Plant Cell Physiol. 45(4): 357–368 (2004)

Rapid Paper

The Multiple-Stress Responsive Plastid Sigma Factor, SIG5, Directs Activation of the *psbD* Blue Light-Responsive Promoter (BLRP) in *Arabidopsis thaliana*

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Transcription in higher plant plastids is performed by two types of RNA polymerases called NEP and PEP, and expression of photosynthesis genes in chloroplasts is largely dependent on PEP, a eubacteria-type multi-subunit enzyme. The transcription specificity of PEP is modulated by six nuclear-encoded sigma factors (SIG1 to SIG6) in *Arabidopsis thaliana*. Here, we show that one of the six sigma factors, *SIG5*, is induced under various stress conditions, such as high light, low temperature, high salt and high osmotic conditions. Interestingly, transcription from the *psbD* blue light-responsive promoter (*psbD*-BLRP) was activated by not only light but also various stresses, and the transcription and the transcriptional activation of *psbD*-BLRP were abolished in a *sig5-2* mutant. This suggests that the PEP holoenzyme containing SIG5 transcribes the *psbD*-BLRP in response to multiple stresses. Since the seed germination under saline conditions and recovery from damage to the PSII induced by high light were delayed in the *sig5-2* mutant, we postulate that SIG5 protects plants from stresses by enhancing repair of the PSII reaction center.

Keywords: *Arabidopsis thaliana* — Chloroplast — *psbD*-BLRP — Sigma factor — Stress response — Transcriptional control.

Abbreviations: bp, base pair(s); DIG, digoxigenin; kb, kilobase(s); kDa, kilodalton(s); PCR, polymerase chain reaction; PSII, photosystem II.

Introduction

Higher plant plastids have their own genetic system based on 120- to 150-kb circular DNA chromosomes encoding about 120 genes (Sugiura 1995, Sato et al. 1999), expression of which is essential for plastid functions such as photosynthesis in chloroplasts (Walbot and Coe 1979, Allison et al. 1996, Stern et al. 1997). Transcription in higher plant plastids is directed by at least two types of RNA polymerases (Stern et al. 1997, Maliga 1998, Hess and Börner 1999). One is a T7/T3 bacteriophage-type RNA polymerase named nuclear-encoded RNA polymerase (NEP) (Lerbs-Mache 1993, Hedtke et al. 1997, Chang et al. 1999, Hedtke et al. 2000) which is considered to transcribe housekeeping gene (Hajdukiewicz et al. 1997, Weihe and Börner 1999). The other is a eubacteria-type RNA polymerase named plastid-encoded RNA polymerase (PEP), and composed of the plastid-encoded core subunits, α, β, β′, β″ (encoded by *rpoA*, *rpoB*, *rpoC1* and *rpoC2*, respectively), and one of the nuclear-encoded sigma (σ) factors (Tanaka et al. 1996, Allison 2000). Since transcripts of many photosynthetic genes were drastically reduced in mutants deficient in the PEP core, PEP is considered to be responsible for the transcription of photosynthesis genes in chloroplasts (Allison et al. 1996, Hajdukiewicz et al. 1997, Serino and Maliga 1998, Krause et al. 2000, Legen et al. 2002).

In general, promoter recognition specificity of eubacteria-type RNA polymerase is determined by the sigma subunits. Most eubacteria have multiple sigma factors to regulate various sets of genes corresponding to specific cellular functions in response to cellular and environmental conditions (Ishihama 2000, Huckauf et al. 2000). Although encoded in the nuclear genome, plastid sigma factors show this multiplicity as found in eubacteria. Thus far, six sigma factor genes have been identified in the nuclear genome of the higher plant *Arabidopsis thaliana* (*SIG1* to *SIG6*) (Tanaka et al. 1997, Isono et al. 1997, Kanamaru et al. 1999, Allison 2000, Fujiwara et al. 2000, Takahashi and Tanaka 2002). Regulation at the transcriptional level is a primary step in controlling gene expression in most

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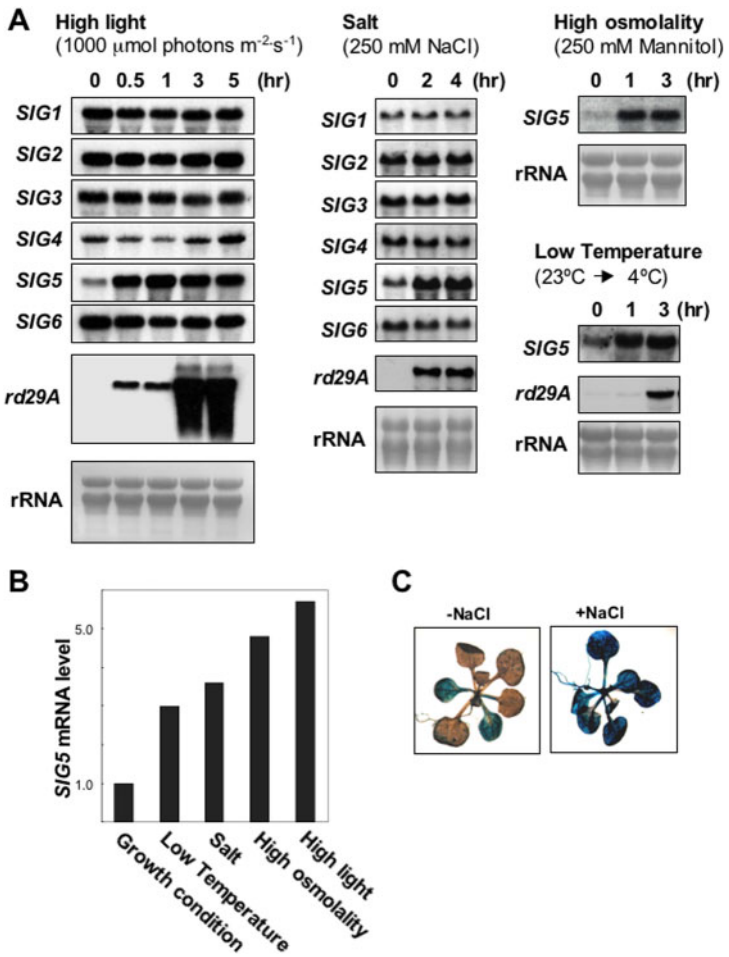

Fig. 1 Induction of *Arabidopsis SIG5* gene in response to various stresses. (A) Northern analyses of *SIG* genes expression under high light (1,000 μmol photons m^{-2} s^{-1}), saline (250 mM NaCl), high osmotic (250 mM mannitol) and low temperature (shift to 4°C) conditions. *Arabidopsis* seedlings (Col) were grown (50 μmol photons m^{-2} s^{-1} of continuous light at 23°C) for 10 or 11 d, and then transferred to the respective stress conditions. Electrophoretic patterns of the blotted rRNA are shown in the lower panels. (B) Induction rates of *SIG5* mRNA levels by the stress treatments. The hybridization density was calculated by subtracting the background pixel density from the pixel density for each band. The relative induction rates of the *SIG5* mRNA were calculated when the expression reached its peak and plotted against time for each stress. The time points were 1, 4, 3 or 3 h after stress application for high light, saline, high osmotic or low temperature stresses, respectively. (C) Saline stress induction of *SIG5* transcription monitored by the *SIG5-uidA* transgene. *SIG5* promoter-*uidA* transgenic *Arabidopsis* plants were grown under normal growth conditions for 3 weeks, and histochemically stained for GUS activity before (–NaCl) and after (+NaCl) the saline stress application (250 mM NaCl for 12 h).

organisms. Although post-transcriptional regulation has been known to play major roles in controlling gene expression in chloroplasts, and thus far only limited roles have been assigned for transcriptional regulation, the identification and characterization of six sigma factor genes in *Arabidopsis* has made it likely that transcriptional regulation is also very important in plant chloroplast gene expression (Kanamaru et al. 2001, Tsunoyama et al. 2002, Privat et al. 2003, Yao et al. 2003). For example, the detailed analyses of a SIG2-deficient mutant (*sig2-1*) revealed that SIG2 was responsible for transcription from several tRNA promoters as well as *psaJ* and one of the *psbD* multiple-promoters (Shirano et al. 2000, Kanamaru et al. 2001, Hanaoka et al. 2003, Nagashima et al. 2004). It has been suggested that SIG2 controls chloroplast development by coordinating plastid translation and chlorophyll biosynthesis (Kanamaru et al. 2001).

The blue light-responsive promoter (BLRP) of the *psbD* operon has been an important subject of chloroplast transcription research (Christopher et al. 1992, Christopher and Mullet 1994). This promoter is highly conserved among higher plant species (Hoffer and Christopher 1997, Nakahira et al. 1998), and activated primarily by blue light mediated by cryptochrome 1 (CRY1) and cryptochrome 2 (CRY2) (Thum et al. 2001). Transcription from the BLRP involves PEP (Allison et al. 1996, Nakahira et al. 1998, Hess and Börner 1999, Kim et al. 1999a), and the response to blue light suggests the possibility that the blue light inducible plastid sigma factor, SIG5, is involved in the BLRP activation (Tsunoyama et al. 2002).

Plants are sessile and have evolved adaptive responses to allow them to survive changes in environmental conditions during growth and development. Plants respond and adapt to abiotic stresses through various biochemical and physiological processes through transcriptional control of many genes which may require the use of a number of transcriptional factors (Shinozaki et al. 2003). Photosynthesis occurs in the chloroplasts which are sensitive to various environmental stresses. Plants have evolved adaptive mechanisms to protect the photosynthetic system from damage, such as cyclic electron flow around PSI, the xanthophyll cycle, the water–water cycle and the reactive oxygen species (ROS) scavenging system (Asada 2000, Müller et al. 2001, Shigeoka et al. 2002, Munekage et al. 2002). However, only limited information has been obtained on chloroplast transcription regulation concerning stress responses.

Because many stress responses are accompanied by transcription regulation by heterogeneous sigma factors among eubacteria, and because plant plastids have a similar genetic

system to eubacteria, we considered the possibility that similar sigma factor regulation is functioning in chloroplasts. In this study, we analyzed the expression of sigma factor genes under various stress conditions. As a result, SIG5 was identified as a stress-responsive sigma factor for chloroplast gene expression. We also identified the T-DNA tagged *sig5-2* mutant, and demonstrated that SIG5 is responsible for the *psbD*-BLRP transcription activation. In fact, this promoter was activated not only by blue light as described (Hoffer and Christopher 1997, Christopher and Hoffer 1998, Thum et al. 2001), but also by various stress conditions. Since we found that seed germination under saline conditions and the recovery of the PSII activity from a high light-induced damage were delayed in the *sig5-2* mutant, we postulate that SIG5 is involved in protection of chloroplasts under various stress conditions through enhancement of the repair mechanism for the PSII reaction center.

Results

SIG5 gene expression is activated by various environmental stresses

In order to identify plastid sigma factors whose expression is influenced by environmental stresses, we performed Northern hybridization analyses of the *Arabidopsis* six sigma factor genes under various stress conditions. *Arabidopsis* plants were grown for 10 or 11 d under light conditions of 50 µmol photons m^{-2} s^{-1} at 23°C prior to each stress treatment. When plants were transferred to high light conditions of 1,000 µmol photons m^{-2} s^{-1}, we found that only *SIG5* transcripts increased significantly (Fig. 1A). Transcript levels reached a maximum within 1 h of high light irradiation, and the induction rate was estimated to be more than fivefold (Fig. 1B). This high level expression continued for a minimum of 5 h (Fig. 1A). High salt treatment (250 mM NaCl) also only induced *SIG5* transcription among the six *SIG* genes, and resulted in about three-fold induction between 2 h and 4 h after stress application (Fig. 1A, B). Osmotic challenge (250 mM mannitol) and low temperature (4°C) also induced *SIG5* transcription in a similar manner (Fig. 1A). A well-analyzed stress-responsive gene, *rd29A*, used as a positive control, showed stress induction as described previously (Yamaguchi-Shinozaki and Shinozaki 1994, Ishitani et al. 1997) (Fig. 1A). We also constructed transgenic plants harboring the *SIG5* promoter region fused to the reporter gene, *uidA* (GUS). We found that salt stress clearly induced GUS activity, implying that such stress responses induce *SIG5* at the transcriptional level (Fig. 1C). The above observations strongly suggested that SIG5 is a multiple-stress responsive sigma factor involved in plastid transcription.

SIG5 expression patterns during seedling development under the non-stress conditions

In the *SIG5* promoter-*uidA* fusion containing transgenic plant, *SIG5* expression was further analyzed under normal growth conditions during seedling development. During the first 3 d after germination, strong GUS staining was observed throughout the plants (Fig. 2A–C). However, GUS activity in the cotyledons disappeared after 4 d, and appeared again after 7 d (compare the open triangles in Fig. 2D–G). In the first true leaves expression was very strong during the early stages of appearance (Fig. 2E, filled triangles), it then disappeared (Fig. 2F) before re-activating (Fig. 2G). We previously observed such rhythmicity of transcriptional activation in *SIG1* and *SIG2* during seedling development (Kanamaru et al. 1999). Our data suggest that a similar periodic activation of *SIG5* transcription occurs during chloroplast development in normally developing plants.

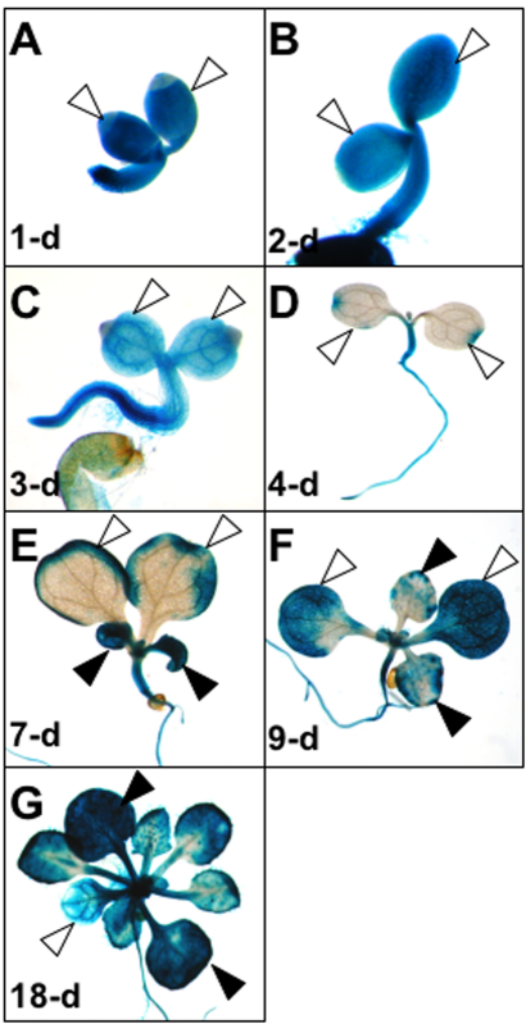

Fig. 2 Monitoring of the *SIG5* expression patterns during leaf development by GUS staining. *SIG5* promoter-*uidA* transgenic *Arabidopsis* plants were grown under normal growth conditions for 18 d. Open arrowheads show cotyledons and filled arrowheads show the first true leaves.

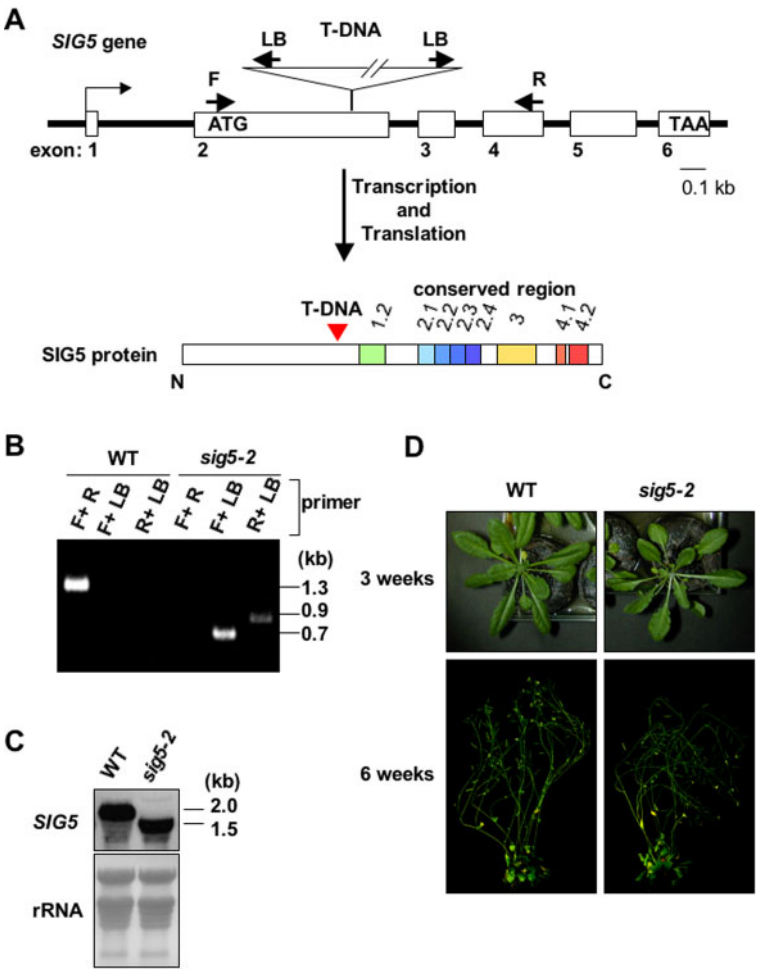

Fig. 3 Identification and analysis of a T-DNA tagged *SIG5* mutant. (A) Schematic representation of the structure of the *SIG5* ORF (At5g24120) and the SIG5 protein. Six exons and the positions of the initiation (ATG) and termination (TAA) codons are indicated. The T-DNA insertion site is indicated by triangles. Arrows indicate direction and location of primers F (SIG5S-Fa), R (SIG5-#1R) and LB. The conserved regions for the sigma 70 family proteins and the T-DNA insertion position are marked for the protein. (B) Confirmation of T-DNA insertion into the *SIG5* ORF by PCR. Genomic DNA from the wild type and the *sig5-2* mutant were used as templates for PCR. Fragments approximately 0.7 kb and 0.9 kb long were amplified between the LB primer located in the T-DNA left border and F or R primer located in *SIG5* ORF. Amplification using the F and R primers resulted in a 1.3 kb fragment only from the wild-type plant (WT). (C) Expression analysis of the *SIG5* mRNA in the wild-type (WT) and the *sig5-2* mutant plants by Northern hybridization. Electrophoretic patterns of blotted rRNA are also shown in the lower panel. (D) Appearance of the *sig5-2* mutant. The wild type (WT) and the *sig5-2* mutant were grown on Jiffy 7 under normal growth conditions.

Identification of a T-DNA tagged SIG5 mutant line

To determine a functional role of SIG5 in *Arabidopsis*, we searched the T-DNA insertion line (SAIL) database provided by Syngenta Biotechnology Inc. (www.tmri.org). We identified one line in which T-DNA was inserted into the *SIG5* coding region (Garlic 1232 H11) of the Columbia ecotype (Col). The T-DNA insertion site of the strain was located in the second exon of the *SIG5* gene, and the position corresponded to the amino terminal side of the conserved region 1.2 (Fig. 3A, Wösten 1998, Fujiwara et al. 2000). The *Arabidopsis* line was self-fertilized several times, and we isolated homozygous plants with the T-DNA insertion in the *SIG5* gene (Fig. 3). The presence of *SIG5* mRNA was assessed by Northern hybridization analysis using a *SIG5* probe that corresponds to the 3′-downstream region of the T-DNA insertion site. Using this probe, no intact *SIG5* mRNA (about 2.0 kb) was detected in the mutant, while a shorter transcript (about 1.5 kb) that was considered to be derived from the mutated allele was detected. Presumably, transcription from the inserted T-DNA sequence produced the aberrant *SIG5* transcript as judged from the transcript size. Even if this transcript was in frame, the product should be non-functional in chloroplasts because of the loss of the N-terminal plastid targeting signal identified in SIG5 (Yao et al. 2003).

Recently, the T-DNA insertion mutation, *sig5-2*, was described by Yao et al. (2003), and they reported that *SIG5* function was essential because a homozygous *sig5-2* mutant exhibited an embryonic lethal phenotype. The fact that we could isolate homozygous *sig5-2* plants apparently contradicts this published report. However, further analysis supported our identification of the mutant as homozygous for *sig5-2*. First, the genomic DNA of the isolated *sig5-2* mutant was characterized by PCR (primer positions indicated in Fig. 3A), confirming that the homozygous insertion of the T-DNA element occurred within the *SIG5* gene (Fig. 3B). Second, our *sig5-2* mutant was backcrossed to Col and outcrossed to the Landsberg ecotype *erecta* (Ler), and no lethal F2 progeny were found. Furthermore, segregation analysis of the BASTA-resistance marker encoded on the T-DNA established the presence of only one T-DNA locus in the genome of this mutant, because one forth of F2 seeds from backcrossed and outcrossed plants were not viable upon treatment with BASTA. This denied the possibility that some secondary mutations suppressed the lethality (data not shown). Thus, our data show that *SIG5* can

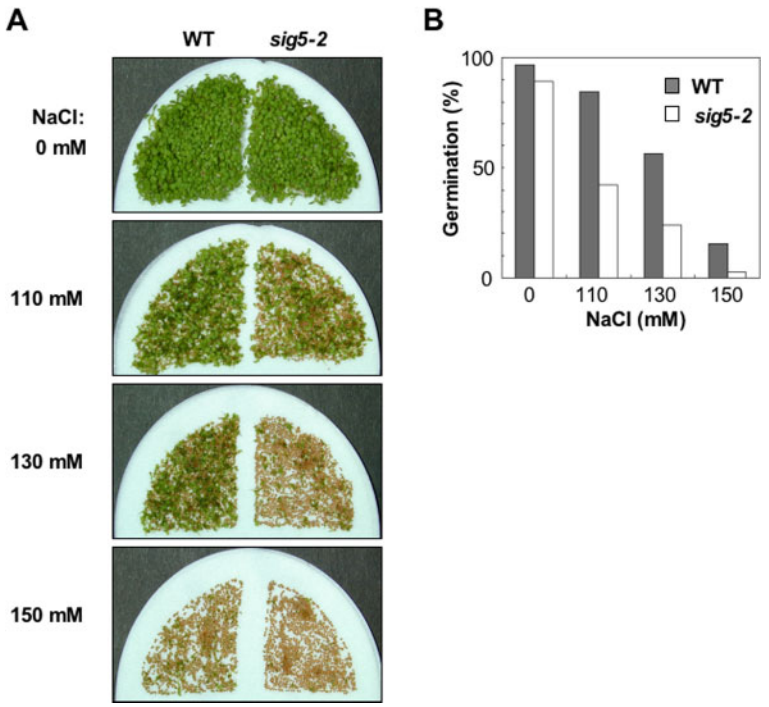

Fig. 4 Enhanced salt sensitivity of the *sig5-2* mutant affecting germination rate. (A) The wild type (WT) and the *sig5-2* mutant were sown and grown on MS medium containing 0, 110, 130 or 150 mM NaCl for 5 d. (B) The germination rates in (A) were calculated as [number of germinated plants]/[number of sown seeds] and shown as a histogram.

be disrupted and that the result in the previous report may come from an unknown element during the experiments or from differences in the plant cultivation conditions.

Sensitivity of the sig5-2 mutant to salinity and high light stresses

The *sig5-2* strain has no visible phenotype under normal growth conditions (Fig. 3D). To investigate the function of SIG5, we analyzed the phenotype of the *sig5-2* mutant under stress (Fig. 4, 5). Since *SIG5* is expressed in early phases of the germination process (Fig. 2A–C), and since *SIG5* was induced under stress conditions (Fig. 1A–C), we wondered if SIG5 has roles in germination under stress conditions. To assess this, we compared the germination rates of the wild-type and mutant strains under saline conditions (Fig. 4). Seeds from the wild type and the mutant were sown on filter papers soaked in germination medium containing various concentrations of NaCl. They were kept in the dark at 4°C for 1 d to break dormancy, and then exposed to 23°C under light for 5 d. On the medium without NaCl, the *sig5-2* mutant did not show any significant difference from the wild type. However, under saline conditions, the germination rate of the *sig5-2* mutant was lower relative to the wild type. In the presence of 110 mM NaCl, the germination rate of the *sig5-2* mutant was about 40% while that of the wild type was about 85%. At 130 mM NaCl, the germination rate of the *sig5-2* mutant decreased to about 20% whereas that of the wild type was about 55%. At the highest concentration of NaCl (150 mM), both the wild type and the mutant hardly germinated. These results strongly suggest that SIG5 and the resulting chloroplast transcription is involved in the germination process under saline stress.

On the other hand, since *SIG5* was rapidly induced by high light treatment, it may be that SIG5 is involved in repair of photo-damaged photosynthetic machinery. To examine this possibility, photoinhibition after high light treatment and a recovery period were estimated by measuring chlorophyll fluorescence (Fig. 5). The wild-type and the mutant plants were

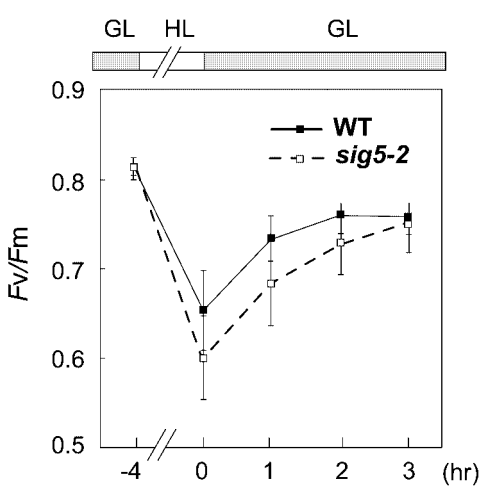

Fig. 5 The recovery processes of PSII photochemical efficiency (F_v/F_m) of the wild-type (WT) and the *sig5-2* mutant after high light irradiation. The wild-type and the *sig5-2* mutant plants were grown under normal growth light conditions (GL; 50 µmol photons m^{-2} s^{-1}), exposed to high light (HL; 1,000 µmol photons m^{-2} s^{-1}) for 4 h, and transferred to the normal light conditions again. The measurements were performed before (–4 h) and after (0 h) the high light treatment, and during the recover process (1–3 h). Data are shown as the means ± SD (n =16).

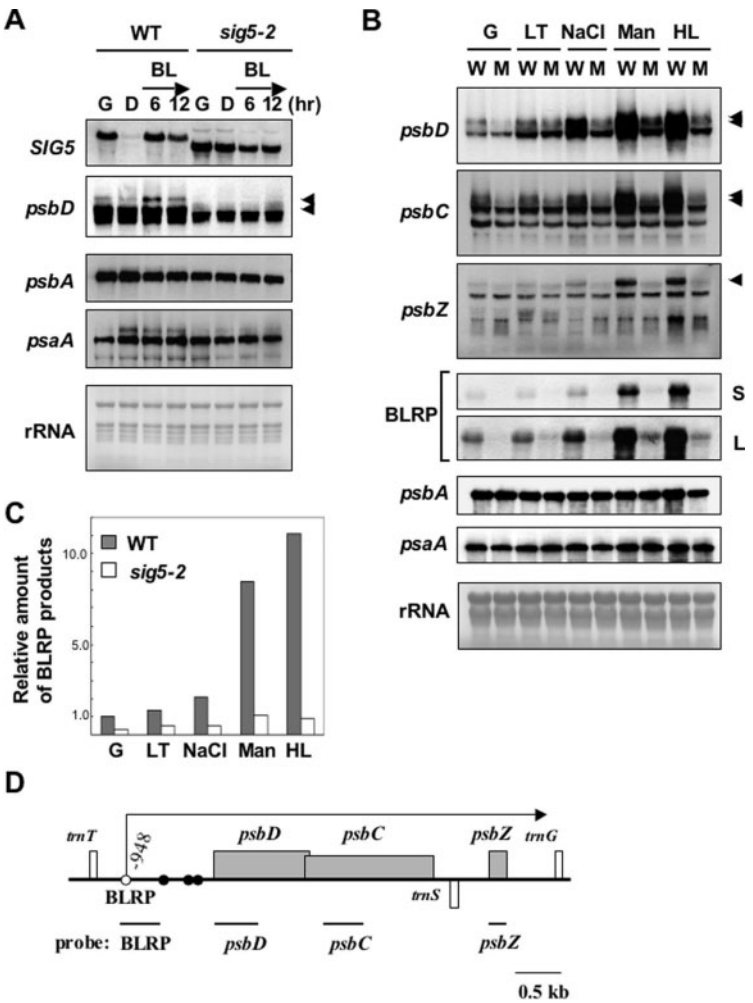

Fig. 6 SIG5 dependent activation of the *psbD*-BLRP. (A) Northern analyses of *SIG5*, *psbD*, *psbA* and *psaA* gene expression during blue-light irradiation. The wild-type (WT) and the *sig5-2* mutant plants were cultivated under the normal growth conditions (lanes G), dark adapted for 16 h (lanes D), and re-exposed to light for 6 or 12 h (lanes 6 and 12) with blue light (25 μmol photons m^{-2} s^{-1}). Triangles indicate the transcripts from the *psbD*-BLRP. Electrophoretic patterns of the blotted rRNA are shown in the lower panel. (B) Northern analyses of the expression of the *psbD* operon detected with the *psbD*, *psbC*, *psbZ* or *psbD*-BLRP (BLRP) specific probes. The wild type (W) and the *sig5-2* mutant (M) plants were grown under normal growth conditions (G), and exposed to low temperature stress (LT; 4°C, 6 h), saline stress (NaCl; 250 mM NaCl, 6 h), high osmotic stress (Man; 250 mM mannitol, 6 h), or high light stress (HL; 1,000 μmol photons m^{-2} s^{-1}, 4 h). For the *psbD*-BLRP specific probe, short (S) and long (L) exposures of the same hybridization filter are shown. Expression of the *psbA* and *psaA* genes was also analyzed under the same conditions. Patterns of the blotted rRNA are shown in the lower panel. (C) Comparison of the *psbD*-BLRP induction rates between the wild-type (WT) and the *sig5-2* mutant. The results with the BLRP probe in (B) were quantified, and the induction ratios were calculated using the wild-type plant without stress as the control. (D) Schematic presentation of the structure of the *psbD* operon. The 5′-end of BLRP product and the other *psbD* transcripts are indicated as an open circle and closed circles, respectively. The locations of specific probes are shown below.

grown for 3 weeks under light (50 μmol photons m^{-2} s^{-1}), then exposed to high light (1,000 μmol photons m^{-2} s^{-1}) for 4 h before being returned to the normal light conditions. The maximal PSII photochemical efficiency, represented by the chlorophyll fluorescence parameter F_v/F_m, decreased after exposure to high light and then increased during the low light recovery period. The high light-induced decrease in F_v/F_m was, to some degree, more pronounced in the mutants than in the wild type. During the low light recovery period, F_v/F_m in the *sig5-2* mutants was lower than in the wild type, indicating the delay in recovery of PSII activity. This result strongly suggests that SIG5 is involved in repair of PSII after photoinhibition.

SIG5 is responsible for the psbD BLRP transcription

Because both *SIG5* and *psbD*-BLRP expression are activated by blue light, it has been suggested that SIG5 is involved in the transcription from the *psbD*-BLRP (Tsunoyama et al. 2002). To test this model, the effects of blue light on *SIG5* and *psbD*-BLRP induction were examined (Fig. 6A). Ten-day-old wild-type and *sig5-2* plants grown under the normal conditions were transferred and kept in the dark for 16 h, and then exposed to blue light (25 μmol photons m^{-2} s^{-1}) for 12 h. Induction of *SIG5* was observed in wild type as described previously (Tsunoyama et al. 2002). The aberrant *SIG5* transcript in the *sig5-2* mutant was constitutively expressed irrespective of the blue light conditions, indicating that the native *SIG5* promoter did not drive the aberrant transcript (Fig. 3). Similarly, while the *psbD*-BLRP transcripts (4.5 kb and 3.7 kb transcripts, the latter resulted from the 3′-prossessing of the longer transcript, indicated by arrowheads in Fig. 6A) were induced in wild type, these *psbD*-BLRP transcripts were not detected in the *sig5-2* mutant. This indicates that *SIG5* is involved in the transcriptional activation of the *psbD*-BLRP.

As *SIG5* was induced by various stresses, we examined whether the *psbD*-BLRP was also induced under these conditions (Fig. 6B). The *psbD* operon is composed of three PSII protein genes, *psbD*, *psbC* and *psbZ*, encoding a D2 reaction center, an internal antenna protein CP43, and a 6.5 kDa protein, respectively (Wallman et al. 1999, Swiatek et al. 2001). Since the *psbD* operon has multiple promoter elements, hybridization probes corresponding to the protein coding regions resulted in complex hybridization patterns (Fig. 6B). Since the

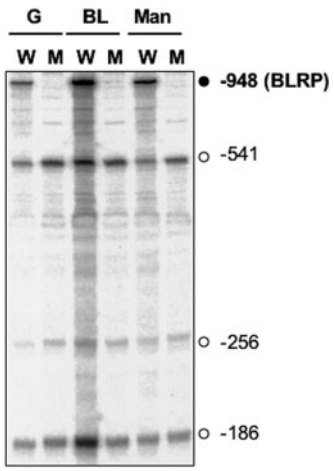

Fig. 7 Transcript mapping of the *psbD* promoter region of the wild-type and the *sig5-2* mutant plants. Transcripts in the *psbD* promoters were analyzed by S1 nuclease protection assay. The wild-type (W) and the *sig5-2* mutant (M) plants were cultivated under normal growth conditions (G), dark-adapted for 16 h, and then re-exposed to blue light (BL; 25 μmol photons m^{-2} s^{-1}) for 6 h, or exposed to high osmotic stress (Man; 250 mM mannitol) for 6 h. The nucleotide positions of the 5′-ends of the transcripts relative to the ATG translation initiator are indicated as circles on the right of the panel. The closed circle indicates the *SIG5*-dependent *psbD*-BLRP.

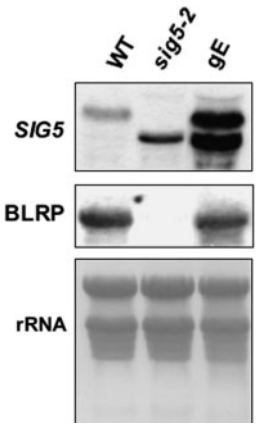

Fig. 8 In vivo complementation of *psbD*-BLRP transcription by *SIG5*. The wild-type (WT), *sig5-2* mutant, and *SIG5*-complemented *sig5-2* (gE) plants were grown under normal growth conditions, and the *SIG5* and *psbD*-BLRP transcripts were analyzed by Northern hybridization. Electrophoretic patterns of the blotted rRNA were shown in the lower panel.

psbD-BLRP is the furthest upstream of the multiple promoters, a hybridization probe that specifically detects the *psbD*-BLRP-driven transcript was designed (Fig. 6D), and used to monitor the expression of *psbD*-BLRP. Eleven-day-old wild-type and *sig5-2* plants were exposed to low temperature, saline, high osmotic and high light stresses, and the gene expression was analyzed by Northern hybridization. All probes detected induction of the *psbD* operon under stress conditions in the wild-type plant (Fig. 6B). Quantification of the estimated BLRP-derived transcript signals showed that the BLRP induction rate, relative to normal growth conditions, was greater than two-, eight- or tenfold under saline, high osmotic or high light conditions, respectively (Fig. 6C). However, in the *sig5-2* mutant, induction of the *psbD* operon was not observed, thus corresponding to the loss of *SIG5* induction in the mutant (Fig. 6B, C). These results showed that *psbD*-BLRP activation under stress conditions was dependent on *SIG5*. The expression of the other two reaction center protein genes for photosystems, *psbA* and *psaA*, was also examined under the same conditions, but the transcript levels remained almost constant, irrespective of the stress conditions and *SIG5* alleles (Fig. 6A, B).

To further confirm that blue light and stresses induce *psbD*-BLRP and that this activation is dependent on SIG5, S1 nuclease protection analysis was performed on the *psbD* promoter region (Fig. 7). It has been shown that this promoter region contains at least four 5′-end sites of the transcript mapped, with respect to the translation initiation site, at the –948 (*psbD*-BLRP), –541, –256 and –186 positions (Hoffer and Christopher 1997, Christopher and Hoffer 1998, Thum et al. 2001, Hanaoka et al. 2003). Recently, the promoter that initiates from the –256 position was shown to be transcribed by PEP containing SIG2 (Hanaoka et al. 2003), but it still remains to be determined if the 5′ ends at the –541 and –186 positions correspond transcription initiation sites or not. As shown in Fig. 7, only the most upstream *psbD*-BLRP (–948) is strongly repressed by the *sig5-2* mutation, and this promoter was clearly activated by blue light as well as osmotic stress in the wild-type plant.

The SIG5 dependence of the *psbD*-BLRP was also confirmed genetically (Fig. 8). We introduced the wild-type genomic copy of *SIG5* into the *sig5-2* mutant, and obtained a *SIG5*-complemented plant line (gE). In this line, the intact and aberrant *SIG5* transcripts were simultaneously detected, and transcription from *psbD*-BLRP was similar to that in the wild-type plant. This result further confirmed the dependence of *psbD*-BLRP on *SIG5*.

Analysis of SIG5 expression and the effects of light signal transduction mutations

It has been shown that all of the *SIG* genes were induced during light illumination after dark-adaptation (Tanaka et al. 1997, Tsunoyama et al. 2002). Using this fact, we further analyzed *SIG5* gene expression in more detail. As shown in Fig. 9A, induction of *SIG5* reached a peak 1–1.5 h after illumination and then gradually decreased, whereas *SIG2* required 2.5 h before strong induction occurred, and the *SIG2* transcript further increased at 6 h after illumination. The other *SIG* genes expressed in a similar pattern with *SIG2* (data not shown). Thus, only *SIG5* expression appeared to be differentially regulated by light. Subsequently, the effects of the light signal transduction mutations, *phyA*, *phyB*, *cry1* and *hy5*, on expression of

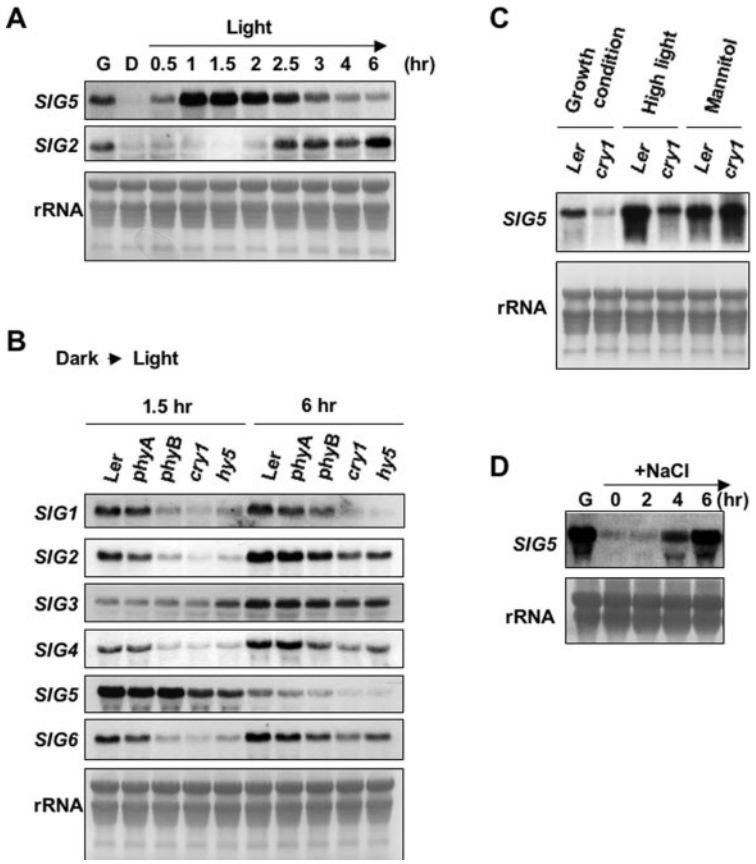

Fig. 9 Effects of light signal transduction mutations on light-dependent and stress-dependent *SIG5* induction. (A) Time course analyses of the light induction of *SIG5* and *SIG2* genes. Wild-type plants (Ler) were cultivated under normal growth conditions (G), dark-adapted for 30 h (D), and re-exposed to normal light. The plants were sampled periodically and analyzed. (B) Northern analyses of the light induction of *SIG* genes in light signal transduction mutants. The wild-type (Ler) and mutant plants (*phyA*, *phyB*, *cry1* and *hy5*) were cultivated under normal growth conditions, dark-adapted for 30 h, and re-illuminated for 1.5 or 6 h to normal light. (C) The effect of the *cry1* mutation on the *SIG5* induction in response to stresses. Inductions of *SIG5* in response to high light and high osmotic stress in wild-type (Ler) plants and the *cry1* mutants were analyzed by Northern hybridization. Seedlings were grown under normal growth conditions, and then exposed to high light (1,000 μmol photons m^{-2} s^{-1}, 0.5 h) or high osmotic stress (250 mM mannitol, 3 h). (D) Northern analysis of the induction of *SIG5* in response to salt stress in the absence of light. The wild-type plants (Ler) were cultivated under normal growth conditions (G), dark-adapted for 30 h (0 time), and then exposed to salt stress (250 mM NaCl) for 2–6 h in the dark. Patterns of blotted rRNA are also shown in the lower panel.

the *SIG* genes were examined 1.5 and 6 h after illumination when *SIG5* and *SIG2* expression reached their peaks, respectively. *SIG5* induction was reduced in the *cry1* and *hy5* mutants but unaffected in *phy* mutants (Fig. 9B). Similarly, other *SIG* gene transcripts tended to be reduced in the *cry1* and *hy5* mutants, but the *SIG1*, *2*, *4* and *6* transcripts were also reduced in the *phyB* mutant (Fig. 9B).

Since cryptochrome 1 is a photoreceptor for blue light, it is reasonable that light induction of *SIG5* is alleviated in the *cry1* mutant. However, as shown in this study, *SIG5* was induced under various stress conditions as well as blue light. To understand the relationship between these two input signals and signal transductions, we examined the effects of *cry1* mutation on *SIG5* induction by high light and high osmotic stresses. As shown in Fig. 9C, *CRY1* was apparently required for rapid high light induction, but not for high osmotic stress induction. The light and stress pathways are surely separate because salt stress-dependent *SIG5* induction was observed even in the absence of light (Fig. 9D). Because this induction was rather weak and slow compared to that by light (Fig. 9A), light signals may somehow enhance the stress signals.

Discussion

In this study, we have discovered that one of the nuclear-encoded plastid sigma factors, SIG5, responds to various stress conditions, and activates the BLRP of the *psbD* operon. Using a *sig5-2* mutant, it was demonstrated that SIG5 actually contributes to repair of the damaged PSII and helps germination under salt stress. It has been demonstrated that plastid sigma factors, and thus plastid transcription, respond to environmental light conditions (Klein and Mullet 1990, Igloi and Kössel 1992, Gruissem and Tonkyn 1993, Mullet 1993, Christopher and Mullet 1994, Satoh et al. 1997, Tanaka et al. 1997, Nakahira et al. 1998, Baena-González et al. 2001, Tsunoyama et al. 2002). However, this is the first report showing that a plastid promoter is activated in response to abiotic environmental stresses through the function of the specific sigma factor. Thus far, only a few linkages have been established between an environmental change and plastid promoter activation. Activation of the *psbD*-BLRP is one such case, and it has been suggested that SIG5 is activated by blue light via the cryptochrome receptors causing *psbD*-BLRP activation (Christopher and Mullet 1994, Hoffer and Christopher 1997, Thum et al. 2001, Tsunoyama et al. 2002). In this study, we demonstrated this relationship and linked environmental stresses to the repair of PSII damages.

The activation of the *psbD*-BLRP drives expression of the *psbD-psbC-psbZ* operon. Under various stress conditions, reduced CO_2 fixation rate and inhibition of photosynthetic electron transport results in light-induced PSII inactivation (photoinhibition; Giardi et al. 1997). Although the main target of

the damage is the D1 reaction center protein (encoded by *psbA* in the chloroplast genome) (Mattoo et al. 1981, Ohad et al. 1985), light induces degradation of another PSII reaction center protein, D2, and the internal antenna protein CP43, encoded by *psbD* and *psbC*, respectively (Christopher and Mullet 1994). Degradation of D1 and CP43 was found to also be enhanced by drought stress (Giardi et al. 1997). Thus, there is considerable validity in that not only de novo synthesis of D1, but also of D2 and CP43 are necessary for repair of PSII under stress conditions. Considering the presence of background transcripts derived from the constitutive promoters in the down-stream of BLRP (Fig. 6, 7), it is reasonable that the effect of the *sig5-2* mutant on the phenotypes is rather mild. However, our data indicates that the recovery of PSII from photodamage is limited by the transcription of the *psbD* operon under the certain stress conditions (Fig. 5, 6). Hence, SIG5 may help this repair process by activating *psbD*-BLRP. As for the D1 protein, protein synthesis is known to be largely controlled by translation regulation, although *psbA* transcription is activated during high light irradiation as well as *psbD* (Baena-González et al. 2001). We have at present no evidence indicating the involvement of *SIG5* in *psbA* transcription, because *psbA* transcript levels were not affected by the *sig5-2* mutation under the conditions examined (Fig. 6B).

The structure and activation mechanism of *psbD*-BLRP have been extensively studied, and two regulatory factors, AGF and PGTF, are currently known. AGF binds just upstream of the –35 promoter element, and is composed of a basic helix-loop-helix DNA binding protein (PTF1; Baba et al. 2001). AGF is considered to activate transcription by binding DNA and positioning the PEP (Allison and Maliga 1995, Kim and Mullet 1995, Kim et al. 1999a). A *ptf1* mutant showed reduction of *psbD*-BLRP transcript accumulation in response to light irradiation, but induction did not disappear in this mutant (Baba et al. 2001). A second complex, PGTF, is a negative regulator, and binds the region further upstream of the AGF binding site. PGTF is phosphorylated dependent on ADP, which results in a decrease of the PGTF/DNA complex (Kim et al. 1999b). Since the *psbD*-BLRP activation vanishes in the *sig5-2* mutant, SIG5 is likely to be a critical determinant in promoter activation, probably because of the direct promoter recognition by a PEP holoenzyme containing SIG5. AGF and PGTF may be positive and negative regulators for this RNA polymerase, respectively.

SIG5 was induced under various stress conditions. Discovering what factors are involved in controlling *SIG5* expression in response to stress is an important question. Analysis of photoreceptor mutants revealed that the light induction of *SIG5* was strongly dependent on CRY1 (Fig. 9B, C). However, *SIG5* activation could occur in the absence of light (Fig. 9D), and it appears that transcriptional changes caused by light and stress signals are independent. However, further study is required to find the signal(s) involving the *SIG5* induction.

The *psbD*-BLRP is conserved among higher plants such as *Arabidopsis*, tobacco, wheat, barley and black pine (Christopher et al. 1992, Hoffer and Christopher 1997), whereas it is not conserved among mosses (Ohyama et al. 1986, Sugiura et al. 2003) and algae (Kowallik et al. 1995, Ohta et al. 2003). Likewise, *SIG5* is only found in higher plants, indicating that the *SIG5*/*psbD*-BLRP stress response system has evolved relatively late during land-plant history.

In conclusion, we postulate that transcriptional control through SIG5 is involved in protection of chloroplasts under various stress conditions through enhancement of PSII reaction center repair. Higher plant chloroplasts have a system for responding to environmental signals and stresses at the transcription level, and this system gives higher plants a survival advantage under harsh natural environments.

Materials and Methods

Plant materials and growth conditions

A. thaliana ecotypes, Columbia (Col) and Landsberg *erecta* (Ler), were used as wild-type plants. The homozygous *sig5-2* mutant was isolated from an *Arabidopsis* T-DNA inserted line (Garlic 1232 H11; Col background) distributed by Syngenta Biotechnology Inc. (SAIL collection: Sessions et al. 2002, www.tmri.org). Light signal transduction mutants, *phyA-201*, *phyB-1*, *hy4-1*(*cry1*) and *hy5-1* (all Ler background), were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, U.S.A.). *Arabidopsis* seeds were sown on either Jiffy 7 (AS Jiffy products) or three layers of filter paper (#540 for top and #2 for middle and bottom, ϕ70mm, Whatman Int. Ltd., Maidstone, England) soaked in Murashige and Skoog (MS) medium and placed on a 0.4% Gelrite (Wako Co. Ltd.) plate containing MS medium (Wako Co. Ltd., Osaka, Japan) without sucrose. After cold treatment at 4°C for 24 h, the seeds were germinated and grown under normal growth conditions (50 μmol photons m^{-2} s^{-1} of continuous light at 23°C). For Northern analyses and S1 protection assay, *Arabidopsis* plants were grown on MS plates for 10 or 11 d, prior to dark or light treatment. For histochemical GUS staining analyses, an *Arabidopsis* transgenic plant containing a *SIG5* promoter-*uidA* fusion (see below) was grown on MS plates for 1–22 d before staining. To determine germination rate under saline stress, *Arabidopsis* seeds were sown on MS media plates containing 0, 110, 130 or 150 mM NaCl, and grown for 5 d. To measure chlorophyll fluorescence parameters, *Arabidopsis* seeds were grown in soil for 3 weeks under normal growth conditions.

Stress treatments

Most stress and light irradiation treatments were performed on 10- or 11-day-old *Arabidopsis* seedlings. The only exception was the use of 3-week-old plants irradiated with high light to measure chlorophyll fluorescence parameters. For saline and high osmotic stress treatments, the seedlings were transferred to five layers of paper filters (#2, ϕ70 mm, Whatman) soaked in MS medium containing 250 mM NaCl or 250 mM mannitol, and incubated under light (50 μmol photons m^{-2} s^{-1}) at 23°C for 1–12 h. For high light treatment, the seedlings were exposed to 1,000 μmol photons m^{-2} s^{-1} at 23°C for 0.5–5 h. To observe the effect of low temperature, the seedlings were incubated at 4°C for 1–6 h under light conditions of 50 μmol photons m^{-2} s^{-1}. For experiments involving blue or white light response, the seedlings were dark-adapted for 16 or 30 h at 23°C, and illuminated for 0.5–12 h with blue LED light (25 μmol photons m^{-2} s^{-1}) or white light (50 μmol photons m^{-2} s^{-1}) at 23°C. To observe the effect of saline stress in the dark, 30 h

dark-adapted seedlings were transferred to MS medium including 250 mM NaCl and further incubated for 2–6 h in the dark.

Preparation of nucleic acids from plant materials

Plant materials were frozen in liquid nitrogen and ground with a Multibeads shocker (Yasui Kikai Co. Ltd., Osaka, Japan). Total DNA was purified using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, U.S.A.). Total RNA for Northern and S1 analyses was purified using TRIzol Reagent (Invitrogen Co., San Diego, CA, U.S.A.) as described by the supplier.

Analysis of the T-DNA insertion site in SIG5

A T-DNA insertion site in *SIG5* ORF of the *sig5-2* mutant was confirmed by PCR using the specific primers, SIG5S-Fa (5′-)GTGATGGGAGTTGTGTC(-3′) and SIG5-#1R (5′-)GACACGAGTGAAGTTTGAAGTTG(-3′) and a primer specific for the left border of the Syngenta T-DNA sequence, LB3 (5′-)TAGCATCTGAATTTCATAACCAATCTCGATACAC(-3′). The PCR reaction was performed for 30 cycles of 94°C for 1 min (for denature), 55°C for 1 min (for annealing) and 72°C for 2 min (for elongation).

Northern analysis and S1 nuclease protection assay

DIG-labeled DNA probes for Northern analyses were prepared using a DIG DNA labeling mixture (Roche, Diagnostics Co., Indianapolis, IN, U.S.A.) and primer sets as described previously (Kanamaru et al. 2000). Primer sets used for synthesis of probes for *SIG1-6*, *psaA*, *psbA*, *psbC*, *psbD* and *psbZ* were described previously (Fujiwara et al. 2000, Nagashima et al. 2004). The *psbD*-BLRP probe was prepared using BLRP-F (5′-)ACCTAACCCATCGAATCATG(-3′) and BLRP-R (5′-)TCACATACACCTTCGATCAC(-3′). The *rd29A* probe was prepared using, rd29A-F (5′-)TCCGGTCAATGAGAAGGATC(-3′) and rd29A-R (5′-)TTAAAGCTCCTTCTGCACCG(-3′). The total amount of RNA loaded in each lane was 0.3 μg for *psaA* and *psbA*, 1 μg for *psbC*, 3 μg for *psbD*, 5 μg for *rd29A*, 10 μg for *SIG2*, *SIG3* and *SIG6*, and 15 μg for BLRP, *psbZ*, *SIG1*, *SIG4* and *SIG5*. RNA blotting, hybridization and detection were performed as described previously (Kanamaru et al. 2000, Nagashima et al. 2004). The image density of the *SIG5* and *psbD*-BLRP transcripts on X-ray films were analyzed using Scion Image (Scion Corporation, Frederick, MA, U.S.A.). The S1 nuclease protection assay was performed as described previously (Hanaoka et al. 2003).

Construction of Arabidopsis transgenic lines

To monitor *SIG5*-promoter activity in situ, we generated a *SIG5* promoter-*uidA* (encoding β-glucuronidase (GUS)) fusion construct using the binary vector plasmid pBI101 (Clontech Labs, Inc., Carlsbad, CA, U.S.A.). A 1.8 kb Col genomic DNA fragment amplified by PCR using the primers, D5-BamHI (5′-)CCGGATCCTTGTAAGATTATGCGTTTAGAG(-3′) and D5-BamHI2 (5′-)ACGGATCCCACAATCTTAAGGCTCAAAAAT(-3′), and an *A. thaliana* P1 clone MLE8, were digested by *Pma*CI and *Bam*HI and cloned into the *Sma*I and *Bam*HI sites of pBluescript II KS+. A *Hin*dIII-*Bam*HI, fragment of the resulting plasmid comprising the *SIG5* promoter region, exon 1, intron 1, and 52 bp of exon 2 of *SIG5*, was cloned into pBI101. The resulting plasmid was introduced into *Arabidopsis* Col by *Agrobacterium*-mediated transformation (Clough and Bent 1998). For genetic complementation of the *sig5-2* mutation, we obtained an *Eco*RV genomic DNA fragment covering the entire *SIG5* and its promoter region from MLE8, and cloned it into the *Sma*I site of pBI101 (Clontech). The resulting construct was introduced into the *sig5-2* mutant as described above. The phenotypically complemented transgenic plant that was generated was designated as gE.

Histochemical analysis of GUS activity

GUS staining of *SIG5* promoter-*uidA* transgenic plants was basically performed as described previously (Kanamaru et al. 1999). Sample tissues were soaked in staining buffer containing 1 mM 5-bromo-4-chloro-3-indoryl-β-D-glucuronide (X-Glc; Rose Scientific Inc., Somerset, NJ, U.S.A.) for 24 h.

Analysis of chlorophyll fluorescence

Chlorophyll fluorescence parameters were measured by using a MINI-PAM portable chlorophyll fluorometer (Walz, Effeltrich, Germany) (Munekage et al. 2002). F_v/F_m was calculated as $(F_m - F_o)/F_m$, where the value of F_m was determined after relaxation of qE ($(F_m - F_m')/F_m'$) for 15 min in the dark.

Acknowledgments

We thank Dr. Nobuyoshi Mochizuki (Kyoto University) for helpful discussions and Yukio Ohashi for technical support. We also thank Syngenta Biotechnology Inc. and Dr. Satoshi Tabata (Kazusa DNA Research Institute) for providing the *sig5-2* T-DNA insertion line pool (Garlic 1232 H11) and the MLE8 P1 clone, respectively. This work was supported by a Grant-in-Aid for Scientific Research to K. T. (No. 14340248) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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(Received: January 20, 2004; Accepted February 9, 2004)