A Bacterial Transgene for Catalase Protects Translation of D1 Protein during Exposure of Salt-Stressed Tobacco Leaves to Strong Light[OA]

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During photoinhibition of photosystem II (PSII) in cyanobacteria, salt stress inhibits the repair of photodamaged PSII and, in particular, the synthesis of the D1 protein (D1). We investigated the effects of salt stress on the repair of PSII and the synthesis of D1 in wild-type tobacco (Nicotiana tabacum ‘Xanthi’) and in transformed plants that harbored the katE gene for catalase from Escherichia coli. Salt stress due to NaCl enhanced the photoinhibition of PSII in leaf discs from both wild-type and katE-transformed plants, but the effect of salt stress was less significant in the transformed plants than in wild-type plants. In the presence of lincomycin, which inhibits protein synthesis in chloroplasts, the activity of PSII decreased rapidly and at similar rates in both types of leaf disc during photoinhibition, and the observation suggests that repair of PSII was protected by the transgene-coded enzyme. Incorporation of [3S]methionine into D1 during photoinhibition was inhibited by salt stress, and the transformation mitigated this inhibitory effect. Northern blotting revealed that the level of psbA transcripts was not significantly affected by salt stress or by the transformation. Our results suggest that salt stress enhanced photoinhibition by inhibiting repair of PSII and that the katE transgene increased the resistance of the chloroplast’s translational machinery to salt stress by scavenging hydrogen peroxide.

Plants depend on light for survival, but light itself and, in particular, light in combination with other environmental stress can be harmful to plants (Boyer, 1982; Murata et al., 2007). Light stress interferes with oxygeneous photosynthesis, a phenomenon known as photoinhibition (Powles, 1984). Some researchers suggested that the primary negative effect of photoinhibition might involve damage to the quinone-binding protein, known now as the D1, in the photochemical reaction center of PSII (Kyle et al., 1984; Ohad et al., 1984; Krieger et al., 1998). The reaction center of PSII contains D1 and D2 proteins, to which all the relevant redox cofactors, including P680, are bound (Nanba and Satoh, 1989; Loll et al., 2005). Vass et al. (1992) have suggested that an excess of light energy might generate oxidative stress that would disrupt the transfer of electrons through PSII by damaging D1. However, the extent of photoinhibition of PSII reflects a balance between the light-induced damage (photodamage) to PSII and the repair of photodamaged PSII. More recently, stimulation of photoinhibition by excess light energy has been explained in terms of the inhibition of the repair of PSII by the oxidative stress that results from absorption of the excess energy (Murata et al., 2007).

Photodamaged PSII is repaired by replacement of damaged D1 by newly synthesized D1 (Kyle et al., 1984; Kettunen et al., 1991; Aro et al., 1993). It seems likely that damaged D1 is cleaved at a site in the stromal D-E loop by DegP, a specific endopeptidase (Haußühl et al., 2001). Then a metalloprotease, designated FtsH (Lindahl et al., 2000), removes the degraded D1 from PSII. At this point, it seems likely that PSII might be disassembled to some extent. The D1-depleted PSII is repaired by introduction of newly synthesized D1 (Kyle et al., 1984; Kettunen et al., 1991; Aro et al., 1993; Kanervo et al., 1998). After the components of PSII have reassembled, with the incorporation of de novo synthesized D1, the complex can function once again in the photosynthetic transport of electrons.

The synergistic effects of various stresses on photoinhibition have been studied extensively in cyanobacteria. Such studies are useful because it is possible to control the level of two or more kinds of stress much more precisely in suspension cultures of microorganisms than it is in plant leaves. For example, the use of Synechocystis sp. PCC 6803 (hereafter, Synechocystis) has allowed researchers to modulate various kinds of stress simultaneously and, thereby, to investigate the mechanisms of the effects of such stresses on the photodamage to PSII (Allakhverdiev and Murata, 2004). In particular, separate examination of the photodamage...
to and repair of PSII in *Synechocystis* has demonstrated that salt stress (Allakhverdiev et al., 2002), oxidative stress (Nishiyama et al., 2001, 2004), and cold stress (Gombos et al., 1994) stimulate the apparent photoinhibition of PSII by inhibiting the repair of PSII and not by accelerating the actual photodamage to PSII. Furthermore, salt stress and oxidative stress inhibit the translation of transcripts of *psbA* genes for the D1. How the salt stress and the oxidative stress are related in the mechanism for the inhibition of the repair and, in particular, the translation of *psbA* transcripts remains to be resolved.

However, cyanobacteria do not contain chloroplasts, and D1 of *Synechocystis* is encoded by a small family of *psbA* genes, namely, *psbA2*, *psbA3*, and *psbA7* (Jansson et al., 1987; Mohamed et al., 1993; Golden, 1994). By contrast, in plants and eukaryotic algae, the *psbA* exists as a single copy in the chloroplast genome. Moreover, the reactive oxygen species (ROS)-scavenging system of higher plants differs from that in cyanobacteria; plant chloroplasts possess two types of ascorbate peroxidase, whereas cyanobacteria contain thioredoxin peroxidase (Yamamoto et al., 1999). In addition, Calvin cycle enzymes of cyanobacteria are resistant to inhibition by hydrogen peroxide (H$_2$O$_2$; Takeda et al., 1995). Thus, results from cyanobacteria cannot necessarily be applied to higher plants.

In plants, the synergistic effects of light stress and other kinds of stress, such as salt stress and oxidative stress, have not been characterized. In previous studies, we introduced the *katE* for catalase of *Escherichia coli* into tobacco (*Nicotiana tabacum*) and into tomato (*Solanum lycopersicon*), with cDNA for a transit peptide of *katE* and *nptII* of *Solanum lycopersicon* (Jansson et al., 1987; Mohamed et al., 1993; Golden, 1994). By contrast, exposure of *katE* transformed plants to the same conditions resulted in less extensive inactivation of PSII, with only 20% inactivation at 50 mM NaCl. At 0.5 M and 1.0 M NaCl, inactivation during the 3-h incubation of leaf discs from wild-type plants fell by 70% of the original activity (Fig. 2, A and B). These results demonstrated that salt stress enhanced the photoinhibition of PSII and that *katE* transformed leaves were more tolerant to salt stress and the *nptII* gene as a marker. The result of PCR amplification indicated that these genes had been inserted into the genome of transformed plants (Fig. 1). In previous studies we demonstrated that *katE*-transgenic tobacco plants synthesized recombinant catalase and contained approximately 3-fold higher levels of catalase activity than wild-type ones (Shikanai et al., 1998). Moreover, in transgenic plants both endogenous and recombinant catalases retained their activity for 24 h under oxidative stress due to an application of paraquat, whereas endogenous catalase in wild-type plants was inactivated by 40% under such conditions (Miyagawa et al., 2000).

We examined the effects of salt stress due to NaCl at various concentrations on PSII activity (Fm’/Fv) during exposure to light stress of leaf discs from wild-type and *katE*-transformed tobacco plants (Fig. 2). When leaf discs of wild-type plants were exposed for 3 h to light at 2,000 μE m$^{-2}$ s$^{-1}$ under low-salt conditions (50 mM NaCl), PSII activity fell by 25% of the original activity. In the presence of 0.5 mM NaCl, inactivation was more marked and occurred more rapidly, such that 70% of the original activity disappeared during incubation for 3 h. In the presence of 1.0 M NaCl, PSII activity declined even more rapidly, with loss of 85% of the original activity over the course of 3 h (Fig. 2A). By contrast, exposure of *katE*-transformed plants to the same conditions resulted in less extensive inactivation of PSII, with only 20% inactivation at 50 mM NaCl. At 0.5 M and 1.0 M NaCl, inactivation during the 3-h incubation resulted in loss of 50% and 75% of the original activity, respectively (Fig. 2B). In darkness, incubation of leaf discs in the presence of 1.0 M NaCl did not inactivate PSII in either type of plant (Fig. 2, A and B). These results demonstrated that salt stress enhanced the photoinhibition of PSII and that *katE* transformed leaves were more tolerant to salt stress.

**RESULTS**

**Effects of Salt Stress on Photoinhibition of PSII**

We characterized *katE*-transgenic tobacco plants in terms of the introduction of the *katE* gene for catalase and the *nptII* gene as a marker. The result of PCR amplification indicated that these genes had been inserted into the genome of transformed plants (Fig. 1). In previous studies we demonstrated that *katE*-transgenic tobacco plants synthesized recombinant catalase and contained approximately 3-fold higher levels of catalase activity than wild-type ones (Shikanai et al., 1998). Moreover, in transgenic plants both endogenous and recombinant catalases retained their activity for 24 h under oxidative stress due to an application of paraquat, whereas endogenous catalase in wild-type plants was inactivated by 40% under such conditions (Miyagawa et al., 2000).

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**Figure 1.** Molecular characterization of *katE*-transgenic tobacco plants by means of PCR amplification of *nptII* and *katE* genes in the genomic DNA isolated from leaves of wild-type and *katE*-transformed tobacco plants. Conditions for PCR and probes for *nptII* and *katE* are described in the text. M, a 100-bp ladder of molecular markers; +C, positive control (pBl101-katE); T, *katE*-transgenic tobacco plants; W, wild-type plants.
than were wild-type leaves. In addition, although exposure of leaf discs separately to light stress and to salt stress inactivated PSII minimally, combination of the two kinds of stress induced marked inactivation of PSII, revealing apparent synergism between the effects of strong light and high concentrations of NaCl.

Effects of Salt Stress on Photoinhibition in the Presence of Lincomycin

To examine the contribution of de novo synthesis of chloroplast genome-encoded proteins to the salt stress-enhanced photoinhibition of PSII, we treated leaves with lincomycin as described in “Materials and Methods.” Figure 2, C and D, shows that inhibition of protein synthesis by lincomycin dramatically accelerated the inactivation of PSII in leaf discs from both wild-type and transgenic plants. Moreover, the inactivation observed in the presence of lincomycin was also unaffected by the concentration of NaCl. However, the extent of inactivation in the presence of lincomycin was only minimal when leaf discs were incubated in darkness in the presence of 1.0 mM NaCl in both types of plant (Fig. 2, C and D). These observations suggested that the protein synthesis de novo might be involved in the synergistic effects of light stress and salt stress during the inactivation of PSII.

Effects of Salt Stress on Levels of D1

We performed western blotting to examine the effects of NaCl on the level of D1 during photoinhibition at 2,000 μE m⁻² s⁻¹ in leaf discs from wild-type and katE-transformed tobacco plants (Fig. 3). The level of D1 decreased as the concentration of NaCl was increased in leaf discs from both wild-type and transformed plants. Incubation of wild-type leaf discs in strong light for 3 h under salt-stress conditions at 50 mM, 0.5 M, and 1.0 M NaCl decreased the level of D1 to 65%, 30%, and 15% of the original level, respectively (Fig. 3C). By contrast, in leaf discs from katE-transformed plants, incubation for 3 h at 50 mM, 0.5 M, and 1.0 M NaCl decreased the level of D1 to 75%, 45%, and 25% of the original level, respectively (Fig. 3D). These results might reflect the respective declines in PSII activity under the same conditions (see Fig. 2B).

Effects of Salt Stress on the de Novo Synthesis of D1

The results in Figures 2 and 3 suggested that the original activity of PSII was maintained by active synthesis of the D1 protein under nonstress conditions (50 mM NaCl), as observed in cyanobacteria. To confirm this hypothesis, we examined the effects of NaCl on the de novo synthesis of D1 in quantitative detail. Figure 4, A and C, shows that, in wild-type thylakoid membranes, the incorporation of [³⁵S]Met reached a maximum level after incubation for 3 h in the presence of 1.0 mM NaCl. However, the incorporation was markedly suppressed, falling to 10% of the original levels, in the presence of 0.5 mM NaCl. However, the incorporation was markedly suppressed, falling to 10% of the original levels, in the presence of 0.5 mM NaCl, which might have reflected the reduced level of PSII activity, under the same conditions, shown in Figure 2A. The synthesis of D1 was totally blocked in the presence of 1.0 mM NaCl.
However, in thylakoid membranes from transformed plants (Fig. 4, B and D), incorporation of [35S]Met similarly reached a maximum level after incubation for 3 h in the presence of 50 mM NaCl, but the rate of synthesis of D1 was reduced by approximately 50% in the presence of 0.5 m NaCl. This reduction reflects the 50% reduction in PSII activity shown in Figure 2B. At 1.0 m NaCl, the synthesis of D1 was inhibited completely. The phenomenon responsible for the inhibitory effect of NaCl on translation remains to be identified in future studies.

Effects of Salt Stress on the Level of the psbA Transcript

To identify the step(s) in the de novo synthesis of D1 that was inhibited by high concentrations of NaCl, we examined the effects of NaCl on the level of the transcript of the psbA gene during photoinhibition in leaf discs of wild-type and katE-transformed tobacco plants at 2,000 ?m E m⁻² s⁻¹ (Fig. 5). The level of the psbA transcript increased rapidly at 50 mM NaCl (Fig. 5, C and D). The presence of 0.5 mM NaCl markedly delayed the increase in the level of the transcript. However, the final level of the transcript, namely, the level after exposure of leaf discs to light at 2,000 ?m E m⁻² s⁻¹ for 3 h, was almost unaffected by 0.5 mM NaCl in leaf discs from both wild-type and katE-transformed plants. At 1.0 mM NaCl almost completely prevented any increase in the level of the transcript. Thus, the katE transgene had no significant effect on the level of the psbA transcript under all tested conditions.

DISCUSSION

In this study, we demonstrated that salt stress due to NaCl stimulated photoinhibition of PSII. Salt stress might have acted directly on sites and/or steps that were essential for the protection of PSII against photoinhibition. However, it also seems possible that salt stress decreased the activity of Rubisco, with resultant inhibition of CO₂ fixation, and a decrease in utilization of NADPH in the Calvin cycle would have stimulated the generation of ROS in PSI (Murata et al., 2007). An increase in ROS concentration might have enhanced the level of photoinhibition.

To examine whether photoinhibition is stimulated by salt stress directly or indirectly via the generation of ROS, we generated the katE-transgenic tobacco plants (Fig. 1), in which the recombinant catalase was synthesized and translocated to chloroplasts. The results in Figure 2, A and B, show that the presence of recombinant catalase alleviated photoinhibition under salt-stress conditions; thus, we concluded that the ROS concentration was probably decreased. These findings may suggest that the indirect effect of salt stress via ROS is involved in the salt-stress-induced stimulation of photoinhibition. However, the direct effect of salt stress on the stimulation of photoinhibition, in combination with the indirect effect, cannot be excluded.

In the presence of lincomycin (Fig. 2, C and D), which inhibits the expression of chloroplast genome-encoded genes, photoinhibition occurs in the absence of repair. Consequently, use of this drug allows separation of photodamage from photoinhibition. We found that salt stress did not have a significant effect on the level of D1 in tobacco leaves. In addition, we confirmed the synergistic negative effects of light stress and salt stress on the PSII complex in tobacco leaves. This observation suggests that salt stress might inhibit repair, with a consequent apparent increase in the extent of photoinhibition. This result reflects a similar phenomenon in cyanobacteria (Allakhverdiev et al., 2002).

Western blotting demonstrated that the level of D1 decreased during photoinhibition under salt stress (Fig. 3). However, the rate of decrease in the level of D1 was lower than the rate of loss of PSII activity. This difference was conspicuous in wild-type plants (Figs. 2A and 3C). The apparent discrepancy between PSII activity and the level of D1 might be explained by the
The antibodies that we used reacted with both active D1 and damaged D1 but before cleavage (Barber and Andersson, 1992; Aro et al., 1993; Allakhverdiev et al., 2002).

When we examined the incorporation of \([\text{35S}]\text{Met}\), we found that the translation of the \(\text{psbA}\) transcript to yield the D1 protein in leaves from wild-type plants was markedly suppressed in the presence of 0.5 M NaCl (Fig. 4C), while such translation was inhibited to a lesser extent in leaves from \(\text{katE}\)-transformed plants under the same conditions (Fig. 4D). The difference, in terms of incorporation of \([\text{35S}]\text{Met}\), the concentration of NaCl (NaCl), and the duration of illumination (h) are indicated above the lanes. Each point and bar represent the average \(\pm s\) of results from three independent experiments.

Northern blotting indicated that there were only insignificant differences, in terms of levels of the \(\text{psbA}\) transcript in the presence of 0.5 M NaCl, between leaf discs from wild-type and \(\text{katE}\)-transformed tobacco plants. Leaf discs were incubated with \([\text{35S}]\text{Met}\) at 2,000 \(\mu\text{E m}^{-2} \text{s}^{-1}\) in the presence of 50 mM, 0.5 M, and 1.0 M NaCl, as indicated. At indicated times, leaf discs were frozen in liquid N\(_2\), ground, and homogenized in isolation buffer (see text for details), and then thylakoid membranes were isolated. The thylakoid membranes were solubilized and proteins were separated by SDS-PAGE as described in the text. Proteins from thylakoid membranes corresponding to 5 \(\mu\)g of Chl were applied to each lane. A, Autoradiography of radioactively labeled proteins from wild-type plants after SDS-PAGE; B, autoradiography of radioactively labeled proteins from \(\text{katE}\)-transformed plants after SDS-PAGE; C and D, quantification of the hybridization signals shown in A and B, respectively. •, 50 mM NaCl; △, 0.5 M NaCl; ■, 1.0 M NaCl. The light intensity (\(\mu\text{E m}^{-2} \text{s}^{-1}\)) application of \([\text{35S}]\text{Met}\), the concentration of NaCl (NaCl), and the duration of illumination (h) are indicated above the lanes. Each point and bar represent the average \(\pm s\) of results from three independent experiments.

In this study, we postulate that the translational machinery in chloroplasts might be inhibited by salt stress via the actions of ROS. The excess ROS, resulting from accumulated electrons in PSI, might attack two potential sites. The first potential site is the site of initiation of translation, at which the reduced form of RB60 activates the translation of \(\text{psbA}\) mRNA by binding to the 5’ untranslated region (Danon and Mayfield, 1994; Zhang and Aro, 2002). The second potential site of disruption is the site of elongation factors are active when their thiol-containing residues are reduced (Kuroda et al., 1996; Yamazaki et al., 2004). It seems also possible that salt stress directly inactivates the translational machinery in chloroplasts, resulting in the inhibition of protein synthesis.
were described elsewhere (Shikanai et al., 1998; Miyagawa et al., 2000). The characteristics of antioxidant enzymes of this transformant were amplified with the forward and reverse primers katE et al., 1991). For genes were designed on the basis of reported sequences (Ossowski, 1998). Seeds of wild-type and *Solanum lycopersicon* Rubisco, was generated as described previously (Shikanai et al., 1998). Seeds of wild-type and katE-transformed tobacco plants were allowed to germinate for 1 week on the wet filter paper in petri dishes, and then seedlings were planted in a mixture of autoclaved perlite and compost soil. Plants were grown in a growth chamber at 60% relative humidity and 25°C with a 16-h-light/8-h-dark cycle under a photon flux density of 300 μE m⁻² s⁻¹. The light intensity (μE m⁻² s⁻¹), the concentration of NaCl (NaCl), and the duration of illumination (h) are indicated above the lanes. Each point and bar represent the average ± s of results from three independent experiments.

(Murata et al., 2007). The relevant sites remain to be identified in future studies.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

The line of katE-transformed tobacco plants (*Nicotiana tabacum 'Xanthi’*) that harbored the katE gene for catalase from *Escherichia coli*, driven by the promoter of the hscSC gene for the small subunit of tomato (*Solanum lycopersicon*) Rubisco, was generated as described previously (Shikanai et al., 1998). Seeds of wild-type and katE-transformed tobacco plants were allowed to germinate for 1 week on the wet filter paper in petri dishes, and then seedlings were planted in a mixture of autoclaved perlite and compost soil. Plants were grown in a growth chamber at 60% relative humidity and 25°C with a 16-h-light/8-h-dark cycle under a photon flux density of 300 μE m⁻² s⁻¹ during the light phase. The characteristics of antioxidant enzymes of this transformant were described elsewhere (Shikanai et al., 1998; Miyagawa et al., 2000).

**Molecular Characterization of Plants**

Genomic DNA was prepared from plant leaves (0.5–1 g fresh weight) by the method of Dellaporta et al. (1983). Specific primers for the katE and *nptII* genes were designed on the basis of reported sequences (Ossowski et al., 1991). For katE, a fragment of 457 bp from positions 923 to 1,379 of the sequence was amplified with the forward and reverse primers 5′-AAAATACTCACCACGGACGTGAC-3′ and 5′-TAATTCGCCGGTTAGT- GTG-3′, respectively. For *nptII*, a fragment of 254 bp from positions 24 to 277 of the sequence was amplified with the forward and reverse primers 5′-GCCAGGTCTCCGCCGCCGCTG-3′ and 5′-GCCAGGTCGCCCTCCTCCCGCTG-3′. The 20-μL amplification mixture contained approximately 0.1 μg of plant genomic DNA, 200 μM dNTPs, 2 mM MgCl₂, 1 μM each primer, and 1 unit of Taq DNA polymerase (Taq; Toyobo). The PCR was conducted with initial incubation at 95°C for 2 min, which was followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1.5 min, and final incubation at 72°C for 7 min.

**Photoinduction and Measurements of Chlorophyll Fluorescence**

Leaf discs (2 cm in diameter) were punched from healthy, fully expanded tobacco leaves from 4-week-old plants, and discs were floated, adaxial side up, in distilled water that contained 50 mM, 0.5M, or 1.0M NaCl and exposed for 1, 2, or 3 h to strong light at 2,000 μE m⁻² s⁻¹ from a tungsten lamp (Luminar Ace LA-150TX; Hayashi Watch-Works) for photoinduction of PSII at 25°C. After photoinduction treatment and subsequent incubation in darkness for 30 min, PSII activity was monitored in terms of chlorophyll (Chl) fluorescence and expressed as Fv/Fm, as determined at 25°C with a fluorescence-monitoring system (Hansatech Instruments).

For experiments in the presence of lincomycin, intact tobacco leaves were incubated with 0.6 mM lincomycin (Sigma) for 2 h in darkness as described by Aró et al. (1993). Leaf discs were then exposed to the stress as described above.

**Western-Blotting Analysis of D1**

Thylakoid membranes were isolated and solubilized from leaves of wild-type and katE-transformed plants as described by Suorsa et al. (2004). Chl concentrations were determined in 80% acetone as described by Mackinney (1941). The thylakoid membranes were immediately frozen in liquid N₂ and stored at −80°C prior to use. After electrophoresis by SDS-PAGE, the separated thylakoid proteins were blotted electrophoretically onto a polyvinylidene fluoride membrane (Dunn, 1986) in a semidy transfer apparatus (Atto). D1 was then detected immunologically with a SuperSignal West Dura Extended Duration Substrate kit (Thermofisher Biotechnology). D1 was detected with rabbit antibodies raised against amino acid residues 55 through 78 in the AB loop of D1 from spinach (*Spinacia oleracea*; Taguchi et al., 1995). As second antibodies, we used horsederish peroxidase-linked antibodies raised in goats against rabbit IgG (Thermofisher Biotechnology). The antibodies raised in rabbits against D1 were kindly provided by Prof. Kimiyuki Satoh (Department of
Incorporation of [35S]Met into Proteins of Thylakoid Membranes

The lower epidermis of tobacco leaf discs (diameter, 2 cm) was gently pressed against coarse sandpaper to facilitate the incorporation of [35S]Met. Leaf discs were then floated on a solution (one disc per 6 ml) that contained 3 nM [35S]Met (>30 TBq mmol⁻¹; Institute of Isotopes) and 0.4% Tween 20. Discs were illuminated at 2,000 μE m⁻² s⁻¹ at 24°C for 1, 2, or 3 h in the presence of 50 mM, 0.5 M, or 1.0 M NaCl. At indicated times, the pulse-labeled leaf discs were rapidly frozen in liquid N₂ and stored until two leaf discs from each time point were combined and thylakoid membranes were isolated with minimal delay. Chl was quantitated and SDS-PAGE was performed. For analysis of radioactivity in proteins of thylakoid membranes, gels were stained, destained, vacuum-dried, and then exposed for autoradiography to phosphor-imager screens for approximately 5 d. The screens were monitored with a phosphor imager (FLA-3000; Fuji Photo Film) and the amount of radioactivity in D1 was estimated densitometrically.

Northern-Blotting Analysis

Northern blotting was performed with the digoxigenin (DIG) system in accordance with the manufacturer’s instructions (Roche Molecular Biochemicals DIG application manual for filter hybridization). Leaf discs that had been exposed to light stress were frozen immediately in liquid N₂ and ground with a pestle and mortar. Total RNA was then extracted with the Sepasol-RNA I super reagent (see above). Five micrograms of total RNA was denatured mixture was fractionated by electrophoresis in MOPS buffer on a 2% agarose gel that contained 2% formaldehyde at 100 mV/1.5 h per gel.

The capillary transfer of RNA, hybridization of blots with DIG-labeled DNA probe, immunological detection, and chemiluminescent detection were performed according to the instruction from Roche Molecular Biochemicals (DIG application manual for filter hybridization). Signals from hybridized mRNAs were detected with a luminescence image analyzer (LAS-1000; Fuji Photo Film). For normalization, staining with ethidium bromide of 25S rRNA allowed as to confirm loading of equivalent amounts of each sample.

Preparation of the Probe for Northern-Blotting Analysis of the psbA Transcript

Total RNA was isolated from tobacco leaves by the CTAB method with the Sepasol-RNA I super reagent (see above). Five micrograms of total RNA was reverse transcribed with an oligo(dT)₁₈; primer, and single-strand cDNA As were prepared with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was then performed with gene-specific primers that were designed on the basis of the sequence of the psbA gene: forward primer, 5'-TTATCCCTACTTCTGACCTATAGG-3' (the underlined sequence is a PsfI site); and reverse primer, 5'-CGATACGGTAGCTGACGGGA-3' (the XbaI site is underlined; positions +245 to +269 and +1,015 to +1,039, respectively, counted from the first ATG of the plastid psbA gene [Sugita and Sugiyama, 1984; EMBL/GenBank/DDJ accession no. X00616]). The amplified 795-bp fragment of DNA that included the coding region of the plastid psbA gene (nucleotides +245 to +1,039, counted from the site of initiation of translation) was cloned into a cloning vector (pBluescript II SK⁺; Stratagene) according to the instructions of Sambrook et al. (1989). The resultant 795-bp fragments of DNA were labeled with DIG-labeled nucleotides (DIG-dUTP) using the PCR DIG probe synthesis kit in accordance with the manufacturer’s instructions (DIG system; Roche Molecular Biochemicals DIG application manual for filter hybridization; Roche Applied Science). The labeled fragments were used as probe.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number X00616.


