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VITAMIN E FUNCTIONS IN PHOTOSYNTHETIC ORGANISMS

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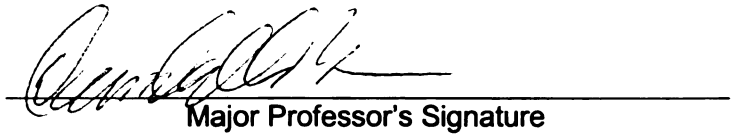

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VITAMIN E FUNCTIONS IN PHOTOSYNTHETIC ORGANISMS

By

Hiroshi Maeda

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ABSTRACT

VITAMIN E FUNCTIONS IN PHOTOSYNTHETIC ORGANISMS

By

Hiroshi Maeda

Tocopherols (vitamin E) are the major class of lipid-soluble antioxidants in biological membranes and produced only by photosynthetic organisms, yet tocopherol functions remain elusive in these organisms. In this study, tocopherol-deficient mutants of *Arabidopsis thaliana* and *Synechocystis* sp. PCC 6803, a model higher plant and cyanobacterium, respectively, have been used to investigate the physiological roles and functional mechanisms of tocopherols in these organisms.

To test a long held assumption that tocopherols are essential for protecting photosynthetic organisms against photooxidative stress, Arabidopsis and Synechocystis tocopherol-deficient mutants were subjected to high intensity light (HL) stress. Surprisingly, the phenotypic and photosynthetic responses of wild-type and tocopherol-deficient mutants were indistinguishable during HL stress in both Arabidopsis and Synechocystis. The tocopherol-deficient Synechocystis mutants became more sensitive than wild-type only when HL stress was combined with treatments of polyunsaturated fatty acids (PUFAs) which induce lipid peroxidation, or norflurazon, a carotenoid biosynthesis inhibitor. These results suggest that tocopherols are indispensable only under extreme lipid peroxidation-induced conditions but not under HL stress alone likely due to an overlapping functionality of tocopherols and carotenoids.

In contrast, the Arabidopsis tocopherol-deficient mutants exhibited dramatic

phenotypes in response to non-freezing low temperature (7.5°C); in comparison to wild-type the mutants grew more slowly, accumulated more anthocyanins in mature leaves, and produced less seed. Significantly, these changes were independent of light and occurred in the absence of photoinhibition or detectable lipid peroxidation, suggesting the mechanisms involved are independent of any photoprotective function of tocopherols. Further analyses revealed that the mutants exhibit rapid reduction in photoassimilate transport from source leaves coincident with callose deposition exclusively in phloem parenchyma transfer cells, potential bottlenecks of photoassimilate transport. Interestingly, the mutant exhibited distinct composition of PUFAs derived from the endoplasmic-reticulum (ER) pathway, which was temporarily and spatially associated with the vasculature specific callose deposition. Finally, these chilling-induced phenotypes were suppressed by the introduction of *fad2* (an ER *ω-6* fatty acid desaturase mutation) in the background of the tocopherol-deficient mutant. These results uncovered new roles for tocopherols in phloem loading and chilling adaptation in *Arabidopsis* and demonstrated that it is mediated through the modulation of extra-plastidic pathway-derived PUFAs.

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Hiroshi Maeda

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***Images in this dissertation are presented in color.**

KEY TO ABBREVIATIONS

18:1	oleic acid
18:2	linoleic acid
18:3	linolenic acid
18:4	stearidonic acid
20:3	eicosatrienoic acid
$^1O_2$	singlet oxygen
^{3}Chl*	triplet state chlorophylls
A	antheraxanthin
A+Z/A+Z+V	de-epoxidation state of xanthophyll cycle carotenoids
ABA	abscisic acid
aos	*allene oxide synthase*
AOX	alternative oxidase
APX	ascorbate peroxidase
Asc	ascorbate
α-T	α-tocopherol
AVED	ataxia with isolated vitamin E deficiency
BHT	butylated hydroxytoluene
β-T	β-tocopherol
Col	Columbia wild-type
DGDG	digalactosyldiacylglycerol
DHAR	dehydroascorbate reductase
DMPBQ,	2,3-dimethyl-6-phytyl-1,4-benzoquinol
δ-T	δ-tocopherol
ELIPs	early light-induced proteins
ER	endoplasmic-reticulum
fad	*fatty acid desaturase*
FOX assay	ferrous oxidation-xylenol orange assay
Fru	fructose
Fv/Fm	maximum photosynthetic efficiency
GGDP	geranylgeranyl-diphosphate
GGDR	GGDP reductase
Glc	glucose
GR	glutathione reductase
GSH	glutathione
gsl5	*glucan synthase like 5*
γ-T	γ-tocopherol
H_2O_2	hydrogen peroxide
HGA,	homogentisic acid
HL	high light
HPAEC	high-pH anion exchange chromatography
HPP	hydroxyphenylpyruvate
HPPD	HPP dioxygenase
HPT	HGA phytyltransferase
JA	jasmonic acid

L•	lipid radicals
LHC	light harvesting complexes
LOH	lipid hydroxides
LOO•	lipid peroxyl radicals
LOOH	lipid hydroperoxides
MDA	malondialdehyde
MDAR	monodehydroascorbate reductase
MEP	2-C-methyl-D-erythritol-4-phosphate
MGDG	monogalactosyldiacylglycerol
MPBQ,	2-methyl-6-phytyl-1,4-benzoquinol
MSBQ	2-methyl-6-solanylbenzoquinone
MT	MPBQ methyltransferase
NF	norflurazon
NPQ	non-photochemical quenching
O_2^-	superoxide
OH•	hydroxyl radical
OPDA	12-oxophytodienoic acid
PAM fluorometer	pulse amplitude modulation fluorometer
paraquat	methyl viologen
PC	phosphatidylcholine
PDP	phytyl-diphosphate
PE	phosphatidylethanolamine
PFD	photo flux density
pgr5	*proton gradient regulation 5*
PLAMs	PLastid Associated membranes
pmr4	*powdery mildew resistant 4*
PQ	plastoquinone
Prx Q	peroxiredoxins Q
PSII	photosystem II
PTOX	plastid terminal oxidase
PUFA	polyunsaturated fatty acid
qE	ΔpH-dependent quenching
qI	photoinhibitory quenching
qT	state transition
ROS	reactive oxygen species
sll0418	Synechocystis MT mutant
slr0089	Synechocystis γ-TMT mutant
slr1736	Synechocystis HPT mutant
slr1737	Synechocystis TC mutant
SOD	superoxide dismutase
Suc	sucrose
sxd1	*sucrose export defective 1*
t-BOOH	*tert*-butyl hydroperoxide
TC	tocopherol cyclase
TEM	transmission electron microscope
V	violaxanthin

VLDL	very low density lipoprotein
vte1,	Arabidopsis TC mutant
vte2	Arabidopsis HPT mutant
vte3	Arabidopsis MT mutant
vte4	Arabidopsis γ-TMT mutant
Ws	Wassilewskija wild-type
Z	zeaxanthin
α-TTP	α-tocopherol transfer protein
γ-TMT,	γ-tocopherol methyltransferase
Φ_{PSII}	quantum yield of PSII

CHAPTER 1: LITERATURE REVIEW

Protective and Acclimation Mechanisms of Plants to Environmental Stress

Because plants are sessile, they are continuously subjected to a variety of environmental stresses, including high intensity light, drought, salinity and low temperatures. These abiotic stresses adversely affect plant growth and development and therefore limit the productivity and geographical distribution of plants. To survive and acquire fitness under such conditions, plants have evolved sophisticated mechanisms to respond, tolerate and adapt to the constantly changing environment.

Protective Mechanisms against High light (HL) Stress

Light is essential for photosynthesis to generate ATP and NADPH, which are required for CO_2 fixation. Under HL stress condition, however, plants absorb more photons than can be utilized for metabolism. This "energy imbalance" between the photosynthetic light reaction and the downstream metabolism causes the accumulation of excessive energy in the photosystems (Huner et al., 1998). Failure to dissipate this excessive energy leads to the generation of reactive oxygen species (ROS) and photooxidative damage to the photosynthetic apparatus (e.g., photoinhibition), which is often manifested as photo-bleaching of leaves (Mullineaux and Karpinski, 2002). To avoid such detrimental effects of HL stress, plants induce various protective mechanisms that minimize the amount of absorbed light, dissipate excessive energy, or detoxify the ROS generated.

To minimize the amount of absorbed light, plants strategically move both their leaves and chloroplasts (Wada et al., 2003), accumulate UV-absorbing compounds such as flavonoids (Li et al., 1993; Winkel-Shirley, 2002), and adjust the size of light harvesting complexes (LHC) (Anderson, 1986). However, these processes are often not

on a timeframe that can cope with the rapidity of light fluctuations and, as a result, excessive photons are absorbed by the photosystems.

Upon light absorption, ground state chlorophylls are excited to singlet state chlorophylls (^{1}Chl*), which transfer energy to photosystem II (PSII) reaction centers to drive photosynthesis. Under HL stress, excitation energy is accumulated in the PSII light harvesting complexes and excess ^{1}Chl* can be converted to triplet state chlorophylls (^{3}Chl*). ^{3}Chl* is a long lived molecular species and can transfer energy to ground state oxygen (O_2) and generate singlet oxygen ($^1O_2$), a highly reactive ROS (Niyogi, 2000). To protect PSII from HL stress, plants induce a mechanism called non-photochemical quenching (NPQ), which quenches ^{1}Chl* and prevents the generation of ^{3}Chl* and hence $^1O_2$ (Asada, 1999; Muller et al., 2001). NPQ consists of three components: ΔpH-dependent quenching (qE), state transition (qT) and photoinhibitory quenching (qI). qE accounts for most NPQ (~80%) and is rapidly induced (within seconds) in response to a buildup of thylakoid ΔpH. The PsbS protein and zeaxanthin, a part of LHCII complex and one of xanthophyll cycle carotenoids, respectively, are critical components of qE (Muller et al., 2001; Holt et al., 2004; Szabo et al., 2005). *Arabidopsis non-photochemical quenching 1* and *4* (*npq1* and *npq4*) mutants are unable to induce NPQ, because *npq1* is defective in violaxanthin de-epoxidase, which converts violaxanthin to zeaxanthin in response to HL, and *npq4* is deficient in PsbS (Niyogi et al., 1998; Li et al., 2000; Li et al., 2002). Both mutants showed reduced fitness in fluctuating HL conditions and increased sensitivity to a short-term HL stress, whereas their response to a long-term high light stress was indistinguishable from wild type (Havaux and Niyogi, 1999; Havaux

et al., 2000; Kulheim et al., 2002). These results indicate that NPQ plays a critical role in short-term photosynthetic regulation.

When the linear photosynthetic electron flow exceeds the capacity of CO_2 fixation, the water-water cycle, chlororespiration and photorespiration serve as alternative electron sinks, all of which use O_2 as an electron acceptor (Ort and Baker, 2002). The water-water cycle reduces O_2 to H_2O at the reducing side of PSI via superoxide (O_2^-) and hydrogen peroxide (H_2O_2) by using the electrons provided through ferredoxin or NADPH (Asada, 1999). The plastid terminal oxidase (PTOX), which is homologous to the mitochondrial alternative oxidase (AOX), has been proposed to be involved in chlororespiration (Joet et al., 2002; Peltier and Cournac, 2002; Kuntz, 2004). PTOX re-oxidizes plastoquinol to plastoquinone and reduces O_2 to H_2O, avoiding over-reduction of the plastoquinone pool. Photorespiration results from an oxygenation reaction catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which ultimately produces CO_2 and NH_3 and consumes ATP and NAD(P)H through a pathway that includes chloroplasts, peroxisomes, and mitochondria (Wingler et al., 2000; Noctor et al., 2002; Bauwe and Kolukisaoglu, 2003).

Cyclic electron flow around PSI also plays an essential role in photosynthetic regulation under HL stress (Munekage et al., 2002; Kramer et al., 2004; Munekaga et al., 2004; Cruz et al., 2005). Electrons reaching the PSI acceptor side are recycled back to plastoquinone through at least two parallel pathways, NAD(P)H dehydrogenase (NDH)- or ferredoxin-dependent pathways. Both pathways generate thylakoid ΔpH, which induces qE (ΔpH-dependent NPQ) and ATP synthesis without NADPH accumulation and thus avoids over-reduction of the PSI acceptor side (Burrows et al., 1998; Shikanai et al.,

1998; Munekage et al., 2002). The *Arabidopsis proton gradient regulation 5* (*pgr5*) mutant, which is defective in the ferredoxin-dependent pathway, exhibited an increased PSI sensitivity to HL stress (Mullineaux and Karpinski, 2002), while inactivation of the NDH-dependent pathway by disruption of the *ndhB* gene in tobacco plants resulted in increased PSII sensitivity under CO_2 limitation (Horvath et al., 2000). The simultaneous disruption of both pathways in *Arabidopsis* resulted in reduced growth rate and chlorophyll content even under normal conditions, suggesting that the PSI cyclic electron flow is a fundamentally essential mechanism for the regulation of photosynthesis (Munekaga et al., 2004).

Cold Tolerance, Adaptation and Acclimation Mechanisms

When plants are shifted to chilling temperatures (2 to 12 °C), tropical and subtropical plants, such as cotton and cucumber, exhibit symptoms of chilling injury, including inhibition of photosynthesis, chlorosis, necrosis and growth cessation (Lyons, 1973). By contrast, cold-hardy species such as *Arabidopsis*, spinach and wheat are able to survive at chilling temperatures. Despite several attempts to isolate and characterize chilling sensitive mutants of *Arabidopsis*, the mechanistic basis of the chilling sensitivity or tolerance remains uncertain (Schneider et al., 1995; Tokuhisa et al., 1997; Tokuhisa et al., 1998; Provart et al., 2003). Interestingly, these cold-tolerant plants also acquire resistance to freezing temperatures by a prior exposure to nonfreezing low temperatures (2 to 8 °C). This process is called cold acclimation (Thomashow, 1999).

Low temperature negatively impacts the activity of Calvin cycle enzymes, which results in an energy imbalance between the light and dark reactions, and hence increases

excitation pressure in the photosystems (Huner et al., 1998). To avoid the generation of excessive energy and ROS, low temperature-treated plants induce various energy dissipation mechanisms similar to what are induced during HL stress (e.g. NPQ and PSI cyclic electron flow; Huner et al., 1998; Foyer et al., 2002; Oquist and Huner, 2003). A rapid down shift of temperature also inhibits export and synthesis of photosynthetic endproducts such as sucrose, which leads to the accumulation of phosphorylated intermediates and subsequently a limitation of free phosphate (Pi) (Krapp and Stitt, 1995; Stitt and Hurry, 2002). This reduced Pi in turn feedback-inhibits photosynthesis and limits the carbon supply for growth and also for the production of osmoprotectants that are required for freezing tolerance (Stitt and Hurry, 2002). In order to restore photosynthesis and continue to supply fixed carbon for growth, plants re-activate Calvin-cycle enzymes and sucrose phosphate synthase (Strand et al., 1997; Strand et al., 1999) and also up-regulate the expression of sucrose transporters involved in phloem loading (Lundmark et al., 2006).

In addition to the dramatic changes in photosynthesis and metabolism, membrane properties are also strongly impacted by low temperatures. Temperature reduction generally rigidifies membranes, whereas freezing-induced dehydration destabilizes membranes through the lamellar-to-hexagonal II phase transition (Steponkus, 1984). To compensate for cold-induced membrane rigidification, plants increase membrane fatty acid unsaturation, which also prevents hexagonal II phase formation and enhances membrane cryostability (Uemura et al., 1995; Nishida and Murata, 1996; Sakamoto and Murata, 2002). Also, in response to cold, the levels of non-bilayer lipids such as monogalactosyldiacylglycerol (MGDG) are decreased and bilayer lipids such as

digalactosyldiacylglycerol (DGDG) are correspondingly increased in the plastid membranes (Uemura and Steponkus, 1997). A variety of membrane-localized peptides and proteins, which include COR15a, lectins, dehydrins, and cryoprotectin, are also produced in response to low temperatures (Thomashow, 1999; Hincha, 2002; Charron et al., 2005). Overexpression of COR15a increased the freezing tolerance of Arabidopsis protoplasts due to a reduced incidence of the lamella-to-hexagonal II phase transition (Steponkus et al., 1998). The overexpression of dehydrins also enhanced freezing tolerance of *Arabidopsis* plants (Puhakainen et al., 2004).

These adaptive responses to low temperatures are regulated through complex transcriptional networks, which include transcription factors such as the C-repeat/dehydration-responsive element-binding factors (CBF/DREB), ZAT12 and the inducer of CBF expression 1 (ICE1) (Stockinger et al., 1997; Liu et al., 1998; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Chinnusamy et al., 2003). Global transcript profiling studies further revealed that these transcription factors regulate a large set of genes related to the aforementioned biochemical changes and also ROS scavenging, hormonal signaling and cell wall modification, etc (Seki et al., 2001; Fowler and Thomashow, 2002; Hannah et al., 2005; Lee et al., 2005; Vogel et al., 2005).

In contrast to the extensively studied transcriptional changes, the initial cold sensing mechanisms and early signals that lead to these transcriptional responses are not as well understood. Membranes have been proposed to be the site of low temperature sensing and signal production. Membrane-localized calcium channels and two-component response regulators have been implicated to play a role in these processes (Plieth et al., 1999; Suzuki et al., 2000; Browse and Xin, 2001; Mikami and Murata, 2003). Other membrane

components, such as phosphatidic acids and lysolipids, have also been proposed to play important roles in both the early response and longer-term adaptation to low temperatures (Ruelland et al., 2002; Welti et al., 2002; Gomez-Merino et al., 2004). However, low temperatures also directly affect the stability of RNA and DNA secondary structures and the activity of enzymes, and thus any or combination of these changes can become the source of low temperature signals.

Reactive Oxygen Species and Antioxidants

Reactive Oxygen Species

Reactive oxygen species (ROS) are the partially reduced forms of molecular oxygen (O_2) and by-products of aerobic metabolic pathways that are localized in different cellular compartments, such as mitochondria, peroxisomes, and chloroplasts. The chloroplast is one of the major site of ROS production during abiotic stress in plants. Over-reduction of the photosynthetic electron transport chain results in the formation of chlorophyll triplets ($^3Chl^*$), which transfer energy to O_2 and generate singlet oxygen ($^1O_2$) at PSII (Niyogi, 2000). Excessive electrons are also transferred to O_2 at the PSI acceptor side, creating superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•) (Apel and Hirt, 2004; Laloi et al., 2004). Under CO_2 limitation, the oxygenation reaction of Rubisco releases glycolate from chloroplasts to peroxisomes, where H_2O_2 is produced upon conversion of glycolate to glyoxylate by glycolate oxidase (Wingler et al., 2000; Noctor et al., 2002). When the mitochondrial electron transport chain is over-reduced such as under low temperature, excess electrons are transferred to O_2, generating O_2^-, H_2O_2 and OH• (Maxwell et al., 1999; Moller, 2001; Rhoads et al., 2006). ROS can also be

generated by several oxidases, such as plasma membrane localized NAD(P)H oxidases and cell wall peroxidases in the apoplastic spaces (Lamb and Dixon, 1997; Wojtaszek, 1997; Apel and Hirt, 2004).

Uncontrolled ROS production triggers unrestricted oxidation of various cellular components and causes enzyme inhibition, lipid peroxidation and DNA and RNA damage (Mittler, 2002; Shulaev and Oliver, 2006). In the presence of transition metal ions such as iron (Fe), ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}) that reacts with H_2O_2 to form OH•. This highly destructive OH• can oxidize both amino acid side chains and peptide backbones, resulting in protein-protein cross linkages and protein fragmentation (Garrison, 1987; Berlett and Stadtman, 1997). OH• can also trigger lipid peroxidation by abstracting allylic hydrogens from polyunsaturated fatty acid (PUFA)-containing lipids and producing lipid radicals (L•), that immediately react with O_2 to form lipid peroxyl radicals (LOO•) (Figure 1.1; Tappel, 1972; Bramley et al., 2000; Schneider, 2005). LOO• can subsequently attack another PUFA generating a second LOO• and propagating a chain reaction of lipid peroxidation that perturbs membrane structures and functions. OH• also attacks deoxyribose bases such as guanine to forms 8-oxo-guanine, which can cause mismatching with adenine during replication. Malondialdehyde (MDA), an end product of lipid peroxidation, also reacts with deoxyribose bases to form a variety of MDA-DNA adducts (Wang and Liehr, 1995; Marnett, 1999).

In addition to the detrimental effect of ROS, these highly reactive ROS molecules also function as signals and regulate various cellular processes (Apel and Hirt, 2004; Mittler et al., 2004). The oxidative burst is a rapid accumulation of O_2^- and H_2O_2 and a

characteristic early response leading to ozone- and pathogen-induced cell death (Lamb and Dixon, 1997; Torres et al., 2002; Overmyer et al., 2003; Torres and Dangl, 2005). H_2O_2 accumulated in growing root hairs and induced by abscisic acid (ABA) during drought stress activates calcium channels and leads to cell expansion and stomata closure, respectively (McAinsh et al., 1996; Pei et al., 2000; Foreman et al., 2003; Jiang et al., 2003). Recently, $^1O_2$ has been implicated as another type of ROS signal that regulates programmed cell death in plants via a pathway mediated through Excecuter1, a novel protein localized in the chloroplasts (op den Camp et al., 2003; Wagner et al., 2004; Laloi et al., 2006).

Water-Soluble Antioxidants

In order to protect cellular components from ROS attack and also tightly regulate the levels of ROS signals, plants have evolved a variety of ROS detoxification mechanisms (Mittler, 2002; Mittler et al., 2004; Foyer and Noctor, 2005). O_2^- generated from the mitochondria and chloroplast electron transport is reduced to H_2O_2 by superoxide dismutases (SODs), which use different metals as co-factors (Alscher et al., 2002). H_2O_2 is further reduced to H_2O by the ascorbate-glutathione system, which composed of two water-soluble antioxidants, ascorbate (Asc, vitamin C) and glutathione (GSH), and a number of enzymes that use Asc and GSH as cosubstrates. These enzymes include ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR) (Noctor and Foyer, 1998; Chew et al., 2003). Oxidized Asc and GSH are regenerated to their reduced forms by reactions using ferredoxin and NAD(P)H as reductants. Peroxiredoxins are also

reported to play roles in the H_2O_2 detoxification in both mitochondria and chloroplasts (Broin et al., 2002; Konig et al., 2002; Dietz, 2003; Dietz et al., 2006). Peroxisomes contain SOD, catalase and APX for detoxification of H_2O_2 produced during photorespiration (Igamberdiev and Lea, 2002).

Lipid-Soluble Antioxidants

In contrast to the well-studied ROS detoxification mechanisms for water-soluble compartments, however, the mechanisms preventing or limiting membrane oxidation are less clear. Because membranes of chloroplasts and cyanobacteria contain high levels of PUFAs and the photosynthetic apparatus, a potential ROS generator, photosynthetic membranes must have extensive mechanisms to protect PUFAs from oxidation. Several peroxiredoxins have been implicated in reducing lipid hydroperoxides (LOOH) to the less toxic lipid hydroxides (LOH) in both plants and cyanobacteria (Gaber et al., 2001; Dietz, 2003). In *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*, specific carotenoids play roles in limiting lipid peroxidation, presumably by directly scavenging LOO• in the plastid membranes (Havaux and Niyogi, 1999; Baroli et al., 2003; Baroli et al., 2004). Tocopherols, a major class of lipid-soluble antioxidants in photosynthetic membranes, are also thought to play important roles in preventing lipid peroxidation (Fryer, 1992; Munne-Bosch and Alegre, 2002). However, while plausible, there is little direct *in vivo* evidence supporting such functions for tocopherols in photosynthetic organisms.

Tocopherols (Vitamin E)

Chemical Structure and Characteristics of Tocopherols

Tocopherols are amphipathic molecules that consist of a polar chromanol head group attached to a hydrophobic phytyl tail. There are four different forms of tocopherols (α, β, γ, and δ–tocopherols) based on the substitution patterns of methyl groups attached to the C5, C7 and C8 positions of the chromanol ring (Figure 1.2; Fernholz, 1938; Kamal-Eldin and Appelqvist, 1996; Bramley et al., 2000; Schneider, 2005). Tocopherols have three chiral centers at the C2, C4' and C8' positions and only *2R*, *4'R*, *8'R*-tocopherols (*RRR*-tocopherols) are synthesized in nature. Tocotrienols (α, β, γ, and δ–tocotrienols) have an unsaturated isoprenoid side chain with double bonds at the C3', C7', and C11' positions and thus contain only one chiral center at the C2 position (Figure 1.2, Kamal-Eldin and Appelqvist, 1996; Bramley et al., 2000; Schneider, 2005). These eight naturally occurring forms, four tocopherols and four tocotrienols, are collectively termed vitamin E.

The hydrophobic phytyl tail allows tocopherols to reside in membranes with the hydroxyl group of the chromanol ring being located near the membrane surface (Niki et al., 1985; Bramley et al., 2000). Methyl groups at position C4' and C8' of the tocopherol phytyl chain fit into a pocket created by the *cis* double bonds of PUFAs, thus allowing tocopherols to tightly associate with PUFAs or PUFA-containing lipids in membranes (Diplock and Lucy, 1973; Erin et al., 1984; Stillwell et al., 1996). These tocopherol-PUFA complexes may reduce membrane permeability, increase membrane protein stability and/or protect membranes against hydrolytic enzymes such as phospholipase A_2 (Lucy, 1972; Kagan, 1989; Grau and Ortiz, 1998; Wang and Quinn, 2000).

Based on *in vitro* studies using organic solutions, liposome and animal cell-derived membranes, tocopherols can both physically and chemically quench $^1O_2$ (Grams and Eskins, 1972; Fahrenholtz et al., 1974; Littarru et al., 1984; Fukuzawa et al., 1998). Although tocopherols are 50 to 100-fold less efficient quenchers than carotenoids, one molecule of α-tocopherol can deactivate roughly 100 molecules of $^1O_2$ before being destroyed (Fahrenholtz et al., 1974; Kamal-Eldin and Appelqvist, 1996; Fukuzawa et al., 1998). Tocopherols also efficiently scavenge various radicals, particularly LOO•. The hydroxyl group attached to the C6 position of the chromanol ring can transfer a hydrogen atom to LOO•, yielding LOOH and the tocopheroxyl radical and thereby terminating the lipid peroxidation chain reaction (Figure 1.1; Tappel, 1962, 1972; Burton and Ingold, 1981; Liebler and Burr, 1992; Ham and Liebler, 1995). The tocopheroxyl radical can be recycled back to the corresponding tocopherol by interaction with other antioxidants such as ascorbate or coenzyme Q in animals (Stoyanovsky et al., 1995; May et al., 1998). It is not yet known if and how such recycling of the tocopherol radical takes place in plants. Alternatively, the tocopheroxyl radical can further react with a second LOO• to form a non-radical product, tocopherol quinonone (Figure 1.1; Liebler and Burr, 1992; Kamal-Eldin and Appelqvist, 1996).

Tocopherols in Animals

Tocopherols were first discovered as the substances that prevented embryo resorption during gestation in female rats (Evans and Bishop, 1922). Subsequently, it was found that dietary vitamin E deficiency in different mammals, such as rat and chicken, also causes male sterility, encephalomalacia (brain softening), and various neurological disfunctions

(Machlin et al., 1977; Shih et al., 1977; Burton, 1994; Brigelius-Flohe et al., 2002). These results clearly indicate that vitamin E is an essential nutrient in mammals.

In mammals, all forms of tocopherols are equally absorbed in the small intestine together with lipids, packaged into large circulating lipoprotein particles named chylomicrons, and transported to the liver (Kayden 1993). In the liver, α-tocopherol is selectively recognized by α-tocopherol transfer protein (α-TTP) and preferentially secreted into very low density lipoprotein (VLDL) for export to the plasma (Kayden and Traber, 1993; Traber et al., 1994; Brigelius-Flohe and Traber, 1999). Mutations in the human α-TTP gene cause a neurodegenerative disease called Ataxia with isolated Vitamin E Deficiency (AVED; Bramley et al., 2000). Likewise,α-TTP deficiency in mice resulted in neurological disfunction (Yokota et al., 2001) and also in the death of embryos at mid-gestation stage (Jishage et al., 2001). These results support an essential role for α-TTP in the transport of α-tocopherol. α-TTP has significantly lower affinity for other forms of tocopherols than α-tocopherol (α-tocopherol, 100%; β-tocopherol, 38%; γ-tocopherol, 9%; δ-tocopherol, 2%; Hosomi et al., 1997). This α-TTP selectivity is likely the molecular basis for the differences in the vitamin E activity of the different tocopherols. The unrecognized tocopherols are subjected to their side-chain degradation via ω-hydroxylation and β-oxidation and are excreted by urine (Brigelius-Flohe et al., 2002).

Because *in vitro* studies demonstrated that α-tocopherol is one of the most efficient lipid-soluble antioxidants in nature (Burton and Ingold, 1981; Kamal-Eldin and Appelqvist, 1996), the essential nutritional roles of vitamin E in mammals are most likely associated with its lipid-soluble antioxidant properties (Tappel, 1962; Burton, 1994;

Kamal-Eldin and Appelqvist, 1996). Consistent with this thesis, vitamin E supplementation suppressed *tert*-butyl-hydroperoxide-induced lipid peroxidation in the rat liver and also significantly reduced atherosclerotic lesions and the generation of isoprostanes, oxidation products of arachidonic acid, in the apolipoprotein E deficient mouse (Ham and Liebler, 1997; Pratico et al., 1998). The vitamin E deficient symptoms, such as ataxia in the α-TTP knockout mouse and muscular dystrophy in the vitamin E deficient chicken, are also coincident with elevated oxidative stress (Shih et al., 1977; Awad et al., 1994; Yokota et al., 2001). Some epidemiological studies have shown that vitamin E supplementation has beneficial effects on diseases associated with oxidative stress, such as atherosclerosis and cancer, while other studies found that vitamin E supplementation has no significant impacts (Brigelius-Flohe and Traber, 1999; Bramley et al., 2000; Brigelius-Flohe et al., 2002).

Recent studies have also suggested that a specific form of tocopherols in animals also can have "non-antioxidant" functions. For instance, α-tocopherol inhibits protein kinase C activity via protein phosphatase 2A activation (Ricciarelli et al., 1998) and also inhibits phospholipase A_2 activity and suppresses the synthesis of arachidonic acid (Pentland et al., 1992; Chandra et al., 2002; Takeda et al., 2004). γ-Tocopherol and its metabolites inhibit cyclooxygenase activity and hence prostaglandin E_2 synthesis (Jiang et al., 2000; Jiang and Ames, 2003). Moreover, α-tocotrienol was proposed to modulate 12-lipoxygenase and suppress glutamate-induced neuronal cell death (Sen et al., 2000; Khanna et al., 2003). Although the underlying mechanisms have not been elucidated, these enzyme activities are differentially modulated by the specific type of tocopherols (e.g. only α-tocopherol but not γ-tocopherol), leading to the hypothesis that tocopherols

also function independently from their antioxidant properties (Rimbach et al., 2002; Schneider, 2005).

Tocopherols in Photosynthetic Organisms

Tocopherol Biosynthesis

Tocopherols (and tocotrienols) are synthesized only in photosynthetic organisms, including all plants and algae, and most cyanobacteria (Powls and Redfearn, 1967; Skinner and Sturm, 1968; Dasilva and Jensen, 1971; Horvath et al., 2006). With the combination of biochemical, genetic and genomic approaches, the tocopherol biosynthetic pathway has recently been fully elucidated in *Arabidopsis thaliana* and *Synechocystis* sp. PCC 6803, a model higher plant and cyanobacterium, respectively (Figure 1.3) (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Shintani et al., 2002; Cheng et al., 2003; Sattler et al., 2003). The plastidic 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway provides phytyl-diphosphate (PDP), the phytyl tail precursor of tocopherols (and also chloroplylls; Lichtenthaler, 1998). Four isopentenyl phosphate (IPP) are combined to produce geranylgeranyl-diphosphate (GGDP) and then converted to PDP by geranylgeranyl-diphosphate reductase (GGDR) (Addlesee et al., 1996; Tanaka et al., 1999). Homogentisate (HGA), the aromatic precursor of tocopherols (and also plastoquinone in plants), is synthesized via the plastidic shikimate pathway and cytosolic tyrosine metabolism (Whistanc and Threlfal, 1970). *p*-Hydroxyphenyl pyruvate (HPP) produced from L-tyrosine degradation is converted to HGA by cytosolic *p*-hydroxyphenyl pyruvate dioxygenase (HPPD) (Garcia et al., 1997; Norris et al., 1998; Dahnhardt et al., 2002; Rippert et al., 2004).

Homogentisate phytyltransferase (HPT) catalyzes the committed step in tocopherol synthesis by condensing HGA and PDP to produce 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) in the plastids (Soll et al., 1980; Collakova and DellaPenna, 2001; Schledz et al., 2001; Savidge et al., 2002). MPBQ is then converted to 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) by MPBQ methyltransferase (MPBQ MT) (Soll et al., 1980, 1985; Shintani et al., 2002; Cheng et al., 2003). Both MPBQ and DMPBQ are substrates for tocopherol cyclase (TC) to produce δ- and γ-tocopherols, respectively (Soll et al., 1985; Porfirova et al., 2002; Sattler et al., 2003), which are then converted to β- and α-tocopherols by γ-tocopherol methyltransferase (γ-TMT) (Dharlingue and Camara, 1985; Shintani and DellaPenna, 1998). The last four steps of tocopherol synthesis occur at the inner envelope of the chloroplasts and presumably other plastids of non-photosynthetic tissues (Soll et al., 1980, 1985). Recent studies using proteomics approaches, however, revealed that TC is also present in plastoglobuli, a lipid monolayer subcompartment extended from the thylakoid membranes (Austin et al., 2006; Vidi et al., 2006; Ytterberg et al., 2006).

Distributions of Tocopherols among Photosynthetic Organisms.

As far as is known, all plants so far investigated produce tocopherols and/or tocotrienols (Sheppard et al., 1993; Horvath et al., 2006). Photosynthetic tissues such as leaves accumulate predominantly α-tocopherol. Non-photosynthetic tissues such as dry seeds, roots, flower petals tend to accumulate more γ-tocopherol than α-tocopherol but there are many exceptions such as sunflower and safflower seeds, which accumulate α-tocopherol as their major form (>95%) (Furuya et al., 1987; Sheppard et al., 1993). Tocotrienols are

found in seeds of various plants, particularly cereals, but generally not in photosynthetic tissues (Sheppard et al., 1993; Horvath et al., 2006). Like photosynthetic tissues of plants, algae and cyanobacteria usually accumulate α-tocopherol as their major constituent, with a few exceptions such as *Anabeana variabilis* accumulating both α- and β-tocopherols (Carr and Hallaway, 1965; Powls and Redfearn, 1967; Skinner and Sturm, 1968; Whistanc.Gr and Threlfal.Dr, 1970; Dasilva and Jensen, 1971). The freshwater unicellular cyanobacteria *Synechococcus elongatus* PCC7942 (*Anacystis nidulans*) is one exceptional cyanobacterium in which no tocopherols were detected (Powls and Redfearn, 1967; Dasilva and Jensen, 1971). Although it is unclear why this particular *Synechococcus* strain does not require tocopherols, the hydroxylated vitamin K_1, 5-hydroxyphylloquinone, accumulated in this cyanobacterium was suggested to complement for the lack of tocopherols (Powls and Redfearn, 1967; Whistanc and Threlfal, 1970). It is also interesting to note that the membranes of *Synechococcus elongatus* PCC7942 do not contain any dienoic or trienoic fatty acids (Wada et al., 1990; Gombos et al., 1997), which may be the alternative reason why tocopherols are not required in this cyanobacterium.

Subcellular Localization of Tocopherols in Photosynthetic Tissues.

With the exception of tocopherols accumulated in oil bodies, the majority of tocopherols are localized in the plastids (Bucke, 1968; Yamauchi and Matsushita, 1976; Wise and Naylor, 1987). In spinach chloroplasts, 2.8 μg tocopherols per mg protein were detected in envelopes, while 1.1 μg per mg protein were present in thylakoids (Lichtenthaler et al., 1981). Considering the lower protein concentration of the envelopes (protein/polar lipid

ratio of 0.65) relative to the thylakoids (protein/polar lipid ratio of 2.0; Heber and Heldt, 1981), 45% of tocopherols are localized in the envelopes and the remaining 55% are in the thylakoids. Within the envelopes, the outer membrane contains slightly more tocopherols (60%) than the inner membrane (40%) (Soll et al., 1985). Within the thylakoids, one tocopherol molecule is present for every sixty to eighty chlorophyll molecules [calculated from the data of (Bucke, 1968; Lichtenthaler et al., 1981; Wise and Naylor, 1987)]. Tocopherols were also detected in the plastoglobuli of old spinach leaves (Lichtenthaler et al., 1981). It is noteworthy that Janiszowska and Korczak (1980) reported that 9 and 6% of total tocopherols were found in the mitochondria and microsome fractions isolated from *Calendula officinalis* leaves, respectively. This could be due to the contamination by chloroplasts as indicated by the presence of some chlorophyll (0.5 to 3%) in these fractions (Janiszowska and Korczak, 1980). Nevertheless, the possible localization of tocopherols outside the chloroplasts needs to be further investigated from different plants under different developmental and environmental conditions.

Proposed Tocopherol Functions in Photosynthetic Organisms.

In photosynthetic organisms, tocopherol levels increase in response to various abiotic stresses. Especially during HL stress, the levels of tocopherols are dramatically elevated in both plants and cyanobacteria (Havaux et al., 2000; Collakova and DellaPenna, 2003; Maeda et al., 2005). Low temperature and drought stress also lead to a 5-fold increase in total tocopherol content (Munne-Bosch et al., 1999; Bergmuller et al., 2003), while salt stress increases tocopherol levels only slightly (20%; Keles and Oncel, 2002).

Tocopherol levels are also increased during senescence, a developmental process which involves oxidative stress (Rise et al., 1989; Thompson et al., 1998). Based on i) the elevated accumulation of tocopherols in response to stress, ii) the evolutional conservation of tocopherol synthesis among oxygenic photosynthetic organisms, iii) the localization of most tocopherols in the plastids, and iv) the chemical characteristics of tocopherols as lipid-soluble antioxidants, it has long been assumed that a primary function of tocopherols is to protect photosynthetic membranes from oxidative stress by acting as lipid-soluble antioxidants (Fryer, 1992; Munne-Bosch and Alegre, 2002). While compelling, such hypotheses are based primarily on correlative and circumstantial evidence and have yet to be rigorously tested *in vivo*.

To address the possible photoprotective role of tocopherols in photosynthetic organisms, Trebst et al. (2002) used an herbicide pyrazolynate, which inhibits HPPD enzyme activity in *Chlamydomonas reinhardtii* and assessed the impact of reduced tocopherol content on HL stress tolerance. The treatment of *Chlamydomonas* with pyrazolynate at 1500 μmole photons m^{-2} s^{-1} reduced tocopherol levels to 20% of controls and induced concomitant degradation of the D1 protein, consistent with the thesis that tocopherols protect PSII against photooxidative stress (Trebst et al., 2002). However, pyrazolynate also inhibits the synthesis of plastoquinone, an essential electron carrier of photosynthesis, but the level of plastoquinone was not quantified in this study. Tanaka et al. (1999) generated antisense tobacco lines for GGDR, which had reduced tocopherol levels to 15 % of wild type. These plants showed delayed growth and pale phenotype under normal condition, and increased sensitivity and elevated lipid peroxidation during HL stress (Tanaka et al., 1999; Grasses et al., 2001; Havaux et al., 2003). However, the

downregulation of GGDR also converted 60 % of chlorophyll phytyl tails to geranylgeranyl tails (Tanaka et al., 1999), which dramatically alters the structure and photochemistry of the photosystems.. Thus, again one cannot exclude that the observed phenotypes in the GGDR-antisense plants are caused by the dramatic changes in the composition of chlorophylls, the major pigments of light harvesting complexes and PS reaction centers, and that impacts on tocopherol levels, lipid peroxidation and photosynthesis are pleiotropic.

Tocopherol Biosynthetic Mutants.

During studies of the tocopherol biosynthetic pathway, mutants disrupting each biosynthetic enzyme have been isolated in both *Arabidopsis* and *Synechocystis* (Figure 1.3; Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Schledz et al., 2001; Porfirova et al., 2002; Shintani et al., 2002; Cheng et al., 2003; Sattler et al., 2003). These mutants only affect tocopherol synthesis and therefore provide ideal systems to directly and specifically investigate tocopherol functions in photosynthetic organisms. The *Arabidopsis vitamin e 2* (*vte2*) mutant and *Synechocytis slr1736* are defective in the HPT enzyme and lack all tocopherols and the pathway intermediates (Figure 1.3; Collakova and DellaPenna, 2001; Sattler et al., 2004). The *vte2* mutants are severely impaired in seed longevity and early seedling development due to the massive and uncontrolled peroxidation of storage lipids (Sattler et al., 2004). This lipid-soluble antioxidant function of tocopherols during seed storage and early seedling development has provided a strong selection pressure for tocopherol biosynthesis during the evolution of seed plants (Sattler et al., 2004). Interestingly, the *vte2* mutants that do survive early

seedling development are virtually indistinguishable from wild type under standard growth conditions, suggesting that tocopherols are dispensable in mature plants in the absence of stress (Sattler et al., 2004). Similarly, the growth and photosynthetic O_2 evolution rates of the *Synechocystis slr1736* mutant were also similar to wild type under standard photoautotrophic growth condition (Collakova and DellaPenna, 2001). These data suggest that a primary function of tocopherols in plants is to control non-enzymatic lipid oxidation during seed storage and early germination and that the absence of tocopherols has no major impact on the growth of photosynthetic cells and tissues under normal conditions.

The *vte1* and *slr1737* mutants of *Arabidopsis* and *Synechocystis*, respectively, are defective in the tocopherol cyclase enzyme, deficient in all tocopherols but, unlike *vte2*, accumulate the redox active biosynthetic intermediate 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) (Figure 1.3; Porfirova et al., 2002; Sattler et al., 2003). The *Arabidopsis vte1* mutant is virtually identical to the wild type under standard growth condition and also in response to short-term cold or heat stresses (Porfirova et al., 2002; Bergmuller et al., 2003; Sattler et al., 2004). Under moderate HL stress (850 μmol photons m^{-2} s^{-1}), *vte1* showed a slight reduction in chlorophyll content and PSII quantum yield (Porfirova et al., 2002). Interestingly, the dramatic *vte2* seedling phenotype was completely attenuated in *vte1*, indicating that the DMPBQ accumulated by *vte1* fully compensates for tocopherols in seedlings (Sattler et al., 2004). This compensation by DMPBQ in the *vte1* mutant also suggested that the *vte1* and *vte2* phenotypes have to be carefully assessed in order to differentiate between effects caused specifically by the absence of tocopherols from those due to the accumulation of DMPBQ.

A maize tocopherol cyclase mutant (*sxd1*, *sucrose export defective 1*) was identified several years prior to *Arabidopsis vte1* not due to its impact on tocopherol synthesis but as a dwarf mutant accumulating carbohydrates and anthocyanins in source leaves. The formation of aberrant plasmodesmata between bundle sheath and vascular parenchyma cells in the mutant suggested blockage in symplastic photoassimilate translocation leads to an impaired sugar export from source leaves (Russin et al., 1996). Cloning of the *SXD1* locus only implicated that the encoded protein is involved in a chloroplast-to-nucleus signaling required for plasmodesmata development (Provencher et al., 2001). It was retrospectively demonstrated that *SXD1* encodes tocopherol cyclase and the maize *sxd1* mutant is indeed tocopherol deficient (Sattler et al., 2003). The *sxd1* carbohydrate accumulation phenotype is intriguing as it suggests an unexpected link between the tocopherol pathway and primary carbohydrate metabolism, though the mechanism involved is unknown. Interestingly, this carbohydrate phenotype did not occur in the orthologous Arabidopsis *vte1* mutant (Sattler et al., 2003) but was observed in *VTE1* RNAi potato lines (Hofius et al., 2004).

Aim of This Study

In contrast to the extensively studied functions of tocopherols in animals, tocopherol functions in photosynthetic organisms have been elusive, with the exception of defining the lipid-soluble antioxidant function of tocopherols in seed longevity and early seedling development. The purpose of this thesis study is to understand tocopherol functions in the photosynthetic tissues including plant leaves and cyanobacteria. To test the long-held assumption that tocopherols protect PUFA-enriched photosynthetic membranes from

oxidative stress, orthologous tocopherol biosynthetic mutants of *Arabidopsis* and *Synechocystis* were subjected to a variety of abiotic stress and ROS-generating or lipid peroxidation-inducing chemical stresses. Detailed phenotypic, biochemical and ultrastructural characterization of the responses uncovered unexpected roles for tocopherols in photosynthetic tissues.

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FIGURES

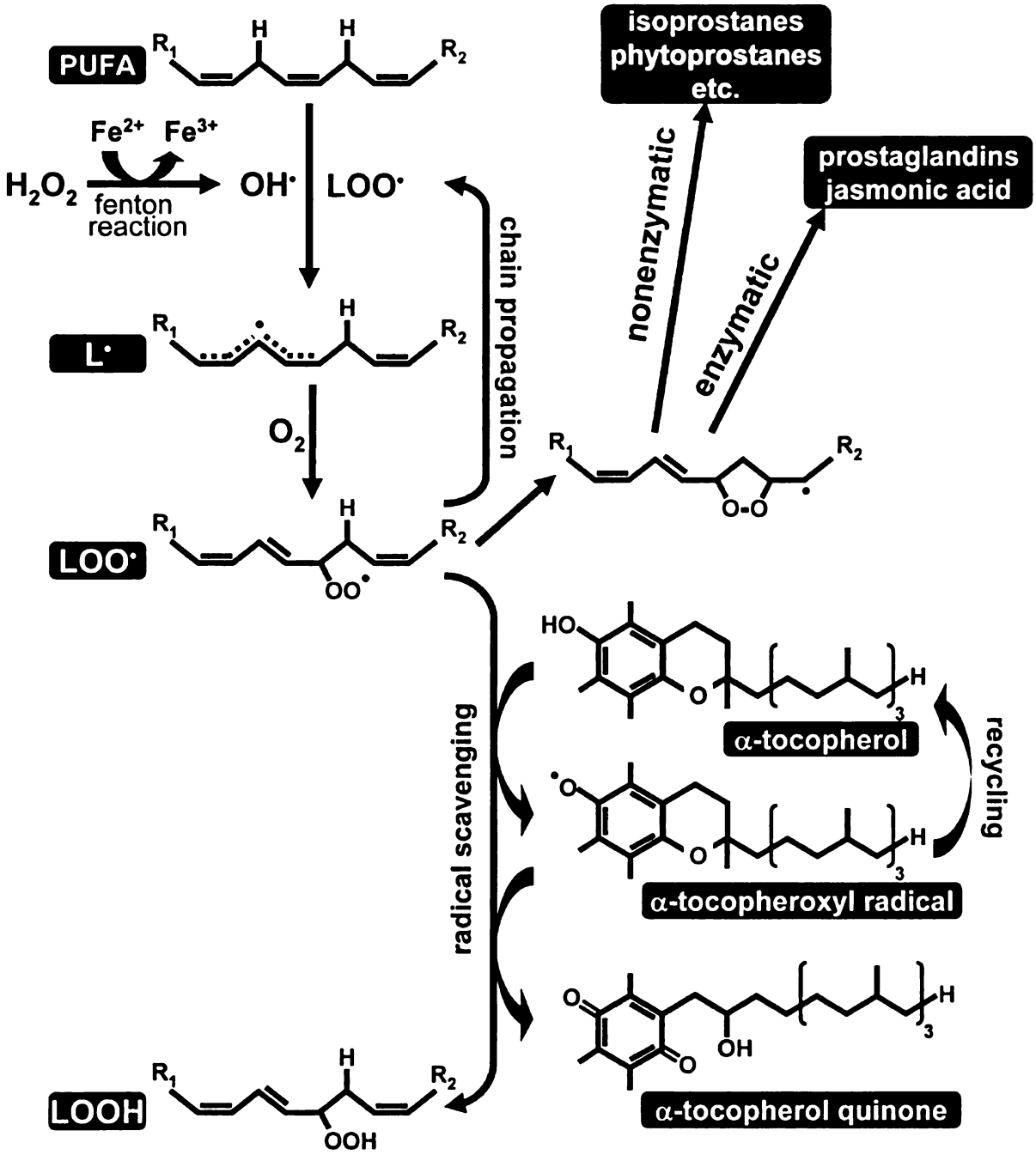
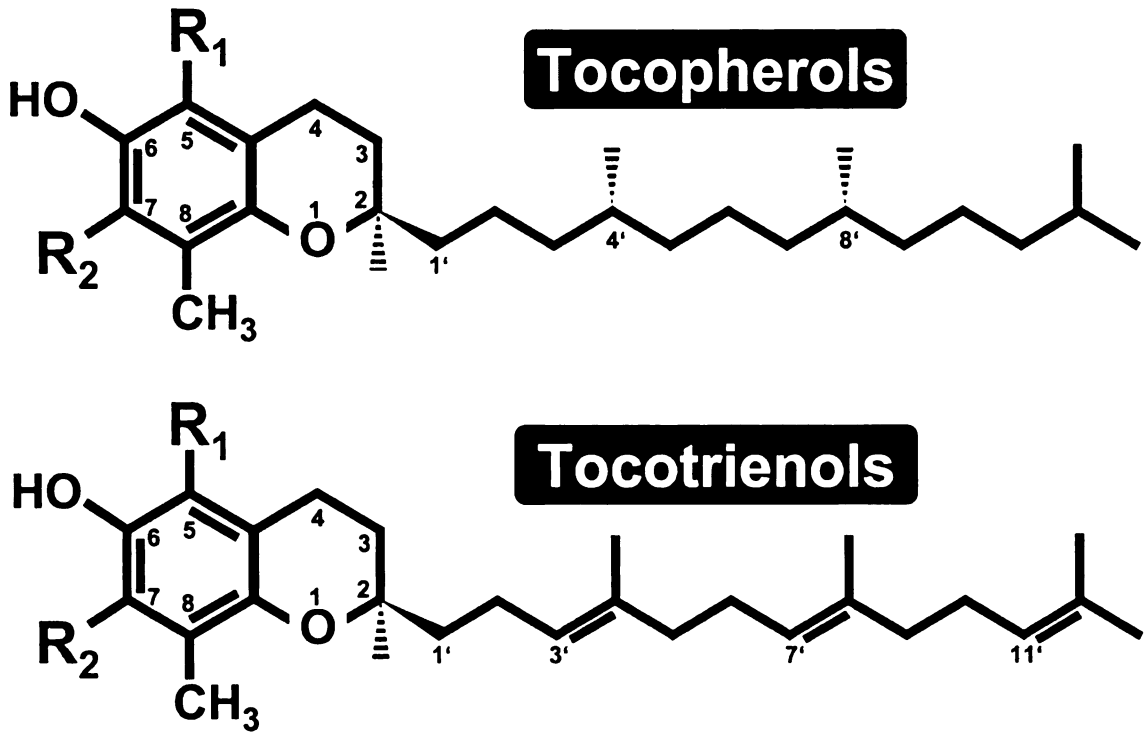


Figure 1.1. Oxydation of Polyundaturated Fatty Acids and Lipid Peroxy Radical Scavenging by Tocopherols.

PUFA, polyunsaturated fatty acid; H_2O_2, hydrogen peroxide; Fe^{2+}, ferrous iron; Fe^{3+}, ferric iron; OH•, hydroxy radical L•, lipid radical; LOO•, lipid peroxy radical; LOOH, lipid hydroperoxide.



tocopherol/ tocotrienol	R_1	R_2
α-	CH_3	CH_3
β-	CH_3	H
γ-	H	CH_3
δ-	H	H

Figure 1.2. The Structures of Tocopherols and Tocotrienols.
The table indicates the number and position of methyl groups present in α-, β-, γ, or δ-tocopherols/tocotrienols.

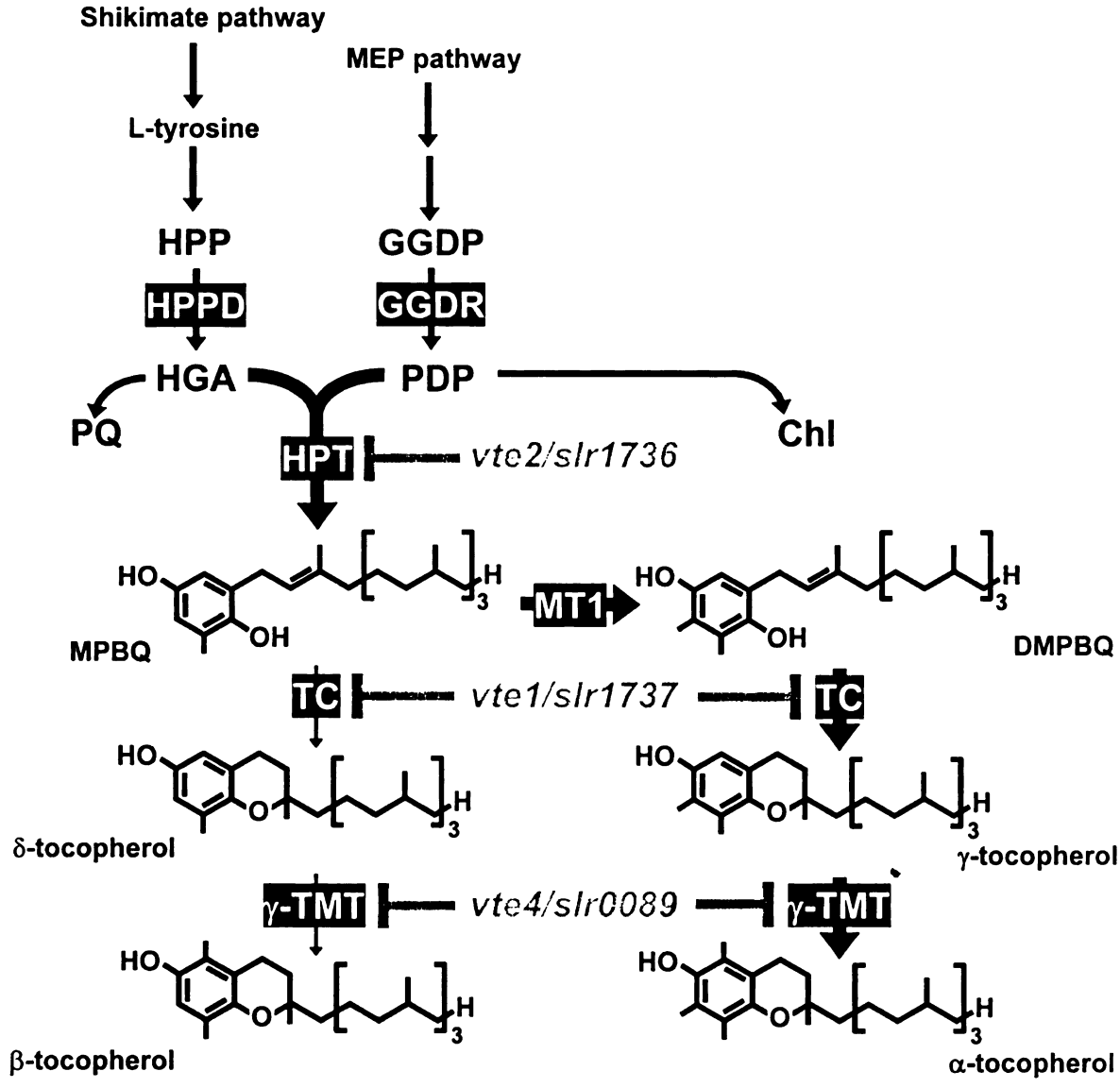


Figure 1.3. Tocopherol Biosynthetic Pathway and Mutants in *Arabidopsis thaliana* and *Synechocystis* sp. PCC6803.

Enzymes are indicated by black boxes and mutations by gray letters and lines. Bold arrows show the primary biosynthetic route in wild type Arabidopsis leaves and Synechocystis. HPP, hydroxyphenylpyruvate; GGDP, geranylgeranyl-diphosphate; PDP, phytyl-diphosphate; HGA, homogentisic acid; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol; MEP, 2-C-methyl-D-erythritol-4-phosphate ; HPPD, HPP dioxygenase; GGDR, GGDP reductase; HPT, HGA phytyltransferase; TC, tocopherol cyclase; MT, MPBQ methyltransferase; γ-TMT, γ-tocopherol methyltransferase; *vte1*, *vte2* and *vte4*, mutants of TC, *HPT* and γ-TMT in Arabidopsis, respectively. *slr1736*, *slr1737* and *slr0089*, mutants of TC, HPT and γ- TMT in Synechocystis,respectively.

CHAPTER 2: TOCOPHEROLS PROTECT *SYNECHOCYSTIS* SP. STRAIN PCC 6803 FROM LIPID PEROXIDATION.

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Author's contributions:

Yumiko Sakuragi generated the *slr1736* mutant, participated in the initial growth experiments and helped with manuscript review. Hiroshi Maeda conducted the remainder of the research and wrote the manuscript. Donald A. Bryant was involved in project development and helped with manuscript review. Dean DellaPenna supervised the entire project and was involved in all aspects of manuscript writing.

ABSTRACT

Tocopherols (vitamin E) are lipid-soluble antioxidants only synthesized by photosynthetic eukaryotes and some cyanobacteria, and have been assumed to play important roles in protecting photosynthetic membranes from oxidative stress. To test this hypothesis, tocopherol-deficient mutants of *Synechocystis* sp. strain PCC 6803 (*slr1736* and *slr1737* mutants) were challenged with a series of reactive oxygen species (ROS)-generating and lipid peroxidation-inducing chemicals in combination with high-light (HL) intensity stress. The tocopherol-deficient mutants and wild type were indistinguishable in their growth responses to HL in the presence and absence of superoxide and singlet oxygen-generating chemicals. However, the mutants showed enhanced sensitivity to linoleic or linolenic acid treatments in combination with HL, consistent with tocopherols playing a crucial role in protecting *Synechocystis* sp. strain PCC 6803 cells from lipid peroxidation. The tocopherol-deficient mutants were also more susceptible to HL treatment in the presence of sub-lethal levels of norflurazon, an inhibitor of carotenoid synthesis, suggesting carotenoids and tocopherols functionally interact or have complementary or overlapping roles in protecting *Synechocystis* sp. strain PCC 6803 from lipid peroxidation and HL stress.

INTRODUCTION

Oxygenic photosynthetic organisms continuously produce oxygen in the presence of light and as such cellular damage from various reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH•), is a constant threat. Photosynthetic organisms have therefore evolved

extensive detoxifying and protective mechanisms, which both limit the production of and potential damage by ROS. Examples include superoxide dismutase (SOD) which reduces O_2^- to H_2O_2, ascorbate peroxidase which reduces H_2O_2 to H_2O, and non-photochemical quenching (NPQ) that quenches singlet state chlorophylls (^{1}Chl*) and harmlessly dissipates excessive excitation energy as heat, thereby reducing $^1O_2$ production (Asada, 1999; Muller et al., 2001).

ROS, such as OH•, can trigger a lipid peroxidation chain reaction by abstracting an allylic hydrogen from polyunsaturated fatty acid (PUFA)-containing lipids producing lipid radicals (L•) that are converted to lipid peroxyl radicals (LOO•) upon O_2 addition. LOO• can subsequently attack another PUFA generating a second LOO• and propagating a chain reaction of lipid peroxidation that perturbs membrane structure and function (Porter, 1986). Given the susceptibility of PUFAs to ROS damage, it seems counter-intuitive that the PUFA-enriched thylakoid membranes would house the photosynthetic machinery, a potential ROS generator. In contrast to the well-studied mechanisms of water-soluble ROS detoxification in photosynthetic organisms (Asada, 1999), the mechanisms preventing or limiting oxidative damage in photosynthetic membranes are less well understood. Several peroxiredoxins have been implicated in reducing lipid hydroperoxides (LOOH) to the less toxic lipid hydroxides (LOH) in both plants and cyanobacteria (Gaber et al., 2001; Dietz, 2003; Gaber et al., 2004). In *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* specific carotenoids have also been shown to play roles in limiting lipid peroxidation, presumably by direct scavenging of free radicals (Havaux and Niyogi, 1999; Baroli et al., 2004). Tocopherols, a second major class of lipid-soluble antioxidants in photosynthetic membranes, are also believed to play

important roles in this process (Fryer, 1992; Munne-Bosch and Alegre, 2002). However, there is surprisingly little direct experimental evidence supporting such functions for tocopherols in photosynthetic organisms.

Tocopherols consist of a polar chromanol head group attached to a hydrophobic phytyl tail, both of which are critical to their roles as lipid-soluble antioxidants. Based on studies in artificial and animal cell-derived membranes, tocopherols can efficiently quench $^1O_2$ and scavenge various radicals (Bramley et al., 2000). The chromanol ring of tocopherols can reduce radicals by the donation of a single electron, resulting in the formation of a relatively stable tocopheroxyl radical, which in animals can be recycled back to the corresponding tocopherol by other antioxidants such as ascorbate or coenzyme Q (Stoyanovsky et al., 1995; May et al., 1998). Subsequent donation of a second electron from the tocopheroxyl radical forms the non-radical product, tocopherol quinone.

The tocopherol biosynthetic pathway has recently been fully elucidated in *Synechocystis* sp. PCC 6803 (Figure 2.1) (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Schledz et al., 2001; Shintani et al., 2002; Cheng et al., 2003; Sattler et al., 2003). Homogentisate phytyltransferase (HPT) catalyzes the committed step in tocopherol synthesis by condensing homogentisate (HGA) and phytyl-diphosphate (PDP) to produce 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ). HGA is produced from hydroxyphenylpyruvate (HPP) by HPP dioxygenase (HPPD). MPBQ is converted to 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) by MPBQ methyltransferase (MPBQ MT). Both MPBQ and DMPBQ are substrates for tocopherol cyclase (TC) to produce δ- and γ-tocopherols, respectively, which are then converted to β- and α-tocopherols by γ-

tocopherol methyltransferase (γ-TMT). During analysis of the biosynthetic pathway in *Synechocystis* sp. PCC 6803, mutants disrupting each biosynthetic enzyme have been isolated and characterized (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Schledz et al., 2001; Shintani et al., 2002; Cheng et al., 2003; Sattler et al., 2003). The tocopherol cyclase (*slr1737*) mutant lacks tocopherols entirely but accumulates the quinonol intermediate, DMPBQ, whereas the HPT (*slr1736*) mutant lacks all tocopherols and pathway intermediates (Figure 2.1) (Collakova and DellaPenna, 2001; Schledz et al., 2001; Sattler et al., 2003).

We have utilized the *slr1736* and *slr1737* mutants to assess the roles that tocopherols play in ROS homeostasis, membrane protection and how tocopherols are functionally integrated into the antioxidant network. In the current study, these mutants were challenged with combinations of chemicals and/or abiotic stresses to induce the formation of different types of ROS, and the ability of the mutants to withstand these stresses was evaluated. The increased sensitivity of tocopherol-deficient mutants to specific treatments indicates that tocopherols play a crucial role in limiting lipid peroxidation in *Synechocystis* sp. PCC 6803 *in vivo*.

RESULTS

Growth of Tocopherol-Deficient Mutants under High Intensity Light and ROS-Generating Conditions

The previously reported tocopherol-deficient *Synechocystis* sp. PCC 6803 mutants containing gene disruptions in homogentisate phytyltransferase (*slr1736)* and tocopherol

cyclase (*slr1737)* were originally isolated and maintained under photomixotrophic conditions, i.e. on glucose-containing media (Collakova and DellaPenna, 2001; Sattler et al., 2003). As described in the accompanying manuscript (Sakuragi et al., 2005), we now know that photomixotrophic selection is lethal for both mutant lines due to a glucose-sensitive phenotype that is a consequence of tocopherol deficiency. Thus, the original *slr1736* and *slr1737* mutant lines isolated had varying genotypes and physiologies presumably due to the unintentional selection of additional secondary suppressors of this glucose-sensitive phenotype. When the *aphII*-containing kanamycin-resistance DNA cartridge was reinserted into the *slr1736* and *slr1737* genes of wild-type *Synechocystis* sp. PCC 6803 and mutant selection was performed under photoautotrophic conditions, fully segregated populations were obtained that were genotypically and physiologically homogenous (Sakuragi et al., 2005). These authentic, photoautotrophically-isolated *slr1736::aphII* and *slr1737::aphII* mutants were used under photoautotrophic conditions for all experiments in the current study.

To test the susceptibility of tocopherol-deficient mutants to high-light (HL) intensity stress, wild type (WT) and the *slr1736* and *slr1737* mutants were initially grown at a relatively low-light (LL) intensity for *Synechocystis* sp. PCC 6803 (15 $\mu E\ m^{-2}\ s^{-1}$), the cells diluted to an appropriate density and transferred to HL (300 $\mu E\ m^{-2}\ s^{-1}$). As shown in Figure 2.2A, HL had little impact on growth of the mutant lines in comparison to WT, indicating tocopherols are dispensable under the HL stress conditions tested.

To investigate further the susceptibility of tocopherol-deficient mutants to additional oxidative stresses, various ROS-generating and stress-inducing chemicals were applied in combination with HL treatment. Paraquat (methyl viologen) causes generation

of O_2^- by transferring electrons from the PSI iron-sulfur clusters to O_2 (Fujii et al., 1990). Treatment with 2 μM paraquat/HL (paraquat in combination with 300 μE m^{-2} s^{-1} HL treatment) slowed the growth of WT and the *slr1736* and *slr1737* mutants to the same degree, while 5 μM paraquat/HL completely inhibited growth of all lines (Figure 2.2B). Similarly, treatment with a sub-lethal concentration (3 μM) of Rose Bengal, a $^1O_2$-generating photosensitizer, in HL also inhibited growth of WT and the *slr1736* mutant to similar degrees (data not shown). These data suggest that tocopherols do not play an essential role in detoxifying or tolerating the damage of O_2^- and $^1O_2$ in *Synechocystis* sp. PCC 6803, or that other compounds or enzymes can compensate for the lack of tocopherols in this regard.

Sensitivity of Tocopherol-Deficient Mutants to Compounds that Enhance Lipid Peroxidation

Tocopherols are known to play a crucial role in protecting animal cells from lipid peroxidation (Ham and Liebler, 1995, 1997) and have been proposed to perform a similar function in photosynthetic organisms (Fryer, 1992; Munne-Bosch and Alegre, 2002). To test this hypothesis, a variety of chemicals were used to induce lipid peroxidation in WT and the tocopherol deficient mutants. PUFAs, which are known to generate LOOH and LOO• by autoxidation reactions in the presence of oxygen (Porter, 1986), have been used to induce lipid peroxidation in yeast (Do et al., 1996) and cyanobacteria (Sakamoto et al., 1998). Linoleic acid ($18:2^{\Delta 9,12}$) and linolenic acid ($18:3^{\Delta 9,12,15}$), hereafter referred to as 18:2 and 18:3 respectively, were applied in combination with HL stress to WT and the

slr1736 and *slr1737* mutants. In the presence of 10 μM 18:2/HL, growth of the *slr1736* mutant ceased after 20 h, whereas WT and the *slr1737* mutant were able to grow as well as untreated controls (Figure 2.2C). Treatment with 10 μM 18:3/HL slowed the growth of all strains similarly during the initial 20 h of growth. At later time points, WT growth rates fully recovered, the *slr1736* mutant ceased to grow while the *slr1737* mutant showed an intermediate growth rate (Figure 2.2D). These data indicate that the tocopherol-deficient mutants are more susceptible to PUFA treatments than WT and that in *Synechocystis* sp. PCC 6803 tocopherols play critical roles in protecting cells from PUFA-induced stress. The intermediate phenotype of the *slr1737* mutant, which lacks tocopherols but accumulates the redox-active pathway intermediate DMPBQ, suggests that DMPBQ can partially compensate for the absence of tocopherols under these conditions.

Only the WT and *slr1736* mutant strains were used for subsequent analyses, and the initial OD_{730} for growth experiments was increased from 0.05 OD_{730} to 0.5 OD_{730} in order to obtain sufficient cells for biochemical analyses. Dose-response curves indicated the ten-fold increase in initial cell concentration required a corresponding increase in PUFA treatment levels to impact growth similarly (data not shown). Treatment of 0.5 OD_{730} cultures with 100 μM 18:3/HL slowed the growth of both the WT and *slr1736* mutant strains in the initial 20 h, while at later time points the *slr1736* mutant ceased to grow and growth of the WT recovered in a fashion similar to that observed in treating 0.05 OD_{730} cultures with 10 μM 18:3/HL (compare Figures 2.2D and 2.3C). The monounsaturated fatty acid, oleic acid ($18:1^{\Delta 9}$), hereafter refer to as 18:1, was used to test whether the toxicity of 18:3 to the *slr1736* mutant was due to the presence of any free

fatty acid in the media (a "detergent effect") or was specific to PUFAs. Both the WT and *slr1736* mutant strains were unaffected by treatment with 100 μM 18:1/HL (data not shown) and were able to grow unaffectedly even in the presence of 500 μM 18:1/HL (Figure 2.3E). These data indicate that the differential effects of 18:3 and 18:2 treatments on the growth of the WT and *slr1736* mutant strains are due to the polyunsaturation of these fatty acids. To test whether other PUFAs can also cause growth inhibition, eicosatrienoic acid ($20:3^{\Delta 11,14,17}$), hereafter referred to as 20:3, was applied at 100 μM, the same concentration of 18:3 that impacted growth of the *slr1736* mutant. Surprisingly, 100 μM 20:3/HL did not show a toxic effect on either the WT or the *slr1736* mutant (Figure 2.3G). These data suggest that factors in addition to the degree of polyunsaturation determine the toxicity of different PUFAs in the tocopherol-deficient mutants.

tert-Butyl hydroperoxide (*t*-BOOH) is a lipid-soluble hydroperoxide that has been used to induce lipid peroxidation in yeast and animal cells (Masaki et al., 1989; Pereira et al., 2003). Dose-response curves indicated growth of both the WT and *slr1736* mutant strains were negatively impacted at 150 μM *t*-BOOH/HL while 200 μM was lethal (data not shown). Growth of the WT and the *slr1736* mutant strains in 150 μM *t*-BOOH/HL was initially inhibited but both recovered rapidly to a similar extent (Figure 2.3I), indicating that tocopherols are not essential for acclimation to *t*-BOOH induced stress.

PUFA Treatments Increase Peroxides in the Growth Media

Because PUFA treatment has previously been shown to cause accumulation of lipid peroxides in yeast (Do et al., 1996) and the cyanobacterium *Synechococcus* sp. PCC 7002 (Sakamoto et al., 1998), the level of total peroxides in the growth media of WT and *slr1736* mutant strains during different treatments were measured using the ferrous oxidation-xylenol orange (FOX) assay (Griffiths et al., 2000; Sattler et al., 2004) and correlated with growth rates. LL, HL, and 18:1/HL treatments did not differentially affect growth of the WT and *slr1736* mutant strains (Figures 2.3A and E) and did not increase the peroxide levels of the media above background levels (Figures 2.3B and F). *t*-BOOH/HL, 18:3/HL and 20:3/HL treatments all resulted in high levels of peroxides in the media but had different impacts on growth. Media-peroxide levels in *t*-BOOH/HL treated WT and *slr1736* mutant cells were elevated at 30 min, returned to background levels by 4 h, but were much lower than in the absence of cells at all time points (Figure 2.3J). Therefore, it appears that both WT and the *slr1736* mutant can rapidly reduce *t*-BOOH, which would explain the limited and similar impact of *t*-BOOH treatment on cell growth of both lines (Figure 2.3I).

Media-peroxide levels in cells treated with 18:3/HL and 20:3/HL were near background levels at 30 min, increased to their highest levels by 4 or 8 h, and decreased thereafter. In the absence of cells, media peroxide levels increased linearly in treatments with both 18:3/HL and 20:3/HL (Figures 2.3D and H). The media peroxides produced during the 18:3/HL treatment were separated into water and lipid phases and more than 90% of the total peroxides were found in the lipid phase (data not shown), indicating the peroxides detected in the media are mainly lipid-derived peroxides. The media peroxide

levels of *slr1736* mutant cells treated with 18:3/HL and 20:3/HL were always equivalent or higher than the levels in treated WT cells. However, despite the apparent correlation of higher medium peroxide levels, especially at early time points, with more severe growth inhibition in *slr1736* mutant cells treated with 18:3/HL, it is clear that media peroxide levels are not the root cause of growth inhibition. Indeed, cells of the WT and the *slr1736* mutant treated with 20:3/HL had media peroxide profiles and levels similar to 18:3/HL treated cells (Figure 2.3H); however, there was no impact on growth of either genotype by 20:3 treatment (Figure 2.3G). This suggests that other processes within the PUFA treated cells, such as the differential incorporation and/or the oxidation of specific fatty acids in membranes contribute to the observed growth inhibition of the *slr1736* mutant.

Incorporation of 18:3 and 20:3 Fatty Acids into Membrane Lipids

The possibility that the toxicity of 18:3/HL may associated with more efficient uptake/incorporation of 18:3 into membranes in comparison to 20:3 was examined by analyzing the esterified fatty acid composition of membrane lipids after 4 h of 18:3/HL and 20:3/HL treatments. 18:3/HL and 20:3/HL treatments both resulted in increased levels of esterified 18:3 and 20:3, respectively, in both WT and the *slr1736* mutant relative to HL controls, though the increase from the 18:3/HL treatment was about three-fold greater than that of 20:3/HL treatment (Figure 2.4). Some incorporated 18:3 also appeared to be further desaturated to stearidonic acid ($18:4^{\Delta 6,9,12,15}$) or elongated to 20:3. As a consequence of the increased incorporation of 18:3 relative to 20:3, the total membrane PUFA content in cells of both the WT and *slr1736* mutant strains was increased significantly by 18:3/HL treatment but only slightly by 20:3/HL treatment

relative to HL controls (Figure 2.4). These results suggest that differential lethality of 18:3/HL and 20:3/HL treatments in the *slr1736* mutant are associated with the more efficient uptake/incorporation of 18:3 relative to 20:3. Because of carry over of exogenously-applied free PUFAs in washed cell pellets, we were unable to assess the relative free PUFA pool sizes of 18:3 and 20:3 treated WT and *slr1736* mutant cells.

Attempts were made to assess the cellular levels of lipid peroxidation by-products, LOOH and LOH, in 18:3/HL-treated cells of the WT and *slr1736* mutant strains using the FOX assay (Griffiths et al., 2000) and HPLC analysis (Sattler et al., 2004), respectively, but the results were inconclusive. LOOH and LOH levels in washed cell pellets did increase several-fold in response to 18:3 and 20:3 treatments, but these increases were highly variable and in all cases paralleled the LOOH and LOH levels detected in the media. Therefore, as with analysis of cellular free PUFA levels, it appears that the high background level of LOOH and LOH in the media of PUFA-treated cells precludes distinguishing and quantifying lipid peroxidation products that were specifically generated in cells or cell membranes.

Changes in Carotenoids, Chlorophyll a, and Tocopherols during HL and 18:3/HL Treatments

The effect of HL and 18:3/HL treatments on photosynthetic pigment composition (carotenoids and chlorophyll *a*) and tocopherols were analyzed by HPLC. In the absence of any treatment (LL grown cells), the total carotenoid and chlorophyll contents of the WT and *slr1736* mutant strains were identical (Figures 2.5A and C at 0 h). Individual

carotenoid levels were also nearly identical with the exception of myxoxanthophyll and zeaxanthin, which were slightly lower and higher, respectively in the *slr1736* mutant in comparison to WT (Figure 2.6 A and C at 0 h). The total carotenoid content of HL treated WT cells was unchanged during the first 20 h (Figure 2.5A) but there was a significant increase in myxoxanthophyll and a corresponding decrease in zeaxanthin and echinenone levels (Figures 2.6A, C and E). By 45 h the total carotenoid content of HL treated WT had increased 20%, mostly due to an increase in myxoxanthophyll content (Figure 2.6A). When WT was subjected to 18:3/HL treatment, the total carotenoid content decreased slightly at 3 h (Figure 2.5A) due to small but significant decreases in myxoxanthophyll and zeaxanthin (Figures 2.6B and D). Total carotenoid levels then increased at 20 h and were 67 % higher by 45 h (Figure 2.5B) due to a large increase in myxoxanthophyll levels and smaller increases in zeaxanthin and beta-carotene (Figures 2.6B, D and H). These data indicate that carotenoid synthesis in WT is up-regulated in response to both HL and 18:3/HL treatments.

The total carotenoid level of *slr1736* mutant cells treated with HL and 18:3/HL were similar to WT for the initial 3 h of treatment and transiently increased at 20 h before decreasing to approximately 80 % of the initial control level by 45 h (Figures 2.5A and B). The decrease in total carotenoid levels in the *slr1736* mutant during HL treatment was due almost entirely to a precipitous drop in myxoxanthophyll levels by 45 h (Figure 2.6A). This drop also occurred in the 18:3/HL treated *slr1736* mutant along with a severe decrease in zeaxanthin levels (Figures 2.6B and D). This reduction in individual and total carotenoid levels in HL-and 18:3/HL-treated *slr1736* mutant cells sharply contrasts with

WT and suggests that in the absence of tocopherols, specific carotenoids in the *slr1736* mutant cells undergo more rapid turnover/degradation than in WT cells.

The chlorophyll *a* contents of the WT and *slr1736* mutant cells during HL treatment were very similar with the exception of 20 h, where the *slr1736* mutant showed a transient increase (Figure 2.5C). This similarity in chlorophyll content is consistent with the growth of WT and the *slr1736* mutant being indistinguishable in HL (Figure 2.3A). When WT was subjected to 18:3/HL treatment, chlorophyll levels initially decreased before recovering by 45 h, in parallel with the increase in total carotenoids (Figures 2.5B and D). In contrast, the chlorophyll content of 18:3/HL-treated *slr1736* mutant cells continuously decreased at all time points to 46 % of the initial value by 45 h (Figure 2.5D), suggesting that impaired growth of the *slr1736* mutant (Figure 2.3C) was coincident with the loss of photosynthetic capacity as reflected by the lower chlorophyll content.

The total tocopherol content was also measured in WT and *slr1736* mutant cells subjected to HL and 18:3/HL treatment (Figures 2.5E and F). No tocopherols were detected in the *slr1736* mutant cells at any time point or treatment, consistent with the nature of the mutation. The tocopherol content of HL-treated WT was reduced approximately 20 % at 3 and 20 h before recovering by 45 h. When WT cells were subjected to 18:3/HL treatment, a more severe reduction in tocopherols was observed after 3 h followed by a sharp increase at 20 and 45 h to twice the initial level. This initial decrease followed by accelerated accumulation of tocopherols during 18:3/HL treatment of WT suggests tocopherols play a key role in the response of *Synechocystis* sp. PCC 6803 to 18:3-induced oxidative stress.

Norflurazon/HL Treatment

The experiments described above (Figure 2.5A) further suggested a possible functional interaction between carotenoids and tocopherols in *Synechocystis* sp. PCC 6803. To assess any potential interaction, carotenoid synthesis was inhibited with norflurazon (NF), a herbicide that specifically inhibits phytoene desaturase (Breitenbach et al., 2001; He et al., 2001). Dose-response experiments indicated that growth of the *slr1736* mutant was much more sensitive to inhibition of carotenoid synthesis at levels as low as 5 μM NF/HL (Figure 2.7A). During treatment with 25 μM NF/HL, WT grew more slowly than HL treatment alone but was still viable, while growth of the *slr1736* mutant was completely abolished after 30 h (Figure 2.7B). Under LL conditions, treatments with 25 μM or 100 μM NF did not affect the growth of either WT or the *slr1736* mutant relative to untreated cells (data not shown). These results indicate that, when *Synechocystis* sp. PCC 6803 cells are subjected to HL-stress, the simultaneous inhibition of both carotenoid and tocopherol synthesis is more deleterious than inhibition of either pathway alone.

Pigment analyses during NF/HL treatment revealed that total carotenoid levels decreased much faster in the *slr1736* mutant cells compared to WT cells (Figure 2.8A). While both WT and the *slr1736* mutant reached a lower steady-state carotenoid level by 20 h of NF/HL treatment, the steady-state carotenoid level in the *slr1736* mutant cells was less than half that of WT cells. Chlorophyll levels were similar in the *slr1736* mutant and WT up to 30 h (Figure 2.8B), but by 45 h the *slr1736* mutant had lost almost all carotenoids and chlorophyll, while WT cells maintained a constant level of both. Because

carotenoid synthesis is presumably inhibited to the same degree by 25 μM NF treatment in WT and the *slr1736* mutant, these results suggest that carotenoids were degraded more rapidly during the NF/HL treatment in the absence of tocopherols; this loss of carotenoids in turn led to bleaching and eventual death of the *slr1736* mutant cells. When individual carotenoids were analyzed during NF/HL treatment all were found to decrease in both WT and the *slr1736* mutant but myxoxanthophyll and beta-carotene decreased to lower levels in the *slr1736* mutant than in WT (Figure 2.9). The combined results of NF/HL treatment on growth and photosynthetic pigments demonstrate that tocopherols and carotenoids play important and complementary roles in protecting *Synechocystis* sp. PCC 6803 cells from HL stress.

DISCUSSION

In contrast to the well-established roles of tocopherols in animals (Brigelius-Flohe and Traber, 1999; Ricciarelli et al., 2002), assessing tocopherol functions in photosynthetic organisms has only recently become experimentally approachable as a result of the complete molecular dissection of the biosynthetic pathway and isolation of mutants in cyanobacteria and plants (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Schledz et al., 2001; Porfirova et al., 2002; Shintani et al., 2002; Cheng et al., 2003; Sattler et al., 2003). The evolutionary conservation of tocopherol synthesis in oxygenic phototrophs, the localization of tocopherols in photosynthetic membranes, and the increased tocopherol accumulation in response to a variety of stresses suggest a key role for tocopherols in photosynthetic organisms during stress (Munne-Bosch and Alegre,

2002; Collakova and DellaPenna, 2003). However, such lines of evidence are circumstantial, and this hypothesis has not yet been rigorously tested.

Light is required for photosynthesis but light intensity in excess of that required for photosynthesis can also create ROS resulting in oxidative damage to the photosystems. Somewhat surprisingly, HL treatment did not differentially affect the growth (Figures 2.2A and 3A), membrane lipid fatty acid composition (Figure 2.4), or chlorophyll *a* content (Figure 2.5C) of the tocopherol-deficient mutants and WT. The only observed differences were total carotenoid levels, which, unlike WT, did not remain elevated in the HL-treated cells of the *slr1736* mutant, primarily due to a severe drop in myxoxanthophyll levels at 45 h (Figures 2.5A and 6A). The results are consistent with those of another tocopherol-deficient mutant in *Synechocystis* sp. PCC 6803 (*slr0090::aphII*, disrupted mutant in the HPPD enzyme) which when grown at 500 μE m^{-2} s^{-1} was also indistinguishable from WT (Dahnhardt et. al., 2002). These combined data indicate that tocopherols are not essential for tolerating/acclimating to moderate (<500 μE m^{-2} s^{-1}) HL conditions in *Synechocystis* sp. PCC 6803.

One could argue that the similar responses of HL-treated cells of the WT and the tocopherol mutants are because the light intensity used (300 μE m^{-2} s^{-1}) was not sufficiently high to require tocopherol function(s), as treatment of *Chlamydomonas reinhardtii* at 1500 μE m^{-2} s^{-1} with an herbicide that inhibits HPPD enzyme activity reduced tocopherol levels to 20% of controls and induced concomitant degradation of the D1 protein (Trebst et al., 2002). However, 300 μE m^{-2} s^{-1} is three times the level needed to saturate photosynthesis in *Synechocystis* sp. PCC 6803, and this condition has

previously been shown to up-regulate both high light-responsive and oxidative stress-related genes (e.g. high-light-inducible proteins, SOD, and glutathione peroxidase) (Hihara et al., 2001; Huang et al., 2002). Another plausible explanation is that other components of the antioxidant network may mitigate ROS damage or compensate for the lack of tocopherols in mutants under the conditions tested. Indeed, like most photosynthetic organisms, *Synechocystis* sp. PCC 6803 contains multiple layers of ROS defenses, including carotenoids, peroxiredoxins, SOD, and catalase-peroxidase (Kaneko et al., 1996), some or all of which may mitigate any damage caused by the lack of tocopherols under the HL conditions tested in this study. Future studies utilizing light intensities approaching full sunlight (2000 $\mu E\ m^{-2}\ s^{-1}$) may provide additional insights into tocopherol functions in photosynthetic organisms.

In order to test the hypothesis that tocopherols play a critical role in tolerance to specific types of ROS or ROS-induced damage, the tocopherol-deficient mutants and WT were subjected to chemical treatments in combination with HL stress to generate different types of ROS. WT and the tocopherol-deficient *slr1736* mutant did not show differential sensitivity to treatment with the $^1O_2$ generating compound Rose Bengal (data not shown). Similarly, paraquat, a O_2^- generator, did not cause differential effects on the growth of WT and tocopherol-deficient mutants (Figure 2.2B). A *Synechococcus* sp. PCC 7942 mutant deficient in SOD showed enhanced sensitivity to paraquat at 100 $\mu E\ m^{-2}\ s^{-1}$, demonstrating that SOD is essential for O_2^- detoxification at moderate light levels (Thomas et al., 1998). These combined data suggest that tocopherols are not crucial for O_2^- or $^1O_2$ detoxification/tolerance in *Synechocystis* sp. PCC 6803 under the conditions tested.

Tocopherols have long been assumed to protect the membranes of oxygenic phototrophs from oxidative stress. To assess this proposed function, the tocopherol-deficient mutants and WT were subjected to treatments known to induce lipid peroxidation. *t*-BOOH is an alkyl peroxide routinely used to induce lipid peroxidation in other systems (Masaki et al., 1989; Pereira et al., 2003). Surprisingly, *t*-BOOH did not differentially impact the *slr1736* mutant and WT (Figure 2.3I), suggesting tocopherols might not be essential in protecting *Synechocystis* sp. PCC 6803 cells from lipid peroxidation. As the role of tocopherols as lipid peroxidation chain reaction terminators is well established *in vitro* and in animal systems (Brigelius-Flohe and Traber, 1999; Wang and Quinn, 2000), this would be most unexpected. The similar and extremely rapid turnover of *t*-BOOH in the media of WT and the *slr1736* mutant (Figure 2.3J) instead suggests that components other than tocopherols function very efficiently in both genotypes to rapidly reduce *t*-BOOH levels *in vivo*. Five peroxiredoxins have been characterized in *Synechocystis* sp. PCC 6803, and four of them, Sll0755, Slr1171, Slr1992 and Sll1621, have been shown to reduce *t*-BOOH efficiently *in vitro* when expressed in *Escherichia coli* (Yamamoto et al., 1999; Gaber et al., 2001; Hosoya-Matsuda et al., 2005). The expression of *slr1171* and *slr1992* are also induced in response to HL (Huang et al., 2002), and it is likely that these peroxiredoxins confer the similar resistance of WT and the *slr1736* mutant to *t*-BOOH treatment.

Unlike *t*-BOOH, the tocopherol-deficient mutants did show enhanced sensitivity to treatments with specific PUFAs. Treatment with 18:3 caused more severe growth inhibition than 18:2, while 18:1 was non-toxic (Figures 2.2C, 2D, 3C, and 3E). These results indicate that the extent of toxicity for eighteen-carbon fatty acids depends on the

degree of polyunsaturation. The results of growth curves and lipid peroxide analyses of the growth media further suggested that oxidation of 18:2 and 18:3 in the medium might be associated with their toxicity. However, in comparing the results from 18:3 and 20:3 treatments, which cause similar levels of lipid peroxides in the medium but have opposite effects on growth (Figures 2.3C, D, G, and H), it is clear that lipid peroxide levels in the medium *per se* are not the primary cause of 18:3 toxicity. The enhanced uptake/incorporation of 18:3 fatty acids into cell membranes relative to 20:3 (Figure 2.4) implies that the 18:3 treatment results in more severe lipid peroxidation inside the cell. This could occur due to elevated levels of free or esterified PUFAs in membranes, either of which could initiate or participate in enhanced auto-catalytic lipid peroxidation in the mutants. Unfortunately, PUFA treatments resulted in such high background levels of free PUFAs and lipid peroxides in media and cell pellets that it was not possible to reproducibly quantify the levels of free PUFAs and esterified or non-esterified lipid peroxidation by-products in PUFA-treated cells. As a consequence we were unable to directly determine whether non-enzymatic or enzyme-mediated lipid oxidation (e.g. lipoxygenases) was enhanced in membranes of tocopherol deficient mutants. Despite these analytical limitations our results are consistent with the hypothesis that tocopherols are critical in protecting *Synechocystis* sp. PCC 6803 from lipid peroxidation.

If tocopherols are crucial for protecting *Synechocystis* sp. PCC 6803 from lipid peroxidation, why is the *slr1737* mutant less sensitive to PUFA/HL treatment than the *slr1736* mutant (Figure 2.2), when both are deficient in tocopherols? The *Arabidopsis vte1* and *vte2* mutants (equivalent to the *slr1737* and *slr1736* mutants, respectively) both had reduced seed longevity but only *vte2* exhibited early seedling developmental defects

and a greater than 100-fold increase in lipid peroxidation during germination (Sattler et al., 2004). The attenuated phenotype of *vte1* relative to *vte2* is consistent with the attenuated phenotype of PUFA-treated *slr1737* relative to *slr1736* mutants (Figure 2.2) and suggests that the quinol intermediate, DMPBQ, that accumulates in the *vte1* and *slr1737*, but not in the *vte2* and *slr1736* mutants, functionally compensates for the absence of tocopherols in many regards, most likely by acting as an alternative lipid-soluble antioxidant. In this regard it is interesting to note that the *Arabidopsis vte1* mutant accumulates slightly but significantly increased levels of glutathione and ascorbate even in the absence of stress (Kanwischer et. al., 2005). Whether these water-soluble antioxidants may also play a role in the attenuated phenotype of the *vte1* is as yet unclear.

Carotenoids are the second major group of lipid-soluble antioxidants in photosynthetic membranes and have been shown to play important roles in protecting plant and green algae during photooxidative stress (Havaux and Niyogi, 1999; Baroli et al., 2003; Baroli et al., 2004). However, with the exception of their structural roles in photosystems, little work has been done to assess other physiological roles of carotenoids in *Synechocystis* sp. PCC 6803. Prior studies have shown that two carotenoid biosynthetic genes (*slr1254* and *slr0940*) are up-regulated during HL stress in WT *Synechocystis* sp. PCC 6803 (Huang et al., 2002), which is consistent with the observed increase in the levels of total and specific carotenoid (myxoxanthophyll being the most prominent) in WT in response to HL (Figures 2.5A and 6 A, G). In contrast, the *slr1736* mutant did not show corresponding increases in total or specific carotenoids during HL and 18:3/HL treatments. These data indirectly but strongly suggest that carotenoids, most likely

myxoxanthophyll, are involved in the adaptation/tolerance of *Synechocystis* sp. PCC 6803 to HL stress and functionally interact with or complement tocopherols.

To assess further the role of carotenoids in adapting to HL stress and any functional interactions between tocopherols and carotenoids, carotenoid synthesis was partially inhibited in WT and the tocopherol-deficient *slr1736* mutant by treatment with NF/HL. Phytoene desaturase (Slr1254), one of two carotenoid biosynthetic enzymes induced in response to HL (Huang et al., 2002), is the enzymatic target of NF (Breitenbach et al., 2001). Treatment with 25 μM NF in HL slowed the growth of WT but had a much more severe impact on growth of the tocopherol-deficient *slr1736* mutant (Figure 2.7B). NF-treated *slr1736* mutant cells also had a steady-state level of carotenoids half that of WT (Figure 2.8A), mainly due to lower levels of myxoxanthophyll and beta-carotene (Figure 2.9). Assuming NF inhibits carotenoid synthesis to a similar degree in both mutant and WT, the higher steady-state level of total carotenoids and better growth rate of WT during NF treatment is due to the presence of tocopherols. These data clearly demonstrate that carotenoids are a key component compensating for the absence of tocopherols during HL stress in the mutant cells. Introduction of other mutations that affect the levels of individual carotenoid species (FernandezGonzalez et al., 1997; Lagarde and Vermaas, 1997; Mohamed and Vermaas, 2004) into the tocopherol-deficient mutant background will further clarify the role(s) of individual carotenoids in the adaptation/tolerance of *Synechocystis* sp. PCC 6803 to HL stress in the absence of tocopherols.

In summary, the enhanced sensitivity of tocopherol-deficient mutants of *Synechocystis* sp. PCC 6803 to specific PUFAs provides physiological and biochemical

evidence that tocopherols are crucial in protecting oxygenic phototrophs from lipid peroxidation *in vivo*. These data are consistent with a recent study of tocopherol-deficient mutants of *Arabidopsis*, which have reduced seed longevity and early seedling developmental defects due to greatly increased lipid peroxidation during germination in the absence of tocopherols (Sattler et al., 2004). From the combined studies in these two model photosynthetic organisms, it can be concluded that a primary function of tocopherols in both eukaryotic and prokaryotic oxygenic photosynthetic organisms is to protect cells from lipid peroxidation. Simultaneous inhibition of carotenoid and tocopherol biosynthesis in *Synechocystis* sp. PCC 6803 clearly demonstrated the two classes of lipid-soluble antioxidants functionally interact or have complementary roles during HL stress. The overlapping functionality of tocopherols and carotenoids in *Synechocystis* sp. PCC 6803 may explain why tocopherols appear to be dispensable during moderate HL stress (up to 500 $\mu E\ m^{-2}\ s^{-1}$) (Figures 2.2A and 3A) (Dahnhardt et al., 2002). However, under extreme and specific stress conditions, such as during PUFA-induced lipid peroxidation in HL, the absence of tocopherols can not be fully compensated by carotenoids, and both lipid-soluble antioxidants are required for survival of *Synechocystis* sp. PCC 6803.

MATERIALS AND METHODS

Chemicals

Oleic acid ($18:1^{\Delta 9}$), linoleic acid ($18:2^{\Delta 9,12}$), linolenic acid ($18:3^{\Delta 9,12,15}$), eicosatrienoic acid ($20:3^{\Delta 11,14,17}$), *tert*-butyl hydroperoxide (*t*-BOOH), paraquat (methyl viologen), Rose Bengal, butylated hydroxytoluene (BHT), and xylenol orange [*o*-cresolsulfonephthalein-

3,3'-*bis*(methylimino-diacetic acid)sodium salt] were purchased from Sigma (St. Louis). Ferrous ammonium sulfate hexahydrate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_20$] was from Aldrich (Germany). Norflurazon was from Chem Service Inc. (California).

Growth Conditions and Chemical Treatments

The construction, photoautotrophic selection and molecular and physiological characterization of authentic *slr1736* and *slr1737* mutants of *Synechocystis* sp. strain PCC 6803 are described in detail in the accompanying manuscript (Sakuragi et al., 2005). WT and mutant strains of *Synechocystis* sp. strain PCC 6803 were grown photoautotrophically in liquid B-HEPES medium, which is BG-11 (Williams, 1988) supplemented with 4.6 mM of HEPES (pH 8.0) and 18 mg L^{-1} ferric ammonium citrate. Growth was at 32 °C with 1% (v/v) CO_2 in air under constant illumination from cool-white fluorescent lamps at 15 μE m^{-2} s^{-1} (low-light, LL) or 300 μE m^{-2} s^{-1} (high-light, HL). Light intensity was measured by a LI-250 Light meter (LI-COR Inc., Nebraska). Cell growth was monitored by the optical density at 730 nm (OD_{730}). For growth and treatments in HL, exponentially growing LL cultures (OD_{730} = 0.7 to 1.0) were diluted to 0.05 or 0.5 of OD_{730} with fresh B-HEPES medium and transferred to the HL condition described above.

Peroxide Analysis

The peroxide contents in media and cell pellets were measured using the ferrous oxidation-xylenol orange (FOX) assay (Griffiths et al., 2000). Aliquots of cultures (500

μl to 1 ml) were collected at different time points and centrifuged at 15,000 *g* for 5 min. The supernatants (60 μl) were mixed with 540 μl of FOX reagent [90% (v/v) methanol, 4 mM BHT, 25 mM sulfuric acid, 250 μM $Fe(NH_4)_2(SO_4)_2$, 100 μM xylenol orange], and incubated for 20 min in darkness, and the A_{560} was measured. The peroxide content was calculated based on a standard curve created by known concentrations of hydrogen peroxide (J. T. Baker, New Jersey).

Lipid Composition Analysis

Cells were collected by centrifugation at 3,500 *g* for 15 min and lipid extracts were prepared as previously described (Hara and Radin, 1978). Esterified fatty acids were selectively methyl-esterified by KOH-catalyzed transesterification as described (Ichihara et al., 1996). Fatty acid methyl esters (FAME) were quantified by gas-liquid chromatography (GLC) using pentadecanoic acid (Sigma, St. Louis) as an internal standard (Rossak et al., 1997).

Carotenoid, Chlorophyll a, and Tocopherol Analyses

The amount of cells equivalent to 10 ml of OD_{730} = 1.0 culture were collected by centrifugation at 8,000 *g* for 5 min and washed twice with 25 mM HEPES buffer, pH 7.0. Carotenoids and tocopherols were extracted in 500 μl of methanol with 1 mg ml^{-1} BHT at 4 °C. After centrifugation and filtration, one hundred microliters was subjected to HPLC (Agilent 1100 series, Agilent, Wilmington, DE) on a Spherisorb ODS-2 5 μm, 250 x 4.6 mm reverse phase column (Column Engineering, Ontario, CA) using a 30 min gradient of

isopropanol (0-10 min, 0%; 10-20 min, 0 to 80%; 20-25 min, 80%; 25-30 min, 80 to 0%) in methanol at a flow rate of 0.75 ml min^{-1}. Photodiode array detection was used to identify each carotenoid species and chlorophyll *a* by their characteristic absorption spectra and their retention times relative to standards. Individual carotenoids and chlorophyll *a* were quantified against a standard equation derived by injection of known amounts of each purified compound. Tocopherols were detected by fluorescence using 290 nm excitation and 325 nm emission and quantified against standard curves generated by commercially available tocopherols (ACROS ORGANICS, NJ).

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FIGURES

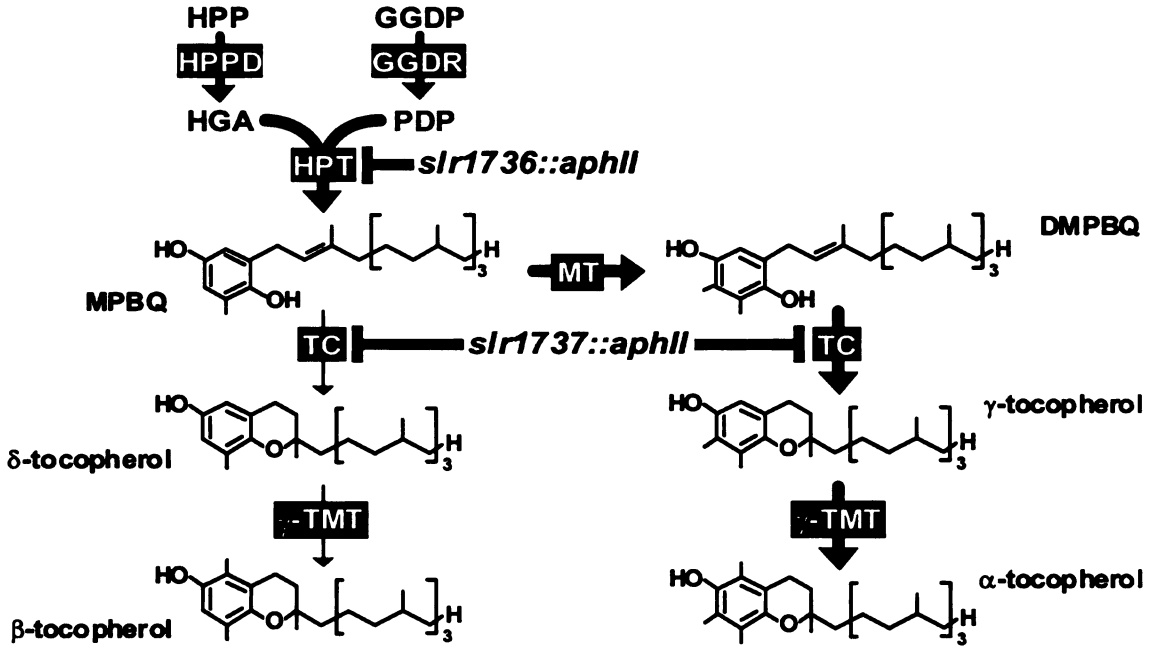

Figure 2.1. Tocopherol Biosynthetic Pathway and Locations of Mutations in *Synechocystis* sp. Strain PCC 6803.

HPP, hydroxyphenylpyruvate; GGDP, geranylgeranyl-diphosphate; PDP, phytyl-diphosphate; HGA, homogentisic acid; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol; HPPD, HPP dioxygenase; GGDR, GGDP reductase; HPT, HGA phytyltransferase; TC, tocopherol cyclase; MT, MPBQ methyltransferase; γ-TMT, γ-tocopherol methyltransferase; *slr1736::aphII* and *slr1737::aphII*, disrupted mutants of HPT and TC, respectively. Bold arrows show the primary biosynthetic route *in vivo*; α-tocopherol is the major tocopherol in *Synechocystis* sp. strain PCC 6803.

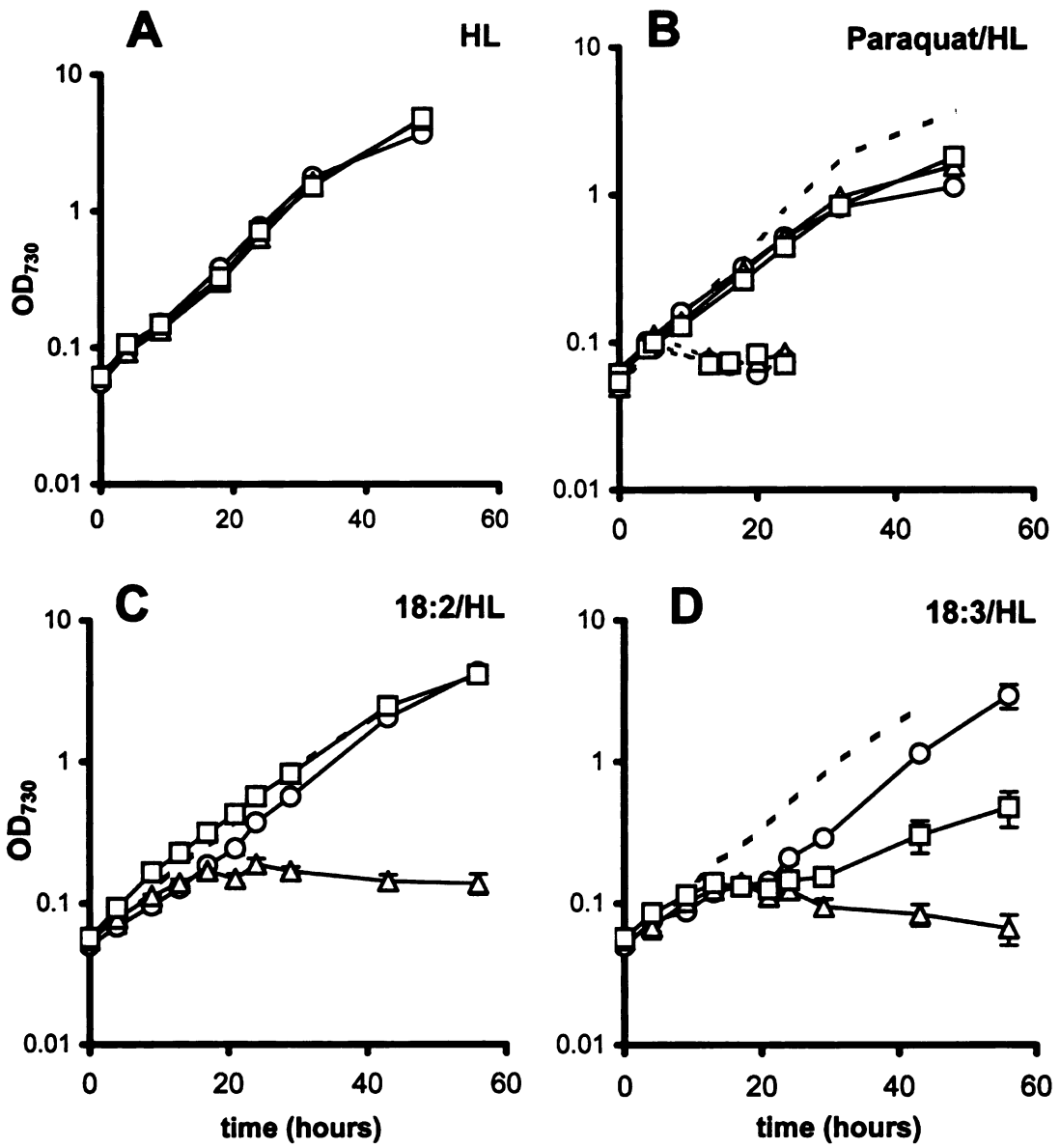

Figure 2.2. Growth Curves of Wild Type (WT) and the Tocopherol-Deficient *slr1736* and *slr1737* Mutants under Different Stress and Chemical Treatments.

WT (circles), *slr1736* mutant (triangles) and *slr1737* mutants (squares) were grown at 32 °C, 1 % (v/v) CO_2 in air under A. HL; B. HL with 2 μM (solid lines) and 5 μM (dotted lines) paraquat; C. HL with 10 μM 18:2; D. HL with 10 μM 18:3. A and B show representative results of at least three independent experiments, while data in C and D are the means ± SD (n = 4). SD in C and D are shown only when larger than symbols. In B, C, and D the HL WT growth curve is shown as a gray dotted line with no symbol for reference.

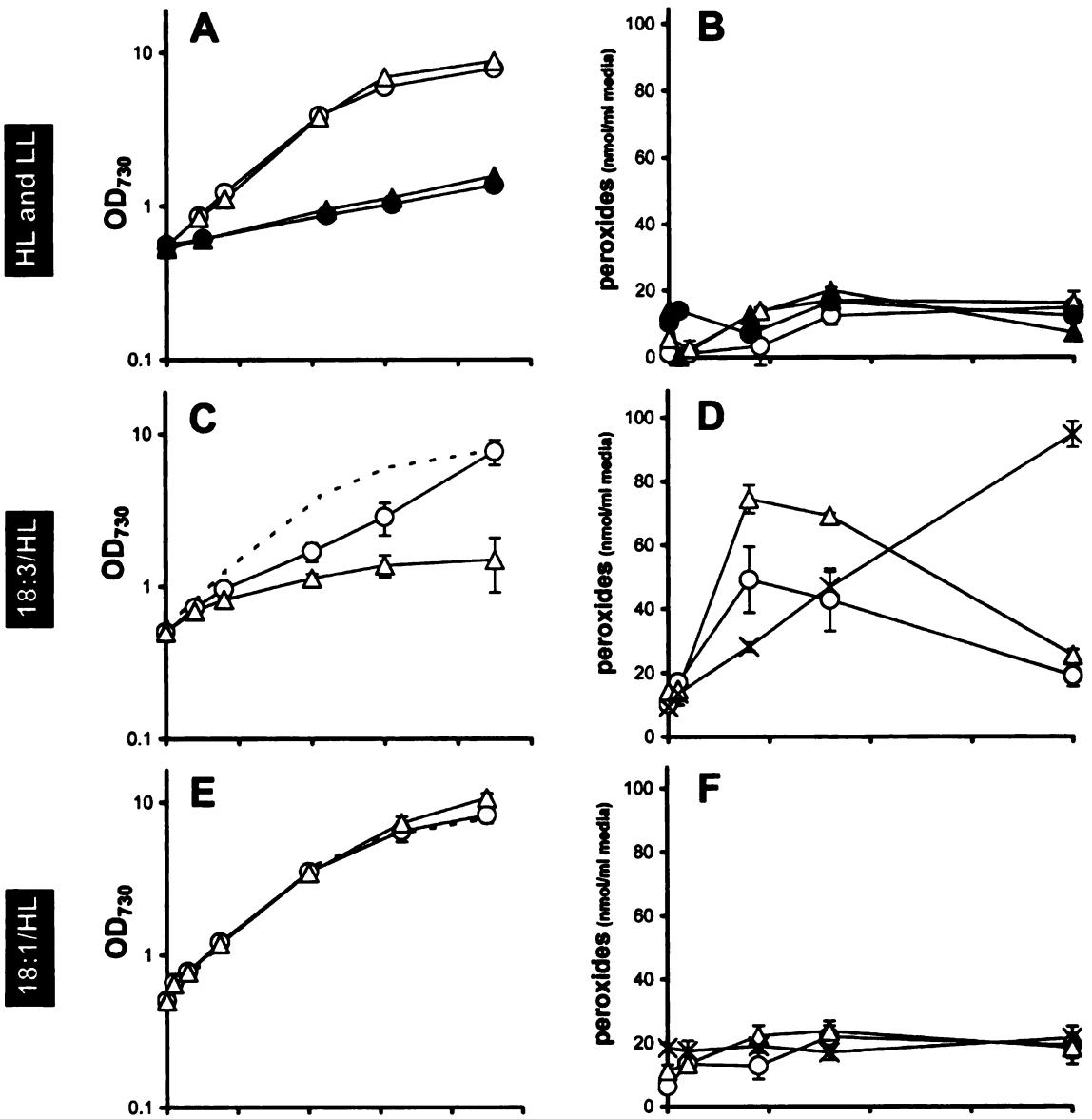

Figure 2.3. Growth Curves and Medium Peroxide Levels of WT and the Tocopherol Deficient *slr1736* Mutant under HL with Various Chemical Treatments.

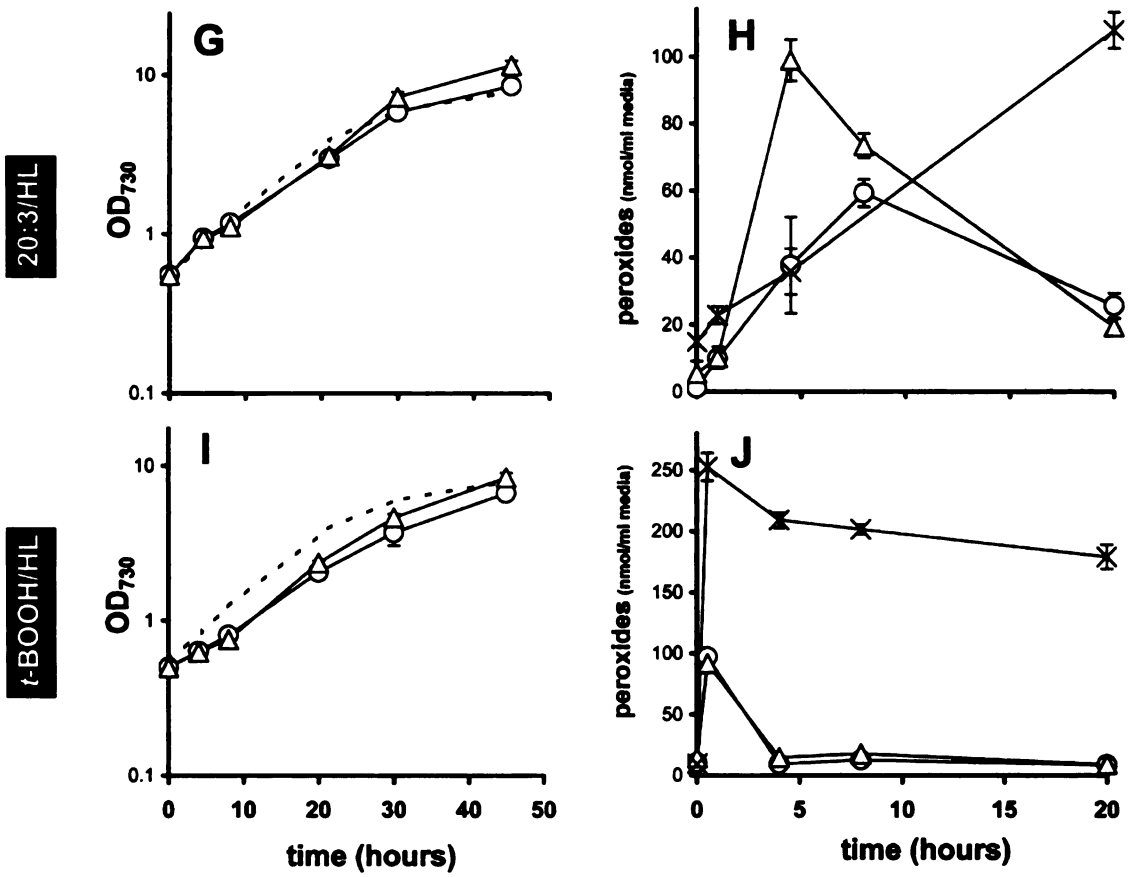

Figure 2.3. (Continued)

WT (circles) and *slr1736* mutant (triangles) were grown at 32 °C, 1 % (v/v) CO_2 in air under HL. A and B, control (HL, open marks; LL, filled marks); C and D, HL with 100 μM 18:3; E and F, HL with 500 μM 18:1; G and H, HL with 100 μM 20:3; I and J, HL with 150 μM *t*-BOOH. A, C, E, G, and I are 45 h growth curves while B, D, F, H, and J are the respective medium peroxide levels during the first 20 h. Peroxide levels before addition of chemicals are shown at 0 h (B, D, F, H, and J). The Y-axis scale of J is different from B, D, F and H. The gray dotted line in C, E, G, and I is the growth curve of HL treated WT from A for reference. Crossed marks in D, F, H, and J are media peroxide levels in the absence of cells. The data shown are the means ± SD of cultures grown in triplicate, except the LL media peroxide levels, which is representative of three independent experiments (B). The SD is shown only when it is larger than the symbols except for the LL media peroxide levels in B.

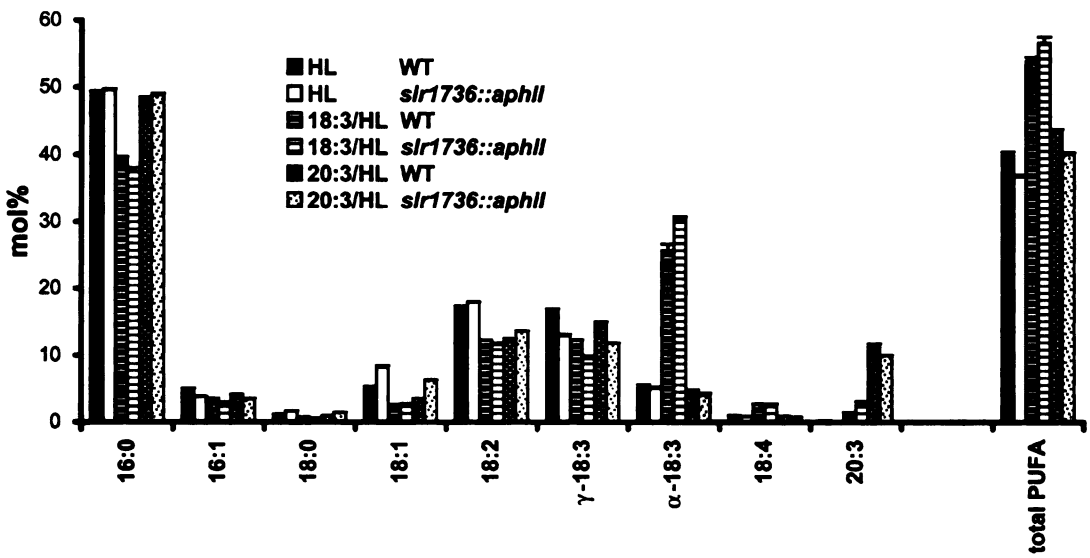

Figure 2.4. Fatty Acid Composition of Total Membrane Lipid Extracts from WT and the Tocopherol-Deficient *slr1736* Mutant after 4 h of Polyunsaturated Fatty Acid (PUFA) Treatment.

The molar percentage of each fatty acid species esterified to membrane lipids is indicated. Total PUFA levels were calculated as sums of 18:2, γ-linolenic acid (γ-18:3), α-linolenic acid (α-18:3), stearidonic acid (18:4) and 20:3 in each genotype. Data shown are the means ± SD (n = 4). SD is shown only when it is larger than symbols.

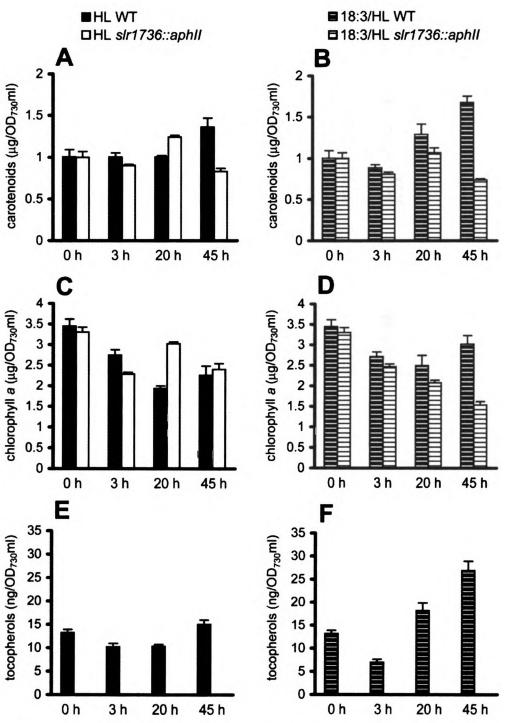

Figure 2.5. Total Carotenoid, Chlorophyll, and Tocopherol Contents in WT and the Tocopherol-Deficient *slr1736* Mutant during HL and 18:3/HL Treatments.

Total carotenoid (A and B), chlorophyll *a* (C and D), and tocopherol (E and F) levels were measured at 0, 3, 20 and 45 h of HL (A, C, and E) and 100 µM 18:3/HL (B, D, and F) treatments at 32 °C, 1 % (v/v) CO_2 in air. Tocopherol was not detected in the *slr1736* mutant in any experiments. Data shown are the means ± SD (n = 3).

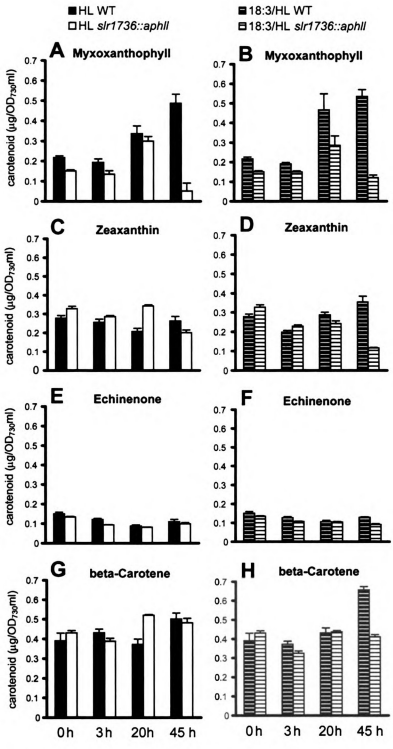

Figure 2.6. Levels of Individual Carotenoids in WT and the Tocopherol-Deficient *slr1736* Mutant during HL and 18:3/HL Treatments.

Myxoxanthophyll (A and B), zeaxanthin (C and D), echinenone (E and F) and beta-carotene (G and H) were measured at 0, 3, 20 and 45 h of HL (A, C, E and G) and 100 µM 18:3/HL (B, D, F and H) treatments at 32 °C, 1 % (v/v) CO_2 in air. Data shown are the means ± SD (n = 3).

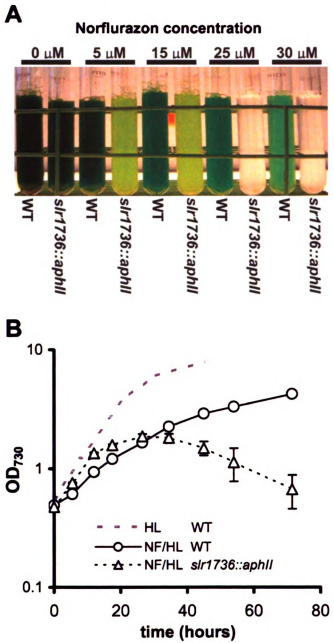

Figure 2.7. Growth of WT and the Tocopherol-Deficient *slr1736* Mutant in the Presence of Norflurazon (NF) in HL.

A. WT and the *slr1736* mutant were grown at the indicated concentration of NF for 90 h under HL at 32 °C, 1 % (v/v) CO_2 in air. B. Growth curves of WT (circles) and the *slr1736* mutant (triangles) during 25 µM NF/HL treatment. Data shown are the means ± SD ($n = 4$). SD is shown only when it is larger than the symbols. The growth curve for the WT under HL was shown as a gray dotted line.

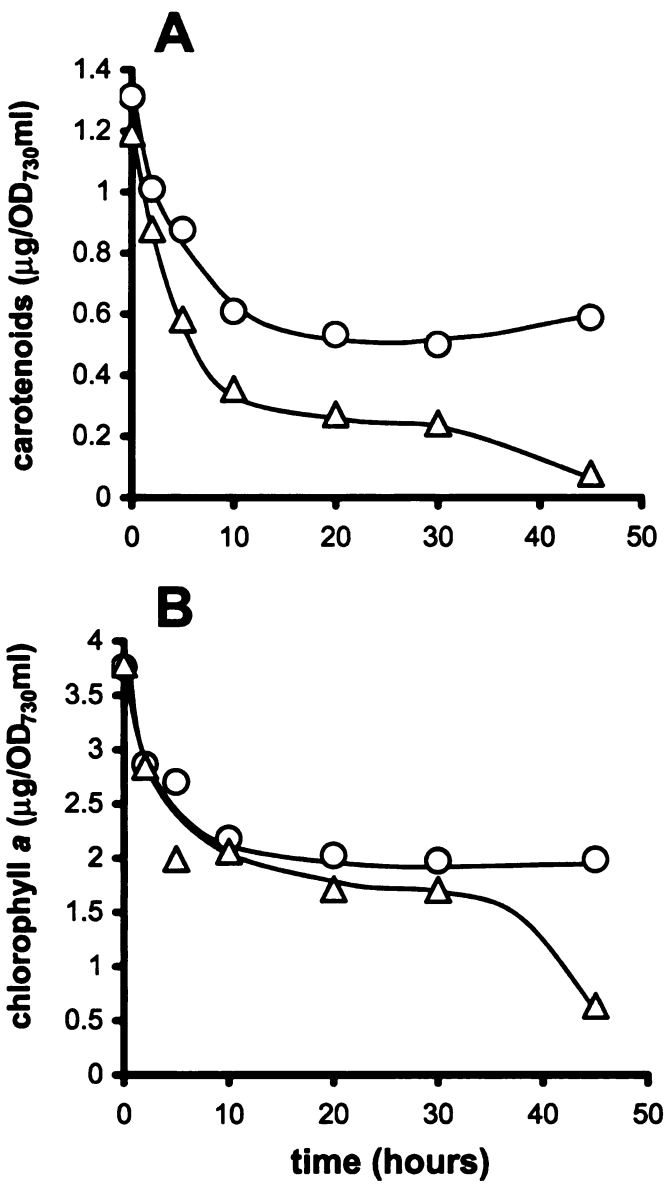

Figure 2.8. Changes in Total Carotenoids and Chlorophyll *a* Contents in the WT and the Tocopherol-Deficient *slr1736* Mutant during Norflurazon (NF)/HL Treatment.

A. Total carotenoids and B. chlorophyll *a* levels of WT (circles) and the *slr1736* mutant (triangles) were measured during 25 μM NF/HL treatment at 32 °C, 1 % (v/v) CO_2 in air. These data are representative of two independent experiments.

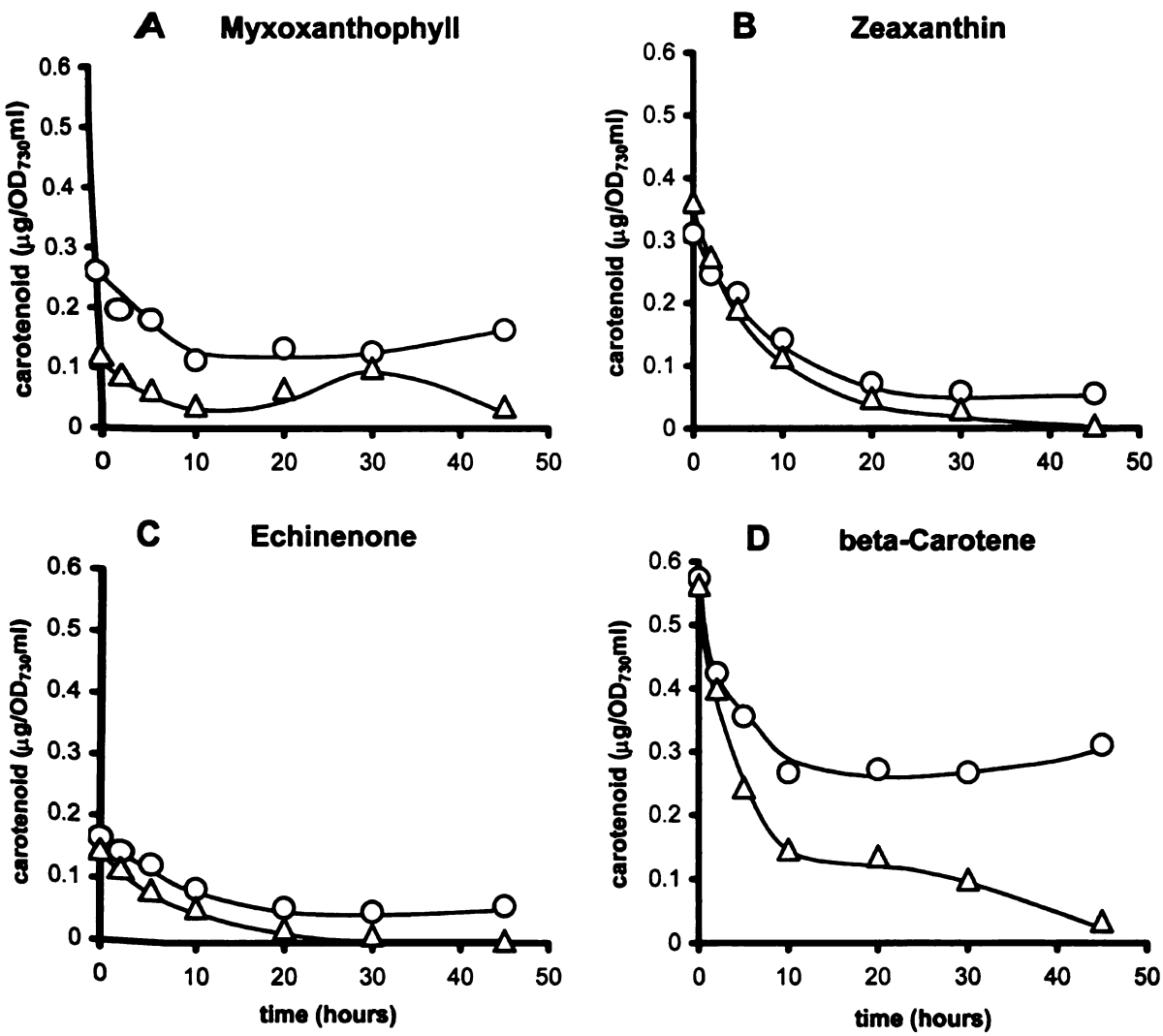

Figure 2.9. Changes in Individual Carotenoids in WT and the Tocopherol-Deficient *slr1736* Mutant During Norflurazon (NF)/HL Treatment.

Myxoxanthophyll (A), zeaxanthin (B), echinenone (C) and beta-carotene (D) were measured during 25 µM NF/HL treatment at 32 °C, 1 % (v/v) CO_2 in air. These data are representative of two independent experiments.

CHAPTER 3: TOCOPHEROLS PLAY A CRUCIAL ROLE IN LOW TEMPERATURE ADAPTATION AND PHLOEM LOADING IN *ARABIDOPSIS.*

The work presented in this chapter has been published:

Hiroshi Maeda, Wan Song, Tammy L. Sage and Dean DellaPenna

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Author's contributions:

Hiroshi Maeda conducted all the research with the exceptions of the diurnal carbohydrate analyses and TEM analyses performed by Wan Song and Tammy L. Sage, respectively. Tammy L. Sage also contributed to writing the microscopy portions of the manuscript. Dean DellaPenna supervised the entire project and involved in all aspects of manuscript writing.

ABSTRACT

To test whether tocopherols (vitamin E) are essential in protection against oxidative stress in plants, a series of *Arabidopsis vitamin E* (*vte*) biosynthetic mutants that accumulate different types and levels of tocopherols and pathway intermediates were analyzed under abiotic stress. Surprisingly subtle differences were observed between the tocopherol-deficient *vte2* mutant and wild type during high light, salinity and drought stresses. However, *vte2,* and to a lesser extent *vte1*, exhibited dramatic phenotypes under low temperature, i.e., elevated anthocyanin levels and reduced growth and seed production. That these changes were independent of light level and occurred in the absence of photoinhibition or lipid peroxidation suggests the mechanisms involved are independent of tocopherol functions in photoprotection. Compared to wild-type, *vte1* and *vte2* had reduced rates of photoassimilate export as early as 6 h into low temperature treatment, elevated soluble sugar levels by 60 h, and increased starch and reduced photosynthetic electron transport rate by 14 days. The rapid reduction in photoassimilate export in *vte2* coincides with callose deposition exclusively in phloem parenchyma transfer cell walls adjacent to the companion cell/sieve element complex. Together these results indicate that tocopherols have a more limited role in photoprotection than previously assumed but play crucial roles in low temperature adaptation and phloem loading.

INTRODUCTION

Tocopherols are the best-studied class of lipid soluble antioxidants and are produced only by photosynthetic organisms including all plants and algae, and some cyanobacteria. Structurally, all four tocopherols (α, β, γ and δ-tocopherols) consist of a chromanol head

group attached to a phytyl tail and differ only in the number and positions of methyl groups on the chromanol ring (Figure 3.1). Tocopherols are amphiphatic molecules and *in vitro* studies using artificial membranes have shown that tocopherols form complexes with specific lipid constituents and physically stabilize membranes (Wassall et al., 1986; Stillwell et al., 1996; Wang and Quinn, 2000; Bradford et al., 2003). Tocopherols can efficiently quench singlet oxygen, scavenge various radicals, particularly lipid peroxy radicals, and thereby terminate lipid peroxidation chain reactions (Liebler and Burr, 1992; Bramley et al., 2000; Schneider, 2005). In animals, vitamin E deficiency results in muscular weakness and neurological dysfunction, which often coincide with elevated lipid peroxidation (Machlin et al., 1977; Yokota et al., 2001). Recent studies have shown that tocopherols also have functions in animals unrelated to their antioxidant activity, such as modulation of cell signaling and transcriptional regulation (Ricciarelli et al., 1998; Jiang et al., 2000; Rimbach et al., 2002; Kempna et al., 2004).

In contrast to the extensive studies of tocopherol functions in animals, we are only beginning to understand tocopherol functions in the photosynthetic organisms in which they are produced. In plants, tocopherols are synthesized and localized in plastid membranes that are also highly enriched in polyunsaturated fatty acids (PUFA) (Bucke, 1968; Soll et al., 1980; Lichtenthaler et al., 1981; Soll et al., 1985; Soll, 1987; Vidi et al., 2006) and increased tocopherol content has been correlated in the response of photosynthetic tissues to a variety of abiotic stresses, including high intensity light (HL), salinity, drought and low temperatures (Munne-Bosch et al., 1999; Keles and Oncel, 2002; Bergmuller et al., 2003; Collakova and DellaPenna, 2003). Such data, together with the evolutionary conservation of tocopherol synthesis among photosynthetic

organisms, has led to the assumption that a primary function of tocopherols is to protect photosynthetic membranes from oxidative stresses by acting as lipid-soluble antioxidants (Fryer et al., 1992; Munne-Bosch and Alegre, 2002). While plausible, such hypotheses are based primarily on correlations and circumstantial evidence and have yet to be rigorously tested *in planta*. The isolation of *Arabidopsis* mutants disrupting steps of the tocopherol biosynthetic pathway provide powerful tools to directly investigate tocopherol functions in plants (Figure 3.1) (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Porfirova et al., 2002; Cheng et al., 2003; Sattler et al., 2003; DellaPenna and Pogson, 2006).

The *vte2* (*vitamin E 2*) mutant is defective in homogentisate phytyl transferase (HPT) and lacks all tocopherols and pathway intermediates (Figure 3.1, Table 3.1). *vte2* mutants are severely impaired in seed longevity and early seedling development due to the massive and uncontrolled peroxidation of storage lipids (Sattler et al., 2004; S.E. Sattler, L. Mene-Saffrane, E.E. Farmer, M. Krischke, M.J. Mueller, and D. DellaPenna, unpublished data), consistent with loss of the lipid-soluble antioxidant functions of tocopherols (Ham and Liebler, 1995, 1997). Interestingly, the *vte2* mutants that do survive early seedling development become virtually indistinguishable from wild type under standard growth conditions (Sattler et al., 2004 and current study), suggesting that unlike seed longevity and germination, tocopherols are dispensable in mature plants in the absence of stress. Consistent with this, constitutive over-expression of *VTE2* in *Arabidopsis* increased total leaf tocopherols 4.5-fold but had no discernible effect relative to wild type on plant growth or chlorophyll and carotenoid content in the absence of stress or under combined nutrient and HL stress (Collakova and DellaPenna, 2003).

The *vte1* mutant is defective in tocopherol cyclase activity and deficient in all tocopherols but unlike *vte2*, accumulates the redox active biosynthetic intermediate 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) (Figure 3.1, Table 3.1, Sattler et al., 2003). When grown at 100 to 120 μmol photon m^{-2} s^{-1} *vte1* plants are virtually identical to wild type at all developmental stages (Porfirova et al., 2002; Sattler et al., 2003; 2004). The lipid peroxidation phenotype observed in germinating *vte2* seedlings was not observed in *vte1* indicating the DMPBQ can fully compensate for tocopherols as a lipid-soluble antioxidant in seedlings (Sattler et al., 2004). Under HL stress (5 days at 850 μmol photon m^{-2} s^{-1}; Porfirova et al., 2002) or a combination of low temperature and HL stress (5 days at 6 to 8°C and 1100 μmol photon m^{-2} s^{-1}; Havaux et al., 2005), *vte1* was nearly identical to wild type for all parameters measured, including lipid peroxidation, with the exception of a slight decrease in maximum photosynthetic efficiency (Fv/Fm). Only under extreme conditions (24 h at 3°C and continuous 1500-1600 μmol photon m^{-2} s^{-1}) did *vte1* show a more rapid induction of lipid peroxidation than wild type, although this difference was transient and after 48 h of treatment lipid peroxidation was similarly elevated in *vte1* and wild type (Havaux et al., 2005). These studies with *vte1* and *vte2* mutants suggest that a primary function of tocopherols is to control non-enzymatic lipid oxidation, especially during seed storage and early germination, and also probably in photosynthetic tissues but only under the most extreme of combined HL and low temperature stress.

Interestingly, a maize tocopherol cyclase mutant (*sxd1*, *sucrose export defective 1*) was identified several years prior to the identification of *Arabidopsis vte1*, not due to its impact on tocopherol synthesis, but because of accumulation of carbohydrates and

anthocyanins in *sxd1* source leaves, which coincided with aberrant plasmodesmata between the bundle sheath and vascular parenchyma cells (Russin et al., 1996). Cloning of the *SXD1* locus did not provide insight into the biochemical activity of the nuclear-encoded chloroplast-localized protein (Provencher et al., 2001) and it is only in retrospect that *SXD1* has been demonstrated to have tocopherol cyclase activity (Sattler et al., 2003). The maize *sxd1* carbohydrate accumulation phenotype was intriguing as it suggested an unexpected link between the tocopherol pathway and primary carbohydrate metabolism, though the mechanism involved was unclear. A similar carbohydrate phenotype did not occur in the orthologous *Arabidopsis vte1* mutant (Sattler et al., 2003) but was observed in *VTE1* RNAi knock-down lines in potato (Hofius et al., 2004).

In the current study, we further define and clarify the physiological role(s) of tocopherols in photosynthetic plant tissues by subjecting and analyzing the response of a suite of *Arabidopsis* tocopherol mutants to a variety of abiotic stresses. We report that in contrast to long-held assumptions about tocopherol functions in plants, tocopherol-deficient mutants are remarkably similar to wild type in their response to most abiotic stresses with the notable exception being an increased sensitivity to non-freezing low temperatures. Detailed physiological, biochemical and ultrastructural data demonstrate that the earliest impact of tocopherol deficiency during low temperature treatment is an inhibition of photoassimilate transport associated with dramatic structural changes in phloem parenchyma transfer cells, a bottleneck for photoassimilate transport. The resulting accumulation of carbohydrates in source leaves impacts the physiology and response of the entire plant to low temperatures.

RESULTS

Tocopherol Biosynthetic (vte) *Mutants Used in This Study*

vte1-1, *vte1-2* and *vte2-1* are previously isolated and characterized ethyl methanesulfonate mutants in the Columbia (Col) ecotype that are deficient in the tocopherol cyclase (*vte1-1* and *vte1-2*) and HPT (*vte2-1*) enzymes (Sattler et al., 2003; 2004, Figure 3.1). *vte2-2* and *vte4-3* are T-DNA insertion mutants in the Wassilewskija (Ws) ecotype in genes encoding HPT and γ-tocopherol methyltransferase (γ-TMT), respectively. Leaves of all mutants, *vte2-1*, *vte2-2*, *vte1-1*, *vte1-2* and *vte4-3*, lack α-tocopherol, the major tocopherol in wild type *Arabidopsis* leaves (Figure 3.1, Table 3.1, Sattler et al., 2003). *vte2-1* and *vte2-2* lack all tocopherols and pathway intermediates. *vte1-1 and vte1-2* lack all tocopherols but accumulate the biosynthetic pathway intermediate DMPBQ at a level comparable to α-tocopherol in Col. The *vte4-3* mutant accumulates γ-tocopherol at an equivalent or slightly higher level than α-tocopherol in Ws. Three to five-week-old plants of all mutant genotypes grown under permissive conditions (12 h 120 μmol photon m^{-2} s^{-1} light at 22°C /12 h darkness at 18°C) were virtually identical to their respective wild type backgrounds, consistent with previous reports that mutations disrupting tocopherol synthesis have little impact on the normal growth of mature plants (Porfirova et al., 2002; Bergmuller et al., 2003; Sattler et al., 2003; 2004).

The Response of Tocopherol-Deficient Mutants to High Intensity Light Stress

High intensity light (HL) stress results in excessive excitation of chlorophyll and consequently generates reactive oxygen species (ROS), which in turn attack various

biochemical targets in the cell including PUFA-enriched photosynthetic membranes. Tocopherols are most abundant in these photosynthetic membranes (Bucke, 1968; Lichtenthaler et al., 1981; Soll et al., 1985) and leaf tocopherol levels increase up to 18-fold during HL stress in *Arabidopsis* (Collakova and DellaPenna, 2003). Therefore, it has been presumed that the elimination of tocopherols from photosynthetic membranes would have dramatic impacts on plant survival during HL stress. To test this hypothesis, Col, *vte2-1*, *vte1-1,* and *vte1-2* were grown for four weeks under permissive conditions and then subjected to two levels of HL stress, 1000 and 1800 μmol photon m^{-2} s^{-1} 16 h light/ 8 h darkness at 22°C (hereafter referred to as HL1000 and HL1800, respectively). HL1000 did not result in differential visible or biochemical phenotypes between *vte2-1* and Col (Supplemental Figure 3.S1). When Col, *vte2-1*, *vte1-1* and *vte1-2* were subjected to HL1800, which approaches the intensity of full sunlight, this led to bleaching of some mature leaves in all genotypes. *vte2-1* had a slight tendency toward more bleached leaves than Col but this was not reproducible or significant, while *vte1-1* and *vte1-2* reproducibly had as many or more bleached mature leaves than Col or *vte2-1* (Figure 3.2A and Supplemental Figure 3.S2). *vte2-2* and *vte4-3* subjected to HL1800 responded similarly to Ws, the corresponding wild type (data not shown).

To assess changes in photosynthetic pigment and tocopherol levels in response to HL stress, the 7th to 9th oldest leaves were harvested before and after 4 days of HL1800 for HPLC analysis. Before HL1800 all levels were similar between genotypes except for slightly lower β-carotene content in *vte2-1* and *vte1-1* relative to Col and the absence of tocopherols in all *vte* genotypes (Supplemental Table 3.S1). After 4 days of HL1800, the *vte* mutants generally had lower total and individual chlorophyll levels than Col but these

differences were not significant in all cases after 4 days of HL1800, even with n = 19 (Figure 3.2B, Table 3.2, Supplemental Figure 3.S2). Total carotenoids were consistently and significantly lower than Col in *vte1-1* and *vte1-2*, but not always in *vte2-1* after 4 days of HL1800. Neoxanthin and violaxanthin were significantly lower in all *vte* mutants, while lutein was significantly lower only in *vte1-1* and *vte1-2*. Interestingly, zeaxanthin was 70 % higher than Col in *vte2-1* but unchanged relative to Col in both *vte1* alleles (Table 3.2).

In vivo chlorophyll *a* fluorescence was also analyzed to assess photosystem II (PSII) function during HL stress. Typically, when plants are under oxidative stress, PSII is inactivated due to enhanced turnover of the D1 protein, a process termed photoinhibition, and maximum photosynthetic efficiency (Fv/Fm) decreases (Bjorkman and Demmig, 1987; Maxwell and Johnson, 2000). Four-week-old Col, *vte2-1*, *vte1-1* and *vte1-2* plants grown under permissive conditions had identical Fv/Fm values of between 0.8 and 0.85, typical values for healthy leaves (Bjorkman and Demmig, 1987; Maxwell and Johnson, 2000, data not shown). After 24 h of HL1800 (8 h HL1800, 8h darkness, and 8h HL1800), a few *vte2-1* leaves showed a dramatic reduction in Fv/Fm (< 0.5), but the majority had values similar to Col and the average Fv/Fm of *vte2-1* was not significantly different from Col, even with n = 30 (Figure 3.2D, Supplemental Figure 3.S2). In contrast, *vte1-1* and *vte1-2* both had more leaves with Fv/Fm < 0.5 and average Fv/Fm values that were significantly lower than Col (Figure 3.2D, Supplemental Figure 3.S2).

These combined results indicate that the elimination of tocopherols in *vte2* has surprisingly little impact on the response of the photosynthetic apparatus to HL stress in

comparison to Col, with the exception of altered xanthophyll cycle carotenoids. Equally surprising is the fact that though the *vte2* and *vte1* genotypes are identical with regard to their tocopherol deficiencies, *vte1* alleles are slightly more susceptible to HL1800 than *vte2*. As the primary biochemical difference between these two genotypes is that *vte1* mutants accumulate the redox active intermediate DMPBQ while *vte2* mutants do not, the presence of DMPBQ in *vte1* may have negative impacts on HL stress tolerance in *Arabidopsis*.

Tocopherol-Deficient Mutants Exhibit a Low Temperature Sensitive Phenotype.

In searching for a condition that more obviously impacts wild type and the tocopherol-deficient mutants in a differential fashion, *vte2-1* and Col plants were subjected to abiotic stress treatments other than HL, including salinity (100, 150 and 200 mM NaCl), drought, and various low temperature treatments. Like HL stress, the salinity and drought stress conditions used also did not result in obvious phenotypic differences between *vte2-1* and Col (Supplemental Figure 3.S1) and further analyses will be required to determine any consequences of tocopherol-deficiency during these stresses. However, when plants were transferred from permissive conditions to non-freezing low temperature conditions, both *vte2-1* and *vte2-2* grew more slowly than their respective wild types, Col and Ws, and their mature leaves changed color to purple (Figure 3.3). These phenotypic differences were consistently observed in conditions ranging from 3 to 12°C and light intensities from 15 to 200 μmol photon m^{-2} s^{-1} (data not shown). Differences were most obvious and consistent under 7.5°C, 12 h 75 μmol photon m^{-2} s^{-1} light/12h darkness (Figure 3.3) and

this low temperature regime (hereafter termed 7.5°C-treated) was used for all subsequent experiments.

Following transfer to 7.5°C, *vte2-1* and Col did not differ in time to bolting (53 ± 4 and 51 ± 3 days, respectively, after transfer to 7.5°C) or number of leaves produced at the start of bolting (32 ± 3 and 31 ± 2 leaves, respectively), indicating that the process of vernalization was not affected by the lack of tocopherols. However, after prolonged growth at 7.5°C *vte2-1* siliques were shorter, produced significantly fewer seeds per silique and per plant compared to Col, and 35 % of the seeds in *vte2-1* siliques were aborted compared to less than 1 % in Col siliques (Figure 3.3E, Table 3.3). These results indicate tocopherols play a crucial role in low temperature adaptation in *Arabidopsis*.

Subjecting *vte1-1* to 7.5°C treatment resulted in a phenotype intermediate between *vte2-1* and Col in terms of overall growth, mature leaf color, silique size, number of seeds/silique, percentage of aborted seed, and seed yield per plant (Figure 3.3, Table 3.3). These phenotypes in 7.5°C-treated *vte4-3* were virtually indistinguishable from wild type (Ws) (Figures 3.3A, B and C). These results indicate that during low temperature adaptation in *Arabidopsis* the quinol biosynthetic intermediate DMPBQ partially compensates for the lack of tocopherols in *vte1-1* while the γ-tocopherol accumulated in *vte4-3* leaves can functionally replace α-tocopherol in this regard.

Photooxidative Damage Is Not Associated with the vte2 Low Temperature Phenotype.

To further examine changes during the time course of 7.5°C treatment, *vte2-1* and Col were subject to detailed comparative biochemical analyses. Plants grown for four weeks

at permissive conditions were transferred to 7.5°C conditions and the 7th to 9th oldest fully expanded rosette leaves were harvested at various time points for analyses. The tocopherol content in Col started to increase after 3 days of 7.5°C treatment reaching levels five-fold higher than initial levels by 28 days, while *vte2-1* lacked tocopherols at all time points (Figure 3.4A).

Consistent with the purple color of mature leaves of 7.5°C-treated *vte2* mutants (Figure 3.3B), *vte2-1* accumulated significantly higher level of anthocyanins than Col after 14 days of 7.5°C (Figure 3.4D). In Col anthocyanins were detected only at 7 days. Because anthocyanin accumulation is often associated with plant responses to stress (Leyva et al., 1995; Chalker-Scott, 1999) and tocopherols are well-characterized lipid-soluble antioxidants in animals (Ham and Liebler, 1995, 1997), it seemed plausible that elevated lipid peroxidation might be occurring in *vte2-1* during low temperature treatment. However, the lipid peroxide levels of *vte2-1* and Col analyzed by the ferrous oxidation xylenol orange (FOX) assay were found to be similar and near background levels at all time points (Figure 3.4B), indicating that the observed phenotypic differences between 7.5°C-treated *vte2-1* and Col are not associated with a detectable increase in lipid peroxidation.

Given the reported localization of tocopherols and tocopherol biosynthetic enzymes to plastids (Bucke, 1968; Soll et al., 1980; Lichtenthaler et al., 1981; Soll et al., 1985; Soll, 1987), it seems reasonable to hypothesize that tocopherol deficiency might affect the components and function of the photosynthetic apparatus during 7.5°C treatment. Under permissive growth conditions, the levels of individual and total photosynthetic pigments (chlorophylls and carotenoids) were nearly identical in *vte2-1*

and Col (Figures 3.4C and E, Supplemental Table 3.S2 at 0 day). The chlorophyll and carotenoid content of both *vte2-1* and Col changed in parallel during the first two weeks of 7.5°C treatment and became significantly different only at 28 days (Figures 3.4C and E, Supplemental Table 3.S2). It is especially noteworthy that zeaxanthin, a xanthophyll cycle carotenoid that accumulates under HL stress (Muller et al., 2001, Table 3.2), was not detectable at any time point in 7.5°C-treated Col and *vte2-1* (Supplemental Table 3.S2), suggesting that the plants were not experiencing photooxidative stress under the low temperature conditions used.

To assess the response of the photosynthetic apparatus to 7.5°C, changes in photosynthetic parameters were analyzed. Fv/Fm was unchanged in both Col and *vte2-1* at any time point (Figure 3.5A), indicating that photoinhibition is not occurring in either genotype during permissive or 7.5°C conditions. The quantum yield of PSII (Φ_{PSII}) was also identical between Col and *vte2-1* under permissive growth conditions (Figure 3.5B at 0 day), suggesting that tocopherol deficiency also does not affect the efficiency of electron transport via PSII in the absence of stress (Genty et al., 1989). During the first 7 days of 7.5°C treatment, Φ_{PSII} responded identically in Col and *vte2-1*: Φ_{PSII}-decreased sharply during the first day followed by a gradual recovery by 7 days. However, at 14 days the *vte2-1* Φ_{PSII} was significantly lower than Col and declined further by 28 days, while the Φ_{PSII} of Col remained stable from day 14 and onward (Figure 3.5B).

Tocopherol-Deficient Mutants Accumulate Carbohydrates During Low Temperature Treatment

The reduced Φ_{PSII} in *vte2-1* after 14 days could result from feedback inhibition of photosynthesis due to the accumulation of downstream carbon metabolites (Goldschmidt and Huber, 1992; Koch, 1996; Paul and Foyer, 2001; Paul and Peliny, 2003). To assess this possibility, starch, glucose, fructose and sucrose contents were analyzed during the time course of 7.5°C treatment. Starch represents the main plastidic carbohydrate storage pool, sucrose and fructose are cytosolic pools, while glucose is present in both subcellular compartments. Col and *vte2-1* had identical carbohydrate contents at the end of the light period under permissive growth conditions (Figure 3.6 at 0 day). During the first 7 days of 7.5°C treatment starch content increased similarly in both Col and *vte2-1* to approximately 120 μmol glucose equivalents/g FW. After 7 days *vte2-1* starch content steadily increased to 680 μmol glucose equivalents/g FW while Col starch levels decreased to near initial levels (Figure 3.6A). Likewise, the glucose, fructose and sucrose content of Col and *vte2-1* increased similarly during the first 3 days of low temperature treatment (Figures 3.6B, C and D), likely as a component of the well-documented cold acclimation response(s) in *Arabidopsis* (Wanner and Junttila, 1999; Taji et al., 2002). After 3 days, Col soluble sugar levels decreased, while *vte2-1* continued to rise reaching 35, 43 and 255 times the initial levels of glucose, fructose and sucrose, respectively, after 28 days of low temperature treatment. The timing of the increase and accumulation of carbohydrates in *vte2-1* is consistent with this being the root cause of the reduction in Φ_{PSII} observed after 14 days at 7.5°C (Figure 3.5B).

To further investigate any differences in carbohydrate accumulation between *vte2-1* and Col during the initial 5 days of low temperature treatment, diurnal changes in carbohydrate content were analyzed one hour before the end of the light and dark cycles. During the 25 h prior to low temperature treatment (Figure 3.7; -25 h, -13 h and -1 h, with 0 h being the transfer of plants to low temperature at the start of the light cycle), starch, glucose, fructose and sucrose content were almost identical in *vte2-1* and Col. These data indicate the lack of tocopherols does not have a significant impact on carbohydrate metabolism under permissive growth conditions. Following transfer to low temperature the soluble sugar content increased similarly in *vte2-1* and Col for the first two diurnal cycles with significant differences first being observed between genotypes at the end of the third low temperature light period (59 h in Figures 3.7B, C and D). In contrast, starch levels did not become significantly different between genotypes until 14 days of low temperature treatment (Figures 3.6A and 7A). The differential elevation of soluble sugars prior to starch accumulation in *vte2-1* indicates that the increase in cytosolic soluble sugars precedes starch accumulation in the chloroplast and that soluble sugars are not being efficiently metabolized or mobilized in 7.5°C-treated *vte2-1*.

The vte2 and* vte1 *Cold Sensitive Phenotypes Are Attenuated in Young Leaves

Mature (7^{th} to 9^{th} oldest) and young (13^{th} to 16^{th} oldest) leaves of *vte2-1* and *vte1-1* mutants showed obvious visible differences in their responses to low temperature; young leaves of *vte2-1* and *vte1-1* did not change their color to purple even after two months of 7.5°C treatment (Figure 3.3C).-Consistent with this visual observations, mature leaves of *vte1-1* had an anthocyanin content 10 % that of *vte2-1* but still higher than Col, while

young *vte2-1* and *vte1-1* leaves accumulated much less anthocyanins compared to their respective mature leaves after 28 days of 7.5°C treatment (Figure 3.8A). Fv/Fm was above 0.8 in all cases, indicating that photoinhibition was not occurring in either young or mature leaves of any genotype (data not shown). The Φ_{PSII} of mature *vte2-1* leaves was reduced to 70 % of mature Col leaves, consistent with Figure 3.5, while the Φ_{PSII} of mature *vte1-1* leaves was only slightly decreased relative to mature Col leaves. However, the Φ_{PSII} of mature and young Col leaves and young *vte2-1* and *vte1-1* leaves were not significantly different (Figure 3.8B). Levels of all carbohydrates in mature *vte2-1* leaves were greatly elevated in comparison to Col, consistent with Figure 3.6. Mature *vte1-1* leaves contained intermediate levels of starch, glucose, fructose and sucrose (51, 53, 68 and 58 % of *vte2-1* levels, respectively) (Figures 3.8C to F). Young *vte2-1* and *vte1-1* leaves contained substantially reduced starch, glucose, and sucrose levels compared to their respective mature leaves. These results indicate that the initiation and development of young *vte2-1* and *vte1-1* leaves under 7.5°C conditions attenuates the biochemical phenotypes observed in mature leaves of both genotypes and that the DMPBQ accumulated in *vte1-1* further suppresses these biochemical phenotypes in both mature and young leaves.

Tocopherol-Deficient Mutants Have Reduced Source Leaf Photoassimilate Export Capacity at Low Temperatures

The reduced seed yield (Table 3.3) and attenuated carbohydrate accumulation in young leaves relative to mature leaves (Figure 3.8) in 7.5°C-treated *vte2-1* suggested impaired translocation of photoassimilates from mature source tissues to young sink tissues. To

test this possibility $^{14}CO_2$ labeling experiments were conducted. Col and *vte2-1* were grown on plates under permissive conditions for three weeks and then transferred to 7.5°C for an additional 7 days. Whole plants were labeled with $^{14}CO_2$ at 7.5°C, transferred to high humidity in darkness at 7.5°C for 2 h to allow for photoassimilate transport and subsequently exposed to a phosphor screen to visualize the movement of ^{14}C labeled photoassimilate. Immediately after labeling >99% of the $^{14}CO_2$ incorporated was present in leaf tissue (data not shown). Col and *vte2-1* incorporated similar amounts of $^{14}CO_2$ into photosynthate suggesting their carbon fixation rates do not differ, consistent with the similar Φ_{PSII} within the first 7 days at 7.5°C (data not shown and Figure 3.5B). Following the 2 h dark period Col had translocated 13.2 % of the ^{14}C labeled photoassimilate fixed in leaves to roots, whereas only 2.7 % was translocated in *vte2-1* (Figure 3.9A). These results demonstrate that *vte2-1* translocates significantly less photoassimilate from source to sink than Col after 7 days of 7.5°C-treatment.

Impaired photoassimilate translocation in 7.5°C-treated *vte2-1* could be due to reduced sink strength or impaired photoassimilate export from source leaves (Gottwald 2000, Stitt 1996, Herbers and Sonnewald 1998). To address these possibilities, phloem exudation experiments were conducted (King and Zeevaart 1974). Col and *vte2-1* were grown for four weeks at permissive conditions and transferred to 7.5°C for an additional 0, 1, 3, or 7 days. Mature (7^{th} to 9^{th} oldest) leaves were excised from plants and labeled with $^{14}CO_2$. The petioles of labeled leaves were then transferred to an EDTA solution to induce phloem exudation and radioactivity in the EDTA solution was determined at various time points (King and Zeevart, 1974). Again, total $^{14}CO_2$ fixed in mature leaves were similar in all genotypes at each time point (Figure 3.9C). Prior to 7.5°C treatment (0

day), Col, *vte1-1* and *vte2-1* leaves exuded similar amounts of labeled photoassimilates, accounting for approximately 34 % of the total $^{14}CO_2$ fixed in each genotype. During 7.5°C treatment, the percent exudation by Col slightly decreased after 3 and 7 days (to 27 and 31 % of the total $^{14}CO_2$ fixed, respectively), whereas that of *vte2-1* was greatly reduced (to 11% and 4% at 3 and 7 days, respectively). Even more intriguingly, exudation in *vte2-1* was significantly lower than Col during the first day of 7.5°C treatment, which corresponds to only 6 h at 7.5°C treatment. The *vte1-1* mutant exuded 17 and 15 % of the total $^{14}CO_2$ fixed after 3 and 7 days at 7.5°C, respectively, levels intermediate between Col and *vte2-1* (Figure 3.9C).

In apoplastic loaders like *Arabidopsis* sucrose is almost the exclusive translocated photoassimilate (Vanbel, 1993). To assess the chemical nature of the labeled compounds exuded from Col and *vte2-1*, phloem exudates were collected and separated by anion-exchange chromatography together with sugar standards. As shown in Figure 3.9B, approximately 85 % of the label in Col and *vte2-1* exudates comigrated with the sucrose standard and 10 % with glucose/fructose standards. The high proportion of sucrose indicates that the label collected is almost entirely from phloem exudate rather than sugars from the cytosol of damaged cells.

Overall, the results obtained from $^{14}CO_2$ labeling experiments indicate that tocopherol deficiency in both *vte1-1* and *vte2-1* results in dramatically reduced capacity of photoassimilate export from source leaves in response to 7.5°C treatment. The rapidity of the reduction in photoassimilate export in 7.5°C-treated *vte2-1* strongly suggests that impairment of photoassimilate export is the root cause of the sugar accumulation phenotype observed in mature leaves of 7.5°C-treated tocopherol-deficient mutants.

Structural Changes in Low Temperature-Treated Tocopherol-Deficient Mutants

Previously, callose was reported to accumulate at the bundle sheath/vascular parenchyma interface of the maize *sxd1* mutant and in vascular tissue of potato *VTE1*-RNAi lines, both of which are defective in tocopherol cyclase (Botha et al., 2000; Hofius et al., 2004). To determine whether callose deposition also occurs in Col, *vte2-1* and *vte1-1,* leaves were harvested at 0, 1, 3 and 13 days of 7.5°C treatment and aniline blue-positive fluorescence assessed. Under permissive conditions, aniline blue-positive fluorescence was absent or sporadic and no significant differences were observed in any genotypes. Aniline blue-positive fluorescence was also not altered in Col during the entire 7.5°C treatment period (e.g., 13 days at 7.5°C, Figure 3.10C). In contrast, aniline blue-positive fluorescence strongly increased in the vascular tissue of 7.5°C-treated *vte2-1* (Figure 3.10), and to a slightly lesser extent *vte1-1* (Supplemental Figure 3.S3). In both *vte2-1* and *vte1-1*, fluorescence initially appeared in a limited number of vascular cells in the petiole as early as 6 h after transfer to 7.5°C conditions (Figures 3.10D and F, Supplemental Figures 3.S3A and B). The number of aniline blue fluorescing cells in the vasculature and their fluorescent intensity subsequently increased in an acropetal fashion in both *vte2-1* and *vte1-1* during the course of 7.5°C treatment (Figure 3.10, Supplemental Figure 3.S3). Intriguingly, the induction, intensity and acropetal spread of vasculature-specific aniline blue positive fluorescence in *vte2-1* at 7.5°C was unaffected by light levels ranging from 1 to 800 μmol photon m^{-2} s^{-1} (Figures 3.11A to F). Aniline blue positive fluorescence was not observed in the vasculature of Col at any light level at

7.5°C (data not shown) and was also absent from the vasculature of both Col and *vte2-1* subjected to HL1800 at 22°C for up to 4 days (Figures 3.11G and H and data not shown).

To confirm whether or not aniline blue-positive fluorescence was cell specific and could be attributed to callose deposition, serial sections of 0 and 14 day 7.5°C-treated Col and *vte2-1* vascular tissue were examined at the level of the transmission electron microscope (TEM). The spatial organization of cells and types of cells comprising the phloem and xylem of both Col and *vte2-1* were identical to what has been previously described for *Arabidopsis* (Haritatos et al., 2000, data not shown). Notably, at day 0 phloem vascular parenchyma cells of both Col and *vte2-1* contained transfer cell wall ingrowths adjacent to sieve elements and companion cells (e.g. Figure 3.12A). 7.5°C-treatment of Col for 14 days did not result in obvious ultrastructural changes in any vascular cell type except for a noticeable increase in phloem parenchyma transfer cell differentiation and transfer cell wall deposition exclusively adjacent to sieve elements and companion cells in all vascular tissue (Figure 3.12B) although to a lesser degree in the midvein.

During the same time course of 7.5°C-treatment in *vte2-1*, changes in cell fine structure occurred exclusively within the phloem parenchyma transfer cells of all vascular traces. Phloem parenchyma transfer cells in 14 day-treated *vte2-1* exhibited irregularly thickened cell wall depositions with ultrastructural features characteristic of callose (Nishimura et al., 2003; Figures 3.12C to F). Large callosic-like masses that dissected the cell lumen corresponded in shape to aniline blue-positive fluorescent regions (compare Figures 3.12C and E to Figures 3.10F, I and L). The callosic-like wall material also formed a sheath around the cells (Figure 3.12D), was deposited over

transfer cell wall ingrowths (Figure 3.12F) and between the end walls of adjoining transfer cells including plasmodesmata (data not shown). Immunolocalization using monoclonal antibodies against callose confirmed the presence of callose at each location (Figures 3.12G to J) and at plasmodesmata between the phloem parenchyma transfer cells and bundle sheath (Figure 3.12K). No immunolabelling was present in controls using secondary antibody only (Figures 3.12D to F) and immunolabelling was rare to absent in all cell types of untreated Col and *vte2-1* and in 14-day 7.5°C-treated Col, including phloem parenchyma transfer cells (e.g. Figure 3.12L).

Serial sections of vascular tissue from Col and *vte2-1* treated at 7.5°C for 3 and 7 days were subsequently examined at the level of the TEM to determine the spatial and temporal development of callose deposition within phloem parenchyma cells. At 3 days, phloem parenchyma transfer cell wall deposition in Col was confined to the sieve element or companion cell boundary (data not shown) but in *vte2-1* wall deposition was present around the entire transfer cell periphery (Figure 3.13A). Cell wall deposition in 3 day-treated *vte2-1* resulted in abnormally thickened and irregular shaped ingrowths with callose-like depositions adjacent to sieve elements and companion cells (Figure 3.13A) that grew increasingly prominent by day 7 (data not shown). In 3-day 7.5°C-treated *vte2-1* positive immunolocalization with monoclonal antibodies to callose was present exclusively at the phloem parenchyma transfer cell wall-sieve element boundary (Figure 3.13B) and included phloem parenchyma transfer cell-sieve element plasmodesmatal connections (Figure 3.13C). In contrast to 14 days, plasmodesmata between bundle sheath and phloem vascular parenchyma cells in 3 day 7.5°C-treated *vte2-1* were continuous and immunonegative for callose (compare Figure 3.12K and 13D).

DISCUSSION

The chemistry of tocopherols as lipid soluble antioxidants and terminators of PUFA free radical chain reactions has been well established from analyses in artificial membranes and animal-derived membrane systems (Liebler and Burr, 1992; Ham and Liebler, 1995). It has long been assumed that similar chemistry occurs in the tocopherol-containing PUFA-enriched membranes of photosynthetic organisms, and this assumption has recently been supported by studies of tocopherol deficient photosynthetic organisms (Sattler et al., 2004; Havaux et al., 2005; Maeda et al., 2005). During the first several days of germination, a period of high oxidative metabolism, *Arabidopsis vte2* seedlings contain levels of oxidized lipids >100-fold higher than wild type or *vte1*, which were indistinguishable (Sattler et al., 2004; S.E. Sattler, L. Mene-Saffrane, E.E. Farmer, M. Krischke, M.J. Mueller, and D. DellaPenna, unpublished data). Thus, a chemical role for tocopherols (or DMPBQ in *vte1*) in limiting lipid peroxidation appears to be conserved between photosynthetic organisms and animals. By 18 days of growth the lipid peroxide levels of *vte2* seedlings had decreased to near that of wild type and *vte1* (Sattler et al., 2004) and became indistinguishable from wild type and *vte1* after four weeks of growth (Figure 3.4B), consistent with tocopherols being dispensable in mature photosynthetic tissues in the absence of stress (Porfirova et al., 2002; Sattler et al., 2003; 2004).

While a chemical role for tocopherols in controlling lipid peroxidation at specific points of the plant life cycle now seems clear, the physiological roles of tocopherols during plant stresses do not. In the current study, we have utilized a suite of *Arabidopsis vte* mutants that accumulate different types and levels of tocopherols and pathway

intermediates (Table 3.1) to directly assess tocopherol specific functions in photosynthetic tissues *in planta* in response to abiotic stress treatments.

A Limited Role for Tocopherols in Protecting* Arabidopsis *Plants from High Intensity Light Stress.

Tocopherols have long been assumed to play crucial roles in HL protection presumably by acting as singlet oxygen quenchers and lipid peroxy radical scavengers (Fryer, 1992; Munne-Bosch and Alegre, 2002; Trebst et al., 2002). In the current study the biochemical and photosynthetic responses of the tocopherol-deficient *vte2* mutant exposed to HL1000 and HL1800 at 22°C for up to eleven days was surprisingly similar to wild type (Figure 3.2, Supplemental Figures 3.S1 and S2). Likewise, the mutation corresponding to *Arabidopsis vte2* in the cyanobacterium *Synechocystis* sp. PCC6803 (*slr1736*) had a similarly limited impact on growth and photosynthesis under permissive growth conditions and during HL stress (Collakova and DellaPenna, 2001; Maeda et al., 2005). In both organisms it is only when HL stress has been combined with other stresses, in combination with lipid peroxidation inducing chemicals in the *Synechocystis slr1736* mutant (Maeda et al., 2005) or in combination with low temperatures (2-3°C) in *Arabidopsis vte2* and *vte1* mutants that differential impacts on photosynthetic parameters or lipid oxidation are observed (Havaux et al., 2005). These combined data indicate that tocopherols are not essential for adaptation and tolerance of photosynthetic tissues subjected to HL stress alone. Such a conclusion runs counter to long-held presumptions that a primary function of tocopherols is to protect photosynthetic tissues against HL stress (Fryer, 1992; Munne-Bosch and Alegre, 2002).

One possible explanation for this surprisingly limited role of tocopherols during HL stress is that other mechanisms compensate for their absence. The zeaxanthin level of HL1800 *vte2* was nearly twice that of Col (Table 3.2). *vte2* (and *vte1*) also had a higher xanthophyll de-epoxidation state (A+Z/A+Z+V) after HL1800 treatment (Table 3.2), as did *vte1* during HL stress combined with low or high temperatures (Havaux et al., 2005). Growth of a tocopherol-deficient *Synechocystis* mutant was also much more susceptible than wild type to treatment with a biosynthetic inhibitor of carotenoid synthesis during HL stress (Maeda et al., 2005). Similarly, a double mutant of *vte1* and *npq1* (*non-photochemical quenching 1*), which cannot accumulate zeaxanthin in response to HL and hence cannot induce non-photochemical quenching (Niyogi et al., 1998), was reported more susceptible than either single mutant to the combination of HL and low temperature stress (Havaux et al., 2005). Conversely, the young *npq1* leaves were also tolerant to short and long term HL stress (up to HL1800) and accumulated higher level of tocopherols than wild type (Havaux et al., 2000; Golan et al., 2006). These data suggest that tocopherols and carotenoids, particularly zeaxanthin, have overlapping functions in protecting photosynthetic organisms against HL stress.

In prior studies it was demonstrated that, although *vte1* and *vte2* are both tocopherol deficient, the two genotypes behaved quite differently during early seedling development: *vte2* exhibited a >100 fold increase in non-enzymatic lipid peroxidation during germination, whereas lipid peroxidation in *vte1* was identical to wild type (Sattler et al., 2004; S.E. Sattler, L. Mene-Saffrane, E.E. Farmer, M. Krischke, M.J. Mueller, and D. DellaPenna, unpublished data). In the current study *vte1* was again found to behave differently from *vte2*. In response to HL1800 stress *vte1* had a slightly, but reproducibly,

higher degree of photoinhibition and higher level of photobleaching than either *vte2* or wild type (Figure 3.2, Table 3.2 and Supplemental Figure 3.S2), suggesting the *vte1* mutation negatively impacts HL stress tolerance beyond its tocopherol deficiency. Why would *vte1* respond so differently from *vte2* during germination and HL1800 given that both mutant genotypes are tocopherol deficient? The most likely explanation resides in the singularly unique biochemical feature of *vte1*: it accumulates the redox active quinol biosynthetic intermediate DMPBQ in place of tocopherols. DMPBQ is absent from *vte2* and Col (Table 3.1 and Sattler et al., 2003) and its presence in *vte1* can clearly have significant, unintended experimental consequences that are independent of the tocopherol deficiency in *vte1*. Thus, when attempting to define tocopherol functions based on *vte* mutant phenotypes one must be careful to delineate genuine tocopherol functions, which would occur in both *vte1* and *vte2*, from potentially confounding artifacts due to the presence of DMPBQ, which would only occur in *vte1*. Such DMPBQ-dependent artifacts can have negative (HL1800), positive (seedling germination) or partially positive (low temperature adaptation) consequences depending on the treatment condition and phenotype assessed. These concerns are not relevant for *vte2*.

Arabidopsis Tocopherol-Deficient Mutants Exhibit a Cold Sensitive Phenotype Independent of Photooxidative Damage.

In contrast to the equivocal results of HL, salinity and drought stress treatments with tocopherol-deficient photosynthetic organisms (Figure 3.2, Supplemental Figures 3.S1 and S2, Porfirova et al., 2002; Havaux et al., 2005; Maeda et al., 2005), both tocopherol-deficient *vte1* and *vte2* genotypes were found susceptible to non-freezing low temperature

treatments in comparison to their respective wild types (Figure 3.3). These results clearly indicate that tocopherols play a critical role in the responses of mature *Arabidopsis* plants to non-freezing low temperatures. Given the well-defined role of tocopherols as lipid soluble antioxidants, we initially hypothesized that tocopherol-deficiency at low temperature would result in increased photooxidative damage relative to wild type and that this might lead to the low temperature sensitive phenotype observed in *vte2*. However, the hallmarks of photooxidative stress and photoinhibition: decreased Fv/Fm and chlorophyll levels and increased zeaxanthin accumulation and lipid peroxidation (Havaux and Niyogi, 1999; Maxwell and Johnson, 2000; Broin and Rey, 2003) were not observed during the first two weeks of low temperature treatment (Figures 3.4B, 4C, 5A, and Supplemental Table 3.S2), the timeframe during which the *vte2* carbohydrate accumulation phenotype fully develops (Figures 3.6 and 7). Likewise, Φ_{PSII}, though altered by low temperature, was identical between wild type and *vte2* during the first week of low temperature treatment (Figure 3.5B). These data indicate that the tocopherol-deficiency has no discernable impact on photosynthesis under the low temperature conditions used and that the low temperature sensitive phenotype of *vte2* is not associated with increased photooxidative damage or photoinhibition due to the absence of tocopherols.

Tocopherols Are Required for Photoassimilate Export from Source Leaves During Low Temperature Adaptation

A well-documented response of plants to low temperatures is the accumulation of soluble sugars and other osmoprotectants, which are critical components for the process of cold

acclimation leading to freezing tolerance (Wanner and Junttila, 1999; Gilmour et al., 2000). The subsequent recovery of photosynthesis and sucrose metabolism is an important component of low temperature adaptation in that it provides carbon to sustain growth under low temperatures (Strand et al., 1997; Strand et al., 1999). During the first two days of low temperature treatment, soluble sugar levels increased similarly in both *vte2* and wild type (Figure 3.7), suggesting tocopherols have little impact on the initial accumulation of soluble sugars in response to low temperature. However, the accumulation of sucrose and other soluble sugars was much higher in *vte2* than wild type after 60 h of low temperature treatment (Figures 3.7B-D), although the rates of photosynthesis and carbon fixation were indistinguishable between the two genotypes until 14 days at low temperature (Figures 3.4B and 9C). *vte2* also reduced soluble sugar levels more slowly at night than wild type after 3 days of low temperature treatment (Figures 3.7B-D). These results suggest that tocopherol deficiency affects carbohydrate utilization/mobilization rather than the supply of fixed carbon from photosynthesis during low temperature adaptation.

$^{14}CO_2$-labeling experiments demonstrated that in comparison to wild type, low temperature-treated *vte2* translocated significantly less ^{14}C-labeled photoassimilates from leaves (source tissue) to roots (sink tissue, Figure 3.9A). The long distance transport of photoassimilates occurs through phloem and the transport rate is determined either by the rate of export from source leaves to phloem (loading) or by removal into sink tissues (unloading) (Vanbel, 1993; Stitt, 1996; Herbers and Sonnewald, 1998). Phloem exudation experiments with excised leaves showed that *vte2* source leaves exported significantly less ^{14}C labeled photoassimilates than wild type as early as 6 h following transfer to low

temperature (Figure 3.9C). The rapidity of this reduction in photoassimilate export in comparison to the elevated sugar accumulation in *vte2* starting at 60 h (compare Figure 3.9 and 7) indicates that impaired photoassimilate export is an early, upstream event in the *vte2* low temperature phenotype and the likely root cause of the elevated sugar accumulation in low temperature-treated *vte2*. Taken together, these analyses demonstrate that tocopherols are required for proper regulation of photoassimilate export from source leaves and thereby play a critical role in low temperature adaptation in *Arabidopsis*.

Previous studies of the maize *sxd1* mutant and potato *VTE1*-RNAi lines (both affecting tocopherol cyclase activity) had suggested a linkage between carbohydrate metabolism and tocopherol biosynthesis, as in both cases carbohydrates accumulated to high levels in mature leaves at normal growth temperatures (19 to 30°C, Russin et al., 1996; Provencher et al., 2001; Hofius et al., 2004). The absence of this phenotype in *Arabidopsis vte1* and *vte2* at 22°C raised questions of the universality of any interaction between tocopherol synthesis and carbohydrate metabolism (Sattler et al., 2003, Figures 3.6 and 7 at 0 day). We now know that tocopherol-deficient *Arabidopsis* mutants do indeed exhibit a phenotype that is analogous to *sxd1* but which is inducible only at low temperatures. Thus, the linkage between tocopherol biosynthesis and carbohydrate metabolism is conserved among all tocopherol-deficient mutants identified in higher plants to date (maize *sxd1*, potato *VTE1*-RNAi and low temperature-treated *Arabidopsis vte1* and *vte2*).

Although the maize *sxd1* mutant and potato *VTE1*-RNAi line suggested tocopherol chromanol ring cyclization was somehow related to regulation of

carbohydrate metabolism, it was unclear whether the phenotype was due to the lack of tocopherols or accumulation of the redox active quinol intermediate DMPBQ (Sattler et al., 2003; Hofius et al., 2004). Analysis of the full suite of *Arabidopsis vte* mutants now allows a conclusive answer to this question. Given that *vte2* lacks DMPBQ (Table 3.1) and exhibits a more severe carbohydrate accumulation phenotype than *vte1* (Figure 3.8), we can conclude that it is the absence of tocopherols rather than accumulation of DMPBQ that causes the carbohydrate accumulation phenotype. The reduced severity of the carbohydrate accumulation phenotype in *vte1* suggests that DMPBQ partially suppresses the low temperature-inducible *vte2* carbohydrate accumulation phenotype.

Tocopherol Deficiency Results in a Cell Specific Response by Phloem Parenchyma Transfer Cells at Low Temperature

The carbohydrate accumulation phenotype of maize *sxd1* was reported to be associated with altered structural features within vascular tissue. Plasmodesmata at the *sxd1* bundle sheath/vascular parenchyma boundary were reported occluded by wall materials (Russin et al., 1996; Provencher et al., 2001) and subsequently suggested to correspond to aniline blue-positive fluorescence (Botha et al., 2000). This structural aberration in *sxd1* plasmodesmata was posited to be the basis of the *sxd1* carbohydrate accumulation phenotype because it would lead to a block in the symplastic movement of photoassimilate. Callose was also observed in vascular tissue of potato *VTE1*-RNAi plants by light microscopy with monoclonal antibodies against β-1,3 glucan (Hofius et al., 2004). In the absence of high-resolution microscopy, Hofius et al. (2004) also suggested this vascular-associated callose somehow interrupts photoassimilate transport. However,

in both the *sxd1* and potato *VTE1*-RNAi studies it was impossible to determine whether callose deposition was a cause or effect of carbohydrate accumulation. A critical observation from the present study is that the low temperature-inducible photoassimilate export defect in *Arabidopsis* tocopherol-deficient mutants is temporally associated with callose deposition in a specific vascular tissue cell type (compare Figures 3.9C and 10). These results are significant as they provide a direct link between defective photoassimilate export and callose deposition (or events tightly associated with callose deposition) in tocopherol-deficient mutants and exclude the possibility that callose deposition is a secondary effect caused by carbohydrate accumulation.

The low temperature-inducible callose deposition in *Arabidopsis vte2* selectively occurred in phloem parenchyma transfer cells (Figures 3.12 and 13). Importantly, initial callose deposition was site specific within these cells and resulted in a callose boundary between the phloem parenchyma transfer cell and sieve element/companion cell complex where transfer cell wall ingrowths occur (Figure 3.13). We saw no evidence of callose deposition or occlusion of plasmodesmata at the bundle sheath-vascular parenchyma boundary during induction of the export defective phenotype in *vte2* (e.g., 3 days of low temperature treatment, Figure 3.13D). However, by 14 days of low temperature treatment, when *vte2* contains high levels of starch and anthocyanins (Figures 3.4D and 6A) and more closely resembles the phenotype of maize *sxd1,* the entire parenchyma transfer cell became encased in a callose sheath associated with abnormally shaped transfer cell wall ingrowths and it is at this point that callose deposition is also observed in *vte2* plasmodesmata at the bundle sheath-vascular parenchyma boundary (Figure 3.12K). When one compares the development, polarity and morphology of transfer cell walls in

7.5°C-treated *vte2* with Col (e.g. compare Figure 3.12G and L), it becomes clear that tocopherols play an important role in transfer cell wall synthesis at low temperatures.

Results from previous structural studies on the minor vein structure of *Arabidopsis* have suggested that phloem parenchyma transfer cells are the site of apoplastic unloading of photoassimilates arriving symplastically from bundle sheath cells (Haritatos et al., 2000). The coincidence in reduction of photoassimilate export with callose deposition in these spatially distinct subcellular sites in the *vte2* mutant during low temperature treatment (Figures 3.9, 10 and 13) provides direct support for the role of transfer cells in photoassimilate export from source leaves via delivery to the phloem apoplast. This callose deposition (or events associated with the callose deposition) in phloem parenchyma transfer cells of 7.5°C-treated *vte2* would form a barrier to symplast-to-apoplast but not symplast-to-symplast transport. The limited export that still occurs in 7.5°C-treated *vte2* source leaves (Figure 3.9) may be due to apoplastic unloading from bundle sheath cells and subsequent loading to the sieve element/companion cell complex (Haritatos et al., 2000). The special characteristics of phloem parenchyma transfer cells that lead them, in comparison to other cell types in the leaf, to be so specifically and differentially impacted by tocopherol-deficiency during low temperature treatment remains to be determined.

Tocopherol Functions in Plant Stress Physiology

In the current study the tocopherol-deficient *vte2* mutant was found to be remarkably similar to wild type in its response to most abiotic stresses with the notable exception of non-freezing low temperature treatments. Tocopherol-deficiency specifically results in

abnormal phloem parenchyma transfer cell wall development at low temperature. This leads to rapid impairment of photoassimilate export that profoundly impacts cellular metabolism and whole plant physiology during both short and long term low temperature treatments. That this occurs in both *vte2* and *vte1* strongly argues that tocopherols play a crucial, previously unrecognized role in low temperature adaptation, specifically in phloem loading. Several studies have suggested that vascular tissues, including vascular parenchyma, are metabolically distinct, sensitive to changing environmental conditions and hence critical sites for stress responses (Orozco-Cardenas et al., 2001; Hibberd and Quick, 2002; Fryer et al., 2003; Koiwai et al., 2004; Narvaez-Vasquez and Ryan, 2004). Our data are consistent with this thesis and suggest tocopherols have important function(s) in regulating the response of these specific cell types to environmental stress, such as low temperatures.

Our findings that photooxidative damage and photoinhibition are not associated with the *vte2* low temperature phenotype and that HL1800 (which approaches full sunlight) at 22°C has little impact on *vte2* compared to wild type suggest a more limited role for tocopherols in protecting plants from photooxidative stress than has been assumed. This seems in direct contradiction with a recent report using *Arabidopsis vte* mutants that concluded tocopherols protect *Arabidopsis* against photoinhibition and photooxidative stress (Havaux et al., 2005). However, the conclusions of this prior work were based entirely on the differential responses of wild type and *vte* mutants exposed to low temperatures (2-8°C) in combination with HL (1000 to 1600 μmol photon m^{-2} s^{-1}) for durations of up to seven days. We now know that such low temperature treatments would rapidly block photoassimilate export in tocopherol-deficient genotypes, but not in wild

type, independent of any light regime imposed (Figures 3.9 to 11) and likely confound any interpretations with respect to previously proposed HL-specific tocopherol functions. Thus, the photoprotective functions of tocopherols in plants remain an open question and a critical reassessment is needed to clarify this issue.

METHODS

Growth Conditions and HL and Low Temperature Treatment

Seeds were stratified for four to seven days (4°C), planted in a vermiculite and soil mixture fertilized with 1 x Hoagland solution, and grown in a chamber under permissive conditions: 12h, 120 µmol photon m^{-2} s^{-1} light at 22°C / 12h darkness at 18°C with 70 % relative humidity. Plants were watered every other day and with 0.5 x Hoagland solution once a week. For HL treatments, four-week-old plants were transferred in the middle of the light cycle to 1800 µmol photon m^{-2} s^{-1} 16h light / 8h darkness at 22°C. For low temperature treatments, three to four-week-old plants were transferred at the beginning of light cycle to 12h 75 µmol photon m^{-2} s^{-1} light / 12h darkness at 7.5°C ($\pm < 3$°C).

Tocopherol, Anthocyanin, Chlorophyll and Carotenoid Analyses

Leaf samples (12-15 mg) were harvested directly into liquid nitrogen at the end of the light cycle and lipids extracted in the presence of 0.01 % (w/v) butylated hydroxytoluene (BHT) using tocol as an internal standard as described (Collakova and DellaPenna, 2001). After phase separation, the aqueous phase was transferred to a new tube, acidified by adding an equal volume of 1N HCl and anthocyanin content measured spectrophotometrically at 520 nm as described (Merzlyak and Chivkunova, 2000). The

lipid phase was used for reverse-phase HPLC analyses to identify and quantify each tocopherol, chlorophyll and carotenoid species as described previously (Collakova and DellaPenna, 2001; Tian and DellaPenna, 2001).

Lipid Peroxide Analysis

Lipid peroxide content was measured using the ferrous oxidation-xylenol orange (FOX) assay as previously described (DeLong et al., 2002; Sattler et al., 2004) with the following modifications. Leaf samples (25-30 mg) harvested at the end of light cycle were immediately extracted with 200 μL of methanol containing 0.01 % (w/v) BHT, 200 μL of dichloromethane, and 50 μL of 150 mM acetic acid using three 3-mm glass beads and a commercial paint shaker. After shaking for 4 min, 100 μL of water and 100 μL of dichloromethane were added for phase separation. Half of the organic phase was incubated with an equal volume of 50 mM triphenyl phosphine in methanol for 30 min to reduce lipid peroxides and half was incubated with an equal volume of methanol for 30 min. The triphenyl phosphine-treated and untreated samples (100 μL) were incubated with 900 μL of FOX reagent [90% (v/v) methanol, 4 mM BHT, 25 mM sulfuric acid, 250 μM ferrous ammonium sulfate, 100 μM xylenol orange] at room temperature for exactly 20 min and A_{560} was measured. Lipid peroxide content was calculated based on a standard curve of hydrogen peroxide as previously described (DeLong et al., 2002).

Chlorophyll Fluorescence Measurements

In vivo chlorophyll *a* fluorescence was measured in the middle of the light cycle using a pulse amplitude modulation (PAM) fluorometer FMS2 (PP Systems, Haverhill, MA).

Attached leaves were dark adapted for at least 15 minutes prior to measurements and fluorescence parameters were determined according to Maxwell and Johnson (2000). Quantum yield of PSII (Φ_{PSII}) was calculated as (F'm-Ft)/F'm, where F'm and Ft are maximum fluorescence and steady state fluorescence in the light, respectively.

Carbohydrate Analyses

Soluble sugar (glucose, fructose and sucrose) and starch levels of leaves were quantified as described (Jones et al., 1977; Lin et al., 1988) with minor modifications. Unshaded leaf tissue (<50mg) was harvested, immediately frozen in liquid nitrogen and extracted twice with 700 μL of 80% ethanol at 80°C. The ethanol extract was evaporated and redissolved in 200 μL of distilled water (Jones et al., 1977). For starch analysis, the extracted leaf residue was ground in 200 μL 0.2N KOH and boiled at 95°C for 45 min. After cooling, the sample was neutralized to pH 5 with 50 μL of 1N acetic acid, centrifuged and 50 μL of supernatant mixed with 492.5 μL of 0.2 M sodium acetate (pH 4.8), 150 μL of H_2O, 4 μL of α-amylase (4 units) and 3.5 μL of amyloglucosidase (2 units) and incubated at 37°C overnight. Glucose, fructose and sucrose levels in the soluble sugar extract and the glucose level of the digested starch extract were determined enzymatically (Jones et al., 1977).

$^{14}CO_2$ Labeling Experiments

For phloem exudation experiments, approximately 20 mature leaves (7^{th} to 9^{th} oldest) were detached in the middle of the day, 0.5 cm of petiole was re-cut under water, the petiole of each leaf was submerged in water and placed in a tightly sealed 10 L glass

chamber. $^{14}CO_2$ was generated in the chamber by adding 3 mL of 0.25 N H_2SO_4 to 0.1 mCi (7 μmol) $NaH^{14}CO_3$ and unlabeled 93 μmol $NaHCO_3$ to give a carbon dioxide concentration of 522 ppm. After labeling for 30 min at 120 μmol photon m^{-2} s^{-1}, the petiole of each leaf was submerged in 0.45 mL of 20 mM disodium-ethylenediaminetetraacetic acid (EDTA) (pH 7.0) and kept in the dark with high humidity to induce phloem exudation (King and Zeevart 1974). All of the aforementioned procedures were performed at 7.5°C for low temperature-treated leaf samples. Exudation of radiolabel into the EDTA solution was then periodically measured over the course of 10 h by liquid scintillation counting (Tri-Carb 2800TR; PerkinElmer, Wellesley, MA, USA). After 10 h of exudation, radiolabel remaining in the leaves was determined by liquid scintillation counting. Total radiolabel fixed per leaf was calculated by adding total radiolabel exuded and remaining in each leaf.

For analyzing translocation of radiolabeled photoassimilates in whole plants, plants were grown on 1/2 MS plates under permissive conditions for 3 weeks and transferred to low temperature conditions for seven days with lids partially ajar to supply atmospheric CO_2. Whole plants were labeled at 7.5°C for 40 min as described above, placed in darkness at 7.5°C and high humidity for 2 h to allow for translocation. Roots were excised from leaves and both exposed to a phosphor screen to visualize the location of radioactivity (Storm; GE Healthcare, UK).

Carbohydrate analysis of phloem exudate was performed by high-pH anion exchange chromatography (HPAEC). Excised leaves were treated as described for phloem exudation experiments except at the end of the initial 2 h exudation period the petiole of each leaf was transferred to 0.45 mL water for 4 h to collect exudates for

analysis. The water exudates were dried under vacuum, dissolved in 50 μL water and 20 μL of the sample was mixed with 5 μL of standards (25 nmol glucose, 50 nmol fructose, 125 nmol sucrose and 100 nmol raffinose) and injected onto the HPAEC. The mixtures were separated on a CarboPac PA-10 column (DIONEX, Sunnyvale, CA, USA) using a 30 min linear gradient of 20 to 140 mM NaOH with a flow rate of 1 mL min^{-1}. One mL fractions were collected and radioactivity was determined by liquid scintillation counting, while sugar standards were detected by pulsed amperometric detection.

Fluorescence and Transmission Electron Microscopy

Leaves were prepared for aniline blue fluorescence microscopy (n = 2 leaves/plant, 4-6 plants/sample time; Martin, 1959) and transmission electron microscopy (TEM; n = 1 leaf/plant, 2-3 plants/sample time; Sage and Williams, 1995) at the same sampling times as above for export studies. The presence or absence of callose was determined using immunolocalization at the level of the TEM as described by Lam et al. (2001) with monoclonal antibodies to β-1, 3 glucan (Biosupplies, Australia). Primary and secondary (anti-mouse IgG gold conjugate 18 nm, Jackson Immunoresearch, West Grove, PA, USA) antibody dilutions were 1: 100 and 1:20 respectively. Incubation time in the 1° and 2° antibodies were 2 and 1 h, respectively. Controls were run by omitting 1° antibody. Images were captured on the Leica MZ 16F fluorescence microscope (Wetzlar, Germany) and the Phillips 201 TEM equipped with an Advantage HR Camera System (Advanced Microscopy Techniques Corp. Danvers, MA, USA).

Statistical Analysis

One-way ANOVA was used for the data in Table 3.2 and Figure 3.2 using genotype as a factor. Two-way ANOVA was used for the data in Figure 3.9C using days of cold treatment and genotype as factors. When significance was observed ($P < 0.05$), pair-wise comparison of least square means was evaluated. SAS software was used for these analyses (SAS Institute, Cary, NC, USA). Student *t* test was used for the rest of the data to compare statistical significance of mutants relative to Col ($P < 0.05$) using Microsoft Excel.

Accession Numbers

Sequence data for the *Arabidopsis thaliana* tocopherol biosynthetic enzymes described in this article can be found in the GenBank nucleotide sequence database under the following accession numbers: *VTE1* (At4g32770), NM119430; *VTE2* (At2g18950), NM179653; *VTE4* (At1g64970), NM105171.

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FIGURES AND TABLES

Table 3.1. Tocopherol and DMPBQ Content in Leaves of Wild Types and Tocopherol Biosynthetic Mutants Grown Under Permissive Conditions.

	α[a]	β	γ	δ	total	DMPBQ
Col	**15.6** ± 3.0	0[b]	**0.6** ± 0.1	0	**16.2** ± 3.0	0
vte2-1[c]	0	0	0	0	0	0
vte1-1[c]	0	0	0	0	0	**19.6** ± 1.3
vte1-2[c]	0	0	0	0	0	**17.3** ± 1.8
Ws	**12.3** ± 2.2	0	0	0	**12.3** ± 2.2	0
vte2-2[d]	0	0	0	0	0	0
vte4-3[d]	0	0	**17.7** ± 1.2	0	**17.7** ± 1.2	0

Plants were grown for four weeks under permissive conditions and mature leaves were harvested for analysis. Data are means ± SD (n = 3 or 4) and are expressed as pmol/mg FW.

[a] α, β, γ and δ indicate α, β, γ and δ-tocopherol, respectively.

[b] 0 indicates the compound was below detection (typically ≤ 0.1 pmol/mg FW).

[c] Col background.

[d] Ws background.

Table 3.2. Content of Photosynthetic Pigments and Tocopherols of Col and the *vte2* and *vte1* Mutants After HL1800 Treatment at 22°C.

	After HL1800			
	Col	***vte2-1***	***vte1-1***	***vte1-2***
Total tocopherols	**1.58** ± 0.25^{a}	**0** ± 0^{b}	**0** ± 0^{b}	**0** ± 0^{b}
Total chlorophylls	**17.28** ± 1.01	**16.60** ± 1.37	**16.51** ± 1.84	**16.21** ± 2.05
Chl*a*	**12.25** ± 0.87	**11.69** ± 1.04	**11.61** ± 1.38	**11.52** ± 1.58
Chl*b*	**5.02** ± 0.26	**4.90** ± 0.35	**4.90** ± 0.51	**4.69** ± 0.50
Chl*a*/Chl*b*	**2.44** ± 0.16	**2.38** ± 0.09	**2.37** ± 0.13	**2.45** ± 0.14
Total carotenoids	**5.37** ± 0.35^{a}	**4.98** ± 0.36^{b}	**4.85** ± 0.54^{b}	**4.75** ± 0.70^{b}
β-car	**0.66** ± 0.08	**0.64** ± 0.07	**0.63** ± 0.09	**0.64** ± 0.11
lutein	**2.45** ± 0.14^{a}	**2.39** ± 0.15^{ab}	**2.26** ± 0.22^{bc}	**2.20** ± 0.27^{c}
N	**0.57** ± 0.03^{a}	**0.51** ± 0.04^{b}	**0.51** ± 0.06^{b}	**0.50** ± 0.07^{b}
V	**1.01** ± 0.12^{a}	**0.57** ± 0.10^{c}	**0.78** ± 0.17^{b}	**0.77** ± 0.22^{b}
A	**0.39** ± 0.04^{ab}	**0.39** ± 0.04^{a}	**0.36** ± 0.04^{bc}	**0.36** ± 0.04^{c}
Z	**0.28** ± 0.05^{b}	**0.48** ± 0.06^{a}	**0.30** ± 0.06^{b}	**0.29** ± 0.03^{b}
A+Z+V	**1.69** ± 0.13^{a}	**1.44** ± 0.12^{b}	**1.44** ± 0.18^{b}	**1.42** ± 0.26^{b}
A+Z/A+Z+V	**0.40** ± 0.05^{c}	**0.61** ± 0.05^{a}	**0.46** ± 0.07^{b}	**0.47** ± 0.07^{b}

Plants were grown for four weeks under permissive growth conditions (120 μmol photon m^{-2} s^{-1}) and pigment contents were analyzed after 4 days of HL (1800 μmol photon m^{-2} s^{-1}) treatment. Data are means ± SD (μg/cm^2, n = 19). When significance is observed between genotypes (ANOVA, $P < 0.05$), pair-wise comparison of least square means is evaluated and non-significant groups are indicated by *a*, *b* or *c* with *a* being the highest group. N, neoxanthin; β-car, β-carotene; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; Chl*b*, chlorophyll *b*; Chl*a*, chlorophyll *a*.

Table 3.3. Yields and Abortion Rates of Seed Produced During Low Temperature (7.5°C) Treatment.

	Col	*vte2-1*	*vte1-1*
seeds / silique	31.1 ± 1.6	19.5 ± 3.4**	27.3 ± 2.5*
aborted seeds / sillique	0.1 ± 0.4	6.8 ± 2.4**	2.0±1.0*
percentage abortion (%)	0.4	34.6**	7.3*
yield (mg seeds / plant)	373 ± 77	87 ± 27**	223[a]

Data are means ± SD (n = 3 for yields, n = 7 for aborted seed). Student's *t* tests relative to Col (*P < 0.05, **P < 0.01).

[a] An average of duplicate plants (225.7 and 219.4 mg seeds/plant)

Supplemental Table 3.S1. Content of Photosynthetic Pigments and Tocopherols of Col and the *vte2* and *vte1* Mutants Grown at Permissive condition.

	Before HL1800			
	Col	***vte2-1***	***vte1-1***	***vte1-2***
Total tococpherols	**0.11** ± 0.01^{a}	**0** ± 0^{b}	**0** ± 0^{b}	**0** ± 0^{b}
Total chlorophylls	**21.90** ± 1.14	**20.51** ± 1.70	**20.15** ± 1.12	**21.26** ± 1.19
Chl*a*	**15.26** ± 0.77	**14.25** ± 1.18	**14.02** ± 0.79	**14.78** ± 0.84
Chl*b*	**6.64** ± 0.37	**6.25** ± 0.53	**6.13** ± 0.33	**6.49** ± 0.35
Chl*a*/Chl*b*	**2.30** ± 0.03	**2.28** ± 0.02	**2.29** ± 0.01	**2.28** ± 0.02
Total carotenoids	**3.56** ± 0.23	**3.32** ± 0.28	**3.28** ± 0.18	**3.44** ± 0.22
β-car	**0.61** ± 0.05^{a}	**0.57** ± 0.03^{b}	**0.55** ± 0.03^{b}	**0.59** ± 0.05^{ab}
lutein	**1.85** ± 0.13	**1.72** ± 0.16	**1.70** ± 0.10	**1.79** ± 0.12
N	**0.59** ± 0.03	**0.55** ± 0.05	**0.54** ± 0.03	**0.57** ± 0.03
V	**0.51** ± 0.04	**0.48** ± 0.05	**0.49** ± 0.03	**0.50** ± 0.03
A	**0** ± 0	**0** ± 0	**0** ± 0	**0** ± 0
Z	**0** ± 0	**0** ± 0	**0** ± 0	**0** ± 0
A+Z+V	**0.51** ± 0.04	**0.48** ± 0.05	**0.49** ± 0.03	**0.50** ± 0.03
A+Z/A+Z+V	**0** ± 0	**0** ± 0	**0** ± 0	**0** ± 0

Plants were grown for four weeks under permissive growth conditions (22°C, 120 μmol photon m^{-2} s^{-1}) and mature leaves were analyzed. Data are means ± SD (μg/cm^2, n = 7). When significance is observed between genotypes (ANOVA, $P < 0.05$), pair-wise comparison of least square means is evaluated and non-significant groups are indicated by *a* or *b* with *a* being the highest group. N, neoxanthin; β-car, β-carotene; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; Chl*b*, chlorophyll *b*; Chl*a*, chlorophyll *a*.

Supplemental Table 3.S2. Content of Individual Photosynthetic Pigments of Col and the *vte2* Mutant During Low Temperature (7.5°C) Treatment.

Days	lutein		β-car		N		total Car	
in cold	Col	*vte2-1*	Col	*vte2-1*	Col	*vte2-1*	Col	*vte2-1*
0	154 ± 16	165 ± 10	66 ± 10	79 ± 6	43 ± 3	46 ± 2	301 ± 32	330 ± 21
1	148 ± 7	167 ± 21	65 ± 4	73 ± 14	39 ± 2	45 ± 6	283 ± 14	319 ± 43
2	154 ± 7	170 ± 8	64 ± 7	72 ± 8	42 ± 2	43 ± 3	297 ± 18	329 ± 19
5	167 ± 8	168 ± 9	63 ± 3	66 ± 4	40 ± 1	42 ± 3	319 ± 14	327 ± 18
7	160 ± 11	160 ± 11	58 ± 4	58 ± 4	42 ± 3	42 ± 3	309 ± 25	309 ± 25
14	159 ± 13	146 ± 26	61 ± 3	54 ± 13	45 ± 2	40 ± 9	313 ± 23	286 ± 54
28	184 ± 14	105 ± 9**	86 ± 13	48 ± 7**	55 ± 3	27 ± 5**	374 ± 30	213 ± 19**

Days	V		A		Z		Chla/Chl*b*	
in cold	Col	*vte2-1*	Col	*vte2-1*	Col	*vte2-1*	Col	*vte2-1*
0	37 ± 3	40 ± 3	1 ± 0	1 ± 0	0 ± 0	0 ± 0	2.32 ± 0.06	2.40 ± 0.04
1	27 ± 3	31 ± 5	4 ± 1	4 ± 2	0 ± 0	0 ± 0	2.37 ± 0.03	2.38 ± 0.03
2	34 ± 2	41 ± 3	3 ± 0	2 ± 1	0 ± 0	0 ± 0	2.39 ± 0.07	2.49 ± 0.03
5	46 ± 3	50 ± 3	2 ± 2	2 ± 1	0 ± 0	0 ± 0	2.30 ± 0.02	2.34 ± 0.05
7	46 ± 8	46 ± 8	4 ± 1	4 ± 1	0 ± 0	0 ± 0	2.28 ± 0.03	2.28 ± 0.03
14	46 ± 4	40 ± 6	2 ± 2	5 ± 2	0 ± 0	0 ± 0	2.32 ± 0.03	2.39 ± 0.02
28	46 ± 3	30 ± 1**	2 ± 1	3 ± 1	0 ± 0	0 ± 0	2.39 ± 0.12	2.60 ±0.18**

Supplemental Table 3.S2. (continued)

Days	Chl*b*		Chl*a*		total Chl	
in cold	Col	*vte2-1*	Col	*vte2-1*	Col	*vte2-1*
0	363 ± 23	388 ± 18	842 ± 75	931 ± 48	1205 ± 98	1319 ± 65
1	320 ± 11	359 ± 41	759 ± 34	854 ± 107	1079 ± 45	1212 ± 148
2	316 ± 6	317 ± 30	753 ± 38	790 ± 68	1069 ± 44	1107 ± 98
5	258 ± 7	262 ± 16	593 ± 18	613 ± 30	851 ± 24	875 ± 46
7	246 ± 17	246 ± 17	559 ± 32	559 ± 32	805 ± 49	805 ± 49
14	251 ± 9	209 ± 39	581 ± 18	499 ± 95	832 ± 26	708 ± 134
28	304 ± 27	138 ± 21**	729 ± 89	357 ± 49**	1033 ± 114	495 ± 69**

Values are expressed as pmol/mg FW. Data are means ± SD (n = 4 or 5). Student's *t* tests of *vte2-1* relative to Col (* $P < 0.05$, ** $P < 0.01$). N, neoxanthin; β-car, β-carotene; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; total Car, total carotenoids; Chl*b*, chlorophyll *b*; Chl*a*, chlorophyll *a*; total Chl, total chlorophylls.

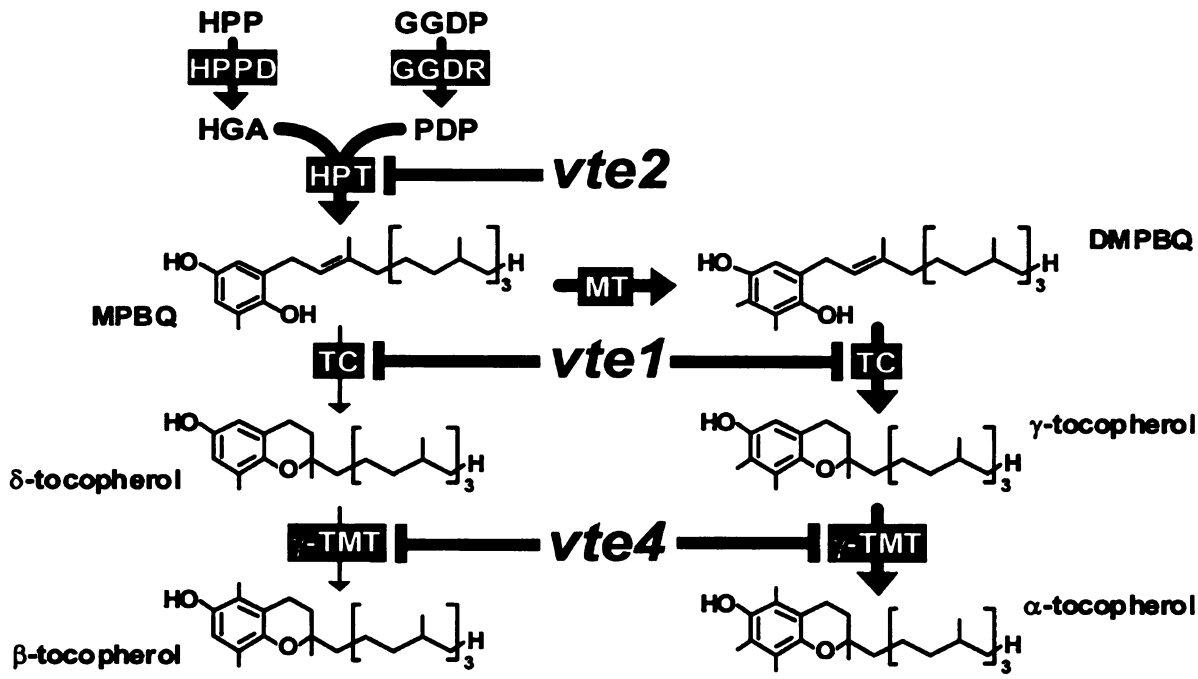


Figure 3.1. Tocopherol Biosynthetic Pathway and *vte* Mutations in *Arabidopsis thaliana*. Enzymes are indicated by black boxes and mutations by gray letters and lines. Bold arrows show the primary biosynthetic route in wild type leaves. HPP, hydroxyphenylpyruvate; GGDP, geranylgeranyl-diphosphate; PDP, phytyl-diphosphate; HGA, homogentisic acid; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol; HPPD, HPP dioxygenase; GGDR, GGDP reductase; HPT, HGA phytyltransferase; TC, tocopherol cyclase; MT, MPBQ methyltransferase; γ-TMT, γ-tocopherol methyltransferase; *vte1*, *vte2* and *vte4*, mutants of TC, HPT and γ-TMT, respectively.

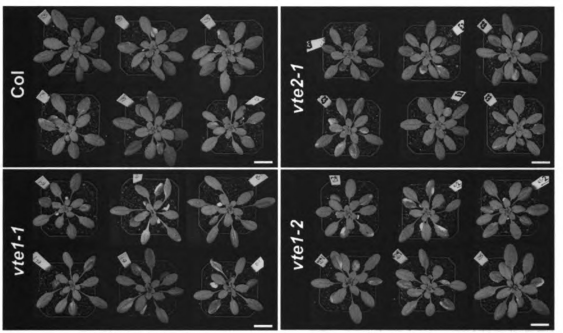


Figure 3.2. Phenotypic and Photosynthetic Responses of Col and the *vte2* and *vte1* Mutants to HL stress.

Plants were grown under permissive conditions for four weeks and then transferred to HL stress in the middle of the day. When significance is observed between genotypes (ANOVA, $P < 0.05$), pair-wise comparison of least square means is evaluated and non-significant groups are indicated by *a*, *b* or *c* with *a* being the highest group.

(A) Six representative plants after 3 days of HL1800. Bars = 2 cm.

(B) and **(C)** Individual values of total chlorophyll (B) and carotenoid (C) contents from 19 leaves after 4 days of HL1800.

(D) Individual values of Fv/Fm from 30 leaves after 24 h of HL1800.

Figure 3.2. (continued)

Figure 3.3. Visible Phenotype of *vte* Mutants During Extended Low Temperature Treatment.

Plants were grown under permissive conditions for three weeks and then subjected to 7.5°C treatment for the indicated time periods.

(A) to **(D)** Representative plants of three-week-old wild type (Col and Ws), *vte1-1* and *vte2-1* (Col background), *vte2-2* and *vte4-3* (Ws background) after 0 day (A), one month (B), two months (C) and four months (D) of 7.5°C treatment. Bars = 2 cm.

(E) Representative siliques from Col, *vte1-1* and *vte2-1* plants after five months of low temperature treatment. Bar = 0.2 cm. Arrows denote aborted seeds in *vte1-1* and *vte2-1* siliques.

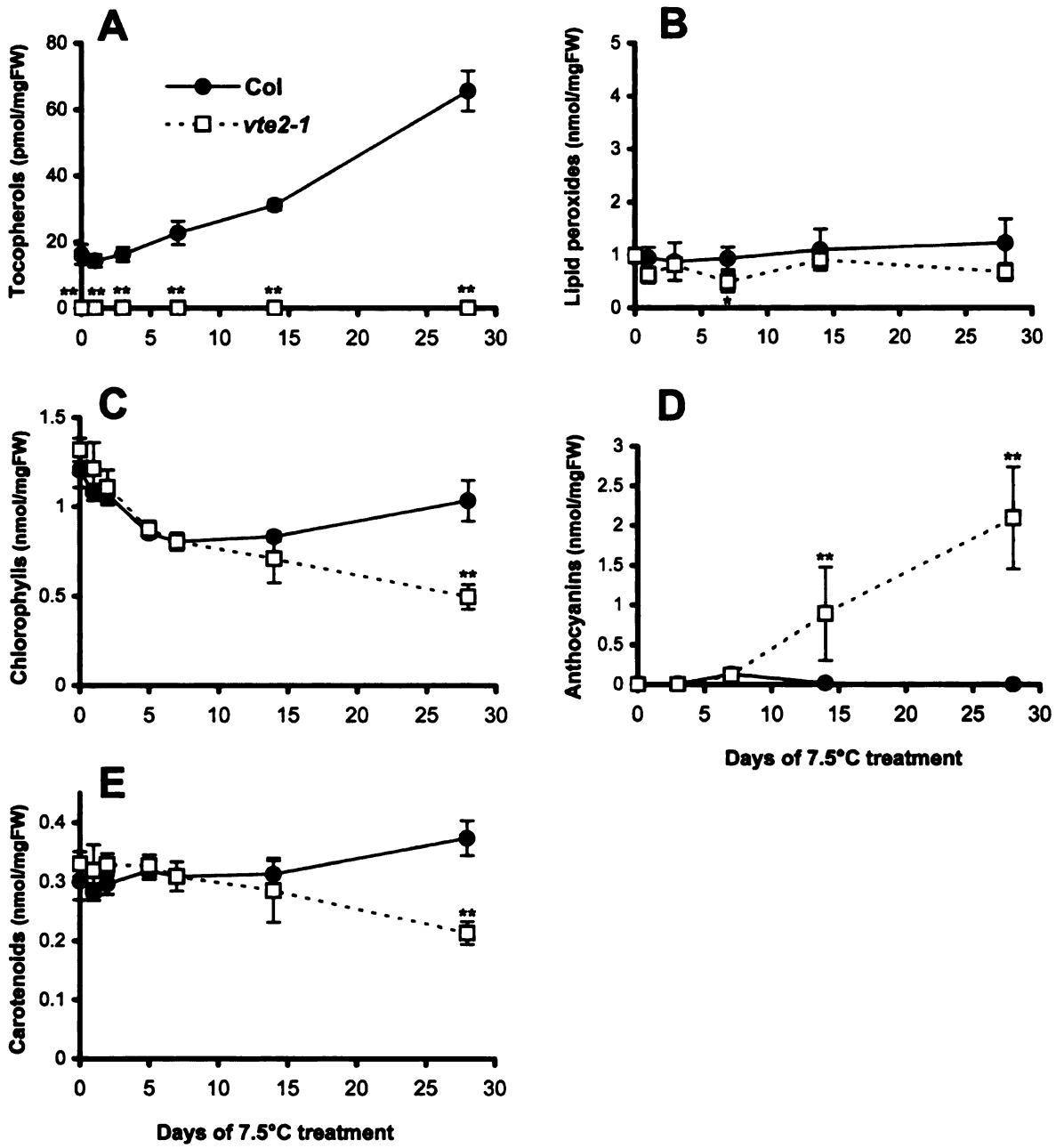


Figure 3.4. Tocopherol, Lipid Peroxide, Anthocyanin, and Photosynthetic Pigment Content of Col and the *vte2* Mutant During Four Weeks of Low Temperature Treatment.
Col (closed circles) and *vte2-1* (open squares) were grown under permissive conditions for four weeks and then transferred to 7.5°C conditions at the beginning of the light cycle for the indicated time. Data are means ± SD (n = 3 or 4). Student's *t* tests of *vte2-1* relative to Col at each time point (*P < 0.05, **P < 0.01).

(A) Total tocopherols

(B) Lipid peroxides

(C) Total chlorophylls

(D) Anthocyanins

(E) Total carotenoids

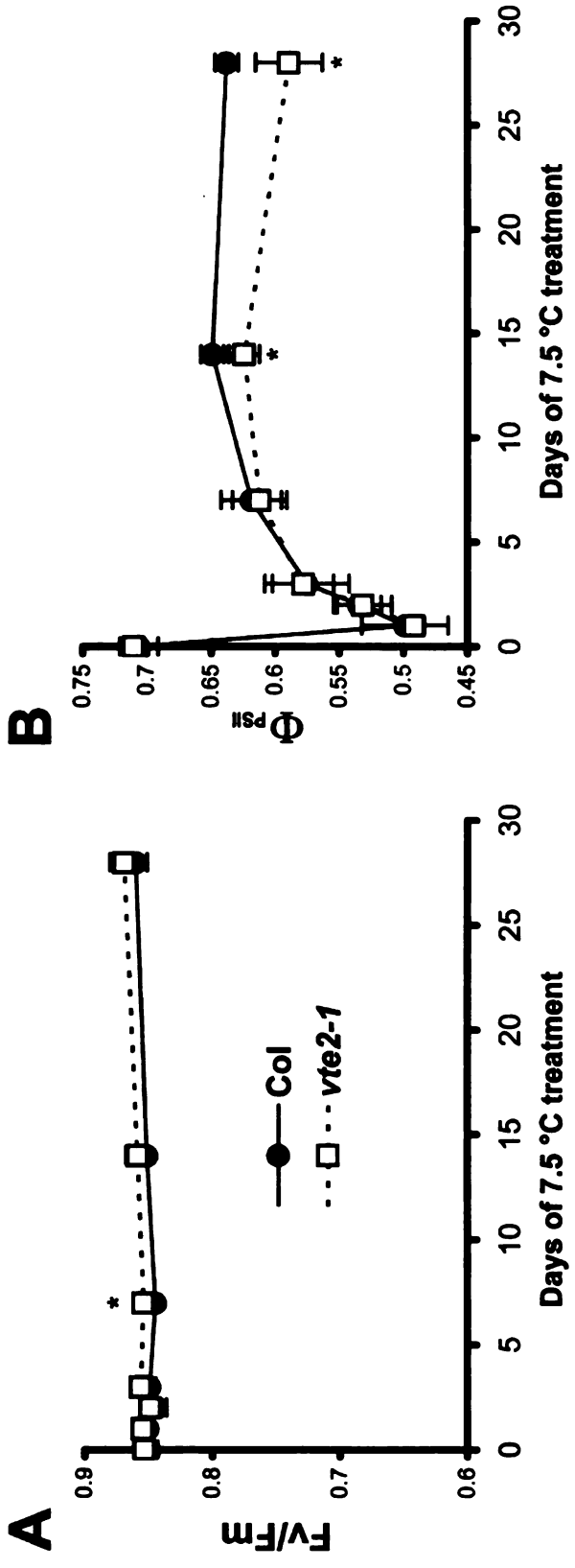


Figure 3.5. Photosynthetic Status of Col and the *vte2* Mutant During Four Weeks of Low Temperature Treatment.

Col (closed circles) and *vte2-1* (open squares) were grown under permissive conditions for four weeks and then transferred to 7.5°C conditions at the beginning of the light cycle for the indicated time. Analysis was conducted in the middle of the light cycle. Data are means ± SD (n = 4). Student's *t* tests of *vte2-1* relative to Col at each time point (*P < 0.05).

(A) Maximum photosynthetic efficiency (Fv/Fm)

(B) Quantum yield of PSII (Φ_{PSII})

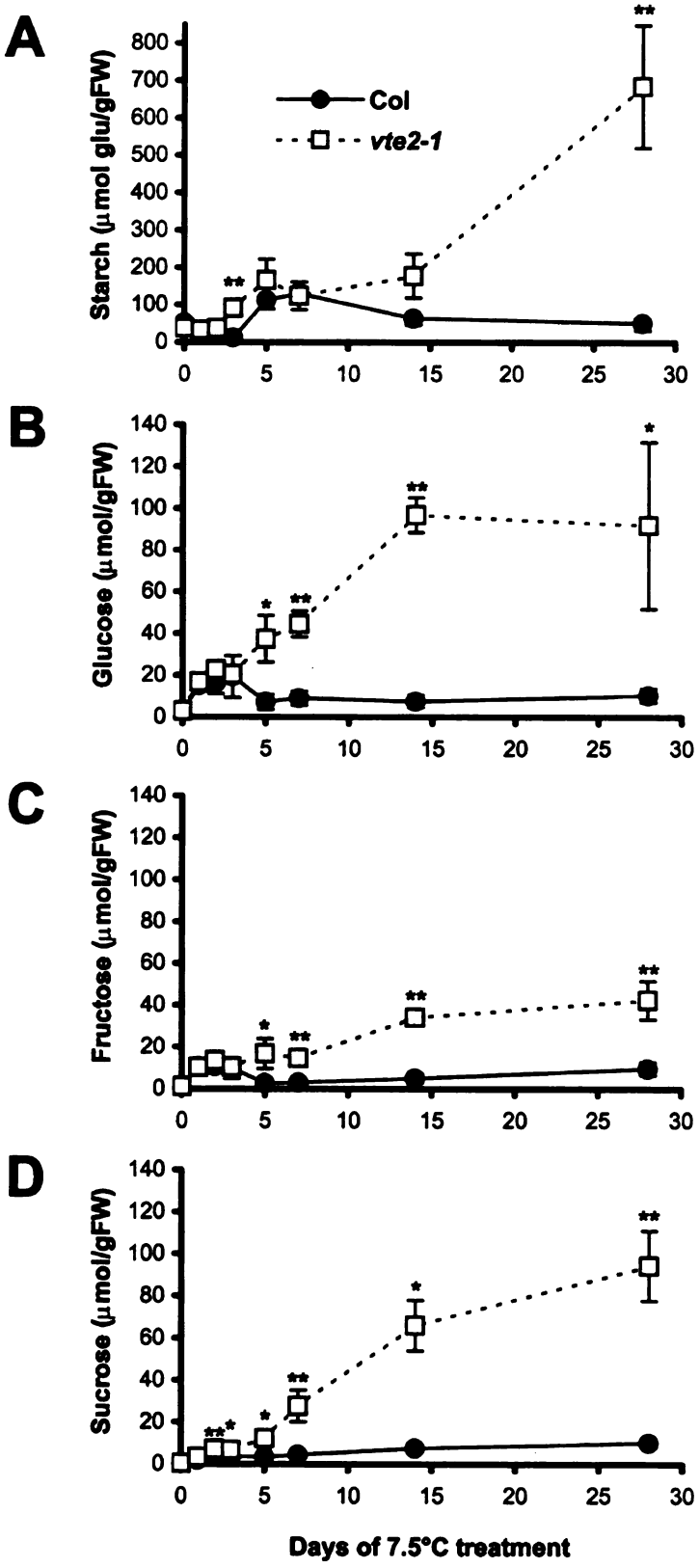


Figure 3.6. Changes in Starch and Soluble Sugar levels in Col and the *vte2* Mutant During Four Weeks of Low Temperature Treatment.

Col (closed circles) and *vte2-1* (open squares) were grown under permissive conditions for four weeks and then transferred to 7.5°C conditions at the beginning of the light cycle for the indicated time. Samples were harvested at the end of light cycles. 0 days of cold treatment indicates the end of the light cycle of the day prior to initiating 7.5°C treatment. Starch is expressed as µmol glucose equivalents / g FW. Data are means ± SD (n = 3 or 4). Student's *t* tests of *vte2-1* relative to Col at each time point (*P < 0.05, **P < 0.01).

(A) Starch
(B) Glucose
(C) Fructose
(D) Sucrose

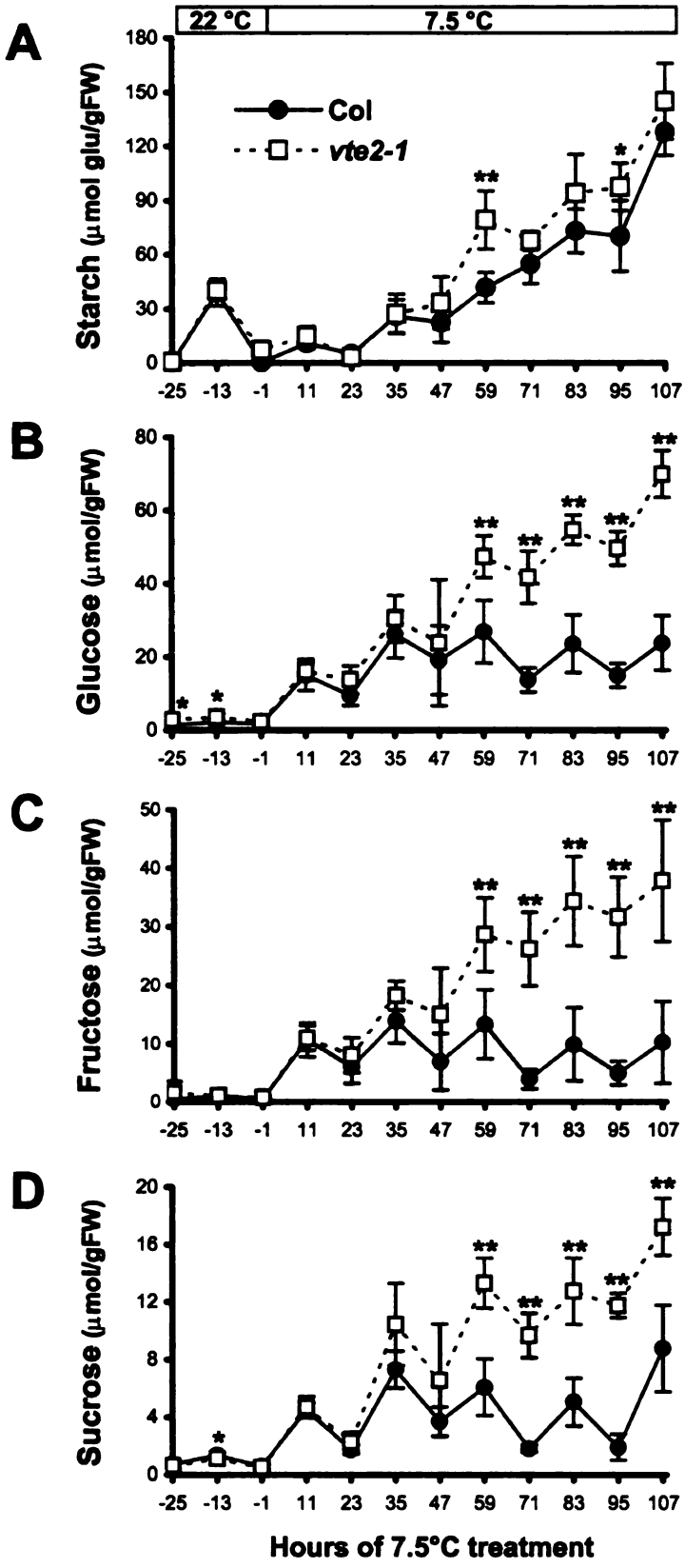


Figure 3.7. Diurnal Changes in Starch and Soluble Sugar levels in Col and the *vte2* Mutant During the First Four Days of Low Temperature Treatment.

Col (closed circles) and *vte2-1* (open squares) were grown under permissive conditions for four weeks and then transferred to 7.5°C conditions at the beginning of the light cycle for the indicated time. Samples were harvested at the end of dark and light cycles. Gray shadows indicate 12 h dark cycles. 0 h of cold treatment indicates the beginning of the first light cycle of low temperature treatment. Starch is expressed as µmol glucose equivalents / g FW. Data are means ± SD (n = 5). Student's *t* tests of *vte2-1* relative to Col at each time point (*P < 0.05, **P < 0.01).

(A) Starch
(B) Glucose
(C) Fructose
(D) Sucrose

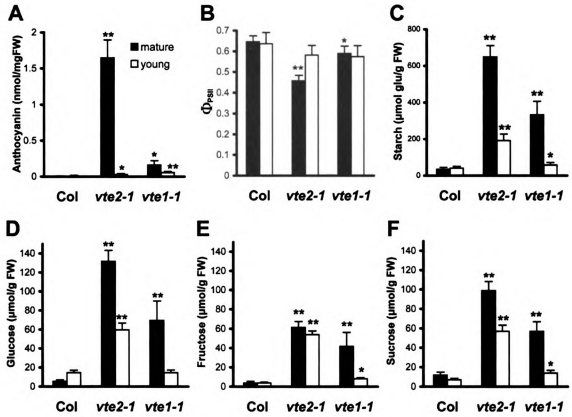


Figure 3.8. Biochemical Phenotypes in Mature and Young Leaves of Col, and the *vte2* and *vte1* Mutants After Four Weeks of Low Temperature Treatment.

Col, *vte2-1* and *vte1-1* mutants were grown under permissive conditions for four weeks and then transferred to 7.5°C conditions at the beginning of the light cycle for an additional four weeks. Mature leaves (7th to 9th oldest, black bars) and young leaves (13th to 16th oldest, white bars) were harvested at the end of the light cycle for analyses in (A), (C), (D), (E) and (F).-Photosynthetic parameter in (B) was measured in the middle of the light cycle. Data are means ± SD (n = 4 or 5). Student's *t* tests of mutant leaves relative to corresponding Col young or mature leaves are indicated (*P < 0.05; **P < 0.01)

(A) Anthocyanin content

(B) Quantum yield of PSII (Φ_{PSII})

(C) Starch content expressed as µmol glucose equivalents / g FW

(D) to **(F)** Glucose, fructose and sucrose content, respectively

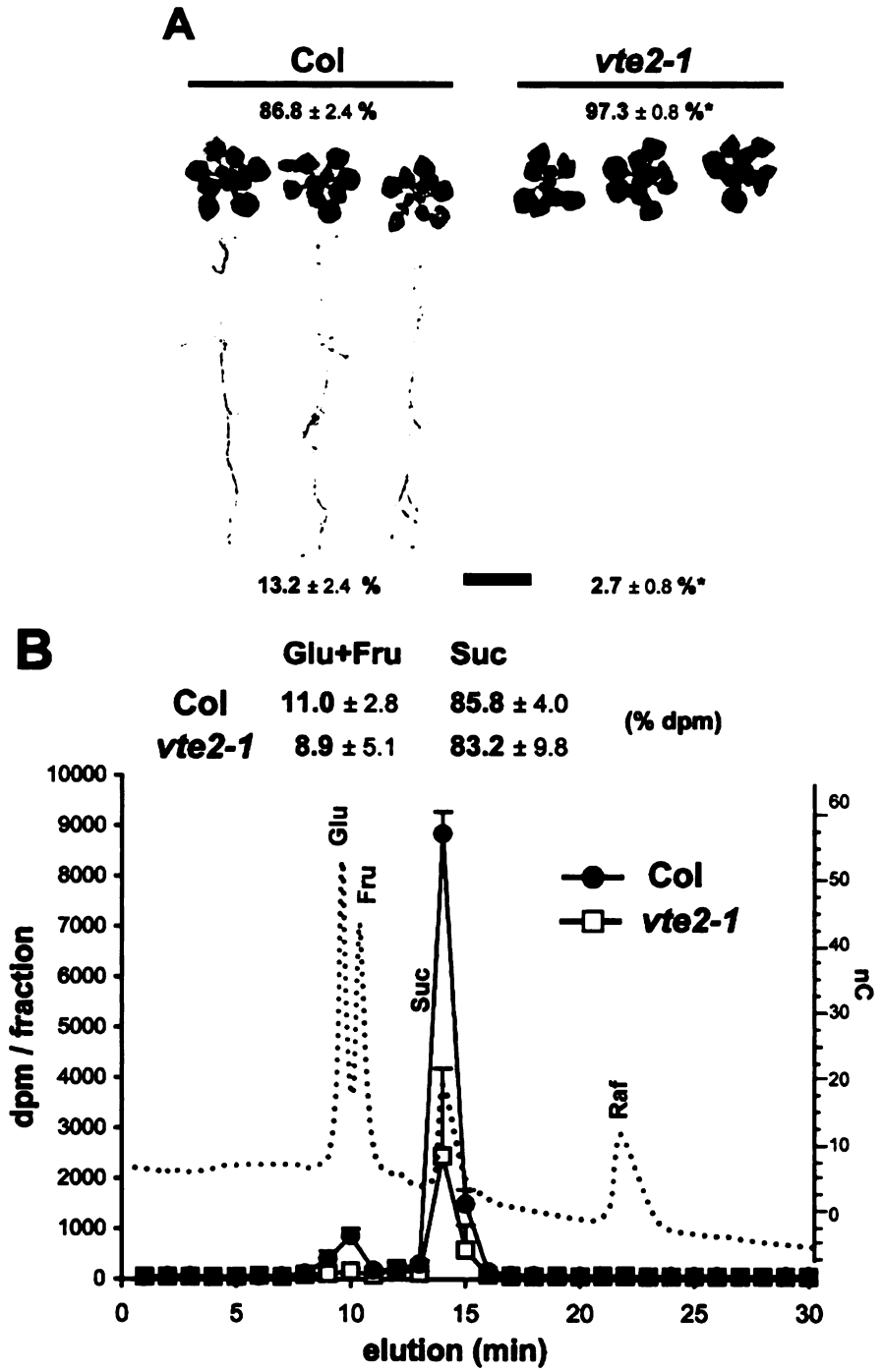


Figure 3.9. Translocation and Export of ^{14}C Labeled Photoassimilates in Low Temperature-Treated Col and the *vte2* and *vte1* Mutants.

(A) ^{14}C labeled photoassimilate translocation of Col and *vte2-1* treated for 7 days at 7.5°C. Percent label detected in leaves (top) and roots (bottom) are indicated as means ± SD (n = 3). Student's *t* tests relative to Col (*P < 0.05, **P < 0.01).

(B) HPLC analysis of phloem exudates collected from mature leaves of Col and *vte2-1* treated for 10 days at 7.5°C. The HPLC trace of sugar standards is shown as dotted grey lines. The percentage of label detected in the glucose/fructose or sucrose fractions are indicated as means ± SD (n = 3). Glu, glucose; Fru, fructose; Suc, sucrose; Raf, raffinose.

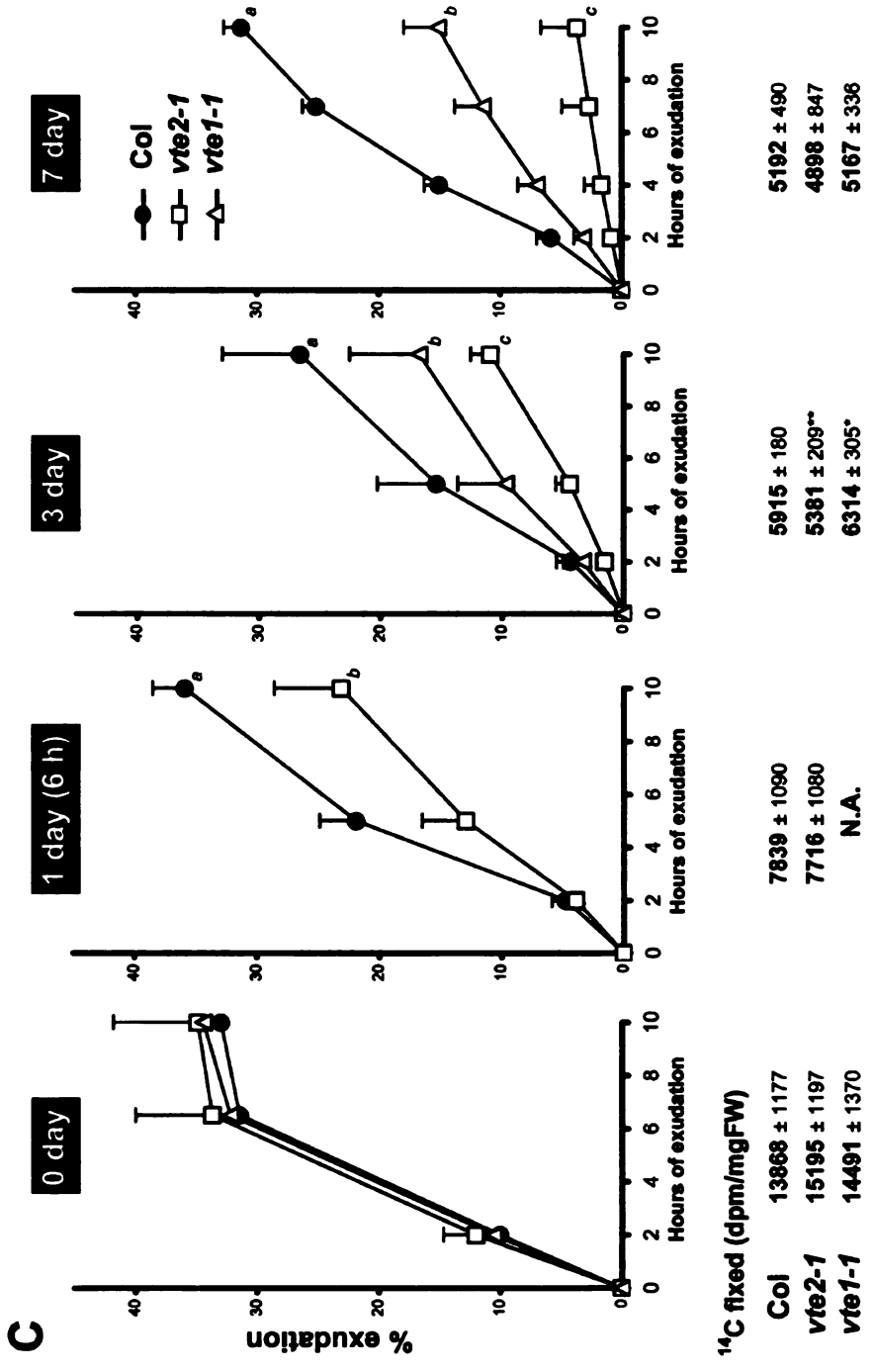


Figure 3.9. (continued)

(C) Phloem exudation of ^{14}C labeled photoassimilates from Col and *vte2-1* and *vte1-1* mature leaves during 7 days of 7.5°C treatment. Total ^{14}C fixed per mg fresh weight of each sample at the indicated time following transfer to 7.5°C is shown below each graph. Data are means ± SD (n = 6 to 8). Two-factor ANOVA using end points (values at 10 h of exudation) indicates interactions are significant ($P < 0.05$, days of 7.5°C treatment and genotype as factors). The pair-wise comparisons of least square means between genotypes at 1, 3 and 7 days of 7.5°C treatment are indicated as *a*, *b* or *c*, while 0 day is not significant. N.A., data not available.

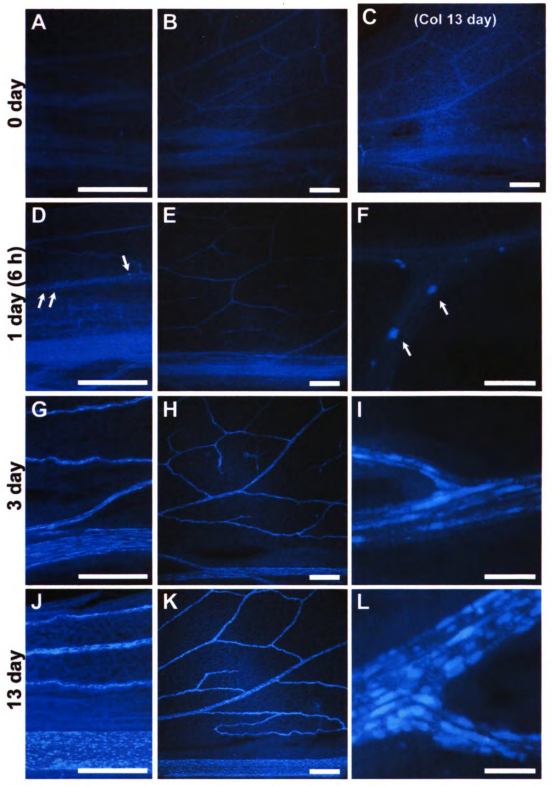


Figure 3.10. Aniline Blue Positive Fluorescence in Leaves of Col and the *vte2* Mutant During Low Temperature Treatment.

Figure 3.10. (Continued)
Col (C) and *vte2-1* (all panels except C) were grown under permissive conditions for four weeks and then transferred to 7.5°C at the beginning of the light cycle. Leaves were harvested in the middle of the day before 7.5°C treatment (0 day; A and B) and after 1 day (6 h; D to F), 3 days (G to I) and 13 days (C and J to L) of 7.5°C treatment and aniline blue positive fluorescence were observed at leaf petioles (A, D, G and J), the lower half of leaves (B, C, E, H and K) and vein junctions (F, I and L). Arrows in (D and F) denote highly fluorescent spots that initially appear in side veins of *vte2-1* petioles after 6 h of 7.5°C treatment. Bars = 50 μm for F, I and L and 500 μm for all other panels.

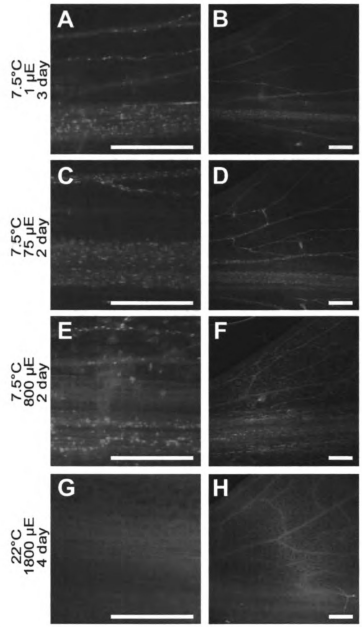


Figure 3.11. Aniline Blue Positive Fluorescence in Leaves of the *vte2* Mutant at Various Light Intensities under Permissive and Low Temperature Conditions.

vte2-1 were grown under permissive conditions for four weeks and then transferred at the beginning of the light cycle to 7.5°C 12h light/12 h darkness at the indicated light levels (A to F) and in the middle of the day to HL1800 at 22°C (G and H). Leaves were harvested in the middle of the day. Aniline blue positive fluorescence was observed at leaf petioles (A, C, E and G) and the lower half of leaves (B, D, F and H). Bars = 500 µm.

(A) and **(B)** 7.5°C at 1 µmol photon m^{-2} s^{-1} for 3 days (54h).

(C) and **(D)** 7.5°C at 75 µmol photon m^{-2} s^{-1} for 2 days (30 h).

(E) and **(F)** 7.5°C at 800 µmol photon m^{-2} s^{-1} for 2 days (30h).

(G) and **(H)** 22°C at 1800 µmol photon m^{-2} s^{-1} for 4 days (72h).

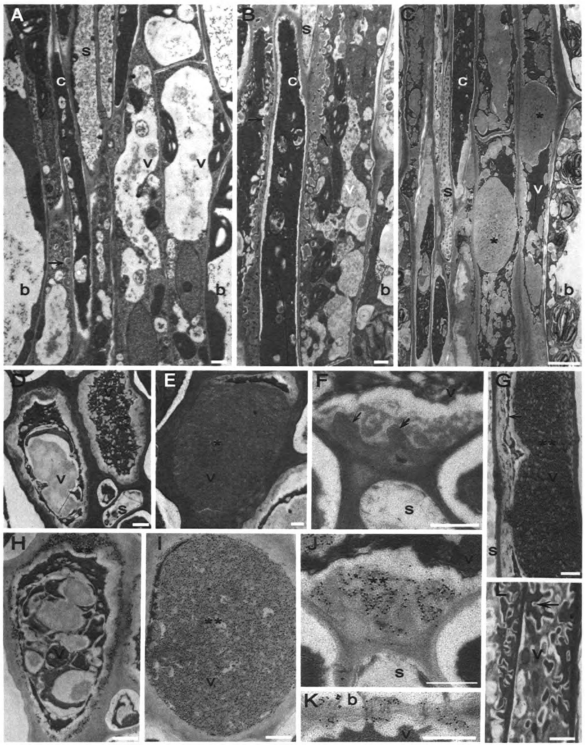


Figure 3.12. Cellular Structure and Immunodetection of Callose in Col and *vte2-1* Before and After 14 Days of Low Temperature Treatment.

Figure 3.12. (Continued)
(G) to (L) are immunolabeled with anti-β-1,3 glucan antibody. (D) to (F) are controls with only 2° antibody. Single arrows denote phloem parenchyma transfer cell wall ingrowths. Double arrows denote abnormal thickening of phloem parenchyma transfer cell wall ingrowths. Single asterisks (*) mark massive wall ingrowths of phloem parenchyma transfer cells. Double asterisks (**) mark wall ingrowths immunolabeled with anti-β-1,3 glucan. Paradermal (C and G) and transverse (E and I) sections show entire phloem parenchyma transfer cell occluded with callose. Paradermal (C and G) and transverse (D and H) sections show the peripheral callose sheath of phloem parenchyma transfer cells. Note callose at boundary between phloem parenchyma transfer cell and sieve element (F and J). Plasmodesmata between bundle sheath (upper cell) and phloem parenchyma transfer cell immunolabeled with anti-β-1,3 glucan (K). b, bundle sheath; c, companion cell; s, sieve element; v, vascular parenchyma transfer cell. Bars = 1 μm (all panels except C) and 5 μm (C)
(A) *vte2-1* before 7.5°C treatment.
(B) to **(L)** *vte2-1* (C to K) and Col (B and L) after 14 days of 7.5°C treatment.

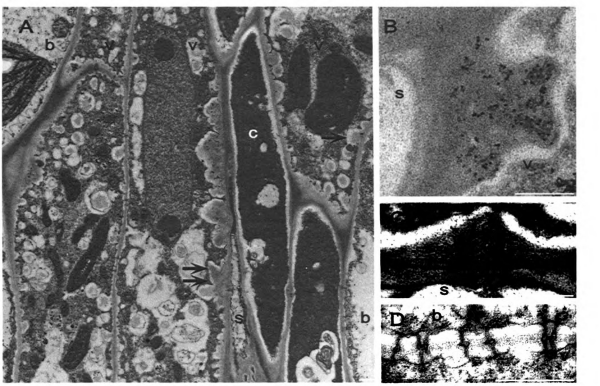
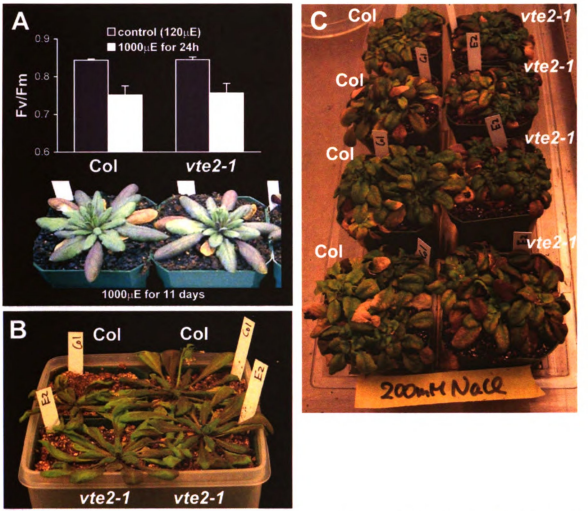


Figure 3.13. Cellular Structure and Immunodetection of Callose in *vte2-1* After 3 Days of Low Temperature Treatment.

(B) to (D) are immunolabeled with anti-β-1,3 glucan antibody. Single arrows denote phloem parenchyma transfer cell wall ingrowths adjacent to bundle sheath. Double arrows denote abnormal thickening of phloem parenchyma transfer cell wall ingrowths adjacent to companion cell. Note that transfer cell wall ingrowths are present around the entire phloem parenchyma transfer cell (A). Transverse section of wall ingrowths immunolabeled with anti-β-1,3 glucan at phloem parenchyma transfer cell and sieve element boundary (B) and plasmodesmata between phloem parenchyma transfer cell (upper cell) and sieve element (C). Plasmodesmata between bundle sheath (upper cell) and phloem parenchyma cell are continuous, lack callose-like wall depositions and are immunonegative for anti-β-1,3 glucan (D). b, bundle sheath; c, companion cell; s, sieve element; v, vascular parenchyma transfer cell. Bars = 0.5 μm



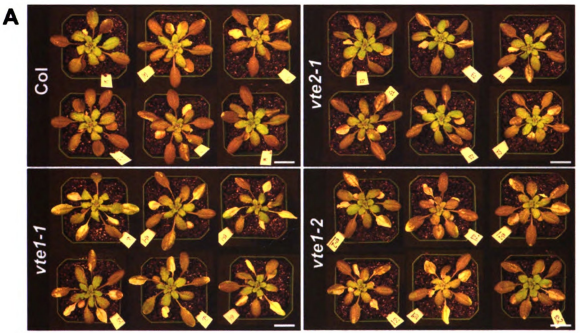
Supplemental Figure 3.S1. Phenotypes of the *vte2* Mutant and Col During HL, Drought and Salinity Stress.

The *vte2* mutant and Col were grown at permissive conditions for four to five weeks prior to the indicated stress treatments.

(A) Four-week-old plants were grown under permissive conditions and then transferred to HL1000 stress (16 h 1000 μmol photon m^{-2} s^{-1} light/8h darkness at 22°C) in the middle of the day. The graph shows the Fv/Fm of Col and *vte2-1* grown under permissive conditions or after 24 h of HL1000. The image shows representative plants of Col and *vte2-1* after 11 days of HL1000.

(B) Five-week-old plants grown under permissive conditions were subjected to drought stress. The image shows representative plants of Col and *vte2-1* that had water withheld for 10 days.

(C) Four-week-old plants grown under permissive conditions were subjected to salinity stress. The image shows representative Col and *vte2-1* plants that were watered with 200 mM NaCl every other day for three weeks.



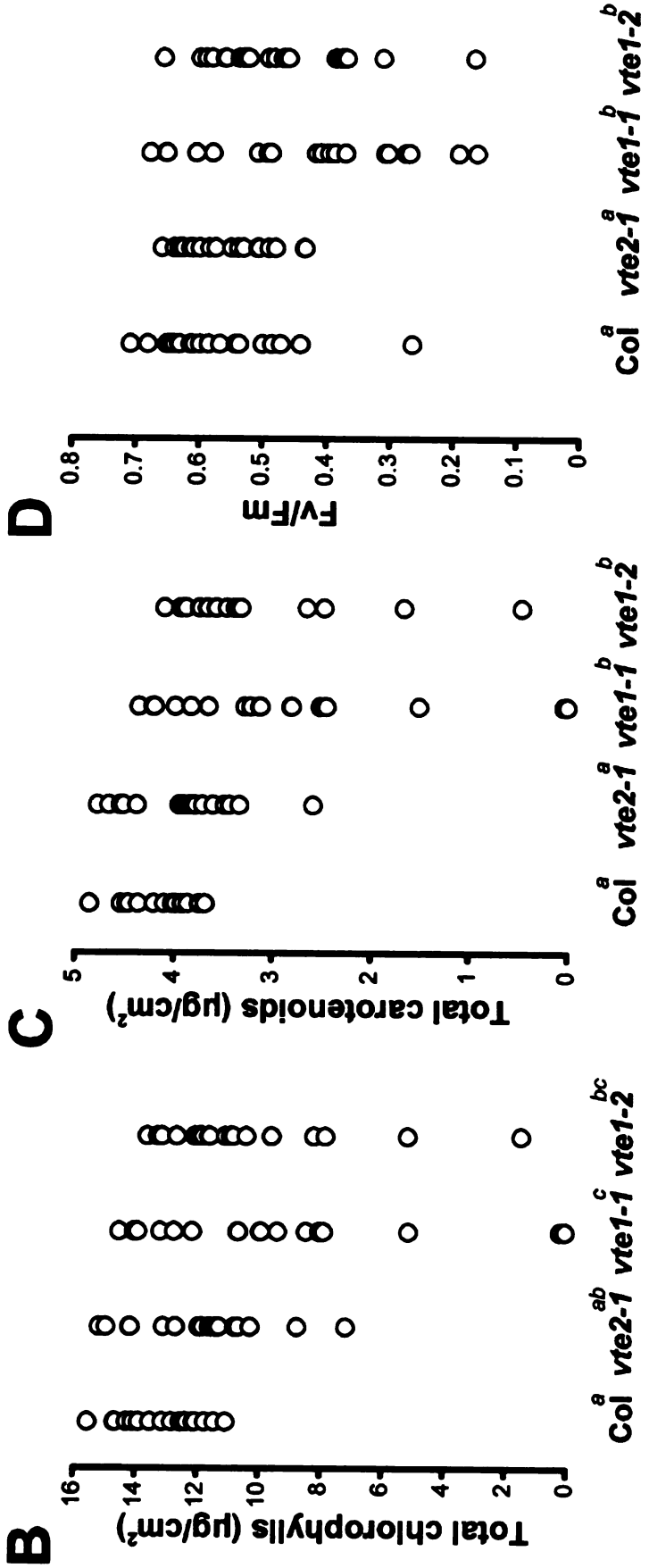
Supplemental Figure 3.S2. Phenotypic and Photosynthetic Responses of Col and the *vte2* and *vte1* Mutants to HL stress.

Plants were grown under permissive conditions for four weeks and then transferred to HL stress in the middle of the day. When significance is observed between genotypes (ANOVA, $P < 0.05$), pair-wise comparison of least square means is evaluated and non-significant groups are indicated by *a*, *b* or *c* with *a* being the highest group.

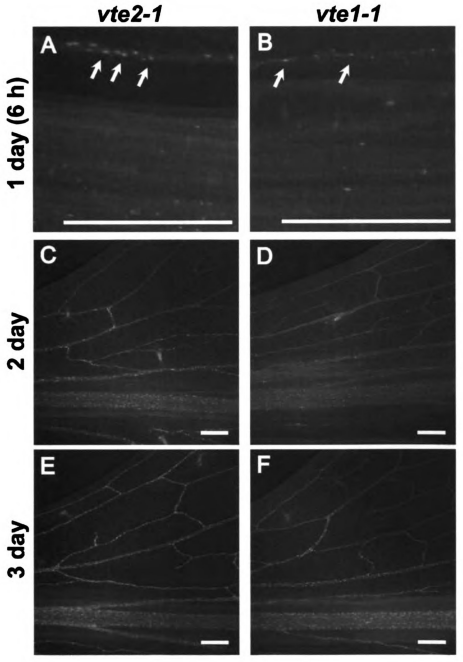
(A) Six representative plants after 3 days of HL1800. Bars = 2 cm.

(B) and **(C)** Individual values of total chlorophyll (B) and carotenoid (C) contents from 19 leaves after 4 days of HL1800.

(D) Individual values of Fv/Fm from 19 leaves after 24 h of HL1800.



Supplemental Figure 3.S2. (continued)



Supplemental Figure 3.S3. Aniline Blue Positive Fluorescence in Leaves of the *vte2* and *vte1* Mutant During Low Temperature Treatment.

vte2-1 (A, C and E) and *vte1-1* (B, D and F) were grown under permissive conditions for four weeks and then transferred to 7.5°C at the beginning of the light cycle. Leaves were harvested in the middle of the day after 1 day (6 h) (A and B), 2 days (C and D) and 3 days (E and F) of 7.5°C treatment and aniline blue positive fluorescence were observed at leaf petioles (A and B), the lower half of leaves (C to F). Arrows in (A and B) denote fluorescence spots initially appeared at side veins of petioles. Bars = 500 μm.

CHAPTER 4: TOCOPHEROLS MODULATE POLYUNSATURATED FATTY ACIDS DERIVED FROM THE EXTRA PLASTIDIC-PATHWAY DURING CHILLING ADAPTATION OF ARABIDOPSIS.

ABSTRACT

Tocopherols (vitamin E) are the major class of lipid-soluble antioxidants in biological membranes and are produced by all plants and algae, and most cyanobacteria, yet their functions in photosynthetic organisms are not well understood. We have previously reported that the *vitamin E deficient 2* (*vte2*) mutant of *Arabidopsis thaliana* is chilling sensitive due to impaired photoassimilate export, which temporally correlated with callose deposition in phloem parenchyma cells. In this study, we found that the introduction of *glucan synthase like 5* (*gsl5*) to the *vte2* background dramatically reduced the callose deposition but did not affect the photoassimilate export phenotype of cold-treated *vte2*, suggesting that the GSL5-dependent vasculature specific callose deposition is not a root cause of the impaired photoassimilate export phenotype. By contrast, the introduction of *fatty acid desaturase 2* (*fad2*), an endoplasmic-reticulum (ER) *ω-6* fatty acid desaturase mutation, to the *vte2* background suppressed nearly all *vte2*-chilling phenotypes, which included both impaired photoassimilate export and vasculature specific callose deposition. Furthermore, in response to low temperature, the *vte2* mutant, in comparison to wild type, exhibited a distinct composition of polyunsaturated fatty acids (PUFAs) (i.e. decreased 18:3 and increased 18:2), which coincided temporally and spatially with the vasculature specific callose deposition. These PUFA alterations occurred primarily in the PUFAs esterified to lipids derived from the ER pathway. Together these results suggest that tocopherols modulate extra-plastidic pathway-derived PUFAs and thereby play a crucial role in phloem loading and chilling adaptation in *Arabidopsis*.

INTRODUCTION

Tocopherols were first discovered as essential nutrients in mammals and together with tocotrienols are collectively known as vitamin E (Evans and Bishop, 1922; Bramley et al., 2000; Schneider, 2005). Tocopherols are well-studied lipid-soluble antioxidants, which quench singlet oxygen and scavenge lipid peroxyl radicals and hence terminate the autocatalytic chain reaction of lipid peroxidation (Tappel, 1972; Fahrenholtz et al., 1974; Burton and Ingold, 1981; Liebler and Burr, 1992; Kamal-Eldin and Appelqvist, 1996). These lipid-soluble molecules are localized in biological membranes and associated with highly unsaturated fatty acids, and thus may also affect membrane properties, such as permeability and stability of membranes (Erin et al., 1984; Kagan, 1989; Stillwell et al., 1996; Wang and Quinn, 2000). Recent studies in mammals have proposed that a specific form of tocopherols (e.g. α-tocopherol but not γ-tocopherol) also has functions unrelated to its antioxidant properties, which include modulation of signaling pathways and regulation of gene expression (Pentland et al., 1992; Ricciarelli et al., 1998; Jiang et al., 2000; Ricciarelli et al., 2002; Rimbach et al., 2002).

Despite the fact that tocopherols are synthesized only in photosynthetic organisms, including all plants and algae, and some cyanobacteria, tocopherol functions in these organisms are not well understood. The tocopherol-deficient *vte2* (*vitamin e 2*) mutant of *Arabidopsis thaliana* is defective in homogentisate phytyl transferase (HPT), the first committed enzyme of the pathway, and lacks all tocopherols and pathway intermediates (Collakova and DellaPenna, 2001; Savidge et al., 2002; Sattler et al., 2004). The *vte2* mutant exhibited reduced seed viability and defective seedling development associated with an elevated lipid peroxidation (Sattler et al., 2004), indicating that tocopherols play

an essential role for seed longevity and early seedling development as lipid-soluble antioxidants. This tocopherol function in seed and seedlings has provided strong selection pressure for the retention of tocopherol biosynthesis during the evolution of seed plants (Sattler et al., 2004).

In contrast to the dramatic *vte2* seedling phenotype, the *vte2* mutants that do survive early seedling development become virtually indistinguishable from wild type under standard growth conditions (Sattler et al., 2004; Maeda et al., 2006), suggesting that tocopherols are dispensable in mature plants in the absence of stress. In leaves, tocopherols are localized in chloroplast membranes and tocopherol levels are elevated in response to a variety of stresses, such as high light, salinity, drought and low temperatures (Munne-Bosch et al., 1999; Collakova and DellaPenna, 2003; Maeda et al., 2006). Based on such correlative and circumstantial evidence together with the evolutionary conservation of tocopherol biosynthesis among photosynthetic organisms, tocopherols had long been assumed to play an essential role in protecting photosynthetic membranes from oxidative stress (Fryer, 1992; Munne-Bosch and Alegre, 2002). Consistent with this hypothesis, the *Arabidopsis vte1* mutant, which is defective in tocopherol cyclase (TC) and lacks all tocopherols but accumulates the redox active pathway intermediates DMPBQ (Porfirova et al., 2002; Sattler et al., 2003), was reported to be more susceptible to a combination of high light and low temperature stress (Havaux et al., 2005). However, a subsequent report demonstrated that the *Arabidopsis* tocopherol-deficient mutants are almost indistinguishable to wild type during high light stress at permissive temperatures but are much more sensitive than wild type to low temperatures (Maeda et al., 2006). Thus, tocopherols are not necessary for

photoprotection but are required for low temperature adaptation in *Arabidopsis*. This low temperature phenotype was independent of light level and was not associated with symptoms of photooxidative stress (i.e. photoinhibition, photobleaching, accumulation of zeaxanthin or lipid peroxides), indicating that the low temperature function of tocopherols is independent of any photoprotective roles of tocopherols (Maeda et al., 2006).

Further analysis of the low temperature responses illustrated that, after transfer to low temperature, tocopherol deficiency initially results in an impairment of photoassimilate export in the *vte2* mature leaves as early as 6 hours after cold treatment. At the same time, unusual deposition of cell wall materials (i.e. callose) occur exclusively in phloem vascular parenchyma cells, possibly creating a bottleneck for photoassimilate transport. The reduced photoassimilate export subsequently leads to carbohydrate and anthocyanin accumulation, feedback inhibition of photosynthesis and growth inhibition of whole plants at low temperature (Maeda et al., 2006). Thus, tocopherols play a crucial role in phloem loading and thereby chilling adaptation in *Arabidopsis* leaves.

The carbohydrate accumulation phenotype of cold-treated *vte2* mature leaves resembled previously reported phenotypes of maize *sucrose export defective 1* (*sxd1*) and potato *SXD1*-RNAi lines, which are also tocopherol deficient and accumulate carbohydrates without cold treatment (Russin et al., 1996; Provencher et al., 2001; Sattler et al., 2003; Hofius et al., 2004). A recent study found that tocopherol-deficient mutants of *Synechocystis* sp. PCC 6803, a unicellular cyanobacterium, also accumulate glycogen, a glucose polymer that is the functional equivalent of starch. Exposure of the *Synechocystis* mutant to external glucose is lethal (Sakuragi et al., 2006). These

combined studies imply that a role for tocopherols in carbohydrate metabolism may be conserved among photosynthetic organisms and it is of great interest to understand the underlying mechanisms. The cold-inducibility of *Arabidopsis vte2* mutant phenotype provides a useful tool to dissect this mechanism.

The aim of this study is to further understand upstream events leading to reduced photoassimilate export in cold-treated *vte2*. A series of mutations affecting callose synthesis and fatty acid desaturation were introduced into the *vte2* background and phenotypic and biochemical responses of the resulting double and triple homozygous mutants were analyzed during low temperature treatment. These results suggest that the vascular specific callose deposition is not a cause of the reduced photoassimilate export and that tocopherol deficiency in *vte2* alters membrane PUFAs derived from the extra-plastidic-pathway, leading to both callose deposition and impaired photoassimilate export at low temperature (Figure 4.1).

RESULTS

gsl5 Attenuates Callose Deposition in vte2 without Suppressing a Photoassimilate Export Phenotype.

To test whether the vasculature specific callose deposition of cold-treated *vte2* causes impaired photoassimilate export, we attempted to genetically eliminate callose deposition by introducing a mutation affecting a callose synthase gene. Arabidopsis has 12 putative glucan synthase-like (*GSL*) genes encoding callose synthases (Hong et al., 2001; Jacobs et al., 2003). GSL5 is the best-characterized callose synthase and is responsible for wounding- and pathogen-responsive callose deposition (Jacobs et al., 2003; Nishimura et

al., 2003). We suspected that GSL5 might also play a role in the cold-induced *vte2* callose deposition. The GSL5 gene was inactivated in the *vte2* mutant background by introducing the *gsl5* mutation, which was originally reported as *powdery mildew resistant 4* (*pmr4*) (Vogel and Somerville, 2000; Nishimura et al., 2003). The resulting *vte2gsl5* double mutant showed visible phenotype similar to Col, *gsl5* and *vte2* under permissive conditions (12h, 120 μmol photon m^{-2} s^{-1} light at 22°C / 12h darkness at 18°C, data not shown). When 4 week-old plants were subjected to 7 days of low temperature treatment (12h 75 μmol photon m^{-2} s^{-1} light / 12h darkness at 7.5°C), *vte2* exhibited expected vasculature-specific callose deposition detected by aniline-blue fluorescence, while no fluorescence signal was detected in the vasculatures of Col and *gsl5* (Figure 4.2A). Interestingly, the vasculature of the *vte2gsl5* double mutant had a substantially reduced level of fluorescence intensity, although a few weak fluorescence spots were still present (Figure 4.2A). This dramatic reduction in *vte2gsl5* callose deposition relative to *vte2* suggests that GSL5 is largely responsible for the cold-induced *vte2* callose deposition.

Given that the level of callose is substantially reduced in *vte2gsl5* relative to *vte2*, one would expect that the impaired photoassimilate transport phenotype of cold-treated *vte2* would be also be attenuated by the *gsl5* mutation. Surprisingly, however, *vte2gsl5* still showed a reduced photoassimilate export capacity comparable to that in *vte2* (Figure 4.2C). Likewise, *vte2gsl5* and *vte2* showed similarly elevated accumulation of soluble sugars and chilling sensitive phenotypes (Figures 2B, and D). These results indicate that the level of callose does not correlate with the capacity of photoassimilate export and the chilling sensitive phenotype of cold-treated *vte2* and suggest that the GSL5-dependent

vascular-specific callose deposition is not a root cause of the impaired photoassimilate export.

vte2 and Col Exhibit Distinct Changes in 18:2 and 18:3 at Low Temperature.

Given the well-characterized chemical nature of tocopherols as lipid-soluble antioxidants (Tappel, 1972; Liebler and Burr, 1992; Kamal-Eldin and Appelqvist, 1996) and their localization in PUFA-enriched chloroplast membranes (Bucke, 1968; Wise and Naylor, 1987), we hypothesized that tocopherol deficiency in *vte2* might impact membrane fatty acid composition. *vte2* and Col were grown under permissive conditions for four weeks, transferred to low temperature conditions and the fatty acid composition of total lipid extracts analyzed from the middle part of the 7th to 9th oldest fully expanded rosette leaves, where all biochemical analyses were previously described (Maeda et al., 2006). Before low temperature treatment, Col and *vte2* had identical total fatty acid composition (0 day in Table 4.1), indicating tocopherol deficiency *per se* does not impact membrane fatty acid composition under permissive growth condition. After transfer to low temperature, however, *vte2* and Col showed distinct changes to their fatty acid compositions. Col gradually increased the molar ratio of α-linolenic acid (18:3), whereas *vte2* increased linoleic acid (18:2) but not 18:3, which gradually decreased in the cold (Table 4.1). As a result, *vte2*, in comparison to Col, showed a significant decrease in the ratio of 18:3/18:2 by as early as 7 days of 7.5°C treatment. Interestingly, the double bond index, an estimation of the total membrane unsaturation level (Falcone et al., 2004), did not differ significantly between genotypes until 14 days of cold treatment (Table 4.1).

Tocopherol Deficiency Reduces 18:3 and Increases 18:2 Derived from the ER Pathway at Low Temperature.

To further examine in which lipid species of the fatty acid composition is affected by the *vte2* mutation at low temperatures, the levels of individual membrane lipid molecules were analyzed using electrospray ionization tandem mass spectrometry (Brugger et al., 1997; Welti et al., 2002; Welti and Wang, 2004). Before low temperature treatment, *vte2* and Col had almost identical lipid species contents and fatty acid composition of each (0h at Figure 4.3A and B). After 14 days of low temperature treatment, the molar ratio of monogalactosyldiacylglycerol (MGDG) decreased and phosphatidylcholine (PC) and phosphatidylethanolamine (PE) increased relative to zero days, but these changes occurred similarly in both *vte2* and Col.

In contrast, the fatty acid compositions of each lipid species exhibited distinct changes in *vte2* and Col. Col increased 36:6 and 34:3 molecular species and correspondingly decreased 36:4 and 34:2 molecular species of PC, PE, and phosphatidylinositol (PI). These changes were not observed in *vte2* and, as a result, *vte2* PC and PE had lower 36:6 and 34:3 and higher 36:4 and 34:2 than Col (Figure 4.3A). According to previous reports (Marechal et al., 1997; Welti et al., 2002), the 36:6, 36:4, 34:3 and 34:2 species mainly consist of the fatty acid pairs of 18:3 (here after indicated as 18:3-18:3), 18:2-18:2, 16:0-18:3 and 16:0-18:2, respectively. Therefore, the decrease in 36:6 (18:3-18:3) and 34:3 (16:0-18:3) and corresponding increase in 36:4 (18:2-18:2) and 34:2 (16:0-18:2) observed in *vte2* phospholipids in comparison to Col are strongly correlated with the 18:3 decrease and 18:2 increase in total fatty acid composition of *vte2* relative to Col (compare Figure 4.3B and Table 4.1). While the level of 36:6 (18:3-18:3)

in the digalactosyldiacylglycerol (DGDG) of Col increased and 34:3 (16:0-18:3) decreased during cold treatment, these changes failed to occur in *vte2* and, as a result, DGDG in *vte2* had lower 36:6 (18:3-18:3) and higher 34:6 (16:0-18:3) levels than Col (Figure 4.3B). Likewise, the MGDG in *vte2* had lower 36:6 (18:3-18:3) and higher 34:6 (16:3-18:3) than Col. As summarized in Figure 4.3C, after transfer to low temperatures, the *vte2* mutant in comparison to Col decreased the level of 18:3 derived from the ER pathway (i.e. 18:3-18:3 of PC, PE, MGDG and DGDG, 16:0-18:3 of PC, PE) and correspondingly increased the level of ER pathway-derived 18:2 (i.e. 16:0-18:2 and 18:2-18:2 of PC and PE). Also, the plastid pathway-derived 18:3 (i.e. 16:0-18:3 of DGDG, 16:0-18:3 of MGDG) were increased in *vte2* relative to Col. These results indicate that tocopherol deficiency significantly impacts the level of PUFAs derived from not only the plastid pathways but also from the ER pathway in response to low temperature.

Distinct PUFA Changes in vte2 Are Temporally and Spatially Associated with Vascular Specific Callose Deposition.

To assess if the observed PUFA changes in *vte2* are related to the vasculature specific callose deposition, tissues from the petiole and the middle of rosette leaves were harvested after 0, 3, 7 and 14 days of cold treatment and total fatty acid composition and aniline blue-positive fluorescence were compared. Consistent with the results from Table 4.1, the middle of *vte2* leaves showed lower 18:3, higher 18:2 levels relative to Col and, as a result, significantly lower 18:3/18:2 ratio after 7 days of cold treatment (Figure 4.4A). The middle of *vte2* leaves also showed detectable callose deposition as early as 7 days of cold treatment (Maeda et al., 2006; Figure 4.4A). Thus, the timing of the PUFA changes

appeared temporally correlated with callose deposition in *vte2* during low temperature treatment. When 18:2 and 18:3 levels were analyzed from the petioles of *vte2* and Col, significant difference in 18:3/18:2 ratio was observed even before cold treatment (0 day at Figures 4.4B), whereas the petioles of *vte2* exhibited callose deposition at 3 days of cold treatment (Maeda et al., 2006; Figure 4.4B). These results suggest that the distinct PUFA changes (i.e. decreased 18:3 and increased 18:2) in *vte2* may be early events leading to the previously reported *vte2* chilling phenotypes including the vasculature specific callose deposition.

Two ω6-Fatty Acid Desaturase Mutations, fad2 and fad6, Suppress the vte2 Chilling Phenotype.

To determine if altered PUFA composition in *vte2* relative to Col is an upstream event leading to the *vte2* chilling phenotype, a series of mutations affecting membrane fatty acid desaturation were introduced to the *vte2* background and impacts on the *vte2* chilling phenotypes assessed. The *fad2* (*fatty acid desaturase 2*) and *fad3* mutants are defective in the ER-*ω6* and *ω3*-fatty acid desaturase enzymes, respectively, and reduce PUFA content predominantly in phospholipids, the major lipid components of extra-plastidic membranes (Miquel and Browse, 1992; Browse et al., 1993; Okuley et al., 1994). The *fad6* and *fad7fad8* mutants are defective in the plastidic-*ω6* and *ω3*-fatty acid desaturase enzymes, respectively, and have reduced PUFAs predominantly in galactolipids and sulfolipids, the major lipid components of plastidic membranes (Browse et al., 1989; Falcone et al., 1994; Mcconn et al., 1994). The selected double or triple homozygous lines containing these *fad* mutations in the *vte2* background allow us to compare the

effects of altering plastidic and extra-plastidic PUFA content on the *vte2* chilling phenotypes.

Under permissive growth conditions, the visible phenotype of all double and triple mutants was virtually indistinguishable from corresponding single or double desaturase mutants (data not shown). When four-week-old plants grown under permissive conditions were subjected to low temperature, the *fad2*, *fad3*, *fad6* and *fad7fad8* mutants also behaved similarly to Col (Figure 4.5). Although the *fad2* and *fad6* mutants are known to be chilling sensitive, our results are consistent with prior reports that *fad6* only shows a low temperature phenotype when plants younger than 13 days are transferred to temperatures lower than 5°C, while the *fad2-1* allele used in this study lacks any low temperature phenotype regardless of plant age due to the leaky nature of this allele under low temperatures (Hugly and Somerville, 1992; Miquel et al., 1993). *vte2* showed the expected chilling sensitive phenotype and accumulated anthocyanins in mature leaves (Figure 4.5; Maeda et al., 2006). *vte2fad3* and *vte2fad7fad8* also similarly accumulated anthocyanins in mature leaves, suggesting the introduction of the *fad3* and *fad7fad8* mutations had little impact on the *vte2* chilling phenotype. Interestingly, low temperature-treated *vte2fad2*, and to a lesser extent *vte2fad6*, showed less anthocyanin accumulation than *vte2* (Figures 4.5).

fad2 and fad6 Suppress the Elevated Soluble Sugar and Reduced Photoassimilate Export Phenotypes of Cold-Treated vte2.

To determine whether *fad2* and *fad6* suppression of the *vte2* visible phenotype is extended to the *vte2* photoassimilate export phenotype (Figure 4.1; Maeda et al., 2006),

soluble sugar levels and the photoassimilate export capacity of the various cold-treated mutant genotypes were analyzed. The *vte2* mutant accumulated elevated levels of soluble sugars compared to Col after 14 days of low temperature treatment consistent with our previous observations (Figure 4.6A; Maeda et al., 2006). *vte2fad3* and *vte2fad7fad8* accumulated elevated levels of soluble sugars equivalent to the level of *vte2*, while the soluble sugar content of *fad2*, *fad3*, *fad6* and *fad7fad8* was similar to Col (Figure 4.6A). The *vte2fad6* double mutant had higher soluble sugar content than Col or *fad6* but was significantly lower than that of *vte2*. The soluble sugar content of *vte2fad2* was much less than the *vte2* level and approached the levels in Col (Figures 6A).

The photoassimilate export capacity of the different genotypes was negatively correlated to their soluble sugar levels (Figure 4.6). The photoassimilate export capacity of *fad2*, *fad3*, *fad6* and *fad7fad8* was similar to Col after 7 days of low temperature treatment, with the exceptions of slightly but significantly lower levels in *fad3* and *fad7fad8* (Figure 4.6B). *vte2* as well as *vte2fad3* and *vte2fad7fad8* had dramatically reduced photoassimilate export capacity compared to Col. The *vte2fad6* and *vte2fad2* double mutants showed significantly higher export capacity than *vte2* with the *vte2fad2* level approaching Col (Figure 4.6B). These results indicate that the introduction of *fad2*, and to a lesser extent *fad6*, but not *fad3* or *fad7fad8*, into the *vte2* background suppress the impaired photoassimilate export phenotype of *vte2*.

fad2 Suppresses the Vasculature Specific Callose Deposition of Cold-Treated vte2.

To test if introduction of the *fad* mutations also affects the vasculature specific callose deposition in cold-treated *vte2* (Figure 4.1; Maeda et al., 2006), 0, 3, and 7 day-cold-

treated plants were harvested and aniline blue positive fluorescence was analyzed. Neither Col nor any of the *fad* mutants (*fad2*, *fad3*, *fad6* and *fad7fad8*) showed vasculature associated aniline blue-positive fluorescence at any time points (Figure 4.7, data not shown). In contrast, aniline blue positive fluorescence appeared at vasculatures of the bottom half of *vte2* leaves at 3 days and spread through the entire leaf at 7 days, as previously reported (Figure 4.7; Maeda et al., 2006). *vte2fad3*, *vte2fad6* and *vte2fad7fad8* showed very similar patterns of aniline blue positive fluorescence to that of *vte2* (first apparent at 3 days in the petioles and then encompassing the entire leaf by 7 days). Interestingly, *vte2fad2* had much less fluorescence than *vte2* at both 3 and 7 days of low temperature treatments and only a few weak fluorescence dots were observed at 7 days (Figure 4.7). These results indicate that *fad2*, but not *fad3*, *fad6* or *fad7fad8*, suppress vasculature specific callose deposition observed in the cold-treated *vte2*.

DISCUSSION

Tocopherols were previously found to play a crucial role in chilling adaptation and phloem loading in Arabidopsis (Maeda et. al., 2006). To obtain a better understanding of the tocopherol function in chilling adaptation, we investigated the early low temperature responses leading to an impaired photoassimilate export in the tocopherol-deficient *vte2* mutant. We first assessed the molecular nature of vasculature specific callose deposition observed in cold-treated *vte2* and its impact on the photoassimilate export phenotype. The introduction of the *gsl5* mutation to the *vte2* background eliminated the majority of callose detected in cold-treated *vte2* (Figure 4.2A), indicating that the *vte2* callose deposition is largely GSL5 dependent (Figure 4.1). Because GSL5 is responsible for

callose deposition in response to wounding (Jacobs et al., 2003), tocopherol deficiency may affect the wound response pathway leading to the activation of the GSL5 enzyme. However, Col and *vte2* showed similar level of wound-induced callose deposition (data not shown), suggesting that tocopherols do not directly influence the wound-signaling pathway leading to the GSL5 activation. Unexpectedly, the elimination of the majority of cold-inducible callose deposition by introduction of the *gsl5* mutation into the *vte2* background did not impact any of *vte2* chilling phenotypes (Figures 4.2). Although the few weak fluorescent signals observed at the vasculature of *vte2gsl5* may still be related, these results indicate that the level of callose does not impact the *vte2* photoassimilate export phenotype and suggest that the GSL5-dependent callose deposition is likely an independent or a downstream event of the impaired photoassimilate export (Figure 4.1).

Interestingly, fatty acid compositions were identical between *vte2* and wild type at permissive growth conditions but tocopherol deficiency in *vte2* resulted in a fatty acid composition distinct from Col at low temperature. Cold-treated *vte2* had a reduced 18:3 and increased 18:2 in comparison to Col (Table 4.1), indicating that the *vte2* PUFA changes are low temperature inducible at least in the middle of leaves. Chronologically, within both the petioles and the middle of *vte2* leaves, the *vte2* PUFA changes are temporally correlated with the vasculature specific callose deposition which was, together with the impaired photoassimilate export phenotype, the earliest detectable phenotype found in our previous study (Figure 4.4; Maeda et al., 2006). It is especially noteworthy that the 18:3/18:2 ratio was lower in the petioles of *vte2* than Col even before cold treatment (0 days in Figure 4.4B), suggesting that the constitutive PUFA changes at petioles may be an immediate consequence of tocopherol deficiency. Furthermore, the

alteration of membrane PUFAs by introducing *fad2* and *fad6* into the *vte2* background suppressed chilling-induced *vte2* phenotypes, including impaired photoassimilate transport and elevated sugar accumulation (Figures 4 and 5). These data provided biochemical and genetic evidence supporting the conclusion that the distinct changes in *vte2* PUFA composition relative to wild-type are upstream events leading to impaired photoassimilate export phenotype during low temperature treatment (Figure 4.1).

This finding raises two interesting questions regarding the function of tocopherols in photoassimilate export and chilling adaptation. The first question relates to the nature of these compositional PUFA changes that lead to impaired photoassimilate export. Based on the comparison of total fatty acid composition between cold-treated *vte2* and Col (Table 4.1), either a decrease in 18:3 or an increase in 18:2 or both are likely key event(s) leading to impairment in photoassimilate export. *ω6*-Fatty acid desaturase mutations (*fad2* and *fad6*), which remove both trienoic and dienoic fatty acids (e.g. both 18:3 and 18:2; Browse et al., 1989; Miquel and Browse, 1992; Falcone et al., 1994; Okuley et al., 1994), partially or fullly suppress the *vte2* phenotype (Figures 4, 5 and 6). On the other hand, *ω3*-fatty acid desaturase mutations (*fad3* and *fad7fad8*), which eliminate only trienoic fatty acids (e.g. 18:3; Browse et al., 1993; Mcconn et al., 1994), do not impact the *vte2* chilling phenotypes (Figures 4, 5 and 6). These results suggest that the changes in 18:2 or both 18:2 and 18:3 levels are likely responsible. When global lipid profiles were compared between cold-treated *vte2* and Col, the 18:2 changes occurred only in 36:4 (18:2-18:2) and 34:4 (16:0-18:2) of phospholipids, that were increased in *vte2* relative to Col (Figure 4.3). Taken together, these biochemical and genetic data

suggest that the alteration in PUFAs derived from the ER pathway may be a key event leading to the photoassimilate export and chilling phenotypes of cold-treated *vte2*.

The second question concerns how tocopherol-deficiency influences membrane PUFA compositions (i.e. increased 18:2 and decreased 18:3). Because tocopherols are the major class of lipid-soluble antioxidants in plastid membranes, the absence of tocopherols in *vte2* may result in increased oxidation/degradation of 18:3 in the plastid membranes and negatively impact photoassimilate export and chilling adaptation of *vte2*. However, lipid profiling analyses showed that the levels of the plastid-pathway-derived 18:3 in cold-treated *vte2* were similar to or even higher than wild type. Although 18:3 of phospholipids were decreased in *vte2* relative to wild type (Figure 4.3B), the degree of total membrane unsaturation estimated by the double bond index (Falcone et al., 2004) was not significantly different between *vte2* and wild type up to 7 days of cold treatment (Table 4.1). Finally, our previous study failed to detect elevated levels of lipid peroxides during low temperature treatment in both *vte2* and wild type (Maeda et al., 2006). These combined results suggest that membrane oxidation is not occurring in the *vte2* mutant during low temperature treatment and that tocopherol functions in chilling adaptation are not by the protection of membrane PUFAs from oxidation.

On the other hand, we found that the characteristic *vte2* PUFA change (i.e. 18:3 decrease and corresponding 18:2 increase) occurred predominantly in the ER-pathway-derived PUFAs (Figure 4.3), indicating that tocopherols affect the composition of PUFAs derived from the ER-pathway. Because the majority of tocopherols are localized in the plastid membranes (Bucke, 1968; Wise and Naylor, 1987), tocopherols may indirectly affect FAD3 activity, which is responsible for the conversion of 18:2 to 18:3 in the ER

membrane (Browse et al., 1993). The most recent study demonstrated that ER membrane has PLastid Associated membranes (PLAMs, Andersson et al., 2006) and tocopherols may be translocated from plastids to ER or tocopherols in the plastid membranes may directly affect activity of enzymes in the ER membrane through the PLAMs. Alternatively, tocopherols may be required for the efficient incorporation of the ER-pathway-derived PUFAs, especially 18:3, into the outer membrane of the plastids. Based on *in vitro* studies, tocopherols have higher affinity to fatty acids with increased levels of unsaturation (Diplock and Lucy, 1973; Erin et al., 1984; Stillwell et al., 1996; Wang and Quinn, 2000). Thus, tocopherol deficient membranes may prefer incorporating 18:2 than 18:3, although they still have to maintain wild type level of total membrane unsaturation in order to acclimate to low temperature (Table 4.1). Plants increase PUFA levels in response to a reduced temperature in order to compensate for the rigidification of membranes (Uemura et al., 1995; Nishida and Murata, 1996; Sakamoto and Murata, 2002) and tocopherols may become important during the process where the PUFA synthesis is elevated. One cyanobacterium which does not produce any tocopherols also does not contain any membrane PUFAs and thus is also extremely chilling sensitive (Powls and Redfearn, 1967; Dasilva and Jensen, 1971; Wada et al., 1990; Gombos et al., 1997). Although the exact mechanism needs to be further investigated, this tocopherol function in the modulation of membrane PUFAs during chilling adaptation may be a fundamental function conserved among photosynthetic organisms.

MATERIAL AND METHOD

Plant Materials and Construction of Double and Triple Homozygous lines

The *pmr4-1*, *fad2-1*, *fad3-1*, *fad6-1*, and fad7-*1fad8-1* mutants were obtained from the Arabidopsis Biological Resource Center (ABRC). The *vte2-1* mutant was previously isolated from ethyl methanesulfonate-mutagenized population and backcrossed to the Col wild type three times (Sattler et al., 2004). All genotypes are in the Col background. The *vte2-1 pmr4-1*, *vte2-1fad2-1*, *vte2-1fad3-1*, *vte2-1fad6-1* double and *vte2-1fad7-1fad8-1* triple mutants were selected from crosses of the respective single or double mutant parents. F_2 progeny homozygous for the *vte2-1* mutation were identified based on their tocopherol deficiency by reverse-phase HPLC (Sattler et al., 2004) and confirmed by genotyping with *vte2-1* CAPs marker, 5'- TTTCACTGGCATCTTGGAGGTAATG -3' and 5'- AAGTGGCAACTGTTTGTAGTAGAAG -3', which generates a 632-bp PCR product with SacI site for the *vte2-1* allele. Plants homozygous for *pmr4-1* were similarly identified based on CAPs maker genotyping (Nishimura et al., 2003). F_2 progeny homozygous for respective *fad* mutation were identified by fatty acid methyl ester analysis using gas-liquid chromatography as described previously (Browse et al., 1986). The total fatty acid composition of *fad2*, *fad3*, *fad6* and *fad7fad8* were almost identical to that of *vte2fad2*, *vte2fad3*, *vte2fad6* and *vte2fad7fad8*, respectively, indicating that the presence of the *vte2* mutation has almost no impact on the fatty acid composition of any *fad* mutant backgrounds (data not shown).

Growth Conditions and Low Temperature Treatment

Seeds were stratified for four to seven days (4°C), planted in a vermiculite and soil mixture fertilized with 1 x Hoagland solution, and grown in a chamber under permissive conditions: 12h, 120 µmol photon m^{-2} s^{-1} light at 22°C / 12h darkness at 18°C with 70 %

relative humidity. Plants were watered every other day and with 0.5 x Hoagland solution once a week. For low temperature treatments, three to four-week-old plants were transferred at the beginning of light cycle to 12h 75 μmol photon m^{-2} s^{-1} light / 12h darkness at 7.5°C (± < 3°C).

Carbohydrate Analyses

Soluble sugar (glucose, fructose and sucrose) levels of leaves were quantified as described (Jones et al., 1977; Lin et al., 1988). Unshaded leaf tissue (<50mg) was harvested, immediately frozen in liquid nitrogen and extracted twice with 700 μL of 80% ethanol at 80°C. The ethanol extract was evaporated and redissolved in 200 μL of distilled water (Jones et al., 1977). Glucose, fructose and sucrose levels in the ethanol extract and the glucose level of the digested starch extract were determined enzymatically (Jones et al., 1977).

Phloem Exudation Experiments

Phloem exudation experiments were conducted according to Maeda et al., (2006) with slight modifications. Seventh and ninth oldest leaves were detached in the middle of the day at the neck of petiole, 0.5 cm of petiole was re-cut under water, the petiole of each leaf was submerged in water and placed in a tightly sealed 10 L glass chamber. $^{14}CO_2$ was generated in the chamber by adding 3 mL of 0.25 N H_2SO_4 to 0.05 mCi (3 μmol) $NaH^{14}CO_3$ and unlabeled 97 μmol $NaHCO_3$ to give a carbon dioxide concentration of 522 ppm. After labeling for 30 min at 120 μmol photon m^{-2} s^{-1}, the petiole of each leaf was submerged in 0.3 mL of 10 mM disodium-ethylenediaminetetraacetic acid (EDTA)

(pH 7.0) and kept in the dark with high humidity for 5 hours to induce phloem exudation (King and Zeevaart, 1974). All of the aforementioned procedures were performed at 7.5°C. The radiolabel exuded into the EDTA solution and also remained in the leaves after 5 h were separately measured by liquid scintillation counting (Tri-Carb 2800TR; PerkinElmer, Wellesley, MA, USA).

Fluorescence Microscopy

Leaves were prepared for aniline blue fluorescence microscopy (n = 2 leaves/plant, 4-6 plants/sample time; Martin, 1959) at the same sampling times as above for export studies. Images were captured on the Leica MZ 16F fluorescence microscope (Wetzlar, Germany).

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Table 4.1. Fatty acid composition of total lipid extracts from *vte2* and Col during 14 days of cold treatment.

	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	18:3/18:2	Double bond index
0 day in cold (28 days-old plants)										
Col	21.6 ± 1.8	3.6 ± 0.4	0.6 ± 0.0	4.5 ± 0.7	1.5 ± 0.1	7.9 ± 0.9	20.7 ± 1.5	39.7 ± 3.4	1.9 ± 0.4	11.4 ± 0.5
vte2	20.8 ± 1.4	3.6 ± 0.2	0.6 ± 0.0	4.9 ± 0.5	1.5 ± 0.1	7.3 ± 0.7	20.8 ± 0.8	40.5 ± 2.1	2.0 ± 0.1	11.6 ± 0.3
4 days in cold										
Col	21.0 ± 2.9	3.4 ± 0.5	0.5 ± 0.1	4.4 ± 1.5	1.0 ± 0.2	7.3 ± 0.9	22.7 ± 1.9	39.7 ± 4.6	1.8 ± 0.1	11.5 ± 0.8
vte2	22.5 ± 1.8	3.6 ± 0.3	0.4 ± 0.0	3.2 ± 1.1	1.0 ± 0.1	7.3 ± 0.6	25.9 ± 1.1	36.0 ± 3.1	1.4 ± 0.2	11.1 ± 0.5
7 days in cold										
Col	22.8 ± 1.8	2.5 ± 0.2	0.3 ± 0.0	3.9 ± 0.9	0.9 ± 0.1	6.3 ± 0.5	22.6 ± 0.8	40.6 ± 2.1	1.8 ± 0.1	11.5 ± 0.4
vte2	25.0 ± 2.6	2.6 ± 0.3	0.3 ± 0.0	3.2 ± 1.1	1.0 ± 0.1	5.7 ± 0.6	27.9 ± 1.1	34.5 ± 3.5 *	1.2 ± 0.1 **	10.8 ± 0.6
14 days in cold										
Col	24.3 ± 1.6	2.0 ± 0.1	0.3 ± 0.0	3.4 ± 0.8	0.8 ± 0.1	4.5 ± 0.6	21.4 ± 1.8	43.4 ± 2.7	2.0 ± 0.3	11.6 ± 0.4
vte2	27.0 ± 0.6	1.9 ± 0.1	0.2 ± 0.0 *	1.8 ± 0.3 **	1.0 ± 0.0	5.2 ± 0.1	31.2 ± 1.1	31.7 ± 0.6 **	1.0 ± 0.1 **	10.4 ± 0.1 **

Values are expressed as molar percent ratios. Student's t-test of Col vs. vte2 (*P<0.05, **P<0.01).

The double bond index is a percentage unsaturated bond of total carbon-carbon bonds.

Data are means ± SD (n = 5)

Increased relative to background genotype

Decreased relative to background genotype

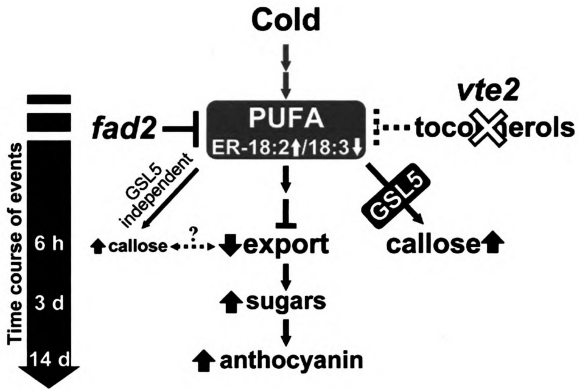

Figure 4.1. A Proposed Time Course of Events Occurring in the cold-treated *vte2* Mutant and Suppression by the *fad2* Mutation.

After transfer to cold, the *vte2* mutant alters PUFA compositions (i.e. increase and decrease in the ER-18:2 and 18:3, respectively), leading to callose deposition in phloem vascular parenchyma (6 hours) and a reduction in photoassimilate export capacity (6 hours) followed by the accumulation of soluble sugars (3 days) and anthocyanins (14 days) in the source mature leaves. The presence of tocopherols in wild type or the introduction of the *fad2* mutation into *vte2* attenuates the increased level of ER-18:2 and thus suppresses the rest of the *vte2* chilling phenotypes. PUFA, polyunsaturated fatty acid; ER-18:2 and 18:3, ER pathway-derived linoleic and linolenic acids, respectively; GSL5, glucan synthase-like 5.

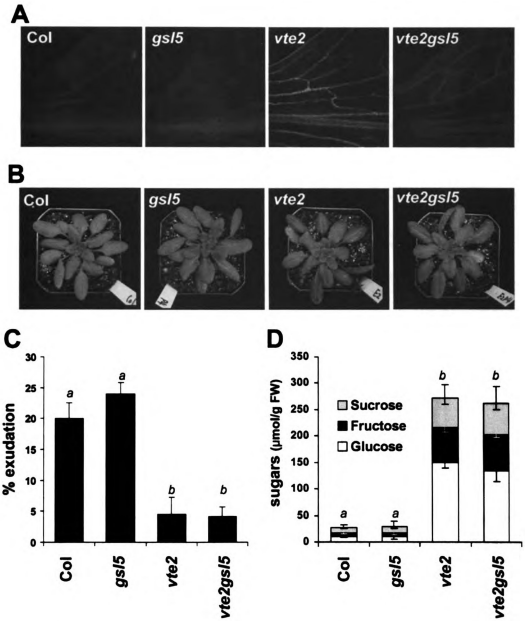

Figure 4.2. Callose Deposition, Whole Plant Phenotypes, ^{14}C-Labeled Photoassimilate Export Capacity, and Soluble Sugar Accumulation of Col, *gsl5*, *vte2* and *vte2gsl5* after Low Temperature Treatment.

All genotypes were grown under standard growth conditions for four weeks and transferred to low temperature (7.5°C) conditions. Aniline-blue positive fluorescence at the lower half of leaves (A) and visible phenotype (B) were observed after one and two weeks of low temperature (7.5°C) treatment, respectively. (C) After one week of 7.5°C treatment, the percent exudation of ^{14}C-labeled photoassimilates was analyzed from mature leaves in the middle of the day. (D) After two weeks of 7.5°C treatment, mature leaves were harvested at the end of light cycle and glucose, fructose and sucrose content analyzed. Data are means ± SD ($n = 5$). Nonsignificant groups are indicated by *a* and *b* ($P < 0.05$).

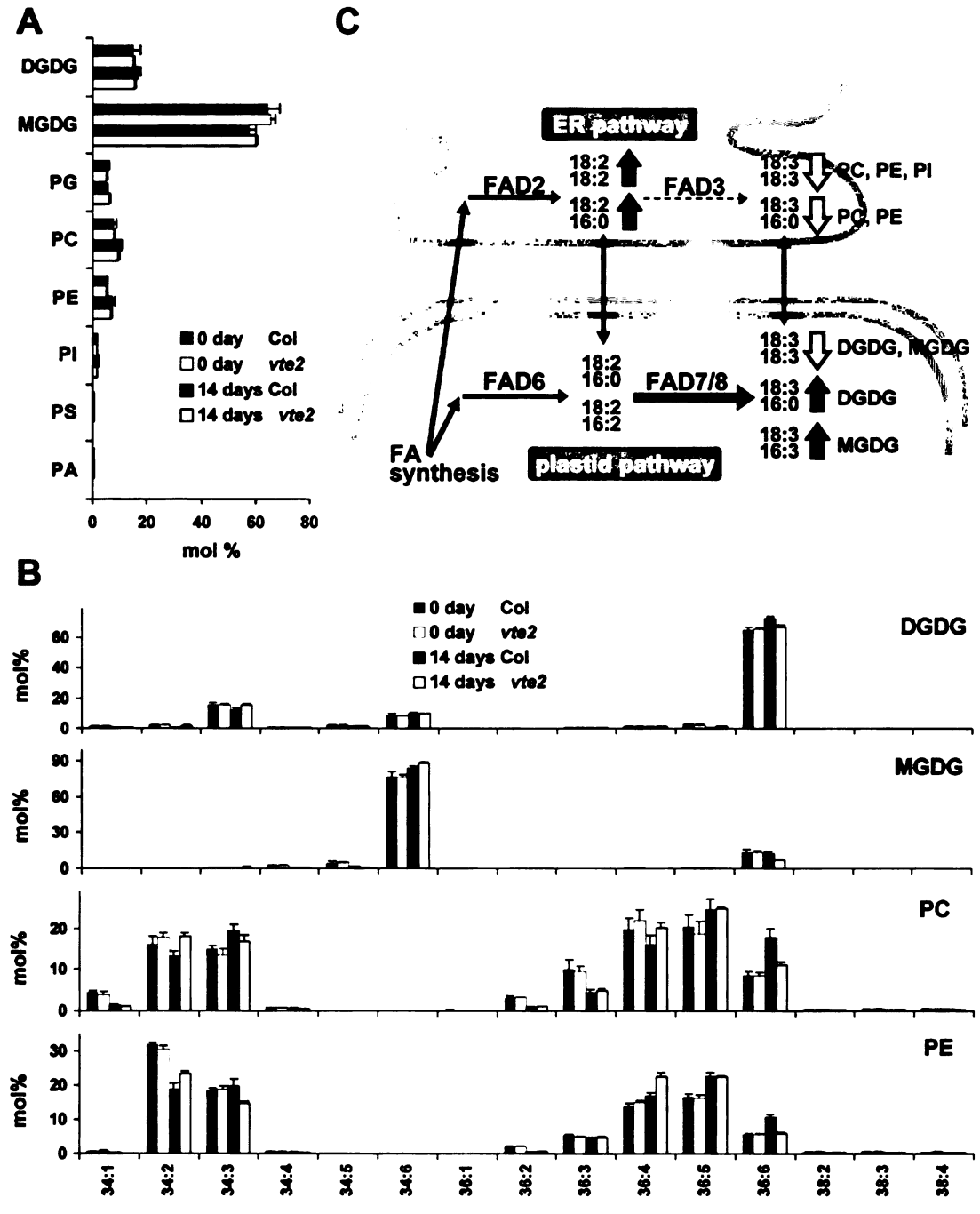

Figure 4.3. Lipid Profiling of Col and *vte2* before and after 14 Days of Cold Treatment.

Col (solid bars) and *vte2* (open bars) were grown under standard growth conditions for four weeks and transferred to low temperature (7.5°C) conditions. Mature leaves were harvested in the middle of the day and total lipids were immediately extracted before (gray bars) and after (black bars) 14 days of cold treatment. Data are means ± SD (n = 5). (A) Mol% of each lipid. (B) Mol% of individual fatty acid pairs within each lipid. (C) A diagram summarizing changes in compositions of each lipid species of *vte2* relative to Col after 14 days of cold treatment.

Figure 4.3. (continued)

DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phoshatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; LysoPG, lysophosphatidylglycerol; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine; FAD, fatty acid desaturase.

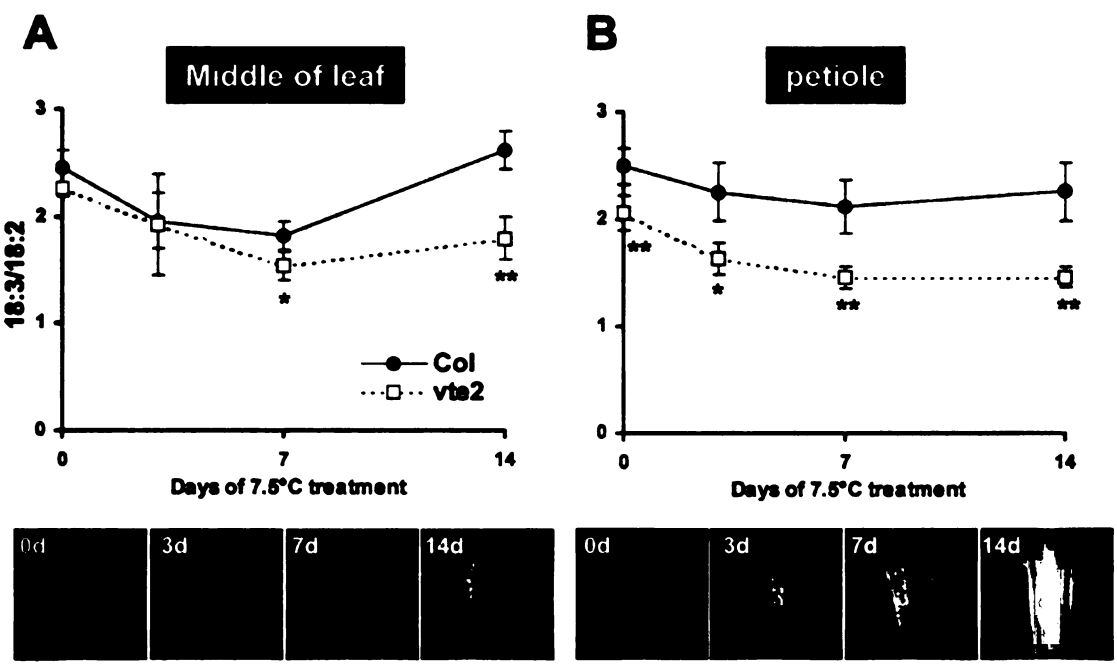

Figure 4.4. 18:3/18:2 Ratio and Callose Deposition at Petioles and the Middle of Leaves of Col and *vte2* during 14 days of Cold Treatment.

Col (closed circles) and *vte2* (open squares) were grown under standard growth conditions for four weeks and transferred to low temperature conditions for the indicated times. Fatty acid composition of total lipid extracts were analyzed from petioles (B) and the middle of leaves (A). Data are means ± SD ($n = 4$). * $P < 0.05$, ** $P < 0.01$ by Student's t test of *vte2* relative to Col at each time point. The bottom pictures show aniline-blue staining of petioles (B) and the middle of leaves (A) of *vte2* for callose visualization at corresponding time points.

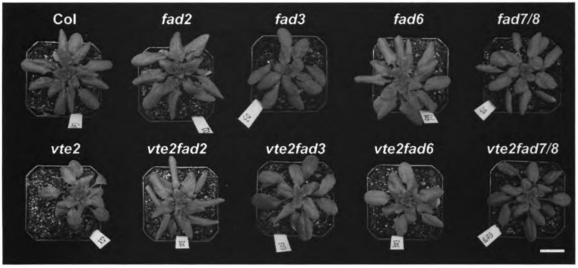

Figure 4.5. Visible Phenotype of Col, *vte2* and a Series of *fad* and *vte2fad* Mutants after Two Weeks of Cold Treatment.

Plants were grown under permissive conditions for four weeks and then transferred to low temperature (7.5°C) conditions for an additional two weeks. Bar = 2 cm.

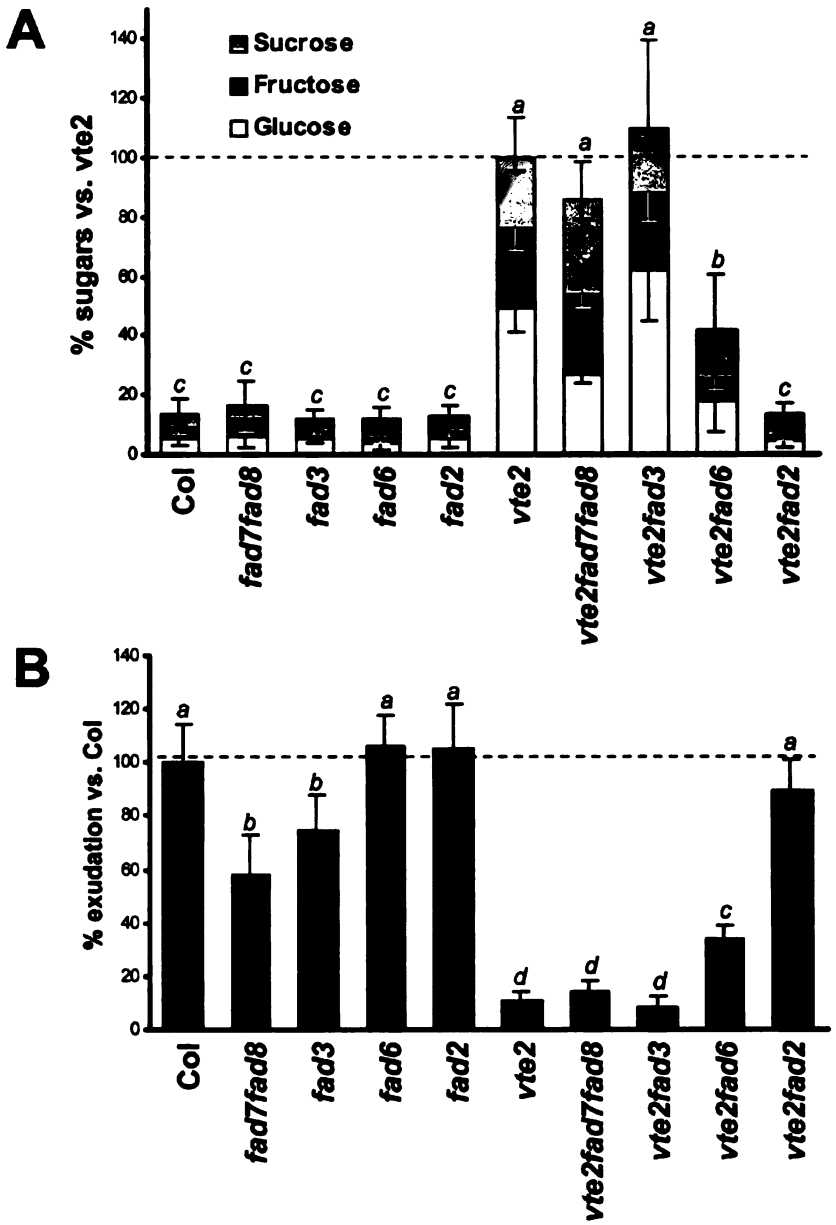

Figure 4.6. Soluble Sugar Content and ^{14}C-Labeled Photoassimilate Export Capacity of Cold-Treated Col, *vte2* and a Series of *fad* and *vte2fad* Mutants.

Plants were grown under permissive conditions for four weeks and then transferred to low temperature condition (7.5°C). (A) After two weeks of cold treatment, the mature leaves were harvested at the end of the light cycle and glucose, fructose and sucrose content analyzed. Values are expressed as a percentage of *vte2*. (B) After one week of cold treatment, the capacity of phloem exudation of ^{14}C-labeled photoassimilates was analyzed in the middle of the day. Values were expressed as percent Col. Data are means ± SD ($n = 5$). Nonsignificant groups are indicated by *a, b, c* and *d*, with *a* being the highest group ($P < 0.05$).

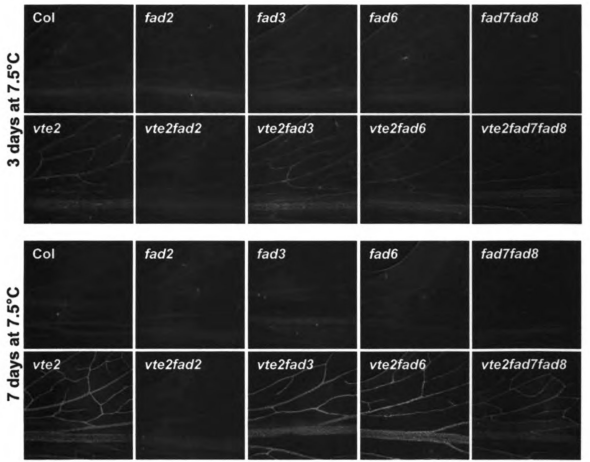

Figure 4.7. Aniline Blue-Positive Fluorescence in Leaves of Col, *vte2* and a Series of *fad* and *vte2fad* Mutants after 3 and 7 days of Cold Treatment.

Plants were grown under permissive conditions for four weeks and then transferred to low temperature (7.5°C) for additional 3 and 7 days. Leaves were harvested in the middle of the day. Aniline blue-positive fluorescence was observed at the lower half of leaves.

CHAPTER 5: SUMMARY AND FUTURE PROSPECTS

Prior to the initiation of the study in this thesis, tocopherol functions in photosynthetic organisms were only speculative due to the lack of tools to directly address this issue. A suite of tocopherol biosynthetic mutants, which produce different levels and types of tocopherols, were isolated in both Arabidopsis and Synechocystis during the gene identification for tocopherol biosynthesis (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Schledz et al., 2001; Porfirova et al., 2002; Shintani et al., 2002; Cheng et al., 2003; Sattler et al., 2003; Sattler et al., 2004). The use of these tocopherol mutants uncovered unexpected roles for tocopherols in photosynthetic organisms, which has led to a better understanding of tocopherol functions and raised many interesting questions.

Tocopherols Have a Limited Role in Photoprotection.

It has long been assumed that tocopherols have an essential role in protecting photosynthetic organisms from photooxidative stress (Fryer, 1992; Munne-Bosch and Alegre, 2002). In the experiments described in Chapters 2 and 3 it si detemonstrated that the elimination of tocopherols by orthologous mutations in both Arabidopsis and Synechocystis PCC6803 have surprisingly subtle impacts on tolerance and adaptation to HL stress. It was only under extreme lipid peroxidation stress conditions, such as PUFA-induced lipid peroxidation stress (Chapter 2) and during early seedling development (Sattler et al., 2004) that these tocopherol-deficient mutants became more susceptible than wild type to oxidative stress. These results suggest that tocopherols are not essential during HL stress under laboratory conditions tested and their function appears limited to the extreme of lipid peroxidation stress.

The early light-induced proteins (ELIPs) or peroxiredoxins Q (Prx Q) have also been proposed to play an important role in HL protection. However, the elimination of ELIPs and Prx Q in Arabidopsis did not lead to a whole plant phenotype during HL stress, likely due to an elevated induction of other compensatory mechanisms (Lamkemeyer et al., 2006; Rossini et al., 2006). Similarly, the lack of tocopherols may also be compensated for by other mechanisms during HL stress. Indeed, a Synechocystis tocopherol-deficient mutant was more susceptible than wild-type to HL stress in the presence of norflurazon, an inhibitor of carotenoid biosynthesis (Figure 2.7). Also, in response to HL stress, Arabidopsis tocopherol-deficient mutants accumulated elevated levels of zeaxanthin, a xanthophyll cycle carotenoid involved in NPQ (Table 3.2; Niyogi et al., 1998; Muller et al., 2001; Holt et al., 2004). These results suggest that enhanced induction(s) of carotenoids or mechanisms that involve carotenoids (e.g. NPQ) compensate for the lack of tocopherols in the mutants. To test this hypothesis further, I have generated Arabidopsis *vte2npq1* and *vte2npq4* double mutants, which are deficient in both tocopherols and zeaxanthin (Niyogi et al., 1998) or PsbS (Li et al., 2000), respectively. Preliminary results showed that the HL susceptibility of these double mutants was similar to the respective *npq* single mutants, suggesting that carotenoids or NPQ do not have overlapping functions with tocopherols or that additional compensatory mechanisms exist in Arabidopsis. The results presented in this thesis uncovered highly versatile mechanisms protecting photosynthetic organisms from HL stress, and suggested that roles of tocopherols in photoprotection have to be investigated as a component of the highly redundant photoprotective mechanisms in the future.

Novel Roles for Tocopherols in Chilling Adaptation.

In contrast to the limited role of tocopherols during HL stress, the dramatic chilling sensitive phenotypes of Arabidopsis tocopherol-deficient mutants indicated that tocopherols play a crucial role in chilling adaptation. Obviously, this tocopherol function cannot be compensated for by other mechanisms. Detailed phenotypic, biochemical and ultrastructural characterization of the chilling phenotype illustrated that tocopherol deficiency results in distinct compositions of PUFAs derived from the ER pathway, leading to both impaired photoassimilate transport and callose deposition specifically at phloem parenchyma cells. Ultimately, tocopherol-deficient mutants accumulate abnormal levels of carbohydrates in source leaves and reduce their sink growth (Figure 4.1). While it became clear that tocopherols have previously unrecognized roles independent of their photoprotective functions, a number of questions remain to be answered in order to further understand tocopherol functions in chilling adaptation.

Are the ultrastructural alterations of the phloem parenchyma a cause or result of impaired photoassimilate export?

Although the callose deposition at the interface between phloem parenchyma and sieve element/companion cell complexes is likely to be intimately associated with impaired photoassimilate export, analyses of *vte2gsl5* indicated that the majority of callose deposited does not impact the *vte2* export phenotype and thus GSL5-dependent callose deposition in cold-treated *vte2* vasculature is likely an independent or downstream event of the export phenotype (Figure 4.1). However, *vte2gsl5* still accumulated residual callose, which is GSL5-independent and clearly produced by other callose synthase(s),

and this may be sufficient to block photoassimilate export in *vte2gsl5*. This possibility can be evaluated by using 2-deoxy-glucose, an inhibitor for callose synthases (Jaffe and Leopold, 1984; Yun et al., 2006), which would inhibit all callose synthases simultaneously and completely eliminate callose. Ultrastructural analyses of the *vte2* mutant treated with 2-deoxy-glucose and also the *vte2gsl5* double mutant will help us to further determine a cause and effect relationship between the phloem parenchyma specific ultrastructural alteration (i.e. callose deposition) and the impaired photoassimilate export observed in cold-treated *vte2*.

How do the distinct PUFA changes in vte2 lead to impaired photoassimilate export?

Based on the reduced level of 18:3 observed in cold-treated *vte2* relative to wild type, we initially hypothesized that tocopherol deficiency may lead to elevated 18:3 degradation in chloroplasts and hence an increase in 18:3-derived oxidation products and signals, such as jasmonic acid (JA). However, we saw no evidence that membrane oxidation is occurring in *vte2* during chilling adaptation; Lipid peroxides were not accumulated and the degree of total membrane unsaturation remained constant in *vte2* during at least the initial one week of low temperature treatment (Figure 3.4 and Table 4.1). Preliminary analysis also failed to detect any increased level of JA, 12-oxophytodienoic acid (OPDA) and other oxidized lipid species (e.g. phytoprostanes, data not shown). Moreover, the introduction of the *fad3* and *fad7fad8* mutations, which eliminate 18:3 synthesis, into the *vte2* background did not suppress any of the *vte2* chilling phenotypes. Thus, it is unlikely that 18:3-derived products are involved in the *vte2* chilling phenotypes. To further confirm this conclusion, the *vte2aos* (*aos*, *allene oxide synthase*) and *vte2fad3fad7fad8*

mutants, which do not produce any JA and/or 18:3 (McConn and Browse, 1996; Park et al., 2002), will be subjected to low temperature treatment and the *vte2* chilling phenotypes will be tested. If *vte2aos* and *vte2fad3fad7fad8* still show a chilling sensitive phenotype similar to *vte2*, we can eliminate an involvement of 18:3-derived signals including JA, OPDA and phytoprostanes in the chilling phenotypes of *vte2*.

Lipid profiling data and the suppression of the *vte2* phenotypes by *fad2* and to a lesser extent *fad6* raised the possibility that alteration in PUFAs derived from the ER pathway may be a key event leading to the *vte2* chilling phenotype. Consistent with this thesis, *fad3* and *fad7fad8*, which constitutively have an increased level of 18:2 and a decreased level of 18:3 compared Col (Browse et al., 1993; McConn et al., 1994), also exhibited a significantly lower capacity of photoassimilate export than Col at low temperatures (Figure 4.6). To further test this hypothesis, lipid profiling analyses for *vte2fad2*, *vte2fad6*, and *vte2fad3* together with respective single mutants will be conducted to assess if there is an inverse correlation between the accumulation of ER-pathway-derived 18:2 and the capacity of photoassimilate export at low temperature. Although it is not clear how the accumulation of the ER-pathway-derived 18:2 negatively impact photoassimilate export from phloem parenchyma cells, activities of membrane proteins, such as sucrose transporters, may be affected by unusual PUFA compositions in cold-treated *vte2*.

How do tocopherols influence PUFAs derived from the ER pathway?

The results presented in Chapter 4 were unexpected and indicated that tocopherols influence the composition of PUFAs derived from the ER pathway during chilling

adaptation. As discussed in Chapter 4, tocopherols may either indirectly affect the activity of FAD3 or be required for efficient incorporation of highly unsaturated fatty acids (e.g. 18:3) into membranes. Because the level of *FAD3* expression correlates with the FAD3 activity in leaves (Shah et al., 1997; Hamada et al., 1998), it is interesting to test if *FAD3* expression is affected in *vte2* relative to Col during low temperature treatment. To investigate the later possibility, wild type and *vte2* will be subjected to treatments that increase PUFA synthesis, such as transferring plants from high to normal temperatures (e.g. 35°C to 22°C, ref). If *vte2* exhibits a lower 18:3/18:2 ratio and photoassimilate export capacity than wild type without being subjected to chilling temperature, it would suggest that tocopherols are required for increased PUFA synthesis rather than low temperature adaptation *per se*.

Alternatively, tocopherols may be translocated to the extra-plastidic membranes and exert their functions outside plastids. Although most previous studies have shown that tocopherols are exclusively localized in the plastid membranes (Bucke, 1968; Wise and Naylor, 1987), the current study revealed that tocopherols affect event(s) occurring outside of the plastids. The most recent study demonstrated that ER membrane has PLastid Associated membranes (PLAMs, Andersson et al., 2006) and tocopherols may be translocated from plastids to ER or tocopherols in the plastid membranes may directly affect activity of enzymes in the ER membrane through the PLAMs. Thus, tocopherol function(s) should not be restricted only in the plastids and we may have to reevaluate the precise localization of tocopherols from different tissues under various environmental conditions.

Is tocopherol function in the modulation of membrane PUFAs conserved among photosynthetic organisms?

Until now, tocopherol-deficient mutants have been reported in four different photosynthetic organisms, which include Arabidopsis, maize, potato, and Synechocystis. Intriguingly, all of these lines exhibited an elevated carbohydrate accumulation phenotype (Russin et al., 1996; Provencher et al., 2001; Hofius et al., 2004; Sakuragi et al., 2006; Chapters 3 and 4, Appendix 3). However, there are a few differences between their carbohydrate phenotypes. For example, Arabidopsis tocopherol-deficient mutants accumulate carbohydrates only under low temperatures (Chapters 3 and 4), whereas maize, potato and Synechocystis mutants exhibit such phenotypes under permissive growth conditions (Russin et al., 1996; Provencher et al., 2001; Hofius et al., 2004; Sakuragi et al., 2006; Appendix 3). It is unclear at this point why the Arabidopsis carbohydrate phenotypes are low temperature inducible. However, PUFA compositions were already altered in the petioles of Arabidopsis tocopherol-deficient mutants under permissive temperatures (Figure 4.4) and it may be that low temperature exacerbates already existing PUFA phenotypes to a threshold level which leads to impaired photoassimilate export and carbohydrate accumulation.

To address such an unanswered question, we have to further understand the mechanisms leading to the carbohydrate accumulation phenotype in these photosynthetic organisms. It is interesting to test if the carbohydrate phenotypes of maize, potato and Synechocystis tocopherol-deficient mutants can be also suppressed by altering PUFA compositions. These experiments will not only provide additional tools to further uncover the underlying mechanisms of tocopherol functions in the PUFA modulation and

carbohydrate metabolism but also address the universality of potentially a novel tocopherol function of photosynthetic organisms.

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APPENDICES

A-1: Preliminary Analyses of the High Light Responses of *Arabidopsis vte* Mutants.

This section presents detailed analyses of the phenotypic and photosynthetic responses of Arabidopsis *vte* mutants to HL stress. The obtained results allowed us to determine the light condition and the time course of HL stress used for the large-scale experiments presented in the Chapter 3.

Visible Phenotype under varied light intensities.

To assess if tocopherol deficiency impacts whole plant survival during HL stress, wild type and tocopherol-deficient mutants were grown for 4 weeks under standard growth conditions (12h, 120 µmol photon m^{-2} s^{-1} light at 22°C / 12h darkness at 18°C) and then transferred to various HL conditions with light intensities ranging from 1000 to 2000 µmol photon m^{-2} s^{-1} (hereafter refer to HL1000 or HL2000). Before HL treatment, the obvious phenotypic differences were not observed among Col, *vte2-1*, *vte1-1*, and *vte1-2* with the exception of *vte1-1* having slightly thinner leaves and longer petioles than other genotypes (Figure A1.1). Similar to the results obtained during HL1000 (Supplemental Figure 3.S1), when Col and *vte2-1* were subjected to HL1500, their leaves changed color to purple after about 48 hours of treatment (data not shown) but no obvious phenotypic difference was observed between genotypes (Figure A1.2). Under HL1800, both Col and *vte2-1* started to show bleached leaves after 24 hours of treatment. Under HL2000, these symptoms appeared earlier and were more severe (Figure A1.2). *vte2-1* had slight tendency towards more bleached leaves than Col but the trend was not reproducible. The degree of these HL symptoms were highly variable between experiments (e.g. compare

Figure 3.2 and Supplemental Figure 3.S2), which is likely caused by environmental factors, such as slight difference in the growth condition before HL treatment (e.g. light intensity, water status). Nevertheless, all genotypes were grown and treated side by side, and thus all HL responses are comparable between genotypes within each experiment.

Time Course Pigment Analyses

To examine changes in photosynthetic pigment contents in response to HL stress, 7th and 9th oldest fully expanded mature leaves were harvested during 5-days time course of HL1600. During HL treatment, total chlorophyll and carotenoid contents were gradually decreased and increased, respectively. However, no obvious genotypic difference was observed (Figure A1.3). The epoxidation state of xanthophyll cycle carotenoids (A+Z/A+Z+V), which reflects the degree of NPQ induction (Niyogi et al., 1998; Muller et al., 2001), was increased from 0.05 to 0.8 during the first day of HL1600 and gradually decreased thereafter. Interestingly, *vte2-1* sustained higher A+Z/A+Z+V value than Col after 3 days of HL1600, suggesting NPQ is being induced in *vte2-1* at higher degree than Col.

Time Course Fv/Fm Measurements

Maximum photosynthetic efficiency (Fv/Fm) was also analyzed during 3 days of HL1600 treatment. All genotypes had Fv/Fm values around 0.8, indicating photosystem II (PSII) is not damaged under standard growth conditions (Figure A1.4). After HL1600, Fv/Fm decreased from 0.8 to around 0.6 during the first day and gradually recovered to 0.7 after 3 days (Figure A1.4A). The Fv/Fm values of all three tocopherol-deficient mutants (*vte2-*

1, *vte1-1*, and *vte1-2*) were slightly lower than Col (*t* test, $P < 0.05$) at the end of the 1st day of HL1600 (8h HL1600) but became similar to Col after 2 and 3 days (Figure A1.4A). To examine initial photosynthetic responses, Fv/Fm was analyzed in Col and *vte2-1* during the first 7 hours of HL1800. Both genotypes showed biphasic reductions in Fv/Fm during the first 2 hours and after 6 hours of treatment. However, these changes in Fv/Fm were almost identical between Col and *vte2-1* (Figure A1.4B).

Quantum yield of PSII (ΦPSII) and non-photochemical quenching (NPQ).

In order to assess photosynthetic efficiency of wild type and tocopherol-deficient mutants, photo flux density (PFD)-dependent quantum yield of PSII (ΦPSII) was analyzed. ΦPSII measures the proportion of absorbed light energy used for photochemistry and hence reflect the efficiency of photosynthetic electron transport from PSII (Genty et al., 1989; Maxwell and Johnson, 2000). Mature leaves of four-weeks-old normally grown plants were illuminated with 100, 500, 1000, and 1500 μmol photon m^{-2} s^{-1} of actinic light provided by a pulse amplitude modulated (PAM) fluorometer and ΦPSII at each light intensity was analyzed. As shown in Figure A1.5A, ΦPSII values were decreased as PFD increased, suggesting that less proportion of absorbed light energy was transferred to photosynthetic linear electron transport as PFD increased. However, these values were not different among genotypes under any PFD, suggesting the presence and absence of tocopherols does not impact the efficiency of photosynthesis.

The degree of NPQ induction was also measured under the same condition. In a reciprocal manner to ΦPSII, NPQ increased as PFD increased (Figure A1.5B), indicating excess energy which could not be used for photosynthetic linear electron transport are

dissipated as heat via NPQ (Maxwell and Johnson, 2000; Moller, 2001). *vte2-1* showed slightly higher NPQ at 1000 and 1500 μmol photon m^{-2} s^{-1} in some experiments (Figure A1.5B) but the differences were not significant. The kinetics of NPQ induction was also analyzed in Col and *vte2-1* during the first 8 and 10 min of 800 and 1500 μmol photon m^{-2} s^{-1} illuminations, respectively. Both Col and *vte2-1* rapidly induced NPQ within a minute of HL treatments but the genotypic differences were not detected (Figure A1.5C).

Based on these preliminary analyses using different light intensities and durations of HL treatment, it became clear that the genotypic differences between wild type and tocopherol-deficient mutants are, if any, very marginal and may be masked by a large variation caused by environmental factors. Thus, large-scale experiments using higher number of replicates were needed to distinguish the genotypic differences from environmental variations. Because a large number of plant samples limit the number of time points and conditions tested, I determined the following conditions for further large-scale analyses as depicted in Figure A1.6. Four-weeks-old plants grown under standard conditions are transferred to HL1800 (16h HL1800/8h dark) in the middle of the day. Fv/Fm is analyzed at the end of the first day (8 h HL) and also in the middle of the second day (8 h HL/8 h dark/8 h HL). Then visible phenotypes are pictured in the middle of the third day when anthocyanin accumulation and any chlorosis become visible. In the middle of the fourth day, photosynthetic pigment and tocopherol contents are analyzed. As described in the Chapter 3, these analyses provided evidence that tocopherols has a limited role under HL stress and other protective mechanisms such as zeaxanthin accumulation may compensate for the lack of tocopherols under HL stress.

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FIGURES

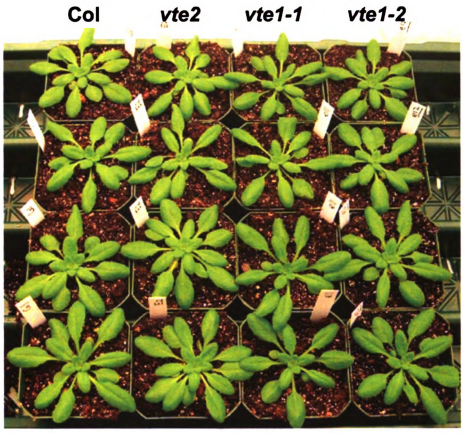

Figure A1.1. Visible Phenotype of Arabidopsis Tocopherol-Deficient *vte* Mutants under Permissive Growth Conditions.
Plants were grown under permissive growth conditions for four weeks and visible phenotype was pictured.

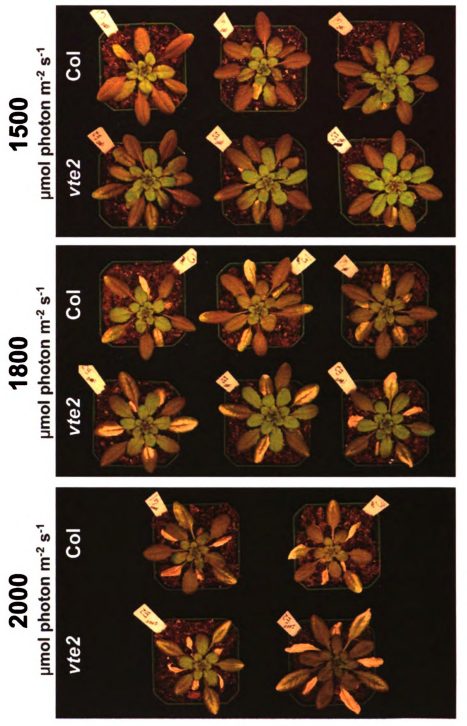

Figure A1.2. Visible Phenotype of Col and the *vte2* Mutant after 3 Days of HL stress with Varied Light Intensities.

Plants were grown under permissive growth conditions for four weeks and then transferred to 1500, 1800, and 2000 µmol photon m^{-2} s^{-1} of HL stress for 3 days.

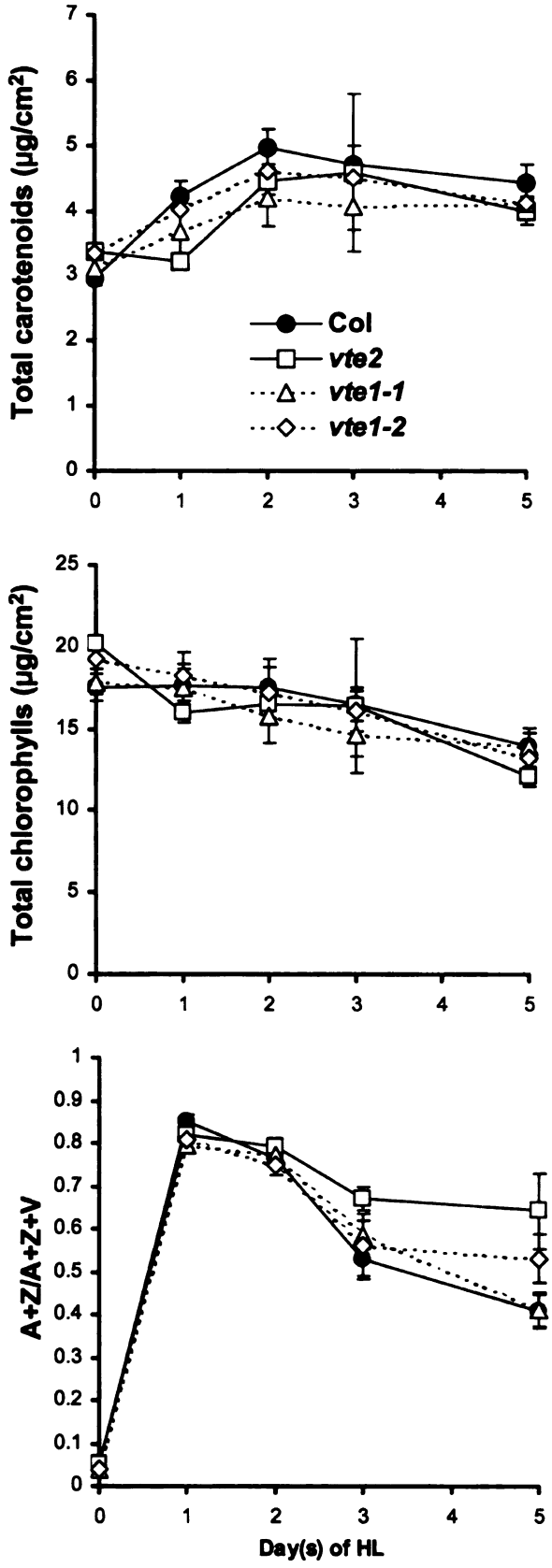

Figure A1.3. Photosynthetic Pigment Contents and Composition of Wild Type and Tocopherol-Deficient Mutants during HL Stress.

Plants were grown under permissive growth conditions for four weeks and then transferred to HL stress (16h, 1600 µmol photon m^{-2} s^{-1}/8h dark) at the beginning of light cycle. Photosynthetic pigments were analyzed at the end of the 1st, 2nd, 3rd and 5th light cycles. Data are means ± SD ($n = 4$). A+Z+V, antheraxanthin + zeaxanthin + violaxanthin.

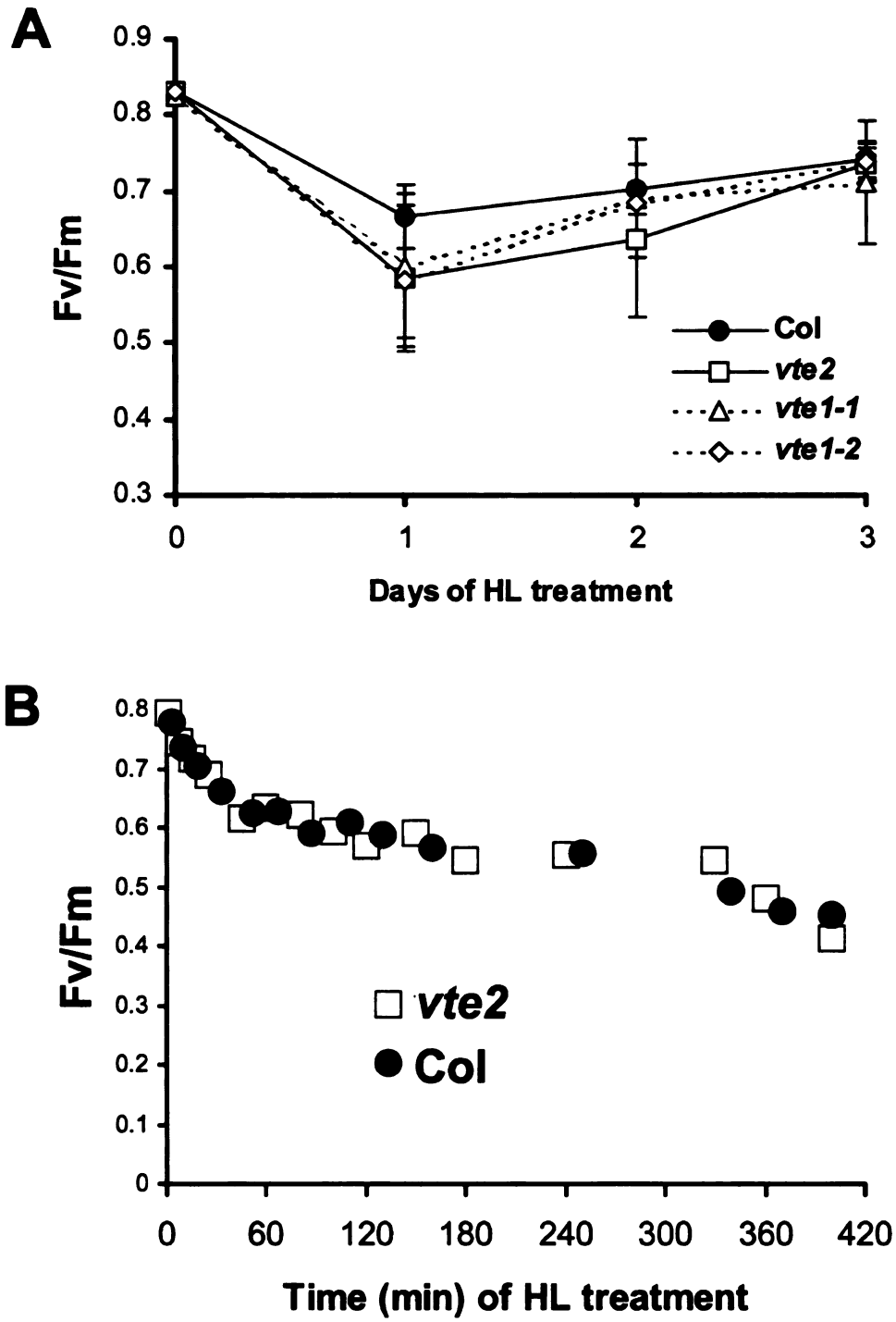

Figure A1.4. Changes in Maximum PSII Photosynthetic Efficiency (Fv/Fm) during HL Stress.

Plants were grown under permissive growth conditions for four weeks and then transferred to HL stress (16h, 1800 μmol photon m^{-2} s^{-1}/8h dark) in the middle of light cycle. Fv/Fm was analyzed at the indicated time points. Data in (A) are means ± SD (n = 12).

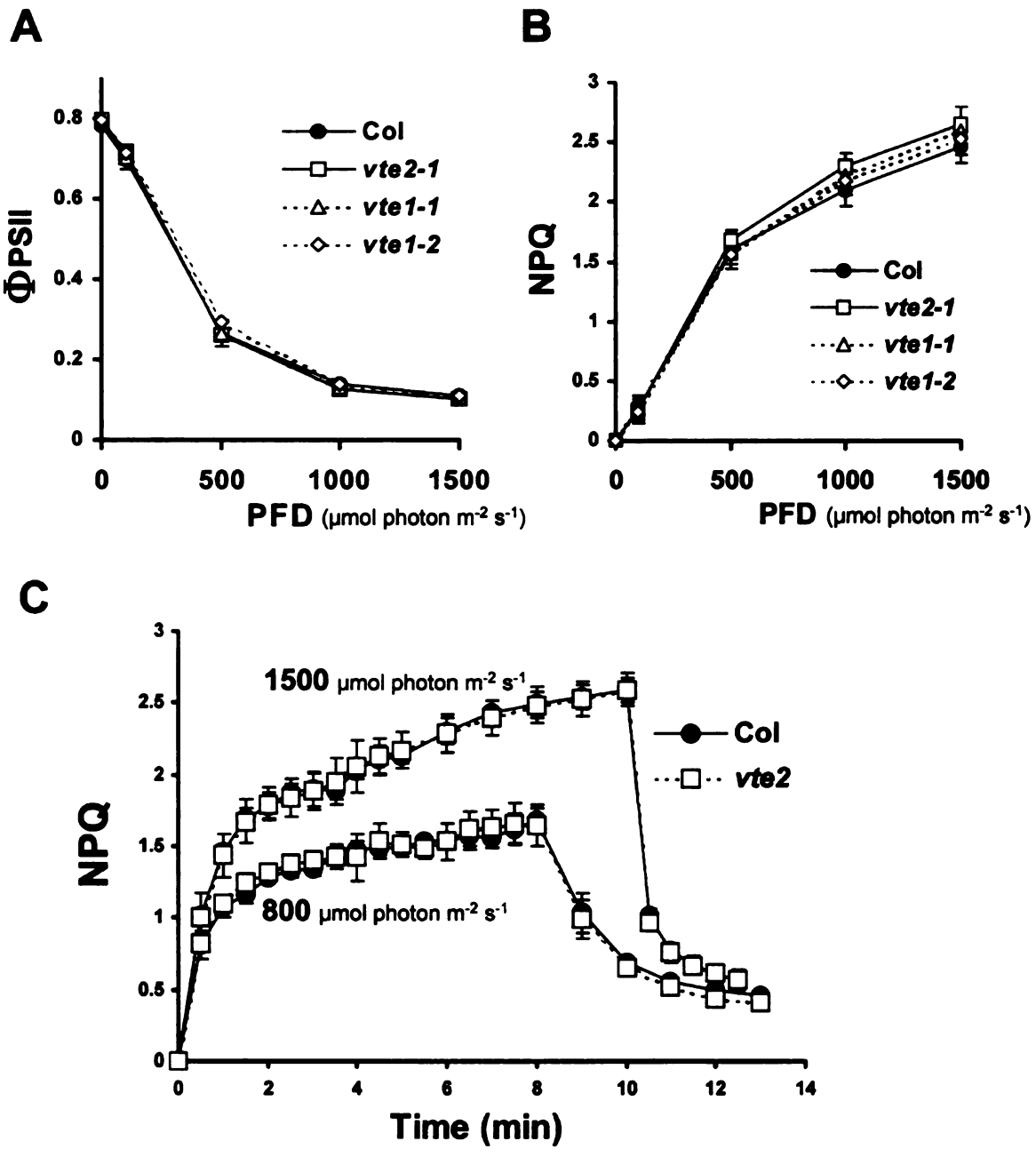

Figure A1.5. Light Dependent-Quantum Yield of PSII (ΦPSII) and Non-Photochemical Quenching (NPQ) and NPQ kinetics of Col and Tocopherol-Deficient Mutants.
Four-weeks-old plants grown under permissive growth conditions were dark-adapted at least 15 min before photosynthetic measurements. ΦPSII (A) and NPQ (B) were measured after 7 min illumination of 100, 500, 1000, and 1500 µmol photon m^{-2} s^{-1} actinic light according to (Maxwell and Johnson, 2000). (C) The Kinetics of NPQ induction was measured during the illumination of 800 and 1500 µmol photon m^{-2} s^{-1} actinic light. Data are means ± SD [n = 4 for (A) and (B), n = 5 for (C)]

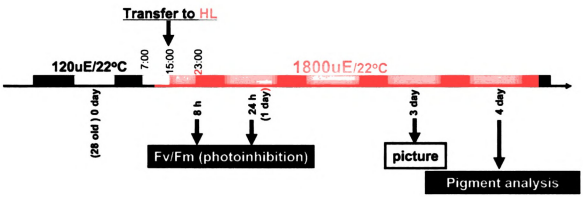

Figure A1.6. The Scheme of a Large-Scale HL Experiment Conducted in the Chapter 3. Plants were grown under permissive conditions (12h, 120 µmol photon m^{-2} s^{-1}/12h dark) for 28 days and then transferred to HL stress (16h, 1800 µmol photon m^{-2} s^{-1}/8h dark) in the middle of the light cycle. Fv/Fm was measured after 8h and 24h of HL stress. Then, visible phenotype was documented after 3days and tissues were harvested for pigment analysis after 4 days.

A-2: Map Based Cloning of Tocopherol Methyltransferase Gene (*VTE3*) in Arabidopsis.

The work presented in this section has been published:

Zigang Cheng, Scott Sattler, Hiroshi Maeda, Yumiko Sakuragi, Donald A. Bryant, and DellaPenna, D. (2003) Highly Divergent Methyltransferases Catalyze a Conserved Reaction in Tocopherol and Plastoquinone Synethesis in Cyanobacteria and Photosynthetic Eukaryotes. *Plant Cell* **15**, 2343-2356

Author's contributions:

Scott Sattler and Hiroshi Maeda carried out the genetic mapping of the *VTE3* locus in Arabidopsis. Hiroshi Maeda also performed quantitation of tocopherols from Synechocystis. Yumiko Sakuragi generated the Synechocystis *sll0418* mutant. Zigang Cheng conducted the rest of the research and wrote the manuscript. Donald A. Bryant was involved in project development. Dean DellaPenna supervised the entire project and was involved in all aspects of manuscript writing.

ABSTRACT

Tocopherols are lipid-soluble compounds synthesized only by photosynthetic eukaryotes and oxygenic cyanobacteria. The pathway and enzymes for tocopherol synthesis are homologous in cyanobacteria and plants except for 2-methyl-6-phytyl-1,4-benzoquinone/2-methyl-6-solanyl-1,4-benzoquinone methyltransferase (MPBQ/MSBQ MT), which catalyzes a key methylation step in both tocopherol and plastoquinone (PQ) synthesis. Using a combined genomic, genetic, and biochemical approach, we isolated and characterized the *VTE3* (vitamin E defective) locus, which encodes MPBQ/MSBQ MT in Arabidopsis. The phenotypes of *vte3* mutants are consistent with the disruption of MPBQ/MSBQ MT activity to varying extents. The ethyl methanesulfonate– derived *vte3-1* allele alters tocopherol composition but has little impact on PQ levels, whereas the null *vte3-2* allele is deficient in PQ and α- and γ-tocopherols. In vitro enzyme assays confirmed that VTE3 is the plant functional equivalent of the previously characterized MPBQ/MSBQ MT (Sll0418) from *Synechocystis* sp PCC6803, although the two proteins are highly divergent in primary sequence. Sll0418 orthologs are present in all fully sequenced cyanobacterial genomes, *Chlamydomonas reinhardtii*, and the diatom *Thalassiosira pseudonana* but absent from vascular and nonvascular plant databases. VTE3 orthologs are present in all vascular and nonvascular plant databases and in *C. reinhardtii* but absent from cyanobacterial genomes. Intriguingly, the only prokaryotic genomes that contain VTE3-like sequences are those of two species of archea, suggesting that, in contrast to all other enzymes of the plant tocopherol pathway, the evolutionary origin of VTE3 may have been archeal rather than cyanobacterial. In vivo analyses of *vte3* mutants and the corresponding homozygous *Synechocystis* sp PCC6803

sll0418::*aphII* mutant revealed important differences in enzyme redundancy, the regulation of tocopherol synthesis, and the integration of tocopherol and PQ biosynthesis in cyanobacteria and plants.

INTRODUCTION

Tocopherols, collectively termed vitamin E, are a class of lipid-soluble compounds that are synthesized only by oxygenic photosynthetic organisms. All tocopherols are amphipathic molecules with polar head groups exposed to the membrane surface and hydrophobic tails that interact with the acyl groups of membrane lipids. Four types of tocopherols (α-, β-, γ-, and δ-tocopherols [α-, β-, γ-, and δ-T, respectively]) are synthesized naturally and differ only in the number and position of methyl substituents on the chromanol ring (Figure A2.1). Tocopherols are essential dietary components for humans and other mammals; as a result, most of our understanding of tocopherol function has been derived from studies in these systems (for reviews, see Hanck, 1985; Brigelius-Flohe and Traber, 1999; Valk and Hornstra, 2000; Brigelius-Flohe et al., 2002; Ricciarelli et al., 2002). Studies in mammals, animal cell cultures, and artificial membranes have shown that tocopherols help maintain membrane structure and integrity (Srivastava et al., 1989), act as antioxidants and free radical scavengers (Tappel, 1962; Jialal and Fuller, 1993; Jialal et al., 2001; Behl and Moosmann, 2002), and perform other nonantioxidant functions related to signaling and transcriptional regulation (Azzi et al., 1995; Grau and Ortiz, 1998; Ricciarelli et al., 2002). The functions of tocopherols in photosynthetic organisms have yet to be determined, but they are likely to include unique functions in addition to those reported in animals (Noctor and Foyer, 1998; Grasses et al., 2001;

Reverberi et al., 2001). Mutant and transgenic approaches in Arabidopsis and *Synechocystis* sp PCC6803 that eliminate tocopherols (Collakova and DellaPenna, 2001; Schledz et al., 2001), replace tocopherols with biosynthetic intermediates (Porfirova et al., 2002; Sattler et al., 2003), or increase tocopherol levels (Collakova and DellaPenna, 2001; Savidge et al., 2002) are beginning to provide insight into tocopherol functions in photosynthetic organisms.

Tocopherols are synthesized by a pathway that is conserved between cyanobacteria and plants (Figure A2.1) (Soll et al., 1980, 1985; Lichtenthaler et al., 1981; Norris et al., 1995, 1998). The conversion of p-hydroxyphenylpyruvate (HPP) to homogentisic acid (HGA) by HPP dioxygenase (HPPD) yields the aromatic head group for both tocopherol and plastoquinone biosynthesis in plants (Norris et al., 1998). The committed step in tocopherol synthesis is the condensation of HGA and phytyldiphosphate by homogentisate phytyltransferase (HPT) to produce the first tocopherol intermediate, 2-methyl-6-phytylbenzoquinone (MPBQ) (Collakova and DellaPenna, 2001; Schledz et al., 2001; Savidge et al., 2002). Biochemical analyses have shown that the steps leading from MPBQ to α-tocopherol are as follows: (1) ring methylation of MPBQ by 2-methyl-6-phytyl-1,4-benzoquinone/2-methyl-6-solanyl-1,4-benzoquinone methyltransferase (MPBQ/MSBQ MT) to yield 2,3-dimethyl-5-phytylbenzoquinone (DMPBQ) (Soll et al., 1985); (2) ring cyclization of DMPBQ by tocopherol cyclase (TC) to yield γ-T (Soll et al., 1985; Arango and Heise, 1998; Porfirova et al., 2002; Sattler et al., 2003); and (3) a second ring methylation by γ-tocopherol methyltransferase (γ-TMT) to yield α-T (Soll et al., 1980; d'Harlingue and Camara, 1985; Shintani and DellaPenna, 1998). Alternatively, MPBQ can be cyclized to form δ-T and

then methylated by γ-TMT to yield β-T. All enzymatic activities for tocopherol synthesis in plants have been localized to the inner chloroplast envelope except HPPD, which is cytosolic (Soll et al., 1980, 1985).

In plants, the lipid-soluble, plastid-localized electron carrier plastoquinone (PQ) is synthesized by the pathway shown in Figure A2.1. As in tocopherol synthesis, the committed step in PQ synthesis is condensation of the aromatic compound HGA with a prenyldiphosphate, solanyldiphosphate, to yield 2-methyl-6-solanylbenzoquinone (MSBQ) by homogentisate solanyltransferase, an activity distinct from HPT (Norris et al., 1995; Collakova and DellaPenna, 2001). MSBQ then is methylated at the same ring position as MPBQ to yield PQ. The similarity of the MPBQ and MSBQ structures has led to the proposal that a single enzyme performs the methylation of both compounds. This has been demonstrated for MPBQ/MSBQ methyltransferase (MPBQ/MSBQ MT) from the cyanobacterium *Synechocystis* PCC6803, which, when cloned and assayed in Escherichia coli, was capable of using both MPBQ and MSBQ as substrates (Shintani et al., 2002). Whether such a multifunctional activity exists in plants is unclear.

Although the plant tocopherol and PQ biosynthetic pathways were elucidated in labeling studies during the 1980s (d'Harlingue and Camara, 1985; Marshall et al., 1985; Soll et al., 1985), the membrane association and low specific activity of most pathway enzymes have hindered their isolation and characterization. Most tocopherol pathway enzymes have been isolated only recently by combining genetic and genomic approaches in *Synechocystis* sp PCC6803 and Arabidopsis, in which the isolation of an enzyme from one organism has facilitated the isolation of the respective ortholog from the other. Sequencing of the *Synechocystis* sp PCC6803 and Arabidopsis genomes has greatly

facilitated this process, so that now, HPPD, HPT, TC, and γ-TMT have been cloned and characterized from both organisms (Norris et al., 1998; Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Shintani et al., 2002; Porfirova et al., 2002; Sattler et al., 2003). Although these studies have shown that these four pathway steps and enzymes are conserved between cyanobacteria and plants, other studies have provided evidence that portions of the tocopherol and PQ pathways are not identical in the two organisms.

One such example is the different requirement of HGA for PQ synthesis in cyanobacteria and higher plants. Although the *Synechocystis* sp PCC6803 and Arabidopsis genomes both encode HPPD enzymes, disruption of HPPD activity in the two organisms yields drastically different phenotypes. A null Arabidopsis HPPD mutant is deficient in both tocopherol and PQ and is seedling lethal (Norris et al., 1998), whereas the orthologous *Synechocystis* sp PCC6803 mutant is viable and lacks tocopherols only (Dahnhardt et al., 2002). This finding suggests that, unlike in plants, the aromatic head group for PQ synthesis in *Synechocystis* sp PCC6803 is not derived from HGA or that there is an alternative route for HGA synthesis in this organism. The different phenotypes of HPPD-deficient Arabidopsis and *Synechocystis* sp PCC6803 mutants indicate that the mere presence of functional orthologs in cyanobacteria and plants does not necessarily equal identical biosynthetic pathways.

The only tocopherol pathway enzyme that has not yet been cloned from plants is MPBQ/MSBQ MT. MPBQ/MSBQ MT activity has been demonstrated in spinach chloroplasts (Soll et al., 1985), and maize and sunflower mutants have been identified with phenotypes consistent with the disruption of MPBQ/MSBQ MT activity (Cook and Miles, 1992; Demurin et al., 1996). MPBQ/MSBQ MT has been cloned and characterized

from *Synechocystis* sp PCC6803 (Shintani et al., 2002), but despite the high degree of evolutionary conservation between plants and cyanobacteria for other tocopherol pathway enzymes, no obvious orthologs could be identified in the completed Arabidopsis and rice genomes and numerous plant EST databases. This finding suggests that, analogous to the different routes to PQ head group synthesis in *Synechocystis* sp PCC6803 and Arabidopsis (Norris et al., 1998; Dahnhardt et al., 2002), MPBQ/MSBQ MT also may differ between cyanobacteria and plants. Here, we report the identification and characterization of a novel MPBQ/MSBQ MT from Arabidopsis that has orthologs in all plant, but not cyanobacterial, databases. The low sequence identity between the cyanobacterial and plant MPBQ/MSBQ MTs suggests that they are nonorthologous, functionally equivalent enzymes that arose independently during the evolution of plants and cyanobacteria.

RESULTS

Search for an Arabidopsis Homolog of Synechocystis sp PCC6803 MPBQ/MSBQ MT Using Genome-Based Approaches

To better understand tocopherol synthesis in plants, we attempted to identify an Arabidopsis ortholog of *Synechocystis* sp PCC6803 MPBQ/MSBQ MT, which had been identified previously as open reading frame (ORF) *sll0418* (Shintani et al., 2002). Sll0418 orthologs were identified using tBLASTn (Basic Local Alignment Search Tool; $P < 10^{-81}$) in the fully sequenced cyanobacterial genomes of *Anabaena* sp PCC7120, *Thermosynechococcus el*ongatus, *Prochlorococcus marinus* MIT9313 and MED4, and *Synechococcus* sp WH8102, in the partially sequenced cyanobacterial genomes of

Synechococcus sp PCC7002 and *Trichodesmium erythraem* IMS101, in the genome and EST databases of the green alga *Chlamydomonas reinhardtii*, and in the raw genome sequence data of the diatom *Thalassiosira pseudonana* (Figure A2.2). However, exhaustive searches of public DNA databases for vascular and nonvascular plants, including the complete Arabidopsis and rice genomes, failed to identify a convincing ortholog of *Synechocystis* sp PCC6803 *sll0418*. The two proteins in the Arabidopsis genome with the greatest similarity to Sll0418 are γ-TMT ($P < 10^{-37}$), which is not active toward MPBQ when assayed in vitro (data not shown), and SMT1 ($P < 10^{-18}$), a sterol methyltransferase that is not targeted to the chloroplast (Diener et al., 2000).

In a parallel approach, 93 predicted proteins in the Arabidopsis genome were identified that contain motifs characteristic of conserved S-adenosylmethionine binding domains in methyltransferases (Kagan and Clarke, 1994). Eleven of these proteins, one of which was γ-TMT, also were predicted to be targeted to the chloroplast, the known subcellular location of plant MPBQ/MSBQ MT activity (Soll et al., 1985). The protein sequences of these putative chloroplast-targeted methyltransferases were aligned with methyltransferases of known functions, including previously characterized methyltransferases involved in tocopherol synthesis (Figure A2.2). The resulting phylogenetic tree indicated that none of the Arabidopsis sequences clustered with the *Synechocystis* sp PCC6803 MPBQ/MSBQ MT clade (Figure A2.2). Given that plant chloroplasts are known to contain MPBQ/MSBQ MT activity (Soll et al., 1985), these combined data suggest that, unlike the other enzymes of the tocopherol pathway, Arabidopsis and *Synechocystis* sp PCC6803 MPBQ/MSBQ MTs share little identity at the level of the primary amino acid sequence.

Identification of Arabidopsis MPBQ/MSBQ MT Mutants and Cloning of the VTE3 Locus

A genetic approach was used concurrently with genomic approaches to identify the MPBQ/MSBQ MT locus in Arabidopsis. A rapid HPLC-based screen of Arabidopsis leaf tissue was developed to identify mutants with altered leaf tocopherol profiles (Sattler et al., 2003). The screening of an ethyl methanesulfonate–mutagenized Arabidopsis population (ecotype Columbia) yielded one mutant line that, compared with the wild type, had reduced levels of α-T and γ-T and greatly increased levels of β-T and δ-T (Table A2.1). This is a biochemical phenotype consistent with a reduction in MPBQ/MSBQ MT activity (Figure A2.1). The mutant was designated *vte3-1* (vitamin E defective).

A map-based cloning approach was used to isolate the *VTE3* locus. *vte3-1* was crossed to wild-type Landsberg erecta, and an F2 population was screened by HPLC for the *vte3-1* phenotype. Seventy-two of 276 F_2 plants were identified as *vte3-1* homozygotes, indicating that *vte3-1* is recessive ($\chi^2 = 0.168$). *vte3-1* was mapped to an ~2-Mb interval at the bottom of chromosome III using simple sequence length polymorphism markers (Figure A2.3A). Two simple sequence length polymorphism markers on BACs T15C9 and T17J13 identified 13 and 2 recombination events for *vte3-1*, respectively, indicating that the *VTE3* locus was located to the right of BAC T17J13 (Figure A2.3A). In the 398-kb interval from T17J13 to the telomere, there are two predicted methyltransferases, At3g63250 and At3g63410. At3g63250 encodes a previously cloned and characterized homocysteine S-methyltransferase (Ranocha et al., 2000). At3g63410 encodes a 338–amino acid (37.9 kD) protein of unknown function that

contains conserved S-adenosylmethionine binding motifs and a predicted 58–amino acid chloroplast transit peptide.

To determine whether the At3g63410 locus encodes VTE3, the corresponding wild-type and *vte3-1* genes were amplified and sequenced. At3g63410 in *vte3-1* contains a C-to-T conversion at nucleotide 281 of the ORF that results in the mutation of Thr-94 to Ile-94 (Figure A2.3B). A second mutant allele, *vte3-2*, was identified from the Salk T-DNA insertion population. *vte3-2* contains a T-DNA insertion in the first exon of At3g63410 at nucleotide 163 of the ORF (Figure A2.3B) and is predicted to result in a complete loss of enzyme activity.

Whole-Plant Phenotypes of vte3-1 and vte3-2

Young *vte3-1* seedlings were slightly smaller than wild-type seedlings (Figure A2.3C) but otherwise were healthy and indistinguishable from the wild type. By contrast, *vte3-2* seedlings were pale green and did not survive beyond 7 days in soil. Therefore, the *vte3-2* allele was maintained as a heterozygote. Progeny from *VTE3-2/vte3-2* germinated fully and segregated at a 3:1 ratio (wild type:mutant; $\chi^2 = 0.172$). Seeds produced from *vte3-1/vte3-1* and *VTE3-2/vte3-2* were indistinguishable from wild-type seeds in size, shape, and germination rates (data not shown), suggesting that the partial or total loss of VTE3 activity does not affect embryo growth and development.

vte3-2 seedlings grown on sterile medium supplemented with 1% sucrose were pale but, unlike soil-grown plants, survived for several weeks and produced sufficient tissue for biochemical analyses. The phenotype of plate-grown *vte3-2* seedlings ranged from albino to those that had pale green newly emerging leaves with older leaves that

were progressively bleached (Figure A2.3C). These observations suggest that, unlike the missense *vte3-1* point mutation, a null mutation of the At3g63410 locus inhibits photosystem assembly/function and causes severe photobleaching.

Analysis of Tocopherols in Leaves and Seeds of vte3 Mutants

Homozygous *vte3-2* mutants are soil lethal. To directly compare the effects of *vte3* mutations on tocopherol synthesis, wild-type and mutant plants were grown on media supplemented with 1% sucrose. The tocopherol profile of wild-type leaves consists of α-T, γ-T, and β-T in an ~90:8:2 ratio (Table A2.1). In *vte3-1* leaves, total tocopherol levels were increased slightly but significantly relative to the wild type, and α-T and γ-T levels were decreased to 33 and 3%, whereas β-T and δ-T were increased to 42 and 22% of total tocopherols, respectively (Table A2.1). These results indicate that the *vte3-1* mutation leads to a >60% reduction in the methylation of MPBQ, which instead is cyclized to δ-T, the majority of which then is methylated further by γ-TMT to yield β-T (Figure A2.1).

Leaf tissue from homozygous *vte3-2* mutants accumulated β-T and δ-T only (Table A2.1), a phenotype consistent with a complete lack of MPBQ/MSBQ MT activity. The absence of α-T and γ-T in *vte3-2* indicates that no redundant MPBQ/MSBQ MT activities exist in Arabidopsis and that the ethyl methanesulfonate–derived *vte3-1* allele is leaky. The total tocopherol level in pale/bleached *vte3-2* leaf tissue was reduced by >50% relative to the wild type, most likely as a result of the reduced flux through the tocopherol pathway or accelerated tocopherol degradation as a result of the severe photobleaching in *vte3-2*. Unlike *vte3-1*, *vte3-2* was found to be semidominant, because leaves of soil-

grown *VTE3-2/vte3-2* plants accumulated significantly increased levels of β-T compared with wild-type leaves (Table A2.1).

In addition to leaves, seeds also accumulate tocopherols. The total tocopherol level of wild-type Arabidopsis seeds was >20-fold higher than that of unstressed soil-grown leaves on a fresh weight basis and was composed of 92% γ-T, 3% α-T, and 5% δ-T (Table A2.2). Seeds of *vte3-1* mutants showed a 15-fold increase in δ-T content, whereas γ-T levels decreased by 31% relative to the wild type (Table A2.2). These data indicate that, as in leaf tissue, the majority of MPBQ in *vte3-1* seeds is not methylated by MPBQ/MSBQ MT but instead undergoes cyclization to produce δ-T. β-T was not detected in the mutant because of the low γ-TMT activity in seeds (Shintani and DellaPenna, 1998).

Because homozygous *vte3-2* mutants are seedling lethal in soil, the effects of the mutation on seed tocopherol synthesis could be determined only in seeds from *VTE3-2/vte3-2* plants. Although only 25% of the seeds obtained from *VTE3-2/vte3-2* plants were homozygous for the *vte3-2* locus, there was an increase in δ-T similar to that seen in homozygous *vte3-1* seeds (Table A2.2). Because *vte3-2* is semidominant in leaf tissue (Table A2.1), we could not determine whether the δ-T produced is only from *vte3-2/vte3-2* seeds or whether *VTE3-2/vte3-2* seeds also contribute to the phenotype. It also is important that not only was seed tocopherol composition altered by the *vte3-1* and *vte3-2* mutations but the levels of total tocopherols in both mutant alleles were increased significantly ($P \leq 0.01$), 38 and 30%, respectively, relative to those in the wild type (Table A2.2). This finding suggests that changes in tocopherol composition caused by

altered MPBQ/MSBQ MT activity affect tocopherol levels either as a result of increased flux through the pathway or as decreased turnover of tocopherols in seeds.

PQ Synthesis Also Is Disrupted in Homozygous vte3-2 Plants

The dramatic difference in the whole-plant phenotypes of *vte3-1* and *vte3-2* (Figure A2.3C), coupled with the fact that plant tocopherol and PQ synthesis are related biochemically (Figure A2.1), prompted us to determine whether PQ synthesis is affected differentially in these mutants. *vte3-1* leaf tissue showed a small (17%) but significant decrease in PQ relative to the wild type (Table A2.1) with a trace amount of MSBQ, the immediate biosynthetic precursor of PQ and substrate for MPBQ/MSBQ MT (Figure A2.4). Leaves of *vte3-2* plants completely lacked PQ and accumulated high levels MSBQ (Table A2.1, Figure A2.4). The small peak at 12.10 min in the *vte3-2* HPLC trace has a spectrum unlike that of any PQ pathway intermediates and is an unrelated compound of low abundance that migrates as a small shoulder of the large PQ peak in the wild type (Figure A2.4, inset).

The combined data from *vte3-1* and *vte3-2* provide genetic evidence that VTE3 is involved in both tocopherol and PQ synthesis in vivo. The severe photobleaching phenotype of *vte3-2* is caused by the PQ deficiency and/or accumulation of MSBQ, rather than by altered tocopherol composition. This finding is in agreement with studies of tocopherol cyclase mutants of Arabidopsis that lack tocopherols and accumulate the biosynthetic intermediate DMPBQ but are phenotypically similar to wild-type plants (Porfirova et al., 2002; Sattler et al., 2003). Finally, the absence of PQ in *vte3-2* indicates that, as with tocopherol synthesis, Arabidopsis does not contain other functionally

redundant activities that catalyze the methylation of MSBQ to form PQ. *VTE3-2/vte3-2* plants accumulated PQ at a level similar to wild-type plants (Table A2.1) with no detectable MSBQ (data not shown), indicating that, unlike the *vte3-2* tocopherol phenotype, *vte3-2* is recessive for the PQ phenotype. While this article was under review, a Ds-tagged *VTE3* allele was reported from a large-scale screen for apg (albino or pale green) mutants in Arabidopsis (Motohashi et al., 2003). Although tocopherols were not analyzed in that study, the Ds-tagged *VTE3* allele also was found to be deficient in PQ (Motohashi et al., 2003).

VTE3 Uses Both MPBQ and MSBQ as Substrates in Vitro

Genetic evidence strongly suggests that VTE3 encodes both MPBQ MT and MSBQ MT activities. To determine the activity of VTE3 against various potential substrates, the full-length *VTE3* protein-coding region was amplified from an Arabidopsis EST clone and engineered for expression in E. coli. Activity assays against various substrates demonstrated that, like *Synechocystis* sp PCC6803 MPBQ/MSBQ MT, VTE3 uses both MPBQ and MSBQ, intermediates in tocopherol and PQ synthesis, respectively, as methylation substrates in vitro (Figure A2.5). Neither *Synechocystis* sp PCC6803 MPBQ/MSBQ MT (Shintani et al., 2002) nor VTE3 (data not shown) used β-T or δ-T as a methylation substrate in vitro. These data demonstrate conclusively that Arabidopsis VTE3 is the functional equivalent of *Synechocystis* sp PCC6803 MPBQ/MSBQ MT.

Analysis of MPBQ/MSBQ MT Activity in Synechocystis sp PCC6803

The *sll0418* ORF in *Synechocystis* sp PCC6803 was shown previously by gene disruption and in vitro enzyme assays to encode a protein with MPBQ/MSBQ MT activity (Shintani et al., 2002). Complete segregation of the mutant locus could not be achieved under mixotrophic growth conditions (glucose-containing medium in the light), suggesting that the total loss of Sll0418 activity was lethal. The merodiploid *sll0418/sll0418::aphII* strain showed a reduced α-T level and a small amount of β-T accumulation, which confirmed a role for Sll0418 in tocopherol synthesis in *Synechocystis* sp PCC6803 in vivo. We have now isolated the homozygous *sll0418::aphII* mutant by selection under photoautotrophic conditions. Apparently, the inability to isolate a homozygous mutant under mixotrophic conditions is the result of a glucose-dependent lethality associated with the disruption of sll0418. A complete description of the homozygous *Synechocystis* sp PCC6803 *sll0418::aphII* mutant and the accompanying phenotype of tocopherol-deficient mutants will be published elsewhere (Y. Sakuragi, H. Maeda, D. DellaPenna, and D.A. Bryant, unpublished data).

The tocopherol content of the homozygous *sll0418::aphII* mutant did not differ significantly from that of the previously described merodiploid strain (Shintani et al., 2002). The homozygous *sll0418::aphII* strain contains 35% of the wild-type tocopherol level and consists primarily of α-T with a small amount of β-T (Table A2.1). The PQ content of the homozygous *sll0418::aphII* strain also is reduced to 70% of wild-type levels, and MSBQ is not detectable (data not shown). These observations are highly significant because they demonstrate the presence of one or more partially redundant MPBQ/MSBQ MT activities in *Synechocystis* sp PCC6803 or the presence of an

alternative biosynthetic route(s) to α-T and PQ. The homozygous *sll0418::aphII* mutant phenotype is in sharp contrast to the corresponding homozygous Arabidopsis *vte3-2* mutant, in which both α-T and PQ synthesis are fully disrupted.

VTE3 and Synechocystis sp PCC6803 MPBQ/MSBQ MT Are Highly Divergent in Primary Sequence

Although VTE3 and *Synechocystis* sp PCC6803 MPBQ/MSBQ MT have conserved enzymatic activities (Figure A2.5), the two proteins are highly divergent in their primary amino acid sequences, with an overall identity of 18% (Table A2.3). The primary regions of significant similarity are in the S-adenosylmethionine binding motifs I, II, and III, domains involved in the binding of the methyl donor (Figure A2.6) (Kagan and Clarke, 1994; Joshi and Chiang, 1998). In addition to a low overall similarity, *Synechocystis* sp PCC6803 MPBQ/MSBQ MT and VTE3 have numerous gaps in their alignment (Figure A2.6). Thus, the VTE3- and *Synechocystis* sp PCC6803–type MPBQ/MSBQ MTs appear to represent nonorthologous, functionally equivalent activities that have evolved independently in the two organisms.

To gain insight into the prevalence of *Synechocystis* sp PCC6803– and VTE3-type MPBQ/MSBQ MTs in nature and their possible evolutionary origins, we expanded our database searches beyond vascular plants and oxygenic cyanobacteria. Orthologs of *Synechocystis* sp PCC6803 MPBQ/MSBQ MT are present in all oxygenic cyanobacterial genome databases but absent from databases representing nonoxygenic phototrophs, nonphotosynthetic eukaryotes, eubacteria, and most photosynthetic eukaryotes, with the exception of *C. reinhardtii* and *T. pseudonana*. The *C. reinhardtii* and *T. pseudonana*

proteins share 45 and 48% identity, respectively, with *Synechocystis* sp PCC6803 Sll0418 (Table A2.3, Figure A2.6). The full-length C. reinhardtii MPBQ/MSBQ MT coding region was amplified from a cDNA library, sequenced, and engineered for the expression and assay of activity in E. coli. As with VTE3 and *Synechocystis* sp PCC6803 MPBQ/MSBQ MT, the C. reinhardtii cyanobacteria-type enzyme was found to use both MPBQ and MSBQ (Figure A2.5) as substrates in vitro but not β-T or δ-T (results not shown). The T. pseudonana protein could not be assayed, because cDNA libraries are not readily available for this organism.

Arabidopsis VTE3 appears to represent a highly conserved gene in vascular plants. The sequences shown in Table A2.3 and Figure A2.6 are representative of the numerous orthologs found in vascular plant databases. Furthermore, VTE3 orthologs also were identified in EST databases of two nonvascular plants, *Physcomitrella patens* and *Marchantia polymorpha*, and in the genome and EST databases of the green alga C. reinhardtii, making the latter the only organism that encodes both types of MPBQ/MSBQ MTs. C. reinhardtii VTE3 is 70% identical to Arabidopsis VTE3 (Table A2.3). Attempts to engineer C. reinhardtii VTE3 for expression and assay of activity in E. coli have been unsuccessful to date. Expanding the search for VTE3 homology beyond the plant kingdom failed to identify VTE3 orthologs in mammalian, fungal, cyanobacterial, or other eubacterial databases. However, apparent orthologs to Arabidopsis VTE3 were identified in two archeal species, *Archaeoglobus fulgidus* and *Halobacterium* sp NRC-1 (Table A2.3). These two archeal "VTE3-like" proteins are ~40% identical to VTE3 proteins from throughout the plant kingdom (Table A2.3, Figure A2.6). However,

although this similarity extends throughout the predicted archeal protein sequences, both archeal VTE3-like proteins lack an ~60–amino acid C-terminal extension that is highly conserved in plants and corresponds to the third exon of the Arabidopsis and rice *VTE3* genes (Figure A2.6). BLAST searches using the protein sequence encoded by the third exon of Arabidopsis VTE3 as a query indicated that this protein domain is unique to eukaryotic VTE3 sequences.

DISCUSSION

In this study, we have reported the identification and characterization of the Arabidopsis *VTE3* locus, which encodes the nonorthologous functional equivalent of *Synechocystis* sp PCC6803 MPBQ/MSBQ MT, a key enzymatic activity in the synthesis of tocopherols and PQ in photosynthetic organisms. Both the Arabidopsis (VTE3-type) and *Synechocystis* sp PCC6803 (cyanobacterial-type) MPBQ/MSBQ MT enzymes have similar in vitro activities toward the tocopherol and PQ biosynthetic intermediates MPBQ and MSBQ, respectively. Despite the striking similarity in their activities, the corresponding proteins in the two organisms are highly divergent in primary sequence and appear to have arisen independently during the evolution of cyanobacteria and plants. Thus, unlike all other tocopherol pathway enzymes in plants, it was not possible to identify Arabidopsis MPBQ/MSBQ MT based on similarity with its cyanobacterial counterpart; instead, a genetic approach was required.

Two Arabidopsis mutants were identified that had phenotypes consistent with a partial or total disruption of MPBQ/MSBQ MT activity. The ethyl methanesulfonate–derived *vte3-1* allele was used to isolate the corresponding *VTE3* locus by positional

cloning. The recessive *vte3-1* mutation substitutes Ile-94 for Thr-94, a residue conserved in all vascular and nonvascular plant VTE3 orthologs (Figure A2.6 and data not shown). *vte3-1* preferentially impairs the methylation of tocopherol substrates in planta and has little effect on the methylation of MSBQ to PQ. Despite the 58% reduction in α-T in *vte3-1* leaves, no significant impact on plant growth and development was observed. *vte3-2* is a null, T-DNA insertion allele that, when homozygous, completely disrupts both MPBQ and MSBQ MT activity in vivo; this results in the absence of α-T in leaves and γ-T in seeds and the accumulation of high levels of β-T and δ-T in each tissue, respectively. Unlike *vte3-1*, *vte3-2* seedlings also lack PQ, accumulate the immediate precursor MSBQ, and are seedling lethal in soil as a result of severe photobleaching. Interestingly, in leaf tissue, *vte3-2* is semidominant for the tocopherol phenotype but recessive for the PQ phenotype.

The Arabidopsis *vte3* mutants do not appear to be the first such mutations identified in plants. Maize and sunflower mutants have been reported previously that, in retrospect, are consistent with the disruption of VTE3 activity in these organisms. Two allelic Mutator transposon-derived, high-chlorophyll-fluorescence maize mutants have been reported that were pale green and seedling lethal (Cook and Miles, 1992). These mutants lacked PQ and α-T in leaves, accumulated what was presumed to be biosynthetic precursors, and had a phenotype strikingly similar to Arabidopsis *vte3-2*. In sunflower, which predominantly accumulates α-T in its seeds, an apparently viable mutant (*tph1*) was identified that accumulated β-T to as much as 40% of total seed tocopherols (Demurin et al., 1996). *tph1* likely contains a mutation in the sunflower VTE3 ortholog

that preferentially affects tocopherol methylation in a manner similar to the Arabidopsis *vte3-1* allele.

Orthologs with high identity to Arabidopsis VTE3 are present in all plant genome and EST databases. Most notably, the spinach VTE3 ortholog had been cloned previously and characterized as an abundant 37-kD chloroplast inner envelope protein (named E37) of unknown function that contained three S-adenosylmethionine binding motifs (Teyssier et al., 1996). A substrate for E37 could not be determined, but the protein was immunologically detectable in both photosynthetic and nonphotosynthetic spinach tissues and in a number of other plant species (Teyssier et al., 1996). Given the facts that the inner envelope is the subcellular site of tocopherol biosynthesis in plants (Soll et al., 1980, 1985) and that spinach E37 is 84% identical to Arabidopsis VTE3 (Table A2.3), it now seems clear that spinach E37 encodes MPBQ/MSBQ MT. The widespread presence of VTE3 orthologs in plant databases and the isolation of maize and sunflower mutants with phenotypes similar to specific Arabidopsis vte3 alleles suggest that this reaction in tocopherol and PQ synthesis is catalyzed by an enzyme that is highly conserved in both structure and function in dicots and monocots.

New Insights into the Synthesis and Regulation of Tocopherols and PQ

Previous studies have demonstrated that tocopherol biosynthetic enzymes are remarkably conserved in both primary sequence and activity in cyanobacteria and plants (Norris et al., 1998; Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Porfirova et al., 2002; Shintani et al., 2002; Sattler et al., 2003). In this regard, the low sequence identity of MPBQ/MSBQ MT in Arabidopsis and *Synechocystis* sp PCC6803 stands in stark

contrast to other pathway enzymes and suggests that although similar, fundamental differences exist in tocopherol and PQ synthesis in cyanobacteria and plants. Several lines of evidence support this hypothesis. The null *vte3-2* mutant lacks α-T and PQ and is seedling lethal, indicating that VTE3 is essential for the synthesis of both compounds in Arabidopsis. By contrast, the analogous *Synechocystis* sp PCC6803 *sll0418::aphII* mutant is viable and has only reduced levels of tocopherols and PQ (Table A2.1). This finding indicates that, unlike Arabidopsis, functional redundancy exists for MPBQ/MSBQ MT in *Synechocystis* sp PCC6803 and that this step in tocopherol and PQ synthesis has diverged significantly in cyanobacteria and plants.

Additional divergence of the two pathways is evident from the phenotypes of HPPD mutants in cyanobacteria and plants. As with VTE3, a null mutation of Arabidopsis HPPD disrupts both tocopherol and PQ synthesis and is seedling lethal (Norris et al., 1998), indicating that the synthesis of both compounds is HGA dependent in plants (Figure A2.1). By contrast, a null HPPD mutation in *Synechocystis* sp PCC6803 results in viable cells that are tocopherol deficient but contain wild-type levels of PQ, indicating that PQ synthesis is HGA independent in *Synechocystis* sp PCC6803 (Dahnhardt et al., 2002). These data demonstrate that although the PQ produced in plants and cyanobacteria is chemically identical, the PQ biosynthetic pathways differ in the two organisms, at least at the steps of aromatic head group synthesis and ring methylation. Therefore, the PQ pathway shown in Figure A2.1 is valid only for photosynthetic eukaryotes, and PQ biosynthesis in cyanobacteria must be reassessed.

Enzymes of the tocopherol pathway often are grouped into two categories: those that contribute to tocopherol flux/accumulation (e.g., HPPD and HPT) and those that

define the tocopherol composition of a tissue (e.g., γ-TMT and TC). Consistent with this classification scheme, overexpression of HPPD (Tsegaye et al., 2002) or HPT (Savidge et al., 2002; Collakova and DellaPenna, 2003) increased total seed tocopherol levels by up to 30 and 75%, respectively, whereas γ-TMT overexpression resulted in the conversion of the large pool of γ-T in seeds to α-T without affecting total seed tocopherol levels (Shintani and DellaPenna, 1998). From these previous studies, one would predict that altering VTE3 activity would affect tocopherol composition without positively affecting total tocopherol flux/accumulation. Therefore, it was quite unexpected to observe, in addition to altered tocopherol composition, a highly significant ($P \leq 0.01$) 31 to 38% increase in total tocopherol levels in *vte3* mutant seeds (Table A2.2). This phenotype suggests the presence of a previously unknown mechanism regulating tocopherol flux or accumulation in wild-type seeds that is disrupted by the vte3 mutations.

The nature of this regulatory mechanism is unclear and theoretically could occur at any time between gene expression and the modulation of enzyme activities in the pathway, but it likely involves the generation or removal of an effector by the vte3 mutations. Because MPBQ and DMPBQ are undetectable in both the wild type and vte3 mutants, these compounds can be excluded as possible effectors. Likewise, γ-T and α-T also can be excluded because altering the seed γ-T: α-T ratio by 80-fold as a result of γ-TMT overexpression did not affect total seed tocopherol levels (Shintani and DellaPenna, 1998). The most likely candidate effector molecule is δ-T, which is undetectable in wild-type seeds and increased in vte3 seeds. Whether δ-T mediates regulation by affecting the kinetic properties of other pathway enzymes or serves as a signal that results in the altered expression of pathway genes remains to be determined. What is clear from the

phenotype of seeds containing *vte3* mutations is that the tocopherol composition of a tissue plays a previously unsuspected role in regulating tocopherol flux and/or accumulation.

The Evolution of VTE3- and Cyanobacteria-Type MPBQ/MSBQ MTs

The low sequence identity of cyanobacteria- and VTE3-type MPBQ/MSBQ MTs suggests that the two classes of enzymes are the result of convergent evolution. The presence of VTE3 orthologs in all angiosperms, in the nonvascular plants P. patens and M. polymorpha, and in the green alga C. reinhardtii suggests that photosynthetic eukaryotes acquired the VTE3-type enzyme before the divergence of green algae and plants some 800 million years ago (O'Kelly, 1992; Lemieux et al., 2000). The absence of VTE3 orthologs from eubacteria (including cyanobacteria) and the presence of VTE3-like orthologs in two archeal species suggest that VTE3 was present in a common ancestor of archea and plants but subsequently was lost from most archeal lineages. Cyanobacteria-type MPBQ/MSBQ MT orthologs are present in all cyanobacterial genomes and in the genomes of two unicellular photosynthetic eukaryotes, *C. reinhardtii* and the diatom *T. pseudonana*. *C. reinhardtii* is unique in being the only organism currently known to encode both cyanobacteria- and VTE3-type MPBQ/MSBQ MTs, suggesting that the common ancestor of green algae and plants had both types of enzymes and that plants subsequently lost the cyanobacteria-type enzyme early in their evolution. *T. pseudonana* also is unique in being the only photosynthetic eukaryote known that does not encode a VTE3-type enzyme in its genome. Whether the common ancestor of plants, green algae, and diatoms contained both types of enzymes and VTE3 was lost in the

diatom lineage will require the sequencing of additional genomes representing early branches of photosynthetic eukaryote evolution.

In summary, we have isolated and characterized an Arabidopsis MPBQ/MSBQ MT that is the nonorthologous functional equivalent of its cyanobacterial counterpart. The Arabidopsis *VTE3* gene encodes a protein and activity in tocopherol and PQ biosynthesis that are highly conserved in photosynthetic eukaryotes. An intriguing question remains regarding when and how this plant enzyme evolved from archea, the third domain of life, whereas all other steps of the plant tocopherol biosynthetic pathway appear to have originated from an endosymbiotic event with a cyanobacterium. Understanding the origin of VTE3 during the evolution of plants will provide new insight into the transfer of genetic information among the three domains of life during the emergence and evolution of the fundamental biological process of photosynthesis.

METHODS

Plant Materials and Growth Conditions

Ethyl methanesulfonate–mutagenized *Arabidopsis thaliana* seeds were obtained from Lehle Seeds (Round Rock, TX). The *vte3-2* T-DNA insertional line was identified by searching the SIGNAL database World Wide Web site (http://signal.salk.edu/cgi-bin/tdnaexpress) at the Salk Institute for Biological Studies (La Jolla, CA). Seeds containing the *vte3-2* mutation were obtained from the ABRC at Ohio State University (http://www.biosci.ohio-state.edu/plantbio/Facilities/abrc/abrchome.htm). Soil-grown plants were kept at 22°C under a 16-h photoperiod. Tissue culture–grown seedlings were

grown at 22/19°C (day/night) with a 12-h photoperiod on 1x Murashige and Skoog (1962) salts (Gibco BRL), pH 5.7, with 1% (w/v) sucrose and 1% (w/v) phytagar.

Identification and Map-Based Cloning of vte3-1

Ethyl methanesulfonate–mutagenized M2 seeds were grown in soil in a 96-well format for 3 to 4 weeks. Total lipids were extracted from leaf tissues according to Collakova and DellaPenna (2001) and subjected to HPLC (1100 series; Agilent, Wilmington, DE) on a Spherisorb ODS-2 5-μm, 250- x 4.6-mm reverse-phase column (Column Engineering, Ontario, CA) using the solvent system described by Sattler et al. (2003). The original *vte3-1* mutant line was backcrossed to wild-type Columbia two times. For mapping purposes, *vte3-1* (in the Columbia background) was crossed to wild-type Landsberg erecta, and the resulting F2 population was screened using reverse-phase HPLC for the *vte3-1* phenotype. Simple sequence length polymorphism markers for BAC clones T15C9 and T17J13 were described by Bell and Ecker (1994). DNA was isolated using Plant DNAZOL (Invitrogen, Carlsbad, CA) and used in a 20-μL PCR.

Analysis of Prenylquinones

Quantitative analyses of tocopherols and plastoquinone (PQ) were performed using normal-phase HPLC. Total lipids were extracted from 65 to 75 mg of tissue culture–grown plants or from 20 mg of dry seeds according to Collakova and DellaPenna (2001). Total lipids were separated on a ReliaSil silica 250- x 4.6-mm normal-phase column (Column Engineering) at 30°C with 1 mL/min hexane and dioxane using the following gradient program: 0 to 15 min, 0.2 to 2% dioxane; 15 to 25 min, 2 to 4% dioxane; 25 to

35 min, dioxane held at 4%; 35 to 40 min, 4 to 0.2% dioxane; and 40 to 60 min, dioxane held at 0.2%. Absorption spectra (190 to 800 nm) and fluorescence signals (excitation at 290 nm, emission at 330 nm) were collected. PQ and tocopherol standard curves were prepared using the same method.

Expression and Assay of 2-Methyl-6-Phytyl-1,4-Benzoquinone/ 2-Methyl-6-Solanyl-1,4-Benzoquinone Methyltransferase in Escherichia coli

Synechocystis sp PCC6803 2-methyl-6-phytyl-1,4-benzoquinone/2-methyl-6-solanyl-1,4-benzoquinone methyltransferase (MPBQ/MSBQ MT) was engineered and expressed in E. coli as described by Shintani et al. (2002). Arabidopsis *VTE3* was amplified by PCR from an Arabidopsis leaf cDNA library using Pwo DNA polymerase (Roche Applied Science, Indianapolis, IN), a forward primer engineered with an NdeI site (underlined) to generate an in-frame ATG (5'-CGGCATATGGCCTCTTTGATGCTCAAC-3'), and a reverse primer (5'-CGGTCAGATGGGTTGGTCTTTGGG-3'). Chlamydomonas reinhardtii MPBQ/MSBQ MT (*Synechocystis* type) was amplified from a C. reinhardtii cDNA library (Davies et al., 1996) using Pwo DNA polymerase, a forward primer engineered with an NdeI site (underlined) to generate an in-frame ATG (5'-CATATGCTTGGGCAATCCCTGC-3'), and a reverse primer (5'-GCACCCGCTCCTTACTTCA-3'). The amplified fragments were ligated to the EcoRV site of pBluescript KS(+) (Stratagene). Inserts were excised with NdeI and BamHI, inserted into the pET30A expression vector digested previously with the same enzymes (Novagen, Madison, WI), and transformed into BL21DE3 (Novagen).

Growth, induction, cell harvesting, and extraction of engineered proteins in E. coli were performed as described by Shintani et al. (2002). Cells carrying an empty pET30A vector also were induced as a negative control. To determine the optimal conditions for the solubilization and activity of each enzyme, each induced protein was solubilized in 50 mM Tris-Cl, pH 8.0, 5 mM DTT, and 1 mM phenylmethylsulfonyl fluoride containing 1% (v/v) of one of the following detergents: Triton X-100, Tween 20, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), and β-D-dodecyl maltoside. After centrifugation at 12,000 rpm, the supernatant was collected, the protein concentration was measured with the Coomassie Protein Assay Reagent (Pierce, Rockford, IL), and aliquots were stored at -80°C until assay.

Enzyme assays were performed as described by Peddibhotla et al. (2002), except that the incubation time for Arabidopsis VTE3 was extended to 6 to 12 h. Enzymes were assayed at detergent concentrations ranging from 0.01 to 0.1% (v/v) final concentration, and the optimal type and concentration of detergent for each solubilized protein were determined by comparing the yield of the radiolabeled products. The optimal detergents and final concentrations for the assays of *Synechocystis* sp PCC6803 MPBQ/MSBQ MT, its ortholog from C. reinhardtii, and Arabidopsis VTE3 were 0.1% (v/v) Tween 20, 0.1% (v/v) β-D-dodecyl maltoside, and 0.1% (v/v) CHAPS, respectively. Chemical synthesis and purification of 2-methyl-6-phytylbenzoquinone substrate were as reported by Peddibhotla et al. (2002). 2-Methyl-6-solanylbenzoquinone substrate was extracted and purified from iris bulbs as described by Henry et al. (1987).

Phylogenetic Analysis

Sequence alignments were performed with CLUSTAL W (MacVector; Genetics Computer Group, Madison, WI) using the BLOSUM 30 protein matrix (for Figure A2.2), the identity protein matrix (for Figure A2.6), and default parameters for gap penalties. The phylogenetic tree was generated using the neighbor-joining method. Ties in the tree are treated randomly, with distances uncorrected and gaps distributed proportionally. Bootstrap measurements were conducted with 1000 iterations.

Accession Numbers

Unless indicated otherwise, all gene accession numbers are from National Center for Biotechnology Information. Rat Gly N-MT, S00112; C. reinhardtii VTE3, AY333781; rice VTE3 (TIGR), TC105417; spinach VTE3, X56963; *Halobacterium* sp NRC-1 VTE3-like protein, NP_280804; *A. fulgidus* VTE3-like protein, NP_069348; yeast ERG6, CAA89944; maize C-24 sterol methyltransferase, AAB70886; Arabidopsis γ-TMT (MIPS; www.mips.biochem.mpg.de), NM_105171; Arabidopsis SMT1, NM_121374; *Synechocystis* sp PCC6803 γ-TMT, ORF Slr0089 (Cyanobase; www.kazusa.or.jp/cyano/cyano.html), BAA10562; *Nostoc punctiforme* γ-TMT, ZP_00110362; *C. reinhardtii* cyanobacteria-type MPBQ/MSBQ MT, AY293576; *Synechocystis* sp PCC6803 MPBQ/MSBQ MT, ORF Sll0418 (Cyanobase), BAA18485; *T. elongatus*, BAC09278; Nostoc sp PCC7120 cyanobacterial MPBQ/MSBQ MT (Joint Genomic Institute [JGI]; http://genome.jgi-psf.org/draft_microbes/nospu/nospu.home.html), contig651; *Trichodesmium erythraem*, ZP_00074879; *P. marinus* MED4, ZP_00105453; *Synechococcus* sp WH8102,

ZP_00116290; *P. marinus* strain MIT9313, ZP_00114022; alfalfa COMT, 1FPQA; and *T. pseudonana* cyanobacteria-type MPBQ/MSBQ MT, assembled from JGI diatom raw genome data (http://genome.jgi-psf.org/thaps0/thaps0.home.html) using the following sequences: PQI43478.x1, TEU52932.y1, SXZ28761.x1, TEU29767.x1, PQI75058.y1, PQI134681.x1, TEU44005.y1, PQJ3077.x2, PQI20677.x1, PQI107604.y1, PQI127568.y1, PQI22068.y1, and PQI117660.y1. The coding region of the *T. pseudonana* consensus sequence was predicted by GENSCAN (http://genes.mit.edu/GENSCAN.html).

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FIGURES

Table A2.1. Analysis of Prenyllipids in Wild-Type and *vte3* Arabidopsis Leaf Tissue and Synechocystis Cell Culture.

Sample	Plastoquinone (pmole/mg)	Total Tocopherol (pmole/mg)	α-Tocopherol (pmole/mg)	β-Tocopherol (pmole/mg)	γ-Tocopherol (pmole/mg)	δ-Tocopherol (pmole/mg)
Arabidopsis plate grown						
VTE3/VTE3	210 ± 14	107 ± 6	95 ± 4 (89%)	3 ± 1 (3%)	9 ± 1 (8%)	0 (0%)
vte3-1/vte3-1	173 ± 9* (33%)	122 ± 8** (42%)	40 ± 2** (3%)	51 ± 2** (22%)	3 ± 1**	27 ± 3**
vte3-2/vte3-2	0**	48 ± 5**	0** (0%)	13 ± 3** (28%)	0** (0%)	35 ± 2** (72%)
Arabidopsis soil grown						
VTE3/VTE3	67.9 ± 9.9	31.5 ± 3.3	29.1 ± 2.7 (92%)	0.5 ± 0.1 (2%)	1.9 ± 0.5 (6%)	0 (0%)
vte3-1/vte3-1	55.3 ± 5.2*	21.5 ± 2.3*	9.7 ± 1.1** (45%)	8.6 ± 0.1** (40%)	1.1 ± 0.5* (5%)	2.1 ± 0.6** (10%)
VTE3-2/vte3-2	72.7 ± 8.6*	28.0 ± 6	24.5 ± 5.2 (88%)	2.2 ± 0.4** (8%)	1.3 ± 0.4 (5%)	0 (0%)
Synechocystis						
SLL0418	233 ± 20	93.3 ± 7.2	87.5 ± 6 (94%)	0 (0%)	5.8 ± 1.2 (6%)	0 (0%)
sll0418	170 ± 1*	28.7 ± 3.9**	27 ± 3.7** (90%)	0.7 ± 0** (2%)	1.0 ± 0.2* (8%)	0 (0%)

Total lipids extracted from 3-week soil-grown or 18-day-old plate-grown Arabidopsis plants or log-phase *Synechocystis* cell cultures were resolved by normal-phase HPLC, and the quantities of individual tocopherols and PQ were determined relative to standards. Values are expressed as the average of quadruplicate analyses ± SD (in pmol/mg fresh weight). Student's *t* test was performed, and statistically significant differences relative to respective controls are indicated by single ($P \leq 0.05$) and double ($P \leq 0.01$) asterisks. Numbers in parentheses indicate the percentage of an individual tocopherol in the total pool. Values for *Synechocystis* are expressed as pmol·OD_{730}^{-1}·mL^{-1}.

Table A2.2. Analysis of Seed Tocopherols in the Wild Type and *vte3* Mutants.

Genotype	Total Tocopherol	α-Tocopherol	γ-Tocopherol	δ-Tocopherol
VTE3/VTE3	853 ± 41	23 ± 1 (3%)	789 ± 37 (92%)	41 ± 3 (5%)
vte3-1/vte3-1	1180 ± 12**	15 ± 1** (1%)	541 ± 4** (46%)	624 ± 7** (53%)
VTE3-2/vte3-2	1120 ± 22*	16 ± 4* (2%)	632 ± 6* (56%)	472 ± 12** (42%)

Total lipids extracted from dry seeds were resolved by normal-phase HPLC, and the quantities of individual tocopherols were determined relative to standards. Values are expressed as the average of quadruplicate analyses ± SD (in pmol/mg dry weight). Student's *t* test was performed relative to the control, and statistically significant differences are indicated by single ($P \leq 0.01$) and double ($P \leq 0.001$) asterisks. Numbers in parentheses indicate the percentage of individual tocopherols in the total pool.

Table A2.3. Pair-Wise Comparison of Cyanobacteria- and VTE3-Type MPBQ/MSBQ MTs.

	Cyanobacteria Type			VTE3 Type				
Species	S.sp	C.r. (I)	T.p.	H.sp	A.fu.	C.r. (II)	S.o.	O.s.
A.t.	18/27	18/28	18/28	39/55	40/56	70/79	85/93	81/89
O.s.	17/27	17/29	18/28	40/56	43/60	65/75	74/83	
S.o.	18/27	16/28	17/28	41/56	41/58	62/71		
C.r. (II)	16/31	16/28	16/27	42/58	40/60			
A.fu.	19/33	21/43	21/36	42/59				
H.sp.	20/31	22/36	20/33					
T.p.	48/66	54/67						
C.r. (I)	45/62							

Pair-wise comparisons of predicted mature proteins (as predicted by PSORT) were performed using CLUSTAL W. Values indicate the percentage identity/similarity in the shorter sequence of each pair. A.fu., *A. fulgidus* VTE3-like protein; A.t., Arabidopsis VTE3; C.r. (I), *C. reinhardtii* cyanobacteria-type ortholog; C.r. (II), *C. reinhardtii* VTE3-type ortholog; H.sp, *Halobacterium* sp NRC-1 VTE3-like protein; O.s., *Oryza sativa* VTE3; S.o., *Spinacia oleracea* VTE3; S.sp, *Synechocystis* sp PCC6803; T.p., *T. pseudonana* SII0418.

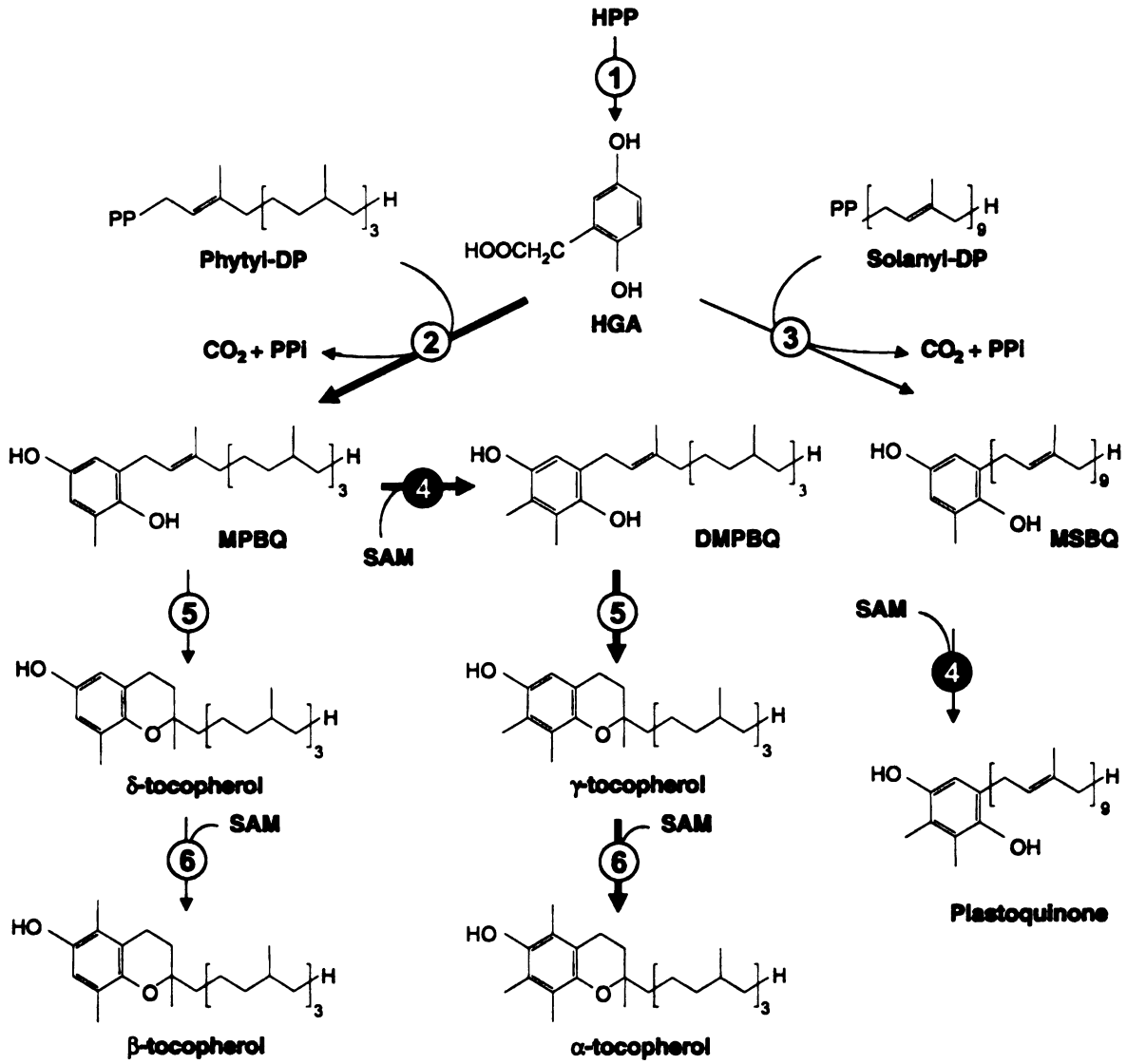

Figure A2.1. The Tocopherol Biosynthetic Pathway in Plants and Cyanobacteria and the PQ Biosynthetic Pathway in Plants.

Boldface arrows represent the steps leading to α-tocopherol, the most abundant tocopherol produced in wild-type Arabidopsis leaves and *Synechocystis* sp PCC6803. DMPBQ, 2,3-dimethyl-5-phytyl-1,4-benzoquinone; HGA, homogentisic acid; HPP, *p*-hydroxyphenylpyruvate; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinone; MSBQ, 2-methyl-6-solanyl-1,4-benzoquinone; phytyl-DP, phytyldiphosphate; SAM, *S*-adenosylmethionine; solanyl-DP, solanyldiphosphate. Enzymes are indicated by circled numbers: 1, HPP dioxygenase (HPPD); 2, homogentisate phytyltransferase (HPT); 3, homogentisate solanyltransferase (HST); 4, MPBQ/MSBQ methyltransferase; 5, tocopherol cyclase (TC); and 6, γ-tocopherol methyltransferase (γ-TMT).

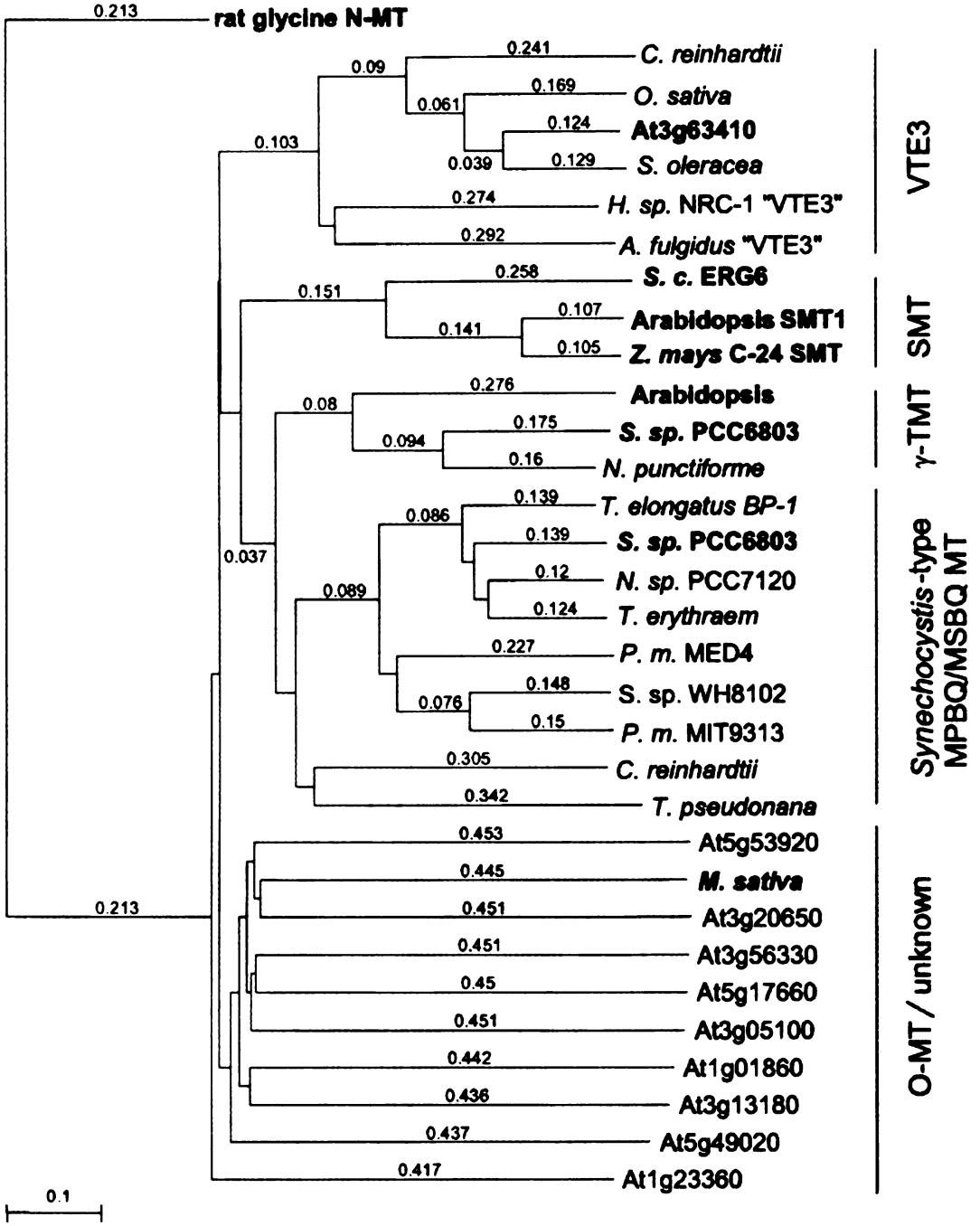

Figure A2.2. Phylogeny of Selected Methyltransferases from Photosynthetic Organisms. Rat Gly *N*-methyltransferase (N-MT) is included as an outgroup. Boldface lettering indicates that the activity of the encoded protein has been demonstrated biochemically. Known or predicted functions for individual clades are indicated at right. *A. fulgidus*, *Archaeoglobus fulgidus*; At3g63410, Arabidopsis VTE3; *H.* sp NRC-1, *Halobacterium* sp NRC-1; *M. sativa*, alfalfa chalcone *O*-methyltransferase (O-MT); *N.* sp PCC7120, *Nostoc* sp. PCC7120; *P. m.* MED4, *Prochlorococcus marinus* MED4; *P. m.* MIT9313, *Prochlorococcus marinus* MIT9313; *S. c.*, *Saccharomyces cerevisiae*; SMT, sterol methyltransferase; *S.* sp PCC6803, *Synechocystis* sp PCC6803; *S.* sp WH8102, *Synechococcus* sp WH8102; *T. elongatus* BP-1, *Thermosynechococcus elongatus* BP-1; *T. erythraem*, *Trichodesmium erythraem* IMS101; γ-TMT, γ-tocopherol methyltransferase; VTE3, plant-type MPBQ/MSBQ MT. All Arabidopsis gene numbers follow the standard genomic nomenclature: At#g#####.

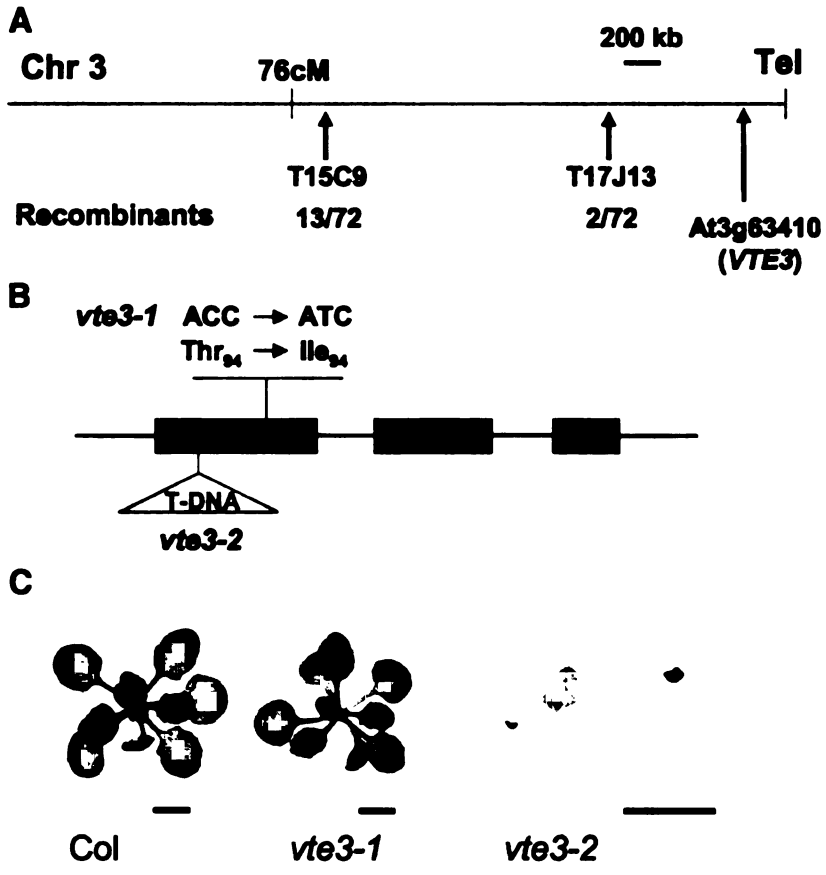

Figure A2.3. Positional Cloning of *VTE3* and Phenotypes of the *vte3* Mutants.
(A) Diagram of chromosome III from ~76 centimorgan (cM) to the telomere (TEL). The number of recombination events is indicated under the markers on BACs T15C9 and T17J13. The *VTE3* locus was delineated further as At3g63410, as described in the text.
(B) Diagram of the *VTE3* genomic clone and the *vte3-1* and *vte3-2* mutations. Black bars represent exons. Thin lines represent untranslated regions and introns. The conversion of Thr-94 to Ile-94 in *vte3-1* is indicated.
(C) Whole-plant phenotypes of plate-grown wild-type and homozygous *vte3-1* and *vte3-2* mutant plants. Col, Columbia wild type. Bars = 2 mm.

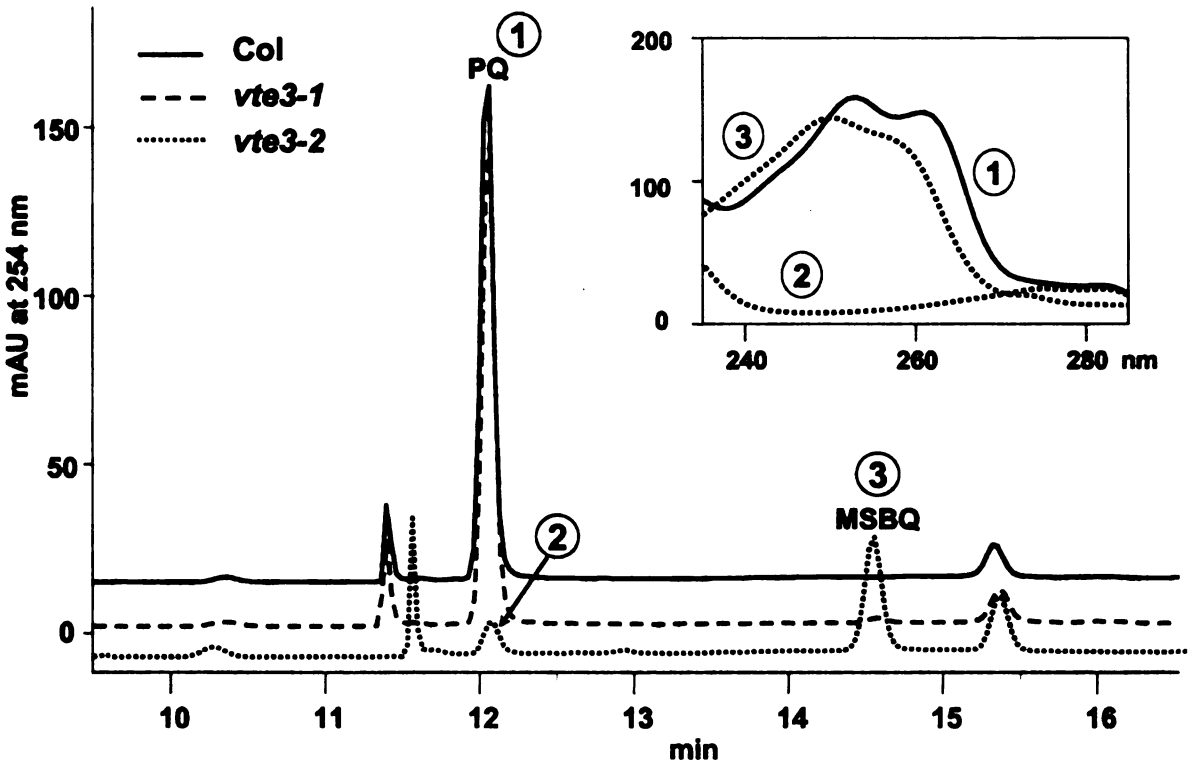

Figure A2.4. HPLC Analysis of PQ and Its Precursor MSBQ in Leaves of the Wild Type and Homozygous *vte3-1* and *vte3-2* Mutants.

Total lipids extracted from leaves of plate-grown wild-type and *vte3* mutant plants were subjected to normal-phase HPLC analysis. The inset shows spectra of the three indicated compounds in the wild type and *vte3-2*. Peak 1, plastoquinone-9 (PQ); peak 2, unknown peak at 12.10 min; peak 3, MSBQ.

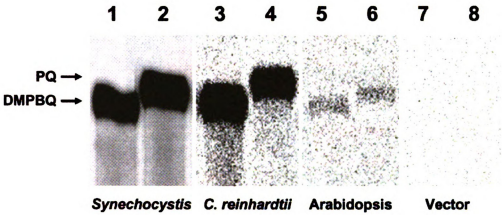

Figure A2.5. Substrate Specificity Assays of *Synechocystis* sp PCC6803 MPBQ/MSBQ MT, the Cyanobacteria-Type *C. reinhardtii* Enzyme, and Arabidopsis VTE3.
The three proteins and an empty vector control were expressed in *E. coli*, solubilized in Tween 20, β-D-dodecyl-maltoside, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, and Triton X-100, respectively, and assayed for activity with MPBQ (lanes 1, 3, 5, and 7) or MSBQ (lanes 2, 4, 6, and 8) as substrates in the presence of ^{14}C-methyl-*S*-adenosylmethionine. Reaction products were extracted and separated by thin layer chromatography. Radiolabeled products were detected by phosphorimaging. Reaction products are indicated by arrows.

Figure A2.6. Alignment of Cyanobacteria- and VTE3-Type MPBQ/MSBQ MTs.

Figure A2.6. (continued)
The transit peptide or signal sequence (as appropriate) was predicted by PSORT for each protein and removed before alignment using CLUSTAL W. The locations of three *S*-adenosylmethionine (SAM I to SAM III) binding domains are indicated by solid bars below the alignment. The asterisk above the alignment indicates the position of Thr-94 in Arabidopsis *VTE3*, which is mutated to Ile-94 in *vte3-1*. Dashed lines indicate gaps in the alignment. Arrowheads indicate the positions of conserved intron:exon junctions in the Arabidopsis and rice *VTE3* genes. Background highlighting is as follows: black, identical in at least seven sequences; red, identical in both archeal VTE3-like proteins and, where indicated, also in other sequences; blue, identical in all cyanobacteria-type MPBQ/MSBQ MT sequences and, where indicated, also in other sequences; yellow, identical in cyanobacteria-type and archeal VTE3-like enzymes only. Red lettering indicates residues identical in at least three eukaryotic VTE3-type MPBQ/MSBQ MT orthologs and, where indicated, also in other sequences. A.fu. "VTE3," *A. fulgidus* VTE3-like protein; A.t. VTE3, Arabidopsis VTE3; C.r. "Sll0418," *C. reinhardtii* ortholog of *Synechocystis* sp PCC6803 Sll0418; C.r. VTE3, *C. reinhardtii* VTE3; H.sp. "VTE3," *Halobacterium* sp NRC-1 VTE3-like protein; O.s. VTE3, *Oryza sativa* VTE3; S.o. VTE3, *Spinacia oleracea* VTE3; S.sp. Sll0418, *Synechocystis* sp PCC6803 MPBQ/MSBQ MT; T.p. "Sll0418," *Thalassiosira pseudonana* ortholog of *Synechocystis* sp PCC6803 Sll0418.

A-3: Characterization of Glucose-Sensitive Phenotype of Tocopherol Mutants in Synechocystis.

The work presented in this section has been published:

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Author's contributions:

Hiroshi Maeda conducted quantitation of tocopherols and helped with manuscript revision. Yumiko Sakuragi conducted the rest of the research and wrote the manuscript. Dean DellaPenna was involved in project development and helped with manuscript revision. Donald A. Bryant supervised the entire project and edited the final manuscript.

ABSTRACT

α-Tocopherol is synthesized exclusively in oxygenic phototrophs and is known to function as a lipid-soluble antioxidant. Here, we report that α-tocopherol also has a novel function independent of its antioxidant properties in the cyanobacterium *Synechocystis* sp. PCC 6803. The photoautotrophic growth rates of wild type and mutants impaired in α-tocopherol biosynthesis are identical, but the mutants exhibit elevated photosynthetic activities and glycogen levels. When grown photomixotrophically with glucose (Glc), however, these mutants cease growth within 24 h and exhibit a global macronutrient starvation response associated with nitrogen, sulfur, and carbon, as shown by decreased phycobiliprotein content (35% of the wild-type level) and accumulation of the *nblA1-nblA2*, *sbpA*, *sigB*, *sigE*, and *sigH* transcripts. Photosystem II activity and carboxysome synthesis are lost in the tocopherol mutants within 24 h of photomixotrophic growth, and the abundance of carboxysome gene (*rbcL*, *ccmK1*, *ccmL*) and *ndhF4* transcripts decreases to undetectable levels. These results suggest that α-tocopherol plays an important role in optimizing photosynthetic activity and macronutrient homeostasis in *Synechocystis* sp. PCC 6803. Several lines of evidence indicate that increased oxidative stress in the tocopherol mutants is unlikely to be the underlying cause of photosystem II inactivation and Glc-induced lethality. Interestingly, insertional inactivation of the *pmgA* gene, which encodes a putative serine-threonine kinase similar to RsbW and RsbT in *Bacillus subtilis*, results in a similar increase in glycogen and Glc-induced lethality. Based on these results, we propose that α-tocopherol plays a nonantioxidant regulatory role in photosynthesis and macronutrient homeostasis through a signal transduction pathway that also involves PmgA.

INTRODUCTION

α-Tocopherol (vitamin E) is a lipid-soluble, organic molecule that is only synthesized by oxygen-evolving phototrophs, including some cyanobacteria and all green algae and plants (Threlfall and Whistance, 1971; Collins and Jones, 1981; Sakuragi and Bryant, 2006). The conservation of α-tocopherol synthesis during the evolution of oxygenic photosynthetic organisms suggests that this molecule performs one or more critical functions. Because α-tocopherol is also an essential dietary component, most of our knowledge of tocopherol functions has been obtained from studies in animals, animal cell cultures, and artificial membranes. Studies in these systems have shown that tocopherols scavenge and quench various reactive oxygen species and lipid oxidation by-products, which would otherwise propagate lipid peroxidation chain reactions in membranes (Kamal-Eldin and Appelqvist, 1996). In addition to these antioxidant functions, several other functions have been reported in mammals. These functions, which are independent of the antioxidant activity of tocopherols and are termed nonantioxidant functions, include transcriptional regulation and modulation of signaling pathways (Chan et al., 2001; Azzi et al., 2002; Ricciarelli et al., 2002; Rimbach et al., 2002).

Tocopherol functions have not yet been clearly defined in oxygenic phototrophs, but it is believed that they likely include some or all of the functions reported in animals, as well as other functions possibly specific to photosynthetic organisms. For example, recent studies with tocopherol-deficient mutants of Arabidopsis (*Arabidopsis thaliana*) demonstrated that tocopherols provide protection against propagation of lipid peroxidation in dormant and germinating seeds and thus are essential for seed longevity

and seedling development (Sattler et al., 2004). α-Tocopherol has been proposed to protect PSII under high light-induced oxidative stress conditions in the green alga *Chlamydomonas reinhardtii* (Trebst et al., 2002). Furthermore, we have previously demonstrated that tocopherol-deficient mutants of *Synechocystis* sp. PCC 6803 grow poorly when challenged with oxidative stress induced by the combination of polyunsaturated fatty acids and high light illumination (Maeda et al., 2005). Therefore, it seems clear that an antioxidant role of α-tocopherol is conserved among the oxygenic phototrophs.

The biosynthesis of α-tocopherol in cyanobacteria occurs as shown in Figure A3.1. Insertional inactivation of the genes encoding each enzyme of the pathway has resulted in a series of mutants in which the content and composition of tocopherol species vary. For example, the *slr0089* mutant accumulates only γ-tocopherol (Shintani and DellaPenna, 1998), the *sll0418* mutant accumulates 30% of the wild-type level of α-tocopherol and a small amount of β-tocopherol (Shintani et al., 2002; Cheng et al., 2003), whereas the *slr1736* mutant lacks all tocopherols (Collakova and DellaPenna, 2001). In light of the established antioxidant activity of α-tocopherol, one would expect that a loss or reduction of α-tocopherol would lead to an obvious phenotypic difference between the wild-type and mutant strains. Intriguingly, however, the tocopherol-deficient *slr1736* mutant was reported to grow similarly to the wild type under both photoautotrophic and photomixotrophic conditions (Collakova and DellaPenna, 2001). These results suggest that α-tocopherol is dispensable for the survival of *Synechocystis* sp. PCC 6803 under the conditions tested (Collakova and DellaPenna, 2001).

In contrast to the results of these previous studies, by reconstructing a series of tocopherol mutants in an isogenic wild-type background, we show here that α-tocopherol is essential for the normal physiology of the cyanobacterium *Synechocystis* sp. PCC 6803. Tocopherol mutants exhibited enhanced photosynthetic activities when grown under photoautotrophic conditions, whereas they lost photosynthetic activity after 24 h and were unable to grow under photomixotrophic conditions (in Glc-containing media). These results demonstrate that α-tocopherol is essential for the survival of *Synechocystis* sp. PCC 6803 under photomixotrophic conditions and suggest a role for α-tocopherol in the regulation of photosynthesis in this cyanobacterium. Further analyses led to the conclusion that oxidative stress is not the major cause of the lethality in cells grown photomixotrophically and that α-tocopherol plays a regulatory role in photosynthesis and macronutrient metabolism in *Synechocystis* sp. PCC 6803 that is independent of its antioxidant properties.

RESULTS

Isolation and Characterization of Isogenic Tocopherol Mutants under Photoautotrophic Conditions

Isogenic mutants deficient in tocopherol biosynthesis were constructed in our laboratory wild-type strain (see "Materials and Methods"). Genomic DNAs extracted from each of the previously isolated tocopherol-deficient mutants (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Shintani et al., 2002) were used for transformation, and the resulting mutants were selected under photoautotrophic growth conditions on the basis of their resistance to kanamycin. Complete segregation of each mutant allele was

confirmed by PCR analysis (Figure A3.2A). Table A3.1 shows the tocopherol content of each homozygous mutant. The tocopherol content of the mutants was similar to that reported previously and further confirmed the targeted gene inactivations (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Cheng et al., 2003). The growth rates of the wild type and mutants were indistinguishable under photoautotrophic growth conditions in liquid B-HEPES medium with 3% (v/v) CO_2 at various light intensities (Figure A3.2B, 50 μmol photons m^{-2} s^{-1}; $\leq$ 5 and 300 μmol photons m^{-2} s^{-1}, data not shown). The data demonstrate that α-tocopherol is not required for the growth of *Synechocystis* sp. PCC 6803 under photoautotrophic conditions, which is consistent with previous studies (Collakova and DellaPenna, 2001; Dähnhardt et al., 2002; Maeda et al., 2005).

The impact of tocopherol deficiency on photosynthesis was investigated in cells grown under photoautotrophic conditions. The oxygen evolution rates for whole cells were measured to assess the PSII activities of the wild-type and tocopherol mutant strains. Each mutant showed an elevated oxygen evolution rate, which was 17% to 32% higher than that of the wild type (Table A3.2). Analyses of the total cellular sugar content of cells, including all sugar residues found in, for example, lipopolysaccharides, nucleic acids, glycoproteins, and glycogen, revealed that the *slr1736* mutant contained 160% of the total sugar level of the wild type when cells were grown photoautotrophically (Table A3.3). In thin-section electron micrographs examined by transmission electron microscopy, the space between thylakoid membranes appeared electron dense and, at most, very small glycogen granules were present in wild-type cells grown under photoautotrophic conditions (Figure A3.3A). In contrast, the spaces between the

thylakoid membranes of the *slr1736* mutant under photoautotrophic conditions were filled with very large glycogen granules (large, electron-transparent oval objects; Figure A3.3B). These results demonstrate that the tocopherol mutants possess elevated photosynthetic activities and accumulate elevated amounts of fixed carbon as glycogen when grown under photoautotrophic conditions.

Tocopherol Mutants Are Sensitive to Glc

Cells grown under photoautotrophic conditions were diluted into fresh B-HEPES medium containing 5 mM Glc, and growth was monitored under photomixotrophic conditions. The mutants grew similarly to the wild type during the initial 12 to 24 h, but all mutants stopped growing after about 24 h in the presence of Glc (Figure A3.2, C and D). After 72 h, the mutants had completely lost viability and could not form colonies even on Glc-free medium (data not shown). The ultrastructure of the *slr1736* mutant cells was dramatically different from that of the wild type when both were grown photomixotrophically. The thylakoid membrane surfaces of the mutant appeared smoother than those of the wild type, and numerous electron-dense oval objects, whose biochemical nature is not yet known, can be seen between thylakoid membranes (compare Figure A3.3, C and D). Under these conditions, the PSII activity in the mutant cells was completely lost by 24 h, whereas the wild type maintained similar PSII activity during the course of measurements (Table A3.2). Furthermore, in the *slr1736* mutant grown under photomixotrophic conditions, no carboxysomes were detectable in thin-section micrographs (see example in Figure A3.3D). These results demonstrate that α-tocopherol is essential for survival as

well as for maintenance of PSII activity and carboxysomes in *Synechocystis* sp. PCC 6803 under photomixotrophic conditions.

Oxidative Stress Is Unlikely to Be the Cause of Glc Lethality in Tocopherol Mutants

Light-dependent inactivation of photosynthesis, termed photoinhibition, is often observed under a variety of environmental stresses, including high-intensity light (Allakhverdiev et al., 1999; Hideg et al., 2000; Trebst et al., 2002; for review, see Aro et al., 1993, and refs. therein). Under such conditions, the PsbA protein, a polypeptide that forms a subunit of the PSII core complexes, is rapidly degraded and PSII activity is lost. Given the established role of α-tocopherol as an antioxidant and the report that it protects PSII from photoinhibition in *C. reinhardtii* (Trebst et al., 2002), we hypothesized that the altered photosynthetic activities and growth capacities in the tocopherol mutants under photomixotrophic conditions resulted from elevated oxidative stress due to the loss of α-tocopherol. However, immunologically detectable PsbA protein levels were essentially identical for the wild type and mutants grown both photoautotrophically and photomixotrophically (Figure A3.4A). Furthermore, the level of immunologically detectable PsbO, a 33-kD protein closely associated with the tetra-manganese cluster of the PSII oxygen evolution complex (Ferreira et al., 2004), was also essentially identical for the wild-type and mutant cells grown both photoautotrophically and photomixotrophically (Figure A3.4A). These results indicate that inactivation of PSII in the tocopherol mutants is not due to damage and degradation of the PsbA and PsbO proteins.

Expression of the *sodB* gene, encoding superoxide dismutase, is known to increase severalfold in response to the presence of various reactive oxygen species, and *sodB* transcripts or SodB are often used as markers for oxidative stress (Hihara et al., 2001; Huang et al., 2002; Ushimaru et al., 2002). Similar and low levels of *sodB* transcripts were detected in the wild-type and *slr1736* mutant cells grown photoautotrophically (Figure A3.4B, 0 h). Following a shift to photomixotrophic growth conditions, *sodB* transcript levels increased gradually and similarly in both the wild type and the *slr1736* mutant (Figure A3.4B, at 4–24 h). These results indicate that the *slr1736* mutant is unlikely to be experiencing oxidative stress beyond that which occurs in wild-type cells. Furthermore, as shown in Figure A3.4, C and D, the cessation of growth observed for the tocopherol mutants under photomixotrophic conditions occurs independently of light intensity within the range from approximately 5 μmol photons m^{-2} s^{-1} to 300 μmol photons m^{-2} s^{-1}. Taken together, these results are not consistent with the hypothesis that oxidative stress causes the inactivation of PSII and growth inhibition observed for the tocopherol mutants under photomixotrophic conditions.

Effect of pH on Glc-Induced Lethality

It is noteworthy that the effect of Glc was dependent on the pH value of the growth medium. The pH in standard B-HEPES medium typically shifted within 48 h from a value of 8.0 to a value between 7.0 and 7.3 due to the supply of 3% CO_2 (v/v). Therefore, the possibility that pH influences the Glc-induced lethality was tested using a modified B-HEPES medium (B-HEPES40, containing 40 mM HEPES) that maintained the pH of the culture within ±0.1 pH units for the duration of the growth experiment. Under

photoautotrophic conditions, the mutants grew similarly to the wild type at all pH values, indicating that the pH shift has little impact on mutants under these conditions (Figure A3.5A). Under photomixotrophic conditions, however, the mutants grew similarly to the wild type at pH 8.0 and 7.6, whereas their growth stopped after 24 h at pH 7.2 and below (Figure A3.5A). The PSII activity of the *slr1736* mutant was higher than the wild type at all pH values under photoautotrophic growth conditions, consistent with the results presented in Table A3.2. In contrast, under photomixotrophic conditions, PSII activity was pH dependent and completely lost at pH 7.0 and below (Figure A3.5B). These results demonstrate that Glc sensitivity and PSII inactivation of tocopherol mutants are pH dependent and occur at approximately pH 7.2 and below.

Glc metabolism leads to the production of NAD(P)H, which feeds electrons into the membrane electron transport chains, driving generation of the membrane electrochemical potential and, as a result, ATP synthesis. This is accompanied by an alkalization of the cytoplasm (Ryu et al., 2004). It has been reported that the cytoplasmic pH value of *Synechocystis* sp. PCC 6803 is neutral or slightly alkaline (pH 6.9–7.5), depending on growth conditions (Katoh et al., 1996). Therefore, it was hypothesized that the combination of the neutral medium pH and increased intracellular pH due to Glc import and metabolism led to a compromised membrane electrochemical potential, thereby abating ATP synthesis and growth in the tocopherol mutant. This possibility was tested by using electron transport chain inhibitors to disrupt electron transport and hence the development of the membrane electrochemical potential. 3-(3',4'-Dichlorophenyl)-1,1-dimethylurea blocks the input of electrons into the photosynthetic electron transport chain by inhibiting PSII activity; methyl viologen withdraws electrons from the

membrane electron transport chain on the acceptor side of PSI, whereas cyanide inhibits cytochrome c oxidase. Regardless of the inhibitors used, the *slr1736* mutant showed similar Glc-induced lethality as observed in the absence of the inhibitors (data not shown). These results suggest that Glc toxicity is probably not associated with increased electron flux through the electron transport chain or with an altered membrane potential.

Altered Macronutrient Metabolism in Tocopherol Mutants

How does Glc cause the death of the tocopherol mutants if not by means of oxidative stress or by a modification of electron flux through the electron transport chain? As shown above, under photoautotrophic conditions, the tocopherol mutants exhibited an enhanced photosynthetic activity and elevated total sugar content (Figure A3.3B; Table A3.3). We hypothesized that such elevation of the intracellular carbon flux would alter the balance between carbon and other macronutrients and that Glc metabolism would exacerbate this metabolic imbalance, perhaps to a level that could impair growth.

It is noteworthy that the tocopherol mutants appeared pale and chlorotic (greenish-yellow) when grown under photomixotrophic conditions. In cyanobacteria, chlorosis is often associated with macronutrient deprivation, such as nitrogen, carbon, sulfur, iron, and phosphate starvation, because of a rapid degradation of phycobiliproteins (PBPs), light-harvesting antenna proteins that can serve as a reserve of fixed carbon and nitrogen (for review, see Grossman et al., 1994). Analysis of the PBP content revealed that tocopherol mutants contained only 35% of the wild-type level of PBPs after 24 h under photomixotrophic conditions (Figure A3.6). Under these conditions, the abundance

of the cpcA transcript, which encodes the α-subunit of phycocyanin, also decreased to undetectable levels (Figure A3.7A).

The *nblA* operon, which comprises the *nblA1* and *nblA2* genes, encodes proteins essential for the regulation of PBP degradation (Baier et al., 2004), whereas the *sbpA* transcript encodes an inducible high-affinity, periplasmic sulfate-binding protein (Laudenbach and Grossman, 1991). The *nblA* and *sbpA* transcripts have previously been shown to accumulate in response to nitrogen and sulfate limitation, respectively, in *Synechocystis* sp. PCC 6803 (Laudenbach and Grossman, 1991; Collier and Grossman, 1994; Richaud et al., 2001). Under photoautotrophic growth conditions, the *slr1736* mutant accumulated slightly higher levels of these transcripts in comparison to the wild type (Figure A3.7A). Under photomixotrophic growth conditions, the *slr1736* mutant accumulated substantially higher levels of the *nblA* and *sbpA* transcripts after 4 h (Figure A3.7A). These data suggest that the *slr1736* mutant is sensing and responding to macronutrient stress and that this stress is greatly accentuated under photomixotrophic conditions. One response is increased PBP degradation in the *slr1736* mutant under photomixotrophic conditions.

It is known that the expression of alternative sigma factors is induced in response to various stresses, including macronutrient limitation for carbon (*sigB* and *sigH*; Caslake et al., 1997; Wang et al., 2004) and nitrogen (sigE; Muro-Pastor et al., 2001). As shown in Figure A3.7B, the transcript levels of these sigma factors were indeed altered in the *slr1736* mutant. For example, the transcript levels of *sigB*, *sigC*, and *sigE* in the *slr1736* mutant appeared slightly higher than those of the wild-type control under photoautotrophic growth conditions, and they increased further (by 4 h) in response to

Glc treatment (Figure A3.7B). These results suggest that the tocopherol mutants are experiencing and transcriptionally responding subtly to macronutrient starvation under photoautotrophic growth conditions and indicate that they are experiencing severe macronutrient starvation related to carbon and nitrogen under photomixotrophic growth conditions. The transcript level for sigI in the *slr1736* mutant did not vary significantly from the wild-type control, whereas *sigD* transcript levels for the mutant and the wild type were highly variable and not reliably reproducible. Interestingly, transcript levels of *sigA* and *sigG* in the *slr1736* mutant gradually decreased to undetectable levels under photomixotrophic growth conditions. *sigA* and *sigG* have been shown to be essential for the survival of this cyanobacterium (Caslake and Bryant, 1996; Huckauf et al., 2000). Therefore, these results indicate that the substantial reduction in the *sigA* and *sigG* transcripts combined with the severe macronutrient starvation response led to the cessation of growth in the tocopherol mutants under photomixotrophic growth conditions at pH 7.0.

Transcript Levels of Inorganic Carbon Metabolism Genes

Given the dramatic differences in carbon assimilation between the tocopherol mutants and wild type under both photoautotrophic and photomixotrophic conditions (Figure A3.3; Table A3.3), the abundance of genes involved in inorganic carbon (Ci) metabolism was also investigated by reverse transcription (RT)-PCR. In the *slr1736* mutant, the transcript levels of carboxysome genes, including *rbcL*, *ccmK1*, and *ccmL* (encoding the large subunit of Rubisco [Pierce et al., 1989] and carboxysome shell proteins [Price et al., 1993], respectively), were identical to those in the wild type under photoautotrophic

growth conditions. In contrast, these transcripts gradually decreased to undetectable levels in the *slr1736* mutant under photomixotrophic growth conditions, whereas those in the wild type were unaffected (Figure A3.7C). As shown by electron micrographs (Figure A3.3D), these results are consistent with the loss of carboxysomes in the *slr1736* mutant under photomixotrophic growth conditions in the *slr1736* mutant. Similarly, the transcript levels of *ndhF4*, encoding a subunit of the constitutive low-affinity CO_2 uptake transporter (Shibata et al., 2001), were not affected under photoautotrophic growth conditions, whereas they gradually decreased to undetectable levels under photomixotrophic conditions in the *slr1736* mutant. *ndhF3*, *ndhR*, and *sbtA* encode a subunit of the low CO_2-inducible high-affinity CO_2 uptake complex, a repressor of *ndhF3*, and the sodium-dependent bicarbonate transporter, respectively (Klughammer et al., 1999; Shibata et al., 2001, 2002). The transcript levels of these genes were constitutively lower in the *slr1736* mutant as compared to the wild type under both photoautotrophic and photomixotrophic growth conditions (Figure A3.7D). These results demonstrate that the abundance of Ci gene transcripts is differentially regulated in the *slr1736* mutant as compared with the wild type.

A pmgA Mutant Also Shows pH-Dependent Lethality under Photomixotrophic Growth Conditions

A previous study identified the *pmgA* gene as a locus responsible for the survival of *Synechocystis* sp. PCC 6803 under photomixotrophic growth conditions (Hihara and Ikeuchi, 1997). Although the underlying mechanism is not completely understood, pmgA has been suggested to play a role in the regulation of Glc metabolism and photosynthesis

in *Synechocystis* sp. PCC 6803 (Hihara and Ikeuchi, 1997). Therefore, a *pmgA* mutant was constructed in the same wild-type genetic background as the tocopherol mutants (see "Materials and Methods"), and the growth of this mutant was compared to the wild type and the *slr1736* mutant under photoautotrophic and photomixotrophic growth conditions at both pH 7.0 and 8.0. Under photoautotrophic conditions at both pH values, the *pmgA* mutant grew similarly to both the wild type and the *slr1736* mutant (Figure A3.8A). Under photomixotrophic growth conditions at pH 7.0, growth of the *pmgA* mutant ceased by 24 h, whereas it continued to grow at pH 8.0 (Figure A3.8B). This pH-dependent growth defect was identical to that observed for the *slr1736* mutant under photomixotrophic conditions (Figure A3.8B). Previously, the *pmgA* mutant was also shown to have higher photosynthetic activity under photoautotrophic conditions (Hihara and Ikeuchi, 1998), suggesting that, like the *slr1736* mutant, the *pmgA* mutant possesses enhanced photosynthetic capacity under photoautotrophic conditions. Therefore, total sugar content of the *pmgA* mutant was measured under photoautotrophic and photomixotrophic conditions. The *pmgA* mutant accumulated twice as much total sugar as the wild type under both photoautotrophic and photomixotrophic growth conditions (Table A3.3), which is very similar to the results observed for the *slr1736* mutant (Table A3.3; see above). The striking similarities between the *slr1736* and *pmgA* mutants lead us to propose that α-tocopherol and PmgA may function in the same signal transduction pathway and participate in the regulation of the photosynthetic activity and macronutrient homeostasis in *Synechocystis* sp. PCC 6803.

DISCUSSION

In this study, we have demonstrated that tocopherol mutants are sensitive to Glc at pH values below approximately 7.4 and are unable to grow under photomixotrophic conditions after 24 h (Figure A3.2, C and D). These results are markedly different from the results reported in a previous study in which the tocopherol mutants grew similarly to the parental wild-type strain under both photoautotrophic and photomixotrophic conditions (Collakova and DellaPenna, 2001). We observed that all of the previously isolated tocopherol mutants (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Shintani et al., 2002) showed colony morphologies that are highly variable with respect to their size and pigmentation when grown under photomixotrophic conditions (data not shown). Inhomogeneous colony morphology typically indicates genotypic heterogeneity within a given population. It is important to note that these mutants were originally isolated and maintained under photomixotrophic conditions (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Shintani et al., 2002), which we now know to be lethal for tocopherol biosynthetic mutants. Therefore, it is highly plausible that the previously isolated populations of tocopherol mutants contain secondary suppressor mutations that were selected for under continuous photomixotrophic conditions. We conclude that the authentic tocopherol mutants described here are Glc sensitive and that α-tocopherol is essential for survival of *Synechocystis* sp. PCC 6803 under photomixotrophic growth conditions at pH values below approximately 7.4.

Due to its antioxidant properties in biological membranes, functions of α-tocopherol are typically discussed in connection with oxidative stress (Kamal-Eldin and

Appelqvist, 1996). Interestingly, in *C. reinhardtii*, an 80% reduction in α-tocopherol levels, due to combined herbicide and high light treatments (1,500 μmol photon m^{-2} s^{-1}), resulted in the complete loss of PSII activity with concomitant degradation of the D1 (PsbA) protein (Trebst et al., 2002). This suggests that α-tocopherol plays an antioxidant role in protecting the structural integrity of PSII during oxidative stress in this green alga. Thus, we initially hypothesized that PSII inactivation in tocopherol mutants under photomixotrophic growth conditions was also related to oxidative stress. However, several lines of evidence indicate that this is not the case. First, the PsbA protein level in tocopherol mutants was not altered, although PSII activity was completely lost (Figure A3.4A). Second, the Glc-sensitive phenotype of tocopherol mutants was light independent and occurred at a wide range of light intensities (approximately 5–300 μmol photons m^{-2} s^{-1}; Figure A3.4, C and D). Third, *sodB* transcript levels, an oxidative stress marker, were identical between the *slr1736* and wild type under both photoautotrophic and photomixotrophic growth conditions (Figure A3. 4B). Last, the deleterious effects of Glc on the *slr1736*, *sll0418*, and *slr0089* mutants were virtually indistinguishable despite the varying compositions and amounts of tocopherols accumulated in each mutant (Table A3.1; Figures A3.2, C and D, and 5A). Should α-tocopherol function solely as a bulk antioxidant, an inverse correlation of susceptibility to Glc and tocopherol content would reasonably be expected. This was not observed, however, and therefore we conclude that Glc-induced PSII inactivation and growth inhibition of tocopherol mutants are not associated with oxidative stress or D1-mediated photoinhibition. Instead, we propose that, in addition to protecting *Synechocystis* sp. PCC 6803 membranes from peroxidation

(Maeda et al., 2005), α-tocopherol also plays a nonantioxidant role in the survival of *Synechocystis* sp. PCC 6803 under photomixotrophic growth conditions at pH 7.0.

Nonantioxidant roles of α-tocopherol are not without precedent. Studies in animal systems have demonstrated nonantioxidant roles for α-tocopherol, including modulation of signaling pathways and transcriptional regulation (Chan et al., 2001; Azzi et al., 2002; Ricciarelli et al., 2002). For example, α-tocopherol has been shown to modulate the phosphorylation state of protein kinase Cα in rat smooth-muscle cells by influencing protein phosphatase 2A activity (Ricciarelli et al., 1998). It has also been demonstrated that α-tocopherol affects the expression of genes encoding liver collagen αI, α-tocopherol transfer protein, and α-tropomyosin collagenase (Yamaguchi et al., 2001; Azzi et al., 2002; Rimbach et al., 2002). Similarly, the loss of α-tocopherol in the *slr1736* mutant constitutively or conditionally altered the abundance of several transcripts, including those encoding components of Ci, nitrogen, and sulfur metabolism (Figure A3.7). Loss of α-tocopherol also resulted in elevated photosynthetic activity in cells grown photoautotrophically as shown by increased PSII activity and total sugar and glycogen content (Tables A3.2 and 3; Figures A3.3B and 5B). These data are consistent with α-tocopherol playing a role in the regulation of photosynthesis and macronutrient metabolism—a role that is independent of its antioxidant properties in *Synechocystis* sp. PCC 6803.

What is the underling mechanism by which α-tocopherol, a small secondary metabolite, could affect such cellular processes on a global scale? In searching for an answer, we focused on the *pmgA* gene, which was previously shown to be essential for the survival of *Synechocystis* sp. PCC 6803 under photomixotrophic growth conditions

(Hihara and Ikeuchi, 1997). An analysis of conserved domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) revealed that the primary structure of PmgA is similar to that of RsbW/RsbT in *Bacillus subtilis* (E value = 6 x e^{-25}), which is a Ser-Thr kinase that acts in the signal transduction cascade that regulates the activity of SigB, a stress-responsive sigma factor in this bacterium (Price, 2000). The *pmgA* mutant showed remarkable similarity to the *slr1736* mutant under both photoautotrophic and photomixotrophic conditions. These included increased levels of total cellular sugars under both growth conditions (Table A3.3), higher oxygen evolution activity than the wild type under photoautotrophic conditions (Hihara and Ikeuchi, 1998), and nearly identical pH-dependent sensitivity to the presence of Glc (Figure A3.8). These combined results demonstrate that both α-tocopherol and PmgA are required for the appropriate regulation of photosynthesis and carbon homeostasis in *Synechocystis* sp. PCC 6803. One possibility is that α-tocopherol and PmgA are both necessary components of a not yet fully characterized signal transduction cascade whose disruption leads to Glc lethality in *Synechocystis* sp. PCC 6803. Recent studies have shown that the His kinase Hik31 is involved in Glc sensing and Glc-induced lethality (Kahlon et al., 2006), whereas the His kinase Hik8 is involved in Glc metabolism and heterotrophic growth in *Synechocystis* sp. PCC 6803 (Singh and Sherman, 2005). Alternatively, α-tocopherol may indirectly influence the activities and functions of the regulatory proteins or proteins involved in Glc metabolism, particularly those associated with the membranes, by affecting membrane integrity (Wang and Quinn, 2000). These possibilities remain to be examined in future studies.

Such a control mechanism for optimal activity of photosynthesis is essential for the normal physiology of *Synechocystis* sp. PCC 6803. We showed here that the *slr1736* mutant accumulated the *nblA1-nblA2* and *sbpA* transcripts slightly higher than the wild type even under photoautotrophic conditions (Figure A3.7A), suggesting that the mutant is already perceiving a macronutrient stress response related to nitrogen and sulfur under photoautotrophic conditions. It is important to note that this did not affect the growth rates or the PBP content of the tocopherol mutants, perhaps because this level of stress is moderate and thus tolerated under photoautotrophic conditions. After transfer to photomixotrophic growth conditions, the intracellular carbon flux increased further as exemplified by the increased total sugar content in the tocopherol mutants (Table A3.3). One could imagine this would inevitably exacerbate the altered macronutrient homeostasis in the *slr1736* mutant. Indeed, under these conditions, the *nblA1-2*, *sbpA*, and *sigE* transcript levels increased dramatically (Figure A3.7, A and B), which parallels the decrease in PBP content and the *cpcA* transcript level (Figures A3.6 and 7A). As a result, the tocopherol mutants showed severe chlorosis and growth defects under these conditions (Figure A3.2, C and D).

Interestingly, the elevated photosynthetic rate observed for the tocopherol mutants eventually ceased after cells were transferred to photomixotrophic growth conditions. PSII activity was completely lost, no carboxysomes were detectable, and *rbcL* and other Ci gene transcript levels decreased substantially by 24 h under these conditions (Table A3.2; Figures A3.3D and 7C). It is well documented in higher plants that the activity of photosynthesis is negatively regulated by the accumulation of carbohydrates. One aspect of such regulation is triggered by hexoses and their metabolites, which function as

signaling molecules and regulate photosynthetic gene expression (for review, see Koch, 1996; Sheen et al., 1999; and refs. therein). Specifically, in *Chenopodium* and maize (*Zea mays*), the addition of Glc induces a large transcriptional down-regulation of *rbcS* (encoding the small subunit of Rubisco; Krapp et al., 1993; Jang and Sheen, 1994), whereas in *Arabidopsis* the level of the OE33 transcript, encoding the 33-kD oxygen-evolving protein, is subject to Glc repression (Zhou et al., 1998). Therefore, it is plausible that a functionally analogous sugar repression mechanism exists and regulates Ci gene transcription in *Synechocystis* sp. PCC 6803. Consistent with these ideas, a Glc-sensitive mutant lacking Hik31 has recently been shown to lack glucokinase activity and, correspondingly, a glucokinase mutant cannot grow in the presence of Glc (Kahlon et al., 2006).

In summary, our efforts in reisolating and characterizing tocopherol mutants under photoautotrophic conditions have yielded new insights into the roles and functions for α-tocopherol in *Synechocystis* sp. PCC 6803. The results described here demonstrate that α-tocopherol is essential for the normal physiology of *Synechocystis* sp. PCC 6803 and suggest that, in addition to its role as an antioxidant, α-tocopherol plays a role in regulating photosynthesis and macronutrient homeostasis that is independent of this antioxidant activity. It is important to note that maize and potato (*Solanum tuberosum*) plants, which are defective in tocopherol cyclase activity and are thus tocopherol deficient, also exhibit large alterations in carbohydrate homeostasis due to impaired sugar metabolism/transport (Provencher et al., 2001; Hofius et al., 2004). Although no biochemical or mechanistic explanation exists for this common phenotype between plants

and cyanobacteria, it seems plausible that a function for α-tocopherol in the regulation of macronutrient homeostasis is conserved between the two groups of oxygenic phototrophs.

METHODS

Growth Conditions and Strains

Isolation of the original *slr1736*, *sll0418*, and *slr0089* mutants under photomixotrophic growth conditions has been described previously (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Shintani et al., 2002). A Glc-tolerant wild-type strain of *Synechocystis* sp. PCC 6803 (Williams, 1988) was used in this study for transformation and isolation of the tocopherol mutants in the absence of Glc (see "Results"). B-HEPES medium, pH 8.0, was used for selection, maintenance, and growth measurements of the wild type and mutants. This medium was prepared by supplementing BG-11 medium (Stanier et al., 1971) with 4.6 mM HEPES-KOH and 18 mg L^{-1} ferric ammonium citrate. B-HEPES40 medium, a modified B-HEPES medium containing 40 mM HEPES to provide greater buffering strength, was used in some experiments that require greater control of the medium pH during growth. The wild type was maintained on solid B-HEPES medium containing 1.5% (w/v) agar and 5 mM Glc, and the photoautotrophically selected tocopherol mutants were maintained on solid B-HEPES medium containing 1.5% (w/v) agar, 50 μg kanamycin mL^{-1}, and, importantly, no Glc. For determination of growth characteristics, late-exponential phase cultures were diluted into fresh liquid B-HEPES medium to $OD_{730\ nm}$ = approximately 0.05 cm^{-1}. The diluted cultures were grown at 32°C with continuous bubbling with air containing 1% or 3% (v/v) CO_2. The $OD_{730\ nm}$ was monitored to measure growth. The medium was supplemented with 5 mM Glc for

photomixotrophic growth conditions. The growth light intensity was 50 μmol photons m^{-2} s^{-1}, unless otherwise specified.

Construction and Isolation of Mutants

The wild type was transformed with genomic DNA extracted from the previously isolated *slr1736*, *sll0418*, and *slr0089* mutants (see above). Segregation of mutant alleles from wild-type alleles was carried out in the absence of Glc and in the presence of 50 μg kanamycin mL^{-1}. Segregation was verified by PCR analysis. Oligonucleotide primers used for PCR analysis were as follows: *slr1736* forward primer (5'-GGCTTCTCCTACCCGGAATTCTACTTCCTG-3'), *slr1736* reverse primer (5'-GCTTTCTAAGTGTACATCTAGACTCCGCCA-3'), *sll0418* forward primer (5'-ATGCCCGAGTATTTGCTTCTGCC-3'), *sll0418* reverse primer (5'-GCACTGCTTTGAACATACCGAAG-3'), *slr0089* forward primer (5'-TCTACCGGAAATTGCCAACTACCA-3'), and *slr0089* reverse primer (5'-CCTAGGAGATTGTGGACTTCAA-3'). The *pmgA* gene was amplified by PCR using forward primer (5'-TTCTCTGTGCCGAAAGCTTCTATG-3') and reverse primer (5'-CACCATGGTGGCGAATTCAGCC-3'). The amplified DNA fragments were digested with HindIII and EcoRI and ligated with pUC19 that had been digested with the same enzymes. An XbaI fragment of pMS266, containing the *aacC1* gene that confers gentamicin resistance, was inserted into the unique SpeI site within the *pmgA* coding region. The resulting plasmid construct was linearized after digestion with EcoRI and used to transform wild-type *Synechocystis* sp. PCC 6803 cells. Transformants were selected on solid medium B-HEPES at pH 8.0 in the presence of 20 μg mL^{-1} gentamicin

at room temperature under moderate light intensity (approximately 50 μE m^{-2} s^{-1}). PCR analyses of the transformants were performed with the same primer pairs described above.

Oxygen Evolution Measurements

Cells grown under photoautotrophic or photomixotrophic conditions for 24 h were harvested by centrifugation at 8,000g at room temperature and resuspended in a 25 mM HEPES buffer, pH 7.0, to obtain an $OD_{730\ nm} = 1.0\ cm^{-1}$. Oxygen evolution was measured immediately after the addition of 1 mM 1,4-benzoquinone and 0.8 mM $K_3Fe(CN)_6$ to the cell suspension. The excitation light intensity was approximately 3 mmol photons m^{-2} s^{-1}. The oxygen concentration was measured polarographically with a Clark-type electrode as described previously (Sakamoto and Bryant, 1998).

Estimation of Relative PBP Content

The relative PBP content of cells was determined by a minor modification of the method of Zhao and Brand (1989). Cells were harvested by centrifugation at 8,000g for 6 min and pellets were resuspended in 25 mM HEPES buffer, pH 7.0, to obtain cell suspensions (2 mL) with $OD_{730\ nm} = 0.5\ cm^{-1}$. These suspensions (1.0 mL) were heated at 100°C for 1 min. The $OD_{635\ nm}$ and $OD_{730\ nm}$ were recorded for unheated and heated samples, and the values were then inserted into the following equation: relative PBP content = $(\Delta OD_{635\ nm} - \Delta OD_{730\ nm})/OD_{730\ nm}$·unheated, where ΔOD indicates $OD_{unheated\ sample} - OD_{heated\ sample}$.

SDS-PAGE and Immunoblotting

Cells were grown under photoautotrophic conditions to the midexponential phase or under photomixotrophic conditions for 24 h, harvested as described above, and resuspended in 25 mM HEPES buffer, pH 7.0, to achieve $OD_{730\ nm} = 100\ cm^{-1}$. Cells were disrupted using an equal volume of glass beads and a home-built bead beater; cold cell suspensions were vigorously shaken four times for 30 s, interrupted by 30-s intervals on ice. An aliquot (10 μL) of each sample was mixed with an equal volume of loading buffer; the mixture was incubated at 65°C for 20 min and applied onto a discontinuous SDS-polyacrylamide gel with 10% (w/v) acrylamide in the separating gel as described (Schägger and van Jagow, 1987). Prof. Eva-Mari Aro kindly provided antibodies raised against amino acids 234 to 242 of the PsbA protein of *Synechocystis* sp. PCC 6803. Prof. Robert Burnap kindly provided antibodies raised against the PsbO protein of *Synechocystis* sp. PCC 6803. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Proteins were detected by immunoblotting by using enhanced chemiluminescence (Amersham Biosciences), according to the manufacturer's specifications.

Isolation of Total RNA and RT-PCR Analyses

Total RNA was isolated and purified from cells using the mini-to-midi RNA isolation kit (Invitrogen). The RNA samples were purified again after DNase digestion. The RNAs obtained were adjusted to a final concentration of 50 ng RNA μL^{-1} and stored at –80°C until used. Transcripts were amplified and detected by using the one-step RT-PCR kit (Qiagen) in the presence of the RNase inhibitor RNAsin (Promega) with target-specific

oligonucleotide primers. The sequences of the primers used for each of the indicated genes will be made available upon request.

Transmission Electron Microscopy

Cells grown under photoautotrophic and photomixotrophic conditions for 24 h were harvested and immediately fixed overnight at 4°C in a 2.5% (v/v) glutaraldehyde solution prepared in 0.1 M cacodylate buffer, pH 7.4. After secondary fixation in a 1% (w/v) osmium tetroxide solution in the cacodylate buffer, the cells were stained with uranyl acetate (2% w/v), followed by dehydration in the following concentrations of ethanol: 50% (v/v), 70% (v/v), 90% (v/v), 95% (v/v) ethanol in water followed by two washes in 100% (v/v) ethanol. The samples were then embedded in Spurr's resin and polymerized overnight at 60°C. Thin sections (approximately 50- to 60-nm thickness) were stained with 2% (v/v) uranyl acetate before examination under a JEM 1200 EXII transmission electron microscope (JEOL).

Total Sugar Assay

Cells were harvested as described above and washed and resuspended in distilled water to achieve the same $OD_{730\ nm}$. The total sugar content of each cell suspension was determined by a previously described colorimetric assay (Dubois et al., 1956). The total sugar content was calculated relative to the $A_{435\ nm}$ for the wild-type cells grown under photoautotrophic and photomixotrophic conditions.

Analysis of Tocopherol Content

The tocopherol content of the wild-type and mutant strains was analyzed as described previously (Cheng et al., 2003).

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Table A3.1. Tocopherol Content of Wild Type and Newly Isolated Tocopherol Mutants.

All values shown are averages and SEs for at least three independent measurements, except for *slr0089*, for which the values are based on a single measurement (% values are expressed relative to wild type as 100%). N.D., Not detectable.

	Tocopherol Content					
	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total	%
	pmol $OD_{730\ nm}^{-1}$ mL^{-1}					
Wild type	80.5 ± 6.0	0.1 ± 0.0	5.8 ± 1.1	N.D.	86.4 ± 5.4	100
slr0089⁻	N.D.	N.D.	8.4	N.D.	8.4	9.7
slr0418⁻	27.2 ± 3.7	0.7 ± 0.1	2.5 ± 0.5	N.D.	30.3 ± 4.1	35
slr1736⁻	N.D.	N.D.	N.D.	N.D.	N.D.	0

Table A3.2. Oxygen Evolution Activities of Wild Type and the Tocopherol Mutants Grown Under Photoautotrophic and Photomixotrophic Conditions for 24 h at 3% CO_2 (v/v), 50 μmol Photons $m^{-2}s^{-1}$, 32°C in B-HEPES Medium.

All values shown are averages and SEs for at least four independent measurements. N.D., Not detectable.

	O_2 Evolution	
	Photoautotrophic Conditions	Photomixotrophic Conditions
	$\mu M\ O_2\ h^{-1}\ OD_{730\ nm}^{-1}$	
Wild type	875 ± 146	1,096 ± 12
slr0089⁻	1,158 ± 129	N.D.
sll0418⁻	1,027 ± 80	N.D.
slr1736⁻	1,065 ± 45	N.D.

Table A3.3. Relative Sugar Content of the Wild Type and *slr1736* and *pmgA* Mutants.

Cells were grown in the absence and in the presence of Glc for 24 h at 3% CO_2 (v/v), 50 μmol photons m^{-2} s^{-1}, 32°C in B-HEPES medium. Equal cell numbers were used for the sugar analysis as described in "Materials and Methods." All values shown are averages of three independent measurements and are expressed as relative to the average value obtained for the wild type under photoautotrophic conditions.

	Photoautotrophic Conditions	Photomixotrophic Conditions
Wild type	1.00 ± 0.0761	2.23 ± 0.342
slr1736$^-$	1.60 ± 0.134	5.07 ± 0.168
pmgA$^-$	2.18 ± 0.465	4.34 ± 0.189

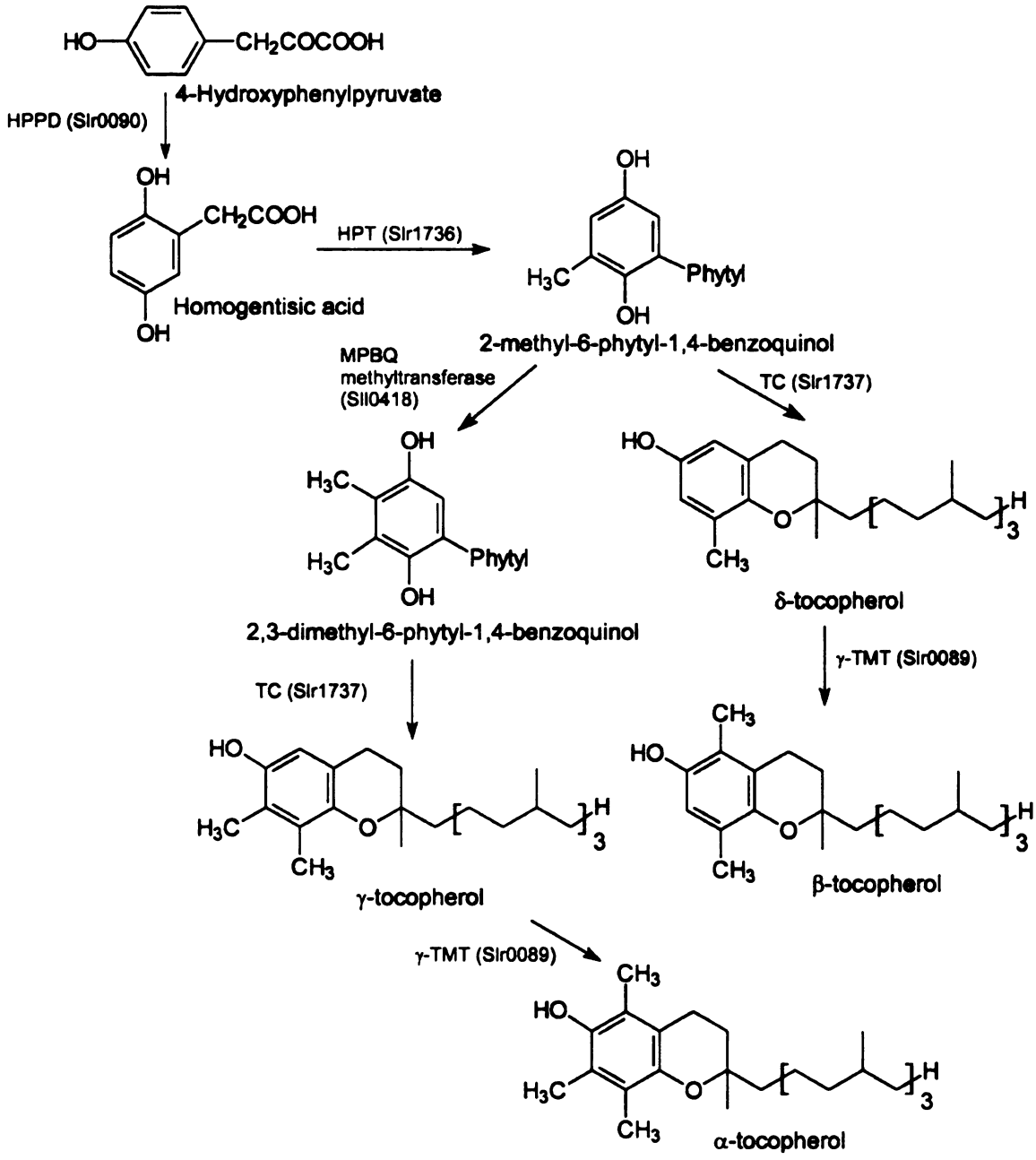

Figure A3.1. Biosynthetic pathway for α-tocopherol in *Synechocystis* sp. PCC 6803.
HPPD, 4-hydroxyphenylpyruvate dioxygenase; HPT, homogentisate phytyltransferase; MPBQ MT, 2-methyl-6-phytyl-1,4-benzoquinone methyltransferase; TC, tocopherol cyclase; γ-TMT, γ-tocopherol methyltransferase.

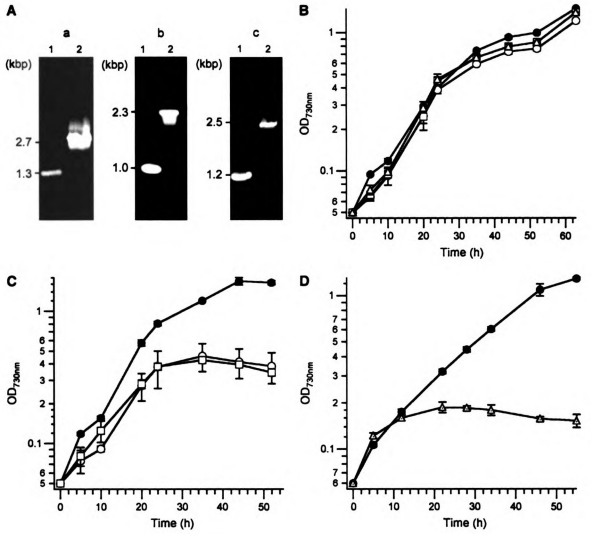

Figure A3.2. Isolation and growth characterization of tocopherol mutants.
A, PCR analysis of the genomic DNA extracted from newly isolated tocopherol mutants selected in the absence of Glc. Lanes 1 and 2 in each image show PCR products amplified from the wild-type and mutant genomic DNA templates, respectively. The DNA fragments amplified from the mutant templates using oligonucleotide primers to *slr0089* (a), *sll0418* (b), and *slr1736* (c) loci (see "Materials and Methods") are 1.3 kb longer than those from the wild-type template because of the insertion of the *aphII* cassette encoding resistance to kanamycin. B, Growth curves of the wild type and tocopherol mutants at 50 μmol photons m^{-2} s^{-1} under photoautotrophic conditions. C and D, Growth curves of the wild type and tocopherol mutants at 50 μmol photons m^{-2} s^{-1} under photomixotrophic conditions. Black circles indicate the wild-type strain; white squares, triangles, and circles indicate the authentic *slr1736*, *sll0418*, and *slr0089* mutant strains, respectively. The data shown for each strain are averages of three independent cultures; SE bars are shown.

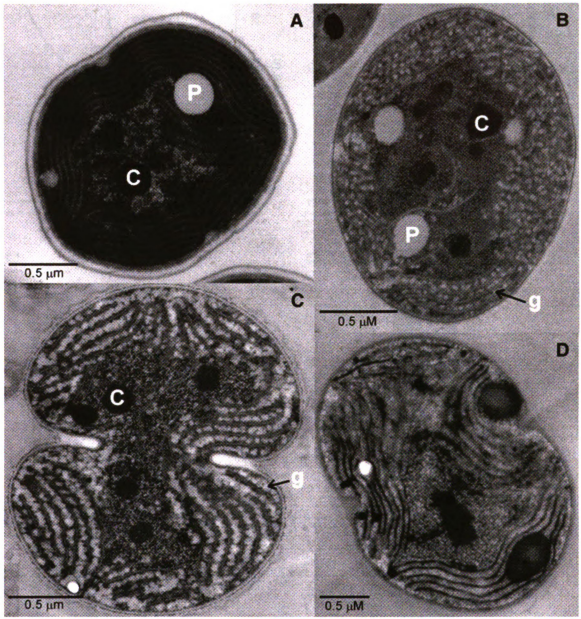

Figure A3.3. Thin-section electron micrographs of *Synechocystis* sp. PCC 6803 strains.
A, *Synechocystis* sp. PCC 6803 wild type. B, *slr1736* mutant grown under photoautotrophic conditions. C, Wild type. D, *slr1736* mutant grown under photomixotrophic conditions for 24 h. Letters C, P, and g indicate carboxysomes, poly-β-hydroxybutyrate, and glycogen granules, respectively. Cells were grown in B-HEPES40 medium, pH 7.0, at 1% (v/v) CO_2 and 50 μmol photons $m^{-2} s^{-1}$.

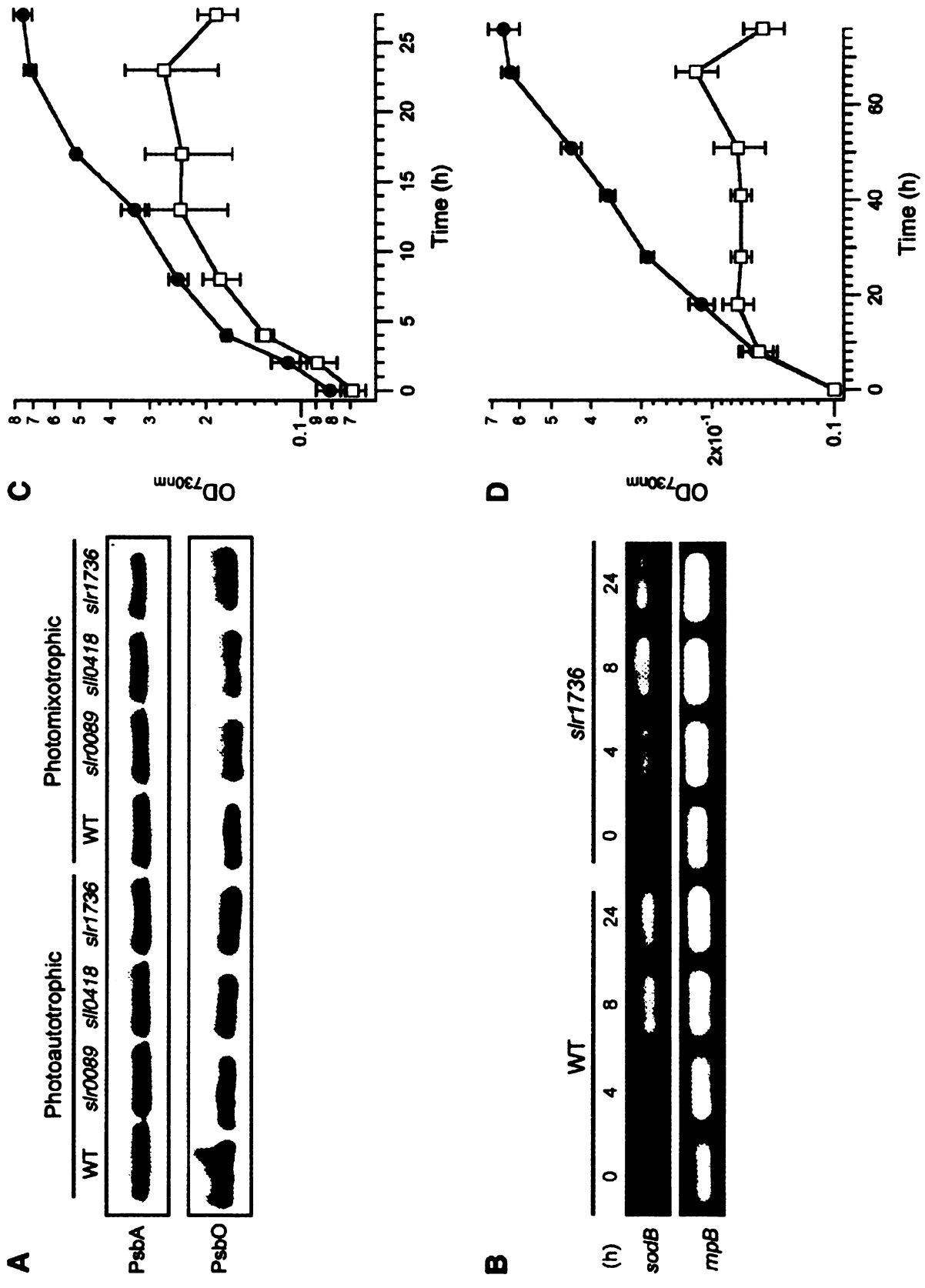

Figure A3.4. Glc sensitivity in the tocopherol mutants is independent of light levels and is not likely to be due to elevated oxidative stress.

Figure A3.4. (continued)
A, Immunoblotting analysis for the PsbA (D1) and PsbO proteins. B, Time-course RT-PCR analysis of the *sodB* transcript in whole cells of wild type and tocopherol mutants grown under photoautotrophic (0 h) and photomixotrophic (4–24 h) conditions at 32°C, 50 μmol photons m^{-2} s^{-1}, with 3% (v/v) CO_2. C and D, Growth curves under photomixotrophic conditions (C) under high light (300 μmol photons m^{-2} s^{-1}) and (D) low light conditions (approximately 5 μmol photons m^{-2} s^{-1}). Black and white symbols indicate the wild type and the *slr1736* mutant, respectively. Proteins from equal amounts of cells (10 μL of cell suspension with $OD_{730\,nm}$ = 100) were loaded for each lane (A). Equal amounts of RNA were used as templates for RT-PCR (B). RT-PCR amplification of the housekeeping *rnpB* RNA was used as the positive control. PCR amplification of the *rnpB* transcripts without the reverse transcription step did not result in product formation (data not shown).

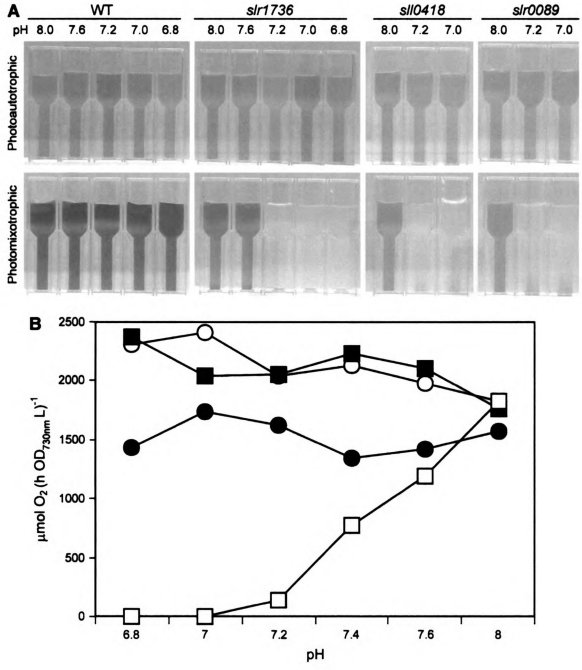

Figure A3.5. pH-dependent Glc-sensitive phenotype of the tocopherol mutants.
A, Cultures of the indicated strains were grown under photoautotrophic and photomixotrophic conditions at 1% CO_2 (v/v), 50 μmol photons m^{-2} s^{-1}, 32°C in B-HEPES40 medium (see "Materials and Methods"). B, PSII-dependent oxygen evolution rates in the wild-type (black symbols) and *slr1736* mutant (white symbols) cells grown under photoautotrophic (circles) and photomixotrophic (squares) conditions.

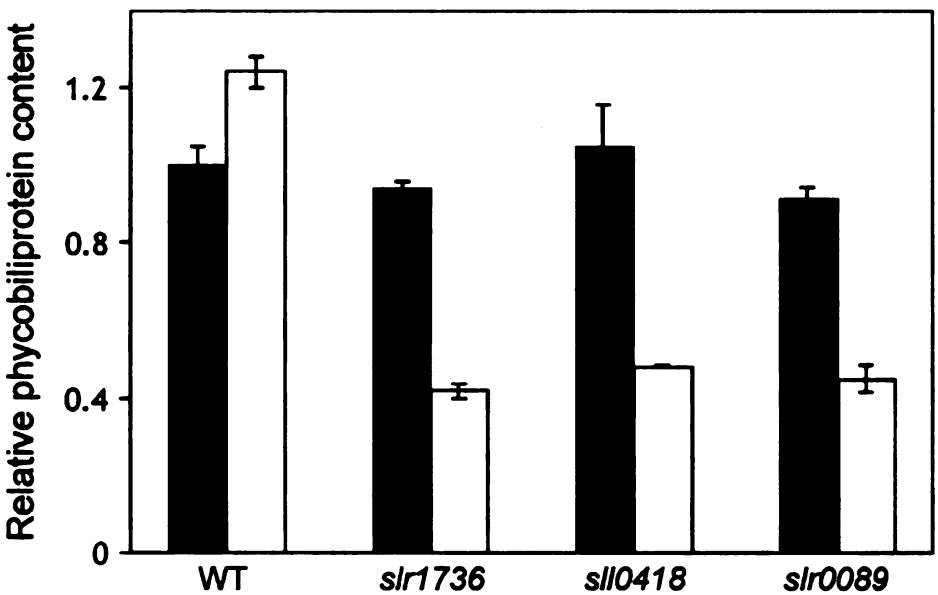

Figure A3.6. PBP content in the wild type and tocopherol mutants.
The wild type and *slr1736*, *sll0418*, and *slr0089* mutants were grown for 24 h under photoautotrophic (black columns) and photomixotrophic (white columns) conditions at 1% (v/v) CO_2, 32°C, pH 7.0, and 50 μmol photons m^{-2} s^{-1}. The data shown for each strain are averages of six independent measurements; SE bars are shown.

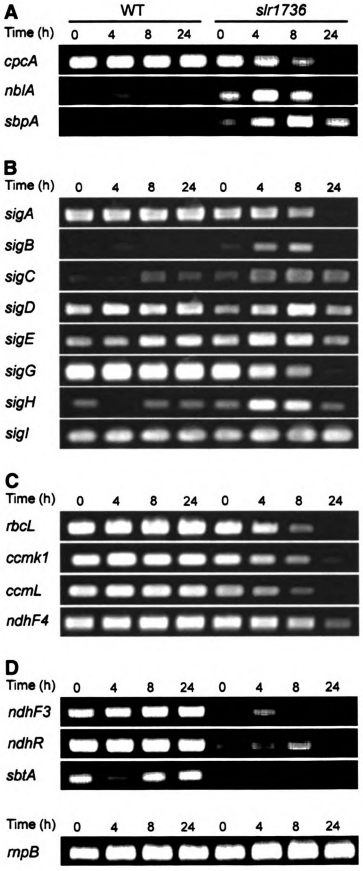

Figure A3.7. Time-course RT-PCR analysis of metabolic genes in the wild type and *slr1736* mutant grown at pH 7.0. Cells were grown under photoautotrophic (shown at 0 h) and photomixotrophic conditions for 4, 8, and 24 h, at 1% (v/v) CO_2, 32 ° C, pH 7.0, and 50 μmol photons m^{-2} s^{-1}.

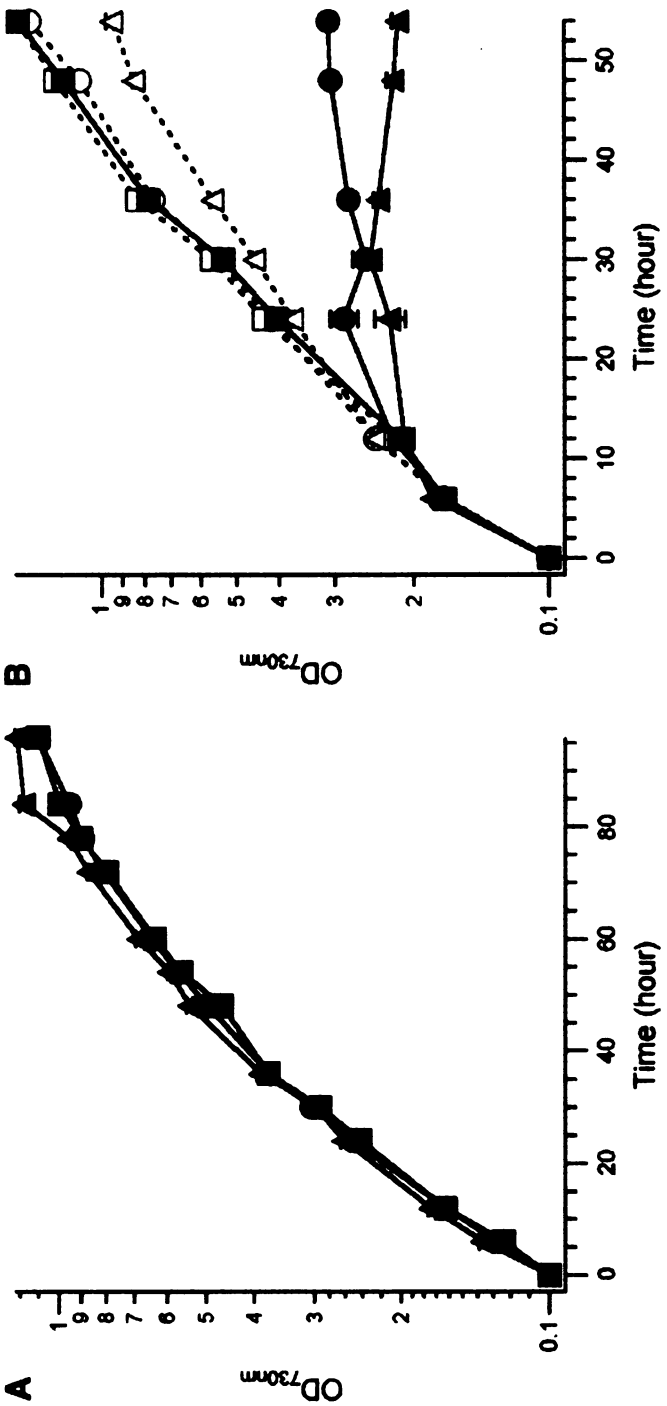

Figure A3.8. Growth analysis of the *pmgA* mutant.
Growth curves (A) determined under photoautotrophic conditions at pH 7.0 and (B) under photomixotrophic conditions at pH 7.0 (black symbols with solid lines) and pH 8.0 (white symbols with dotted lines) are shown. Squares, circles, and triangles represent the wild type, *slr1736*, and *pmgA* mutants, respectively. The growth curves recorded at pH 8.0 in the absence of Glc coincided with those at pH 7.0 (data not shown). Cells were grown in B-HEPES40 medium, pH 7.0, 1% (v/v) CO_2, 32° C, 50 μmol photons $m^{-2} s^{-1}$, at 32° C. The data shown for each strain are averages of three independent cultures; SE bars are shown.