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# Complementation of the Arabidopsis *pds1* Mutation with the Gene Encoding *p*-Hydroxyphenylpyruvate Dioxygenase

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**Plastoquinone and tocopherols are the two major quinone compounds in higher plant chloroplasts and are synthesized by a common pathway. In previous studies we characterized two loci in Arabidopsis defining key steps of this biosynthetic pathway. Mutation of the *PDS1* locus disrupts the activity of *p*-hydroxyphenylpyruvate dioxygenase (HPPDase), the first committed step in the synthesis of both plastoquinone and tocopherols in plants. Although plants homozygous for the *pds1* mutation could be rescued by growth in the presence of homogentisic acid, the product of HPPDase, we were unable to determine if the mutation directly or indirectly disrupted HPPDase activity. This paper reports the isolation of a cDNA, pHPPD, encoding Arabidopsis HPPDase and its functional characterization by expression in both plants and *Escherichia coli*. pHPPD encodes a 50-kD polypeptide with homology to previously identified HPPDases, including 37 highly conserved amino acid residues clustered in the carboxyl region of the protein. Expression of pHPPD in *E. coli* catalyzes the accumulation of homogentisic acid, indicating that it encodes a functional HPPDase enzyme. Mapping of pHPPD and co-segregation analysis of the *pds1* mutation and the HPPD gene indicate tight linkage. Constitutive expression of pHPPD in a *pds1* mutant background complements this mutation. Finally, comparison of the HPPD genomic sequences from wild type and *pds1* identified a 17-bp deletion in the *pds1* allele that results in deletion of the carboxyterminal 26 amino acids of the HPPDase protein. Together, these data conclusively demonstrate that *pds1* is a mutation in the HPPDase structural gene.**

Plastoquinone and tocopherols are the two major classes of chloroplastic, lipid-soluble quinone compounds in higher plants. Plastoquinone is best known for its role as an electron carrier between PSII and the Cyt $b_6/f$ complex, and to a lesser extent as an electron carrier for NAD(P)H-plastoquinone oxidoreductases (Berger et al., 1993). In mammals, which cannot synthesize plastoquinone or tocopherols, $\alpha$-tocopherol (vitamin E) is an essential dietary component (Mason, 1980) and has a well-documented role as a membrane-associated free radical scavenger (for review, see Liebler, 1993). In plants, tocopherols are also presumed to function as membrane-associated antioxidants and as structural components of membranes, although evidence supporting these roles is limited (for review, see Hess, 1993).

Figure 1 shows the pathway for plastoquinone and tocopherol biosynthesis in plants. The first step of this pathway, common to the synthesis of both plastoquinone and tocopherol, is the formation of HGA from HPP by the enzyme HPPDase (EC 1.13.11.27). HPPDase catalyzes a complex, irreversible reaction involving the introduction of two molecules of oxygen, and decarboxylation and rearrangement of the side chain (Fig. 1). HPPDase is generally present at low levels in plant tissues and has only recently been purified to homogeneity from a plant source (Garcia et al., 1997).

Although mammals and nonphotosynthetic bacteria cannot synthesize plastoquinone or tocopherols, they do nonetheless contain HPPDase enzymatic activity. This activity is often present at very high levels and is involved in Phe and Tyr degradation. HPPDase has been purified from several mammalian and bacterial sources (Wada et al., 1975; Lindstedt et al., 1977; Roche et al., 1982; Endo et al., 1992), and in all cases the active enzyme was found to be a homodimer or, less commonly, a homotetramer, with subunits of approximately 40 to 48 kD. As a result of the central role HPPDase serves in aromatic amino acid metabolism in mammals and plastidic quinone synthesis in plants, a class of competitive inhibitors of HPPDases collectively known as triketones has been developed and used for a variety of clinical and agricultural purposes (Lindstedt et al., 1992; Schultz et al., 1993; Secor, 1994). In humans, the triketone 2-(2-nitro-4-trifluromethylbenzoyl)-1,3-cyclohexanedione and related compounds are used as an alternative to liver transplantation in patients with the otherwise fatal hereditary disorder tyrosinemia type I. This disorder results from a deficiency in the last enzyme of Tyr catabolism (Lindstedt et al., 1992; Gibbs et al., 1993) and 2-(2-nitro-4-trifluromethylbenzoyl)-1,3-cyclohexanedione treatment inhibits liver HPPDase activity, blocking formation of HGA and its subsequent breakdown to the toxic intermediates succinylacetoacetate and succinylacetone. In plants, triketones such as sulcotrione (2-[4-chloro-2-nitrobenzoyl]-5,5-dimethylcyclohexane-1,3-dione) are effective bleaching herbicides. Their mode of action arises from a direct inhibition of plastoquinone and tocopherol synthesis and an indirect inhibition of carotenoid desatu-

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Abbreviations: HGA, homogentisic acid; HPP, *p*-hydroxyphenylpyruvate; HPPDase, *p*-hydroxyphenylpyruvate dioxygenase.

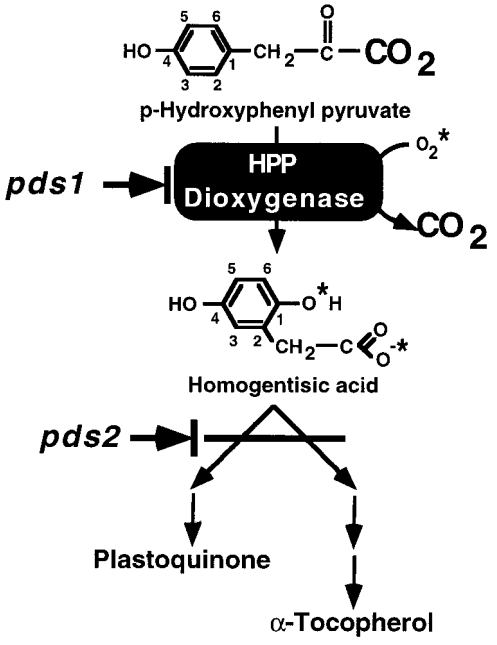


**Figure 1.** The plastoquinone and α-tocopherol biosynthetic pathway in higher plants. For clarity, not all biosynthetic steps are shown and only the HPPDase reaction is shown in detail. The $CO_2$ lost and molecular oxygen introduced by HPPDase are indicated with a larger font and asterisks, respectively. The conjugated rings of HPP and HGA are numbered to indicate rearrangement of the side chain. The locations of the *pds1* and *pds2* mutations in the pathway are indicated.

ration (Mayonado et al., 1989; Schultz et al., 1993; Secor, 1994). The latter results in accumulation of the carotenoid biosynthetic intermediate phytoene and photooxidation of the plastid.

A genetic basis for the effects of triketones on plant carotenoid synthesis was suggested by the identification of two Arabidopsis mutations that disrupt *p*hytoene *d*esaturation (*pds* mutations) but do not map to the phytoene desaturase enzyme locus (Norris et al., 1995). Previous work demonstrated that mutations in either the *PDS1* or *PDS2* loci resulted in plants deficient in tocopherol and plastoquinone biosynthesis and, as a secondary effect of this deficiency, disruption of carotenoid desaturation (Norris et al., 1995). The *pds* mutations thus provide genetic evidence that plastoquinone is an essential component for carotenoid biosynthesis in plants and provide insight into plastidic quinone synthesis and function. The biochemical basis of the *pds1* mutation was hypothesized to be a disruption in the HPPDase structural gene because the mutant phenotype could be biochemically complemented by growth on medium supplemented with the product (HGA) but not the substrate (HPP) of the HPPDase enzyme. However, despite this compelling evidence, it could not be determined whether the *pds1* mutation directly or indirectly affected the HPPDase enzyme (Norris et al., 1995).

To functionally test the hypothesis that the Arabidopsis *pds1* mutation is the result of a lesion in the structural HPPDase gene, it is necessary to isolate and functionally characterize Arabidopsis HPPDase cDNAs and the corresponding wild-type and mutant HPPDase alleles. In this paper we report the isolation and characterization of a cDNA encoding HPPDase from Arabidopsis and demonstrate the activity of the protein when expressed in *Escherichia coli*. Linkage of the HPPD gene and the *pds1* mutant was demonstrated by both mapping and co-segregation analysis. Sequence analysis of the wild-type and mutant HPPDase genomic sequences identified a small deletion that produces a truncated protein in the mutant. Finally, we show functional complementation of the *pds1* mutant phenotype when the HPPDase cDNA is constitutively expressed. Combined, these data conclusively demonstrate that *pds1* is a mutation of the HPPDase gene.

## MATERIALS AND METHODS

### cDNA and Genomic DNA Isolation and DNA Sequence Analysis

A BLAST search (Altschul et al., 1990) of plant DNA sequence databases with various bacterial and mammalian HPPDase sequences identified a truncated Arabidopsis cDNA (accession no. T20952) with homology to the carboxy terminus of human HPPDase (accession no. X72389). The 460-bp insert from this expressed sequence tag was used as a probe to screen $4 \times 10^5$ plaques of the Arabidopsis PRL2 library (Newman et al., 1994). Eighty-one individual plaques were collected for further evaluation and detailed characterization was performed on 32 isolates, of which four full-length clones were sequenced. Isolate 18 was chosen for further studies and renamed pHPPD. A pHPPD probe was made by labeling a *Sal*I/*Not*I fragment of pHPPD using the Random Prime kit (Boehringer Mannheim).

Genomic DNAs for use as substrates for PCR were isolated from wild-type and *pds1* genotypes (both are ecotype Wassilewskija [Ws]) by the modified minipreparation method (DellaPorta et al., 1983). Two sets of primers were used to amplify genomic copies of the HPPDase gene from wild-type Ws tissue: SN418T7+10 (5′-CGTCCGAGTTTTAGCAGAGTTGG-3′) and SN418MF+11 (5′-AGAGCCAGATGTTGTAGCCC-3′) for the first 1000 bp of the gene, and SN418T7+4 (5′-CCAATTCGCAGAGTTC-3′) and SN418MF+12 (5′-CGTTTTAAATGAGATGTTGTATAAC-3′) for the last 700 bp of the gene. Similarly, for the *pds1* mutant, two sets of primers were used: SN418T7+10 and SN418MF+1b (5′-CAGATGTTGTAGCCCT-3′) for the first 1000 bp of the gene, and SN418T7+4 and SN418MF+12 for the last 700 bp of the gene. In both cases, the two amplified genomic fragments overlap by about 200 bp.

Three independent sets of PCR reactions were performed for each fragment amplification. PCR products were analyzed by gel electrophoresis, and equal concentrations of each were pooled, purified, and used directly for sequencing. DNA sequencing was performed using a dye deoxy terminator cycle sequencing kit (Applied Biosystems) and an automated DNA sequencer (model 310, Applied Biosystems). DNA-sequence analysis was done using both DNAStar and MacVector (International Biotechnologies, Inc., New Haven, CT).

### Protein Overexpression

For cloning purposes a *Nco*I site was introduced 5′ of the ATG start codon by changing the A at position −1 to a C using PCR-based mutagenesis with the two oligonucleotides 5′-TGTAAAACGACGGCCAGT-3′ and 5′-GTTGGTGAAATCCATGGGCCACCAAAACGC-3′. The amplified product was ligated into the pCRII vector (Invitrogen, San Diego, CA), generating clone SN507. A 1.49-kb *Nco*I/*Bam*HI fragment from SN507 was ligated into the pET15b vector (Novagen, Madison, WI), generating pET-HPPD. pET15b and pET-HPPD were transformed into *Escherichia coli* cell line BL21(DE3) (Novagen) via electroporation. HPLC analysis of bacterial cultures for the presence of HGA was performed according to published procedures (Denoya et al., 1994). HGA was identified in extracts based on comparison of retention time and spectra to a HGA (Sigma) standard with a Hewlett-Packard series 1100 chromatograph and photodiode array detector.

### Linkage Analysis

Co-segregation of the *pds1* and HPPDase loci was determined by restriction fragment-length polymorphism linkage analysis using pHPPD as probe. $F_2$ progeny heterozygous for the *pds1* mutation were selected from a cross between *PDS1/pds1* (ecotype Ws) and *PDS1/PDS1* (ecotype Columbia [Col]). Digestion of Ws and Col genomic DNA with *Nco*I gave a restriction fragment-length polymorphism for the pHPPD probe. Genomic DNA for co-segregation analysis was isolated from $F_2$ progeny by the modified minipreparation method (DellaPorta et al., 1983). The digested DNA was separated on a 0.6% agarose gel and transferred to a nylon membrane (Micron Separations, Westborough, MA). The blots were hybridized with the pHPPD probe and washed two times at room temperature for 15 min with 2× SSC, 0.1% SDS and two times at 55°C for 25 min in 1× SSC, 0.1% SDS.

### Plant Transformation

Clone SN500 was generated by subcloning a 1.5-kb *Kpn*I/*Hin*dIII fragment containing the complete coding region of pHPPD into the plant-transformation shuttle vector pART7 (Gleave, 1992). After partial digestion of SN500 with *Not*I, a 4.4-kb fragment containing the cauliflower mosaic virus promoter, pHPPD coding sequences, and an OCS terminator was isolated and ligated into the binary plant-transformation shuttle vector pART27 (Gleave, 1992), generating clone SN506. SN506 was electroporated into *Agrobacterium tumefaciens* strain C58 and used to transform wild-type Arabidopsis (ecotype Ws) via vacuum infiltration (Bent et al., 1994). Seed was collected from individual $T_1$ plants, surface sterilized, and plated on MS2 medium (Norris et al., 1995) with 100 mg/L carbenicillin, 60 mg/L kanamycin, and 10 mg/L benomyl. Kanamycin-resistant $T_2$ seedlings were transferred to soil and grown to maturity, and $T_3$ seed was harvested. For complementation analysis, kanamycin-resistant $T_2$ plants were crossed with *PDS1/pds1* heterozygotes. The resulting $F_1$ seeds were surface sterilized and plated on MS2 medium with 60 mg/L kanamycin. Kanamycin-resistant $F_1$ seedlings were transferred to soil and grown as described above. Developing $F_2$ seeds in siliques of mature $F_1$ plants were scored for the homozygous albino mutant *pds1* phenotype as described previously (Norris et al., 1995). The $F_2$ seeds were also collected at maturity, surface sterilized, and plated on MS2 medium with and without 60 mg/L kanamycin and then scored for both kanamycin resistance and the *pds1* mutant phenotype.

## RESULTS

### Isolation and Characterization of an Arabidopsis HPPDase cDNA

Genes and cDNAs encoding HPPDase have been identified from several mammalian, fungal, bacterial, and plant sources (Gershwin et al., 1987; Endo et al., 1992, 1995; Hummel et al., 1992; Ruetschi et al., 1993; Coon et al., 1994; Denoya et al., 1994; Wilson et al., 1994; Wintermeyer et al., 1994; Kaneko et al., 1995; Wyckoff et al., 1995; Garcia et al., 1997) and show between 25% and 95% identity at the amino acid level. A computer search of the plant DNA databases, including 20,000 random Arabidopsis cDNAs (Newman et al., 1994), was conducted using human and bacterial HPPDase sequences as the query. This search identified a 460-bp truncated Arabidopsis cDNA (single-underlined DNA sequence in Fig. 2) with significant homology to the carboxy terminus of previously identified HPPDases. This partial cDNA was used as a probe to isolate a full-length cDNA that was named pHPPD. The first ATG of pHPPD begins an open-reading frame encoding a 50-kD protein of 445 amino acids (Fig. 2). The putative Arabidopsis HPPDase protein has from 17% to 27% amino acid identity with bacterial, fungal, and animal HPPDases and between 58% and 70% amino acid identity with two other plant HPPDases. The estimated 50-kD size of the Arabidopsis HPPDase protein closely approximates that reported for other HPPDases, which range from 40 to 48 kD.

### Functional Analysis of the Arabidopsis HPPDase Protein

The protein-sequence homology of the putative Arabidopsis HPPDase to other HPPDases suggested that it encodes an HPPDase enzyme. To test this hypothesis, pHPPD was overexpressed in *E. coli* and functionally analyzed. *E. coli* harboring the pET-HPPD construct developed a dark-brown color, whereas cultures containing the empty pET15b vector did not (data not shown). A similar dark-brown coloration was reported when the gene encoding HPPDase from *Streptomyces avermitilis* was expressed in *E. coli* (Denoya et al., 1994). This brown coloration is caused by the accumulation of ochronotic pigment, which forms upon the oxidative polymerization of HGA. To verify that the brown coloration in *E. coli* expressing pET-HPPD was the result of plasmid-mediated HGA production, cell-free supernatants from *E. coli* cultures containing the empty pET15b vector and pET-HPPD were analyzed by HPLC for the presence of HGA (Fig. 3). A HGA standard eluted at 7.9

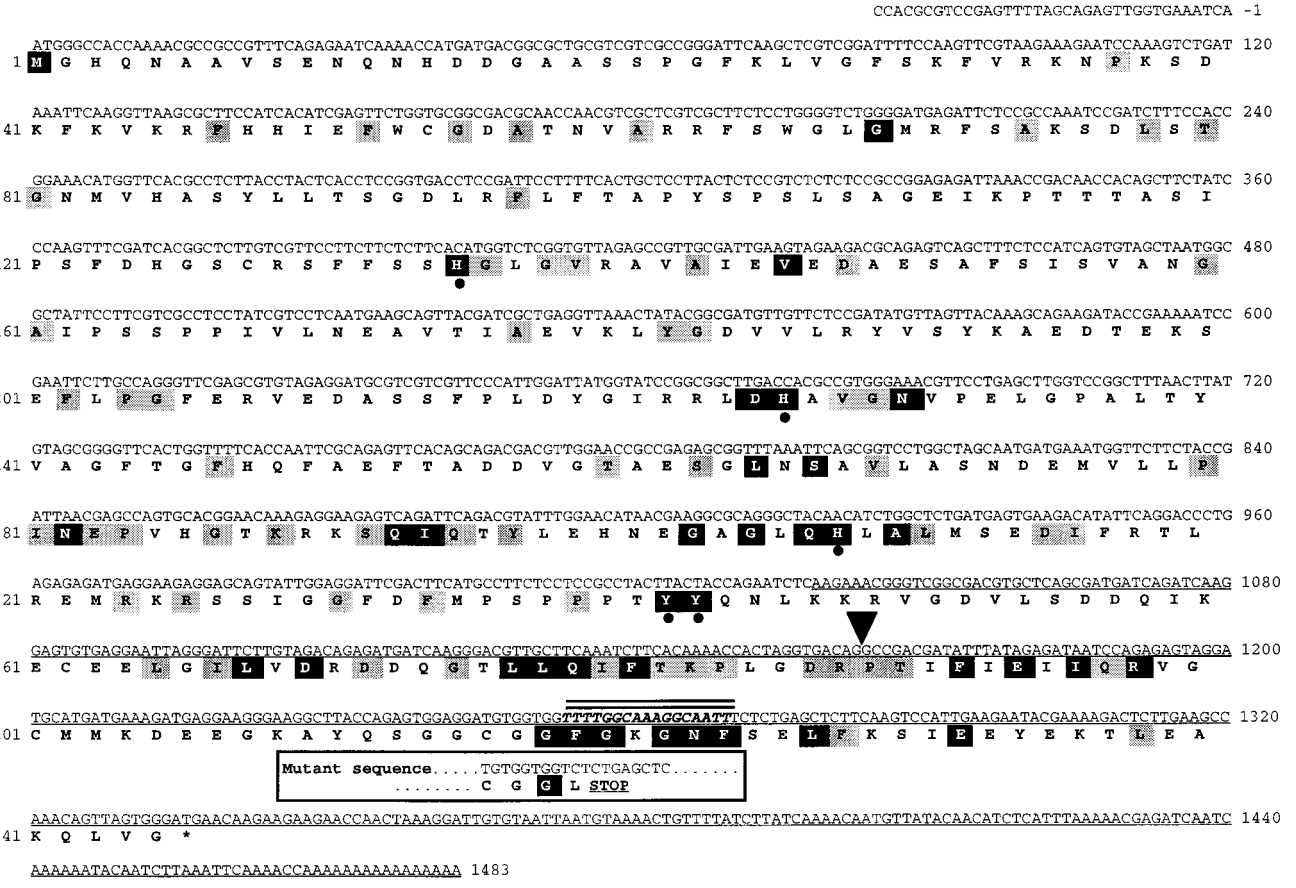


**Figure 2.** Nucleotide and deduced amino acid sequence of the Arabidopsis HPPDase cDNA pHPPD. The protein sequence is shown in boldface underneath the nucleotide sequence (accession no. AF000228). The nucleotide sequence of the originally identified, truncated expressed sequence tag (accession no. T20952) is indicated by a single underline. Alignments were performed to13 other HPPDase proteins (accession nos. AJ000693, D64004, L38493, U11864, U87257, S69666, M59289, M59429, Z50016, X72389, D29987, M18405, and D13390). Arabidopsis HPPDase amino acid residues showing identity in 9 of the other 13 HPPDase proteins are indicated with shaded boxes. Amino acid residues identical in all 14 HPPDase sequences are denoted with black boxes. The five conserved Tyr and His residues postulated to form the HPPDase ferric iron center are indicated by filled dots. The location of the single 107-bp intron in the HPPDase genomic sequences of Ws and *pds1* is denoted by an inverted, filled triangle. The 17-bp deletion in the HPPDase gene in *pds1* is denoted by a boldface, italic DNA sequence and two overhead lines. The Ws and *pds1* HPPDase gene and protein sequences are identical up to the deletion. The consequence of this mutation at the protein level is indicated in the box below the deletion.

min and had the spectra and absorbance maximum (291 nm) shown in Figure 3B. The pET-HPPD culture filtrate had a prominent peak that co-migrated with the HGA standard (Fig. 3A) and had a spectrum and absorbance maximum that were identical to those of the HGA standard (Fig. 3B). The pET15b control culture lacked a peak at 7.9 min and had a minor peak at 7.7 min, with a spectrum and absorbance maxima (271, 280, and 287 nm) that indicated that it was not HGA (Fig. 3B). These results indicate that Arabidopsis pHPPD encodes a functional HPPDase enzyme.

### Mapping, Molecular Complementation, and Genomic Sequence Analysis

In previous work we demonstrated that the biochemical basis of the Arabidopsis *pds1* mutation is an inability to convert HPP to HGA (Fig. 1; Norris et al., 1995). The *PDS1* gene product could therefore be the HPPDase enzyme, a regulator of HPPDase expression or activity, or a cofactor required for HPPDase activity. Three complementary approaches were undertaken to determine whether the gene identified by the *pds1* mutation encodes HPPDase: cosegregation of the *pds1* mutation and HPPD gene, functional complementation of the *pds1* mutant with the wild-type pHPPD cDNA, and DNA-sequence analysis of the wild-type and mutant HPPD alleles.

The *pds1* mutation was previously mapped to chromosome 1 between *distorted1* and *chlorina1* (Norris et al., 1995). Recombinant inbred lines (Lister and Dean, 1993) were used to determine the chromosomal location of the HPPDase gene, which was localized in the region of *PDS1* on chromosome 1 (data not shown). For finer mapping, segregation analysis of the *pds1* mutation and a restriction fragment-length polymorphism for the HPPDase gene

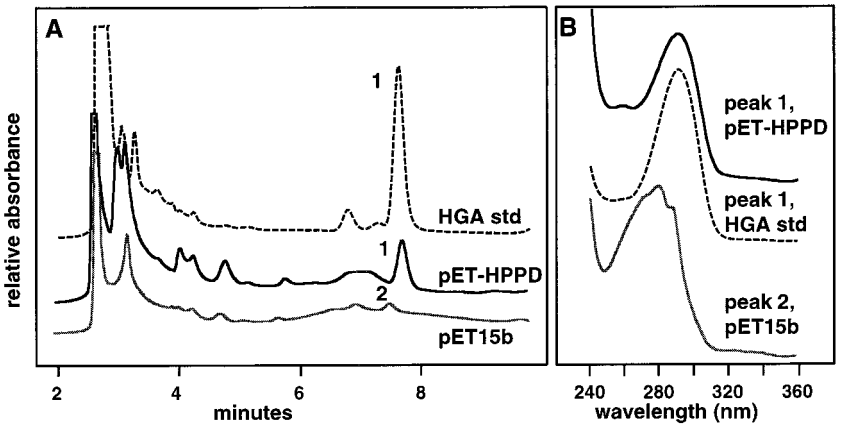


**Figure 3.** Expression of Arabidopsis HPPDase cDNA in *E. coli*. A, HPLC analysis of a HGA standard in Luria-Bertani broth is shown in the top plot. The middle and bottom plots are cell-free extracts from cultures of *E. coli* harboring the pET-HPPD construct and the pET15b construct, respectively. B, Absorption spectra of peaks 1 and 2 from A. Peak 1, HGA standard and co-migrating peak in medium of pET-HPPD; peak 2, unidentified compound in pET15b.

showed no recombinations in 38 *PDS1/pds1* lines, indicating that the two were linked within 4 centimorgans (data not shown). Together, these data indicate that the *PDS1* locus and HPPDase gene are linked in the Arabidopsis genome.

Molecular complementation of the *pds1* mutation with the Arabidopsis pHPPD cDNA was undertaken to determine if the *pds1* mutant could be rescued by constitutive overexpression of the wild-type HPPDase protein. A transcriptional fusion of the cauliflower mosaic virus 35S promoter and the full-length pHPPD cDNA in the sense orientation was used to transform wild-type Arabidopsis (Ws) plants. Three independent transgenic lines constitutively overexpressing HPPDase were selected and crossed with *PDS1/pds1* heterozygotes. Fifty percent of the resulting kanamycin-resistant $F_1$ progeny from these crosses were also heterozygous for the *pds1* mutation. These kanamycin-resistant, *pds1* heterozygous $F_1$ plants were then selfed, and segregation of their $F_2$ progeny for both kanamycin resistance and the *pds1* phenotype was determined (Table I). $\chi^2$ analysis shows that the ratio of green to white embryos in each line is statistically significant for a 15:1 ratio (Table I), indicating that the *pds1* mutant phenotype was complemented by the presence of the overexpressed pHPPD cDNA in all plant lines analyzed. Loss of the transgene should restore a 3:1 green:white ratio to such plants. This hypothesis was verified by analyzing the $F_1$ plants that were 100% kanamycin sensitive; one-half of which contained $F_2$ progeny segregating 100% green (*PDS1/PDS1*) and half of which segregated 3:1 green:white (*PDS1/pds1*) (data not shown). These data demonstrate that overexpression of a wild-type HPPDase protein in the *pds1* mutant background complements the mutation and suggest that the molecular basis of the *pds1* mutation is a disruption in the HPPDase gene.

To determine the molecular basis of the *pds1* mutation, the HPPDase genomic DNA sequences from wild-type Ws Arabidopsis (accession no. AF060481) and *pds1* mutant tissues were determined by direct sequencing of PCR-amplified products. Both HPPDase genomic sequences contain a single 107-bp intron of identical sequence between positions 1162 and 1163 of the HPPD cDNA sequence in Figure 2. The coding frames of the wild-type and *pds1* HPPDase alleles were completely identical with the exception of a 17-bp deletion (5′-TTTTGGCAAAGGCAATT-3′) in the *pds1* HPPD gene from nucleotides 1254 to 1270 of the wild-type cDNA sequence in Figure 2. This deletion causes a frame shift and substitution of a Leu for the conserved Phe at position 419, followed immediately by a stop codon (Fig. 2). This stop codon results in the deletion of the remaining 26 amino acids from the carboxy-terminal end of the protein. This result defines the molecular basis of the *pds1* mutation as a mutation in the structural HPPD gene.

## DISCUSSION

The plastids of higher plants accumulate large amounts of two biosynthetically related quinone compounds: plastoquinones and tocopherols. Plastoquinones are fundamentally important components of the photosynthetic electron-transport chain, whereas tocopherols are thought to be important for free radical scavenging and protection from oxidative stress. Plastoquinone and tocopherols share a common biosynthetic pathway that has been elucidated for some time (Fig. 1). Recently, genetic insight into the

**Table I.** *Segregation analysis of progeny from plants heterozygous for both an HPPDase transgene and the pds1 mutation*

| Transgenic Plant Line | Green:White | $\chi^{2a}$ | Kanamycin Resistant: Kanamycin Sensitive | $\chi^{2b}$ |
|---|---|---|---|---|
| 105-1-6 | 125:8 | 0.9[c] | 135:35 | 1.76[d] |
| 107-2-2 | 95:10 | 1.38[e] | 79:30 | 0.37[c] |
| 113-1-8 | 187:10 | 0.80[c] | 133:51 | 0.72[c] |

[a] $\chi^2$ values were calculated for a 15:1 ratio. [b] $\chi^2$ values were calculated for a 3:1 ratio. [c] $P > 0.3$. [d] $P > 0.1$. [e] $P > 0.2$.

pathway has been obtained, primarily because of the isolation and characterization of mutations in Arabidopsis that disrupt two key steps of plastidic quinone biosynthesis (Norris et al., 1995). One of these mutations, *pds1*, was shown to affect the activity of HPPDase, the committed step of plastidic quinone biosynthesis (Fig. 1). To further understand the nature of the *pds1* mutation, we have isolated and functionally analyzed cDNAs and genomic clones encoding HPPDase from Arabidopsis.

Computer database searches with mammalian and bacterial HPPDase sequences identified a single truncated Arabidopsis expressed sequence tag with significant homology to the carboxyl domains of other HPPDases. This expressed sequence tag was used to isolate a full-length Arabidopsis cDNA clone, pHPPD. Comparison of the putative Arabidopsis HPPDase protein sequence with HPPDase protein sequences from 13 other diverse species identified 37 conserved residues clustered primarily in the carboxy region of the protein (Fig. 2). Presumably, these highly conserved residues are important for substrate binding or the catalytic mechanism of HPPDases. Five Tyr and His residues, postulated to form a ferric iron center in HPPDases (Denoya et al., 1994), are also conserved in the putative Arabidopsis HPPDase (Fig. 2).

To determine whether the putative Arabidopsis HPPDase cDNA encoded a functional HPPDase enzyme, the open-reading frame of this cDNA was expressed in *E. coli.* As shown in Figure 3, *E. coli* cultures expressing pHPPD accumulate a compound that co-migrates with, and has a spectrum identical to, the HGA standard (Fig. 3). *E. coli* containing a control plasmid without the HPPDase open-reading frame lacks this peak (Fig. 3A). In addition to HPPDase-dependent HGA accumulation, pHPPD expression in *E. coli* resulted in accumulation of ochronotic pigment, an oxidative polymerization product of HGA. Similar results were reported when a *S. avermitilis* HPPDase was expressed in *E. coli* (Denoya et al., 1994). These data demonstrate that the Arabidopsis cDNA pHPPD encodes a functional HPPDase enzyme.

As discussed previously, Arabidopsis plants homozygous for the *pds1* mutation are unable to synthesize both plastoquinone and tocopherols because of an inability to convert HPP to HGA (Fig. 1). Although it was clear from previous work that the *pds1* mutation affected HPPDase activity, we could not determine whether the *pds1* mutation directly or indirectly affected the HPPDase enzyme (Norris et al., 1995). Isolation of Arabidopsis pHPPD provided the means for directly testing the hypothesis that *pds1* is a disruption in the HPPDase gene by mapping, molecular complementation, and DNA sequence analysis.

Linkage analysis indicated that the gene corresponding to Arabidopsis pHPPD maps near (±4 centimorgans) the *pds1* mutation (data not shown). Transgenic plants overexpressing the pHPPD cDNA were generated in a wild-type background and crossed with plants heterozygous for the *pds1* mutation. Kanamycin-resistant $F_1$ plants were selected and selfed, and the resulting $F_2$ plants were scored. Failure of the transgene to functionally complement the *pds1* mutation would result in $F_2$ progeny that segregate 3:1 green: white (wild type to mutant), whereas functional complementation by the transgene would result in $F_2$ progeny that segregate 15:1 green:white, assuming that the transgene and the *pds1* mutation were not linked. Table I shows that the $F_2$ green-to-white segregation ratios from crosses of *pds1* heterozygotes to three independent, parental transgenic lines are statistically significant for a 15:1 ratio. These data provide genetic evidence that constitutive expression of the pHPPD transgene complements the *pds1* mutation.

Sequence analysis of the HPPDase gene from both wild-type and homozygous *pds1* mutant plants was performed to define the molecular basis of the *pds1* mutation. As shown in Figure 2, the wild-type and *pds1* HPPD alleles are identical in sequence with the exception of a 17-bp deletion in the *pds1* HPPD allele. This deletion results in a substitution of Leu for the highly conserved Phe at position 419, followed immediately by a stop codon. The consequence of this deletion at the protein level is the loss of 26 carboxy-terminal amino acids from the HPPDase protein, including a tight cluster of several amino acids that are conserved in all HPPDase proteins in the database. The null phenotype of the *pds1* mutant suggests that these 26 carboxy-terminal residues are essential for HPPDase enzymatic activity. Most significantly, these data define the molecular basis of the *pds1* mutation as a lesion in the structural HPPD gene.

In conclusion, we have identified and characterized Arabidopsis cDNA and genomic clones encoding HPPDase. The functional expression of the Arabidopsis HPPDase cDNA in *E. coli* demonstrates that it encodes a functional HPPDase enzyme. Constitutive expression of the protein encoded by the Arabidopsis HPPDase cDNA is sufficient to restore wild-type pigmentation to plants homozygous for the *pds1* mutation. Finally, and most significantly, we have shown that the *pds1* HPPD gene contains a small deletion that results in the elimination of a portion of the carboxy terminus of the protein. These results demonstrate conclusively that the nature of the *pds1* mutation in Arabidopsis is a mutation in the gene encoding the HPPDase enzyme. Future studies will determine the consequences of overexpressing the wild-type HPPDase enzyme in plants. Continued analysis of other plastoquinone and tocopherol biosynthetic mutants, such as *pds2* (Norris et al., 1995), will also provide valuable information concerning the subcellular location and regulation of tocopherol and plastoquinone synthesis in plants.

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