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Disruption of phytoene desaturase gene results in albino and dwarf phenotypes in *Arabidopsis* by impairing chlorophyll, carotenoid, and gibberellin biosynthesis

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Carotenoids play an important role in many physiological processes in plants and the phytoene desaturase gene (*PDS3*) encodes one of the important enzymes in the carotenoid biosynthesis pathway. Here we report the identification and analysis of a T-DNA insertion mutant of *PDS3* gene. Functional complementation confirmed that both the albino and dwarf phenotypes of the *pds3* mutant resulted from functional disruption of the *PDS3* gene. Chloroplast development was arrested at the proplastid stage in the *pds3* mutant. Further analysis showed that high level of phytoene was accumulated in the *pds3* mutant. Addition of exogenous GA_3 could partially rescue the dwarf phenotype, suggesting that the dwarf phenotype of the *pds3* mutant might be due to GA deficiency. Microarray and RT-PCR analysis showed that disrupting *PDS3* gene resulted in gene expression changes involved in at least 20 metabolic pathways, including the inhibition of many genes in carotenoid, chlorophyll, and GA biosynthesis pathways. Our data suggest that the accumulated phytoene in the *pds3* mutant might play an important role in certain negative feedbacks to affect gene expression of diverse cellular pathways.

***Keywords*:** *Arabidopsis thaliana*, phytoene desaturase, albino, dwarf, microarray analysis

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Introduction

Carotenoids, the membrane-bound lipid-soluble pigments including carotene and xanthophyll, are a class of C_{40} hydrocarbon compounds formed through the condensation of isoprenoids [1]. They play an important role in a large number of physiological processes in plants. Carotenoids act as accessory pigments in photosynthesis and form the basic structural units of photosynthetic antennae. They also serve as photo-protection agents by quenching singlet oxygen that might otherwise damage chlorophyll. Moreover, carotenoids serve as precursors in the biosynthesis of vitamin A and abscisic acid (ABA). Many clinical studies have demonstrated that carotenoids function as free radical quenchers in many physiological pathways [2].

Although carotenoids are synthesized in plastids, all the enzymes involved in their biosynthesis are encoded by nuclear genes. As shown in Figure 1, DMAPP is synthesized via the mevalonate (MVA) pathway or non-MVA pathway before being converted into geranylgeranyl

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Abbreviations: GA (Gibberellic acid); ABA (Abscisic acid); PDS (phytoene desaturase); CLA1 (1-deoxyxylulose 5-phosphate synthase); DXR (1-deoxyxylulose 5-phosphate reductoisomerase); HMGR (3-hydroxy-3-methylglutaryl coenzyme A reductase); IPI (isopentenyl pyrophosphate: dimethyllallyl pyrophosphate isomerase); GGPS (geranylgeranyl pyrophosphate synthase); CHS (chalcone synthase); GGRS (geranylgeranyl reductase); CPS (copalyl diphosphate synthase); KS (ent-Kaurene synthase); PSY (phytoene synthase); ZDS (z-carotene desaturase); LYC (lycopene cyclase)

pyrophosphate (GGPP) through a series of condensation reactions. GGPP is then shuttled into three routes: 1) to form β-carotene, which eventually leads to the synthesis of ABA [1, 3]; 2) to form gibberellins (GAs) [4] and 3) to form chlorophyll [5]. Phytoene desaturation is an important step in the β-carotene biosynthesis pathway. The enzyme responsible for this step is phytoene desaturase (PDS), the target of a commercially important herbicide Norflurazon [6]. The *PDS* gene was first cloned from cyanobacterium [7]. Homologous *PDS* genes were then cloned from soybean [8], pepper [9], tomato [10, 11], *Arabidopsis* [12] and maize [13]. These homologous proteins share about 60% identity and 75% similarity to the cyanobacterium PDS in amino acid sequences.

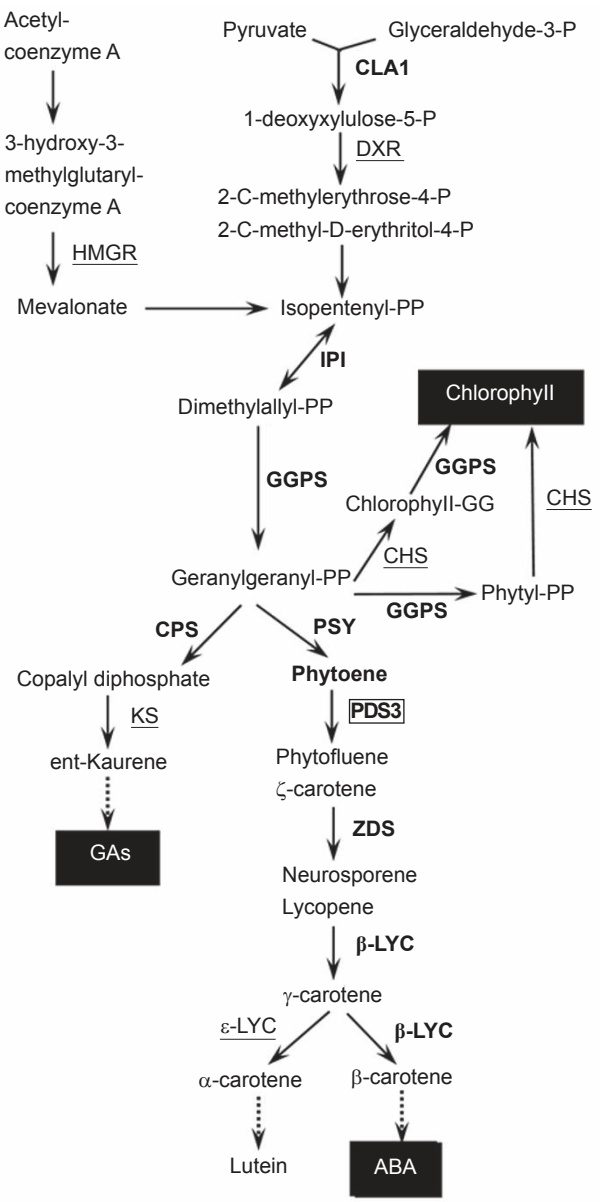

Figure 1 Carotenoid, chlorophyll and GA biosynthesis pathways. The enzymes for which the expression of their corresponding coding genes was quantitatively analyzed by RT-PCR in this paper are boldfaced, and the other enzymes are underlined. PDS3 is boldfaced and bracketed.

The phytoene desaturation step in carotenoid biosynthesis has also been studied through the use of mutants in *Arabidopsis*. Genetic screen for phytoene desaturation defect resulted in the isolation of two related mutants, *pds1* and *pds2*, both defective in phytoene desaturation and exhibiting the albino phenotype [14, 15]. The fact that the *PDS1* and *PDS2* genes encode 4-hydroxyphenylpyrurate dioxygenase and prenylphytyltransferase respectively [14, 15], suggests that plastoquinone is a critical intermediate electron carrier for electron transport in the phytoene desaturation reaction. Another *Arabidopsis* mutant, *immutans*, accumulates phytoene in variegated white tissues. The IMMUTANS gene encodes plastid terminal oxidase, the disruption of which prevents oxidation during chlorophyll biosynthesis and results in the accumulation of phytoene [16, 17]. However, mutations in the gene encoding the phytoene desaturase itself (designated *PDS3*) [14] have not been reported. Expression analysis of the gene (*PDS3*) encoding the phytoene desaturase indicated that it is highly regulated by light [18, 19] in tomato. Nevertheless, analysis of the *immutans* mutant showed that the expression of *PDS3* gene was not necessarily correlated to the amount of pigments in the cells [20].

We have obtained a new recessive albino mutant from a collection of T-DNA insertion mutants of *Arabidopsis* generated in our center. Molecular evidence demonstrated that the mutant phenotype resulted from a T-DNA insertion within the *PDS3* gene. Further analysis showed that the mutant accumulated phytoene, and exogenous GA_3 could partially rescue the dwarf phenotype of the mutant. Molecular genetic and the microarray techniques were used to investigate the role of phytoene desaturation and the underlying causes of albino and dwarf phenotypes in the *pds3* mutant.

Material and Methods

Mutagenesis and growth of Arabidopsis

Arabidopsis of Columbia ecotype (col-0) were grown at 22 °C under long-day conditions. The floral dip method [21] was used to generate transgenic *Arabidopsis* with *Agrobacterium* GV3101 harboring pSKI015 [22]. Harvested seeds were screened on 1/2 MS medium containing 10 μg/mL of PPT (DL-Phosphinothricin). Green seedlings were transferred to soil and placed under the same conditions as described above. For determination of GAs, as well as for RT-PCR, HPLC microarray, and microscope analyses, the seedlings were transferred onto 1/2 MS medium in flask instead of soil.

Identifying the flanking sequences of the insertions

Genomic DNA was extracted from *Arabidopsis* leaves by the CTAB method [23] and served as the template for TAIL-PCR [24] in the amplification of flanking regions. The DNA fragments amplified by TAIL-PCR were recovered from agarose gels and purified before sequencing using an ABI 377 automated DNA sequencer (Applied Biosystems, USA). The output sequences were then searched against the *Arabidopsis* genome sequence database (GenBank) using BLAST to localize the position of the T-DNA insertion as described previously [25].

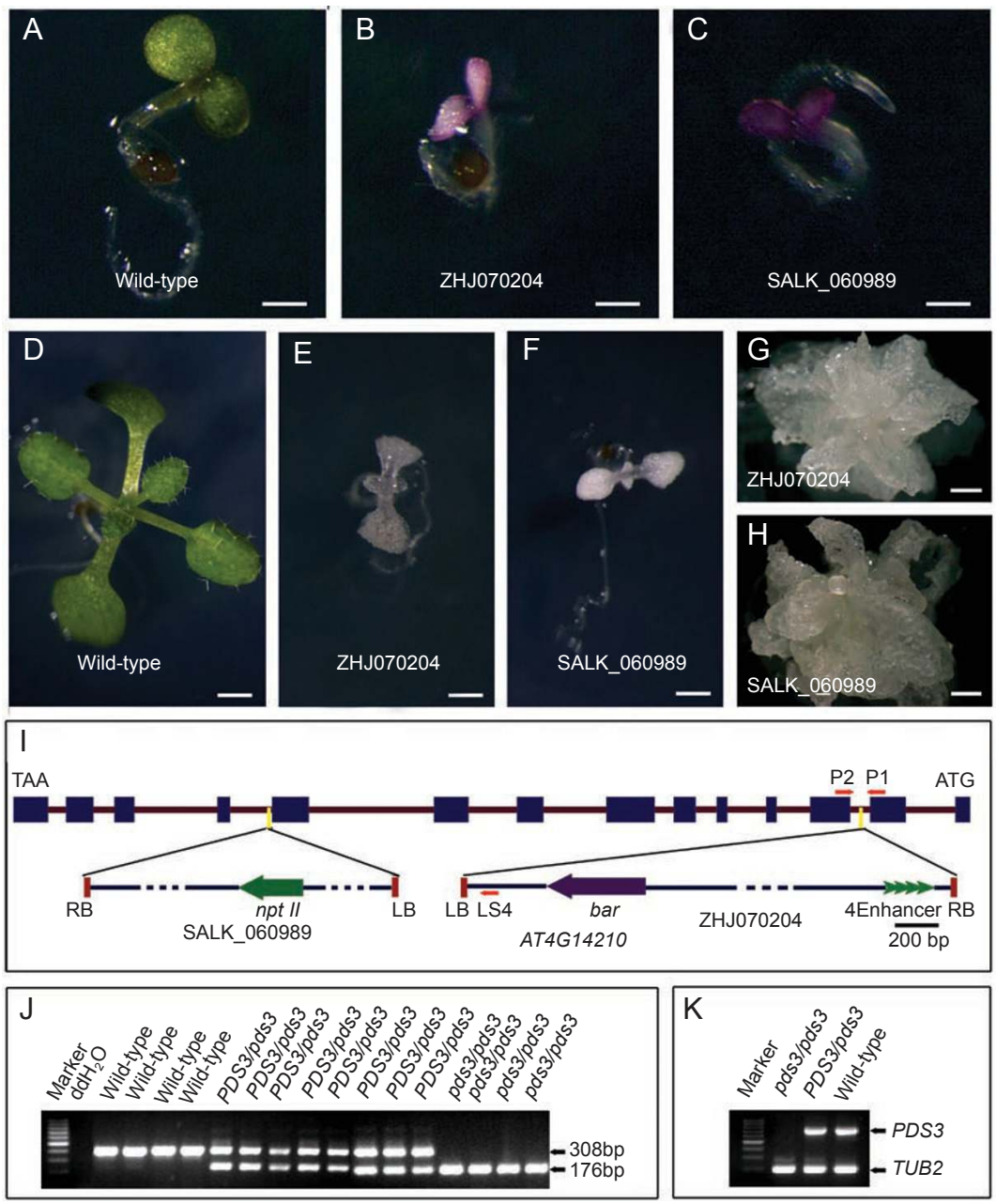

Figure 2 Phenotypes of the *pds3* mutant. After cold treatment at 4 ºC for three days, wild-type and the *pds3* mutant seeds were grown at 22±2 ºC under long-light conditions and compared at different developmental stages. **(A)** 5-day-old wild-type. **(B)** 5-day-old ZHJ070204. **(C)** 5-day-old SALK_060689. **(D)** 21-day-old wild-type. **(E)** 21-day-old ZHJ070204. **(F)** 21-day-old SALK_060689. **(G)** 42-day-old ZHJ070204. **(H)** 42-day-old SALK_060689. **(I)** Schematic representation of T-DNA insertion sites in the mutants of ZHJ070204 and SALK_060689. **(J)** Co-segregation analysis of the *pds3* mutant. **(K)** Expression analysis of the *PDS3* gene in wild-type and the *pds3* mutant. The β-tubulin gene *TUB2* was used as an internal control. Scale bar = 1 mm.

Co-segregation analysis

The primers LS4, P1 and P2 were designed to check the T-DNA insertion in the *pds3* mutant (Figure 2I). The primer sequences used were: LS4: 5′-TTG GTA ATT ACT CTT TCT TTT CCT CC-3′; P1: 5′-AGA GGA CGA CAT GGT TCA CAG T-3′; P2: 5′-CGG TCA ATG CTG GAG CTA GTA T-3′. Five pmol of each of the three primers and 20 ng of genomic DNA were present in 25 μL of the PCR reaction mix. Following denaturation at 95 ºC for 2 min, the reaction was then subjected to 30 cycles of denaturation (94 ºC for 30 sec), annealing (58 ºC for 30 sec) and extension (72 ºC for 45 sec). PCR products were then analyzed on 2% agarose gels.

Cloning of the Arabidopsis PDS3 *gene*

Two primers were designed based on the available cDNA sequence (Accession No: AF360196) to amplify the *Arabidopsis PDS3* gene, 5′-ATG GTT GTG TTT GGG AAT GTT TCT GCG GCG A-3′ and 5′-TCA TGA TGA TGA TAC TGT TGC CTC CGA CAA CTT-3′. Using 10 ng of first-strand cDNA from wild-type *Arabidopsis* leaves as the template, PCR was performed in a 50-μL volume with 20 pmol of each primer and high-fidelity *Pfu* DNA polymerase. Following a one-minute initial denaturation step at 94 ºC, the amplification went through 30 cycles consisting of denaturation at 94 ºC for 30 sec, annealing at 65 ºC for 30 sec, followed by extension at 72 ºC for 2 min. The amplified product was recovered from low-melting point agarose gel and then ligated into the *Eco*R V site of pBS after purification. Recombinant plasmids containing the *PDS3* gene in both sense (designated as pBPDS3) and anti-sense orientations (designated as pAPDS3) were identified through sequencing.

Arabidopsis transformation

After being treated with the appropriate restriction enzymes, the insert from recombinant plasmid pBPDS was recovered from low-melting point agarose gels, purified and then ligated into the plant expression vector pWM101, to generate pWPDS3 (Figure 3A). *PDS3-pds3* heterozygous mutant plants were used as the recipients for *Agrobacterium*-mediated transformation by the floral dip method. The seeds of transgenic plants were screened on 1/2 MS medium containing 25 μg/ml of hygromycin and the surviving seedlings were transferred into soil after a one-week recovery period on non-selective MS medium.

Two primers were designed based on the *HPTII* gene sequence in pWM101 and used to confirm the success of the transformation. The primer sequences were: 5′-AGG AAT CGG TCA ATA CAC TAC A-3′ and 5′-ACT ATC GGC GAG TAC TTC TAC A-3′. PCR was performed in 25 μL volumes with 5 pmol of each primer and 10 ng of genomic DNA. Following a two-minute initial denaturation step at 95 ºC, the amplification proceeded through 30 cycles consisting of denaturation at 94 ºC for 30 sec, annealing at 58 ºC for 30 sec, followed by extension at 72 ºC for 1 min. PCR products were analyzed on 1% agarose gels.

Quantitative RT-PCR analysis

Total RNA was extracted from the leaves of 4-week-old plants (WT, *PDS3-pds3*, *pds3-pds3* mutants) using Qiagen RNAeasy Plant Mini Prep kits (Qiagen, USA). First-strand cDNA synthesis was performed in a 20-μL-reaction volume consisting of 3 μg of total RNA, oligo-dT_{12-18} and SuperScript™ reverse transcriptase (GIBCO BRL). The reaction was allowed to proceed at 42 ºC for 50 min before being terminated by treatment at 70 ºC for 15 min. Quantitative RT-PCR analysis was performed using the following primers: TUB2 (β-tubulin): 5'-GTT CTC GAT GTT GTT CGT AAG-3' and 5'-TGT AAG GCT CAA CCA CAG TAT-3'; *CPS*: 5′-AAG TGT CCG CAG ATG TAT TC-3′ and 5′-ATA TAG TTG CAG CCG CTA AG-3′; *PDS3*: 5′-TTG CAG TGG AAG GAA CAC TC-3′ and 5′-ACT CTT AAC CGT GCC ATC GT-3′; *CLA1*: 5′-GAG TAT GCT CGA GGA ATG ATA A-3′ and 5′-AGA CGA ATA GAT TGC ACA GAA G-3′; *IPI*: 5′-ATG CTG TTC AGA GAC GAC TCA T-3′ and 5′-TCA CCA GCT CCT TAA GCT CTT C-3′; *GGPS*: 5′-ATC CAC GAA GCG ATG CGT TAC T-3′ and 5′-CAC TTC CTC CAC CAA CAA TAG C-3′; *PSY*: 5′-GAA CCG AAG TAG AAG AAT TG-3′ and 5′-GAT CAT CGA AGT TCT GGT AT-3′; *ZDS*: 5′-AGA TAG AGG TGG CAG AAT CC-3′ and 5′-GGT GTT AGA ACG CAC TGA AG-3′; *LYC*: 5′-TGG TCT GGT GCT GTT GTC TAT G-3′ and 5′-GCT CGT CTT CCT CAA TCC TCT T-3′; *GGRS*: 5′-CAT CGA GCG TAA GAT GGA CAA T-3′ and 5′-GCG ACA CAT CAT CTC CAA CAT A-3′; *PDS1*: 5′-AAG TAG AAG ACG CAG AGT CA-3′ and 5′-ATC AGA GCC AGA TGT TGT AG-3′; *IM* (*immutans*): 5′-CCT TAC AGA CTC GGT TAT TA-3′ and 5′-TTA CTG GTC TTC GAG TAT TG-3′.

RT-PCR was performed in a 25 μL reaction volume consisting

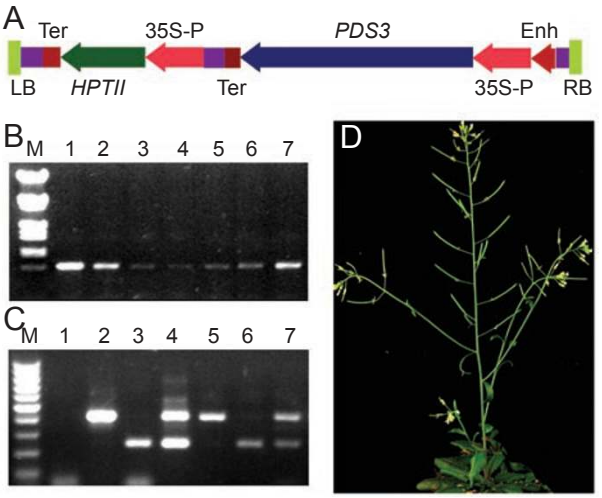

Figure 3 Phenotype rescue of the *pds3* mutant by a wild type *PDS3* transgene. **(A)** The pWPDS3 construct. Different elements of the vector are represented by boxes with different colours; the transcriptional orientation is indicated by arrow. LB, the left border of T-DNA; RB, the right border of T-DNA; Ter, CaMV 35S ployA; 35S-P, CaMV 35S promoter; *HPTII*, hygromycin phosphotransferase gene; *PDS3*, wild-type phytoene desaturase gene; Enh, CaMV 35S enhancer. **(B)** PCR amplification of *HPT II* genes. M: λ/*Eco*R I + *Hin*d III marker; 1-7: seven independent plants transformed with pWPDS3. The 568-bp band is amplified from *HPT II* genes. **(C)** Genotype characterization of the three transgenic plants using the three primers LS4, P1 and P2. M: 100-bp ladder; 1: blank control; 2: wild-type; 3: the *pds3-pds3* homozygous mutant; 4: *PDS3-pds3* heterozygous mutant; 5-7: three independent plants of different genotypes were segregated from heterozygous *PDS3-pds3* mutant transformed with pWPDS3. **(D)** The phenotype of *pds3-pds3* homozygous plant was rescued by transforming with pWPDS3.

of 0.25 μL of the first-strand cDNA product and 5 pmol each primer pairs, with one pair for a specific gene and the other for the tubulin gene (internal control). PCR mixes were subjected to 24 cycles (with the exception of 30 cycles for the *CPS* gene and 28 cycles for the *PDS1* and *IM* genes). Following a one-minute initial denaturation step at 94 ºC, the amplification consisted of denaturation at 94 ºC for 30 sec, annealing at 54 ºC for 30 sec, followed by extension at 72 ºC for 1 min. The products were analyzed on 1.5% agarose gels and the relative intensity of each band was determined and compared. For each gene analyzed, RT-PCR was repeated independently for three times, the average relative intensity counts were calculated and *t*-tests for pairwise comparisons of means were carried out.

HPLC analysis of carotenoids

One-month-old wild-type *Arabidopsis* seedlings were transferred onto MS medium containing 30 μg/mL of Norflurazon and allowed to grow for two weeks before being collected for carotenoid extraction. Meanwhile, six-week-old wild-type and the *pds3* mutant plants were also collected for carotenoid extraction. The protocol used for the extraction of lipid-soluble carotenoids was slightly modified from the previous report [14]. Carotenoids were separated by reverse-phase HPLC on a ZOR BAX 300SB C18 column (4.6 mm ID×250 mm, Gilson, France) using a 45-min gradient of ethyl acetate (0 to 100%) in acetonitrile-water (9:1) at a flow rate of 1 mL per min. Retention times relative to known standards, using detection at both 296 and 440 nm, were used to identify the chlorophylls and carotenoids. Since no phytoene standard was available in this laboratory, carotenoids extracted from wild-type *Arabidopsis* treated with the herbicide Norflurazon, in which phytoene was accumulated [26], were used as standards.

Microarray analysis

Microarray experiments and data analysis were performed according to a previously established protocol [27]. Briefly, 100 μg of total RNA from the *pds3* mutants and wild-type seedlings was used to synthesize Cy3- and Cy5-labeled cDNA, respectively. A combination of wild-type cDNA and an equal amount of mutant cDNA was used to simultaneously probe a glass slide containing duplicate arrays in three microarray experiments. Each experiment was repeated with reverse labeling.

Electron microscope analysis

Sections of leaf tissue were prepared for EM observation according to a previously described protocol [28] with minor modifications. Briefly, *Arabidopsis* leaf tissue was fixed with glutaraldehyde, then fixed with osmium tetroxide and dehydrated in an ethanol series before being infiltrated with Spurr (Dow Chemical Co., USA). Polymerization was conducted at 70 ºC for 8 h. Specimens were sliced to yield ultra-thin sections (LKB-8800, Sweden) and then stained with uranyl acetate and alkaline lead citrate before being examined with a JEM-100S transmission electron microscope.

Results

Isolation of a new albino mutant

From a collection of T-DNA insertion mutants of *Arabidopsis* that we recently generated, T_2 seeds of 2 500 lines were screened for observable phenotypes. One line (ZHJ070204) with an albino phenotype was further analyzed. The homozygous mutant was completely albino and unable to grow in soil. However, it could survive and grow on 1/2 MS medium containing 1% sugar. While germinating normally, the mutant had purple cotyledons that were gradually bleached to complete white with extended growth period (Figure 2B, 2E, 2G). The mutant was greatly retarded in development, displaying a severe dwarf phenotype with small rosette leaves. No bolting was observed in any of these cultured mutant plants (Figure 2G).

The mutant phenotype co-segregates with the T-DNA insertion

As a first step in revealing the molecular nature of the mutation, the flanking sequences of the T-DNA insertion site were TAIL-PCR-amplified and sequenced. One insertion was located in the second intron of the phytoene desaturase gene (At4g14210) in this mutant (Figure 2I). We designated this mutant as *pds3* for reasons discussed in the following sections. When T3 seeds from different T2 green lines were planted on MS medium without PPT selection, the χ^2 test showed that the number of green plantlets vs. that of albino was 3:1, suggesting that the phenotype was caused by a single recessive mutation.

To check whether the T-DNA insertion co-segregates with the albino phenotype, DNA from T3 progenies were used as templates for PCR analysis (Figure 2J). Primers were designed to amplify a 308-bp fragment from wild-type plant and a 176-bp fragment from the homozygous mutant. In all heterozygous mutants, both fragments were amplified, indicating that the T-DNA insertion in the *PDS3* gene co-segregated with the albino phenotype (Figure 2J). SALK_060989, a SALK T-DNA line with a T-DNA insertion in the tenth intron of At4g14210 (Figure 2I) from the *Arabidopsis* Biological Resource Center [29], displayed the similar phenotypes with ZHJ070204 (Figure 2C, 2F, 2H). These data further support that the albino phenotype was caused by the T-DNA insertion. This conclusion is also consistent with the fact that the *PDS3* transcript was absent in the albino mutant as shown by RT-PCR analysis (Figure 2K).

The PDS3 gene complements the albino and dwarf phenotypes

To find out if the disruption of *PDS3* was responsible for the albino phenotype, we attempted to rescue the phenotype by introducing a cDNA of the *PDS3* gene driven by the 35S promoter back into the mutant. When the vector harboring the wild type *PDS3* gene was constructed (Figure 3A) and transformed into heterozygous *pds3* plants, we were able to obtain progeny homozygous for *pds3* yet with the wild type phenotype (Figure 3B-D). These transgenic plants homozygous for *pds3* carried a new *PDS3* transgene (Figure 3C).

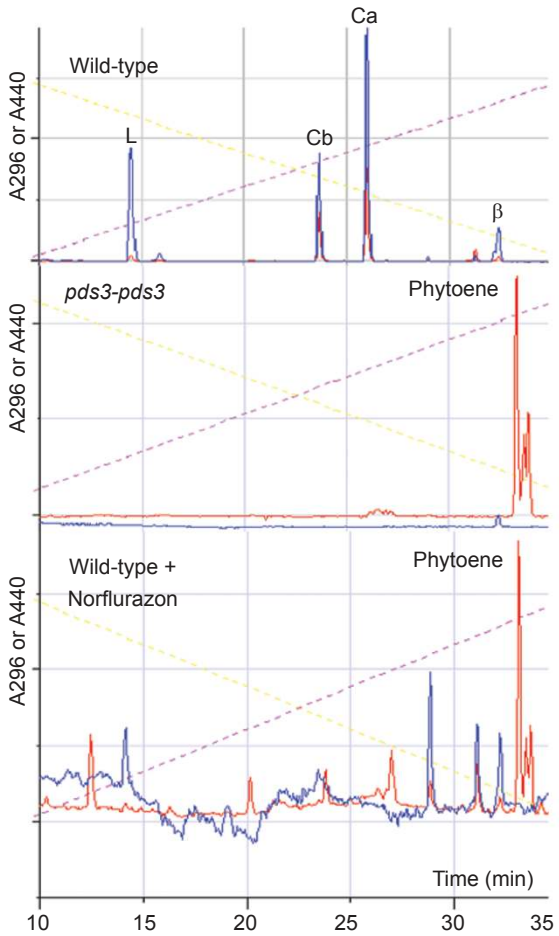

Figure 4 HPLC analysis of carotenoids in leaves from wild-type, *pds3* mutant and Norflurazon-treated wild-type plants. The absorbance spectra at 296 nm and 440 nm are indicated by red and blue lines, respectively. L, lutein; Cb, chlorophyll b; Ca, chlorophyll a; β, β-carotene.

Further phenotype analysis of the rescued plants indicated that the *PDS3* gene could fully complement the albino phenotype and all other associated abnormalities, including dwarfism, lethality, and infertility (Figure 3D). To further confirm the contribution of the *PDS3* gene disruption to the observed phenotype of the *pds3* mutant, an RNAi transgene for *PDS3* under the strong constitutive 35S promoter was constructed and introduced into wild type *Arabidopsis*. All the RNAi transgenic plants examined exhibited, to different extent, similar phenotypes to that of the *pds3* mutant (Supplemental information, Figure S1). This result further confirmed that the phenotype of our initial *pds3* mutant was strictly due to the disruption of *PDS3* gene, thus providing the basis for designation of the mutant as *pds3*.

High levels of phytoene accumulate in the pds3 mutant

HPLC was performed to examine carotenoid and other pigment contents in the *pds3* mutant. The analysis showed that the chlorophyll and β-carotene peaks detected in the wild type plants were absent in the *pds3* mutant (Figure 4). Furthermore, the three main peaks that were observed in the *pds3* mutant occurred at the same position where phytoene would normally appear in plants treated with the herbicide Norflurazon (Figure 4). The three peaks are probably the cis- and trans-isoforms of phytoene. These data indicated that phytoene accumulated in the *pds3* mutant due to the loss of phytoene desaturation activity, which also led to the absence of chlorophyll and β-carotene.

Dwarf phenotype of pds3 might result from GA deficiency

The finding that the *pds3* mutant was severely dwarfed, together with the fact that GGPP is required for the biosynthesis of carotenoid, chlorophyll and GAs, raised the possibility that GA biosynthesis in the *pds3* mutant was disrupted. Semi-quantitative RT-PCR analysis showed that expression of the gene coding for copalyl diphosphate synthase (CPS), the first enzyme in the committed GA biosynthesis pathway, was largely reduced in the homozygous

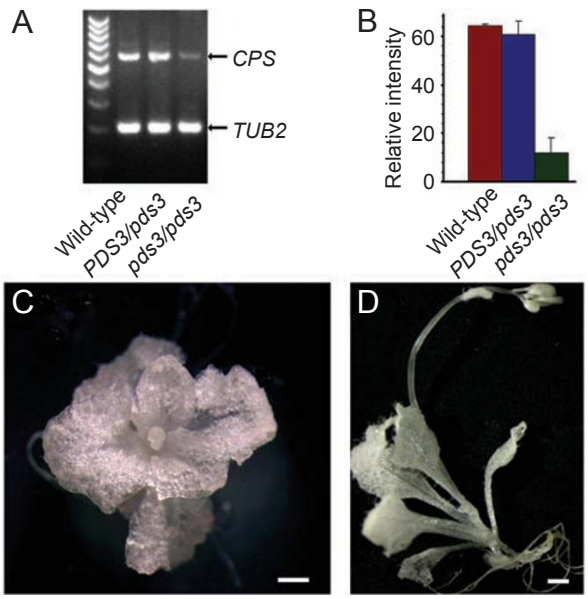

Figure 5 The *pds3* mutant might be deficient of GAs. **(A, B)** Semi-quantitative analysis of *CPS* gene expression by RT-PCR. M: 100-bp ladder; *TUB2*: β-tubulin gene as the internal control. **(C)** 35-day-old *pds3* seedling grown on 1/2 MS medium. **(D)** 35-day-old *pds3* seedling transferred to 1/2 MS medium containing 1 μg/mL of exogenous GA_3 after growing on 1/2 MS for two weeks. Scale bar = 1 mm.

pds3 mutant but not affected in the wild-type or heterozygous *pds3* plants (Figure 5A and 5B). This suggested that the *pds3* mutation affected the expression of genes involved in commitment to GA biosynthesis. We then tested whether exogenous GA could rescue the dwarf phenotype of the *pds3* mutant. We observed that after transferring the *pds3* mutant plants to 1/2 MS plate containing 1 μg/mL of exogenous GA_3, these plants grew bigger, exhibited elongated petioles and normal bolting, and were capable of setting flowers in contrast to those grown on the 1/2 MS without GA_3 (Figure 5C and 5D). In comparison, the *pds3* plants without exogenous GAs were infertile with degenerate stamens and pistils (data not shown). Taken together, these results indicate that the dwarf phenotype in the *pds3* mutant might be caused by the deficiency of GAs.

Disrupting PDS3 gene results in inhibition of many other genes involved in carotenoid, chlorophyll, and GA biosynthesis pathways

To systematically examine possible effects of *pds3* mutation on other related biosynthetic pathways, nine representative genes were chosen for semi-quantitative RT-PCR analysis (shown in Figure 1). These genes include *CLA1* [30], *IPI*, *GGPS*, *PSY*, *ZDS* and *LYC* that are directly involved in the carotenoid biosynthesis pathway, *GGRS* that is required in the side-branched chlorophyll biosynthesis, and *PDS1* and *IM* that are functionally related to phytoene desaturation. The results showed that the nine genes shared a similar pattern of expression. The expression level of most genes was comparable between wild-type and heterozygous *pds3* plants but clearly reduced in the homozygous *pds3* mutant plants (Figure 6). For example, the expression of *GGPS* and *PSY* in the *pds3* mutant was significantly lower than that in the wild-type plant. These data indicated that the expression of many genes involved in the carotenoid, GA, and chlorophyll biosynthesis pathways was lowered by disruption of the *PDS3* gene.

Metabolic pathways and nuclear encoded chloroplastic genes were affected in the pds3 mutant

To further investigate effects of the mutated *PDS3* gene on other metabolic pathways, we compared the genome expression profile of the *pds3* mutant seedlings with that of the wild type using microarray analysis. The microarray contained a total of 9 216 ESTs representing approximately 6 120 unique genes [27]. A total of 596 unique genes exhibited twofold or more differential expression in *pds3* in comparison with wild-type, with 330 being up-regulated and 266 down-regulated. Of the 596 differentially regulated genes, 392 have known functions or share sequence similarity with genes of known functions, whereas the rest 204 were genes with unknown function (Supplemental information, Table S1). Of the 392 genes with known or predicted functions, 126 or about 21% of them can be clas-

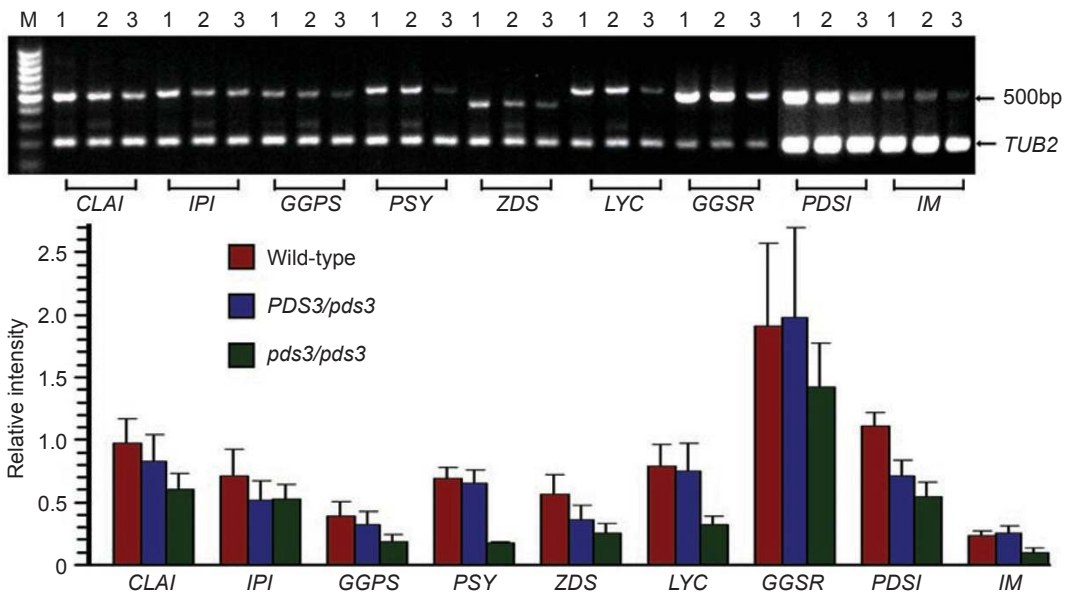

Figure 6 Expression analysis of genes involved in related biosynthetic pathways in wild-type (1), *PDS3-pds3* heterozygous mutant (2), and *pds3-pds3* homozygous mutant (3). M: 100-bp ladder; *TUB2*: β-tubulin gene as the internal control. Primers designed for *IPI* gene can be used to amplify both *AtIPI1* and *AtIPI2* transcripts.

Table 1 Number of genes involved in different functional groups up-regulated or down-regulated by at least two-fold in the *pds3* mutant

Pathways	Gene No.	Representative genes [a]
Down-regulated		
Calvin cycle	9	Rubisco small subunit 1b, 2b, 3b, enolase, 2 glyceraldehyde 3-phosphate dehydrogenase, fructose-bisphosphate aldolase, nucleotide sugar epimerase, phosphoribulokinase
Photosynthetic light reactions	12	7 chlorophyll a / b binding proteins, photosystem I reaction center subunit II, IV, V, 2 proteins involved in electron transport
Glycolysis	6	β-amylase, alcohol dehydrogenase, lactate dehydrogenase, enolase, fructose-bisphosphate aldolase, phosphorglyerate mutase
Trichloroacetic acid cycle	3	Citrate synthase, pyruvate dehydrogenase, isocitrate dehydrogenase
Cell wall synthesis	4	3 glucosyltransferase, UDP-glucose:glycoprotein glucosyltransferase
Biosynthesis of terpenoid or chlorophylls	5	Mevalonate diphosphate decarboxylase, CLA1 protein, geranylgeranyl reductase, protoporphyrinogen oxidase, lupeol synthase protein
Phenylpropanoid biosynthesis	5	Glutathione-conjugate transporter, 2 GSTs, 2 cytochrome P450
Protein synthesis in chloroplast	3	2 chloroplast 50S ribosomal protein, chloroplast initiation factor
Protein synthesis in cytoplasm	13	5 60S ribosomal proteins, 3 other ribosomal proteins, arginyl-tRNA synthetase, 2 translation initiation factor, 2 RNA helicases
Ubiquitin - proteasome pathway	4	2 ubiquitin-conjugating enzyme E2, 26S proteasome subunit, 26S proteasome regulatory subunit
Amino acid synthesis pathways	4	Glycine dehydrogenase, Proline synthetase, phosphoribosylglycinamide synthetase, Δ-1-pyrroline 5-carboxylase synthetase
Transcription factors	10	CCR4-associated factor, MADS box transcription factors, DNA binding protein CCA1, transcriptional adaptor, myb-related protein, ring-box protein, bHLH transcription factor, MYB-related transcription factor, bZIP transcription factor TGA1, basic leucine zipper transcription activator
Up-regulated		
Cell wall degradation and cell wall proteins	12	Xyloglucan endo-1,4-β-D-glucanase, pectinacetylesterase, pectinesterase, XET, 2 xylosidases, β-galactosidase, 2 peroxidases, 2 glycine-rich proteins, proline-rich protein
Ethylene biosynthsis and ethylene responsive genes	4	ACC synthase, ACC oxidase, ethylene responsive element binding factor, ethylene-respon sive protein
Stress tolerance	8	Drought-induced protein, wound-responsive protein, water stress-induced protein, salt-induced protein, aluminum-induced protein, harpin-induced protein, nematode-resistance protein, cold-acclimation protein
Protein synthesis in cytoplasm	5	60S ribosomal protein, 40S ribosomal protein, other ribosomal protein, chaperonin, translation initiation factor
Nitrogen assimilation	3	Glutamate dehydrogenase, glutamine synthetase, cysteine synthase
Branched-chain amino acid catabolism	5	3 branched-chain alpha keto-acid dehydrogenases, branched-chain amino acid aminotransferase, aminotransferase
Sulfur assimilation	4	2 GSTs, sulfate transporter, cysteine synthase
Transcription factors	7	C2H2 zinc finger transcription factor, transcription regulator protein, 2 DNA-binding proteins, transcriptional co-repressor SIN3A, transcription factor-like protein light-induced protein, zinc-finger protein Lsd1

[a] Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; GST, glutathione S-transferase; XET, xyloglucan endotransglycosylase; ACC, 1-aminocyclopropane-1-carboxylic acid.

sified into 20 different metabolic pathways, 12 of which are repressed while 8 are enhanced (Table 1), according to criteria previously described [27].

After searching against MAtDB [31], 93 out of the 596 differentially-expressed genes were found to code for products that are targeted to chloroplast, with 34 be-

ing up-regulated and 59 down-regulated (Supplemental information, Table S2). Among these nuclear encoded chloroplastic genes, seven chlorophyll-binding protein genes and three photosystem protein genes, involved in the photosynthesis and the Calvin cycle, were down-regulated, indicating that both light and dark reactions were repressed (Table 1). These data are consistent with the notion that lack of carotenoids and chlorophyll in the albino *pds3* mutant would result in the repression of chlorophyll binding protein genes and photosynthesis. Further, the expression level of *CLA1* and *GGRS* from the microarray analysis was also significantly lower in the *pds3* mutant than that in the wild-type plant (Table 1), which is well corroborated with the RT-PCR analysis shown above (Figure 6).

Chloroplast development is arrested at the proplastid stage in the pds3 mutant

To examine the development status of the plastids in the *pds3* mutant, mesophyll cell plastids from 5 d, 30 d and 90 d old *pds3* mutant seedlings were analyzed by transmission electron microscopy. The data showed that in wild-type *Arabidopsis* chloroplasts and other plastids including the thylakoid membrane system developed normally in light (Figure 7A). In contrast, no mature plastids were found in the *pds3* mutant. Only undifferentiated proplastids were found in the mesophyll cells at all three developmental stages examined, while no thylakoid membrane system formed in any of the cells examined (Figure 7C-D). Cells became increasingly vacuolated during development, which was also observed in another albino mutant *apg2* [32]. These results indicated that proplastids in the mesophyll cells of the *pds3* mutant were arrested at the undifferentiated stage and had lost the ability to develop into mature chloroplasts.

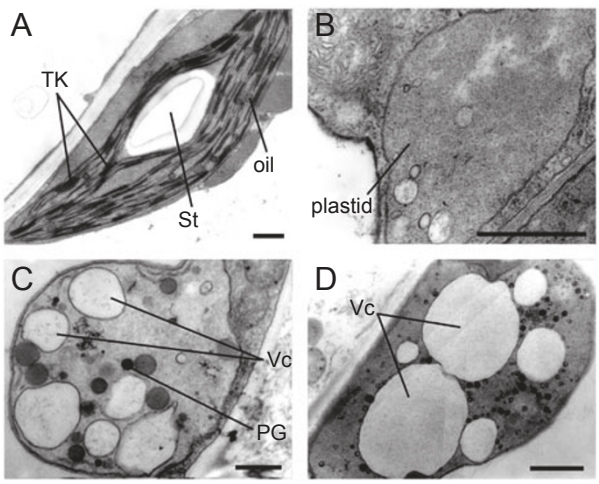

Figure 7 The effect of the *pds3* mutation on plastid development. **(A)** wild-type. **(B)** Plastids in leaf mesophyll cells of purple seedlings 5 days after germination. **(C)** Plastids in leaf mesophyll cells of albino seedlings 30 days after germination. **(D)** Plastids in leaf mesophyll cells of albino seedlings 90 days after germination. St: starch granule; Tk: thylakoid; Oil: oil drops; PG: plastoglobule. Scale bar = 1.2 μm.

DISCUSSION

Disruption of the PDS3 gene resulted in failure of phytoene desaturation, albino and dwarf phenotypes in Arabidopsis

Carotenoid biosynthesis is an important metabolic pathway in *Arabidopsis*. The biosynthesis of chlorophylls, phytohormones, vitamins, and some important accessory pigments for photosynthesis such as β-carotene and xanthophylls all relates to the carotenoid biosynthesis pathway. Phytoene desaturase (PDS3) plays an important role in carotenoid biosynthesis. Two *Arabidopsis* PDS-related mutants, *pds1* and *pds2*, with albino phenotypes, were found to accumulate high levels of phytoene, the substrate of PDS3, in their leaves [14, 15]. However, mutations in *PDS1* and *PDS2* genes only affected the availability of the electron carrier plastoquinone required for the phytoene desaturase function, but not the PDS3 enzyme itself. In our study, the albino mutant *pds3* was obtained and analyzed. The *pds3* mutant displayed albino and dwarf phenotypes with characteristics that included slow growth, small leaves, short petioles, absence of bolting and flowering, and infertility. Molecular and genetic evidence showed that an insertion of T-DNA into the *PDS3* gene co-segregated with the mutant phenotype (Figure 2). Further analysis demonstrated that an intact copy of *PDS3* gene complemented the *pds3* mutant phenotype (Figure 3) and that knock-down of *PDS3* by RNAi in transgenic *Arabidopsis* resulted in variegated or albino, semi-dwarf or dwarf phenotypes (Supplemental information, Figure S1). Together with the evidence from the HPLC analysis showing that phytoene accumulated in the *pds3* mutant (Figure 4), we concluded that the albino and dwarf phenotypes resulted from the disruption of the *PDS3* gene.

The pds3 mutation affects expression of about 1/10 of genes and causes multiple phenotypes

In order to investigate the molecular basis for the *pds3* mutant phenotypes, microarray analysis of *pds3* mutant seedlings was performed in comparison with wild-type. Our results indicated that expression of about 1/10 of genes was either up- or down-regulated by two-fold or more in the *pds3* mutant (Supplemental information, Table S1). Among these differentially regulated genes, ninety-three encode chloroplast-destined proteins, with thirty-four

genes being up-regulated and fifty-nine down-regulated (Supplemental information, Table S2). This significant change in gene expression for chloroplast proteins is consistent with the severely retarded development of the plastids (Figure 7), and with a previous report that nuclear encoded photosynthetic genes were repressed in bleached carotenoid-deficient mutant [33]. At least 26 pathways were found to be light-regulated in *Arabidopsis* [27]. In *pds3*, at least 20 metabolic pathways were affected. Interestingly, compared to the results of gene expression profiling in wild-type *Arabidopsis*, many light-regulated pathways were completely disrupted by the *pds3* mutation. Due to the lack of chlorophylls, photosynthesis in *pds3* was not functional; and photosynthesis-related pathways, such as photoreaction, photosystems, the Calvin cycle and chlorophyll biosynthesis, were all suppressed (Table 1 and Supplemental information, Table S2). Without photosynthesis, the mutant plants could only survive by taking carbons directly from the growth medium. Consequently, glycolysis and the Kreb's Cycle were suppressed while N- and S-assimilation related pathways were induced (Table 1 and Supplemental information, Table S2). Furthermore, the *pds3* mutation resulted in a burst of induction of genes encoding stress-related proteins (Table 1 and Supplemental information, Table S2).

Dwarfism might be caused by a deficiency of GAs

The *pds3* mutant showed a severe dwarf phenotype including retarded growth, short petioles, and absence of bolting (Figure 2G-2H). There are two potential explanations for the dwarfism. One hypothesis is that the dwarfism results from an energy deficiency due to the disruption of photosynthesis in the *pds3* mutant; alternatively loss-of-function in *PDS3* gene may have impeded the biosynthesis of GAs, which is closely related to the β-carotene biosynthesis pathway (Figure 1). When exogenous GAs were added to the medium, the *pds3* mutant, while still albino, showed normal bolting and extended petioles (Figure 5D). In addition, expression of the *CPS* gene, coding for the first enzyme in the committed GA biosynthesis pathway, was found to be down-regulated in the *pds3* mutant, which probably led to a reduced production of GAs (Figure 5A and 5B). Therefore, the dwarfism of the *pds3* mutant might be due to a deficiency of GAs.

Possible mechanisms of gene expression changes in the pds3 mutant

In the *pds3* mutant, not only the carotenoid pathway genes encoding downstream enzymes after phytoene desaturation, such as *ZDS* and *LYC*, were down-regulated, but also were the genes encoding upstream enzymes such as *CLA1*, *IPI*, *GGPS* and *PSY*, as well as those involved in the related GA and chlorophyll pathways, such as *CPS* and *GGRS*. Two possible mechanisms could be involved. First, photo-oxidative damage due to the lack of carotenoids may have inhibited the expression of not only photosynthetic genes but also other genes involved in carotenoid and GA biosynthesis pathways. It is reported that expression of nuclear-encoded photosynthetic genes could be inhibited through at least three signaling pathways based on the developmental status of the plastids [34]. Recently, an analysis on the Norflurazon-treated *Arabidopsis* seedlings using an 8200-EST microarray showed that expression of 182 genes was repressed by three-fold or more [35]. In our microarray analysis, expression of 83 genes including many photosynthetic genes was repressed by three-fold or more (Supplemental information, Table S1). Some of these photosynthetic genes were repressed in a similar pattern with those in Norflurazon-treated seedlings, indicating that the chloroplast-nucleus signaling is functional in the *pds3* mutant [35]. Therefore, since carotenoid biosynthesis and initial steps of GA biosynthesis occur in plastids, it is possible that expression of the involved genes could be reduced in the *pds3* mutant due to the photo-oxidative damage. Second, there might be a negative feedback mechanism in the carotenoid biosynthesis pathway. It was reported that the expression of *GA5* and *GA4* in leaves, stems and flower buds was noticeably inhibited by GA treatment in *Arabidopsis*, suggesting the existence of a negative feedback regulation in GA biosynthesis [36]. Similarly, blocking the haem branch could feedback-regulate upstream genes in the tetrapyrrole biosynthesis pathway, which in turn inhibits the chlorophyll branch, another branch in the same pathway [37]. It is interesting to note that biosyntheses of both tetrapyrrole and GAs represent branches of the carotenoid biosynthesis pathway. In this study, the expression of *GGPS*, *PSY* and *CPS* was shown to be reduced in the *pds3* mutant. *GGPS* and *PSY* are located upstream of *PDS3* in the carotenoid biosynthesis pathway, and *CPS* encodes the first enzyme in the GA biosynthesis pathway. GGPP, the common substrate for both *PSY* and *CPS*, is synthesized through the catalysis by GGPS. Hence, there might be a feedback mechanism in the pathway that leads to a reduced production of GAs when the function of the *PDS3* gene is disrupted.

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(**Supplementary information** is linked to the online version of the paper on the Cell Research website.)