An Arabidopsis Pentatricopeptide Repeat Protein, SUPPRESSOR OF VARIEGATION7, Is Required for FtsH-Mediated Chloroplast Biogenesis[1][W][OA]

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The Arabidopsis (*Arabidopsis thaliana*) *yellow variegated2* (*var2*) mutant has green- and white-sectored leaves due to loss of VAR2, a subunit of the chloroplast FtsH protease/chaperone complex. Suppressor screens are a valuable tool to gain insight into VAR2 function and the mechanism of *var2* variegation. Here, we report the molecular characterization of *004-003*, a line in which *var2* variegation is suppressed. We found that the suppression phenotype in this line is caused by lack of a chloroplast pentatricopeptide repeat (PPR) protein that we named SUPPRESSOR OF VARIEGATION7 (SVR7). PPR proteins contain tandemly repeated PPR motifs that bind specific RNAs, and they are thought to be central regulators of chloroplast and mitochondrial nucleic acid metabolism in plants. The *svr7* mutant has defects in chloroplast ribosomal RNA (rRNA) processing that are different from those in other *svr* mutants, and these defects are correlated with reductions in the accumulation of some chloroplast proteins, directly or indirectly. We also found that whereas *var2* displays a leaf variegation phenotype at 22°C, it has a pronounced chlorosis phenotype at 8°C that is correlated with defects in chloroplast rRNA processing and a drastic reduction in chloroplast protein accumulation. Surprisingly, the cold-induced phenotype of *var2* cannot be suppressed by *svr7*. Our results strengthen the previously established linkage between *var2* variegation and chloroplast rRNA processing/chloroplast translation, and they also point toward the possibility that VAR2 mediates different activities in chloroplast biogenesis at normal and chilling temperatures.

Variegation mutants are powerful tools to gain insight into mechanisms of chloroplast biogenesis (Sakamoto et al., 2003; for review, see Rodermel, 2002; Yu et al., 2007). The Arabidopsis (*Arabidopsis thaliana*) *yellow variegated2* (*var2*) mutant has green cotyledons but its true leaves are green and white sectored; sectoring is produced in homozygous recessive plants. The green sectors contain normal-appearing chloroplasts, while the white sectors contain abnormal plastids that lack pigments and developed lamellar structures (Chen et al., 1999). Heteroplastidic cells that have some normal-appearing chloroplasts are also found in *var2* white tissues, indicating that individual plastids are affected differently by the nuclear mutation (i.e. *var2* acts in a plastid-autonomous manner; Chen et al., 1999). Because the tissues of *var2* have a uniform genetic constitution, a major question is why some chloroplasts are able to bypass the requirement for VAR2 during chloroplast biogenesis. Why isn't the mutant all white?

The *VAR2* gene (also designated *AtFtsH2*) encodes an FtsH ATP-dependent metalloprotease that is targeted to thylakoid membranes (Chen et al., 2000; Takechi et al., 2000). In *Escherichia coli* and yeast, mitochondrial FtsH proteins play a central role in protein quality control and cellular homeostasis (Ito and Akiyama, 2005; Koppen and Langer, 2007). The best understood function of FtsH in photosynthetic organisms is the degradation of photodamaged D1 reaction center proteins during the PSII repair cycle (Lindahl et al., 2000; Adam and Clarke, 2002; Bailey et al., 2002; Sakamoto et al., 2002; Silva et al., 2003; Kamata et al., 2005; Nixon et al., 2005; Zaltsman et al., 2005a, 2005b; Yoshioka et al., 2006; Cheregi et al., 2007; Kato et al., 2009). Other substrates of chloroplast FtsH include unassembled cytochrome b_6f Rieske FeS proteins (Ostersetzer and Adam, 1997) and light-harvesting complex II (LHCII) proteins (Zelisko et al., 2005). In cyanobacteria, FtsH acts on a variety of unassembled PSII subunits (Komenda et al., 2006). FtsH might mediate other plastid activities as well, inasmuch as it has been implicated in membrane fusion and/or translocation events (Hugueney et al., 1995), and appears to be a component of signal transduction chains that are elicited in response to pathogens (Seo et al., 2000), light

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(Tepperman et al., 2001), and temperature (Chen et al., 2006).

We have proposed a threshold model to explain the mechanism of *var2* variegation (Yu et al., 2004, 2005, 2007). According to this model, chloroplast biogenesis requires a threshold of FtsH activity, and the threshold varies from plastid to plastid because of intrinsic differences in the metabolic profiles of the 100+ developing plastids in a typical Arabidopsis mesophyll cell (e.g. due to gradients, sometimes steep, of CO_2, light, chlorophyll, and photosynthesis across the thickness of a leaf; Smith et al., 1997). The plastids in a cell might also have variable amounts of the various FtsH proteins. It is proposed that subthreshold FtsH activities result in an arrest of chloroplast development; consequently, white plastids are formed that generate white cells and white sectors in the developing leaf. Threshold activities, on the other hand, result in the formation of chloroplasts and green sectors. Our working hypothesis is that thresholds in *var2* are achieved by FtsH activities per se and/or by the activities of factor(s)/process(es) that are able to compensate for a lack of VAR2.

To gain insight into the compensating mechanisms that allow green sector formation in *var2*, we and others have carried out second-site suppressor screens (Park and Rodermel, 2004; Miura et al., 2007; Yu et al., 2008). These screens have led to the identification of a number of recessive suppressor genes. These include *ClpC2*, the gene for a chloroplast ClpC/Hsp100 chaperone (Park and Rodermel, 2004); *SUPPRESSOR OF VARIEGATION1* (*SVR1*), which codes for a chloroplast pseudouridine synthase (Yu et al., 2008); *SVR2*, also designated *ClpR1* (Koussevitzky et al., 2007b), the gene for a subunit of the chloroplast ClpP/R protease complex (Yu et al., 2008); *FUG1*, which encodes the chloroplast translation factor IF-2 (Miura et al., 2007); and *SCO1*, which codes for the chloroplast translation factor EF-G (Miura et al., 2007). Interestingly, most of these suppressors are involved in the coupled processes of chloroplast ribosomal RNA (rRNA) processing and translation. This has given rise to the hypothesis that reductions in chloroplast translation are able to compensate for a lack of VAR2 by lowering the threshold demand for FtsH in the population of developing chloroplasts. Another mechanism for suppression has recently come to light with the demonstration that ectopic expression of the heterotrimeric G protein α-subunit (GPA1) suppresses *var2* variegation, perhaps by regulating the expression of nuclear genes for FtsH proteins (Zhang et al., 2009).

In this report, we characterize another *var2* suppressor (designated *004-003*) and demonstrate that suppression of variegation in this line is due to down-regulated expression of *SVR7*, encoding a chloroplast-localized pentatricopeptide repeat (PPR) protein. We show that *svr7* mediates defects in chloroplast rRNA processing that are different from those reported for *svr1* and *svr2* (Yu et al., 2008) and that processing proceeds by a hierarchy of events, as highlighted by double mutant analyses. The defects in rRNA processing are accompanied by decreases in the accumulation of some chloroplast proteins, consistent with the idea that decreased protein synthesis is able to suppress FtsH-mediated defects in chloroplast biogenesis. Interestingly, examination of *004-003* revealed that there is a unique genetic interaction between *svr7* and *var2* at chilling temperatures (8°C): at this temperature, *var2* displays a pronounced chlorosis in newly emerging tissues that cannot be bypassed by the loss of SVR7 activity. Taken together, the data presented here reinforce our earlier findings of a strong genetic interaction between chloroplast rRNA processing/translation and VAR2 function and raise the intriguing possibility that VAR2 mediates chloroplast biogenesis via independent processes that act at normal and chilling temperatures.

RESULTS

Phenotype of the *var2* Suppressor Line, *004-003*

We have previously reported the isolation of *var2* suppressors using ethyl methanesulfonate mutagenesis (Park and Rodermel, 2004) and T-DNA activation tagging (Yu et al., 2008). One of the suppressors in our collection of activation-tagged mutants (designated *004-003*) is the topic of this paper. Backcrosses between *004-003* and wild-type Columbia showed that the suppressor gene in *004-003* behaves in a recessive manner. We named the suppressor gene locus *SVR7*, and the mutant allele in *004-003* was designated *svr7-1*. *svr7-1* single mutants were isolated in the F2 progeny of the backcross using derived cleaved-amplified polymorphic sequence primers (Neff et al., 1998) to genotype the *VAR2* locus (Park and Rodermel, 2004). Figure 1A shows that the *004-003* double mutant (*var2-5 svr7-1*) resembles the *svr7-1* single mutant and that both are smaller and more pale green than the wild type and *var2-5*. The reductions in chlorophyll content in *004-003* and *svr7* are significant (approximately 25%; Fig. 1B) but do not reflect an underlying perturbation in photosystem structure or stoichiometry, since chlorophyll *a*/*b* ratios are not appreciably altered (Fig. 1C).

The *var2-5* allele is caused by a missense mutation and is leaky, inasmuch as the mutant plants accumulate *VAR2* mRNAs and low amounts of the mutant protein (Chen et al., 2000). Its variegation phenotype is less severe than that of *var2-4*, which has a defect in mRNA splicing and approximates the null phenotype (Yu et al., 2004). To test whether the suppression of variegation in *004-003* is allele specific, we generated *svr7-1 var2-4* double mutants. Figure 1D shows that these plants are not variegated and that they resemble *004-003*. Taken together, the data in Figure 1 indicate that *svr7-1* is epistatic to *var2* and that suppression of *var2* variegation by *svr7-1* is not allele specific.

Map-Based Cloning of *SVR7*

Cosegregation analysis indicated that the suppression of variegation phenotype in *004-003* is not linked

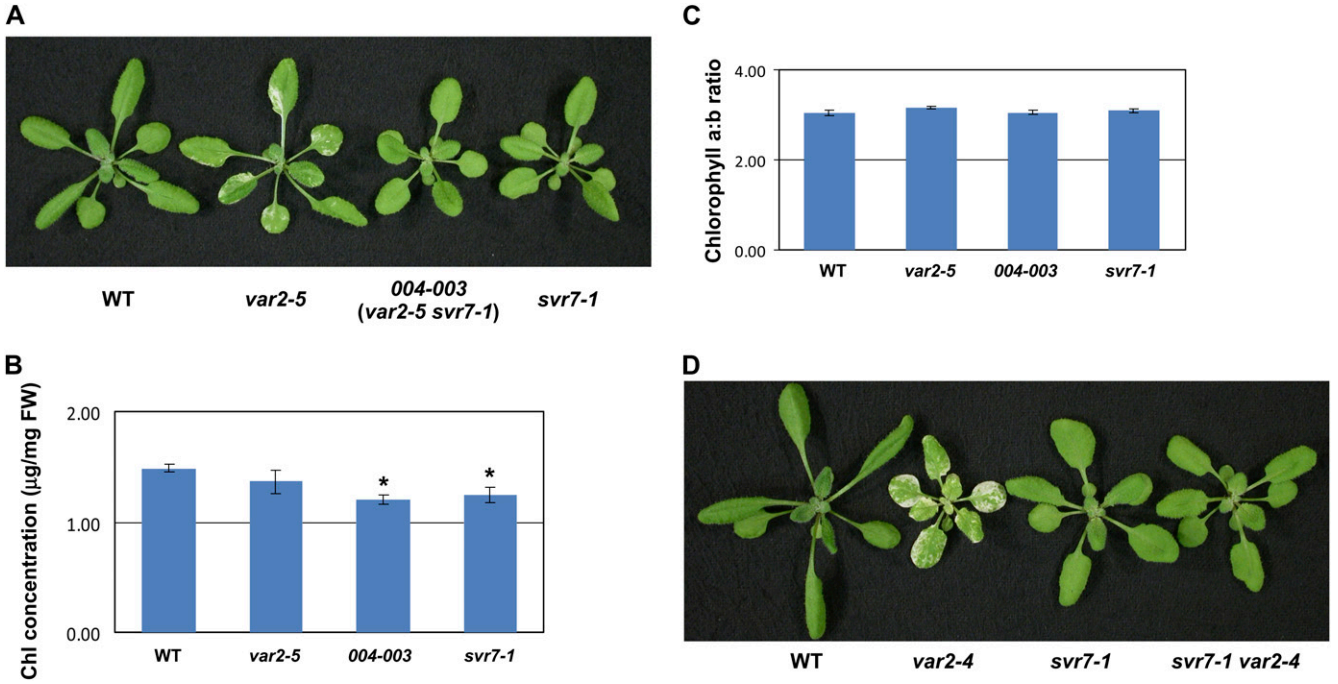

Figure 1. Phenotypes of double and single suppressor mutants. A, Representative 3-week-old wild-type (WT), *var2-5*, *004-003* (*var2-5 svr7-1*), and *svr7-1* plants. B, Chlorophyll contents of 2-week-old wild-type, *var2-5*, *004-003*, and *svr7-1* plants. Asterisks indicate significant differences ($P < 0.01$). FW, Fresh weight. C, Chlorophyll *a*/*b* ratios of 2-week-old wild-type, *var2-5*, *004-003*, and *svr7-1* plants. D, Representative 3-week-old wild-type, *var2-4*, *svr7-1*, and *svr7-1 var2-4* plants.

to a T-DNA insert but, rather, is caused by a recessive mutation at another site (data not shown). Hence, we turned to methods of positional cloning to identify the *SVR7* locus (see "Materials and Methods"). In brief, this method involved first mapping the gene to an approximately 76-kb interval between *FCA5#3* and *FCA6#2* and then determining the genomic DNA sequences of candidate nuclear genes for chloroplast proteins in this interval in the mutant *svr7-1* plants (Fig. 2). One of these genes (At4g16390) was found to contain a series of short (1–5 bp) deletions and a few point mutations in a discrete region of the gene. The At4g16390 gene model contains a single exon and codes for a protein of 702 amino acids (approximately 78.2 kD), and the mutations would be predicted to generate a premature stop codon and a truncated translation product (317 amino acids) lacking important functional domains of the protein (described below). Hence, we tentatively designated At4g16390 as *SVR7*.

Complementation of *004-003* by *SVR7*

If suppression of variegation in *004-003* is due to a loss of functional SVR7, it should be possible to restore the variegation phenotype by overexpression of *SVR7* in *004-003*. To test this hypothesis, a full-length *SVR7* genomic DNA sequence was cloned into a binary T-DNA vector, and the construct was transformed into *004-003*. Figure 3A shows that the transgenic plants (*004-003 P35S:SVR7*) have a variegated phenotype, similar to *var2-5*. Figure 3B shows that the overexpression plants are indeed transformed in that they have dramatically enhanced *SVR7* mRNA levels when compared with the *004-003* plants. Taken together with the prediction that a translation product lacking the C-terminal half of SVR7 would be generated in *svr7-1* (Fig. 2), the extremely low levels of *SVR7* transcripts in *004-003* suggest that *svr7-1* is a molecular null, or at least that it approximates the null phenotype. We conclude from the data in Figure 3 that disruption of At4g16390 is responsible for the suppression of *var2* variegation in *004-003*.

SVR7 Is a PPR Protein with an SMR Domain

Analyses using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) revealed that SVR7 has all the hallmarks of a P subclass PPR protein (Lurin et al., 2004; Quevillon et al., 2005; Schmitz-Linneweber and Small, 2008). PPR proteins contain degenerate PPR motifs (approximately 31–36 amino acids) each of which contains a pair of antiparallel α-helices (Small and Peeters, 2000). PPR motifs are organized as tandem arrays in the protein, and they assemble into a superhelical structure that is able to bind an RNA ligand with high specificity (Delannoy et al., 2007). PPR proteins have been subdivided into P and PLS classes that are distinguished from one another on the basis of differences in their PPR sequences as well as on the presence of various motifs in their C termini (Lurin et al., 2004).

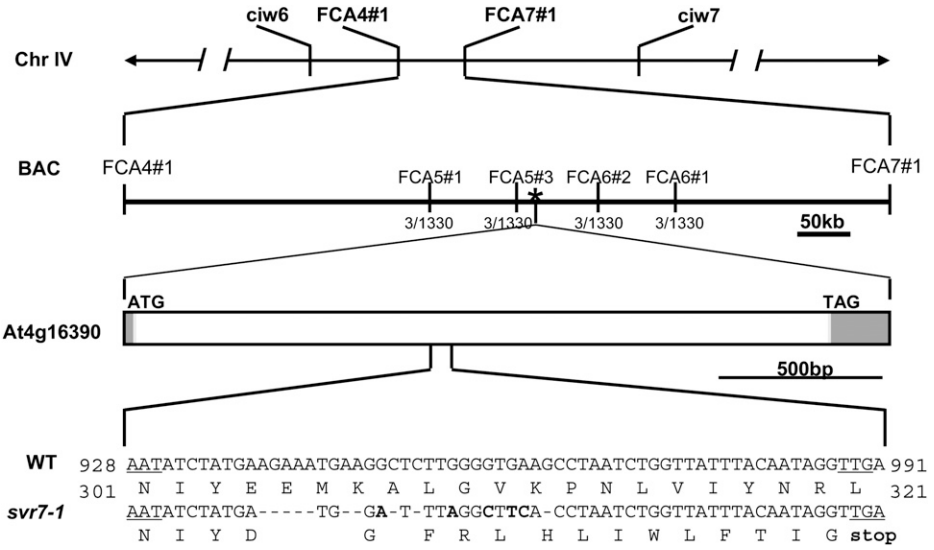

Figure 2. Map-based cloning of *SVR7*. *SVR7* was mapped by positional cloning as described in "Materials and Methods." The gene was fine-mapped between *FCA5#3* and *FCA6#2* using markers described in Supplemental Table S1 (indicated by vertical lines). The number of recombinants is shown under each marker; 665 F2 plants (1,330 chromosomes) were examined. Genomic sequencing of candidate genes between *FCA5#3* and *FCA6#2* led to the identification of *SVR7* (At4g16390). The position of *SVR7* is indicated by an asterisk. There is a single exon in the *SVR7*/At4g16390 gene model (indicated by the box). The gray parts of the box in the gene model represent the 5′ and 3′ untranslated regions. The deletions (1–5 bp) in *svr7-1* are shown as dashed lines, and the point mutations are indicated in boldface.

The C-terminal motifs are thought to define sites for binding of effectors (such as proteins or nucleic acids; Lurin et al., 2004; Delannoy et al., 2007).

Figure 4A shows that SVR7 contains 10 PPR motifs, nine of which occur in a tandem array, spanning amino acid residues 176 to 491. SVR7 also contains an SMR (for small MutS related) domain near its C terminus. The SMR domain was first identified in the C-terminal region of the *Synechocystis* sp. MutS2 protein (Moreira and Philippe, 1999), and it is also present in the *E. coli* SMR protein (Fig. 4A). MutS2 might be involved in the inhibition of homologous recombination (Pinto et al., 2005), but the function of the *E. coli* SMR protein is unclear.

In Arabidopsis, at least eight proteins have an architecture similar to SVR7 (i.e. tandem arrays of PPR motifs + an SMR domain; Fig. 4A). These include GUN1, which is a key regulator of chloroplast-to-nucleus retrograde signaling (Koussevitzky et al., 2007a), and pTAC2, which copurifies with plastid transcriptionally active chromosomes (pTACs; Pfalz et al., 2006). Figure 4B shows that among the eight proteins, SVR7 is most closely related to At5g46580 (38% amino acid identity), while GUN1 is most similar to pTAC2.

Chloroplast Localization of SVR7

Organelle-targeting algorithms predict that SVR7 has an N-terminal chloroplast transit peptide of 53 amino acids (Emanuelsson et al., 2007). To confirm that SVR7 resides in the chloroplast, an SVR7/GFP fusion (*P35S*:*SVR7*:*GFP*) was generated and transiently expressed in wild-type Arabidopsis leaf protoplasts; confocal microscopy was used to monitor the fluorescence signals. Figure 5, A to C, reveals that the green fluorescence signals from GFP are present in the cytoplasm of cells transformed with a control construct lacking *SVR7* (*P35S*:*GFP*); these signals do not merge with the red autofluorescence signals from chloroplasts. By contrast, when *P35S*:*SVR7*:*GFP* is expressed (Fig. 5, D–F), the green fluorescence signals colocalize with chloroplasts, some as discrete foci within the organelle (as indicated by merging of the red and green fluorescence signals). These data indicate that SVR7 is a plastid protein.

Disruption of *SVR7* Results in Chloroplast rRNA Processing Defects

Chloroplast rRNA genes (23S, 16S, 4.5S, and 5S) constitute an operon (*rrn* operon) in the chloroplast genome (Fig. 6A). The maturation of chloroplast rRNAs involves a series of endonuclease-cleavage and exonuclease-trimming events (Bollenbach et al., 2005). Casual inspection of an ethidium bromide-stained denaturing gel of RNAs from *004-003* (Fig. 3B) reveals that the stoichiometry of rRNA bands is disrupted in this line. To examine this in greater detail, RNA gel-blot analyses were performed on RNAs from *004-003* and *svr7-1* using rRNA gene-specific probes (Fig. 6B). Blots of total leaf Arabidopsis RNAs probed with 23S rDNA contain seven readily detectable bands (Fig. 6B, lane WT): the 3.2-kb band is the 23S-4.5S dicistronic precursor; the 2.9-, 2.4-, and 1.7-kb bands

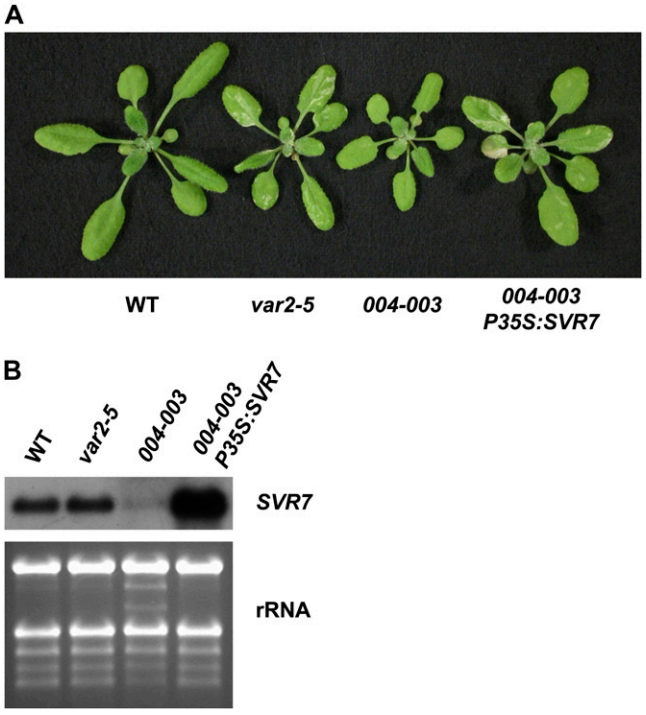

Figure 3. Complementation of *004-003*. A, Representative 3-week-old wild-type (WT), *var2-5*, *004-003*, and *004-003 P35S:SVR7* plants. B, RNA gel-blot analysis of *SVR7* mRNAs in wild-type, *var2-5*, *004-003*, and *004-003 P35S:SVR7* plants. Equal amounts of RNA (3 μg) extracted from 3-week-old seedlings were loaded onto each lane of a formaldehyde gel and transferred to a nylon membrane. The RNA gel blot was probed with ^{32}P-labeled *SVR7* cDNAs. The ethidium bromide-stained RNA gel is shown as a loading control.

are processing intermediates; and the 1.2-, 1.0-, and 0.5-kb bands are the mature forms of 23S rRNA (Bollenbach et al., 2005; Yu et al., 2008). Figure 6B shows that *var2-5* and the wild type have similar banding patterns, but that in *004-003* and *svr7-1*, the levels of the 2.9- and 2.4-kb rRNAs are significantly increased while those of the 1.7-, 1.2-, and 1.0-kb rRNAs are significantly decreased. The metabolism of chloroplast 16S rRNA and 4.5S rRNA is also perturbed in *004-003* and *svr7-1*, inasmuch as the 16S rRNA precursor and the 23-4.5S dicistronic precursor accumulate in these lines (Fig. 6B). Figure 6C shows that the 23S rRNA profile is normalized in the *004-003* complementation line (*004-003 P35S:SVR7*), indicating that the defect in chloroplast rRNA processing in *004-003* is caused, either directly or indirectly, by a lack of SVR7.

One question that arises is whether the defects in chloroplast rRNA processing are unique to *svr7-1*. To address this question, we examined 23S rRNA accumulation patterns in three other suppressor mutants: *svr1-1* (Yu et al., 2008), *svr3-1*, and *svr8-1*. The suppressor genes in the latter two lines have not yet been cloned, but fine-mapping studies indicate that they do not map to other *svr* loci or to genes for PPR proteins. Figure 6D shows that 23S rRNA processing is defective in all four *svr* lines and that each line has a unique rRNA profile. Although we cannot rule out that these defects are secondary effects of other, more general perturbations in chloroplast function, the data suggest that each mutant defines a distinct step in chloroplast rRNA processing, either directly or indirectly.

We next carried out western immunoblotting analysis to investigate the impact of *svr7* on chloroplast protein accumulation (Fig. 6E; Supplemental Fig. S2A). On a fresh weight basis, the levels of three representative chloroplast DNA-encoded proteins we examined (the D1 protein of PSII, the large subunit of Rubisco, and the α-subunit of the ATP synthase) are reduced to approximately 50% of those of the wild type in *004-003* and *svr7-1* (Fig. 6E). By contrast, the levels of most nucleus-encoded chloroplast proteins (Lhcb2, PsbP protein of PSII, and the Rieske Fe-S protein of the cytochrome b_6f complex) are not reduced in *004-003* and *svr7-1*, with the exception of the PSI PsaF protein, which appears to be reduced to approximately 50% of the wild-type level (Fig. 6E). As anticipated, sharp reductions in VAR2 in *var2-5* and *004-003* are accompanied by decreased amounts of the AtFtsH1 protein, likely because of turnover of unassembled subunits of the AtFtsH complex (Sakamoto et al., 2003; Yu et al., 2004). Considered together, the data in Figure 6E indicate that the accumulation of some chloroplast proteins, especially those of chloroplast origin, is impaired in the *svr7* background. However, at present, it is not clear whether *SVR7* mutation directly, or indirectly, leads to this impairment.

Chilling Sensitivity of *var2*

Chloroplast rRNA processing and translation are coupled events, and analyses of various *var2* suppressor lines have indicated that there is a genetic interaction between VAR2 function and these processes (Miura et al., 2007; Yu et al., 2008). A critical aspect of chloroplast translation is its importance under chilling temperatures (Tokuhisa et al., 1998; Rogalski et al., 2008). Therefore, we felt that it might be instructive to examine the responses of *var2*, *svr7-1*, and the *004-003* double mutant to chilling stress. For these experiments, we grew the plants at 22°C for 3 weeks and then transferred them to 8°C for another 4 weeks. At both temperatures, the plants were maintained under continuous light (100 μmol m^{-2} s^{-1}). Figure 7A shows that leaves that develop under chilling stress are green in wild-type plants but uniformly chlorotic in *var2-5*. The same is true of the severe allele, *var2-4*. These data indicate that the phenotype of *var2* is temperature dependent: variegation at normal growth temperatures and a chilling-induced chlorosis.

We also examined the chilling response of *var1*, which lacks the AtFtsH5 subunit of the AtFtsH complex (Sakamoto et al., 2002). Figure 7B shows that the basal parts of *var1* leaves that develop at 8°C are yellow but that, as the leaves expand, their tips eventually turn green. This suggests that chloroplast bio-

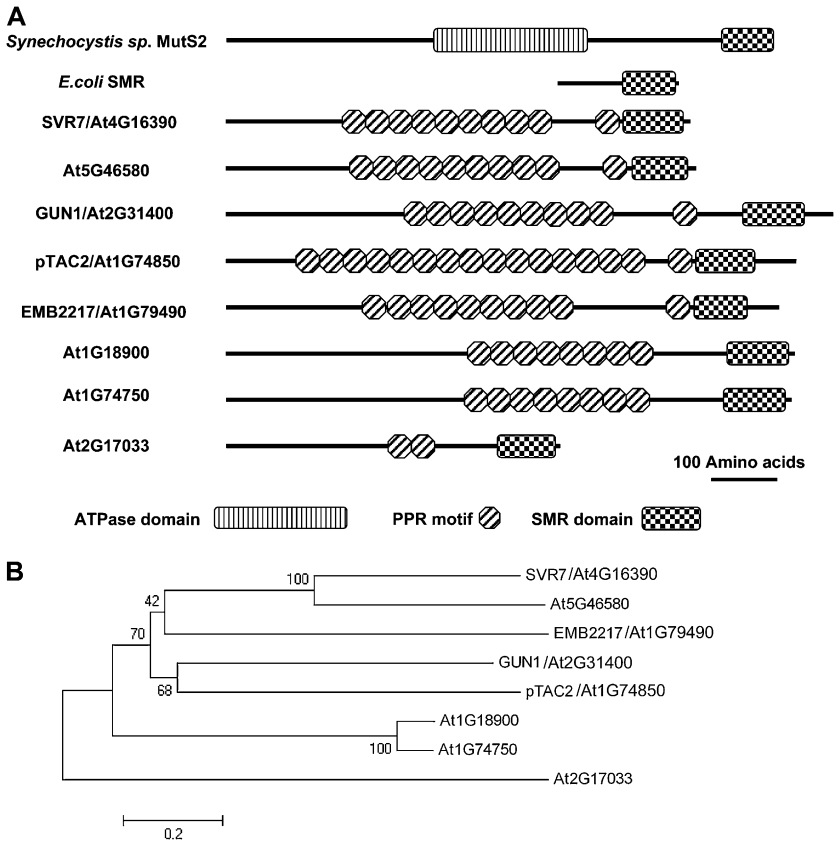

Figure 4. Protein sequence analysis of SVR7. A, Eight Arabidopsis PPR proteins have an architecture similar to SVR7. Full-length *Synechocystis* sp. MutS2 (P73625) and *E. coli* SMR (P0A8B2) protein sequences were obtained from the UniProt protein database (www.uniprot.org). Protein sequences of Arabidopsis genes were obtained from The Arabidopsis Information Resource (www.arabidopsis.org). InterProScan (www.ebi.ac.uk/Tools/InterProScan/; Quevillon et al., 2005) was used to identify conserved domains. B, Phylogenetic relatedness of Arabidopsis PPR proteins containing an SMR domain. Full-length protein sequences were obtained as in A. The phylogenetic tree was constructed using MEGA4 software (Tamura et al., 2007).

genesis in *var1* is delayed at 8°C. The less severe phenotype of *var1* versus *var2* at 8°C is in agreement with the lower abundance of VAR1 (AtFtsH5) than VAR2 (AtFtsH2) in AtFtsH complexes and with the relatively weak phenotype of *var1* versus *var2* under normal growth conditions (Sakamoto et al., 2002).

We next tested the response of *svr7-1* and *004-003* (*var2-5 svr7-1*) to chilling stress. Figure 7C reveals that the leaves of *004-003* are chlorotic at 8°C, similar to *var2-5*, while the leaves of *svr7-1* are all green at this temperature. In fact, chlorophyll concentrations at 8°C are reduced approximately 25% in *svr7-1* versus the wild type at 8°C (similar to the reductions observed at 22°C; Fig. 1B) and sharply reduced to less than 5% of normal in *var2-5* and *004-003* (Fig. 7D). Accompanying these changes are drastic alterations in chloroplast rRNA profiles: whereas temperature does not alter these profiles in *svr7-1* and the wild type (the profiles in Figs. 6B and 7E are similar), the overall levels of 23S and 16S rRNAs are greatly reduced in both

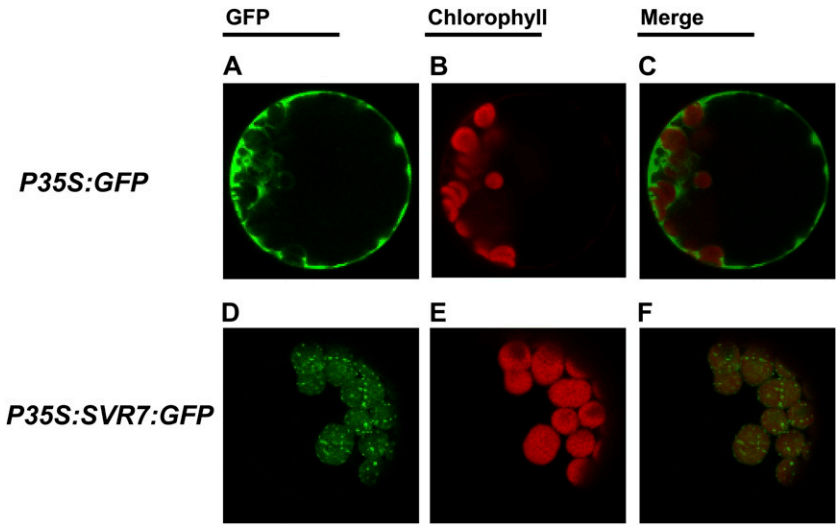

Figure 5. Chloroplast localization of SVR7. Arabidopsis leaf protoplasts from wild-type Columbia were transiently transformed with a control vector *P35S:GFP* (A–C) or with a *P35S:SVR7:GFP* vector (D–F). Green fluorescence signals (A and D) and chlorophyll autofluorescence signals (B and E) were monitored by confocal microscopy. Merged images of A and B and of D and E are shown in C and F, respectively. A representative image of a single protoplast is shown in each panel.

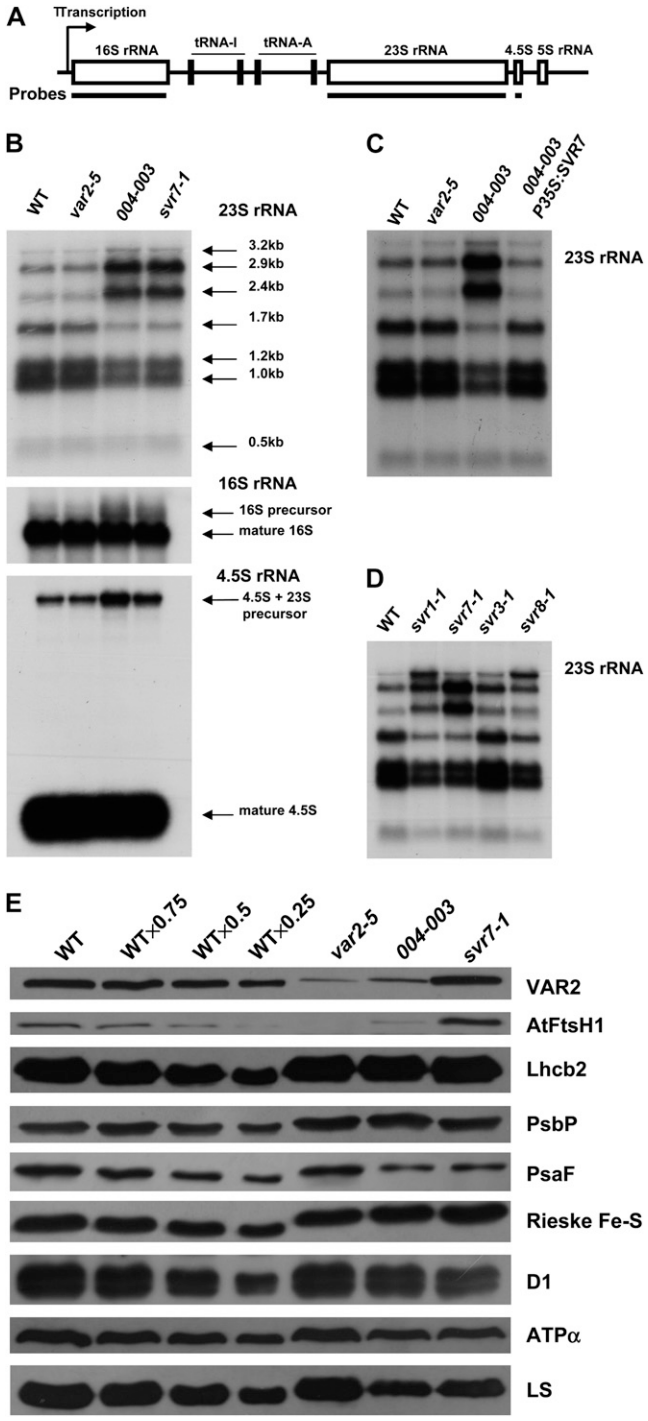

Figure 6. Chloroplast rRNA processing defects and protein accumulation in *004-003* and *svr7-1*. A, Structure of the chloroplast *rrn* operon in Arabidopsis. Thick solid lines under each rRNA gene represent the probes used for the RNA gel-blot analyses in B to D. Equal amounts of total leaf RNA (2 μg) from 3-week-old seedlings were loaded on the gel. See Supplemental Figure S1 for photographs of the ethidium bromide-stained gels in B to D. B, Accumulation of chloroplast 23S, 16S, and 4.5S rRNAs in the wild type (WT), *var2-5*, *004-003*, and *svr7-1*. C, Accumulation of 23S rRNAs in the wild type, *var2-5*, *004-003*, and *004-003 P35S:SVR7* (the *004-003* complementation line; see Fig. 3). D, Accumulation of 23S rRNAs in the wild type and four *var2* suppressor lines (*svr1-1*, *svr7-1*, *svr3-1*, and *svr8-1*). *SVR1* codes for a chloroplast pseudouridine synthase (Yu et al., 2008), while molecular characterization of *svr3-1* and *svr8-1* has not yet been reported (F. Yu, X. Liu, and S. Rodermel, unpublished data). E, Accumulation of chloroplast proteins in wild-type, *var2-5*, *004-003*, and *svr7-1* plants. Total leaf protein from 3-week-old seedlings was loaded onto the gels based on an equal fresh weight basis. See text for description of proteins; see Supplemental Figure S2A for the quantification of immunoblots.

var2-5 and *004-003*, with precursor forms accumulating at the expense of mature forms (Fig. 7E).

The dramatic changes in chlorophyll accumulation and chloroplast rRNA processing prompted us to assess the patterns of chloroplast protein accumulation in cold-developed tissues (Fig. 7F; Supplemental Fig. S2B). Figure 7F shows that some proteins, when compared on a fresh weight basis, are modestly reduced in amount in *svr7-1*, including the plastid DNA-encoded D1, LS, and ATPα proteins and the nuclear DNA-encoded PsaF protein. These findings are similar to those found at 22°C (Fig. 6E), indicating that chloroplast protein synthesis in not affected by temperature in *svr7-1*. In contrast, the levels of most chloroplast proteins are drastically reduced in the chlorotic tissues of *var2-5* and *004-003* (many are present at lower than 25% of wild-type levels). The only exception appears to be AtFtsH1, which is accumulated at around the wild-type level in *004-003* and is more abundant in cold-developed *svr7-1* single mutant tissues. Taken together, Figure 7F suggests a much greater impact of chilling temperature on chloroplast protein accumulation in *var2-5* and *004-003*, although we are not clear if this is directly or indirectly the consequence of *VAR2* mutation.

In summary, the data in Figures 3 and 6 show that at 22°C, *004-003* and *svr7-1* have similar visual and molecular phenotypes, as monitored by chlorophyll concentrations, chloroplast rRNA profiles, and chloroplast protein accumulation profiles. This suggests that *svr7-1* is epistatic to *var2* at this temperature (i.e. a loss of SVR7 is able to bypass the requirement for VAR2 during chloroplast biogenesis). The data in Figure 7, on the other hand, show that this is conditional, inasmuch as *svr7* does not suppress the chilling-induced chlorosis phenotype of *var2*, nor does it suppress the *var2*-mediated defects in rRNA processing and protein accumulation at the low temperature. This suggests that *var2-5* is epistatic to *svr7-1* at 8°C (i.e. a loss of SVR7 does not compensate for a loss of VAR2 in chloroplast development).

Genetic Interactions between *svr7* and *svr2*

In addition to *svr1* (Fig. 6D), we have previously shown that *svr2* has a chloroplast rRNA processing defect (Yu et al., 2008). SVR2 is a subunit of the chloroplast ClpP/R protease, a stromal enzyme that is thought to be responsible for most protein turnover in the plastid (Adam and Clarke, 2002; Adam et al.,

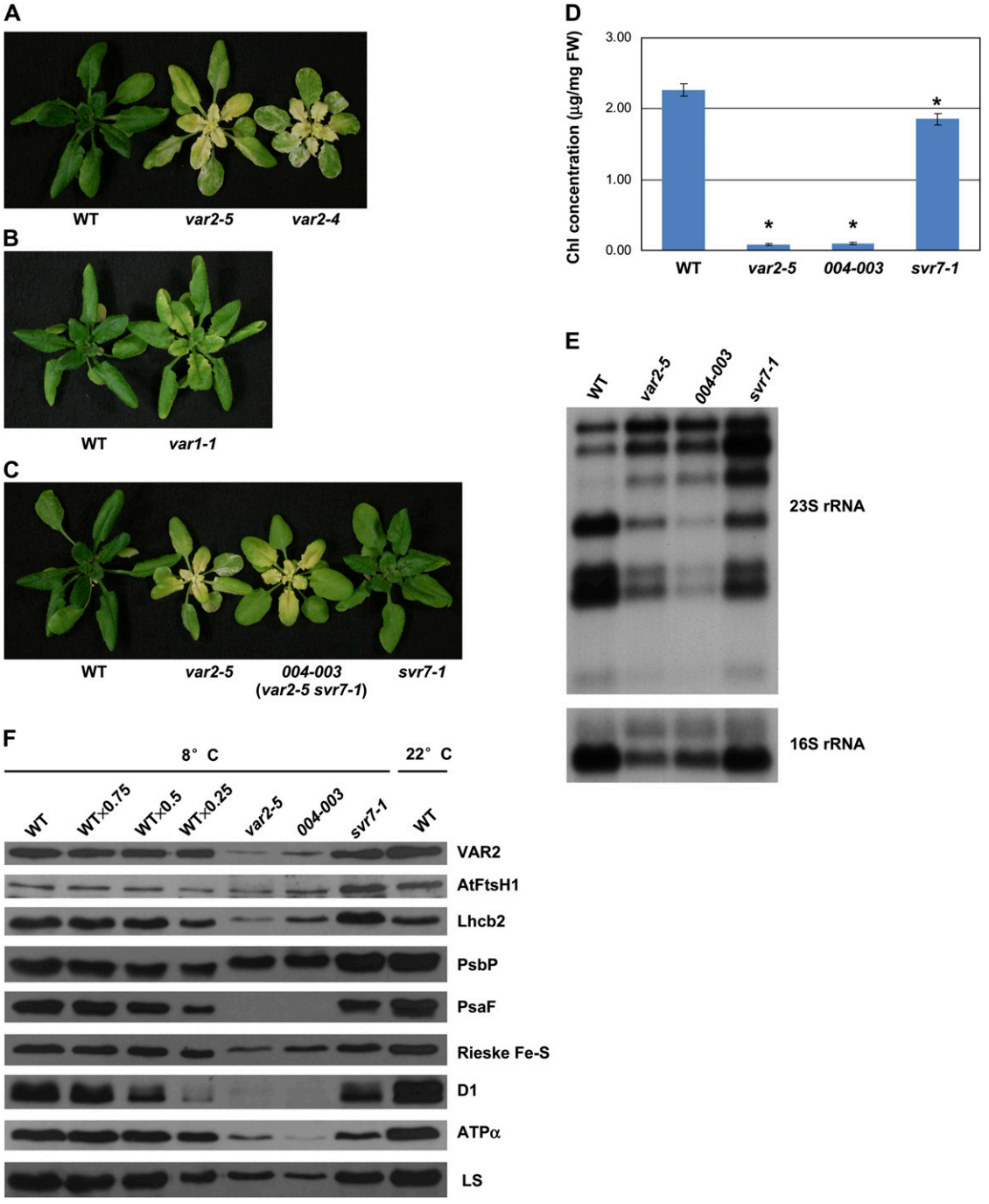

Figure 7. Chilling-induced chlorosis in *var2*. Plants were grown at 22°C for 3 weeks and then transferred to 8°C for another 4 weeks before photographing. At both temperatures, plants were grown under continuous light (100 μmol m^{-2} s^{-1}). A, Phenotypes of representative wild-type (WT), *var2-5*, and *var2-4* plants. B, Phenotypes of representative wild-type and *var1-1* plants. C, Phenotypes of representative wild-type, *var2-5*, *004-003*, and *svr7-1* plants. D, Chlorophyll contents of cold-developed tissues of wild-type, *var2-5*, *004-003*, and *svr7-1* plants. Asterisks indicate significant differences ($P < 0.01$). FW, Fresh weight. E, RNA gel-blot analysis of chloroplast 23S rRNA and 16S rRNA accumulation in the plants in C. Equal amounts of RNA (2 μg) were loaded on the gel. See Supplemental Figure S1 for a photograph of the ethidium bromide-stained gel. F, Accumulation of chloroplast proteins in leaves of wild-type, *var2-5*, *004-003*, and *svr7-1* plants developed at 8°C. Total leaf protein was extracted and loaded on a fresh weight basis for immunoblotting. See Supplemental Figure S2B for the quantification of immunoblots.

2006). As a first step to understand how SVR proteins are involved in chloroplast rRNA processing, we generated *svr2 svr7* double mutants. These were identified using a PCR-based assay (data not shown).

Figure 8A shows that the *svr2 svr7* double mutant and *svr2-1* single mutants have similar growth characteristics and morphologies and that these are markedly different in *svr7-1*. Figure 8B reveals that the 23S rRNA profiles of *svr2-1* and *svr7-1*, although similar, have different precursor stoichiometries and that the profile of the double mutant resembles that of *svr2-1*, not *svr7-1*. Taken together, these data indicate that *svr2* is epistatic to *svr7*. One interpretation consistent with these data is that SVR2 and SVR7 function in the same process or pathway of chloroplast rRNA processing and that SVR2 is involved in a more upstream event than SVR7.

DISCUSSION

Function of SVR7

Our analyses have shown that SVR7 is not essential for chloroplast development but that loss of SVR7 is able to suppress *var2*-mediated defects in chloroplast biogenesis in a temperature-dependent manner. To gain insight into these observations, perhaps the first question concerns the function of SVR7. PPR proteins are central regulators of RNA metabolism in organelles, and they bind RNA via the PPR motif (Delannoy et al., 2007; Schmitz-Linneweber and Small, 2008, Stern et al., 2010). Because PPR motifs do not have known catalytic sites, PPR proteins are thought to act as adaptors to facilitate interaction between an effector molecule and a specific RNA substrate (Delannoy et al., 2007). Consistent with this idea, a number of PPR proteins contain C-terminal sequences that confer specific functions. An increasing number of substrate RNAs and effectors of PPR proteins have been identified either biochemically or genetically (Schmitz-Linneweber and Small, 2008, Stern et al., 2010).

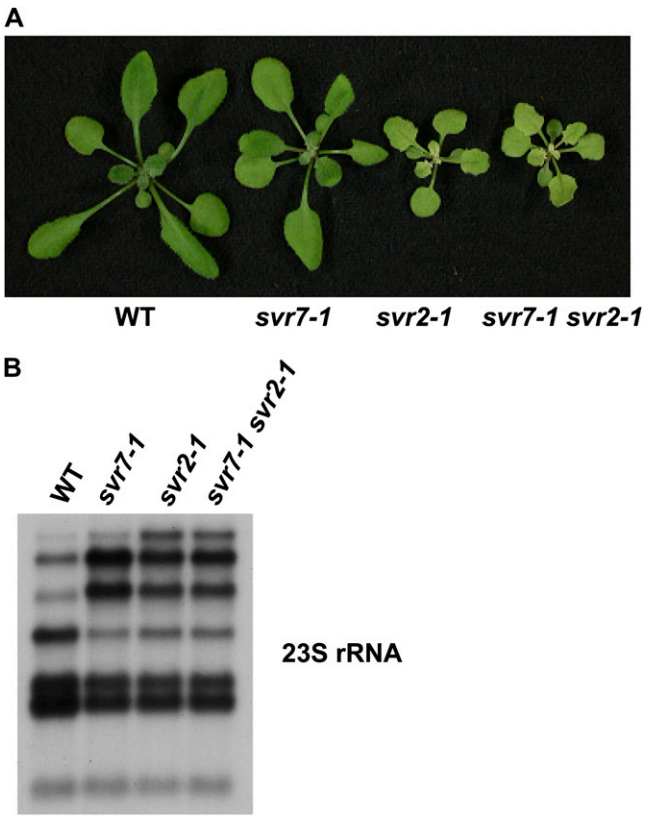

Figure 8. Genetic interaction between *svr7* and *svr2*. A, Phenotypes of representative 3-week-old wild-type (WT), *svr7-1*, *svr2-1*, and *svr7-1 svr2-1* plants growing under normal conditions. B, RNA gel-blot analysis of chloroplast 23S rRNA accumulation in the plants in A. Equal amounts of RNA (2 μg) extracted from 3-week old seedlings were loaded on the gel. See Supplemental Figure S1 for a photograph of the ethidium bromide-stained gel.

Our studies have shown that SVR7 has canonical PPR motifs and a conserved C-terminal SMR domain. Support for the idea that SVR7 binds RNA comes from studies of the radish (*Raphanus sativus*) P67 protein (Echeverria and Lahmy, 1995; Lahmy et al., 2000). The sequence of P67 is very similar to SVR7, and it was identified in radish extracts as a nuclear protein with in vitro binding activity to a fragment of nuclear pre-rRNA, designated rBP (Echeverria and Lahmy, 1995). However, subsequent studies showed that P67 resides in the plastid rather than the nucleus-cytoplasm, suggesting that rBP is not the physiological binding partner of P67 (Lahmy et al., 2000). Because the nucleotide sequence of rBP does not share homology with any chloroplast RNA species, Lahmy et al. (2000) proposed that the interaction between P67 and its RNA binding partner might be directed by the secondary or tertiary structure of the RNA fragment. This RNA has yet to be defined biochemically.

SVR7 is one of at least eight Arabidopsis PPR proteins that have an SMR domain in their C terminus (Fig. 4A). Two of these, pTAC2 and GUN1, have been partially characterized. pTAC2 is a component of pTACs that have been isolated from Arabidopsis and mustard (*Brassica juncea*; Pfalz et al., 2006), while GUN1 has received much attention as a putative central integrator of retrograde signaling pathways (Susek et al., 1993; Koussevitzky et al., 2007a). Because GUN1 colocalizes with pTAC2 in discrete foci within chloroplasts (reminiscent of the punctuate GFP signals of SVR7; Fig. 5), it has been suggested that GUN1, like pTAC2, is associated with sites of transcriptionally active plastid chromosomes (Koussevitzky et al., 2007a). In support of this hypothesis, the SMR domain of GUN1 is able to bind DNA (Koussevitzky et al., 2007a). This is similar to the SMR domains of the human B3BP/Nedd4-binding protein 2 and the eubacterial *Thermus thermophilus* MutS2, both of which have nicking and endonuclease activities in addition to DNA binding activity (Fukui et al., 2007; Diercks et al., 2008).

Given the demonstrated biochemical functions of PPR and SMR domains, perhaps the simplest interpretation of our data is that SVR7 is directly involved

in chloroplast rRNA processing. For example, the PPR domain could bind an rRNA species and the SMR domain could bind a nuclease that acts on the rRNA. However, until the biochemistry is completed, we cannot rule out the possibility that the chloroplast rRNA processing defects in *svr7* are secondary effects of a more general impairment in chloroplast function. Against this notion are our observations that many of the *var2* suppressors isolated to date are impaired in chloroplast rRNA processing and that the processing defects in these lines are unique. These findings suggest that each of the different SVR proteins mediates a distinct step of rRNA metabolism, either directly or indirectly. In support of this idea is our finding from double mutant analyses that *svr2* is epistatic to *svr7* (Fig. 8), which suggests that the defects in rRNA processing mediated by SVR7 are downstream of those mediated by SVR2. This further implies that chloroplast rRNA processing proceeds by a hierarchy of events.

In summary, our data indicate that characterization of *var2* suppressors is a powerful and facile tool to identify factors that are involved in the poorly understood process of chloroplast rRNA processing. This is underscored by the fact that very few proteins have been identified with a defined role in this process. These include one endoribonuclease, CSP41, which is involved in 23S rRNA processing (Yang et al., 1996; Yang and Stern, 1997; Beligni and Mayfield, 2008) and two 3′-5′ exoribonucleases, PNPase and RNR1 (Walter et al., 2002; Kishine et al., 2004; Bollenbach et al., 2005; Sauret-Güeto et al., 2006).

Model of Suppression of Variegation by *svr7*

We have proposed that a threshold of VAR2 activity is required early in chloroplast biogenesis in the developing leaf and that plastids respond differently to a lack of VAR2, depending on such factors as substrate availability and the presence of compensating factors (Yu et al., 2004, 2007). As was the case for *svr1* and *svr2* (Yu et al., 2008), *svr7* is able to suppress variegation in both *var2-5* and the null allele, *var2-4*. Therefore, suppression is not likely the consequence of a direct interaction between SVR7 and VAR2. Rather, we propose that loss of SVR7 changes the functional state of the chloroplast in such a manner that the threshold of VAR2 activity needed to ensure proper chloroplast biogenesis is lowered.

Evidence for how this might occur is provided by several *var2* suppressors that have pointed to a fundamental linkage between the suppression of variegation and an impairment in chloroplast rRNA processing and/or chloroplast translation (Miura et al., 2007; Yu et al., 2008). rRNA processing, ribosome assembly, and translation are coupled events in chloroplasts, and defects in one are accompanied by defects in the others (Shen and Bremer, 1977; Keus et al., 1984; Barkan, 1993; Leal-Klevezas et al., 2000; Walter et al., 2002; Bellaoui et al., 2003; Williams and Barkan, 2003; Bollenbach et al., 2005; Sauret-Güeto et al., 2006; Schmitz-Linneweber et al., 2006; Watkins et al., 2007; Yu et al., 2008). Our findings with *svr7*, as well as with a number of other *svr* lines that include *svr3* and *svr8* (Fig. 6), thus strengthen the linkage between chloroplast rRNA processing/translation and suppression of variegation. This linkage is highlighted by the observation that variegation is suppressed in *var2* seedlings that are grown in the presence of inhibitors of chloroplast translation, such as chloramphenicol and spectinomycin (Yu et al., 2008).

What is the relationship between VAR2 and chloroplast rRNA processing/translation? One possibility is that the interaction is direct, as in yeast mitochondria, where the inner membrane m-AAA complex (composed of FtsH homologs) directly regulates mitochondrial translation (Nolden et al., 2005). VAR2 might also exert its effect on chloroplast translation in a more indirect manner (e.g. by influencing the activities of proteins that are involved in rRNA processing, ribosome assembly, or translation). Because FtsH proteins act as proteases/chaperones and are important in protein quality control (for review, see Adam and Clarke, 2002; Sakamoto, 2006; Koppen and Langer, 2007), another possibility is that translation is influenced by VAR2 control of protein translocation across the membrane, as found with the i-AAA (Yme1) FtsH homolog in yeast mitochondria (Rainey et al., 2006). This could result, for example, in reduced accumulation of imported proteins of nuclear origin necessary for chloroplast gene expression, resulting in reduced translation. A lack of VAR2 might also result in an accumulation of misfolded polypeptides and protein aggregates, with deleterious effects on organelle development. Such effects are thought to explain mitochondrial abnormalities in mutants of human and yeast that lack mitochondrial FtsH proteins (Nolden et al., 2005). Suppressors such as *svr1*, *svr2*, and *svr7* might thus reduce this load by decreasing the amount of protein produced, thus lowering the threshold of VAR2 activity required for folding/degradation. A related hypothesis is that decreased chloroplast protein synthesis in the suppressors causes a reduction in photosynthetic protein accumulation, which, in turn, might decrease ROS production at PSII and hence the amount of VAR2 needed for D1 turnover and photoprotection.

Other mechanisms of *var2* suppression based on an impairment of chloroplast translation have been discussed in detail (Yu et al., 2008). These mechanisms will not be recapitulated here but, in brief, proceed from the premise that translation affects the duration of chloroplast biogenesis, the propagation of a retrograde (plastid-to-nucleus) signal, or the synthesis of a chloroplast DNA-encoded substrate of VAR2 that regulates chloroplast development. Given the large number of possibilities, and the likelihood that there is not a single mechanism of suppression applicable to all *svr* mutants, a future challenge will be to describe suppression mechanisms at the molecular level.

VAR2 Mediates Chloroplast Biogenesis in a Temperature-Dependent Manner

One of the central findings of this paper is that *var2* displays a leaf variegation phenotype at 22°C but a chlorotic phenotype at 8°C (Fig. 7). *var1* has similar temperature-dependent phenotypes (Fig. 7). Our suppressor studies further revealed that the *svr7* mutation is able to suppress the leaf variegation phenotype but not the chilling-induced chlorosis phenotype. These observations are consistent with molecular data (chloroplast rRNA profiling and chloroplast protein accumulation) showing that *var2-5* is epistatic to *svr7-1* at 8°C, while the converse is true at 22°C (i.e. *svr7-1* is epistatic to *var2-1*; Figs. 6 and 7).

One hypothesis consistent with our data is that VAR2 mediates a single process during chloroplast biogenesis and that this process is only modestly impaired at 22°C, resulting in a variegation phenotype, but severely impaired at 8°C, resulting in chlorosis. According to this scenario, a lack of SVR7 activity would be sufficient to compensate for the mild VAR2 defect, perhaps by decreasing chloroplast protein synthesis, but not sufficient to compensate for the severe VAR2 defect. A second hypothesis is that VAR2 mediates two different processes, one that occurs at normal growth temperatures and one that occurs at chilling temperatures. In this model, a lack of SVR7 activity would be able to compensate for the defective *var2*-mediated process that is required at 22°C but not for the defective process that is required at 8°C.

Chilling-induced chlorosis is a frequent symptom of chilling injury and can be caused by different mechanisms (Tokuhisa et al., 1998). While it is premature to speculate on the role of VAR2 in chloroplast development at low temperature, it is interesting that chilling-induced chlorosis has frequently been associated with a loss of chloroplast translation. For example, early studies showed that several maize (*Zea mays*) mutants that display chilling-induced chlorosis, including M-11 (Millerd et al., 1969), *hcf7* (Barkan, 1993), and *v16* (Hopkins and Elfman, 1984), have defects in chloroplast ribosome accumulation and/or translation that are more pronounced at low temperature. A chilling-sensitive Arabidopsis mutant (*chs1*) has reduced chloroplast protein accumulation at low temperatures (Schneider et al., 1995), and chilling-induced chlorosis of the Arabidopsis *paleface* mutant, which defines the gene for a homolog of the yeast 18S rRNA methylase (DIM1), is caused by a chilling-sensitive step in chloroplast rRNA processing that inhibits ribosome assembly (Tokuhisa et al., 1998). More recently, it has been demonstrated that a tobacco (*Nicotiana tabacum*) transplastomic line that is devoid of chloroplast ribosomal protein L33 is sensitive to cold stress (Rogalski et al., 2008).

In addition to chilling-induced effects on chloroplast translation, it has been known for some time that chilling stress results in a decrease in the de novo synthesis and degradation of D1 (Salonen et al., 1998), another process that involves VAR2 activity. This decrease has recently been correlated with ribosome pausing and a delayed translation elongation of *psbA* mRNAs that results in decreased D1 synthesis (Grennan and Ort, 2007).

Given that rRNA processing and chloroplast protein accumulation are drastically impaired in *var2* (Fig. 7) and that D1 turnover is also perturbed in *var2* (Kato et al., 2009), it is tempting to speculate that a loss of VAR2 accentuates a cold-induced impairment in one or more of these processes. The latter would be consistent with the suggestion that VAR2 plays an important role in chloroplast biogenesis beyond its extensively studied role in D1 turnover (Chen et al., 2000; Zaltsman et al., 2005a).

MATERIALS AND METHODS

Plant Material and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) mutants used in this study are in the Columbia ecotype background. Plants were grown at either 8°C or 22°C under continuous illumination with a light intensity of approximately 100 μmol m^{-2} s^{-1}. The *svr1-1* single mutant (derived from the *TAG-FN* suppressor line) has been described (Yu et al., 2008). Other single mutants used in this study include *svr7-1*, *svr3-1*, and *svr8-1*, which were derived from the suppressor lines *004-003* (this work), *TAG-11*, and *023-005*, respectively (F. Yu, X. Liu, and S. Rodermel, unpublished data).

Chlorophyll Determination

Two-week-old seedlings were harvested, weighed, and finely ground in liquid N_2. Total chlorophyll was extracted with 95% ethanol, and chlorophyll concentrations and chlorophyll *a*/*b* ratios were calculated according to Lichtenthaler (1987).

Positional Cloning

Procedures for positional cloning have been described in detail (Park and Rodermel, 2004). In brief, an F2 mapping population was generated by crossing *svr7-1* with Landsberg *erecta*. Bulked segregant analysis (Lukowitz et al., 2000) was then performed using a pool of DNAs from 96 F2 plants having an *svr7-1* phenotype, and the mutant gene was mapped between simple sequence length polymorphism markers *ciw6* and *ciw7* on chromosome IV (Bell and Ecker, 1994). The gene was fine-mapped using markers that were designed based on insertion/deletion or single nucleotide polymorphisms between Landsberg *erecta* and Columbia ecotypes (Jander et al., 2002; Supplemental Table S1). A total of 665 F2 plants (1,330 chromosomes) were used in fine-mapping the suppressor gene.

Complementation of *004-003*

A genomic DNA fragment encompassing the full-length At4g16390 (*SVR7*) sequence was amplified by *pfu* Turbo DNA polymerase (Stratagene) using primers 16390F and 16390R, and the amplified fragment was cloned into the *Bam*HI site of pBluescript KS+. Following verification of the *SVR7* sequence, the *Bam*HI fragment was subcloned into a modified pBI121 vector (Yu et al., 2004); sequences in this vector are driven by the cauliflower mosaic virus 35S promoter. The resulting construct was transformed into *Agrobacterium tumefaciens* by electroporation, and the floral dip method was used to transform *004-003* plants (Clough and Bent, 1998). After transformation, T1 seeds were collected and grown on plates in a medium containing 1× Murashige and Skoog salts, 1% Suc, and 0.8% agar (pH 5.7) supplemented with kanamycin (50 mg L^{-1}). The phenotype of the complementation lines was confirmed in the T2 generation.

Confocal Microscopy of SVR7 Expression

A construct was made containing the full-length *SVR7* gene fused at its C terminus to the open reading frame of *eGFP* in the vector pTF486 (Yu et al.,

2008). Genes are driven by the cauliflower mosaic virus 35S promoter in this construct. Primers 16390F and 16390GFPR were used to amplify the *SVR7* sequence using *pfu* Turbo DNA polymerase (Stratagene). The PCR product was digested with *Bam*HI and *Nco*I and cloned in-frame into pTF486. The resulting construct (designated *P35S*:*SVR7*:*GFP*) was sequenced to verify that the open reading frame of *eGFP* was correctly fused to the 3′ end of *SVR7*. A control construct lacked the *SVR7* sequence (designated *P35S*:*GFP*). Methods for transient expression of wild-type Arabidopsis leaf protoplasts have been described (Yoo et al., 2007; Yu et al., 2008). Confocal microscopy (Leica TCS NT) with a fluorescein isothiocyanate-tetramethyl rhodamine isothiocyanate filter combination was used to capture fluorescence signals of GFP and chlorophyll autofluorescence.

DNA, RNA, and Protein Manipulations

Leaf DNAs were isolated using the cetyl-trimethyl-ammonium bromide method (Wetzel et al., 1994), and total leaf RNAs were purified using the Trizol RNA reagent (Invitrogen). Radiolabeled probes were generated according to Yu et al. (2008). Northern-blot analyses were performed as described by Wetzel et al. (1994). Total leaf proteins from 2-week-old seedlings were extracted, and western immunoblot analyses were carried out using a series of antibodies to representative proteins, as described previously (Yu et al., 2008). The quantification of western-blot signals was carried out using QuantityOne software (Bio-Rad).

Generation of *svr2 svr7* Double Mutants

The *svr7-1* single mutant was crossed with *svr2-1* (Yu et al., 2008), and genomic DNAs were isolated from the F2 progeny. The genotype of the F2 plants at these two loci was determined by PCR-based assays: PCR with primers 004-003F and 004-003R was used to determine the genotype of the *SVR7* locus, whereas PCR with primers 49970F2 and 49970R2 was used to determine the genotype at the *SVR2* locus (Yu et al., 2008).

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At4g16390, BAC43491; At5g46580, NP_199470; At2g31400, NP_180698; At1g18900, NP_173324; At1g74850, NP_177623; At1g74750, NP_177613; At1g79490, NP_178067; At2g17033, NP_849962; *E. coli* SMR, NP_416834; *Synechocystis* sp. PCC 6803 MutS2, NP_440990.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Ethidium bromide staining of RNA gels.

Supplemental Figure S2. Quantification of immunoblot signal intensities.

Supplemental Table S1. Primers used in this study.

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