Rapid Paper

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

Akitomo Nagashima 1, Mitsumasa Hanaoka 1, Toshiharu Shikanai 2, Makoto Fujiwara 1, Kengo Kanamaru 1, 3, Hideo Takahashi 1, 4 and Kan Tanaka 1, 5

1 Laboratory of Molecular Genetics, Department of Molecular Biology, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-0032 Japan
2 Laboratory of Plant Molecular Biology, Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama-cho, Ikoma-shi, Nara, 630-0101 Japan

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, kilo-base(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...
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). 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.
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 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 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...
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. We examined this possibility, photo-inhibition 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. 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.

**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 recovery process (1–3 h). Data are shown as the means ± SD (n=16).
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 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. (B) Northern analyses of the psbD-BLRP 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.
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′-ends 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
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 (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 CO₂ 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 Mul-let 1995; Kim et al. 1999a). A pfl1 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.
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′-GTGATGGGAATGTCGATAC(-3′)) and SIG5S-4R (5′-GACCGAAGTGAAGTTGAAGTTG(-3′)) and a primer specific for the left border of the Syngenta T-DNA sequence. LB3 (5′-TAGCAGTGAATCTACATAACCAATCTCGATAC(-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., Indiana-Polis, 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 (Fujiiwara et al. 2000, Nagashima et al. 2004). The psbD-BLRP probe was prepared using BLRP-F (5′-ACCTAACCCATCGAATCATG(-3′)) and BLRP-R (5′-GTACATACCTCTGGATCAC(-3′)). The rd29A probe was prepared using, rd29A-F (5′-TCCGGTCAATGAGAAGGATC(-3′) and rd29A-R (5′-TCTAGGCTGCCAGCAGC(-3′)). The total amount of DNA 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′-CTGGATCTTGAAGTTGAC(-3′)) and D5-BamHI (5′-ACCTAACCCATCGAATCATG(-3′), and an A. thaliana P1 clone MLE8, were digested by PstI and BamHI and cloned into the Smal and BamHI sites of pBluescript II K+S. A HindIII-BamHI fragment, resulting of the 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 EcoRV genomic DNA fragment covering the entire SIG5 and its promoter region from MLE8, and cloned it into the Smal 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 GEL.

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-indolyl-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'/Fm was calculated as (Fm − F) / Fm where the value of Fm was determined after relaxation of qE (Fm − Fm') 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.

References


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