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Comprehensive analysis of *AHL* homologous genes encoding AT-hook motif nuclear localized protein in rice

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The AT-hook motif is a small DNA-binding protein motif that has been found in the high mobility group of non-histone chromosomal proteins. The *Arabidopsis* genome contains 29 genes encoding the AT-hook motif DNA-binding protein (AHL). Recent studies of *Arabidopsis* genes (*AtAHLs*) have revealed that they might play diverse functional roles during plant growth and development. In this report, we mined 20 *AHL* genes (*OsAHLs*) from the rice genome database using *AtAHL* genes as queries and characterized their molecular features. A phylogenetic tree revealed that OsAHL proteins can be classified into 2 evolutionary clades. Tissue expression pattern analysis revealed that all of the *OsAHL* genes might be functionally expressed genes with 3 distinct expression patterns. Nuclear localization analysis using transgenic *Arabidopsis* showed that several OsAHL proteins are exclusively localized in the nucleus, indicating that they may act as architectural transcription factors to regulate expression of their target genes during plant growth and development. [BMB reports 2011; 44(10): 680-685]

INTRODUCTION

Most biological processes including cell division, metabolism, homeostasis, and responses to developmental and environmental changes are regulated at the level of gene transcription. Several positive or negative transcription factors that recognize specific DNA sequences on the promoters of target genes play key roles in gene expression regulation. To orchestrate the complex biological processes involved, most transcription factors interact with other sequence-specific DNA-binding proteins, chromatin remodeling and modification complexes, and the general transcription machinery (1).

The AT-hook motif is a small DNA-binding protein motif that was first described in the high mobility group (HMG) non-histone chromosomal protein HMG-I/Y (2). The HMG is a group of chromosomal proteins that help with transcription, replication, recombination, and DNA repair (3, 4). There are 3 distinct classes of HMG proteins: HMGA (formerly HMG-I/Y), HMGB (formerly HMG-1/2), and HMGN (formerly HMG-14/17). Plants contain genes belonging to the HMGA and HMGB families, whereas HMGN proteins so far have been found exclusively in vertebrates (4). Since the first discovery of the AT-hook DNA-binding motif in HMGA, this motif has been found in many nuclear DNA-binding proteins including the histone H1 domain, homeodomain, SET domain, and both PHD and zinc finger proteins, from a wide range of eukaryotes. AT-hooks have an invariant core peptide motif of Arg-Gly-Arg-Pro (i.e., R-G-R-P) flanked on either side by basic amino acid residues (Arg and Lys) and repetitive Pro residues (5). The HMGA proteins bind, via the AT hooks, to the minor groove of stretches of AT-rich DNA, but recognize particular DNA structures rather than the nucleotide sequence itself. In contrast, common transcription factors with DNA-binding activity such as homeodomain, leucine zipper, and helix-loop-helix primarily bind to the major groove of duplex DNA (5, 6).

HMGA proteins containing the AT-hook DNA-binding motif not only play a functional role as nuclear architecture by binding to the nuclear matrix or scaffold, but also play a role in transcription regulation by acting as accessory factors influencing the association of transcription factors with chromatin during the formation of an enhanceosome on a promoter and/or enhancer regions of target genes (4, 6-9). Based on the bioinformatic analysis of AT-hook motif DNA-binding proteins from various eukaryotes, Aravind and Landsman (1998) also proposed that AT-hook motif proteins may play a role in translocating regulatory chromosomal proteins to scaffold-associated regions (SARs) or matrix-associated regions (MARs). The mammalian HMGA non-histone proteins participate in a wide variety of cellular processes, including regulation of inducible gene transcription, integration of retroviruses into chromo-

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somes, the induction of neoplastic transformation, and promotion of metastatic progression of cancer cells. Diverse mammalian and viral genes were regulated in either a positive or negative fashion by the HMGA proteins *in vivo* (3, 6).

Accumulating evidence also reveals that plant AT-hook DNA-binding proteins play crucial roles in governing growth, development, and differentiation, such as gibberellin (GA) homeostasis (10), jasmonate signaling (11), leaf longevity (12), photomorphogenesis (13, 14), flowering (14) and reproductive organ patterning and differentiation (15). There is further evidence that AT-hook proteins regulate expression levels of target genes by binding to the *cis*-element on the target promoters (10, 11, 15, 16). These results suggest that AT-hook DNA-binding proteins play diverse functional roles via transcriptional regulation of target genes during the plant life cycle. Due to sequence redundancy among AT-hook DNA-binding protein genes, often no visible phenotypes are observed from single loss-of-function mutants, whereas multiple mutants from the genetic crossings among genes sharing sequence homology can show obvious abnormalities as compared to wild-type or single mutants (13, 14). However, ectopic overexpression of individual *AHL* genes often has a severe effect on plant growth and development (12-15).

The *Arabidopsis* genome contains 29 different genes encoding AT-hook motif nuclear-localized proteins (AHL) (9, 10). Among them, the functional roles of several *AHL* genes have been explored by using loss-of-function and/or gain-of-function mutants (12-15). Several genes containing the AT-hook motif also have been identified from rice (16, 17), however, their specific functions have not yet been studied. Here, we report identification of 20 different *AHL* genes (hereafter *OsAHL*) from the rice genome database by using *Arabidopsis AHL* genes (hereafter *AtAHL*) as queries. We analyzed their expression patterns; various tissues and the responses of etiolated seedlings to light exposure. A phylogenetic tree revealed that OsAHL proteins can be classified into 2 distinct groups, as previously reported in *Arabidopsis*. Nuclear localization of several OsAHL proteins was also determined in the transgenic *Arabidopsis*.

RESULTS AND DISCUSSION

The rice genome contains 20 different *AHL* homologous genes encoding AT-hook motif nuclear localized protein

Since identification and characterization of the first *Arabidopsis* gene (*AtAHL*) that encodes the AT-hook motif DNA-binding protein and belongs to the HMGA group (18, 19), a total of 29 *AtAHL* genes have been identified from its genomic sequence (9, 10). As an initial step toward understanding the functional roles of *AtAHL* homologous genes in rice (*OsAHL*), we mined *OsAHL* genes from the rice genome database using *AtAHL* genes as queries. As a result, we identified 20 *OsAHL* genes and generated a tree to reveal the phylogenetic relationship among the rice genes, including *AtAHL* genes (Fig. 1). The phylogenetic tree revealed that *OsAHL* genes can be classified into 2 major evolutionary clades consisting of groups A and B, as previously described in *Arabidopsis* (9, 10). Groups A and B contain 11 and 9 *OsAHL* genes, respectively. Up to now, functional studies were performed on several *AtAHL* genes, all belonging to group B, such as *AGF1/AHL25*, *AGF2/AHL15* (10), *ESC/ORE7/AHL27* (12), *GIANT KILLER/AHL21* (15), *SOB3/AHL29* (13), and *AHL22* (14). *AGF1/AtAHL25* and *AGF2/AtAHL15* play a role in GA homeostasis by negatively regulating a GA biosynthesis gene *AtGA3ox1* encoding GA 3-oxidase (10). *OsAHL3* and Os*AHL7* are closely related to the *AGF*

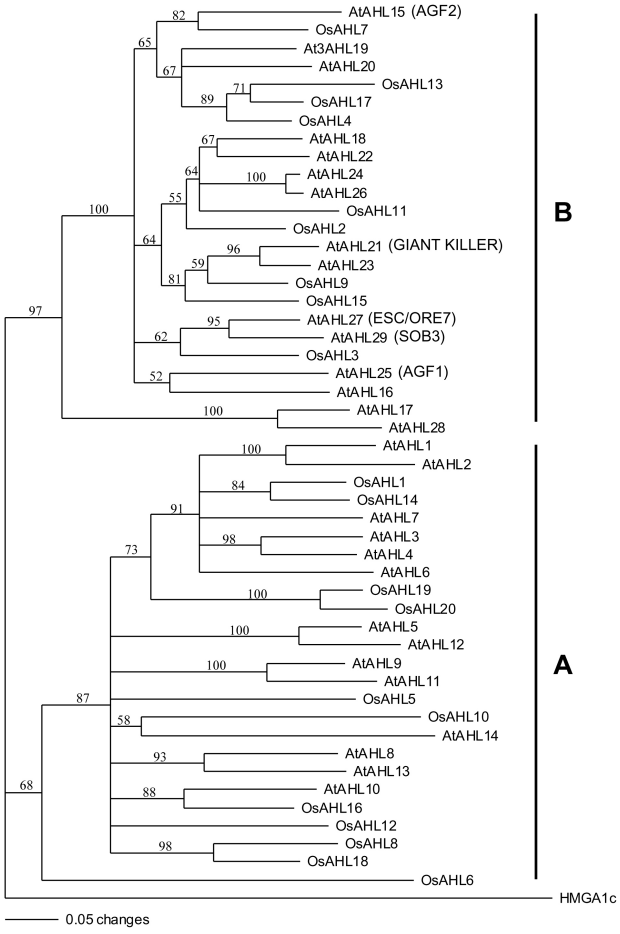


Fig. 1. A phylogenetic tree of AT-hook DNA-binding proteins from *Arabidopsis* and rice. Relationship of OsAHL and AtAHL amino acid sequences was inferred from neighbor joining (NJ) analysis using the mean character difference. All indel regions in the aligned amino acid sequences were excluded from the NJ analysis. Supporting values for tree nodes were estimated with 1,000 bootstrap replicates. The numbers above the tree branches indicate bootstrap values. Human HMGA1c encoded by *HMGA1* (GenBank accession No. AF176039; 6) was used as an outgroup. The NJ analysis revealed 2 major clades (clades A and B) in the OsAHL and AtAHL amino acid sequences. The clade B was well grouped with high bootstrap value (97%), whereas the clade A was supported with relatively low bootstrap value.

genes (Fig. 1), indicating that those rice genes are likely to be functional orthologs of *Arabidopsis* counterparts. Overexpression of *GIANT KILLER/AHL21* in *Arabidopsis* resulted in pleiotropic defects in reproductive organ patterning and differentiation (15). It would be interesting to test whether the biological function has been conserved between *GIANT KILLER/AHL21* and its rice homolog *OsAHL*9 (Fig. 1). Due to sequence redundancy among *AtAHL* genes, a single loss-of-function mutation was not sufficient to obtain phenotypical changes as a clue to its function (14). Therefore, the phylogenetic tree will be informative in the functional analysis of Os*AHL* genes on the basis of sequence redundancy (Fig. 1).

Informed by our phylogenetic relationship analysis, we performed multiple sequence alignment for the group A and B proteins. AT-hook DNA-binding proteins contain at least an AT-hook motif and an unknown PPC (plants and prokaryotes conserved) domain that includes a hydrophobic region and was described as DUF296 (domain of unknown function) in InterProScan (URL http://www.ebi.ac.uk/Tools/pfa/iprscan/). Proteins with PPC domains are found in bacteria, Archaea, and plants, but not in animals. However, the hydrophobic PPC domain-containing proteins in bacteria and Archaea have no AT-hook motif (9). Most OsAHLs belonging to group A have 2 AT-hook motifs containing the invariant Gly-Arg-Pro core flanked by Arg and Pro amino acid residues (Supplementary Fig. 1). However, OsAHL6, -19, and -20 contain a single AT-hook motif, missing the motif on either the first (OsAHL19 and -20) or the second position (OsAHL6). The middle and C-terminal parts containing a PPC-like domain were highly conserved among the A group proteins. In case of OsAHL6, the C-terminal region was highly conserved, whereas the middle part close to the hydrophobic region had 6 amino acid residues of deletion and was also fairly variable compared to other A group proteins. Due to the sequence variations observed in the AT-hook motif and the middle part of OsAHL6, it may be diverged from the remaining proteins belonging to A group (Fig. 1). The hydrophobic region in the PPC domain is known to be indispensable for nuclear localization (9). OsAHL proteins belonging to group B, except for OsAHL13, have a single AT-hook motif in their whole peptide (Supplementary Fig. 2). The hydrophobic amino acid-rich region was also identified from the C-terminal part of the group B proteins. The overall sequence homology in the PPC domain was low between group A and group B, although short stretches of amino acid residues were conserved between the groups. Although a functional role of the PPC domain is not apparent at present, it may play a role in DNA-protein or protein-protein interactions in the nucleus during chromatin packaging and remodeling, and enhanceosome formation (4, 6).

Subcellular localization of OsAHL proteins in transgenic *Arabidopsis*

Plant AT-hook DNA-binding proteins have been reported to be localized in the nucleus (8-10, 12, 13, 20). AHL1, an *Arabidopsis* AT-hook DNA-binding protein for which the subcellular localization has been characterized in detail, was mainly localized in the nucleoplasm and was concentrated in the boundary region between euchromatin and heterochromatin during interphase. An *in vitro* DNA binding assay further revealed that AHL1 binds to MAR (matrix attachment region) and the AT-rich sequence through the AT-hook motif, indicating that AHL1 may play a role in the positioning of chromatin fibers in the nucleus as a MAR binding protein. In addition, AHL1 was specifically localized on the chromosomal surface throughout mitosis (9).

Up to now, subcellular localization of rice AT-hook DNA-binding proteins has not been reported. To examine the subcellular localization of OsAHL proteins and to gain insight into their functions as DNA-binding proteins, we determined their subcellular localization in transgenic *Arabidopsis*. To do this, we cloned 4 genes (2 of each from groups A and B; Fig. 1) -OsAHL3, OsAHL5, OsAHL8 and OsAHL9 - into the binary vector pCAMBIA1303 (URL http://www.cambia.org) to make OsAHL-GUS-GFP fusion proteins. OsAHL5 and -8 contain a typical motif for their nuclear localization, 119 (101 for OsAHL8)-KKKRGRPRK-127 (109 for OsAHL8). OsAHL3 and -9 also contain a putative nuclear localization motif, 98 (82 for OsAHL9)-RRPR-102 (85 for OsAHL9). The PSORT program (URL http://psort.hgc.jp/) predicted a high possibility that OsAHL5 and -8 could be localized in the nucleus.

We analyzed the subcellular localization of OsAHL proteins using *Arabidopsis* seedlings at T_1 generation. To determine the nuclear location, we stained the root or leaf tissues with DAPI.

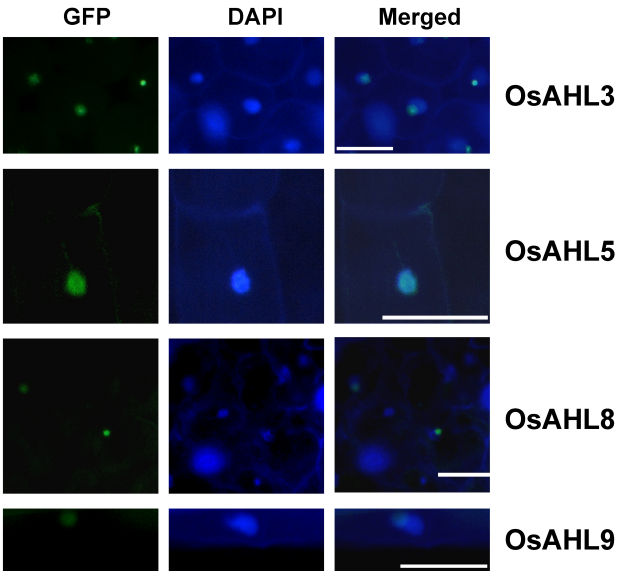


Fig. 2. Subcellular localization of OsAHL-GUS-GFP fusion proteins in transgenic Arabidopsis seedlings. After DAPI staining of whole seedlings, the GFP image was obtained using fluorescence microscopy and then DAPI and GFP images were merged. Scale bar = 50 μm.

In all cases, OsAHL-GFP proteins were exclusively localized in the nucleus with different nuclear localization patterns among the OsAHL proteins investigated (Fig. 2). GFP signals for either OsAHL5 or OsAHL8 were ubiquitously detected throughout the nucleus, whereas OsAHL3 and OsAHL9 were concentrated in a specific location of the nucleus (Fig. 2). AtAHL1 protein with *in vitro* MAR binding activity was concentrated in the boundary region between euchromatin and heterochromatin during the interphase (9). Therefore, our data imply that all OsAHLs analyzed here may serve specific functions in the nucleus as architectural transcriptional regulators (5)

Gene expression pattern of OsAHL genes in various rice tissues

Most genes encoding AT-hook DNA-binding proteins were expressed ubiquitously in the plant tissues analyzed, although the expression levels often varied significantly among different tissues (10, 14, 18, 21, 22). In plants and animals, HMGA genes are often highly expressed in the proliferative state of the cells and tissues or during embryonic development (6, 18, 22). A maize HMGA gene showed a high expression level during endoreduplication in developing endosperm cells (23). To obtain insight into the functional roles of *OsAHL* genes during growth and development of rice, we analyzed their gene expression patterns in various rice tissues. Comparison of gene expression pattern data among different rice tissues may provide useful information to consider functional redundancy, along with sequence similarity data. Total RNA, isolated from immature seeds, flowers, flag leaves, mature leaves, stems, seedling shoots, and seedling roots was subjected to RT-PCR analysis. The majority of *OsAHL* genes were expressed ubiquitously in all tissues investigated (Fig. 3) with significant variation among different tissues, as reported in other plant species (18, 19, 21, 22). Among them, *OsAHL2, -3, -9, -15*, and *-17* showed much higher expression levels in roots than in other plant tissues. Meanwhile, *OsAHL4* and -11 were specifically expressed in roots (Fig. 3). Based on oligonucleotide microarray technology, diverse databases for rice gene expression profiling are available, including Genevestigator, Rice Expression Profile Database, and Gene Expression Atlas in RiceGE (24). Our tissue expression data for *OsAHL* genes were also quite comparable to the results from the microarray-based gene expression profiling data (Supplementary Fig. 3). Taken together, from our tissue expression data (Fig. 3) and *in silico* analyses using the gene expression profile databases, we estimate that 20 OsAHL proteins are likely encoded by expressed and functional genes in the rice genome.

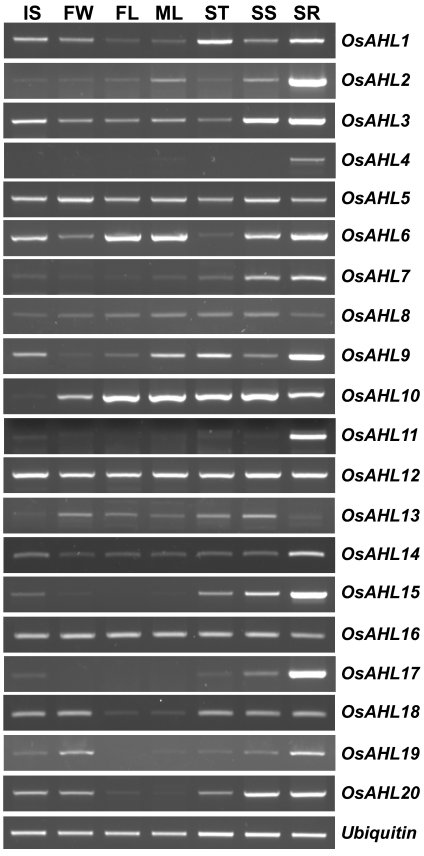


Fig. 3. Expression pattern of AT-hook DNA-binding protein genes in various tissues of rice. Total RNA was isolated from immature seeds (IS), flower (FW), flag leaf (FL), mature leaf (ML), stem (ST), seedling shoot (SS) and seedling root (SR) and used for RT-PCR analysis. Ubiquitin was used as an internal control for PCR.

Expression patterns of *OsAHL* genes in response to light exposure of etiolated seedlings

Light is a key environmental factor governing plant growth and development through light quantity/quality perception and downstream signaling. According to an *in vitro* analysis, a putative AT-hook DNA-binding protein from rice interacted with a functionally defined AT-rich element in the oat phytochrome A3 gene promoter (16). To examine the effect of light on transcriptional change in *OsAHL* genes, etiolated seedlings grown for 7 days under dark condition were exposed to light condition for 12 h, and then transferred to dark condition for 24 h. We performed semi-quantitative RT-PCR using total RNA purified from the samples and repeated the RT-PCR experiments using independent samples. However, light had no effect on transcriptional change for all *OsAHL* genes under our experimental conditions (Supplementary Fig. 4), indicating that change in expression of *OsAHL* genes caused by light might be regulated at post-transcriptional and/or post-translational levels. Post-translational modifications of HMGA proteins regulate their biochemical functions in higher eukaryotes including mammals and plants. *AGF1/AHL25* is known to be necessary for negative feedback of GA biosynthesis. However, its transcriptional level was not affected by treatment with either GA or uniconazole P (10). HMGA proteins can be phosphorylated by several protein kinases, such as CDC2 kinase and mi-

togen-activated protein kinases, and can also be acetylated and methylated (3, 4, 6, 20).

MATERIALS AND METHODS

Plant materials and experimental conditions

A rice cultivar (*Oryza sativa cv.* Dongjin) was used for this series of experimental procedures. The surface-sterilized rice seeds were germinated on agar-solidified 1 × MS media and grown for 7 days in a growth chamber (25°C, 16 h light/8 h dark cycle). The seedlings were transplanted to soil and further grown in the greenhouse during the summer season. Total RNA was isolated from various tissues at the 7-day-old seedling stage (seedling shoot and seedling root), heading stage (flag leaf, mature leaf, and stem), and flowering stage (flower and immature seed at 10 days after flowering). To examine changes of gene expression in etiolated seedlings upon light exposure, 7-day-old etiolated seedlings were exposed to light conditions for 12 h and then transferred to dark conditions for 24 h.

Sequence analysis

Sequence information for genes encoding AT-hook motif nuclear localized proteins (*AHL*) of *Arabidopsis* was obtained from The *Arabidopsis* Information Resource (TAIR) (TAIR9 version; URL http://www.*Arabidopsis*.org/). To identify *AHL* homologues in rice, we used *Arabidopsis AHL* genes as queries for the BLASTN and BLASTX searches against NCBI nr database, Knowledge-based Oryza Molecular biological Encyclopedia (KOME) (URL http://cdna01.dna.affrc.go.jp/cDNA/), and Rice Genome Annotation Project database (URL http://rice.plantbiology.msu.edu/). We analyzed the sequences with the ExPASy Molecular Biology Server (URL http://kr.expasy.org), the BLAST program (25), and BOXSHADE (version 3.2; URL http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic analysis was performed with the Molecular Evolutionary Genetics Analysis (MEGA) software (version 4.0.; 26), and a phylogenetic tree was inferred by the neighbor-joining tree method. The resulting tree topology was evaluated in bootstrap analyses based on 1,000 replicates. Multiple sequence alignment was performed using BLOSUM as a weight matrix, neighbor joining as a clustering method. Values for open (or end) gap penalty and extending (or separation) gap penalty were 10 and 0.05, respectively.

Isolation of total RNA from rice tissues and semiquantitative reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA was isolated from rice seedlings or tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The isolated total RNA was directly used for RT-PCR analysis after treatment with RQ1 RNase-free DNase I (Promega, Madison, WI, USA). We used the purified total RNA (5 μg) for first strand cDNA synthesis with Moloney Murine Leukemia Virus-reverse transcriptase (Invitrogen). The conditions for PCR amplification were as follows: 96°C, 5 min for initial denaturation followed by 94°C for 15 sec, 55°C for 30 sec, and 72°C for 1 min (total 25-35 cycles) with 10 min of final extension at 72°C. The primers for semi-quantitative RT-PCR are listed in Supplementary Table 1.

Subcellular localization of rice AHL proteins in transgenic *Arabidopsis*

The primers used in this study for plasmid construction are listed in Supplementary Table 2. *Bg*/II and *Spe*I restriction sites were introduced into the primers for easy cloning of the PCR products. We obtained cDNA through PCR reactions using PrimeSTAR DNA polymerase (Takara, Otsu, Japan) and a pair of primers. The PCR products were subcloned into the pTOP TA V2 vector (Enzynomics, Daejon, Korea) after A-tailing. The complete nucleotide sequences of the PCR products were determined to check for PCR error. The restriction fragment of the PCR product was subcloned into the *Bg*/II/*Spe*I site of pCAMBIA1303 binary vector (CAMBIA, Canberra, Australia), resulting in translational fusion with the GUS-GFP reporter. The constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation and introduced into *Arabidopsis* ecotype Col-0 plants using the floral dip method (27). Transgenic plants were selected on MS plates containing hygromycin (40 μg/ml) and subjected to GFP fluorescence detection using a fluorescence microscope (Carl Zeiss, DE/Axio Imager A1). DAPI (4,6-diamidino-2-phenylindole, dihydrochloride) was used to stain nuclei. For DAPI staining of the nuclei, the transgenic seedlings were submerged into 1 μg/ml DAPI and 0.2% (v/v) Triton X-100 for 1 h, and then rinsed with water.

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