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IDENTIFICATION AND FUNCTIONAL ANALYSES OF HOMOGENTISATE PHYTYLTRANSFERASE; A KEY ENZYME INVOLVED IN TOCOPHEROL SYNTHESIS IN PHOTOSYNTHETIC ORGANISMS

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By

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ABSTRACT

IDENTIFICATION AND FUNCTIONAL ANALYSES OF HOMOGENTISATE PHYTYLTRANSFERASE; A KEY ENZYME INVOLVED IN TOCOPHEROL SYNTHESIS IN PHOTOSYNTHETIC ORGANISMS

By

Eva Collakova

Tocopherols are lipid soluble antioxidants collectively known as Vitamin E, an essential component of the human diet. Tocopherols consist of a polar chromanol ring and a hydrophobic prenyl chain derived from homogentisate (HGA) and phytyl diphosphate (PDP), respectively. Condensation of these two compounds represents the committed step in tocopherol biosynthesis catalyzed by homogentisate phytyltransferase (HPT). HPTs in *Synechocystis sp.* PCC 6803 (SynHPT) and *Arabidopsis thaliana* (AtHPT) were identified based on their sequence similarity to chlorophyll synthases, which also utilize PDP. Both SynHPT and AtHPT showed prenyltransferase activity *in vitro* with HGA and PDP as preferred substrates. Neither enzyme was active with solanesyl diphosphate, the prenyl substrate used in plastoquinone-9 biosynthesis. Plastoquinone-9 levels were unaffected in *Synechocystis sp.* PCC 6803 HPT disruption mutant, which showed a complete absence of tocopherols. These results suggest existence of separate polyprenyltransferase activities involved tocopherol and plastoquinone synthesis in *Synechocystis sp.* PCC 6803.

Tocopherol levels fluctuate during plant growth, possibly due to an altered expression of genes involved in tocopherol synthesis and accumulation. Because HPT catalyzes the committed step of tocopherol biosynthesis, it is likely that HPT is a

regulated enzyme, activity of which may limit tocopherol synthesis in non-stressed and stressed plants. This hypothesis was addressed by generating transgenic Arabidopsis plants constitutively overexpressing AtHPT (35S::*HPT1*). Increases in HPT specific activity correlated with elevated total tocopherol levels observed in transgenic lines under normal and high-light stress conditions. Non-stressed 35S::*HPT1* lines accumulated a 4.4-fold and 40 % increase in total tocopherol levels in leaves and seeds, respectively, relative to wild type plants. Total tocopherol levels in 35S::*HPT1* increased 2- to 3.8-fold relative to wild type during high light stress. These results indicate that HPT activity limits tocopherol biosynthesis in non-stressed and stressed wild type Arabidopsis plants. Stress resulted in an up-regulation of HPT and several other enzymes involved in tocopherol accumulation and concomitantly in a dramatic increase in total tocopherol levels in both wild type and 35S::*HPT1*, suggesting that HPT is not the only enzyme involved in regulation of tocopherol biosynthesis. The presence of high levels of other tocopherols in stressed plants also suggested a possible limitation of α-tocopherol synthesis by other tocopherol-related enzymes. Identification of the steps limiting tocopherol synthesis is crucial for understanding the regulation of the pathway, which can be applied in plant metabolic engineering of the tocopherol biosynthetic pathway.

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KEY TO ABBREVIATIONS

12-OPDA - 12-oxo-phytodienoic acid
13-HPOT - 13-hydroperoxy-9,11,15-octadecatrienoic acid
35S::*γ-TMT* - transgenic lines overexpressing γ-TMT
35S::*HPT1* - transgenic lines overexpressing HPT
α-TE - α-tocopherol equivalents
α-TTP - α-tocopherol transfer protein
γ-TMT - γ-tocopherol methyltransferase
ADP - adenosine diphosphate
ATBC - Alpha-Tocopherol Beta-Carotene
AtHPT - *Arabidopsis thaliana* homogentisate phytyltransferase
ATP - adenosine triphosphate
BLAST - basic local alignment search tool
bp - base pair
CaMV - cauliflower mosaic virus
cDNA - complementary deoxyribonucleic acid
CDP - cytidine diphosphate
CPME - 4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol
CTP - cytidine triphosphate
cyt b-559 HP - high potential form of cytochrome b-559
DAHP - 3-deoxy-D-arabino-heptulosonate-7-phosphate
DHQ - 3-dehydroquinate
DHS – dehydroshikimate
DMAPP - dimethylallyl diphosphate
DMGBQ - 2,3-dimethyl-6-geranylgeranyl-1,4-benzoquinol
DMPBQ - 2,3-dimethyl-6-phytyl-1,4-benzoquinol
DMPQ-9 - 2-demethylplastoquinol-9
DXP - 1-deoxy-D-xylulose-5-phosphate
DXPS - 1-deoxy-D-xylulose-5-phosphate synthase
E4P - erythrose-4-phosphate
E. coli - *Escherichia coli*
EF1α - elongation factor 1α
EPSP - 5-enolpyruvylshikimate-3-phosphate
FW - fresh weight
G3P - glyceraldehyde-3-phosphate
GGDP - geranylgeranyl diphosphate
GGDR - geranylgeranyl diphosphate reductase
GGPS1 - geranylgeranyl diphosphate synthase 1
Glc – glucose
HEPES - [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid]
HGA - homogentisate or homogentisic acid
HGAD - homogentisate dioxygenase
HPFS - Health Professionals Follow-up Study
HPLC - high performance liquid chromatography
HPP - *p*-hydroxyphenylpyruvate
HPPD - *p*-hydroxyphenylpyruvate dioxygenase
HPT - homogentisate phytyltransferase
IPP - isopentenyl diphosphate
IU - international units
JA – jasmonate
Km^r - kanamycin resistance

LDL - low density lipoproteins
MECP - 2-C-methyl-D-erythitol-2,4-cyclodiphosphate
MEP - 2-C-methyl-D-erythritol-4-phosphate
MESP - 2-C-methyl-D-erythrose-4-phosphate
MGBQ - 2-methyl-6-geranylgeranyl-1,4-benzoquinol
MPBQ - 2-methyl-6-phytyl-1,4-benzoquinol
MPBQ MT - 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase
mRNA - messenger ribonucleic acid
NADPH - reduced nicotinamid adenosyl diphosphate
nd - not detected
NHS - Nurses' Health Study
NMR - nuclear magnetic resonance
ORF - open reading frame
PCPME - 2-phospho-4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol
PCR - polymerase chain reaction
PDS - phytoene desaturation
PDP - phytyl diphosphate
PEP – phosphoenolpyruvate
PLA_2 - phospholipase A_2
prenyl-DP - prenyl diphosphate
PKC - protein kinase C
PP2A - protein phosphatase 2A
PQ-9 - plastoquinone-9
PS – photosystem
PUFA - polyunsaturated fatty acid
ROS - reactive oxygen species
rRNA - ribosomal ribonucleic acid
SAM - S-adenosyl-L-methionine
SD - standard deviation
SDP - solanesyl diphosphate
SDS - sodium dodecylsulfate
SELECT - Selenium and Vitamin E Cancer Prevention Trial
***sn*-1** - stereospecifically numbered-1
SynHPT - *Synechocystis* sp. PCC 6803 homogentisate phytyltransferase
TAP - α-tocopherol associated protein
TAT - tyrosine transaminase
TC - tocopherol cyclase
TLC - thin-layer chromatography
Toc – tocopherol
Tyr – tyrosine
UV – ultraviolet
VLDL - very low density lipoproteins

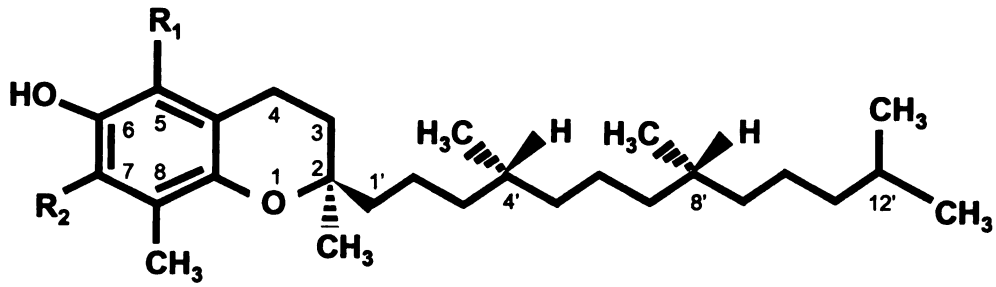
INTRODUCTION

TOCOPHEROL AND TOCOTRIENOL STRUCTURES

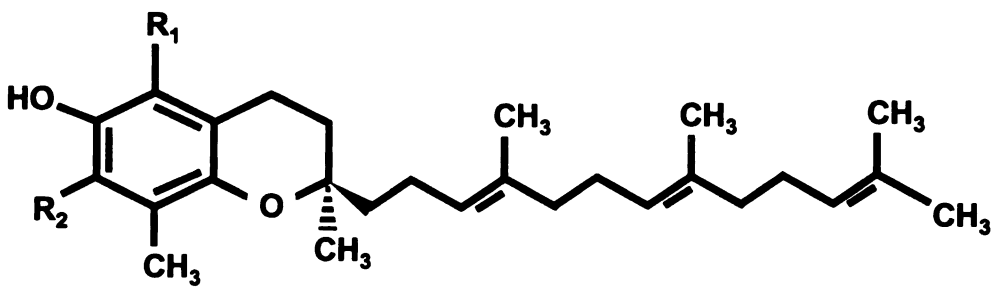
Vitamin E comprises a group of lipid-soluble antioxidants known as tocopherols and tocotrienols, which are synthesized by photosynthetic organisms. Other organisms, including humans, cannot make tocopherols and therefore acquire vitamin E from their diets (Brigelius-Flohe and Traber, 1999; Bramley et al., 2000). Tocopherols can quench and scavenge various reactive oxygen species including singlet oxygen and superoxide radicals and also can deactivate alkyl peroxy radicals formed in the lipid bilayers. These combined activities protect polyunsaturated fatty acids from lipid peroxidation (Fukuzawa et al., 1982; Fukuzawa and Gebicki, 1983; Munne-Bosch and Alegre, 2002a).

Tocopherols are amphipathic compounds consisting of a polar chromanol ring that is responsible for their antioxidant properties and a 15-carbon prenyl chain, which contributes to their lipophilic character. Based on the number and relative positions of the individual methyl groups attached to the chromanol ring, four different tocopherol vitamin isomers (vitamers), α, β, γ, and δ, are synthesized in photosynthetic organisms (Figure 1). Besides the methyl substituent on carbon 8 of the chromanol ring, which is present in all natural tocopherols, two additional positions, carbons 5 and 7, can be methylated. If neither of the two positions is methylated then the resulting vitamer will be δ-tocopherol. A single methylation on carbon 5 yields β-tocopherol, while methylation on carbon 7 results in the formation of γ-tocopherol. Methylation of both carbons yields α-tocopherol.

Tocotrienols have a chromanol ring that is identical to that in the corresponding tocopherols. Tocopherols and tocotrienols differ only in the degree of saturation in their prenyl chains (Figure 1). Tocopherols contain a saturated tail derived from phytyl diphosphate (PDP). In contrast, tocotrienols have an unsaturated isoprenoid tail based on geranylgeranyl diphosphate (GGDP). In both tocopherols and tocotrienols, the prenyl



Tocopherols



Tocotrienols

	R_1	R_2	Relative vitamin E activity (%)
α-tocopherol (tocotrienol)	CH_3	CH_3	100 (30)
β-tocopherol (tocotrienol)	CH_3	H	50 (5)
γ-tocopherol (tocotrienol)	H	CH_3	10 (ND)
δ-tocopherol (tocotrienol)	H	H	3 (ND)

Figure 1. Natural tocopherols and tocotrienols and their relative vitamin E activities. α-Tocopherol has the highest vitamin E activity in animals as determined by the rat fetal resorption assay. Only α-tocotrienol showed significant vitamin E activity of all tocotrienols. Vitamin E activities of β-, γ-, and δ-tocotrienols was very low or below detection (ND - not detected) (Food and Nutrition Board, 2000).

chain is attached to carbon 2 of the chromanol head-group. The resulting configuration of all natural tocopherols is R, R, R (2-R, 4'-R, 8'-R). Similarly, prenyl chains of all naturally synthesized tocotrienols are also present in a single, 2-R and 3', 7', and 11' *trans* configuration (Kamal-Eldin and Appelqvist, 1996). However, chemical synthesis of tocopherols yields up to eight possible stereoisomers of each tocopherol (RRR, RSR, RRS, RSS, SRR, SSR, SRS, SSS, also known as *all rac*-α-tocopherol), which vary significantly in their vitamin E activity (Eitenmiller, 1997; Food and Nutrition Board, 2000). Because only 2R isomers are retained in plasma, these stereoisomers exhibit significant vitamin E activity, while 2S isomers do not. The biological activity of *all rac*-α-tocopherol is approximately 50% of the biological activity of RRR-α-tocopherol, the most potent form of vitamin E. RRR-β-, -γ-, and -δ-tocopherols also show lower vitamin E activity than RRR-α-tocopherol due to their reduced retention in plasma. Biological activity of individual tocopherols is currently expressed in terms of α-tocopherol equivalents (α-TE). According to this definition, 1 mg of RRR-α-, -β-, -γ-, and -δ-tocopherols have biological activities of 1; 0.5; 0.1; and 0.03 mg α-TE, respectively (Bramley et al., 2000; Food and Nutrition Board, 2000). These values were determined using the rat fetal resorption-gestation assay (Food and Nutrition Board, 2000).

TOCOPHEROL ANTIOXIDANT PROPERTIES *IN VITRO*

Vitamin E functions are associated predominantly with the antioxidant and lipophilic properties of the molecule. The free hydroxyl group on the chromanol ring and prenyl tail at carbon 2 with R stereochemistry are required for maximal tocopherol antioxidant function and membrane localization, respectively (Nishikimi et al., 1980; Fukuzawa et al., 1981; Kamal-Eldin and Appelqvist, 1996). Several mechanisms for tocopherol action in terms of antioxidant properties have been proposed. These mechanisms include quenching and

scavenging of various radicals such as reactive oxygen species (ROS) and lipid peroxy radicals (Fryer, 1992; Liebler, 1993; Liebler, 1998; Munne-Bosch and Alegre, 2002a).

Quenching

Tocopherols are effective quenchers of singlet molecular oxygen ($^1O_2$), which can cause damage to membranes (Fahrenholtz et al., 1974; Yamauchi and Matsushita, 1979b; Neely et al., 1988). Quenching of $^1O_2$ by tocopherols occurs through resonance energy transfer and involves a physical deactivation of $^1O_2$ (Fahrenholtz et al., 1974; Foote et al., 1974; Stevens et al., 1974; Neely et al., 1988). An electron from the tocopherol molecule is donated to the $^1O_2$ molecule to form an excited charge transfer complex that dissociates into tocopherol and triplet (ground state) molecular oxygen ($^3O_2$) (Yamauchi and Matsushita, 1977). The quenching rate was proposed to be dependent on the polarity of the solvent used for these in vitro studies because hydrophobic solvents inhibited formation of these charge transfer complexes (Fahrenholtz et al., 1974; Neely et al., 1988). The type of tocopherol also affected the rate of radical quenching. α-Tocopherol was the most effective $^1O_2$ quencher, followed by β-, γ-, and δ-tocopherols, in a hydrocarbon solvent that mimicked a natural membrane environment (Neely et al., 1988). Similarly, α-tocopherol was the most effective quencher of the perferryl ions in lecithin liposomes (Fukuzawa et al., 1982). A single molecule of α-, β-, γ-, δ-tocopherol, and tocol was able to protect 220, 120, 100, 30, and 20 molecules of polyunsaturated fatty acids (PUFAs), respectively, from lipid peroxidation (Fukuzawa et al., 1982). Based on kinetic studies utilizing autosensitized rubrene photooxidation, it was estimated that a single molecule of α-tocopherol quenches ~ 120 molecules of $^1O_2$ prior to its degradation (Fahrenholtz et al., 1974).

Scavenging

Scavenging of reactive oxygen species (ROS) and alkyl peroxy radicals is based on the chemical reaction of a tocopherol molecule with the target radical molecule. Tocopherols can scavenge ROS including $^1O_2$, superoxide anion (O_2^-), and hydroxyl ($OH^\cdot$) radical and perhydroxyl (OH_2^-) anion (Foote et al., 1974; Yamauchi and Matsushita, 1979b; Nishikimi et al., 1980; Fukuzawa and Gebicki, 1983; Fukuzawa et al., 1997). Electron spin resonance studies have demonstrated that scavenging of radicals by tocopherols involves the donation of a hydrogen atom from tocopherols to the radical and formation of a tocopheroxyl radical (Scarpa et al., 1984; Kagan and Packer, 1994; Singh et al., 1998). Several other intermediates and final tocopherol degradation products have also been identified. Scavenging of ROS by tocopherols usually results in the formation of tocopheryl hydroperoxydienones and hydroxytocopherones of varying stability that disintegrate into tocopheryl quinone and its epoxides in hydrocarbon solvents and model membranes (Yamauchi and Matsushita, 1979b; Nishikimi et al., 1980; Fukuzawa and Gebicki, 1983; Neely et al., 1988; Singh et al., 1998). Besides tocopheryl quinone formation, nitration of γ-tocopherol to yield 5-NO_2-γ-tocopherol was also observed during scavenging of NO_2^- radicals (Singh et al., 1998).

Tocopherols are potent protectors of PUFAs from lipid peroxidation in spite of a relatively low tocopherol to PUFA ratio in membranes. This ability of tocopherols to protect lipids from peroxidation may be due in part to recycling of tocopheroxyl radicals to tocopherols via the ascorbate/glutathione/NADPH recycling system (Nishikimi et al., 1980; Scarpa et al., 1984; Liebler, 1993; Kagan and Packer, 1994). Scavenging of lipid peroxy radicals is the primary mechanism by which tocopherols protect polyunsaturated fatty acids (PUFAs) from lipid peroxidation (Liebler, 1993). Figure 2 shows an example of peroxidation of the PUFA linolenic acid, the scavenging of the corresponding lipid peroxy

radical by α-tocopherol, and recycling the α-tocopheroxyl radical back to α-tocopherol. Lipid peroxidation is initiated by removing a hydrogen atom from PUFA by an electron-deficient free radical, causing the formation of an alkyl radical, which is rearranged to a conjugated diene. Interaction of the conjugated diene with molecular oxygen results in the formation of a lipid peroxy radical. In the absence of chain-breaking antioxidants such as tocopherols, these lipid peroxy radicals can remove hydrogen atoms from other PUFAs, resulting in the formation of new radicals and propagation of lipid peroxidation. Tocopherols are able to neutralize the highly reactive lipid peroxy radicals by donating a hydrogen atom from their chromanol hydroxyl group thus leading to the termination of lipid peroxidation by the formation of relatively stable alkyl hydroperoxide and tocopheroxyl radical (Fukuzawa et al., 1981; Fukuzawa et al., 1982; Scarpa et al., 1984). In plants, alkyl hydroperoxides can be metabolized to a wide variety of lipid alcohols and epoxides, jasmonate, n-hexenal, or traumatic acids (Schaller, 2001; Blee, 2002; Howe and Schilmiller, 2002).

Tocopheroxyl radicals are stabilized by delocalization of unpaired electrons on the aromatic structure of the chromanol rings (Kamal-Eldin and Appelqvist, 1996; O'Malley, 2002). There are several ways to eliminate these radicals *in vitro*. Tocopheroxyl radicals do not interact with PUFAs, but they can be converted back to tocopherols via ascorbate/glutathione/NADPH recycling system or react with another radical (Scarpa et al., 1984; Kagan and Packer, 1994; Munne-Bosch and Alegre, 2002a). Reaction of a tocopheroxyl radical with a lipid peroxy radical yields tocopherol/lipid adducts. Tocopheroxyl radicals may react together to generate tocopherol dimers and trimers, while interaction of a tocopheroxyl radical with ROS yields a variety of oxidation products including epoxy tocopherones and epoxy tocopheryl quinones (Kamal-Eldin and Appelqvist, 1996; Liebler et al., 1996; Liebler, 1998). The extent of tocopherol recycling or whether all these oxidation products are formed *in vivo* is not known. In chloroplasts, the final products of tocopherol oxidation were identified as tocopheryl quinol and the

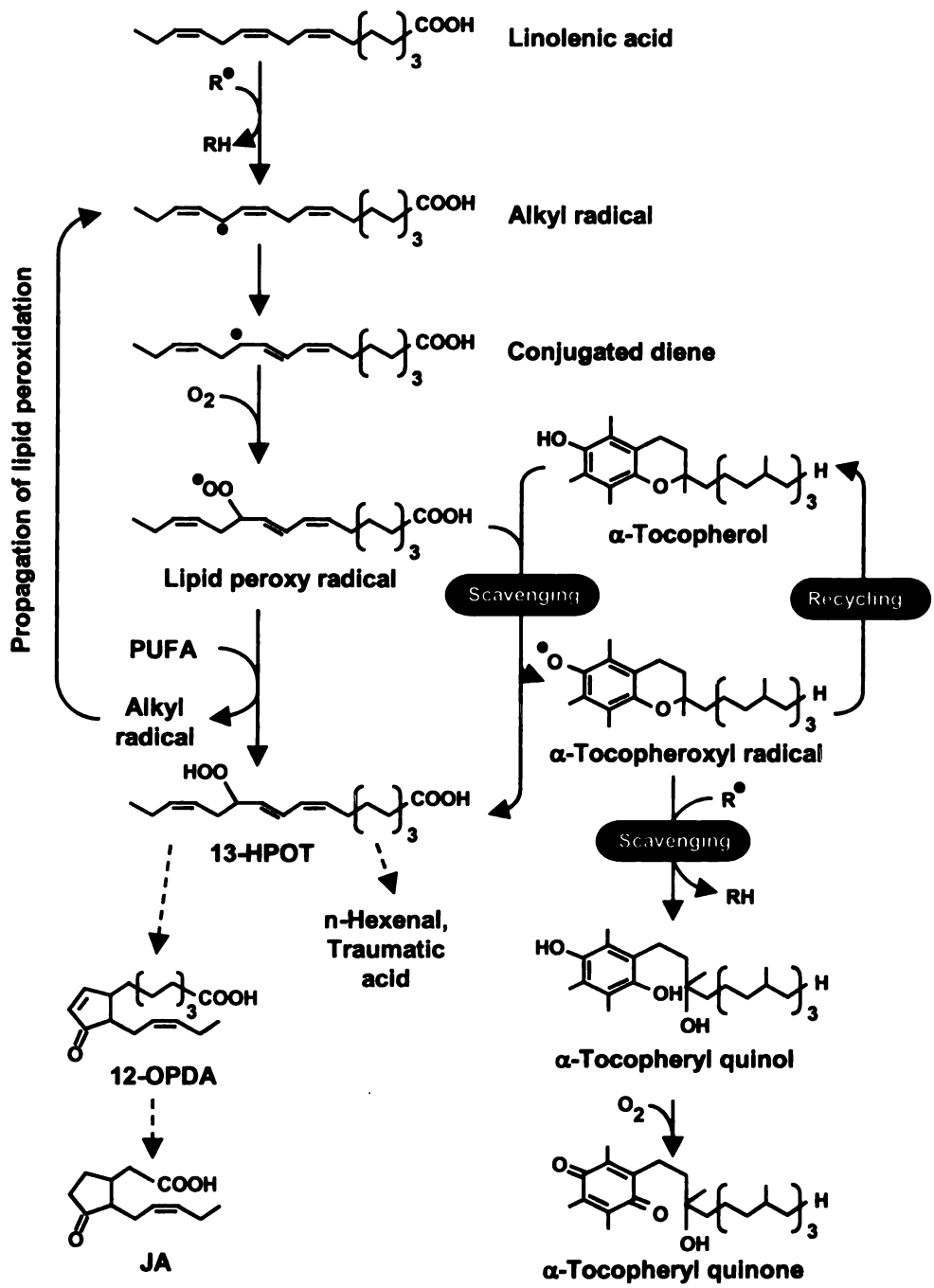


Figure 2. Example of lipid peroxidation and chain-breaking antioxidant activity of tocopherols. Abbreviations: electron-deficient radical (R˙); polyunsaturated fatty acid (PUFA); 13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT); 12-oxo-phytodienoic acid (12-OPDA), jasmonate (JA)

corresponding quinone (Figure 2), which may be involved in cyclic photosynthetic electron transport around photosystem II (Yamauchi and Matsushita, 1979a; Michalski and Kaniuga, 1981; Kruk and Strzalka, 1995; Fryer, 1992; Munne-Bosch, 2002a].

TOCOPHEROLS AND MEMBRANE STABILIZATION

A single *cis* double bond in a fatty acid introduces a "kink" in the hydrocarbon chain of the fatty acid, interrupting some Van der Waals interactions with other fatty acids in the membrane. Therefore, high levels of unsaturated fatty acids in the lipid bilayer result in increased fluidity of such membranes, which is desirable at low temperatures in terms of increasing the tolerance of living organisms to chilling (Wada et al., 1990; Gombos et al., 1992; Wada et al., 1993; Ishizaki-Nishizawa et al., 1996; Los and Murata, 1998). Small amphipathic molecules such as tocopherols and cholesterol fit between the gaps formed by unsaturated fatty acids in the membranes and increase membrane rigidity by interacting with these fatty acids (Stillwell et al., 1996). In 1973, Diplock and Lucy proposed a model describing possible membrane-stabilizing interactions of α-tocopherol with arachidonic acid as an example of a polyunsaturated fatty acid (PUFA) based on the complementary shapes of these two compounds by using molecular models. The methyl groups at C-4' and C-8' of the vitamin fit in the pockets created by Δ^5 and Δ^{11} *cis* double bonds of the arachidonic acid such that both polar parts of the tocopherol and phospholipid face the same direction where they interact with the water-soluble cellular components. Using fluorescent probes, it has been demonstrated that the chromanol ring of α-tocopherol does not protrude into the lipid-water interphase (Bisby and Ahmed, 1989). Diplock and Lucy (1973) also proposed that at least two *cis* double bonds interrupted by a methylene group are required for pocket formation and that the straight chains of the fully saturated fatty acids do not provide the pockets to fit the phytyl methyl groups of tocopherols. Ideally, four double bonds would provide two pockets for the interaction of both C-4' and C-8' methyl groups of the

tocopherol, which would lead to the stabilization of the lipid bilayers and contribute to the rigidity of membranes (Diplock and Lucy, 1973).

To support the hypothesis of membrane stabilization by α-tocopherol, it was necessary to demonstrate physical interaction of α-tocopherol with membrane lipids. Erin et al. (1984) showed that α-tocopherol can form stable complexes with free saturated and polyunsaturated fatty acids in ethanol or when incorporated into phosphatidylcholine liposomes or sarcoplasmic reticulum membranes *in vitro*. In addition, this interaction was independent of the length of these fatty acids, but it did increase with the degree of fatty acid unsaturation (Erin et al., 1984), consistent with the hypothesis proposed by Diplock and Lucy (1973). The following *in vitro* studies have demonstrated that α-tocopherol exerted certain effects on various physical properties of the model membranes, including membrane stability and rigidity as well as the transition of gel to liquid-crystalline structure of membranes (Wassall et al., 1986; Stillwell et al., 1996; Wang and Quinn, 2000).

Stillwell et al. (1996) compared the effects of α-tocopherol and cholesterol on the properties of membranes composed of phosphatidylcholine containing saturated stearic acid in the *sn*-1 position and various unsaturated fatty acids in the *sn*-2 position. Both cholesterol and α-tocopherol broadened and lowered the main transition of gel to liquid-crystalline structure of the membrane (Stillwell et al., 1996). Similar effects were observed when α-tocopherol was added to the model multilamellar membrane consisting of egg yolk lysophosphatidylcholine with $[^2H_{31}]$-palmitic acid at the *sn*-2 position (Wassall et al., 1986). Cholesterol and α-tocopherol had different effects on the membranes containing different types of unsaturated fatty acids (Stillwell et al., 1996). α-Tocopherol had a larger and more complex effect than cholesterol on the membranes containing a high content of PUFAs with a double bond before the Δ^5 position, suggesting that its mode of interaction with fatty acids in lipid bilayers is different from that of cholesterol. α-Tocopherol decreased the permeability to protons in all types of membranes tested, while cholesterol was effective only with less unsaturated fatty acids. α-Tocopherol was shown to exert the highest effect

on membranes composed of highly unsaturated fatty acids, which coincides with its role in protecting these PUFAs from lipid peroxidation (Stillwell et al., 1996).

It appears that α-tocopherol associates preferentially with PUFAs and this interaction causes an increase in membrane rigidity. In all these *in vitro* studies, phospholipid model membranes simulating animal membranes and non-physiologically high concentrations of α-tocopherol were used (Erin et al., 1984; Wassall et al., 1986; Stillwell et al., 1996; Wang and Quinn, 2000). No direct *in vivo* evidence or studies using membranes resembling the high galactolipid content of chloroplasts are available. Photosynthetic organisms regulate their membrane fluidity through controlling unsaturation of fatty acids (Wada et al., 1990; Gombos et al., 1992; Wada et al., 1993; Ishizaki-Nishizawa et al., 1996; Los and Murata, 1998) and the contribution of α-tocopherol to the regulation of membrane fluidity in plants is unclear. Cold-treated plants tend to have reduced α-tocopherol levels relative to the plants grown at optimal temperatures (Fryer et al., 1998; Leipner et al., 1999), which is expected because lower α-tocopherol levels translate into higher membrane fluidity. However, this observation could also be a result of α-tocopherol degradation due to oxidative damage during cold stress. Mutants and transgenic plants with the altered levels and composition of α-tocopherol and PUFAs should be helpful in addressing this question.

VITAMIN E FUNCTION IN ANIMALS

Tocopherol function in animals is mostly connected to their antioxidant properties, especially their ability to protect PUFAs from lipid peroxidation. In animals, vitamin E contributes to the normal muscular, neural, and immune function and was originally found as a factor essential during pregnancy. In recent years, additional roles for α-tocopherol in cellular signaling have been proposed in animals that may be unrelated to their antioxidant

properties because these functions are not performed by other tocopherols exhibiting similar antioxidant activities.

Antioxidant Function

The ability of tocopherols to quench and scavenge various free radicals in model membranes predetermines their antioxidant activity in living organisms. In fact, there is an inverse correlation between tocopherol levels and the degree of oxidative damage in membranes. Male mouse livers, which were highly susceptible to oxidative damage induced by 2-butoxyethanol, showed lower vitamin E levels than the less susceptible female mice and rat livers (Siesky et al., 2002). In addition, tocopherol supplementation had protective effects on lipids in different animal cells and tissues, in which oxidative damage was induced by treatments with various prooxidants. For example, hydrogen peroxide-induced oxidative stress resulted in a significant decrease in PUFA levels relative to control and H_2O_2/vitamin E-treated guinea pig brains (Celik and Ozkaya, 2002). Vitamin E also inhibited lipid peroxidation and cell death exerted by paraquat in isolated hepatocytes (Watanabe et al., 1986). Skin treatment with cumene hydroperoxide caused an increased radical adduct formation in keratocytes of vitamin E-deficient relative to vitamin E-sufficient mice (Schvedova et al., 2002). Mitochondria in rat livers perfused with butylhydroperoxide showed increased lipid peroxidation, which was inhibited by the addition of α-tocopherol (Ham and Liebler, 1997). The degree of α-tocopherol degradation was similar in both vitamin E treated and non-treated rat livers, indicating that the lipid peroxidation inhibition occurred due to supplementary α-tocopherol (Ham and Liebler, 1997). Collectively, these studies suggest that tocopherols reduce oxidative damage by protecting PUFAs from lipid peroxidation in animals.

Roles in Intracellular Signaling

In animals, vitamin E has been proposed to affect signaling by modulating activities of some components of the signal transduction pathways involved in regulation of processes, such as cell proliferation, apoptosis, and inflammation (Azzi et al., 2000; Meydani, 2001; Ricciarelli et al., 2001). Inflammatory processes are associated with a phospholipase A_2 (PLA_2)-dependent release of arachidonic acid, followed by an induction of lipoxygenases and cyclooxygenases, enzymes producing intermediates in oxylipin synthesis (Balsinde et al., 1999). Vitamin E inhibits PLA_2 activity in a non-competitive manner by affecting physical properties of the membrane rather than directly interacting with the enzyme (Grau and Ortiz, 1998). In contrast, vitamin E up-regulated PLA_2 activity in heart myoblasts and megakaryocytes (Tran et al., 1996; Chan et al., 1998). In mouse macrophages, cyclooxygenase activity and prostaglandin formation can be induced by lipid peroxidation and inhibited by α-tocopherol through its antioxidant function (Beharka et al., 2002). Induction of platelet aggregation and cell proliferation involve protein phosphatase 2A (PP2A) inhibition and subsequent protein kinase C (PKC) activation. α-Tocopherol has been shown to inhibit PKC activity by altering the PKC phosphorylation state by activating PP2A in platelets and smooth muscle cells at the post-translational level (Ricciarelli et al., 1998; Azzi et al., 2001; Freedman and Keaney, 2001). In mammary epithelial cells, all tocotrienols, δ-tocopherol, but not α-, β-, and γ-tocopherols, were effective inhibitors of PKCα activity and mitogenesis, suggesting an antioxidant-independent mechanism for these vitamin E forms (Sylvester et al., 2001).

Apoptosis occurs through the induction of caspase cascade and also involves modulation of PKC activity (Nicholson and Thornberry, 1997; Bang et al., 2001; Neuzil et al., 2002). Differential effects of different vitamin E forms on apoptosis in distinct experimental systems have been observed, which could be a result of the existance of different PKC isoforms (Yu et al., 1999; Bang et al., 2001; Neuzil et al., 2002; Uemura et

al., 2002). Both α- and β-tocopherols efficiently scavenged reactive oxygen species, reducing the degree of oxidized LDL-induced apoptosis in vascular endothelial cells, but only α-tocopherol was the most effective form of vitamin E inhibiting caspase-3 activity (Uemura et al., 2002). In human breast cancer cells, all tocotrienols and δ-tocopherol efficiently induced apoptosis, while α-, β-, and γ-tocopherols were ineffective even at high concentrations (Yu et al., 1999). These differences in biological activities of the individual forms of vitamin E point out a mechanism of tocopherol action beyond its antioxidant properties (Yu et al., 1999; Azzi et al., 2001; Meydani, 2001; Ricciarelli et al., 2001; Sylvester et al., 2001; Uemura et al., 2002).

A non-antioxidant α-tocopherol function may also involve an effect on gene expression in animals. Both α- and δ-tocopherols induced expression of the gene encoding α-tocopherol transfer protein (Fechner et al., 1998), which is responsible for specific transport of α-tocopherol to lipoproteins in the liver (Arita et al., 1997). In rat vascular smooth muscle cells, α-tocopherol, but not β-tocopherol, induced expression of α-tropomyosin, which may play a role in early stages of atherosclerosis progression, by an antioxidant-independent mechanism (Aratri et al., 1999). Neither study showed whether α-tocopherol effects on the expression of these genes were direct or indirect. There is ample evidence that the lipid soluble vitamins A and D directly regulate gene expression and similar roles have been proposed for the structurally similar α-tocopherol (Carlberg, 1999). In recent years, α-tocopherol associated protein (TAP) has been shown to specifically bind α-tocopherol, but not other tocopherols (Stocker et al., 1999; Yamauchi et al., 2001). Upon binding α-tocopherol, TAP translocated from cytoplasm to the nucleus and activated reporter gene expression, suggesting that TAP-α-tocopherol complex may function as a transcriptional activator (Yamauchi et al., 2001).

Vitamin E And Degenerative Disease Prevention

A positive correlation between oxidative damage and progression of certain degenerative disease has been suggested. The ability of vitamin E to prevent oxidative damage is thought to play a key role in decreasing the risk of cardiovascular disease and certain types of cancer. Coronary heart disease is associated with the formation of atherosclerotic depositions due to oxidized cholesterol present in low density lipoproteins (LDL). Vitamin E may prevent atherogenesis by reducing oxidized cholesterol levels in LDL (Buring and Hennekens, 1997; Brigelius-Flohe et al., 2002). Oncogenesis is initiated by DNA damage caused by free radicals. Because tocopherols are able to quench and scavenge free radicals, they may reduce the risk of cancer by preventing incidence of DNA mutagenesis (Bramley et al., 2000; Brigelius-Flohe et al., 2002; Schwenke, 2002). Non-antioxidant vitamin E functions may also provide a molecular basis for its mechanism in chemoprevention of these diseases (Buring and Hennekens, 1997; Azzi et al., 2000; Azzi et al., 2001; Meydani, 2001; Ricciarelli et al., 2001). This mechanism involves modulating signal transduction pathways associated with the progression of atherosclerosis and cancer, such as platelet aggregation, cell division, and apoptosis (Freedman and Keaney, 2001; Tang and Meydani, 2001; Uemura et al., 2002).

Several studies have demonstrated positive effects of increased vitamin E intakes on reducing atherosclerosis and cancer in animals. α-Tocopherol supplementation to experimental animals helped to reduce plasma peroxide levels and subsequently atherosclerotic plaque formation, thus decreasing the risk of heart disease (Smith and Kummerow, 1989; Wojcicki et al., 1991; Schwenke et al., 2002). Increased α-tocopherol intakes were correlated with a decreased DNA damage and the inhibition of tumor growth in rodents (Fleshner et al., 1999; Factor et al., 2000; Slamenova et al., 2002). The beneficial effects of vitamin E were also associated with the inhibition of cell proliferation and induction of tumor cell apoptosis *in vivo* (Barnett et al., 2002; Malafa et al., 2002). While

all these studies suggested that high levels of vitamin E reduced the risk of these degenerative diseases, conclusive evidence for beneficial vitamin E effects in humans is still lacking.

Two types of clinical studies have been performed to assess whether increased vitamin E intakes help to reduce the risk of heart disease and cancer in humans: observational (prospective) studies and randomized trials. In the observational studies, a large group of healthy individuals was divided into sub-groups based on the amount of consumed vitamin E they reported along with other health-related information in questionnaires. In randomized trials, a large group of randomly selected participants was given daily a pharmacological dose of vitamin E or a placebo. In both types of trials, various end-points such as death or manifestation of disease was observed within several years and statistical analyses were performed to correlate high tocopherol consumption or plasma levels with the decreased risk of the disease.

The Health Professionals Follow-up Study (HPFS), the Iowa Women's Health Study, and the Nurses' Health Study (NHS) are examples of prospective cohort trials (Rimm et al., 1993; Stampfer et al., 1993; Kushi et al., 1996). In the first two trials, a statistically significant decrease in the incidence of myocardial infarction or mortality was observed in participants with the highest vitamin E intakes (Rimm et al., 1993; Kushi et al., 1996). In contrast, the NHS study did not show this type of correlation (Stampfer et al., 1993). Inconclusive results were also obtained from randomized trials. The Cambridge Heart Antioxidant Study and the Secondary Prevention with Antioxidants of Cardiovascular Disease in Endstage Renal Disease study suggested that pharmacological doses of RRR-α-tocopherol (up to 800 IU daily) are beneficial for patients with heart disease in terms of reducing the incidence of myocardial infarction, ischemic stroke, and other heart disease-related symptoms (Stephens et al., 1996; Boaz et al., 2000). In other studies, high α-tocopherol intakes had no effect on cardiovascular disease progression (The ATBC Cancer Prevention Study Group, 1994; The HOPE Study Investigators, 2000).

Several observational and randomized trials attempting to demonstrate a correlation between high vitamin E intakes and a decreased risk of various cancer types have also been conducted. In many trials, high vitamin E consumption was associated with the decreased risk of some cancers, while others failed to show this correlation. Some observational studies showed the inverse relationship between increased vitamin E consumption and the risk of non-melanoma skin, esophageal, and epithelial ovarian cancers (Bidoli et al., 2001; Bollschweiler et al., 2002; Davies et al., 2002). In the Cancer Prevention Study II, significantly lower mortality rates were associated only with regular vitamin E supplementation periods exceeding ten years in patients suffering from bladder, but not stomach cancer (Jacobs et al., 2002a; Jacobs et al., 2002b). Controversial results were also obtained in the NHS and HPFS studies, in which high vitamin E intakes reduced the risk of colon cancer in men, but not in women (Wu et al., 2002). In the randomized ATBC study, besides fewer new cases of prostate cancer in the smokers receiving α-tocopherol relative to the placebo group, there was no overall positive effect of vitamin E supplementation on the gastric and lung cancer incidence or the mortality rate (The ATBC Cancer Prevention Study Group, 1994; Malila et al., 2002).

Brigelius-Flohe et al. (2002) pointed out that some factors such as dose and form of vitamin E, initial vitamin E levels and genetic pre-disposition for the disease of selected individuals, age and selection of individuals and stage of the disease have not been the same or considered in these trials, which may explain some of the controversial results. Negative results could also be due to low amounts of vitamin E or relatively short follow-up periods used in some studies. For example, consumption 50 mg of *all rac* α-tocopheryl acetate daily for up to 8 years was probably too low to prevent the incidence of most cancers in the ATBC study (The ATBC Cancer Prevention Study Group, 1994). Results from the ongoing randomized trials, such as SELECT (the SELenium and vitamin E Cancer prevention Trial), should provide more conclusive evidence than some previous trials about

the potential beneficial effects of vitamin E in degenerative disease chemoprevention (Klein et al., 2001).

α-Tocopherol Transport And Vitamin E Deficiency in Humans

In humans, lipids and tocopherols are absorbed, assembled into chylomicrons in the intestine and transported to the liver through the lymphatic system (Traber and Arai, 1999). Discrimination between α-tocopherol and other forms of vitamin E occurs only after several hours of tocopherol ingestion in the liver, where α-tocopherol transfer protein (α-TTP) specifically binds α-tocopherol, which is subsequently packed into very low density lipoproteins (VLDL) and distributed to the target organs through the bloodstream (Traber et al., 1990; Traber and Arai, 1999). In addition to α-TTP, other proteins capable of binding α-tocopherol have recently been identified in rats and humans (Stocker et al., 1999; Voegele et al., 2002). Afamin is a protein found in human plasma able to specifically and efficiently bind both α- and γ-tocopherols at physiological concentrations. This albumin-type protein has been proposed to play similar roles in the transport of the two tocopherols in the bloodstream as chylomicrons and VLDL (Voegele et al., 2002). In contrast, α-tocopherol-associated protein (TAP) belongs to a family of lipophillic ligand-binding proteins and is most likely involved in regulation of gene expression (Stocker et al., 1999; Yamauchi et al., 2001).

Genetic or metabolic disorders in any of these steps of tocopherol absorption and transport may result in vitamin E deficiency, which is associated with severe neurological disorders including cerebral ataxia, myopathies, and sensory problems in humans (Brigelius-Flohe and Traber, 1999). α-TTP specifically facilitates α-tocopherol transport in parenchymal hepatocytes, resulting in high plasma levels of α-tocopherol, but not other forms of vitamin E (Arita et al., 1997). Patients with a non-functional α-TTP show low α-tocopherol levels in plasma due to the impaired α-tocopherol transport in liver (Traber and

Arai, 1999). Besides defective genes encoding α-TTP and possibly other α-tocopherol transport proteins, additional disorders such as lipid malabsorption or hyperlipidemia may result in vitamin E deficiency (Burton and Traber, 1990; Traber and Arai, 1999). Because α-tocopherol absorption efficiency is dependent on lipid intake, vitamin E deficiency may be a secondary effect in patients suffering from lipid absorption disorders. Hyperlipidemic patients may have normal plasma α-tocopherol levels, but they are actually deprived of vitamin E because their abnormally high lipid levels contribute to the reduced α-tocopherol/lipid ratio (Burton and Traber, 1990; Traber and Arai, 1999).

OCCURRENCE AND SUBCELLULAR LOCALIZATION OF VITAMIN E

Vitamin E can be found nearly in all living organisms. However, only photosynthetic organisms such as cyanobacteria, algae, and plants are able to synthesize tocopherols and tocotrienols. Exceptions include some members of the cyanobacterial species such as *Anacystis*, *Synechococcus*, and *Prochlorococcus*, which completely lack and are unable to synthesize tocopherols because their genomes do not contain the necessary tocopherol biosynthetic genes (Dasilva and Jensen, 1971; Thomas et al., 1998; Sattler and DellaPenna, 2003). The lack of tocopherols coincides with the traces or absence of PUFAs observed in some of these cyanobacteria (Stanier and Cohen-Bazire, 1977; Merritt et al., 1991). These observations are consistent with the proposed roles of tocopherols as protectors of PUFAs from lipid peroxidation (Liebler, 1993). Most cyanobacteria synthesize PUFAs and α-tocopherol and may accumulate some of the prenylchromanol and quinol precursors of α-tocopherol (Dasilva and Jensen, 1971; Henry et al., 1987; Merritt et al., 1991; Wada et al., 1993; Shintani and DellaPenna, 1998). In plants, tocopherol and tocotrienol levels and composition vary widely among different tissues and between species. Tocopherols accumulate in photosynthetic tissues, flowers, fruits, and seeds of all plants,

while tocotrienols have been found in gymnosperms and in the seeds of monocots (Piironen et al., 1986; Franzen et al., 1991; Bramley et al., 2000; Ching and Mohamed, 2001).

In leaves and pine needles, α-tocopherol is the predominant form present, but small amounts of γ-tocopherol may accumulate as well (Piironen et al., 1986; Franzen and Haas, 1991; Molina-Torres and Martinez, 1991; Shintani and DellaPenna, 1998). In photosynthetic tissues, tocopherols are localized mostly in membranes and plastoglobuli of chloroplasts (Griffiths et al., 1967; Lichtenthaler et al., 1981; Soll et al., 1984; Soll et al., 1985). In spinach chloroplasts, α-tocopherol comprised approximately 64%, 20%, and 34% of the total prenylquinones in envelopes, thylakoids, and plastoglobuli, respectively (Lichtenthaler et al., 1981). When expressed on a protein basis, the outer envelope of spinach chloroplasts contained 50% more α-tocopherol than the inner envelope and α-tocopherol was the major prenylquinone in both envelopes (Soll et al., 1984; Soll et al., 1985).

Plant seeds contain the highest tocopherol levels of all tissues, with γ-tocopherol being the major contributor to the total tocopherol in seeds of many plants including soybean, mung bean, canola, maize, and Arabidopsis (Franzen and Haas, 1991; Shintani and DellaPenna, 1998; Yoshida et al., 1998; Goffman and Mollers, 2000; Goffman and Bohme, 2001). In contrast, sunflower, safflower, and wheat seeds accumulate predominantly α-tocopherol (Bramley et al., 2000; Delgado-Zamarreno et al., 2001). Other tocopherols, namely β- and δ-tocopherols, are present at small quantities in most seeds (Delgado-Zamarreno et al., 2001; Goffman and Bohme, 2001; Goffman and Mollers, 2000). High levels of tocotrienols can be found in seeds of monocots such as rice, maize, and palm (Goffman and Bohme, 2001; Eitenmiller, 1997; Franzen and Haas, 1991), primary needles and young seedlings of gymnosperms (Franzen and Haas, 1991; Franzen et al., 1991) and some fruits and berries (Piironen et al., 1986). Within the seed, tocopherols and tocotrienols accumulate in different parts. In oat, tocopherols and tocotrienols accumulated preferentially in the germ and endosperm, respectively (Peterson, 1995). Tocopherol

analysis of dissected seeds of three different soybean cultivars showed that the axis had the highest tocopherol content, followed by the cotyledons and seed coat (Yoshida et al., 1998). Because cotyledons accounted for ~ 93% of total seed weight, they contributed to the greatest extent to the final tocopherol levels of soybean seeds. In general, γ-tocopherol was the predominant tocopherol in all parts of the soybean seed, followed by δ- and α-tocopherols (Yoshida et al., 1998).

PLANT TOCOPHEROL, TOCOTRIENOL, AND PLASTOQUINONE-9 BIOSYNTHESIS

Tocopherols consist of a chromanol ring and a 15-carbon prenyl chain derived from homogentisate (HGA) and phytyl diphosphate (PDP), respectively. In tocotrienol synthesis, geranylgeranyl diphosphate (GGDP) is used instead of PDP as a prenyl tail, while plastoquinone-9 (PQ-9) is synthesized from HGA and solanesyl diphosphate (SDP). HGA and these prenyl diphosphates (prenyl-DP) are synthesized by two independent pathways (Figures 3 and 4). In plants, HGA originates from shikimate pathway in the cytosol (Figure 3) and PDP is synthesized from isopentenyl diphosphate (IPP) derived from 1-deoxy-D-xylulose-5-phosphate (DXP) in plastids (Figure 4).

HGA Biosynthesis - Shikimate Pathway And Tyrosine Degradation

Shikimate biosynthesis is found only in microorganisms and plants. In plants, the shikimate pathway provides intermediates for the synthesis of aromatic amino acids (tyrosine, phenylalanine, and tryptophan), lignin, and phenylpropanoids and phenolic compounds involved in protection of plants from pathogens (Herrman, 1995; Herrman and Weaver, 1999). Up to 20% of the photosynthetically fixed carbon can be directed to the plant shikimate pathway (Herrmann, 1995). In plants, all enzymes of the pathway are predicted to be chloroplast localized because they contain chloroplast targeting sequences

(Herrmann and Weaver, 1999). Shikimate synthesis starts with the condensation of two activated compounds, phosphoenolpyruvate (PEP) and erythrose-4-P (E4P) catalyzed by 3-Deoxy-D-arabino-heptulosonate-7-P (DAHP) synthase to yield DAHP (Figure 3). Antisense suppression of transketolase, which catalyzes E4P formation, resulted in a reduction of aromatic amino acid and tocopherol levels in transgenic tobacco, confirming that shikimate pathway provides aromatic precursors for tocopherol biosynthesis (Henkes et al., 2001). Genes encoding several isoforms of DAHP synthase have been cloned from a variety of plants including potato, tobacco, Arabidopsis, and tomato (Dyer et al., 1989;

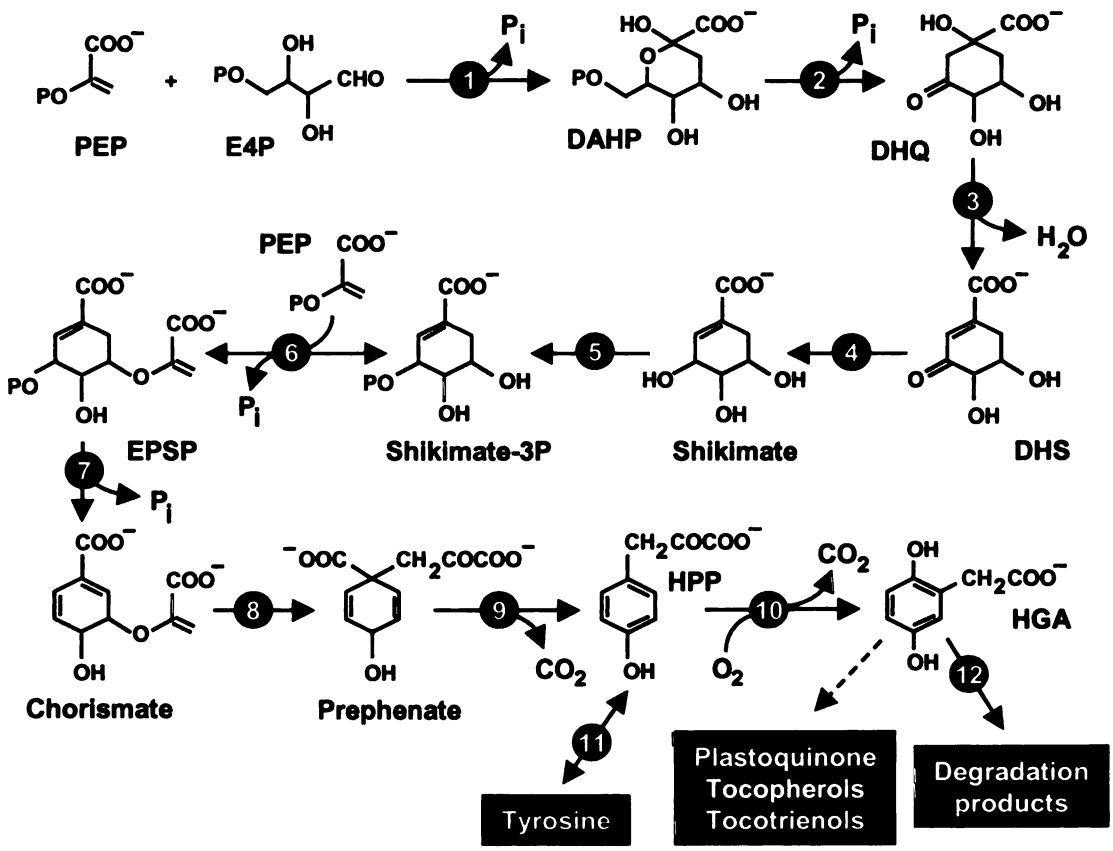


Figure 3. Shikimate biosynthetic pathway. The shikimate pathway leads to the formation of homogentisate (HGA) used in the tocopherol, tocotrienol, and PQ-9 biosynthesis in photosynthetic organisms. The dashed arrow represents multiple biosynthetic steps. Enzymes are represented by numbered circles: 1. 3-deoxy-D-arabino-heptulosonate-7-P (DAHP) synthase; 2. 3-dehydroquinate (DHQ) synthase; 3. DHQ dehydratase; 4. shikimate dehydrogenase; 5. shikimate kinase; 6. 5-enolpyruvylshikimate-3-P (EPSP) synthase; 7. chorismate synthase; 8. chorismate mutase; 9. prephenate dehydrogenase; 10. *p*-hydroxyphenylpyruvate (HPP) dioxygenase; 11. tyrosine transaminase (TAT); 12. HGA dioxygenase

Keith et al., 1991; Wang et al., 1991; Gorlach et al., 1993a). 3-Dehydroquinate (DHQ) synthase is an oxidoreductase involved in the formation of DHQ from DAHP (Bartlett and Satake, 1988). Tomato DHQ synthase is similar to the bacterial homologues and the DHQ synthase cDNA was shown to functionally complement an *E. coli* mutant lacking DHQ synthase activity (Bischoff et al., 1996).

DHQ is converted in two steps to shikimate by a single enzyme, dehydroquinase/shikimate dehydrogenase (Mousdale et al., 1987; Bonner and Jensen, 1994). In tobacco, the N terminus of this enzyme contains the dehydroquinase activity, while the C terminus contains the shikimate dehydrogenase activity (Bonner and Jensen, 1994). Several wound-inducible shikimate dehydrogenase isozymes have been identified in pepper leaves (Diaz and Merino, 1998). The next enzyme of the pathway is shikimate kinase, which uses ATP to phosphorylate the hydroxyl at carbon 3 on the shikimate molecule. Shikimate kinase is encoded by a single gene in tomato and is processed to a mature form in chloroplasts (Schmid et al., 1992). The product of this enzyme, shikimate-3-P, is a substrate for 5-enolpyruvylshikimate-3-P (EPSP) synthase, cDNAs for which have been isolated from several plant species (Shah et al., 1986; Gasser et al., 1988; Gasser and Klee, 1990). EPSP synthase is the target of the herbicide glyphosate and many plant species have been subjected to metabolic engineering to improve their resistance to this herbicide (Steinrucken and Amrhein, 1980; Shah et al., 1986).

After the condensation of shikimate-3-P and PEP, EPSP is dephosphorylated to chorismate by the last enzyme of the shikimate pathway, chorismate synthase (Hawkes et al., 1990). Chorismate synthase genes have been isolated from *Synechocystis* sp. PCC 6803 and tomato (Gorlach et al., 1993b; Schmidt et al., 1993). Chorismate synthase is targeted and processed in chloroplasts and only mature forms of this enzyme are functional (Henstrand et al., 1995; Braun et al., 1996). Chorismate is the last product of the shikimate pathway and it is used as a substrate in the biosynthesis of many primary and secondary metabolites. Chorismate mutase plays a central role in the biosynthesis of tyrosine and

phenylalanine as it catalyzes the first committed step in the synthesis of these aromatic amino acids, conversion of chorismate to prephenate. In Arabidopsis, two out of three isoforms of chorismate mutase contain chloroplast targeting sequences (Mobley et al., 1999). Expression studies suggested that the three isoforms play different roles in regulating chorismate mutase activity during development and pathogen attack in Arabidopsis (Mobley et al., 1999).

Prephenate dehydrogenase oxidizes prephenate to form *p*-hydroxyphenylpyruvate (HPP), a direct biosynthetic precursor of tyrosine and HGA. Transamination of HPP yields tyrosine, which is used in the biosynthesis of proteins or aromatic compounds (Lopukhina et al., 2001). Excess tyrosine may be converted back to HPP because the transamination is a reversible reaction. Radiotracer studies showed that HPP and tyrosine were incorporated into tocopherols and plastoquinones in chloroplasts isolated from a variety of plants, suggesting that HPP used for tocopherol and plastoquinone biosynthesis can be formed not only directly from prephenate, but also from tyrosine (Whistance and Threlfall, 1970; Fiedler et al., 1982; Schulz et al., 1993).

In plant tocopherol, tocotrienol, and plastoquinone synthesis, HPP is converted to HGA by HPP dioxygenase (HPPD), a cytosolic enzyme that requires Fe^{2+}, ascorbate, and molecular oxygen for its optimal activity (Fiedler et al., 1982; Schulz et al., 1993; Norris et al., 1995; Garcia et al., 1997; Norris et al., 1998; Garcia et al., 1999; Tsegaye et al., 2002). In Arabidopsis, HPPD is encoded by a single nuclear gene, *PDS1*, disruption of which is lethal due to a lack of plastoquinone, tocopherols, chlorophylls, and carotenoids (Norris et al., 1995; Norris et al., 1998). The lack of carotenoids and chlorophylls is a pleiotrophic rather than a direct effect of the deficiency in HPPD activity. Plastoquinone is required as an electron acceptor in the phytoene desaturation reactions in carotenoid biosynthesis (Mayer et al., 1990). Because Arabidopsis *pds1* mutants are unable to synthesize carotenoids and tocopherols, the major lipid-soluble antioxidants in chloroplasts, they are photobleached (Norris et al., 1995). In contrast to the Arabidopsis *pds1* mutants,

Synechocystis sp. PCC 6803 HPPD disruption mutants also lack tocopherols, but have normal levels of PQ-9 and other prenyllipids (Dahnhardt et al., 2002). These results suggest that HPPD is required only for tocopherol biosynthesis and PQ-9 biosynthesis most likely occurs through an HPPD-independent pathway in *Synechocystis* sp. PCC 6803 (Dahnhardt et al., 2002).

Prenyl-DP Biosynthesis - Non-mevalonate Pathway

Until 1993, it was widely accepted that all isoprenoids are derived from the acetate/mevalonate pathway (Goldstein and Brown, 1990). In recent years, a novel pathway for isoprenoid synthesis has been discovered in plants and other organisms, including bacteria (Rohmer et al., 1993; Eisenreich et al., 1996; Schwender et al., 1996; Lichtenthaler et al., 1997b; Disch et al., 1998; Proteau, 1998). In photosynthetic organisms, this mevalonate-independent, or 1-deoxy-D-xylulose-5-phosphate (DXP), pathway (Figure 4) is the major source of isopentenyl diphosphate (IPP) used for the synthesis of all plastidic isoprenoids (carotenoids, isoprene, and side chains of tocopherols, chlorophylls, plastoquinones, etc.), while the mevalonate pathway is used for the synthesis of cytosolic isoprenoids (sterols, sesquiterpenes, and triterpenes) (Eisenreich et al., 1998; Lichtenthaler, 1998; Logan et al., 2000). However, a minimal IPP exchange between cytosol and chloroplasts might occur (Arigoni et al., 1997; Estevez et al., 2000; Nagata et al., 2002).

The initial step in IPP synthesis is the condensation of glyceraldehyde-3-phosphate (G3P) and pyruvate for the synthesis of the five-carbon compound DXP (Rohmer et al., 1996; Arigoni et al., 1997). This reaction is catalyzed by a transketolase-like enzyme, DXP synthase, which utilizes thiamin diphosphate as a cofactor. Genes encoding DXP synthase have been cloned and functionally characterized from a variety of plants including Arabidopsis, bell pepper, tomato, and peppermint (Mandel et al., 1996; Bouvier et al., 1998;

Lange et al., 1998; Estevez et al., 2000; Lois et al., 2000). In Arabidopsis, DXP synthase is encoded by a single nuclear gene, *CLA1* and the corresponding *cla1* mutants

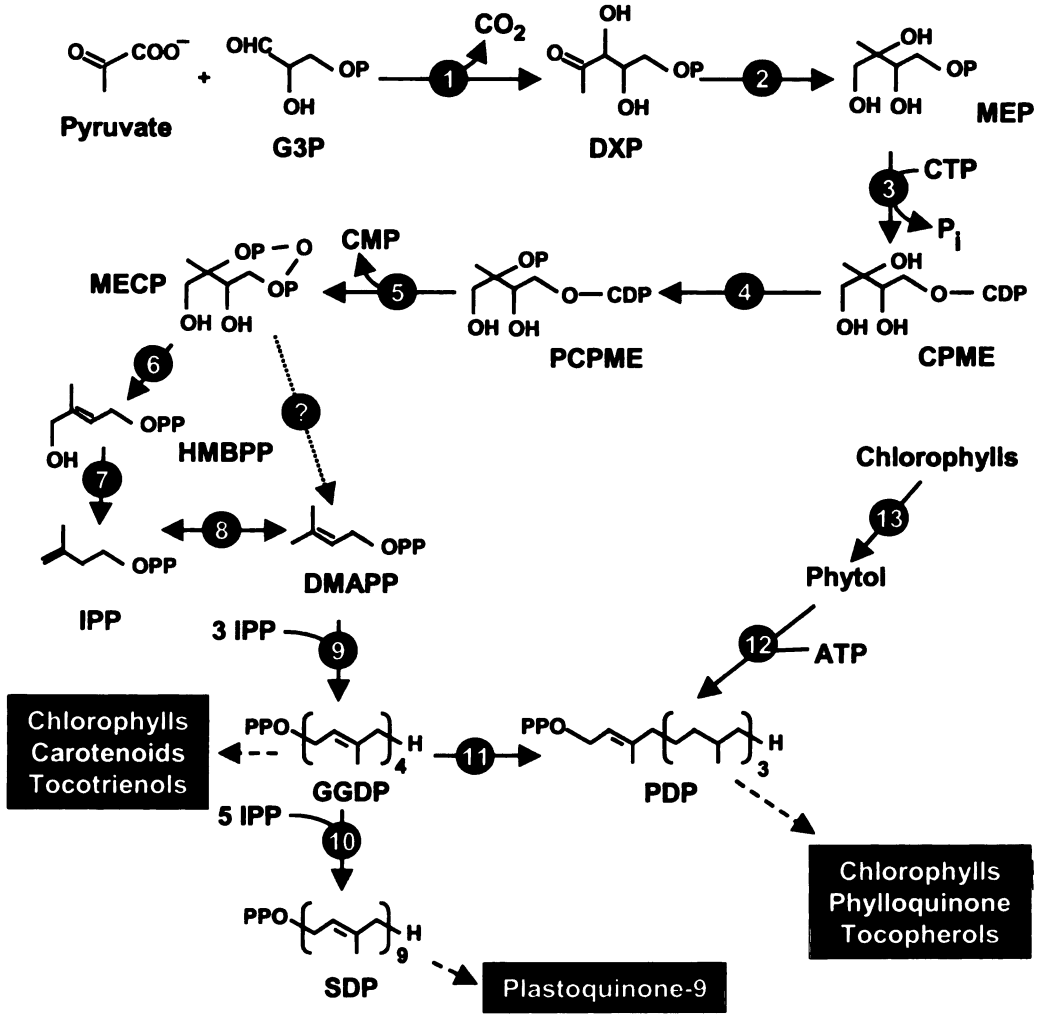


Figure 4. The plastidic 1-deoxy-D-xylulose-5-phosphate (DXP) biosynthetic pathway. The DXP pathway leads to the formation of the prenyl diphosphates phytyl diphosphate (PDP), geranylgeranyl diphosphate (GGDP), and solanesyl diphosphate (SDP) used for tocopherol, tocotrienol, and PQ-9 synthesis. Dashed arrows represent multiple steps. Enzymes involved are depicted as white numbers in black circles: 1. 1-deoxy-D-xylulose-5-P (DXP) synthase; 2. DXP reductoisomerase; 3. 2-C-methyl-D-erythritol (MEP) cytidyltransferase; 4. 4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol (CPME) kinase; 5. 2-C-methyl-D-erythitol-2,4-cyclodiphosphate (MECP) synthase; 6. MECP reductase 7. (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) reductase; 8. IPP/DMAPP isomerase; 9. solanesyl diphosphate (SDP) synthase; 10. geranylgeranyl diphosphate (GGDP) synthase 1; 11. GGDP reductase; 12. prenol:ATP kinase; 13. chlorophyllase. Additional abbreviations: 2-phospho-4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol (PCPME)

show an albino phenotype as a result of impaired chlorophyll and carotenoid biosynthesis (Mandel et al., 1996; Estevez et al., 2000). The *CLA1* gene is expressed in virtually all tissues, but the highest *CLA1* expression levels are observed in young developing tissues (Estevez et al., 2000).

DXP reductoisomerase catalyzes the molecular rearrangement of DXP and a reduction of the resulting unstable intermediate 2-C-methyl-D-erythrose-4-phosphate (MESP) to yield 2-C-methyl-D-erythritol-4-phosphate (MEP) (Takahashi et al., 1998). Similar to the phenotype of Arabidopsis *cla1* mutants, peppermint transgenic plants with low DXP reductoisomerase expression due to cosuppression of the endogenous DXPR gene were small and chlorotic, indicating that DXP reductoisomerase is also essential for normal plant growth and development (Mahmoud and Croteau, 2001). The next enzyme in IPP biosynthesis, MEP cytidyltransferase, is involved in the formation of 4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol (CPME) (Rohdich et al., 1999; Rohdich et al., 2000a). CPME is subsequently phosphorylated by CPME kinase to form 2-phospho-4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol (PCPME) (Luttgen et al., 2000; Rohdich et al., 2000b). 2-C-methyl-D-erythitol-2,4-cyclodiphosphate (MECP) synthase catalyzes the conversion of PCPME to MECP (Herz et al., 2000).

In bacteria, MECP is converted to IPP and dimethylallyl diphosphate (DMAPP) in two steps by the reductases GcpE and LytB through the intermediate (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) (Altincicek et al., 2002). In plants, these enzymes remain to be identified. NMR studies have demonstrated that IPP synthesis precedes DMAPP synthesis in plants (Arigoni et al., 1999). Similar to the mevalonate pathway, IPP isomerase is thought to catalyze a reversible conversion of IPP to DMAPP in plants, but branching at HMBPP may also occur in bacteria (Altincicek et al., 2002). The genome of the cyanobacterium *Synechocystis* sp. PCC 6803 lacks any apparent homologues of IPP isomerase and it is not clear whether this enzyme is absent from this organism or structurally distinct from the known IPP isomerases and therefore

unrecognizable in BLAST searches (Ershow et al., 2000). The steps subsequent to IPP synthesis involve a family of related prenyl diphosphate (prenyl-DP) synthases (Chen et al., 1994). In geranylgeranyl diphosphate (GGDP) synthesis, chloroplastic GGDP synthase 1 combines one molecule of DMAPP with three molecules of IPP in a head-to-tail manner (Okada et al., 2000). GGDP can be used directly as a prenyl-DP precursor in tocotrienol biosynthesis in seeds of monocots and gymnosperms or it can serve as a substrate in SDP and PQ-9 synthesis (Lichtenthaler et al., 1997a). Alternatively, GGDP can be reduced to PDP by a multifunctional GGDP reductase (GGDR) identified in cyanobacteria and plants (Soll et al., 1983; Addlesee et al., 1996; Keller et al., 1998; Addlesee and Hunter, 1999). In chloroplasts, PDP can also be formed from phosphorylation of phytol with ATP, which is catalyzed by an unknown soluble kinase present in the stroma (Soll et al., 1984).

Tocopherol, Tocotrienol, And Plastoquinone Biosynthetic Pathways

Approximately thirty years ago, radiotracer studies using isolated chloroplasts and seedlings of various plants delineated possible tocopherol, tocotrienol, and plastoquinone biosynthetic intermediates and enzymatic activities (Threlfall and Whistance, 1971; Schulze-Siebert et al., 1987). The tocopherol, tocotrienol, and plastoquinone biosynthetic pathways are shown in Figure 5. The first committed step in tocopherol, tocotrienol, and plastoquinone synthesis involves the condensation of the aromatic precursor HGA and PDP, GGDP, or SDP catalyzed by homogentisate prenyltransferase/decarboxylases (Threlfall and Whistance, 1971; Hutson and Threlfall, 1980; Soll et al., 1980; Schulze-Siebert et al., 1987; Collakova and DellaPenna, 2001). The products of these prenylation reactions are CO_2, inorganic pyrophosphate, and the first prenylquinol intermediates in the respective tocopherol and plastoquinone pathways leading to 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) and 2-demethylplastoquinol-9 (DMPQ-9), respectively (Hutson and

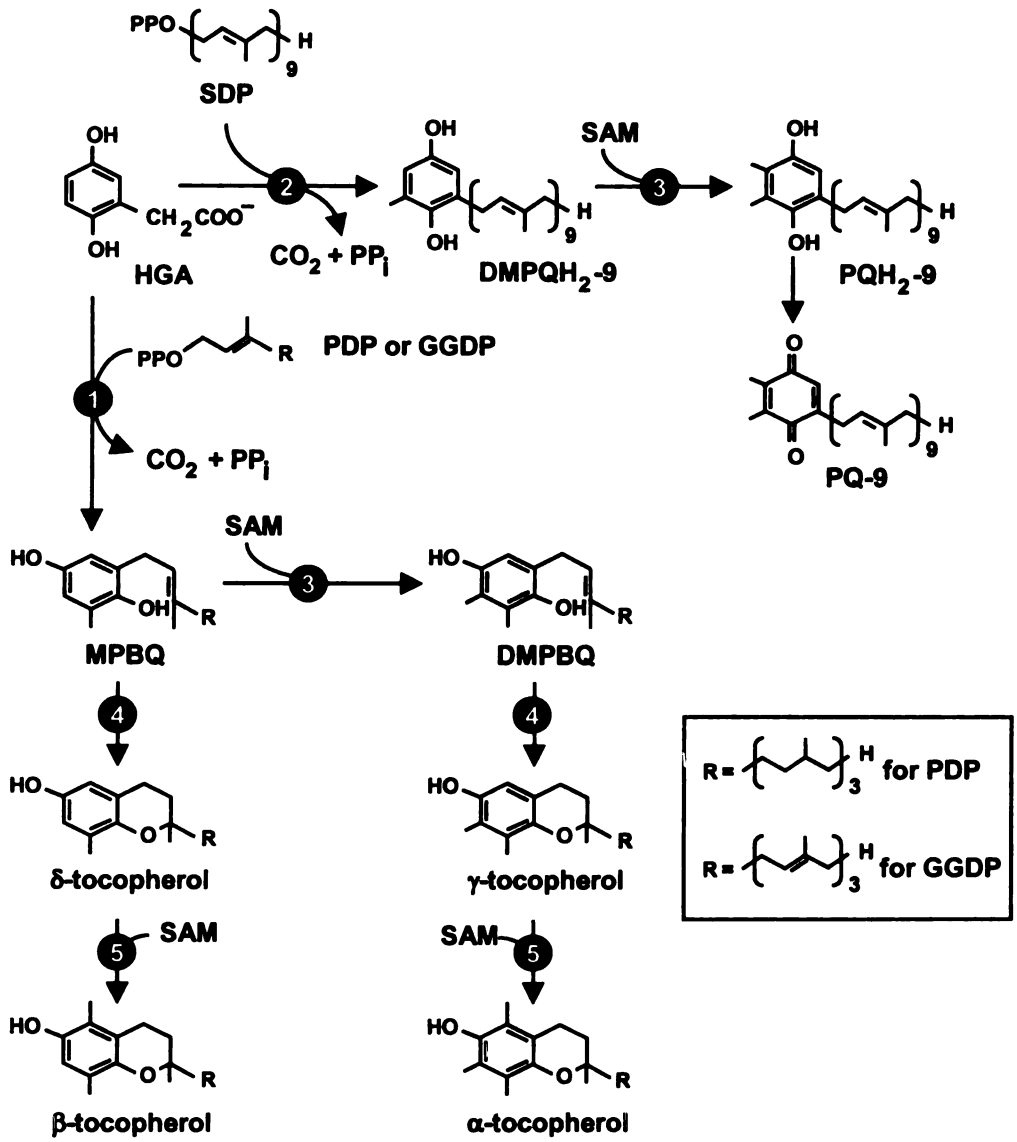


Figure 5. Tocopherol, tocotrienol, and plastoquinone-9 biosynthesis in cyanobacteria and plants. Enzymes are shown as numbers in black circles. 1. homogentisate (HGA) prenyltransferase(s); 2. HGA solanesyltransferase; 3. 2-methyl-6-prenyl-1,4-benzoquinol (MPBQ) methyltransferase(s) (MT); 4. tocopherol cyclase (TC); 5. γ-tocopherol methyltransferase (γ-TMT). Other abbreviations: solanesyl diphosphate (SDP); phytyl diphosphate (PDP), geranylgeranyl diphosphate (GGDP); demethyl plastoquinol-9 ($DMPQH_2$); plastoquinol-9 (PQH_2); plastoquinone-9 (PQ-9); 2,3-dimethyl-6-prenyl-1,4-benzoquinol (DMPBQ); S-adenosyl-L-methionine (SAM)

Threlfall, 1980; Soll et al., 1980; Marshall et al., 1985; Collakova and DellaPenna, 2001). In tocotrienol synthesis, a similar reaction would yield 2-methyl-6-geranylgeranyl-1,4-benzoquinol (MGBQ) (Threlfall and Whistance, 1971).

Biochemical studies using isolated chloroplasts and radiolabeled precursors of tocopherol and plastoquinone biosynthesis could not distinguish between separate prenyltransferase activities (Schulze-Siebert et al., 1987). Phytylation of HGA occurred primarily in the envelopes, whereas prenylation of HGA with SDP took place both in the envelopes and thylakoids, suggesting that separate prenyltransferase activities might be responsible for tocopherol and plastoquinone synthesis (Soll et al., 1980). However, these differences could also be due to different availability of the endogenous substrates in the two membranes. Arabidopsis homogentisate phytyl/prenyltransferase was postulated to be encoded by the *PDS2* gene because disruption of *PDS2* led to the same albino phenotype as the *pds1* mutation (Norris et al., 1995). The Arabidopsis *pds2* mutants did not accumulate detectable levels of tocopherols, PQ-9, chlorophylls, or carotenoids. Norris et al. (1995) proposed that the *PDS2* gene product may represent a homogentisate phytyl/ prenyltransferase involved in tocopherol and PQ-9 biosynthesis. Chapter 1 reports cloning and characterization of the gene encoding homogentisate phytyltransferase in *Synechocystis* sp. PCC 6803 and *Arabidopsis thaliana*. This work was also published in Plant Physiology (Collakova and DellaPenna, 2001). Genetic and biochemical data presented in Chapter 1 suggested that homogentisate phytyltransferase (HPT) is involved only in tocopherol biosynthesis and that a separate homogentisate solanesyltransferase (HST) must exist in these two photosynthetic organisms. Similarly, tocopherol and tocotrienol synthesis may involve separate prenyltransferase activities utilizing PDP and GGDP, respectively, to prenylate HGA in monocots, which accumulate tocotrienols. Many plant species contain several different genes encoding putative prenyltransferases that may be involved in tocopherol or tocotrienol synthesis (DellaPenna et al., 2000). However, I have demonstrated that the *Synechocystis* HPT can utilize both PDP and GGDP, suggesting that a single

enzyme can contain both prenyltransferase activities (Collakova and DellaPenna, 2001). This observation was surprising because *Synechocystis* sp. PCC 6803 does not accumulate any detectable tocotrienol levels (Collakova and DellaPenna, 2001). More research is required to address this question in plants.

Some of the subsequent steps in tocopherol, tocotrienol, and plastoquinone synthesis may also involve the same types of enzymes and intermediates. The three prenylquinols formed by the HGA prenyltransferases can be methylated to 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ), 2,3-dimethyl-6-geranylgeranyl-1,4-benzoquinol (DMGBQ), and plastoquinol-9, respectively (Soll and Schult, 1979; Soll et al., 1980; Schultz et al., 1985; Shintani et al., 2002). These reactions may be catalyzed by a single methyltransferase (MT), which utilizes S-adenosyl-L-methionine (SAM) as a methyl donor. In *Synechocystis* sp. PCC 6803, the MT encoded by the ORF SLL0418 can efficiently methylate both MPBQ and DMPQ-9 *in vitro*, indicating that the methylation of these two prenylquinols may be performed by the single enzyme MPBQ/DMPQ-9 MT (Shintani et al., 2002). SLL0418 disruption mutants showed altered tocopherol composition and β-tocopherol in addition to α-tocopherol, the predominant tocopherol form in *Synechocystis* sp. PCC 6803, was detected. Consistent with the hypothesis that SLL0418 is also involved in PQ-9 synthesis in this cyanobacterium, only partial sll0418 disruption mutants were isolated presumably because PQ-9 is essential for survival of photosynthetic organisms (Shintani et al., 2002).

Spinach chloroplasts can catalyze all three methylation reactions when radiolabeled SAM and the corresponding 2-methylprenylquinols are used as substrates (Soll and Schult, 1979; Soll et al., 1980; Schultz et al., 1985). Based on studies with purified chloroplast envelopes and thylakoids, the prenylquinol methylation reaction in tocopherol synthesis has been associated with the envelope membranes, while that in PQ-9 synthesis occured in both envelopes and thylakoids, suggesting that separate MT activities may operate in plant tocopherol and PQ-9 synthesis (Soll et al., 1980; Schultz et al., 1985). Geranylgeranylated

precursors were preferred substrates relative to the phytylated ones for the methyltransferase(s) present in spinach chloroplasts (Soll and Schultz, 1979). Because no tocotrienols have been detected in spinach, it is possible that GGDP is not used *in vivo* to prenylate HGA or that any newly formed tocotrienol precursors are efficiently reduced to tocopherol precursors (Collakova and DellaPenna, 2001). These questions are further discussed in Chapter 1.

The next steps in tocopherol and tocotrienol synthesis are catalyzed by tocopherol cyclase (TC), a relatively non-specific enzyme that can utilize both single and double methylated tocopherol intermediates and both phytylated and geranylgeranylated intermediates as substrates (Stocker et al., 1996; Porfirova et al., 2002). Cyclization of MPBQ and DMPBQ yields δ- and γ-tocopherol, respectively, whereas cyclization of MGBQ and DMGBQ results in the formation of δ- and γ-tocotrienols, respectively. TC activity has been shown in *Anabeana variabilis* and bell pepper chromoplasts (Stocker et al., 1993; Stocker et al., 1994) and TC has been cloned in *Synechocystis* sp. PCC 6083, Arabidopsis, and maize (Porfirova et al., 2002; Sattler and DellaPenna, 2003). TC is the best studied enzyme of the tocopherol and tocotrienol biosynthetic pathway in terms of reaction mechanism and substrate specificity. The prenylquinol enters the active site of the enzyme with its tail (Stocker et al., 1996). The chromanol ring is formed by a nucleophillic attack of carbon 1 phenolic hydroxyl group on the protonated double bond of the phytyl tail (Stocker et al., 1994). Tocopherol cyclase requires prenylquinol substrates, a prenyl tail in the 6th position on the benzoquinol ring, the double bond in the (*E*) configuration, and prenyl tails longer than 10 carbons (Stocker et al., 1993; Stocker et al., 1994; Stocker et al., 1996). The last step of tocopherol synthesis involves methylation of γ- and δ-tocopherols to α- and β-tocopherols, respectively. In Arabidopsis and *Synechocystis*, these reactions are catalyzed by γ-tocopherol methyltransferase (γ-TMT), a membrane-bound enzyme that utilizes SAM as a methyl donor and requires an intact chromanol ring with carbon 5

available for methylation (Shintani and DellaPenna, 1998). Unfortunately, tocotrienols have not been used as substrates to test γ-TMT substrate specificity.

TOCOPHEROL FUNCTION IN PLANTS

Antioxidant Function

Chloroplasts are the major source of $^1O_2$ and O_2^- in plant cells (Mittler, 2002). In chloroplasts, $^1O_2$ originates by the interaction of ground-state molecular oxygen ($^3O_2$ - triplet oxygen) and excited triplet chlorophyll during photosynthesis. During this reaction, the energy is exchanged between these two molecules and ground-state singlet chlorophyll and excited $^1O_2$ are formed (Melis, 1999; Mittler, 2002). Excess light energy may result in generation of excited triplet chlorophyll when the photosynthetic electron transport is overreduced. On the other hand, ferredoxin and other components of the photosynthetic electron transport are generators of O_2^- radicals (Mittler, 2002). In chloroplasts, tocopherols are most likely involved in O_2^- scavenging rather than directly in $^1O_2$ quenching, which is efficiently performed by carotenoids and superoxide dismutase (Yamauchi and Matsushita, 1979a; Michalski and Kaniuga, 1981). Carotenoids are prone to degradation by the products of lipid peroxidation and one mechanism, by which tocopherols can indirectly reduce $^1O_2$ levels, is to prevent carotenoid degradation by inhibiting lipid peroxidation. In isolated spinach chloroplasts, visible light-induced lipid peroxidation was accompanied by α-tocopherol disappearance and α-tocopheryl quinone formation, suggesting the active oxidation of α-tocopherol (Yamauchi and Matsushita, 1979a). Illumination of detached cold-treated tomato leaves led to a several-fold increase of α-tocopherol and α-tocopheryl quinone levels relative to leaves stored at room temperature (Michalski and Kaniuga, 1981). Because cold-treated tomato leaves contain inactive

superoxide dismutase, another important scavenger of O_2^- in plant chloroplasts, O_2^- oxidized α-tocopherol to form α-tocopheryl quinone to a greater extent in cold-treated leaves than in the fresh leaves containing active superoxide dismutase. Addition of exogenous α-tocopherol reduced malondialdehyde formation in isolated chloroplasts, suggesting that α-tocopherol is involved in protection of membrane lipids from peroxidation in plant chloroplasts (Michalski and Kaniuga, 1981).

Interaction of tocopherols with other antioxidants may play an important role in protecting plants from photooxidative damage. Finckh and Kunert (1985) subjected nine different plant species to an herbicide-induced lipid peroxidation. A decrease in oxidative damage correlated well with high tocopherol and ascorbate levels. Mustard and sicklepod plants, which contained tocopherols and ascorbate in the ratio of 1 to 10 - 15 showed the lowest degree of cell damage. Although action of other protective mechanisms could not be excluded, the authors suggested that tocopherols were recycled by ascorbate because the plants that showed high tocopherol and ascorbate levels, as well as a certain ratio of these two antioxidants, were most resistant to oxidative damage (Finckh and Kunert, 1985). The Arabidopsis *vtc-1* mutant, which has levels of ascorbate reduced to ~ 60% of the corresponding wild type levels, was more sensitive to lipid peroxidation and accumulated less α-tocopherol and β-carotene than wild type during drought stress (Munne-Bosch and Alegre, 2002b). Ascorbate, which is present in the cytosol, is unlikely to scavenge ROS within membranes and the reduced α-tocopherol and β-carotene levels in the *vtc-1* mutant can be caused by impaired recycling (Munne-Bosch and Alegre, 2002b). Because ascorbate is required as a cofactor for the tocopherol biosynthetic enzyme HPPD (Fiedler et al., 1982), low tocopherol levels in the *vtc-1* mutant may also be a result of impaired tocopherol biosynthesis. Because tocopherols protect β-carotene by scavenging lipid peroxy radicals (Yamauchi and Matsushita, 1979a), reduced β-carotene levels in this mutant may be due to a secondary effect of insufficient tocopherol levels. Direct evidence that

ascorbate plays a key role in recycling of lipid-soluble antioxidants such as α-tocopherol and β-carotene is still lacking.

Plants have developed numerous antioxidant systems to protect them from oxidative damage. Oxidative stress is associated with up-regulation of multiple antioxidants (carotenoids, ascorbate, glutathione, or anthocyanins) and ROS detoxifying enzymes (glutathione reductase, ascorbate peroxidase, catalase, or superoxide dismutase) (Leipner et al., 1999; Ye and Gressel, 2000). Tocopherol levels also increase in plants exposed to various abiotic stress conditions such as high light, low temperature, drought, and high salinity (Munne-Bosch and Alegre, 2002a). Cultivars showing increased tolerance to stress accumulate increased α-tocopherol levels relative to the stress-sensitive cultivars (Gossett et al., 1994; Streb et al., 1998; Leipner et al., 1999). Mountain plants growing at high altitudes are exposed to low temperatures and high irradiance. Several alpine plant species growing at high altitudes showed increased tocopherol levels relative to their counterparts growing at low altitudes or lowland plants (Wildi and Lutz, 1996; Streb et al., 1998). High light and/or low temperatures led to an increase in α-tocopherol levels in other plants including Arabidopsis and maize (Fryer et al., 1998; Havaux and Niyogi, 1999; Leipner et al., 1999; Havaux et al., 2000; Havaux and Kloppstech, 2001). Drought and/or high light stress also resulted in a several-fold increase in α-tocopherol levels in rosemary and wheat leaves (Bartoli et al., 1999; Munne-Bosch and Alegre, 2000). It appears that various types of oxidative stress lead to an increase in α-tocopherol levels in a variety of plants, which, along with other antioxidant systems, may protect those plants from oxidative damage and death. However, this correlation has not been shown in all studies.

In some instances, α-tocopherol levels decreased or did not change with oxidative stress (Gossett et al., 1994; Havaux et al., 2000; Kranner et al., 2002). These observations can be explained in part by the progressive degradation of tocopherols, which may occur when oxidative stress becomes detrimental to the plant. α-Tocopherol levels gradually increased in Arabidopsis leaves exposed to high light intensities from 250 to 1,000 μE, but

rapidly decreased when the plants were exposed to 1,500 μE (Havaux et al., 2000). An eight-month desiccation of the resurrection plant *Myrothamnus flabellifolia* resulted in a nearly complete depletion of the α-tocopherol levels as well as other antioxidants, which were recovered upon rehydration (Kranner et al., 2002). It is also possible that the stress conditions used in some studies were not high enough to induce tocopherol synthesis in some plant species or that these plants use other components of the antioxidant system to cope with particular types of stress. Cotton plants treated with 150 mM NaCl did not accumulate increased tocopherol levels, but showed increased glutathione reductase, peroxidase, and superoxide dismutase activities (Gossett et al., 1994).

Abiotic stress is not the only factor affecting tocopherols in plants. Tocopherol levels and composition change during different stages of plant growth and development (Franzen and Haas, 1991; Franzen et al., 1991; Molina-Torres and Martinez, 1991, Tramontano et al., 1992). These changes may contribute to optimal plant growth and development by controlling the levels of reactive oxygen species and lipid peroxidation. During seed germination, light induces the synthesis of various components of photosynthetic apparatus including chlorophylls, carotenoids, and plastoquinone in developing chloroplasts, which are becoming photosynthetically active and generate high ROS levels (Lichtenthaler, 1969). Light also induced tocopherol accumulation in developing barley seedlings, thus most likely protecting these chloroplasts from oxidative damage (Lichtenthaler, 1969). Tocopherol levels and composition vary among seeds and seedlings of different plant species. In general, the tocopherol, which predominates in dry seeds, is also initially found in germinating seeds and primary leaves or needles of angiosperms or gymnosperms (Franzen and Haas, 1991; Franzen et al., 1991). The levels of α-tocopherol, the major tocopherol in gymnosperm seeds, gradually increased in developing Norway spruce seedlings (Franzen and Haas, 1991; Franzen et al., 1991). γ-Tocopherol was the major tocopherol present in the seeds and primary leaves of 1-, 2-, and 3-day old monocot and dicot seedlings (Franzen and Haas, 1991). In these seedlings, the

gradual decrease in γ-tocopherol levels was accompanied by an increase in the levels of α-tocopherol (Franzen and Haas, 1991), the major tocopherol present in mature monocot and dicot leaves (Piironen et al., 1986; Molina-Torres and Martinez, 1991; Tramontano et al., 1992; Shintani and DellaPenna, 1998).

Aging and senescence are programmed stress-inducing processes involving chlorophyll, nucleic acid, protein, and lipid degradation and nutrient mobilization from senescing parts of plants to developing leaves (Buchanan-Wollaston, 1997). These processes are also associated with an increase in lipid peroxidation, a decrease in photosynthetic activity and chlorophyll levels, and changes in antioxidant status (Munne-Bosch et al., 2001; Munne-Bosch and Alegre, 2002c). α-Tocopherol accumulation increased with both leaf and plant age in soybean, pinto bean, and *Xanthium strumarium* leaves (Tramontano et al., 1992; Molina-Torres and Martinez, 1991). In addition, an increase in γ-tocopherol levels with age was also observed in soybean plants (Tramontano et al., 1992). Rise et al. (1989) reported that α-tocopherol levels increased in the senescing leaves in a variety of plant species, which was also correlated with elevated chlorophyll degradation. It has been postulated that the phytol tail released by chlorophyllase during chlorophyll degradation is phosphorylated in chloroplasts and the resulting phytyl diphosphate used for α-tocopherol synthesis in senescing leaves (Soll et al., 1980; Rise et al., 1989). Other studies showed that α-tocopherol levels decreased during aging and senescence in several Mediterranean plant species (Munne-Bosch et al., 2001; Munne-Bosch and Alegre, 2002c). These Mediterranean field-grown plants were also subjected to a combination of high light and drought stress and the reduced α-tocopherol levels observed were likely due to increased tocopherol degradation.

Because plants regulate tocopherol levels and composition as a response to oxidative stress, specific tocopherols may play important roles in controlling oxidative damage during plant growth and development. Several cyanobacterial and plant mutants or transgenic plants showing altered tocopherol levels have been isolated and characterized (Henry et al.,

1986; Norris et al., 1995; Tanaka et al., 1999). Because some mutants were also impaired in the synthesis of other vitally important compounds, photobleached phenotypes observed in these organisms could not be ascribed to the lack of tocopherols. Geranylgeranyl diphosphate reductase (GGDR) is a multifunctional enzyme involved in the conversion of GGDP to PDP, a substrate used for tocopherol, phylloquinone, and chlorophyll biosynthesis (Soll et al., 1983; Addlesee et al., 1996; Keller et al., 1998; Addlesee and Hunter, 1999). Besides tocopherols, a *Scenedesmus* mutant lacking GGDR activity or tobacco GGDR antisense plants were unable to synthesize chlorophylls and phylloquinone (Henry et al., 1986; Tanaka et al., 1999). Similarly, the Arabidopsis *PDS1* gene encodes *p*-hydroxyphenylpyruvate dioxygenase (HPPD), an enzyme involved in tocopherol and plastoquinone biosynthesis (Norris et al., 1995). The homozygous *pds1* mutants in Arabidopsis was seedling-lethal and required a carbon source for its growth and completely lacked tocopherols, plastoquinone-9, chlorophylls, and carotenoids (Norris et al., 1995).

In *Synechocystis* sp. PCC 6083, disruption of HPPD or HPT completely abolished tocopherol synthesis, while the levels of other prenyllipids were unaffected (Collakova and DellaPenna, 2001; Dahnhardt et al., 2002; Savidge et al., 2002). The absence of tocopherols had no effects on the growth or photosynthesis under normal or high-light conditions in these mutants (Collakova and DellaPenna, 2001; Dahnhardt et al., 2002; Savidge et al., 2002). Some cyanobacterial species naturally lack tocopherols as indicated by the absence of several tocopherol biosynthetic genes in their genomes, which suggests that tocopherols are not required for normal growth in these organisms (Dasilva and Jensen, 1971; Thomas et al., 1998; Sattler et al., 2003). However, tocopherols may protect photosynthetic organisms from specific types of stress. Experiments using the *Synechocystis* and Arabidopsis HPT mutants are underway in Dr. Dean DellaPenna's laboratory to address this question.

Roles in Signaling

In animals, eicosanoid signaling is associated with the phospholipase A_2-dependent release of arachidonic acid, a precursor in the synthesis of various oxylipins involved in inflammatory processes (Balsinde et al., 1999). Tocopherols can affect phospholipase A_2 activity and regulate oxylipin production in animals (Tran et al., 1996; Chan et al., 1998; Grau and Ortiz, 1998). In plants, linolenic acid is cleaved off by an analogous phospholipase and used for the synthesis of phyto-oxylipins including jasmonate (JA) in octadecanoid signal transduction pathway (Schaller, 2001; Blee, 2002; Howe and Schilmiller, 2002). Other enzymes downstream of phospholipases, which include cytochromes P450, lipoxygenases, and other oxygenases, are also functionally similar in animals and plants, indicating a resemblance between animal eicosanoid and plant octadecanoid signaling (Blee, 2002). Although there is no evidence that tocopherols may directly modulate enzyme activities involved in plant octadecanoid signaling, the possibility exists that they may affect plant signal transduction pathways by mechanisms involving their antioxidant properties.

ROS are by-products of aerobic metabolism considered to be harmful to chloroplasts. ROS levels or production are controlled by a variety of means, including antioxidant levels. However, animals and plants also produce ROS as signaling molecules to regulate various cellular processes including stress responses, plant-pathogen interaction, and programmed cell death (Mittler, 2002). Tocopherols may affect some of these processes by controlling ROS and lipid hydroperoxide levels in plants (Munne-Bosch and Alegre, 2002a). Lipid peroxidation propagation is associated with the formation of a lipid peroxy radical, which can interact with another PUFA to generate a lipid hydroperoxide and another lipid peroxy radical (Figure 2). For example, if the target molecule is linolenic acid, the resulting lipid hydroperoxide formed during lipid peroxidation will be 13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOD), which is also a product of 13-lipoxygenase and a

substrate for several enzymes including allene oxide synthase involved in JA biosynthesis (Schaller, 2001; Howe and Schilmiller, 2002; Blee, 2002). Uncontrolled propagation of lipid peroxidation could lead to a massive JA and other oxylipin synthesis during oxidative stress. As chain-breaking antioxidants, tocopherols can scavenge lipid peroxy radicals to form lipid hydroperoxides such as 13-HPOD. Therefore, by scavenging 13-HPOD radicals and by controlling the levels of lipid peroxidation, tocopherols may interfere with the octadecanoid pathway signaling by inhibiting synthesis of jasmonate and other oxylipins.

Roles in Photosynthesis

The oxidation products of α-tocopherol, α-tocopheryl quinol and quinone (Figure 2) have been suggested to be involved in photosynthetic electron transport (Lichtenthaler, 1993; Kruk and Strzalka, 1995). α-Tocopheryl quinol/quinone can be oxidized/reduced to the corresponding quinone/quinol and this redox state change may allow the molecules to perform similar roles as ubiquinones and plastoquinones in electron transport. Several *in vitro* studies have suggested that α-tocopheryl quinone may participate as an electron carrier in cyclic electron transport of photosystem II (PS II) in chloroplasts (Michalski and Kaniuga, 1981; Kruk et al., 2000). Stable picrylhydrazyl radicals can specifically oxidize and extract α-tocopherol present in chloroplasts. α-Tocopherol oxidation with the picrylhydrazyl radicals resulted in an inhibition of PS II activity, which was partially reversed by the addition of α-tocopherol to isolated chloroplasts (Michalski and Kaniuga, 1981). It was proposed that at least two α-tocopherol pools exist independently in chloroplasts and that only ~ 70% of α-tocopherol may function in PS II electron transport (Michalski and Kaniuga, 1981).

Experiments with the *Scenedesmus obliquus* GGDP reductase mutant suggested that α-tocopherol and its quinone may be involved in the cyclic electron transport chain

around PS II when the PS II is over-reduced (Kruk et al., 2000). Besides tocopherols, this mutant also lacks the high-potential form of cytochrome b-559 (cyt b-559 HP), which participates in the cyclic electron transport in PS II (Henry et al., 1986; Kruk et al., 2000). Both PQ-A and α-tocopherol reduced b-559 HP, while α-tocopheryl quinone oxidized the reduced form of this cytochrome in wild type *Scenedesmus obliquus*, but not in the mutant, which suggests a specific interaction between these prenyllipids and the cyt b-559 HP. α-Tocopheryl quinone also efficiently quenched chlorophyll fluorescence in thylakoid membranes isolated from both wild type and mutant, suggesting that this function was independent of the cyt b-559 HP and that α-tocopheryl quinone interacts with another component of the cyclic electron transport besides cyt b-559 HP (Kruk et al., 2000). Although all these studies suggest that α-tocopherol and α-tocopheryl quinone may be involved in cyclic electron transport, no studies have been performed to confirm whether this function exists *in vivo*.

NUTRITIONAL GENOMICS

Natural Sources of Dietary Vitamin E

As mentioned before, humans cannot synthesize tocopherols and must acquire vitamin E from their diets, mainly from plants and their products (Brigelius-Flohe and Traber, 1999; Bramley et al., 2000). Plant seed oils are the major source of dietary vitamin E in the average American diet (Eitenmiller, 1997). Soybean oil, the most commonly consumed plant oil in the world, contains up to 115 mg of total tocopherols per 100 g oil (Eitenmiller, 1997; Bramley et al., 2000). However, the predominant tocopherol in soybean oil is γ-tocopherol, which has only 10% the biological activity of α-tocopherol. Therefore, the maximal vitamin E activity of soybean oil is relatively low, only 20 mg α-TE per 100 g oil (Eitenmiller, 1997; Bramley et al., 2000). Seeds of other plants such as canola, corn, and

Arabidopsis also accumulate predominantly γ-tocopherol and their vitamin E activity is also very low (Eitenmiller, 1997; Bramley et al., 2000; Goffman and Mollers, 2000; Goffman and Bohme, 2001). Oils with the highest percentage and content of α-tocopherol have the highest vitamin E activity. These oils include safflower, sunflower, and wheat germ oil, vitamin E activities of which varies between 41 and 174 mg α-TE per 100 g of oil, respectively (Eitenmiller, 1997). In most vegetables, berries, and fruits, α-tocopherol is the predominant form of vitamin E, but its levels are very low, between 1 and 6 mg α-tocopherol per 100 g fresh weight (Piironen et al., 1986; Ching and Mohamed, 2001).

Currently, the recommended dietary intakes for α-tocopherol are 15 mg (35 μmol) of RRR-α-tocopherol daily (Food and Nutrition Board, 2000). The maximal vitamin E activities of spinach leaves, soybean oil, sunflower oil, and wheat-germ oil are 3.7, 20, 63, and 174 mg α-TE per 100 g fresh weight or oil, respectively (Ching and Mohamed, 2001; Eitenmiller, 1997; Bramley et al., 2000). Based on these values, it can be estimated that one should daily consume one of the following to meet the dietary requirements for α-tocopherol: 405 g fresh spinach leaves, 75 g soybean oil, 24 g sunflower oil, or 8.6 g wheat-germ oil. Pharmacological doses of up to 800 IU (537 mg of α-TE) of RRR-α-tocopherol or its acetate were administered daily to the patients suffering from atherosclerosis in the heart disease prevention trials CHAOS and SPACE (Stephens et al., 1996; Boaz et al., 2000), while the upper tolerable limit for α-tocopherol is 1000 mg per day (Food and Nutrition Board, 2000). Even the best natural α-tocopherol sources are clearly insufficient to provide these extremely high doses of vitamin E. The limitation of α-tocopherol in the average American diet may be overcome through metabolic engineering of tocopherol biosynthesis in plants.

Regulation And Metabolic Engineering of Tocopherol Biosynthesis in Plants

Enzymes of tocopherol biosynthetic pathway can be divided into two groups. The first group includes enzymes that may be involved in regulating flux through the pathway and subsequently total tocopherol levels. This group contains some enzymes committed to the tocopherol biosynthetic pathway as well as the enzymes of the shikimate and non-mevalonate pathways. The second group includes only three enzymes determining tocopherol composition, namely TC, MPBQ MT and γ-TMT (Grusak and DellaPenna, 1999). It is desirable both to increase total tocopherol levels and to alter tocopherol composition in plants towards α-tocopherol, the most potent form of vitamin E. In order to genetically manipulate plants to produce elevated α-tocopherol levels, it is important to understand the regulation of tocopherol biosynthetic pathway, identify enzymatic steps limiting tocopherol synthesis, and clone and study the relevant genes. The regulation of tocopherol biosynthesis appears to be a very complex process and may involve several enzymes of the pathway.

In plants, the shikimate pathway (Figure 2) provides chorismate, an aromatic precursor in the biosynthesis of aromatic amino acids, tocopherols, plastoquinones, phylloquinones, salicylic acid and other phenolics involved in plant pathogen defense and cell wall lignification (Herrman, 1995; Herrman and Weaver, 1999; Diaz et al., 2001). This pathway is probably regulated at the transcriptional level in plants and can be globally up-regulated during biotic and abiotic stresses (Gorlach et al., 1995; Jones et al., 1995; Bischoff et al., 1996; Batz et al., 1998; Diaz and Merino, 1998; Diaz et al., 2001). Unfortunately, the effects of the shikimate pathway up-regulation on tocopherol levels was not investigated in these reports. However, at least two enzymes downstream of chorismate, TAT and HPPD, have been shown to regulate HGA production and tocopherol levels in plants (Sandorf and Hollander-Czytko, 2002; Lopukhina et al., 2001; Klebler-Janke and Krupinska, 1997; Tsegaye et al., 2002). In plant chloroplasts, TAT catalyzes transamination

of tyrosine to HPP and its activity can be induced by a variety of factors including coronatine, wounding, various octadecanoids, high light, UV light, and the herbicide oxyfluorfen in wild type Arabidopsis (Lopukhina et al., 2001; Sandorf and Hollander-Czytko, 2002). Elevated α- and γ-tocopherol levels were associated with the increase in TAT protein levels induced by all these elicitors (Sandorf and Hollander-Czytko, 2002). In the Arabidopsis *dde1* mutant impaired in JA synthesis due to a block in the octadecanoid enzyme 13-OPDA reductase 3, TAT levels were lower and tocopherol levels were higher than in wild type. The high total tocopherol content in the non-stressed *dde1* mutant did not correlate with the low TAT levels, which suggests that TAT is not the only factor affecting regulation of tocopherol synthesis (Sandorf and Hollander-Czytko, 2002).

HPPD activity may also be involved in regulation of tocopherol synthesis because HPPD mRNA levels were elevated during stress senescence in barley leaves (Klebler-Janke and Krupinska, 1997). HPPD catalyzes the conversion of HPP to HGA and its gene has been cloned and characterized from several plant species (Garcia et al., 1997; Norris et al., 1998; Klebler-Janke and Krupinska, 1997; Garcia et al., 1999). The HGA pool may limit tocopherol synthesis because the addition of HGA to safflower cultures moderately stimulated tocopherol synthesis in spite of a rapid HGA oxidation in the media (Furuya et al., 1987). Thus, HPPD activity may also limit tocopherol synthesis in other plants (Tsegaye et al., 2002). Constitutive HPPD overexpressing in Arabidopsis resulted in a 10-fold increase in HPPD specific activity, but only a 10 to 30 % increase in total tocopherol levels in both leaves and seeds. The minor effect of HPPD overexpression on tocopherol levels might be due to enhanced degradation of HGA in transgenics. It is also possible that HPPD does not present a significant limitation for tocopherol biosynthesis (Tsegaye et al., 2002).

In plastids, the non-mevalonate pathway provides PDP, which is used as a substrate in tocopherol biosynthesis (Rohmer et al., 1993; Schwender et al., 1996; Lichtenthaler et al., 1997b; Proteau, 1998). Feeding phytol alone to safflower cell cultures induced tocopherol

synthesis to even a higher extent than HGA, indicating that phytol pool is also limiting for tocopherol biosynthesis (Furuya et al., 1987). Genes encoding some of the enzymes of the non-mevalonate pathway providing the IPP pool for the plastidic isoprenoid synthesis have been cloned and some of these genes overexpressed in plants (Lange et al., 1998; Estevez et al., 2000; Estevez et al., 2001; Mahmoud and Croteau, 2001). Only the first enzyme of the DXP pathway, DXP synthase, has been studied in terms of tocopherol biosynthesis (Estevez et al., 2001). Constitutive overexpression of DXP synthase in Arabidopsis leaves resulted in elevated levels of several plastidic isoprenoids. Tocopherol levels increased up to 2-fold in leaves of transgenics relative to wild type plants, indicating that DXP synthase limits tocopherol synthesis in Arabidopsis chloroplasts (Estevez et al., 2001). Therefore, tocopherol synthesis may be regulated in part by the availability of HGA and phytol or PDP. In chloroplasts, these precursors are also used in the syntheses of other prenyllipids such as plastoquinone, phylloquinone, and chlorophyll (Threlfall and Whistance, 1971; Schulze-Siebert et al., 1987; Oster et al., 1997). Because of the involvement of HGA and PDP in multiple biosynthetic pathways, the actual regulation most likely occurs at the level of the next biosynthetic steps, the prenylation reactions that are catalyzed by structurally and functionally related prenyltransferases.

HPT catalyzes the first committed step of tocopherol biosynthesis, the condensation of HGA and PDP (Hutson and Threlfall, 1980; Soll et al., 1980; Marshall et al., 1985; Collakova and DellaPenna, 2001; Savidge et al., 2002). A recent report of seed-specific overexpression of HPT in Arabidopsis seeds demonstrated a 75% increase in total seed tocopherol levels, suggesting that HPT activity is a limiting factor for tocopherol biosynthesis in Arabidopsis seeds (Savidge et al., 2002). Because the seed-specific napin promoter was used, the effects of HPT overexpression on tocopherol levels in leaves could not be determined (Savidge et al., 2002). In Chapter 2 (Collakova and DellaPenna, 2003), I described and discussed the effects of constitutive HPT overexpression on the levels of tocopherols and other prenyllipids in Arabidopsis leaves and seeds. HPT overexpressers

(35S::*HPT1*) showed up to a 100-fold increase in HPT mRNA levels and a 10-fold increase in HPT activity relative to wild type. These increases in HPT expression resulted in a 4.4-fold and 40% increase in total tocopherol levels in leaves and seeds, respectively. The levels of other prenyllipids was not affected by increasing flux to the tocopherol pathway (Collakova and DellaPenna, 2003).

In Chapter 3, I describe the use of high light stress to increase flux through the tocopherol biosynthetic pathway. During high light stress, total tocopherol levels rapidly increased in both wild type and 35S::*HPT1* leaves. Stressed 35S::*HPT1* leaves accumulated elevated total tocopherol levels relative to the corresponding wild type leaves. The increase in total tocopherol levels was most likely due to an up-regulation of HPT mRNA levels and activity and increased expression of two other tocopherol-related genes, TAT and HPPD. Enhanced tyrosine and chlorophyll degradation have been proposed to provide increased pool of HGA and PDP that could be used to synthesize tocopherols in stressed plants (Chapter 3).

The last three enzymes of the pathway are involved in the controlling tocopherol composition in plants. Tocopherol cyclase catalyzes the formation of the first tocopherols, γ- and δ- tocopherols (Stocker et al., 1993; Stocker et al., 1994; Stocker et al., 1996; Porfirova et al., 2002; Sattler et al., 2003). Based on the kinetic studies of HGA incorporation in yellow bell pepper chromoplasts, TC does not seem to limit tocopherol biosynthesis in chromoplasts (Arango and Heise, 1998). MPBQ MT methylated MPBQ to DMPBQ and the corresponding gene has been cloned from *Synechocystis* ps. PCC 6803 (Shintani et al., 2002). The Arabidopsis MPBQ MT is currently being cloned and characterized in Dr. DellaPenna's laboratory. The Arabidopsis γ-TMT gene has been cloned and overexpressed in leaves and seeds (Shintani and DellaPenna, 1998). Wild type Arabidopsis leaves accumulated predominantly α-tocopherol, while the major tocopherol present in seeds was γ-tocopherol. In Arabidopsis leaves, γ-TMT overexpression had no effect on total tocopherol levels or composition. In contrast, overexpression of γ-TMT in

Arabidopsis seeds resulted in the conversion of more than 95% of the γ-tocopherol to α-tocopherol. Similar to leaves, total tocopherol content remained unchanged in the transgenic seed. These results indicated that γ-TMT was a key enzyme controlling seed tocopherol composition, but had no effect on flux through the pathway (Shintani and DellaPenna, 1998). If the entire pool of γ-tocopherol can be methylated to α-tocopherol in HPT overexpressers (Savidge et al., 2001), then a combined overexpression of HPT and γ-TMT will increase vitamin E activity in Arabidopsis seeds up to 15-fold.

CHAPTER 1

ISOLATION AND FUNCTIONAL ANALYSIS OF HOMOGENTISATE PHYTYLTRANSFERASE FROM *SYNECHOCYSTIS* SP. PCC 6803 AND *ARABIDOPSIS THALIANA*

Abstract

Tocopherols, collectively known as Vitamin E, are lipid soluble antioxidants synthesized exclusively by photosynthetic organisms and are required components of mammalian diets. The committed step in tocopherol biosynthesis involves condensation of homogentisic acid and phytyl diphosphate (PDP) catalyzed by a membrane-bound homogentisate phytyltransferase (HPT). HPTs were identified from *Synechocystis sp.* PCC 6803 and *Arabidopsis thaliana* based on their sequence similarity to chlorophyll synthases, which utilize PDP in a similar prenylation reaction. HPTs from both organisms used homogentisic acid and PDP as their preferred substrates *in vitro* but only *Synechocystis* HPT was active with geranylgeranyl diphosphate as a substrate. Neither enzyme could utilize solanesyl diphosphate, the prenyl substrate for plastoquinone-9 synthesis. In addition, disruption of *Synechocystis* HPT function causes an absence of tocopherols without affecting plastoquinone-9 levels, indicating that separate polyprenyltransferases exist for tocopherol and plastoquinone synthesis in *Synechocystis*. Surprisingly, the absence of tocopherols in this mutant had no discernible effect on cell growth and photosynthesis.

Introduction

Tocopherols are a group of amphipathic compounds synthesized only by photosynthetic organisms. The best characterized and probably most important function of tocopherols is to act as recyclable chain-reaction terminators of polyunsaturated fatty acid

free radicals generated by lipid oxidation. Tocopherols have a well-documented role in mammals both as an essential nutrient (vitamin E) and general antioxidant (Fryer, 1993; Liebler, 1998; Brigelius-Flohe and Traber, 1999). A similar, though less well documented antioxidant role is also proposed for tocopherols in photosynthetic organisms (Fryer, 1992; Niyogi, 1999).

From a biosynthetic perspective, tocopherols are members of a large, multifunctional family of lipid soluble compounds called prenylquinones that also include tocotrienols, plastoquinones, and phylloquinones (vitamin K_1). Structural features shared by all prenylquinones include hydrophobic prenyl tails of various lengths attached to aromatic head-groups that can reversibly change their redox states. Tocopherols contain a chromanol head-group and lipophillic tail derived from the 20-carbon alcohol phytol, while plastoquinones contain a quinone head-group and isoprenoid tails of 40, 45, or 50 carbons. Such structural features are essential for the diverse biochemical and physiological roles fulfilled by various prenylquinones.

The committed step in the synthesis of all prenylquinones is the condensation of various aromatic precursors and prenyl diphosphate (prenyl-DP) substrates in reactions catalyzed by a small family of related polyprenyltransferases (Lopez et al., 1996). Most aromatic and prenyl-DP substrates are utilized by more than one polyprenyltransferase (Figure 6). For example, the aromatic compound homogentisic acid (HGA) is used for condensation with PDP, geranylgeranyl diphosphate (GGDP), or solanesyl diphosphate (SDP) in tocopherol, tocotrienol, and plastoquinone synthesis, respectively, while PDP is used as the isoprenoid-derived tail in the synthesis of tocopherols, phylloquinones, and chlorophylls (Threlfall and Whistance, 1971; Schulze-Siebert et al., 1987; Oster et al., 1997). Thus, polyprenyltransferases act at biosynthetic branch-points and are potential key regulatory enzymes for the synthesis of many essential compounds in photosynthetic organisms.

In plant chloroplasts, the synthesis of tocopherols and plastoquinones is closely related. Biochemical studies have shown that condensation of HGA with PDP or SDP yields 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) and 2-demethylplastoquinol-9, the first prenylquinol intermediates in tocopherol and plastoquinone-9 (PQ-9) synthesis, respectively (Hutson and Threlfall, 1980; Soll et al., 1980; Marshall et al., 1985). Although

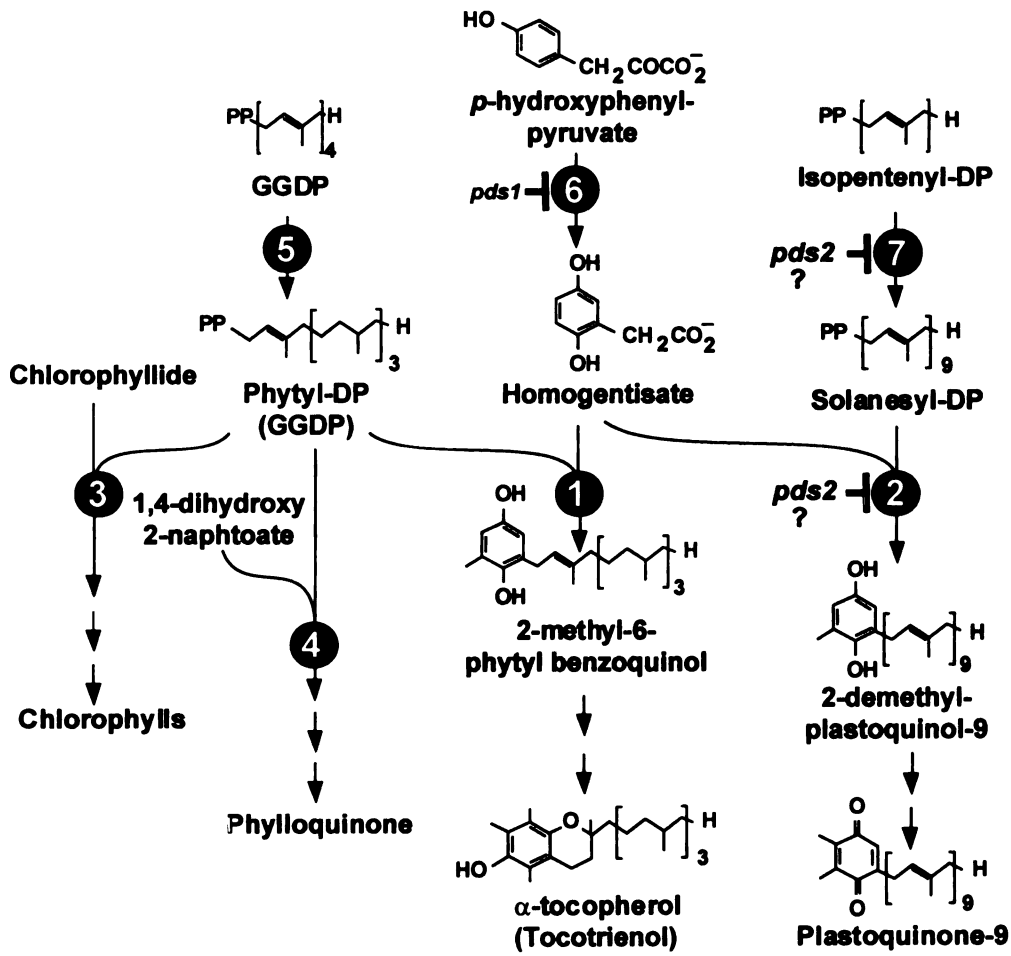


Figure 6. Generalized overview of prenylquinone biosynthetic pathways in photosynthetic organisms. Prenylation steps and substrates in tocopherol and plastoquinone synthesis are shown in detail, while those for other prenyllipids are incomplete for clarity. Aromatic and prenyl-DP substrates are shared among the various polyprenyltransferases (see text for details). Enzymes are depicted as numbers in black circles: 1. Homogentisate phytyltransferase; 2. Homogentisate solanesyltransferase; 3. Chlorophyll synthase; 4. 1,4-dihydroxy-2-naphthoate phytyltransferase; 5. GGDP reductase; 6. p-hydroxyphenylpyruvate dioxygenase; 7. SDP synthase. Compounds in parentheses indicate where GGDP may be used in place of PDP by HPT resulting in a tocotrienol product.

these studies could not distinguish whether one or more polyprenyltransferases catalyzed these reactions, it was suggested that separate enzymes might be involved (Schulze-Siebert et al., 1987). In contrast, recent genetic data from *Arabidopsis thaliana* suggested involvement of a single polyprenyltransferase activity in tocopherol and PQ-9 synthesis. Two loci were identified, *PDS1* and *PDS2* (*PDS* = Phytoene DeSaturation), which when mutated, decreased the levels of both tocopherols and plastoquinones below detection (Norris et al., 1995), consistent with the disruption of enzymes shared in their synthesis. The *PDS1* locus has been cloned and encodes *p*-hydroxyphenylpyruvate dioxygenase (HPPD) (Norris et al., 1998), which catalyzes formation of HGA. The *pds2* mutation was proposed to disrupt another shared pathway enzyme, most likely a polyprenyltransferase, which could utilize either PDP or SDP as substrates for condensation with HGA (Norris et al., 1995). Unlike *PDS1*, the *PDS2* locus has not yet been cloned.

As an alternative to purifying the membrane-bound *PDS2* gene product or walking to the *PDS2* locus, we attempted to clone an orthologous gene from the cyanobacterium *Synechocystis sp.* PCC 6803, which also synthesizes α-tocopherol. In this paper, we report the cloning and functional analysis of gene products from *Synechocystis* and Arabidopsis encoding polyprenyltransferases specific to tocopherol biosynthesis. We also present biochemical and physiological characterization of the corresponding *Synechocystis* polyprenyltransferase knockout mutant, which completely lacks tocopherols.

Results

Identification And Disruption of A Polyprenyltransferase Involved in Tocopherol Biosynthesis in *Synechocystis sp.* PCC 6803

Due to the metabolic synteny observed for the prenyllipid biosynthetic pathways in photosynthetic organisms, we decided to utilize a genomics-based approach to identify the gene encoding the homogentisate polyprenyltransferase involved in tocopherol synthesis,

first from cyanobacteria, and subsequently from plants. We hypothesized that this polyprenyltransferase would show some similarity to previously characterized polyprenyltransferases from cyanobacteria and plants that utilize similar prenyl-DPs as substrates.

Chlorophyll synthase is a polyprenyltransferase that attaches PDP or GGDP to the tetrapyrrole core of chlorophyllide during chlorophyll biosynthesis (Lopez et al., 1996; Oster et al., 1997). The *Synechocystis* chlorophyll synthase ORF (ChlG, GenBank accession number BAA10281) was used to query CyanoBase, which contains the complete *Synechocystis sp.* PCC 6803 genome sequence (Kaneko et al., 1996). Several ORFs showing varying degrees of similarity were identified and SLR1736 was selected as a putative HPT based on its protein identity to ChlG (21%) and the presence of prenyl diphosphate- and divalent cation-binding motifs characteristic of polyprenyltransferases (Lopez et al., 1996; Figure 7A). SLR1736 is also a highly hydrophobic protein (Figure 7B), as would be expected for a membrane-bound HPT (Soll et al., 1980; Soll et al., 1984).

To test the hypothesis that SLR1736 is involved in tocopherol biosynthesis, a disruption mutant (SLR1736::Km^r) was generated by homologous recombination of the kanamycin cassette-disrupted SLR1736 gene into the wild-type SLR1736 locus (Figure 8). If the SLR1736::Km^r mutation disrupted HPT activity, one would expect a complete absence of tocopherols and their prenylquinol intermediates. HPLC analysis shows that wild type *Synechocystis* lipid extracts contain predominantly α-tocopherol (Figure 9 and Table I). In contrast, α-tocopherol and its prenylchromanol and quinol precursors are absent from SLR1736::Km^r lipid extracts (Figure 9, Table I and data not shown), consistent with the hypothesis that SLR1736 encodes a polyprenyltransferase involved in tocopherol synthesis.

As shown in Figure 6, the various polyprenyltransferases in photosynthetic organisms utilize many of the same aromatic and prenyl-DP substrates. Homogentisic acid is the aromatic precursor in both tocopherol and plastoquinone synthesis and PDP is a

substrate for tocopherol, phylloquinone, and chlorophyll polyprenyltransferases (Threlfall and Whistance, 1971; Schulze-Siebert et al., 1987; Oster et al., 1997). Given this biosynthetic relationship, disrupting SLR1736 activity could directly or indirectly affect the synthesis of other prenylated compounds in pathways that also utilize these substrates. To determine the effect of the SLR1736 gene disruption on the synthesis of other prenylated

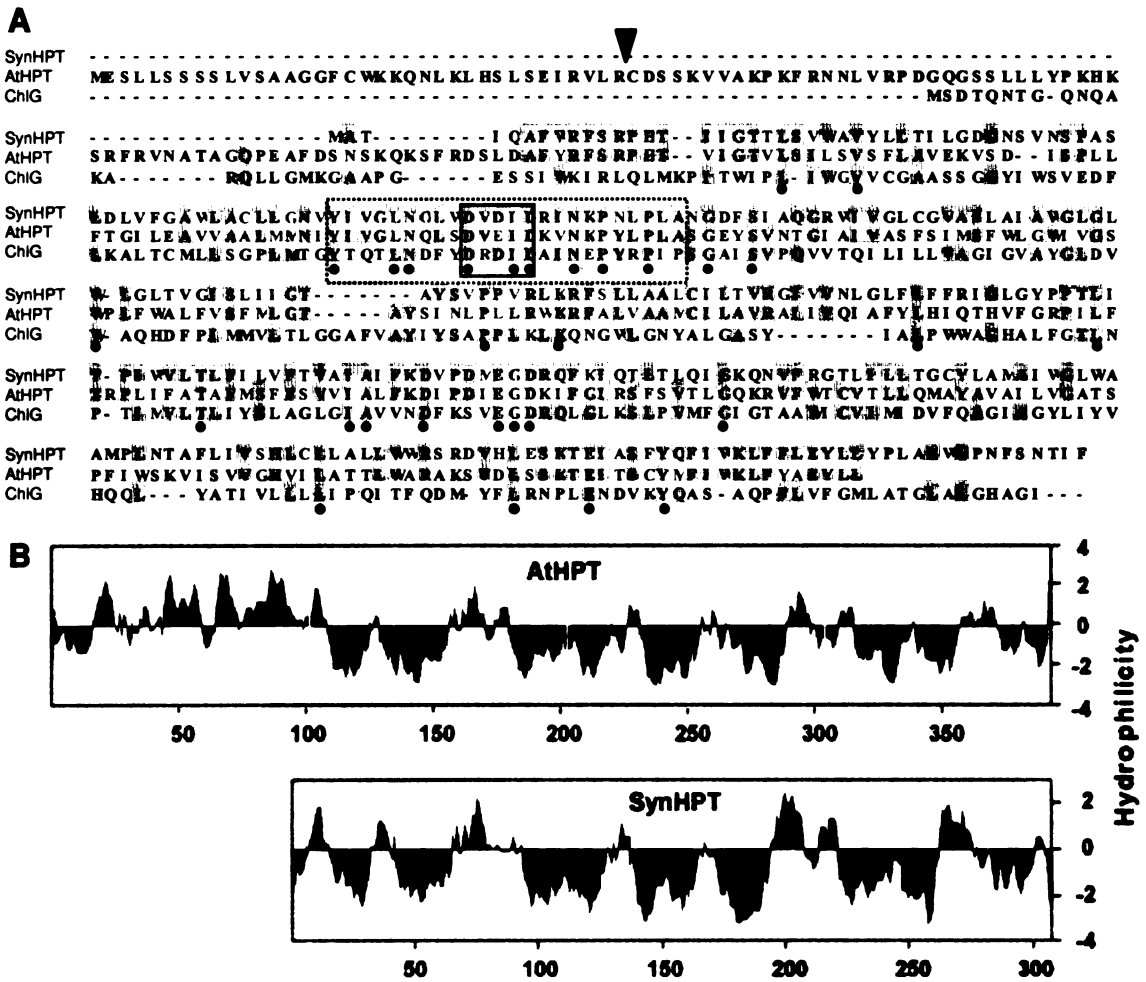


Figure 7. Alignment of *Synechocystis* and Arabidopsis polyprenyltransferases. A, Homogentisate phytyltransferases from *Synechocystis sp.* PCC 6803 (SynHPT, GenBank accession number S74813) and Arabidopsis (AtHPT, AF324344) share 41% protein identity, while SynHPT and ChlG (*Synechocystis sp.* PCC 6803 chlorophyll synthase, BAA10281) share 22% protein identity. Residues conserved in at least two of three sequences are shaded in gray, while residues identical in all three proteins are labeled by black dots. The conserved prenyl-DP and divalent cation binding domains are indicated by dashed and solid boxes, respectively. The predicted AtHPT chloroplast targeting domain cleavage site is indicated by a black arrow. B, Kyte/Doolittle hydrophillicity profiles of AtHPT and SynHPT. The two profiles are nearly identical. Negative values indicate hydrophobicity.

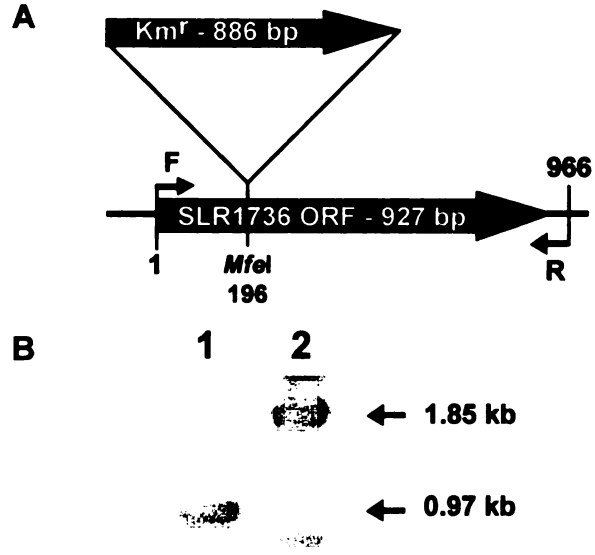


Figure 8. Construction of the *Synechocystis* PCC 6803 SLR1736::Km^r mutant. A, Simplified scheme of the wild type SLR1736 ORF in the *Synechocystis* genome. Insertion of a kanamycin (Km^r) cassette into *Mfe*I site of the SLR1736 ORF and the SLR1736F and R PCR primers (F and R, small arrows) are indicated. B, Autoradiograph of the PCR products amplified from wild type (lane 1) and the SLR1736::Km^r mutant (lane 2) genomic DNA. No wild type copies of SLR1736 were detected in the SLR1736::Km^r mutant.

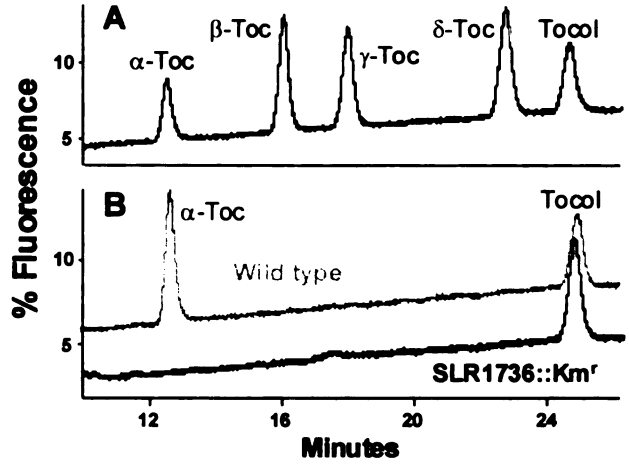


Figure 9. HPLC traces of tocopherol standards and lipids extracted from wild type *Synechocystis sp.* PCC 6803 and SLR1736::Km^r. Equivalent weights of fresh cells were extracted for the analysis shown. Tocopherol analysis was performed on a normal phase column using 8% di-isopropyl ether in hexane as a solvent. A, Separation of α-, β-, γ-, and δ-tocopherol (Toc = tocopherol) and tocol standards. B, Wild type cells accumulate predominantly α-tocopherol (gray trace). No tocopherols were detected in the SLR1736::Km^r mutant (black trace). Tocol was used as an internal standard.

Table I. *Prenyllipid contents of photoautotrophically grown wild type Synechocystis sp. PCC 6803 and the SLR1736::Km^r mutant.* Other than the absence of tocopherols in the SLR1736::Km^r mutant, no significant differences were observed between wild type and mutant cells for the presented parameters. Each value is the mean ± SD of at least five separate measurements per experiment. Each experiment was repeated at least three times. [a] mmol mol^{-1} chlorophyll *a*; [b] mmol mg^{-1} cells

Prenyllipid	wild type	SLR1736::Km^r
α-tocopherol [a]	18.7 ± 1.4	not detected
Plastoquinone-9 [a]	31.6 ± 4.3	28.4 ± 1.7
Phylloquinone [a]	13.8 ± 1.1	12.5 ± 1.2
Chlorophyll a [b]	4.3 ± 0.3	4.6 ± 0.4

compounds, we analyzed plastoquinone, phylloquinone, and chlorophyll levels in the SLR1736::Km^r mutant relative to wild type. No significant differences were observed in the levels of these compounds (Table I).

Biochemical Characterization of the SLR1736 Gene Product

The SLR1736::Km^r phenotype strongly suggests that SLR1736 encodes a polyprenyltransferase specific to tocopherol synthesis. To determine the activity and substrate specificity of the SLR1736 gene product, HGA polyprenyltransferase assays were performed using SLR1736 protein expressed in *E. coli*. These assays are based on TLC separation and subsequent autoradiography or HPLC separation of prenylated quinones formed from radioactive HGA and various unlabeled prenyl-DPs in the presence of a putative polyprenyltransferase.

When various prenyl-DPs at the same molar concentrations were tested as potential substrates for the SLR1736 protein, PDP was used most efficiently, though GGDP could also be utilized (Figures 10, 11B and 11D). The amount of geranylgeranylated benzoquinone product formed was approximately 18% that of the phytylated product. No products were observed when SDP, the prenyl-DP substrate for PQ-9 synthesis, was used

(Figure 10). In the case of PDP, the main reaction product co-migrated with *2'-trans*-MPBQ in both TLC and HPLC analyses (Figures 10 and 11B).

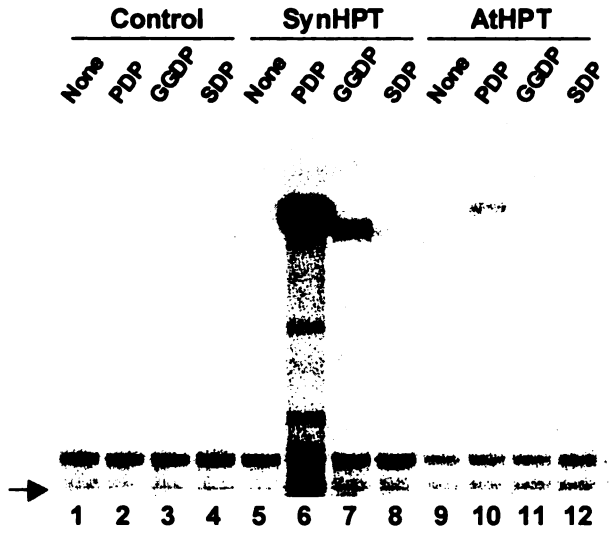


Figure 10. Homogentisate polyprenyltransferase assays. Individual reactions contained the indicated prenyl-DP and protein extracts from *E. coli* expressing empty vector or the indicated phytyltransferases. Radiolabeled prenylquinol reaction products were extracted, oxidized to corresponding quinones, separated by TLC, and subjected to autoradiography. SynHPT can utilize both PDP and GGDP as prenyl-DP substrates (lanes 6 and 7), while AtHPT can only use PDP (lane 10). Neither enzyme could catalyze condensation of HGA and SDP (lanes 8 and 12). No prenylquinone products were detected in control reactions (lanes 1 - 5, and 9). The arrow indicates the origin.

We also observed a couple of minor products on TLC and HPLC (Figure 10 and 11). On TLC, a band (Figure 10, lane 6, $R_f \sim 0.3$) likely corresponds to the quinol form of MPBQ, since intensity of this band increases when the samples are not oxidized with AgO prior to TLC (data not shown). A small radioactive peak eluting before the major *2'-trans*-MPBQ peak was also observed in HPLC analysis (Figure 11B). This peak probably corresponds to *2'-cis*-MPBQ formed by isomerization of the *trans*-isomer as previously reported (Hutson and Threlfall, 1980; Henry et al., 1987). It is unlikely that this peak represents the corresponding quinol as quinols are eluted much later than quinones in the HPLC system used. Due to their low abundance, further analyses of these minor peaks could not be performed.

When GGDP was used as a substrate the SLR1736 enzyme also produced a major and minor product. Unfortunately, 2-methyl-6-geranylgeranyl-1,4-benzoquinone (MGBQ), the expected product of HGA and GGDP condensation, was not available. However, indirect evidence suggests that the major GGDP reaction product is MGBQ. First, consistent with previous reports (Soll and Schultz, 1979; Hutson and Threlfall, 1980), this product migrates slightly slower than its phytylated counterpart in the TLC system used (Compare lanes 6 and 7 of Figure 10). Moreover, the chromatographic properties of the major product in normal-phase HPLC are consistent with those previously published for MGBQ (Hutson and Threlfall, 1980). Specifically, it elutes 9 minutes after the internal control *2'-trans*-MPBQ (Figure 11C), which is in good agreement with the previously reported relative elution difference between MPBQ and MGBQ (Hutson and Threlfall, 1980). As with PDP, an unknown minor GGDP product migrating prior to the major peak is also present in HPLC analysis. Unfortunately, neither GGPD products are produced in sufficient quantity to allow further analytical characterization. Based on the combined results of these polyprenyltransferase assays and the tocopherol-specific phenotype of the SLR1736::Km^r mutant, the protein encoded by the SLR1736 gene was named SynHPT, for *Synechocystis* Homogentisate PhytylTransferase.

Identification And Characterization of An HPT Homologue from *Arabidopsis thaliana*

To identify an HPT homologue from plants, we used the SynHPT protein sequence as a database query for BLAST searches (Altschul et al., 1990). A single predicted Arabidopsis gene on chromosome 2 (BAC clone F19F24) containing regions of significant similarity to SynHPT was identified. Subsequently, the corresponding cDNA was isolated from an Arabidopsis seed cDNA library and fully sequenced. The predicted protein encoded by this cDNA (GenBank accession number AF324344) shares 41% identity with SynHPT. In addition, both proteins have remarkably similar hydrophobicity profiles and

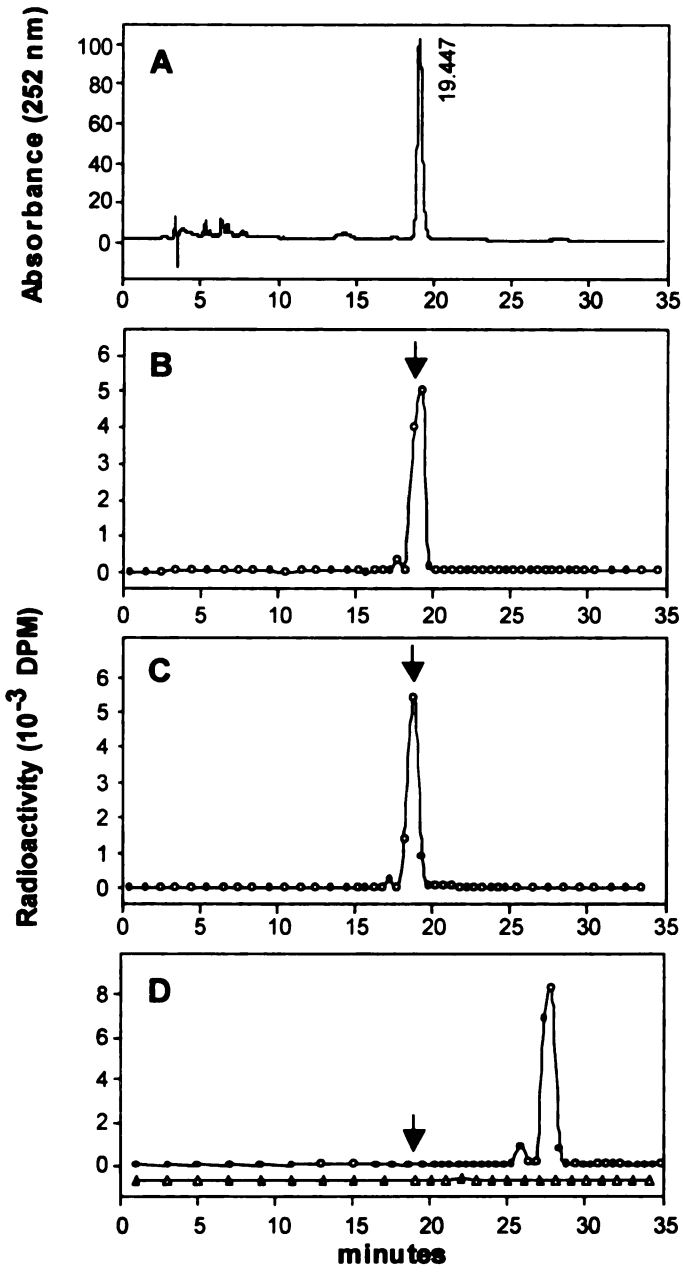


Figure 11. Normal phase HPLC separation of radiolabeled prenylquinones produced from HGA and prenyl-DP substrates by HPTs. Homogentisate polyprenyltransferase reactions were performed in a total volume of 0.5 mL for SynHPT with PDP (panel B) and 5 mL for SynHPT with GGDP, and AtHPT with PDP or GGDP (panels C, D, or E, respectively) as described in *Materials and Methods*. Elution of the internal standard *2'-trans*-MPBQ was monitored at 252 nm, while that of the prenylquinones formed during the assay was monitored by scintillation counting of collected fractions. The UV traces are not shown for B, C and D for clarity, but when aligned with the radioelution profiles shown, the major radiolabeled phytylated products co-chromatographed with authentic *2'-trans*-MPBQ standard (indicated by arrows). A, Elution of *2'-trans*-MPBQ; B and C, SynHPT and AtHPT catalyzed formation of *2'-trans*-MPBQ from HGA and PDP; D, SynHPT catalyzed formation of MGBQ from HGA and GGDP (circles). AtHPT did not produce a product with HGA and GGDP as substrates (triangles).

contain prenyl-diphosphate and divalent cation binding motifs conserved in both location and sequence (Figure 7). The Arabidopsis protein also contains an additional 95 amino acid N-terminal extension that is not present in SynHPT. The first 36 amino acids of this domain exhibit features of a chloroplast targeting sequence (Emanuelsson et al., 1999), consistent with the reported chloroplast envelope localization of HPT activity in plants (Soll et al., 1980; Soll et al., 1984). The Arabidopsis protein was tentatively named AtHPT for *Arabidopsis thaliana* Homogentisate PhytylTransferase.

In order to determine the activity and substrate specificity of the putative AtHPT and compare it to SynHPT, AtHPT was expressed in *E. coli* and HGA polyprenyltransferase assays were performed. Like SynHPT, AtHPT catalyzed condensation of HGA and PDP to form *2'-trans*-MPBQ as a major product and was not active with the substrates HGA and SDP. Unlike SynHPT, no products were observed when HGA and GGDP were used as substrates (Figures 10 and 11D). To test whether this difference between the two enzymes was due to the presence of chloroplast targeting sequences in AtHPT, we also tested two truncated versions of the protein. One truncation removed the predicted 36 amino acid chloroplast transit peptide, while the second removed 95 N-terminal amino acids not present in SynHPT. Neither truncation altered the specific activity or substrate specificity of AtHPT (results not shown).

The specific activity of AtHPT expressed in *E. coli* was approximately 3% that of SynHPT expressed from the same vector. Several explanations are plausible for this difference, including decreased protein stability, poor protein expression in *E. coli* due to codon bias, or an improper lipid environment relative to that of chloroplasts. Neither AtHPT nor SynHPT could be visualized on coomassie-stained gels following induction, indicating both are expressed at low levels in *E. coli*. Addition of lipids extracted from Arabidopsis leaves or seeds to reactions had no discernible effect on AtHPT activity (data not shown). Finally, addition of Tween 80 and CHAPS to final concentrations of 0.2, 0.5, 1, or 2% moderately stimulated both AtHPT and SynHPT activities without appreciably

changing their specific activities relative to each other (data not shown). It appears that a combination of lower expression and/or lower stability of AtHPT relative to SynHPT may be the cause of limited AtHPT activity in *E. coli*.

Physiological Consequences of Tocopherol Deficiency in *Synechocystis sp.* PCC 6803

Given the importance of tocopherols in free radical scavenging, a photosynthetic organism lacking tocopherols might be expected to be compromised in growth or exhibit increased sensitivity to high light stress. To address this question, we compared growth of the tocopherol deficient SLR1736::Km^r mutant and wild type *Synechocystis* under low light or high light conditions (~ 30 and 110 μE, respectively). Surprisingly, the doubling times of both strains under photoautotrophic or heterotrophic conditions in both low and

Table II. *Growth and O_2 evolution rates of wild type Synechocystis sp. PCC 6803 and SLR1736::Km^r.* Doubling times and photosynthetic activity of wild type and mutant cells are similar. Each value is the mean ± SD of three independent measurements in a representative experiment. Each experiment was repeated at least three times. [a] The cells were grown at high light (105 - 110 μE); [b] The cells were grown photoautotrophically, O_2 evolution rates are in mmol O_2 mg^{-1} chlorophyll h^{-1} and measured at 5 mE.

Parameter	wild type	SLR1736::Km^r
Growth, doubling time (h) [a]		
Photoautotrophic	22 ± 3	19 ± 3
Photoheterotrophic	12 ± 1	13 ± 1
Oxygen evolution rates [b]		
Whole chain	156 ± 6	163 ± 8

high light were comparable (Table II and data not shown). Whole-chain oxygen evolution measured at 0.75, 2 and 5 mE was also found to be similar in both strains, indicating that the initial rates of photosynthesis in SLR1736::Km^r and wild type are comparable (Table II).

Discussion

Due to the conserved evolution of photosynthetic organisms, many biosynthetic pathways in cyanobacteria and plants are often quite similar (Whistance and Threlfall, 1970; Marechal et al., 1997). The rapid growth of EST and genome databases in a wide variety of organisms allows plant biochemists to utilize such inter-kingdom conservation in their research more effectively. In particular, the availability of the fully sequenced *Synechocystis sp.* PCC 6803 genome in a searchable on line database, CyanoBase (Kaneko et al., 1996), coupled with straightforward gene disruption methods for analysis of gene function in this organism (Williams, 1988) makes *Synechocystis* an attractive system to complement studies of tocopherol synthesis in plants. Two tocopherol biosynthetic enzymes, GGDP reductase (Addlesee et al., 1996; Keller et al., 1998) and γ-tocopherol methyltransferase (Shintani and DellaPenna, 1998) have already been cloned and characterized from *Synechocystis* and used successfully as database probes to identify orthologs from Arabidopsis. The γ-tocopherol methyltransferases from both organisms were shown to have nearly identical activities (Shintani and DellaPenna, 1998). We have employed a similar genomics-based approach to identify and characterize a third enzyme of the tocopherol pathway from *Synechocystis* and Arabidopsis, HGA phytyltransferase, and assess whether this step in tocopherol synthesis is also conserved between cyanobacteria and plants.

In photosynthetic organisms, condensation of HGA with either a 20 or 45 carbon prenyl-DP is the branch point in tocopherol and plastoquinone synthesis, respectively. Early biochemical studies established that the tocopherol and plastoquinone pathways are remarkably similar in oxygenic cyanobacteria, algae, and plants (Whistance and Threlfall, 1970). Although these studies could not distinguish separate polyprenyltransferase activities for tocopherol and plastoquinone synthesis, it was suggested that separate prenylation enzymes might be involved (Schulze-Siebert et al., 1987). More recently, genetic analysis of the pathways in Arabidopsis identified two loci whose mutant

phenotypes are consistent with the disruption of enzymes shared in the synthesis of tocopherols and plastoquinones (Norris et al., 1995). Indeed, this was found the case for the *PDS1* locus, which encodes HPPD, the enzyme that produces the aromatic head-group HGA in both the plastoquinone and tocopherol pathways (Norris et al., 1998). The *PDS2* locus was suggested to encode a similarly shared polyprenyltransferase that could utilize either PDP or SDP for tocopherol and plastoquinone synthesis, respectively (Norris et al., 1995). The cloning of plant and cyanobacterial HPTs now allows us to directly address the nature of polyprenyltransferases involved in tocopherol and plastoquinone synthesis in oxygenic photosynthetic organisms.

The *Synechocystis* SLR1736::Km^r mutant lacks tocopherols but accumulates wild type levels of PQ-9, consistent with the existence of separate HGA polyprenyltransferases in tocopherol and plastoquinone synthesis. This conclusion is also supported by enzymatic studies showing that SynHPT can utilize the 20 carbon tocopherol substrates, PDP or GGDP, but is inactive with the 45 carbon PQ-9 substrate, SDP. Thus, it appears that *Synechocystis* contains a single, polyprenyltransferase specific to tocopherol synthesis, SynHPT, and a separate, yet to be characterized, polyprenyltransferase specific to plastoquinone synthesis.

In Arabidopsis, the prenyltransferase reaction involving HGA and PDP substrates appears to be nearly identical to that in *Synechocystis*. The AtHPT and SynHPT enzymes share 61% protein similarity and both enzymes use PDP as their preferred prenyl-DP substrate *in vitro* to form *2'-trans*-MPBQ as a major product. This is in agreement with previous biochemical studies of HPT activity in isolated spinach, lettuce, and pea chloroplasts where MPBQ was the only product detected (Hutson and Threlfall, 1980; Soll et al., 1980; Marshall et al., 1985). Like SynHPT, AtHPT did not generate detectable prenylquinone products with SDP as a substrate, suggesting Arabidopsis likely contains separate polyprenyltransferases for tocopherol and plastoquinone synthesis. An Arabidopsis HPT knockout mutant is needed to rigorously address this question.

In considering the nature of homogentisate polyprenyltransferase reactions in plants, it is important to note that our original goal of cloning the Arabidopsis *PDS2* locus has not been achieved. Tocopherol and plastoquinone levels are reduced below detection in *pds2,* leading to the hypothesis that *PDS2* encodes a polyprenyltransferase shared in tocopherol and plastoquinone synthesis (Norris et al., 1995). However, given that AtHPT is a phytyltransferase encoded by a single-copy gene on chromosome 2 while *PDS2* maps to chromosome 3, this proposal now seems unlikely. If *PDS2* is not a polyprenyltransferase shared in tocopherol and plastoquinone synthesis, what does it encode? One explanation is that *PDS2* encodes an enzyme specific to plastoquinone synthesis (i.e., HGA solanesyltransferase or SDP synthase) and that the absence of tocopherols in *pds2* is a pleiotropic effect of this mutation. In this scenario, the absence of plastoquinone, the main lipid soluble electron carrier in plastids, results in such high levels of oxidative stress in *pds2* that any tocopherols produced are rapidly oxidized and degraded, and hence undetectable. Alternatively, plastoquinone may be a cofactor required for the synthesis of tocopherols and its absence arrests tocopherol synthesis. Regardless of mechanism, it appears likely that the tocopherol deficiency in *pds2* is an indirect, rather than a direct effect of the *pds2* mutation.

Though SynHPT and AtHPT are similar in their substrate specificities, there is one notable exception: SynHPT can use both PDP and GGDP as substrates, while AtHPT only uses PDP. The utilization of both PDP and GGDP as substrates by a polyprenyltransferase is not unprecedented. An analogous reaction occurs in chlorophyll biosynthesis where chlorophyll synthase can attach either PDP or GGDP to the tetrapyrrole moiety, and in cyanobacteria, PDP is the preferred substrate (Oster et al., 1997). We observed a similarly strong preference of SynHPT for PDP over GGDP. The use of GGDP by SynHPT *in vivo* would yield tocotrienol intermediates and endproducts that only differ from their tocopherol counterparts in having an unsaturated rather than saturated hydrophobic tail. This would necessitate subsequent enzymes in the pathway being active

towards geranylgeranylated substrates. At least one other tocopherol biosynthetic enzyme from cyanobacteria has been shown to utilize both phytylated and geranylgeranylated intermediates *in vitro*, tocopherol cyclase from *Anabaena variabilis* (Stocker et al., 1996). However, *Synechocystis sp.* PCC 6803 does not accumulate tocotrienols, suggesting that any geranylgeranylated intermediate produced by SynHPT is either efficiently reduced (most likely by GGDP reductase), or that GGDP is not a substrate *in vivo*. Additional work is required to delineate the *in vivo* substrate(s) and product(s) of SynHPT.

Within the limits of our assay sensitivity (~ 3 % of PDP product levels), AtHPT did not utilize GGDP as a substrate. Other researchers also failed to demonstrate condensation of HGA and GGDP using isolated spinach, lettuce, and pea chloroplasts (Hutson and Threlfall, 1980; Soll et al., 1980). These data are consistent with the general observation that dicots do not produce tocotrienols (Piironen et al., 1986; Franzen and Haas, 1991). However, many monocots and gymnosperms do produce both tocopherols and tocotrienols (Piironen et al., 1986; Franzen et al., 1991; Franzen and Haas, 1991) and we speculate that HPTs from such organisms would utilize GGDP and PDP as substrates, analogous to SynHPT. Phylogenetic analysis of polyprenyltransferases from various photosynthetic organisms shows that HPTs from cyanobacteria, monocots and dicots form separate groups (Figure 12), which probably represents taxonomic differences, although it may in part reflect differences in the substrate specificities of these enzymes. As with *Synechocystis*, we would also anticipate that pathway enzymes after HPT in monocots would be active toward both geranylgeranylated and phytylated intermediates. The substrate specificity of tocopherol biosynthetic enzymes from monocots have not been characterized, however, in spinach (a dicot), where enzymology of the pathway has been best-studied, later methyltransferases of the pathway are active toward various geranylgeranyl intermediates (Soll and Schultz, 1979). It appears that at some point in evolution, dicots, like monocots, could likely produce tocotrienols but have lost this ability as their HPTs have evolved substrate specificity for PDP over GGDP.

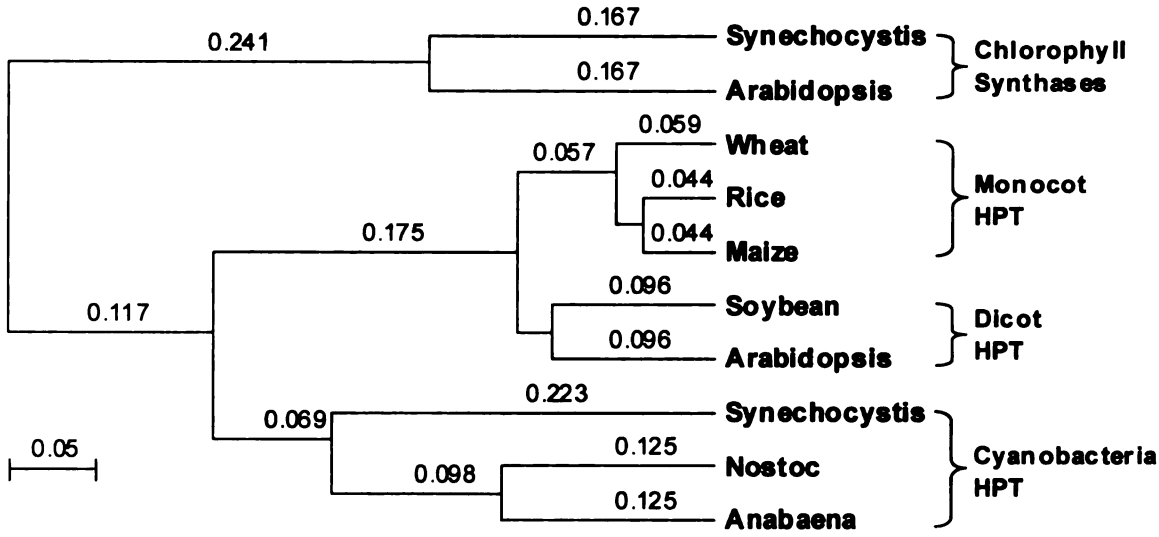


Figure 12. Phylogenetic analysis of various prenyllipid polyprenyltransferases. Sequence alignment and phylogenetic analysis were performed using MacVector software (Genetic Computer Group, Madison, WI). Numbers indicate distances between protein sequences estimated by the uncorrected *p*-distance method. Chlorophyll synthases form a separate clade from homogentisate phytyltransferases. Within HPTs, cyanobacterial, monocot, and dicot HPTs also form distinct sub-groups.

Numerous studies suggest that tocopherols are important antioxidants involved in photoprotection of plants. Tocopherol levels correlate well with the degree of oxidative stress in numerous plant species grown under various stress conditions, including high light, drought, and low temperatures (Havaux et al., 2000; Wildi and Lutz, 1996; Streb et al., 1998; Bartoli et al., 1999; Munne-Bosch and Alegre, 2000). Given this suggestive role of tocopherols in antioxidant and photoprotective function, a mutation that eliminates tocopherol synthesis in a photosynthetic organism would be anticipated to increase sensitivity to oxidative stress, reducing growth or viability of this organism under stressful conditions. Various mutants and transgenic plants with decreased tocopherol levels have been reported to exhibit photobleaching phenotypes and compromised growth (Henry et al., 1986; Norris et al., 1995; Tanaka et al., 1999). However, in all these cases, other prenyllipids such as phylloquinone, chlorophylls, carotenoids, or plastoquinone were affected in addition to tocopherols (Henry et al., 1986; Norris et al., 1995; Tanaka et al.,

1999). Therefore, it was impossible to specifically attribute these phenotypes to tocopherol deficiency. The tocopherol-specific phenotype of SLR1736::Km^r provides a unique tool to begin to specifically address the question of tocopherol function in photosynthetic organisms.

Surprisingly, SLR1736::Km^r growth rates under photoautotrophic and photoheterotrophic conditions in low and high light were indistinguishable from wild type. Mutant and wild type whole-chain oxygen evolution rates were also similar. The observation that the absence of tocopherols did not appreciably affect growth, photosynthetic electron transport, and tolerance to high light stress seemingly contradicts the concept that tocopherols are essential lipid soluble antioxidants. However, there are several possible explanations for these apparently incongruous results. First, α-tocopherol is not the only antioxidant present in photosynthetic membranes. Photosynthetic organisms have evolved multiple mechanisms for protection from oxidative stresses (carotenoids, ascorbate, superoxide dismutases, etc.) which if up-regulated, could partially or fully compensate for the absence of tocopherols in SLR1736::Km^r under certain conditions. Alternatively, tocopherols may protect from a specific type of lipid peroxidation or at a particular site. Finally, the limited oxidative treatments used in this report may not be sufficient to produce detectable differences between wild type *Synechocystis* and SLR1736::Km^r at the level of culture growth rates. A detailed analysis of membrane lipids, lipid peroxidation products, and other component of oxidative stress compensation and adaptation are needed to discern any effects of tocopherol deficiency in SLR1736::Km^r.

Finally, one potential application of the described work would be to allow engineering of elevated tocopherol levels in food crops for nutritional purposes. Given the central position of HPT in tocopherol synthesis, it seems likely that the enzyme may be an important step for controling flux into the pathway. A crucial observation is that eliminating HPT activity in *Synechocystis* does not affect the levels of other biosynthetically related compounds (plastoquinone, phylloquinone, and chlorophylls). This suggests that altering

HPT enzyme levels in plants may also be tocopherol-specific and have little effect on the synthesis of other prenylquinone compounds in the plastid. Experiments are underway to positively modify AtHPT expression in Arabidopsis to test whether the activity is a target for engineering tocopherol levels in plants.

Materials and Methods

Chemicals and Bacterial Strains. Prenyl-DPs were more than 99% pure. PDP was kindly provided by Dr. Stephanie Sen (Purdue University, Indianapolis, IN), while GGDP and SDP were purchased from American Radiolabeled Chemicals (St. Louis, MO). (U-^{14}C)-HPP (0.6 - 1.5 μM) was prepared from (U-^{14}C)-tyrosine (specific activity 464 mCi mmol^{-1}, Amersham, Arlington Heights, IL) as described (Schulz et al., 1993). Tocol was a gift from H. Banno (Eisai Company, Japan). A mixture of various *cis*- and *trans*-methyl-phytyl-1,4-benzoquinone isomers was synthesized by Dr. Daniel Liebler (University of Arizona, Tucson, AZ). *2'-trans*-2-methyl-6-phytyl-1,4-benzoquinone was purified from the mixture by a combination of TLC and HPLC (Henry et al., 1987). Plastoquinone-9 was extracted from *Iris hollandica* bulbs and purified by both TLC (Pennock, 1985) and HPLC (Johnson et al., 2000). Wild type *Synechocystis* sp. PCC 6803 was grown on BG-11 plates or liquid media (Williams, 1988) either photoheterotrophically (with 15 mM glucose) or photoautotrophically (without glucose) at 20 - 30 μE and 30 °C unless otherwise stated. *Synechocystis* cells were sub-cultured at least three times in liquid media prior to growth experiments. *E. coli* strains DH5α (Stratagene, La Jolla, CA) and BL-21 (DE3) (Novagen, Milwaukee, WI) were used for conventional sub-cloning and protein expression, respectively.

Plasmids and Mutants. Primers 5'-TATTCATATGGCAACTATCCAAGCTTTTTG-3' (SLR1736F) and 5'-GGATCCTAATTGAAGAAGATACTAAATAGTTC-3' (SLR1736R)

containing engineered *Nde*I and *Bam*HI sites (underlined) and Vent DNA polymerase (New England Biolabs, Beverly, MA) were used to amplify the SLR1736 ORF (GenBank accession number BBA17774) from *Synechocystis* genomic DNA. The amplified fragment was sub-cloned into the *Eco*RV site of pBluescript II KS (+) to generate pKS1736. pKS1736 was digested with *Mfe*I and ligated with the *Eco*RI digested kanamycin resistance cassette from pUC4K (Taylor and Rose, 1988). Two constructs with opposite orientation of the kanamycin resistance cassette relative to the SLR1736 ORF were used to transform wild type *Synechocystis* and generate disruption mutants by homologous recombination (Williams, 1988). Transformants were sub-cultured on kanamycin containing media for several plating cycles and the absence of wild type SLR1736 gene copies was confirmed by PCR using SLR1736F and R primers followed by Southern blot analysis (Figure 8A and B). Because the two orientation disruption mutants were phenotypically indistinguishable (data not shown), that with the Km^r cassette in the same orientation as the SLR1736 ORF, referred to hereafter as the SLR1736::Km^r mutant (Figure 8A), was used for further analyses. The *Nde*I-*Bam*HI fragment from pKS1736 encoding the entire SLR1736 ORF was ligated into *Nde*I-*Bam*HI digested pET30b (Novagen) to create pSynHPT, which was transformed into BL-21 (DE3) cells for protein expression.

The SLR1736 protein sequence was used to search the *A. thaliana* database and identify a single genomic clone, F19F24, containing a homologous sequence. Primers 5'-TTGTTTTCAGGCTGTTGTTGCAGCTCTC-3' and 5'-CGTTTCTGACCCAGAGTTACAGAGAATG-3' were used to amplify a 977-bp fragment from F19F24 for use as a probe to screen an Arabidopsis seed cDNA library (a gift of Dr. John Ohlrogge, Michigan State University, East Lansing, MI). The longest of 15 positive clones was sequenced and shown to encode a protein similar to SLR1736 that was designated AtHPT. For protein expression purposes the full-length clone encoding AtHPT was amplified using primers (5'-CCATGGAGTCTCTGCTCTC-3' and 5'-

GGATCCCAAGCAGAGACTTCTTTACC-3') and sub-cloned into *Nco*I-*Bam*HI digested pET3d vector (Novagen) to generate pAtHPT.

Prenyllipid Analysis. Fifteen to twenty mg of 14-day old plate-grown, *Synechocystis sp.* PCC 6803 cells were harvested, their lipids extracted (Bligh and Dyer, 1959) and dissolved in 100 μL of hexane or ethyl acetate. Ten μL of each sample was withdrawn for chlorophyll determination (Lichtenthaler, 1987), while 50 μL was subjected to HPLC (Hewlett Packard 1100, Wilmington, DE) on a LiChrosorb 5 Si60A 4.6 X 250 mm normal phase column (Phenomenex, Torrance, CA) at 42 °C as described (Syvaoja et al., 1986). Tocopherols were detected by fluorescence using 290 nm excitation and 325 nm emission. For plastoquinone and phylloquinone analysis, separation was achieved on a reverse phase column (Spherisorb 5 ODS2 4.6 X 250 mm, Waters, Marlborough, MA) as described (Johnson et al., 2000). Plastoquinone-9 and phylloquinone were detected at 250 nm and 275 nm, respectively.

Homogentisate polyprenyltransferase assay. Each 0.2 mL reaction contained freshly prepared (U-^{14}C)-HPP (~ 0.2 μM, specific activity 464 mCi mmol^{-1}), 50 mM HEPES (pH 7.6), 4 mM $MgCl_2$, 50 mM potassium ascorbate, 100 μM KF, 0.2 % (w/v) CHAPS, and 0.1 mg of total protein extracted from *E. coli* expressing HPPD (Norris et al., 1995). Individual reactions contained 100 μM PDP, GGDP, or SDP and the insoluble protein fraction from *E. coli* expressing pSynHPT (0.03 mg protein), pAtHPT (1 mg protein), or the empty pET vector (0.03 or 1 mg protein). Reactions were incubated for 1 hour at room temperature, extracted with two volumes of methanol:chloroform (1:1), and any newly formed prenylquinols were oxidized with AgO (Pennock, 1985). The organic phase was transferred to a fresh tube, evaporated to dryness, dissolved in ethyl acetate, and subjected to TLC on silica gel (J.T.Baker, Phillisburg, NJ) developed with 20% ethyl ether in petroleum

ether (Pennock, 1985). Labeled prenylquinones were detected by autoradiography for 14 days.

For HPLC analyses, the polyprenyltransferase assays were performed as described above except that larger volumes were used for the individual reactions to ensure formation of sufficient products: 0.5 mL for SynHPT with PDP and 5 mL for SynHPT with GGDP, and AtHPT with PDP or GGDP. At the end of incubation, reactions were spiked with *2'-trans*-MPBQ and extracted prenyllipids resolved by TLC as above. The areas corresponding to prenylquinones (R_f 0.36 - 0.67) were scraped from the TLC plates, eluted with ethyl ether, dried under nitrogen, and dissolved in hexane. Samples were then subjected to HPLC on a normal phase column (LiChrosorb 5 Si60A, 4.6 X 250 mm) using 0.1% dioxane in hexane as a mobile phase to separate various methyl-phytyl benzoquinone isomers (Henry et al., 1987). For geranylgeranylated quinone products, a mobile phase consisting of 0.15% dioxane in iso-octane was used (Hutson and Threlfall, 1980). Prenylquinones were detected at 252 nm. Eluents were collected at 30- to 60-second intervals and the associated radioactivity determined by liquid scintillation counting.

Growth curves. Wild type *Synechocystis* and the SLR1736::Km^r mutant were inoculated to a final OD_{730} of 0.05 in 50 mL of liquid BG-11 medium and grown at 30 °C with vigorous shaking in four possible combinations: with or without 15 mM glucose and at 20-30 (low light) or 105-110 (high light) μE. The OD_{730} was measured every 6-12 hours and used to calculate cell density (Williams, 1988).

Oxygen evolution. Liquid cultures of photoautotrophically grown wild type and mutant *Synechocystis* cells were washed twice and resuspended in fresh BG-11 medium at a concentration of 3 mg chlorophyll per mL. The cells were exposed to three different high light intensities for 5 minutes (0.75, 2 and 5 mE). Oxygen measurements were performed with a Clark-type electrode at 25° C using a Hansatech CB1-D3 recording unit with Minirec

recording software (Hansatech Instruments, King's Lynn, England). The oxygen evolution rate was calculated from the slope within the linear region of the curves.

Phylogenetic analysis. Sequence alignment (ClustalW alignment using the BLOSUM series matrix) and subsequent phylogenetic analysis were performed using MacVector software (Genetic Computer Group, Madison, WI). The N-terminal 96-amino acid extension of AtHPT and the corresponding N-termini of the other polyprenyltransferases were not included in the phylogenetic analysis. The following protein sequences were used: *Synechocystis* ChlG (BAA10281), Arabidopsis ChlG (S60222), *Synechocystis* HPT (S74813), Nostoc HPT (480 - 1445 bp of contig 566), Anabaena HPT (6672 - 7625 bp of contig c295), rice HPT (AX046728), maize HPT (AX046716), Arabidopsis HPT (AF324344), soybean HPT (AX046734), and wheat HPT (BE471221 - overlapped BE471221 and BG604641 corresponded to the C-terminal part of AtHPT starting at Asp-160). For phylogenetic analysis, distances between the amino acid sequences were estimated by using the uncorrected *p*-distance method with gaps distributed proportionally. The best tree was constructed by the Unweighted Pair-Group Method with Arithmetic Mean with random tie breaking. Bootstrapping (10,000 reps.) confirmed the confidence of the best tree structure.

CHAPTER 2

HOMOGENTISATE PHYTYLTRANSFERASE ACTIVITY IS LIMITING FOR TOCOPHEROL BIOSYNTHESIS IN ARABIDOPSIS

Abstract

Tocopherols are essential components of the human diet and synthesized exclusively by photosynthetic organisms. These lipophilic antioxidants consist of a chromanol ring and a 15-carbon tail derived from homogentisate (HGA) and phytyl diphosphate (PDP), respectively. Condensation of HGA and PDP, the committed step in tocopherol biosynthesis, is catalyzed by homogentisate phytyltransferase (HPT). To investigate whether HPT activity is limiting for tocopherol synthesis in plants, the gene encoding *Arabidopsis thaliana* HPT, *HPT1*, was constitutively overexpressed in *Arabidopsis*. In leaves, *HPT1* overexpression resulted in a ten-fold increase in HPT specific activity, and a 4.4-fold increase in total tocopherol content relative to wild type. In seeds, *HPT1* overexpression resulted in a four-fold increase in HPT specific activity and a total seed tocopherol content that was 40% higher than wild type, primarily due to an increase in γ-tocopherol content. This enlarged pool of γ-tocopherol was almost entirely converted to α-tocopherol by crossing *HPT1* overexpressing plants with lines constitutively overexpressing γ-tocopherol methyltransferase (γ-TMT). Seed of the resulting double overexpressing lines had a 12-fold increase in vitamin E activity relative to wild type. These results indicate that HPT activity is limiting in various *Arabidopsis* tissues and that total tocopherol levels and vitamin E activity can be elevated in leaves and seeds by combined overexpression of the *HPT1* and *γ-TMT* genes.

Introduction

Tocopherols, collectively known as vitamin E, are a class of lipid soluble antioxidants synthesized exclusively by photosynthetic organisms. Tocopherols are essential components of the human diet as they perform numerous critical functions

including quenching and scavenging various reactive oxygen species and free radicals and protecting polyunsaturated fatty acids from lipid peroxidation (Fukuzawa and Gebicky, 1983; Neely et al., 1988; Fryer, 1993; Bramley et al., 2000). Due to these and other activities, dietary tocopherols are thought to play an important role in improving immune function and in limiting the incidence and progression of several degenerative human diseases including certain types of cancer, cataracts, neurological disorders, and cardiovascular disease (Brigelius-Flohe and Traber, 1999; Bramley et al., 2000; Pryor, 2000).

In plants, indirect evidence suggests that tocopherols perform antioxidant and radical quenching functions similar to those in animals (Fryer, 1992), and that tocopherols may have additional roles related to photosynthesis (Munne-Bosch and Alegre, 2002a). Plants alter their tocopherol levels during development (Molina-Torres and Martinez, 1991; Tramontano et al., 1992) and in response to a variety of stresses, including high light, low temperature, drought, and salt stress (Gossett et al., 1994; Streb et al., 1998; Leipner et al., 1999; Havaux et al., 2000; Munne-Bosch and Alegre, 2000). In addition, during leaf senescence, a process accompanied by chlorophyll degradation and oxidative damage in photosynthetic membranes, there is an increased accumulation of tocopherols (Rise et al., 1989). These combined studies suggest that the synthesis of tocopherols is highly regulated during plant growth and development.

Tocopherols are amphipatic molecules, consisting of a polar chromanol head group and a lipophilic isoprenoid tail derived from HGA and PDP, respectively. The tocopherol biosynthetic pathway is depicted in Figure 13. In plants, the aromatic precursor of tocopherols, HGA, is synthesized from *p*-hydroxyphenyl pyruvate (HPP) by a cytosolic HPP dioxygenase (HPPD) (Norris et al., 1995; Garcia et al., 1997; Garcia et al., 1999). The biosynthetic source of HPP in tissues is unclear as HPP can originate from the shikimate pathway via prephenate or by transamination of tyrosine (Threlfall and

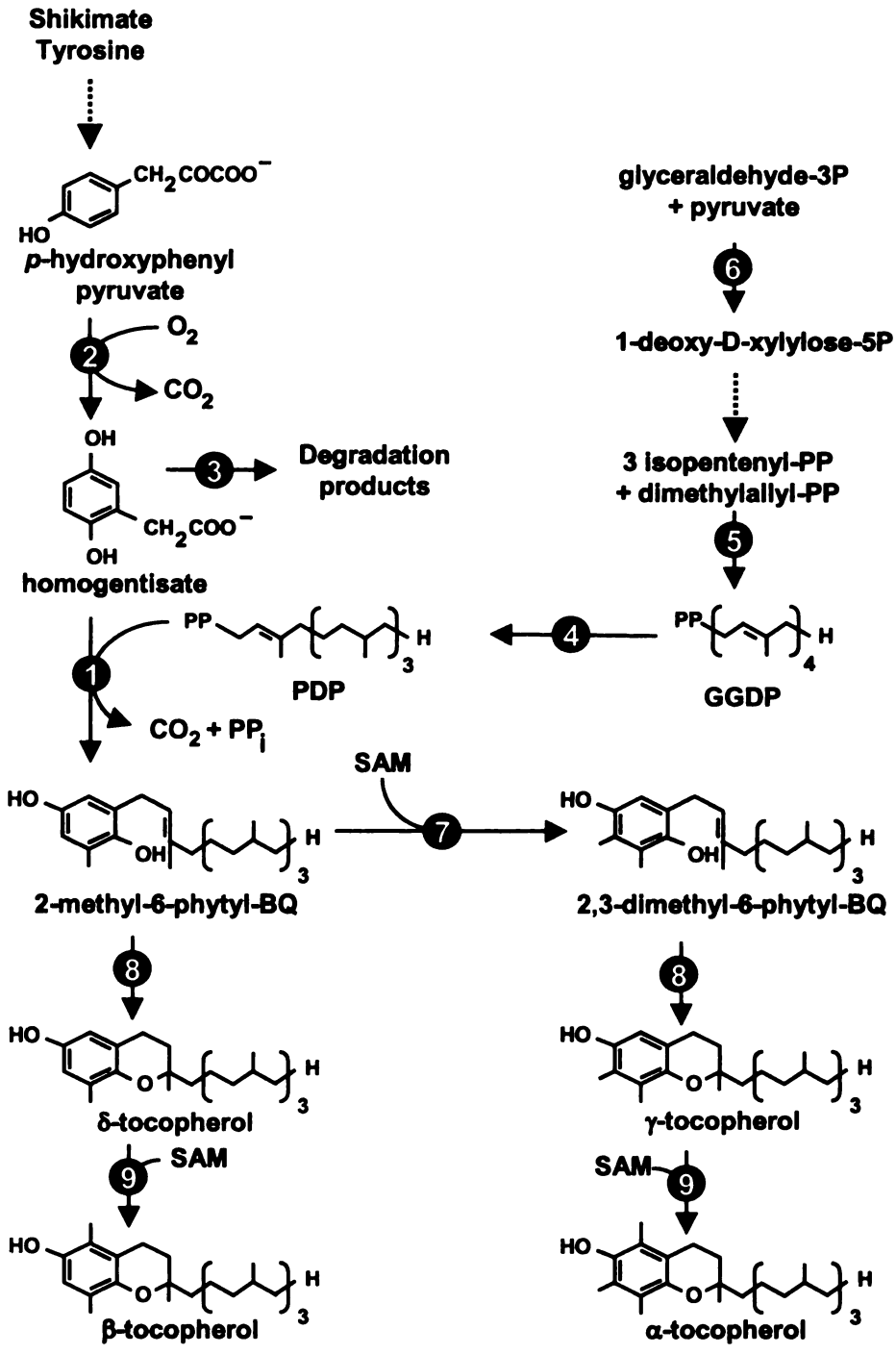


Figure 13. The tocopherol biosynthetic pathway in plants. Dashed arrows represent multiple steps. Enzymes are indicated by circled numbers: (1) homogentisate phytyltransferase, (2) *p*-hydroxyphenylpyruvate dioxygenase, (3) homogentisate dioxygenase, (4) geranylgeranyl diphosphate reductase, (5) geranylgeranyl diphosphate synthase, (6) 1-deoxy-D-xylulose-5-phosphate synthase, (7) 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase, (8) tocopherol cyclase, (9) γ-tocopherol methyltransferase

Whistance, 1971; Fiedler et al., 1982; Garcia et al., 1999; Lopukhina et al., 2001). Phytyl diphosphate is formed by the sequential action of the plastidic geranylgeranyl diphosphate synthase 1 (GGPS1) and geranylgeranyl diphosphate reductase (GGDR) (Kuntz et al., 1992; Addlesee et al., 1996; Keller et al., 1998; Addlesee and Hunter, 1999; Okada et al., 2000). As with other plastid-synthesized isoprenoids, PDP is derived from the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway (Eisenreich et al., 1998; Lichtenthaler, 1998).

In photosynthetic organisms, condensation of HGA and PDP, the committed step in tocopherol biosynthesis, is catalyzed by homogentisate phytyltransferase (HPT) (Soll et al., 1980; Soll et al., 1984; Soll, 1987; Collakova and DellaPenna, 2001; Savidge et al., 2002). The product of this reaction, 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ), is the first prenylquinol intermediate in the pathway and can be methylated to 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) by MPBQ methyltransferase (MPBQ MT) (Soll and Schultz, 1979; Hutson and Threlfall, 1980; Soll and Schultz, 1980; Marshall et al., 1985; Soll, 1987; Shintani et al., 2002). Both MPBQ and DMPBQ are substrates for tocopherol cyclase (TC) to yield the first tocopherols of the pathway, γ-tocopherol and δ-tocopherol, respectively (Stocker et al., 1996; Arango and Heise, 1998; Porfirova et al., 2002). Both γ- and δ-tocopherol can be methylated by γ-tocopherol methyltransferase (γ-TMT) to yield α- and β-tocopherol, respectively (D' Harlingue A and Camara, 1985; Shintani and DellaPenna, 1998).

Due to the importance of vitamin E in human and plant physiology, the tocopherol biosynthetic pathway has become a focus for plant metabolic engineering. In order to successfully manipulate the tocopherol content and/or composition of various plant tissues, enzymes with high flux coefficients must be identified in the pathway. This requires the cloning of individual tocopherol biosynthetic enzymes and a detailed understanding of the molecular and biochemical regulation of individual steps of the pathway. In recent years, several genes encoding enzymes directly or indirectly involved in tocopherol biosynthesis have been cloned and overexpressed in plants to test whether they are limiting for

tocopherol synthesis in various tissues (Garcia et al., 1997; Norris et al., 1998; Shintani and DellaPenna, 1998; Garcia et al., 1999; Collakova and DellaPenna, 2001; Estevez et al., 2001; Schledz et al., 2001; Savidge et al., 2002; Tsegaye et al., 2002).

Precursor feeding studies with safflower cell cultures suggested that levels of HGA, PDP, or both might be limiting for flux through the tocopherol biosynthetic pathway (Furuya et al., 1987). HPPD produces HGA from HPP and has been cloned and characterized from a variety of plants (Garcia et al., 1997; Norris et al., 1998; Garcia et al., 1999). Overexpression of HPPD in *Arabidopsis* leaves and seeds resulted in a 10-fold increase in HPPD specific activity, but only a 10 to 30% increase in total tocopherol levels (Tsegaye et al., 2002). This result may be due to enhanced degradation of HGA in transgenics or to a low flux coefficient of HPPD in the tocopherol pathway (Tsegaye et al., 2002). Plastidic phytol levels may also be limiting for tocopherol synthesis as feeding phytol to safflower cell cultures stimulated tocopherol levels to an even higher extent than HGA (Furuya et al., 1987). Other studies suggest that DXP pathway-derived IPP, which is used for the synthesis of phytol and PDP, may limit isoprenoid synthesis in *Arabidopsis* chloroplasts (Estevez et al., 2001). Constitutive overexpression of the first enzyme of the DXP pathway, DXP synthase (DXPS) in *Arabidopsis* leaves resulted in elevated levels of several plastidic isoprenoids including tocopherols, which were increased up to 2-fold relative to wild type (Estevez et al., 2001).

Arabidopsis leaves accumulate predominantly α-tocopherol, while the major tocopherol present in *Arabidopsis* seeds is γ-tocopherol (Shintani and DellaPenna, 1998). This difference was shown to be the result of low seed γ-TMT activity as overexpression of γ-TMT in *Arabidopsis* seeds led to the conversion of more than 95% of the γ-tocopherol to α-tocopherol. In these experiments, total tocopherol levels remained unchanged in the transgenic seed. These results indicated that γ-TMT was a key enzyme controlling seed tocopherol composition, but had no effect on flux through the pathway (Shintani and DellaPenna, 1998).

Homogentisate phytyltransferase is one of the most recent tocopherol biosynthetic enzyme to be cloned and characterized (Collakova and DellaPenna, 2001; Schledz et al., 2001; Savidge et al., 2002). Homogentisate phytyltransferase catalyzes the committed step of tocopherol biosynthesis (Figure 13), making it a likely candidate for an enzyme with a high flux coefficient. To test whether HPT activity is limiting for tocopherol synthesis in different tissues, HPT was overexpressed in *Arabidopsis* leaves and seeds and the resulting transgenic lines were characterized at both the molecular and biochemical levels. In addition, simultaneous overexpression of HPT and γ-TMT in *Arabidopsis* leaves and seeds was performed to determine whether the transgenic phenotypes conferred by these two genes were additive.

Results

Biochemical And Molecular Characterization of Wild Type And 35S::*HPT1* Plants

Using a genomics-based approach, we have recently cloned *HPT1*, the gene encoding HPT in *Arabidopsis*, which catalyzes the condensation of HGA and PDP in tocopherol synthesis (Collakova and DellaPenna, 2001). Because both HGA and PDP are substrates for other prenyltransferases (Threlfall and Whistance, 1971; Schulze-Siebert et al., 1987; Oster et al., 1997) and HPT activity is low in plant chloroplasts, we hypothesized that HPT could be a highly regulated enzyme with a high flux coefficient (Collakova and DellaPenna, 2001). To assess whether HPT activity is limiting for tocopherol synthesis in different tissues, the enzyme was expressed under the control of the Cauliflower Mosaic Virus 35S rRNA (CaMV 35S) promoter in *Arabidopsis*. Sixty-six independent primary transformant lines (35S::*HPT1*) were generated and analyzed (data not shown). 35S::*HPT1* lines showing antibiotic resistance segregation ratios consistent with a single insertion locus and exhibiting leaf tocopherol levels higher than wild type were taken to homozygosity.

To address the question of whether HPT activity was limiting for tocopherol synthesis in photosynthetic tissue, tocopherol composition and levels were analyzed in leaves of wild type and homozygous 35S::*HPT1* lines. Plants overexpressing *HPT1* contained 3 to 4.4-fold higher total tocopherol levels than wild type that were highly significant ($p < 0.01$, Figure 14). Both wild type and 35S::*HPT1* leaves contained predominantly α-tocopherol, though γ-tocopherol levels were also elevated in 35S::*HPT1*. Wild type leaves accumulated α-tocopherol at 19.1 ± 1.5 pmol mg^{-1}, while α-tocopherol levels in leaves of 35S::*HPT1* ranged between 55 and 80 pmol mg^{-1}. Leaf γ-tocopherol content was 0.6 ± 0.1 pmol mg^{-1} in wild type and 1 - 9 pmol mg^{-1} in *HPT1* overexpressers. The levels of β- and δ-tocopherols in both wild type and 35S::*HPT1* were below detection. MPBQ and DMPBQ, prenylquinol intermediates in tocopherol biosynthesis, were not detectable in wild type and 35S::*HPT1* leaves (data not shown). These results suggest that

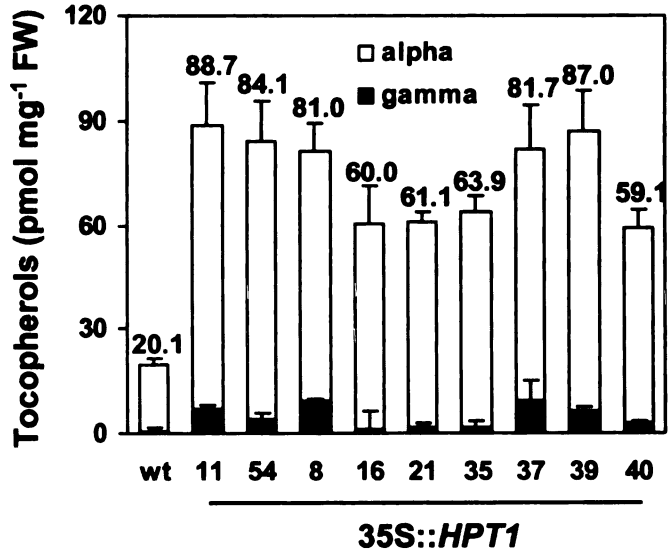


Figure 14. Tocopherol composition and levels in leaves of 4-week old wild type and homozygous 35S::*HPT1 Arabidopsis* plants. Thirty mg of leaf tissue was extracted and individual tocopherols were separated and quantified by reverse phase HPLC. Each line is represented as an average ± SD of tocopherol levels in 3 plants analyzed in duplicate. Total tocopherol levels in pmol mg^{-1} leaf are indicated above the error bar of each line. Total tocopherol levels of 35S::*HPT1* lines were significantly higher than wild type levels ($p < 0.01$). β- and δ-tocopherols were not detected.

MPBQ MT and TC are not limiting activities for tocopherol synthesis in leaf tissue. Two independent transgenic lines exhibiting relatively high α- and γ-tocopherol levels, 35S::*HPT1*-11 and -54 (Figure 14), were selected for subsequent analyses.

To confirm that the increased tocopherol levels in transgenic lines were due to elevated HPT activity resulting from overexpression of *HPT1*, HPT mRNA levels and specific activity were measured in wild type and transgenic lines. Real time PCR showed that HPT mRNA levels were extremely low in wild type and 20- to 100-fold higher in 35S::*HPT1* lines (data not shown). Using isolated chloroplasts, prenyltransferase assays demonstrated that HPT specific activity in 2, 4, and 6 week old wild type plants was also very low (0.11 - 0.34 pmol h^{-1} mg^{-1} protein) and that transgenic lines had four- to ten-fold higher HPT specific activity than the corresponding wild type plants (Table III). In general, the highest HPT specific activity in both wild type and 35S::*HPT1* was observed

Table III. *HPT specific activity in chloroplasts isolated from 2, 4, and 6 week old wild type and 35S::HPT1-11 and -54 leaves.* HPT specific activity was determined using radiolabeled HGA and unlabeled PDP and expressed as an average ± SD of 2 - 3 independent experiments performed in triplicate. Values in parentheses represent fold increases in the specific activity of transgenics compared to wild type plants of the corresponding age. HPT specific activity in leaves of both 35S::*HPT1* overexpressers was significantly higher than wild type ($p < 0.05$). Though there was a general trend of HPT specific activity decreasing in older relative to younger plants in each line, this was only statistically significant between 2 and 4 week old wild type plants ($p < 0.01$)*.

Plant age	Specific activity in pmol h^{-1} mg^{-1} protein (fold change)		
[weeks]	wild type	35S::HPT1-11	35S::HPT1-54
2	0.34 ± 0.06*	1.69 ± 0.39 (5.1)	1.36 ± 0.11 (4.1)
4	0.11 ± 0.03*	1.14 ± 0.48 (10.3)	0.84 ± 0.27 (7.6)
6	0.15 ± 0.10	0.71 ± 0.41 (4.7)	0.75 ± 0.36 (5.0)

in chloroplasts isolated from young, 2 week old plants. The specific activity of both wild type and *HPT1* overexpressing lines decreased approximately by 2-fold by 6 weeks of age. However, the elevated specific activity in transgenics relative to wild type was maintained

throughout plant development (Table III). These results are consistent with previous findings that HPT activity is highest in young plants and diminishes with age (Hutson and Threlfall, 1980).

In addition to HPT, the enzymes shown in Figure 13, HPPD, HGAD, GGPS1, GGDR, and γ-TMT are also directly or indirectly involved in tocopherol synthesis. Given the elevated levels of tocopherols in 35S::*HPT1* lines, it is possible that the expression of other enzymes involved in tocopherol synthesis might also be indirectly affected in the transgenics. To test this hypothesis, the mRNA levels of all available genes of the pathway in wild type and 35S::*HPT1* leaves were measured. With the exception of the 20- to 100-fold increase in HPT mRNA levels in 35S::*HPT1*, no significant differences in the expression of these other tocopherol-related genes were observed between wild type and 35S::*HPT1 Arabidopsis* plants (data not shown).

Effects of *HPT1* Overexpression on Tocopherol Levels in *Arabidopsis* Seed

Molecular and biochemical analyses of the tocopherol biosynthetic pathway in leaves of wild type and 35S::*HPT1* transgenic plants demonstrated that HPT expression and activity are limiting for tocopherol synthesis in *Arabidopsis* leaf tissue. To test whether HPT is also limiting in seeds, total and individual tocopherol levels were determined in seeds of wild type and homozygous 35S::*HPT1* plants. Total tocopherol levels in wild type *Arabidopsis* seeds were 838 ± 18 pmol mg^{-1} seed. In 8 of the 9 homozygous 35S::*HPT1* lines shown in Figure 3, 35S::*HPT1*-16 being the exception, highly significant increases ($p < 0.001$) in total seed tocopherol content were observed. The best *HPT1* overexpressing lines (11 and 54) contained up to 37% higher seed tocopherol levels than wild type, corresponding to an additional 310 pmol tocopherols mg^{-1} seed (Figure 15). The majority of this increase was due to elevation of γ-tocopherol levels, the predominant tocopherol in wild type *Arabidopsis* seeds, though in some transgenic lines, δ-tocopherol levels also

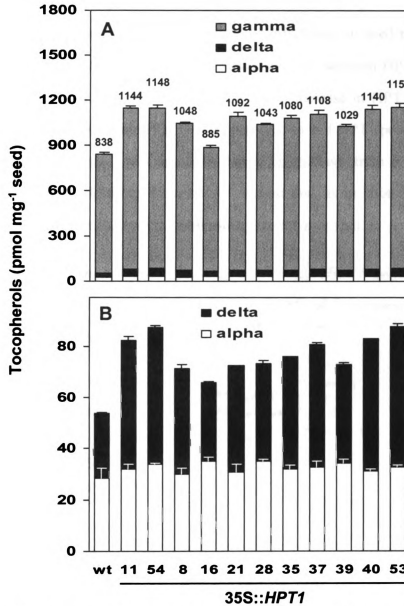


Figure 15. Tocopherol composition and levels in seed of wild type and homozygous 35S::*HPT1 Arabidopsis* plants. Seed was extracted and analyzed for tocopherols by normal phase HPLC. Tocopherol levels are expressed as an average ± SD of 3 analyses per each line in a representative experiment. Total tocopherol levels in pmol mg^{-1} seed are indicated above the error bar of each line. *Arabidopsis* seeds accumulate predominantly γ-tocopherol, which increased up to 40% in 35S::*HPT1* compared to wild type. The levels of α-tocopherol did not change significantly, whereas δ-tocopherol increased up to 2-fold in some transgenic lines. β-Tocopherol was not detected. With the exception of 35S::*HPT1*-16, statistical significance for total tocopherol levels of all transgenic lines relative to wild type was $p < 0.001$.

doubled. α-Tocopherol levels did not change significantly (Figure 15).

We have previously demonstrated that 35S::*HPT1*-11 and -54 have significantly higher HPT specific activity relative to wild type (Table III). HPT specific activity in seeds was also determined to assess whether the observed differences in seed tocopherol levels between wild type and 35S::*HPT1* lines were correlated with increased HPT activity. Both transgenic lines (-11 and -54) exhibited nearly a 4-fold increase in HPT specific activity relative to wild type seeds (Table IV). When compared to leaf HPT specific activities, the HPT specific activity in seeds was similar to that of chloroplasts from 6-week old plants (Tables III and IV). Collectively, these results suggest that, as in photosynthetic tissues, HPT activity is limiting for tocopherol synthesis in *Arabidopsis* seed.

Table IV. *HPT specific activity in seed of wild type and 35S::HPT1 overexpressers (lines #11 and 54).* Protein extracts from dry *Arabidopsis* seeds were used to determine HPT specific activity as described. Values are an average ± SD of 3 independent experiments performed in triplicate. HPT specific activity in seed of both 35S::*HPT1* overexpressers was significantly higher than wild type ($p < 0.01$).

Line	HPT specific activity [pmol h^{-1} mg^{-1} protein]
wild type	0.17 ± 0.05
35S::HPT1-11	0.66 ± 0.10
35S::HPT1-54	0.67 ± 0.08

Tocopherol Analysis of *Arabidopsis* Leaves And Seeds Overexpressing Both *HPT1* And *γ-TMT* Transgenes

Constitutive *HPT1* overexpression in *Arabidopsis* led to a maximal 4.4-fold and 40% increase in total leaf and seed tocopherol levels, respectively. The increase in leaves was primarily due to an elevation in α-tocopherol levels and to a lesser extent γ-tocopherol. In *Arabidopsis* seed, γ-tocopherol was the major contributor to the 40% increase (Figure 15). This is a result of a limitation in seed γ-TMT activity, which catalyzes methylation of

γ- and δ-tocopherols to α- and β-tocopherols, respectively, and this metabolic block can be overcome by overexpressing γ-TMT during seed development (Shintani and DellaPenna, 1998).

To test whether the traits conferred individually by γ-TMT and HPT overexpression are additive, individual 35S::*HPT1* and 35S::*γ-TMT* lines were crossed, double homozygotes selected, and their leaf and seed tocopherol content and composition determined. The levels of individual tocopherols in leaves of wild type, double overexpressers, and the corresponding single transgene homozygous parent lines are shown in Figure 16. The total leaf tocopherol levels of 35S::*HPT1*–11 and –54 in Figure 16 are somewhat lower than in Figure 14. Leaf tocopherol content is extremely sensitive to environmental stimuli and plant developmental stage (Munne-Bosch and Alegre, 2002a), and these differences are consistent with the normal range of inter-experiment variation observed in tocopherol content (data not shown).

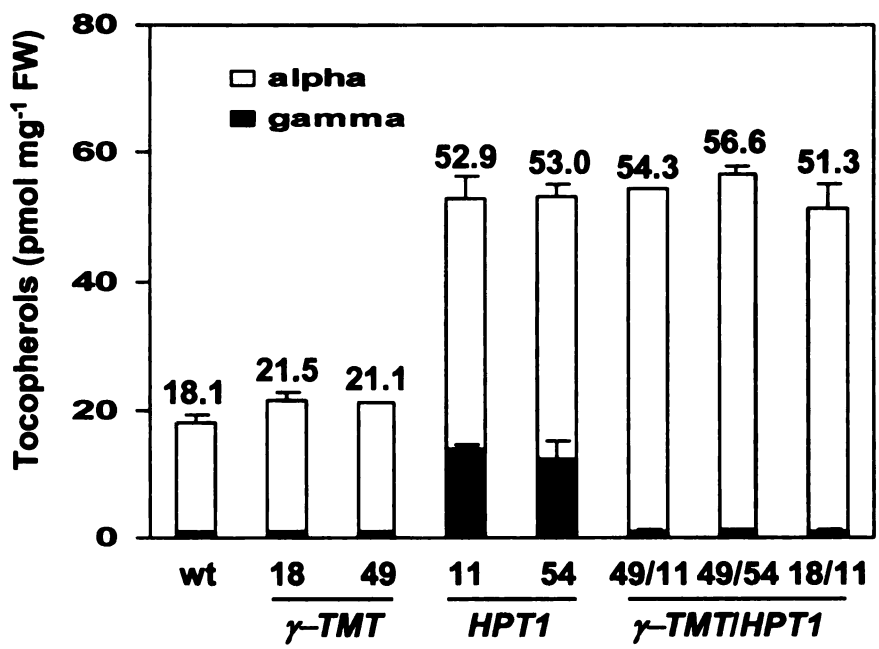


Figure 16. Tocopherol composition and levels in leaves of 5-week old wild type, 35S::*γ-TMT*, 35S::*HPT1*, and double 35S::*γ-TMT*/35S::*HPT1* overexpressers. Leaf tissue (~ 70 mg) was extracted and individual tocopherols were separated and quantified by normal phase HPLC. Each line is represented as an average ± SD from 3 plants. Total tocopherol levels of 35S::*HPT1* and 35S::*γ-TMT*/35S::*HPT1* plants were significantly higher than wild type levels ($p < 0.005$). γ-tocopherol in double transgenics was methylated to α-tocopherol.

There was no significant difference in tocopherol composition or levels between wild type and 35S::*γ-TMT* leaves. However, the leaf γ-tocopherol present in 35S::*HPT1*-11 and -54 was converted to α-tocopherol in 35S::*HPT1*/35S::*γ-TMT* double homozygotes (Figure 16). Similarly, in seed of double overexpressers, nearly the entire pool of γ- and δ-tocopherols was methylated to α- and β-tocopherols, respectively (Figure 17A and B). These data indicate that the traits conferred by each single transgene are additive.

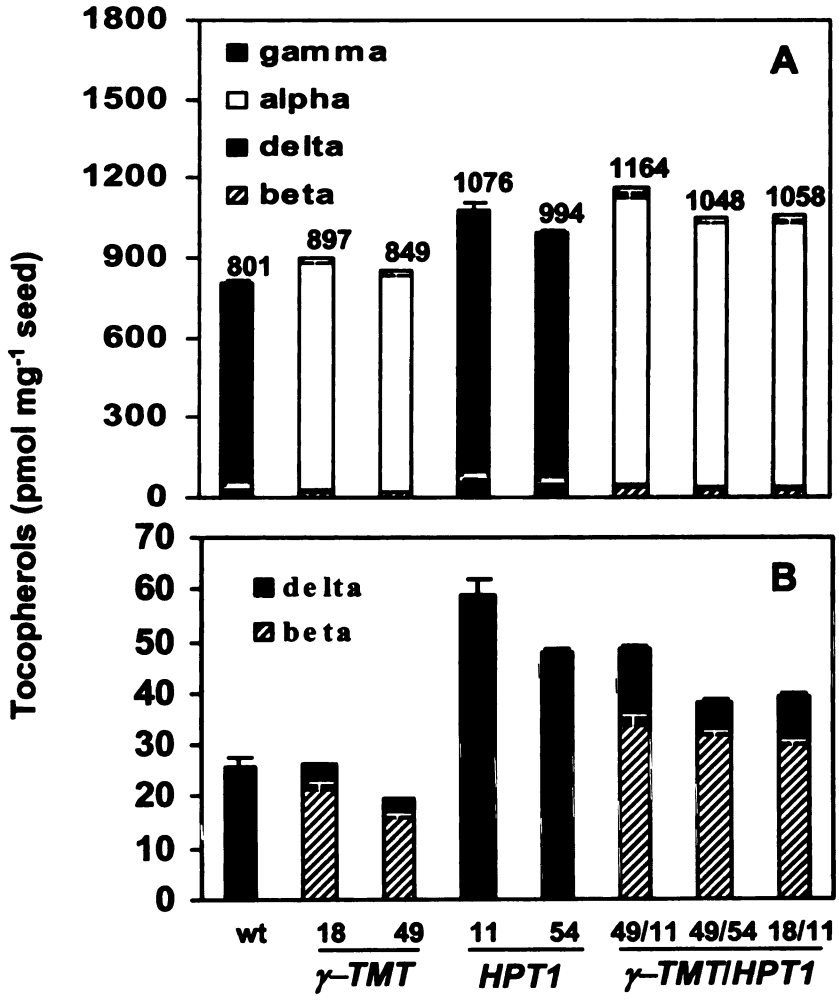


Figure 17. Tocopherol composition and levels in seed of wild type, 35S::*γ-TMT*, 35S::*HPT1*, and 35S::*γ-TMT*/35S::*HPT1* overexpressers. A, α-, β-, γ-, and δ- tocopherols, B, β- and δ- tocopherols. Tocopherol analysis of mature dry seed was performed as described in *Materials and Methods*. Each line is presented as an average of 3 independent measurements of 3 replicates. Total tocopherol levels of 35S::*HPT1* and 35S::*γ-TMT*/35S::*HPT1* lines were significantly higher than wild type ($p < 0.05$). The majority of γ- and δ- tocopherols was converted to α- and β- tocopherols in 35S::*γ-TMT*/35S::*HPT1* overexpressers.

Effects of Increased Tocopherol Levels on Chlorophyll, Carotenoid, And Plastoquinone-9 Content in 35S::*HPT1*

In plastids, the HGA and IPP (as GGDP or PDP) used in tocopherol synthesis are also utilized in the synthesis of chlorophylls, carotenoids, and plastoquinone-9 (PQ-9) (Threlfall and Whistance, 1971; Schulze-Siebert et al., 1987; Oster et al., 1997). Several studies suggest that the plastidic GGDP pool originating from the DXP pathway may be limiting for isoprenoid synthesis (Fray et al., 1995; Shewmaker et al., 1999; Estevez et al., 2001). Increased flux through the tocopherol pathway may therefore have an effect on the levels of other compounds formed from GGDP or HGA. To test this hypothesis, levels of HGA-derived PQ-9 and two major GGDP-derived compounds, chlorophylls and carotenoids, were measured in leaves and seeds of wild type and 35S::*HPT1*-11 and -54. No statistically significant differences in total chlorophyll, carotenoids, or PQ-9 levels were observed between wild type and transgenic plants (Table V). In leaf, the average chlorophyll

Table V. *Total chlorophyll, carotenoids, and PQ-9 levels in wild type and 35S::HPT1 Arabidopsis leaves and seeds.* In leaves, chlorophyll and carotenoid levels were determined in 90% methanol as described previously (Lichtenthaler, 1987). Twenty mg of seed was extracted and pigments were analyzed by reverse phase HPLC. Lutein comprised > 95% of total seed carotenoids (data not shown) and its levels were taken as total carotenoid levels. Chlorophylls and PQ-9 were not detected (nd) in dry *Arabidopsis* seed. Chlorophyll and carotenoid levels in 35S::*HPT1* leaves and seeds were comparable to wild type levels. Pigment levels are an average ± SD of 3 measurements.

	Leaf content			Seed content	
Line	Chlorophyll (nmol mg^{-1})	Carotenoid (nmol mg^{-1})	PQ-9 (pmol mg^{-1})	Chlorophyll and PQ-9	Carotenoid (pmol mg^{-1})
wild type	1.00 ± 0.06	0.43 ± 0.04	82 ± 8	nd	30.3 ± 0.2
HPT1-11	1.01 ± 0.10	0.45 ± 0.04	80 ± 5	nd	28.3 ± 0.6
HPT1-54	0.98 ± 0.08	0.43 ± 0.05	83 ± 4	nd	35.9 ± 0.2

and carotenoid levels were ~ 1 nmol mg^{-1} and 0.44 nmol mg^{-1} tissue, respectively. In wild type and 35S::*HPT1*, PQ-9 levels were ~ 82 pmol mg^{-1} leaf tissue. In seed, chlorophylls and PQ-9 were not detected, while carotenoid levels were ~ 31 pmol mg^{-1} seed (Table V).

Discussion

Homogentisate phytyltransferase is a likely candidate for an enzyme with a high flux coefficient in tocopherol biosynthesis. HPT utilizes HGA and PDP to catalyze formation of the first committed prenylquinol intermediate in the tocopherol biosynthetic pathway, MPBQ. Because HGA and PDP are also used in the synthesis of plastoquinones, phylloquinones, and chlorophylls (Threlfall and Whistance, 1971; Schulze-Siebert et al., 1987; Oster et al., 1997) and HPT acts at the branch-point for these plastidic prenyllipid pathways, it could be a key enzyme controlling flux through the tocopherol pathway. To test this hypothesis, the *Arabidopsis HPT1* cDNA was constitutively expressed under the control of the CaMV 35S promoter. Significant increases in HPT specific activity and total tocopherol levels were observed in both leaf and seed of 35S::*HPT1* plants compared to wild type.

In leaves, *HPT1* overexpression resulted in a 4.4-fold increase in total tocopherols, the highest percentage increase in engineering leaf tocopherol content yet reported in *Arabidopsis*. Three other enzymes affecting tocopherol biosynthesis, DXPS, HPPD, and γ-TMT, have been overexpressed in *Arabidopsis* leaves with varying degrees of success in altering leaf tocopherol levels. Estevez et al. (2001) reported significantly elevated levels of several plastidic isoprenoids, including a 2-fold increase in tocopherols, when DXPS was constitutively expressed in *Arabidopsis* leaves. Only a 30% increase in total leaf tocopherol content was observed in transgenic *Arabidopsis* lines overexpressing HPPD (Tsegaye et al., 2002). Constitutive overexpression of γ-TMT in *Arabidopsis* had no effect on tocopherol composition or total tocopherol levels in leaves (Shintani and DellaPenna, 1998). With the

exception of γ-TMT, it appears that HPT, DXPS, and HPPD activities all limit tocopherol accumulation to some degree in wild type *Arabidopsis* leaves.

In seeds, *HPT1* overexpression resulted in accumulation of 37% more total tocopherols than wild type, which corresponds to an additional 310 pmol mg^{-1} seed versus 70 pmol mg^{-1} tissue for the 4.4 fold increase in leaf tocopherol levels. The increase in the absolute tocopherol levels in leaf tissue appears less significant than in seed until one considers the relative water content of the two tissues. Because *Arabidopsis* leaves contain 91% water, total tocopherol levels increased from 230 to 1010 pmol mg^{-1} dry weight in wild type and 35S::*HPT1* leaves, respectively. In contrast, because mature *Arabidopsis* seed contain 11% water, total tocopherol content increased from 940 to 1290 pmol mg^{-1} dry weight in wild type and 35S::*HPT1* leaves, respectively. Thus, overexpressing *HPT1* elevated the HPT specific activity of seeds and leaves to similar levels, leading to similar final levels of total tocopherols in the two tissues but a proportionately higher impact on tocopherol accumulation in leaves. These data suggests that HPT has a relatively high flux coefficient in both leaves and seed, though indirect evidence suggests HPT activity is still limiting for tocopherol synthesis in 35S::*HPT1* seeds. Savidge et al. (2002) reported that *HPT1* overexpression driven by the strong, seed-specific napin promoter resulted in a 75% increase in seed tocopherol levels relative to wild type, which would correspond to approximately 1650 pmol mg^{-1} dry weight. Although HPT activity was not determined in these studies (Savidge et al., 2002), the higher tocopherol levels obtained are consistent with reports that the CaMV 35S promoter is less effective in developing seed than the napin promoter (Eccleston and Ohlrogge, 1998). Despite these differences, both studies demonstrate that HPT activity is limiting for tocopherol synthesis in wild type *Arabidopsis* seed and is a key enzyme regulating tocopherol accumulation in plant tissues.

By alleviating the HPT limitation in the tocopherol pathway, it is possible that other enzyme activities downstream of HPT (TC, MPBQ MT, or γ-TMT) or their substrates might become limiting in 35S::*HPT1* plants. A limitation in TC activity would result in

accumulation of DMPBQ, while a limitation in MPBQ MT activity would lead to the increased levels of β- and δ-tocopherols. If both enzymes were limiting, MPBQ in addition to DMPBQ and β- and δ-tocopherols would accumulate. None of these compounds were detected in leaves of wild type or 35S::*HPT1* plants. In 35S::*HPT1* seed, δ-tocopherol levels were doubled, but still accounted for less than 5% of the total tocopherol pool. These data indicate that neither TC nor MPBQ MT are limiting in 35S::*HPT1* seeds and leaves. Recent radiotracer studies using HGA and PDP also suggested that TC and MPBQ MT are not limiting for α-tocopherol synthesis in chromoplasts of yellow pepper (Arango and Heise, 1998).

In wild type *Arabidopsis* seeds, but not in leaves, γ-TMT activity has previously been shown to be limiting for α-tocopherol synthesis (Shintani and DellaPenna, 1998). In leaves of 35S::*HPT1*, γ-tocopherol levels were up to 15-fold higher than wild type, while a 37% increase in γ-tocopherol levels was observed in 35S::*HPT1* seeds. To test whether γ-TMT or S-adenosyl-L-methionine (SAM) were limiting in 35S::*HPT1*, the previously characterized 35S::*γ-TMT* lines (Shintani and DellaPenna, 1998) were crossed to 35S::*HPT1* lines and the resulting double homozygous transgenic progeny selected and analyzed for tocopherol content and composition. Overexpression of γ-TMT in the 35S::*HPT1* background resulted in almost complete methylation of the excess γ-tocopherol to α-tocopherol in both leaves and seeds. The persistence of small quantities of γ-tocopherol in 35S::*γ-TMT* and the double transgenics may reflect a distinct functional role for γ-tocopherol or indicate that a fraction of the γ-tocopherol pool is not accessible for further methylation. Collectively, these data indicate that the biochemical phenotypes conferred individually by HPT and γ-TMT overexpression are additive in both seeds and leaves, and that SAM is not limiting for α-tocopherol synthesis in 35S::*HPT1* lines.

Altering flux through a metabolic pathway can have unanticipated effects on compounds synthesized by biochemically related pathways, especially when some of the substrates are shared among these pathways (Fray et al., 1995; Shewmaker et al., 1999;

Estevez et al., 2001). Overexpression of DXPS in *Arabidopsis* leaves increased the levels of several isoprenoid-derived compounds including carotenoids, chlorophylls, and tocopherols, suggesting that availability of chloroplastic GGDP, a common precursor in their biosynthesis, may be limiting in wild type plants (Estevez et al., 2001). In canola seed, overexpression of phytoene synthase led to a 50-fold increase in carotenoids and a 50% decrease in tocopherol levels relative to wild type (Shewmaker et al., 1999). Constitutive overexpression of the GGDP-utilizing carotenoid biosynthetic enzyme phytoene synthase in tomato caused chlorosis and dwarfism, most likely by redirecting the limited GGDP pool from the gibberellin and chlorophyll biosynthetic pathways to carotenoid synthesis (Fray et al., 1995). In light of these reports, the effects of *HPT1* overexpression on the levels of other HGA- and GGDP-derived compounds in *Arabidopsis* tissues were also assessed.

Although we did not directly measure gibberellin levels in leaves of 35S::*HPT1* plants, gibberellin metabolism appeared to be unaffected as no obvious differences in growth, flowering time, dormancy, or physical appearance were observed relative to wild type (data not shown). PQ-9, chlorophyll, and carotenoid levels in leaf and seed were also unaffected by *HPT1* overexpression in *Arabidopsis*. One molecule of GGDP is used in the synthesis of each chlorophyll molecule, while carotenoids contain two molecules of GGDP or "GGDP equivalents". The total chlorophyll and carotenoid content in leaves corresponds to 1900 pmol mg^{-1} tissue of "GGDP equivalents"; the 70 pmol mg^{-1} increase in incorporation of "GGDP equivalents" into leaf tocopherols in 35S::*HPT1*-11 is negligible by comparison. In mature wild type *Arabidopsis* seeds, this composition is reversed, with tocopherols predominating (up to 1000 pmol mg^{-1} seed) and carotenoids being a minor component (~ 30 pmol mg^{-1} seed). The 310 pmol mg^{-1} seed increase in "GGDP equivalents" incorporated into tocopherols in 35S::*HPT1*-11 seed is highly significant relative to the level of carotenoids, but apparently did not affect GGDP availability for carotenoid synthesis. This increased demand for GGDP in seeds may be compensated in part by increased flux through the DXP pathway. A similar phenomenon

was observed in *Brassica* seed engineered for elevated carotenoid synthesis (Shewmaker et al., 1999). There seems to be a relatively high flexibility for flux through the seed DXP pathway leading to GGDP, likely by feedback regulation.

Outlook for Metabolic Engineering of Vitamin E Levels in Crops

Tocopherols are essential components of the human diet. Due to the relatively low levels of α-tocopherol, the most effective form of vitamin E, in most commonly consumed vegetables and oils (Eitenmiller, 1997; Bramley et al., 2000; Ching and Mohamed, 2001), α-tocopherol is limiting in the average American diet (Grusak and DellaPenna, 1999; Horwitt, 2001). It is recommended that 35 μmol (15 mg) of α-tocopherol be consumed daily (Food and Nutrition Board, 2000). Although *Arabidopsis* is not of direct agricultural importance, it has become a model for metabolic engineering of the tocopherol pathway and can be used to extrapolate the impact of engineering on dietary vitamin E intakes. In Table VI we estimate the vitamin E activity of wild type and various transgenic lines reported in this study in terms of the α-tocopherol equivalents (α-TE) per "serving size" of 100 g leaves

Table VI. *Vitamin E activity in wild type, single, and double overexpressers.* Vitamin E activity was calculated for each line using the absolute levels of individual tocopherols from Figures 16 and 17 and the relative biological activities of R,R,R-tocopherols. One mg of α-, β-, γ-, and δ-tocopherol corresponds to 1, 0.5, 0.1, and 0.03 mg α-TE, respectively (Food and Nutrition Board, 2000).

	Vitamin E activity (mg α-TE per 100 g tissue)	
Line	leaves	seeds
wild type	0.75	3.9
35S::HPT1	1.81	5.2
35S::γ-TMT	0.87	35.6
35S::HPT1/35S::γ-TMT	2.39	47.4

and seeds (for definition of α-TE see Table VI). In leaves of 35S::*HPT1*/35S::*γ-TMT* double overexpressers, which accumulate predominantly α-tocopherol, vitamin E activity was 3.2-fold higher than wild type. Because γ-tocopherol is the major tocopherol in mature wild type *Arabidopsis* seed, the vitamin E activity is relatively low (3.9 mg α-TE) despite the high levels of total tocopherols. In seed of 35S::*HPT1*/35S::*γ-TMT* lines, α-tocopherol is the major tocopherol and the vitamin E activity increased 12-fold relative to wild type (Table VI). Similar increases in seed vitamin E activity are anticipated for genetically engineered crops (Grusak and DellaPenna, 1999).

Materials And Methods

Chemicals. Tocopherol standards and tocol were purchased from Matreya (Pleasant Gap, PA). Phytyl diphosphate was a gift from Dr. Stephanie Sen (Purdue University, West Lafayette, IN). [U-^{14}C]HPP was prepared from L-[U-^{14}C]tyrosine (sp. activity 464 mCi mmol^{-1}, Amersham, Arlington Heights, IL) according to Schulz et al. (1993). Minor modifications were introduced to obtain more concentrated [U-^{14}C]HPP. Briefly, L-[U-^{14}C]tyrosine was dried under a stream of nitrogen and dissolved in the original volume of phosphate buffer (0.5 M, pH 6.5). Catalase (Roche, Indianapolis, IN) and L-amino acid oxidase type IV (Sigma, St. Louis, MO) were both added to the final concentration 0.4 mg mL^{-1}. After 2 hours of incubation at room temperature, [U-^{14}C]HPP was purified on an ion exchange column (Dowex, Sigma) equilibrated with 0.1 N HCl and used immediately for prenyltransferase assays.

Generation of *HPT1* overexpressing lines. A cDNA encoding HPT was excised from pSK*HPT1* (Collakova and DellaPenna, 2001) by digestion with *Eco*RI and *Kpn*I and sub-cloned into *Eco*RI and *Kpn*I digested pART7 (Gleave, 1992). The resulting construct was mobilized by *Not*I digestion and ligation into the pART27-based vector pMLBART (Gleave,

1992), which contains the *bar* gene for selection of transformed plants. This construct was introduced into *Agrobacterium tumafaciens* and used to transform wild type *Arabidopsis* plants (ecotype Columbia) by the floral dip method (Clough and Bent, 1998) to obtain 35S::*HPT1* T_0 sense plants.

Sixty-six independent transformants were selected by glufosinate (120 mg L^{-1}) resistance and analyzed for tocopherol content. Seeds of lines exhibiting elevated leaf tocopherol levels were harvested and subjected to segregation analysis. All T_2 35S::*HPT1* transgenic plants segregating 3:1 for BASTA resistance were carried through the next generation and plants homozygous for BASTA resistance were selected for further experiments. Plants overexpressing *γ-TMT* (Shintani and DellaPenna, 1998) under the control of 35S CaMV promoter were crossed with 35S::*HPT1* and double homozygotes were selected based on their dual resistance to kanamycin and BASTA. Transgenic lines, 35S::*HPT1*-11 and -54 and 35S::*γ-TMT*-18 and -49 were used for these crosses.

Prenyllipid analyses of transgenic plants. For leaf prenyllipid analyses, plants were grown in a 16-hour photoperiod (70 - 100 μE) at 22/19 °C day/night cycle for 4 to 5 weeks. For tocopherol analysis, lipids from 30 to 35 mg of leaf tissue or 10 to 13 mg of seeds were extracted in the presence of butylated hydroxytoluene (2 mg mL^{-1}) to prevent tocopherol degradation (Bligh and Dyer, 1959). Tocol was used as an internal standard. For leaf analysis, tocopherols were separated on a reverse phase HPLC (C_{18}, ODS2, 4.6 X 250 mm, Column engineering, Ontario, CA; Shimadzu VP HPLC system, Japan) using an isocratic solvent system of 5% (v/v) isopropanol in methanol at 2 mL min^{-1}. For leaf and seed analyses, where separation of β- and γ-tocopherols was required, a normal phase HPLC system (LiChrosorb 5 Si60A 4.6 X 250 mm Silica column, Column engineering, Ontario, CA and HP 1100 series HPLC system, Agilent Technologies, Palo Alto, CA) and a 10-minute isocratic method using 17% di-isopropyl ether in hexane (v/v) at 42 °C at 2 mL min^{-1} were utilized. Tocopherols were detected by fluorescence at 290 nm excitation and 325 nm

emission. Chlorophylls, carotenoids, and PQ-9 were analyzed as described (Collakova and DellaPenna, 2001). For PQ-9 analysis, 50 mg of leaves or 15 mg of seeds was used for extractions. Detection limit for PQ-9 was 0.04 pmol in seed.

Real time PCR. To obtain sufficient amounts of tissue, plants (wild type and 35S::*HPT1* #11 and 54) were grown at a 10-hour photoperiod at 75 - 100 μE for 6 weeks. Tissue from 6 representative plants was harvested 2 - 3 hours after the start of the light cycle and immediately frozen in liquid nitrogen. Total RNA was isolated and any contaminating genomic DNA was removed by treatment with RQ1-RNase free DNase (Promega, Madison, WI). Ten μg of total RNA was reverse transcribed to generate cDNA in two 50-μL reactions for each sample using a TaqMan kit according to manufacturer recommendations (Applied Biosystems, Foster City, CA). An aliquot of cDNA corresponding to 100 - 200 ng of total RNA was used in each TaqMan real-time PCR assays (Applied Biosystems). Elongation factor EF1α was used to normalize RNA concentrations. Standard curves were constructed for each gene and used to calculate the corresponding mRNA concentrations. Sequences of the primers and probes used in the real-time PCR assays and their optimal final concentrations are available upon request.

Prenyltransferase assays. Homogentisate phytyl transferase assays were performed using crude chloroplast preparations or seed protein extracts from wild type plants and 35S::*HPT1* #11 and 54 lines grown as described for real-time PCR assays. Chloroplasts were prepared from 25 - 30 g of leaf tissue. Tissue was disrupted using a blender in a buffer containing 0.6 M sorbitol, 0.5 M HEPES pH 8.4, 4 mM EDTA, 4 mM EGTA, 10 mM Na_2CO_3, 0.2% Bovine Serum Albumin (w/v), 1 μM benzamidine (BA), and 5 μM 4-aminocaproic acid (4-ACA). Chloroplasts were filtered through 2 layers of miracloth and centrifuged at 4,000 g for 5 minutes at 4 °C. Crude chloroplasts were washed once in a

buffer containing 50 mM HEPES pH 7.6, 4 mM $MgCl_2$, 1 μM BA, and 5 μM 4-ACA and once more in the same buffer lacking proteinase inhibitors.

Each prenyltransferase reaction (0.1 mL) contained 0 or 100 μM PDP, approximately 5 μM [U- ^{14}C]HPP (1 μCi), 20 μg HPPD protein, and chloroplasts corresponding to about 0.45 – 0.65 mg of protein (0.10 – 0.15 mg of chlorophyll) in HPT reaction buffer (50 mM HEPES pH 7.6, 4 mM $MgCl_2$, 1 mM KF, 0.2% (v/v) TWEEN 80, and 50 mM potassium ascorbate). Reactions were incubated at room temperature for 2 hours with shaking and quinones were extracted with two volumes of acetone: light petroleum ether (1:1 v/v). After centrifugation, the ether phase was loaded on a 4-mL Silica column (pore size 60 μm, Sigma) and quinones were eluted with 4 volumes of 20% (v/v) diethyl ether in petroleum ether. After drying, 5 mL of scintillation liquid was added to each sample and radioactivity was determined by scintillation counting. Background activities obtained from control reactions containing ^{14}C labeled HGA without exogenous PDP were subtracted from the activities when both substrates were added, which was always at least 3.5-fold higher than the background activities to obtain HPT specific activity. Chloroplasts were extracted with 90% methanol (v/v) and centrifuged to determine chlorophyll concentration (Lichtenthaller, 1987). Proteins present in the remaining pellet were solubilized by NaOH in the presence of SDS and determined by the method of Lowry (Stoscheck, 1990).

In seeds, protein extracts were prepared as follows: Dry mature seeds were ground in 50 mM HEPES (pH 7.6) containing 4 mM $MgCl_2$ and centrifuged. The resulting pellet of water insoluble proteins was resuspended in HPT reaction buffer to a protein concentration of 20 - 30 mg mL^{-1}. HPT assays containing 2 - 3 mg seed protein were performed in a volume of 0.2 mL as described for chloroplasts. Seed HPT specific activities were about 3-fold higher than the corresponding background activities.

CHAPTER 3

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HOMOGENTISATE PHYTYLTRANSFERASE ACTIVITY LIMITS TOCOPHEROL SYNTHESIS IN STRESSED *ARABIDOPSIS THALIANA* LEAVES

Abstract

Tocopherols are amphipathic antioxidants synthesized exclusively by photosynthetic organisms. Tocopherol levels change significantly during plant growth and development and in response to stress, likely as a result of altered expression of pathway-related genes. Homogentisate phytyltransferase (HPT) is a key enzyme limiting tocopherol biosynthesis in unstressed Arabidopsis leaves (Collakova and DellaPenna (2003) Plant Physiol. 131: 632-642). Wild type and transgenic Arabidopsis plants constitutively overexpressing HPT (35S::*HPT1*) were subjected to high light stress (up to 1 mE) for up to 15 days and tocopherol levels, composition, and expression of several tocopherol pathway-related genes were determined. High light stress resulted in an 18- and 8-fold increase in total tocopherol content in wild type and 35S::*HPT1* leaves, respectively, with levels in 35S::*HPT1* being 2- to 4-fold higher than wild type at all experimental time points. Increased total tocopherol levels correlated with elevated HPT mRNA levels and HPT specific activity in 35S::*HPT1* and wild type leaves, suggesting that HPT activity limits total tocopherol synthesis during high light stress. In addition, substrate availability, and expression of pathway enzymes prior to HPT also contribute to increased tocopherol synthesis during stress. The accumulation of high levels of β-, γ-, and δ-tocopherols in stressed tissues suggested that the methylation of phytylquinol and tocopherol intermediates limit α-tocopherol synthesis during stress. Overexpression of γ-tocopherol methyltransferase in the 35S::*HPT1* background resulted in nearly complete conversion of γ- and δ-tocopherols to α- and β-tocopherols, respectively, indicating that γ-tocopherol methyltransferase activity limits α-tocopherol synthesis during high light stress.

Introduction

Tocopherols are a group of lipid soluble antioxidants collectively known as vitamin E that are essential components of animal diets. Dietary vitamin E is required for maintaining proper muscular, immune, and neural function and may be involved in reducing the risk of cancer, cardiovascular disease, and cataracts in humans (Pryor, 2000; Brigelius-Flohe et al., 2002). In plants, tocopherols are believed to protect chloroplast membranes from photo-oxidation and help to provide an optimal environment for the photosynthetic machinery (Fryer, 1992; Munne-Bosch and Alegre, 2002a). Many of the proposed tocopherol functions in animals and plants are related to their antioxidant properties, the most prominent of which is protection of polyunsaturated fatty acids from lipid peroxidation by quenching and scavenging various reactive oxygen species (ROS) including singlet oxygen, superoxide radicals, and alkyl peroxy radicals (Fukuzawa and Gebicky, 1983; Munne-Bosch and Alegre, 2002a).

Tocopherols are only synthesized by photosynthetic organisms and consist of a polar chromanol ring and a 15-carbon lipophilic prenyl chain derived from homogentisic acid (HGA) and phytyl diphosphate (PDP), respectively (Figure 18). In plants, HGA is formed from *p*-hydroxyphenyl pyruvate (HPP) by the cytosolic enzyme HPP dioxygenase (HPPD) (Garcia et al., 1997; Norris et al., 1998; Garcia et al., 1999). Based on radiotracer studies, HPP can originate either from prephenate or tyrosine by the shikimate pathway, but the relative contribution of these two precursors to the total HPP pool is unknown (Threlfall and Whistance, 1971; Fiedler et al., 1982; Lopukhina et al., 2001). In plastids, isopentenyl diphosphate (IPP) derived from the 1-deoxyxylulose-5-phosphate (DXP) pathway (Eisenreich et al., 1998; Lichtenthaler, 1998) is utilized by geranylgeranyl diphosphate synthase 1 (GGPS1) for the synthesis of geranylgeranyl diphosphate (GGDP) (Okada et al., 2000). Three of the four double bonds in the GGDP molecule are reduced to form PDP through partially reduced intermediates by a multifunctional GGDP reductase (GGDR)

(Addlesee et al., 1996; Keller et al., 1998). Alternatively, PDP can be generated from phytol and ATP by a kinase activity present in chloroplast stroma (Soll et al., 1980).

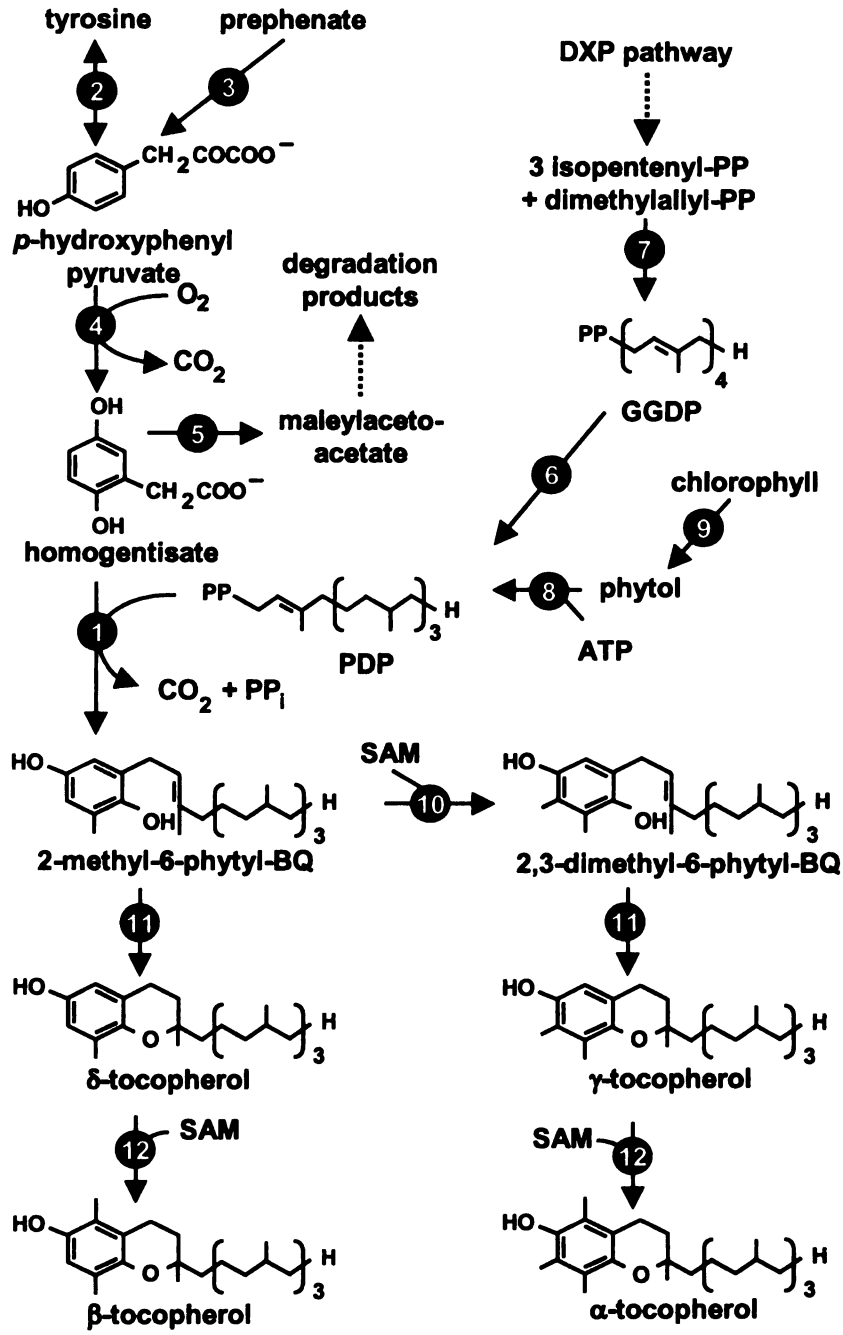

Figure 18. Tocopherol biosynthesis in plants. Dashed arrows represent multiple steps. Enzymes are indicated as circled numbers: 1. homogentisate phytyltransferase, 2. tyrosine transaminase, 3. prephenate dehydrogenase, 4. *p*-hydroxyphenylpyruvate dioxygenase, 5. homogentisate dioxygenase, 6. geranylgeranyl diphosphate reductase, 7. geranylgeranyl diphosphate synthase 1, 8. unspecified kinase, 9. chlorophyllase, 10. 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase, 11. tocopherol cyclase, 12. γ-tocopherol methyltransferase

HPT is a membrane-bound chloroplast enzyme, which catalyzes the committed step of tocopherol biosynthesis, the condensation of HGA and PDP, to form 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) (Soll, 1987; Collakova and DellaPenna, 2001; Savidge et al., 2002). MPBQ can be methylated to 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) by MPBQ methyltransferase (MPBQ MT) (Marshall et al., 1985; Soll, 1987; Shintani et al., 2002). MPBQ and DMPBQ can be cyclized by tocopherol cyclase (TC) to form δ- and γ-tocopherol, respectively (Stocker et al., 1996; Arango and Heise, 1998; Porfirova et al., 2002). The last enzyme of the pathway, γ-tocopherol methyltransferase (γ-TMT), catalyzes methylation of γ- and δ-tocopherol to α- and β-tocopherol, respectively (D' Harlingue and Camara, 1985; Shintani and DellaPenna, 1998).

In plants, tocopherol levels and composition vary in different tissues and fluctuate during development and in response to abiotic stresses. Dry and germinating seeds of many plants accumulate predominantly γ-tocopherol, while α-tocopherol is the major tocopherol in leaves, which may reflect distinct roles of individual tocopherols in these tissues (Bramley et al., 2000; Franzen and Haas, 1991; Shintani and DellaPenna, 1998). Significant increases in leaf α-tocopherol levels are observed during aging and senescing of plants (Rise et al., 1989; Molina-Torres and Martinez, 1991; Tramontano et al., 1992), possibly to protect cellular components from increased oxidative stress (Munne-Bosch and Alegre, 2002c). Enhanced tocopherol accumulation also occurs in response to a variety of abiotic stresses including high light, drought, salt, and cold and may provide an additional line of protection from oxidative damage (Havaux et al., 2000; Munne-Bosch and Alegre, 2002a).

While there is a growing body of knowledge about the individual enzymes required for tocopherol biosynthesis in plants, the mechanisms that regulate the overall pathway and result in differential tocopherol content and composition during plant development or stress remain poorly understood. Regulation of tocopherol biosynthesis in senescing and stressed plants may occur at multiple steps of the pathway. HPPD activity limits tocopherol

synthesis in non-stressed Arabidopsis plants (Tsegaye et al., 2002) and HPPD mRNA levels are up-regulated in senescing barley leaves (Klebler-Janke and Krupinska, 1997). Similarly, various biotic and abiotic stresses elevate tyrosine aminotransferase (TAT) mRNA and protein levels and enzyme activity in Arabidopsis (Lopukhina et al., 2001; Sandorf and Hollander-Czytko, 2002). Whether other steps of the tocopherol pathway are also involved in the regulation of tocopherol biosynthesis during stress remains to be determined.

It has been recently demonstrated that HPT activity limits tocopherol synthesis in non-stressed Arabidopsis leaves (Collakova and DellaPenna, 2003). The gene encoding HPT, *HPT1*, has been cloned from *Synechocystis* sp. PCC 6803 and *Arabidopsis thaliana* (Collakova and DellaPenna, 2001; Schledz et al., 2001). Overexpression of HPT in Arabidopsis increased leaf and seed tocopherol content by 4.4-fold and 75%, respectively (Collakova and DellaPenna, 2003; Savidge et al., 2002). The current study was undertaken to further define the role of HPT in regulating tocopherol biosynthesis in stressed photosynthetic tissues. By combining high light stress with molecular and biochemical analyses, we have also identified additional enzymes and/or substrates that limit α-tocopherol synthesis in stressed Arabidopsis leaves.

Results

The Biochemical Response of Wild Type and 35S::*HPT1* Plants To High Light Stress

Stress is associated with increased total tocopherol levels in a variety of plants (reviewed in Munne-Bosch and Alegre, 2002a). We have shown previously that HPT activity is limiting for tocopherol synthesis in non-stressed Arabidopsis leaves (Collakova and DellaPenna, 2003). To investigate whether HPT activity also limits tocopherol synthesis in stressed Arabidopsis leaf tissue, 6-week-old wild type and two well-

characterized 35S::*HPT1* lines (lines 11 and 54, Collakova and DellaPenna, 2003) were subjected to high light stress (0.8 - 1 mE) for up to 15 days and tocopherol content and composition were analyzed during the treatment. The general response to stress was monitored by assessing anthocyanin accumulation and the decrease in chlorophyll and carotenoid levels (Figure 19A and B). Total anthocyanin levels rapidly increased from

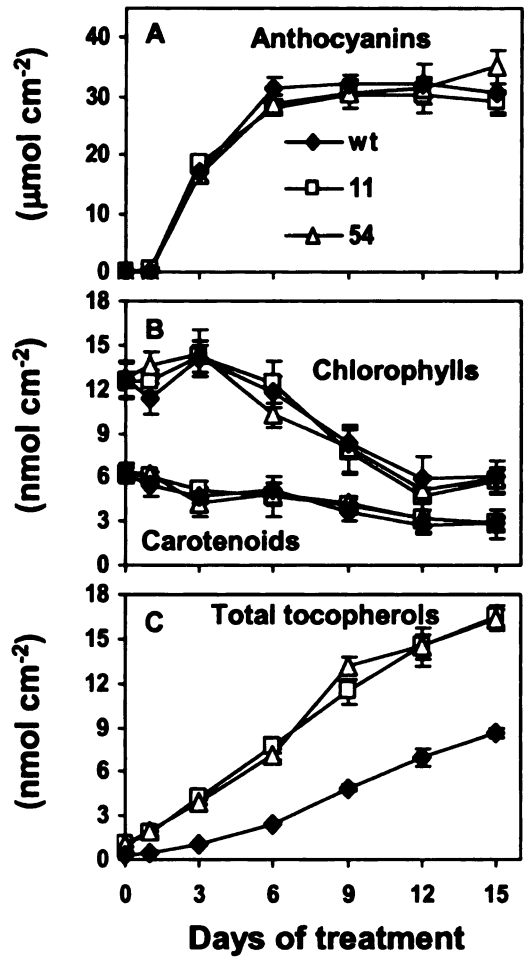

Figure 19. Total anthocyanin, chlorophyll, carotenoid, and tocopherol levels in stressed wild type and 35S::*HPT1* leaves. Plants of the indicated genotypes were grown in a 10/14 hour light/dark cycle at 75 to 100 µE for 6 weeks and then transferred to approximately 900 µE growth conditions. A, Anthocyanin accumulation in leaves of stressed wild type and 35S::*HPT1* Arabidopsis plants. Anthocyanin synthesis increased within the first 3 days of high-light stress and reached high steady-state levels after 6 days of high light treatment. B, Chlorophyll and carotenoid degradation in leaves of stressed wild type and 35S::*HPT1* plants. Total chlorophyll and carotenoid levels decreased gradually to approximately 50 % of the initial levels in all stressed lines. C, Total tocopherol levels in leaves of stressed wild type and 35S::*HPT1* plants. High-light stress resulted in a significant elevation of total tocopherol levels in both 35S::*HPT1* transgenic and wild type plants.

below detection to approximately 35 μmol cm^{-2} leaf area by day six and were maintained at this level throughout the stress treatment (Figure 19A). Total chlorophyll and carotenoid levels decreased gradually during the 15-day stress treatment to approximately half of their initial levels (Figure 19B). There were no significant differences in chlorophyll, carotenoid, or anthocyanin content between wild type and 35S::*HPT1* throughout the course of the experiment (Figure 19A and B).

Total tocopherol levels increased in a near linear manner during exposure of both wild type and 35S::*HPT1* plants to high light ($R^2 \geq 0.97$, Figure 19C). Prior to stress treatment, the total tocopherol levels of 35S::*HPT1* leaves were 3-fold higher than wild type (1.06 ± 0.22 and 0.36 ± 0.05 nmol cm^{-2} leaf area, respectively). In response to 15 days of high light stress, total tocopherol levels increased 24-fold to 8.7 ± 0.3 nmol cm^{-2} leaf area in wild type, while the total tocopherol levels of 35S::*HPT1* increased 15.5-fold to 16.4 ± 0.8 nmol cm^{-2} leaf area. After 15 days, the respective final total tocopherol levels in non-stressed wild type and 35S::*HPT1* leaves were 0.49 ± 0.04 and 2.00 ± 0.50 nmol cm^{-2} leaf area, respectively. At any time point during stress, total tocopherol levels in 35S::*HPT1* were 1.9- to 3.8-fold higher than wild type ($p < 0.006$, Figure 19C), suggesting that HPT activity limits tocopherol synthesis in stressed wild type Arabidopsis leaves.

HPT Expression And Enzyme Activity in Unstressed And Stressed Wild Type And 35S::*HPT1*

We have shown previously that non-stressed 35S::*HPT1* Arabidopsis plants accumulated 20- to 100-fold higher *HPT1* mRNA levels than wild type and showed 4- to 10-fold increases in HPT specific activity, which resulted in up to 4.4-fold increased tocopherol levels in leaves of transgenic lines compared to wild type (Collakova and DellaPenna, 2003). In non-stressed wild type leaves, average HPT mRNA levels ranged from 0.3 to 0.6 fmol mg^{-1} total RNA during the 12-day experimental time course (Figure

20A). Consistent with our previous study (Collakova and DellaPenna, 2003), HPT mRNA levels in non-stressed 35S::*HPT1* leaves were at least 20-fold higher than wild type and ranged from 7 to 15 fmol mg^{-1} total RNA during the course of the experiment (Figure 20B).

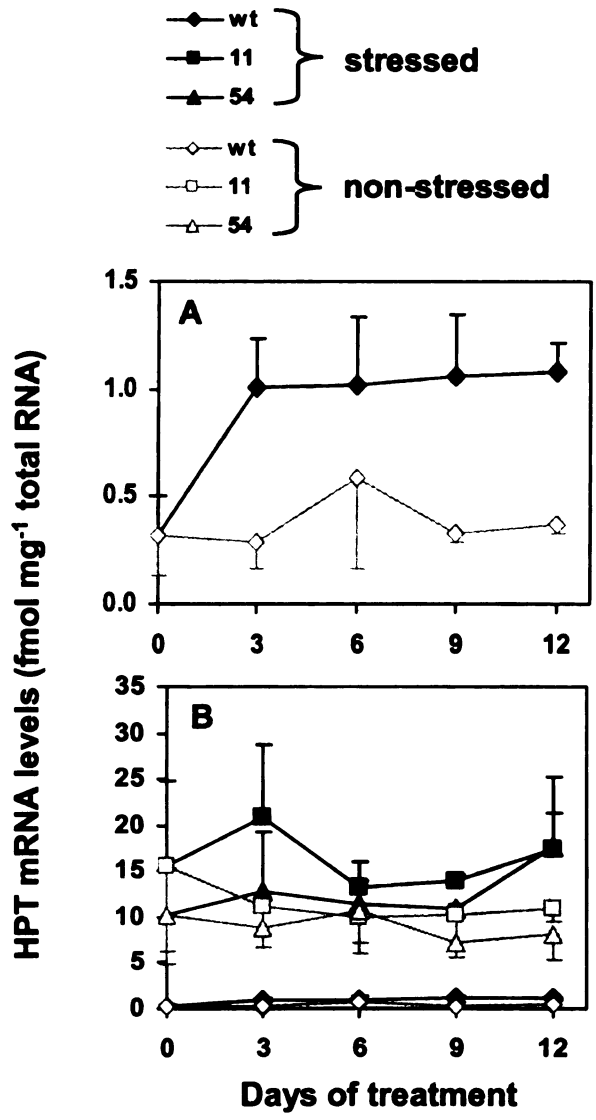

Figure 20. HPT expression in control and stressed wild type and 35S::*HPT1* plants. Plants were grown and stressed as in Figure 2, total RNA extracted, and HPT mRNA levels determined by real-time PCR. Data are normalized for EF-1α mRNA levels and presented as average ± SD of three independent experiments. A, Wild type HPT mRNA levels. B, Wild type and 35S::*HPT1* HPT mRNA levels. High light stress resulted in an up-regulation of HPT mRNA levels in wild type, while no trend was observed in stressed 35S::*HPT1* and the corresponding control plants.

To assess any correlation between elevated total tocopherol levels and changes in HPT expression or activity during stress, HPT mRNA levels and specific activity were determined in non-stressed and stressed wild type and 35S::*HPT1* plants. HPT mRNA levels were induced up to 3.5-fold in wild type by 3 days of high light treatment and remained elevated throughout the course of the experiment (Figure 20A). No clear trend was observed for HPT mRNA levels in stressed 35S::*HPT1* lines (Figure 20B), though there was significant biological variation in HPT mRNA levels in both wild type and 35S::*HPT1* plants during stress treatments (Figure 20A and B). Consistent with prior studies, HPT specific activity in the absence of high light stress was 6-fold higher in 35S::*HPT1* lines compared to wild type (Figure 21; $p < 0.0005$). In response to 3 and 6 days of high light stress, HPT specific activity in wild type and 35S::*HPT1* lines increased approximately 3-fold and up to 4.4-fold relative to their respective unstressed controls. In 35S::*HPT1*, the relative HPT specific activity after 3 days of stress was 9-fold that of

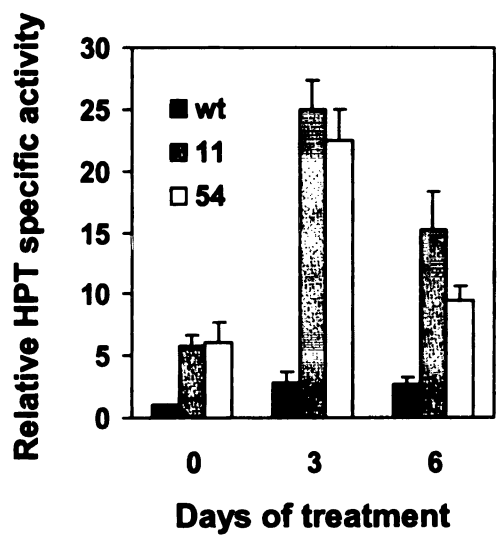

Figure 21. Relative HPT specific activity in control and high light stressed wild type and 35S::*HPT1* Arabidopsis chloroplasts. Six week-old plants were transferred to high light (0.8 - 1 mE) for three and six days, chloroplasts isolated, and assayed for HPT activity. Results from two independent experiments are presented as an average ± SD of the activity increase relative to wild type non-stressed chloroplasts (0.15 ± 0.10 pmol h^{-1} mg^{-1} protein). High light stress resulted in the strong induction of HPT activity relative to control plants in both wild type and 35S::*HPT1*. Relative HPT specific activity in 35S::*HPT1* was significantly higher than in wild type ($p < 0.0005$).

comparably treated wild type. After 6 days of high light stress, HPT specific activity was 5.6- and 3.5-fold higher than the corresponding wild type for 35S::*HPT1* lines 11 and 54, respectively (Figure 21).

Changes in mRNA Levels of Other Tocopherol Pathway-Related Genes in Non-Stressed And Stressed Wild Type And 35S::*HPT1* Leaves

In addition to HPT, several other tocopherol biosynthetic enzymes (TAT, HPPD, HGAD, GGPS1, GGDR, TC, and γ-TMT) may play roles in regulating tocopherol synthesis in Arabidopsis leaves. To assess the role of these enzymes in regulating tocopherol accumulation during high light stress, their mRNA levels were measured in wild type and 35S::*HPT1* leaves using real-time PCR (Figure 22). TAT and HPPD catalyze formation of the tocopherol biosynthetic precursors HPP and HGA, respectively, whereas HGAD is involved in HGA degradation (Figure 18). GGPS1 and GGDR catalyze synthesis of PDP, a prenyl substrate used in tocopherol biosynthesis, while TC and γ-TMT are involved in regulating tocopherol composition (Figure 18). The steady-state mRNA levels of TAT, HPPD, HGAD, GGPS1, and γ-TMT in non-stressed tissues were similar in the different genotypes and varied between 1 and 6 fmol mg^{-1} total RNA during the 12-day experimental time course (Figure 22A, B, C, D, G). GGDR showed the highest steady-state mRNA levels of all tested genes and fluctuated between 20 and 30 fmol mg^{-1} total RNA in non-stressed Arabidopsis leaves (Figure 22E). TC mRNA levels were quite low (0.3 - 0.6 fmol mg^{-1} total RNA) and comparable to wild type HPT mRNA levels (Figures 22F and 21A). Although leaf mRNA levels of most tested genes varied 2- to 3-fold across all genotypes and time-points the overall trends indicated relatively constant steady-state mRNA levels for each gene and the differences observed probably represent the biological variation of the system (Figure 22).

In response to high light treatment, TAT, HPPD, and HGAD mRNA levels were elevated several-fold in both wild type and 35S::*HPT1* lines (Figure 22A, B, and C),

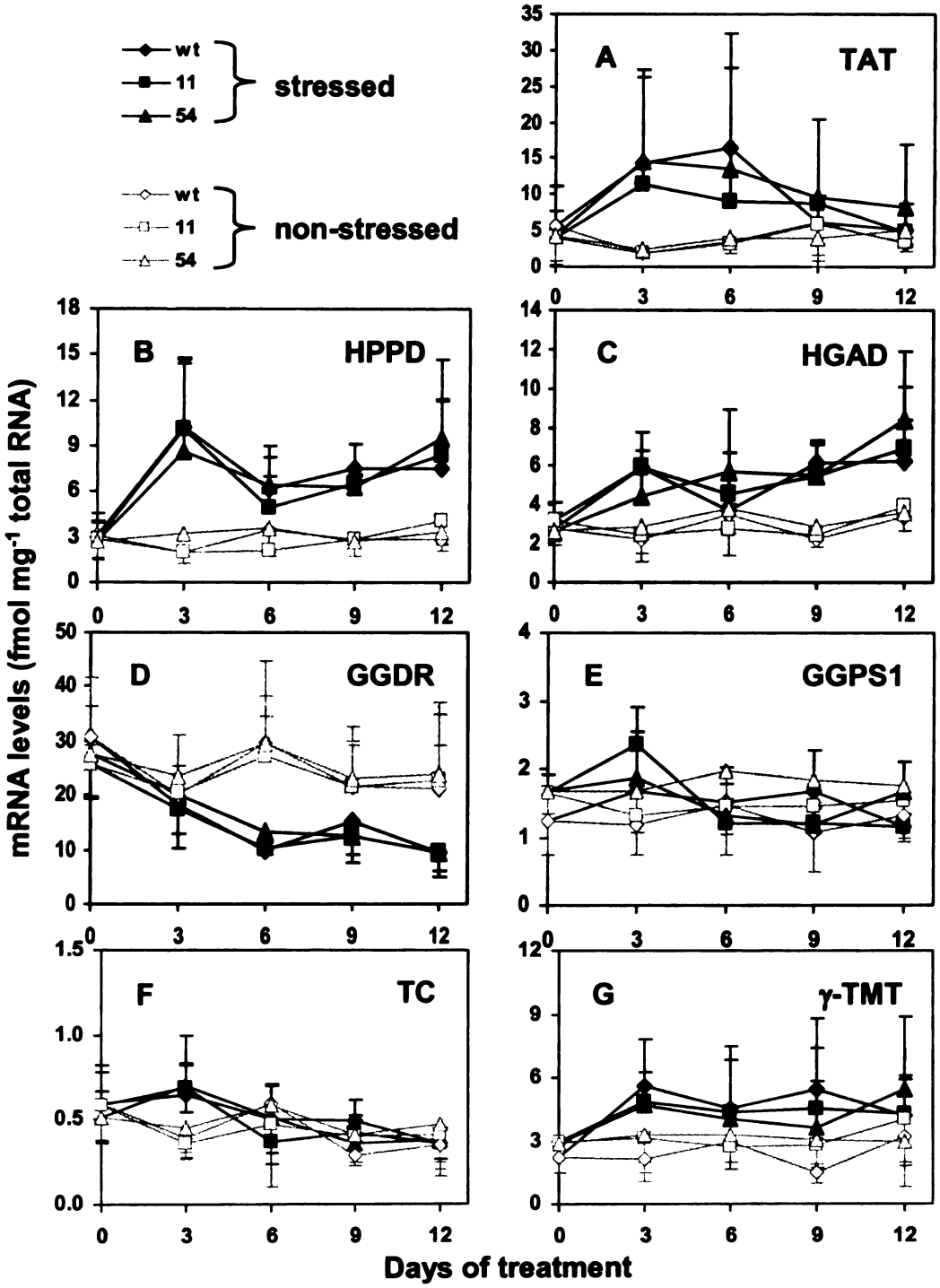

Figure 22. Expression of other tocopherol-related genes in control and stressed wild type and 35S::*HPT1* leaves. Experiments were performed as described in Figure 3. A, tyrosine transaminase (TAT); B, *p*-hydroxyphenyl pyruvate dioxygenase (HPPD); C, homogentisate dioxygenase (HGAD); D, geranylgeranyl diphosphate synthase 1 (GGPS1); E, geranylgeranyl diphosphate reductase (GGDR); F, tocopherol cyclase (TC); G, γ-tocopherol methyltransferase (γ-TMT). TAT, HPPD, and HGAD mRNA levels increased, while GGDR mRNA levels decresed during stress. GGPS1, TC, and γ-TMT mRNA levels were not up-regulated in response to stress.

suggesting that these enzymes may also be involved in regulating tocopherol levels during stress. TAT mRNA levels increased 3- to 5-fold relative to non-stressed leaves within the first 6 days of high light stress before returning near control levels by the end of the time course (Figure 22A). HPPD and HGAD mRNA levels were elevated throughout the time course of stress treatment (Figure 22B and C) and in this regard showed expression profiles similar to HPT mRNA levels in stressed wild type (Figure 20A). The maximal increase for HGAD and HPPD in stressed relative to non-stressed plants was 2.7- and 5.4-fold, respectively (Figure 22B and C). GGPS1, TC, and γ-TMT steady-state mRNA levels were not significantly altered in wild type or 35S::*HPT1* leaves in response to high light stress (Figure 22E, F, and G). The GGDR expression profile was unique in showing a downward trend during high light stress (Figure 22D). As with HPT, mRNA levels of the tocopherol pathway-related genes showed a large biological variation between experiments, especially in stressed plants. Consistent with our previous study (Collakova and DellaPenna, 2003), there were no significant differences between genotypes for mRNA levels of these genes in stressed or unstressed plants (Figure 22). These results suggest that increased HPT expression and tocopherol levels in 35S::*HPT1* transgenic lines does not have a significant impact on the expression of other tocopherol pathway-related genes.

Analysis of Individual Tocopherols And Phytylquinols in Non-Stressed And Stressed Wild Type And Transgenic Plants

Like most plants, Arabidopsis can synthesize four different tocopherols, α-, β-, γ-, and δ-tocopherols from the phytylquinols MPBQ and DMPBQ by the routes shown in Figure 18. The current study (Figure 19C, 20, and 21) and previous report (Collakova and DellaPenna, 2003) make it clear that HPT activity is a major limitation in total tocopherol synthesis and accumulation in both non-stressed and stressed leaves. Analyses of tocopherol and phytylquinol levels and compositions were performed to identify steps subsequent to HPT that might limit α-tocopherol synthesis in non-stressed and stressed

leaves of wild type and 35S::*HPT1* Arabidopsis plants. Non-stressed wild type Arabidopsis leaves accumulate more than 95% α-tocopherol and less than 5% γ-tocopherol (Collakova and DellaPenna, 2003). In non-stressed 35S::*HPT1* leaves, α- and γ-tocopherols constituted 90% and 10% of total tocopherol levels, respectively. β-Tocopherol, and δ-tocopherol were below detection levels in non-stressed wild type and 35S::*HPT1* Arabidopsis leaves. The phytylquinols MPBQ and DMPBQ were also below detection in non-stressed and stressed wild type and 35S::*HPT1* leaves (data not shown), suggesting that TC activity is not limiting for tocopherol synthesis during normal growth or high light stress.

Tocopherol composition changed significantly in both wild type and 35S::*HPT1* leaves during high light stress. Though α-tocopherol was still the major tocopherol in stressed leaves, β-, γ-, and δ-tocopherols accumulated to high levels as well (Figure 23). This effect was most pronounced in 35S::*HPT1* leaves after 15 days of stress treatment, where α-tocopherol constituted only 57% of total tocopherols, as opposed to 84 % in wild type (Figure 23A). This difference was due to the presence of high levels of other tocopherols in 35S::*HPT1* leaves throughout the experimental time course. β- and δ-tocopherols accumulate when MPBQ is directly cyclized to yield δ-tocopherol rather than being methylated to DMPBQ (Figure 18). Collectively, β- and δ-tocopherols accounted for 0.48 nmol cm^{-2} leaf area (5.6% of total tocopherols) in stressed wild type, but accounted for 3 and 6 nmol cm^{-2} leaf area (20 and 35% of total tocopherols) in stressed 35S::*HPT1* lines 54 and 11, respectively (Figure 23C and D). Similarly, stressed 35S::*HPT1* lines accumulated γ-tocopherol, the immediate precursor of α-tocopherol, to a greater extent than wild type (Figure 23B and D). After 15 days of high light stress, γ-tocopherol constituted 10% of total tocopherols in wild type and 14 and 17% in 35S::*HPT1*-11 and -54, respectively (Figure 23B and D). These results collectively indicate that both the methylation of MPBQ to DMPBQ and γ-tocopherol to α-tocopherol limit α-tocopherol synthesis in stressed 35S::*HPT1* leaves.

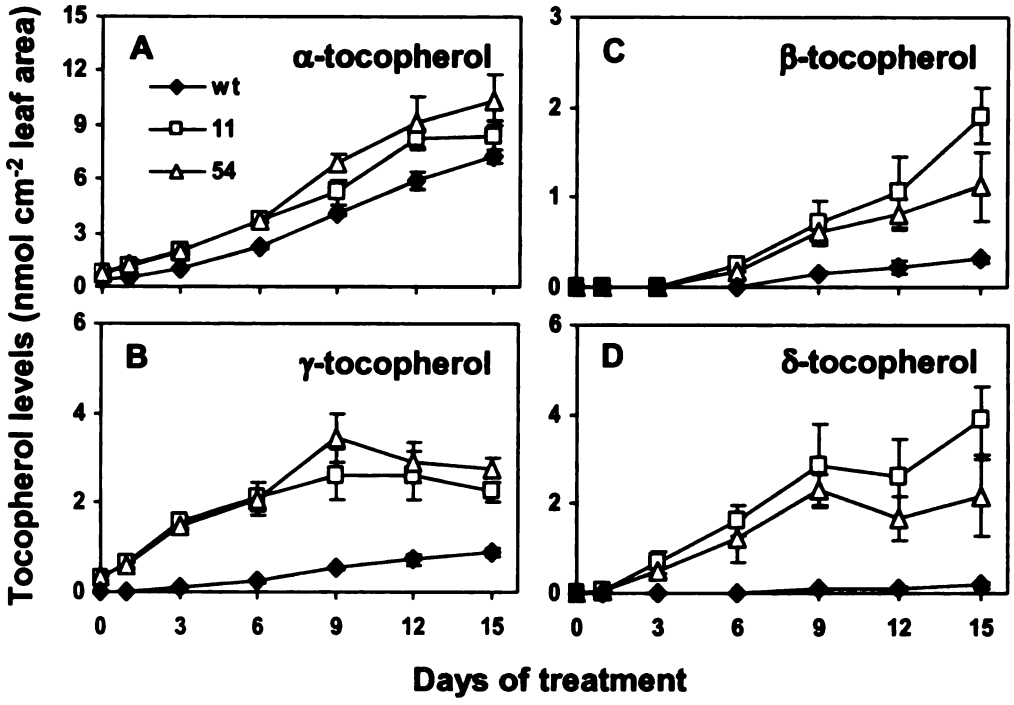

Figure 23. Tocopherol levels and composition in stressed wild type and 35S::*HPT1* plants. A, α-tocopherol B, γ-tocopherol C, β-tocopherol D, δ-tocopherol. Leaves of stressed 35S::*HPT1* plants accumulated slightly more α-tocopherol than those of wild type plants. Large differences are evident in the levels of β-, γ-, and δ-tocopherol between 35S::*HPT1* and wild type.

To distinguish between a limitation in γ-TMT activity and SAM levels, 35S::*HPT1* lines were crossed to previously characterized lines overexpressing γ-TMT (35S::*γ-TMT*), which should eliminate any limitation to γ-TMT activity *in vivo* (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2003). We have shown previously that under optimal growth conditions, excess γ-tocopherol synthesized in 35S::*HPT1* was methylated to α-tocopherol in 35S::*HPT1*/35S::*γ-TMT* double overexpressers (Collakova and DellaPenna, 2003). During high light stress, γ-TMT overexpression resulted in reduced γ- and δ-tocopherol levels in 35S::*γ-TMT* and 35S::*HPT1*/35S::*γ-TMT* lines relative to wild type and 35S::*HPT1*, respectively, without affecting total tocopherol levels (Figure 24). In stress treated 35S::*HPT1*/35S::*γ-TMT* double overexpressers, the change in tocopherol composition was significant as γ- and δ-tocopherols constituted less than 5% of total

tocopherols as opposed to 34% in stressed 35S::*HPT1*. This change was only marginal in 35S::*γ-TMT* parental lines, where γ- and δ-tocopherols accounted for less than 6% of total tocopherols compared to 14% in wild type leaves during high light stress (Figure 24). These results suggest that γ-TMT activity and not SAM levels limits α- and β-tocopherol synthesis in stressed wild type and 35S::*HPT1* plants.

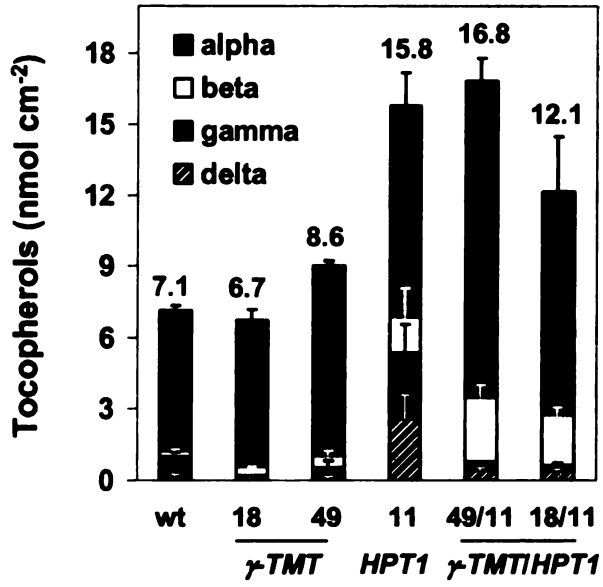

Figure 24. Tocopherol levels and composition in stressed wild type, 35S::*HPT1*/35S::*γ-TMT* double overexpressers, and the corresponding transgenic parents. Plants were stressed for 15 days and individual tocopherols were analyzed by normal phase HPLC. After 15 days of high light stress, 35S::*HPT1* leaves accumulated 2-fold higher total tocopherol levels relative to wild type. The conversion of γ- and δ-tocopherols to α- and β-tocopherols occurred to a much greater extent in 35S::*HPT1*/35S::*γ-TMT* than in 35S::*HPT1*, suggesting that γ-TMT activity limits α- and β-tocopherol synthesis in 35S::*HPT1* leaves during high light stress.

Discussion

Chloroplasts generate increased levels of excited chlorophylls and ROS including singlet oxygen, superoxide anions, and hydroxyl radicals during stress (Fryer, 1992; Mittler, 2002). Plants have developed numerous highly regulated mechanisms for protection from excessive ROS including a variety of different antioxidants and ROS

detoxifying enzymes that are generally up-regulated in their levels and/or activity during stress (Foyer et al., 1994). Tocopherols are thought to represent a key antioxidant protecting polyunsaturated fatty acids from photooxidation by quenching and scavenging ROS and alkyl peroxy radicals generated during photosynthesis (Fryer, 1992). In plants, tocopherol levels can change significantly depending on tissue type, developmental stage, and environmental conditions (Rise et al., 1989; Havaux et al., 2000; Munne-Bosch and Alegre, 2002a). We have used light stress as a treatment to increase tocopherol synthesis in wild type and 35S::*HPT1* plants in order to address basic questions about regulation of the pathway in response to stress.

We have shown previously that phytylation of HGA is a limiting step in tocopherol biosynthesis in non-stressed Arabidopsis leaves (Collakova and DellaPenna, 2003). To determine whether HPT activity is also limiting for tocopherol synthesis under stress conditions, wild type and 35S::*HPT1* plants were subjected to high light stress and analyzed. Stressed 35S::*HPT1* leaves accumulated 2- to 4-fold elevated total tocopherol levels relative to wild type throughout the experimental time course. These increases correlated with significantly elevated HPT mRNA levels and HPT specific activity in 35S::*HPT1* compared to wild type leaves during high light stress. Collectively, these results indicate that, as in unstressed leaves, HPT activity limits tocopherol synthesis in stressed wild type leaves. It appears that HPT limits plant tocopherol biosynthesis under both normal and stressed conditions.

While a several-fold difference in total tocopherol levels between stressed wild type and 35S::*HPT1* plants was maintained during the experimental time course, the 15-day high light treatment also resulted in an 18- and 8-fold increase in wild type and 35S::*HPT1*, respectively, relative to unstressed controls. In non-stressed wild type and 35S::*HPT1* plants, tocopherol levels nearly doubled during the 15-day time course of the experiment. This increase is likely due to aging of the 6-week-old plants during the experiment as increasing plant age is associated with a modest increase in total tocopherol content (Rise et

al., 1989; Molina-Torres and Martinez, 1991; Tramontano et al., 1992). Despite this increase, the absolute tocopherol levels in unstressed wild type and 35S::*HPT1* plants remained very low relative to stressed plants. In wild type leaves, both HPT mRNA levels and HPT specific activity were up-regulated in response to high light treatment throughout the experimental time course. In stressed 35S::*HPT1* lines, HPT mRNA levels were not significantly elevated, whereas HPT specific activity was, suggesting that the HPT enzyme may also be regulated at the post-transcriptional level during high light stress. Collectively, these results indicate that elevated HPT expression and activity contribute significantly to the increased tocopherol accumulation in stressed wild type and 35S::*HPT1* leaves.

Although HPT specific activity is clearly a key factor regulating total tocopherol synthesis during stress, it appears that other factors are also involved. This hypothesis is supported by the observation that HPT specific activity did not correlate with the linear increase in total tocopherol levels at all time points. First, HPT specific activity was similar in wild type leaves stressed for 3 and 6 days, though total tocopherol levels nearly doubled during this period. Similarly, in 35S::*HPT1* lines, HPT specific activity was reduced significantly in 6-day stressed plants relative to those stressed for 3 days, whereas tocopherol levels doubled during this time. Finally, though HPT specific activity increased nearly 3-fold in wild type leaves stressed for 6 days, it still did not reach the levels of non-stressed 35S::*HPT1* plants, yet total tocopherol levels in these stressed wild type plants were 2.3-fold higher than non-stressed transgenics. These results suggest that one or more HPT-independent limitation(s) that cannot be overcome by *HPT1* overexpression alone in non-stressed Arabidopsis plants are alleviated by high light stress treatment. Increased tocopherol levels in stressed leaves may simply be due to the increased availability of substrates for tocopherol synthesis as well as up-regulation of other tocopherol pathway enzymes upstream of HPT.

HPT catalyzes the committed step of tocopherol synthesis, condensation of two potentially limiting substrates, HGA and PDP. The aromatic precursor HGA is derived

from prephenate or tyrosine, both products of the shikimate pathway (Threlfall and Whistance, 1971; Fiedler et al., 1982; Lopukhina et al., 2001). In plants, the shikimate pathway has been shown to be regulated at the transcriptional level and globally up-regulated during biotic and abiotic stresses (Gorlach et al., 1995; Herrman and Weaver, 1999; Diaz et al., 2001). Thus, the shikimate pathway may provide an increased pool of prephenate that is at least partially utilized for elevated tocopherol synthesis in stressed plants. In addition, tyrosine is released during protein degradation and may also provide an increased pool of HPP available for tocopherol synthesis during high light stress. Elevated tyrosine catabolism in stressed plants is consistent with our findings that TAT, HPPD, and HGAD mRNA levels were all up-regulated during stress in both wild type and 35S::*HPT1*. These results are consistent with prior studies that TAT expression was up-regulated in Arabidopsis leaves during various biotic and abiotic stresses (Lopukhina et al., 2001; Sandorf and Hollander-Czytko, 2002) and that HPPD mRNA levels were elevated during senescence-induced stress in barley leaves (Klebler-Janke and Krupinska, 1997). Although increases in HPPD mRNA levels are associated with elevated tocopherol levels during stress, HPPD activity is probably a minor component regulating tocopherol biosynthetic pathway because constitutive HPPD overexpression alone or in a *HPT1* overexpressing background had no effect on total tocopherol levels in high light stressed Arabidopsis leaves (data not shown).

As with HGA, there are two known metabolic sources of the other HPT substrate, PDP. First, excess PDP required for tocopherol biosynthesis may be synthesized *de novo* from DXP pathway-derived IPP during stress. In plastids, flux through the DXP isoprenoid biosynthetic pathway is regulated at the level of DXP synthase and DXP reductoisomerase activities, which have been shown to limit isoprenoid biosynthesis in non-stressed plants (Estevez et al., 2001; Mahmoud and Croteau, 2001). Enzymes downstream of the DXP pathway, such as GGPS1 and GGDR were not up-regulated at mRNA levels in response to high light stress, suggesting that they are not major regulators of tocopherol

biosynthesis or, if they are, the activities of these two enzymes are translationally or post-translationally regulated. The second potential source of PDP is phytol, which is derived from chlorophyll degradation, a process involving several steps including the removal of the phytol tail by chlorophyllase (Matile et al., 1999; Tsuchiya et al., 1999). Chlorophyllase gene expression is induced by treatments with stress-associated compounds such as coronatine and methyl jasmonate (Tsuchiya et al., 1999). A correlation between chlorophyll degradation and tocopherol accumulation during leaf senescence has also been reported (Rise et al., 1989). We observed a large decrease in chlorophyll levels in wild type and 35S::*HPT1* during high light stress, which coincided with the rise in tocopherol levels. If one only considers degradation of existing chlorophyll, the maximal pool of phytol released during the 15-day stress treatment would allow for the synthesis of ~ 6 nmol cm^{-2} leaf area of total tocopherols in wild type and 35S::*HPT1* leaves. While this possibility is intriguing, it is still not clear what contribution chlorophyll degradation makes to tocopherol biosynthesis during high light stress because we lack direct evidence for the incorporation of chlorophyll-derived phytol into tocopherols.

The increase in total tocopherol levels was paralleled by a corresponding increase in α-tocopherol levels in response to high light stress. However, large increases in the levels of other tocopherols in stressed wild type and 35S::*HPT1* plants suggested that steps downstream of HPT that regulate tocopherol composition, including TC, MPBQ MT, and γ-TMT, may limit α-tocopherol accumulation during stress. The absence of the phytylquinol intermediates MPBQ and DMPBQ suggests that TC activity is sufficient in both stressed and unstressed plants and the phytylquinol intermediates are rapidly converted to the corresponding tocopherols, or rapidly degraded by unknown mechanisms. The fact that TC mRNA levels are very low and not up-regulated significantly during stress suggests TC is either very active and/or very stable in stressed Arabidopsis leaves. The presence of β- and δ-tocopherols in stressed wild type and transgenic Arabidopsis leaves suggests that either MPBQ MT activity or SAM limits α-tocopherol synthesis in these plants. This

limitation appears to be more severe in 35S::*HPT1* leaves, which accumulate β- and δ-tocopherol at several-fold higher levels than wild type at all time points during stress. Based on these results, it appears that the excess MPBQ produced by HPT in stressed transgenic plants is not methylated to DMPBQ, but rather cyclized to form δ-tocopherol.

The final enzyme of the tocopherol pathway is γ-TMT, which utilizes SAM to generate α- and β-tocopherol from γ- and δ-tocopherol, respectively (D'Harlingue and Camara, 1985; Shintani and DellaPenna, 1998). Relatively high levels of γ- and δ-tocopherols were detected in stressed wild type and much higher levels in 35S::*HPT1* leaves, suggesting that γ-TMT activity or SAM may be limiting for α-tocopherol synthesis during high light stress. To differentiate these possibilities, tocopherol composition and levels were analyzed in stressed transgenic plants overexpressing *HPT1* and *γ-TMT* in combination (35S::*HPT1*/35S::*γ-TMT*). Conversion of nearly the entire pool of γ- and δ-tocopherols to α- and β-tocopherols, respectively, in stressed 35S::*HPT1*/35S::*γ-TMT* indicates that γ-TMT activity, and not SAM, limits α-tocopherol biosynthesis during stress. The persistence of small but similar amounts of γ- and δ-tocopherols in stressed leaves of wild type and all transgenic genotypes implies that these tocopherols are either not accessible to γ-TMT or accumulate at these levels for a distinct functional role.

The current study represents an initial step toward understanding the molecular and metabolic regulation of tocopherol biosynthesis during high light stress in plants. We have focused on determining the changes in steady-state tocopherol levels and pool sizes and correlating this with changes in gene expression for various steps of the pathway. Because we cannot yet address tocopherol turnover, our data do not represent total tocopherol synthesis or fluxes through the pathway. HPT catalyzes HGA phytylation, the first and committed step in the tocopherol pathway, and limits total tocopherol synthesis in both non-stressed and stressed wild type Arabidopsis plants. Although stressed leaves showed increased HPT specific activity relative to non-stressed leaves, HPT activity is not the only component that contributes to the elevated total tocopherol levels observed during high light

stress. The differential production and availability of aromatic and prenyl diphosphate precursors in non-stressed and stressed plants may also play an important role in regulating tocopherol levels under various growth conditions. Up-regulation of the shikimate pathway and protein and tyrosine degradation may all contribute to the HPP pool used for elevated tocopherol synthesis in stressed relative to non-stressed plants. Similarly, elevated chlorophyll degradation and *de novo* isoprenoid synthesis may both contribute to the increased PDP pool needed for tocopherol synthesis during high light stress. All enzymes downstream of HPT (TC, MPBQ MT and γ-TMT) regulate leaf tocopherol composition. Our results indicate that methylation of both MPBQ and γ-tocopherol, but not the cyclization of tocopherol intermediates, also appears to limit α-tocopherol synthesis in stressed wild type and 35S::*HPT1* Arabidopsis leaves.

Materials And Methods

Plant growth and high-light stress. The following lines were used in this study: wild type *Arabidopsis thaliana* (ecotype Columbia) and the previously characterized homozygous transgenic lines 35S::*γ-TMT*-18 and -49 (Shintani and DellaPenna, 1998) and 35S::*HPT1*-11 and -54, and the corresponding homozygous double overexpressers obtained by crossing these lines (Collakova and DellaPenna, 2003). To obtain a sufficient amount of tissue for all analyses, seeds were planted 3 - 4 cm apart and grown at a 10-hour photoperiod at 75 - 100 μE (22/19 °C day/night cycle) for 6 weeks. At 6 week age, plants were transferred to high light (0.8 - 1 mE, 10-hour photoperiod) for stress experiments or maintained at 75 - 100 μE. To exacerbate oxidative stress, a combination of high light, low nutrient, and water stress was used such that plants grown at high light were watered every 2 days with distilled water. For simplicity, this combination of abiotic stresses is referred to high light stress. At indicated time-points, tissue was harvested for HPLC, real-time PCR, or HPT enzyme assays, within 3 to 5 hours after the start of the light cycle.

Prenyllipid and anthocyanin analyses. Each genotype was represented by three plants and each plant was analyzed twice. Three representative leaf discs (total surface area: 1.507 cm^2, 30 - 35 mg of fresh weight) per plant were harvested and subjected to lipid extraction (Bligh and Dyer, 1959). After phase separation, an aliquot of the aqueous phase was acidified with 0.1 N HCl and the amount of anthocyanins present was measured spectrophotometrically at 520 nm as described previously (Merzlyak and Chivkunova, 2000). The organic phase was dried under vacuum and lipids were dissolved in hexane. An aliquot of the resulting lipid extract was dried, dissolved in 90% (v/v) methanol, and used to determine chlorophyll and carotenoid content by the spectrophotometric method of Lichtenthaler (1987). Tocopherols were analyzed and quantified by normal phase HPLC as described (Collakova and DellaPenna, 2003).

Real-time PCR. Tissue harvested from three representative plants was ground in liquid nitrogen, total RNA isolated, and real-time PCR was performed as previously described (Collakova and DellaPenna, 2003). The procedure was modified such that 3 μg of total RNA for each sample was reverse transcribed in triplicate. Each reaction was diluted with 2 volumes of water and cDNA corresponding to 200 ng of total RNA was subjected to real-time PCR using ABI PRISM Sequence Detection System 7000 (Applied Biosystems, Foster City, CA). Standard curves were constructed for each gene and used to determine the absolute mRNA levels. Relative mRNA levels of elongation factor EF-1α were used to normalize each sample. EF-1α mRNA levels varied over a 2.5-fold range during the high light treatment with no apparent trend (data not shown). Results from three independent experiments are presented as average ± SD for each line and time point. Taqman probes, primers and their final optimized concentrations are presented in Table VII.

Table VII. *Probes, primers and their final optimal concentrations used for real-time PCR.* Each Taqman probe was labeled with 6-fluorescein at the 5'- and the quencher TAMRA at the 3'-end.

Enzyme	Primers/ Probes	Sequence (5' -> 3')	Conc. [nM]
HPT	Forward	TCTCTAAAAGACTTCTGTTTGCTATTCG	900
	Reverse	AGTCGAGATTTCGGGTTAATGC	900
	Probe	AAAGCCTCAGGCTGACCCGCAGT	175
HPPD	Forward	CAAGGAGTGTGAGGAATTAGGGATT	300
	Reverse	TCGTCGGCCTGTCACCTAGT	300
	Probe	ATGATCAAGGGACGTTGCTTCAAATCTTCA	150
HGAD	Forward	ACCAACCATCGAGGAGGAAA	900
	Reverse	TAAATACAGTCCTTACAGCACCAACTG	900
	Probe	ATGACAAAATCAAGCAAGGCCACACCA	175
TC	Forward	GTATTTGAGCCTCATTGGCAGAT	900
	Reverse	TCACCGCCCCATTCTATCC	900
	Probe	ATGGCAGGAGGCCTTTCCACAGG	200
GGPS1	Forward	TCCGGTGAGAGTGGTTCGAG	900
	Reverse	CTTGACCCGCCACTAACCC	900
	Probe	TTGGAGAATTGGCTAAAGCGATAGGAACAGA	200
GGDR	Forward	CACTTAGGGAACACCCAACCA	900
	Reverse	ACTTACTATGAGGATTTAGCTGAGATGTATG	900
	Probe	AGAAATCCGGCGACACATCATCTCCA	150
TAT	Forward	AACCGAAAGCCAACGTTTTG	900
	Reverse	TCTTGTAGATGGAGCGGACTAGGT	600
	Probe	TTCCGAGTCCCGGCTTCCCATG	200
γ-TMT	Forward	GGCCAAGAGAGCCAATGATC	900
	Reverse	CGCATCCGCAACTTGGA	900
	Probe	CGGCTGCTCAATCACTCTCTCATAAGGCT	200
EF-1α	Forward	CGAACTTCCATAGAGCAATATCGA	300
	Reverse	GCATGGGTGTTGGACAAACTT	300
	Probe	ACCACGGTCACGCTCGGCCT	175

Prenyltransferase assays. Homogentisate phytyl transferase assays were performed using [U-^{14}C]HGA and PDP as substrates and crude chloroplast preparations from wild

type and transgenic plants as described previously (Collakova and DellaPenna, 2003). Each high-light experiment involved analysis of control (0 days) and stressed (3 and 6 days) wild type and 35S::*HPT1* plants. Chloroplast isolation and HPT activity assays of all plants were performed the same day to ensure equal treatment of all samples in the experiment. Thus, a set of 6-week-old plants grown under non-stress conditions were transferred to high light and 3 days later another set of non-stressed plants was transferred to high light. After additional 3 days, chloroplasts were isolated from a set of non-stressed, 3-day-stressed, and 6-day-stressed wild type and 35S::*HPT1* plants. HPT assays with plants stressed for more than 6 days could not be reliably performed because of inconsistent and insufficient chloroplast yields obtained from such plants. HPT specific activity was calculated by subtracting background HPT activities obtained from control reactions containing radiolabeled HGA without exogenously added PDP from the HPT activities when both substrates were present. HPT specific activity is presented as relative HPT specific activity, which is expressed as a fold difference relative to HPT specific activity of non-stressed wild type chloroplasts.

SUMMARY AND FUTURE DIRECTIONS

Homogentisate phytyl transferase plays an important role in vitamin E biosynthesis, because it catalyzes the committed step of the tocopherol biosynthetic pathway, condensation of HGA and PDP. The isolation and biochemical characterization of the genes encoding HPT in *Synechocystis* sp. PCC 6803 and *Arabidopsis thaliana* were described in Chapter 1. Phenotypic characterization of the *Synechocystis* sp. PCC 6803 HPT disruption mutant lacking tocopherols (SLR1736::Km^r) is also presented. In Chapters 2 and 3, molecular and biochemical characterization of Arabidopsis transgenic plants overexpressing HPT were presented to address the question whether HPT activity is limiting for tocopherol synthesis in non-stressed and stressed Arabidopsis plants.

I identified the genes encoding SynHPT and AtHPT based on their similarity to chlorophyll synthase from *Synechocystis* sp. PCC 6803, an analogous prenyltransferase in chlorophyll biosynthesis. Both SynHPT and AtHPT utilized HGA and PDP, but not SDP, to yield MPBQ indicating that HPT is specific to tocopherol, but not plastoquinone biosynthesis. This hypothesis was further supported by the finding that the tocopherol-deficient mutant SLR1736::Km^r showed wild-type PQ-9 levels. The gene encoding homogentisate solanesyltransferase in cyanobacteria and plants remains to be identified. There are several ORFs encoding prenyltransferases similar to SynHPT in *Synechocystis* genome, which has been fully sequenced. In *Synechocystis* sp. PCC 6803, the ORFs SLR1518 and SLL0057 encode 1,4-dihydroxy-2-naphtoate phytyltransferase involved in phylloquinone synthesis and chlorophyll synthase, respectively (Addlesee et al., 1996; Reategui et al., 1998; Addlesee and Hunter, 1999). The only remaining putative HST ORF is SLR0096, which shows the highest homology to the *E. coli* hydroxybenzoate octaprenyltransferase (Figure 25), which is known to catalyze the condensation of 4-hydroxybenzoate and octaprenyl diphosphate in the biosynthesis of ubiquinone, an electron carrier involved in mitochondrial respiration (Soballe and Poole, 1999).

Several lines of evidence suggest that SLR0096 encodes putative HST. Because *Synechocystis* sp. PCC 6803 does not synthesize ubiquinones, both respiratory and photosynthetic electron transport involve PQ-9 as an electron carrier in this cyanobacterium (Friedrich and Weiss, 1997). In addition to SLR0096, *Synechocystis* genome contains

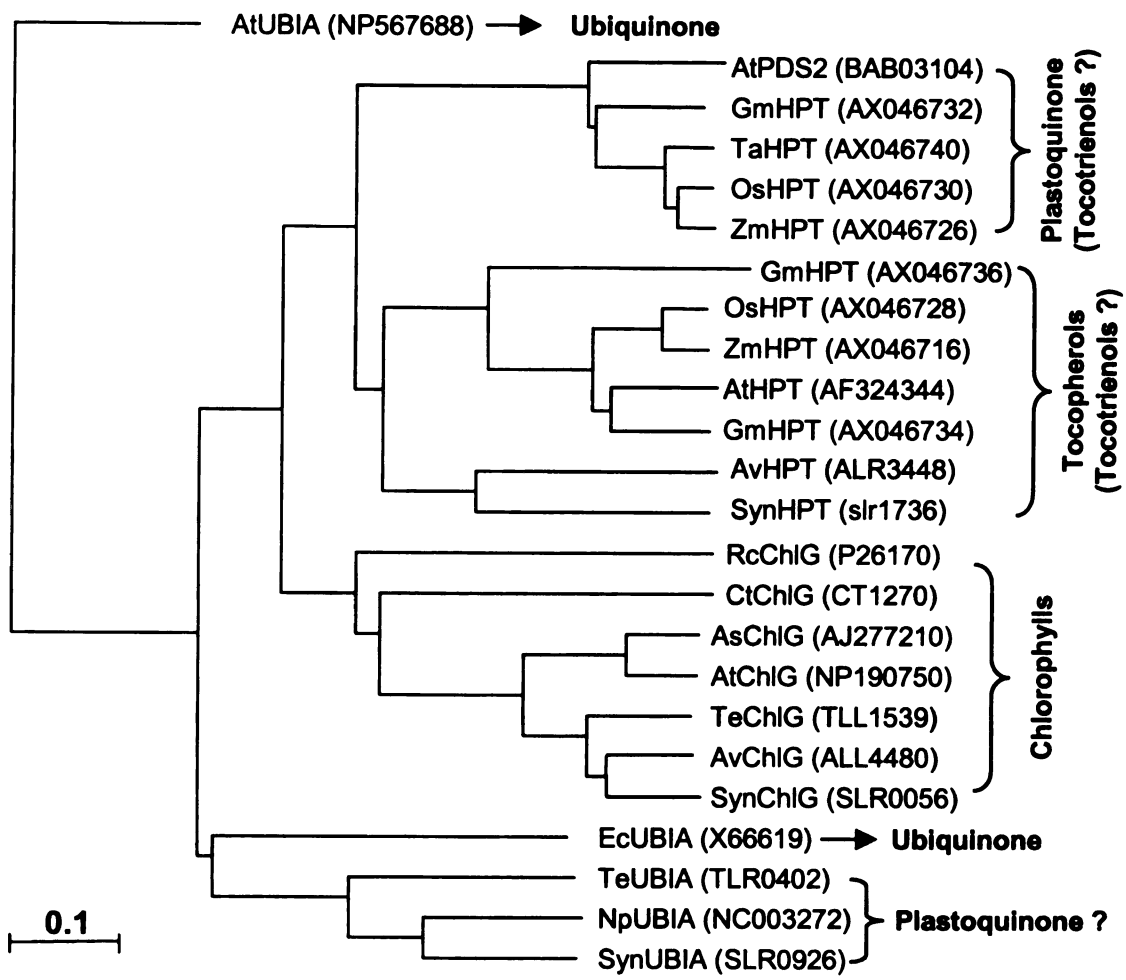

Figure 25. Phylogenetic analysis of related prenyltransferases involved in tocopherol, tocotrienol, plastoquinone, chlorophyll, and ubiquinone biosynthesis. Numbers in parentheses represent GenBank accession numbers or ORF numbers from the online database CyanoBase. Analysis was performed as described for Figure 12 in Chapter 1. Arabidopsis UbiA was used as an outgroup. The transit peptides were not included in the analysis. Plants: As – *Avena sativa*, At - *Arabidopsis thaliana*, Gm – *Glycine max*, Os – *Oryza sativa*, Ta – *Triticum aestivum*, Zm – *Zea mays*; Eubacteria: *Anabaena* sp. PCC 7120, Ct – *Chlorobium tepidum* TLS, Ec – *Escherichia coli*, Nos – *Nostoc* sp. PCC 7120, Rc – *Rhodobacter capsulatus*, Syn – *Synechocystis* sp. PCC 6803, Te – *Thermosynechococcus elongatus* BP-1

other ORFs that show remarkable similarity to other *E. coli* ubiquinone biosynthetic enzymes such as UbiX and UbiH (Dahnhardt et al., 2002). PQ-9 synthesis in *Synechocystis* sp. PCC 6803 does not require HPPD, the enzyme involved in the formation of the aromatic precursor HGA (Dahnhardt et al., 2002). HPPD ORF is absent from the genomes of cyanobacteria that can synthetize PQ-9, but not tocopherols (Sattler et al., 2003). Thus, PQ-9 synthesis is independent of HPPD and HGA and probably proceeds through a ubiquinone-like pathway using chorismate as a precursor and the ubiquinone-like solanesyltransferase SLR0096 in this cyanobacterium. Initially, feeding experiments using various radiolabeled aromatic precursors in tocopherol and plastoquinone synthesis including tyrosine, HPP, HGA, chorismate, prephenate, and hydroxybenzoate should be performed with wild type *Synechocystis* sp. PCC 6803 and the deletion HPPD mutant Δslr0090 (Dahnhardt et al., 2002) to delineate the cyanobacterial PQ-9 biosynthetic pathway.

In contrast to *Synechocystis* sp. PCC 6803, HPPD plays a key role in plant plastoquinone biosynthesis by providing HGA for HST (Norris et al., 1995; Norris et al., 1998; Garcia et al., 1998). In Arabidopsis, HST is probably encoded by the *PDS2* gene, disruption of which leads to the lethal albino phenotype identical to that of the *pds1* mutants (Norris et al., 1995). It is unlikely that the *PDS2* gene encodes SDP synthase or other enzymes of isoprenoid synthesis upstream of SDP synthase, disruption of which would also result in a lethal albino phenotype (Estevez et al., 2000; Mahmoud et al., 2001), because the *PDS2* gene product strongly resembles the prenyltransferases. Phylogenetic analysis of prenyltransferases from several plants has identified a separate clade containing putative HSTs, which includes PDS2. This clade is more closely related to HPTs than to other prenyltransferases such as 4-hydroxybenzoate polyprenyltransferases (ubiquinone synthesis) or chlorophyll synthases (Figure 25). Confirmation that the *PDS2* gene encodes HST can be accomplished by cloning the *PDS2* gene, showing the prenyltransferase activity

using radiolabeled HGA and SDP, and functionally complementing the Arabidopsis *pds2* mutant.

SynHPT, but not AtHPT, was also able to catalyze the condensation of HGA and GGDP *in vitro* to form MGBQ, a prenylquinol precursor in tocotrienol biosynthesis. These results were surprising because *Synechocystis* sp. PCC 6803 does not accumulate detectable levels of tocotrienols. Thus, the geranylgeranyl tails of tocotrienols or their prenylquinol precursors may be efficiently reduced by GGDP reductase (ChlP) in *Synechocystis* sp. PCC 6803 (Keller et al., 1998). Further research is required to determine the significance of the GGDP utilization for tocopherol/tocotrienol biosynthesis *in vivo* and the *Synechocystis* GGDR mutant, which is unable to produce PDP may be helpful in addressing this question. The presence of tocotrienols in this mutant would suggest that SynHPT can use GGDP as a substrate *in vivo*.

Besides AtHPT, we have identified numerous putative prenyltransferases in other plants (DellaPenna et al., 2000). Based on their sequence similarity, these prenyltransferases fall into two main functional clades (Figure 25). First, as mentioned above, proteins similar to PDS2 are probably solanesyltransferases involved in PQ-9 synthesis. Second, sub-clades of closely related monocot and dicot prenyltransferases similar to AtHPT are most likely involved in tocopherol biosynthesis. Some of the monocot HSTs or HPTs may represent prenyltransferases that utilize HGA and GGDP as substrates in tocotrienol biosynthesis. Additional partial clones that were identified in some monocots and are not shown in the phylogenetic tree may be responsible for tocotrienol synthesis. Biochemical analysis needs to be done to reveal substrate specificities of these putative prenyltransferases. All these enzymes should be tested for the prenyltransferase activity *in vitro* using PDP, GGDP, and SDP. To determine whether dicots retained their ability to synthesize tocotrienols, some of the confirmed monocot geranylgeranyltransferases may be overexpressed in a dicot such as Arabidopsis. If successful, this experiment will represent the first step in the engineering of tocotrienol synthesis in plants. Tocotrienols have

cholesterol lowering properties in animals (Packer et al., 2001) and engineering dicot crops for high tocotrienol content would provide novel sources of dietary tocotrienols.

In addition to wild type Arabidopsis, the geranylgeranyltransferase cDNAs should also be transformed into the tocopherol-free background of the HPT T-DNA tagged Arabidopsis mutant or GGDR antisense transgenic Arabidopsis plants, both isolated in Dr. Dean DellaPenna's laboratory (unpublished data). These plants may be particularly helpful in determining whether the geranylgeranyl tail of tocotrienols or their intermediates is converted to phytyl tail of tocopherols by GGDR and in testing for both geranylgeranyl and phytyltransferase activities *in vivo*. Tocotrienols are present in seeds, but not in leaves in monocots (Franzen and Haas, 1991), which suggests that the two types of tissues may be metabolically distinct. These distinction could be a result of the differences in GGDP availability or expression of the tocotrienol biosynthetic genes in the two tissues. Therefore, both leaf and seed tocotrienol analyses should be performed in these transgenic plants.

Regulation And Metabolic Engineering of Tocopherol Biosynthesis in Arabidopsis

HPT catalyzes phytylation of HGA and appears to be a highly regulated enzyme in the tocopherol biosynthetic pathway. In Chapters 2 and 3, I presented data showing that HPT activity is limiting for tocopherol synthesis in non-stressed wild type Arabidopsis leaves and seeds as well as in leaves subjected to high light stress. Overexpression of HPT in Arabidopsis resulted in increased HPT mRNA levels and activity and subsequently in elevated total tocopherol levels in non-stressed leaves and seeds compared to wild type. Similarly, stressed 35S::*HPT1* leaves exhibited increased HPT activity and accumulated up to 3.8-fold increased total tocopherol levels relative to stressed wild type Arabidopsis leaves. Analysis of prenylquinol and tocopherol composition in stressed wild type and 35S::*HPT1* leaves was useful in identifying two other enzymatic steps that limit α-tocopherol synthesis, MPBQ and γ-tocopherol methylation reactions. Collectively, these data contribute

significantly to the current knowledge about the regulation of tocopherol synthesis. However, there are several questions that need to be addressed about the regulation of tocopherol biosynthetic pathway under normal and stress conditions.

From the developmental point of view, HPT activity decreases with age in non-stressed wild type and 35S::*HPT1* leaves as young, 2-week old plants exhibited higher HPT activity than 4- and 6-week old plants. However, a more detailed analysis during initial stages of plant development (during and within the first 2 weeks after seed germination) and in senescing plants (more than 8 weeks old) would complement the current knowledge about tocopherol biosynthetic pathway and its regulation. Wild type Arabidopsis seeds contain approximately 4-fold more tocopherols than leaves, when expressed on a dry weight basis. Seed germination is accompanied by an increase in oxidative stress as photosynthetic apparatus begins to function (Lichtenthaler, 1969). Similarly, leaf senescence is also associated with increased oxidative stress in chloroplasts (Munne-Bosch and Alegre, 2002c). Increased oxidative stress translates into increased tocopherol degradation and a need for tocopherol synthesis to sustain oxidative damage. As described in Chapter 2, dry Arabidopsis seeds contain detectable HPT activity, suggesting that HPT may contribute to the *de novo* tocopherol synthesis in young developing seedlings. It would be interesting to study the role of HPT in tocopherol synthesis and to identify other possible bottlenecks of the pathway in germinating seeds, developing seedlings, and senescing plants by using wild type and 35S::*HPT1* Arabidopsis lines.

In Chapter 3, steady-state levels of tocopherols and their prenylquinol intermediates in control and high light stressed wild type and 35S::*HPT1* leaves are used to shed some light into regulation of the tocopherol pathway. There are several processes that may contribute to the steady-state tocopherol levels in chloroplasts: tocopherol biosynthesis, degradation, and recycling. Recycling of the tocopheroxyl radicals back to tocopherols has been demonstrated *in vitro*, but there is only indirect evidence that it occurs *in vivo* (Munne-Bosch and Alegre, 2002b). The lack of evidence for the *in vivo* tocopheroxyl radical

recycling is primarily due to inability to distinguish between tocopherol recycling and scavenging of ROS by ascorbate (Kagan and Packer, 1994).

Tocopherol oxidation yields the corresponding tocopheryl quinone. In non-stressed tissues, α-tocopheryl quinone comprises from 4 to 10% α-tocopherol levels (Lichtenthaler, 1969; Lichtenthaler et al., 1981; Michalski and Kaniuga, 1981). The levels of α-tocopheryl quinone increased up to 3-fold in stressed tomato leaves relative to non-stressed leaves (Michalski and Kaniuga, 1981). Therefore, total tocopherol levels presented for the high light time course for both wild type and 35S::*HPT1* are likely underestimated and do not represent total tocopherol synthesis because tocopheryl quinone levels were not measured. In addition, the steady-state tocopherol levels alone cannot be used to determine flux through the pathway, hence a detailed flux analysis in non-stressed and stressed wild type and 35S::*HPT1* leaves should be performed. This analysis would require an effective separation, identification, and quantitation of major tocopherol biosynthetic precursors and breakdown products. Arabidopsis tocopherol biosynthetic mutants in HPT, TC, MPBQ MT, and γ-TMT, which are currently available in Dr. Dean DellaPenna's laboratory, should be helpfull in identifying the individual intermediates in tocopherol biosynthesis and degradation.

Real-time PCR experiments suggested that some tocopherol biosynthetic genes are up-regulated during high light stress. All genes encoding the enzymes of the shikimate pathway were also transcriptionally up-regulated during biotic and abiotic stress (Jones et al., 1995; Bischoff et al., 1996; Diaz and Merino, 1998; Gorlach et al., 1995; Batz et al., 1998). To obtain a complete picture about the transcriptional regulation of the whole pathway during stress, all genes, including these from the shikimate and DXP pathways as well as the genes encoding TAT, chlorophyllase, and tocopherol enzymes presented in Figures 3, 4, and 5 should be used in a microarray experiment. Total RNA isolated from leaves of non-stressed wild type Arabidopsis plants and those subjected to various stress treatments including high light and JA can be reverse-transcribed, labeled, and utilized to

probe the microarray slide. A global induction of transcription of the tocopherol-related genes in stressed plants would indicate an existence of one or more transcriptional regulators in tocopherol biosynthesis.

Valuable data contributing to the understanding of the regulation of tocopherol biosynthetic pathway in Arabidopsis plants under normal and stressed conditions are presented in this dissertation. As outlined in this section, additional research is required to further characterize mechanisms that regulate plant tocopherol levels. Besides studying these mechanisms, future tocopherol research should also focus on determining role(s) of tocopherols in plants and interaction of tocopherols with other antioxidants.

APPENDIX

EXPERIMENTAL PROCEDURES

In this section, procedures used in this study are described in a great detail such that all experiments presented can be repeated using these protocols without the necessity of consulting other publications. Unpublished observations or problems that one may encounter when using some of the procedures or their limitations are also presented. All relevant primers, their sequences, and use are summarized in Tables VIII and IX.

Chemicals And Standards

Large-scale quinone extraction. The purified PQ-9 standard was used to create standard curves for PQ-9 quantification in *Synechocystis* sp. PCC 6803, while 2-DMPQ-9 was used as an internal standard for quantitative quinone determinations. PQ-9 and 2-DMPQ-9 standards were extracted from *Iris hollandica* bulbs and purified by both TLC (Pennock, 1985) and HPLC (Johnson et al., 2000). PQ-9 can also be extracted from leaves of many plants such as spinach, peas, or ficus, but due to the lack of contaminating chlorophylls, *Iris hollandica* bulbs are an ideal quinone source. In addition, other quinones such as PQ-8, 2-DMPQ-8, and ubiquinones can also be obtained from these bulbs. Bulbs were cleaned and cut into small pieces. Tissue was frozen in liquid nitrogen and stored at - 80 °C or used immediately for lipid extractions. After adding 100 mL of 50% methanol (v/v) to 50 g of bulbs, tissue was homogenized in a blender. The homogenate was transferred to a clean glass beaker containing 50 mL of chloroform. After 5 - 10 minute incubation at room temperature, the homogenate was filtered through 6 layers of miracloth and the filtrate was centrifuged at 8000 g for 10 minutes. The organic phase (bottom phase) was dried using a rotary evaporator at 30 °C, lipids were dissolved in 1 - 2 mL of ethylacetate and quinols

were oxidized by shaking with 50 mg of silver oxide in glass tubes at room temperature for 2 h. Lipids were subjected to separation by TLC (Silica) in 20% ethyl ether (v/v) in petroleum ether. PQ-8 and -9 migrated together in this TLC system (R_f 0.88; right below β-carotene). 2-DMPQ-8 and -9 also migrated together, but at R_f 0.77. Silica corresponding to the two areas was scraped and transferred to 5 mL syringe columns; quinones were then eluted with 5 volumes of ethyl ether, dried under nitrogen, and subjected to reverse phase HPLC. The following gradient was used to purify these prenylquinones (Johnson et al., 2000): isocratic flow from 100% methanol for 10 minutes changed to a gradient of 3% (v/v) methanol in isopropanol within next 20 minutes, which was held for additional 10 minutes, all at flow 0.5 mL min^{-1}. The column was equilibrated for 15 minutes with 100% methanol at 2 mL min^{-1}. Quinones were dried, dissolved in 0.5 mL of hexane, and stored at - 20 °C for at least 3 years without detectable degradation. Purity of the quinones was confirmed by HPLC and the PQ-9 concentration was determined spectrophotometrically at 255 nm in hexane using ε 18,030 M^{-1} cm^{-1} (Pennock, 1985).

BG-11 medium. BG-11 liquid medium can be prepared as follows: One liter of BG-11 contained 10 mL 100X BG-11 and 1 mL of each 1000X solutions. 100X BG-11 without iron, phosphate, carbonate had149.6 g $NaNO_3$, 7.5 g $MgSO_4$, 3.6 g $CaCl_2.2H_2O$, 0.6 g citric acid, 1.12 mL 0.25 M NaEDTA pH 8, 100 mL trace minerals per 1 L. Trace minerals were present at 2.86 g H_3BO_3, 1.81 g $MnCl_2.4H_2O$, 0.22 g $ZnSO_4.7H_2O$, 0.39 g $Na_2MoO_4.2H_2O$, 0.079 g $CuSO_4.5H_2O$, 0.049 g $Co(NO_3)_2.6H_2O$ per 1 L. Other components included 1000X stocks of 6 mg mL^{-1} ferric ammonium citrate, 20 mg mL^{-1} Na_2CO_3, 30.5 mg mL^{-1} K_2HPO_4. For agar plates, 10 mL 1M TES/NaOH buffer pH 8.2, 3 g sodium thiosulfate, and 10 g phytagar were added. For photoheterotrophically grown cyanobacteria, glucose was added to a final concentration 15 mM.

Plasmids And Mutants

Generation of constructs, mutants, and transgenic plants. Primers SLR1736F and R (Table VIII) were used to amplify the SLR1736 ORF (GenBank accession number BBA17774) from *Synechocystis* genomic DNA using Vent DNA polymerase (New

Table VIII. PCR primer pairs used in this study.

Name	Sequence (from 5' to 3') of primer pairs	This primer pair was used to:
SLR1736F SLR1736R	TATTCATATGGCAACTATCCA AGCTTTTTG GGATCCTAATTGAAGAAGAT ACTAAATAGTTC	Amplify SLR1736 ORF in Synechocystis and to show the absence of wild type copies of this ORF in the SLR1736::Km^r mutant.
AT1736F AT1736B	TTGTTTTCAGGCTGTTGTTGC AGCTCTC CGTTTCTGACCCAGAGTTAC AGAGAATG	Amplify a part of AtHPT genomic sequence for use as a probe to screen an Arabidopsis cDNA library.
HPTF HPTB	CCATGGAGTCTCTGCTCTC GGATCCCAAGCAGAGACTTC TTTACC	Amplify full-length HPT1 cDNA for protein expression in E. coli.
HPTΔF HPTB	AGCCATGGGTTGTGATTC GGATCCCAAGCAGAGACTTC TTTACC	Create a truncated version of AtHPT lacking the first 37 aa.
HPT96 HPTB	CCATGGACTCGTTAGATGCG GGATCCCAAGCAGAGACTTC TTTACC	Create a truncated version of AtHPT lacking the first 96 aa.

England Biolabs, Beverly, MA). The following program was used: 95 °C for 5 minutes (1 cycle); 95 °C for 45 seconds, 45 °C for 45 seconds, and 68 °C for 45 seconds (5 cycles); 95 °C for 45 seconds, 52 °C for 45 seconds, and 72 °C for 45 minutes (30 cycles); 72 °C for 10 minutes (1 cycle). Note that no PCR products were obtained without the 5-cycle "priming". ATG in the SLR1736F represents the start codon of the SLR1736 ORF. The *Nde*I and *Bam*HI sites (underlined) were engineered in the two primers to facilitate further sub-cloning procedures for protein expression. The amplified SLR1736 ORF was sub-

cloned into the *Eco*RV site of pBluescript II KS (+). The resulting construct pKS1736 and pUC4K (Taylor and Rose, 1988) were digested with *Mfe*I and *Eco*RI. Ligation of pKS1736 with the kanamycin resistance cassette released from pUC4K yielded two constructs, one with the same (pKS-1736-KanF) and one with the opposite (pKS-1736-KanR) orientation of the kanamycin resistance cassette relative to the SLR1736 ORF. Both constructs were used to transform wild type *Synechocystis* sp. PCC 6803 and generate disruption mutants by homologous recombination (Williams, 1988). Wild type cells (0.1 mL, 10^9 cells mL^{-1}, OD_{730} of 0.25 corresponds to 10^8 cells mL^{-1}) were mixed with the knockout construct DNA (~ 1 μg) and incubated at 20 - 30 μE at 30 °C with shaking. After a 4-hour incubation, the cells were pelleted and plated on sterile nitrocellulose filters (without detergent) placed onto BG-11 plates with 15 mM glucose and without kanamycin. After 2 days of incubation at 20 - 30 μE at 30 °C, the filters were transferred onto fresh BG-11 plates containing 15 mM glucose and 15 μg mL^{-1} kanamycin. Selected transformants were sub-cultured on kanamycin containing media for 6 plating cycles and the absence of wild type SLR1736 gene copies in the mutants was confirmed by PCR using SLR1736F and R primers followed by Southern blot analysis. Because there was no phenotypic difference between the two orientation disruption mutants (data not shown), the disruption mutant derived from pKS-1736-KanF (referred to hereafter as the SLR1736::Km^r mutant) was used for further analyses.

A single genomic clone, F19F24, was identified in the *Arabidopsis thaliana* database based on its protein similarity to the SLR1736 protein. Primers AT1736F and B (Table VIII) were used to amplify a 977-bp fragment from F19F24 with the following PCR program: 95 °C for 5 minutes (1 cycle); 95 °C for 45 seconds, 54 °C for 45 seconds, and 72 °C for 1 minute (30 cycles); 72 °C for 10 minutes (1 cycle). The resulting PCR product was utilized as a probe to screen a pBluescript II SK-based Arabidopsis seed cDNA library (a gift of Dr. John Ohlrogge, Michigan State University, East Lansing, MI). The longest of 15 positive clones (#1) was sequenced (see Table IX for sequencing primers) and

designated pSK*HPT1* (Figure 26A), which is also labeled as pSK-AT1736. The *HPT1* gene encodes a prenyltransferase similar to SLR1736, AtHPT.

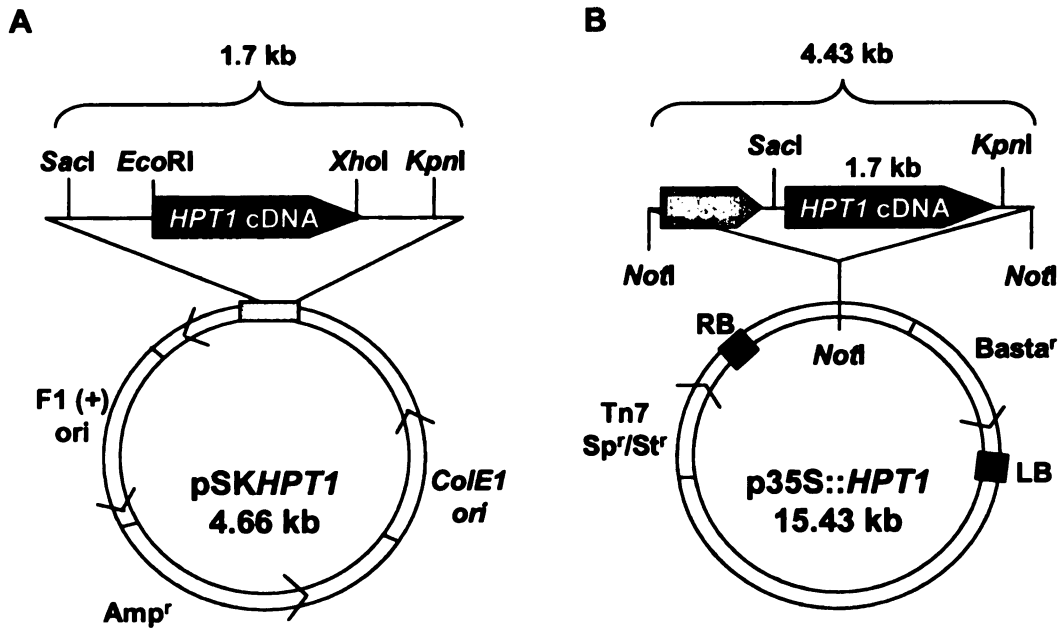

Figure 26. Constructs used in this study. A, pSK*HPT1* B, p35S::*HPT1*. Amp – ampicillin; kb – kilobases; LB – left border; ori – origin of replication; RB – right border; Sp – spectinomycin; St – streptomycin

For protein expression in *E. coli*, the entire SLR1736 ORF was released from pKS1736 by *Nde*I and *Bam*HI digestion and sub-cloned into *Nde*I/*Bam*HI digested pET30b (Novagen) to create pSynHPT. Similarly, primers HPT1 and 2 (Table VIII) were used to amplify the full-length Arabidopsis *HPT1* gene from pSK*HPT1*. To facilitate sub-cloning into the pET vectors, the *Nco*I and *Bam*HI sites were introduced into these primers (Table VIII, underlined). The PCR product was sub-cloned into the *Eco*RV site of pBluescript II KS (+) to generate pKS-HPT. After digestion of pKS-HPT and pET3d with *Nco*I and *Bam*HI and ligation, pAtHPT was generated. The pSynHPT and pAtHPT constructs were transformed into BL-21 (DE3) cells (Novagen, Milwaukee, WI) to express both prenyltransferases for the enzyme assays. For enzyme activity purposes, two additional truncated versions of AtHPT were used. The first truncation removed the first 37

amino acids corresponding to a transit peptide predicted by PSORT (primers used: HPTΔF and HPTB) and the second truncation removed the first 96 amino acids corresponding to a putative transit peptide missing from SynHPT, (primers used: HPT96 and HPTB).

Table IX. Sequencing primers used in this study.

Primers for SynHPT	
1736T3S	CCAGAGTAAGGCTAATAAGCAC
1736T7S	TGACCGCATCAATAAGCC
Primers for AtHPT	
AT1736T3C	CGTTTATGAGCTTTTTCTCTGTCG
AT1736T7C	GACATATTTTTGCAGTCTGCC
0AT1736F	TTCGAGTAAAGTTGTCGC
1F1736A	AGAGACTCGTTAGATGCG
2FAT1736	TTTCACTGGCATCTTGGAGG
3FAT1736	TGCAGCAATGTGTATCCTCG
4FAT1736	CTCGCAACAACTTTGTGGGC
0AT1736R	TGCCAATGGAAGATAGGG
1RAT1736	TTCCAACAAAACCCACCAGC
2RAT1736	AAAATGCTAAGCACTGTGCC
3RAT1736	GTACCGAGCATGAAACTCAC
4R1736	GCCACTGCGTTTATGAGC
4R1736A	AGTGGCGAAAATAAGAGG
5RAT1736	CAAGCAGAGACTTCTTTACCC

To generate transgenic plants in Arabidopsis overexpressing the *HPT1* gene, p35S::*HPT1* (Figure 26B) was created by sub-cloning the cDNA encoding HPT from pSK*HPT1* into pART7 and then into pMLBART, a pART27-based vector (Gleave, 1992). Both pSK*HPT1* and pART7 were subjected to digestion with *Sac*I and *Kpn*I and subsequent ligation to yield pART*HPT1*. Digestion of pART*HPT1* with *Not*I released a fragment containing the CaMV 35S rRNA promoter followed by the *HPT1* gene in the sense orientation. This fragment was ligated into the *Not*I site of pMLBART containing the *bar* gene for selection of transformed plants on glufosinate. The resulting construct p35S::*HPT1* was introduced into *Agrobacterium tumafaciens*. 35S::*HPT1* T_0 sense plants

were obtained by transforming wild type Arabidopsis plants (ecotype Columbia) with *Agrobacterium* containg p35S::*HPT1* using the floral dip method (Clough and Bent, 1998).

Briefly, the floral dip medium contained 5% sucrose and 0.05% Silwet L-77. Plants were submerged into this medium for ~ 5 seconds and left covered overnight in dark to keep high humidity. T_1 seeds were planted in soil and seedlings were sprayed with the glufosinate solution (120 mg of ammonium glufosinate and 0.25 mL of silwett per 1 liter of water) three times after their germination to ensure the elimination of wild type seedlings: before and after most of them developed the first pair of true leaves and one more time after the sixth leaf development (it takes about 5 days to visually observe the effects of the herbicide sensitivity). Surviving plants (66 independent transformants) were analyzed for tocopherol content. Lines 35S::*HPT1*-8, 11, 16, 21, 35, 37, 53, 54 showed increased leaf tocopherol levels and their T_2 plants segregated 3:1 for glufosinate resistance. These lines were carried through to the homozygosity. Lines 35S::*HPT1*-11 and -54 were selected for further analyses and for the crosses with 35S::*γ-TMT*-18 and –49, the two best characterized Arabidopsis lines overexpressing *γ-TMT* under the control of 35S CaMV promoter (Shintani and DellaPenna, 1998).

Seed from crosses #9 (35S::*HPT1*-11 X 35S::*γ-TMT*-49), #14 (35S::*HPT1*-54 X 35S::*γ-TMT*-18), #16 (35S::*HPT1*-54 X 35S::*γ-TMT*-49), and #20 (35S::*HPT1*-11 X 35S::*γ-TMT*-18) were surface sterilized, plated on MS plates containing 50 μg mL^{-1} Km, transferred to soil, and sprayed with glufosinate after 5 days of adaptation to soil to select for dual resistance. These plants were hemizygous for Km and glufosinate resistance. Their seed was germinated on plates and 50 - 60 seedlings for each cross were selected for dual resistance as described above. Seeds of each of these plants were harvested individually and planted on soil. After glufosinate selection, seeds of the lines homozygous for glufosinate resistance were plated on MS plates containing Km. Double homozygotes were selected for crosses #9, #14, #16, and #20. In crosses #14 and #16, Km and

glufosinate resistances were linked, suggesting that 35S::*HPT1*-54, 35S::*γ-TMT*-18, and 35S::*γ-TMT*-49 are localized on the same chromosome.

Oxygen Evolution Rates

Oxygen evolution rates. Liquid cultures of photoautotrophically grown wild type and mutant *Synechocystis* cells were washed twice with fresh BG-11 medium and resuspended in BG-11 medium. For O_2 evolution experiment, the cells were diluted to a concentration of 3 mg chlorophyll per mL. The cells were incubated in the dark for 1 h. Cell suspension (1 mL) was placed into the cell and O_2 consumption was measured in the dark for one minute. Subsequently, the cells were exposed to high light for 5 minutes and three different light intensities were used: 0.75, 2 and 5 mE. O_2 measurements were performed with a Clark-type electrode at 25 °C using a Hansatech CB1-D3 recording unit with Minirec recording software (Hansatech Instruments, King's Lynn, England). The O_2 evolution rate was calculated from the slope within the linear region of the curves.

Gloves must be worn while handling the electrode. The electrode was prepared as follows: A square of 1.5 cm cigarette paper was pre-wet in saturated KCl solution, placed on the top of the electrode, and then covered by a same sized piece of S4 PTFE membrane (Hansatech Instruments). The small rubber O-ring was used to secure the paper and membrane to the electrode without any bubbles or membrane damage. The electrode was attached to the cell and the large rubber O-ring was used to prevent leakage from the cell at the junction with the electrode. Temperature was controlled by a thermostat, while a magnetic stirrer was used to ensure homogeneity of the cell suspension. The electrode was allowed to equilibrate for 1 - 2 h in air-saturated water or until a stable maximal baseline (1400 - 1600 U) was observed. Sodium dithionate was added to the cell to remove O_2 and to determine the minimal baseline (~ 40 U). If the electrode was prepared properly, the response to sodium thiosulfate should be very fast and the reading of the lower baseline

should be obtained within less than 1 minute. Because sodium dithionate tends to bind to the electrode, it was quickly removed by washing with air-saturated water. O_2 evolution rates obtained from dark measurements were subtracted from the rates obtained during the high light treatment and the resulting values represent the net O_2 evolution rates. The following equation was used to calculate O_2 evolution rates [μmol O_2 mg^{-1} Chlorophyll h^{-1}]:

$$O_2 \text{ evolution rate} = (S \times 60 \times 60 \times dy)/(\Delta y \times C),$$

where S is the solubility of O_2 in water at 25 °C and corresponds to 0.253, dy is the slope of the linear response obtained from measurements collected every second, Δy can be obtained by subtracting the minimal baseline from the stable maximal baseline, and C is the total chlorophyll amount in mg.

Prenyllipid Analyses

Tocopherol analyses. Wild type *Synechocystis* and the SLR1736::Km^r mutant was grown on BG-11 plates either photoheterotrophically (with 15 mM glucose) or photoautotrophically (without glucose) at 20 - 30 μE at 30 °C for 2 weeks. The cells (15 - 20 mg) were harvested and their lipids were extracted in eppendorf tubes by adding 200 μL of 2:1 methanol:chloroform (v/v). Tocol (50 - 100 ng) was added and the cells were disrupted by grinding with a pestle. Chloroform (70 μL) and water (200 μL) were added and the tubes were centrifuged at 13,000 rpm for 5 minutes after vortexing. The organic phase was transferred to a fresh tube and dried under vacuum. The lipids were dissolved in 100 μL of hexane and 10 μL of each sample was withdrawn for chlorophyll determination (Lichtenthaler, 1987), while 50 μL of the lipid extract was subjected to HPLC (Hewlett Packard 1100, Wilmington, DE) on a LiChrosorb 5 Si60A 4.6 X 250 mm normal phase column (Phenomenex, Torrance, CA) at 42 °C and 1 mL min^{-1} using a gradient of isopropyl

ether and hexane (v/v) according to Syvaoja et al. (1986). Within the first 30 minutes, isopropyl ether content was increased from 8% to 18% and kept at 18% for an additional 10 minutes. The column was then equilibrated with 8% of isopropyl ether in hexane (v/v) for 15 minutes. Tocopherols were detected by fluorescence using 290 nm excitation and 325 nm emission.

To analyze tocopherol content in non-stressed wild type and transgenic Arabidopsis leaves and seeds, plants were grown in a 16-hour photoperiod (70 - 100 μE) at 22/19 °C day/night cycle. Lipids from 30 - 35 mg of leaf tissue or 10 - 15 mg of seeds were extracted in the presence of butylated hydroxytoluene (2 mg mL^{-1}) to prevent tocopherol degradation (Bligh and Dyer, 1959). Tocol was used as an internal standard. For leaf analysis, tocopherols were separated on a reverse phase HPLC (C_{18}, ODS2, 4.6 X 250 mm, Column engineering, Ontario, CA; Shimadzu VP HPLC system, Japan) using an isocratic solvent system of 5% (v/v) isopropanol in methanol at 2 mL min^{-1}. For seed analysis, a 10-minute isocratic method using 17 % di-isopropyl ether in hexane (v/v) at 42 °C at 2 mL min^{-1} was utilized on a normal phase HPLC system (LiChrosorb 5 Si60A 4.6 X 250 mm Silica column, Column engineering, Ontario, CA and HP 1100 series HPLC system, Hewlett Packard, Wilmington, DE). Tocopherols were detected by fluorescence at 290 nm excitation and 325 nm emission.

Real-Time PCR

Real-time PCR. Plants (wild type and 35S::*HPT1* #11 and 54) were grown at a 10-hour photoperiod at 75 - 100 μE for 6 weeks. Tissue from three representative plants was harvested 2 - 3 hours after the start of the light cycle and immediately frozen in liquid nitrogen. Total RNA was isolated and any contaminating genomic DNA was removed by treatment with RQ1-RNase free DNase (Promega, Madison, WI) and re-extracted with phenol and chloroform to remove the DNase. Total amount of 9 μg of total RNA was

reverse transcribed to generate cDNA in three 30-μL reactions for each sample using a TaqMan kit according to manufacturer recommendations (Applied Biosystems, Foster City, CA) in a 96-well format. Briefly, each 30-μL reaction contained 3 μL 10X RT buffer, 6.6 μL 25 mM $MgCl_2$, 6 μL dNTPs, 1.5 μL oligo dT_{16}, 1.2 μL RNasin, 0.75 μL Multiscribe Reverse Transcriptase™, and 3 μg total RNA (in 10.95 μL water). The reactions were pre-incubated at 25 °C for 10 minutes and reverse transcription was carried out at 48 °C for 30 minutes. The enzyme was inactivated by incubation of samples at 95 °C for 10 minutes. An aliquot of cDNA corresponding to 200 ng of total RNA was used in each TaqMan real-time PCR assays (Applied Biosystems). Each 30-μL reaction contained 15 μL of 2X mastermix purchased from Applied Biosystems, 3 μl of each primer and the corresponding probe, and 6 μL cDNA. Standard curves were constructed for each gene and used to calculate the corresponding mRNA concentrations.

Prenyltransferase Assays

Prenyltransferase Assays Using *E. coli* Protein Extracts

Preparation of the *E. coli* protein extracts (adapted from pET vector manual). *E. coli* cells harboring pET1736, pETAtHPT, pACHPPD, and vectors alone (pET3d and pET30b) were grown overnight at 30 °C with shaking. Each of these cultures was used to inoculate 50 mL LB media containing appropriate antibiotics (50 mg L^{-1} kanamycin for pET30b and pETAtHPT and 100 mg L^{-1} ampicillin for pET3d, pET1736, and pACHPPD). The cells were grown for 3 - 4 h at 30 °C with shaking until the cell density reached OD_{600} 0.6. With the exception of the cells harboring pACHPPD, 240 mg L^{-1} IPTG was added to induce protein expression from the lacZ operone. Cells containing pET1736 and pET3d were shaken at 30 °C, while those transformed with pETAtHPT, pACHPPD, and pET30b were transferred to room temperature. After 4h of shaking, the cells were washed once with

the ice-cold HPT reaction buffer (50 mM HEPES (pH 7.6) containing 4 mM $MgCl_2$) and sonicated on ice by 3 or 4 short, 10-second pulses at 4 - 5 output control using a microtip probe (W-220F sonicator, Ultrasonics, INC., Plainview, NY). After centrifugation, the protein pellets (all expressed proteins were previously found to be associated with insoluble inclusion bodies) were washed twice and resuspended in the HPT reaction buffer. These crude *E. coli* protein extracts were stored at - 20 °C for up to 3 weeks and used for the following prenyltransferase assays.

Preparation of U-[^{14}C] HPP. U-[^{14}C] HPP was prepared from U-[^{14}C] tyrosine (sp. activity 464 mCi $mmol^{-1}$, Amersham, Arlington Heights, IL) according to Schulz et al. (1993) with minor modifications. Briefly, 2 mL of 0.5 M potassium phosphate buffer (pH 6.5), bovine liver catalase (Boehringer Mannheim, Germany), and L-amino acid oxidase type IV (Sigma, St. Louis, MO) were added to 1 mL of U-[^{14}C] tyrosine (10 μCi). The final concentration of both enzymes was 0.4 mg mL^{-1}. After 2 hours of incubation at room temperature, U-[^{14}C] HPP was purified on an ion exchange column (Dowex, Sigma) equilibrated with 0.1 N HCl as follows: the first 2 - 3 drops were discarded and the next 3 mL were collected. HPPD assays using eluted fractions and HPPD demonstrated that the first 3 mL of eluate contained most U-[^{14}C] HPP (data not shown). The eluted solution pH was adjusted to ~ 7 with KOH and used immediately for prenyltransferase assays.

Prenyltransferase assays. Each 0.2 mL reaction was performed in the HPT reaction buffer (50 mM HEPES (pH 7.6) and 4 mM $MgCl_2$) containing 100 μM KF, 0.2 % (w/v) CHAPS, 0.1 mg of total protein extracted from *E. coli* expressing HPPD, and freshly prepared 0.2 μM U-[^{14}C] HPP and 50 mM potassium ascorbate. To test HPT substrate specificity, individual reactions contained either 100 μM PDP, GGDP, or SDP and the insoluble protein fraction from *E. coli* expressing pSynHPT (0.03 mg protein), pAtHPT (1 mg protein), or the corresponding empty vectors (0.03 or 1 mg protein from cells

transformed with pET3d and pET30b, respectively). Reactions were incubated for an hour at room temperature, extracted with two volumes of methanol:chloroform (1:1), and any newly formed prenylquinols were subsequently oxidized at room temperature with AgO for 2 h with shaking (Pennock, 1985). The organic phase was transferred to a fresh tube, evaporated to dryness, dissolved in ethyl acetate, and subjected to TLC on silica gel (J.T.Baker, Phillisburg, NJ). The TLC plate was developed with 20% (v/v) ethyl ether in petroleum ether (Pennock, 1985), dried, and subjected to autoradiography. The film was exposed for 14 days.

For HPLC analysis, the polyprenyltransferase assays contained all-of-the-above components except that larger reaction volumes were needed to ensure formation of detectable prenylquinone products. The following final reaction volumes were used: 0.5 mL for SynHPT with PDP and 5 mL for SynHPT with GGDP, AtHPT with PDP, and AtHPT with GGDP. After 2 hours of incubation at room temperature, the reactions were spiked with non-labeled *2'-trans*-MPBQ used as a carrier and internal standard and extracted prenyllipids were separated by TLC as described above. For each sample, silica corresponding to the area of R_F 0.36 - 0.67 was scraped from the TLC plates, eluted with 5 mL of ethyl ether, dried under nitrogen, and dissolved in 100 μL of hexane. Samples (50 μL) were then injected on a normal phase HPLC column (LiChrosorb 5 Si60A, 4.6 X 250 mm; Shimadzu) to separate various methyl-phytyl benzoquinone isomers in 0.1% (v/v) dioxane in hexane (Henry et al., 1987). For geranylgeranylated quinone products, a mobile phase consisting of 0.15% dioxane (v/v) in iso-octane was used (Hutson and Threlfall, 1980). Isocratic separation was performed at 30 °C with a constant flow of 2 mL min^{-1} and the prenylquinones were detected at 252 nm for both methods. Eluents were collected at 30- to 60-second intervals and the associated radioactivity was determined in 5 mL of scintillation liquid by scintillation counting.

Prenyltransferase Assays Using Arabidopsis Chloroplasts And Seed Protein Extracts

Preparation of crude chloroplasts and seed protein extracts. Wild type and 35S::*HPT1*-11 and -54 Arabidopsis plants were used for all the prenyltransferase assays. To determine HPT specific activity during plant development, chloroplasts were prepared from 25 - 30 g of leaf tissue of 2, 4, and 6 week old plants. For the high-light experiment, 6-week-old wild type and 35S::*HPT1* plants were either kept at normal light conditions or transferred to high light for 6 days. Three days later, some of the non-stressed plants were transferred to high light for 3 days, so that analysis of control plants and the plants stressed for 3 and 6 days was performed the same day to ensure equal treatment of all samples in the experiment. All procedures were performed at 4 °C and all buffers were pre-chilled and kept on ice. Tissue was disrupted in 200 mL of buffer containing 0.5 M HEPES pH 8.4, 0.6 M sorbitol, 4 mM EDTA, 4 mM EGTA, 10 mM Na_2CO_3, 0.2% BSA (w/v), 1 μM benzamidine (BA), and 5 μM 4-aminocaproic acid (4-ACA) using a blender. Crude chloroplasts were filtered through two layers of miracloth and centrifuged at 4,000 g for five minutes. Chloroplasts were washed in the HPT reaction buffer containing 1 μM BA and 5 μM 4-ACA. After centrifugation, an additional wash with the HPT reaction buffer without the proteinase inhibitors BA and 4-ACA was performed and the chloroplast membranes were resuspended in the HPT reaction buffer to a final protein concentration of 5.5 - 6.5 mg mL^{-1}. Seed protein extracts were prepared as follows: Dry mature seeds (~ 100 mg) were ground in 1 mL of the HPT reaction buffer using a mortar and pestle and centrifuged. The resulting pellet of water insoluble proteins was washed once and resuspended in the HPT reaction buffer to a protein concentration of 20 - 30 mg mL^{-1}. Preliminary HPT assays showed that over 95% of HPT activity was associated with the pellet (data not shown).

Prenyltransferase assays using Arabidopsis chloroplasts and seed protein extracts. To obtain more concentrated U-[^{14}C] HPP, U-[^{14}C] tyrosine (1 mL, 50 μCi, specific activity

464 mCi $mmol^{-1}$) was dried under a stream of nitrogen and dissolved in 0.8 mL of phosphate buffer (0.5 M, pH 6.5). U-[^{14}C] HPP was prepared and purified as described above. For HPT assays in non-stressed and stressed chloroplasts, each prenyltransferase reaction (0.1 mL) was performed in HPT reaction buffer containing approximately 5 μM U-[^{14}C] HPP (1 μCi), 0 or 100 μM PDP, 1 mM KF, 0.2% (v/v) TWEEN 80, 50 mM potassium ascorbate, 20 μg HPPD protein, and chloroplasts corresponding to 0.45 – 0.65 mg of protein (0.10 – 0.15 mg of chlorophyll for non-stressed plants). HPT assays using seed protein extracts were performed as these using chloroplasts except that each reaction (performed in a reaction volume 0.2 mL) contained 2 - 3 mg seed protein. Reactions were incubated at room temperature for 2 hours with shaking and quinones were extracted with 2 volumes of acetone:light petroleum ether (1:1 v/v). After centrifugation, the quinones (in ether phase) were purified on hand-made 4-mL Silica columns (pore size 60 μm, Sigma) in 10-mL syringes and eluted directly into scintillation vials with 4 volumes of 20% (v/v) diethyl ether in petroleum ether. After drying, 5 mL of scintillation liquid was added to each vial and radioactivity was determined by scintillation counting. Background activities obtained from control reactions containing ^{14}C-labeled HGA with no exogenously added PDP were subtracted from the activities when both substrates were added, which was always at least 3.5- and 3-fold higher than the background activities in chloroplasts and seeds, respectively. HPT specific activity was calculated as PDP-stimulated HPT activity per mg of protein. Chloroplasts and seed protein extracts were extracted with 90 % methanol (v/v) and centrifuged. Total chlorophyll concentrations were determined spectrophotometrically on the 90 % methanol (v/v) chloroplast extracts (Lichtenthaller, 1987). Proteins present in the remaining pellet were solubilized by NaOH in the presence of SDS and determined by the method of Lowry (Stoscheck, 1990) in a 96-well format. Briefly, three 4-mm beads (Sigma) and 0.2 mL 1N NaOH were added. The protein pellets were disrupted by using a paint shaker for 5 minutes, incubated at 37 °C for 10 minutes, and this procedure was repeated three times. The resulting NaOH protein extract was briefly centrifuged and 80 μL

of the supernatant was added to a fresh tube containing 80 μL 10% SDS. After adding 240 μL copper reagent and 400 μL water, the samples were incubated 10 minutes at room temperature and 200 μL 0.2 N Folin reagent (Sigma, sold as 2N solution) was added. The absorbance was measured at 750 nm against a blank consisting of all reagents lacking proteins. The standard curve was constructed using BSA as a standard. The copper reagent must be prepared fresh and contains 2 g Na_2CO_3, 0.04 g $CuSO_4.5H_2O$, and 0.02 g sodium potasium tartrate per 30 mL water.

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