Leaf Variegation of Thylakoid Formation1 Is Suppressed by Mutations of Specific α-Factors in Arabidopsis

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Thylakoid Formation1 (THF1) has been shown to play roles in chloroplast development, resistance to excessive light, and chlorophyll degradation in Arabidopsis (Arabidopsis thaliana). To elucidate mechanisms underlying THF1-regulated chloroplast development, we mutagenized thf1 seeds with ethyl methanesulfonate and screened second-site recessive mutations that suppress its leaf variegation phenotype. Here, we characterized a unique suppressor line, 42-6, which displays a leaf virescent phenotype. Map-based cloning and genetic complementation results showed that thf1 variegation was suppressed by a mutation in α-FACTOr6 (SIG6), which is a plastid transcription factor specifically controlling gene expression through the plastid-encoded RNA polymerase. Northern-blot analysis revealed that plastid gene expression was down-regulated in not only 42-6 and sig6 but also, thf1 at the early stage of chloroplast development. Interestingly, mutations in SIG2 but not in other α-factors also suppressed thf1 leaf variegation. Furthermore, we found that leaf variegation of thf1 and var2 could be suppressed by several virescent mutations, including yellow seedling1, brz-insensitive-pale green2, and nitric oxide-associated protein1, indicating that virescent mutations suppress leaf variegation. Taken together, our results which might be triggered by defects in plastid gene transcription.

Chloroplast biogenesis is a key process for the transition of plant growth from heterotrophy to autotrophy. Chloroplasts can arise from proplastids or other various types of plastids, such as etioplasts and chromoplasts, under the light condition. It is well known that chloroplast development is controlled by the coordinated expression of the plastid and nuclear genes. Although significant progress has been made toward our understanding of molecular mechanisms on chloroplast development, it still remains largely unclear how plastid and nuclear gene expressions are coordinately regulated. Leaf-variegated mutants, which display green sectors and nongreen sectors in the same leaf, are recognized as an ideal system for investigating the mechanisms of chloroplast formation (Liu et al., 2010; Putarjunan et al., 2013). The prominent characteristic of variegation mutants is that the severity of leaf variegation depends on environmental and developmental cues. To date, a number of variegation mutants have been reported in Arabidopsis (Arabidopsis thaliana; for review, see Yu et al., 2007; Putarjunan et al., 2013). Among these mutants, variegated1 (var1), var2, and immutans (im) are well characterized. VAR1 and VAR2 encode Filamentation temperature-sensitive H5 (FtsH5) and FtsH2 subunits, respectively, of the FtsH metalloprotease complex, whereas IM encodes plastid terminal oxidase, which transfers electrons from the plastoquinol to molecular oxygen (Carol et al., 1999; Wu et al., 1999; Chen et al., 2000; Sakamoto et al., 2002). The FtsH protease composed of types A (FtsH1 and FtsH5/VAR1) and B (FtsH2/VAR2 and FtsH8) subunits plays an important role in degradation of the photodamaged D1 protein in the PSII repair cycle and probably acts as a molecular chaperone in chloroplasts as well (Bailey et al., 2002; Sakamoto et al., 2003). IM has been shown to be a versatile quinol oxidase that is involved in regulation of the plastoquinone pool and carotenoid biosynthesis (Wetzol et al., 1994; Wu et al., 1999; Aluru and Rodermel, 2004). Both ftsH2/var2 and im mutants display more severe leaf variegation in high light than in low light (Rosso et al., 2009). It seems that the leaf variegation phenotype is associated with the defect in biochemical functions of FtsH2 and IM in photoreaction processes, particularly with the activity of PSII. Wang et al. (2004) reported that loss of function of Thylakoid Formation1 (THF1), a homolog of cyano-bacterial photosystem I29 (psb29), led to leaf variegation in Arabidopsis. Our previous data revealed that the
thf1 variegation phenotype resulted from the reduced activity of FtsH protease (Zhang et al., 2009; Wu et al., 2013). How THF1 mutations down-regulate the level of FtsH protease remains unknown.

Suppressor screening in the var2 and thf1 genetic backgrounds has been shown to be a powerful strategy to elucidate the mechanism underlying variegated leaf formation, and a number of suppressor genes has been identified to date. These suppressors include recessive genes coding for Caseinolytic protease C1 (ClpC1), ClpC2, ClpR1, and ClpR4 subunits of chloroplast ClpP/R protease (Park and Rodermel, 2004; Yu et al., 2008; Wu et al., 2013), pseudouridine synthase (Suppressor of Variegation1 [SVR1]; Yu et al., 2008), plastid ribosomal proteins L11 (Wu et al., 2013) and L24 (SVR8; Liu et al., 2013), a chloroplast translation initiation factor2 (Fugari1 [FUG1]; Miura et al., 2007), a chloroplast translation elongation factor G (Snowy cotyledon1 [SCO1]; Miura et al., 2007), a type A-like translation elongation factor (SVR3; Liu et al., 2010a), a pentatricopeptide repeat protein (SVR7; Liu et al., 2010b), a functionally unknown protein localized in chloroplasts (SVR4; Yu et al., 2011), and chloroplast peptide deformylase (Peptide deformylase 1B [PDF1B]; Adam et al., 2011). All of these variegation suppressor proteins are localized in chloroplasts, and most of them function directly in the process of chloroplast gene expression, such as ribosomal RNA (rRNA) processing and protein translation. However, overexpressing FtsH8 and G protein α-subunit1 rescues the leaf variegation phenotype of var2 and thf1 mutants (Yu et al., 2004; Zhang et al., 2009). Based on these findings, a threshold level of FtsH protease is suggested to be required for normal chloroplast development (Chen et al., 2000; Yu et al., 2004). In the suppressor lines, it is plausible to assume that the FtsH threshold level needed for chloroplast development is lowered, because mutations impair chloroplast gene expression and subsequently, delay chloroplast development (Yu et al., 2008). However, not all mutants that are defective in chloroplast development are able to suppress leaf variegation, indicating that the suppression of leaf variegation is regulated in a complicated and specific manner (Liu et al., 2010c). This is consistent with the previously reported proteomic data showing that the proteome of the suppressor line is very similar to that of the single suppressor mutant, providing a systemic view on the mechanism of leaf variegation suppression (Wu et al., 2013).

In this study, we isolated another unique suppressor gene of thf1, namely α-factor6 (sig6). α-factors are bacterial transcription factors that bind to the core RNA polymerase and initiate RNA synthesis (Helmann and Chamberlin, 1988). Most promoters of housekeeping genes are recognized by the primary α-factor α20 in Escherichia coli. Consistently, plant factors have been reported to form a complex with plastid-encoded RNA polymerase (PEP) in vitro and in vivo (Troxler et al., 1994; Liu and Troxler, 1996; Homann and Link, 2003; Suzuki et al., 2004). The Arabidopsis genome encodes six plastid-localized α-factors (SIGs; SIG1–SIG6) that are clustered into the same superfamily of α20 and implement the same function as those in bacteria (Gruber and Gross, 2003; Lysenko, 2007). Physiological and genetic evidence revealed that different α-factors function redundantly and specifically to regulate plastid gene expression in Arabidopsis (Kanamur et al., 2001; Privat et al., 2003; Yao et al., 2003; Nagashima et al., 2004; Tsunoyama et al., 2004; Favory et al., 2005; Ishizaki et al., 2005; Loschelder et al., 2006; Zghidi et al., 2007). For example, loss of function of SIG2 or SIG6 resulted in slow chloroplast development because of inhibition of plastid gene expression, and the sig2 sig6 double mutant showed a very severe defect in chloroplast development and was unable to grow in nature (Shirano et al., 2000; Hanaoka et al., 2003; Ishizaki et al., 2005; Woodson et al., 2012). SIG5 was reported to control circadian rhythms of transcription of several chloroplast genes (Noordally et al., 2013). In addition, α-factors have been shown to play an important role in anterograde and retrograde signaling between the nucleus and plastids (Woodson et al., 2012; Oh and Montgomery, 2013). Thus, it is clear that α-factors play multiple roles in plastid gene expression and signal transduction in higher plants. Here, we showed that the thf1 variegation phenotype was suppressed by mutations in SIG2 or SIG6 but not in other SIGs, indicating the specificity of SIG genes in controlling chloroplast development.

RESULTS

SIG6 Mutations Suppress Leaf Variegation of thf1

To dissect the genetic network regulating the thf1-mediated chloroplast development, we mutagenized 2 mL of thf1 seeds with ethyl methanesulfonate and screened for suppressor lines that do not display the leaf variegation phenotype. In total, eight suppressor lines were isolated from 8,000 independent M2 lines. Here, we characterized the suppressor line 42-6. Compared with the wild type, thf1 produced variegated leaves, whereas the suppressor line 42-6 displayed a virescent phenotype in newly emerging leaves (Fig. 1A). Chlorophyll content analysis showed that thf1 and 42-6 mutants contained about 90% and 93%, respectively, of the wild type in mature leaves (Fig. 1B). The slow growth rate of thf1 was not altered in 42-6 (Fig. 1C). As previously reported, transmission electron microscopy analysis showed that stacked and stromal thylakoid membranes were observed in the green sector but not in the yellow/white sector of thf1 leaves (Fig. 1D). In addition, there was no difference in the ultrastructure of chloroplasts from 42-6 and wild-type mature leaves (Fig. 1D). These results reveal that the second mutation in 42-6 rescues chloroplast development in the yellow sectors of thf1.

To clone the suppressor gene, 42-6 was first backcrossed with thf1 for three generations. Genetic analysis showed that thf1 leaf variegation was suppressed by a single recessive gene in 42-6. Then, 42-6 was crossed with Landsberg erecta (Ler) to generate the F2 mapping population. Map-based cloning results...
revealed that the suppressor gene was located within the 94-kb region, which contains 19 annotated genes, between two simple sequence length polymorphism (SSLP) markers (T1J8 and T2N18) on chromosome II (Fig. 2A). We sequenced all of these genes and identified a single nucleotide substitution from G to A in the fifth exon of the gene locus AT2G36990, which encodes SIG6. The G-to-A mutation resulted in a genetic code switch from the codon of Trp to the stop codon (Fig. 2A). These results suggest that the function of SIG6 is disrupted in 42-6.

The single sig6 mutant was isolated from 42-6 and named sig6-3. The transcript of SIG6 was not detected in sig6-3 by northern-blot analysis with the genespecific probe (Fig. 2B), indicating that sig6-3 is a null mutant. As previously reported (Ishizaki et al., 2005; Woodson et al., 2012), sig6-3 cotyledons were yellow/white at the early growth stage but gradually turned to green 8 d after germination (Supplemental Fig. S1). In addition, the newly emerging leaf of sig6-3 showed a slightly virescent phenotype, which was observed in 42-6. These data suggest that sig6 is epistatic to thf1 with regard to chloroplast development.

To confirm the map-based cloning result, the genomic DNA of SIG6 driven by the cauliflower mosaic virus 35S promoter was transformed into 42-6. As shown in Figure 2C, overexpressing SIG6 in 42-6 resulted in leaf variegation, indicating that our map-based cloning result is correct. Taken together, our data reveal that sig6 is a suppressor for thf1 leaf variegation.

Hypersensitivity of thf1 to Excessive Light Is Not Suppressed by sig6

The thf1 mutant was reported to be hypersensitive to excessive light (Wang et al., 2004; Keren et al., 2005). To test whether sig6 was able to suppress the sensitivity of thf1 to high light, we examined the value of variable PSII fluorescence (Fv)/maximum PSII fluorescence (Fm) in the dark-adapted state, which indicates the maximum quantum yield of PSII photochemistry (PSII activity), using detached leaves of 30-d-old plants. The Fv/Fm value was reduced more dramatically in thf1 and 42-6 than in the wild type and sig6 in excessive light (Fig. 3). When the leaves were treated by high light for 4 h, PSII activity was almost undetectable in thf1 and 42-6, whereas it remained at one-half of the original value in the wild type and sig6. These data indicate that SIG6 mutations have no effect on plant sensitivity to excessive light. During the dark recovery period, the value of Fv/Fm was recovered much slower in thf1 and 42-6, whereas it remained at one-half of the original value in the wild type and sig6. These results imply that sig6 cannot suppress thf1 hypersensitivity to high light.

SIG6 Is Predominantly Expressed in Green Tissues

To profile SIG6 expression, the GUS gene driven by the SIG6 promoter was introduced into wild-type plants. GUS activity was analyzed using 4-d-old
Plastid Gene Expression Is Regulated Differentially by THF1 and SIG6

It is well known that σ-factors initiate gene transcription by recognizing a specific set of promoters for the PEP in higher plants (Isono et al., 1997; Fujiwara et al., 2000). To elucidate the mechanism by which chloroplast development in thf1 was rescued by sig6, we analyzed plastid gene expression in wild-type, thf1, sig6, and 42-6 seedlings at the early stage of chloroplast development. Our results showed that, in 4-d-old seedlings, transcript levels of the examined PEP-dependent genes were significantly lower in thf1 and sig6 than in the wild type (Fig. 5, A and B), whereas those of nuclear-encoded RNA polymerase (NEP)-dependent genes increased in thf1 and sig6 compared with the wild type (Fig. 5C). This result is consistent with the general observation that the inhibition of PEP-dependent gene expression promotes the expression of NEP-dependent genes (Kanamaru et al., 2001; Zhou et al., 2009; Yagi et al., 2012). Interestingly, transcript levels of the PEP-dependent genes accumulated quite similarly between thf1 and sig6 and apparently lower in 42-6 than in the thf1 and sig6 single mutants (Fig. 5; Supplemental Fig. S2), indicating that THF1 and SIG6 regulate PEP-dependent gene expression in a redundant manner. In 8-d-old seedlings, the difference in expression of the most plastid genes examined was small between the wild type and mutants, whereas expression of some genes including cytochrome b6 and glutamyl tRNA was obviously lower in 42-6 and sig6 than in the wild type and thf1. These results indicate that SIG6 has a more profound influence on plastid gene expression than THF1. In addition, we also examined expression of some nuclear genes with products that are targeted to chloroplasts. The mRNA levels of genes encoding light harvesting complex PSII subunit6, light harvesting complex PSI subunit1, and ribose bisphosphate carboxylase small chains were not apparently affected by

Figure 2. Identification of the suppressor gene sig6. A, Map-based cloning of the sig6 gene. The suppressor gene was mapped to the 94-kb region between two SSLP markers (T1J8 and T2N18) on chromosome II (Chr II). A substitution of G to A (red arrow) was identified in the gene At2g36990, which encodes SIG6. B, Northern-blot analysis of the transcript levels of SIG6 in 30-d-old wild-type (WT), thf1, 42-6, and sig6-3 plants. The level of rRNA was used as a loading control. The molecular weight of the SIG6 transcript is about 2 kb. C, Complementation of 42-6 by the SIG6 genomic DNA. Transgenic plants display the same leaf variegation as thf1.

Figure 3. Hypersensitivity of thf1 to high light is not rescued by sig6. The values of $F_v/F_m$ were measured in detached leaves from 30-d-old wild-type (WT), thf1, 42-6, and sig6 plants grown in soil under an approximately 100 μmol photons m$^{-2}$ s$^{-1}$ photoperiod (16-h-light/8-h-dark cycle) at 22°C. Error bars indicate ± so ($n=10$).
mutations of THF1 and SIG6 in 4- and 8-d-old seedlings compared with the wild type (Fig. 5D). Thus, our results suggest that plastid gene expression is regulated by THF1 and SIG6 in a redundant manner at the early stage of chloroplast development.

SIG2 Mutations Suppress thf1 Leaf Variegation

The Arabidopsis genome has six σ-factor genes: SIG1 to SIG6. To investigate whether other σ-factors are involved in suppressing thf1 leaf variegation, we identified other sig single mutants from the Arabidopsis Biological Resource Center seed stocks and made their double mutants with thf1 through a genetic cross. In agreement with previous reports (Shirano et al., 2000; Privat et al., 2003; Favory et al., 2005; Zghidi et al., 2007; Lai et al., 2011), sig2 displayed a pale-green phenotype, whereas sig1, sig3, sig4, and sig5 had no visible phenotypes (Fig. 6A). Except for sig2 thf1, other double mutants sig1 thf1, sig3 thf1, sig4 thf1, and sig5 thf1 still exhibited the leaf variegation phenotype (Fig. 6B). These results indicate that, other than sig6, a loss-of-function mutation sig2 can also suppress thf1 leaf variegation.

Virescent Mutants Suppress thf1 Variegation

Because both sig2 and sig6 mutants display the leaf virescent phenotype, we tested whether other virescent mutations suppress thf1 leaf variegation. To do this, we selected three virescent mutants (no-associated protein1 [noa1; Guo et al., 2003], yellow seedling1 [ys1; Zhou et al., 2009], and brz-insensitive-pale green2 [bpg2; Komatsu et al., 2010]) and constructed their double mutants with thf1. Similar to that previously reported, chloroplast development was slower in cotyledons and young leaves of 8-d-old ys1, noa1, and bpg2 seedlings compared with the wild type (Fig. 7A). In 30-d-old plants, the virescent phenotype was observed mainly in newly emerging noa1 and bpg2 leaves but not in ys1 leaves (Fig. 7A). The thf1 noa1, thf1 ys1, and thf1 bpg2 double mutants did not display variegated leaves (Fig. 7B), suggesting that leaf variegation can be suppressed by virescent mutations.

Leaf Variegation of var2 Is Suppressed by sig6 and ys1

To test whether suppressor genes for thf1 are functional in var2, we made var2 sig6 and var2 ys1 double mutants. As shown in Figure 8, both sig6 and ys1 were able to suppress var2 variegation, supporting our previous results that suppressor genes for thf1 variegation are functional in var2 and vice versa. These data also suggest that THF1 and VAR2 function in the same pathway to regulate chloroplast development.

DISCUSSION

In higher plants, plastid genes are transcribed by two different types of RNA polymerase, namely NEP and PEP. PEP is responsible for transcribing photosynthetic genes, such as psbA and psbD. Miura et al. (2010) reported that numerous genes related to photosynthesis and chloroplast functions were repressed in the white sectors of var2. A dramatic decrease in plastid gene expression was also observed in the etiolated seedlings of thf1 (Wu et al., 2011). It remains unclear whether leaf variegation is associated with the
defect in plastid gene expression. The genetic screen for suppressor genes of var2 and thf1 variegation revealed that chloroplast development is highly linked to several key biological processes in chloroplasts, such as protein quality control monitored by Clp protease, ribosomal assembly, and protein translation. The ultimate influence of these suppressors may be converged directly or indirectly on plastid gene expression. Although suppressor genes of leaf variegation have been shown to function in almost every step of plastid gene expression, not all genes involved in this process are able to suppress leaf variegation. Thus, it is possible that leaf variegation results from a specific defect in plastid gene expression.

In this study, we identified a unique suppressor gene sig6 for thf1-mediated leaf variegation. Consistent with previous reports (Ishizaki et al., 2005; Woodson et al., 2012), the identified sig6 mutant displays a clear virescent phenotype, particularly in cotyledons and newly emerging leaves. In Arabidopsis, σ-factors have been suggested to be localized in plastids (Isono et al., 1997; Fujiwara et al., 2000; Hara et al., 2001; Yao et al., 2003). Bioinformatic analysis showed that σ-factors are conserved only in the C-terminal region that is responsible for the promoter recognition, whereas the N-terminal region is highly variable both in length and sequence, indicating that individual plastid σ-factors have a distinctive role in temporally and spatially regulating plastid gene expression. Indeed, genetic analysis showed that SIG2 and SIG6, which function in a partially redundant manner, are essential for plastid gene expression during chloroplast development, whereas other σ-factors are involved in specific gene expression for maintaining photosynthetic efficiency under various conditions (Nagashima et al., 2004; Shimizu et al., 2010; Noordally et al., 2013). Consistently, our data showed that sig6 and sig2, which exhibit a virescent phenotype, but not sig1, sig3, sig4, and sig5, which have no obvious phenotype in chloroplast development, suppressed thf1 leaf variegation. Recently, our proteomic analysis revealed that the proteome of chloroplasts was more severely affected by virescent mutations than variegation mutations (Wu et al., 2013). Thus, it seems that plastid gene expression specifically regulated by SIG2 and SIG6 is related to leaf variegation.

Figure 5. Northern-blot analysis of gene expression in 4- and 8-d-old wild-type (WT), thf1, 42-6, and sig6 seedlings grown on one-half-strength MS supplemented with 1% (w/v) Suc under an approximately 100 μmol photons m⁻² s⁻¹ photoperiod (16-h-light/8-h-dark cycle) at 22°C. A, mRNA levels of the PEP-dependent, photosynthetic-related plastid genes. B, mRNA levels of the PEP-dependent plastid transfer RNA (tRNA) genes. C, mRNA levels of the NEP-dependent genes. D, mRNA levels of the nuclear-encoded genes. The level of 25S rRNA is shown as a loading control. The molecular weights of gene products are shown on the right. accD, Carboxyltransferase beta subunit; atpB, ATP synthase beta subunit; Lhca1, light harvesting complex PSI subunit1; Lhcb6, light harvesting complex PSII subunit6; petB, cytochrome b6; rbcL, ribulose 1,5-bisphosphate carboxylase/oxygenase; RbcS, ribose 1,5-bisphosphate carboxylase small chains; rpoB, RNA polymerase beta subunit; trnE, glutamic tRNA; trnQ, glutamine tRNA; trnW, tryptophan tRNA.

Figure 6. Mutations in SIG2 but not in other SIGs suppress thf1 leaf variegation. A, Phenotypes of 30-d-old wild-type (WT), thf1, sig1, sig2, sig3, sig4, and sig5 plants. B, Phenotypes of 30-d-old thf1 and its double mutants with sig1 to sig5. All plants were grown in soil under short-day (8-h-light/16-h-dark cycle) conditions with a light intensity of 100 μmol photons m⁻² s⁻¹.
To date, a number of recessive genes has been reported to suppress var2 leaf variegation (Park and Rodermel, 2004; Mirua et al., 2007; Yu et al., 2008, 2011; Liu et al., 2010a, 2010b; Adam et al., 2011; Wu et al., 2013). It is evident that the majority of them are of a virescent leaf phenotype. These mutants include clpC1, clpR1, fug, sbo1, pdf1b, svr3, svr4, and svr7 (Miura et al., 2007; Yu et al., 2008, 2011; Liu et al., 2010a, 2010b; Adam et al., 2011). This raises an interesting question of whether all virescent mutants are able to suppress leaf variegation. In this study, we tested three reported virescent mutants, namely noa1, bpg2, and ys1. Genetic analysis showed that noa1, bpg2, and ys1 were epistatic to thf1. Additional investigation is required to make such a conclusion.

How virescent mutants suppress leaf variegation is a fascinating question. According to the threshold model of FtsH protease, which has gained molecular and genetic evidence to explain the heterogeneity of chloroplast development (Yu et al., 2004), either an increase in the FtsH level or a decrease in the threshold of the FtsH level is able to suppress leaf variegation. It was proposed that the threshold of FtsH protease is reduced, because the period of chloroplast development in virescent mutants is extended (Yu et al., 2007; Liu et al., 2010c). However, proteomic and western-blot assays revealed that the level of FtsH protease in the suppressor line (thf1 clpR4) was actually higher than that in the variegation mutant thf1 (Wu et al., 2013). Up-regulation of FtsH levels by suppressor genes can take place at a transcriptional and/or posttranscriptional level, such as by increasing FtsH gene expression, preprotein import into plastids, and subsequent protein folding and complex assembly (Wu et al., 2013). In this study, we found that transcriptional levels of PEP-dependent genes were much lower in the suppressor line 42-6 than in the single thf1 and sig6 mutants at the early stage of chloroplast development, providing molecular evidence to support that plastid development is delayed in the suppressor line of leaf variegation. Again, western-blot analysis showed that the level of the VAR1 subunit of FtsH protease was higher in the suppressor lines, including 42-6, thf1 ys1, and thf1 noa1, than in thf1 (Supplemental Fig. S3). Thus, FtsH protease may no longer be a limiting factor for chloroplast development in suppressor lines. Interestingly, both SIG2 and SIG6 have been reported to be involved in retrograde signaling from chloroplasts to the nucleus (Woodson et al., 2012). It is possible that retrograde signaling is involved in suppression of leaf variegation.

The nature for FtsH protease to regulate chloroplast biogenesis still remains unknown. The best characterized function of FtsH protease is in degradation of the photodamaged D1 protein during PSII repair (Lindahl et al., 2000; Bailey et al., 2002; Kato and Sakamoto, 2009). The extent of variegation was positively correlated with an increase in excitation pressure (Rosso et al., 2009), suggesting that loss of function of VAR2 leads to chloroplast photooxidation and subsequently, impairs chloroplast biogenesis. The controversy is that a defect in D1 degradation does not necessarily lead to leaf variegation (Miura et al., 2007), and prolamellar body formation of etioplasts is severely inhibited when var2 is grown in the dark (Wu et al., 2013). This evidence indicates that the role of VAR2 in D1 degradation is not directly associated with early chloroplast development. In addition, it is suggested that the role of FtsH protease in vesicle fusion and/or maintaining thylakoid membranes might be involved in chloroplast biogenesis (Hugueney et al., 1995; Kato et al., 2012). Considering that leaf variegation of thf1 is attributed to a significant decrease in FtsH protease activity (Zhang et al., 2009) and that the identified genes suppressing leaf variegation of thf1 and var2 can be mutually replaced (Wu et al., 2013), molecular mechanisms for leaf variegation formation will be focused on how VAR2 is involved in thylakoid membrane formation in the future.
Materials and Methods

Plant Material and Growth Conditions

All experiments were performed with Arabidopsis (Arabidopsis thaliana) ecotypes Columbia-0 and Ler. Seeds were germinated and grown on 0.7% (w/v) agar plates containing one-half-strength Murashige and Skoog medium (MS) with 1% (w/v) Suc. For additional analysis, plants were transferred to soil and maintained under the short-day (8 h of light/16 h of dark) or long-day (16 h of light/8 h of dark) conditions, with a light intensity of 100 μmol photons m⁻² s⁻¹ at 21°C. All of the primers used in this study are listed in Supplemental Table S1. The transfer DNA insertion mutant of sig4 (Salk_146777) was identified from the Arabidopsis Biological Resource Center seed stocks. Other single mutants used in this study were previously described: thf1 (Wang et al., 2004), ysl1 (Zhou et al., 2009), bg2 (Komatsu et al., 2010), noa1/noa1 (Guo et al., 2003), sig1 (Lai et al., 2011), sig2 (Oh and Montgomery, 2013), sig3 (Zhigidi et al., 2007), and sig5 (Tsunoyama et al., 2004).

Map-Based Cloning

Suppressors were identified by screening M2 seeds that were mutagenized by 0.1% (w/v) ethyl methanesulfonate in the thf1 genetic background. The suppressor line 42-6 was crossed to Ler, and the F2 seedlings were used as mapping populations. The mutation gene was mapped using SSLP markers, and information is available at The Arabidopsis Information Resource (http://www.arabidopsis.org).

Plasmid Construction and Transformation

Plasmids were constructed using the Gateway Cloning System (Invitrogen). PCR products were cloned into AtSIG6 genomic and promoter regions were cloned into the pENTRY SD/D-TOPO entry vector with gene-specific primers. The genomic fragment of SIG6 was then recombined into the pGW2B destination vector, whereas the promoter fragment was recombined into the pGWBD destination vector (Research Institute of Molecular Genetics, Shimane University). The constructs were then transformed into the Agrobacterium tumefaciens GV3101 strain and introduced into Arabidopsis plants by the floral dip method (Clough and Bent, 1998). Transformants were selected on one-half-strength MS plates containing 50 mg L⁻¹ kanamycin. Transgenic lines were confirmed by reverse transcription PCR or PCR using gene-specific primers.

Transmission Electron Microscopy

Samples were obtained from the leaves of 30-d-old plants grown in short-day (8 h of light/16 h of dark) conditions. The samples were fixed in 4% (w/v) glutaraldehyde, stained, and examined as described by Harris et al. (1994).

Gene Expression Analysis

Total RNA was isolated using the RNAgents Total RNA Isolation System (Promega) in accordance with the manufacturer’s instructions; 0.5 μg of total RNA was added to 10 μL of reverse transcription reaction, and 1 μL of complementary DNA was used as template for reverse transcription PCR. Northern-blot analysis was performed using gene-specific probes as described by Huang et al. (2000). Five to ten micrograms of total RNA was separated by denaturing agarose gel electrophoresis.

Chlorophyll Analysis and Chlorophyll Fluorescence Analysis

Chlorophyll was extracted from samples that were kept in 75% (v/v) acetone at 4°C for 24 h. Total chlorophyll content and chlorophyll a/b ratios were calculated according to Lichtenthaler, 1987. Chlorophyll fluorescence was measured with an Imaging PAM 101 (Wartz, and Fv/Fm values were calculated according to the manufacturer’s instructions. Before the measurement, plants were maintained in the dark for 10 min, and the initial Fv/Fm values were recorded. Plants were then exposed to high light (800 photons m⁻² s⁻¹) for 4 h using a halogen light source.

Data from this article can be found in the Arabidopsis Genome Initiative under accession numbers TFH1, AT2g20890l; YSI, AT3g22660; NOA1/NOA1, AT; 3g47450; BPG2, AT3g57180l; SIG1, AT1g64860; SIG2, AT1g28540; SIG3, AT3g53920; SIG4, AT5g13730; SIG5, AT5g24120; and SIG6, AT2g36990.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phenotypes of wild-type, thf1, 42-6, and sig6-3 cotyledons.

Supplemental Figure S2. Quantification of the northern-blot results in Figure 5.

Supplemental Figure S3. Western-blot analysis of VAR1 content in wild-type, thf1, 42-6, sig6, thf1ypl1, ysl1, ysl1/noa1, and noa1 leaves.

Supplemental Table S1. List of the primers used in this study.

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Leaf Variegation and Plastid Gene Expression


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