White Leaf Sectors in yellow variegated2 Are Formed by Viable Cells with Undifferentiated Plastids1[C][W][OA]

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The yellow variegated2 (var2) is one of the best-characterized Arabidopsis (Arabidopsis thaliana) mutants showing leaf variegation. Leaf variegation of var2 results from the loss of an ATP-dependent metalloprotease, FtsH2, which is a major component of the FtsH heterocomplex in thylakoid membranes. While the functional role of FtsH2 in protein quality control has been extensively studied, the physiological state of plastids in white tissues of the var2 is not well characterized. Here we show that the white tissue in var2 is neither the result of photobleaching nor enhanced senescence. Visualization of plastids by plastid-targeted green fluorescent protein revealed that plastids in the white sector are distinct and have undifferentiated characteristics. The plastids are also distinct in that they contain large nucleoids, a complex structure of plastid DNA and proteins, that are typically found in undifferentiated plastids. Comparative analyses of protein profiles from green and white tissues suggested that the difference was observed in the proteins related to photosynthesis but not due to proteins of other organelles. Thus, cells in the white tissue are viable and their defect is limited to plastid function. The plastid accumulates normal levels of chloroplast transcripts, whereas a substantial repression of nuclear-encoded photosynthetic genes was evident in the white sector. Based upon these results, we inferred that the white sectors in var2 are made by viable cells that have plastids arrested in thylakoid formation. A proposed model to form the variegated sector in var2 is provided.

Leaf variegation is occasionally observed in higher plants. It raises a fundamental question of why and how two sectors containing different cell types, a green cell with normal-appearing chloroplasts and a white cell with abnormal plastids, can be formed in leaf tissue. Leaf variegation has been recognized as a genetic trait since early twentieth century (for review, see Kirk and Tilney-Bassett, 1978). Several recessive genes responsible for the variegation phenotype have been identified in model systems such as maize (Zea mays), Arabidopsis (Arabidopsis thaliana), and tomato (Lyco persicum esculentum; e.g. Han et al., 1992; Carol and Kuntz, 2001). Studies from these model systems have shown that leaf variegation has various causes. On the other hand, variegation is affected not only by leaf development but also in response to biotic and abiotic circumstances, resulting in the formation of complex patterns (for review, see Kirk and Tilney-Bassett, 1978; Sakamoto, 2003; Yu et al., 2007). Such unstable and unpredictable patterns for the appearance of white sectors hinders our ability to elucidate the physiological control of this phenomenon.

We have been studying yellow variegated1 (var1) and var2 in Arabidopsis as a model to study the mechanisms of leaf variegation. The responsible genes, VAR1 and VAR2, encode FtsH5 and FtsH2 metalloproteases, respectively (Chen et al., 2000; Takechi et al., 2000; Sakamoto et al., 2002). FtsH is a membrane-anchored ATP-dependent metalloprotease that degrades various chloroplastic proteins (for review, see Ito and Akiyama, 2005; Adam et al., 2006; Sakamoto, 2006). Arabidopsis contains nine FtsH isomers in plastoplasts and FtsH exists in thylakoid membranes as a heterocomplex formed by at least two type isomers (A and B, represented by FtsH1/5 and FtsH2/8, respectively; Sakamoto et al., 2003; Yu et al., 2004; Zaltsman et al., 2005b). A loss of FtsH2 or FtsH5 in var1 or var2 mutants is partially compensated for by FtsH8 and FtsH1, respectively (Yu et al., 2004, 2005). A nonlethal phenotype of leaf variegation in var1 and var2 is at least attributable to the redundancy of FtsH gene family. In Escherichia coli, FtsH occurs as a single copy essential gene (for review, see Ito and Akiyama, 2005).

Mounting evidence indicates that FtsH plays a dual role in chloroplasts. First, given the fact that FtsH degrades D1 protein of PSII reaction center both in vivo and in vitro in a light-dependent manner (Lindahl et al., 2000; Bailey et al., 2002; Sakamoto et al., 2004), FtsHs were suggested to participate in the PSII repair cycle. Second, presence of plastids without thylakoids in cells of white sectors, as revealed by electron microscopy (Chen et al., 2000; Takechi et al., 2000;
Sakamoto et al., 2002), suggests that FtsHs act on the formation of thylakoid membranes. Based on this, a threshold model was proposed in which thylakoid development is cell-autonomously determined by the overall FtsH levels (Yu et al., 2005; Zaltsman et al., 2005a). A genetic approach to screen suppressors of var2 leaf variegation has been conducted and revealed some of the factors involved in this process (Park and Rodermel, 2004; Miura et al., 2007). These investigations suggest that the balance between the synthesis and degradation of chloroplast proteins affects leaf variegation.

Despite these observations, the physiological properties of white sectors in var2 remain uncharacterized. It is apparent that white sectors do not simply represent dead tissues, as their phenotypes are apparently different from those of cell death mutants (e.g. Mach et al., 2001; Gray et al., 2002; Mateo et al., 2004). Since leaf variegation is occasionally found in nature, we hypothesize that the formation of variegated sectors may be beneficial for plants. In this study, we focus on plastids in the white sectors of var2 and successfully visualized them with GFP. Studies on the accumulation of mRNAs and proteins showed that both chloroplast and nuclear genes were expressed in the white tissues, although the levels of photosynthetic proteins appeared to decrease. Furthermore, we showed that plastid DNAs in the white tissues were organized as nucleoids that were typically detected in undifferentiated plastids. Collectively, these results demonstrate that the white sectors of var2 are active tissues that are formed by viable cells with undifferentiated plastids. An integrated model of forming undifferentiated plastids in the white sectors is discussed.

RESULTS

White Sectors in Variegated Leaves Are Not Stained by Evans Blue

Histochemical staining was performed to determine whether white sectors of var2 contained necrotic tissue. Excised leaflets from var2-1 and wild-type plants were vacuum infiltrated with an Evans blue solution for specific staining of dead cells. With the exception for cut sites, no tissues were stained in either variegated var2-1 leaves nor wild type (Fig. 1). When leaflets were used from an accelerated cell death mutant (accelerated cell death2 [acd2]; Mach et al., 2001) as a positive control, staining was apparent subsequent to a coronatine treatment that induced cell death. These data allowed us to conclude that white sectors are not simply comprised of dead cells and led us to further characterize white cells. Evans blue stain was carried out with var2-1 leaflets that were illuminated under high light (800 μmol m⁻² s⁻¹ for 4 h). Blue stains were never observed in either white or green sectors (data not shown), suggesting that white tissues are not the results of photooxidative damage.

Plastids in the White Sectors Can Be Visualized by GFP

We attempted to visualize chloroplasts of var2 with GFP analysis. A transgenic plant expressing a plastid-targeted L12-GFP protein (an N-terminal transit peptide from Oryza sativa ribosomal protein L12 was fused to GFP) was crossed to var2-1. Individuals expressing L12-GFP in var2-1 were obtained in the F₂ population and F₂ progeny was subjected to further analysis. Thin sections of leaf tissues of var2-1/L12-GFP showed that L12-GFP was expressed in both green and white sectors. Similar to wild type, overlapping detection of GFP signals with chlorophyll autofluorescence indicated that L12-GFP was localized in chloroplasts of green sectors (Fig. 2A). We also detected L12-GFP signals representing plastids throughout the white sectors. Mesophyll protoplasts were prepared from var2-1/L12-GFP leaves to carefully observe L12-GFP. As shown in Figure 2B, organelle structures like undifferentiated plastids were detected in the white protoplasts. Their morphologies were variable but mostly smaller than the chloroplasts in green sectors, and occasionally contained stromule-like structures. Thus, cells in the white sectors are active and retain plastids with intact envelopes.

Mitochondria in the White Sectors Can Be Visualized by GFP

We also attempted to visualize mitochondria of var2 by using a mitochondria-targeted GFP. A transgenic
plant expressing a mitochondria-targeted DIPS-GFP, containing N-terminal mitochondrial presequence from Arabidopsis mitochondrial ATP-δ subunit, was crossed to var2-1. Similar to the results from L12-GFP, DIPS-GFP was detected as rod- and occasionally string-like structures representing mitochondria in both green and white sectors. Mitochondrial morphologies detected by DIPS-GFP were indistinguishable between green and white sectors (Fig. 2B). These results again suggest that white cells are not dead, and that they contain viable mitochondria.

**Protein Accumulation in the White and Green Sectors**

To observe the difference between white and green sectors, we next characterized their respective protein profiles. Green and white sectors were dissected from 6-week-old leaves (Fig. 3A) and subjected to protein extraction and SDS-PAGE. The result shown in Figure 3B (proteins were loaded on equal fresh weight) displayed that white sectors appeared to contain less proteins than green sectors, but overall protein profiles were, if not all, similar. Immunoblot analyses confirmed that white sectors contain substantially reduced amounts of chloroplastic proteins, D1 (reaction center protein of PSII), PsbO (subunit of oxygen evolving system of PSII), and Lhcb1 (light-harvesting complex [LHC] proteins of PSII; Fig. 3C). The reduction of Rubisco large subunit and LHCII was also evident in the silver-stained SDS-PAGE (Fig. 3B, black arrowheads). Conversely, we noticed an increased accumulation of nonplastid proteins, Phb5 (mitochondrial prohibitin complex protein), BiP (endoplasmic reticulum luminal binding protein), and β-tubulin in white sectors. This was most likely attributable to the fact that the blots were prepared by a fresh weight basis; since developed thylakoid membranes were lacking, white sectors contained a larger number of cells than the green cells. Likewise, several proteins that appeared to increase in the white sectors were detectable in the silver-stained gel (Fig. 3B, white arrowheads). However, when normalized by β-tubulin as a control, the steady-state levels of Phb5 and BiP were not significantly altered between Columbia (Col), green, and the white sectors of var2 (Fig. 3D). Based upon these observations, we inferred that cells in the white and green sectors are indistinguishable in the accumulation of nonchloroplastic proteins.

We also compared chloroplastic protein accumulation between Col and green sectors of var2-1. Chloroplasts were purified by a Percoll step gradient from Col and var2-1 mature leaves and soluble proteins were prepared using phenol extraction and ammonium
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Plastid DNA Accumulation and Observation of Plastid Nucleoids

The presence of plastid mRNAs apparently indicated that plastid DNAs exist in the white sectors, but it remained unclear whether the quality and quantity of plastid DNAs are equivalent between the two sectors. To examine this, we first performed semiquantitative PCR to assess the plastid DNA level. Similar to the results obtained by RT-PCR, when primers specific for both nuclear and plastid genes were used, DNA levels were indistinguishable between Col, and green and white sectors of var-2-1 (Supplemental Fig. S2).

We considered that the observation of nucleoids was useful to know the state of plastids in the white sectors, since plastid nucleoids are subject to dynamic change during chloroplast development (Kuroiwa et al., 1981; Nemoto et al., 1988). We prepared leaf cross sections of mature leaves from var-2-1 and stained them by a DNA-specific fluorescent dye, 4′,6-diamidino-2-phenylindole (DAPI). In comparison to green sectors, plastid nucleoids were indistinguishable between the two sectors (Fig. 3). In contrast, both NEP- and PEP-transcribed genes (accD and rpoB by NEP and psbA and rbcL by PEP) appeared to be transcribed and their transcripts stably accumulated in white tissues. Steady-state levels of the corresponding transcripts were comparable between Col, green, and white sectors. Given a very limited amount of D1 and large subunit of Rubisco in the white sectors, these proteins appeared to be regulated at the posttranscriptional level. A control experiment using actin2 showed that no significant difference was present for nonphotosynthetic nuclear genes (Fig. 4).

Accumulation of Transcripts for Photosynthetic Genes in White Tissues

The presented data so far implied that the difference between the green and white sectors is limited to the state of plastid development. We therefore examined the expression of photosynthetic genes, encoded both in chloroplast and nuclear genomes, by semiquantitative reverse transcription (RT)-PCR. Three types of photosynthetic genes, namely (1) nuclear genes, (2) plastid genes transcribed by a nuclear-encoded RNA polymerase (NEP), and (3) plastid genes transcribed by a plastid-encoded RNA polymerase (PEP) were selected as an index (Fig. 4). In plastids, PEP is predominantly responsible for transcription of photosynthetic genes, whereas NEP mainly mediates the transcription of housekeeping genes (Kanamaru and Tanaka, 2004).

We found that transcripts of nuclear-encoded genes (psbO and rbcS) decreased within white sectors. This is consistent with the result of protein accumulation (Fig. 3). In contrast, both NEP- and PEP-transcribed genes (accD and rpoB by NEP and psbA and rbcL by PEP) appeared to be transcribed and their transcripts stably accumulated in white tissues. Steady-state levels of the corresponding transcripts were comparable between Col, green, and white sectors. Given a very limited amount of D1 and large subunit of Rubisco in the white sectors, these proteins appeared to be regulated at the posttranscriptional level. A control experiment using actin2 showed that no significant difference was present for nonphotosynthetic nuclear genes (Fig. 4).

Accumulation of psbA and rbcL mRNAs in the white plastids is consistent with our previous observation (Takechi et al., 2000), and demonstrates that the plastids are active. In contrast, the decrease of nuclear-encoded psbO and rbcS mRNAs suggested that a retrograde regulation occurs in the white sectors.
DAPI fluorescence was obviously strong in white sectors (Fig. 5A). A similar result was obtained when another fluorescent dye (SYBR Green) was used (data not shown). To obtain a more detailed fluorescent image, var2-1 leaflets showing variegation were fixed and thin sections were prepared by Technovit resin. Examination of sections after DAPI staining revealed that organelle nucleoids were organized differently between the two sectors (Fig. 5A).

To confirm whether the detected DAPI signals resulted from plastid nucleoids and to examine the plastid nucleoids in detail, we prepared protoplasts from var2-1/L12-GFP plants. The protoplasts were subjected to GFP detection, chlorophyll autofluorescence, and another DNA-specific fluorescent dye Hoechst 33342, which can stain DNA in living cells. Merged images of GFP and Hoechst 33342 signals showed that plastid nucleoids in the white cells were larger than those in green cells (Fig. 5B). Each nucleoid body in the green cells was detected as a small dot and single chloroplasts were observed to contain many nucleoids. Conversely, nucleoids in the white plastids consisted of a few large bodies that resembled those typically observed in undifferentiated plastids (Kuroiwa et al., 1981; Nemoto et al., 1988). Thus, the white sectors of var2 seem to contain cells with undifferentiated plastids throughout leaf development.

**DISCUSSION**

**Detailed Characterization of the White Sectors in var2**

Leaf variegation in var1 and var2 mutants, which is caused by the loss of FtsH2 (type B) or FtsH5 (type A), respectively, has been extensively studied by several laboratories. Characterization on two types of chloroplast FtsHs, which are encoded by duplicated genes and whose expression is coordinately regulated, implicated that overall FtsH levels correlate with the degree of white sectors. These observations led to the threshold model that explains leaf variegation (Yu et al., 2004 and see below). Although these studies highlighted a question regarding the variegation mechanism, the resulting white sectors were largely neglected and remained uncharacterized.

Therefore, in this study we dissected and compared the green and white sectors of var2 morphologically and biochemically. First, we assumed that the white sectors are made by living cells that possess distinct plastid structures. Collective observations from Evans blue staining, visualization of plastids by GFP, and the accumulation of proteins, mRNAs, and DNAs supported this assumption. Second, we attempted to clarify that the white sectors are neither the consequence of photobleaching, senescence, nor cell death. In contrast to the necrotic tissue in coronatine-treated acd2, white sectors were never stained by Evans blue and therefore exclude the possibility that white cells are associated with enhanced senescence. In addition, light has no effect on Evans blue stain. This result is consistent with the observation by Zaltsman et al. (2005a), where they demonstrated that the degree of white sectors is unaffected by light intensity and that the sector formation does not result from photobleaching. Thus, although plastids in the white cells apparently lack photosynthetic capacity, they otherwise lack any additional abnormalities.

It is noteworthy to recognize that photobleaching does not contribute to the formation of white sectors. We previously interpreted that the variegation of var2 was partly due to photoinhibition. This was concluded because FtsH plays a key role in the repair cycle of PSII
and particularly in the quality control of D1 protein (Lindahl et al., 2000; Sakamoto et al., 2002; Komenda et al., 2006). Without FtsH2, the var2 mutants become susceptible to high light and result in the decrease of PSII activity (Bailey et al., 2002; Sakamoto et al., 2004). Similar to this, certain photosynthesis mutants were known to be more susceptible to photoinhibition than wild type and the photoinhibition often leads to cell death (Havaux and Niyogi, 1999; Ifuku et al., 2005). The presented data in this study strongly suggest that the white sectors in var2 mutants differ from photobleaching in these mutants, and that the high light sensitivity of var2 is solely characteristic to green sectors. Thus, our observations support the conclusion by Zaltsman et al. (2005a) that the chloroplastic FtsHs are involved in two independent processes, namely, thylakoid differentiation and maintenance. However, whether this dual role of FtsH is completely separable or not remains unclear. For example, we observed that a suppressor of leaf variegation (fug1) mitigates high light sensitivity (Miura et al., 2007).

Formation of the White Sectors: An Integrated Model

Based on the proposed threshold model, the fate of chloroplast development in var2 may be explained as illustrated in Figure 6. Differentiation of plastids into chloroplasts is light dependent. At a particular stage of leaf development, plastids are cell-autonomously destined to permit the formation of thylakoids. If sufficient FtsHs are provided at this stage, then the plastids enter this process and become normal-appearing chloroplasts. In contrast, insufficient FtsHs at this stage prevent plastids from developing thylakoid membranes. As notified by Zaltsman et al. (2005a), the difference of the FtsH levels at this stage may be caused by noise in gene expression. Cells with the plastids that fail to develop thylakoids still undergo leaf development and consequently result in variegated sectors. In this model, the decisive role of FtsH must be executed at the particular stage. This is concluded since our observations of the white sectors and similar observations from other researchers demonstrate that the variegation pattern is irreversible once developed (Zaltsman et al., 2005a). Characterization of a rice virescent mutant that shows temperature-dependent irreversible chlorosis after a particular developmental stage is in good accordance with this notion (Kusumi et al., 2004; Sugimoto et al., 2004). Although the responsible genes for the chlorotic phenotype in this mutant are unrelated to FtsH, it again implies the important stage in leaf development.

Plastid nucleoids sometimes allow us to assess chloroplast status, because they vary in their size, number,
Figure 6. A possible mechanism of the white sector formation in var mutants. During leaf development, plastids in a leaf primordium (left) become plastids (middle) that are distinguishable in the distribution and number of nucleoids (circles). Differentiation of the plastid into the chloroplast accompanies thylakoid formation, which is light and FtsH dependent. At a certain stage, the level of the chloroplastic FtsH determines whether or not the plastid becomes competent for thylakoid formation. A sufficient level of FtsH allows the plastid to develop chloroplasts comparable to the wild type (bottom right). If not, the plastid becomes arrested at later developmental stages (top right). The undifferentiated plastid in the white sector sometimes contains vacuolated structures. [See online article for color version of this figure.]

and distribution in stroma in various developmental stages (Kuroiwa et al., 1981; Nemoto et al., 1988). A remarkable nucleoid structure detected in the white plastids, together with irregular plastid morphologies revealed by GFP, suggests that plastids in the white sectors are undifferentiated (Fig. 6). At an early stage of chloroplast formation from proplastids, nucleoids are large and attach to chloroplast envelope membranes, and the number of nucleoids increases. At a later developmental stage, the nucleoids attach to thylakoid membranes and constitute tight complexes with various proteins (for review, see Sato, 2001). In senescent leaves, nucleoids disappear in the initial stage of the chloroplast degradation process (Oldenburg and Bendich, 2004; Rowan et al., 2004). Although circumstantial, these results suggest that plastids from white sectors are different from known plastid types such as amyloplasts and etioplasts.

Possible Involvement of a Retrograde Signaling in the Sector Formation

We previously revealed that chloroplast transcripts (psbA, rbcL, and atpB) accumulate normally regardless of the degree of variegation (Takechi et al., 2000). A careful dissection of the white sectors in this study now confirms this fact for both Pep- and Nep-transcribed genes. In contrast to this, a sharp decrease was found in the expression of nuclear-encoded photosynthesis genes (rbcS and psbO). Our preliminary microarray analysis also implicated that genes involved in photosynthesis are down-regulated in the white sectors (Y. Kato and W. Sakamoto, unpublished data). Several explanations can be drawn from these results. First, plastids themselves in the white sectors retain their potential to develop thylakoid membranes. This suggests that the irreversible fate of the white sectors in var2 is controlled by nuclear genes. Second, a lack of photosynthetic proteins in the white plastids is likely under the posttranscriptional regulation that is known to play important roles in chloroplasts (Wollman et al., 1999). Finally, repression of nuclear genes in the white tissues is similar to that observed in norflurazon-treated leaves (Susek et al., 1993; Gray et al., 2003). This leads to an intriguing hypothesis that a retrograde signaling pathway is involved in the sector formation (Nott et al., 2006).

To further characterize the mechanism of leaf variegation, a molecule that accumulates preferentially in var2 needs to be identified. To date, a comparative analysis of protein profiles from green and white sectors failed to reveal any difference due to the normalization problem (as evidenced in Fig. 3 and unpublished data [Y. Kato and W. Sakamoto]). Additionally, the decisive role of FtsH in thylakoid formation also needs to be clarified. Although there are many causes for variegation, the physiological status in the white sectors may be similar to each other. Future studies on other variegated plants, as well as var2, may broaden our view on plastid differentiation.

MATERIALS AND METHODS

Plant Materials and Growth Condition

Arabidopsis (Arabidopsis thaliana) Col ecotype was used as a wild-type plant in this study. Unless specifically noted, plants were germinated and grown on 0.7% (w/v) agar plates containing Murashige and Skoog medium supplemented with Gamborg’s vitamins (Sigma-Aldrich) and 1.5% (w/v) Suc. Plants were maintained under 12-h light (approximately 60 μmol m⁻² s⁻¹) at a constant temperature of 22°C. For further analysis, plants were transferred to soil after 4 weeks and were maintained under 12-h light (100 μmol m⁻² s⁻¹) at a constant temperature of 22°C. A transgenic plant expressing a plastid-targeted GFP, L12-GFP, under the control of cauliflower mosaic virus 35S promoter, was kindly provided by Shinichi Ariamura (Ariamura et al., 1999). A transgenic plant expressing a mitochondria-targeted GFP (DIPS-GFP) under control of the 35S promoter was generated previously (Sakamoto and Hoshino, 2004). These transgenic plants were crossed with var2-1 and a resulting F2 individual exhibiting the variegated phenotype with GFP signals was selected. F2 plants were selfed and further analyses were conducted in the F3 progeny homozygous for the transgene. Seeds of var2-1 originated from the Arabidopsis Biological Resource Center and seeds for acl2-2 were kindly provided by Stephan Hortensteiner.

Evans Blue Staining

Excised leaflets from Col, var2-1, and acl2-2 mutants (6 weeks old under 12-h light, 100 μmol m⁻² s⁻¹) were vacuum infiltrated with 0.1% (w/v) Evans blue (purchased from Fluka) for 10 min. After staining, the leaves were washed three times with distilled water until they were fully decolorized. Leaves were subsequently observed with an Olympus SZ61 stereoscopic microscope. For the induction of cell death in acl2-2, 5 μL of 3 mM coronatine (Sigma-Aldrich) dissolved in methanol was spotted onto the leaf of acl2-2. Plants were maintained under 12-h light (100 μmol m⁻² s⁻¹) for 24 h after the induction of cell death.

Protoplast Preparation

Fully expanded leaves of Col and var2-1 (7–8 weeks old under 12-h light, 100 μmol m⁻² s⁻¹) were used for the preparation of protoplasts. Excised leaves were gently suspended in enzyme solution (0.1% [w/v] cellulase Onozuka R10 [Yakult], 0.05% [w/v] Pectolyase Y-23 [Kyowa Chemical Products], 400 mM mannitol, 10 mM CaCl₂, 20 mM KCl, 5 mM EGTA, 20 mM MES pH 5.5).
5.7). After incubation at room temperature for 1 h, protoplasts were collected by centrifugation at 60g for 1 min and washed with ice-cold wash buffer (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 2 mM MES pH 5.7). After centrifugation at 60g for 1 min, the isolated protoplasts were resuspended with 500 μL of wash buffer and used for microscopic observation.

Protein Extraction, SDS-PAGE, and Immunoblot Analysis
Leaflets were harvested from 6-week-old Col and var2-1 plants, and green and white sectors of var2-1 were dissected. Samples were immediately frozen in liquid nitrogen and pulverized with a microtome homogenizer. Total proteins were extracted by adding 10 times volume of extraction buffer (125 mM Tris-Cl pH 6.8, 2% [v/v] SDS, 5% [v/v] glycerol, 5% [v/v] 2-mercaptoethanol, 0.05% [w/v] bromo phenol blue). After centrifugation at 14,000g for 10 min, equally loaded supernatants (based on fresh weights) were subjected to SDS-PAGE analysis. The resolved gels were stained using the PlusOne Silver Staining kit, protein (GE Healthcare), according to the manufacturer’s instructions. For immunoblot analysis, total proteins were electroblotted onto polyvinylidene difluoride membrane (ATTO) after SDS-PAGE and membranes were blocked with 5% (w/v) skim milk in 50 mM sodium phosphate buffer (pH 7.5) containing 155 mM NaCl and 0.05% (v/v) Tween 20 (PBST buffer) for 1 h. After two washes with PBST buffer, the membranes were incubated with either anti-FtsH2 (dilution 1:5,000), anti-PsbO (dilution 1:5,000), anti-Lhcb1 (dilution 1:5,000), anti-D1 (dilution 1:5,000), anti-BiP (dilution 1:2,000; gift from Ikuko Hara-Nishimura; Hatano et al., 1997), anti-Phb5 (dilution 1:2,000; gift from Ikuko Hara-Nishimura; Hatano et al., 1997), or anti-tubulin (aC-18) antibodies (dilution 1:1,000; Santa Cruz). The membranes were incubated with secondary antibodies and detections were performed as previously described (Nakamoto et al., 2003). Relative amounts of signals from immunoblots were quantified by the NIH Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/), normalized by the signal from β-tubulin, and calibrated based upon the protein amount in the Col background. To isolate chloroplasts, 2 g of fully expanded leaves from 7- to 8-week-old Col and var2-1 were ground in a blender with homogenization buffer (330 mM mannitol, 50 mM HEPES, 2 mM NaCl, EDTA). Homogenates were then filtered through gauze and the recovered extracts were centrifuged at 600g for 2 min. The recovered pellet was resuspended in 1 mL of homogenization buffer and overlaid on three Percoll gradients (10%, 40%, 80% [v/v]) and centrifuged at 2,500g for 2 min. The recovered pellet was resuspended in 1 mL of homogenization buffer and overlaid on three Percoll gradients (10%, 40%, 80% [v/v]) and centrifuged at 2,500g for 15 min. Intact chloroplasts were recovered from the interface between the 40% and 80% gradient and used for further analysis. The 2-DE sample preparation of chloroplastic soluble proteins was carried out as described in Wang et al. (2006). Soluble proteins were isoelectric focused using immobiline DryStrips, pH 4 to 7 (GE Healthcare). Isoelectric focused samples were then subjected to SDS-PAGE analysis in a 10% polyacrylamide gel. Sample loadings were normalized relative to the amount of total chlorophyll.

RNA Isolation and Semiquantitative RT-PCR
Green and white sectors of var2-1 were dissected and total RNA was isolated from 4-week-old Col and var2-1 mutant reverse transcribed with the QIAGEN RNeasy Plant Mini kit. Semiquantitative RT-PCR assays with gene-specific primers were performed using total RNA (final concentration 2 ng μL−1 to each RT-PCR reaction) as a template according to the methods of Wang et al. (2006). Gene-specific primers and PCR reaction were described above.

DNA Staining and Microscopic Observation
Thin segments of leaves from approximately 6-week-old var2-1 mutants were fixed in 2.5% (v/v) glutaraldehyde and stained with 1 μg mL−1 DAPI to prepare Technovit sections. Thin segments of the leaf tissues were fixed in 45% (v/v) ethanol, 5% (v/v) acetic acid, and 1.8% (v/v) formaldehyde, dehydrated by a dilution series of ethanol and embedded in Technovit 7100 resin (Kulzer). Thin sections of leaves (3 μm) were subsequently stained with DAPI. We used the vital stain Hoechst 33342 (Invitrogen; final concentration 1 μg mL−1) for DNA staining in viable protoplast cells. Samples were visualized with an Olympus BX61 light microscope with a disc scan unit (Olympus) that was Col, and dissected green and white sectors of var2-1 were extracted in DNA extraction buffer (200 mM Tris-HCI, 250 mM NaCl, 25 mM EDTA, 5% [w/v] SDS, pH 7.5). Samples were subsequently centrifuged at 15,000g for 10 min and nucleic acids in the supernatant were precipitated by an equal volume of isopropanol. Semiquantitative PCR assays were performed using the isolated DNAs (final concentration 2 ng μL−1 to each PCR reaction) as the template. Gene-specific primers and PCR reaction were described above.

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

Supplemental Figure S1. Chloroplast protein profiles as studied by 2-DE analysis.

Supplemental Figure S2. Semiquantitative PCR analysis in leaf tissues of Col, and green and white sectors.

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