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Genetic Dissection of Carotenoid Synthesis in Arabidopsis Defines Plastoquinone as an Essential Component of Phytoene Desaturation

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Carotenoids are C_{40} tetraterpenoids synthesized by nuclear-encoded multienzyme complexes located in the plastids of higher plants. To understand further the components and mechanisms involved in carotenoid synthesis, we screened Arabidopsis for mutations that disrupt this pathway and cause accumulation of biosynthetic intermediates. Here, we report the identification and characterization of two nonallelic albino mutations, *pds1* and *pds2* (for *phytoene desaturation*), that are disrupted in phytoene desaturation and as a result accumulate phytoene, the first C_{40} compound of the pathway. Surprisingly, neither mutation maps to the locus encoding the phytoene desaturase enzyme, indicating that the products of at least three loci are required for phytoene desaturation in higher plants. Because phytoene desaturase catalyzes an oxidation reaction, it has been suggested that components of an electron transport chain may be involved in this reaction. Analysis of *pds1* and *pds2* shows that both mutants are plastoquinone and tocopherol deficient, in addition to their inability to desaturate phytoene. Separate steps of the plastoquinone/tocopherol biosynthetic pathway are affected by these two mutations. The *pds1* mutation affects the enzyme 4-hydroxyphenylpyruvate dioxygenase because it can be rescued by growth on the product but not the substrate of this enzyme, homogentisic acid and 4-hydroxyphenylpyruvate, respectively. The *pds2* mutation most likely affects the prenyl/phytyl transferase enzyme of this pathway. Because tocopherol-deficient mutants in the green alga *Scenedesmus obliquus* can synthesize carotenoids, our findings demonstrate conclusively that plastoquinone is an essential component in carotenoid synthesis. We propose a model for carotenoid synthesis in photosynthetic tissue whereby plastoquinone acts as an intermediate electron carrier between carotenoid desaturases and the photosynthetic electron transport chain.

INTRODUCTION

Carotenoids are a diverse group of lipophilic pigments synthesized in plants, fungi, and bacteria. In photosynthetic tissues, carotenoids function as accessory pigments in light harvesting and play important roles in photoprotection by quenching free radicals, singlet oxygen, and other reactive species (Siefermann-Harms, 1987). In the plastids of nonphotosynthetic tissues, high levels of carotenoids often accumulate, providing the intense orange, yellow, and red coloration of many fruits, vegetables, and flowers (Pfander, 1992). Epoxy carotenoids also serve as biosynthetic precursors for the plant hormone abscisic acid (Rock and Zeevart, 1991). In addition to their many functions in plants, carotenoids and their metabolites also have important functions in animals, where they serve as the major source of vitamin A (retinol), and have been implicated as providing protection from some forms of cancer due to their antioxidant activities (Krinsky, 1989).

In plants, carotenoids are synthesized and accumulate exclusively in plastids via the pathway shown in Figure 1. The first committed step in carotenoid synthesis is the condensation of two molecules of the C_{20} hydrocarbon geranylgeranyl pyrophosphate by the enzyme phytoene synthase to form the colorless C_{40} hydrocarbon, phytoene. In oxygenic photosynthetic organisms (e.g., plants, algae, and cyanobacteria), phytoene undergoes two sequential desaturation reactions, catalyzed by phytoene desaturase, to produce ζ-carotene through the intermediate phytofluene. Subsequently, ζ-carotene undergoes two further desaturations, catalyzed by ζ-carotene desaturase, to yield the red pigment lycopene. Lycopene is cyclized to produce either α-carotene or β-carotene, both of which are subject to various hydroxylation and epoxidation reactions to yield the carotenoids and xanthophylls most abundant in photosynthetic tissues of plants, namely, lutein, β-carotene, violaxanthin, and neoxanthin.

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The genes encoding the first two enzymes of the pathway, phytoene synthase and phytoene desaturase, have been isolated and studied from a number of plant and bacterial sources in recent years (reviewed in Sandmann, 1994). Phytoene desaturase has been the most intensively studied, in part because it is a target for numerous commercially important herbicides and because the phytoene desaturation reaction is thought to be a rate-limiting step in carotenoid synthesis (Chamovitz et al., 1993). Molecular and biochemical studies suggest that two types of phytoene desaturase enzymes have evolved independently (Sandmann, 1994): the *crtI* type, named for the corresponding locus in *Rhodobacter capsulatus*, is found in anoxygenic photosynthetic organisms; and the *pds* (for *phytoene desaturation*) type is found in oxygenic photosynthetic organisms. *crtI*-type enzymes catalyze either three or four dehydrogenations of phytoene to neurosporene or lycopene, respectively, whereas the *pds* type catalyzes only two dehydrogenations to ζ-carotene. Little homology is found between the two types of enzymes in their amino acid sequences, although significant homology exists among members of each class. Despite these dissimilarities, all phytoene desaturase enzymes contain a dinucleotide binding domain (flavin adenine dinucleotide [FAD] or NAD/NADP), which in *Capsicum annum* has been shown to be a FAD (Hugueney et al., 1992). Presumably, the bound dinucleotide is reduced during desaturation and reoxidized by an unknown reductant present in the plastid or bacterium.

Several lines of evidence have suggested a role for quinones in the phytoene desaturation reaction in higher plants. Using isolated daffodil chromoplasts, Mayer et al. (1990) demonstrated that in an anaerobic environment, oxidized quinones were required for the desaturation of phytoene, whereas reduced quinones were ineffective. Further supporting evidence comes from studies with the triketone class of herbicides (e.g., sulcotrione), which causes phytoene accumulation in treated tissues but, unlike the well-studied pyridazone class (e.g., norflorazon), does not directly affect the phytoene desaturase enzyme. Rather, triketone herbicides competitively inhibit 4-hydroxyphenylpyruvate (OHPP) dioxygenase (Schulz et al., 1993; Secor, 1994), an enzyme common to the synthesis of both plastoquinone and tocopherols, suggesting that one or more classes of quinones play a role in carotenoid desaturation reactions (Beyer et al., 1994).

To further investigate carotenoid biosynthesis in higher plants, we have taken a genetic approach to studying the pathway by isolating mutants from Arabidopsis that are blocked in carotenoid synthesis. We report the isolation and characterization of two nonallelic mutations in Arabidopsis that affect phytoene desaturation, *pds1* and *pds2*. Surprisingly, neither is a mutation in the phytoene desaturase enzyme, thus defining two additional loci required for phytoene desaturation in Arabidopsis. The reported findings show that both *pds1* and *pds2* are defective in plastoquinone/tocopherol biosynthesis and provide conclusive evidence that plastoquinone is an essential component in phytoene desaturation.

RESULTS

Isolation and Pigment Analysis of Two Arabidopsis Mutants Defective in Carotenoid Biosynthesis

In other systems (e.g., maize and tomato), plants homozygous for defects in the early stages of carotenoid synthesis (e.g., before β-carotene) do not survive when grown in soil, due to abscisic acid deficiency, severe photooxidative damage, and the resulting inability to harvest light energy and fix carbon (Bartley and Scolnik, 1994). Isolation of such mutations necessitates the design of screening procedures to identify plants heterozygous for the recessive mutations. We have found most recessive, pigment-deficient Arabidopsis mutants that do not survive in soil can be grown to near maturity in tissue culture on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with sucrose (MS2 medium). Under these conditions, photosynthesis and chloroplast development are essentially dispensable, and all the energy and nutritional needs of the plant are supplied by the medium.

The hallmark phenotype for disruption of a biosynthetic pathway is the accumulation of an intermediate compound before the site of blockage. Such blockage of the carotenoid pathway can be mimicked chemically by growth of wild-type plants on MS2 medium containing the herbicide norflorazon, an inhibitor of the phytoene desaturase enzyme (Figure 1), which causes accumulation of phytoene in treated tissues (Britton, 1979). Figure 2A shows C_{18} reverse-phase HPLC analysis of the carotenoids that accumulate in wild-type Arabidopsis leaves; Figure 2B shows the analogous pigment profile for norflorazon-treated wild-type leaves. Spectral analysis of the strongly absorbing 296-nm peak at 33 min in norflorazon-treated wild-type tissue shows absorbance maxima at 276, 286, and 298 nm, which is diagnostic for phytoene (Figure 2E).

To identify mutations affecting the carotenoid pathway, >450 lines from the Feldmann T-DNA–tagged Arabidopsis population (Forsthoefel et al., 1992) were initially selected for analysis based on their segregation for pigment mutations (see Methods). Seeds from these lines were grown on MS2 medium, the segregating pigment mutants were identified, and tissue was harvested from individual plants for pigment analysis. Although numerous mutant lines with severe pigment deficiencies were identified, only two were found to be putative carotenoid biosynthetic mutants. Figures 2C and 2D show pigment analysis of albino homozygous mutants from these two mutant lines. The low absorbance at 440 nm in Figures 2B to 2D demonstrates that, like norflorazon-treated wild-type tissue, both mutants lack all chlorophylls and carotenoids that normally accumulate in wild-type tissue (compare with Figure 2A). However, unlike the wild type, both mutants contain a peak with a retention time at ~33 min that absorbs strongly at 296 nm. The retention time and absorbance of the 33-min peak in both mutants correspond to the phytoene peak in pigment extracts of norflorazon-treated wild-type tissue (Figure 2B).

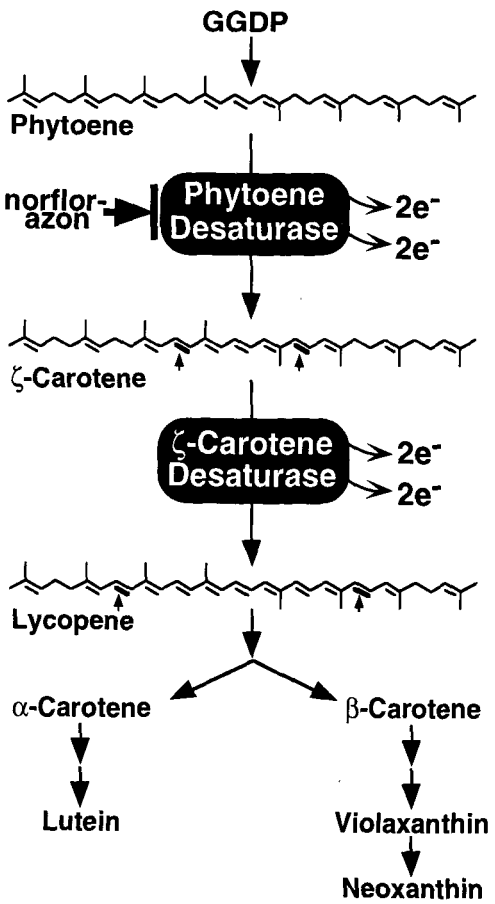

Figure 1. Early Steps of Carotenoid Biosynthesis in Plants.

Small arrows indicate the position of newly formed double bonds (shown in bold). The site of inhibition of the herbicide norflorazon is shown. Subsequent to α- and β-carotene formation, only the xanthophylls that accumulate to appreciable levels in leaf tissue are shown. For clarity, not all intermediates or enzymes are shown. GGDP, geranylgeranyl pyrophosphate; $2e^-$, two electrons.

Spectral analysis of the 33-min peak from both mutants is shown in Figures 2F and 2G and is virtually identical to the spectra of phytoene from norflorazon-treated wild-type tissue (Figure 2E) as well as the published spectra for phytoene. These results confirmed the chemical identity of the accumulating compound in both mutant lines as phytoene, so the mutations were designated *pds1* and *pds2*, because both disrupt phytoene desaturation.

Genetic Analysis of *pds1* and *pds2*

The genetic nature of both mutations was determined by analyzing seeds resulting from selfing either *pds1* or *pds2* heterozygous plants. Table 1 shows segregation analysis for both mutations. Before desiccation, F_1 seeds were scored as either green (wild type or heterozygous) or white (homozygous). A 3:1 segregation ratio was observed, indicating that both *pds1* and *pds2* are inherited as single recessive nuclear mutations. Allelism tests were performed to determine if *pds1* and *pds2* represent multiple alleles of a single locus or different loci. These tests were performed by crossing plants heterozygous for the *pds1* mutation with plants heterozygous for the *pds2* mutation, as shown in Table 1. Fifty-three embryos from several independent and reciprocal crosses were scored as above, and all embryos were found to be green, as would be expected for nonallelic mutations. These results suggest that *pds1* and *pds2* are two loci that disrupt phytoene desaturation in Arabidopsis.

Because both *pds1* and *pds2* mutants are inhibited in the desaturation of phytoene, it seemed likely that one might be a mutation in the phytoene desaturase enzyme, which had previously been mapped to chromosome 4 between *agamous* (*ag*) and *brevipedicellus* (*bp*) (Wetzel et al., 1994). To test this hypothesis, the *pds1* and *pds2* mutations were mapped relative to visible markers. The results of the mapping data are summarized in Figure 3. The *pds1* mutation was mapped to chromosome 1, ~7 centimorgans below *distorted trichomes1* (*dis1*), whereas the *pds2* mutation was mapped to the top of chromosome 3, ~7 centimorgans above *long hypocotyl2* (*hy2*) (Franzmann et al., 1995). These data establish that neither *pds1* nor *pds2* maps to the phytoene desaturase enzyme locus (designated the *PDS3* locus in Figure 3) and prove that neither is a mutation in the structural gene for phytoene desaturase. These results reveal that the products of at least three loci are required for phytoene desaturation in Arabidopsis. Although isolated from a T-DNA–tagged population, segregation for the T-DNA–encoded kanamycin resistance marker and genomic DNA gel blot analysis using the entire T-DNA insertion element as a probe indicated that neither mutation is the result of a detectable T-DNA insertion event (data not shown).

Homozygous *pds1* Mutants Can Be Rescued by Homogentisic Acid, an Intermediate in Plastoquinone and Tocopherol Biosynthesis

Previous research has suggested a role for quinones in phytoene desaturation, leading us to investigate the quinone biosynthetic pathway in the *pds1* and *pds2* mutants. The early stages of plastoquinone/tocopherol synthesis were functionally analyzed in *pds1* and *pds2* mutants by growth in the presence of two compounds intermediate in the pathway, OHPP and homogentisic acid (HGA) (Figure 4A). Wild-type and albino *pds1* or *pds2* plants were first germinated on MS2 medium and then transferred to MS2 supplemented with either OHPP or HGA. *pds2* and wild-type plants showed no visible change when grown on either OHPP or HGA, remaining albino and green, respectively (data not shown). Figure 5C shows, however, that greening occurred when *pds1* plants

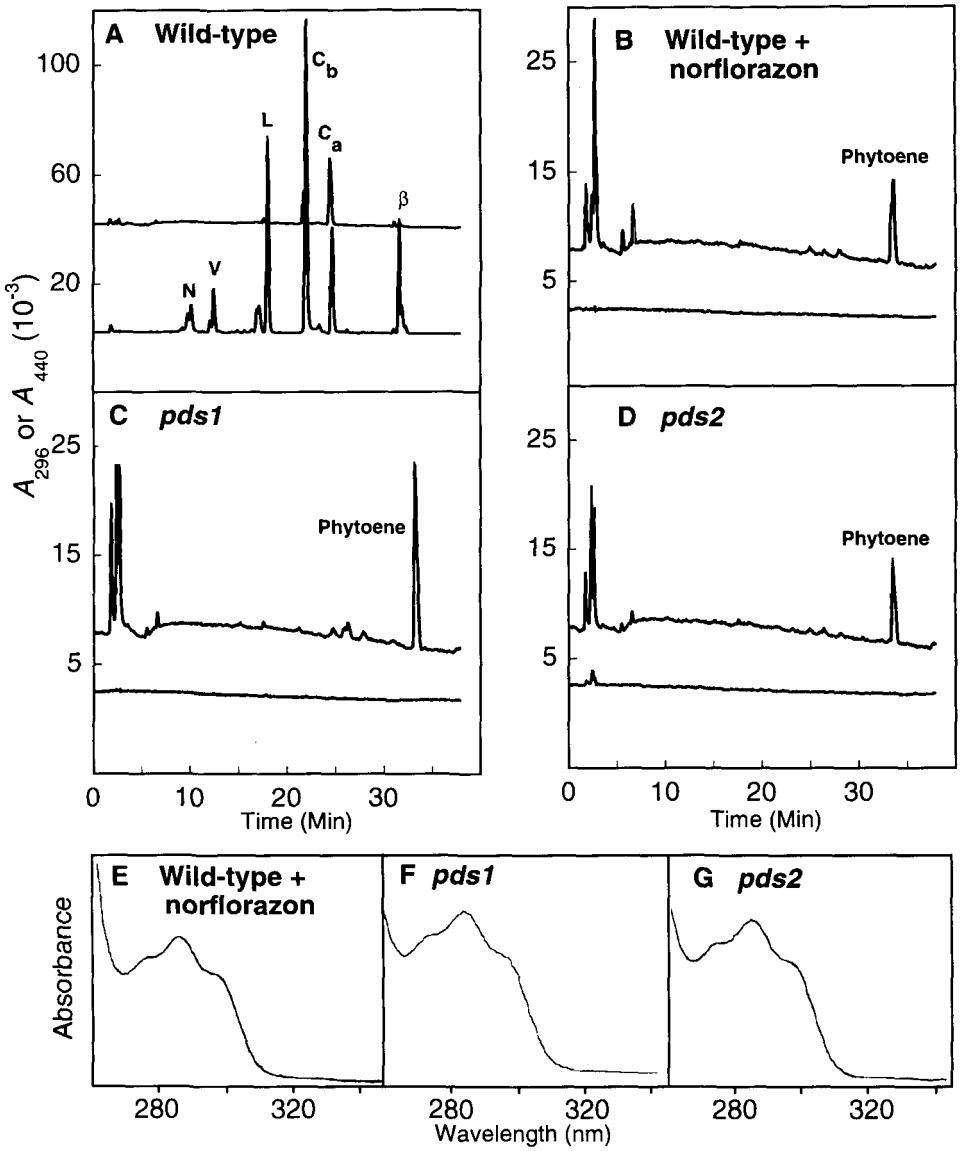

Figure 2. Pigment Analysis of Wild-Type, Norflorazon-Treated Wild-Type, *pds1*, and *pds2* Tissues.

In **(A)** to **(D)**, C_{18} HPLC separation of lipid soluble pigments from tissue culture–grown plants is shown. The top and bottom traces in each panel represent absorbance at 296 and 440 nm, respectively. Each profile represents pigments extracted from 10 mg fresh weight of tissue.
(A) Wild type. N, neoxanthin; V, violaxanthin; L, lutein; C_b, chlorophyll *b*; C_a, chlorophyll *a*; β, β-carotene.
(B) Norflorazon-treated wild-type tissue.
(C) Homozygous *pds1* mutant tissue.
(D) Homozygous *pds2* mutant tisssue.
(E) to **(G)** Spectra of the 296-nm peak at 33 min from **(B)** through **(D)**, respectively.

were transferred to medium containing HGA. *pds1* plants remained albino when transferred to medium containing OHPP, as shown in Figure 5B. For comparison, a wild-type Arabidopsis plant of the same age grown on MS2 medium is shown in Figure 5A. Figures 5D to 5F show HPLC analysis at 440 nm of carotenoids extracted from plants like those in Figures 5A to 5C.

The pigment profiles of wild-type plants and *pds1* mutants grown on OHPP are similar to the profiles of plants grown on MS2 medium shown in Figures 2A and 2C, respectively. Comparison of the pigment profiles in Figures 5D and 5F indicates that growth in the presence of HGA is able to restore qualitatively a wild-type carotenoid profile to albino, homozygous *pds1* plants. Although no chlorophyll *a* is detectable in Figure 5F,

chlorophyll *a* has been detected at varying levels in equivalent extracts of *pds1* grown in the presence of HGA. These results argue that the *pds1* mutation affects the enzyme OHPP dioxygenase, because *pds1* mutants are not altered when grown on the substrate of this enzyme, OHPP, but produce wild-type pigments when grown on the product of this enzyme, HGA (Figure 4A). The complementation of *pds1* with HGA also suggests that intermediates or end products of this pathway (plastoquinone and/or tocopherols; Figure 4) are necessary components for phytoene desaturation in plants.

HPLC Analysis Argues That *pds2* Is Also a Mutation in the Plastoquinone/Tocopherol Biosynthetic Pathway

The fact that *pds2* mutants were unaffected by growth on either OHPP or HGA suggests that this mutation affects either a stage of quinone synthesis after OHPP dioxygenase (Figure 4A) or a different pathway required for phytoene desaturation. To address these possibilities, the later stages of the plastoquinone/tocopherol pathway were analyzed in *pds1* and *pds2* tissue utilizing C_8 HPLC to resolve total lipid extracts and identify three separate classes of quinones: ubiquinone, plastoquinone, and α-tocopherol (vitamin E) (Figure 4B). Ubiquinone and plastoquinone perform analogous electron transport functions in the mitochondria and chloroplast, respectively, but are synthesized by different pathways in separate subcellular compartments (Goodwin and Mercer, 1983), making ubiquinone an ideal internal control in these analyses.

Figure 6 shows the C_8 HPLC analysis of lipid-soluble extracts from norflorazon-treated wild-type, *pds1*, and *pds2* tissue. In norflorazon-treated wild-type tissue (Figure 6A), peaks 3 and 4 were identified as ubiquinone-9 and plastoquinone-9, respectively, based on retention time (26 and 27 min), optical spectra, and mass spectra. The molecular mass of protonated plastoquinone-9 was 749.7, whereas that of protonated ubiquinone-9 was 795.8. Norflorazon-treated wild-type tissue contained a peak (peak 1) with a retention time of 13.5 min, which was identified as α-tocopherol based on the retention time of a standard. However, optical spectroscopy and mass spectrometry demonstrated that peak 1 was composed of two major components: α-tocopherol (1a) and an unidentified compound (1b). The molecular mass of protonated α-tocopherol was 431, whereas that of the unidentified compound was determined to be 412 (data not shown), clearly demonstrating the presence of two compounds in peak 1.

This quinone analysis demonstrates that the herbicide norflorazon, which specifically inhibits the phytoene desaturase enzyme, does not affect synthesis of the HGA-derived quinones, plastoquinone-9 and α-tocopherol. *pds1* and *pds2* tissues (Figures 6B and 6C) contain ubiquinone-9 (peak 3) but lack plastoquinone-9 (peak 4). In addition, although both *pds1* and *pds2* contain a peak at 13.5 min, optical spectroscopy and mass spectrometry data demonstrate that this peak in both mutants lacks α-tocopherol (1a) and is composed solely of the compound 1b (data not shown). Therefore, homozygous *pds1* and *pds2* plants accumulate ubiquinone-9 but lack both plastoquinone-9 and α-tocopherol. This is consistent with the *pds1* mutation affecting OHPP dioxygenase (Figure 4A), as suggested by the rescue of the mutation by HGA. These results also indicate that *pds2* affects a step common to the biosynthesis of both compounds, but after HGA, providing further evidence for a role for HGA-derived quinones in phytoene desaturation.

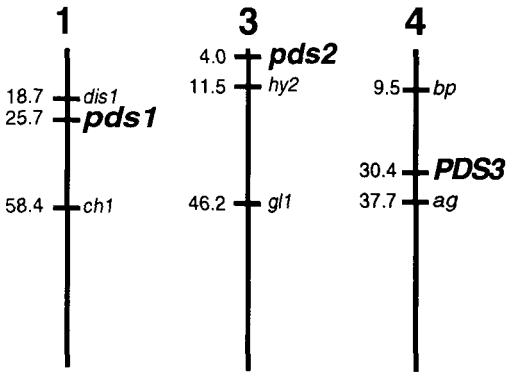

Figure 3. Physical Map Positions of *pds1*, *pds2*, and *PDS3*, the Phytoene Desaturase Gene.

The numbers 1, 3, and 4 represent three of the five linkage groups of Arabidopsis. For clarity, only the linkage groups relevant to this study are shown. This map was modified from Franzmann et al. (1995) and Wetzel et al. (1994). *ag, agamous*; *bp, brevipedicellus*; *ch1, chlorina1*; *dis1, distorted trichomes1*; *gl1, glabrous1*; *hy2, long hypocotyl2*.

Table 1. Segregation Analysis and Allelism Tests for *pds1* and *pds2*

Cross	Total Embryos	Green Embryos	White Embryos	χ^{2a}
PDS1/pds1 × *PDS1/pds1*	194	146	48	0.01[b]
PDS2/pds2 × *PDS2/pds2*	224	173	51	0.60[c]
PDS1/pds1 × *PDS2/pds2*	53	53	0	

[a] χ^2 values calculated for a 3:1 ratio.
[b] $P > 0.9$.
[c] $P > 0.3$.

DISCUSSION

pds1 and *pds2* Are Two Quinone Biosynthetic Mutants That Are Also Defective in Phytoene Desaturation

To further current understanding of the desaturation mechanisms of carotenoid biosynthesis in higher plants, we isolated

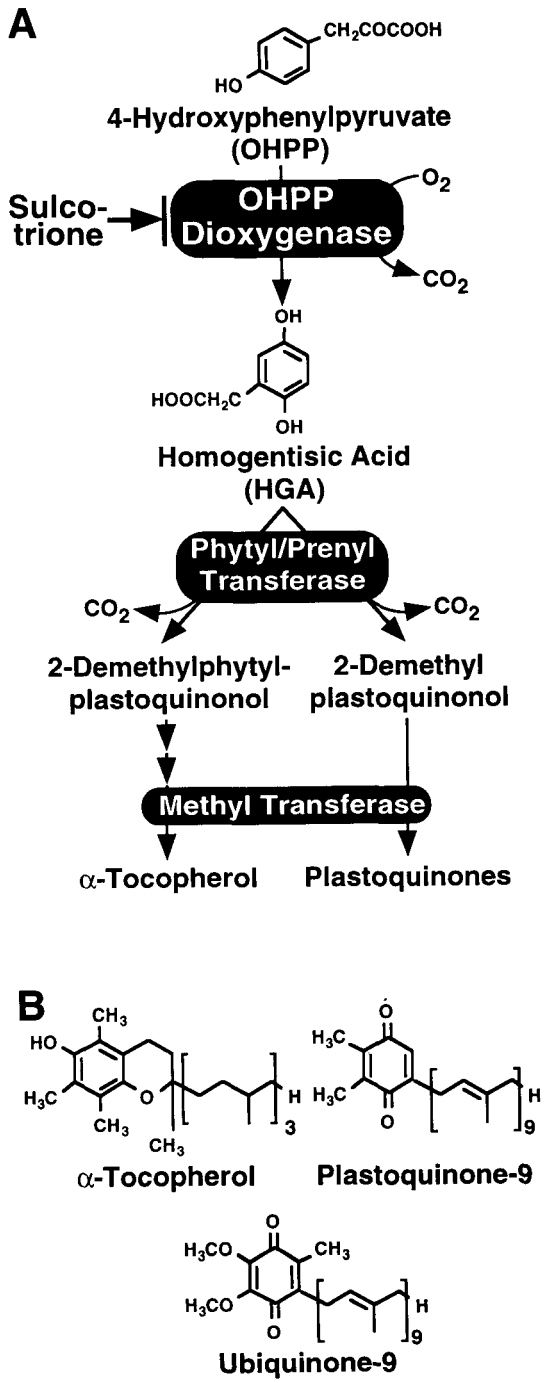

Figure 4. The Plastoquinone and α-Tocopherol Biosynthetic Pathways.

(A) Enzymes and chemical structures of selected intermediates in the pathway are shown. The site of inhibition for the herbicide sulcotrione is shown. Only one methylation takes place in the synthesis of plastoquinone. The three arrows following 2-demethylphytylplastoquinonol represent two methylation steps and a cyclization step in α-tocopherol synthesis whose exact reaction sequence is not known. For clarity, not all intermediates, structures, or enzymes are shown.
(B) Structures of three classes of quinone compounds.

and characterized two nonallelic, nuclear-encoded recessive mutations, *pds1* and *pds2*, that disrupt the desaturation of phytoene in Arabidopsis. Neither mutation maps to the phytoene desaturase locus (Figure 3), indicating that phytoene desaturation in Arabidopsis requires the products of at least three genes, *PDS1*, *PDS2*, and *PDS3*. Other evidence, in addition to these mutant studies, supports the hypothesis that phytoene desaturation in higher plants is a complex biochemical process requiring components in addition to the phytoene desaturase enzyme. For example, biochemical studies with two classes of herbicides that inhibit phytoene desaturation by different mechanisms support this hypothesis (Sandmann et al., 1989; Schulz et al., 1993). In vitro studies using isolated daffodil chromoplasts demonstrated that phytoene desaturation has an absolute requirement for oxidized artificial quinones in anaerobic environments, leading to the proposal that an electron transport chain is involved in the desaturation reaction (Mayer et al., 1990). Taken together, these findings suggest that additional plastidic components are required for phytoene desaturation and that *pds1* and *pds2* define genetically two such components. In light of these studies, we investigated the possibility that quinone biosynthesis was affected by the *pds1* and *pds2* mutations.

To determine whether either *pds* mutation affects the early steps of the plastoquinone/tocopherol pathway, chemical complementation tests were performed by incorporating the biosynthetic intermediates OHPP or HGA into growth medium. *pds1* mutants were complemented by HGA but not by OHPP (Figure 5), consistent with the hypothesis that the *pds1* mutation affects OHPP dioxygenase (Figure 7). These results also suggest that one or more end products of the plastoquinone/tocopherol pathway are required for the desaturation of phytoene. *pds2* mutants were not affected by growth on either HGA or OHPP, suggesting that the *pds2* mutation affects either a later stage of the plastoquinone/tocopherol pathway or another biosynthetic pathway also required for phytoene desaturation.

To gain additional information about the *pds1* mutation and to determine whether *pds2* also affects plastoquinone/tocopherol biosynthesis, three major classes of plant quinones were analyzed: plastoquinone, α-tocopherol, and ubiquinone. Although ubiquinone and plastoquinone are similar in structure and function (Figure 4B), they are synthesized by different pathways in mitochondria and chloroplast, respectively (Goodwin and Mercer, 1983). Norflorazon-treated wild-type, *pds1*, and *pds2* tissues all accumulated ubiquinone (Figure 6), confirming transmission electron microscopy data showing intact mitochondria in these tissues (data not shown). Norflorazon-treated wild-type plants also contained both plastoquinone and α-tocopherol, indicating that photobleached tissues arising from chemical blockage of the phytoene desaturase enzyme are still capable of synthesizing both compounds (Figure 6A). Figure 6B shows that *pds1* mutant tissue lacks both α-tocopherol and plastoquinone, consistent with a mutation affecting OHPP dioxygenase (Figure 4A). Interestingly, *pds2* mutant tissue also lacks plastoquinone and α-tocopherol (Figure 6C),

indicating that the *pds2* mutation also affects synthesis of both compounds. Therefore, we conclude that the *pds2* mutation must affect a step of the pathway after OHPP dioxygenase.

Figure 4A shows the plastoquinone/tocopherol biosynthetic pathway branching with the addition of prenyl and phytyl tails to HGA for plastoquinone and tocopherols, respectively. This reaction was proposed to be catalyzed by two separate enzymes, a prenylase/decarboxylase and a phytylase/decarboxylase (Threlfall and Whistance, 1971; Soll et al., 1985; Soll, 1987). However, our analysis of the *pds2* mutation does not support this hypothesis. Three lines of evidence argue for a single enzyme catalyzing both prenylation and phytylation coupled to decarboxylation, as shown in Figure 4A. First, biochemical complementation and end point analysis demonstrate that the biosynthetic reaction affected by the *pds2* mutation is common to both plastoquinone and tocopherol synthesis,

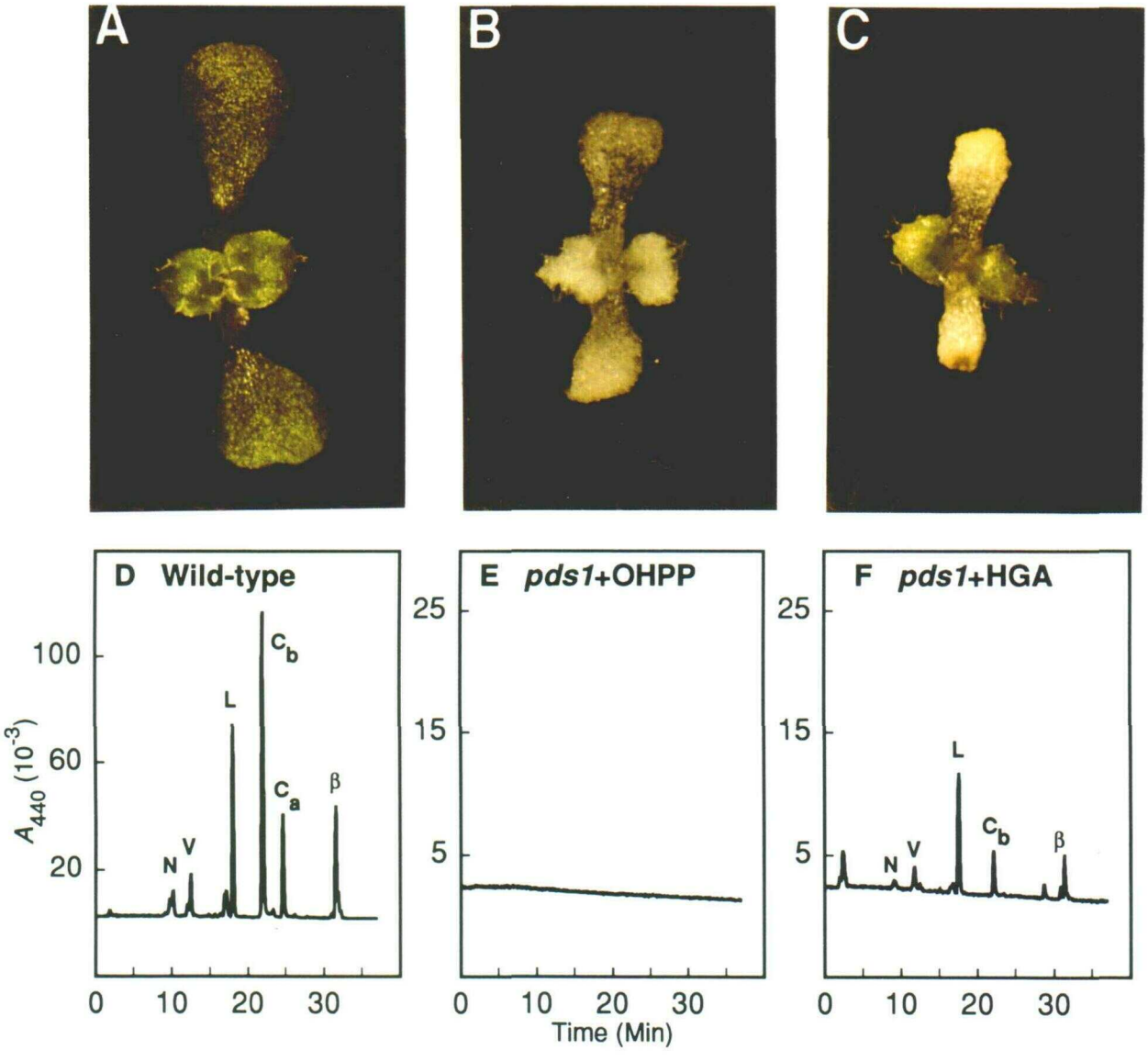

Figure 5. Complementation of *pds1* with HGA.

(A) Wild-type plants on MS2 medium.
(B) Homozygous *pds1* mutants on MS2 medium supplemented with 100 μM OHPP.
(C) Homozygous *pds1* mutants on MS2 medium supplemented with 100 μM HGA.
(D) to **(F)** C_{18} HPLC separation of lipid-soluble pigments from plants equivalent to those in **(A)** to **(C)**, respectively. Absorbance at 440 nm is shown. Each profile represents pigments extracted from 10 mg fresh weight of tissue. Abbreviations are as given in the legend to Figure 2.

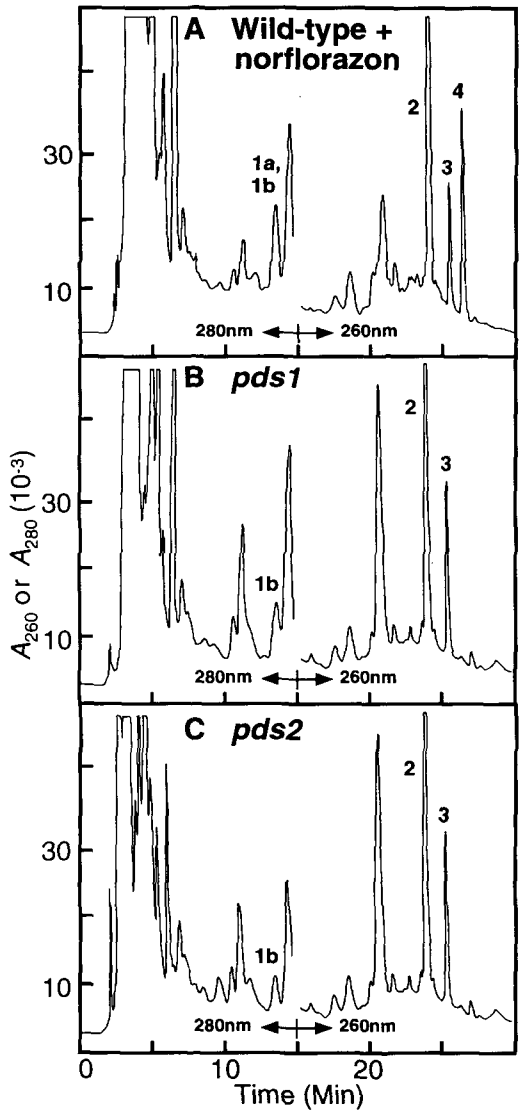

Figure 6. Quinone Analysis of Norflorazon-Treated Wild-Type, *pds1*, and *pds2* Tissue.

C_8 HPLC analysis of quinones is shown. The profiles represent lipids extracted from 500 mg fresh weight of tissue. The HPLC traces represent absorbance at 280 nm for the first 15 min and absorbance at 260 nm for the second 15 min. 1a and 1b, α-tocopherol and comigrating contaminant, respectively; 2, phytoene; 3, ubiquinone-9; 4, plastoquinone-9.
(A) Norflorazon-treated wild type.
(B) Homozygous *pds1* mutant tissue.
(C) Homozygous *pds2* mutant tissue.

after the synthesis of HGA. Second, two allelic maize mutants defective in the common methyl transferase enzyme of the quinone pathway (refer to Figure 4A) are green and viable in soil (Cook and Miles, 1992), suggesting that the *pds2* mutation affects a step before methylation. Finally, the possibility that a separate HGA decarboxylase enzyme, common to the synthesis of both plastoquinone and tocopherols, would be disrupted by *pds2* was tested by growth in the presence of the hypothetical decarboxylated intermediate of HGA, 2,5-dihydroxytoluene. Neither mutant was complemented by this compound, indicating that a decarboxylated intermediate does not exist (data not shown). These experiments suggest that a single enzyme, which is affected by the *pds2* mutation, is responsible for the concerted decarboxylation and prenylation/phytylation of HGA (Figure 7). However, the possibility that *pds2* affects a common regulator or a common subunit of separate prenylase/decarboxylase and phytylase/decarboxylase enzymes cannot be excluded; further analysis of the *pds2* mutant is required to shed light on this key step of the pathway.

Quinones and Electron Transport Chains Provide a Unifying Mechanism for Carotenoid Desaturations

The studies reported here demonstrate conclusively that plastoquinone and/or tocopherols are essential components in the phytoene desaturation reaction in higher plants. Given its well-characterized role as a lipid-soluble electron carrier, plastoquinone seems the obvious candidate as an electron carrier in phytoene desaturation. This hypothesis is supported by mutants in the green alga *Scenedesmus obliquus*, which are unable to synthesize tocopherols but still synthesize wild-type levels of carotenoids and plastoquinone (Bishop and

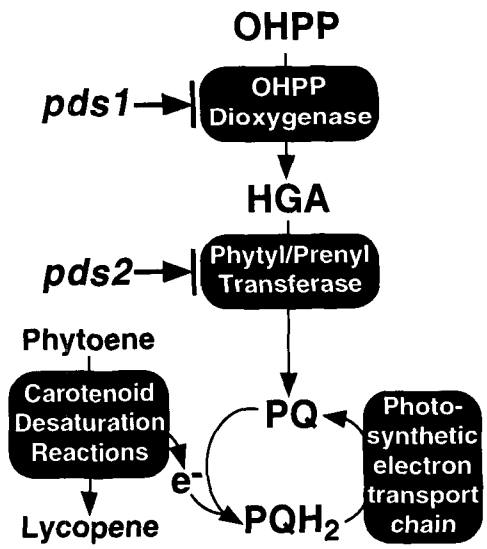

Figure 7. Model for the Involvement of Plastoquinone in Carotenoid Desaturations.

Proposed interactions of plastoquinone, carotenoid desaturation enzymes, and the photosynthetic electron transport chain. The steps proposed to be affected by the *pds1* and the *pds2* mutations are shown. Carotenoid desaturation reactions refer to those reactions carried out by phytoene desaturase and ζ-carotene desaturase, as shown in Figure 1. These enzymes require plastoquinone as an intermediate electron carrier, in this example, to the photosynthetic electron transport chain. e^-, electron; PQ, plastoquinone; PQH_2, plastoquinol.

Wong, 1974; Henry et al., 1986). These *Scenedesmus* mutants demonstrate clearly that tocopherols are not required for carotenoid synthesis, allowing us to conclude that plastoquinone is essential for phytoene desaturation in photosynthetic tissue.

It remains to be determined whether plastoquinone is oxidizing the phytoene desaturase–$FADH_2$ directly or whether other intermediate electron carriers are present between plastoquinone and the phytoene desaturase–$FADH_2$. The mechanism whereby plastoquinol is subsequently reoxidized to plastoquinone is also open to speculation; however, in green tissues, the photosynthetic electron transport chain is an obvious candidate, as shown in Figure 7. Utilizing plastoquinone as an electron acceptor would provide a high degree of flexibility in the system that reoxidizes plastoquinol, thereby allowing plastoquinone to act as a "universal adapter molecule" for carotenoid desaturation reactions in a variety of plant tissues. Thus, in tissues in which the photosynthetic electron transport chain may not be functioning (e.g., fruits and flowers), plastoquinol could be oxidized by a tissue-specific redox component, such as that proposed for daffodil flowers, an NADPH-dependent plastoquinone oxidoreductase (Mayer et al., 1992). Finally, given the similarity of the phytoene and ζ-carotene desaturation reactions (Figure 1), we propose that plastoquinone is also an electron carrier in the desaturation reactions mediated by the yet-to-be-isolated ζ-carotene desaturase in higher plants.

Molecular and biochemical characterization of phytoene desaturase from a variety of organisms suggests that this enzyme has arisen via independent evolution in oxygenic photosynthetic organisms and anoxygenic photosynthetic organisms (reviewed in Sandmann, 1994). Despite the dissimilarities of the two enzyme classes, our data suggest a common reaction mechanism. Organisms containing the *pds*-type enzyme utilize plastoquinone as an electron carrier. Because organisms containing the *crtI*-type phytoene desaturase lack plastoquinone (Gabellini et al., 1982), we hypothesize that this latter class of desaturases utilizes the closely related compound ubiquinone as its electron carrier (see Figure 4B), replacing plastoquinone in the model shown in Figure 7. A ubiquitous function for quinones as electron carriers for desaturation reactions is supported by the observation that, despite their sequence divergence and independent evolution, *pds*-type enzymes are active when expressed in *Escherichia coli*, which lacks plastoquinone but contains ubiquinone and the respiratory electron transport chain; likewise, *crtI*-type enzymes are active when expressed in plants and targeted to chloroplasts, which lack ubiquinone but contain plastoquinone and the photosynthetic electron transport chain (Fraser et al., 1992; Misawa et al., 1993). These data support our thesis that quinones function as universal adapter molecules for carotenoid desaturation reactions, in this case allowing different electron transport chains (i.e., photosynthetic or respiratory) to be utilized by carotenoid desaturases expressed in heterologous organisms.

In conclusion, we identified two novel carotenoid biosynthetic mutations in Arabidopsis, *pds1* and *pds2*, that result in the accumulation of phytoene as the sole carotenoid, although neither is a mutation of the gene encoding the phytoene desaturase enzyme. We demonstrated that the *pds1* and *pds2* mutations affect different steps of tocopherol/plastoquinone biosynthesis and in this manner disrupt phytoene desaturation. In combination with previously identified tocopherol-deficient mutants that synthesize both carotenoids and plastoquinone, the *pds1* and *pds2* mutants demonstrate conclusively that plastoquinone is an essential component in carotenoid desaturations in higher plants. These studies also suggest a mechanistic link between carotenoid desaturation reactions, plastoquinone, and the photosynthetic electron transport chain in green tissue. Additional studies of these and other phytoene-accumulating mutants in higher plants will shed light on the integration of carotenoid biosynthesis and electron transport components in photosynthetic and nonphotosynthetic tissues.

METHODS

Mutant Screen

The Feldmann T-DNA–tagged population of *Arabidopsis thaliana* (Forsthoefel et al., 1992) was screened for mutants segregating for pigment defects (albino, yellow, yellow-green, or pale green) by scoring T_3 seedlings germinated on agar-solidified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2% sucrose and 0.01% Nitsch and Nitsch vitamins (Sigma; MS2 medium). Additionally, mature green siliques of T_3 plants were dissected to identify pigment mutants in T_4 progeny. Depending on the method of identification, either T_3 or T_4 seed from identified lines were surface sterilized and grown on MS2 medium for subsequent HPLC pigment analysis.

Plant Tissue

Plant tissue for carotenoid extraction was grown on sterile MS2 medium at 22°C under constant light of ~30 μE m^{-2} sec^{-1}. The tissue was harvested, frozen in liquid nitrogen, and stored at −80°C until carotenoid extractions were performed. Plants grown in the presence of the herbicide norflorazon were grown as above on MS2 plates containing 10 mM norflorazon. 4-Hydroxyphenylpyruvate (OHPP; Sigma) was resuspended in water at a concentration of 100 mM and then added to MS2 medium at a final working concentration of 100 mM. Homogenistic acid (HGA; Sigma) was resuspended in 30% acetone at a concentration of 100 mM and then added to MS2 medium at a final working concentration of 100 μM.

Carotenoid Analysis

Plant tissue was placed in a microcentrifuge tube and ground with a micropestle in 200 mL of 80% acetone. Ethyl acetate (120 mL) was added, and the mixture was vortexed. Water (140 mL) was added, and the mixture was centrifuged for 5 min. The carotenoid containing upper phase was then transferred to a fresh tube and vacuum dried in a centrifugal evaporator (model RC1010; Jouan, Inc., Winchester, VA). The dried extract was resuspended in ethyl acetate at a concentration

of 0.5 mg fresh weight of tissue per microliter and either analyzed immediately by HPLC or stored at −80°C under nitrogen.

Carotenoids were separated by reverse-phase HPLC analysis on a Spherisorb ODS2 5-micron C_{18} column, 25 cm in length (Phase Separations Limited, Norwalk, CT) using a 45-min gradient of ethyl acetate (0 to 100%) in acetonitrile-water-triethylamine (9:1:0.01 [v/v]), at a flow rate of 1 mL per min (Goodwin and Britton, 1988). Carotenoids were identified by retention time relative to known standards, with detection at both 296 and 440 nm. When needed, absorption spectra for individual peaks were obtained with a photodiode array detector (model 1040A; Hewlett Packard, Palo Alto, CA) and compared with published spectra or available standards.

Quinone Analysis

Quinones were extracted from tissue using a method modified from Bligh and Dyer (1959). Frozen plant tissue was ground in a mortar with three volumes of chloroform and six volumes of methanol and transferred to a test tube. Water and additional chloroform were added until a biphasic mixture was obtained. The quinone-containing chloroform phase was then collected. To increase yields, the aqueous phase was back-extracted with chloroform; the two chloroform phases were pooled and then filtered through Whatman No. 3 filter paper. The resulting filtrate was dried under a constant stream of nitrogen. Once dried, the pellet was resuspended in methanol at a concentration of 10 mg fresh weight per microliter and immediately analyzed by HPLC. Quinones were resolved by reverse-phase HPLC analysis on a LiChrosorb RP-8 5-micron column, 25 cm in length (Alltech, San Jose, CA), using an isocratic solvent of 10% H_2O in methanol for the first 14 min, at which time the solvent was switched to 100% methanol for the remainder of the run (modified from Lichtenthaler, 1984). The flow rate was 1 mL per min for the duration.

Peaks were identified based on the retention time of known standards, with detection at 280 nm for α-tocopherol and 260 nm for plastoquinone and ubiquinone, as well as by absorption spectra from a Hewlett Packard 1040A photodiode array detector. When needed, fractions represented by individual chromatographic peaks were collected and submitted to the Southwest Environmental Health Science Center's Analytical Core Laboratory for mass spectral analysis. Results were obtained using a tandem mass spectrometer (model TSQ7000; Finnigan Corp., San Jose, CA) equipped with an atmospheric pressure chemical ionization source operated in the positive ion mode. The instrument was set to unit resolution, and the samples were introduced into the source in a 0.3 mL per min methanol stream and ionized using a 5-kV discharge.

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