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Original Paper

ATL54, a ubiquitin ligase gene related to secondary cell wall formation, is transcriptionally regulated by MYB46

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Abstract We previously characterized Arabidopsis Tóxicos en Levadura54 (ATL54), a ubiquitin ligase associated with secondary cell wall formation. The *ATL54* gene is co-expressed with secondary wall-associated genes, and the knock-out of *ATL54* up-regulates the expression of cellulose, lignin, and xylan biosynthetic genes in apical stem portions of four-week-old plants. Here, we report the tissue-level localization patterns and the regulation of *ATL54* expression. The *β-glucuronidase* (*GUS*) reporter gene driven by the *ATL54* promoter was significantly expressed in interfascicular fibers, xylary fibers, and vessels in inflorescence stems. A transient transfection assay using *Arabidopsis* T87 cells showed that the expression of the firefly luciferase gene driven by the *ATL54* promoter was activated by MYB46, which is a key transcriptional activator of secondary wall formation. In addition, the electrophoretic mobility of *ATL54* promoter fragments was shifted by a recombinant MYB46 protein. These results indicate that *ATL54* expression is regulated by MYB46, and support the view that ATL54 has a role in secondary wall formation.

Key words: *Arabidopsis thaliana*, E3 ubiquitin ligase, secondary cell wall formation, transcription factor.

Lignified secondary cell walls are essential for vascular plants to live on land (Boerjan et al. 2003; Umezawa 2010; Zhong and Ye 2009). The major components of lignified secondary cell walls are cellulose, hemicelluloses, and lignin. Biosynthesis of these components is highly coordinated. Several transcription factors have been shown to have important roles in the coordinated transcriptional activation of secondary wall biosynthetic genes in some plant species, including *Arabidopsis*, *Eucalyptus*, pine and poplar (Demura and Fukuda 2007; Zhong et al. 2010, 2011). In *Arabidopsis*, a number of NAM/ATAF/CUC (NAC) domain transcription factors have been identified as master regulators of secondary wall formation in vessel elements and fiber cells (Kubo et al. 2005; Mitsuda et al. 2007; Yamaguchi et al. 2008, 2011; Yamaguchi and Demura 2010; Zhong et al. 2006). These NAC transcription factors regulate the expression of secondary wall biosynthetic genes together with downstream MYB, NAC, and KNOTTED1-like homeobox (KNOX) family transcription factors (Bhargava et al. 2013; Ko et al. 2009; McCarthy et al. 2009; Nakano et al. 2010; Öhman et al. 2013; Zhong et al. 2007, 2008; Zhou et al. 2009). Among the MYB transcription factors, MYB46 and MYB83 redundantly regulate the expression of the genes responsible for biosynthesis of all three major secondary wall components—cellulose, xylan, and lignin—in vessel elements and fiber cells (Ko et al. 2009; McCarthy et al. 2009; Zhong et al. 2007).

Yamaguchi et al. (2008) have previously suggested

Abbreviations: ATL, Arabidopsis Tóxicos en Levadura; CaMV, cauliflower mosaic virus; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; GUS, β-glucuronidase; MBP, maltose binding protein; NAC, NAM/ATAF/CUC; NST, NAC secondary wall thickening promoting factor; PMSF, phenylmethylsulfonyl fluoride; RAFL clones, RIKEN *Arabidopsis* full-length cDNA clones; TMV, tobacco mosaic virus; VND, vascular-related NAC domain.

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the possibility that VASCULAR-RELATED NAC-DOMAIN7 (VND7), a key regulator of secondary wall biosynthesis and programmed cell death in protoxylem vessels, is regulated by the proteasome-mediated protein degradation pathway. Protein degradation via the ubiquitin-proteasome pathway is initiated by conjugation of ubiquitin to target proteins by ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) (Moon et al. 2004). Target-specific ubiquitin ligases are important for selective protein degradation. Thus far, however, ubiquitin ligases specific to VND7 and other secondary wall-associated transcription factors have not been identified.

In our previous study (Noda et al. 2013), we searched the ATTED-II database (Obayashi et al. 2009) for genes encoding RING finger family proteins that were co-expressed with secondary wall-associated transcription factor and enzyme genes, because RING finger proteins are the most frequent catalytic subunits of E3 ubiquitin ligases (Mazzucotelli et al. 2006). As a result, we found that *Arabidopsis Tóxicos en Levadura54* (*ATL54*, At1g72220) showed the highest correlation coefficients for several secondary wall-associated genes (Noda et al. 2013). A ubiquitination assay using a recombinant ATL54 protein showed that ATL54 had E3 ubiquitin ligase activity. The expression of secondary wall biosynthetic genes in apical stem portions of four-week-old plants was up-regulated by knock-out of *ATL54*, whereas the expression of *Xylem Cysteine Peptidase1* (*XCP1*), a gene involved in programmed cell death in tracheary elements (Funk et al. 2002), was down-regulated in middle stem portions of both *ATL54*-overexpressed and *ATL54*-knock-out mutants (Noda et al. 2013). However, it remains unclear how the expression of *ATL54* is transcriptionally regulated. In other previous studies, it has been shown that overexpression of either *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*) or *MYB46*, both of which are genes of master regulators of secondary wall formation, results in a significant increase in the expression level of *ATL54* (Ko et al. 2009; Mitsuda et al. 2005). Considering that MYB46 is a transcription factor downstream of NST1 (Zhong et al. 2007), we hypothesized that ATL54 is downstream of MYB46. In this paper, we report the tissue-level expression patterns of *ATL54* and the direct regulation of *ATL54* expression by MYB46.

Materials and methods

Vector construction

For a tissue-level expression pattern analysis, an *ATL54* promoter fragment (2,075 bp upstream from the start codon) was amplified from genomic DNA of wild-type *Arabidopsis thaliana* (ecotype Col-0) by PCR. The fragment was subcloned into pENTR/D-TOPO (Invitrogen Japan KK, Tokyo, Japan) and transferred to pBGGUS (Kubo et al. 2005) using LR Clonase II (Invitrogen) to obtain pBGGUS-*ATL54*p, in which the *ATL54* promoter fragment was fused with the *β-glucuronidase* (*GUS*) reporter gene.

For a dual luciferase transient transfection assay, the *MYB46* full-length cDNA fragment was PCR-amplified from the RIKEN *Arabidopsis* full-length cDNA clone (RAFL clone; RIKEN BioResource Center) and inserted into the *Kpn*I and *Sac*I sites of the p35SG effector plasmid (Fujimoto et al. 2000; Yamaguchi et al. 2010) to obtain p35SG-*MYB46*, in which the *MYB46* cDNA was fused with the cauliflower mosaic virus (CaMV) 35S promoter and the Ω sequence of the tobacco mosaic virus (TMV). An *ATL54* promoter fragment (1,031 bp 5′ upstream from the start codon) was PCR-amplified and inserted into the *Hin*dIII site of the p190LUC reporter plasmid (Fujimoto et al. 2000; Mitsuda et al. 2010) to obtain p190LUC-*ATL54*p, in which the *ATL54* promoter fragment was fused with the CaMV 35S minimal promoter, the Ω sequence of TMV, and the firefly luciferase gene.

For an electrophoretic mobility shift assay (EMSA), the full-length cDNA fragment of *MYB46* was inserted into the *Nde*I and *Eco*RI sites of the pMAL-c5X vector (New England Biolabs Japan Inc, Tokyo, Japan) to obtain pMAL-*MYB46*. pMAL-DC-6myc (Zhang et al. 2005) was recombined with pENTR-gus (Invitrogen) using LR Clonase II to obtain pMAL-*GUS*-6myc.

The sequences of all constructs described above were confirmed by DNA sequencing. All primers used for the vector construction or the DNA sequencing are listed in Table 1.

Tissue-level expression pattern analysis

Transformation of *Arabidopsis* wild-type plants with pBGGUS-*ATL54*p and cultivation were performed according to the method of Kubo et al. (2005). Stem internodes near cessation of elongation, non-elongating stem internodes, and hypocotyls were obtained from four-week-old transgenic plants (T_2 generation). GUS staining was carried out according to the method of Pyo et al. (2004). After staining, samples were fixed for 1 h with FAA (3.7% formaldehyde, 5% acetic acid, and 50% ethanol) and embedded in 4% agarose. Approximately 80 μm-thick cross sections were cut from the samples using a ZERO-1 micro-slicer (Dosaka EM, Kyoto, Japan). The sections were counterstained with phloroglucinol-HCl and viewed under a light microscope (BX51; Olympus, Tokyo, Japan). Two-week-old seedlings of the transformants were GUS-stained and immersed in a mixture of 6 : 1 (v/v) ethanol/acetic acid overnight, and then observed under a stereomicroscope (SZX-10; Olympus).

Dual luciferase transient transfection assay

A. thaliana T87 suspension-cultured cells were obtained from the RIKEN BioResource Center, and maintained in mJPL3 medium (Ogawa et al. 2008) at 23°C under a long-day condition (16 h light, 8 h dark) with rotary shaking at 120 rpm. According to the method of Fujimoto et al. (2005), protoplasts of *Arabidopsis* T87 cells were prepared and co-transformed

Table 1. Oligonucleotides used in this study

Name	Sequence (5′ to 3′)*	Purpose
pENTR-ATL54p_f	CACCTATTTAGTATGGTAAAAACAACAAACCAAT	For subcloning *ATL54* promoter fragment to insert into pBGGUS
ATL54p_r	CCTCGCCATTAAAGAGAACACTCAAAACAC	For subcloning *ATL54* promoter fragment to insert into pBGGUS
*Kpn*I-MYB46_f	TAGGTACCATGAGGAAGCCAGAGGTA	For amplifying *MYB46* cDNA to insert into p35SG
*Sac*I-MYB46_r	CCGAGCTCTCATATGCTTTGTTTGAAGTTG	For amplifying *MYB46* cDNA to insert into p35SG
*Hin*dIII-ATL54p_f	AGAAGCTTCCTGAAAATTAAAAGAAACAATTGC	For amplifying *ATL54* promoter fragment to insert into p190LUC
*Hin*dIII-ATL54p_r	CCAAGCTTTAAAGAGAACACTCAAAACAC	For amplifying *ATL54* promoter fragment to insert into p190LUC
*Nde*I-MYB46_f	TACATATGAGGAAGCCAGAGGTAGCC	For amplifying *MYB46* cDNA to insert into pMAL-c5X
*Eco*RI-MYB46_r	CCGAATTCTATGCTTTGTTTGAAGTTGAAG	For amplifying *MYB46* cDNA to insert into pMAL-c5X
M13 forward (−20)	GTAAAACGACGGCCAG	For DNA sequencing of pENTR/D-TOPO and p190LUC
M13 reverse	CAGGAAACAGCTATGAC	For DNA sequencing of pENTR/D-TOPO
TrbcSf1	ATGCATTAAAAGCTGAAACAGTTATTTAG	For DNA sequencing of pBGGUS
GUSr2	GCGATCCAGACTGAATGCCCAC	For DNA sequencing of pBGGUS
p35Sf3	AGGAAGGTGGCTCCTACAAATGCCATC	For DNA sequencing of p35SG
Tnosr1	TGATAATCATCGCAAGACCGGCAAC	For DNA sequencing of p35SG
luc rev (GL primer 2)	CTTTATGTTTTTGGCGTCTTCC	For DNA sequencing of p190LUC
malE primer	GGTCGTCAGACTGTCGATGAAGCC	For DNA sequencing of pMAL-c5X
pMAL-5 reverse	TGTCCTACTCAGGAGAGCGTTCAC	For DNA sequencing of pMAL-c5X
ATL54p −163 to −412_f	TAAAGTCTTATTTTTTTCTTTTGCAGTCT	For amplifying the *ATL54* promoter fragment to use for EMSA
ATL54p −163 to −412_r	AGAAAGATGAAAATTTCTTCTCATCTTAG	For amplifying the *ATL54* promoter fragment to use for EMSA
ATL54p −413 to −662_f	ACATAAAACCTTATTATTAATAAGTCTTTATTCTC	For amplifying the *ATL54* promoter fragment to use for EMSA
ATL54p −413 to −662_r	ACTAGATTTCTTAGATCTGAAAAAAAAATG	For amplifying the *ATL54* promoter fragment to use for EMSA

* Restriction sites are underlined.

with 10 μg each of p35SG-*MYB46*, p190LUC-*ATL54*p, and a reference plasmid containing the *Renilla* luciferase gene driven by the CaMV 35S promoter. p35SG and p190LUC without inserts were used as negative controls. Transformed protoplasts were incubated at 23°C overnight in mJPL3 medium containing 0.4 M mannitol and 0.4 M glucose instead of sucrose. Luciferase activity in the protoplasts was determined with a Dual-Luciferase Reporter Assay System (Promega KK, Tokyo, Japan) and a GloMax 20/20n Luminometer (Promega).

Electrophoretic mobility shift assay (EMSA)

Escherichia coli strain BL21 Star (DE3) (Invitrogen) was transformed with pMAL-*MYB46* or pMAL-*GUS*-6myc. The *E. coli* cells were cultured in LB medium, and expression of the recombinant proteins fused with MBP was induced by addition of 0.1 mM isopropyl β-D-thiogalactopyranoside at mid-log phase (A_{600}=0.4–0.6). The cells were then grown for 8 h at 16°C and collected by centrifugation at 2,000×g at 4°C. The collected cells were resuspended in buffer A [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM dithiothreitol (DTT), 5% 2-methylpentane-2,4-diol, and Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics KK, Tokyo, Japan)] and lysed by sonication. The lysate was centrifuged at 10,000×g for 30 min at 4°C, and the recombinant protein in the supernatant was purified using amylose resin (New England Biolabs) according to the manufacturer's protocol. The fractions containing the recombinant GUS protein (MBP-GUS) were desalted using a Sephadex G-25 (GE Healthcare Japan, Tokyo, Japan) column. The fractions containing the recombinant MYB46 protein (MBP-MYB46) were diluted 3-fold with buffer B [20 mM Bis-tris HCl (pH 6.5), 1 mM EDTA, 1 mM PMSF, 2 mM DTT, and 5% 2-methylpentane-2,4-diol], and were further purified on a Waters 650E Advanced Protein Purification System (Waters Corporation, Milford, MA, USA) connected with a MonoQ 5/50 GL anionic exchange column (GE Healthcare) that had been pre-equilibrated with buffer B. The protein was eluted from the column with a NaCl linear gradient (0.05–1 M). The fractions were desalted using a Sephadex G-25 column.

Two *ATL54* promoter fragments, 163–412 and 413–662 bp upstream of the start codon of *ATL54*, were amplified by PCR using the primers listed in Table 1. The amplified DNA fragments were labeled with biotin using a Biotin 3′ End DNA Labeling Kit (Thermo Fisher Scientific KK, Yokohama, Kanagawa, Japan). The labeled probes were heated at 65°C for 5 min, and immediately chilled on ice. Aliquots of the probes (20 fmol) were incubated for 30 min at 4°C with 5 μg of MBP-

MYB46 or MBP-GUS in the binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 50 ng μl^{-1} poly(dI–dC), 2.5% glycerol, and 5 mM $MgCl_2$]. For competition experiments, unlabeled *ATL54* promoter fragments and Unlabeled EBNA DNA (LightShift Chemiluminescent EMSA Kit; Thermo Fisher Scientific) were added to the reactions as specific and non-specific competitors, respectively. Polyacrylamide gel electrophoresis (PAGE), blotting, and detection of biotin-labeled DNA fragments were performed as previously described (Yamaguchi et al. 2011).

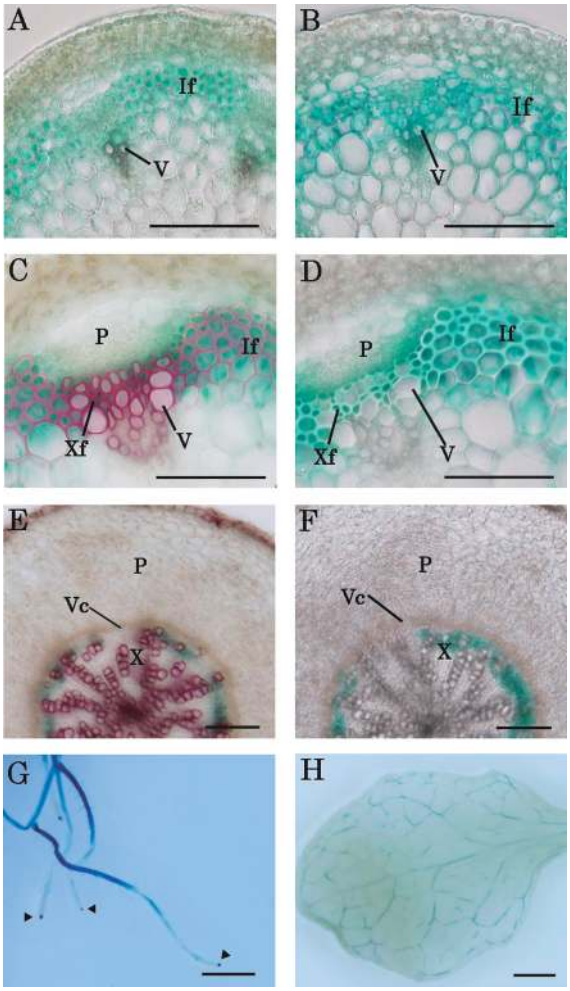

Figure 1. Expression pattern of *ATL54*. The *GUS* reporter gene driven by the *ATL54* promoter was expressed in *Arabidopsis* plants. Various organs were stained and examined by microscopes. Because similar patterns were observed in two lines of the transformants, the results of one line are shown in this figure as representatives. (A–F) Cross sections of a stem internode near cessation of elongation (A, B), a non-elongating stem internode (C, D), and a hypocotyl (E, F) with GUS and phloroglucinol-HCl double staining (A, C, E) or only GUS staining (B, D, F). (G) GUS-stained root. Arrowheads indicate root apices. (H) GUS-stained developing leaf. V, vessel element; If, interfascicular fiber; Xf, xylary fiber; P, phloem; X, xylem; Vc, vascular cambium. Bars = 100 μm in (A–F) and 1 mm in (G, H).

Results

Tissue-level expression pattern of ATL54

To investigate the tissue-level expression patterns of *ATL54*, an *ATL54* promoter fragment was fused to the *GUS* reporter gene, and the construct was introduced into *A. thaliana* plants. Stem internodes and hypocotyls of the transformants were used for GUS staining and counterstained with phloroglucinol-HCl, by which lignified cell walls are stained magenta. Two lines of the transformants exhibited similar GUS staining patterns as follows. GUS signal was detected in unlignified interfascicular fibers, xylary fibers and differentiating vessels in the internodes near cessation of elongation (Figure 1A, B). In the non-elongating internodes, GUS

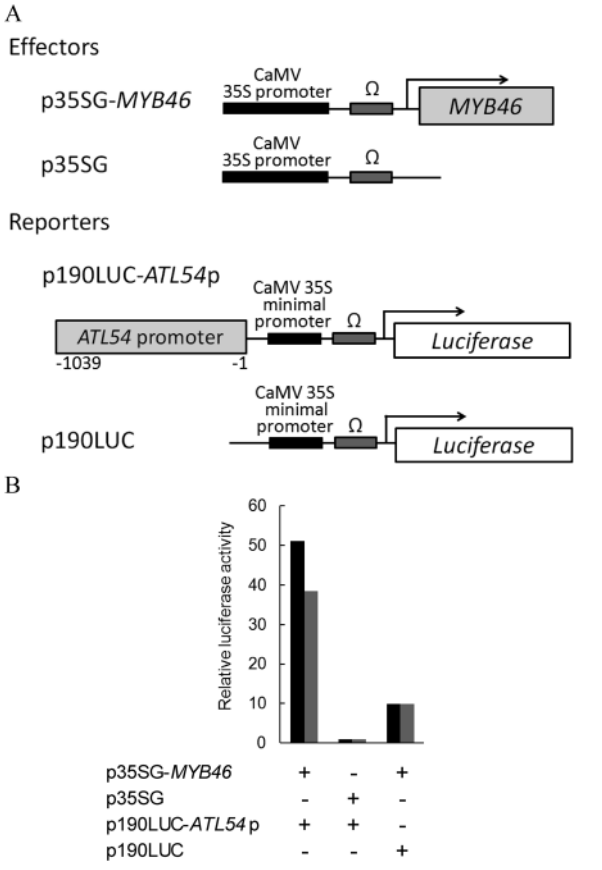

Figure 2. Transactivation analysis of the *ATL54* promoter by MYB46. An effector, a reporter and a reference plasmid were co-transfected into *Arabidopsis* T87 protoplasts using polyethylene glycol solution. After incubation, the protoplasts were lysed and luciferase activity was measured. (A) Schematic diagrams of the effector and reporter constructs used for the dual luciferase transient transfection assay. The effector construct contained the CaMV 35S promoter and the Ω sequence of TMV with or without the *MYB46* gene. The reporter construct contained the CaMV 35S minimal promoter and the Ω sequence of TMV upstream of the firefly luciferase reporter gene, with or without the *ATL54* promoter. (B) Relative luciferase activity. The activity was normalized to the reference *Renilla* luciferase activity. Values of duplicate assays for each sample are shown. The value of the pair of p35SG and p190LUC-*ATL54*p was set to 1.

signal was detected in lignifying interfascicular and xylary fibers, but not in mature vessels (Figure 1C, D). In the hypocotyls, GUS staining was seen in the inner side of the cambium (Figure 1E, F). GUS was also expressed in the roots of seedlings (Figure 1G). GUS staining became faint toward the distal portions of roots, but root apices were strongly stained (Figure 1G). Developing leaves from seedlings showed a discontinuous pattern of GUS staining in the veins (Figure 1H).

Transactivation of ATL54 *by MYB46*

To test whether ATL54 is downstream of MYB46, we performed a transactivation analysis. An effector plasmid containing the full-length *MYB46* cDNA driven by the CaMV 35S promoter and a reporter plasmid containing the firefly luciferase gene driven by the *ATL54* promoter were co-transfected into *Arabidopsis* T87 protoplasts (Figure 2A). The protoplasts transformed with the effector containing *MYB46* showed 40-fold higher luciferase activity compared with those transformed with the control effector without *MYB46* (Figure 2B). The reporter containing the *ATL54* promoter caused 4-fold higher activity than the reporter without the *ATL54* promoter (Figure 2B). These results indicated that *ATL54* was transactivated by MYB46.

Binding activity of the MYB46 protein to the ATL54 *promoter*

Next, we investigated whether MYB46 directly binds to the *ATL54* promoter using an electrophoretic mobility shift assay (EMSA). Both the −163 to −412 bp and the −413 to −662 bp *ATL54* promoter fragments labeled with biotin showed clear band shifts in the presence of a recombinant MBP-MYB46 protein (Figure 3). When the corresponding unlabeled promoter fragments were added, the shifted bands were weakened in a concentration-dependent manner, and bands of free probes became visible (Figure 3). On the other hand, addition of an EBNA DNA fragment, a non-specific competitor, did not weaken the band shift of either probe (Figure 3). These results showed that MBP-MYB46 bound to the *ATL54* promoter fragments in a sequence-specific manner. MBP-GUS did not cause a band shift (Figure 3), indicating that MYB46 was responsible for the interaction between the probes and the recombinant protein. Together, these results demonstrated that MYB46 can bind to the *ATL54* promoter.

Discussion

Our previous study suggested that ATL54 may be involved in the regulation of programmed cell death as well as secondary wall formation during xylogenesis (Noda et al. 2013). In this study, the tissue-level expression pattern analysis demonstrated that *ATL54* was expressed in stem interfascicular fibers, xylary fibers, and vessels (Figure 1A–D). In addition, the discontinuous pattern of GUS staining observed in developing leaves (Figure 1H) is typical of genes specifically expressed in cells differentiating into xylem vessels (Funk et al. 2002; Yamaguchi et al. 2008). These results demonstrate that *ATL54* is significantly expressed in cells where secondary

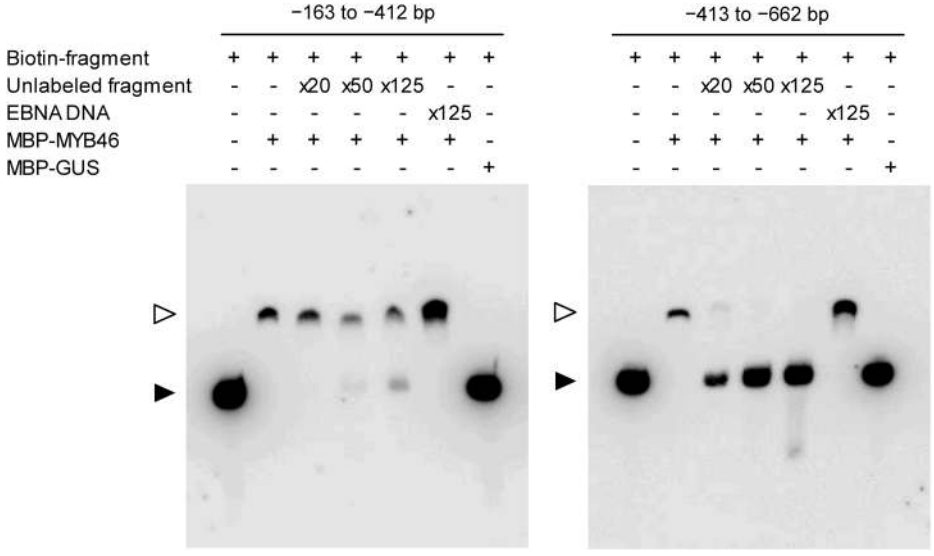

Figure 3. Electrophoretic mobility shift assay (EMSA) of the recombinant MYB46 protein and the *ATL54* promoter fragments. The MYB46 protein fused with MBP was incubated with the biotin-labeled −163 to −412 bp and −413 to −662 bp *ATL54* promoter fragments, and the products were subjected to PAGE and blotting, followed by detection of chemiluminescence. A 20-, 50-, or 125-fold excess of unlabeled fragments or a 125-fold molar excess of EBNA DNA over the biotin-labeled fragment was used for competition experiments. MBP-GUS was used as a negative control for the recombinant MYB46 protein. The assays were repeated three times, and similar results were obtained. Black and white arrowheads indicate bands of free probes and shifted bands, respectively.

wall formation and programmed cell death should occur. On the other hand, *ATL54* is also expressed in root apices where secondary walls do not exist (Figure 1G), suggesting that ATL54 may also be involved in cellular processes other than secondary wall formation.

Our EMSA experiment showed that MYB46 directly bound to two *ATL54* promoter fragments, i.e. the −163 to −412 bp and −413 to −662 bp fragments (Figure 3). This result indicates that MYB46 can bind to multiple sites in the *ATL54* promoter. Recently, several researchers have proposed MYB46-binding elements such as MBSII (GTTAGGT; Ramírez et al. 2011), M46RE [(A/G)(G/T)-T(A/T)GGT(G/A); Kim et al. 2012], and SMRE [ACC(A/T)A(A/C)(T/C); Zhong and Ye 2012]. We found three distinct motifs similar to these proposed MYB46-binding elements in the *ATL54* promoter region used in EMSA (−163 to −662; see Supplemental Figure S1). MYB46 might bind to these motifs, but this will require further experimental validation.

The dual luciferase transient transfection assay demonstrated that *ATL54* was transactivated by MYB46 in vivo (Figure 2B). Combined with the results of the EMSA experiment, our results indicate that *ATL54* expression is directly regulated by MYB46. MYB46 is known to be a positive regulator of secondary wall biosynthesis (Ko et al. 2009; Zhong et al. 2007), while it was shown that overexpression of *MYB46* did not activate *XCP1* and *XCP2*, the genes related to programmed cell death during xylogenesis (Zhong et al. 2007). In our previous study, we showed that the expression of secondary wall biosynthetic genes in apical stem portions was up-regulated by knock-out of *ATL54* (Noda et al. 2013), suggesting that ATL54 may act as a negative regulator of secondary wall biosynthesis. Spatially and temporally accurate lignin deposition and vessel element differentiation are known to be controlled by the action of both negative and positive regulators (Yamaguchi et al. 2010; Zhao et al. 2008; Zhao and Dixon 2011). Considering that ATL54 is a ubiquitin ligase (Noda et al. 2013), ATL54 might cooperate with other positive regulators and contribute to optimization of secondary wall formation via degradation of transcription factors and/or other regulatory proteins involved in the process. The regulation of *ATL54* expression during programmed cell death remains to be investigated.

In summary, *ATL54* is expressed in fiber cells and vessel elements in which secondary cell walls accumulate. *ATL54* expression is directly regulated by MYB46, a key transcriptional activator of secondary wall formation. These facts strongly suggest that ATL54 plays a specific role in secondary wall formation. Identification of proteins ubiquitinated by ATL54 may help us to uncover a regulatory system for secondary wall formation by proteasome-mediated protein degradation.

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