Remobilization of Phytol from Chlorophyll Degradation Is Essential for Tocopherol Synthesis and Growth of Arabidopsis

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Phytol from chlorophyll degradation can be phosphorylated to phytyl-phosphate and phytyl-diphosphate, the substrate for tocopherol (vitamin E) synthesis. A candidate for the phytyl-phosphate kinase from *Arabidopsis thaliana* (At1g78620) was identified via a phylogeny-based approach. This gene was designated *VITAMIN E DEFICIENT6* (*VTE6*) because the leaves of the Arabidopsis *vte6* mutants are tocopherol deficient. The *vte6* mutant plants are incapable of photoautotrophic growth. Phytol and phytyl-phosphate accumulate, and the phytyl-diphosphate content is strongly decreased in *vte6* leaves. Phytol feeding and enzyme assays with Arabidopsis and recombinant *Escherichia coli* cells demonstrated that VTE6 has phytyl-P kinase activity. Overexpression of *VTE6* resulted in increased phytyl-diphosphate and tocopherol contents in seeds, indicating that *VTE6* encodes phytyl-phosphate kinase. The severe growth retardation of *vte6* mutants was partially rescued by introducing the phytol kinase mutation *vte5*. Double mutant plants (*vte5 vte6*) are tocopherol deficient and contain more chlorophyll, but reduced amounts of phytol and phytyl-phosphate compared with *vte6* mutants, suggesting that phytol or phytyl-phosphate are detrimental to plant growth. Therefore, VTE6 represents the missing phytyl-phosphate kinase, linking phytol release from chlorophyll with tocopherol synthesis. Moreover, tocopherol synthesis in leaves depends on phytol derived from chlorophyll, not on de novo synthesis of phytyl-diphosphate from geranylgeranyl-diphosphate.

INTRODUCTION

Tocochromanols (vitamin E), a group of prenyl quinol lipids accumulating in cyanobacteria and chloroplasts of plants and green algae, are involved in the protection against oxidative stress and in adaptation to low-temperature conditions (Sattler et al., 2004; Havaux et al., 2005; Maeda et al., 2006). Depending on the side chain, three classes of tocochromanols can be distinguished, with tocopherols carrying a phytyl chain, tocotrienols harboring a geranylgeranyl chain, and plastochromanol-8 (PC-8) with a solanesyl chain. The biosynthesis of tocopherols includes the condensation of homogentisate with phytyl-diphosphate (phytyl-PP) catalyzed by homogentisate phytyl transferase (VTE2) (Collakova and Della-Penna, 2001; Savidge et al., 2002). In some plants, in particular monocotyledons, geranylgeranyl-diphosphate (GG-PP) is used for condensation with homogentisate by an alternative enzyme, homogentisate geranylgeranyl transferase, leading to the synthesis of tocotrienols (Cahoon et al., 2003; Yang et al., 2011). Subsequent methylation reactions and closure of the second ring by tocopherol cyclase (VTE1) result in the production of the four forms of tocopherol (α, β, γ, and δ) differing by the numbers and positions of the methyl groups on the chromanol ring (Dörmann, 2007; Hunter and Cahoon, 2007; Maeda and DellaPenna, 2007). PC-8 is produced from plastoquinol-9 via cyclization by VTE1 (Kleinig and Liedvogel, 1978; Mène-Saffrané et al., 2010; Zbierzak et al., 2010).

While the biosynthesis of the tocopherol head group has been studied in detail, less is known about the origin of the phytol moiety for tocopherol synthesis. Phytyl-PP is believed to largely originate from isoprenoid de novo synthesis via reduction of three double bonds in GG-PP. The corresponding enzyme, geranylgeranyl reductase (GGR), from plants has been characterized (Soll et al., 1983; Keller et al., 1998). Later, an alternative pathway for phytyl-PP production via phosphorylation of free phytol to phytyl-monophosphate (phytyl-P) and phytyl-PP was described (Ischebeck et al., 2006; Valentin et al., 2006). Free phytol can be derived from chlorophyll turnover and breakdown, in particular during senescence or chlorotic stress. Chlorophyll degradation starts with the removal of the magnesium cation, yielding pheophytin (Schelbert et al., 2009). Subsequently, phytol is cleaved from pheophytin by pheophytin pheophorbide hydrolase (PPH). Interestingly, GGR from *Arabidopsis thaliana* is capable of reducing the geranylgeranyl moiety in both GG-PP and the geranylgeranylated form of chlorophyll (Keller et al., 1998). Chlorophyll synthase can utilize phytyl-PP or GG-PP for prenylation of chlorophyllide (Soll et al., 1983). As the pool sizes and turnover rates of the isoprenyl-diphosphates in plants are unknown, the exact routes for chlorophyll and tocopherol synthesis are unclear. Increasing attention has been paid to the link of chlorophyll degradation and tocopherol accumulation. Overexpression of PPH in seeds resulted in a modest increase in tocopherol (Zhang et al., 2014).

These results suggested that manipulation of expression of genes involved in chlorophyll breakdown reveals minor effects on seed tocopherol levels and that a PPH-independent pathway for chlorophyll dephytylation might exist in seeds.

The first enzyme of phytol phosphorylation, phytol kinase, was isolated from Arabidopsis (*VTE5*, At5g04490) and *Synechocystis* (slr1652) (Valentin et al., 2006). The Arabidopsis *vte5* mutant contains 65 and 85% of tocopherol in leaves and seeds, respectively, compared with the wild type. However, it remained unclear to what extent phytol phosphorylation or the GG-PP-based de novo pathway contributes to overall phytyl-PP synthesis. Furthermore, the identity of the second enzyme of the phosphorylation pathway, phytyl-P kinase, remained unknown.

In prokaryotes, the genes of a common biosynthetic pathway are often localized in close proximity in the genome, e.g., in operons. Therefore, the sequences of enzymes of one pathway in plants can be identified by analyzing the prokaryotic genome structure, provided that the pathway of interest is present in both plants and bacteria. The phytol kinase is predicted to be present in all plants, algae, and bacteria containing chlorophyll and carrying out oxygenic photosynthesis (Ischebeck et al., 2006; Valentin et al., 2006). In agreement with this prediction, genes encoding phytol kinase were identified in Arabidopsis and *Synechocystis*, and related sequences were found in many other plants and photosynthetic bacteria (Valentin et al., 2006). The SEED database, harboring sequences of a large number of bacterial and plant genomes, provides the means to search for genes in subsystems (groups of genes with a related function or participating in a common pathway) (Overbeek et al., 2005; Seaver et al., 2014). Analysis of the subsystem encompassing functions involved in the metabolism of long-chain isoprenoids, including phytol kinase *VTE5*, undecaprenyl pyrophosphate synthetase (UppS), GGR, and chlorophyll synthase, revealed the presence of genes encoding a protein of unknown function (COG1836, pfam01940) that localize close to other isoprenoid genes in many bacterial genomes. Among Archaea, COG1836 sequences cluster with different genes of isoprenoid synthesis. In some cyanobacteria (*Anabaena* and *Nostoc*), orthologs of COG1836 are located in the genome close to phytol kinase, and in certain bacteria (*Pelodyction*, *Symbiobacterium*, and *Thermoplasma*) they are C-terminally fused to an ortholog of phytol kinase. Sequence orthologs of COG1836 are also present in *Synechocystis* (sll0875) and in Arabidopsis (At1g78620) (Seaver et al., 2014).

Here, we describe the characterization of the Arabidopsis At1g78620 gene and the corresponding insertional mutant plants. Biochemical and physiological analyses reveal that At1g78620 encodes phytyl-P kinase and that the phytol phosphorylation pathway represents the most relevant route of phytyl-PP synthesis for tocopherol production in leaves. Furthermore, we show that the phytol phosphorylation pathway is essential for plant growth and development.

RESULTS

Tocopherol Accumulation in Leaves Depends on Phytol Released from Chlorophyll

Tocopherol accumulates in leaves during stress or senescence (Collakova and DellaPenna, 2003). Phytyl-PP required for tocopherol synthesis can be derived from GG-PP from plastidial isoprenoid de novo synthesis or by phosphorylation of free phytol from chlorophyll hydrolysis. To determine the relative contribution of the phytol phosphorylation pathway for phytyl-PP synthesis destined for tocopherol production in leaves, the tocopherol contents in Arabidopsis wild type and the *pheophorbide a oxygenase1* (*pao1*) mutant were determined during senescence. Chlorophyll degradation in the *pao1* mutant is significantly retarded, due to a mutation in pheophorbide a oxygenase (PAO1) (Pruzinská et al., 2003, 2005) (Figure 1A). While total tocochromanol strongly accumulates during dark-induced senescence of detached leaves in the wild type, it remains at a very low amount in *pao1* leaves (Figure 1B), indicating that all tocopherol synthesis during leaf senescence is based on phytol derived from chlorophyll degradation. In *pao1* mutant seeds, the tocopherol content is reduced by 60% (Figure 1C). This result suggests an important role for phytyl-PP derived from chlorophyll degradation in the seeds, in accordance with previous findings (Valentin et al., 2006).

The COG1836 Sequence At1g78620 Shows Similarity with Cytidylyltransferases

At1g78620 from Arabidopsis shows high similarity with COG1836 sequences from the genomes of cyanobacteria (*Anabaena* and *Nostoc*) and other photosynthetic prokaryotes (*Archaeoglobus*) where it clusters in close proximity with genes of isoprenoid metabolism (Seaver et al., 2014). COG1836 amino acid sequences from bacteria and plants can be organized into different groups. The first group includes sequences from Streptophyta (including At1g78620), Chlorophyta, and Cyanobacteria. A second group contains a second Arabidopsis sequence (At5g19930) and COG1836 sequences that form translational fusions with VTE5 proteins from Firmicutes and Chlorobi (Supplemental Figure 1). The two archaeal COG1836 sequences from *Archaeoglobus* and *Thermoplasma* can be placed between the At1g78620- and At5g19930-related COG1836 sequences. The At1g78620 protein sequence is more closely related to *Synechocystis* sll0875 (43.8% identity) than to At5g19930, a gene encoding a protein of unknown function (30.5%). At1g78620 belongs to the DUF92 family of proteins of unknown function harboring several transmembrane α-helices (Seaver et al., 2014). BLAST searches in the GenBank protein database revealed that At1g78620 shows low similarity to cytidylyltransferases, including At3g45040 (24.3%), a putative dolichol kinase (Valentin et al., 2006) (Supplemental Figure 1). At1g78620 is weakly related to phytol kinases from different organisms, including Arabidopsis (At5g04490/VTE5, 17.5% identity). The phytol kinase/VTE5 sequences cluster into a cyanobacterial/plant group containing At5g04490 (VTE5) and a related sequence (At5g58560, VTE5-like) (Fitzpatrick et al., 2011) and the second branch containing the phytol kinase domains that form fusion proteins with COG1836 sequences from Chlorobi and Firmicutes (Supplemental Figure 1) (Valentin et al., 2006).

The At1g78620 Protein Localizes to Chloroplast Envelopes

The At1g78620 sequence harbors an ~70-amino-acid-long N-terminal extension absent from other COG1836 proteins (e.g.,
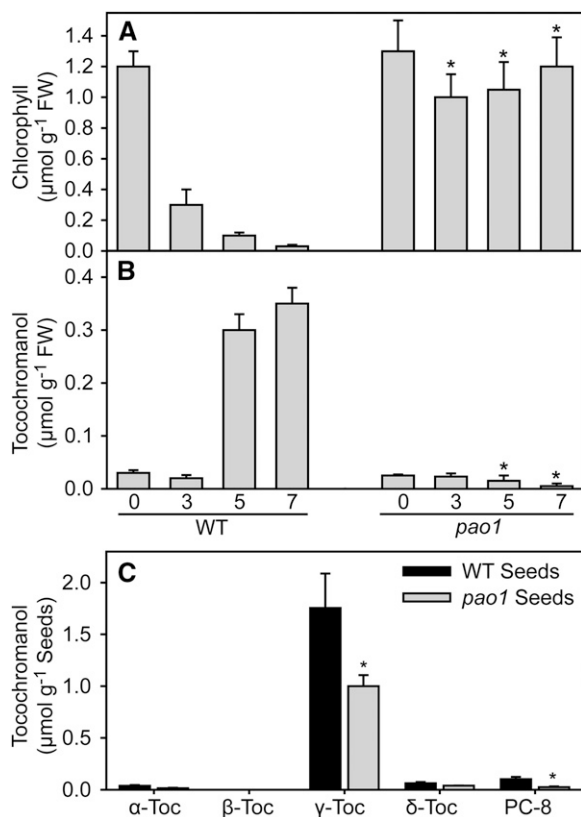
Figure 1. Tocopherol Accumulation in Leaves and Seeds of the Wild Type and *pao1* Mutant.

(A) Chlorophyll contents in leaves of the wild type and *pao1* mutant. Detached leaves were incubated on wet filter paper in the darkness for 3, 5, and 7 d.

(B) Tocochromanol (tocopherol and plastochromanol-8) contents in leaves of the wild type and *pao1*.

(C) Tocochromanol (tocopherol and plastochromanol-8) contents in seeds of the wild type and *pao1*.

Data represent mean and SD of three to four measurements. Values significantly different from the wild type; *P < 0.05; Student's *t* test. Toc, tocopherol.

Synechocystis sll0875) and At5g19930 (Supplemental Figure 1). Sequence analysis with ChloroP1.1 (http://www.cbs.dtu.dk/) (Emanuelsson et al., 1999) revealed the presence of a putative N-terminal transit peptide of 65 amino acids predicted to target the protein to the chloroplast. To test the subcellular localization experimentally, the full-length At1g78620 sequence was C-terminally fused to YFP, and this construct introduced into *Agrobacterium tumefaciens*. After infiltration into *Nicotiana benthamiana* leaves, subcellular localization was observed in protoplasts by confocal laser scanning microscopy. Yellow fluorescence was observed as a ring-like structure surrounding the chlorophyll autofluorescence, indicating that At1g78620 localizes to the chloroplasts envelopes (Supplemental Figure 2). This result is in agreement with the identification of the spinach (*Spinacia oleracea*) ortholog of At1g78620 in isolated chloroplast envelopes by a proteomics approach (Ferro et al., 2002).

Arabidopsis At1g78620 Insertional Mutant Plants Are Incapable of Photoautotrophic Growth

To study the role of At1g78620 in growth and phytol lipid synthesis, two Arabidopsis insertional mutant lines were obtained. The mutant plants (pst15134 and pst00121) carry transposons in the first exon of the At1g78620 gene (Figure 2A). Expression analysis by RT-PCR revealed that the At1g78620 mRNA was not detectable in the two mutant lines, suggesting that they represent null alleles (Figure 2B). The heterozygous plants of the two mutant lines grow similarly to the wild type. Homozygous pst15134 and pst00121 mutant plants are only viable on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with sucrose (Figure 2C). The plants are characterized by a stunted growth and short petioles and show a pale-green leaf color. They have a weakly developed or missing root system. Later, the plants produce a bushy rosette with small leaves but do not bolt and flower and therefore do not produce seeds. The plants die shortly after transfer to soil.

At1g78620 Mutant Plants Are Tocopherol-Deficient

To study the role of At1g78620 in tocopherol metabolism, the amounts of the different forms of tocopherol and of PC-8 were measured by fluorescence HPLC in leaves of homozygous At1g78620 mutant plants grown on MS medium with sucrose for 6 weeks. Arabidopsis wild-type leaves mainly contain α-tocopherol and low amounts of the other tocopherol forms and of PC-8 (Figure 2D). In contrast, tocopherol and PC-8 levels were significantly reduced in the leaves of the two At1g78620 mutant lines, mostly due to the reduction in α-tocopherol by ~98 to 100% (Figures 2D and 4A, respectively). Therefore, the mutation in the gene At1g78620 causes severe tocochromanol deficiency, and the corresponding mutant lines were renamed *vte6-1* (pst15134) and *vte6-2* (pst00121) for *vitamin E deficient6*.

Longevity of Homozygous *vte6* Seeds Is Compromised

Previous studies showed that seed longevity of the tocopherol-deficient mutants *vte1* and *vte2* was strongly compromised (Sattler et al., 2004). This defect was associated with increased nonenzymatic oxidation of storage lipids in the tocopherol-deficient seeds of *vte2*. To study the seed longevity of the *vte6* mutants, ~250 seeds each from heterozygous *vte6-1* and *vte6-2* plants were either freshly harvested or stored for more than 3 months at room temperature. The seeds were germinated on sucrose-supplemented MS medium. The average germination rate was calculated and the genotype of the germinated plants was determined by PCR of genomic DNA. The total germination rate was 96% ± 3%, 76% ± 6%, and 91% ± 3% for freshly harvested seeds from wild-type and heterozygous *vte6-1* and *vte6-2* plants, respectively. It was significantly reduced in segregating *vte6-2* seeds compared with the wild type after storage, when 67% ± 8% (*vte6-2*) and 89% ± 5% (wild type) of seeds germinated. The germination rate in freshly harvested segregating *vte6-1* seeds was significantly lower (76% ± 6%) than that of wild-type seeds, yet was not altered after storage (76% ± 7%).

A segregation of 50% heterozygous plants, 25% wild-type, and 25% homozygous mutant plants would be expected for the seeds
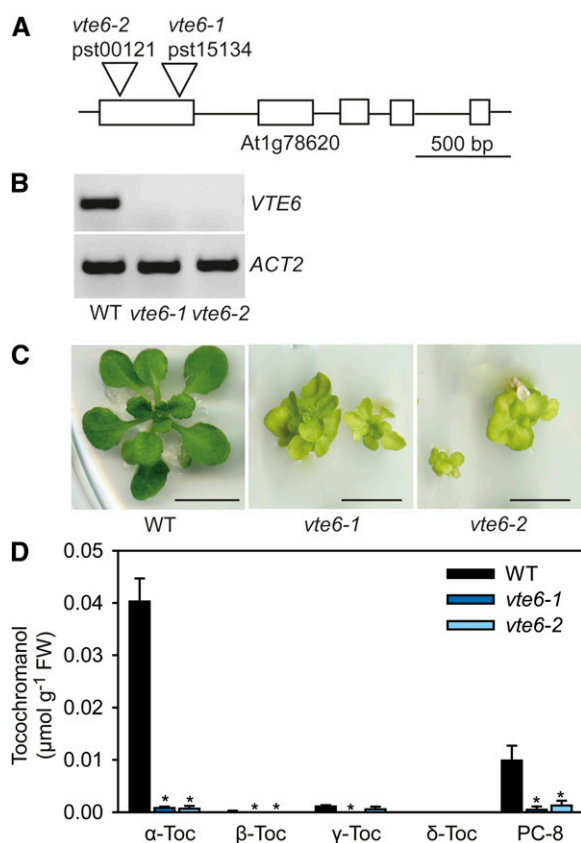
Figure 2. Isolation and Tocochromanol Content of the *vte6* Mutant from Arabidopsis.

(A) Map of the *VTE6* (At1g78620) locus of Arabidopsis with exons (boxes) showing the positions of transposon insertions in the *vte6* mutant alleles. Bar = 500 bp.

(B) Expression analysis of *VTE6* in Arabidopsis *vte6* mutant plants. The RT-PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Actin (*ACT2*) was used as control.

(C) Growth of Arabidopsis *vte6-1* and *vte6-2* mutant plants. Plants of the wild type and the two *vte6* mutant alleles were grown on MS medium with sucrose for 6 weeks. The *vte6* mutant plants are homozygous as revealed by PCR of genomic DNA.

(D) Different forms of tocopherol (α-Toc, β-Toc, γ-Toc, and δ-Toc) and PC-8 were measured by fluorescence HPLC in leaves of the wild type, *vte6-1*, and *vte6-2* grown on MS medium with sucrose for 6 weeks. Data show mean and sp of three measurements. Values significantly different from the wild type; *P < 0.05; Student's *t* test.

harvested from heterozygous *vte6-1* or *vte6-2* plants. However, only 11% of homozygous seedlings was retrieved from freshly harvested segregating *vte6-1* or *vte6-2* seeds, indicating that homozygous mutant seeds show significantly reduced viability (Figure 3). When seeds of heterozygous *vte6-1* or *vte6-2* plants were stored for 3 months at room temperature, germination of homozygous seeds was abolished. Only heterozygous or wild-type segregant seeds were able to germinate after storage. Therefore, similar to *vte1* and *vte2*, homozygous *vte6* seeds reveal a strong reduction in seed longevity, indicating that the *VTE6* gene is essential for maintenance of seed viability.

The *vte6* Mutation Affects Chlorophyll Content and Results in the Accumulation of Phytol and Fatty Acid Phytyl Esters

The strong reduction in tocopherol in leaves of the *vte6-1* and *vte6-2* mutant plants (Figures 2D and 4A) was expected to affect the amounts of other phytol-derived lipids, including chlorophyll. In agreement with the yellowish leaf color, the chlorophyll contents in *vte6-1* and *vte6-2* were significantly reduced as compared with the wild type (Figure 4B). The measurement of free phytol revealed a significant increase ($\sim$3-fold) in the *vte6-1* and *vte6-2* leaves (Figure 4C). Previous results showed that free phytol can be used for the synthesis of fatty acid phytyl esters in chloroplasts of Arabidopsis. Quantification of fatty acid phytyl esters showed a significant increase in different molecular species, in particular 12:0-phytol, 14:0-phytol, and 16:0-phytol in *vte6-1* and *vte6-2* (Figure 4D).

VTE6 Harbors Phytyl-P Kinase Activity

The in vivo activity of VTE6 was investigated by feeding phytol to seedlings of Arabidopsis wild type and *vte6-1*. Seedlings were incubated in buffer (control) or in buffer containing phytol, then seedlings were harvested and phytyl-P and phytyl-PP quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Figure 5A). Control plants incubated in buffer contained low amounts of phytyl-P. The amounts of phytyl-PP were high in the wild type, but extremely low in *vte6-1* plants. Phytol feeding resulted in a strong increase of phytyl-P in *vte6-1*, but not in the wild type. In contrast, the phytyl-PP content in the wild type strongly increased (>4-fold) after phytol feeding, but not in *vte6-1*, where phytol feeding had almost no effect on the amount of phytyl-PP. In fact, the phytyl-PP content in *vte6-1* was only 5% compared with the wild type.

Next, total protein was isolated from leaves of Arabidopsis wild-type, *vte6-1*, and *vte6-2* plants. Phytyl-P kinase assays were done employing phytyl-P and CTP as substrates, and phytyl-PP was quantified by LC-MS/MS. The phytyl-P kinase activity with wild-type protein was significantly (3 to 6 times) higher compared with *vte6-1* and *vte6-2* (Figure 5B).

Phytol can be taken up and converted into phytyl-P by *Escherichia coli* cells expressing VTE5 (Valentin et al., 2006); this system was employed to demonstrate the activity of recombinant VTE6. To this end, the two cDNAs for VTE5 and VTE6 were introduced into *E. coli* as single constructs or in combination. After induction, the expression of VTE5 and VTE6 proteins was detected by immunoblot analysis (Supplemental Figure 3). Phytol was added to the *E. coli* cultures, and after 3 h, phytyl-P and phytyl-PP were extracted and quantified by LC-MS/MS. The amount of phytyl-P in VTE6-expressing cells was very low, suggesting that VTE6 might not harbor phytol kinase activity (Figure 5C). Cells expressing both VTE5 and VTE6 produced phytyl-P, albeit less than cells expressing only VTE5. The amounts of phytyl-PP in cells expressing either VTE5 or VTE6 were very low. However, when the two proteins were coexpressed in *E. coli*, large amounts of phytyl-PP accumulated (Figure 5C).

Coupled enzyme assays with protein extracts from *E. coli* expressing either VTE5 or VTE6 or expressing both proteins were performed employing phytol and CTP as substrates. Only minor amounts of phytyl-PP were produced with protein extracts from
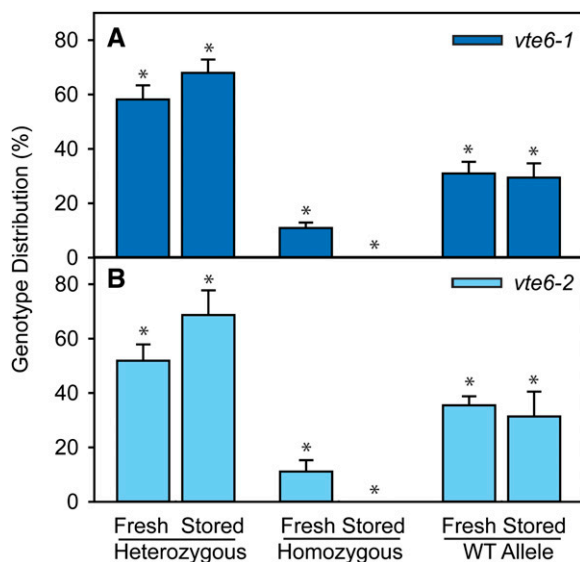
Figure 3. Decreased Germination Capacity of *vte6* Seeds after Storage.

(A) Germination of seeds from a heterozygous *vte6-1* plant.
(B) Germination of seeds from a heterozygous *vte6-2* plant.
The seeds were germinated on MS medium with sucrose. Freshly harvested seeds stored for 3 months at room temperature were used. The genotype of the seedlings was determined by PCR of genomic DNA. The P values (asterisks) calculated from χ^2 tests for the deviations of the genotype distributions of fresh or stored *vte6-1* or *vte6-2* seeds from the expected segregation (50:25:25) were <0.01.

VTE5- or VTE6-expressing cells, indicating the presence of low phytol kinase and phytyl-P kinase activity in *E. coli*. Protein from cells coexpressing VTE5 and VTE6 showed significantly (~3-fold) higher activity (Figure 5D). Taken together, phytol feeding experiments and enzyme assays with Arabidopsis wild-type and *vte6* mutant plants, and with recombinant *E. coli* cells expressing VTE5/VTE6, demonstrate that VTE6 harbors phytyl-P kinase activity.

The Growth Defect of *vte6-1* Is Alleviated in the *vte5-2* *vte6-1* Double Mutant

The strong growth retardation of the *vte6-1* and *vte6-2* mutant plants is in contrast with other tocopherol-deficient mutants (*vte1*, *vte2*, *vte4*, and *vte5*) because growth of these mutants under normal conditions is very similar to the wild type (Porfirova et al., 2002; Bergmüller et al., 2003; Maeda et al., 2006; Valentin et al., 2006). As an exception, the *vte3-2* mutant shows reduction of both tocopherol and plastoquinol-9 due to a mutation in the methyl-transferase VTE3; therefore, *vte3-2* plants are incapable of photoautotrophic growth (Cheng et al., 2003). The tocopherol-deficient mutants *vte1*, *vte2*, *vte4*, and *vte5* can grow on soil and produce fertile seeds, while *vte6-1* and *vte6-2* cannot grow photoautotrophically and are infertile. Therefore, the strong growth reduction of *vte6* mutant plants cannot be explained by tocopherol deficiency per se, but must be caused by other factors. For example, it is possible that due to the block in phytyl-P phosphorylation, the accumulation of phytyl-P in *vte6* plants might have negative effects on chloroplast development and growth, in contrast to other tocopherol-deficient plants, where such accumulation would not be expected. To investigate this potential scenario, and to suppress the accumulation of phytyl-P in *vte6* plants, the *vte5* mutation (phytol kinase) was introduced into the *vte6-1* background. The Arabidopsis *vte5-1* mutant carrying a premature stop codon in the *VTE5* gene was previously isolated from a chemically mutagenized population (Valentin et al., 2006). An independent mutant allele (pst12490, *vte5-2*) was obtained that carries a transposon insertion in the first exon behind the start codon of the *VTE5* gene (Figure 6A). Analysis of gene expression by RT-PCR revealed the absence of *VTE5* mRNA in *vte5-2* (Figure 6B), indicating that the *vte5-2* mutation represents a null allele. After crossing of homozygous *vte5-2* and heterozygous *vte6-1* plants, double homozygous plants were identified by PCR in the F2 population. When grown on MS medium with sucrose, *vte5-2 vte6-1* plants produce larger and greener leaves in contrast with *vte6-1* plants (Figure 6C). The double mutant plants survive after transfer to soil. Figures 6D and 6E show 6-, 9-, and 12-week-old *vte5-2 vte6-1* plants grown on soil. The morphology of the plants is stunted and bushy with many leaves. Double mutant plants can grow on soil for longer than 4 months. At this stage, wild-type and *vte5-2* plants have died. Therefore, *vte5-2 vte6-1* plants show a stay-green phenotype with strongly delayed senescence and extended lifetime. Old leaves do not turn yellow, but remain green and eventually wither and die (Figure 6E). These results indicate that the strong growth retardation and the incapability to grow on soil of the *vte6-1* single mutant can be partially complemented by introducing the *vte5-2* mutation into the genetic background of *vte6-1*.

Tocopherol, Phytol, and Fatty Acid Phytyl Esters in the *vte5-2 vte6-1* Double Mutant

Wild-type, *vte5-2*, *vte6-1 vte6-2*, and *vte5-2 vte6-1* plants were grown on MS medium with sucrose for 6 weeks, and leaves were harvested for the measurement of tocochromanols, chlorophyll, phytol, and fatty acid phytyl esters. Leaves of *vte5-2* contain ~50% less tocopherol than wild-type leaves under these conditions (Figure 4A). Tocopherol levels in leaves of *vte6-1*, *vte6-2*, and *vte5-2 vte6-1* are below the detection limit. The reduced amount of tocopherol in *vte6-1*, *vte6-2*, and in the double mutant *vte6-1 vte5-2* was accompanied by a significant decrease in PC-8 levels compared with the wild type (Figures 2D and 4A). The chlorophyll content is only slightly decreased in *vte5-2 vte6-1* compared with the wild type and *vte5-2*, but it much is higher than in *vte6-1* and *vte6-2* (Figure 4B). Free phytol accumulates in leaves of *vte6-1* and *vte6-2* to ~3 nmol g^{-1} fresh weight (FW), while the amounts of phytol are unchanged in *vte5-2* and *vte5-2 vte6-1* compared with the wild type (Figure 4C). In addition, the different forms of fatty acid phytyl esters are increased in leaves of *vte5-2*, *vte6-1*, *vte6-2*, and *vte5-2 vte6-1* compared with the wild type, especially 12:0-phytol, 14:0-phytol, and 16:0-phytol (Figure 4D).

Phytyl-P Accumulates in the *vte6-1* Mutant, but Not in the *vte5-2 vte6-1* Double Mutant Plants

The severe growth retardation of *vte6-1* was partially rescued in the *vte5-2 vte6-1* double mutant, suggesting that alterations in phytol, phytyl-P, or phytyl-PP metabolism might be causal for the growth reduction of *vte6-1*. Phytyl-P, phytyl-PP, and GG-PP were
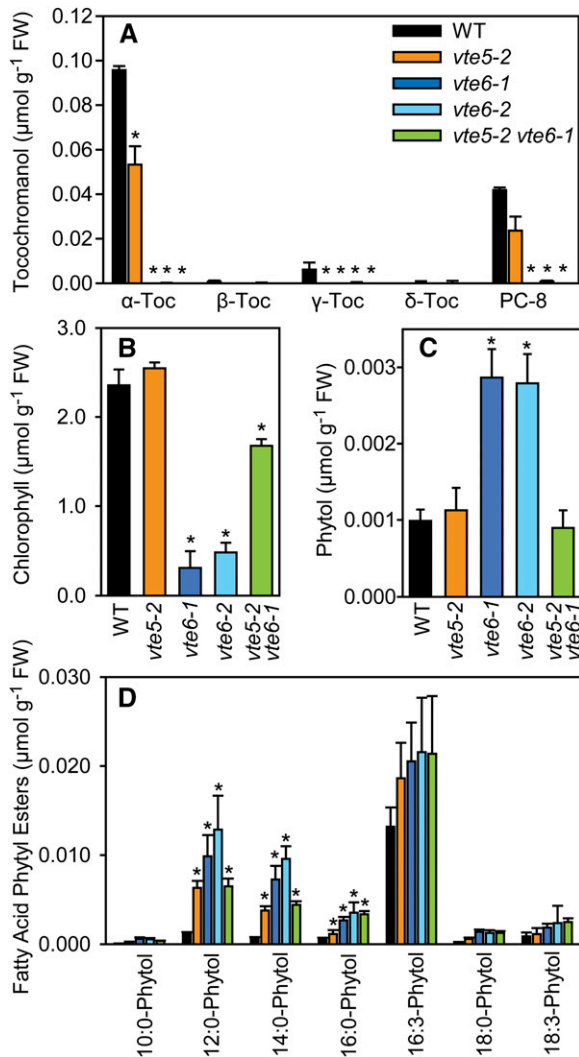
Figure 4. Tocopherol, Chlorophyll, Phytol, and Fatty Acid Phytyl Esters in the Wild Type, *vte5-2*, *vte6-1*, *vte6-2*, and *vte5-2 vte6-1*.

(A) Tocopherol (α-Toc, β-Toc, γ-Toc, and δ-Toc) and PC-8 were measured by fluorescence HPLC.

(B) Chlorophyll was measured photometrically.

(C) The amount of free phytol was quantified by gas chromatography-mass spectrometry.

(D) Fatty acid phytyl esters were measured by direct infusion quadrupole time-of-flight tandem mass spectrometry.

Plants (wild type, *vte5-2*, *vte6-1*, *vte6-2*, and *vte5-2 vte6-1*) were grown on MS medium containing sucrose for 6 weeks. Leaves were harvested and analyzed for their lipid content. Mean and SD of four measurements are shown. Significantly different from the wild type; *$P < 0.05$. Chlorophyll content in *vte5-2 vte6-1* was also significantly different from *vte6-1* (Figure 4B); Student's *t* test.

quantified by LC-MS/MS in the leaves of plants grown on MS medium with sucrose. Arabidopsis leaves of wild-type plants contain around 15 nmol g^{-1} FW of phytyl-PP. The phytyl-P content in the wild type is extremely low (0.1 nmol g^{-1} FW). The phytyl-PP contents are significantly decreased in *vte5-2*, *vte6-1*,

vte6-2, and *vte5-2 vte6-1* mutant leaves to 6 to 8 nmol g^{-1} FW (Figure 7). The phytyl-P contents in *vte6-1* and *vte6-2* are significantly increased by ~4- and 10-fold, respectively, compared with the wild type, *vte5-2*, and *vte5-2 vte6-1*. These results show that the increase in the amount of phytyl-P in *vte6-1* is suppressed in the *vte5-2 vte6-1* double mutant, suggesting a negative impact of phytyl-P accumulation on plant growth. The amounts of GG-PP were ~2 nmol g^{-1} FW for all lines, indicating that the content of GG-PP is much lower than that of phytyl-PP and that it is not affected by changes in phytyl-P or phytyl-PP metabolism. Furthermore, these results are consistent with the presumed function of VTE6 as phytyl-P kinase because a block in phytyl-P kinase activity in *vte6-1* and *vte6-2* is expected to cause an accumulation of phytyl-P associated with a decrease in phytyl-PP.

Overexpression of *VTE6* Results in Increased Phytyl-PP and Tocopherol Levels in Seeds

VTE6 was overexpressed under the control of the CaMV 35S promoter in Arabidopsis wild-type plants, and the seeds of these plants were analyzed for isoprenyl-phosphates and tocochromanols. In the VTE6-overexpressing lines VTE6#3 and VTE6#4, phytyl-PP levels were significantly increased (509 and 514 nmol g^{-1} seeds) compared with the empty vector control line (247 nmol g^{-1} seeds) (Figure 8A). Phytyl-P and GG-PP levels were unaffected by overexpression of VTE6 (Figure 8A). Interestingly, the γ-tocopherol content was significantly increased in the VTE6 overexpression lines (1.5 and 1.6 µmol g^{-1} seeds) compared with the empty vector line (1.3 µmol g^{-1} seeds) (Figure 8B). Therefore, overexpression of *VTE6* in seeds results in the accumulation of phytyl-PP, which can be used for prenylation of homogentisate by VTE2, finally causing an increase in tocopherol biosynthesis.

DISCUSSION

Tocopherol (vitamin E) is an important antioxidant in plants and animals and an essential component of the human diet. Work in recent years has established the pathways for tocopherol and tocotrienol synthesis, including the prenylation of homogentisate with a phytyl or geranylgeranyl group, respectively, with subsequent methylation and cyclization reactions (Hunter and Cahoon, 2007; Mène-Saffrané and DellaPenna, 2010). For a long time it was believed that the phytyl moiety for tocopherol synthesis exclusively originates from GG-PP via reduction by GGR. However, in the past years it became clear that tocopherol synthesis also depends on phytyl-PP derived from phytol released during chlorophyll breakdown (Figure 9). Two kinase reactions are required for the conversion of phytol into phytyl-PP (Ischebeck et al., 2006). While the gene for phytol kinase (VTE5) was characterized (Valentin et al., 2006), the identity of the second kinase remained unknown. Here, we present the characterization of a novel protein as candidate for phytyl-P kinase (VTE6). The sequence for *VTE6* was identified based on its colocalization with orthologs of *VTE5* and other genes of isoprenoid lipid metabolism in genomes of prokaryotic photosynthetic organisms, its coexpression pattern with genes of tocopherol metabolism, and its predicted localization in the chloroplast (Seaver et al., 2014). Furthermore, the VTE6 sequence displays weak similarity to cytidylyltransferases and to isoprenoid lipid kinases (Supplemental Figure 1).
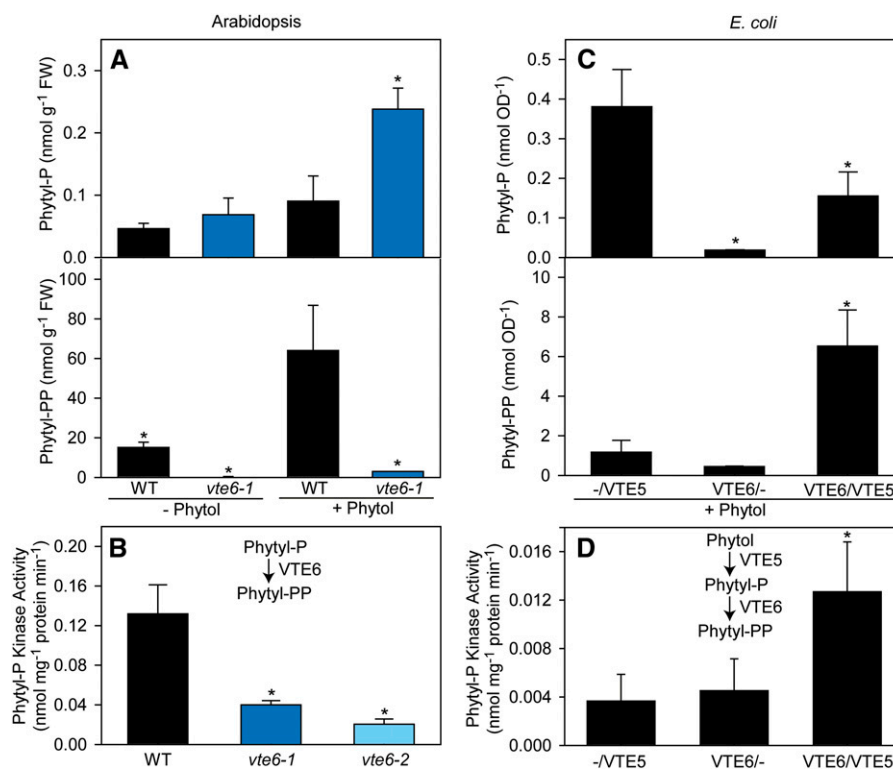
Figure 5. VTE6 Harbors Phytyl-P Kinase Activity.

(A) Phytol feeding experiments with Arabidopsis plants. Wild-type and *vte6-1* plants were incubated in buffer without phytol or with 0.1% (v/v) phytol for 48 h, and isoprenyl-phosphates were isolated.

(B) Phytyl-P kinase assay with protein extracts from Arabidopsis wild-type, *vte6-1*, and *vte6-2* leaves employing phytyl-P and CTP as substrates.

(C) Phytol feeding experiment with recombinant *E. coli* cells. Protein expression in *E. coli* cells harboring plasmids with either VTE5 (-/VTE5), VTE6 (VTE6/-) or both cDNAs (VTE6/VTE5) was induced and the cells incubated in the presence of phytol before extraction of isoprenyl-phosphates.

(D) Coupled phytol kinase/phytyl-P kinase assay with protein extracts from recombinant *E. coli* cells. Total protein was isolated from *E. coli* expressing VTE5 (-/VTE5), VTE6 (VTE6/-), or both proteins (VTE6/VTE5) and used for a coupled phytyl kinase/phytyl-P kinase assay employing phytol and CTP as substrates. Isoprenyl-phosphates (phytyl-P and phytyl-PP) were measured by LC-MS/MS. Data represent mean and sd of three to four measurements. Significantly different from the wild type (**[A]** and **[B]**) or to *E. coli* expressing only VTE5 (**[C]** and **[D]**); *P < 0.05; Student's *t* test.

Phytyl-P Kinase Activity

Phytyl-P kinase catalyzes the conversion of phytyl-P into phytyl-PP. Feeding experiments with Arabidopsis wild-type and *vte6-1* seedlings showed that phytol is taken up by the plants and converted into phytyl-P by VTE5. Phytyl-PP levels were strongly increased in wild-type seedlings, while phytyl-PP levels were extremely low in *vte6-1* plants after phytol feeding (Figure 5). On the other hand, phytyl-P, the precursor of phytyl-PP, increased in *vte6-1*, but not in the wild type. Furthermore, enzyme assays with Arabidopsis leaf protein showed that phytyl-P kinase activity in *vte6-1* is ~3- to 4-fold lower compared with the wild type.

Feeding experiments and enzyme assays with recombinant *E. coli* cells confirmed the results obtained with Arabidopsis plants. After phytol feeding, phytyl-P but not phytyl-PP accumulated in VTE5-expressing cells. In VTE5/VTE6 coexpressing cells, the accumulation of phytyl-P was attenuated, while phytyl-PP accumulated, indicating that a certain proportion of phytyl-P was converted into phytyl-PP by VTE6. In cells expressing only VTE6,

neither phytyl-P nor phytyl-PP accumulated, indicating that VTE6 does not harbor phytol kinase activity, but is dependent on the presence of phytyl-P, which can only be provided by VTE5 but not by other *E. coli* proteins in this system.

A coupled enzyme assay with total protein extracts from *E. coli* and phytol and CTP as substrates showed that phytyl-PP synthesis depends on the presence of VTE6. Taken together, these experiments conclusively demonstrate that VTE6 converts phytyl-P into phytyl-PP, in agreement with the hypothesis that *VTE6* encodes phytyl-P kinase.

Phytyl-PP for Tocopherol Biosynthesis in Leaves Is Almost Exclusively Provided by the Phytol Phosphorylation Pathway

Two mutant alleles, *vte6-1* and *vte6-2*, were isolated which both showed reduced growth and changes in phytol lipid contents. Furthermore, the *vte6-2* mutant was backcrossed to the wild type (see Methods), and a homozygous F2 plant was obtained that showed the same growth and lipid phenotypes as *vte6-1*. These experiments demonstrate that the strong growth retardation and
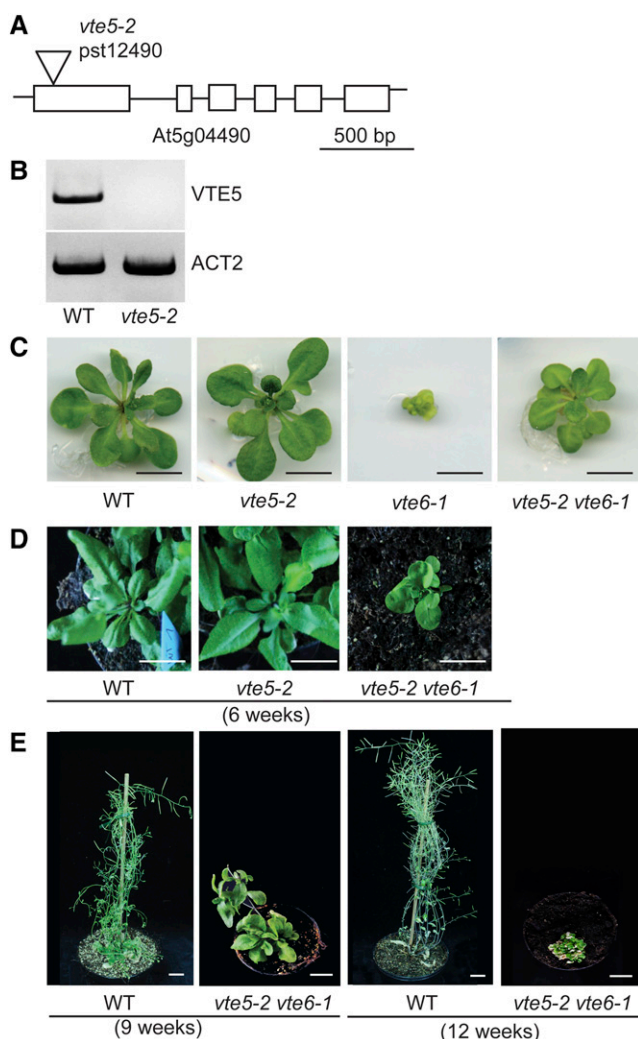
Figure 6. The *vte5-2 vte6-1* Double Mutant.

(A) The *vte5-2* mutant carries a transposon insertion in the first exon of *VTE5* (At5g04490, phytol kinase). Exons are indicated by boxes. Bar = 500 bp.
(B) Expression analysis of the *vte5-2* mutant. The RT-PCR products for *VTE5* and *ACT2* (control) were separated by agarose gel electrophoresis and stained with ethidium bromide.
(C) Growth of wild-type, *vte5-2*, *vte6-1*, and *vte5-2 vte6-1* plants on MS medium with sucrose. Plants are 6 weeks old. Bar = 1 cm.
(D) Growth of wild-type, *vte5-2*, and *vte5-2 vte6-1* plants on soil for 6 weeks. Bar = 2 cm.
(E) Wild-type and *vte5-2 vte6-1* plants grown on soil for 9 or 12 weeks. Bar = 2 cm

the phytol lipid changes are genetically linked with the *vte6* mutations.

Phytyl-phosphates and geranylgeranyl-phosphates are important intermediates for the synthesis of chlorophyll, tocopherol, phylloquinol, and carotenoids in plant leaves, but their measurement was hindered by their low abundance. Employing an LC-MS/MS-based strategy, the amounts of phytyl-P, phytyl-PP, and GG-PP were measured in leaves and seeds of Arabidopsis. Phytyl-P levels in wild-type leaves are extremely low ($\sim$0.1 nmol g^{-1} FW), while phytyl-PP and GG-PP amount to 15 or 2 nmol g^{-1}

FW, respectively (Figure 7). Phytyl-P accumulates in the *vte6-1* mutant, in accordance with the role of VTE6 as phytyl-P kinase, while the phytyl-PP level is reduced to 50% compared with the wild type. As *vte6-1* represents a null mutation (Figure 2), the residual amount of phytyl-PP presumably is derived from GG-PP reduction by GGR. Interestingly, the reduction of phytyl-PP to 50% of wild-type levels is associated with a severe decrease in tocopherol contents in leaves (by $\sim$98 to 100% compared with the wild type), highlighting the relevance of VTE6 for tocopherol synthesis. Therefore, the residual content of phytyl-PP in *vte6* cannot be used for tocopherol synthesis. Maybe different pools of phytyl-PP exist in the chloroplast, derived from phytol phosphorylation or from GGR, and only the phytyl-PP derived from phytol phosphorylation might be accessible for tocopherol synthesis. The amount of GG-PP was not decreased in leaves of *vte6-1* but was much lower than that of phytyl-PP, indicating that GG-PP reduction to phytyl-PP cannot compensate for the loss of phytyl-P phosphorylation activity to provide phytyl-PP as substrate for tocopherol synthesis. Therefore, GG-PP produced via isoprenoid de novo synthesis plays a negligible role in providing the phytyl-moiety for tocopherol synthesis in leaves.

Phytyl-PP Synthesized by VTE6 Is Used for Tocopherol Biosynthesis in Seeds

Seeds of the Arabidopsis phytol kinase mutant *vte5-1* contain only 20% of wild-type tocopherol levels (Valentin et al., 2006). Therefore, it was considered that the bulk of tocopherol is synthesized from phytyl-PP that originates from the phytol phosphorylation pathway. Intriguingly, overexpression of *VTE6* in Arabidopsis results in a 2-fold increase in phytyl-PP and in increased tocopherol levels in seeds. This finding is in agreement with the hypothesis that *VTE6* encodes phytyl-P kinase and that it provides phytyl-PP for seed tocopherol synthesis. Furthermore, this result suggests that phytyl-PP might be limiting for tocopherol synthesis in seeds.

Longevity in *vte6* Mutant Seeds Is Impaired

Seeds of the *vte2* mutant are completely devoid of tocopherol and exhibit a severe reduction in longevity. Accelerated ageing at high temperature and high humidity causes a strong defect in germination rate of *vte2* seeds, which was associated with non-enzymatic peroxidation of seed lipids, caused by the lack of antioxidant capacity (Sattler et al., 2004). However, apart from a delay in seedling development and rare cotyledon defects, *vte2* plants develop normally, grow to full size, and produce seeds. The *vte5-1* mutant contains 20 and 35% of tocopherol in the seeds and leaves, respectively, compared with the wild type and also exhibits normal plant development and growth (Valentin et al., 2006). Similarly, germination of *vte5-2* seeds was not affected. In contrast, *vte6-1* and *vte6-2* plants can only grow on sucrose-supplemented MS medium, are pale-green and dwarfed, and do not produce seeds. Homozygous *vte6* seeds do not germinate after storage for more than 3 months. These plants are infertile, and homozygous *vte6* mutant seeds cannot be selected from seeds of a heterozygous plant because the seeds cannot be distinguished. Therefore, it was not possible to analyze homozygous *vte6* seeds for lipid contents. Attempts to measure tocopherol, phytol, or isoprenyl-phosphates in
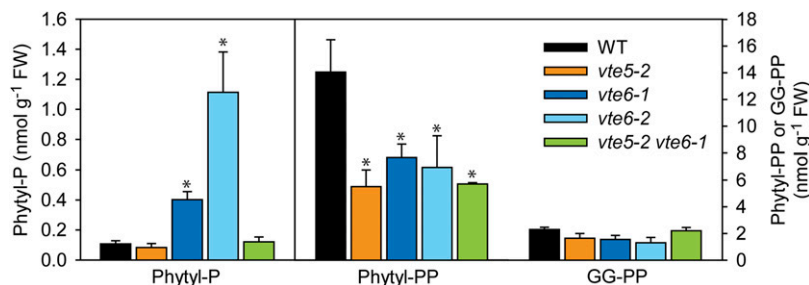
Figure 7. Quantification of Isoprenyl-Phosphates in Wild-Type, *vte5-2*, *vte6-1*, *vte6-2*, and *vte5-2 vte6-1* Plants.

Plants were grown on MS medium with sucrose and leaves were harvested after 6 weeks. Isoprenyl-phosphates were measured by LC-MS/MS. Data show mean and SD of four to five measurements. Significantly different from the wild type; *P < 0.05; Student's *t* test.

single, segregating seeds of a heterozygous *vte6* plant were not successful. However, the fact that longevity is affected in *vte6* seeds, similar to *vte2* seeds, suggests that *vte6* seeds also suffer from a severe reduction in tocopherol content.

The Deficiency in Growth and Development of *vte6* Plants Is Unrelated to the Degree of Membrane Lipid Unsaturation

Tocopherol plays a crucial role during the adaptation of growth under low-temperature conditions (Maeda et al., 2006). Tocopherol-deficient mutants including *vte2* show increased sugar and carbohydrate contents in the leaves, accompanied by callose deposition at plasmodesmata in phloem parenchyma transfer cells, when grown at low temperature. Biochemical and genetic evidence showed that this effect is unrelated to oxidative stress, but it is associated with reduced desaturation of linoleic acid (18:2) to α-linolenic acid (18:3), particularly in phosphatidylcholine (PC). Therefore, when grown under normal or low temperature, PC in *vte2* contains a reduced amount of 18:3 compared with the wild type. The mechanism of how tocopherol deficiency affects lipid desaturation remains unclear. It was possible that the severe growth retardation of the *vte6* mutant was related to the low temperature phenotype of *vte2* plants. Measurements of membrane lipids revealed that the proportion of monogalactosyldiacylglycerol is reduced in *vte6-1* and *vte6-2*, while the relative amount of PC is increased (Supplemental Figure 4). This result indicates a general decrease in the amounts of thylakoid lipids, albeit much less severe as the decrease in chlorophyll. The molecular species of 36:6 (18:3/18:3) and 36:5 (18:3/18:2) are the predominant PC molecules carrying two C18 fatty acids. The *vte6-1* and *vte6-2* plants contain higher amounts of 36:6 PC than the wild type, while the amounts of 36:5 PC remain similar. Therefore, *vte6* plants contain a higher degree of unsaturated C18 fatty acids in PC than the wild type, in contrast to *vte2*, which contains lower amounts of 36:6 compared with the wild type (Maeda et al., 2006). This result indicates that the severe growth retardation of *vte6* is unrelated to the low-temperature phenotype of the *vte2* mutant.

Phytyl Lipids in the *vte5* and *vte6* Mutants

The *vte5-1* mutant of Arabidopsis was described previously (Valentin et al., 2006). We used a second mutant allele (*vte5-2*),

which grows like the wild type and shows normal chlorophyll contents. The amount of tocopherol is reduced, as was shown for *vte5-1*, indicating that the insertion in *vte5-2* is causal for the tocopherol deficiency, in analogy to *vte5-1*. The amount of tocopherol is strongly reduced in leaves of *vte6* (<5%), while *vte5* leaves contain considerable more tocopherol (*vte5-1*, 35% of the wild type, Valentin et al., 2006; *vte5-2*, 50% of the wild type, Figures 4 and 6). As *vte5-2* represents a null allele, a second phytol kinase activity might exist in the chloroplasts, which could explain the residual capacity for the conversion of phytol into phytyl-P and the absence of phytol accumulation in *vte5-2*. On the other hand, VTE6 presumably is the only phytyl-P kinase in chloroplasts. Therefore, the complete block of the phytol phosphorylation pathway in *vte6* might lead to the accumulation of phytyl-P and at the same time of phytol because phytol phosphorylation by VTE5 (and additional phytol kinase activities) might be downregulated, possibly by a feedback mechanism, to avoid the uncontrolled accumulation of phytyl-P.

The Severe Impairment of Growth and Development in *vte6* Mutants Is Alleviated in *vte5 vte6* Double Mutants

To test the hypothesis whether the block in sequestration of phytol to phytyl-P and subsequently to phytyl-PP is related to the strong growth retardation of the *vte6-1* mutant, a genetic test was conducted by generating a double mutant of *vte5-2* and *vte6-1*. Interestingly, growth of homozygous *vte5-2 vte6-1* plants is strongly improved compared with *vte6-1* single mutant plants. Similar to *vte6-1*, *vte5-2 vte6-1* double mutant plants show strongly reduced amounts of tocopherol (Figure 4A).

The amount of phytyl-P is increased in *vte6-1* leaves (0.4 nmol g^{-1} FW) but is reduced in *vte5-2 vte6-1* to levels similar to the wild type and *vte5-2* (around 0.1 nmol g^{-1} FW) (Figure 7). The amounts of free phytol follow a similar pattern, because phytol is unaffected in *vte5-2* and *vte5-2 vte6-1*, while it is strongly accumulated in *vte6-1* compared with the wild type (Figure 4C). Therefore, the introduction of the *vte5-2* mutation into the *vte6-1* mutant background strongly improves growth, associated with the suppression of the accumulation of phytol and phytyl-P. This pattern of changes is in accordance with the expected result of combining mutations in phytol kinase and phytyl-P kinase activities in a single plant.
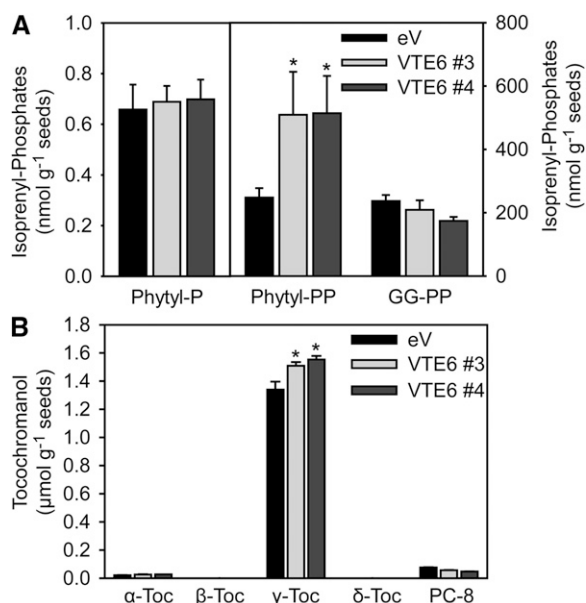
Figure 8. Quantification of Isoprenyl-Phosphates and Tocopherol in *VTE6* Overexpression Lines.

Seeds of Arabidopsis wild-type plants, empty vector (eV) control, and two lines overexpressing *VTE6* (VTE6#3 and VTE6#4) were harvested from plants simultaneously grown on soil. Data show mean and SD of three to four measurements. Significantly different from the wild type; *P < 0.05; Student's *t* test.
(A) Isoprenyl-phosphates were isolated from 20 mg of seeds and quantified by LC-MS/MS.
(B) Tocopherol (α-Toc, β-Toc, γ-Toc, and δ-Toc) and PC-8 were measured by fluorescence HPLC.

The fact that phytyl-PP in *vte6-1* and *vte5-2 vte6-1* is reduced compared with the wild type, but that chlorophyll content in *vte5-2 vte6-1* is strongly increased, indicates that the reduction in phytyl-PP content per se does not become limiting for chlorophyll synthesis in *vte5-2 vte6-1*, as well as in *vte6-1*. It is possible that phytol or phytyl-P levels, which are increased in *vte6-1*, affect chlorophyll synthesis activity, e.g., by competing with phytyl-PP for binding to chlorophyll synthase or by interfering with other important chloroplast pathways.

The relative amounts of galactolipids in *vte6* are reduced to only a minor extent (monogalactosyldiacylglycerol drops from 42 to 33 to 36%, while digalactosyldiacylglycerol and sulfoquinovosyldiacylglycerol are not changed; Supplemental Figure 4), indicating that the overall effect on chloroplast membrane lipids is much less severe than the decrease in chlorophyll content. Furthermore, not all chloroplast lipids are decreased. Some lipids increased in *vte6*, in particular phytol, phytyl-P, and fatty acid phytyl esters. Therefore, the block in *vte6* preferentially affects phytol-containing lipids. On the other hand, the amounts of PC-8 in *vte6-1* and *vte6-2* were reduced. PC-8 synthesis is not directly linked with phytyl-PP metabolism. It is possible that the block in phytol phosphorylation might affect tocochromanol synthesis (head group and/or prenyl side chain production) by feedback inhibition (see above), which would explain the general

decrease in the levels of all tocochromanols in *vte6*. In line with this scenario, PC-8 also decreased in the *vte5-2* mutant, which has normal chlorophyll levels (Figure 4), and in the seeds of the *pao1* mutant (Figure 1).

The *vte5-2 vte6-1* plants contain significantly higher chlorophyll contents than *vte6-1* (Figure 4B). Therefore, the strong reduction in *vte6* growth is primarily not caused by tocopherol deficiency or reduction in chlorophyll synthesis capacity, but might rather be associated with the accumulation of free phytol or phytyl-P. It has previously been shown that the accumulation of free alcohols is detrimental to membrane integrity (Löbbecke and Cevc, 1995). Therefore, the accumulation of phytol might destabilize chloroplast membranes affecting chlorophyll and galactolipid synthesis in *vte6-1*. In addition, it is possible that phytyl-P is toxic per se or that the sequestration of phytol into the phytyl-P pool is harmful for
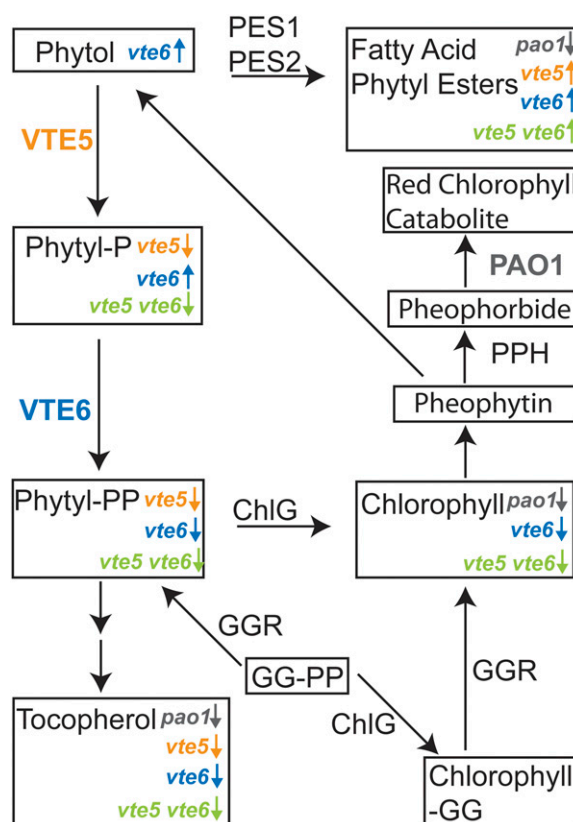

Figure 9. Phytol Metabolism in Arabidopsis.

Phytol is derived from chlorophyll degradation via PPH or by an unknown dephytylase. PAO1 is involved in further pheophorbide catabolism. Phosphorylation by VTE5 and VTE6 results in the production of phytyl-PP, which serves as precursor for tocopherol synthesis. Phytol is also used for the synthesis of fatty acid phytyl esters by phytyl ester synthases (PES1 and PES2) (Lippold et al., 2012). GG-PP or phytyl-PP can be used to synthesize geranylgeranyl-chlorophyll (chlorophyll-GG) or chlorophyll, respectively, by chlorophyll synthase (ChlG). Chlorophyll-GG is reduced to chlorophyll by GGR. The contribution of de novo synthesis via direct reduction of GG-PP to phytyl-PP by GGR is low. Alterations in metabolite or lipid contents in the respective mutants are indicated by arrows (upregulation or down-regulation).

plant metabolism. In this regard, it is interesting to note that phytyl-P represents an alcohol phosphate analogous to sphingobase-1-phosphates (e.g., sphingosine-1-phosphate), which are known signaling molecules in plants and animals (Lynch et al., 2009). It is also possible that phytyl-P-derived compounds accumulate in *vte6* with detrimental consequences for plant growth.

Synechocystis harbors a protein sequence (sll0875) highly related to Arabidopsis VTE6 (Supplemental Figure 1). A gene knockout strategy was employed to mutagenize sll0875 in *Synechocystis* by homologous recombination. However, screening of transformed *Synechocystis* cells after repeated rounds of selection on antibiotic medium revealed the absence of fully segregated homozygous mutant cells, indicating that this gene is essential. Therefore, the phosphorylation pathway of phytol is crucial for Arabidopsis and *Synechocystis*, and this essential function is independent of tocopherol deficiency.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* transposon insertion lines *vte6-1* (pst15134, 13-0604-1) and *vte6-2* (pst00121, 54-0912-1) were obtained from the RIKEN Bioresource Center (Tsukuba, Japan). Seeds of heterozygous *vte6-1* and *vte6-2* plants were germinated on MS medium containing 2% (w/v) sucrose (Murashige and Skoog, 1962). Homozygous seedlings were selected by PCR using the primers bn233 and bn234 (genomic *VTE6* locus, 1255 bp); bn232 and bn234 (*vte6-1*, 970 bp); and bn130 and bn234 (*vte6-2*, 1230 bp). The phenotype of the line *vte6-2* (pst00121) was complicated by the fact that heterozygous plants were severely stunted, originating from a second site mutation. After backcrossing this plant with the RIKEN transposon donor line Ds13 (N8521), a *vte6-2* plant devoid of the secondary mutation was obtained.

The *vte5-2* (At5g04490; pst12490, 11-6074-1) transposon mutant allelic to *vte5-1* deficient in phytol kinase (Valentin et al., 2006) was obtained from the RIKEN Bioresource Center. Homozygous *vte5-2* plants were isolated by PCR screening (genomic locus, bn771, bn772, 1352 bp; insertion, bn771, bn232, 1080 bp). Heterozygous *vte6-1* and homozygous *vte5-2* plants were crossed, and plants heterozygous for *vte6-1* and homozygous for *vte5-2* were selected in the F2 progeny, and double homozygous *vte5-2 vte6-1* plants in the F3, by PCR screening.

The *pao1* mutant (At3g44880, SALK_111333) was obtained from Stefan Hörtensteiner, University of Zürich, Switzerland (Pruzinská et al., 2005). Plants were grown at 150 µmol m^{-2} s^{-1} light at 16 h light per day, 20°C, and 55% relative humidity.

RT-PCR

RNA was extracted from leaves, and cDNA synthesis and RT-PCR were performed as described previously (Lippold et al., 2012). Oligonucleotides used for RT-PCR of *VTE6*, *VTE5*, and *ACT2* are described in Supplemental Table 1.

Generation of Overexpression Lines

To generate transgenic Arabidopsis lines overexpressing *VTE6*, the full-length coding sequence of At1g78620/*VTE6* was amplified from *VTE6* cDNA (pda00492, RIKEN) by PCR adding restriction sites using the oligonucleotides bn327 (*Xma*JI) and bn328 (*Sal*I) (Supplemental Table 1). The PCR products were ligated into the cloning vector pGEM-T Easy (Promega). The plasmid was digested with *Xma*JI and *Sal*I (*VTE6*) and the insert ligated into the *Spe*I and *Sal*I sites of the binary vector pLH9000 (DNA Cloning Service), yielding pLH9000-VTE6 for expression in plants under the control of the CaMV 35S promoter.

The plasmid was transferred into *Agrobacterium tumefaciens* (GV3101-pMP90) by electroporation. Arabidopsis wild-type Col-0 plants were transformed by floral dipping (Clough and Bent, 1998). Transgenic seeds were selected by fluorescence microscopy based on the expression of the DsRed marker gene. Overexpression of *VTE6* was confirmed by RT-PCR.

Measurement of Chlorophyll, Tocopherol, Phytol, and Fatty Acid Phytyl Esters

Chlorophyll was quantified photometrically (Porra et al., 1989). For the extraction of tocopherol, fatty acid phytyl esters, and free phytol, 50 mg of leaf tissue was frozen in liquid nitrogen and ground to a fine powder (Precellys homogenizer). The ground tissue was resuspended in 500 µL diethylether and phase separation achieved by centrifugation after adding 250 µL of 0.2 M H$_3$PO$_4$/1 M KCl. The organic phase was harvested and dried under air flow. Tocopherol was measured by fluorescence HPLC on a diol column (Zbierzak et al., 2010). Free phytol was quantified by gas chromatography-mass spectrometry after derivatization with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide using oleyl alcohol (Sigma-Aldrich) as internal standard (Ischebeck et al., 2006).

Fatty acid phytyl esters were quantified using 17:0-phytol, synthesized from heptadecanoic acid (17:0) and phytol (Ischebeck et al., 2006). Fatty acid phytyl esters were detected by scanning for the neutral loss of the mass of 278.2974 (C$_{20}$H$_{38}$, [phytol-H$_2$0]) by direct infusion-tandem mass spectrometry experiments (Agilent 6530 Accurate Mass Q-TOF). The samples were infused via an HPLC-ChipCube nanospray ion source in chloroform/methanol/300 mM ammonium acetate (300:665:35, v/v/v) at 1 µL min^{-1} (Welti et al., 2002). The Q-TOF mass spectrometer was operated in positive ion mode with a fragmentor voltage of 270 V. Membrane glycerolipids were isolated and quantified as described (Gasulla et al., 2013).

Quantification of Isoprenyl-Phosphates by Liquid Chromatography-Mass Spectrometry

Isoprenyl-phosphates were extracted from leaves (50 mg) using isopropanol/50 mM KH$_2$PO$_4$/acetic acid (1:1:0.025, v/v/v) (Larson and Graham, 2001). Internal standards (0.1 nmol each of decanyl-phosphate and decanyl-diphosphate) were synthesized and purified (Joo et al., 1973) and added to the lipid extracts. Isoprenyl-phosphates were separated on a Nucleodur C8 column (50 mm × 4.6 mm, 1.8 µm; Macherey and Nagel), using a gradient of acetonitril and 5 mM aqueous ammonium acetate. The concentration of acetonitril was increased from 20 to 100% in 25 min. Mass spectrometry was performed on an Agilent 6530 Accurate Mass Q-TOF instrument with JetStream ESI source. Isoprenyl-phosphates were analyzed in negative ion mode after collision-induced dissociation, by precursor ion scanning for the phosphate group (HPO$_3$$^-$, *m/z* 78.9591). The parental ions of the isoprenyl-phosphates [M-H]$^-$ were phytyl-P (*m/z* 375.2664), phytyl-PP (*m/z* 455.2328), and GG-PP (*m/z* 449.1858) as previously described (Valentin et al., 2006).

Expression of VTE6 and VTE5 Proteins in *Escherichia coli*

The coding sequences of *VTE6* and *VTE5* were amplified from Arabidopsis cDNA, and specific restriction sites were added using the primer pairs CB1/CB2 and CB3/CB4 (Supplemental Table 1), respectively. The fragments were subcloned into pJet2.1 (ThermoFisher Scientific) and verified by sequencing. After digestion with *Bam*HI/*Not*I (*VTE6* fragment) or *Nde*I/*Fse*I (*VTE5* fragment), the fragments were ligated into the multiple cloning sites 1 or 2 of the expression vector pETDuet-1 (Novagen, Merck Millipore), which is designed for coexpression of two genes. Thus, *VTE6* was N-terminally fused to a 6x His-tag sequence, while *VTE5* was C-terminally fused to an S-tag. In addition to the pETDuet-1 plasmid containing both the *VTE6* and *VTE5* cDNAs, plasmids carrying either *VTE6* or *VTE5* were also generated. The plasmids were transformed into *E. coli* Rosetta (DE3) cells, and

ampicillin-resistant transformants were selected. Cells were grown in Luria-Bertani medium at 37°C and 200 rpm until an OD_{600} of 0.7 was reached. Protein expression was induced with 1 mM isopropylthio-β-galactoside. The fusion proteins were detected by immunodetection after blotting using horseradish peroxidase-conjugated antibodies specific for His-tag (Miltenyi Biotec) and S-tag (Merck Millipore) for VTE6 and VTE5, respectively.

In Vivo Phytol Feeding

Arabidopsis seedlings (the wild-type, *vte6-1*, and *vte6-2*) were grown on MS medium with 2% (w/v) sucrose for 6 weeks and then transferred to 20 mL of 20 mM MES-KOH, pH 6.5, containing 0.1% (v/v) phytol (Sigma-Aldrich) in an Erlenmeyer flask. The flasks were incubated under continuous light (100 μmol photons m^{-2} s^{-1}) for 48 h while shaking before extraction of isoprenyl-phosphates.

Feeding of *E. coli* cells with synthetic phytol was done as described by Valentin et al. (2006) with some modifications. Briefly, protein expression was induced using 1 mM isopropylthio-β-galactoside and the cells grown overnight at 16°C. Cells were harvested, washed, and transferred to 5 mL of Luria-Bertani medium containing carbenicillin (50 μg/mL), 5 mM phytol, and 0.2% toluene. The cells were incubated for 3 h at 30°C and harvested by centrifugation and the cell pellet used for isoprenyl-phosphate extraction.

In Vitro Phytol Kinase and Phytyl-P Kinase Assays

Proteins were isolated from Arabidopsis leaves and *E. coli* cell pellets as previously described (Ischebeck et al., 2006). For the phytyl-P kinase assay, 10 nmol of phytyl-P (Isoprenoids) in ethanol were transferred to a 1.5-mL microfuge tube and dried under air flow. Afterwards, 300 μg crude protein extract was mixed with 10 nmol CTP and 8 μL assay buffer (20 mM $MgCl_2$, 50 mM Na-orthovanadate, and 0.25% CHAPS) to a final volume of 100 μL. For the coupled phytol kinase/phytyl-P kinase assay, 1 nmol of phytol instead of phytyl-P was used in the reaction. The reaction was incubated for 30 min at 30°C and terminated by freezing in liquid N_2. Isoprenyl-phosphates were isolated and analyzed using LC-MS/MS as described above.

Subcellular Localization Studies of YFP-Tagged VTE6 Protein

For subcellular localization studies, the coding sequence (without stop codon) of *VTE6* was PCR amplified from Arabidopsis cDNA with gene-specific primers (CB157/CB158; Supplemental Table 1) carrying Gateway recombination sites and recombined into the pUBC-YFP binary vector (Grefen et al., 2010) for C-terminal fusion of *VTE6* to *YFP*, driven by the Ubiquitin10 promoter. The fusion construct *YFP:VTE6* was verified by sequencing. Agrobacterium strain GV3101 was transformed with the plasmid and infiltrated into *Nicotiana benthamiana* leaves for transient expression of YFP:VTE6 fusion proteins. After 48 h, protoplasts were isolated for localization studies by a confocal laser scanning microscope (Zeiss LSM 510) as described in detail earlier (Breuers et al., 2012).

Phylogenetic and Statistical Analysis

Amino acid sequences of VTE5- and VTE6-related proteins from Arabidopsis, *Synechocystis*, *Synechococcus*, *Anabaena*, *Nostoc*, *Chlorobium*, *Pelodyction*, *Symbiobacterium thermophilum*, *Thermoplasma acidophilum*, and *Archaeoglobus fulgidus* were obtained from GenBank. Sequence alignments were done using ClustalW, and phylogenetic trees were constructed based on the neighbor-joining method with the bootstrap test with MEGA 6 (Tamura et al., 2013). A text file of the alignment is available as Supplemental Data Set 1.

Statistical analyses were performed using Microsoft Excel 2010. Values are significantly different according to Student's *t* test ($P < 0.05$). The significance of differences in genotypes between the segregating population and the expected distribution (Figure 3) was calculated according to the χ^2 test ($P < 0.01$). At least three biological replicates were used for the biochemical analyses.

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under Arabidopsis locus identifiers At5g04490 and At1g78620, for *VTE5* and *VTE6*, respectively.

Supplemental Data

Supplemental Figure 1. Phylogenetic Relationship of COG1836-Like and Phytol Kinase Sequences.

Supplemental Figure 2. Subcellular Localization of VTE6 to the Chloroplast Envelope Membranes.

Supplemental Figure 3. Expression of VTE5 and VTE6 in *Escherichia coli*.

Supplemental Figure 4. Membrane Glycerolipids in Leaves of the *vte6* Mutant.

Supplemental Table 1. Oligonucleotides Used in This Study.

Supplemental Data Set 1. Text File of the Alignment Corresponding to the Phylogenetic Analysis in Supplemental Figure 1.

AUTHOR CONTRIBUTIONS

P.D., G.H. M.E., A.P.M.W., and A.D.H. designed the experiments. K.v.D., G.H., C.P., M.E., and M.A. performed the research. A.D.H. contributed new analytic and computational tools. All authors analyzed data. P.D., K.v.D., M.E., A.P.M.W., and A.D.H. wrote the article.

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