

# Mutations in *SUPPRESSOR OF VARIEGATION1*, a Factor Required for Normal Chloroplast Translation, Suppress *var2*-Mediated Leaf Variegation in *Arabidopsis* <sup>W</sup>

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**The *Arabidopsis thaliana* yellow variegated2 (*var2*) mutant is variegated due to lack of a chloroplast FtsH-like metalloprotease (FtsH2/VAR2). We have generated suppressors of *var2* variegation to gain insight into factors and pathways that interact with VAR2 during chloroplast biogenesis. Here, we describe two such suppressors. Suppression of variegation in the first line, TAG-FN, was caused by disruption of the nuclear gene (*SUPPRESSOR OF VARIEGATION1* [SVR1]) for a chloroplast-localized homolog of pseudouridine ( $\Psi$ ) synthase, which isomerizes uridine to  $\Psi$  in noncoding RNAs. *svr1* single mutants were epistatic to *var2*, and they displayed a phenotypic syndrome that included defects in chloroplast rRNA processing, reduced chloroplast translation, reduced chloroplast protein accumulation, and elevated chloroplast mRNA levels. In the second line (TAG-IE), suppression of variegation was caused by a lesion in SVR2, the gene for the ClpR1 subunit of the chloroplast ClpP/R protease. Like *svr1*, *svr2* was epistatic to *var2*, and *clpR1* mutants had a phenotype that resembled *svr1*. We propose that an impairment of chloroplast translation in TAG-FN and TAG-IE decreased the demand for VAR2 activity during chloroplast biogenesis and that this resulted in the suppression of *var2* variegation. Consistent with this hypothesis, *var2* variegation was repressed by chemical inhibitors of chloroplast translation. In planta mutagenesis revealed that SVR1 not only played a role in uridine isomerization but that its physical presence was necessary for proper chloroplast rRNA processing. Our data indicate that defects in chloroplast rRNA processing are a common, but not universal, molecular phenotype associated with suppression of *var2* variegation.**

## INTRODUCTION

Variegation mutants are ideal models to gain insight into the mechanisms of chloroplast biogenesis (reviewed in Rodermel, 2002; Sakamoto, 2003; Yu et al., 2007). A growing number of variegation mutants have been characterized at the molecular level, primarily in *Arabidopsis thaliana*, maize (*Zea mays*), and tomato (*Solanum lycopersicum*) (reviewed in Yu et al., 2007). Our laboratory has focused on *Arabidopsis* variegations, one of which is *yellow variegated2* (*var2*) (Martínez-Zapater, 1993; Chen et al., 1999). Sectoring in *var2* is due to the action of a nuclear recessive mutation. Whereas cells in the green leaf sectors and cotyledons of *var2* contain morphologically normal chloroplasts, cells in the yellow and white sectors are heteroplasmic and contain vacuolated plastids with few organized lamellae in addition to some normal-appearing chloroplasts (Chen et al., 1999).

VAR2 codes for a chloroplast FtsH metalloprotease (designated VAR2/FtsH2) (Chen et al., 2000; Takechi et al., 2000). This protein belongs to a large class of AAA (for ATPase associated

with various cellular activities) proteins that are ubiquitous among prokaryotes and eukaryotes. AAA proteins contain one or more AAA cassette domains (~200 to 250 amino acids), which act as ATP-powered molecular motors to drive processes of protein denaturation, disassembly, and translocation (Sauer et al., 2004). It is thought that proteins destined for destruction by FtsH are denatured by the AAA cassette, allowing degradation by the proteolytic domain to occur (Sauer et al., 2004).

In *Arabidopsis*, there are 12 *FtsH* genes (Sokolenko et al., 2002; Sakamoto et al., 2003; Yu et al., 2004). All are located in chloroplasts except FtsH3, -4, and -10, which are targeted to mitochondria (Lindahl et al., 1996; Chen et al., 2000; Sakamoto et al., 2002, 2003; Yu et al., 2004); FtsH11 appears to be dual-targeted to both chloroplasts and mitochondria (Urantowka et al., 2005). Chloroplast FtsH proteins are present in thylakoid membrane complexes that are composed (at a minimum) of two phylogenetic pairs of proteins that are at least partially functionally redundant (viz, FtsH2/8 and FtsH1/5). FtsH2 and -8 are interchangeable with one another, as are FtsH1 and -5 (Sakamoto et al., 2003; Yu et al., 2004, 2005; Zaltsman et al., 2005b). However, FtsH2 and -8 are not interchangeable with FtsH1 and -5.

The functions of FtsH have been most extensively investigated in *Escherichia coli*, in which both chaperone and protease activities have been identified (Suzuki et al., 1997). Chloroplast FtsH mediates a variety of functions that are important for chloroplast biogenesis and maintenance (Chen et al., 1999; Zaltsman et al.,

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2005a; Yu et al., 2007). The best understood of these is a role in the D1 turnover process, during which FtsH participates in the degradation of photodamaged D1 proteins of photosystem II (PSII) (Lindahnl et al., 2000; Bailey et al., 2002; Sakamoto et al., 2002; Silva et al., 2003; Chen et al., 2006; Yoshioka et al., 2006).

We have proposed a threshold model to explain the mechanism of *var2* variegation (Yu et al., 2004, 2007). In this model, a threshold of FtsH-containing complexes is required for normal chloroplast function and green sector formation. This model has received widespread support from morphological, biochemical, and double mutant studies (Zaltsman et al., 2005b; Kato et al., 2007). To better understand VAR2 function and the underlying mechanism of *var2* leaf variegation, we have conducted genetic suppressor screens and isolated a number of mutants that modify the *var2* variegation phenotype (Park and Rodermel, 2004). Our long-range goal is to use *var2* suppressors/enhancers as a tool to gain understanding of the pathways and processes that regulate chloroplast biogenesis. Toward this goal, we plan to generate a comprehensive genetic interactions map, based on *var2* suppressors, and then use this map to provide guidance in the design of experiments to test hypotheses about mechanisms of chloroplast development. The utility of this approach is illustrated by our demonstration that *var2* variegation can be suppressed by mutations in *ClpC2*, the gene for a chloroplast Hsp100 chaperone (Park and Rodermel, 2004). This provided evidence of a linkage between FtsH and Clp protease function in chloroplasts, thus opening up possibilities for future research into chloroplast protease networks.

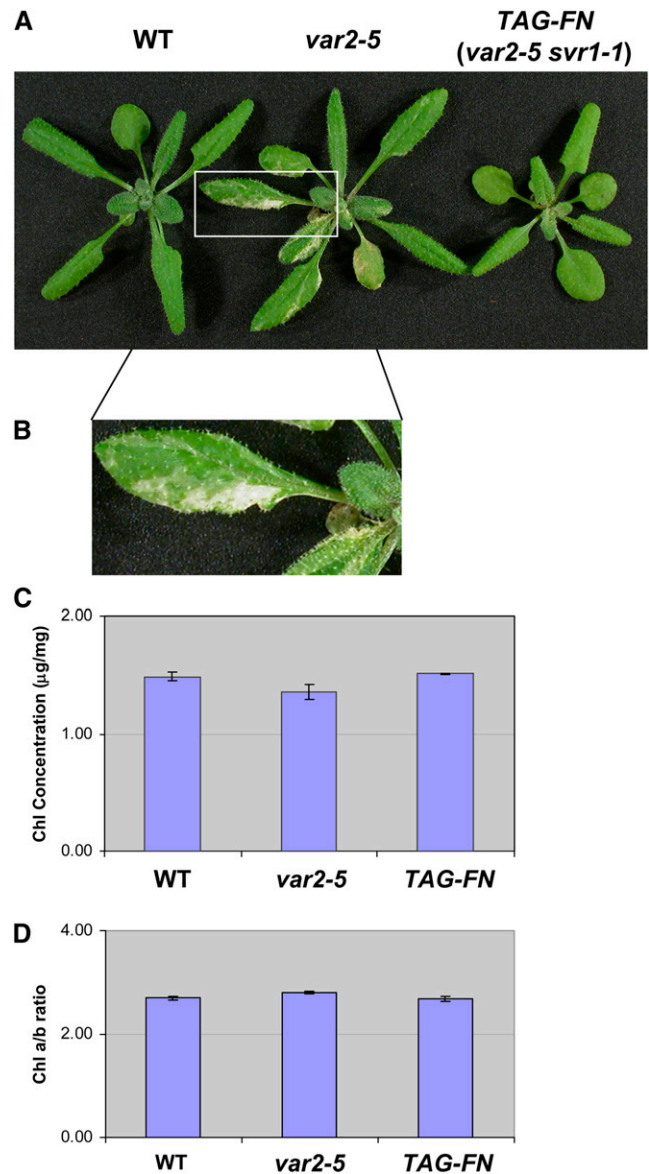
Here, we characterize two *var2* suppressors generated by T-DNA tagging (designated *TAG-FN* and *TAG-IE*). We show that mutation of a chloroplast-localized pseudouridine synthase, designated *SUPPRESSOR OF VARIATION1 (SVR1)*, is responsible for the suppression of variegation in the *TAG-FN* line. This enzyme isomerizes uridine to pseudouridine ( $\Psi$ ) in non-coding RNAs. Suppression of variegation in the second line (*TAG-IE*) is due to mutation of *SVR2*, a nuclear gene for the ClpR1 subunit of the chloroplast ClpP/R protease. This is thus another example of linkage between FtsH and Clp protease function in chloroplasts. The suppressor lines, as well as the *svr1* and *svr2* single mutants, condition defects in chloroplast rRNA processing, chloroplast translation, and chloroplast protein accumulation. We present a model of *var2* variegation suppression whereby reduced rates of plastid protein synthesis decrease the demand for FtsH in developing chloroplasts, allowing more plastids to overcome a threshold and turn green in a *var2* background. Confirmation of this model comes from the finding that variegation is suppressed by growth of *var2* in the presence of chemical inhibitors of chloroplast translation.

## RESULTS

### Isolation of *var2* Suppressors by T-DNA Activation Tagging

We previously reported the isolation of *var2* suppressors using ethyl methanesulfonate mutagenesis (Park and Rodermel, 2004). Here, we used T-DNA activation tagging (Weigel et al., 2000) as an alternative mutagenesis approach. This was accomplished by transforming plasmid pSKI015 (activation tagging vector) into

*var2-5*, a weak *var2* allele (Chen et al., 2000). We observed a number of lines in which the T1 and/or T2 generations had altered variegation phenotypes due to gain-of-function or loss-of-function mutations. Figures 1A and 1B show a line (designated *TAG-FN*) in which the T1 plant was variegated but the T2



**Figure 1.** Phenotype of *TAG-FN*, a *var2-5* Suppressor.

(A) Representative 4-week-old wild-type, *var2-5*, and *TAG-FN* plants. The plants were maintained at 22°C under continuous light ( $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ).

(B) Enhanced magnification of a portion of *var2-5* [(A)], illustrating the extent of leaf variegation; *var2-5* is a weak allele (low amount of variegation). (C) and (D) Chlorophyll contents (on a fresh weight basis) (C) and chlorophyll *a/b* ratios (D) in leaves from 2-week-old wild-type, *var2-5*, and *TAG-FN* seedlings. Rosette leaves were pooled from individual plants for each determination. Each bar represents an average  $\pm$  SD of three different pooled leaf samples.

plants were variegated or all green in an ~3:1 ratio (variegated: nonvariegated). This indicated that suppression of variegation in this line was due to a recessive (loss-of-function) mutation. We named this locus *SVR1*; the mutant allele in *TAG-FN* was designated *svr1-1*. *TAG-FN* plants grew somewhat more slowly than wild-type plants (Figure 1A), but they attained a normal stature at flowering. Mature leaves of the *TAG-FN* plants had normal chlorophyll concentrations, chlorophyll *a/b* ratios (Figures 1C and 1D), and chloroplast anatomies (see Supplemental Figure 1 online). These observations suggested that chloroplast biogenesis was not grossly altered in the *TAG-FN* suppressor line.

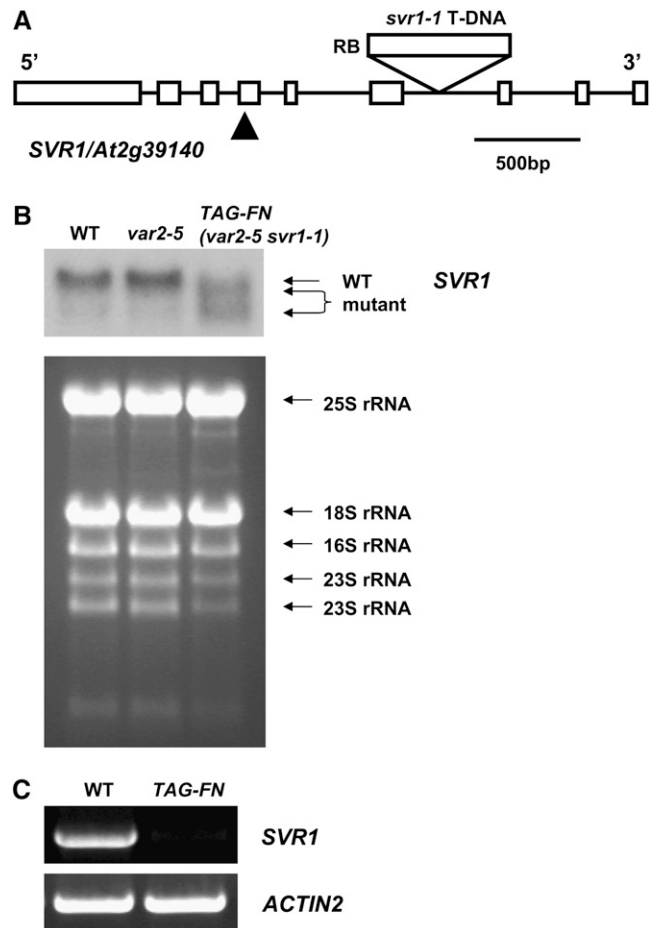
### Identification of the Suppressor Gene in *TAG-FN*

To identify the suppressor gene in *TAG-FN*, we performed cosegregation analysis to establish the linkage between the T-DNA insert(s) and the mutant phenotype. DNA gel blot analyses revealed that a 6.5-kb *Hind*III band cosegregated with the suppression-of-variegation phenotype (see Supplemental Figure 2 online). This band was isolated by plasmid rescue, and the rescued plasmid was sequenced. We recovered a 92-bp *Arabidopsis* genomic sequence flanking the T-DNA right border in the rescued plasmid that corresponded to a portion of intron 6 of *At2g39140* on chromosome 2. This suggested that *At2g39140* was *SVR1*. *At2g39140* contains nine exons and eight introns (Figure 2A) and has been annotated as the gene for a 410-amino acid protein (45.1 kD) that bears resemblance to members of the *E. coli* pseudouridine synthase family of enzymes.

Figure 2B shows that *TAG-FN* (*var2-5/var2-5 svr1-1/svr1-1*) contained two forms of *SVR1* mRNAs. The sizes of these transcripts were smaller than the mature-sized mRNAs in the wild type and *var2-5* (i.e., truncated *SVR1-1* transcripts were produced in *TAG-FN*). This conclusion was confirmed by RT-PCR analyses showing that full-length *SVR1* transcripts did not accumulate in *TAG-FN* (Figure 2C). It is possible, therefore, that *svr1-1* translation products retained function (at least in part) and that *svr1-1* was a knockdown, rather than a knockout, allele. The subsequent isolation of a more severe *SVR1* allele (*svr1-2*; described in Figure 5 below) lent support to this conclusion.

### *SVR1* Is the *var2* Suppressor Locus: Complementation of *TAG-FN*

Complementation tests were performed to confirm that suppression of *var2* variegation in *TAG-FN* was due to the disruption of *SVR1* expression. Because *TAG-FN* was homozygous recessive at the *SVR1* and *VAR2* loci (*svr1-1/svr1-1 var2-5/var2-5*), we reasoned that overexpression of a wild-type copy of *SVR1* in *TAG-FN* might mask the *svr1* defect, thus restoring a variegation phenotype. To test this hypothesis, we transformed *TAG-FN* with an overexpression construct that contained a full-length *SVR1* cDNA driven by the cauliflower mosaic virus (CaMV) 35S promoter. Consistent with our hypothesis, the transgenic plants were variegated (Figures 3A and 3B). This was likely a consequence of *SVR1* overexpression, inasmuch as the transformants had dramatically increased *SVR1* transcript levels (Figure 3C). These data support



**Figure 2.** Identification of the Suppressor Gene in *TAG-FN*.

**(A)** The location of the T-DNA insert in *At2g39140* is shown. This gene is designated *SVR1*, and the insertion allele is designated *svr1-1*. Boxes represent exons and lines denote introns. The T-DNA insert is not drawn to scale. The triangle shows the location of the conserved nine-amino acid sequence in the active site (see Figure 6B). RB, right border.

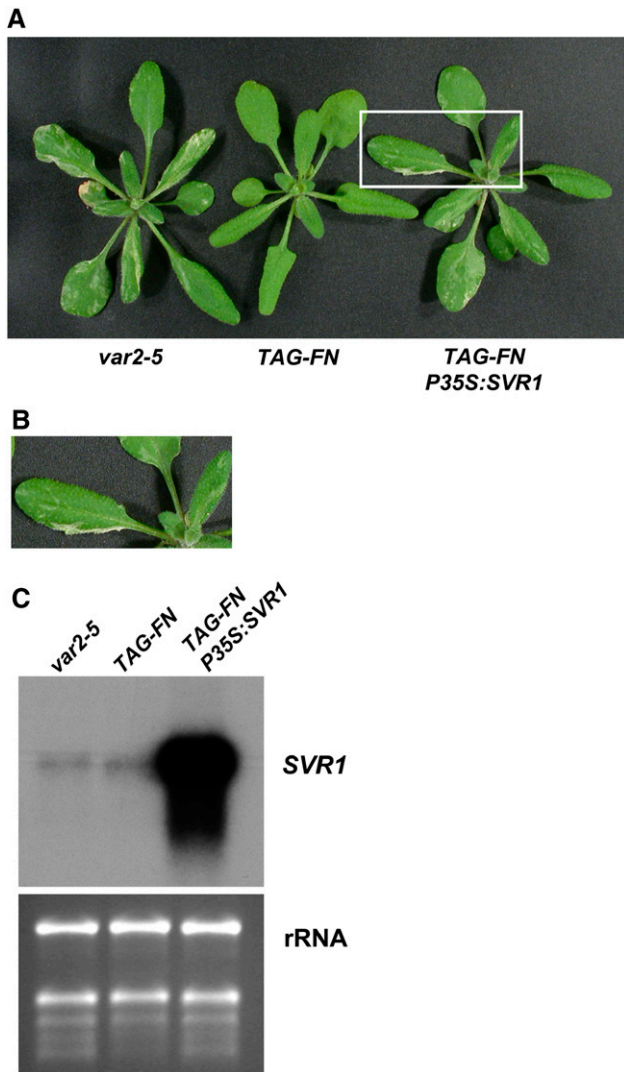
**(B)** Representative RNA gel blot of *SVR1* mRNA accumulation in leaves from 4-week-old wild-type, *var2-5*, and *TAG-FN* seedlings (as in Figure 1A). Rosette leaves were pooled from individual plants for each determination. Equal amounts of total leaf RNA (8  $\mu$ g) were electrophoresed through formaldehyde gels and transferred to nylon filters; the filters were probed with  $^{32}$ P-labeled *SVR1* cDNA. Arrows show the sizes of the wild-type versus two smaller *SVR1* mRNAs (mutant) in *TAG-FN*. Ethidium bromide staining of rRNAs is shown as a loading control.

**(C)** Full-length *SVR1* transcript accumulation in the wild type and *TAG-FN*. Total leaf RNA was extracted and reverse-transcribed. *SVR1* cDNA was detected using primers that flank the *SVR1* gene. Amplification of *ACTIN2* served as a control. The primers are listed in Supplemental Table 1 online.

the notion that a defect in *SVR1* was responsible for the suppression of *var2-5* variegation in the *TAG-FN* double mutants.

### Genetic Interactions between *svr1-1* and Alleles of *var2*

To obtain *svr1-1* single mutants (i.e., *svr1-1/svr1-1 VAR2/VAR2* seedlings), we backcrossed *TAG-FN* to wild-type Columbia and



**Figure 3.** Complementation of *TAG-FN*.

**(A)** *TAG-FN* was transformed with a construct containing *SVR1* driven by the CaMV 35S promoter (*TAG-FN P35S:SVR1*; designated the *TAG-FN* complementation line). A representative T2 plant is shown. Controls are *var2-5* and *TAG-FN*.

**(B)** Enhanced magnification of a portion of the *TAG-FN* complementation line (boxed in **[A]**) showing that the leaves are variegated.

**(C)** Representative RNA gel blot of *SVR1* mRNA accumulation in *var2-5*, *TAG-FN*, and the *TAG-FN* complementation line (conducted as in Figure 2B).

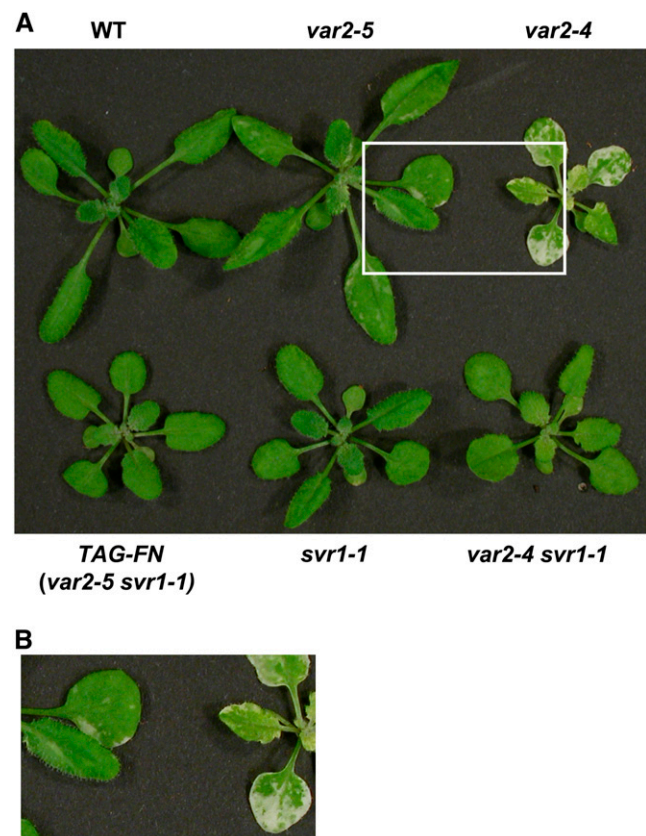
selfed the F1; the F2 were genotyped using derived cleaved amplified polymorphic sequence primers that detected mutant *var2-5* sequences (Park and Rodermel, 2004). Figure 4A shows that *svr1-1* single mutants were not variegated and that they resembled *TAG-FN* in morphology and growth habit. This indicated that *svr1-1* was epistatic to *var2-5*.

The *var2-5* allele contains a missense mutation in the AAA cassette domain of the protein (Chen et al., 2000). It is a leaky allele and accumulates *VAR2* mRNA and protein; its variegation

phenotype is less severe than that of *var2-4*, a putative null allele (Chen et al., 2000) (Figures 4A and 4B). *var2-4* lacks detectable *VAR2* mRNA and protein due to a splice site mutation. To examine the allele specificity of *svr1-1*-mediated variegation suppression, we crossed *svr1-1* with *var2-4*. Figure 4A shows that the double mutants (*svr1-1/svr1-1 var2-4/var2-4*) were not variegated and that they resembled *TAG-FN*. This indicated that the mechanism of *svr1-1* suppression was not allele-specific and, in addition, that suppression of variegation did not require the presence of the *VAR2* protein.

#### Identification of *svr1-2*, a Molecular Null Allele

Because we could not rule out the possibility that *svr1* was a leaky allele, we sought to identify a true knockout allele of this gene in the Salk collection of T-DNA insertion mutants (<http://signal.salk.edu/cgi-bin/tdnaexpress>; Alonso et al., 2003).



**Figure 4.** Genetic Interactions between *TAG-FN* and Two *var2* Alleles.

**(A)** *TAG-FN* was crossed with the wild type, and *svr1-1/svr1-1* single mutants were isolated in the F2; they resembled *TAG-FN*. The *svr1-1* single mutants were crossed with *var2-4*: the F1 plants (*var2-4/var2-4 svr1-1/svr1-1*) were not variegated, indicating that *svr1-1* was able to suppress the variegation phenotype of *var2-4*. Shown are the wild type, *var2-4*, the *svr1-1* single mutants (i.e., *svr1-1/svr1-1* seedlings), and the double mutants (*var2-4/var2-4 svr1-1/svr1-1*). *var2-5* and *TAG-FN* are also shown for comparison.

**(B)** Enhanced magnification of the boxed portion of **(A)** showing that *var2-4* is a more severe allele than *var2-5*.

Several lines were identified, one of which (Salk\_013085) had an insertion in the first exon of the gene (Figure 5A). Homozygous plants of this line (designated *svr1-2*) were yellow-green and significantly reduced in size compared with wild-type and *svr1-1* plants (Figure 5B). The leaves of *svr1-2* also remained pale green throughout their development.

Supplemental Figure 3B online shows that *svr1-2* was able to suppress the variegation phenotypes of *var2-5* (leaky allele) and *var2-4* (null allele); both double mutants resembled *svr1-2* single mutants. This confirmed (1) that loss-of-function mutations in *SVR1* were epistatic to *var2* and (2) that interactions between *SVR1* and *VAR2* proteins were not required for the suppression of *var2* variegation phenotypes.

Taken together, the observations in Figure 5 and Supplemental Figure 3B online indicated that *svr1-2* was a more severe allele than *svr1-1*. Consistent with this idea, *SVR1* transcripts could not be detected in this line by RNA gel blot analysis (data not shown). This indicated that *svr1-2* was a molecular null allele.

### SVR1 Encodes a Pseudouridine Synthase

As mentioned earlier, At2g39140 has been annotated as a member of the *Arabidopsis* pseudouridine synthase gene family.  $\Psi$  is the most abundant modified nucleotide in noncoding RNA species, including rRNAs, tRNAs, small nuclear RNAs, and small nucleolar RNAs; it is not normally found in mRNAs (Charette and Gray, 2000; Ofengand, 2002). The isomerization of uridine to  $\Psi$  is catalyzed by pseudouridine synthase (Charette and Gray, 2000).

Phylogenetic analyses were undertaken as a first step toward determining whether *SVR1* was a bona fide pseudouridine synthase. Pseudouridine synthases are best characterized in *E. coli*, in which there are 11 family members. These enzymes mediate uridine-to- $\Psi$  isomerization in tRNAs (designated *Tru* genes) and rRNAs (designated *Rlu* genes, for ribosomal 50S rRNAs, and *Rsu* genes, for ribosomal 30S rRNAs) (Koonin, 1996; Ofengand, 2002; Kaya and Ofengand, 2003). *E. coli* pseudouridine synthases diverge widely in amino acid sequence, yet they fold into similar

structures (Mueller, 2002). They also share a conserved nine-amino acid motif at the active site that includes a universally conserved Asp residue. This Asp is perfectly conserved not only among *E. coli* pseudouridine synthases but also among all other pseudouridine synthases identified to date, ranging from bacteria to yeast to humans (Ofengand, 2002). The frequency of amino acids within this motif in pseudouridine synthases from diverse sources is illustrated in Figure 6B.

To determine the complement of pseudouridine synthases in *Arabidopsis*, we searched the databases for sequences with similarity to each of the 11 *E. coli* proteins. Twenty-two homologous sequences were found. Of these, 17 contained the active site motif with the perfectly conserved Asp; these included *SVR1*. The phylogenetic relatedness of the *E. coli*, *Arabidopsis*, and *Chlamydomonas* pseudouridine synthases is illustrated in Figure 6A. The tree is complex but notable for the relative lack of phylogenetic pairs that have arisen as a consequence of duplications that occurred during the evolution of the *Arabidopsis* genome, as seen in many *Arabidopsis* gene families, including *FtsH* (Yu et al., 2007). On the basis of our phylogenetic analyses, we concluded that *SVR1* bore all of the hallmarks of a true pseudouridine synthase.

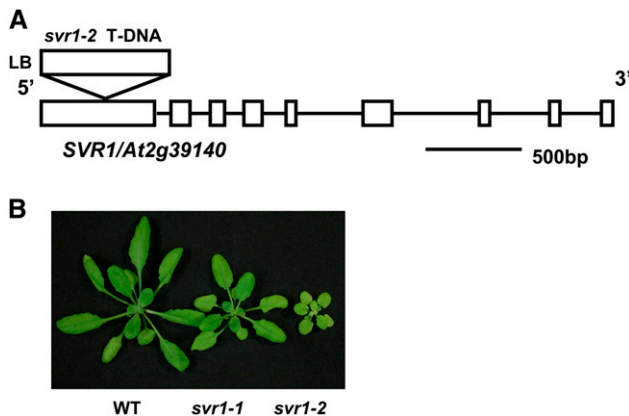
### SVR1 Is Localized in the Plastid

*SVR1* was predicted to be plastid-localized based on the presence of a putative transit peptide of 35 amino acids. To confirm this localization, we examined the intracellular location of a transiently expressed P35S:*SVR1*:GFP (for green fluorescent protein) fusion construct in protoplasts isolated from wild-type *Arabidopsis* leaves; the control was a P35S:GFP fusion construct. Fusion protein expression was monitored by confocal microscopy. Figures 7A to 7C show control protoplasts that were transformed with GFP alone: green fluorescence was present in the cytoplasm (Figure 7A) but was not associated with the chloroplast (as shown by chlorophyll autofluorescence; Figure 7B); consistent with this apparent lack of congruity, the two fluorescence images did not merge (Figure 7C). By contrast, green fluorescence from the *SVR1*:GFP fusion protein (Figure 7D) matched the pattern of chlorophyll autofluorescence (Figure 7E); this was confirmed by the merging of the two images (Figure 7F). These data indicated that *SVR1* was a chloroplast protein.

To determine whether *SVR1* is expressed in plastid types other than chloroplasts, we conducted RNA gel blot analyses using RNAs from various tissue types (Figure 7G). As anticipated, *SVR1* was highly expressed in chloroplast-containing tissues; we suspect that floral expression was from the sepals. *SVR1* mRNAs also accumulated in amyloplast-containing root tissues but not in etioplast-containing tissues of dark-grown seedlings. We concluded that *SVR1* was important for chloroplast development and maintenance but that it likely functioned in other plastid types as well.

### Defects in SVR1 Mediate Alterations in Chloroplast rRNA Processing

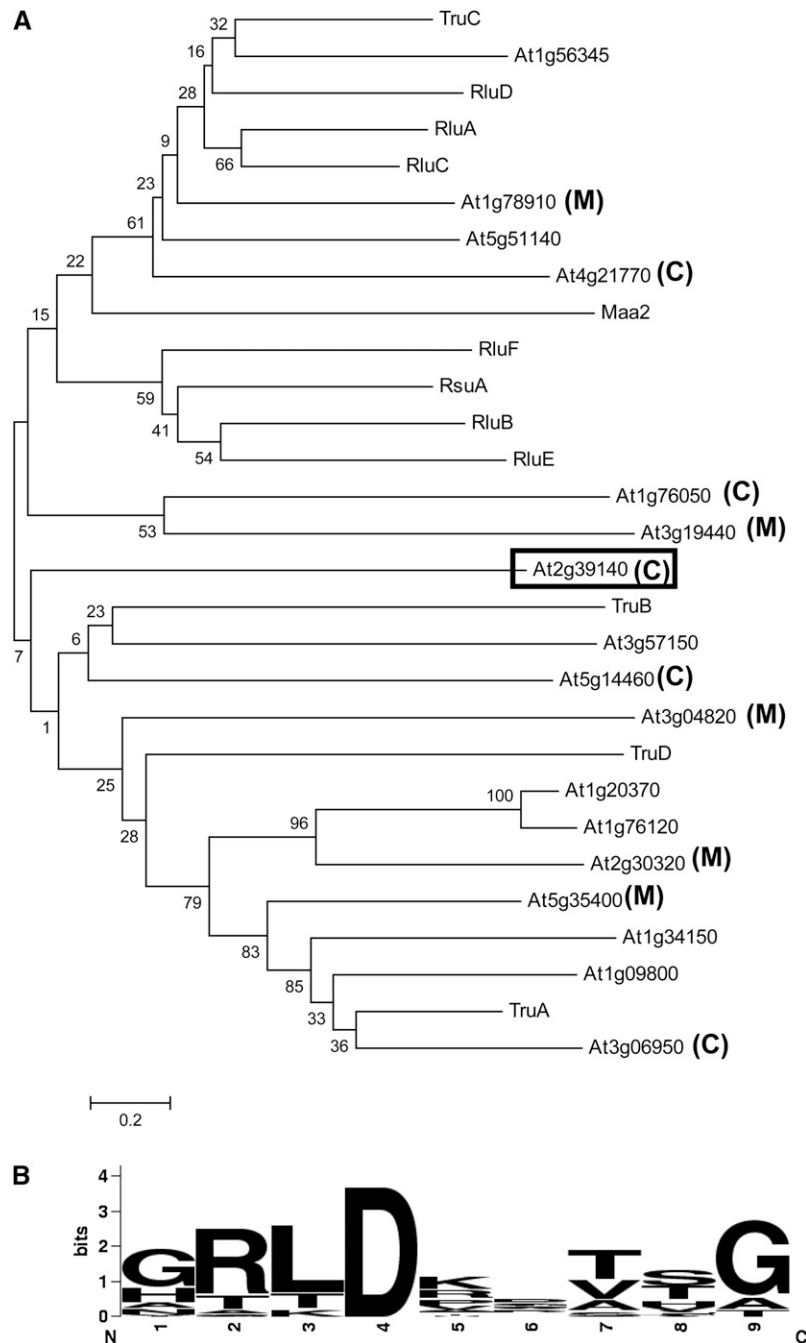
rRNAs are the most abundant RNA species in plant cells, and plastid rRNAs (23S, 16S, 5S, and 4.5S) and cytosolic rRNAs (25S, 18S, 5.8S, and 5S) can be easily observed on ethidium



**Figure 5.** Identification of *svr1-2*, a Molecular Null Allele.

(A) Salk\_013085 contains a T-DNA insertion in the first exon of At2g39140 (*SVR1*); we designated this line *svr1-2*.

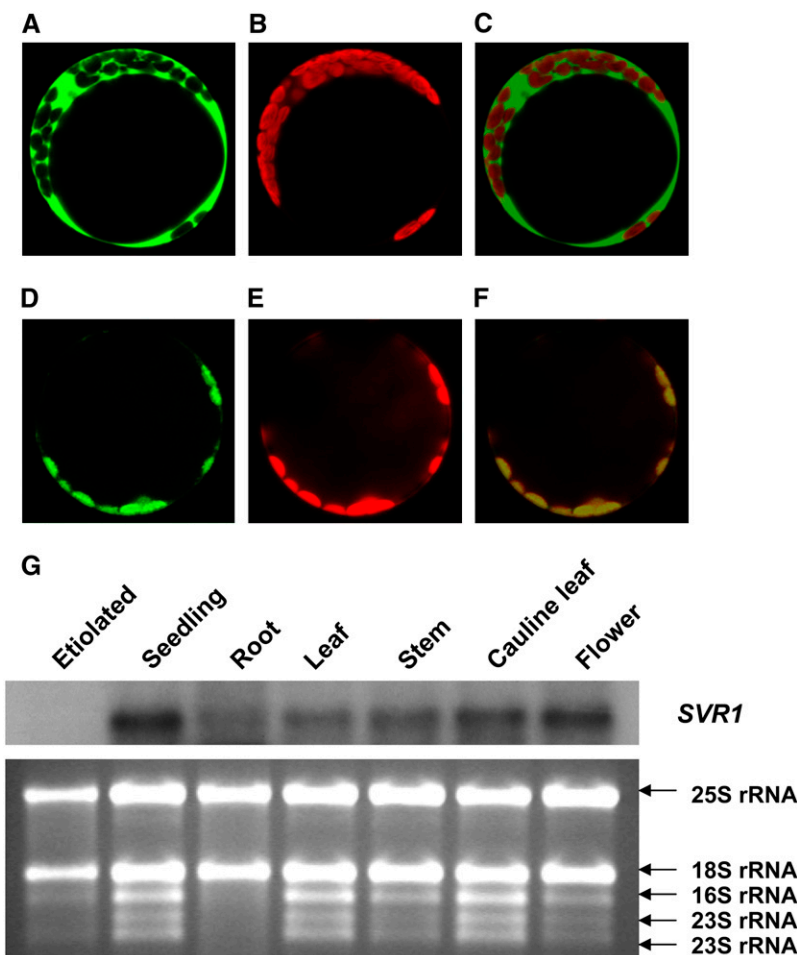
(B) Phenotypes of wild-type and homozygous *svr1-1* and *svr1-2* plants.



**Figure 6.** Phylogenetic Analysis of Pseudouridine Synthases.

**(A)** Phylogenetic tree of pseudouridine synthases from *E. coli* (*Tru*, *Rlu*, and *Rsu* gene products), *Arabidopsis* (*At* gene products), and *Chlamydomonas* (*Maa2* gene product). Full-length protein sequences were obtained from the National Center for Biotechnology Information and The Arabidopsis Information Resource, aligned, and analyzed using MEGA 2.1 (Kumar et al., 2001). Genes for 17 pseudouridine synthase-like proteins were found in the *Arabidopsis* genome; five of these had chloroplast targeting sequences (C) and five had mitochondrial targeting sequences (M). Targeting sequences were identified using TargetP and ChloroP (Emanuelsson et al., 2007). All 17 are expressed at the level of mRNA. The *SVR1* (*At2g39140*) gene product is boxed, and bootstrap values are shown at nodes. Supplemental Data Set 1 online contains the alignment used to generate this tree.

**(B)** An active site Asp residue is perfectly conserved among pseudouridine synthases from all sources, including 11 enzymes from *E. coli*, 10 from yeast, *Maa2* from *Chlamydomonas*, *SVR1* from *Arabidopsis*, and dyskerin from human. The Asp is embedded within a nine-amino acid sequence that is also highly conserved. All protein sequences were retrieved from GenBank, and the graph was constructed using WebLogo 3 (Crooks et al., 2004); the larger the letter, the more conserved the amino acid.



**Figure 7.** Localization and Expression of SVR1.

(A) to (F) Leaf protoplasts were isolated from wild-type *Arabidopsis* leaves and transiently transformed with a control GFP vector (A) to (C) or a SVR1:GFP vector (D) to (F). Green fluorescence from GFP (A) and (D) and chlorophyll autofluorescence (B) and (E) were monitored by confocal microscopy. (C) and (F) show merged images of (A) and (B) and (D) and (E), respectively. Each panel shows a single protoplast.

(G) Expression of *SVR1* mRNAs was examined in the indicated tissues from wild-type plants by RNA gel blot analysis (performed as in Figure 2B). The filter was probed with  $^{32}\text{P}$ -labeled *SVR1* cDNAs. The ethidium bromide-stained gel is shown as a loading control.

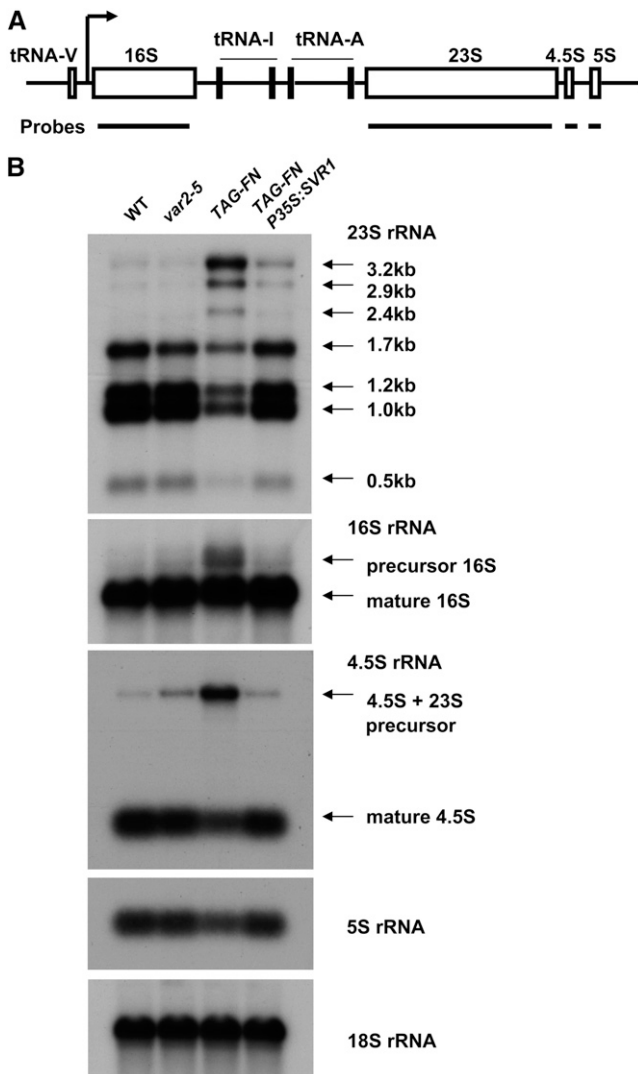
bromide-stained, denaturing agarose gels (Leaver and Ingle, 1971; Kössel et al., 1985). Casual observation of RNA gels such as those in Figures 2 and 3 revealed that chloroplast rRNA metabolism was likely affected in the *TAG-FN* suppressor line, inasmuch as the abundant 1.2- and 1.0-kb 23S rRNA species were reduced in amount compared with the wild type and *var2-5* (Figure 2).

To examine the patterns of chloroplast rRNA accumulation in *TAG-FN* in greater detail, we conducted RNA gel blot analyses using gene-specific probes. The genes for chloroplast rRNAs (23S, 16S, 5S, and 4.5S) are located in the large inverted repeat region of land plant chloroplast genomes, where they form part of the *rnr* operon (Figure 8A). This operon, which also includes two tRNA genes, is transcribed as a large polycistronic RNA that is processed by endoribonucleases and exoribonucleases to yield the mature rRNA and tRNA species (Bollenbach et al., 2005).

Figure 8B shows that there was a dramatic shift in the stoichiometry of rRNAs complementary to the 23S probe in *TAG-FN*

compared with the wild type, *var2-5*, and the *TAG-FN* complementation line. This shift was due to a marked enhancement in levels of the three largest 23S rRNAs (the 3.2-kb precursor and the 2.9- and 2.4-kb mature rRNA species) and to a sharp decline in levels of the four smaller 23S rRNA species (the 1.7-kb processing intermediate and the mature 0.5-, 1.0-, and 1.2-kb rRNA species). Maturation of the 4.5S, 5S, and 16S rRNAs was also perturbed in *TAG-FN*: levels of the mature rRNAs were decreased, while those of the precursor rRNAs were increased. Cytosolic 18S rRNA levels were normal in all plants, suggesting that the accumulation of cytosolic rRNAs was not affected in the mutants.

Considered together, the data in Figure 8B indicated that *TAG-FN* was impaired in chloroplast rRNA processing and that this defect was rescued in the *TAG-FN* complementation line. This supported the idea that defects in chloroplast rRNA processing can be mediated by *svr1-1*. This was confirmed by examining profiles of chloroplast rRNA accumulation in the *svr1-1* and *svr1-2*



**Figure 8.** Chloroplast rRNA Processing Is Altered in *TAG-FN*.

**(A)** The structure of the chloroplast rRNA (*rrn*) operon in *Arabidopsis*. In addition to four rRNA genes, the operon contains genes for tRNA-I (Ile) and tRNA-A (Ala); each tRNA gene has a large intron. tRNA-V (Val) is immediately upstream from the 5' transcription start site of the operon, marked by the arrow. The probes used in the RNA gel blot analyses in **(B)** are shown as thick black lines under the individual rRNA genes.

**(B)** Accumulation of chloroplast rRNAs. RNA gel blot experiments were conducted as in Figure 2B using equal amounts of total cell RNAs (2  $\mu$ g) from leaves of 4-week-old wild-type, *var2-5*, *TAG-FN*, and the *TAG-FN P35S:SVR1* complementation line. The blots were probed with gene-specific sequences for plastid rRNAs (see **[A]**) and for the cytosolic 18S rRNA; the primers for these probes are listed in Supplemental Table 1 online. Ethidium bromide staining (rRNA control) of each gel is shown in Supplemental Figure 7 online. Sizes and precursor identifications are in agreement with previous reports (Bollenbach et al., 2005).

single mutants. Supplemental Figure 3A online shows that these profiles were qualitatively the same as those in *TAG-FN* (Figure 8B) but that there were quantitative differences in rRNA accumulation between *svr1-1* and *svr1-2*. In particular, *svr1-2* had enhanced accumulation of the three largest 23S rRNAs and of the 16S and 4.5S rRNA precursors as well as compensating decreases in the levels of the four smaller 23S rRNAs and of the mature 16S and 4.5S rRNAs. These observations were consistent with the notion that *svr1-2* was a more severe allele than *svr1-1*.

### Nuclear and Plastid Gene Expression in *svr1* Mutants

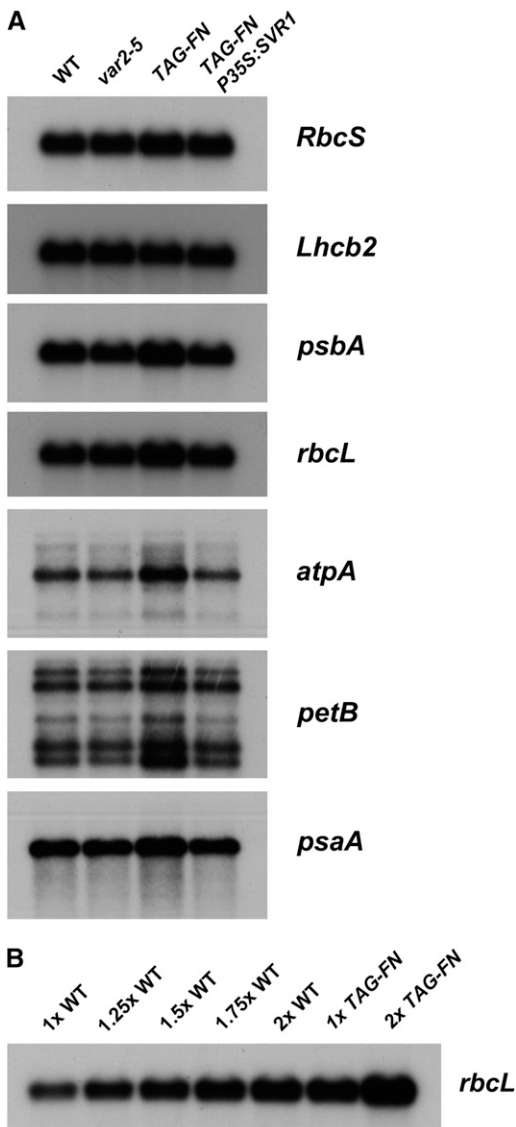
One question that arose was whether the alterations in plastid rRNA processing were indicative of a more global defect in RNA metabolism in *svr1* plants. Were patterns of mRNA accumulation affected? To address this question, we performed RNA gel blot analyses using representative nuclear and plastid genes as probes. These included *rbcL* (encoding the ribulose-1,5-bisphosphate carboxylase/oxygenase [Rubisco] large subunit; LS), *psbA* (encoding the D1 protein of PSII), *psaA* (encoding the PsaA protein of PSI), *atpA* (encoding the  $\alpha$ -subunit protein of the proton ATP synthase), and *petB* (encoding cytochrome *f* of the cytochrome *b<sub>6</sub>f* complex). Two nuclear genes for plastid proteins served as controls: *RbcS* (encoding the Rubisco small subunit) and *Lhcb2* (encoding the major light-harvesting chlorophyll *a/b* binding protein of PSII).

Figure 9A shows that the transcript profiles of the two nuclear genes were quantitatively and qualitatively similar in the wild type, *var2-5*, *TAG-FN*, and the *TAG-FN* complementation line. The patterns of mRNA accumulation were also qualitatively similar for each of the plastid genes. This indicated that mRNA processing per se was not likely altered in *TAG-FN*, since genes that were transcribed as polycistronic mRNAs, then processed to their mature forms (such as *atpA* and *petB*), had qualitatively similar mRNA accumulation profiles. It was notable, however, that plastid mRNA levels were increased in *TAG-FN* by about twofold, as estimated by comparing the amount of *rbcL* mRNA in *TAG-FN* to a titration series of *rbcL* mRNA accumulation in wild-type plants (Figure 9B). These increases were mediated by *svr1-1*, inasmuch as they were reversed in the *TAG-FN* complementation line. Considered together, the data in Figure 9 suggested that SVR1 played a global role in plastid mRNA accumulation, perhaps by affecting rates of transcription and/or turnover.

We were next interested in determining whether a lack of SVR1 affected chloroplast protein accumulation. For these experiments, we conducted immunoblot studies (Figure 10) on leaf proteins from the wild type, *var2-5*, *TAG-FN*, *svr1-1*, and *svr1-2* using antibodies against representative photosynthetic proteins: D1, PsbP, and Lhcb2 (all from PSII); PsaF (from PSI); the Rieske Fe-S protein (from the cytochrome *b<sub>6</sub>f* complex); ATP $\alpha$  (the  $\alpha$ -subunit of the proton ATPase); and LS. We also probed the blots with antibodies against VAR2 and FtsH1.

Consistent with the nearly normal chlorophyll levels and chlorophyll *a/b* ratios in *TAG-FN* and *var2-5* (Figures 1C and 1D), the data in Figure 10 showed that chloroplast protein levels were very similar in *var2-5*, *TAG-FN*, and *svr1-1*. Exceptions were VAR2 and FtsH1, which were dramatically reduced in amount in *var2-5* and *TAG-FN*. This was as expected for *var2-5*: FtsH1, FtsH2





**Figure 9.** Patterns of Nuclear and Chloroplast mRNA Accumulation.

**(A)** Expression of representative nuclear and plastid genes in the wild type, *var2-5*, *TAG-FN*, and the *TAG-FN* complementation line. Total cell RNA was isolated from leaves of 3-week-old seedlings grown at 22°C under continuous light ( $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). RNA gel blot analyses with the indicated probes were conducted as in Figure 2B. Ethidium bromide staining of each RNA gel is shown in Supplemental Figure 7 online.

**(B)** Total cell RNA from the wild type and *TAG-FN* was loaded on an agarose gel in a dilution series, and RNA gel blot analysis was conducted using an *rbcL* probe.

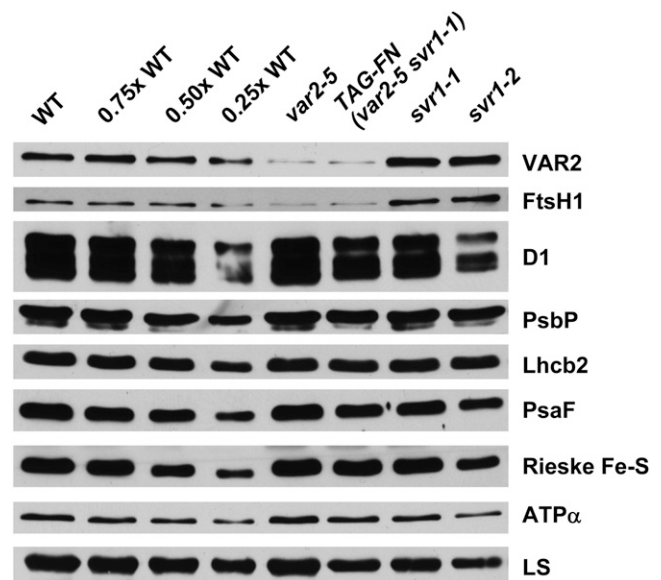
(VAR2), FtsH5, and FtsH8 interact to form oligomeric complexes in the thylakoid membrane, and when one of these proteins is produced in excess, it is degraded to match the abundance of the others (Yu et al., 2004, 2005). The fact that VAR2 and FtsH1 were coordinately reduced in amount in *TAG-FN* indicated that suppression of *var2* variegation in this line was not due to an enhancement of VAR2 and/or of FtsH1 protein abundance.

In contrast with *svr1-1*, some of the plastid proteins in Figure 10 were markedly reduced in amount. For instance, we consistently observed reductions of  $\sim 50\%$  in D1, PsaF, the Rieske Fe-S protein, ATP $\alpha$ , and LS. This suggested that SVR1 was capable of influencing chloroplast protein accumulation.

#### Inhibition of Chloroplast Translation Suppresses *var2* Variegation

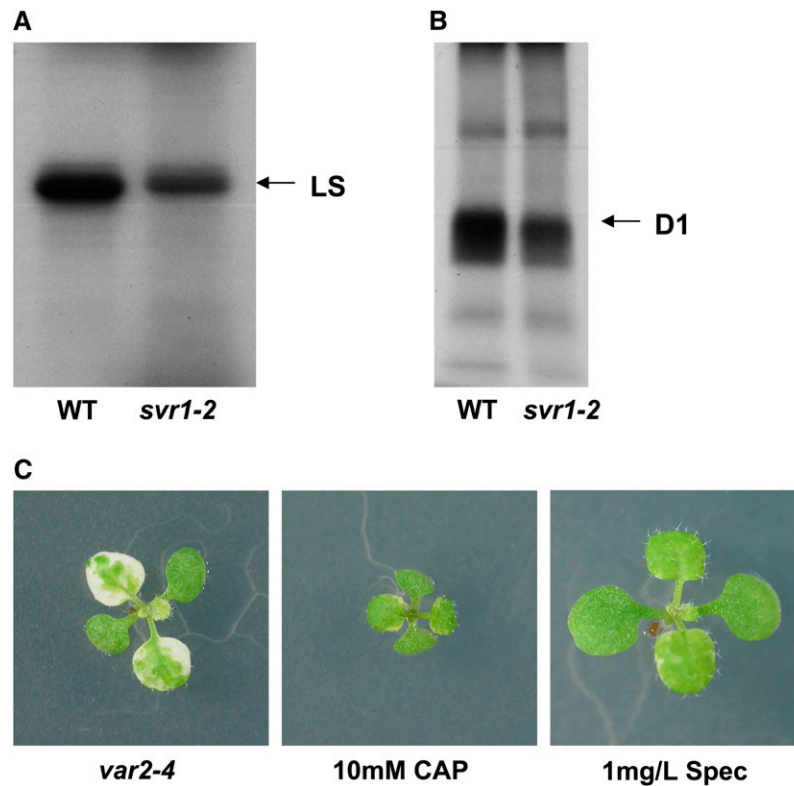
The data in Figures 9 and 10 were consistent with the idea that defects in *SVR1* resulted in higher than normal chloroplast mRNA levels but decreased abundance of at least some chloroplast proteins. This suggested that translation was impaired in the *svr1* mutants. This might not be unexpected, since chloroplast rRNA processing defects of the sort documented for *svr1* (Figure 8; see Supplemental Figure 3 online) have previously been correlated with defects in plastid ribosome assembly and translation (Bollenbach et al., 2005; Koussevitzky et al., 2006). To test this hypothesis, we pulse-labeled wild-type and *svr1-2* plants with [ $^{35}\text{S}$ ]Met for 20 min. In this system, the LS is the most abundant labeled protein in the soluble fraction and the D1 protein of PSII is the most abundant labeled protein in the membrane fraction (Rodermel et al., 1988; Pesaresi et al., 2001). Figures 11A and 11B show that the amounts of labeled LS and D1 that accumulate during the pulse were markedly reduced in *svr1-2*. This was consistent with the notion that chloroplast translation was reduced in *svr1-2*.

To test whether translation defects were able to suppress *var2* variegation, we treated *var2-4* (a null allele) with chemical



**Figure 10.** Patterns of Protein Accumulation.

Immunoblot analyses were conducted on total cell proteins from leaves of the wild type, *var2-5*, *TAG-FN*, *svr1-1*, and *svr1-2*; the plants were grown as in Figure 9. Following electrophoresis, the proteins were transferred to a nitrocellulose membrane and probed with polyclonal antibodies against the proteins indicated. The wild-type samples were loaded in a dilution series, and other lanes were standardized to equal fresh weights of leaf tissue.



**Figure 11.** Chloroplast Translation and Suppression of *var2* Variegation.

**(A)** and **(B)** Pulse-labeling of chloroplast proteins. Young, expanding leaves from wild-type and *svr1-2* seedlings were pulse-labeled with [<sup>35</sup>S]Met for 20 min. Total soluble proteins **(A)** or membrane proteins **(B)** were extracted, and equal cpm of trichloroacetic acid-precipitable proteins were subjected to SDS-PAGE analysis. Labeled bands were detected by autoradiography. Rubisco LS was the most prominent labeled membrane protein, and the D1 protein of PSII was the most prominent labeled membrane protein.

**(C)** Translation inhibitor experiments. *var2-4* seedlings were grown in the presence or absence of chloramphenicol (10 mM CAP) or spectinomycin (1 mg/L Spec). *var2* cotyledons are green, while the true leaves are variegated (Chen et al., 1999).

inhibitors of chloroplast translation, including chloramphenicol, which prevents the binding of amino acyl-tRNA to the A site on the 50S subunit of 70S ribosomes (Pestka, 1971), and spectinomycin, which prevents translocation of the peptidyl-tRNA from the A site to the P site on the 30S subunit of 70S ribosomes (Vázquez, 1979). We found that high concentrations of these inhibitors were lethal but that sublethal concentrations were able to suppress the variegation phenotype of *var2-4* (Figure 11C).

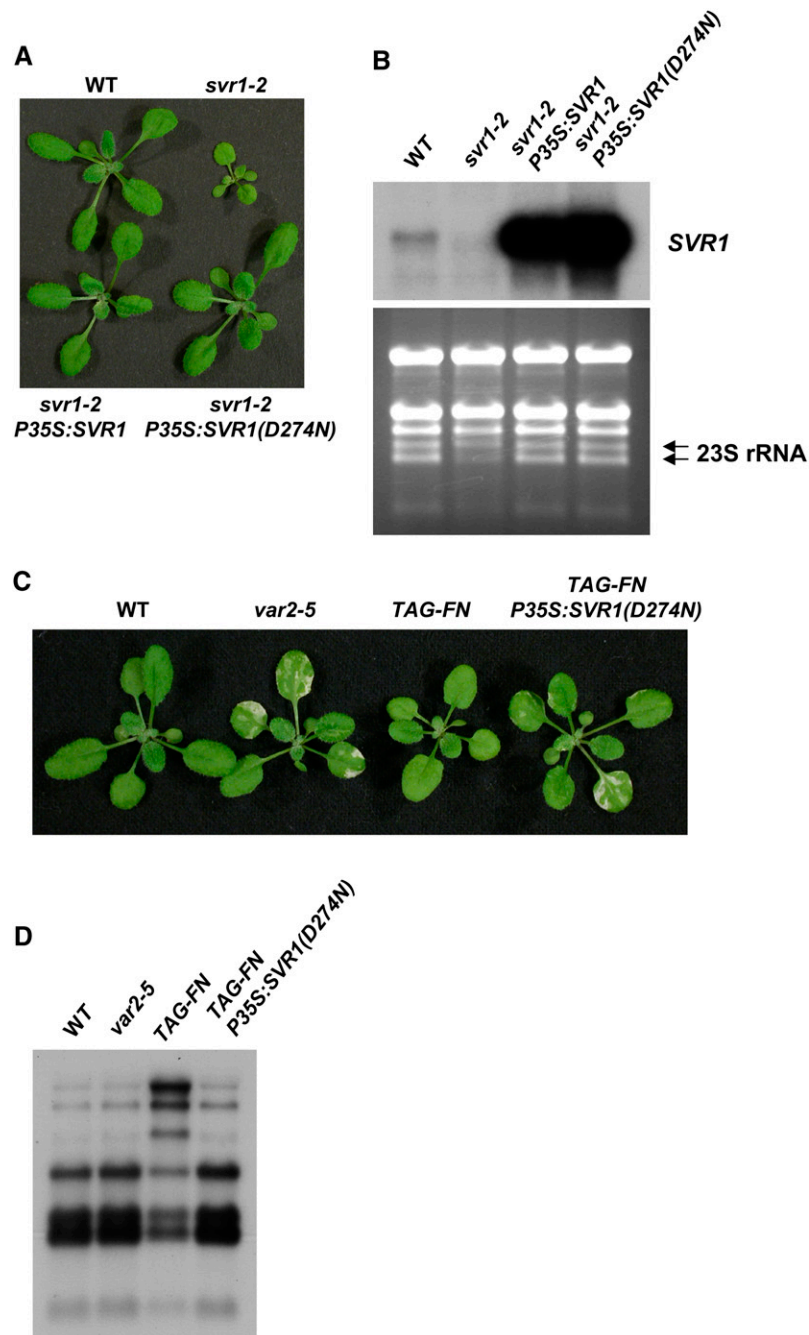
#### Mutagenesis of the Perfectly Conserved Asp in the Active Site of SVR1

As mentioned earlier, pseudouridine synthases contain a short stretch of nine amino acids in their active sites that includes a perfectly conserved Asp residue (Koonin, 1996; Ofengand, 2002) (Figure 6B). Mutations of this residue abolish the ability of pseudouridine synthase to isomerize uridine to  $\Psi$  in *E. coli* (Ofengand, 2002). This Asp corresponds to amino acid 274 of the SVR1 gene product.

We wanted to take advantage of our molecular null allele (*svr1-2*) to ask whether in vitro mutagenized copies of SVR1 that contained substitutions of this residue could complement the

*svr1-2* defect, thus normalizing the mutant phenotype. Figure 12A shows results from a representative experiment in which Asp-274 was mutagenized in vitro to Asn (D274N), a substitution of a charged residue with a similarly sized, uncharged residue. The mutant form of SVR1 was transformed into *svr1-2* under the control of the CaMV 35S promoter; the transformants were designated P35S:SVR1(D274N). Transformation of *svr1-2* with a wild-type SVR1 cDNA served as a control (transformants were designated P35S:SVR1). Figure 12A shows that both sets of transgenic plants resembled the wild type (i.e., both constructs were able to rescue *svr1-2*). This correlated with high levels of mRNA expression from both transgenes (Figure 12B). Figure 12B further shows that the 23S rRNA processing defects in *svr1-2* were reversed in the overexpressors. This is consistent with our earlier data showing that SVR1 is necessary for normal chloroplast rRNA processing.

Figure 3 shows that overexpression of wild-type SVR1 was able to restore a variegated phenotype to the TAG-FN suppression line. To test whether the active site Asp-274 was necessary for this restoration, we transformed TAG-FN with the D274N construct. Figure 12C shows that the transformants were variegated. This suggested that D274N, like wild-type SVR1, was able



**Figure 12.** Site-Directed Mutagenesis of the Conserved Asp-247 of SVR1.

**(A)** Representative 3-week-old wild-type, *svr1-2*, *svr1-2* transformed with a P35S-driven wild-type copy of *SVR1* (*P35S:SVR1*), and *svr1-2* transformed with a P35S-driven mutant *SVR1*, designated *P35S:SVR1(D274N)*, plants. In the mutant, Asp-274 was altered to Asn.

**(B)** RNA gel blot analysis of RNA from leaves of the plants in **(A)**. The experiments were conducted as in Figure 2B; the blot was probed with labeled *SVR1* sequences. The arrows label the two bands corresponding to 23S rRNA, and the ethidium bromide-stained gel is shown as a loading control.

**(C)** Representative 3-week-old wild-type, *var2-5*, TAG-FN, and TAG-FN transformed with the P35S-driven mutant *SVR1* construct, designated *P35S:SVR1(D274N)*, plants.

**(D)** RNA gel blot analysis of RNA from leaves of the plants in **(C)**. The experiment was conducted as in Figure 2B; the blot was probed with the 23S rRNA sequences described in Figure 8A. The ethidium bromide-stained gel of rRNAs is shown in Supplemental Figure 7 online.

to complement the *svr1-1* defect in *TAG-FN*. Also like the *TAG-FN* lines in which wild-type *SVR1* was overexpressed (Figure 8B), the 23S rRNA-processing defect in *TAG-FN* was normalized in the *D274N* overexpression lines (Figure 12D). This indicated that Asp-274, which is required for the isomerization of uridine to  $\Psi$ , was not required for plastid rRNA processing. However, plastid rRNA processing appeared to require the presence of the *SVR1* protein per se (Figure 12B).

### Another *var2* Suppressor, *svr2*, Is Impaired in Chloroplast rRNA Processing

During the course of our *var2* suppressor screens, we identified a T-DNA-tagged line (designated *TAG-IE*) with a chloroplast rRNA processing defect similar to that of *svr1* (Figure 13E). *TAG-IE* plants were smaller than normal and had a pronounced virescent phenotype, with newly emerged leaves having a bright yellow coloration (Figure 13A). We found that *TAG-IE* suppressed *var2* in a recessive manner and that the *TAG-IE* suppressor phenotype cosegregated with a complex T-DNA structure (see Supplemental Figure 4 online). DNA gel blot analyses revealed that this structure was likely composed of multiple rearranged T-DNAs. We named the suppressor locus *SVR2* and the mutant allele in *TAG-IE* *svr2-1*. Homozygous *svr2-1* plants had the same virescent phenotype as *TAG-IE*, indicating that *svr2-1* was epistatic to *var2-5* in *TAG-IE*.

Due to the complex nature of the T-DNA insertion in *svr2-1*, we failed to isolate the gene via plasmid rescue and thus turned to map-based cloning procedures. Bulked segregant analysis of a mapping population obtained by crossing *TAG-IE* with Landsberg *erecta* showed that *svr2-1* was genetically linked to molecular markers *ciw1* and *nga80* on chromosome 1. Using a candidate gene approach, we noted that this region of chromosome 1 contained genes for a number of putative and identified plastid proteins, including At1g49970, the gene for the ClpR1 subunit of the chloroplast Clp protease complex. It has been suggested that Clp protease core complexes (325 to 350 kD), which are composed of ClpP/R/S subunits (Peltier et al., 2001), are involved in general protein turnover in plastids (Adam and Clarke, 2002; Adam et al., 2006). We focused our attention on *ClpR1* because previously described *clpR1-1* and *clpR1-2* mutants have a virescent phenotype similar to that of *svr2-1* (Koussevitzky et al., 2006).

To test whether *svr2-1* defined the gene for ClpR1, we performed allelism tests between *svr2-1* and *clpR1-2*, a T-DNA insertion allele (Salk\_088407) (Koussevitzky et al., 2006). We found that *clpR1-2* and *svr2-1* failed to complement one another, suggesting that they were allelic. To confirm this hypothesis, we examined whether the *ClpR1* gene in *TAG-IE* contained a T-DNA structure. This was accomplished by PCR analysis of genomic DNA from *TAG-IE* using three pairs of primers that spanned the *Arabidopsis ClpR1* gene (Figure 13B). Figure 13C shows that the F2 and R2 primer pair failed to amplify a genomic DNA fragment, consistent with the idea that an insertion resided between these two primers. We also failed to detect a *ClpR1* transcript in *TAG-IE* via RT-PCR using *ClpR1* primers that would have amplified the entire gene (Figure 13D). This indicated that the *clpR1* allele in *TAG-IE* was likely a molecular null. Considered together, the data in Figure 13 supported the idea that *SVR2* encoded ClpR1.

Molecular characterization of *clpR1-1* (a null allele) has been performed in the Chory laboratory (Koussevitzky et al., 2006). Their studies showed that this mutant has many similarities to *svr1*. First, it accumulates higher molecular weight forms of chloroplast 23S and 4.5S rRNAs, suggesting that it is impaired in chloroplast rRNA processing. Second, it has normal levels of mRNA from several representative plastid genes but reduced protein accumulation. To explain these findings, Koussevitzky et al. (2006) suggested that chloroplast rRNA maturation and translation are linked and that chloroplast translation is inhibited in *clpR1-1*. Again, this is similar to our conclusions regarding *svr1-2*.

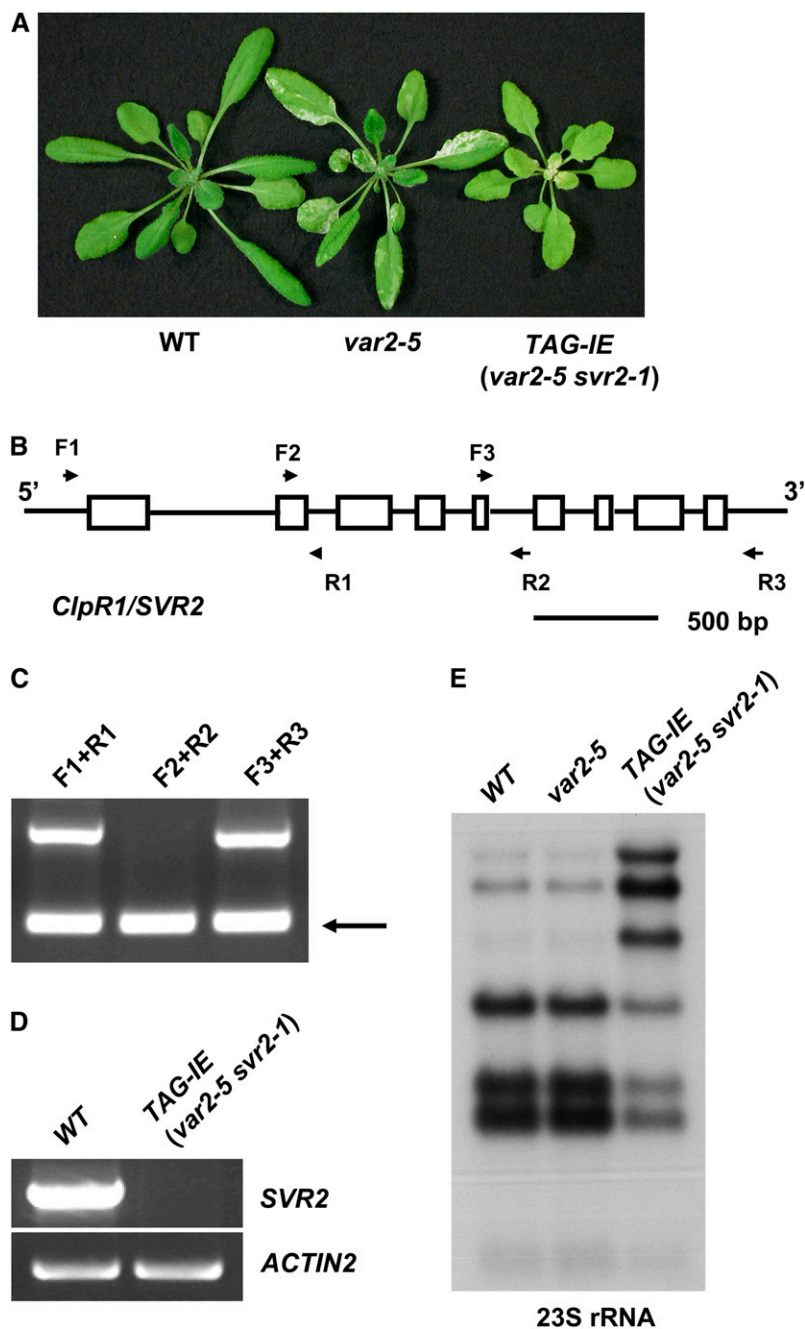
### Are Plastid rRNA Defects Common in *var2* Suppressors?

The question arose whether defects in plastid rRNA processing were universally associated with the suppression of *var2* variegation. To address this question, we examined chloroplast 23S rRNA profiles in various suppressors of *var2* we had generated (see Methods); the suppressors described below were all recessive. Supplemental Figure 5 online shows that three suppressors had profiles that resemble the wild type (and *var2-5*): *TAG-11*, *ems2505*, and *ems2544*. By contrast, other suppressor lines had 23S rRNA processing defects similar to those in *TAG-FN*, *svr1-2*, and *TAG-IE*. These included *clpC1*, a mutant that lacks the ClpC1 chaperone, which could suppress *var2* variegation at early stages of seedling development (see Supplemental Figure 6 online). We concluded that not all *var2* suppressors had defects in chloroplast rRNA processing.

## DISCUSSION

### Chloroplast rRNA Processing and Translation Are Perturbed in *svr1* and *svr2*

The molecular players that mediate plastid rRNA processing are poorly understood, but they include two 3' to 5' exoribonucleases. One is a polynucleotide phosphorylase (PNPase) that is involved in 23S rRNA processing as well as the metabolism of tRNAs and mRNAs (Walter et al., 2002; Sauret-Güeto et al., 2006), and the other is a homolog of *E. coli* RNase R (RNR1) that is involved in the maturation of 23S, 16S, and 5S rRNAs (Kishine et al., 2004; Bollenbach et al., 2005). Other factors that affect chloroplast rRNA maturation are defined by a handful of mutants that accumulate rRNA intermediates. These include the following: (1) maize *high chlorophyll fluorescence7* (Barkan, 1993), maize *mc1* (Watkins et al., 2007), and *Arabidopsis white cotyledon* (Yamamoto et al., 2000), all of which accumulate primarily 16S rRNA precursors; (2) *Chlamydomonas ac20*, which is defective in 23S rRNA maturation (Holloway and Herrin, 1998); (3) *Arabidopsis dal* (for *dag*-like, from the *differentiation and greening* mutant of snapdragon [*Antirrhinum majus*]) (Chatterjee et al., 1996), which accumulates 16S and 23S precursor rRNAs (Babiychuk et al., 1997; Bisanz et al., 2003); and (4) tomato *dcl* (for *defective chloroplasts and leaves*), in which 4.5S rRNA processing is defective (Bellaoui et al., 2003); 4.5S rRNA processing is also impaired in *Arabidopsis* mutants with down-regulated *DCL* gene expression (Bellaoui and Grussem, 2004).



**Figure 13.** Another *var2* Suppressor, *SVR2*, Affects Chloroplast rRNA Processing.

(A) Representative 4-week-old wild-type, *var2-5*, and *TAG-IE (var2-5 svr2-1)* plants.

(B) The gene structure of *ClpR1/SVR2*. The three sets of PCR primers used to identify the site of the T-DNA insertion are shown. The insertion is complex and resides between primers F2 and R2.

(C) Determination of the T-DNA insertion site in *ClpR1/SVR2*. Genomic DNA was extracted from *TAG-IE*, and PCR was performed using three sets of overlapping primers to the *Arabidopsis ClpR1* gene (Koussevitzky et al., 2006), as indicated in (B). The primers F2 and R2 were not able to amplify DNA, indicating that the insertion resided between them. The arrow designates an internal PCR control (see Methods).

(D) The expression of *ClpR1/SVR2* mRNA in the wild type and *TAG-IE*. Total cell RNA was extracted from the wild type and *TAG-IE* and subjected to RT-PCR analysis using the F1 and R3 primers; these primers should amplify a full-length *ClpR1/SVR2* cDNA.

(E) RNA gel blot analysis of 23S rRNA accumulation in the wild type, *var2-5*, and *TAG-IE*. The blotting was performed as in Figure 2B using total leaf RNA from 3-week-old seedlings; the blot was probed with 23S RNA sequences.

These mutants define genes whose products affect chloroplast rRNA processing. Several of these genes have been cloned, including *DCL* and *DAL*, which code for novel chloroplast proteins (Bellaoui et al., 2003; Bisanz et al., 2003), and *RNC1*, which codes for a maize group II splicing factor that mediates RNA binding but does not have endonuclease activity (Watkins et al., 2007). Mutants deficient in *RNC1* (*rnc1*) are illustrative of the principle that mutants with defects in chloroplast rRNA processing need not define genes whose products are directly involved in this process; rather, processing defects are frequently found in mutants with primary lesions in ribosome assembly and/or function (Keus et al., 1984; Barkan, 1993; Leal-Klevezas et al., 2000; Bellaoui et al., 2003; Williams and Barkan, 2003; Bollenbach et al., 2005; Schmitz-Lineweber et al., 2006). An early demonstration of this principle (Shen and Bremer, 1977) was the observation that rRNA maturation is defective in *E. coli* cells treated with chloramphenicol, an inhibitor of translation on 70S ribosomes. In a complementary manner, PNPase and RNR1 mutants, which have primary defects in chloroplast rRNA processing, have pleiotropic effects that include reduced rates of chloroplast translation and protein accumulation (Walter et al., 2002; Bollenbach et al., 2005; Sauret-Güeto et al., 2006).

One of the distinguishing molecular phenotypes of *svr1* is its impairment in the processing of all four plastid rRNA species (23S, 16S, 5S, and 4.5S). These defects were accompanied by above-normal levels of chloroplast mRNAs but by decreased rates of chloroplast protein synthesis and by reduced accumulation of chloroplast proteins. We conclude that *svr1* mediates defects in chloroplast rRNA processing either directly or indirectly. We propose that a similar explanation applies to *svr2* (Koussevitzky et al., 2006).

It might be noted that the defects in chloroplast rRNA processing in *svr1* were accompanied by chloroplast mRNA levels that exceeded those found in the wild type; *rbcL* mRNA levels, for example, were approximately doubled (Figure 9). Close inspection of the RNA gel blot data described by Koussevitzky et al. (2006) reveals that the levels of some chloroplast mRNAs are also elevated in *clpR1-1*. Although we do not understand the reason for these increases, our data are consistent with Barkan's (1993) early observation that the accumulation of chloroplast mRNA species is frequently altered in mutants with chloroplast ribosome defects.

### Possible Role of SVR1 in Chloroplast RNA Metabolism

In this report, we found that suppression of *var2* variegation in *TAG-FN* is caused by the disruption of *SVR1*, the gene for a plastid pseudouridine synthase. The isomerization of uridine-to- $\Psi$  occurs posttranscriptionally, and although the precise function of  $\Psi$  in noncoding RNAs is unclear, it has been proposed to play a structural role and might also be involved in peptidyl transfer during translation (Lane et al., 1992; Charette and Gray, 2000; Ofengand, 2002).

One of the central findings of the research reported here is that mutation of the conserved Asp in the active site of SVR1 does not affect chloroplast rRNA processing but that correct rRNA processing requires SVR1 protein accumulation (Figure 12). Our

working hypothesis is that Asp is required to isomerize uridine to  $\Psi$  but that SVR1 per se is necessary for rRNA processing. For instance, SVR1 could be involved in RNA binding as a component of a ribonucleoprotein complex that directly or indirectly affects rRNA metabolism. In support of this idea, the chloroplast Maa2 (for maturation of *psaA*) pseudouridine synthase from *Chlamydomonas* is necessary for *trans* splicing of group II introns in chloroplast mRNAs as well as for the isomerization of uridine (Perron et al., 1999). However, site-directed mutagenesis of several well-conserved amino acids of Maa2 (including the active site Asp) revealed that isomerization activity is not required for *trans* splicing, but splicing requires the physical presence of the protein (Perron et al., 1999). Similar results were reported for *E. coli* *TruB* and *RluD* pseudouridine synthase mutants (Gutgsell et al., 2000, 2001, 2005) as well as for yeast rRNA pseudouridine synthase Cbf5p (Lafontaine et al., 1998). By analogy to Maa2, it is thus possible that the reductions in translation that we observed in *svr1* plants might be due to a defect in splicing of a chloroplast mRNA for a protein required for chloroplast ribosome assembly/function. It might be noted that in addition to pseudouridine synthases, other examples of enzymes that modify noncoding RNAs and whose functions extend beyond this modification activity include *E. coli* methyltransferases RlmA<sup>1</sup> (Liu et al., 2004), a tRNA m<sup>5</sup>U54-methyltransferase (Persson et al., 1992), and rRNA methylases from yeast (the *DIM1* gene product) and *Arabidopsis* chloroplasts (the *PALEFACE1* gene product) (Lafontaine et al., 1995; Tokuhsa et al., 1998).

Based on phylogenetic considerations, SVR1 bears all of the hallmarks of a bona fide pseudouridine synthase. However, we have not directly demonstrated that the enzyme has isomerization activity. Even if it is ultimately demonstrated that SVR1 does not have pseudouridine synthase activity, our data clearly suggest that the presence of the protein is required for normal rRNA processing. Such a situation would be consistent with the idea that enzymes involved in plastid RNA metabolism are frequently derived from antecedents with different roles in RNA metabolism. A well-known example of this phenomenon is RNC1, which is a component of a chloroplast ribonucleoprotein complex that promotes the splicing of group II introns (Watkins et al., 2007). RNC1 contains two ribonuclease III domains, but amino acids that are essential for ribonuclease III activity are missing in the protein. Mutant and biochemical analyses have revealed that the role of RNC1 in splicing involves RNA binding but not endonucleolytic activity. Another example of this general phenomenon is CRS2, a maize group II intron splicing factor that lacks its ancestral peptidyl-tRNA hydrolase activity (Ostheimer et al., 2005).

### Model of Suppression of *var2* Variegation

Suppression of *var2* variegation by *svr1* and *svr2* is likely to be indirect, inasmuch as our genetic analyses showed that *svr1-2* and *svr2-1* are able to suppress *var2-4* (a null allele). This suggests that the physical presence of the VAR2 protein is not necessary for suppression; this does not rule out an involvement of SVR1 or SVR2 with other components of the FtsH complex. Taken together, our data suggest that suppression of variegation in both *svr1* and *svr2* is caused by a phenotypic syndrome that

includes defects in chloroplast rRNA processing, reduced chloroplast translation, reduced chloroplast protein accumulation, and elevated chloroplast mRNA levels. We propose that there is a fundamental interaction between chloroplast translation and *var2*-mediated leaf variegation. This was confirmed by treatment of *var2* with chloroplast translation inhibitors such as chloramphenicol and spectinomycin, both of which are able to suppress variegation (Figure 11).

One hypothesis of variegation suppression proceeds from the premise that a reduction in the rate of chloroplast translation prolongs the process of chloroplast biogenesis, allowing more time to accumulate a threshold of factors that are able to compensate for a lack of *var2* (i.e., before the decision is made to turn white or green). (Intermediate plastid types are not seen in *var2* tissues [Chen et al., 1999]). For example, these factors could enhance photoprotection or other *var2*-mediated plastid developmental processes. We propose that this prolongation would be especially important during early leaf development, when plastid divisions are occurring and proplastids are being converted into chloroplasts (Yu et al., 2004).

A second hypothesis is that the crucial element in variegation suppression is chloroplast translation per se and that reduced translation affects the expression of nuclear genes and/or plastid genes. It is well established that reductions in plastid translation are sensed by the nucleus via a poorly defined retrograde signaling pathway (Oelmüller et al., 1986; Adamska, 1995; Gray et al., 1995; Yoshida et al., 1998; Sullivan and Gray 1999, 2002; Sugimoto et al., 2004). In this pathway, reductions in chloroplast translation elicit the transmission of a signal from the plastid to the nucleus that results in altered transcription of nuclear genes, primarily those for chloroplast proteins. Therefore, it is possible that suppression of *var2* variegation is caused by an altered chloroplast proteome that allows the developing plastid to compensate for a lack of VAR2.

In a third scenario, variegation suppression is caused by reduced translation of a chloroplast DNA-encoded factor. For example, this factor could be a substrate of VAR2 that normally acts to promote early chloroplast biogenesis but that later inhibits this process if it is not degraded. The inhibitor would be present in *var2* (causing white sector formation), but its production would be reduced to such an extent in *svr1* and *svr2* plants that normal chloroplast biogenesis could proceed even in the presence of small amounts of the inhibitor. This hypothesis, like the others, would be consistent with the idea of a threshold (e.g., some plastids would have reached a threshold of the plastid factor, while others would not have).

Previous work from our laboratory demonstrated that mutations in the nuclear gene for ClpC2, a chloroplast Hsp100 chaperone, are potent suppressors of *var2* variegation (Park and Rodermel, 2004). It has been suggested that ClpC2 functions both as a regulatory subunit of the Clp protease complex and independently as a molecular chaperone (Adam et al., 2006; Sakamoto, 2006). Our finding that mutations in another subunit of the Clp protease (ClpR1) are able to suppress *var2*-mediated variegation establishes a second linkage between FtsH and Clp function in chloroplasts. Our suppressor studies to date thus open up possibilities for further research into chloroplast protease networks.

In this study, we found that defective chloroplast rRNA processing is a common, but not universal, molecular phenotype of *var2* suppressors and that this phenotype is accompanied by alterations in chloroplast translation and protein accumulation. We conclude that this class of suppressor should provide valuable insight into the factors that mediate plastid rRNA maturation, ribosome assembly, and function (Manuell et al., 2004). In support of this idea, Sakamoto's group (Miura et al., 2007) recently reported that *var2* variegation is suppressed by defects in chloroplast elongation factor G (*SCO1* locus) and chloroplast translation initiation factor 2 (*FUG1* locus).

## METHODS

### Plant Material

All *Arabidopsis thaliana* plants used in this study are in the Columbia ecotype background. They were grown at 22°C under continuous light of  $\sim 100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . The mutant *var2-4* and *var2-5* alleles have been described (Chen et al., 2000). Suppressor lines included *TAG-FN* (this study), *TAG-IE* (this study), *TAG-11* (X. Liu, F. Yu, and S. Rodermel, unpublished data), all obtained by activation tagging (below); and *ems2505* (S. Park and S. Rodermel, unpublished data) and *ems2544* (Park and Rodermel, 2004), both obtained by ethyl methanesulfonate mutagenesis. *svr1-2* was identified from a Salk line (Salk\_013085); a T-DNA insertion allele of *svr2* was also obtained from a Salk line (Salk\_088407) (Koussevitzky et al., 2006). The *clpC1* mutant was described by Sjögren et al. (2004). In some experiments, *var2-4* seeds were grown on Murashige and Skoog plates in the presence or absence of inhibitors of chloroplast translation (10 mM chloramphenicol or 1 mg/L spectinomycin) under the conditions described above.

### Activation Tagging

The *var2-5* allele was used for the activation tagging experiments. An *Agrobacterium tumefaciens* strain containing the activation tagging vector pSKI015 was kindly provided by Joanne Chory at the Salk Institute. Bolting *Arabidopsis* plants were transformed by the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). T1 plants were germinated on soil, and transgenic plants were selected by spraying with a 1:2000 dilution of Finale (AgrEvo), which contains 5.78% (w/v) ammonium glufosinate. The T1 plants were self-fertilized, and analyses were performed on T2 and subsequent generation plants.

### Map-Based Cloning

The suppressor gene in *TAG-IE* was mapped by bulked segregation analysis (Lukowitz et al., 2000) using a pool of 112 F2 seedlings from a cross between *TAG-IE* and Landsberg *erecta*. The gene was fine-mapped using sets of codominant simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994). The suppressor gene resides between SSLP markers *ciw1* and *nga280*, described by Lukowitz et al. (2000). Three pairs of PCR primers were used for confirmation of the site of T-DNA insertion in the suppressor gene (F1 and R1, F2 and R2, and F3 and R3); these are listed in Supplemental Table 1 online. PCR primers MRO11F and MRO11R were used as an internal PCR control (see Supplemental Table 1 online).

### Phylogenetic Analysis

Full-length protein sequences for 17 pseudouridine synthases from *Arabidopsis*, 11 pseudouridine synthases from *Escherichia coli*, and the

Maa2 pseudouridine synthase from *Chlamydomonas reinhardtii* were obtained from the National Center for Biotechnology Information GenBank. The sequences were aligned with ClustalX (see alignment in Supplemental Data Set 1 online), and a phylogenetic tree was constructed using default settings of MEGA 2.1 (Kumar et al., 2001). Bootstrap analysis was performed using 1000 trials.

### Plasmid Construction and Transformation

To complement the *TAG-FN* mutant, a full-length At2g39140 (*SVR1*) cDNA was amplified by Plantium *pf*x DNA polymerase (Invitrogen) using primers FNCB and FNCR (see Supplemental Table 1 online). The PCR product was digested with *SacI* and cloned into the *SacI* site of pBlue-script KS+. The resulting construct was sequenced to confirm the *SVR1* sequence. The *SacI* fragment was then transferred into a modified pBI121 plasmid (Yu et al., 2004), and the resulting construct was transformed into *Agrobacterium* by electroporation. *Arabidopsis* transformation was performed as described above (Clough and Bent, 1998).

To generate a GFP-tagged *SVR1* fusion protein (*SVR1:GFP*) for transient expression in protoplasts, At2g39140 (*SVR1*) cDNA was amplified using primers FNCBF and FNGFPR (see Supplemental Table 1 online) and cloned into vector pTF486 at the *Bam*HI and *Nco*I sites, creating a C-terminal GFP fusion. Transcription is driven by the CaMV 35S promoter (P35S) in this vector. Protoplasts from wild-type *Arabidopsis* leaves were isolated and transiently transformed with the P35S:*SVR1:GFP* construct or, alternatively, with a GFP vector control (P35S:GFP) according to Yoo et al. (2007). Fluorescence from GFP and autofluorescence from chlorophyll were monitored by confocal microscopy.

### DNA and RNA Gel Blot Analyses

Genomic DNA was isolated and DNA gel blot analyses were performed as described by Wetzel et al. (1994). Total cell RNA isolation from *Arabidopsis* leaf tissues using the Trizol RNA reagent (Invitrogen) and RNA gel blot analyses were also performed as described by Wetzel et al. (1994). Primers used to generate the probes used in this work are listed in Supplemental Table 1 online. Most probes were labeled by random priming, but the 4.5S and 5S probes were labeled by the end-labeling method (Sambrook et al., 1989). RT-PCR was performed as described (Yu et al., 2004) using the primers listed in Supplemental Table 1 online.

### Plasmid Rescue

For plasmid rescue, genomic DNA was extracted from 100 mg of *Arabidopsis* tissue and resuspended in 30  $\mu$ L of deionized water (Wetzel et al., 1994). A 10- $\mu$ L aliquot of this suspension was digested with various restriction enzymes for 8 h in 30- $\mu$ L reactions, and the digests were extracted with phenol-chloroform followed by chloroform. DNA was precipitated with ethanol and resuspended in 20  $\mu$ L of deionized water. This suspension was split into two 10- $\mu$ L fractions and ligated overnight using T4 DNA ligase (Invitrogen) at either 14°C or room temperature. The two ligation reactions were combined and extracted with phenol-chloroform, followed by chloroform, and the DNA was precipitated with ethanol and resuspended in 10  $\mu$ L of deionized water. One microliter of the final suspension was used for transformation using recombination-deficient *E. coli* SURE competent cells (Stratagene). Rescued plasmids were selected on Luria-Bertani plates containing 100 mg/L ampicillin.

### Protein Manipulations

*Arabidopsis* leaves were weighed and frozen in liquid N<sub>2</sub>. Frozen leaves were then homogenized in 2 $\times$  SDS buffer and incubated at 65°C for 2 h. After centrifugation at 14,000 rpm for 10 min, the supernatants were

loaded onto 12% SDS polyacrylamide gels based on equal fresh weights (Yu et al., 2004). SDS-PAGE was performed based on Laemmli (1970) as described by Yu et al. (2004, 2005). Polyclonal antibodies used in this study were directed against VAR2 (Chen et al., 2000), FtsH1 (Yu et al., 2005), D1 (Yu et al., 2005), Rieske Fe-S protein (Yu et al., 2005), and the ATPase  $\alpha$ -subunit (Yu et al., 2005). Antibodies against PsbP and PsfA were from Agrosera and were kindly provided by Poul Erik Jensen at the University of Copenhagen. The SuperSignal West Pico chemiluminescence kit (Pierce) was used for signal detection on the immunoblots.

Labeling of proteins *in vivo* was performed as described previously (Rodermeil et al., 1988; Pesaresi et al., 2001). In brief, [<sup>35</sup>S]Met (50  $\mu$ Ci) was applied to the surface of expanding *Arabidopsis* leaves, and the leaves were vacuum-infiltrated for 10 s. After a 20-min pulse of the label, total proteins were isolated (as described above) and equal cpm were loaded onto 12.5% SDS polyacrylamide gels. The gels were dried using a Bio-Rad Geldryer and exposed to autoradiograph film.

### Chlorophyll Analysis

The first pair of rosette leaves of 2-week-old seedlings was harvested and weighed. Leaves were ground in liquid N<sub>2</sub>, and chlorophyll was extracted with 95% ethanol. Total chlorophyll contents and chlorophyll *a/b* ratios were calculated as described (Lichtenthaler, 1987).

### Site-Directed Mutagenesis

The full-length At2g39140 (*SVR1*) cDNA was amplified using primers FNCBF and FNCR and cloned into pBlue-script KS+ at the *Bam*HI site. Asp-274 was then mutated *in vitro* using the QuikChange site-directed mutagenesis kit (Stratagene). The primers used for mutagenesis were DNF and DNR (see Supplemental Table 1 online). The wild-type and mutated forms of the *SVR1* cDNA were cloned into the *Bam*HI site of pB003 (provided by Dr. David Oliver, Iowa State University); transcription was driven by the CaMV 35S promoter in this vector. The constructs were introduced into *Arabidopsis* by the methods described above.

### Electron Microscopy

For transmission electron microscopy, leaf samples were obtained from 7-d-old seedlings grown as described above. The samples were fixed, stained, and examined as described by Horner and Wagner (1980).

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *SVR1/At2g39140*, NP\_181447; *ClpR1/SVR2/At1g49970*, NP\_564560; *At1g09800*, NP\_172451; *At1g20370*, NP\_564112; *At1g34150*, NP\_564438; *At1g56345*, NP\_176031; *At1g76050*, NP\_177732; *At1g76120*, NP\_565126; *At1g78910*, NP\_178012; *At2g30320*, NP\_180591; *At3g04820*, NP\_187133; *At3g06950*, NP\_187351; *At3g19440*, NP\_188575; *At3g57150*, NP\_191274; *At4g21770*, NP\_193908; *At5g14460*, NP\_196950; *At5g35400*, NP\_198390; *At5g51140*, NP\_199927; *Maa2*, CAB62387; *RsuA*, AAC75244; *RluB*, AAC74351; *RluE*, AAC74219; *RluF*, AAC76992; *RluA*, AAC73169; *RluC*, AAC74170; *RluD*, AAC75643; *TruC*, AAC75833; *TruB*, AAC76200; *TruA*, AAC75378; *TruD*, AAC75787.

### Supplemental Data

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

**Supplemental Figure 1.** The Ultrastructure of Chloroplasts in the Wild Type, *var2-5*, and *TAG-FN*.



**Supplemental Figure 2.** Linkage Analysis of *TAG-FN* Double Mutants.

**Supplemental Figure 3.** Characterization of *svr1-2*.

**Supplemental Figure 4.** Linkage Analysis of *TAG-IE* Double Mutants.

**Supplemental Figure 5.** Chloroplast 23S rRNA Accumulation in *var2* Suppressors.

**Supplemental Figure 6.** The Suppression of *var2-5* by *clpC1*.

**Supplemental Figure 7.** Ethidium Bromide Staining of RNA Gels.

**Supplemental Table 1.** Primers Used in This Study.

**Supplemental Data Set 1.** Alignment Used to Generate the Phylogenetic Tree in Figure 6.

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