INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Localization and Expression of Carotenoid Biosynthetic Enzymes in Endosperms of *Zea mays* and *Oryza sativa*

By

Jia Yu

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

1999

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

UMI Number: 9917719

Copyright 1999 by
Yu, Jia

All rights reserved.

UMI Microform 9917719
Copyright 1999, by UMI Company. All rights reserved.

This microform edition is protected against unauthorized
copying under Title 17, United States Code.

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

© 1999

JIA YU

All Rights Reserved

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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

4/10/98
Date

Eleanor Waxman
Chair of Examining Committee

February 1, 1999
Date

Horst Schulz
Executive Officer

Supervisory Committee

The City University of New York

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Abstract

Localization and Expression of Carotenoid Biosynthetic Enzymes in Endosperms of *Zea mays* and *Oryza sativa*

By

Jia Yu

Advisor: Professor Eleanore T. Wurtzel

Carotenoids are essential pigments existing in all green tissues, that also accumulate in the endosperm of maize (*Zea mays*) but not in rice (*Oryza sativa*). To investigate the difference in carotenoid accumulation between maize and rice, I carried out parallel studies where I measured carotenoid levels, and expression and localization of two carotenoid biosynthetic enzymes. To study the expression of the carotenoid enzymes phytoene synthase (PSY) and phytoene desaturase (PDS), I generated antisera specific to maize PSY and PDS proteins. The antisera detected antigens in both maize and rice; the antigens were predicted to be the processed forms as found in plastids.

In developing maize endosperm, the onset of carotenoid accumulation appeared to correlate with expression of PSY between 10 to 20 days after pollination suggesting that PSY may be a rate limiting enzyme in the carotenoid biosynthetic pathway during early stages of endosperm development. Differences in PSY level detected in various maize tissues suggested that PSY accumulation is under tissue-specific regulation. PSY and

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

PDS proteins were detected in an amyloplast fraction of maize endosperm. Using confocal microscopy, PSY was localized to the envelope, and PDS was present both on the envelope and in the stroma of maize endosperm amyloplasts.

In rice, PSY accumulated at a similar level in embryo and endosperm, but was not detectable in leaf. PSY was not associated with an amyloplast fraction of rice endosperm. Using western analysis and confocal microscopy, I showed that PSY protein was present on the envelope of plastids in the rice embryo, but was found in the cytoplasm of rice endosperm cells. These results suggest that the compound amyloplasts in rice endosperm may be unable to retain the PSY protein. The improper localization of PSY may be the critical reason that carotenoids do not accumulate in rice endosperm.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ACKNOWLEDGMENTS

I am deeply grateful to my advisor Dr. Eleanore Wurtzel for her patience, constant advice, support and encouragement. I am also grateful to Dr. D. Basile, Dr. M. Brody, Dr. D. Calhoun, Dr. T. Jensen and Dr. M. Philipp for their guidance, advice, earlier reviews of the thesis, and kind service on my thesis committee.

I would like to acknowledge Dr. D. Basile for his technical support and the permission in using the fluorescent microscope. I thank Dr. G. Aisemberg for assistance and advice in confocal microscopy. I am also grateful to Dr. E.R. Macaguo (Department of Biology, Columbia University) for the permission to operate the confocal microscope. I would like to thank Mr. M. Baxter for his assistance in use of the electron microscope. I am especially grateful to Dr. M. Cervantes-Cervantes for his critical reading and correction of the thesis. I thank Ms. R.B. Luo and Dr. Z-h Li for critical reading of the thesis. I would like to acknowledge members of Dr. Wurtzel's lab for their friendship and help in the maize field work. I thank Mr. P. Matthews and Dr. A. Yoganathan for their friendship and advice in research.

I am grateful to Dr. T. Jensen for providing teaching assistantships and fellowships. I am also grateful to Dr. J. Valdovinos for his kindness and advice. I thank Mr. D. Cain for his kind help in growing corn in the maize field and in the green house. I am also grateful to Dr. H. Schulz for his advice and support. I thank Ms. S. Weintraub for her kindness and

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

help. I acknowledge Ms D. Vitanza for her administrative efforts. Thanks are extended to graduate students in the Department of Biological Sciences for their friendship.

I am extremely grateful to my husband, Yi Zhang. Without his love, support and patience, I would not be able to finish the dissertation. I am also grateful to my dear grandparents, Ruth and Tom Yu, for their encouragement and support. I especially thank my mother, Yiping Zhang and my stepfather B-Y Du for their strong help and understanding. I would like to thank my in-laws for providing special care for my baby son. I would like to express my appreciation to my lovely son Raymond, who brings me lots of joy and encouragement so that I feel strong to complete my thesis.

The research project was funded by the Rockefeller Foundation International Rice Biotechnology Program; the City University of New York--CUNY HEAT and PSCUNY Programs; the National Institutes of Health-Minority Biomedical Research Support Program; the National Science Foundation; the McKnight Foundation and the American Cancer Society.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF ABBREVIATIONS

aa	amino acid
ABA	abscisic acid
AIM	amyloplast isolation medium
AP	alkaline phosphatase
Arg	Argentina
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
Btl	Brittle-1 protein
CBB	Coomassie Brilliant Blue-R
ChrA	carotenoid binding protein A
Cpn60	chaperonin 60
DAF	days after flowering
DAP	days after pollination
DMAPP	dimethallyl diphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetra acetic acid
FITC	fluorescein isothiocyanate
FPP	farnesyl pyrophosphate
GGPP	geranylgeranyl pyrophosphate
GGPS	geranylgeranyl pyrophosphate synthase
GPP	geranyl pyrophosphate

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

GUS	β-glucuronidase
HYD	carotene hydroxylase
Hsp70	heat shock protein 70
IgG	immunoglobulin G
IPP	isopentenyl pyrophosphate
IPTG	isopropyl-β-D-thio-galactopyranoside
kb	kilobase
kDa	kilodalton
LB	Luria broth
LCYB	lycopene β cyclase
LCYE	lycopene ϵ cyclase
LHC	light harvesting system
lw	lemon white
NBT	nitro blue tetrazolium
PAGE	polyacrylamide gel electrophoresis
PAP	plastid-lipid-associated protein
PBS	phosphate buffered saline
PDS	phytoene desaturase
PIPES	piperazine-N,N'-bis-[2-ethanesulfonic acid]
PMSF	phenylmethylsulfonyl fluoride
PS	photosystem
PSY	phytoene synthase

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

PPPP	prephytoene pyrophosphate
RDA	recommended dietary allowance
RT-PCR	reverse transcriptase-polymerase chain reaction
SBEII	starch debranching enzyme II
SDS	sodium dodecyl sulfate
Sh	Shrunken protein, sucrose synthase
vp	viviparous
w3	white3
Wc	white cap
Wx	waxy
y	yellow
Y1	Yellow1
ZDS	ζ-carotene desaturase

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

TABLE OF CONTENTS

TITLE.....i

COPYRIGHT.....ii

APPROVAL.....iii

ABSTRACT.....iv

ACKNOWLEDGMENTS.....vi

LIST OF ABBREVIATIONS.....viii

TABLE OF CONTENTS.....xi

LIST OF TABLES.....xvi

LIST OF FIGURES.....xvii

Chapter 1. Introduction.....1

1.1 Chemistry and roles of carotenoids.....1

1.1.1 Chemistry of carotenoids.....1

1.1.2 Roles of carotenoids in animals.....3

1.1.3 Distribution and functions of carotenoids in plants.....4

1.2 Biosynthesis of carotenoids.....8

1.2.1 Cytosolic and plastidial pathways for IPP synthesis.....8

1.2.2 Carotenoid biosynthetic pathway.....10

1.3 Molecular and genetic studies of carotenoid biosynthesis in higher plants.....13

1.3.1 Regulation of phytoene synthase and phytoene desaturase expression.....13

1.3.2 Localization of phytoene synthase and phytoene desaturase.....18

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1.3.3 Carotenoid biosynthesis in maize endosperm....20

1.4 Carotenoid biosynthesis in rice endosperm....21

1.5 Objectives....22

1.5.1 Significance....22

1.5.2 Specific aims....24

Chapter 2. Expression of phytoene synthase in maize....33

2.1 Introducton....33

2.2 Materials and methods....33

2.2.1 Maize materials....33

2.2.2 Pigment extraction....35

2.2.3 Carotenoid quantification....35

2.2.4 Protein extraction....36

2.2.5 SDS-PAGE electrophoresis....36

2.2.6 Western analysis....37

2.2.7 Protein concentration determination....38

2.2.8 Protein sequence analysis....38

2.2.9 Preparation of *E. coli* lysate....38

2.2.10 Rabbit screening for pre-immune responses....39

2.2.11 Antigen preparation....39

2.2.12 Immunization protocol....40

2.2.13 Titering antisera....40

2.3 Results....43

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

2.3.1 Carotenoid analysis in developing maize endosperm....43

2.3.2 Production of a polyclonal antiserum against maize PSY....44

2.3.3 Dosage effect of *Y1* on PSY....46

2.3.4 Temporal regulation of PSY expression in developing endosperm....46

2.3.5 Expression of PSY in maize endosperm, embryo and leaf....49

2.4 Discussion....49

2.4.1 Carotenoid accumulation in maize endosperm....49

2.4.2 Characterization of anti-PSY antiserum....50

2.4.3 PSY expression in selective mutants of maize....51

2.4.4 Tissue specific control of PSY expression in maize....52

2.4.5 Temporal regulation of PSY expression in maize endosperm....52

Chapter 3. Expression of phytoene synthase in rice....76

3.1 Introduction....76

3.2 Materials and methods....77

3.2.1 Rice materials....77

3.2.2 Protein extraction....77

3.2.3 SDS-PAGE electrophoresis and western analysis....78

3.3 Results....78

3.3.1 PSY expression in rice endosperm, embryo and leaf tissues....78

3.3.2 Temporal regulation of PSY expression in rice endosperm....79

3.4 Discussion....80

3.4.1 Tissue specific expression of PSY....80

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

3.4.2 Developmental regulation of PSY expression....81

Chapter 4. Immunocytochemical localization of phytoene synthase in maize and rice endosperm and embryo....87

4.1 Introduction....87

4.2 Materials and methods....88

4.2.1 Maize materials....88

4.2.2 Rice materials....88

4.2.3 Protein extraction, SDS-PAGE electrophoresis and western analysis....88

4.2.4 Histological methods....89

4.2.5 Amyloplast isolation from maize and rice....90

4.3 Results....92

4.3.1 Localization of PSY in maize endosperm....92

4.3.2 Localization of PSY in embryo of maize and rice....94

4.3.3 Localization of PSY in rice endosperm....95

4.4 Discussion....97

Chapter 5. Production of polyclonal antisera against maize PDS....121

5.1 Introduction....121

5.2 Materials and methods....123

5.2.1 Materials....123

5.2.2 Subcloning of maize *Pds*....123

5.2.3 Over expression of the T7-PDS recombinant protein....126

5.2.4 Purification of the fusion protein....126

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

5.2.5 Immunization....127

5.3 Results....128

5.3.1 Overexpression of PDS fusion protein....128

5.3.2 Localization of PDS in maize B73 endosperm....130

5.4 Discussion....130

5.4.1 Specificity of maize anti-PDS antiserum....130

5.4.2 Localization of PDS in maize endosperm....131

Chapter 6. Summary and perspectives....140

6.1 Regulation of phytoene synthase expression....140

6.2 Expression of phytoene desaturase....145

Appendix....148

A. Antibodies used in western analysis and immunocytochemical localization....148

B. Figure and file description....150

C. Description of confocal microscopic images....153

D. Recombinant plasmids constructed....156

Bibliography....157

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF TABLES

Table 1-1. Maize mutants affecting chlorophyll, endosperm pigmentation, and carotenoid accumulation.....32

Table 2-1. Maize mutants used in western analysis of PSY expression.....42

Table 6-1. Carotenoid content from various higher plants.....147

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF FIGURES

1-1. General carotenoid biosynthetic pathway....25

1-2. Electron micrographs of plastids....26

1-3. Pathways generating isopentenyl diphosphate (IPP)....27

1-4. The biochemical pathways using GGPP as a common precursor....27

1-5. Amino acid sequence alignments of phytoene synthase....28

1-6. Structure of a mature maize kernel....30

1-7. The carotenoid and ABA biosynthetic pathway in *Zea mays*....31

2-1. Carotenoid analysis of developing endosperms....55

2-2. Western analysis--characterization of the anti-phytoene synthase antibody....57

2-3. Western analysis--phytoene synthase expression in maize wild type and mutant *Yellow 1* (*Y1*) endosperm....58

2-4. Western analysis--phytoene synthase accumulation in maize endosperm of normal and mutant from various loci....59

2-5. Western analysis--Y1 dosage effect on PSY expression....61

2-6. Western analysis--PSY expression in developing endosperm of B73 inbred, 1993 field....62

2-7. Western analysis--PSY expression in developing endosperm of A632 inbred, 1993 field....64

2-8. Western analysis--PSY expression in developing endosperm of Argentina inbred, 1993 field....66

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

2-9. Western analysis--PSY expression in developing endosperm of B73 inbred, 1996 field....68

2-10. Western analysis--PSY expression in developing endosperm of A632 inbred, 1996 field....70

2-11. Western analysis--PSY expression in developing endosperm of Argentina inbred, 1996 field....72

2-12. Western analysis--PSY expression in various tissues of maize B73 inbred....74

3-1. Western analysis--phytoene synthase expression in rice and maize endosperm....82

3-2. Western analysis--phytoene synthase expression in various tissues of rice IR36 inbred....83

3-3. Western analysis--phytoene synthase accumulation in developing endosperm of rice IR36 inbred....84

3-4. Western analysis--phytoene synthase accumulation in developing endosperm of rice Cypress inbred....86

4-1. PSY expression in in endosperm and embryo of maize B73 and rice IR36....100

4-2. Western analysis--phytoene synthase expression in amyloplast fractions of maize and rice endosperm....102

4-3. Localization of PSY in maize B73 endosperm dissected at 17 DAP....104

4-4. Western analysis--Expression of Bt1 in maize B73 endosperm....106

4-5. Brittle-1 localization in maize B73 endosperm dissected at 17 DAP....107

4-6. Immunofluorescent localization of PSY in maize B73 embryo dissected at 17 DAP....109

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

4-7. Immunofluorescent localization of PSY in rice embryo dissected at 7 DAF.....111

4-8. Immunofluorescent localization of PSY in rice IR36 endosperm dissected at 7 DAF.....113

4-9. Brittle-1 localization in rice IR36 endosperm dissected at 7 DAF.....115

4-10. Confocal microscopic images--Semithin sections of endosperm and embryo of maize.....117

4-11. Confocal microscopic images--Semithin sections of endosperm and embryo of rice.....119

5-1. Construction of the ZMPDS fusion protein in *E. coli*.....133

5-2. Overexpression of ZMPDS fusion protein in *E. coli*.....135

5-3. Western analysis of phytoene desaturase expression in maize.....137

5-4. Localization of PDS in maize B73 endosperm dissected at 17 DAP.....138

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter 1. Introduction

Carotenoids are a group of natural pigments bearing yellow, orange, and red colors, although some are colorless. They are synthesized *de novo* by higher plants, algae, fungi, photosynthetic and non-photosynthetic bacteria, but not by humans and other animals. In higher plants, all carotenoid biosynthetic enzymes are nuclear-encoded and the enzymatic activities reside in plastids. Due to the biological and physiological importance of carotenoids, carotenoid biosynthesis has been studied for many years in several organisms using approaches of biochemistry, chemistry, genetics, and molecular biology.

1.1 Chemistry and roles of carotenoids

1.1.1 Chemistry of carotenoids

Carotenoids are widely distributed in higher plants, algae, fungi and bacteria, and certain carotenoids are found in animals and humans. Approximately 600 carotenoids have been chemically identified from various organisms [for reviews, see (Britton and Goodwin, 1982; Bartley *et al.*, 1994; Sandmann, 1994; Cunningham and Gantt, 1998)]. In general, carotenoids are composed of eight isoprene units (see Figure 1-1) joined together in a "head-to-tail" manner to form a symmetrical backbone. Carotenoids vary in their chemical structures based on the number of conjugated carbon-carbon double bonds along the backbone and the modifications of their end groups, such as cyclization at one

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

and/or both ends and oxygenation on the cyclic structures. Therefore, they are categorized into linear, cyclic and oxygenated carotenoids. Phytoene is the first colorless C_{40} carotenoid on the pathway. Its linear structure has two configurations: 15Z, or 15,15'-*cis*, and all E, or all-*trans* (Bramley, 1985). In higher plants and fungi, 15-*cis*-phytoene is the predominant form (Than *et al.*, 1972). In nonphotosynthetic bacteria, 15-*trans*-phytoene is predominant (Kushwaha *et al.*, 1980). The insertion of a series of sequential conjugated C-C double bonds to the phytoene chromophore occurs through a number of desaturation reactions (Figure 1-1). Carotenoids such as phytofluene, ζ-carotene, neurosporene and lycopene, are the desaturation products, and are all in *trans* configuration (Bramley, 1985). In addition, different numbers of C-C double bonds are responsible for the various light absorption properties and in turn, give colors to these pigments, ranging from light yellow to yellow to red. Lycopene, a red pigment, undergoes cyclization to produce monocyclic or dicyclic carotenoids bearing β- and ϵ-rings (Goodwin, 1980; Spurgeon and Porter, 1980; Cunningham and Gantt, 1998). A major monocyclic carotenoid intermediate of the cyclization reaction is γ-carotene, (β,ψ-carotene), and the principal bicyclic carotenoids are β-carotene (β,β-carotene), and α-carotene (β,ϵ-carotene). Hydroxylation of the cyclic carotenes gives rise to the oxygenated pigments which comprise the major groups of carotenoids with yellow color, such as the xanthophylls, which accumulate in certain organisms (Britton and Goodwin, 1982). Introduction of hydroxyl groups may occur at the C-3 position of either or both rings on cyclic carotenes. Hydroxylation of γ-carotene and β-carotene gives rise to rubixanthin and zeaxanthin, respectively. Hydroxylation of α-carotene gives rise to lutein.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1.1.2 Roles of carotenoids in animals

Carotenoids play important roles in various organisms in their normal growth and development, participating in their adaptive responses to environmental challenges (Zhu *et al.*, 1997). In humans and other animals, β-carotene and other structurally related carotenoids that contain β-ionone ring(s) are the major dietary sources of vitamin A. Among the 600 carotenoids that have been identified, some have pro-vitamin A activity (Olson, 1994). Dietary provitamin A compounds are converted to vitamin A or retinol in the small intestine through either a central cleavage or an excentric cleavage (Olson, 1989). Vitamin A is an essential nutrient for humans and animals because humans and animals lack the enzymes to synthesize provitamin A compounds *de novo*. Vitamin A could be provided in the diet as a commercial source, and provitamin A carotenoids could be provided in the diet. Typical provitamin A carotenoids include β-carotene, α-carotene and γ-carotene, among others (Krinsky *et al.*, 1990). β-carotene is the major precursor of vitamin A and it shows the highest pro-vitamin A activity. Vitamin A is crucial for normal development in animals. β-carotene prevents cellular damage arising from oxidation; it also minimizes the risk of developing certain types of cancers, plays important roles in the human immune system (Underwood and Arthur, 1996). Retinol, vitamin A, a derivative of carotenoids, is a crucial molecule for normal vision. As an essential nutrient, dietary vitamin A content is required at a level between 0.4 to 1.3 mg daily. The Recommended Dietary Allowances (RDA) for vitamin A and provitamin A carotenoids have been established. The demand for β-carotene is between 2.4 to 7.8 mg

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

daily, and other provitamin A carotenoids are required between 4.8 to 15.6 mg daily. Insufficient intake of pro-vitamin A carotenoids leads to some severe health problems. Each year, about five million children in south Asia suffer from xerophthalmia, with 5% of them becoming blind (Sommer, 1988). Vitamin A deficiency is also related to the development of diarrhea, measles and respiratory diseases (Grant, 1991).

1.1.3 Distribution and functions of carotenoids in plants

Carotenoids protect photosynthetic organisms against harmful photooxidation. They are structural components of the photosynthetic antennae and reaction center complexes. They are involved in the thermal dissipation of excess light energy captured by light-harvesting antennae (Demmig-Adams, 1990). Carotenoids are precursors of abscisic acid, a plant hormone which participates in the control of water retention and modulates development and stress responses (Koornneef, 1986). The brilliant colors of flowers (rose, *Eschscholtzia*, *Narcissus*, etc) and fruits (citrus, pepper, pine-apple, tomato, etc) are due to the carotenoids. Carotenoids in seeds attract pollinators and are agents of seed dispersal (Goodwin, 1980).

In higher plants, carotenoids are synthesized and accumulated in various types of plastids and among different tissues. Plastids are organelles originated from proplastids, but each type of plastid has a unique structure and performs different functions during plant development (Tilney-Bassett, 1989). Examples are chloroplasts in photosynthetic tissues,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

chromoplasts in flowers, fruits and roots, and amyloplasts in storage tissues such as endosperm and roots (see Figure 1-2).

In the chloroplasts, where photosynthesis takes place, carotenoids are present in photosystem I (PS I) and photosystem II (PS II) (Goodwin, 1976). Together with chlorophylls, carotenoids are usually associated with pigment-protein complexes in reaction centers and light-harvesting systems, and are integral constituents of the thylakoid membrane (Siefermann-Harms *et al.*, 1985). In general, the features of carotenoids fall into three major themes: (1) a photo-protective role in the reaction centers, (2) a supplementary light-harvesting property of xanthophylls and (3) the function of zeaxanthin in the xanthophyll cycle. In the PS I reaction center, β-carotene is the predominant carotenoid, with only trace amounts of α-carotene. In the light-harvesting complex of PS I (LHC I), antheraxanthin and zeaxanthin are present and involved in the "xanthophyll cycle." The reaction center of PS II contains only β-carotene. Carotenoids found in LHC II of PS II are lutein, violaxanthin and neoxanthin. The light-harvesting function of carotenoids such as zeaxanthin is a singlet-singlet energy transfer process. After carotenoids are excited by light, energy is transferred from the excited singlet state of carotenoid to chlorophyll to initiate photosynthesis (Cogdell and Frank, 1987). The photoprotective role of carotenoids is related to the triplet-triplet energy transfer reaction in chloroplasts. Energy of triplet chlorophyll is transferred to the ground state carotenoid to prevent the formation of singlet-oxygen ($^1O_2$). Carotenoids are also capable to quench $^1O_2$ molecules directly in the pigment-protein complexes. If the excited state of chlorophyll is not rapidly

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

quenched, a reaction occurs with molecular oxygen to form an extremely reactive singlet oxygen molecule which reacts with and damages many cellular components such as lipids. A number of studies provide evidence that conversion of violaxanthin into zeaxanthin is involved in the indirect chlorophyll fluorescence quenching--an important role of the xanthophyll cycle (Demmig-Adams, 1990). When plants grow under excessive light, violaxanthin is de-epoxidated to form zeaxanthin--the excitation energy competitor, therefore, the excessive energy can be removed in the photosynthetic apparatus (for review, see Demmig-Adams and Adams, 1993).

The chromoplast is another type of plastid that is specialized for the production and accumulation of yellow, orange and red carotenoids in flower petals, fruits, and storage tissues such as carrots and sweet potatoes. In chromoplasts of tomato fruit, the major carotenoid accumulated is lycopene. In chromoplasts of red pepper fruit, the carotenoids accumulated are cyclopentane carotenoids such as capsanthin and capsorubin (Camara *et al.*, 1982). In chromoplasts of daffodil flowers, carotenes are the major carotenoids accumulated (Kreuz *et al.*, 1982). Several studies show that carotenoids are associated with proteins in the internal concentric pigment-bearing structures such as globular, fibrillar, membranous, reticular-tubular or crystalline membranes (Bathgate *et al.*, 1985; Cervantes-Cervantes *et al.*, 1990; Emter *et al.*, 1990; Smirra *et al.*, 1993). The role of carotenoids in daffodil flower chromoplasts is to act as visual attractants of insects facilitating pollination.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Amyloplasts are mature non-green plastids derived from proplastids and specialized as a storage resource for carbohydrates in the form of starch. This type of plastid is present in storage tissues such as maize and rice endosperm and in many roots (Tilney-Bassett, 1989). An amyloplast is delimited by a double membrane, and contains an internal stroma occupied by starch granules (Miernyk, 1985). In the stroma there is no well-developed internal membrane system which resembles the chloroplast thylakoid or chromoplast membrane grana (Miernyk, 1985). Instead, starch granules occupy most of the space in amyloplasts. Both maize and rice are grain crops that contain amyloplasts in their endosperm cells. In contrast to maize endosperm plastids where starch is stored in simple granules, starch in rice endosperm plastids is stored in compound granules (Badenhuizen, 1969). In rice endosperm, several starch granules touch each other and remain separated by narrow layers of stroma (Badenhuizen, 1969). Many varieties of maize accumulate colored carotenoids in their kernels, predominantly in the endosperm and the embryo. The carotenoids accumulated in normal yellow kernels are mainly xanthophylls such as zeaxanthin, lutein and β-cryptoxanthin (for review, see Goodwin, 1980). The endosperm of several maize mutants accumulates various carotenoid intermediates due to the block of some steps of the carotenoid biosynthetic pathway (Robertson, 1975a).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1.2 Biosynthesis of carotenoids

1.2.1 Cytosolic and plastidial pathways for IPP formation

Carotenoids are derived from the C_5 compound IPP, a common precursor for many isoprenoid derived compounds (Kleinig, 1989). IPP biosynthetic pathways have been elucidated based on the intermediates and their chemical structures. In plant cells, isoprenoids are found in various cell compartments such as cytoplasm/endoplasmic reticulum (for sterol), mitochondria (for ubiquinone) and plastids (for carotenoid, chlorophyll, plastoquinone, tocopherol) (Lütke-Brinkhaus and Kleinig, 1987; Kleinig, 1989; Liedvogel and Kleinig, 1984). Synthesis of IPP in the cytoplasm involves acetyl-CoA via the mevalonate pathway (Figure 1-3). Early steps include the condensation of three molecules of acetyl-CoA to form β-hydroxyl-β-methylglutaryl-CoA (HMG-CoA), which is then reduced to mevalonate, which in turn is phosphorylated and decarboxylated to form IPP. However, synthesis of IPP in plastids, where carotenoids are synthesized, occurs via a different mechanism. Results indicate that a non-mevalonate pathway employing glycolytic enzymes and a pyruvate dehydrogenase complex are involved (Liedvogel and Bauerle, 1986). An alternate non-mevalonate pathway to form IPP in green algae was first proposed by Schwender *et al.*, 1996 (Figure 1-3). Incorporation of ^{13}C was found in IPP, the common precursor molecule for isoprenoid synthesis. By using ^{13}C-NMR spectroscopy, it was shown that ^{13}C from [1-^{13}C]-glucose was incorporated into several isoprenoids, such as phytol, lutein, β-carotene, the prenyl chain of plastoquinone

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

in cellular compartments. Higher plant monoterpenes and diterpenes are also formed by the alternative pathway (Eisenreich *et al.*, 1996; Eisenreich *et al.*, 1997; Lichtenthaler *et al.*, 1997a; Lichtenthaler *et al.*, 1997b). The result also implies that there is a common pathway for the synthesis of several plant isoprenoids in plastids.

IPP undergoes isomerization catalyzed by IPP isomerase to form dimethallyl diphosphate (DMAPP). Thereafter, IPP reacts with its isomer DMAPP to synthesize the C_{10} molecule geranyl diphosphate (GPP) catalyzed by GPP synthase; an additional IPP molecule is added to form the C_{15} molecule farnesyl diphosphate (FPP) catalyzed by FPP synthase. In the cytoplasm, FPP is used to produced the C_{30} molecule squalene, and further to produce sterols. In organelles, FPP reacts with an additional IPP to form C_{20} geranylgeranyl pyrophosphate (GGPP) catalyzed by GGPP synthase.

GGPP is an essential precursor which branches off for several biosynthetic pathways and reactions (Figure 1-4), such as synthesis of the C_{20} lipid moiety in prenylated proteins that act as membrane anchors; plastoquinone, which is involved in electron transport; the phytol chain, which helps maintain chlorophyll integrity for photosynthesis; phytoene, for carotenoid synthesis; gibberellin, a plant growth regulator controlling seed germination, cell elongation, cell division, and development of flowers and fruits. All of these pathways will compete for the common precursor GGPP [for review, see (Chappell, 1995)].

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1.2.2 Carotenoid biosynthetic pathway

The carotenoid biosynthesis pathway begins with the head-to-head condensation of two molecules of GGPP to form phytoene. Phytoene is the first specific carotenoid on the carotenoid biosynthetic pathway. The formation of phytoene is catalyzed by a bifunctional enzyme, phytoene synthase (PSY). PSY converts GGPP to the intermediate prephytoene pyrophosphate (PPPP), then phytoene. PSY has been studied in various organisms such as *Erwinia*, *Rhodobacter* (Armstrong *et al.*, 1990; Armstrong *et al.*, 1989), the cyanobacterium *Synechococcus* PCC7942 (Chamovitz *et al.*, 1991), tomato (Ray *et al.*, 1987; Bartley *et al.*, 1992; Bramley *et al.*, 1992; Bartley and Scolnik, 1993; Giuliano *et al.*, 1993; Fraser *et al.*, 1994; Misawa *et al.*, 1994; Fray *et al.*, 1995), pepper (Römer *et al.*, 1993; Hugueney *et al.*, 1996), daffodil (Schledz *et al.*, 1996; Bonk *et al.*, 1997), *Arabidopsis* (von Lintig *et al.*, 1997), maize (Buckner and Robertson, 1993; Buckner *et al.*, 1996) and melon (Karvouni *et al.*, 1995). To date, active PSY enzyme has been successfully purified only from *Capsicum* chromoplasts (Dogbo *et al.*, 1988) using a GGPP analog coupled to an affinity chromatography matrix. In tomato, there are two PSY proteins expressed from two different *psy* genes. As for all the higher plant carotenoid enzymes–PDS, ZDS, LCY and other enzymes, each PSY protein is encoded by a nuclear gene and the gene product contains a transit peptide which is cleaved upon import to plastids, the site of carotenoid biosynthesis (Figure 1-5).

Phytoene undergoes a sequence of desaturation reactions to produce the pink-colored

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

carotenoid lycopene. Each desaturation step introduces a carbon-carbon double bond into the chromophore, which in turn gives rise to the change of light absorption properties of the carotenoid. Desaturation reactions are catalyzed by desaturase enzymes whose activities differ among various organisms. It has been reported that there are two types of phytoene desaturase enzymes (Sandmann, 1994), the PDS type and the CrtI type. The PDS type enzyme catalyzes two desaturation steps to form ζ-carotene through the intermediate phytofluene, and is present in oxygenic photosynthetic organisms such as higher plants, algae and cyanobacteria. The CrtI phytoene desaturase is named after the corresponding locus in *Rhodobacter capsulatus*, and is found in anoxygenic photosynthetic organisms. This type of enzyme catalyzes either three or four desaturation steps to form neurosporene or lycopene, respectively. Both types of phytoene desaturase contain a dinucleotide binding domain (FAD or NAD/NADP) at the amino terminal region whose function has been associated with the coupling of desaturation and electron transport systems (Norris *et al.*, 1995). In addition, all plant PDS proteins possess transit peptides of approximately 110 amino acid residues (Bartley *et al.*, 1991) that lead the proteins to plastids where the catalytic activities reside and that are cleaved upon plastid import. Phytoene desaturase has been investigated in a variety of organisms such as *Erwinia* (Armstrong *et al.*, 1990; Armstrong *et al.*, 1989; Fraser *et al.*, 1992; Linden *et al.*, 1991; Misawa *et al.*, 1993), *Rhodobacter* (Armstrong *et al.*, 1989; Bartley and Scolnik, 1989), the fungus *Neurospora crassa* (Schmidhauser *et al.*, 1990), *Phycomyces* (Ruiz-Hidalgo and Benito, 1997), the cyanobacterium *Synechococcus* PCC7942 (Chamovitz *et al.*, 1991; Schneider *et al.*, 1997), tomato (Pecker *et al.*, 1992; Fraser *et al.*,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1994; Mann *et al.*, 1994), pepper (Hugueney *et al.*, 1992), daffodil (Al-Babili *et al.*, 1996), *Arabidopsis* (Scolnik and Bartley, 1993), maize (Hable and Oishi, 1995; Li *et al.*, 1996; Hable *et al.*, 1998), and soybean (Bartley *et al.*, 1991). PDS proteins of higher plants are homologous to cyanobacterial PDS, but not to the ones in bacteria or fungi (Bartley *et al.*, 1991; Hugueney *et al.*, 1992; Pecker *et al.*, 1992).

Later steps in the pathway are catalyzed by several other enzymes. In plants and cyanobacteria, ζ-carotene desaturase (ZDS) converts ζ-carotene to the red pigment lycopene through the intermediate neurosporene. Lycopene undergoes cyclization catalyzed by lycopene β-cyclase (LCYB) to yield β-carotene, or by lycopene-ϵ-cyclase (LCYE) and LCYB to form α-carotene via γ-carotene [for review see (Cunningham and Gantt, 1998)]. Both carotenes are subject to hydroxylation and epoxidation catalyzed by α- or β-hydroxylases and epoxidases to form xanthophylls such as lutein, neoxanthin, and violaxanthin. ABA is derived from xanthophylls (oxygenated carotenoids) (Parry *et al.*, 1990; Parry and Horgan, 1992). Maize mutants blocking ABA synthesis, *vp1* and *vp14*, were characterized (McCarty *et al.*, 1989; McCarty *et al.*, 1991; Schwartz *et al.*, 1997). Vp1 was found to be a transcription activator in ABA-perception in seeds (McCarty *et al.*, 1991). Vp14 was shown to catalyze the cleavage of epoxycarotenoids (violaxanthin and/or neoxanthin) to produce apo-aldehyde and xanthoxin (Schwartz *et al.*, 1997).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1.3 Molecular and genetic studies of carotenoid biosynthesis in higher plants

Various aspects of carotenoid biosynthesis have been investigated to date. Gene expression of several enzymes (PSY, PDS and others) in the pathway has been analyzed at transcriptional, post-transcriptional, translational, and/or post-translational levels in different organisms.

1.3.1 Regulation of phytoene synthase and phytoene desaturase expression

A number of genes coding for PSY and PDS enzymes have been studied at both the transcript and protein levels. Developmental and environmental regulation of gene expression have been analyzed in certain plants. In tomato (*Lycopersicon esculentum*), two *psy* genes have been isolated (Bartley *et al.*, 1992; Bartley and Scolnik, 1993). Both *psy1* and *psy2* are expressed during tomato development. RT-PCR results have shown that *psy1* transcripts are abundant in seedlings and ripe fruits, while *psy2* transcripts are higher in leaves (Bartley and Scolnik, 1993). Treatment with Norflurazon, an inhibitor blocking carotenoid synthesis at the PDS step, triggered the expression of both *psy1* and *psy2* at the transcript level. PSY expression was low in tomato roots where less carotenoid accumulated. The amount of *Psy1* transcript increased during flower development. During fruit ripening, carotenoid levels increase and PSY expression increases dramatically. Using a monoclonal antibody against tomato PSY, a 38 kDa single polypeptide was detected, and it was localized in the stromal fraction in the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

chromoplasts of the ripe fruit (Misawa *et al.*, 1994). The transit peptide of tomato PSY in the ripe fruit is about 9 kDa, which represents 80 amino acid residues. An *in vitro* import study showed that tomato PSY1 is expressed as a 47 kDa precursor protein, and targeted into pea chloroplasts as a 42 kDa mature protein (Bartley *et al.*, 1992). It has been proposed that import assays with heterologous systems might not be reliable for correct processing of mature proteins (Karvouni *et al.*, 1995). In addition, PSY1 protein is peripherally associated with the chromoplast membrane. Tissue specific regulation of phytoene synthesis was observed. As reported (Bramley *et al.*, 1992), the pTOM5 gene encodes the PSY isoform that is expressed in flowers and ripening fruits, but not in green fruits and leaves. The two PSY genes differ in the promoter sequence and in the number of exons.

The expression of PSY has been studied in fruit chromoplasts of *Capsicum annuum* (Hugueney *et al.*, 1996). Results revealed that PSY expression was developmentally regulated. *Psy* transcript was lower in leaves and young fruits, but higher in ripe fruits. Expression of PSY was also induced after fungal elicitor treatment and wounding of fruit. PSY transcript was relatively low in leaves and young fruits in comparison to the higher level in fruits (Römer *et al.*, 1993). PSY transcripts of different sizes have been observed in pepper leaves and fruits which suggested that the regulation of PSY expression in pepper was tissue specific. In addition, post-transcriptional modification may be involved (Römer *et al.*, 1993). Immunochemical localization results showed that PSY was localized in the stroma of both chromoplast and chloroplast (Camara *et al.*, 1989).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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

In daffodil (*Narcissus pseudonarcissus*) flowers, there is a transitional process in which the chloroplasts are converted into chromoplasts with accumulation of carotenes (322 to 2899 μg/g dry weight, about 8 fold increase as compared to levels in green leaves and 6 fold increase as compared to that in green bulbs) (Schledz *et al.*, 1996). During the transition, PSY transcript levels (analyzed by Northerns) were not detectable in green leaves, high in the early flower stage, then slightly decreased; but PSY protein levels increased in the early flower stage, then decreased (Schledz *et al.*, 1996). In addition, PSY activity required the association of the enzyme to galactolipids (Schledz *et al.*, 1996). Using an antibody against daffodil PSY, two isoforms of PSY were identified. The active PSY form was present on the chromoplast membrane, while the inactive PSY form resided in the chromoplast stroma associated with a soluble Cpn60 protein complex (Bonk *et al.*, 1997). To become active, the soluble form of PSY protein must be dissociated from the Cpn60 chaperonin and must be associated with galactolipids.

In maize (*Zea mays*), PSY is encoded by the *Y1* gene (Buckner *et al.*, 1996). The dominant *Y1* transcript was highly expressed in embryo, seedling and endosperm while

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the mutant *y1* transcript was normally expressed in the embryo and seedling, but was not expressed in the endosperm. PSY expression at the transcript level is developmentally regulated (Li, 1998).

In chloroplasts, carotenoids are required to form the photosynthetic apparatus to conduct photosynthesis. Light stimulates the conversion of etioplasts to chloroplasts and induces carotenoid accumulation in maize seedlings (Albrecht and Sandmann, 1994). If maize leaves were treated with herbicides and light, no change in PSY activity was detected but IPP isomerase activity was induced (Albrecht and Sandmann, 1994); in tomato, light causes an increase in *Psy* transcripts in seedlings (Bartley and Scolnik, 1993; Giuliano *et al.*, 1993). In developing white mustard (*Sinapis alba*) seedlings, RT-PCR results showed that PSY transcript was up-regulated by light, while PDS and GGPS expression levels were not (von Lintig *et al.*, 1997). Similar experiments carried out in *Arabidopsis thaliana* seedlings showed that certain light qualities induced *Psy* transcript, but not carotenoid content. Results suggested that light conditions allowing chlorophyll synthesis could lead to an increase of carotenoid content (von Lintig *et al.*, 1997).

In daffodil flower chromoplasts, PDS proteins are present as two distinct forms similar to PSY. Using polyclonal antibodies against daffodil PDS, both forms were identified (Al-Babili *et al.*, 1996). The functional form of PDS was located on the chromoplast membrane, whereas the inactive form of PDS resided in the stroma of chromoplast as a complex with the chaperonin Hsp70. During daffodil flower formation, levels of Cpn60

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

and Hsp70 were induced (Al-Babili *et al.*, 1996; Bonk *et al.*, 1996). It has been reported that both Cpn60 and Hsp70 are involved in the protein rearrangement in the chromoplast during flower development. An *in vitro* import study of daffodil PDS showed that the protein translocates into the chromoplast, but remains in the stromal fraction (Bonk *et al.*, 1997).

In transgenic tomato and tobacco plants, transcription of a *Pds* promoter-*gus* fusion gene was differentially controlled (Corona *et al.*, 1996). In transgenic tomato anthers, petals, and ripening fruits, where chromoplasts are present, transcript levels of the *Pds* promoter-*gus* construct were high. In comparison, in transgenic tobacco fruits and petals, where chromoplasts do not exist, *Pds* promoter-*gus* transcript levels were low. In tobacco seedlings treated with herbicides which inhibit carotenoid and chlorophyll biosynthesis, *Pds* promoter-*gus* expression was induced (Corona *et al.*, 1996). The above results imply that different feedback regulatory mechanisms might exist in various tissues depending upon the type of plastid present.

In transgenic tomato plants expressing the *Psy1* antisense RNA, carotenoid levels were greatly reduced in comparison to normal tomato plants (Bird *et al.*, 1991). When gibberellin content was analyzed in transgenic tomatoes, an increase of gibberellin was observed. Results suggest that GGPP is consumed by transgenic tomatoes to synthesize gibberellins (Fraser *et al.*, 1995).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

When the *Psy1* gene was overexpressed in transgenic tomato seedlings, chlorophyll levels were decreased and plants showed dwarfness (Fray *et al.*, 1995). The explanation may be that channelling of GGPP, a common precursor, to synthesize carotenoids, affects biosynthesis of gibberellins and phytol (Fray *et al.*, 1995).

In maize endosperm amyloplasts, where carotenoids are synthesized and accumulated, expression of PDS at the transcript level, as assayed by RT-PCR and Northerns, was fairly constant during early seed development (10 to 16 DAP) (Li et al., 1996; Hable *et al.*, 1998). PDS protein accumulation was also found to be constant throughout seed development (14 to 30 DAP) when plastid membrane-enriched proteins from endosperms were analyzed using an anti-PDS antibody (Hable *et al.*, 1998).

1.3.2 Localization of phytoene synthase and phytoene desaturase

The carotenoid biosynthetic enzymes are thought to be organized in multi-enzyme complexes (Cunningham and Gantt, 1998). Phytoene synthase complex contains IPP isomerase, GGPS and PSY. This complex is localized membrane-peripherally (Camara, 1993), and its product is phytoene, a C_{40} compound that is the first specific intermediate for the carotenoid biosynthetic pathway. Both PDS and ZDS enzymes are membrane-associated, and might comprise another enzyme complex (for review, see Cunningham and Gantt, 1998).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

In higher plants, all carotenoid biosynthetic enzymes are nuclear-encoded. These proteins are synthesized as precursors on membrane-free polysomes in the cytoplasm, then imported into plastids as mature proteins. Thereafter, enzymes undergo folding, oligomeric assembly, translocation and membrane insertion in plastids. These processes are chaperonin-assisted (Bonk *et al.*, 1997).

Chromoplasts do not possess thylakoids, which is the major site of carotenoid requirement and function in chloroplasts. Instead, depending on the chromoplast type, there is some unique membrane system formed during the chloroplast to chromoplast transition. Immunochemical localization study showed that PSY was localized in the stroma of chromoplast as well as chloroplast in pepper fruits (Camara *et al.*, 1989).

A comparison of the N-terminal amino acid sequences of eukaryotic and prokaryotic PSY proteins (Figure 1-5) indicates that eukaryotic PSY polypeptides are larger than those from prokaryotic PSY proteins. The existence of the transit sequence in eukaryotic PSY targets the PSY protein to plastids. An *in vitro* import study revealed that translated PSY protein was cleaved and localized into thylakoid membrane fraction of pea chloroplasts (Bonk *et al.*, 1997).

In vitro import study using a soybean *Pds* cDNA showed that the expressed PDS protein (63.9 kDa) was processed to a smaller size (59 kDa) after import into thylakoid membrane of pea chloroplasts (Bartley *et al.*, 1991). Incorporation of IPP and GGPP *in*

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

vitro with chloroplast fraction showed that PSY and PDS were associated with the envelope membrane from spinach chloroplasts (Lütke-Brinkhaus *et al.*, 1982; Kreuz *et al.*, 1982). In cyanobacteria, PDS was found to be associated with the thylakoids (Serrano *et al.*, 1990). Immunoglod localization and immunological studies showed that the majority of PDS was localized in thylakoid membranes from tobacco chloroplasts (Linden *et al.*, 1993). In daffodil chromoplasts, PDS was reported to be associated with envelope membrane and within stroma (Al-Babili *et al.*, 1996).

1.3.3 Carotenoid biosynthesis in maize endosperm

Maize is an essential plant to study carotenoid biosynthesis and accumulation. It has been widely used in genetic studies. In the mature endosperm of the maize seed (Figure 1-6), carotenoids accumulate to about 5-12 μg/g fresh weight (Yu and Wurtzel, unpublished). The major endosperm carotenoids are xanthophylls such as zeaxanthin, β-cryptoxanthin, and lutein (Goodwin, 1980). These pigments condition the yellow endosperms in maize. There are several recessive mutations that block carotenoid biosynthesis at various steps (Figure 1-7), thus leading to the accumulation of different carotenoid intermediates in their endosperms, embryos and leaves (Robertson, 1975a; Neil *et al.*, 1986; Anderson and Robertson, 1960; Fong *et al.*, 1983). The mutants (Table 1-1) having these features are: *w3*, *vp2*, *vp5*, *vp7*, and *vp9*, and their embryos undergo precocious germination (Robertson, 1955; Robertson, 1975a). Plants carrying the mutations *lw1*, *cl1*, *y1*, *y3*, and *y11*, do not show vivipary, and do not accumulate either carotenoids or their precursors. It

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

is known that four distinctive mutations, *w3*, *vp2*, *vp5*, and *vp9*, block the desaturation steps in the carotenoid biosynthetic pathway. Biochemical studies revealed that *vp2* and *vp5* mutant seeds accumulated phytoene and phytofluene; *w3* mutants accumulated phytoene and phytofluene; *vp9* mutants accumulated ζ-carotene (Neil *et al.*, 1986). Recent molecular studies show that the *vp5* locus encodes PDS (Hable and Oishi, 1995; Hable *et al.*, 1998; Li *et al.*, 1996). White cap (*Wc1*) is a dominant white allele. Kernels are whitest with one dominant allele, and more yellow with fewer dominant alleles. White endosperms do not accumulate any carotenoids nor precursors (Robertson, 1955; Robertson, 1975a). The cl_1 mutant is one of white albino mutants of maize (Robertson, 1975a). The mutant shows white endosperms and albino seedlings. It is known that cl_1 mutant seedlings do not accumulate carotenoids (Robertson, 1966). *Clm1* is a dominant modifier of cl_1 (Robertson, 1966). *Clm1* alters the phenotype of cl_1 seedlings so that they appear pale green or green, but *Clm1* does not affect the phenotype of the cl_1 endosperm (Robertson, 1966).

1.4 Carotenoid biosynthesis in rice endosperm

Rice (*Oryza sativa*) is one of the leading food crops in the world and lacks endosperm carotenoids. Milled rice, the edible portion after removal of aleurone and embryo tissues, does not accumulate either carotenes or other carotenoid intermediates (Juliano, 1972). Rice does possess the carotenogenesis genes in its genome, since photosynthesis takes place in leaves and other green tissues where carotenoids are essential components in the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

chloroplast. It has been reported that carotenes and tocopherol (vitamin E) are present in rice embryo and aleurone layers (bran and polish) (Sechi and Rossi-Manaresi, 1958). Lipids such as squalene and sterols also have been found in rice bran and embryo (Akiya, 1962). In immature rice endosperm (10-15 DAP), a feeding experiment with [1-^{14}C] IPP showed incorporation of radioactivity into the C_{15} farnesol, the C_{20} GGPP and the C_{30} squalene, but not into any C_{40} carotenoids. Results suggest that immature rice endosperms are capable to synthesize C_{15}, C_{30} isoprenoids and C_{20} GGPP, but not C_{40} phytoene (Burkhardt *et al.*, 1997). Using [^{14}C] GGPP and [^{14}C] phytoene, no carotenoids were detected (Burkhardt *et al.*, 1997). This suggests that immature rice endosperms are not capable of converting GGPP or phytoene to carotenoid intermediates. In rice endosperm transformed with a daffodil *psy* cDNA, colorless phytoene accumulated indicating that transgenic rice is capable of converting GGPP to phytoene (Burkhardt *et al.*, 1997). Using the RT-PCR technique, results showed that both *psy* and *pds* genes were expressed in rice leaf, embryo and endosperm tissues at the transcriptional level (Wurtzel *et al.*, 1996). The reason why rice endosperm does not accumulate any carotenoids still remains unclear.

1.5 Objectives

1.5.1 Significance

To improve the pro-vitamin A content in rice endosperm, it is necessary to determine what is blocking rice endosperm carotenoid biosynthesis. Maize endosperm does

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

accumulate carotenoids. Both rice and maize are monocotyledonous plant cereals whose endosperm contains amyloplasts where starch molecules are stored. A parallel study of carotenoid biosynthesis in rice and maize endosperm could develop the foundation for understanding mechanisms controlling carotenoid biosynthesis in amyloplast-bearing endosperm tissue.

One hypothesis is that carotenoid biosynthesis is regulated differently in maize and rice endosperms. The lack of endosperm carotenoids in rice may be due to the improper expression and/or localization of phytoene synthase, the first key enzyme specific to the carotenoid biosynthesis pathway.

Therefore, study toward understanding regulation of PSY expression is essential, and in turn provides information on regulation of the entire carotenoid biosynthetic pathway. The pattern of PSY protein accumulation in various tissues in maize and rice will reveal any possibility that PSY protein is tissue specifically accumulated. The regulation of PSY expression has been studied in certain dicotyledonous plants, but little is known in grain crops such as maize and rice. There are no results from any prior studies that show the localization of PSY protein in maize and rice endosperm cells.

1.5.2 Specific aims

The specific objectives of this investigation are to answer the following questions:

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1. How is PSY protein accumulated during endosperm development in various inbred lines of maize and rice?

2. What is the pattern of carotenoid accumulation in maize endosperms during seed development? Is there any correlation between carotenoid accumulation and PSY expression in developing maize endosperms?

3. Is PSY protein accumulation varied in a tissue-specific manner in leaf, embryo, and endosperm of maize and rice?

4. Where does PSY protein localize in endosperm and embryo cells of maize and rice?

5. Where does PDS protein localize in maize endosperm cells?

Results of the current investigation will be presented as follows:

In chapter 2, expression of PSY protein in maize is presented; in chapter 3, expression of PSY in rice endosperm and embryo is presented; in chapter 4, immunocytochemical localization of PSY in maize and rice endosperm and embryo is presented; in chapter 5, production of a polyclonal antiserum against maize PDS and the primary results of localization of PDS are presented.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

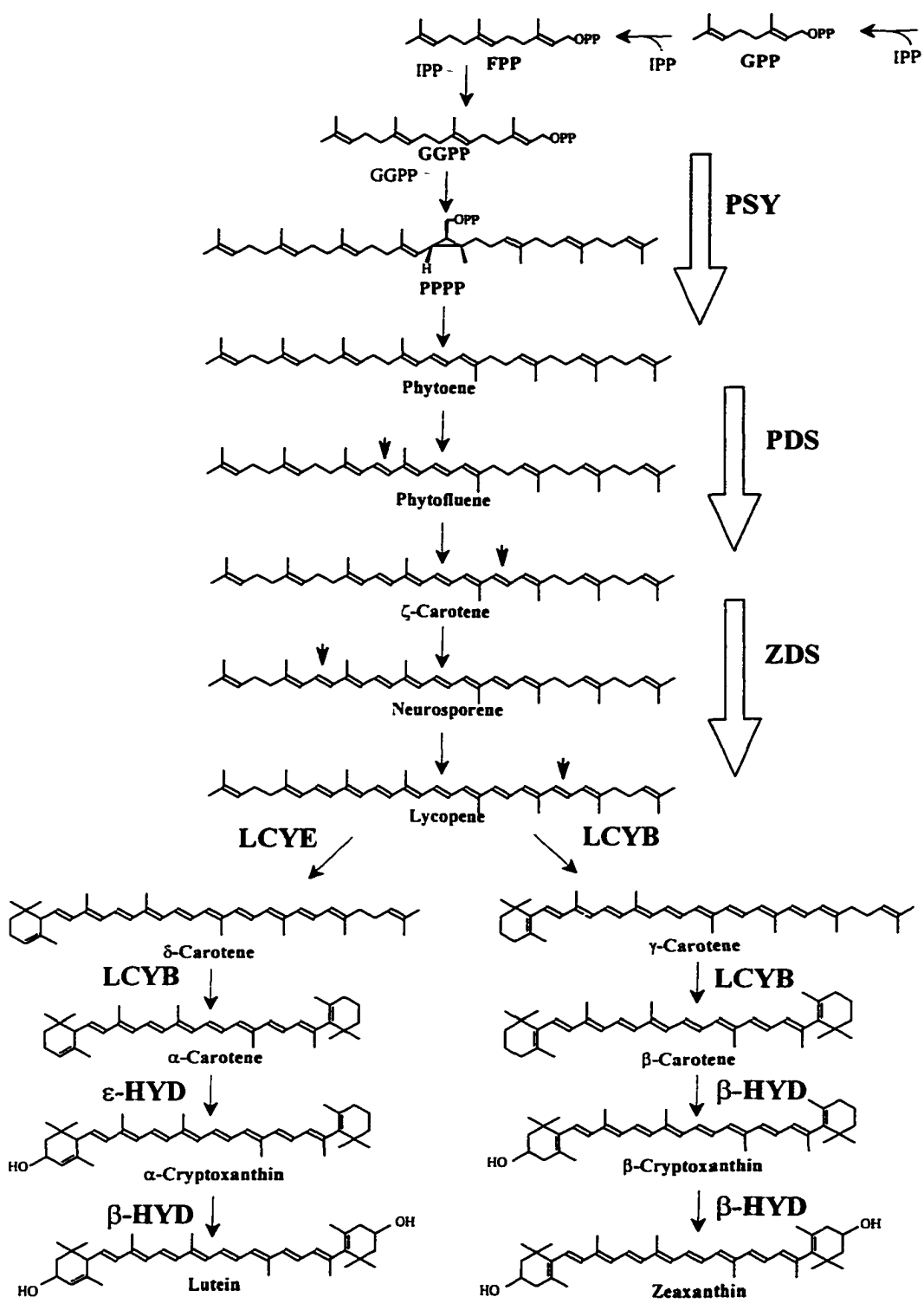


Figure 1-1. General carotenoid biosynthetic pathway.
(Acknowledgment to Dr. A. Yoganathan for drawing this pathway)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

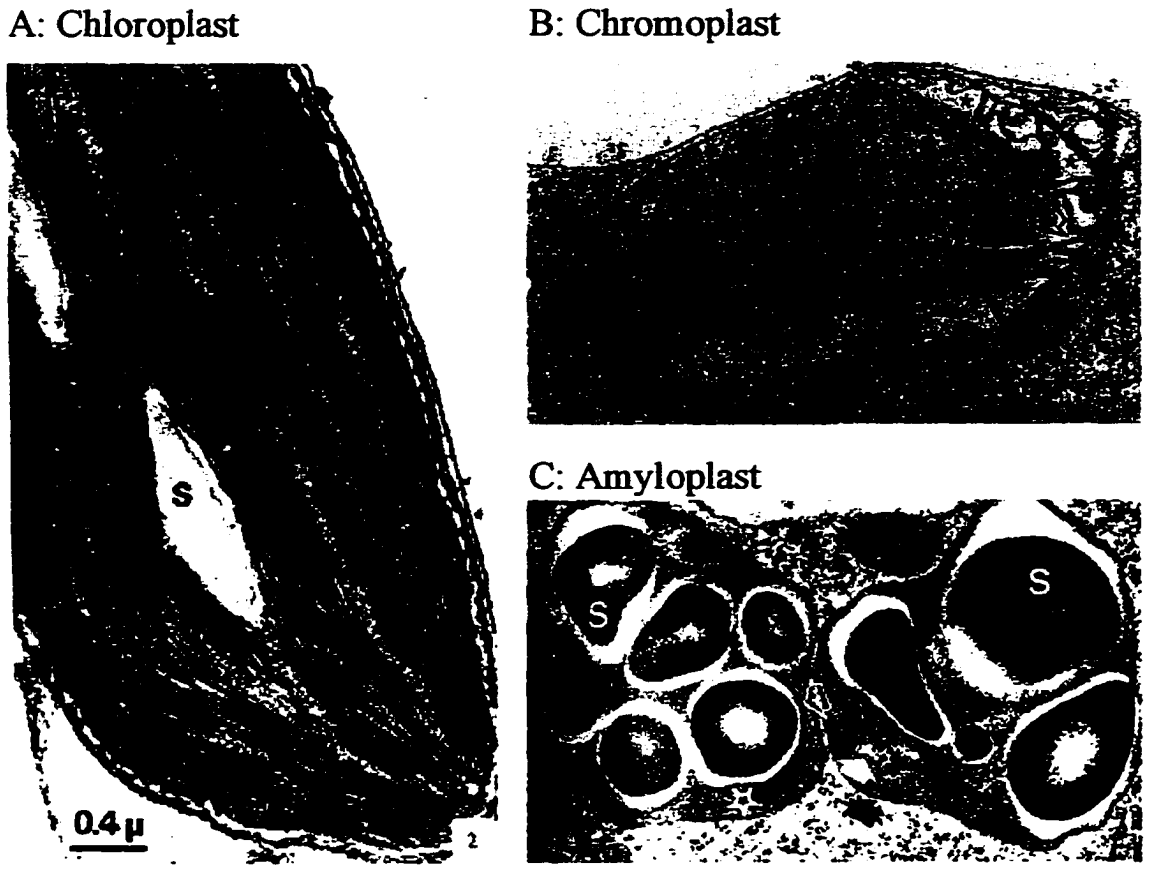


Figure 1-2. Electron micrographs of plastids
(A): Chloroplast of maize leaf. G, grana stacks; S, starch grain. (Bachmann *et al.*, 1969).
(B): Chromoplast of tomato fruit. Stars, lycopene crystals. (Gunning and Steer, 1996).
(C): Amyloplast of soybean seed. S, starch grain. (Gunning and Steer, 1996).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

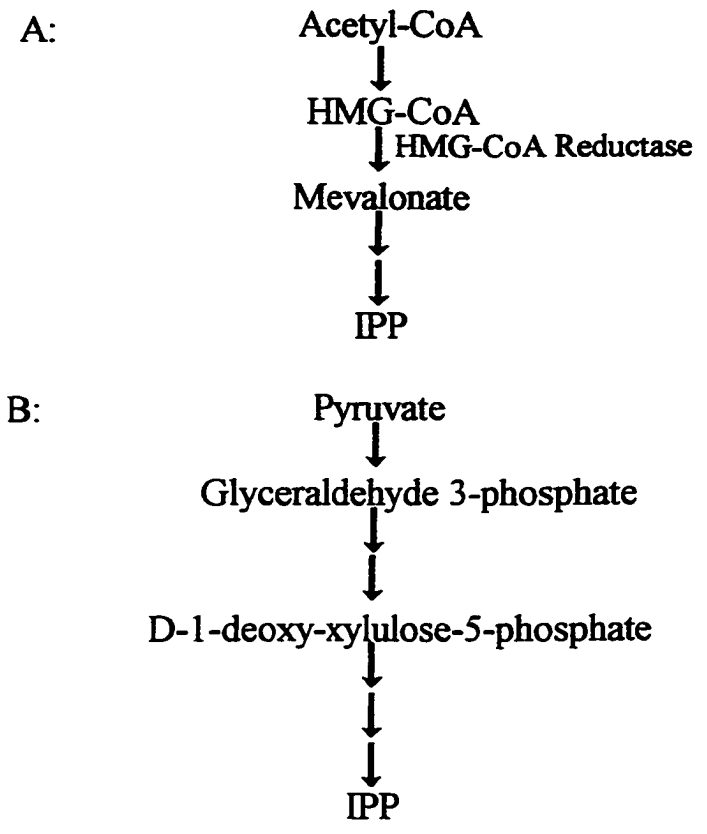


Figure 1-3: Pathways generating isopentenyl diphosphate (IPP).
A: Biosynthesis of IPP from the mevalonate pathway.
B: Biosynthesis of IPP via the pyruvate/glyceraldehyde pathway.

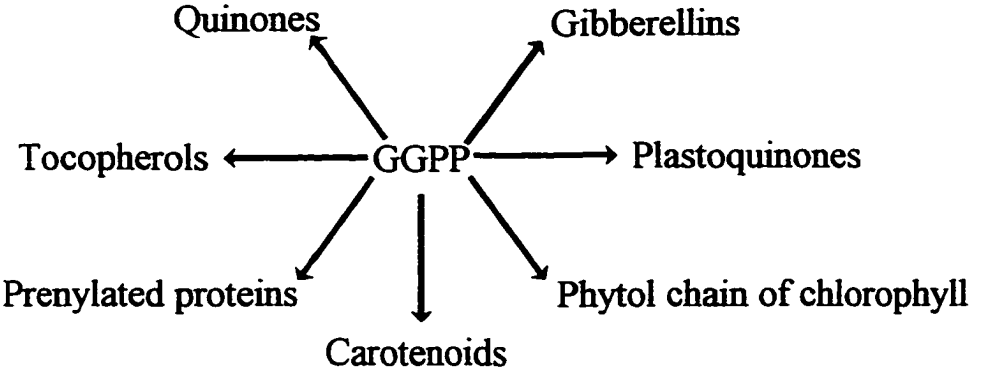


Figure 1-4: The biochemical pathways using GGPP as a common precursor.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 1-5. Amino acid sequence alignment of phytoene synthase proteins.

Asterisks indicate identical residues and dots indicate similar residues.

ZM, maize, GenBank #U32636; *NP*, daffodil, #P53797; *CA*, pepper, #X68017; *AT*, *Arabidopsis*, #413732; *SY*, *Synechocystis*, #S45340

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

```
ZM          MA--IILVRAASPGLSAAD-----------------SISHQGTLQCST--    29
NP          MV--VAILRVVSAIEIPIRL---GF-----SEANWRFSSPKYDNLGRKKSR    41
CA          MS--VALLWVVSPC---DVSNGTGFLVSVREGNRIFDSSGRRNLAC-NER    44
AT          MSSSVAVLWVATSSLNPDPMNNCG-LVRVLESSRLFS-------PCQNQR    42

ZM          LLKTKRPAARRWMPCSLLGLHPWEAGRPSPAVYSSLPVNPAGEAVVSSEQ    79
NP          LSVYSLYTTSKYACVGF----EAENNGKF-LIRSSLVANPAGEATISSEQ    86
CA          IKRGGGKQ--RWSFGSYLGGAQTGSGRKFSV-RSAIVATPAGEMTMSSER    91
AT          LNKGKKKQIPTWS-SSFV----RNRSRRIGVVSSSLVASPSGEIALSSEE    87
SY                                                  MANGQI      6
                                                          ...
ZM          KVYDVVLKQAALLKRQLRTPVLDA---RPQDMDMPRNG---LKEAYDRCG   123
NP          KVYDVVLKQAALVKDQTKSSRKSTDVK--PDIVLPGTVYLL-KDAYDRCG   133
CA          MVYDVVLRQAALVKRQLRSTD-ELDVKK--DIPIPGTLGLL-SEAYDRCS   137
AT          KVYNVVLKQAALVNKQLRSSSYDLDVKKPQDVVLPGSLSLLVGEAYDRCG   137
SY          SPQRAVTKPQSWWLTSEPRPSLMLQLPKPSPSAKPCAS---VEEAYEICR    53
             .. .*.*..... .                  *         .**..*

ZM          EICEEYAKTFYLGTMLMTEERRRAIWAIYVWCRRTDELVDGPNANYITPT   173
NP          EVCAEYAKTFYLGTLLMTPERRRAIWAIYVWCRRTDELVDGHNASHITPS   183
CA          EVCAEYAKTFYLGTMLMTPERRKAIWAIYVWCRRTDELVDGPNASHITPA   187
AT          EVCAEYPKTFYLGTLLMTPERRKAIWAIYVWCRRTDELVDGPNASHITPM   187
SY          QVTAQYAKTFYLGTLLMPPAKRRAIWAIYLWCRRTDELVDGPQAATTTPE   103
            .. ..*.*******.**. ..*.******.**********..*. .**

ZM          ALDRWEKRLEDLFTGRPYDMLDAALSDTISRFPIDIQPFRDMIEGMRSDL   223
NP          ALDRWEARLEDLFAGRPYDMFDAALSDTVSRFPVDIQPFMDMVEGMRMDL   233
CA          ALDRWEDRLEDVFSGRPFDMLDAALSDTVSKFPVDIQPFRDMIEGMRMDL   237
AT          ALDRWEARLEDLFRGRPFDMLDAALADTVARYPVDIQPFRDMIEGMRMDL   237
SY          TLDHWERRLEGIFAGQPQDDADVALVDTLETFPLDIQPFRDMIAGQRMDL   153
            .**.** ***..* *.* *  *.** **.. .*.*****.**..* * **

ZM          RKTRYNNFDELYMYCYYVAGTVGLMSVPVMGIATESKATT---ESVY---   267
NP          KKSRYKNFDELYLYCYYVAGTVGLMSVPVMGIAPESLAEA---ESVY---   277
CA          RKSRYRNFDELYLYCYYVAGTVGLMSVPIMGIAPESKATT---ESVY---   281
AT          KKSRYQNFDDLYLYCYYVAGTVGLMSVPVMGIDPKSKATT---ESVY---   281
SY          YRSRYQTFEELDLYCYRVAGTVGLMSSAVLGVDTGNGQAPWQPDAVYIPQ   203
             ..**..*..* .*** ********* ...*... . ...   ..**

ZM          SAALALGIANQLTNILRDVGEDARRGRIYLPQDELAQAGLSDEDIFKGVV   317
NP          NAALALGIANQLTNILRDVGEDARRGRIYLPQDELAEAGLSDEDVFTGKV   327
CA          NAALALGIANQLTNILRDVGEDARRGRVYLPQDELAQAGLSDEDIFAGRV   331
AT          NAALALGIANQLTNILRDVGEDARRGRVYLPQDELAQAGLSDEDIFAGKV   331
SY          EEAIALGVANQLTNILRDVGEDVERGRIYLPLEDLERFNYSEQDLLNGVN   253
            ..*.***.**************. ***.*** ..*.  . *..*.. *

ZM          TNRWRNFMKRQIKRARMFFEEAERGVNELSQASRWPVWASLLLYRQILDE   367
NP          TDKWRSFMKRQIKRARTFFEQAEKGVTELSQASRWPVWASLLLYRQILDE   377
CA          TDKWRIFMKKQIQRARKFFDEAEKGVTELSAASRWPVLASLLLYRRILDE   381
AT          TDKWRNFMKMQLKRARMFFDEAEKGVTELSAASRWPVWASLLLYRRILDE   381
SY          DDRWRSLMKFEIDRARHYFEDAERGIRALNRDARWPVWTALMLYKGILDV   303
            ...** .** ...*** .*..**.*. .*. ..**** ..*.**. ***

ZM          IEANDYNNFTKRAYVGKGKKLLALPVAYGKSLLLPCSLRN---GQT       410
NP          IEANDYNNFTKRAYVSKVKRLAALPLAYGKSLLIPLSLRPPSLSKA       423
CA          IEANDYNNFTKRAYVSKPKKLIALPIAYAKSLVPSTRT--------       419
AT          IEANDYNNFTKRAYVGKVKKIAALPLAYAKSVLKTSSSRLSI----       423
SY          IEANNYNVFNRRAYVPTPKKLLYLPVAW---------LRAQVL---       337
            ****.** *..**** . *..  **.*.
```

Fig. 1-5

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

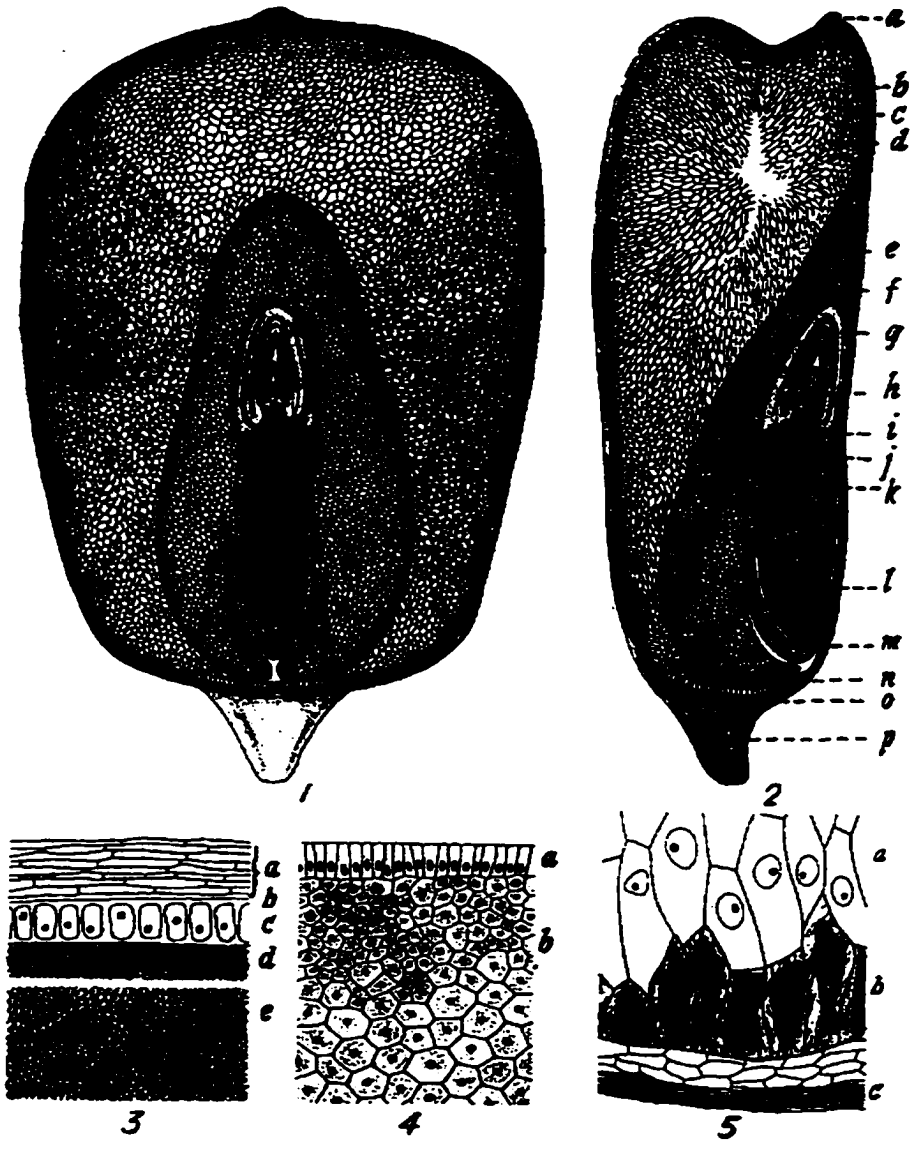


Figure 1-6. Structure of a mature maize kernel.
1 and 2, vertical sections in two planes of a mature kernel of corn, showing arrangement of organs and tissues. a, silk scar; b, pericarp; c, aleurone; d, endosperm; e, scutellum; f, glandular layer of scutellum; g, coleoptile; h, plumule with stem and leaves; i, first internode; j, lateral seminal root; k, scutellar node; l, primary root; m, coleorhiza; n, basal conducting cells of endosperm; o, brown abscission layer; p, pedicel or flower stalk. 3, enlarged section through pericarp and endosperm. a, pericarp; b, nucellar membrane; c, aleurone; d, marginal cells of endosperm; e, interior cells of endosperm. 4, enlarged section of scutellum. a, glandular layer; b, interior cells. 5, vertical section of the basal region of endosperm. a, ordinary endosperm cells; b, thick-walled conducting cells of endosperm; c, abscission layer. (Figure taken from Kiesselbach, 1980)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

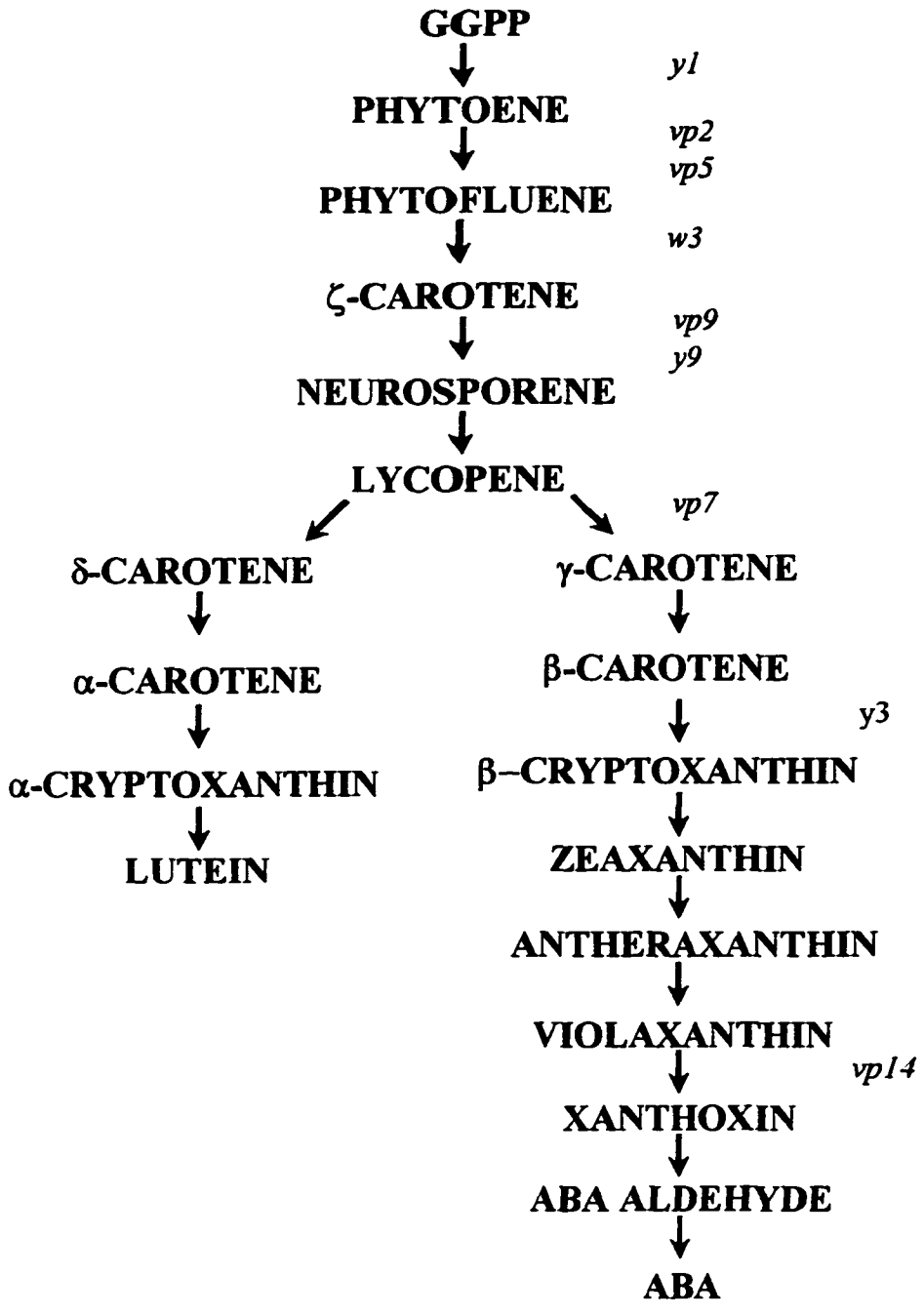


Figure 1-7. The carotenoid and ABA biosynthetic pathways in *Zea mays*. Left side represents the enzymes in the early pathway; right side represents the mutant alleles cooresponding to certain blocks in the pathway.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 1-1. Maize mutants affecting chlorophyll, endosperm pigmentation, and carotenoid accumulation. N/D represents that no carotenoids were detected. (Neuffer *et al.*, 1997)

Locus	*Map position	Endosperm phenotype	Seedling phenotype	**Carotenoid accumulated	Reference (allele)
vp5	1S	white	albino	phytoene	(Robertson, 1952)
vp2	5S	white	albino	phytoene	(Eyster, 1931b; Eyster, 1931a)
vp9	7S	pale yellow	albino	ζ-carotene	(Robertson, 1955)
w3	2L	pale yellow	albino	phytoene, phytofluene	(Demerec, 1923; Lindstrom, 1924)
y1	6L	white	green	N/D	(Correns, 1901)
y3	2S	pale yellow	green	N/D	(Perry and Sprague, 1936)
y9	10S	pale yellow	green	phytoene, phytofluene, ζ-carotene	(Robertson, 1975b)
y11		pale yellow	green	N/D	(Sprague, 1987)
lw1	1L	pale yellow	albino	N/D	(Tulpule, 1954)
wc1	9L	white	green	N/D	(Kulkarni, 1927)
y12		pale yellow	green	N/D	(Sprague, 1987)
vp7	5S	pink	albino	lycopene	(Sprague, 1936)
cl1	3S	pale yellow	albino	N/D	(Everett, 1949)
Clm1	8	yellow	green	N/D	(Everett, 1949)

*Map position: Chromosome Map Position

**Carotenoid accumulated in endosperms: see reference (Mayfield *et al.*, 1986)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter 2. Expression of phytoene synthase in maize

2.1 Introduction

To investigate regulation of carotenoid biosynthesis in maize endosperm, I have focused attention on the first step specific to the pathway, the synthesis of phytoene (see Fig. 1-1). Phytoene synthesis is mediated by the enzyme PSY, encoded by the *Y1* gene. I developed a polyclonal antibody specific for PSY from maize. I used this antibody to assay PSY protein in developing endosperm of several inbred lines varying in carotenoid accumulation. I also examined accumulation of total colored carotenoids. My results show that at early developmental stages (between 10 and 20 DAP) PSY might be rate-limiting in the carotenoid biosynthetic pathway. Results will be presented and discussed in the following sections.

2.2 Materials and Methods

2.2.1 Maize materials

Maize (*Zea mays*) seeds of B73 and A632 lines were obtained from Dr. S. Briggs (Pioneer Hi-Bred, Johnston, Iowa); Argentina high carotenoid line was from Dr. P. Scolink; mutants from the Maize Coop (Urbana, IL), Dr. D.S. Robertson (Iowa State University) and Dr. J.D. Smith (Texas A&M University). Maize plants were grown under

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

summer field conditions in the maize field at the Black Rock Forest (Cornwall, New York) in 1991, 1993 and 1994, and at the Maize Genetic Station in Pelham Bay Park (Bronx, New York) in 1996. Ears of three lines were harvested at 5, 10, 15, 20, 25, 30, and 35 or 40 DAP. Ears of other mutant lines were collected at about 20 DAP. The pericarp was removed, and the endosperm and embryo were separated. Unfertilized ovules were dissected from unfertilized ears. Endosperms, embryos, and unfertilized ovules were quickly frozen in liquid nitrogen, then stored at -80°C prior to protein and pigment extraction.

Maize mutant materials used in western analysis were from 1994 stocks. For *Y1* dosage effect analysis, endosperm materials were from 1991 stocks (Table 2-1). For developmental accumulation of PSY protein and pigments analysis, endosperm samples were from 1993 stocks as following: B73 line 93024 sibling cross, A632 line 93027 sibling cross, and Argentina high carotenoid line 93037 sibling cross. For pigment analysis, 10 DAP endosperms of Argentina line were from 1994 stock as 94015 sibling cross. For developmental accumulation of PSY experiment, samples were from 1996 stocks as following: B73 line 96004 sibling cross, A632 line 96010 sibling cross, and Argentina high carotenoid line 96016 sibling cross. For amyloplast isolation and tissue fixation, B73 endosperm at 17 DAP was from 96004 sibling cross.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

2.2.2 Pigment extraction

Carotenoids were extracted from developing endosperms of B73, A632 and Argentina high carotenoid line lines as described in Wurtzel (Wurtzel, 1992) with modification. All samples were from the 1993 field (as indicated above), except 10 DAP endosperm of Argentina high carotenoid line, which was from the 1994 field. Approximately 1 gram of endosperm dissected at various DAP was ground in 2 ml acetone. Extracts were placed on ice for 15 minutes in the dark, and the upper acetone phase was transferred to a new tube; the extraction was repeated with 2 ml acetone, and the upper phases were combined. An equal amount of petroleum ether was added, and the mixture was centrifuged at 10,000 x g at 4°C for 15 minutes. The upper phase was transferred to a new tube, and dried under nitrogen gas. The pigment was solubilized in 1 ml petroleum ether. Absorbance was scanned between 200 nm and 800 nm, and A_{452} was recorded. Results were repeated with three or four replica for each developmental stage.

2.2.3 Carotenoid quantification

Colored carotenoids were quantified using the absorbance reading and the average absorption coefficient ($A^{1\%}_{1cm}$) (an average $A^{1\%}_{1cm}$ value of 2500 was used for the extract containing a mixture of carotenoids) as described in Schiedt and Liaaen-Jensen, 1995.

$$\textbf{[carotenoids] mg/ml} = (\textbf{Absorbance}_{452} \times 1000)/(A^{1\%}_{1cm} \times 100)$$

The standard deviation was calculated based on the repeated results. Carotenoid amounts

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

were compared to the published data in (Lee *et al.*, 1981).

2.2.4 Protein extraction

Maize protein from endosperm and embryo was extracted according to the method described in Wurtzel (Wurtzel *et al.*, 1987) with modifications: 0.5 g endosperm and embryo tissues were ground in 400 μL 2X SDS buffer [100 mM Tris pH 6.8; 200 mM DTT; 4% (w/v) SDS; 20% (v/v) glycerol; 0.2% (w/v) bromophenol blue] in eppendorf tubes with plastic pestles. The homogenate was centrifuged at 12,000 x g at 4°C for 5 minutes, then the supernatant was transferred to new eppendorf tubes. Extracts were boiled for 5 minutes, centrifuged at 12,000 x g at 4°C for 5 minutes, samples were aliquoted and stored at -20°C for further experiments.

2.2.5 SDS-PAGE electrophoresis

Proteins samples were heated in a boiling water bath for 1 minute, centrifuged at 12,000 x g at 4°C for 2 minutes, supernatant was transferred to new eppendorf tubes, then subjected to a 10% or 12% SDS-PAGE gel (0.75 mm thick x 80 mm wide x 50 mm high) in a Mighty Small II apparatus (Hoefer Scientific Instruments, San Franscisco, CA). Each gel was run in the gel running buffer [25 mM Tris; 192 mM glycine; 1% (w/v) SDS] at 25 mA at room temperature for two hours. After electrophoresis was completed, gels were stained with Coommassie Blue, and destained in 10% (v/v) acetic acid solution.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Duplicate (unstained) gels were incubated in transfer buffer [25 mM Tris; 192 mM glycine; and 20% (v/v) methanol] for 10 minutes at room temperature with gentle shaking on a rotary platform. Proteins were transferred to a piece of nitrocellulose membrane (80mm x 50mm) in a Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA) containing transfer buffer, at 750 mA at 4°C for 1 hour (Sambrook *et al.*, 1989).

2.2.6 Western analysis

Blots were blocked in 3% (w/v) BSA in TBST [10 mM Tris pH 8; 150 mM NaCl; 0.1% (v/v) Tween 20], or 5% (w/v) nonfat dry milk in TBST at room temperature for 30 minutes, probed with primary antisera (see Appendix A-1) diluted in TBST for 30 minutes, washed three times with TBST for 5 minutes each, incubated for 30 minutes with a goat anti-rabbit IgG alkaline phosphate conjugated secondary antibody diluted in TBST (see Appendix A-1), washed three times with TBST for 5 minutes each, and blotted dry with layers of Kimwipes. To detect alkaline phosphate activity, 33 μL of 50 mg/ml NBT and 16.5 μL of 50 mg/ml BCIP were mixed in 5 ml alkaline phosphate buffer (100 mM Tris pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$), and reactions were stopped in deionized water. Blots and stained gels were photographed on an EagleEye II Still Video System (Stratagene, La Jolla, CA).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

2.2.7 Protein concentration determination

To measure total protein concentration, the method of Lowry was carried out using the Lowry Protein Assay Kit (Sigma Diagnostics, St. Louis, MO) following the vendor's protocol, with minor modifications: BSA standard at 25 μg/ml was added to a standard group to create a calibration curve from 25 μg/ml to 1,000 μg/ml of BSA. Absorbance values were measured on a Lambda 2 UV/VIS Spectrophotometer (Perkin-Elmer, Foster, CA).

2.2.8 Protein sequence analysis

Sequence analysis and homology comparisons were performed using PCGene software (Intelligenetics). Amino acid alignments were carried out using the CLUSTAL program (Higgins and Sharp, 1988). PSY protein sequences used for the alignments are as follows: maize [Genbank accession number # U32636, (Buckner *et al.*, 1996)], daffodil [#P53797, (Schledz *et al.*, 1996)], *Arabidopsis* [NCBI #413732, (Bartley *et al.*, 1994)], pepper [EMBL #X68017, (Römer *et al.*, 1993)], *Synecchocystis sp.* [#S45340, (Martinez-Ferez *et al.*, 1994)].

2.2.9 Preparation of *E. coli* lysate

Bacterial strain BL21(DE3) (Novagen Inc., Madison, WI) was inoculated into a 3 L flask

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

containing 500 ml LB and grown at 37°C overnight with gyratory shaking at 250 rpm. The overnight culture was chilled on ice for 10 minutes, contrifuged at 5,000 x g at 4°C for 15 minutes, and the cell pellet resuspended in 50 ml TE buffer (50 mM Tris-HCl pH 8; 2 mM EDTA pH8.0). The suspension was sonicated at 50% duty and output control setting of 5 for 3 x 20 seconds using a Vibra Cell Sonicator (Sonic Material, Inc., Danbury, CT). After sonication, unbroken cells were removed by centrifugation. The concentration of the lysate was determined using the Lowry Protein Assay Kit (Sigma Diagnostics, St. Louis, MO). The lysate stock was stored at -20°C.

2.2.10 Rabbit screening for pre-immune responses

To test if rabbits showed any response to maize endosperm proteins, immunoblots containing 40 μg protein extract from maize endosperm were probed with bleed from ten rabbits prior to immunization. The bleeds were diluted at 1:100 and 1:1,000 in TBST supplemented with *E. coli* BL21(DE3) lysate to a final concentration of 0.3 mg/ml. Two rabbits (#3445 and #3448) that showed the least degree of response were chosen for immunization.

2.2.11 Antigen preparation

A partial B73 maize Y1 cDNA obtained from B. Buckner (clone 12A33A) was subcloned as a BglII-XhoI fragment into the BamHI-XhoI sites of the Novagen vector, pPET23c(+)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(E. Wurtzel, unpublished). After transformation of BL21(DE3)pLysS and induction, approximately 800 μg inclusion body fraction containing the Y1 fusion protein [including 12 aa of T7-Tag and 70% of Y1 protein (aa#124--aa#410), Genbank accession number #U32636] was separated on a 12% SDS-PAGE gel (110 mm wide x 140 mm high x 1.5 mm thick) at 40 mA for 4 hours at room temperature (E. Wurtzel, unpublished). A small gel slice was stained using Commassie blue staining method in order to determine the position of the fusion protein band. To prevent shrinking, the remaining gel portion was incubated in 40% methanol on a shaker. The fusion protein band (about 50% yield) was excised, and immersed in 5 ml of 125 mM NaCl.

2.2.12 Immunization protocol

Each rabbit was injected with 100 μg Y1 fusion protein preparation at week one, two, three, and five. The immununization was conducted by the Lampire Biological Laboratory Inc. (Ottsville, PA). The first test bleed was prepared at week seven. Production bleeds were prepared every month afterwards. For western analysis and localization experiments, bleed from rabbit #3445 (3/30/94, five weeks after immunization) was used.

2.2.13 Titering antisera

Antisera were titered using Y1 fusion protein at concentrations 1 ng, 10 ng, 100 ng, 1 μg

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

and T7-Tag protein (100 ng) spotted onto a piece of nitrocellulose. Dot blot analysis was carried out. Antisera were diluted in TBST at 1:100; 200; 500; 1,000; 2,000; 5,000; 10,000; and 20,000. *E. coli* lysate BL21(DE3) was incubated with the primary and secondary antisera at a final concentration of 0.3 mg/ml for 30 minutes, respectively. The optimal dilution was determined to be 1:10,000 from serum obtained.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 2-1. Maize mutants used in western analysis of PSY expression.
Phenotype indicates the appearance of endosperm which shows the yellow (Y) and white (W) or pale yellow (P) segregation on one ear. Cross represents the origin of the seed materials: self, self cross; sib, sibling cross. B73 and Arg are the lines used to cross with the mutant lines to maintain "isogenic" genetic backgrounds. Arg, high carotenoid line Argentina. JDS, JD Smith, represents another source of mutant seeds.

Locus	Genotype	Phenotype	Progenitor stock	Seed source	Cross
vp5	*vp5/+ (B73)*	seg Y+W	86004	JD Smith	94021-8 self
vp5	*vp5/+ (Arg)*	seg Y+W	86004	JD Smith	94024-3 self
vp5	*vp5-Mum/+ (B73)*	seg Y+W	87-869-7	D. Robertson	94019-11 self
vp5	*vp5-Mum/+ (Arg)*	seg Y+W	87-869-7	D. Robertson	94025-2 self
vp5	*vp5/+ (JDS)*	seg Y+W	93 1123-6	JD Smith 94	94033-4 self
vp5	*vp5-Mum/+ (JDS)*	seg Y+W	93 1178-4	JD Smith 94	94034-7 self
vp2	*vp2/+*	seg Y+W	87433	D. Robertson	94001 self
w3	*w3/+*	seg Y+W	89-723-2	Maize Coop	94032-23 self
vp9	*vp9/+*	seg Y+P	89-2169-1	Maize Coop	94028-17 self
vp9	*vp9-Mum/+*	seg Y+P	87436	D. Robertson	94027-13 self
y3	*y3/+*	seg Y+P	82-269-1	Maize Coop	94030-5 self
y9	*y9/+*	seg Y+P	86-1659-1	Maize Coop	94031-7 self
y11	*y11/+*	seg Y+P	89-671-1	Maize Coop	94037-10 self
y1	*y1/y1*	W	89-656-3	Maize Coop	91050-17 sib
y1	*Y1/Y1*	Y	89-1855-1	Maize Coop	91051-4 sib
y1	*y1/Y1*	seg Y+W	89-656-3 (*y*) 89-1855-1 (*Y*)	Maize Coop	91050 X 91051-10
y1	*Y1/y1*	seg Y+W	89-656-3 (*y*) 89-1855-1 (*Y*)	Maize Coop	91051-10 X 91050
lw1	*lw1/+*	seg Y+P	89-682-2	Maize Coop	94029-17 self
wc1	*wc1/+*	seg W+Y	91-1871-1	Maize Coop	94045A-3 self
wc1	*wc1/+ (B73)*	seg W+Y	93-2405-1	Maize Coop	94042A X 94004

JD Smith: Texas, A&M University
D. Robertson: Iowa State University, Ames, Iowa

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

2.3 Results

2.3.1 Carotenoid analysis in developing endosperm of maize

To establish when carotenoid accumulation takes place during endosperm development, total colored carotenoids were measured according to the characteristic wavelength of 452 nm from three lines varying in amount of total carotenoids. The carotenoid content in the "orange endosperm" Argentina line (Arg) after 10 DAP was higher than the content in either B73 or A632 lines (Figure 2-1). In the Argentina line, the carotenoid level of endosperm at 30 DAP was 11±0.5 μg/g fresh weight (see Figure 2-1A). However, the carotenoid content was 5.5±1 μg/g fresh weight in B73 endosperm at 30 DAP (Figure 2-1B), and 5±3 μg/g fresh weight in A632 endosperm at 30 DAP (Figure 2-1C). The accumulation of carotenoid in the endosperm appeared to start about 10 DAP for all the three lines. From 10 DAP to 20 DAP, the accumulation of carotenoid in the Argentina endosperm increased dramatically, and the carotenoid content reached a maximum level at 20 DAP. In comparison, the carotenoid content in B73 and A632 lines increased gradually from 10 DAP to 30 DAP, and the carotenoid level (5 μg/g fresh weight) at 30 DAP of these yellow lines was only about one half of the carotenoid content of the Argentina line (11 μg/g fresh weight) after 20 DAP. But at 20 DAP, which is about the stage when fresh corn is eaten, the difference between the Argentina and the B73/A632 lines was about four fold.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

2.3.2 Production of a polyclonal antiserum against maize PSY

Because the antibody from a dicot PSY (Capsicum) (see Appendix A-2) did not recognize maize PSY protein (data not shown), a polyclonal antiserum against maize PSY was developed. The specificity of the antiserum was tested using both *Y1* fusion protein and maize endosperm protein extract in the western analysis (Figure 2-2). The polyclonal antiserum detected a 33.3 kDa single antigenic signal (lane 1 in Figure 2-2), that is the same size as the recombinant bacterial protein containing a truncated maize PSY fused to the N-terminus of the T7 gene 10 product (E. Wurtzel, unpublished). In maize endosperm, anti-PSY antiserum recognized a specific antigen, and the size was about 38 kDa according to the migration pattern on a denatured SDS-PAGE gel (lane 2 in Figure 2-2). Sequence homology analysis of PSY between four genes of higher plants and one strain of cyanobacterium showed the additional amino acid residues--considered as transit sequences in the PSY proteins of higher plants (Figure 1-5). Molecular weights of PSY precursor proteins were between 40 kDa and 48 kDa, while mature PSY proteins were between 38 kDa and 42 kDa. The transit sequences are highly diverged among different PSY protein precursors. The molecular weight of the unprocessed maize PSY protein is estimated about 46.7 kDa, and the estimated size for the mature PSY protein is 37.0 kDa calculated by PCGene. Therefore, the 38 kDa PSY antigen detected in maize endosperm by western analysis may represent the mature PSY found in plastids.

The *Y1* gene has been cloned and the sequence shown to encode PSY on the basis of

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

sequence homology with other PSY proteins (Buckner *et al.*, 1996). To demontrate that the anti-PSY antiserum was specific to the PSY protein, the signals were compared in endosperms of *Y1* and *y1* genotypes. Western analysis showed that PSY expression is reduced dramatically in the PSY mutant endosperm (*y1y1y1*) in comparison to the normal endosperm (*Y1Y1Y1*) as shown in Figure 2-3A, whereas a duplicate blot probed with an anti-Sh antiserum showed the same level of accumulation of sucrose synthase regardless of *Y1* genotype (Figure 2-3B). This result shows the protein detected by the anti-PSY antiserum is related to the *Y1* locus. Results in Figure 2-3 show that the polyclonal antiserum against PSY is specific to the PSY protein in maize endosperm. Therefore, this antiserum can be used to study PSY expression in maize.

To test whether other loci regulate PSY expression, a series of western analyses were carried out using the anti-PSY antiserum against proteins extracted from several carotenoid mutant endosperms compared with normal segragating endosperms. The viviparous mutants *vp2*, *vp5*, *vp5-Mum*, *vp9*, *vp9-Mum*, and *vp12* (data not shown for *vp12*), did not show any difference of PSY expression at the protein level compared with the normal segregating endosperms (Figure 2-4). Other mutants *lw1*, *w3*, *y3*, *y9*, and *y11* were also examined for PSY expression (Figure 2-4). The dominant White Cap mutant (*wc1*) was tested for PSY expression as shown in Figure 2-4. Results revealed that PSY protein was expressed in all the mutant endosperm and the normal endosperm tested. Therefore, none of these loci appear to regulate expression of PSY in endosperm.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

2.3.3 Dosage effect of Y1 on PSY expression

It is known that *Y1* has a dosage effect on carotenoid accumulation in maize endosperm (Randolph and Hand, 1940). In the triploid maize endosperm tissue, a yellow endosperm is of one of three genotypes *Y1Y1Y1*, *Y1Y1y1* and *Y1y1y1*. With the increase in number of *Y1* alleles, the carotenoid level also increases (Randolph and Hand, 1940). Difference in coloration of the endosperm can be observed from the seeds possessing various genotypes (data not shown). To test if *Y1* has a dosage effect on PSY expression at the protein level, I examined the PSY accumulation in endosperms having various copies of *Y1* or *y1* alleles. As shown in Figure 2-5A, PSY expression was constant in the endosperm bearing one, two or three *Y1* alleles, but barely detectable in the endosperm of *y1y1y1* genotype. Using anti-Sh as a control (shown in Figure 2-5B), the expression of Sh remained constant in the endosperm of all the four genotypes. Results show that the *Y1* dosage has no effect on PSY protein level.

2.3.4 Temporal regulation of PSY expression in developing endosperm

To examine if PSY expression is regulated during development of the maize endosperm, western analysis was carried out in three maize line lines, B73, A632, and Argentina grown during two field seasons, 1993 and 1996. Figure 2-6A shows the expression of the 38 kDa PSY protein in developing endosperm of B73, a yellow endosperm line grown in 1993. For unfertilized ovules and developing endosperms collected between 5 to 40 DAP,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the expression starts to appear at 10 DAP, reaches a peak level at 15 DAP, then gradually decreases from 20 DAP to maturity. Using anti-Sh to probe a replicate gel, the accumulation of the level of the 96 kDa Sh protein markedly increased from 10 to 15 DAP and further increased until maturity (Figure 2-6B). Equivalent amount of protein was loaded for each developmental stage, as shown in Figure 2-6C; a replicate gel was stained with Commassie Blue to show the amount of protein at each time point. The same experiment was performed with another yellow endosperm line, A632. As shown in Figure 2-7A, PSY protein accumulation appeared to follow a similar pattern to the one in B73. PSY protein started to accumulate at 10 DAP, reached a maximal level at 15 DAP, then declined after 20 DAP. As seen for B73, using anti-Sh, the amount of the 96 kDa Sh protein increased from 10 to 15 DAP but did not change greatly after 15 DAP as shown in Figure 2-7B. Figure 2-7C shows the stained replicate gel indicating that approximately equal amounts of protein were loaded for each time point. I also tested PSY expression in the high carotenoid line (Argentina). Results from samples of 1993 are shown in Figure 2-8. PSY expression was detected at 10 DAP, reached a maximal level between 15 to 20 DAP, and remained constant thereafter (Figure 2-8A). Figure 2-8B shows the expression of Sh, which was similar to that in B73 and A632.

To test the reproducibility of PSY protein accumulation during endosperm development in different years, we repeated the experiment using plants grown in 1996. In the B73 endosperms collected between 10 and 40 DAP, the level of PSY protein accumulation remained high from 10 and 20 DAP, then gradually decreased from 25 DAP onward (see

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 2-9A). Using anti-Sh to probe a replicate gel, the accumulation of the 96 kDa Sh protein appeared to decrease during endosperm development (see Figure 2-9B). A stained gel showed the amount of proteins for each DAP as shown in Figure 2-9C. When examining 1996 samples of A632 endosperms collected between 10 and 40 DAP, levels of PSY protein increased from 10 DAP to 15 DAP, then reduced after 25 DAP (see Figure 2-10A). Using anti-Sh antiserum to probe a replicate blot, Sh protein accumulation reduced gradually during seed development (see Figure 2-10B). Figure 2-10C showed a stained gel indicating similar amount of protein for each DAP. I also tested PSY expression in the Argentina line using 1996 endosperms collected between 10 and 40 DAP. PSY protein accumulation reached a peak level between 15 and 20 DAP, and remained constant throughout endosperm development as shown in Figure 2-11A. The accumulation of Sh protein decreased throughout the development as shown in Figure 2-11B. A stained gel showed the amount of protein loaded for each time point (see Figure 2-11C). Induction between 10 and 15 DAP was seen for Sh protein accumulation in developing endosperms collected in 1993, whereas 1996 samples did not show this expected induction (Wurtzel *et al.*, 1987). This suggests that 10 DAP samples from 1996 may have been overloaded, hence overestimating the level of PSY at 10 DAP in the 1996 samples.

Comparing the PSY protein accumulation between yellow lines B73/A632 and the high carotenoid line Argentina, the results clearly showed that PSY expression in Argentina line is different from those in the other two yellow lines B73 and A632 using samples

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

from both 1993 and 1996. In all three lines, PSY was not detectable until 10 DAP. PSY levels decreased after 20 DAP in B73 and A632 endosperms, but remained constant in Arg endosperms after 20 DAP.

2.3.5 Expression of PSY in maize endosperm, embryo and leaf

To examine PSY expression in different tissues of maize, proteins were extracted from endosperm and embryo of B73 line at 20 DAP and from seedlings of B73 line at the 3 to 5 leaf stage. For equivalent amounts of total proteins loaded, western analysis showed that the level of PSY expression is high in endosperm, barely detectable in embryo, and undectable in leaf (Figure 2-12). This result suggests that PSY expression is regulated in a tissue specific manner among the tissues of endosperm, embryo, and leaf.

2.4 Discussion

2.4.1 Carotenoid accumulation in maize endosperm

In maize, carotenoids accumulate in various tissues such as leaves, embryos and endosperms. In leaves, the carotenoid content ranges about 70 to 1000 μg/g fresh weight determined spectrophotometrically (Mayfield *et al.*; Gavazzi *et al.*, 1974; Bachmann *et al.*, 1973; Robertson and Anderson, 1961). In embryos, total carotenoids are approximately 1.6 μg/g fresh weight (Yu and Wurtzel, unpublished). To compare the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

carotenoid content of maize endosperm with embryos and leaves, carotenoid levels were examined in developing endosperm of three lines, B73, A632, and Arg. B73 and A632 are two yellow endosperm lines, and carotenoid levels in mature endosperms were about 5 μg/g fresh weight. Arg is a high carotenoid line, and endosperms show an "orange" color. When total carotenoids were assayed, the maximal level was about 11 μg/g fresh weight in mature endosperms. One question can be raised–what is the relationship between carotenoid accumulation in various tissues and PSY protein level? The following sections of discussion will answer the above question.

2.4.2 Characterization of anti-PSY antiserum

To compare carotenoid accumulation with PSY accumulation, a polyclonal antiserum against maize PSY was generated. Using this antiserum, a single antigenic signal was detected from the maize endosperm protein extract. Western analysis showed that the antigen was reduced in *y1* mutant endosperm (*y1y1y1*) and present in *Y1* endosperm (*Y1Y1Y1*). Since PSY is the product of the maize *Y1* locus, this result shows that the antigen detected by the anti-PSY antiserum represents the maize PSY protein. The apparent molecular weight of this antigen was 38 kDa. As deduced from the DNA sequence, the PSY precursor is composed of 410 aa residues, and its molecular mass is predicted to be about 46.5 kDa. Based on comparison with the sequence of the *Synechocystis* PSY, the putative transit peptide of maize PSY should consist of 73 aa residues or 8 kDa, and the mature PSY would be predicted to be 337 aa residues or 37

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

kDa. The estimated size is consistent with the 38 kDa as calculated from the SDS-PAGE gel. Therefore, the antigen detected is predicted to be the processed form found in plastids.

2.4.3 PSY expression in selective mutants of maize

There are several recessive and dominant mutations that block carotenoid biosynthesis at various steps, thus leading to accumulation of different carotenoid intermediates (Neil *et al.*, 1986; Anderson and Robertson, 1960). Using western analysis, endosperms of selected mutants were analyzed to determine whether there was any affect on PSY expression. Results showed that there was no difference in the PSY protein level in endosperms of the mutants and segregating normal endosperms. White Cap (*Wc1*) is a dominant white allele. Kernels are the whitest with three dominant alleles, and appear more yellow with fewer dominant alleles. Results showed that PSY protein level was not affected by this allele. Similar results were obtained from other mutants, such as *lw1, w3, y3, y9* and *y11*. Results demonstrate that these loci do not regulate PSY expression. It has been reported that the enzymatic activity of PSY in plastids requires certain auxiliary factors such as molecular chaperonin and membrane components (Bonk *et al.*, 1997). These loci may encode regulatory proteins governing protein modification and/or translocation.

It is known that Y1 has dosage effect on carotenoid accumulation in maize endosperms

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(Randolph and Hand, 1940). Western analysis showed that PSY protein was reduced in *y1y1y1* endosperm, but showed the equivalent amounts in protein extracts from endosperms bearing various numbers of the *Y1* allele. Therefore, the increased amount of carotenoid in endosperms with various genotypes may be due to the elevated activity of PSY but not due to differences in the PSY protein level.

2.4.4 Tissue specific control of PSY expression in maize

Carotenoid biosynthesis takes place in various tissues. The significant accumulation of carotenoids is tissue specifically regulated in other organisms (Sandmann, 1994). Both PSY transcript and protein levels increase when carotenoid level is increased in a particular tissue. In maize, results show that the level of PSY expression is higher in endosperm, lower in embryos, and not detectable in leaves. Given that only one PSY gene has been detected in maize (Buckner *et al.*, 1996), these results suggest that the PSY expression may be regulated tissue-specifically. When comparing differences in carotenoid levels in endosperm and the adjacent embryo tissue, there is a correlative difference in PSY level. However, the high level of carotenoids in leaves does not correlate with an increased level of PSY, rather, PSY in leaves is surprisingly undetectable.

2.4.5 Temporal regulation of PSY expression in maize endosperm

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

GGPP is a common precursor to several biochemical pathways in higher plants. This compound must be channeled into those pathways. The first step specific to the carotenoid biosynthesis is the formation of phytoene through head-to-head condensation of two molecules of GGPP, catalyzed by PSY. It is important to understand how PSY expression is regulated. During maize endosperm development, the number of the amyloplasts triples (McCullough *et al.*, 1989), and the carotenoid level increases 5-10 fold between 10-20 DAP in various lines. In two yellow lines, B73 and A632, total colored carotenoid content continues to increase after 20 DAP until maturity. RT-PCR analysis showed that *Psy* transcript accumulation increased between 10 to 15 DAP for both B73 and A632. In the Argentina line, the *Psy* transcript level was constant between 10-20 DAP (Li, 1998). The high level of *Psy* transcripts at 10 DAP may contribute to a higher level of PSY and corresponding increased carotenoid accumulation during endosperm development in the Arg line. Western analysis showed that PSY accumulation for 1993 endosperms of B73 and A632 started at 10 DAP, reached the highest amount between 15-20 DAP, then declined after 25 DAP. Comparison of PSY transcript and total colored carotenoid accumulation indicates that the lower level of *Psy* transcript at 10 DAP may be responsible for lower amount of PSY which would lead to lower carotenoid accumulation in yellow maize endosperms; the higher amount of *Psy* transcript at 10 DAP as seen in the Arg line, might contribute to the higher level of PSY and corresponding higher levels of carotenoid accumulated in Arg endosperms. In addition, comparison of PSY protein accumulation and the total carotenoid accumulation indicates that the lower level of PSY protein after 20 DAP may be responsible for lower amount of

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

carotenoid accumulation in maize endosperms at the developmental stages later than 25 DAP. When analyzing the Argentina line, the continuous high level of PSY beginning at 10 DAP prompts the carotenoid accumulation at the maximal amount throughout endosperm development. Therefore, PSY may be the rate limiting enzyme during the early developmental stage (between 10 to 20 DAP) in the carotenoid biosynthetic pathway in maize endosperm.

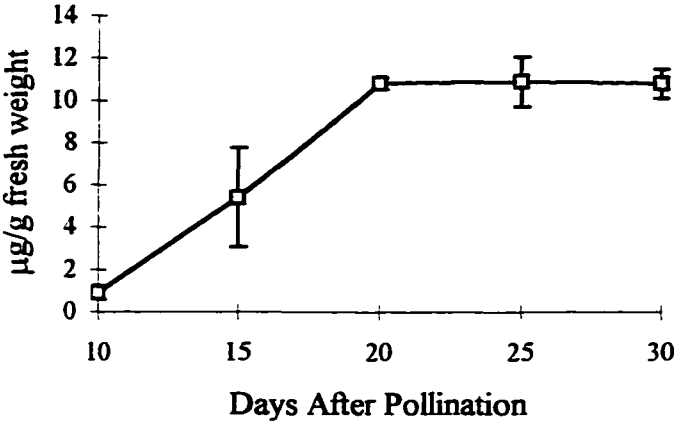
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 2-1. Carotenoid analysis of developing endosperms.

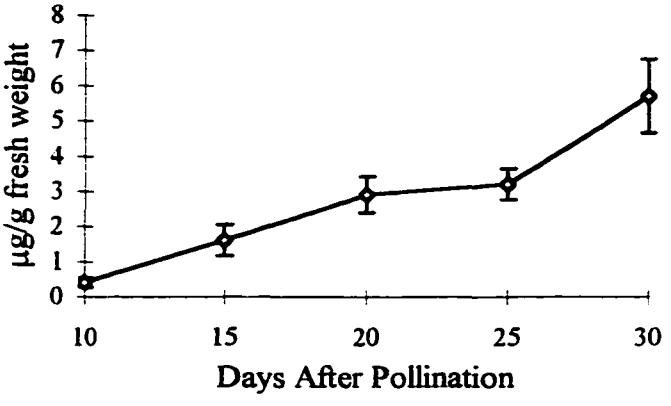
Carotenoids were extracted from endosperm dissected at various DAP from (A)B73, (B)A632 and (C) Argentina lines. Total carotenoid content is represented by μg/g fresh weight. Each point represents the average of three or four replicas. Bars represent standard error for each time point. Samples were from 1993, except for the Argentina sample at 10 DAP that was from 1994.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

A: Arg



B: B73



C: A632

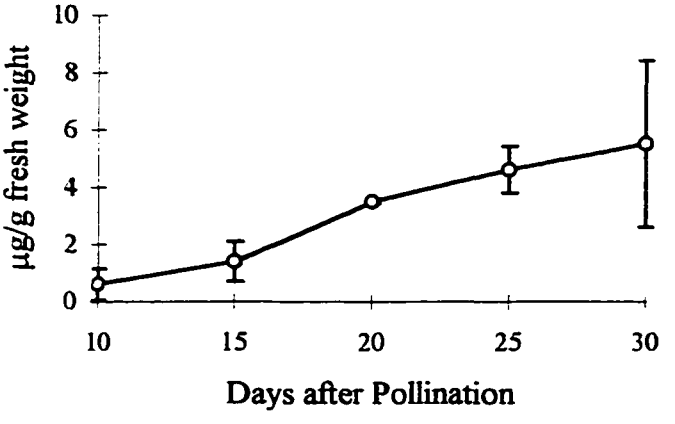


Fig. 2-1

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

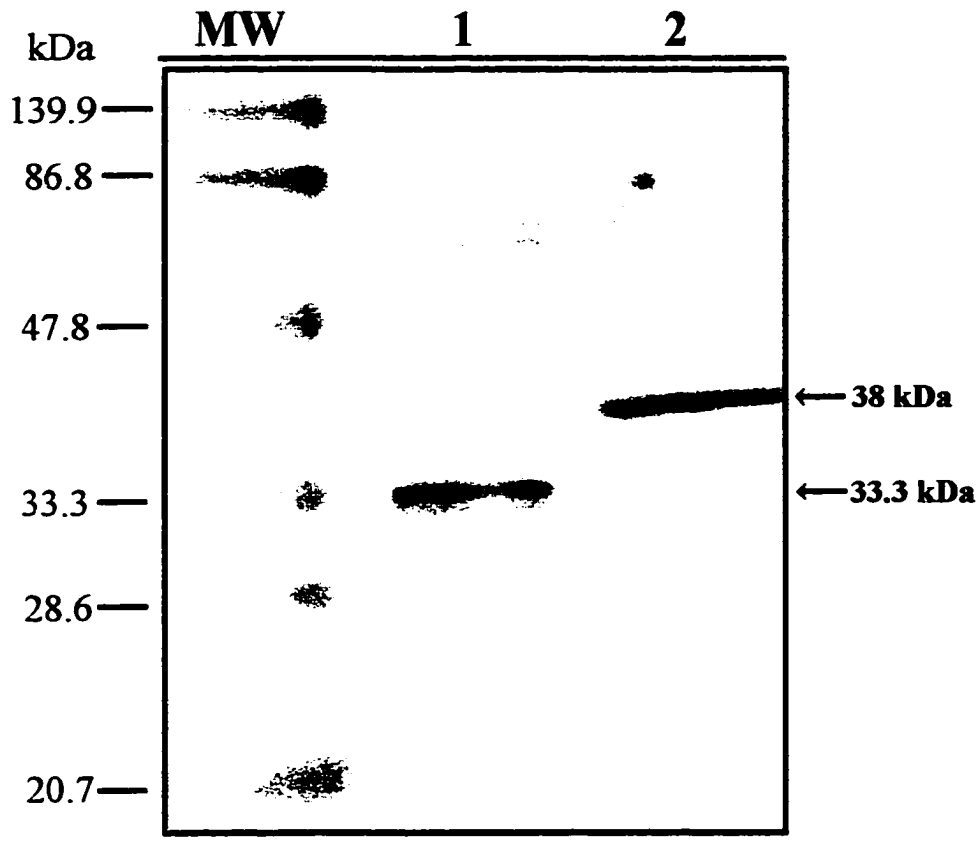


Figure 2-2. Western analysis--characterization of the anti-phytoene synthase antiserum. Lane 1, Y1 fusion protein, purified from *E. coli*, that was used to immunize rabbits; lane 2, total protein extract of maize endosperm dissected at 20 DAP; MW, protein molecular weight standards in kDa. Immunoblot was probed with anti-maize PSY polyclonal antiserum (see also Appendix A).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

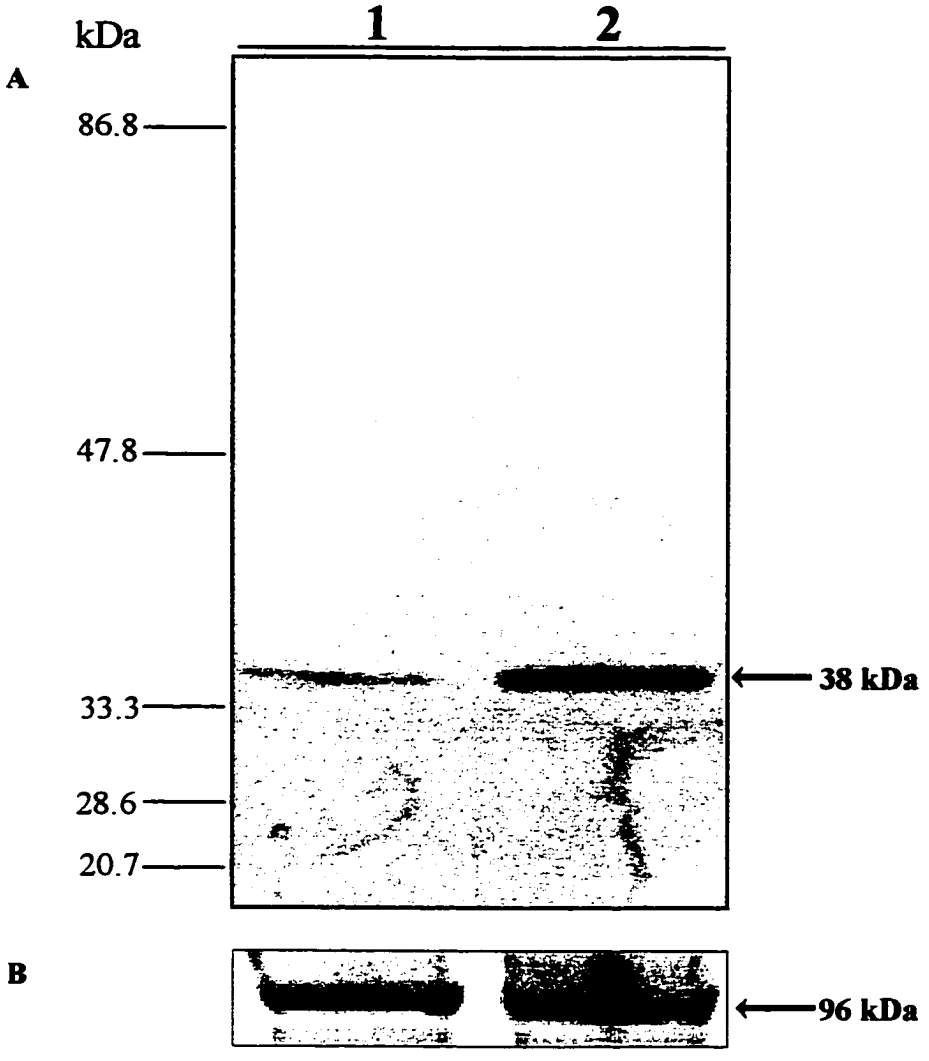


Figure 2-3. Western analysis--Phytoene synthase expression in normal (*Y1*) and mutant (*y1*) maize endosperm.
Lane 1, protein extracted from white endosperm (*y1y1y1)* at 20 DAP; lane 2, protein extracted from normal yellow endosperm (*Y1Y1Y1)* at 20 DAP.
(A) Immunoblot probed by anti-PSY antiserum.
(B) Immunoblot probed by anti-Sh antiserum contained a duplicate set of samples as in (A).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 2-4. Western analysis--phytoene synthase accumulation in segregating maize endosperms of normal and mutant carotenoid phenotypes.

Proteins were extracted from 20 DAP normal and mutant endosperms segregating on ears of various genotypes shown at the top of each panel. Immunoblots were probed with the anti-PSY antiserum. (+) represents the dominant phenotype, (-) represents the recessive phenotype for the endosperm with each given genotype. Each panel represents a separate gel except for the *lw*/+ samples added to the left of the top panel.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

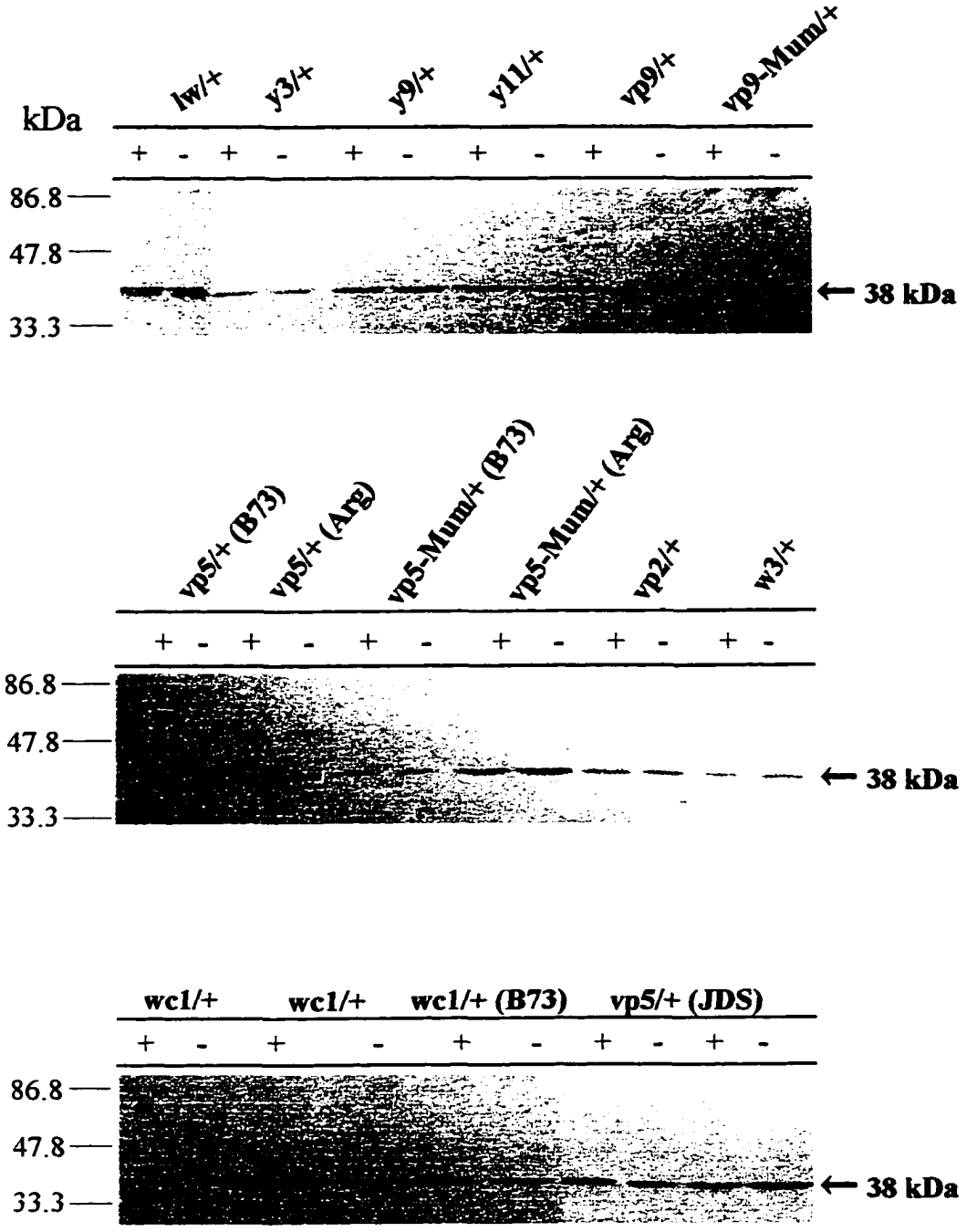


Fig. 2-4

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

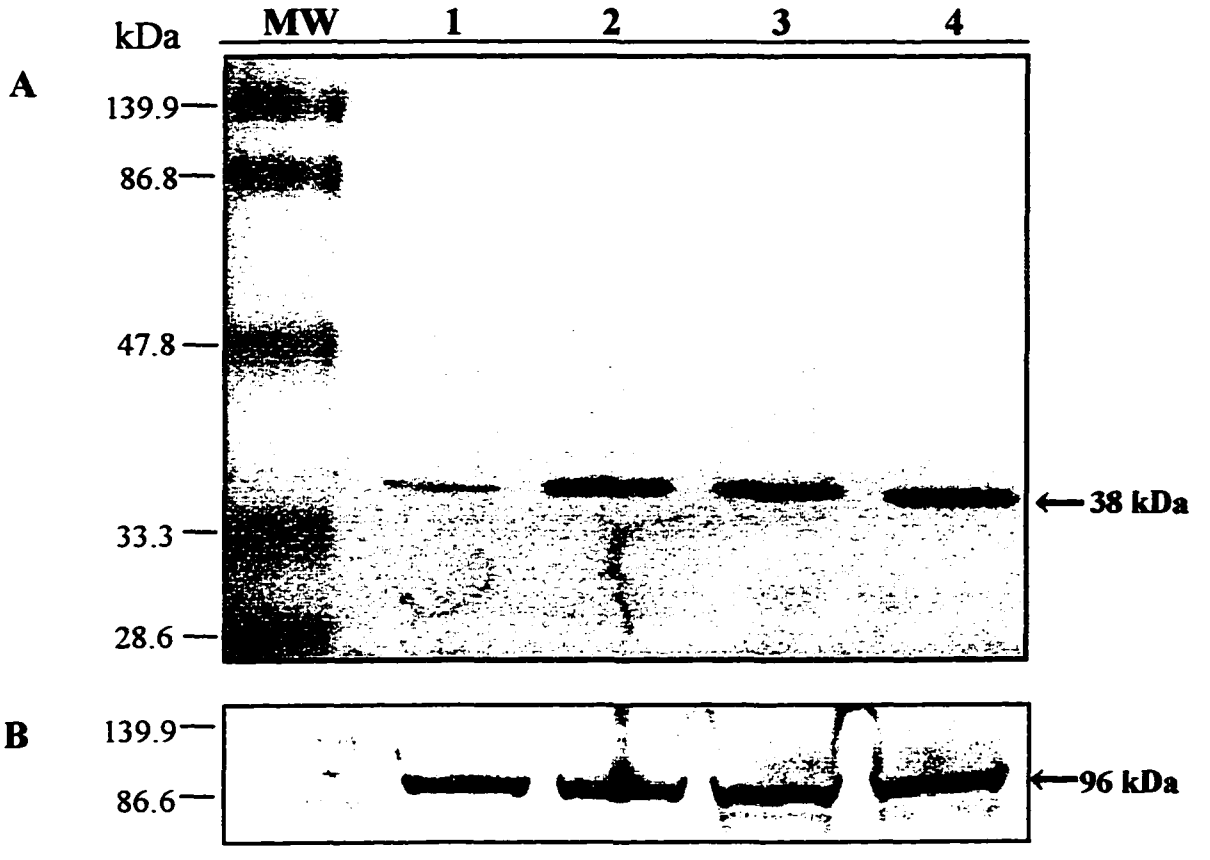


Figure 2-5. Western analysis--*Y1* dosage effect on PSY expression.
Proteins were extracted from endosperm harvested at 23 DAP that contained various copies of *Y1* or *y1* allele.
Genotypes of endosperm: Lane 1, *y1y1y1*; lane 2, *Y1y1y1*; lane 3, *Y1Y1y1*; lane 4, *Y1Y1Y1*.
(A) Immunoblot probed by anti-PSY antiserum.
(B) Immunoblot probed by anti-Sh antiserum contained a duplicate set of samples as (A).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 2-6. Western analysis--PSY expression in developing endosperm of B73 maize grown in the 1993.

Replicate gels containing proteins extracted from developing endosperm dissected at various DAP shown by corresponding numbers at the top. * indicates that maternal tissue may also be present in the endosperm sample. MW indicates protein molecular weight standards in kDa. 0 represents unfertilized ovules.

(A) Immunoblot probed by anti-PSY antiserum.

(B) Immunoblot with probed by anti-Sh antiserum.

(C) Coomassie blue stained SDS-PAGE gel.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

B73
1993 Field

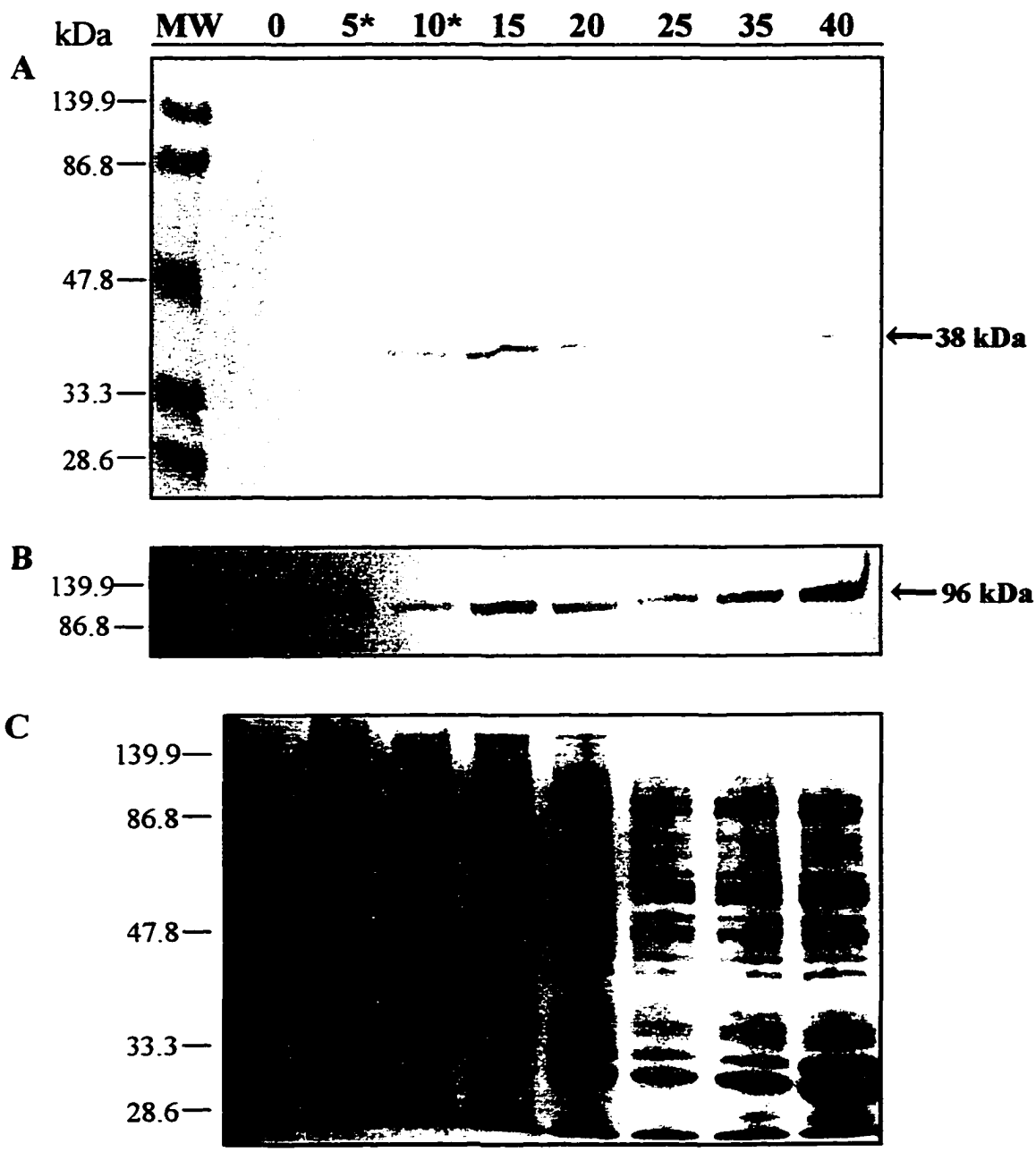


Fig. 2-6

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 2-7. Western analysis--PSY expression in developing endosperm of A632 maize grown in 1993.

Replicate gels containing proteins extracted from developing endosperm dissected at various DAP shown by corresponding numbers at the top. * indicates that maternal tissue may also be present in the endosperm sample. MW indicates protein molecular weight standards in kDa. 0 represents unfertilized ovules.

(A) Immunoblot probed by anti-PSY antiserum.

(B) Immunoblot probed by anti-Sh antiserum.

(C) Coomassie blue stained SDS-PAGE gel.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

A632
1993 field

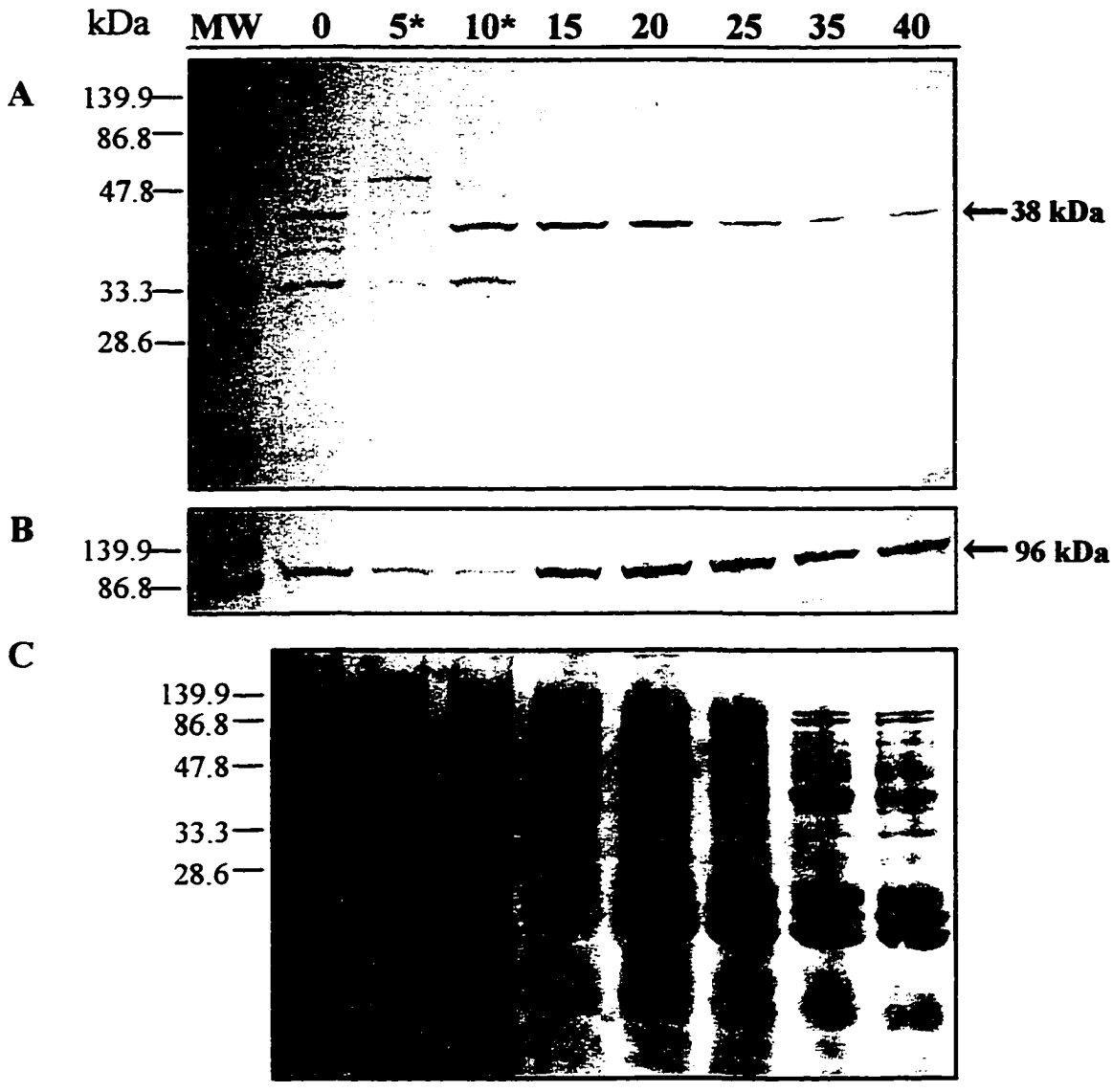


Fig. 2-7

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 2-8. Western analysis--PSY expression in developing endosperm of Argentina maize grown in 1993.

Replicate gels containing proteins extracted from developing endosperm dissected at various DAP shown by corresponding numbers at the top. * indicates that maternal tissue may also be present in the endosperm sample. MW indicates protein molecular weight standards in kDa. 0 represents unfertilized ovules.

(A) Immunoblot probed by anti-PSY antiserum.

(B) Immunoblot probed by anti-Sh antiserum.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Arg
1993 Field

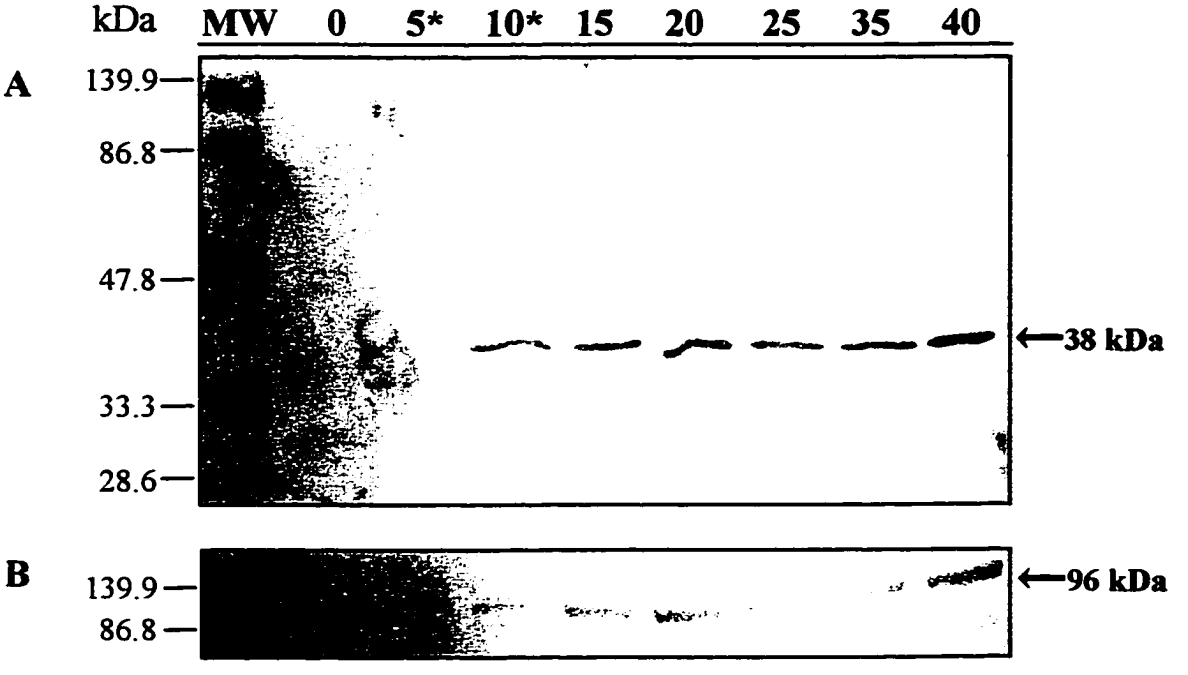


Fig. 2-8

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 2-9. Western analysis--PSY expression in developing endosperm of B73 maize grown in 1996.

Replicate gels containing proteins extracted from developing endosperm dissected at various DAP shown by corresponding numbers at the top. * indicates that maternal tissue may also be present in the endosperm sample. MW indicates protein molecular weight standards in kDa.

(A) Immunoblot probed by anti-PSY antiserum.

(B) Immunoblot probed by anti-Sh antiserum.

(C) Coomassie blue stained SDS-PAGE gel.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

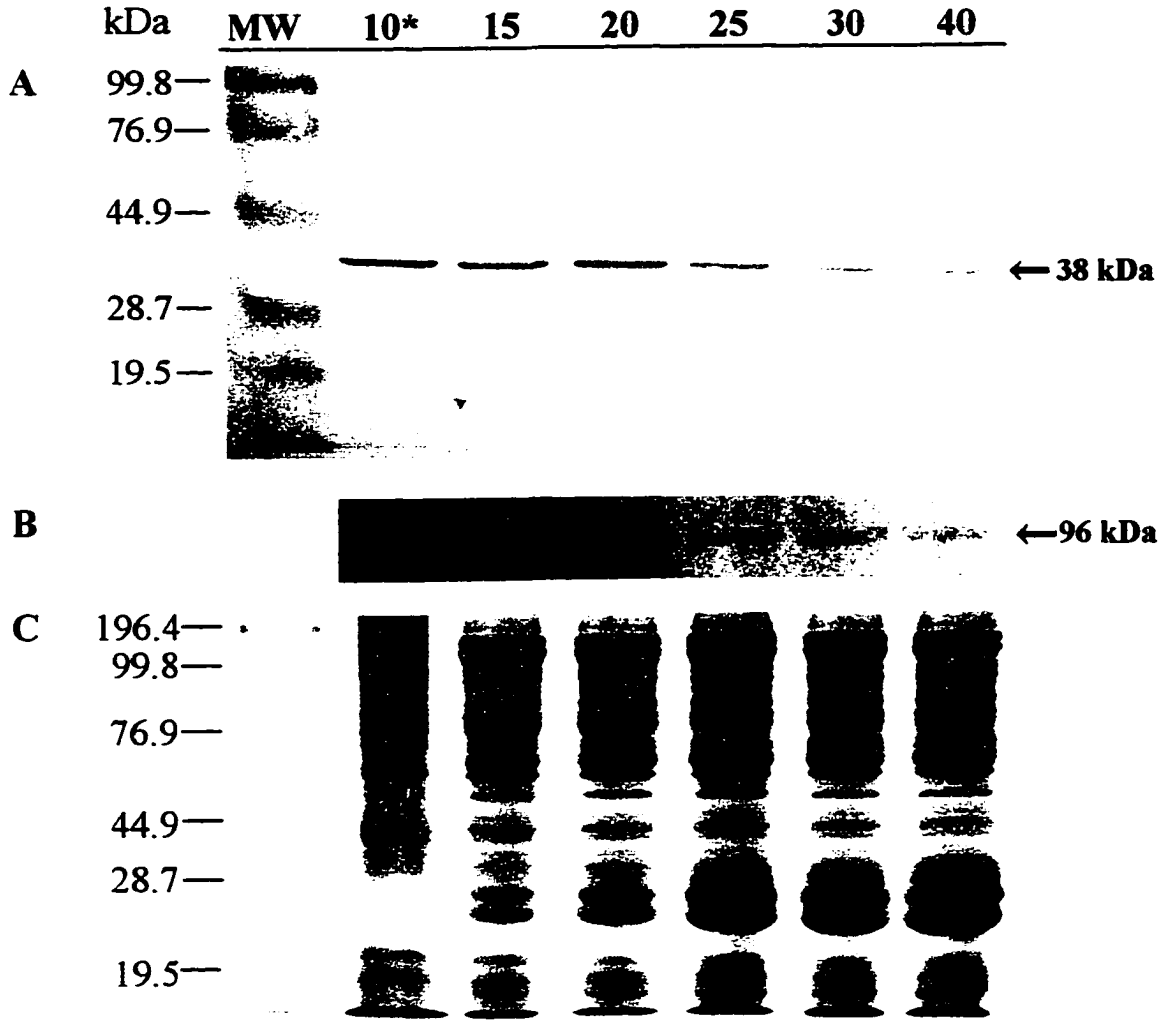


Fig. 2-9

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 2-10. Western analysis--PSY expression in developing endosperm of A632 maize grown in 1996.

Replicate gels containing proteins extracted from developing endosperm dissected at various DAP shown by corresponding numbers at the top. * indicates that maternal tissue may also be present in the endosperm sample. MW indicates protein molecular weight standards in kDa.

(A) Immunoblot probed by anti-PSY antiserum.

(B) Immunoblot probed by anti-Sh antiserum.

(C) Coomassie blue stained SDS-PAGE gel.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

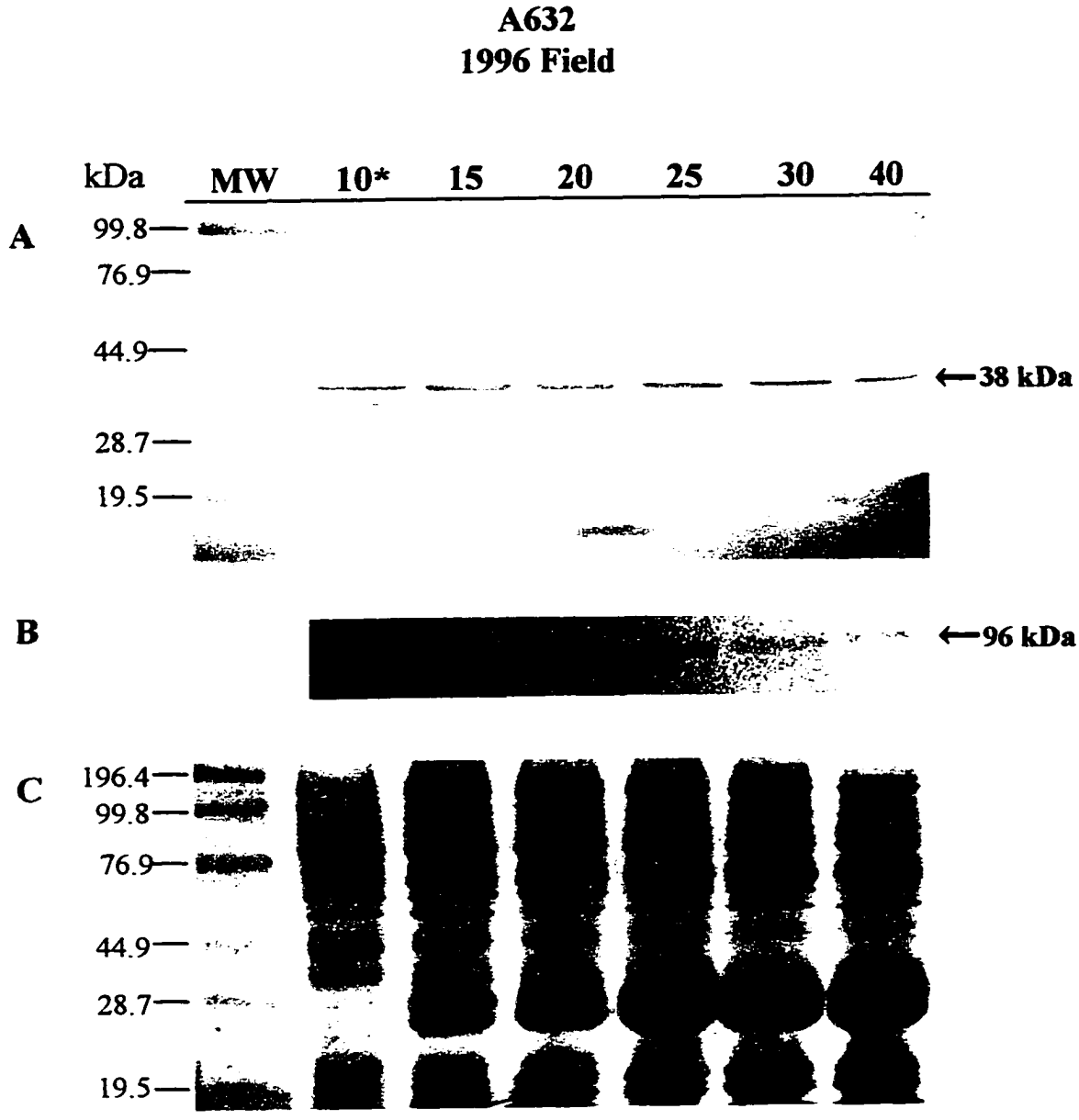


Fig. 2-10

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 2-11. Western analysis--PSY expression in developing endosperm of Argentina maize grown in 1996.

Replicate gels containing proteins extracted from developing endosperm dissected at various DAP shown by corresponding numbers at the top. * indicates that maternal tissue may also be present in the endosperm sample. MW indicates protein molecular weight standards in kDa.

(A) Immunoblot probed by anti-PSY antiserum.

(B) Immunoblot probed by anti-Sh antiserum.

(C) Coomassie blue stained SDS-PAGE gel.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

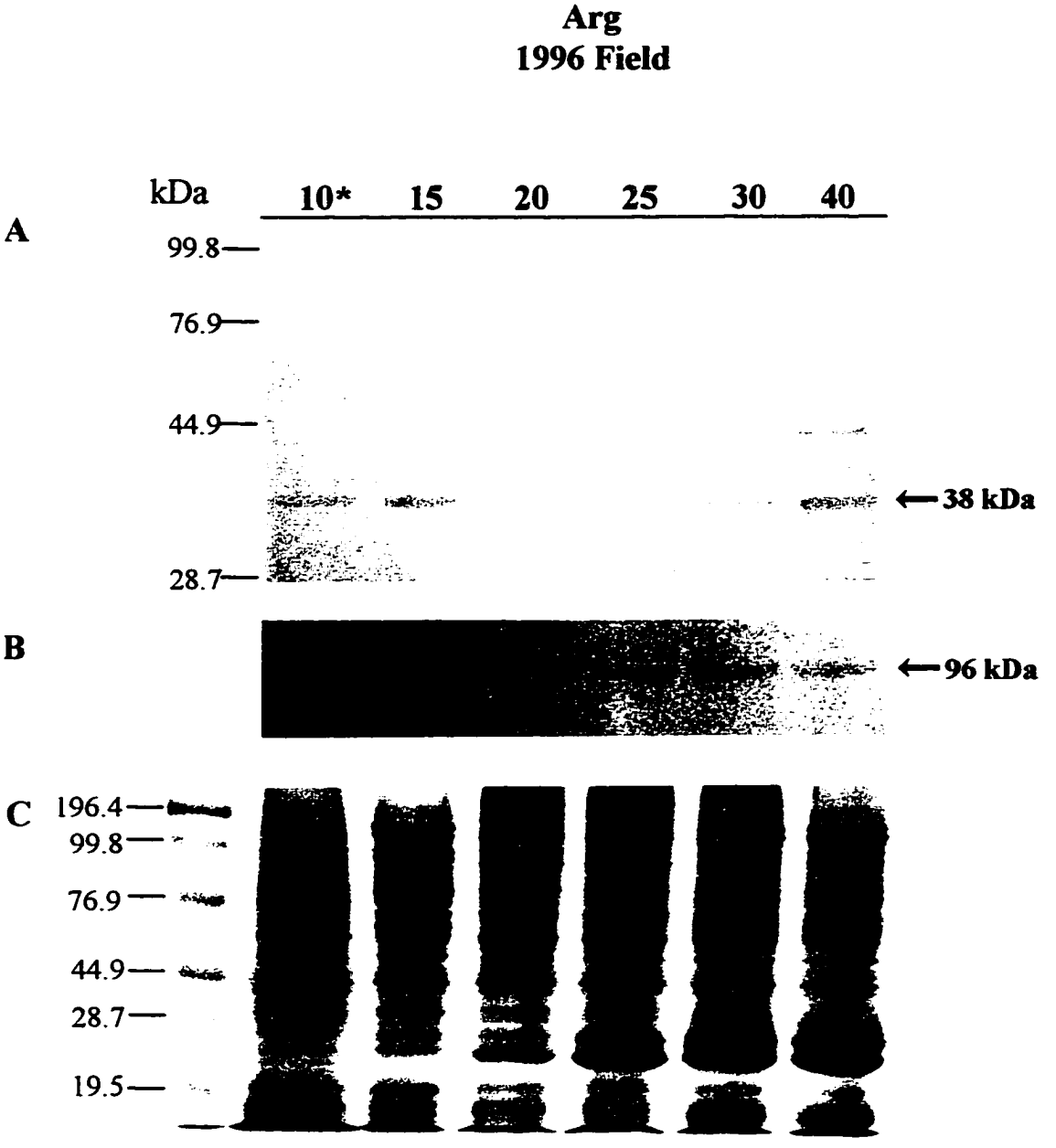


Fig. 2-11

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 2-12. Western analysis--Phytoene synthase expression in various tissues of maize B73.

Proteins were extracted from tissues of seedlings at the 3-5 leaf stage (lane 1); seed at 20 DAP (lane 2); endosperm at 20 DAP (lane 3); embryo at 20 DAP (lane 4). Twenty μg protein extracts were loaded in each lane. Immunoblot was probed by anti-PSY antiserum. The arrow points to the PSY antigen.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

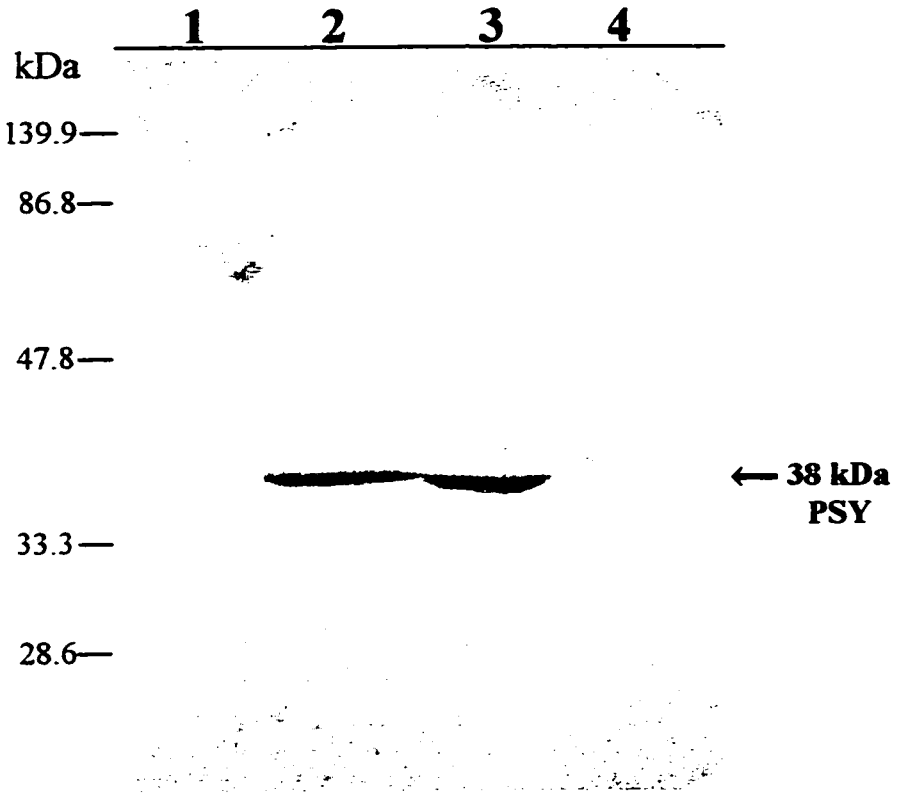


Fig. 2-12

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter 3. Expression of phytoene synthase in rice endosperm

3.1 Introduction

Rice is one of the leading food crops in the world and lacks endosperm carotenoids. Rice provides a large proportion of the total nourishment of the worldwide population. Rice endosperm does not accumulate any carotenes nor other carotenoid intermediates. It has been reported that carotenes and tocopherol (vitamin E) are present in rice embryo and aleurone layers (bran and polish) (Sechi and Rossi-Manaresi, 1958). Lipids such as squalene and sterols also have been found in rice bran and embryo (Akiya, 1962). In immature rice endosperm (10-15 DAP), a feeding experiment with [1-^{14}C] IPP showed incorporation of radioactivity into the C_{15} farnesol, the C_{20} GGPP and the C_{30} squalene, but not into any C_{40} carotenoids. Using [^{14}C] GGPP and [^{14}C] phytoene, no carotenoids were detected. In rice endosperm transformed with a daffodil *psy* cDNA, colorless phytoene accumulated (Burkhardt *et al.*, 1997). Rice does possess the carotenogenesis genes in its genome, since photosynthesis takes place in leaves and other green tissues where carotenoids are essential components in the chloroplast. Both *psy* and *pds* genes are expressed in rice leaf, embryo and endosperm tissues at the transcriptional level which has been analyzed using RT-PCR (Wurtzel *et al.*, 1996). The reason why rice endosperm does not accumulate any carotenoids still remains unclear.

The maize-specific anti-PSY antiserum was used to test whether PSY is expressed in rice

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

endosperm tissue and if so, how is the expression pattern compared with PSY expression in maize. Furthermore, PSY expression in rice leaf and embryo tissues, which accumulate carotenoids, were also examined.

3.2 Materials and Methods

3.2.1 Rice and maize materials

Rice (*Oryza sativa*) IR36 line (from S. McCouch, Cornell University) was grown in the research greenhouse at Lehman College (Bronx, New York). Controlled pollination was performed (Yoganathan, 1998). Seeds were harvested at 5, 7, 10, 15, and 20 DAF. The seed coat was removed, and then the endosperm and embryo were separated. For mature seeds, some maternal tissue may have remained since the seed coat could not be removed entirely. Tissues were quickly frozen in liquid nitrogen, and then stored at -80°C. The seeds of the variety Cypress, at various stages of development, were provided by Dr. Steve Linscombe (Louisiana Rice Research Station, Crowley, LA). Maize B73 endosperm was used as described in Chapter 2.

3.2.2 Protein extraction

Rice proteins from seed, endosperm and embryo were extracted according to the method described in Wurtzel (Wurtzel *et al.*, 1987) with modifications: 0.5 g endosperm/seed or

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

0.2 g embryo tissues were ground in 500 μL or 200 μL 2X SDS buffer [100 mM Tris pH 6.8; 200 mM DTT; 4% (w/v) SDS; 20% (v/v) glycerol; 0.2% (w/v) bromophenol blue] in eppendorf tubes with plastic pestles. The homogenate was centrifuged at 12,000 x g at 4°C for 5 minutes, then the supernatant was transferred to new eppendorf tubes. Extracts were boiled for 5 minutes, centrifuged at 12,000 x g at 4°C for 5 minutes to remove pellets, and samples aliquoted and stored at -20°C for further experiments.

To extract proteins from leaves of rice IR36 rice, seedlings at the 3-5 leaf stage were ground in 2X SDS as described in Chapter 2.

3.2.3 SDS-PAGE electrophoresis and western analysis

Methods in this section follow the ones described in Chapter 2--section 2.2.5 and 2.2.6.

3.3 Results

3.3.1 PSY expression in rice endosperm, embryo and leaf tissues

The antibody against maize PSY detected a single antigenic band from the proteins extracted from rice endosperm (Figure 3-1). The molecular weight of this band (lane 1) is the same as the 38 kDa band detected in maize endosperm (lane 2). Thus the antibody raised against maize PSY appears to recognize the PSY from rice endosperm. The same

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

size antigen was also detected in total protein extracted from rice seed and embryo (Figure 3-2). However, no antigen was detected from the leaf protein extract, as previously found for maize (see chapter 2).

3.3.2 Temporal regulation of PSY expression in rice endosperm

To evaluate the PSY protein accumulation during endosperm development, equal amounts of protein extracted from developing rice IR36 endosperm (5, 8, 10, 15, and 20 DAF) were subjected to western analysis using the maize anti-PSY antiserum. As shown in Figure 3-3A, rice PSY protein was expressed throughout endosperm development from 5 to 20 DAF. PSY protein levels slightly reduced during endosperm development. Using anti-Sh as a control shown in Figure 3-3B, Sh protein was nearly constantly expressed during endosperm development. Another rice line, Cypress, was also examined and found to show a similar pattern of PSY accumulation during seed development. As shown in Figure 3-4, PSY was found throughout endosperm development and a slight reduction was seen late in endosperm development. These western analysis results showed that PSY was present throughout endosperm development in two rice lines; there was no significant temporal control of PSY expression at the protein level in rice endosperm of two line lines after 5 DAF.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

3.4 Discussion

3.4.1 Tissue-specific expression of PSY

It has been reviewed (see Chapter 1) that rice is capable to synthesize and accumulate carotenoids in photosynthetic tissues. The *Psy* gene is transcribed in rice endosperm (Yoganathan, 1998). To investigate the lack of carotenoid accumulation in rice endosperm, PSY protein accumulation was analyzed in various rice tissues. It appears that a PSY antigen identical in size to that in maize is expressed in endosperm tissue and the level of expression is similar in endosperm and embryo of rice. This pattern is different to PSY expression in maize--high in endosperm, low in embryo. PSY protein was not detectable in leaves from both maize and rice.

In rice endosperm, no carotenoids accumulated even though *Psy* was transcribed (Wurtzel *et al.*, 1996) and PSY protein accumulated. In maize, PSY may be rate-limiting for carotenoid biosynthesis. Transgenic rice endosperm overexpressing a daffodil *Psy* cDNA could accumulate phytoene (Burkhardt *et al.*, 1997). All of the above indicates that PSY in rice endosperm may not function properly. Possible explanations include 1) PSY expression is not sufficient; 2) PSY protein is not active; or 3) PSY protein is not at the proper site.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

3.4.2 Developmental regulation of PSY expression

To determine whether PSY expression in rice endosperm was regulated during seed development, two rice line lines, IR36 and Cypress, were examined. Results of western analysis indicated that PSY expression was at the highest level at the early developmental stage, then marginally declined as development progressed. While *Psy* transcript is constant during seed development, there does not appear to be any induction of PSY protein levels in developing rice endosperms, unless this occurs prior to the first stage tested (Wurtzel *et al.*, 1996). In maize, induction of PSY accumulation at 10 DAP might lead to higher accumulation of carotenoids during endosperm development. If induction of PSY levels early in endosperm development is critical for carotenoid accumulation, then the lack of PSY induction in rice endosperm might be a contributing factor to the absence of endosperm carotenoids.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

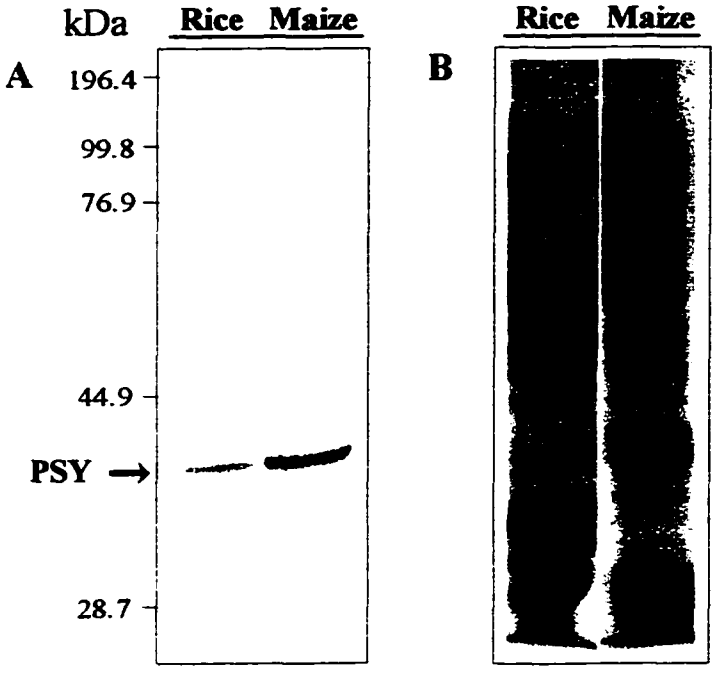


Figure 3-1. Western analysis--Phytoene synthase expression in rice and maize endosperm.
Proteins were extracted from rice IR36 endosperm at 10 DAF and maize B73 endosperm at 20 DAP.
(A) Immunoblot probed by maize specific anti-PSY antiserum.
(B) SDS-PAGE gel showing samples corresponding to (A).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

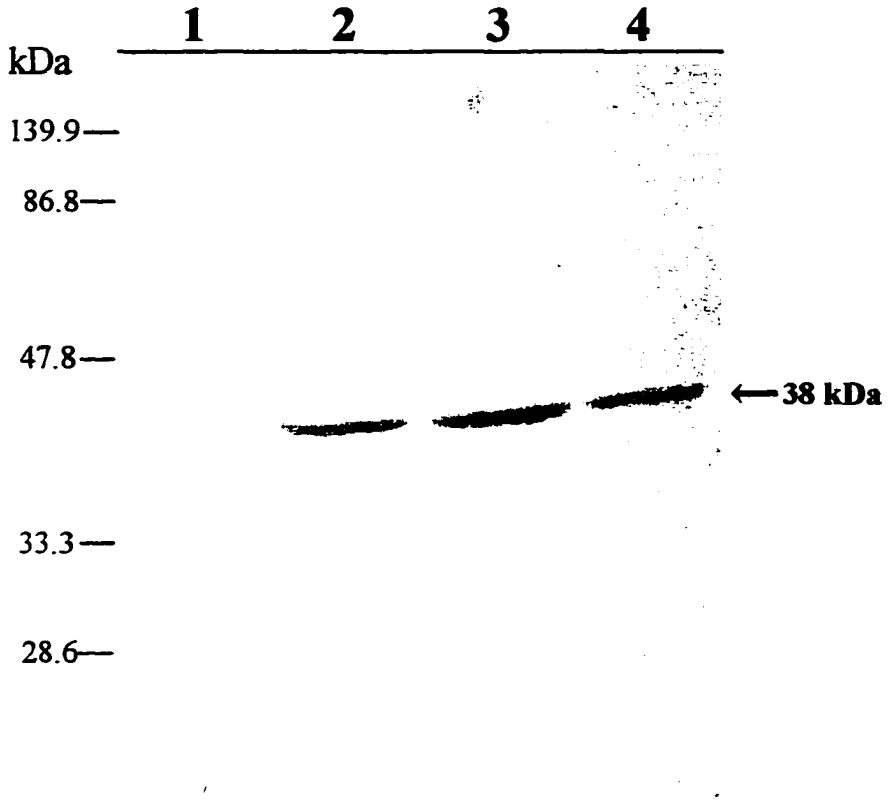


Figure 3-2. Western analysis--Phytoene synthase expression in various tissues of rice IR36.
Proteins were extracted from tissues of seedlings grown to the 3-5 leaf stage and of seed at 10 DAF. Lane 1, leaf; lane 2, seed; lane 3, endosperm; lane 4, embryo. Forty μg protein extract was loaded in each lane. The immunoblot was probed with the anti-PSY antiserum.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 3-3. Western analysis--Phytoene synthase accumulation in developing endosperm of rice IR36.

Replicate gels containing proteins extracted from endosperm at various days after flowering (DAF) shown at the top.

(A) Immunoblot probed by maize-specific anti-PSY antiserum.

(B) Immunoblot probed by maize-specific anti-Sh antiserum.

(C) SDS-PAGE gel stained with Coomassie Blue.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

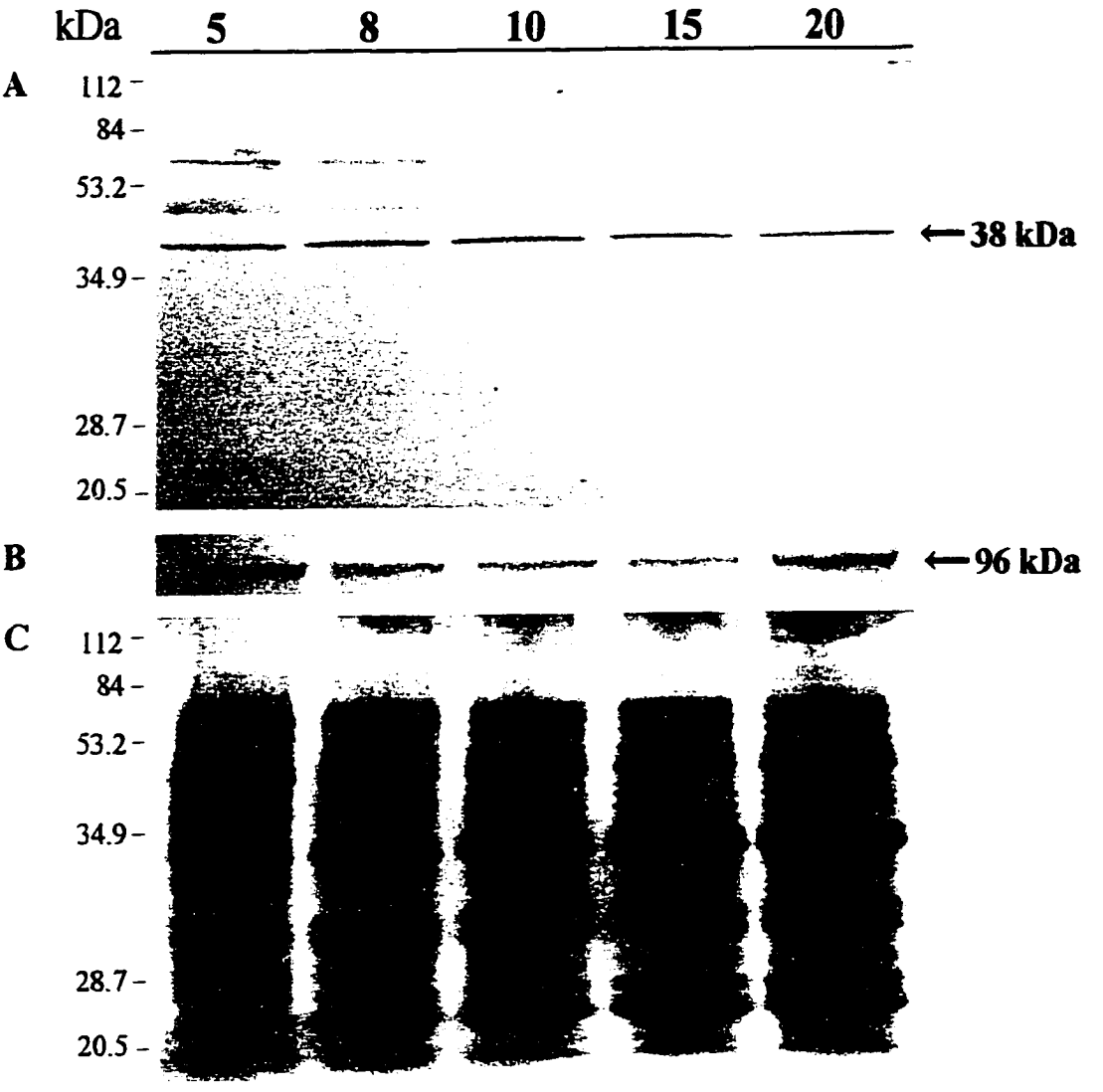


Fig. 3-3

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

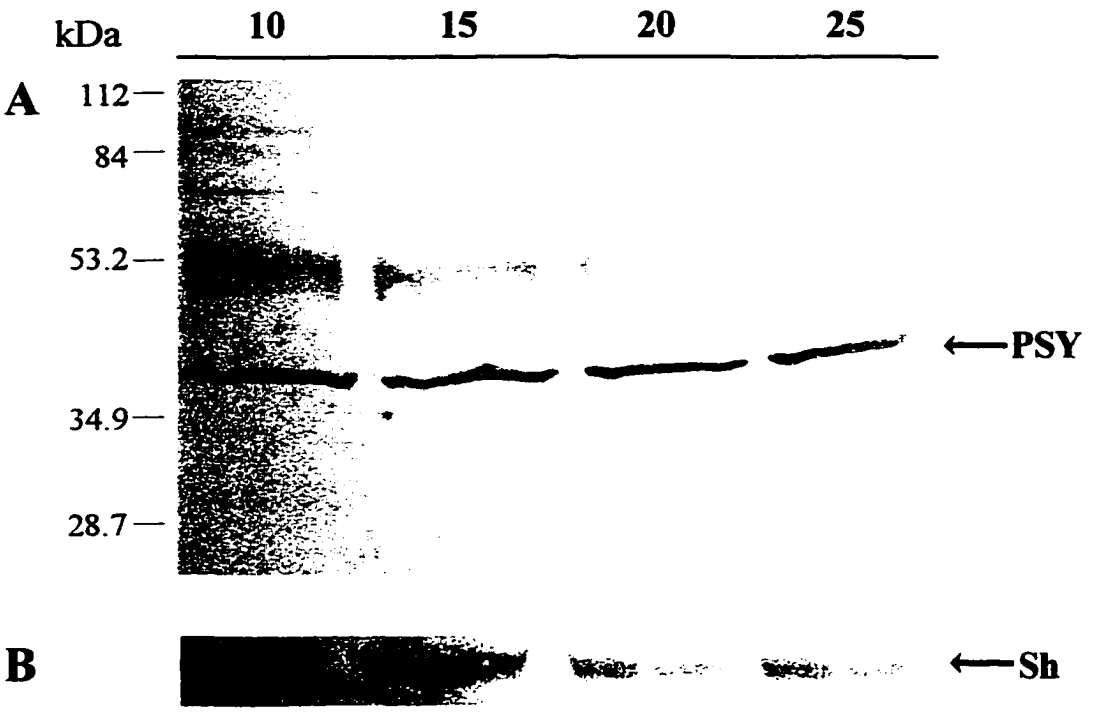


Figure 3-4. Western analysis --Phytoene synthase accumulation in developing endosperm of Cypress rice.
Replicate gels containing proteins extracted from endosperm at various DAF shown at top.
(A) Immunoblot probed with a maize specific anti-PSY antiserum.
(B) Immunoblot with a maize specific anti-Sh antiserum contained a duplicate set of samples as (A).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter 4. Immunocytochemical localization of PSY in maize and rice endosperm

4.1 Introduction

In higher plants, all carotenoid biosynthetic enzymes are nuclear gene encoded. These proteins are synthesized as precursors on membrane-free polysomes in the cytoplasm, then imported into plastids as mature proteins.

The western analysis results shown in Chapter 2 and Chapter 3 indicate that PSY is present in protein extracts prepared from both maize and rice endosperm tissues. The unprocessed maize PSY is predicted to be 46.7 kDa, clearly distinguishable from the 38 kDa which is the predicted size of the processed, plastid-localized enzyme. By comparison, the 38 kDa size of both the rice and maize antigens detected with the PSY antiserum would suggest that these are processed and/or found in the rice and maize plastids, respectively. One question can be raised, that is--if the rice PSY protein is present, is it in the right location in endosperm cells? There are no prior results for localization of PSY in maize or rice endosperm cells. To investigate the subcellular localization of PSY protein in endosperm cells of maize and rice, immunocytochemical localizations of PSY were conducted. Results are presented and discussed.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

4.2 Materials and Methods

4.2.1 Maize materials

Maize (*Zea mays*) B73 seeds were obtained from Dr. S. Briggs (Pioneer Hi-Bred, Johnston, IA). Plants were grown under summer field conditions in the maize field at the Black Rock Forest (Cornwall, New York) and at the Maize Genetic Station in Pelham Bay Park (Bronx, New York). For amyloplast isolation and tissue fixation, B73 endosperms and embryos at 17 DAP were obtained from a 96004 sibling cross.

4.2.2 Rice materials

Rice (*Oryza sativa*) IR36 (from S. McCouch, Cornell University) was grown in the research greenhouse at Lehman College (Bronx, New York). Controlled pollinations were performed (Yoganathan, 1998). Seeds were harvested at 7 DAF. The seed coat was removed, then the endosperm and embryo were separated.

4.2.3 Protein extraction, SDS-PAGE electrophoresis and Western analysis

Methods used in this chapter are the same as described in Chapter 2 and Chapter 3 (see related sections).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

4.2.4 Histological methods

Tissue fixation

Endosperms and embryos were dissected from seeds of the maize B73 line at 17 DAP and the rice IR36 at 7 DAF. Tissues were immersed in 50 mM sodium phosphate buffer (pH 7.2) according to Zhang *et al.* (Zhang and Boston, 1992) with modifications. Several slices (2 mm x 4 mm x 1 mm) for each tissue were fixed in 3% glutaldehyde in sodium phosphate buffer either for 2 hours at room temperature or overnight at 4°C. Fixed slices were washed with sodium phosphate buffer at room temperature three times for 15 minutes each, dehydrated at room temperature through a series of ethyl alcohol 10%, 25%, 50%, 70%, and 95% (v/v) for 10 minutes each, then twice in 100% ethyl alcohol for 10 minutes each time. Slices were infiltrated in 25%, 50%, and 75% (v/v) LR white (Polysciences, Warrington, PA) (v/v) in ethyl alcohol at room temperature for 2 hours each, and then in 100% LR white at room temperature over night. Slices were transferred to 100% LR white in beam capsules (Polysciences, Warrington, PA) and polymerized at 65°C for 24 hours.

Sectioning

One μm semi-thin sections were prepared using glass knives on an Ultra Microtome (Leica). Sections were collected onto precoated glass slides (Polysciences, Inc., Warrington, PA) and air dried.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Immunofluorescent labeling

All steps were carried out at room temperature. Slides were rehydrated in PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4] for 15 minutes, blocked in 0.5% (w/v) BSA in PBS for 30 minutes, incubated with primary antiserum diluted at 1:25 in BSA/PBS over night in a moist chamber, washed with PBS three times (5 minutes each), labeled with the goat anti-rabbit IgG-FITC conjugate (Sigma Diagonostics, St. Louis, MO) for 2 hours, washed with PBS three times at 5 minutes each. Slides were air dried, and mounted with coverslips using Aqua Poly/Mount as an anti-fading agent (Polysciences, Inc., Warrington, PA). For negative controls, primary antisera were replaced with pre-immune serum diluted at 1:100 in PBS; or with 0.5% (w/v) BSA in PBS. Slides were observed using a fluorescent microscope with a blue filter, excitable at 488 nm. Photographs were taken under a MRC-600 Laser Scanning Confocal Imaging System (Bio-Rad, Hercules, CA) at Columbia University. Images were processed using Adobe Photoshop software (Adobe System, Inc.).

4.2.5 Amyloplast isolation from maize and rice endosperm

Method I–Amyloplasts were isolated according to the method described in Mu-Forster and Wasserman (Mu-Forster and Wasserman, 1998) with modifications. Protease inhibitor solutions (final concentrations:7 mM DTT; 10 mM benzamidine and 50 mM 2-amino-n-caproic acid; 1.1 mM PMSF) were freshly added to AIM buffer (0.8 M sorbitol; 50 mM HEPES pH 7.5; 1 mM EDTA pH 8; 1 mM KCl; 2 mM $MgCl_2$; and 0.1% BSA).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Ten grams of endosperm from B73 line were dissected and were immersed in 20 ml AIM buffer, and endosperms sliced into half using a razor blade. Slices were covered with buffer for 30 minutes on ice to release amyloplasts. The suspension surrounding the endosperm slices was carefully transferred to a Falcon tube and kept on ice. The endosperm was further sliced, and the suspension was collected. The pooled suspension samples were centrifuged at 100 x g in a GPR centrifuge (Beckman Instruments, Palo Alto, CA) at 4°C for 10 minutes, the supernatant discarded, and the pellet resuspended at 1 g/ml in DB buffer (50 mM Tris-HCl pH 7; 10 mM EDTA pH 8; 10% glycerol; and 1.25 mM DTT, final concentration, added immediately before use). Finally the amyloplast-enriched pellet was stored at -20°C.

Method II--All steps were carried out at 0-4°C. The method was described in Wurtzel (Wurtzel *et al.*, 1987) for nuclei isolation with modification (Wurtzel, unpublished). Endosperm of maize B73 line line was dissected from one 17 DAP ear and placed in HB, Homogenate Buffer [1 M hexylene glycol; 20% (w/v) glycerol; 10 mM $MgCl_2$; 250 mM sucrose; 10 mM NaCl; 10 mM PIPES pH 7.2] with protease inhibitor solutions (final concentrations: 5 mM DTT; 10 mM benzamidine and 50 mM 2-amino-n-caproic acid; 1.0 mM PMSF) added immediately before use. The PMSF stock solution (0.1 M) used was in isopropanol. The mixture was ground with a prechilled mortar and pestle. The homogenate was filtered through a 200 μm Nitex screen (Tetko, Inc., Elmsford, NY). The filtrate was transferred onto 5 ml of an 80% Percoll solution [500 mM hexylene glycol; 400 mM $MgCl_2$; 250 mM sucrose; 10 mM NaCl; 10 mM PIPES pH 7.2; Percoll 80%]

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

and centrifuged for 5 minutes at 3,000 x g in a J2-21 M/E centrifuge (Beckman Instruments, Palo Alto, CA) using a swinging bucket rotor (GH 3.7). A yellow band at the HB:Percoll interface was collected in an equal volume of the wash buffer (HB without glycerol) with protease inhibitor stock solutions (added immediately before use), centrifuged at 3,000 x g for 5 minutes, and the pellet was washed again. Finally the amyloplast-enriched pellet was solubilized in wash buffer at 100 µl/g starting material, aliquoted and stored at -20°C.

To isolate rice amyloplasts, **Method I** was used. About 300 mg rice endosperm at 7 DAP were immersed in 20 ml AIM buffer supplemented with protease inhibitor solutions and amyloplasts purified as described and resuspended in 1.5 ml DB buffer. The fraction was tested positively by iodine staining which stains starch.

Amyloplast fractions in DB buffer (Method I) from maize and rice were stored at -20°C. Before subjecting to SDS-PAGE gel, aliquots were and mixed with equal volumes of 2XSDS sample buffer, then spun at 14,000 x g for 2 min to remove insoluble material, then boiled for 5 min, supernatant used for gel electrophoresis.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

4.3 Results

4.3.1 Localization of PSY in maize endosperm

Maize endosperm and embryo accumulate carotenoids. For a yellow carotenoid-containing endosperm line such as B73, immature endosperms at 20 DAP contain carotenoids at 3.0 μg/g fresh weight (fw) (Chapter 2), and embryo accumulates carotenoids at 1.6 μg/g (fw) (data not shown). Rice embryo (from mature seeds) accumulates carotenoids at 0.7 μg/g (dry weight), but rice endosperm does not contain any carotenoids (Luo and Wurtzel, unpublished).

When phytoene synthase (PSY) protein was analyzed in embryo and endosperm from maize and rice, PSY was present in the protein extracts from all of the above tissues, although the level in maize embryo was barely detectable (shown in Figure 4-1). Between rice embryo and endosperm, PSY levels were relatively similar; but between maize embryo and endosperm, PSY levels were greater in endosperm than in embryo.

To examine whether PSY protein was present in endosperm plastids, amyloplast fractions from maize and rice endosperms were isolated according to Method I, and western analysis was conducted using a maize-specific anti-PSY antiserum. In maize endosperm, PSY protein accumulated in endosperm protein extracts as well as in amyloplast-enriched fractions as shown in Figure 4-2A. This result was confirmed by isolating amyloplasts

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

using Method II (not shown). In contrast, PSY was present in the rice endosperm protein extract, but not detectable in the amyloplast-enriched (Method I) fraction (Figure 4-2B).

To further investigate the location of PSY protein in maize and rice endosperm cells, I carried out an immunocytochemical localization experiment using the maize specific anti-PSY polyclonal antiserum and FITC-conjugated IgG. The confocal microscopic image of 1 μm semithin sections of maize endosperm (Figure 4-3A) showed fluorescence on the envelope of the endosperm amyloplasts and no signal was present in the stroma of the amyloplasts, nor the cytoplasm, nor the nuclei. The fluorescence of the cell wall was also seen due to the autoflourescence characteristic of cell wall materials. With a negative control which was labeled with FITC only, there was no fluorescence signal detectable except the auto-fluorescent cell wall network (Figure 4-3B). These results clearly show that PSY protein is associated with the envelope of maize amyloplasts.

To further demonstrate that the PSY signal was on the plastid envelope, tissues were tested with anti-Bt1 antiserum. The *Brittle1* (*Bt1*) gene encodes a membrane translocator that is involved in starch biosynthesis and is located on the plastid envelope (Sullivan and Kaneko, 1995; Sullivan *et al.*, 1991; Li *et al.*, 1992). Figure 4-4 shows the Bt1 signal obtained from total maize endosperm proteins. When this antiserum was used for confocal microscopy (see Figure 4-5A), the Bt1 protein was found associated with the envelope of the amyloplasts of maize endosperm cells. Therefore the signal detected with the anti-PSY antiserum is clearly on plastid envelope membranes.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

4.3.2 Localization of PSY in embryo of maize and rice

Embryo is a diploid tissue as compared to the triploid endosperm although both tissues accumulate carotenoids. We carried out a parallel PSY localization study in maize and rice embryo tissues. As shown in Figure 4-6A, the PSY signal is associated with the plastid envelope in maize embryo cells, while no signal is detected using the pre-immune bleed and FITC (shown in Figure 4-6B). When rice embryo sections were analyzed, the results showed that PSY is also localized on the membrane of the rice embryo plastids as shown in Figure 4-7A, whereas no signal was detectable using the pre-immune bleed and FITC only (Figure 4-7B). Therefore, our results clearly show that PSY protein, detected by western analysis, is localized on the plastid membrane of both the maize and rice embryo cells.

4.3.3 Localization of PSY in rice endosperm

When rice endosperm tissue was tested for PSY localization (Figure 4-8A), we found that the pattern of fluorescence in rice endosperm appeared mostly close to the cell wall; at some point among the amyloplasts, the fluorescence branches off the cell wall and extends into the cell. The fluorescence in the cytoplasm appeared external to the amyloplasts, thus the boundary of the amyloplast is roughly seen. But the fluorescence was not observed surrounding the amyloplasts completely. Thus the PSY protein seems only accumulated in the cytoplasm. Probing with the FITC conjugated anti-rabbit IgG

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

alone showed the auto-fluorescent cell wall network (Figure 4-8B). This result is consistent with the western analysis (Figure 4-2) which showed rice PSY in total endosperm extracts, but not in the amyloplast fractions, while maize PSY was found in both fractions. An anti-Bt1 antiserum was also used to test the localization of Bt1 protein in rice endosperm. As shown in Figure 4-9A, Bt1 protein was mainly localized around the amyloplasts. This result is similar to the one observed in maize amyloplasts, where Bt1 protein was in the envelope of amyloplasts as reported (Sullivan and Kaneko, 1995; Sullivan *et al.*, 1991; Li *et al.*, 1992).

Cellular structures of maize and rice endosperm show some major differences (Figure 4-10 and 4-11). The pictures provide the details of cellular components at the light microscopic level. In endosperm cells of both maize and rice, amyloplasts where starch grains develop are the most dominant organelles. In maize 17 DAP endosperm, each cell contains amyloplasts; cytoplasmic space among amyloplasts is clearly observed as shown in Figure 4-10A; a central localized nucleus is observed in most endosperm cells. Amyloplasts of maize endosperm are simple units which are usually round or oval. The structure of rice endosperm cells about 7 DAF is shown in Figure 4-11A. Rice endosperm cells are elongated; most of the space within the cells is filled with starch granules, and very limited cytoplasm is pushed toward the cell wall, nuclei were not observed in the endosperm at 7 DAF. The starch grains in rice endosperm are compound starch grains which are of irregular shape. On the contrary, embryo cells of maize and rice do not show great difference (Figure 4-10B and 4-11B). These cells are relatively small, contain large

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

central nuclei and less abundant plastids in the cells.

4.4 Discussion

Results presented in this chapter show for the first time, the subcellular localization of PSY protein in cereal plants, maize and rice. Confocal microscopic images clearly point out that the localization of PSY is restricted to the envelope of amyloplasts, but not to the interior of the amyloplasts in maize endosperm cells. PSY also localizes to the envelope of plastids in maize and rice embryo cells. However, in rice endosperm, which appears to contain a processed form of PSY, the protein is not found associated with amyloplasts as determined both by amyloplast fractionation and by confocal microscopy.

It is reported that the PSY protein exists in two forms in daffodil flowers, an active membrane associated form and an inactive stromal form (Bonk *et al.*, 1997; Schledz *et al.*, 1996). The PSY detected on the envelope of the maize amyloplasts may be the active form. It is also reported that the function of PSY in the plastids depend on the proper packaging--association of PSY to molecular chaperonins (Bonk *et al.*, 1997). The post-translational regulation would be different between amyloplasts and chromoplasts. Therefore, the localization of PSY appears different between the maize endosperms–on the envelope and the daffodil flowers chromoplasts–in the membrane and stroma.

The structure of rice endosperm cells, at about 7 DAF, shows that cells are elongated;

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

most of the space within the cells is filled with amyloplasts, and a very limited cytoplasm is pushed toward the cell wall, nuclei were not observed in the endosperm at 7 DAF. The amyloplasts found in rice endosperm were compound and of irregular shape. On the contrary, embryo cells of maize and rice do not show great differences in sizes and cellular components. Cells in these tissues are relatively small, contain large central nuclei and are less abundant in number of plastids.

Rice endosperms do not accumulate any carotenoids nor phytoene (Burkhardt *et al.*, 1997). However, *Psy* is transcribed (Wurtzel *et al.*, 1996), and translated in rice endosperms. Thus, it is a critical point to understand why the PSY in rice endosperms can not convert GGPP to phytoene. Using the anti-PSY antiserum raised against the maize PSY protein, confocal microscopic images clearly show clusters of strong fluorescence associated with the protein pockets in the cytoplasm of the rice endosperm cells. This is in contrast to the localization results with maize endosperm. The rice PSY does not localize to the envelope of amyloplasts in rice endosperms.

In rice embryo cells, the signals are also detected on the envelope of the plastids. It is reported that the plastids in the rice embryo display a limited thylakoid system, and a type of plastid also found in rice aleurone cells, but not in the endosperm cells (Bechtel and Pomeranz, 1977). Using the antiserum against maize Bt1, the Bt1 antigen was found to be associated with the envelope of the amyloplasts of both maize and rice endosperm. These results suggest that the PSY protein targets to the amyloplasts in maize endosperm cells,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

but obviously does not localize to the amyloplasts in rice endosperm cells. It has been found that certain proteins such as light-harvesting chlorophyll a/b-protein complex polypeptides and apoproteins of LHCII were not retained in the chloroplasts after being translocated and processed, but rather accumulated in cytoplasm in *Chlamydomonas reinhardtii* (Wolfe *et al.*, 1997; Komine *et al.*, 1996; Park and Hoober, 1997; White *et al.*, 1996). Therefore, there is a possibility that rice PSY protein was imported into amyloplast, but then exported out to the cytoplasm.

Rice transformation with a full length *Psy* cDNA from daffodil showed that phytoene accumulated in transgenic rice endosperms *in vivo*, and the functional PSY protein from the transgenic rice endosperms was detected by the anti-PSY antiserum originated from daffodil (Burkhardt *et al.*, 1997). In nontransgenic rice endosperms, *Psy* was expressed at both transcriptional (Wurtzel *et al.*, 1996) and translational levels, but PSY protein only accumulated in the cytoplasm. Import of PSY protein to the envelope of the compound amyloplasts of rice may be the most critical step that causes the absence of carotenoid in the rice endosperm.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 4-1. PSY expression in endosperm and embryo of maize B73 and rice IR36.

A, western blot probed with an anti-PSY antiserum; B, stained gel corresponding to the samples used in the western blot.

Lane 1, total protein from rice endosperm at 10 DAF; lane 2, total protein from rice embryo at 10 DAF; lane 3, total protein from maize embryo at 20 DAP; lane 4, total protein from maize endosperm at 20 DAP.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

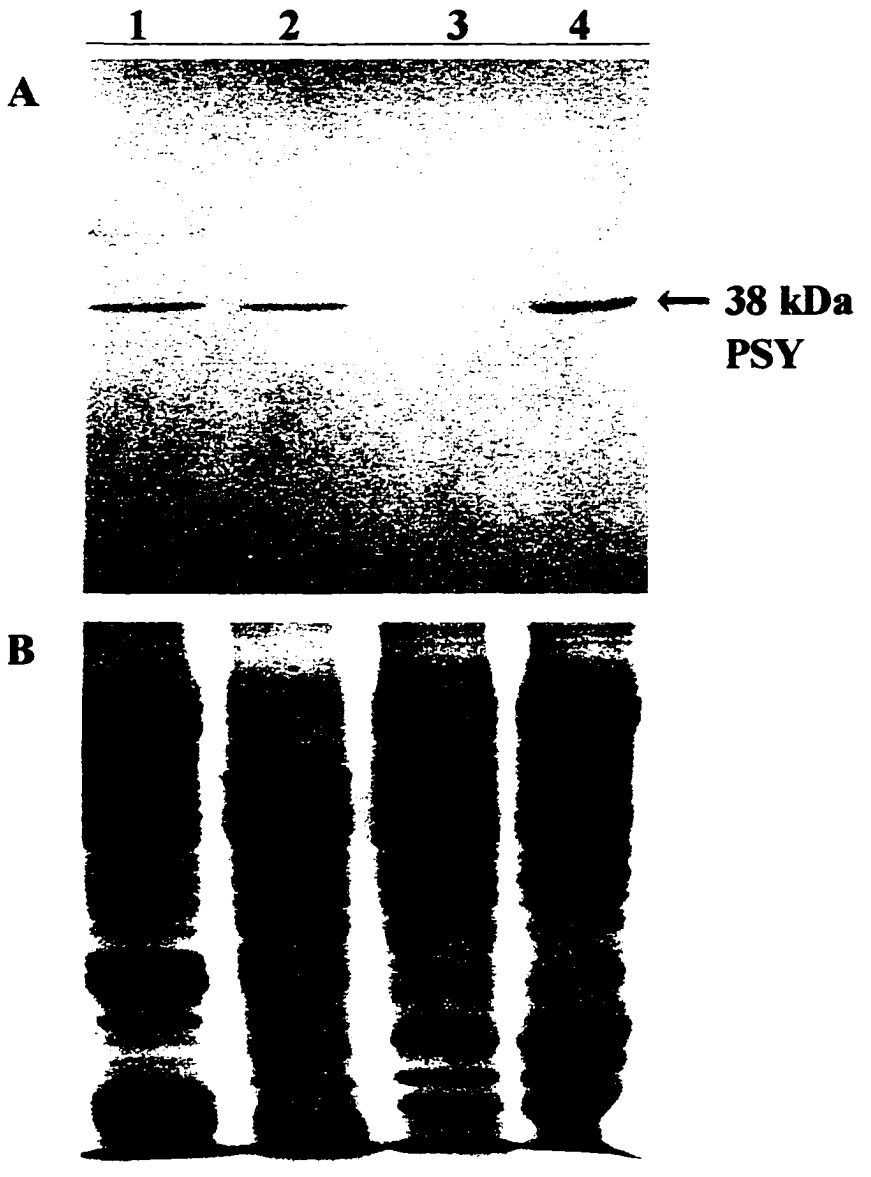


Fig. 4-1

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 4-2. Western analysis--Phytoene synthase expression in amyloplast fractions of maize and rice endosperm.

Top panel, maize B73; bottom panel, rice IR36.

Lane 1, amyloplast fraction (isolated using **Method I**); lane 2, endosperm protein extract.

(A) SDS-PAGE gel.

(B) Immunoblot probed with the maize specific anti-PSY antiserum.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

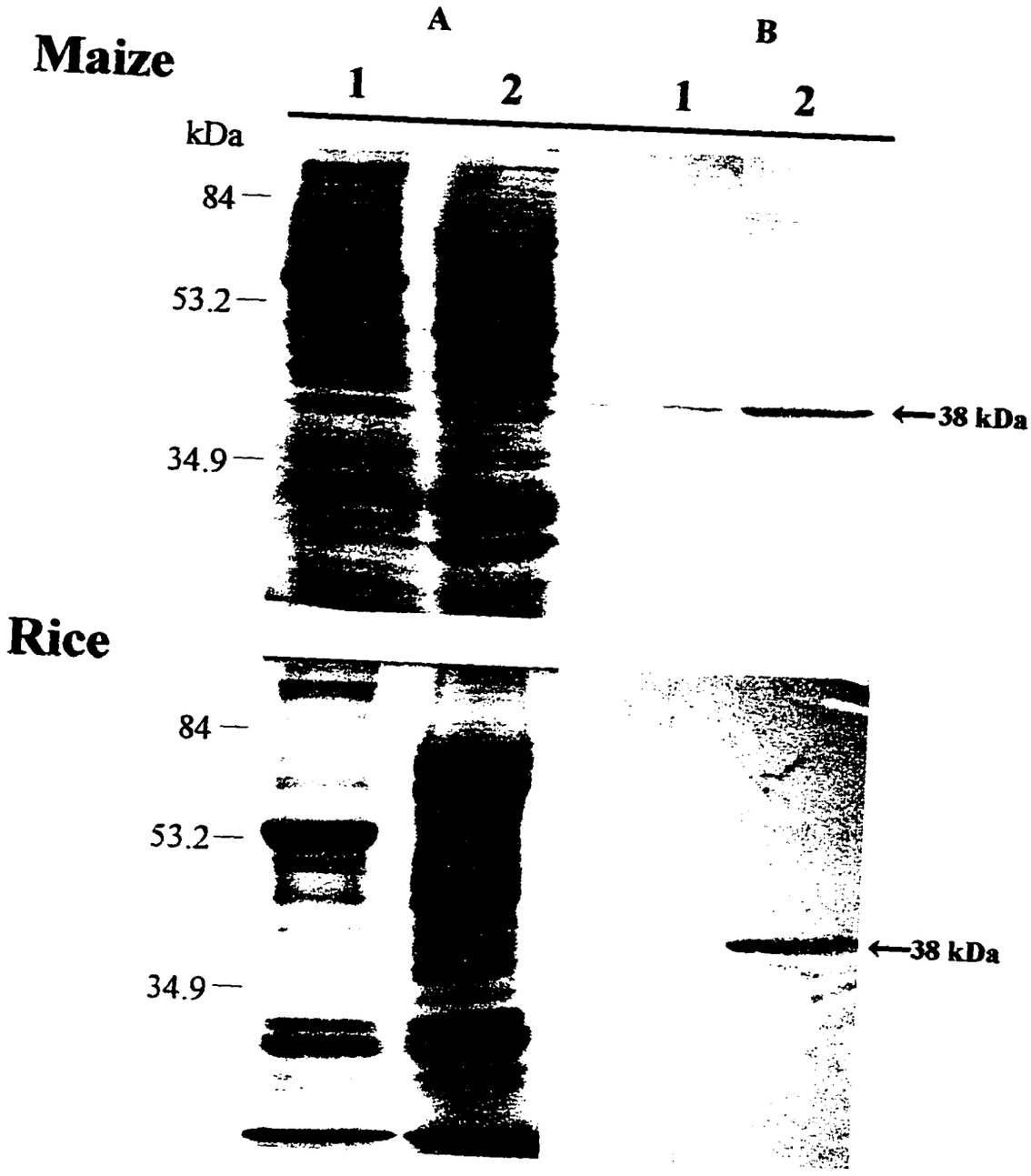


Fig. 4-2

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 4-3. Localization of PSY in maize B73 endosperm dissected at 17 DAP.

A, labeled with anti-PSY antiserum and anti-rabbit IgG FITC conjugate.

B, negative control labelled with anti-rabbit IgG FITC conjugate only.

Bar represents 50 μm in A, and 100 μm in B.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

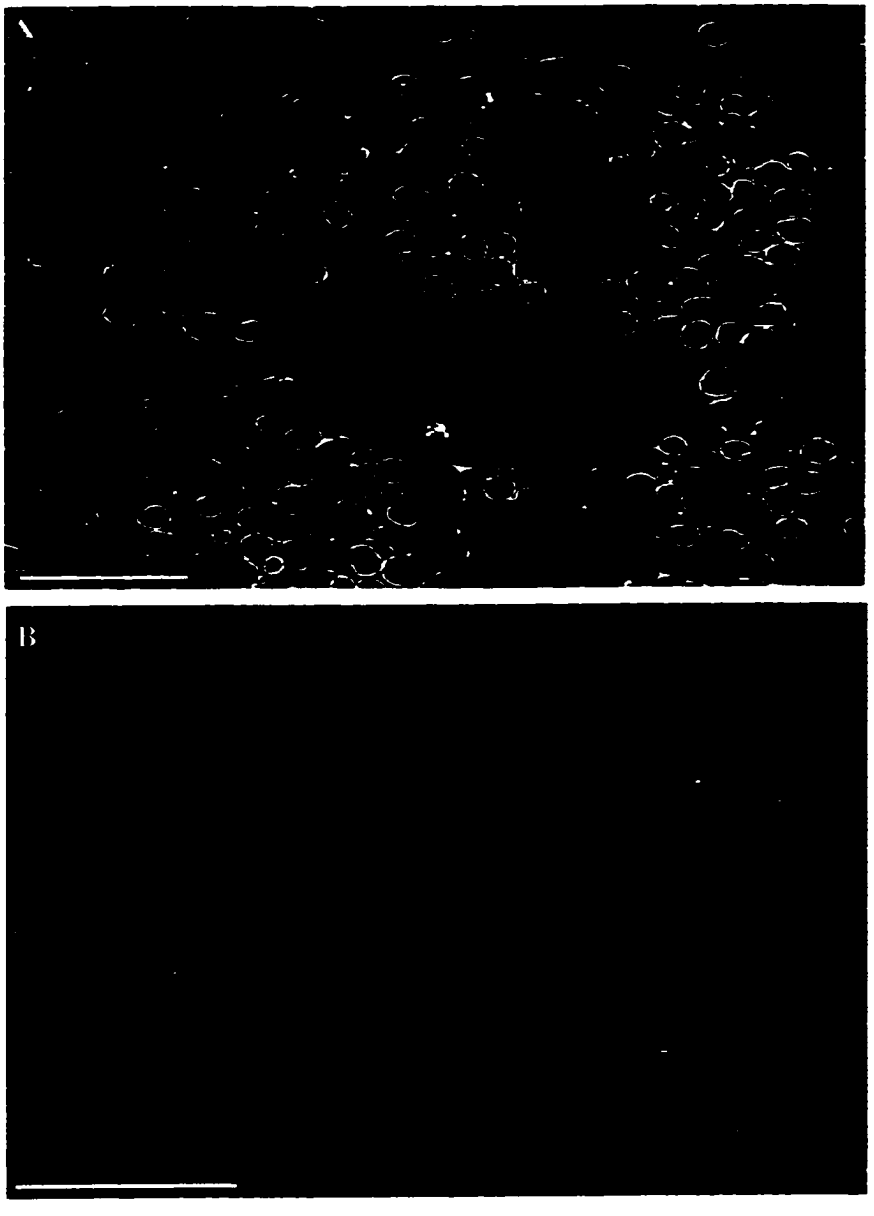


Fig. 4-3

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

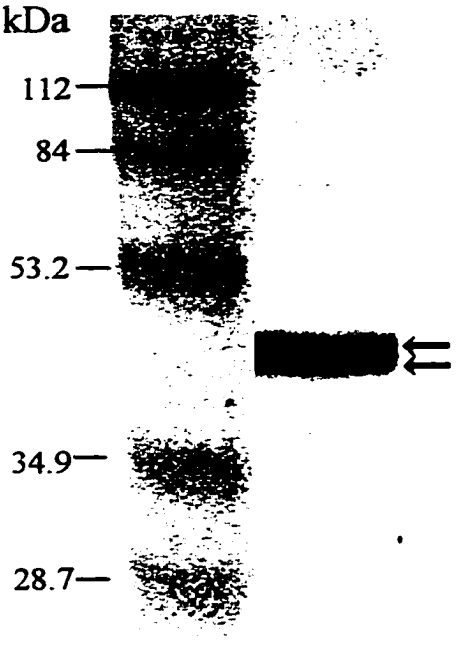


Figure 4-4. Western analysis--Expression of Bt1 protein in maize B73 endosperm.
Lane 1, Protein molecular weight standards shown in kDa; lane 2, total endosperm proteins extracted at 20 DAP.
Immunoblot was probed with a maize specific anti-Bt1 antiserum.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 4-5. Brittle-1 localization in maize B73 endosperm dissected at 17 DAP.

A, labeled with anti-Bt1 antiserum and anti-rabbit IgG FITC conjugate.

B, negative control labeled with anti-rabbit IgG FITC conjugate only.

Bar represents 100 μm in A, and 100 μm in B.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

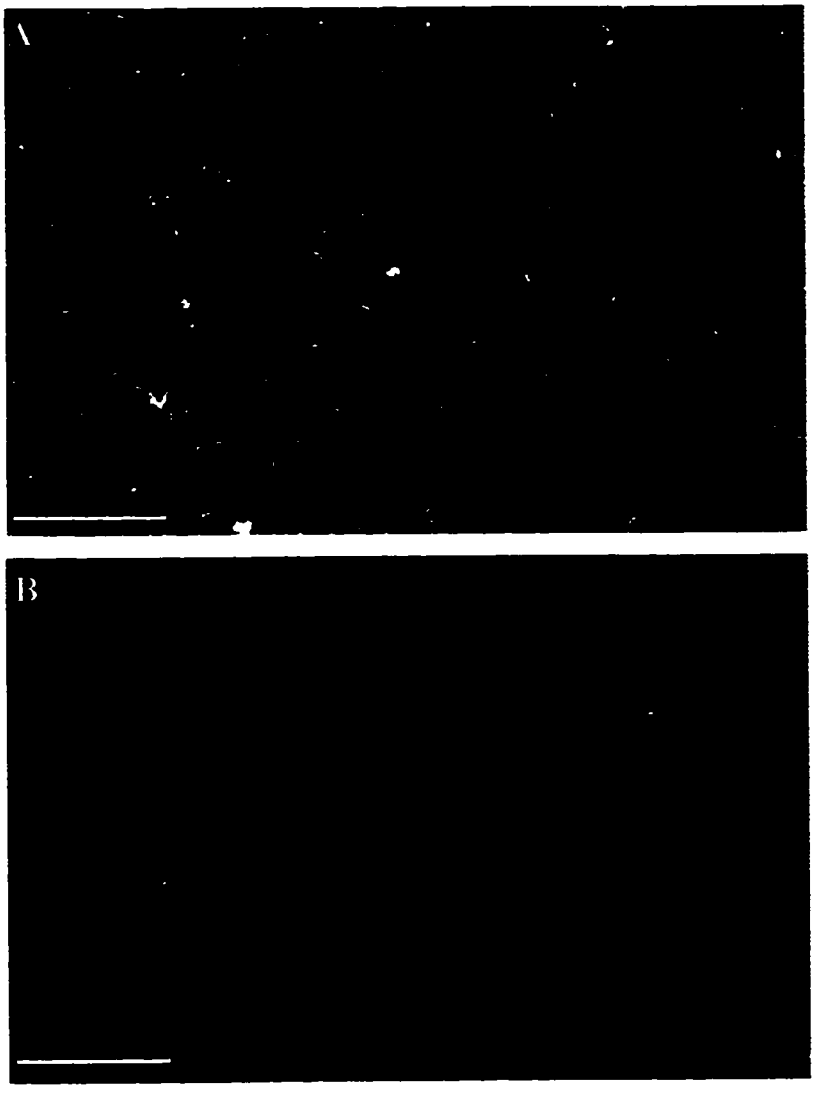


Fig. 4-5

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 4-6. Immunofluoresent localization of PSY in maize B73 embryo dissected at 17 DAP.

A, section incubated with anti-PSY antiserum and anti-rabbit IgG FITC conjugate.

B, control section incubated with the pre-immune serum and anti-rabbit IgG FITC conjugate.

Bar represents 50 μm in A, and 50 μm in B.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

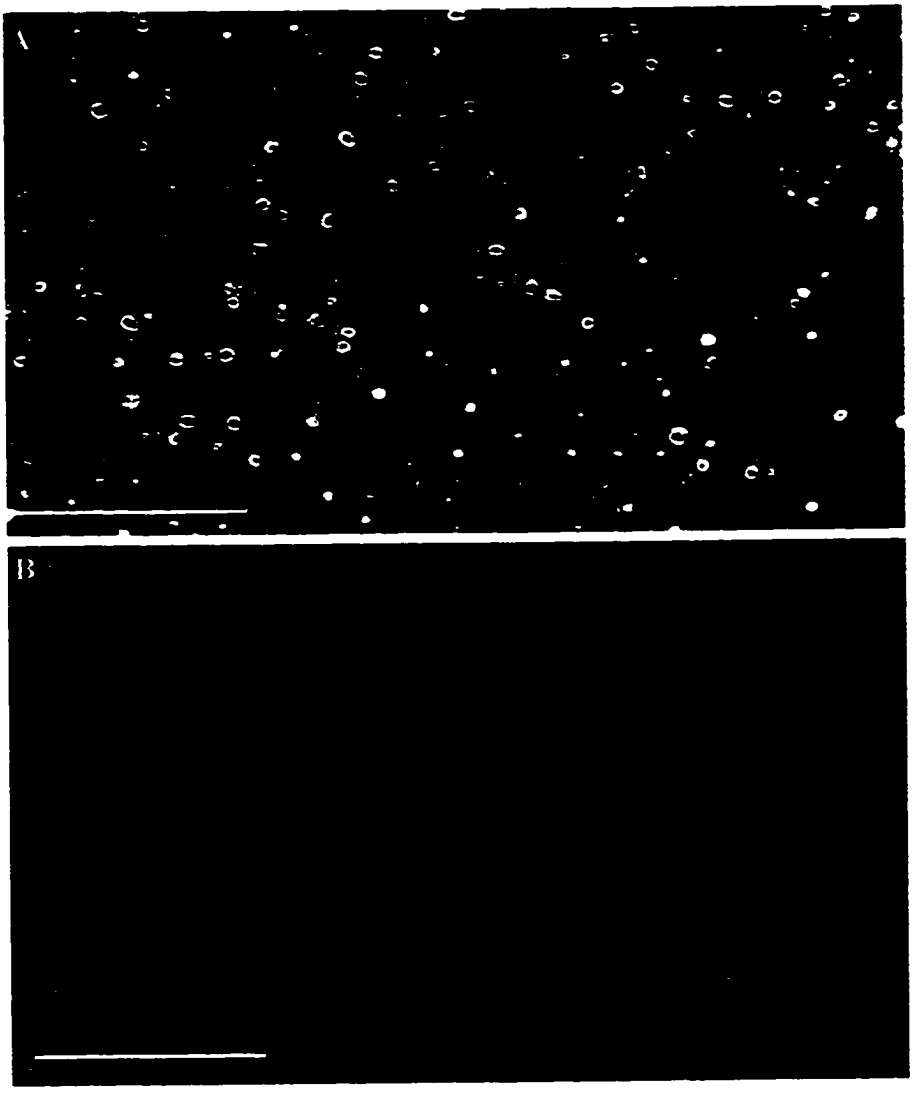


Fig. 4-6

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 4-7. Immunofluoresent localization of PSY in rice IR36 embryo dissected at 7 DAF.

A, section incubated with anti-PSY antiserum and anti-rabbit IgG FITC conjugate.

B, control section incubated with the pre-immune serum and anti-rabbit IgG FITC conjugate.

Bar represents 50 μm in A, and 50 μm in B.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

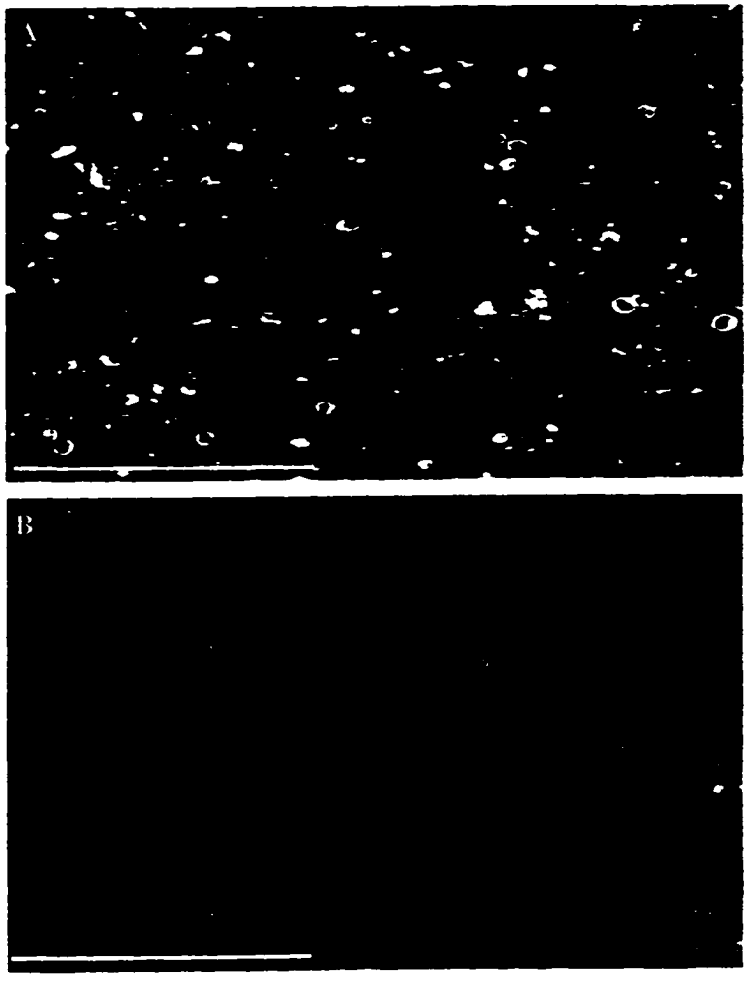


Fig. 4-7

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 4-8. Immunofluoresent localization of PSY in rice IR36 endosperm dissected at 7 DAF.

A, section incubated with anti-PSY antiserum and anti-rabbit IgG FITC conjugate.

B, control section incubated with anti-rabbit IgG FITC conjugate only.

Bar represents 50 μm in A, and 50 μm in B.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

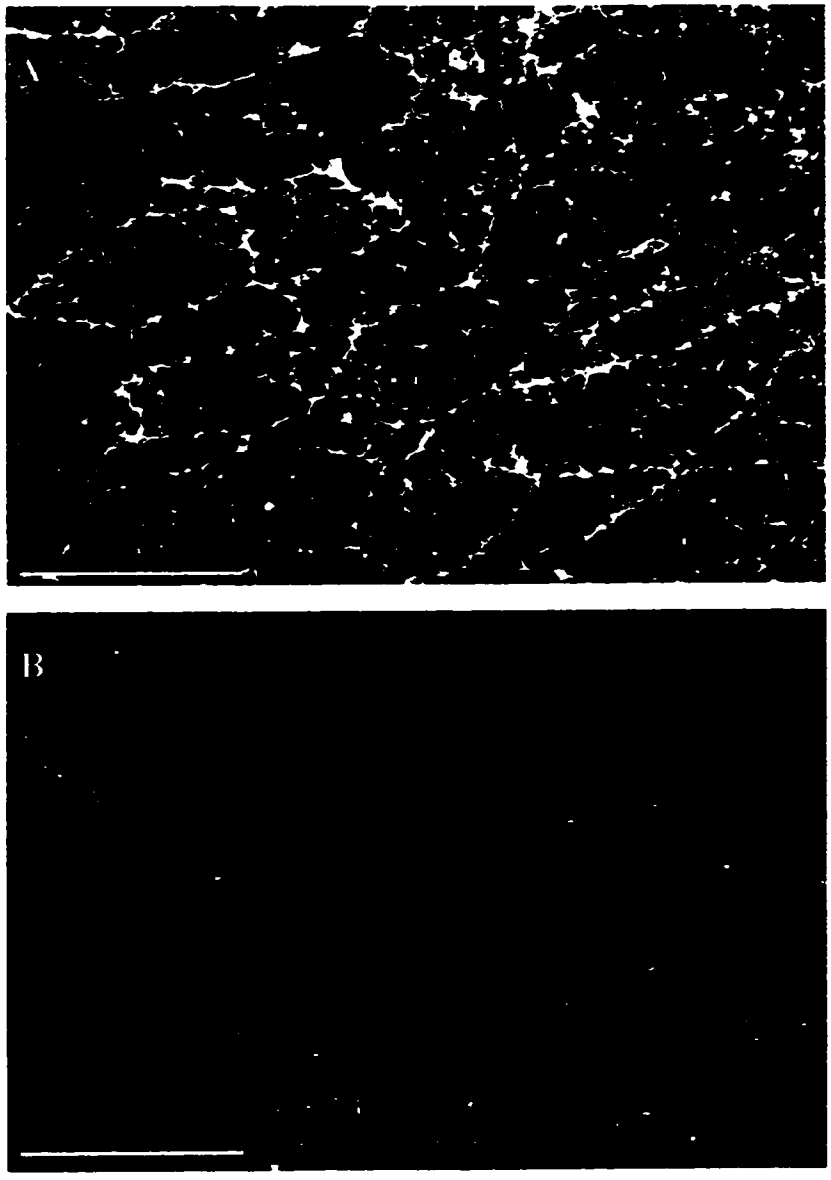


Fig. 4-8

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 4-9. Brittle-1 localization in rice IR36 endosperm dissected at 7 DAF.

A, labelled with anti-Bt1 antiserum and anti-rabbit IgG FITC conjugate.

B, negative control labelled with anti-rabbit IgG FITC conjugate only.

Bar represents 50 μm in A, and 50 μm in B.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

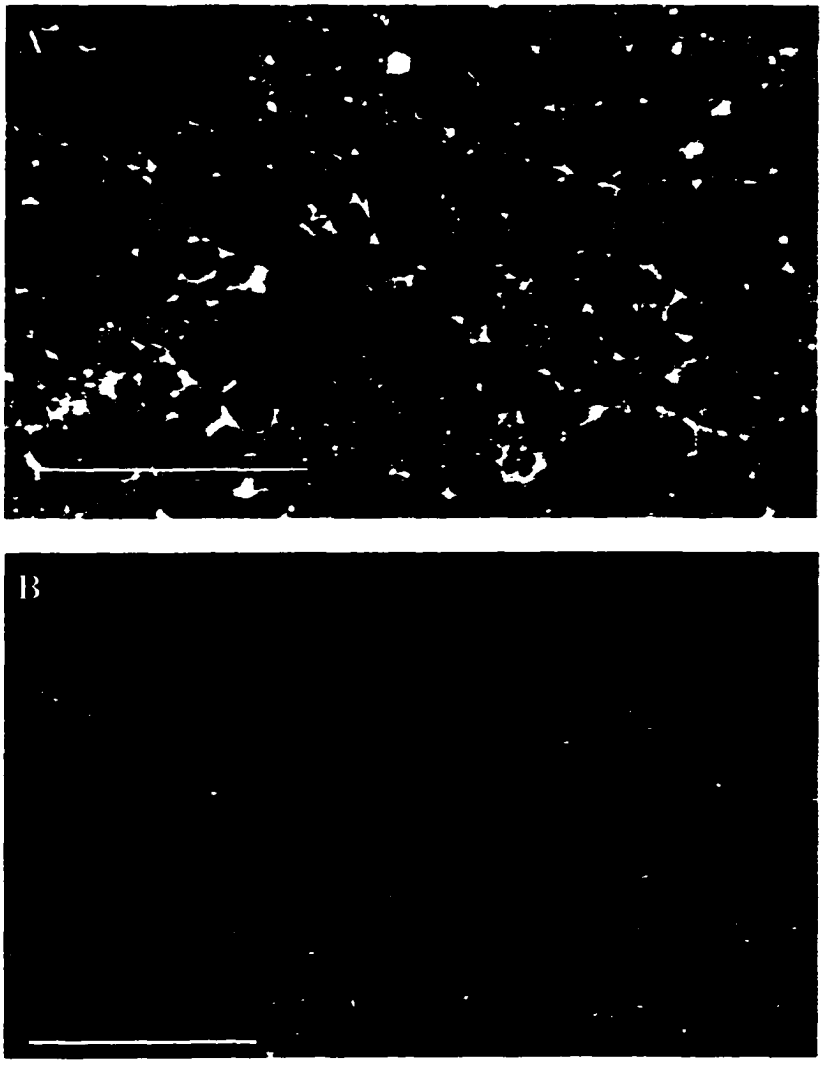


Fig. 4-9

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 4-10. Confocal microscopic images--Semithin sections of endosperm and embryo of maize viewed under bright light field.

Maize B73 endosperm (A) and embryo (B) dissected at 17 DAP.

N, nucleus; CW, cell wall; P, plastid.

Bar represents 50 μm in A and 50 μm in B.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

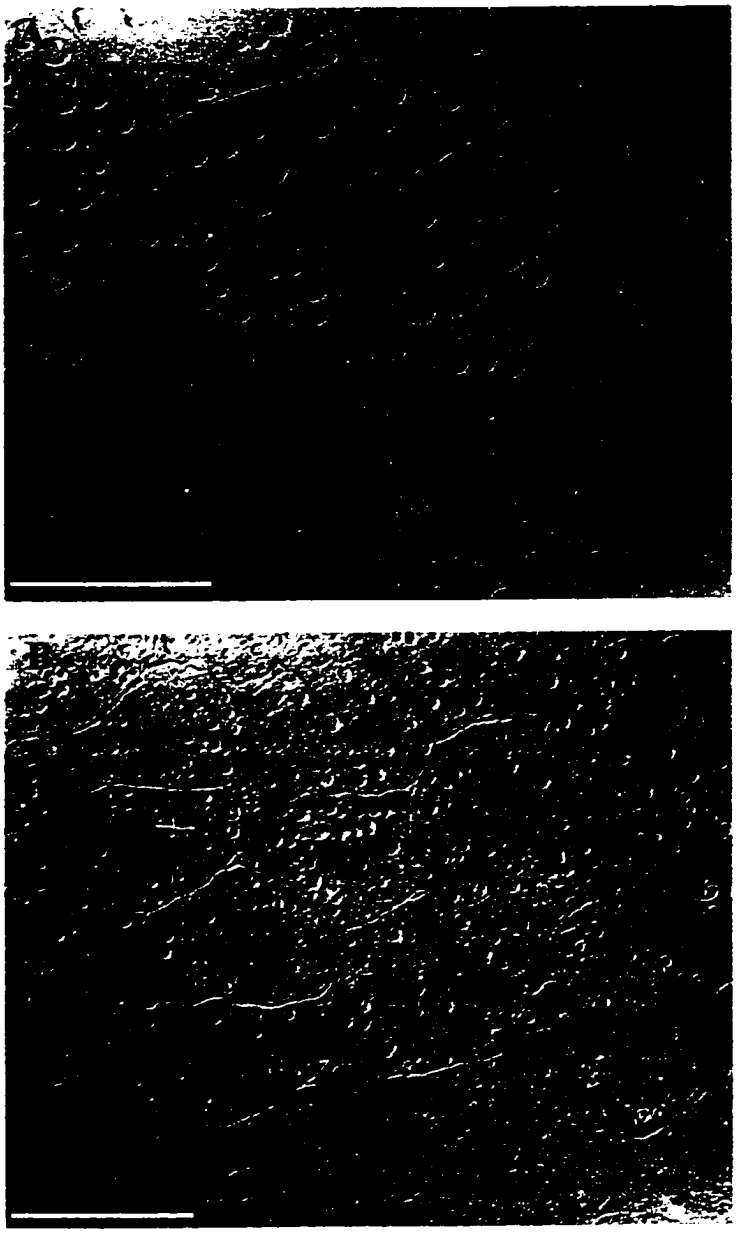


Fig. 4-10

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 4-11. Confocal microscopic images--Semithin sections of endosperm and embryo of rice viewed under bright light field.

Rice IR36 endosperm (A) and embryo (B) dissected at 7 DAF.

N, nucleus; CW, cell wall; P, plastid.

Bar represents 50 μm in A and 50 μm in B.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

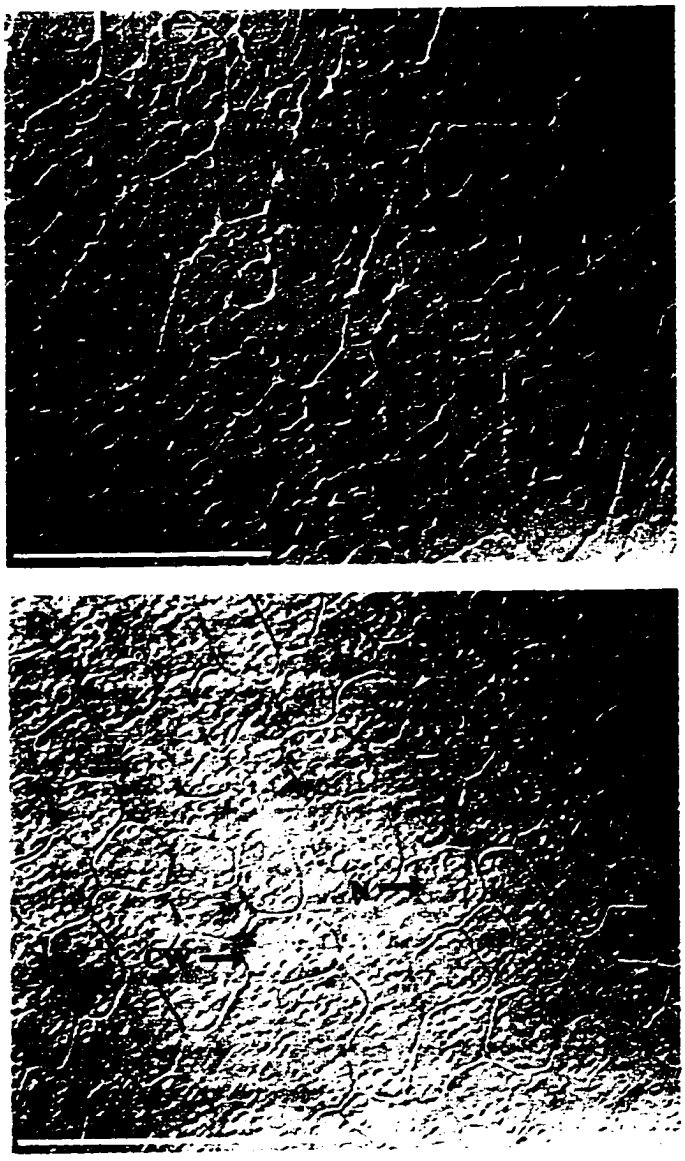


Fig. 4-11

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter 5. Production of maize specific anti-PDS antiserum

5.1 Introduction

Phytoene undergoes a sequence of four desaturation reactions to produce the pink-colored carotenoid lycopene. Each desaturation step introduces a carbon-carbon double bond into the chromophore, which in turn gives rise to the change of light absorption properties of the carotenoid. Desaturation reactions are catalyzed by desaturase enzymes whose activities differ among various organisms. It has been reported that there are two types of phytoene desaturase enzymes (Sandmann, 1994), the PDS type and the CrtI type. The PDS type enzyme catalyzes the first two desaturation steps to form ζ-carotene through the intermediate phytofluene, and is present in oxygenic photosynthetic organisms such as higher plants, algae and cyanobacteria; the second two desaturation steps are catalyzed by zetacarotene desaturase (Albrecht *et al.*, 1995; Linden *et al.*, 1994). The CrtI phytoene desaturase is named after the corresponding locus in *Rhodobacter capsulatus*, and is found in anoxygenic photosynthetic organisms, nonphotosynthetic bacteria and fungi. Enzymes in this group catalyze either three or four desaturation steps to form neurosporene or lycopene, respectively. Both types of phytoene desaturase contain a dinucleotide binding domain (FAD or NAD/NADP) at the amino terminus and functions to integrate desaturation with electron transfer cascades (Norris *et al.*, 1995). The dinucleotide binding domain is also present in ZDS proteins (Albrecht *et al.*, 1995; Linden *et al.*, 1994). All plant PDS proteins possess transit peptides of approximately 110

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

amino acid residues (Bartley *et al.*, 1991) that lead the proteins to plastids where the catalytic activities reside. PDS proteins of higher plants are homologous to cyanobacterial PDS, but not to the ones in bacteria or fungi (Bartley *et al.*, 1991; Hugueney *et al.*, 1992; Pecker *et al.*, 1992).

Previously, a Capsicum specific anti-PDS antiserum (kindly provided by Dr. B. Camara, IBMP, France) was used to examine PDS expression in maize and rice. Unfortunately, the antibody was not specific for the study in maize and rice (data not shown). In this chapter, I describe efforts to produce a monocot-specific antiserum for PDS, by expressing in *E. coli* a recently isolated maize *Pds* cDNA [GenBank #U37285 (Li *et al.*, 1996)]. A 1.85 kb cDNA fragment (nt#148–nt#2009) comprising 91% of the maize *Pds* gene was subcloned into an expression vector to produce a bacterial fusion protein. The purified fusion protein was used to immunize rabbits to produce a maize specific anti-PDS polyclonal antiserum.

The characterization of the anti-PDS antiserum will be presented in this chapter. In addition, preliminary results of expression of PDS in maize and localization of PDS in maize endosperm cells will be presented.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

5.2 Materials and Methods

5.2.1 Materials

Maize amyloplasts were isolated (Method I) from B73 endosperm (96004 sibling cross) at 17 DAP as described in Chapter 4. Yellow daffodil flowers were collected from the garden at Lehman College. Proteins were extracted from the yellow daffodil flower petals using the method described in Chapter 2. For the immunolocalization experiment, sections were prepared from B73 endosperm (96004 sibling cross) at 17 DAP as described in Chapter 4.

5.2.2 Subcloning of maize *Pds*

Construction of a recombinant plasmid pPET23a-MPds33

Plasmid pMPds33 in pBluescript SK^- (kindly provided by Z. Li, GenBank #U37285) was purified from 20 ml overnight culture of *E. coli* XL-Blue using the Wizard *Plus* Miniprep DNA Purification System (Promega, Madison, WI) according to the vendor's protocol. DNA concentration was determined by measuring absorbance at 260 nm on a Lambda 2 UV/VIS Spectrophotometer (Perkin-Elmer, South Plainfield, NJ) where 1 OD_{260} = 50 μg/ml. Purified plasmid DNA was digested with *Bgl*II and *Xho*I (Gibco BRL, Gaithersburg, MD) according to the manufacturer's protocol. Digestion products were separated on a 0.7% low melting point agarose gel in 0.5X TAE buffer (40 mM Tris

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Acetate; 1 mM Na_2EDTA pH 8) with ethidium bromide to a final concentration 0.5 μg/ml. A 1.85 kb *Bgl* II-*Xho*I cDNA fragment (nt#148-nt#2009) comprising 91% maize *Pds* (Genbank #U37285) (Li *et al.*, 1996) was excised from the agarose gel with a razor blade under UV light, and then purified using the GeneClean II Kit (BIO 101, Vista, CA) following the vendor's protocol. Two μg of an expression vector pET23a(+) (Novagen Inc., Madison, WI) was digested with *Bam*HI and *Xho*I, and the 3.6 kb cleaved vector was purified using the GeneClean Kit. To generate a recombinant construct, 30 ng of the purified *Bgl*II-*Xho*I *Pds* DNA (1.85 kb) and 50 ng of the purified *Bam*HI-*Xho*I fragment (3.6 kb) of pET23a were ligated with T4 DNA ligase (Gibco BRL, Gaithersburg, MD) at 16°C overnight following the method described in Sambrook (Sambrook *et al.*, 1989).

Bacterial transformation

To prepare *E. coli* competent cells of NovaBlue and BL21(DE3)pLysS, 10 ml LB were inoculated with single fresh bacterial colonies until absorbance at 600 nm of 0.6 was reached. Cells were chilled on ice for 10 minutes, then centrifuged down to pellet the cells at 3,000 rpm at 4°C for 15 minutes. Cells were suspended in 20 ml ice cold 100 mM $CaCl_2$, then collected after centrifugation at 3,000 rpm at 4°C for 10 minutes. The cell pellet was resuspended in 1 ml ice cold 100 mM $CaCl_2$.

Ten ng ligation products (pET23a-MPds33) described above or 1 ng vector plasmid DNA were mixed with 200 μl freshly prepared competent cells of a non-expression host, NovaBlue (Novagen, Inc., Madison, WI). The mixture was kept on ice for 30 minutes.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Following a 45 second heat shock at 42°C, the mixture was chilled on ice for 5 minutes, 800 µl LB added, and the mixture incubated at 37°C for 45 minutes. A 200 µl suspension was spread on LB solid medium supplemented with ampicilin (50 µg/ml) and chloramphenicol (34 µg/ml). Bacteria were incubated at 37°C overnight for growth of transformants.

Confirmation of the recombinant plasmid pPET23a-MPds33

The recombinant plasmid pPET23a-MPds33 was isolated from 10 ml overnight culture of transformants. Plasmid DNA was cleaved with *Eco*RI or *Hin*dIII to check the digestion pattern. In addition, the purified recombinant plasmid pPET23a-MPds33 was partially sequenced to confirm the in-frame junction (Figure 5-1). Double stranded DNA sequencing was performed using the Sequenase™ Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH) according to the manufacture's protocol. T7 primer [5'-d(TAATACGACTCACTATAGGG)-3'] was used in the sequence reactions. Reaction products were separated on a Baseace™ Vertical Sequencer (Stratagene, La Jolla, CA). Gels were analyzed on the PhosphorImager™ 445SI (Molecular Dynamics, Sunnyvale, CA).

The confirmed plasmid pPET23a-MPds33 and the pET23a vector (as a negative control) were transformed into an expression host, BL21(DE3)pLysS (Novagen Inc., Madison, WI) that contained an inducible bacteriophage T7 RNA polymerase gene.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

5.2.3 Over expression of the T7-PDS recombinant protein

A single colony from a freshly streaked plate was picked to inoculate 1 ml LB containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) and grown overnight at 37°C. Fifty ml LB containing both antibiotics in a 250 ml Erlenmeyer flask were inoculated with 0.5 ml of the overnight culture. Absorbance at 600 nm was measured every 20 minutes to generate a growth curve. To induce the expression of the recombinant protein, IPTG was added at various time points to the growing culture to a final concentration of 0.4 mM. One ml of cells were harvested at 15 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr and overnight after induction. Total cell proteins were extracted and dot blot analysis was carried out. Protein extracts were separated on SDS-PAGE, and a novel protein about 63 kDa was identified after the gel was stained. The optimal induction was carried out when OD_{600} was between 0.2 to 0.6. The optimal expression condition was to harvest cells 4 hr after induction.

5.2.4 Purification of the fusion protein

Cells from 3 x 500 ml culture in a 3 L Erlenmeyer flask were harvested 4 hours after the induction (OD_{600} about 0.760), kept on ice for 10 minutes, centrifuged at 5,000 x g at 4°C for 15 minutes, the cell pellet was resuspended in TE buffer (50 mM Tris pH 8; EDTA 2 mM) containing protease inhibitor stock solutions (5 mM DTT; 10 mM benzamidine and 50 mM 2-amino-n-caproic acid; 1.1 mM PMSF), incubated with lysozyme (1 mg/ml) and

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Triton X-100 (0.1% v/v) at 30°C for 15 minutes, and frozen at -80°C overnight. The suspension was thawed in a 25°C water bath, sonicated at 50% duty and output control setting of 5 for 3 x 20 seconds using a Vibra Cell Sonicator (Sonic Materials, Inc., Danbury, CT). The suspension was centrifuged at 5,000 x g at 4°C for 15 min. The pellet was washed twice with TE plus protease inhibitors and then solublized in 1 ml of 2x SDS protein sample buffer (see Chapter 2). From 1.5 liters of induced culture, about 2 mg fusion protein with approximately 50% purity was yielded.

5.2.5 Immunization

Approximately 3 mg protein extract containing the fusion protein were subjected to two 12% SDS-PAGE gels. Proteins were separated at 12 mA for 16 hr at room temperature. About 1.2 mg of the fusion protein were excised from the SDS gels. Gel slices were combined in 1 ml of 125 mM NaCl. For each of three immunization projects, prior to immunization, 10 rabbits were prescreened to test for preexisting antigenic responses to maize proteins. From the prescreened group, two rabbits were chosen for immunization. Each rabbit received 500 μg PDS fusion protein for each injection (conducted by Lampire Biological Inc., Piperville, PA). The immunization schedule was the same as the one used for the production of the PSY antiserum (see Chapter 2). After the first test bleed was tested (four weeks after the initial immunization), each rabbit was boosted with 500 μg antigen biweekly, and a test bleed were prepared two weeks later. Production bleed (20 ml) was obtained (8/28/97) from rabbit #6079 (8/28/97) two months after the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

immunization initiation.

5.3 Results

5.3.1 Overexpression of PDS fusion protein

To generate an antibody against maize PDS, subcloning of Mpds33 into pET23a was designed as shown in Figure 5-1A. The *Bam*HI-*Xho*I cleaved vector and a *Bgl*II-*Bam*HI fragment of Mpds33 containing 1.85kb were fused in-frame to generate a recombinant plasmid pPET-MPDS33 as shown in Figure 5-1B. In this construct, sequence encoding part of the PDS transit peptide (nt #1–nt#147 corresponding to aa #1-aa#17) is missing. The recombinant plasmid was first transformed into NovaBlue, a non-expression host, and the plasmid was sequenced to check the proper junction of the vector and *Pds*. As shown in Figure 5-1C, partial DNA sequence shows the correct junction and a small region of maize *Pds*.

The fusion protein was over expressed in BL21(DE3)pLysS. A polypeptide from the bacterial inclusion bodies with an apparent molecular mass of 63 kDa (Figure 5-2A, lane 1) was observed on the SDS-PAGE gel. This protein comprised about 50% of the total bacterial proteins and was consistent with the predicted size of 62.2 kDa. The fusion protein was also observed in the total bacterial cell extract (lane 3) at a lower level compared to the fusion protein detected from the bacterial inclusion body (lane 1). From

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the bacterial cells without the *Pds* fusion construct pPET23a-MPds33, no fusion protein was detected as expected (Figure 5-2A, lane 2). Using an anti-T7-Tag monoclonal antibody, a protein identical in size to the fusion protein was detected from the purified inclusion body fraction (Figure 5-2B, lane 1) and from the total cell extract (Figure 5-2B, lane 3), whereas no signal was detected from the cell extract containing only the host cell proteins (Figure 5-2B, lane 2). After six rabbits from three independent projects were immunized with the gel purified fusion protein, only one rabbit (#6079) showed immune response against maize proteins (data not shown). This bleed was characterized for the anti-PDS antibody activity. As shown in Figure 5-3, the fusion protein was recognized by this antiserum (lane 1). When maize endosperm protein extract was used, there was no strong specific reaction present (data not shown). Therefore, the maize amyloplast was isolated (Method I) to enrich the target protein, and used for western analysis. An antigenic signal with an apparent size of 53 kDa was detected (lane 2) when the PDS antiserum was used as a probe of the western blot. A daffodil flower protein extract was also subjected to western analysis, and a protein with similar migration to the maize amyloplast protein was identified (lane 3). When 8 μg of the antiserum activity was mixed with 12 μg fusion protein, the antigenic signals detected on the western blot were greatly reduced (data not shown). This suggests that the antiserum specifically recognized the PDS protein.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

5.3.2 Localization of PDS in maize B73 endosperm

The PDS protein was detected in an amyloplast-enriched endosperm protein fraction, but was undetectable when total endosperm proteins were assayed. Therefore, another approach, immunofluorescent localization, was applied to test if the antiserum could react with any potential antigens in the endosperm cell. The preliminary result showed signals both on the envelope and in the interior of amyloplasts (Figure 5-4).

5.4 Discussion

5.4.1 Specificity of maize anti-PDS antiserum

Several antisera were tested for potential study of PDS expression in maize. These antisera obtained from other labs were raised against PDS originated from different organisms including *Capsicum* (provided by Dr. Camara), *Synechoccocus* (provided by Dr. Hirschberg) and synthetic MAP peptides (Yu and Wurtzel, unpublished). However, none of these antisera showed good immunological reaction with the maize or rice PDS proteins (data not shown). Recently, Hable *et al.* generated a maize PDS antiserum and reported that it reacted with a 55 kDa protein that was only detectable using plastid fractions from endosperm and embryo (Hable *et al.*, 1998). The antiserum was not specific and recognized multiple antigens on western blots; only the 55 kDa antigen level was greatly reduced after antiserum activity was inhibited when incubated with the fusion

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

protein (Hable *et al.*, 1998). Therefore, after a maize PDS cDNA was cloned (Li *et al.*, 1996), it was used to generate a recombinant protein in *E. coli*, then the over expressed fusion protein was injected into rabbits to produce polyclonal antisera. One bleed (PDS polyclonal antiserum) with immunoactivity was obtained. Results show that the PDS polyclonal antiserum can specifically detect the PDS fusion protein (about 63 kDa) accumulated in the bacterial inclusion body. A major signal (about 53 kDa) from an amyloplast fraction of maize endosperm and from total proteins extracted from daffodil flower were detected by this PDS antiserum. The size of the maize PDS antigen (53 kDa) is about the same as the size (53.5 kDa) of the mature PDS protein deduced from the cDNA sequence (Li *et al.*, 1996). This PDS antiserum also recognized an antigen in the protein extract of daffodil flower, and the size (about 53 kDa) was similar to the 52 kDa PDS protein detected by a daffodil anti-PDS antiserum (Al-Babili *et al.*, 1996).

5.4.2 Localization of PDS in maize endosperm

Maize PDS was found to be associated with amyloplast envelope and stroma in endosperm. *In vitro* import and immunological studies showed that the daffodil PDS protein has two forms, active--membrane bound form, and inactive--soluble form (Bonk *et al.*, 1997; Schledz *et al.*, 1996). Several *in vitro* studies, using purified thylakoids from *Synechocystis* (Dogbo *et al.*, 1988) and isolated chloroplasts from green pepper fruits and radish seedlings (Grumbach and Britton, 1984), showed that PDS protein is an integral protein of the thylakoid membrane. Therefore, PDS localization in maize endosperm

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

amyloplasts is similar to that in daffodil flower chromoplasts. My results support that the PDS may have two forms in the amyloplasts in maize endosperm.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 5-1. Construction of pPET23a-MPds33 for expression of PDS fusion protein.

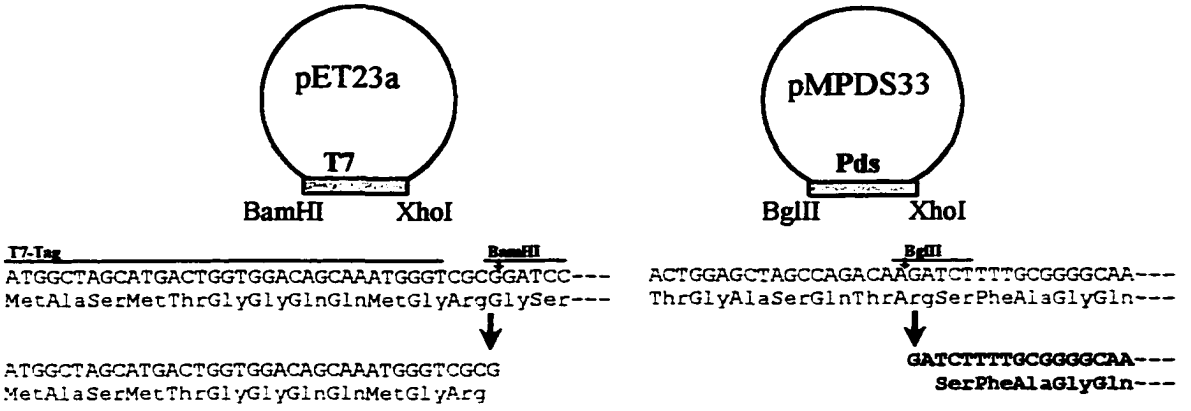
A: Plasmids pPET23a and pMPds33 used for ligation.

B: Partial DNA and amino acid sequences showing the junction of the construct of pPET23a-MPds33.

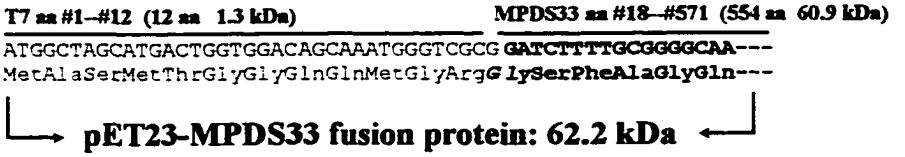
C: DNA sequence of junction.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

A:



B:



C:

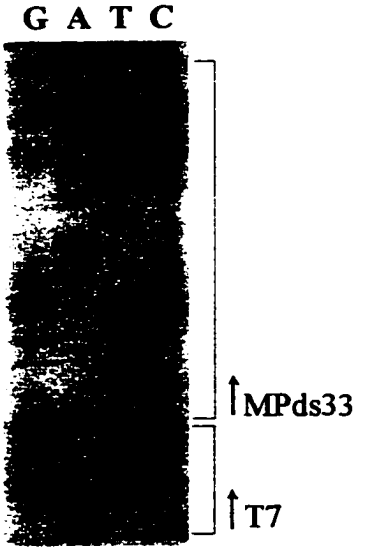


Fig. 5-1

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 5-2. Overexpression of PDS fusion protein in *E. coli*.

Lane 1, protein extract prepared from inclusion body fraction purified from BL21(DE3)pLysS transformed with the recombinant plasmid pPET23a-MPds33.

Lane 2, total protein extract from host cells BL21(DE3)pLysS without pPET23a-Mpds33.

Lane 3, total protein extract from BL21(DE3)pLysS transformed with the recombinant pPET23a-Mpds33.

(A) SDS-PAGE gel.

(B) Immunoblot probed with the anti-T7 monoclonal antibody.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

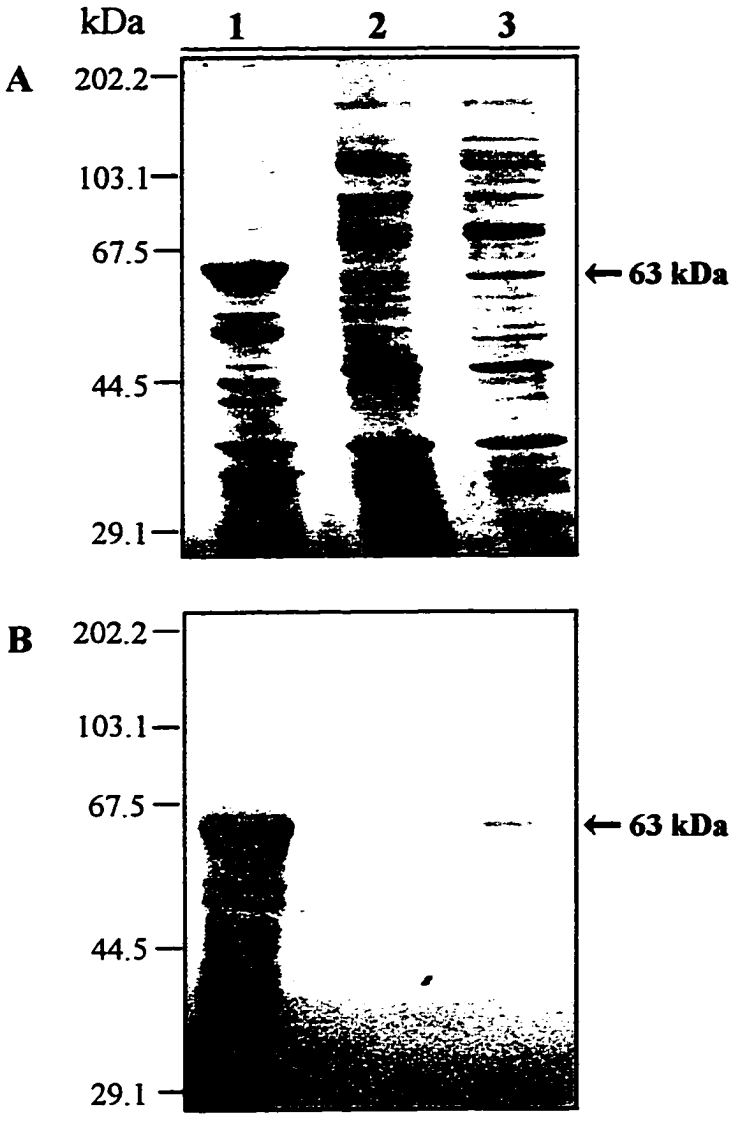


Fig. 5-2

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

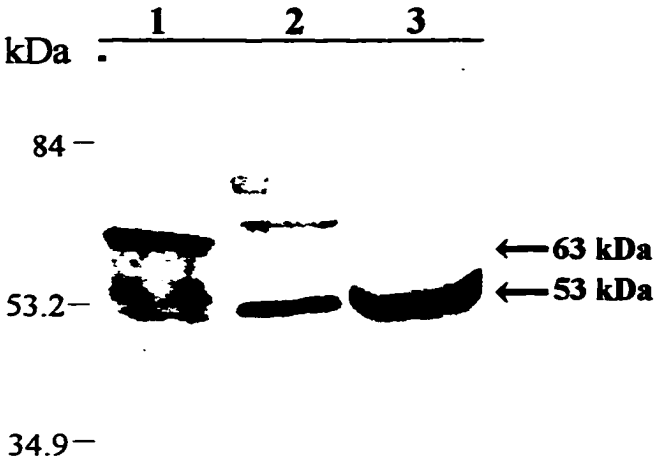


Figure 5-3. Western analysis of phytoene desaturase expression. Immunoblot probed with anti-PDS antiserum. Lane 1, PDS fusion protein expressed in *E. coli*; 2, maize B73 endosperm amyloplast fraction; 3, daffodil flower total protein extract.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

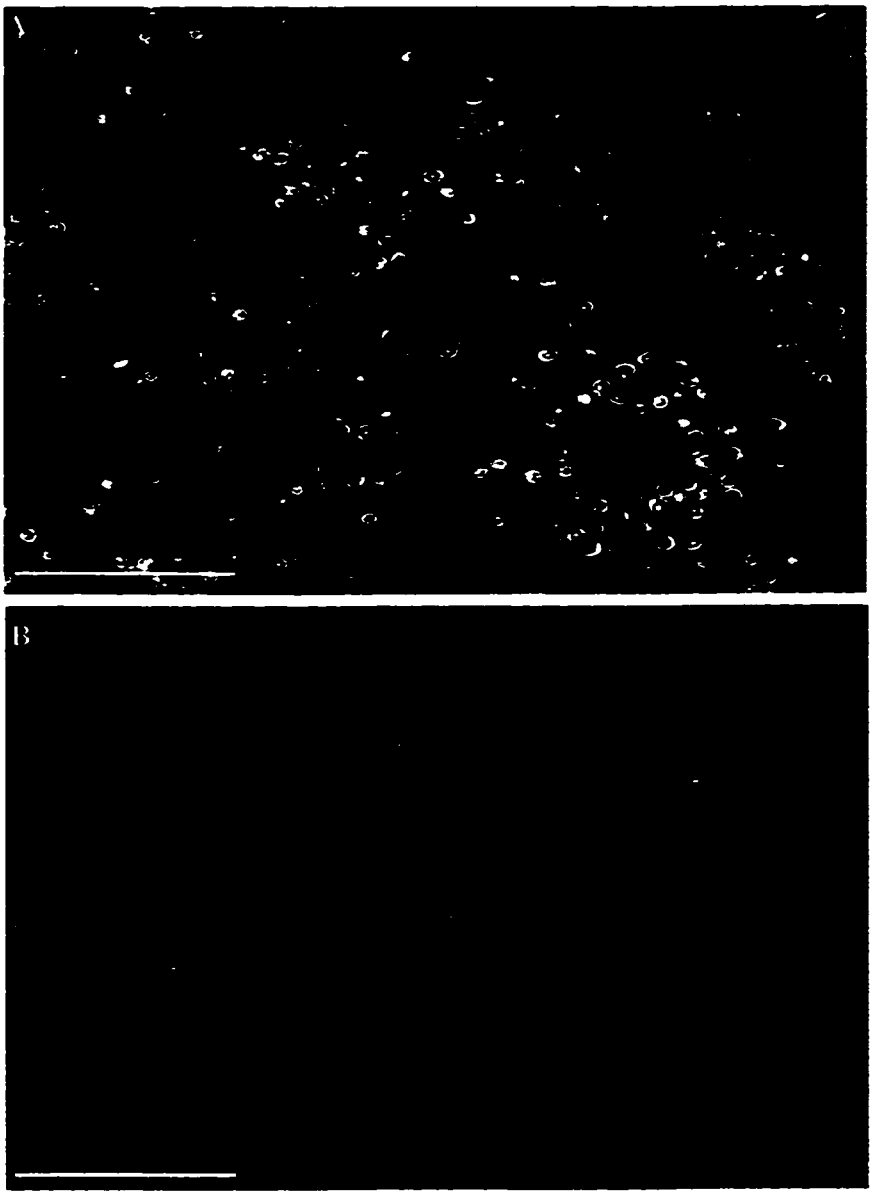
Figure 5-4. Localization of PDS in maize B73 endosperm dissected at 17 DAP.

A, probed with anti-PDS antiserum and anti-rabbit IgG FITC conjugate.

B, negative control probed with anti-rabbit IgG FITC conjugate only.

Bar represents 100 μm in A and 100 μm in B

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter 6. Summary and future perspectives

Using techniques in biochemistry, cell biology, immunology and molecular biology, I have analyzed carotenoid accumulation during seed development in maize. I studied expression of phytoene synthase, the first enzyme in the carotenoid biosynthetic pathway, during endosperm development and in various tissues of maize and rice. I also conducted immunocytochemical localization of phytoene synthase in amyloplasts of maize and rice endosperms. I developed a polyclonal antibody against maize phytoene desaturase, characterized the anti-PDS antibody, and studied the expression and localization of PDS in maize amyloplasts. The importance of the above results will be summarized, and future studies will be discussed.

6.1 Regulation of phytoene synthase expression

Study of regulation of carotenoid biosynthesis has been ongoing for many years. Many genes in the carotenoid biosynthetic pathway have been cloned and characterized. Regulation of gene expression has been studied with respect to development, tissue-specificity, plastid specificity and environmental effects. In maize endosperm, PSY expression correlated with colored carotenoid accumulation during the early developmental stage (between 10 to 20 DAP). Therefore, PSY might be the rate-limiting enzyme in the pathway. PSY expression is temporally controlled in maize--levels of PSY protein were higher between 10-20 DAP, then decreased throughout endosperm

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

development in B73 and A632 lines. In rice, accumulation of PSY protein declined gradually during seed development. In addition, PSY expression showed a tissue specific manner in maize and rice. In maize, PSY expression was higher in endosperm, low in embryo, undetectable in leaf. In rice, PSY accumulation was fairly similar in endosperm and embryo, but undetectable in leaf. The tissue specific expression of PSY protein was observed in several higher plants. In daffodil flower chromoplasts, PSY was found in two forms, an active form associated with chromoplast membrane, and an inactive form coupled with Cpn60, but not Hsp70, in chromoplast stroma (Al-Babili *et al.*, 1996). An *in vitro* import experiment of PSY import into pea chloroplasts showed that PSY targeted to the thylakoid membrane but very little associated with the chloroplast envelope (Bonk *et al.*, 1997). In maize endosperm amyloplasts, PSY appears to be only in the envelope of amyloplasts. The difference of the PSY localization in maize endosperm amyloplast and daffodil flower chromoplast could be due to the different structure and internal components in plastids. Daffodil flower chromoplasts and maize endosperm amyloplasts are different types of plastids. It is known that daffodil chromoplasts differentiated from chloroplasts at an early stage of flower development. In maize, amyloplasts derive from proplastids without developing a significant internal membrane system. Therefore, maize PSY may only target to amyloplast envelope. Various mutants of maize show different phenotypes of leaf and endosperm carotenoids. *Wc1* is a dominant mutant blocking carotenoid accumulation in maize endosperms. Since PSY protein is expressed in *Wc1* endosperms, this locus might encode other factors involved in PSY import, such as chaperonins or carotenoid-binding proteins in plastids. Using available antisera against

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the above proteins, their involvement in carotenoid accumulation in maize mutants can be examined through western analyses and immunocytochemical localization.

In rice endosperms, amyloplasts appear to be compound in comparison to simple ones in maize. PSY seems not to localize in amyloplasts, but rather remains in the cytoplasm. From western analysis, rice PSY has the similar molecular mass as maize PSY. If the PSY that accumulates in rice cytoplasm is the processed form, we could assume that the protein has been imported to amyloplasts, but then exported out to the cytoplasm. It has been reported that the light-harvesting chlorophyll a/b-protein complex (LHCII) polypeptides were found in the chloroplast envelope as well as in small cytoplasmic vacuoles in *Chlamydomonas reinhardtii* (Wolfe *et al.*, 1997). These studies indicated that the proteins were translocated into the chloroplast and processed, but were not retained in the chloroplast. These studies also suggested that if the rate of synthesis of LHCII polypeptides exceeded that of chlorophyll, import was aborted (Wolfe *et al.*, 1997). Similar results were reported using apoproteins (Lhcb) of LHCII (Komine *et al.*, 1996; Park and Hoober, 1997; White *et al.*, 1996). If other components required for proper localization are not ready, then PSY might not localize to the proper destination. In rice amyloplasts, starch deposit starts early in seed development. After 10 DAF, endosperms become very starchy. Timing for other activities such as auxillary factors required for protein import in amyloplasts could be critical. As reported, carotenoids accumulated in plastids are in complexes containing carotenoid-binding proteins (Cervantes-Cervantes *et al.*, 1990). If these proteins do not function synchronizingly with PSY protein expression

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

and/or import, nonfunctional PSY protein would accumulate in region(s) other than amyloplast membrane. That would be one explanation for the accumulation of PSY in the cytoplasm in rice endosperm cells. To study where PSY protein accumulated in the cytoplasm of endosperm cells, electron microscopy with higher magnification and resolution will provide more cellular details. Therefore, localization of PSY at the electron microscopic level will be an important experiment for further study of PSY in both maize and rice. Western analyses using maize PSY antiserum showed that rice PSY is 38 kDa. It is not known whether the 38 kDa PSY protein in rice is a mature protein or a precursor protein. If the unprocessed rice PSY is close to 38 kDa, western analysis would not distinguish the two forms. Therefore, the following experiments will be necessary to answer the above questions.

To confirm the antigenic polypeptide recognized from rice protein extract using maize PSY antiserum is a real PSY, the maize anti-PSY antibody could be used to screen a rice cDNA expression library to pull out a rice *Psy* cDNA. After a full length cDNA is obtained, sequencing information will reveal any homology among other *Psy* genes. In addition, the size of rice PSY protein could be predicted from the cDNA sequence. Furthermore, comparison between prokaryotic PSY and rice PSY deduced protein sequences could demonstrate if rice PSY protein contains a transit peptide region. Or otherwise a rice specific antibody has to be generated using isolated rice *Psy* cDNA. Another approach to test where rice PSY protein is localized, the transit sequence of the rice *Psy* gene could be fused with green-fluorescent protein (GFP). The recombinant

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

plasmid of PSY-GFP could be transformed into *Agrobacterium tumefaciens* by electroporation. Then rice endosperm cells could be co-cultured with *A. tumefaciens* and transformed rice plants regenerated. Visualization of the fluorescense could tell where the recombinant protein PSY-GFP targets in rice endosperm cells.

Rice does produce carotenoids in green tissues even though it does not accumulate any carotenoids nor precursors in endosperm. Transgenic rice plants expressing a daffodil *Psy* cDNA accumulated phytoene but no other carotenoids in endosperm (Burkhardt *et al.*, 1997). Overexpression of the daffodil *Psy* cDNA in transgenic rice did not overcome the lack of carotenoid synthesis in rice endosperm. Therefore, carotenoid biosynthetic enzymes, PSY and/or others, may not function properly nor efficiently in rice endosperm. Results from the current study strongly support that PSY may not function due to its improper localization in rice endosperm.

Contents of carotenoids differ in various plants, tissues, and plastids. As shown in Table 6-1, marigold flower petals accumulate about 20 mg/g carotenoids; daffodil flower petals accumulate 2.8 mg/g carotenoids; pepper fruits accumulate 75-825 μg/g carotenes; ripe tomato fruits accumulate 284 μg/g carotenoid; tomato seedlings contain 668.1 μg/g carotenoids; maize seedlings contain 72-1079 μg/g carotenoids; while mature maize endosperms accumulate 5-12 μg/g carotenoids. The great differential in carotenoid content of various plants and tissues, ranging several orders of magnitude, suggests the potential for significant enhancement of maize endosperm carotenoid content.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Accumulation of carotenoids is related to the combination of several elements including level of carotenoid biosynthetic enzymes, carotenoid binding proteins, lipids in plastids, and molecular chaperonin in plastids. Such a goal would require a deeper understanding of the factors involved in promoting carotenoid biosynthesis and accumulation, specifically in maize endosperm.

6.2 Expression of phytoene desaturase

Although PSY might be the rate-limiting step in the carotenoid biosynthetic pathway, there might be other steps that play regulatory roles. Therefore, it is important to understand how PDS expression is controlled in maize and rice. A monocot specific antiserum is necessary to analyze PDS expression in both maize and rice. An amyloplast-enriched fraction showed the mature PDS protein detected by western analysis. Preliminary result of PDS localization showed two locations of this protein; one form is in the stroma and the other is in the envelope of amyloplasts in maize endosperms. This result is consistent to PDS localization in daffodil flower chromoplasts (Bonk *et al.*, 1997). In rice endosperms, PSY has been found not associated with amyloplasts. It will be interesting to know where PDS protein is localized in rice endosperms. Since there is no rice specific anti-PDS antiserum available, the maize PDS antiserum could be used to test whether and how PDS protein is accumulated in rice.

In maize, several mutants blocking the carotenoid pathway have been tested for PSY

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

expression by western analyses. Except for *y1*, none of the loci examined seems to regulate PSY expression. Using the anti-PDS antiserum, endosperms with those mutations could be analyzed for any possible regulatory effects on PDS expression. It is known that *vp5* encodes PDS in maize (Hable and Oishi, 1995; Hable *et al.*, 1998; Li *et al.*, 1996). RT-PCR analysis showed that *Pds* transcript level was dramatically reduced in *vp5-Mum* endosperm (Li *et al.*, 1996). In this study, there were no antigenic signals detected by the anti-PDS antiserum when protein extracts from normal and mutant endosperms of *vp5-Mum* were used. It was reported that PDS expression was low in maize endosperms, and only the plastid fraction could give positive signals on western analyses (Hable *et al.*, 1998). Therefore, amyloplast fractions could be isolated from endosperms with those mutations, then different pattern of PDS protein accumulation would be expected between normal and mutant endosperms.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 6-1: Carotenoid contents from various higher plants.

Plant	Tissue	Carotenoid(s)*	Content (μg/g fw)	Reference
rice	endosperm	N/D		Wurtzel *et al.*, 1998 Burkhardt *et al.*, 1997
rice	embryo	carotenoids	0.7**	Luo and Wurtzel, unpublished
maize	endosperm	carotenoids	5-12	Chapter 2
maize	embryo	carotenoids	1.6	Yu and Wurtzel, unpublished
maize	seedling	carotenoids	1079 300 213 71.6	Mayfield *et al.*, 1986 Gavazzi *et al.*, 1974 Bachmann *et al.*, 1973 Robertson and Anderson, 1961
daffodil	flower petal	carotenes	2855.3	Schledz *et al.*, 1996
marigold	flower petal	carotenoids	20000.5**	Delgado-Vargas and Paredes-López, 1997
pepper	fruit	carotenoids	75-825	Mozafar, 1994
tomato	seedling	β-carotene xanthophylls	242.4 425.7	Fraser *et al.*, 1994
tomato	ripe fruit	lycopene	284 969**	Giuliano *et al.*, 1993 Harris *et al.*, 1969

N/D: not detected
*specific carotenoids listed represent the major (>90%)carotenoids
**represents dry wt

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Appendix

A: Antisera used in western analysis and immunocytochemical localization. Antisera were diluted in TBST for western analysis experiments, and in PBS for immunocytochemical labeling.

A-1: Antibodies used in experiments described in this thesis.

Antisera	Animal source	Dilution factor	Origin
Anti-PSY (maize)	rabbit #3445 (3/30/94)	1:10,000 (w) 1:100 (c)	Yu and Wurtzel
Anti-PDS (maize)	rabbit #6079 (8/28/97)	1:1,000 (w) 1:100 (c)	Yu and Wurtzel
Anti-Sh (maize)	rabbit #1--3rd	1:12,000	Dr. B. Burr Brookhaven National Laboratory
Anti-Bt1 (maize)	rabbit #2991-p2	1:5,000 (w) 1:100 (c)	Dr. T. Sullivan University of Wisconsin
Anti-T7-Tag	mouse	1:3,000	Novagen Inc. (Madison, WI)
Anti-mouse IgG AP conjugate	rat	1:7,500	Promega (Madison, WI)
Anti-rabbit IgG AP conjugate	goat	1:8,000	Promega (Madison, WI)
Anti-rabbit IgG FITC conjugate	goat	1:50 or 1:100	Sigma (St. Louis, MO)
PSY-preimmune serum	rabbit #3445	1:100	Lampire Biological Inc. (Piperville, PA)

(w): dilution used in western analyses
(c): dilution used in confocal microscopic experiments

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

A-2: Antisera used in experiments not described in this thesis.

Antisera	Animal source	Dilution factor	Origin
Anti-CrtI (*Rhodobacter capsulatus*)	rabbit (bleed "O")	1:16,000	Dr. E.T. Wurtzel
Anti-GGPS (Capsicum)	rabbit	1:5,000	Dr. B. Camara IBMP, France
Anti-PSY (Capsicum)	rabbit	1:5,000	Dr. B. Camara IBMP, France
Anti-PDS (Capsicum)	rabbit	1:5,000	Dr. B. Camara IBMP, France
Anti-PDS (*Synechococus*)	rabbit	1:2,000	Dr. J. Hirschberg The Hebrew University of Jerusalem, Israel
Anti-PAP	rabbit	1:5,000	Dr. R. Schantz IBMP, France
Anti-Wx (maize)	rabbit	1:8,000	Dr. S. Wessler University of Georgia, Georgia
Anti-SBEII (maize)	rabbit	1:10,000	Dr. C. Mu-Forster & Dr. B.P. Wasserman Rutgers State University, New Jersey

IBMP: Institut De Biologie Moleculaire des Plantes

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

B: Figure and file descriptions.

Fig#	Fig File Name	Original Data File #	File Description	Experiment Date	Lab Book #
2-1	2-1.xls		Carotenoid determination	0794-0695	5
2-2	2-2.prs		Characterization of anti-PSY antiserum	042894	4
2-3	2-3.prs	001.tiff 002.tiff	PSY expression in *Y1* mutant	042794	4
2-4	2-4.prs	02039507.tiff 02039506.tiff	PSY expression in maize mutants	111094 011795	4
2-5	2-5.prs	001.tiff 002.tiff	Dosage effect of Y1	042794 042094	4
2-6	2-6.prs	98032206.tiff 98032207.tiff 98101901.tif	PSY B73-1993	050494	4
2-7	2-7.prs	98032702.tiff 98032703.tiff 98101902.tiff	PSY A632-1993	080994	4
2-8	2-8.prs	98032205.tiff 98032208.tiff	PSY Argentina-1993	050694	4
2-9	2-9.prs	01229804.tiff 01229801.tiff	PSY B73-1996	012098	9
2-10	2-10.prs	01229806.tiff 01229807.tiff 01299803.tiff	PSY A632-1996	012098	9
2-11	2-11.prs	01229802.tiff 01229805.tiff arg96w.tiff	PSY Arg-1996	012098	9
2-12	2-12.prs	98032202.tiff	PSY expression in tissues of maize	050694	4

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Fig#	Fig File Name	Original Data File #	File Description	Experiment Date	Lab Book #
3-1	3-1.prs	97052703.tiff	PSY expression in endosperm of maize and rice	052297	9
3-2	3-2.prs	98032201.tiff	PSY expression in tissues of rice	050694	4
3-3	3-3.prs	IRPSY96.tiff IR36SH.tiff IRSTAIN.tiff	PSY expression-IR36	100394	6
3-4	3-4.prs	98032203.tiff 98032204.tiff	PSY expression-Cypress	100794	6
4-1	4-1.prs	97050804.tiff 97050803.tiff	PSY in endosperm and embryo of maize and rice	052297	9
4-2	4-2.prs	97061402.tiff 97052701.tiff	PSY in amyloplasts in maize and rice	052297 061297 041297	9
4-3	4-3A.prs 4-3B.prs	33.pic 27.pic	Localization of PSY in B73 endosperm	012698 110397	10
4-4	4-4.prs	98032701.tiff	Bt1 in B73 endosperm	092597	9
4-5	4-5A.prs 4-5B.prs	23.pic 27.pic	Bt1 localization in B73 endosperm	012698 110397	10
4-6	4-6A.prs 4-6B.prs	5.pic 7.pic	Localization of PSY in B73 embryo	012698	10
4-7	4-7A.prs 4-7B.prs	15.pic 19.pic	Localization of PSY in IR36 embryo	110397	10
4-8	4-8A.prs 4-8B.prs	Red1.pic 31.pic	Localization of PSY in IR36 endosperm	110397 012298	10
4-9	4-9A.prs 4-9B.prs	29.pic 31.pic	Bt1 localization in IR36 endosperm	012298	10

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Fig#	Fig File Name	Original Data File #	File Description	Experiment Date	Lab Book #
4-10	4-10A.prs 4-10B.prs	34.pic 6.pic	Confocal images of endosperm and embryo of maize	012698	10
4-11	4-11A.prs 4-11B.prs	10.pic 14.pic	Confocal images of endosperm and embryo of rice	110397	10
5-1	5-1.prs	gel19.gel	Construction of ZMPDS fusion protein	101996	8
5-2	5-2.prs	97010702.tiff 97010701.tiff	Overexpression of PDS fusion protein	010697	8
5-3	5-3.prs	97100102.tiff	PDS expression	093097	8
5-4	5-4A.prs 5-4B.prs	25.pic 27.pic	PDS localization in B73 endosperm	110397	10

gel: ImageQT DNA sequencing file
pic: Confocal microscopic image file (open as "RAW"; 712 x 568 in Photoshop program)
prs: Harvard Graphic presentation file (Harvard Graphics 3.0)
tiff: Photoshop image file
xls: Excel file

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

C: Description of confocal microscopic images.

Maize ED, maize B73 endosperm dissected at 17 DAP; Maize EM, maize embryo dissected at 17 DAP; Rice ED, rice IR36 endosperm dissected at 7 DAF; Rice EM, rice IR36 embryo dissected at 7 DAF.

F-blue: fluorescence with blue filter; Bright: Bright light field

Experimental sets were: 1/26/98 (1.pic-8.pic, 21.pic-24.pic, 33.pic-34.pic); 1/22/98 (29.pic-32.pic); 11/03/97 (9.pic-20.pic, 25.pic-28.pic, Red1.pic)

Data file	Fig # in thesis	Tissue	Sera	Lens x	Light	Zoom
1.pic		Maize ED	PSY	25	F-blue	1
2.pic		Maize ED	PSY	25	Bright	1
3.pic		Maize ED	Pre-immune (PSY)	25	F-blue	1
4.pic		Maize ED	Pre-immune (PSY)	25	Bright	1
5.pic	4-6A	Maize EM	PSY	40	F-blue	1.3
6.pic	4-10B	Maize EM	PSY	40	Bright	1.3
7.pic	4-6B	Maize EM	Pre-immune (PSY)	40	F-blue	1.3
8.pic		Maize EM	Pre-immune (PSY)	40	Bright	1.3
9.pic		Rice ED	PSY	40	F-blue	1.3
10.pic	4-11A	Rice ED	PSY	40	Bright	1.3
11.pic		Rice ED	Pre-immune (PSY)	40	F-blue	1.3
12.pic		Rice ED	Pre-immune (PSY)	40	Bright	1.3
13.pic		Rice EM	PSY	40	F-blue	1
14.pic	4-11B	Rice EM	PSY	40	Bright	1
15.pic	4-7A	Rice EM	PSY	40	F-blue	1.5

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Data file	Fig # in thesis	Tissue	Sera	Lens x	Light	Zoom
16.pic		Rice EM	PSY	40	Bright	1.5
17.pic		Rice EM	PSY	40	F-blue	1
18.pic		Rice EM	PSY	40	Bright	1
19.pic	4-7B	Rice EM	Pre-immune (PSY)	40	F-blue	1
20.pic		Rice EM	Pre-immune (PSY)	40	Bright	1
21.pic		Maize ED	Bt1	40	F-blue	1
22.pic		Maize ED	Bt1	40	Bright	1
23.pic	4-5A	Maize ED	Bt1	25	F-blue	1
24.pic		Maize ED	Bt1	25	Bright	1
25.pic	5-4A	Maize ED	PDS	25	F-blue	1
26.pic		Maize ED	PDS	25	Bright	1
27.pic	4-3B 4-5B 5-4B	Maize ED	anti-rabbit IgG FITC conjugate only	25	F-blue	1
28.pic		Maize ED	anti-rabbit IgG FITC conjugate only	25	Bright	1
29.pic	4-9A	Rice ED	Bt1	40	F-blue	1
30.pic		Rice ED	Bt1	40	Bright	1
31.pic	4-8B 4-9B	Rice ED	anti-rabbit IgG FITC conjugate only	40	F-blue	1
32.pic		Rice ED	Anti-rabbit IgG FITC conjugate only	40	Bright	1
33.pic	4-3A	Maize ED	PSY	40	F-blue	1

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Data file	Fig # in thesis	Tissue	Sera	Lens x	Light	Zoom
34.pic	4-10A	Maize ED	PSY	40	Bright	1
Red1.pic	4-8A	Rice ED	PSY	40	F-blue	1

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

D: Recombinant plasmids constructed

D-1: PDS constructs

Plasmid Constructed	Original Clone	Fragment (kb)	PDS Nucleotides # Amino acids #	Cloning Vector (kb)	Chapter in Thesis
pPET23-MPDS33	pMPDS33	BglII-XhoI (1.85 kb)	#148-2009 #18-571	pPET23a BamHI-XhoI (3.6 kb)	5
pPET23-MPDS73	pMPDS73	SstI-XhoI (1.3 kb)	#608-1914 #202-571	pPET23b SacI-XhoI (3.6 kb)	
pPET23-MPDS218	pMPDS218	SstI-XhoI (1.7 kb)	#209-1914 #69-571	pPET23b SacI-XhoI (3.6 kb)	
pPET23-MPDS97	pMPDS97	SstI-XhoI (1.9 kb)	#132-1914 #44-571	pPET23b SacI-XhoI (3.6 kb)	

D-2: LTK constructs

Plasmid Constructed	Original Fragment	Fragment (kb)	LTK Nucleotides # Amino acids #	Cloning Vector (kb)	Chapter in Thesis
pPET23-LTK229	LTK PCR-229	SalI-SstI (1.18 kb)	#1-1180 #1-394	pPET23c SalI-SstI (3.6 kb)	
pPET23-LTK230	LTK PCR-230	SalI-SstI (1.24 kb)	#1-1240 #1-414	pPET23c SalI-SstI (3.6 kb)	

All junctions confirmed by sequencing.
Original PDS clones and LTK fragments were from Dr. Z-h Li.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Bibliography

Akiya, T. (1962). Components of unsaponifiable matter of rice bran oil. Agr. Biol. Chem. (Tokyo) *26*, 180.

Al-Babili, S., von Lintig, J., Haubruck, H., and Beyer, P. (1996). A novel, soluble form of phytoene desaturase from *Narcissus pseudonarcissus* chromoplasts is Hsp70-complexed and competent for flavinylation, membrane association and enzymatic activation. The Plant Journal *9*, 601-612.

Albrecht, M., and Sandmann, G. (1994). Light-stimulated carotenoid biosynthesis during transformation of maize etioplasts is regulated by increased activity of isopentenyl pyrophosphate isomerase. Plant Physiology *105*, 529-534.

Albrecht, M., Klein, A., Hugueney, P., Sandmann, G., and Kuntz, M. (1995). Molecular cloning and functional expression in *E. coli* of a novel plant enzyme mediating ζ-carotene desaturation. FEBS Letters *372*, 199-202.

Anderson, I.C., and Robertson, D.S. (1963). Chlorophyll and carotenoid contents of some pigment mutants in corn. (Abstr.) Plant Physiology *38*, iii.

Anderson, I.C., and Robertson, D.S. (1960). Role of carotenoids in protecting chlorophyll from photodestruction. Plant Physiology *35*, 531-534.

Armstrong, G. A., Alberti, M., Leach, F., and Hearst, J. E. (1989). Nucleotide sequence, organization, and nature of the protein products of the carotenoid biosynthesis gene cluster of *Rhodobacter capsulatus*. Mol Gen Genet *216*, 254-68.

Armstrong, G. A., Alberti, M., and Hearst, J. E. (1990). Conserved enzymes mediate the early reactions of carotenoid biosynthesis in nonphotosynthetic and photosynthetic prokaryotes. Proc Natl Acad Sci U S A *87*, 9975-9.

Bachmann, M. D., Robertson, D. S., and Bowen, C. C. (1969). Thylakoid anomalies in relation to grana structure in pigment-deficient mutants of *Zea mays*. J. Ultrastructure Res *28*, 435-451.

Badenhuizen, N. P. (1969). The Biogenesis of Starch Granules in Higher Plants (New York: Appleton-Century-Crofts).

Bartley, G. E., Scolnik, P. A., and Giuliano, G. (1994). Molecular biology of carotenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol *45*, 287-301.

Bartley, G. E., Viitanen, P. V., Bacot, K. O., and Scolnik, P. A. (1992). A tomato gene

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

expressed during fruit ripening encodes an enzyme of the carotenoid biosynthesis pathway. J Biol Chem *267*, 5036-9.

Bartley, G. E., Viitanen, P. V., Pecker, I., Chamovitz, D., Hirschberg, J., and Scolnik, P. A. (1991). Molecular cloning and expression in photosynthetic bacteria of a soybean cDNA coding for phytoene desaturase, an enzyme of the carotenoid biosynthesis pathway. Proc Natl Acad Sci U S A *88*, 6532-6.

Bartley, G. E., and Scolnik, P. A. (1995). Plant carotenoids: pigments for photoprotection, visual attraction, and human health. Plant Cell *7*, 1027-1038.

Bartley, G. E., Schmidhauser, T. J., Yanofsky, C., and Scolnik, P. A. (1990). Carotenoid desaturases from *Rhodobacter capsulatus* and *Neurospora crassa* are structurally and functionally conserved and contain domains homologous to flavoprotein disulfide oxidoreductases. J Biol Chem *265*, 16020-4.

Bartley, G. E., and Scolnik, P. A. (1989). Carotenoid biosynthesis in photosynthetic bacteria. Genetic characterization of the *Rhodobacter capsulatus* CrtI protein [published erratum appears in J Biol Chem 1989 Oct 25;264(30):18260]. J Biol Chem *264*, 13109-13.

Bartley, G. E., and Scolnik, P. A. (1993). cDNA cloning, expression during development, and genome mapping of *Psy2*, a second tomato gene encoding phytoene synthase. J Biol Chem *268*, 25718-25721.

Bathgate, B., Purton, M. E., Grierson, D., and Goodenough, P. W. (1985). Plastid changes during the conversion of chloroplasts to chromoplasts in ripening tomatoes. Planta *165*, 197-204.

Bechtel, D. B., and Pomeranz, Y. (1978). Ultrastructure of the mature ungerminated rice (*Oryza sativa*) caryopsis. The starchy endosperm. Amer. J. Bot. *65*, 684-691.

Bird, C. R., Ray, J. A., Fletcher, J. D., Boniwell, J. M., Bird, A. S., Teulieres, C., Blain, I., Bramley, P. M., and Schuch, W. (1991). Using Antisense RNA to study gene function: Inhibition of carotenoid biosynthesis in transgenic tomatoes. Biotechnology *9*, 635-639.

Bonk, M., Tadros, M., Vandekerckhove, J., Al-Babili, S., and Beyer, P. (1996). Purification and characterization of chaperonin 60 and heat-shock protein 70 from chromoplast of *Narcissus pseudonarcissus*. Involvement of heat-shock protein 70 in a soluble protein complex containing phytoene desaturase. Plant Physiology *111*, 931-939.

Bonk, M., Hoffmann, B., Von Lintig, J., Schledz, M., Al-Babili, S., Hobeika, E., Kleinig, H., and Beyer, P. (1997). Chloroplast import of four carotenoid biosynthetic enzymes *in*

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

vitro reveals differential fates prior to membrane binding and oligomeric assembly. Eur J Biochem *247*, 942-50.

Bramley, P. M. (1985). The in vitro biosynthesis of carotenoids. In Advances in lipid research., R. Paoletti and D. Kritchevsky, eds. (New York: Academic Press, Inc.), pp. 243-279.

Bramley, P., Teulieres, C., Blain, I., Bird, C., and Schuch, W. (1992). Biochemical characterization of transgenic tomato plants in which carotenoid synthesis has been inhibited through the expression of antisense RNA to pTOM5. Plant J *2*, 343-349.

Britton, G., and Goodwin, T. W. (1982). Carotenoid Chemistry and Biochemistry (New York: Pergamon Press).

Buckner, B., San Miguel, P., and Bennetzen, J. L. (1996). The *y1* gene of maize codes for phytoene synthase. Genetics *143*, 479-488.

Buckner, B., and Robertson, D. S. (1993). Cloning of carotenoid biosynthetic genes from maize. Methods Enzymol *214*, 311-23.

Burkhardt, P. K., Beyer, P., Wunn, J., Kloti, A., Armstrong, G. A., Schledz, M., von Lintig, J., and Potrykus, I. (1997). Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. Plant J *11*, 1071-8.

Camara, B. (1993). Plant phytoene synthase complex: component enzymes, immunology, and biogenesis. Methods Enzymol *214*, 352-365.

Camara, B., Bousquet, J., Cheniclet, C., Carde, J. P., Kuntz, M., Evrard, J. L., and Weil, J. H. (1989). Enzymology of Isoprenoid Biosynthesis and Expression of Plastid and Nuclear Genes During Chromoplast Differentiation in Pepper Fruits (*Capsicum annuum*). In Physiology, biochemistry and genetics of nongreen plastids, C. Boyer, J. Shannon and R. Hardison, eds. (Washington DC: American Society of Plant Physiology), pp. 141-156.

Camara, B., Bardat, F., and Monéger, R. (1982). Sites of biosynthesis of carotenoids in *Capsicum* chromoplast. Eur J Biochem *127*, 255-258.

Cervantes-Cervantes, M., Hadjeb, N., Newman, L. A., and Price, C. A. (1990). ChrA is a carotenoid-binding protein in chromoplasts of *Capsicum annuum*. Plant Physiology *93*, 1241-1243.

Chamovitz, D., Pecker, I., and Hirschberg, J. (1991). The molecular basis of resistance to the herbicide norflurazon. Plant Mol Biol *16*, 967-974.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chappell J. (1995). Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Annu Rev plant Physiol Plant Mol Biol *46*, 521-547.

Cogdell, R. J., and Frank, H. A. (1987). How carotenoids function in photosynthetic bacteria. Biochem. Biophys. Acta *895*, 63-79.

Corona, V., Aracri, B., Kosturkova, G., Bartley, G. E., Pitto, L., Giorgetti, L., Scolnik, P. A., and Giuliano, G. (1996). Regulation of a carotenoid biosynthesis gene promoter during plant development. Plant J *9*, 505-12.

Correns, C. (1901). Bastrade zwischen Maisrassen, mit besonderer Berucksichtigung der Xenien. Bibl. Bot. *53*, 1-161.

Cunningham, F. X. J., and Gantt, E. (1998). Genes and enzymes of carotenoid biosynthesis in plants. Annu. Rev. Plant Physiol. Mol. Biol. *49*, 577-583.

Delgado-Vargas, F. and Paredes-López, O. (1997). Enzymatic treatment to enhance carotenoid content in dehydrated marigold flower meal. Plant Foods for Human Nutrition *50*, 163-169.

Demerec, M. (1923). Inheritance of white seedlings in maize. Genetics *8*, 561-593.

Demmig-Adams, B., and Adams, W. W. (1993). The xanthophyll cycle., R. G. Alscher and J. L. Hess, eds. (London: CRC Press).

Demmig-Adams, B. (1990). Carotenoids and photoprotection in plants: A role for the xanthophyll zeaxanthin. Biochimica et Biophysica Acta *1020*, 1-24.

Dogbo, O., Laferrière, A., D'Harlingue, A., and Camara, B. (1988). Carotenoid Biosynthesis: isolation and characterization of a bifunctional enzyme catalyzing the synthesis of phytoene. Proceedings of the National Academy of Sciences *85*, 7054-7058.

Eisenreich, W., Sagner, S., Zenk, M. H., and Bacher, A. (1997). Monoterpenoid essential oils are not of mevalonoid origin. Tetrahedron letters *38*, 3889-3892.

Eisenreich, W., Menhard, B., Hylands, P. J., Zenk, M. H., and Bacher, A. (1996). Proc. Natl. Acad. Sci. USA *93(13)*, 6431-6436. Studies on the biosynthesis of taxol: the taxane carbon skeleton is not of mevalonoid origin.

Emter, O., Falk, H., and Sitte, P. (1990). Specific carotenoids and proteins as prerequisites for chromoplast tubule formation. Protoplasma *157*, 128-135.

Everett, H. L. (1949). A genic series controlling chloroplast pigments production in

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

diploid *Zea mays* L. Proc. Natl. Acad. Sci. USA *35*, 628-634.

Eyster, W. H. (1931a). Vivipary in maize. Genetics *16*, 574-590.

Eyster, W. H. (1931b). Heritable characters of maize. XLII. Reduced endosperm. J. Hered. *22*, 251-252.

Fong, F., Koehler, O. E., and Smith, J. D. (1983). Fluridone induction of vivipary during maize seed development., J. E. Kruger and D. E. LaBerge, eds. (Boulder, Colorado: Westview Press).

Fraser, P. D., Misawa, N., Linden, H., Yamano, S., Kobayashi, K., and Sandmann, G. (1992). Expression in *Escherichia coli*, purification, and reactivation of the recombinant *Erwinia uredovora* phytoene desaturase. The Journal of Biological Chemistry *267*, 19891-19895.

Fraser, P., Truesdale, M., Bird, C., Schuch, W., and Bramley , P. (1994). Carotenoid biosynthesis during tomato fruit development. Evidence for tissue specific gene expression. Plant Physiology *105*, 405-413.

Fraser, P., Hedden, P., Cooke, D., Bird, C., Schuch, W., and Bramley, P. (1995). The effect of reduced activity of phytoene synthase on isoprenoid levels in tomato pericarp during fruit development and ripening. Planta *196*, 321-326.

Fray, R. G., Wallace, A., Fraser, P. D., Valer, D., and Hedden, P. (1995). Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. Plant J. *8*, 693-701.

Giuliano, G., Bartley, G. E., and Scolnik, P. A. (1993). Regulation of carotenoid biosynthesis during tomato development. Plant Cell *5*, 379-387.

Gavazzi, G., Piccardo, C., and Manzoni, L. (1975). A study of the effects of temperature and nutrients on the expression of chlorophyll mutants in maize. Z. Pflanzenphysiol. Bd. *75*, 381-391.

Goodwin, T. W. (1980). The Biochemistry of the Carotenoids, 2nd Edition, Volume I. (London: Chapman & Hall).

Goodwin, T. W. (1976). Chemistry and biochemistry of plant pigments, 2nd Edition, Volume 1 (New York: Academic Press).

Grant, J. P. (1991). The State of the World's Children (Oxford: Oxford University Press).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Gregonis, D. E., and Rilling, H. C. (1974). The stereochemistry of trans-phytoene synthesis. Some observations on lycopersene as a carotene precursor and a mechanism for the synthesis of cis- and trans-phytoene. Biochemistry *13*, 1538-42.

Grumbach, K. H., and Britton, G. (1984). Carotenoid localization and biosynthesis in radish seedlings (*Raphanus sativus*) grown in the presence or absence of bleaching herbicides. In Advances in Photosynthesis Research. Volume IV, C. Sybesma, ed. (Boston: Martinus Nijhoff/Dr. W. Junk Publ.).

Gunning, B. E. S., and Steer, M. W. (1996). Plant cell biology: Structure and function. (Boston: Jones and Bartlett Publisher).

Hable, W. E., Oishi, K. K., and Schumaker, K. S. (1998). *Viviparous-5* encodes phytoene desaturase, an enzyme essential for abscisic acid (ABA) accumulation and seed development in maize. Mol Gen Genet *257*, 167-76.

Hable, W. E., and Oishi, K. K. (1995). Maize Phytoene Desaturase maps near the *viviparous5* locus. Plant Physiology *108*, 1329-1330.

Harris, W. M. and Spurr, A. R. (1969). Chromoplasts of tomato fruits. I. Ultrastructure of low-pigment and high-beta mutants. Carotene analysis. Amer J Bot *56*, 369-379.

Higgins, D. G., and Sharp, P. M. (1988). CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene *73*, 237-244.

Hugueney, P., Romer, S., Kuntz, M., and Camara, B. (1992). Characterization and molecular cloning of a flavoprotein catalyzing the synthesis of phytofluene and zeta-carotene in Capsicum chromoplasts. Eur J Biochem *209*, 399-407.

Hugueney, P., Bouvier, F., Badillo, A., Quennemet, J., d'Harlingue, A., and Camara, B. (1996). Developmental and stress regulation of gene expression for plastid and cytosolic isoprenoid pathways in pepper fruits. Plant Physiology *111*, 619-626.

Juliano, B. O. (1972). The rice caryopsis and its composition., D. D. Houston, ed. (St. Paul, Minnesota: American Association of Cereal Chemists).

Karvouni, Z., John, I., Taylor, J. E., watson, C. F., Turner, A. J., and Grierson, D. (1995). Isolation and characterization of a melon cDNA encoding phytoene synthase. Plant Molecular Biology *27*, 1153-1162.

Kiesselbach, T. A. (1980). The structure and Reproduction of Corn. (Lincoln, Nebraska: University of Nebraska Press).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Kleinig, H. (1989). The role of plastids in isoprenoid biosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology *40*, 39-59.

Komine, Y., Park, H., Wolfe, G. R., and Hoober, J. K. (1996). Secretory granules in the cytoplasm of a wall-less mutant of *Chlamydomonas reinhardtii* contain processed light-harvesting complex apoproteins and HSP70. J. Photochem. Photobiol. B: Biol. *36*, 301-306.

Koornneef, M. (1986). Genetic aspects of abscisic acid. In A Genetiuc Approach to Plant Biochemistry, A. D. Blonstein and P. J. King, eds. (New York: Springer-Verlag), pp. 35-54.

Kreuz, K., Beyer, P., and Kleinig, H. (1982). The site of carotenogenic enzymes in chromoplasts from *Narcissus pseudonarcissus* L. Planta *154*, 66-69.

Krinsky, N., Russett, M. D., Handelman, G. J., and Snodderly, D. M. (1990). Structural and geometrical isomers of carotenoids in human plasma. J. Nutr. *120*, 1654-1662.

Kulkarni, C. G. (1927). Inheritance studies of white-capping in yellow dent maize. Pap. Mich. Acad. Sci. Art Lett. *6*, 253-273.

Kushwaha, S.C., Kates, M., and Weber, H.J. (1980). Exclusive formation of all-trans-phytoene by a colorless mutant of *Halobacterium halobium*. Can J Microbiol *26*, 1011-1014.

Lee, C., McCoon, P., and LeBowitz, J. (1981). Vitamin A value of sweet corn. J. Agric. Food Chem *29*, 12941295.

Li, H. M., Sullivan, T. D., and Keegstra, K. (1992). Information for targeting to the chloroplastic inner envelope membrane is contained in the mature region of the maize *Bt1*-encoded protein. J Biol Chem *267*, 18999-9004.

Li, Z. H., Matthews, P. D., Burr, B., and Wurtzel, E. T. (1996). Cloning and characterization of a maize cDNA encoding phytoene desaturase, an enzyme of the carotenoid biosynthetic pathway. Plant Mol Biol *30*, 269-79.

Li, Z. H. (1998). Ph.D. Thesis: Molecular cloning and characterization of phytoene desaturase cDNA and Leucine-rich Repeat Protein Kinase cDNA from Maize. (New York: The Graduate School and University Center of the City University of New York).

Lichtenthaler, H. K., Rohmer, M., and Schwender, J. (1997a). Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. Physiologia Plantarum *101*, 643-652.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Lichtenthaler, H. K., Schwender, J., Disch, A., and Rohmer, M. (1997b). Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. FEBS Lett *400*, 271-4.

Liedvogel, B., and Bauerle, R. (1986). Fatty-acid synthesis in chloroplasts from mustard (*Sinapis alba* L.) cotyledons:formation of acetyl coenzyme A by intraplastid glycolytic enzymes and a pyruvate dehydrogenase complex. Planta *169*, 481-489.

Linden, H., Lucas, M. M., de Felipe, M. R., and Sandmann, G. (1993). Immunogold localization of phytoene desaturase in higher plant chloroplasts. Physiol Plant *88*, 229-231.

Linden, H., Misawa, N., Saito, T., and Sandmann, G. (1994). A novel carotenoid biosynthesis gene coding for zeta-carotene desaturase: functional expression, sequence and phylogenetic origin. Plant Mol Biol *24*, 369-79.

Linden, H., Misawa, N., Chamovitz, D., Pecker, P., Hirschberg, J., and Sandmann, G. (1991). Functional complementation in *Escherichia coli* of different phytoene desaturase genes and analysis of accumulated carotenes. Z Naturforsch *46c*, 1045-1051.

Lindstrom, E. W. (1924). Complementary genes for chlorophyll development in maize and their linkage relations. Genetics *9*, 305-326.

Lütke-Brinkhaus, F., Liedvogel, B., Kreuz, K., and Kleinig, H. (1982). Phytoene synthase and phytoene dehydrogenase associated with envelope membranes from spinach chloroplasts. Planta *156*, 176-180.

Lütke-Brinkhaus, F., and Kleinig, H. (1987). Formation of isopentenyl diphosphate via mevalonate does not occur within etioplasts and etiochloroplasts of mustard (*Sinapis alba* L.) seedlings. Planta *171*, 406-411.

Lütke-Brinkhaus, F., Liedvogel, B., and Kleinig, H. (1984). On the biosynthesis of ubiquinones in plant mitochondria. Eur J Biochem *141*, 537-541.

Mann, V., Pecker, I., and Hirschberg, J. (1994). Cloning and characterization of the gene for phytoene desaturase (*Pds*) from tomato (*Lycopersicon esculentum*). Plant Mol Biol *24*, 429-34.

Martinez-Ferez, I., Fernandez-Gonzalez, B., Sandmann, G., and Vioque, A. (1994). Cloning and expression in *Escherichia coli* of the gene coding for phytoene synthase from the cyanobacterium *Synechocystis* sp. PCC6803. Biochim. Biophys. Acta *1218(2)*, 145-152.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Mayfield, S.P., Nelson, T., and Taylor, W.C. (1986). The fate of chloroplast proteins during photooxidation in carotenoid-deficient maize leaves. Plant Physiology *82*, 760-764.

McCarty, D. R., Hattori, T., Carson, C. B., Vasil, V., Lazar, M., and Vasil, I. K. (1991). The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. Cell *66*, 895-905.

McCarty, D. R., Carson, C. B., Stinard, P. S., and Robertson, D. S. (1989). Molecular Analysis of *viviparous-1*: An Abscisic Acid-Insensitive Mutant of Maize. The Plant Cell *1*, 523-532.

McCullough, A. J., Gengenbach, B. G., and Jones, R. L. (1989). Plastid genome amplification and expression in developing endosperm, Volume 2, C. D. Boyer, J. C. Shannon and R. C. Hardison, eds. (Maryland: American Society of Plant Physiologists).

Miernyk, J. A. (1985). Isolation and characterization of nongreen plastids, H. F. Linskens and J. F. Jackson, eds. (Berlin: Springer-Verlag).

Misawa, N., Yamano, S., Linden, H., de Felipe, M. R., Lucas, M., Ikenaga, H., and Sandmann, G. (1993). Functional expression of the *Erwinia uredovora* carotenoid biosynthesis gene *crtI* in transgenic plants showing an increase of beta-carotene biosynthesis activity and resistance to the bleaching herbicide norflurazon [published erratum appears in Plant J 1994 Feb;5(2):309]. Plant J *4*, 833-40.

Misawa, N., Truesdale, M. R., Sandmann, G., Fraser, P. D., Bird, C., Schuch, W., and Bramley, P. M. (1994). Expression of a tomato cDNA coding for phytoene synthase in Escherichia coli, phytoene formation in vivo and in vitro, and functional analysis of the various truncated gene products. J Biochem (Tokyo) *116*, 980-5.

Mozafar, A. (1994). Plant vitamins: agronomic, pharmalogical and nutritional aspects. (Boca Raton: CRC Press).

Mu-Forster, C., and Wasserman, B. P. (1998). Surface localization of Zein storage proteins in starch granules from maize endosperm. Plant Physiol. *116*, 1563-1571.

Neil, S. J., Horgan, R., and Parry, A. D. (1986). The carotenoid and abscisic acid content of viviparous kernels and seedlings of *Zea mays* L. Planta *169*, 87-96.

Neuffer, M. G., Coe, E., and Wessler, S. (1997). Mutants of Maize (New York: Cold Spring Harbor Laboratory Press).

Norris, S. R., Barrette, T. R., and DellaPenna, D. (1995). Genetic dissection of carotenoid

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

synthesis in *Arabidopsis* defines plastoquinone as an essential component of phytoene desaturation. The Plant Cell *7*, 2139-2149.

Olson, J. A. (1989). Provitamin A function of carotenoids: the conversion of beta-carotene into vitamin A. J Nutr *119*, 105-8.

Olson, J. A. (1994). Absorption, transportation, and metabolism of carotenoids in humans. Pure Appl. Chem. *66*, 1011-1016.

Park, H., and Hoober, J. K. (1997). Chlorophyll synthesis modulates retention of apoproteins of light-harvesting complex II by the chloroplast in *Chlamydomonas reinhardtii*. Physiol. Plant. *101*, 135-142.

Parry, A. D., Babiano, M. J., and Horgan, R. (1990). The role of *cis*-carotenoids in humans. Pure Appl. Chem. *182*, 118-128.

Parry, A. D., and Horgan, R. (1992). Abscisic acid biosynthesis in roots I. The identification of potential abscisic acid precursors, and other carotenoids. Planta *187*, 185-191.

Pecker, I., Chamovitz, D., Linden, H., Sandmann, G., and Hirschberg, J. (1992). A single polypeptide catalyzing the conversion of phytoene to zeta-carotene is transcriptionally regulated during tomato fruit ripening. Proc Natl Acad Sci USA *89*, 4962-4966.

Perry, H. S., and Sprague, G. F. (1936). A second-chromosome gene, *Y3*, producing yellowing endosperm color in maize. J. Am. Soc. Agron. *28*, 990-996.

Randolph, L. F., and Hand, D. B. (1940). Relation between carotenoid content and number of genes per cell in diploid and tetraploid corn. J Agr Res *60*, 51-64.

Ray, J., Bird, C. R., Maunders, M., Grierson, D., and Schuch, W. (1987). Sequence of pTom5, a ripening related cDNA from tomato. Nucleic Acids Research *24*, 10587.

Robertson, D. S. (1975a). Survey of the albino and white-endosperm mutants of maize. J Hered *66*, 67-74.

Robertson, D. S. (1975b). Phenotypic and genetic studies of a new mutant of yellow endosperm in maize. J. Hered. *66*, 127-130.

Robertson, D. S., Bachmann, M. D., and Anderson, I. C. (1966). Role of carotenoids in protecting chlorophyll from photodestruction-II. Study on the effect of four modifiers of the albino *cl1* mutant of maize. Photochem Photobiol *5*, 797-805.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Robertson, D. S., and Anerson, I. C. (1961). Temperature-sensitive alleles of the Y1 locus in maize. J. Heredity *LII*, 53-60.

Robertson, D. S. (1952). The genotype of the endosperm and embryo as it influences vivipary in maize. Proc. Natl. Acad. Sci. USA *38*, 580-583.

Robertson, D. S. (1955). The genetics of vivipary in maize. Genetics *40*, 745-760.

Römer, S., Hugueney, P., Bouvier, F., Camara, B., and Kuntz, M. (1993). Expression of the genes encoding the early carotenoid biosynthetic enzymes in *Capsicum annuum*. Biochem Biophys Res Commun *196*, 1414-1421.

Ruiz-Hidalgo, M. J., and Benito, E. P. (1997). The phytoene dehydrogenase gene of *Phycomyces*: regulation of its expression by blue light and vitamin A. Mol. Gen. Genet. *253*, 734-744.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A laboratory manual, 2nd Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Sandmann, G., and Böger, P. (1989). Inhibition of carotenoid biosynthesis by herbicides. In target sites of herbicide action, P. Böger and G. Sandmann, eds. (Boca Raton, Florida: CRC Press), pp. 25-44.

Sandmann, G. (1994). Carotenoid biosynthesis in microorganisms and plants. Eur J Biochem *223*, 7-24.

Schiedt, D., and Liaaen-Jensen S. (1975). Isolation and analysis. In Carotenoids. Vol 1A. Britton, G., Liaaen-Jensen, S., and Pfander, H. P., eds. (Berlin:Birkhauser Verlag).

Schledz, M., Al-Babili, S., von Lintig, J., Haubruck, H., Rabbani, S., Kleinig, H., and Beyer, P. (1996). Phytoene synthase from *Narcissus pseudonarcissus*: functional expression, galactolipid requirement, topological distribution in chromoplasts and induction during flowering. The Plant Journal *10*, 781-792.

Schmidhauser, T. J., Lauter, F. R., Russo, V. E., and Yanofsky, C. (1990). Cloning, sequence, and photoregulation of *al-1*, a carotenoid biosynthetic gene of *Neurospora crassa*. Mol Cell Biol *10*, 5064-70.

Schneider, C., Boger, P., and Sandmann, G. (1997). Phytoene desaturase: heterologous expression in an active state, purification, and biochemical properties. Protein Expr Purif *10*, 175-9.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Schwartz, S.H., Tan, B.C., Cage, D.A., Zeevaart, J.A.D., and MaCarty, D.R. (1997). Specific oxidative cleavage of carotenoids by VP14 of maize. Sci. *276*, 1872-1874.

Schwender, J., Seemann, M., Lichtenthaler, H. K., and Rohmer, M. (1996). Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains of chlorophylls and plastoquinone) via a novel pyruvate/glyceraldehyde 3-phosphate non-mevalonate pathway in the green alga *Scenedesmus obliquus*. Biochem J *316*, 73-80.

Scolnik, P. A., and Bartley, G. E. (1993). Phytoene desaturase from *Arabidopsis*. Plant Physiol *103*, 1745.

Sechi, A. M., and Rossi-Manaresi (1958). The vitamin contents in a variety of Italian rice and its byproducts. J. Vitaminology *4*, 114-117.

Serrano, A., Gimenez, P., Schmidt, A., and Sandmann, G. (1990). Immunocytochemical localization and functional determination of phytoene desaturase in photoautotrophic prokaryotes. J Gen Microbiol *136*, 2465-2469.

Siefermann-Harms, D., Fritz, B., and Ninnemann, H. (1985). Evidence for a pterin-derivative associated with the molybdenum cofactor of *Neurospora crassa* nitrate reductase. Photochem Photobiol *42*, 771-778.

Smirra, I., Halevy, A. H., and Vainstein, A. (1993). Isolation and characterization of a chromoplast-specific carotenoid-associated protein from *Cucumis sativus* corollas. Plant Physiol. *102*, 491-496.

Sommer, A. (1988). New imperatives for an old vitamin (A). J. Nutri. *119*, 96-100.

Sprague, G. F. (1987). *y11* and *y12*. Maize Genet. Coop. Newslett. *61*, 96.

Sprague, G. F. (1936). The relation of moisture content and time of harvest to germination of immature corn. J. Am. soc. Agron. *28*, 472-478.

Spurgeon, S. L., and Porter, J. W. (1980). Carotenoids, Volume 4, P. K. Stumpf and E. E. Conn, eds. (New York: Academic Press).

Sullivan, T. D., and Kaneko, Y. (1995). The maize *brittle1* gene encodes amyloplast membrane polypeptides. Planta *196*, 477-84.

Sullivan, T. D., Strelow, L. I., Illingworth, C. A., Phillips, R. L., and Nelson, O. E., Jr. (1991). Analysis of maize *brittle-1* alleles and a defective Suppressor-mutator induced mutable allele. Plant Cell *3*, 1337-48.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Than, A., Bramley, P. M., Davis, B. H., and Rees, A. F. (1972). Stereochemistry of phytoene. Phytochemistry *11*, 3187-3192.

Tilney-Bassett, R. A. E. (1989). The diversity of the structure and function of higher plants plastids., C. D. Boyer, J. C. Shannon and R. C. Hardison, eds.: The American Society of Plant Physiologists).

Tulpule, S. H. (1954). A study of pleiotropic genes in maize. Am. J. Bot. *41*, 294-301.

Underwood, B. A., and Arthur, P. (1996). The contribution of vitamin A to public health. The FASEB Journal *10*, 1040-1049.

von Lintig, J., Welsch, R., Bonk, M., Giuliano, G., Batschauer, A., and Kleinig, H. (1997). Light-dependent regulation of carotenoid biosynthesis occurs at the level of phytoene synthase expression and is mediated by phytochrome in *Sinapis alba* and *Arabidopsis thaliana* seedlings. Plant J. *12*, 625-634.

White, R. A., Wolfe, G. R., Komine, Y., and Hoober, J. K. (1996). Localization of light-harvesting complex apoproteins in the chloroplast and cytoplasm during greening of *Chlamydomonas reinhardtii* at 38°C. Photosynthesis Res. *47*, 267-280.

Wolfe, G. R., Park, H., Sharp, W. P., and Hoober, J. K. (1997). Light-harvesting complex apoproteins in cytoplasmic vacuoles in *Chlamydomonas reinhardtII* (Chlorophyta). J. Phycol. *33*, 377-386.

Wurtzel, E. T. (1992). Use of a *Ds* chromosome breaking element to examine maize *Vp5* expression. J Hered *83*, 109-113.

Wurtzel, E. T., Burr, F. A., and Burr, B. (1987). DNase I hypersensitivity and expression of the *Shrunken-1* gene of maize. Plant Mol Biol *8*, 251-264.

Wurtzel, E. T., Li, Z.-h., Luo, R., Matias, D., Mozoub, D., Matthews, P. D., Upasani, V. N., Valdez, G., Yoganathan, A., and Yu, J. (1996). Research towards improvement of the pro-vitamin A (carotenoid) content of rice endosperm. Intern Rice Res Notes *21*, 43-44.

Yoganathan, A. (1998). Ph.D. Thesis: Isolation, expression and functional analysis of a cDNA encoding phytoene desaturase, a carotenoid biosynthetic enzyme from rice, *Oryza sativa* L. (New York: The Graduate School and Universtiy Center of the City University of New York).

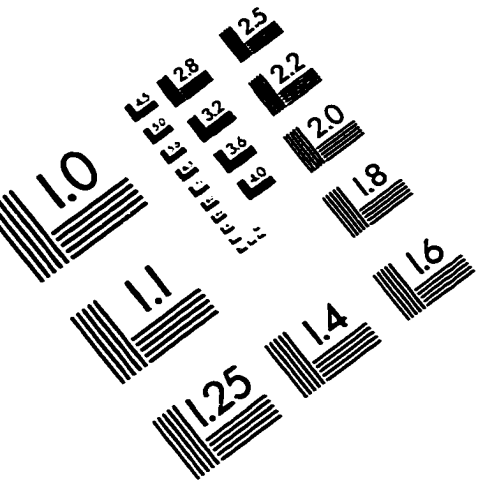
Zhang, F. And Boston, R.S. (1992). Increases in binding protein (BiP) accomany changes in protein body morphology in three high-lysine mutants of maize. Proplasma *171*, 142-152.

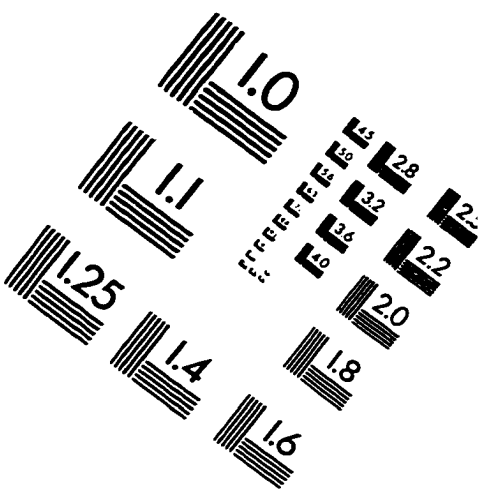
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

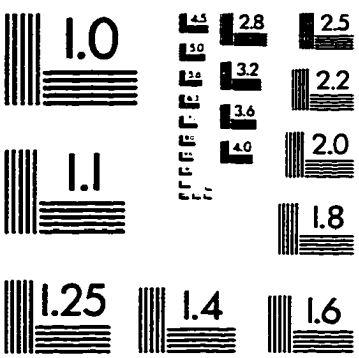
Zhu, X. F., Suzuki, K., Okada, K., Tanaka, K., and Nakagawa, T. (1997). Cloning and functional expression of a novel geranylgeranyl pyrophosphate synthase gene from *Arabidopsis thaliana* in *Escherichia coli*. Plant Cell Physiol. *38*, 357-361.

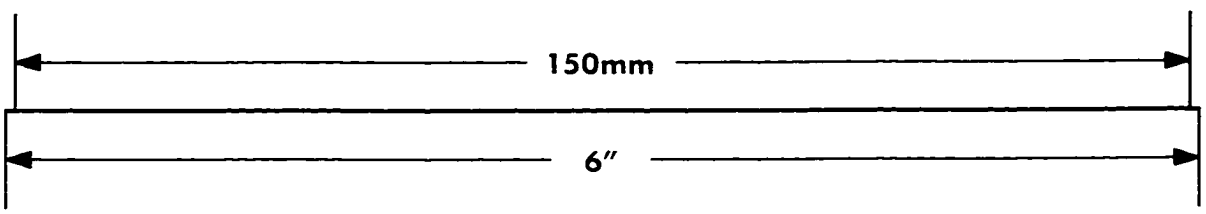
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

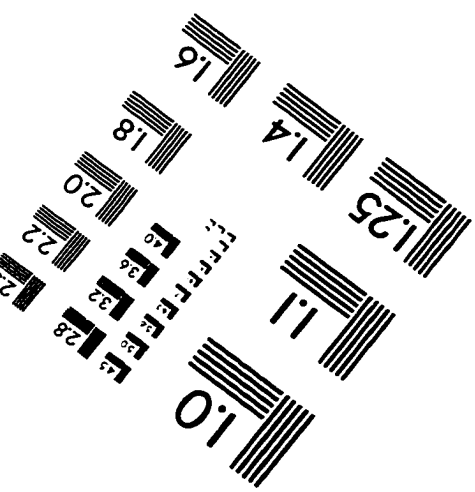
IMAGE EVALUATION TEST TARGET (QA-3)

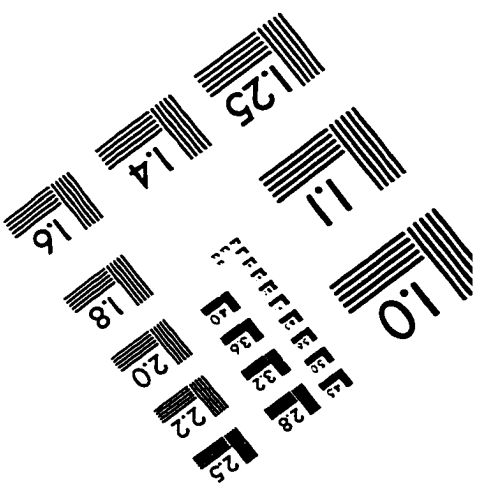












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.