure 1-2). The gene encoding ZDS in the cyanobacterium *Anabaena* PCC7120 (GenBank # D26095) has been cloned by heterologous complementation (Albrecht et al., 1995; Linden et al., 1994; Linden et al., 1993a). The function of a higher-plant type ζ-carotene desaturase in the cyanobacterium *Synechocystis* PCC6803 has been characterized (Breitenbach et al., 1998). In higher plants, a few *Zds* cDNAs have been cloned: *Arabidopsis,* (GenBank # U38550) (Scolnik and Bartley, 1995a), *Narcissus* (Genbank # AJ224683) (Al-Babili et al., 1998) and *Capsicum annuum* (GenBank # 2129927) (Albrecht et al., 1995). The *Arabidopsis Zds* cDNA was isolated using a PCR product as probe; primer design was based on the sequence of a putative *Arabidopsis Zds* EST clone (GenBank # T46272) (Scolnik and Bartley, 1995a). *Zds* from *Capsicum annuum* was isolated by screening a pepper cDNA library using a heterologous probe from *Arabidopsis* (Albrecht et al., 1995). For *Narcissus*, primers were designed based on the sequence of the *Arabidopsis Zds*. A daffodil *zds* PCR product was used to screen a *Narcissus* flower cDNA library and a *Zds* clone with a missing 5' end was isolated. A full length *Narcissus Zds* clone was obtained by a nested PCR method (Al-Babili et al., 1998). Amino acid sequence comparisons show that the cyanobacterium *Anabaena* ZDS has higher homology with the CRTI-type desaturase than with the ZDS from *Capsicum* (Albrecht et al., 1995), but the ZDS in the cyanobacterium, *Synechocystis* is more similar to the plant type ZDS (Breitenbach et al., 1998). In higher plants, ZDS also has low homology with the PDS amino acid sequence, suggesting that PDS and ZDS in higher plants might have evolved from a progenitor

desaturase from bacteria, and then diverged into two groups of desaturases (Albrecht et al., 1995). The detailed sequence comparisons of ZDS will be discussed in Chapter 2, as this work deals with the cloning of maize *Zds*.

Lycopene cyclase convert the pink pigment lycopene into α- or β-carotene. There are two kinds of lycopene cyclase: lycopene β-cyclase (LCYB) and lycopene ε-cyclase (LCYE) (Figure 1-1A, B). The formation of β-carotene is catalyzed by LCYB, which symmetrically forms two β-ionone rings at each end of lycopene. On the other hand, α-carotene synthesis is catalyzed by the dual action of lycopene ε-cyclase, which forms an ε-ionone ring at one end of lycopene, and lycopene β-cyclase, which introduces a β-ionone ring on the other end (Bishop et al., 1995; Cunningham et al., 1996; Hirschberg et al., 1995). Several genes encoding lycopene β-cyclase have been cloned from bacteria and several dicotyledonous plants. The gene for lycopene β-cyclase was first isolated from *Erwinia uredovora* (Misawa et al., 1990) as part of a cluster of carotenoid genes which includes *crtB* (PSY), *crtE* (GGPPS), *crtI* (CRTI) and *crtY* (LCYB). In cyanobacteria, the gene encoding LCYB was cloned by genetic complementation of a mutant strain resistant to the LCY inhibitor 2-(4-methylphenoxy) triethylamine hydrochloride (MPTA) (Cunningham et al., 1993; Cunningham et al., 1994). The lycopene β-cyclase gene from *Arabidopsis* was cloned based on an EST (GenBank # Z29211) which has 43% amino acid identity and 63% similarity with *Synechococcus* LCY (Scolnik and Bartley, 1995b). cDNAs encoding LCYB have been also isolated from pepper (Hugueney et al., 1995), tobacco (Hirschberg, GenBank # X81787), tomato (Hirschberg, GenBank # X81787), and *Narcissus* (Al-Babili et al., 1996b). Genes for

LCYE have been isolated from *Arabidopsis* (Cunningham et al., 1996); and from lettuce (Cunningham and Gantt, 1998).

In recent years, genes encoding enzymes that catalyze formation of hydroxy-, epoxy- and oxy-, or de-epoxy derivatives of carotenes have been cloned (Bouvier et al., 1998a; Lotan and Hirschberg, 1995; Misawa et al., 1995a; Sun et al., 1996). Using α-carotene as a substrate, ε-carotene hydroxylase and β-carotene hydroxylase catalyze the formation of α-cryptoxanthin and lutein; β-carotene hydroxylase catalyzes the synthesis of zeaxanthin from β-carotene, via β-cryptoxanthin (Bouvier et al., 1998a; Pogson et al., 1996). The gene for β-carotene hydroxylase has been isolated from *Arabidopsis*, *Capsicum* and, recently, from the cyanobacterium *Synechocystis* sp. (Bouvier et al., 1998a; Masamoto et al., 1998; Sun et al., 1996). The gene encoding, β-C_4-oxygenase, the enzyme that converts β-carotene to the keto-carotenoid canthaxanthin, has been cloned from the green alga *Haematococcus pluvialis* (Lotan and Hirschberg, 1995). Another gene responsible for the biosynthesis of asthaxanthin, *crtW*, or called *crtO*, have been cloned from the marine bacterium *Agrobacterium aurantiacum* and green alga *Haematococcus plurialis* (Harker and Hirschberg, 1997; Misawa et al., 1995a). cDNAs encoding the zeaxanthin epoxidase in *Nicotiana plumbaginifolia* (Marin et al., 1996) and the violaxanthin de-epoxidase in romaine lettuce (Bugos and Yamanoto, 1996) have also been identified. These latter enzymes play roles in the "xanthopyll cycle" (Demmig-Adams and Adams, 1992).

1.3 Localization of carotenogenic enzymes

In higher plants, genes encoding enzymes for carotenoid biosynthesis are nuclear-encoded; the enzymes are translated as precursors on cytoplasmic ribosomes, and imported into plastids, at which point their transit peptides are removed (Bartley and Scolnik, 1995; Bartley et al., 1994; Sandmann, 1994). Carotenoids are synthesized in different plastid types such as: (1) chloroplasts, found in photosynthetic tissues; (2) chromoplasts, found in fruits and flowers; and (3) amyloplasts, found in storage tissues such as seed endosperm, stems, and roots. Biosynthesis of carotenoids has been carried out *in vitro* using chromoplast or chloroplast membranes from several higher plants (Beyer et al., 1989; Beyer et al., 1985; Jones and Porter, 1985; Kreuz et al., 1982; Lutke-Brinkhaus et al., 1982). These studies have shown that carotenogenic enzymes present in plastids, are either localized to the stroma, or bound to the thylakoids, peripherally or integrally. But, the precise localization of carotenogenic enzymes in different types of plastids still remains controversial. Lütke-Brinkhaus *et al.*, found that PSY and PDS are associated with the chloroplast envelope membrane from spinach chloroplasts (Lutke-Brinkhaus et al., 1982). In *Rhodobacter*, *Synechocystis* and *Anabaena*, PDS was found to be membrane-associated by immunocytochemical localization (Serrano et al., 1990). Linden *et al.*, found that the majority of PDS is associated with the thylakoid membrane of tobacco and spinach chloroplasts, using Western analysis and immunocytochemical localization (Linden et al., 1993b). GGPPS and phytoene synthase have been localized in the stroma of the plastids of *Capsicum*, when using immunocytochemical methods (Camara, 1993). PSY has been found in two forms: either loosely bound to the thylakoid membranes, or as an inactive soluble form in the stroma of *Narcissus pseudonarcissu* (Schledz et al., 1996).

Recently, several studies have focused on the import of nuclear-encoded carotenogenic enzymes into internal compartments of the plastids. In a pea chloroplast import experiment, it was found that GGPPS was soluble in the stroma, while phytoene synthase was bound to the thylakoids upon import (Bonk et al., 1997). Interestingly, phytoene desaturase and lycopene cyclase which rely on membrane affiliation for enzyme activity, also remained soluble in the stoma of chloroplasts. It was found that the soluble forms of phytoene synthase, phytoene desaturase and lycopene cyclase were as a high-molecular-mass complex, that could be imported to the stroma, and that could also bind to the thylakoid membrane via the chloroplast 60 kDa chaperonin (Cpn 60) (Bonk et al., 1997). A soluble but inactive form of phytoene desaturase has also been found bound to the 70 kDa heat shock protein (Hsp 70) in the stroma of *Narcissus* chromoplasts, while the active form was membrane bound (Al-Babili et al., 1996b).

Based on the data described above, it is generally considered that the nuclear-encoded carotenoid biosynthetic enzymes are targeted to the plastids by an N-terminal transit peptide, which is cleaved upon import; the native protein is folded and assembled with the help of a chaperonin protein. Enzymatic complexes which include GGPPS and phytoene synthase, are membrane-peripherally localized (Bonk et al., 1997). Phytoene desaturase and ζ-carotene desaturase might also constitute another enzyme complex present on thylakoid membranes for desaturation of phytoene (Al-Babili et al., 1996a; Bonk et al., 1997; Cunningham and Gantt, 1998).

Because of the hydrophobic character of both carotenoids and the carotenoid biosynthetic

enzymes, the biochemical purification and characterization of carotenogenic biosynthesis enzymes has been a difficult task. Some enzymes have been purified (Dogbo and Camara, 1987), however they are usually unstable and impure. Recent efforts have focused on the use of molecular genetic tools to study the carotenoid biosynthesis pathway. The molecular descriptions of carotenoid biosynthesis genes can provide a powerful indirect approach for predicting structural properties of the gene products.

1.4 Regulation of carotenoid biosynthesis

The regulation of carotenoid biosynthesis varies according to plant, tissue type, and developmental stage (Bartley and Scolnik, 1995; Sandmann, 1994). Induction of carotenoid biosynthesis by light has been observed during transition from etioplasts to chloroplasts in seedlings of corn, peas, and radish (Cohen and Goodwin, 1962; Grumbach, 1983; Henshall and Goodwin, 1969). This effect is mediated by phytochrome; the synthesis of carotenoids is stimulated by red light and it can be prevented by a flash of far red light (Rau, 1983). Ruban *et al.* found that the concentration and composition of leaf xanthophylls were affected by light intensity (Ruban et al., 1994). Albrecht *et al.* showed that light-stimulated synthesis of carotenoid correlated with increased activity of IPP isomerase but not increased activity of GGPPS and PSY, when maize leaves, were treated with the herbicide norflurazon and exposed to white light (Albrecht and Sandmann, 1994). More recently, von Lintig *et al.* (1997) showed that both continuous far-red and red light were able to increase phytoene synthase mRNA levels in wild-type *Arabidopsis* seedlings, which might suggest that expression of the *Psy* gene may be

regulated by both light-labile and light-stable phytochromes. However, under continuous far-red light, the expression of *Psy* didn't elevate in a *phyA* mutant, showing that phytochrome A mediates the increase of *Psy* transcript levels under these light conditions. On the other hand, the red light response was normal in a *phyB* mutant, indicating that the expression of *Psy* is not regulated by phytochrome B, but by other light-stable phytochromes. The up-regulation of the expression of *Psy* does not necessarily induce an increase in carotenoid content, since the activity of PSY may not increase at the same time. Carotenoid content increases significantly only when plants were irradiated with light qualities which increase the biosynthesis of chlorophyll, such as continuous red light, continuous blue light and continuous white light (von Lintig et al., 1997).

The accumulation of certain kinds of carotenoids in chromoplasts of fruits and flowers is developmentally regulated. In the bell pepper, the increase of mRNA levels of the GGPPS gene was correlated with increased GGPPS activity during fruit ripening (Kuntz et al., 1992). The increased expression of *Ggpps*, *Psy*, *Pds* and *Css* was only observed during pepper fruit ripening (Hugueney et al., 1996). In *Narcissus pseudonarcissus*, the PSY mRNA level was high in flowers as compared to green leaves where no signal was detected. During flower development the PSY transcript level was constant, while there was an increase in PSY protein levels (Schledz et al., 1996). In tomato, the mRNA levels of *Psy* and *Pds* increase and the mRNA levels of *Lcy* decreased during the "breaker stage" of fruit ripening (Giuliano et al., 1993; Pecker et al., 1996). Therefore, the accumulation of the pink pigment lycopene in tomato fruit is presumably due to a down-regulation of the lycopene cyclase gene at the "breaker stage" of fruit development.

The synthesis of carotenoids is also affected by other isoprenoid pathways, because many end-products of different isoprenoid pathways share common substrates like GPP, FPP and GGPP. For example, GGPP is also a precursor of some plant secondary metabolites, such as gibberellins, phytoalexins, diterpenes, plastoquinones, tocopherols, phytol, taxol, etc. (McGarvey and Croteau, 1995). It is thought that several GGPPS enzymes are involved and encode dedicated enzymes for different products of the isoprenoid pathway (Bartley and Scolnik, 1995). Some evidence also suggests that regulation of carotenoid accumulation may be "upstream," even before GGPP. For instance, light induction of IPP isomerase activity has been shown to account for the increase of carotenoid content in maize leaves (Albrecht and Sandmann, 1994). In *E. coli*, expression of an exogenous IPP isomerase can also lead to elevated levels of phytoene, lycopene and β-carotene (Kajiwara et al., 1997).

Several lines of research show that environmental factors such as nutrition, pH, temperature, stress and oxygen levels may also affect the transcriptional level of carotenoid biosynthesis genes (Britton, 1988; Hugueney et al., 1996). Bouvier et al., found that transient oxidative stress can synchronously induce the mRNA level of several carotenogenic genes such as *Ggps*, *Psy*, *Pds*, *Lcy* and *Ccs* (Bouvier et al., 1998b). Despite much research toward understanding the regulation of the carotenoid biosynthetic pathway, the exact molecular mechanisms controlling carotenoid biosynthesis still remains to be understood.

1.5 Maize and rice mutants blocked in the carotenoid biosynthetic pathway

Many mutations that block the carotenoid biosynthesis pathway have been found in maize (Figure 1-4). Classical genetic experiments have shown that mutations that block the carotenoid biosynthetic pathway are caused by recessive, dominant or suppressor alleles. Two classes of albino maize mutants have been identified: Class I mutants, which are characterized by having a white or pale-yellow endosperm (*e.g.*, *vp2, vp5, vp7, vp9, w3 y9, lw, lw2, lw3* and *cl*), and Class II mutants, which are characterized by normal yellow color endosperm (*e.g.*, *w, w2, w4, w5*) (Robertson, 1975). Most of Class I mutations such as *vp2, vp5, vp7, vp9, w3* and *y9* have been mapped to genetic loci and linked with certain steps of carotenoid biosynthesis on the basis of accumulated intermediates (Neill et al., 1986). Albino mutants *lw*, *lw2*, and *lw3* are not viviparous, and no carotenoid precursors can be detected in their mutant tissues. Seedling phenotypes of *cl* mutants are white to green seedlings, depending upon alleles of the modifier *Clm*. Little work has been done with Class II mutations. Most Class I mutations are viviparous, *i.e.* seeds germinate on the immature corn ear because of the lack of the carotenoid-derived hormone ABA, which is required to maintain seed dormancy. If rescued prior to desiccation, these mutant seeds grow into albino seedlings, a result of photooxidation of chlorophyll and aberrant chloroplast development due to the absence of the photoprotective carotenoids (Robertson, 1975; Neill et al., 1986). Because they lack chlorophyll and carotenoids, albino seedlings will die gradually without proper photosynthesis.

Class I mutants *vp2, vp5, vp7, vp9* and *w3* have been found that have altered carotenoid

contents in both endosperm and leaves and they map to chromosomes 5, 1, 5, 7, and 2, respectively (Maize Database: http://www.agron.missouri.edu/). Tissues of the recessive mutants *vp2* and *vp5* can accumulate phytoene and phytofluene (Robertson et al., 1978), as shown by TLC analysis; while by using HPLC it was shown that only phytoene was detected (Neill et al., 1986). Mutant *vp7* accumulates the pink pigment lycopene, and some γ- and δ-carotene (Neill et al., 1986), suggesting that *vp7* might regulate expression of *Lcy*. Mutant *vp9* accumulates ζ-carotene (Neill et al., 1986). *w3* has been found to accumulate phytoene and phytofluene and small amounts of ζ-carotene (Robertson et al., 1978). *y9* and its allele *y12* are reported as non-lethal viviparous mutants which can accumulate phytoene, phytofluene, and ζ-carotene; hence they have a pale yellow mutant endosperm, although they can grow into green seedlings (Robertson, 1975). *y8*, which maps to chromosome 7, is a non-viviparous mutant having a pale yellow endosperm; and the homozygous recessive seeds can grow into green seedlings (Robertson, 1975; Maize Genome database: htpp://www.agron.missouri.edu). The *vp5* locus has been found to encode phytoene desaturase (Hable et al., 1998; Li et al., 1996), and *y1* has been found to encode phytoene synthase (Buckner et al., 1996). Other loci, such as *vp2*, *vp7*, *w3*, *vp9*, *y9* and the non-lethal mutants *y8* have yet to be functionally characterized.

There are several reports on genetic analysis of viviparous rice mutants (Iwashita, 1970; Yatou and Iida, 1994). Some researchers have found that viviparous rice is probably controlled by multiple genes. Recent studies by Dr. O. Yatou (Institute of Radiation Breeding, National Institute of Agrobiological Resource, MAFF; Ibaraki, Japan), showed that the viviparous rice lines 84NMEMdr2 and 90KHEMdr1 are controlled by single

recessive genes (Yatou and Iida, 1994). These mutants were obtained by treating Nihonmasari and Koshihikari rice cultivars with EMS (ethyl methanesulfonate), gamma-irradiation, and exposure to thermal neutrons. These two mutants show the viviparous phenotype with precocious germination and albino seedlings (Yatou and Iida, 1994). In maize, the viviparous mutants are blocked in either carotenoid or ABA biosynthesis. For example, viviparous mutants *vp2, vp5, vp7, vp9* show decreased levels of ABA and produce albino seedlings (Neill et al., 1986). Viviparous mutants affecting only ABA biosynthesis do not show an albino phenotype. For instance, *vp1* has been shown to be an ABA-responsive transcription activator, in which the levels of carotenoids and ABA are normal; however, while aleurone anthocyanin is suppressed in *vp1* mutants, the seedlings are green (McCarty et al., 1989; McCarty et al., 1991). The *vp14* mutant was impaired in carotenoid cleavage; homozygous seedlings are green but exhibit markedly higher rates of water loss with respect to those of the normal plants (Tan et al., 1997). Since the 84NMEMdr2 and 90KHEMdr1 are rice viviparous mutants with albino seedlings, they may be blocked in carotenoid biosynthesis. It would be interesting to conduct biochemical or molecular studies of these two rice lines in order to better understand the functions of the corresponding mutant proteins in relation to carotenoid biosynthesis in rice.

1.6 Objectives

1.6.1 Long-term goals

Rice is one of the most important food staples worldwide, yet it lacks carotenoids in the endosperm; nevertheless, the photosynthetic tissues of rice contain carotenoids as accessory light harvesting pigments. Therefore, rice has the genes required for carotenoid biosynthesis. Vitamin A deficiency is a serious problem in developing countries especially Southeast Asia and Central America, where rice is a major staple. Recent (Ye et al., 2000) efforts to express plant and bacterial genes encoding pathway enzymes in rice have led to moderate accumulation of carotenoids in rice endosperm. Further improvement of endosperm carotenoid content will require a thorough understanding of the regulation of the pathway in endosperm from gene expression of pathway enzymes to substrate availability. Unlike rice, maize stores carotenoids in the endosperm, therefore maize can be used as a model system to study the regulation of carotenoid biosynthesis in endosperm.

1.6.2 Specific aims of the work

Until now, only a few genes involved in carotenoid biosynthesis from maize and rice have been isolated. For example, *Psy* and *Pds*, which encode enzymes for the early steps in the carotenoid biosynthetic pathway have been isolated both from rice and maize (Buckner et al., 1996; Li et al., 1996; Yoganathan, 1998). Much less is known about the maize and rice genes related to later steps of the pathway. It is also unclear how the entire pathway is regulated in monocot endosperm. Maize can store carotenoid in the endosperm, and maize contains low levels of provitamin A comparing with non-provitamin A carotenoids. Cloning genes of later steps in the pathway and studying their

expression in maize and rice will help us understand the molecular basis of carotenoid accumulation in endosperm. As discussed above, phytoene, the first specific precursor of all carotenoids, is converted to lycopene through four desaturase reactions catalyzed by PDS and ZDS. ζ-carotene desaturase (*Zds*) has not been isolated from maize and rice. To help further understand how the carotenoid biosynthetic pathway is regulated in maize and rice, cloning the *Zds* cDNA becomes a necessity. What is the catalytic function of maize ζ-carotene desaturase? For studying ZDS protein accumulation patters, a ZDS antibody is needed. Maize mutants play important roles in the study of carotenoid biosynthesis and further biochemical studies of mutants, such as *vp2, vp7, vp9, w3, y8,* and *y9* will certainly help understanding of the whole pathway. One hypothesis is that maize *Zds* might be linked to the maize mutation that is blocked in the desaturation of ζ-carotene. To link *Zds* with a specific mutant, it is necessary to investigate what is the expression of *Zds* at the RNA levels. Since plastoquinone has been shown to play roles in phytoene desaturation (Mayer et al., 1990; Norris et al., 1995; Schulz et al., 1993), one may ask, what are the possible functions of these maize carotenoid biosynthetic loci, especially for those accumulating phytoene, in the plastoquinone biosynthetic pathway? As mentioned before, two rice viviparous mutants have been reported recently: 90KHEMdr1 and 84NMEMdr1 (Yatou and Iida, 1994). Since most viviparous mutations in maize usually block the carotenoid biosynthetic pathway, are these mutants blocked in the carotenoid biosynthetic pathway?

In an attempt to answer the above questions, the following research was undertaken:

1. A full length ζ-carotene desaturase cDNA was isolated from maize, and its function

was identified (Chapter 2).

2. Several maize carotenoid mutants were biochemically characterized to link maize *Zds* to *vp9* (Chapter 2).

3. Sequence analysis of the rice ζ-carotene desaturase cDNA and genomic DNA were carried out (Chapter 3).

4. Expression of maize ζ-carotene desaturase in *E. coli* (Chapter 4).

5. A chemical complementation study on the intersection of the plastoquinone and carotenoid biosynthetic pathway was made to identify which mutation might confer blocks in the plastoquinone biosynthetic pathway (Chapter 5).

6. The viviparous rice lines 84NMEMdr2 and 90KHEMdr1 were characterized to test whether there was accumulation of certain intermediates of the carotenoid biosynthetic pathway (Chapter 5).

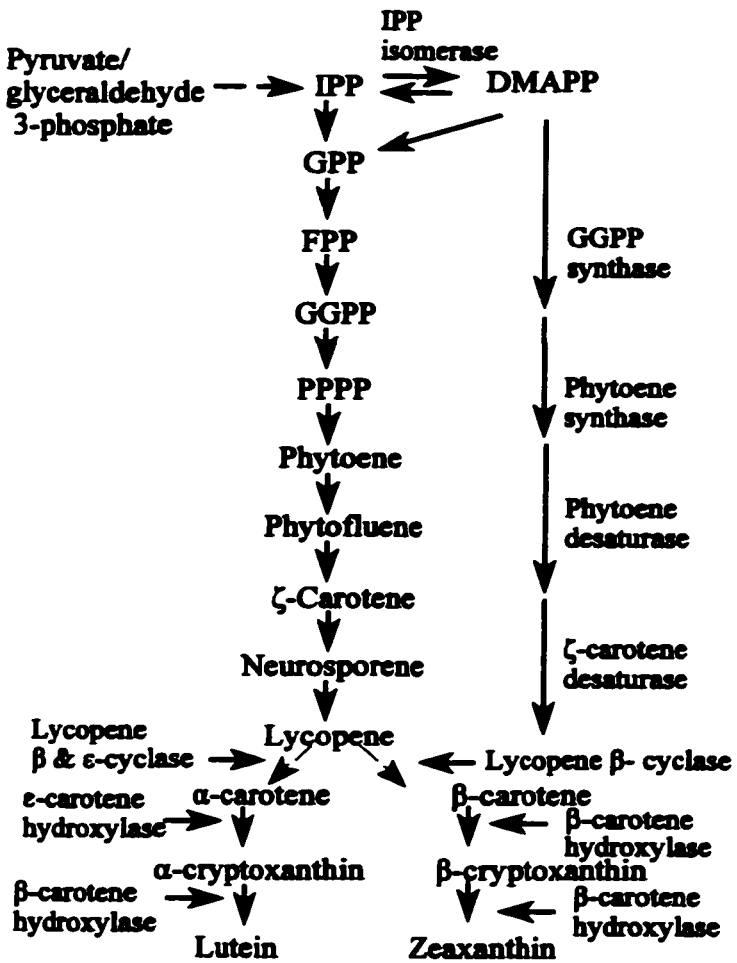


Figure 1-1A. General carotenoid biosynthetic pathway in higher plants. IPP, isopentenyl pyrophosphate; GPP, geranylgeranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; PPPP, prephytoene diphosphate.

Figure 1-1B. General carotenoid biosynthetic pathway in higher plants with chemical structures. Small arrows within the chemical structures indicate the bond position of dehydrogenation. (Dr. A. Yoganathan is acknowledged for drawing this pathway)

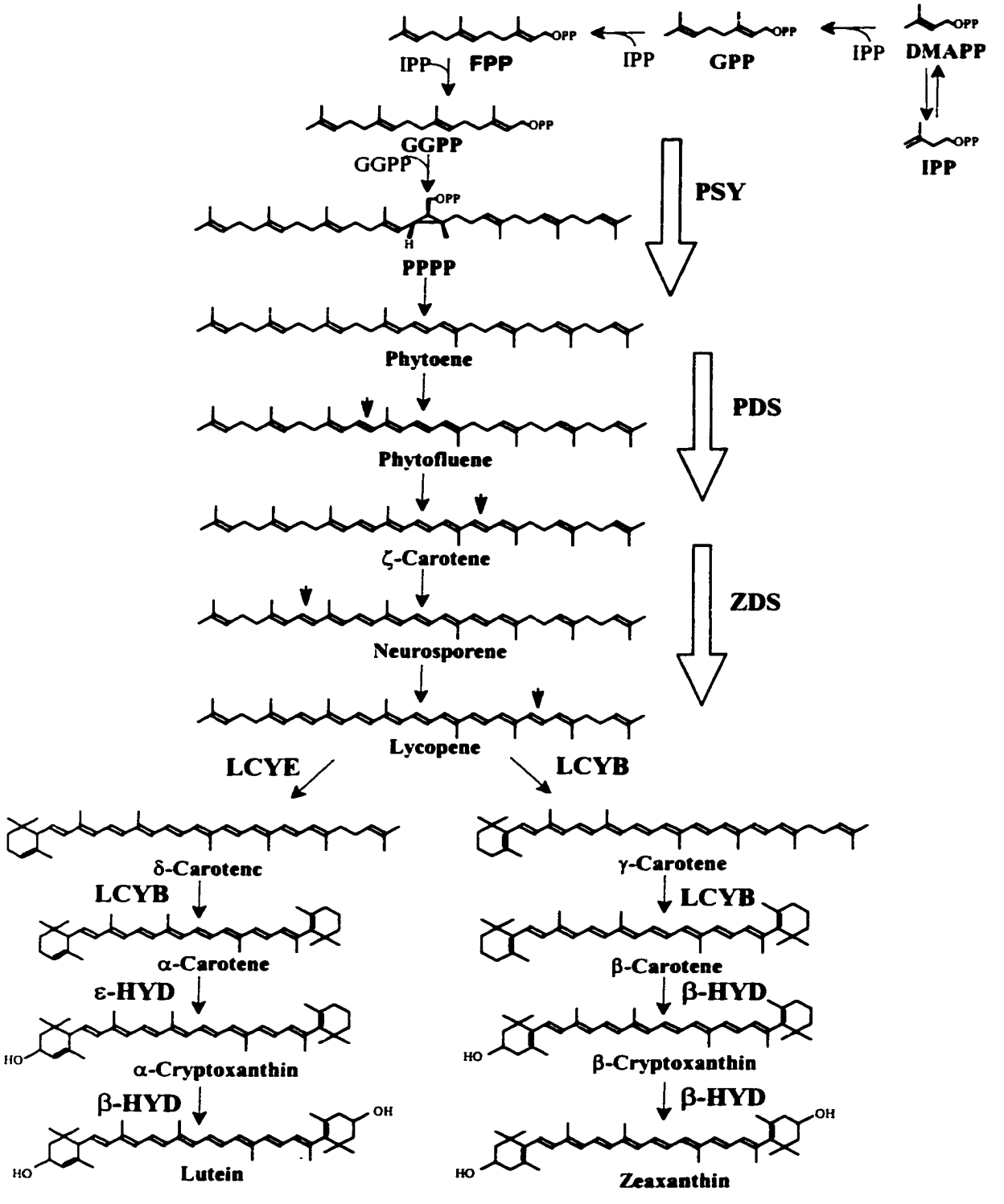
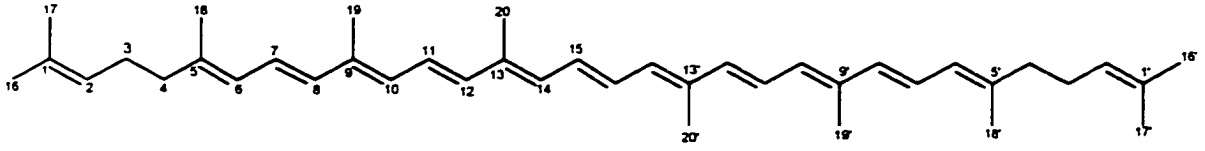


Figure 1-1B.



Lycopene

β-carotene

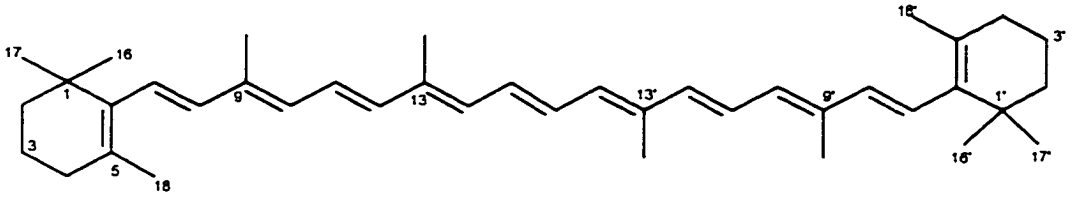


Figure 1-2. Basics structure and numbering scheme of lycopene and β-carotene.

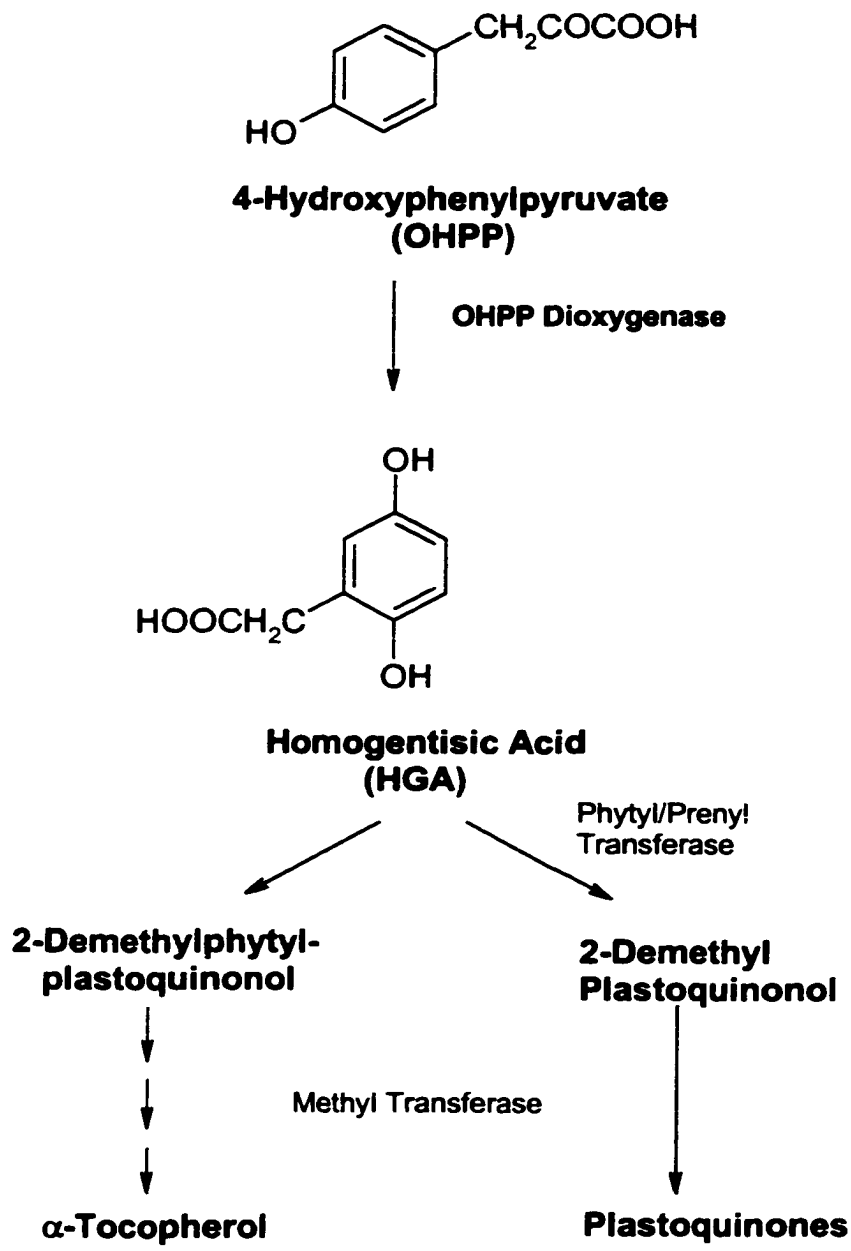


Figure 1-3. Outline of the plastoquinone and α-tocopherol biosynthetic pathway.

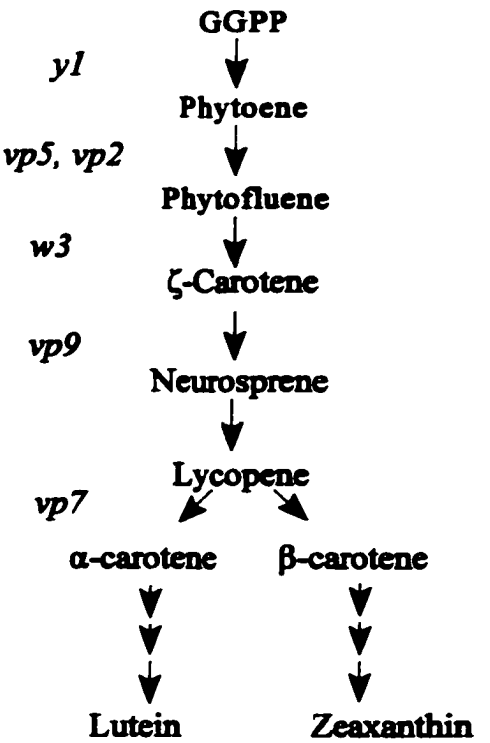


Figure 1-4. Genetic loci associated with the carotenoid biosynthetic pathway in maize. Loci are shown to left of the steps at which their gene products are thought to act.

Chapter 2. The link between a cDNA encoding ζ-carotene desaturase and genetic loci associated with carotenoid biosynthesis in maize[1]

2.1 Abstract

In higher plants, carotenoid biosynthesis takes places in plastids of different architectures and functions. Maize genetics is a useful tool for dissecting regulation in different tissues harboring different plastid types. There are numerous carotenoid mutations, some of which confer accumulation of pathway intermediates or completely block the pathway; there are dominant, recessive, and suppressor alleles, and some mutations show tissue-specific phenotypes. This rich collection of mutants represents putative structural and regulatory genes, though the functions of their gene products have yet to be identified. To this end, I have isolated a maize endosperm cDNA (2,265 nt) encoding ζ-carotene desaturase (ZDS) which is involved in carotenoid biosynthesis. ZDS participates in the early part of the pathway where the first specific pathway intermediate, phytoene, undergoes four sequential desaturations to produce lycopene. The maize *Zds* gene product was functionally tested in *E. coli* and shown to mediate desaturation of ζ-carotene to lycopene via neurosporene. RFLP mapping and transcript analyses suggested that maize ZDS may be encoded by the *vp9* locus on the short arm of chromosome 7. HPLC (High Pressure Liquid Chromatography) together with TLC (Thin Layer Chromatography)

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[1]The content of this chapter has been submitted for publication in the journal Molecular and General Genetics. The *Zds* sequence was submitted to GenBank and reported as: Luo and Wurtzel (1999).

analysis, confirmed that ζ-carotene accumulates in endosperm homozygous for either *vp9* or *y9*, but that neither ζ-carotene nor any other carotenoid intermediate accumulates in endosperm homozygous for *y8*, a carotenoid locus mapping to 7S, near *zds*. Other carotenoid desaturation mutants were also tested by chemical complementation; it was discovered that *vp2*, which conditions phytoene accumulation, blocks biosynthesis of plastoquinones, components of the electron transfer chain associated with the desaturation steps of carotenoid biosynthesis

2.2 Introduction

Carotenoids are a large class of yellow, red, and orange pigments derived from isoprenoids; they are produced in certain bacteria and fungi, and by all plants and cyanobacteria. In higher plants, they are synthesized in plastids by nuclear-encoded enzymes (Cunningham and Gantt, 1998). Carotenoids are essential for plant growth and development; they function as accessory pigments in photosynthesis, as photoprotectors preventing photooxidative damage, and as precursors to the plant hormone abscisic acid (ABA). The presence of carotenoids in endosperm tissue of certain plants also adds to their nutritional value; in humans and animals, dietary carotenoids are essential precursors to Vitamin A and to retinoid compounds needed in animal morphogenesis (Bendich and Olson, 1989; Lee et al., 1981).

The C_{20} isoprenoid GGPP (geranylgeranyl pyrophosphate) is the first precursor to biosynthesis of carotenoids, and to a variety of other isoprenoid-derived pathways,

including the biosynthesis of gibberellins, the phytol chain of chlorophyll, prenylquinones, tocopherols, and many secondary metabolites such as taxol and casbene (Chappell, 1995). The condensation of two GGPP molecules is catalyzed by PSY (phytoene synthase) to produce phytoene, the first compound specific to the carotenoid biosynthetic pathway (Cunningham and Gantt, 1998). Phytoene undergoes four sequential desaturation steps, increasing the number of conjugated double bonds in the central chromophore, and resulting in the carotenoid intermediate lycopene. In fungi and nonphotosynthetic bacteria, the four desaturation reactions are catalyzed by a single enzyme, designated as the "CRTI" type. In plants and cyanobacteria, these steps are catalyzed by two enzymes, PDS (phytoene desaturase) and ZDS (ζ-carotene desaturase), each mediating two steps. The plant/cyanobacterial PDS and ZDS enzymes are more similar in amino acid sequence to each other than to the CRTI-type bacterial enzyme. However, a bacterial type enzyme has been found in one cyanobacterium, *Anabaena*, though it only catalyzes a two-step desaturation from the ζ-carotene product of a companion PDS enzyme; other cyanobacteria do possess the plant-type ZDS (Breitenbach et al., 1998; Linden et al., 1993a). The desaturation steps require oxygen, and as demonstrated in a chromoplast *in vitro* system, are coupled to an electron transport chain, with oxygen being the final acceptor and involving a membrane-bound plastoquinone (Beyer et al., 1989; Mayer et al., 1990; Mayer et al., 1992; Norris et al., 1995). In different organisms, additional factors required for enzyme activity include chaperonins (Al-Babili et al., 1996b; Bonk et al., 1996) and galactolipids (Schledz et al., 1996), while accumulation or sequestration of the carotenoid biosynthetic endproducts, has been found to be regulated through lipid composition (Rabbani et al., 1998) and/or

specific carotenoid binding proteins (for example, see (Cervantes-Cervantes et al., 1990)).

Clearly, many factors may be involved in regulating carotenoid biosynthesis, expression of the component enzymes, and accumulation of pathway intermediates and endproducts. While carotenoids can be synthesized in plastids of different architecture and function, such as endosperm amyloplasts and leaf chloroplasts, less is known of the tissue-specific and plastid-specific differences in pathway localization and overall regulation. Maize is a wonderful model system to explore these differences because of the plentiful collection of mutations affecting the carotenoid pathway in endosperm and leaves. In particular are the viviparous mutants that are deficient in ABA due to the carotenoid biosynthetic block and manifest precocious germination in developing seeds in addition to an albino phenotype in germinated seedlings (Robertson, 1975). Other mutations affecting the pathway include recessive, dominant, and suppressor alleles; these are putative structural and regulatory loci, some of which have tissue-specific phenotypes and most of which have not been characterized with respect to function (Neill et al., 1986; Robertson et al., 1966; Robertson, 1975; Treharne et al., 1966). A number of mutations appear to affect the desaturase steps of the pathway because they condition accumulation of desaturation intermediates such as phytoene and ζ-carotene. However, there are more mutations than predicted enzymes. Therefore, in isolating genes encoding enzymes of the carotenoid biosynthetic pathway in maize, attempts are being made to link them to known loci to distinguish structural and regulatory genes from those encoding ancillary factors. This is being accomplished through RFLP mapping and gene structure and transcript analysis using normal and mutant alleles (Li et al., 1996).

Here I report the isolation and characterization of a maize cDNA encoding a functional ZDS. Analysis of *Zds* transcripts in normal and mutant tissue combined with RFLP analysis has allowed identifications of the structural locus. Using HPLC analysis and chemical complementation, other loci were tested to establish their roles in the desaturation reactions of the maize carotenoid biosynthetic pathway.

2.3 Materials and methods

2.3.1 Plant materials

Rice (*Oryza sativa*) IR36 and maize (*Zea mays*) inbred line B73 were grown in the greenhouse (20-25°C). Green shoots were collected after 7-10 days, immediately frozen in liquid nitrogen, and stored at -80°C until used. Maize mutants *vp9-Mum*, *vp2*, *w3*, *vp5*, *vp7* and *y9* were grown in the experimental field at Pelham Bay Park, Bronx, New York, while *y8* was grown in the greenhouse. Mutant and normal endosperms of *vp9-Mum*, *y9* and *y8* were dissected from maize harvested at 20 DAP, frozen in liquid nitrogen and stored at -80°C until used.

2.3.2 Cloning and sequencing

A rice EST clone #S14426 (GenBank # D48291, see also Chapter 3) showing 75 % nucleotide identity to *Arabidopsis Zds* was fully sequenced (GenBank #AF054629) and found to encode a 1632 nt cDNA insert having 74% nucleotide identity and 81% amino

acid identity (94% similarity) to the *Arabidopsis Zds* cDNA, indicating that the EST encoded rice ZDS. Two primers were designed based on a rice EST sequence: primer #202, nt 57-84, 5'-GCATTCCTACGAACTAACCAACTCAAGG-3'; and primer #203, nt 474-447, 5'-GGTGCTTACATAGAGACCTCCCTACACC-3'. A 417-bp RT-PCR product was amplified from rice IR36 leaf RNA (Logemann et al. 1987) as follows: 1 μg total RNA was used as template for cDNA first strand synthesis (Gibco-BRL SuperScript ™ preamplification system), then, 1 μl of cDNA product (ca. 0.2-0.8 ng) was utilized in a 25 μl PCR reaction (20 mM Tris-HCl, pH 8.4; 50 mM KCl; 2.5 mM $MgCl_2$; 0.2 mM of each dNTP; 0.5 μM of each primer; 0.04 u/μl *Taq* polymerase [Gibco-BRL, Gaithersburg, MD]). The PCR conditions were as follows: 1 cycle at 94°C (3 min); 35 cycles at 94°C (30 s), 68°C (30 s), 72°C (30 s); and one cycle at 72°C (10 min). After DNA sequence confirmation, the 417 bp RT-PCR product was used as a hybridization probe to screen one million clones of a λgt11 maize endosperm cDNA library (Fontes et al., 1991) according to Sambrook et al.(Sambrook et al., 1989). Hybridization was performed in 40 % (v/v) formamide, 6 X SSC, 1X Denhardt's solution, and 1 mM EDTA, at 42 °C overnight; filters were washed twice with 2 X SSC at room temperature and twice with 0.1 X SSC and 0.1% (w/v) SDS at 50°C, 15 min. Eight positive λ-phage clones, whose insert sizes varied from 1.4-kb to 2.2-kb, were obtained. The 2.2-kb *Eco*RI insert was subcloned into the *Eco*RI site of pBluescript II SK(–) vector and the resulting plasmid was named pMzds48 (See its map in Figure 2-1). Supercoiled pMzds48 plasmid DNA was obtained by $CsCl_2$ equilibrium density centrifugation. A plasmid deletion series was produced by double digesting pMzds48 with *Sal*I and *Kpn*I followed by 5'-deletion using the Exonuclease III and Mung Bean Nuclease Deletion Kit, according to the

instructions of the manufacturer (Stratagene, La Jolla, CA). Sequencing of one strand was carried out manually by using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corporation); the second strand was sequenced in an automated sequencer (Molecular Biology Core Facility, Oregon Regional Primate Research Center, Beaverton, OR 97006). The resulting maize *Zds* sequence was deposited as GenBank # AF047490. Other *Zds* sequences used for comparisons were plant-type, from *Capsicum* (GenBank # 2129927); *Narcissus* (GenBank # AJ224683); *Synechocystis* (GenBank #D90914), and bacterial-type, from *Anabaena* (GenBank # D26095). The maize *Pds* sequence used for comparison was GenBank # U37285. Sequence comparisons were carried out using BLAST programs (Altschul et al., 1997; Tatusova and Madden, 1999).

2.3.3 Expression and functional complementation of maize *Zds*

To test for functional complementation of the maize *Zds* cDNA, the plasmid pACCRT-EBP, conferring ζ-carotene accumulation, was first constructed. A 2.0-kb *Pvu*II-*Pvu*II fragment containing a maize *Pds* cDNA was removed from pMPDS3 (Li et al., 1996), both ends were filled in with Klenow and inserted into the *Bam*HI site (filled in with Klenow) of pACCRT-EB (Linden et al., 1991). The resulting plasmid pACCRT-EBP (see its map in Figure 2-2) carries *Erwinia uredovora crtE*, encoding GGPP synthase, and *crtB*, encoding phytoene synthase and maize phytoene desaturase. Next, pMzds48 was digested with *Not*I, the 5'-end filled in with Klenow and digested with *Xba*I. A 5' deletion from *Xba*I to the 5' end of maize *Zds* was performed with the Exonuclease III and Mung Bean Nuclease Deletion Kit (Stratagene, La Jolla, CA). One of the pMzds48-deleted

plasmids, pMzds-107 from which 106 bp had been deleted, was confirmed to be in frame with *lacZ* (See the junction sequence in Figure 2-3). *E. coli* XL-1 Blue cells carrying pACCRT-EBP were transformed with pMzds-107. Ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) were used to select transformants containing both pMZDS-107 and pACCRT-EBP, since pMzds-107 carries only an Amp^R marker, and pACCRT-EBP possesses only a Chl^R marker. Co-transformed cell cultures were grown overnight in 100 ml LB at 37°C supplemented with 1mM isopropylthio-β-D-galactoside (IPTG) and antibiotics. Pigment extraction and HPLC analysis were slightly modified from Pecker *et al.* (Pecker et al., 1996). Overnight cultures of *E. coli* transformed with the appropriate plasmids were centrifuged at 3000 rpm, 4 ° C for 15 min. Pellets were suspended in 10 ml acetone, incubated at 65°C for 30 min and centrifuged at 11,000*g* at 4°C for 15 min. The volumes of supernatants were reduced to 1-2 ml by blowing nitrogen. One-hundred μl of the final extract (in acetone) was injected into a 25 cm X 4.6 mm SphereClone ODS (1) C_{18} column (Phenomenex, Torrance, CA) by using acetonitrile/H_2O (9:1, v/v) and 100% ethyl acetate in a 25 min linear gradient, at 1 min/ml flow rate. The HPLC separation was conducted in a Waters HPLC system with a 600 Controller and pump, a 996 Photodiode Array Detector, and a 717 Autosampler.

2.3.4 Southern blotting

Maize B73 leaf genomic DNA (4 μg) was digested with restriction enzymes, run in an 0.8% (w/v) agarose gel, transferred to nitrocellulose and hybridized as described in Wurtzel et al. (Wurtzel et al., 1987) and exposed to a phosphorimager screen (Molecular

Dynamics, Sunnyvale, CA).

2.3.5 RT-PCR analysis of *Zds* expression in maize endosperm

RNA extraction and estimated concentrations were done according to Li et al. (Li et al., 1996). One μg total RNA was used for first strand as template for cDNA synthesis (Gibco-BRL Superscript ™ preamplification system) in a 20 μl reaction. Afterwards, 1 μl (ca. 0.2-0.8 ng) cDNA product was utilized in a 25 μl RT-PCR reaction containing 20 mM Tris-HCl pH 8.4; 50 mM KCl; 2.5 mM $MgCl_2$; 0.2 mM of each dNTP, 0.5 μM of each primer; and 0.04 μ/μl *Taq* polymerase (Gibco-BRL). RT-PCR primers for amplifying maize *Zds* (GenBank # AF047490) were primer #279 (nt 1408-1426), 5'-GGTTGGGTTGGATAACCTT-3' and primer #262 (nt 1712-1695), 5'-TCTGATCAGGCCTGAATG-3'; and for amplification of maize *Pds* (GenBank# U37285), were primer #110 (nt 1377-1396), 5'-GGAACTGTGAAACACTTCGC-3' and primer #111 (nt 1904-1885), 5'-GAAACCTTCGATAGGTGACC-3'. The PCR conditions for *Zds* were: 1 cycle at 94°C (3 min); 35 cycles at 94°C (30 s), 68°C (30 s), 72°C (1 min); and one cycle at 72°C (10 min). The PCR conditions for *Pds* were: 1 cycle at 94°C (3 min); 40 cycles at 94°C (30 s), 52°C (30 s), 72°C (30s); and one cycle at 72°C (10 min). RT-PCR products were applied onto a 1.6% (w/v) agarose gel, at 100 volts for about 2 hrs. For amplification of genomic DNA, 0.1 μg of maize B73 genomic DNA was used as a template in 25 μl reactions; PCR conditions were as described above.

2.3.6 TLC and HPLC analyses of normal and mutant maize endosperms

Extraction of carotenoids for TLC and HPLC analyses was done by grinding 0.5-1 g mutant endosperm, dissected at 20 DAP, to a powder form in liquid nitrogen, followed by addition of 10 ml of acetone, incubation at 65°C for 30 min, then centrifugation at 10,000*g* for 15 min. The supernatant was collected and reduced to 1-2 ml by blowing nitrogen. For TLC analysis, the mobile phase was ethyl ether/hexane, 60:40 (v/v), and the stationary phase was silica Gel 60 F_{254} (Kieselgel 60 F_{254}, Merck, Darmstadt, Germany). One ml of the final extract was applied in multiple applications on the TLC plates, air drying between each application. For HPLC analysis, 50 µl of the extracts were applied to the column. The HPLC solvent and column were the same as described above, except that the HPLC system used was a Waters 2690 Separations Module with a Waters 996 Photodiode Array Detector.

2.3.7 Chemical complementation of maize mutants

Developing seeds, harvested at 20 DAP, were surface-sterilized for 5 min in a solution of 10% (v/v) household bleach and 10% ethanol (v/v), and rinsed several times with sterilized water. Embryos were dissected under a sterile hood and planted in Murashige and Skoog medium (cat. 11117-066, Gibco-BRL), supplemented with sucrose at 20 g/liter and varying concentrations (0, 0.001, 0.01, 0.1 or 1 mM) of homogentisic acid (HGA) or 4-hydroxyphenylpyruvate (OHPP). Twenty seeds of each maize mutant at each condition were grown for one week in a 26°C growth chamber, with a cycle of 12 h light/12 h darkness.

2.4 Results and discussion

2.4.1 Isolation of maize *Zds* cDNAs

A rice EST clone (#S14426, GenBank # D48291) with 518 nt published sequence showed 75 % nucleotide identity to *Arabidopsis Zds*; it was fully sequenced as further discussed in chapter 3 (GenBank #AF054629) and found to contain a 1632 nt cDNA insert having 74% nucleotide identity and 81% amino acid identity (94% similarity) to the *Arabidopsis Zds* cDNA, indicating that the EST encoded rice ZDS. Primers were designed according to the rice EST sequence, amplified a 417 bp RT-PCR product from rice leaf mRNA which was used to probe a maize endosperm cDNA library, whereby 8 positive clones were obtained. The clones were hybridized to maize genomic DNA digested with *Hin*dIII, *Eco*RI or *Bam*HI and all showed the same pattern (data not shown). The longest, 2.265 kb clone, was subcloned into pBluescript II SK (–), designated as pMzds48, and used for further sequencing and functional analysis. The pMzds48 nucleotide sequence and the deduced amino acid sequence are shown in Figure 2-4. This 2.265 kb maize *Zds* cDNA (GenBank # AF047490) contains untranslated 5'- and 3'- regions of 205 and 350 nt respectively, and an open reading frame predicted to encode a 570-amino acid, 63.1-kDa protein based on the position of the first ATG codon at nt 206 and a stop codon at nt 1916. There are no stop codons upstream of the first ATG, and based on sequence comparisons with other ZDS sequences (Figure 2-5), it is unlikely that there is another upstream methionine codon.

2.4.2 Maize *Zds* sequence comparison with other genes

The deduced amino acid sequence of maize ZDS was compared with other available ZDS amino acid sequences (Figure 2-5) and that of the rice EST used to isolate the maize cDNA. The maize *Zds* sequence was found to have 87% nucleotide identity and 95% amino acid identity (98% similarity) to the rice *Zds* sequence, although the rice EST proved to be missing some nucleotides at the 5' end. When compared to the *Zds* sequence from daffodil, a more distantly related monocot than rice, the nucleotide identity was 76%, while the amino acid identity was 85% with 94% similar residues. Comparing *Zds* DNA and deduced amino acid sequences between maize (a monocot) and *Arabidopsis* (a dicot), I found nucleotide identity of 73%, and an amino acid identity of 82% with 91% similar residues. The amino acid identity with *Capsicum*, another dicot was 87% with 92% similarity. When the maize *Zds* sequence was compared with the corresponding sequence from the cyanobacterium *Synechocystis*, they shared 74% nucleotide identity, 65% amino acid identity (79% similarity). Maize ZDS, compared with a CRTI (bacterial) type ZDS from *Anabaena*, showed 18% amino acid identity and 29% similarity. Therefore, Based on the nucleotide homology, the maize *Zds* shows higher homology to the sequence of the plant-type rather than bacterial type gene.

The deduced amino acid sequence of maize ZDS revealed a typical dinucleotide binding domain (residues 71-101) (Figure 2-6B) that was present in PDS (Li et al., 1996). The similar enzymatic reaction of ZDS and PDS was reflected in the sequence similarity of the mature proteins, which showed an amino acid identity of about 32% and a similarity

of 50%.

A transit sequence for plastid targeting was predicted for residues 1-64 by comparing the maize ZDS sequence with that of the cyanobacterium *Synechocystis* (GenBank #D90914). The predicted transit peptide for daffodil, another monocot, is also 64 residues, and for pepper and *Arabidopsis* are 79 and 56 residues, respectively. The predicted transit peptides of maize ZDS is markedly different from that of PDS (Figure 2-6C). The predicted molecular mass of the mature maize ZDS polypeptide found in plastids is 56.3 kDa (506 amino acids) based on the sequence from nt 401-1918, as compared to 56.5 predicted for the mature daffodil ZDS (Figure 2-6A).

2.4.3 Functional analysis of maize ZDS using HPLC

Function of the maize ZDS cDNA gene product was confirmed by heterologous expression in *E. coli*. To accomplish this, the *Zds* cDNA insert of pMzsd48 was first subjected to 5'-end deletion of nt 1-106 to produce an in-frame translational fusion with *lacZ*. The resulting construct was designated as pMzds-107. Next, *E. coli* cells were transformed with pACCRT-EBP, which carries *crtE* and *crtB*, genes for GGPPS (GGPP synthase) and PSY from *Erwinia uredovora*, and the gene for PDS from maize; together these three genes confer accumulation of ζ-carotene in the *E. coli* host cells. To confirm activity of the cloned ZDS cDNA, cells carrying pACCRT-EBP were transformed with pMzds-107 and induced with IPTG. Function of maize ZDS in *E. coli* was expected to confer accumulation of neurosporene and lycopene, products of ζ-carotene desaturation.

The carotenoid products extracted from transformants were subjected to HPLC, and identified based on retention times, spectrophotometric profiles and comparison with known standards. As shown in the top panel of Figure 2-7A, cells transformed only with pACCRT-EBP accumulated ζ-carotene (retention time of 18.1 min); the characteristic spectrophotometric profile for ζ-carotene is shown in Figure 2-7, panel B. The lower panel of Figure 2-7A, shows the HPLC chromatogram of pigments extracted from *E. coli* cells that were induced with IPTG following transformation with both pACCRT-EBP and pMzds-107. In addition to the residual level of ζ-carotene, new peaks corresponding to neurosporene (17.0 min) and lycopene (16.0 min) were identified; the corresponding spectra are shown in panel B of Figure 2-7. We can't exclude the possibility that the neurosporene peak may contain pro-lycopene, the *cis*-lycopene product observed by Bartley et al. (1999) that has similar spectral properties as neurosporene. The appearance of the expected enzyme products indicate that the maize *Zds* cDNA encoded a functional enzyme. However, little lycopene accumulated, and much ζ-carotene remained, suggesting that maize ZDS, unlike maize PDS (Li et al., 1996) functioned inefficiently in this *E. coli* host. Another construct was also made by removing the first 392 nt (corresponding to the first 62 of 64 codons of the putative transit peptide). After co-transforming *E. coli* cells with this construct and pACRRT-EBP, the HPLC results showed no significant increase in the level of neurosporene or lycopene (data not shown). This indicates that the transit peptide is not responsible for the inefficient production of lycopene by maize ZDS expressed in *E. coli*, but some other factor. Similar results were obtained when the pepper ZDS was expressed in *E. coli* (Albrecht et al., 1995).

Since ZDS and PDS share some structural similarities, and desaturases in higher plants, fungi, and bacteria can catalyze two, three, or four desaturation reactions, a test was also conducted whether maize ZDS might use phytoene as a substrate. Cells accumulating phytoene were generated by transforming *E. coli* with pACCRT-EB, a plasmid carrying the *Erwinia* genes *crtE*, for GGPPS; and *crtB*, for phytoene synthase. Cells containing pACCRT-EB were transformed with pMzds-107, but only phytoene was detected by HPLC, whether or not the pMzds-107 plasmid was present (data not shown). This experiment verified that ZDS uses only ζ-carotene as a substrate, and will not catalyze desaturation of phytoene. So ZDS encoded by this maize cDNA, like ZDS in other higher plants, can only catalyze two desaturation reactions from ζ-carotene to lycopene via neurosporene.

2.4.4 Southern hybridization and RPLP mapping of maize *zds*

To determine how many copies of the *zds* gene are present in maize, pMzds48 was hybridized to maize B73 genomic DNA digested with several restriction enzymes (Figure 2-8). Though there were two or three bands in DNAs digested with *Xho*I, *Hin*dIII, *Eco*RI or, *Bam*HI, there was only one band in the *Kpn*I digestion, suggesting that *zds* may be a single copy gene. Hybridization of either the maize ZDS cDNA or the rice EST against rice genomic DNA also revealed single bands for DNAs digested by different restriction enzymes (Figure 2-9). Together these results suggest that *zds* may be a single copy gene in both maize and rice.

Maize *zds* was mapped to chromosome position by RFLP analysis using a Tx303 X CO159 population (UMC, University of Missouri-Columbia Maize RFLP laboratory, MO) and a T232 X CM37 population (B. Burr, Brookhaven National Laboratory, Upton, NY). In RFLP mapping, two distinct inbred lines are used to detect any polymorphism of a particular unmapped gene. To this effect, the gene of the interest and known gene markers are used as probes in Southern blotting. If the gene of interest co-segregates with the marker genes, it can be mapped to the corresponding locus of the markers in the chromosome (Burr et al., 1988). According to RFLP analysis of the Tx303 x CO159 population, *zds* mapped to the short arm of chromosome 7 (7S) in bin 7.02 between *asg34* and *asg49*; these loci flank *vp9*, a locus associated with blocks in ζ-carotene desaturation. Mapping results in the T232 X CM37 population gave similar results, where *zds* mapped to 7S closely linked to *o2* and *in1*. These two loci flank *y8*, while *vp9* is about 5 map units proximal. RFLP mapping from two different sources established that *zds* maps to maize chromosome 7 at two different loci; these results might be due to the lack of accuracy of the RFLP technique.

2.4.5 Transcript levels of *Zds* in the maize mutants *vp9-Mum*, *y8*, and *y9* and their normal endosperm counterparts

Based on the mapping results, it is suspected that either *y8* or *vp9* might be the *zds* structural gene; such a distinction might be manifested as reduced *Zds* transcript levels when comparing mutant and normal alleles of these loci. To test whether either *y8* or *vp9* might encode or regulate *Zds*, RT-PCR analysis was used to compare transcript levels in

normal (yellow) and mutant (white or light yellow) endosperms segregating for either *y8* or *vp9-Mum* (a *Mutator*-induced allele of *vp9*). Normal and mutant endosperms segregating for *y9* were also included in the analysis since homozygous *y9* endosperms were reported to accumulate ζ-carotene as summarized in a review by Robertson (Robertson, 1975). Since *y9* mapped to chromosome 10S (Maize Database: http://www.agron.missouri.edu/) and *zds* mapped to 7S, *y9* was not a candidate for the *zds* structural gene but could encode a regulator of ζ-carotene desaturation or *Zds* expression. RNA was extracted from normal and mutant endosperms dissected from ears segregating for one of the three mutant alleles and used as a template for amplification of the *Zds* transcript by RT-PCR. Primers were chosen to flank an intron; the presence of an intron was confirmed by amplification from B73 genomic DNA, which yielded a 540 bp product (Figure 2-10A, lane 1) as compared with a 303 bp product (Figure 2-10A, lane 2) obtained from a B73 leaf mRNA sample; no products were obtained if reverse transcriptase or either primer were omitted from the RT-PCR reaction (Figure 2-10A, lanes 3-5). As shown in Figure 2-10B (top), *Zds* transcripts were amplified from each of the three mutant endosperms and compared with amplification of RNA isolated from their normal segregating counterparts; only in the *vp9-Mum* material was there a reduction seen in amplification products as compared to the transcript level in the normal endosperm. As a comparison, *Pds* transcripts were amplified as before (Li et al., 1996), and no difference in *Pds* amplification products were seen in any of the three mutant genotypes tested (Figure 2-10B, bottom). Taken together with the mapping results, it is consistent that the *vp9* locus encodes ZDS; *y8* likely encodes some other factor required for carotenoid accumulation in endosperm. From these results, it appears that *y9* does not

regulate *Zds* transcript accumulation, but may have some other role in the desaturation process.

2.4.6 Carotenoid content of maize mutants *vp9-Mum*, *y8*, and *y9* and their normal endosperm counterparts

Since much of the earlier carotenoid identifications were limited to TLC analysis, I decided to confirm identification of accumulating intermediates for mutant alleles of loci linked to ζ-carotene desaturation; this further enabled to test whether *y8* had any role in ζ-carotene desaturation. Both TLC and HPLC were used to test for accumulation of ζ-carotene in each of the above mutants. The TLC results shown in Table 1 indicate that both *y9* and *vp9-Mum*, but not *y8*, endosperms had a pigment matching the R_f value for ζ-carotene isolated from *E. coli* cells carrying pACCRT-EBP, the plasmid conferring ζ-carotene accumulation. The normal segregating endosperms did not accumulate ζ-carotene, as expected, but instead accumulated a compound tentatively identified as a xanthophyll. Further confirmation of these results were obtained from the HPLC analysis shown in Figure 2-11. In extracts from the *vp9-Mum* and *y9* mutants, a ζ-carotene peak (1) was detected at 13. 0 min, the same retention time as the standard, but no corresponding peak was found for the extract from *y8* mutant endosperms. The xanthophyll peak (2), with a retention time of 6.5 min, was present only in normal endosperms of *vp9-Mum*, *y9* and *y8* seeds. Therefore, the HPLC results confirm the TLC results and together indicate that *y8* is unrelated to ζ-carotene desaturation, whereas *vp9* and *y9* are involved in the ζ-carotene desaturation steps.

2.4.7 Chemical complementation of the maize mutants *vp2*, *w3*, *vp5*, *vp7*, and *vp9*

Since *vp9* is likely to be the *zds* structural gene, it is unclear what is the role of *y9* in ζ-carotene desaturation. Similarly, for desaturation of phytoene to ζ-carotene, there are also loci that can be attributed to the PDS structural gene (e.g. *vp5*), and others (e.g. *vp2*, *w3)* which do not regulate transcript accumulation but for which recessive alleles may confer phytoene accumulation (Li et al., 1996). It is possible that some of these loci might regulate or control biosynthesis of plastoquinones, known participants in the electron transfer reactions associated with carotene desaturation steps. In Arabidospsis, *pds1* conditions phytoene accumulation due to a block in the biosynthesis of plastoquinones, specifically at the step mediated by 4-hydroxyphenylpyruvate dioxygenase, an enzyme catalyzing the synthesis of homogentisic acid (HGA) from 4-hydroxyphenylpyruvate (OHPP) (Norris et al., 1995; Norris et al., 1998). Therefore, a chemical complementation experiment was carried out to test whether any of the maize desaturation mutants were blocked at this step of the plastoquinone biosynthetic pathway. *vp7* (that confers lycopene accumulation) and *vp5* were included as negative controls. Unfortunately the *y9* mutant could not be tested as it affects only the endosperm phenotype. Mutant embryos (twenty each) homozygous for *vp2*, *w3*, *vp5*, *vp7*, or *vp9* were placed in medium containing increasing and identical amounts of HGA or OHPP. Without either chemical added, all of the mutant embryos germinated into albino seedlings or into pink seedlings in the case of *vp7* (Table 2). However, when 0.1 or 1 mM HGA was supplemented, the *vp2* embryos germinated into pale green and green seedlings, respectively. No other mutant tested showed this effect. Regardless of the amount of OHPP added (0 to 1 mM), all of the

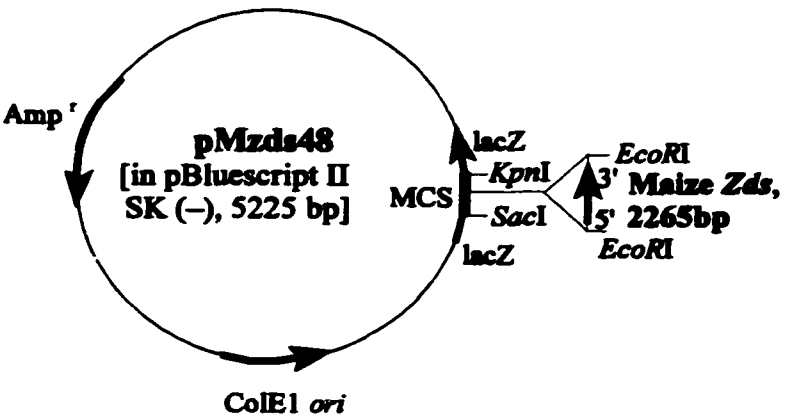
seedlings retained their mutant phenotype. Ten *vp2* mutant plants in 0.1 or 1 mM HGA that had become pale green or green were transplanted to Murashige and Skoog medium lacking HGA. After 3-4 days, the seedlings gradually reverted to white. This confirmed that the HGA deficiency causes *vp2* mutant plants to be albino and that the *vp2* locus, but none of the other loci tested, is involved in biosynthesis of plastoquinones at the step leading to biosynthesis of HGA. From these results I propose that maize *vp2*, as *pds1* in *Arabidopsis*, encodes or regulates the expression of 4-hydroxyphenylpyruvate dioxygenase.

2.4.8 Summary

I have isolated and sequenced a cDNA encoding a ZDS from maize using a fragment of the corresponding gene from rice. In both maize or rice, the gene is likely to be single copy. The maize cDNA was functionally tested in *E. coli* and demonstrated to encode a two-step desaturase. With the ZDS encoding cDNA, combined with RFLP analysis, transcript and HPLC analysis of maize carotenoid mutants, I was able to further characterize the potential function of several of the maize genetic loci that are linked to the carotenoid desaturation steps. I showed that *vp9* and *y9* mutants accumulate ζ-carotene, while *y8* mutants are completely blocked in the carotenoid pathway and do not accumulate any intermediate. It appears that the *vp9* locus is the likely candidate for the *zds* structural gene, while *y9* affects ζ-carotene desaturation in some unknown manner. Of the other desaturation mutants tested, *vp2* was found to be blocked in the biosynthesis of HGA, an intermediate of plastoquinone biosynthesis, while *w3* was not, despite its

conditioning of phytoene accumulation.

A. Map of pMZDS48



B. MCS (Mutiple cloning site)

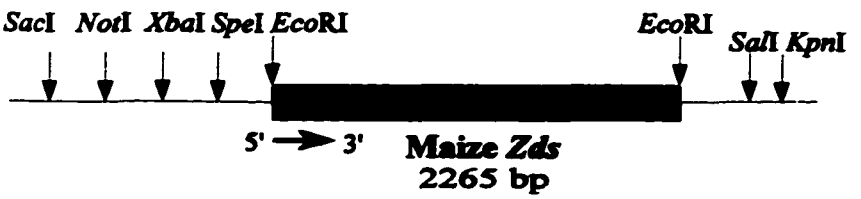


Figure 2-1. Map of pMzds48 in pBluescript II SK (-) vector. A, Map of pMzds48; B, MCS (Multiple cloning site) of pMzds48. Arrows indicate direction of transcription.

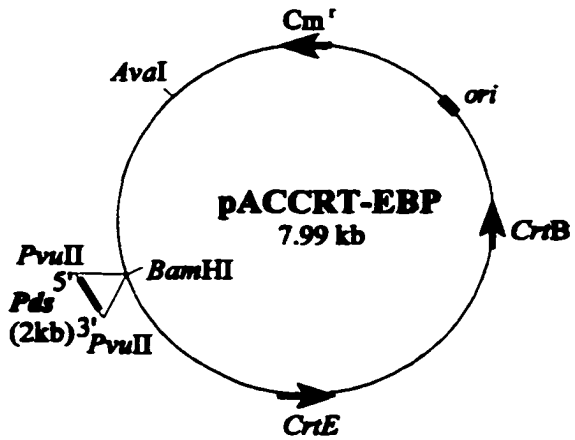
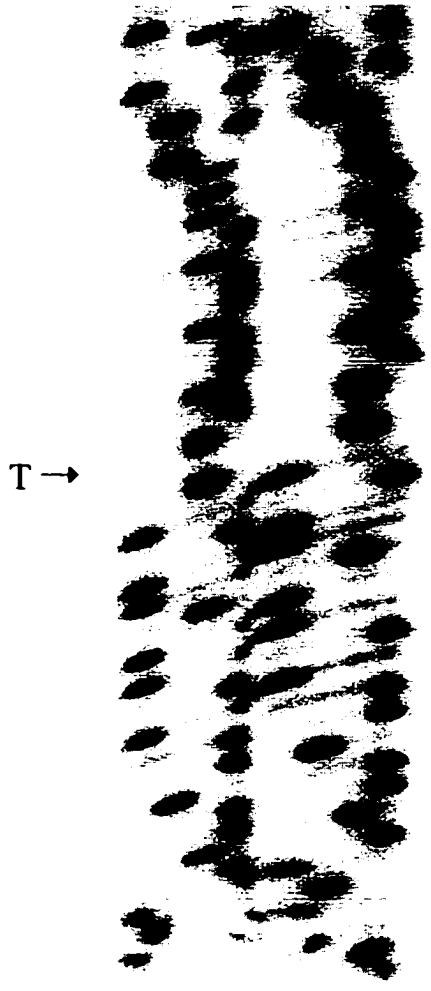


Figure 2-2. Map of pACCRT-EBP. Plasmid pACCRT-EB was kindly provided by Dr. N.Misawa, *Pds* was isolated from maize by Dr. Zhouhui Li in our laboratory. Sites of *Bam*HI and *Pvu*II were destroyed upon subcloning.

Figure 2-3. Junction sequence of insert and vector of pMzds-107. The bold nucleotides are from pBluescript II SK (–), the bold and italicized nucleotides are T3 primer sequence; underlined nucleotides are from pMzds48; in the autoradiograph, "G" with the arrow indicates the first "g" in pMzds48 in the junction sequence.



Junction sequence:

T3 promoter→ *SacI*

ATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCC

MetThrMetIleThrProSerCysLysLeuThrLeuThrLysGlyAsnLysSerTrpSerSer

β-Galactosidase→

pBluescript II SK (-)

SacII 107 127

ACCGCGGTGGCG TTCTCCTCCTCTTTACACGCT

ThrAlaValAla PheSerSerSerLeuHisAla

pBluescript II SK(-) **pMzds48**

Figure 2-3.

Figure 2-4. Nucleotide and amino acid sequences of the maize *Zds* cDNA. A, pMzds48 (GenBank # AF047490) nucleotide and amino acid sequences. The deduced amino acid sequence is shown as single letters under the nucleotide sequence. Bold letters show the putative dinucleotide binding domain. The underlined amino acids indicate the putative transit peptide. B, pMzds48 sequence in GenBank format.

```
   1  CCCTGCCACGACGCCCGCGACAAATCCCTGCGCGACGGCATCTTCGCCTCCCATCCCCTC
  61  CCAGCTTCCCCTCCCACTCCGGCCCTCACACAAATTGCCCCTCTTCTTCTCCTCCTCTTT
 121  ACACGCTGCCGACCACGGCTGCCGCCAACCACCCGCCCCACCCGTCCACCGCTGCCGAGT
 181  GCTAGCCATTTGGAGCTGCCGCGCCATGGCGTCCGTGGCCGCCACCACCACGCTGGCACC
                                  M  A  S  V  A  A  T  T  T  L  A  P
 241  GGCACTCGCCCCGCGCCGGGCGCGGCCAGGGACTGGGCTCGTGCCGCCGCGCCGGGCCTC
  13    A  L  A  P  R  R  A  R  P  G  T  G  L  V  P  P  R  R  A  S
 301  GGCCGTCGCTGCTCGCTCGACCGTAACGTCTCCGACATGGCGTCAACGCTCCCAAAGGTT
  33    A  V  A  A  R  S  T  V  T  S  P  T  W  R  Q  R  S  Q  R  L
 361  ATTCCCACCCGAGCCAGAGCACTACAGGGGCCCGAAGCTCAAGGTGGCCATCATAGGGGC
  53    F  P  P  E  P  E  H  Y  R  G  P  K  L  K  V  A  I  I  G  A
 421  AGGCCTTGCGGGCATGTCCACCGCTGTTGAGCTCTTGGACCAGGGCCATGAGGTTGATTT
  73    G  L  A  G  M  S  T  A  V  E  L  L  D  Q  G  H  E  V  D  L
 481  GTACGAGTCCCGTCCGTTTATCGGTGGCAAGGTTGGCTCCTTTGTTGACAGGCAAGGAAA
  93    Y  E  S  R  P  F  I  G  G  K  V  G  S  F  V  D  R  Q  G  N
 541  CCATATCGAGATGGGGCTGCATGTGTTCTTCGGGTGCTACAGCAATCTCTTCCGCCTCAT
 113    H  I  E  M  G  L  H  V  F  F  G  C  Y  S  N  L  F  R  L  M
 601  GAAGAAGGTTGGCGCTGATAATAATCTGCTGGTGAAGGAACATACCCATACTTTTGTAAA
 133    K  K  V  G  A  D  N  N  L  L  V  K  E  H  T  H  T  F  V  N
 661  TAAAGGGGGCACGATTGGTGAACTTGATTTTCGGTTCCCGGTGGGAGCTCCGTTACATGG
 153    K  G  G  T  I  G  E  L  D  F  R  F  P  V  G  A  P  L  H  G
 721  CATTCAAGCATTCCTAAGAACTAATCAGCTCAAGGTTTATGATAAAGCAAGAAATGCAGT
 173    I  Q  A  F  L  R  T  N  Q  L  K  V  Y  D  K  A  R  N  A  V
 781  TGCTCTTGCCCTTAGTCCAGTTGTTCGGGCTCTGGTTGATCCTGATGGTGCATTGCAGCA
 193    A  L  A  L  S  P  V  V  R  A  L  V  D  P  D  G  A  L  Q  Q
 841  AGTGCGGGACTTGGATGATATAAGTTTCAGTGATTGGTTCATGTCCAAAGGGGGTACTCG
 213    V  R  D  L  D  D  I  S  F  S  D  W  F  M  S  K  G  G  T  R
 901  GGAGAGTATCACAAGAATGTGGGATCCTGTTCGTTACGCTTTGGGTTTCATTGACTGTGA
 233    E  S  I  T  R  M  W  D  P  V  R  Y  A  L  G  F  I  D  C  D
 961  TAATATCAGTGCACGTTGCATGCTTACTATTTTCACCTTGTTTGCCACAAAGACAGAGGC
 253    N  I  S  A  R  C  M  L  T  I  F  T  L  F  A  T  K  T  E  A
1021  ATCCCTGTTACGCATGTTAAAGGGTTCACCTGATGTTTACTTAAGTGGTCCAATAAAGAA
 273    S  L  L  R  M  L  K  G  S  P  D  V  Y  L  S  G  P  I  K  K
1081  GTATATAACAGACAGGGGTGGTAGGTTTCACTTAAGGTGGGGATGCAGAGAGGTTCTCTA
 293    Y  I  T  D  R  G  G  R  F  H  L  R  W  G  C  R  E  V  L  Y
1141  TGAGAAGTCACCTGATGGAGAGACCTATGTTAAGGGCCTTCTACTCACCAAGGCTACAAG
 313    E  K  S  P  D  G  E  T  Y  V  K  G  L  L  L  T  K  A  T  S
1201  TAGAGAGATAATCAAAGCTGATGCATACGTCGCAGCCTGTGATGTTCCAGGTATCAAAAG
 333    R  E  I  I  K  A  D  A  Y  V  A  A  C  D  V  P  G  I  K  R
1261  ATTACTTCCATCAGAATGGAGGGAGTGGGAAATGTTTGACAATATCTACAAGTTAGATGG
 353    L  L  P  S  E  W  R  E  W  E  M  F  D  N  I  Y  K  L  D  G
1321  TGTCCCTGTTGTCACTGTCCAGCTCCGCTACAACGGATGGGTCACTGAACTTCAAGATTT
 373    V  P  V  V  T  V  Q  L  R  Y  N  G  W  V  T  E  L  Q  D  L
1381  GGAGAAATCAAGACAACTGCAAAGGGCGGTTGGGTTGGATAACCTTTTGTACACGGCGGA
 393    E  K  S  R  Q  L  Q  R  A  V  G  L  D  N  L  L  Y  T  A  D
1441  TGCAGACTTTTCCTGTTTTTCGGACCTTGCTCTCTCATCTCCTGCTGATTACTACATTGA
 413    A  D  F  S  C  F  S  D  L  A  L  S  S  P  A  D  Y  Y  I  E
1501  AGGGCAAGGTTCCCTGATCCAAGCTGTGCTGACTCCTGGAGATCCATACATGCCATTGCC
 433    G  Q  G  S  L  I  Q  A  V  L  T  P  G  D  P  Y  M  P  L  P
1561  AAACGAGGAGATCATTAGTAAGGTTCAAAAGCAGGTTGTAGAACTGTTCCCATCTTCCCG
 453    N  E  E  I  I  S  K  V  Q  K  Q  V  V  E  L  F  P  S  S  R
1621  GGGCTTAGAAGTTACATGGTCCAGTGTGGTAAAGATCGGACAATCGCTGTACCGTGAGGC
 473    G  L  E  V  T  W  S  S  V  V  K  I  G  Q  S  L  Y  R  E  A
1681  TCCTGGAAACGACCCATTCAGGCCTGATCAGAAGACGCCCGTTAAAAACTTCTTCCTCTC
 493    P  G  N  D  P  F  R  P  D  Q  K  T  P  V  K  N  F  F  L  S
1741  TGGATCTTACACGAAACAGGACTACATCGACAGCATGGAAGGAGCAACTCTCTCCGGCAG
 513    G  S  Y  T  K  Q  D  Y  I  D  S  M  E  G  A  T  L  S  G  R
1801  GCGAACGTCGGCCTACATCTGCGGTGCCGGGGAGGAGCTGCTGGCCCTCCGAAAGAAGCT
 533    R  T  S  A  Y  I  C  G  A  G  E  E  L  L  A  L  R  K  K  L
1861  ACTCATCGACGACGGCGAGAAGGCGCTGGGGAACGTTCAAGTCCTGCAGGCTAGCTGAAC
 553    L  I  D  D  G  E  K  A  L  G  N  V  Q  V  L  Q  A  S  -
1921  AACCCCTCCTGCACTGCAGAGAAGCTTGGATCTTTCCAACCACACATACATGCTGGAATG
1981  GACAAACCAACCAACCATTGTCTTTTCTCGCTTCAGGGTGCTGGCGATTCCCGCAGCAAC
2041  CTCCTGTGTATCGTATCCAATTTGAGCATTAGATCTGCCCCCCCCCCCCTGCAGGCGTTTC
2101  TTTCCTATCCCTGATCCGAGAAGCAGGGTGTAGTCTAGGTGGCTGGCATACGGGATTACA
2161  TCAGGCAGTGTGTAAGTTCAGCTGGAACTCGATTGGTAATTGGGATGGATGATTGATGAT
2221  ATATATATAGCACACACTGTTCTTGCGTCTTGCAAAAAAAAAAAA
```

Figure 2-4A.

B

```
LOCUS       AF047490     2265 bp    mRNA            PLN
DEFINITION  Zea mays zeta-carotene desaturase mRNA, complete cds.
ACCESSION   AF047490
KEYWORDS    .
SOURCE      maize.
 ORGANISM   Zea mays
            Eukaryota; Viridiplantae; Charophyta/Embryophyta group;
            Embryophyta; Tracheophyta; seed plants; Magnoliophyta; Liliopsida;
            Poales; Poaceae; Zea.
  AUTHORS   Luo,R. and Wurtzel,E.T.
  TITLE     Direct Submission
 FEATURES             Location/Qualifiers
    source          1..2265
                    /organism="Zea mays"
                    /db_xref="taxon:4577"
                    /chromosome="7"
                    /map="ASG34-ASG49"
    CDS             206..1918
                    /codon_start=1
                    /product="ζ-carotene desaturase"
                    /translation="MASVAATTTLAPALAPRRARPGTGLVPPRRASAVAARSTVTSPT
                    WRQRSQRLFPPEPEHYRGPKLKVAIIGAGLAGMSTAVELLDQGHEVDLYESRPFIGGK
                    VGSFVDRQGNHIEMGLHVFFGCYSNLFRLMKKVGADNNLLVKEHTHTFVNKGGTIGEL
                    DFRFPVGAPLHGIQAFLRTNQLKVYDKARNAVALALSPVVRALVDPDGALQQVRDLDD
                    ISFSDWFMSKGGTRESITRMWDPVRYALGFIDCDNISARCMLTIFTLFATKTEASLLR
                    MLKGSPDVYLSGPIKKYITDRGGRFHLRWGCREVLYEKSPDGETYVKGLLLTKATSRE
                    IIKADAYVAACDVPGIKRLLPSEWREWEMFDNIYKLDGVPVVTVQLRYNGWVTELQDL
                    EKSRQLQRAVGLDNLLYTADADFSCFSDLALSSPADYYIEGQGSLIQAVLTPGDPYMP
                    LPNEEIISKVQKQVVELFPSSRGLEVIWSSVVKIGQSLYREAPGNDPFRPDQKTPVKN
                    FFLSGSYTKQDYIDSMEGATLSGRRTSAYICGAGEELLALRKKLLIDDGEKALGNVQV
                    LQAS"
BASE COUNT      525 a    603 c    585 g    552 t
ORIGIN
        1 ccctgccacg acgcccgcga caaatccctg cgcgacggca tcttcgcctc ccatcccctc
       61 ccagcttccc ctcccactcc ggccctcaca caaattgccc ctcttcttct cctcctcttt
      121 acacgctgcc gaccacggct gccgccaacc acccgcccca cccgtccacc gctgccgagt
      181 gctagccatt tggagctgcc gcgccatggc gtccgtggcc gccaccacca cgctggcacc
      241 ggcactcgcc ccgcgccggg cgcggccagg gactgggctc gtgccgccgc gccgggcctc
      301 ggccgtcgct gctcgctcga ccgtaacgtc tccgacatgg cgtcaacgct cccaaaggtt
      361 attcccaccc gagccagagc actacagggg cccgaagctc aaggtggcca tcataggggc
      421 aggccttgcg ggcatgtcca ccgctgttga gctcttggac cagggccatg aggttgattt
      481 gtacgagtcc cgtccgttta tcggtggcaa ggttggctcc tttgttgaca ggcaaggaaa
      541 ccatatcgag atggggctgc atgtgttctt cgggtgctac agcaatctct tccgcctcat
      601 gaagaaggtt ggcgctgata ataatctgct ggtgaaggaa catacccata cttttgtaaa
      661 taaagggggc acgattggtg aacttgattt tcggttcccg gtgggagctc cgttacatgg
      721 cattcaagca ttcctaagaa ctaatcagct caaggtttat gataaagcaa gaaatgcagt
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      841 agtgcgggac ttggatgata taagtttcag tgattggttc atgtccaaag ggggtactcg
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      961 taatatcagt gcacgttgca tgcttactat tttcaccttg tttgccacaa agacagaggc
     1021 atccctgtta cgcatgttaa agggttcacc tgatgtttac ttaagtggtc caataaagaa
     1081 gtatataaca gacaggggtg gtaggtttca cttaaggtgg ggatgcagag aggttctcta
     1141 tgagaagtca cctgatggag agacctatgt taagggcctt ctactcacca aggctacaag
     1201 tagagagata atcaaagctg atgcatacgt cgcagcctgt gatgttccag gtatcaaaag
     1261 attacttcca tcagaatgga gggagtggga aatgtttgac aatatctaca agttagatgg
     1321 tgtccctgtt gtcactgtcc agctccgcta caacggatgg gtcactgaac ttcaagattt
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     1441 tgcagacttt tcctgttttt cggaccttgc tctctcatct cctgctgatt actacattga
     1501 agggcaaggt tccctgatcc aagctgtgct gactcctgga gatccataca tgccattgcc
     1561 aaacgaggag atcattagta aggttcaaaa gcaggttgta gaactgttcc catcttcccg
     1621 gggcttagaa gttacatggt ccagtgtggt aaagatcgga caatcgctgt accgtgaggc
     1681 tcctggaaac gacccattca ggcctgatca gaagacgccc gttaaaaact tcttcctctc
     1741 tggatcttac acgaaacagg actacatcga cagcatggaa ggagcaactc tctccggcag
     1801 gcgaacgtcg gcctacatct gcggtgccgg ggaggagctg ctggccctcc gaaagaagct
     1861 actcatcgac gacggcgaga aggcgctggg gaacgttcaa gtcctgcagg ctagctgaac
     1921 aacccctcct gcactgcaga gaagcttgga tctttccaac cacacataca tgctggaatg
     1981 gacaaaccaa ccaaccattg tcttttctcg cttcagggtg ctggcgattc ccgcagcaac
     2041 ctcctgtgta tcgtatccaa tttgagcatt agatctgccc ccccccctg caggcgtttc
     2101 tttcctatcc ctgatccgag aagcagggtg tagtctaggt ggctggcata cgggattaca
     2161 tcaggcagtg tgtaagttca gctggaactc gattggtaat tgggatggat gattgatgat
     2221 atatatatag cacacactgt tcttgcgtct tgcaaaaaaa aaaaa
```

Figure 2-4B.

Figure 2-5. ZDS protein comparison. ZM, *Zea mays*; CA, *Capsicum annuum*; NP, *Narcissus pseudonarcissus*; AT, *Arabidopsis thaliana*; SS; *Synechocystis*. "*" shows that a position in the alignment is perfectly conserved; "." shows that a position is well conserved; and "-" shows that a position in the alignment is not matched. The bold letters indicate the dinucleotide binding domain.

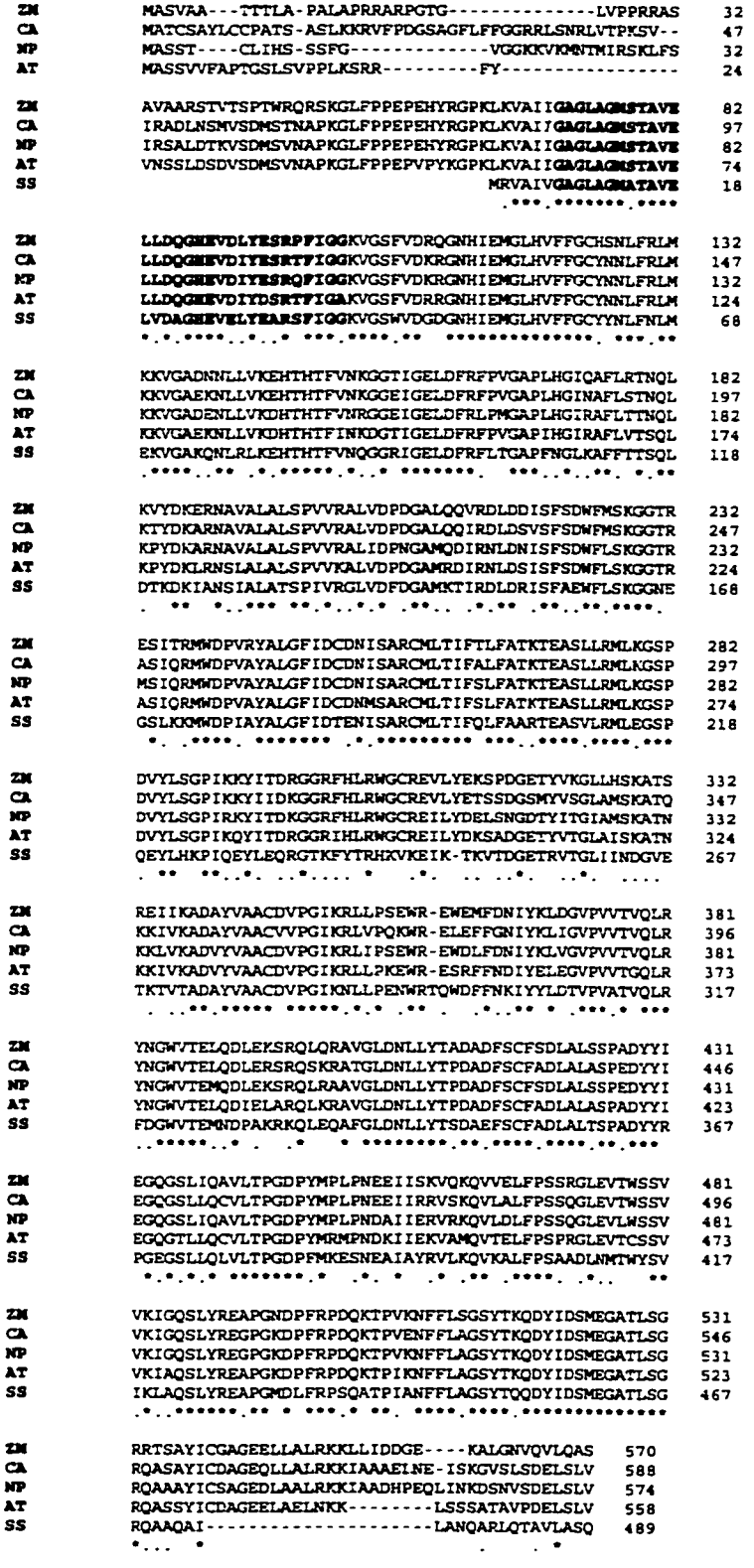


Figure 2-5.

Figure 2-6. Alignments of ZDS amino acids sequences. A, N-terminus and transit peptide of ZDS. B, Sequence comparison of dinucleotide binding domains of ZDS and PDS in higher plants. Asterisks mark identical residues and dots mark similar residues, and the line is a separator of ZDS and PDS. C, Comparison of transit peptide sequence of maize ZDS and PDS (GenBank # U37285). ZM, *Zea mays* (GenBank # AF047490); CA, *Capsicum annuum* (GenBank # 2129927); NP, *Narcissus pseudonarcissus* (GenBank # AJ224683); AT, *Arabidopsis thaliana* (GenBank # U38550); SS; *Synechocystis* (GenBank # D90914)].

A. N-terminus and transit peptide of ZDS

```
ZM          MASVAA---TTTLA-PALAPRRARPGTG--------------LVPPRRAS    32
CA          MATCSAYLCCPATS-ASLKKRVFPDGSAGFLFFGGRRLSNRLVTPKSV--    47
NP          MASST----CLIHS-SSFG-------------VGGKKVKMNTMIRSKLFS    32
AT          MASSVVFAPTGSLSVPPLKSRR---------FY-----------------    24
SS          M                                                     1
            **.          .   .   .

ZM            AVAARSTVTSPTWRQRSKGLFPPEPEHYRGPKLKVAII    70
CA            IRADLNSMVSDMSTNAPKGLFPPEPEHYRGPKLKVAII    85
NP            IRSALDTKVSDMSVNAPKGLFPPEPEHYRGPKLKVAII    70
AT            VNSSLDSDVSDMSVNAPKGLFPPEPVPYKGPKLKVAII    62
SS                                              RVAIV     6
                . . . .*... ...********..*.****.***.
```

B. Comparison of dinucleotide binding domian of ZDS and PDS

```
ZM (ZDS)     71   GAGLAGMSTAVELLDQGHEVDLYESRPFIGG   101
CA (ZDS)     86   GAGLAGMSTAVELLDQGHEVDIYESRTFIGG   116
NP (ZDS)     71   GAGLAGMSTAVELLDQGHEVDIYESRQFIGG   101
AT (ZDS)     63   GAGLAGMSTAVELLDQGHEVDIYDSRTFIGA   93
SS (ZDS)      7   GAGLAGMATAVELVDAGHEVELYEARSFIGG   37
ZM (PDS)    103   GAGLAGLSTAKYLADAGHKPILLEARDVLGG   133
CA (PDS)    117   GAGLGGLSTAKYLADAGHKPILLEARDVLGG   147
NP (PDS)    104   GAGLAGLSTAKYLADAGHKPILLESRDVLGG   134
AT (PDS)     99   GAGLAGLSTAKYLADAGHKPLLLEARDVLGG   129
SS (PDS)      7   GAGLAGLACAKYLADAGFTPVVLERRDVLGG   37
                  ****.*...*..*.*.*........* ..*.
```

C. Comparison of ZDS and PDS transit peptide in maize

```
ZM (ZDS)     1    MASVAATTTL APALAPRRAR PGTGLVPPRR30
ZM (PDS)     1    MDTGCLSSMN ITGASQTRSF AGQLPPQRCF30
                  *                     *

ZM (ZDS)    31   ASAVAARSTV TSPTWRQRSK GLFPPEPEHY      60
ZM (PDS)    31   ASSHYTSFAV KKLVSRNKGR RSHRRHPALQ 60
                  **       *                  *

ZM (ZDS)    61   RGPKL----- ---------- ---------- ------
ZM (PDS)    61   VVCKDFPRPP LESTINYLEA GQLSSFFRNS ERPSKP     96
```

Figure 2-6.

Figure 2-7. Functional complementation of maize *Zds*. A, HPLC analysis of pigments extracted from *E. coli* with pACCRT-EBP (top) or pACCRT-EBP and pMzds107 (bottom); B, spectra of ζ-carotene (Z), neurosporene (N), lycopene (L).

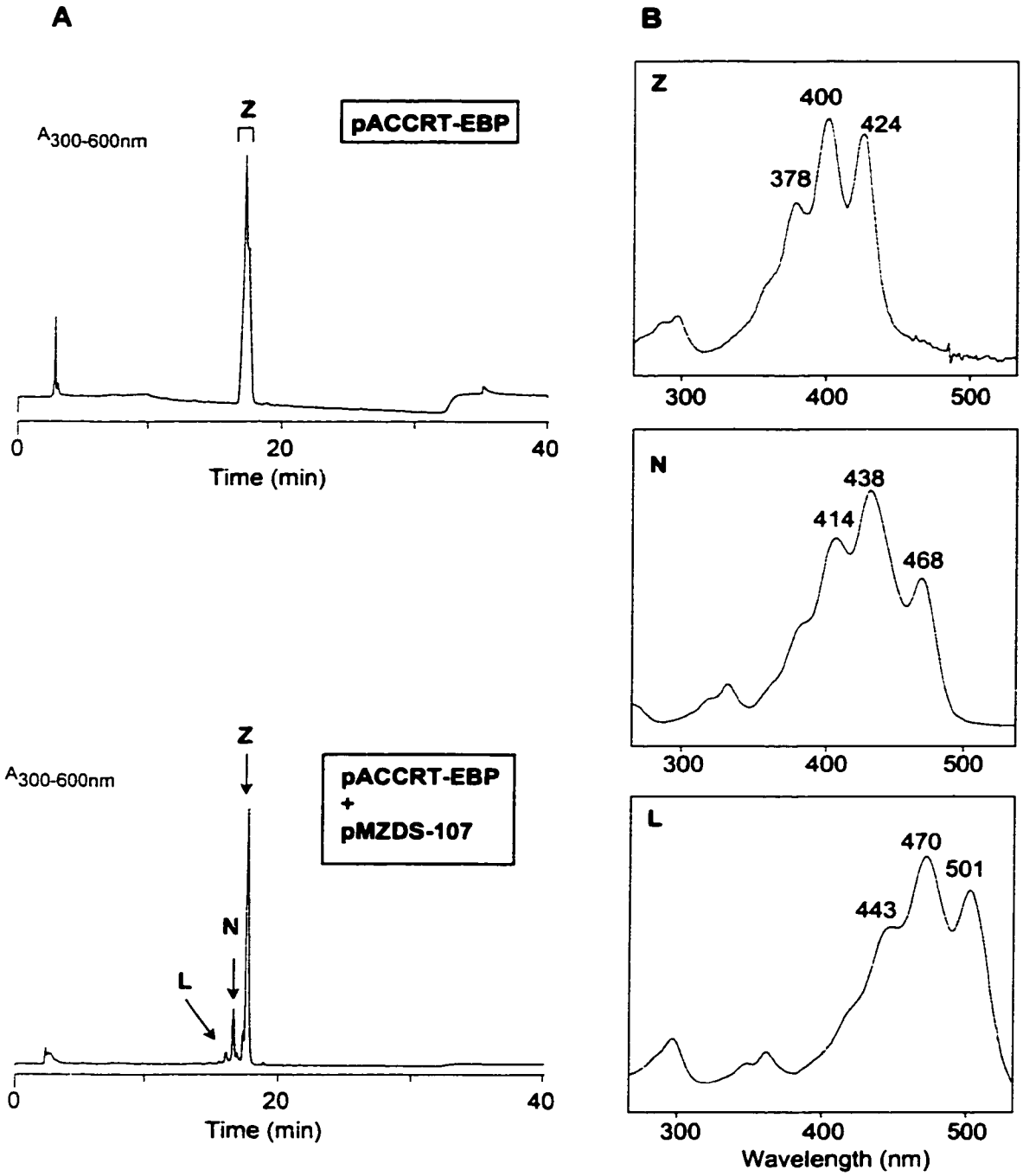


Figure 2-8. Hybridization of maize *zds* to maize B73 genomic DNA digested with *Xho*I (X), *Kpn*I (K), *Hin*dIII (H), *Eco*RI (E), or *Bam*HI (B). M, molecular weight marker.

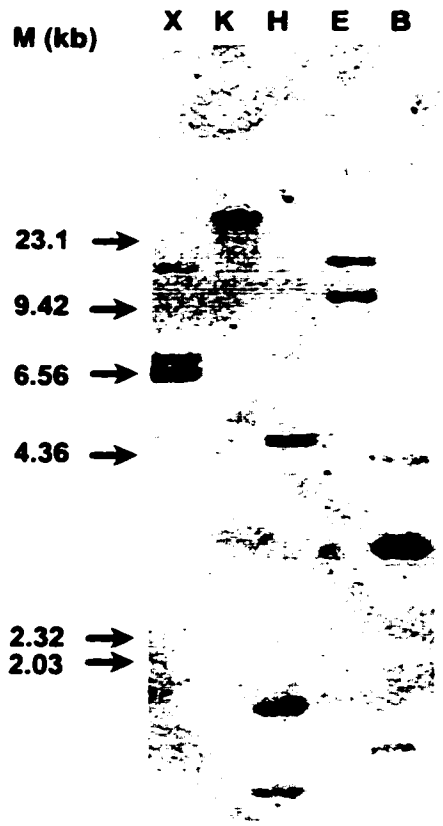


Figure 2-8.

Figure 2-9. Southern hybridization of the rice 417-bp RT-PCR product and the pMzds48 cDNA clone with rice genomic DNA; H: *Hin*dIII, E: *Eco*RI, B: *Bam*HI.

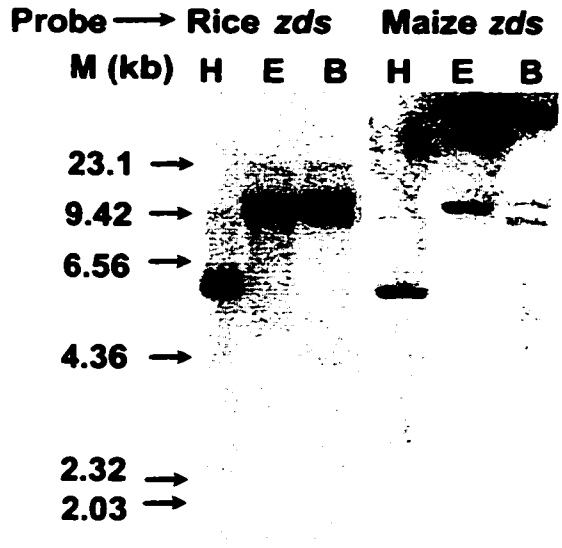


Figure 2-9.

Figure 2-10. RT-PCR of *Zds* and *Pds* from maize mutants *vp-9 mum, y9* and *y8;* and *Zds* RT-PCR amplification control experiments. RT-PCR. A, *Zds* RT-PCR amplification of 1, B73 leaf genomic DNA template; 2, B73 leaf mRNA template; 3, no reverse transcriptase added; 4, forward primer 262 only; 5, reverse primer 279 only. MW: molecular weight marker, 100bp DNA ladder (Gibco BRL). B. RT-PCR of *Zds* and *Pds* from normal and mutant endosperms segregating for genotypes indicated. W, white or light yellow homozygous mutant endosperm; Y, yellow normal endosperm (homozygous or heterozygous for the dominant allele).

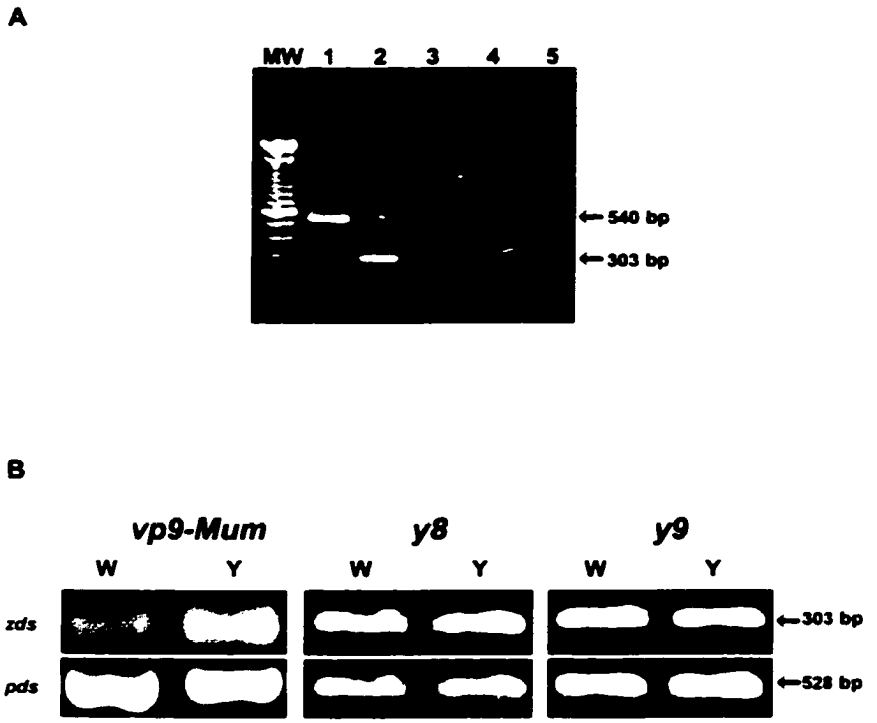


Figure 2-10.

Table 1. R_f value of carotenoids pigments from *vp9-Mum*, *y9* and *y8*.

	Endosperm types	ζ-carotene (R_f values)	Xanthophyll (R_f values)
E. coli (pACCRT- EBP)	—	0.959	—
vp9-Mum (mutant)	Mutant	0.959	—
	Normal	—	0.281
y9 (mutant)	Mutant	0.954	—
	Normal	—	0.279
y8 (mutant)	Mutant	—	—
	Normal	—	0.291

Figure 2-11. HPLC analysis of carotenoids from A, endosperm segregating for *vp9-Mum*, *y9* or *y8*. WK, white or light yellow homozygous mutant endosperm. YK, yellow normal endosperm (homozygous or heterozygous for the dominant allele). B, HPLC analysis of ζ-carotene standard from *E. coli* (pACCRT-EBP) extract. Peak 1, ζ-carotene; peak 2, xanthophyll. A_{400}, absorbance at 400 nm.

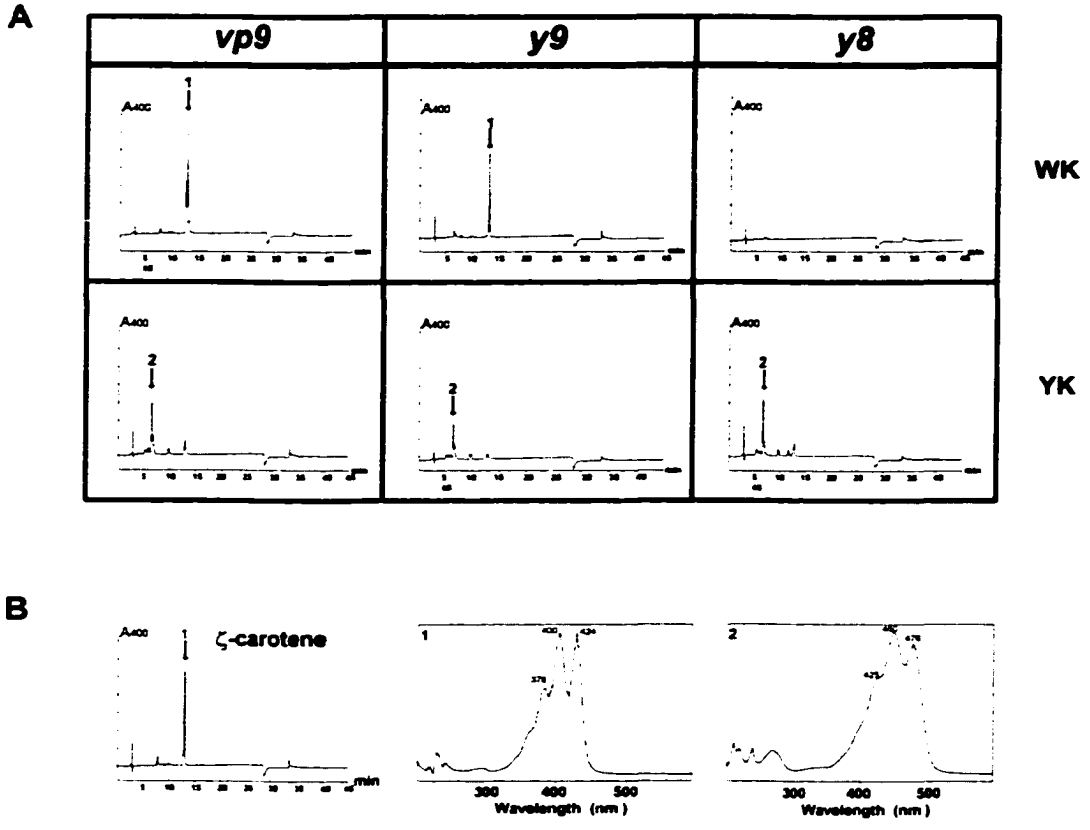


Table 2. Phenotypes of the plants utilized in the chemical complementation experiments.

Maize mutants	Phenotypes of plants supplemented with different concentrations of homogentisic acid (μM)				
	0	1	10	100	1000
vp2	white	white	white	**pale green**	**green**
w3	white	white	white	white	white
vp5	white	white	white	white	white
vp7	pink	pink	pink	pink	pink
vp9	white	white	white	white	white

Chapter 3. Preliminary studies on rice ζ-carotene desaturase cDNA and genomic DNA

3.1 Introduction

As mentioned in chapter 2 (section 2.3.2), the isolation of the maize *Zds* cDNA required characterization of a rice EST clone #S14426 (GenBank # D48291) that showed high homology to dicot ZDS protein. The DNA sequence of this cDNA and a related genomic DNA fragment are presented in this chapter. A 1.6-kb segment of the rice *Zds* cDNA was sequenced, but the 5'-end of this rice *Zds* cDNA was found to be missing. In an attempt to obtain a full length rice *Zds* cDNA, a rice *zds* genomic clone was isolated from a BAC library and partially sequenced.

3.2 Methods

3.2.1. Sequence of the partial rice *Zds* cDNA

The rice *Zds* EST clone S14426 (GenBank # D48291) was double digested with *Sal*I and *Not*I, with release of a 1.6-kb insert. Sequencing of this clone was conducted by primer walking, either manually using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical corporation), or by automated sequencing (Molecular Biology Core, Oregon Regional Primate Research Center, Beaverton, OR 97006).

3.2.2. Studier hybridization, subcloning and partial sequence of a rice *zds* BAC clone

A rice *zds* BAC clone, LM13, was isolated by Dr. Vivek Upasani using the 417 bp rice RT-PCR product as the probe to screen a rice (*Japonica*: variety, Lemont) BAC library which was constructed by using a 7.4 kb pBeloBAC 11 vector [Southern Crop Improvement Facility, Texas A&M University, College Station, TX] (Zhang et al., 1996; Zhang et al., 1997; Zhu et al., 1997). From clone LM13, an 11.3-kb *Eco*RI fragment, pRgzds2, was subcloned by Mr. Anthony Shcherbina. For identification of the 5' end of the *Zds* gene, the 2.2-kb insert of pMzds48 was digested with *Eco*RI followed by *Ava*I. This 2.2-kb *Zds* fragment was cut with *Ava*I into 4 fragments. These *Ava*I fragments were used as probes in a hybridization experiment with the rice *Zds* BAC subclone pRgzds2 immobilized on a nitrocellulose membrane. Random-labeled *Ava*I fragments were boiled in 200 µl 0.5 M NaOH for 15 min, then hybridized with pRgzds2, which was previously digested by *Bam*HI and *Eco*RI and run in a 0.7% (w/v) agarose gel and transferred onto a nitrocellulose membrane. Hybridization conditions were: 10 ml Studier hybridization buffer (0.5 M NaCl, 0.1 M NaH_2PO_4, 0.1 M Tris, 2 mM EDTA, 1% (w/v) SDS), at 68 °C overnight. Then, the nitrocellulose membrane was washed in 2X SSC at room temperature, 15 min, twice; 0.1X SSC-0.1% (w/v) SDS, at 50 °C, 15 min, twice. The images were exposed to a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) for 2 hrs. Sequencing of pRzds6.6 was conducted by primer walking by automated sequencing.

3.3 Results

3.3.1. Sequence of a partial rice *Zds* cDNA and sequence comparison with maize *Zds*

The rice *Zds* EST clone S14426 (GenBank # D48291) sequence available from GenBank was only a partial sequence (518 bp). Therefore, I proceeded to obtain the entire sequence of this rice *Zds* EST and found that it is 1.6-kb. The sequence of this *Zds* clone is shown in Figure 3-1 and deposited into GenBank as # AF054629. This 1.6-kb rice cDNA clone was sent for RFLP mapping by Dr. S. McCouch in Department of Plant Breeding, Cornell University; it was found that rice *zds* mapped to RG477, on chromosome 7.

The nucleotide sequence comparison of the rice *Zds* cDNA versus the maize *Zds* is shown in Figure 3-2. The nucleotide homology between rice and maize *Zds* shows 77.3% identity. Based on the alignment with maize *Zds*, 600-700 bp nucleotides are missing at the 5'-end of the rice *Zds* cDNA clone.

The homology of the predicted amino acid sequence available for rice ZDS compared to the maize ZDS shows a 90.0% identity, and a 92.6% similarity (Figure 3-3). The amino acid homology of between maize and rice ZDS proteins is higher than between maize and other dicot ZDS proteins, which makes sense from an evolutionary standpoint. Based on the comparison between the deduced sequence of the maize and rice ZDS proteins, the putative transit peptide that is essential for plastid targeting, and the dinucleotide binding domain that is crucial for function are missing in the rice ZDS sequence.

3.3.2 Rice *zds* BAC clone 5' mapping, subcloning and partial sequencing

In an attempt to obtain the missing 5' sequence of rice ZDS, I tried to identify the 5'-end using a rice genomic DNA *zds* BAC clone. From the Southern hybridization experiment shown in Figure 2-9, and the 77.3% homology between the maize and rice *Zds* cDNAs and the nucleotide sequence of the incomplete rice *Zds*, it can be predicted that the full-length maize *Zds* cDNA should hybridize with BAC clone pRgzds2, which should contain more of the 5'-end sequence of the rice *Zds*. To properly orient clone pRgzds2, the 2.2-kb insert of pMzds48 was sequentially digested with *Eco*RI and *Ava*I. This 2.2-kb insert possesses three *Ava*I sites, as shown in Figure 3-4. These *Ava*I fragments and the entire insert were used as probes to hybridize with the rice *zds* BAC subclone pRgzds2. As it can be seen in Figure 3-4, probe 1 (the 2.2-kb pMzds48 full insert) hybridized to 6.6 and 3.6 kb; probes 2 and 3 hybridized only with the 6.6-kb fragment while probe 4 hybridized only to the 3.6-kb fragment. Since probes 2 and 3 are located toward the 5'-end, it can be inferred that the 5'-end of the rice *zds* maps to the 6.6-kb *Eco*RI/*Bam*HI fragment of pRgzds2. Therefore this 6.6-kb *Eco*RI/*Bam*HI fragment was subcloned into the *Eco*RI and *Bam*HI sites of pBluescript II SK (–) to construct the clone pRzds6.6. When sequenced using a primer designed from the rice *Zds* EST clone S14426 sequence (GenBank # D48291), pRzds6.6 was shown to overlap 278 bp nucleotides with the EST clone (Figure 3-6B). The pRzds6.6 partial sequence, obtained by primer walking with primer #306 (5'-GATCCCACATCCTTGTGA-3', Appendix IC) is shown in Figure 3-5. Based on a previous restriction map of pRgzds2 and preliminary sequencing of pRzds6.6,

the orientation of the pMzds48 insert to pRgzds2 is shown in Figure 3-4. Clone pRzds6.6, which is 6.6 kb, was expected to contain the missing 5' end of rice *Zds* and its promoter. The alignment of pRzds6.6 and rice EST clone S14426 (Genbank # D48291) is shown in Figure 3-6A, and B. The sequence of pRzds6.6, including the region overlapping with the rice *Zds* EST clone S14426 (GenBank # D48291), was used to conduct a BLAST search by NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). The overlapping region (278 bp) picked up other higher plant ZDS genes, but the remaining sequence (233 bp) of pRzds6.6 appeared to be an intron; this later 233 bp sequence subjected to a BLAST search (using "BLOSUM62" matrix), showed no similarity with any homologous protein sequence (Henikoff, 1992). Therefore, the missing 5' sequence must be further upstream. Based on the sequence of this intron, two primers (primer #324 and primer #325, Appendix IC) were designed, but further sequencing was unsuccessful.

3.3.3 Summary

Preliminary studies on a rice *Zds* EST have provided a partial sequence. Compared with pMzds48 (Figure 3-3), the rice *Zds* is missing the putative transit peptide and the dinucleotide binding domain at the 5'-end, so this rice EST clone S14426 is not a full length cDNA clone, and is not predicted to be functional. Isolation of the genomic DNA BAC clone pRzds6.6, which is 6.6 kb in length has raised the opportunity to obtain the full sequence of the rice *Zds* cDNA, including its promoter sequence.

Figure 3-1. Sequence of the rice *Zds* partial cDNA S14426. A. Nucleotides sequence of the rice *Zds* partial cDNA S14426 (GenBank #AF054629). B. GenBank format of rice *Zds* cDNA S14426.

A

```
   1  CCCACGCGTC CGCCCACGCG TCCGGATTGG TGAACTTGAT TTTCGGTTTC 50
  51  CTGTGGGAGC TCCGTTACAT GGTATCCAAG CATTCCTACG AACTAACCAA 100
 101  CTCAAGGTTT ATGATAAAGC AAGAAATGCC GTTGCTCTTG CTCTAAGCCC 150
 151  AGTTGTTCGA GCTCTTGTTG ATCCAGATGG TGCATTGCAG CAAGTACGGG 200
 201  ATTTGGATGA TGTAAGTTTC AGCGATTGGT TCTTGTCGAA AGGTGGTACT 250
 251  CGAGAGAGCA TCACAAGGAT GTGGGATCCT GTTGCCTATG CTCTTGGTTT 300
 301  CATTGACTGT GATAATATCA GTGCACGTTG CATGCTTACC ATTTTCACTC 350
 351  TGTTTGCCAC AAAAACAGAG GCATCTTTAT TACGCATGCT AAAGGGTTCA 400
 401  CCTGATGTTT ATCTGAGTGG TCCAATAAAG AAGTACATAA CAGACAGGGG 450
 451  TGGTAGGTTT CACCTGAGGT GGGGATGTAG GGAGGTTCTC TATGATAAGT 500
 501  CACCTGATGG GGAAACCTAT GTTAAAGGCC TTCTCCTATC CAAGGCTACA 550
 551  AGTAGAGAGA TAATCAAAGC AGATGCATAT GTCGCAGCTT GTGATGTCCC 600
 601  GGGGATCAAA AGACTTTTAC CTTCTGAATG GAGGCAATGG GATACATTTG 650
 651  ACAACATCTA CAAGTTAGAT GGTGTTCCTG TAGTCACAGT ACAGCTTCGT 700
 701  TATAATGGAT GGGTTACAGA ACTTCAAGAT TTGGAGAAAT CAAGACAACT 750
 751  GAAAAAGGCA GTTGGCTTGG ATAATCTTCT CTACACTCCA GATGCAGATT 800
 801  TTTCATGTTT TTCAGACCTT GCACTTTCAT CTCCTGCTGA CTACTACATT 850
 851  GAAGGACAAG GTTCCTTGAT CCAAGCTGTG CTAACCCCTG GCGATCCTTA 900
 901  CATGCCATTG CCGAATGAGG AGATAATTAG CAAGGTTCAA AAGCAGGTCT 950
 951  TAGAATTGTT CCCGTCATCA CAAGGCTTGG AACTTACATG GTCGAGTGTG 1000
1001  GTGAAAATCG GTCAATCATT GTACCGCGAG TCACCAGGAA ATGATCCATT 1050
1051  TAGACCTGAT CAAAAGACAC CAGTTAAAAA CTTCTTCCTG TCTGGCTCTT 1100
1101  ACACAAAACA GGACTACATT GACAGCATGG AAGGGGCAAC TCTCTCAGGC 1150
1151  AGGAGAACCG CGGCCTACAT CTGTGGTGCA GGAGAGGAGC TGCTTCGCCC 1200
1201  TCCGAAAGAA GCTCATTGTC GACGACAGCG GAGAAGGCCA GGGGTAAGGT 1250
1251  CGACGGCCCT TCAGACAAGC TGAGCTTCCT CAAATGACAC ATGCTGGAGT 1300
1301  GAGTGGATTG CTATGCCCAA AACAAAAACA GCTTCCTGGG TGTAGTAGGC 1350
1351  GATTTCCGCA GCGACTCTCA TGTAAATCCT ACTTGATTGA GCATTTAGGT 1400
1401  CCAATCTGCT GCTGCCCTTT TTGCCTTGAC ACGATCGTTC GTTCGCCCGT 1450
1451  CAATGGTGTG TTCTTCGTTA TTGTGAATTT GTGATTGGGA ACCAAAGGTG 1500
1501  GCATACGGGA TTACATCAGG CAGCGTGTGT TTTGTTCAGC TTAACCGATC 1550
1551  ATTGAACCCA TTGATGATGA TGATGATGTT TATATAGTGC ACACATCACT 1600
1601  TAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AA 1632
```

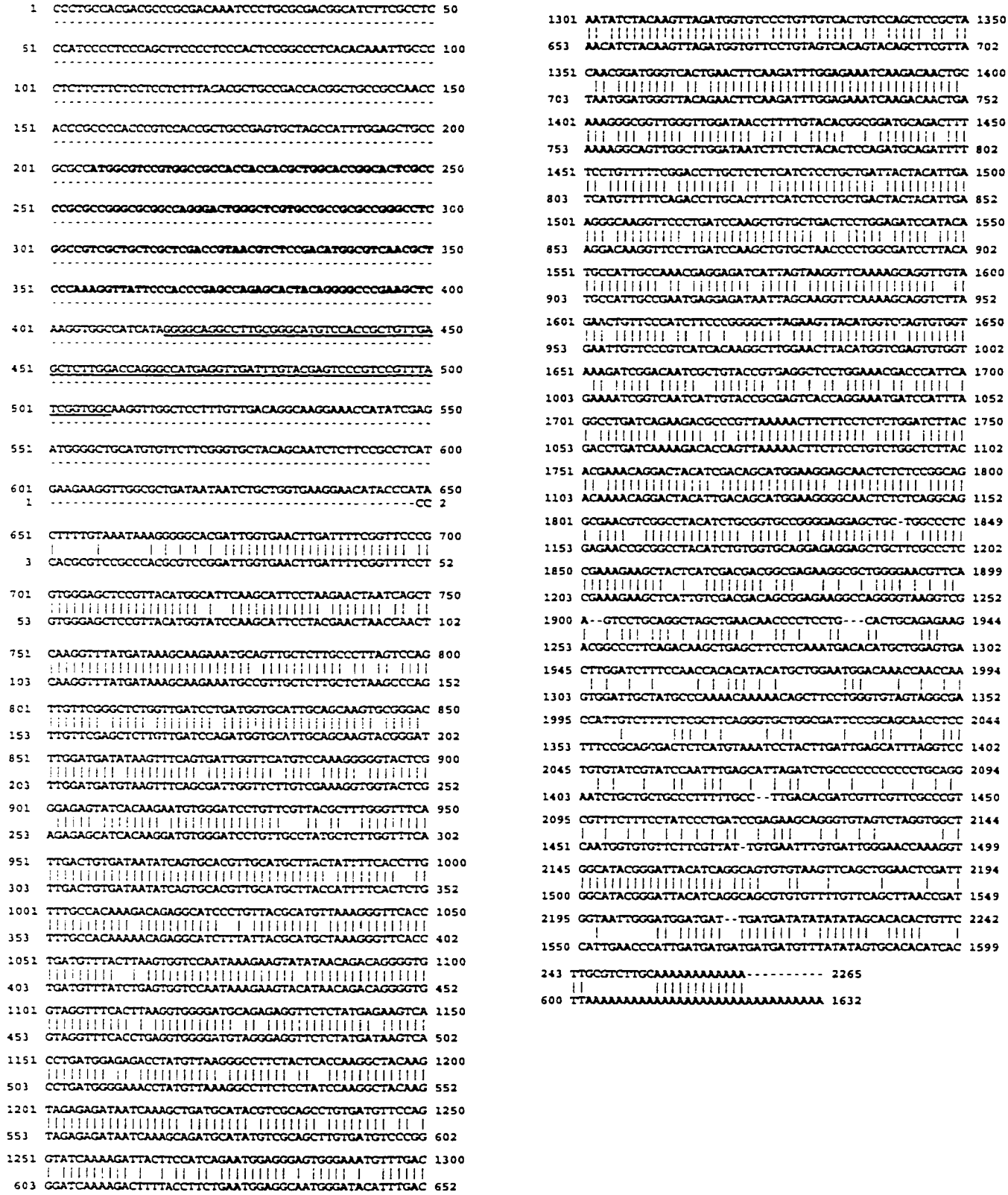
Figure 3-1A.

B

```
LOCUS       AF054629     1632 bp    RNA             PLN      01-JUL-1998
DEFINITION  Oryza sativa zeta-carotene desaturase precursor (zds) gene,partial
    cds.
ACCESSION   AF054629
SOURCE      Oryza sativa;
ORGANISM    Oryza sativa
            Eukaryota; Viridiplantae; Charophyta/Embryophyta group;
            Embryophyta; Tracheophyta; seed plants; Magnoliophyta;
Liliopsida; Poales; Poaceae; Oryza.
REFERENCE   1  (bases 1 to 1632)
  AUTHORS   Luo,R.B. and Wurtzel,E.T.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-MAR-1998) Biological Sceince, Lehman, 250 Bedford Park Blvd.
           West, Bronx, NY 10468, USA
FEATURES      Location/Qualifiers
  source      1..1632
              /organism="Oryza sativa"
              /cultivar="Nipponbare"
              /db_xref="taxon:4530"
     gene     <1..1273
              /gene="zds"
     CDS      <1..1273
              /gene="zds"
              /codon_start=2
              /product="zeta-carotene desaturase precursor"
              /translation="PRVRPRVRIGELDFRFPVGAPLHGIQAFLRTNQLKVYDKARNAV
              ALALSPVVRALVDPDGALQQVRDLDDVSFSDWFLSKGGTRESITRMWDPVAYALGFID
              CDNISARCMLTIFTLFATKTEASLLRMLKGSPDVYLSGPIKKYITDRGGRFHLRWGCR
              EVLYDKSPDGETYVKGLLLSKATSREIIKADAYVAACDVPGIKRLLPSEWRQWDTFDN
              IYKLDGVPVVTVQLRYNGWVTELQDLEKSRQLKKAVGLDNLLYTPDADFSCFSDLALS
              SPADYYIEGQGSLIQAVLTPGDPYMPLPNEEIISKVQKQVLELFPSSQGLELTWSSVV
              KIGQSLYRESPGNDPFRPDQKTPVKNFFLSGSYTKQDYIDSMEGATLSGRRTAAYICG
              AGEELLRPPKEAHCRRQRRRPGVRSTALQTS"
BASE COUNT      452 a    345 c    390 g    445 t
ORIGIN
    1 cccacgcgtc cgcccacgcg tccggattgg tgaacttgat tttcggtttc ctgtgggagc
   61 tccgttacat ggtatccaag cattcctacg aactaaccaa ctcaaggttt atgataaagc
  121 aagaaatgcc gttgctcttg ctctaagccc agttgttcga gctcttgttg atccagatgg
  181 tgcattgcag caagtacggg atttggatga tgtaagtttc agcgattggt tcttgtcgaa
  241 aggtggtact cgagagagca tcacaaggat gtgggatcct gttgcctatg ctcttggttt
  301 cattgactgt gataatatca gtgcacgttg catgcttacc attttcactc tgtttgccac
  361 aaaaacagag gcatctttat tacgcatgct aaagggttca cctgatgttt atctgagtgg
  421 tccaataaag aagtacataa cagacagggg tggtaggttt cacctgaggt ggggatgtag
  481 ggaggttctc tatgataagt cacctgatgg ggaaacctat gttaaaggcc ttctcctatc
  541 caaggctaca agtagagaga taatcaaagc agatgcatat gtcgcagctt gtgatgtccc
  601 ggggatcaaa agacttttac cttctgaatg gaggcaatgg gatacatttg acaacatcta
  661 caagttagat ggtgttcctg tagtcacagt acagcttcgt tataatggat gggttacaga
  721 acttcaagat ttggagaaat caagacaact gaaaaaggca gttggcttgg ataatcttct
  781 ctacactcca gatgcagatt tttcatgttt ttcagacctt gcactttcat ctcctgctga
  841 ctactacatt gaaggacaag gttccttgat ccaagctgtg ctaacccctg gcgatcctta
  901 catgccattg ccgaatgagg agataattag caaggttcaa aagcaggtct tagaattgtt
  961 cccgtcatca caaggcttgg aacttacatg gtcgagtgtg gtgaaaatcg gtcaatcatt
 1021 gtaccgcgag tcaccaggaa atgatccatt tagacctgat caaaagacac cagttaaaaa
 1081 cttcttcctg tctggctctt acacaaaaca ggactacatt gacagcatgg aaggggcaac
 1141 tctctcaggc aggagaaccg cggcctacat ctgtggtgca ggagaggagc tgcttcgccc
 1201 tccgaaagaa gctcattgtc gacgacagcg gagaaggcca ggggtaaggt cgacggccct
 1261 tcagacaagc tgagcttcct caaatgacac atgctggagt gagtggattg ctatgcccaa
 1321 aacaaaaaca gcttcctggg tgtagtaggc gatttccgca gcgactctca tgtaaatcct
 1381 acttgattga gcatttaggt ccaatctgct gctgcccttt ttgccttgac acgatcgttc
 1441 gttcgcccgt caatggtgtg ttcttcgtta ttgtgaattt gtgattggga accaaaggtg
 1501 gcatacggga ttacatcagg cagcgtgtgt tttgttcagc ttaaccgatc attgaaccca
 1561 ttgatgatga tgatgatgtt tatatagtgc acacatcact taaaaaaaaa aaaaaaaaaa
 1621 aaaaaaaaaa aa
```

Figure 3-1B.

Figure 3-2. Comparison of maize and rice *Zds* nucleotide sequences. Top, pMzds48 nucleotide sequence (GenBank # AF047490); bottom , rice EST *Zds* sequence (GenBank # AF054629). Bold letters are nucleotides in pMzds48 encoding the putative transit peptide; underlined letters are nucleotides encoding the putative dinucleotide binding domain of maize *Zds*.



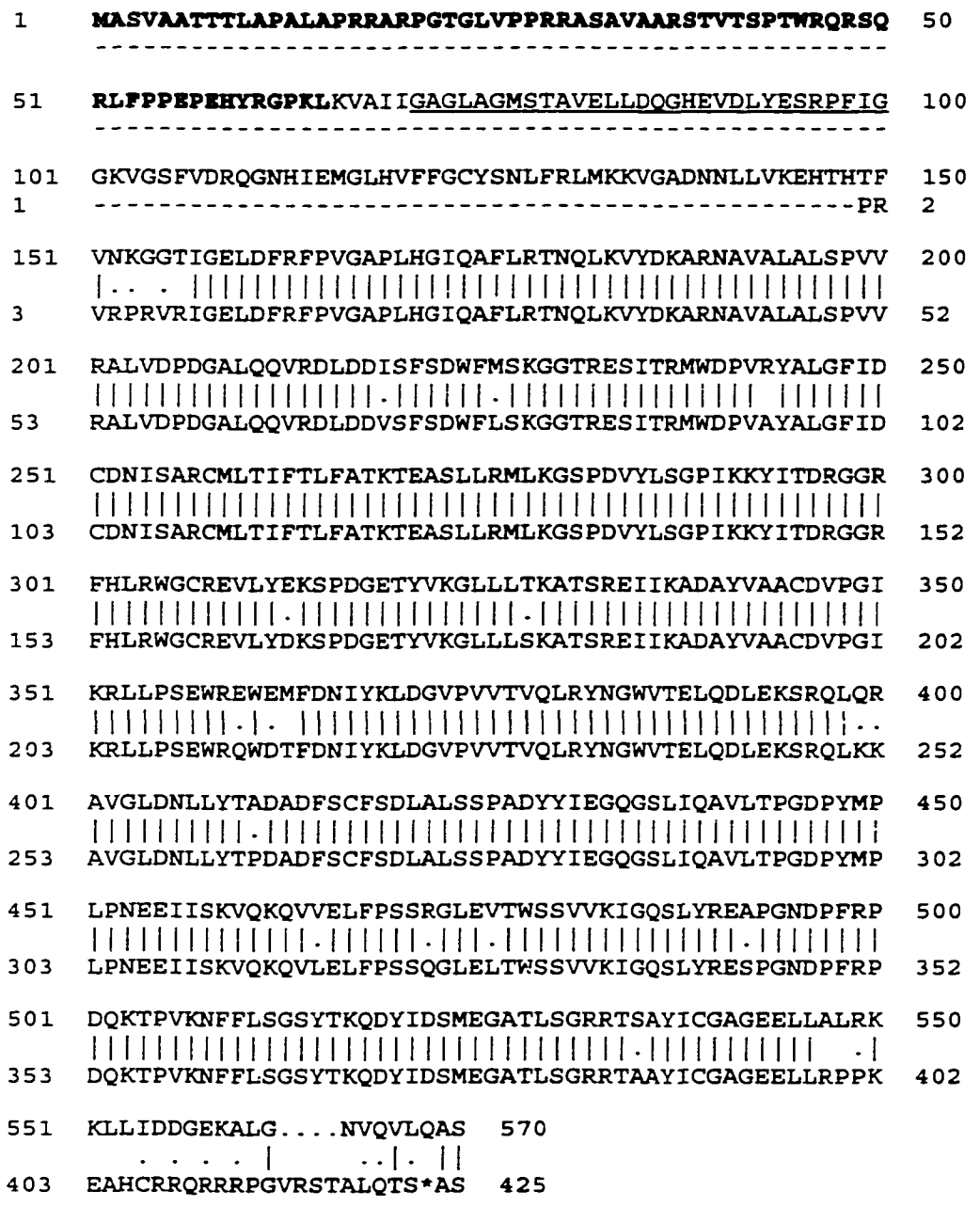


Figure 3-3. Comparison of maize and rice ZDS predicted amino acid sequences. Top, pMzds48 sequence; bottom, rice *Zds* sequence. Bold letters mark the putative transit peptide; underlined letters indicate the putative dinucleotide binding domain of maize *Zds*. "|" indicate identical residues and "." indicate similar residues.

Figure 3-4. Hybridization of *Ava*I-derived probes of maize *Zds* cDNA restriction fragments with the rice genomic DNA BAC clone pRgzds2. A, *Ava*I; B, *Bam*HI; E, *Eco*RI. Probe 1-4 are restriction fragments, indicate on the bottom left map. Probe 4, consisting of 1,368-bp from the 3' end of maize *Zds* cDNA, is actually a mixture of two *Ava*I fragments, 720-bp and 648-bp. The arrow shows direction of transcription, based on the hybridization results of lower right panel.

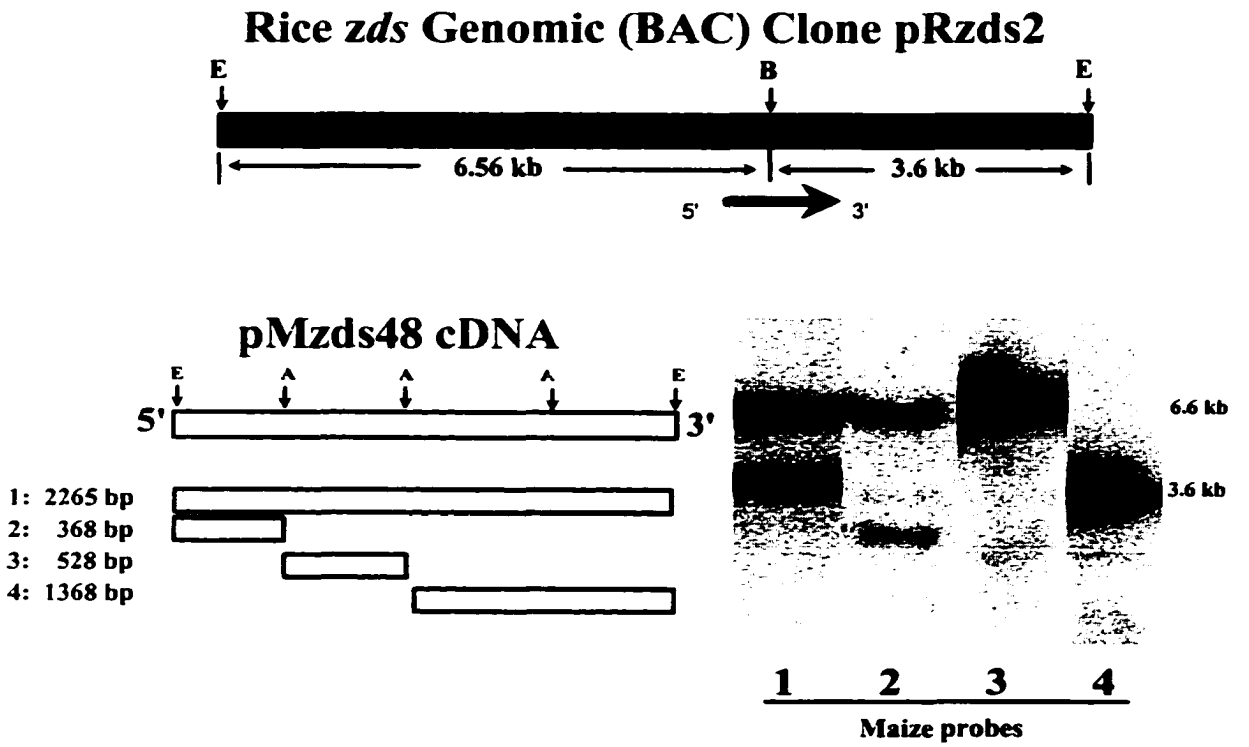


Figure 3-4.

```
  1  GATCCCACAT CCTTGTGATG CTCTCTCGAG TACCACCTTT CGACAAGAAC CAATCGCTGA
 61  AACTTACATC ATCCAAATCC CGTACTTGCT GCAATGCACC ATCTGGATCA ACAAGAGCTC
121  GAACAACTGG GCTTAGAGCA AGAGCAACGG CATTTCTTGC TTTATCATAA ACCTTGAGTT
181  GGTTAGTTCG TAGGAATGCT TGGATACCAT GTAACGGAGC TCCCACAGGA AACCGAAAAT
241  CAAGTTCACC AATCCGGACG CGTGGGCAGA TCGCAGTGGT TAATCCTTAT AGAATCCTTA
301  ACAAATATGC TTATCACCCA TAAATCTTCA ACTTGAACTA TAACAACGAT TTACAACAAT
361  CCATATCACA TTCTATCTGG TGACATGGCC ATCAAGAACT ATCTACGCCA CTTGCATATA
421  TACACCCGTA GGAAGACCCC ACTTTCTTTT GATTCATGAC ATTCCATCCC CTGAATCCTC
481  CTCCTTTGAT TTATCACCTT CTGCACTATT C
```

Figure 3-5. Sequence (511 bp) of the *zds* genomic DNA clone pRzds6.6 (GenBank # AF086803). The underlined nucleotides indicate primer #306 (Appendices IC) used to obtain the pRzds6.6 sequence which actually began at nucleotide 31. Sequence in bold (nt 1-nt 278) represents the region overlapping with the rice *Zds* cDNA sequence (GenBank #AF054629, nt1-nt 278). Sequence from nt 279 to nt 511 represents an intron.

Figure 3-6. The alignment of pRzds6.6 and rice EST clone S14426 (GenBank # D48291). A, The cartoon of the alignment of the pRzds6.6 and the rice EST clone S14426. Dark box is exonic region, and open box denotes intronic region. B, Sequence of the alignment of pRzds6.6 and rice EST clone S14426. The underlined and bolded nucleotides indicates primer #306.

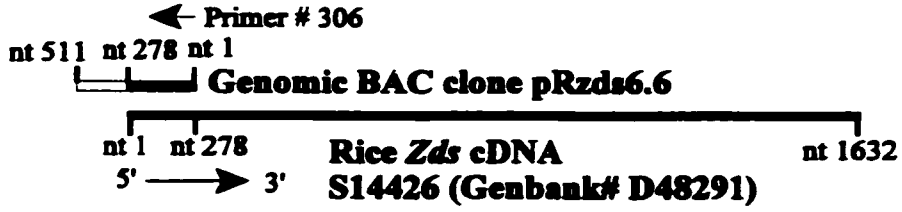


Figure 3-6A.

```
          427          417          407          397          387
S14426    TATTGGACCA CTCAGATAAA CATCAGGTGA ACCCTTTAGC ATGCGTAATA

          377          367          357          347          337
S14426    AAGATGCCTC TGTTTTTGTG GCAAACAGAG TGAAAATGGT AAGCATGCAA

          327          317          307          297          287
S14426    CGTGCACTGA TATTATCACA GTCAATGAAA CCAAGAGCAT AGGCAACAGG
pRzds6.6                                                      G

          277          267          257          247          237
S14426    ATCCCACATC CTTGTGATGC TCTCTCGAGT ACCACCTTTC GACAAGAACC
pRzds6.6  ATCCCACATC CTTGTGATGC TCTCTCGAGT ACCACCTTTC GACAAGAACC
          2            12           22           32           42
          Primer#306→

          227          217          207          197          187
S14426    AATCGCTGAA ACTTACATCA TCCAAATCCC GTACTTGCTG CAATGCACCA
pRzds6.6  AATCGCTGAA ACTTACATCA TCCAAATCCC GTACTTGCTG CAATGCACCA
          52           62           72           82           92

          177          167          157          147          137
S14426    TCTGGATCAA CAAGAGCTCG AACAACTGGG CTTAGAGCAA GAGCAACGGC
pRzds6.6  TCTGGATCAA CAAGAGCTCG AACAACTGGG CTTAGAGCAA GAGCAACGGC
          102          112          122          132          142

          127          117          107          97           87
S14426    ATTTCTTGCT TTATCATAAA CCTTGAGTTG GTTAGTTCGT AGGAATGCTT
pRzds6.6  ATTTCTTGCT TTATCATAAA CCTTGAGTTG GTTAGTTCGT AGGAATGCTT
          152          162          172          182          192

          77           67           57           47           37
S14426    GGATACCATG TAACGGAGCT CCCACAGGAA ACCGAAAATC AAGTTCACCA
pRzds6.6  GGATACCATG TAACGGAGCT CCCACAGGAA ACCGAAAATC AAGTTCACCA
          202          212          222          232          242

          27
S14426    ATCCGGACGC GTGGGCGGAC GCGTGTG
pRzds6.6  ATCCGGACGC GTGGGCAGAT CGAAGTGGTT AATCCTTATA GAATCCTTAA
          252          262          272          282          292

pRzds6.6  CAAATATGCT TATCACCCAT AAATCTTCAA CTTGAACTAT AACAACGATT
          302          312          322          332          342

pRzds6.6  TACAACAATC CATATCACAT TCTATCTGGT GACATGGCCA TCAAGAACTA
          352          362          372          382          392

pRzds6.6  TCTACGCCAC TTGCATATAT ACACCCGTAG GAAGACCCCA CTTTCTTTTG
          402          412          422          432          442

pRzds6.6  ATTCATGACA TTCCATCCCC TGAATCCTCC TCCTTTGATT TATCACCTTC
          452          462          472          482          492

pRzds6.6  TGCACTATTC
          502
```

Figure 3-6B.

Chapter 4. Expression of maize ζ-carotene desaturase in *E. coli*.

4.1 Introduction

To study ZDS protein expression, ZDS-specific antibodies would be useful for study of the carotenoid biosynthetic pathway. To raise maize ZDS-specific antibodies, the maize *Zds* cDNA was cloned into a pET vector. Expression of *Zds* will be presented in this chapter.

4.2.Methods

4.2.1 Production of a maize ZDS fusion protein in a pET vector

pMzds48 (see restriction enzyme map in Figure 2-1) was digested with *Xba*I, filled in with Klenow, then digested with *Spe*I. A 347-bp fragment was removed from pMzds48 by using the Exonuclease III and Mung Bean Nuclease Deletion Kit (Stratagene, La Jolla, CA) to form the construct pMzds348. pMzds348 was double digested with *Not*I and *Xho*I, and ligated into the pET23a vector (Novagene, Inc. Milwaukee, WI) *Not*I-*Xho*I site. This construct was designated as pET23a-Mzds348 (Figure 4-1). In pET23a-Mzds348, the maize *Zds* fragment is in frame with the T7 promoter of the pET23a vector, and the maize ZDS can be expressed as a fusion protein with a T7·Tag (the 11 amino acids of the T7 gene 10 peptide) at the N-terminal. pET23a-Mzds348 was maintained in the non-expression host *E. coli* NovaBlue. For expression of the maize ZDS fusion

protein, the expression host *E. coli* BL21 (DE3) pLysS was transformed with the pET23a-Mzds348 construct. For expression of the fusion protein, a single colony was picked up from a freshly streaked plate and inoculated in to 2 ml LB containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol, incubated with shaking at 37 °C until the A_{600} reached 0.6 to 1.0. The culture was stored at 4 °C overnight; cells were collected by centrifugation for 30s in a microcentrifuge (14,000 rpm), resuspended in 2 ml of fresh LB medium with the same antibiotics, and inoculated into 50 ml medium until A_{600} reached 0.6. Samples were removed from an uninduced control, and IPTG added to a final concentration of 1 mM. Cells (1 ml) were collected by centrifugation at 30 min intervals up to 4 hrs following IPTG addition. Cell pellets were resuspended in 50 μl sample buffer (0.125M Tris, pH 6.8; 3% [v/v] glycerol; 4 % [w/v] SDS; 0.2 M DTT; 0.001% [w/v] bromophenol blue) and frozen at -20 ° C. After samples from all time points were collected, they were boiled for 3 min, and 10 μl was loaded on a 10% SDS-PAGE mini-gel (0.75 mm thick x 80 mm wide x 50 mm high) in 1X running buffer (25 mM Tris, 192 mM glycine and 1% (w/v) SDS) at 25 mA. Proteins were transferred to an nitrocellulose membrane in transfer buffer (25 mM Tris; 192 mM glycine; and 20% [v/v] methanol) at 750 mA at 4°C for 2 hours. Western analysis was carried out by using as a primary antibody, a T7·Tag monoclonal antibody (diluted 1:7500) (Novagen, Inc.) , and as a secondary antibody, an anti-mouse alkaline phosphatase-conjugated IgG (diluted 1:8000) (Promega).

4.3 Results

4.3.1 Expression of maize ZDS in *E. coli*

To overexpress maize ZDS in *E. coli* and obtain enough recombinant maize ZDS protein to raise antibodies for studying the levels of maize ZDS protein during plant development, pMzds48 was subcloned in-frame (Figure 4-1) into the pET23a expression vector (Novagen, Madison, WI) which contains a T7 promoter and T7·Tag (the 11 amino acids of the T7 gene 10 peptide) (Figure 4-1). A T7·Tag antibody is a commercially available mouse monoclonal antibody directed against the gene 10 product, so it can detect the expression of the maize ZDS fusion protein.

The expected size of the maize ZDS fusion protein encoded by the pET23a vector was 60.8 kDa. A protein of around 60.8 kDa was detected by the T7 monoclonal antibody in Western blots (Figure 4-2). This result shows that maize ZDS can be expressed in pET23a-Mzds348 under the T7 promoter. This western blot shows the expression of maize ZDS in *E. coli* whole-cell extracts, but analysis of inclusion body or supernatants fractions, gave similar patterns (data not shown). From the coomassie brilliant blue stained SDS-PAGE gel, the fusion protein was not detected. Hence, these conditions were not optimal for purifying the ZDS protein needed for antibody production.

4.4 Discussion

In order to raise maize ZDS specific antibodies, the maize *Zds* clone was introduced into an appropriate *E. coli* host. As shown in Figure 4-1, maize *Zds* was indeed in-frame with T7 promoter of pET23a. The size detected for the fusion protein was expected to be 60.8

kDa because of 2.9 kDa from vector sequences represented by aa# 1 to 26 from pET23a (including 11 aa from T7·Tag and 13 aa from the multiple cloning site) and 2 aa from pBluescript II SK (-), plus 57.9 kDa (523 aa) expected from ZDS (aa# 48 to aa# 570. In reality, a ZDS fusion protein about the expected size was produced in the host cell *E. coli* BL21 (DE3) pLysS (Figure 4-2). Although the amount of antigen was approximately constant during the time course of IPTG-induction (30 min to 240 min), the total cellular protein increased over time, so the relative amount of the fusion protein decreased over time. This result suggests that, although maize ZDS can be expressed in the pET system, the host *E. coli* cell does not overexpress the fusion protein under the condition used. It might be worth to try to optimizing ZDS expression by growing the induced culture at temperatures less than 37 °C to get to soluble, active ZDS protein. It is worth trying to transform pET23a-Mzds348 into a different expression host cell line to induce the over expression of maize ZDS. It might also be worth trying other expression vectors, such as pGEX, with control of a *tac* promoter (Amersham, Piscatway, NJ) to express the entire protein as a fusion with glutathione S-transferase. In daffodil, it was shown that PDS and PSY can be overexpressed in insect cells, and corresponding antibodies generated (Al-Babili et al., 1996b; Schledz et al., 1996); this could also be done with the maize *Zds* for the overproduction of the ZDS fusion protein.

Figure 4-1. Map and junction sequence of pET23a-Mzds348. A. Autoradiography of the DNA sequence at the junction between the *Zds* cDNA and pET23a vector of pET23a-Mzds348. The arrow indicates the first "G' in the *Zds* cDNA derived from pMzds348. B, map of the pET23a-Mzds348 construct; C, nucleotide and amino acid sequences of pET23a-Mzds348, at the junction of insert and vector. Bold nucleotides (without underline) mark the region of the T7 promoter; underlined (nonbold) nucleotides mark the region encoding the T7 gene 10 peptide; bold and underlined nucleotides or amino acids mark the sequence of pMzds348. In the ZDS fusion protein construct 5' end, there are 72 nt (nt 239 to nt 167) from the pET23a vector and 6 nt (bold and italicized nucleotides) derived from pBluescript II SK(-) when the *Zds* cDNA was cut out from the parent plasmid pMzds48. Arrow indicates the direction of transcription. Nonbold numbers indicate nucleotide numbering system from pET23a vector while bold number indicates nucleotide numbering from maize *Zds* (GenBank # AF047490).

A

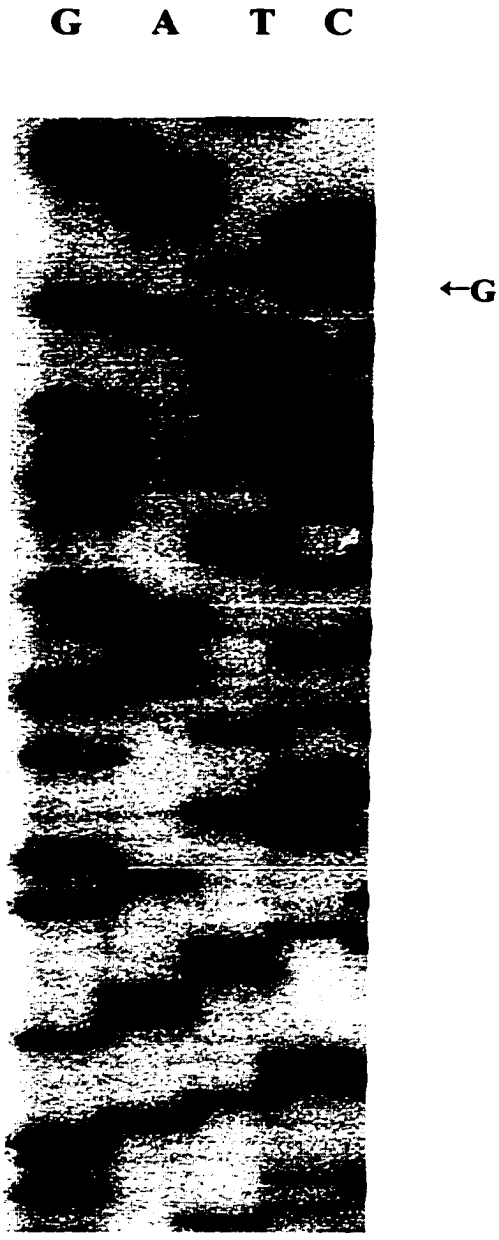
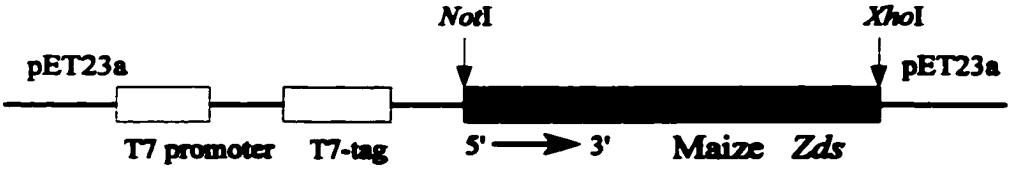


Figure 4-1A.

B



C

```
AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG AGACCACAAC GGTTTCCCTC
                      T7 promoter
                                                239          229
TAGAAATAAT TTTGTTTAAC TTTAAGAAGG AGATATACAT ATGGCTAGCA TGACTGGTGG
                                             MetAlaSerMet ThrGlyGly
                                                   T7·Tag
219          209          199          189          179          169          348
ACAGCAAATG GGTCGCGGAT CCGAATTCGA GCTCCGTCGA CAAGCTTGCG GCCGCTCTAG
 GlnGlnMet GlyArgGlySer GluPheGlu LeuArgArg GlnAlaCysGly ArgSerSer 117

CTCCCAAAGG TTATTCCCA
 SerGlnArg LeuPhePro 223
    Maize  Zds
```

Figure 4-1B,C.

Figure 4-2. Times course of ZDS fusion protein expression. Western blot probed with T7·Tag antibody. T7·Tag is 31 kDa positive control supplied by Novagen, Inc. Proteins were extracted from *E. coli* cells at times after IPTG induction as indicated.

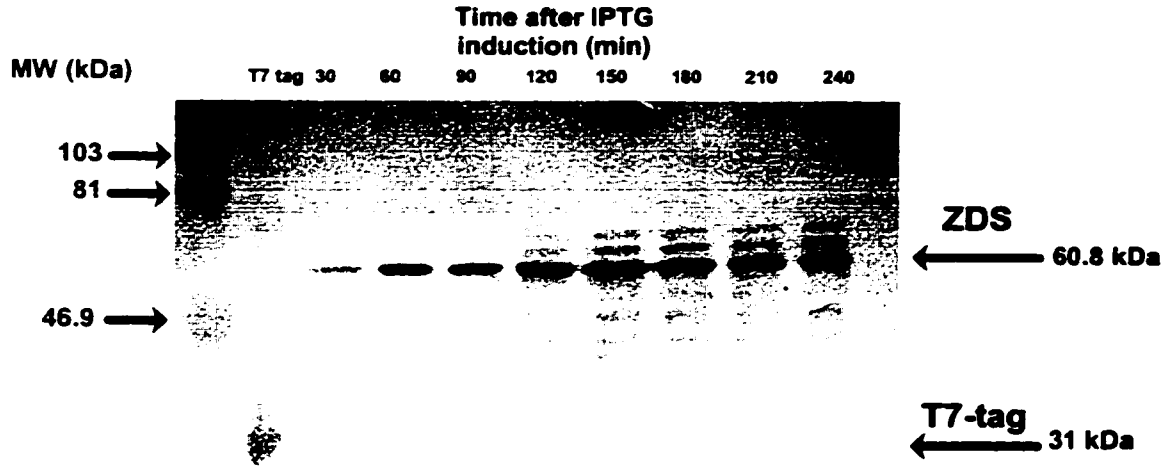


Figure 4-2.

Chapter 5. A viviparous rice mutant is blocked in carotenoid biosynthesis

5.1 Abstract

A rice mutant was reported that showed embryo vivipary combined with an albino seedling phenotype. Such a phenotype is typical of mutations affecting carotenoid accumulation. To test this possibility, pigments were extracted from endosperm, embryo, and leaves of a viviparous albino rice mutant and subjected to HPLC (High Pressure Liquid Chromatography) analysis. It was found that in embryo and albino seedlings, a carotenoid intermediate, phytoene, accumulated. This suggested that the viviparous mutation was due to a block in the carotenoid biosynthetic pathway at the phytoene desaturation step. This first rice mutant found with a block in the carotenoid pathway will be useful for studies of the rice carotenoid biosynthetic pathway.

5.2 Introduction

Carotenoids are a structurally diverse group of pigments; they function as accessory pigments in photosynthesis, as photoprotectors preventing photooxidative damage, and as precursors to the plant hormone, abscisic acid (ABA) (Cunningham and Gantt, 1998; Robertson et al., 1966). Mutations blocking the pathway lead to accumulation of intermediates and typically have pleiotropic phenotypic effects. For example, vivipary or precocious embryo germination is a consequence of a carotenoid-derived ABA

deficiency; the lack of photoprotective carotenoids in green tissue leads to an albino phenotype when mutant seedlings germinate in high light and chlorophyll is destroyed (Robertson, 1975; Neill et al., 1986). Albino mutants are not always carotenoid deficient, since blocks in chlorophyll biosynthesis would also lead to an albino phenotype. The albino mutants that are defective in carotenoid biosynthesis can be distinguished from those affecting chlorophyll biosynthesis, since at low light carotenoid deficient seedlings will accumulate chlorophyll. Furthermore, mutations specifically blocking the chlorophyll pathway do not exhibit a viviparous phenotype, whereas carotenoid mutants do, especially if a biosynthetic intermediate accumulates. Lastly, viviparous mutants that give rise to green seedlings are apparently defective only in ABA biosynthesis or ABA perception.

In an effort to study the regulation of carotenoid accumulation in rice, I searched for putative carotenoid mutants. Of interest were mutants that exhibited vivipary combined with an albino seedling phenotype. Such mutants were described (Yatou and Iida, 1994) and I decided to test whether they accumulated any intermediates of the carotenoid biosynthetic pathway. One candidate was identified and I report that it represents the first rice mutant defective in carotenoid biosynthesis on the basis of accumulation of a pathway intermediate in leaf and embryo tissue.

5.3 Materials and Methods

5.3.1 Plant materials

A mutant of *Oryza sativa* L., 84NMEMdr2 (Yatou and Iida, 1994) that segregated viviparous seeds which germinated into albino seedlings, were grown in the greenhouse with supplemental lighting at Lehman College, CUNY. Albino seedlings were collected and stored at -80°C until used. Endosperms and embryos were dissected from mature seeds.

5.3.2 Carotenoid extraction and HPLC analysis

Albino leaf, embryo and endosperm derived from mutant seeds segregating from self-pollination of a plant heterozygous for the mutation 84NMEMdr2 were used for extraction of carotenoids under low light using a modified method (Britton, 1985; Fraser et al., 1994; Yatou and Iida, 1994). Because of the easily discerned albino plant phenotype, all leaves were homozygous for the mutation, whereas embryos and endosperm were from a mixture of seeds, approximately 25% of which were homozygous for the mutation. One g of frozen tissue (stored at -80°C) was ground in liquid N_2, suspended in 10 mL methanol and centrifuged at 9000 x g, for 10 min at 4°C. After addition of 1 mL 60% (w/v) KOH, the supernatant was heated at 65°C for 20 minutes. The mixture was extracted three times in 10% (v/v) diethylether in hexane, the organic phase evaporated under N_2, dissolved in 700 µL methanol and stored in amber vials at -20°C. A Waters HPLC system with 600 controller and pump, 996 Photodiode Array

Detector, and WISP 717 autosampler were used to separate samples by reverse phase chromatography on a Spherisorb-ODS (5 μ, 250x4.6 mm) column, eluted with acetonitrile:methanol:isopropanol (85:10:5, v/v/v) (Sandmann et al. 1993) at a flow rate of 1 mL/min. Peaks were identified by spectrophotometric profiles and retention times and comparison with authentic standards.

5.4. Results and discussion

Evidence of a carotenoid mutant of rice was suggested by the phenotype of a mutant, 84NMEMdr2, described by Yatou and Iida (Yatou and Iida, 1994), for which viviparous seeds germinated into albino seedlings. Such a phenotype is characteristic of the maize viviparous mutants such as *vp5*, *vp2*, and *w3;* these recessive mutations condition a white endosperm/albino seedling phenotype as compared to the normal yellow endosperm/green seedling phenotype (Robertson, 1975). In those mutants, seeds are viviparous because of the absence of the carotenoid precursors of ABA, needed to promote seed dormancy late in seed development; seedlings are albino because absence of photoprotective carotenoids leads to photodestruction of chlorophyll and abnormal chloroplast development (Robertson et al., 1978). As shown in Figure 5-1A, the HPLC chromatogram of a leaf extract prepared from a rice plant homozygous for this mutant allele, contained a major peak (peak 1), identified as phytoene based on the characteristic spectral profile shown in Figure 5-1B. Phytoene is the first compound specific to the carotenoid biosynthetic pathway and is synthesized by the enzyme phytoene synthase

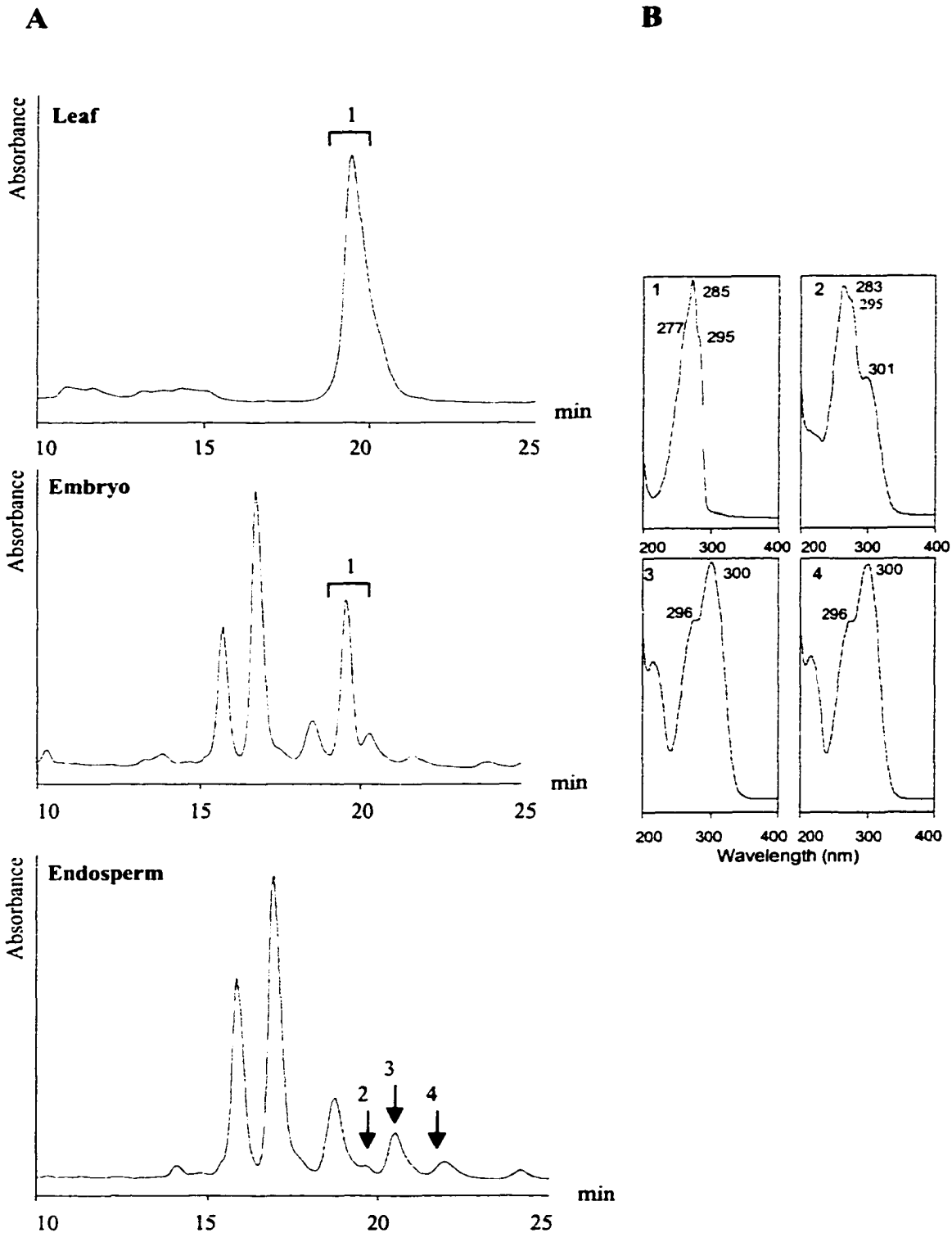
(Cunningham and Gantt, 1998).

Pigments were then extracted from embryo and endosperm dissected from mature seeds obtained from a self-pollinated plant heterozygous for the 84NMEMdr2 mutation. The extracted compounds were analyzed by HPLC and compared with that of the mutant leaf tissue. For lack of a visible phenotype, it was not possible to separate the predicted 25% homozygous mutant seeds from normal seeds in a segregating population. However, phytoene could still be detected in extracts prepared from dissected embryos, but not from endosperms. As shown in Figure 5-1A, the carotenoid intermediate phytoene (peak 1) was detected in embryo and leaf extracts, but not in endosperm extracts. Peaks 2-4 detected in the endosperm extracts, had similar retention times as for peak 1, but were not identified as phytoene because their spectrophotometric profiles, shown in Figure 5-1B, did not match that of phytoene (peak 1). HPLC analysis was conducted for another rice mutant 90KHEMdr1; none of the precursors in the carotenoid pathway were found in either albino leaves, embryo or endosperm (data not shown).

The viviparous phenotype of the 84NMEMdr2 rice mutant was apparently caused by a block in phytoene desaturation leading to a deficiency in the carotenoid precursor of ABA. It is surprising that phytoene only evidenced in embryo and leaf tissue, and not in endosperm. Either the mutation is tissue-specific or the endosperm produces no carotenoid whatsoever, such that even under conditions where the pathway is blocked, no intermediates are detected.

In summary, I describe the first rice mutant defective in carotenoid biosynthesis; the accumulation of phytoene suggests that the pathway is blocked in the desaturation of phytoene to ζ-carotene (Li et al., 1996) which is mediated by the enzyme phytoene desaturase (PDS). The desaturation is a redox reaction linked to an electron transport chain involving plastoquinones (Norris et al., 1995). Therefore, the block in carotenoid biosynthesis could represent a defect in a component required for function or expression of PDS or for plastoquinone biosynthesis.

Figure 5-1. HPLC separation of pigments extracted from leaf, embryo and endosperm of rice mutant 84NMEMdr2. (A) Eluted peaks detected within 200-600 nm; (B) Spectrophotometric profiles of all peaks eluting near phytoene (peak 1). Numbers correspond to peaks in (A).



Chapter 6. Summary and future prospects of the work

6.1 Maize and rice ζ-carotene desaturase

Using various molecular and biochemical techniques, I report the successful isolation of a 2.2-kb maize cDNA (pMzds48) encoding ZDS. An open reading frame of 2,060 bp encodes a protein with a calculated molecular weight of 63.07 kDa. The predicted amino acid sequence of maize ZDS includes an N-terminal transit peptide of 64 residues and a dinucleotide binding domain of 31 residues. After expression in *E. coli* cells accumulating ζ-carotene, maize ZDS was active and could, albeit inefficiently, catalyze the conversion of ζ-carotene into neurosporene and lycopene. A construct for expression of a maize ZDS fusion protein was prepared. Southern analysis and RFLP mapping of maize and rice *zds* indicated that there might be only one copy of *zds* in the maize and rice genomes. RT-PCR together with TLC and HPLC analyses suggest that maize *zds* is more likely to be encoded by *vp9* and not by *y8*. A 1.6-kb cDNA *Zds* clone from rice was sequenced; and a rice *zds* genomic DNA BAC clone was also obtained.

6.2 Mutants that are blocked in the carotenoid biosynthetic pathway

Since the desaturation steps leading to biosynthesis of carotenoids are linked to a respiratory redox pathway using plastoquinone as an electron carrier, homogentisic acid (HGA), a key intermediate in the biosynthesis of tocopherol and plastoquinone, was

treated for rescue of mutants *vp2, w3, vp5, vp7 and vp9* albino seedlings. Only *vp2* mutant plants were rescued with HGA, demonstrating that the *vp2* locus plays a role in the biosynthesis of plastoquinones needed for the carotenoid desaturation steps. HPLC analysis of the rice mutant, 84NMEMdr2, showed that it was blocked in the desaturation of phytoene to ζ-carotene.

6.3 Future prospects of the work

Many carotenoid biosynthetic genes have been isolated and characterized in higher plants. Significant advances have been made in understanding the regulation of the carotenoid biosynthetic pathway in higher plants. On the other hand, genes encoding carotenoid biosynthetic enzymes, especially in rice and maize, still need to be isolated and studied. Because the rice *Zds* cDNA is incomplete, more sequencing of the 6.6-kb rice genomic BAC clone pRzds6.6 provides the opportunity for obtaining the missing 5'-end of a rice *Zds* cDNA and for identifying the promoter region of rice *Zds* which would be useful for further study of *Zds* gene regulation. It might be worthwhile to obtain the full length of rice cDNA by 5'-RACE based on sequence of the partial rice cDNA sequence. Since it was recently found that pepper ZDS functions as a monomer when using lipophilic quinones as a cofactor (Breitenbach, et al., 1999), it would be interesting to purify an high active maize or rice ZDS (with the full length cDNA) and study enzyme kinetics and role of cofactors such as FAD, NAD, NADP or lipophilic quinones. The full-length maize *Zds* cDNA would be useful for studying the ZDS active site by site-specific

mutagenesis. Since transcripts of *Psy* and *Pds* have been detected in rice leaf, embryo and endosperm (Wurtzel et al., 1996), it would be interesting to determine the *Zds* transcript level in different specific tissues of rice, especially in the endosperm. Similarly the *Zds* RNA levels could be determined in developing endosperm as correlated with carotenoid accumulation. The maize *Zds* expression plasmid, pET23a-Mzds48, will aid ZDS antibody development for future investigations of ZDS protein accumulation profiles in maize and rice.

Whether the carotenoid biosynthesis pathway is shut down completely in rice endosperm still remains unknown. The expression levels of *Zds* RNA and protein in rice can help to determine the existence of enzymes needed for the steps up to and including ζ-carotene desaturation. ZDS antibodies could also be used to localize ZDS in plastids, particularly in the endosperm amyloplast, by immunocytochemical methods. Since it has been proposed that ZDS with PDS and LCY form a multienzyme complex (Cunningham and Gantt, 1998), differential fluorescent labeling of PDS, ZDS and LCY antibodies can directly provide evidence of the existence of the complex. With the goal of improving carotenoid contents of cereal endosperms by genetic engineering, the maize *Zds* cDNA together with other cDNAs such as maize *Pds*, can be utilized for stable transformation in genetic engineering in cereals.

Maize *zds* mapping, HPLC and RT-PCR experiments on the maize mutants *vp9*, *y8* and *y9* may indicate that *vp9* most probably encodes maize ZDS. It is more reasonable that

vp9 is a *zds* locus since *vp9* but not *y8* mutant tissues can accumulate ζ-carotene. Southern hybridization can be performed by using maize *Zds* cDNA as probe to detect if there is any DNA level differences between *vp9* mutant and its normal endosperm. Maize ZDS antibodies, whenever available, can be used to detect whether there is a low level of ZDS protein in the *vp9* mutant embryo and endosperm, compared to the normal embryo and endosperm. Using transposon tagging, it is possible to isolate *vp9* cDNA, and further confirms if *vp9* encodes ζ-carotene desaturase.

Since the gene for OHPP dioxygenase (GenBank accession no. AF000228) had been isolated in *Arabidopsis* (Norris et al., 1998), based on its sequence information, further screening of the maize cDNA library can lead to isolation of the full length maize OHPP dioxygenase cDNA. More investigations are needed to test whether the gene product of *vp2* is OHPP dioxygenase.

HPLC analysis was used to characterize the rice mutant 84NMEMdr2. It is the first viviparous rice carotenoid mutant that has ever been identified. Since it accumulates phytoene in the mutant embryo and leaf, its phytoene desaturase transcripts and protein levels can be compared with the mutant and normal tissues. It also might be worth using chemical complementation, like in *vp2*, to rescue the rice mutant 84NMEMdr2.

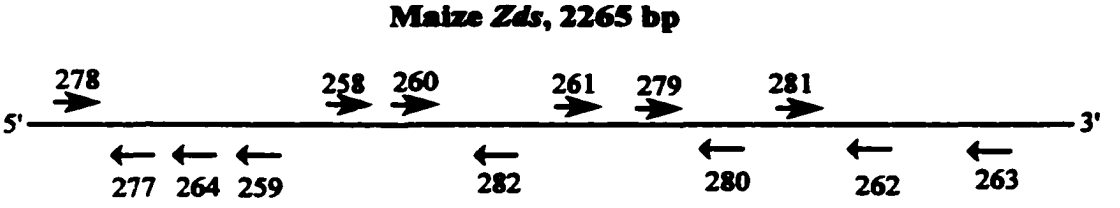
Appendices

IA. Primers.

Lab primer No.	Primer sequence	Description	Page & Figure cited
202 (forward)	5'-GCATTCCTACGAACTAACCAACTCAAGG-3'	rice EST *Zds* clone (GenBank # D482910), nt 57-nt 84, for RT-PCR	pp. 35
203 (reverse)	5'-GGTGCTTACATAGAGACCTCCCTACACC-3'	rice EST *Zds* clone (GenBank # D4829), nt 447-nt 474, for RT-PCR	pp. 35
279 (forward)	5'-GGTTGGGTTGGATAACCTT-3'	Maize *Zds* (GenBank # AF047490), nt 1408-nt 1426, for RT-PCR	pp. 38 & Figure 2-10
262 (reverse)	5'-TCTGATCAGGCCTGAATG-3'	Maize *Zds* (GenBank # AF047490), nt 1695-nt 1712, for RT-PCR	pp. 38 & Figure 2-10
110 (forward)	5'-GGAACTGTGAAACACTTCGC-3'	Maize *Pds* (GenBank # U37285), nt 1377-nt 1396, for RT-PCR	pp. 38 & Figure 2-10
111 (reverse)	5'-GAAACCTTCGATAGGTGACC-3'	Maize *Pds* (GenBank # U37285), nt 1904-nt 1885, for RT-PCR	pp. 38 & figure 2-10
306	5'-GATCCCACATCCTTGTGA-3'	Rice pRzds6.6 (GenBank # AF086803), nt 150-nt 167, for sequencing	pp. 77, Figure 3-5, 3-6B

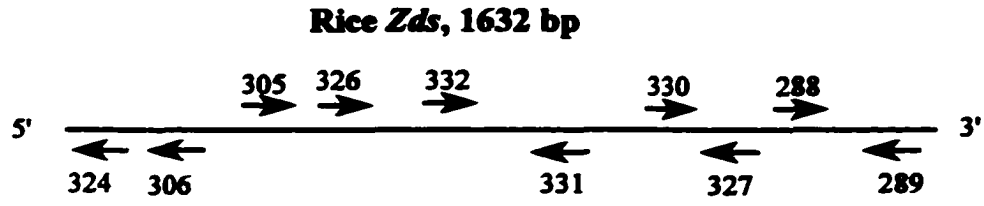
*nt: nucleotide

IB. Primers used for sequencing the maize *Zds* (GenBank # D482910).



Lab primer No.	Primer sequence	Description Maize *Zds* (GenBank # AF047490)
278 forward	CACAAATTGCCCCTCTTCTT	nt 89-nt 108
277 reverse	CATGTCGGAGACGTTACG	nt 322-nt 339
264 reverse	CAAGAAGAGGAGAGGCCGGA	nt 333-nt 370
259 reverse	CGATATGGTTTCCTTGCCTGTC	nt 527-nt 548
258 forward	CTCTGGTTGATCCTGATGGTGCAT	nt 810-nt 833
260 forward	TGTTTGCCACAAAGACAGAG	nt 999-nt 1018
282 reverse	CAGGTGAACCCTTTAACAT	nt 1034-nt 1052
261 forward	GAAAGATAATCAAAGCTGATG	nt 1203-nt 1223
279 forward	GTTGGGTTGGATAACCTT	nt 1409-nt 1426
280 reverse	ACAGTTCTACAACCTGCT	nt 1590-nt 1607
281 forward	GTGTGGTAAAGATCGGACAA	nt 1644-nt 1663
262 reverse	TCTGATCAGGCCTGAATG	nt 1695-nt 1712
263 reverse	GTTTGTCCATTCCAGCATGT	nt 1967-nt 1970

IC. Primers used for sequencing the rice *Zds* (GenBank # AF054629).



Lab primer No.	Primer sequence	Description Rice *Zds* (GenBank #AF054629)
325	GGAATGCTTGGATACCATGT	nt 67- nt 86
324 reverse	GCAACGGCATTTCTTGCTTT	nt 116-nt 135
306 reverse	GTACCCACATCCTTGTGA	nt 261-nt 278
305 forward	ATTGCAGCAAGTACGGGATT	nt 184-nt 203
326 forward	TGCTTACCATTTTCACTCTGT	nt 333 -nt 353
332 forward	CTGATGGGGAAACCTATG	nt 504-nt 521
331 reverse	TCTCCTCATTCGGCAATG	nt 907-nt 924
330 forward	GTGAAAATCGGTCAATCATTG	nt 1004-nt 1024
327 reverse	CACCCAGGAAGCTGTTTT	nt 1328-nt 1345
288 forward	GGGTGTAGTAGGCGATTT	nt 1345-nt 1362
289 reverse	GCAGATTGGACCTAAATGC	nt 1398-nt 1416

IIA. Constructs.

Constructs	Description	Chapter cited	Page No.	GenBank accession no.
pMzds48	maize *Zds*, 2.2-kb cDNA	2	40	# AF047490
pMzds-107	construct for the maize *Zds* function analysis (106 nt deleted)	2	Not shown	—
pACCRT-EBP	construct carries *Erwinia crtE*, *crtB* and maize *Pds*	2	52, Figure 2-7	—
pMzds348	construct for maize *Zds* fusion protein expression	2	Not shown	—
pET23a-Mzds348	construct for maize *Zds* fusion protein expression	2	91, Figure 4-1	—
pRzds6.6	rice *Zds* genomic DNA clone, derived from BAC clone LM13, subclone pRgzds2	2	Not shown	# AF086803

IB. Clone sheets for above.

1. pMzds48

Clone Number/Name: MZDS (48) (maize *Zds* cDNA, renamed as pMzds48)

Constructed by: RuiBai Luo
Purified by: RuiBai Luo
Date constructed: 5/ 12 /1996
(MONTH)/ (DAY)/ (YEAR)
Storage Location-
Box Number: 3 Position: 5-I

Clone description: Sequence of pMzds48 is GenBank # AF047490 and reported in Luo thesis as Figure 2-4. Cloning strategy: Using 417 bp rice RT-PCR (Based on nt 57-474 rice EST clone S14426 (Genbank # D48291) as product as probe screened maize λgt11 cDNA library. Subcloned into pBluescript II SK (-), *EcoR* I site (a full length cDNA clone)
Cited in journal: See Luo thesis as Figure 2-4
Cloning vector used: pBluescript II SK (-) Vector size: 2.96 kb
Organism source of gene: maize * Insert size: 2265 bp
Restriction enzyme(s) to release insert: *Eco*RI
Lab Notebook to reference: Ruibai's note #1.
Concentration: 0.5 µg/ul
E. coli Antibiotic markers: X amp; __tet; __ chloramphenicol; (50 µg/ml Amp)
Strain Transformed into: XL-1Blue Frozen glycerols, strain box # 7-5-E

*Reference for the λgt11 cDNA library: Fontes., et al., 1991, Plant Cell, Vol 3, pp.463-496)

2. pMzds-107

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

Constructed by: RuiBai Luo
Purified by: RuiBai Luo
Date constructed: 7/ 3/ 1997
(MONTH)/ (DAY)/ (YEAR)

Storage Location-
Box Number: 3 Position: 6 B

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

3. pACC-EBP

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

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

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

4. pMzds348

Clone Number/Name: Mzds348 (Start from 347 nt of Maize ZDS cDNA)

Constructed by: RuiBai Luo
Purified by: Ruibai Luo
Date constructed: 8 (MONTH)/ /18 (DAY)/ /1997 (YEAR)
Storage Location-
Box Number: 3 Position: A-10

Clone description: pMzds48 (GenBank # AF047490) was digested by *Xba*I,filled in by Klenow, then digested by *Spe*I, 5' deleting 347 bp into Mzds348. (*Xba*I and *Spe*I are in the MCS). Junction sequence:ctagc|tccaaa=vector|mzds348
Cited in journal: Luo thesis, chapter 4
Cloning vector used: pBluescript II SK (-) Vector size: 2.96 kb
Organism source of gene: maize Insert size: 1918 bp
Restriction enzyme(s) to release insert: *Not*I and *Xho*I
Lab Notebook to reference: Ruibai #1, #2.
Concentration: 0.05 µg/ul
E. coli Antibiotic markers: X amp; __tet; __ chloramphenicol; (50 µg/ml Amp)
Strain Transformed into: XL-1 Blue Frozen glycerols, strain box # 7-6-D

5. pET23a-Mzds348

Clone Number/Name: pet23a-mzds348 (maize *zds* expression clone)

Constructed by: RuiBai Luo
Purified by: Ruibai Luo
Date constructed: 8/ (MONTH)/ 18/ (DAY)/ 1997 (YEAR)
Storage Location-
Box Number: 3 Position: 7C

Clone description: pMzds48 (GenBank # AF047490) was digested by *Xba*I, filled in by Klenow, then digested by *Spe*I, 5' deleting 347bp into Mzds348. The insert was released by *Not*I and *Xho*I , and insert into pet 23a *Not*I-*Xho*I site. (Junction sequence :tgc|ggc,cgc, tct, agc, tcc, caa, agg=pet 23a/zds)
Cited in journal: Luo thesis, chapter 4, Figure 4-1.
Cloning vector used: pET23a Vector size: 3.666 kb
Organism source of gene: maize Insert size: 1918 bp
Restriction enzyme(s) to release insert: *Not*I and *Xho*I
Lab Notebook to reference: Ruibai #1, #2.
Concentration: 0.05 µg/ul
E. coli Antibiotic markers: X amp; __tet; __ chloramphenicol; (50 µg/ml Amp)
Strain Transformed into: NovaBlue Frozen glycerols, strain box # 7-4-I

6. pRzds6.6

Clone Number/Name: RZDS6.6 (Rice *Zds* genomic DNA, renamed as pRzds6.6)

Constructed by: Ruibai Luo
Purified by: Ruibai Luo
Date constructed: 8 (MONTH) / 22 (DAY) /1997 (YEAR)
Storage Location-
Box Number: 3 Position: B7

Clone description: A rice *zds* BAC clone, LM13, was isolated by Dr. Vivek Upasani using the 417 bp rice RT-PCR product as the probe to screen a rice (*Japonica*: variety, Lemont) BAC library which was constructed by using a 7.4 kb pBeloBAC 11 vector [Southern Crop Improvement Facility, Texas A&M University, College Station, TX]. From clone LM13, an 11.3-kb *Eco*RI fragment, pRgzds2, was subcloned by Anthony Shcherbina. pRgZDS2 (rice bac clone "# 84" by Anthony) was digested by *Bam*HI, and *Eco*RI, the 6.6 kb fragment was released, and it was subcloned into *Bam*HI-*E co*RI site of pBluescript II SK (-), Genbank# AF086803.
Cited in journal: **BAC-library**: Zhang, H. B., Choi, S., Woo, S S., Li, Z., and Wing, R. A. (1997). Construction and characterization of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. Mol Breeding *2*, 11-24. Luo thesis chapter 3, Figure 3-5, 3-6
Cloning vector used: pBluescript II SK (-) Vector size: 2.96 kb
Organism source of gene: rice Insert size: 6.6 kb
Restriction enzyme(s) to release insert: *Bam*HI and *E co*RI
Lab Notebook to reference: Antoiny #7.
Concentration: 0.2 µg/ul
E. coli Antibiotic markers: X amp; __tet; __ chloramphenicol; (50 µg/ml Amp)
Strain Transformed into: XL-1 Blue Frozen glycerols, strain box # 7-6-C

III. Plant materials, field information, genotypes and phenotypes.

Table 3. Plant materials for TLC and HPLC analyses (Chapter 2).

Maize mutants	Field No.	Genotype	Endosperm phenotype	Plant phenotype
vp9-Mum	97044 *(self)*	*vp9-Mum/B73*	segregates yellow and pale yellow endosperm	segregates green and albino seedlings
y12	97054-10 (sib)	*y12/y12*	pale yellow endosperm	green seedlings
y8	GH98006 (sib)	*y8/y8*	yellow endosperm	green seedlings

IV. Table 4. Plant materials for chemical complementation experiments (Chapter 2).

Maize mutants	Field No.	Genotype	Endosperm phenotype	Plant phenotype
vp2	95001-16 (self)	*vp2*/+ (B73)	segregates white and yellow endosperm	segregates green and albino seedlings
vp5	95024 (self)	*vp5*/+ (B73)	segregates white and yellow endosperm	segregates green and albino seedlings
vp7	95037 (self)	*vp7*/+	segregates pink and yellow endosperm	segregates green and albino seedlings
w3	95034 (self)	*w3*/+ (B73)	segregates white and yellow endosperm	segregates green and albino seedlings
vp9-Mum	95030 (self)	*vp9-Mum*/+ (B73)	segregates yellow and pale yellow endosperm	segregates green and albino seedlings
y9	95025 (self)	*y9*/+ (B73)	segregates yellow and pale yellow endosperm	green seedlings

V. Figures and file descriptions.

Figure #	Original data file name	Figure file name	Experiment date	File description	Notebook #
1-1a, b		1-1a.wpd, 1-1b.wpd		carotenoid biosynthetic pathway	
1-2		1-2.wpd		structure of lycopene and β-carotene	
1-3		1-3.wpd		plastoquinone biosynthetic pathway	
1-4		1-4.wpd		maize mutants & carotenoid pathway	
2-1		2-1.wpd		map of pMzds48	1
2-2		2-2.wpd		map of pACCRT-EBP	1
2-3	619971.tif	2-3.wpd	June 19,1997	sequencing the junction of pMzds107	1
2-4a,b	mzdstral.txt	2-4a.wpd, 2-4b.wpd	Oct. 11, 1997	sequence of pMzds48	1
2-5	zdsagn4.txt	2-5.wpd	Mar. 17,1998	ZDS protein sequence comparison	1
2-6		2-6.wpd		N-terminal transit peptide comparison	1
2-7	hplc1.ppt	2-7.ppt	Oct 16., 1997	functional complementation of pMzds107	6
2-8	rzds.prs	2-8.wpd	June,1996	southern hybridization	1
2-9	rsms.prs	2-9.wpd	Nov.,1996	southern hybridization	1

Figure #	Original data file name	Figure file name	Experiment date	File description	Notebook #
2-10	RT-PCR.prs	2-10.wpd	Feb10 &18.,1998	RT-PCR of maize mutant *vp9-mum, y8, & y9*	5
2-11	HPLC2.PRS	2-11.ppt	Jan. 19, 1998	HPLC analysis of mutant *vp9, y8, & y9*	6
3-1a, b		3-1a.wpd, 3-1b.wpd		sequence of rice *Zds* cDNA S14426	
3-2		3-2.wpd		nucleotide sequence comparison of *Mzds* & *Rzds*	
3-3		3-3.wpd		amino acid sequence comparison of MZDS & RZDS	
3-4	bacsou.prs	3-4.prs	Aug. 17, 1997	studier hybridization	7 (Antoniy's)
3-5	rzdsbac.seq	3-5.wpd	Sep., 1997	sequence of pRzds6.6	3
3-6a, b		3-6a.wpd 3-6b.wpd		sequence alignment of pRzds6.6 with rice *Zds* cDNA S14426	
4-1	816971.tif	4-1.wpd	Aug. 16, 1997	sequencing the junction of pET23a-Mzds348	1
4-2	wpet.prs	4-2.wpd	Dec. 14, 1997	ZDS expression in *E. coli*	2
5-1	HPLC3.prs	5-1.ppt	Mar. 2, 1995	HPLC analysis of rice mutant 84NMEMdr2	6

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