Chlorophyll Degradation: The Tocopherol Biosynthesis-Related Phytol Hydrolase in Arabidopsis Seeds Is Still Missing[1][C][W][OPEN]

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Phytyl diphosphate (PDP) is the prenyl precursor for tocopherol biosynthesis. Based on recent genetic evidence, PDP is supplied to the tocopherol biosynthetic pathway primarily by chlorophyll degradation and sequential phytol phosphorylation. Three enzymes of Arabidopsis (*Arabidopsis thaliana*) are known to be capable of removing the phytol chain from chlorophyll in vitro: chlorophyllase1 (CLH1), CLH2, and pheophytin pheophorbide hydrolase (PPH), which specifically hydrolyzes pheophytin. While PPH, but not chlorophyllases, is required for in vivo chlorophyll breakdown during Arabidopsis leaf senescence, little is known about the involvement of these phytol-releasing enzymes in tocopherol biosynthesis. To explore the origin of PDP for tocopherol synthesis, seed tocopherol concentrations were determined in Arabidopsis lines engineered for seed-specific overexpression of PPH and in single and multiple mutants in the three genes encoding known dephytylating enzymes. Except for modestly increasing tocopherol content observed in the PPH overexpressor, none of the remaining lines exhibited significantly reduced tocopherol concentrations, suggesting that the known chlorophyll-derived phytol-releasing enzymes do not play major roles in tocopherol biosynthesis. Tocopherol content of seeds from double mutants in NONYELLOWING1 (NYE1) and NYE2, regulators of chlorophyll degradation, had modest reduction compared with wild-type seeds, although mature seeds of the double mutant retained significantly higher chlorophyll levels. These findings suggest that NYEs may play limited roles in regulating an unknown tocopherol biosynthesis-related phytol hydrolase. Meanwhile, seeds of wild-type overexpressing NYE1 had lower tocopherol levels, suggesting that phytol derived from NYE1-dependent chlorophyll degradation probably doesn't enter tocopherol biosynthesis. Potential routes of chlorophyll degradation are discussed in relation to tocopherol biosynthesis.

Vitamin E tocochromanols are lipidic antioxidants found in photosynthetic organisms that exist as two alternate classes, tocopherols and tocotrienols, which differ in the degree of saturation of the hydrophobic C20 prenyl side chain classes. Among these two classes, four forms occur that differ in methylation of the hydrophilic tocochromanol head group (Sattler et al., 2004). The initial step of tocopherol biosynthesis is the condensation of the aromatic head group precursor homogentisate and the prenyl tail precursor phytyl diphosphate (PDP). This reaction is catalyzed by a plastid-localized enzyme, homogentisate PDP transferase (HPT; Soll et al., 1980; Collakova and DellaPenna, 2001). PDP for tocopherol biosynthesis is either provided through direct reduction of geranylgeranyl diphosphate (Keller et al., 1998) or from chlorophyll-bound phytol through chlorophyll hydrolysis and subsequent conversion of free phytol into PDP by two consecutive kinase reactions (Fig. 1; Rise et al., 1989; Goffman et al., 1999; Matile et al., 1999; Kräutler, 2002; Hörtensteiner, 2006). The first of these phosphorylation steps was shown to be catalyzed by vitamin E pathway5 (VTE5; Valentin et al., 2006).

Seeds of the Arabidopsis (*Arabidopsis thaliana*) *vte5* mutant have only about 20% of wild-type concentrations of vitamin E, while containing 3-fold more free

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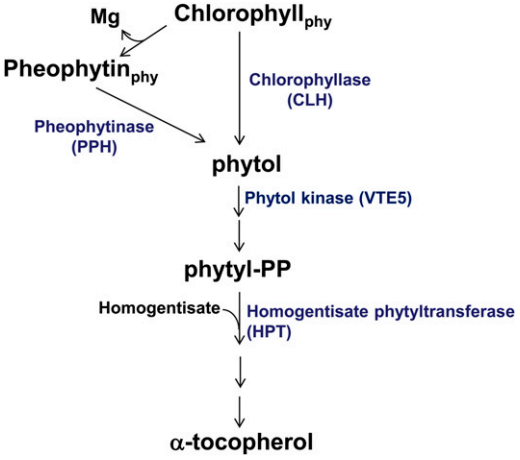

Figure 1. The substrate PDP directing toward tocopherol biosynthesis is primarily derived from chlorophyll degradation. Two phytol-releasing activities are known, i.e. CLH catalyzing release from chlorophyll and PPH dephytylating pheophytin. Phytol is then converted to PDP by sequential kinase reactions catalyzed by VTE5 and a second, unknown kinase. Condensation of PDP and homogentisate by HPT marks the initial reaction of tocopherol biosynthesis. phy, Phytyl. [See online article for color version of this figure.]

phytol compared with seeds of wild-type plants (Valentin et al., 2006). In addition, it has been shown that tocopherol accumulation in *Brassica napus* seeds correlates with chlorophyll breakdown during seed development (Valentin et al., 2006). Therefore, it was concluded that in Arabidopsis, the 80% of PDP that is used for VTE5-dependent tocopherol biosynthesis in seeds arises from free phytol released during chlorophyll degradation. Chlorophyll degradation is an important catabolic process that is catalyzed by a multistep pathway and occurs during leaf senescence and fruit ripening. An early reaction of the chlorophyll degradation pathway is dephytylation. The true identity of the enzyme(s) associated with phytol release has only recently been revealed. It was long believed that chlorophyllase (CLH) is responsible for phytyl hydrolysis, yielding chlorophyllide and free phytol (Heaton and Marangoni, 1996; Takamiya et al., 2000; Hörtensteiner, 2006). However, analysis of the two CLHs in Arabidopsis, AtCLH1 and AtCLH2 (Tsuchiya et al., 1999; Takamiya et al., 2000), indicated that the AtCLH isoforms are neither chloroplast localized nor essential for senescence-related chlorophyll breakdown (Schenk et al., 2007). These findings are consistent with the observation that not all molecularly identified CLHs contain a predicted chloroplast transit peptide (Jacob-Wilk et al., 1999; Tsuchiya et al., 1999). As a consequence, subcellular compartments distinct from plastids were considered to be additional sites of chlorophyll degradation (Takamiya et al., 2000). By contrast, results obtained from *Citrus* spp. suggested that CLH functions as a rate-limiting enzyme in chlorophyll catabolism within the chloroplast and is controlled by posttranslational regulation (Harpaz-Saad et al., 2007; Azoulay Shemer et al., 2008). Additionally, work in Arabidopsis indicated that *clh2* mutants showed a slight delay in chlorophyll degradation compared with *clh1* and wild-type plants (Schenk et al., 2007).

More recently, a novel plastid-localized enzyme, pheophytin pheophorbide hydrolase (PPH), was shown to be essential for chlorophyll breakdown during leaf senescence in Arabidopsis. PPH catalyzes the dephytylation of pheophytin rather than chlorophyll, resulting in pheophorbide and free phytol as the products (Schelbert et al., 2009). *pph* mutants are unable to degrade chlorophyll during senescence and therefore exhibit a stay-green phenotype in leaves. Altogether, these data reflect the complexity of the process of chlorophyll dephytylation and raise the question whether any of these activities may be related to tocopherol biosynthesis.

Recently, Gregor Mendel's green cotyledon gene *stay-green* (*SGR*), encoding a chloroplast-localized protein, was shown to be required for the initiation of chlorophyll breakdown (Armstead et al., 2007; Sato et al., 2007). Like in many plant species (Hörtensteiner, 2009), NON-YELLOWING1 (NYE1; also named SGR1), the Arabidopsis homolog of SGR, plays an important positive regulatory role in chlorophyll degradation during senescence, because NYE1 overexpression resulted in either pale-yellow leaves or even albino seedlings, while *nye1* mutants retain chlorophyll during senescence (Ren et al., 2007). In addition, the second isoform of NYE in Arabidopsis, NYE2 (also named SGR2), is a negative regulator of chlorophyll degradation in senescent leaves (Sakuraba et al., 2014). By contrast, both enzymes positively contribute to chlorophyll breakdown during seed maturation (Delmas et al., 2013). NYE1 and NYE2 were shown to interact at light-harvesting complex II (LHCII) with other chlorophyll catabolic enzymes, including PPH. This suggests that SGRs might function as scaffold proteins in the formation of a catabolic multienzyme complex regulating chlorophyll degradation

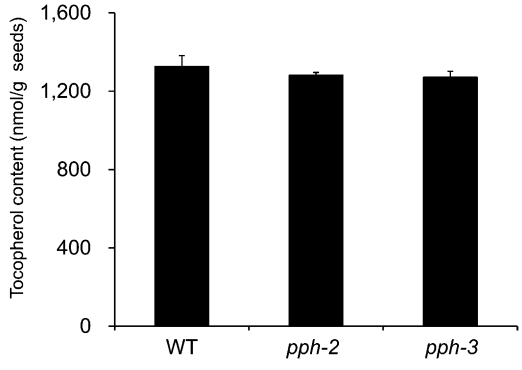

Figure 2. Deficiency of PPH does not result in a decrease in total tocopherol in mature Arabidopsis seeds. Total tocopherol concentrations of mature seeds from the wild type (WT) and two independent T-DNA insertion lines (*pph-2* and *pph-3*) are shown. Data represent mean and SE of at least three biological replicates.

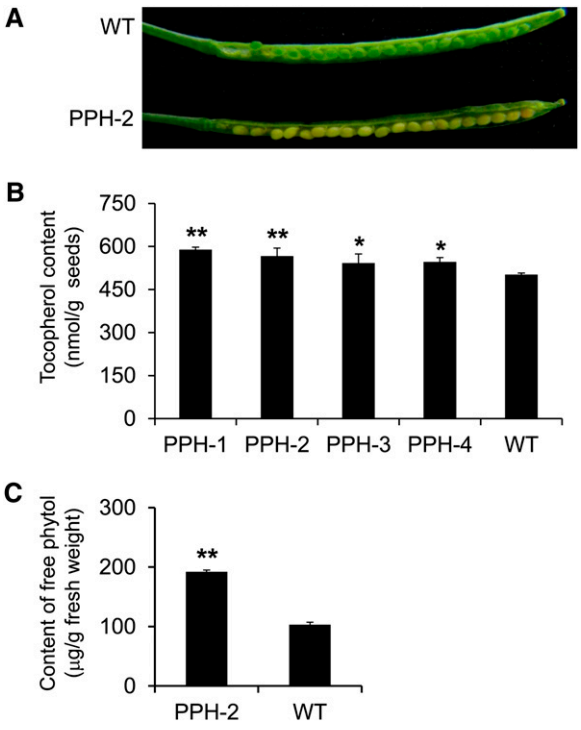

Figure 3. Impact of seed-specific overexpression of PPH on seed morphology and contents in total tocopherol and free phytol. A, Siliques of the wild type (WT) and one wild-type line overexpressing PPH seed, specifically (PPH-2) at 12 DAP. Note that compared with the wild type, PPH-2 seeds contain less chlorophyll. B, Mature seeds from four independent PPH-overexpressing lines (PPH-1–PPH-4) have slightly increased total tocopherol concentrations. C, Free phytol content of seeds from the wild type and PPH-2 at 12 DAP. Data represent mean and SE of at least three biological measurements. $^{**}P < 0.01$, $^{*}P < 0.05$; Student's *t* test.

(Sakuraba et al., 2012, 2014). Whether NYE1 and NYE2 may also affect CLH function remains unclear, but their role as a key regulators for chlorophyll degradation raises the question whether NYEs may also play a role in tocopherol biosynthesis.

Here, by employing Arabidopsis transferred DNA (T-DNA) insertion or nonsense mutants that are defective in known chlorophyll degradation-associated genes, and by PPH or NYE1 overexpression, we provide genetic and physiological evidence that neither CLHs nor PPH plays a major role in tocopherol biosynthesis in Arabidopsis seeds.

RESULTS

Only a Small Portion of Free Phytol Derived from PPH-Mediated Dephytylation Seems Able to Enter Tocopherol Biosynthesis in Arabidopsis

Recent studies indicated that during leaf senescence, chloroplast-localized PPH is involved in dephytylating the Mg-free chlorophyll pigment pheophytin, yielding pheophorbide and free phytol (Schelbert et al., 2009). At the same time, in Arabidopsis, the majority of PDP, one of the two substrates for tocopherol biosynthesis, was shown to be provided from chlorophyll-derived phytol after stepwise phosphorylation catalyzed by VTE5 and an unknown phytyl monophosphate kinase (Ischebeck et al., 2006; Valentin et al., 2006). To investigate whether PPH is involved in seed tocopherol biosynthesis, total tocopherol concentrations were determined in seeds from T-DNA null mutants for *PPH* (*pph-2* and *pph-3*; Fig. 2; see Supplemental Fig. S1 for knockout mutant confirmation). Mutant tocopherol concentrations were nearly indistinguishable from the wild type, suggesting that either PPH is irrelevant for chlorophyll dephytylation during seed maturation or the timing of PPH-mediated chlorophyll degradation and tocopherol biosynthesis probably do not overlap. To investigate this further, Arabidopsis PPH was overexpressed under the control of the strong seed-specific glycinin1 promoter in wild-type plants, allowing PPH expression earlier than during wild-type seed maturation (Supplemental Fig. S2). Developing seeds of PPH-overexpressing lines started at 9 d after pollination (DAP) to turn yellow earlier than wild-type seeds (at 15 DAP; Fig. 3A), indicating premature and/or increased chlorophyll degradation in these transgenic seeds. Consistent with this, the content of free phytol was about 2-fold higher in transgenic seeds compared with seeds from nontransformed plants (Fig. 3C). Total tocopherol levels were also elevated in developing and mature seeds as well as in PPH-overexpressing plants, although among different tested transgenic lines, the highest increase in mature seeds was only about 17% compared with the wild type (Fig. 3B), while the tocopherol content increased rapidly in developing seeds of PPH-overexpressing plants (PPH-1) between 6 and 9 DAP relative to seeds from wild-type plants (Fig. 4; Supplemental Fig. S3), suggesting that at least a portion

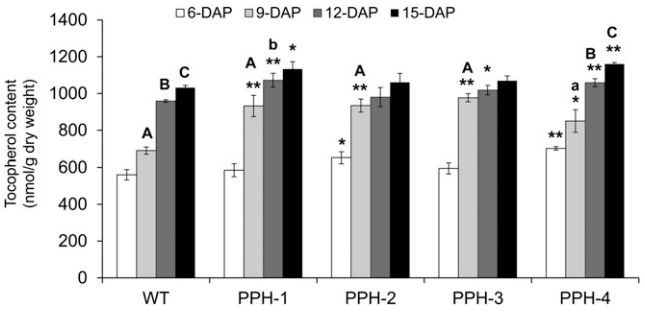

Figure 4. Total tocopherol content increases rapidly in developing seeds of *PPH*-overexpressing lines between 6 and 9 DAP. Tocopherol content was determined in the wild type and four independent PPH-overexpressing lines (PPH-1–PPH-4) at 6, 9, 12, and 15 DAP. Data represent mean and SE of at least three measurements. For the statistical difference between the wild type and each of four independent PPH-overexpressing lines (PPH-1–PPH-4) at the same developmental stage, double asterisks indicate $P < 0.01$ and a single asterisk indicates $P < 0.05$, and for the statistical difference between stages for each line, A, B, and C indicates $P < 0.01$ and a, b, and c indicates $P < 0.05$ (Student's *t* test).

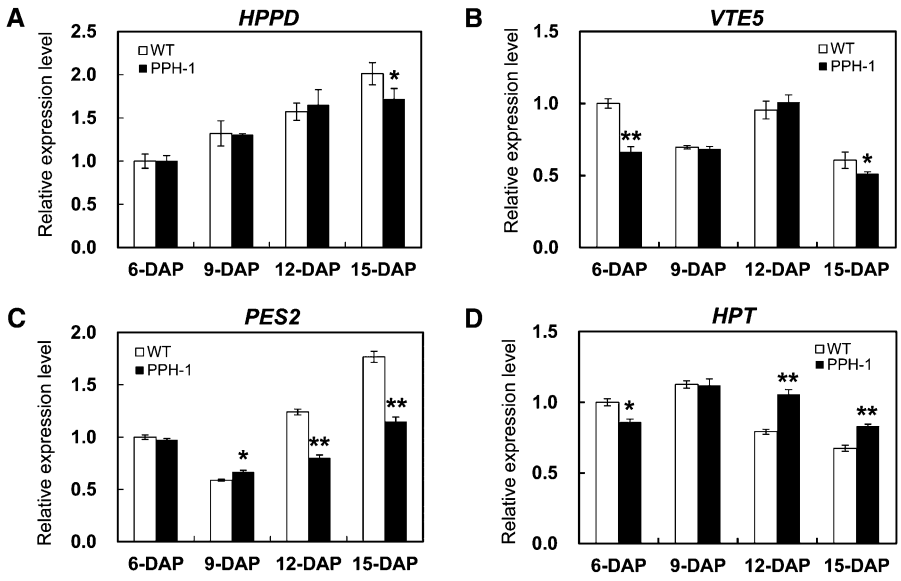

Figure 5. Analysis of expression of some genes involved in tocopherol biosynthesis in seeds of one biological replicate. The expression of the indicated genes was determined by real-time PCR in seeds of the wild type (WT) and a PPH-overexpressing line (PPH-1) at 6, 9, 12, and 15 DAP. A, *HPPD*. B, *VTE5*. C, *PES2*. D, *HPT*. Data represent mean and SE of three technical measurements. Gene expression was normalized to At5G09810. $^{**}P < 0.01$, $^{*}P < 0.05$; Student's *t* test.

of phytol from PPH-mediated chlorophyll dephytylation is able to enter the tocopherol biosynthetic pathway. Consistently, the timing of PPH-mediated chlorophyll degradation and tocopherol biosynthesis is more likely overlapped, because unlike the expression of some genes for tocopherol biosynthetic proteins that is high only at the onset of maturation based on publicly available transcriptome datasets of developing seeds of Arabidopsis (Supplemental Fig. S4) and soybean (*Glycine max*; Collakova et al., 2013), the expression of *PPH* was relatively high before and during seed maturation (Supplemental Fig. S4).

Altered Expression Levels of Genes for Some Tocopherol Metabolic Enzymes Account for Only Small Increases in Tocopherol Content in PPH-Overexpressing Seeds

Given that overexpression of PPH led to a large increase in free phytol, but only small increases in seed tocopherol (Fig. 3B), we examined the expression profiles of genes involved in the pathway for biosynthesis of homogentisate (HGA), the substrate for production of tocopherol, in seeds at different developmental stages to see if enhanced PPH expression affects the expression of these genes. The HGA used in tocopherol biosynthesis is derived from *p*-hydroxyphenylpyruvate via a decarboxylation by hydroxyphenylpyruvate dioxygenase (HPPD; Garcia et al., 1999). As analyzed by quantitative PCR, expression levels of *HPPD* increased steadily following seed development but without differences between PPH-overexpressing and wild-type plants at the early stages (Fig. 5A; Supplemental Fig. S5). Further, we examined whether enhanced PPH expression impacts the expression of genes for enzymes that metabolize free phytol, i.e. VTE5 and PHYTOL ESTER SYNTHASE2 (PES2), which is involved in the formation of fatty acid phytyl esters (Lippold et al., 2012). Especially at 6 DAP, suppression of *VTE5* expression was observed in PPH-overexpressing seeds compared with the wild type (Fig. 5B; Supplemental Fig. S5). *PES2* expression was significantly suppressed at 12 and 15 DAP and upregulated at 9 DAP in the PPH-overexpressing line relative to wild-type plants (Fig. 5C; Supplemental Fig. S5). By contrast, expression of the first committed enzyme of tocopherol biosynthesis, HPT (Collakova and DellaPenna, 2001), was increased at 12 DAP upon PPH overexpression (Fig. 5D; Supplemental Fig. S5). As a conclusion, the lower expression of *HPPD* indicates a likely lower availability of HGA, and the significantly suppressed expression of *VTE5* may account for the only small increases in tocopherol content in seeds of PPH-overexpressing lines.

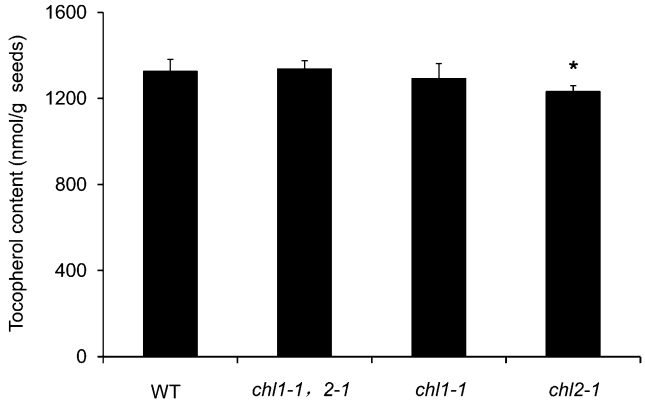

Figure 6. CLH1 and CLH2 deficiency does not result in a decrease in total tocopherol concentration in mature Arabidopsis seeds. Total tocopherol concentration of mature seeds from the wild type (WT) and *clh1-1* and *clh2-1* single and *clh1-1,2-1* double mutants was assayed. Data represent mean and SE of at least three biological replicates. $^{*}P < 0.05$; Student's *t* test.

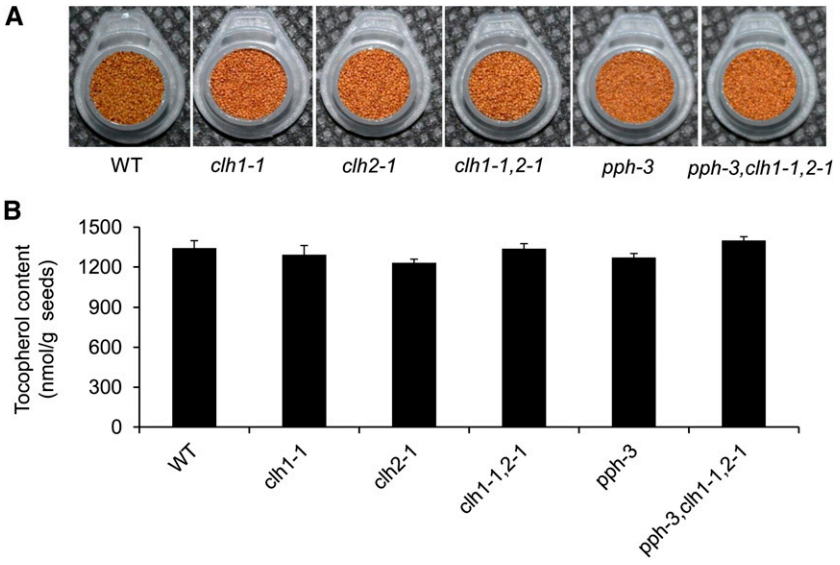

Figure 7. Simultaneous absence of CLHs and PPH does not alter tocopherol content in mature seeds. A, Phenotype of mature seeds from the wild type (WT) and different mutants. B, Total tocopherol in seeds of the wild type and different mutants at the onset of maturation (12 DAP). Data in B represent mean and SE of at least three biological replicates.

Total Tocopherol Content Is Not Reduced in clh1-1, clh2-1, and clh1-1,2-1 Seeds

The above experiments indicated that in wild-type plants, only a small portion of free phytol generated by PPH during seed maturation is likely entering tocopherol biosynthesis. Next, seeds of Arabidopsis plants disrupted in CLH1 or CLH2, or both (Supplemental Fig. S1; Schenk et al., 2007), were analyzed to explore whether seed tocopherol biosynthesis is related to CLH. The total tocopherol content in seeds of the different mutants was not different than that in wild-type seeds (Fig. 6), indicating that, like PPH, CLH1 and CLH2 are also not involved in tocopherol biosynthesis in Arabidopsis seeds.

CLH1, CLH2, and PPH Do Not Function Redundantly for Tocopherol Biosynthesis in Arabidopsis

All three Arabidopsis enzymes, i.e. CLH1, CLH2, and PPH, have been shown to be capable of catalyzing dephytylation of chlorophyll in vitro. To explore the possibility of functional redundancy of PPH and CLHs in supplying free phytol for tocopherol biosynthesis, triple mutants were generated between clh1-1,2-1 and pph-2 (pph-2,clh1-1,2-1) or pph-3 (pph-3,clh1-1,2-1). Progenies of F2 populations were genotyped by PCR (Supplemental Fig. S6), and the expression level of corresponding genes in triple mutants was measured by reverse transcription-PCR (Supplemental Fig. S1). Subsequently, dark-induced senescence was analyzed in leaves. After 5 d, most chlorophyll was retained in the triple mutants as well as in pph, while clh1-1,2-1 turned yellow like the wild type (Supplemental Fig. S7). However, the color and total tocopherol levels of mature seeds were almost indistinguishable between the different lines (Fig. 7). Despite this fact, tocopherol or chlorophyll biosynthesis may be delayed in some of the mutants during seed maturation. To analyze this possibility, total tocopherol and chlorophyll contents were determined in developing seeds at an earlier stage of maturation (12 DAP). Again, total tocopherol and chlorophyll levels were indistinguishable between

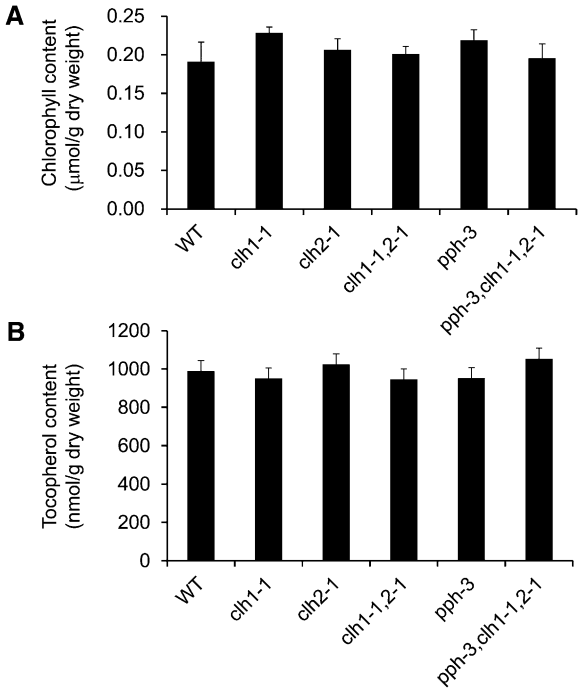

Figure 8. Simultaneous absence of CLHs and PPH does not alter tocopherol and chlorophyll contents in developing seeds on the onset of maturation. The chlorophyll content (A) and total tocopherol content (B) in seeds of the wild type (WT) and different mutants at the onset of maturation (12 DAP). Data represent mean and SE of at least three biological replicates.

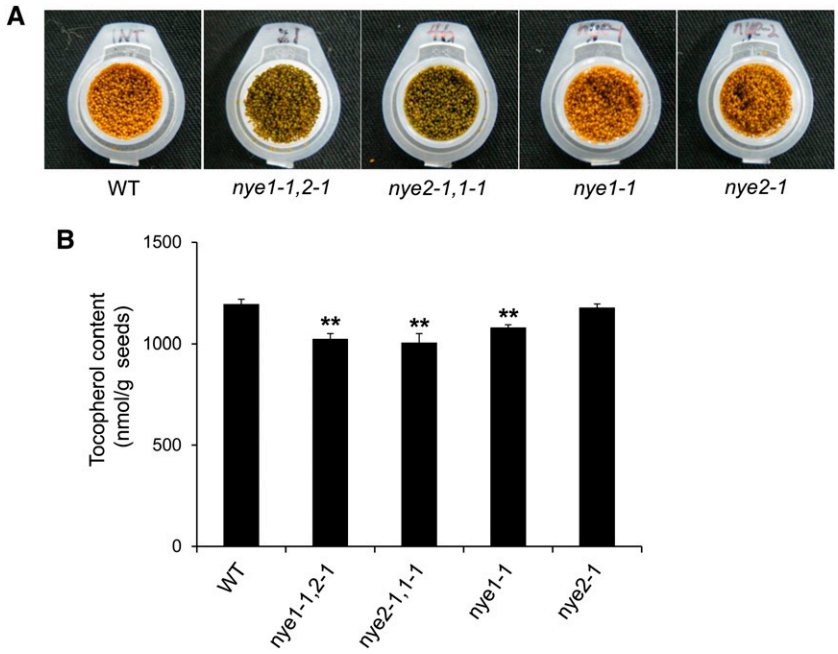

Figure 9. Deficiency of NYEs has only a small impact on seed tocopherol. A, Phenotype of mature seeds from the wild type and different mutants. Note that seeds of *nye1-1,nye2-1* retain chlorophyll and thus exhibit a stay-green phenotype. B, Total tocopherol content in mature seeds from the wild type and different nye mutants is shown. Data represent mean and SE of at least three biological replicates. $^{**}P < 0.01$; Student's *t* test. WT, Wild type.

the different genotypes (Fig. 8). Based on findings that the Arabidopsis vte5 mutant is defective in phytol kinase activity (Valentin et al., 2006), it has become apparent that the primary route of PDP synthesis in Arabidopsis is via reduction of geranylgeranyl bound to chlorophyll rather than by direct reduction of geranylgeranyl diphosphate to form PDP. Thus, our results indicate that unknown hydrolytic activities distinct from PPH and CLH seem to be responsible for producing phytol from chlorophyll for tocopherol biosynthesis, although the role of PPH and CLHs in the phytol supply for tocopherol biosynthesis in leaves remains to be investigated.

Total Tocopherol Concentrations Are Modestly Reduced in Seeds from Plants Disrupted in NYEs

SGR1 (NYE1) was recently shown to be crucial for the initiation of chlorophyll breakdown by interacting directly or indirectly with chlorophyll catabolic enzymes at LHCII during senescence in leaves (Sakuraba et al., 2012). As a consequence, *nye1-1*, a mutant deficient in NYE1, shows a stay-green leaf phenotype, i.e. it does not degrade chlorophyll (Ren et al., 2007). Considering the possibility that NYE1 might also affect a putative tocopherol biosynthesis-related phytol hydrolase, we analyzed the *nye1-1* mutant in respect to tocopherol biosynthesis. In Arabidopsis, another gene (At4g11910) encoding a plastid-localized protein has high sequence similarity to *NYE1* (Ren et al., 2007; Aubry et al., 2008) and was named NYE2 (or SGR2). NYE2 was recently shown to have an opposite role in leaf senescence (Sakuraba et al., 2014). By contrast, during seed maturation, NYE1 and NYE2 are functionally redundant homologs in the regulation of chlorophyll degradation, because a *nye1-1,2-1* double mutant, but not single mutants, retains chlorophyll in seeds (Delmas et al., 2013; Fig. 9A). We analyzed total tocopherol content in seeds of *nye1-1*, *nye2-1*, and *nye1-1,2-1* plants. Compared with wild-type seeds, tocopherol concentrations

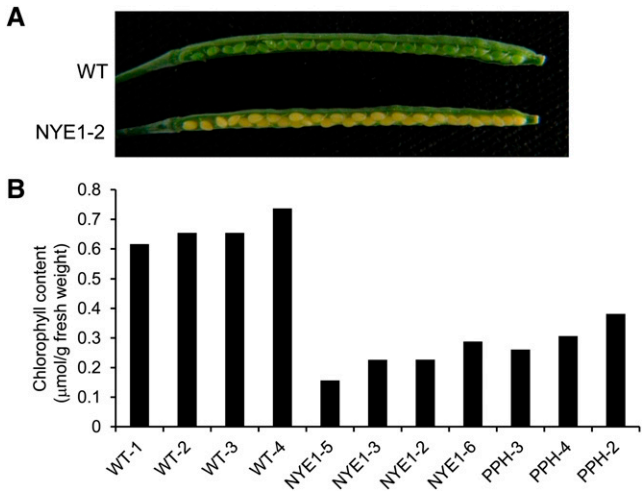

Figure 10. Impact of seed-specific overexpression of NYE1 on seed morphology. A, Siliques of the wild type and one line overexpressing NYE1 seed specifically (NYE1-2) at 12 DAP. Note that, compared to the wild type, NYE1-2 seeds contain less chlorophyll. B, Chlorophyll quantification in seeds of the wild type and lines overexpressing NYE1 or PPH at 12 DAP. Note that chlorophyll content in seeds of overexpressing NYE1 or PPH is less than that of wild-type seeds at the same developmental stage. WT, Wild type.

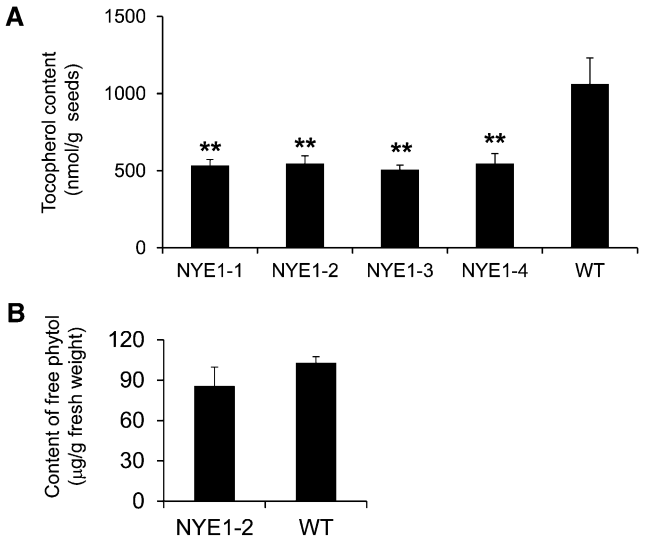

Figure 11. Impact of seed-specific overexpression of NYE1 on total tocopherol and free phytol contents. A, Levels of total tocopherol content in mature seeds of the wild type (WT) and four independent NYE1-overexpressing lines (NYE1-1–NYE1-4). B, Free phytol content in developing seeds from the wild type and NYE1-2 at 12 DAP. Data represent mean and SE of at least three biological replicates. $^{**}P < 0.01$; Student's *t* test.

in *nye2* seeds were unaltered, but *nye1* and *nye1-1,2-1* had, respectively, about 10% and 14% decreased tocopherol content to significantly different level (Fig. 9B). This indicated that *NYE* genes play a limited role in regulating tocopherol biosynthesis in Arabidopsis seeds.

Seed-Specific Overexpression of *NYE1* Accelerates Chlorophyll Degradation But Decreases Tocopherol Accumulation in Arabidopsis

To further examine a possible role of NYEs in tocopherol production, *NYE1* was overexpressed in ecotype Columbia under the control of the strong seed-specific glycinin1 promoter (Supplemental Fig. S8). Developing seeds of transgenic lines started to turn yellow earlier compared with those of wild-type plants at 6 DAP (Fig. 10A). This was consistent with the chlorotic phenotype observed in leaves from plants engineered to constitutively overexpress *NYE1* (Ren et al., 2007). At 12 DAP, seeds of overexpressing NYE1 lines accumulated similar levels of chlorophyll as PPH overexpressing plants but significantly less than that of wild-type seeds at the same developmental growth stage (Fig. 10B). Surprisingly, total tocopherol content of mature seeds was reduced by about 50% in different independent NYE1-overexpressing plants (Fig. 11A). At the same time, the content of free phytol in developing seeds of NYE1-overexpressing plants was not significantly different from that of wild-type plants at the same developmental stage (Fig. 11B).

DISCUSSION

It has been reported that the majority of PDP for tocopherol biosynthesis in Arabidopsis seeds is derived from chlorophyll degradation, i.e. from free phytol, which, after two-step phosphorylation by VTE5 and a second kinase, enters tocopherol biosynthesis (Ischebeck et al., 2006; Valentin et al., 2006). However, little is known about tocopherol biosynthesis-related phytol hydrolyzing activities. In this study, single and multiple Arabidopsis mutants deficient in the known phytol hydrolases, i.e. CLH and PPH, were shown to be unaltered in seed tocopherol content as well as in degreening during seed ripening. This indicates that a different, so far unknown, dephytylating activity is involved in chlorophyll degradation to provide phytol for tocopherol biosynthesis. Consistent with this, only a modest increase in total seed tocopherol concentrations was observed in lines overexpressing PPH, despite enhanced chlorophyll breakdown in these lines.

In leaves, PPH and other chlorophyll catabolic enzymes have been shown to interact with NYE1 and NYE2 (Sakuraba et al., 2012, 2014), indicating that the activity of PPH depends on these NYEs. To test the role of NYEs in chlorophyll breakdown during seed ripening, we analyzed single and double *nye* mutants and NYE1-overexpressing lines. As for *pph* and *clh*

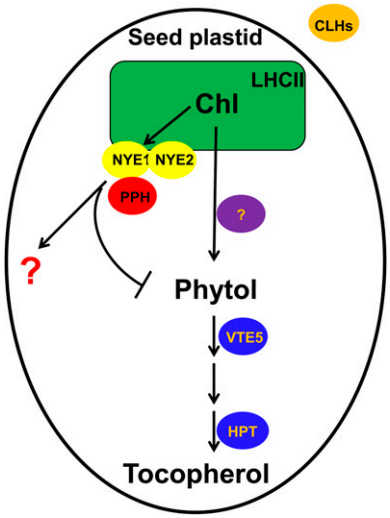

Figure 12. Tentative model of the relation of chlorophyll breakdown to tocopherol biosynthesis in seeds of Arabidopsis. Destabilization of LHCII by NYEs is essential for chlorophyll degradation. Furthermore, two activities, i.e. CLH (supposed to be localized in cytosol) and PPH (localized in plastid), have been shown to be able to release phytol. The data presented here indicate that neither CLH nor PPH provide phytol for tocopherol biosynthesis. Instead, we postulate a novel, NYE-independent dephytylase that is active in seeds and that produces free phytol, which, after two-step phorphorylation involving VTE5, is fed into tocopherol biosynthesis. The curved line is representative of NYE/PPH-derived phytol seemingly does not contribute to the pool of PDP used for tocopherol synthesis. Chl, Chlorophyll.

mutants, seed tocopherol concentrations were only slightly altered in *nye* mutants, indicating that NYEs play a slight role in seed tocopherol biosynthesis. This observation was even more evident given the fact that *nye1-1,2-1* double mutants retain substantial amounts of seed chlorophyll (Delmas et al., 2013), i.e. supposedly providing less free phytol for tocopherol biosynthesis. By contrast, NYE1 overexpression caused about 50% reduced seed tocopherol concentrations, despite the fact that chlorophyll degradation was considerably accelerated in these lines.

Together, these data suggest that although overexpression of NYE1 or PPH accelerates chlorophyll breakdown in seeds, under wild-type conditions, neither of these activities contributes substantially to tocopherol biosynthesis. The fate of NYE/PPH-derived phytol in respective overexpressors remains unknown, but it seemingly does not contribute to the pool of PDP used for tocopherol synthesis (Fig. 12). Notably, NYE1 overexpression resulted in about 50% reduced tocopherol content. One possible explanation is NYE overexpression activated the utilization of free phytol and reduced its availability for tocopherol biosynthesis. Meanwhile, PPH overexpression led to a 2-fold increase of free phytol but only slightly increased tocopherol content. Consistent with this, an increase in free phytol in leaves of the *pes1pes2* mutant caused by blocking the capacity of PESs did not result in an increase in the amount of leaf tocopherol (Lippold et al., 2012). A possible explanation is the existence of two distinct pools of phytol, one pool that is targeted for tocopherol biosynthesis, while a substantial amount of phytol released from chlorophyll is metabolized by alternative, tocopherol-independent pathways. The fate of free phytol may be related to the availability of tocopherol metabolic enzyme activities. Though enzyme activities are regulated at multiple levels, the low gene expression level and the altered gene expression of some tocopherol metabolic enzymes may account for only small increases in tocopherol concentrations in PPH-overexpressing seeds. As shown in this study, developing seeds of PPH-overexpressing plants between 6 and 9 DAP display high levels of tocopherol biosynthesis. During this time, expression of the gene encoding VTE5 was severely suppressed, indicating that the two kinases, including VTE5, required for activating free phytol to PDP are likely limiting in tocopherol biosynthesis in seeds that overexpress PPH. Thus, even though more phytol was available due to increased chlorophyll degradation in PPH-overexpressing plants, synthesis of tocopherol was not enhanced. Alternatively, another possible explanation is that degradation of chlorophylls is not a limiting step for tocopherol biosynthesis in Arabidopsis seeds, i.e. phytol levels are already sufficient in wild-type seeds.

In addition to questioning the role of known chlorophyll phytol-releasing enzymes for tocopherol biosynthesis, our data imply the existence in seeds of an unknown dephytylating activity, tentatively termed dephytylase, that is distinct from PPH and CLHs (Fig. 12). This NYE likely plays a limited role in regulation of this dephytylase, because the majority of tocopherol synthesis occurred even in seeds of the *nye* double mutant. The nature of the dephytylase remains unknown, and its molecular identification is needed to determine the extent of its involvement in tocopherol biosynthesis-related phytol formation.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The following T-DNA insertion lines were employed in this study: *pph-2*, *pph-3* (Schelbert et al., 2009), *clh1-1*, *clh2-1*, *clh1-1,2-1* (Schenk et al., 2007), and *nye1* (Ren et al., 2007). In addition, SALK_003830C (designated *nye2-1*) was obtained from the Arabidopsis Biological Resource Center (Alonso et al., 2003). *nye1-1,2-1*, *pph-2,clh1-1,2-1*, and *pph-3,clh1-1,2-1* double and triple mutants were produced by crossing. Double and triple homozygous F2 plants were identified by PCR using T-DNA and gene-specific primers as listed in Supplemental Table S1.

All plants were grown on soil with a photoperiod of 16-h light/8-h darkness at 22°C. Alternatively, after surface sterilization with 50% (v/v) household bleach (5.5%–6.5% sodium hypochlorite) for 3 min and 50% (v/v) ethanol for 5 min, seeds were washed four times with deionized water and grown on plates containing Murashige and Skoog medium with 1.5% (w/v) Suc and 0.9% (w/v) agar with 100 mg L^{-1} ampicillin. To induce senescence, detached leaves from 3- to 4-week-old plants were placed on wet filter paper and incubated in darkness at ambient temperature for 5 to 7 d.

Seed-Specific Expression

To overexpress *PPH* or *NYE1* seed specifically in Arabidopsis (*Arabidopsis thaliana*), the previously described binary vector pBinGlyRed3 (Zhang et al., 2013) was employed. The open reading frames of *PPH* and *NYE1* were PCR amplified using primers as listed in Supplemental Table S1. All PCR reactions were conducted using Phusion polymerase (New England Biolabs). The resulting *PPH* product was cloned as an *Eco*RI/*Sma*I fragment into pBinGlyRed3 between the strong seed-specific soybean (*Glycine max*) glycinin1 promoter and the 3′ untranslated region, yielding pGlyPPH. Likewise, the *NYE1* was cloned as an *Eco*RI/*Xho*I fragment, resulting in pGlyNYE1. Transformation of pGlyPPH and pGlyNYE1 into wild-type Arabidopsis ecotype Columbia was performed by the *Agrobacterium tumefaciens*-mediated floral dip method as previously described (Clough and Bent, 1998). Transgenic seeds were selected by fluorescence of a DsRed marker (Jach et al., 2001).

Gene Expression Analysis by Quantitative Real-Time PCR

Total RNA was isolated from developing seeds using a modified cetyltrimethyl-ammonium bromide-LiCl method as reported (Li et al., 2006). Total RNA (2,000 ng) was first treated with 1 μL of DNase (Thermo) and then used for reverse transcription with the Synthesis Super Mix (TransGen) for first-strand complementary DNA synthesis. For quantitative reverse transcription-PCR analysis, 3.5 μL of first-strand complementary DNA (corresponding to 10 ng of RNA) was used as template in a 15-μL reaction with the gene-specific primers as listed in Supplemental Table S1. Three technical replicates were performed on a Bio-Rad CFX96 Real-Time System. Statistical analysis was done with the LigReg software as described (Ramakers et al., 2003). The amount of target gene amplified was normalized to the abundance of the constitutive *actin7* (At5G09810) gene using primers as shown in Supplemental Table S1.

Chlorophyll Measurement

For estimation of the total chlorophyll content, approximately 100 mg of dark-induced dried leaves or 6 mg of dried seeds was weighed and then ground in liquid nitrogen. Six microliters of extracting solution containing 3 mL of each acetone and hexane with 10 mM KOH was added, and after vigorous shaking in the dark and centrifugation for 10 min at 10,000*g*, chlorophyll was determined in the supernatant according to the method of Arnon (1949).

Tocopherol Content and Free Phytol Analysis

After the addition of appropriate amounts of 5,7-dimethyltocol (Matreya) as internal standard, total tocopherol was extracted from about 5 mg of dried mature seeds with 2 mL of methanol:dichloromethane (9:1, v/v) containing 0.01% (w/v) butylated hydroxytoluene. HPLC analyses and quantification of total tocopherol were conducted as previously described (Yang et al., 2011). For determination of free phytol, total lipid was extracted from developing seeds and silylated (Lippold et al., 2012), and the total content of free phytol was quantified by gas chromatography-mass spectrometry (Shimadzu GCMS-QP2010 Plus) according to a described procedure (Ischebeck et al., 2006).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. RT-PCR analysis of *CLH1*, *CLH2*, and *PPH* gene expression in developmental stage of silique from different mutants.

Supplemental Figure S2. Expression of *PPH* in wild type and PPH-overexpressing lines.

Supplemental Figure S3. Expression of *PPH* in wild type and a PPH overexpressing line during seed development at 6-,9-,12-,15-days after DAP.

Supplemental Figure S4. Schematic representation of relative expression of tocopherol synthesis relevant genes from publicly available transcriptomic data sets from Arabidopsis developing seeds.

Supplemental Figure S5. Analysis of expression of some genes involved in tocopherol biosynthesis in seeds.

Supplemental Figure S6. Genotyping of different mutants by PCR.

Supplemental Figure S7. Characterization of simultaneous absence of CLHs and PPH during dark-induced senescence.

Supplemental Figure S8. Expression of *NYE1* in wild type and NYE1 overexpressing lines.

Supplemental Table S1. List of primers used in this study.

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