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Unexpected roles of plastoglobules (plastid lipid droplets) in vitamin K_1 and E metabolism

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Tocopherol (vitamin E) and phylloquinone (vitamin K_1) are lipid-soluble antioxidants that can only be synthesized by photosynthetic organisms. These compounds function primarily at the thylakoid membrane but are also present in chloroplast lipid droplets, also known as plastoglobules (PG). Depending on environmental conditions and stage of plant development, changes in the content, number and size of PG occur. PG are directly connected to the thylakoid membrane via the outer lipid leaflet. Apart from storage, PG are active in metabolism and likely trafficking of diverse lipid species. This review presents recent advances on how plastoglobules are implicated in the biosynthesis and metabolism of vitamin E and K.

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Introduction

Tocopherols (vitamin E) and phylloquinone (vitamin K_1) are essential nutrients for humans and can only be synthesized by photosynthetic organisms such as cyanobacteria and plants [1]. Tocopherols and phylloquinone belong to the family of prenylquinone compounds that also includes plastoquinone (PQ) and its chromane derivative plastochromanol (PC8). The family consists of lipid-soluble molecules, which act as antioxidants preventing lipid peroxidation in the thylakoid membrane and quenching harmful reactive oxygen species (ROS) [2–4]. Phylloquinone and PQ also act as electron carriers in photosystem I (PSI) and II (PSII), respec-tively. Phylloquinone deficiency causes a reduction of PSI and increase of PQ leading to the unbalanced functioning of PSII [5,6]. Phylloquinone is present in the PsaA and PsaB subunits of the PSI complex. Phylloquinone, located at A_1 acceptor locus, receives an electron from the chlorophyll a acceptor molecule (A_0) and then donates an electron to the membrane-associated iron-sulfur protein acceptor cluster (F_X, F_A/F_B)[5,7–10].

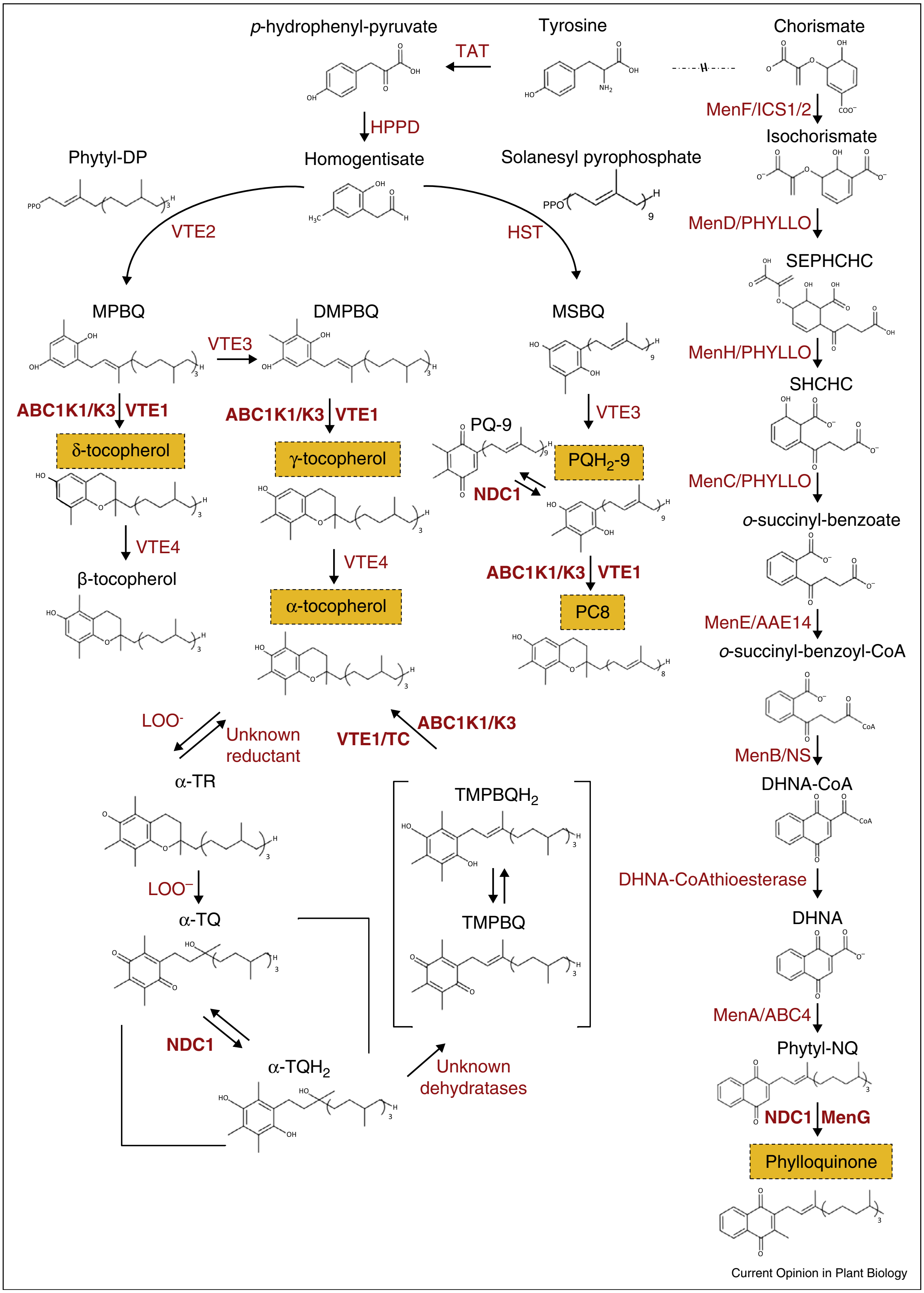
Tocopherols belong to the eight-member tocochromanol family, consisting of four tocopherols and four tocotrienols. Tocopherols have a phytyl side chain whereas tocotrienols have an unsaturated geranylgeranyl side chain with three double-bonds. α-, β-, γ- and δ only differ in the number and position of the methyl groups in the hydrophilic chromane group. α-, β-, γ- and δ-tocopherols and tocotrienols have many activities in plants as detailed in recent reviews [11,12] and highlighted in subsequent sections. These compounds also act as important lipid soluble antioxidants in the diet of mammals (including humans) and in this context are collectively referred to as vitamin E. The vitamin E activity of the different compounds ranges from zero for δ-tocotrienol and γ-tocotrienol to 100% for α-tocopherol, the most active form.

The majority of the chloroplast enzymes implicated in the biosynthesis of prenylquinones have been localized at the inner membrane of the chloroplast envelope [13–15], with the exception of part of the phylloquinone biosyn-thetic pathway that is compartmentalized in peroxisomes [16,17] (Figure 1).

However, recent studies have demonstrated that several important steps in the metabolic pathways localize at plastoglobules (PG) [26,27].

Vitamin K_1 is synthesized *de novo* from a naphthoquinone ring derived from chorismate in the shikimate pathway and a prenyl side chain derived from phytyldiphosphate [10,28] (Figure 1). Similarly, the tocopherol benzoquinone group is a product of the shikimate pathway and prenyl side chains originate from plastidic isoprenoid pathway [4,14,22,29]. The benzoquinone head group is derived from the homogentisic acid (HGA) that is formed from p-hydroxyphenylpyruvic acid by *p*-hydroxyphenylpyruvic acid dioxygenase (HPPD)[6]. The isoprenoid phytyl or geranylgeranyl (in the case of tocotrienols) tail derives from the 1-deoxy-D-xylulose- 5-phosphate pathway located in the plastid. All the subsequent steps in the tocopherol synthesis have been reported to occur in the inner envelope of the chloroplast [22,30,31] (Figure 1).

Figure 1



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Plastoglobules change with plant developmental stages and function as microdomains for (prenyl-) lipid metabolism The photosynthetic light reactions take place at the chloroplast thylakoid membranes. Thylakoid membranes are composed mainly of galactolipids but also contain prenylquinones as well as chlorophylls and carotenoids that are mostly attached to thylakoid membrane proteins [32,33]. At the curved stromal margins of thylakoid mem-branes, PG may emerge and constitute thylakoid mem-brane microdomains [34–37].

PG were long thought to be just storage droplets for excess neutral lipids. However, recent studies show that PG also play important metabolic roles in the metabolism of prenyl lipids [26,28,37–39]. PG consist of an outer, polar lipid monolayer, which is contiguous with the thylakoid outer lipid leaflet [38,40]. The PG core contains neutral lipids such as prenylquinones (tocopherol, DMPBQ, phylloquinone, PQ, carotenoids, fatty acid phythyl esters, and triacylglycerols). The PG surface is studded with proteins. Among these are the fibrillins (FBN), previously known as plastoglobulins (PGL). FBNs are considered structural proteins [40], that are also known or predicted to be involved in various lipid metabolic pathways [26].

PG numbers, size and lipid content change, depending on environmental conditions and plant developmental stage [20]. These changes at the ultrastructural level (visible by electron microscopy) correlate with the remodeling of lipid content of the thylakoid membrane. Moreover, PG partic-ipate in remodeling by metabolizing and storing lipid molecules. Their role in trafficking from and to the thyla-koid is inferred from the existence of PG-thylakoid contact sites that may serve as conduits for lipid molecules.

Vitamin K_1 in the thylakoids and plastoglobules

Phylloquinone is present mainly in the thylakoid membrane and, two molecules localize to each PSI complex. However, it has been demonstrated through chloroplast isolation and subplastidial fractionation that phylloquinone is not restricted to PSI. Around a third of the total phyllo-quinone was found to be located in PG, suggesting that PG function as a reservoir for excess phylloquinone [6].

In the final step of phylloquinone biosynthesis, AtMENG functions as the methylase for 2-phytyl-1,4-naphthoqui-none (Phytyl-NQ) [6] (Figure 2). The *AtmenG* knock-out mutant is devoid of phylloquinone but accumulates Phytyl-NQ. Unexpectedly, the lack of phylloquinone does not provoke a drastic phenotype as reported previously for the *abc4* mutant, in which the absence of phylloquinone led to a lethal albino phenotype [5]. *AtmenG* only exhibited reduced growth and anthocyanin accumulation under normal light condition, besides there was a slight decrease in photosyn-thetic efficiency upon high light (HL) treatment due to accelerated degradation of PSI [6]. It therefore appears that the Phytyl-NQ precursor of phylloquinone is able to re-place phylloquinone under non-stress conditions.

Strangely, the mutant of the PG protein NDC1 (NAD(P)H-dependent:quinone oxidoreductase) has a molecular phenotype that is similar to that of *atmenG*. Lipidomics analysis in Arabidopsis showed that the most significant difference between the *ndc1* mutant and the wild type (wt) consisted in the absence of phylloquinone in wt and the presence of the Phytyl-NQ precursor in *ndc1* [28]. An ortholog of NDC1 exists in the green alga *Chlamydomonas reinhardtii*. In this organism the NDA2 NAD(P)H:quinone oxidoreductase, takes over the function of the NDH complex in cyclic electron flow and

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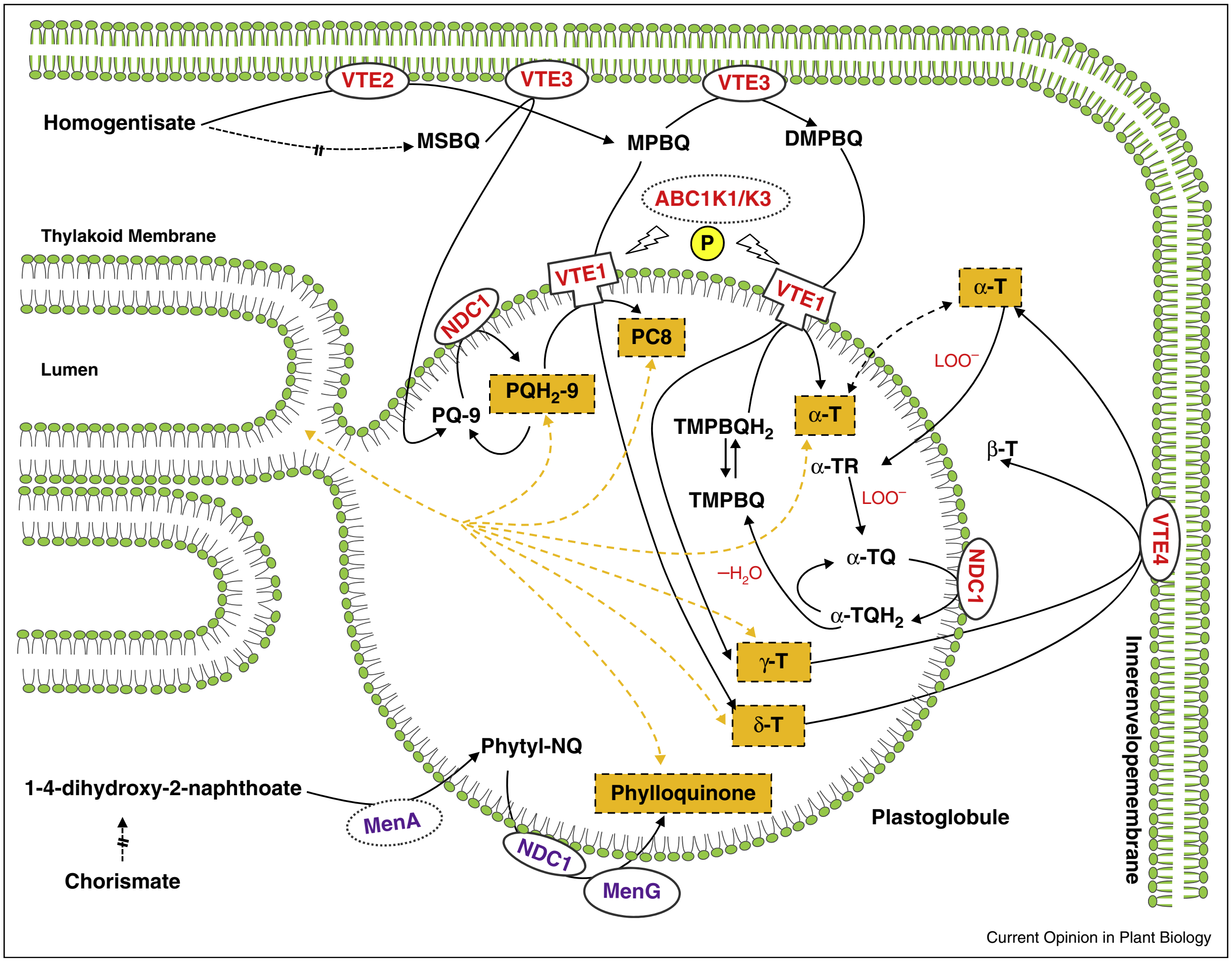
Overview of the prenylquinone biosynthetic pathways in Arabidopsis. Phylloquinone *de novo* synthesis. Four Men enzymes are responsible for the conversion of chorismate to *o*-succinyl-benzoate. They are referred to as MenF, D, H and C, respectively [16–18]. The three next steps consist of the activation of *o*-succinyl-benzoate into *o*-succinyl-benzonyl-CoA followed by the formation of the naphtoate ring. Then, production of the DHNA-CoA is compartmentalized in peroxisomes where MenE, B and H act sequentially [16,17]. The pathway then returns to the plastid where the CoA moiety of DHNA-CoA is removed, and conversion to DHNA is catalyzed by a thioesterase hotdog-fold enzyme [19]. DHNA phytyltransferase (MenA) converts DHNA to Phytyl-NQ. Phytyl-NQ is methylated at the C3-position of the naphthoquinone moiety by the MenG gene yielding phylloquinone [6]. Tocopherol *de novo* synthesis: the benzoquinone head group is derived from the shikimate pathway and the phytyl tail from the plastidic 1-deoxy-D-xylulose-5-phosphate isoprenoid pathway. Phytyl-DP prenylation by homogentisate phytyltransferase (HPT/VTE2) converts HGA into MPBQ [20,21••]. TC/VTE1 catalyzes chromanol ring formation in MPBQ leading to the formation of δ-tocopherol. Alternatively, MPBQ methyltransferase (encoded by the Arabidopsis VTE3 locus) may methylate MPBQ to give DMPBQ. Cyclization DMPBQ by VTE1 leads to the formation of γ-tocopherol. The addition of a methyl group to the sixth position of the chromanol ring by VTE4 is responsible for conversion of δ-tocopherols and γ-tocopherols into β-tocopherols and α-tocopherols, respectively [4,11,21••,22–25]. Enzymes are shown in red and enzymes which are discussed in more detail are shown in bold letters. The prenylquinone metabolites highlighted in yellow are probably synthesized at the plastoglobule. *Abbreviations*: TAT, tyrosine aminotransferase; HPPD, p-hydroxyphenyl-pyruvate dioxygenase; HST, homogentisic acid solanesyl transferase; VTE, enzymes of vitamin E synthesis; LOO-, lipid peroxy radical; TC, tocopherol cyclase; Men, menaquinone synthesis; ICS 1/2, isochorismate synthase 1 and 2; AAE14, acyl-CoA activating enzyme isoform 14; NS, naphtoate synthase; DHNA-CoA, 1,4-dihydroxy-2-naphthoyl-CoA; DHNA-CoA thioesterase, 1,4-dihidroxy-2naphthoyl-CoA thioesterase; DHNA, 1,4-dihydroxy-2-naphthoate; Phytyl-NQ, 2-phytyl-1,4-naphthoquinone; ECHld, enoyl-CoA hydratase/isomerase; DP, diphosphate; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinone; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinone; MSBQ, 2-methyl-6-solanesyl-1,4-benzoquinol; PQH_2-9, plastoquinol; PQ-9, plastoquinone; PC8, plastochromanol; SEPHCHC, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate; SHCHC, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate; NDC1, NAD(P)H-dependent:quinone oxidoreductase; ABC1K1/K3, activity of bc1 kinases 1 and 3 complex; $TMPBQH_2$, Trimethylbenzoquinol; TMPBQ, Trimethylbenzoquinone; α-TQH_2, α-tocopherol quinol; α-TQ, α-tocopherol quinone; α-TR, α-tocopheroxyl radical.*Source*: Adapted from Eugeni Piller *et al.* [21••,26].

Figure 2



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Topology of biosynthetic pathways and enzymes of Vitamin E and K metabolism in plastoglobules. Enzymes in red participate in α-tocopherol metabolism, including its redox cycle, as well as the conversion of plastoquinone to plastochromanol. Enzymes in purple participate in phylloquinone metabolism. In light orange connectors represent bidirectional trafficking of metabolites between plastoglobules and thylakoids. Final products are highlighted in orange. MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol; MSBQ, 2-methyl-6-solanesyl-1,4-benzoquinol; PQ-9, plastoquinone; PQH_2-9, plastoquinol; PC8, plastochromanol-8; α-T, alpha-tocopherol; α-TR, alpha-tocopheroxyl radical; α-TQ, alpha-tocopherolquinone; TMPBQ, trimethylbenzoquinone; $TMPBQH_2$, trimethylbenzoquinol; γ-T, gamma-tocopherol; δ-T, delta-tocopherol; β-T, beta-tocopherol; Phytyl-NQ, 2-phytyl-1,4-naphthoquinone.

chlororespiration [10,28]. NDC1 in Arabidopsis is a mem-ber of a family of seven homologs. Apart from NDC1 that is present in chloroplasts the homologs have been local-ized in mitochondria. However, NDC1 may be dually localized and also be present in mitochondria [28]. NDC1 does not appear to play a role in a major electron pathway. Yet it keeps the PQ in PG reduced resulting in an overall reduced state of total chloroplast PQ. The consequences of this for chloroplast Redox signaling have not yet been studied. How NDC1 is implicated in the AtmenG-de-pendent methylation step of phylloquinone synthesis remains a mystery. It will be interesting to see whether NDC1 is uniquely required for phylloquinone in Arabi-dopsis or whether this is also the case in other species [16,17,19].

Tocopherol cyclase (VTE1) located at plastoglobules fulfills diverse roles in tocopherol metabolism

A large proportion of VTE1 (tocopherol cyclase — TC) is localized at PG [41•]. This was one of the first indications that PG function not only in the storage but also partici-pate in the metabolism of tocopherol [15,20] (Figure 2). The *vte1* mutant lacking TC and the *vte2* mutant lacking the homogentisate phytyl transferase are both devoid of α-tocopherol. Instead of α-tocopherol, *vte1* accumulates its precursor DMPBQ and also lacks PC8 [21••,42•]. It has been suggested that in *vte1* DMPBQ can substitute for α-tocopherol, for example, in seed germination [43]. However, under HL *vte1* displays a drop in PSII efficien-cy. Under HL, α-tocopherol concentrations increase in wt but in vte1 the DMPBQ concentrations remain constant [4]. This supports the idea that the accumulation of DMPBQ under HL is not sufficient to completely replace α-tocopherol and assume its membrane protective role in the thylakoid membranes [2,44,45]. *vte2* displayed a simi-lar phenotype under short-term HL stress as *vte1* [2]. In *vte2*, both tocopherols and DMPBQ are entirely absent. However, in *vte2* PC8 may partially assume the antioxi-dant protection role of α-tocopherol attenuating or sup-pressing perturbed phenotypes under stress. In support of this notion, the absence of both tocopherol and PC8 in the *vte1/vte2* double mutant leads to a dramatic reduction in seed longevity and seedling viability [42•]. The role of VTE1 in the production of PC8 is clearly apparent in overexpressing lines that show a massive increase of PC8 [42•]. Moreover, much of the PC8 accumulates in PG.

VTE1 and NDC1 enzymes are directly implicated in the redox cycle of α-tocopherol The oxidation of α-tocopherol involves donation of two electrons. First, a lipid peroxy radical (LOO^{-}) is reduced to a more stable hydroperoxide (LOOH) yielding α-toco-pheroxyl radical (α-TR). Subsequently, α-TR is oxidized to α-tocopherol quinone (α-TQ) by LOO^{-}. α-TQ accu-mulates in PG. A PG-based repair system enables the reconversion of the oxidation products to tocopherol [4,21••,37,46,47] (Figure 2).

Initially, α-TQ is reduced to yield α-tocopherol quinol (α-TQH_2), a reaction that is facilitated by NDC1. This is not surprising because NDC1 has a wide substrate spec-ificity [28]. The following step consists of the conversion of α-TQH_2 to trimethylbenzoquinol ($TMPBQH_2$) by an unknown dehydratase. Finally, the conversion of $TMPBQH_2$ to α-tocopherol is carried out by VTE1 [4,15,21••,46] (Figure 2). The presence of a large propor-tion of VTE1 suggests that it may be committed to tocopherol recycling in PG rather than to *de novo* synthesis in this compartment.

ABC1-like kinases affect prenyl lipid composition of the chloroplast ABC1 (activity of bc1 complex)-like kinases are essential regulators of ubiquinone synthesis in bacteria and mitochondria of all lineages [26]. In plants, surprisingly, a group of 8 ABC1-like kinases family are present in chloroplasts, six of which are associated with PG. By analogy to bacterial and mitochondrial ABC1-like kinases implication in pre-nylquinone metabolism was proposed and demonstrated using reverse genetic experimentation in conjunction with lipidomics [41•,44,48••]. The ABC1K1 homolog was iden-tified earlier as PGR6 (proton gradient regulation 6). The *pgr6* mutant has a high fluorescence phenotype indicative of a defect in the formation of the proton gradient across the thylakoid membrane. Lipidomics analysis revealed that the *abc1k1/pgr6* mutant is defective in prenyl lipid metabolism. Under HL conditions, *abc1/pgr6* failed to accumulate α-tocopherols, γ-tocopherols and δ-tocopherols to the high levels observed in wt. At the same time, *abc1k1/pgr6* showed a striking 6-fold increase of α-TQ, the oxidation product of α-tocopherol over wt under HL. These findings indicated a perturbation in tocopherol metabolism at the level of VTE1. The kinase nature of ABC1K1/PGR6 and the common localization in PG suggested that VTE1 might be a substrate of ABC1/PGR6. This was confirmed by *in vitro* experimentation. Moreover, VTE1 is a known phosphoprotein containing a phosphor-ylation hotspot near its N-terminus [44]. However, the range of other metabolic effects (reduced levels of β-carotene and lutein, altered PQ/PQH_2 ratio) observed in the mutant suggests that ABC1K1/PGR6 has many more targets. This molecular phenotype resulted in reduced photosynthetic electron transport, strong but reversible photodamage under HL and decrease of carbon fixation. Thus, the mutant had lower photosynthetic rates which led to reduced starch production and perturbation in the starch and sugar ratios after recovery from initial photodamage [48••,49].

Another ABC1 homolog in PG, ABC1K3 has a mutant phenotype related to that of ABC1K1/PGR6, but photosynthetic efficiency was as in wt. *abc1k3* was affected in prenylquinone composition but, unlike *abc1k1*, α-tocopherol accumulation was as in wt. In *abc1k3*, α-TQ over accumulated under HL and PC8 was sharply reduced under all conditions. These results suggest that, ABC1K1 specifically regulates tocopherol recycling and plastochromanol production, functions that have been attributed to PG-localized VTE1. Like ABC1K1, ABC1K3 kinase phosphorylates VTE1 *in vitro*, which may provide a basis for regulation of VTE1 activity *in vivo*.

Under HL stress conditions, gene expression of ABC1K3 and VTE1 were highly correlated. At the protein level, however, Martinis *et al.* [44,48••] showed significantly lower contents of VTE1 in the *abc1k1/pgr6* and *abc1k3* mutants than in wt. These data suggest that ABC1K1/PGR6 and ABC1-K3 stabilize VTE1 levels. However, these data differ from the data by Lundquist *et al.* [50•] that show no difference in VTE1 levels between the *abc1k1/abc1k3* double mutant and wt. Currently, the reasons for this discrepancy are not known. The ABC1K1 and ABC1-K3 kinases have been shown to interact with each other forming a protein complex (ABC1K1/3 complex). Analysis of the *abc1k1/abc1k3* double mutant revealed additional defects suggesting a premature senes-cence phenotype by activation of jasmonate pathways, including chlorophyll and PSII degradation [50•].

Conclusions

PG functions have been reported throughout all stages of plant development, from plastid biogenesis to senescence and fruit maturation [5]. This review focuses on the role of PG in phylloquinone synthesis and tocopherol metabolism including the Redox cycle and accumulation under HL [4,26,28,37–39,46]. Astonishingly, NDC1 a PG enzyme with no apparent link to phylloquinone synthesis is required for its accumulation in Arabidopsis. The mechanism of NDC1 is currently unknown.

It is important to note that not only metabolic enzymes reside at the PG but also regulatory ABC1-like kinases that affect tocopherol accumulation and Redox recycling under HL. VTE1 is the likely target of the ABC1K1 and ABC1-K3 kinases. However, these kinases probably have far more targets judged by the multitude of processes that are perturbed in the single and double *abc1k1* and *abc1-k3* mutants.

How can PG exert their role in remodeling chloroplast prenyl lipid composition? It is hard to imagine that remodeling could take place if the thylakoid and PG did not form a continuum, in which the PG serve as a functional membrane microdomain bearing an arsenal of specific enzymes [26,39,40,41•]. Implicitly, trafficking of metabolites between PG and the thylakoid membrane is required. The conduit formed by the common outer membrane leaflet is likely the 'bottle-neck' in metabolite trafficking between the thylakoid and the PG. A lot remains to be discovered as PG are clearly tied into a larger metabolic network that includes pathways beyond prenylquinone metabolism, affecting the metabolisms of galactolipids [51], carotenoids [52], chlorophyll, sugars and more.

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