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CLONING AND CHARACTERIZATION OF CAROTENOID HYDROXYLASES IN *ARABIDOPSIS THALIANA*

By

Li Tian

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
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ABSTRACT

CLONING AND CHARACTERIZATION OF CAROTENOID HYDROXYLASES IN *ARABIDOPSIS THALIANA*

By

Li Tian

Carotenoids are isoprenoid-derived pigments that are synthesized by bacteria, fungi, and plants. Oxygenated carotenoids, xanthophylls, are essential components of the light harvesting complexes and play critical roles in photoprotection. Lutein and zeaxanthin are dihydroxy xanthophylls produced from their corresponding carotene precursors by the action of β- and ε-ring carotenoid hydroxylases. β-hydroxylases add hydroxyl groups to the β-rings of carotenes and have been cloned from several bacteria and plants. ε-hydroxylase hydroxylates on ε-rings and was genetically defined by the *lut1* mutation in Arabidopsis, but not yet cloned. The main goal of this dissertation was to clone and characterize the Arabidopsis ε-hydroxylase gene by molecular, genomic, and genetic approaches. The *in vivo* functions of β- and ε-hydroxylases as well as xanthophylls were studied in parallel.

We proposed three hypotheses for the identity of the Arabidopsis ε-hydroxylase/*LUT1* locus: 1) it is a hydroxylase evolved from and related to the β-hydroxylase. 2) it is an independently evolved hydroxylase and divergent from the β-hydroxylase. 3) it is an ancillary protein that modifies the existing β-hydroxylase to function towards ε-rings. In an attempt to test the first hypothesis, I screened cDNA and genomic libraries from Arabidopsis using the Arabidopsis β-hydroxylase cDNA as a

probe. As a result of the screening, I cloned and characterized a second β-hydroxylase in Arabidopsis (Chapter 2). The encoded protein shares high identity to the previously reported Arabidopsis β-hydroxylase 1 and functions efficiently on β-rings (but not ε-rings). Neither β-hydroxylase cDNA maps to the *LUT1* locus, and the genomic region encompassing the *LUT1* locus does not contain a third related hydroxylase. In order to test the second and third hypotheses, I identified the *LUT1* gene by map-based cloning and found that it encodes a cytochrome P450 type monooxygenase (Chapter 3).

In order to address the individual and overlapping functions of the three Arabidopsis carotenoid hydroxylase activities *in vivo*, T-DNA knockout mutants corresponding to β-hydroxylase 1 and 2 (*b1* and *b2*, respectively) were isolated and all possible hydroxylase mutant combinations were generated together with *lut1* (Chapter 4). Carotenoid composition in leaf and seed, NPQ induction and amplitude were analyzed in these mutants. Overall, the hydroxylase mutants provide insight into the unexpected overlapping activity of carotenoid hydroxylases *in vivo*.

I have also used mutations in *Arabidopsis thaliana* that selectively eliminate (and substitute) specific xanthophylls in order to study their function(s) *in vivo* (Chapter 5). These include two lutein-deficient mutants, *lut1* and *lut2*, the epoxy xanthophyll-deficient *aba1* mutant, and the *lut2aba1* double mutant. Photosystem stoichiometry, antenna sizes, and xanthophyll cycle activity have been related to alterations in non-photochemical quenching of chlorophyll fluorescence (NPQ). Altered NPQ in xanthophyll biosynthetic mutants is explained by disturbed macro-organization of LHC II and reduced PS II-antenna size in the absence of the optimal, wild type xanthophyll composition.

This dissertation is dedicated to my parents, Jinxiang Tian and Baoqin Chang.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor, Dr. Dean DellaPenna, for his guidance, encouragement, and patience during my graduate study. Dean has helped me to become a better scientist and a mature person. It has always been a privilege to work in his lab. I would also like to thank my committee members, Drs. Katherine Osteryoung, Christoph Benning, and John Ohlrogge for their advises, suggestions, and stimulating discussions.

Barry, Heiko, Dave, Max, and Steve, former postdocs in the lab, have guided me through the early years of my graduate school and taught me useful molecular biology and photobiology techniques. I would like to thank Dr. Tom Newman for instructions on Real Time PCR and Dr. Jan Zeevaart for helping me with ABA analysis. Maria and Valeria have provided excellent technical support for my research.

I would also like to thank Joe, Craig, and the rest of the DellaPenna lab members for teaching me interesting American slang. I will always remember the sunny summer days when I went rock-climbing with Craig, Dave, Max, and Steve. I also enjoyed collecting fossils and rocks with Heiko. I missed the fun times and good food that I had with Molly, Haiming, and Xiaoxia in Reno. Woonbong and Xiaohua have been my inspiration for playing tennis. Dean showed me the way to the putting green. Uwe gave me my very first sailing lesson. I want to thank Elena for inviting me to her wonderful parties, Laura for sharing books with me, and Eva for always being a friend.

There has been emotional ups and downs over the past few years, but I am so grateful that I have my loving parents and sister (and my very cute nephew Tongtong) who have always truly believed in me and offered me their love and support.

I am pleased to finally see a closure for my graduate study and am excited to welcome a new beginning.

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Introduction

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1.1.1 Terpenoid biosynthesis

Terpenoids are one of the major groups of secondary metabolites that are produced by plants. All terpenoids, including those derived from both primary and secondary metabolism, are constructed from the five-carbon isoprene unit, isopentenyl pyrophosphate (IPP) (Table 1.1). Terpenoids are also known as isoprenoids because of these isoprene building blocks. The common precursors for biosynthesis of all terpenoids are IPP and dimethylallyl pyrophosphate (DMAPP). Two major biosynthetic pathways that operate in different subcellular compartments lead to IPP production for different groups of terpenoids in plants (Figure 1.1). Cytoplasmic IPP is derived from the mevalonate (MVA) pathway and leads to the synthesis of sesquiterpene (C_{15}) and triterpene (C_{30}). The plastidic IPP biosynthetic pathway begins with condensation of glyceraldehyde-3-phosphate (G3P) and pyruvate, and gives rise to monoterpene (C_{10}), diterpene (C_{20}), and tetraterpene (C_{40}) classes of terpenoids, the latter of which includes tocopherols, phylloquinones, and carotenoids.

1.1.2 The non-mevalonate pathway for carotenoid biosynthesis in plastids

The classic mevalonate pathway was discovered in the 1950's and has been studied in detail in fungi, animals, and plants (Goodwin, 1971; Schroepfer, 1981). The mevalonate pathway was considered to be the sole source of IPP for plant isoprenoid

Table 1.1. Classification of terpenoids in higher plants.

Isoprene	Carbon	Class name	Examples
1	5	hemiterpene	isoprene
2	10	monoterpene	limonene, menthol
3	15	sesquiterpene	phytoalexin, abscisic acid
4	20	diterpene	taxol, casbene, phytol, gibberellin
6	30	triterpene	brassinosteroid
8	40	tetraterpene	carotenoid, tocopherol, phylloquinone
>8	>40	polyterpene	rubber, plastoquinone, ubiquinone

Figure 1.1. Isoprenoid biosynthesis in higher plants. Biosynthetic enzymes of both mevalonate and DXP pathways and their locations in the cell are indicated. HMG-CoA, hydroxymethylglutaryl CoA; MVA, mevalonate; MVP, 5-phosphomevalonate; MVPP, 5-diphosphomevalonate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl pyrophosphate; DXP, 1-deoxyxylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; ME-cPP, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate ; HMBPP, hydroxymethyl-butenyl 4-diphosphate.

AACT, acetoacetyl CoA thiolase (EC 2.3.1.9); HMGS, HMG-CoA synthase (EC 4.1.3.5); HMGR, HMG-CoA reductase (EC 1.1.1.88); MVK, MVA kinase (EC 2.7.1.36); PMK, MVP kinase (EC 2.7.4.2); PMD, MVPP decarboxylase (EC 4.1.1.33); IDI, IPP isomerase (EC 5.3.3.2); GPS, GPP synthase (EC 2.5.1.1); FPS, FPP synthase (EC 2.5.1.10); GGPS, GGPP synthase (EC 2.5.1.29); DXS, DXP synthase (EC 4.1.3.37); DXR, DXP reductoisomerase (EC 1.1.1.267); CMS, CDP-ME synthase (EC 2.7.7.60); CMK, CDP-ME kinase (EC 2.7.1.148); MCS, ME-2,4cPP synthase (EC 4.6.1.12); HDS, HMBPP synthase; IDS, IPP/DMAPP synthase.

This figure is adapted from Figure 1 in **Rodríguez-Concepción and Boronat** (2002). Plant Physiol. **130,** 1079-1089.

Mitochondria
Ubiquinones
FPP
FPS
x2
DMAPP
IPP
Cytoplasm
Acetyl-CoA
AACT
Acetoacetyl-CoA
HMGS
HMG-CoA
HMGR
MVA pathway
MVK
MVA
PMK
MVP
PMD MVPP
DMAPP
IDI
IPP
x3
x2
FPS
GGPS
GGPP
Cytokinins
Prenylation of proteins
FPP
Polyprenols
Dolichol
Sesquiterpenes
Phytosterols
Brassinosteroids
Plastid
G3P + pyruvate
DXS
DXP
DXR
MEP
CMS
DXP pathway
CDP-ME
CMK
CDP-MEP
MCS
ME-cPP
HDS
HMBPP
IDS
IDI
IPP
DMAPP
Isoprene
GPS
x3
GGPS
GPP
Chlorophylls
Carotenoids
Plastoquinones
GGPP
Tocopherols
Gibberellins
Phylloquinones

Figure 1.1

biosynthesis for almost four decades until the discovery of non-mevalonate pathway in bacteria and plants in the late 80's and early 90's (Flesch and Rohmer, 1988; Rohmer et al., 1993; Schwender et al., 1996). Incorporation of ^{13}C-labelled glucose, acetate, pyruvate, or erythrose into the hopanoids and/or ubiquinones in several bacteria (*Zymomonas mobilis*, *Methylobacterium fujisawaense*, *Escherichia coli*, and *Alicyclobacill acidoterrestris*) yielded a labeling pattern not compatible with the MVA pathway and suggested a novel route for the early steps of isoprenoid biosynthesis. It was suggested that the C5 isoprene unit was produced from the condensation of a C2 unit derived from pyruvate decarboxylation and a triose phosphate (Rohmer et al., 1993; Horbach et al., 1993). Subsequently, similar labeling studies were undertaken in plants (Schwender et al., 1996) and indicated the presence of a pathway other than the MVA pathway for IPP biosynthesis in higher plants. This new IPP biosynthetic pathway has been called the "Rohmer pathway" after the scientist who first discovered the pathway, or the "Alternative pathway" to indicate that it is an alternative to the classic mevalonate pathway. Following identification of the key metabolites in the pathway, it is also called the "Pyruvate/glyceraldehyde 3-phosphate (G3P) pathway" (the substrate), the "Deoxyxylulose 5-phosphate (DXP) pathway" (the first pathway intermediate), or the "2-C-methyl-D-erythritol 4-phosphate (MEP) pathway" (the second pathway intermediate).

Combined molecular biology, genetics, biochemistry, organic chemistry, and bioinformatics approaches have been applied to first define the DXP pathway in plants and then identify the genes of the pathway in bacteria and plants. The first step in the DXP pathway is the head to head condensation of G3P and pyruvate to form 1-deoxy-D-xylulose-5-phosphate (DXP) by DXP synthase (DXPS). DXP is then reduced by DXP

reductoisomerase (DXPR) to form MEP. *DXPS* and *DXPR* have been cloned from a number of plants and bacteria (Mandel, 1996; Kuzuyama et al., 1998; Lois et al., 1998; Takahashi et al., 1998). MEP is then converted to 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (ME-2,4cPP) in three enzymic steps catalyzed by CDP-ME synthase, (CMS), CDP-ME kinase (CMK), and ME-2,4cPP synthase (MCS), encoded by *ygbP*, *ychB*, and *ygbB*, respectively, in *E. coli* (Figure 1.1.; Rohdich et al., 1999; Herz et al., 2000; Lüttgen et al., 2000). CMS condenses MEP and CTP to form 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME). CDP-ME is phosphorylated by CMK to form 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate (CDP-ME2P). The CMP molecule is then eliminated from CDP-ME2P to produce ME-2,4cPP by MCS.

As a biochemically and genetically well studied organism, *E.coli* has been a target for DXP pathway research and all the pathway genes have been initially cloned from *E.coli* (reviewed in Rohmer, 1999; Lichtenthaler, 2000). Comparative genomics has facilitated the isolation of DXP pathway genes in plants. All plant DXP pathway enzymes have putative signal peptides for chloroplastic localization (reviewed in Lichtenthaler, 1999; Rodríguez-Concepción and Boronat, 2002). The DXP pathway is the source of IPP for the biosynthesis of carotenoids and other chloroplastic metabolites, such as tocopherols and gibberellins. Therefore, the regulation of the DXP pathway may be an important control point in quantitative production of carotenoids and other plastidic isoprenoids.

1.1.3 Carotenoid biosynthesis in higher plants

Carotenoids are best known as colorants that are widespread in plants and microorganisms. Carotenoids are also present in animals where they are not synthesized but rather obtained from food. More than 700 different carotenoids are now known (Britton, 1998). This part of the introduction will focus on the reactions and enzymes that belong to the main carotenoid biosynthetic pathway common to all higher plants, namely leading from phytoene to neoxanthin synthesis. Additional reactions and enzymes are involved in the production of a large variety of carotenoids that accumulate in microorganisms and will not be reviewed in this chapter.

The light absorbing properties of carotenoids derive from their conjugated double bond system in which π-electrons are delocalized over the entire polyene chain. This conjugated double bond system is also called a chromophore due to its function in light absorption. Carotenoids with 3-5 conjugated double bonds absorb light in the UV region and are colorless. Carotenoids with seven or more conjugated double bonds absorb light in the visible region, which give such carotenoids their yellow, orange, or red colors.

In plants, carotenoids are essential components of the photosystem reaction centers and the light harvesting complexes. β-carotene is mainly associated with the reaction centers while lutein, violaxanthin, and neoxanthin are associated with the light harvesting complexes. Carotenoid biosynthesis is localized in the chloroplast and the pathway enzymes are present at very low abundance. Most carotenoid enzymes are associated with the thylakoid membrane and are sensitive to detergent treatment, which makes purification of carotenoid biosynthetic enzymes difficult and in only a few cases has been successful (Camara and Moneger, 1981; Beyer et al., 1985). Using a molecular biology approach and elegant *E. coli* based screening and complementation systems

(Cunningham et al., 1994; Lotan and Hirschberg, 1995), genes for carotenoid biosynthesis have been isolated from various sources in recent years, including *E.coli*, cyanobacteria, green algae, and higher plants (Figure 1.2; Table 1.2).

The first committed step in carotenoid biosynthesis is the formation of phytoene from two geranylgeranyl pyrophosphate (GGPP) molecules by phytoene synthase (PSY) (Figure 1.3). The condensation of two GGPP molecules requires the abstraction of one hydrogen from the C(1) position of each GGPP and results in a 15-*cis* double bond in phytoene in higher plants. The *PSY* gene has been cloned and found to be structurally and functionally conserved in plants and bacteria (Sandmann, 1994; Schledz et al., 1996). The enzymes from both organisms contain conserved prenyl transferase domains that resemble squalene synthase, an enzyme that catalyzes the condensation of two farnesyl pyrophosphate (FPP) molecules to form squalene (Sandmann, 1994; Schledz et al., 1996). *In vitro* analysis with daffodil PSY showed that the enzyme requires galactolipids for catalytic activity. In addition, both soluble and membrane-bound forms of PSY are detected in the daffodil chromoplast stroma (Schledz et al., 1996). The soluble form of PSY is inactive and becomes activated upon membrane attachment. The active membrane-bound form of PSY is consistent with the presence of lipophilic phytoene and the existence of subsequent carotenoid biosynthetic steps in the thylakoid membrane. Although the plastid HSP70 was highly induced during flower development and previously found to be associated with phytoene desaturase, its association with PSY was not observed in daffodil chromoplasts (Al-Babili et al., 1996; Schledz et al., 1996).

Phytoene is colorless because of its short (triene) chromophore. The formation of colored carotenoids requires the extension of the conjugated double bond system by a

Figure 1.2. Map locations of carotenoid biosynthetic genes in Arabidopsis. *PSY*, phytoene synthase; *PDS*, phytoene desaturase; *ZDS*, ζ-carotene desaturase; *CCR1*, carotenoid isomerase 1; *CCR2*, carotenoid isomerase 2; *LYCβ*, lycopene β-cyclase; *LYCε*, lycopene ε-cyclase; *βCH1*, β-carotene hydroxylase 1; *βCH2*, β-carotene hydroxylase 2; *LUT1*, ε-hydroxylase; *ZEP*, zeaxanthin epoxidase; *VDE*, violaxanthin deepoxidase.

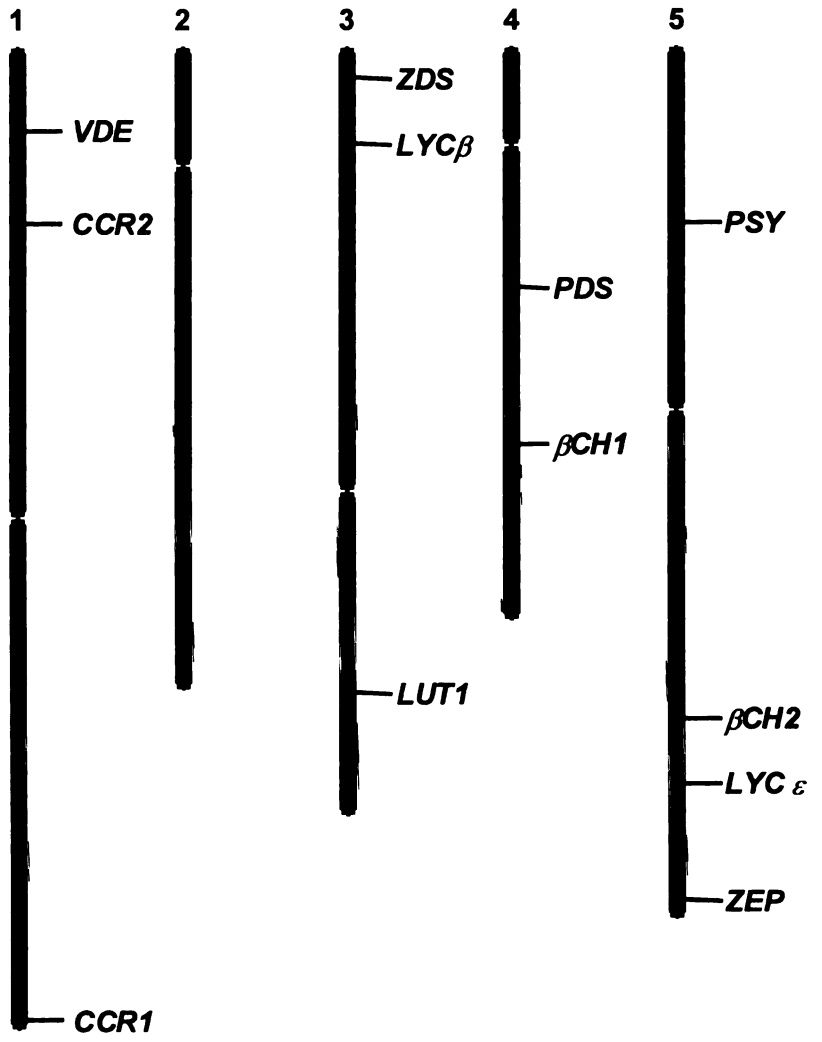


Figure 1.2

Table 1.2. Arabidopsis carotenoid biosynthetic genes.

Gene	Enzyme	Identifier	Location (Chromosome)	Mutant
PSY	phytoene synthase	At5g17230	5	n.a.
PDS	phytoene desaturase	At4g14210	4	n.a.
ZDS	ζ-carotene desaturase	At3g04870	3	n.a.
CCR1	carotenoid isomerase 1	n.a.	1	*ccr1*
CCR2	carotenoid isomerase 2	At1g06820	1	ccr2
LYCβ	lycopene β-cyclase	At3g10230	3	n.a.
LYCε	lycopene ε-cyclase	At5g57030	5	*lut2*
βCH1	carotenoid β-hydroxylase 1	At4g25700	4	*b1*
βCH2	carotenoid β-hydroxylase 2	At5g52570	5	*b2*
εCH	carotenoid ε-hydroxylase	At3g53130	3	*lut1*
ZEP	zeaxanthin epoxidase	At5g67030	5	*npq2 (aba1)*
VDE	violaxanthin deepoxidase	At1g08550	1	*npq1*

n.a., not available.

Figure 1.3. Carotenoid biosynthesis in higher plants. The Arabidopsis mutations that block specific steps of the pathway are indicated. *lut1*, *lut2*, *aba1* (*npq2*), *npq1*, *ccr1* and *ccr2* are mutations in ε-hydroxylase, lycopene ε-cyclase, zeaxanthin epoxidase, violaxanthin deepoxidase, carotenoid isomerase 1, and carotenoid isomerase 2, respectively. *pds1* and *pds2* disrupt phytoene desaturation reactions but are not the mutations in the phytoene desaturase gene.

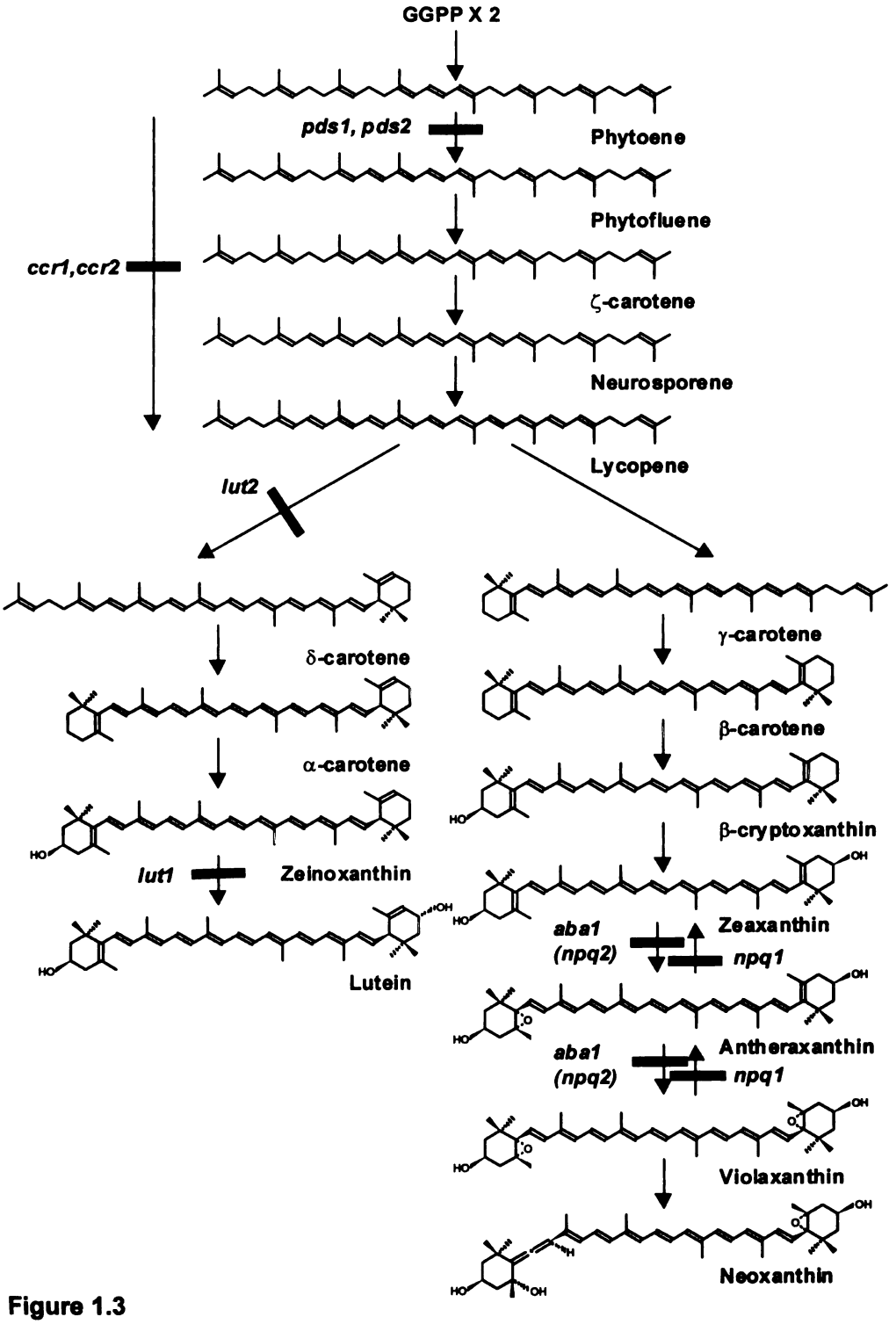

GGPP X 2
pds1, pds2
Phytoene
Phytofluene
ccr1,ccr2
ζ-carotene
Neurosporene
Lycopene
lut2
δ-carotene
α-carotene
lut1
Zeinoxanthin
Lutein
γ-carotene
β-carotene
β-cryptoxanthin
aba1
(npq2)
Zeaxanthin
npq1
aba1
(npq2)
Antheraxanthin
npq1
Violaxanthin
Neoxanthin

Figure 1.3

series of desaturation reactions. The phytoene desaturation reactions proceed with elimination of two hydrogen atoms and formation of a double bond. Four successive desaturations take place alternatively on each half of phytoene to yield phytofluene, ζ-carotene, neurosporene, and lycopene, respectively. The desaturation reactions are catalyzed by a single enzyme phytoene desaturase (CRT I) in bacteria and fungi. In higher plants, these four double bonds are introduced by the combined action of two enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) (Figure 1.4). PDS requires flavin adenine dinucleotide (FAD) as a cofactor and plastoquinones are intermediate electron acceptors in the reaction in higher plants (Norris et al., 1995; Schledz et al., 1996). Bleaching herbicides like norflurazon, can inhibit PDS directly and therefore prevent the synthesis of colored carotenoid and cause accumulation of colorless phytoene (Britton, 1998). Another class of herbicides, triketones, also causes accumulation of phytoene and bleaching of tissue by inhibiting the biosynthesis of the electron transporter, plastoquinone (Britton, 1998) (Figure 1.4). It was proposed that bacteria share a common desaturation mechanism with higher plants, although bacteria use ubiquinones rather than plastoquinones as electron acceptors. This hypothesis was supported by the experimental evidence that the plant PDS gene can be expressed and is active in *E. coli*; bacterial *CRTI* can be transformed into and still be active in plants (Bartley et al., 1999; Ye et al., 2000).

The Arabidopsis *immutans* (*im*) mutant disrupts a plastidic terminal oxidase that is homologous to mitochondrial alternative oxidase and shows a variegated phenotype (Carol et al., 1999). The albino sectors of the *im* mutants contain decreased levels of carotenoids and increased levels of phytoene suggesting a role for IM protein in the

phytoene desaturation reactions. The alternative oxidase in mitochondria oxidizes the ubiquinone pool using molecular oxygen as a terminal acceptor. Because PDS requires plastoquinone for activity, it was proposed that IM protein acts as a terminal oxidase and is associated with the transport of electrons from PDS (Carol et al., 1999) (Figure 1.4).

In nature, lycopene and many other colored carotenoids exist in the all-*trans* configurations. However, the biosynthetic precursor phytoene is synthesized in the15-*cis* configuration. Therefore, isomerization of the C (15,15') double bond must occur at a certain stage of carotenoid biosynthesis. The existence of a potential carotenoid isomerase enzyme was suggested from the phenotype of recessive mutations in tomato (Tomes et al., 1953) and *Scenedesmus* (Ernst and Sandmann, 1988), which accumulate prolycopene (7Z,9Z,7'Z,9'Z tetra-*cis*-lycopene), poly-*cis*-isomers of phytofluene, ζ-carotene, and neurosporene. This hypothesis was confirmed by cloning of isomerase genes from both tomato and Arabidopsis (Isaacson et al., 2002; Park et al., 2002). The *tangerine* mutant of tomato (*Lycopersicon esculentum*) produces fruits that are orange instead of red and accumulate prolycopene (7Z, 9Z, 7'Z, 9'Z tetra-*cis*-lycopene) instead of all-*trans* lycopene. Using map-based cloning, the gene encoding tangerine, *CRTISO*, was isolated. It encodes a redox-type enzyme that is structurally related to the bacterial PDS. *E.coli* cells transformed with the Erwinia GGPP synthase (*GGPS*) and *PSY* genes and Synechococcus PCC7942 *PDS* gene accumulated 15-*cis*-ζ-carotene. When the tomato *ZDS* gene was introduced into this *E.coli* background, prolycopene (7Z, 9Z, 7'Z, 9'Z tetra-*cis*-lycopene) was produced. Expressing both of the tomato *ZDS* and *CRTISO* genes in the 15-*cis*-ζ-carotene producing *E. coli* strain led to significant accumulation of all-*trans*-lycopene. This result further confirmed the isomerization activity of CRTISO.

Figure 1.4. Carotenoid desaturation reactions in plants and bacteria. Proposed integration of desaturation reactions with electron transport carriers in plants is indicated.

Figure 1.4.

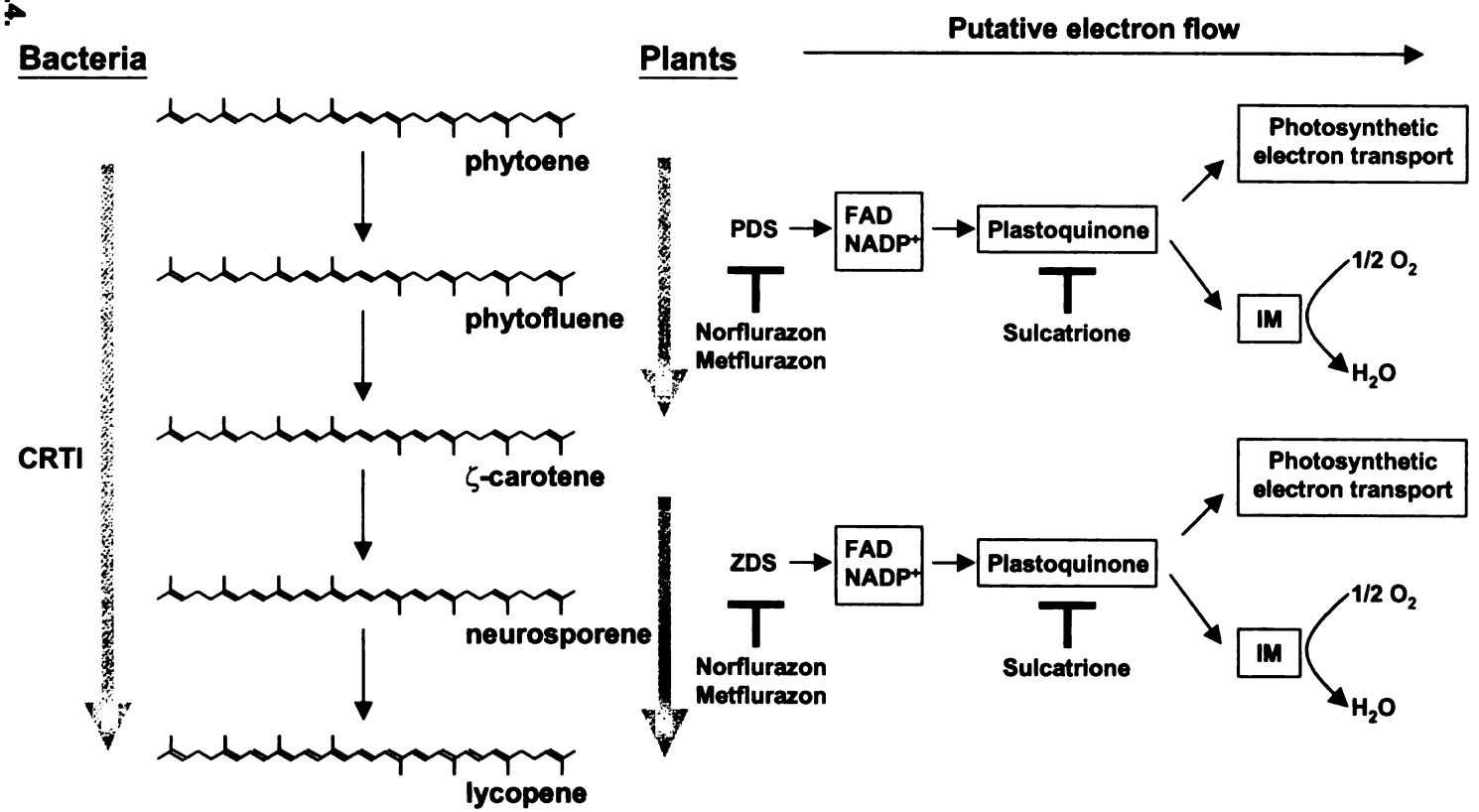

Bacteria
Plants
Putative electron flow
phytoene
phytofluene
ζ-carotene
neurosporene
lycopene
CRTI
PDS
ZDS
Norflurazon
Metflurazon
FAD
NADP+
Plastoquinone
Sulcatrione
Photosynthetic electron transport
IM
1/2 O2
H2O

Figure 1.4. Carotenoid desaturation reactions in plants and bacteria. Proposed integration of desaturation reactions with electron transport carriers in plants is indicated.

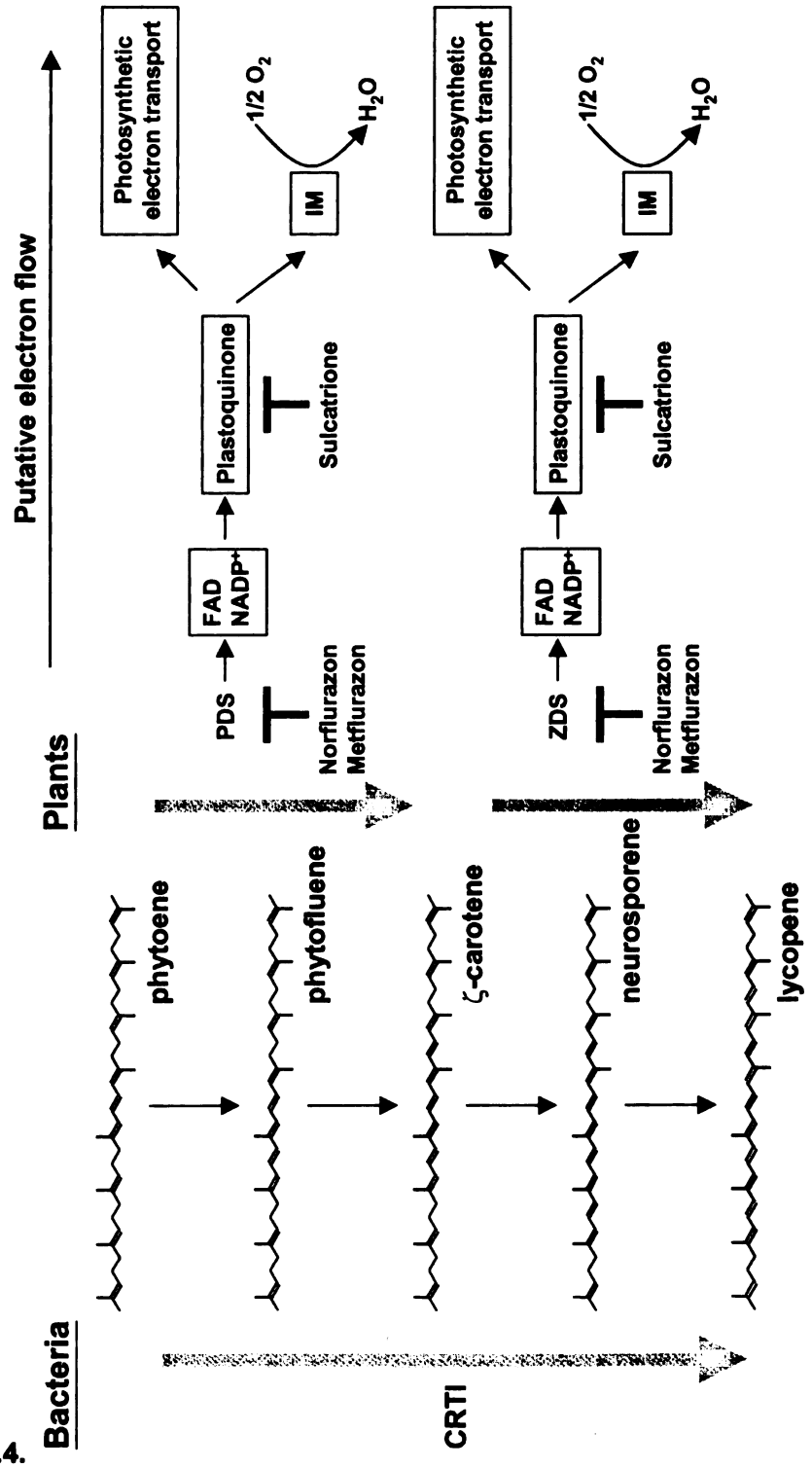

Putative electron flow
Plants
Bacteria
Photosynthetic electron transport
1/2 O_2
H_2O
IM
Plastoquinone
Sulcatrione
FAD
$NADP^+$
PDS
Norflurazon
Metflurazon
ZDS
phytoene
phytofluene
ζ-carotene
neurosporene
lycopene
CRTI

Figure 1.4.

Orthologs of *CRTISO* were also identified in Arabidopsis and cyanobacteria (Masamoto et al., 2001; Park et al., 2002). Characterization of the carotenoid isomerase in tomato and Arabidopsis also raised the possibility of photoisomerization of prolycopene to form all-*trans*-lycopene (Isaacson et al., 2002; Park et al., 2002). This hypothesis was supported by the fact that carotenoid isomerase deficiency in Synechocystis mutant was compensated by illumination (Masamoto et al., 2001). Evolutionarily, the occurrence of carotenoid isomerase has been correlated with the presence of the poly-*cis*-desaturation pathway that utilizes PDS and ZDS enzymes in higher plants and cyanobacteria.

The subsequent cyclization of lycopene is an important branching point for carotenoid biosynthesis. It is thought that the straight chain end group of lycopene is first folded to the desired shape upon binding of the substrate by the cyclase enzyme, followed by electrophilic attack at the C(2) position and formation of the C(1)-C(6) bond to close the ring (Figure 1.5). Loss of a proton at the C(6) position leads to the formation of the β-ring; alternatively, loss of a proton at C(4) forms the ε-ring (Figure 1.5). Carotenoids with two β-end groups (e.g. β-carotene and zeaxanthin) and one β- and one ε- end group (e.g. lutein) are most abundant in plants and perform critical functional roles. The formation of two symmetrical β-end groups is catalyzed by lycopene β-cyclases. Lycopene ε-cyclases are related enzymes that only catalyze the formation of one ε-ring and in combination with lycopene β-cyclase, α-carotene (β,ε-carotene) is produced in higher plants (Cunningham et al., 1996). Carotenoids with two ε-rings are uncommon in plants and only accumulate to substantial levels in lettuce.

Lycopene β- and ε- cyclases have been cloned from various plants; both enzymes show high similarity at the protein sequence level and likely evolved from the same

Figure 1.5. Lycopene cyclization mechanism. The flexible acyclic end group of the substrate is first folded into the required shape and then a concerted electrophilic attack occurs at the C2 position, which closes C1-C6 bond to form the ring. A loss of H_A results in the formation of the β-ring and an alternative loss of H_B leads to the formation of the ε-ring.

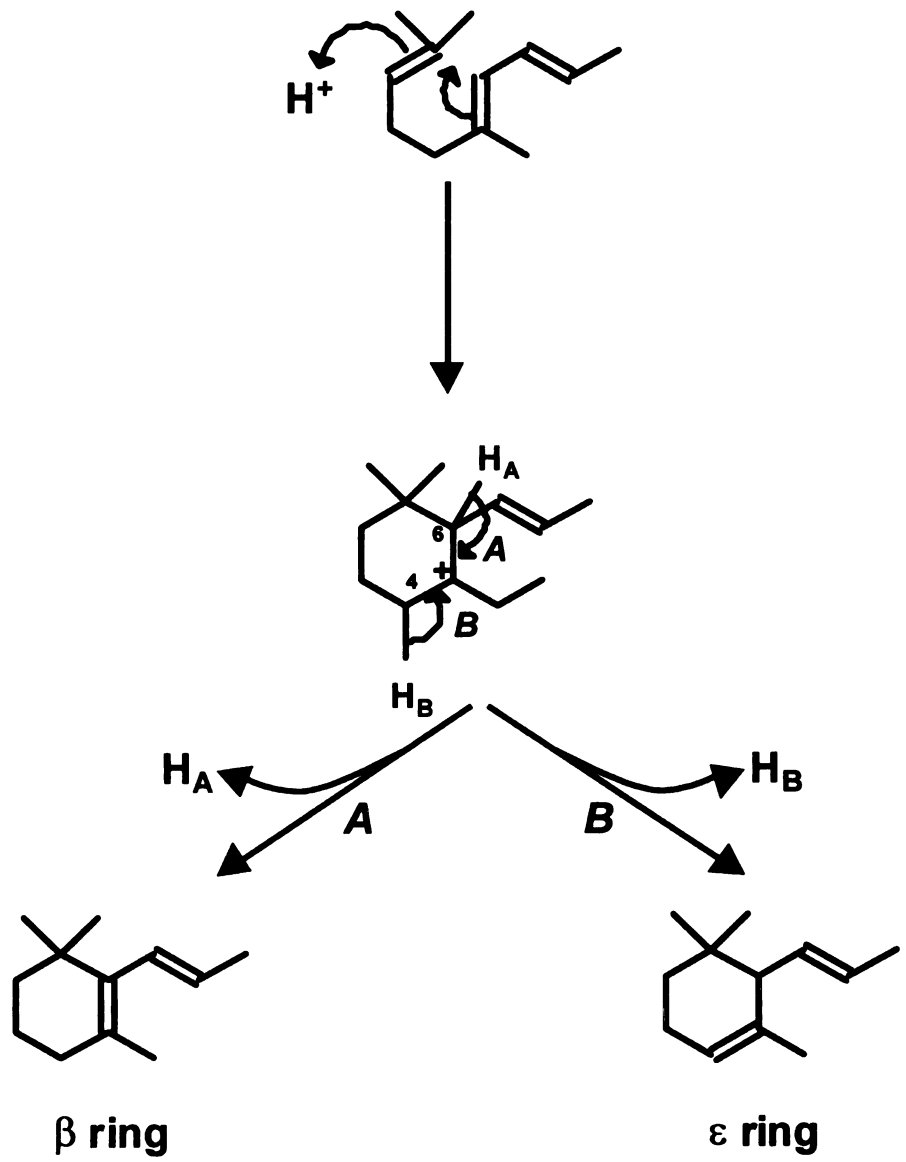


Figure 1.5

ancestor (Cunningham et al., 1994; Hugueney et al., 1995; Cunningham et al., 1996). The ε-cyclase that catalyzes the formation of two ε-rings has been cloned from Romaine lettuce (Krubasik and Sandmann, 2000). By using a domain swapping approach, a six-amino acid domain was identified for determining whether one or two ε-rings are produced (Cunningham and Gantt, 2001). Further mutagenesis studies showed that a single histidine residue in the lettuce ε-cyclase is the determining factor for the formation of two ε-rings (Cunningham and Gantt, 2001).

In addition to cyclization of end groups, another key control point for carotenoid biosynthesis is the introduction of various oxygen functional groups into carotenes to produce the oxygenated derivatives of carotenes, xanthophylls. Oxygenation reactions are catalyzed by a large and diverse group of enzymes, such as hydroxylases, ketolases, and epoxidases. A wide range of xanthophylls is formed from these reactions, including the most abundant carotenoids in photosynthetic plant tissues: lutein, neoxanthin, and violaxanthin. The most common oxygenation reactions are the hydroxylation of β-carotene (β,β-carotene) and α-carotene (β,ε-carotene) to form zeaxanthin (β,β-carotene-3,3'-diol) and lutein (β,ε-carotene-3,3'-diol), respectively. Early isotope labeling studies with ^{18}O showed that the hydroxyl groups in these reactions originated from molecular oxygen rather than from water (Yamamoto et al., 1962). Both β- and ε-ring hydroxylations take place by direct replacement of the hydrogen atom at the C(3) position of the ring structure by a hydroxyl group. Hydroxylations on the C(3) carbon of the ionone ring is stereospecific in all cases. All β-ring hydroxyl groups (e.g. β-cryptoxanthin, zeaxanthin, and the β-ring of lutein) have the same chirality and are designated as 3R. The chirality of C(3) on the ε-ring is opposite to β-ring, but is also

designated as 3R according to the sequence rule (Britton, 1998).

β-hydroxylase enzymes have been cloned from a range of bacteria, fungi, and higher plants (Misawa et al., 1990; Hundle et al., 1993; Sun et al., 1996; Bouvier et al., 1998; Masamoto et al., 1998; Tian and DellaPenna, 2001). Although all β-hydroxylases carry out the same reaction, they can be clearly categorized into three groups based on their primary structures. Eukaryotic β-hydroxylases are highly conserved and share less than 30% protein identity with β-hydroxylases from non-photosynthetic bacteria. The β-hydroxylase of *Synechocystis* PCC6803 (a cyanobacteria) lacks similarity to plant and non-photosynthetic bacterial β-hydroxylases with the exception of conserved histidine residues (Masamoto *et al.*, 1998). *Synechocystis* β-hydroxylase is more closely related to bacterial β-carotene ketolases (β-ketolase), suggesting that it may have evolved independently of the plant and bacterial β-hydroxylases.

As a group, the three classes of β-hydroxylases have overall low protein identity but contain conserved histidine regions that have been previously identified in the membrane fatty acid desaturases (Shanklin et al., 1994). Like the fatty acid desaturases, the β-hydroxylases are non-heme di-iron proteins that require iron, ferredoxin, and ferredoxin oxido-reductase for activity. They are able to directly oxidize the carbon atoms on the ionone ring structures. In contrast to the well-studied β-hydroxylases, the enzyme involved in ε-ring hydroxylation has only been genetically identified by the *lut1* mutation in Arabidopsis (Pogson et al., 1996), and at the time of this writing is the only remaining gene in the main carotenoid biosynthetic pathway in plants to be cloned (Figure 1.3). The publication of the Arabidopsis genome in 2000 allowed thorough database searches with known β-hydroxylase sequences from bacteria, fungi, and higher

plants, but did not identify a possible *LUT1* ortholog. Furthermore, *lut1* did not map to either of the two cloned β-hydroxylases in Arabidopsis (Tian and DellaPenna, 2001). The isolation and characterization of the *LUT1* gene is a major focus of this dissertation and will be discussed in chapter 3.

In photosynthetic and non-photosynthetic bacteria, green algae, fungi, and higher plants, there are also carotenoids with keto functional groups, the ketocarotenoids, such as echininone (β,β-carotene-4-one), canthaxanthin (β,β-carotene-4,4'-dione), and astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione). As with β-hydroxylases, β-C-4-oxygenases that catalyze the formation of the keto-groups have been cloned from different phyla (Misawa et al., 1994; Lotan and Hirschberg, 1995; Fernandez-Gonzalez et al., 1997). Green algal and bacterial β-C-4-oxygenases are similar in sequence while β-C-4-oxygenases from plants have high similarity to plant and bacterial β-hydroxylases. β-C-4-oxygenases from cyanobacteria define a third distinct group of ketolase enzymes that are similar to bacterial PDS (Fernandez-Gonzalez et al., 1997). Despite this wide sequence diversity, the β-C-4-oxygenases from bacteria, green algae, and higher plants are non-heme di-iron oxygenases, analogous to the β-hydroxylases in higher plants.

The xanthophyll cycle consists of a cyclic sequence of interconversions between zeaxanthin, antheraxanthin (5,6-epoxy-5,6-dihydro-β,β-carotene-3,3'-diol), and violaxanthin (5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol). These reactions are catalyzed by two enzymes, zeaxanthin epoxidase (ZEP) and violaxanthin deepoxidase (VDE), which localize to different chloroplast subcellular compartments. ZEP localizes to the stromal side of the thylakoid membrane and is constitutively active. Genes encoding ZEP have been cloned from various plants (Bouvier et al, 1996; Marin et

al., 1996). ZEP is a monooxygenase that requires O_2 and is active at approximately pH 7.5. The *ZEP* gene from *Capsicum annuum* was expressed in *E. coli* and shown to require NADPH, ferredoxin, and ferredoxin: NADP oxido-reductase for activity (Bouvier et al., 1996), similar to the β-hydroxylases. ZEP specifically utilized zeaxanthin and antheraxanthin as substrates, while the β-ring of lutein was not a substrate *in vitro* (Bouvier et al., 1996). This result is consistent with the fact that lutein epoxide does not normally accumulate *in vivo*. VDE catalyzes the reverse reactions of ZEP. Deepoxidation of violaxanthin to form zeaxanthin and antheraxanthin is induced by excessive light and is catalyzed by VDE (reviewed in Niyogi, 1999).

When absorption of light exceeds the ability of plants to fix CO_2, a higher than normal pH gradient builds up across the thylakoid membrane due to photosynthetic electron transport. VDE is located within the thylakoid membrane and is most active at a pH around 5.2, which occurs with the acidification of the thylakoid lumen due to the buildup of large H^+ gradient under excessive light. VDE was purified from lettuce, and ascorbate and monogalactosyldiacylglyceride (MGDG) are required for VDE activity *in vitro* (Rockholm and Yamamoto, 1996). ZEP and VDE were the first plant enzymes identified that belong to the lipocalin family, a group of proteins that bind small lipophilic molecules and have conserved β-barrel tertiary structures (Bugos et al., 1998).

Further modifications of antheraxanthin and violaxanthin in pepper lead to rearrangement of the epoxy end groups to give rise to the allenic end group of neoxanthin and 6-oxo-κ end group of capsanthin and capsorubin. The neoxanthin synthase gene has been cloned from both tomato and potato and shows high sequence identity to lycopene cyclases (Al-Babili et al., 2000; Bouvier et al., 2000). The formation of capsanthin and

capsorubin is catalyzed by capsanthin capsorubin synthase (CCS). CCS from *Capsicum annuum* also belongs to the lycopene cyclase family. This enzyme converts antheraxanthin or violaxanthin to capsanthin or capsorubin, respectively, by a mechanism similar to lycopene cyclases (Bouvier et al., 1994). Other modifications of carotenoids include the degradation of C_{40} carotenoid and generation of apocarotenoids (degradation from one end), e.g. abscisic acid, and diapocarotenoids (degradation from both ends), e.g. bixin and crocetin.

1.1.4 Cytochrome P450 monooxygenase

1.1.4.1 Cytochrome P450 monooxygenase and carotenoid biosynthesis

Cytochrome P450 is a superfamily of proteins that metabolizes a large variety of physiological important compounds, e.g. steroids and fatty acids (reviewed in Ortiz de Montellano, 1995). In plants, cytochrome P450s play important roles in secondary metabolism, such as in the synthesis of phenylpropanoids. Cytochrome P450s also catalyze the degradation of exogenous compounds (reviewed in Chapple, 1998). About 300 cytochrome P450 enzymes are identified in the *Arabidopsis thaliana* genome, which constitute one of the largest families of enzymes in this model organism.

There is some circumstantial evidence that cytochrome P450 enzymes may be involved in carotenoid biosynthesis in photosynthetic organisms. In the green algae *Haematococcus pluvialis*, biosynthesis of astaxanthin is carried out by two different pathways: the addition of two keto-groups followed by two hydroxyl-groups or two

hydroxylation steps followed by two ketolation steps. Astaxanthin biosynthesis in Haematococcus requires oxygen, NADPH, and Fe^{2+}, common cofactors for cytochrome P450 type oxygenases, suggesting that the biosynthesis of astaxanthin might involve a cytochrome P450 enzyme. Treatment of cells with the cytochrome P450 specific inhibitor ellipticine inhibited the carotenoid hydroxylase activity and only the diketo product canthaxanthin accumulated (Schoefs et al. 2001). Although these data strongly suggested that carotenoid synthesis in Haematococcus involves a cytochrome P450 type enzyme, direct demonstration of a cytochrome P450 carotenoid biosynthetic activity in the organism has not been shown.

Cytochrome P450 enzymes are structurally a diverse group but contain some conserved motifs involved in catalysis. The most highly conserved regions in cytochrome P450s are the PERF motif, the K-helix, and the heme-binding domain (Poulos, 1995). The consensus sequence that binds the proximal face of the heme, FxxGxxxCxG, contains a conserved cysteine that is the fifth ligand of the heme iron. The non-conserved region of the cytochrome P450 proteins usually binds the substrate and one of the several known redox partners. By definition, cytochrome P450s are grouped into a family if their amino acid sequences are >40% identical and are further grouped into subfamilies if the identity is >55%. In the standard cytochrome P450 nomenclature, cytochrome P450 enzymes start with CYP followed by a number designating the family, then a capital letter designating subfamily (e.g. CYP86B). Within subfamilies, allelic variants that are less than 3% different are designated with an additional number after the subfamily designation, e.g. CYP86B1.

1.1.4.2 Localization of the plant cytochrome P450s

Cytochrome P450 monooxgenases have molecular mass ranging from 45 to 62 kDa with an average of 55 kDa. The majority of cytochrome P450s reside in or are associated with the endoplasmic reticulum. Although a few mitochondrial cytochrome P450s have been found in the mammalian adrenal cortex, no plant mitochondrial-localized cytochrome P450s have been discovered thus far. Recently, several lines of evidence have indicated that plant cytochrome P450s also localize to the chloroplast.

The first direct evidence for a chloroplast-localized cytochrome P450 was the identification of CYP86B1. CYP86B1 has an unknown function but the protein sequence shows a putative chloroplast transit peptide. Using import assays with isolated pea chloroplast, CYP86B1 has been localized to the outer envelope of chloroplast (Watson et al., 2001). Two cytochrome P450 enzymes involved in oxylipin (oxygenated fatty acids) metabolism are allene oxide synthase (AOS, CYP74A) and fatty acid hydroperoxide lyase (HPL, CYP74B). AOS catalyzes the synthesis of 12,13-epoxyoctadecatrienoic acid (EOT), an intermediate in jasmonic acid biosynthesis, from 13-hydroperoxide linoleinic acid (13-HPOT). HPL alternatively converts 13-HPOT to 12-oxo-*cis*-9-dodecenoic acid (ODA), which leads to the production of C6 aldehydes. Although both AOS and HPL belong to the CYP74 family, they are targeted to different membranes of the chloroplast envelope by different pathways (Froehlich et al. 2001). Tomato (*Lycopersicon esculentum*) AOS contains a putative chloroplast targeting peptide and inserts into the inner envelope of chloroplast. Its targeting process requires both ATP and protease-sensitive components on the outer face of the chloroplast. HPL, on the other hand, does

not have an N-terminal chloroplast targeting peptide and its insertion to the outer envelope does not require ATP and is not protease sensitive (Froehlich et al. 2001). CYP86B1 and tomato AOS and HPL are the only known activities of chloroplast cytochrome P450s in higher plants. Out of the 272 known Arabidopsis cytochrome P450s, only nine are predicted to be chloroplast targeted, most of these have unknown functions (Table 1.3).

1.1.4.3 Cytochrome P450 functions in plants and monooxygenation reaction mechanism

Based on their functions, cytochrome P450s in plants can be generally divided into two classes: those involved in biosynthesis and those involved in detoxification and degradation. Cytochrome P450s play important roles in the biosynthesis of a bewildering array of secondary metabolites, such as lignins, terpenoids, flavonoids, sterols, isoflavonoids, and furanocoumarins. Cytochrome P450s are also involved in the synthesis of several plant hormones, such as jasmonic acid (JA), gibberellic acid (GA), brassinosteroid, and auxin (reviewed in Schuler, 1996). The first characterized plant cytochrome P450 was cinnamate-4-hydroxylase, which converts *trans*-cinnamic acid to p-coumaric acid (Russell and Conn, 1967). Besides the indispensable roles in biosynthesis, cytochrome P450s are also essential in detoxifying herbicides (Schuler, 1996).

Cytochrome P450 enzyme catalyzes monooxygenation in a multistep mechanism. The substrate first binds to the active site of the cytochrome P450 enzyme close to the

Table 1.3. Arabidopsis cytochrome P450s with predicted chloroplast localization.

Gene	Subfamily	Function
At1g31800	97A3	unknown
At1g74550	98A9	unknown
At3g53130	97C1	unknown
At4g13310	71A20	unknown
At4g36220	84A1	ferulate-5-hydroxylase
At4g37340	81D3	unknown
At5g05260	79A2	unknown
At5g25120	71B11	unknown
At5g42650	74A	allene oxide synthase

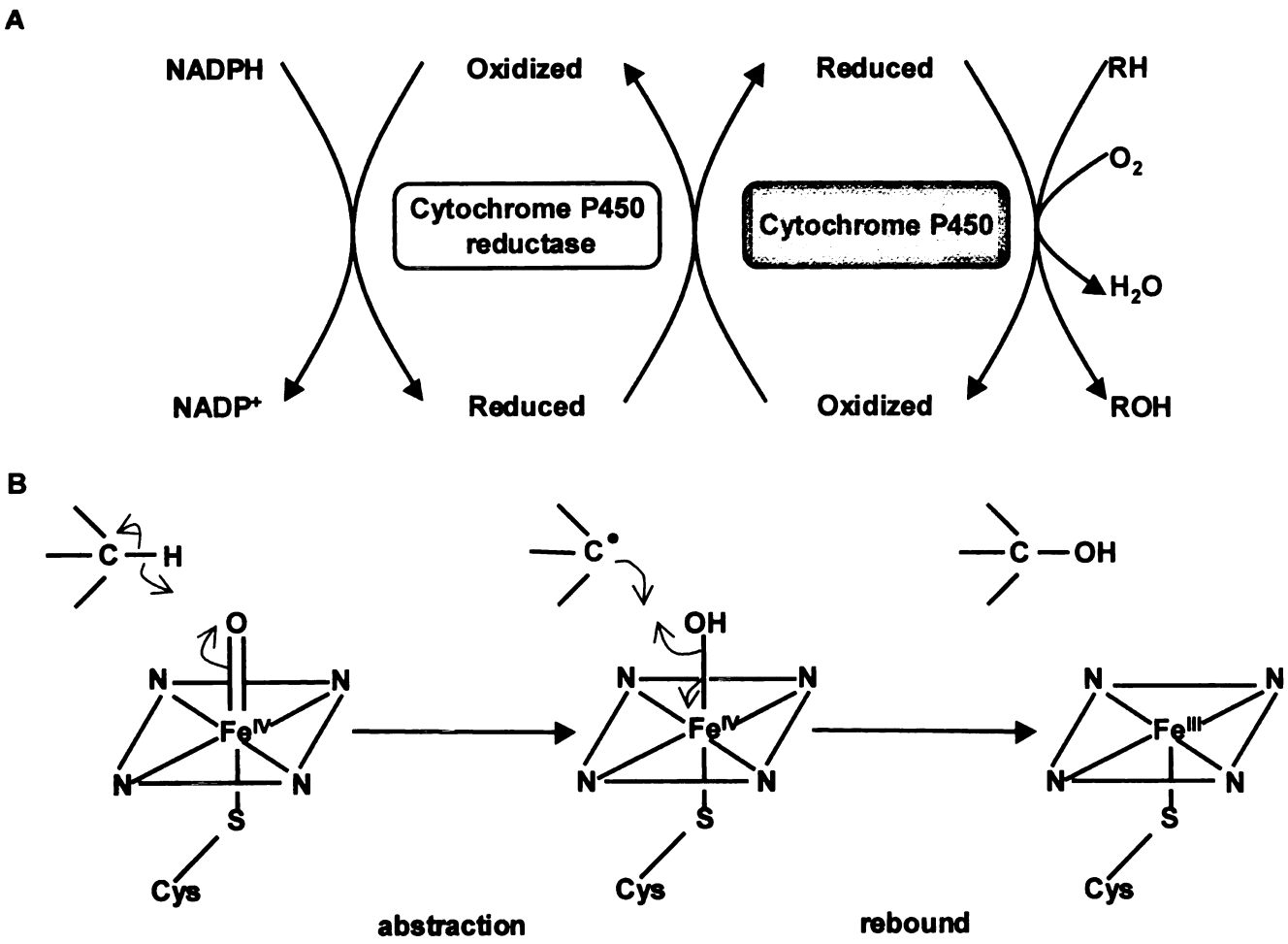
iron center but not directly ligated to the iron. Molecular oxygen then binds to the reduced heme iron, which resulted from electron transfer. A hydrogen atom from the substrate is subsequently abstracted by an oxoiron species (Fe^{IV}=O), and finally, a rapid collapse of the resulting carbon radical-hydroxyferryl complex to give the hydroxylated product and the resting-state enzyme (Figure 1.6). NADPH-cytochrome P450 oxido-reductase (CPR), a membrane-bound flavoprotein, is required and essential for the transfer of electrons during the cytochrome P450 catalytic cycle.

1.1.4.4 Arabidopsis NADPH-Cytochrome P450 reductases (CPR)

Cytochrome P450s require CPR for function. There is only one CPR in animals (Ortiz de Montellano, 1995), while in plants there are multiple forms of CPRs present. This is maybe because of the requirement of specific cytochrome P450s in plants or because cytochrome P450s and hence CPRs localize to different subcellular compartments in plant cells. It is also possible that plant CPRs are developmental stage or tissue specific. Arabidopsis CPRs were cloned using a functional approach by complementing yeast deficient of CPR with Arabidopsis cDNAs (Urban et al. 1997). Two Arabidopsis CPRs were identified from screening and designated as ATR1 (At4g24520) and ATR2 (At4g30210), respectively. Both reductases contain the signature FMN-, FAD-, and NADPH- binding domains for cytochrome P450 and support the activity of cinnamate 4-hydroxylase (CYP73A5) *in vitro* (Urban et al. 1997). ATR1 and ATR2 are 63% identical at the amino acid level and are conserved in their C-terminal sequences but not their N-termini. ATR2 contains a stretch of amino acids that are

Figure 1.6. Cytochrome P450 reaction mechanism. A. Generalized scheme for Cytochrome P450 enzyme reaction. B. The proposed hydrogen abstraction-oxygen rebound mechanism for cytochrome P450 hydroxylation.

Figure 1.6


A
NADPH
Oxidized
Reduced
RH
O_2
Cytochrome P450 reductase
Cytochrome P450
H_2O
$NADP^+$
Reduced
Oxidized
ROH
B
C H
O
N
Fe^{IV}
S
Cys
C•
OH
C OH
Fe^{III}
abstraction
rebound

serine/threonine rich and predicted to be chloroplast-targeted (Urban et al., 1997; Hull and Celenza, 1999). A third putative CPR was identified from database searching and designated as ATR3 (At3g02280). However, its expression and activity have not yet been tested.

1.1.4.5 Current genomics approach for cytochrome P450 characterization

The sequencing of the Arabidopsis genome has identified 272 cytochrome P450s (http://www.biobase.dk/P450/p450.shtml), most with unknown functions. Genome sequencing and high-throughput functional genomics are tools that have been developed for Arabidopsis and allow scientists to specifically mutagenize genes of interest and study their functions *in vivo*. Using a reverse genetics approach, T-DNA insertional lines for several cytochrome P450 genes have been isolated by several groups, but only one mutant showed a visible phenotype that affected normal growth. The others still need to be analyzed more closely for possible biochemical alterations or changes in primary and/or secondary metabolism.

Another approach for cytochrome P450 characterization has been to correlate the expression of specific cytochrome P450s with physiological and developmental states. The impact of developmental stage on cytochrome P450 expression was investigated using microarray technology (Xu et al., 2001). Many cytochrome P450s on the microarray showed tissue specific expression patterns, which may facilitate selective study of the role of certain cytochrome P450s in tissue-specific metabolic pathways. However, most of the highly expressed cytochrome P450s still have unknown functions.

A larger scale microarray experiment with different conditions and clustering analysis (e.g. self-organizing map) will be more informative because cytochrome P450 with known function in a cluster may suggest a potential function of the other unknown cytochrome P450s. Chapter 3 of this dissertation described the isolation and functional characterization of CYP97C1, a cytochrome P450 enzyme involved in carotenoid biosynthesis.

1.1.5 Model for possible carotenoid biosynthetic enzyme complexes

Carotenoids are hydrophobic compounds, therefore the carotenoid biosynthetic enzymes are predicted to be membrane-bound or membrane-associated. A "phytoene synthase" complex, which contains IPP isomerase, GGPS, and PSY, was isolated from chromoplasts of higher plants (e.g. daffodil flowers and bell peppers) (Camara and Moneger, 1981; Kleinig and Beyer, 1985). The complex was readily solubilized suggesting that it was only loosely (peripherally) attached to the thylakoid membrane. Biochemical analysis also showed that imported PSY is present in both soluble and membrane-bound forms in daffodil chromoplasts (Schledz et al., 1996). The soluble protein is inactive while the membrane-bound PSY is active. The presence of active PSY in the thylakoid membrane is consistent with the lipophilic character of the end product, phytoene.

Based on the above experimental evidence, the physical properties of carotenoids, and predicted localization and hydrophobicity of carotenoid biosynthetic enzymes, an overall model for carotenogenic complexes was proposed by Cunningham and Gantt

(1998) (Figure 1.7). In this hypothetical model, a single IPP isomerase, two GGPS, and one PSY form a peripheral complex that associates with the thylakoid membrane. IPP substrates are supplied to and phytoene are produced from this peripheral "phytoene synthase complex". The phytoene produced is then transferred to PDS, which is a part of a proposed integral membrane complex including PDS, ZDS, and lycopene cyclases. Because only β,β-carotene and β,ε-carotene are normally formed in most of the plants, it was proposed that there are two types of complexes in the thylakoid membrane, one containing two lycopene β-cyclases to produce β,β-carotene (the β,β-complex) and one containing one β- and one ε- cyclase to form β,ε-carotene (the β,ε-complex). A complex with two ε-cyclases is not hypothesized due to the lack of ε,ε-carotene in most of the plants.

This model suggested a fundamental mode for carotenogenic enzyme interactions in product generation. However, *cis*-isomers of phytoene need to be converted to all-*trans* lycopene before they could be converted to the cyclized products. A more refined model (Cunningham, 2002) added the isomerase enzyme to both β,β- and β,ε-complexes (Figure 1.7). However, there is no direct evidence for the existence of membrane carotenoid complexes hence the hypothesis has not been proven. A key factor lacking in the model is the accommodating ZEP, VDE, and the hydroxylases. How are these enzymes in the later part of the pathway organized? Are they involved in the same complexes as the cyclases? The lack of accumulation of reaction intermediates (such as phytoene, ζ-carotene, and lycopene) has been considered as a consequence of the substrate channeling in the hypothetical enzyme complexes. However, it is also likely that the carotenogenic enzymes have low Kms and high affinity for various substrates,

Figure 1.7. Proposed model for carotenogenic complexes in chloroplasts of plants. IPI, isopentenyl pyrophosphate isomerase; GGPS, geranylgeranyl pyrophosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LHCB, lycopene β-cyclase; LHCE, lycopene ε-cyclase; CRTISO, carotenoid isomerase. This figure is adapted after **Cunningham and Gantt.** (1998) Ann. Rev. Plant Physiol. Plant Mol. Biol. **49,** 557-583.

Stroma
8 IPP
IPI
GGPS
GGPS
PSY
phytoene
Thylakoid membrane
LCYB
ZDS
PDS
PDS
CRTISO
LCYE
β,β complex
β,ε complex

Figure 1.7.

and this could also explain why no accumulation of intermediates are observed. A more refined model for carotenogenic complex formation is expected with a better understanding of carotenoid enzyme properties. Chapter 4 of this dissertation will address this question in reference to hydroxylases from a molecular genetic perspective.

1.2 Regulation and metabolic engineering of the carotenoid pathway

1.2.1 Regulation of carotenoid biosynthesis

Carotenoids share the common biosynthetic precursor, IPP, with the synthesis of many other biologically important terpenoids. Therefore, the regulation of carotenoid formation must also involve the branching or channeling of isoprene units into the carotenoid pathway. The non-mevalonate pathway provides the isoprene building blocks in plastids and may regulate the production of carotenoids by apportioning carbon through carotenoid biosynthesis. Mutation in the *CLA1* gene from Arabidopsis, a gene that encodes the transketolase DXPS, impeded the development of chloroplasts, caused an albino phenotype, and led to a severe reduction in carotenoid and chlorophyll levels (Mandel et al., 1996). The *CapTKT2* gene cloned from pepper fruit encodes a plastid transketolase. It catalyzes the formation of DXP from pyruvate and G3P *in vitro*, and therefore proved to be a DXPS (Bouvier et al., 1998a). *CapTKT2* expression was up-regulated during the chloroplast to chromoplast transition, which correlated with the massive accumulation of carotenoids in ripening bell peppers (Bouvier et al., 1998a). In addition, it has been observed in tomato fruits that the expression of the *DXPS* gene

:orrelates with the ripening-associated massive accumulation of carotenoids (Lois et al., ?000). Expression analysis in tomato also showed that the *DXPS* gene has a similar :xpression pattern as *PSY1*, a key regulatory gene in the carotenoid biosynthetic pathway. Moreover, feeding of DXP induced the expression of both *DXPS* and *PSY1* in tomato ruit (Lois et al., 2000). The combined results from Arabidopsis, pepper, and tomato ;uggest that the non-mevalonate pathway may be involved in the regulation of flux into he carotenoid pathway and therefore should be taken into consideration in studying of :arotenoid biosynthesis. It has also been recognized that PSY is a major quantitative :ontrol point for carotenoid biosynthesis, which will be discussed in detail in the ollowing sections on metabolic engineering.

Carotenoids are essential components of the photosystem reaction centers and the ight harvesting complexes in chloroplasts. The regulation of carotenoid biosynthesis in green tissue is therefore likely related to and integrated with chloroplast development, :hlorophyll formation, the level of carotenoid binding proteins, and thylakoid membrane ipid composition. The mechanism for this complicated regulation is still not well understood. However, light is known to be a key factor in the regulation of carotenoid biosynthesis in chloroplasts. Transferring Arabidopsis or tomato plants from low light to high light induced a shift in the ratio between lycopene β- and ε- cyclase mRNA levels (Hirschberg, 2001). This result not only suggests that xanthophyll composition is regulated by light but also indicates a modulation of carotenoid biosynthesis at the branch of α- and β- carotene in the carotenoid pathway (Hirschberg, 2001). A key regulatory step in xanthophyll composition at the β,β- and β,ε- branches (at the level of the ε- cyclase enzyme) has previously been suggested by the Arabidopsis *lut2* mutant. *lut2* is a

disruption in ε-ring cyclization in which lutein biosynthesis is abolished and β-carotene derived xanthophylls accumulated to a much greater amount than wild type (Pogson et al., 1996). Finally, transgenic Arabidopsis overexpressing the lycopene ε-cyclase gene produced almost twice as much lutein as wild type providing direct evidence for the key regulatory role of the cyclization enzymes and the ε-cyclase in partitioning carbon between α- and β- branches (Pogson and Rissler, 2000).

The regulation of carotenoid biosynthesis in chromoplasts has been more extensively studied than chloroplasts (Bramley, 2002). Tomato and pepper fruits and marigold flowers have been used as model systems for studying chromoplast carotenoid biosynthesis. Experimental data from all three systems have shown that several of the pathway genes are transcriptionally up-regulated during the large increase in carotenoid biosynthesis in each system. For example, the mRNA levels for PSY and the desaturases were induced 10- to 20- fold during tomato ripening (Giuliano et al., 1993); carotenoid accumulation in cultivars of marigold that differ in their carotenoid levels by 100 fold is correlated with the mRNA levels of the pathway genes (Moehs et al., 2001, Appendix A). Post-transcriptional regulation of carotenoid genes was reported in daffodil chromoplasts where PSY was inactive in its soluble form but became active when it was bound to the membrane (Schledz et al., 1996). In addition, carotenoid-associated and binding proteins play important roles in the sequestration of carotenoids and affect the accumulation of carotenoids within chromoplasts post-transcriptionally (Vishnevetsky et al., 1999).

1.2.2 Metabolic engineering

Carotenoids have diverse functions related to human health; they are required for normal vision and are associated with a reduction in the occurrence and severity of several degenerative diseases including some cancers. Certain carotenoids are limiting in some diets and associated with increased risk of age-related macular degeneration. While carotenoid supplements are available, the efficiency of absorption of supplements varies. Carotenoids in food are facilitated in their absorption during digestion where dietary lipids enhance the solubility of carotenoids. Engineering of the carotenoid pathway in plants has been proposed as a method to elevate specific carotenoids in the food supplies or to provide plant-based production of specific carotenoids for industrial or agricultural purposes.

One strategy for metabolic engineering of carotenoids is to increase the total amount of existing carotenoids in plants or a specific plant tissue. PSY catalyzes the first committed step for carotenoid biosynthesis and may direct carbon flow from the "bulk" IPP pool into the carotenoid pathway. Therefore, it has been considered as a regulatory point for the pathway and used as a target for metabolic engineering by several groups. The differing outcomes of apparently similar experimental approaches highlight the large gaps in our understanding of carotenoid synthesis in plants. Overexpressing PSY gene in tomatoes had little impact on fruit carotenoid levels and resulted in dwarfism (Fray et al., 1995). This is because GGPP is also the precursor for gibberellin biosynthesis (Figure 1.1). Overexpressing PSY led to a decrease in gibberellin synthesis in green tissue, and hence dwarfism (Fray et al., 1995).

One of the most successful examples of carotenoid engineering to date is using a bacterial PSY gene (*Erwinia uredovora*; *crtB*) fused to a seed specific promoter and

expressed in the canola (*Brassica napus*) seeds. This led to a more than 50-fold increase in total seed carotenoids (Shewmaker et al., 1999). This increased level of carotenoids is comparable or even higher than certain high carotenoid producing flowers or fruits. However, the increase in the carotenoid pool impacted other compounds that also use IPP and GGPP as precursors, such as tocopherols and chlorophylls in the developing seeds. Sterols are synthesized through the cytosolic mevalonate pathway and their levels were not changed in the transgenic seeds. The fatty acid composition in transgenic canola seeds was also slightly changed with relative more oleic acid (18:1) than linoleic acid (18:2) and linolenic acid (18:3).

Metabolic engineering of the carotenoid pathway was also accomplished by shifting to another carotenoid gene product in tomato. A bacterial (*Erwinia uredovora*) phytoene desaturase gene was constitutively expressed in tomato (Römer et al., 2000). β-carotene content was increased about three-fold at the expense of lycopene but total carotenoid levels were reduced, suggesting a feedback inhibition of the pathway. Levels of other GGPP-derived isoprenoids did not significantly change in this experiment.

Another general metabolic engineering strategy is to transfer the entire carotenoid biosynthetic pathway into a tissue that is devoid of carotenoids. Mammals do not synthesize carotenoids and depend solely on dietary intake for carotenoids. β-carotene (provitamin A) is the precursor of retinol, a critical visual pigment for humans (Simpson and Chichester, 1981). There is no β-carotene present in the endosperm of rice (*Oryza sativa*), therefore provitamin A deficiency is a severe problem in the countries where rice is the major staple food (Ye et al., 2000). Immature rice endosperm does produce GGPP, the precursor for phytoene biosynthesis. Therefore, the entire β-carotene biosynthetic

pathway was engineered into rice endosperm by transforming vectors containing daffodil (*Narcissus pseudonarcissus*) *PSY* and lycopene β-cyclase open reading frames, and a bacterial phytoene desaturase (*Erwinia uredovora*; *crtI*) gene (Ye et al., 2000). As a result, β-carotene was produced to a substantial amount in the transgenic lines. In addition, lutein and zeaxanthin were also unexpectedly accumulated in the transformed endosperm indicating lycopene ε-cyclase and the hydroxylases may have been constitutively expressed or induced in the transgenic rice lines. β-carotene accumulating rice endosperm sets a good example of engineering crop plants for improved nutritional value.

Besides providing plant food with increased carotenoid content for humans, engineering of the carotenoid biosynthetic pathway can also enhance stress tolerance of plants. The Arabidopsis β-hydroxylase 1 gene was overexpressed under the constitutive 35S promoter in wild type Arabidopsis (Davison et al., 2002). This led to accumulation of xanthophyll cycle carotenoids at twice the levels in wild type plants and an increase in the total carotenoids without affecting the lutein levels. Sucrose gradient fractionation of pigment protein complexes indicated that the extra xanthophylls produced reside mainly in the LHC II complex. The transgenic plants were tested for stress tolerance by switching growth condition to high light (1000 μmol photon $m^{-2}s^{-1}$) and high temperature (40°C) for two weeks. Two to four times more zeaxanthin were accumulated in the transgenic plants when compared to wild type controls. As a consequence, the transgenic plants showed less negative effects from stress than wild type as indicated by decreased anthocyanin accumulation and a 30% decrease in lipid peroxidation, as measured by malondialdehyde (MDA) production. Although zeaxanthin is involved in

nonphotochemical quenching (NPQ), the constitutive accumulation of zeaxanthin did not lead to an increase in the NPQ capacity in the engineered plants. Therefore, increased NPQ is not likely the cause for increased stress tolerance. Earlier findings showed that zeaxanthin plays a role in preventing lipid peroxidation (Havaux and Niyogi, 1999). Therefore, the transgenic plants most probably tolerate stress by preventing lipid peroxidation through the action of zeaxanthin. Genetic manipulation of a single enzyme (β-hydroxylase 1) in the carotenoid biosynthetic pathway leads to stress tolerance and suggests it may be a route for producing more stress tolerant crops.

1.3 Carotenoid functions in higher plants

1.3.1 Zeaxanthin as a membrane stabilizer

Thylakoid membranes are bilayers of amphipathic di-acyl galactolipids in which proteins are imbedded. Thylakoid membranes contain high amounts of polyunsaturated galactolipids and lack cholesterol or other sterols. Therefore, thylakoid membranes are relatively fluid and thermolabile system, however, they are also sensitive targets of singlet oxygen and reactive oxygen species during high light stress. Terpenoid molecules in the thylakoid membrane include tocopherols, chlorophylls, carotenoids, and plastoquinones. It has been suggested that α-tocopherol plays a role in membrane stabilization that is analogous to cholesterol (Fukuzawa et al., 1977), although no direct evidence for this hypothesis has been shown.

In vitro studies have shown that the orientation of zeaxanthin in lipid bilayers is

with the long axis of zeaxanthin perpendicular to the thylakoid membrane surface and the polar β-end groups anchored at both sides of the membrane (Gruszecki and Sielewiesiuk, 1991). It is suggested that zeaxanthin acts as a "rivet" spanning the entire thylakoid membrane bilayer and linking the two halves of the bilayer. Zeaxanthin accumulates to high levels in excess light conditions through the deepoxidation of the violaxanthin. Therefore, it is possible that the operation of xanthophyll cycle correlates the light stress and regulation of thylakoid membrane fluidity and thermostability by zeaxanthin.

Barley leaves were exposed to a photon flux density (PFD) of 1,000 μmol $m^{-2}s^{-1}$ for several minutes, which leads to a conversion of violaxanthin to zeaxanthin without the induction of lipid peroxidation (Tardy and Havaux, 1997). The light treatment led to a decrease in thylakoid membrane fluidity as measured by electron spin resonance (ESR) spectroscopy. The rigidification of the thylakoid membrane correlated with an increase in zeaxanthin and was blocked by dithiothreitol (an inhibitor of violaxanthin deepoxidase). Membrane fluidity recovered in the dark when zeaxanthin was re-epoxidized to violaxanthin. Such a phenomenon was not observed in the *aba1* mutant of Arabidopsis, which lacks the xanthophyll cycle and constitutively accumulates zeaxanthin (Tardy and Havaux, 1997). There is also an increased thermostability in the light treated barley leaves, possibly because the stabilization effect by zeaxanthin counteracts the effect of heat induced fluidity increase of membrane lipids. Plants may have developed this mechanism to quickly protect from high light induced membrane destruction by producing membrane stabilizing zeaxanthin molecules through the xanthophyll cycle.

1.3.2 Carotenoid functions in light harvesting and photoprotection

1.3.2.1 Light harvesting

The earth's biosphere derives its energy from sunlight through photosynthesis. Plants, algae, and photosynthetic bacteria have evolved efficient systems to harvest the light from sun and use this energy to drive metabolic reactions required for life. The main photosynthetic pigments in higher plants are chlorophylls (Chls) and carotenoids. In the chloroplasts of plants, Chls and carotenoids are embedded in the photosystems (PS) that are localized in the thylakoid membranes. Two different photosystems (PS I and PS II) are linked together by an electron transport chain. PS II is a large membrane complex containing pigments for light harvesting and pigment binding proteins. It oxidizes water to produce oxygen and transfers electrons by photochemical reactions. PS I functions as a light-dependent plastocyanin-ferredoxin oxido-reductase that accepts the electrons from the electron transport chain and reduces $NADP^+$ to generate NADPH. Each PS consists of two closely linked components: a reaction center and a light harvesting complex (LHC). LHCs consist of antenna pigments (Chls and carotenoids). Upon absorption of light, antenna pigments are converted into the excited singlet state. Energy from excited singlet antenna pigments can be transferred to neighboring pigment molecules via resonance transfer and eventually delivered to the reaction center Chl a. The absorbed light energy is then converted into chemical energy and provides organic chemicals for plants (photochemistry, qP). In addition to photochemistry, the excited light energy absorbed by antenna pigments can be emitted as chlorophyll fluorescence or dissipated as heat (non-radiative decay).

1.3.2.2 The role of carotenoids in photoprotection

In addition to being used in photosynthesis or decaying to the ground state, triplet chlorophyll (^{3}Chl) can be formed from singlet chlorophyll (^{1}Chl) through intersystem crossing. ^{3}Chl has a longer lifetime than ^{1}Chl (ms time scale vs. ns time scale) therefore is able to react with O_2 to produce singlet O_2 ($^1O_2$), posing a high potential for photooxidative damage to the PSs. Excessive light energy can prolong the half-life of ^{1}Chl and consequently increase the probability of producing more ^{3}Chl and $^1O_2$ in the LHCs. Generation of $^1O_2$ in the LHCs is harmful because the thylakoid membrane (where LHCs are located) is enriched in polyunsaturated fatty acid (PUFA), which are highly susceptible to $^1O_2$ attack and can initiate peroxyl radical chain reactions. The damage caused by $^1O_2$ and its reactive products can decrease the efficiency of the PS and lead to photoinhibition.

Carotenoids in the LHCs have excited singlet and triplet energy states that are lower than Chls and are able to accept excitation energy directly from ^{3}Chl to prevent the formation of $^1O_2$ (Niyogi, 1999). The excited triplet carotenoid has very low energy therefore is unable to generate other reactive species and instead dissipates energy as heat to convert to the ground state harmlessly. In addition to quenching $^1O_2$, carotenoids are also involved in other mechanisms that protect the photosystem from photodamage, the most notable being nonphotochemical quenching (NPQ).

1.3.2.3 The role of carotenoids in NPQ

Thermal dissipation of excessive light energy is measured as nonphotochemical quenching of chlorophyll fluorescence (NPQ or qN). NPQ is a major mechanism protecting plants from photooxidative damage. Both qP and NPQ can minimize chlorophyll fluorescence, decrease chlorophyll fluorescence life time and excitation energy decay by the ^{3}Chl pathway. When reactive molecules are inevitably generated, despite the operation of NPQ and qP, carotenoids, plastid-localized tocopherols, ascorbate, glutathione, and other antioxidative enzymes can protect the photosynthetic apparatus from the various reactive oxygen species. Other protective mechanisms that prevent plants from photodamage include: alteration in the leaf surface to increase light reflection, decreases in transmittance of light to the chlorophyll containing cells, and leaf and chloroplast movements to decrease the light absorption. However, zeaxanthin-associated NPQ is both more ubiquitous and rapid compared to these mechanisms.

NPQ consists of three major components, qE, qT, and qI. qE is the principal and most rapidly inducible and reversible component of NPQ. qE is energy dependent, activated by lowered thylakoid lumen pH, and can rapidly relax within seconds to minutes. qT relies on state transitions of LHC II between PSII and PSI and is a slower component than qE. It relaxes in tens of minutes. A phosphokinase activated under high light phosphorylates the LHC IIb polypeptides, which uncouples LHC II from PS II and causes association of LHC II with PS I. Experimental evidence has suggested that qT is not essential for photoprotection in higher plants but is required for NPQ in algae (Niyogi, 1999). The slowest component of NPQ is qI, which is also called photoinhibition. qI is involved in longer term adaptation to light stress and relaxes within

a range of hours. qI could be a combination of photodamage and photoprotection and is the least characterized NPQ component.

It has been firmly established from combined molecular, genetic, and biochemical studies that qE requires a lower pH in the thylakoid lumen, the xanthophyll cycle carotenoid (zeaxanthin), and the PsbS protein (Müller et al., 2001). The mechanism of qE will be described in the following sections. Lutein has also been proposed to be involved in NPQ based on studies with the Arabidopsis *lut2* mutation, which affects the lycopene ε-cyclase gene and is unable to synthesize lutein (Pogson et al., 1996; Pogson et al., 1998). Detailed characterization of LHC size and stability established that lutein deficiency in the *lut2* mutant also affects the assembly and stability of LHC II. The conclusion from such studies is that although lutein is necessary for efficient NPQ, it is not directly involved in the mechanism of NPQ (Lokstein et al., 2002).

1.3.2.4 NPQ is measured by way of chlorophyll fluorescence

Excitation energy is consumed by three processes: photochemistry, chlorophyll fluorescence, and thermal dissipation. Photochemistry includes charge separation at the reaction center and electron transport via a series of carriers. Chlorophyll fluorescence is the emission of photons by radiative de-excitation of the excited chlorophyll molecules, while non-radiative thermal dissipation of excitation energy occurs via heat loss (NPQ). Either increased consumption of excited energy by the photochemical pathway or increased dissipation as heat leads to increased quenching of chlorophyll fluorescence. NPQ can therefore be determined by measuring chlorophyll fluorescence during a brief

pulse of light that saturates photochemistry. Chlorophyll fluorescence is measured using a pulse amplified modulation (PAM) fluorometer. After irradiation, the chlorophyll fluorescence changes over time, which is called Kautsky effect (Kautsky and Hirsch, 1934). The PAM-fluorescence technique allows one to obtain information on chlorophyll fluorescence induction kinetics *in vivo*.

Chlorophyll fluorescence within a sample is excited by strong light pulses from a blue light emitting detector (L.E.D.) with a short wavelength red emission peak of 650 nm. The longer wavelength components of the L.E.D. light are eliminated through a short pass filter (λ<670 nm). Electronic circuits pulse the L.E.D. illumination and synchronously amplify the resulting pulses and detected by a photo diode detector that has been protected by a long pass filter (λ>700 nm). The filtered signal is then recorded.

1.3.2.5 The mechanism of qE

qE is associated with changes in chlorophyll fluorescence lifetime distributions and a three-state model was proposed for PS II (Gilmore et al., 1995; Gilmore et al., 1998). When the thylakoid lumen is not acidified and PS II is not active, the lifetime of chlorophyll fluorescence is broad and designated as the W state. Upon illumination, a pH gradient forms across the thylakoid lumen and results in the conversion of the W state to a much shorter and narrower X state. Binding of xanthophyll (zeaxanthin or antheraxanthin) further converts PS II from the X state to an active quenching Y state (Gilmore et al., 1995; Gilmore et al., 1998).

The xanthophyll cycle

The so-called xanthophyll cycle involves reversible epoxidation/deepoxidation of zeaxanthin to antheraxanthin and violaxanthin under normal light conditions and deepoxidation of violaxanthin to antheraxanthin and zeaxanthin under high light stress. In order to address the involvement of xanthophyll cycle carotenoids in NPQ, mutants that affect xanthophyll metabolism and/or NPQ capacity have been isolated from Arabidopsis and characterized in detail. The Arabidopsis *npq1* mutation has a disrupted violaxanthin deepoxidase and when exposed to strong light, is unable to convert violaxanthin to antheraxanthin and zeaxanthin, even after prolonged high light treatment (Niyogi et al. 1998). NPQ capacity is also reduced in the *npq1* mutant relative to wild type plants after high light treatment. Characterization of the *npq1* mutant provided evidence that zeaxanthin is required for most of the qE component of NPQ (Niyogi et al., 1998). As mentioned previously, lutein is indirectly involved in NPQ and the introduction of the *lut2* mutation into the *npq1* background completely abolished qE (Niyogi et al., 2001). These studies further confirm that both zeaxanthin and lutein are necessary for maximal NPQ.

In several algal groups, such as the diatoms, the dinophytes, and the haptophytes, the xanthophyll cycle is replaced by conversion of diadinoxanthin and diatoxanthin, the diadinoxanthin cycle (Lohr and Wilhelm, 1999). Because only one of the ionone ring of diadinoxanthin carries an epoxy group, this cycle only contains one deepoxidation/epoxidation step. The coexistence of both the xanthophyll cycle and diadinoxanthin cycle was observed under high light stress conditions in the diatom

Phaeodactylum tricornutum (Lohr and Wilhelm, 1999). Recently, it has been shown that in the parasitic plant *Cuscuta reflexa*, neoxanthin is replaced by lutein-5,6-epoxide (Bungard et al., 1999). Under high light, lutein-5,6-epoxide is deepoxidized to lutein. This reaction is probably catalyzed by violaxanthin deepoxidase, which has been shown to use lutein-5,6-epoxide as a substrate. This interesting discovery also supports a role for lutein in photoprotection.

The role of the PsbS protein in NPQ

The *npq* mutants in Arabidopsis have been invaluable tools for studying NPQ. As described earlier, *npq1* mutation blocks violaxanthin to zeaxanthin conversion under high light and defines a key role of zeaxanthin in NPQ. Similarly, another *npq* mutant, *npq4*, has defined a key role for the PsbS protein. PsbS is a pigment binding protein in photosystem II, which when mutated in the *npq4* mutant resulted in a semidominant NPQ phenotype (Li et al., 2000). The *npq4* mutant showed compromised NPQ activity in response to excessive light, similar to the *npq1* mutant. However, *npq4* has a zeaxanthin level similar to wild type. The *npq4* mutant lacks the ΔpH and zeaxanthin dependent conformational change in the thylakoid membrane as measured by a light-induced spectral change at 535nm. Although NPQ was decreased in the *npq4* mutant, light harvesting and photosynthesis were not affected (Li et al., 2000). Global chlorophyll fluorescence lifetime distribution analysis on wild type and *npq4-1* plant indicated that the pH-dependent formation of the X state is not present in the *npq4-1* mutant where PsbS protein is absent (Li et al., 2002b). Furthermore, overexpression of the *PsbS* gene

led to a two-fold increase in qE indicating that PsbS is a limiting factor in qE capacity (Li et al., 2002c). The *PsbS* overexpressing lines showed a higher resistance to photoinhibition compared to wild type although the xanthophyll pool size was similar in *npq4* and wild type.

While the role of PsbS protein in qE is indispensable, it is still not clear how PsbS is involved in NPQ. It has been proposed that the PsbS protein binds H^+ and/or de-epoxidized xanthophylls and switches the LHC II to a high quenching state (Li et al., 2000). One effort to identify the amino acid binding sites for PsbS function is site-directed mutagenesis (Li et al., 2002a). Predicted PsbS protein sequences from nine different species of land plants were aligned. Eight putative H^+-binding amino acids are highly conserved in the plants and predicted to face the thylakoid lumen where H^+ accumulates upon high light stress. Each individual amino acid was mutagenized and the modified *PsbS* gene was transformed into a null *npq4* mutant. Mutations that affect E122 and E226 had the most dramatic phenotype and all qE was abolished in the E122E226 double mutant despite the presence of wild type levels of mutant PsbS proteins. These results indicate that the symmetrical pair E122 and E266 is essential for PsbS function and further support that H^+ binding to PsbS is critical for qE (Li et al., 2002a).

The possible involvement of other proteins in qE is still unclear. The PS II minor antenna proteins CP29 and CP26 have been suggested to be involved in qE due to their location between the inner antenna and the major LHC II (Bassi et al., 1997). The role of CP29 and CP26 in energy dissipation was investigated using antisense plants that depressed the expressions of each protein independently (Andersson et al., 2001). Both antisense lines were capable of highly efficient photosynthesis and were still able to

perform qE to a significant level, although there were changes in the rate of NPQ induction and amplitude. Thus, CP29 and CP26 are unlikely to be the sites for NPQ (Andersson et al., 2002).

Careful characterization of the chloroplast *ycf9* gene in both Chlamydomonas and tobacco indicated that it encodes a PS II core subunit, PsbZ, and it is involved in maintaining the stability of the PS II-LHC II supercomplex (Swiatek et al., 2001). PsbZ deficient tobacco plants exhibited greatly reduced capacity for NPQ under excessive light and/or decreased temperature. PsbS protein accumulation is unaltered in the PsbZ mutant. The total carotenoid pool is larger in the PsbZ mutant relative to wild type and showed a dramatically altered xanthophyll cycle that has a stronger increase in the deepoxidized state and is recalcitrant to re-epoxidation during dark relaxation (Swiatek et al., 2001). In addition, PsbZ is highly conserved through evolution; it is present even in organisms that lack a xanthophyll cycle and do not accumulate zeaxanthin, PsbS protein, or CP29 protein, but are still capable of NPQ for photoprotection (Campbell et al., 1998). This result suggests that PsbZ might be the key player for NPQ in phycobillisome containing organisms.

1.4 Approaches taken in this dissertation for characterization of carotenoid hydroxylases

As described in section 1.1.3, the ε-ring hydroxylase has been genetically defined by the *lut1* mutation in Arabidopsis but has not been cloned (Pogson et al., 1996). Attempts to isolate a gene that is related to β-hydroxylases but functions with ε-rings by

screening of Arabidopsis cDNA and genomic libraries and database searching using β-hydroxylase as a query were not successful (Tian and DellaPenna, 2001). Though a second β-hydroxylase gene was identified and characterized in these studies, its activity was similar to β-hydroxylase 1 in that it effectively hydroxylated β-rings but was poorly active toward ε-ring. Furthermore, neither of the β-hydroxylase genes mapped to the *lut1* interval. Map-based cloning was therefore adopted to isolate the *LUT1* gene during the course of this dissertation research (Chapter 3). The *in vivo* functions of the β- and ε-hydroxylases and possible interactions and overlapping functions were studied by isolating T-DNA knockout mutants of the two Arabidopsis β-hydroxylase genes (*b1* and *b2*), generating and characterizing various mutant combination lines defective in *b1*, *b2*, and *lut1*. The effects of the resulting carotenoid composition modifications on NPQ induction kinetics were measured by chlorophyll fluorescence in the different carotenoid hydroxylase mutants (Chapter 4).

1.4.1 Map-based cloning of the *lut1* mutation

When no additional information is available, the gene disrupted by the mutation can be cloned by physically mapping the mutant locus to a specific chromosomal location and using functional complementation to demonstrate the gene identified encodes the targeted protein. In order to map a novel mutation to a well-defined chromosomal region, linkage analysis is carried out between the mutation and the previously mapped markers. The markers being used include traditional morphological markers that give a visible phenotype and molecular markers, such as Cleaved Amplified Polymorphic Sequences

(CAPS) and Simple Sequence Length Polymorphisms (SSLP) markers. It has been estimated that Col and Ler differ by 0.5% to 1% at the DNA level (Hauser et al. 1998), due to point mutations, insertions, or deletions that randomly occurred in one ecotype but not in the other. Arabidopsis contains tandem repeats of one-, two-, or three- nucleotide motifs. These repeat sequences are usually polymorphic in different ecotypes because of variations in the number of repeats introduced by DNA polymerase. All these natural differences between ecotypes can be conveniently utilized to generate molecular markers. CAPS is a method using PCR to detect the restriction site polymorphorism between two ecotypes. The PCR amplified fragments are digested with specific restriction enzymes and the polymorphism is then revealed by agarose gel electrophoresis. SSLP method takes advantage of the variation in the numbers of repeat units in different ecotypes and the polymorphic fragments are directly detected by PCR followed by agarose gel electrophoresis.

To map a mutation that was generated in one ecotype, the mutant is crossed to a plant of another ecotype that is wild type for that locus and the F_1 progeny is allowed to self-pollinate. The segregating F_2 generation is then used to analyze for linkage between the mutation and the molecular markers for the two different ecotypes. The recombination frequency between a mutation and a molecular marker is the number of the chromosomes scored as mutant ecotype divided by the total number of chromosomes analyzed. However when converting the recombination frequency to map distance, two factors need to be considered for calculation. One is that when two independent recombination events occurred between two markers, no recombination event is actually scored. The other one is that a recombination event can influence the probability of a

second recombination event occurring in its vicinity, which is called interference. In Arabidopsis, a map distance is estimated by the formula (Kosambi function) D=25xln[(100+2r)/(100-2r)], where r is the recombination rate expressed in its percentage form and D is the map distance in centiMorgans (cM) (Koornneef and Stam, 1992).

The *lut1* mutation that genetically defines the ε-hydroxylase in Arabidopsis is an EMS mutation in the Columbia ecotype background (Pogson et al., 1996). A homozygous *lut1* mutant was crossed with a wild type (*LUT1*) *Lansberg erecta* plant. F_1 progeny were self-pollinated and the homozygous *lut1* plants in the F_2 population were used to analyze for linkage between *lut1* and various molecular markers (CAPS and SSLP). The *LUT1* locus was initially mapped at 67 cM ± 3 cM on chromosome 3 (Tian and DellaPenna, 2001). Fine mapping of the *LUT1* locus is subsequently carried out to clone the *LUT1* gene and is discussed in detail in Chapter 3.

1.4.2 T-DNA insertional mutants as a tool for *in vivo* function study

During the course of this dissertation, the genome of Arabidopsis was fully sequenced (The Arabidopsis Genome Initiative, 2000) and led to the development of many important tools to study gene function. Once the genome sequence is known, large scale T-DNA insertional mutagenesis becomes a convenient tool for "reverse genetics". T-DNA insertion often causes a null mutation and offers an efficient way to study the function of gene products *in vivo*. T-DNA insertion allows targeting of individual genes in a gene family and the stable maintenance of the mutant genotype and phenotype

because the mutant can be kept in the heterologous state if the homozygous plant is lethal. With the availability of large population of T-DNA insertional mutants and the effectiveness of the PCR-based knockout screening approaches and DNA sequencing, T-DNA insertional mutagenesis has been a preferred approach for gene silencing over antisense.

Briefly, the screening process for T-DNA insertional mutants starts with vacuum infiltration of the wild type Arabidopsis plants with Agrobacterium harboring a binary T-DNA vector. Transformed plants are selected by antibiotic resistance. Transformed T_1 plants are grouped and T_2 seeds are collected from grouped T_1 plants. The samples are further consolidated to even smaller number of pools, which is more convenient for management and screening. Genomic DNA is extracted from pooled plants. DNA is further pooled together to form a limited number of superpools that are convenient for PCR reactions. DNA from superpools are used as templates and screened with one T-DNA border primer and one gene specific primer. The T-DNA knockout mutants are verified with gene specific primers. Once a positive hit is identified, it can be sequenced with the T-DNA border primer to identify the insertion site. The same pair of primers is used to screen the DNA sub-pools that constitute the superpool until an individual mutant is identified. The homozygous mutant is isolated and the genotype and phenotype confirmed with various analytical methods. Several T-DNA populations have been made available to public through the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/abrc/tdna_lines.html). With the advance of high-throughput sequencing, different groups have also sequenced large numbers of T-DNA insertions from the available T-DNA knockout mutant populations. Researchers can simply search

the T-DNA mutant population with a DNA sequence of interest to identify potential T-DNA mutant. One such site used in this research is the SIGAL website at the Salk Institute (http://signal.salk.edu/cgi-bin/tdnaexpress).

Two β-hydroxylase genes have been cloned from Arabidopsis and represent the only identifiable β-hydroxylases in the genome (Sun et al., 1996; Tian and DellaPenna, 2001). During the course of this research, a single antisense β-hydroxylase transgene was employed to attempt to study the function of both β-hydroxylase 1 and 2 *in vivo* (Rissler and Pogson, 2001). The two β-hydroxylases are 79% identical at the nucleic acid level and a concern with this approach is that a single antisense gene might not be able to effectively eliminate expression of both genes. As discussed in Chapter 4, this appears to be the case because the T-DNA knockout mutants differ significantly in phenotype from the β-hydroxylase 1 antisense plants. T-DNA knockout mutants that correspond to disruptions of the respective β-hydroxylases were isolated during this dissertation study and crossed to each other and to the *lut1* mutant. The effects of multiple hydroxylase deficiency on carotenoid composition and possible interactions and overlapping functions of the hydroxylases will be discussed in Chapter 4.

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CHAPTER 2

Characterization of a second carotenoid β-hydroxylase gene from Arabidopsis and its relationship to the *LUT1* locus

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Abstract

Xanthophylls are oxygenated carotenoids that perform critical roles in plants. β-carotene hydroxylases (β-hydroxylases) add hydroxyl groups to the β-rings of carotenes and have been cloned from several bacteria and plants, including Arabidopsis. The *lut1* mutation of Arabidopsis disrupts ε-ring hydroxylation and has been suggested to identify a related carotene hydroxylase that functions specifically on ε-ring structures. We have used library screening and genomics-based approaches to isolate a second β-hydroxylase genomic clone and its corresponding cDNA from Arabidopsis. The encoded protein is 70% identical to the previously reported Arabidopsis β-hydroxylase 1. Phylogenetic analysis indicates a common origin for the two proteins, however, their different chromosomal locations, intron positions and intron sizes suggest their duplication is not recent. Although both hydroxylases are expressed in all Arabidopsis tissues analyzed, β-hydroxylase 1 mRNA is always present at higher levels. Both cDNAs encode proteins that efficiently hydroxylate the C-3 position of β-ring containing carotenes and are only weakly active towards ε-ring containing carotenes. Neither β-hydroxylase cDNA maps to the *LUT1* locus, and the genomic region encompassing the *LUT1* locus does not contain a third related hydroxylase. These data indicate that the *LUT1* locus encodes a protein necessary for ε-ring hydroxylation but unrelated to β-hydroxylases at the level of amino acid sequence.

Introduction

Carotenoids are isoprenoid-derived pigments that are synthesized by bacteria, fungi and plants. In plants, carotenoids are synthesized and localized in plastids. In chloroplasts, carotenoids are essential components of the photosynthetic antenna and reaction center complexes and serve as precursors for abscisic acid biosynthesis (Rock and Zeevaart, 1991). In chromoplasts of nonphotosynthetic tissues, carotenoids can accumulate to high levels where they provide color to flowers and fruits and act as attractants for pollinators (Goodwin, 1986). In addition to these roles in plants, the carotenoid pathway is a target for herbicides (Bramley, 1994) and certain carotenoids play a role in human nutrition as provitamin A (Solomons and Bulux, 1993).

Carotenoids can be divided into two major groups: carotenes and xanthophylls. Carotenes are linear or cyclized hydrocarbons such as lycopene, α-carotene and β-carotene. Xanthophylls are oxygenated derivatives of carotenes and can contain epoxy, keto or hydroxy groups. Lutein (β,ε-carotene-3,3'-diol), a dihydroxy α-carotene derivative, is the most abundant carotenoid in photosynthetic plant tissue while the analogous dihydroxy β-carotene derivative, zeaxanthin, is an important component of the xanthophyll cycle (Demmig-Adams *et al.*, 1996).

Carotenoid hydroxylases catalyze the formation of lutein and zeaxanthin from α-carotene and β-carotene, respectively. β-hydroxylases add hydroxyl groups to the number 3 carbon of both β-rings and have been cloned from several photosynthetic eukaryotes, including Arabidopsis, tomato and algae (reviewed in Cunningham and Gantt, 1998). Biochemical studies of pepper fruit β-hydroxylase show that the enzyme is a nonheme diiron protein containing conserved histidine motifs (Bouvier *et al.*, 1998) also found in

the membrane fatty acid desaturase family (Shanklin *et al.*, 1994). *In vitro* reconstitution experiments indicate that pepper β-hydroxylase requires iron, ferredoxin and ferredoxin oxido-reductase for activity and uses activated oxygen to break the C-H bond and introduce a hydroxyl group (Bouvier *et al.*, 1998).

β-hydroxylases have also been cloned from various photosynthetic and non-photosynthetic bacteria (Masamoto *et al.*, 1998; Misawa *et al.*, 1990; Hundle *et al.*, 1993). Although all β-hydroxylases carry out the same reaction, they can be clearly categorized into three groups based on their primary structure. Eukaryotic β-hydroxylases are highly conserved and share less than 30% protein identity with β-hydroxylases from non-photosynthetic bacteria. The β-hydroxylase of *Synechocystis* PCC6803 (a cyanobacteria) shares little similarity to plant and non-photosynthetic bacterial β-hydroxylases with the exception of conserved histidine residues (Masamoto *et al.*, 1998). *Synechocystis* β-hydroxylase is more closely related to bacterial β-carotene ketolases (β-ketolase), suggesting that it may have evolved independently of the plant and bacterial hydroxylases.

One feature distinguishing plants and green algae from other carotenoid containing organisms is the synthesis of ε-ring containing carotenoids, for example, α-carotene (β,ε-carotene). ε-Rings differ from β-rings in the position of the double bond on the ring structure (Figure 2.2). α-Carotene is converted to lutein by hydroxylation at carbon 3 on both the β and ε rings. Cloned Arabidopsis β-hydroxylase efficiently hydroxylates carbon 3 of β-rings but showed almost no activity towards carbon 3 of ε-rings (Sun *et al.*, 1996). In addition, the chirality of ε ring hydroxylation is opposite to that of β-ring hydroxylation (Britton, G., 1990), suggesting that a distinct activity is

involved. Genetic data further support the existence of a second hydroxylase as the *lut1* mutation of Arabidopsis disrupts ε-ring hydroxylation without affecting β-hydroxylation (Pogson *et al.*, 1996), suggesting that the *LUT1* locus encodes a protein necessary for ε-hydroxylase activity.

We propose three hypotheses for the identity of the Arabidopsis ε-hydroxylase/*LUT1* locus: 1) it is a hydroxylase evolved from and related to the β-hydroxylase, 2) it is a hydroxylase evolved independently and therefore divergent from the β-hydroxylase, or 3) it is an ancillary protein that modifies the existing β-hydroxylase to function towards ε-rings. To test these hypotheses, we attempted to clone the Arabidopsis ε-hydroxylase by molecular, genomic and genetic approaches. In this paper, we report the isolation and characterization of a second, related β-hydroxylase from Arabidopsis and its relationship to the *LUT1* locus.

Materials and methods

Screening of *Arabidopsis thaliana* genomic and cDNA libraries

An Arabidopsis CD4-8 Landsberg genomic library and CD4-13, CD4-14 size selected cDNA libraries were obtained from the Arabidopsis Biological Resource Center (Columbus, Ohio). Approximately 200,000 plaques from the genomic library were screened using the Arabidopsis β-hydroxylase 1 cDNA as a probe (Sun *et al.*, 1996). Prehybridization was performed in 6X SSPE, 0.5% nonfat dry milk, 0.5% SDS at 60°C for four hr and hybridization with α-^{32}P labeled β-hydroxylase 1 insert (500,000 cpm/ml

hybridization solution) at the same temperature overnight. Filters were washed twice at 55°C in 1X SSC, 0.1% SDS (low stringency wash) for 30 min and exposed to X-ray film for 16 hr. Following film development, the membranes were washed twice in 0.1X SSC, 0.1% SDS at 65°C (high stringency wash) for 30 min and again exposed to X-ray film for 16 hr. Plaques showing strong signals at low stringency and partial loss of signal at high stringency were picked and purified. Plaques showing equal signals at both low and high stringency were used as positive controls. Phage DNA was purified according to standard methods (Sambrook *et al.*, 1988). The cDNA library was screened in a similar manner using the β-hydroxylase 1 insert as a probe and putative clones were isolated and sequenced.

DNA sequencing and analysis

DNA sequencing was performed using Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit and an ABI 310 automated DNA sequencer (Perkin Elmer Applied Biosystems, Foster City, CA). cDNA and genomic clones were fully sequenced by primer walking. DNA sequence analyses were carried out with MacVector™ 7.0 software (Eastman Kodak Company, Rochester, NY).

RNA isolation

One-month-old Arabidopsis leaf, stem, root, flower and silique tissues were harvested, frozen in liquid nitrogen and ground to a fine powder. Three ml of homogenization buffer/gm tissue (0.2 M sodium borate, 30 mM EGTA, 1% SDS, 1%

deoxycholate, 2% polyvinylpyrrolidone 40,000, 10 mM DTT) was preheated to 65°C and one volume 1:1 (v:v) phenol:chloroform was added. The mixture was vortexed for several min and the phases were separated by centrifuging at 10,000g for 20 min at room temperature. The aqueous phase was extracted twice with one volume of chloroform, adjusted to 2 M LiCl and incubated at 4°C overnight. The resulting precipitate was collected by centrifuging at 10,000g for 20 min at room temperature. The pellet was washed once with 2 M LiCl and resuspended in 1ml TE, 0.5%SDS and reprecipitated by adding 0.1 volume 2 M potassium acetate (pH 5.5) and two volumes of ethanol at room temperature for 10 min. The precipitate was pelleted at 10,000g for 20 min at 4°C then resuspended in 1 ml DEPC treated water. RNA was quantified spectrophotometrically and stored at –80°C. Polysaccharides from total RNA preparation were removed as described (Ausubel *et al.*, 1995).

cDNA synthesis and TaqMan PCR assay

Reverse transcription reactions were conducted in triplicate using the TaqMan Gold RT-PCR kit (Perkin Elmer Applied Biosystems, Foster City, CA) according to the manufacture's protocol. Ten μg of total RNA was used as template in each reverse transcription reaction. cDNA products from triplicate reactions were pooled together for TaqMan analysis.

TaqMan probes and primers (shown in Table 2.1) were designed from the cDNA sequences of β-hydroxylase 1, β-hydroxylase 2 and a housekeeping gene, Elongation Factor 1α (EF-1α), using Primer Express software (Perkin Elmer Applied Biosystems,

Table 2.1. TaqMan probes and primers used in this study.

β-hydroxylase 1	Forward	CTCGTGCACAAGCGTTTCC
	Reverse	GGCGACCTTTCGGAGGTAA
	Probe	TGTAGGTCCCATCGCCGACGTCC
β-hydroxylase 2	Forward	TTCTCCGCAAACCACCCTATA
	Reverse	ACGTCGGAAGCCGTTGAAT
	Probe	CCACCGCAGTTTTCCCTCCATCTCT
EF-1α	Forward	CGAACTTCCATAGAGCAATATCGA
	Reverse	GCATGGGTGTTGGACAAACTT
	Probe	ACCACGGTCACGCTCGGCCT

Foster City, CA). Reporter dye and quencher dye for the TaqMan probes were FAM (6-carboxyfluorescein) and TAMRA (6-carboxytetramethylrhodamine), respectively. TaqMan probe and primer concentrations were optimized by determining the minimum probe and primer concentrations that gave the highest amplification for each probe target. The optimal TaqMan probe, forward primer and reverse primer concentrations were: EF-1α, 175 nM, 300 nM, 300 nM; β-hydroxylase 1, 225 nM, 50 nM, 50 nM; β-hydroxylase 2, 225 nM, 300 nM, 300 nM. TaqMan PCR assays for each gene target were performed in triplicate on cDNA samples from reverse transcription reactions or plasmids standards containing genes of interest using an ABI Prism 7700 Sequence Detection system (Perkin Elmer Applied Biosystems, Foster City, CA). For each 30 µl TaqMan reaction, 2 µl cDNA template (out of a 100 µl reverse-transcription reaction containing 10 µg total RNA) was mixed with 15 µl TaqMan Universal PCR Master Mix (2X), 3 µl forward primer, 3 µl reverse primer, 3 µl TaqMan probe and 4 µl H_2O. PCR parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min.

Data analysis of TaqMan PCR assay

TaqMan PCR results were captured and analyzed using Sequence Detector Software (SDS version 1.7; Perkin Elmer Applied Biosystems, Foster City, CA). After analyzing the raw data, an amplification plot for each sample was generated, showing the increase in the reporter dye fluorescence (ΔRn) with each cycle. In the initial cycles of PCR, there is little change in fluorescence signal, which defines the baseline above which a fixed fluorescence threshold is set. The threshold cycle (C_T), representing the cycle

number at which the fluorescence passed the fixed threshold, was then calculated for each amplification plot. C_T values of all the samples were exported into Microsoft Excel worksheets for further analysis.

Standard curves were generated using serial dilutions of known molar quantities of β-hydroxylase 1 or β-hydroxylase 2 cDNA. Quantification of β-hydroxylase 1 and β-hydroxylase 2 transcripts in each tissue were accomplished by comparing the C_T values to their standard curves, respectively. The molar amount of both transcripts (per 0.2 μg total RNA) from the same tissue could then be directly compared (Figure 2.4). The house-keeping gene EF-1α has its lowest expression (largest C_T value) in floral tissue. Relative EF-1α message in different tissues was calculated by arbitrarily setting floral EF-1α equal to 1.

Computer analyses of β-hydroxylase sequences

Deduced amino acid sequences of β-hydroxylases from plants and bacteria were aligned using the ClustalW (version 1.4) algorithm (Thompson *et al.*, 1994). This alignment was used to construct a neighbor-joining tree with the *Synechocystis* PCC6803 β-hydroxylase as the outgroup (MacVector™ software, version 7.0; East Kodak Company, Rochester, NY). Residues prior to the start codon of *Agrobacterium aurantiacum* were not used for calculation of distance, i.e. chloroplast targeting sequences of plant β-hydroxylases were not included for distance calculation. Positions with gaps were also excluded from the calculation. Distances were corrected for multiple substitutions (Kimura, 1980). GenBank accession numbers of the β-hydroxylases are

given in the legend to Figure 2.5. Bootstrap analysis was performed to test the reliability of the branches (10,000 replicates).

HPLC analysis of enzymatic activities in carotenoid-producing *E.coli*

β-Carotene, ε,ε-carotene and δ-carotene producing *E. coli* strains were generously provided by Dr. Cunningham (University of Maryland, College Park, Maryland; Cunningham *et al.*, 1994; Cunningham *et al.*, 1996). Plasmids expressing the open reading frames of the β-hydroxylase 1 and 2 cDNAs were transformed into a β-carotene producing *E. coli* strain (Sun *et al.* 1996). Fifty ml of *E. coli* culture was grown in LB media at 37°C to an O.D. $_{550}$ of 0.8, at which time the expression of fusion protein was induced with 0.4 mM IPTG at 28°C for an additional twelve hr. The cells were collected by centrifugation and resuspended in 400 μl H_2O to which 750 μl 2:1 methanol: chloroform (v:v) was added. After centrifugation, the chloroform phase containing carotenoid pigments was recovered, transferred to a fresh eppendorf tube and dried under vacuum. The carotenoid containing residue was resuspended in ethyl acetate, clarified by centrifugation and resolved by chromatography on a C_{18} reverse phase HPLC column (Waters, Milford, MA). Mobile phases were A: acetonitrile: H_2O: triethylamine (900:99:1, v:v:v) and B: ethyl acetate. The column was equilibrated at 1 ml/min flow rate with buffer A and the following gradient initiated after sample injection: 0 to 31.0 min: 0% B to 66.6% B; 31.0 to 31.2 min: 66.6% B to 100% B. 100% B buffer was maintained for an additional two min at which time the column was reequilibrated with 100% buffer A. Fifty μl samples were injected and data was collected at 440 nm, 477 nm and 296 nm.

Mapping of the *lut1* mutation

Homozygous *lut1* mutants (Columbia) were crossed with wild type *Landsberg erecta* and F_2 progeny homozygous for the *lut1* mutation were selected by HPLC analysis of leaf carotenoids as described (Pogson *et al.*, 1996). Genomic DNA was isolated from individual F_2 plants (Dellaporta, 1983) and further purified by phenol:chloroform extraction and precipitation prior to PCR amplifications. The *lut1* mutation was mapped relative to CAPS (Cleaved Amplified Polymorphic Sequences) markers (Konieczny and Ausubel, 1993). CAPS PCR reactions were performed in 50 µl volumes containing PCR buffer, 3 mM $MgCl_2$, 0.125 mM dNTP mix, 2.5 U of Taq DNA polymerase, 30 pmol of each primer and 50-100 ng genomic DNA template. Following preincubation at 94°C for 3 min, a PCR program (annealing at 55°C for 15 s, extension at 72°C for 30 s, denaturation at 94°C for 15 s) was performed for 40 cycles.

Results

Isolation of cDNAs encoding a novel Arabidopsis β-carotene hydroxylase

In plants, β-hydroxylase adds hydroxyl groups to the 3 and 3' ring carbons of β-carotene to form zeaxanthin. A cDNA encoding this enzyme has been previously cloned from Arabidopsis by color complementation of a β-carotene containing *E.coli* strain (Sun *et al.* 1996). This cDNA, β-hydroxylase 1, was used as a probe to screen an Arabidopsis cDNA library for related clones. Of the 48 clones identified, most were truncated

versions of the β-hydroxylase 1 cDNA except for two, which appeared to encode a second related hydroxylase in Arabidopsis. The newly identified clone was designated β-hydroxylase 2.

Figure 2.1 shows a comparison of the deduced protein sequences of β-hydroxylase 1 and 2. The proteins share 70% identity at the primary sequence level. The chloroplast transit peptide prediction software ChloroP v1.1 (Center for Biological Sequence Analysis, The Technical University of Denmark, Denmark) predicts a chloroplast transit peptide cleavage site between Val-52 and Glu-53 in both β-hydroxylase sequences, consistent with the presumed chloroplastic location of carotenoid biosynthetic enzymes (Cunningham and Gantt, 1998). The putative transit peptides show the highest divergence and their removal increases the primary sequence identity of the two hydroxylases to 81%.

Activity and substrate specificity of the β-hydroxylase 2 protein

The β-hydroxylase 1 cDNA was previously shown to be highly active toward β rings and weakly active toward ε rings in *E.coli* strains engineered to produce carotenoids containing these ring structures (Sun *et al.*, 1996). The β-hydroxylase 2 cDNA was similarly introduced into *E.coli* strains engineered to accumulate β or ε ring containing carotenes and the activity towards each ring structure was determined by HPLC.

When expressed in a β-carotene accumulating background, the β-hydroxylase 2 cDNA produced 90% zeaxanthin (β,β-carotene-3,3'-diol), and 6% of the monohydroxylated intermediate, β-cryptoxanthin (β,β-carotene-3-ol) (Figure 2.2A). The

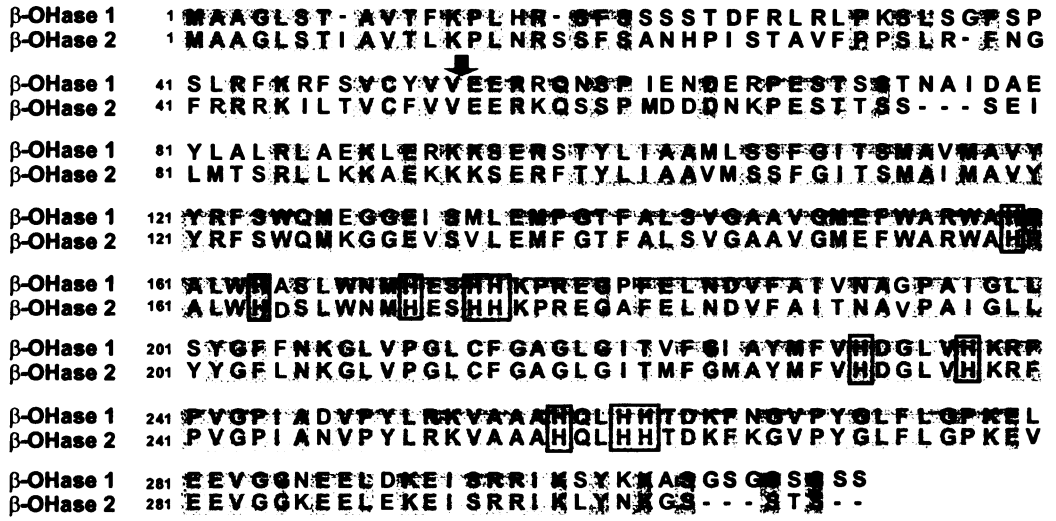


Figure 2.1. Alignment of proteins encoded by the Arabidopsis β-hydroxylase 1 and 2 cDNAs. Residues identical between β-hydroxylase 1 and 2 (β-OHase 1 and β-OHase 2, respectively) are shaded. Conserved histidine residues are boxed. The black arrow indicates the predicted cleave site of the transit peptide in both sequences.

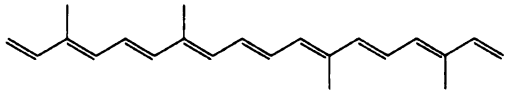
β-hydroxylase 1 cDNA gave a similar conversion in this background (data not shown). When the β-hydroxylase 2 cDNA was expressed in a δ-carotene accumulating *E.coli* background (δ-carotene, ε,ψ-carotene, which has a single ε-ring structure), only a small amount (<6%) was converted to the hydroxylated product (ε,ψ-carotene-3-ol) (Figure 2.2B), again similar to what is observed with the β-hydroxylase1 cDNA (data not shown). Another ε-ring containing carotenoid (ε,ε-carotene which has ε-rings at both ends of the polyene chain) was also tested and similarly low levels of ε-ring hydroxylation were observed with both β-hydroxylase cDNAs (data not shown).

Structural comparisons of β-hydroxylase 1 and β-hydroxylase 2 genes

The β-hydroxylase 1 cDNA was also used to screen an Arabidopsis genomic library and clones encoding both the β-hydroxylase 1 and β-hydroxylase 2 genes were identified and fully sequenced. Their structures are compared in Figure 2.3A. Both genomic clones contain six introns of differing size and sequence that are conserved in position in the two genes. Subsequent to sequencing by our laboratory, BAC clones containing both β-hydroxylase genes were sequenced by the Arabidopsis Genome Initiative Project, hence their chromosomal locations are known (Figure 2.3B). The β-hydroxylase 1 gene is located on chromosome 4 at 75 cM (GenBank accession number AF125577), while the β-hydroxylase 2 gene is located on chromosome 5 at 105 cM (GenBank accession number AB025606).

Expression of β-hydroxylase 1 and 2 genes in different Arabidopsis tissues

Figure 2.2. HPLC elution profiles of β-hydroxylase 1 and 2 cDNAs expressed in β or ε ring-producing *E. coli*. A. HPLC elution profiles of carotenoids in the β-carotene-accumulating *E. coli* strain (gray trace) and after transformation with the β-hydroxylase 2 expression plasmid (black trace). B. HPLC elution profiles of carotenoids in the δ-carotene-accumulating *E. coli* strain (gray trace) and after transformation with the β-hydroxylase 2 expression plasmid (black trace). The chemical structures of the compounds of each peak are shown below their names.

R: 

C. The chemical structures of β and ε-rings.

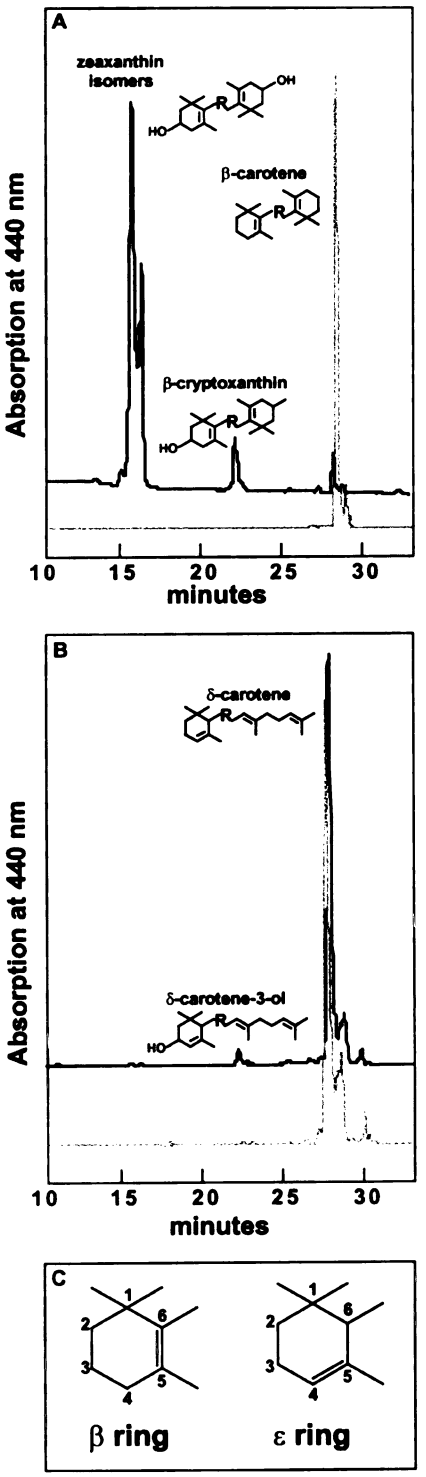
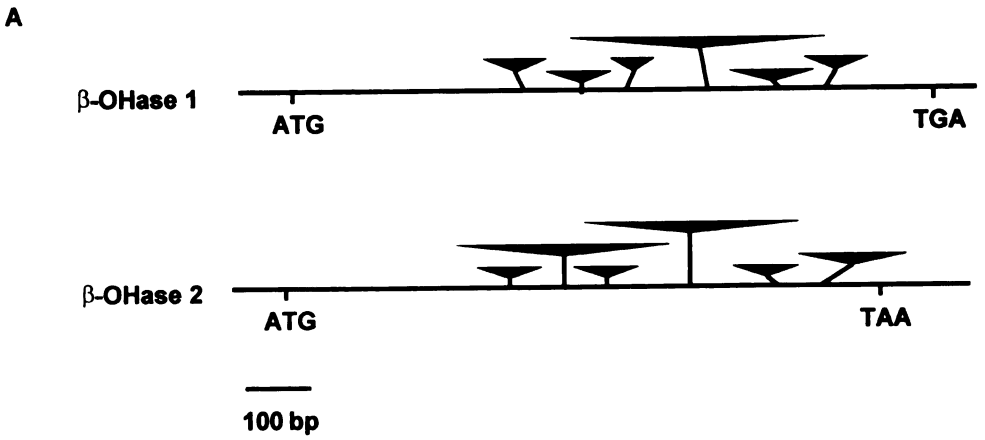

A
zeaxanthin isomers
β-carotene
β-cryptoxanthin
Absorption at 440 nm
10 15 20 25 30
minutes
B
δ-carotene
δ-carotene-3-ol
Absorption at 440 nm
10 15 20 25 30
minutes
C
β ring
ε ring

Figure 2.2

Figure 2.3. A. Schematic diagrams of β-hydroxylase 1 and 2 genomic clones. Exons and introns are drawn to scale with their positions and sizes indicated by straight lines and triangles extracted from the exon sequences, respectively. B. Map positions of β-hydroxylase 1, β-hydroxylase 2 and *LUT1* locus. β-hydroxylase 1 and 2 map positions were obtained from the *Arabidopsis thaliana* Database. The map position of *lut1* was determined by linkage to CAPS markers. The diagram is not to scale.


A
β-OHase 1
ATG
TGA
β-OHase 2
ATG
TAA
100 bp

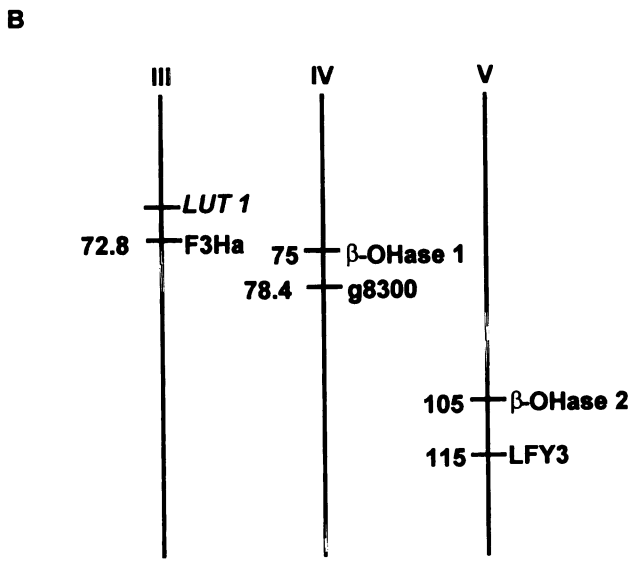

B
III
IV
V
LUT 1
72.8
F3Ha
75
β-OHase 1
78.4
g8300
105
β-OHase 2
115
LFY3

Figure 2.3

Initial attempts to determine β-hydroxylases transcripts level in various Arabidopsis tissues by Northern blotting were not successful due to the fact that both genes are expressed at extremely low levels. We therefore adopted the more sensitive TaqMan RT-PCR method for expression analyses (Heid *et al.*, 1996). Quantification was performed using a standard curve of each target sequence.

Both hydroxylase mRNAs are most abundant in Arabidopsis leaf tissue with β-hydroxylase 1 mRNA approximately twenty fold higher than β-hydroxylase 2 mRNA (Figure 2.4). In the other tissues examined, β-hydroxylase 1 mRNA was always more abundant, ranging from seventeen to seventy fold higher than β-hydroxylase 2 mRNA. EF-1α transcript levels varied less than 50% between all tissues with the exception in roots, where EF-1α mRNA levels were 3.4 fold higher than in flowers.

Mapping of the *LUT1* locus relative to the β-hydroxylase 1 and 2 genes

The *lut1* mutation defines a locus whose disruption reduces ε ring hydroxylation more than 90% relative to wild type (Pogson *et al.*, 1996). To determine if either β-hydroxylase gene showed linkage to the *LUT1* locus, linkage analyses were performed using CAPS markers (Konieczny and Ausubel, 1993). The location of both β-hydroxylase genes is known from the Arabidopsis genome sequencing program and the CAPS markers nearest to each (g8300 and LFY3 for β-hydroxylase 1 and 2, respectively) were used to test for linkage in a F_2 population segregating for the *lut1* mutation. Recombination rates between the *LUT1* locus and both β-hydroxylase linked CAPS markers exceeded 50% indicating that neither β-hydroxylase is linked to the *lut1*

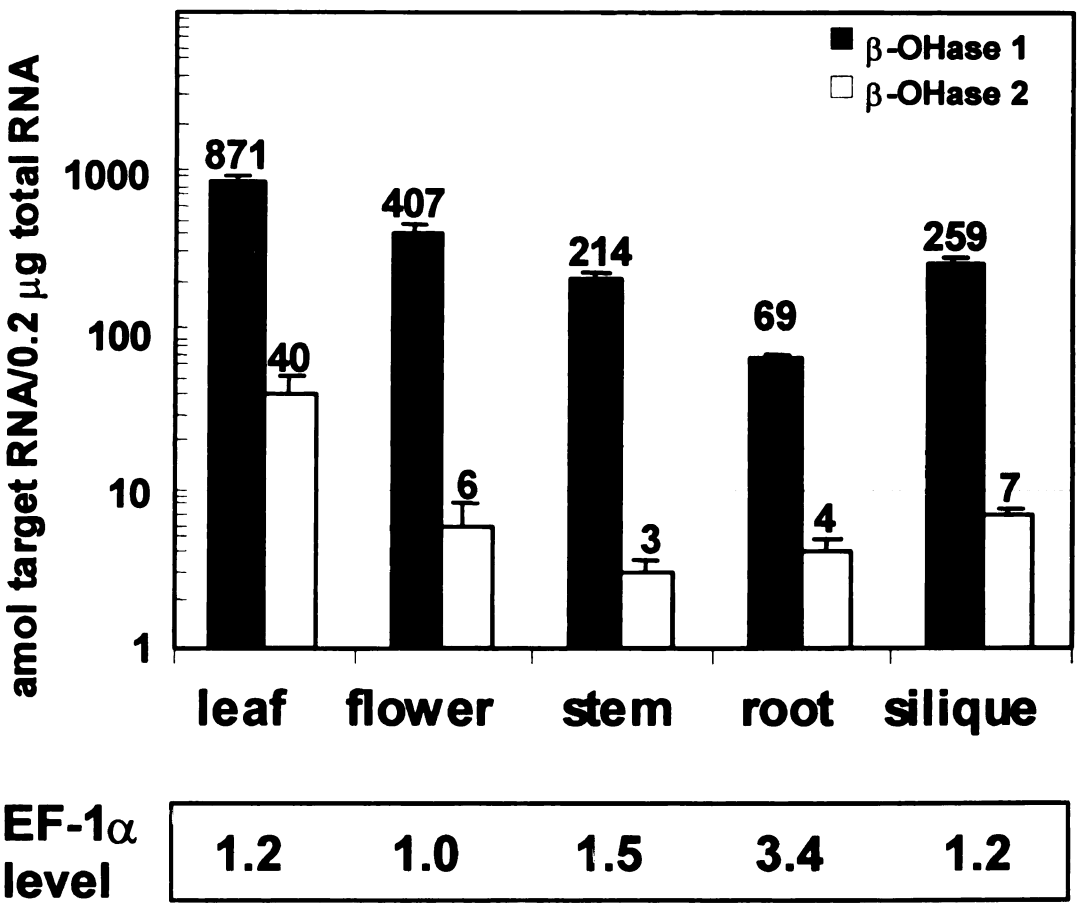


Figure 2.4. β-hydroxylase 1 and 2 mRNA levels in Arabidopsis leaf, flower, stem, root and silique tissues. The amol amount of target RNAs per 0.2 μg total RNA for each gene in different tissues are shown on top of the bar graphs. Relative EF-1α expression levels are indicated below the corresponding tissues.

mutation. Mapping of the *lut1* mutation was carried out with the same F_2 population using twelve pairs of CAPS markers distributed over the five Arabidopsis chromosomes. The *LUT1* locus maps at 67 cM +/- 3 cM on chromosome 3.

Discussion

In plants, carotene hydroxylases are key enzymes responsible for the formation of xanthophyll cycle carotenoids and lutein, the most abundant carotenoid in higher plant photosystems. Carotene hydroxylases add a hydroxyl group to the 3 position of β- and ε-rings. β-ring hydroxylases have been cloned and analyzed from several organisms and shown to be highly active against β-rings and only weakly active against ε-rings. All carotene hydroxylases are nonheme diiron proteins, contain the conserved histidine residues indicated in Figure 2.1 and, at least for plants, have been shown to require ferredoxin oxidoreductase for their activity (Bourier *et al.* 1998).

Despite the fact that hydroxylated ε-ring carotenoids (e.g. lutein) are the most abundant xanthophylls in plants, a hydroxylase enzyme specific for ε-rings has not yet been cloned. It has been proposed from considerations of reaction stereochemistry that there exists a hydroxylase enzyme that acts specifically on ε-rings (Britton, 1990). In Arabidopsis, this activity has been genetically identified by the *lut1* mutation, which disrupts ε-ring hydroxylation and causes accumulation of the monohydroxy pathway intermediate, zeinoxanthin (β,ε-carotene, with only the β-ring hydroxylated), at the expense of the normal dihydroxy product, lutein (Pogson *et al.*, 1996).

We propose three hypotheses for the nature of the ε-ring hydroxylase activity in plants. Given the similarity of the β and ε ring substrates and hydroxylation enzyme reaction mechanisms, one possibility would be that the Arabidopsis ε-hydroxylase is an enzyme related to and evolved from the β-hydroxylase. By analogy, Arabidopsis β and ε cyclases both function on lycopene, form similar ring structures (β and ε rings) and show 58% similarity at the amino acid level. An alternative hypothesis is that though the ε-hydroxylase has a function similar to the β-hydroxylase, it evolved independently and therefore would show little homology with the β-hydroxylase. This has precedent in that β-hydroxylases from bacteria and plants have similar activities but little sequence homology. A final possibility is that the "ε-ring hydroxylase" defined by the *lut1* mutation does not have hydroxylation activity *per se* but is an ancillary protein that modifies the endogenous β-hydroxylase enzyme activity to efficiently function on ε-rings.

In an attempt to test the first hypothesis and isolate an ε-hydroxylase cDNA related to the β-hydroxylase enzyme, we screened cDNA and genomic libraries from Arabidopsis using the Arabidopsis β-hydroxylase cDNA as probe. A second, novel hydroxylase cDNA class with 70% protein identity to the Arabidopsis β-hydroxylase 1 cDNA was isolated. Although phylogenetic analysis (Figure 2.5) and protein sequence alignment (Figure 2.1) indicate that the two hydroxylases were derived from a common ancestor, comparison of their gene structures and sequences indicate they have been diverging for some time. Like all known carotene-hydroxylases, the new hydroxylase has ten conserved histidine residues localized in the hydrophilic domains of the protein,

Figure 2.5. Neighbor-joining tree for deduced amino acid sequences of β-hydroxylase genes from plants and bacteria. β-hydroxylase sequence from *Synechocystis* PCC6803 was used as the outgroup. Branch lengths are drawn to scale. Bootstrap values are indicated (10,000 replicates). GenBank accession numbers of the sequences are as follows.

A.a.: *Agrobacterium aurantiacum* (D58420) A.l.: *Alcaligenes* sp. (D58422)

A.t.: *Arabidopsis thaliana* (β-OHase 1, U58919; β-OHase 2, AB025606)

C.a.: *Capsicum annuum* (β-OHase 1, Y09225; β-OHase 2, Y09722)

C.u.: *Citrus unshiu* (AF296158) E.h.: *Erwinia herbicola* (S52982)

E.u.: *Erwinia uredovora* (D90087) F.a.: *Flavobacterium* ATCC 21588 (U62808)

H.p.: *Haematococcus pluvialis* (AF082326)

L.e: *Lycopersicon esculentum* (β-OHase 1, Y14809; β-OHase 2, Y14810)

N.p.: *Narcissus Pseudonarcissus* (AJ278882) P.m.: *Paracoccus marcusii* (Y15112)

SS: *Synechocystis* sp. PCC6803 (D90906, sll1468) T.e.: *Tagetes erecta* (AF251018)

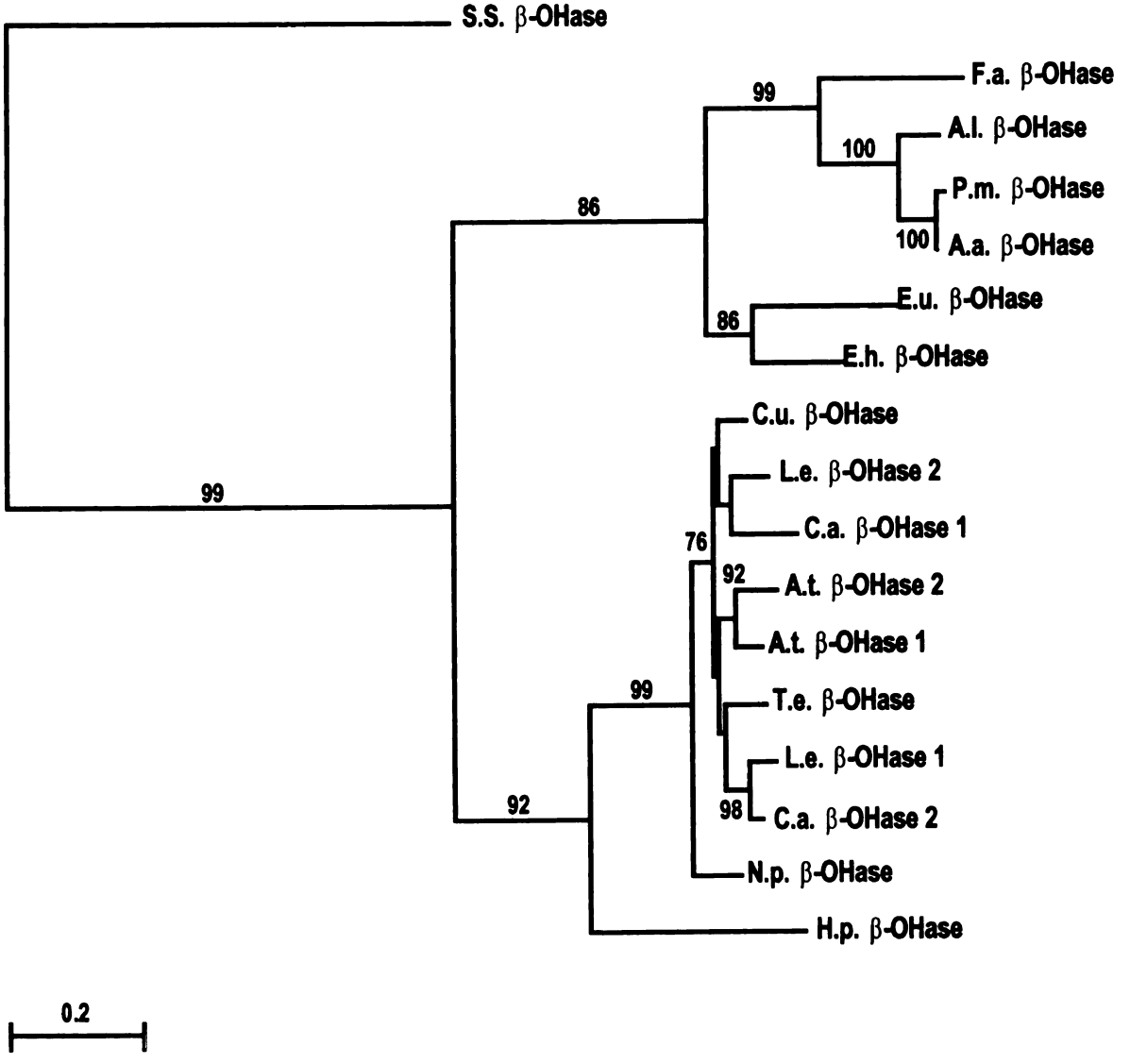

S.S. β-OHase
F.a. β-OHase
A.l. β-OHase
P.m. β-OHase
A.a. β-OHase
E.u. β-OHase
E.h. β-OHase
C.u. β-OHase
L.e. β-OHase 2
C.a. β-OHase 1
A.t. β-OHase 2
A.t. β-OHase 1
T.e. β-OHase
L.e. β-OHase 1
C.a. β-OHase 2
N.p. β-OHase
H.p. β-OHase
99
100
86
100
86
99
76
92
99
92
98
0.2

Figure 2.5

which are thought to coordinate the reactive iron atoms (Bouvier *et al.*, 1998). Although there is an overall high identity between the new hydroxylase and the Arabidopsis β-hydroxylase 1 protein, significant amino acid changes do exist between the proteins (Figure 2.1), which could be sufficient to alter substrate specificity and enzyme activity.

To determine whether the new hydroxylase was an ε- or β-ring hydroxylase, its activity, expression and chromosomal location relative to the *lut1* mutation were examined. When expressed in *E.coli* strains producing various cyclic carotenoids, the new hydroxylase showed high activity against β-ring substrates but very low activity against ε-ring substrates, similar to the β-hydroxylase 1 cDNA. Both hydroxylases are expressed at low levels in root, leaf, flower, silique and stem tissues and could only be detected by PCR based methods. β-hydroxylase 1 transcripts were more abundant than β-hydroxylase 2 transcripts in all tissues analyzed. Finally, neither of the β-hydroxylase genes in Arabidopsis maps to the *LUT1* locus indicating neither encodes the activity defined by the *LUT1* locus.

The presence of two β-hydroxylases in a single plant system has been reported in pepper, where both enzymes were shown to be active towards β-rings (Bouvier *et al.*, 1998). Pepper β-hydroxylase 1 expression was induced during the chloroplast to chromoplast transition in fruit, however, tissue-specific expression patterns of the two hydroxylases were not examined. Our data show that both Arabidopsis hydroxylases were expressed in all the tissues, and β-hydroxylase 1 has a steady-state message level ten to fifty fold higher than β-hydroxylase 2 (Figure 2.4). We are currently investigating the expression patterns of the two hydroxylases under stress conditions.

We have cloned and characterized a second member of the β-hydroxylase gene family in Arabidopsis. Several lines of evidence allow us to conclude this second gene encodes an active β-hydroxylase enzyme and not the ε-hydroxylase. Most notably, the encoded protein is highly active against β-rings, but not ε-rings, and does not map to the *LUT1* (ε-hydroxylase) locus. Our data are consistent with the *LUT1* locus encoding either a third, divergent hydroxylase in Arabidopsis or an ancillary protein that modifies existing β-hydroxylase(s) to be active toward ε-rings. Our exhaustive library screening and database analyses suggest that if *LUT1* does encode an enzyme with ε-hydroxylase activity, it likely evolved independently of the β-hydroxylases as no additional β-hydroxylase paralogs are present in the recently completed Arabidopsis genome (The Arabidopsis Genome Initiative, 2000). Indeed, it is most likely that *LUT1* encodes an ancillary protein that modifies existing β-hydroxylase activities, as the genome sequence encompassing the *LUT1* locus does not contain proteins with the conserved histidine motifs characteristic of all known carotene-hydroxylases. We are currently fine mapping the *lut1* mutation in preparation for map-based cloning of this locus.

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CHAPTER 3

The Arabidopsis *LUT1* locus encodes a carotenoid ε-hydroxylase that is a member of the cytochrome P450 family

Abstract

Lutein is the most abundant carotenoid in photosynthetic tissues and its occurrence is highly conserved in higher plants. Lutein acts as a structural component of the light harvesting complexes and plays an essential role in photoprotection. β- and ε-hydroxylases catalyze the formation of lutein from α-carotene (β,ε-carotene). In the EMS-derived *lut1* mutants, lutein accumulation is decreased ~80% and the immediate biosynthetic precursor zeinoxanthin (β,ε-carotene, with only the β-ring hydroxylated) accumulates. These biochemical characteristics suggest that *lut1* is a mutation affecting ε-hydroxylase. We cloned the *LUT1* gene by map-based cloning and found that it encodes a cytochrome P450 type monooxygenase. An additional null *lut1* T-DNA knockout allele (*lut1-3*) was identified. The phenotype of *lut1-3* confirmed that the EMS-derived *lut1-1* and *lut1-2* alleles are leaky. The deduced LUT1 protein sequence has a putative chloroplast transit peptide and a transmembrane domain, consistent with its predicted thylakoid membrane localization. Putative orthologs of *LUT1* are present in monocots, dicots, and diatoms. LUT1 represents a novel class of carotenoid hydroxylase that has evolved independently from and uses a different mechanism than the non-heme di-iron β-hydroxylases. We suggest that the evolution of these different hydroxylation mechanisms may be due to differing redox chemistry and/or substrate specificity. Characterization of LUT1 activity will provide additional information on carotenoid hydroxylation reaction mechanisms and possible carotenogenic complex formation on the thylakoid membrane. Isolation of *LUT1* will also facilitate metabolic engineering for lutein production in plants and other organisms.

Introduction

Plant carotenoids are C_{40} compounds derived from eight isoprene units. Carotenoids are essential structural and functional components of the photosynthetic antenna and reaction center complexes and specific carotenoids serve as precursors for the synthesis of abscisic acid. In addition to their roles in plants, some carotenoids play an essential role in human nutrition as provitamin A (reviewed in Cunningham and Gantt, 1998). Based on their structures, carotenoids can be grouped into two classes: the carotenes, which are linear, or cyclized hydrocarbons and the xanthophylls, which are various oxygenated derivatives of carotenes. One xanthophyll, lutein (3R, 3'R-β,ε-carotene-3,3'-diol), is the most abundant carotenoid in all plant photosynthetic tissues, where it plays an important role in light harvesting complex II (LHC II) assembly and functions in photoprotection. Zeaxanthin (3R, 3'R-β,β-carotene-3,3'-diol) is a structural isomer of lutein and is a critical component in the mechanism of non-photochemical quenching (reviewed in Niyogi, 1999; Hirschberg, 2001).

The committed step in lutein and zeaxanthin biosynthesis is the cyclization from lycopene to form α- and β- carotene, respectively (Figure 3.1). α-carotene has one ε-ring and one β-ring, while β-carotene has two β-rings. The ε- and β- rings differ from each other by the position of double bond on each ring structure (Britton, 1998). This small difference has profound consequences for the structural and photochemical properties of α- and β- carotene and their xanthophyll derivatives (Niyogi, 1999). Lutein and zeaxanthin are produced from α- and β- carotene, respectively, by a class of enzymes known as carotenoid hydroxylases. β-hydroxylases add hydroxyl groups to carbon 3 of

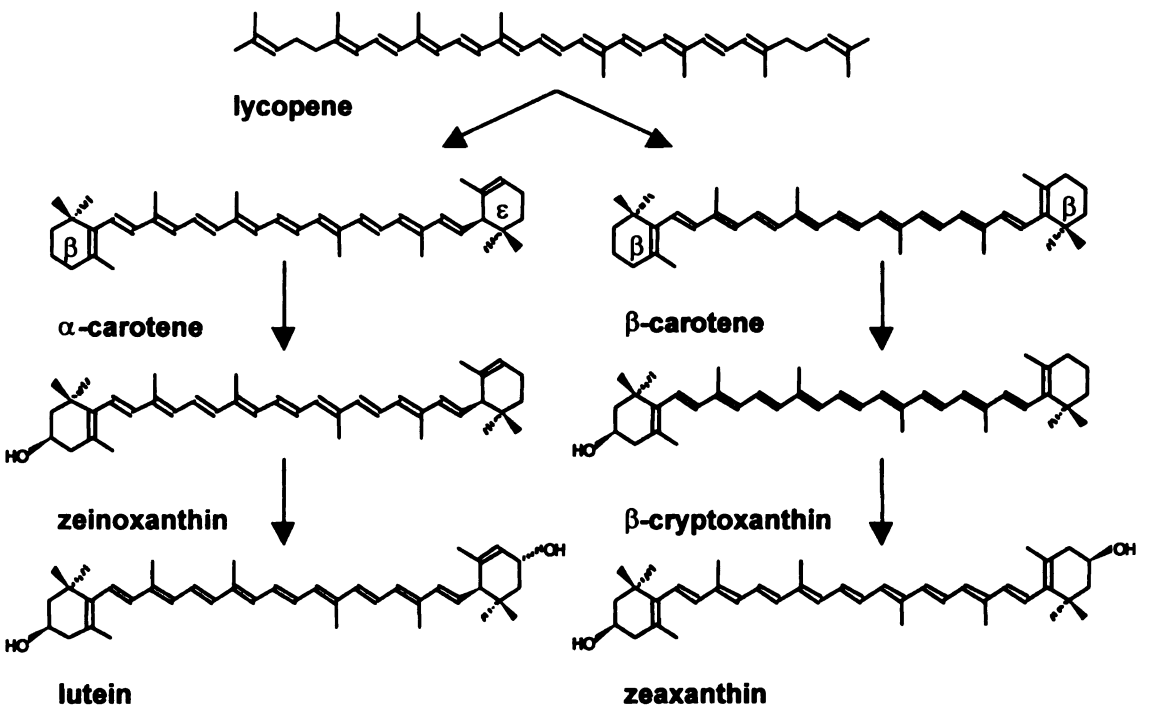


Figure 3.1. Carotenoid hydroxylation reactions.

the β-rings of β-carotene to form zeaxanthin, while hydroxylations of carbon 3 on the β- and ε-ring of α-carotene catalyzed by β- and ε- hydroxylase, respectively, yield lutein (Figure 3.1).

Based on the configuration of carbon 3 (C-3) and the requirement for molecular oxygen, carotenoid hydroxylation reactions were predicted to be catalyzed by mixed function oxygenase, such as cytochrome P450s (Walton et al., 1969; Britton, 1998). β-hydroxylases have been cloned from a variety of photosynthetic and non-photosynthetic bacteria, fungi, and higher plants (reviewed in Cunningham and Gantt, 1998) through functional complementation and heterologous hybridization. In all three phyla, the deduced β-hydroxylase amino acid sequences do not encode cytochrome P450 monooxygenases but rather are non-heme di-iron proteins and have a different hydroxylation mechanism than heme-binding cytochrome P450 enzymes (Shanklin et al., 1994). Biochemical and mutagenesis studies with pepper (*Capsicum annum*) β-hydroxylases have confirmed that the enzymes require iron, ferredoxin, and ferredoxin oxido-reductase for activity and all of the ten conserved iron-coordinating histidines are required for activity (Bouvier et al., 1998). Although all β-hydroxylase enzymes efficiently hydroxylate β-rings, they work poorly on ε-rings *in vitro* (Sun et al., 1996).

Early isotope labeling studies have shown that hydroxylation reactions are stereospecific (Walton et al., 1969; Milborrow et al., 1982). The chirality of C-3 carbon in the ε-ring of lutein is opposite to that in the β-ring. This difference in chirality may explain why β-hydroxylase functions poorly on ε-ring and was an initial suggestion that two different hydroxylases are needed for the β- and ε- ring hydroxylations. Genetic

studies in Arabidopsis have demonstrated the presence of an ε-ring specific hydroxylase based on mutant analysis and biosynthetic precursor accumulation (Pogson et al., 1996). Mutation of the *LUT1* locus decreases the production of lutein by 80% and results in accumulation of the monohydroxyl immediate precursor zeinoxanthin, a classic phenotype for a mutation affecting a biosynthetic enzyme. ε-ring hydroxylation was specifically blocked in *lut1* because production of β-carotene derived xanthophylls was unaffected. It was therefore proposed that *lut1* is a mutation specific for ε-hydroxylation (Pogson et al., 1996).

Attempts to clone an ε-ring specific hydroxylase by sequence-based homology to β-hydroxylase in Arabidopsis were not successful, though a paralog for the β-hydroxylase 1 gene (β-hydroxylase 2) was identified and characterized (Tian and DellaPenna, 2001). Like cloned β-hydroxylases from all three phyla, β-hydroxylase 2 is highly active toward the β-ring but only weakly active toward the ε-ring. In addition, a thorough search of the fully sequenced Arabidopsis genome did not identify an additional gene that bears significant homology to the two cloned β-hydroxylases, suggesting that the ε-hydroxylase defines a structurally distinct carotenoid hydroxylase family. Furthermore, neither of the two Arabidopsis β-hydroxylases mapped to the *LUT1* locus in Arabidopsis (Tian and DellaPenna, 2001). We report here identification of the *LUT1* locus by positional cloning and show that LUT1 defines a new class of carotenoid hydroxylases in nature.

Results

Fine mapping of the *LUT1* locus

We previously mapped *LUT1* to the bottom arm of chromosome 3 at 67 ±3 cM (Tian and DellaPenna, 2001). For fine mapping of the *LUT1* locus, 530 plants homozygous for the *lut1* mutation were identified from approximately 2,000 plants in a segregating F_2 mapping population. Using both CAPS and SSLP markers, *LUT1* was initially localized to an interval spanning two BAC clones (F8J2 and T4D2) and was further delineated to an interval of 100 kb containing 30 predicted proteins (Figure 3.2). As with all other carotenoid biosynthetic enzymes, the *LUT1* gene product is predicted to be targeted and localized to the chloroplast. Within the 100 kb interval containing *LUT1*, six proteins were predicted as being chloroplast-targeted by the TargetP prediction software (http://www.cbs.dtu.dk/services/TargetP). One of these chloroplast-targeted proteins, At3g53130, was a member of the cytochrome P450 monooxygenase family (CYP97C1). Cytochrome P450s are heme-binding proteins that insert a single oxygen atom into substrates, e.g. hydroxylation reactions. Therefore we considered At3g53130 to be a strong candidate for *LUT1*.

Identification of *LUT1*

The identity of At3g53130 as *LUT1* was demonstrated by complementation analysis. Homozygous *lut1* mutants were transformed with a 4116 bp genomic fragment from wild type Columbia (the background of *lut1*) containing the At3g53130 coding region, 959 bp upstream of the start codon, and 690 bp downstream of the stop codon.

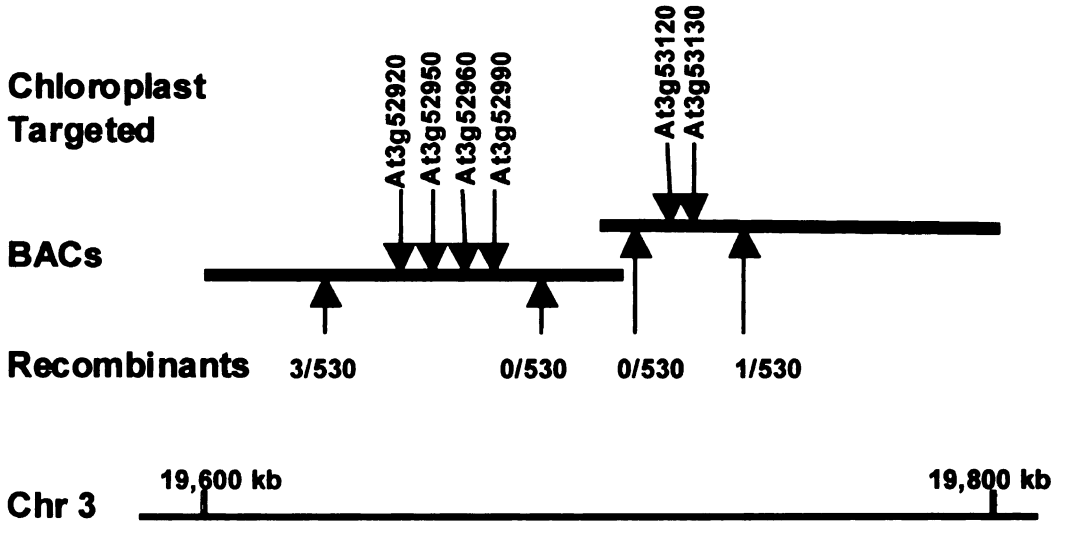


Figure 3.2. Positional cloning of the *LUT1* gene. 530 homozygous *lut1* F_2 plants were used as mapping population. *lut1* was delineated to a region on two BAC clones F8J2 and T4D2 within 100 kb on chromosome 3. The number of recombinants between *lut1* and molecular markers are shown below the BAC clones. Predicted chloroplast targeted proteins within this interval are shown on top of the BAC clones.

Eight independent transformants were selected by Basta resistance and all showed a wild type lutein level when analyzed by HPLC (data not shown). These data indicate that At3g53130 genomic DNA can complement the *lut1* mutation.

To determine the nature of the *lut1* mutations, we sequenced both original EMS-derived alleles of *lut1* (Pogson et al., 1996). The *lut1-2* allele contains a **G** to A mutation at the highly conserved exon/intron splice junction (5' AG/GT, frequency 67%, 67%/**100%**, 100%) that would cause an error in RNA splicing and lead to production of a non-functional protein (Figure 3.3A). The *lut1-1* allele was sequenced but no mutations were identified in the coding region. However, a rearrangement was identified by southern analysis in the upstream region of the *lut1-1* allele but was not characterized further (data not shown). A third *lut1* allele, *lut1-3*, was identified by screening of a T-DNA knockout population using At3g53130-specific primers. A homozygous knockout mutant was obtained that had a T-DNA insertion in the sixth intron of the *LUT1* gene (Figure 3.3A). HPLC analysis of leaf carotenoids from *lut1-3* showed a complete lack of lutein and accumulation of the monohydroxyl intermediate zeinoxanthin (Figure 3.4). This result indicates that the previously EMS-derived *lut1-1* and *lut1-2* alleles are indeed leaky for ε-hydroxylase function. Overall, the combined complementation of the *lut1* mutation, splice site mutation of the *lut1-2* allele, and phenotype of an At3g53130 T-DNA knockout mutant confirm At3g53130 is the *LUT1* locus.

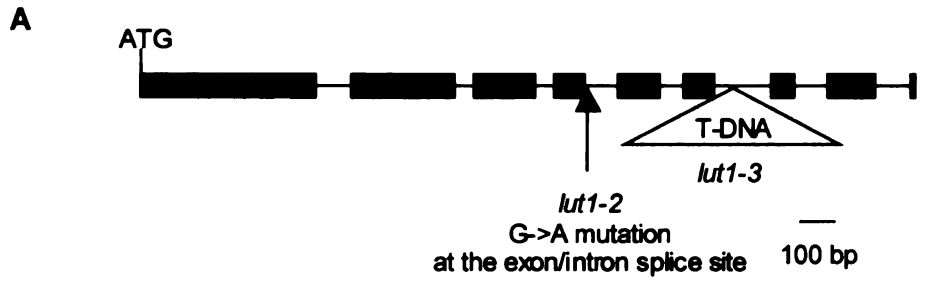
LUT1 encodes a chloroplast-targeted cytochrome P450 with a single transmembrane domain

Figure 3.3B shows the deduced amino acid sequence of LUT1. The chloroplast transit peptide prediction software ChloroP v 1.1 (http://www.cbs.dtu.dk/services/ChloroP/) predicts a chloroplast transit peptide that is cleaved between Arg-36 and Ser-37 (Figure 3.3B). The prediction of a chloroplast localization for LUT1 is consistent with the subcellular localization of carotenoid biosynthesis in higher plants (Cunningham and Gantt, 1998).

The deduced amino acid sequence of LUT1 also contains several features characteristic of cytochrome P450 enzymes. Cytochrome P450 monooxygenases have a consensus sequence of (A/G)GX(D/E)T(T/S) that forms a binding pocket for molecular oxygen with the invariant Thr residue playing a critical role in oxygen binding in both prokaryotic and eukaryotic cytochrome P450s (Chapple, 1998). In the deduced LUT1 protein sequence, this oxygen-binding pocket is highly conserved (single underline in Figure 3.3B). The conserved sequence around the heme-binding cysteine residue for cytochrome P450 type enzymes is FXXGXXXCXG, which is also observed in LUT1 (double underline in Figure 3.3B).

β-hydroxylases have four hydrophobic regions that are predicted to be transmembrane helices. A model by Cunningham and Gantt (1998) suggested that the four helices cross the thylakoid membrane and the di-iron binding sites facing the stroma. The topology of LUT1 was predicted from its deduced amino acid sequence and a hydropathy plot (Figure 3.3C). Unlike the β-hydroxylases, LUT1 only has a single predicted transmembrane domain (25 aa) located in the C-terminus of the protein (Figure 3.3C).

Figure 3.3. A. Identification of the *lut1* mutations. Overview of the intron-exon organization of *LUT1* and the mutations in *lut1-2* and *lut1-3*. B. Deduced amino acid sequence of LUT1. The putative cleavage site for chloroplast targeting sequence is indicated by arrow. The molecular oxygen binding pocket and the cysteine motif are single and double underlined, respectively. C. Predicted membrane topology of LUT1. The hydrophilicity plot is drawn with Kyte/Doolite method (window size = 19 aa). The predicted transmembrane domain is shaded in gray.



B

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                                                  *
   1  MESSLFSPSSSSYSSLFTAKPTRLLSPKPKFTFSIRSSIEKPKPKLETNS
  51  SKSQSWVSPDWLTTLTRTLSSGKNDESGIPIANAKLDDVADLLGGALFLP
 101  LYKWMNEYGPIYRLAAGPRNFVIVSDPAIAKEVLRNYPKYAKGLVAEVSE
 151  FLFGSGFAIAEGPLWTARRRAVVPSLHRKYLSVIVERVFCKCAERLVEKL
 201  QPYAEDGSAVNMEAKFSQMTLDVIGLSLFNYNFDSLTTDSPVIEAVYTAL
 251  KEAELRSTDLLPYWKIDALCKIVPRQVKAEKAVTLIRETVEDLIAKCKEI
 301  VEREGERINDEEYVNDADPSILRFLLASREEVSSVQLRDDLLSMLVAGHE
 351  TTGSVLTWTLYLLSKNSSALRKAQEEVDRVLEGRNPAFEDIKELKYITRC
 401  INESMRLYPHPPVLIRRAQVPDILPGNYKVNTGQDIMISVYNIHRSSEVW
 451  EKAEEFLPERFDIDGAIPNETNTDFKFIPFSGGPRKCVGDQFALMEAIVA
 501  LAVFLQRLNVELVPDQTISMTTGATIHTTNGLYMKVSQR
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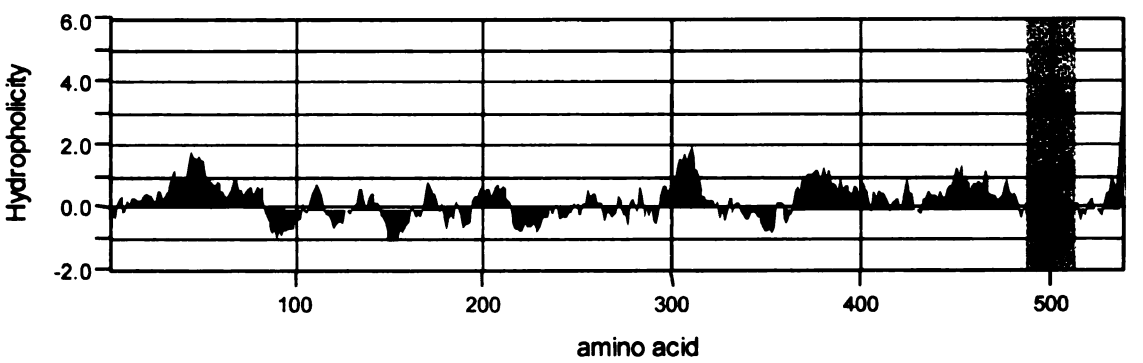


Figure 3.3

Characterization of *lut1* mutant alleles

Carotenoids were extracted from four-week old wild type, *lut1-2*, and *lut1-3* plants, and separated on HPLC (Figure 3.4). The T-DNA derived *lut1-3* mutant shows a complete absence of lutein, consistent with a null mutation in the ε-hydroxylase gene. The EMS-derived *lut1-2* allele contains 5% of wild type levels suggesting the mutation is leaky (Table 3.1).

The leakiness of *lut1-2* allele was further characterized by RT-PCR reactions using primers spanning the mutation site (Figure 3.5). *lut1-2* accumulates the same size amplification product as the wild type, suggesting that a certain amount of message is spliced correctly and results in functional protein. Additional amplification products are not present in the *lut1-2* mutant. The leakiness of the *lut1-1* allele can be explained by a rearrangement upstream from the start codon (not shown), which possibly affects the promoter sequence and transcriptional efficiency.

Enzymatic activities of LUT1

Two approaches have been adopted to express the *LUT1* gene and analyze its activities towards different substrates *in vitro*, although neither has been successful to date. One approach is to express the *LUT1* gene in a yeast expression system that contains the Arabidopsis cytochrome P450 reductase (ATR2) (Pompon et al., 1996). An alternative approach being performed is to express both *LUT1* and Arabidopsis cytochrome P450 reductase (Hull and Celenza, 1999) in carotenoid producing *E.coli*

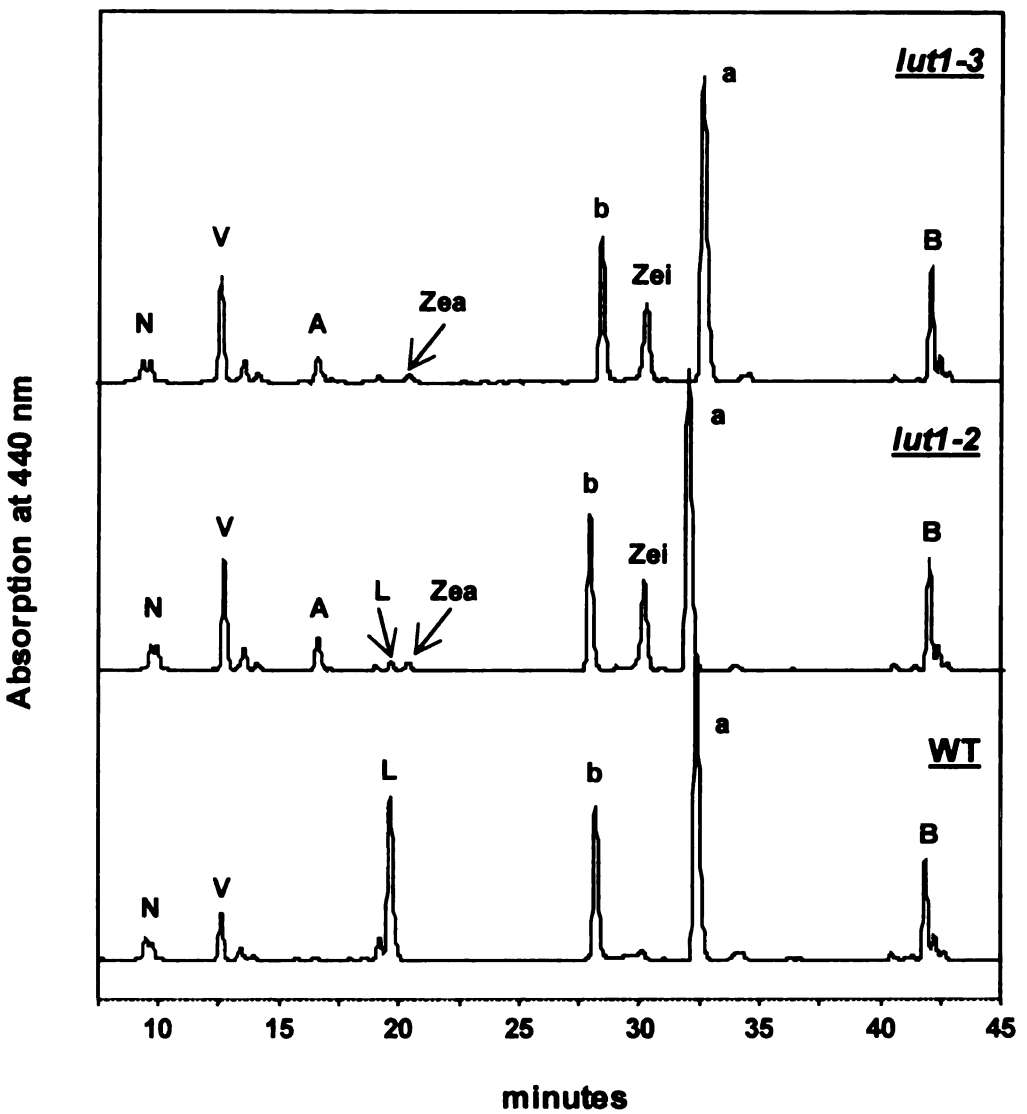


Figure 3.4. HPLC elution profiles of wild type, *lut1-2*, and *lut1-3* plants. N, neoxanthin; V, violaxanthin; A, antheraxanthin; L, lutein; Zea, zeaxanthin; b, chlorophyll b; a, chlorophyll a; Zei, zeinoxanthin; B, β-carotene.

Table 3.1. Wild type and mutant carotenoid composition in leaf tissue quantified by HPLC.

	Lutein	Zeinoxanthin	β-carotene	Neoxanthin	Violaxanthin	Antheraxanthin	Zeaxanthin	β-xanthophylls	Total Carotenoids
WS	158.1±6.5 (52)	n.d.	81.4±6.3 (27)	28.7±2.4 (9)	36.8±5.4 (12)	n.d.	n.d.	65.6±10.1 (21)	305.1±7.3
Col	158.8±12.2 (50)	n.d.	85.2±4.8 (27)	32.8±3.3 (10)	37.8±4.8 (12)	n.d.	n.d.	70.6±2.4 (22)	314.6±16.2
lut1-2	7.8±1.0 (3)	64.5±2.3 (21)	90±3.9 (29)	35.4±5.3 (11)	78.9±3.2 (25)	28.7±4.1 (9)	8.3±2.3 (3)	114.3±12.3 (48)	313.5±8.0
lut1-3	n.d.	62.6±10.7 (21)	87.5±2.7 (29)	26.4±1.8 (9)	86.9±4.0 (29)	28.7±2.4 (10)	9.4±1.4 (3)	113.3±5.2 (51)	301.4±11.9

The amount of carotenoid is expressed as mmol pigment/mol Chl a+b. Each value is the mean of six experiments ± S.D., with the relative molar percentage of each carotenoid given in parentheses. Values marked with the same characters are not significantly different from each other within a column (student's t-test, P> 0.05). n.d., not detected.

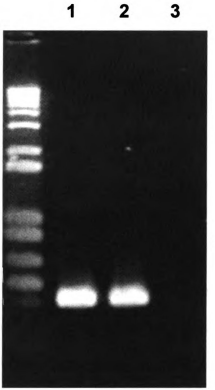


Figure 3.5. RT-PCR reactions using *LUT1* specific primers. Total RNA templates used in lane 1,2 are wild type, *lut1-2*, respectively. Lane 3 is a blank control for the PCR reactions.

strains. Modifications are currently being made in order to obtain ε-hydroxylase activities *in vitro*.

In order to test the activity of LUT1 (ε-ring hydroxylase) towards β- and ε- rings, the full length *LUT1* cDNA was subcloned into the yeast pYeDP60 vector (Pompon et al. 1996) and expressed in the yeast WAT11 strain that has been engineered to express an Arabidopsis cytochrome P450 reductase (Urban et al. 1997). Empty pYeDP60 vector transformed WAT11 cells were used as negative controls throughout the expression studies. The microsomal fraction was prepared from WAT11 cells expressing either the pYeDP60-*LUT1* construct or the empty pYeDP60 vector. Carbon monoxide (CO) difference spectrum was measured for both microsomal preparations. We were unable to obtain CO spectra for LUT1. In retrospect, this is likely due to the absence of an ER targeting sequence on LUT1. The mature protein is not targeted to the membranes therefore it is not present in the microsomal fraction prepared. Currently, experiments are carried out to engineer an ER targeting sequence onto the *LUT1* coding sequence.

A second approach being taken is to engineer both full length and truncated LUT1 (without the chloroplast targeting sequence) into an Arabidopsis cytochrome P450 reductase containing plasmid for expression in *E.coli* (Hull and Celenza, 1999). These constructs are being transformed into *E.coli* strains engineered to accumulate carotenoids with different ring structures (Cunningham et al., 1994). Hydroxylation of carotene substrates has not yet been observed in these *E.coli* strains. It is possible that LUT1 also requires a specific lipid (e.g. MGDG) for activity analogous to that reported for the violaxanthin deepoxidase enzyme (Rockholm and Yamamoto, 1996). We are currently incorporating various lipids into the reaction mixtures.

Putative *LUT1* orthologs

LUT1 was designated as CYP97C1 according to the cytochrome P450 nomenclature (http://www.biobase.dk/P450). By definition, cytochrome P450s are grouped into a family if their amino acid sequences are >40% identical and are further grouped into subfamilies if the identity is >55%. There are two additional Arabidopsis proteins that belong to the CYP97 family: CYP97A3 (At1g31800) and CYP97B3 (At4g15110) with 49% and 42% identity to LUT1, respectively. CYP97A3 is predicted to be chloroplast targeted while CYP97B3 is apparently cytosolic.

Various EST and genomic databases were searched using the deduced amino acid sequence of *LUT1* as a query. There are two other dicot proteins that belong to different CYP97 subfamiles: CYP97B1 from pea (*Pisum Sativum*) and CYP97B2 from soybean (*Glycine max*) are 44% and 42% identical to At3g53130, respectively, suggesting that they are possible LUT1orthologs. We were also able to identify an ortholog for LUT1 (AAK20054) in the monocot plant *Oryza sativa*. This rice protein is 70% identical to LUT1. In addition, there is also a CYP97 family member (37% identical to LUT1; AAL73435) in diatom (*Skeletonema costatum*, CYP97E1), the function of which is unknown.

Discussion

Lutein is the most abundant carotenoid in photosynthetic tissues of plants and

plays essential roles in light harvesting and photoprotection (Niyogi, 1999). β- and ε-hydroxylases add hydroxyl groups to the β- and ε- ring of α-carotene to form lutein. β-hydroxylases have been extensively studied and characterized in the past several years. In contrast, the ε-hydroxylase is only genetically defined by the *lut1* mutation in Arabidopsis (Cunningham and Gantt, 1998). Several lines of evidence have confirmed that At3g53130 is the *LUT1* gene and encodes the carotenoid ε-ring hydroxylase that is a member of the cytochrome P450 family. Cytochrome P450 heme-binding monooxygenases carry out a mechanistically distinct type of hydroxylation reaction from non-heme di-iron β-hydroxylases, which suggests that LUT1 is a novel hydroxylase that has evolved independently from β-hydroxylases.

EMS-derived *lut1* mutants are leaky

lut1-1 and *lut-2* were previously isolated from a screen of EMS-generated mutants (Pogson et al., 1996). Both alleles still accumulated lutein, suggesting that these EMS-derived mutants might be leaky. Cloning of the *LUT1* gene enabled the isolation of a knockout mutant (*lut1-3*) using a reverse genetics approach. The *lut1-3* mutant completely lacks lutein (Figure 3.4), confirming that the EMS-derived *lut1-1* and *lut1-2* alleles are leaky and that LUT1 is the only activity that carries out ε-ring hydroxylation *in vivo*. Sequencing analysis has shown that *lut1-2* is a mutation in an intron-exon splicing site (Figure 3.3A). This intron is 98 bp long and would shift the opening reading frame and cause a premature stop codon if not correctly spliced. RT-PCR reactions using primers spanning the *lut1-2* mutation site showed that *LUT1* transcripts still accumulate

in the *lut1-2* mutant (Figure 3.5). This result indicates that some of the *LUT1* mRNA is correctly spliced and functional protein is synthesized, which explains the leakiness of LUT1 function in the *lut1-2* mutant. An "unspliced mRNA" (with the fourth intron) was not observed in the *lut1-2* amplification products. A possible splicing site is identified in the fourth intron, which may cause an alternative splicing and result in a product with similar size to *LUT1*. Southern analysis has shown that there is a rearrangement upstream of the *LUT1* gene in the *lut1-1* mutant (data not shown), which may affect the efficiency of the promoter and result in reduced LUT1 mRNA and activity in *lut1-1*.

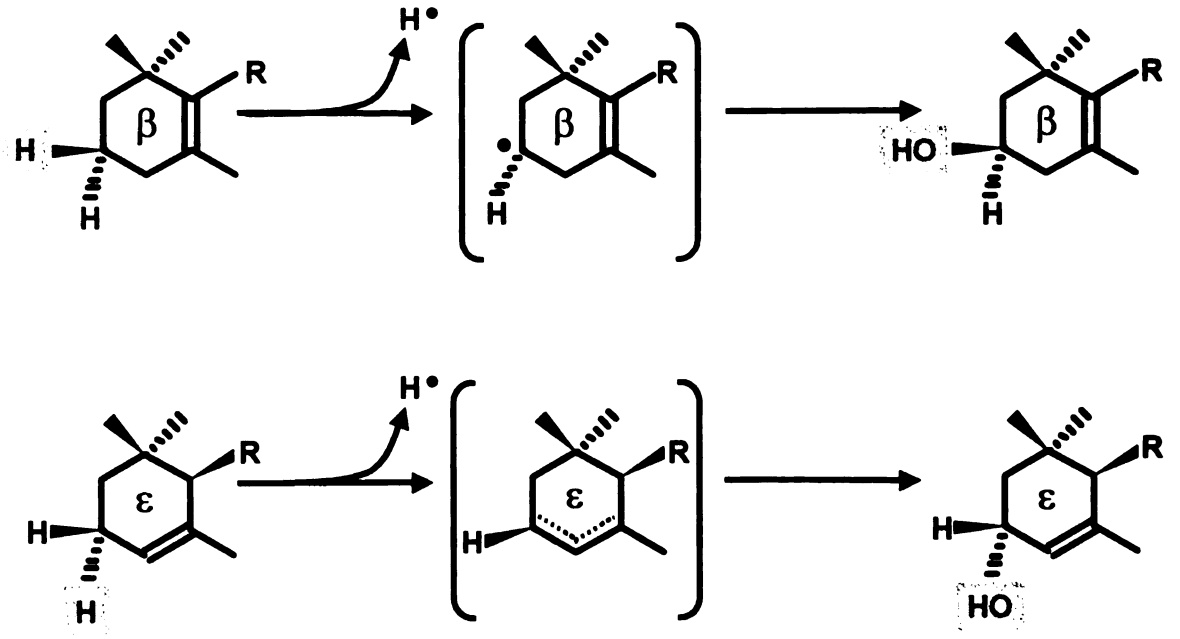
Different hydroxylation mechanisms for β- and ε-ring hydroxylase

Carotenoid β- and ε-hydroxylase add hydroxyl groups to the β- and ε- rings, respectively. β- and ε-ring are very similar and differ from each other only in the placement of a double bond on the ring structure (Figure 3.6A). While both enzymes use nearly identical substrates, why are two different types of monooxygenases required for the hydroxylation reactions?

Both β- and ε- ring hydroxylations require the abstraction of a hydrogen atom from the C-3 position of the ring structure (Figure 3.6A). However, it is relatively easier to withdraw a hydrogen atom from the C-3 of an ε-ring than from a β-ring (dissociation energy 86 kcal/mol vs. 100 kcal/mol; Berkowitz et al., 1994), because the C-3 on ε-ring is an allylic carbon while the C-3 carbon on β-ring is not (Figure 3.6A). Therefore, cleavage of C-H bond at the C-3 position of an ε-ring requires less energy due to the resonance stabilization of the allylic radical product. The β-ring requires a stronger

Figure 3.6. A. The hydroxylation reactions of β- and ε- ring. R, polyene chain. B. 3-D structures of α- and β- carotene substrates for hydroxylation reactions.

A



B

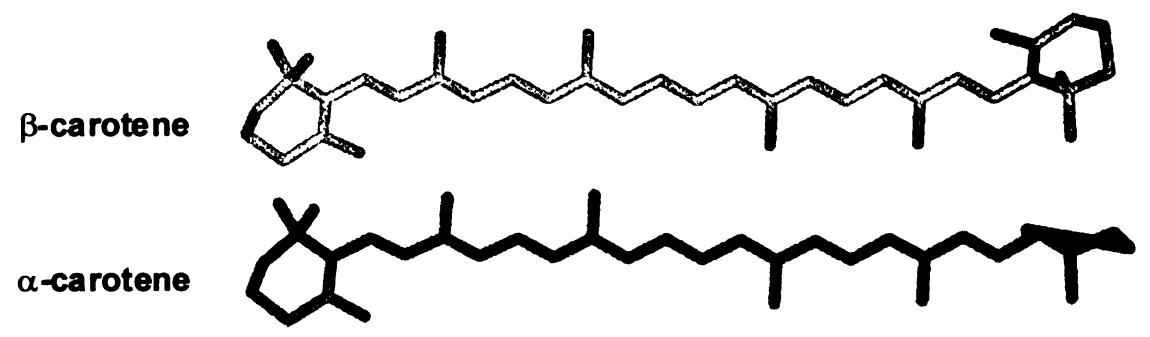
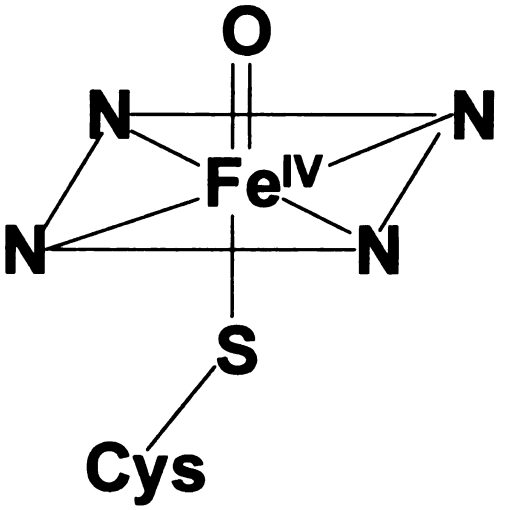


Figure 3.6

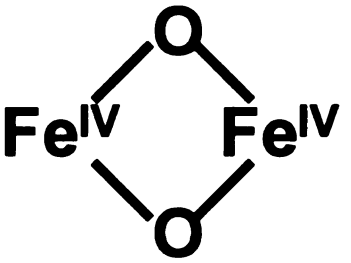
oxidant for hydrogen abstraction.

This raises the possibility that non-heme di-iron β-hydroxylases are stronger oxidants than the cytochrome P450 enzymes. The currently accepted mechanism for hydrocarbon hydroxylation involves two reactions: first, abstraction of a hydrogen atom from the substrate by an oxoiron species (Fe^{IV}=O), and secondly, a rapid collapse of the resulting carbon radical-hydroxyferryl complex to give the hydroxylated product and the resting-state enzyme (Groves et al., 1978). Both cytochrome P450 and di-iron monooxygenases hydroxylate substrates by a hydrogen atom abstraction/oxygen rebound mechanism with a short-lived iron-oxo intermediate. The key cytochrome P450 intermediate is an Fe^{IV}=O porphyrin π-radical cation (Ortiz de Montellano, 1995), while the key di-iron monooxygenase intermediate is a di-Fe^{IV} unit with bridging oxos (Shanklin and Cahoon, 1998) (Figure 3.7). Experimental evidence have shown that both enzymes are able to add oxygen to inactivated C-H bonds (Sono et al., 1996; Yoshizawa, 2000) and it is not clear which enzyme produces a stronger oxidant in the "activated" intermediate. Therefore, the requirement of two fundamentally different types of hydroxylases for the two rings can not be simply explained by their oxidation capacities.

Another key factor that determines enzyme catalysis is access to substrates. Neither the β- nor the ε- hydroxylase has been crystallized, therefore the precise conformations of their substrate binding sites are not known. However, when comparing the carotene substrate structures, the β-ring still has a double bond conjugated to the hydrocarbon backbone, thus, the β-ring is restrained to the same plane as the hydrocarbon backbone. On the other hand, the double bond in the ε-ring is not conjugated to the backbone and has relatively free rotation along the C6'-C7' carbon (Figure 3.6B). One



Heme binding cytochrome P450 monooxygenase



Non-heme di-iron monooxygenase

Figure 3.7. Oxidizing intermediates for cytochrome P450 and non-heme di-iron monooxygenases.

explanation for the hydroxylase substrate specificity is that the substrate binding pocket of β-hydroxylase can only accommodate a straight chain hydrocarbon, such as the β-ring structure; an ε-ring that is tilted from the hydrocarbon backbone can not fit in the β-hydroxylase binding site. It is *vise versa* for the ε-hydroxylase. Another explanation for the substrate specificity is that both hydroxylases can bind to the β- and ε- ring substrates and abstract a hydrogen atom from C-3 carbon, but the hydroxylation reaction is stereospecific, i.e., the ring substrate has to be at the "correct" conformation for the oxidation reaction to occur. In addition, β- and ε-hydroxylases may differ in their membrane topologies (Cunningham and Gantt, 1998; Figure 3.3C), which may contribute to their substrate specificity.

Evolution of β- and ε-hydroxylases

Over 700 carotenoids have been identified in nature and constitute a structurally diverse group of compounds. The polyene chain in carotenoids has been conserved through evolution while the end groups have not and show a wide variety of modifications. Specific carotenoid end groups are necessary for their recognition and/or binding to proteins and orientation in the thylakoid membrane, which further determine the biological diversities of carotenoid functions in different organisms.

Carotenoids with β,β- and β,ε-end groups are prevalent in plants and perform critical roles in light harvesting and photoprotection. One of the most common modifications on β,β- and β,ε-end groups are hydroxylation reactions to form various xanthophylls. Carotenoid β-hydroxylases have been cloned from various photosynthetic

and non-photosynthetic bacteria, green algae, and higher plants. They have invariably been shown to be di-iron enzymes. From phylogenetic studies based on the deduced amino acid sequences, β-hydroxylases can be clearly grouped into three clads: plant and nonphotosynthetic bacterial β-hydroxylases are related but share less than 30% protein identity, while the cyanobacterial β-hydroxylases are more closely related to the bacterial β-ketolases (Tian and DellaPenna, 2001).

The present data have shown that plant ε-hydroxylases evolved independently of β-hydroxylases. Putative ε-hydroxylase orthologs have been identified from both monocot (rice) and dicots (soybean and pea) plants via database search. There is also a cytochrome P450 protein in diatom (*Skeletonema costatum*, CYP97E1) that belongs to the same family as *LUT1* and is possibly related to ε-hydroxylase. The question remains: what is the evolutionary origin of the ε-hydroxylase in plants?

The substrate for the ε-hydroxylase is α-carotene (β,ε-carotene) and ε-hydroxylase may have evolved in parallel to α-carotene synthesis. α-carotene is unique to land plants and green algae that gave rise to land plants. In prokaryotes, α-carotene has only been found in Prochlorococcus and Acaryochloris (Hess et al., 2001; Miyashita et al., 1997). The development of photosystem II (PSII) marked an important evolution of carotenoid biosynthesis. Chlorophyll b (Chl b) is mainly associated with PSII. Prochlorococcus is a Chl b-containing cyanobacteria that lives in the ocean. Two Prochlorococcus strains, MED4 (low Chl b/a ratio) and MIT9313 (high Chl b/a ratio) are representatives of high- and low- light adapted ecotypes. This genus is distinct from other cyanobacteria by the presence of high concentration of α-carotene. Both MED4 and MIT9313 genomes contain two genes encoding for putative lycopene β- and ε- cyclases.

β-hydroxylase orthologs have also been identified from these two prochlorococcus. However, no lutein was detected in the pigment profile and no potential ε-hydroxylase identified in database searches.

Future perspectives

Current models (Cunningham and Gantt, 1998) suggests that two types of desaturase/cyclase complexes exist in the thylakoid membrane, one with two β-cyclases and the other with one β- one ε-cyclase. Following this model, carotenoid hydroxylases in higher plants may also form complexes. β-hydroxylase 1 and/or 2 may form one complex and work specifically on β,β-rings, while ε-hydroxylase may form a complex with either β-hydroxylase 1 or 2 and preferably utilizes β,ε-ring carotenoid as substrate. Cloning of the ε-hydroxylase now allows for the possibility of generating an ε-hydroxylase antibody and elucidation of possible hydroxylase complexes.

In higher plants, studies using the lutein deficient Arabidopsis *lut1* and *lut2* mutants showed that in addition to light induced trans-thylakoid pH change and zeaxanthin accumulation, lutein is also required for efficient nonphotochemical quenching (Lokstein et al., 2002). Lutein is essential for optimizing photosynthetic antenna structure, stability, therefore efficiently using harvested light under normal and high light growth conditions, respectively. Cloning of the ε-hydroxylase gene will allow one to manipulate lutein production *in vivo*. Lutein is also known for protecting against damage from reactive oxygen species and minimizing the occurrence and/or severity of certain chronic diseases in human. Elevated lutein production will increase the nutritional

values of plant food and promote human health.

Methods

Positional cloning of *LUT1*

lut1-2 (ecotype Columbia) was crossed to *Lansberg erecta*. The resulting F_1 plants were self-pollinated and homozygous *lut1* mutants were identified in a segregating F_2 mapping population by a Thin Layer Chromatography (TLC) screening method. Carotenoid samples were extracted as described (Tian and DellaPenna, 2001), resuspended in ethyl acetate, spotted on a C_{18} TLC plate (J.T. Baker, Phillipsburg, New Jersey), and developed in 90:10 (v:v) hexane: isopropanol. Homozygous *lut1* F_2 plants were identified by the characteristic appearance of an extra yellow band due to their accumulation of zeinoxanthin that is absent in wild type plants.

lut1 was initially mapped to 67 ± 3 cM on chromosome 3 (Tian and DellaPenna, 2001). The *lut1* interval on chromosome 3 was searched for CAPS and SSLP markers using the TAIR search engine
http://www.arabidopsis.org/servlets/Search?action=new_search&type=marker
In order to achieve additional molecular markers for fine-mapping, SSLP markers were designed based on the INDEL information obtained from the Cereon website: http://godot.ncgr.org/Cereon/. Linkage analysis was performed between *lut1* and the molecular markers to delineate *lut1* to a narrow region on chromosome 3.

DNA extraction and PCR reactions

A diameter of 0.5 mm leaf area of three weeks old Arabidopsis plant was excised and pulverized in a 1.5 ml microcentrifuge tube with blue pestle (Nalge Nunc International). 150 µl DNAzol reagent (Invitrogen, Carlsbad, CA) was added and more grinding was carried out until no green leaf tissue was visible in the solution. The tube is incubated at room temperature with shaking for 5 min. 150 µl chloroform was added to the tube and mixed well. The mixture was further incubated at room temperature for another 5 min. The extracts were then centrifuged at 13,000g for 10 min. The upper phase was transferred to a fresh microcentrifuge tube and the DNA was precipitated with 75 µl 100% ethanol at room temperature for 5 min. The DNA was centrifuged at 10,000g for 5 min. The supernatant was discarded and the pellet was washed with 300 µl 75% ethanol. Following ethanol wash, the ethanol-DNA mix was centrifuged at 10,000g for 5 min. The supernatant was discarded and the pellet was dried briefly under vacuum. Finally, the DNA was resuspended in 100 µl 8 mM NaOH. The pH of the DNA solution was adjusted to 7.5 by adding 15.9 µl of 0.1 M HEPES.

PCR reactions were performed with 1 µl of genomic DNA in a 20 µl reaction mixture. The PCR program is 94°C for 3 min, 60 cycles of 94°C for 15 s, 50°C-60°C (the annealing temperature is optimized for each specific pairs of primers) for 30 s, 72°C for 30 s, and finally 72°C for 10 min. A portion of the PCR product (6 µl) was then separated on 3% agarose gel.

Complementation of *lut1* with wild type gene

An Arabidopsis cosmid library (Meyer et al., 1996) was screened and several cosmids that span both T4D2 and F8J2 BAC clones were identified. At3g53130 genomic DNA was obtained by digesting the cosmid DNA with . The resulting fragment was first subcloned into Hind III and BamH I digested pART7 vector. The pART7-At3g53130 construct was digested with NotI and subcloned into pMLBART vector that has Basta as a selection marker (Gleave, 1992). The identity of the construct was confirmed by both digestion patterns and amplification with At3g53130 specific primers. Agrobacterium strain GV3101 was transformed with the construct using freeze-thaw method (Chen et al., 1994). Agrobacterium DNA was extracted and its identity was confirmed by restriction enzyme digestion followed by gel-electrophoresis. Homozygous *lut1* plants were transformed with agrobacterium by Floral Dip method (Clough and Bent, 1998).

Knockout mutant isolation

At3g53130 specific primers (forward, 5'-CTTCCTCTTCTTACTCTTCTCTCTTCACT-3'; reverse, 5'-AAGAACGATGGATGTTATAGACTGAAATC-3') were sent to the University of Wisconsin Arabidopsis T-DNA knockout facility for screening knockout mutants of the *LUT1* gene. A single knockout line was identified and isolated as described (http://www.biotech.wisc.edu/Arabidopsis/). A homozygous mutant line was identified by PCR and designated *lut1-3* (Figure 3.3). A second knockout mutant (Salk_042859) was identified through the Salk T-DNA express website (http://signal.salk.edu) and

designated as *lut1-4*. *lut1-4* has a T-DNA insert 117 bp upstream of the *LUT1* start codon.

HPLC and RT-PCR analyses of the *lut1* mutants

Wild type Columbia and WS plants as well as *lut1-2* (Columbia background) and *lut1-3* (WS background) mutants were grown under 120 μmol photons $m^{-2}s^{-1}$ for four weeks. The same leaf area from each plant was excised by a cork borer. Total carotenoids were extracted and quantified as previously described (Tian and DellaPenna, 2001). Total RNA was extracted from Columbia and *lut1-2* plants and RT-PCR reactions were performed (Tian and DellaPenna, 2001). Primers for the PCR reactions are forward, 5'-TTGTGTAAGATAGTCCCGAGACAGG-3' and reverse, 5'-GGATGAGGATAGAGACGCATTGAC-3'.

Construct for yeast expression

The cDNA of At3g53130 was reverse transcribed from wild type Columbia total RNA and gene specific primers used for PCR reactions are 5'-AAGGATCC**ATG**GAGTCTTCACTCTTTTCTC-3' (start codon in bold, BamHI site is underlined) and 5'-AAGAATTC**TTA**CCTTTGGCTCACCTTCATATAC-3' (stop codon in bold, EcoRI site is underlined). The PCR reaction was performed using Pfu polymerase and the PCR program was 94°C for 3 min, then 35 cycles of 94°C 30 s, 60°C 30 s, and 72°C 2 min, the PCR reaction was continued with 72°C 10 min and holded at 4°C. Yeast expression vector pYeDP60 was kindly provided by Drs. Pompon and Urban

(Pompon et al. 1996). Full length At3g53130 cDNA was digested with both BamHI and EcoRI and ligated into BamHI and EcoRI digested pYeDP60 vector.

Yeast transformation and microsomal preparation

pYeDP60-At3g53130 plasmid was transformed into yeast WAT11 strain as previously described (Schiestl and Gietz, 1989). For expression studies, one colony was picked from the pYeDp60-At3g53130 plate and added to 50 ml SGI media and grown at 30°C for about 24 hrs with shaking. Microsomal fraction was subsequently prepared following procedures in Ralston et al. (2001).

Construct for *E.coli* expression

An N-terminal modified form (without chloroplast targeting sequence) of Arabidopsis cytochrome P450 reductase (ATR2) open reading frame was subcloned into the pCWori$^+$ vector (Hull and Celenza, 1999). This plasmid was expressed in *E.coli* and resulted in high yield of a functional reductase, which supports CYP79B2 activity *in vitro* (Hull and Celenza, 1999). At3g53130 full length cDNA was amplified from Columbia total RNA using Pfu polymerase with forward (5'-AAGGATCC**ATG**GAGTCTTCACTCTTTTCTC-3'; BamH I site underlined and start codon in bold) and reverse (5'-AAGAATTC**TTA**CCTTTGGCTCACCTTCATA-3'; EcoR I site underlined and stop codon in bold) primers. At3g53130 without chloroplast targeting sequence was amplified with forward (5'-

AAGGATCC**ATG**TCCTCCATTGAGAAACCCA-3'; BamH I site underlined and start codon in bold) and reverse (5'-AAGAATTC**TTA**CCTTTGGCTCACCTTCATA-3'; EcoR I site underlined and stop codon in bold) primers. The amplification products were digested with BamH I and EcoR I and ligated into pre-digested (BamH I and EcoR I) pBluescript SK^+ vector. The resulting plasmids were digested with Pvu II and the DNA inserts were ligated into blunt-ended $pCWori^+$-ATR2 plasmids. Plasmids harboring both At3g53130 and ATR2 were transformed into carotenoid accumulating *E.coli* strains, pAC-DELTA (producing carotene with a single ε-ring), pAC-BETA04 (producing β,β-carotene), and pAC-EPS04 (producing ε,ε-carotene) (Cunningham et al., 1994). Growth of *E.coli* and carotenoid extractions were performed as described (Cunningham et al., 1994).

Acknowledgements

We thank Drs. Dennis Pompon and Philippe Urban for providing the yeast strain WAT11 and pYeDP60 vector. We also thank Dr. Clint Chapple for helpful discussion on cytochrome P450 assays. The University of Wisconsin Arabidopsis T-DNA knockout facility provided mutant screening service and the Salk Institute Genomic Analysis Laboratory provided sequence-indexed Arabidopsis T-DNA insertion mutants.

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CHAPTER 4

Functional analysis of β- and ε- ring carotenoid hydroxylases in *Arabidopsis thaliana*

The work presented in this chapter has been published:

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Abstract

Lutein and zeaxanthin are dihydroxy xanthophylls that perform critical roles in photosystem structure and function and are produced from their corresponding carotene precursors by the action of β- and ε-ring carotenoid hydroxylases. Two genes encoding β-ring hydroxylases (β-hydroxylase 1 and 2) have been identified in the Arabidopsis genome and are highly active towards β-rings but only weakly active toward ε-rings. A third distinct activity required for ε-ring hydroxylation has been defined by mutation of the *LUT1* locus, but not yet cloned. In order to address the individual and overlapping functions of the three Arabidopsis carotenoid hydroxylase activities *in vivo*, T-DNA knockout mutants corresponding to β-hydroxylase 1 and 2 (*b1* and *b2*, respectively) were isolated and all possible hydroxylase mutant combinations were generated. β-hydroxylase single mutants do not exhibit obvious growth defects and have limited impact on carotenoid compositions relative to wild type, suggesting the encoded proteins have a significant degree of functional redundancy *in vivo*. Surprisingly, the *b1b2* double mutant, which lacks both known β-hydroxylase enzymes, still contains significant levels of β-carotene-derived xanthophylls suggesting additional β-ring hydroxylation activity exists *in vivo*. The phenotype of double and triple hydroxylase mutants indicates at least a portion of this activity resides in the *LUT1* gene product. The single, double, and triple hydroxylase mutants do not alter the total amount of carotenoids accumulated in leaf tissue but cause progressively more severe alterations in carotenoid composition, NPQ induction, and NPQ amplitude. Despite the severe reduction of β-carotene-derived xanthophylls (up to 90% in the *lut1b1b2* triple mutant), the double and triple hydroxylase

mutants still contain at least 50% of the wild type amount of hydroxylated β-rings. This suggests it is the presence of minimal amounts of hydroxylated β-rings, rather than minimal amounts of specific β-carotene-derived xanthophylls, that are essential for LHC II assembly and function *in vivo*. The carotenoid profiles in wild type seeds, and the effect of single and multiple hydroxylase mutations are distinct from that in photosynthetic tissues indicating the activities of each gene product differs in the two tissues. Overall, the hydroxylase mutants provide insight into the unexpected overlapping activity of carotenoid hydroxylases *in vivo*.

Introduction

Xanthophylls are oxygenated carotenoids that perform a variety of critical roles in photosystem structure and assembly, light harvesting, and photoprotection. The xanthophyll content of photosynthetic plant tissues is highly conserved through evolution with lutein (L) being the most abundant followed by β-carotene, neoxanthin (N), and violaxanthin (V). Zeaxanthin (Z) and antheraxanthin (A) accumulate to high levels in response to high light stress. L is a critical structural component of the Light Harvesting Complex II (LHC II) trimers, while Z, a structural isomer of L, is best known for its role in non-photochemical quenching (NPQ). NPQ is a measurement of the dissipation of excess light energy absorbed by the photosystems and is one of the major mechanisms that protect plants from photooxidative damage. Energy dependent NPQ requires a pH change in the thylakoid lumen, the PsbS protein, and accumulation of Z (and A) that results from deepoxidation of V (Li et al., 2000; Müller et al., 2001). L has also been found to be necessary for efficient NPQ, although its role is thought to be more indirect than Z and A (Pogson et al., 1998; Lokstein et al., 2002).

L and Z are dihydroxy xanthophylls that are derived from α-carotene (β,ε-carotene) and β-carotene (β,β-carotene), respectively, by the addition of hydroxyl groups to the 3, 3' position of both rings (Figure 4.1). L and Z have identical chemical formulas and similar structures but small differences in their ring structures, conjugated double bond systems, and hydroxylation stereochemistry allow for distinct and specialized roles in photosystem structure, light harvesting, and photoprotection (Demmig-Adams et al., 1996; Horton et al., 1996; Niyogi et al., 1998). The enzymes that mediate carotenoid ring

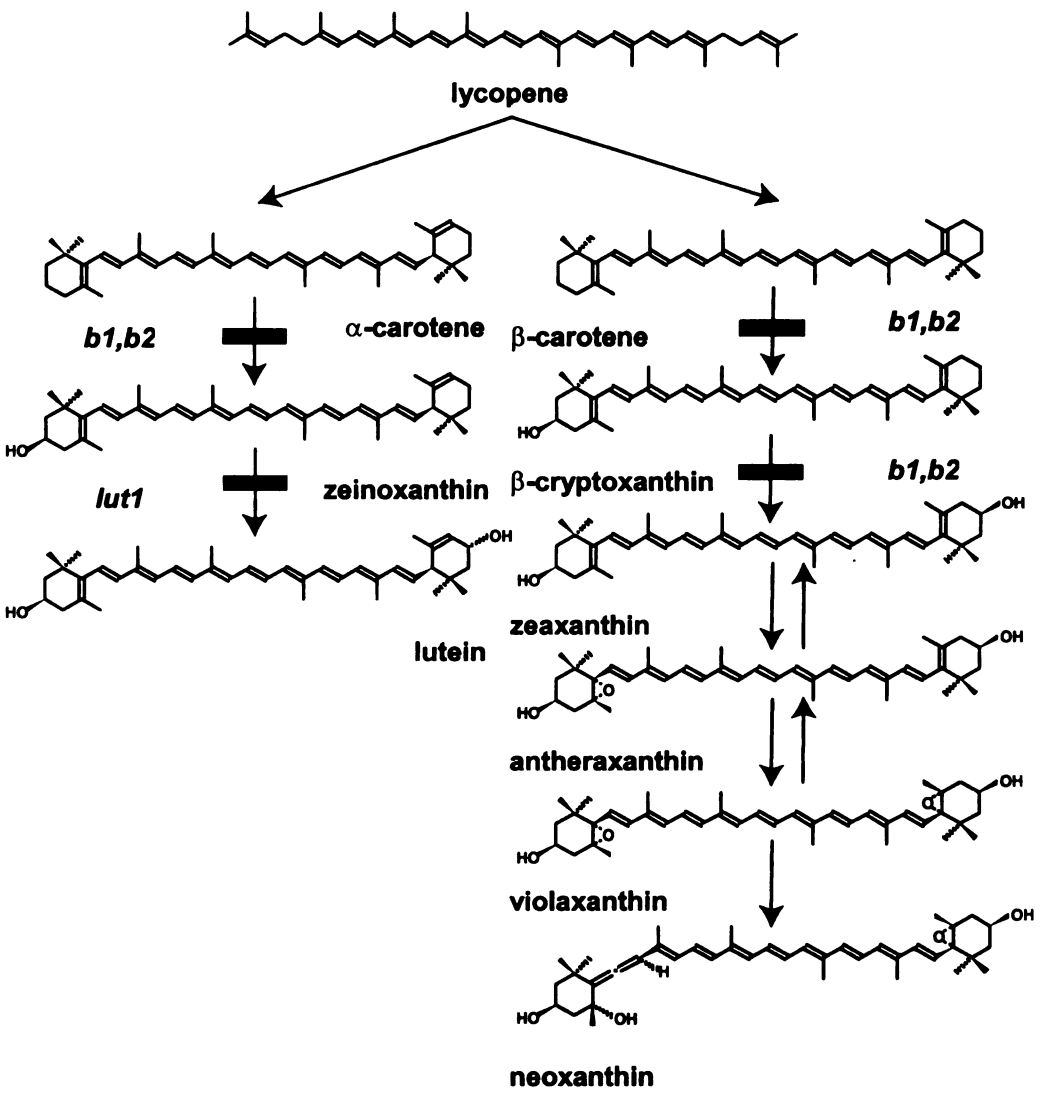

Figure 4.1. Xanthophyll biosynthesis in Arabidopsis. The positions of the *lut1*, *b1* (*CrtR-b1*), and *b2* (*CrtR-b2*) mutations in the pathway are indicated.

hydroxylation reactions are key for the biosynthesis of these two functionally important xanthophylls (Figure 4.1). Z is widespread in bacteria, fungi, and plants and β-hydroxylases involved in Z synthesis have been cloned and characterized from all three phyla. All are non-heme di-iron oxidases that contain conserved histidine motifs required for activity (Bouvier et al., 1998). However, enzymes from the three phyla have otherwise low protein identity, are thought to have evolved independently, but efficiently catalyze hydroxylation of both β-rings of β-carotene to form Z (Misawa et al., 1990; Hundle et al., 1993; Bouvier et al., 1998; Masamoto et al., 1998). The formation of L from α-carotene requires the action of a second hydroxylase, the ε-hydroxylase, in addition to a β-hydroxylase (Britton, 1998). The ε-hydroxylase has not yet been cloned from any organism, but has been genetically identified in *Arabidopsis thaliana* (Pogson et al., 1996).

With regard to the genetics and molecular biology of carotenoid hydroxylation, *Arabidopsis thaliana* is the best-characterized plant system. Two genes encoding β-hydroxylases (β-hydroxylase 1 and 2) are present in the Arabidopsis genome (Sun et al., 1996; Tian and DellaPenna, 2001). The predicted mature proteins share 81% protein identity and are coordinately expressed although β-hydroxylase 1 mRNA levels are always much higher than β-hydroxylase 2 mRNA. When expressed *in vitro*, both β-hydroxylases are highly active towards β-rings and function poorly with ε-ring containing substrates (Sun et al., 1996; Tian and DellaPenna, 2001). Mutational studies have also identified the *LUT1* locus as being essential for hydroxylation of ε-rings in Arabidopsis. Plants homozygous for the *lut1* mutation show an 80% reduction in L levels and accumulate the immediate monohydroxy precursor zeinoxanthin. The *lut1* mutation

does not affect β-ring hydroxylation (Pogson et al., 1996), and *lut1* does not map to either β-hydroxylase 1 or 2 locus. A thorough analysis of the Arabidopsis genome also failed to identify additional paralogs similar to β-hydroxylases from bacteria, cyanobacteria, or plants (Tian and DellaPenna, 2001). These combined data suggest that LUT1 defines a novel class of carotenoid hydroxylase enzymes and that β-hydroxylase 1, β-hydroxylase 2, and LUT1 may represent the full complement of carotenoid hydroxylases in Arabidopsis.

The *lut1* mutation was the first demonstration that by manipulating carotenoid hydroxylase activities *in vivo*, one can modify both the types and amounts of xanthophylls that accumulate in plants. More recently, the β-hydroxylase 1 gene was constitutively overexpressed and antisensed in Arabidopsis leaf tissue. Overexpression resulted in a two-fold increase in xanthophyll cycle carotenoids (V+A+Z) without affecting L levels (Davison et al., 2002). Expression of a β-hydroxylase 1 antisense construct reduced the level of V and N in leaf tissue without affecting L, however, it was unclear which of the closely related β-hydroxylase genes were affected and to what levels (Rissler and Pogson, 2001).

In order to elucidate the *in vivo* functions of carotenoid hydroxylases, T-DNA knockout mutations in the β-hydroxylase 1 and 2 genes were isolated. Homozygous β-hydroxylase mutants were studied singly, in combination, and introduced into the previously isolated *lut1* mutant background in order to determine any specific or overlapping function(s) of the β- and ε- hydroxylases *in vivo.* The effects of various mutant genotypes on carotenoid biosynthesis, xanthophyll composition, and NPQ capacity are presented.

Results

Isolation of Arabidopsis T-DNA insertion mutants for β-hydroxylases and generation of double and triple mutant combinations

In order to study the *in vivo* functions of β-hydroxylase 1 and 2, plants containing T-DNA insertion alleles of each gene were isolated. Segregation of the kanamycin resistance marker indicated both mutant lines were segregating for a single Mendelian locus. Lines homozygous for each insertion were identified and designated *b1* and *b2*, for β-hydroxylase 1 and 2, respectively (*CrtR-b1* and *CrtR-b2* according to standard nomenclature; Hirschberg, 2001). The genome insertion sites were determined by sequencing products amplified from each mutant. The β-hydroxylase 1 gene (At4g25700) contains an insertion in the first intron 375 bp downstream from the start codon, while the β-hydroxylase 2 gene (At5g52570) contains an insertion in the fourth exon 527 bp downstream from the start codon (Figure 4.2A).

β-hydroxylases are non-heme di-iron proteins that contain ten conserved histidines required for iron binding and activity. The mutation of any one of the ten histidines resulted in a complete loss of activity (Bouvier et al., 1998). In the *b1* mutant, all ten histidines are downstream of the T-DNA insertion site. Five of the ten conserved histidines are downstream of the T-DNA insertion site in the *b2* mutant. Both mutants are therefore expected to result in a complete loss of functional protein. RT-PCR reactions using primer pairs designed to span the T-DNA insertion site of each mutant locus were

Figure 4.2. A. β-hydroxylase 1 and β-hydroxylase 2 insertional mutations (*b1* and *b2*, respectively). Exons are represented as filled boxes and introns as lines connecting the boxes. The sizes of the boxes and lines are drawn to scale. Locations of forward and reverse primers for β-hydroxylase 1 and 2 genes as well as the T-DNA insertion site are indicated. B. RT-PCR reactions with RNA extracted from wild type (WS) and homozygous *b1* and *b2* knockout mutants. Lane 1, 4 and 7, RT-PCR products from WS. Lanes 2, 5 and 8, RT-PCR products from *b1*. Lanes 3, 6 and 9, RT-PCR products from *b2*. Arabidopsis ε-cyclase specific primer pairs were used in lanes 1-3. β-hydroxylase 1 specific primer pairs were used in lanes 4-6. β-hydroxylase 2 specific primer pairs were used in lanes 7-9.

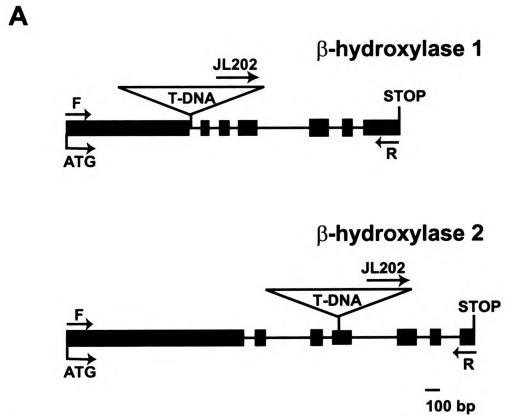
A
β-hydroxylase 1
JL202
T-DNA
STOP
F
ATG
R
β-hydroxylase 2
JL202
T-DNA
STOP
F
ATG
R
100 bp
B
1 2 3 4 5 6 7 8 9

Figure 4.2

used to determine whether full-length transcripts were produced in each genotype. The Arabidopsis lycopene ε-cyclase gene (At5g57030) was used as a control to demonstrate the integrity of all RNA preparations (Figure 4.2B). Both β-hydroxylase mRNAs were detected in WS and β-hydroxylase 1 and 2 mRNA was detected in *b2* and *b1* mutants, respectively. However, no amplification products were observed for β-hydroxylase 1 and 2 in the *b1* and *b2* mutants, respectively (Figure 4.2B). These results confirm that transcripts spanning the T-DNA insertion sites are not present in the respective knockout mutants.

Homozygous *b1* and *b2* mutants grown under moderate light conditions did not exhibit a whole plant phenotype differing from wild type (Figure 4.3). This result suggests significant functional redundancy exists between the two Arabidopsis β-hydroxylases. To further address this question, we attempted to generate a *b1b2* double mutant. Because no additional β-hydroxylase paralogs are present in the Arabidopsis genome, if β-hydroxylase 1 and 2 were the only gene products with β-hydroxylation activity, one would expect a complete absence of β-carotene derived xanthophylls and likely lethality in a *b1b2* double mutant. *b1* and *b2* mutants were crossed and viable, homozygous *b1b2* double mutants were identified by PCR at the expected frequency in F_2 progeny. This unexpected result suggests that other gene products must also have β-hydroxylation activity in Arabidopsis.

Although β-hydroxylase 1 and 2 are most active towards β-rings, each can also add a hydroxyl group to ε-rings *in vitro*, though at much lower efficiency (Sun et al., 1996; Tian and DellaPenna, 2001). Therefore, it is conceivable that the reciprocal could be true for the presumed ε-hydroxylase encoded by *LUT1*; that it can also function

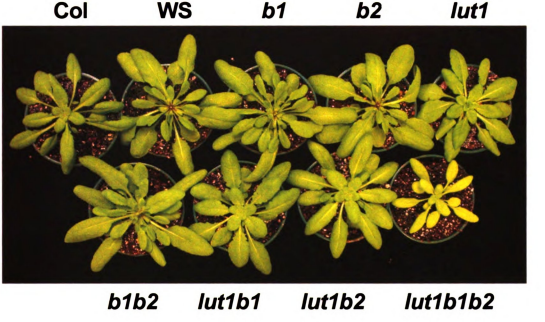

Figure 4.3. Six-week old wild type and mutant Arabidopsis plants. The plants shown were grown under normal light conditions (120-150 μmol photons m^{-2} s^{-1}) with a 12 hr photoperiod. Top row (from left to right): Col, WS, *b1*, *b2*, *lut1*. Bottom row (from left to right): *b1b2*, *lut1b1*, *lut1b2*, *lut1b1b2*.

Images in this dissertation are presented in color.

towards β-rings *in vivo*. In order to determine whether and to what extent LUT1 is functionally redundant with β-hydroxylase 1 and 2, the *b1* and *b2* mutations were introduced into the *lut1* mutant background, individually and in combination. *lut1* is in the Columbia (Col) background while the *b1* and *b2* mutations are in WS. Therefore, both Col and WS were used as controls in all experiments containing combinations of *lut1* with *b1* and *b2*. In order for a *lut1b1*, *lut1b2*, or *lut1b1b2* mutant phenotype to be considered significantly different from wild type, it must therefore be significantly different from both parental wild type phenotypes.

Homozygous *lut1b1* and *lut1b2* double mutants were selected from the corresponding F_2 populations. Neither double mutant showed a visible deleterious whole plant phenotype compared to wild type (Figure 4.3). In order to generate a line deficient in all three hydroxylase activities, *lut1b1* and *lut1b2* plants were crossed and viable homozygous *lut1b1b2* triple mutants were identified in the F_2 progeny. Unlike all other mutant combinations, the *lut1b1b2* triple mutant exhibited an obvious whole plant phenotype and was paler and smaller than either wild type parental ecotype (Figure 4.3).

Expression of carotenoid hydroxylases and lycopene cyclases genes in wild type and various mutant genotypes

To determine the effect of various mutant combinations on expression of the remaining unmutated hydroxylases and the two lycopene cyclase enzymes, mRNA levels for β-hydroxylase 1, β-hydroxylase 2, lycopene β-cyclase, and lycopene ε-cyclase were determined in wild type and different mutant genotypes. Because expression of

carotenoid biosynthetic genes is quite low in photosynthetic tissues, we developed TaqMan real time PCR assays for these studies. β-hydroxylase 1 and 2 mRNAs were not detected in genotypes that contain disruptions in these genes (Figure 4.4A and B), consistent with the RT-PCR analysis in Figure 4.2B. The steady-state β-hydroxylase 1 mRNA level was similar to wild type for all mutant genotypes that contain a functional β-hydroxylase 1 gene. β-hydroxylase 2 mRNA was also similar to wild type in the *lut1* mutant, but was increased approximately 50% and 30% relative to wild type in the *b1* and *lut1b1*, respectively. The expression of lycopene β- and ε-cyclase genes was also analyzed to determine whether specific hydroxylase mutant combinations affected cyclase gene expression. Both β- and ε-cyclase mRNA levels were nearly identical to wild type in all mutant genotypes except in the *lut1b1b2* triple mutant, where the β-cyclase mRNA level was 20% lower than wild type (Figure 4.4C and D).

Leaf pigment compositions in wild type and various mutant genotypes

Wild type and mutant genotypes were grown under 120-150 μmol photons m^{-2} s^{-1} for six weeks and their carotenoid compositions were quantified by HPLC. The level of total carotenoids in the mutant genotypes was not significantly different from their respective wild type controls when expressed per mole chlorophyll or fresh weight, except that the *lut1b1b2* triple mutant had 20% less total carotenoids on a fresh weight basis (data not shown). However, though carotenoid levels were unchanged, carotenoid compositions were significantly altered in most mutant lines (Table 4.1). The *b2* mutant was the least affected of all genotypes and did not differ significantly from WS, with the

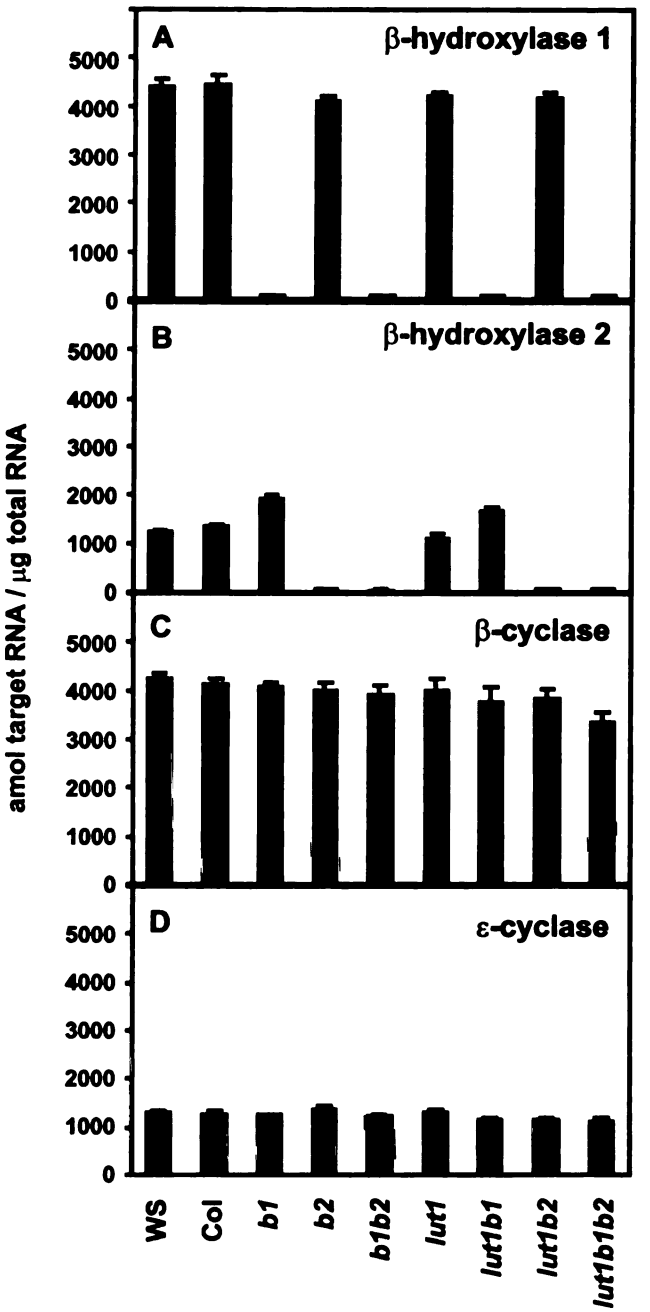

Figure 4.4. TaqMan Real Time PCR analysis of β-hydroxylase 1, β-hydroxylase 2, lycopene β-cyclase, and lycopene ε-cyclase mRNA levels in wild type and mutant genotypes. A. β-hydroxylase 1. B. β-hydroxylase 2. C. lycopene β-cyclase. D. lycopene ε-cyclase. Data are shown as means + S.D. (n=4).

Table 4.1. Wild type and mutant carotenoid composition in leaf tissue quantified by HPLC.

	Lutein	Zeinoxanthin	β-carotene	Neoxanthin	Violaxanthin	Antheraxanthin	Zeaxanthin	β-xanthophylls	Total Carotenoids
WS	100±9.0 (54)a	-	31.0±2.5 (17)a	30.5±5.1 (16)a	23.1±4.2 (13)a	-	-	53.6±6.7 (29)a	184.5±15.0a
Col	104.9±6.9 (58)a	-	31.2±6.7 (17)a	25.7±2.5 (14)a,b	20.8±3.9 (11)a,b	-	-	46.5±4.6 (25)a	182.5±14.2a
b1	118.5±6.2 (64)b	-	33.7±5.5 (18)a	16.9±2.0 (9)c	16.1±0.6 (9)b	-	-	33.0±3.5 (18)b	185.1±7.7a
b2	104.3±3.1 (60)a	-	26.9±2.9 (15)a	23.4±2.4 (13)b	21.2±1.0 (12)a	-	-	44.6±3.0 (25)a	175.8±7.8a
b1b2	130.9±4.7 (74)c	-	34.3±4.7 (19)a	3.2±1.1(2)d	8.4±1.7 (5)c	-	-	11.6±2.7 (7)c	176.8±4.1a
lut1	20.4±3.6(11)d	35.5±3.1 (20)a	29.8±6.2 (17)a	21.9±3.2 (12)b,e	46.5±4.1 (26)d	19.2±2.9 (11)a	5.5±1.0 (3)a	93.1±6.4 (52)d	178.8±16.7a
lut1b1	13.2±1.5 (7)e	44.1±3.6 (25)b	40.5±2.6 (23)b	19.3±1.8 (11)c,e	35.8±2.1 (20)e	20.5±3.3 (11)a	6.3±1.1 (3)a	81.9±4.6 (46)e	179.7±6.0a
lut1b2	11.5±2.2 (6)e	32.8±5.0 (18)a	41.1±3.2 (22)b	20.2±1.3 (11)c,e	49.1±1.6 (27)d	22.3±2.7 (12)a	6.7±1.0 (4)a	98.3±5.3 (53)d	183.8±6.9a
lut1b1b2	14.1±1.5 (8)e	88.1±8.6 (50)c	64.0±5.3 (36)c	1.6±0.1 (1)f	8.5±1.2 (5)c	-	-	10.1±1.7 (6)c	176.3±6.9a

The amount of carotenoid is expressed as mmol pigment/mol Chl a+b. Each value is the mean of six experiments ± S.D., with the relative molar percentage of each carotenoid given in parentheses. Values marked with the same characters are not significantly different from each other within a column (student's t-test, P> 0.05).

exception of a small decrease in N. The *b1* mutant has a much more severe phenotype with 30% and 45% decreases in V and N, respectively, and an 18% increase in L relative to WS. β-carotene was unchanged (Table 4.1) and the monohydroxy xanthophyll β-cryptoxanthin (β,β-carotene-3-ol) was not detected in *b1* or *b2* (data not shown). With the exception of increased L levels, the leaf carotenoid phenotype of the *b1* mutant is similar to that reported for a constitutive Arabidopsis β-hydroxylase 1 antisense transgene (Rissler and Pogson, 2001). The *lut1* mutant profile is consistent with previously published data (Pogson et al., 1996). *lut1* has an 80% decrease in L, accumulates the monohydroxy xanthophyll zeinoxanthin (β,ε-carotene-3-ol), and has a two-fold larger xanthophyll cycle carotenoid pool (V+A+Z) compared to Col. β-carotene and N levels were not significantly different in *lut1* compared to Col.

The homozygous *b1b2* double mutant was quite informative with regard to the role of various carotenoid hydroxylases in Arabidopsis. The most surprising result is that, though both β-hydroxylase activities are eliminated in the *b1b2* mutant, β-ring hydroxylated xanthophylls (L, V, N) are still synthesized, albeit at lower levels than WS or either single mutant. N and V are reduced 90% and 65%, respectively, and there is a 30% increase in L relative to WS. As with the single β-hydroxylase mutants, β-carotene is unchanged relative to wild type and β-cryptoxanthin does not accumulate. That *b1b2* has a more severe carotenoid phenotype than either single β-hydroxylase mutant indicates that the two mutations are additive and there is significant functional redundancy between β-hydroxylase 1 and 2. β-hydroxylase 1 and 2 are clearly the major β-ring hydroxylation activities as their combined absence decreases the levels of β-carotene derived xanthophylls nearly 80%. However, β-carotene derived xanthophylls are

still produced at 20% of wild type levels indicating there is an additional and previously undescribed activity in Arabidopsis that is also capable of producing β-carotene derived xanthophylls.

The carotenoid compositions in the *lut1b1* and *lut1b2* double mutants are quite different from their corresponding single mutant parents. This result indicates there is functional compensation or redundancy between LUT1 and the two β-hydroxylases. As with the single β-hydroxylase mutants, *b1* has a much greater effect on carotenoid composition than *b2* when each is introduced into the *lut1* background. The level of zeinoxanthin is increased 25%, V and L levels are reduced by 25% and 35%, respectively, and β-carotene is increased by 35% in *lut1b1* relative to *lut1*. The only carotenoids significantly changed in *lut1b2* are β-carotene and L.

An even more dramatic alteration in carotenoid composition was observed in the *lut1b1b2* triple mutant. *lut1b1b2* has the highest level of β-carotene in any genotype and more than twice the level of either wild type parent. There are severe reductions in N (95%) and V (60%) relative to either wild type and unlike *lut1*, *lut1b1*, or *lut1b2*, Z and A are absent in the triple mutant. β-carotene derived xanthophylls (V+Z+A+N) account for only 5% of the total carotenoid pool in the triple mutant *versus* 25-30% in wild type. L is unchanged in the triple mutant relative to the *lut1b1* and *lut1b2* double mutants, but zeinoxanthin is more than doubled relative to any other *lut1* containing genotype.

Seed pigment compositions of wild type and mutants

In order to determine the consequence of the various hydroxylase mutant genotypes on seed carotenoid composition, carotenoids from dry seeds of wild type and the various mutant genotypes were extracted and quantified by HPLC. In general, Arabidopsis seeds contain five to ten-fold less total carotenoids than leaf tissue on a dry weight basis. The major carotenoid in wild type seed is L. A and Z are present at high levels in seeds compared to leaf tissue but unlike leaf tissue, there is little β-carotene accumulation in seed. WS seeds have twice the level of total carotenoids as Col due to significant increases in all carotenoids except A and Z (Table 4.2). These data indicate significant quantitative genetic variations for seed carotenoid levels exist between Arabidopsis ecotypes.

The level of total carotenoids in *b1*, *b2*, and *lut1* mutant seeds does not differ significantly from their corresponding wild type controls. As with leaf tissue, the *b1* mutation has a more significant impact on seed carotenoid composition than *b2*. L is unchanged while Z, V, and N are significantly decreased and A is increased in *b1* seed relative to wild type. The carotenoid composition of *b2* seed is quite different from both *b1* and wild type with a 10% decrease in L, a nearly two-fold increase in A, and 30% increase in Z. Unlike *b1*, V and N in *b2* seed do not differ significantly from wild type. The differential effect of the two single mutations in seeds is most obvious in the total β-carotene derived xanthophylls accumulated: the *b1* mutation decreases while the *b2* mutation increases the level of total β-carotene derived xanthophylls in seed.

The phenotype of the *lut1* mutation in seeds differs dramatically from that in leaves. The most obvious difference is that L levels are only reduced 30% in seeds *versus* 80% in leaves and there is no zeinoxanthin accumulation in *lut1* seed. These surprising

Table 4.2. Wild type and mutant carotenoid composition in seed quantified by HPLC.

	Lutein	β-carotene	Neoxanthin	Violaxanthin	Antheraxanthin	Zeaxanthin	β-xanthophylls	Total Carotenoids
WS	108.9±5.2 (76)a	0.4±0.03 (0.3)a	7.5±0.2 (5)a	15.8±0.9 (11)a	4.8±0.4 (3)a	5.2±0.1 (4)a	33.3±1.2 (23)a	142.6±6.6a
Col	52.6±2.4 (75)b	0.1±0.03 (0.2)b	2.9±0.2 (4)b	3.0±0.2 (4)b	6.5±0.8 (9)b	4.6±0.3 (7)a,b	17.0±0.9 (24)b	69.7±3.7b
b1	111.0±2.7 (80)a	0.4±0.08 (0.3)a	4.2±0.3 (3)c	11.4±0.4 (8)c	7.1±0.2 (5)b,c	4.0±0.1 (3)b	26.7±0.8 (19)c	138.1±3.7a
b2	96.6±4.4 (72)c	0.3±0.04 (0.2)a	6.3±0.6 (5)a	15.8±1.0 (12)a	8.5±0.5 (6)c	6.8±0.3 (5)c	37.4±1.5 (28)d	134.3±6.7a
b1b2	77.1±2.0 (90)d	0.4±0.05 (0.4)a	1.7±0.1 (2)d	1.3±0.2 (2)d	2.3±0.2 (3)d	3±0.2 (3)d	8.3±0.4 (10)e	85.7±2.3c
lut1	35.9±2.9 (57)e	0.4±0.02 (0.6)a	4.9±0.4 (8)c	12.3±1.2 (20)c,e	5.6±0.5 (9)a,b	4.0±0.2 (6)b	26.8±1.5 (42)c	63.1±5.1b
lut1b1	77.2±2.0 (83)d	0.6±0.05 (0.7)a	4.2±0.3 (4)c	6.5±0.5 (7)f	3.5±0.3 (4)e	1.5±0.1 (2)e	15.7±0.8(17)b	93.6±2.7d
lut1b2	67.4±0.3 (68)f	0.4±0.02 (0.4)a	8.1±0.7 (8)a	14.1±0.5 (14)a,e	6.0±0.4 (6)b	3.5±0.2 (4)d	31.7±1.1 (32)a	99.4±1.6d
lut1b1b2	50.7±3.7 (78)b	2.3±0.2 (3.5)c	1.3±0.3 (2)d	4.1±0.7 (6)b	3.5±0.6 (5)e	3.5±0.4 (5)d	12.4±1.3 (19)f	65.4±4.2b

The amount of carotenoid is expressed as nmol pigment/g seed. Each value is the mean of three experiments ± S.D., with the relative molar percentage of each carotenoid given in parentheses. Values marked with the same characters are not significantly different from each other within a column (student's t-test, P> 0.05).

results can be understood by considering the total amount of L produced in leaf and seed as a percentage of dry weight. In Col leaves, the molar amount of L synthesized is ten times that in seeds (data not shown). Thus, all things being equal, the residual or secondary ε-hydroxylase activity that allows production of 20% wild type L levels in *lut1* leaf tissue is apparently sufficient to fully convert the much lower level of zeinoxanthin in seeds to L. N and V are increased in *lut1* seeds while A and Z are almost unchanged. This contrasts with the large accumulation of A and Z in *lut1* leaf tissue relative to wild type (Table 4.1).

Unlike the single mutant parent lines, the carotenoid levels of *b1b2* seed are reduced 40% relative to wild type (WS). However, as in leaf tissue, β-carotene derived xanthophylls are still present at 10% of wild type levels despite the fact that both β-hydroxylase activities are eliminated in the *b1b2* double mutant. There is also a dramatic shift in carotenoid composition in the *b1b2* mutant with Z, A, V, and N reduced 40%, 50%, 90%, 80%, respectively, relative to wild type.

Interpretation of seed carotenoid data from *lut1b1*, *lut1b2*, and *lut1b1b2* is more difficult due to the large quantitative differences in total and individual carotenoid levels between the two parental ecotypes (Col and WS). However, assuming the loci responsible for these quantitative differences are segregating independently of the mutant hydroxylase loci, F_2 progeny should segregate randomly for quantitative differences in seed carotenoids. To minimize the impact of any quantitative loci, twenty F_2 individuals homozygous for *lut1b1*, *lut1b2*, and *lut1b1b2* were selected and their seeds pooled for analyses, which should average out quantitative differences in the progeny while maintaining selection for the respective mutant loci. Consistent with this hypothesis,

lut1b1 and *lut1b2* double mutants have similar amounts of total carotenoids relative to each other and are intermediate relative to their Col and WS parents. *lut1b2* has higher levels of individual and total β-carotene derived xanthophylls compared to *lut1b1*, while the *lut1b1* mutant has a higher level of L than *lut1b2*. The total carotenoid level of the *lut1b1b2* triple mutant seed is 35% lower than either *lut1b1* or *lut1b2*, but L still accounts for 80% of total carotenoids. Most β-carotene derived xanthophylls are significantly reduced in the triple mutant relative to *lut1b1* or *lut1b2* and there is a four to twenty-fold increase in β-carotene relative to other genotypes (Table 4.2).

Photosynthetic efficiency and NPQ capacities in leaves of wild type and mutant genotypes under normal light conditions

Leaf carotenoid compositional analysis showed that production of α- and β-carotene derived xanthophylls could be severely impacted by disrupting one or more hydroxylase activity (Table 4.1). To determine the effect of modified xanthophyll profiles on photosystem efficiency and NPQ, chlorophyll fluorescence was measured in leaves of each mutant genotype grown under moderate light conditions (120-150 μmol photons m^{-2} s^{-1}). The Fv/Fm ratio (a measurement of maximum photosystem II photochemical efficiency) of wild type (Col and WS), *lut1*, *b1*, and *b2* mutants did not differ significantly, but *b1b2*, *lut1b1*, *lut1b2*, and *lut1b1b2* had lower Fv/Fm ratios compared to wild type (Table 4.3). These results suggest that the altered carotenoid composition resulting from elimination of more than one hydroxylase activity can affect the efficient transfer of absorbed light energy to the photosystem II reaction center. Chl a/b ratios in

Table 4.3. Chl a/b ratios and maximum PS II efficiencies (Fv/Fm) in wild type and mutant leaves.

	Chl a/b	Fv/Fm
WS	2.47±0.07a	0.846±0.017a
Col	2.52±0.06a	0.841±0.006a
b1	2.54±0.08a	0.837±0.013a
b2	2.50±0.05a	0.847±0.006a
b1b2	2.63±0.05b	0.824±0.011b
lut1	2.61±0.06a	0.843±0.010a
lut1b1	2.64±0.02b	0.827±0.010b
lut1b2	2.67±0.03b	0.826±0.015b
lut1b1b2	2.74±0.02c	0.821±0.007b

Values marked with the same characters are not significantly different from each other within a column (student's t-test, P> 0.05).

b1 and *b2* mutants do not significantly differ from wild type but are significantly increased in the *lut1* mutant as previously reported (Lokstein et al., 2002) and also in all the double mutants. The *lut1b1b2* triple mutant has the most drastically affected Chl a/b ratio, and is significantly higher than all the other genotypes (Table 4.3).

As with wild type seed carotenoid composition, significant genetic variation was observed for NPQ between WS and Col. WS has both a slower induction rate and lower NPQ amplitude compared to Col. The *b2* mutant has NPQ induction and amplitude similar to WS while the *b1* mutant, where the relatively highly expressed β-hydroxylase 1 gene is disrupted, has a slower induction and ~10 % reduction in NPQ amplitude. This impact on NPQ is consistent with the greater impact of the *b1* mutation on xanthophyll cycle carotenoids (Table 4.1). Light activation of NPQ is also delayed in *lut1* and NPQ amplitude is reduced, consistent with previous reports (Pogson et al., 1998; Lokstein et al., 2002).

In *b1b2*, there is a synergistic effect of the two β-hydroxylase mutations on both carotenoid composition and NPQ. NPQ induction and amplitude are reduced to a much greater extent in *b1b2* than in either single mutant parent (Figure 4.5A). In *lut1b1* and *lut1b2* double mutants, the NPQ phenotypes of the single mutants are additive and likely result from the combined deficiency of both L and xanthophyll cycle carotenoids in these double mutants. That the *lut1b1* phenotype is more severe than the *lut1b2* phenotype can be predicated from the respective single mutants. The *lut1b1b2* triple mutant has a very rapid initial induction of NPQ, however the NPQ level obtained is greatly reduced compared to wild type and all other mutant genotypes (Figure 4.5B).

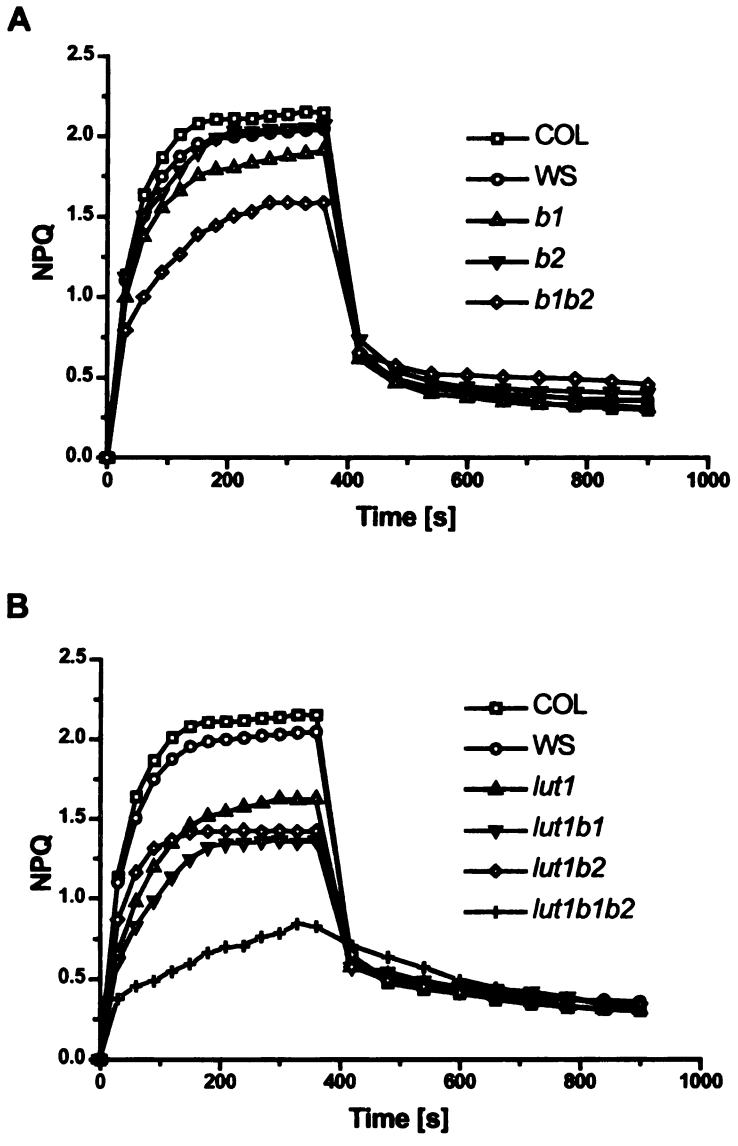

Figure 4.5. NPQ analysis of wild type and mutant genotypes. A. Wild type (WS and Col) and *b1*, *b2* and *b1b2* mutants. B. Wild type (WS and Col) and *lut1*, *lut1b1*, *lut1b2*, *lut1b1b2* mutants. Chlorophyll fluorescence was measured during 6 min of illumination with a PFD of 500 μmol photons m^{-2} s^{-1} followed by 9 min of dark relaxation. Each data point is the mean of six independent experiments and all S.D. are within the symbol sizes.

Discussion

In plants, carotenoid hydroxylases catalyze the formation of α- and β-carotene derived xanthophylls, which perform a variety of critical roles in the photosynthetic apparatus. Three genes that participate in carotenoid hydroxylation reactions have been identified in the Arabidopsis genome through combined molecular and genetic analyses. Among these, two β-hydroxylases have been cloned and shown to be highly active against β-rings and weakly active against ε-rings (Sun et al., 1996; Tian and DellaPenna, 2001). We have isolated T-DNA knockout mutants in the β-hydroxylase 1 and 2 genes and shown that expression of mRNA for each gene is fully eliminated. A third Arabidopsis gene product required for ε-ring hydroxylation has been genetically defined by the *lut1* mutation but has not yet been cloned (Pogson et al., 1996). The effects of these three carotenoid hydroxylase mutations individually and in various combinations have been used to assess the roles, interactions, or functional redundancies of the hydroxylase activities *in vivo*.

LUT 1 is the major ε-ring hydroxylating activity *in vivo*

The effect of mutating the *LUT1* locus, the presumed ε-hydroxylase, on leaf carotenoid composition has been described previously (Pogson et al., 1996; Lokstein et al., 2002) and has the most dramatic effects of any single hydroxylase mutation. The fact that ε-ring hydroxylation is reduced by 80% in *lut1*, and zeinoxanthin accumulates indicates the *LUT1* gene product is the primary ε-ring hydroxylation activity in

photosynthetic tissue. The absence of β,ε-carotene-3'-ol (α-carotene with a single hydroxyl group on the ε-ring) in *lut1* or any other mutant line studied suggests that β-ring hydroxylation precedes ε-ring hydroxylation during L synthesis. Both *lut1* alleles (*lut1-1* and *lut1-2*) are EMS-derived and still contain 15-20% of wild type L levels. This suggests that either both alleles are leaky and the L made is from residual LUT1 activity or, if the alleles are null, that there is a second ε-ring hydroxylation activity in Arabidopsis. The cloning of *LUT1* and identification of a definitive null mutation in the locus is required to distinguish between these possibilities.

β-hydroxylase 1 and 2 are functionally redundant *in vivo*

Neither *b1* nor *b2* exhibits a whole plant phenotype suggesting that significant functional redundancy exists between the two β-hydroxylases. This observation is consistent with our inability to identify β-hydroxylase mutants in various EMS mutant screens (data not shown). β-hydroxylase 1 and 2 are closely related at the level of protein sequence, have similar activities *in vitro* and are coordinately expressed in all wild type tissues analyzed, although β-hydroxylase 1 mRNA is always present at much higher levels than β-hydroxylase 2 mRNA (Tian and DellaPenna, 2001). The high relative expression level of β-hydroxylase 1 suggests it might be responsible for the majority of β-ring hydroxylation activity in leaf tissue. The alterations to carotenoid composition in the β-hydroxylase single mutants support this hypothesis: β-carotene-derived xanthophylls are more severely affected in *b1* than in *b2* (Table 4.1; Figure 4.5).

The function of β-hydroxylase 2 is difficult to discern by single mutant analysis, because it is not significantly different from wild type in carotenoid composition and NPQ function. However, β-hydroxylase 2 is clearly both functionally additive and complementary to β-hydroxylase 1 *in vivo*. The *b1b2* double mutant has a more severe effect on β-ring hydroxylations than the *b1* mutation alone (Table 4.1) indicating that β-hydroxylase 2 can compensate to a significant degree for the absence of β-hydroxylase 1 activity in the *b1* mutant. Moreover, β-hydroxylase 2 is also able to compensate for the loss of both LUT1and β-hydroxylase 1 (in the *lut1b1* double mutant) to almost the same extent as β-hydroxylase 1 in the *lut1b2* background (Table 4.1). These data indicate that although β-hydroxylase 2 is expressed at a much lower level than β-hydroxylase 1 in wild type, it plays an important role in functional redundancy in the carotenoid pathway. The functional redundancy of β-hydroxylase 2 in the *b1* and *lut1b1* mutants may be enhanced in part by a small increase in β-hydroxylase 2 expression (Figure 4.4B).

The fact that different *b1* and *b2* containing carotenoid mutant genotypes are preferentially affected in the accumulation of specific carotenoids (Table 4.1), indicates that while the two β-hydroxylases can partially compensate for each other, they are not entirely equivalent and likely have specialized functions. This is most apparent when a β-hydroxylase 1 or 2 deficiency is present in wild type or the *lut1* background (Table 4.1). While the biochemical basis for two closely related but functionally distinct β-hydroxylase enzymes in Arabidopsis is unknown, tomato and pepper have also maintained two homologous β-hydroxylase genes, suggesting this may be a common theme in carotenoid synthesis in plants.

β-carotene-derived xanthophylls are still produced in the *b1b2* double mutant indicating additional β-ring hydroxylation activity exists *in vivo*

A major and unexpected outcome of carotenoid hydroxylase double mutant analyses is that when both known Arabidopsis β-hydroxylase activities are eliminated in the *b1b2* double mutant, β-carotene-derived xanthophylls are still produced at significant levels. The 80% reduction in β-carotene-derived xanthophylls in *b1b2* confirms that β-hydroxylase 1 and 2 are the predominant β-carotene hydroxylation activities *in vivo*. However, β-carotene-derived xanthophylls still account for 7% of the total carotenoids in the *b1b2* mutant *versus* 29% in wild type. In addition, β-ring hydroxylation is also necessary for α-carotene to be converted to L, and L is produced at even higher levels in the *b1b2* double mutant than wild type. The synthesis of β-carotene derived xanthophylls and the accumulation of L in *b1b2* indicates an additional, previously unknown, β-ring hydroxylation activity exists *in vivo*. These data raise the question: what is the nature of this additional β-ring hydroxylation activity?

Three hypotheses can be proposed for the additional β-ring hydroxylation activity present in the *b1b2* background. (1) It is a third, novel β-hydroxylase enzyme unrelated at the protein sequence level to known β-hydroxylases. (2) It is a secondary, intrinsic activity of the *LUT1* gene product (ε-hydroxylase) capable of β-ring hydroxylation *in vivo*. (3) It is a second LUT1-like enzyme that is active toward both types of rings. Searches of the Arabidopsis genome using representative β-hydroxylase protein sequences from all three phyla as queries failed to identify additional homologs beyond

β-hydroxylase 1 and 2 (data not shown). Therefore, if the additional activity resides in a third β-hydroxylase enzyme, it would represent a new structural class of β-hydroxylases in nature. The possibility that LUT1 might hydroxylate β-rings in addition to ε-rings *in vivo* has precedent as both β-hydroxylase 1 and 2 can also hydroxylate ε-rings *in vitro*, although quite poorly relative to β-ring substrates (Sun et al., 1996; Tian and DellaPenna, 2001). A reciprocal activity could also be true for LUT1. Finally, if LUT1 does not have a secondary β-hydroxylase activity, it is possible that a LUT1 paralog exists that is active towards both β- and ε- rings. This would be analogous to the two highly conserved Arabidopsis β-hydroxylases, however this LUT1 paralog would likely be a minor component of total hydroxylase activity because it cannot fully compensate for the ε-ring hydroxylation deficiency in *lut1*. Clearly, isolation of *LUT1* and biochemical characterization of its product will serve to delineate these various hypotheses.

Double and triple mutants indicate overlapping functions and interactions between the β- and ε- hydroxylases

In order to investigate any overlapping functions or interactions between the β- and ε- hydroxylases of Arabidopsis, the *b1* and *b2* mutations were introduced into the *lut1* background to generate the corresponding double mutants. As with the single *b1* and *b2* mutants, *b1* has a more pronounced effect in the *lut1* background than *b2*. *lut1b1* has decreased V and increased zeinoxanthin relative to *lut1*. β-hydroxylase 1 and 2 also appear to have a low level of ε-ring hydroxylation activity *in vivo* as elimination of either activity in the *lut1* background further decreases the level of L (Table 4.1).

The failure to eliminate L in *lut1b1* and *lut1b2* may be due to a low level of ε-ring hydroxylation activity *in vivo* from the single functional β-hydroxylase still present in each double mutant. To further delineate these possibilities, we attempted to create a *lut1b1b2* triple mutant. Assuming no other carotenoid hydroxylases are present in Arabidopsis and the *lut1-2* allele is null, one would expect the triple mutant to lack all xanthophylls and likely be lethal as a result. Surprisingly, homozygous *lut1b1b2* mutants were isolated at the expected ratio from crosses and though paler green than wild type, they were viable and set seed. Because the L level in *lut1b1b2* (where both β-hydroxylase 1 and 2 activities are eliminated) is not further reduced relative to *lut1b1* or *lut1b2*, we conclude that β-hydroxylase 1 and 2 do not contribute significantly to ε-ring hydroxylation *in vivo*. The L synthesized in *lut1b1b2* must therefore be due to either residual ε-ring hydroxylation activity from the mutant LUT1 enzyme or another ε-hydroxylase. In either case, LUT1 or a second ε-hydroxylase must also be active toward β-rings as β-ring hydroxylation still occurs at significant levels in *lut1b1b2* (Figure 4.6).

Total hydroxylation activity and the synthesis of specific xanthophylls are differentially affected in the hydroxylase mutants

The impact of hydroxylase deficiencies has been discussed thus far based on the relative changes to specific carotenoid (e.g. violaxanthin) or groups of α- and β- carotene derived xanthophylls in each genotype (Table 4.1). In the case of double and triple mutants, the effects on individual xanthophylls can be quite extreme. However, focusing only on the species or groups of xanthophylls affected leads to underestimation of total β-

ring hydroxylation activity because hydroxylation of the β-rings of zeinoxanthin and L are overlooked. A more accurate accounting of total hydroxylation activity is to consider the total hydroxylation of each ring type (total moles of hydroxyl groups introduced onto β- and ε-rings, respectively, see Figure 4.6). From this perspective, though the *b1* mutant significantly decreases N and V (Table 4.1), the level of hydroxylated β-rings produced by the mutant is hardly affected and total ring hydroxylation (β- plus ε-rings) is unchanged relative to wild type (Figure 4.6). This is even more apparent in *b1b2* where β-carotene derived xanthophylls are reduced 80% but β-ring hydroxylations are only reduced 25% and total hydroxylation is not significantly changed relative to wild type. These data suggests an even higher degree of functional redundancy among the hydroxylases with regard to their ability to perform β- and/or ε-ring hydroxylations in the absence of another. The ability of each hydroxylase to functionally compensate for another in the production of specific xanthophylls is clearly much more limited (Table 4.1).

The differential effect of hydroxylase deficiencies on total hydroxylation levels and the production of specific xanthophylls suggests a level of biochemical regulation in the production of different xanthophylls that has not previously been considered. Whether this regulation stems from innate differences in the substrate preference and turnover rates of each hydroxylase or is due to the differential ability of hydroxylases to participate in biosynthetic complexes for the production of certain xanthophylls is unknown. It is also possible that specific molecular or biochemical regulatory mechanisms could be activated in the mutants to preferentially provide for a minimum level of β-ring hydroxylation.

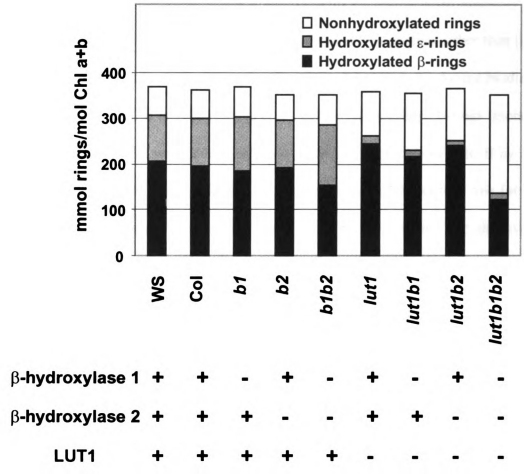

Figure 4.6. Hydroxylation levels of β- and ε-rings in all genotypes. The molar amount of hydroxylated β- and ε-rings and non-hydroxylated rings were calculated from the leaf tissue carotenoid composition for each genotype. The presence or absence of each of the three known carotenoid hydroxylases in each genotype is indicated by a plus or minus sign, respectively.

The surprisingly high level of total β-ring hydroxylation retained in all mutant genotypes suggests that hydroxylated β-rings, rather than specific xanthophylls, might be the structural and/or functional minimal requirements for photosystem assembly *in vivo*. This is consistent with a recent report that 3-hydroxy-β-end groups, rather than β-carotene derived xanthophylls *per se*, are the minimal requirements for the binding of xanthophylls to LHC II *in vitro* (Phillip et al., 2002). Hydroxylated β-rings (mono- or di-hydroxy xanthophylls) were able to promote the *in vitro* assembly of LHC II by facilitating the correct folding of LHC II proteins. In light of this critical role for 3-hydroxy-β-end groups in LHC II assembly, it is easier to understand why the levels of β-ring hydroxylations, rather than specific xanthophylls, are maintained *in vivo* in the carotenoid hydroxylase mutants. An extreme example of this principle is *lut1b1b2*, which has the lowest level of β-carotene derived xanthophylls of any genotype (Table 4.1). In *lut1b1b2*, zeinoxanthin (which contains a single 3-hydroxy-β-end group) is increased for more than two-fold relative to *lut1* and accounts for 75% of the total 3-hydroxy-β-end groups.

Modified xanthophyll composition in the hydroxylase mutants correlates with alterations in NPQ induction kinetics

In addition to their essential roles in LHC II assembly and photosystem structure, xanthophylls also have important functions in photoprotection. The severity of xanthophyll compositional changes in the different hydroxylase mutant genotypes coincides with their degree of compromised NPQ induction kinetics (Table 4.1; Figure

4.5). Other carotenoid biosynthetic mutants previously used to study xanthophyll function in Arabidopsis *in vivo*, *lut1*, *lut2*, *npq1*, *aba1* (*npq2*) and their double mutants, block the production of one or more xanthophylls (Niyogi et al., 1998; Pogson et al, 1998; Lokstein et al., 2002). The carotenoid hydroxylase mutants are unique in that the synthesis of multiple xanthophylls are attenuated to different degrees, rather than eliminated. This allows one to modify the types and levels of specific xanthophylls involved in light harvesting and photoprotection under normal growth conditions.

Because Chl b and N are primarily associated with LHC II (Ruban et al., 1999), the increased Chl a/b ratio and decreased N levels in the double and triple hydroxylase mutants (Tables 4.1 and 4.3) are consistent with a reduction in LHC II levels. These data are also consistent with the assembly or stability of the peripheral LHC II being affected, and are correlated with impaired NPQ induction and amplitude in the double and triple hydroxylase mutants (Figure 4.5). In addition, the Fv/Fm ratios in all double and triple hydroxylase mutants are significantly decreased, indicating the quantum efficiency of photosystem II is also affected due to their altered carotenoid compositions. Similar observations have been reported for the *lut1*, *lut2*, *aba1*, and *lut2aba1* mutants (Tardy and Havaux, 1996; Pogson et al., 1998; Lokstein et al., 2002). The most severe impact on NPQ and quantum efficiency occurs in the *lut1b1b2* mutant where L and xanthophyll cycle carotenoids are reduced over 80% relative to wild type. Given the continuum of xanthophyll changes in the full suite of hydroxylase mutants, it will be quite informative for our understanding of xanthophyll functions to assess the responses of the hydroxylase mutants to high light stress.

Insights into carotenoid pathway regulation in photosynthetic tissues

In addition to differentiating the overlapping functions of carotenoid hydroxylases *in vivo*, the phenotypes of hydroxylase mutants provide further insight into levels of regulation that exist in the carotenoid pathway. The extreme changes in xanthophyll composition are not the result of a corresponding alteration in total hydroxylation activity (Figure 4.6) but rather a large shift in the accumulation of carotene cyclization products in each mutant line (Figure 4.7). The increased L level in *b1b2* relative to wild type indicates synthesis of β,ε-carotene branch carotenoids is enhanced when β-ring hydroxylation is blocked. Likewise, syntheses of β,β-carotene branch carotenoids are enhanced 60% relative to wild type when ε-ring hydroxylation is blocked in *lut1*. Most significantly, in the *lut1b1b2* triple mutant, where all three hydroxylases are absent, the accumulation of β,ε- and β,β- carotenoids returns to a wild type ratio (Figure 4.7). These results indicate that one response of the carotenoid pathway to the absence of one or more hydroxylases is to shift synthesis between the β,ε- and β,β- carotene branches of the pathway; a regulation that can only take place at the level of carotenoid cyclases (Figure 4.1). This regulation appears to be translational or post-translational as mRNA levels for both the β- and ε-lycopene cyclase are essentially unchanged in all hydroxylase mutant genotypes (Figure 4.4C and D). A model put forth by Cunningham and Gantt (1998) proposed that β,ε- or β,β- carotene production is determined by the formation/stability of different carotenoid desaturase/cyclase complexes in the thylakoid membrane. Our data are consistent with the carotenoid hydroxylases participating in these proposed complexes to determine the relative levels of β,ε- or β,β- carotene produced in leaf tissue. An alternative explanation is that the hydroxylase deficiencies differentially affect the

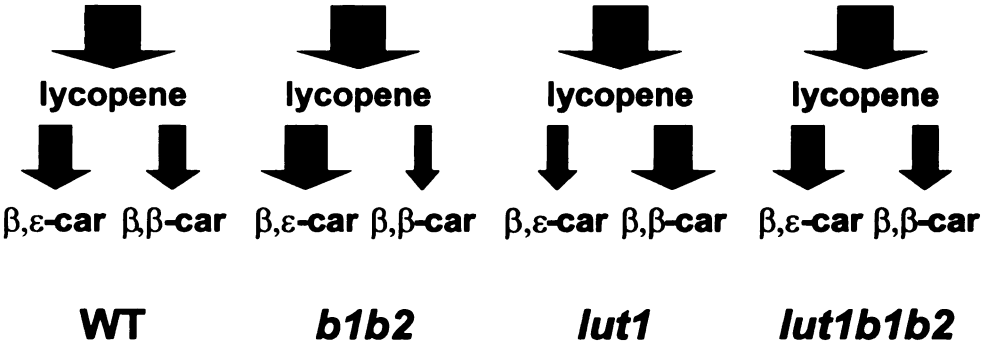

Figure 4.7. Accumulation of β,β-carotene and β,ε-carotene branch carotenoids in leaves of wild type and selected hydroxylase mutants. The total relative amount of carotenoids accumulated through lycopene and the β,β- and β,ε-cyclization branches of the pathway are indicated by arrows. The width of an arrow is proportional to the percentage of each carotenoid accumulated. The arrows leading to lycopene are identical in width because the total amount of carotenoids produced in each line do not differ significantly (refer to Table 4.1).

substrate specificity/affinity of the cyclases by feedback regulation due to the altered xanthophylls accumulated in each mutant line.

The impact of hydroxylase deficiencies on seeds carotenoid compositions

The seed carotenoid content of mutants was also analyzed to determine the penetration of each mutant phenotype in a non-photosynthetic tissue. Lutein is the main carotenoid in both wild type leaves and seeds (50% and 80%, respectively). β-carotene accounts for about 15% of the total carotenoids in wild type leaf tissue (Table 4.1) but is present only in trace amounts in seeds (Table 4.2). This difference may be due to more efficient hydroxylation of β-carotene in seeds in the absence of the carotenoid binding proteins present in photosynthetic tissue or to enhanced turnover of β-carotene during seed development relative to leaf tissue. In general, the effect of β-hydroxylase mutations on seed carotenoid composition is similar to that in leaves. The *b1* mutation has a more significant impact than *b2* and the *b1b2* double mutant is more severe than either single mutant but still synthesizes β-carotene derived xanthophylls. The most surprising result from studies of mutant seed is that unlike *lut1* leaves, *lut1* seeds do not accumulate zeinoxanthin, which is a signature phenotype for the *lut1* mutation in leaves. It is possible that unlike leaf tissue, zeinoxanthin in *lut1* seed is degraded or unbound zeinoxanthin is more readily accessible to the residual ε-ring hydroxylation activity of LUT1 or a second ε-ring hydroxylation enzyme.

In contrast to leaf tissue, where individual and total carotenoids do not statistically differ between ecotypes, WS seed accumulates more than twice the carotenoid level of

Col (Table 4.2). Interpreting the impact of combined *lut1, b1*, and *b2* mutations on seed carotenoid profiles is confounded by the segregation of other quantitative loci in the Col (*lut1*) and WS (*b1* and *b2*) parental ecotypes.The only genotype combinations that can be directly compared are *lut1b1* and *lut1b2*, which were taken from seeds pooled from twenty independent homozygous F_2 plants. As observed in leaf tissue, the *b1* mutation has a more significant impact in the *lut1* background than *b2*. Like leaves, β-carotene derived xanthophylls are still synthesized at reduced levels in *b1b2* and *lut1b1b2* seeds, despite the absence of both known β-hydroxylases. These results indicate that, an additional β-ring hydroxylation activity is also present in non-photosynthetic tissue.

Conclusions

Our data reveal the multifunctional nature of carotenoid hydroxylases, provide insights into the overlapping functions and interactions of the three known hydroxylases in Arabidopsis and the regulation of carotenoid ring hydroxylations *in vivo*. The hydroxylase mutants also present additional examples of the remarkable flexibility of plant photosystems with regard to carotenoid composition and will be important tools for furthering our understanding of xanthophyll functions in plants, especially in response to high light stress. Future studies will also focus on cloning of the *LUT1* gene to determine the biochemical nature of this novel ε-hydroxylase and assess its role in the synthesis of α- and β- carotene derived xanthophylls.

Methods

Identification of T-DNA mutant lines and construction of double and triple mutant combinations

The *b1* and *b2* plants were identified from the T-DNA insertion mutant population available through the University of Wisconsin Biotechnology Center (Madison, Wisconsin; http://www.biotech.wisc.edu/Arabidopsis/) using the screening strategy previously described (Krysan et al., 1999). The T-DNA border primer was used for sequencing amplification products to determine the site of insertion into each gene (Figure 4.2A).

Homozygous *b1* and *b2* plants were crossed and individual F_1 seeds were grown and self-fertilized to obtain the F_2 generation. The *b1* and *b2* genes are located on chromosome 4 and 5, respectively and therefore segregate independently in an F_2 population. The genotype of the F_2 individuals was determined by PCR using β-hydroxylase 1 and 2 specific primers as well as T-DNA border primer and homozygous *b1* and *b2* plants selected. The primer sequences for screening and identifying homozygous β-hydroxylase 1 and 2 T-DNA knockout mutants are: β-hydroxylase 1 (forward, 5'-TTAAACGCTTTTCTGTCTGTTACGTCGTC-3'; reverse, 5'-TTGTGATTGTAGGTCACTCCCGATCATAG-3') and β-hydroxylase 2 (forward, 5'-AATAGAAGTGGAGTGATTCGCTGTCGATG-3'; reverse, 5'-AAGGACACATCGGTTCCAGAAGAAATAAG-3'), respectively.

Homozygous *b1* and *b2* single mutants were crossed with each other and to the EMS mutagenized *lut1-2* mutant. Homozygous *b1b2*, *lut1b1*, and *lut1b2* were selected

from the segregating F_2 population. Crosses were then performed between homozygous *lut1b1* and *lut1b2* plants and *lut1b1b2* triple mutants were isolated from the F_2 population.

RT-PCR and TaqMan real time PCR assay

Total RNA was extracted from wild type (WS), *b1*, and *b2*, respectively, as described (Tian and DellaPenna, 2001). Ten μg of total RNA was used as template and first-strand cDNA synthesis was conducted using the SuperScript Preamplification System (Invitrogen, Carlsbad, CA) according to manufacture's protocol. Subsequent amplification of the first-strand cDNA was performed using Taq DNA polymerase (Promega, Madison, WI) and a MJ Research DNA Engine^{TM} thermal cycler (Waltham, MA). Primers specific for lycopene ε-cyclase (forward, 5'-CGAACAAAAGAATCTCGCC-3'; reverse, 5'-AAACCCTTGCCACATCCT-3') were used as a control to test the integrity of all plant RNA samples. Primer pairs spanning T-DNA insertion sites in β-hydroxylase 1 and 2 open reading frames, β-hydroxylase 1 (forward, 5'-AACCGCCGTTACATTCAAACC-3'; reverse, 5'-ACCTACAGGGAAACGCTTG-3') and β-hydroxylase 2 (forward, 5'-TTCTCCGCAAACCACCCTATA-3'; reverse, 5'-CCAACTCTTCTTTTCCTCCCACTTC-3') were used to determine whether full-length transcripts were synthesized in the corresponding mutants. The PCR program for amplification was: 94°C for 3 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and

72°C for 2 min, and a final extension at 72°C for 10 min. The PCR products were analyzed on a 1% agarose gel.

TaqMan real time PCR assay was adopted for quantification of steady-state mRNA levels in the wild type and hydroxylase mutant plants. First-strand cDNA products from three independent reverse transcription reactions were pooled and used for TaqMan analysis. TaqMan probes and primers (Table 4.4) were designed from cDNA sequences of Arabidopsis β-hydroxylase 1 (AY113923), β-hydroxylase 2 (AY117225), lycopene β-cyclase (U50739), and lycopene ε-cyclase (U50738) using Primer Express software (Perkin Elmer Applied Biosystems, Foster City, CA). Reporter (5' end) and quencher (3' end) dyes for the TaqMan probes are FAM and TAMRA, respectively. TaqMan real time PCR assay conditions and analysis were performed as previously described (Tian and DellaPenna, 2001).

Plant material and HPLC analysis of pigment content

Two wild type Arabidopsis thaliana ecotypes (Col and WS) and homozygous *b1*, *b2*, *lut1*, *b1b2*, *lut1b1*, *lut1b2*, *lut1b1b2* plants were grown in soil at 120-150 μmol photons m^{-2} s^{-1} under a 12hr day: 12hr night (21°C: 18°C) cycle in growth chambers. Carotenoids from six-week old leaf tissue were extracted in a 96-well format. A well-exposed leaf from each plant was selected and same-size leaf area was excised using a cork borer. A leaf disk was allocated in every tube in a 96-well rack (Dot Scientific Inc., Burton, MI), and 300 μl of 60:40 (v:v) Acetone : Ethyl Acetate followed by 200 μl distilled water was added. Three 4 mm glass beads (Fisher Scientific, Pittsburgh, PA)

Table 4.4. Sequences of TaqMan probes and primers.

Gene	Primer/Probe	5'-3' sequence
β-hydroxylase 1	forward	CTCGTGCACAAGCGTTTCC
	reverse	GGCGACCTTTCGGAGGTAA
	probe	TGTAGGTCCCATCGCCGACGTCC
β-hydroxylase 2	forward	CCCATTGCCAACGTTCCTTA
	reverse	TGTCTGTGTGGTGTAGCTGGTG
	probe	CTTCGAAAGGTCGCCGCCGC
β-cyclase	forward	TCATTACTGGCACGGATTCTTG
	reverse	GAGCGACAACCCGAAGACC
	probe	TCCAGGCTGTTTCTCCCGGAACTG
ε-cyclase	forward	CCTTTGGTGCTGCCGC
	reverse	CTTCAGACAAAGATCTCACAACTGAAT
	probe	AGCATGGTACATCCCGCAACAGGC

were added to each tube, plate was sealed, fastened onto a commercial paint shaker (H.E.R.O. Industries, Burnaby, B.C., Canada) and shaken for 5 min at the maximum speed. The 96 well plate was then centrifuged in a Sorvall RT centrifuge (Kendro Laboratory Products, Newtown, CT) at 3,750g for 5 min to obtain phase separation. The resulting upper Ethyl Acetate phase was transferred into an Eppendorf tube and dried under vacuum. Once samples were dried, they were resuspended in 100 µl Acetonitrile:H_2O:Triethylamine (900:99:1, v:v:v) for HPLC analysis. Seed carotenoid extractions were performed following similar procedures except that Methanol:Chloroform (1:1, v:v) was used as extraction solvent and the 500 ng of Tocol (2-methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol) standard was added to the extraction buffer and used to calculate recovery. HPLC separation of the carotenoids and quantitative analysis of carotenoid compositions were performed as described (Tian and DellaPenna, 2001).

Chlorophyll content (Chl a, Chl b, and total) of 11 cm diameter leaf discs were quantified spectrophotometrically as described (Porra et al., 1989). Briefly, 1 ml of dimethylformamide was added to each leaf disc and extraction was carried out at room temperature for 2 hr in darkness. The extraction mixture was centrifuged, the supernatant was transferred to a 1 ml cuvette, and absorption at 647 nm and 664 nm was measured and Chl a and Chl b content were calculated as Chl a = 12 A_{664}-3.11A_{647} and Chl b = 20.78A_{647}-4.88A_{664}, respectively.

Chlorophyll fluorescence measurements

Six-week old wild type and mutant Arabidopsis plants were dark-adapted overnight prior to chlorophyll fluorescence measurements. *In vivo* chlorophyll fluorescence was measured using a pulse amplitude modulation fluorometer (FMS2, PP Systems, Haverhill, MA, U.S.A.) using attached leaves that had not been shaded during growth. Fv/Fm is the maximum photochemical efficiency of PSII in the dark-adapted state. NPQ was determined as Fm-Fm'/Fm' (Lokstein et al., 2002).

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes.

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CHAPTER 5

Xanthophyll Biosynthetic Mutants of *Arabidopsis thaliana*: Altered Nonphotochemical Quenching of Chlorophyll Fluorescence is due to Changes in Photosystem II Antenna Size and Stability

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Abstract

Xanthophylls (oxygen derivatives of carotenes) are essential components of the plant photosynthetic apparatus. Lutein, the most abundant xanthophyll, is attached primarily to the bulk antenna complex, LHC II. We have used mutations in *Arabidopsis thaliana* that selectively eliminate (and substitute) specific xanthophylls in order to study their function(s) *in vivo*. These include two lutein-deficient mutants, *lut1* and *lut2*, the epoxy xanthophyll-deficient *aba1* mutant and the *lut2aba1* double mutant. Photosystem stoichiometry, antenna sizes and xanthophyll cycle activity have been related to alterations in non-photochemical quenching of chlorophyll fluorescence (NPQ). Non-denaturing PAGE indicates reduced stability of trimeric LHC II in the absence of lutein (and/or epoxy xanthophylls). Photosystem (antenna) size and stoichiometry is altered in all mutants relative to wild type. Maximal ΔpH-dependent NPQ (qE) is reduced in the following order: WT > *aba1* > *lut1* ≈ *lut2* > *lut2aba1*, paralleling reduction in PS II antenna size. Finally, light-activation of NPQ shows that zeaxanthin and antheraxanthin present constitutively in *lut* mutants are not qE active, and hence, the same can be inferred of the lutein they replace. Thus, a direct involvement of lutein in the mechanism of qE is unlikely. Rather, altered NPQ in xanthophyll biosynthetic mutants is explained by disturbed macro-organization of LHC II and reduced PS II-antenna size in the absence of the optimal, wild-type xanthophyll composition. These data suggest the evolutionary conservation of lutein content in plants was selected for due to its unique ability to optimize antenna structure, stability and macro-organization for efficient regulation of light-harvesting under natural environmental conditions.

Introduction

Carotenoids (carotenes and their oxygenated derivatives, the xanthophylls) are essential components of the photosynthetic apparatus in higher plants and their composition comprising lutein, β-carotene, violaxanthin (V) as well as its deepoxidation products and neoxanthin, is highly conserved throughout the plant kingdom. Lutein is the most abundant carotenoid and is bound primarily to LHC II, the major light-harvesting chlorophyll (Chl) *a/b*-binding complex of photosystem II (PS II). LHC II is composed of mixed trimers of the closely related *Lhcb1-3* gene products (Jansson, 1994). A 3.4 Å resolution structural model for trimeric LHC II reveals that in addition to 12 Chls, two xanthophylls (assumed to be luteins) are centrally located between two membrane-spanning α-helices in each monomeric subunit (Kühlbrandt et al., 1994). Biochemical analyses further indicate the presence of one neoxanthin and up to one V molecule per LHC II monomer (Croce et al., 1999; Ruban et al., 1999). The minor LHCs of PS II (comprising the *Lhcb4-6* gene products, also termed CP29, CP26 and CP24, respectively) as well as LHC I proteins also bind xanthophylls to a varying extent (Thayer et al., 1992; Bassi et al., 1993; Jansson, 1994; Ruban et al., 1999). The various xanthophylls play pivotal, synergistic roles in the photosynthetic apparatus (Siefermann-Harms, 1987; Paulsen, 1995; Niyogi, 1999). Xanthophylls are required for stable assembly of pigment-protein complexes *in vitro* and *in vivo* (reviewed in Paulsen, 1995), act as accessory light-harvesting pigments (Siefermann-Harms, 1987), and along with β-carotene are of paramount importance for photoprotection (for a recent review see Niyogi, 1999).

The primary photoprotective process in plants is the nonradiative dissipation of energy absorbed in excess of what can be utilized in photosynthesis (reviewed in Niyogi, 1999). The phenomenon can be readily visualized as nonphotochemical quenching of Chl fluorescence (NPQ). NPQ is a cooperative phenomenon - which despite intensive study - is not yet fully understood at the mechanistic level. Several processes contribute to NPQ. The major component (often termed qE, for energization-dependent quenching) is rapidly reversible and associated with acidification of the thylakoid lumen, or trans-thylakoidal ΔpH (for a review see Horton et al., 1994). Studies of plants with altered antenna composition and isolated LHCs *in vitro* indicate that qE arises in the photosystem II antenna system (Rees et al., 1989; Lokstein et al., 1993; Briantais, 1994; Härtel et al., 1996).

Transthylakoidal ΔpH induces a variety of alterations to LHC II pigments and proteins that collectively contribute to qE. A major consequence is protonation of specific LHC amino acid residues that is proposed to cause conformational changes in antenna organization facilitating qE *in vivo*. ΔpH-mediated quenching can be mimicked *in vitro* with isolated LHCs and has been used to establish the link between pH-induced LHC II aggregation and quenching (Horton et al., 1994). In the same system, added xanthophylls, most notably zeaxanthin (Z), antheraxanthin (A) and V, have differing effects on pH-induced aggregation/quenching that are consistent with models proposed for qE. Zeaxanthin production by the xanthophyll cycle in response to high-light stress has been correlated with NPQ (for reviews see Demmig-Adams, 1990; Pfündel and Bilger, 1990). Violaxanthin deepoxidase (VDE) is activated by low pH and converts V (di-epoxide), via A (mono-epoxide) into Z (epoxide-free) (see Pfündel and Bilger, 1990). Z epoxidase

catalyzes the reverse reactions (in the dark or under low light) and is inhibited by low pH (Pfündel and Bilger, 1990). Thus, a second consequence of the ΔpH generated in high light is the net formation of Z (+A) by the xanthophyll cycle. The combined protonation of LHCs and production/interaction of Z (+A) with LHCs are two major factors that cooperatively facilitate qE *in vivo*.

Genetic analyses have substantiated and further defined the *in-vivo*-roles of specific xanthophylls and other, novel components of NPQ in algae and plants (Niyogi, 1999). Mutants defective in VDE activity (e.g., the *Arabidopsis thaliana npq1* mutant) are unable to form Z in response to high light. The *npq1* mutant retains a rapid, initial pH-dependent component of NPQ but is defective in a slower phase and has reduced maximal amplitude compared to the wild type (WT) (Niyogi et al., 1997; Niyogi et al., 1998). Conversely, mutations disrupting Z epoxidase activity (e.g., the *A. thaliana aba1* mutant) accumulate constitutively high levels of Z, display accelerated NPQ induction and, surprisingly, have decreased NPQ amplitude. Together *aba1* and *npq1* have confirmed and delineated the role for Z (+A) and the xanthophyll cycle in NPQ *in vivo*. A third mutation in *A. thaliana* , *npq4,* eliminates the qE component of NPQ despite having an antenna pigment and protein composition, xanthophyll cycle activity and photosynthetic rate indistinguishable from WT. The *NPQ4* locus encodes the psbS (CP22) protein and defines this protein as essential for NPQ (Li et al., 2000).

Another class of mutations shown to affect NPQ, the *lut* mutants, are defective in specific aspects of lutein synthesis (Pogson et al., 1996). Lutein levels are reduced by approximately 80% in *lut1* due to a disruption in ε-ring hydroxylation and as a result zeinoxanthin, the immediate biosynthetic precursor of lutein, accumulates. The *lut2*

mutant is defective in ε-ring cyclization and devoid of lutein and all other α-carotene-derived xanthophylls. In addition to their lutein deficiency, both *lut* mutants also accumulate elevated levels of β-carotene-derived xanthophyll cycle pigments (V, A and Z). Similar to *npq1*, NPQ induction is retarded in both *lut* mutants and maximal amplitude is decreased, suggesting a previously unsuspected role for lutein in NPQ (Pogson et al., 1998).

As a group, the *A. thaliana* xanthophyll biosynthetic mutants are particularly well suited for probing the function(s) of individual xanthophylls *in vivo* and for providing insight into the mechanistic contribution of specific xanthophylls to NPQ. The current study was performed to provide a more detailed understanding of the various functions of xanthophylls within the light-harvesting system, the ability and limits of various xanthophylls to functionally complement for each other and the mechanistic basis for altered NPQ in the mutants. The common theme emerging from this work is that altered NPQ in xanthophyll biosynthetic mutants is due primarily to significant changes in LHC II-stability, PS II antenna size and superstructure.

Materials and methods

Plant Materials and Pigment Analysis

Wild type *A. thaliana thaliana* and the *lut1*, *lut2*, *aba1* and *lut2aba1* mutants (Pogson et al., 1998) were grown in soil:vermiculite (3:1) at 100 μmol photons m^{-2} s^{-1} under a 10:14-h light/dark cycle (21/19 °C) in growth chambers. Leaves from 6-8-week

old plants (prior to bolting) were used for all experiments. Pigment isolation and analyses were performed as described (Pogson et al., 1996) except that tissue was frozen in liquid nitrogen immediately following treatments and stored at -80 °C until extraction.

Photosystem Stoichiometry and Antenna Size Measurements

Isolation of thylakoid membranes and non-denaturing PAGE ("green gels") was performed as described (Dörmann et al., 1995). PS II and PS I concentrations were assessed from the amplitude of light minus dark absorption changes of freshly prepared thylakoid membrane suspensions at 320 nm (Q_A) and 700 nm (P_{700}) (Melis and Brown, 1980). Functional Chl *a/b*-antenna sizes of PS I and PS II were obtained from analyses of the kinetics of P_{700} photooxidation and Q_A-photoreduction, respectively (Melis, 1989).

Chlorophyll Fluorescence Measurements

In vivo Chl fluorescence was determined using a pulse amplitude modulation fluorometer (FMS 2, PP Systems, Haverhill, MA, USA) from attached leaves as previously described (Lokstein et al., 1993). Fluorescence parameters are according to (van Kooten and Snel, 1990). $F_v/F_m = (F_m-F_0)/F_m$ is the maximum photochemical efficiency of PS II, in the dark-adapted state. NPQ was quantified as (F_m/F_m')-1. Photon flux densities (PFDs) were measured with a quantum sensor (Li-Cor, LI-189A; Lincoln, NE, USA).

Results

Photochemical Efficiency and Nonphotochemical Chlorophyll Fluorescence Quenching

Maximum photochemical efficiency of PS II (measured as F_v/F_m) was similar in WT and the *lut1* and *lut2* mutants, slightly lower in *aba1* and significantly lowered in *lut2aba1* (Table 5.1). The lower F_v/F_m in *aba1* and *lut2aba1* most likely arise from a higher PS I contribution to F_0 and/or the presence of Z as a constitutive quencher of PS II fluorescence (refer also to later sections). NPQ induction kinetics for all genotypes at 500 μmol photons m^{-2} s^{-1} actinic light are shown in Figure 5.1A. As has been previously observed (Pogson et al., 1998), when compared to WT (half-rise time, $t_{1/2}$ = 73 s) NPQ induction is retarded in *lut1* ($t_{1/2}$ = 87 s) and *lut2* ($t_{1/2}$ = 105 s) and considerably accelerated in *aba1* ($t_{1/2}$ = 11 s) and *lut2aba1* ($t_{1/2}$ = 14 s). Maximal NPQ levels attained at 500 μmol photons m^{-2} s^{-1} are reduced in the order: WT > *aba1* > *lut1* ≈ *lut2* > *lut2aba1*. With the exception of *lut2aba1*, NPQ induction in all genotypes can be described by a single exponential rise, indicating that only one process, qE, contributes to NPQ at this actinic light level. However, at 500 μmol photons m^{-2} s^{-1} the *lut2aba1* curve shows, in addition to the fast initial rise, a second slower component. This second component is associated with a significant increase in F_0 (relative to the initial dark-adapted F_0 value) after relaxation of qE (data not shown) and hence is attributable to photoinhibition. This photoinhibitory component is not observed in the other genotypes following illumination with 500 μmol photons m^{-2} s^{-1}.

Table 5.1. Chl *a/b*-ratios, photosystem stoichiometries, antenna sizes and maximum photosystem II efficiencies (F_V/F_M) in *A. thaliana* WT and xanthophyll mutants.

	WT	*lut1*	*lut2*	*aba1*	*lut2aba1*
Chl *a/b*-ratio	2.72	2.88[a]	2.86[a]	3.04	3.23
F_v/F_m	0.840	0.843	0.846	0.805	0.783
Chl *a+b*/P_{700}	774	616	548	663	627
Chl *a+b*/Q_A	527	409	351	466	319
PS II/I (Q_A/P_{700})	1.47	1.51	1.56	1.42	1.97
Chl *a+b*/PS II	377[a]	275	213	373[a]	203
Chl *a+b*/PS I	220[b]	202[a]	202[a]	289	229[b]

Unless otherwise indicated all values are significantly different between genotypes (t-test, significance level = 0.05). N > 30 for Chl *a/b*-ratio and F*v*/F*m*. N = 3 - 9 for all other measurements. [a,b]not significantly different.

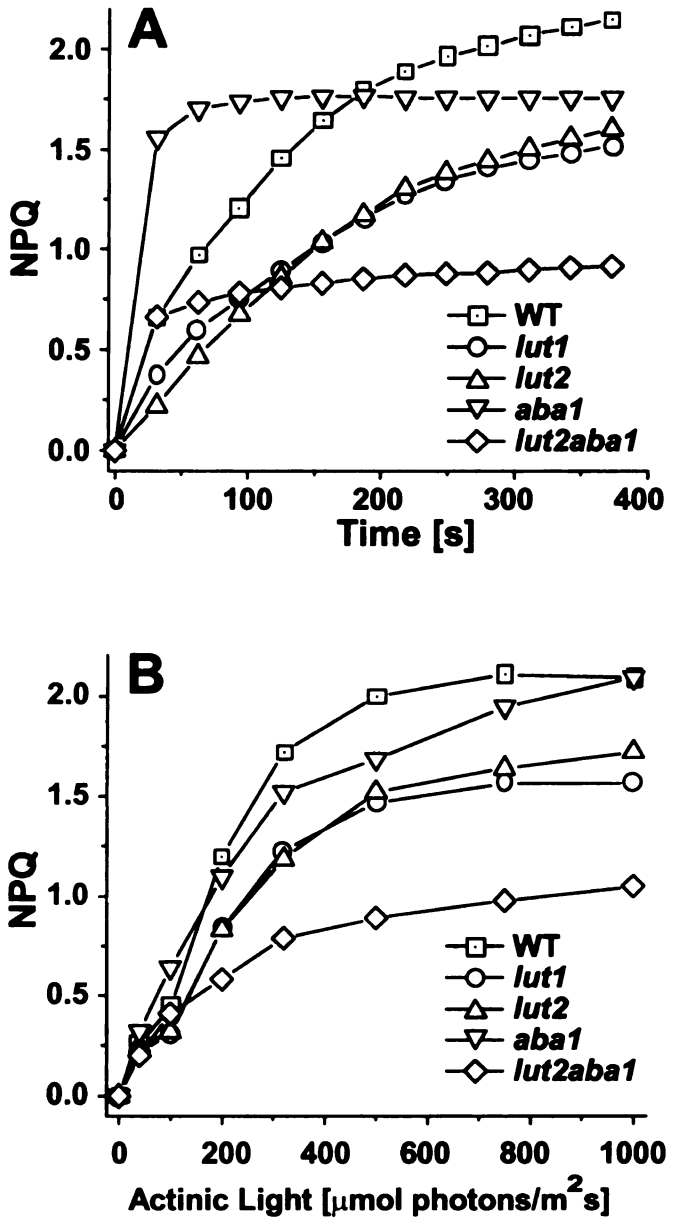

Figure 5.1. (A) Nonphotochemical fluorescence quenching (NPQ) in leaves of *A. thaliana* WT and xanthophyll biosynthesis mutants, as a function of time of exposure to 500 μmol photons m^{-2} s^{-1}. (B) NPQ as a function of actinic light intensity. All plants were dark-adapted for 30 min prior to light exposure. Each data point is the mean of at least three separate experiments. S.D. are within the symbol size.

Figure 5.1B displays the maximal NPQ levels reached after 6 min of illumination as a function of incident PFD for all genotypes. Wild type, *lut1* and *lut2* curves start to saturate at ~500 μmol photons m^{-2} s^{-1} while the *aba1* and *lut2aba1* curves continuously increase and are not saturated even at 1000 μmol photons m^{-2} s^{-1}. The maximal NPQ levels reached at 1000 μmol photons m^{-2} s^{-1} decrease in the order: WT ≈ *aba1* > *lut2* > *lut1* > *lut2aba1*. This altered order (relative to 500 μmol photons m^{-2} s^{-1}) is due to a genotype-dependent, differential increase in the photoinhibitory component of NPQ (increase in F_0) at higher light levels as the capacity of qE build-up is exhausted (Figure 5.1B and data not shown). At higher PFDs this additional slower inducible NPQ-component, indicative of susceptibility to photoinhibition, increases in the order: WT < *lut1* < *lut2* < *aba1* < *lut2aba1*.

Kinetics of Violaxanthin Deepoxidation in WT, *lut1* and *lut2*

A pivotal role for Z (and A) in NPQ is well-established (Demmig-Adams, 1990; Horton et al., 1994; Pfünder and Bilger, 1994; Härtel et al., 1996; Niyogi, 1999). Therefore, we first investigated whether the compromised NPQ development in *lut* mutants might be due to altered xanthophyll cycle activity and/or V+A+Z pool sizes. Time-dependent changes in the relative amounts of V, A and Z in leaves of plants dark-adapted for 24 h and then exposed to high light (1000 μmol photons m^{-2} s^{-1}) are shown in Figure 5.2. All data are expressed as mmol of the respective xanthophyll mol^{-1} Chl *a*+*b* instead of the more conventional % V+A+Z pool, as the V+A+Z pools in *lut1* and *lut2* were 2.5 and 3.8 fold larger, respectively, than in WT (Table 5.2 and Figure 5.2).

Table 5.2. Carotenoid composition of *A. thaliana* WT and xanthophyll mutant leaves.

	WT	*lut1*	*lut2*	*aba1*	*lut2aba1*
Lutein[a]	139	26	-	117	-
Neoxanthin	38	35[b]	35[b]	-	-
β-Carotene	81	91	114[b]	98	112[b]
Zeinoxanthin	-	50	-	-	-
V+A+Z[c]	33	81	124[b]	122[b]	191
Σ Carotenoids	291[b]	284[b]	273	337	303

Unless otherwise indicated all values are significantly different between genotypes (t-test, significance level = 0.05). N > 30 for all measurements.

[a] All carotenoid contents are expressed as mmoles carotenoid/mol Chl *a+b*.

[b] Not significantly different.

[c] Pool size of the xanthophyll-cycle pigments V, A and Z.

Figure 5.2. Kinetics of V deepoxidation via A to Z in leaves of *A. thaliana* WT (A) and the *lut1* (B) and *lut2* (C) mutants exposed to a PFD of 1000 μmol photons m^{-2} s^{-1}. All plants were dark-adapted for 24 h prior to light exposure. □, violaxanthin; Δ, zeaxanthin; O, antheraxanthin. Each data point represents the mean of at least five separate experiments. S.D. are within the symbol size. Note the different abscissa scales.

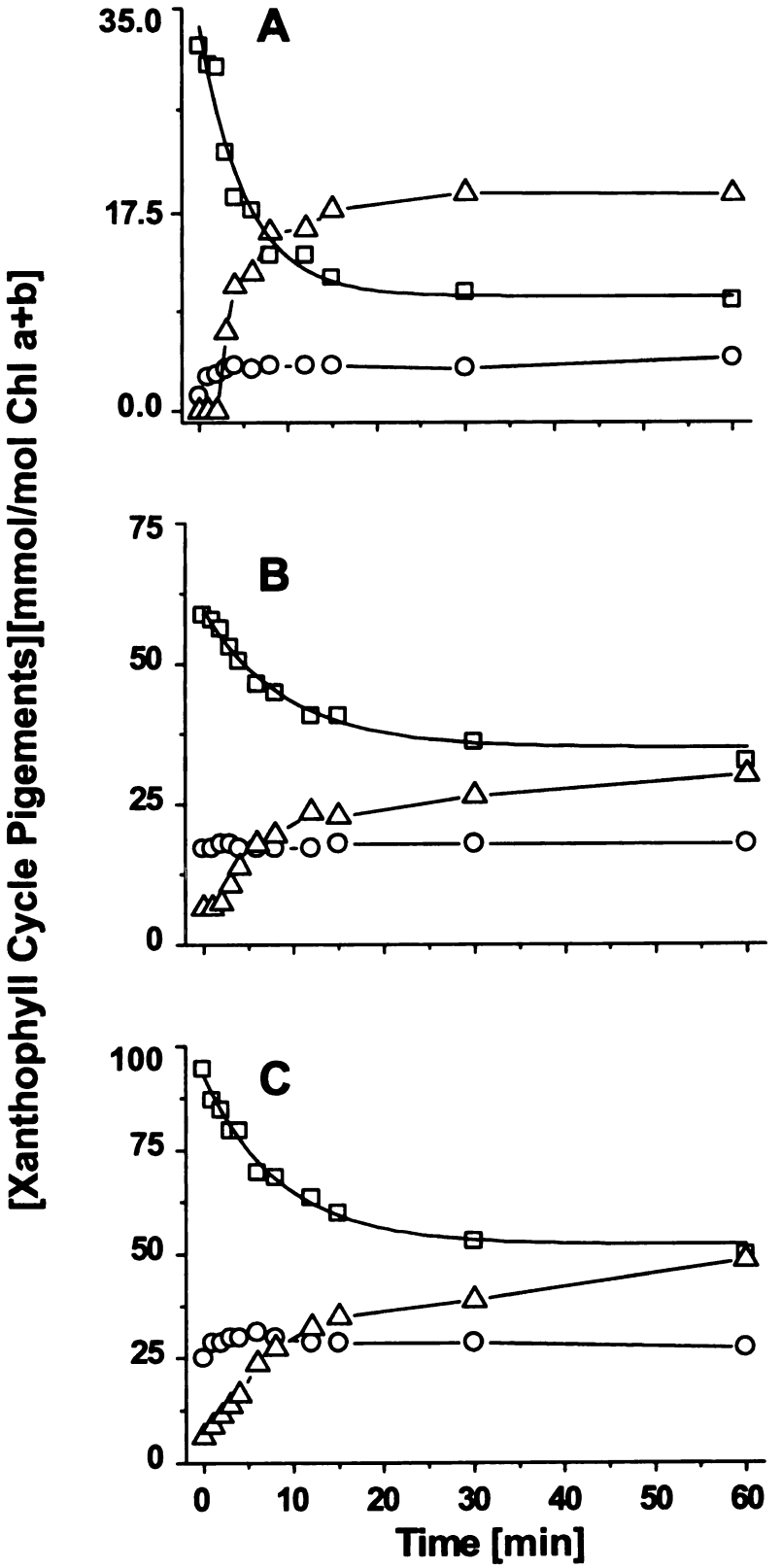
A
B
C
35.0
17.5
0.0
75
50
25
0
100
75
50
25
0
0
10
20
30
40
50
60
Time [min]
[Xanthophyll Cycle Pigements][mmol/mol Chl a+b]

Figure 5.2

In dark-adapted WT plants (Figure 5.2A), Z was absent and only traces of A were detectable. The 60 min light treatment resulted in deepoxidation of 21.5 mmol V to 18.2 and 3.3 mmol Z and A, respectively, with 11.6 mmol V (35%) being recalcitrant to deepoxidation. Both *lut* mutants (Figure 5.2B,C) have larger V+A+Z pools than WT and contained high levels of A and Z in the dark-adapted state (17.1 and 24.9 mmol A and 6.5 and 6.2 mmol Z for *lut1* and *lut2*, respectively). The 60 min light treatment had little effect on A levels but caused deepoxidation of 26 and 44.8 mmol V (mainly to Z) with 36.6 and 40.7 mmol V being recalcitrant to deepoxidation in *lut1* and *lut2*, respectively. While these deepoxidation levels as attained after 60 min illumination are much higher than in WT, when one considers the time dependent product formation (Z+A) and that NPQ is near maximal after 6 minutes of light treatment in all genotypes (Figure 5.1), the xanthophyll cycle activity that may be relevant to NPQ is remarkably similar in all genotypes. During the 6 min treatment leading to maximal NPQ 14.4, 11.1 and 22.1 mmol Z+A are formed with 50% product formation times of 2.5, 3.1 and 2.7 min, for WT, *lut1* and *lut2*, respectively. In the light of these minor differences in rates and product formation, it seems unlikely that altered xanthophyll cycle activity is the primary cause of compromised NPQ in *lut* mutants.

The data in Figure 5.2 suggest that the large differences in deepoxidized and recalcitrant V between *lut* mutants and WT originate from the existence of *at least* two functionally distinct sub-pools of V (and A+Z for the mutants) with differential accessibility by VDE and Z epoxidase. One sub-pool is bound to the more peripherally located xanthophyll (V, A, Z) binding sites of LHC-proteins and hence, is accessible by the xanthophyll cycle enzymes. A second "protected" sub-pool (11.6 mmol V in WT and

60.2 and 71.8 mmol V+A+Z in *lut1* and *lut2,* respectively) is bound to sites within LHC II and inaccessible by the xanthophyll cycle enzymes. In the case of the *lut* mutants, a significant proportion of this recalcitrant V+A+Z sub-pool is apparently substituting for lutein in the internally located lutein-binding sites of LHC II (Kühlbrandt et al., 1994) and unavailable to the xanthophyll cycle during light stress. Pigment compositional analysis of isolated LHCs from WT and the mutants support this conclusion (refer to later sections and Table 5.3). Finally and most significantly, the large amount of protected, internally located Z+A (23.6 and 31.1 mmol *lut1* and *lut2,* respectively) appears to not be NPQ-active. To further test this hypothesis we performed NPQ light-activation experiments in WT and the *lut* mutants.

Light-Activation of Nonphotochemical Quenching in WT, *lut1* and *lut2*

Light-activation of NPQ and its correlation with xanthophyll cycle activity is a well-established phenomenon (Rees et al., 1989). Leaves of dark-adapted WT and *lut* mutants were subjected to 500 μmol photons m^{-2} s^{-1} for 6 min (inducing V deepoxidation and NPQ build-up), subsequently dark-adapted for 2 min (sufficient to relax the qE component of NPQ but not to re-epoxidate the newly formed Z+A) and then subjected to a second high-light treatment (Figure 5.3).

NPQ induction in *lut1* and *lut2* during the initial illumination period is delayed compared to WT, consistent with the data in Figure 5.1A. However, during the second illumination period, NPQ induction in both *lut* mutants is similar to WT and is complete in all three genotypes in less than 30 sec. This observation reveals the functional

Table 5.3. Pigment composition of *A. thaliana* WT and xanthophyll mutant light-harvesting complexes as resolved by non-denaturing electrophoresis as displayed in Figure 5.4.

	Chl *b*[a]	lutein	neoxanthin	VAZ	ΣCarotenoids
WT-LHC II_3	5.4	1.8	1.2	0.2	3.2
WT-LHC_{mono}	5.4	1.7	1.1	0.2	3.0
lut1-LHC_{mono}	5.3	0.3	1.0	1.5	2.8
lut2-LHC_{mono}	5.5	n.d.[b]	1.3	2.0	3.3
aba1-LHC_{mono}	5.8	1.5	n.d.	1.5[c]	3.0
lut2aba1-LHC_{mono}	5.9	n.d.	n.d.	3.5[c]	3.5

[a] All pigments are expressed as per 7 molecules Chl *a*; $n = 3$, S.D. are about 10 %.

[b] Not detectable.

[c] Only zeaxanthin.

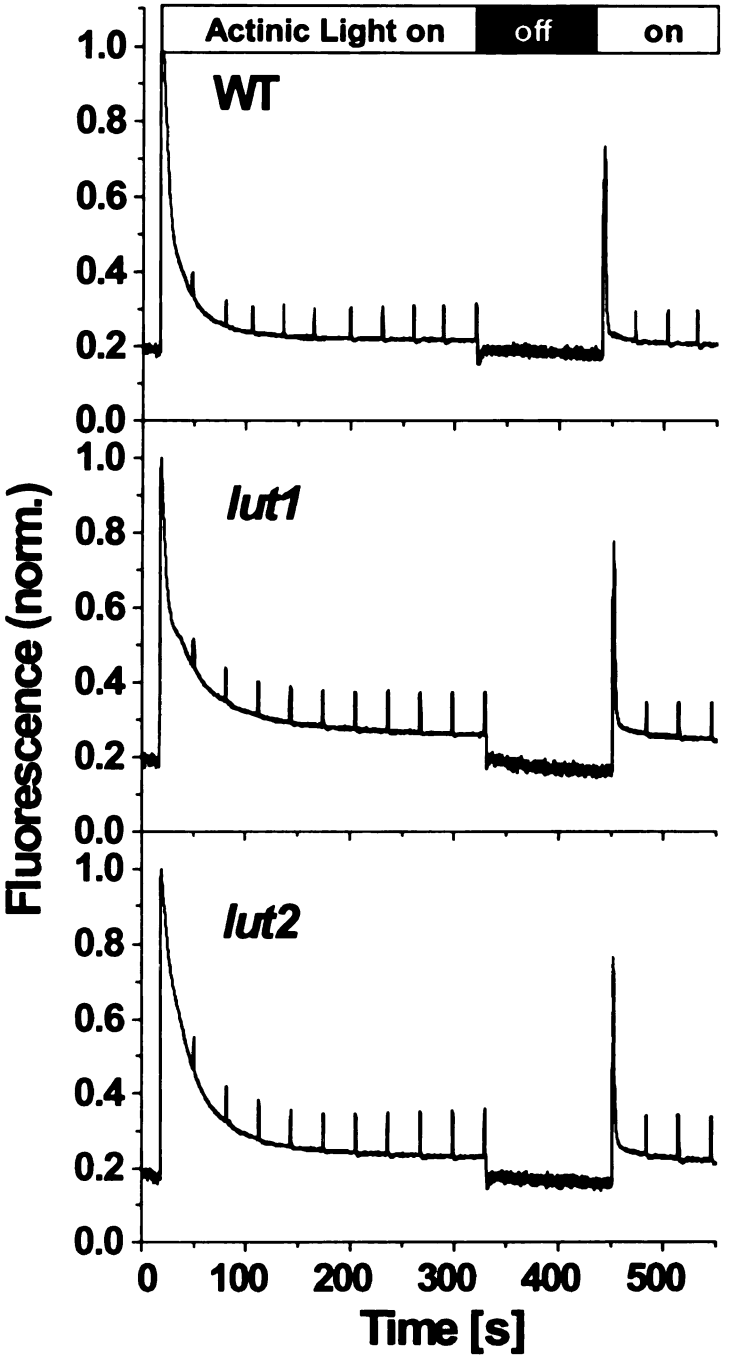

Figure 5.3: Light-activation of NPQ in leaves of *A. thaliana* WT, the *lut1* and *lut2* mutants. Dark-adapted leaves were illuminated with a PFD of 500 μmol photons m^{-2} s^{-1} for 6 min followed by 2 min darkness and a second illumination.

significance of the different pools of xanthophyll cycle pigments (V+A+Z) in the *lut* mutants. Only Z (and possibly A) *formed by xanthophyll cycle activity during the first illumination period* is NPQ active. This xanthophyll cycle (and NPQ-) active V+A+Z is bound most likely to peripheral pigment binding sites of the LHCs. The large amounts of constitutively present Z (and A) in dark-adapted *lut* mutants (bound to the lutein-binding sites of LHC II) do not contribute to fast NPQ induction. Moreover, the same can be logically inferred for lutein normally bound to these sites in WT. Thus, the hypothesis that lutein is directly involved in the mechanism of NPQ (Niyogi et al., 1997; Pogson et al., 1998) is not supported. An alternative explanation for compromised NPQ in the *lut* mutants (and in xanthophyll-deficient *A. thaliana* mutants in general) would be more indirect and result from alterations in LHC assembly/stability.

Assembly, Composition and Stability of Light-Harvesting Complexes in Xanthophyll Mutants

In vitro reconstitution studies have shown that xanthophylls are indispensable for stable assembly of monomeric LHCs (Paulsen, 1995). In order to investigate assembly/stability of pigment-protein complexes in the various xanthophyll mutants, thylakoid membranes were solubilized in mild (non-ionic) detergents and subjected to non-denaturing PAGE ("green gels", Figure 5.4). Most striking is the considerably reduced stability of trimeric LHC II in all xanthophyll mutants. Whereas the *lut1* mutant retains a minor fraction of LHC II-trimers (10-20 % of the WT level, corresponding approximately to its residual lutein content), only a very faint trimeric LHC II band is

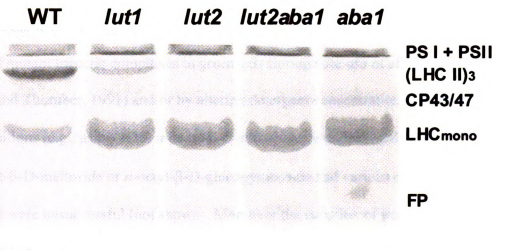

Figure 5.4. Non-denaturing ("green") gel electrophoretic separation of pigment-protein complexes from *A. thaliana* thylakoid membranes of the indicated genotypes. Note that the LHC_{mono} band also contains LHC I and minor LHC II complexes, FP designates free pigment. The pigment composition of the LHC bands is given in Table 5.3.

observed for *aba1*. The LHC II trimer band is absent in *lut2* and *lut2aba1*, both of which are completely deficient in lutein. Disappearance of trimeric LHC II is accompanied by a concomitant increase of a band attributable to LHC II monomers (including also monomeric LHC I and minor LHCs II, i.e., CP24, CP26 and CP29) in all mutant genotypes. The free pigment band (almost exclusively carotenoids, V+A+Z as well as zeinoxanthin in *lut1*) is least pronounced in WT, markedly stronger in all xanthophyll mutants and most prominent in the *lut2aba1* double mutant. Attempts to improve the yield of mutant trimeric complexes in green gels through the use of alternate gel systems (Peter and Thornber, 1991) and/or by altering detergent concentrations and/or compositions (e.g., using SDS, *n*-decyl-β-D-maltoside, *n*-undecyl-β-D-maltoside, *n*-dodecyl-β-D-maltoside or *n*-octyl-β-D-glucopyranoside and various combinations thereof) were unsuccessful (not shown). Moreover the isolation of pure (trimeric) LHC II with standard procedures (Burke et al., 1978; Krupa et al., 1987) also varying solubilization conditions (e.g., 0.2 to 0.7 % Triton X-100 or 0.05 to 1.0 % *n*-dodecyl-β-D-maltoside) proved to be impossible, too.

The bands corresponding to trimeric LHC II (WT) and monomeric LHCs (all genotypes, note that the latter contains also the minor LHCs II and LHC I) were excised and their pigment content analyzed by HPLC. The pigment composition of the bands is given in Table 5.3. WT trimeric LHC II, WT monomeric LHC and *aba1* monomeric LHC pigment compositions were similar to those previously reported (Connelly et al., 1997). In the *lut1* and *lut2* monomeric LHCs lutein is replaced on a nearly equimolar basis by xanthophyll cycle pigments (V+A+Z). In *lut2aba1* lutein, V and neoxanthin are replaced by Z.

Photosystem Stoichiometry and Antenna Size in Xanthophyll Mutants

It has been shown previously that the extent of NPQ can be correlated with PS II antenna size (Härtel et al., 1996). The xanthophyll mutants showed an increasing Chl *a/b* ratio in the order: WT < *lut1* ~ *lut2* < *aba1* < *lut2aba1* (Table 5.1). Since Chl *b* is associated exclusively with LHCs (mainly LHC II), the Chl *a/b* ratios are consistent with a reduction in LHC II levels. However, alterations in photosystem stoichiometry alone or in combination with altered antenna sizes could bring about similar changes in Chl *a/b* ratios. To further address these possibilities, photosystem stoichiometry and antenna size were analyzed in all genotypes (Table 5.1).

Light-induced absorption difference spectra of thylakoid membrane preparations were used to determine the concentrations of Q_A and P_{700} as a measure of PS II and PS I reaction centers, respectively (Melis and Brown, 1980). The corresponding PS antenna sizes were derived from the kinetics of Q_A-photoreduction and P_{700}-photooxidation (Melis, 1989).

Lack of lutein leads to an increase in the ratio of PS II to PS I in the order: *lut2aba1* > *lut2* > *lut1* > WT. Conversely, the lack of epoxidized xanthophylls (neoxanthin and V) in *aba1* leads to a decrease in the ratio of PS II to PS I relative to the WT. With regard to PS I antenna size (Chl *a*+*b*/PS I), no clear tendency is observed in relation to a specific xanthophyll deficiency. However, the lack of lutein is strongly correlated with a reduction in PS II antenna size (Chl *a*+*b*/PS II) in the following order: WT ~ *aba1* > *lut1* > *lut2* > *lut2aba1*. Remarkably, this reduction in PS II antenna size

parallels the decrease in maximum attainable NPQ (as measured at 500 μmol photons m^{-2} s^{-1} actinic illumination (cp. Figure 5.1A) to avoid interference by photoinhibition)

Discussion

Xanthophylls are indispensable, integral components of plant photosynthetic pigment-protein complexes. In addition to their accessory light-harvesting function, they are required for stable assembly of both antenna and reaction center complexes as well as for effective photoprotection (see Paulsen, 1995). The ability of plants to dissipate excess excitation energy (measurable as NPQ) is of paramount importance for photoprotection and has been correlated with light-induced trans-thylakoidal ΔpH and Z formation (Rees et al, 1989; Demmig-Adams, 1990; Härtel et al., 1996; Niyogi, 1999). It has also been proposed that the xanthophyll cycle intermediate, A, may be involved in NPQ (Gilmore and Yamamoto, 1993).

Mutations in *A. thaliana* have been useful in advancing our understanding of NPQ in plants and fall into two general classes: those that affect NPQ without altering the pigment compositions of unstressed leaves (e.g. *npq1* and *npq4*) and those that affect NPQ but also alter pigment compositions in unstressed leaves (e.g. *lut1*, *lut2*, *aba1* and their double mutants). In *aba1*, NPQ induction is more rapid but attains lower amplitude than in WT, while in *lut1* and *lut2* both the induction and amplitude of NPQ are negatively impacted (Figure 5.1A and Pogson et al., 1998). These effects are additive in *lut1aba1* and *lut2aba1* double mutants indicating the *aba1* and *lut* mutations affect independent processes related to NPQ (Figure 5.1A and Pogson et al., 1998). These

general observations can be extended to green algae - analogous mutations in *Chlamydomonas reinhardtii* show similarly compromised NPQ (Niyogi et al., 1997). Such studies have led to the proposal that in addition to light-induced trans-thylakoidal ΔpH and Z formation, lutein is also required for efficient NPQ, either directly or indirectly, by an unknown mechanism (Niyogi et al., 1997; Pogson et al., 1998). In the current study we have compared and contrasted aspects of photosystem structure and function across four *A. thaliana* xanthophyll mutant lines (*lut1*, *lut2*, *aba1* and *lut2aba1*) and WT in an attempt to identify a common mechanism(s) whereby xanthophyll modifications differing from WT - in particular lutein deficiency - influence NPQ.

Given the importance and effects of the xanthophyll cycle (in particular Z formation) in NPQ build-up, it would seem logical that any alterations in xanthophyll cycle pigment pool size (V+A+Z), especially the levels of Z and A prior to light stress, would have an impact on NPQ induction. This is indeed the case; however, instead of positively affecting NPQ as one might expect, the high constitutive levels of Z (and A) and ~3-fold increase in V+A+Z pools in *lut* mutants coincide with compromised NPQ induction kinetics and amplitudes. Perhaps the compromised NPQ in the mutants is due to an alteration in xanthophyll cycle activity/kinetics, rather than absolute xanthophyll cycle pigment levels. This appears not to be the case either, as xanthophyll cycle activity (V to Z conversion rates and amounts) during the timeframe of NPQ induction was remarkably similar in *lut* mutants and WT.

The experiments in Figure 5.2 show that approximately 65 % of the enlarged V+A+Z pool in *lut* mutants is "protected" from xanthophyll cycle activity. This is most likely due to these pigments occupying binding sites in LHCs that would normally (in

WT) bind lutein. Given the role of Z (and possibly A) as NPQ-active xanthophylls, one would again anticipate that any Z (or A) would increase NPQ or at least predispose the system to NPQ. However, NPQ light activation experiments clearly show that the large amounts of Z+A (occupying lutein sites) in the *lut* mutants do not contribute to NPQ indicating that, as in WT, only Z (and A) produced by the xanthophyll cycle is active in NPQ in *lut* mutants. The observations are significant for several reasons. First, they provide direct evidence that the location of a particular xanthophyll (e.g. Z and A) within the pigment-protein complexes is as important - if not more important - than the identity of the pigment for determining its role in NPQ. Second, since Z (and A) incorporated into lutein-binding sites do not directly participate in NPQ, it follows that the lutein that normally occupies these sites in WT also does not directly participate in NPQ. Still, the question remains: If lutein is not directly involved in NPQ and substituted Z (and A) is not "NPQ-active", by what mechanism does the absence of lutein in *lut* mutants affect NPQ?

Although xanthophyll biosynthetic mutations affect many aspects of the plant photosynthetic apparatus, when taken together, our studies of LHC stability, antenna size and photosystem stoichiometry indicate that the effect of xanthophyll compositional changes on NPQ results primarily from alterations to the PS II antenna. Non-denaturing PAGE analysis of pigment-protein complexes indicates LHC II trimer stability is substantially reduced across the spectrum of *A. thaliana* xanthophyll mutants studied, though most severely in those that lack lutein (Figure 5.4). This is consistent with previous reports of LHC II instability in a α-carotenoid-free mutant of the green alga *Scenedesmus obliquus* (Bishop, 1996; Heinze et al., 1997). It is most likely that the

destabilization of LHC II trimers in *A. thaliana* xanthophyll mutants is brought about by subtle structural changes in monomeric LHC II units due to the binding of V+A+Z instead of lutein.

A clear inverse correlation exists between the maximal attainable NPQ and the reduction of functional PS II antenna size across the full spectrum of mutants studied (Figure 5.1 and Table 5.1). We propose that this is the primary, underlying lesion that affects NPQ in all xanthophyll biosynthetic mutants. The lack of a WT xanthophyll composition leads to instability of LHC II trimers. This reduction of LHC II trimer stability is likely translated into a disruption of optimal, higher-order, macro-organization of the PS II-antenna system (and probably less connectivity between PS II centers) and hence, is responsible for the compromised NPQ. This is consistent with previous findings that NPQ decreased in parallel with the reduction of PS II antenna size in a Chl *b*-less barley mutant (Lokstein et al., 1993; Briantais, 1994; Härtel et al., 1996). Interestingly, Chl *b*-less barley mutants grown under an intermittent light regime (Härtel et al., 1996) show a NPQ phenotype that closely resembles the *A. thaliana npq4* mutant (Li et al., 2000). Intermittent light grown Chl *b*-less barley mutants are devoid of all major and minor LHCs but retain WT levels of the psbS protein (Bossmann et al., 1997), while the *npq4* mutant is unaffected in PS II-antenna composition but completely lacks psbS (Li et al., 2000). This indicates that both psbS and an optimally organized and functional PS II antenna system are required for maximum NPQ build-up.

The xanthophyll composition of plant photosynthetic pigment-protein complexes is remarkably conserved across evolution. This is presumably due to strong selection pressure for retention of these particular xanthophylls in order to enable specific

function(s) and/or the resultant fitness of land-based photosynthetic organisms. In particular, lutein is the most abundant thylakoid carotenoid and its presence is diagnostic for land-based photosynthetic organisms. Moreover, lutein is present in the branch of algae that presumably gave rise to land plants. Thus, the initial identification of lutein-deficient *A. thaliana* mutants, which lacked an obvious phenotype, and the subsequent generation of xanthophyll double mutants that lacked all WT xanthophylls with relatively minor observable whole plant phenotypes were both surprising and puzzling (Pogson et al., 1996). If, as the single and double *A. thaliana* xanthophyll mutations imply, the plant photosynthetic apparatus is so flexible with regard to xanthophyll composition, why do all plants produce lutein, have such a highly conserved xanthophyll composition and finally, why doesn't one find plants in nature with xanthophyll profiles similar to the *A. thaliana* mutants?

The observation that all single and double *A. thaliana* xanthophyll biosynthetic mutants are defective in aspects of NPQ suggested that plants with "non-WT" xanthophyll compositions are impaired in their ability to cope with light in excess of what is needed for photosynthesis (Pogson et al., 1998). The current study clearly shows that although other xanthophylls (in particular V, A and Z) are able, to a certain degree, to assume the structural role of lutein in the mutants, the resulting "non-WT" xanthophyll compositions severely compromise antenna size, stability and function. These "replacement" xanthophylls do not provide the same unique combination of antenna stability, flexibility and function that lutein does to fulfill the requirement of land plants for fast and effective regulatory and protective responses under rapidly fluctuating environmental conditions. In this light, the strong selection for maintenance of a specific

xanthophyll composition in plant thylakoids during evolution is easily understood. This may also explain why only descendents of green algae with their seemingly redundant manifold of Chl *a/b*-binding and xanthophyll containing antenna complexes that confer unique adaptive flexibility (the xanthophyll cycle and NPQ) have ever successfully conquered land.

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CHAPTER 6

Future directions

Over the past decade, most of the carotenoid biosynthetic genes have been cloned from various organisms and some of which were further characterized for enzyme activity and function (reviewed in Cunningham and Gantt, 1998; Hirschberg, 2001). However, much remains to be learned about the mechanisms of carotenoid biosynthetic enzymes and reactions. This dissertation focused on cloning of the ε-hydroxylase gene (Chapter 2 and 3) and has addressed the overlapping functions of the carotenoid hydroxylases in Arabidopsis (Chapter 4) as well as xanthophyll functions (especially lutein) in photosystem structure and photoprotection (Chapter 5). During the course of this dissertation research, many more questions and hypotheses were generated than could be answered and tested. In order to understand the mechanism of carotenoid hydroxylation reactions, the developmental responses of plants towards carotenoid hydroxylase deficiency, and the fitness of hydroxylase mutants under various stress conditions, the following future experiments are proposed to further our knowledge in these areas. Experiments proposed in section 6.1.1 and 6.1.2 have been performed and preliminary results obtained during the course of this dissertation research. Future research should be focusing on the mechanism of hydroxylation reactions (6.3.) and the response of various hydroxylase mutants to the stress conditions (6.4.).

6.1. Developmental responses to carotenoid deficiency in Arabidopsis hydroxylase mutants

6.1.1. ABA biosynthesis in the hydroxylase deficient mutants

ABA (an apocarotenoid) is a sesquiterpene derived from C_{40} carotenoids. ABA is a plant growth regulator involved in induction of seed dormancy and adaptation to a variety of stresses (reviewed in Giraudat et al., 1994). The regulation of the stress adaptation process is partially due to *de novo* synthesis of ABA. Therefore, understanding ABA synthesis is essential for understanding plant stress accommodation. A direct correlation between ABA production and epoxy-carotenoids, violaxanthin and neoxanthin, has been established by labeling experiment and characterization of the *aba* mutant (Creelman et al., 1987; Rock and Zeevaart, 1991).

ABA deficient mutants have played critical roles in studying ABA biosynthesis and function (Milborrow, 2001). Some of these mutants were isolated due to their wilting or viviparous phenotypes. Previously identified mutants in the carotenoid biosynthetic pathway have also shown viviparous phenotypes, e.g. VP5 mutant in maize is ABA deficient and is a lesion in the phytoene desaturase gene; VP3 and VP7 may encode lycopene cyclases in maize, etc. In addition, the *aba1* mutation in Arabidopsis is a mutation of the zeaxanthin epoxidase gene.

The β-hydroxylase double mutant *b1b2* and hydroxylase triple mutant *lut1b1b2* isolated during this dissertation research produce very low amounts of the ABA precursors, violaxanthin and neoxanthin (less than 5% and 2% of total carotenoids, respectively; refer to Table 4.1). Unlike the ABA deficient mutants, the hydroxylase mutants do not show wilting or germination defective phenotypes. This result suggests that although produced in very limited amount, the violaxanthin and neoxanthin pool in the hydroxylase mutants is able to produce sufficient ABA for seed germination and other ABA-related physiological processes under normal growth conditions.

In order to test this hypothesis, the ABA level in wild type WS and Col, as well as *b1b2* and *lut1b1b2* mutants were measured in collaboration with Dr. Jan Zeevaart (Plant Research Laboratory, Michigan State University). It has been previously observed that the wilting of leaves induces a sudden and rapid increase in ABA content ("stress" ABA; Milborrow, 1981). Therefore, ABA levels in both normal growth and water stressed plants (for 5 hr) were measured. Our preliminary data showed that *b1b2* and *lut1b1b2* mutants accumulate similar amount of ABA compared to the wild type (~0.2 μg/g tissue) under normal growth condition. When these plants were subject to drought stress, the ABA level in wild type plants were induced up to 10 fold, while only a very moderate increase (~0.1 μg/g tissue) was observed in the hydroxylase mutants. These results indicate that although the hydroxylase mutants can synthesize sufficient ABA for normal growth, stressed induced ABA production is greatly reduced in these mutants. The carotenoid compositions of drought-stressed plants (wild type, hydroxylase mutants, and *aba1-5* control plant) are currently being characterized.

6.1.2. Chloroplast ultrastructures in the hydroxylase mutants

The increased Chl a/b ratios in the hydroxylase double and triple mutants (Table 4.3, Chapter 4) suggested an alternation in photosystem stoichiometry due to the presence of dramatically decreased amounts of xanthophylls. In order to determine whether the xanthophyll deficiency affects the structure of the photosynthetic apparatus, chloroplasts in the wild type and mutants mesophyll cells were visualized by transmission electron microscopy (TEM).

TEM study revealed abnormal chloroplast ultrastructures in both types of hydroxylase mutants. *b1b2* mutant showed reduced thylakoid grana stacking, which indicates that the structure of the photosynthetic apparatus has been adversely affected by xanthophyll deficiency and xanthophylls are involved in grana stacking either directly or indirectly. Xanthophylls mainly bind to the light harvesting complex proteins in the thylakoid membrane. Chl b deficient mutants grown under intermittent light lack LHC proteins and have thylakoid membranes organized into large parallel arrays, indicating that LHC proteins are necessary for grana stacking (Król et al., 1995). Therefore, the relative levels of LHC proteins in the wild type and hydroxylase mutant plants should be quantified with specific antibodies.

Unlike the *b1b2* double mutant, *lut1b1b2* triple mutant has a normal grana stacking pattern, however there is a large increase in starch accumulation (as indicated by the size of the starch grains) compared to wild type plants. Starch accumulation is frequently observed under stress conditions such as phosphate deficiency, magnesium deficiency, high light etc (Fischer et al., 1998; Weston et al., 2000; Zakhleniuk et al., 2001). The triple mutant was grown under moderate light with nutrients identical to wild type control plants, it is possible that the triple mutant was more stressed than wild type plants under such growth conditions. Starch accumulation could be the consequence of decreased degradation or increased synthesis. Therefore, starch content during a prolonged dark period will be measured in the triple mutant. To further analyze the cause of starch accumulation in the hydroxylase triple mutant, the enzymes involved in starch synthesis and degradation, soluble sugar levels, and anion contents (e.g. phosphate) should also be tested.

6.1.3. Lipid composition in the hydroxylase mutants

Membrane lipids form bilayers in the cell. Chloroplast membranes primarily contain galactolipids. MGDG accounts for more than 50% of the lipids in the thylakoid membrane and LHC II has a high affinity for MGDG. As mentioned in section 6.1.2, the structure of the thylakoid membrane has been altered in the hydroxylase mutants, which suggests that lipid levels, esp. MGDG, may be correspondingly altered in these mutants. In addition, MGDG is also essential for binding of violaxanthin and zeaxanthin produced by the xanthophyll cycle to the LHC proteins (Morosinotto et al., 2002). The carotenoid composition change in the hydroxylase mutants, drastically decreased violaxanthin and neoxanthin in particular, may affect the membrane lipid composition and membrane fluidity; therefore, these parameters in the hydroxylase mutants should be further analyzed.

6.2. Mutants screening

T-DNA knockout (null) mutants for β-hydroxylase 1 and 2 (*b1* and *b2*, respectively) were isolated and crossed to generate *b1b2* double mutant, in which both β-hydroxylase activity were eliminated (Chapter 4). The *b1b2* double mutant was further introduced into the *lut1* (EMS mutation, now known to be leaky) background in order to address the possible overlapping function of the hydroxylases.

The *LUT1* gene was cloned during this dissertation research and a corresponding knockout mutant was isolated. In the leaves of the *lut1* knockout mutant, there is no lutein accumulation indicating it is a null allele of the ε-hydroxylase gene. Although the previously generated *lut1b1b2* triple mutant was informative as to the overlapping hydroxylase functions, we were unable to eliminate the possible residual function of the ε-hydroxylase due to the potential leakiness of the *lut1* EMS mutation. It is therefore critical to cross the *lut1* knockout mutant to *b1b2* and screen for putative triple hydroxylase knockout mutant. Both HPLC and PCR based screening are currently employed to isolate the putative homozygous triple mutant.

In the *lut1b1b2* triple mutant, the production of lutein and β-carotene derived xanthophylls was greatly reduced. However, the amount of zeinoxanthin, a monohydroxyl precursor of lutein, was more than doubled in the triple mutant compared to wild type. Zeinoxanthin bears one hydroxylated β-ring and may partially compensate for the lack of lutein and β-carotene derived xanthophylls. In order to address this question, *lut2* mutation was introduced to the *b1b2* background. *lut2* is a mutation in the ε-cyclase gene, in which zeinoxanthin and lutein production is entirely abolished. β-hydroxylase 2 gene (At5g52570) and ε-cyclase gene (At5g57030) locate to the bottom arm of chromosome 5 and are tightly linked (only 7-8 cM apart), therefore they are not independently segregating. Out of 288 F_2 plants from *lut2* and *b1b2* cross, only heterozygous mutants are isolated due to this tight linkage. The heterozygous mutants are currently growing on soil and the F_3 generation will be screened for possible homozygous *lut2b1b2* mutant.

6.3. Structural and mechanistic basis of the β- and ε-hydroxylases

The substrates for β- and ε- ring hydroxylase are very similar except for the double bond position on their ring structures. The previously cloned β-hydroxylases are non-heme diiron type monooxygenase (Bouvier et al., 1998). Like the membrane fatty acid desaturases, they require ferredoxin, NADPH, NADPH-ferredoxin oxido-reductase for activity. ε-hydroxylase gene was cloned during this dissertation research and shown to be a cytochrome P450 type monooxygenase that requires NADPH, O_2, and CPR for activity. However, the underlying mechanisms for these different types of hydroxylation reactions are still not known. In the future, biochemical analyses of substrate specificity and enzyme kinetics should be carried out to further characterize β- and ε- ring hydroxylase activity. In addition, crystallization of both types of hydroxylases will provide valuable information on substrate binding, enzyme conformation and will further our knowledge on the reaction mechanisms. A better understanding of the hydroxylation reactions will also add insights into possible carotenogenic complexes proposed by Cunningham and Gantt (1998).

ε-hydroxylase can be engineered into bacteria, such as cyanobacteria, to produce lutein that is not normally present. Metabolic engineering in plants can be carried out by either abolishing ε-hydroxylase gene expression to divert carbon flow into β-carotene production. Lutein is only synthesized in land plants and green algae. Putative homologs of ε-hydroxylases have been identified in monocots, dicots, and diatoms. An important question remains to be addressed is how the ε-hydroxylase evolved.

6.4. Hydroxylase mutants response to environmental stresses

In chapter 4 and 5 of this dissertation, the function of xanthophylls in NPQ was addressed using mutants deficient in carotenoid biosynthetic genes. Carotenoids have also established their roles as antioxidants to scavenge reactive oxygen species, especially under stress conditions (Mittler, 2002). When plants are exposed to high light, free radicals and oxidizing species are generated and released within the chloroplasts. Future studies on the hydroxylase mutants should focus on characterizing the responses of these plants to high light stress. Other abiotic stresses, such as salt, drought and desiccation, temperature etc. should also be applied and mutant responses characterized.

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APPENDIX A

Analysis of carotenoid biosynthetic gene expression during marigold petal development.

The work presented in this appendix has been published:

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Abstract

Marigold (*Tagetes erecta* L.) flower petals synthesize and accumulate carotenoids at levels greater than 20 times that in leaves and provide an excellent model system to investigate the molecular biology and biochemistry of carotenoid biosynthesis in plants. In addition, marigold cultivars exist with flower colors ranging from white to dark orange due to >100-fold differences in carotenoid levels, and presumably similar changes in carbon flux through the pathway. To examine the expression of carotenoid genes in marigold petals, we have cloned the majority of the genes in this pathway and used these to assess their steady state mRNA levels in four marigold cultivars with extreme differences in carotenoid content. We have also cloned genes encoding early steps in the biosynthesis of isopentenyl pyrophosphate (IPP), the precursor of all isoprenoids, including carotenoids, as well as two genes required for plastid division. Differences among the marigold varieties in the expression of these genes suggest that differences in mRNA transcription or stability underlie the vast differences in carotenoid synthesis and accumulation in the different marigold varieties.

Introduction

Carotenoids are a large family of C_{40} isoprenoid pigments that are found in all higher plants as well as many bacteria and fungi. Over 600 different carotenoid structures have been identified (Starub, 1987). Carotenoids play essential roles in photosynthesis in the green tissues of higher plants. They have structural and functional roles in the light harvesting antennae and serve as photoprotective compounds by quenching triplet chlorophyll and singlet oxygen derived from excess light energy (Demmig-Adams and Adams, 1996). The role of the carotenoids that accumulate to high levels in many fruits and flowers is thought to be to serve as visual attractants for insects and animals to aid in pollination and seed dispersal.

Interest in carotenoid synthesis has also been stimulated by the various roles that carotenoids play in the human diet (DellaPenna, 1999; Hirschberg, 1999). Some β-ring containing carotenoids are precursors of vitamin A in the human diet. Vitamin A deficiency is a significant cause of blindness and early mortality, particularly in countries where rice, which is low in vitamin A, is the main staple in the diet. In addition, diets rich in carotenoids, such as lycopene, that lack β rings and thus do not have provitamin A activity have other health-promoting effects (Mayne, 1996). The nutritional benefits of carotenoids have led to metabolic engineering of crops deficient in carotenoids for increased production of these compounds (Burkhardt et al., 1997; Shewmaker et al., 1999; Ye et al., 2000).

The first committed step in the biosynthesis of carotenoids is the head to head condensation of two molecules of geranylgeranyl pyrophosphate to form the colorless

intermediate phytoene. This reaction is catalyzed by the enzyme phytoene synthase (Harker and Hirschberg, 1998). In plants, four subsequent desaturation reactions are catalyzed by two enzymes, phytoene desaturase and ζ-carotene desaturase, that each introduces two symmetrical double bonds to yield lycopene. Subsequently, the ends of the linear carotenoid lycopene can be cyclized and various oxygen functions introduced to form the xanthophylls.

Although the biochemical reactions that lead to the synthesis of the carotenoids have been known since the mid-1960s, the majority of the genes encoding enzymes of the pathway have only been identified in the past decade (reviewed by Cunningham and Gantt, 1998). Carotenoid biosynthesis occurs in the plastids, yet all of the genes are nuclear encoded. Biochemical characterization of the enzymes responsible for carotenogenesis has been hampered by the fact that many of them are membrane associated and difficult to purify in an active state. In addition, the steady state mRNA levels of many of the enzymes are very low in photosynthetic tissues and can only be detected by very sensitive techniques such as RT-PCR (Giuliano et al., 1993).

During the development of fruits and flowers, plastids differentiate into organelles referred to as chromoplasts that are specialized for the sequestration of lipophilic molecules such as carotenoids (Camara et al, 1995). The process of carotenoid accumulation in chromoplasts has been studied in a number of non-photosynthetic plant tissues such as tomato and pepper fruits, and daffodil flowers. During chromoplast development in tomato fruit, it is known that differential transcriptional regulation of carotenoid biosynthetic genes plays a critical role in determining the amounts and types of carotenoids that accumulate. While the mRNAs encoding the genes phytoene synthase

and phytoene desaturase are strongly induced during fruit development (Giuliano et al., 1993), the mRNAs encoding cyclization enzymes are down-regulated (Pecker et al., 1996), consistent with the accumulation of lycopene in tomato fruits. Similar evidence for the importance of phytoene synthase and phytoene desaturase in the regulation of carotenoid accumulation in pepper fruits has also been obtained (Bouviet et al., 1994).

In the current study, we characterize the carotenoid biosynthetic pathway in marigolds, *Tagetes erecta* L. Marigolds are a self-fertile, diploid species (2n=24) best known for their large showy flowers. Marigold varieties exist that range in petal color from white to dark orange. The pigmentation of the flowers is due to the massive synthesis and accumulation of carotenoids during the 7-10 days of petal development. The dark orange colored varieties contain concentrations of carotenoids that are up to 20-fold greater than in marigold leaves and 20 times the concentration of carotenoids found in ripe tomato fruit. For this reason, marigolds are grown commercially as a source of carotenoids and a meal made from the petals is used as an animal feed supplement (Delgado-Vargas and Paredes-Lopez, 1997).

The massive increase in the concentration of carotenoids in marigold petals in orange varieties and the existence of numerous, genetically distinct mutants that cloning from marigold and expression analysis of many of the genes required for carotenoid and IPP biosynthesis as well as two of the genes required for plastid division. Differences in the expression of these genes during petal development among four varieties that accumulate vastly different amounts of carotenoids yields insights into the role transcript abundance accumulate lower amounts of petal carotenoids makes marigold an excellent system to examine the regulation of flux through this pathway. In tomatoes and peppers,

other experimental systems used to study carotenoid biosynthesis; the available mutants generally do not affect flux but rather are blocked at individual biosynthetic steps of the pathway. This highlights the advantage of using marigold petals as an experimental system to study flux through the carotenoid pathway. In this work, we describe the plays in carotenoid biosynthesis and chromoplast development in this system.

Materials and methods

Plant materials

Marigold seeds were from the W. Atlee Burpee Company, Clinton, Iowa. They include Dark Orange Lady (wild type) and three mutant cultivars, Golden Lady (yellow flowers), Primrose Climax (pale yellow flowers), and French Vanilla (white flowers). Petal development was identical in the various cultivars and arbitrarily divided into six stages from which RNA and carotenoids were extracted. Stage 1 petals were approximately 2-4 mm in length, stage 2 petals were approximately 5-7 mm in length, stage 3 and stage 4 petals were approximately 8-10 and 13-15 mm in length, respectively. Stage 5 petals were defined as partially opened flowers (> 2 cm), while stage 6 petals represented the petals from fully opened flowers. Color development in wild type marigold petals is first visible at stage 3.

Molecular techniques

Because carotenoid accumulation in marigold petals increases most rapidly in stages 3 and 4, a petal cDNA library was made from poly A RNA isolated from stage 3 and 4 petals from the variety Dark Orange Lady (cDNA library was constructed as a service by Stratagene, La Jolla, CA). Total RNA was isolated by LiCl precipitation (Sambrook et al., 1989) and poly A RNA was obtained by two passes of the RNA over an oligo-dT cellulose column.

Hybridization of the marigold lambda library with *Arabidopsis* genes as heterologous probes was performed at 50° C using 6X SSPE; filters were washed in 2X SSC, 0.1% SDS at 50° C. Plaque screening and isolation was done according to standard techniques (Sambrook et al., 1989). Single clone excision and mass excision of the lambda library to yield phagemids were done as recommended the manufacturer (Stratagene, La Jolla, CA). Isolated plasmids were sequenced by primer walking on both strands using an ABI dRhodamine cycle sequencing kit. Marigold cDNAs isolated using *Arabidopsis* heterologous probes were phytoene synthase, phytoene desaturase, ζ-carotene desaturase, lycopene ε-cyclase and β-carotene hydroxylase. A marigold α tubulin cDNA was identified by random sequencing of marigold library clones and used as a constitutive control.

To analyze steady state mRNA levels for the described marigold genes, poly A RNA was isolated as described above from pools of stage 1 and 2 petals, stage 3 and 4 petals, and stage 5 and 6 petals of the indicated cultivars. Tissue was harvested in liquid nitrogen and either used immediately for RNA isolation or stored at –80° C. Poly A RNA prepared by two passes of the RNA over an oligo-dT cellulose column was separated by electrophoresis on 1% MOPS-formaldehyde agarose gels (2 μg/lane) and blotted to nylon

membranes (Micron Separations, Westborough, MA). Probes were prepared from the isolated marigold gene fragments by random primer labeling (Gibco BRL, Rockville, MD). Equivalent numbers of counts were used for all hybridizations. Northern blots were hybridized overnight at 42° C in 30% formamide and washed under low stringency condition using 2X SSC at 50° C. An exposure was made using a phosphorimager (Molecular Bioimager, BioRad, Hercules, CA); subsequently, the blots were washed using stringent conditions of 0.1X SSC at 60 ° C and an additional exposure was made. The northern blots using the *Arabidopsis* 1-deoxy-D-xylulose reductoisomerase EST (ABRC EST clone 120E8T7) and the marigold α-tubulin genes as probes were washed at 1X SSC at 55° C.

Color complementation

Marigold genes encoding lycopene β cyclase, 1-deoxy-D-xylulose synthase, IPP isomerase, GGDP synthase, and the plastid division genes MinD and FtsZ were isolated by functional expression in *E. coli* that had been engineered to express various carotenoids (Cunningham et al., 1994). Mass excision of the marigold lambda library and color complementation screening were performed as described (Cunningham et al., 1996). *E. coli* (strain DH5α) was grown in LB supplemented with chloramphenicol to maintain the carotenoid-gene containing plasmid and with ampicillin (100μg/ml) to maintain the marigold library plasmids. Marigold lycopene β cyclase was identified as a yellow, β-carotene containing colony in a background of pink, lycopene accumulating colonies due to the presence of pAC-LYC (Cunningham et al., 1994). Marigold 1-deoxy-

D-xylulose synthase was identified in the same screen as a much darker red colony in a background of paler pink colonies (Moehs et al., 1998). Marigold IPP isomerase and the plastid division gene MinD were isolated during a low temperature screen. Zeaxanthin producing *E. coli* cells (harboring pAC-ZEAX, Sun et al., 1996) grew more slowly than control *E. coli* when incubated at 18 °C. Marigold IPP isomerase and marigold MinD were selected based on the observation that the presence of these genes allowed zeaxanthin containing *E. coli* cells to grow more rapidly and accumulate more pigment than in their absence. Marigold GGDP synthase was isolated using a derivative of pAC-BETA (Cunningham et al., 1996) whose *E. herbicola* GGDP synthase had been deleted. *E. coli* cells harboring this plasmid are colorless. When the marigold library was introduced into this background, rare yellow, β-carotene producing colonies were expressing a complementing marigold GGDP synthase. The marigold plastid division gene FtsZ was isolated fortuitously during a color complementation screen.

Carotenoid extraction, quantification, and HPLC analysis

Carotenoids were extracted from leaves and from different stages and different varieties of marigold petals according to Pogson et al. (1996). Following saponification, the extract was washed free of alkali and the organic phase was dried under vacuum. Absorption determinations were made in ethanol at 444 nm using an extinction coefficient of 2500. HPLC separation of carotenoids was performed as described by Norris et al. (1995).

Results

Isolation of marigold genes

Figure A.1 depicts the pathway and enzymes for carotenoid biosynthesis in plants leading from glyceraldehyde-3-phosphate and pyruvate to lutein and zeaxanthin. The genes in the pathway that we have cloned from marigold are listed in Table A.1. Phytoene synthase, phytoene desaturase, ζ-carotene desaturase, lycopene β-cyclase, lycopene ε-cyclase, and β-carotene hydroxylase are genes specific to the carotenoid. Additional cDNAs cloned include 1-deoxy-D-xylulose synthase (DXP synthase), IPP isomerase, and GGDP synthase that catalyze steps of the central isoprenoid pathway common to the synthesis of carotenoids and other plastid derived terpenoids such as tocopherols, plastoquinone, and chlorophylls. Cloning of these cDNAs was accomplished either by using the corresponding *Arabidopsis* cDNAs as heterologous probes, or by using a function-based *E. coli* cloning technique termed color complementation (Cunningham and Gantt, 1998).

Marigold cDNAs encoding phytoene synthase, phytoene desaturase, ζ-carotene desaturase, lycopene ε-cyclase, and β-carotene hydroxylase were isolated using *Arabidopsis* cDNAs as heterologous probes. In general, the marigold genes were highly homologous to the corresponding *Arabidopsis* genes at the protein level with the percent identity ranging between 70 and 80%. The identity is even higher when the less conserved N-terminal putative plastid targeting signals are not considered. Identity at the DNA level was lower, averaging about 60%. Table A.1 lists characteristics of the isolated

Figure A.1. Non-mevalonate isoprenoid and carotenoid biosynthetic pathway in plants. Enzymes are abbreviated as follows: DXPS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; ?, additional unknown enzymes of non-mevalonate pathway; IPP, isopentenyl pyrophosphate isomerase; GGDP, geranylgeranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; Beta cyc, β-ring cyclase; Eps cyc, ε-ring cyclase; Beta hydrox, β-ring hydroxylase; Eps hydrox, ε-ring hydroxylase.

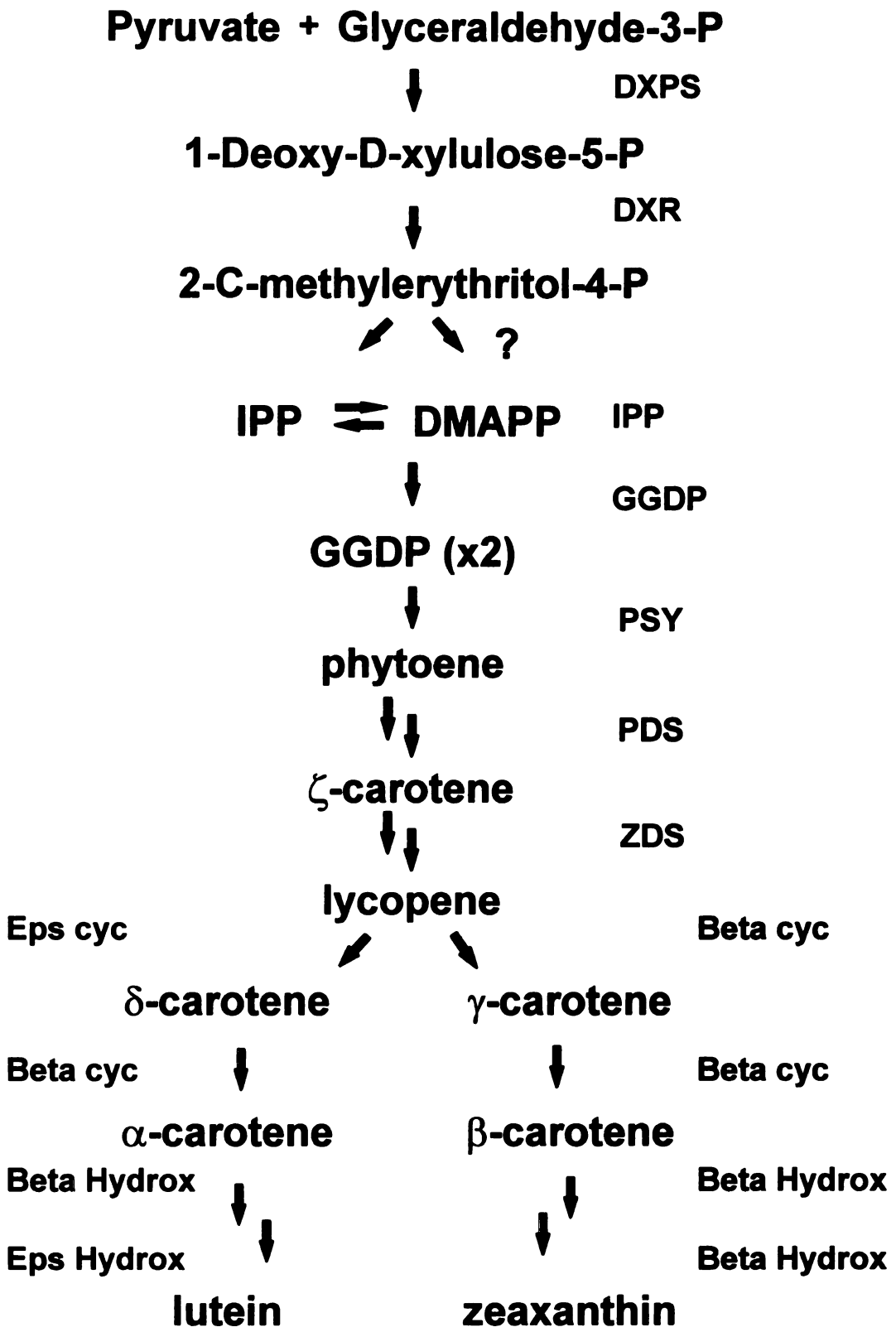

Pyruvate + Glyceraldehyde-3-P
DXPS
1-Deoxy-D-xylulose-5-P
DXR
2-C-methylerythritol-4-P
?
IPP
DMAPP
IPP
GGDP
GGDP (x2)
PSY
phytoene
PDS
ζ-carotene
ZDS
lycopene
Eps cyc
Beta cyc
δ-carotene
γ-carotene
Beta cyc
Beta cyc
α-carotene
β-carotene
Beta Hydrox
Beta Hydrox
Eps Hydrox
Beta Hydrox
lutein
zeaxanthin

Figure A.1

Table A.1. Information about the genes discussed in this report including the number of nucleotides of each clone, the number of amino acids encoded by each cDNA, and the accession number is presented. Abbreviations: DXPS, 1-deoxy-D-xylulose-5-phosphate synthase; IPPI, isopentenyl pyrophosphate isomerase; GGDPS, geranylgeranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-desaturase; Beta Cyc, β-ring cyclase; Eps Cyc, ε-ring cyclase; Beta Hydrox, β-ring hydroxylase; FtsZ and MinD, plastid division genes FtsZ and MinD, respectively.

cDNA	Nucleotides	Amino acids	% Protein identity with Arabidopsis homologue	Accession number
DXPS	2291	725	67	AF251020
IPPI	987	232	83	AF251011
GGDPS	1176	361	66	AF251012
PSY	1371	422	70	AF251015
PDS	1861	551	79	AF251014
ZDS	2061	526	77	AF251013
Beta Cyc	1906	512	74	AF251017
Eps Cyc	1830	517	69	AF251016
Beta Hydrox	996	308	65	AF251018
FtsZ	1357	410	76	AF251346
MinD	1164	296	75	AF251019

marigold cDNAs.

Color complementation was used to isolate marigold genes encoding 1-deoxy-D-xylulose synthase (DXP synthase), IPP isomerase, GGDP synthase, and lycopene β–cyclase, as well as the plastid division genes MinD and FtsZ. This technique relies on the ability of *E. coli* to accumulate various carotenoids when carotenoid biosynthetic genes are expressed from a plasmid (Cunningham et al., 1996). A second plasmid from the library of interest can be introduced into this background with a different selectable marker and a compatible replicon to enable the selection of colonies with the desired phenotype. This method has proven to be effective for isolating carotenoid biosynthetic genes from a number of organisms (Cunningham and Gantt, 1998). The gene encoding lycopene β-cyclase was isolated using *E. coli* containing the plasmid pAC-LYC, which caused the bacteria to accumulate lycopene, resulting in a pink appearance of the colonies. The mass-excised marigold cDNA library was introduced into this background and four colonies of approximately 3.5×10^5 colonies screened were identified based on their yellow color. Subsequent sequencing of the marigold library plasmids confirmed these cDNAs encode marigold lycopene β-cyclase.

Interestingly, several dark red colonies were also identified in the course of this screen. These colonies accumulated greater than two fold more lycopene (data not shown) than the pink pAC-LYC containing *E. coli.* Sequencing of several of these colonies identified the presence of a gene with strong homology to *Arabidopsis* 1-deoxy-D-xylulose synthase (DXP synthase), the first committed step in an alternative, non-mevalonate route to isopentenyl pyrophosphate (Lichtenthaler, 1999). This enzyme catalyzes a transketolase-like reaction in which pyruvate and glyceraldehyde-3-phosphate

are condensed to form 1-deoxy-D-xylulose. The fact that the presence of the marigold DXP synthase more than doubled the amount of lycopene that accumulated in *E. coli* screen suggests that the activity of this enzyme is limiting for carotenoid synthesis in *E. coli*. Similar results have been reported by Harker and Bramley (1999) who found that overexpression of DXP synthases from *Bacillus subtilis* and *Synechocystis* sp.6803 in lycopene producing *E. coli* likewise led to an increase in lycopene accumulation. It is interesting to speculate that the activity of this enzyme catalyzes a rate-limiting step in plant plastid isoprenoid biosynthesis, although this has not been definitively shown. Certainly, its activity is necessary as evinced by the albino phenotype of *Arabidopsis* mutants lacking this enzyme (Mandel et al., 1996).

Additional functional expression screens were used to isolate the remaining genes discussed in this report. Marigold GGDP synthase was isolated using a deletion derivative of pAC-BETA (Cunningham et al., 1996). pAC-BETA contains the *E. herbicola* genes encoding GGDP synthase, phytoene synthase, phytoene desaturase, and lycopene β cyclase, and consequently *E. coli* containing this plasmid accumulates β carotene and is yellow in color. Colorless *E. coli* colonies result when they are transformed with a derivative of pAC-BETA from which the GGDP synthase has been deleted. We introduced the marigold library into this GGDP synthase deficient background and identified two colonies that were yellow in a background of approximately 3×10^5 non-pigmented colonies. These colonies contained marigold library plasmids expressing a complementing GGDP synthase gene.

Finally, a low-temperature screen led to the isolation of marigold genes encoding the isoprenoid enzyme IPP isomerase and the plastid division protein MinD. This screen

was based on the observation that *E. coli* engineered to accumulate zeaxanthin (Sun et al., 1996) grow more slowly at 18° C than *E. coli* harboring the vector plasmid alone and accumulated significantly less zeaxanthin than when they were grown at 37° C. The basis of this temperature dependent phenotype is unknown. When the marigold cDNA library was transformed into zeaxanthin containing *E. coli*, we identified numerous rapidly growing, highly pigmented colonies in a background of pale, slow growing colonies. Plasmids isolated from several of these colonies were sequenced, and similarity searches against the publicly available databases revealed IPP isomerase homologous sequences, as well as a marigold gene with similarity to the *E. coli* MinD protein which is required for proper cell division in *E. coli* (de Boer et al., 1989). A marigold homologue of the *Arabidopsis* plastid division gene *AtFtsZ1-1* was also identified fortuitously during an unrelated expression screen. Antisense inhibition of *AtFtsZ1-1* expression in *Arabidopsis* has been shown to greatly reduce plastid division, leading in some cases to mesophyll cells containing a single enlarged plastid (Osteryoung et al., 1998). This and other evidence indicates that bacterial and plastid division are evolutionarily related (Osteryoung and Pyke, 1998). The basis of increased growth of *E. coli* harboring the marigold plastid division gene MinD may lie in its complementation of a cold-sensitive aspect of *E. coli* cell division.

The majority of the genes we isolated appear to represent full-length cDNAs with the exception of phytoene synthase which lacks a small number of amino acids including an initiating methionine at its N-terminus when aligned with the corresponding *Arabidopsis thaliana* protein. Analysis of the conceptual translation of all of the reported cDNAs with the chloroplast targeting signal recognition program ChloroP (Emanuelsson

et al.,1999) indicated that each contained a high confidence plastid targeting signal with the exception of the marigold ε-cyclase and the marigold IPP isomerase. When the corresponding *Arabidopsis* ε-cyclase and IPP isomerase were similarly analyzed with ChloroP, the ε-cyclase was found to contain a high confidence plastid targeting signal while the IPP isomerase did not. Since the products of these genes are expected to be chloroplast targeted, the plastid targeting of these and the other enzymes will have to be experimentally confirmed.

Analysis of carotenoid levels in different varieties of marigolds

Total carotenoids were extracted from the leaves and petals of marigold varieties whose petals ranged in pigmentation from white to dark orange. Flowers of the varieties examined are depicted in Figure A.2B; the stages of petal development are shown in Figure A.2A. While the four varieties contained vastly different amounts of carotenoids in their petals, all of the varieties contained approximately equal quantities of carotenoids in their leaves (Figure A.3). An HPLC profile of the carotenoids found in the leaves of the wild-type dark orange cultivar is shown in Figure A.4A.This profile is similar to that observed in most higher plant chloroplasts (Ryberg et al., 1993) where lutein is the most abundant carotenoid, followed in abundance by β-carotene, violaxanthin, and neoxanthin. An identical profile is also observed for leaves of the other three marigold cultivars (data not shown).This indicates that the mutation(s) that alter flux through the carotenoid Figure A.2.pathway in petals of the different varieties do not affect carotenoid synthesis in leaves.

Figure A.2. A. Stages of marigold petal development are depicted. Petal development is identical in the various cultivars and was arbitrarily divided into six stages from which RNA and carotenoids were extracted. B. Fully expanded flowers from the four marigold cultivars discussed in this report are shown.

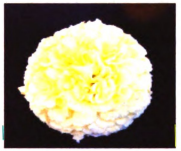
Images in this dissertation are presented in color.

A

1 2 3 4 5 6

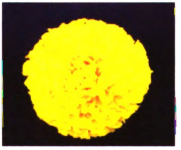
Stages of Marigold Petal Development

B



French Vanilla

Primrose Climax



Golden Lady

Dark Orange Lady

Marigold Cultivars

Figure A.2

In contrast to leaves, the cultivars differ dramatically in the accumulation of carotenoids in their petals. During wild type petal development, there is both a quantitative and a qualitative change in the carotenoids that accumulate. As shown in Figure A.3, there is a sharp induction of carotenoid biosynthesis in wild type petals that is evident by stage 3 and continues until the petals are fully expanded. This induction leads to the accumulation of greater than 20-fold more carotenoids in petals than in the leaves and more than a 100-fold increase during petal development. In contrast, the other varieties show a much-reduced induction of carotenoid biosynthesis, and carotenoid accumulation does not appear to increase beyond stages 3 and 4. The ratios of carotenoids present in marigold petals are also different from those found in leaves. Figure A.4B shows the HPLC profile of an unsaponified extract of carotenoids from petals of the wild type cultivar. The large peaks eluting between 30 and 35 minutes are the carotenoid lutein esterified on its ring hydroxyl groups to various fatty acids. Saponification of this extract yields lutein almost exclusively as shown in Figure A.4C. Esterification of carotenoids is frequently seen in chromoplasts (Camara et al.,1995) and the identification of esterified carotenoids in marigold petals has been previously reported (Quackenbush and Miller, 1972; Gau et al. ,1983; Rivas, 1989). Thus, in wild-type petals there is both a strong induction of overall synthesis and an alteration of the carotenoid pathway in favor of the α-carotene-derived xanthophylls, which contain a β and an ε cyclic end group, as opposed to the β-carotene-derived xanthophylls, which contain two β cyclic end groups.

All of the varieties in this study accumulate primarily esterified lutein in their petals, albeit in vastly different amounts. This indicates the mutations resulting in the

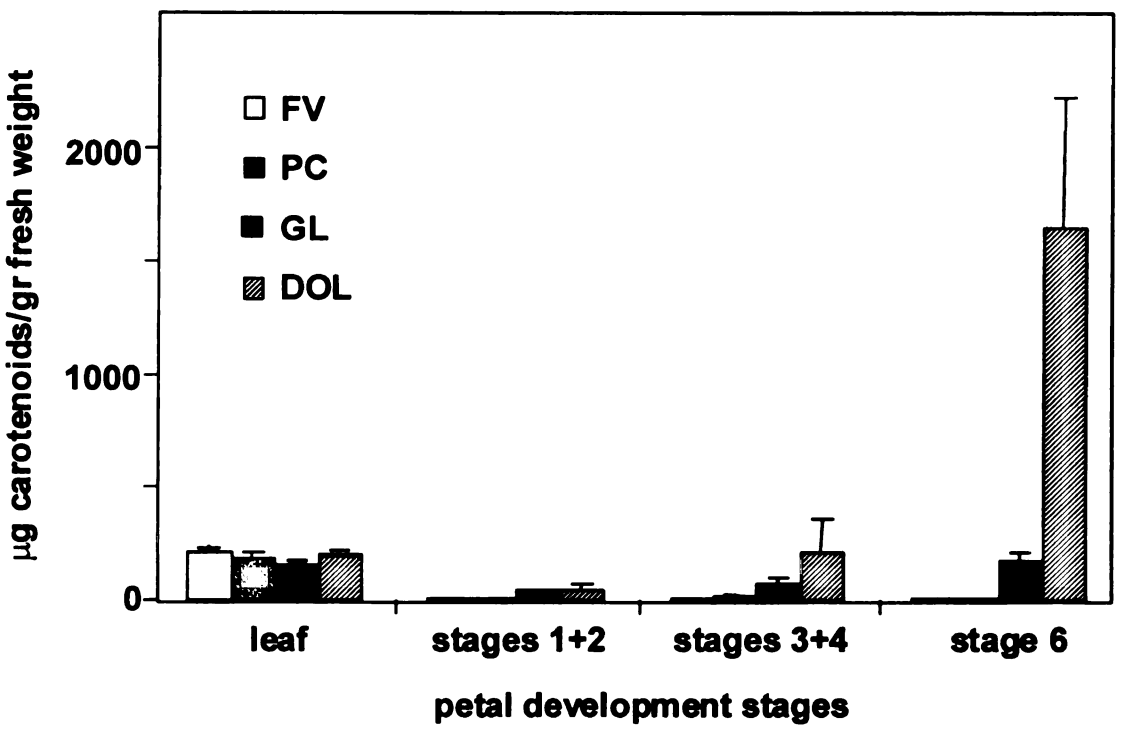


Figure A.3. Carotenoid levels in leaves and during various stages of petal development in the different marigold cultivars. FV, French Vanilla; PC, Primrose Climax; GL, Golden Lady; DOL, Dark Orange Lady.

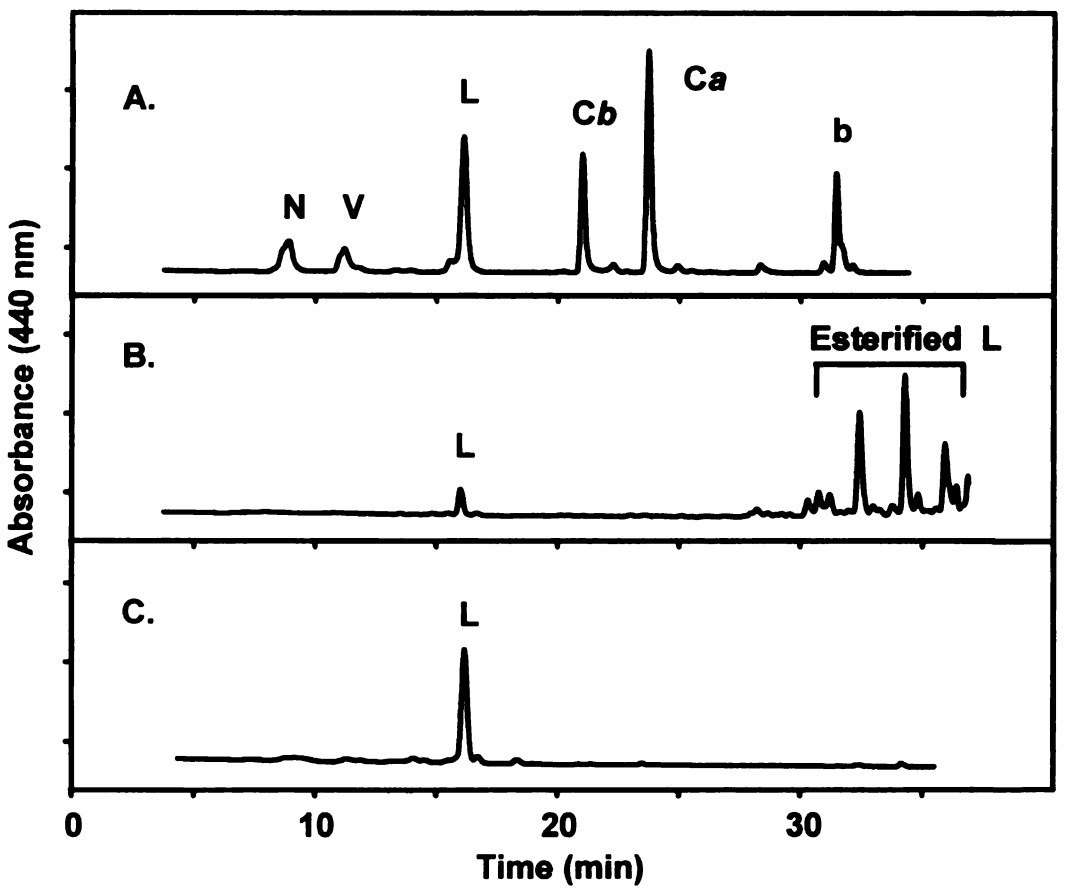


Figure A.4. HPLC analysis of pigments from marigold leaves and petals of the cultivar Dark Orange Lady. A. Profile of leaf pigments. B. Profile of an unsaponified extract of stage 6 petals. C. Profile of a saponified extract of stage 6 petals. Abbreviations: N, neoxanthin; V, violaxanthin; L, lutein; Esterified L, esterified lutein; C*b*, Chlorophyll *b*; C*a*, Chlorophyll *a*; b, β-carotene.

Primrose Climax, Golden Lady and French Vanilla varieties are not only petal specific but also primarily quantitative in their effect, that is they do not block individual steps of the carotenoid pathway or cause accumulation of biosynthetic intermediates. This suggests that differences in the expression pattern of carotenoid biosynthetic pathway genes among the different varieties would relate primarily to the regulation of flux through the pathway and not to the specific type of carotenoid produced.

Expression analysis of isolated genes

To determine the steady state mRNA levels of the genes we have cloned, poly A RNA was isolated by oligo-dT cellulose chromatography from each of the four marigold varieties and from different petal developmental stages. Equal amounts of poly A RNA (2μg/lane) were probed with equivalent numbers of counts from labeled probes made from each of the genes. In Figures A.5 and A.6, the ratio of expression of each of the isoprenoid and carotenoid biosynthetic genes at petal developmental stage 3 and 4 relative to their expression in wild type leaves is shown. The Northern blots are shown as insets to allow an assessment of the relative expression levels of all of the genes to be made. Based on the hybridization with a marigold α-tubulin, the expression of the characterized genes in the French Vanilla and the Primrose Climax varieties may be somewhat over-estimated since α-tubulin mRNAs in petals of these cultivars are slightly higher than wild type.

It is apparent that in the dark orange variety the majority of the genes described, including early steps in the isoprenoid pathway as well as the genes specific for all steps

of the carotenoid pathway, accumulate to considerably higher levels during petal development compared to their accumulation in leaves. In addition, a comparison among the different varieties allows one to draw several conclusions regarding the contribution of transcript abundance of particular pathway steps to regulating flux through the carotenoid pathway. As expected from its position as the first committed enzymatic step in the carotenoid pathway, the expression of phytoene synthase appears to be most closely correlated with the levels of carotenoids and hence flux into the pathway. Despite the nearly ten-fold difference between the amount of carotenoids in fully expanded petals of the yellow flowers of Golden Lady and the orange flowers of Dark Orange Lady (Figure A.3), the abundance of transcripts of pathway genes beyond phytoene synthase is nearly identical. However, phytoene synthase steady state mRNA levels were reduced at least four-fold relative to the wild type dark orange variety (Figure A.6). The conclusion that the expression and/or stability of phytoene synthase transcripts is a key requirement for the accumulation of carotenoids is also supported by its virtually undetectable expression in the varieties with pale yellow or white flowers. These varieties have much reduced expression of most of the characterized genes, but of the carotenoid pathway-specific genes; phytoene synthase mRNA levels are reduced to the greatest extent. Interestingly, the expression of the DXP synthase, the first step in the non-mevalonate route to IPP is also greatly reduced in the mutant cultivars whereas the expression of later steps (deoxy-xylulose reductoisomerase and IPP isomerase) are much less affected (Figure A.5). This suggests that the general production of IPP precursors may also be reduced in these cultivars.

Among the other carotenoid pathway genes analyzed, it is particularly noteworthy

Figure A.5. Northern blots of RNA from stage 3 and 4 petals of each of the indicated cultivars and from leaves of the Dark Orange Lady cultivar. Approximately 2 μg of poly A RNA per lane were loaded. Bar graphs represent expression levels of the indicated genes relative to their expression in leaf tissue. Abbreviations: DXPS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; IPP, isopentenyl pyrophosphate isomerase; GGDP, geranylgeranyl diphosphate synthase; FV, French Vanilla; PC, Primrose Climax; GL, Golden Lady; DOL, Dark Orange Lady.

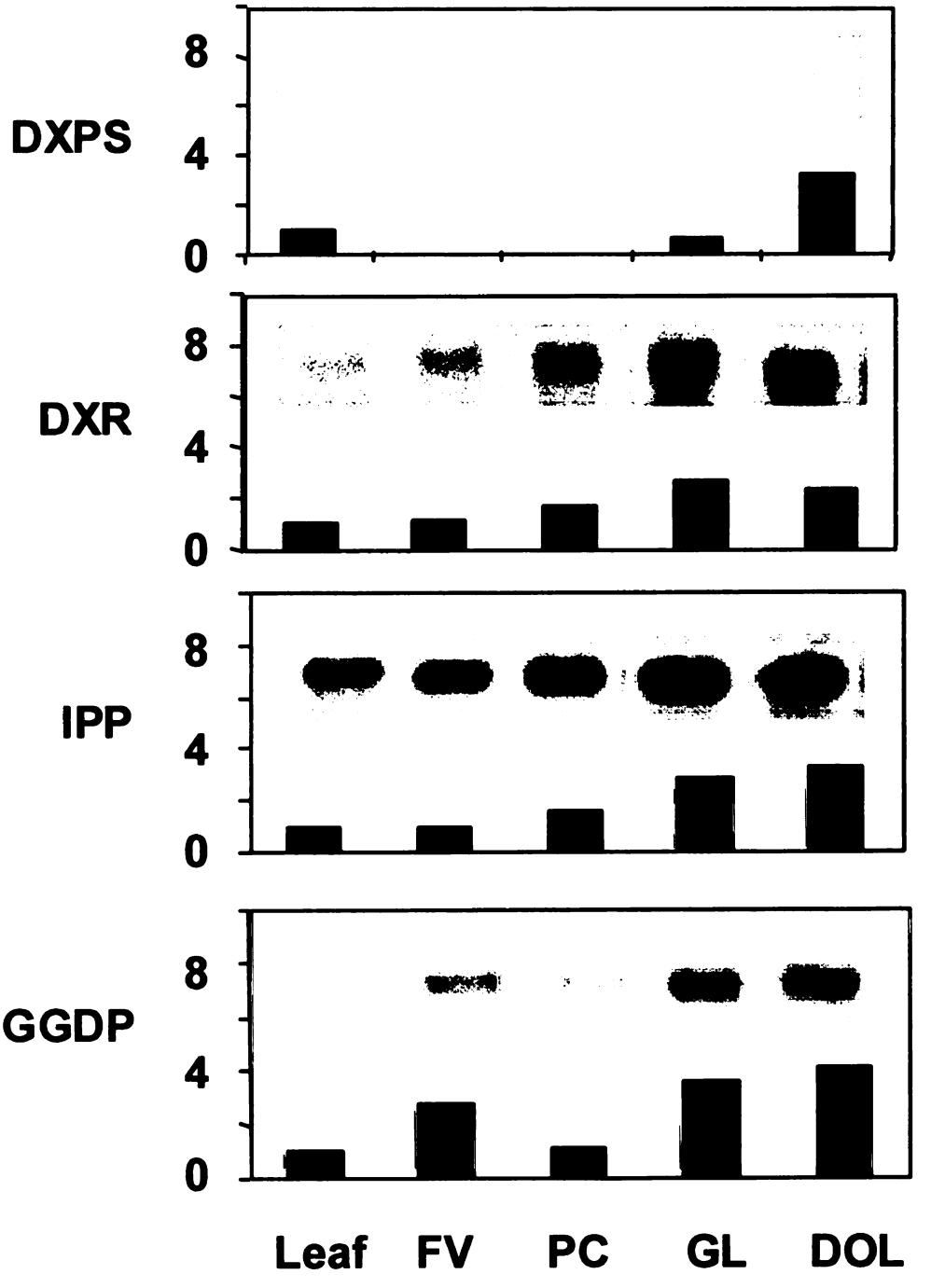


Figure A.5

Figure A.6. Northern blots of RNA from stage 3 and 4 petals of each of the indicated cultivars and from leaves of the Dark Orange Lady cultivar. Approximately 2 μg of poly A RNA per lane were loaded. Bar graphs represent expression levels of the indicated genes relative to expression in leaf tissue. Abbreviations: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-desaturase; Beta cyc, β-ring cyclase; Eps cyc, ε-ring cyclase; Hydrox, β-ring hydroxylase; TUB, α-tubulin; FV, French Vanilla; PC, Primrose Climax; GL, Golden Lady; DOL, Dark Orange Lady.

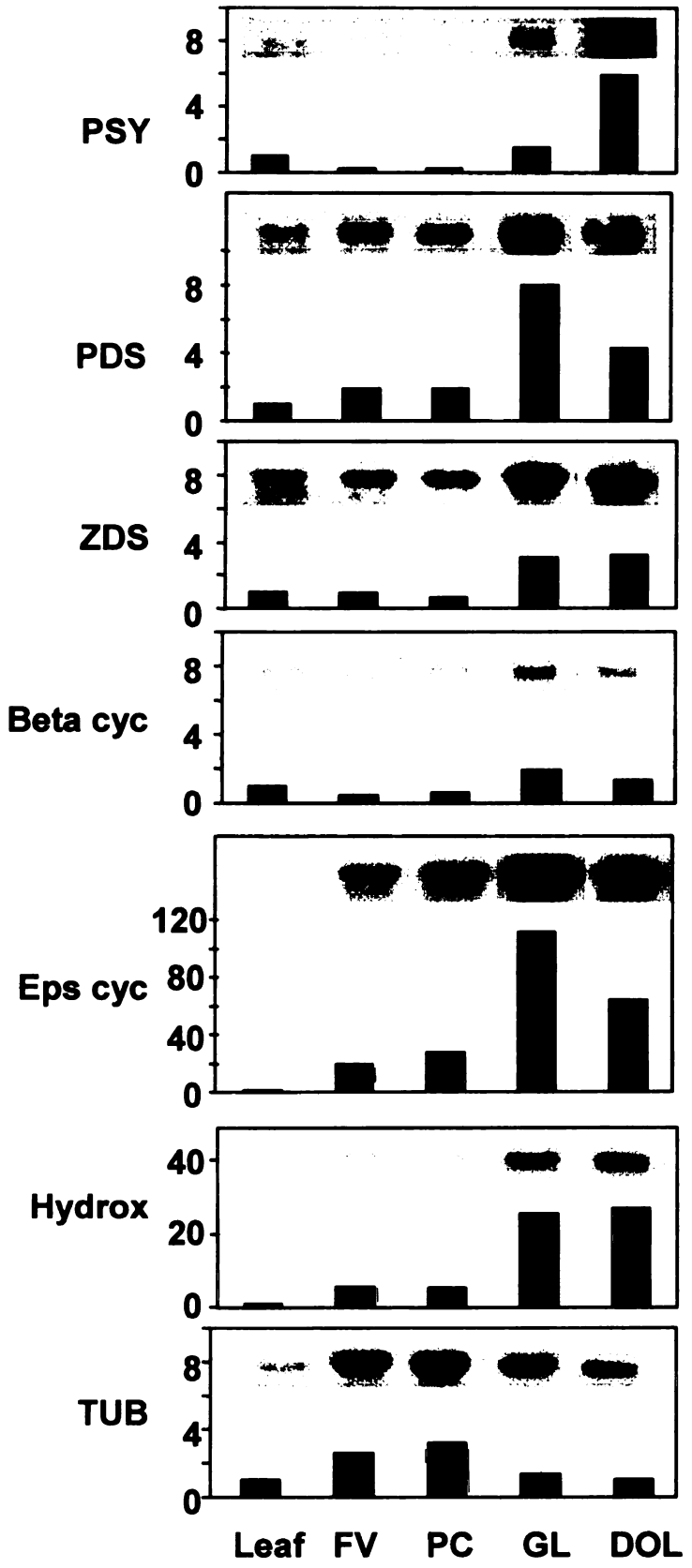

PSY
8
4
0
PDS
8
4
0
ZDS
8
4
0
Beta cyc
8
4
0
Eps cyc
120
80
40
0
Hydrox
40
20
0
TUB
8
4
0
Leaf
FV
PC
GL
DOL

Figure A.6

that while the expression of the β-cyclase is not induced during petal development, the expression of the ε-cyclase is the most strongly induced of the examined genes. Esterified lutein, with one β and one ε ring, is the predominant carotenoid synthesized by petals, while carotenoids having two β rings are much reduced compared to leaves (Figure A.4). Thus the increase in the abundance of the mRNA for ε-cyclase is consistent with a recent model (Cunningham et al., 1996) proposing that the level of ε-cyclase activity controls the degree to which the intermediate lycopene is diverted into the branch of carotenoids having one ε and one β ring. The ε-cyclase gene is up regulated as well in the pale varieties, although not to the same extent as in the yellow and orange flowered varieties. This reinforces the notion that the pale varieties are altered in their flux through the carotenoid pathway but do not differ qualitatively in the type of carotenoid that accumulates in the petals from the yellow and orange varieties. Somewhat surprising, however, is the strong increase in abundance of the β-hydroxylase gene in the yellow and orange varieties, particularly since the transcript for the β-cyclase gene is not similarly increased. An *Arabidopsis* mutant lacking β-hydroxylase activity has been isolated (Pogson et al., 1996) however the gene has not yet been cloned. The *Arabidopsis* β-hydroxylase gene will hydroxylate ε rings but only at a low level compared to its natural β ring substrate (Sun et al., 1996). The marigold β-hydroxylase is 76 % identical to the *Arabidopsis* enzyme at the amino acid level excluding the first 50 amino acids that are predicted to be a plastid-targeting signal. It has a similarly low activity towards ζ rings (data not shown). Attempts to clone a marigold gene related to the marigold β-hydroxylase that would have a high level of ε-hydroxylase activity have been

unsuccessful.

Interestingly, transcripts encoding two proteins involved in plastid division also increased in abundance during petal development in the yellow and orange flower varieties. Recent characterization of the role of the *Arabidopsis* FtsZ proteins suggests that both a cytosol-localized and a plastid-targeted form of these proteins are required for plastid division in plants (Osteryoung et al., 1998). We have cloned a marigold homologue of the *Arabidopsis* plastid-targeted FtsZ gene and Figure A.7 shows its expression in the various marigold varieties. In addition, we show the expression of a second gene also involved in plastid division that represents an eukaryotic ortholog of the *E. coli* gene ***minD***. In *E. coli*, MinD is required for proper placement of the FtsZ division ring (de Boer et al., 1989). Recent research suggests that the eukaryotic MinD ortholog performs an analogous function in plastid division (Colletti et al., 2000). While the expression of both of these genes is virtually undetectable in leaves, FtsZ is increased in abundance about ten fold in yellow and orange flowers, while MinD is increased about 2-fold in these varieties. In the French Vanilla and Primrose Climax varieties, the accumulation of these transcripts is not increased. These results suggest that petal development involves alterations in plastid division in marigold and that defects in the expression of plastid division genes are correlated with decreased carotenoid biosynthesis in pale flower varieties.

Discussion

Marigold flowers range in color from white to dark orange, yet these differences

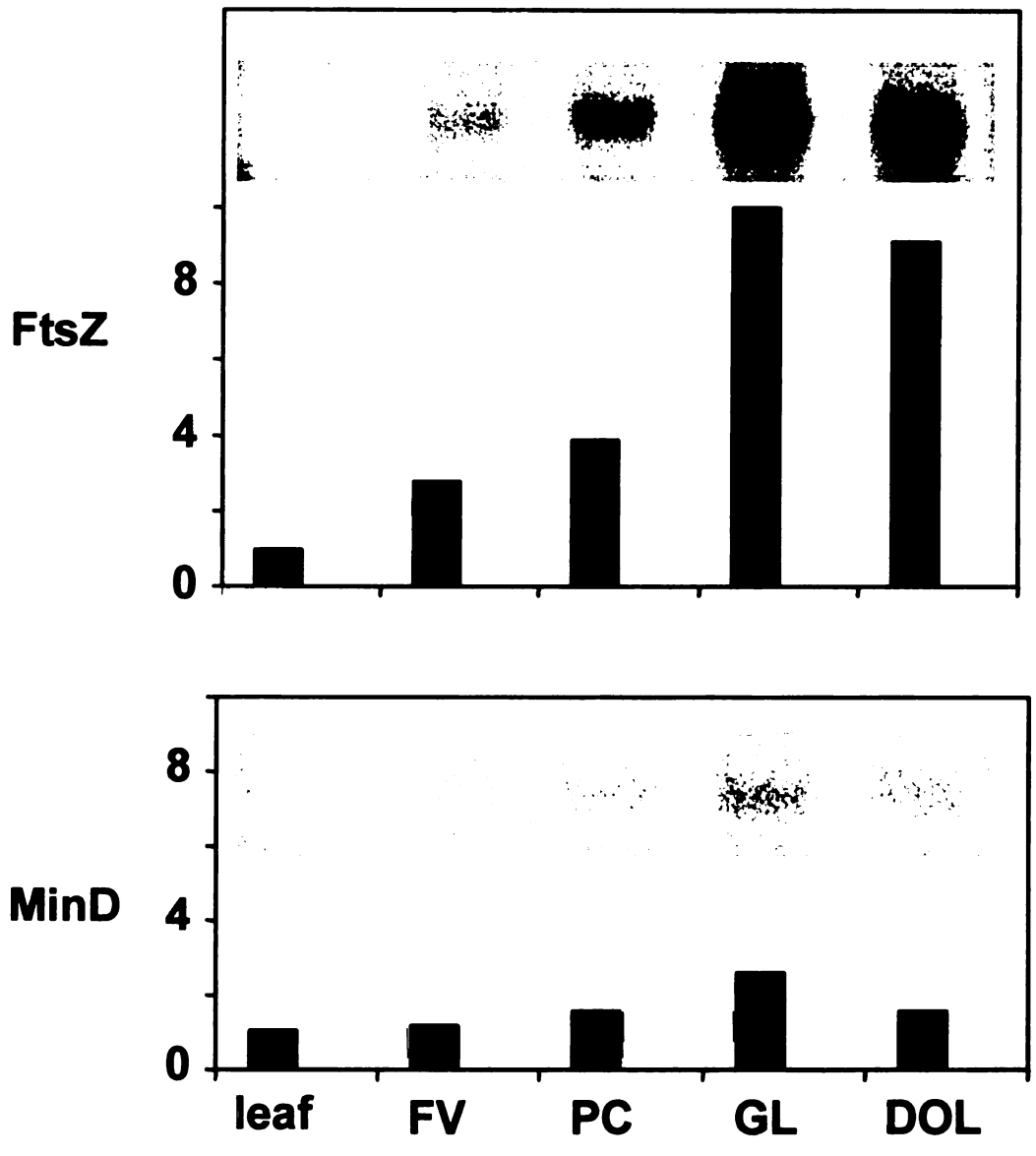


Figure A.7. Northern blots of RNA from stage 3 and 4 petals of each of the indicated cultivars and from leaves of the Dark Orange Lady cultivar. Approximately 2 μg of poly A RNA per lane were loaded. Bar graphs represent expression levels of the indicated plastid division genes relative to expression in leaf tissue. Abbreviations: FV, French Vanilla; PC, Primrose Climax; GL, Golden Lady; DOL, Dark Orange Lady.

in color are due not to the accumulation of different carotenoids or pathway intermediates but rather due to the accumulation of vastly different amounts of the same carotenoid, lutein. During petal development lutein is esterified on each of its hydroxyl groups with various fatty acids, principally myristate and palmitate (Gau et al., 1983; Rivas, 1989) and stored in globules in the chromoplast. In order to identify molecular correlates of carotenoid accumulation and chromoplast development, we have cloned genes from marigold involved in isoprenoid and carotenoid biosynthesis as well as genes involved in plastid division. It is apparent that in the varieties French Vanilla and Primrose Climax, which accumulate very low amounts of carotenoids in their petals, the accumulation of transcripts of all carotenoid pathway genes, isoprenoid biosynthetic genes and at least two genes involved in plastid division, are considerably reduced compared to the wild type orange flowers of the variety Dark Orange Lady. Although the nature of the mutations in these varieties is unknown, this wholesale down-regulation of an entire pathway suggests that they are regulatory in nature and affect the transcription or stability of the messages of multiple genes during petal development. In contrast, the variety Golden Lady is distinguished from the variety Dark Orange Lady primarily by a reduced accumulation of the transcripts for phytoene synthase and DXP synthase. It should be noted that low levels of all of the enzymes are likely to be present even in the pale varieties since low levels of esterified lutein (1-2% of wild type) are still found in these varieties.

In contrast to petals, the synthesis of carotenoids in leaves appears to be unaffected in all varieties. This raises the question whether there exist petal-specific isoforms of the enzymes required for carotenoid biosynthesis and plastid division or

whether the down-regulation of the pathway in petals is due to the tissue-specific mis-regulation of single genes. At present, we cannot say, although studies in other organisms suggests multiple genes or small gene families are common for several of the steps involved in isoprenoid and carotenoid biosynthesis. Indeed, in *Arabidopsis* six different GGDPS genes have been identified several of which are localized to different subcellular compartments (Okata et al., 1999) and two differentially regulated phytoene synthase genes exist in tomato (Bartley and Scolnik, 1993). Tomato Psy-1 appears to be responsible for carotenoid biosynthesis in fruits, while Psy-2 synthesizes foliar carotenoids (Fraser et al., 1999). These two enzymes are 95% identical in their amino acid sequence, however antisense inhibition of Psy-1 abolishes carotenoid accumulation in fruits only (Bramley et al., 1992). In our experiments, low stringency hybridization with the marigold ε-cyclase gene revealed a lower molecular weight transcript specifically in leaves, suggesting the presence of at least a second ε-cyclase gene (data not shown). Data from genomic southern analysis for several pathway steps are consistent with the presence of small gene families in marigold (data not shown). Thus, it is likely that multiple genes exist in marigold for many of the steps in the pathways we have examined.

Somewhat surprisingly, the DXP synthase mRNA showed the lowest absolute accumulation of the genes we examined. This gene encodes the first step in the recently identified non-mevalonate route to plastid isoprenoids and is necessary for the synthesis of carotenoids and other plastid synthesized isoprenoids (Mandel et al., 1996; Bouvier et al., 1998). It remains to be seen for this and the other genes we isolated how closely the level of gene expression is correlated with protein abundance and enzyme activity.

Attempts are in progress to raise antibodies to several of the marigold enzymes to address these questions.

Cunningham et al. (1996) determined that *Arabidopsis thaliana* ε-cyclase catalyzes the introduction of a single ε ring onto the linear lycopene, while the β-cyclase introduced two β rings onto lycopene to produce β-carotene. Carotenoids with two ε rings are rare in nature. These results have led Cunningham and Gantt (1998) to propose that there may exist two multienzyme complexes in plastid membranes, one containing two β-cyclase enzymes, while the other contains one β- and one ε-cyclase. Since the ε-cyclase mRNA is the most strongly up regulated of the studied genes and lutein is the predominant petal carotenoid, we expect that marigold chromoplast membranes would be enriched in carotenogenic complexes containing ε-cyclase. Other studies have suggested that there is a channeling of intermediates through a multienzyme complex during carotenoid biosynthesis (Candau et al., 1991; Camara, 1993) and the interaction of multiple enzymes has also been found in the synthesis of another abundant class of plant pigments, the flavonols (Burbulis and Winkel-Shirley, 1999).

The up regulation of two genes required for plastid division in two of the studied marigold varieties and the lack of induction of these genes in the pale-colored marigold varieties suggests a correlation between carotenoid accumulation and chromoplast replication. Given that plastids are the site for both synthesis and storage of carotenoids, it is likely that plastid numbers or size would increase to accommodate or allow high level synthesis and accumulation of carotenoids. FtsZ (filamentous temperature-sensitive) was first identified in *E. coli*; mutants in this gene cause long undivided filamentous cells to form (Lutkenhaus and Addinall, 1997). In *Arabidopsis*, both plastid-targeted and

presumed cytoplasmic FtsZ homologues exist and both are required for plastid division (Osteryoung et al., 1998). MinD, likewise, is a bacterial division gene first identified in *E. coli* and plays a role in determining the site of cell division (de Boer et al., 1989). Antisense repression of the Arabidopsis homologue leads to asymmetric plastid division in leaves and petals, indicating a role for the gene in placement of the plastid division apparatus (Colletti et al., 2000). The process of plastid differentiation and division during petal development has not been extensively characterized although one published study (Pyke and Page, 1998) suggests that there is little plastid division during petal development in *Arabidopsis*. However, Pyke (1997) has presented evidence for a large increase in plastid number and reduction in plastid size during the chloroplast to chromoplast differentiation process in ripening tomato fruit. Further work will be needed to examine plastid differentiation and division in the petals of the different varieties of marigolds and its relationship to carotenoid accumulation.

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