Characterization of Tocopherol Cyclases from Higher Plants and Cyanobacteria. Evolutionary Implications for Tocopherol Synthesis and Function

Scott E. Sattler, Edgar B. Cahoon2, Sean J. Coughlan3, and Dean DellaPenna*

Department of Biochemistry and Molecular Biology, Biochemistry Building, Michigan State University, East Lansing, Michigan 48824–1319 (S.E.S., D.D.P.); and DuPont Crop Genetics, Experimental Station, Wilmington, Delaware 19880–0402 (E.B.C., S.J.C.)

Tocopherols are lipophilic antioxidants synthesized exclusively by photosynthetic organisms and collectively constitute vitamin E, an essential nutrient for both humans and animals. Tocopherol cyclase (TC) catalyzes the conversion of various phytyl quinol pathway intermediates to their corresponding tocopherols through the formation of the chromanol ring. Herein, the molecular and biochemical characterization of TCs from Arabidopsis (VTE1 [VITAMIN E 1]), Zea mays (SXD1 [Sucrose Export Deficient 1]) and Synechocystis sp. (slr1737) are described. Mutations in the VTE1, SXD1, or slr1737 genes resulted in both tocopherol deficiency and the accumulation of 2,3-dimethyl-6-phytyl-1,4-benzoquinone (DMPBQ), a TC substrate. Recombinant SXD1 and VTE1 proteins are able to convert DMPBQ to γ-tocopherol in vitro. In addition, expression of maize SXD1 in a Synechocystis sp. PCC6803 slr1737 knockout mutant restored tocopherol synthesis, indicating that TC activity is evolutionarily conserved between plants and cyanobacteria. Sequence analysis identified a highly conserved 30-amino acid C-terminal domain in plant TCs that is absent from cyanobacterial orthologs. The localization of tocopherols and most of the tocopherol biosynthetic enzymes in plastid membranes supports the cyanobacterial origins of the pathway in plants (Soll et al., 1980, 1985; Lichtenthaler et al., 1981; Fryer, 1992; Kruk and Strzalka, 1995; Arango and Heise, 1998a).

Although comparatively little is known about tocopherol functions in photosynthetic organisms, the physiological importance of these molecules in human and other animal systems has been studied extensively. The complete absence of dietary tocopherols, for example, results in chronic wasting, death, and fetal reabsorption in rats (Bramley et al., 2000). Less severe tocopherol dietary deficiencies in humans and animal models are associated with numerous degenerative diseases such as atherosclerosis, arthritis, some cancers, vision maladies, weakened immune system, and neuromuscular abnormalities (Brigelius-Flohe and Traber, 1999; Bramley et al., 2000; Ricciarelli et al., 2001).

Among the best characterized functions of tocopherols in cells is their ability to scavenge and quench reactive oxygen species and lipid-soluble by-products of oxidative stress (Brigelius-Flohe and Traber, 1999; Bramley et al., 2000; Ricciarelli et al., 2001). Tocopherols are unique in this regard to other phenolic antioxidants, such as hydroxyquinones, which must donate two electrons to attain a stable structure. Tocopherols can also donate two electrons, which results in opening of the chromanol ring to form the corresponding tocopheroxyl radical. These com-

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1 This work was supported in part by the Michigan State University Center for Novel Plant Products.
2 Present address: U.S. Department of Agriculture-Agricultural Research Service Plant Genetics Research Unit, Donald Danforth Plant Science Center, 975 N. Warson Rd., St. Louis, MO 63132.
3 Present address: Agilent Technologies Inc., Little Falls Site, 2850 Centreville Rd, Wilmington, DE 19808.
* Corresponding author; e-mail dellapen@msu.edu; fax 517–353–9334.
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combined molecular characteristics allow tocopherols to protect polyunsaturated fatty acids from lipid peroxidation by scavenging lipid peroxyl radicals that propagate lipid peroxidation chain reactions in membranes (Burton et al., 1986; Liebler, 1993). Though direct evidence is lacking, tocopherols are thought to play similar roles in protecting the polyunsaturated fatty acid-rich plastid membrane from lipid peroxidation.

Recent studies in mammalian systems have demonstrated additional biological activities of tocopherols that are independent of their antioxidant functions. The underlying mechanisms for these effects are the modulation of signal transduction pathways by specific tocopherols and, in some instances, transcriptional activation of gene expression mediated by tocopherol-binding proteins (Brigelius-Flohe and Träger, 1999; Sen et al., 2000; Chan et al., 2001; Ricciarelli et al., 2001; Yamauchi et al., 2001; Clement et al., 2002; Nobata et al., 2002). Modulation of the protein kinase C signaling cascade and eicosanoid synthesis are two well-characterized examples of the antioxidant-independent effects of tocopherols in mammalian systems (Greenberglevy et al., 1993; Tran et al., 1996; Azzi et al., 2002). Although direct experimental evidence is lacking for antioxidant-independent tocopherol activities in plants, these data raise the possibility that tocopherols may also have roles in plants that extend beyond their proposed antioxidant functions.

Though the functions of tocopherols in plants remain an open question, much has been learned about tocopherol synthesis and the pathway enzymes during the past 5 years (Norris et al., 1998; Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Schledz et al., 2001; Porfirova et al., 2002; Savidge et al., 2002; Shintani et al., 2002). Tocopherol synthesis draws substrates from two separate metabolic pathways, aromatic amino acid metabolism and isoprenoid synthesis. Homogentisic acid, an intermediate in aromatic amino acid degradation and the head group of tocopherols, is produced from \( p \)-hydroxyphenylpyruvate by the cytosolic enzyme \( p \)-hydroxyphenylpyruvate dioxygenase (HPPD; Garcia et al., 1997, 1999; Norris et al., 1998; Dahnhardt et al., 2002). The isoprenoid-derived phytyl tail of tocopherols is a product of the plastid-localized 1-deoxy-D-xylulose-5-phosphate pathway (Eisenreich et al., 1998; Lichtenthaler, 1998). The remaining steps in tocopherol synthesis occur within the inner envelope of the chloroplast and include a phytol transferase, two different methyltransferases, and a ring-producing enzyme, the tocopherol cyclase (TC; Soll et al., 1980, 1985; Arango and Heise, 1988a).

The TC adds a second oxygen-containing ring at the junction between the aromatic head group and phytol tail to create a two-ring structure known as a chromanol ring (Fig. 1), which is essential for resonance stabilization of tocopheryl radicals after single-electron transfer. Previous work has characterized TC activity in chloroplasts and chromoplasts of higher plants and in cyanobacteria (Soll, 1979; Soll and Schultz, 1980; Soll et al., 1985; Stocker et al., 1993, 1994, 1996; Arango and Heise, 1998b). The primary substrate of the TC is reduced (quinol form) 2,3-dimethyl-6-phytyl-1,4-benzoquinone (DMPBQ), which is converted to \( \gamma \)-tocopherol by TC (Soll, 1979; Soll and Schultz, 1980; Soll et al., 1985). However, the enzyme characterized from *Anabaena* sp. PCC7120 has also been shown to cyclize other 6-prenyl-1,4-benzoquinol substrates in vitro (Stocker et al., 1996). In this report, we describe the isolation and functional characterization of TCs from Arabidopsis, maize (*Zea mays*), and *Synechocystis* sp. PCC6803 and discuss the evolutionary implications of tocopherol cyclization for both tocopherol synthesis and function. The identification and characterization of TC from Arabidopsis were recently reported by Porfirova et al. (2002).

**RESULTS**

**Isolation and Characterization of vte1 Mutants**

To further understand the tocopherol pathway in higher plants, an HPLC-based screen of Arabidopsis
leaf tissue was developed to isolate mutants with tocopherol profiles that differ from wild type. Arabidopsis leaves accumulate approximately 10 ng $\alpha$-tocopherol mg$^{-1}$ fresh weight and 0.2 ng $\gamma$-tocopherol mg$^{-1}$ fresh weight under standard growth conditions (see “Materials and Methods”). Numerous mutants were identified from an ethyl methanesulfonate (EMS)-mutagenized population, including two mutants that were devoid of tocopherols in leaf tissue (Fig. 2A). Genetic complementation tests confirmed that the mutants were allelic (data not shown). The mutants were designated vte1-1 and vte1-2 (vitamin e).

The visible phenotypes of both vte1 mutants did not significantly differ from wild type when grown

under normal laboratory conditions (see “Materials and Methods”). Although several possibilities could result in a tocopherol-deficient phenotype, the two most likely are a loss of HPT activity or a loss of TC activity (Fig. 1). Assuming no genetic redundancy, a mutation disrupting either gene would result in a tocopherol-deficient phenotype, but the two classes of mutations should be readily distinguishable by the intermediates that accumulate. A defect in the TC should result in the accumulation of the DMPBQ, whereas a mutation in HPT would not accumulate tocopherol pathway prenyl quinone intermediates (Fig. 1). To understand the biochemical basis of vte1-1 and vte1-2, prenyl quinones were isolated from each mutant and analyzed by HPLC (Fig. 3A). A novel peak with a retention time and spectrum consistent with the prenyl quinone DMPBQ (Hutson and Threlfall, 1980; Marshall et al., 1985; Johnson et al., 2000) was observed in the vte1-1 and vte1-2 mutants but not in wild type (Fig. 3A). This compound was purified by HPLC and a mass of 415 D, the mass of DMPBQ, was determined by fast atom bombardment mass spectroscopy (data not shown). These combined characteristics indicate that the novel compound that accumulates in vte1-1 and vte1-2 mutants is DMPBQ, the substrate of the TC.

In addition to green tissues, seeds also contain tocopherols, but instead of $\alpha$-tocopherol predominating as in leaves, $\gamma$-tocopherol accumulates due to low $\gamma$-TMT activity (Shintani and DellaPenna, 1998). Lipids were extracted from the seeds of wild-type and vte1 mutants, and the tocopherols were analyzed by HPLC (Fig. 4). Tocopherols were absent in vte1-1 seed, but vte1-2 seeds contained approximately 25% of the tocopherol level in wild type (84.9 $\pm$ 0.8 versus 322.5 $\pm$ 7.4 ng total tocopherols mg$^{-1}$seed in vte1-2 and wild type, respectively), suggesting vte1-2 is a weaker allele than vte1-1.

A map-based cloning approach was undertaken to isolate the gene encoding the TC from Arabidopsis. vte1-1 was crossed to Landsberg erecta, and 1,100 individuals from an F2 population were used to map the VTE1 locus to a 140-kb interval on the bottom of chromosome 4. Analysis of the genes within this interval identified At4g32770, encoding an unknown protein of 488 amino acids that contains a putative N-terminal chloroplast transit peptide of 68 amino acids. Previously, At4g32770 and the Synechocystis sp. PCC6803 protein slr1737 were identified as homologs of SXD1 (Saccharomyces Cerevisiae D1) from maize (Provencher et al., 2001). slr1737 is a protein of unknown function and is in the same operon as slr1736, which encodes HPT (Collakova and DellaPenna, 2001; Schledz et al., 2001; Savidge et al., 2002), another enzyme of the tocopherol biosynthetic pathway (Fig. 1). At4g32770 was fully sequenced in both mutants, and each was found to contain a nonsense mutation. The vte1-1 mutation creates a premature
stop codon at amino acid 237 (Trp to stop), whereas the vte1-2 mutation creates a premature stop codon at amino acid 465 (Gln to stop). Both mutations are base transitions, G to A and C to T, respectively, which is consistent with the mutagenic properties of EMS. The identification of At4g32700 as the Arabidopsis TC concurs with a recent report by Porfirova et al. (2002).

Sequence Analysis and Evolutionary Origin

BLAST searches revealed that SXD1, VTE1, and slr1737 share a high degree of amino acid sequence similarity (Table I) with other proteins in the nonredundant GenBank database: three proteins of unknown function in the cyanobacteria Anabaena sp. PCC7120, N. punctiforme, and Synechococcus sp. PCC7002. SXD1 is a chloroplast-targeted protein of unknown function that had been identified previously based on a mutation causing a defect in symplastic photosynthetic transport near the site of phloem loading within the minor veins of maize leaves (Russin et al., 1996; Provencher et al., 2001). When the SXD1 and VTE1 chloroplast transit peptides are removed, SXD1, VTE1, and the four cyanobacterial proteins share long stretches of amino acid identity.

The four cyanobacterial proteins are assumed to be orthologs of VTE1 because the cyanobacterial genomes each contain obvious orthologs of the four other known genes of the tocopherol pathway: HPPD, HPT, MPBQ methyltransferase, and γ-TMT. VTE1 and the cyanobacterial orthologs exist as single genes within their respective sequenced genomes. There are several other cyanobacteria whose genomes also have been sequenced: Prochlorococcus marinus.

Figure 3. HPLC analysis of the prenyl quinones from wild-type and mutant Arabidopsis, maize, and Synechocystis sp. PCC6803. Lipids were extracted from Arabidopsis, maize, and Synechocystis sp. PCC6803, and total prenyl quinones were isolated by thin-layer chromatography (TLC) and then analyzed by normal phase HPLC (see "Materials and Methods") A, Arabidopsis. Solid line, Columbia wild type; dotted line, vte1-1; gray line, vte1-2. B, Maize. Solid line, Wild type; dotted line, sxd1. C, Synechocystis sp. PCC6803. Solid line, Wild type; dotted line, Δslr1737 insertional mutant; gray line, SXD1 cDNA expressed in the Δslr1737 mutant background. Insets, Spectra of the peak labeled DMPBQ. Phyllo, Phylloquinone; PQ, Plastoquinone.

Figure 4. HPLC analysis of seed tocopherols in wild-type Arabidopsis, vte1-1, and vte1-2. Total seed lipids were extracted, and the tocopherols present were separated by reverse phase HPLC and detected using a fluorescence detector; 290-nm excitation and 325-nm emission. Tocol, a synthetic tocopherol, was used as an internal recovery standard. Solid line, Columbia wild type; dotted line, vte1-1; gray line, vte1-2. Retention times of α-, δ-, and γ-tocopherol and tocol were determined by HPLC analysis of tocopherol standards.
rinus MED4, *P. marinus* MIT9313, *Synechococcus* sp. PCC7002 WH8102, and *Thermosynechococcus elongatus* BP-1. The genomes of these organisms lack obvious VTE1 orthologs and obvious orthologs for HPPD, HPT, and γ-TMT (refer to pathway in Fig. 1). Thus, it appears likely that only a subgroup of cyanobacteria have evolved the ability to synthesize tocopherols. VTE1, SXD1, and the four cyanobacterial orthologs lack any previously described protein motifs. There are numerous plant expressed sequence tags (ESTs) in the public database that share high similarity with VTE1 and SXD1, and full-length sequences of the *M. truncatula* and barley VTE1 orthologs were obtained from EST assemblies. These four representative plant sequences are more conserved than the four cyanobacterial sequences (Table I). Sequence alignment of the plant and the cyanobacterial protein sequences identified a highly conserved 30-amino acid carboxyl domain in the plant VTE1 orthologs (starting at Thr-458 of Arabidopsis VTE1) that is absent from the cyanobacteria proteins (Fig. 5). The last five amino acids of this carboxyl domain (KPPGL) are invariant among the plants represented, which include the bryophyte *P. patens*, monocots, and dicots. With the exception of vascular and nonvascular VTE1 orthologs, this 30-amino acid domain was not found in other proteins in the nonredundant database. Interestingly, the *C. reinhardtii* VTE1 ortholog has a shortened version of the carboxyl domain (Fig. 5) and lacks the last 12 amino acids (starting at Leu-477 of Arabidopsis VTE1), including the invariant KPPGL motif. The vte1-2 mutation causes premature termination of VTE1 and deletion of 24 amino acids of the conserved carboxyl domain.

**TC Function in Arabidopsis, Maize, and *Synechocystis* sp. PCC6803**

To confirm that the VTE1 orthologs are required for tocopherol synthesis in plants other than Arabidopsis, lipids were isolated from leaves of the *sxd1* mutant and analyzed for tocopherols by HPLC. As with *vte1-1* and *vte1-2*, leaves of the *sxd1* mutant lack tocopherols, whereas wild-type maize leaves contain both α- and γ-tocopherols (Fig. 2B). Prenyl quinones from leaves of wild-type maize and the *sxd1* mutant were also analyzed by HPLC. This analysis indicated that like the *vte1* mutants, *sxd1* contained a prenyl quinone that was absent from wild type (Fig. 3B) with an absorbance spectrum and retention time identical to the DMPBQ that accumulated in *vte1* mutants.

To show that this gene family has an identical function in cyanobacteria and plants, an insertion mutant, Δslr1737, was created in the slr1737 open reading frame (ORF) of *Synechocystis* sp. PCC6803.

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**Table 1** Pairwise comparisons of VTE1 orthologs from plants and cyanobacteria

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<th>Synceho</th>
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<td>55</td>
<td>61</td>
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**Figure 5.** Alignment of the carboxy termini of TC orthologs from plants and cyanobacteria. The asterisk above the At4g32770 protein sequence denotes the position of the vte-1 mutation. *Anabaena, Anabaena* sp. PCC7120 (all0245); Chlamy, *Chlamydomonas reinhardtii*; Medicago, *M. truncatula*; Nostoc, *N. punctiforme* (506-74); Physcomitrella, *Physcomitrella patens*; *Synecho, Synechococcus* sp. PCC7002. The *P. patens*, rice (Oryza sativa), and wheat (Triticum aestivum) sequences are partial sequences obtained from ESTs. Dark shading, Amino acid identity; light shading, Amino acid similarity. The threshold for amino acid consensus identity or similarity is 51%.
immediately after (Fig. 2). The two unknown peaks detected in a wild-type tocopherol profile to described in “Plant Physiol. Vol. 132, 2003 2189
standards. The14C incorporation into separated by TLC, and radiolabeled products were detected by phosphor imager analysis. Products were identified by comigration with standards. The 14C incorporation into γ-tocopherol was quantified densitometrically and expressed as pixels per microgram of total protein.

through homologous recombination. The growth rate of ∆slr1737 was indistinguishable from wild type (data not shown). Lipids were extracted from ∆slr1737 and wild-type cells and analyzed for tocopherol composition by HPLC. ∆slr1737 lacked α-tocopherol, similar to the vte1 and sxdl mutants (Fig. 2). The two unknown peaks detected in ∆slr1737 immediately after α-tocopherol are also present in the tocopherol-deficient mutants ∆slr1736 and ∆slr0090 (Dahnhardt et al., 2002) and do not correspond to tocopherols or tocopherol pathway intermediates. HPLC analysis of prenyl quinones in ∆slr1737 and wild type showed that, like vte1 and sxdl, ∆slr1737 accumulates DMPBQ (Fig. 3C).

As further proof that the cyanobacterial and plants genes are functionally equivalent, a SXD1 cDNA expression cassette was transformed into the ∆slr1737 line. Lipids were isolated from the cells and analyzed by HPLC for tocopherols. The transformed cells contained α-tocopherol (Fig. 2C) and did not accumulate DMPBQ (Fig. 3C). Thus, expression of SXD1 restored a wild-type tocopherol profile to ∆slr1737, indicating that SXD1 is able to functionally complement ∆slr1737. Hence, these cyanobacterial and plant genes have not only high sequence similarity but also functional equivalency, suggesting a common evolutionary ancestry.

To determine the activity of the VTE1 protein and its maize and cyanobacterial orthologs, we expressed VTE1, SXD1, and slr1737 in Escherichia coli using the pET expression system. Lysates from E. coli expressing either VTE1 or SXD1 were able to convert [14C]2,3-dimethyl-6-phytyl-1,4-benzoquinol into γ-tocopherol (Fig. 6). This result conclusively demonstrates that both genes encode an enzyme with TC activity. Activity was not observed with the slr1737 protein expressed in E. coli for reasons that are unknown.

Carbohydrate Assimilation in vte1

Although SXD1 and VTE1 have similar enzymatic activities (Fig. 6) and primary biochemical phenotypes (tocopherol deficiency and DMPBQ accumulation, Figs. 2 and 3), sxdl was initially isolated because of a secondary phenotype, a Suc transport defect (Russin et al., 1996; Provencher et al., 2001). To determine whether vte1 caused a similar Suc transport defect, Glc, Suc, and starch levels were analyzed in mature leaves from 4-week-old vte1-1 plants. The leaves were sampled at the end and at the beginning of the photoperiod. There were no significant differences between wild-type and vte1-1 leaves for Glc, Suc, and starch at the beginning or end of the photoperiod (Table II). Thus, unlike the sxdl mutant, carbohydrate metabolism in mature leaves appears unaffected by the vte1 mutation. The molecular and biochemical similarities of the VTE1 and SXD1 proteins suggest the difference in sxdl and vte1 carbohydrate metabolism phenotypes in maize and Arabidopsis reflects additional roles of tocopherols or the tocopherol pathway beyond antioxidant chemistry, rather than simply a difference in the enzymatic activity of the SXD1 and VTE1 proteins.

**DISCUSSION**

In this report, we have shown that the three proteins, VTE1, SXD1, and slr737 from a dicot, monocot,

![Figure 6. TC activity of proteins expressed in E. coli. E. coli cell lysates from cells overexpressing the empty pET vector or pET engineered to express TC proteins from Arabidopsis, maize, and Synecocystis sp. PCC6803 were incubated with radiolabeled 2,3-methyl-6-phytyl-1,4-benzoquinol (3 methyl 14C) for 4 h as described in “Materials and Methods.” Total lipids were extracted, separated by TLC, and radiolabeled products were detected by phosphor imager analysis. Products were identified by comigration with standards. The 14C incorporation into γ-tocopherol was quantified densitometrically and expressed as pixels per microgram of total protein.](image-url)

Table II. Analysis of Glc, Suc, and starch in wild-type Arabidopsis and vte1-1

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<tr>
<td>Glc</td>
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<td>2.46 ± 0.58</td>
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<tr>
<td>Suc</td>
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<td>0.16 ± 0.16</td>
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<tr>
<td>Starch*</td>
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* Starch is expressed as nanomoles of Glc monomers per milligram fresh wt.
and cyanobacterium, respectively, function as TCs. Mutations in the TC gene from each organism result in identical primary biochemical phenotypes, a block in tocopherol synthesis, and accumulation of DMPBQ, the endogenous substrate for the TC. In addition, the SXD1 and VTE1 proteins expressed in E. coli were able to convert DMPBQ to γ-tocopherol. Finally, expression of maize SXD1 was sufficient to complement the tocopherol-deficient phenotype of the Synechocystis sp. PCC6803 slr1737 deletion mutant (Δslr1737). This result demonstrates that slr1737 and SXD1 are functionally equivalent and that the biochemical activity of TCs has been evolutionarily conserved between plants and cyanobacteria. Our finding that At4g32770 encodes a functional TC in Arabidopsis concurs with a recent report by Porfirova et al. (2002).

**Sequence Analysis and Evolutionary Implications of the TC Family**

The TCs (VTE1, SXD1, and slr1737) share significant amino acid similarity with each other and define an evolutionarily conserved gene family that includes putative orthologs in a large number of other plants and cyanobacteria. VTE1 orthologs were not identified in databases of fungal, animal, or non-photosynthetic bacterial species, none of which are known to produce tocopherols. Full-length sequences of two additional VTE1 orthologs from plants (barley and M. truncatula) and three from cyanobacteria (N. punctiforme, Anabaena sp. PCC7120, and Synechococcus sp. PCC7002) were identified in the public databases. Although all VTE1 proteins share a high degree of amino acid similarity, they are devoid of any previously described protein motifs, with the exception of ubiquitous phosphorylation and myristolation motifs. All VTE1 orthologs are hydrophobic proteins with low pIs and a high number of conserved Trp residues (Provencher et al., 2001). These characteristics are consistent with the TC activity characterized in Anabaena variabilis being membrane associated (Stocker et al., 1993, 1994, 1996).

Although plant and cyanobacterial TCs exhibit a high degree of protein sequence similarity, plant orthologs have additional N- and C-terminal domains that are absent in the four cyanobacterial TCs. The N-terminal domains of plant VTE1 orthologs are poorly conserved and are predicted to encode chloroplast transit peptides that would target each protein to the chloroplast. The N-terminal sequence of SXD1 has been demonstrated experimentally to be required for import into plastids (Provencher et al., 2001). HPT and γ-TMT, two other tocopherol biosynthetic enzymes, are also predicted to be chloroplast targeted (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Schledz et al., 2001; Savidge et al., 2002), and chloroplast localization of the TC is consistent with the reported localization of TC activity and tocopherol synthesis in plastids (Soll et al., 1985; Arango and Heise, 1998a).

In contrast to the N-terminal domain of plant TCs, the 30-amino acid C-terminal domain is highly conserved between angiosperms and the moss P. patens. This evolutionary conservation suggests an important function for this domain in tocopherol synthesis in plants, whereas the absence of the sequence from the four cyanobacterial VTE1 orthologs suggests that the domain is not an absolute requirement for TC enzymatic activity per se. The restriction of this C-terminal domain to vascular and nonvascular plants suggests it arose relatively recently in progenitors of land plants rather than in the endosymbiotic cyanobacteria that gave rise to plastids (Goksöy, 1967). The shortened C-terminal domain present in the C. reinhardtii TC further suggests evolution took place in an ancestor common to C. reinhardtii and plants (i.e. before the split of Chlorophyta and Charophyta; Karol et al., 2001). The vte1-2 mutation causes deletion of the majority of the conserved C-terminal domain, and tocopherols fail to accumulate in vte1-2 leaf tissue but reach 25% of wild-type levels in seeds, indicating the truncated protein retains at least partial activity in vivo. The vte1-2 phenotype suggests the C-terminal domain plays a more significant role in TC activity/function in leaf chloroplasts than in the plastids of seeds. The complementation of Δslr1737 by SXD1 suggests that the presence of the C-terminal domain does not affect TC activity and function in cyanobacteria. The relevance of this highly conserved carboxyl domain for TC function in plants requires further investigation.

**The sxdl and vte1 Phenotypes**

A surprising phenotype of the sxdl mutants is a block in Suc export from leaves and an accumulation of anthocyanins and starch in leaf blades (Russin et al., 1996). This pleiotropic phenotype results from aberrant plasmodesmata at the interface between the bundle sheath cells and the vascular parenchyma cells surrounding the minor veins. These defective plasmodesmata block symplastic transport of Suc to the phloem and, hence, cause the Suc export defect. Unlike sxdl, vte1-1 and vte1-2 do not accumulate anthocyanins in leaves, do not accumulate starch in cells surrounding the leaf veins, and do not have stunted growth (data not shown). In addition, the similar Suc, Glc, and starch levels in mature leaves of vte1-1 and wild type (Table II) indicate that a functional Suc export pathway is present in vte1. However, this most obvious difference in phenotype between sxdl and vte1 is not entirely unexpected considering the anatomical and physiological differences between C3 and C4 plants. Maize bundle sheath cells contain chloroplasts that differ morphologically and physiologically from the chloroplasts of mesophyll cells (Evert et al., 1977a; Fahn, 1990).
Maize bundle sheath plastids have few grana, little PSII activity, high NADP-malate decarboxylase activity, and accumulate starch (Evert et al., 1977a, 1977b, 1978; Fahn, 1990). Arabidopsis lacks a physiologically equivalent cell type to the C4 bundle sheath cell. Chloroplasts within the analogous cells surrounding the minor veins in Arabidopsis do not differ significantly from the chloroplasts of mesophyll cells (Hari-tatos et al., 2000). Mutants of the Arabidopsis SUC2 gene, which encodes a Suc-H+/H2-symporter required for apoplastic phloem loading, share striking similarities to sxd1 mutants (Gottwald et al., 2000). suc2 mutants accumulate anthocyanins and starch in their cotyledons, have stunted growth, and are seedling lethal in soil (Gottwald et al., 2000). Thus, the absence of the sxd1 Suc export phenotype in vte1 suggests that disruption of VTE1 in Arabidopsis either does not affect Suc export, most likely because of the fundamentally different mechanisms of Suc export in maize and Arabidopsis, or that the effect is too small to be observable as an analogous whole-plant phenotype in Arabidopsis.

In sxd1 mutants, the link between the production of aberrant plasmodesmata in the BS parenchyma cells and the defect in symplastic transport of Suc was straightforward and easy to rationalize (Provencher et al., 2001). However, the mechanistic link between the disruption of a gene encoding a chloroplast protein of unknown function (SD1) and defective Suc transport was not so obvious. Provencher et al. (2001) raised the possibility that the sxd1 mutation exerts its pleiotropic phenotype by disrupting or altering a signal from the chloroplast to the nucleus. The nature of this signal was unknown, and cloning of the SD1 locus did not provide further insight. Although the precise nature of this signal remains unclear, the demonstration that SD1 encodes a TC and determination of the primary sxd1, vte1, and Δsdr1737 biochemical phenotypes now greatly limits the possibilities.

The absence of tocopherols in vte1 and Δsdr1737 does not cause a pleiotropic phenotype analogous to sxd1. vte1 mutants are indistinguishable from wild type under normal growth conditions, and Δsdr1736 and Δsdr1737 mutants grow at rates identical to wild type (Collakova and DellaPenna, 2001). These data suggest that the sxd1 phenotype is not simply due to the absence of tocopherols as lipophilic antioxidants because similar effects would be observed in the vte1, Δsdr1737, and Δsdr1736 mutants, which also lack tocopherols. In addition, the DMPBQ that accumulates in vte1, sxd1, and Δsdr1737 mutants can still act as an antioxidant by donating a pair of electrons and then being recycled by reduction back to the quinol form of DMPBQ (Kruk et al., 1994; Kruk and Strzalka, 1995; Liebler and Burr, 2000). Thus, even in the absence of tocopherols, the sxd1 mutant is not entirely deficient in membrane-associated antioxidants.

Influencing membrane fluidity is another potential function of tocopherols, and this could be related to the plasmodesmatal defect in sxd1 mutants. However, the consensus from cell fractionation studies is that tocopherols are localized exclusively in plastid membranes (Lichtenhager et al., 1981; Fryer, 1992; Kruk and Strzalka, 1995), and although it cannot be excluded, it is unlikely that tocopherols or DMPBQ would be present in plasma membranes to directly impact plasmodesmatal development. However, the bundle sheath cell is a specialized cell type, and direct physical connections between plasmodesmata and chloroplasts through the endoplasmic reticulum have been reported in maize bundle sheath cells (Evert et al., 1977a, 1977b). This potential association between the chloroplast and plasmodesmata would provide a means for tocopherols or prenyl quinones to impact membrane fluidity at the plasma membrane. Still, it is difficult to envision a mechanism whereby membrane fluidity would alter plasmodesmatal development. If diminished antioxidant capacity or altered membrane fluidity is not the cause of the sxd1 phenotype, the question still remains: How does disruption of TC activity result in the pleiotropic sxd1 phenotype?

In addition to the well-defined role of tocopherols as antioxidants, specific tocopherols, tocotrienols, and their oxidized products have been demonstrated to have biological activities in mammalian systems that are independent of their antioxidant functions. The unifying theme for these antioxidant-independent activities is the modification or modulation of various signal transduction pathways (Brigelius-Flohe and Traber, 1999; Ricciarelli et al., 2001; Clement et al., 2002). The effects of tocopherols on the protein kinase C signaling cascade and the synthesis of eicosanoids in mammals have been well characterized. α-Tocopherol posttranslationally inhibits the activity of protein kinase C in several mammalian systems (Chan et al., 2001; Azzi et al., 2002; Clement et al., 2002). Plant genomes also contain protein kinase C homologs and other components of this signaling pathway. Tocopherols also have been shown to posttranslationally inhibit the activity of phospholipase A2 (Tran et al., 1996; Chandra et al., 2002), cyclooxygenase (COX-2; Jiang et al., 2000; Wu et al., 2001), and lipoxygenase-5 (Greenberglevy et al., 1993; Wang et al., 2000; Ricciarelli et al., 2002). These enzymes are involved in the production of eicosanoid signaling molecules (prostaglandins, prostacyclins, thromboxanes, and leukotrienes) from polyunsaturated fatty acids in mammals (Ohuchi and Levine, 1980; Tran et al., 1996; Kim et al., 2001). Like eicosanoids in animals, jasmonic acid and other oxylipins in plants are synthesized from polyunsaturated lipids by the action of lipoxygenase(s) and phospholipase(s) (Blee, 2002; Howe and Schimiller, 2002). A third example of α-tocopherol-dependent, antioxidant-independent signal transduction in mammals is the transcriptional activator TAP...
(tocopherol-associated protein). Upon the binding of α-tocopherol by TAP, the complex is translocated into the nucleus, where it has been shown to activate the transcription of transgenes (Yamauchi et al., 2001).

Although tocopherols have not yet been shown to affect protein kinase C signaling, transcriptional regulation, or the synthesis of jasmonic acid or other oxylipins in plants, studies in mammalian systems suggest plausible mechanisms whereby the absence of TC activity (and, hence, tocopherols) could affect signaling and result in the pleiotropic sxdI phenotype. Thus, it is possible that many tocopherol functions will be universal, including the roles tocopherols play in modulating signal transduction pathways or acting as signals themselves. Although the downstream events of signal transduction pathways would not necessarily be evolutionarily conserved between plants and mammals, many of the core components and biochemical motifs of signal transduction pathways are. We suggest that the sxdI phenotype is the first evidence that tocopherols act as signaling molecules or modulators of signaling in plants. Tocopherols, tocopherol derivatives, or tocopherol pathway intermediates may provide or modulate signals required for the development of maize bundle sheath vascular parenchyma plasmodesmata, analogous to the effects of tocopherols in mammalian signaling. Alternatively, the DMPBQ that accumulates in sxdI may interfere with an endogenous signaling pathway required for the process. Several groups have provided evidence that the redox status of the chloroplast, which is monitored through the plastoquinone (PQ) pool, regulates nuclear-encoded photosynthetic gene expression (Pfannschmidt et al., 1999a, 1999b, 1999c; Allen et al., 2000; Allen and Pfannschmidt, 2000; Kujat and Oettl, 2000; Li and Sherman, 2000; Pursiheimo et al., 2001; Trebibsh and Danon, 2001; Yang et al., 2001). DMPBQ has the same 2,3-dimethyl-1,4-benzoquinone head group as PQ and could interfere with redox signaling through the PQ pool. Another more remote possibility is that the SXDI protein has an unknown substrate in addition to DMPBQ, and this product is required for signal transduction in maize. Although none of these models can be excluded based on the present data, they do provide a framework for future study.

The observation that Arabidopsis vte1 mutants do not exhibit phenotypes analogous to sxdI suggests that the downstream signal transduction events impacted by tocopherol deficiency differ between monocots and dicots. The pathways leading to maize bundle sheath vascular parenchyma plasmodesmata formation may either be absent or not equivalent in Arabidopsis, or the effects are too subtle to be observed at the whole-organism level as in sxdI. Experiments to assess the whole-genome responses of Arabidopsis vte1 mutants are under way.

**MATERIALS AND METHODS**

**Growth Conditions and Seed Stocks**

Arabidopsis plants were grown at 22°C under a 12-h photoperiod (120 μE) in a vermiculite and potting soil mixture. M₃ EMS-mutagenized Arabidopsis seeds (Columbia ecotype) were purchased from Lehle Seed (Round Rock, TX). vte1-2 was backcrossed to wild type three times, and vte1-2 was backcrossed twice. Maize (Zea mays) plants were grown under greenhouse conditions in the same soil mixture and fertilized biseweekly with 20-20-20 fertilizer. The maize sxdI-2 allele used in this publication was isolated through the Trait Utility System for Corn (Pioneer Hybrids, Johnston, IA). The sxdI-2 Mu insertion site and mutant phenotype were described previously (Provencher et al., 2001). Synechocystis sp. PCC6803 was grown on BG-11 media photosynthetically or photonmixotrophically (BG11 media containing 15 mM Glc) on plates or in liquid culture at 30°C and 50 to 70 μE light.

**Tocopherol Analysis**

For tocopherol analyses, total lipids were extracted from 30 to 35 mg of Arabidopsis or maize leaf tissue or 15 to 20 mg of plate-grown Synechocystis sp. PCC6803 cells (Bligh and Dyer, 1959; Collakova and DellaPenna, 2001), and dissolved in 100 μL of methanol or hexane. Methanol extracts (50 μL) were subject to HPLC (Agilent 1100 series, Agilent, Wilmington, DE) on a Spherisorb ODS-2 5-μm, 250 × 4.6-mm reverse phase column (Column Engineering, Ontario, CA) at 28°C with a flow rate of 2 mL min⁻¹ with 95% (v/v) methanol and 5% (v/v) isopropanol. Hexane extracts (50-μL volume) were subjected to HPLC on a ReliaSil Silica 5-μm, 250 × 4.6-mm normal phase column (Column Engineering) at 42°C with a flow rate of 2 mL min⁻¹ with 85% (v/v) hexane and 15% (v/v) isopropanol ether. Tocopherols were detected by fluorescence using 290-nm excitation and 325-nm emission.

**Analysis of Prenyl Quinones**

One gram of Arabidopsis or maize leaf tissue and a 500-μL culture of Synechocystis sp. PCC6803 (OD₅₇₀ = 0.8) were harvested and total lipids extracted (Collakova and DellaPenna, 2001). Prenyl quinones were purified by TLC as described by Pennock (1985) and eluted with diethyl ether. After drying, the samples were resuspended in 500 μL of hexane. A 100-μL aliquot was dried, reconstituted in 75 μL of isopropanol, and subjected to HPLC on a reverse phase column (described above) under conditions previously described by Johnson et al. (2000). Alternatively, 60 μL of hexane extract was subjected to HPLC on a normal phase column (described above), at 30°C with a 1 mL min⁻¹ flow rate using 0.1% (v/v) dioxane in hexane. Prenyl quinones were detected by A₂₅₄ using a diode array detector.

**Map-Based Cloning of vte1**

PCR-based markers were designed using INDEL or SNP from the Cereon Arabidopsis Polymorphism and Landsberg erecta Sequence Collection (Cereon Genomics LLC, Cambridge, MA; Jander et al., 2002). DNA was extracted from 1- to 2-mm developing leaves using Plant DNAzol (Invitrogen, Carlsbad, CA) following the protocol of the manufacturer. Alternatively, DNA was isolated from 1- to 2-mm developing leaves in 1.1-mL tubes arrayed in a 96-well format. Two 4-mm glass beads were added to tubes along with 200 μL of 10 mM Tris (pH 8.0) and 200 μL of chloroform. The tubes were shaken with a commercial paint shaker for 5 min and centrifuged at 1,750 rpm for 10 min. One microliter of the aqueous phase or resuspended DNA from DNazol extractions was used in a 20-μL PCR reaction.

**Sequence Analysis**

All DNA sequences other than vte1 mutant alleles were obtained from public databases using BLAST: wheat (Triticum aestivum; BQ19951), rice (Oryza sativa; AU021770), Phycomitrella patens (BJ164574), M. truncatula (BF641171 and TC48011), barley (Hordeum vulgare; TC33553 and TC32886), Arabidopsis (AF302188), and maize (AF302187). A TC prefix denotes sequences obtained from The Institute for Genomic Research. All others have GenBank accession numbers. The cyanobacteria and algae sequences were
obtained from their respective genome sequencing projects. *Synechocystis* sp. PCC6803 and *Anabaena* sp. PCC7120 sequences were obtained from Cyanobase (http://www.kazusa.or.jp/cyano/cyano.html). *Chlamydomonas reinhardtii* Nostoc sequences were obtained from the Joint Genome Institute (http://www.jgi.doe.gov). The *Synechococcus* sp. PCC7002 sequence was obtained from National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Alignments were performed using MacVector 7.0 (Oxford Publishing, London), which includes the ClustalW algorithm.

**Construction of slr1737, SXD1, and VTE1 Protein Expression Vectors**

Primers 5'-CATATGACCCCTAATTTATCCTTTTGTG-3' (F1), 5'-CATATG-GCAAGAATTCCTGCTTAAACTCTG-3' (F2), 5'-CTCAGTTATAGCACGGGTG-GGTGTT-3' (R5), and turbo Pfu (Stratagene, La Jolla, CA) were used to PCR amplify the VTE1 cDNA from a seed cDNA library (a gift from Dr. John Ohlrogge, Michigan State University, East Lansing). F2 was used to amplify the full-length cDNA, and F2 was used to amplify a truncated version of the cDNA that encodes a version of the protein lacking chloroplast transit peptide. PCR products were cloned into the Smal site of pBluescript II SK- for SXD1 and slr1737. The two versions of the VTE1 cDNA were cloned as Ndel-Xhol fragments (sites underlined in primers) into Ndel and Xhol sites of the pET30b expression vector (Novagen, Madison, WI).

Primers 5'-CATATGAAATTTATCCGGCCCCAACGTGGTAC-3' (F1) and 5'-GGATCTTAAAGCAATACAAAAACAGGC-3' (R5), and turbo Pfu were used to amplify the slr1737 gene from *Synechocystis* sp. PCC6803 genomic DNA. The PCR product was cloned into the EcoRV site of pBluescript II SK- to PCR amplify the 5'-flanking portions. The PCR product was cloned into the Ndel and EcoRI sites of the pET30b expression vector (Novagen). The fidelity of all constructs was confirmed by sequencing.

**Generation of Δslr1737**

Primers 5'-CTCTATGTTATCATCAGGGTGCC-3' (F3) and 5'-GGAGATTTAGCAGGACGATGGCT-3' (R4) and Pfu polymerase (Stratagene) were used to PCR amplify the 5' region flanking slr1737 from *Synechocystis* sp. PCC6803 genomic DNA. Primers 5'-ATAAATATCTCTGATCTCCCGAGAATAA-CATGCTCTTTTGTG-3' (F3) and 5'-ACCTGTCCTTCAACACTATT-3' (R5) and Pfu polymerase (Stratagene) were used to PCR amplify the 3' region flanking slr1737 from *Synechocystis* sp. PCC6803 genomic DNA (underlined nucleotides indicate an added BsiWI restriction site). The 5' and 3'-flanking PCR products were joined through re-amplification with Pfu polymerase using Primers F3 and R4 to generate a contiguous 1,041-bp fragment containing a BsiWI site separating the 5' and 3'-flanking portions. The PCR product was cloned into the pPCR-Script AMP vector (Stratagene) to generate pSLR1737-5'flank. The aadA gene encoding spectinomycin adenyltransferase was inserted as a BsiWI fragment into the corresponding site of pSLR1737-5'flank. The resulting plasmid was then used to replace ORF slr1737 by homologous recombination (Williams, 1988). Combinant lines were selected by spectinomycin resistance, and replacement of the slr1737 ORF with the aadA gene was confirmed by PCR.

**Complementation of Δslr1737 by Expression of the Maize SXD1 cDNA**

A vector, designated pStynExp-2, was used to express SXD1 in *Synechocystis* sp. PCC6803, pStynExp-2 was derived from pPCR-Script AMP (Stratagene) and contained the *Synechocystis* sp. PCC6803 psbA2 promoter linked to a multicloning site to Tn9, which encodes chloramphenicol acetyltransferase. To facilitate homologous recombination, the promoter and multicloning site were flanked by St12- and 429-bp sequences from the slr2699 locus. Primers 5'-TTTTTTTTTGTGATCAGGCGCGATACGGGCTACC-3' (F5) and 5'-TTTTTTTTGATCGATCAGGCGCTTCTGGTTGAACAT-TAG-3' (R5) and Pfu polymerase were used to PCR amplify the coding sequence of the mature maize SXD1 polyepitope from the SXD1 cDNA. The PCR product was cloned into the Ndel and BsoII restriction sites of the multicloning site behind the psbA2 promoter. The resulting plasmid was used to transform Δslr1737 through homologous recombination of the slr2699 locus. Recombination events were selected by chloramphenicol resistance, and the introduction of the maize SXD1 cDNA into slr1737 knockout mutants was confirmed by PCR analysis of genomic DNA isolated from chloramphenicol- and spectinomycin-resistant (selection for Δslr1737) resistant cell lines.

**TC activity from *Escherichia coli*-Expressed Proteins**

C45 (DE3) cells containing the relevant pET vectors engineered to express TCs from the three organisms were grown at 30°C in 50 mL of Luria-Broth culture (50 mg mL⁻¹ kanamycin) to mid-log phase (0.4-0.6 OD₆₀₀nm) then 1 mM isopropyl-β-D-galactoside were added and grown at 15°C for 18 h (Miroux and Walker, 1996). Cells were harvested by centrifugation and washed with 100 mM KHPO₄ (pH 7.8) and 4 mM MgCl₂. The cells were resuspended in 1 mL of 100 mM KHPO₄ (pH 7.8), 4 mM MgCl₂, 4 mM dithiothreitol, and 4 mM glutathione, and sonicated six times for a duration of 10 s at 40 Hz. Dodecyl maltoside was added to 0.08 mM, and the cell lysate was shaken gently for 1 h at 4°C. Radiolabeled DMBPQ substrate was prepared by enzymatic labeling of MPBQ with 14C-adenosyl-l-Met (Amersham, Piscataway, NJ) using heterologously expressed *Synechocystis* sp. PCC6803 MPBQ methyl transferase (Shintani et al., 2002). The labeled DMBPQ was purified from the reaction by TLC (Pennon, 1985). Each 100-μL TC assay contained 4.5 × 10⁻⁴ μL of 2.9μCi 3-dimethyl-6-phytyl-1,4-benzoquinol. The substrate was reduced with 2 μg of NaBH₄ immediately before the assay. Each reaction contained 100 mM KHPO₄ (pH 7.8), 4 mM MgCl₂, 0.8 mM dithiothreitol, 0.8 mM glutathione, 0.8 mM dodecyl maltoside, 20 mM ascorbic acid, 1 mM cyclodextrin, and 20 μL of cell lysate from *E. coli* expressing SXD1, VTE1, or slr1737. The dodecyl maltoside and cyclodextrin were added first to solubilize the substrate. The reactions were sparged with nitrogen gas and gently shaken for 4 h at room temperature. Total lipids were extracted in extraction buffer containing 1 mg mL⁻¹ butylated hydroxytoluene and unlabeled 20 μg mL⁻¹ γ-tocopherol as a carrier. The prenyl lipids were separated by TLC, and the presence of the unlabeled prenyl quinone and tocopherol standards was determined by staining with Emmeric-Engel reagent (Pennon, 1985). 14C-labeled compounds were detected by a 4-d exposure to a low-energy Phosphorimage screen (Molecular Dynamics, Piscataway, NJ) and analyzed using a Phosphorimeter (Molecular Dynamics).

**Analysis of Glc, Suc, and Starch in Leaves**

Mature leaves from 4-week-old Arabidopsis plants were harvested at the end and the beginning of the photoperiod. Sugars were extracted from leaf tissue in 80% (v/v) ethanol at 80°C for 30 min. Starch was also extracted from the cleared leaf tissue using 0.2 μM KOH at 95°C for 45 min and neutralized with 1 μM acetic acid to pH 5.0. The sugars and starch levels were measured enzymatically through the conversion of NAD to NADH by Glc-6-phosphate dehydrogenase and observed at 340 nm as described (Stitt et al., 1989).

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**LITERATURE CITED**


**Tocopherol Cyclase**

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