Disruption of the Homogentisate Solanesyltransferase Gene Results in Albino and Dwarf Phenotypes and Root, Trichome and Stomata Defects in Arabidopsis thaliana

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Abstract

Homogentisate solanesyltransferase (HST) plays an important role in plastoquinone (PQ) biosynthesis and acts as the electron acceptor in the carotenoids and abscisic acid (ABA) biosynthesis pathways. We isolated and identified a T-DNA insertion mutant of the HST gene that displayed the albino and dwarf phenotypes. PCR analyses and functional complementation also confirmed that the mutant phenotypes were caused by disruption of the HST gene. The mutants also had some developmental defects, including trichome development and stomata closure defects. Chloroplast development was also arrested and chlorophyll (Chl) was almost absent. Developmental defects in the chloroplasts were consistent with the SDS-PAGE result and the RNAi transgenic phenotype. Exogenous gibberellin (GA) could partially rescue the dwarf phenotype and the root development defects and exogenous ABA could rescue the stomata closure defects. Further analysis showed that ABA and GA levels were both very low in the pds2-1 mutants, which suggested that biosynthesis inhibition by GAs and ABA contributed to the pds2-1 mutants’ phenotypes. An early flowering phenotype was found in pds2-1 mutants, which showed that disruption of the HST gene promoted flowering by partially regulating plant hormones. RNA-sequencing showed that disruption of the HST gene resulted in expression changes to many of the genes involved in flowering time regulation and in the biosynthesis of PQ, Chl, GAs, ABA and carotenoids. These results suggest that HST is essential for chloroplast development, hormone biosynthesis, pigment accumulation and plant development.

Introduction

HST is an important enzyme that catalyzes the condensation of the tyrosine-derived aromatic compound, homogentisate (HGA), with the isopenoid, all-trans-nonaphenyl diphosphate solanesyl diphosphate, to form 2-Me-6-solanesyl-1,4-benzoquinol (also known as 2-demethylplastoquinol-9) [1]. This branch-point compound directly leads to the biosynthesis of either vitamin E or the photosystem II (PSII) mobile electron transport co-factor, PQ [2,3]. Indirectly, it also links a number of other diverse, important metabolic pathways, including carotenoid biosynthesis, which uses PQ as a co-factor for phytoene desaturase, and ABA biosynthesis, which is derived from the breakdown of carotenoids [4–6]. Consequently, HST is likely to affect plant growth and development. While the homogentisate and solanesyl pathways are critical for forming prenylated electron carrier molecules, such as PQ in chloroplasts and ubiquinone in mitochondria (Figure 1, modified from Motohashi et al.) [7], the C20 diterpene geranylgeranyldiphosphate (GGPP) stands at the crux of a number of other biosynthetic pathways important to plant survival and adaptation. GGPP is also used as a substrate in the formation of dolichol (required for protein glycosylation) and phytol side chains during Chl biosynthesis. Changes in fluxes through many of these different pathways could influence other pathways through feedback regulation, changes to higher order regulatory genes and changes to the balance between substrate “draw”, which depends on tissue-specific and organ-specific gene expressions, and expression timing. The feedback regulation balances between substrate “draw” and expression timing are known for GA biosynthesis, the 2-C-methyl-derythritol-4-phosphate (MEP) pathway and for carotenoid biosynthesis [8,9].

PQ transports electrons from PSII to cytochrome b 6f in the light harvesting reactions of photosynthesis [1,10] and is the immediate electron acceptor (co-factor) in the -carotene to lycopene stage during carotenoid and ABA biosynthesis and in the desaturation of phytoene and ζ-carotene (Figure 1). A certain quinone/hydroquinone balance is necessary for optimal phytoene desaturation. PQ is enriched in chloroplasts and is also found in Golgi membranes and in minor quantities in microsomes [11]. PQ is not detectable in mitochondria; instead the mobile quinone electron carrier, ubiquinone, dominates in mitochondria. The presence of PQ in the cytosol has also been reported, which probably reflects its transport from the site of synthesis to its final location [11,12].
In higher plants, GAs can regulate seed germination, stem elongation, leaf expansion, trichome development and plant fertility (stamen elongation, pollen release and germination and pollen tube growth) [8,13–15]. GAs are mainly synthesized by the MEP/isoprenoid pathway and this biosynthesis can be divided into three stages [16–18]. The \( \text{GA1} \) and \( \text{GA2} \) genes play an important role in the conversion of GGPP to ent-kaurene and the Arabidopsis \( \text{ga1} \) mutant is a GA-responsive male-sterile dwarf due to the disruption of GA biosynthesis. Accumulation of the 1st stage enzyme, cyclase \( \text{ent}-\text{copalyl diphosphate synthase (CPS, also known as GA1)} \), in \( \text{Escherichia coli} \), the conversion of GGPP to copalyl diphosphate (CDP) in \( \text{GA1} + \text{GGPP synthase} \) transgenic \( \text{E. coli} \) and the transgenic complementation of Arabidopsis \( \text{ga1-3} \) mutants, confirmed that the \( \text{GA1} \) gene locus, U11034, encodes CPS [19]. The Arabidopsis \( \text{ga2} \) mutant is also a GA-deficient dwarf because it contains mutated ent-kaurene synthase (KS) and has impaired CDP conversion to ent-kaurene [20].

Genes encoding HST or its homologs have been isolated and identified in Arabidopsis and many other plants [2,4,21–23]. The HST gene, \( \text{VTE2} \), was first isolated from \( \text{Glycine max} \) and the constitutive expression of \( \text{VTE2-2} \) in transgenic Arabidopsis resulted in a significant 13% tocopherol increase compared to the transgenic vector control plants [22]. Previous studies have explored the function of the Arabidopsis \( \text{HST} \) by utilizing \( \text{pds2} \) mutants and transgenic technology [2,4,21,22]. Enzyme assay results for cell expression of the \( \text{HST} \) gene in \( \text{E. coli} \) suggested that HST catalyzes the first step in PQ biosynthesis [2]. Overexpression of the \( \text{HST} \) gene improved prenyl lipid, PQ and tocopherol levels in transgenic Arabidopsis [2,22]. Disruption of the \( \text{HST} \) gene produced an albino phenotype and caused a deficiency in the synthesis of PQ and tocopherol by affecting the prenyl/phytyl transferase enzyme [4]. The in-frame 6 bp deletion in the coding region of the \( \text{HST} \) gene caused an albino phenotype and the expression of the \( \text{HST} \) gene in this \( \text{pds2} \) mutant turned albino plants into green plants [21].

Recently, we screened and found a new recessive albino knockdown mutant called \( \text{pds2-1} \). TAIL-PCR and DNA sequencing showed that the mutant phenotype was caused by a T-DNA insertion in the \( \text{HST} \) gene. To date, phenotypic analysis of HST has been restricted to an albino phenotype, which results from disruption to chloroplast development and photosynthetic pigment biosynthesis. However, in addition to pigment biosynthesis, HST indirectly regulates carotenoid and ABA biosynthesis, but analyses of the developmental and the physiological changes due to \( \text{HST} \) gene blockage have not been reported. Detailed analysis of \( \text{pds2-1} \) indicated that \( \text{HST} \) knockdown impaired carotenoid and ABA biosynthesis, as well as GA biosynthesis and auxin content, which resulted in severe developmental defects. However, the application of exogenous GA3 and ABA to \( \text{pds2-1} \) partially restored the wild-type phenotype. Our new mutant line and analytical data confirm that HST is essential for carotenoid, ABA, and GA biosynthesis and that it plays a critical role in plant growth and development.

**Materials and Methods**

**Plant Materials and Growth Conditions**

*Arabidopsis thaliana* (ecotype Columbia-0) plants were grown in soil or on \( \frac{1}{2} \) strength Murashige and Skoog (MS) plates (pH 5.7) containing 0.8% agar and 3% sucrose at 22°C and 65% humidity under a 16 h light (100 \( \mu \text{mol m}^{-2} \text{s}^{-1} \))/8 h dark cycle. Insertion mutants were obtained from a transgenic experiment via the transformation of Arabidopsis by the floral dip method [24] using *Agrobacterium tumefaciens* GV3101 that contained pART27 [25]. Self-pollinated seed recovered from 16 independent transgenic events was screened on \( \frac{1}{2} \) strength MS plates containing 50 mg L\(^{-1}\) of kanamycin. Then the surviving seedlings were transferred to soil to...
generate seeds at 22°C under long-day conditions. For PCR, RT-PCR, Enzyme-linked immunosorbent assays (ELISA), RNA-sequencing and microscale analyses, the seedlings were grown on ½ MS plates containing 3% sucrose.

**TAIL-PCR**

The CTAB method was used to isolate genomic DNA from WT seedlings and heterozygous Arabidopsis. Genomic DNA was used as a template for the TAIL-PCR, which was carried out using a Genome Walking Kit (TAKARA) according to the manufacturer’s instructions. The three gene-specific primers used in TAIL-PCR were: \( R_1 \) (5’-ATGGGCTGCTTCCAGCTTCGCGGCTTT-3’), \( R_2 \) (5’-GTCGTCGCTTCCAGCTTCGCGGCTTT-3’), and \( R_3 \) (5’-GTCGTCGCTTCCAGCTTCGCGGCTTT-3’). The 

**Co-segregation Analysis**

Three primers were designed and used to analyze the segregation pattern of the T-DNA insertion into the \( HST \) gene in seedlings that showed a mutant phenotype. The analysis was performed on DNA extracted from plates of WT and heterozygous mutant seedlings and the primer sequences were: \( hst-g1 \) (5’-CTATCTCCTCAAAAATAAGAGAAGAAG-3’); \( hst-g2 \) (5’-CATCTCCACAAAATAAGAGAAGAAG-3’); and \( hst-g3 \) (5’-GTGTCGCTCTCATACTTCGCGGTTTTCG-3’). The PCR products were sequenced using R3 as the sequencing primer. The next sequencing results were searched against the Arabidopsis genome sequence database (GenBank) using BLAST to localize the position of the T-DNA insertion.

**Cloning of the \( HST \) Gene and Binary Vector Construction**

Based on the \( HST \) cDNA (Accession No.: NM_00116137.1) sequence, a pair of primers: \( hst-1 \) (5’-ACAGGAAATGACGAGGACAGAAAGAAG-3’) and \( hst-2 \) (5’-CTAGAGAAAAGGCGAAAAGATG-3’), \( hst-g2 \) (5’-CATCTCCACAAAATAAGAGAAGAAG-3’) and \( hst-g3 \) (5’-GTGTCGCTCTCATACTTCGCGGTTTTCG-3’), were designed to obtain the DNA sequence that included the coding region of the \( HST \) gene. Total RNA was isolated from 3-week-old Arabidopsis wild-type plants for use as a control gene along with two primers: \( UBQ-f \) (5’-CCGCGGAAATCAATGGAATGTTTATG-3’) and \( UBQ-r \) (5’-CATATGAAAAGAAGGATAACGG-3’). RT-PCR reactions were performed using a Gel Image Analysis System.

**RT-PCR and Quantitative Real-Time RT-PCR (qRT-PCR)**

For RT-PCR, WT and albino Arabidopsis plant leaves were harvested after 3 weeks growth on agar medium containing 3% sucrose. Total RNA was extracted using TRIzol Reagent (Biomed). The cDNA was synthesized with the reverse transcriptase (Takara) and oligo d(T) primer. The RT-PCR reactions were performed using \( 35S_{prom}::HST \) as an internal control gene along with two primers: \( UBQ-f \) (5’-GAGCCGAAAATCAATGGAATGTTTATG-3’) and \( UBQ-r \) (5’-CATATGAAAAGAAGGATAACGG-3’). RT-PCR images were captured using a Gel Image Analysis System.

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**qRT-PCR**

qRT-PCR analysis was used for the expression analyses and was performed on 7-week-old WT plants at different developmental stages and on a variety of tissues. It was also performed on 7-week-old WT or \( pds2-1 \) mutant plants for gene expression analyses. The system used was an ABI 7500 system with SYBR green detection. For analyses of \( HST \) expression in different tissues and developmental stages of WT Arabidopsis, \( UBQ10 \) was used as an internal control. For analyses of gene expression in \( pds2-1 \) mutants, \( TUB2 \) was used as an internal control. The two-step thermal cycling profile used was 15 s at 95°C and 1 min at 68°C. The qRT-PCR reactions were performed in biological triplicates using total RNA samples extracted from three independent plant materials grown under identical growth conditions. The comparative \( \Delta \Delta CT \) threshold cycle method [27] was used to evaluate the relative quantities of each amplified product in the samples. All primers used in the qRT-PCR analyses are reported in Supplementary Table S1.

**RNA Sequencing**

The whole plants of 3-week-old WT plants and WT Arabidopsis plants were harvested and used for RNA extraction. The experiment was then repeated following the same collection scheme, thus providing two distinct biological replicates. Total RNA was extracted using TRIzol reagent following the manufacturer’s instructions and was treated with RNase-free DNase I (NEB) to remove contaminated genomic DNA. The mRNA was isolated from the total RNA using Dynabeads oligo(dT) (Invitrogen). An illumina library was constructed using the NEB mRNA library prep kit instruction manual. The cDNA library was sequenced for paired ends on the Illumina Hiseq2000 platform at the Beijing Center for Physical and Chemical Analysis (Beijing, China). The raw reads were filtered by removing adaptor sequences, empty reads and low quality reads containing more than 50% bases with Q<30 using FASTX-Toolkit with methods described previously [28-30]. The resulting high-quality reads were mapped onto the Arabidopsis thaliana reference genome (TAIR 10) using TopHat (v2.0.5) [31]. Gene expression levels were measured as FPKM (frags per kilobase of exons per million mapped reads). EdgeR outputs the T-statistic and the p-values for each gene. Differential expression was estimated and tested using the edgeR software package (R version: 2.14, edgeR version: 2.3.52) [32]. We then calculated the FDR, estimated fold change (FC) and the FC (fragments per kilobase of exon model per million mapped reads).
tools, found at the agriGO website (http://bioinfo.cau.edu.cn/agriGO/), were used to analyze the differential expression genes during the functional annotation and enrichment analysis.

Chl and Carotenoid Assays
Chl and carotenoids were extracted from the abovementioned parts of 40-day-old Arabidopsis, as described previously [33]. Absorbance (A) of the final solution at 663, 647 and 470 nm was measured and the concentrations of Chl a (Ca), Chl b (Cb) and carotenoids (Cc) were calculated as follows [34]:

\[
Ca = 12.21 \times A_{663} - 2.81 \times A_{446};
\]

\[
Cb = 20.13 \times A_{646} - 5.03 \times A_{663};
\]

\[
Cc = (1000 \times A_{470} - 3.27 \times Ca - 104 \times Cb).
\]

ELISA Analyses
Initially, four plant hormones: ABA, GA₃, IAA and ZR, were measured on the aerial parts of 3-week-old WT and pds2-1 plants by a previously described ELISA method [35].

β-Glucuronidase Staining
Histochemical β-Glucuronidase staining in \(HST_{pro:GUS}\) transgenic plants was performed as described previously [36].

Transmission Electron Microscope (TEM) and scanning electron microscope (SEM) Assays
Leaves harvested from 3-week-old WT and pds2-1 plants were stained with uranyl acetate and observed with a TEM (Hitachi).

Results
Phenotype Analysis of the Albino Mutant
\(Arabidopsis\) thaliana was transformed by the binary vector, pART27, so that a small number (16) of T-DNA insertion lines could be generated. Seedlings from self-pollinated plants, selected on ½-strength Murashige and Skoog plates containing kanamycin and sucrose, were mainly green, but one plate contained several albino plants. Progeny from heterozygous albino plants were mainly green, but progeny derived from self-pollinated heterozygotes segregated 3:1 into green and albino colored siliques (Figure S1). The cotyledons of all the young homozygous progeny from the albino type plants were purple on the selection medium (Figure S2 A), which suggested that Chl may be low or absent and that anthocyanin pigments may have accumulated. Most homozygous albino mutants gradually faded to white with continued growth on the selection medium. A small number of plants also showed the purple coloration on the rest of their plant parts (Figure 2 A–C). All albino seedlings died when they were grown on soil or ½ strength MS medium without sucrose, but in ½ MS medium with 3% sucrose, albino seedlings produced albino leaves (and even translucent stem tissues) and only one main shoot over their whole life cycle (Figure 2 B,E,F). Low resolution microscopy indicated that the albino plants had shorter roots, fewer root hairs, fewer and smaller leaves with shorter petioles and a reduced trichome density (Figure 2 B–F; Figure S2; Figure S3). Microscope or SEM was used to investigate the leaf surface morphology (trichomes and stomata) of pds2-1 and WT plants in greater detail. The results showed that pds2-1 trichomes were shorter in length and most (~80%) trichomes had two branches instead of three (Figure 3 A,B; Figure S3; Figure S4). Their stomata had larger openings with unusual swollen structures surrounding the opening (Figure 3 C–F), which suggested that pds2-1 stomata may close abnormally.

Albino and Dwarf Phenotypes are Caused by a T-DNA Insertion into \(HST\)
We hypothesized that the albino pds2-1 phenotype was caused by a T-DNA insertion because the albino plants were obtained from transgenic Arabidopsis T-DNA insertion lines and the green and albino plants of \(T_1\), \(T_2\) and \(T_3\) segregated in a 3:1 ratio on nonselective medium. To determine the insertion site within the Arabidopsis genome, DNA was isolated from \(T_3\) heterozygous plants and non-transformed WT plants (as a negative control) for the TAIL-PCR (Figure S5). The TAIL-PCR and sequencing analysis indicated that the T-DNA insertion was located at Chr3:3782298, which is within the second intron of the Arabidopsis \(HST\) gene (At3g11945) (Figure 2 H).

To check whether the T-DNA insertion was responsible for the albino phenotype, DNA was isolated from WT, heterozygous (green) and homozygous (white) Arabidopsis plants for PCR analysis. Primers, hst-g1 and hst-g2 (Table S1) amplified a full-length 969 bp fragment of \(HST\) gDNA from the WT and heterozygous plants. In contrast, primers R3 and hst-g2 amplified a 466 bp fragment from the heterozygous and homozygous pds2-1 plants. These results indicated that the T-DNA insertion in the \(HST\) gene co-segregated with the albino phenotype (Figure 2J). To analyze the transcription of \(HST\) in the heterozygous and homozygous pds2-1 plants, RT-PCR was conducted using gene specific primers: hst-1 and hst-2. The results showed that \(HST\) expression was eliminated in the homozygous plants (Figure 2 J), which suggested that albino plants were \(HST\) null mutants and that the mutant phenotype was caused by a T-DNA insertion into the \(HST\) gene.

The \(HST\) Gene is Highly Expressed in Green Tissues
To understand the breadth of the role played by the \(HST\) gene, we re-examined the expression pattern of \(HST\) in WT Arabidopsis using qRT-PCR. The qRT-PCR assays revealed that \(HST\) was expressed at high levels in stems and leaves, but at relatively lower levels in the flowers and roots of adult plants (Figure 3 A). Furthermore, different \(HST\) expression levels were detected in the leaves at different developmental stages. The \(HST\) transcript levels were highest in non-senescent leaves, but they gradually decreased as the leaves began to senesce (Figure 3 B).

To further examine the \(HST\) expression patterns in different plant tissues, a 813 bp \(HST\) promoter sequence, upstream of the transcription start site, was fused to a GUS-coding sequence and this led to the appearance of \(HST_{pro:GUS}\) transgenic Arabidopsis plants. In 40-day-old seedlings, GUS activity was highly detected in green tissues, such as leaves and stems (Figure 4 C–F). Notably, GUS activity was detected at a high level in adult green leaves, but at decreased levels in senescent leaves (Figure 4 J). These results suggested that \(HST\) was highly expressed in green tissues and had an important role to play in their development.
Figure 2. Analysis of *pds2-1* mutant plants. (A) WT and homozygous *pds2-1* (T3) seedlings with purple cotyledons grown on ½ strength MS medium for 4 days. (B) Adult homozygous *pds2-1* mutant (T3) and WT plants grown on ½-strength MS medium for 4 weeks. (C) A purple T3 *pds2-1* mutant seedling. Scale bar = 1 mm. (D) Inflorescences and flower buds from 8-week-old WT and *pds2-1* plants. (E) Roots from *pds2-1* and WT plants. (F) Adult *pds2-1* plant grown on ½ strength MS medium for 8 weeks. Scale bar = 2 mm. (G) Adult inflorescence of a *pds2-1* mutant plant. Scale
HST is Required for Pigment Accumulation, Proplastid Growth and Thylakoid Membrane Formation

Total Chl and carotenoid (Car) contents were examined in pds2-1 and WT plants. The calculated results showed that Chl and Car were almost absent in the homozygous pds2-1 mutant plants. Chl a decreased to 0.55% of the WT level, Chl b to 1.86% and total Chl to 0.93% (Table 1), while carotenoids were reduced to 0.24% of the WT level. The Chl a/b ratio also decreased dramatically in pds2-1 mutants and was only 26.90% of the WT level. As a result,

Figure 3. SEM and TEM of mesophyll cells and leaf surfaces of pds2-1 mutant plants. (A) SEM analysis of trichomes from pds2-1. Scale bar = 100 μm. (B) SEM analysis of the trichomes from a WT plant. Scale bar = 50 μm. (C,D) SEM analysis of stomata from a pds2-1 plant (C) and a WT plant (D). Scale bars = 5 μm. (E,F) TEM analysis of a pds2-1 plant (E) and a WT plant (F). Scale bars = 25 μm. (G) TEM analysis of a mesophyll cell from a pds2-1 plant. Scale bar = 5 μm. (H) TEM analysis of a mesophyll cell from a WT plant. Scale bar = 2 μm. (I) TEM analysis of chloroplasts from a pds2-1 plant. PG: plastoglobule. Scale bar = 0.5 μm. (J) TEM analysis of chloroplasts from a WT plant. St: starch granule; Tk: thylakoid; Oil: oil drops. Scale bar = 1 μm.

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the Chl/Car ratio increased to 20.79 in the pds2-1 mutants (Table 1). These results showed that disruption of the HST gene caused significant reductions in Chl and carotenoid levels and that carotenoids were affected more seriously than Chl in pds2-1 mutants.

To assess the effect of pds2-1 mutations on chloroplast development, plastids from the first leaves of 30-day-old seedlings were examined by TEM. The data showed that the chloroplasts in pds2-1 were smaller than those in the wild type and lacked starch granules, oil drops and thylakoids, but contained many densely

Figure 4. Expression analysis of the HST gene in WT Arabidopsis. (A) Tissue-specific expression as determined by qRT-PCR. The UBQ10 gene was used as an internal control. Values are means ± SD (n = 3). Rt: roots; RL: rosette leaves; CL: cauline leaves; St: stems; Fl, flowers. (B) HST transcript levels at different leaf development stages. NS: no senescence; ES: early senescence; LS: late senescence. (C–J) Representative GUS expressions in HSTpro:GUS transgenic Arabidopsis. (C) 5-day-old etiolated seedling; (D) 15-day-old seedling; (E) mature leaf of a 4-week-old plant; (F) mature silique; (G) root; (H) inflorescence and flowers; (I) trichome from a stem; (J) leaves at different developmental stages.

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stained globule structures that resembled plastoglobulins (Figure 3 G–J). These results suggested that proplastids in the mesophyll cells of the pds2-1 mutant have lost the ability to develop into mature chloroplasts. The Rubisco large subunit levels were also highly reduced in pds2-1 when total protein was analyzed by SDS-PAGE (Figure S6) and this was consistent with the albino phenotype and the chloroplast development defects in pds2-1. These data suggested that HST played an important role in proplastid growth and thylakoid membrane formation.

An anti-sense HST-RNAi vector was used to transform Arabidopsis WT plants and six transgenic Arabidopsis lines were analyzed for phenotype and chloroplast defects (Figure S7 A). Leaves from one adult RNAi transgenic line (Anti-HST) were stained green color than the WT plant leaves. The HST transcripts were significantly down-regulated in this line compared to the WT plants and the darker green RNAi lines (Figure S7 B,C). The levels of Chl a and b were also significantly lower in Anti-HST plants compared to the WT lines (Figure S7 D).

The HST Gene Rescues the pds2-1 Mutant Phenotype

HST cDNA, driven by the 35S promoter, was inserted into the genome of the pds2-1 heterozygous mutant. Transgenic seeds were screened on 1/5 MS plates containing 3% sucrose and 20 mg L⁻¹ hygromycin and six independent lines were selected. Homozygous pds2-1 plants with 35S::::HST were confirmed by PCR (Figure 5 A–C). T₂ progeny of pds2-1 that were homozygous for 35S::::HST displayed a phenotype that was similar to the WT plants (Figure 5 D–J). These results showed that the HST gene driven by the 35S promoter could completely rescue the mutant phenotype.

Severe Declines in GA and ABA Content Were Seen in pds2-1 Leaves

ELISA were performed to determine the concentrations of ABA, GA₃, zeatin riboside (ZR) and IAA in the aerial parts of 3-week-old WT and pds2-1 plants. A substantial decrease in ABA (40%), GA₃ (61.5%) and ZR (69.8%) concentrations occurred in pds2-1 plants compared to WT plants (Table 2) but the ratio of GA₃:ABA was increased from 0.0621 in WT to 0.0923 in pds2-1 (Table 2). This disruption of the HST gene also led to a substantial increase (by 41.6%) in the IAA content of pds2-1.

Gene Transcriptions That Specified Carotenoids, GA, ABA, Trichomes and Roots Were Depressed in pds2-1

Previous reports indicated that albino mutants blocked the MEP pathway and led to a decrease in carotenoid, GA and ABA biosynthesis [35]. Therefore, we investigated whether disruption of the HST gene changed the expression of genes involved in these pathways using qRT-PCR. DAX, DAX, LYC, IM, PST and ZDS are involved in the MEP pathway and in the biosynthesis of carotenoids [38–40]. GGPS encodes an enzyme involved in isoprenoid biosynthesis [41] and GGRS encodes a protein with geranylgeranyl reductase activity [42]. The PDS1 gene (encoding the p-hydroxyphenylpyruvate) plays an important role in the synthesis of both plastocyanin and tocopherols in plants [43]. GA1, GA2 and GA3 are involved in GA biosynthesis [19,20,44]; ABA1 plays an important role in ABA accumulation [43] and GL2, WAVE1 and WAVE2 are involved in trichome and root hair initiation [46]. The results showed that transcription of all these selected genes was significantly lower in pds2-1 plants compared to WT plants and the key gene in ABA biosynthesis, ABA1, was almost absent in the pds2-1 plants (Figure 6).

A Mutation in HST Promotes Early Flowering in pds2-1

Although the growth of homozygous mutants was severely retarded, the pds2-1 albino plants could bolt when grown on 1/5 MS containing 3% sucrose (Figure 7 A). All the homozygous plants had flower-like structures that never matured into normal flowers (Figure 7 A,B and D). Therefore, pds2-1 had to be propagated in the heterozygous state, which suggested that the gene was essential for plant development. Moreover, flowering time was disrupted in pds2-1 plants and they flowered earlier than the WT plants (Figure 7 A,C). This conclusion is supported by the light green-colored anti-sense HST-RNAi line, anti-L2, which also displayed an early flowering phenotype (Figure 7 D). Transcripts for two flowering genes, FLC and GAI, were also significantly depressed in pds2-1 plants, but CO, SOC1 and FT were unaffected by the mutation (Figure 7 E).

HST Disruption Affects the Expression of Many Photosynthetic Genes, Cellular Components and Biological Processes

RNA sequencing was carried out to further explore the effects of the HST gene on metabolic pathways. A total of 1254 unique genes exhibited two fold or greater differential expression in pds2-1 plants compared to WT plants, with 351 genes up-regulated and 903 genes down-regulated. A total of 222 differentially expressed genes were then identified with a false discovery rate (FDR) of less than 0.05 (Table S2). Gene Ontology (GO) was used to analyze the differentially expressed genes by functional annotation and enrichment analysis, which required the use of singular enrichment analysis (SEA) and the Parametric Analysis of Gene set Enrichment tool (PAGE) [47]. In total, 212 of the 222 differentially expressed genes were assigned to at least one term in GO under the different biological process categories (molecular function, subcellular structure/components and biological process) and the number of significant GO terms was 138. In pds2-1 mutants, the expression levels of many genes related to cellular structure/components, including thylakoids, plastids, chloroplasts, photosynthetic membrane, plastocyanine, plastoglobules, light-harvesting complexes, organelles, the cytoplasm and the apoplast, were dramatically reduced (Table S3). Moreover, the expression levels of molecular components involved in photosynthesis, the light reaction, pigment metabolic processes and processes such as

| Table 1. Chl and carotenoids contents of WT and pds2-1 mutant plants. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Arabidopsis | Chl a (mg L⁻¹) | Chl b (mg L⁻¹) | Car (mg L⁻¹) | Chl (mg L⁻¹) | Chl a/b | Chl/Car |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| WT | 19.03±0.40 | 8.01±1.86 | 5.17±0.82 | 27.04±2.76 | 2.60±0.93 | 5.29±0.38 |
| pds2-1 | 0.10±0.01** | 0.15±0.01** | 0.01±0.00** | 0.25±0.01** | 0.70±0.03** | 20.79±3.25** |

Leaves from 40-day-old plants were sampled to determine the Chl and carotenoid contents. ** indicates significant differences of the means at P<0.01 between WT plants and pds2-1 mutant plants for each parameter measured (n=4). doi:10.1371/journal.pone.0094031.t001
the regulation of catalytic activity, response to stimuli, and response to stress, had also decreased significantly. When differentially expressed genes involved in cellular components were submitted to the PAGE tool after RNA sequencing, 22 GO terms (including thylakoid, chloroplast, photosynthetic membrane, etc.) were significantly over-represented in the hierarchical tree (Figure 8; Table S4).

GA$_3$ and ABA partially rescued the pds2-1 mutant phenotype

The 14-day-old pds2-1 seedlings were transferred onto K$_2$MS medium containing 3% sucrose and 10 $\mu$M GA$_3$. Two weeks later, the leaf petioles and roots of all the tested pds2-1 plants were longer than those grown without GA$_3$ and root hair density had increased (Figure 9 A–D, G). The pistils of pds2-1 mutants grew longer with GA$_3$ supplementation, but flower buds did not develop into mature flowers (Figure 9 E). Stem trichomes were obvious on pds2-1 mutant plants when treated with GA$_3$ (Figure 9 F). The pds2-1 plants treated with GA$_3$ also had extended petioles, were larger and grew faster than those without GA$_3$ and they produced more branches and inflorescences (Figure 9 G,H).

ABA plays an important role in regulating stomata closure in plants. To find out whether the stomata structural defect in pds2-1 plants was caused by ABA absence, 14-day-old pds2-1 seedlings were transferred onto K$_2$MS medium supplemented with ABA. Two days later, the leaves of the mutant plants were harvested and the stomata structure observed using light microscopy. The stomata closed normally in pds2-1 mutants with exogenous ABA (Figure 9 J–K), which suggested that the stomata closure defect was caused by an ABA deficiency in pds2-1 mutants.

**Discussion**

In this study, we identified an albino mutant, pds2-1, and showed that disruption to the HST gene resulted in an albino phenotype and developmental defects. Homozygous pds2-1 mutants all died when they were grown on soil or $\frac{1}{2}$MS medium without sucrose, but produced albino leaves or white inflorescences when 3% sucrose was included. Other Arabidopsis mutants exhibited a similar albino phenotype when DXR, PDS or the IspH homologue of an isoprene biosynthetic gene were disrupted [9,33,48,49].

RT-PCR and GUS staining showed that HST was expressed in almost all the plant tissues and at higher levels in green tissues. HST protein contains a putative 69 aa chloroplast transit peptide at its N-terminus. Subcellular localization analysis of HST in a

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Table 2. Plant hormone concentrations in pds2-1 and WT seedlings grown on $\frac{1}{2}$ MS plates.

| Plant hormone | WT (ng g$^{-1}$ FW) | pds2-1 (ng g$^{-1}$ FW) | pds 2-1:WT | P-value |
|---------------|---------------------|--------------------------|------------|---------|
| ABA           | 138.38 ± 2.95       | 59.58 ± 2.68             | 0.431      | <0.0001 |
| GA$_3$        | 8.95 ± 0.24         | 5.5 ± 0.15               | 0.615      | <0.0001 |
| ZR            | 15.94 ± 0.56        | 11.12 ± 0.43             | 0.698      | <0.0001 |
| IAA           | 56.49 ± 2.14        | 79.98 ± 2.18             | 1.416      | 0.0002  |

Three-week-old seedlings were sampled to determine the levels of four plant hormones. P-values represent significant differences of the means between WT and pds2-1 tissues (n = 3) after comparing them using Student's t-test. FW: fresh weight. doi:10.1371/journal.pone.0094031.t002

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Figure 5. HST gene rescues the phenotype of pds2-1 mutant plants. (A) PCR analysis of transgenic Arabidopsis using the HST-f and HST-r primers. The 2,860 bp band represents HST gDNA and the 1,202 bp band represents the HST coding domain sequence in transgenic Arabidopsis. M: DNA marker; WT: wild type; L1-5: five independent plants transformed with 35Spro::HST. (B) PCR analysis of transgenic Arabidopsis using the R3 and hst-g2 primers. The 466 bp band represents the T-DNA insertion in the HST gene. (C) PCR analysis of transgenic Arabidopsis using the hst-g1 and hst-g2 primers. Absence of the 969 bp band shows that L4 is a pds2-1 homozygous mutant. (D) Representative phenotype of pds2-1 homozygous plants rescued by transforming them with 35Spro::HST.

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previous report showed that HST was targeted at chloroplasts [21]. In this study, TEM analysis demonstrated that pds-1 mutant chloroplasts were smaller and developmentally malformed. Our study confirmed previous reports, which showed that HST disruption resulted in an albino phenotype in Arabidopsis [4,21]. We measured Chl levels to confirm the negative effect of a mutated HST gene on the photosystem and found that the pds-1 mutant had significantly reduced Chl levels, which was consistent with the reduced expression we measured for key genes involved in chloroplast biosynthesis. Moreover, SDS-PAGE analysis of the total proteins in pds-2-1 plants also confirmed that the photosystem was destroyed and that RuBisCo was not produced after total proteins in pds-2-1 plants also confirmed that the photosystem was destroyed and that RuBisCo was not produced after total proteins in pds-2-1 plants also confirmed that the photosystem was destroyed and that RuBisCo was not produced after.

HST affects GAs biosynthesis. The pds-2-1 mutants showed a pronounced dwarf phenotype and trichome and root hair development defects. The pds-2-1 mutants showed a pronounced dwarf phenotype and trichome and root hair development defects. The pds-2-1 mutants showed a pronounced dwarf phenotype and trichome and root hair development defects.

Carotenoids play important roles in many physiological processes and in chloroplast biogenesis. They are also precursors of ABA. biochemical analysis of the pds-2-1 mutant plants revealed that carotenoids, ABA and PQ levels were dramatically reduced in plant leaves. HST is a critical enzyme in the PQ biosynthesis pathway. PQ is both a PSII electron carrier and a critical component that links carotenoid and ABA biosynthesis [4–6] with many pathways through direct and indirect mechanisms. PQ is both a PSII electron carrier and a critical component that links carotenoid and ABA biosynthesis [4–6] with many pathways through direct and indirect mechanisms. PQ is both a PSII electron carrier and a critical component that links carotenoid and ABA biosynthesis [4–6] with many pathways through direct and indirect mechanisms.

Figure 6. qRT-PCR analyses of genes involved in the carotenoid, GA and ABA biosynthetic pathways and in the regulation of trichome and root hair development. Significant differences of the means (± SD) between WT plants and pds2-1 plants (n = 3) are indicated by * (P <0.05) and ** (P <0.01). The Arabidopsis TUB2 gene was used as an internal control.

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Figure 7. Reduced expression of HST results in an early flowering phenotype. (A) Typical 22-day-old pds2-1 and WT seedlings. Black arrows indicate floral structures on bolting pds2-1 plants. (B) Onset of early flowering in three pds2-1 plants compared with WT plants. (C) Anti-HST early flowering phenotype. (D) Expression analysis by qRT-PCR of genes involved in regulating flowering time in pds2-1 plants. Significant differences of the means (± SD) between WT plants and pds2-1 plants (n = 3) are indicated by * (P <0.05). The Arabidopsis TUB2 gene was used as an internal control. FLC: Flowering Locus C; GI: Gigantea; CO: Constans; SOC1: Supressor of Overexpression of Constance1.

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GA levels decreased in *pds2-1* mutants. ABA is believed to act as an antagonist to GAs during plant growth and development [61,62]. ABA biosynthesis mutants have an early flowering phenotype [61,63] and ABA can delay flowering onset by up-regulating the expression of *FLC*, as occurs in Arabidopsis [64]. In fact, although the levels of both hormones fell in *pds2-1* seedlings, the overall ratio of GAs:ABA increased by 48.6% (Table 2). Genetic analyses of the interaction between GAs and ABA shows that GAs play a major rate-limiting role in floral promotion [61]. Our findings, together with an earlier report, suggested that the increased ratio of GA:ABA in *pds2-1* mutants had maintained the predominant role played by GA and caused the triggering of the early transition from vegetative to reproductive growth. A second interpretation is that *HST* disruption affected other hormone levels. Earlier reports showed that a number of hormones, including GAs, ABA and auxins, played important roles in regulating flowering time [65]. For example, exogenous IAA was able to induce flowering in long day plants [66]. Our ELISA results showed that auxin levels (IAA) increased by 41.6% in the *pds2-1* mutant compared to the WT plants. Hence, the early flowering phenotype displayed by *pds2-1* mutant plants could also be due to their increased IAA levels. In summary, disruption of the *HST* gene promoted flowering by affecting the levels of several plant hormones.

Screening of a small Arabidopsis T-DNA population uncovered a novel albino mutant *pds2-1* with a T-DNA insertion in the *HST* gene. This gene is known to play an important role in PQ biosynthesis. The *pds2-1* mutation resulted in a typical carotenoid-deficient phenotype, with reduced PQ, abnormal chloroplast development, reduced photo-protection and reduced PS II
activity. These findings confirmed that the *HST* gene plays an important role in pro-plastid growth and thylakoid membrane formation. However, the mutation also produced novel IAA-enhanced, ABA-deficient and GA-deficient phenotypes that had not been reported previously. Furthermore, GA$_3$ and ABA application partially rescued the mutant phenotype. Other novel phenotypes not previously reported for this gene include short roots and petioles, fewer leaf numbers, root hairs and trichomes, more swollen stomata, an early flowering date and high expression of *HST* gene in green tissues. Moreover, qRT-PCR and RNA sequencing confirmed that the mutant had a blocked MEP pathway and the genes that played a role in GA biosynthesis did not function. This detailed analysis of the *pds2-1* mutant paves the way for a comprehensive study of the physiological regulation of carotenoids, chloroplast components and other physiological processes by major plant hormones through the use of a combination of genetic, biochemical and molecular approaches. *HST* will probably lie at the heart of these processes as it appears to impact directly and indirectly on so many hormones and biological processes.

Figure 9. Exogenous application of GA$_3$ or ABA to *pds2-1* mutants. (A) Root hair of a *pds2-1* mutant plant after 10 μM GA$_3$ treatment. Scale bar = 1 mm. (B) Root hair of a *pds2-1* mutant plant without GA$_3$ treatment. Scale bar = 1 mm. (C) Representative petiole lengths from *pds2-1* mutants with exogenously applied GA$_3$ (GA$_3$+) or without GA$_3$ (GA$_3$–). Scale bar = 1 mm. (D) Petiole length analyses of *pds2-1* plants with or without GA$_3$ treatment. A total of 30 leaves (from 10 plants) were measured for each sample. ** indicates significantly different means (± SD) at $P<0.01$ compared to those without GA$_3$ treatment. (E) The longer flower bud pistil seen on *pds2-1* mutant plants after GA$_3$ treatment. Scale bar = 0.5 mm. (F) Trichomes (arrows) on a *pds2-1* mutant stem after GA$_3$ treatment. (G) Sizes of bolting *pds2-1* mutant plants grown on $\frac{1}{2}$ strength MS medium with or without GA$_3$ treatment. (H) Mature *pds2-1* mutant plant showing multiple flowing shoots after GA$_3$ treatment. (I) Usual stomata structure of a WT plant without ABA treatment. Scale bar = 5 μm. (J) Swollen open stomata structure of a *pds2-1* mutant without ABA treatment. Scale bar = 5 μm. (K) Closed stomata structure of a *pds2-1* mutant after 10 μM ABA treatment. Scale bar = 5 μm.

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**Supporting Information**

**Figure S1** Seed segregation in a silique from a heterozygous *pds2-1* mutant plant. (TIF)

**Figure S2** Microscopic analysis of root hair from the *pds2-1* mutant and WT Arabidopsis. (TIF)

**Figure S3** Trichome phenotype of leaves from *pds2-1* and WT Arabidopsis. (TIF)

**Figure S4** Microscopic and statistical analyses of stomata from the *pds2-1* mutant and WT Arabidopsis. (TIF)

**Figure S5** Representative TAIL-PCR analysis of heterozygous (*PDS2/pds2*) plants. (TIF)

**Figure S6** SDS-polyacrylamide gel electrophoresis showing loss of a major protein subunit (Rubisco) in *pds2-1*. (TIF)

**Figure S7** Gene expression and pigments in HST RNAi transgenic lines. (TIF)

**Table S1** Primers used in RT-qPCT analyses. (DOC)

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