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Functional Modeling Identifies Paralogous Solanesyl-diphosphate Synthases That Assemble the Side Chain of Plastoquinone-9 in Plastids*§

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Background: Plastid isoforms of solanesyl-diphosphate synthase catalyze the elongation of the prenyl side chain of plastoquinone-9.

Results: Corresponding mutants display lower levels of plastoquinone-9 and plastochromanol-8 and display intact levels of vitamin E.

Conclusion: Plastochromanol-8 originates from a subfraction of non-photoactive plastoquinol-9 and is not essential for seed longevity.

Significance: Viable plastoquinone-9 mutants are invaluable tools for understanding plastid metabolism.

It is a little known fact that plastoquinone-9, a vital redox cofactor of photosynthesis, doubles as a precursor for the biosynthesis of a vitamin E analog called plastochromanol-8, the physiological significance of which has remained elusive. Gene network reconstruction, GFP fusion experiments, and targeted metabolite profiling of insertion mutants indicated that Arabidopsis possesses two paralogous solanesyl-diphosphate synthases, AtSPS1 (At1g17050) and AtSPS2 (At1g17050), that assemble the side chain of plastoquinone-9 in plastids. Similar paralogous pairs were detected throughout terrestrial plant lineages but were not distinguished in the literature and genomic databases from mitochondrial homologs involved in the biosynthesis of ubiquinone. The leaves of the atsp2 knock-out were devoid of plastochromanol-8 and displayed severe losses of both non-photoactive and photoactive plastoquinone-9, resulting in near complete photoinhibition at high light intensity. Such a photoinhibition was paralleled by significant damage to photosystem II but not to photosystem I. In contrast, in the atsp1 knock-out, a small loss of plastoquinone-9, restricted to the non-photoactive pool, was sufficient to eliminate half of the plastochromanol-8 content of the leaves. Taken together, these results demonstrate that plastochromanol-8 originates from a subfraction of the non-photoactive pool of plastoquinone-9.

Plastoquinone-9 (2,3-dimethyl-6-solanesyl-1,4-benzoquinone) is a vital redox cofactor for oxygenic photoautotrophs. It is required during photosynthesis for electron transfer and proton translocation in thylakoid membranes, as well as for respiration in cyanobacteria and as a necessary oxidant for the desaturation of the carotenoid precursor phytoene (1–3). Furthermore, it is via the redox state of plastoquinone-9 in thylakoid membranes that plants and cyanobacteria monitor the balance of photosystem II and photosystem I activity and adjust the expression of some plastid and nuclear genes (4, 5).

Plastoquinone-9 is a bipartite molecule made up of a redox active benzoquinone ring attached to a solanesyl (C45) chain (Fig. 1). Plants derive the benzenoid moiety from homogentisate, which is prenylated and decarboxylated in the inner envelope of chloroplasts, yielding 2-methyl-6-solanesyl-1,4-benzoquinol (6, 7). The latter is then methylated to give plastoquinol-9 (Fig. 1). Analogy with the assembly of the polyprenyl side chain of ubiquinone in proteobacteria and mitochondria indicates that the biosynthesis of the plastoquinone-9 solanesyl moiety proceeds from the trans-long chain prenyl-diphosphate synthase-catalyzed elongation of a C15–C20 allylic diphosphate precursor. The corresponding activity has therefore been tentatively attributed in Arabidopsis and rice to plastid-targeted solanesyl-diphosphate synthases, products of the At1g17050 and Os05g0582300 genes, respectively (8–10), although there is no direct evidence that such enzymes indeed participate in plastoquinone-9 biosynthesis. A peculiarity of the biosynthetic pathway of plastoquinone-9 in photosynthetic eukaryotes is
that it intertwines with that of tocopherols (Fig. 1). First, the hydroxychroman moiety of tocopherols also originates in part from homogentisate (6). Second, the methyltransferase that decorates the benzoquinol ring intermediate also functions in the biosynthesis of tocopherols (11). Lastly, plastoquinol-9 itself can serve as a substrate for tocopherol cyclase to yield plastochromanol-8 in plastoglobules (12, 13). Although it has received considerably less attention than tocopherols, plastochromanol-8 is thought to function as an antioxidant in plants. This seems a valid possibility, because plastochromanol-8 contains the same hydroxychroman ring as \( \alpha \)-tocopherol (Fig. 1), and hydroxyplastochromanol-8, formed as a result of singlet oxygen scavenging by plastochromanol-8, has been shown to accumulate in leaves during high light stress and aging (14). In particular, there is evidence that the seeds of an Arabidopsis mutant unable to synthesize either plastochromanol-8 or tocopherols exhibit increased lipid oxidation during desiccation and quiescence, resulting in lower seed longevity compared with those of another mutant that is devoid of tocopherols only (15). However, it is unclear to what extent plastochromanol-8 contributes to the antioxidant arsenal of plant tissues when tocopherols are present, as would normally happen in nature. In fact, studying the specific roles of plastoquinone-9 and plastochromanol-8 by means of straightforward loss-of-function strategies has invariably proved vexing. Not only is the biosynthesis of plastoquinone-9 and plastochromanol-8 seemingly impossible to disconnect from that of tocopherols, but all of the tocopherol biosynthetic mutants identified to date, namely those corresponding to 4-hydroxyphenylpyruvate dioxygenase, homogentisate solanesyl transferase, and 2-methyl-6-solanesyl-1,4-benzoquinol methyltransferase (Fig. 1), are albino and seedling-lethal (3, 11, 16).

In this study, we demonstrated that Arabidopsis thaliana possesses two solanesyl-diphosphate synthases involved in the assembly of the plastoquinone-9 side chain. We then examined the impact of a deficit of plastoquinone-9, plastoquinol-9, and plastochromanol-8 in the leaves and seeds of cognate mutants, the tocopherol biosynthetic capabilities of which are intact.

**MATERIALS AND METHODS**

**Chemicals and Reagents**—Ubiquinone-10 was from Sigma-Aldrich. \( \alpha \)-Tocopherol was from Acros Organics, and \( \delta \)-tocopherol and \( \gamma \)-tocopherol were from Matreya, LLC. Ubiquinone-9, plastoquinone-9, and plastochromanol-8 standards were extracted from Candida utilis, Synechocystis sp. PCC 6803, and the seeds of Camelina sativa, respectively, and HPLC-purified. Quinol standards were synthesized chemically from the reduction of their corresponding quinone forms using sodium borohydride. Calibration solutions were quantified spectrophotometrically using the molar extinction coefficients of 14,600 M\(^{-1}\)cm\(^{-1}\) at 275 nm for ubiquinone, 15,200 M\(^{-1}\)cm\(^{-1}\) at 255 nm for plastoquinone-9, 3260 M\(^{-1}\)cm\(^{-1}\) at 292 nm for \( \alpha \)-tocopherol, 3510 M\(^{-1}\)cm\(^{-1}\) at 298 nm for \( \delta \)-tocopherol, and 3810 M\(^{-1}\)cm\(^{-1}\) at 298 nm for plastochromanol-8 (17) and the absorbance value \( E_{1\text{cm}} \) (1%) at 296 nm = 55.5 for plastochromanol-8 (18). High fidelity PCR amplifications were performed with Phusion polymerase (Finnzymes), and PCR genotyping experiments were performed with GoTaq polymerase.
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(Promega). All DNA constructs were verified by sequencing. Unless mentioned otherwise, all other reagents were from Fisher Scientific.

**Plant Material and Growth Conditions—*Arabidopsis***

T-DNA insertion mutant SALK_126948 (Atig78510; AtSPS1) and SALK_064292 (Atig17050; AtSPS2) were obtained from the Arabidopsis Biological Resource Center at the Ohio State University (19). Seeds were allowed to germinate in *vitro* on Murashige and Skoog solid medium and transferred to potting mix in a growth chamber at 22 °C in 16-h days (110 μmol mètres⁻² s⁻¹) for 4 weeks. The double knock-out was obtained from the F2 segregating progeny resulting from the cross between the individual SALK_126948 and SALK_064292 homozygous T-DNA mutants. For the measurements of chlorophyll fluorescence and metabolite quantification during acclimation to high light, plants were grown directly on soil and SALK_064292 (At1g17050; AtSPS2) was amplified using primers 5'-ATCATTCTTTCGAGGTTATACAA-3' and 5'-ATGTTGAACCGCTTGCTGCAACT-3'/*H11032* (forward) and 5'-CGCGACCTTGCTTTCGACTTTA-3'/*H11032* (reverse) for the native promoter, a *AtSPS1* fragment 48 h later. For imaging in *Arabidopsis* plants, the pCAMBIA-AtSPS1-GFP construct was stably introduced using the floral dip method (22).

**Terpenoid Conjugates Analyses—*Arabidopsis*** leaf (36–94 mg of fresh weight) and seed (35–45 mg) samples were spiked with 6.2–6.9 nmol of ubiquinone-10 and homogenized in 0.5 ml of 95% (v/v) ethanol using a 5-ml Pyrex tissue grinder. For the quantification of plastoquinol-9 in illuminated leaves (determination of photoreactive pool), care was taken to maintain exposure to high light intensity until tissues were completely disrupted. Trial experiments showed that there was no post-mortem photoreduction of plastoquinone-9. The grinder was rinsed with 0.2 ml of 95% (v/v) ethanol, and the wash was combined with the original extract. The sample was then centrifuged (5 min at 18,000 × g) and immediately analyzed by HPLC on a 5 μm Supelco Discovery C-18 column (250 × 4.6 mm, Sigma-Aldrich) thermostatted at 30 °C and developed in isocratic mode at a flow rate of 1.5 ml min⁻¹ with methanol. Plastoquinone-9 and ubiquinone-10 were detected spectrophotometrically at 255 and 275 nm, respectively. Plastoquinol-9, plastochromanol-8, and tocopherols were detected fluorometrically (290 and 330 nm for excitation and emission, respectively). Retention times were 5.3 min (β-tocopherol), 5.9 min (γ-tocopherol), 6.6 min (α-tocopherol), 14.3 min (plastoquinol-9), 28.4 min (plastochromanol-8), 40.7 min (plastoquinone-9), and 45.1 min (ubiquinone-10). Compounds were quantified according to their corresponding external calibration standards, and data were corrected for recovery of the ubiquinone-10 internal standard. Ubiquinone-9 was quantified as described previously (23).

**Analysis of Photosystems—Illuminating of maximum quantum efficiency of photosystem II on whole *Arabidopsis*** plants was performed using a FluorCam 700 MF system (Photon Systems Instruments) using the quenching analysis effect settings. Pulses of actinic light and continuous illumination were generated by two arrays, each made up of 345 620 nm-LEDs. Plants were dark-adapted for 20 min before each experiment. Fluorescence parameters were calculated as follows: $F_v/F_m = (F_m - F_o)/F_m$, where $F_v$ is the calculated variable fluorescence, $F_m$ is the maximal fluorescence measured immediately after the saturating pulse, and $F_o$ is the initial fluorescence of dark-adapted tissues; $R_{lo} = F_o/F_m$, where $F_o$ is the fluorescence decrease from $F_m$ to $F_o$ (the steady state fluorescence measured after 3 s of continuous illumination); non-photochemical quenching (NPQ) = $(F_{m} - F_{m}^\prime)/F_{m}^\prime$, where $F_{m}^\prime$ is the maximal fluorescence measured after a 3-s adaptation to actinic light; photoinhibition = $1 - (F_v/F_m)/(F_v/F_m^\prime)$, where $F_v$ and $F_m^\prime$ are the variable and maximal fluorescence, respectively, measured after 2, 24, and 48 h of exposure to actinic light at 800 μmol mètres⁻² s⁻¹ followed by 20 min of recovery in the dark. The
RESULTS

Modeling of Gene Co-expression Network Predicts That Arabidopsis Possesses Two Solanesyl-Diphosphate Synthases That Functionally Intersect in Plastids—BLASTp searches of Arabidopsis genomic databases using either Escherichia coli octaprenyl-diphosphate synthase (lspB) or Saccharomyces cerevisiae hexaprenyl-diphosphate synthase (Coq1) as queries, both of which assemble the side chain of ubiquinone in their respective hosts (25, 26), detected three proteins that bear the molecular attributes of trans-long chain diphosphate synthases, At1g78510, At1g17050, and At2g34630. As previous reports indicate that all three of these proteins harbor solanesyl-diphosphate synthase activity (9, 27, 28), we shall call them hereafter, for purpose of clarity, AtSPS1, AtSPS2, and AtSPS3, respectively. Modeling of a high stringency network made up of the top 100 genes (0.45% of the 22,263 expressed loci in the ATTED-II database) that co-express with AtSPS1, AtSPS2, and AtSPS3 generated six gene clusters (Fig. 2). Three of those clusters corresponded to genes that uniquely co-express with AtSPS1, AtSPS2, or AtSPS3, two to genes that co-express either with AtSPS1 and AtSPS2 or with AtSPS1 and AtSPS3, and one to genes that co-express with all three AtSPS members (Fig. 2). (Supplemental Data File 1 provides a list of these genes including their correlation rank and functional annotations.) Subcellular localization data compiled from the SUBA and TAIR databases were then overlaid on each cluster (Fig. 2). Remarkably, AtSPS1 and AtSPS2 emerged from such a reconstruction as top co-expressors of each other, number 2 and number 4, respectively (supplemental data File 1), sharing more than half of their co-expressing genes (Fig. 2). The co-expressors of AtSPS1 and AtSPS2 also displayed strikingly similar patterns of subcellular distribution, including a marked prevalence for genes that encode for plastid-targeted proteins (Fig. 2). In sharp contrast, 93% of the genes that co-expressed with AtSPS3 clustered uniquely with this enzyme (Fig. 2). Over one-third of those were found to encode for proteins targeted to the mitochondrion (Fig. 2), agreeing with the role of AtSPS3 as a ubiquinone biosynthetic enzyme in this organelle (23). Taken together, these data imply that AtSPS1 and AtSPS2 belong to the same functional network. The latter is not solely distinct from the AtSPS3 functional network but is also intimately linked to plastids. Although such a model fits with the demonstration that AtSPS2 is targeted to plastids, it conflicts with the previous assumption that AtSPS1 localizes to the endoplasmic reticulum (8, 9).

AtSPS1 Is Targeted Exclusively to Plastids—To reexamine the subcellular localization of AtSPS1, a 3089-bp genomic fragment comprising the AtSPS1 sequence (minus its stop codon)
as well as its 5'-untranslated region and native promoter was cloned in-frame to the 5'-end of GFP. Confocal laser scanning microscopy of the transiently expressed AtSPS1-GFP construct in tobacco leaf tissues showed a pattern of green pseudocolor that co-localized with the autofluorescence of chlorophyll in plastids (Fig. 3, A–C). Untransformed plastids confirmed that the fluorescence attributed to GFP was not merely due to an overflow of the red pseudocolor into the green pseudocolor (Fig. 3, D). Imaging of the leaves of T1 transgenics demonstrated that again the fluorescence of GFP was localized in plastids (Fig. 3, D–F). A comparison with the leaves of wild type Arabidopsis point to those that are paralogous. Bootstrap values out of 100 replications are given next to each branching. AtSPS1 (ABF58968.1), AtSPS2 (NP_173148.2), ATSPS3 (AAM13005.1), B. distachyon (XP_001762387.1, XP_001753309.1), C. merolae (NP_171068.1, XP_002985427.1; 2, XP_001753309.1), Cucumis sativus (1, XP_004137246.1; 2, XP_004134571.1), Danio rerio (NP_001071665.1), D. melanogaster (NP_733425.1), Gallus gallus (XP_418592.3), Glycine max (1, XP_003543174.1; 2, XP_003546747.1; 3, XP_003525839.1), Homo sapiens DPS (AAAD28559.1), Hordeum vulgare (1, BAK00672.1; 2, BAK05302.1), Neurospora crassa (XP_959949.1), N. sp. PCC 7120 (NP_481410.1), Oryza sativa (SPS1, NP_001058362.1; SPS2, XP_003559297.1), P. patens (1, XP_001014989.1), P. patens (2, XP_001753309.1), P. patens (3), Prochlorococcus marinus (Yp_001014989.1), S. cerevisiae HPS (EGA88084.1), S. moellendorffii (1, XP_002985427.1; 2, XP_002979762.1; 3, XP_002977539.1), Trichodesmium erythraeum (YP_720124.1), Vitis vinifera (1, XP_002856665.1; 2, XP_002268222.2), Zea mays (1, ACN25661.1, 2, NP_001149100.1, 3, ACCG39955.1), DPS, decapeynyl-diphosphate synthase; HPS, hexaprenyl-diphosphate synthase; SPS, solanesyl-diphosphate synthase.

Duplication of Solanesyl-diphosphate Synthase of Plastids Is Widespread in Land Plants—Database mining of fully sequenced genomes pointed to cyanobacterial trans-long chain prenyl-diphosphate synthases as the closest prokaryotic relatives of AtSPS1 and AtSPS2. Notably, like metazoa and fungi, cyanobacteria have only one gene coding for this class of enzymes, whereas without exception plants have several. For instance, the green alga Chlamydomonas reinhardtii, the red alga Cyanidioschyzon merolae, barley, grape, tomato, and other crops have several dupl...
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AtSPS1 and AtSPS2 Contribute to the Biosynthesis of Plastoquinone-9 and Plastochromanol-8—A T-DNA mutant corresponding to an insertion located in the first exon of AtSPS1 (SALK_126948) was identified using the T-DNA Express gene mapping tool and confirmed by DNA genotyping (Fig. 5, A and B). RT-PCR analysis using a primer pair designed to amplify a cDNA region spanning from the end of the second exon to the beginning of the sixth exon confirmed that the *atsps1* locus was null (Fig. 5, A and C). A tandem T-DNA insertion was similarly identified in the sixth exon of AtSPS2 (SALK_064292), but RT-PCR amplification of a cDNA region located upstream of the predicted insertion revealed that the cognate locus was not null (Fig. 5, D–F). Sequencing of a PCR fragment encompassing the junction between the T-DNA border and the last exon located the insertion precisely at the second nucleotide position of the AtSPS2 stop codon (Fig. 5D). Conceptual translation of the cognate mRNA resulted in a chimeric protein formed from the in-frame C-terminal fusion between AtSPS2 and a T-DNA encoded polypeptide.

HPLC analyses of prenylated benzoquinones and tocochromanols showed that the content in total plastoquinone-9, quinone (oxidized) + quinol (reduced) forms, was decreased by 11 and 57% in the leaves of the *atsps1* and *atsps2* mutants, respectively, compared with that of wild type plants (Fig. 6A). The level of plastochromanol-8, the product of plastoquinol-9 cyclization, was decreased by about 35% in the *atsps1* mutant (Fig. 6A). For each *atsps* mutant, these differences in plastoquinone-9 and plastochromanol-8 levels still strictly co-segregated with the T-DNA insertion after three back-crosses. Total tocopherol levels were either undistinguishable or, owing to a ~20% increase in α-tocopherol content, slightly higher than those of the wild type in the *atsps1* mutant and *atsps2* mutant, respectively (Fig. 6B). No statistically significant differences were observed in the levels of ubiquinone-9 between the mutants and the wild type control (Fig. 6C). Dry seeds of the *atsps1* and *atsps2* mutants accumulated 11 and 36% less plastochromanol-8 than their wild type counterparts, respectively (Fig. 6D). Although *atsps2* seeds contained about 40% more α-tocopherol than wild type and *atsps1* seeds, such an increase was counterbalanced by a similar decrease in δ-tocopherol, resulting in no statistically significant difference in total tocopherol accumulation (Fig. 6E).

The plastoquinone-9 and ubiquinone-9 levels of dry seeds were below the detection threshold. The *atsps1* *atsps2* double homozygous mutant displayed a prominent albino phenotype (Fig. 7, A and B) and was devoid of plastoquinone-9 and plastochromanol-8 (Fig. 7C). As observed for the *atsps1* and *atsps2* single mutants, it did how-
ever retain the ability to accumulate tocopherols to wild type levels (Fig. 7C). The *atsps1 atsps2* double knock-out survived *in vitro* on an external carbon source and under low illumination but never developed beyond a couple of leaves. These plants died quickly when transferred to soil, and we therefore deemed the *atsps1 atsps2* knock-out seedling-lethal. Taken together, these data indicate that AtSPS1 and AtSPS2 are involved in the biosynthesis of plastoquinone-9. Moreover, because plastoquinone-9 and plastochromanol-8 is not functional. Most importantly, the deficit or absence of plastochromanol-8 in the *atsps1 atsps2* double homozygous mutant, it can also be deduced that the chimeric AtSPS2 mRNA in SALK_064292 mutant (*atsps2*) is not functional. Most importantly, the deficit or absence of plastochromanol-8 was undetectable in the *atsps1 atsps2* double homozygous mutant, which was markedly decreased in the *atsps1* mutant, representing a mere 43% of the photoactive pool of control plants (Fig. 8A). The quantity of non-photoactive plastoquinone was decreased by 15% as compared with the wild type control in the *atsps1* mutant and by up to 60% in the *atsps2* knock-out (Fig. 8B). The ratio of plastoquinol-9 to plastoquinone-9 of the *atsps1* knock-out was not statistically different from that of the wild type, whereas it was markedly decreased in the *atsps2* mutant, representing a mere 43% of the photoactive pool of control plants (Fig. 8A). The ratio of plastoquinol-9 to plastoquinone-9 of the *atsps1* knock-out was not statistically different from that of the wild type, whereas it was markedly decreased in the *atsps2* mutant, representing a mere 43% of the photoactive pool of control plants (Fig. 8A).

**The Photoactive and Non-photoactive Pool Sizes of Plastoquinone-9 Are Differently Altered in the *atsps1* and *atsps2* Mutants**—Because the quantities of plastoquinone-9 and plastochromanol-8 were altered to markedly differing extents in each *atsps* mutant, we sought to determine whether such deficiencies were related to the size of the photoactive pool of plastoquinone or to the non-photoactive one or to both. To that end, we quantified plastoquinone-9 and plastoquinol-9 in the leaves of plants placed in the dark and then exposed to saturating light intensity. In this method, the amount of plastoquinone the redox status of which responds to changes in light regime represents the size of the photoactive pool; it is calculated from the difference between the levels of plastoquinol-9 measured in the light and that measured in the dark. Conversely, the quantity of plastoquinone-9 that does not re-oxidize in the dark and that of plastoquinone-9 that is not reduced in the light represent the non-photoactive pool. These measurements showed that the pool size of photoactive plastoquinone in the *atsps1* knock-out was not statistically different from that of the wild type, whereas it was markedly decreased in the *atsps2* mutant, representing a mere 43% of the photoactive pool of control plants (Fig. 8A). The ratio of non-photoactive plastoquinone was decreased by 15% as compared with the wild type control in the *atsps1* mutant and by up to 60% in the *atsps2* knock-out (Fig. 8B). The ratio of plastoquinol-9 to plastoquinone-9 of the *atsps1* knock-out was not statistically different from that of the wild type (1.68 ± 0.51 for the WT versus 1.36 ± 0.20 for *atsps1*; *p* analysis of variance = 0.27). On the other hand, this ratio was significantly lower in the *atsps2* knock-out (0.80 ± 0.19; *p* analysis of variance = 0.003), indicating that the redox state of non-photoactive plastoquinone had been altered in these plants. It should be noted that the *atsps1* knock-out, in which the deficit of plastoquinone-9 has an impact solely on its non-photoactive

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**FIGURE 6. Levels of prenylated quinones and tocochromanol in *Arabidopsis* leaves and seeds.** Extracts from leaves of 4-week-old plants grown at 120 μE·m⁻²·s⁻¹ and seeds were analyzed by HPLC in spectrophotometric detection mode for plastoquinone-9 (PQ-9) and ubiquinone-9 (UQ-9) or fluorometric detection mode for plastochromanol-8 (PC-8) and tocopherols (α/γ-TC). A, plastoquinone-9 (open bars) and plastochromanol-8 (black bars) in leaves. B, α- and γ-tocopherols in leaves. C, ubiquinone-9 in leaves. D, plastochromanol-8 in seeds. E, tocopherols in seeds. Data are the means of 8–12 replicates ± S.E. for the measurements of plastoquinone-9, plastochromanol-8, and α- and γ-tocopherols in leaves, 5 replicates ± S.E. for that of ubiquinone-9 in the wild type and the *atsps1* and *atsps2* mutants, duplicates ± S.E. for that of ubiquinone-9 in the *atsps1 atsps2* double knock-out, and 3–6 replicates ± S.E. for plastochromanol-8 and tocopherols in seeds. Differing asterisk annotations indicate that the corresponding values are significantly different as determined by Fisher’s least significant difference test (*p* < 0.05) from an analysis of variance.

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**Solanesyl-diphosphate Synthases in Plastids**
At a high light regime, the more slowly than the of the induction of chlorophyll a fluence showed that wild type and atsps plants grown at moderate light intensity (120 μE·m^{-2}·s^{-1}) displayed overall similar photosynthetic performances (Fig. 9C). Maximum quantum efficiencies (Fv/Fm) of photosystem II were typical of non-stressed Arabidopsis leaves (33), ranging from 0.77 for the wild type to 0.76 and 0.75 for the atsps1 and atsps2 knock-outs, respectively (Fig. 9C and Table 1). Similarly, calculations of the variable fluorescence decrease ratio (Rd), which correlates linearly with net CO2 assimilation and assesses potential photosynthetic activity (34), did not reveal any defects for either of the atsps mutants (Table 1). Somewhat lower values for NPQ, i.e. the protective dissipation of excess chlorophyll excitation as heat (35), were observed for both mutants (Table 1). Upon exposure to high light intensity (800 μE·m^{-2}·s^{-1}), however, the fluorescence emission of the atsps2 knock-out differed noticeably from that of the atsps1 mutant and wild type control (Fig. 9D–F). The Fv/Fm, R50, and NPQ values of the atsps2 mutant collapsed after only 2 h of strong illumination (Table 1). Already at this point, photoinhibition, i.e. the irreversible loss of photosystem II efficiency, was five times higher in the atsps2 mutant than in the atsps1 and wild type plants (Table 1). By 24 h of high light treatment, the Fv/Fm value of the atsps2 knock-out had dropped to 0.31, point ing to a critical defect of photosystem II, and by 48 h of treatment all of the fluorescence parameters indicated that photosynthesis was barely operating (Table 1). It is noteworthy that up until 48 h of exposure to high light intensity, NPQ was the sole calculated fluorescence parameter that differed markedly between the atsps1 mutant and the wild type (Table 1). Thus, this observation provides evidence that non-photoactive plastocyanine-9, the pool of which is altered independently of that of its photoactive counterpart in the atsps1 knock-out (Fig. 8B), is involved in NPQ. Immunodetection of the reaction center polypeptide D1 showed that photoinhibition of the atsps2 knock-out at high light intensity was paralleled by a loss of the core of photosystem II complex (Fig. 9G). In contrast, measurements of P700 absorption indicated that the photo-oxidation of
photosystem I in the atsps2 mutant was only marginally lower than that of the atsps1 knock-out and wild type control, under either a normal or high light regime (Fig. 10, A and B). Immunodetection of the photosystem I subunit PsaA did not reveal any significant differences between the atsps mutants and the wild type (Fig. 10C). Altogether, these data indicate that the bulk of the photodamage in the atsps2 knock-out was specific to photosystem II. In a parallel experiment designed to compare

### Table 1

#### Calculated fluorescence parameters of wild type, atsps1, and atsps2 plants

Values of maximum quantum efficiency of photosystem II ($F_v/F_m$), variable fluorescence decrease ratio ($F_{v/0}$), NPQ, and percentages of photoinhibition were calculated based on fluorescence measurements like those shown in Fig. 11. Data are means of 20 measurements ± S.D.

<table>
<thead>
<tr>
<th>Fluorescence parameters</th>
<th>WT</th>
<th>atsps1</th>
<th>atsps2</th>
</tr>
</thead>
<tbody>
<tr>
<td>120 μM/s⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F_v/F_m$</td>
<td>0.77 ± 0.01</td>
<td>0.76 ± 0.01</td>
<td>0.75 ± 0.03</td>
</tr>
<tr>
<td>$F_{v/0}$</td>
<td>2.12 ± 0.06</td>
<td>2.31 ± 0.03</td>
<td>2.11 ± 0.02</td>
</tr>
<tr>
<td>NPQ</td>
<td>1.24 ± 0.02</td>
<td>1.08 ± 0.02</td>
<td>1.12 ± 0.01</td>
</tr>
<tr>
<td>Photoinhibition (%)</td>
<td>3.0 ± 2.1</td>
<td>3.2 ± 2.4</td>
<td>15 ± 3.7</td>
</tr>
</tbody>
</table>

| 800 μM/s⁻¹ (2 h)        |         |         |         |
| $F_v/F_m$                | 0.75 ± 0.02 | 0.74 ± 0.02 | 0.64 ± 0.02 |
| $F_{v/0}$                | 1.85 ± 0.04 | 1.65 ± 0.05 | 1.23 ± 0.03 |
| NPQ                     | 1.63 ± 0.03 | 1.42 ± 0.03 | 0.97 ± 0.02 |
| Photoinhibition (%)     | 3.2 ± 2.4 | 3.0 ± 2.1 | 15 ± 3.7 |

| 800 μM/s⁻¹ (24 h)       |         |         |         |
| $F_v/F_m$                | 0.72 ± 0.04 | 0.70 ± 0.05 | 0.31 ± 0.05 |
| $F_{v/0}$                | 1.61 ± 0.07 | 1.44 ± 0.05 | 0.32 ± 0.03 |
| NPQ                     | 1.43 ± 0.03 | 0.87 ± 0.03 | 0.52 ± 0.03 |
| Photoinhibition (%)     | 6.5 ± 3.3 | 7.9 ± 3.4 | 59 ± 17.2 |

| 800 μM/s⁻¹ (48 h)       |         |         |         |
| $F_v/F_m$                | 0.71 ± 0.03 | 0.62 ± 0.04 | 0.15 ± 0.07 |
| $F_{v/0}$                | 1.42 ± 0.08 | 1.24 ± 0.07 | 0.21 ± 0.03 |
| NPQ                     | 1.21 ± 0.05 | 0.83 ± 0.05 | 0.41 ± 0.07 |
| Photoinhibition (%)     | 7.9 ± 3.8 | 18.5 ± 5.3 | 80 ± 27.4 |

The absence of plastochromanol-9 does not alter seed longevity in the atsps1 atsps2 Double Knock-out—Having shown that the atsps mutants accumulated tocopherols to wild type levels, we investigated how in this context a deficit in antioxidant activity restricted to that of plastochromanol-8 would impact seed longevity. As the duration of desiccation and storage had been shown to be key determinants for the germination of tocochromanol biosynthetic mutants in Arabidopsis (15), tests of accelerated aging were performed on F2 seeds resulting from the cross between the homozygous atsps1 and atsps2 mutants. All segregating genotypes were therefore rigorously harvested at the same time and subjected to the same treatments. Surprisingly, the germination rates of both the aged and non-aged populations were similar, and the cognate seedlings
segregated according to the ratios expected for two unlinked genes in canonical Mendelian inheritance (Table 2). Such a finding was particularly remarkable in the case of the double atspo1 atspo2 knock-outs, indicating that the absence of plasto chromanol-8 had no visible impact on seed longevity and germination. Neither the seedlings of the double atspo1 atspo2 knock-out nor those of its atspo parents exhibited the failure to expand cotyledons that is typically associated with tocochromanol deficiencies (data not shown). None of the aged vte2-1 mutant seeds, which are devoid of tocopherols and display a marked decrease in longevity (20), germinated, thus verifying the efficiency of the aging procedure in our hands (Table 2).

DISCUSSION

Confusion has long prevailed regarding the identity of the Arabidopsis solanesyl-diphosphate synthase that makes the side chain of plastoquinone-9. The corresponding activity was initially attributed to gene At1g78510 (AtSPS1) and then to gene At1g17050 (AtSPS2), whereas AtSPS1 was reassigned to the biosynthesis of the ubiquinone-9 side chain (8, 9, 27).

Further complicating the picture, a third trans-long chain prenyl-diphosphate synthase, the product of gene At2g34630 (AtSPS3), was recently identified in Arabidopsis and shown to be capable of producing solanesyl diphosphate (28). Nevertheless, direct evidence of function in plants was missing all along, leading to the common belief that each of these three enzymes could function in the biosynthesis of both plastoquinone-9 and ubiquinone-9 (27, 28, 37).

Functional modeling shows that AtSPS1 and AtSPS2 are linked with each other as well as with plastid metabolism, whereas AtSPS3 is a marked outlier. Coinciding with these findings, AtSPS1 and AtSPS2 both displayed canonical plastid pre-
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sequences (Fig. 12), and previous studies have indeed confirmed that AtSPS2 is targeted exclusively to chloroplasts (8, 9, 38, 39). The localization of GFP-fused proteins demonstrates that this strict plastid localization holds true for AtSPS1 as long as the cognate gene is expressed under the control of its native promoter.

Our data show that the atsps1 and atsps2 knock-outs both display lower levels of plastoquinone-9. The fact that the atsps1 atsps2 double knock-out is devoid of plastoquinone-9 and cannot grow photoautotrophically rules out that an additional trans-long chain prenyl-diphosphate synthase contributes to the biosynthesis of plastoquinone-9. Importantly, the absence of AtSPS1 and AtSPS2 does not impact the level of ubiquinone-9. This result is supported by the observation that in tomato the trans-long chain prenyl-diphosphate synthases that elongate the allylic diphosphate precursors of ubiquinone-10 and plastoquinone-9, respectively, cannot complement the silencing of each other (31). The correct functional assignment of the AtSPS members is therefore as follows. (i) AtSPS1 and AtSPS2 contribute exclusively to the biosynthesis of plastoquinone-9. The corresponding genes descend from cyanobacteria and are most probably in-paralogs, i.e. originate from a gene duplication event that postdates the speciation of Arabidopsis. Similar duplications of solanesyl-diphosphate synthase occurred multiple times throughout terrestrial plant lineages resulting in in-paralogs that have been readily mistaken in most genomic databases for the trans-long prenyl-diphosphate synthases required for the biosynthesis of ubiquinone. (ii) AtSPS3 is dedicated to the biosynthesis of ubiquinone-9, being part of a distinct phylogenetic clade that regroups COQ1-type proteins from other eukaryotic organisms, photosynthetic or not.

It is evident from our data that AtSPS2 bears most of the plastoquinone-9 biosynthetic flux. The atsps2 knock-out lacks close to two-thirds of its photoactive and non-photoactive pools of plastoquinone-9, and exposure to high light exacerbates the deficit, leading to specific and irreversible damage to photosystem II. Although comparatively small, the loss of plastoquinone-9 in the atsps1 knock-out is remarkable, for it is restricted to the non-photoactive pool and is sufficient to wipe out about half of the plastochromanol-8 content of leaves. Thus, two notable features of the architecture of plastochromanol-8 biosynthesis in photosynthetic tissues emerged from these observations. First, plastochromanol-8 originates predominantly, if not exclusively, from the non-photoactive pool of plastoquinone-9, agreeing with the co-occurrence of three plastochromanol-8 biosynthetic enzymes, NAD(P)H quinone oxidoreductase C1, tocopherol cyclase, and ABC1-like kinase, in plastoglobules (Fig. 1) (13, 40 – 43). Second, only a fraction of non-photoactive plastoquinol-9 is cyclized into plastochromanol-8. The leaves of the atsps2 mutant, in which plastochromanol-8 is undetectable while the standing pool of non-photoactive plastoquinol-9 still represents three times the quantity of

**TABLE 2**

**Segregation ratios of the atsps1 x atsps2 F2 population**

Three-month-old seeds were subjected to accelerated aging and then vernalized, whereas control seeds were vernalized without prior aging treatment. Aged and control seeds were then allowed to germinate on Murashige and Skoog solid medium containing sucrose in 16-h days (110 μE m−2 s−1) at 22 °C. Germinated plants were scored and genotyped after 3 weeks. Numbers of plants calculated from the Mendelian segregation ratios are shown in parentheses.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control (Expected)</th>
<th>Aged (Expected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtSPS1/AtSPS1; AtSPS2/AtSPS2</td>
<td>13 (13)</td>
<td>15 (17)</td>
</tr>
<tr>
<td>AtSPS1/AtSPS1; atsps2/AtSPS2</td>
<td>29 (26)</td>
<td>32 (33)</td>
</tr>
<tr>
<td>atsps1/AtSPS1; AtSPS2/AtSPS2</td>
<td>24 (26)</td>
<td>28 (33)</td>
</tr>
<tr>
<td>atsps1/AtSPS1; atsps2/AtSPS2</td>
<td>53 (52)</td>
<td>75 (67)</td>
</tr>
<tr>
<td>atsps1/atsps1; atsps2/AtSPS2</td>
<td>21 (26)</td>
<td>34 (33)</td>
</tr>
<tr>
<td>atsps1/atsps1; atsps2/AtSPS2</td>
<td>24 (26)</td>
<td>23 (33)</td>
</tr>
<tr>
<td>atsps1/atsps1; AtSPS2/AtSPS2</td>
<td>16 (13)</td>
<td>15 (17)</td>
</tr>
<tr>
<td>atsps1/AtSPS1; atsps2/atsps2</td>
<td>12 (13)</td>
<td>22 (17)</td>
</tr>
<tr>
<td>atsps1/AtSPS1; atsps2/atsps2</td>
<td>15 (13)</td>
<td>18 (17)</td>
</tr>
<tr>
<td>Not germinated</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>208 (208)</td>
<td>267 (267)</td>
</tr>
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**Aging treatment control**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control (Expected)</th>
</tr>
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<tbody>
<tr>
<td>vte2−1/vte2−1</td>
<td>77</td>
</tr>
<tr>
<td>Not germinated</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
</tr>
</tbody>
</table>

**FIGURE 12.** Sequence alignment of AtSPS1 (ABFS8968) and AtSPS2 (NP_173148) with their octaprenyl-diphosphate synthase homologue in *E. coli* (EcISP; YP_491372). Note the N-terminal extensions of the plant enzymes (in green boxes) as predicted by both WoLF PSORT and TargetP 1.1 to encode plastid targeting peptides. AtSPS1 and AtSPS2 share 80% identical residues, pointing to a recent gene duplication. Identical residues are shaded in black and similar ones in gray. Dashes symbolize gaps introduced to maximize alignment.
plastochromanol-8 of wild type plants, exemplifies the most extreme case of such an arrangement. Our data show that this low level of cyclization does not result from a side effect of the loss of plastoquinone-9 on tocopherol cyclase activity, for the atsp1 atsp2 double mutant, as well as its atsp parents, accumulates tocopherols to wild type levels. We propose instead that the access of tocopherol cyclase to plastoquinol-9 is restricted severely in plastoglobules, possibly in a fashion similar to the protein crowding that hinders the diffusion of plastoquinone-9 molecules in thylakoid membranes (44). In support of such a scenario, there is mounting evidence that plastoquinol-9 is not only not very abundant sites for lipids as thought initially, but are authentic plastidial subcompartments derived from thylakoid membranes and associated with many enzymes and structural proteins (41, 45, 46).

Although the occurrence of plastochromanol-8 in plant tissues has been known for almost 5 decades, its physiological significance is particularly unclear. On the one hand, the genetic dissection of tocochromanol biosynthesis in Arabidopsis demonstrates that the presence of plastochromanol-8 in the seeds of tocopherol-deficient mutants mitigates oxidative damage to lipids and its subsequent negative effects on germination (15). On the other hand, one could argue that the occurrence of plastochromanol-8 in seeds that are devoid of tocopherols does not a priori occur in wild type plants. It appears instead that the amount of tocopherol in seeds dwarfs that of plastochromanol-8. Thus, calculations from our data and those of others (15, 12) show that the pool size of plastocho-

Acknowledgments—We thank Dr. E. Cahoon for the gift of the vte-2-1 seeds, Hardik Kundariya for generating the Arabidopsis atsp1 atsp2 double knock-out, and Dr. A. L. Ducluzeau for taking the pictures of the Arabidopsis plants.

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