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Editing of *accD* and *ndhF* chloroplast transcripts is partially affected in the *Arabidopsis vanilla cream1* mutant

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Abstract The *vanilla cream1* (*vac1*) albino mutant is defective in a gene encoding a chloroplast-localized pentatricopeptide repeat protein of the DYW subgroup. However, the carboxyl-terminal DYW motif is truncated in VAC1. To identify *vac1*-specific phenotypes, we compared 34 chloroplast RNA editing sites and ~90 chloroplast gene expression patterns among wild type, *vac1* and another albino mutant *ispH*, which is defective in the plastid isoprenoid biosynthesis pathway. We found that the editing of *accD* and *ndhF* transcripts is partially affected in *vac1*. In addition, steady-state levels of chloroplast rRNAs are significantly decreased in *vac1*. The expression of plastid-encoded RNA polymerase transcribed genes is down-regulated, whereas the expression of nucleus-encoded RNA polymerase transcribed genes is up-regulated in *vac1*. Although the development and function of mutant chloroplasts are severely impaired, steady-state mRNA levels of nucleus-encoded photosynthetic genes are not affected or are only slightly decreased in *vac1*. The *ZAT10* gene encodes a transcription factor and its expression is down-regulated by norflurazon treatment in wild type. This norflurazon effect was not observed in *vac1*. These results suggest that the VAC1 protein may be involved in plastid-to-nucleus retrograde signaling in addition to its role in chloroplast RNA editing and gene expression. A defect in a key biosynthetic pathway can have many indirect effects on chloroplast gene expression as is seen in the *ispH* mutant. Similarly, the *vac1* mutant has pleiotropic molecular phenotypes and most of which may be indirect effects.

Keywords *Arabidopsis* · Chloroplast · Albino · Pentatricopeptide repeat protein · Chloroplast gene expression · Chloroplast RNA editing

Introduction

Chloroplasts have their own genome and gene expression machinery. The chloroplast genome of higher plants contains ~120 genes, which are usually organized in polycistronic transcription units (Sugita and Sugiura 1996; Sato et al. 1999). In contrast, chloroplasts contain about 3,000 proteins. Thus more than 95% of chloroplast proteins are encoded by nuclear genes, synthesized in the cytosol and imported into chloroplasts post-translationally. Therefore, development and functions of chloroplasts are highly dependent on nuclear genes. The coordination of nuclear and chloroplast gene expression is also important for chloroplast biogenesis (Mullet 1988; Leon et al. 1998).

The expression and regulation of chloroplast genes are very different from that of nuclear genes. There are two types of RNA polymerase involved in transcribing chloroplast genes: a plastid-encoded multimeric RNA polymerase (PEP) and a nucleus-encoded RNA polymerase (NEP). The subunits of PEP are encoded by *rpoA*, *rpoB*, *rpoC1* and *rpoC2*, which are similar to those of eubacterial RNA polymerase (Hu and Bogorad 1990; Igloi and Kössel 1992). In contrast, NEP is a phage-type monomeric RNA polymerase (Hedtke et al. 1997; Hess and Börner 1999). It has been suggested that these RNA polymerases are responsible for the transcription of distinct types of plastid

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genes. In general, PEP is involved in the transcription of photosynthesis genes whereas NEP preferentially transcribes housekeeping genes (Hajdukiewicz et al. 1997; Liere and Maliga 1999). The molecular mechanisms of selective usage of PEP and NEP in chloroplast gene expression are still unknown. Glutamyl-tRNA has been proposed to mediate the usage of PEP and NEP during chloroplast biogenesis (Hanaoka et al. 2005). Although the involvement of RNA polymerases in transcribing chloroplast genes has been identified, promoter sequences recognized by PEP and NEP have yet to be established. Recent studies on PEP and NEP promoters indicate that the usage of PEP and/or NEP promoters is very complicated and seems to be species-specific (Swiatecka-Hagenbruch et al. 2007, 2008).

The complexity of chloroplast gene expression system is not limited to the transcription level. The expression of chloroplast genes is also highly regulated at the post-transcriptional level (Sugita and Sugiura 1996). Many chloroplast genes are transcribed as polycistronic transcription units. These transcripts have to go through extensive modifications post-transcriptionally. For instance, endonucleolytic cleavage of di or polycistronic transcripts is required for efficient translation of mRNAs (Barkan et al. 1994; Sugiura et al. 1998). Other transcript maturation processes including RNA splicing and RNA editing are essential in higher plant chloroplasts (Maier et al. 1996; Sugita and Sugiura 1996). It appears that the gene expression system in plastids is more complicated than that of cyanobacteria and the other prokaryotes. It is not clear why plant plastids have evolved to have such complex transcriptional and post-transcriptional processes.

The complex chloroplast gene expression machinery requires the involvement of many proteins encoded by the nuclear genes. Recently, many chloroplast-localized pentatricopeptide repeat (PPR) proteins have emerged as primary nuclear factors that are involved in chloroplast gene expression and RNA metabolism in higher plants. PPR proteins are defined by tandem repeats of a degenerate 35 amino acid motif (Small and Peeters 2000). The PPR family is one of the largest protein families in plants, which contains ~450 and 477 PPR proteins in *Arabidopsis* and rice, respectively. A large majority (~80%) of the *Arabidopsis* and rice PPR genes are intronless (O'Toole et al. 2008). Moreover, most of the PPR proteins are predicted to localize to chloroplasts or mitochondria (Lurin et al. 2004). The PPR family is divided into P and PLS subfamilies based on the presence of classic PPR (P) motif and longer (L) or shorter (S) variant PPR motifs in the tandem arrays of PPR (Lurin et al. 2004). The PLS subfamily seems to be specific to land plants and is further divided into PLS, E and DYW subclasses based on the presence of E or DYW motifs in the C-terminal sequences (O'Toole et al. 2008; Schmitz-Linneweber and Small 2008). The huge number of different PPR proteins in plants indicates that most of the PPR proteins may have functions that are specific to plants.

PPR proteins have been shown to be involved in the transcription of chloroplast genes (Pfalz et al. 2006), RNA splicing (Schmitz-Linneweber et al. 2006; de Longevialle et al. 2007, 2008), RNA cleavage (Hashimoto et al. 2003; Meierhoff et al. 2003; Hattori et al. 2007; Okuda et al. 2009), RNA editing (Kotera et al. 2005; Okuda et al. 2007, 2008, 2009; Chateigner-Boutin et al. 2008; Cai et al. 2009; Hammani et al. 2009; Robbins et al. 2009; Yu et al. 2009; Zhou et al. 2009), translation (Fisk et al. 1999; Williams and Barkan 2003; Schmitz-Linneweber et al. 2005), and RNA stabilization (Yamazaki et al. 2004; Beick et al. 2008; Pfalz et al. 2009). These findings indicate that the PPR protein family has acquired essential roles in organelle gene expression in plants. The molecular bases underlying these PPR related functions are largely unknown. PPR proteins may directly bind to RNA or recruit effectors to the correct sites of target transcripts to perform splicing, editing, processing and translation (Lurin et al. 2004; Schmitz-Linneweber and Small 2008). Thus, PPR proteins have been proposed as sequence-specific RNA binding factors that are involved in post-transcriptional processes in organelles (Delannoy et al. 2007). In addition to organelle gene expression, PPR protein might also affect nuclear gene expression. For instance, GUN1 is a PPR protein that has been implicated in plastid-to-nucleus signaling (Koussevitzky et al. 2007). It is not yet known how the chloroplast-localized GUN1 PPR protein regulates nuclear gene expression.

To identify nuclear genes that are involved in chloroplast development and function, we have isolated a collection of pigmentation mutants in *Arabidopsis*. One of the albino mutants, *vanilla cream1* (*vac1*), is caused by a T-DNA insertion in an intronless PPR gene. The *vac1* mutant is allelic to *ecb2*, which has been shown to be totally impaired in editing of the 794th nucleotide from the AUG of *accD* mRNA (Yu et al. 2009). In order to identify the *vac1*-specific molecular phenotypes, we have included another albino mutant *ispH* as a reference line in gene expression and RNA editing analyses. The *ispH* is a null mutant of 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR) from the plastid nonmevalonate pathway of isoprenoid biosynthesis (Hsieh and Goodman 2005). Our studies revealed that some molecular phenotypes are common but others are distinct between the *vac1* and *ispH* albino mutants. One of the distinct features is that the editing efficiency of two sites in *accD* and one site in *ndhF* is partially reduced in *vac1*. We also analyzed the editing of *accD* and *ndhF* transcripts in *ecb2* and found similar results. In addition to distinct features in chloroplast

RNA editing, steady-state levels of rRNAs are specifically reduced in *vac1*. Moreover, the expression of NEP-transcribed genes is up-regulated, whereas steady-state mRNA levels of PEP-transcribed genes are decreased in *vac1*.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis vac1* albino mutant was originally isolated from ACR3 promoter-GUS transgenic lines (Hsieh and Goodman 2002). Progeny of the ACR3p-GUS 73 line segregated green and albino plants in a ratio of 3:1. This line was later named *vanilla cream1* (*vac1*). Genetic analysis confirmed that the *vac1* albino mutant co-segregated with the T-DNA insertion. TAIL-PCR analysis was used to identify the genomic flanking sequence of *vac1* T-DNA mutant (Liu et al. 1995). *Arabidopsis thaliana* ecotype Columbia-0, *vac1* and *ispH* mutants were 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 growth chamber at a light intensity of 80 μmolm^{-2} s^{-1} on a 16 h light/8 h dark cycle at 23°C. For norflurazon treatment, progeny of heterozygous *vac1* and *ispH* mutants were grown on regular MS medium for 6 days to identify homozygous albino segregants. 6-day-old homozygous albino plants were then transferred to MS medium with or without 5 μM NF for 6 days. As a control, 6-day-old wild type *Arabidopsis* plants grown under a normal condition were transferred to MS medium with or without 5 μM NF for 6 days. Total RNA extracted from these samples was used for northern blot analysis.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured using a pulse-amplitude-modulated (PAM) fluorometer (Walz, Germany). Minimal chlorophyll fluorescence (F_0) was measured under measuring light (650 nm) after 10 min of dark adaptation. Maximal chlorophyll fluorescence (F_m) was measured during a 1-s pulse of saturating white light (2,500 μmol m^{-2} s^{-1}). The maximum quantum yield of PSII electron transport was calculated using the following equation: $F_v/F_m = (F_m - F_0)/F_m$, where F_v is variable chlorophyll fluorescence. For the extraction of chlorophylls and carotenoids, 2-week-old *Arabidopsis* seedlings were harvested and homogenized with 80% acetone (v/v). The determination of total chlorophylls and carotenoids in 3 independent samples of wild-type, *vac1* and *ispH* seedlings grown in tissue culture was conducted as described (Lichtenthaler and Wellburn 1983).

Genomic Southern, RNA gel blot and RT-PCR analyses

Arabidopsis genomic DNA was extracted using a standard urea extraction buffer. For genomic Southern blot analysis, ten microgram of total DNA from 2-week-old wild-type, *vac1* mutant and *vac1* complemented line 9-2 was digested with *Xho* I. The following primers were used to make DIG-labeled single-stranded DNA probe for *VAC1*: 5′-TACT GGATATTCAGAGCGGG-3′, 5′-GTATCCATTCTAATC TCCAC-3′. Forty micrograms of total RNA extracted from 2-week-old *Arabidopsis* wild type and *vac1* albino mutants grown in tissue culture were used for RNA gel blot analysis to detect the *VAC1* transcripts. The same probe used for *VAC1* genomic Southern blot analysis was used to detect the *VAC1* transcripts in RNA gel blot analysis. The other primers used to make DIG-labeled probes for RNA gel blot analyses are listed in Supplemental Table S1. DIG probe labeling, pre-hybridization, hybridization, wash conditions and detection were performed according to Roche's DIG Application Manual for Filter Hybridization. For RT-PCR analysis, total RNA was isolated from various organs of 6-week-old *Arabidopsis* grown in soil or 3-day-old etiolated seedlings exposed to light for 0, 1, 2 and 4 h using a phenol extraction protocol (Jackson and Larkins 1976). 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 *VAC1* genes. The following primers were used for RT-PCR analyses. *VAC1*, 5′-TAGCGATGACAAGTACCATC-3′, 5′-GTATCCATTCTAATCTCCAC-3′; *UBQ10*, 5′-CGAT TACTCTTGAGGTGGAG-3′, 5′-AGACCAAGTGAAGT GTGGAC-3′. The primers used to make probes for 4.5S, 5S, 16S and 23S rRNAs were designed as described (Kishine et al. 2004). Chloroplast RNA editing sites were analyzed by RT-PCR and direct sequencing. Total RNA extracted from 2-week-old wild type, *vac1* and *ispH* was used for RT-PCR with primers encompassing 34 known chloroplast RNA editing sites (Chateigner-Boutin and Small 2007). Excerpts of sequencing chromatograms for all 34 editing sites were shown in Fig. 9 and Supplemental Fig. S5.

Analysis of *accD* (S265L), *accD* (3′UTR) and *ndhF* (S97L) RNA editing efficiency

TOPO cloning kit (Invitrogen, Carlsbad, CA) was used to clone the amplified cDNAs derived from RT-PCR products harboring the *accD* (S265L), *accD* (3′UTR) and *ndhF* (S97L) editing sites from wild type, *vac1* and *ispH*. Plasmids prepared from one hundred independent white colonies of each sample (total 900 clones) were

sequenced to determine the editing efficiency of *accD* (S265L), *accD* (3′UTR) and *ndhF* (S97L). For the nucleotide at *accD* (S265L) editing site, 0 C and 100 T (100% edited) in wild type, 38 C and 62 T (62% edited) in *vac1*, and 19 C and 81 T in *ispH* (81%) were detected. For the nucleotide at *accD* (3′UTR) editing site, 45 C and 55 T (55% edited) in wild type, 92 C and 8 T (8% edited) in *vac1* and 52 C and 48 T (48% edited) in *ispH* were detected. For the nucleotide at *ndhF* (S97L) editing site, 5 C and 95 T (95% edited) in wild type, 51 C and 49 T (49% edited) in *vac1* and 13 C and 87 T (87% edited) in *ispH* were detected.

Transmission electron microscopy

The leaf samples from 2-week-old wild type or *vac1* albino plants 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).

Complementation of *Arabidopsis vac1* mutant

A 3.3 kb *VAC1* (At1g15510) genomic clone encompassing the entire predicted 5′ intergenic region (422 bp), the *VAC1* open reading frame, and the 3′ intergenic region (281 bp) was amplified by PCR and cloned into the *Kpn* I site of the binary vector pCambia1301, which contains a hygromycin selectable marker. The resulting construct was transformed into kanamycin resistant heterozygous *vac1* (+/−) plants by floral dip (Clough and Bent 1998). Successful transformants were selected from T1 plants grown on MS medium containing kanamycin and hygromycin. Genomic DNA extracted from green kanamycin^R/hygromycin^R primary transformants was used to determine the genotype of the *VAC1* locus (+/− or −/−) by genomic Southern. Six of the 24 green hygromycin^R primary transformants tested were *vac1* (−/−) homozygous. In the T2 generation, about 100 seeds from each line were germinated on a hygromycin selective medium and all hygromycin^R seedlings were green, an indication that the *VAC1* genomic clone complements the albino phenotype in all lines. Genotypic and phenotypic characterizations of line 9-2, a representative T3 homozygous line for the *VAC1* transgene and *vac1* (−/−) locus are shown in Fig. 3c, d.

GFP fusion proteins

The computer program ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) predicts that the length of VAC1 transit peptide is 52 amino acids. According to this prediction, the N-terminal cDNA sequence encoding the first 72 amino acids of VAC1 was 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). The primers used are: 5′-CCTTCCATG GCGTCTTCTGCTCAAAG-3′, 5′-CTCTAGGCCTATTC GCGCAGAGTCCATGTA-3′. The resulting construct, which encodes the first 72 N-terminal amino acids of VAC1 fused to GFP, was transformed into *Arabidopsis* protoplasts and observed under confocal laser scanning microscope 510 META Zeiss.

Results

Isolation and phenotypic analysis of *Arabidopsis vanilla cream1* mutant

To isolate mutants impaired in chloroplast development and function, we generated a collection of *Arabidopsis* T-DNA insertion lines and screened for plants displaying albino, pale green or pale yellow phenotypes. One of the isolated mutants, *vanilla cream1* (*vac1*), exhibits albino to pale yellow phenotype. Homozygous *vac1* plants are albino lethal so the mutant is maintained as a heterozygous line. Progeny from a self-pollinated heterozygous plant segregate green and albino plants in a ratio of 3:1 on a half MS plus sucrose medium, i.e. the albino phenotype is inherited as a monogenic recessive mutation (Fig. 1a). The *vac1* albino plants are lethal under either normal or low light condition. Some *vac1* albino plants can grow to develop flower bud-like structures on a tissue culture medium (Fig. 1b). However, these flower buds never grow to mature before the plant dies. In 2-week-old *vac1* mutants, total chlorophylls and carotenoids are 8.9 and 22.2%, respectively, of the amounts in wild-type plants (Fig. 1c). In contrast, the content of photosynthetic pigments was almost undetectable in the *ispH* albino plants, which are null mutants of the *IspH* gene encoding 1-hrdroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR) of the plastid nonmevalonate pathway for isoprenoid biosynthesis (Hsieh and Goodman 2005). In parallel with their photosynthetic pigment content, the PSII maximum quantum yield of *vac1* mutants was about 8.7% of wild-type plants, whereas the photosynthetic activity was not detectable in the *ispH* albino plants (Fig. 1d).

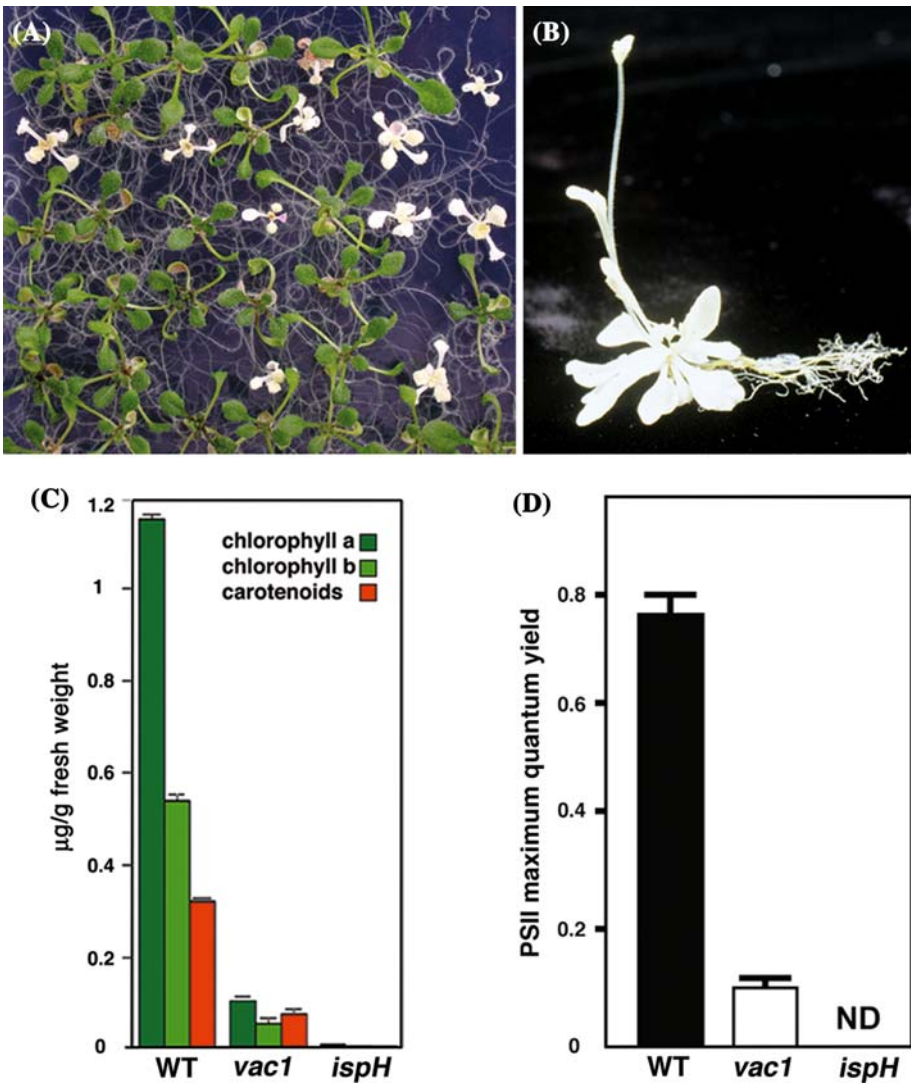

Fig. 1 Phenotypic analyses of *Arabidopsis vac1* albino mutant. **a** Progeny of a self-pollinated heterozygous *vac1* mutant segregate green and albino plants in a ~3:1 ratio on a non-selective medium. Plants shown are 2 weeks old. **b** A representative *vac1* albino plant grown on a ½ MS plus 2% sucrose medium for 6 weeks. **c** Photosynthetic pigment content of 2-week-old wild type, *vac1* and *ispH* seedlings. Levels of chlorophylls and carotenoids were low in the *vac1* albino plants and almost undetectable in the *ispH* albino mutants. **d** PSII maximum quantum yield (F_v/F_m), measured by chlorophyll fluorescence analysis of wild type, *vac1* and *ispH* plants. Chlorophyll fluorescence of *ispH* albino plants was not detectable (*ND*). Values shown are means of six samples ± SE

Ultrastructures of *vac1* mutant chloroplasts

Transmission electron microscopic analysis of *vac1* leaf sections revealed that the development of chloroplast is impaired in the mutant. In contrast to wild-type chloroplasts, which are lens-shaped containing well-organized inner thylakoid membrane systems, the morphology of *vac1* mutant chloroplasts range from round- or amoeboid-shape and completely lack of thylakoids to lens-shaped with the development of few stacking and non-stacking thylakoids (Fig. 2). These various types of mutant chloroplasts may exist in the same or different cells. The mutant chloroplasts without thylakoids are usually filled with large vesicles. In addition, densely stained globule aggregates are frequently observed in the *vac1* mutant chloroplasts. These observations are consistent with the phenotypes that the *vac1* mutants still retain certain amounts of photosynthetic pigments and some photosynthetic activity.

Molecular characterization of the *vac1* locus

Genetic analysis of the *vac1* mutants has confirmed that the albino phenotype co-segregates with the T-DNA insertion. To identify the *vac1* locus, we used thermal asymmetric interlaced (TAIL)-PCR to analyze the genomic flanking sequence of the *vac1* T-DNA mutant. Sequence analysis of the TAIL-PCR products revealed that the T-DNA insertion is located in the open reading frame of At1g15510 (Fig. 3a). RNA gel blot analysis confirmed that ~2.6 kb transcripts of the predicted At1g15510 gene were detected in the wild type but not in the *vac1* albino plants (Fig. 3b). Since the RNA gel blot analysis cannot detect very low abundant transcripts, we conducted RT-PCR analysis to show that there is no trace of *VAC1* transcripts left in the mutant (Fig. 3c). These results suggest that *vac1* is a null allele of the At1g15510 gene. To further prove that we have identified the allele responsible for the *vac1* albino mutant, we introduced a 3.3 kb genomic clone

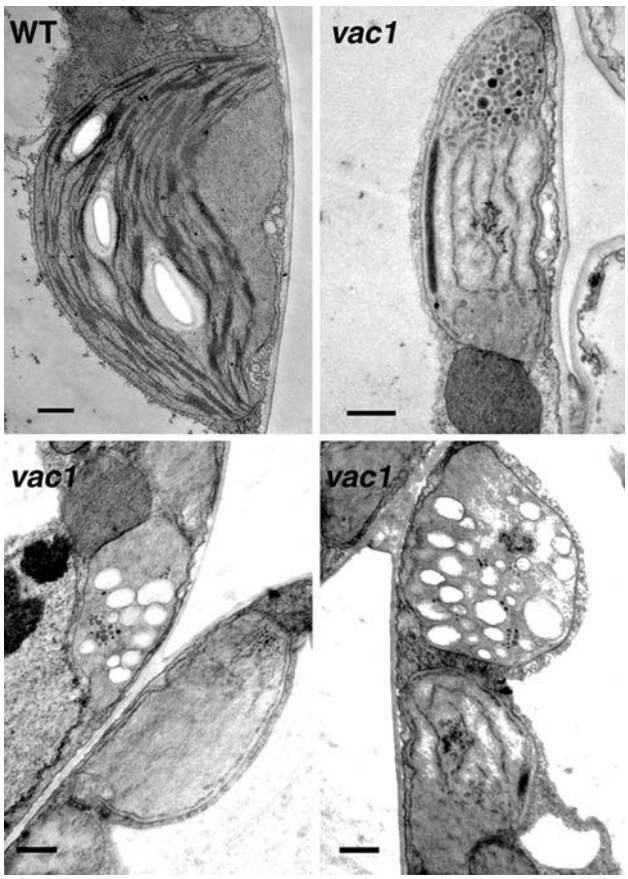

Fig. 2 Transmission electron micrographs of wild type (*WT*) and *vac1* mutant chloroplasts. Sections are from the first leaves of 2-week-old *Arabidopsis* plants grown in tissue culture. Various mutant chloroplasts containing large vesicles, densely stained globule aggregates and thylakoid-like structures were observed in the mesophyll cells of *vac1* albino plants. *Scale bars* are 500 nm

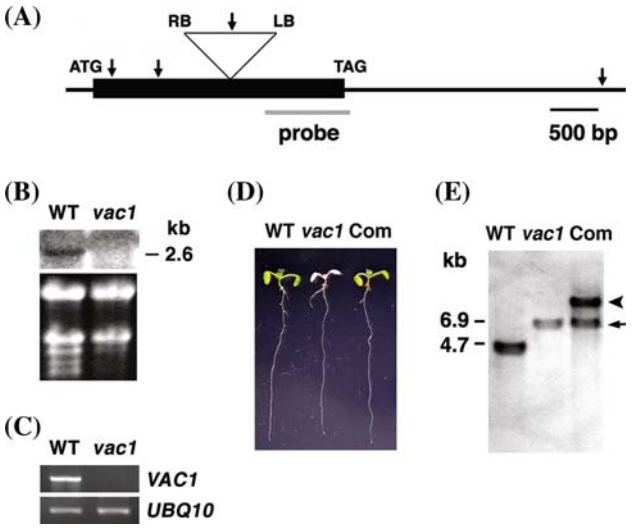

Fig. 3 Molecular characterization of *vac1* locus. **a** Schematic diagram of *Arabidopsis VAC1* gene. Arrows indicate *Xho* I restriction sites. The *black box* indicates the only exon of *VAC1*. The T-DNA (*white triangle*) is not drawn to scale. **b** RNA gel blot analysis. Forty micrograms of total RNA extracted from 2 weeks old wild type (*WT*) and *vac1* albino plants were used for RNA gel blot analysis to detect the transcripts of *VAC1*. **c** RT-PCR analysis of *VAC1* and *ubiquitin 10* (*UBQ10*) mRNAs in 2-week-old wild type and *vac1*. **d** Seven-day-old *Arabidopsis* wild-type (*WT*), *vac1* albino mutant and *VAC1* complemented (*Com*) seedlings. **e** Genomic Southern blot analysis (*Xho* I digested). The arrow indicates the *vac1* mutant allele and the arrowhead indicates the *VAC1* transgenic allele in a complemented (*Com*) line

encompassing the predicted open reading frame of the *VAC1* gene and its putative promoter into the mutant. The *VAC1* gene driven by its own native promoter was able to complement the *vac1* albino mutant. The phenotype and genotype of a representative complementation line are shown in Fig. 3d and e, respectively. These results confirm that the *vac1* albino phenotype is caused by a loss-of-function mutation in the *VAC1* gene.

VAC1 encodes a chloroplast-localized PPR-DYW protein

Arabidopsis VAC1 is an intronless gene that encodes a PPR protein (Fig. 4a). The amino acid sequence and domain composition of VAC1 PPR protein are most similar to those in the DYW subgroup (O'Toole et al. 2008). However, the VAC1 PPR protein has a truncated DYW domain. The other reported PPR-DYW proteins end with a conserved DYW or DFW tri-peptide (Supplemental Fig. S1). The VAC1 PPR protein is predicted to localize to the chloroplast (http://www.cbs.dtu.dk/services/ChloroP/). To verify the prediction, we fused the first 72 amino acids of VAC1 protein, which encompass the putative transit peptide, to the N-terminus of a green fluorescent protein (GFP). The VAC1-GFP fusion construct driven by a cauliflower mosaic virus (CaMV) 35S promoter was transformed into *Arabidopsis* protoplasts. Confocal microscopy was used to observe the fluorescent signals 24 h after transformation. The green fluorescent signals derived from the VAC1-GFP fusion protein co-localized with the autofluorescent signals of chlorophylls in the chloroplasts (Fig. 4b). These results suggest that the *Arabidopsis* VAC1 PPR protein is localized to the chloroplast.

Light induction of *Arabidopsis VAC1* gene

We used semi-quantitative RT-PCR analysis to examine the expression patterns of *VAC1* in 6-week-old wild type *Arabidopsis* plants grown in soil. The expression of *VAC1* is ubiquitous as the *VAC1* transcripts were detected in all organs examined. Levels of *VAC1* transcripts are low in roots and high in leaves, stems, flowers and siliques (Fig. 4c). To test the effects of light on the expression of *VAC1*, we treated 3-day-old *Arabidopsis* etiolated

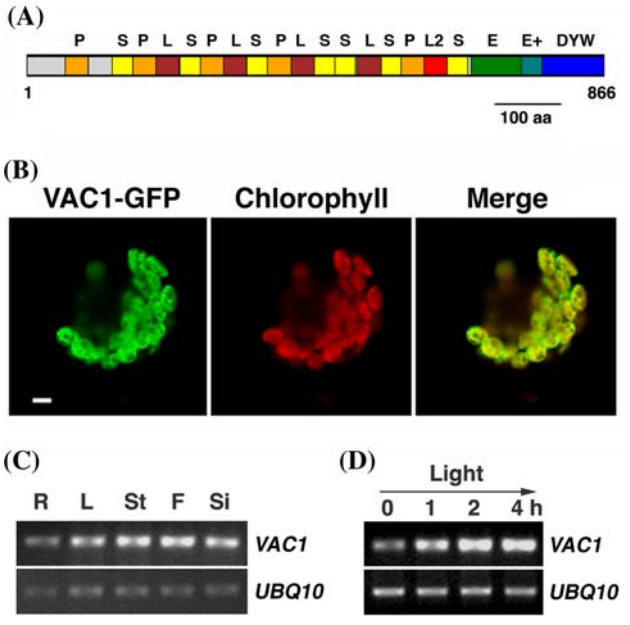

Fig. 4 **a** Schematic diagram of domain composition of *Arabidopsis* VAC1 protein. *P* PPR motif, *S* shorter variant of PPR motif, *L* longer variant of PPR motif, *E*, *E+* and *DYW* motifs are also indicated above the diagram. **b** Chloroplast localization of *Arabidopsis* VAC1 PPR protein. *Arabidopsis* protoplasts were transformed with a 35S:VAC1-GFP construct, which encodes the first N-terminal 72 amino acids of VAC1 fused to GFP. Chloroplasts were visualized by red chlorophyll autofluorescence. The green fluorescent signals of VAC1-GFP co-localized with the red fluorescent signals of chlorophylls (merge). *Scale bar* is 10 μm. **c** RT-PCR analysis of *VAC1* and *ubiquitin 10* (*UBQ10*) mRNAs in 6-week-old wild type *Arabidopsis* plants grown in soil. *R* roots, *L* leaves, *St* stems, *F* flowers, *Si* siliques. **d** RT-PCR analysis of *VAC1* and *ubiquitin 10* (*UBQ10*) mRNAs in 3-day-old etiolated seedlings exposed to light for 0–4 h. The expression of *VAC1* is rapidly induced by light

seedlings with light for 0, 1, 2 and 4 h. Total RNA extracted from these samples was analyzed by semi-quantitative RT-PCR to detect the expression levels of *VAC1*. Low levels of *VAC1* mRNA were detected in 3-day-old etiolated *Arabidopsis* seedlings (Fig. 4d, 0 h light). Exposure to light for 1 h rapidly increased the accumulation of *VAC1* transcripts and higher levels of *VAC1* mRNA were detected in seedlings treated by light for 2 and 4 h (Fig. 4d).

Chloroplast gene expression profiles of wild type, *vac1* and *ispH* albino mutants

Many chloroplast protein-coding genes are organized as clusters and are co-transcribed as polycistronic messages. The expression of these polycistronic genes has to go through extensive posttranscriptional processing. To further identify *vac1*-specific molecular phenotypes, we used RNA gel blot analysis to compare the abundance and banding patterns of chloroplast transcripts in wild type, *vac1* and *ispH* mutants. The *psb* genes encode subunits of the photosystem II protein complex and the *pet* genes encode subunits of the photoelectron transport cytochrome b6f protein complex. Compared to those of wild type, steady-state levels of *psbA*, *psbB-psbH-petB-petD*, *psbD-psbC*, *psbE-psbF-psbL-psbJ*, *psbK-psbI*, *psbM* and *psbZ* mRNAs are significantly decreased in the *vac1* mutant (Fig. 5a). These effects are specific to the *vac1* albino mutants, because the expression of most of the *psb* genes is only slightly affected in the *ispH* albino plants (Fig. 5a). In addition to the difference in transcript levels, the banding patterns of *psbE-psbF-psbL-psbJ*, *psbK-psbI* and *psbM* transcripts in *vac1* are different from those of wild type and *ispH*. The intensity of each individual band of the *psbE-psbF-psbL-psbJ* transcripts is differentially regulated in *vac1* compared to those of wild type and *ispH*. Although the abundance of *psbK-psbI* transcripts is significantly decreased in *vac1*, at least three unique high molecular weight *psbK-psbI* transcripts were specifically detected in *vac1* but not in wild type and *ispH*. These results suggest that the abundance and expression patterns of chloroplast *psb* transcripts are significantly and specifically affected in the *vac1* albino mutant.

The *psa* genes encode subunits of the photosystem I protein complex. The *psaA*, *psaB* and *rps14* genes are co-transcribed in the same operon. Compared to those of wild type, steady-state levels of *psaA-psaB-rps14* transcripts are significantly decreased in both *vac1* and *ispH* albino mutants (Fig. 5b). These results suggest that the down-regulation of *psaA-psaB-rps14* transcripts is not specific to the *vac1* mutant. The *ndh* genes encode subunits of the NADH dehydrogenase. The *psaC* and *ndhD* genes are located in the same operon. Compared to those of wild type and *ispH*, steady-state levels of high molecular weight precursor transcripts of *psaC-ndhD* are specifically up-regulated in the *vac1* albino mutant (Fig. 5b). In contrast to *ndhD*, the expression patterns of most *ndh* genes are similar between the *vac1* and *ispH* albino mutants (Supplemental Fig. S2). Compared to those of wild type, levels of *psaI* transcripts are slightly decreased in both *vac1* and *ispH* mutants (Fig. 5b). By contrast, steady-state levels of *psaJ* mRNAs are specifically down-regulated in the *vac1* mutant (Fig. 5b). Whereas the abundance and expression patterns of *petA*, *petG* and *petL* transcripts are similar between *vac1* and *ispH* albino mutants, steady-state levels of *petN* mRNA are significantly decreased in *vac1*, compared to those of wild type and *ispH* (Fig. 5c).

The chloroplast *atp* genes are organized into two transcriptional units in *Arabidopsis*. One cluster of genes has the order *atpB-atpE* and the other cluster has the order *atpI-atpH-atpF-atpA*. We designed probes corresponding to the coding regions of these chloroplast-encoded ATP synthase subunit genes to detect the abundance and banding patterns

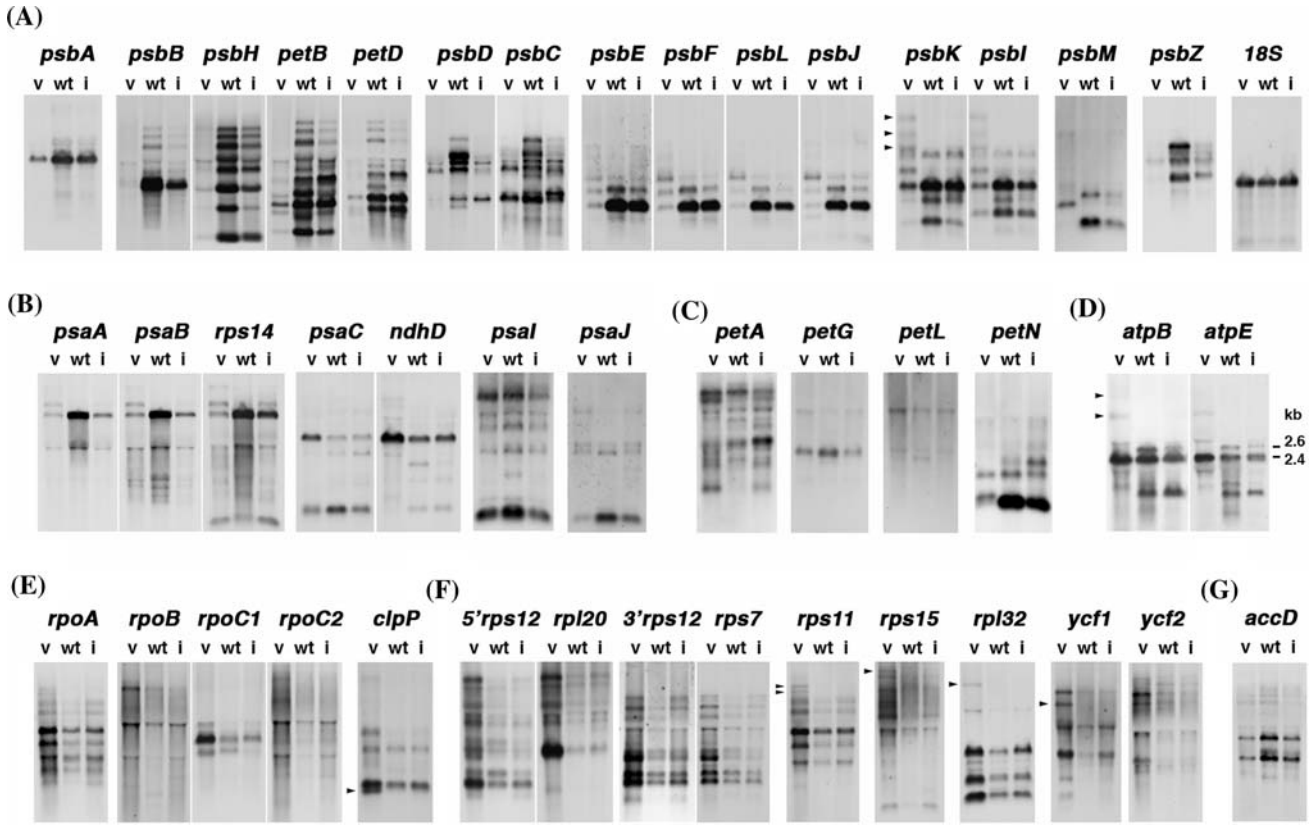

Fig. 5 RNA gel blot analysis of chloroplast genes in wild type (*wt*), *vac1* (*v*) and *ispH* (*i*) mutants. **a** Steady-state levels of *psbA, psbB-psbH-petB-petD, psbD-psbC, psbE-psbF-psbL-psbJ, psbK-psbI, psbM* and *psbZ* transcripts are significantly decreased in *vac1*. A replicate gel was used to detect the expression levels of nuclear 18S rRNA as a loading control. **b** Steady-state levels of *psaA-psaB-rps14* transcripts are significantly decreased in both *vac1* and *ispH* albino mutants. Transcripts of *psaC-ndhD* and *psaJ* are differentially regulated in *vac1* and *ispH*. The expression patterns of *psaI* are similar between *vac1* and *ispH*. **c** Differential regulation of *pet* genes in *vac1* and *ispH*. Steady-state levels of *petN* transcripts are significantly decreased in *vac1*. **d** In *atpB-atpE* operon, the PEP-transcribed ~2.6 kb transcripts are almost undetectable, whereas the NEP-transcribed ~2.4 kb transcripts are increased in *vac1*. **e** Steady-state levels of *rpoA, rpoB, rpoC1, rpoC2* and *clpP* transcripts are increased in *vac1*. **f** Up-regulation of *5′rps12-rpl20, 3′rps12-rps7, rps11, rps15, rpl32, ycf1* and *ycf2* transcripts in *vac1*. *Arrowheads* indicate high molecular weight transcripts that specifically appear in *vac1*. **g** Steady-state levels of *accD* transcripts in wild type, *vac1* and *ispH*

of these polycistronic transcripts by RNA gel blot analyses. We found that the expression patterns of *atpB-atpE* transcripts are specifically affected in the *vac1* mutant (Fig. 5d). In the *atpB-atpE* operon, 2.6 and 2.4 kb transcripts are transcribed by PEP and NEP, respectively (Swiatecka-Hagenbruch et al. 2007). In the *vac1* mutant, steady-state levels of NEP-transcribed 2.4 kb *atpB-atpE* transcripts are increased compared to those of wild type and *ispH*, whereas the PEP-transcribed 2.6 kb *atpB-atpE* transcripts are almost undetectable (Fig. 5d). Moreover, there are at least two additional high molecular weight *atpB-atpE* transcripts that specifically accumulate in the *vac1* mutant (Fig. 5d). We used sense and antisense probes located in the *atpB-atpE* operon and its adjacent gene *rbcL* to demonstrate that these high molecular weight transcripts in *vac1* have the same orientation as *atpB-atpE* transcripts (Supplemental Fig. S3). In contrast to the *atpB-atpE* transcripts, the expression patterns of *atpI-atpH-atpF-atpA* in *vac1* are similar to those of *ispH* (Supplemental Fig. S2). Compared to those of wild type, steady-state levels of *atpI* transcripts are significantly decreased in both *vac1* and *ispH* albino mutants, whereas the abundance of *atpH*, *atpF* and *atpA* transcripts is not affected (Supplemental Fig. S2).

The plastid-encoded RNA polymerase consists α, β, β' and β'' subunits, which are encoded by the *rpoA*, *rpoB*, *rpoC1* and *rpoC2* genes, respectively. Compared to those of wild type and *ispH*, steady-state levels of *rpoA*, *rpoB*, *rpoC1* and *rpoC2* transcripts are increased in the *vac1* mutant (Fig. 5e). Similarly, steady-state levels of *clpP* transcripts are specifically up-regulated in the *vac1* mutant (Fig. 5e). In addition, a novel low molecular weight *clpP* transcript was specifically detected in the *vac1* mutant (Fig. 5e). The *rps* and *rpl* genes encode ribosomal proteins of small and large subunits, respectively. The *Arabidopsis*

rps12 gene is split into two parts, which belong to two different operons in the chloroplast genome. The 5′ and 3′ *rps12* transcripts have to go through trans-splicing to form mature transcripts. Compared to those of wild type and *ispH*, steady-state levels of *5′rps12-rpl20*, *3′rps12-rps7*, *rps11*, *rps15* and *rpl32* are significantly increased in the *vac1* mutant (Fig. 5f). The *rpl33* and *psaJ* genes are located in the same operon. Similar to that of *psaJ*, steady-state levels of *rpl33* transcripts are specifically down-regulated in the *vac1* mutant (Supplemental Fig. S2). The expression levels of the other *rps* and *rpl* genes are similar between the *vac1* and *ispH* albino mutants (Supplemental Fig. S2). The *ycf* genes encode proteins of unknown functions in the chloroplast. Compared to those of wild type and *ispH*, steady-state levels of *ycf1* and *ycf2* transcripts are specifically increased in the *vac1* mutant (Fig. 5f). Moreover, the expression patterns and the intensity of each individual band of *ycf3* and *ycf5* transcripts in *vac1* are significantly different from those of wild type and *ispH* (Supplemental Fig. S2). The *accD* gene encodes the β subunit of acetyl-CoA carboxylase. Steady-state levels of *accD* transcripts are decreased in both *vac1* and *ispH* (Fig. 5g).

Levels of chloroplast rRNA are significantly reduced in *vac1*

The chloroplast rRNA genes are organized as a 16S-23S-4.5S-5S operon similar to that of prokaryotes (Fig. 6a). Ethidium bromide staining of total RNA extracted from 2 weeks old *vac1* albino plants reveals that levels of chloroplast ribosomal RNA are significantly reduced in the *vac1* albino mutant compared to that of wild type and *ispH* (Fig. 6b bottom). We used RNA gel blot analysis to examine the expression patterns and steady-state levels of 16S, 23S, 4.5S and 5S rRNAs in wild type, *vac1* and *ispH* mutants. Compared to those of wild type and *ispH*, steady-state levels of 16S, 23S, 4.5S and 5S rRNAs are significantly decreased in the *vac1* mutant (Fig. 6b, c). Despite the dramatic difference in transcript abundance, the banding patterns of chloroplast 4.5S and 23S rRNAs in *vac1* are similar to those of wild type and *ispH* (Fig. 6c). These results suggest that steady-state levels of chloroplast rRNAs are specifically affected in the *vac1* mutant but not in the *ispH* albino plants. In addition to rRNAs, we also used RNA gel blot analysis to examine the expression of chloroplast tRNA genes. It has been suggested that the expression of chloroplast *trnE* gene is dependent on PEP and $tRNA^{Glu}$ can specifically repress the activity of NEP during the late phase of chloroplast development (Hanaoka et al. 2005). Compared to those of wild type, steady-state levels of *trnE* transcripts are slightly decreased in *ispH* and dramatically reduced in *vac1* (Fig. 6d). Similarly, steady-state levels of *trnH, trnQ, trnR, trnT, trnW* and *trnY* transcripts are also significantly decreased in *vac1* (Fig. 6d). By contrast, steady-state levels of *trnC, trnF, trnI, trnL, trnfM, trnN, trnP* and *trnV* transcripts are decreased to similar levels in both *vac1* and *ispH* compared to those of wild type (Supplemental Fig. S2).

Distinct regulation of nucleus-encoded photosynthetic genes in *vac1* and *ispH*

Ribulose-bisphosphate carboxylase (Rubisco), the key enzyme of photosynthetic CO_2 fixation, is composed of large and small subunits, which are encoded by *rbcL* in the chloroplast and *rbcS* in the nucleus, respectively. Compared to those of wild type and *ispH*, steady-state levels of *rbcL* transcripts are significantly decreased in *vac1* (Fig. 7). By contrast, steady-state levels of *rbcS* transcripts are not affected in *vac1* but are significantly decreased in *ispH* (Fig. 7). Similarly, compared to those of wild type, steady-state levels of *CAB* transcripts are only slightly reduced in *vac1* but are dramatically decreased in *ispH* (Fig. 7). The *vac1* mutants are albino lethal, which only retain limited ability in photosynthesis (Fig. 1). It is unexpected to observe that the amounts of *rbcS* and *CAB* transcripts are not or only slightly affected in the *vac1* albino plants. We further examined the expression levels of several nucleus-encoded *psa* and *psb* genes in wild type, *vac1* and *ispH* mutants by northern blot analysis. Interestingly, the results are very similar to those of *rbcS* and *CAB*. Compared to those of wild type, steady-state levels of *psaD, psaE, psaF, psaL, psbO, psbP, psbR, psbS, psbW, psbX* and *psbY* transcripts are not or only slightly affected in *vac1* but are significantly decreased in *ispH* (Fig. 7).

The VAC1 PPR protein is involved in retrograde regulation of *ZAT10*

Norflurazon (NF) is a phytoene desaturase inhibitor that will block the synthesis of carotenoids. The signaling pathways (e.g. *gun* mutants) involved in NF-initiated plastid-to-nucleus retrograde regulation of *CAB* and *rbcS* genes have been established (Susek et al. 1993; Koussevitzky et al. 2007). To examine if the VAC1 PPR protein is involved in plastid-to-nucleus retrograde regulation, we used NF to treat wild type, *vac1* and *ispH* albino plants. Six days old wild-type *Arabidopsis*, *vac1* and *ispH* plants grown under normal condition were transferred to medium with or without NF for 6 days (Supplemental Fig. S4). Total RNA extracted from these samples was subject to northern blot analysis to examine the expression of retrograde-regulated genes, *CAB*, *rbcS*, *ZAT10* and *ZAT12*

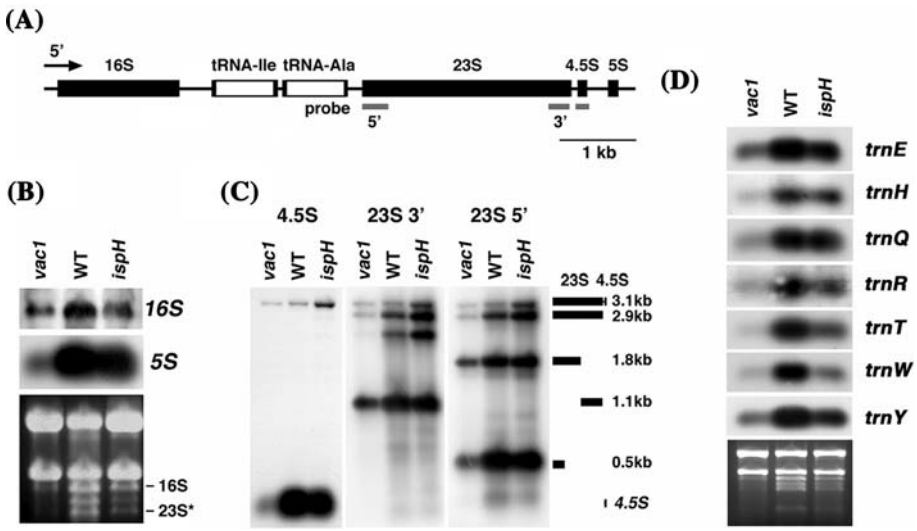

Fig. 6 RNA gel blot analysis of chloroplast rRNA genes in wild type (*WT*), *vac1* and *ispH*. **a** Schematic diagram of chloroplast rRNA genes. **b** RNA gel blot analysis to detect levels of 16S and 5S rRNA. 23S* indicates breakdown products of 23S rRNA. **c** RNA gel blot analysis to detect levels of 4.5S and 23S rRNA. The structures and sizes of detected transcripts are indicated on the right. **d** Steady-state levels of *trnE, trnH, trnQ, trnR, trnT, trnW* and *trnY* transcripts are significantly decreased in *vac1*

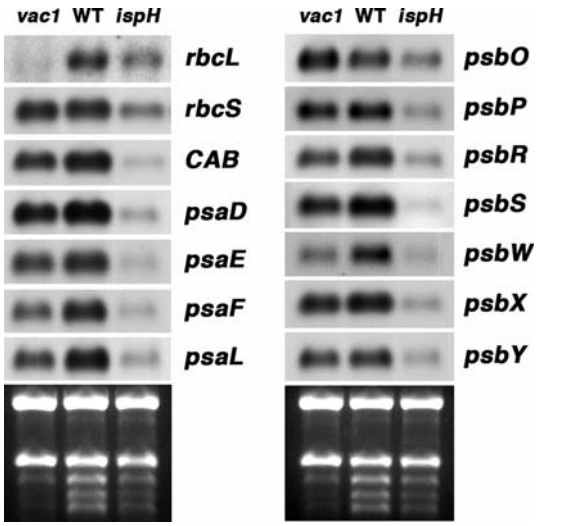

Fig. 7 RNA gel blot analysis of chloroplast and nuclear genes in wild type (*WT*), *vac1* and *ispH*. Compared to those of wild type, steady-state levels of chloroplast-encoded *rbcL* transcripts are significantly decreased, whereas nucleus-encoded *rbcS*, *CAB*, *psaD, psaE, psaF, psaL, psbO, psbP, psbR, psbS, psbW, psbX* and *psbY* transcripts are not or only slightly decreased in *vac1*

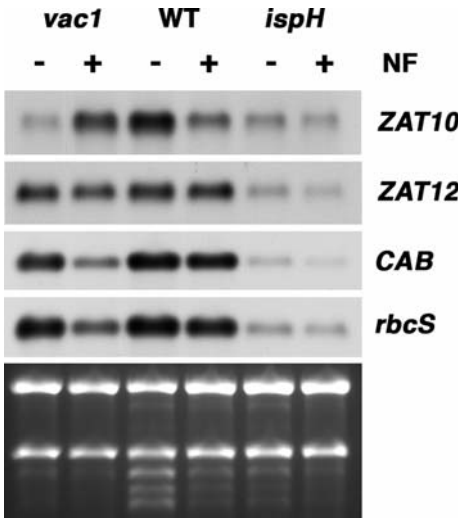

Fig. 8 RNA gel blot analysis of *ZAT10, ZAT12, CAB* and *rbcS* transcripts in wild type (*WT*), *vac1* and *ispH* plants treated with (+) or without (−) norflurazon (*NF*). NF treatment represses the expression of *ZAT10* in wild type and *ispH* but not in *vac1*

(Susek et al. 1993; Pogson et al. 2008). After NF treatment, steady-state levels of *ZAT10* mRNA are decreased in wild type and *ispH* (Fig. 8). Interestingly, this NF-initiated retrograde regulation of *ZAT10* does not exist in *vac1*. Instead of down-regulation, steady-state mRNA levels of *ZAT10* are significantly increased in *vac1* after NF treatment (Fig. 8). By contrast, steady-state levels of *CAB*, *rbcS* and *ZAT12* mRNAs are further decreased in both *vac1* and *ispH* albino mutants after NF treatment (Fig. 8). These results suggest that the VAC1 PPR protein may be required for the specific retrograde regulation of *ZAT10* initiated by NF treatment.

The *vac1* mutant is defective in *accD* and *ndhF* RNA editing

We used RT-PCR and bulk sequencing of the amplified cDNAs to examine all 34 chloroplast RNA editing sites in 2-week-old wild type, *vac1* and *ispH* mutants. We found that the editing of two sites in *accD* and one site in *ndhF* was significantly reduced in *vac1* (Fig. 9a). One of the *accD* editing sites is located at the 794th nucleotide from the AUG of the *accD* mRNA. C-to-U editing at this position results in an S265L amino acid change. The other *accD* editing site is located in the 3′UTR. In *ndhF*, C-to-U editing at the 290th nucleotide from the AUG leads to an S97L amino acid change. To determine the editing

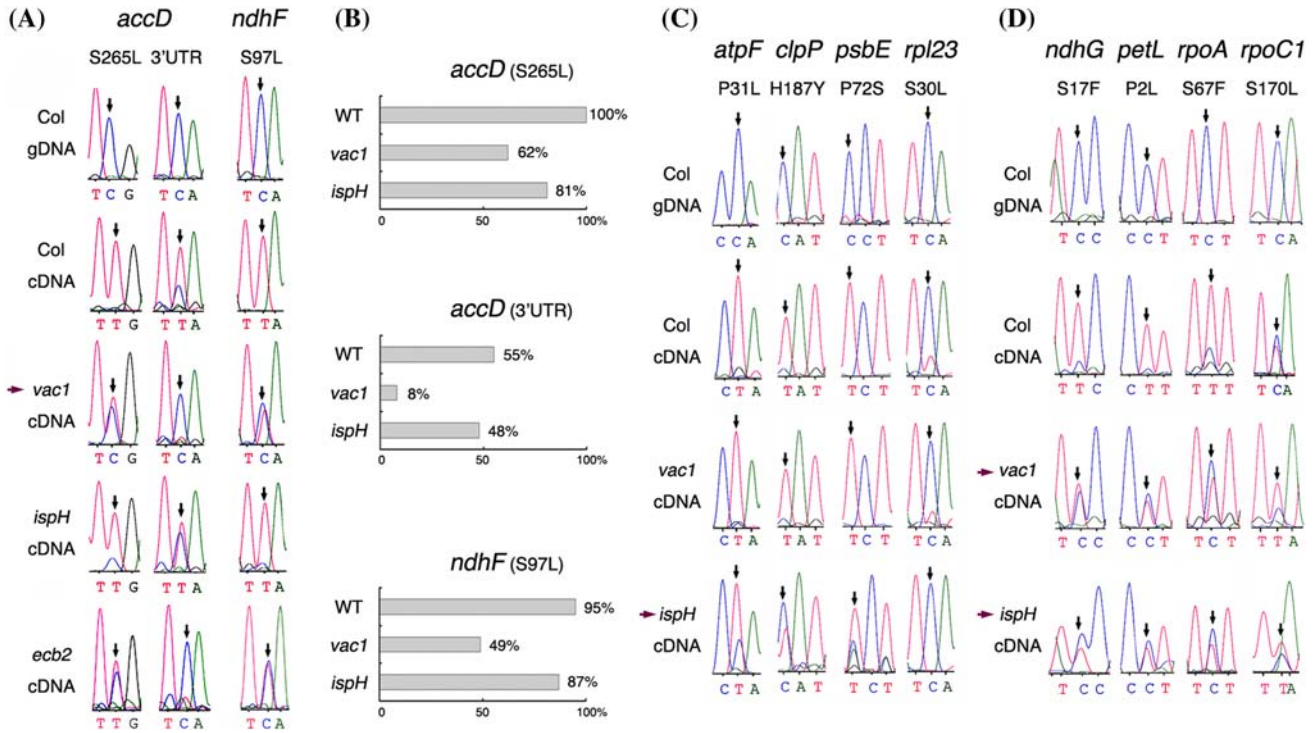

Fig. 9 RNA editing analysis in wild type Columbia (*Col*), *vac1* and *ispH*. **a** Editing of *accD* and *ndhF* transcripts is specifically affected in *vac1*. **b** Editing efficiency of *accD* and *ndhF* transcripts in wild type, *vac1* and *ispH*. **c** Editing of *atpF, clpP, psbE* and *rpl23* transcripts is affected in *ispH*. **d** Editing of *ndhG, petL, rpoA* and *rpoC1* is affected in both *vac1* and *ispH*. Sequence chromatograms of PCR amplified genomic DNA (gDNA) or cDNA of the codon encompassing the editing sites are shown. The position of RNA editing indicated on the *top* represents the name of transcripts and the resulting amino acid change in wild type. *Black arrows* indicate the editing sites

efficiency of these sites, we cloned the RT-PCR products and randomly sequenced 100 independent clones from each sample. Compared to that of wild type, the editing efficiency of these three sites was significantly decreased in *vac1* and was only slightly reduced in *ispH* (Fig. 9a, b). The *accD* (S265L) editing site is 100%, 62% and 81% edited in wild type, *vac1* and *ispH*, respectively (Fig. 9a, b). The editing of *accD* (3′UTR) site is partial in wild type (55%) and *ispH* (48%), but is dramatically reduced in *vac1* (8%; Fig. 9a, b). Similarly, the editing efficiency of *ndhF* (S97L) site is 95%, 49% and 87% in wild type, *vac1* and *ispH*, respectively (Fig. 9a, b).

Finding obtained here that the editing of *accD* (S265L) site is partially impaired in *vac1* is different from its allelic mutant *ecb2* (Yu et al. 2009). We obtained the *ecb2* (SALK_112251) line from the *Arabidopsis* Biological Resource Center (ABRC) and examined the editing of *accD* (S265L), *accD* (3′UTR) and *ndhF* (S97L) sites in the albino plants. Our results indicate that the editing profiles of these sites in *ecb2* are very similar to those of *vac1* (Fig. 9a, compare *vac1* to *ecb2*).

By comparing the editing profiles among WT, *vac1* and *ispH*, we have identified several RNA editing sites that are specifically affected in the *ispH* albino mutant. These RNA editing sites are located in *ClpP* (H187Y), *atpF* (P31L), *psbE* (P72S), and *rpl23* (S30L) transcripts (Fig. 9c). In addition, the editing of *ndhG* (S17F), *petL* (P2L), *rpoA* (S67F) and *rpoC1* (S170L) is reduced to similar levels in both *vac1* and *ispH* albino mutants (Fig. 9d). The profiles of the other RNA editing sites are similar among wild type, *vac1* and *ispH* (Supplemental Fig. S5).

Discussion

Editing of *accD* and *ndhF* is partially reduced, but not completely lost in *vac1*

The DYW proteins have been suggested to be involved in chloroplast RNA editing (Salone et al. 2007). We have examined all 34 known chloroplast RNA editing sites in wild type, *vac1* and *ispH*. This comparison allows us to identify editing defects that are specific to *vac1* or *ispH* albino mutants. We found that the editing of *accD* (S265L), *accD* (3′UTR) and *ndhF* (S97L) is specifically reduced, but not completely abolished in *vac1*. In contrast, it has been shown that the editing of *accD* (S265L) is completely abolished in a mutant (e.g. *ecb2*) that is allelic to *vac1* (Yu et al. 2009). To solve the discrepancy, we obtained the *ecb2* (SALK_112251) line from ABRC and analyzed the

editing of *accD* and *ndhF* transcripts in the mutant. We found that the results are similar to those of *vac1*. The editing of *accD* (S265L) is partially reduced rather than completely abolished in *ecb2*. Thus the conclusion that the albino phenotype of *ecb2* is caused by totally impaired in editing of *accD* (S265L) is incorrect. Moreover, it has been shown that a complete loss of *accD* (S265L) editing does not affect chloroplast development and the appearance of *Arabidopsis* (Robbins et al. 2009).

The *accD* gene encodes the β-carboxyl transferase subunit of acetyl-CoA carboxylase, which is important for fatty acid synthesis. It has been shown that the *accD* gene is essential and *accD* knockout plants are pale in tobacco (Kode et al. 2005). The editing of *accD* (3′UTR) is dramatically reduced in *vac1*. It is possible that the editing defect in the 3′UTR of *accD* mRNA may affect its stability or translation, which in turn may affect chloroplast development and function in *vac1*. Nonetheless, further experiments are required to test this possibility.

Similarity between *vac1* and PEP-related mutants

Steady-state mRNA levels of NEP-transcribed genes (e.g. *rpoA*, *rpoB*, *rpoC1*, *rpoC2*, *clpP*, *ycf1*, *ycf2* and some ribosomal protein genes) are increased, whereas the abundance of PEP-transcribed transcripts (e.g. *rbcL*, *psaA*, *psaB* and *psb* genes) is decreased in *vac1*. This type of molecular phenotypes has been observed in *ys1* (Zhou et al. 2009), *clb19* (Chateigner-Boutin et al. 2008), *dg1* (Chi et al. 2008), *ptac* (Pfalz et al. 2006), *rpo* and mutants that do not accumulate PEP (Hess et al. 1993; Allison et al. 1996; Hajdukiewicz et al. 1997; Silhavy and Maliga 1998; De Santis-Maciossek et al. 1999; Krause et al. 2000; Legen et al. 2002). In addition, very long transcripts detected by probes derived from several chloroplast genes were present in *vac1*, which is similar to those observed in *clb19* and PEP mutants (Legen et al. 2002; Chateigner-Boutin et al. 2008). For instance, high molecular weight *atpB-atpE* transcripts similar to those observed in *clb19* were detected in *vac1*. However, our analyses reveal that the orientation of these high molecular weight transcripts in *vac1* is different from those in *clb19*. The majority of *Arabidopsis* chloroplast genes are transcribed from multiple NEP and PEP promoters (Swiatecka-Hagenbruch et al. 2007). Those very long transcripts may be transcribed from cryptic NEP promoters. The complex chloroplast gene expression patterns observed in *vac1* may be derived from selective usage of PEP and NEP promoters, and/or defects in RNA metabolism.

Glutamyl-tRNA has been proposed to mediate a switch in RNA polymerase use during chloroplast biogenesis (Hanaoka et al. 2005). The plastid-encoded glutamyl-tRNA can bind to and inhibit the transcriptional activity of NEP in vitro (Hanaoka et al. 2005). We used RNA gel blot analyses to examine the steady-state levels of *trn* transcripts in wild type, *vac1* and *ispH*. These analyses allow us to detect polycistronic and unspliced transcripts of most *trn* genes. The banding patterns of *trn* transcripts in *vac1* are similar to those of wild type and no obvious defects in tRNA splicing were observed. Interestingly, steady-state levels of some *trn* transcripts, including those of *trnE*, are specifically decreased in *vac1*. Low levels of glutamyl-tRNA in *vac1* may relieve the inhibition of NEP, which may in part explain the up-regulation of NEP-transcribed transcripts in *vac1*.

Most of the molecular phenotypes of *vac1* are similar to those of PEP-related mutants. One of the exceptions is the expression of *accD* gene. It has been shown that the abundance of NEP-transcribed *accD* transcripts is increased in *clb19* and *ys1* mutants (Chateigner-Boutin et al. 2008; Zhou et al. 2009). Interestingly, steady-state levels of *accD* transcripts are slightly decreased in the *vac1* mutant. Unlike the *clb19* and *ys1* mutants, the editing of *accD* transcripts is specifically affected in *vac1*. It is possible that these editing defects may affect the stability of *accD* mRNA or expression *accD* gene.

Editing defects in *rpoA* and *rpoB* transcripts may affect PEP activity and cause profound effects on chloroplast gene expression (Zhou et al. 2009; Chateigner-Boutin et al. 2008). We did not observe specific editing defects in transcripts of *rpoA*, *rpoB* and *rpoC1* in *vac1*. The efficiency of *rpoA* RNA editing is reduced to about 50% in both *vac1* and *ispH*. Similarly, we did not observe significant difference in the editing of *rpoB* and *rpoC1* transcripts between *vac1* and *ispH*. Thus the incomplete editing of *rpoA*, *rpoB* and *rpoC1* cannot be accounted for the albino phenotype of *vac1*. Still, it is possible that the *rpoA*, *rpoB*, *rpoC1* and *rpoC2* transcripts are not properly edited in *vac1* at a site that has not been reported yet. We used RT-PCR to amplify cDNAs covering the entire coding regions and parts of the 5′ and 3′UTR of *rpoA*, *rpoB*, *rpoC1* and *rpoC2* in wild type and *vac1*. We compared the amplified cDNA sequences to genomic DNA and did not find new editing sites in these genes (data not shown). It is unlikely that editing defects in *rpoA*, *rpoB*, *rpoC1* or *rpoC2* transcripts are responsible for the *vac1* phenotypes. However, we cannot exclude the possibility that the VAC1 protein may be involved in regulating PEP activity via mechanisms other than RNA editing.

Common and distinct molecular phenotypes between two albino mutants

The primary defect of *ispH* is in the plastid isoprenoid biosynthetic pathway. We found that a defect in a key biosynthetic pathway such as the *ispH* mutant can have

many indirect effects on chloroplast gene expression and RNA editing. Similarly, we expect to see many secondary effects in the *vac1* albino mutant. We tried to rule out the indirect or nonspecific effects in *vac1* by comparing to another albino mutant *ispH*. This comparison allows us to distinguish common and distinct molecular phenotypes between two different albino mutants. For instance, a dramatic reduction in the abundance of *psaA-psaB* transcripts is common in both *vac1* and *ispH* (Fig. 5b). It is likely that the down-regulation of these genes is due to some common secondary effects occurring in both albino mutants. This type of non-specific effects cannot be found by only comparing the *vac1* mutant to wild type.

By contrast, we have uncovered several molecular phenotypes that are specific to *vac1* or *ispH*. For example, the abundance and expression patterns of many chloroplast *psb* transcripts are only slightly affected in *ispH* but are dramatically affected in *vac1*. The abundance of 23S, 4.5S and 5S rRNAs and the editing of *accD* and *ndhF* transcripts are specifically reduced in *vac1*. These *vac1*-specific molecular phenotypes can be attributed to the loss of VAC1 protein rather than the general photo-oxidative defects commonly occur in albino plants.

In addition to common photo-oxidative damages occurred in albino mutants, it is possible that different albino mutants may have specific redox and/or oxidative stress signals. These common and specific signals may be involved in regulating the expression of chloroplast and nuclear genes. For instance, the specific defects in chloroplast *psb* gene expression in *vac1* may be caused by signals specifically generated in *vac1* but not in *ispH*. Another example is the expression of nucleus-encoded *psa* and *psb* genes in *vac1*. It is well known that the functional state of chloroplasts may signal changes in the expression of nuclear genes (Nott et al. 2006; Woodson and Chory 2008). Chloroplast development and function are impaired in *vac1*. It is expected that the expression of nucleus-encoded photosynthetic genes will be down-regulated in *vac1*. Interestingly, compared to those of wild type, steady-state mRNA levels of *CAB*, *rbcS* and many nucleus-encoded *psa* and *psb* genes are significantly decreased in *ispH* but not or are only slightly decreased in *vac1*. These results suggest that distinct plastid-to-nucleus retrograde signaling pathways may exist in *vac1* and *ispH*, respectively.

It is likely that multiple plastid-to-nucleus signaling pathways may exist in plants. One of the examples is the GUN signaling pathway involved in NF-initiated retrograde regulation of *CAB* and *rbcS* (Susek et al. 1993; Mochizuki et al. 2001; Larkin et al. 2003; Koussevitzky et al. 2007). The expression of several non-photosynthetic genes including *ZAT10* and *ZAT12* is also subject to plastid-to-nucleus retrograde regulation (Pogson et al. 2008). The *ZAT10* gene encodes a zinc finger transcription factor and its expression is regulated by drought, salt, cold and high light (Sakamoto et al. 2000, 2004; Gong et al. 2001; Lee et al. 2002; Rossel et al. 2007). We found that NF treatment significantly represses the expression of *ZAT10* in wild type. This NF effect still exists in *ispH* but not in *vac1*. By contrast, the NF effect on the repression of *CAB* and *rbcS* is not affected in *vac1*. These results suggest that the VAC1 protein is not involved in the GUN signaling pathway. Instead, the VAC1 PPR protein may be specifically involved in an unknown retrograde regulation of *ZAT10* initiated by NF. It will be interesting to further examine if VAC1 is directly or indirectly involved in specific retrograde signaling pathways.

Possible roles of the VAC1 PPR protein

The VAC1 PPR protein has a truncated DYW domain, whereas the other PPR-DYW proteins end with a very conserved tri-peptide DYW or DFW. This suggests that VAC1 could act differently from the other PPR-DYW proteins. The *vac1* knockout mutant has global defects in chloroplast gene expression. It remains unclear if the phenotypes of *vac1* are caused by a severe editing defect in *accD* 3′UTR. The molecular phenotypes in *vac1* could be explained by defects in translation or PEP activity. Since the accumulation of chloroplast rRNAs is significantly reduced, the function of chloroplast ribosome may be compromised in *vac1*. A defect in ribosome will directly affect the translation of chloroplast proteins and causes a global effect on chloroplast gene expression. Nevertheless, more studies are required to unravel the exact molecular mechanism of VAC1.

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