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Leaf Variegation of thylakoid formation\textsuperscript{1} is Suppressed by Mutations of Specific Sigma Factors in \textit{Arabidopsis}

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One-sentence Summary:

Loss-of-function mutations in the specific plastidic transcriptional factors, Sig6 and Sig2, suppress \textit{thf1} and \textit{var2} leaf variegation in \textit{Arabidopsis}.
Footnotes:

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Thylakoid Formation1 (THF1) has been demonstrated to play roles in chloroplast development, resistance to excessive light and chlorophyll degradation in Arabidopsis. 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 new 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 SIGMA FACTOR6 (SIG6), which is a plastid transcription factor specifically controlling gene expression via the plastid-encoded RNA polymerase. Northern blot analysis revealed that plastid gene expression was down-regulated not only in 42-6 and sig6 but also in thf1 at the early stage of chloroplast development. Interestingly, mutations in SIG2 but not in other sigma 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 provide new insights into thf1-mediated leaf variegation, which might be triggered by defects in plastid gene transcription.
INTRODUCTION

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 expression are coordinately regulated. Leaf-variegated mutants, which display green sectors and non-green sectors in the same leaf, are recognized as an ideal system for investigating the mechanisms of chloroplast formation (Liu et al., 2010b; 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 (reviewed by Yu et al., 2007; Putarjunan et al., 2013). Among these mutants, variegated1 (var1), var2 and immutans (im) are well characterized. VAR1 and VAR2 encode FtsH5 and FtsH2 subunits of the FtsH metalloprotease complex, respectively, whereas IM encodes plastid terminal oxidase (PTOX), 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 type A (FtsH1 and FtsH5/VAR1) and type 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 molecular chaperones in chloroplasts too (Bailey et al., 2002; Sakamoto et al., 2003). IM has been demonstrated to be a versatile quinol oxidase that is involved in regulation of the plastoquinone pool and carotenoid biosynthesis (Wetzel et al., 1994; Wu et al., 1999; Aluru et al., 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 photosystem II (PSII). Wang et al.
(2004) reported that loss-of-function of *Thylakoid Formation1* (*THF1*), a
homolog of cyanobacterial *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
demonstrated to be a powerful strategy to elucidate the mechanism underlying
variegated-leaf formation, and a number of suppressor genes have been
identified to date. These suppressors include recessive genes coding for
ClpC1, ClpC2, ClpR1 and ClpR4 subunits of chloroplast ClpP/R protease
(Park and Rödermel, 2004; Yu et al., 2008; Wu et al., 2013), pseudouridine
synthase (*SVR1*) (Yu et al., 2008), plastid ribosomal proteins L11 (*PRPL11*)
(Wu et al., 2013) and L24 (*SVR8*) (Liu et al., 2013), a chloroplast translation
initiation factor 2 (*FUG1*) (Miura et al., 2007), a chloroplast translation
elongation factor G (*SCO1*) (Miura et al., 2007), a TypA-like translation
elongation factor (*SVR3*) (Liu et al., 2010a), a pentatricopeptide repeat protein
(*SVR7*) (Liu et al., 2010c), a functionally unknown protein localized in
chloroplasts (*SVR4*) (Yu et al., 2011), and chloroplast peptide deformylase
(*PDF1B*) (Adam et al., 2011). All these variegation suppressor proteins are
localized in chloroplasts, and most of them function directly in the process of
chloroplast gene expression, such as rRNA processing and protein translation.
On the other hand, overexpressing *FtsH8* and *GPA1* (the G-protein alpha
subunit) 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 since 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., 2010b). 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 new suppressor gene of thf1, namely
sigma factor6 (sig6). Sigma factors are bacterial transcription factors that bind
to the core RNA polymerase and initiate RNA synthesis (Helmann et al., 1988).
Most promoters of housekeeping genes are recognized by the primary sigma
factor σ70 in E. coli. Consistently, plant sigma 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
sigma factors (SIG1-SIG6) that are clustered into the same superfamily of σ70
and implement the same function as those in bacteria (Gruber et al., 2003;
Lysenko, 2007). Physiological and genetic evidence revealed that different
sigma factors function redundantly and specifically to regulate plastid gene
expression in Arabidopsis (Kanamaru 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 due
to inhibition of plastid gene expression, and the sig2 sig6 double mutant
showed 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., 2013). SIG5 was reported to control circadian rhythms of
transcription of several chloroplast genes (Noordally et al., 2013). In addition,
sigma factors have been demonstrated to play an important role in
anterograde and retrograde signaling between the nucleus and plastids
(Woodson et al., 2013; Oh and Montgomery, 2014). Thus, it is clear that sigma factors play multiple roles in plastid gene expression and signal transduction in higher plants. Here, we showed that the \textit{thf1} variegation phenotype was suppressed by mutations in \textit{SIG2} or \textit{SIG6} but not in other \textit{SIGs}, indicating of the specificity of \textit{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 (EMS) and screened for suppressor lines that do not display the leaf variegation phenotype. A total of eight suppressor lines were isolated from 8000 independent M2 lines. Here, we characterized a suppressor line 42-6. Compared to the wild type (WT), 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% of WT 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 (TEM) 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 WT 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 these genes and identified a single nucleotide substitution from G to A in the fifth exon of the gene locus AT2G36990, which encodes SIGMA FACTOR6 (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 was named sig6-3. The transcript of SIG6 was not detected in sig6-3 by Northern blot analysis with the gene specific probe (Fig. 2B), indicating that sig6-3 is a null mutant. As previously reported (Ishizaki et al., 2005; Woodson et al., 2013), sig6-3 cotyledons were yellow/white at the early growth stage, but gradually turned into green 8 days after germination (Supplemental Fig. S1). In addition, the newly emerging leaf of sig6-3 showed a slightly virescent phenotype as 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 hypersensitivity to excessive light (Wang et al., 2004; Keren et al., 2009). To test whether sig6 was able to suppress the sensitivity of thf1 to high light, we examined the value of Fv/Fm, which indicates the maximum quantum yield of PSII photochemistry (PSII activity), using detached leaves of 30-day-old plants. The Fv/Fm value was reduced more dramatically in thf1 and 42-6 than in WT 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 while remained half of the original value in WT 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 than in WT and sig6, whereas there was no statistically significant difference in the recovery of Fv/Fm between 42-6 and thf1. Thus, these results imply that sig6 cannot suppress thf1 hypersensitivity to high light.
**SIG6 is Predominantly Expressed in Green Tissues**

To profile SIG6 expression, the β-glucuronidase (GUS) gene driven by the SIG6 promoter was introduced into WT plants. GUS activity was analyzed using 4-day-old seedlings grown in the dark or light and 8-week-old mature plants. Our results showed that the activity of the SIG6 promoter was high in all green tissues, such as young seedlings, leaves, flowers and siliques, but not in non-photosynthetic roots (Fig. 4A-C). By contrast, GUS activity was detected only in the cotyledon from etiolated seedlings (Fig. 4D). We also examined the expression of SIG6 in various tissues or under different stresses using Northern blot analysis with specific probes against SIG6. Consistent with the results of GUS analysis, SIG6 mRNA accumulated abundantly in all tissues except for the root (Fig. 4E). SIG6 expression was induced moderately by NaCl and cold stresses but significantly suppressed by ABA (Fig. 4F). Thus, our data suggest that SIG6 is predominantly expressed in photosynthetically active tissues, and can be up- or down-regulated by different stimuli.

**Plastid Gene Expression is Regulated Differentially by THF1 and SIG6**

It is well known that sigma factors initiate gene transcription via recognizing a specific set of promoters for the plastid-encoding RNA polymerase (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 WT, thf1, sig6 and 42-6 seedlings at the early stage of chloroplast development. Our results showed that in 4-day-old seedlings transcript levels of the examined PEP-dependent genes were significantly lower in thf1 and sig6 than in WT (Fig. 5A and 5B), whereas those of NEP-dependent genes increased in thf1 and sig6 compared to the WT (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 (Zhou et al., 2009; Kanamaru et al., 2001; 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 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-day-old seedlings, the difference in expression of the most plastid genes examined was small between WT and mutants, whereas expression of some genes (petB and trnQ) was obviously lower in 42-6 and sig6 than in WT 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 whose products are targeted to chloroplasts. The mRNA levels of genes encoding the light harvesting complex photosystem II subunit 6 (Lhcb6), light harvesting complex photosystem I subunit 1 (Lhca1) and ribose bisphosphate carboxylase small chains (RbcS) were not apparently affected by mutations of THF1 and SIG6 in 4- and 8-day-old seedlings, compared to WT (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 sigma factor genes, SIG1-SIG6. To investigate whether other sigma factors is involved in suppressing thf1 leaf variegation, we identified other sig single mutants from the ABRC seed stocks and made their double mutants with thf1 via 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, while 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 beside sig6 a loss-of-function mutation sig2 can also suppress thf1 leaf variegation.
Virescent Mutants Suppress \textit{thf1} Variegation

Since both \textit{sig2} and \textit{sig6} mutants display the leaf virescent phenotype, we tested whether other virescent mutations suppress \textit{thf1} leaf variegation. To do this, we selected three virescent mutants, \textit{no-associated protein 1 (noa1)} (Guo et al., 2003), \textit{yellow seedling 1 (ys1)} (Zhou et al., 2009) and \textit{brz-insensitive-pale green 2 (bpg2)} (Komastu et al., 2010), and constructed their double mutants with \textit{thf1}. Similar to previously reported, chloroplast development was slower in cotyledons and young leaves of 8-day-old \textit{ys1}, \textit{noa1} and \textit{bpg2} seedlings compared to WT (Fig 7A). In 30-day-old plants, the virescent phenotype was observed mainly in newly emerging \textit{noa1} and \textit{bpg2} leaves but not in the \textit{ys1} leaves (Fig. 7A). The \textit{thf1 noa1}, \textit{thf1 ys1} and \textit{thf1 bpg2} double mutants did not display variegated leaves (Fig. 7B), suggesting that leaf variegation can be suppressed by virescent mutations.

Leaf Variegation of \textit{var2} is Suppressed by \textit{sig6} and \textit{ys1}

To test whether suppressor genes for \textit{thf1} is functional in \textit{var2}, we made \textit{var2 sig6} and \textit{var2 ys1} double mutants. As shown in Figure 8, both \textit{sig6} and \textit{ys1} were able to suppress \textit{var2} variegation, supporting our previous results that suppressor genes for \textit{thf1} variegation are functional in \textit{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 demonstrated to function almost in 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 new suppressor gene sig6 for thf1-mediated leaf variegation. Consistent with previous reports (Ishizaki et al., 2005; Woodson et al., 2013), the identified sig6 mutant displays a clear virescent phenotype, particularly in cotyledons and newly emerging leaves. In Arabidopsis, sigma factors have been suggested to be localized in plastids (Isono et al., 1997; Fujiwara et al., 2001; Hara et al., 2001; Yao et al., 2003). Bioinformatic analysis showed that sigma 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 sigma factors have a distinctive role in temporally and spatially regulating plastid gene expression. Indeed, genetic analysis demonstrated that SIG2 and SIG6 that function in a partially redundant manner
are essential for plastid gene expression during chloroplast development while other sigma 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 that exhibit a virescent phenotype but not sig1, sig3, sig4 and sig5 that 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.

To date, a number of recessive genes have been reported to suppress var2 leaf variegation (Park and Rodermel, 2004; Mirua et al., 2007; Yu et al., 2008; Liu et al., 2010a; Liu et al., 2010c; Adam et al., 2011; Yu 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, sco1, pdf1b, svr3, svr4 and svr7 (Miura et al., 2007; Yu et al., 2008; Liu et al., 2010a; Liu et al., 2010c; Adam et al., 2011; Yu et al., 2011). This raises an interesting question whether all virescent mutants are able to suppress leaf variegation. In the present study, we tested three reported virescent mutants, namely noa1, bpg2 and ys1. Genetic analysis showed that noa1, bpg2 and ys1 were epistatic to thf1. Further 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 since the period of chloroplast development in virescent mutants is extended (Yu et al., 2007; Liu et al., 2010b). 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 via 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, thf1ys1 and thf1noa1 than in thf1 (Supplemental Fig. S3). Thus, FtsH protease may be no longer 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., 2013). 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 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.
METHODS

Plant Material and Growth Conditions
All experiments were performed with *Arabidopsis thaliana* ecotypes Columbia (Col-0) and Landsberg erecta (Ler). Seeds were germinated and grown on 0.7% (w/v) agar plates containing half MS with 1% sucrose. For further analysis, plants were transferred to soil and maintained under the short day condition (8 h light/16 h dark) or long day condition (16 h light/8 h dark) with light intensity of 100 μmol photons m⁻² sec⁻¹ at 21 ℃. All of the primers used in this study are listed in Supplemental Table online. The T-DNA insertion mutant of *sig4* (Salk_146777) was identified from the ABRC seed stocks. Other single mutants used in this study were previously described: *thf1* (Wang *et al*., 2004), *ys1* (Zhou *et al*., 2009), *bpg2* (Komastu *et al*., 2010), *noa1/nos1* (Guo *et al*., 2003), *sig1* (Lai *et al*., 2011), *sig2* (Oh and Montgomerz, 2014), *sig3* (Zghidi *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% 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 simple sequence length polymorphism (SSLP) markers, whose information is available at The Arabidopsis Information Resource (TAIR: [http://www.arabidopsis.org](http://www.arabidopsis.org)).

Plasmid Construction and Transformation
Plasmids were constructed using the Gateway cloning system (Invitrogen). PCR products from *AtSIG6* genomic and promoter regions were cloned into the pENTR SD/D-TOPO entry vector with gene-specific primers. The genomic fragment of *SIG6* was then recombined into the pGWB2 destination vector while the promoter fragment was recombined into the pGWB3 destination.
vector (Research Institute of Molecular Genetics, Shimane University, Japan). 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 1/2 MS plates containing 50 mg/L kanamycin. Transgenic lines were confirmed by RT-PCR or PCR using gene specific primers.

**Transmission Electron Microscopy**

Samples were obtained from the leaves of 30-day-old plants grown in short-day conditions (8 h light/16 h night). The samples were fixed in a solution of 4% glutaraldehyde, then 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 manufacture’s instructions. Point five μg total RNA was added to 10 μL of reverse transcription reaction, and 1 μL of the cDNA was used as template for RT-PCR. Northern blot analysis was performed using gene specific probes as described by Huang et al. (2000). Five to ten μg of total RNA was separated by denaturing agarose gel electrophoresis.

**Chlorophyll Analysis and Chlorophyll Fluorescence Analysis**

Chlorophyll were extracted samples with 75% acetone keeping at 4 °C for 24 h. Total chlorophyll content and chlorophyll a/b ratios were calculated according to Lichtenthaler (1987). Chl fluorescence was measured with an Imaging PAM 101 (Watzs, Germany), and Fv/Fm values were calculated according to the manufacturer’s instructions. Prior to the measurement, plants were maintained in the dark for 10 min, and the initial Fv/Fm values were recorded based on measuring Fm and Fo. Plants were then exposed to high light (800 photons m⁻²
sec\(^{-1}\)) for 4 h using a halogen light source.

**Accession Numbers**

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: *THF1*, At2g20890; *YS1*, At3g22690; *NOA1/NOS1*, At3g47450; *BPG2*, At3g57180; *SIG1*, At1g64860; *SIG2*, At1g08540; *SIG3*, At3g53920; *SIG4*, At5g13730; *SIG5*, At5g24120; *SIG6*, At2g36990.

**SUPPLEMENTAL DATA**

**Supplemental Figure S1.** Phenotypes of WT, *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 WT, *thf1*, 42-6, *sig6*, *thf1ys1*, *ys1*, *thf1noa1*, and *noa1* leaves.

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Figure 1. Characterization of 42-6, one of the suppressor lines for thf1 leaf variegation. (A) Phenotypes of 30-day-old WT, thf1 and 42-6 plants grown under 8 h light/16 h dark conditions with light intensity of 100 μmol photons m$^{-2}$ sec$^{-1}$ at 22 °C. Bar = 1 cm. The two magnified images showed variegation of the thf1 leaf marked by the rectangle and virescence of 42-6 new leaves marked by the square, respectively. (B) Chlorophyll content of leaves from 30-day-old WT, thf1 and 42-6 plants shown in (A). Error bars indicate ± SD (n = 3). (C) Leaf number of 60-day-old of WT, thf1 and 42-6 grown under 8 h light/16 h dark conditions. Error bars indicate ± SD (n = 10). Asterisks indicate significant differences in leaf number between the WT and mutant plants (p < 0.001, Student’s t test). (D) Ultrastructure of chloroplasts from mature leaves of 30-day-old WT, thf1 white sectors, thf1 green sectors and 42-6 plants. Bar = 1 μm.
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 simple sequence length polymorphism (SSLP) markers, T1J8 and T2N18 on chromosome 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-day-old 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 Fv/Fm were measured in detached leaves from 30-day-old WT, thf1, 42-6 and sig6. Error bars indicate the standard deviation (n = 10).
**Figure 4.** Expression patterns of AtS/G6. (A-D) Analysis of GUS activity driven by the native S/G6 gene promoter in 4-day-old seedlings (A), leaves (B), flowers (C) and 4-day-old dark grown seedling (D). (E) Northern blot analysis of S/G6 transcripts (2.0 kb) in various tissues from 8-day-old seedlings and 8-week-old plants. The level of 25S rRNA was shown as a loading control. (F) Northern blot analysis of S/G6 transcripts (2.0 kb). Eight-day-old seedlings were treated with deficiency of phosphate (-P), 50 μM ABA, 100 mM NaCl, 10 polyethyleneglycol 6000 (PEG), 100 mM H₂O₂ and 6 °C (cold) for 12 hours. CK, without treatment. The level of 25S rRNA was shown as a loading control.
Figure 5. Northern blot analysis of gene expression in 4- and 8-day-old WT, thf1, 42-6 and sig6 seedlings grown on half MS media supplemented with 1% sucrose under an approximately 100 μmol photons m^{-2} sec^{-1} photo-period (16 h light/8 h dark) at 22 °C. (A) mRNA levels of the PEP-dependent photosynthetic-related plastid genes. (B) mRNA levels of the PEP-dependent plastid tRNA genes. (C) mRNA levels of the NEP-dependent genes. (D) mRNA levels of the nuclear encoded genes. The level of 25S rRNA was shown as a loading control. The molecular weight of gene products was shown on the right side of each panel.
Figure 6. Mutations in SIG2 but not other SIGs suppress thf1 leaf variegation. (A) Phenotypes of 30-day-old WT, thf1, sig1, sig2, sig3, sig4 and sig5 plants. (B) Phenotypes of 30-day-old thf1 and its double mutants with sig1-sig5. All plants were grown in soil under short day (8 h light/16 h dark) conditions with
Figure 7. Leaf variegation of *thf1* is suppressed by virescent mutants. (A) The virescent phenotype of 8-day-old (upper panel) and 30-day-old (lower panel) yellow seedling 1 (*ys1*), no-associated protein 1 (*noa1*) and brz-insensitive-pale green 2 (*bpg2*) plants, compared to WT. (B) Phenotypes of 30-day-old *thf1, thf1 noa1, thf1 ys1* and *thf1 bpg2* plants grown in soil under short day (8 h light/16 h dark) conditions with light intensity of 100 μmol photons m⁻² sec⁻¹ at 22 °C. Bar = 1 cm. Red arrows indicate the yellow sectors of *thf1* leaves.
Figure 8. Leaf variegation of var2 is suppressed by sig6 and ys1. Phenotypes of 30-day-old var2, var2 sig6 and var2 ys1 plants grown in soil under short day (8 h light/16 h dark) conditions with light intensity of 100 μmol photons m$^{-2}$ sec$^{-1}$ at 22 °C. Bar = 1 cm.