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Original Article

In vitro identification of DNA-binding motif for the new zinc finger protein AtYY1

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The functional characterization of novel transcription factors identified by systematic analysis remains a major challenge due to insufficient data to interpret their specific roles in signaling networks. Here we present a DNA-binding sequence discovery method to *in vitro* identify a G-rich, 11-bp DNA-binding motif of a novel potential transcription factor AtYY1, a zinc finger protein in *Arabidopsis*, by using polymerase chain reaction-assisted *in vitro* selection and surface plasmon resonance analysis. Further mutational analysis of the conserved G bases of the potential motif confirmed that AtYY1 specifically bound to these conserved G sites. Additionally, genome-wide target gene analysis revealed that AtYY1 was involved in diverse cellular pathways, including glucose metabolism, photosynthesis, phototropism, and stress response.

Keywords surface plasmon resonance; *in vitro* selection; DNA-binding motif; zinc finger; AtYY1

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Introduction

The systematic differential expression analysis of cell signaling and function, like proteomics and genomics, reveals that numerous new proteins, genes, and even non-coding RNAs are involved in different pathways [1–3]. However, the current information regarding how these new molecules operate in different pathways is still incomplete due to insufficient functional data. Among those newly identified proteins, transcription factors are critical to decipher the signaling pathways of different biological processes due to their important roles in converting environmental signals to gene expression regulation.

The *Arabidopsis* Yin Yang 1 (AtYY1, At4g06634.1) was newly identified in our laboratory as a plant C2H2-type zinc finger transcription factor with both transcriptional repression and activation domains (unpublished data). AtYY1 has four consecutive C2H2-type zinc finger domains and another separate C2H2-type zinc finger domain without a plant-specific QALGGH motif. AtYY1 is different from typical plant C2H2-type zinc finger proteins, which are known to have one or several zinc finger domains with a unique plant-specific QALGGH motif separated by a long spacer. The uniqueness of AtYY1 may indicate its interesting evolutionary origin in *Arabidopsis* and its important role. An expression pattern analysis of *AtYY1* demonstrated that it was inhibited by the dark and induced after salt stress (unpublished data). However, studies of how AtYY1 was involved in light regulation and salt stress and of the other signaling pathways in which AtYY1 may be involved were inconclusive. Identification of the core DNA-binding sequences of AtYY1 would shed new light on its possible functions and related pathways.

Conventional methods of core DNA-binding sequence identification mainly employ polymerase chain reaction (PCR)-based selection and gel shift assay to determine and verify the DNA-binding sequence recognized by transcription factors or DNA-binding proteins [4–10]. With these methods, core DNA-binding sequences were validated by the specific interaction of radio-labeled DNA fragments with the protein of interest. Nevertheless, the low efficiency of these classical methods is inadequate for the high-throughput demand of systematic functional analysis of newly identified transcription factors or DNA-binding proteins. Moreover, the radioactivity involved in these methods limits their applications in a regular laboratory due to expense and regulatory concerns.

Here a non-radioactive DNA-binding motif discovery method was developed to identify and characterize the core DNA-binding motif of a potential light-regulated transcription regulator of *Arabidopsis*, AtYY1. The possible signaling pathway of AtYY1 was then revealed by genome-wide target gene analysis (see **Fig. 1** for experimental strategy). The selection and amplification-binding assay (SAAB) [11] was employed to systematically select and sequence DNA oligos with high affinity for the novel zinc finger protein AtYY1

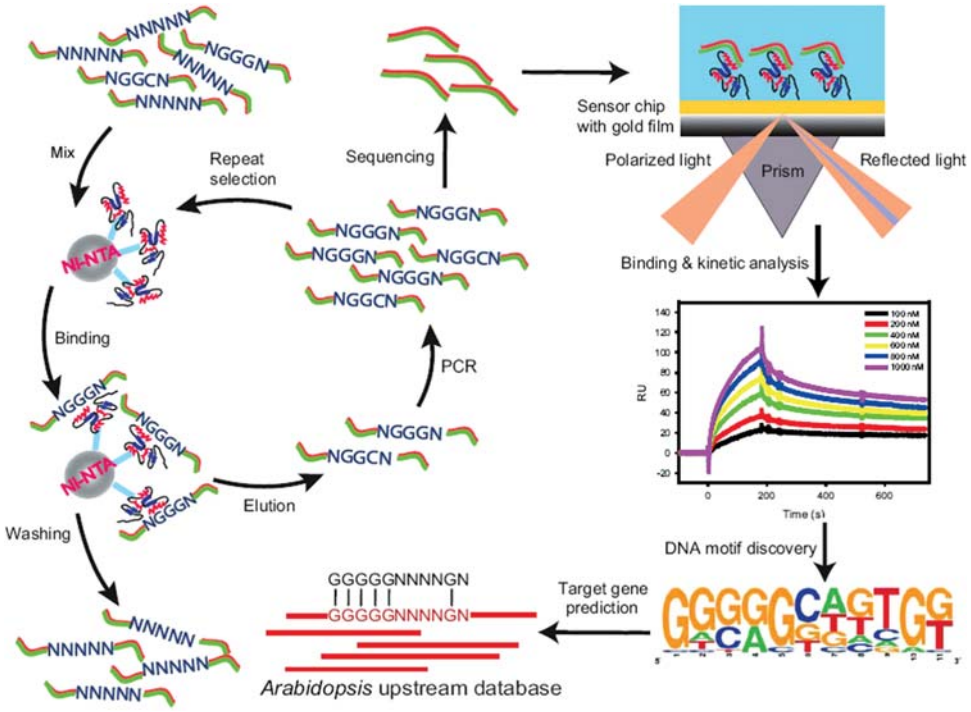

Figure 1 DNA motif discovery process A random DNA oligo library was chemically synthesized and applied to the AtYY1-immobilized Ni-NTA column for six rounds of repeated binding DNA selection. Unbound DNA was washed away, and the bound DNA was eluted and amplified for further selection. The final selected DNA was submitted to sequencing and resynthesized for AtYY1 binding validation using Biacore systems (GE Healthcare). Based on the AtYY1 binding and kinetic analyses, an AtYY1 DNA-binding motif was discovered by MEME and STAMP and further authenticated by mutational analysis of these conserved sequences. Target gene analysis was performed by finding an exact match of the AtYY1-binding sequences in the *Arabidopsis* upstream database.

from a random DNA library. These DNA oligos were chemically synthesized for further validation of the specific interaction with AtYY1 by surface plasmon resonance (SPR) using Biacore systems (GE Healthcare, Buckinghamshire, UK). The AtYY1-binding DNA motif was discovered by motif searching among these validated DNA oligo sequences using a two-motif search package, MEME [12] and STAMP [13]. The AtYY1-binding specificity of the core-binding sequence and its mutants was also evaluated by Biacore systems. Finally, target genes and the possible function of AtYY1 were predicted from search results of the AtYY1 DNA-binding motif in the *Arabidopsis* upstream sequence database.

Materials and Methods

Recombinant AtYY1 production and purification

The cDNA of AtYY1 was amplified by reverse transcription (RT)-PCR from total RNA of 2-week-old *Arabidopsis* seedlings. The forward and reverse primers were 5′-CGGAATTCATGGATCATCAAAATTATCAATACC-3′ and 5′-CAGGTCGACCTAATCTTCATACTCGGTC-3′, respectively. The underlined GAATTC and GTCGAC sequences are the *Eco*RI and *Sal*I recognition sites, respectively. The *Eco*RI–*Sal*I RT-PCR fragment was inserted into the pET-32a(+) vector (Novagen, Madison, USA). *Escherichia coli* BL21(DE3) cells were transformed with pET32a-AtYY1, grown in Luria–Bertani medium at 37°C until the absorbance at 600 nm (A_{600}) reached 0.6, and then induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3–5 h at 25°C.

The lysis buffer contained 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 20 mM imidazole, 1 mg/ml lysozyme, and 1 mM phenylmethanesulfonyl fluoride. The lysate was collected by centrifugation at 4°C and 12,000 *g* for 15 min after three cycles of a 3-s sonication with a 5-s interval. The lysate was filtered through a 0.45-μm filter and applied to an immobilized metal ion affinity chromatography (IMAC) column (Ni-NTA Superflow; Qiagen, Hilden, Germany) equilibrated with lysis buffer. The column was first washed with 10 column volumes (CV) of lysis buffer, followed by 10 CV of washing buffer (50 mM NaH_2PO_4, pH 8.0, 1 M NaCl, 40 mM imidazole, 5 mM β-mercaptoethanol, 0.2% Tween-20, and 5% ethanol) for a more stringent wash. Purified AtYY1 was eluted from the washed column with 10 CV of elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 150 mM imidazole) and stored at −80°C in 100-μl aliquots.

SAAB assay

A total of 100 μl of a 50% suspension of Ni-NTA resin was added to a 1.5-ml Nanosep centrifugal tube and rinsed three

times with 400 μl of ultra-pure water. A total of 80 μl of the His-tagged AtYY1 elution fraction from the previous section was diluted with 520 μl of ultra-pure water and applied to the Ni-NTA resin. The unbound fraction was removed by centrifugation at 100 *g* and 4°C for 1 min. Before binding to DNA, the immobilized His-tagged AtYY1 was rinsed twice with phosphate-buffered saline (PBS; pH 7.4). The AtYY1-bound Ni-NTA column was then equilibrated with binding buffer [10 mM Tris-HCl, pH 7.5, 75 mM NaCl, 6% glycerol, 1% NP-40, and 10 μM $ZnCl_2$ supplemented with 1 mg/ml bovine serum albumin (BSA) and 30 μg/ml poly dI-dC before use].

A random DNA library was prepared by chemically synthesizing the single-strand DNA GAGAGGATCCAGTCA GCATG-N19-CTCAGCCTCGAGAATTCGACA from Integrated DNA Technologies (Coralville, USA), in which N19 is a random 19-mer DNA fragment, and enzymatically extending it to create double strands by using the Klenow fragment (New England BioLabs, London, UK) according to the manufacturer's instructions. The Klenow extension products were further extracted with phenol/chloroform/isoamyl alcohol (25/24/1) and precipitated with 2.5 volumes of ethanol and 1/10 of a volume of 3 M sodium acetate.

Resolubilized random DNA (15 μl) was diluted in 100 μl of binding buffer and then applied on PBS-equilibrated Ni-NTA resin and incubated at 4°C for 30 min. Next, 400 μl of washing buffer I (15 mM Tris-HCl, pH 7.5, 75 mM NaCl, 0.15% Triton-X100, and 1% BSA) and washing buffer II (15 mM Tris-HCl, pH 7.5, 75 mM NaCl, 0.15% Triton-X100) were each applied twice to the AtYY1–DNA complex-bound Ni-NTA column. Finally, the Ni-NTA resin was resuspended in 50 μl of ultra-pure water after washing with 400 μl of ultra-pure water and heated in boiling water for 10 min to elute any DNA bound by the immobilized AtYY1. The eluted DNA solution was removed from the beads by centrifugation at 4°C and 10,000 *g* for 1 min and was ready for PCR amplification.

Fifteen microliters of eluted DNA was amplified with 16 cycles of PCR (5 min at 94°C; 3 cycles of 20 s at 96°C, 1 min at 52°C, 1.5 min at 72°C; 13 cycles of 10 s at 96°C, 30 s at 52°C, 1 min at 72°C; 10 s at 72°C) using forward primer ARP-F (5′-GAGAGGATCCAGTCAGCATG-3′) and reverse primer ARP-R (5′-TGTCGAATTCTCGA GGCTGAG-3′). The PCR products (15 μl) were subjected to a second SAAB selection. The SAAB selection was repeated six times. The final SAAB-selected DNA population was cloned into the pMD19-T simple vector (Takara, Dalian, China) and sequenced.

SPR binding and kinetic assay

The CM5 chip (GE Healthcare, Buckinghamshire, UK) for the immobilization of AtYY1 was activated according to the procedure provided by the manufacturer. Twenty microliters of AtYY1 (50 μg/ml) was diluted in 180 μl of 10 mM sodium acetate (pH 4.0) and then applied using the Biacore X100 (GE Healthcare). Unreacted amine groups on the CM5 chip were blocked with 1 M ethanolamine. Each selected DNA was chemically synthesized and subjected to binding analysis in a volume of 300 μl and at a concentration of 1 μM. For kinetic analyses, the DNA was further diluted to 0.8, 0.6, 0.4, 0.2, and 0.1 μM. The CM5 chip was regenerated with 1 M NaCl, 0.2 M guanidine HCl, and 20% ethylene glycol. The SPR data analysis was performed with built-in Biacore software. Four DNA sequences including the metal-responsive elements (MRE and MRE-S) [14,15], the CAAT box (CAAT) [16–18], and the Myb core sequence (MYB) [19] were used as negative controls. Their sequences were listed in **Supplementary Table S1**.

DNA motif search and AtYY1 target gene analysis

The sequences of all SAAB selected DNA were submitted to the motif searching software package MEME suite [12] to find conserved motifs. Sequence data with weights or without weights were subjected to the MEME suite in which the weights were calculated by normalization of the AtYY1-binding capacity of each DNA. The sequence logo of the DNA motif was drawn by Weblogo [20]. Different DNA motifs were aligned to find their conserved pattern using STAMP [13].

Mutational analysis was based on the conserved pattern generated by STAMP, and all conserved G bases were mutated to A or T. As a protein negative control, His-tag alone was expressed, purified, and immobilized on the CM5 chip using the same protocol of AtYY1 purification and immobilization. To find the possible target gene of AtYY1, a DNA motif and its corresponding region in the individual SAAB-selected DNA were submitted to FIMO analysis [12] to locate the matching sequence fragment in the *Arabidopsis* upstream sequence database. Functional annotation of the individual target gene was extracted from gene records in the NCBI protein database.

Results

PCR-assisted *in vitro* selection of AtYY1-binding DNA

Recombinant His-tagged AtYY1 was successfully expressed in *E. coli* BL21(DE3) cells from a pET32a construct. Soluble, recombinant AtYY1 was extracted from BL21(DE3) cells after induction with 0.8 mM IPTG and then purified with a Ni-NTA column to >80% purity as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. IMAC-purified AtYY1 was then immobilized again on the Ni-NTA column to select DNA sequences that specifically bound AtYY1. Finally, 40

DNA sequences were selected as AtYY1-binding DNA candidates from a 19-bp random DNA library; their AtYY1-binding capacities were further validated by SPR (**Supplementary Table S2** and **Fig. 2**). Thirty-three of the 40 DNA sequences had significant, specific binding with AtYY1, and 18 of the 33 DNA sequences had at least a 2-fold greater binding capacity to AtYY1 in comparison with the negative control DNAs (**Fig. 2** and **Supplementary Figure S1**).

To confirm the binding specificity between AtYY1 and the selected DNA, six DNA sequences with the highest binding capacities were chosen for further evaluation by kinetic analysis using the Biacore X100. All six DNAs showed concentration-dependent association and dissociation curves, which indicated specific interactions between AtYY1 and all six DNAs (**Fig. 3** and **Supplementary Fig. S2**). The kinetic parameter-like dissociation constant K_d was calculated using the built-in curve fitting function of the Biacore evaluation software, and the K_ds of all six DNAs were $\sim 10^{-7}$ M, which was comparable to the existing K_d between protein and DNA. Additionally, the association and dissociation rates of all six DNAs were similar to each other.

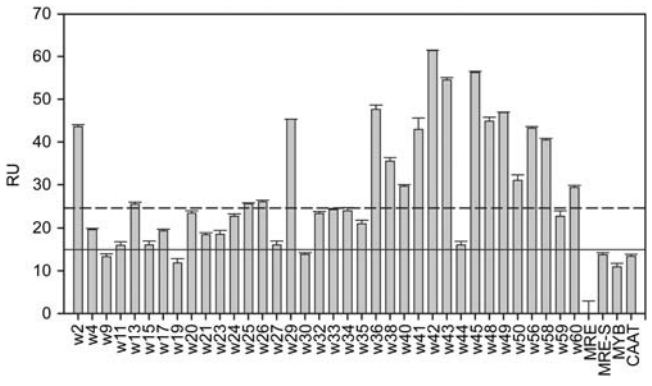

Figure 2 Validation of the SAAB-selected DNA by SPR The AtYY1-binding capacity was calculated from the average of the highest SPR binding value (RU) of three independent repeats. The MRE, MRE-S, MYB and CAAT negative controls were unrelated DNAs recognized by other transcription factors. The bar height above the solid line indicates that the AtYY1-binding capacity of the DNA was significantly greater than the control ($P < 0.01$). The bar height above the dotted line indicates that the AtYY1-binding capacity was 2 folds greater than the negative controls.

AtYY1 DNA-binding motif discovery

Based on these validation results, 36 DNA sequences with significant AtYY1-binding capacity were submitted to the DNA motif searching software package MEME. The most likely DNA motif in these DNAs was a G-rich, 11-bp motif [**Fig. 4(A–C)**]. To increase the quality of the motif search result, 22 DNAs with the AtYY1-binding capacity that was at least 2 folds greater than the control DNA were used for the second motif search. The most likely DNA motif in these 22 DNAs was still the G-rich, 11-bp motif. In addition, we used a weighted motif search method in MEME to optimize the DNA motif search result. The weight of each DNA sequence was calculated by normalization of the AtYY1-binding capacity of different DNAs. When weights are used, the contribution of the AtYY1-binding DNA sequences to the DNA motif search will be proportional to their weights. The DNA motif from the weighted alignment between 33 DNAs with significant AtYY1-binding capacity was still the G-rich, 11-bp motif (**Supplementary Figure S3**). If we increased the weight cut-off value from 0.2 to 0.7, there were 12 groups of DNA with an increased weight distribution and a decreased number of DNA in the group (**Supplementary Figure S3**). The conserved DNA motif in different DNA groups was similar, and the consensus sequence pattern among these motifs that were computed from STAMP was still the G-rich, 11-bp motif with conserved Gs at 1–5 and 10 positions [**Fig. 4(C)**]. In comparison, there were several zinc finger DNA-binding motifs that had the G-rich sequence pattern in the STAMP motif database including the DNA-binding motifs of zinc finger protein MZF1_1-4 and MZF1_5-13 [21].

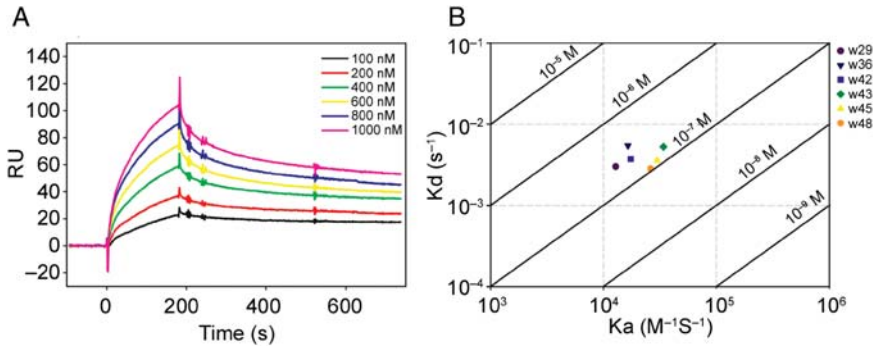

Figure 3 Kinetic analysis of DNA binding by AtYY1 (A) SPR kinetic analysis of W29 binding by AtYY1. The different concentrations of W29 were shown in the inset. (B) Kinetic parameters of six DNAs with the highest AtYY1-binding capacity. The diagonal lines at different positions indicate the measurement of the dissociation constant, K_d.

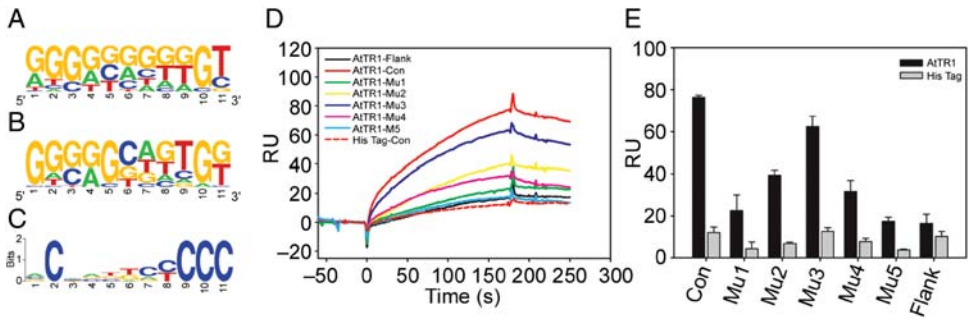

Figure 4 DNA binding motif discovery and mutational analysis of AtYY1 (A,B) The 11-bp DNA motif generated by MEME with 36 DNAs that are significantly bound by AtYY1 and 22 DNAs that are bound at least 2 fold greater by AtYY1. The sequence logos were created by Weblogo [20] using the base frequency at different positions. (C) DNA motif comparison results from STAMP. The height of different bases indicates the degree of conservation of the base at each position. (D,E) Mutational analysis of the conserved DNA motif. AtYY1 and the His-tag alone were immobilized in different channels on a CM5 chip in the Biacore 3000 system. All DNA concentrations were 1 μM. The binding between different mutants and AtYY1 or the His-tag is shown as a solid line of different colors or a dotted line. Additionally, the AtYY1 and His-tag binding capacity to the conserved DNA sequence and its mutants is shown in (E). Con, control; Mu1-5, mutants 1-5; flank, no DNA addition.

Mutational analysis of the putative AtYY1-binding motif

Subsequently, we used SPR to confirm that the specific interaction between these selected DNAs and AtYY1 was mediated by the proposed G-rich, 11-bp DNA motif. The most frequent sequence of GGGGGCAGTGG in the proposed DNA motif from 22 DNAs with at least a 2-fold increase in the AtYY1-binding capacity was considered the most probable consensus AtYY1-binding core sequence. The striking differences in the AtYY1-binding capacity of this DNA sequence and its mutants at different positions of the conserved G residues [**Fig. 4(D,E)** and **Supplementary Table S3**] demonstrated that these conserved Gs in the postulated DNA motif significantly contributed to the specific binding. In addition, the affinity tag at the N-terminus of AtYY1 showed no significant binding capacity for the DNA motif and its mutations. Thus, the specific AtYY1-binding capacity of SAAB-selected DNA was not significantly mediated by the His-tag but by AtYY1 itself.

Genome-wide target gene analysis of AtYY1

Next, we used the consensus sequence of GGGGGCAGTGG to search for the exact matching fragment in the *Arabidopsis* upstream database of FIMO [12]. The FIMO result showed a dentin sialophosphoprotein-related gene with an unknown function whose expression product was predicted to be located in the chloroplast; it had the same sequence in its upstream sequence. These search results could not provide sufficient information to suggest the function of AtYY1. If the MEME-predicted AtYY1 potential binding sequences of the six DNAs with the highest binding capacity were used to search for matching sequences in the *Arabidopsis* upstream database, there were 19 genes with a perfectly matched upstream sequence (**Supplementary Table S4**). These genes are known to be involved in several pathways including photosynthesis, phototropism, protein phosphorylation, ubiquitin-mediated protein degradation, saccharide metabolism, transcription, and salt stress. These predictions were also consistent with our lab's unpublished data indicating that AtYY1 was induced in photosynthesis and salt stress.

Discussion

Here we present a non-radioactive method to identify and verify the DNA-binding motif of the novel potential transcription factor AtYY1, which belongs to the C2H2-type zinc finger protein family in *Arabidopsis*. The DNA-binding motif of AtYY1 that was selected and validated *in vitro* is important for dissecting the biological function of this uncharacterized zinc finger protein. AtYY1 is a unique C2H2-type zinc finger protein in *Arabidopsis* that lacks a QALGGH motif in the zinc finger region, which is similar to the C2H2-type zinc finger from animals. This uniqueness of AtYY1 could indicate its distinct role in *Arabidopsis*. A BLAST analysis of the zinc finger domain of AtYY1 also revealed its high level of conservation with the YY1 family. YY1 is a ubiquitous C2H2-type zinc finger protein that functions both as a transcriptional activator and repressor that is involved in diverse biological processes [22,23].

Our lab's unpublished data also indicated that AtYY1 plays roles in several biological processes including a light-related pathway and stress response, and it could be a potential transcriptional repressor or activator. This observation is supported by the functional annotation of AtYY1 in the Conserved Domain Database [24], which shows that AtYY1 has a putative transcriptional repressor domain at the C terminus that regulates the G2/M transition. It was also reported that a phosphorylation modification at S284

in AtYY1 was identified after phosphoproteomic screening of *Arabidopsis* [25]. Although the function of this serine phosphorylation was not explored in that report, the phosphorylation of C2H2-type zinc finger proteins usually play an additional regulatory role in DNA-binding activity or the ability to interact with binding partners [26,27]. Combining the expression pattern data and function similarity search, we could conclude that AtYY1 could be involved in various pathways as a potent transcriptional activator or repressor. However, the limited characterization of AtYY1 still restricts our understanding of its exact function in *Arabidopsis*. *In vitro* selection of the DNA-binding motif of an unknown protein such as AtYY1 could provide a meaningful starting point to dissect the biological function of these proteins.

The G-rich zinc finger protein DNA-binding motif can also be found in other C2H2-type zinc finger proteins like MZF1, which is preferentially expressed in differentiating myeloid cells and regulates transcription during hemopoietic development [21,28]. MZF1 has 13 zinc finger domains; domains 1–4 preferentially bind to NGGGGA and domains 5–13 preferentially bind to NKAGGGGNA. Interestingly, domains 1–8 of MZF1 also have a highly similar amino acid sequence to the zinc finger domain of AtYY1.

In conclusion, we have selected the *in vitro* DNA-binding motif, an 11-bp, G-rich sequence of GGGGGNNNNGN, of recombinant AtYY1 using SAAB and validated the binding capacity of DNA containing this motif. Mutations of the conserved G in this motif largely decrease or diminish the protein-binding ability. A genome-wide target gene analysis based on this motif also reveals several potential AtYY1-regulated genes involved in photosynthesis, phototropism, salt stress, and other processes. These results provide functional insights into the DNA-binding properties of AtYY1 as a novel transcriptional factor. Our future work will focus on the confirmation of the binding motif and potential downstream genes of AtYY1 *in vivo* by using chromatin immunoprecipitation method.

Supplementary Data

Supplementary data are available at *ABBS* online.

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