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The chloroplast protein BPG2 functions in brassinosteroid-mediated post-transcriptional accumulation of chloroplast rRNA

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SUMMARY

Brassinazole (Brz) is a specific inhibitor of the biosynthesis of brassinosteroids (BRs), which regulate plant organ and chloroplast development. We identified a recessive pale green Arabidopsis mutant, *bpg2-1* (*Brz-insensitive-pale green 2-1*) that showed reduced sensitivity to chlorophyll accumulation promoted by Brz in the light. *BPG2* encodes a chloroplast-localized protein with a zinc finger motif and four GTP-binding domains that are necessary for normal chloroplast biogenesis. *BPG2*-homologous genes are evolutionally conserved in plants, green algae and bacteria. Expression of *BPG2* is induced by light and Brz. Chloroplasts of the *bpg2-1* mutant have a decreased number of stacked grana thylakoids. In *bpg2-1* and *bpg2-2* mutants, there was no reduction in expression of *rbcL* and *psbA*, but there was abnormal accumulation of precursors of chloroplast 16S and 23S rRNA. Chloroplast protein accumulation induced by Brz was suppressed by the *bpg2* mutation. These results indicate that BPG2 plays an important role in post-transcriptional and translational regulation in the chloroplast, and is a component of BR signaling.

Keywords: brassinosteroid, BR biosynthesis inhibitor Brz, GTPase, chloroplast biogenesis, chloroplast rRNA, processing.

INTRODUCTION

The plant brassinosteroids (BRs) brassinolide, castasterone, teasterone, and so on, are essential for plant growth and development. The most active BR, brassinolide (BL), was first isolated from pollen of *Brassica napus* (Grove *et al.*, 1979), and since then, more than 50 BRs have been isolated from other plant species (Bajguz and Tretyn, 2003). Molecular characterization of Arabidopsis BR biosynthetic mutants has revealed the important role of BRs in photomorphogenesis, leaf development, stem elongation, root elongation, pollen tube growth, xylem differentiation, sterility and senescence.

deetiolated2 (*det2*) was first thought to be an abnormal photomorphogenesis mutant, and was later identified as the first mutant deficient in BR biosynthesis (Chory *et al.*, 1991).

DET2 encodes a steroid 5α reductase involved in BR biosynthesis that can also catalyze mammalian steroid 5α reduction (Fujioka *et al.*, 1997; Li *et al.*, 1997). *det2* has a dwarf phenotype with dark-green round leaves and short inflorescences in the light, and a short hypocotyl and open cotyledons in the dark. In addition to these developmental characteristics, dark-grown *det2* mutants also show increased expression of light-induced photosynthetic genes and their translated proteins encoded by the nuclear and chloroplast genomes. These results suggest that BR deficiency regulates chloroplast gene expression, as photosynthetic genes are normally not expressed in the dark. Based on the *det2* phenotype, several BR-deficient mutants have been isolated, such as the BR biosynthesis mutants *dwf4* (Azpiroz *et al.*, 1998; Choe *et al.*, 1998) and *cpd* (Szekeres *et al.*, 1996), as well as BR-insensitive mutants such as the BR signaling mutants *bri1* (Clouse *et al.*, 1996; Li and Chory, 1997) and *bin2* (Li *et al.*, 2001; Li and Nam, 2002). These BR mutants generally show abnormal development in the light and de-etiolation in the dark. Previous characterization of the chloroplast in BR mutants has been limited, but it is necessary to further analyze the relationship between chloroplast development and BR.

Brassinazole (Brz) is a triazole compound that specifically inhibits BR biosynthesis by blocking the cytochrome P450 steroid C-22 hydoxylase encoded by *DWF4*/*CYP90B1* (Asami *et al.*, 2000, 2001). In the dark, Brz-treated Arabidopsis has open cotyledons and a short hypocotyl similar to BR-deficient mutants (Nagata *et al.*, 2000). After growth in the dark for 40 days, plants treated with Brz develop true leaves with epidermal cells, guard cells, trichomes, palisade parenchyma cells and spongy parenchyma cells. This phenotype in Arabidopsis can be rescued by addition of BR (Asami and Yoshida, 1999).

Recently, the mechanism of BR signal transduction in plant development has been analyzed in detail using chemical genetics to screen for mutants with altered responses to Brz in darkness at the germination stage. When grown in medium containing Brz, wild-type plants had short hypocotyls, but a mutant identified by the screen, *Brz-insensitive-long hypocotyl 1* (*bil1-D*) had a long hypocotyl in the dark (Asami *et al.*, 2003). *bil1-D* has the same mutation as *brassinazole-resistance 1-1D* (*bzr1-1D*), and *BZR1* encodes a functional transcription factor with dual roles in regulating BR biosynthesis genes and growth responses (Wang *et al.*, 2002; He *et al.*, 2005). BES1 was isolated from the mutant *bri1-EMS suppressor 1 (bes1-D*), and is a semi-dominant suppressor of *bri1*. *BES1* encodes a close homolog of *BZR1*/*BIL1* but regulates BR response genes in plant development (Yin *et al.*, 2002).

Here, we isolated and characterized a recessive Arabidopsis mutant, *bpg2*, which has pale green cotyledons and is insensitive to Brz-induced promotion of greening. *BPG2* encodes a chloroplast protein that specifically regulates accumulation of 16S and 23S rRNA but not mRNA from the chloroplast genome. Brz-inducible protein accumulation in chloroplasts is suppressed by the *bpg2* mutation. We discuss the important role of BPG2 in chloroplast development in BR signaling.

RESULTS

Isolation of the *bpg2* mutant

Brz binds directly to the cytochrome P450 steroid C-22 hydroxylase encoded by the *DWF4* gene, and specifically inhibits BR biosynthesis (Asami *et al.*, 2000, 2001). Brz treatment reduces BR content in plant cells and causes the same de-etiolation and dwarf phenotype as the BR-deficient mutant. In addition to these morphological changes, Brz treatment also induced chloroplast gene expression in the dark for both wild-type and the BR-deficient mutant (Nagata *et al.*, 2000). These results and research on BR-deficient mutants suggest that BR plays a role in regulating chloroplast development. In the light, Brz also promotes greening of cotyledons of wild-type Arabidopsis. If the pale green phenotype of a mutant is independent of BR signaling, the pale color will be restored to darker green by Brz. Pale green mutants that are not recoverable by Brz may have decreased or disrupted BR signaling for chloroplast regulation.

We screened approximately 10 000 Arabidopsis activation-tagged lines (Nakazawa *et al.*, 2003) and isolated a recessive mutant, *Brz-insensitive-pale green2-1* (*bpg2-1*), which retained pale green cotyledons when grown with Brz in the light (Figure 1c,d). The cotyledons of *bpg2-1* seedlings were paler green that those of wild-type seedlings on media containing various concentrations of Brz (Figure 1a–d).

For detailed analysis of cotyledon greening, endogenous levels of chlorophyll *a* and *b* in wild-type and *bpg2-1* seedlings were measured with or without Brz in the light (Figure 1e,f). *bpg2-1* accumulated approximately half the amount of chlorophylls *a* (Figure 1e) and *b* (Figure 1f) compared to wild-type seedlings. In wild-type seedlings, endogenous chlorophyll *a* and *b* levels were increased by Brz treatment, but were not increased in *bpg2-1* seedlings. When grown on soil, *bpg2-1* produced pale green semi-dwarf rosette leaves (Figure 1h) and inflorescences (Figure 1j). This phenotype differed from the dwarf phenotype of the BR-deficient mutant *det2* and the BR-insensitive mutant *bri1*.

In general, BR-deficient mutants have a short hypocotyl in the dark, but the *bpg2-1* hypocotyl was elongated, as in the wild-type (data not shown). This indicates that BR biosynthesis was normal in the *bpg2-1* mutant, and that BPG2 is not involved in BR biosynthesis. Furthermore, when *bpg2-1* was grown with Brz in the dark, *bpg2-1* showed the same short hypocotyl as the wild-type plants (data not shown). These results suggest that Brz binds to cytochrome P450 C-22 hydroxylase and inhibits BR biosynthesis in *bpg2-1*.

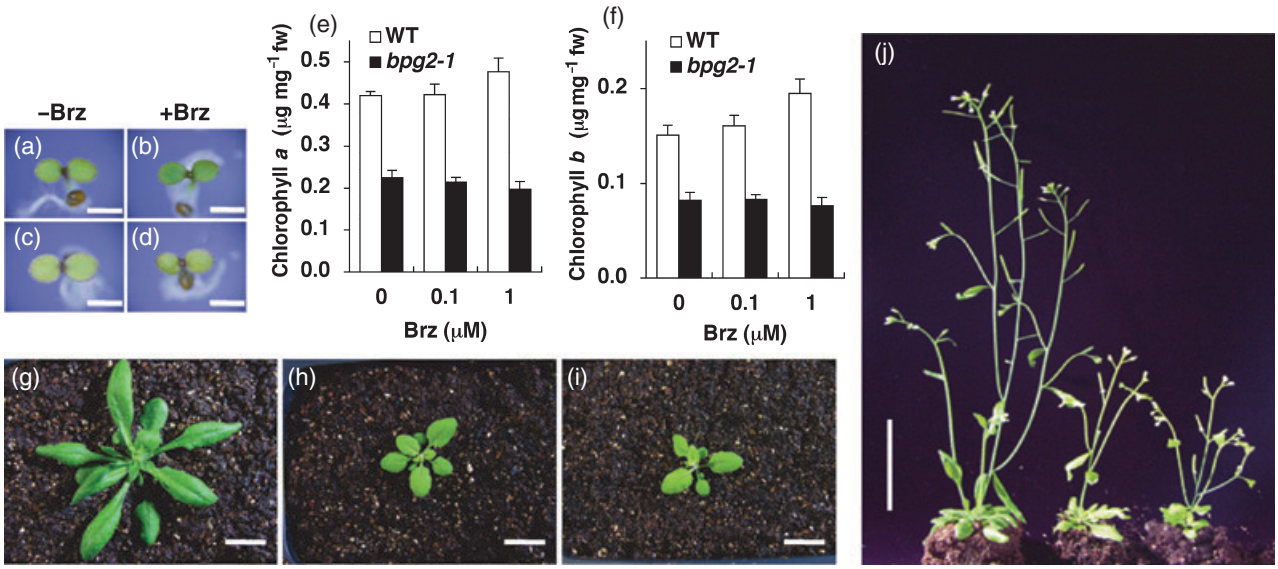

Figure 1. Phenotype of *bpg2* mutants.
(a–d) Cotyledons of wild-type (a, b) and *bpg2-1* (c, d) grown on half-strength MS medium under long days (16 h light/8 h dark) without Brz (a, c) or with 1 μM Brz (b, d) for 4 days. Scale bars = 1 mm.
(e, f) Endogenous contents of chlorophyll *a* (e) and chlorophyll *b* (f) of wild-type (WT) and *bpg2-1* plants grown without Brz (0 μM) or with Brz (0.1 and 1 μM) for 4 days under long days (16 h light/8 h dark). Error bars indicate SE.
(g–i) Wild-type (g), *bpg2-1* (h) and *bpg2-2* (i) seedlings grown under long days (16 h light/8 h dark) on soil for 2 weeks. Scale bars = 10 mm.
(j) Wild-type, *bpg2-1* and *bpg2-2* plants grown under long days (16 h light/8 h dark) on soil for 3 weeks. Scale bar = 5 cm.

bpg2-1 is thus insensitive to Brz effects, especially with respect to chloroplast regulation, and the semi-dwarf phenotype might be a secondary effect of chloroplast deficiency. From these analyses, it can be inferred that, after the initial perception of BR by the receptor BRI1, BR signaling can be separated into at least two phases: developmental regulation and chloroplast regulation, and that BPG2 appears to play a major role in chloroplast regulation by BR signal transduction.

BPG2 is a GTPase that is evolutionally conserved in plants, green algae and bacteria

Co-segregation of the Brz-insensitive, pale green phenotype with a selection marker after back-crossing with the wild-type indicated that *bpg2-1* was a recessive mutant with a single T-DNA insertion. To identify the *bpg2-1* mutation, we amplified a T-DNA insertion site on the *bpg2-1* genome by TAIL-PCR (Liu *et al.*, 1995) with the left border of T-DNA-specific primers and a combination of degenerate primers, and isolated the fragment. The identified T-DNA insertion site was in the third intron of *At3g57180* (Figure 2a). PCR results indicated that *bpg2-1* lacked an enhancer region of T-DNA (data not shown) and was a recessive mutant, suggesting that the *bpg2-1* phenotype was caused by disruption of *At3g57180* by the T-DNA insertion. Expression of full-length *At3g57180* in the *bpg2-1* mutant was not detected by RT-PCR (Figure 2b). To confirm that disruption of *At3g57180* is responsible for the *bpg2-1* mutant, we isolated the knockout mutant *bpg2-2* (SALK_068713) from a mutant pool of T-DNA insertion lines obtained from the Arabidopsis Biological Resource Center (Figure 2a). RT-PCR indicated that expression of *At3g57180* was also very low in the *bpg2-2* mutant (Figure 2b), and a pale green phenotype similar to *bpg2-1* was observed (Figures 1i,j and 3e,f,k–m).

BLAST searches for the BPG2 amino acid sequence identified similar genes in Arabidopsis (AGI codes At4g10620, unknown protein; At3g47450, RIF1/NOS1/NOA1) (Flores-Pérez *et al.*, 2008), rice (*Oryza sativa*), *Medicago truncatula*, grape (*Vitis vinifera*), the moss *Physcomitrella patens*, and the green algae *Ostreococcus lucimarinus* and *Chlamydomonas reinhardtii* (Figure 2c). Further searches suggested that some bacteria had *BPG2*-homologous genes that included a YqeH-type GTPase in Gram-positive bacteria such as *Bacillus subtilis* (Uicker *et al.*, 2007; Loh *et al.*, 2007; Figure 2c). The YqeH-type GTPase of bacteria has a GTP-binding domain with a G4-G1-G2-G3 motif and an N-terminal putative zinc finger motif with a conserved $CXXCX_nCXXC$ sequence (Loh *et al.*, 2007). The four GTP-binding domains and the zinc finger motif were also found in a putative BPG2 amino acid sequence (Figure 2d).

To confirm that disruption of the GTPase homologous gene caused the *bpg2-1* and *bpg2-2* mutant phenotype, the *BPG2* candidate cDNA was placed under the control of the CaMV 35S promoter and transformed into *bpg2-1* and *bpg2-2* by *Agrobacterium*-mediated transformation. The

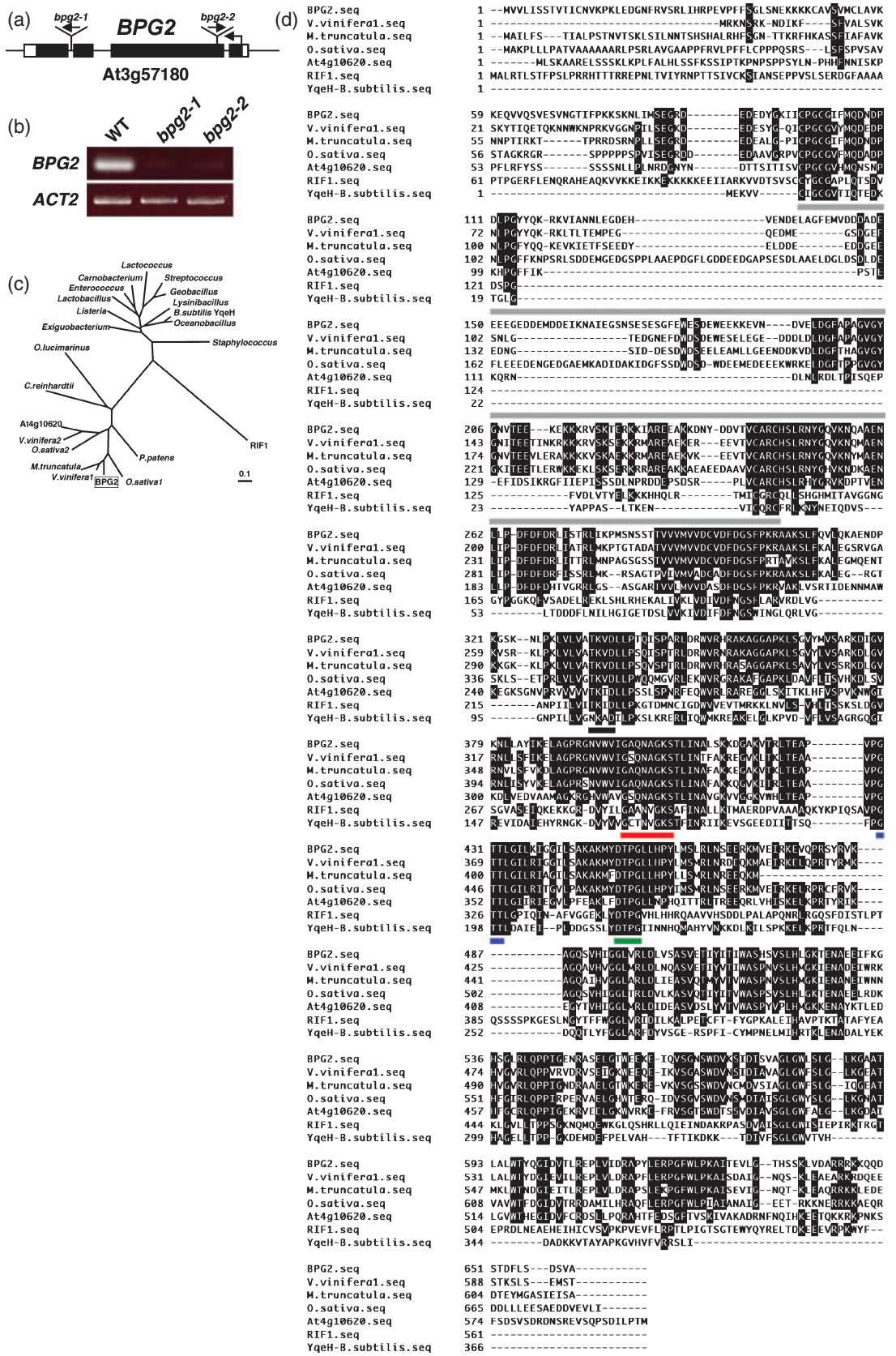

Figure 2. Identification and structure of BPG2.
(a) Gene structure of *BPG2* indicating T-DNA insertions causing mutations. T-DNA causing the *bpg2-1* mutation was inserted 1922 bp upstream of the start codon (ATG). T-DNA causing the *bpg2-2* mutation was inserted 113 bp downstream of the start codon.
(b) RT-PCR analysis of *BPG2* expression in wild-type (WT), *bpg2-1* and *bpg2-2*. *ACT2* was used as an internal control.
(c) Phylogenic analysis of the relationship between BPG2 and BPG2 homologs in plants, green algae and Gram-positive bacteria. GenBank accession numbers: *Oryza sativa 1*, CM000143; *O. sativa 2*, NM_001064237; *Vitis vinifera 1*, CU459251; *V. vinifera 2*, CU459220; *Medicago truncatula*, AC158502; *Physcomitrella patens*, XM_001758456; *Ostreococcus lucimarinus*, XM_001418245; *Chlamydomonas reinhardtii*, XM_001700742; *Listeria monocytogenes*, NC_003210; *Exiguobacterium sibiricum*, NC_010556; *Lactobacillus casei*, NC_008526; *Enterococcus faecium*, NZ_AAAK03000016; *Lactococcus lactis*, NC_009004; *Streptococcus sanguinis*, NC_009009; *Geobacillus thermodenitrificans*, NC_009328; *Lysinibacillus sphaericus*, NC_010382; *Staphylococcus haemolyticus*, NC_007168; *Oceanobacillus iheyensis*, NP_692909; *Bacillus subtilis*, Z99117.
(d) Sequence alignment of BPG2 and BPG2 homologs in plants and *Bacillus subtilis* YqeH. Colored bars under the sequence indicate the zinc finger domain (gray) and the GTP-binding motifs G4 (black), G1 (blue), G2 (red) and G3 (green).

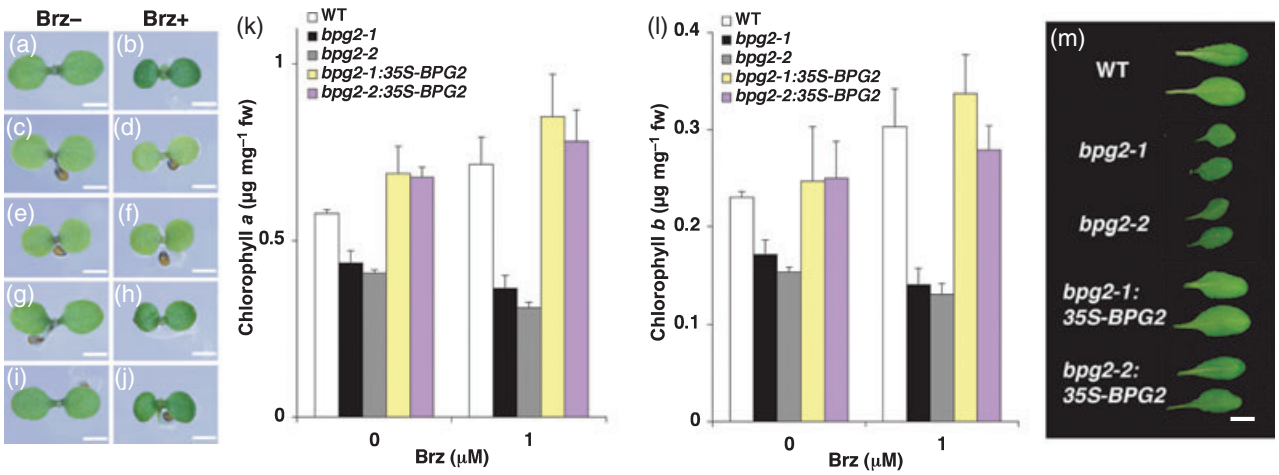

Figure 3. Effect of Brz on *bpg2-1*, *bpg2-2*, a complementation line of *bpg2-1* and a complementation line of *bpg2-2*.
(a–j) Cotyledons of wild-type (a, b), *bpg2-1* (c, d), *bpg2-2* (e, f), a complementation line of *bpg2-1* (g, h) and a complementation line of *bpg2-2* (i, j) grown on half-strength MS medium under long days (16 h light/8 h dark) without Brz (a, c, e, g, i) or with 1 μM Brz (b, d, f, h, j) for 5 days. Scale bars = 1 mm.
(k, l) Endogenous contents of chlorophyll *a* (k) and chlorophyll *b* (l) of wild-type (WT), *bpg2-1*, *bpg2-2*, a complementation line of *bpg2-1* (*bpg2-1:35S-BPG2*) and a complementation line of *bpg2-2* (*bpg2-2:35S-BPG2*) grown without Brz (0 μM) or with Brz (1 μM) for 4 days under long days (16 h light/8 h dark). Error bars indicate SE.
(m) Rosette leaf morphology of 3-week-old plants of wild-type (WT), *bpg2-1*, *bpg2-2*, *bpg2-1:35S-BPG2* and *bpg2-2:35S-BPG2*. Scale bar = 1 cm.

resulting *bpg2-1:35S-BPG2* and *bpg2-2:35S-BPG2* plants showed a normal green phenotype, confirming that decreased chlorophyll *a* and *b* levels in *bpg2-1* and *bpg2-2* were rescued by *BPG2* (Figure 3g–j,m).

The *bpg2:35S-BPG2* transformants also showed an increase in chlorophyll levels following Brz treatment, and rescue of Brz sensitivity in *bpg2-1* and *bpg2-2* (Figure 3k,l). Furthermore, the semi-dwarf rosette leaves of 3-week-old *bpg2-1* and *bpg2-2* were rescued by *BPG2* (Figure 3m and Table 1). Thus, these results show that the normal *BPG2* gene was able to complement the *bpg2* mutant and rescue the wild-type phenotype.

To investigate the contribution of the various domains to the role of BPG2 in chloroplast development, conserved amino acids in the zinc finger N-terminus (C98A, G100A, C101A and G102A), zinc finger C-terminus (C242A, R244A and C245A) and in the GTP-binding motifs G4 (K335A and D337A), G1 (G404A and K405A), G2 (T431A and T432A) and G3 (D450A and G453A) were replaced by alanine (Figure 4a), and constructed under the control of the CaMV 35S promoter and fused with the gene encoding green fluorescent protein (GFP) of the pGWB5 vector (Nakagawa *et al.*, 2007). These *35S::mutated BPG2-GFP* fusion constructs were transformed into the *bpg2-1* mutant (Figure 4b–g). Mutants *bpg2-1* with *35S::BPG2-GFP* (Figure 4h) and *bpg2-2* with *35S::BPG2-GFP* (Figure 4i) showed a wild-type normal green phenotype compared to the *bpg2-1* pale green phenotype (Figure 4j). However, when mutated *BPG2* genes

Table 1 Leaf sizes of wild-type, *bpg2-1*, *bpg2-2*, *bpg2-1:35S-BPG2* and *bpg2-2:35S-BPG2*

Plant	Leaf width (mm)	Leaf length (mm)	Ratio (length:width)
Wild-type	10.34 ± 0.59	18.45 ± 0.87	1.80 ± 0.07
bpg2-1	9.69 ± 0.28	14.05 ± 0.39	1.46 ± 0.04
bpg2-2	9.57 ± 0.34	13.40 ± 0.63	1.41 ± 0.07
bpg2-1:35S-BPG2	12.27 ± 0.67	21.95 ± 1.19	1.82 ± 0.11
bpg2-2:35S-BPG2	11.11 ± 0.38	23.12 ± 1.01	2.12 ± 0.16

Data are means ± SE (*n* = 12 for each genotype).

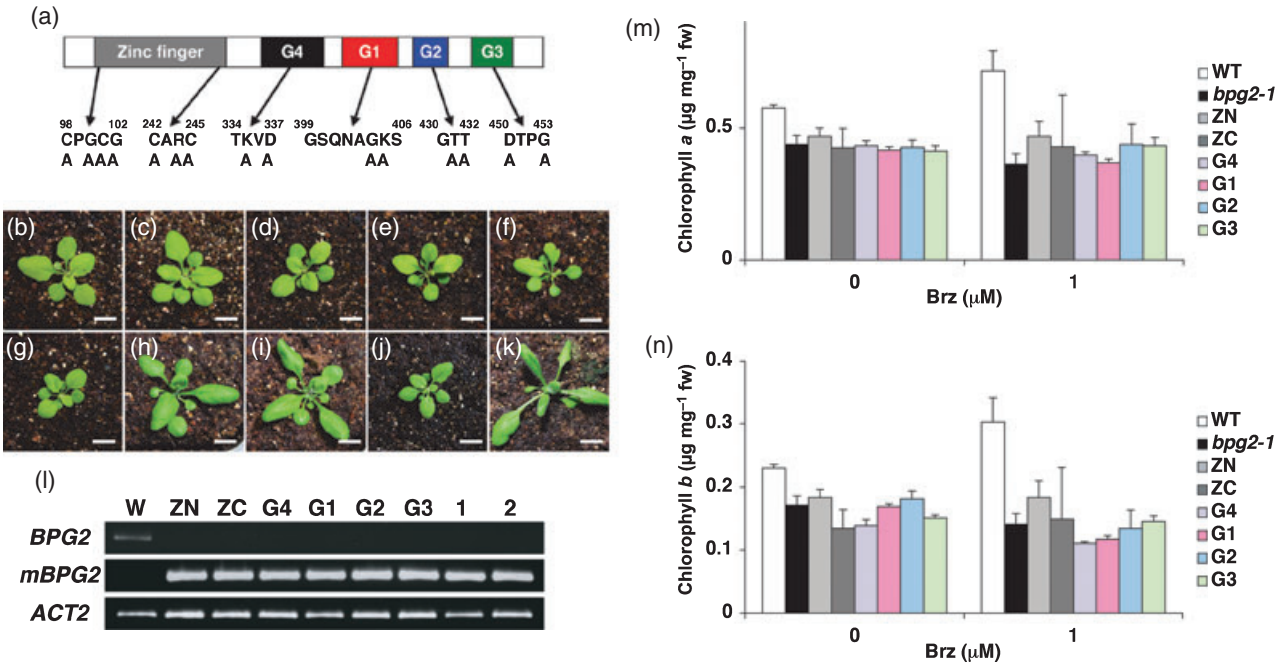

Figure 4. Phenotype of *bpg2-1* transformed with the wild-type BPG2 gene and the BPG2 gene mutated in the zinc finger domain and GTP-binding motifs.
(a) Predicted domain structure of BPG2 with targeted mutagenesis in the zinc finger motif or GTP-binding domains. The conserved amino acid sequences were changed to alanine.
(b–g) *bpg2-1* plants transformed with *35S::mutated BPG2-GFP* with mutations in the zinc finger N-terminus (b), the zinc finger C-terminus (c), and the G4 (d), G1 (e), G2 (f) and G3 domains (g). The *35S::wild-type BPG2–GFP* construct was transformed into *bpg2-1* (h) and *bpg2-2* (i). Control plants that were not transformed were *bpg2-1* (j) and wild-type Arabidopsis Col-0 (k). These plants were grown under long days (16 h light/8 h dark) on soil for 2 weeks.
(l) RT-PCR analysis of expression of *BPG2* and mutated *BPG2* in transformed *bpg2-1* and wild-type. Expression of each mutated *BPG2* was detected in *bpg2-1* transformed with *BPG2* cDNA mutated in the zinc finger N-terminus (ZN), the zinc finger C-terminus (ZC), and the G4, G1, G2 and G3 domains. Each mRNA was amplified using *bpg2*-specific and GFP-specific primers. Expression of wild-type *BPG2* was detected in wild-type Arabidopsis but not in transformed *bpg2-1* plants. *ACT2* was used as an internal control.
(m, n) Endogenous contents of chlorophyll *a* (m) and chlorophyll *b* (n) of wild-type (WT), *bpg2-1* and the six transformants grown without Brz (0 μM) or with Brz (1 μM) for 4 days under long days (16 h light/8 h dark). Error bars indicate SE.

driven by the CaMV 35S promoter were expressed in the *bpg2-1* mutant (Figure 4l), all six transformants remained pale green and could not be restored to the wild-type phenotype (Figure 4b–g). Furthermore, chlorophyll levels in the transformants remained low and Brz sensitivity was not rescued in *bpg2-1* and *bpg2-2* (Figure 4m,n). These results suggest that the GTP-binding motifs and zinc finger motif play important roles in chloroplast development and are regulated by BPG2.

Localization of BPG2 and function in chloroplast differentiation

To determine the subcellular localization of the BPG2 protein, a translational *BPG2-GFP* fusion was expressed under the control of the constitutive CaMV 35S promoter and introduced into wild-type Arabidopsis (Figure 4h). GFP fluorescence was detected in chloroplasts of guard cells of *35S::BPG2-GFP* plants (Figure 5b,c), and the signal was merged with chlorophyll autofluorescence (Figure 5a–d). These results suggest that the BPG2 protein is localized in chloroplasts.

The pale green phenotype of *bpg2* mutants and localization of BPG2 protein suggest that BPG2 plays a role in chloroplast morphology. To analyze the role of BPG2 in chloroplast differentiation, electron microscope observations of the wild-type and the *bpg2-1* mutant were performed (Figure 5i,j). Abnormal chloroplasts were observed in *bpg2-1* leaves. While 3-week-old wild-type chloroplasts had stacked grana thylakoids (Figure 5i), plastids of the *bpg2-1* mutant had fewer stacked grana in the thylakoids, more starch grains, and more and larger plastoglobules (Figure 5j). These results suggest that BPG2 is necessary for normal chloroplast differentiation.

Tissue-specific and light-regulated expression of *BPG2*

To analyze the possible function of BPG2 in plastids in various tissues, expression of *BPG2* under various conditions was examined using RT-PCR (Figure 6). The *BPG2* gene was highly expressed in stems, petioles, rosette leaf blades, cauline leaves and flowers of 3-week-old wild-type plants, but only faintly in roots (Figure 6a). As *BPG2* gene expression was found in all green tissues, the effect of light on the

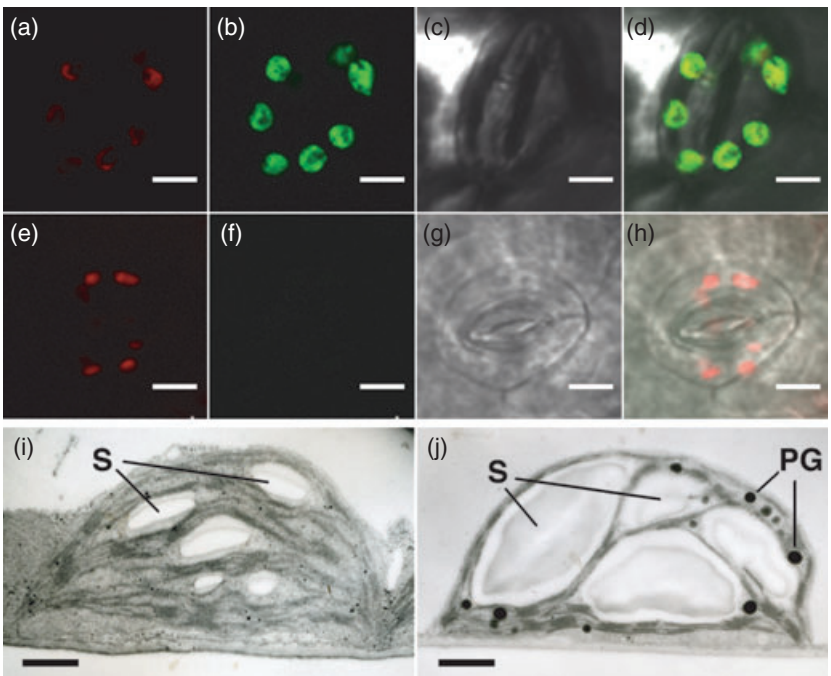

Figure 5. Localization of BPG2 protein in chloroplasts, and morphology of the chloroplast in *bpg2-1*.
(a–h) Confocal laser scanning microscopy of guard cells in *35S::BPG2-GFP* transformants (a–d) and wild-type plants (e–h). Plants were grown for 2 weeks on half-strength MS medium containing kanamycin. (a, e) Red autofluorescence of chlorophyll. (b, f) Green fluorescence of GFP. (c, g) Bright-field images. (d) Merged image of (a–c). (h) Merged image of (e–g). Scale bars = 5 μm.
(i, j) Electron microscopy of wild-type (i) and *bpg2-1* (j) chloroplasts in rosette leaves. Plants were grown on soil for 3 weeks under long-day conditions. PG, plastoglobule; S, starch granule. Scale bars = 1 μm.

expression of *BPG2* was analyzed using total RNA isolated from seedlings harvested at 0, 0.5, 1, 2 and 4 h after transfer of dark-grown plants to light (Figure 6b). In light-stimulated plants, two nuclear-encoded genes, *CAB*, the light-harvesting chlorophyll *a*/*b* binding protein, and *rbcS*, the small subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), began to be expressed after 0.5 and 2 h of light stimulation (Figure 6b), and expression of *BPG2* continued after 2 h of light treatment. This is consistent with the expression patterns of *CAB* and *rbcS* (Figure 6b).

Dark-grown BR-deficient mutants expressed chloroplast genes, such as *CAB* and *rbcS* (Chory *et al.*, 1991; Szekeres *et al.*, 1996), and dark-grown wild-type plants treated with Brz accumulated more Rubisco protein than the wild-type without Brz (Nagata *et al.*, 2000). To study the effect of BR on *BPG2* gene expression, we performed RT-PCR analysis of wild-type plants grown in the dark with Brz (Figure 6c), and found that expression of *CAB* and *rbcS* was increased by Brz. *DWF4* encodes cytochrome P450 (CYP90B1), and its expression is increased by feedback mechanisms in BR-deficient mutants. These expression levels showed that Brz treatment of dark-grown wild-type caused BR deficiency and promoted chloroplastic gene expression in the dark (Figure 6c). In the Brz-treated tissues, *BPG2* gene expression actually increased (Figure 6c), suggesting that *BPG2* gene expression is negatively regulated by BR and positively by light in green organs.

Expression of genes encoded by the chloroplast genome of the *bpg2* mutant

The plastid genome of Arabidopsis encodes approximately 87 open reading frames (ORFs) and four rRNAs on 154 kbp of DNA (Arabidopsis Genome Initiative, 2000). Transcriptional, post-transcriptional and translational regulatory mechanisms in chloroplasts have been analyzed (Leister, 2003), but molecular mechanisms for chloroplast regulation by brassinosteroid remain unknown. To investigate the function of BPG2 responsible for the pale green phenotype, we performed expression analysis of chloroplast-encoded photosynthesis genes by Northern blot analysis using wild-type and *bpg2* plants (Figure 7). No reduction in expression of chloroplast-encoded *rbcL*, the large subunit of Rubisco, and *psbA*, a D1 protein of photosystem II, was found in *bpg2* mutants compared to the wild-type in seedlings (Figure 7a) or rosette leaves at the reproductive stage (Figure 7b). There was also no reduction in expression of *CAB* and *rbcS* in the mutants (Figure 7a,b). Brz stimulated increased expression of *psbA*, *rbcL*, *CAB* and *rbcS* in *bpg2* mutants to the same degree as in wild-type (Figure 7c).

Essential role of BPG2 for chloroplast rRNA maturation

The chloroplast genome encodes 16S and 23S rRNA. These rRNAs are encoded in a single operon with three tRNAs, and are expressed as a 7.4 kb precursor that is post-transcriptionally processed (Figure 8a) (Strittmatter and Kössel, 1984). We performed Northern blot analysis of chloroplast rRNA in wild-type and *bpg2* mutants at the seedling and reproductive stages (Figure 8b,c) using the specific probes (I–V) indicated in Figure 8a.

When blots were analyzed using a 16S rRNA-specific probe (probe I), the levels of mature 16S rRNA transcript (1.5 kb) were lower in *bpg2* mutants compared with the wild-type at both seedling and reproductive stages (Figure 8b,c).

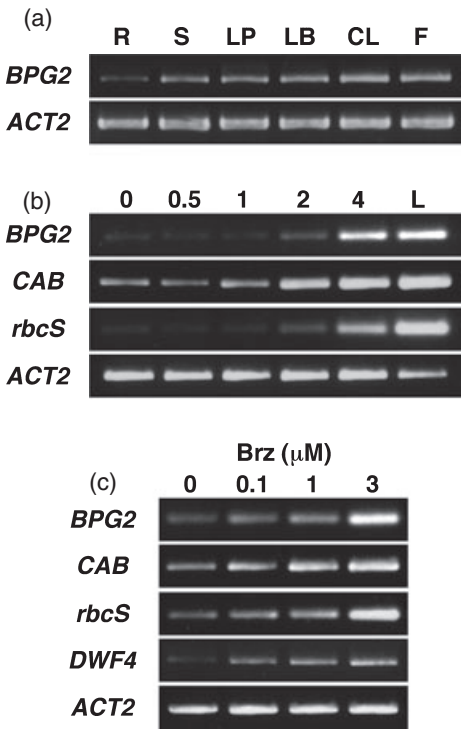

Figure 6. Inducible expression of *BPG2* by light and Brz.
(a) RT-PCR analysis of *BPG2* gene expression in various organs: root (R), stem (S), rosette leaf blade (LB), rosette leaf petiole (LP), cauline leaves (CL) and flowers (F) of wild-type Arabidopsis. *ACT2* was used as an internal control.
(b) RT-PCR analysis of expression of *BPG2*, *CAB* and *rbcS* after exposure to light. Total RNAs were extracted from the wild-type germinated in the dark for 7 days and exposed to light for 0, 0.5, 1, 2 or 4 h, and from 7-day-old wild-type plants grown under long-day conditions (16 h light/8 h dark) (L).
(c) RT-PCR analysis of expression of *BPG2*, *CAB*, *rbcS* and *DWF4* in plants treated with Brz. Total RNAs were extracted from wild-type germinated in the dark for 7 days with 0, 0.1, 1 or 3 μM Brz.

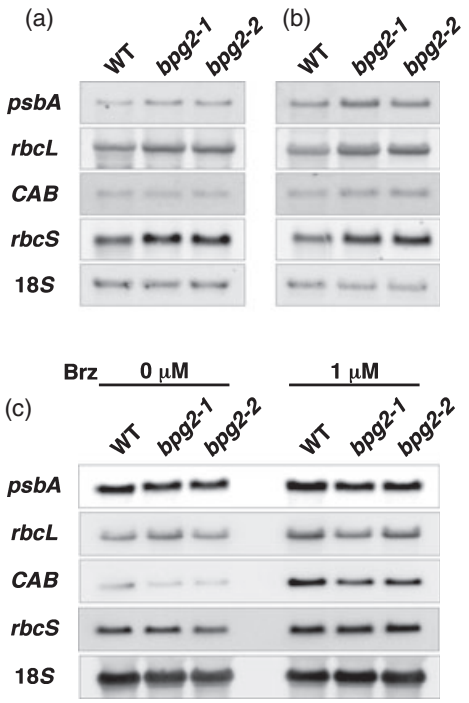

Figure 7. Chloroplast gene expression in *bpg2* mutants.
Total RNA was extracted from light grown 4-day-old seedlings (a), 3-week-old rosette leaves (b) and dark-grown 5-day-seedlings (c) of wild-type (WT), *bpg2-1* and *bpg2-2*. Northern blot analysis was performed using probes for *psbA* and *rbcL*, encoded in the chloroplast, and *rbcS*, *CAB* and 18S rRNA, encoded in the nucleus.
(c) Seedlings were germinated with 0 or 1 μM Brz in the dark.

The accumulation of a 1.7 kb precursor transcript was higher in *bpg2* than in wild-type. Mature 16S rRNA is generated by endonucleolytic cleavage of the intergenic space of a primary transcript, approximately 180 bp downstream of the mature 16S 3′ end. To identify the 1.7 kb RNA band as pre-16S rRNA, blots were analyzed using probe II, which is specific to the intergenic spacer of the 16S rRNA flanking region. Probe II detected 1.7 kb RNA in *bpg2* mutants, but not in the wild-type, suggesting that pre-16S rRNA accumulates in *bpg2* (Figure 8b,c).

When blots were analyzed with a 23S rRNA-specific probe (probe III), 23S rRNA accumulated as seven major transcripts, i.e. 3.2, 2.9, 2.4, 1.7, 1.2, 1.0 and 0.5 kb bands (Figure 8a). At both seedling and reproductive stages, no differences in the size of the seven transcripts between wild-type and the *bpg2* mutant were observed. The 3.2 kb band, which represents a 23S–4.5S di-cistronic precursor, accumulated at levels that were approximately three times greater in *bpg2* mutants than in the wild-type at the seedling stage (Figure 8b), and approximately 8.5 times greater in *bpg2* mutants at the reproductive stage (Figure 8c). The 2.4 kb band decreased in *bpg2* at the seedling stage (Figure 8b), but the 2.9 and 2.4 kb bands increased four- and eightfold, respectively, in *bpg2* mutants at the reproductive stage (Figure 8c). The levels of the 1.2 and 1.0 kb bands, which are produced by 'hidden breaks' after incorporation into ribosomes, did not differ between *bpg2* mutants and the wild-type (Figure 8b,c).

When blots were analyzed using 4.5S and 5S rRNA-specific probes (probes IV and V), the 3.2 kb band that represents the 23S–4.5S precursor was also detected in *bpg2* mutants (Figure 8b,c). Pre-matured precursor bands were not detected in *bpg2* using the 5S rRNA-specific probe, but decreased levels of matured 5S rRNA were found in *bpg2* mutants at the seedling stage, with increased levels of matured 5S rRNA in *bpg2* mutants at the reproductive stage (Figure 8b,c). These results suggest that BPG2 protein plays an important role in processing or maturation of chloroplast rRNA.

Decreased accumulation of chloroplast proteins in *bpg2*

To test whether abnormal rRNA processing or maturation in *bpg2* chloroplasts has an effect on chloroplast protein

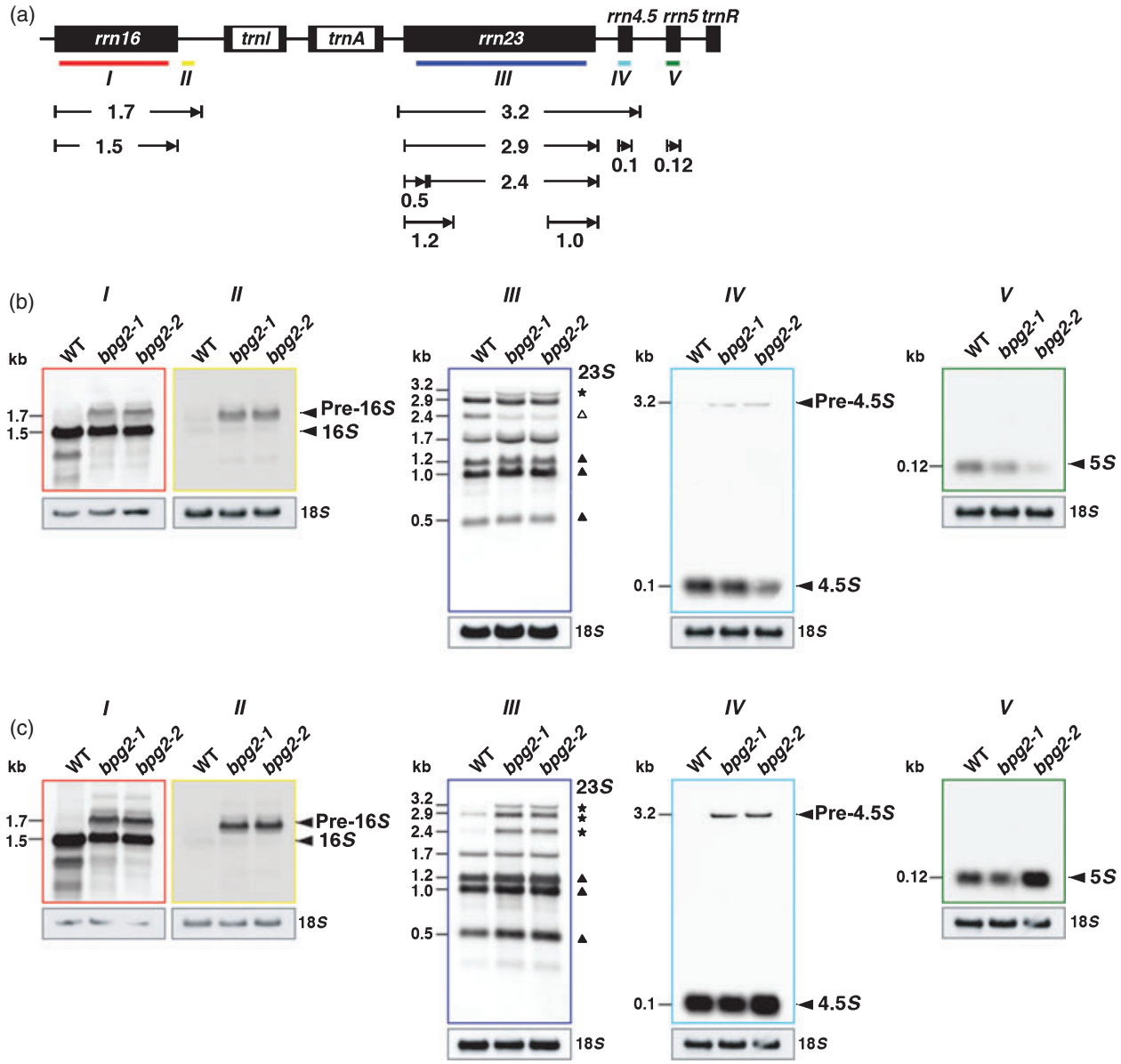

Figure 8. Accumulation of pre-mature chloroplast rRNA in *bpg2*.
(a) Diagram of the rRNA operon, and sizes of the transcripts (kb) shown in (b, c). The locations of probes (I–V) used for Northern blot analyses are indicated by color bars: I (red), II (yellow), III (purple), IV (blue) and V (green).
(b) Northern blot analysis of 4-day-old seedlings of wild-type (WT), *bpg2-1* and *bpg2-2*. An 18S standard was used to determine equal loading. The 3.2 kb band that is increased in the mutants is indicated by an asterisk and the 2.4 kb band that is decreased in the mutants is indicated by an open triangle. Transcripts with a hidden break are indicated by filled triangles.
(c) Northern blot analysis of 3-week-old rosette leaves of wild-type (WT), *bpg2-1* and *bpg2-2*. The bands of 3.2, 2.9 and 2.4 kb that are increased in the mutants are indicated by asterisks, and transcripts with a hidden break are marked by filled triangles.

accumulation, total protein from *bpg2* mutants and wild-type was analyzed by immunoblotting (Figure 9). The level of the photosystem II D1 protein encoded by *psbA* was markedly lower in *bpg2* mutants than in the wild-type at both seedling (Figure 9c) and reproductive stages (Figure 9d). The level of the thylakoid light-harvesting chlorophyll *a*/*b* binding protein (LHCP) encoded by *CAB* was slightly decreased in *bpg2* in both seedling (Figure 9e) and rosette leaves (Figure 9f). Accumulation of the Rubisco large subunit (LSU) protein encoded by *rbcL* and the Rubisco small subunit (SSU) protein encoded by *rbcS* was lower in *bpg2* mutants at both seedling (Figure 9g) and reproductive stages (Figure 9h). These results show that translation of chloroplast proteins encoded by the chloroplast genome decreased in *bpg2* chloroplasts.

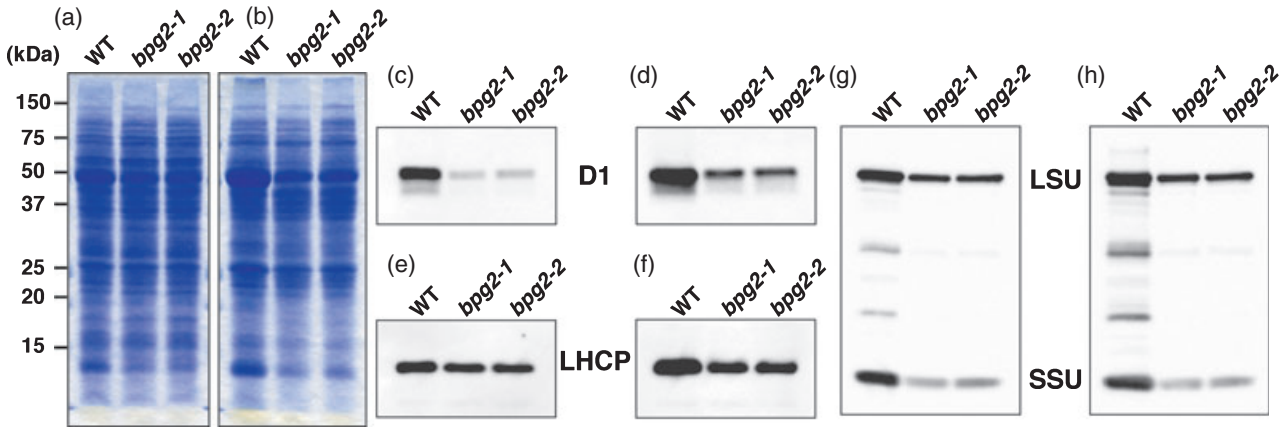

Figure 9. Decreased accumulation of proteins from genes encoded on the chloroplast genome in *bpg2*.
Total protein was prepared from 4-day-old seedlings (a, c, e, g) and 3-week-old rosette leaves (b, d, f, h) of wild-type (WT), *bpg2-1* and *bpg2-2*. (a, b) Gel stained with Coomassie brilliant blue.
(c–h) Immunoblot analyses were performed using polyclonal antibodies against photosystem II D1 protein (c, d), LHCP protein (e, f) and Rubisco LSU and SSU (g, h).

Chloroplast protein accumulation was not increased by Brz in bpg2 mutants

To analyze BR-mediated BPG2 function on chloroplast protein accumulation, we performed an immunoblot analysis of seedlings of wild-type and *bpg2* mutants in the light with and without Brz (Figure 10). In the wild-type, the level of Rubisco LSU protein from Brz-treated seedlings increased approximately 1.4-fold and that of D1 protein increased approximately 1.3-fold compared to that of seedlings that were not treated with Brz (Figure 10a,b). In *bpg2* mutants, the levels of Rubisco LSU protein and D1 protein in Brz-treated seedlings were the same as those in seedlings seedlings that were not treated with Brz (Figure 10a,b). In the wild-type, the levels of nuclear-encoded LHCP protein and Rubisco SSU protein were also increased by Brz treatment (Figure 10c,d). The effect of BPG2 deficiency on the level of LHCP protein was much smaller than that on D1 and Rubisco LSU.

DISCUSSION

BPG2 functions as a translational regulator of brassinosteroid signaling

BRs and their biosynthesis inhibitor Brz can regulate not only plant development but also chloroplast development. The dark-grown BR-deficient mutant *det2* and dark-grown wild-type plants treated with Brz showed photomorphogenesis and expression of the photosynthetic genes *rbcS* and *CAB* (Chory *et al.*, 1991; Asami *et al.*, 2000, 2001), and increased accumulation of Rubisco LSU and SSU protein (Nagata *et al.*, 2000). Although the physiological relationships between BR and chloroplast regulation are clear, the molecular mechanism has not been revealed.

Here, we screened a Brz-insensitive pale green mutant, *bpg2*, and found that the phenotype was caused by disruption of a novel chloroplast protein containing a putative zinc finger motif and GTP-binding domains. Brz was shown to induce greening of wild-type Arabidopsis, but the pale green phenotype of the *bpg2* mutant could not be recovered by Brz. Brz increased endogenous chlorophyll levels in wild-type Arabidopsis, but chlorophyll synthesis was not increased in the *bpg2* mutants (Figure 1e,f).

Sodium nitroprusside (SNP) is a nitric oxide donor that has been shown to induce greening in wild-type Arabidopsis (Flores-Pérez *et al.*, 2008). When both wild-type Arabidopsis and *bpg2* mutants were treated with SNP under the same conditions as for Brz treatment described in Figure 1, the chlorophyll content of *bpg2* mutants was increased (Figure S1). These results suggest that the Brz-insensitive pale green phenotype of *bpg2* specifically depends on BR signaling. Furthermore, the abnormal chloroplast ultrastructure observed for *bpg2-1* by electron microscopy (Figure 5j and Figure 6), together with the reduced *BPG2* gene induction in response to Brz, suggest that the BPG2 protein plays an important role in regulation of plastid differentiation under BR signal transduction. The present paper shows the effect of a chloroplast morphogenesis mutant on BR signaling.

Although *bpg2-1* showed semi-dwarf leaves and inflorescences, the shape was restored to wild-type by transformation with the wild-type *BPG2* gene (Figure 3 and Table 1). These results suggest that BPG2 could affect plant development as a consequence of regulation of chloroplast development and photosynthesis by BPG2 itself.

A working hypothesis for BPG2 function in chloroplast regulation is shown in Figure S2. In *bpg2* mutants, there was no reduction in expression of *psbA* and *rbcL* (Figure 7a,b), but accumulation of D1 protein encoded by the *psbA* gene and Rubisco LSU protein translated from the *rbcL* gene was decreased compared to the wild-type (Figure 9c,d,g,h). In

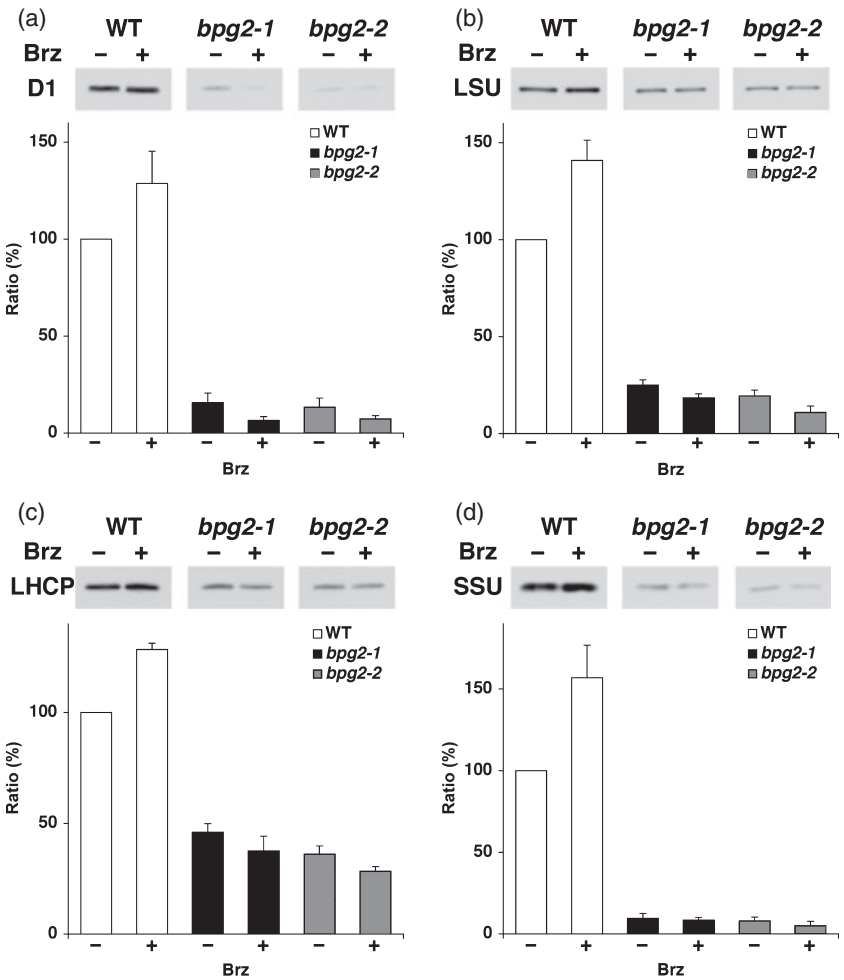

Figure 10. Accumulation of chloroplast proteins was not increased by Brz in *bpg2*.
Total protein was prepared from wild-type (WT), *bpg2-1* and *bpg2-2* germinated in the light for 3 days with 0 or 1 μM Brz. Immunoblot analyses were performed using polyclonal antibodies against photosystem II D1 protein (a), LHCP protein (b) and Rubisco LSU (c) and SSU (d). Error bar indicates SE.

contrast to these chloroplast-encoded proteins, there was a lower ratio of reduction for the nuclear-encoded protein LHCP in *bpg2* mutants relative to the wild-type (Figure 9e,f). In the *bpg2* mutant, we identified abnormal accumulations of pre-16S rRNA and pre-23S rRNA, and abnormal fragmentation of 23S rRNA compared to wild-type (Figure 8). This fragmentation of 23S rRNA is also widespread in bacteria.

In many bacteria, rRNA splicing and fragmentation are tightly related to quality control of rRNA during assembly of the ribosomal subunits, and have been shown to be important for cell viability (Cheng and Deutscher, 2003; Evguenieva-Hackenberg, 2005). Bacterial pre-23S and pre-16S rRNA are spliced after poly-cistronic transcription by the endoribonuclease RNase III (Evguenieva-Hackenberg, 2005). Post-transcriptional regulation of mRNA has also been analyzed in detail for bacteria. Bacterial mRNA is generally encoded without introns, and the full-length mRNA is not regulated by splicing but is controlled by degradation with exoribonuclease (Kennell, 2002). The post-transcriptional regulation of bacterial mRNA and rRNA is considered to be controlled by two different systems. As the chloroplast gene expression system is considered to be similar to the prokaryotic system, the abnormal levels of chloroplast protein in *bpg2* may be regulated by accumulation of abnormal rRNA.

Finally we analyzed the effect of Brz on chloroplast protein accumulation in the light-germinated *bpg2* mutant. For the wild-type, accumulation of chloroplast genome-encoded Rubisco LSU protein and D1 protein clearly increased under Brz treatment in the light (Figure 10). In contrast, Brz had little effect on chloroplast protein accumulation in the light-germinated *bpg2* mutant (Figure 10). These results showed that disruption of *BPG2* function interferes with the stimulation of chloroplast protein translation or accumulation by Brz. Thus, BPG2 may regulate chloroplast protein translation and/or accumulation according to the regulation of chloroplast rRNA maturation in BR signal transduction. Although unspliced pre-16S and pre-23S rRNA could be clearly detected in the *bpg2* mutant, normally spliced 16S and 23S rRNAs appeared to be the major products. Nonetheless, the levels of D1 and RubisCo LSU proteins were greatly reduced in *bpg2* mutants. Enhanced inhibition of translation has also been detected in an Arabidopsis mutant of *RNR1*, an exoribonuclease for chloroplast rRNA, but the molecular

mechanism of the protein decreasing in the *rnr1* mutant has yet to be elucidated (Bollenbach *et al.*, 2005). In *bpg2*, abnormal ribosomes with unspliced rRNA could limit the rate of translation, and repeated translational delay might cause the greater reduction of protein accumulation that is observed in these mutants.

ClpB3 and ClpC1 are thought to act as molecular chaperones for chloroplast protein folding, and the mutants *clpB3* and *clpC1* showed a pale green phenotype and reduced accumulation of photosynthetic protein complexes (Myouga *et al.*, 2006; Nakagawara *et al.*, 2007). Under conditions where levels of Rubisco LSU protein and D1 protein increased in response to Brz treatment of the wild-type in the light, no induction of these chloroplast proteins was observed in *clpB3* and *clpC1* mutants (Figure S3a). The results thus suggest that ClpB3 and ClpC1 might be new members of the group of chloroplast protein regulatory factors involved in BR signaling. Chloroplast protein regulation by BR might be controlled not only by BPG2 but also by a number of other players. Nevertheless, the lower chloroplast protein accumulation in the *bpg2* mutant (Figure 10) and the higher induction of *BPG2* gene expression by Brz in comparison with *ClpB3* and *ClpC1* (Figure S3b) suggests that BPG2 plays an especially important role for chloroplast protein regulation in BR signal transduction.

BPG2 as a novel regulator of chloroplast rRNA processing

The *BPG2* gene encodes a putative 660 amino acid sequence (Figure 2d). A further search showed that *BPG2* homologues are found in Gram-positive bacteria, such as *Listeria monocytogenes*, *Lactococcus lactis* and *B. subtilis* (Figure 2c). The *B. subtilis* YqeH proteins have been recently characterized (Loh *et al.*, 2007; Uicker *et al.*, 2007), and found to possess a highly conserved zinc finger motif ($CXXCX_nCXXC$) that has previously been found in ribosomal proteins and may participate in protein-RNA interaction (Anand *et al.*, 2006; Uicker *et al.*, 2007). Arabidopsis BPG2 has a putative zinc finger motif and GTP-binding domains that are similar to those of YqeH (Figure 2d). We constructed a mutated cDNA of *BPG2* in which conserved amino acids of the zinc finger motif and four GTP-binding domains were replaced by with alanine, and transformed the *bpg2-1* mutant with the mutated *BPG2 cDNA* (Figure 4a). All six transformants showed a pale green phenotype (Figure 4b–g,m,n), which was not rescued by the mutated *BPG2*. These results suggested that the zinc finger motif and GTP-binding domains are necessary for BPG2 function, and possibly regulate chloroplast biogenesis.

In this paper, we have shown the accumulation of pre-16S rRNA and pre-23S rRNA in *bpg2* mutants (Figure 8). In Arabidopsis, factors related to chloroplast rRNA processing have been isolated. An Arabidopsis mutant *rnr1* lacking exoribonuclease showed accumulation of pre-16S, pre-23S and pre-4.5S rRNA (Kishine *et al.*, 2004; Bollenbach *et al.*, 2005). The levels of 23S rRNA processed at hidden breaks of 1.2, 1.0, and 0.5 kb were decreased in the *rnr1* mutant but accumulated in *bpg2* mutants at similar levels to the wild-type (Figure 8b,c). Pre-16S rRNA and pre-23S–4.5S rRNA di-cistronic processing intermediates accumulated in the Arabidopsis *dal1* mutant (Bisanz *et al.*, 2003). In *dal1*, expression of *CAB* and *rbcL* decreased in comparison to the wild-type. Unlike *dal1*, expression of *CAB* and *rbcL* in *bpg2* mutants did not differ from that in the wild-type (Figure 7a,b).

As described previously, *B. subtilis* YqeH is homologous to BPG2, and YqeH-depleted cells accumulate pre-16S rRNA (Loh *et al.*, 2007; Uicker *et al.*, 2007). GTP binding domains G4-G1-G2-G3 are highly conserved between *B. subtilis* YqeH and Arabidopsis BPG2 (Figure 2d). YqeH is a member of the Era/Obg family, which is involved in assembly of ribosomal subunits (Matsuo *et al.*, 2007). In Arabidopsis, at least one homolog to YqeH has been identified, under three gene names (*RIF*/*NOS*/*NOA*), and the knock-out phenotype was pale green leaves. From analysis of *rif* mutants, it appears that RIF1 protein is involved in post-transcriptional up-regulation of isoprenoid biosynthesis proteins in chloroplasts (Flores-Pérez *et al.*, 2008). NOS protein was found to bind specifically to GTP and had GTP hydrolysis activity (Moreau *et al.*, 2008). A chimeric YqeH protein comprising the transit peptide of AtNOA1 and bacterial GsYqeH from *Geobacillus* complemented the pale green phenotype of the *Atnoa1* mutant. From these analyses, it is not possible to establish whether RIF1, NOS1 and NOA1 are involved in both regulation of the chloroplast ribosome as well as regulation of chloroplast rRNA. However, our studies suggest that the BPG2 protein has a novel function in regulating chloroplastic 16S and 23S rRNA maturation, and these results have not yet been analyzed by previous authors, using plant YqeH homologous proteins. The relationship between BPG2 function and ribosomal regulation promises to be very interesting, and these analyses will clarify the molecular mechanism of chloroplast protein synthesis in the future.

A homologous gene of *BPG2* and *RIF*/*NOS*/*NOA*, *At4g10620*, has also been identified, and the GTP binding domains G4-G1-G2-G3 are conserved in the three genes (Figure 2c,d). Based on hydropathicity plot analysis, N-terminal hydrophobic amino acid sequences in BPG2 and RIF/NOS/NOA were identified that were predicted to be chloroplast transit peptides. By contrast, an N-terminal sequence of the At4g10620 protein was predicted to be hydrophilic, indicating that At4g10620 protein is not transported into the chloroplast. This suggests that, with respect to functional homology, BPG2 might be closer to RIF/NOS/NOA than to At4g10620.

BLAST searches with the BPG2 amino acid sequence revealed that *BPG2*-homologous genes are widespread in dicot and monocot plants, including Arabidopsis, rice,

Medicago truncatula and grape (Figure 2c,d). BPG2 homologs are also present in the green algae *O. lucimarinus* and *C. reinhardtii*, and Gram-positive bacteria, such as *L. monocytogenes*, *L. lactis* and *Bacillus subtilis* (Figure 2c). These results suggest that the *BPG2*-homologous gene family might have been conserved during evolution, before symbiosis of ancestral green algae into higher plants. rRNA fragmentation and processing has been found widely in bacteria and extensively researched, although the enzymatic machinery has not yet been elucidated. The evolutionary conservation between BPG2 and the proteins of many plant organelles and bacteria (Figure 2c,d) can be used to elucidate mechanisms of rRNA processing and translational regulation.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana Columbia-0 (Col-0) was used as the wild-type. For cotyledon analysis, plants were germinated and grown on half-strength MS medium (Duchefa, http://www.duchefa.com) containing 1.5% sucrose and 0.9% phytoagar (Duchefa), with or without Brz. Germinated plants were transferred to soil. Conditions in the growth chamber were 16 h light (100 μE m^{-2} sec^{-1} white light)/8 h dark at 22°C.

Measurement of chlorophylls *a* and *b*

Chlorophyll was extracted from 3-day-old seedlings grown in the light (100 μE m^{-2} sec^{-1}) under long days (16 h light/8 h dark). Plants were homogenized in 80% v/v acetone. The chlorophyll content of the centrifuged supernatants was determined at 645 and 663 nm. Chlorophyll *a* and *b* content were determined as described previously by Arnon (1949):

$$\text{Chlorophyll } a\ (\mu\text{g/mg fresh weight}) = \frac{(12.7A_{663} - 2.59A_{645})}{\text{mg fresh weight}}$$

$$\text{Chlorophyll } b\ (\mu\text{g/mg fresh weight}) = \frac{(22.9A_{645} - 4.67A_{663})}{\text{mg fresh weight}}$$

TAIL-PCR

To identify the flanking genomic sequence of the T-DNA of pPCVICEn4HPT, we performed thermal asymmetric interlaced PCR (TAIL-PCR) as described previously (Liu *et al.*, 1995). Genomic DNA was extracted from 3-week-old Arabidopsis rosette leaves using nucleon PHYTOpure PLANT DNA extraction (Amersham, http://www5.amershambiosciences.com/). The T-DNA flanking sequence was amplified using the T-DNA-specific primers LB150 (5′-CACGTCGAAATAAAGATTTCCG-3′) for the TAIL1 reaction, LB100 (5′-CCTATAAATACGACGGATGC-3′) for the TAIL2 reaction and LB50 (5′-ATAATAACGCTGCGGACATCT-3′) for the TAIL3 reaction, and degenerate primers AD2 (5′-NGTCGASWGANAWGAA-3′) or AD5 (5′-SSTGGSTANATWATWCT-3′) for all three reactions (S = G or C, W = A or T, N = A, G, C or T).

Generation of BPG2–GFP transgenic plants

The *BPG2* cDNA was amplified from wild-type Col-0 cDNA by RT-PCR using KOD-plus DNA polymerase (Toyobo, http://www.toyobo.co.jp). The PCR product was cloned into the pENTR®/D-TOPO™ vector using the pENTR™ directional TOPO® cloning kit (Invitrogen, http://www.invitrogen.com/). Site-directed mutagenesis for *BPG2* was performed as described previously (Higuchi *et al.*, 1988), and PCR products of mutated *BPG2* genes were cloned into the pENTR®/D-TOPO™ vector. Using Gateway technology (Invitrogen), the resulting pENTR-BPG2 and pENTR-mutated BPG2 vectors were further cloned into the binary vector pGWB5 (Nakagawa *et al.*, 2007), which contains a CaMV 35S promoter. The generated constructs *35S::BPG2-GFP* and *35S::mutated BPG2–GFP* were transformed into wild-type Col-0, *bpg2-1* or *bpg2-2* using the *Agrobacterium*-mediated floral dip method. Transgenic plants were screened on half-strength MS agar plates containing 25 μg ml^{-1} kanamycin.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. The chlorophyll *a* and *b* contents for wild-type and the *bpg2* mutant were increased by the nitrate oxide donor SNP.

Figure S2. Possible function of the BPG2 protein in wild-type and the *bpg2* mutant.

Figure S3. Accumulation of chloroplast proteins was not increased by Brz in *clp* mutants, but *BPG2* mRNA was highly induced by Brz in comparison with *CLP* mRNAs.

Appendix S1. Supplementary experimental procedures for RT-PCR, Northern blot analysis, immunoblot analysis of chloroplast proteins, confocal laser scanning microscopy, and electron microscopy.

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