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ORIGINAL ARTICLE

Mutations in circularly permuted GTPase family genes *AtNOA1/RIF1/SVR10* and *BPG2* suppress *var2*-mediated leaf variegation in *Arabidopsis thaliana*

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Abstract Leaf variegation mutants constitute a unique group of chloroplast development mutants and are ideal genetic materials to dissect the regulation of chloroplast development. We have utilized the Arabidopsis *yellow variegated* (*var2*) mutant and genetic suppressor analysis to probe the mechanisms of chloroplast development. Here we report the isolation of a new *var2* suppressor locus *SUPPRESSOR OF VARIEGATION* (*SVR10*). Genetic mapping and molecular complementation indicated that *SVR10* encodes a circularly permuted GTPase that has been reported as *Arabidopsis thaliana* NITRIC OXIDE ASSOCIATED 1 (AtNOA1) and RESISTANT TO INHIBITION BY FOSMIDOMYCIN 1 (RIF1). Biochemical evidence showed that SVR10/AtNOA1/RIF1 likely localizes to the chloroplast stroma. We further demonstrate that the mutant of a close homologue of *SVR10/AtNOA1/RIF1*, *BRASSINAZOLE INSENSITIVE PALE GREEN 2* (*BPG2*), can also suppress *var2* leaf variegation. Mutants of *SVR10* and *BPG2* are impaired in photosynthesis and the accumulation of chloroplast proteins. Interestingly, two-dimensional blue native gel analysis showed that mutants of *SVR10* and *BPG2* display defects in the assembly of thylakoid membrane complexes including reduced levels of major photosynthetic complexes and the abnormal accumulation of a chlorophyll-protein supercomplex containing photosystem I. Taken together, our findings suggest that SVR10 and BPG2 are functionally related with VAR2, likely through their potential roles in regulating chloroplast protein homeostasis, and both SVR10 and BPG2 are required for efficient thylakoid protein complex assembly and photosynthesis.

Keywords Chloroplast development · Leaf variegation · VAR2 · Genetic suppressor · SVR10/AtNOA1/RIF1

Introduction

Chloroplasts perform an array of essential functions including photosynthesis and the biosynthesis of myriads of regulatory and metabolic molecules such as phytohormones, amino acids, and lipids (Sakamoto et al. 2008). To facilitate these critical functions, higher plants devote a significant portion of their proteomes to the chloroplast. Different estimates put the chloroplast proteome at about 3000 proteins and the vast majority of these proteins are products of nuclear genes for chloroplast proteins (NGCPs) (Leister 2003; Jarvis and Robinson 2004). Investigations of individual NGCP have greatly improved our understanding of how chloroplast development is regulated. However, the proper development of the chloroplast is apparently under complex regulations, as evident from the spectrum of chloroplast defects such as pale green, yellow, variegation, or albino that can result from mutations in NGCPs. In addition, how these genes interact genetically to regulate the development of the chloroplast is still not fully understood.

Leaf variegation mutants are unique tools for investigating chloroplast development and one of the most well-

Yafei Qi and Jun Zhao have equally contributed to this paper.

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studied variegation mutants in *Arabidopsis thaliana* is the *yellow variegated* (*var2*) mutant (Liu et al. 2010; Putarjunan et al. 2013). *var2* plants have green cotyledons, but variegated true leaves contain green and albino sectors (Martínez-Zapater 1993; Chen et al. 1999). The green sectors of *var2* contain chloroplasts that are morphologically normal. In contrast, highly vacuolated plastids lacking internal thylakoid membranes accumulate in the albino sectors (Chen et al. 1999). *VAR2* encodes a chloroplast thylakoid protein AtFtsH2 belonging to the FtsH family of AAA proteins (ATPases associated with diverse cellular activities) (Chen et al. 2000; Takechi et al. 2000). Interestingly, the mutant of another chloroplast *FtsH* homologue *VAR1/AtFtsH5* also gives a leaf variegation phenotype in the *var1* mutant (Sakamoto et al. 2002). The genetic identifications of VAR2/AtFtsH2 and VAR1/AtFtsH5, along with biochemical evidence established that FtsH proteins are integral proteins in thylakoid membranes (Lindahl et al. 1996).

In *Escherichia coli*, FtsH is an ATP-dependent membrane metalloprotease processing numerous substrates (Herman et al. 1995; Kihara et al. 1995; Tomoyasu et al. 1995; Akiyama et al. 1996; Ogura et al. 1999). FtsH proteins form complexes that are anchored in the plasma membranes in prokaryotes (Akiyama et al. 1995; Niwa et al. 2002; Boehm et al. 2012). In photosynthetic organisms, including cyanobacteria and higher plant chloroplasts, FtsH homologues were shown to be involved in the degradation of photosystem II (PSII) reaction center D1 protein (Lindahl et al. 2000; Bailey et al. 2002; Silva et al. 2003). In Arabidopsis, at least two types of FtsH proteins, type A subunits (AtFtsH1 and VAR1/AtFtsH5) and type B subunits (VAR2/AtFtsH2 and AtFtsH8) participate in the FtsH complex formation in thylakoid membranes (Yu et al. 2004, 2005; Zaltsman et al. 2005).

Although it is generally believed that thylakoid membrane FtsH complexes are involved in the degradation of damaged photosynthetic subunits, accumulating evidences point to a role for their involvement in the regulation of chloroplast development. First, thylakoid FtsH proteins are essential for chloroplast development and double mutants of AtFtsH1 and VAR1/AtFtsH5, as well as VAR2/AtFtsH2 and AtFtsH8, display an albino phenotype (Yu et al. 2005; Zaltsman et al. 2005). Second, it was shown that the abnormal plastids observed in the albino sectors of *var2* leaves represented undifferentiated plastids, suggesting that the chloroplast development is blocked (Kato et al. 2007). Third, genetic suppressor analyses indicate that VAR2/AtFtsH2 is genetically linked to the process of protein synthesis in the chloroplast, pointing to a functional linkage between FtsH and protein translation machinery in chloroplasts (reviewed in Putarjunan et al. 2013). Last but not least, the recent findings that the conserved zinc binding sites, which were thought to be essential for the proteolytic activities of FtsH proteins, were not required for the functioning of VAR2/AtFtsH2 and AtFtsH8, further suggested that thylakoid FtsH complexes may participate in other aspects of chloroplast development (Zhang et al. 2010).

The intriguing nature of leaf variegation points to both the complexity and plasticity of chloroplast development. To address the mechanisms of leaf variegation and the genetic regulation of chloroplast development, we have utilized the Arabidopsis *yellow variegated* (*var2*) mutant and a genetic suppressor screen approach. We have previously characterized a series of *SUPPRESSOR OF VARIEGATION* (*SVR*) loci and begun to form a snapshot of the complex genetic regulatory networks that govern chloroplast development (Yu et al. 2008; Liu et al. 2010, 2013). Here we report the isolation of a new *var2* suppressor locus *SVR10*. The cloning of *SVR10* revealed that it encodes a chloroplast protein belonging to the family of circularly permuted GTPases. Loss-of-function mutants of *SVR10* and its close homologue, *BRZ INSENSITIVE PALE GREEN2* (*BPG2*) can suppress the leaf variegation phenotype of *var2*. In addition, *svr10* and *bpg2* mutants are compromised in photosynthesis and chloroplast protein accumulation, consistent with their putative roles in chloroplast ribosome assembly. Surprisingly, we uncovered assembly defects of thylakoid photosynthetic protein complexes in both *svr10* and *bpg2* mutants. Our findings further validate a potential functional link between chloroplast ribosome assembly and VAR2-mediated protein quality control in chloroplasts and also suggest a role for SVR10 and BPG2 in the regulation of thylakoid protein complex assembly.

Materials and methods

Plant materials and growth condition

Arabidopsis strains used in this research are all in the Columbia-0 (Col-0) background. *svr10-1* was isolated in this study. *var2* mutant alleles, *var2-4* and *var2-5,* have been described (Chen et al. 2000). *bpg2-2* (SALK_068713) has been described (Komatsu et al. 2010). Arabidopsis seeds were sowed on either commercial soil mix (Pindstrup, Denmark), or on half-strength Murashige and Skoog medium (1/2 MS) (Caisson Laboratories, UT, US) and stratified for 2 days, before placed in a growth room maintained at 22 °C. All plants were cultivated under continuous illumination ($\sim$100 μmol m^{-2} s^{-1}).

Nucleotide acids manipulations

Arabidopsis DNA isolation was performed with CTAB method (Wetzel et al. 1994). Total cellular RNAs were

isolated from fresh leaf tissues of two-week-old plants with TRIzol reagent (Invitrogen, CA, USA). For semi-quantitative RT-PCR analysis, cDNA was generated with Transcriptor First Strand cDNA synthesis kit following the manufacturer's instructions (Roche, Switzerland). All primers used in this study are listed in Supplementary Table S1.

Map-based cloning of *SVR10*

Map-based cloning was performed as described (Lukowitz et al. 2000). In brief, *svr10-1* was crossed with Arabidopsis Landsberg *erecta* (L*er*) ecotype to generate a mapping population composed of 475 plants showing pale-green mutant phenotype in the F2 generation. Bulked segregant analysis using pooled DNA samples from 95 plants of the mapping population located the mutation site in *svr10-1* to a region between SSLP markers F13I12#1 and T8P19#1 on chromosome 3. Additional molecular markers based on Indel or SNP polymorphisms between L*er* and Col were designed to fine map *svr10-1* mutation. Detailed information of the molecular markers used in this study is listed in Supplementary Table S1.

Plasmid constructions and plant transformation

Full-length cDNA of *SVR10*/At3g47450 was amplified with *pfu* Turbo DNA polymerase (Agilent Technologies, CA, USA) using primers 47450F and 47450R. *SVR10-FLAG* was generated with primers 47450F and Flag-Rev-SVR10. PCR fragments were cloned into the pBlueScript KS+, sequenced, and subsequently cloned into a binary vector pBI111L (Yu et al. 2004). These binary vectors were used to transform *svr10-1* or *svr10-1 var2-5* using the floral dip method (Clough and Bent, 1998). Transgenic lines were screened on 1/2 MS medium containing 50 mg L^{-1} kanamycin.

Chlorophyll fluorescence imaging

Chlorophyll fluorescence parameters were measured with Open FluorCam FC800-O (Photon Systems Instruments, Czech Republic) following the manufacturer's manual. In brief, three-week-old Arabidopsis plants were dark adapted for 15 min before F_0 (minimum fluorescence) was measured. 50 % light intensity of SuperPulse was used to determine the F_M (maximum fluorescence). F_V/F_M was calculated as $(F_M - F_0)/F_M$.

Intact chloroplasts isolation and fractionation

Chloroplast isolation was performed as described with some minor modifications (Kunst 1998). Three-week-old dark-adapted Arabidopsis plants were homogenized in HB buffer (Kunst 1998). Intact chloroplasts were isolated through a two-step Percoll gradient (40–80 %) centrifugation (Adhikari et al. 2011). Stroma and membrane fractions were separated by passing intact chloroplasts through a 24-gauge syringe over 50 times (Qi et al. 2012). Intact chloroplasts, stroma, and membrane fractions equivalent to the same amount of chlorophyll were resolved on a 10 % SDS-PAGE gel for immunoblot analyses.

Immunoblotting analysis

Proteins separated in SDS-PAGE gel were transferred on a nitrocellulose membrane (0.45 μm) with a semi-dry transfer device (Bio-Rad, CA, USA). Membranes were blocked with 5 % nonfat dry milk powder in TBST buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 % Tween 20), and incubated with specific antibodies, and followed by secondary antibodies conjugated horseradish peroxidase. Signals were detected with Immun-Star chemiluminescence solutions and ChemiDoc Imaging System (Bio-Rad, CA, USA).

Blue native PAGE analysis

Blue Native PAGE analysis was performed as described (Järvi et al. 2011). Briefly, thylakoid membrane fractions equivalent to 20 μg chlorophyll were solubilized with 1.0 % (w/v) of β-dodecylmaltoside (β-DM) in 25BTH20G buffer and resolved on 3–14 % gradient native PAGE with a constant voltage of 70 V at 4 °C. For 2D-PAGE analysis, excised BN-PAGE lanes were denatured in 125 mM Tris-HCl pH 6.8, 4 % SDS and 1 % β-mercaptoethanol for 1 h. Each denatured lane was laid on top of 12 % SDS-PAGE and electrophorese at room temperature. 2D gels were stained with Coomassie brilliant blue R250 and scanned with GS-800 scanner (Bio-Rad, CA, USA).

Results

The isolation of the *svr10-1* mutant

To gain insight into the mechanisms of *yellow variegated* (*var2*)-mediated leaf variegation and the genetic regulation of chloroplast development, we generated a large-scale activation tagging mutant population and screened for genetic suppressors of *var2* (Yu et al. 2008). A series of *var2-5* suppressors were identified and the corresponding suppressor gene loci were named as *SUPPRESSOR OF VARIEGATIONs* (reviewed in Putarjunan et al. 2013). Here we report the isolation of a new *var2-5* suppressor line, designated as *051-002*. The suppressor locus in *051-002*

was named as *SUPPRESSOR OF VARIEGATION10* (*SVR10*) and the genotype of the suppressor line *051-002* was thus *var2-5 svr10-1*. The phenotype of the single mutant *svr10-1* resembled that of *var2-5 svr10-1*, displaying a pale-green leaf color and a small stature (Fig. 1a). In addition, *svr10-1* can also suppress the leaf variegation phenotype of a stronger *var2* mutant allele, *var2-4*, suggesting the suppression of *var2*-mediated leaf variegation by *svr10-1* is not allele specific (Fig. 1b). These results indicate that the pale-green phenotype of *svr10-1* is capable of preventing the formation of green and white leaf sectors in *var2* mutants.

The molecular cloning of *SVR10*

Genetic analysis revealed that the pale-green phenotype of *svr10-1* was likely caused by a nuclear recessive mutation, and it was not linked with an insertion of the activation tagging T-DNA. A positional cloning strategy was utilized to map the mutation site in *svr10-1*. A F2 mapping population was generated by crossing *svr10-1* (Col-0 background) with the Arabidopsis L*er* ecotype. The initial bulked segregant analysis using molecular markers distributed on all five Arabidopsis chromosomes placed the mutation site in *svr10-1* on chromosome III between markers F13I12#1 and T8P19#1 (Fig. 2a). Further fine mapping narrowed the physical interval containing the mutation site down to a ~122 kb region between markers F13I12#1 and F1P2#2 (Fig. 2a). The pale-green coloration of the *svr10-1* mutant suggested that *SVR10* likely codes for a chloroplast protein. Seven putative chloroplast protein-coding genes were predicted by TargetP in the final 122 kb physical interval (Emanuelsson et al. 2000). The genomic DNA regions of these seven genes were amplified by PCR and sequenced to determine whether any mutation occurred. Interestingly, several PCR amplifications of At3g47450 failed to yield the expected products in *svr10-1* (Fig. 2b). Further sequencing analyses showed that a 648 bp deletion occurred in At3g47450 in *svr10-1*, consistent with the failed PCR amplifications (Fig. 2a, b; Supplementary Fig. S1a). This 648 bp deletion covered the majority of exon 9 (96 bp out of 114 bp), and the entire sequences of intron 9, 10, and 11, and exon 10, 11, and 12 (Fig. 2a, d; Supplementary Fig. S1a). In addition, a 13 bp sequence of unknown origin was present immediately after the remaining sequences of exon 9 (Supplementary Fig. S1a). Semi-quantitative RT-PCR revealed that the deletion did not abolish At3g47450 transcripts entirely in *svr10-1* (Fig. 2c). A shorter version of At3g47450 transcript that in theory can be translated into a truncated form of SVR10 containing the first 346 amino acids and two additional amino acids residues before a predicted stop codon was detected in *svr10-1* (Fig. 2c, d; Supplementary Fig. S1b).

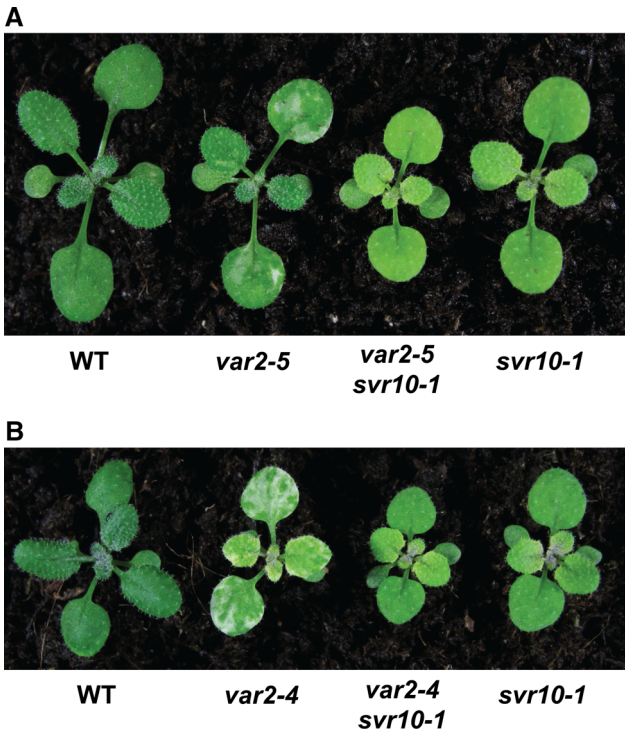


Fig. 1 Phenotypes of the *var2* suppressor mutant *svr10-1*. **a** Representative two-week-old seedlings of wild type (WT), *var2-5*, *var2-5 svr10-1* double mutant, and *svr10-1* single mutant. **b** Representative two-week-old seedlings of wild type, *var2-4*, *var2-4 svr10-1*, and *svr10-1*. Plants were maintained under continuous light (~100 μmol m^{-2} s^{-1}) at 22 °C

To confirm that the pale-green phenotype of *svr10-1* was caused by the mutation identified in At3g47450, an overexpression vector (*P_{35S}::At3g47450*) in which a full-length wild-type At3g47450 cDNA was placed under the control of the constitutive CaMV 35S promoter was generated and transformed into *svr10-1* and *var2-5 svr10-1*, respectively. In *svr10-1* background, the pale-green phenotype was effectively complemented by the expression of wild-type At3g47450 in transgenic plants (*svr10-1 P_{35S}::At3g47450*; Fig. 3a). As expected, At3g47450 transcripts were elevated in the *svr10-1* complementation lines (Fig. 3b). In *var2-5 svr10-1* background, the overexpression of wild-type At3g47450 restored the variegated phenotype of *var2-5* (Fig. 3c, d), validating that the suppression of *var2-5* by *svr10-1* is due to the mutation in At3g47450. These results show that At3g47450 is *SVR10*, and the mutation of *SVR10*/At3g47450 leads to a chlorophyll accumulation defect and the suppression of *var2*-mediated leaf variegation.

SVR10 is a chloroplast stroma protein

Previous studies have identified SVR10 as a potential Arabidopsis nitric oxide synthase (AtNOS1) localized to

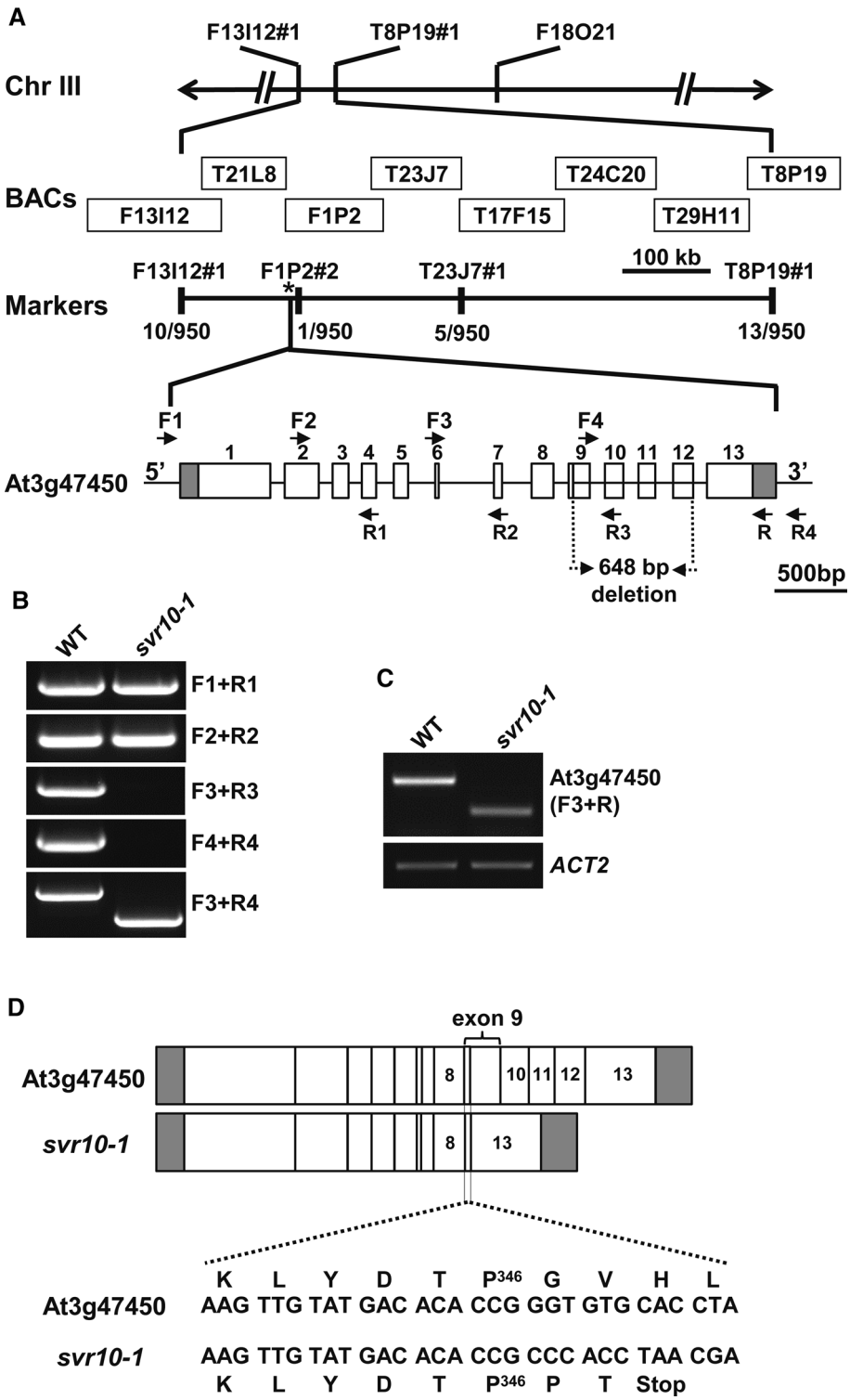


Fig. 2 The molecular cloning of *SVR10*. **a** Schematic representation of the map-based cloning of *SVR10*. Illustrated was the genetic interval in which *SVR10*/At3g47450 reside. The numbers of recombinants are listed under each marker. Detailed information of all the molecular markers used in this study is listed in Supplementary Table S1. The position of *SVR10*/At3g47450 was marked by the *asterisk*. In the gene model of At3g47450, *gray boxes* represented 5′ and 3′ untranslated regions (UTRs), and *white boxes* and *solid lines* represent exons and introns, respectively. Location of a 648 bp deletion found in *svr10-1* was marked. Positions of the primers used in **b** and **c** were marked by *arrows*. **b** Verification of the deletion in *svr10-1* using genomic DNA PCRs with various primer combinations. **c** Semi-quantitative RT-PCR detection of a partial cDNA fragment of At3g47450 in wild type and *svr10-1*. Expression of the *ACT2* gene served as the internal control. **d** Model of the transcription and translation product of At3g47450 in the wild type and *svr10-1*

the mitochondria (Guo et al. 2003; Guo and Crawford 2005). However, more recent reports point to a role for SVR10 as a member of GTPases family and SVR10 has been since renamed NITRIC OXIDE ASSOCIATED 1 (AtNOA1) (Crawford et al. 2006; Zemojtel et al. 2006; Moreau et al. 2008). In addition, SVR10 was also identified as chloroplast localized RESISTANT TO INHIBITION BY FOSMIDOMYCIN 1 (RIF1) (Flores-Pérez et al. 2008).

To determine the sub-chloroplast localization of SVR10/AtNOA1/RIF1, a tagged version of SVR10 with a FLAG

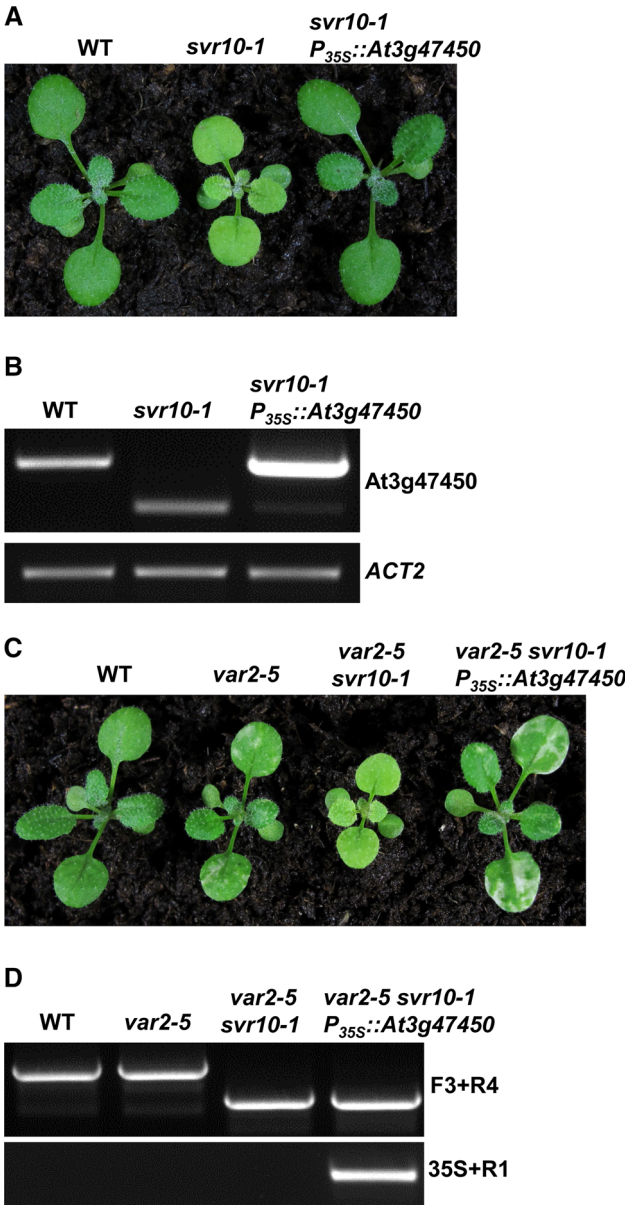


Fig. 3 Complementation of *svr10-1*. **a** Two-week-old seedlings of wild type, *svr10-1,* and a representative *svr10-1 P*$_{35S}$*::At3g47450* line. **b** Semi-quantitative RT-PCR analysis of At3g47450 transcripts accumulation in plants shown in **a**. **c** Two-week-old seedlings of wild type, *var2-5*, *var2-5 svr10-1*, and a representative *var2-5 svr10-1 P*$_{35S}$*::At3g47450* line. **d** Confirmation of the genotypes of plants shown in **c** via genomic DNA PCR using indicated primers

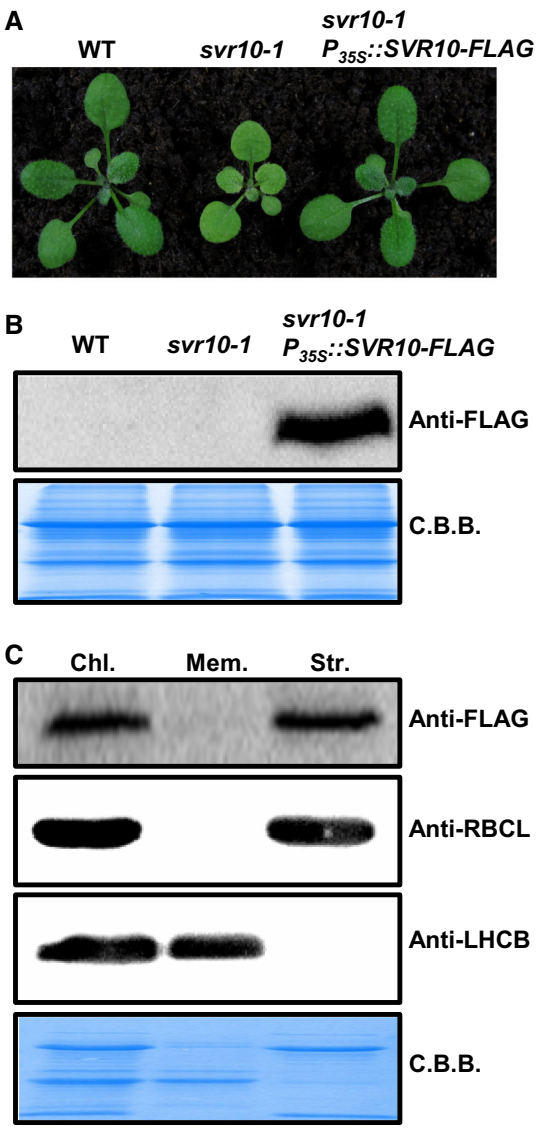


Fig. 4 SVR10 is a chloroplast stroma protein. **a** Two-week-old seedlings of wild type, *svr10-1*, and a representative *svr10-1 P*$_{35S}$*::SVR10-FLAG* line. **b** Immunoblot analysis of the expression of SVR10-FLAG fusion proteins in plant shown in **a**. **c** Detection of SVR10-FLAG in the chloroplast stroma protein fractions. Protein samples of intact chloroplasts (Chl.), thylakoid membrane fractions (Mem.), and stroma fractions (Str.) were purified from *svr10-1 P*$_{35S}$*::SVR10-FLAG* lines. Samples were normalized to equal amount of chlorophyll and subjected to immunoblot analyses. Antibodies against to RBCL and LHCB were used as stroma and thylakoid controls, respectively. Coomassie Brilliant Blue (C. B. B.)-stained gels served as loading controls in **b** and **c**

epitope fused at its C-terminus (SVR10-FLAG) driven by the 35S promoter was introduced into the *svr10-1* background. Expression of SVR10-FLAG fully complemented the mutant phenotype of *svr10-1*, suggesting that the SVR10-FLAG fusion protein is functional in planta (Fig. 4a). Accumulation of SVR10-FLAG fusion protein was confirmed by immunoblotting with a monoclonal anti-FLAG antibody (Fig. 4b). Intact chloroplasts were isolated from SVR10-FLAG lines and fractionated into total membranes and soluble stroma fractions. As expected, light-harvesting chlorophyll *a*/*b* binding protein (LHCB) was detected only in the membrane fractions, while Rubisco large subunit (RBCL) was detected in the soluble fractions (Fig. 4c). Similar to the signals of RBCL, SVR10-FLAG was detected in the stroma fractions but not the membrane fractions (Fig. 4c). These data indicated that

SVR10/AtNOA1/RIF1 can be localized to the chloroplast stroma.

Phylogenetic analysis of SVR10 and SVR10-like proteins

SVR10/AtNOA1/RIF1 belongs to a unique subfamily of GTPases, called the circularly permuted GTPases (cGTPases) (Bourne et al. 1991). G domains in cGTPase subfamily are arranged in an order of G4-G5-G1-G2-G3, instead of the more common order of G1-G2-G3-G4-G5 (Anand et al. 2006). In Arabidopsis, two other proteins, BRZ-INSENSITIVE-PALE GREEN 2 (BPG2, At3g57180) (27 % identities and 50 % similarities) and At4g10620 (24 % identities and 39 % similarities), shared high homologies with SVR10/AtNOA1/RIF1. Sequences of proteins that are homologous to SVR10/AtNOA1/RIF1, BPG2, or At4g10620 from various photosynthetic organisms and a non-photosynthetic bacterium *Bacillus subtilis* were obtained from NCBI. The evolutionary relationship and putative cellular localization of these SVR10-like proteins were analyzed (Fig. 5a). In Arabidopsis, SVR10/AtNOA1/RIF1 and BPG2 have putative chloroplast transit peptide, while At4g10620 was predicted to be targeted to the mitochondrion (Emanuelsson et al. 2000). Homologues of SVR10/AtNOA1/RIF1, BPG2, and At4g10620 from other eukaryotic species we examined all had putative mitochondrion or chloroplast transit peptide (Fig. 5a; Emanuelsson et al. 2000). Phylogenetic tree constructed from these SVR10-like proteins showed that orthologous proteins of SVR10/AtNOA1/RIF1, BPG2, or At4g10620 are present in both dicot (Arabidopsis) and monocot species (maize and rice), suggesting this complement of genes is likely conserved between dicotyledonous and monocotyledonous plants (Fig. 5a).

Next, we examined the tissue expression profiles of Arabidopsis *SVR10/AtNOA1/RIF1*, *BPG2,* and At4g10620 via semi-quantitative RT-PCR. *SVR10* and *BPG2* showed similar expression patterns with stronger expressions in photosynthetic tissues, consistent with the chloroplast localization of their gene products (Fig. 5b). In contrast, At4g10620 showed a more ubiquitous expression profile as its transcripts were detected in all tissues examined (Fig. 5b).

Mutations in *BPG2* suppresses *var2*

Given that both *SVR10/AtNOA1/RIF1* and *BPG2* code for chloroplast proteins, we next tested the genetic interactions between *var2* and *BPG2*. A T-DNA insertion mutant allele,

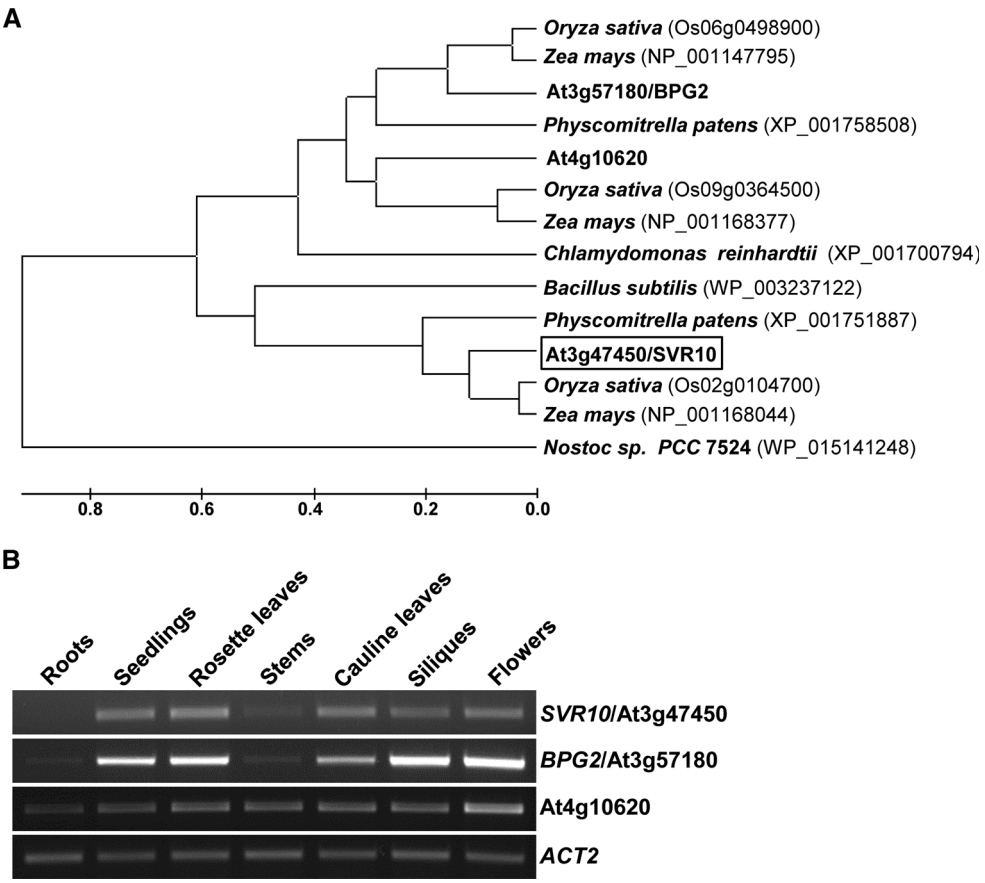


Fig. 5 Phylogenetic and expression analyses of *SVR10* and its homologous genes. **a** Phylogenetic tree of homologs of SVR10 and SVR10-like proteins from Arabidopsis, *Oryza sativa*, *Zea mays*, *Physcomitrella patens*, *Chlamydomonas reinhardtii*, *Nostoc sp.* PCC 7524, and *Bacillus subtilis*. Full-length protein sequences obtained from National Center for Biotechnology Information (NCBI) were aligned and analyzed with MEGA5 (Tamura et al. 2011). **b** Tissue expression patterns of *SVR10*, *BPG2,* and At4g10620 analyzed via semi-quantitative RT-PCR. Total cellular RNAs were extracted from different Arabidopsis tissues. cDNAs were synthesized from 1 μg of DNase I treated RNAs. Expression of *ACT2* gene was used as a control

bpg2-2 (SALK_068713, Komatsu et al. 2010) was obtained from the European Arabidopsis Stock Centre (NASC) and crossed with *var2-5*. As shown in Fig. 6a, *bpg2-2* displayed a smaller stature and a pale-green appearance, similar to *svr10-1*. Double mutants of *var2-5 bpg2-2* resembled *bgp2-2* single mutants and did not exhibit leaf variegation, suggesting that *bpg2-2* could suppress *var2* (Fig. 6b). The suppression of *var2* leaf variegation by both *svr10-1* and *bpg2-2* suggests that SVR10/AtNOA1/RIF1 and BPG2 may be involved in similar processes in the chloroplast, consistent with the high degree of conservation between these two proteins.

Photosynthetic parameters are compromised in *svr10-1* and *bpg2-2*

The leaf color phenotypes of *svr10-1* and *bpg2-2* suggest that *SVR10* and *BPG2* are involved in chloroplast development and photosynthesis. To further demonstrate the impact of mutations of *SVR10* and *BPG2* on photosynthesis, we examined chlorophyll fluorescence parameters in wild type, *svr10-1,* and *bpg2-2*. Whole plant chlorophyll fluorescence imaging showed that compared to wild type, both the F_0 and F_M values were increased in *svr10-1* and *bpg2-2* (Fig. 7a; Table 1). Meanwhile, F_V/F_M, a major

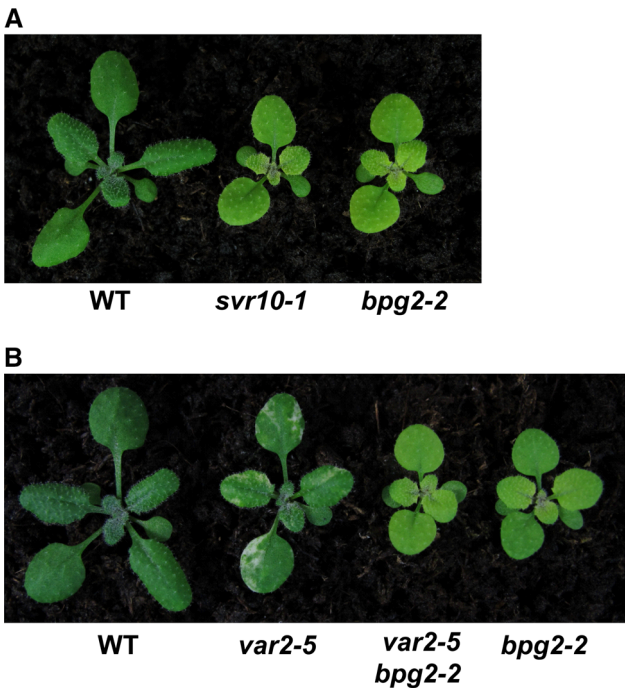


Fig. 6 *bpg2-2* suppresses *var2*-mediated leaf variegation. **a** Two-week-old seedlings of wild type, *svr10-1*, and *bpg2-2*. **b** Two-week-old seedlings of wild type, *var2-5*, *var2-5 bpg2-2* double mutant, and *bpg2-2*

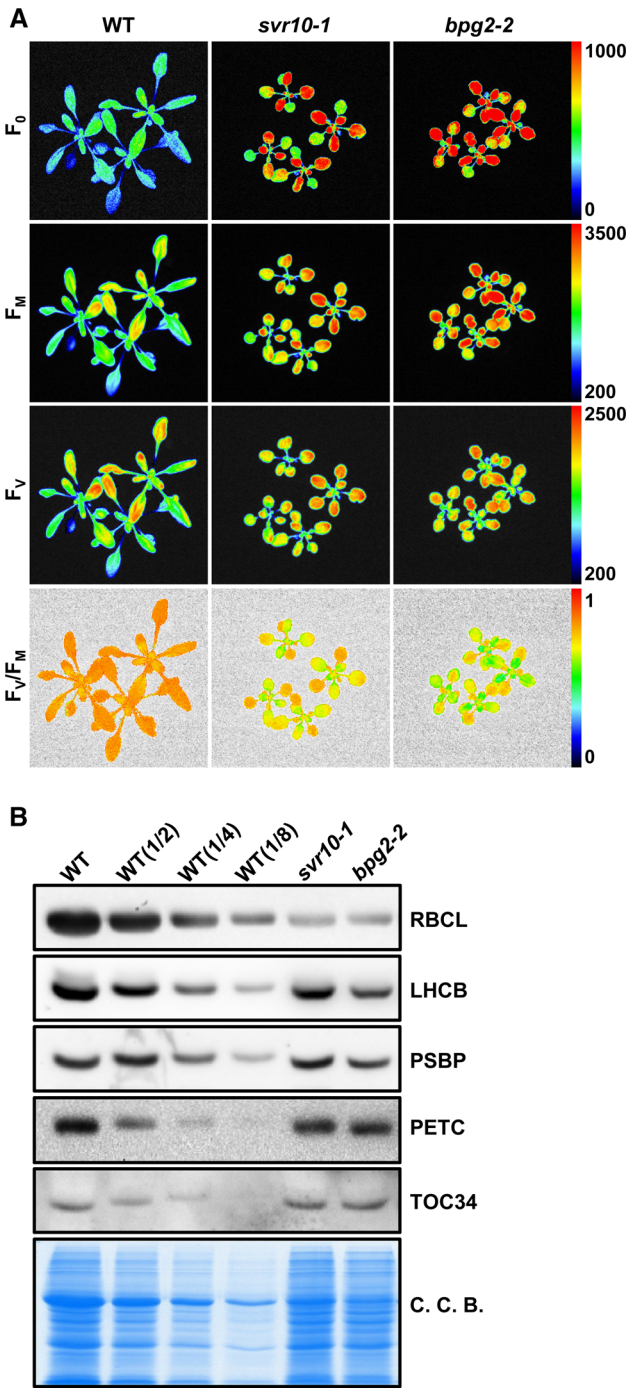


Fig. 7 Analyses of chlorophyll fluorescence and accumulation of chloroplast proteins in wild type, *svr10-1*, and *bpg2-2*. **a** Whole plant chlorophyll fluorescence images of three-week-old wild type, *svr10-1*, and *bpg2-2*. Plants were pseudo-colored according to the F_0, F_M, F_V, and F_V/F_M values. **b** Accumulation of representative chloroplast proteins in wild type, *svr10-1*, and *bpg2-2*. Total leaf proteins were extracted from two-week-old seedlings. Protein samples were normalized based on tissue fresh weight. Immunoblots were performed using polyclonal antibodies against RBCL, LHCB, PSBP, PETC, and TOC34. The C. B. B.-stained gel served as a loading control

Table 1 Chlorophyll fluorescence parameters of wild type, *svr10-1,* and *bpg2-2*

Genotype	Leaf number	F_0	F_M	F_V	F_V/F_M
WT	2nd	315.15 ± 15.55	1717.99 ± 76.00	1402.84 ± 61.59	0.81 ± 0.01
	6th	347.89 ± 78.55	1796.69 ± 345.62	1448.80 ± 269.03	0.81 ± 0.01
svr10-1	2nd	534.37 ± 27.70	2190.97 ± 144.63	1656.60 ± 118.37	0.76 ± 0.01
	6th	896.75 ± 31.11	2385.29 ± 46.32	1488.54 ± 39.95	0.63 ± 0.02
bpg2-2	2nd	685.34 ± 89.36	2287.86 ± 158.48	1602.52 ± 93.38	0.70 ± 0.03
	6th	1102.31 ± 77.39	2499.60 ± 133.57	1397.28 ± 87.16	0.59 ± 0.01

Measurements were made on the second and the sixth true leaves of three-week-old plants. Data were presented as mean ± standard deviation of three biological replicates

F_0, minimum fluorescence in dark-adapted state; F_M, maximum fluorescence in dark-adapted state; F_V, variable fluorescence in dark-adapted state; F_V/F_M, maximum PSII quantum yield

indicator of PSII activity was decreased in *svr10-1* and *bpg2-2* compared to that of wild type (Fig. 7a; Table 1). In addition, in line with previous reports, wild type displayed a nearly uniform F_V/F_M of ~0.82 in both developing and mature leaves (Fig. 7a; Table 1; Chi et al. 2012). To the contrast, the decline of F_V/F_M value was more prominent in developing leaves compared to that of mature leaves in both *svr10-1* and *bpg2-2* (Fig. 7a; Table 1). This pattern of F_V/F_M is consistent with the virescent phenotype of *svr10-1* and *bpg2-2*.

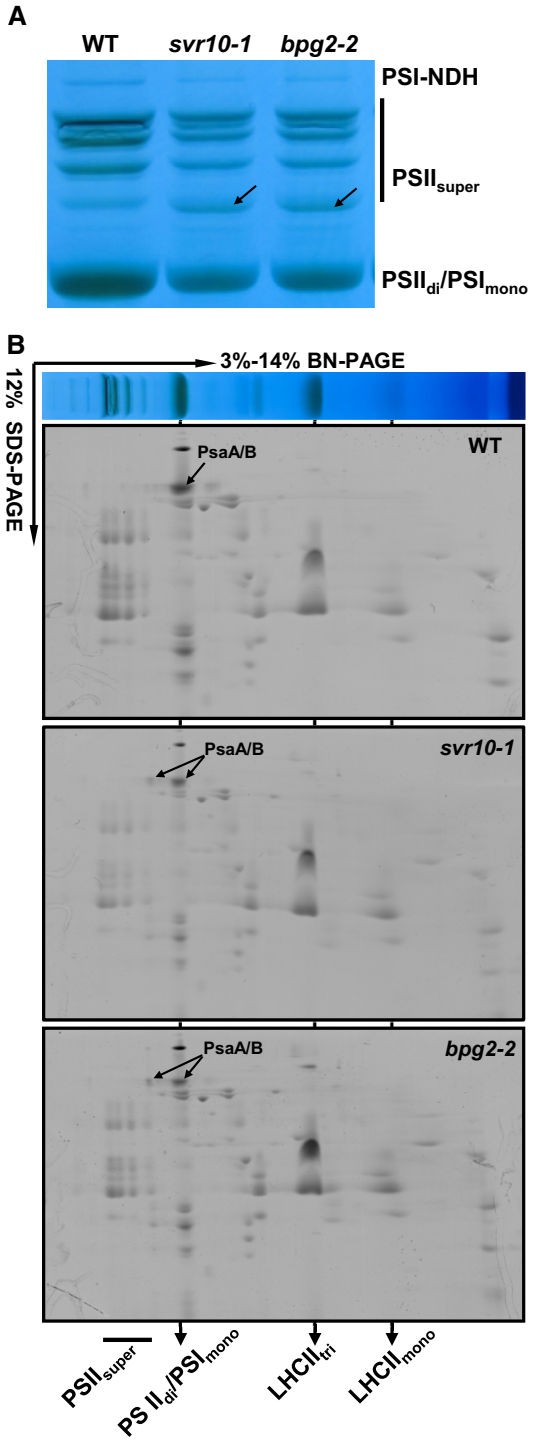
Next, we examined the accumulation of chloroplast proteins in wild type, *svr10-1,* and *bpg2-2* using immunoblot analysis. The amount of plastid genome encoded RBCL was greatly reduced to less than 1/8 of wild-type level in *svr10-1* and *bpg2-2* (Fig. 7b). On the other hand, the accumulations of chloroplast proteins encoded by nuclear genes, including LHCB, 23 kDa protein of the oxygen evolving complex of the photosystem II (PSBP) and Rieske iron-sulfur protein (PETC) were reduced to lesser degrees (Fig. 7b). Surprisingly, the levels of TOC34 (a chloroplast outer envelope membrane translocon complex protein) in *svr10-1* and *bpg2-2* were similar to that of wild-type level, suggesting that the chloroplast protein import apparatus is not grossly impaired in the mutants. Overall, these results suggest that chloroplast protein accumulation is compromised in *svr10-1* and *bpg2-2*, and the loss of SVR10 or BPG2 has a greater impact on proteins translated in the chloroplast.

Thylakoid membrane protein complexes assembly defects in *svr10-1* and *bpg2-2*

To further analyze the defects of chloroplasts in *svr10-1* and *bpg2-2*, we compared the accumulations of photosynthetic complexes *svr10-1* and *bpg2-2* to that of wild type using the blue native PAGE (BN-PAGE). With our BN-PAGE system, several forms of large PSII supercomplexes can be readily identified in the wild type, similar to a previous report (Fig. 8a; Fu et al. 2007). In *svr10-1* and *bpg2-2* mutants, we observed a general trend of decreased PSII supercomplexes formation (Fig. 8a). The most dominant band in BN-PAGE, which corresponds to PSII dimer and PSI monomer, was also reduced in *svr10-1* and *bpg2-2* (Fig. 8a). Interestingly, an abnormal protein-chlorophyll complex that is slightly smaller than the smallest wild-type form of PSII supercomplex was observed in *svr10-1* and *bpg2-2* (Fig. 8a). To corroborate the first dimension results, we resolved protein subunits of photosynthetic complexes with second dimension SDS-PAGE (Fig. 8b). The presence of at least four PSII supercomplexes was observed in wild type (Fig. 8b). The second dimension gel further confirmed that the abundance of PSII supercomplexes was reduced in *svr10-1* and *bpg2-2* (Fig. 8b). Moreover, the second dimension gel also indicated that the abnormal protein-chlorophyll complex present in *svr10-1* and *bpg2-2* contained PSI reaction center proteins PsaA/B, suggesting that this abnormal complex is a form of PSI, and the size of this abnormal form of PSI was clearly larger than PSI monomer (Fig. 8b). Taken together, these data showed that thylakoid membrane chlorophyll-protein complexes assembly is defective in *svr10-1* and *bpg2-2* and an abnormal form of thylakoid complex containing PSI subunits over-accumulates in the two mutants. These findings are consistent with the leaf color phenotype and compromised photosynthetic parameters of *svr10-1* and *bpg2-2*.

Discussion

Chloroplasts are the locale for photosynthesis and many other essential metabolic processes, and the proper development of the chloroplast requires the participation of a large number of NGCPs (Leister 2003; Jarvis and Robinson 2004). Due to their pale green, yellow, variegated, or albino leaf color phenotypes, mutants defective in NGCPs and chloroplast development constitute the most conspicuous category of mutants in genetic screens, and are instrumental for elucidating the mechanisms of chloroplast



◀**Fig. 8** Accumulation of photosynthetic complexes in wild type, *svr10-1,* and *bpg2-2*. **a** Blue native PAGE analysis of photosynthetic complexes in wild type, *svr10-1*, and *bpg2-2*. **b** Excised BN-PAGE lanes were denatured and resolved on 12 % SDS-PAGE for 2D analysis. Thylakoid membranes isolated from each genotype were resolved on 3–14 % blue native PAGE. The abnormal PSI-chlorophyll supercomplexes in *svr10-1* and *bpg2-2* were indicated by *black arrows* in **a**. Other complexes were marked at the side of the gel. $PSII_{di}$, PSII dimer; PSI_{mono}, PSI monomer; $PSII_{super}$, PSII supercomplexes; PSI-NDH, PSI-NAD(P)H dehydrogenase supercomplex; LHC_{tri}, Light-harvesting complex II trimer; LHC_{mono}, Light-harvesting complex II monomer

development. Among these mutants, variegation mutants represent a unique group and have long attracted attention (Rédei 1967). We have used the Arabidopsis variegation mutant *yellow variegated* (*var2*) as a tool to investigate the genetic wiring of chloroplast development. The functions of VAR2/AtFtsH2 and related thylakoid FtsH proteins have been thought to be related to their potential protease activities (Lindahl et al. 2000). However, an ever-increasing body of evidence points to the notion that VAR2/AtFtsH2 is necessary for proper chloroplast development, especially when the chloroplast is undertaking normal translation. Genetic suppressor data from various groups have established that defects that cause a reduction in chloroplast translation, including mutations of translation initiation factors and factors that are involved in the chloroplast ribosomal RNA processing, are sufficient for the suppression of the characteristic leaf variegation phenotype of *var2* mutants (Miura et al. 2007; Yu et al. 2008; Liu et al. 2010, 2013). The most direct genetic evidence linking translation and *var2* suppression came as we demonstrated that mutations in *SVR8*, coding a chloroplast ribosomal protein L24, can suppress *var2* leaf variegation (Liu et al. 2013). In addition, direct inhibition of chloroplast translation with drugs also led to the suppression of leaf variegation in *var2* (Yu et al. 2008). In this work, continuing our *var2* genetic suppressor work, we established that mutations of two closely related chloroplast cGTPases SVR10/AtNOA1/RIF1 and BGP2, can suppress *var2* leaf variegation. SVR10/AtNOA1/RIF1 and BPG2 share high homology with bacterial YqeH proteins. In *Bacillus subtilis*, YqeH participates in biogenesis of the 30S ribosome (Loh et al. 2007; Uicker et al. 2007). In Arabidopsis, BPG2 binds to chloroplast 16S and 23S rRNA and may regulate their processing (Kim et al. 2012). When a bacterial YqeH was fused at its N-terminus with a chloroplast transit peptide and targeted to the chloroplast, it was sufficient to rescue the *atnoa1* mutant phenotype, suggesting a functional conservation between SVR10/AtNOA1/RIF1 and bacterial YqeH (Flores-Pérez et al. 2008). SVR10/AtNOA1/RIF1 and BPG2 in chloroplast ribosomal assembly is in agreement with our previous findings that many *SVR* loci we cloned code for proteins that participate in the regulation of different aspects of chloroplast protein homeostasis. Identification of *SVR10/AtNOA1/RIF1* and *BPG2* as *var2* suppressor loci provides further support for the functional link between the general status of chloroplast translation and the need for VAR2/

AtFtsH2. Based on available data, it is likely that the essential nature of thylakoid FtsH activities is conditional. Their functions are essential for chloroplast development under normal translation but become dispensable when the translation capacities are compromised. In a sense, a robust chloroplast translation may prove to be a burden for the chloroplast as mis-folding and mis-assembly events may occur, which necessitates protein quality control mechanism(s) including the involvement of VAR2/AtFtsH2. The findings that the putative proteolytic activities of VAR2/AtFtsH2 and AtFtsH8 were not required for their roles in chloroplast development raise an interesting question regarding additional functions of VAR2/AtFtsH2 in chloroplast (Zhang et al. 2010).

Recent studies showed that mutations in *AtNOA1/RIF1*, *BPG2*, and a number of other genes encoding proteins that are involved in regulating chloroplast gene expression could genetically suppress another well-studied Arabidopsis variegation mutant, *thylakoid formation1* (*thf1*), which is defective in the Arabidopsis counterpart of cyanobacterial Psb29 protein (Wang et al. 2004; Huang et al. 2006; Hu et al. 2015; Ma et al. 2015). Together with previous reports that FtsH level is reduced in *thf1* mutant, THF1 and VAR2/AtFtsH2 may be functionally related during chloroplast biogenesis, consistent with the similar variegation phenotypes of the two mutants (Zhang et al. 2009; Wu et al. 2013). However, the exact mechanisms behind the suppression of both *var2* and *thf1* are still not clear and the relationship between THF1 and VAR2/AtFtsH2 warrants further investigations.

The *var2* suppressor locus *SVR10*, which was previously identified as *AtNOS1*, *AtNOA1,* and *RIF1*, has been the focus of much research in the past decade (Guo et al. 2003; Crawford et al. 2006; Flores-Pérez et al. 2008). This locus was first identified as potentially coding for the plant nitric oxide (NO) synthase, which catalyzes the direct synthesis of NO from arginine and it was shown that AtNOS1 may be targeted to the mitochondria (Guo et al. 2003; Guo and Crawford 2005). However, the NO synthase activity of AtNOS1 has since been disputed and the locus was renamed *AtNOA1* (Crawford et al. 2006; Moreau et al. 2008). Although it is still generally believed that NO level is reduced in *atnoa1* mutants and that AtNOA1 is involved in NO homeostasis directly or indirectly, AtNOA1 is not considered as the enzyme that is directly responsible for the synthesis of NO. One of the early findings regarding AtNOA1 was that *atnoa1* mutants displayed a pronounced pale-green leaf color, suggesting a role for *AtNOA1* in chloroplast development (Guo et al. 2003). Consistent with this notion, AtNOA1 was identified as RIF1 in a genetic screen for resistance to the inhibitory effect of fosmidomycin, which disrupts the MEP-derived plastid isoprenoids biosynthesis pathway in the chloroplast and GFP-fused RIF1 was targeted to the chloroplast (Flores-Pérez et al. 2008). In this study, we identified *svr10-1* mutant through our *var2* suppressor screen, and confirmed that *svr10-1* represents a new mutant allele of *AtNOA1/RIF1*. The mutation of *SVR10*, as well as its close homologue *BPG2*, was able to suppress *var2*-mediated leaf variegation. We further showed that photosynthetic activities and chloroplast protein accumulation were all compromised in the *svr10-1* mutant compared with wild type. Using a functional FLAG-tagged AtNOA1/RIF1/SVR10, we demonstrated biochemically that AtNOA1/RIF1/SVR10 is likely located in the chloroplast stroma (Fig. 7c). Taken together, both published records and our data suggest that SVR10 is likely a chloroplast protein and is intimately involved in chloroplast development. However, currently the relationship between AtNOA1/RIF1/SVR10 and NO homeostasis is still not clear.

Interestingly, we observed thylakoid protein complex assembly defects in both *svr10-1* and *bpg2-2*. In general, major thylakoid membrane complexes, including PSII supercomplexes, PSI monomer, and PSII dimer were reduced in *svr10-1* and *bpg2-2* mutants, consistent with reduced photosynthetic capacities and chloroplast protein accumulation. However, we observed the accumulation of an abnormal form of supercomplex containing PSI in both mutants, suggesting that the assembly of thylakoid membrane protein complexes was reprogramed in response to the lack of SVR10 and BPG2. Similar complexes have been reported and they may be one consequence caused by the over-reduction of chloroplast plastoquinone pool (Pesaresi et al. 2002; Armbruster et al. 2010). Considering that both SVR10 and BPG2 may be involved in chloroplast ribosome assembly and translation, it is possible that compromised translation may create a physiological state that necessitates an adaptive response of thylakoid protein complex assembly process.

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