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Chloroplast localization of methylerythritol 4-phosphate pathway enzymes and regulation of mitochondrial genes in *ispD* and *ispE* albino mutants in Arabidopsis

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Abstract Plant isoprenoids are derived from two independent pathways, the cytosolic mevalonate pathway and the plastid methylerythritol 4-phosphate (MEP) pathway. We used green fluorescent fusion protein assays to demonstrate that the Arabidopsis MEP pathway enzymes are localized to the chloroplast. We have also characterized three Arabidopsis albino mutants, *ispD-1*, *ispD-2* and *ispE-1*, which have T-DNA insertions in the *IspD* and *IspE* genes of the MEP pathway. Levels of photosynthetic pigments are almost undetectable in these albino mutants. Instead of thylakoids, the *ispD* and *ispE* mutant chloroplasts are filled with large vesicles. Impairments in chloroplast development and functions may signal changes in the expression of nuclear, chloroplast and mitochondrial genes. We used northern blot analysis to examine the expression of photosynthetic and respiratory genes in the *ispD* and *ispE* albino mutants. Steady-state mRNA levels of nucleus- and chloroplast-encoded photosynthetic genes are significantly decreased in the albino mutants. In contrast, transcript levels of nuclear and mitochondrial genes encoding subunits of the mitochondrial electron transport chain are increased or not affected in these mutants. Genomic Southern blot analysis revealed that the DNA amounts of mitochondrial genes are not enhanced in the *ispD* and *ispE* albino mutants. These results support the notion that the functional state of chloroplasts may affect the expression of nuclear and mitochondrial genes. The up-regulation of mitochondrial genes in the albino mutants is not caused by changes of mitochondrial DNA copy number in Arabidopsis.

Keywords Arabidopsis · Albino · Chloroplast · Isoprenoid biosynthesis · Methylerythritol 4-phosphate pathway · Mitochondrial gene expression

Introduction

Isopentenyl diphosphate (IPP) and its allyl isomer dimethylallyl diphosphate (DMAPP) are the basic five-carbon units for all isoprenoids. In plants, IPP and DMAPP are synthesized via two independent pathways, the cytosolic mevalonate (MVA) pathway and the plastid methylerythritol 4-phosphate (MEP) pathway. In the cytosolic MVA pathway, IPP and DMAPP are derived from three molecules of acetyl-CoA and the conversion of 3-hydroxy-3-methylglutaryl (HMG) CoA to MVA by HMG-CoA reductase is the limiting step of the pathway. It is believed that ubiquinones, sesquiterpenes, sterols, triterpenes and polyterpenes are synthesized via the cytosolic MVA pathway in plants (Lichtenthaler 1999; Rodriguez-Concepcion and Boronat 2002; Suzuki et al. 2004).

The MEP pathway was originally discovered in bacteria (Rohmer et al. 1993). Most, if not all, enzymes involved in the MEP pathway have been identified in *Escherichia coli* (Sprenger et al. 1997; Lois et al. 1998; Takahashi et al. 1998; Rohdich et al. 1999, 2002, 2003; Herz et al. 2000; Luttgen et al. 2000; Hecht et al. 2001; Adam et al. 2002). Pyruvate and glyceraldehyde-3-phosphate, instead of acetyl-CoA, are the precursors of the MEP pathway. In the first step, the 1-deoxy-D-xylulose 5-phosphate (DOXP)

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synthase (DXS) converts pyruvate and glyceraldehyde-3-phosphate to DOXP, which also serves as a precursor of vitamins B1 (thiamine) and B6 (pyridoxal) in bacteria (White 1978; Sprenger et al. 1997). DOXP is then converted to 2-C-methyl-D-erythritol 4-phosphate (MEP) by the DOXP reductoisomerase (DXR). With the addition of CTP, MEP is converted to 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) by CDP-ME synthase (CMS or IspD). CDP-ME is then phosphorylated by CDP-ME kinase (CMK or IspE) and the product, 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate (CDP-ME2P), is converted to IPP and DMAPP in consecutive steps catalyzed by enzymes encoded by the *IspF*, *IspG*, and *IspH* genes (Fig. 1a). This pathway is called the MEP pathway, because MEP is the first committed compound in bacteria. However, it has been shown that the biosynthesis of vitamin B6 is derived from ribulose-5-phosphate from the pentose phosphate pathway in Arabidopsis (Tambasco-Studart et al. 2005, 2007). Thus DOXP, instead of MEP, may be the first committed precursor in the MEP pathway in plants.

The Arabidopsis genome contains genes encoding homologs of the *E. coli* MEP pathway enzymes (Rodriguez-Concepcion and Boronat 2002). Predictions by the TargetP program (http://www.cbs.dtu.dk/services/TargetP/) indicate that the Arabidopsis MEP pathway enzymes all possess a transit peptide for chloroplast localization. The subcellular localization of some Arabidopsis MEP pathway enzymes has been demonstrated. For instance, the chloroplast localization of Arabidopsis DXR and IspG has been established by green fluorescent protein (GFP) fusion studies (Carretero-Paulet et al. 2002; Querol et al. 2002). We have previously shown that the Arabidopsis IspH is a chloroplast stromal protein by organelle fractionation and immunoblot analysis (Hsieh and Goodman 2005). In addition to Arabidopsis, the chloroplast localization of the other plant MEP

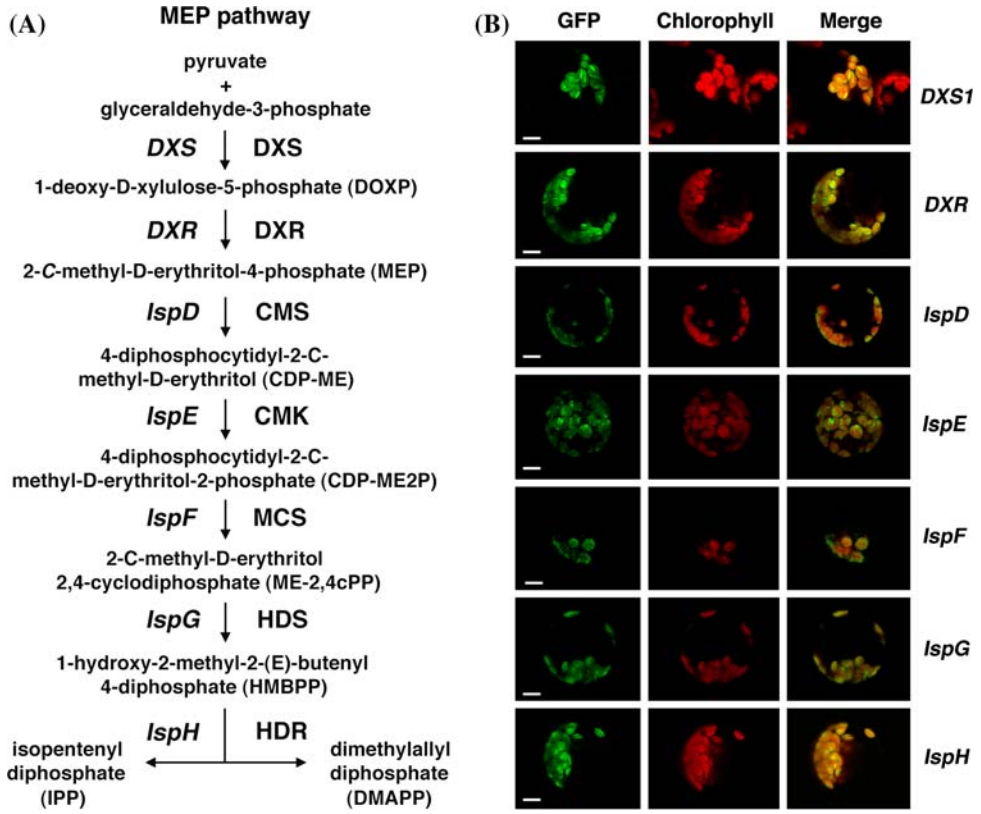

Fig. 1 Subcellular localization of Arabidopsis methylerythritol 4-phosphate (MEP) pathway enzymes. (**a**) The MEP pathway of plastid isoprenoid biosynthesis in Arabidopsis. Enzymes of the MEP pathway are indicated on the right and the names of their corresponding genes are indicated on the left of each step. DXS1, DOXP synthase1; DXR, DOXP reductoisomerase; CMS, CDP-ME synthase (IspD); CMK, CDP-ME kinase (IspE); MCS, ME-2,4cPP synthase (IspF); HDS, HMBPP synthase (IspG); HDR, HMBPP reductase (IspH). (**b**) Chloroplast localization of Arabidopsis MEP pathway enzymes. Arabidopsis protoplasts were transformed with the indicated transit peptide-GFP fusion constructs, and the localization of green fluorescent signals was examined at 24 h after transformation by confocal laser scanning microscopy. Chloroplasts were visualized by red chlorophyll autofluorescence. In *DXS1*, one transformed protoplast showing green fluorescence and part of two non-transformed neighboring protoplasts (dark, no green fluorescence) were shown. The autofluorescence of chlorophylls (red) was observed in all three protoplasts. A representative transformed protoplast showing green fluorescence and red chlorophyll autofluorescence was shown for the other GFP fusion constructs. Scale bars are 10 μm

pathway enzymes has been established for DXS in tomato (Lois et al. 2000) and IspD and IspF in *Ginkgo biloba* (Kim et al. 2006a, b).

Because carotenoids and the phytol side chain of chlorophylls are derived from the plastid MEP pathway, plants carrying mutations in the MEP pathway genes are expected to have a pigmentation phenotype. It has been shown that null mutants of the MEP pathway genes are albino lethal in Arabidopsis. For instance, the Arabidopsis *cla-1* albino mutant is caused by loss-of-function of the *DXS1* gene (Mandel et al. 1996; Estevez et al. 2000). In contrast, Arabidopsis *DXS1* overexpressers have increased levels of various isoprenoids including chlorophylls, carotenoids, abscisic acids and gibberellins (Estevez et al. 2001). A mass screening for seedling lethal mutants from collections of T-DNA and transposon insertion lines has identified albino mutants disrupted in the *DXS*, *DXR*, and *IspD* genes, but these lines have not been further verified or characterized (Budziszewski et al. 2001). In Arabidopsis *IspD* antisense plants, levels of photosynthetic pigments and the GA precursor *ent*-kaurene are significantly reduced (Okada et al. 2002). Arabidopsis *clb4* albino mutants are defective in the *IspG* gene (Gutierrez-Nava et al. 2004). Arabidopsis *ispH* null mutants are albino lethal (Guevara-Garcia et al. 2005; Hsieh and Goodman 2005). Recently, we have also shown that the Arabidopsis *ispF* null mutants are albino lethal (Hsieh and Goodman 2006). In addition to loss-of-function studies on the MEP pathway mutants, constitutive overexpression of the tomato *IspH* in Arabidopsis results in increased carotenoid levels (Botella-Pavia et al. 2004). An increased accumulation of chlorophylls and carotenoids was also observed in Arabidopsis *DXR*-overexpressing lines (Carretero-Paulet et al. 2006).

Chloroplasts and mitochondria are highly interdependent in several biochemical pathways (Raghavendra and Padmasree 2003). However, we know very little about the interactions between these two organelles at the gene expression level. There are three genomes compartmented in nucleus, chloroplasts and mitochondria inside a plant cell. Thus co-ordination of gene expression among these genomes is important in plant cells. Despite having their own genomes, the biogenesis and functions of chloroplasts and mitochondria require the involvement of many nuclear genes (Leon et al. 1998; Leister 2005). Moreover, functional states of chloroplasts and mitochondria may affect the expression of nuclear genes via retrograde signaling pathways (Leister 2005; Nott et al. 2006). It is not clear if the expression of mitochondrial genes is affected in the chloroplast MEP pathway mutants.

Here we used GFP fusion protein assays to demonstrate that all the Arabidopsis MEP pathway enzymes are localized to the chloroplast. In addition, we showed that homozygous T-DNA insertion mutants *ispD-1*, *ispD-2* and *ispE-1* are albino lethal. The development of thylakoids is completely abolished and levels of photosynthetic pigments are almost undetectable in these mutants. To study the interactions between chloroplasts and mitochondria, we used the *ispD* and *ispE* albino mutants to examine the effects of dysfunctional chloroplasts on the expression of nuclear, chloroplast and mitochondrial genes. Interestingly, steady-state mRNA levels of some nuclear and mitochondrial genes encoding subunits of the mitochondrial electron transport chain complexes are increased or not affected in these albino mutants. Our results support the notion that the inter-organellar crosstalk between chloroplasts and mitochondria also occurs at the gene expression level.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 was grown on half strength Murashige and Skoog (MS) plates [MS salts (Sigma), pH adjusted to 5.7 with 1 N KOH, 0.8% (w/v) agar] containing 2% sucrose, or in soil in the greenhouse on a 16 h light/8 h dark cycle at 23°C. Seeds of *ispD-1* (SALK_042163), *ispD-2* (SALK_030640) and *ispE-1* (SALK_107310) were obtained from the Arabidopsis Biological Resource Center. Determination of total chlorophylls and carotenoids in 2-week-old Arabidopsis seedlings grown in tissue culture was conducted as described (Lichtenthaler and Wellburn 1983).

Analyses of DNA and RNA

Arabidopsis total RNA was isolated using a phenol extraction protocol (Jackson and Larkins 1976). For RNA gel blot analysis, a gene-specific digoxigenin (DIG)-labeled single-stranded DNA probe was generated by PCR (Myerson 1991). Primers used for making DIG-labeled gene-specific probes are listed in Supplementary Table 1. Primers for making probes to detect the expression of *psaN* (U32176), *psbA* (X79898), *psbP* (X98108), *CAB* (X03909), *rbcL* (U91966) and *rbcS* (X13611) genes were designed as described (Motohashi et al. 2001). For light induction experiments, 3-day-old etiolated seedlings exposed to light for 0, 1, 2 and 4 h were used for RNA extraction. One microgram of total RNA treated with DNase I was used as a template for first-strand cDNA synthesis in a volume of 20 µl with 1 µl of Superscript III RT (Invitrogen). The PCR regime was 30 s at 94°C, 30 s at 55°C and 1 min at 72°C with 25 cycles for the *UBQ10* and 30 cycles for the *IspD* and *IspE* genes. The following primers were used for RT-PCR analysis to examine the effects of light on the

expression of *IspD* and *IspE*. *IspD*, 5′-ATGGCGATGCT TCAGACGAA-3′, 5′-GATTTCCTGAAGTCCACTGTA-3′; *IspE*, 5′-ATGGCAACGGCTTCTCCTCC-3′, 5′-GAGCTCA TTTGCCGCCCAGAG-3′; *UBQ10*, 5′-CGATTACTCTTG AGGTGGAG-3′, 5′-AGACCAAGTGAAGTGTGGAC-3′. Arabidopsis genomic DNA was extracted using a standard urea extraction buffer (Hsieh et al. 1998). For genomic Southern blot analysis, one microgram of total DNA from 2-week-old *ispD-1*, *ispD-2*, *ispE-1* and wild-type plants was digested with *Bam* HI. The same probes used to detect *cob* and *cox1* transcripts in RNA gel blot analyses were used in genomic Southern blot analyses. Primers 5′-GACAGACTG AGAGCTCTTTC-3′ and 5′-ACAGGTATCGACAATGAT CC-3′ were used to make DIG-labeled probe to detect the nuclear 18S rDNA gene. DIG probe labeling, pre-hybridization, hybridization, wash conditions and detection were performed according to Roche's DIG Application Manual for Filter Hybridization.

Transmission electron microscopy

The leaf samples were fixed in 4% glutaraldehyde, 100 mM sodium cacodylate (pH7.2) for 16 h at 4°C, and postfixed with 1% osmium tetroxide in the same buffer for 6 h at 4°C. The fixed samples were dehydrated through a series of alcohol solutions and embedded in Spurr resin. Ultrathin sections were cut on a Reichert Ultracut-S (Leica Microsystems, Bannockburn, IL) and stained with uranyl acetate and lead citrate and viewed with a transmission electron microscope, JEOL 1200EX (JEOL USA, Peabody, MA).

GFP fusion proteins

The N-terminal cDNA sequences encoding amino acids encompassing putative transit peptides of DXS1, DXR, IspD, IspE, IspF, IspG and IspH were amplified by PCR, digested with *Nco* I and *Stu* I, and cloned into the N-terminus of a GFP expression vector driven by a CaMV 35S promoter (Chiu et al. 1996). Primers used for making these GFP fusion constructs are listed in Supplementary Table 2. The resulting constructs encode the first 60, 90, 100, 100, 60, 50 and 52 N-terminal amino acids of DXS1, DXR, IspD, IspE, IspF, IspG and IspH, respectively, fused to GFP. These GFP fusion constructs were transformed into Arabidopsis protoplasts and observed under confocal laser scanning microscope 510 META Zeiss. As a control, the GFP empty-vector was also transformed into Arabidopsis protoplasts and observed under confocal laser scanning microscope. The green fluorescent signal of GFP empty-vector was mainly observed in the cytosol and nucleus of transformed Arabidopsis protoplasts (data not shown) (Chiu et al. 1996).

Results

Chloroplast localization of the Arabidopsis MEP pathway enzymes

All Arabidopsis MEP pathway enzymes are predicted to have an N-terminal transit peptide for chloroplast localization (http://www.cbs.dtu.dk/services/TargetP/). To provide experimental evidence for the subcellular localization of the Arabidopsis MEP pathway enzymes, we fused the N-terminal regions encompassing putative transit peptides of DXS1, DXR, IspD, IspE, IspF, IspG and IspH to the N-terminus of a reporter green fluorescent protein (GFP). The resulting GFP fusion constructs were transformed into Arabidopsis protoplasts and observed under confocal laser scanning microscope. The green fluorescent signals of these GFP fusion proteins co-localized with the auto-fluorescence of chlorophylls (Fig. 1b). These results demonstrate that the putative transit peptides of the Arabidopsis MEP pathway enzymes are able to target the reporter GFP to the chloroplast.

Molecular characterization of *ispD-1*, *ispD-2* and *ispE-1* mutants

Because carotenoids and the phytol side chain of chlorophylls are derived from the plastid MEP pathway, plants defective in this pathway are expected to have a reduced pigmentation phenotype. We obtained seeds of *ispD-1* (SALK_042163), *ispD-2* (SALK_030640) and *ispE-1* (SALK_107310) T-DNA insertion mutants from the Arabidopsis Biological Resource Center. The T-DNA insertion sites in *IspD* and *IspE* genes were initially confirmed by PCR with T-DNA and gene-specific primers (data not shown). Genomic Southern blot analyses were used to verify the T-DNA insertions of these mutant lines (Supplementary Fig. 1). Homozygous *ispD-1*, *ispD-2* and *ispE-1* plants are albino lethal and progeny from a self-pollinated heterozygous plant segregate green and albino plants in a ratio 3:1 on a non-selective medium, i.e. the albino phenotype is inherited as a recessive mutation (Fig. 2a). Schematic diagrams of Arabidopsis *IspD* and *IspE* genes and their T-DNA insertion sites are shown in Fig. 2b. RNA gel blot analyses revealed that truncated *IspD* transcripts were detected in the *ispD-1* albino plants, whereas *IspD* transcripts were undetectable in the *ispD-2* mutant (Fig. 2c). These results indicate that the T-DNA insertion mutant *ispD-2* is a null mutant. In the *ispD-1*

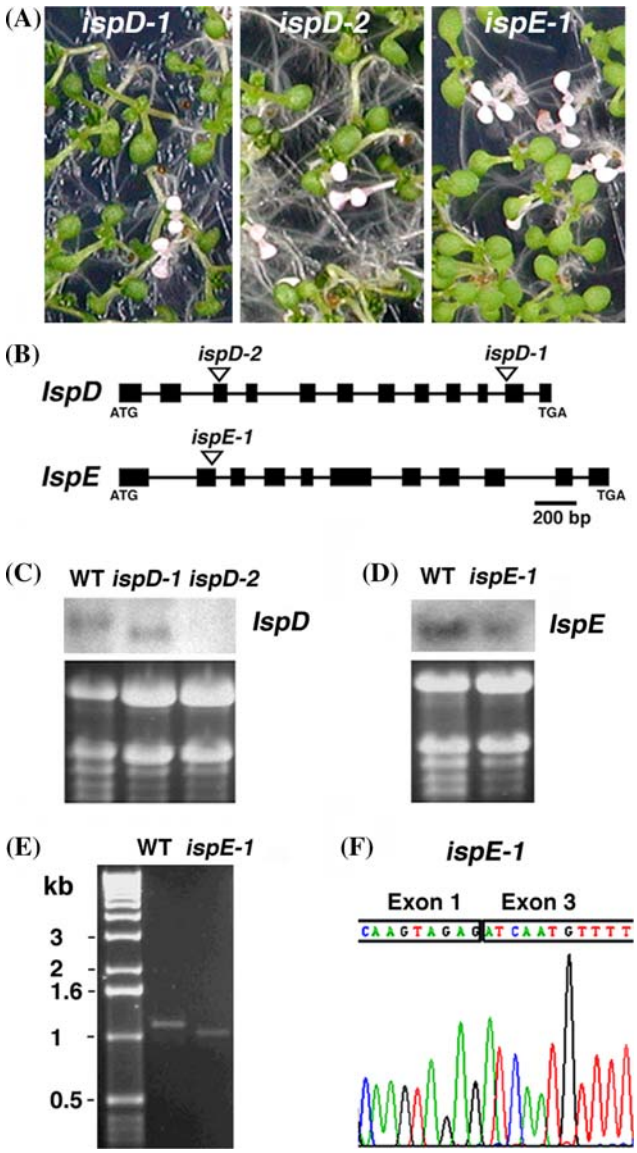

Fig. 2 Molecular characterization of Arabidopsis *ispD-1*, *ispD-2* and *ispE-1* mutants. (**a**) Progeny of self-pollinated *ispD-1*, *ispD-2* and *ispE-1* heterozygous plants segregate 3 green: 1 albino on MS plus sucrose medium. The *ispD-1*, *ispD-2* and *ispE-1* homozygous plants are albino lethal. Plants shown are 7-day-old. (**b**) Schematic diagram of Arabidopsis *IspD* (At2g02500) and *IspE* (At2g26930) genes. Black boxes indicate exons and solid lines indicate introns. Open triangles indicate T-DNA insertion sites. (**c** and **d**) RNA gel blot analyses of *IspD* and *IspE* genes. Ten micrograms of total RNA extracted from 2-week-old Arabidopsis wild-type (WT), *ispD-1*, *ispD-2* and *ispE-1* plants were used for RNA gel blot analysis to detect transcripts of *IspD* and *IspE*. The ethidium bromide-stained agarose gel of the same samples is shown at the bottom. (**e**) RT-PCR analysis of the *IspE* gene in wild type and *ispE-1* mutants. The *ispE-1* mutant cDNA is 96 nucleotides shorter than that of wild type. (**f**) Sequence analysis of *ispE* mutant cDNA. Exon 1 is followed by exon 3 indicating that the entire second exon has been spliced out in the *ispE-1* mutant transcript

mutant, the T-DNA insertion site is located in the eleventh exon of the *IspD* gene. It is possible that the first 10 exons of the *IspD* gene are correctly spliced and remain stable to accumulate as the truncated *IspD* transcript in *ispD-1* plants. It is likely that the truncated *IspD* transcript in the *ispD-1* mutant is either non-translatable or the translated protein is unstable or not functional.

In *ispE-1* albino mutants, the T-DNA insertion site is located in the second exon of the *IspE* gene (Fig. 2b). RNA gel blot analysis revealed that the size of the *ispE-1* mutant transcripts is similar to that of wild type (Fig. 2d). RT-PCR analysis with primers designed to amplify the full length *IspE* cDNA was used to examine the *IspE* transcripts in both wild type and *ispE-1* mutants. The results indicated that the amplified *ispE-1* mutant cDNA was slightly shorter than that of wild type (Fig. 2e). Sequence analysis of *ispE-1* mutant cDNA revealed that the entire second exon (96 nucleotides) of the *IspE* gene is missing in the mutant (Fig. 2f). It is possible that the T-DNA insert and the entire second exon of the *IspE* gene have been spliced out in the *ispE-1* mutant. The accumulated *ispE-1* mutant transcripts may be non-translatable or the translated peptide is unstable or dysfunctional in the mutant.

Ultrastructures of *ispD-1*, *ispD-2* and *ispE-1* mutant chloroplasts

In *ispD-1*, *ispD-2* and *ispE-1* albino plants, total chlorophylls and carotenoids are less than 1% of their amounts in wild-type plants (Table 1). The maximum quantum yield of photosystem II, which may represent the photosynthetic activity, was not detectable in the *ispD* and *ispE* albino mutants (data not shown). Loss of photosynthetic pigments may affect the development of chloroplasts. We used transmission electron microscopy to observe the morphology of wild-type and mutant chloroplasts in 2-week-old mesophyll cells. Compared to the well-developed thylakoid membranes in wild-type chloroplasts, *ispD-1*, *ispD-2* and *ispE-1* mutant chloroplasts are completely devoid of thylakoids. Instead, the mutant chloroplasts are filled with large vesicles (Fig. 3). We also compared the morphology of chloroplasts in leaf vascular tissues of wild-type and *ispD-2* plants. Since some of the vascular tissues are consisted of young and actively dividing cells, chloroplasts at

Table 1 Photosynthetic pigment content of Arabidopsis *ispD* and *ispE* mutants

	Chlorophyll a	Chlorophyll b	Carotenoids
Wild type	802.6 ± 58.8	291.2 ± 39.7	205.2 ± 13.6
ispD-1	4.6 ± 0.4	6.2 ± 0.3	1.8 ± 0.3
ispD-2	4.0 ± 0.5	5.0 ± 0.7	1.6 ± 0.2
ispE-1	3.2 ± 0.5	1.2 ± 0.1	0.7 ± 0.2

Values shown are μg/g fresh weight ± SE

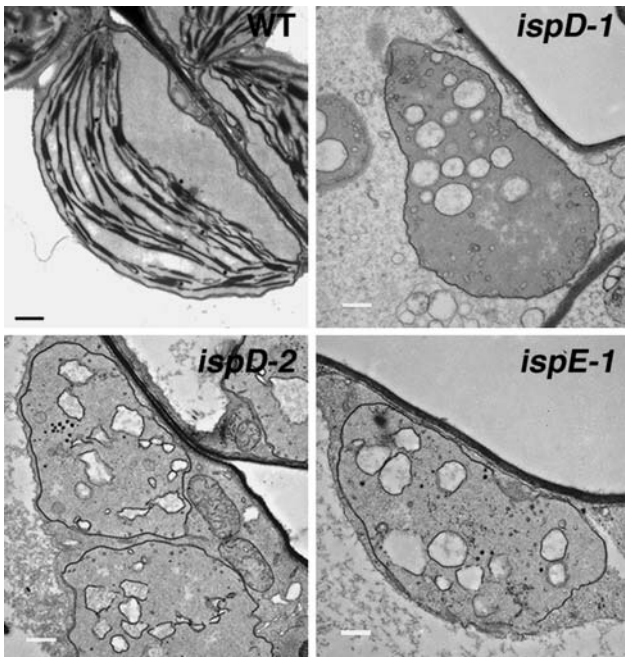

Fig. 3 Ultrastructures of chloroplasts in 2-week-old wild type (WT), *ispD-1*, *ispD-2* and *ispE-1* mesophyll cells. The mutant chloroplasts are completely devoid of thylakoids. Scale bars are 500 nm

early developmental stages may be observed in these cells. In wild-type leaf vascular cells, despite the fact that chloroplasts are smaller than those of mesophyll cells, the thylakoid membrane systems are highly developed (Fig. 4a). In contrast, the mutant *ispD-2* leaf vascular cells only contain proplastid- or amoeboid plastid-like structures consisting of a few invaginations of the inner membrane and a small number of flattened sacs (Fig. 4b). These observations suggest that the development of *ispD-2* mutant chloroplasts is arrested at an early stage.

Light induction of *IspD* and *IspE* genes

We used RNA gel blot analysis to examine the expression patterns of *IspD* and *IspE* genes in various organs of 6-week-old wild-type Arabidopsis plants grown in soil. Steady-state levels of *IspD* mRNA were high in leaves and flowers, medium in stems and low in roots and siliques (Fig. 5a). Similarly, the *IspE* transcripts accumulated in all organs and relatively lower levels of *IspE* mRNA were detected in roots and siliques (Fig. 5a). Light plays an important role in regulating the biosynthesis of photosynthetic pigments. Because the photosynthetic pigments are mainly derived from the MEP pathway, light may also have a role in regulating the expression of *IspD* and *Isp E* genes. We have previously shown that the expression of *IspD* and *IspE* is induced by light in 2 weeks old Arabidopsis plants (Hsieh and Goodman 2005). To mimic natural growth conditions, we examined the effects of light on the expression of Arabidopsis *IspD* and *IspE* genes in 3 days old etiolated seedlings by RT-PCR. The transcript levels of *IspD* and *IspE* were low in 3-day-old etiolated seedlings and exposure to light for 1, 2 and 4 h significantly increased the accumulation of *IspD* and *IspE* transcripts (Fig. 5b). These results suggest that light can rapidly induce the expression of Arabidopsis *IspD* and *IspE* genes in etiolated seedlings.

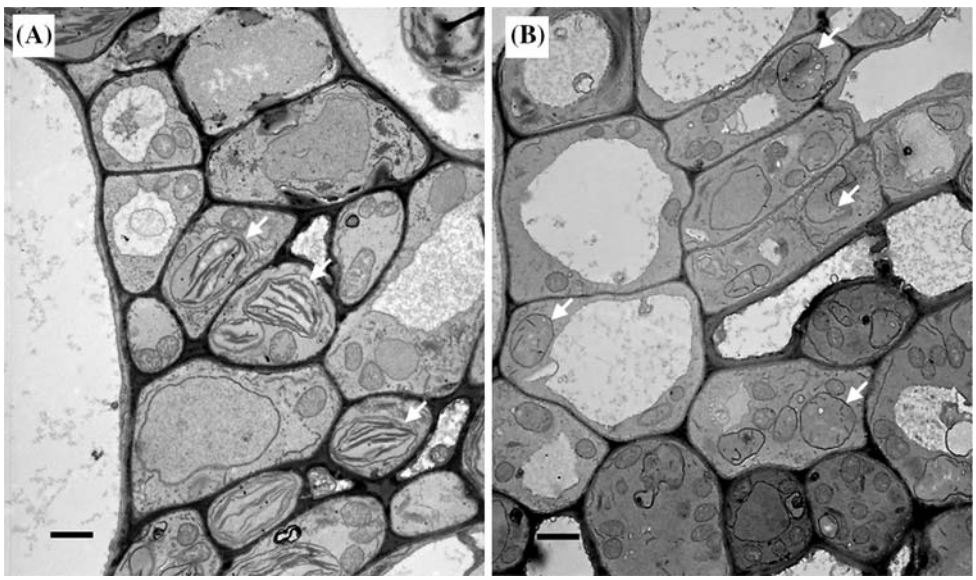

Fig. 4 Transmission electron micrographs of 2-week-old Arabidopsis leaf vascular cells in wild type (**a**) and *ispD-2* (**b**). Arrows indicate young chloroplasts in wild type (**a**), and proplastid- or amoeboid plastid-like structures in *ispD-2* (**b**). Scale bars are 1 μm

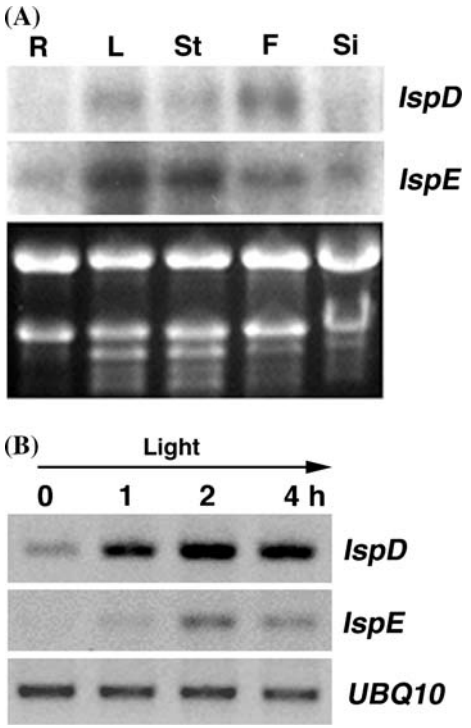

Fig. 5 Expression and regulation of Arabidopsis *IspD* and *IspE* genes. (**a**) RNA gel blot analyses of *IspD* and *IspE* genes in various organs. Ten micrograms of total RNA extracted from roots (R), leaves (L), stems (St), flowers (F) and siliques (Si) of 6-week-old Arabidopsis grown in soil were used for RNA gel blot analyses to detect the transcripts of *IspD* and *IspE*. A representative ethidium bromide-stained agarose gel of the same samples is shown at the bottom. (**b**) Light induction of *IspD* and *IspE* genes. Total RNA extracted from 3-day-old etiolated seedlings treated with light for 0, 1, 2 and 4 h was used for RT-PCR analyses to monitor the accumulation of *IspD*, *IspE* and *UBQ10* transcripts. The numbers of PCR cycles are 25 for *UBQ10* and 30 for *IspD* and *IspE*

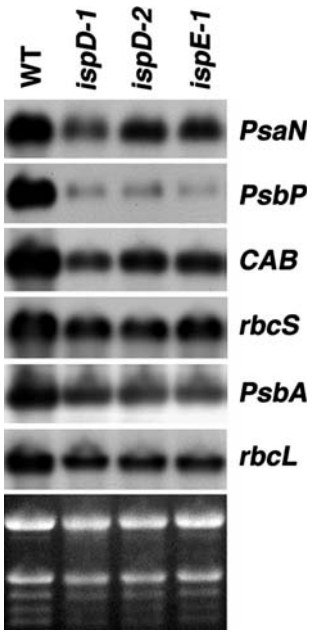

Fig. 6 Steady-state mRNA levels of photosynthetic genes are decreased in Arabidopsis *ispD-1*, *ispD-2* and *ispE-1* mutants. Five micrograms of total RNA extracted from 14-day-old wild type (WT), *ispD-1*, *ispD-2* and *ispE-1* Arabidopsis seedlings were used for RNA gel blot analyses to detect the transcripts of *PsaN*, *PsbP*, *CAB*, *rbcS*, *PsbA* and *rbcL* genes. A representative ethidium bromide-stained agarose gel of the same samples is shown at the bottom. *PsaN*, *PsbP*, *CAB* and *rbcS* are nuclear genes. *PsbA* and *rbcL* are chloroplast-encoded genes

Expression of photosynthetic genes in *ispD-1*, *ispD-2* and *ispE-1* mutants

It is known that the functional state of chloroplasts will affect the expression of a set of nuclear genes encoding chloroplast-localized proteins via retrograde signaling (Nott et al. 2006). We examined the expression of several nucleus- and chloroplast-encoded photosynthetic genes in wild type, *ispD-1*, *ispD-2* and *ispE-1* mutants. The nuclear genes examined are the N subunit of photosystem I (*psaN*), the 23 kD protein of the oxygen-evolving complex of photosystem II (*psbP*), the light harvesting chlorophyll a/b-binding protein (*CAB*) and the small subunit of ribulose-bisphosphate carboxylase (*rbcS*). The chloroplast-encoded genes are *psbA* and *rbcL*, which encodes the D1 protein of photosystem II and the large subunit of ribulose-bisphosphate carboxylase, respectively. Compared with the wild type, steady-state levels of *psaN, psbP, CAB*, *rbcS*, *psbA* and *rbcL* mRNAs were significantly decreased in the *ispD-1*, *ispD-2* and *ispE-1* mutants (Fig. 6). These results indicate that the expression of photosynthetic genes is down-regulated in these albino mutants.

Effects of *ispD* and *ispE* mutants on the expression of mitochondrial genes

In addition to photosynthetic genes, we also examined the expression of nuclear and mitochondrial genes encoding subunits of complex I to complex V of the electron transport chain (Fig. 7). Interestingly, steady-state mRNA levels of mitochondrial electron transport chain genes are either increased or not affected in the *ispD-1*, *ispD-2* and *ispE-1* mutants. Compared to those of wild type, transcript levels of nuclear genes encoding 40 kD subunit of complex I, succinate dehydrogenase (SDH2) of complex II, 14 kD subunit of complex III, coxVc subunit of complex IV, and atpδ′ subunit of complex V were increased in the *ispD-1*, *ispD-2* and *ispE-1* mutants (Fig. 7a). Moreover, the expression of nuclear gene *AOX1a*, which encodes the alternative oxidase, was also induced in the *ispD-1*, *ispD-2* and *ispE-1* mutants (Fig. 7a).

In addition to nuclear genes, we also examined the effects of *ispD* and *ispE* albino mutants on the expression

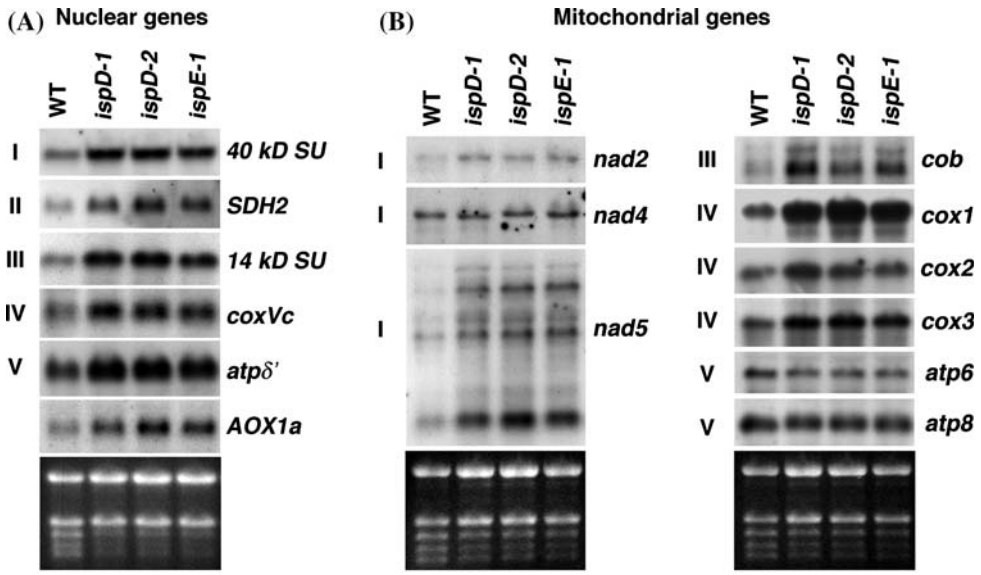

Fig. 7 Steady-state mRNA levels of mitochondrial electron transport genes are increased or unaltered in Arabidopsis *ispD-1*, *ispD-2* and *ispE-1* mutants. Five micrograms of total RNA extracted from 14-day-old wild type (WT), *ispD-1*, *ispD-2* and *ispE-1* Arabidopsis seedlings were used for RNA gel blot analyses to detect the transcripts of nuclear (**a**) and mitochondrial (**b**) genes encoding subunits of electron transport complexes. A representative ethidium bromide-stained agarose gel of the same samples is shown at the bottom of each panel. *40 kD SU, nad2, nad4* and *nad5* are complex I genes. *SDH2* is a complex II gene. *14 kD SU* and *cob* are complex III genes. *coxVc*, *cox1, cox2* and *cox3* are complex IV genes. *atpδ', atp6* and *atp8* are complex V genes. Transcripts of these respiratory genes, except *nad4*, *atp6* and *atp8*, are increased in the *ispD* and *ispE* mutants. Steady-state mRNA levels of *AOX1a*, which encodes the alternative oxidase, are also increased in the mutants

of electron transport genes encoded by the mitochondrial genome. The mitochondrial genes examined are: *nad2*, *nad4* and *nad5* encoding subunits of NADH dehydrogenase (complex I); *cob* encoding cytochrome b of cytochrome bc1 complex (complex III); *cox1*, *cox2* and *cox3* encoding subunits of cytochrome c oxidase (complex IV); *atp6* and *atp8* encoding subunits of ATP synthase (complex V). Compared to those of wild type, steady-state mRNA levels of *nad2*, *nad5*, *cob*, *cox1*, *cox2* and *cox3* were significantly increased in the *ispD* and *ispE* mutants. By contrast, the accumulation of *nad4*, *atp6* and *atp8* transcripts was not affected in the mutants (Fig. 7b).

Effects of *ispD* and *ispE* mutants on the amounts of mitochondrial DNA

It has been suggested that changes in mitochondrial gene copy number may contribute to the accumulation of transcripts (Hedtke et al. 1999). In addition to RNA gel blot analysis, we used genomic Southern blot analysis to investigate the amounts of mitochondrial genes in Arabidopsis *ispD-1*, *ispD-2* and *ispE-1* albino mutants. According to the results of RNA gel blot analyses (Fig. 7 b), we chose two highly induced mitochondrial genes, *cob* and *cox1*, to examine their DNA copy numbers by genomic Southern blot analysis. The same *cob* and *cox1* probes as in the RNA gel blot analyses were used for Southern blot analyses to detect the mitochondrial DNA. The nuclear gene 18S rDNA was also detected in the same blot as a control. These Southern blot analyses revealed that the intensity of each hybridized band was similar between wild type and the albino mutants (Fig. 8). These results indicate that the amounts of mitochondrial DNA are unaltered in the *ispD* and *ispE* albino mutants.

Discussion

There are seven enzymes involved in the MEP pathway of chloroplast isoprenoid biosynthesis in Arabidopsis. Since the plant MEP pathway is compartmentalized in the chloroplast, the nucleus-encoded MEP pathway enzymes have to be synthesized in the cytosol and targeted to the chloroplast. Here we have provided experimental evidence to demonstrate that enzymes of the Arabidopsis MEP pathway are localized to the chloroplast by transit peptide-GFP fusion studies (Fig. 1b). The existence of cytosolic MVA pathway and plastid MEP pathway for the synthesis of IPP and DMAPP inside a plant cell raises an obvious question as to whether and, if so, how these two pathways interact with each other. It has been proposed that the crosstalk between Arabidopsis MVA and MEP pathways mainly occurs at the post-transcriptional levels (Laule et al. 2003).

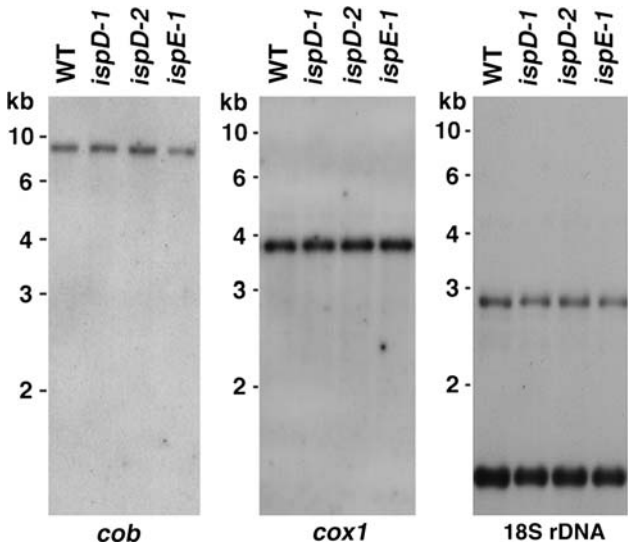

Fig. 8 Southern blot analyses of mitochondrial *cob* and *cox1* genes. Equal amounts of total genomic DNA extracted from 2-week-old wild type (WT), *ispD-1*, *ispD-2* and *ispE-1* mutant seedlings were digested with *Bam* HI and subjected to Southern blot analysis to detect the mitochondrial genes *cob* and *cox1* in two replicates. The same membrane used for *cox1* was stripped and reprobed with the nuclear gene 18S rDNA. There is a *Bam* HI restriction site in the 18S rRNA gene that is located in the region of the probe

The transport of IPP and geranyl diphosphate from plastids to cytosol has been shown to occur in plants (Bick and Lange 2003). The albino lethal phenotype of *ispD*, *ispE* (Fig. 2a) and the other MEP pathway mutants (Mandel et al. 1996; Budziszewski et al. 2001; Gutierrez-Nava et al. 2004; Guevara-Garcia et al. 2005; Hsieh and Goodman 2005, 2006) suggests that the influx of isoprenoid precursors from cytosol to chloroplasts may be very limited in Arabidopsis.

It is known that the functional and developmental states of chloroplast may affect the expression of nuclear genes via retrograde regulation (Nott et al. 2006; Koussevitzky et al. 2007). The Arabidopsis *ispD* and *ispE* albino mutants have lost their photosynthetic functions and the mutant chloroplasts are arrested during early developmental stages. It is likely that retrograde signals derived from these impaired mutant chloroplasts may affect the expression of nuclear genes encoding plastid-localized proteins. Indeed, the expression of nucleus-encoded photosynthetic genes *PsaN*, *PsbP*, *CAB* and *rbcS* is down-regulated in these albino mutants (Fig. 6). In contrast, the expression of nuclear genes, *40 kD SU*, *SDH2*, *14 kD SU*, *coxVc*, *atpδ′* and *AOX1a*, encoding mitochondrion-localized proteins is up-regulated in these albino mutants (Fig. 7a). It is not clear if the down-regulation of nuclear genes encoding plastid-localized proteins and the up-regulation of nuclear genes encoding mitochondrion-localized proteins are mediated via the same mechanism(s). It will be interesting to test if any of the retrograde signals derived from dysfunctional chloroplast is also involved in the up-regulation of nuclear genes encoding mitochondrion-localized proteins.

Chloroplasts and mitochondria are essential organelles inside the plant cell. Several important reactions, e.g. photosynthesis and respiration, take place in these organelles. Mitochondrial metabolism and chloroplast photosynthetic carbon assimilation are highly interdependent (Raghavendra and Padmasree 2003). However, very little concerning the crosstalk between chloroplasts and mitochondria at the gene expression level has been documented. Previous studies in barley *albostrians* mutant, which is deficient in plastid ribosomes, have revealed that both transcript levels and DNA amounts of some mitochondrial genes are increased in the albino tissue (Hedtke et al. 1999; Emanuel et al. 2004). We used Arabidopsis *ispD* and *ispE* albino mutants to examine the effects of dysfunctional chloroplasts on the expression of mitochondrial genes. Similar to the results of barley *albostrians* mutant, steady-state mRNA levels of some mitochondrial genes are increased in these albino mutants (Fig. 7b). We chose *cob* and *cox1* genes to analyze their DNA amounts, because they are located in different regions of mitochondrial genome and their transcript levels are significantly increased in the *ispD-1*, *ispD-2* and *ispE-1* mutants. In contrast to the results observed in the barley *albostrians* mutant, the DNA copy numbers of *cob* and *cox1* genes are unaltered in these albino mutants (Fig. 8). These results suggest that the quantity of mitochondrial DNA is not related to the increased levels of mitochondrial transcripts in the Arabidopsis *ispD* and *ispE* mutants. The mechanisms underlying the interorganellar crosstalk may be different between Arabidopsis and barley.

It is not clear why the expression of mitochondrial genes is up-regulated in these albino mutants. Chloroplasts and mitochondria are the major energy producing sites inside a plant cell. The energy source derived from photosynthesis has been abolished in the Arabidopsis *ispD-1*, *ispD-2* and *ispE-1* albino mutants. These albino plants may therefore activate the expression of mitochondrial electron transport genes to compensate for the loss of photosynthesis. Retrograde signals derived from impaired chloroplasts may be involved in the up-regulation of nuclear genes encoding mitochondrion-localized proteins, which, in turn, may coordinately up-regulate the expression of mitochondrial genes. In addition, chloroplasts and mitochondria are highly interdependent in many biochemical pathways. Some intermediate metabolites derived from plastids may serve as signaling molecules to directly affect the accumulation of mitochondrial transcripts.

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