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An R2R3-type transcription factor gene *AtMYB59* regulates root growth and cell cycle progression in *Arabidopsis*

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MYB proteins play important roles in eukaryotic organisms. In plants, the R1R2R3-type MYB proteins function in cell cycle control. However, whether the R2R3-type MYB protein is also involved in the cell division process remains unknown. Here, we report that an R2R3-type transcription factor gene, *AtMYB59*, is involved in the regulation of cell cycle progression and root growth. The AtMYB59 protein is localized in the nuclei of onion epidermal cells and has transactivation activity. Expression of *AtMYB59* in yeast cells suppresses cell proliferation, and the transformants have more nuclei and higher aneuploid DNA content with longer cells. Mutation in the conserved domain of AtMYB59 abolishes its effects on yeast cell growth. In synchronized *Arabidopsis* cell suspensions, the *AtMYB59* gene is specifically expressed in the S phase during cell cycle progression. Expression and promoter-GUS analysis reveals that the *AtMYB59* gene is abundantly expressed in roots. Transgenic plants overexpressing *AtMYB59* have shorter roots compared with wild-type plants (*Arabidopsis* accession Col-0), and around half of the mitotic cells in root tips are at metaphase. Conversely, the null mutant *myb59-1* has longer roots and fewer mitotic cells at metaphase than Col, suggesting that *AtMYB59* may inhibit root growth by extending the metaphase of mitotic cells. AtMYB59 regulates many downstream genes, including the *CYCB1;1* gene, probably through binding to MYB-responsive elements. These results support a role for *AtMYB59* in cell cycle regulation and plant root growth.

Keywords: MYB protein, transcription factor, cell cycle, root growth

Introduction

MYB proteins are characterized by a DNA-binding MYB domain located in the N-terminus. The MYB domain usually contains one, two or three conserved motifs of around 50 amino acids. These repeated motifs have a helix-helix-turn-helix conformation and may facilitate interaction with the target DNA. The first *MYB* gene identified in plants was C1 from maize, which is involved in anthocyanin biosynthesis [1]. Since then,

several *MYB* genes have been isolated [2-5]. Recently, Chen *et al*. [6] found 198 *MYB* genes from *Arabidopsis* and 183 from rice. Plant MYB proteins can be classified into three types: the R2R3-type MYB, with two repeats; the R1R2R3-type MYB, with three repeats; and a third type, usually containing a single repeat or an atypical repeat. Among the MYB proteins from *Arabidopsis*, 126 are R2R3-type and 5 are R1R2R3-type. The remaining 67 are MYB-related or atypical types [6].

MYB proteins play important roles in multiple aspects of plant growth and development [2-5]. Several MYB proteins may be involved in secondary metabolism [7-9]. AtMYB0/GL1 is required for leaf trichome differentiation [10]. AtMYB66/WER plays roles in epidermal cell patterning [11]. *MYB* genes also have functions in lateral meristem initiation [12]. All the above functions are known from studying R2R3-type MYB proteins. Plant R1R2R3-type MYB proteins have also been studied,

and they may play roles in regulating cell cycle progression, similar to the roles played by the three vertebrate MYB proteins with three repeats [2, 13, 14]. The unusual MYB protein AtMYBCDC5 may also be involved in cell cycle control [15]. However, whether the R2R3-type MYB proteins are involved in cell cycle regulation is not known.

Cell cycle progression is important for cell proliferation and for plant growth and development. In general terms, the cell cycle is divided into four phases, including G1, S (DNA replication), G2 and M (mitosis). G1 and G2 are two phases during which the cell determines whether it will go into the following S phase or M phase. The mechanism for cell cycle control is highly conserved among different organisms, and the major regulators of the plant cell cycle have been identified, including cyclin-dependent kinases (CDKs), cyclins, CDK-inhibitory proteins, the WEE kinase and components of the RB/E2F/DP pathway [16-18]. In *Arabidopsis*, two classes of CDKs and 49 cyclins have been identified. A-type cyclins are important for the S-G2-M phase control, and B-type cyclins may regulate the G2 to M transition. D-type cyclins may control the G1 to S transition [16]. CDKs and cyclins can form kinase complexes, which phosphorylate various substrates, leading to the progression of the cell cycle. All the components of the cell cycle progression may be regulated at various levels and by different developmental or environmental factors [17, 18]. Plant hormones, including auxin, cytokinin, brassinosteroids, gibberellin and abscisic acid, also affect the cell cycle and cell division [18].

Previously, we analyzed the ethylene receptor-regulated genes using a microarray approach, and identified one *MYB* gene [19]. In the present study, we investigated the function of this R2R3-type transcription factor gene, *AtMYB59*. We found that this gene affects cell growth by influencing DNA replication and cell division in yeast cells and regulates root development by altering the progression of mitosis in the root tip cells. AtMYB59 may function through regulation of its downstream target genes.

Results

AtMYB59 gene expression, protein localization and transcriptional activation

AtMYB59 (At5g59780) was identified during a microarray analysis for ethylene receptor-regulated genes [19] and was investigated further. It encodes an R2R3-type MYB protein of 236 amino acids. The two repeats are located at the N-terminus from amino acids 9 to 59 and 62 to 110. The *AtMYB59* gene is highly expressed in roots but is barely detectable in stems, leaves, flowers and young siliques (Figure 1A). To further examine the specific expression pattern of *AtMYB59* in plants, the 1.6 kb promoter region of the gene was used to drive expression of the *GUS* gene in a plant expression vector, pCAMBIA1301. The transgenic plants exhibited GUS activity in hypocotyls and root systems, including root hairs, vascular tissue and the root tip meristem region (Figure 1B). GUS staining was also observed in some points along the leaf edge and the pedicel supporting the silique (Figure 1B, upper middle and upper right). These results indicate that *AtMYB59* is abundantly expressed in roots. It may also be detected in other organs at different developmental stages.
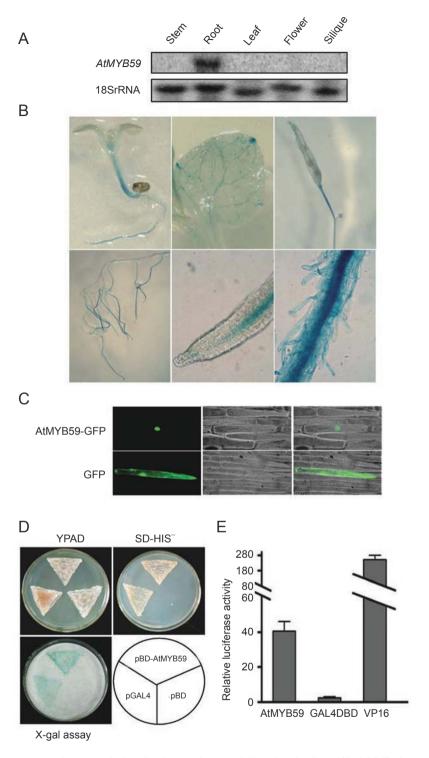
The subcellular localization of the AtMYB59 protein was studied. Figure 1C shows that the AtMYB59-GFP protein is localized in the nuclei of onion epidermal cells, whereas the GFP control protein is observed in the cytoplasm. This result indicates that AtMYB59 is a nuclear protein.

The transcriptional activation ability of the AtMYB59 protein was investigated using a yeast assay system. The AtMYB59 coding region was fused to the GAL4 DNA-binding domain to generate pBD-AtMYB59. This plasmid, together with the positive control pGAL4 and the negative control pBD vector, was transformed into the yeast strain YRG-2, and the growth status of the transformants was examined. Figure 1D shows that all the transformants grew well on the YPAD normal medium. However, only the positive control pGAL4 and the pBD-AtMYB59 transformants were able to grow on the selection medium SD-HIS$^-$ (Figure 1D, upper right panel). In the X-gal assay, the yeast cells harboring pGAL4 and pBD-AtMYB59 turned blue (Figure 1D, lower left panel). These results indicate that AtMYB59 can activate the expression of the *HIS3* and *LacZ* marker genes in the yeast cell, implying that AtMYB59 has transcriptional activation activity.

The transcriptional activation ability of AtMYB59 was also examined in an *Arabidopsis* protoplast system (Figure 1E). The effector AtMYB59 protein had moderate transactivation activity in activating the reporter *LUC* gene, whereas the positive control effector VP16 had very high activity. The negative control GAL4BD protein had no transcriptional activation ability. These results indicate that AtMYB59 has transcriptional activation activity in plant cells.

AtMYB59 affects cell division of yeast cells

Yeast is a model system for studying gene function, and MYB-related proteins have been found in yeast cells [20]. We investigated the function of AtMYB59 using

Figure 1 *AtMYB59* gene expression, protein localization and transcriptional activation. **(A)** *AtMYB59* expression in different organs of *Arabidopsis* plants. **(B)** GUS staining of transgenic plants harboring the *GUS* reporter gene driven by the 1.6 kb promoter of the *AtMYB59* gene. **(C)** Subcellular localization of the AtMYB59-GFP protein in onion epidermal cells. The *GFP* plasmid was transformed as a control. The photographs were taken in the dark for green fluorescence (left), in bright light for morphology (middle) and in combination (right). **(D)** Transactivation activity of AtMYB59 in the yeast assay system. Transformants harboring pBD-AtMYB59, the positive control pGAL4 or the negative control pBD vector were streaked onto YPAD (upper left) or SD-HIS⁻ (upper right) for growth examination. The *LacZ* marker gene was examined by X-gal assay (lower left). **(E)** Transactivation ability of AtMYB59 in *Arabidopsis* protoplasts. VP16 was used as a positive control. The GAL4 DNA-binding domain (GAL4DBD) was used as a negative control. The *LUC* gene was used as a reporter.

the yeast system. The *AtMYB59* gene in the pESPM-AtMYB59 plasmid was driven by the NMT promoter and can be activated in the absence of thiamine (–VB1) (Figure 2A). Under inductive conditions (–VB1) on a plate, the proliferation of the transformants harboring the *AtMYB59* gene was severely inhibited in comparison with the transformants harboring the control vector (Figure 2B, 2C). Under normal non-inductive conditions (+VB1), both the transformants containing the *AtMYB59* gene and those containing the vector grew well. The yeast transformants under inductive conditions were also stained with 4,6-diamidino-2-phenylindole (DAPI) and observed under a fluorescence microscope. The results in Figure 2D show that, whereas the yeast cells containing the vector had a normal phenotype, the yeast cells containing the *AtMYB59* gene exhibited a significant change in morphology. The latter cells were ~threefold longer than the vector-transformed cells (Figure 2D, 2E, right panel), and more than 20% of the *AtMYB59*-transformed cells had double nuclei or abnormal nuclei (Figure 2E, left panel). AtMYB59 was further mutated at four highly conserved amino acid positions (S48G, R50S, R52S, W53G), and the mutated AtMYB59 showed no significant effects on yeast cell growth compared with the pESPM vector control (Figure 2F). All these results indicate that the *AtMYB59* gene specifically suppresses cell proliferation by affecting cell division and cell growth.

Because the *AtMYB59*-transformed cells showed defects in cell nuclei distribution, we further measured the DNA content in these cells using flow cytometry. Figure 3A (upper panel) shows that normal vector-transformed cells had two peaks in DNA content during the cell cycle. The first peak represents cells at the G2 phase, whereas the second peak indicates cells that have divided but have not yet separated from each other. Therefore, the cells in the second peak have twice the DNA content of a single cell but remain at the G2 phase. These features did not change during the culture. However, the *AtMYB59*-transformants showed significant alterations in DNA content during cell division (Figure 3A, lower panel). Several peaks at different DNA contents were observed in addition to the normal peak, suggesting the presence of cells with aneuploid DNA content (Figure 3A, lower panel). During progression of the culture, more than 70% of the *AtMYB59*-transformed cells showed variation in DNA content (Figure 3B, left panel). An increasing number of cells undergoing apoptosis were also observed in the *AtMYB59*-containing yeast cells but not in control cells (Figure 3A, lower panel; 3B, right panel). These results further indicate that the expression of AtMYB59 in yeast cells disturbs DNA synthesis and/or chromosome separation and hence affects the cell division process.

AtMYB59 is specifically expressed in the S phase of the cell cycle

Because AtMYB59 affects the cell division process, we examined its gene expression during cell cycle progression using a suspension culture. Several cell cycle marker genes were also examined. Figure 4 shows that *CYCD3;1* gene expression starts during the S phase and extends to the G2/M phase; *CDKA;1* is constantly expressed during the cell cycle; *CDKB1;1* is abundantly expressed in the S to G2 phases; and the *Histone H4* gene is highly expressed in the G1 to S phases. The expression patterns of these marker genes are consistent with those previously reported [21]. Compared with the marker genes, *AtMYB59* was strongly expressed in the S or S to G2 phases (Figure 4). In the G2 to M phases and the G1 phase, the *AtMYB59* gene was only weakly expressed. The higher expression of the *AtMYB59* gene in the S phase suggests that AtMYB59 may play roles in this phase or the following phase.

AtMYB59 suppresses root growth and affects cell cycle progression

To study the *AtMYB59* gene function *in vivo*, an expression vector harboring the 35S-driven *AtMYB59* gene was transformed into *Arabidopsis* plants. Two homozygous lines with higher *AtMYB59* expression, 3 and 11, were selected for further analysis (Figure 5A). Two T-DNA insertion mutants, *myb59-1* and *myb59-2*, were also identified; they had insertion sites in the third exon and the 3′-untranslated region, respectively (Figure 5B). As shown in Figure 5B, *myb59-1* is a knockout mutant, while *myb59-2* is a knockdown mutant. Seedlings of the two overexpressing lines L3 and L11, *myb59-1*, *myb59-2*, and *Arabidopsis* accession Col-0 (Col) were compared. Figure 5C and D show that the two *AtMYB59*-overexpressing lines had shorter roots and that *myb59-1* had longer primary roots when compared with Col. However, *myb59-2* did not show a significant difference in root growth. The aerial parts appeared not to have significant differences among these compared plants. The cross-sections of leaves from these plants were also examined, and no significant difference was found (Supplementary information, Figure S1B). These results indicate that At-MYB59 may inhibit root growth.

Short roots in the *AtMYB59*-overexpressing plants may result from altered cell size in the root. However, when cross-sections of the roots in the elongation zone were examined, no significant difference in root cell structure or size was observed among the *AtMYB59*-overexpressing plants, the mutants and Col (Supplementary information, Figure S1A). Confocal microscopic observation did not reveal significant differences in root
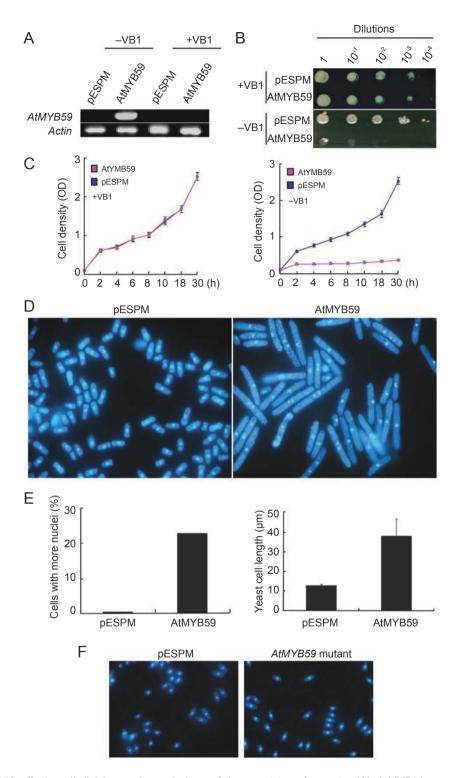
Figure 2 AtMYB59 affects cell division and morphology of the yeast transformants. **(A)** *AtMYB59* expression in pESPM-AtMYB59 transformants (AtMYB59) with (+VB1) or without VB1 (–VB1). The transformants (pESPM) harboring the pESPM vectors were used as a control. **(B)** Dilution analysis of the yeast transformants harboring pESPM-AtMYB59 or the pESPM vector with (+VB1) or without VB1 (–VB1) on EMM plates. **(C)** Growth of the yeast transformants harboring pESPM-AtMYB59 or the pESPM vector in EMM liquid culture with (+VB1) or without VB1 (–VB1). Optical density at 595 nm was measured. **(D)** Phenotypic comparison of the yeast transformants containing pESPM-AtMYB59 or the pESPM vector in the absence of VB1. The cells were stained with DAPI. **(E)** Comparison of cell length and proportions of the cells with more nuclei. The cells from **(D)** were examined. **(F)** Effects of the *AtMYB59* mutant gene on yeast transformants in the absence of VB1.
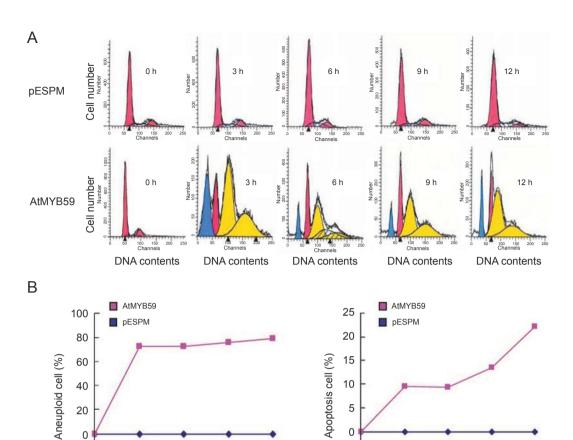
Figure 3 Flow cytometric analysis of the yeast transformants harboring pESPM-AtMYB59. **(A)** Comparison of the flow cyto-metric profiles of the transformants harboring pESPM-AtMYB59 or the pESPM vector. Red peaks indicate normal G2 cells. Yellow peaks indicate aneuploid cells. Blue peaks indicate cells in apoptosis. **(B)** Proportions of cells with aneuploid DNA content and in apoptosis. The percentage was derived from **(A)**.
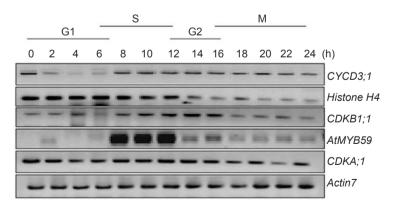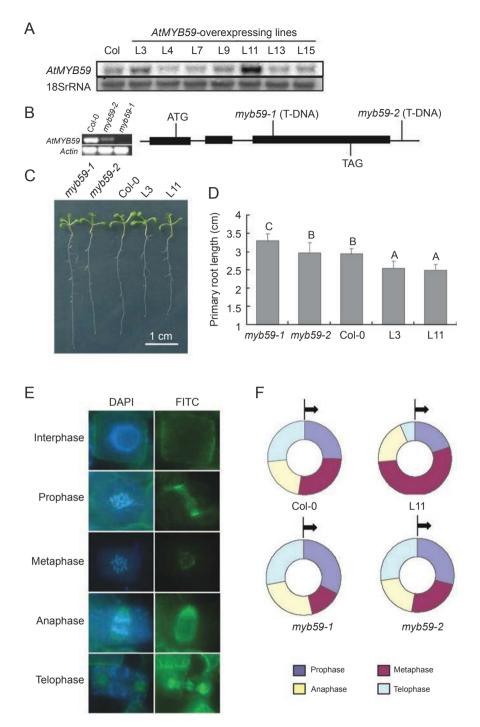

Figure 4 *AtMYB59* expression during cell cycle progression of *Arabidopsis* suspension cultures. RT-PCR was performed to examine the expression of *AtMYB59* and four other marker genes and the control gene *Actin7*. The same images as those in Zhang *et al*. [47] for the marker genes were used. The cell cycle phases (G1, S, G2 and M) are labeled on top of the panel.

Figure 5 Overexpression of *AtMYB59* affects root growth and cell division of root tip cells. **(A)** *AtMYB59* expression in various *AtMYB59* transgenic lines. **(B)** Identification of the *AtMYB59* T-DNA insertion mutants *myb59-1* and *myb59-2*. The left panel shows *AtMYB59* expression in *myb59-1* and *myb59-2*. The right panel shows the T-DNA insertion sites in the *AtMYB59* genomic region. **(C)** Root growth of the *AtMYB59*-transgenic lines L3 and L11, Col, *myb59-1* and *myb59-2*. **(D)** Comparison of root lengths of the two *AtMYB59*-transgenic lines, *myb59-1*, *myb59-2*, and Col plants. Seedlings (Three3-day-old) seedlings were transferred onto plates and grown vertically for 10 days. Bars indicate SD. (n = 20). Different letters above each column indicate significant differences (P < 0.01) between lines according to one-way ANOVA (SPSS 10.0). **(E)** Cell division phases in *Arabidopsis* root tip cells. Chromosome behavior was revealed by DAPI staining. Tubulin distribution was revealed by an FITC immunofluorescence assay. Five phases are shown. **(F)** Proportions of the mitotic cells at each phase in root tips of the *AtMYB59* transgenic line L11, *myb59-1*, *myb59-2*, and Col plants. In total, around 100 mitotic cells were examined for each plant, and the mitotic phase of each cell was identified based on comparison with the phases in **(E)**.

cell length among these plants (data not shown).

Because the *AtMYB59* gene is specifically expressed in the S phase of the cell cycle, affects yeast cell division and inhibits root growth (Figures 2-5), we then examined whether the cell division phase was altered in root tips of the *AtMYB59*-overexpressing plants and of the mutants *myb59-1* and *myb59-2* in comparison with those of Col. Root tips were stained with DAPI and assayed for FITC immunofluorescence to reveal DNA and tubulin distribution, respectively. Four phases of the cells at mitosis can be found, i.e., prophase, metaphase, anaphase and telophase (Figure 5E). A cell at interphase is also shown for comparison (Figure 5E). The phases of the cells at mitosis were identified, and the percentage of cells at each phase was calculated. Figure 5F shows that, in Col root tips, the mitotic cells at each phase were in similar percentages (21%–27%). In the two T-DNA insertion mutants, *myb59-1* had 14% of cells at metaphase and *myb59-2* had 24% at metaphase. These percentages were lower than the 27% at metaphase in Col. However, in the *AtMYB59*-overexpressing line, a very high percentage of cells were at metaphase (54%) and a very low percentage of cells were at telophase (Figure 5F). These results indicate that AtMYB59 changes the behavior of cells at mitosis, possibly through disturbance of metaphase.

AtMYB59 regulates downstream genes and has DNA binding specificity

Because AtMYB59 affected root development and the cell division process, we then performed an Affymetrix genechip analysis using the *AtMYB59*-overexpressing line L11 and Col to identify the genes regulated by this transcription factor. Among the differentially expressed genes, *CYCB1;1* and a *NAM*-like gene (At5g14000) were upregulated in the *AtMYB59*-overexpressing line L11 but were downregulated in the two *myb59* mutants (Figure 6A). The *CYCB1;1* gene mainly functions during the G2 to M transition of the cell cycle [22]. *NAM*-like genes usually play roles in meristem formation and lateral root formation [19, 23]. In addition, the *AtMYB59* gene affected the expression of the cytokinin signal-transduction gene *ARR16* (Figure 6A). These genes, together with another ~150 genes identified from the genechip analysis (Supplementary information, Tables S1 and S2), may contribute to the function of the *AtMYB59* gene in plant cell cycle progression and root growth. It should be noted that elimination of *AtMYB59* expression in *Atmyb59-1* only reduced – it did not abolish – the expression of the three genes, suggesting that AtMYB59 plays a partial role in regulation of these genes.

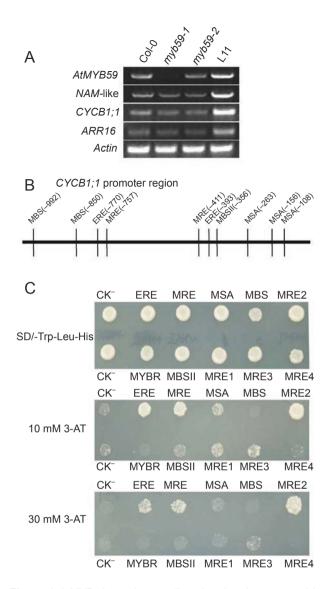Because the *CYCB1;1* gene was regulated by the R2R3-type AtMYB59, we further examined whether

Figure 6 AtMYB59 regulates cell cycle-related genes and has DNA binding specificity. **(A)** Expression of the *NAM*-like gene (At5g14000), *CYCB1;1* and *ARR16* in the *AtMYB59*-transgenic line L11, *myb59-1*, *myb59-2*, and Col plants. RT-PCR was used for examination. **(B)** Potential MYB-binding elements in the promoter region of the *AtMYB59* gene. MSA has the AACGG core sequence. MBSII: TAACTAAC. MRE: AACCaaa. MBS: CAACtg. Two ethylene-responsive elements (ERE: ATTTCAAA) are also indicated. **(C)** DNA binding specificity of the AtMYB59 in a yeast one-hybrid assay. The effector pAD-AtMYB59 and the reporter pHIS2, harboring different *cis*-elements (three tandem repeats) plus the *HIS3* gene, were co-transformed into yeast cells. The growth of the transformants was examined on SD/-Trp-Leu-His or 3-AT plates. The *cis*-element in each transformant is labeled on the top or bottom of each panel. CK⁻ indicates negative control cells harboring pAD-AtMYB59 and the pHIS2 vector without any *cis*-element. MSA: CCCAACGGT. MRE1: CCGGCAGT-TAGGAT. MRE2: TATAACGGTTTTTT. MRE3: TCTAACCTAC-CA. MRE4: TCTCACCTACC. MYBR: TGGTTAG. Other element sequences are the same as those in **(B)**.

the promoter region of *CYCB1;1* has any MYB protein-binding elements and whether AtMYB59 can bind these elements. The 1.6 kb promoter region of the *CYCB1;1* gene was analyzed using the PlantCare program and based on previous studies [24]. Eight cis-elements, including three MSA sequences (AACGG), one MBSII sequence (TAACTAAC), two MRE sequences (AACC) and two MBS sequences (CAAC), were identified as potential MYB protein-binding elements (Figure 6B). Two ethylene-responsive elements (ERE) were also identified (Figure 6B). A yeast one-hybrid assay was used to test whether AtMYB59 can bind to five of these elements (MSA (–156), CCCAACGGT; MBSII (–356), TAACTAAC; ERE (–393 and –770), ATTTCAAA; MRE (–411), AACCAAA; MBS (–850 and –992), CAACTG). Other elements (MYBR, MRE1, MRE2, MRE3 and MRE4) that have previously been identified for other MYB proteins were also examined for AtMYB59 binding. Each of the pHIS2 reporter plasmids harboring different elements plus the reporter gene *HIS3*, together with the effector plasmid pAD-AtMYB59, was transformed into yeast cells. Growth of the transformants on 3-AT indicates activation of the *HIS3* gene and binding of AtMYB59 to the corresponding *cis*-elements. Figure 6C shows that all the transformants grew well on SD/-Trp-Leu-His. However, only the transformants harboring the MRE, ERE and MRE2 (TATAACGGTTTTTT)-controlled reporter gene grew well on 3-AT, suggesting that AtMYB59 binds to these three elements but not to the other elements examined (Figure 6C, middle and lower panels). ERE represents an ethylene-responsive element, and the binding of AtMYB59 to this sequence may indicate that AtMYB59 has multiple regulatory roles by binding to different *cis*-elements in the *CYCB1;1* gene.

Discussion

In vertebrate cells, the MYB proteins regulate cell cycle progression at the G1 to S transition or function by binding telomeres [14, 25, 26]. In tobacco plants, the R1R2R3-type MYB proteins NtmybA1, NtmybA2 and NtmybB play roles in cell cycle progression by binding to *cis*-elements and regulating cyclin and *NACK1* genes [2, 13]. However, the first two proteins appear to be expressed at the G2 to M phase and function as activators in tobacco BY2 cells, whereas the latter protein (NtmybB) may be constantly expressed during the cell cycle and functions as a repressor [13]. The present R2R3-type AtMYB59 also plays roles in cell cycle progression in both yeast cells and plant cells. In yeast cells, AtMYB59 may suppress cell division by affecting DNA synthesis in the S phase, since a large proportion of yeast cells with

aneuploid DNA content were observed. Uneven separation of the replicated chromosomes may also result in the aneuploid DNA content in yeast cells. Some *AtMYB59*-overexpressing cells had two nuclei and appeared to be three times longer than the control cells. These transformed cells may have had normal chromosome separation and nuclei formation, but the two daughter cells could not separate from each other, leading to longer cells. This phenomenon further suggests that the cell division process is altered in *AtMYB59*-transformed yeast cells, probably due to the inability to form a cell plate between the two daughter cells or due to other unknown mechanisms. It is also possible that the change in cell division in *AtMYB59*-transformed yeast cells is due to the interference of AtMYB59 with yeast CDC5, which shows similarity to the c-MYB protein and is involved in cell cycle progression [27].

In plants, AtMYB59 appears to be a functional activator of transcription. It is specifically expressed during the phases S to G2 of the cell cycle in cell suspensions. This timing of expression is slightly later than those of the R1R2R3-type *MYB* genes in animal cells but earlier than that of the two tobacco genes *NtmybA1* and *NtmybA2*, suggesting that AtMYB59 has specific roles in cell cycle progression. In root tips of *AtMYB59*-transgenic plants, a high proportion of mitotic cells were at metaphase, and a low proportion of mitotic cells were at metaphase in *myb59-1*. However, the cell size and other characteristics related to cell division appeared not to be changed (data not shown). It is possible that S/G2 phase expression of the *AtMYB59* gene may shorten the S/G2 phase and then cause a significant increase in the proportion of metaphase cells and the length of metaphase. AtMYB59 may affect cell division through regulation of the *CYCB1;1* gene by direct binding to the DNA elements in its promoter region (Figure 6).

Other *MYB* genes have been found to be expressed at different phases of the cell cycle. Three *MYB* genes have peak expressions at the S phase, whereas five other *MYB* genes are mainly expressed at the M phase [28, 29]. Two other *MYB* genes are expressed at the G1 and G2 phases [29]. Detailed characterization of these genes should determine their roles in cell cycle progression. *AtCDC5*, an *Arabidopsis* homolog of yeast *cdc5*, plays roles in the cell cycle that are similar to those of yeast cdc5 [15, 30]. The AtCDC5 MYB domain has two repeats that are only distantly related to those of the R2R3-type MYB domain [5, 15]. Another unusual MYB protein (At2g03470) has also been identified during characterization of the promoter region of the *Arabidopsis CYCB1;1* gene [31]. This protein can function as a transcription activator and bind to the AACGG element of the *CYCB1;1* gene, sug-

gesting a role in cell cycle regulation [31]. These studies suggest that multiple MYB proteins, including R1R2R3-type, R2R3-type and unusual types, may function during the cell cycle progression of plant cells.

Root growth and development are initiated from the root apical meristem and are regulated by transcription factors [32]. Mutations of SHR and SCR result in shorter roots. SHR may specify endodermal cell fate, whereas SCR mediates the division of the cortex/endodermal initial daughter cell [33, 34]. Another MYB coiled-coil transcription factor gene, *APL*, may be necessary for phloem cell formation [35]. The *AtMYB59* gene studied herein is abundantly expressed in the vascular tissue of roots, consistent with the expression pattern reported by Li *et al*. [36]. Transgenic *Arabidopsis* overexpressing *AtMYB59* exhibited shorter roots, and *myb59-1* had longer roots than Col, indicating that AtMYB59 may inhibit root growth and function as a negative regulator. Because the dividing root tip cells of the *AtMYB59*-overexpressing plants had a high proportion at metaphase and the proportion of cells at each phase may reflect the relative duration of each phase [21], the dividing cells in root tips of *AtMYB59*-transgenic plants may have an extended metaphase. This relative extension of the metaphase of root tip cells during mitotic cell division likely resulted in the slower root growth of the AtMYB59-transgenic plants. The disturbance in cell division may be caused by an alteration in the cyclin gene *CYCB1;1* and in many other genes, since AtMYB59 regulates the expression of these genes (Figure 6A; Supplementary information, Table S1 and S2). Changes in gene expression and/or activity of the cell cycle components have been found to affect root growth and differentiation [22, 37]. The inhibitory effect of AtMYB59 on root growth is consistent with a recent report by Petroni *et al*. [38], who found that another MYB protein (AtMYB11) delays root growth and other developmental processes by reducing the proliferation activity of meristematic cells [38].

Abundant expression of the *AtMYB59* gene in roots correlates with its roles in root growth regulation. *AtMYB59* is also slightly expressed in aerial plant organs. However, no significant phenotypic change was observed in the aerial parts of the overexpressing lines or the mutant lines. The reason for this remains unknown. In the two mutants, the residual *AtMYB59* expression differs, whereas the examined downstream genes seem to be expressed at similar levels. This phenomenon may be due to the fact that AtMYB59 only partially regulates the expression of these genes. However, phenotypic changes in *myb59-1* are well correlated with loss of *AtMYB59* expression, suggesting that other genes regulated by *AtMYB59* are also involved in the process in addition to the tested genes.

Overall, we have demonstrated a role for the R2R3-type MYB protein AtMYB59 in cell division and root growth. Further study should reveal more about the function of AtMYB59 in plants.

Materials and Methods

Plant materials and treatments

Seeds of wild-type *Arabidopsis* (*Arabidopsis thaliana,* ecotype Columbia, Col-0), and transgenic plants or mutants were sowed on MS plate after sterilization, stratified at 4 ℃ for 3 days and germinated at 22 ℃ under continuous illumination. Seedlings (14-day-old) of these plants were harvested for RNA isolation. Wild-type plants (2-month-old) grown in soil were collected for organ-specific expression analysis.

For root growth comparison, 3-day-old seedlings were transferred to plates for vertical growth. The lengths of the primary roots from 20 plants were measured. The experiment was repeated independently three times, and the results were identical. The result from one experiment is presented.

RNA isolation, northern hybridization and RT-PCR analysis

Total RNA isolation and hybridization were carried out according to the description by Zhang *et al*. [39]. *AtMYB59* cDNA probe was synthesized using templates amplified with primers 5′-CCG GTT TAA GGA CAA ACC GGG-3′ and 5′-TCA GAA ATT CCA AAC GCA ATC-3′. After stripping the probes, the same blots were re-hybridized with 18S rDNA gene to examine the RNA loading.

For *AtMYB59*-regulated gene expression analysis, two independent sets of total RNAs were used for cDNA synthesis and then RT-PCR analysis. The results were consistent, and only one set of results was presented. Specific primers for each gene were as follows: for *AtMYB59*, 5′-AAC ATG GGA AAG TTC CTT GG-3′ and 5′-CCA TGT TCA AAA CTT AGA GG-3′; for *NAM*-like (At5g14000), 5′-GAT TTG CGT CGG CTA AGG TGT CT-3′ and 5′-ATC TCA ATT CTT CAT CAT CTC CC-3′; for *CYCB1;1*, 5′-ACT GTG CAA AGC TGT TGG CGT AT-3′ and 5′-TTC GTC CTT GGA GTA TTT CTT TCG T- 3′; for *ARR16*, 5′-TGG AGT ATT TGG GTT TGG GAG AT-3′ and 5′-CCA GGC ATA CAG TAA TCG GTG AT-3′; for *actin7* gene, 5′-GGA CCT GAC TCA TCG TAC TC-3′ and 5′-TAC AGT GTC TGG ATC GGA GG-3′.

Construction of AtMYB59 expression vector and transformation

The coding sequence of *AtMYB59* gene was obtained from *Arabidopsis* by RT-PCR using primers 5′-ATA GAA TTC ATG GAT TAC GAG GCA TCA AG-3′ and 5′-ACT GTC GAC TCA GAA ATT CCA AAC GCA ATC-3′. The PCR product was digested with *Bam*HI and *Sac*I, cloned into the plant expression vector pBI121 and confirmed by sequencing. *AtMYB59* was driven by the CaMV 35S promoter. Plant transformation was performed using the vacuum infiltration method. The transgenic seeds were screened on MS plates containing 50 µg/ml kanamycin and the homozygous T3 seeds were analyzed further.

For promoter-GUS analysis, 1.6 kb promoter region of the *AtMYB59* gene was amplified using specific primers 5′-GCA TGC GAC CAA ACC CAA CTA ATC AC-3′ and 5′-GGA TCC AGA

GAG AGA GAG AGA GAG AG-3′, sequenced and cloned into the *Bam*HI and *Xho*I sites of the plant expression vector pCambia1301 to drive the *GUS* reporter gene expression. This construct was transformed into *Arabidopsis*, and the transgenic plants were selected on hygromycin. GUS staining was performed to examine GUS activity.

Identification of T-DNA insertion mutants for the AtMYB59 gene

Two T-DNA insertion lines (N460129 for *myb59-1* from NASC, European *Arabidopsis* Stock Center, and Salk_044751 for *myb59-2* from ABRC at Ohio State University) for the *AtMYB59* gene were requested and the homozygous plants were identified by PCR. The primers used for screening homozygous mutants *myb59-1* were F: 5′-GAA ATG ACC TGG TTT TGG TTG TAG-3′ and R: 5′-ATG TTG GAG AAG CCA GAG GAG G-3′ for the gene, and pCA161F: 5′-CCG GAC ATG AAG CCA TTT ACA AT-3′ from the T-DNA region. The primers used for the *myb59-2* mutant were LP: 5′-TGG GAA CTG ATC TTT TAT CAT GGA-3′ and RP: 5′-GGC TCA AGA GAA GAA GCG ACC-3′ from the gene, and the primers LBb1: 5′-GCG TGG ACC GCT TGC TGC AAC T-3′ from the T-DNA region. These two mutants were further identified by RT-PCR.

Subcellular localization of AtMYB59-GFP in onion epidermal cells

The AtMYB59 coding region was amplified with primers 5′-ATA GGA TCC ATG GAT TAC GAG GCA TCA AG-3′ and 5′-ATC GTC GAC GAA ATT CCA AAC GCA ATC C-3′, sequenced and fused to the 5′- end of *GFP* in transient expression vector, resulting in the pUC-AtMYB59-GFP construct. The *AtMYB59-GFP* was driven by the CaMV 35S promoter. The pUC-GFP vector, controlled by the 35S promoter, was used as a control. The two constructs were introduced into onion epidermal cells with a Biolistic Particle Delivery System (BioRad). The fluorescence of GFP in onion epidermal cells was visualized under a Confocal microscope (Olympus FV500).

Transcriptional activation activity of the AtMYB59 protein

The coding sequence of *AtMYB59* was obtained by PCR using the primers 5′-ATA GAA TTC ATG GAT TAC GAG GCA TCA AG-3′ and 5′-ACT GTC GAC TCA GAA ATT CCA AAC GCA ATC-3′. The PCR products were cloned into the vector containing the GAL4 DNA-binding domain to obtain pBD-AtMYB59. The pBD-AtMYB59, the positive control pGAL4, and the negative control pBD vector were all transformed into the yeast strain YRG2. The transformed strains were confirmed by PCR, and then streaked on YPAD or SD/HIS⁻ plates. The transcription activation activity of the protein was evaluated according to their growth status [40].

The transcriptional activation ability was also examined in *Arabidopsis* protoplast system according to our previous report [41]. A plasmid containing 5X UAS and 35S promoter upstream of a reporter gene encoding a firefly luciferase (LUC) was used as a reporter. The GAL4 DNA-binding domain (BD)-coding sequence was fused to the transcription factor gene *AtMYB59* and inserted into the pRT107 to generate effector plasmids pRT-BD-AtMYB59. The fusion genes were under the control of 35S promoter. The BD sequence was also fused to VP16 gene to generate positive control effector plasmid. The pRT107 containing the BD sequence was used as negative control. The reporter plasmids and the effector plasmids were transfected through PEG-mediated transfection. A pPTRL plasmid that contained a CaMV 35S promoter and a luciferase gene from *Renilla* was used as an internal control [42]. Luciferase assays were performed with the Promega Dual-luciferase reporter assay system and the GloMax™20/20 Luminometer.

DNA binding specificity of the AtMYB59 in yeast one-hybrid assay

Three copies of each potential *cis*-acting DNA element in tandem repeat, with cohesive ends, were synthesized (Sengon of Shanghai, China), annealed and ligated into the *Sac*I and *Mlu*I sites of the reporter plasmid pHIS2. The putative *cis*-elements in the promoter region of the *Arabidopsis CYCB1;1* gene were predicted using the PlantCARE program. The nucleotide sequences of the two strands of these potential elements or other known MYB-binding elements were as follows: for ERE, 5′-CATTTc aaaATTTcaaaATTTcaaaA-3′ and 5′-CGCGTtttgAAATtttgAAATtttgAAATGAGCT-3′; for MBS, 5′-CCAACtgCAACtgCAACtgA-3′ and 5′-CGCGTcaGTTGcaGTTGcaGTTGGAGCT-3′; for MRE, 5′-CAACCaaaAACCaaaAACCaaaA-3′ and 5′-CGCGTtttGGTTtttGGTTtttGGTTGAGCT-3′; for MSA, 5′-CcccAACGgtcccAACGgtcccAACGgtA-3′ and 5′-CGCGTacCGTTgggacCGTTgggacCGTTgggGAGCT-3′; for MBS II [24], 5′-CTAACTAACTAACTAACTAACTAACA-3′ and 5′-CGCGTGTTAGTTAGTTAGTTAGTTAGTTAGAGCT-3′; for MYBR [43], 5′-CTGGTTAGTGGTTAGTGGTTAGA-3′ and 5′-CGCGTCTAACCACTAACCACTAACCAGAGCT-3′; for MRE1, 5′-CCCGGCAGTTAGGATCCGGCAGTTAGGATCCGGCAGTTAGGATA-3′ and 5′-CGCGTATCCTAACTGCCGGATCCTAACTGCCGGATCCTAACTGCCGGAGCT-3′; for MRE2 [44], 5′-CTATAACGGTTTTTTTATAACGGTTTTTTTATAACGGTTTTTTA-3′ and 5′-CGCGTAAAAAACCGTTATAAAAAAACCGTTATAAAAAAACCGTTATAGAGCT-3′; for MRE3 [45], 5′-CTCTAACCTACCATCTAACCTACCA TCTAACCTACCAA-3′ and 5′-CGCGTTGGTAGGTTAGATGGTAGGTTAGA TGGTAGGTTAGAGAGCT-3′; for MRE4 [46], 5′-CTCTCACCTACCTCTCACCTACC TCTCACCTACCA-3′ and 5′-CGCGTGGTAGGTGAGAGGTAGGTGAGA GGTAGGTGAGAGAGCT-3′; the underlined sequences represent the cohesive ends after annealing. These DNA elements were located upstream the nutritional reporter gene *HIS3*. The sequence of the inserts was confirmed by sequencing. The *AtMYB59* gene was cloned into the plasmid pAD-GAL4-2.1 and the effector plasmid pAD-AtMYB59 was made. The effector and the reporter plasmids were transformed into yeast cells and the transformants were examined for their growth on SD/-Trp-Leu-His plate or this plate plus 3-AT based on the instruction of the BD Matchmaker™ Library Construction & Screening Kits. The pAD-AtMYB59 construct and the pHIS2 vector were co-transformed into yeast cells (Y187) as a negative control.

Affymetrix Genechip analysis

Two biological replicates were used for the analysis. Total RNAs from 12-day-old seedlings of the *AtMYB59*-transgenic line L11 and the wild-type Col plants were used for cDNA probe synthesis and Affymetrix Genechip hybridization analysis. Genechips (Affymetrix ATH1-121501) were used and the experiments were

performed by the Shanghai Biochip Co Ltd. The genes with at least twofold difference in expression between the *AtMYB59*-overexpressing plants and the Col are listed in Supplementary information, Table S1 for upregulated genes and in Supplementary information, Table S2 for downregulated genes. Several genes were further examined by RT-PCR method.

Yeast transformation, growth and observation

The coding region of the *AtMYB59* was inserted in expression vector pESPM and the pESPM-AtMYB59 was constructed. This recombinant plasmid and the pESPM vector were transformed into the fission yeast Leu⁻ strain SPQ-01 by lithium acetate-mediated method. Cells were selected on EMM agar plates with thiamine at 30 ºC. The transformant was cultured in liquid EMM medium to mid-exponential phase with thiamine at 30 ºC. The cells were washed three times with the EMM only to remove the thiamine and to de-repress the NMT1 promoter that drives the *AtMYB59* gene, and then cultured at 30 ºC for 22 h. The growth of the yeast cells was examined by measuring the optical density of the suspensions or observed under a light microscope. The transformed mid-exponential yeast cells were streaked onto the plates with or without thiamine, with different dilutions, and incubated at 30 ºC. The grown of the yeast cells was observed after 60 h. The yeast cells harboring the pESPM vector were used as a control.

Expression of the *AtMYB59* gene in the transformed yeast cells was examined by RT-PCR using primers 5′-CCG CTC GAG ATG AAA CTT GTG CAA GAA G-3′ and 5′-CGG GAT CCC TAA AGG CGA CCA CTA CCA TG-3′. Yeast *actin* gene was examined using primers 5′-CAG CCA CTT TCT CAA ATC AG-3′ and 5′-GCC AAG GGT CAC TAC AC-3′.

AtMYB59 mutation was generated in the peptide RTGK-SCRLRWVNYL at positions S48G, R50S, R52S and W53G. Two primers, *Sal*I-MYB59: 5′-GTC GAC ATG AAA CTT GTG CAA GA-3′ and mutmyb59-1: 5′-AAC CCC ACT TAA ACT GCA ACC CTT-3′, were used to amplify the 5′-part of the mutant gene. Two primers, *Bam*HI-MYB59: 5′-GGA TCC CTA AAG GCG ACC ACT A-3′ and mutmyb59-2: 5′-AAG GGT TGC AGT TTA AGT GGG GT-3′, were used to amplify the 3′-part of the mutant gene. The two fragments harboring the mutation sites were used for PCR amplification with the *Sal*I-MYB59 and *Bam*HI-MYB59 primers to obtain the full-length of the mutant gene. The mutant gene was further cloned into pESPM vector to test the mutation effects in yeast transformants.

The yeast cells were stained with DAPI (1 mg/ml) to reveal the DNA and examined under an Olympus fluorescence microscope.

Flow cytometry analysis

The yeast cells harboring the pESPM-AtMYB59 or the pESPM vector were grown at 30 ºC for 12 h in EMM medium containing thiamine. The cultures were washed six times with the EMM medium without thiamine to de-repress the NMT1 promoter, and diluted to less than 5×10^6 cells/ml in EMM, and then incubated at 30 ºC. Samples were taken at 0, 3, 6 and 9 h and analyzed with a FACS Caliber cytometer (B-D Corporation, USA).

Staining and observation of cell division phase in Arabidopsis root tip cells

Whole seedlings grown on MS plates for 4 days were fixed in PEM buffer (50 mM Pipes, 5 mM $MgCl_2$, 5 mM EGTA, pH 6.9)

containing 4% paraformaldehyde for 1 h. After washing with PEM buffer, the samples were digested with 1% cellulase plus 0.1% pectase for 5 min. The root tips were cut and cells were spread out by pressing cover glasses gently. The materials were extracted with PEM buffer containing 0.1% Triton X-100 for 30 min. After washing, the materials were further incubated with PBSA containing 0.1% BSA for 10 min, and then incubated with mouse anti-tubulin antibody (Sigma T4026) (1:1 000 dilution) at 4 ºC overnight. The materials were incubated with horse anti-mouse antibody (1:500 dilution) labeled by FITC at 37 ºC for 2 h, and then stained with DAPI. The cells were observed under an Olympus fluorescence microscope, and the cell division phase was identified and counted.

Synchronization of cell suspensions and RT-PCR analysis of marker genes

Arabidopsis cell suspension was synchronized following the description by Zhang *et al.* [47]. RT-PCR analysis and primers of the cell cycle marker genes were based on the descriptions, and the experiments were performed at the same time and the same images were used [47]. For *AtMYB59*, the 5′- and 3′-coding sequences were used as primers for amplification.

Acknowledgments

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)