The tetratricopeptide repeat-containing protein slow green1 is required for chloroplast development in Arabidopsis

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Abstract

A new gene, SG1, was identified in a slow-greening mutant (sg1) isolated from an ethylmethanesulphonate-mutagenized population of Arabidopsis thaliana. The newly formed leaves of sg1 were initially albino, but gradually became pale green. After 3 weeks, the leaves of the mutant were as green as those of the wild-type plants. Transmission electron microscopic observations revealed that the mutant displayed delayed proplastid to chloroplast transition. The results of map-based cloning showed that SG1 encodes a chloroplast-localized tetratricopeptide repeat-containing protein. Quantitative real-time reverse transcription–PCR data demonstrated the presence of SG1 gene expression in all tissues, particularly young green tissues. The sg1 mutation disrupted the expression levels of several genes associated with chloroplast development, photosynthesis, and chlorophyll biosynthesis. The results of genetic analysis indicated that gun1 and gun4 partially restored the expression patterns of the previously detected chloroplast-associated genes, thereby ameliorating the slow-greening phenotype of sg1. Taken together, the results suggest that the newly identified protein, SG1, is required for chloroplast development in Arabidopsis.

Key words: Albino, Arabidopsis thaliana, chloroplast development, proplastid to chloroplast transition, slow greening, tetratricopeptide repeat-containing protein.

Introduction

The chloroplast is a crucial organelle in higher plants. It is essential for fixation of CO₂ and also for biosynthesis of carbon skeletons, fatty acids, pigments, and amino acids from inorganic nitrogen (Staehelin, 2000). Plastids are generally believed to have originated from a unicellular photosynthetic bacterium, which was incorporated into a eukaryotic host cell (Dyall, 2004). During evolution, most of the genes encoded by the bacterial ancestor were transferred to the host nuclear genome. For example, the plastid genome of Arabidopsis thaliana encodes ~100 proteins; however, >2000 proteins are encoded by the nuclear genes that function in the chloroplast (Abdallah et al., 2000; Richly and Leister, 2004; Cui, 2006). Consequently, normal plastid development depends on the coordination of nuclear and plastid signals. This coordination is accomplished by nuclear signals that regulate the expression of plastid-encoded and nuclear-encoded plastid proteins, and also by signals sent from the developing plastids to the nucleus.

Plastids send signals to the nucleus via retrograde signaling, which operates through four distinct signal transduction pathways that are dependent on tetrapyrrole biosynthesis, plastid gene expression, the plastid redox state, or reactive oxygen species (ROS) (Surpin et al., 2002; Nott et al., 2006; Kakizaki et al., 2009). The identification and characterization...
of the genomes uncoupled (gun) mutants (gun1–gun6), in which the developmental status of plastids and the nuclear-encoded chloroplast genes are uncoupled, have considerably enhanced understanding of retrograde signalling (Susek et al., 1993; Mochizuki et al., 2001; Larkin et al., 2003; Woodson et al., 2011). An example of this uncoupling is the abnormally high expression level of light-harvesting chlorophyll ab-binding protein 1 (LHCBI.1) in gun mutants when chloroplast development is blocked by the herbicide norflurazon {4-chloro-5-(methylamino)-2-[3-(trifluoromethyl)phenyl]-3-(2H)-pyridazinone} (Susek et al., 1993; Mochizuki et al., 2001).

The process of chloroplast biogenesis is a complex plastic process, involving the interaction of environmental, cellular, and temporal factors (Pogson and Albrecht, 2011). The most influential environmental factor is light—in the absence of light, proplastids change into etioplasts (Robertson and Laetsch, 1974). Cellular factors include factors inside and outside the plastids. Most of the identified cellular factors are chloroplast-localized proteins involved in protein import, chloroplast gene transcription, RNA maturation, and protein translation and assembly (Pogson and Albrecht, 2011). Temporal factors such as embryo maturation can also influence chloroplast development (Albrecht et al., 2008; Kim et al., 2009; Pogson and Albrecht, 2011).

In flowering plants, the development and activity of chloroplasts differ between cotyledons and true leaves. In cotyledons, plastids partially develop during embryogenesis; however, their development is arrested during seed maturation and dormancy. Cotyledons serve primarily as storage organs until the seedling becomes autotrophic; the cotyledons may then develop chloroplasts (Mansfield and Briarty, 1996; Charuvì et al., 2012). In contrast, the chloroplasts of true leaves differentiate directly from the proplastid present in the shoot apex, and their primary function is photosynthesis (Charuvì et al., 2012). In developed leaves, chloroplasts are further propagated by fission, similar to that observed in bacteria (Pyke, 1997; Leon et al., 1998; Pogson and Albrecht, 2011). These differences have been examined in mutants having chloroplast defects restricted either to the cotyledons or to the true leaves. For instance, the snowly cotyledon (sco) mutant group has chlorotic or bleached cotyledons but green true leaves (Albrecht et al., 2006, 2008, 2010; Shimada et al., 2007). Conversely, immutans (im) and variegated2 (var2) mutants have green cotyledons but chlorotic true leaves (Aluru et al., 2006; Kato et al., 2007; Liu et al., 2010).

In the past decade, studies of chloroplast development mutants have enhanced our understanding of the transition from proplastids to chloroplasts within true leaves. A useful Chloroplast mutant has disturbed expression of chloroplast-related genes and displayed delayed chloroplast differentiation. The findings suggest that SG1 is required for chloroplast development in Arabidopsis.

### Materials and methods

#### Plant materials and growth conditions

*Arabidopsis* ecotype Columbia (Col) was used as the wild type (WT). T-DNA insertion lines SALK_046229C and SALK_026339 were obtained from the ABRC (Ohio State University). The seeds of gun1-1, gun4-1, and gun5-1 were kindly provided by Professor Enrique Lopez Juez (University of London, UK). All plants were grown at room temperature (22–25 °C) under long-day conditions (16h light/8h dark). To inhibit photoinhibition, sg1 was grown under 80 mmol m⁻² s⁻¹ white light. For phenotype identification, all plants were grown under conditions similar to that used for sg1. Otherwise, the plants were grown under 125 mmol m⁻² s⁻¹ white light.

#### Mutant isolation and mapping

The sg1 mutant was isolated from an ethylmethanesulphonate (EMS)-mutagenized M₂ population with a Col background. The SG1 locus was mapped by using individuals of an F₂ population derived from a cross between sg1 and WT Ler (Landsberg erecta). After PCR amplification, genetic markers were scored as simple sequence length polymorphism (SSLPs) or cleaved amplified polymorphic sequences (CAPSs) (Michaels and Amasino, 1998). The markers and restriction enzymes used to reveal the polymorphisms are detailed in Supplementary Tables S3 and S4 available at JXB online. MIE15 was a CAPS marker cleaved by the MseI-restricted enzyme. Map distances were calculated according to Kosambi (1943).

#### Transmission electron microscopy

Immediately after harvest, the sixth leaves from plants at different growth stages were cut into small pieces, and fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) for 4h at 4 °C. The samples were rinsed and incubated in 1% OsO₄ for 12h at 4 °C. Then the samples were rinsed with phosphate buffer (pH 7.0), infiltrated with a graded series of epoxy resin in epoxy-propane, and then embedded in Epon 812 resin. Thin sections (~50nm) were obtained by using an ultramicrotome (Leica). The sections were stained in 2% uranyl acetate (pH 5.0), followed by 10nm lead citrate (pH 12), and viewed under a transmission electron microscope (JEM-1230). All images in this study were processed using Adobe Photoshop and Image J software.

#### Complementation test and overexpression of SG1

For the complementation test, the region between ~1500bp upstream of SG1 and the entire genomic fragment of SG1 was PCR-amplified by using the primers SG1-P1F (ACG CGT CGA CGT CTT GGC CTT TTA GTA GTT TAA TG) and SG1-P1R (GG GGT ACC...
GTG CTC CTC ACT ACC AC), and cloned into the binary vector pCAMBIA1300 by using the SalI and KpnI enzymes (underlined regions indicate the introduced SalI and KpnI sites, respectively). For overexpression of SG1, the full-length genomic DNA of SG1 (introns are absent in SG1) was amplified by using the primers SG1-P2F (GC TCT AGA GCA TGA TTT CGT CTC TCT CAG) and SG1-P1R, and cloned into the binary vector pCAMBIA1300-GFP (green fluorescent protein) by using the XbaI and KpnI enzymes. The resulting plasmids pSG1::SG1::GFP and p35S::SG1::GFP were transferred into Agrobacterium tumefaciens strain GV3101 (Koncz and Schell, 1986), and transformed into sgl or WT plants by using the floral dip method (Clough and Bent, 1998). Transformed plants were selected on Murashige and Skoog (MS) medium containing 25 mg L\(^{-1}\) hygromycin.

Subcellular localization of GFP proteins

The constructed p35S::SG1::GFP plasmid was transformed into Arabidopsis protoplasts to observe the transient expression of the fusion protein. Meanwhile the p35S::GFP vector was transformed as a control. The procedures for protoplast isolation and plasmid transformation were described by Kim and Somers (2010). The transformed protoplasts were observed by using confocal microscopy. A 488 nm argon ion laser line was used for excitation of GFP and chlorophyll, while 505–515 nm and 650 nm emission filters were used for simultaneously capturing GFP and chlorophyll fluorescence, respectively, by using an Olympus FV1000MPE2 confocal microscope.

Chlorophyll detection

Total chlorophyll was determined in triplicate according to the method described by Lichtenthaler and Wellburn (1983). Extracts were obtained from the sixth leaves or seedlings at different growth stages. Approximately 0.2 g of fresh tissue was homogenized in 5 ml of 80% acetone for 12 h in darkness. Spectrophotometric quantification was carried out in a Gene Quant spectrophotometer (GE Healthcare), using the following calculations: Chl \(a\) = 12.21 × \(A_{663}\) - 2.81 × \(A_{646}\), and Chl \(b\) = 20.13 × \(A_{646}\) - 5.03 × \(A_{663}\) (\(\mu\)g ml\(^{-1}\)).

Real-time reverse transcription–PCR

Total RNA was extracted from 0.5 g of plant tissue by using the E.Z.N.A Plant RNA Kit (Omega) according to the manufacturer’s instructions, with the addition of an RNase-free DNase I treatment (Omega). The cDNAs were synthesized from 1 mg of total RNA using the Prime Script™ RT Reagent Kit (Perfect Real Time; Takara). All of the quantitative real-time reverse transcription–PCR (qRT–PCR) measurements were performed using an MX 3000 Real-time PCR system (Stratagene) with SYBR Premix Ex Taq (Takara, Japan), according to the manufacturer’s instructions. The housekeeping gene \(\beta\)-tubulin was used as a normalization control. The relative expression was calculated by using the formula 2\(^{-\Delta\Delta Ct}\). All the experiments were performed for each biological replicate. The primer sequences for qRT–PCR are provided in Supplementary Table S1 available at JXB online. The results suggest that sgl affects chloroplast development during the early stages of seedling growth.

Differentiation of proplastids into chloroplasts

The delayed-greening phenotype of sgl implies defective chloroplast development. Transmission electron microscopy was used to examine the chloroplast ultrastructures of the sixth leaves of 3-, 4-, and 5-week-old sgl mutant plants (exhibiting albino, pale-green, and green leaves, respectively). In wild-type plants grown under normal conditions, the proplastid to chloroplast transition occurs at the shoot apical meristem during the early stages of development (Charuvi et al., 2012). Thus, in the present study, the chloroplasts of WT leaves at the same developmental stages were already differentiated and crescent-shaped, and contained

Results

Identification of a slow-greening mutant

To identify the genes involved in chloroplast development, a slow-greening mutant, designated sgl, was isolated from an EMS-mutagenized population of Arabidopsis. The initial rosette leaves of sgl were completely albino, but gradually became green (Fig. 1A). At ~3 weeks post-emergence, the leaves of the mutant were as green as those of the WT (Fig. 1A). The slow-greening phenotype was apparent in other newly formed organs of sgl, including the stems, inflorescences, and siliques. The young inflorescences and siliques of sgl were white or pale green, and became green as they matured (Fig. 1B, C). These observations are consistent with a pigment deficiency in sgl, and therefore the levels of chlorophyll \(a\) and chlorophyll \(b\) were measured at different growth stages of leaf development. The sixth leaves of 3-, 4-, and 5-week-old sgl mutant plants (exhibiting albino, pale-green, and green leaves, respectively) and the corresponding Col leaves were used. Consistent with their phenotypes, the chlorophyll contents increased as the leaves turned green; when the sixth leaves of 5-week-old sgl mutants became green, the chlorophyll contents were markedly higher than those of the albino leaves, but lower than those of the WT (see Supplementary Table S1 available at JXB online). Over time, the chlorophyll contents of the mutant and wild-type leaves became comparable. At all growth stages, the mutant plants were smaller than the WT (Fig. 1A).

Many chloroplast-development mutants of Arabidopsis can grow well when supplied with sucrose as a carbon source (Koch, 1996; Chi et al., 2008; Yu et al., 2009); furthermore, they may show abnormal embryo development (Uwer et al., 1998; Apuya et al., 2001; Kobayashi et al., 2007). To investigate whether SG1 is involved in chloroplast development, sgl seedlings were grown on MS medium without or with 2% sucrose, and the sgl embryogenesis of sgl homozygotes was observed. It was determined that sucrose partially alleviated the albino phenotype of sgl, and also that embryogenesis of sgl in heterozygote plants was delayed (see Supplementary Fig. S1 available at JXB online). The results suggest that sgl affects chloroplast development during the early stages of seedling growth.
well-developed thylakoid membranes with grana stacks (Fig. 2A, E, I). Starch grains were lacking in the chloroplasts of 3-week-old WT plants (Fig. 2A), but were present in the chloroplasts of 4- and 5-week-old plants (Fig. 2E, I). The albino leaves of 3-week-old sg1 seedlings contained few well-developed crescent-shaped chloroplasts, but many smaller, abnormal, irregularly shaped chloroplasts, similar to proplastids (Fig. 2B–D). These abnormal chloroplasts could be classified into three types according to their morphologies. The first type was rounded and highly vacuolated, with almost no thylakoid membrane, and appeared undifferentiated (Fig. 2B). The second type had fewer vacuoles and easily observable thylakoid membranes (Fig. 2C), possibly representing an intermediate form between the proplastid and chloroplast. The third type had discontinuous thylakoid membranes, resembling chloroplasts at an early stage of development (Fig. 2D). The pale-green leaves of 4-week-old sg1 seedlings contained differentiated chloroplasts that were smaller and had fewer thylakoid membranes than did chloroplasts of WT seedlings at the same growth stage (Fig. 2F–H); these chloroplasts resembled WT chloroplasts at an early stage of development. Some of the thylakoid membranes were discontinuous and some were arranged as grana stacks (Fig. 2F–H). Similar to the WT, the green leaves of 5-week-old sg1 plants contained chloroplasts with well-developed thylakoid membranes and grana stacks, and well-developed starch grains (Fig. 2J–L). The conversion of proplastids to chloroplasts in sg1 occurred over a period of ~3 weeks. These results suggest the delayed transition of proplastids to chloroplasts in sg1, consistent with a slow-greening phenotype.

Gene cloning and complementation of sg1

To verify whether sg1 is a nuclear recessive mutant, the M3 generation families of sg1 were crossed reciprocally with wild-type plants. Plants in the F1 generations of sg1 (♂)×Col (♀) and Col (♂)×sg1 (♀) were as green as the WT. The offspring of F1 plants from both of the crosses segregated in a 3:1 ratio (see Supplementary Table S2 available at JXB online). These results suggest that sg1 is a single recessive gene mutation with nuclear inheritance.

A map-based cloning approach was used to identify the mutated gene, by crossing sg1 with the Landsberg erecta (Ler) ecotype of Arabidopsis, to generate an F2 mapping population. SSLP markers (see Supplementary Table S3 available at JXB online) were selected for primary determination of the linkage group. On the basis of 21 F2 plants, it was concluded that SG1 was located on the upper arm of chromosome 3, in the interval between the markers NGA162 (7.24%) and GAPAb (16.24%) (Fig. 3A). Additional InDel markers and a CAPS marker (Fig. 3A; Supplementary Table S4 available at JXB online) were used to refine the position of SG1. On the basis of 146 F2 plants, the location of SG1 was narrowed down to an ~110 kb region between the markers MIE15 and MYF24. In this region, only four genes encoding proteins predicted to be involved in chloroplast localization were identified, namely AT3G18230,
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Sequence analysis of the open reading frames of these four candidate genes revealed the existence of a single G to A mutation in base pair 542 from the start codon ATG of *AT3G18420* genomic DNA; this mutation caused a conversion of arginine to lysine in amino acid 181 of SG1 protein (Fig. 3A). The expression level of SG1 in the *sg1* mutant was determined, and it was shown that the G to A substitution did not affect mRNA accumulation (Fig. 3C). To confirm that the *SG1* gene is *AT3G18420*, the *sg1* mutant was genetically complemented with the full-length *AT3G18420* cDNA under control of the promoter (1500 bp upstream of the open reading frame) of *AT3G18420*. A total of 22 T1 transgenic plants were screened for *pSG1::SG1* with a *sg1* background. Subsequent phenotypic observations confirmed that the complemented mutants had WT traits (Fig. 3D). Further, the chlorophyll contents of 28-day-old rescued transgenic plants were similar to those of the WT (Fig. 3B), and ultrastructural examination of the chloroplasts from the sixth leaves of these plants revealed that they were well developed and similar to those of the WT (Fig. 3F). These results indicate that *AT3G18420* can complement the chloroplast differentiation defects in the *sg1* mutant, thereby further suggesting that *AT3G18420* is responsible for the *sg1* phenotypes. Two T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center. The T-DNA insertion harboured 296 bp upstream of the ATG translation start codon in the SALK_046229C line, and 74 bp downstream of the TAA translation stop codon in the SALK_026339 line. The results of RT–PCR indicated that the *SG1* gene could be expressed in both T-DNA lines, without apparent effects on the chloroplasts.

To investigate further the function of *SG1* in chloroplast development, a plasmid containing the full-length cDNA of *AT3G18420*, under control of the Cauliflower mosaic virus 35S promoter (CaMV 35S), was constructed, which was transformed to the WT. Thirty-one T1 overexpressing (*OE*) transgenic plants were screened, and no visible phenotypic effects were observed (Fig. 3C, D). The chlorophyll contents were measured and the ultrastructures of three *OE* lines were examined; it was determined that the content of chlorophyll *a* was slightly higher than that of WT plants (Fig. 3B). The chloroplasts of the *OE* lines were somewhat irregularly shaped, and had a slightly higher proportion of thylakoid membranes, especially in the grana; these characteristics may be responsible for the higher chlorophyll *a* content (Fig. 3B, 3C).

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**Fig. 2.** Transmission electron micrographs of chloroplasts from the sixth leaves of 3- to 5-week-old Col and *sg1* plants. (A, E, I) Chloroplast structures from the wild-type plants. (B–D), (F–H), and (J–L) Chloroplast structures from the *sg1* mutant plants. Cp, chloroplast; SG, starch grain; Thy, thylakoid; Gr, grana thylakoids. Bars=1 μm.
The results indicate that SG1 plays an important role in chloroplast development.

**Encoding of a conserved, widely expressed, chloroplast-localized TPR-containing protein by SG1**

Analysis of the complete Arabidopsis sequence by using BLAST revealed that the nuclear genome contains a single copy of the SG1 gene. The results of phylogenetic analysis and protein alignments indicated that SG1 was conserved during the evolutionary process; further, it shares significant identity with the (hypothetical) Arabidopsis proteins—AT2G37400, AT3G53560, AT5G02590, and AT3G09490—which are chloroplast lumen common family proteins (see Supplementary Fig. S2 available at JXB online). SG1 encodes a putative polypeptide of 316 amino acids, with four TPR motifs (see Supplementary Fig. S2B available at JXB online). To confirm the subcellular localization of SG1, the transient expression of the p35S-SG1-GFP plasmid was examined in living Arabidopsis protoplasts. As a control, Arabidopsis protoplasts were transformed with a plasmid containing only GFP, under control of the 35S promoter. The GFP signals were observed by using confocal laser-scanning microscopy. In the absence of transformation, only chlorophyll autofluorescence was detected (Fig. 4A). After transformation with the control vector, GFP signals accumulated ubiquitously in the cytosol (Fig. 4B). In contrast, in the p35S-SG1-GFP-transformedoplasts, GFP signals were coincident with chlorophyll autofluorescence; further, some SG1–GFP fusion protein was observed accumulated as spots in the chloroplast, which may be important for its function (Fig. 4C).

Next, the expression profiles of SG1 was examined. The mRNAs were isolated from different tissues of WT plants, and the SG1 expression level was detected by using qRT–PCR. The housekeeping gene β-tubulin, the expression levels of which remain similar across different tissues, was used to normalize different samples. The expression level of SG1 in 10-day-old seedlings was arbitrarily set to 1. It was determined that SG1 was widely expressed in all Arabidopsis tissues (Fig. 4D). The highest expression levels were observed in young siliques and flower clusters, while the lowest expression level was observed in roots (Fig. 4D). SG1 was ubiquitously
expressed throughout the plant; however, its expression was preferentially associated with green tissues, particularly newly formed tissues. These results indicate that SG1 localizes to the chloroplast and exhibits ubiquitous expression.

**Disrupted expression levels of genes associated with chloroplast development, photosynthesis, or chlorophyll biosynthesis**

The TPR or TPR-related motif-pentatricopeptide repeat (PPR) proteins are reported to be involved in chloroplast gene expression (Pfalz et al., 2006; Chi et al., 2008; Su et al., 2012). Since abnormal chloroplast development was observed in sgl, the effect of the loss of SGLI on the expression of chloroplast-related genes was investigated using qRT–PCR. The transcription levels of plastid-encoded polymerases (PEPs) and nucleus-encoded polymerases (NEPs), which transcribe chloroplast genes, nuclear-encoded chloroplast genes, and chlorophyll biosynthesis genes, were examined in sgl plants, and also in the corresponding WT leaves (Fig. 5A–D). Three PEP genes, namely those encoding two members of photosystem II complexes (psbA and psbB) and a RuBisCO large subunit (RbcL), were selected (Fig. 5A). Three NEP genes were also selected, namely an accD, which encodes a carboxytransferase β subunit of the acetyl-CoA carboxylase (ACCase) complex, ycf2.2, which encodes a predicted chloroplast-localized ATP-binding protein, and rpoB, which encodes a chloroplast DNA-dependent RNA polymerase B subunit (Fig. 5B). Three nuclear-encoded chloroplast genes: RuBisCO small subunit (RbcS), light-harvesting chlorophyll a/b-binding protein (CAB2/LHCBI.1), and oxygen evolving polypeptide 1 (Psbo) were detected (Fig. 5C). Genes that are crucial to chlorophyll biosynthesis, namely CAO (encoding chlorophyllide-a oxygenase), HEMAI (encoding glutamyl-tRNA reductase 1), and PORB (encoding protochlorophyllide oxidoreductase B), were also selected for detection (Fig. 5D). The expression levels of psbA, RbcL, accD, Psbo, and PORB showed very similar tendencies. The expression levels of RbcL, accD, and PORB (especially RbcL and accD) were markedly lower in the albino leaves of mutant plants than in the WT; however, the expression levels of all five genes gradually increased as the leaves became green, to reach higher levels than those of the WT (Fig. 5A–D). Nevertheless, the expression of the rpoB gene showed the opposite expression pattern. Its expression level was markedly higher in albino leaves than in the leaves of the WT; further, the expression level gradually decreased as the leaves grew, but remained slightly higher in green leaves than in the leaves of the WT (Fig. 5B). The expression of psbB, ycf2.2, RbcS, and CAO was a little higher in sgl albino leaves than in the WT. These four genes and HEMAI (lower in albino leaves) decreased in pale-green leaves while they increased when the leaves turned green (Fig. 5A–D). The expression level of CAB2 was lower in albino leaves than in the leaves of the WT; however, in pale-green and green leaves, it increased to approximately the same level as in the leaves of the WT (Fig. 5C). Thus, the expression levels of all four types of genes were affected in sgl. In addition, the soluble proteins were profiled in leaves of sgl mutant plants at different growth stages. It was determined that albino leaves of mutant...
plants contained significantly lower amounts of RbcL and RbcS (Fig. 5E). As the leaves became green, the amounts of these two proteins gradually increased (Fig. 5E). The increase in RbcL was in accordance with the transcript data. On the other hand, the expression level of RbcS did not change in the albino leaves, despite a significant decrease in the level of encoded protein. Furthermore, in the green leaves of sg1, the expression levels of RbcL and RbcS were higher than those in the corresponding WT leaves; however, the protein levels were lower than those of the corresponding WT leaves. Therefore, SG1 may also be involved in chloroplast protein biosynthesis and/or degradation.

Genetic interaction of GUN1 and GUN4 with SG1

To investigate further the putative pathways in which SG1 may be involved in chloroplast development, sg1 gun1 and sg1 gun4 double mutants were constructed by crossing sg1 with gun1-1 and gun4-1 mutants. Interestingly, both sg1 gun1 and sg1 gun4 double mutants alleviated the delayed-greening phenotype of sg1 (Fig. 6A). The leaves of the sg1 gun1 double mutant were of a similar green colour to the leaves of the WT plants; however, their development was considerably delayed relative to that in sg1 plants, but slightly delayed in comparison with the WT (Figs 2A, E, I, 6C). In 3- to 5-week-old sg1 gun1 double mutants, the development of chloroplasts in the sixth leaves was almost the same as in WT plants (Fig. 2A, E, I; Fig. 6C, upper panels). In the 3-week-old sg1 gun4 double mutant, prophase chloroplasts formed, but the abundance of thylakoid membranes was lower than that in the corresponding WT plants (Fig. 2A, E, I; Fig. 6C, lower panels). Further,
these chloroplasts were similar to those present in the sixth leaves of 4-week-old \textit{sg1} plants (Fig. 2G, H; Fig. 6C, lower panels). Mature chloroplasts were present in the sixth leaves of 4-week-old and 5-week-old \textit{sg1 gun1} plants (Fig. 6C, lower panels).

The expression levels of chloroplast-related genes were also quantified in 2-week-old \textit{Col}, \textit{sg1}, \textit{gun1}, \textit{sg1 gun1}, and \textit{sg1 gun4} plants. To eliminate the influence of green cotyledons, these were removed before RNA isolation was performed. The results showed that both \textit{gun1} and \textit{gun4} mutations in the \textit{sg1} background affected the expression of chloroplast-related genes compared with those in \textit{sg1}. For example, both double mutants increased the expression levels of \textit{RbcL} and \textit{accD} (expressed at very low levels in \textit{sg1}), and decreased the expression level of \textit{rpoB} (expressed at much higher levels in \textit{sg1}) (Fig. 7A–D). Also, the chlorophyll biosynthesis genes \textit{CAO} and \textit{HEMA1} were increased in both double mutants compared with the WT and \textit{sg1} mutant (Fig. 7A–D). The levels of soluble proteins were profiled in 2-week-old \textit{Col}, \textit{sg1}, \textit{gun1}, \textit{sg1 gun1}, \textit{gun4}, and \textit{sg1 gun4} plants (with cotyledons removed). Consistent with the observations of the alleviated phenotypes, the amounts of \textit{RbcL} and \textit{RbcS} in the double mutant plants were significantly higher than were those in \textit{sg1}, but lower than those in the WT (Fig. 7E).

Subsequently the \textit{sg1 gun5} double mutant was constructed, and it was revealed that the mutation of \textit{GUN5} (with a function similar to that of \textit{GUN1} and \textit{GUN4} in retrograde signalling) did not alleviate the \textit{sg1} phenotype. On the contrary, it slightly enhanced the albino phenotypes of \textit{sg1} (see Supplementary Fig. S3 available at JXB online). Taken together, the present results indicate that the \textit{gun1} and \textit{gun4} mutations in \textit{sg1} may partially restore the disordered expression patterns of chloroplast relative genes, thereby alleviating the \textit{sg1} phenotypes.
Discussion

TPR-containing proteins comprise a common group of proteins that participate in protein–protein interactions or assembly of multiprotein complexes. The TPR domain consists of a degenerate, 34 amino acid sequence, which is present in tandem arrays of 3–16 motifs (D’Andrea and Regan, 2003; Whitfield and Mainprize, 2010). The TPR proteins have been found to be involved in many diverse processes within eukaryotic cells, including synaptic vesicle fusion (Young et al., 2003), peroxisomal targeting and import (Brocard and Hartig, 2006; Fransen et al., 2008), and mitochondrial and chloroplast import (Baker et al., 2007; Mirus et al., 2009). In the present study, a novel chloroplast-localized TPR-containing protein, SG1, was identified which is required for chloroplast development.

Influence of TPR protein SG1 mutation on normal chloroplast development

A large number of TPR-containing proteins are predicted to target to either mitochondria or chloroplasts (Small and Peeters, 2000). Consistent with their localization, many TPR proteins, for example pTAC2, Nac2, Toc64, Pyg7, and LAP1, have been reported to be involved in chloroplast development (Boudreau et al., 2000; Sohrt and Soll, 2000; Peng et al., 2006; Pfalz et al., 2006; Stockel et al., 2006; Kalanon and McFadden, 2008). These TPR proteins regulate chloroplast development in many ways, including gene expression (pTAC2), mRNA processing (Nac2), and protein transport and assembly (Toc64, Pyg7, and LAP1). SG1 is also required for chloroplast development; however, the mechanism of regulation differs from those of previously reported TPR proteins. First, and most importantly, SG1 is only required for the early stage of chloroplast development; once the plant has grown, the chloroplast becomes normal, and the seedling can grow photoautotrophically. In contrast, many previously reported TPR mutations, such as *ptac2* and *pyg7*, are lethal (Pfalz et al., 2006; Stockel et al., 2006). Secondly, previously reported TPR protein regulation of chloroplast gene expression showed high specificity; for example, pTAC2 directly regulates the expression of PEP genes (Pfalz et al., 2006). In contrast, the *sg1* mutant showed disrupted expression of PEP genes, NEP genes, nuclear-encoded chloroplast genes, and chlorophyll biosynthesis genes. It could not be determined whether SG1 was involved in mRNA processing, protein transport, or assembly. However, the inconsistency between the mRNA levels and protein levels of RbcL and RbcS in the *sg1* mutant suggests that SG1 may be involved in protein biosynthesis or degradation in chloroplast development.

The present protein alignment data indicated that the mutation site of SG1 is located within the first TPR motif; this site is not a conserved site in the motif, but is conserved in its homologous sequences in *Arabidopsis* (see Supplementary Fig. S2B available at *JXB* online). The mutation may not affect the protein–protein interaction scaffolds formed by
the TPR motif, but affect the proper function of SG1 in chloroplast development. The gene analysis showed that the expression of chloroplast-related genes was changed in the process of the $sgl$ albino leaf becoming green; for example, $RbcL$ and $accD$ which were decreased in albino leaf were up-regulated, while $rpoB$ which was increased in albino leaf was down-regulated (Fig. 5A–D). These changes may re-establish a new balance among the chloroplast-related genes in the $sgl$ mutant and result in the leaf turning green. Further studies should be conducted to clarify the mechanisms by which SG1 regulates chloroplast development.

**Mutation of GUN1 and GUN4 ameliorating $sgl$ phenotypes**

The chloroplast developmental status has been shown to control a set of nuclear genes that encode chloroplast-localized proteins via a process known as retrograde signalling (Surpin et al., 2002). GUN genes (including GUN1–GUN6) are important for sending signals to the nucleus, to regulate nuclear-encoded chloroplast gene expression (Suszek et al., 1993). In the present study, the $gun1$ and $gun4$ mutations ameliorated the slow-greening phenotypes of $sgl$ (Fig. 6A), and the leaves of $sgl$ $gun1$ and $sgl$ $gun4$ double mutants showed higher chlorophyll contents than did the leaves of $sgl$ mutant plants, at all growth stages (see Supplementary Table S1 available at JXB online). These results indicate that GUN1 and GUN4 genetically interact with SG1. GUN1 and GUN4 are both important factors in the retrograde signalling pathway of chloroplast development. Therefore, to investigate whether other components of retrograde signalling interact with SG1, the $sgl$ $gun5$ double mutant was constructed, and it was revealed that $sgl$ $gun5$ did not alleviate the $sgl$ phenotypes (see Supplementary Fig. S3 available at JXB online). Possible explanations are that GUNs play very important but differing roles in retrograde signalling; SG1 genetically interacts with GUN1 and GUN4 through their differing roles from GUN5 in retrograde signalling; or that the mutation of GUN1 and GUN4 ameliorates $sgl$ phenotypes through other roles in chloroplast development, apart from their involvement in the retrograde signalling pathway.

The present gene expression data revealed that $gun1$ and $gun4$ mutation in plants with the $sgl$ background altered the disturbed expression pattern of chloroplast-related genes in $sgl$, which partially restore the imbalanced expression of chloroplast-related genes caused by $sgl$ mutation. The expression changes brought by $gun1$ and $gun4$ are not identical, and were therefore capable of ameliorating $sgl$ phenotypes to different degrees. Further, the results of protein analysis showed that the contents of $RbcL$ and $RbcS$ in $sgl$ $gun1$ and $sgl$ $gun4$ double mutants were significantly higher than were those in the $sgl$ mutant. The abundance of $RbcL$ and $RbcS$ was higher in $sgl$ $gun1$ than in $sgl$ $gun4$ (Fig. 7E), possibly indicating the different degrees to which $gun1$ and $gun4$ restore the phenotypes of $sgl$. Taken together, the results indicate that $gun1$ and $gun4$ can partially restore the imbalance of chloroplast-related genes in $sgl$, thereby alleviating the defective phenotypes.

**Supplementary data**

Supplementary data are available at JXB online.

Figure S1. Early growth of $sgl$ favoured by sucrose, and delayed embryogenesis during $sgl$ seed development.

Figure S2. Phylogenetic analysis and amino acid sequence alignment of SG1 TPR domains.

Figure S3. The phenotypes of the $sgl$ $gun5$ double mutant.

Table S1. Chlorophyll contents of leaves from different genotypes at different growth stages.

Table S2. The segregated ratio of different phenotypic seedlings in the $F_1$ offspring of a reciprocal cross between Col and $sgl$.

Table S3. Primers for markers used in first mapping.

Table S4. Primers for markers used for fine mapping.

Table S5. Primers for qRT–PCR.

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