Isolation and characterization of photosystem 2 *PsbR* gene and its promoter from drought-tolerant plant *Prosopis juliflora*

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Abstract

Prosopis juliflora is a hardy plant tolerant to drought, salinity, extremes of soil pH, and heavy metal stress. We isolated and characterized a photosystem 2 (PS2) gene *PsbR* (*Pj PsbR*) and its promoter. Northern analysis for *Pj PsbR* in *P. juliflora* leaves under 25 % polyethylene glycol stress showed steady decrease in transcript level at 12, 24, and 48 h after stress application. Under 90 mM H_2O_2 stress, transcript level dropped drastically at 12 h, but increased again compared to the control at 24 h. A 1.7 kb fragment upstream the 5' UTR of this gene (putative promoter) was isolated and analyzed *in silico*. Several putative *cis*-acting DNA elements were identified in this sequence.

Additional key words: H_2O_2; Northern analysis; photosystem 2; polyethylene glycol; proteins; transcript level; water deficit.

Introduction

Despite reports that photosystem 2 (PS2) is highly drought resistant (Yordanov *et al*. 2003), as the water stress increases, photosynthetic electron transport through PS2 is inhibited (Chakir and Jenson 1999). Several *in vivo* studies demonstrated that water deficit results in damages of the PS2 oxygen evolving complex (Lu and Zhang 1999, Skotnica *et al*. 2000) and of the PS2 reaction centres associated with the degradation of D1 protein (Cornic 1994). Thus PS2 may play a key role in the response of photosynthesis to environmental perturbations and the study of various protein components of PS2 might reveal stress response mechanisms in a plant. Collet *et al*. (2003) reported rapid reduction in transcript amount of PS2 protein coding gene, *PsbR*, during dehydration. We report the isolation and initial characterization of a PsbR protein coding cDNA and its promoter from drought-tolerant plant *Prosopis juliflora*. The cDNA clone was obtained from a cDNA library created from drought-stressed leaf tissue of *P. juliflora* and was the single most abundant cDNA clone in the 1 750 sequenced EST set (Suja *et al*. 2007).

PsbR is a low molecular mass PS2 protein found only in green algae and higher plants and encoded by nuclear genes. The PsbR protein precursor has a molecular mass of 12.8–14.6 kDa and consists of 126–141 amino acids. PsbR is an intrinsic protein and does not bind any cofactors (Ljungberg *et al*. 1986). Based on biochemical studies (Ljungberg *et al*. 1984, 1986, Mishra and Ghanotakis 1993) and sequence analysis (Lautner *et al*. 1988, Webber *et al*. 1989), the majority of the PsbR protein is purportedly located on the lumenal side of the thylakoid membrane. PsbR may provide a binding site for the extrinsic PsbP (23 kDa) protein to the thylakoid membrane (Ljungberg *et al*. 1986). Sequence analysis shows that the N-terminal region of PsbR is highly charged, providing scope to form ion bridges with extrinsic proteins. Thus, the charged domain together with the transmembrane span might make the PsbR protein function as a docking protein (Suorsa *et al*. 2006, De Las Rivas *et al*. 2007).

P. juliflora is an extremely drought tolerant tree of *Fabaceae* (Burkart and Simpson 1977). An introduced species in India, it has subsequently spread aggressively across the country. This species grows in very hot dry climates, with temperatures up to 48 °C and annual precipitation from 150 to 750 mm. It is found from sea level to 1 500 m above sea level. The roots penetrate to great depths in the soil searching for required water. If root growth is not obstructed, the tree can grow in a variety of soils, including saline and alkaline areas and in sandy and rocky soils (Drake 1993). *P. juliflora* can withstand high leaf-to-air vapour pressure deficit (VPD) (Shirke and Pathre 2004) and is tolerant to heavy metals, *e.g.* cadmium, chromium, and copper (Senthil Kumar *et al*. 2005, Sinha *et al*. 2005).

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Materials and methods

Isolation of the cDNA clone and *in silico* analysis: A cDNA library was created from the leaf tissue of drought stressed two-month-old *P. juliflora* seedlings (Suja *et al*. 2007). The relative water content (RWC) of the *P. juliflora* plants used for creation of the cDNA library was 64.4 %. 1 750 random ESTs were sequenced from this library and the single most abundant cDNA amongst the sequenced ESTs was found to code for PS2 protein PsbR. 13 clones of this cDNA were found in the library. Upon BLAST analysis, these clones showed maximum similarity to the PS2 10 kDa protein (PsbR) from *Nicotiana tabacum* (Genbank ID: Q40519). The full-length cDNA was sequenced completely and re-named *Pj PsbR* (Genbank ID: EU170654). Open reading frames (ORFs) were identified in this cDNA using ORF finder at NCBI (www.ncbi.nlm.nih.gov) and the longest ORF with a predicted start and stop codon was identified as the putative coding region. Protein targeting predictions were done using the *Target P 1.1 Server* (Nielsen *et al*. 1997, Emanuelsson *et al*. 2000). Molecular mass predictions were made using *Prot Param* (Gasteiger *et al*. 2005). *HMMTOP* (Tusnády and Simon 1998, 2001) was used for making putative transmembrane predictions. Multiple sequence alignments were done using the *ClustalW* program at NPS (Network Protein Sequence analysis). The conserved domains present in the ORFs were identified using the NCBI Conserved Domain Database (*NCBI-CDD* – Bauer *et al*. 2007).

Northern analysis of *Pj PsbR*: Two-month-old *P. juliflora* seedlings were acclimated for 72 h in half-strength MS (Murashige and Skoog 1962) nutrient solution and then subjected to the stress treatment. For drought stress, the plants were kept in half-strength MS containing 25 % *PEG 6000* and leaf tissue was frozen at 12, 24, and 48 h after stress application. For oxidative stress, the acclimatized plants were kept in half-strength MS containing 90 mM H_2O_2 and leaf tissue was frozen at 12, 24, and 48 h after stress application. Total RNA from *P. juliflora* leaf tissue was isolated according to Chomczynski and Sacchi (1987). The expression patterns of *Pj PsbR* in *P. juliflora* leaf tissue under PEG stress and H_2O_2 stress were studied using Northern analysis. Full length cDNA amplified using UTR specific primers *Pj PsbR* 5'UTR F (GTGAGCGGTGTGGTGTGTT), and *Pj PsbR* 3'UTR R (GGGAAAACTAGGCTCATAA) was used as the probe (PCR conditions: 1′ pre-amplification at 94 °C, 30 PCR cycles of 30 s at 94 °C, 40 s at 60 °C, 1′ and 30 s at 72 °C, a final extension of 10′ at 72 °C). The Northern experiments were carried out as described in Mehta *et al*. (2005).

Isolation of the 5′ upstream region of *Pj PsbR*: The 5′ upstream regions of *Pj PsbR* were isolated using TAIL-PCR (Liu *et al*. 1995). In this, PCR is carried with 3 long nested gene-specific primers designed close to the 5′ end of the cDNA [*Pj PsbR* GSP1 (CTGGCGACGACCT TGAAGGAAG), *Pj PsbR* GSP2 (AGGGAAGGAAGGC CTCTCACTG), *Pj PsbR* GSP3 (GACGGAAGCCAT CACAGAGGAC)], and short degenerate primers of arbitrary sequence (primers AD1 − NGTCGASW GANAWGAA, AD2 – TGWGNAGSANCASAGA, AD3 – WGTGNAGWANCANAGA, and AD4 − STTGNT ASTNCTNTGC). An elaborate thermal cycling program, composed of "supercycles", each consisting of one low stringency cycle and two high stringency cycles, allows only sequence-specific primers to be amplified. The TAIL-PCR reaction products were separated on adjacent lanes in a 1.5 % agarose gel and distinct PCR products showing a difference in size corresponding to the relative positions of the nested gene-specific primers were identified. The specific products obtained were gel-eluted and sequenced. The putative *cis*-acting DNA elements in the isolated 5′ regions were identified using the *PLACE* database (Higo *et al*. 1999).

Results

The full length cDNA clone of *Pj PsbR* is 608 bp long with a predicted ORF of 420 bp. A chloroplast targeting peptide with a length of 39 amino acids was predicted using the *TargetP 1.1 Server*. Thus the mature protein may contain about 100 amino acids, with a molecular mass around 10.3 kDa. Trans-membrane predictions showed a transmembrane domain close to its C-terminus. Domain search of the putative ORF showed the presence of pfam domain − pfam04725. A multiple sequence alignment of *Pj PsbR* with PsbR proteins from other plants such as *Nicotiana tabacum* (Q40519), *Solanum tuberosum* (P06183), *Arachis hypogaea* (ABC46708), *Trifolium pratense* (AAQ24852), *Arabidopsis thaliana* (NP_178025), and *Brassica rapa* (ABL97960) revealed a high similarity between them. 57.86 % amino acids were identical, while 15.71 % were strongly similar, and 7.14 % were weakly similar. Only 19.29 % amino acids were different (Fig. 1).

Northern analysis for *Pj PsbR* in *P. juliflora* leaves under 25 % PEG stress showed steady decrease in transcript level at 12, 24, and 48 h after stress application. Under 90 mM H_2O_2 stress, the transcript level dropped drastically at 12 h, but increased again compared to the control at 24 h. The expression level dropped again at 48 h (Fig. 2).

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                    10         20         30         40         50         60
                     |          |          |          |          |          |
Q40519       MASTVMS----LKPAPFSVEKTAVKGLPSLARSSSSFRVQASGVKKLKTDKPYGINGSMS
P06183       MASTVMSS-LSLKPT-FTLEKTSVKGLPSLARSSSSFKVVASGVKKLKTDKPYGINGSMA
ABC46708     -----------LKPAPFTAERSSVRGIPSLSRASSSFKVVASGGKKIKTDKPYGINGGMN
AAQ24852     MASSVMAS-VSLKPTPFMVEKSSVRGLPNLSRP---FRVVASGVKKIKTDTPYGTGGGMD
Pj PsbR      MASSVMAS-VSLKPAPFTVEKSAVRGLPSLSRSSSSFKVVASGGKKIKTDKPYGINGGMN
NP_178025    MAASVMLSSVTLKPAGFTVEKTAARGLPSLTRARPSFKIVASGVKKIKTDKPFGINGSMD
ABL97960     MAASLMLSSVTLKPAGFTVEKMSVRGLPSLTRAPSSFKIVASGVKKIKTDKPFGVNGSMD
                        ***: *  *: :.:*:*.*:*.   *:: *** **:***.*:* .*.*

                    70         80         90        100        110        120
                     |          |          |          |          |          |
Q40519       LRDGVDASGRKQKGKGVYQFVDKYGANVDGYSPIYNTDDWSPSGDVYVGGTTGLAIWAVT
P06183       LRDGVDASGRKPKGKGVYQYVDKYGANVDGYSPIYNTDEWSPSGDVYVGGTTGLAIWAVT
ABC46708     LRDGLDASGRKGKGKGVYQFVDKYGANVDGYSPIYDQKDWSPTGDVYTGGTTGLAIWAVT
AAQ24852     LPNGLDASGRKQKGKGVYQFVDKYGANVDGYSPIYEPKDWSPTGDVYTGGTTGLAIWAVT
Pj PsbR      LVEGVDSSGRKGKGKGVYQFVDKYGTNVDGYSPIYNTEDWSPSGDVYVGGTTGLAIWAVT
NP_178025    LRDGVDASGRKGKGYGVYKYVDKYGANVDGYSPIYNENEWSASGDVYKGGVTGLAIWAVT
ABL97960     LRDGLDASGRKGKGYGVYKFVDKYGANVDGYSPIYNEDEWSASGDVYKGGVTGLAIWAVT
             * :*:*:**** ** ***::*****:*********: .:**.:**** **.*********

                    130        140
                     |          |
Q40519       LVGILAGGALLVFNTSALAQ
P06183       LVGILAGGALLVYNTSALAQ
ABC46708     LVGLLAGGALLVYNTSALSQ
AAQ24852     LAGLLAGGALLVYNTSALSQ
Pj PsbR      LAGLLAGGALLVYNTSALAQ
NP_178025    LAGILAGGALLVYNTSALAQ
ABL97960     LAGILAGGALLVYNTSALAQ
             *.*:********:*****:*
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Fig. 1. Multiple sequence alignment of Pj PsbR protein with those from other higher plants. Numbers indicate the position of amino acids from the N-terminus. * − identical; : − strongly similar; . − weakly similar amino acids. The alignment revealed high similarity between the proteins analyzed. Accession nos. Q40519 (*Nicotiana tabacum*), P06183 (*Solanum tuberosum*), ABC46708 (*Arachis hypogaea*), AAQ24852 (*Trifolium pratense*), NP_178025 (*Arabidopsis thaliana*), and ABL97960 (*Brassica rapa*).

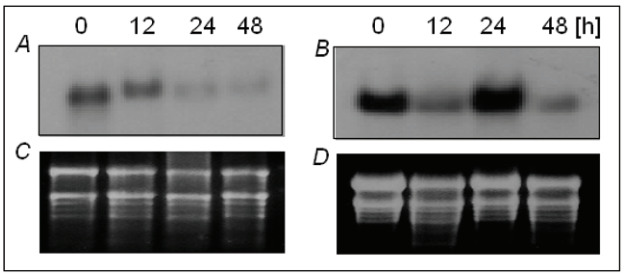

Fig. 2. Northern analysis of *Pj PsbR* under stress conditions. Samples were collected at 0, 12, 24, and 48 h of stress application. (*A*) Transcript analysis of *Pj PsbR* in *P. juliflora* leaves under 25 % PEG stress. (*B*) *Pj PsbR* expression in *P. juliflora* leaves at 90 mM H_2O_2 stress. (*C, D*) Ethidium bromide-stained gels prior to transfer.

Isolation of the 5′ upstream region of *Pj PsbR* and *in silico* analysis of the putative promoter: A fragment of approximately 1.7 kb was amplified from *P. juliflora* genomic DNA by TAIL-PCR product as the 5′ upstream region for *Pj PsbR*. It was sequenced and found to have a 78 bp overlap with the *Pj PsbR* cDNA and was thus confirmed to be a specific amplification product. The 1 714 bp promoter fragment was re-amplified from *P. juliflora* genomic DNA (Genbank ID: EU170655). The gel electrophoresis of TAIL-PCR products is shown in Fig. 3.

The first ATG of the longest ORF was considered as the putative translation start site (TSS) and numbered +1. Only one ABRE related element, MACGYGB was found (–128) in the sequence. The ACGT core motif, involved in dehydration response, was found at positions −1 489, −1 352, −1 210, −1 136, −759, −753, −413, and −128. ATAGAA, an element known as "Box II", which was found in the tobacco plastid *atpB* gene promoter and conserved in several non-consensus type II promoters of plastid genes, and is important for the activity (Kapoor and Sugiura 1999) was found at −395 position. Several CAAT boxes were also present (−1 661, −1 655, −1 538, −1 369, −1 319, −1 248, −1 198, −1 129, −676, −627, −478, −382, −263, −115, and –83). The tetra nucleotide YACT, a key component in mesophyll-specific expression was found at positions −1 195, −1 100, −1 047, −904, −738, −360, −290, −253, −95, −65, and −60. A *cis*-acting element involved in auxin responsiveness, CATATG, was found at −1 419, −1 393, and −1 091. An element involved in circadian regulation, CAANNNN ATC, was located at positions −1 323, −676, and −382.

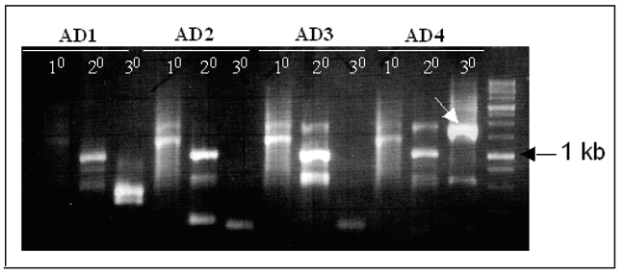

Fig. 3. TAIL–PCR amplification products of *Pj PsbR*. Primary, secondary, and tertiary TAIL-PCR reactions using the degenerate primers AD1, AD2, AD3, and AD4 and *Pj PsbR*-specific nested reverse primers are shown. *The fragment indicated by arrow* was cloned and sequenced; it had sequence overlap with the 5′ end of the *Pj PsbR* cDNA, confirming that it is specific amplification product.

Discussion

Transmembrane predictions for *Pj PsbR* showed a transmembrane domain close to its C-terminus. The hydrophobic C-terminus of the mature protein may serve as a non-cleavable transfer domain, which together with the N-terminal transit peptide direct the protein into the chloroplast and across the thylakoid membrane (Lautner *et al*. 1988, Webber *et al*. 1989). ClustalW alignment of the translated *Pj PsbR* ORF with PsbR proteins from other plant species reveals the highly conserved nature of this protein across families such as Solanaceae (Q40519, P06183), Fabaceae (ABC46708, AAQ24852), and Brassicaceae (NP_178025, ABL97960). Northern analysis for *Pj PsbR* in *P. juliflora* leaves under 25 % PEG stress showed steady decrease in transcript level at 12, 24, and 48 h after stress application. Down-regulation of *PsbR* gene under drought stress has been previously reported in rice (Zhou *et al*. 2006). In the resurrection plant *Xerophyta humilis*, the level of *PsbR* transcript decreases steadily during dehydration and is up-regulated within 12 h of re-hydration (Collett *et al*. 2003). Suorsa *et al*. (2006) characterized the *Arabidopsis* T-DNA insertion mutant for *psbR* and demonstrated the requirement of the PsbR protein for stable association of the OEC (Oxygen Evolving Complex) subunit PsbP with the PS2 core. Their results showed that the absence of PsbR diminishes the oxygen evolution capacity of thylakoid membranes, indicating that PsbR is essential for optimization of photosynthetic water splitting and electron transfer in PS2. The electron transfer from PS2 to the plastoquinone pool was hampered in the *psbR* mutant. It is possible that the transcription of PsbR gene is regulated in response to the water availability, water being a limiting factor in photosynthesis. As drought stress prolongs, the reduction in transcript level of *Pj PsbR* could be an energy conservation mechanism in the absence of water.

Because PS2 is responsible for the water-splitting, oxygen-evolving functions of photosynthesis, and drives the strongest oxidizing reaction known to occur in nature, loss of PS2 function correlates with the loss of power to generate potentially damaging reactants and, therefore, PS2 may act as a sensor for stress (Shi and Schroder 2004). While the genes previously reported to be up-regulated in abiotic stresses were predominant in the *P. juliflora* cDNA library, the single most abundant cDNA amongst the sequenced ESTs coded for PS2 protein PsbR (13 clones). The data imply that the photosynthetic machinery in *P. juliflora* was intact and functioning at 64.4 % RWC. Actually, the functional classification of the 1 750 ESTs showed that genes involved directly or indirectly in photosynthesis comprise the main category. The reasons for abundance of *PsbR* cDNA over other photosynthetic genes need to be further analyzed.

Promoter elements of eukaryotic genes are generally divided into two classes; core-promoter elements needed for basal transcription initiation; and gene-specific regulatory elements located upstream (Roeder 1998). The core promoter is located at or near the transcription initiation site, and generally defined as the DNA region bound by the pre-initiation complex (PIC), which consists of RNA polymerase and general transcription factors. TATA boxes are essential in most of the core promoters. In some TATA-less promoters, other promoter elements such as Initiator (Inr) element, downstream promoter element (DPE), TFIIB recognition element (BRE), *etc*. were shown to compensate for the loss of a TATA box (Smale and Baltimore 1989, Burke and Kadonaga 1996, Lagrange *et al.* 1998). Nakamura *et al.* (2004) analyzed 232 plant promoters for the presence of TATA box near the transcription start site. Their results revealed that frequency of the TATA-less promoters was remarkably higher in photosynthesis genes compared to other functional category genes. PS1 genes were shown to seldom utilize TATA boxes. In *Pj PsbR,* the TATA box nearest to the translation start site was found at –557. It is possible that *Pj PsbR* also uses core promoter modules other than TATA box. Core promoter diversity could be a critical

A *cis*-acting element, TATTCT (termed '−10 promoter element') found in the promoter of barley chloroplast *psbD* gene (Thum *et al* 2001), which encodes a PS2 reaction centre chlorophyll-binding protein, was found at –75. The GATA box, found at 12 locations, is reported to be present in the promoter of *Petunia* chlorophyll *a/b* binding protein, *Cab22* gene, and is required for high level, light regulated, and tissue specific expression (Gilmartin *et al.* 1990). The *cis*-acting element GRWAAW, involved in light-regulated gene expression (Villain *et al*. 1996) was found in 9 locations. Several elements involved in pollen-, root-, and nodule-specific expression were also found in the 5′ upstream region of *Pj PsbR*. The TATA box nearest to the translation start site was found at –557.

factor for the regulated expression of plant genes. Further characterization of this promoter would throw light into the possible functions of this gene.

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