

IN THIS ISSUE

Vitamin E–Defective Mutants of Arabidopsis Tell Tales of Convergent Evolution

Tocopherols (collectively known as vitamin E) are lipophilic antioxidants that are essential in the human diet. Not surprisingly, most of what is known about the biological functions of tocopherols comes from studies of mammalian systems, yet they are synthesized only by photosynthetic eukaryotes and some oxygenic cyanobacteria. Tocopherols belong to a diverse group of compounds known as prenylquinones, which also includes the photosynthetic electron carrier plastoquinone. These compounds have aromatic redox-active head groups that are exposed to the membrane surface and hydrophobic prenyl tails that are embedded in the membrane. Tocopherols generally are believed to play an important antioxidant role against lipid free radicals generated as a result of aerobic metabolism and to help prevent human diseases associated with oxidative stress, such as cardiovascular disease, cancer, chronic inflammation, and neurological disorders (Ricciarelli et al., 2002).

There is growing evidence that tocopherols play other important roles in signaling and gene regulation in animals that are not related to their antioxidant function (Ricciarelli et al., 2002). In plants, tocopherols are synthesized at the inner chloroplast envelope and are believed to have an important function in protecting the photosynthetic apparatus from toxic free radicals generated during photosynthesis (Porfirova et al., 2002). To date, there is very little evidence for signal transduction-related or other nonantioxidant roles in plants. One of the only studies to suggest such a function is based on the characterization of the *sucrose export defective1* (*sxd1*) mutant of maize. The *sxd1* mutant was identified originally based on a block in sucrose transport from leaves caused by the aberrant formation of plasmodesmata between bundle sheath cells and vascular parenchyma cells (Provencher et al., 2001). When

the *sxd1* locus was cloned, it was found to encode a chloroplast protein of unknown function, leading to the hypothesis that SXD1 somehow functions in signaling from the chloroplast. Sattler et al. (2003) subsequently showed that the maize *SXD1* gene encodes tocopherol cyclase, which catalyzes the conversion of phytyl quinol intermediates to their corresponding tocopherols and is essential for the biosynthesis of all four tocopherol products (Figure 1). These authors postulated that tocopherols might modulate signals required specifically for

the development of maize bundle sheath vascular parenchyma plasmodesmata, and tocopherol cyclase mutants of Arabidopsis and *Synechocystis* sp PCC 6803 do not show a similar phenotype because they lack a cell type physiologically equivalent to the C4 bundle sheath cell (Sattler et al., 2003). However, direct evidence of a role for tocopherols in signaling or gene regulation is lacking.

In plants, tocopherols and plastoquinone are synthesized from the precursor homogentisic acid (HGA) (Figure 1). The first

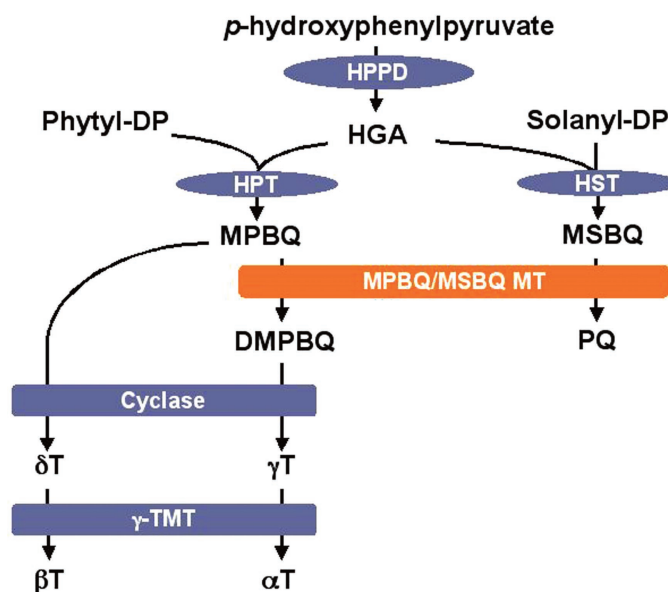


Figure 1. Tocopherol and Plastoquinone Biosynthetic Pathways in Higher Plants.

The pathway shown for tocopherol synthesis is identical in plants and cyanobacteria, but cyanobacteria appear to have an alternative route for plastoquinone (PQ) synthesis (see text). α -Tocopherol is the most abundant tocopherol produced in wild-type Arabidopsis leaves and *Synechocystis* sp PCC 6803. Cyclase, tocopherol cyclase; DMPBQ, 2,3-dimethyl-5-phytyl-1,4-benzoquinone; HGA, homogentisic acid; HPPD, *p*-hydroxyphenylpyruvate dioxygenase; HPT, homogentisate phytyltransferase; HST, homogentisate solanyletransferase; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinone; MPBQ/MSBQ MT, MPBQ/MSBQ methyltransferase; MSBQ, 2-methyl-6-solanyle-1,4-benzoquinone; phytyl-DP, phytyl-diphosphate; SAM, S-adenosyl-L-Met; solanyle-DP, solanyle-diphosphate; γ -TMT, γ -tocopherol methyltransferase.

IN THIS ISSUE

committed step in plastoquinone synthesis is the condensation of HGA with solanyl-diphosphate by the enzyme homogentisate solanyltransferase to yield 2-methyl-6-solanylbenzoquinol (MSBQ), which is further methylated in the aromatic ring to produce plastoquinone. The first committed step in tocopherol biosynthesis is the condensation of HGA with phytyl-diphosphate by the enzyme homogentisate phytyltransferase to yield 2-methyl-6-phytylbenzoquinol (MPBQ), which then undergoes ring methylation followed by ring cyclization to yield γ -tocopherol; a second ring methylation yields α -tocopherol. Alternatively, MPBQ can undergo the cyclization directly to form δ -tocopherol and the second ring methylation step to yield β -tocopherol.

There are distinct features of these pathways in higher plants relative to cyanobacteria. For example, in *Arabidopsis*, a null mutation in the enzyme that produces HGA leads to deficiencies in tocopherol and plastoquinone and to seedling lethality (Norris et al., 1998), suggesting that HGA is the only precursor for both compounds in this higher plant. By contrast, an orthologous *Synechocystis* sp PCC 6803 mutant lacks tocopherols but is not affected in plastoquinone synthesis and is viable (Dahnhardt et al., 2002), suggesting the existence of an alternative route for plastoquinone biosynthesis in cyanobacteria.

In *Synechocystis* sp PCC 6803, the first methylation of MPBQ en route to γ - and α -tocopherol and the methylation of MSBQ to yield plastoquinone (both at position 3 in the substrate aromatic ring) can be performed by the same enzyme, MPBQ/MSBQ methyltransferase (MT) (Shintani et al., 2002). This is the only tocopherol enzyme that has not yet been characterized in higher plants. In this issue of *The Plant Cell*, **Cheng et al. (pages 2343–2356)** report the cloning and characterization of MPBQ/MSBQ MT from *Arabidopsis*. The authors use a combination of genetic, biochemical, and genomic approaches to elucidate the evolution of the tocopherol and plastoquinone biosynthetic pathways in cyanobacteria and photosynthetic eukaryotes.

Cheng et al. first undertook a comprehensive search of numerous plant DNA databases and *Arabidopsis* predicted protein sequences in an attempt to identify a higher plant homolog of *Synechocystis* sp PCC 6803 MPBQ/MSBQ MT, which had been cloned and characterized previously by Shintani et al. (2002). This failed to identify any sequence that could be classified as a homolog of the *Synechocystis* sp PCC 6803 protein, despite evidence that plant chloroplasts contain MPBQ/MSBQ MT activity (Soll et al., 1985), providing the first hint that *Arabidopsis* and *Synechocystis* sp PCC 6803 MPBQ/MSBQ MT amino acid sequences likely share a low degree of similarity. The authors then made use of an HPLC-based screen to identify *Arabidopsis* mutants with altered leaf tocopherol profiles from an ethyl methanesulfonate-mutagenized population (Sattler et al., 2003). They identified a mutant line, designated *vitamin E defective3-1* (*vte3-1*), that exhibited reduced levels of γ - and α -tocopherol and increased levels of δ - and β -tocopherol, which is consistent with a reduction in MPBQ/MSBQ MT activity (Figure 1). The *VTE3* locus was isolated via map-based cloning, and the *vte3-1* sequence was found to contain a single nucleotide change resulting in the mutation of a Thr to an Ile. A second mutant allele, designated *vte3-2*, was isolated from the Salk T-DNA population and contains an insertion predicted to result in the complete loss of enzyme activity. Motohashi et al. (2003) recently identified a third allele of *VTE3* in a *Ds* transposon-tagged line of *Arabidopsis*. This mutant, designated *albino* or *pale green mutant1*, was found to be deficient in plastoquinone, but MT activity of the encoded protein was not verified in vitro and tocopherol levels were not assessed in *apg1* mutant tissue.

Cheng et al. showed that *VTE3* has MT activity with either MPBQ or MSBQ as the substrate in vitro and, like *Synechocystis* sp PCC 6803 MPBQ/MSBQ MT, it does not methylate δ - or β -tocopherol. A comparison of the *vte3-1* and *vte3-2* mutants suggested that it methylates both MPBQ and MSBQ in vivo but that the two sub-

strates have different requirements for *VTE3* amino acid residues for optimal enzyme activity. The single amino acid substitution in the *vte3-1* mutant did not abolish enzyme activity but preferentially impaired methylation of the tocopherol substrate MPBQ. This resulted in a significant reduction in α - and γ -tocopherol in leaves, but it had little effect on the methylation of the plastoquinone intermediate MSBQ. Plastoquinone levels were reduced only slightly, and the growth and development of seedlings were affected only mildly in this mutant relative to the wild type. By contrast, *vte3-2* mutant seedlings were pale green and did not survive for >1 week on soil. Analysis of this mutant showed a complete lack of α - and γ -tocopherol and plastoquinone in leaves, suggesting that *VTE3* is responsible for the methylation of both MPBQ and MSBQ in vivo and, interestingly, that *Arabidopsis* lacks functional redundancy for both of these enzyme activities.

A comparison of protein amino acid sequences confirmed that *Arabidopsis* *VTE3* is not an ortholog of the *Synechocystis* sp PCC 6803 MPBQ/MSBQ MT, because the two proteins share only 18% identity in their primary amino acid sequences. However, biochemical and genetic analyses showed that *Arabidopsis* *VTE3* represents the functional equivalent of *Synechocystis* sp PCC 6803 MPBQ/MSBQ MT. This finding is quite interesting from an evolutionary perspective, because all other enzymes of the tocopherol pathway are highly conserved between higher plants and cyanobacteria (Sattler et al., 2003). Together with their localization in plastid membranes, this suggests a cyanobacterial origin for most of these enzymes in higher plants, as postulated by Goksoyr (1967). Cheng et al. found that *VTE3*-type sequences are present in all available angiosperm databases and also in the moss *Physcomitrella patens*, the liverwort *Marchantia polymorpha*, and the green alga *Chlamydomonas reinhardtii* but not in cyanobacterial or other eubacterial genomes. Intriguingly, apparent orthologs to *VTE3* (~40% identical to the higher plant

IN THIS ISSUE

protein sequences) were found in two archaeal genomes. This finding suggests that convergent evolution acting on a protein of archaeobacterial rather than cyanobacterial origin gave rise to the higher plant MPBQ/MSBP MT enzyme VTE3. Notably, the archaeal sequences were found to be missing a C-terminal extension that was highly conserved among all of the eukaryotic VTE3-like proteins; thus, it appears to correspond to a protein domain unique to the eukaryotic protein. By contrast, sequences similar to the *Synechocystis* sp PCC 6803 gene were found only in cyanobacterial genomes and in two unicellular photosynthetic eukaryotes, *C. reinhardtii* and the diatom *Thalassiosira pseudonana*.

An intriguing observation is that VTE3 appears to have no functional redundancy in *Arabidopsis* and is required for the biosynthesis of both tocopherols and plastoquinone, the latter of which also is required for seedling viability. Yet, the cyanobacteria-type enzyme evidently was lost from higher plant genomes early in their evolutionary history. This suggests key differences in the requirements for this enzymatic step in most photosynthetic eukaryotes relative to prokaryotic cyanobacteria, requirements that were met by the ancestral eukaryotic VTE3-type MT, which was recruited to fulfill this function in higher plant chloroplasts, and that were not met by the cyanobacteria-type MT, which was lost subsequently from most eukaryotic genomes. Interestingly, *Synechocystis* sp PCC 6803 appears to have redundancy in MPBQ/MSBP MT activity,

because a homozygous null mutant of the *Synechocystis* sp PCC 6803 VTE3 homolog still contains approximately one-third of the wild-type levels of both α -tocopherol and plastoquinone. A more detailed study of these enzymes in *C. reinhardtii*, the only organism found to contain both types of MPBQ/MSBP MT, *T. pseudonana*, a eukaryotic organism that contains only the cyanobacteria-type enzyme, and archaea, which contain only a VTE3-like ortholog that lacks the eukaryotic C-terminal domain, might help explain the evolutionary history and biochemical requirements of these pathways in higher plants.

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