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Structural Analysis and Identification of *Cis*-Elements of Rice *osRACD* Gene

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Abstract The *osRACD* gene correlated with fertility transformation in the photoperiod sensitive genic male sterile rice (PGMR), Nongken 58S, encoded a rice (*Oryza sativa* L. ssp. *japonica*) small GTPase belonging to the Rac/Rho family. Inverse PCR was performed to amplify a fragment about 1.4 kb in 5′ upstream region of the *osRACD* promoter. Deletion mutation and gel mobility shift assay characterized two fragments (–799 to –686 nt, and –686 to –431 nt) in the *osRACD* promoter that could be involved in its transcriptional regulation. When these two deletion fragments were used as probe respectively, a retarded band appeared in the nuclear extracts of fertile 58S rice under short day (58S-SD). Whereas no retarded band was shown in the nuclear extracts of sterile 58S rice under long day (58S-LD). Competition assay indicated that the factors in the retarded bands binding to these two fragments were the same *trans*-acting factor (termed rice factor, RF). The binding affinity of RF was affected by phosphorylation and was higher in SD-growth rice than that of LD-growth rice.

Key words photoperiod-sensitive male genic sterile rice; *osRACD* gene; photoperiod fertility transformation; *cis*-element

Sexual reproduction in many plants is sensitive to the change of day-long and photoperiod. It was discovered by Shi *et al*. [1] in 1973 that the natural mutant Nongken 58S is infertile under long day while being fertile under short day. In recent two decades, much work has been done to probe into its molecular mechanism from various perspectives, such as physiology, genetics as well as molecular biology [2,3]. The previous study showed that physiological changes of 58S rice could be dependent on combinational regulation of light regulatory genes and their associated binding factors. The light-dependent transcriptional regulation is mostly controlled by light-responsive elements in promoter region. So the isolation of critical genes controlling photoperiod fertility transformation in 58S rice, and the identification of their light-responsive elements, are essential for the elucidation of light-induced signal transduction pathways.

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In our previous study, by employing the modified method of mRNA difference display, a new gene, *osRACD*, was isolated from the photoperiod sensitive genic male sterile rice (PGMR), Nongken 58S [4]. The homologous comparison of amino acid residues and the analysis of the tertiary structure showed that *osRACD* encoded a small GTP-binding protein involved in Rac/Rho subfamily. In mammals, Rho GTPases are molecular switches that control a number of signal transduction pathways leading to the morphogenesis of cytoskeleton [5], cell proliferation and differentiation [6,7], gene expression and reactive oxygen species production [8]. In plants, Rho/Rac GTPases are also involved in many signal regulatory pathways. Since the first Rac protein in plant was found in 1993 [9], 52 *Rac* genes from 17 sorts of plants have been cloned [10–12]. Plant *Rac* gene was also called as Rop(lant) (Rho-related GTPases from plant), which belongs to a distinct subfamily of the Rho family. In *Arabidopsis*, studies using the pollen-specific Rop1AT and its homologues show that Rop acts as a central switch to control pollen tube polar growth via modulating the dynamics of tip actin and the formation of tip-focused calcium gradients [13]. Another *A. thaliana* Rac-like protein was confirmed to being involved in a signaling

complex that includes the CLAVATA1 receptor-like kinase (RLK) with leucine-rich repeat (LRR) domain which regulates the balance between proliferation and differentiation at the shoot meristem[14]. Further support for the role of plant Rac protein in regulation of cell polar growth came from our studies of DN-osRACD in transgenic *Arabidopsis* [15]. In pollen germination *in vitro*, Dominant negative (DN)-osRACD appears to inhibit pollen tube polar growth. In transgenic 58S rice via SD-treatment, DN-osRACD also apparently reduced pollen fertility.

However, the tissue-specific expression pattern of *osRACD* revealed by RT-PCR analysis showed high expression of *osRACD* transcripts in 58S-SD rice spikelets [4]. This was further confirmed by using *in situ* hybridization (Xu *et al*., unpublished data), in which *osRACD* transcripts were localized in anther and germinating pollen, supporting a role for osRACD protein in the regulation of anther morphogenesis and pollen maturation. Besides, since *osRACD* gene was activated under short day, its expression might be regulated by light [4]. A pea (*Pisumsativum*) small GTPase gene, *pra2*, which belongs to the YPT/Rab family, has been demonstrated as one of the genes whose expressions are mediated by phytochrome [16]. A 12-bp sequence in the *pra2* promoter might confer light down-regulation [17].

Here we demonstrated by gel mobility shift assay that two fragments in the *osRACD* promoter could be involved in regulation of its expression. Furthermore, the *trans*-acting factors binding these two fragments were possibly regulated by phosphorylation. These results showed that the small GTP-binding protein osRACD was a critical regulator in photoperiod fertility transformation of 58S rice.

Materials and Methods

Materials

The rice samples (*Oryza sativa* L. *ssp*. *japonica*) were kindly provided by Prof. Mu TM (Hubei Academy of Agricultural Sciences), which included 58N, the normal late *japonica* rice and 58S, and the natural mutant of PGMR. 58S rice has photoperiod fertility transformation property, respectively treated with long day (LD, light/dark: 15/9) and short day (SD, light/dark: 9/15). The lowest temperature in light is 28 °C, while in dark, the lowest temperature is 25.1 °C. 58S rice under LD and SD are named as 58S-LD and 58S-SD respectively.

osRACD cDNA was prepared by Mi ZY (Institute of Genetic and Developmental Biology, Chinese Academy of Sciences). Restriction enzymes were purchased from Gibco. T4 DNA ligase and *Taq* DNA polymerase were purchased from Promega.

Isolation of the *osRACD* promoter by inverse PCR (IPCR)

The genome DNA of 58S rice was purified according to the manufacturer's instructions of Magic MiniPreps DNA purification system (Promega), and was further digested with restriction enzymes *Nhe*I, *Kpn*I, *Xho*I and *Sna*I respectively. A ligation using a final DNA concentration of 0.002 mM was performed overnight, the ligated DNA was purified and concentrated to 20 μl by ethanol precipitation. Using the purified and ligated DNA (0.5 μg) as template, PCR was performed with primer IP1, 5′-CTGAGGAAACTCATTGGTGCAGC-3′, and IP2, 5′-GGAGGTGTAGGAGATGAGCATGC-3′. PCR condition was as following: denaturing at 94 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, synthesis at 72 °C for 1 min; a final extension at 72 °C for 10 min. With the amplified product diluted 50-fold as template, the second PCR was performed with primer IP3, 5′-TGTATAATTACGCTAGATGCCACCGG-3′, and IP4, 5′-GTGTAGGAGATGAGCATGCAGGTC-3′. The condition was as following: denaturing at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, synthesis at 72 °C for 1 min; a final extension at 72 °C for 10 min (Fig. 1). Amplified products were cloned into plasmid pGEM-T-easy, and sequenced by Sangon Company.

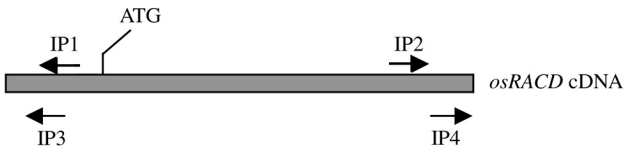

Fig. 1 Map of cloning *osRACD* promoter with IPCR

DNA probe for gel mobility shift assay

The DNA probe used in gel mobility shift assay was synthesized by end-labeling. Restriction endonuclease *Bcl*21, *Csp*45I, *Dra*I, *Xba*I, *Hae*III and *Kpn*I (Gibco) cleaved *osRACD* promoter to obtain six deletion fragments: BC (–1139 to –799), CD (–799 to –686), DX (–686 to –431), XH (–431 to –193), HD (–193 to –25) and DK (–25 to +476).

Extraction of nuclear protein

Rice spikelets were homogenized with a mortar in buffer A [1 M 2-methyl-2,4-pentanediol, 10 mM pH 7.6 HEPES/KOH, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 0.5% Triton X-100] and filtered through a single-layer nylon cloth. The filtrate was centrifuged at 1600 *g* for 10 min to isolate the nuclei. The pellet was carefully resuspended and washed twice in buffer B (0.5 M 2-methyl-2,4-pentanediol, 10 mM HEPES/KOH, pH 7.6, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM PMSF), and centrifuged at 1600 *g* for 5 min. The pellet was resuspended again in lysis buffer C (15 mM HEPES/KOH, pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF) to which a one-tenth volume of 4 M ammonium sulfate was added before gently rocked at 4 °C for 30 min. Insoluble materials were removed by centrifugation at 36,000 rpm for 2 h. The supernatant was saturated to 75% with ammonium sulfate, then centrifuged at 20,000 *g* for 20 min. Finally, the pellet was dissolved in buffer D (25 mM HEPES/KOH, pH 7.6, 100 mM KCl, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF). The sample was dialyzed in 1000-fold volume of buffer D overnight to remove the ammonium sulfate.

Gel mobility shift assay

The gel mobility shift assay was performed with 4% non-denaturing polyacrylamide gel electrophoresis at 5 V/cm. Before electrophoresis, the reaction mixture (2 ng DNA probe, 4 μg of carrier DNA [poly(dIdC)–poly(dIdC)], 8 μg of nuclear protein, 25 mM HEPES/KOH, pH 7.8, 1 mM EDTA, 10% glycerol, 0.5 mM DTT, 60 mM KCl, 0.3 mM PMSF) was incubated at 25 °C for 15 min.

Results

Isolation and structural analysis of the *osRACD* promoter

IPCR technology was applied broadly in constructing genome walking library and the isolation of 5′ upstream region of genes. To isolate the *osRACD* promoter, we used nested PCR with two pairs of primers to amplify a fragment of about 1.8 kb from 58S rice genome, which was digested with *Xho*I and ligated (Fig. 2). The fragment was demonstrated to comprise about 400 bp 5′ upstream region of *osRACD* gene and an approximately 1.4 kb section of *osRACD* promoter containing the TATA box and CAAT box.

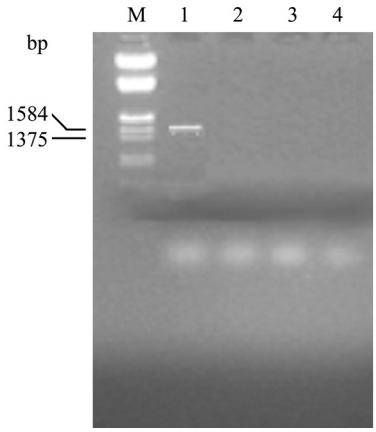

Fig. 2 Electrophoresis map of second amplified products by IPCR

M, λDNA/*Eco*RI+*Hin*dIII marker; 1–4 were digested by different enzymes respectively. 1, *Xho*I; 2, *Nhe*I; 3, *Kpn*I; 4, *Sna*I.

Fig. 3 shows the nucleotide sequence of the *osRACD* promoter, TAAT box and CAAT box were located in –27 nt and –47 nt from the transcriptional initiation site respectively. Using Plant CARE software, we found several LRE (light-responsive element)-homologous sequences in the 5′ upstream region of the *osRACD* gene. Besides, several putative regulatory elements (AuxRR-core, pollen1LELAT52) are also present within the *osRACD* promoter (Table 1). Given the central role of light-responsive elements in transcriptional regulation, the identifications of light-responsive elements and their associated binding factors are an important step toward understanding regulatory mechanisms that operate during photoperiod fertility transformation. Except for light-responsive elements, the existence of Pollen1LELAT52 [18] in 5′ upstream sequence of the *osRACD* gene could confer high-level pollen-specific *osRACD* gene expression.

Preparation of restriction fragment of the *osRACD* promoter

To identify the light-responsive elements in the 5′ upstream region of the *osRACD* gene and their associated *trans*-acting factors, gel mobility shift assays with six DNA fragments were carried out, BC (–1139 to –799), CD (–799 to –686), DX (–686 to –431), XH (–431 to –193), HD (–193 to –25), DK (–25 to +476) (Fig. 4), these fragments were respectively generated by restriction enzyme, *Bcl*I, *Csp*45I, *Dra*I, *Xba*I, *Hae*III and *Kpn*I.

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CGATCGACAT AATGATCACC TATTGATTAT CAAGATGTTG TTTTTTTGGA AAACAATGTT   -1092
AGTATAGTCA TTTTGTTCTG AACTAGCATG GTGGCCCGCA CAGATTGCTC GGCTAGCACT   -1032
GTTATATTTT CTCACATAAA TAACATATAT ATTTTCATAT ATTATTATTA GAATGGCAAC    -972
ATAATTTGAA ATTATGTACT AAGTAGAACA AACTAACCAA TATATAATTT TTATATTGTA    -912
TTGTATACAC GTGATAGTTA TCAATTATTT TTTTAATTCT GAATTTTAGT TATTTTGAAT    -852
TGTATTTCTA TATGGACTAT GAATTCCTCT TCCAATATTC TTTATTTTTT AATTCGAAGT    -792
TCCATTATTT ACTCCTATTT GAATATTTTT TTAGTTTCAA ATTTTTGTTA TTTGTAAATT    -732
ATATTTCTAT ATAAACTCTA TAATATTGTT TCAATATGCC TTATTTTAAA TTTTAAATTT    -672
TAGTTATTTA TAAATTGTAT TCCTATATGG TCTCTAAACT CTTCTTCTTC AATATTTTTA    -612
TTTTTAAATT CCAAATTCAA TTATTTCTAA ACTCTTTCTA AAGTGTATTT CTACATGGAC    -552
TGTAAACTCT ACTTTTACTT TTTTCTTATT TTTAATTCCA ACTTTCAATT CTTTTAAATT    -492
CCTTTATGAA CTCTAAACTT TATGAATTTT AATTATCTCT AAATTGTATT CCTATATGGA    -432
CTCTAGACTC TTCTTCCCAT ATTCCTTAAT TTTTAATTTT ATTTATTTCT AAAATGTATT    -372
TCTATATGGA CTTTGACTAT ACTTTTATTT TTTAAATTTT AAATTTCAAT TAAGTCTAAA    -312
TCGTATTCCT TTATGGACTC TTCTTCCAAT ATTTTTTTAA TTTCTAATTT TAGCTATTTA    -252
AATTGTATTT CTATATGGAC TCGTTTTTTA TTTTTTTTAT TAATATGAGA ATTTTTAGGC    -192
CGTGAGAGCG AACATGGAGA CTCTTTTTTT ATTATTTTGA TATGTTAATA GATTGTAACC    -132
CCTAATAAAA AAAATCATAC AAAATGTATG TCATTTTGAA GAAACAATGA TATATGTAGA     -72
TCTCATCAAC CATAGATGAA AAACCTCAAT CTTTAAGGTG GTATTTTAAA TAGCTTTCTT     -12
ATGTTTTAAT CTA
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Fig. 3 Partial sequence of the *osRACD* promoter

Regulatory *cis*-elements that are found in known light-responsive gene promoters are indicated in gray. The transcription initiation site is boxed. TAAT box is underlined.

Table 1 *Cis*-Elements found at the 5′-upstream region of *osRACD* gene

Element	Position[a]	Sequence	Reference
CAAT box	–47	-CAATCT-	
TAAT box	–27	-TAATAA-	
G box	–905	-CACGTG-	Martinez-Garcia *et al*. [19]
BoxI	–758, –509	-TTCAA-	Kuhlemeier *et al*.[20]
AT1 box (AT rich element)	–622	-AATATTTTTATT-	Datta *et al*. [21]
I-box	–84	-GATA/TA-	Borello *et al.* [22]
AuXRR-core	+102	-GGTCC-	Sakai *et al*.[23]

[a] The distance (in bp) from the putative transcription start site.

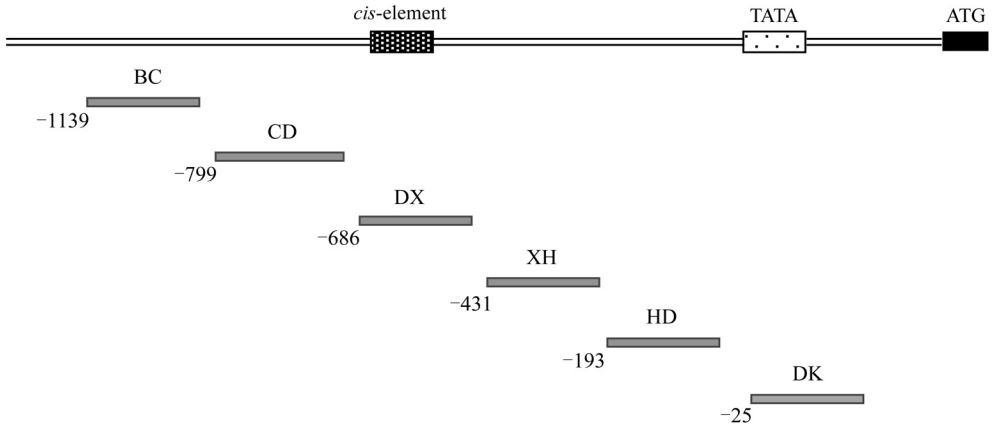

Fig. 4 Six deletion fragments for the gel mobility shift assay

Identification of *cis*-element of the *osRACD* promoter by gel mobility shift assay

[α-^{32}P]dCTP was filled in the end of six restriction fragments by end-labeling to generate DNA probe for gel mobility shift assay. BC was 340 bp in length containing a G-box sequence, CD had 103 bp of the section of the gene and contained the first BoxI-like sequence, DX was 255 bp long and contained the second BoxI-like motif and AT-rich element, and HD was 168 bp long and contained the I-box and transcriptional initiation site of *osRACD* gene. The gel mobility shift assay was performed with nuclear protein extracts from 58S-SD and 58S-LD spikelets using the six fragments as probe. A single band had slower mobility in the nuclear protein extracts from 58S-SD spikelets using the CD or DX fragment as probe, whereas no retarded band appeared in the nuclear protein extracts from 58S-LD spikelets (Fig. 5, 6). In addition, no retarded band appeared in the nuclear protein extracts from 58S-LD and 58S-SD spikelets when using the BC, XH, or DX fragment as probe. This indicated that the 5′ upstream region of the *osRACD* gene has two binding sites (–799 to –686, –686 to –431) for some factors, which only exist in fertile 58S-SD rice spikelets. The binding activity of nuclear protein from 58S-SD spikelets to the two fragments was significantly higher than that of nuclear protein from 58S-LD spikelets, suggesting an involvement of the protein in light down-regulation. Besides, in the gel mobility shift assay using HD as the probe, a retarded band appeared in nuclear protein of both 58S-SD and 58S-LD spikelets (Fig. 7), suggesting that HD did not confer the response to light. The retarded band could be generated by transcription initiation complex, since HD contained a TAAT box.

Competition experiments were performed to confirm that the bands shared by CD and DX were due to interactions with the same *trans*-acting factor. The CD fragment was used as radiolabeled probe, and the DX fragment was used as competitor. When the competitor DNA was added, the binding activity of nuclear protein of 58S-SD spikelets

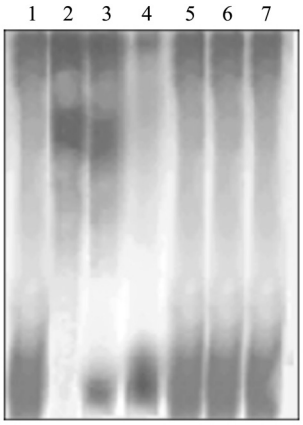

Fig. 5 Gel mobility shift assay with nuclear protein extracts from 58S-SD and 58S-LD spikelets using CD as a probe

1 and 5, free probes; 2, probe plus nuclear protein from 58S-SD spikelets; 3 and 4, probe plus nuclear protein from 58S-SD spikelets and unlabeled CD as competitor DNA (50-fold, 200-fold); 6, probe plus nuclear protein from 58S-LD spikelets; 7, probe plus nuclear protein from 58S-LD spikelets and 200-fold of unlabeled CD as competitor DNA.

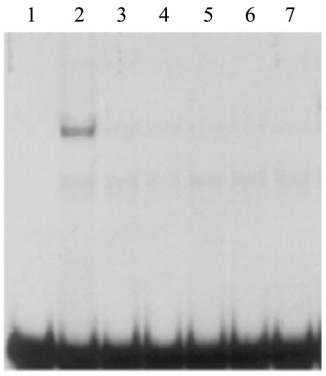

Fig. 6 Gel mobility shift assay with nuclear protein extracts from 58S-SD and 58S-LD spikelets using DX as a probe

1 and 4, free probes; 2, probe plus nuclear protein from 58S-SD spikelets; 3, probe plus nuclear protein from 58S-SD spikelets and unlabeled DX as competitor DNA (200-fold); 5, probe plus nuclear protein from 58S-LD spikelets (7.5 mg); 6, probe plus nuclear protein from 58S-LD spikelets (37.5 mg); 7, probe plus nuclear protein from 58S-LD spikelets (7.5 mg) and unlabeled CD as competitor DNA (200-fold).

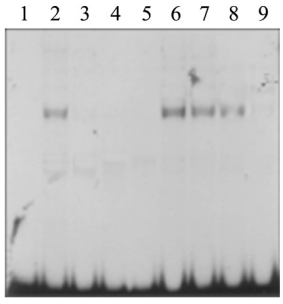

Fig. 7 Gel mobility shift assay with nuclear protein extracts from 58S-SD and 58S-LD spikelets using HD as a probe

1 and 5, free probes; 2, probe plus nuclear protein from 58S-SD spikelets; 3 and 4, probe plus nuclear protein from 58S-SD spikelets and unlabeled HD as competitor DNA (100-fold, 200-fold); 6, probe plus nuclear protein from 58S-LD spikelets; 7, 8, and 9, probe plus nuclear protein from 58S-LD spikelets and unlabeled HD as competitor DNA (100-fold, 200-fold, and 300-fold, respectively).

to the CD fragment was reduced (Fig. 8). This result indicated that the *trans*-acting factors binding to CD were the same as the factors shown in Fig. 6. The particular factor (rice factor, termed RF) binding to two sites in the 5′ upstream region of the *osRACD* gene (–799 to –686, –686 to –431). Since CD and DX both contained a BoxI-like motif, we concluded that RF could be bound to the Box-I-like sequences, although the binding sites extended in both the 5′ and 3′ directions. The presumption has been further verified by DNaseI footprinting assay of DX fragment, that contains the second BoxI-like motif (Xu *et al.*, unpublished data).

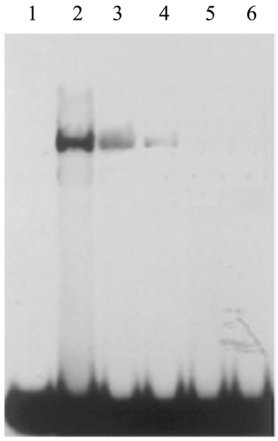

Fig. 8 Gel mobility shift assay with CD as a probe and DX as competitor

1, free probe; 2, probe plus nuclear protein from 58S-SD spikelets; 3, 4, 5, and 6, probe plus nuclear protein from 58S-SD spikelets and unlabeled DX as competitor DNA (50-fold, 100-fold, 200-fold, and 300-fold, respectively).

To elucidate the regulation of the binding activity, an additional retardation assay was carried out with the CD fragment as the probe. Nuclear protein extracts from 58S–SD spikelets were treated with CIP, both in the presence and absence of NaF, a known inhibitor of CIP. The bound band was reduced in intensity by CIP treatment and was unchanged following the addition of NaF (Fig. 9), suggesting that RF binding was dependent on their phosphorylation status.

Discussion

The further study of *osRACD* promoter region showed that a Mybcore (CNGTTR) sequence exits in the –665 bp from the putative transcription initiation site of the *osRACD*

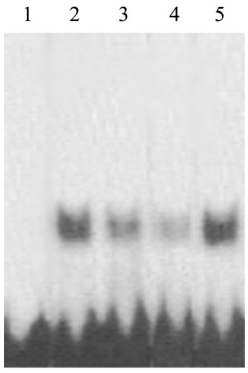

Fig. 9 Gel mobility shift assay with CD as a probe and nuclear extracts from 58S-SD spikelets that were treated with CIP

1, free probe; 2, probe plus nuclear protein from 58S-SD spikelets; 3, treated with 0.1 U CIP for 30 min; 4, treated with 1 U CIP for 30 min; 5, treated with 1 U CIP in the presence of 5 mM NaF.

gene. Although it is not clear if the element and its associated Myb protein are involved in light-dependent transcription regulation of *osRACD* gene, the most well-established role of plant Myb proteins is in the control of genes in the flavonoids biosynthetic pathway [24–26], however, flavonoids are required for male gametophyte development in some species, which are thought to be an important structural and signaling regulator in pollen germination [27,28]. The further evidence has shown that GTP-binding protein is also involved in regulating the flavonoids biosynthetic pathway [29]. Though it remains to be determined if *osRACD* protein depends on regulating the flavonoid biosynthetic pathway to influence pollen sterility, this finding is fascinating us.

The photoperiod-dependent expression of *osRACD* gene in 58S rice [4] indicated that some *trans*-acting factors regulated by light/dark cycle could bind to *osRACD* promoter region. The *trans*-acting factors and the associated *cis*-acting element of the light regulatory genes were always located in their promoter regions. The identification of protein-DNA interactions in the transcriptional regulation of light-responsive genes is essential for the elucidation of light-induced signal transduction pathways. In our study, several LRE-homologous sequences were present in the 5′ upstream region of *osRACD* gene. From these sequences, five elements (an AT-rich element, two BoxI motif, a G-box sequence, an I-box motif) were chosen for the derivation of deletion fragments that were subsequently employed *in vitro* analyses. In the *in vitro* assays, two regions in *osRACD* promoter (–799 to –686,

–686 to –431) contain the essential *cis*-elements for gene expression. They were bound by some specific *trans*-acting factors (termed RF) only in the nuclear protein of 58S-SD, whereas in the nuclear protein of 58S-LD rice, RF lost their binding activities. The binding activity of RF only appeared in short-day condition, suggesting that the activity was controlled by the photoperiod. The dephosphorylation experiment showed that RF binding was modulated by phosphorylation and dephosphorylation. Phosphorylative control of transcription factor binding affinities has been reported, the ability of numerous DNA-binding proteins to bind to target sequences was also modulated by phosphorylation [30]. Given this, SD-treatment maintains the phosphorylation level of RF, conferring SD-specific expression of *osRACD* gene in 58S rice.

Though it has been demonstrated that the *osRACD* promoter contains three binding regions (CD, DX and HD) for some transcription factors, only CD (–799 to –686) and DX fragment (–686 to –431) could bind with some SD-inducible *trans*-acting factors. Competition assay between CD and DX fragment indicated that they contained the same core sequence motif. Sequence analysis between CD and DX revealed that they shared a typical BoxI motif (TTTCAA), two BoxI-like motif dispersed in the region from –761 to –753 (AGTTTCAA) and from –512 to –505 (ACTTTCAA). BoxI motif was first found in the pea *rbcS-3A* promoter [20]. The *rbcS-3A* promoter is one of these promoters that confer tissue specific and light regulated gene expression. In *rbcS-3A* promoter, several conserved light-responsive elements (BoxI-III, BoxII*, BoxIII*) were identified by *in vitro* protein-binding experiments [31,32]. A further sequence similarity search of the two BoxI-like elements (–761 to –753, –512 to –505, in *osRACD* promoter) in the motif database PLACE for plant *cis*-acting regulatory DNA element, revealed an apparent similarity with BoxIII (ATCATTTTCACT, *Pisum sativum*) [31,32]. Box-III was shown to interact with a nuclear regulatory protein, GT-1 [33]. GT-1 not only binds to Box-III, but also to an additional five *cis*-element of the *rbcS-3A* gene with the consensus sequence [34]. Besides, two light-responsive enhancer elements EE-1 (AGATTTTCA) and EE-2 (CGACTTACGAA) were also identified in *cah1* promoter [35]. Though GT-1 typically binds to the consensus sequence G(A/G)(A/T)AA(A/T), given the difference between Box-III and the consensus sequence, the comparison between several conserved light-responsive elements and the two BoxI-like motif in the region of *osRACD* promoter revealed their sequence homology. ANTTNC could be another recognizing site to GT-1 (Fig. 10). GT-1 is a typical transcription factor regulated by phosphorylation [30], the binding characteristic of GT-1 is very similar to that of RF. Detailed amino acid sequence comparisons revealed that GT-1 is closely related to Myb proteins that function as transcription activators in diverse organisms ranging from animals to plants [36]. The finding of Mybcore (–655) in the *osRACD* promoter constructs a potential link between Mybcore and BoxI-like motif. DNase I footprinting assay was further performed with the DX fragment to detect RF-binding site (5′-TTAATTCCAACTTTCAATTCT-3′), which contains the second Box-I-like motif in the *osRACD* promoter (Xu *et al.*, unpublished data). It is derived from these results that the transcriptional activation of *osRACD* gene under short day could be correlated with the BoxI-like motif. At present, the identification of CD and DX using deletion function assay in the transgenic plants, has been done, the initial data showed the deletion of CD fragment would cause a lower level expression of report gene in light. In view of this, we initially recognize that CD and DX fragments contain the positive *cis*-elements to the *osRACD* gene.

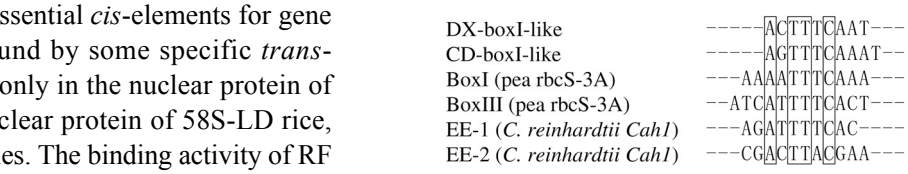

Fig. 10 Comparison of the nucleotide sequences between the two potential *cis*-elements in the *osRACD* promoter and several conserved light-responsive elements

Conserved nucleotides among these elements are boxed.

References

1 Shi MS. Discovery and preliminary analysis of a photoperiod-sensitive genic male sterile rice. Chinese Agriculture Science, 1985, 2: 44–48

2 Zhang Q, Shen BZ, Dai XK, Mei MH, Maroof MAS, Li ZB. Using bulked extremes and recessive class to map genes for photoperiod-sensitive genic male sterility in rice. Proc Natl Acad Sci USA, 1994, 91(18): 8675–8679

3 Li ZB. Studies on the photoperiod sensitive genic male sterility in rice and its utilization in breeding. Wuhan: Hubei Science Press (In Chinese), 1995, 89-362

4 Mi ZY, Wang SS, Wu NH. Isolation of *osRACD* gene encoding a small GTP-binding protein from rice. Chinese Science Bulletin, 2002, 47(20): 1673

5 Tapon N, Hall A. Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. Curr Opin Cell Biol, 1997, 9(1): 86–92

6 Lamaze C, Chuang TH, Terlecky LJ, Bokoch GM, Schmid SL. Regulation of receptor-mediated endocytosis by Rho and Rac. Nature, 1996, 382: 177–179

7 Schmalzing G, Richter HP, Hansen A, Schwarz W, Just I, Aktories K.

Involvement of the GTP-binding protein Rho in constitutive endocytosis in *Xenopus laevia* oocytes. J Cell Biol, 1995, 130(6): 1319–1332
8 Ono E, Wong HL, Kawasaki T, Hasegawa M, Kodama O, Shimamoto K. Essential role of the small GTPase Rac in disease resistance of rice. Proc Natl Acad Sci USA, 2001, 98(2), 759–764
9 Yang Z, Watson JC. Molecular cloning and characterization of Rho, a ras-related small GTP-binding protein from the garden pea. Proc Natl Acad Sci USA, 1993, 90(18): 8732–8736
10 Borg S, Brandstrup B, Jensen TJ, Poulsen C. Identification of new protein species among 33 different small GTP-binding proteins encoded by cDNAs from *Lotus japonicus*, and expression of corresponding mRNAs in developing root nodules. Plant J, 1997, 11(2): 237–250
11 Winge P, Brembu T, Bones AM. Cloning and characterization of rac-like cDNAs from *Arabidopsis thaliana*. Plant Mol Biol, 1997, 35(4): 483–495
12 Li H, Wu G, Ware D, Davis KR, Yang Z. Arabidopsis Rho-related GTPases: Differential gene expression in pollen and polar localization in fission yeast. Plant Physiol, 1998, 118(2): 407–417
13 Kost B, Lemichez E, Spielhofer P, Hong Y, Tolias K, Carpenter C, Chua NH. Rac homologues and compartmentalized phosphatidylinositol 4,5-bisphosphate act in a common pathway to regulate polar pollen tube growth. J Cell Biol, 1999, 145(2): 317–330
14 Trotochaud AE, Hao T, Wu G, Yang Z, Clark SE. The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. Plant Cell, 1999, 11(3): 393–406
15 Ye JR, Wu NH. Fertility analysis of the *Arabidopsis* transformed with antisense rice *osRACD* gene. Progress in Nature Science, 2003, 13(6): 424-428
16 Nagy F, Schafer E. Phytochromes control photomorphogensis by differentially regulated, interacting signaling pathways in higher plants. Annu Rev Plant Biol, 1993, 53: 329–355
17 Inaba T, Nagano Y, Sakakibara T, Sasaki Y. Identification of a *cis*-regulatory element involved in phytochrome down-regulated expression of the pea small GTPase gene *pra2*. Plant Physiol, 1999, 120(2): 491–500
18 Bate N, Twell D. Functional architecture of a late pollen promoter: Pollen-specific transcription is developmentally regulated by multiple stage-specific and co-dependent activator elements. Plant Mol Biol, 1998, 37(5): 859–869
19 Martinez-Garcia JF, Huq E, Quail PH. Direct targeting of light signals to a promoter element bound transcription factor. Science, 2000, 288(5467): 859–863
20 Kuhlemeier C, Fluhr R, Green PJ, Chua NH. Sequences in the pea rbcS-3A gene have homology to constitutive mammalian enhancers but function as negative regulatory elements. Genes Dev, 1987, 1(3): 247–255
21 Datta N, Cashmore AR. Binding of a pea nuclear protein to promoters of certain photoregulated genes is modulated by phosphorylation. Plant Cell, 1989, 1(11): 1069–1077
22 Borello U, Ceccarelli E, Giuliano G. Constitutive, light-responsive and circadian clock- responsive factors compete for the different I-box elements in plant light-regulated promoters. Plant J, 1993, 4: 611–619
23 Sakai T, Takahashi Y, Nagata T. Analysis of the promoter of auxin-inducible gene, parC, of tobacco. Plant Cell Physiol, 1996, 37(7): 906–913
24 Lipsick JS. One billion years of Myb. Oncogene, 1996, 13(2): 223–235
25 Jin H, Martin C. Multifunctionality and diversity within the plant *MYB*-gene family. Plant Mol Biol, 1999, 41(5): 577–585
26 Martin C, Paz-Ares J. MYB transcription factors in plants. Trends Genet, 1997, 13(2): 67–73
27 Quattrocchio F, Wing JF, Leppen H, Mol J, Koes RE. Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. Plant Cell, 1993, 5(11): 1497–1512
28 van der Meer IM, Stam ME, van Tunen AJ, Mol JN, Stuitje AR. Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility. Plant Cell, 1992, 4(3): 253–262
29 Ortega X, Polanco R, Castaneda P, Perez LM. Signal transduction in lemon seedlings in the hypersensitive response against *Alternaria alternata*: Participation of calmodulin, G-protein and protein kinases. Biol Res, 2002, 35(3-4): 373–383
30 Marechal E, Hiratsuka K, Delgado J, Nairn A, Qin J, Chait BT, Chua NH. Modulation of GT-1 DNA-binding activity by calcium-dependent phosphorylation. Plant Mol Biol, 1999, 40(3): 373–386
31 Khoudi H, Vezina LP, Mercier J, Castonguay Y, Allard G, Laberge S. An alfalfa rubisco small subunit homologue shares *cis*-acting elements with the regulatory sequences of the RbcS-3A gene from pea. Gene, 1997, 197: 343–351
32 Kuhlemeier C, Cuozzo M, Green PJ, Goyvaerts E, Ward K, Chua NH. Localization and conditional redundancy of regulatory elements in *rbcS-3A*, a pea encoding the small subunit of ribulose-bisphosphate carboxylase. Proc Natl Acad Sci USA, 1988, 85: 4662–4666
33 Green PJ, Kay SA, Chua NH. Sequence-specific interactions of a pea nuclear factor with light-responsive elements upstream of the *rbcS-3A* gene. EMBO J, 1987, 6: 2543–2549
34 Green PJ, Yong MH, Cuozzo M, Kano-Murakami Y, Silverstein P, Chua NH. Binding site requirements for pea nuclear protein factor GT-1 correlate with sequences required for light-dependent transcriptional activation of the *rbcS-3A* gene. EMBO J, 1988, 7: 4035–4044
35 Kucho K, Yoshioka S, Taniguchi F, Ohyama K, Fukuzawa H. *Cis*-Acting elements and DNA-binding proteins involved in CO_2-responsive transcriptional activation of *Cah1* encoding a periplasmic carbonic anhydrase in *Chlamydomonas reinhardtii*. Plant Physiol, 2003, 133: 783–793
36 Nagano Y. Several features of GT-factor trihelix domain resemble those of the Myb DNA-binding domain. Plant Physiol, 2000, 124: 491–494

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