Characterization of the Light-Responsive Promoter of Rice Chloroplast psbD-C Operon and the Sequence-Specific DNA Binding Factor

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The transcription of rice plastid psbD-psbC genes encoding photosystem II reaction center protein D2 and chlorophyll a-binding protein CP43 is closely regulated by light. To elucidate the sequence requirement for the light-responsive promoter of psbD-psbC operon, transcriptional analysis of the rice promoter was performed with deleted mutants and site-directed mutants in vitro. Deletion of −546−−100 upstream sequences resulted in 4- to 5-fold decrease in the transcription rate. Further deletion of −99−−40 conserved region of repeated sequences resulted in 2-fold decrease in the transcription rate. The core light-responsive promoter requires “−10” element but not “−35” element for accurate initiation of basal transcription. No downstream promoter element was found in the +4−+111 region. The competitive gel-retardation experiments revealed the presence of DNA-binding protein in the rice chloroplasts, which interacts specifically with the −60−−37 repeated sequences. Southwestern blot analysis further demonstrated that the binding factor is composed of 36-kDa polypeptide(s).

Key words: Chloroplast — Light-responsive — psbD-C promoter — Rice (Oryza sativa) — Trans-acting factor.

The psbD-C operon of rice plastids is composed of the photosynthetic genes psbD and psbC, flanking by two open reading frames orf100 and orf62 of unknown function (Chen et al. 1994, Hiratsuka et al. 1989). The psbD gene encodes photosystem II reaction-center protein D2, which forms a heterodimer with protein D1 to bind chlorophyll, phylloquinone, and quinone. This gene overlaps by 17 bp the psbC gene encoding chlorophyll a-binding protein CP43 that serves as a light-harvesting core antenna (Alt et al. 1984, Holschuh et al. 1984, Rasmussen et al. 1984). Light has been reported to play a critical role in the expression of the psbD-C operon in rice (Chen et al. 1995), barley (Sexton et al. 1990), and wheat (Wada et al. 1994). The promoter of this operon, located immediately upstream of orf100, has been found to be blue light-responsive in monocot and dicotyledonous plants (Christopher et al. 1992). In the illu-

| Abbreviations: LRP, light-responsive promoter; Ta, threonine attenuator. |

| Plasmid construction—The rice plastid EcoRI/MaeI fragment, extending from −546 to +111 (region Y as described in Chen et al. 1994, also see Fig. 1), was inserted into the BamHI site of pTZ19-Ta to generate p546. pTZ19-Ta contains the factor-independent transcription terminator from E. coli threonine attenuator (Chen and Orozco 1988). To construct the 5′-deletion clones, p546 was digested at both SphI and XbaI sites present in the multiple cloning sites upstream of the plastid DNA insert. Deletion with exonuclease III was carried out from the XbaI end, followed by religation to yield plasmids p99, p68, p17, p7, and p1, which contain the deletion region spanning from −546 to −1. Plasmid p99, extending from −99 to +111, was further utilized as parental construct for in vitro mutagenesis using a site-directed |
transglutamase kit (Clontech Laboratories, Inc. CAL. U.S.A.). Trans Oligo Affil I/Bgl II was used as a selection primer. Three base-substitution clones p33CC, p12CG, and p4CG, one insertion clone p22+ CT, and three internal deletion clones Δ (4/46): deletion from +4 to +46, Δ (88/111): deletion from +4 to +87, and Δ (88/111): deletion from +88 to +111 were thus generated.

**Rice chloroplast isolation and high-salt extract preparation**—Rice (Oryza sativa L. cv. Tainong 67) seeds were imbibed at 28°C in the dark for two days, then planted in vermiculite and transferred to a growth chamber under a 12 h dark/12 h light cycle (250 μmol m−2 s−1), cool white fluorescent tube (P96T12/CW/VHO, Sylvania, Danvers, MA, U.S.A.) plus incandescent bulbs (Sylvania 40W]). To isolate chloroplasts, the apical 5–7 cm portions of leaves and sheath from the two-week-old rice seedlings were harvested and homogenized in five times volume of grinding buffer (50 mM HEPES, pH 8.0, 1 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, 330 mM sorbitol, 5 mM sodium ascorbate). After filtration through two layers of Miracloth (Calbiochem), the filtrate was briefly centrifuged at 22,000 g. The pellet was suspended in a small volume of grinding buffer and overlaid on top of 30%/60% sucrose step gradient in grinding buffer. The gradient was prepared in a 35-ml centrifugation tube and consisted of 30%/60% sucrose step gradient in grinding buffer. The gradient was prepared in a 35-ml centrifugation tube and consisted of 30%/60% sucrose step gradient in grinding buffer. Following centrifugation at 22,000 rpm for 30 min with a Hitachi SRP28 rotor, the green interface layer of chloroplasts was collected.

**Promoter analysis with in vitro transcription**—Transcriptionally active chloroplast extract was prepared from spinach essentially as described by Orozco et al. (1985). The transcription reaction (40 μl) containing spinach high-salt extract (approximately 50 μg of protein), 370 Kbp a-32P[γGTP] (Amersham International plc. England), and 500 ng of supercoiled DNA template of various mutant plasmids was performed at 28°C for 1 h. DNA of pTZ19-Ta was used as a negative control. The in vitro transcription products were phenol extracted and analyzed by electrophoresis on a SDS-polyacrylamide gel (10%). The molecular size markers (Pharmacia Biotech AG, Switzerland) were run in parallel with the sample. After running, the gel was subjected to autoradiography essentially the same as described by Chen et al. (1993). The gel was equilibrated with blotting buffer (25 mM Tris-HCl, pH 8.3, 190 mM glycine, 0.1% SDS) for 30 min, and then electroblotted onto a nitrocellulose membrane (BA85, Schleicher & Schuell, Germany). The membrane blot was then incubated with renaturation buffer (10 mM HEPES, pH 7.5, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 1 mM MgCl₂, 0.1 mM ZnSO₄, and 5% nonfat milk) at 4°C for 24 h. After washing with TNE-50 buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT), the blot was incubated in TNE-50 buffer containing either the double-stranded 24-bp probe WT (1.5 × 10⁶ cpm ml⁻¹) or the 24-bp mutant probe MT (2.6 × 10⁵ cpm ml⁻¹), together with poly(dI-dC)-poly(dI-dC) (10 μg ml⁻¹) for 1 h at 25°C. The blot was then washed twice with TNE-50 at room temperature for 10 min followed by autoradiography. A 31-bp polylinker fragment (LINKER) obtained by EcoRI/XbaI digestion of pUC19 was also utilized as control probe (4.1 × 10⁶ cpm ml⁻¹).

**Results**

Analysis of cis-acting elements of the rice psbD-C LRP—To elucidate the sequence requirement for the psbD-C light-responsive promoter, rice plastid EcoRI-MaeI fragment extending from −546 to +111 (Fig. 1), containing the light-responsive promoter and a distal non light-responsive promoter, was fused with a threonine attenuator (Ta) of pTZ19-Ta to yield plasmid p546. As exogenous DNA template for in vitro transcription with spinach chloroplast high-salt extract, both of the two promoters in this minigene construct were utilized by chloroplast RNA polymerase to initiate transcription. Ta was effectively recognized by chloroplast RNA polymerase for termination. The in vitro transcription products of 213–215 nt shown in the autoradiogram of Fig. 2 corresponded to those transcribed from the LRP. Another transcription product of 363 nt corresponding to that transcribed from the distal non light-responsive promoter was also obtained (data not shown). The products transcribed from the LRP in vitro by spinach chloroplast extract, however, revealed less heterogeneity at the 5' termini than those transcribed by rice chloroplasts in vivo (Chen et al. 1994).
Fig. 1 Nucleotide sequence of the rice psbD-C light-responsive promoter region. The multiple transcription initiation sites are marked with asterisks and +1 corresponds to the most abundant transcript generated in vivo. The short direct repeats of AAAG, AAGT and GAC are marked with horizontal arrows. The putative "-35" and "-10" sequences are underlined. The 5' end of each 5'-deletion construct is indicated with a rectangular arrow. The substitution/insertion clones are indicated with vertical arrows pointing to the bases substituted or the position for insertion. The region underlined with dashed line is used to make oligoprobe WT. The coding region of psbD starts from +566.

Plasmid p546 was then subjected to exonuclease III deletion to give a series of 5'-deletion clones p99, p68, p40, p15, p7, and p1. The constructs were analyzed for their transcriptional activity. Plasmid p99, containing a deletion spanning from -546 to -100 but retaining the highly conserved region upstream of the psbD-C light-induced transcript 5' termini, was transcribed accurately from LRP to give the products of 213~215 nt (Fig. 2, Fig. 3B). The decrease in the band intensity indicated that the sequences between -546 and -100 were able to enhance the promoter activity of LRP 4- to 5-fold. Plasmid p68, with more deletion containing part of A-rich sequences of the conserved region ( -99 to -69), gave rise to less 213~215 nt products (about 50%) compared to p99. Additional removal of AAAG repeats and AAGT repeats in p40 resulted in slight decrease in promoter strength compared to that of p68. Removal of the putative "-35" element in p15 reduced about 50% of the promoter strength. Further deletion of the multiple transcription initiation sites covering the putative "-10" in plasmids p7 and p1 resulted in complete loss of the LRP promoter activity. This observation indicates the AT-rich sequences of the multiple transcription initiation sites containing the "-10" element do function as the core promoter element for psbD-C LRP.

It was suspected that core promoter element present downstream the transcription start sites. To test this possibility, internal deletion of sequences from +4 to +46, +4 to +87, and +88 to +111 was performed by site-directed mutagenesis of p99. Three deletion constructs Δ(4/46), Δ(4/87), and Δ(88/111) were thus obtained for transcription analysis. Fig. 3A shows that deletion of the downstream sequences spanning from +4 to +111 did not affect the transcription. The sizes of the transcripts produced by these deletion constructs are smaller than that produced by the parental construct p99, according to the internal deletion length. This result indicates the absence of the downstream promoter element for psbD-C LRP in this region.

Base substitution and insertion were then performed to dissect the core promoter sequences and the flanking sequences. Substitution of the putative "-35" from TTGAAT into CCGAAT (plasmid p33CC) reduced the promoter strength about 30-40% (Fig. 3B). This observation, together with the result of the deletion construct p15 (Fig. 2) indicate the putative "-35" is not essential for the core promoter function. Substitution in the AT-rich region of the multiple transcription start sites (plasmids p12CG and p4CG) completely abolished the promoter activity, indicating the essential role of this core promoter region. It is very interesting that insertion of two bases CT between "-10" and "-35" regions (plasmid p22+CT) greatly enhanced the promoter activity (Fig. 3B). The spacing between "-10" and "-35" was extended to 17 bp, which is the optimal spacing for prokaryotic promoter (Hawley and McClure 1983) and many chloroplast promoters (Hanley-Bowdoin and Chua 1987).
Light-responsive \textit{psbD-C} promoter and \textit{trans} factor

Fig. 2 Transcriptional analysis of p546 harbouring rice \textit{psbD-C} LRP and the relevant 5'-deletion constructs with spinach chloroplast extract. pTZ19-Ta was used as control template. Parental construct p546 contained rice plastid DNA fragment extending from $-546$ to $+111$, Clones p99, p68, p40, p15, p7, and p1 are deletion clones with 5' ends starting from $-99$, $-68$, $-40$, $-15$, $-7$ and $+1$ respectively. Arrow indicates the transcripts. The molecular size markers are indicated at the left margin in nucleotides (nt).

Fig. 3 Transcriptional analysis of the internal deletion constructs (A) and the substitution/insertion constructs (B) with spinach chloroplast extract. pTZ19-Ta was used as control template. $\Delta(88/111)$, $\Delta(4/46)$, and $\Delta(4/87)$ are clones containing internal deletion from $+88$ to $+111$, from $+4$ to $+46$, and from $+4$ to $+87$ respectively, p33CC is the clone with 2-bp substitution of putative "$-35$". p12CG is the clone with 2-bp substitution of "$-10$". p4CG is the clones with 2-bp substitution at $-4$ and $-3$ of AT-rich region. p22+CT is the clone with 2-bp insertion between putative "$-35$" and "$-10$". Arrows indicate the transcripts. The molecular size markers are indicated at the left margin in nucleotides (nt).
Fig. 4 Detection of the rice chloroplast proteins interacting with the conserved repeated sequences of \textit{psbD-C} LRP by gel retardation assay. (A) The end-labeled 24-bp oligoprobe WT (1 ng) containing the AAAG, AAGT and GAC repeat elements (from position $-60$ to $-37$) was incubated either in the absence (lane 0) or presence (lane 1) of 3\,$\mu$g of chloroplast protein fraction. The resulting DNA-protein complexes were resolved on a native 5\% polyacrylamide gel. In a parallel experiment, the chloroplast protein fraction was pretreated with proteinase K for 15 min at 25\,°C prior to the binding reaction (lane 2). (B) The rice chloroplast protein fraction was preincubated at 0\,°C, 37\,°C, 50\,°C, 60\,°C, and 70\,°C for 20 min, followed by binding reaction. The free probe (F) and the retarded bands (B1, B2, B3, B4) of bound complexes are indicated at the left margin.

50- to 100-fold molar excess over probe concentration (lanes 6, 7), indicating that AAAG element is very critical for complex formation. The AAGT repeats competed two retarded bands B3 and B4 but not B1 or B2 (lane 10). The GAC repeats competed all four retarded bands (lane 13), but less efficiently compared to the AAAG repeats. The importance of AAAG element is further demonstrated by using a "mutant" probe MT for the binding reaction (Fig. 5C). Two AAAG elements were specifically mutated to a heterologous sequence in this probe. Fig. 5C demonstrated that the retarded bands B2, B3, and B4 disappeared nearly completely when using MT as binding probe. Only B1 band is revealed in the autoradiogram.

To further characterize the sequence-specific binding protein in rice chloroplasts, Southwestern blot analysis was carried out. The ammonium sulfate fraction of chloroplast high-salt extract was denatured first, followed by SDS-polyacrylamide gel electrophoresis. The separated proteins on the gel were electroblotted onto nitrocellulose membrane and renatured. The sequence-specific binding activity of the protein was then tested by incubating the membrane blots with various probes (Fig. 6). Even after overexposure of the autoradiogram, no complex formation was detected when both negative controls MT and LINKER were employed as the binding probes. In contrast, the protein band at approximately 36-kDa position exhibited strong binding.

Fig. 5 Gel-retardation competition analysis showing the specific protein binding to the conserved repeated sequences of \textit{psbD-C} LRP. (A) Sequences of the oligoprobe WT, the WT subfragments including AAAG repeats, AAGT repeats, and GAC repeats, as well as "mutant" probe MT used in the gel retardation experiments. The repeated sequences are underlined or overlined. (B) Competition binding experiments using end-labeled WT (1 ng) as a probe were performed with 5\,$\mu$g of rice chloroplast protein fraction in the absence (lane 1) or presence of varying amounts (5-, 25-, and 100-fold molar excess) of each unlabeled oligonucleotide competitors indicated above lanes 2~13. Lanes 14 and 15 are binding experiments in the presence of 100-fold molar excess of unlabeled pBR322/MspI digests and \lambda/HindIII digests respectively. (C) Binding experiments was performed using labeled "mutant" probe MT (1 ng). The probe was incubated without (lane 1) or with (lane 2) 5\,$\mu$g of rice chloroplast protein fraction. All binding reactions in (B) and (C) contained 2\,$\mu$g of poly(dI-dC). The free probe (F) and the retarded bands (B1, B2, B3, B4) of bound complexes are indicated at the left margin.
The arrow indicates the 36 kDa-protein band interacting specifically with probe WT. The protein size markers are indicated at the left margins.

activity with probe WT. The data strongly suggest that the rice chloroplast 36-kDa protein is the major factor that binds specifically to the 24-bp sequence of the rice psbD-C LRP.

Discussion

In this work, the functional analysis of the rice psbD-C LRP by exonuclease III deletion mapping and site-directed mutagenesis with in vitro transcription in chloroplast extract has been carried out. Our results suggest the presence of a remote sequence element between positions −546 to −100, which enhances the rate of transcription from LRP 4- to 5-fold (Fig. 2). This observation has not been reported in the studies of tobacco (Allison and Maliga 1995) or barley (Kim and Mullet 1995), since only 80 to 121 bp upstream of the light-responsive transcription initiation sites were analyzed in those studies. The sequences between −99 and −69, corresponding to the so-called PGT box of barley LRP or A-rich sequences of tobacco LRP, were found to enhance the rate of transcription about 2-fold (Fig. 2). Our data is consistent with the in vivo study in tobacco that A-rich sequences act to enhance the promoter strength. However, Kim and Mullet (1995) reported that deletion of barley PGT box had little influence on the promoter strength by in vitro transcription assay.

Removal of the upstream sequences including “−35” (TTGAAT) or 2-bp substitution of “−35” to give CCGAAAT resulted in reduced transcription rate (Fig. 2, Fig. 3B); however, it still allows accurate transcription from the same sites utilized by the full LRP promoter. Further deletion of the “−10” element or base substitution in the AT-rich region covering the multiple transcription initiation sites caused complete loss of promoter activity (Fig. 2, Fig. 3B). Taken together, these data suggest that the basal psbD-C LRP activity requires “−10” element but not “−35” element. This atypical type of chloroplast promoters have been observed in some chloroplast genes including mustard rps16 (Neuhaus et al. 1989), tobacco rpl32 (Vera et al. 1992), Chlamydomonas atpB (Klein et al. 1992) and rbcL (Klein et al. 1994). Klein et al. (1994) reported the activity of the atypical Chlamydomonas rbcL promoter was enhanced by a remote sequence element located downstream the core promoter. However, in our study, internal deletion analysis spanning from −4 to +111 (Fig. 3A) ruled out the possibility that sequences downstream of the core promoter act to enhance transcription from LRP. Several evidence point to the existence of at least two different forms of chloroplast RNA polymerases (Greenberg et al. 1984, Igloi and Kossel 1992). The E. coli-like RNA polymerase, with core subunits encoded by plastid rpo genes, has been well reported (Little and Hallick 1988, Hu et al. 1991). This polymerase recognizes the prokaryotic-like promoter containing −35/−10 elements. Recently a non-E. coli-like RNA polymerase, nuclear-encoded, has been discovered in spinach with unknown promoter specificity (Lerbs-Mache 1993). This enzyme might be involved in the transcription of the above-described atypical chloroplast promoter lacking the “−35” element.

The competitive gel retardation assays (Fig. 5) revealed the presence of sequence-specific DNA-binding protein in the rice chloroplasts, which interacts with the highly conserved region upstream of the core LRP (positions −60 to −37). The AAAG element appears to play a pivotal role in specific DNA-protein interaction. The existence of the chloroplast proteins interacting with the conserved region of LRP has been recently found in barley (Kim and Mullet 1995) and tobacco (Allison and Maliga 1995). The barley chloroplast factor AGF, which interacts with the AAG-rich region, is concluded to be distinct from the plastid sigma-like factor (Tiller and Link 1993, Troxler et al. 1994).

Our Southwestern blot analysis (Fig. 6) further demonstrated that the rice chloroplast protein binding to the conserved repeated sequences is composed of 36-kDa polypeptide(s). In order to understand whether this DNA-binding protein is related to the light-regulatory components that activated specifically the LRP, as suggested by Wada et al. (1994) from in vitro transcription analysis with the wheat psbD-C LRP, we have carried out a gel retardation
assay with etioplast extract from dark-grown rice. However similar band patterns were observed as compared with that using chloroplast extract (data not shown), suggesting that the sequence-specific factor may not be directly under light control. Further purification and characterization of the protein factor will help to give more insight into the molecular mechanism of the light-responsive transcription of psbD-C genes.

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References


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