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Functional analysis of the rice rubisco activase promoter in transgenic *Arabidopsis*

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ABSTRACT

To gain a better understanding of the regulatory mechanism of the rice rubisco activase (*Rca*) gene, variants of the *Rca* gene promoter (one full-length and four deletion mutants) fused to the coding region of the bacterial reporter gene β-glucuronidase (GUS) were introduced into *Arabidopsis* via *Agrobacterium*-mediated transformation. Our results show that a 340 bp fragment spanning from −297 to +43 bp relative to the transcription initiation site is enough to promote tissue-specific and light-inducible expression of the rice *Rca* gene as done by the full-length promoter (−1428 to +43 bp). Further deletion analysis indicated that the region conferring tissue-specificity of *Rca* expression is localized within a 105 bp fragment from −58 to +43 bp, while light-inducible expression of *Rca* is mediated by the region from −297 to −58 bp. Gel shift assays and competition experiments demonstrated that rice nuclear proteins bind specifically with the fragment conferring light responsiveness at more than one binding site. This implies that multiple *cis*-elements may be involved in light-induced expression of the rice *Rca* gene. These works provide a useful reference for understanding transcriptional regulation mechanism of the rice *Rca* gene, and lay a strong foundation for further detection of related *cis*-elements and *trans*-factors.

1. Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco), the most abundant protein in the world [1], catalyzes the first step of photosynthetic carbon assimilation. CO_2 fixed by rubisco is converted into carbohydrate, which provides most of the carbon resources and energy needed by life on earth [2,3]. However, newly assembled rubisco is inactive and needs to be activated by rubisco activase (RCA) before it can fix CO_2 [2]. RCA was discovered in a nuclear gene mutant of *Arabidopsis thaliana*. So far, it has been isolated from more than 10 species, including C3 [4–11], C4 plants [12] and green algae [13]. Most of these plant species have two isoforms of activase that derive from the same transcript precursor by alternative splicing [14]. Under physiological conditions, they form a functional complex that has ATP hydrolase activity. Accompanying with the ATP hydrolyzation, the complex facilitates the release of sugar phosphates from the rubisco active site and accelerates the carbamylation of rubisco. These events result in a decrease in the association constant of CO_2 and prompt the carboxylation and oxygenation of ribulose bisphosphate (RuBP) [3,15–17].

Several studies have shown that expression of the *Rca* gene is tissue-specific and light-inducible in addition to being affected by development, the circadian clock, and phytohormones [18–20]. As expected, transcription of *Rca* is highly coordinated with that of rubisco [18]. However, the mechanism by which such coordination is achieved is still unknown. Although they share some known regulatory elements in their promoters, the coordination of their expression involves sequences unique to the respective genes [21]. To gain a better understanding of the coordination in expression of *rubisco* and *Rca*, more work on the mechanism of the transcriptional regulation is needed. Compared to the plethora of research on the small subunit gene promoter of *rubisco*, only a few studies in spinach [21] and *Arabidopsis* [22] have focused on the *Rca* gene promoter. Here, we report our study on the transcriptional regulation of *Rca* in *Oryza sativa*, an important food crop and an excellent plant model for genetic research. We isolated the rice *Rca* gene promoter and probed its function by deletion analysis based on expression of the β-glucuronidase (GUS) reporter gene in transgenic *Arabidopsis*. Promoter regions for tissue-specific and light-inducible expression of the rice *Rca* gene were identified. Furthermore, combinations of these promoter fragments and nuclear extracts from native rice were analyzed by gel-shift assays. Our results provide a useful reference for understanding the molecular mechanism of transcriptional regulation of the *Rca* gene in rice.

2. Materials and methods

2.1. DNA constructs

For construction of *Rca-uidA* fusions, the full-length *Rca* promoter *SH*(−1428 ∼ +43) was amplified by using primers P1

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(5′-acagtcgacaaatatacaagttcaag-3′) and P2(5′-tcgaagcttgtagaggatcact-3′) from genomic DNA of *O. sativa* (Japonica). The PCR products were then digested with *SalI* and *HindIII* (the restriction sites are underlined) before subcloning. A series of 5′ deletion mutants of the *Rca* promoter, *BH* (−1191 ∼ +43), *PH* (−741 ∼ +43) and *EH* (−297 ∼ +43) were generated by double-digesting *SH* with *BamHI/HindIII*, *PstI/HindIII* and *EcoRI/HindIII*, respectively. Truncated promoter *NH*(−58 ∼ +43) was amplified by using primers P3 (5′-tccgaattccatggcgacgtccaat-3′) and P2, then digested with *EcoRI* (the restriction sites are underlined) and *HindIII*. All of the promoter fragments were fused with the *uidA* reporter gene in the binary vector *pCAMBIA1381Z* (Cambia, Australia) digested with corresponding restriction enzymes.

2.2. Plant transformation

Binary vector constructs were transformed into *Agrobacterium tumefaciens* strain GV3101pMP90 [23] by freeze–thaw. *Agrobacterium*-mediated transformation of *Arabidopsis* ecotype Columbia was performed by the floral dip method [24]. The seeds harvested from infiltrated plants were screened on selection plates containing Murashige and Skoog salts [25] and 40 mg/L Hygromycin B (Amresco, USA). Resistant plants were confirmed by amplification of a 1065 bp fragment of the *uidA* gene with primers P4 (5′-atcaggaagtgatggagcatc-3′) and P5 (5′-atggtatcggtgtgagcgtcg-3′). T2 seeds of confirmed plants were harvested and used in the following experiments.

2.3. Histochemical staining and quantitative analysis of GUS activity

T2 seeds were germinated on MS plate containing 40 mg/L Hygromycin B and 7-days old seedlings were used for GUS staining. Fresh samples were immersed in 100 mM sodium phosphate buffer (pH 7.0) containing 1% Triton X-100, 1% DMSO, 10 mM EDTA and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide, and incubated overnight at 37 °C in darkness [26]. The staining solution was then removed and the samples were dehydrated using 75% ethanol.

For quantitative analysis, 7-days old seedlings were homogenized in 200 μL extraction buffer (50 mM sodium phosphate buffer [pH 7.0], 10 mM EDTA, 0.1% Triton X-100, 0.1% sarkosyl, 10 mM β-mercaptoethanol) and clarified by centrifugation at 12000 rpm for 5 min at 4 °C. A 100 μL sample of the supernatant was assayed for GUS activity in extraction buffer containing 1 mM 4-methylumbelliferyl-β-D-glucuronide [26]. Fluorescence was measured using the VersaFluor™ Fluorometer System (Bio-Rad, USA). Protein concentrations were determined using the DU800 spectrophotometer (Beckman Coulter, USA).

2.4. Northern blot analysis

For dot-blot analysis, 3-weeks old transgenic plants received 48 h dark treatment or plus 24 h continuous illumination before sampling. Total RNA was isolated from leaves using TRIzol® Reagent (Invitrogen, USA) and 10 μg total RNA from each sample was directly applied onto a Hybond-N+ nylon membrane(GE Healthcare, UK). For preparation of probes used in northern blot analysis, the *uidA* gene was amplified by primers P4 and P5 (see section 2.2) using the plasmid *pCAMBIA1381Z* as a template. The full length of the*16s rRNA* gene (1491 bp) was amplified by primers P6 (5′-tctcatggagagttcgatcc-3′) and P7 (5′-aaaggaggtgatccagcc-3′) using *Arabidopsis* genomic DNA as the template. Probe labeling and hybridization were carried out by using the DIG high prime DNA labeling and detection starter kit I (Roche, Germany). Hybridization signals were examined by chemiluminescence using CSPD as the substrate.

2.5. Nuclear protein extraction and gel shift assay

Oryza sativa (Japonica) plants were grown at 28 °C with 16 h/8 h light/dark photoperiod for 3 weeks. Green leaves that had fully spread were used for extraction of nuclear protein using the CelLytic™ PN Isolation/-Extraction Kit for plant leaves (Sigma–Aldrich, USA). The probes EN1 and EN2 for gel shift assays were prepared by PCR using primer pairs EN1 forward(5′-ggtgaattctgtggtgagga-3′)/EN1 reverse (5′-gatccacagcatagcatgac-3′) and EN2 forward(5′-gtcatgctatgctgtggatc-3′)/EN2 reverse (5′-tcgccatggaagttggaggc-3′), respectively. Terminal labeling of probes and gel shift assays were carried out using the DIG Gel Shift Kit, 2nd Generation (Roche, Germany). Binding reactions contained 1 ng DIG-labeled probe, 1 μg poly[d(I-C)], 5 μg nuclear extracts, along with 20 mM HEPES(pH7.6), 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 30 mM KCl and 0.2% Tween-20. The mixture was incubated for 15 min at room temperature under light then loaded onto 3.5% acrylamide gels in 0.25×TBE. After electrophoresis, the gels were examined by chemiluminescence using CSPD as the substrate.

3. Results

3.1. Prediction and computer analysis of the rice Rca promoter

Based on the genomic sequence of *Oryza sativa* (Japonica) obtained from NCBI (GenBank ID: AC137064), a 1.5 kb fragment upstream of the ATG start codon of *Rca* was designated as the promoter region (Fig. 1). The transcription initiation site predicted by BDGP (Berkeley Drosophila Genome Project) [27] is located 67nt upstream of the ATG start codon. A CAAT box (CAAT) at −45nt and a TATA box motif (TTATTT) at −32nt are included in the putative basic promoter (Fig. 1).

By searching against the PLACE [28] and PlantCARE [29] databases, several *cis*-elements related to light-regulation were found (Fig. 1). In addition to elements like G-box [30], I-box [30], GATA box [31,32], GT-1 box [31,33] which have been identified experimentally in other promoter research, there is a SORLIP-1 (sequences over-represented in light-induced promoter) element which is defined by bioinformatics method due to its high frequency appearance in light induced gene promoter [34]. The functions of these elements in light inducible expression of rice *Rca* gene need further confirmation.

3.2. Analysis of the Rca promoter in transgenic Arabidopsis

To analyze the function of the *Rca* promoter, five variants of the promoter – one full-length and four deletion mutants – were subcloned into a binary vector *pCAMBIA1381Z* [35] and transformed into *Arabidopsis*. They were named as *SH*(−1428 ∼ +43), *BH* (−1191 ∼ +43), *PH* (−741 ∼ +43), *EH*(−297 ∼ +43) and *NH* (−58 ∼ +43) depending on the restriction enzyme site at their 5′-end (Fig. 1). Histochemical staining of 7 days old green seedlings grown in 12 h/12 h light/dark photoperiod showed that the full-length promoter and the deletion mutants were all able to effectively promote *uidA* gene expression. The staining was observed in cotyledons, newly-emerged true leaves and hypocotyls, but not in roots (Fig. 2A). This is coincident to the expression pattern of rubisco activase reported before [19].

To test the light-inducible characteristic of the *Rca* promoter, seedlings of transgenic lines were grown in the dark for 7 days and subsequently examined. Like green seedlings, etiolated seedlings of the NH line showed staining in the cotyledons and the hypocotyls, but not in roots (Fig. 2B). Under dark conditions, full-length (*SH*) and other deletion mutants (*EH*, *PH*, *BH*) of the *Rca* promoter could not drive any *uidA* expression in the hypocotyls, and

Fig. 1. The *Rca* promoter sequence in *Oryza sativa* (Japonica). 1560 bp fragment upstream of the ATG start codon of *Rca* was designated as the promoter region. The putative transcription start site and the start codon (ATG) are indicated in capital letters in bold. Putative light regulated *cis*-elements, the CAAT box and the TATA box are shadowed. The primer sequences used for PCR amplification of the complete promoter are underlined. The starting points of promoter deletion constructs are marked with arrow-lines.

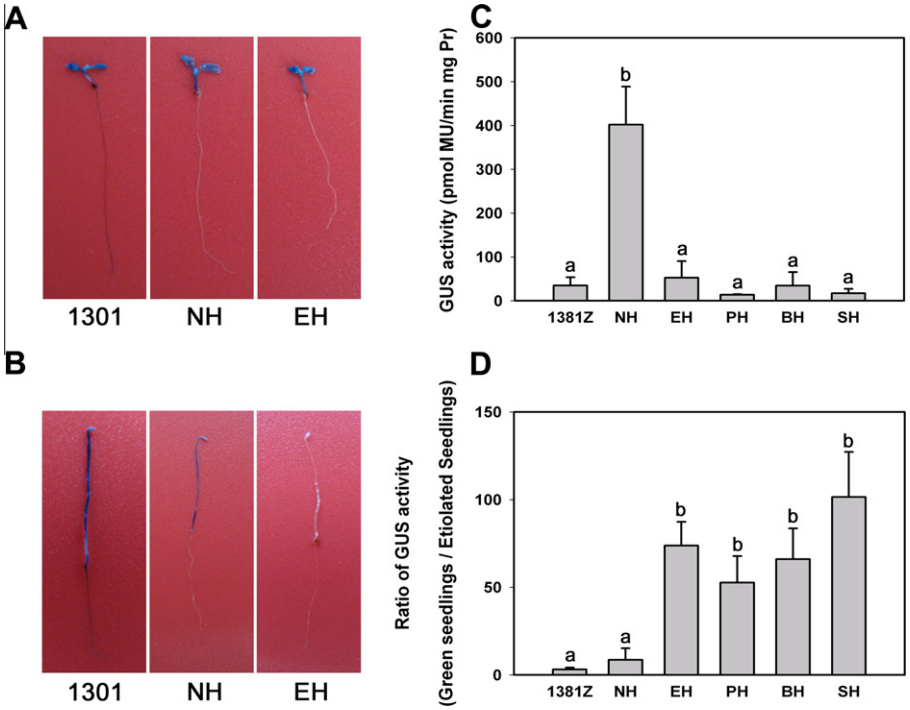

Fig. 2. GUS activity in transgenic *Arabidopsis*. (A) Histochemical staining of green seedlings grown in 12 h/12 h light/dark photoperiod; (B) Histochemical staining of etiolated seedlings; (C) Quantitative analysis of GUS activity in etiolated seedlings; (D) Ratio of GUS activities of green seedlings and etiolated seedlings. Seven-days old seedlings were used for examining GUS activity. NH, EH, PH, BH, SH: transgenic lines expressing *NH-uidA, EH-uidA, PH-uidA, BH-uidA, SH-uidA* constructs, respectively; 1301: transgenic line expressing the *35S-uidA* construct; 1381Z: promoterless control. For each construct, 3–5 lines were examined. The results were analyzed by two-tailed *t*-tests. Samples marked with various letters were significantly different ($P < 0.05$).

drove only weak expression in the etiolated cotyledons. Quantitative analysis indicated that except for NH, there is no significant difference among these transgenic lines and the promoterless control (*pCAMBIA1381Z*) (Fig. 2C).

Ratio of GUS activity between green seedlings and etiolated seedlings were also used for analyzing the light-inducible characteristic of different *Rca* promoter fragment (Fig. 2D). The ratio of GUS activity between green seedlings and etiolated seedlings expressing the *NH* construct was low, and it was not significantly different compared to the promoterless control (*pCAMBIA1381Z*). However, in transgenic lines expressing *SH*, *BH*, *PH* and *EH* constructs, the ratios of GUS activity between green seedlings and etiolated seedlings were significantly higher than NH line and the promoterless control. Based on these results, it can be inferred that the key *cis*-element responsible for light-induced expression is located in the promoter fragment ranging from −297 to −58 bp, which is included in the *EH* but not the *NH* region.

Expression of the *uidA* gene was also assessed in the adult plants of transgenic *Arabidopsis* by Northern blot analysis (Fig. 3). After 48 h of dark treatment, accumulation of *uidA* transcripts in rosetta leaves was detectable only in the NH transgenic lines and the *35S-uidA* control (*pCAMBIA1301*). Illumination for the following 24 h was enough to induce expression of the *uidA* gene in EH, PH, BH and SH transgenic lines. These results were consistent with those observed in young seedlings.

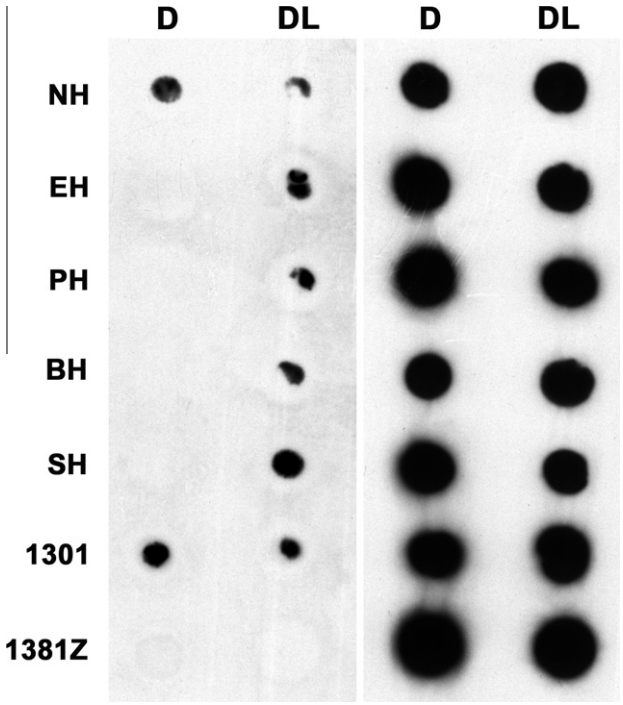

Fig. 3. GUS gene expression in transgenic *Arabidopsis*. Three-weeks old plants grown in 12 h/12 h light/dark photoperiod were treated with 48 h dark (D) and 24 h light following 48 h dark (DL). Ten microgram of total RNA isolated from rosette leaves was directly loaded onto the nylon membrane and hybridized with Digoxigenin-labeled probe of the GUS-coding region (left) and 16s rRNA (right). NH, EH, PH, BH, SH: transgenic lines expressing *NH-uidA, EH-uidA, PH-uidA, BH-uidA, SH-uidA* constructs, respectively; 1301: transgenic line expressing the *35S-uidA* construct; 1381Z: promoterless control. Seven to ten plants were sampled for each line.

3.3. Interaction between the Rca promoter and rice nuclear protein

Gel shift experiments were carried out to investigate the interaction between the 240 bp promoter fragment (*EN*) ranging from −297 to −58 bp and rice nuclear proteins. Two bands were observed when separated on a 3.5% acrylamide gel (data not shown). Then, two probes *EN1* (−297 ∼ −172) and *EN2*

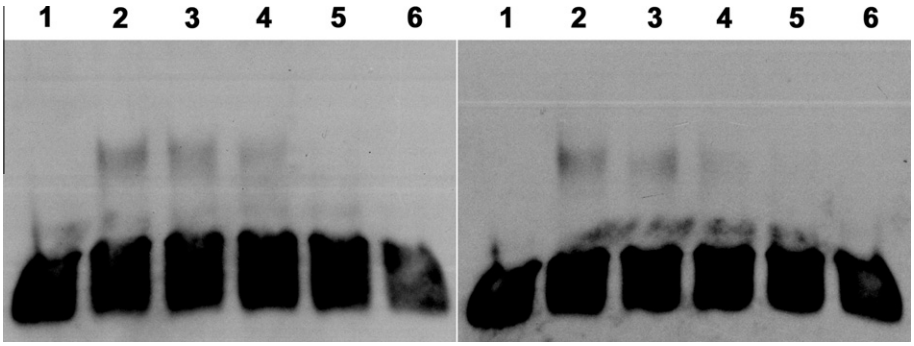

Fig. 4. Gel shift assays of EN1 (left) and EN2 (right). Assay conditions included 5 μg of rice leaf nuclear extract proteins, 1 μg poly[d(I-C)], 1 ng Digoxigenin-labeled probe. Lane 1, free probe. Lanes 2–6, plus nuclear extract. Lanes 3–6, plus 10×, 25×, 50×, 100× competitors.

(−192 ∼ −58), which covered the *EN* region and had a 20 bp overlap with each other, were prepared by PCR amplification. They were labeled by Digoxigenin and incubated for 15 min at room temperature with 5 μg nuclear protein prepared from green leaves of rice. After electrophoresis, both *EN1* and *EN2* showed retarded bands when compared with free probes. Addition of non-labeled probe into the reaction mixture led to gradual disappearance of the bands, which suggested that the binding between the probe and nuclear extracts was specific (Fig. 4).

4. Discussion

For a long time, the simple, quick and high-efficiency *Arabidopsis* transformation system has been used for rice gene function research. In addition to studies on protein-coding genes, several works on functional analysis of rice promoters in transgenic *Arabidopsis* have been reported in recent years [36,37]. In our study, the expression of the reporter gene under a full length rubisco activase promoter was observed to be tissue-specific and light-inducible in transgenic *Arabidopsis*, which is coincident to the native gene expression pattern of rubisco activase in rice [19]. So it is feasible to study the function of rice *Rca* promoter in detail by using transgenic *Arabidopsis* system.

Deletion analysis of the promoter showed that at different developmental stages, a 297 bp fragment upstream from the transcriptional initiation site (*EH*) is enough for tissue-specific and light-inducible expression of the reporter gene in transgenic *Arabidopsis*. In spinach and *Arabidopsis*, minimal promoters conferring *Rca* gene expression were identified to be 294 and 317 bp region upstream the transcription initiation site respectively. This suggested that in both monocotyledons and dicotyledons, *cis*-elements sufficient to confer organ-specific and light-regulated transcription of *Rca* genes are localized proximal to the transcription start site [21,22].

Although it has been demonstrated that tissue specificity and light regulation are separately encoded in the *Rca* promoter [21], there is no definite report about promoter elements conferring tissue-specific expression of the *Rca* gene yet. Here, we found that the *NH* region spanning from −58 to +43 of the rice *Rca* promoter, the putative basic promoter containing a CAAT box and a TATA box, is enough to mediate the tissue-specific expression of reporter gene in transgenic *Arabidopsis* without showing light-inducing properties. Corresponding sequences in the *Arabidopsis Rca* promoter is not sufficient to drive detectable transcription of the *uidA* gene at all [22]. This implies different regulation mechanism of tissue specific expression of *Rca* in rice and *Arabidopsis*.

By GUS staining, we observed that the etiolated cotyledons of all transgenic lines were stained weakly, but hypocotyls of etiolated seedlings were stained negatively except for NH lines. It implies that expression of the *uidA* gene in cotyledons and hypocotyls are regulated separately. Same mechanism may exist in dicotyledon *Arabidopsis*, the accumulation of little *Rca* transcripts in etiolated cotyledons was also observed in *Arabidopsis Rca* promoter research [22].

In the *EN* region conferring light-inducible expression (−297 to −58 bp), several conserved light responsive elements were found (Fig. 1). Among them, the G-box has been identified to be essential for the expression of the spinach *Rca* gene [21] and the *Arabidopsis rbcS-1A* gene [38] in leaves. A consensus G-box was also found in the *Arabidopsis Rca* minimal promoter regulated by light and the circadian clock [22], but its function is not confirmed yet. Gel shift assays identified that there are multiple combinations between rice nuclear extracts and *EN* fragment. To examine the possibility of the involvement of above-mentioned elements in the regulation of rice *Rca* expression, *EN* was divided into two fragments, *EN1* (−297 to −172 bp) without any putative light-responsive element and *EN2* (−192 to −58 bp) within the conserved G-box and other putative light-regulated elements. Gel shift assays showed that both of them bound specifically with rice nuclear proteins, suggesting that not only light-inducible elements have been reported, but also some unknown elements are involved in the light-regulation of the rice *Rca* gene. More work should be done to identify the important *cis*-elements that regulate *Rca* gene expression. A thorough understanding of the function of the rice *Rca* gene promoter can be helpful for not only probing the coordination mechanism between *Rca* and rubisco, but also its exploitation in biotechnological applications.

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