

Chlorophyll Synthase under Epigenetic Surveillance Is Critical for Vitamin E Synthesis, and Altered Expression Affects Tocopherol Levels in Arabidopsis¹[OPEN]

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Chlorophyll synthase catalyzes the final step in chlorophyll biosynthesis: the esterification of chlorophyllide with either geranylgeranyl diphosphate or phytyl diphosphate (PDP). Recent studies have pointed to the involvement of chlorophyll-linked reduction of geranylgeranyl by geranylgeranyl reductase as a major pathway for the synthesis of the PDP precursor of tocopherols. This indirect pathway of PDP synthesis suggests a key role of chlorophyll synthase in tocopherol production to generate the geranylgeranyl-chlorophyll substrate for geranylgeranyl reductase. In this study, contributions of chlorophyll synthase to tocopherol formation in Arabidopsis (*Arabidopsis thaliana*) were explored by disrupting and altering expression of the corresponding gene *CHLOROPHYLL SYNTHASE* (*CHLSYN*; At3g51820). Leaves from the homozygous *chlsyn1-1* null mutant were nearly devoid of tocopherols, whereas seeds contained only approximately 25% of wild-type tocopherol levels. Leaves of RNA interference lines with partial suppression of *CHLSYN* displayed marked reductions in chlorophyll but up to a 2-fold increase in tocopherol concentrations. *Cauliflower mosaic virus35S*-mediated overexpression of *CHLSYN* unexpectedly caused a cosuppression phenotype at high frequencies accompanied by strongly reduced chlorophyll content and increased tocopherol levels. This phenotype and the associated detection of *CHLSYN*-derived small interfering RNAs were reversed with *CHLSYN* overexpression in *rna-directed rna polymerase6* (*rdr6*), which is defective in RNA-dependent RNA polymerase6, a key enzyme in sense transgene-induced small interfering RNA production. *CHLSYN* overexpression in *rdr6* had little effect on chlorophyll content but resulted in up to a 30% reduction in tocopherol levels in leaves. These findings show that altered *CHLSYN* expression impacts tocopherol levels and also, show a strong epigenetic surveillance of *CHLSYN* to control chlorophyll and tocopherol synthesis.

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The tocopherol form of vitamin E is synthesized in plastids by condensation of the saturated C20 isoprenoid phytyl diphosphate (PDP) with homogentisate through the activity of homogentisate phytyltransferase (HPT; Soll et al., 1980; Collakova and DellaPenna, 2003). PDP is the product of the reduction of geranylgeranyl by the enzyme geranylgeranyl reductase (GGR; Keller et al., 1998). In vitro assays of Arabidopsis (*Arabidopsis thaliana*) GGR have shown that this enzyme can directly convert geranylgeranyl diphosphate (GGDP) to PDP or use geranylgeranyl-chlorophyll as a substrate (Keller et al., 1998). To be of physiological significance, the latter pathway would require the prenylation of chlorophyllide with geranylgeranyl from GGDP by chlorophyll synthase activity and the release of phytol formed by GGR activity with geranylgeranyl-chlorophyll (Fig. 1). The released phytol would then require two phosphorylation steps to generate the PDP substrate for tocopherol synthesis. The existence and quantitative significance of this indirect pathway for tocopherol production in Arabidopsis are supported by the identification of the *VITAMIN E PATHWAY GENE5* (*VTE5*)-encoded phytol kinase that catalyzes the first phosphorylation step of phytol released from chlorophyll (Valentin et al., 2006).

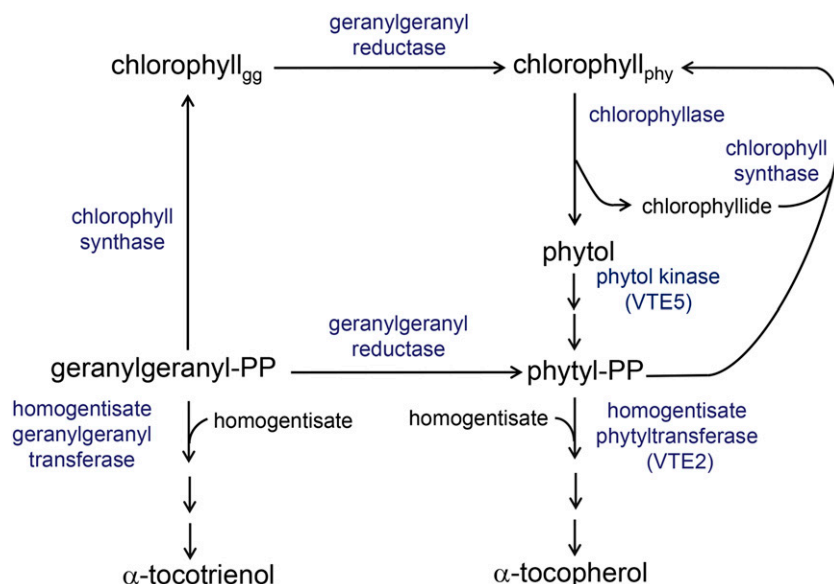


Figure 1. Pathways for de novo PDP synthesis and tocopherol biosynthesis in photosynthetic organisms. Tocopherol biosynthesis is initiated by the prenylation of homogentisate with PDP catalyzed by HPT. PDP is synthesized by the reduction of geranylgeranyl either in the diphosphate form or as the hydrophobic sidechain of chlorophyll by the activity of GGR, and recent evidence has pointed to that at least 80% of PDP synthesis proceeds through the reduction of geranylgeranyl bound to chlorophyll in *Arabidopsis* (Ischebeck et al., 2006; Valentin et al., 2006). This pathway for PDP synthesis requires an array of enzymes in addition to GGR, including chlorophyll synthase to esterify geranylgeranyl to chlorophyll, chlorophyllase to release phytol from chlorophyll, and two phytol kinases, including VTE5, to convert released phytol to PDP. The esterification of chlorophyllide with GGDP catalyzed by chlorophyll synthase is the primary reaction for chlorophyll biosynthesis, and esterification of chlorophyllide with PDP is a secondary reaction for chlorophyll synthesis catalyzed by chlorophyll synthase using PDP derived from chlorophyll degradation. An alternative fate for GGDP is condensation with homogentisate for biosynthesis of the tocotrienol form of vitamin E (Cahoon et al., 2003; Yang et al., 2011). chlorophyll_{gg}, Chlorophyll linked to a geranylgeranyl moiety; chlorophyll_{phy}, chlorophyll linked to a phytol moiety.

The *Arabidopsis vte5-1* mutant accumulates only 35% of wild-type levels of tocopherols in leaves and 20% of wild-type levels of tocopherols in seeds (Valentin et al., 2006). Similarly, disruption of the *Synechocystis* sp. PCC 6803 homolog of VTE5 (encoded by open reading frame slr1652) results in a 50% reduction of tocopherol levels. The *Arabidopsis* and *Synechocystis* sp. PCC 6803 mutants also accumulate free phytol (Valentin et al., 2006).

Tocopherol synthesis has been a major target for biotechnological improvement of the antioxidant content and nutritive value of plants (Karunanandaa et al., 2005; Raclaru et al., 2006). Significant progress has been made in these efforts by enhancing expression of genes for enzymes, such as HPT (Soll et al., 1980; Collakova and DellaPenna, 2003). However, the indirect pathway of PDP formation likely represents a bottleneck for achieving maximal tocopherol production, particularly in green seeds, such as *Arabidopsis*, soybean (*Glycine max*), and rapeseed (*Brassica napus*; Karunanandaa et al., 2005; Raclaru et al., 2006; Seo et al., 2011; Yang et al., 2011; Zhang et al., 2013). For example, HPT overexpression in these seeds, even with the combined enhancement of homogentisate synthesis, has failed to achieve high levels of tocopherol enhancement (Savidge et al., 2002; Karunanandaa et al., 2005). This can be attributed, in part, to the complexity of PDP production

through the chlorophyll-linked pathway. As such, a more in-depth understanding of the steps in this pathway is likely to provide important insights to guide tocopherol biofortification efforts.

Central to the indirect pathway of PDP synthesis for tocopherol production is the activity of chlorophyll synthase to generate the geranylgeranyl-chlorophyll substrate for GGR. Although chlorophyll synthase has been extensively studied for its role in chlorophyll synthesis, the importance of this enzyme for tocopherol synthesis has not been previously examined. Chlorophyll synthases from a variety of photosynthetic organisms have been shown to use not only PDP as a substrate for prenylation of chlorophylls *a* and *b* but also, GGDP as a substrate (Oster and Rüdiger, 1997; Oster et al., 1997; Wu et al., 2007). In fact, the enzyme encoded by the single chlorophyll synthase gene in *Arabidopsis* (CHLOROPHYLL SYNTHASE [CHLSYN]; At3g51820) displays higher *in vitro* activity with GGDP than with PDP for the esterification of chlorophyllide to generate chlorophyll (Oster and Rüdiger, 1997). The *Synechocystis* sp. PCC 6803 chlorophyll synthase, however, has higher activity with PDP than with GGDP (Oster et al., 1997).

Previous studies that have targeted chlorophyll synthase for genetic manipulation in plants have only

focused on chlorophyll synthesis and not on tocopherol synthesis. For example, a missense mutation in chlorophyll synthase in rice (*Oryza sativa*) was shown to reduce enzymatic activity and chlorophyll content in leaves as well (Wu et al., 2007). In addition, antisense suppression of the tobacco (*Nicotiana tabacum*) chlorophyll synthase gene resulted in coordinate reduction of transcript levels for genes encoding enzymes involved in synthesis of the chlorophyllide porphyrin ring and chlorophyll binding proteins (Shalygo et al., 2009).

In this report, we explore the in planta contributions of chlorophyll synthase to tocopherol synthesis by examination of a transfer DNA (T-DNA) mutant for *CHLSYN* and by up- or down-regulation of this gene. Our findings provide additional support for the quantitative importance of the indirect biosynthetic pathway for the tocopherol precursor PDP in Arabidopsis leaves and seeds. They also show that altered chlorophyll synthase activity inversely impacts tocopherol levels in Arabidopsis leaves. Furthermore, it was discovered that *CHLSYN* is subject to strong epigenetic surveillance that affects both chlorophyll and tocopherol biosynthesis, because *CHLSYN* overexpression induces the production of small interfering RNAs (siRNAs) at a very high frequency.

RESULTS

Leaves and Seeds of an Arabidopsis Chlorophyll Synthase Mutant Are Deficient in Tocopherol

A T-DNA insertion mutant for chlorophyll synthase (SALK_134433; designated *chlsyn1-1*) was characterized to assess the role of this enzyme in tocopherol biosynthesis. The T-DNA insertion of *chlsyn1-1* was confirmed to be present in the last exon of the Arabidopsis *CHLSYN* gene (Fig. 2A). The heterozygous T-DNA insertion line, which was confirmed by PCR analysis, displayed a segregation ratio of 3:1 for green seeds:yellow seeds in siliques. Although seed abortion was not detected in siliques, no plants homozygous for

the T-DNA insertion were obtained from soil-sown seeds from self-pollinated plants genotyped as heterozygous *chlsyn1-1* plants. However, albino plants genotyped as homozygous *chlsyn1-1* plants were recovered by germination of these seeds on media containing 2% (w/v) Suc at the expected segregation ratio of 1:3. These plants lacked detectable *CHLSYN* transcript as assessed by reverse transcription (RT)-PCR analysis of leaf RNA (Fig. 2B). Homozygous *chlsyn1-1* plants retained viability with vigorous growth on Suc-containing media for >5 weeks, although they were deficient in chlorophyll. Analysis of leaves of plants at weekly intervals from 1 to 5 weeks of growth revealed only low tocopherol concentrations in homozygous *chlsyn1-1* plants relative to wild-type plants (Fig. 3; Supplemental Fig. S1). At 2 weeks of growth, homozygous *chlsyn1-1* plants had 10% of wild-type tocopherol concentration, which was the highest ratio during the 5-week growth period. Leaves of homozygous *chlsyn1-1* plants at this growth stage had 50% of wild-type total fatty acid concentration (Supplemental Fig. S2), indicating that plastids are still metabolically active, despite chlorophyll deficiency. These results suggest a strong link between chlorophyll synthase activity and vitamin E production in Arabidopsis.

To gain additional evidence in support of this conclusion, genetic complementation studies of *chlsyn1-1* were conducted. For these studies, the Arabidopsis *rna-directed rna polymerase6* (*rdr6*) mutant was transformed with a *CHLSYN* complementary DNA (cDNA) under control of a *Cauliflower mosaic virus35S* (CaMV35S) promoter and linked to a DsRed fluorescent protein selection marker. This background was used to mitigate the high frequency of *CHLSYN* silencing observed with overexpression of this gene in Arabidopsis Columbia-0 (Col-0), which is described in more detail below. Transgenic lines were then crossed into heterozygous *chlsyn1-1* plants. After selection and genetic confirmation, DsRed-positive F₃ plants homozygous for the *chlsyn1-1* allele displayed growth and pigmentation identical to those of wild-type plants (Fig. 4A), indicating that At3g51820 is responsible in vivo for chlorophyll synthesis, which was expected. Plants homozygous for the

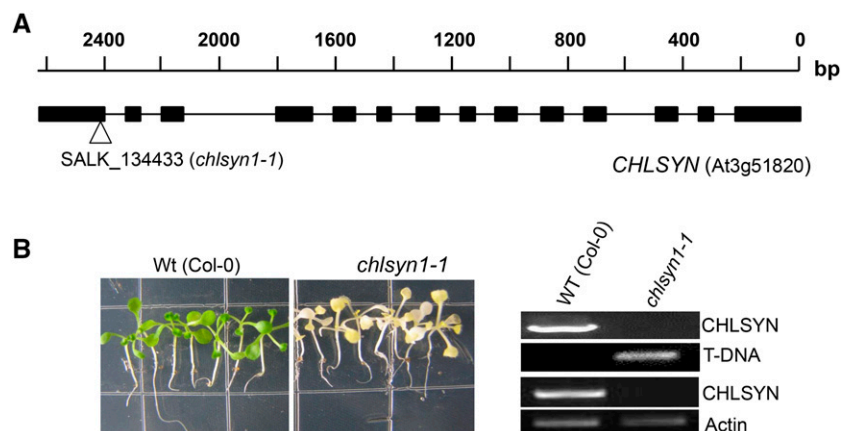


Figure 2. Gene structure and characterization of the mutant for *CHLSYN*. A, Gene structure of *CHLSYN* and T-DNA insertion site in SALK134433 (*chlsyn1-1*). Black bars represent exons, and lines indicate introns. The white triangle indicates the T-DNA insertion site. B, Plants shown are wild-type (WT) and null mutant *chlsyn1-1*, which were genetically and transcriptionally confirmed by PCR and RT-PCR, respectively. For RT-PCR analysis, the *UBC* gene (At5g25760) was used as an internal control.

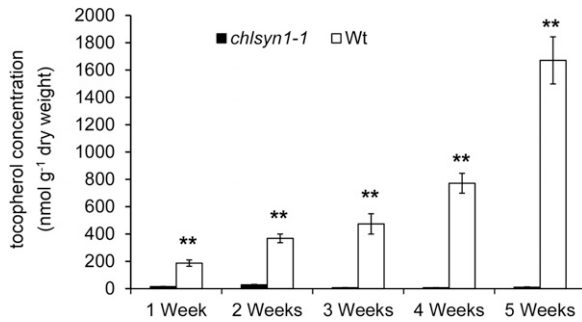


Figure 3. Tocopherol accumulation is reduced in leaves of the null mutant of *chlsyn1-1*. Measurements of tocopherol concentrations were conducted weekly with leaves from 1- to 5-week-old plants. Data represent means \pm SE of three independent biological replicates. Wt, Wild type; **, $P < 0.01$ (Student's *t* test).

chlsyn1-1 allele but segregating for the transgene (Fig. 4, B and C) provided a tool for additional examination of the involvement of chlorophyll synthase in tocopherol synthesis. DsRed-positive seeds from the two lines corresponding to *chlsyn1-1* complementation had tocopherol content comparable with that in wild-type seeds. However, non-DsRed seeds from the two lines corresponding to a lack of *chylsyn1-1* complementation had tocopherol contents at 20% and 26% of wild-type seed content (Fig. 4D). These reductions in tocopherol content are nearly the same as those previously reported in the *vte5* mutant (Valentin et al., 2006) and provide additional support for a central role of chlorophyll synthase in tocopherol biosynthesis.

RNA Interference-Mediated Silencing of CHLSYN Leads to Enhancement of Tocopherol Content in Arabidopsis Leaves

As shown in the model presented in Figure 1, chlorophyll synthase is not only required for phytol synthesis by GGR, but also, it catalyzes a biosynthetic reaction for chlorophyll formation that competes for PDP with HPT, the initial tocopherol biosynthetic enzyme. As such, it is not clear how alteration of CHLSYN would affect tocopherol biosynthesis. To examine this question, RNA interference (RNAi) suppression of CHLSYN was initially conducted. As expected for a gene encoding a central enzyme in chlorophyll synthesis, suppression of CHLSYN resulted in extensive chlorosis in transformants (Fig. 5, A and B). This phenotype, although not visually observed in 5-d-old seedlings, was strongly apparent in 5-week-old plants. In lines RNAi-13 and RNAi-14 with the most extreme chlorotic phenotype, chlorophyll levels were reduced in leaves at 5 and 7 weeks of growth by up to 65% relative to those in leaves of wild-type plants of the same age. Reductions in chlorophyll concentrations in leaves of transformants were accompanied by a general inverse correlation in

tocopherol concentrations (Fig. 5C). In lines RNAi-13 and RNAi-14, for example, tocopherol concentrations were 1.5- to 2-fold higher in leaves of 5- and 7-week-old plants relative than those in wild-type plants of the same age.

Overexpression of Arabidopsis Chlorophyll Synthase Triggers CHLSYN Sense Suppression

To complement the studies above, experiments were also conducted to examine the effects of overexpression of CHLSYN on tocopherol concentrations in Arabidopsis leaves. For these experiments, a CHLSYN cDNA was expressed under control of the constitutive CaMV35S promoter. Unexpectedly, approximately 90% of the transgenic plants obtained displayed varying degrees of chlorosis in leaves after 2 weeks of growth. As determined by RT-PCR, CHLSYN transcript was detected in leaves of 5-d-old seedling in four lines selected for more detailed characterization. However, no transcript was detectable in leaves of 5-week-old plants in any of these lines (Fig. 6A). Consistent with this, chlorophyll concentrations in leaves of these lines ranged from 25% to 30% of those in

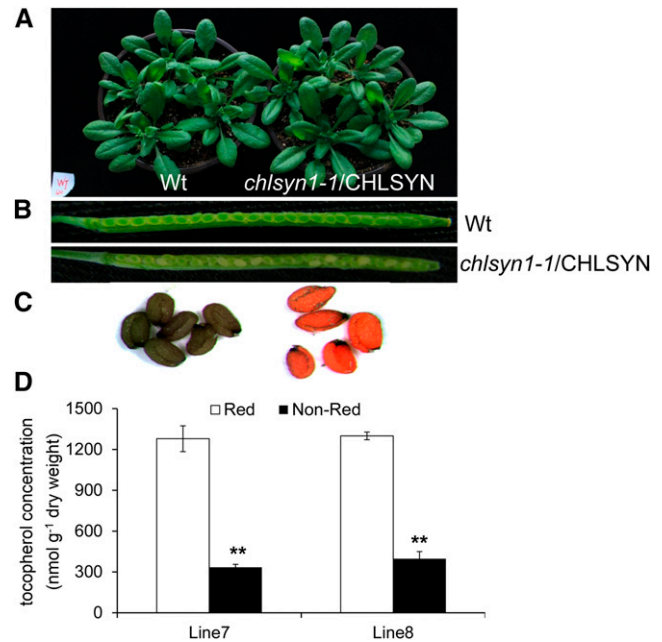


Figure 4. Tocopherol accumulation is reduced in mature seeds of *chlsyn1-1*. Seedlings and seed morphologies (A and B) of *chlsyn1-1* plants with genetic complementation of *AtCHLSYN*. Also shown are green seeds lacking DsRed fluorescence corresponding to non-complemented seeds (C, left), seeds with DsRed fluorescence corresponding to complemented seeds (C, right), and comparison of tocopherol concentrations in seeds with *chlsyn1-1* complementation (D; red) and non-complemented *chlsyn1-1* (D; nonred) seeds. Data represent means \pm SE of three independent biological replicates. Wt, Wild type; **, $P < 0.01$ (Student's *t* test).

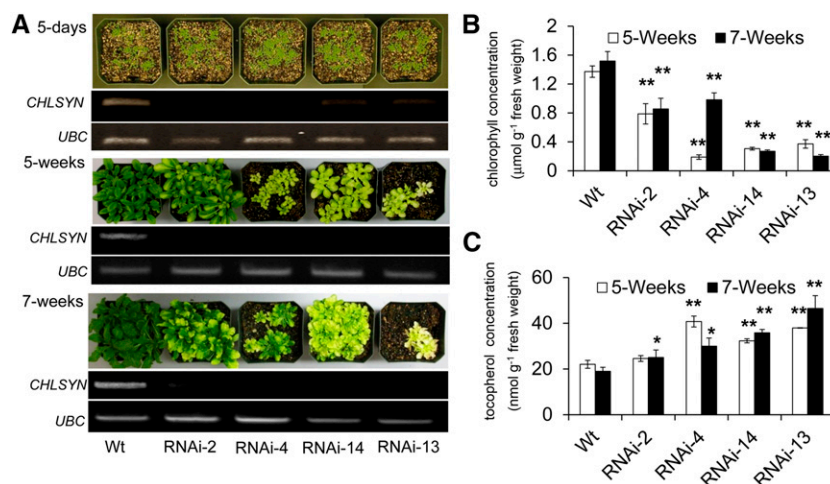


Figure 5. RNAi suppression of *CHLSYN* results in reduced chlorophyll accumulation but enhanced tocopherol concentrations. A, Plants shown are Col-0 plants and Col-0 plants transformed with the pH8*CHLSYN* RNAi construct at 5 d, 5 weeks, and 7 weeks grown under a 14 h of light/10 h of dark cycle. Semiquantitative RT-PCR analysis of the *CHLSYN* expression level in leaves from corresponding plants. The wild type (Wt) and RNAi-2, RNAi-4, RNAi-13, and RNAi-14 are representative of plants of Col-0 and independent transgenic events of Col-0 plants transformed with the pH8*CHLSYN* RNAi construct, respectively. The gene encoding *UBC* (At5g25670) was used as an internal control in RT-PCR analyses. Chlorophyll (B) and tocopherol (C) concentrations of leaves from Col-0 plants and Col-0 plants transformed with the pH8*CHLSYN* RNAi construct from sampling of 5- and 7-week-old plants. Data represent means \pm SE of three independent biological replicates. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test).

leaves of wild-type plants of the same age (Fig. 6B). However, *CHLSYN* suppression and leaf chlorosis were not stable over the entire lifecycle for two of these lines. For example, *CHLSYN* transcript was detectable in leaves of lines Wt/*SYN3* and Wt/*SYN7* in 7-week-old plants, and sharp increases in leaf chlorophyll concentration were measured in Wt/*SYN7* between 5 and 7 weeks of age.

The effects of chlorophyll reductions on tocopherol concentrations in leaves of lines engineered for *CHLSYN* overexpression were similar but larger than those detected in leaves of plants engineered for *CHLSYN* RNAi suppression (Fig. 6C). In leaves of lines Wt/*Syn8* and Wt/*Syn13*, which displayed the largest reductions in leaf chlorophyll concentrations relative to those of leaves of wild-type plants, tocopherol concentrations were 2- to 3-fold higher than those in leaves of wild-type plants in both 5- and 7-week-old plants.

Overexpression of *CHLSYN* Decreases Tocopherol Content of Leaves

The observation of high rates of *CHLSYN* cosuppression triggered by overexpression of this gene was suggestive of sense transgene-induced posttranscriptional gene silencing similar to that reported for *ARGONAUTE1* (Mallory and Vaucheret, 2009). This process was shown to be dependent primarily on *RDR6*, *SUPPRESSOR OF GENE SILENCING3*, and *SILENCING DEFECTIVE5* (Mallory and Vaucheret,

2009). Based on this prior report, the CaMV35S-*AtCHLSYN* construct described above was introduced into the *Arabidopsis rdr6* mutant that is defective in sense transgene-induced posttranscriptional gene silencing. In >30 independent lines obtained from this transformation, no leaf chlorosis phenotype was observed (Fig. 7A). Consistent with this, the *CHLSYN* transcript was elevated in four randomly selected transformants (Fig. 7B). Additional examination of RNA isolated from leaves of overexpression lines in the Col-0 and *rdr6* backgrounds revealed high accumulation of *CHLSYN*-derived siRNAs in Col-0 that were not detectable in the *rdr6* background (Fig. 8). Measurements conducted on extracts from leaves of four independent overexpression lines in the *rdr6* background revealed no significant change in chlorophyll concentrations but approximately 30% reduction in tocopherol concentrations (Fig. 7, C and D) relative to leaves of non-transformed *rdr6* plants. These results together with those from *CHLSYN*, RNAi, or sense suppression indicate a negative correlation between *CHLSYN* expression levels and tocopherol concentrations in *Arabidopsis* leaves.

DISCUSSION

Previous findings from the *vte5* mutant have highlighted the importance of metabolic flux of GGDP through chlorophyll for synthesis of the phytol side-chain of tocopherol by the activity of GGR in *Arabidopsis* (Valentin et al., 2006). Central to this pathway is

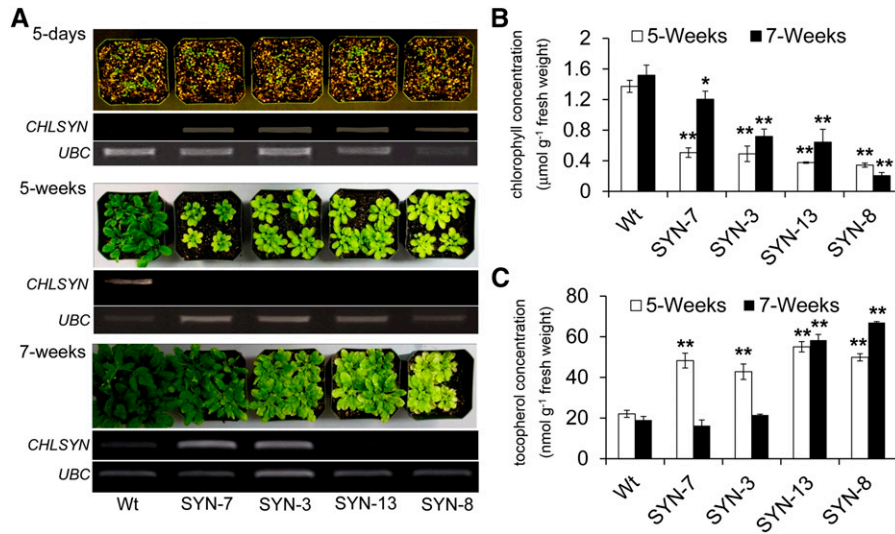
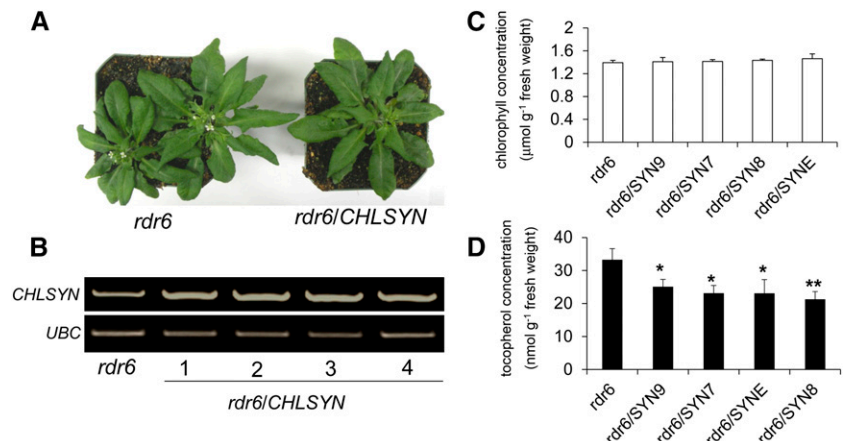


Figure 6. Overexpression of *CHLSYN* triggers sense suppression and alters chlorophyll and tocopherol concentrations in leaves. A, Plants shown are Col-0 and Col-0 expressing chlorophyll synthase under control of the CaMV35S promoter at 5 d, 5 weeks, and 7 weeks of age and maintained in a 14 h of light/10 h of dark cycle. Semiquantitative RT-PCR analysis of expression of chlorophyll synthase in leaves from these plants is shown in A, middle. The wild type (Wt) and Wt/SYN-7, Wt/SYN-3, and Wt/SYN-13 are representative of plants of Col-0 and four independent transgenic chlorophyll synthase overexpression lines, respectively. The gene encoding *UBC* (At5g25670) was used as an internal control in RT-PCR analysis. Shown are chlorophyll (B) and tocopherol (C) concentrations of leaves from Wt plants and plants engineered for chlorophyll synthase overexpression from sampling of 5- and 7-week-old plants. Data represent means \pm SE of three independent biological replicates. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test).

chlorophyll synthase, which has the dual role of catalyzing the attachment of geranylgeranyl as a diphosphate substrate to chlorophyllide to initiate the synthesis of phytol and catalyzing the attachment of released phytol as a diphosphate substrate to chlorophyllide for synthesis of the major chlorophyll forms used in photosynthesis (Oster et al., 1997; Fig. 1). The studies presented here were conducted to examine the quantitative importance of chlorophyll synthase in tocopherol synthesis and explore its use as a target for altering tocopherol levels in leaves. A useful tool for our studies was a *CHLSYN* T-DNA insertion mutant *chlsyn1-1*. Although lacking chlorophyll, *chlsyn1-1*

seedlings were viable when grown on Suc and had 10% of wild-type tocopherol concentration. This mutant coupled with the use of a *CHLSYN* overexpression transgene linked to a DsRed fluorescence marker also provided a unique approach for investigating the quantitative significance of chlorophyll synthase in seed tocopherol synthesis. Plants homozygous for the *chlsyn1-1* allele but segregating for the overexpression transgene were phenotypically similar to wild-type plants in appearance and leaf tocopherol concentrations. Seeds from these plants could be readily segregated based on the DsRed marker: genetically complemented *chlsyn1-1* seeds were fluorescent,

Figure 7. Overexpression of *CHLSYN* in *rdr6* background results in modest reductions of leaf tocopherol concentrations. A, Plants shown are *rdr6* and *rdr6* transformed with *CHLSYN* under control of the CaMV35S promoter. B, Expression of *CHLSYN* in *rdr6* plants and *rdr6* plants from four independent events overexpressing *CHLSYN* is confirmed by RT-PCR (upper) and expression of an internal control gene (*UBC*; At5g25670; lower). Shown are measurements of chlorophyll (C) and tocopherol (D) concentrations of leaves from 3-week-old plants from *rdr6* and four independent events of *rdr6* transformed with *CHLSYN*. Data represent means \pm SE of three independent biological replicates. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test).



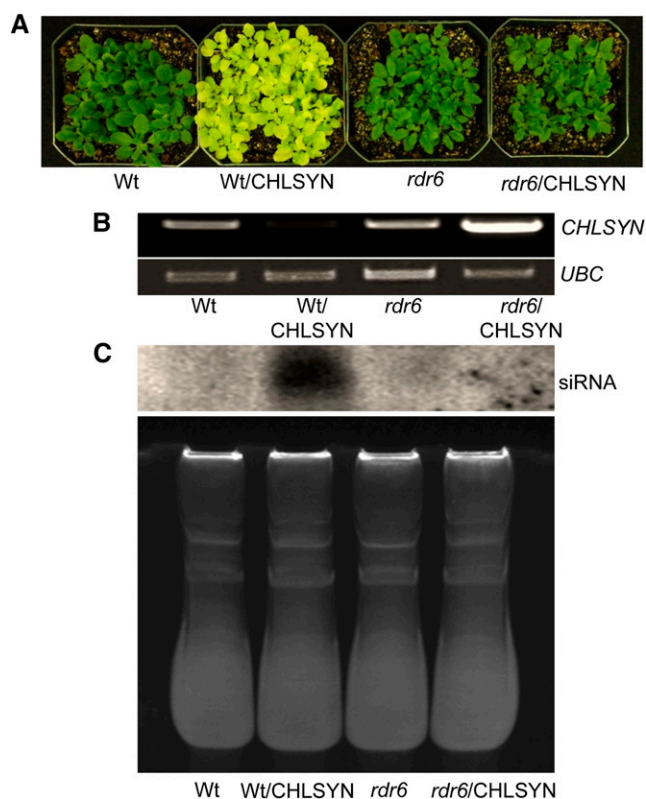


Figure 8. Sense suppression line accumulates *CHLSYN*-specific siRNAs. A, Plants shown from left to right are the wild type (Wt), sense suppression (Wt/*CHLSYN*), *rdr6*, and *rdr6* transformed with *CHLSYN* (*rdr6/CHLSYN*) under control of the CaMV35S promoter. Also, RT-PCR analysis of *CHLSYN* transcript levels is shown in B. Shown in C, top is the accumulation of *CHLSYN* siRNA (21–22 nucleotides) in leaves from Wt and *rdr6* plants nontransformed or engineered for CaMV35S-mediated overexpression of *CHLSYN*. C, bottom shows ethidium bromide staining of low-molecular weight RNA.

whereas seeds without the transgene and homozygous for the *chlsyn1-1* allele were not fluorescent. The latter seeds, lacking a functional chlorophyll synthase, had approximately 20% of the tocopherol concentration of wild-type seeds, which is identical to the tocopherol reductions measured previously in *vte5-1* seeds. In addition to providing useful information in our studies, a similar approach using DsRed as a screenable marker for complementation should have general use for examining seed phenotypes in mutants with compromised vegetative growth.

Because chlorophyll synthase has dual functions in phytol and chlorophyll synthesis (Oster et al., 1997; Ischebeck et al., 2006; Lin et al., 2014), the impact of suppressed or enhanced *CHLSYN* expression on tocopherol concentrations could not be easily predicted. Our findings indicate that suppression of *CHLSYN* expression increases tocopherol concentrations in leaves, whereas enhanced expression of this gene reduces tocopherol concentrations in leaves (Fig. 9). These observations can largely be attributed to the role

of chlorophyll synthase in phytol attachment to chlorophyllide. Partial suppression of *CHLSYN*, which was achieved by RNAi or sense suppression, would likely reduce the competition between HPT (the initial step in tocopherol synthesis) and chlorophyll synthase for PDP. The result would be increased availability of PDP for tocopherol synthesis. The increases in tocopherol concentrations may indicate that some level of chlorophyll turnover occurs in leaves that can provide additional PDP for the tocopherol biosynthetic pathway in the *CHLSYN* suppression lines. Conversely, enhanced expression of *CHLSYN* may shunt additional PDP to chlorophyll synthesis at the expense of tocopherol production. Because chlorophyll concentrations are >100-fold higher than tocopherol concentrations in leaves, increased PDP flux into chlorophyll may have quantitative significance for tocopherol concentrations but little measurable effect on chlorophyll concentrations, which was observed in our studies.

An unexpected finding was the high incidence of *CHLSYN* suppression in response to the introduction of the CaMV35S-*AtCHLSYN* overexpression transgene in Arabidopsis Col-0. This effect was overcome by the use of the Col-0 *rdr6* mutant as the overexpression background. Because *RDR6* is required for generation of transgene-induced siRNAs and because *CHLSYN* overexpression in Col-0 resulted in formation of *CHLSYN*-specific siRNAs at very high frequency, the observed effect is clearly epigenetic based and suggests that chlorophyll biosynthesis is under the stringent surveillance of epigenetic machinery in Arabidopsis. Notably, the easily visualized chlorotic phenotype associated with *CHLSYN* overexpression may provide a useful tool for studies of siRNA-mediated gene silencing.

Our findings with *chlsyn1-1* together with previous findings from *vte5-1* (Ischebeck et al., 2006; Valentin et al., 2006) indicate that flux of geranylgeranyl through chlorophyll is the primary route of phytyl formation for tocopherol synthesis. However, in both

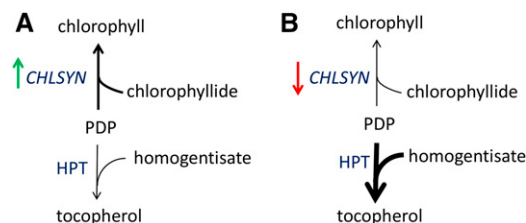


Figure 9. Model for the effects of altered chlorophyll synthase expression on tocopherol concentrations in Arabidopsis leaves. As indicated, HPT, which catalyzes the initial reaction in tocopherol biosynthesis, competes for the PDP substrate with chlorophyll synthase. Based on results from RNAi and cosuppression lines, reduced expression of *CHLSYN* increases PDP concentrations for HPT, leading to enhanced tocopherol production in Arabidopsis leaves (A). Conversely, based on results from overexpression lines, increased expression of *CHLSYN* decreases PDP concentrations for HPT, leading to reduced tocopherol production in Arabidopsis leaves (B).

mutants, seeds retain approximately 20% of wild-type tocopherol levels, and in leaves of the *vtc5-1* mutant, 35% of wild-type tocopherol levels are detected (Valentin et al., 2006). These findings are consistent with lower but significant contributions of direct conversion of GGDP to PDP for tocopherol synthesis. It is likely that this pathway is also a major contributor to tocopherol production in *CHLSYN* suppression lines. Like Arabidopsis, seeds from many other species, including commercially important oilseeds, such as rapeseed and soybean, retain chlorophyll throughout most of their development. As such, it is expected that the chlorophyll route of phytol synthesis is quantitatively important for tocopherol production in seeds of these species. However, many nongreen oilseeds are also enriched in tocopherol. Castor bean (*Ricinus communis*) seeds, for example, have reported tocopherol concentrations of approximately $600 \mu\text{g g}^{-1}$ seed weight (Velasco et al., 2005), which is 200 to $300 \mu\text{g g}^{-1}$ higher than tocopherol concentrations typically found in rapeseed and soybean seeds (Karunanandaa et al., 2005). Because castor bean and other nongreen seeds lack chlorophyll, it is possible that GGR in these seeds has evolved for more efficient direct conversion of GGDP to PDP. Tocopherol biosynthesis in these seeds also does not compete with chlorophyll biosynthesis for PDP, similar to the metabolic scenario in leaves of Arabidopsis *CHLSYN* suppression lines. Overall, more complete understanding of phytol formation in nongreen seeds may provide clues for enhancing tocopherol concentrations in green seeds through metabolic routes that bypass chlorophyll. This is important for tocopherol biofortification efforts in green seeds, which to date, have been relatively ineffective by approaches such as HPT overexpression (Savidge et al., 2002; Karunanandaa et al., 2005), likely because of insufficient pools of PDP.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type and mutant Arabidopsis (*Arabidopsis thaliana*) lines used for these studies were of the Col-0 ecotype. SALK_134433, a *chlsyn1-1* heterozygous T-DNA insertion line, was obtained from the Arabidopsis Biological Resource Center (Alonso et al., 2003). Seeds of SALK_134433 were surface sterilized, grown on one-half-strength Murashige and Skoog medium agar plates supplemented with 2% (w/v) Suc and 100 mg L^{-1} of ampicillin, and grown under a 16-h-light ($100 \mu\text{mol m}^{-2} \text{ s}^{-1}$)/8-h-dark cycle at 22°C. Plants were genotyped by two PCR reactions using a gene-specific primer pair (P1, 5'-AAACTGCAAAATGGATATGCCG-3' and P2, 5'-AACAAAAACATTCTGAAAGCAGAG-3') to determine homozygosity and primer P2 and the T-DNA left border primer PLB (5'-TGGTTCACGTAGTGGCCATCG-3') to test for the presence of the T-DNA. Cosuppression lines and RNAi-mediated gene silencing lines grown on soil were maintained at 22°C and 50% humidity under conditions with a 14-h-light ($100 \mu\text{mol m}^{-2} \text{ s}^{-1}$)/10-h-dark cycle. Unless indicated, plants were grown on soil under a long-day regime at 22°C and 50% humidity with a 16-h-light ($100 \mu\text{mol m}^{-2} \text{ s}^{-1}$)/8-h-dark cycle.

Vector Construction

All PCR amplifications were conducted with Phusion Polymerase (New England Biolabs), and products were verified by sequencing. Arabidopsis

CHLSYN was expressed under control of the CaMV35S promoter in wild-type Arabidopsis and mutant *rdm6*. This construct was prepared by PCR amplification of the Arabidopsis flower cDNA library with forward and reverse oligonucleotides that contain added restriction enzyme sites (underlined): 5'-TATACAATTTGGTTCTATGACTTCGATTCAC-3' and 5'-TATTCTCAGTCAGTGTGGGATGCTAATGCC-3'. The PCR reaction product was digested with *MfeI/XhoI* and ligated into *EcoRI/XhoI* sites of the binary vector pBin35SRed to produce pCaMV35S-CHLSYN with the insert flanked 5' by the CaMV35S promoter and 3' by the 3' untranslated region of the nopaline synthase gene. This vector contains a DsRed fluorescent protein marker for selection of transgenic seeds.

An AtCHLSYN RNAi suppression construct was generated using the pHellgate8 RNAi Gateway Vector System (Helliwell and Waterhouse, 2003). A 349-bp fragment was amplified from the *AtCHLSYN* 3' untranslated region using forward and reverse oligonucleotides: 5'-CACCACCTTCTCAAGGACCCTGTCAAATACG-3' and 5'-CAGAATAACATAAACCCCAATCTCG-3', and the product was TOPO cloned into pENTR-TOPO clone vector (Invitrogen) to yield pENT-CHLSYN. The resulting plasmid was then moved into the destination vector pHellgate8 by LR recombination reaction according to the manufacturer's protocol (Invitrogen) to generate the final RNAi plasmid, pH8CHLSYN.

Arabidopsis Transformation and Selection

Binary vectors pCaMV35S-CHLSYN and pH8CHLSYN were introduced into *Agrobacterium tumefaciens* C58 by electroporation. Arabidopsis (Col-0) plants or *rdm6* mutants were transformed with the recombinant *Agrobacterium tumefaciens*-containing vector of pCaMV35S-CHLSYN, and Col-0 plants were transformed with recombinant *A. tumefaciens*-harboring pH8CHLSYN by use of the floral dip method as described (Clough and Bent, 1998). Transgenic seeds transformed with pCaMV35S-CHLSYN were identified by DsRed fluorescence as described previously (Jach et al., 2001; Pidkowich et al., 2007), and plants transformed with pH8CHLSYN were selected by resistance to Basta.

HPLC Analysis of Tocochromanols

For measuring tocopherol content of leaves from different types of plants, the procedure was used as described by Yang et al. (2011) with modification for vitamin E extraction: the freshly harvested or dried leaves were homogenized in 2 mL of 9:1 (v/v) methanol:dichloromethane containing 0.01% (w/v) butylatedhydroxytoluene. After 20 min of incubation and centrifugation, the supernatant was then recovered for HPLC analysis as described by Yang et al. (2011); 5,7-dimethyltolcol (Matreya) was added to each sample as an internal standard before extraction. Sample components were identified by mobility relative to standards and quantified relative to the internal standard.

Chlorophyll Measurement

The method for spectrophotometric determination of chlorophyll concentrations was as described (Strain et al., 1971). For extraction of chlorophyll, freshly harvested leaf tissue was weighed and homogenized in liquid nitrogen, and subsequently, it was extracted in 3 volumes of 80% (v/v) acetone containing $1 \mu\text{M}$ KOH. After centrifugation for 2 min at $16,000g$, supernatants were combined and used for spectrophotometric analysis.

Gene Expression Analysis

Total RNA was isolated from Arabidopsis leaves using the RNeasy Plant Kit (Qiagen) following the manufacturer's protocol. Total RNA ($1 \mu\text{g}$) was first treated with DNase (Roche), and then it was reverse transcribed for synthesizing the first-strand cDNA using SuperScript III Reverse Transcriptase (Life Technologies) and oligo(dT) primer according to the manufacturer's instructions. The resulting first-strand cDNA template of each sample was adjusted to the same concentration according to the PCR signal generated from the internal standard housekeeping gene *Ubiquitin-Conjugating Enzyme* (*UBC*; At5g25670; Czechowski et al., 2005). For semiquantitative RT-PCR analysis, a $2\text{-}\mu\text{L}$ aliquot of each adjusted first-strand cDNA was used as the template to amplify *AtCHLSYN* or the gene for the *UBC* (At5g25670) in a $20\text{-}\mu\text{L}$ reaction. The primers used for the amplification of *AtCHLSYN*-derived cDNA were 5'-CTCAACACTGTCTCCACCATCC-3' and 5'-GCAAAATTTCCAACCCATCC-3'. The primers used for

the amplification of the *UBC*-derived cDNA were 5'-ATGCAGGCATCAAGAGCGCGACTGT-3' and 5'-CACCGCCTTCGTAAGGAGTCTCCGA-3'.

Small RNA Northern Blot

RNA isolation and hybridization were performed as described by Park et al. (2002). The cDNA template for synE siRNA detection was amplified using primer sets forward: 5'- ATGACTTCGATTCTCAAC-3' and reverse: 5'-TCAGTGTGCGATGCTAATGCC-3'. P³²-labeled probes were further prepared by random prime labeling using the Ready-to-Go DNA Labeling Beads Kit (GE Healthcare).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Morphologies of Col-0 and *chlsyn1-1*.

Supplemental Figure S2. Total fatty acid accumulation in leaves from Col-0 and *chlsyn1-1* plants.

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