Identification of a Cis-Acting Element of ART1, a C2H2-Type Zinc-Finger Transcription Factor for Aluminum Tolerance in Rice1[OA]

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Rice (Oryza sativa) is one of the most aluminum (Al)-tolerant species among small-grain cereals. Recent identification of a transcription factor AL RESISTANCE TRANSCRIPTION FACTOR1 (ART1) revealed that this high Al tolerance in rice is achieved by multiple genes involved in detoxification of Al at different cellular levels. ART1 is a C2H2-type zinc-finger transcription factor and regulates the expression of 31 genes in the downstream. In this study, we attempted to identify a cis-acting element of ART1. We used the promoter region of SENSITIVE TO AL RHIZOTOXICITY1, an Al tolerance gene in the downstream of ART1. With the help of gel-shift assay, we were able to identify the cis-acting element as GGNT/g/a/CV(C/A/g/S(C/G)). This element was found in the promoter region of 29 genes among 31 ART1-regulated genes. To confirm this cis-acting element in vivo, we transiently introduced this element one or five times tandemly repeated sequence with 35S minimal promoter and green fluorescent protein reporter together with or without ART1 gene in the tobacco (Nicotiana tabacum) mesophyll protoplasts. The results showed that the expression of green fluorescent protein reporter responded to ART1 expression. Furthermore, the expression increased with repetition of the cis-acting element. Our results indicate that the five nucleotides identified are the target DNA-binding sequence of ART1.

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SENSITIVE TO AL RHIZOTOXICITY1 (STAR1) and STAR2 encode an ATP-binding and a transmembrane domain of a bacterial-type ATP-binding cassette transporter, respectively. The complex between STAR1 and STAR2 transports UDP-Glc, which is supposed to be used for modification of the cell wall although the exact mechanism remains unknown (Huang et al., 2009). Recently, Nrat1 (Nramp Al transporter 1), belonging to Nramp (natural resistance-associated macrophage protein) family, was reported to be required for Al tolerance in rice (Xia et al., 2010). Nrat1 is localized at the plasma membranes of all root cells and functions as a transporter for uptake of trivalent Al ion in rice, which is required for a prior step of final Al detoxification through sequestration of Al into vacuoles (Xia et al., 2010). The expression of all these ART1-regulated genes is specifically up-regulated by Al (Yamaji et al., 2009; Xia et al., 2010). However, most downstream genes have not been functionally characterized.

In this study, we investigated a cis-acting element of ART1 by using gel-shift assay and transient expression analysis. We have been successful in identification of cis-acting element of ART1, which is present in the promoter regions of 29 genes out of 31 genes regulated by ART1.

RESULTS AND DISCUSSION

Identification of a Cis-Acting Element on the STAR1 Promoter with Gel-Shift Assay

STAR1 is one of the downstream genes regulated by ART1 (Yamaji et al., 2009). Previously, a yeast (Saccharomyces cerevisiae) one-hybrid assay showed that the ART1 protein interacted with promoter regions of STAR1 between −436 and −298 from the translation start site (Yamaji et al., 2009), indicating the presence of cis-acting element(s) in this region. To establish the gel-shift assay system, we produced recombinant ART1 protein using wheat germ cell-free protein synthesis system (Takai et al., 2010), and designed a probe covering the region between −446 and −288 (STAR1 full) from start codon of STAR1 (Fig. 1A). A gel-shift assay (in vitro) yielded a band at the size of ART1/Digoxigenin (DIG)-labeled probe complex (Fig. 1B, lane 1). This band disappeared in the presence of 100 ng (lane 2) and 500 ng (lane 3) non-DIG-labeled probe as a competitor. To confirm that the band shift was caused by binding of ART1 protein, super gel-shift assay with an anti-ART1 antibody was performed. The gel-shift assay was performed in the presence of anti-ART1 antibody and with 0 (lane 4), 100 ng (lane 5), and 500 ng (lane 6) of non-DIG-labeled probe as a competitor. The black and gray arrowheads indicate the position of shifted band of ART1/DIG-labeled probe complex and ART1/DIG-labeled probe/anti-ART1 antibody complex, respectively. The white asterisks indicate free probe signals.

Figure 1. Schematic diagram of the probes used for gel-shift assay and identification of the DNA-binding region of the ART1 transcription factor. A, Schematic diagram of the eight probes on the promoter of STAR1 for the gel-shift assay. B, Identification of DNA-binding region of ART1 on STAR1 promoter region. Lanes 1 to 3, Binding of ART1 protein to STAR1 full probe (−446 to −288) in the absence (lane 1) and presence of 100 ng (lane 2) and 500 ng (lane 3) non-DIG-labeled probe as a competitor. Lanes 4 to 6, Super gel-shift assay. The gel-shift assay was performed in the presence of anti-ART1 antibody and with 0 (lane 4), 100 ng (lane 5), and 500 ng (lane 6) of non-DIG-labeled probe as a competitor. The black and gray arrowheads indicate the position of shifted band of ART1/DIG-labeled probe complex and ART1/DIG-labeled probe/anti-ART1 antibody complex, respectively. The white asterisks indicate free probe signals.

previous findings obtained by yeast one-hybrid assay (Yamaji et al., 2009).

To narrow this region, we designed two probes; STAR1-1 (−507 to −358 from the start codon) and STAR1-2 (−368 to −208) covering the region identified above (Fig. 1A). Gel-shift assay showed that ART1 protein was bound with STAR1-2 probe, but not with STAR1-1 (Fig. 2A). This is also supported by the presence of self competitor of the STAR1-2 probe that inhibited the interaction between ART1 protein and STAR1-2 probe (Fig. 2A). These results indicate that the cis-acting sequence
recognized by ART1 protein is present in the region between −368 and −208 of STAR1 promoter region.

Since both STAR1 full (−464 to −288) and STAR1-2 (−368 to −208) probes were able to bind ART1 protein, the candidate region should be between −368 and −288. To confirm this region, gel-shift assay with a probe (STAR1-3, −368 to −288) was performed. STAR1-3 was able to be bound with ART1 protein and super shifted in the presence of anti-ART1 antibody (Fig. 2B). To further narrow the target region of STAR1 promoter, we divided STAR1-3 region into three parts (Fig. 1A, probes 1–3). Gel-shift assay showed that only probe 2 (−358 to −319) was able to bind ART1 protein, whereas probe 1 (−386 to −347) and probe 3 (−330 to −291) was not able to bind ART1 protein (Fig. 2B). These results indicate that the region between −358 and −319 in the STAR1 promoter contains cis-acting element of ART1. This is further confirmed by competition and super shift experiments (Fig. 2C, lanes 5–8).

The candidate region was further narrowed by gel-shift assay with a new probe (probe 4, −368 to −329). A complex between probe 4 and ART1 protein was detected at the similar size as that of probe 2 (Fig. 2C, lane 1). Presence of non-DIG-labeled probe 4 inhibited the formation of this complex (Fig. 2C, lane 2). Presence of ART1 antibody caused super shift of this complex (Fig. 2C, lane 3). This result combined with that from probes 1 and 2 indicated that the cis-element

Figure 2. Further identification of the DNA-binding site of the ART1. A, Binding of ART1 protein to STAR1-1 probe (−368 to −208) and STAR1-2 probe (−507 to −358) on the STAR1 promoter in the absence or presence of 500 ng non-DIG-labeled probes as a competitor. B, Binding of ART1 protein to STAR1-3 probe (−368 to −288), probe 1 (−386 to −347), probe 2 (−358 to −319), probe 3 (−330 to −291) on the STAR1 promoter in the absence or presence of 500 ng non-DIG-labeled probes as a competitor and/or anti-ART1 antibody. C, Binding of ART1 protein to probe 4 (−368 to −329), probe 2 (−358 to −319) on the STAR1 promoter in the absence or presence of 500 ng non-DIG-labeled probes as a competitor and/or anti-ART1 antibody. The black and gray arrowheads indicate the position of shifted band of ART1/DIG-labeled probe complex and ART1/DIG-labeled probe/anti-ART1 antibody complex, respectively. The white asterisks indicate free probe signals.

Figure 3. DNA-binding affinity of the ART1 protein to a 21-bp fragment (−352 to −329) of the STAR1 promoter. A, Scheme of probes with two nucleotide substations each. Lowercase shows nucleotides substituted. B, Binding of ART1 protein to STAR1 cis1 probes in the absence or presence of 500 ng non-DIG-labeled mutated probes (STAR1 cis1 and STAR1 M1-M9) as a competitor. Black arrowhead indicates the position of shifted band of ART1/DIG-labeled STAR1-cis1 probe complex. The white asterisks indicate free probe signals.
region recognized by ART1 is located between −358 and −329 of STAR1 promoter region.

To identify the cis-acting element within this region, we prepared the narrowest 30-bp probe STAR1-cis1 (−358 to −329) and a series of probes (STAR1-M1 to -M9) by substitution of two or six bases at the position between −352 and −329 in STAR1-cis1 (Fig. 3A). The binding ability of ART1 protein with these substituted probes was examined by the competition assay. STAR1-M1, -M2, or -M9 as a competitor was able to inhibit the interaction of ART1 protein and STAR1-cis1 probe (Fig. 3B). In contrast, presence of probes (STAR1-M5 and -M6) was not able to inhibit the binding to ART1 protein (Fig. 3B). Probes of STAR1-M3, -M4, -M7, and -M8 showed weak inhibitory effect on ART1 binding. These results indicate that ART1 protein is mainly bound to the core region (GTCC) between −342 and −339 of STAR1 promoter.

Since two substitutions for each probe were made in above experiment, there is a possibility that the nucleotides of two sides are also included in the cis-acting element. We therefore consider the putative cis-acting element to be GGTCCT. We produced DIG-labeled probes with single mutation at this part (PM1–PM18; Fig. 4A) and performed binding assay with ART1 protein (Fig. 4B). All substitutions of the nucleotide at the position of −343 and −342 (probes PM1–6) resulted in no binding with ART1 protein, indicating that these nucleotides (GG) are critical for ART1 binding (Fig. 4B). By contrast, substitution of T to C (probe PM9) at the position of −341 did not affect the binding to ART1 protein although substitution to G and A (probes PM7 and 8) at the same position resulted in weakened signal. Substitution of C to A and G (probes PM10 and 11) at the position of −340 did not affect the binding to the ART protein, whereas that to T (probe PM12) resulted in loss of the binding (Fig. 4B). Probes PM13 and 14...
with substitution of C to A and T at the position of −339 did not show ability to bind ART protein (Fig. 4B), but probe PM15 with substitution to G showed strong binding to ART1 protein. Finally, substitution of T to A, C and G (probes PM16 to 18) resulted in stronger signal of ART1/DIG-labeled probe complex compared with STAR1-cis1 probe (Fig. 4B), indicating that this nucleotide is not important for the recognition. Taken together, all these results indicate that the cis-acting core element of ART1 is GGN(T/g/a/C)V(C/A/g)S(C/G)

Table I. *The number and position of ART1 cis-element in ART1 regulated genes*

Position of ART1 cis-element indicate 2-kb promoter region from start codon of these genes.

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<tr>
<th>Cell wall maintenance and root elongation</th>
<th>RAP-DB Homolog</th>
<th>Arabidopsis Homolog</th>
<th>Description</th>
<th>No. of ART1 Cis-Element</th>
<th>Position of ART1 Cis-Element</th>
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<td>Os01g08692000</td>
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sequences, localized between −343 and −339 from translation start site of STAR1 gene. However, the ART1-binding affinity of nucleotides with small character is weaker than those with large characters.

In Vivo Confirmation of ART1 Cis-Acting Element

To confirm the cis-acting element identified by gel-shift assay, we introduced single or five repeated cis-acting element fused with cauliflower mosaic virus (CaMV) 35S minimal (−46) promoter (Fang et al., 1989) and GFP as a reporter gene (Fig. 5A) into tobacco (Nicotiana tabacum) protoplasts. As an effector, ART1 genomic DNA fragment including 2-kb-long own promoter was coexpressed as well as red fluorescent protein gene DsRed-monomer under the control of CaMV 35S promoter as an internal control (Fig. 5A). Transient expression assay showed no expression of ART1 in the protoplasts not introducing ART1 effector plasmid, but showed similar expression level of ART1 in the protoplasts introducing ART1 effector plasmid, irrespectively of repetition of the cis-acting element on the reporter plasmid (Fig. 5B). In the absence of ART1 effector, the expression of GFP as a reporter gene was at the level of background (Fig. 5C). In the presence of ART1 effector, the expression of GFP was higher in protoplasts expressing pentameric cis-acting element than monomeric one (Fig. 5C). These results demonstrated that the ART1 interacts with the cis-acting element and the transcriptional activation potential is enhanced by the repetition of the cis-acting element.

Search of Cis-Acting Element in All Downstream Genes Regulated by ART1

We used promoter region of STAR1 for identification of cis-acting element of ART1 in above experiments. Since ART1 regulates 31 genes (Yamaji et al., 2009), we therefore, examined whether the cis-acting element found in STAR1 also exists in other genes by searching the promoters (up to 2 kb from start codon) of all these genes. The cis-acting element was found in the promoter region of 29 genes out of 31 genes regulated by ART1 (Table I). Furthermore, the cis-acting element is present in the multiple positions of the same promoter region. For example, in the promoter region of STAR1, the cis-acting element was found in −343, −711, −735, and −972. In STAR2 promoter, two copies of the cis-acting element are present in the region −211, −1,168, and in Nrat1 promoter, there are three copies in the −490, −1,364, and −1,382. These results suggest that ART1 activates the expression of these genes by binding to the same target sequence.

The expression of ART1 is not induced by Al (Yamaji et al., 2009), but the expression of the downstream genes is up-regulated by Al. For example, the expression of STAR1 and STAR2 was induced by approximately 6- to 10-fold by a short exposure (2 h) to Al (Huang et al., 2009). The expression of Nrat1 is also up-regulated by approximately 8 times by 3 h exposure to Al (Xia et al., 2010). These findings suggest that activation of ART1 by Al is required in vivo to induce the expression of downstream genes. ART1 is localized at the nuclei and this localization is unaffected by Al (Yamaji et al., 2009). It would be an interesting topic to next elucidate the signal transduction pathway from Al perception to activation of ART1.

MATERIALS AND METHODS

Preparation of Recombinant ART1 Protein

Recombinant ART1 protein was produced by using cell-free protein expression system. Protein synthesis of ART1 using wheat (Triticum aestivum) germ extract was performed essentially according to the method described by ENDEXT technology protocol (CellFree Sciences Co., Ltd.). For construction of ART1 vector for the cell-free system, the open reading frame of ART1 was amplified by PCR from rice (Oryza sativa cv ‘Koshihikari’) root cDNA. Primer pairs used for amplification and introduction of restriction sites were 5’-ACTATGATGAGTGCACGGCACCAGATGCAACAAAC-3’ and 5’-CATGTCGCTATCCCATATTCTCTCCG-3’. The plasmid was constructed with PCR-amplified DNA fragments containing ART1 coding region cloned into the Spel and Ncol site of the wheat germ expression vector pEU3b (Sawasaki et al., 2002). Two microliters of the high-purity plasmid DNA (1 μg/μL) was added to a tube containing the transcription premix solution (CellFree Sciences Co., Ltd.) for transcription reaction. The mixture was incubated at 37°C for 6 h in an incubator. After cooled down to the room temperature, the translation mixture was resuspended and 10 μL of the mixture was added into 10 μL of WEPRO 3240 (wheat germ extract solution for translation reaction). The mixed solution (20 μL) was then transferred to the bottom of the single-break strip well containing SUB-AMIX (206 μL) to form bilayers. After incubation at 15°C for 20 h, the mixture is used as recombinant ART1 protein for gel-shift assay.

Gel-Shift Assay

Gel-shift assay was performed essentially according to the method described by DIG gel shift kit, second generation protocol (Roche Applied Science). DNA-binding reaction was carried out in a 20-μL volume containing 25 mM HEPES-KOH (pH 7.6), 40 mM KCl, 0.1% (w/v) Nonidet P-40, 10 mM ZnCl2, 1 μg Poly(dI-dC), 0.1 M Tris-Cl (pH 9.5), and 40 ng of DIG-labeled probe, and 63 ng recombinant ART1 protein as prepared above. For super shift assay, 1 μL of anti-ART1 antibody (Yamaji et al., 2009) was added to the above solution. After incubating for 30 min at 20°C, the mixture was subjected to electrophoresis with an 8% native PAGE.

The transfer of above DIG-labeled probes from native PAGE to a positively charged nylon membrane was performed with a semidyblotting system (30 min at 144 mA). The transferred probes were then fixed to the membrane by cross linking with UV light for 3 min at 120 mJ. The membrane was placed in a washing buffer (0.1 M maleic acid, 0.3% (w/v) Tween 20, pH 7.5) for 5 min at room temperature. The washed membrane was slowly shaken for 30 min in 50 mL of blocking solution (1% (w/v) blocking reagent, 0.1% maleic acid, 0.15 μM NaCl, pH 7.5). The membrane was then transferred to 10 mL of anti-DIG antibody solution (0.75 μL/mL anti-DIG-AP, 1% (w/v) blocking reagent, 0.1% maleic acid, 0.15 μM NaCl, pH 7.5) and slowly shaken for 30 min. The membrane was washed twice each for 15 min in 100 mL of washing buffer and equilibrated in 10 mL of detection buffer (0.1 μL Tris-HCl, 0.1 μM NaCl, pH 9.5) for 5 min. The membrane was placed with DNA side face up on a hybridization bag and applied 1 mL of disodium 3-(4-methoxyphenylphosphoryl)l,2-dioxoatane-3,2′-(5′-chloro)tricyclo[3.3.1.13,7]decane-4y] (5′-chloro)tricyclo[3.3.1.13,7]decane-4y] phenyl phosphate working solution [1 μg/mL disodium 3-(4-methoxyphosphoryl)l,2-dioxoatane-3,2′- (5′-chloro)tricyclo[3.3.1.13,7]decane-4y] phenyl phosphate solution, 0.1 μL Tris-HCl, 0.1 μM NaCl, pH 9.5] and incubated for 5 min at room temperature and for a further 10 min at 37°C to enhance the luminescent reaction. Chemiluminescence signal was detected using LAS-1000 (FUJIFILM Corporation).

Isolation of Tobacco Leaf Protoplasts

The seeds of tobacco (Nicotiana tabacum cv ‘Petit Havana SR1’ were sown on filter paper moistened with deionized water in a petri dish, and germinated
at 25°C under a 16-h/8-h light/dark cycle. After 10 d, the seedlings were transferred to a plastic mesh floating on a one-fifth Hoagland culture solution in a 1.5-L plastic container. After a further 14-d growth, the plants were transferred to a 3.5-L plastic pot (six plants per pot) containing one-fifth Hoagland solution. The plants were grown in a growth chamber at 25°C under a 16-h/8-h light/dark cycle.

For isolation of protoplast, young tobacco leaves were cut into small pieces with a razor and incubated in an enzyme mix solution (1% [w/v] cellulase onozuka RS, 0.3% [w/v] macerozyme R-10, 20 mg MES-KOH, 20 mg CaCl₂, 400 mg mannitol, pH 5.6) with shaking at a slow speed (20 rpm) in the dark at 20°C. After 15 h, the digested leaves were filtered through kimwipe in a funnel into a falcon tube, followed by centrifugation at 100 g for 5 min. After the supernatant was removed, the pellet was resuspended in a 20-mL washing solution (10 mg MES-KOH, 20 mg CaCl₂, 400 mg mannitol, pH 5.6) and washed once with the washing solution. The protoplasts were finally collected by centrifugation at 100 g for 5 min and resuspended in a MaMg solution (10 mg MES-KOH, 30 mg MgCl₂, 400 mg mannitol, pH 5.6; Negruțiu et al., 1987) and used for following transient expression experiment.

DNA Transfer to Protoplasts

For transient assay in tobacco protoplasts, the CaMV 35S minimal promoter (−66 to −46) was used (Fang et al., 1989). GFP was used as a reporter gene. One- or five-repeated coint-acting element fused with 35S minimal promoter was synthesized and incubated for 10 min at 95°C for synthesis of the double-strand DNA fragments. The fragments generated were then inserted into upstream of GFP and the NOS terminator in pBlueScript vector. For construction of a translational ART1 with the ART1 promoter as an effector, genomic fragment containing a 2-kb upstream region and the coding region of ART1 (have no intron) was amplified by PCR from rice (cv ‘Nipponbare’) genomic DNA. Primer pairs used for amplification were

\[ 5'-GAACCTTGGCCCTTGTAACCCG-3' \] and \[ 5'-GAACCTTGGCTTCTGCAGTGTAACCCG-3' \].

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AB357984 (ART1).

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LITERATURE CITED


Identification of the ART1 Cis-Element