

Identification of a *cis*-acting regulatory motif recognized by MYB46, a master transcriptional regulator of secondary wall biosynthesis

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Abstract While many aspects of primary cell wall have been extensively elucidated, our current understanding of secondary wall biosynthesis is limited. Recently, transcription factor MYB46 has been identified as a master regulator of secondary wall biosynthesis in *Arabidopsis thaliana*. To gain better understanding of this MYB46-mediated transcriptional regulation, we analyzed the promoter region of a direct target gene, *AtC3H14*, of MYB46 and identified a *cis*-acting regulatory motif that is recognized by MYB46. This MYB46-responsive *cis*-regulatory element (M46RE) was further characterized and shown to have an eight-nucleotide core motif, RKTWGGTR. We used electrophoretic mobility shift assay, transient transcriptional activation assay and chromatin immunoprecipitation analysis to show that the M46RE was necessary and sufficient for MYB46-responsive transcription. Genome-wide analysis identified that the frequency of M46RE in the

promoters were highly enriched among the genes upregulated by MYB46, especially in the group of genes involved in secondary wall biosynthesis.

Keywords *cis*-acting motif · MYB46 · Secondary wall biosynthesis · Transcription factor

Introduction

Secondary cell wall is a structure found in many plant cells, located between the primary cell wall and the plasma membrane. The cell starts producing thick secondary cell wall after the primary cell wall biosynthesis is complete and the cell has stopped expanding (Lerouxel et al. 2006; Somerville 2006; Zhong and Ye 2007). Secondary cell wall consists mainly of cellulose, hemicellulose and lignin. Secondary cell wall is a defining feature of the cells in xylem fibers and vessels that provide mechanical support for their growing body and serve as a conduit for long-distance transport of water/solutes, respectively (Fukuda

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1997; Ye 2002). In addition, secondary wall from plant biomass is a major source of lignocellulosic material and one of the most promising sources of sugars for liquid biofuel production (Han et al. 2007).

Formation of secondary wall requires a coordinated transcriptional activation of the genes involved in the biosynthesis of secondary wall components such as cellulose, hemicellulose and lignin. However, our understanding of the transcriptional regulatory network for secondary wall biosynthesis is limited. Recent studies on NAC and MYB transcription factors have provided an insight into the complex network of transcriptional regulation of secondary wall biosynthesis (Zhong and Ye 2007). ANAC012/SND1 (At1g32770) functions as a potential regulator of secondary wall thickening in xylary fibers (Zhong et al. 2006; Ko et al. 2007). Several MYB transcription factors were also identified as important regulators of secondary wall biosynthesis in *Arabidopsis*. MYB46, a direct target of ANAC012/SND1, is capable of inducing secondary wall formation (Zhong et al. 2007; Ko et al. 2009). Overexpression of MYB46 results in ectopic deposition of secondary walls in the cells that are normally parenchymatous, while suppression of its function reduces secondary wall thickening (Zhong et al. 2007; Ko et al. 2009). However, the mechanism underlying MYB46-mediated transcriptional regulation is still largely unknown, mainly due to the lack of information on downstream targets of MYB46. Information on *cis*-acting regulatory elements (i.e., transcription factor binding motifs) that are recognized by MYB46 will facilitate the identification of the target genes of MYB46, which are involved in transcriptional regulation of secondary wall biosynthesis.

The goal of the current study is to identify *cis*-regulatory motifs in MYB46-mediated transcriptional regulation. Recently, we have identified *AtC3H14* (At1g66810) as a direct target of MYB46 (Ko et al. 2009). *AtC3H14* is a plant specific CCCH-type zinc finger protein in *Arabidopsis* (Wang et al. 2008) and one of the 68 CCCH-type zinc finger proteins in *Arabidopsis* having two identical C-x8-C-x5-C-x3-H motifs (TZF; Tandem CCCH Zinc Finger) (Wang et al. 2008). However, the exact function of *AtC3H14* is not characterized yet. We then carried out a series of electrophoretic mobility shift assays (EMSA) using MYB46 protein and *AtC3H14* promoter sequences with various deletions. The analysis narrowed down the MYB46 protein binding region to a 25-nucleotide fragment and identified a core MYB46-responsive *cis*-regulatory element (M46RE). Using *in vivo* transient activation analysis, we confirmed that the M46RE is necessary and sufficient for MYB46-responsive transcription. Genome-wide analysis of promoter sequences in *Arabidopsis* revealed that the most of the MYB46-induced secondary wall biosynthetic genes have one or more M46REs in their promoter region. We

discuss the significance of this motif in the transcriptional regulation of secondary wall biosynthesis.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana, ecotype Columbia (Col-0), was grown on soil in a growth chamber (16 h light/8 h dark) at 23°C. Rosette leaves of three-week-old *Arabidopsis* were used in transcriptional activation analysis (described below).

Protein expression and purification

MYB46 was fused in frame with GST and expressed in *Escherichia coli* strain Rosetta gami (Novagen). The induction of the recombinant MYB46-GST proteins was performed by adding 0.3 mM IPTG (isopropyl β -D-thiogalactopyranoside) for 16 h of incubation at 16°C. The recombinant proteins were purified using Glutathione immobilized particles (MagneGST; Promega) and used for EMSA.

Electrophoretic mobility shift assay (EMSA)

DNA fragments for EMSA were obtained by PCR-amplification or synthesized and labeled with [γ - 32 P]ATP using T4 polynucleotide kinase (NEB, <http://www.neb.com/>). The end-labeled probes were purified with Microspin S-200 HR column (GE Healthcare, <http://www.gelifesciences.com/>). The labeled DNA fragments were incubated for 25 min with 50 ng of MYB46-GST in a binding buffer [10 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM MgCl₂, 100 μ g/mL BSA, and 50 ng/ μ L poly(dI-dC)]. Five percent polyacrylamide gel electrophoresis (PAGE) was used to separate the recombinant protein-bound DNA fragments from the unbound ones. The gel was dried, and placed in a film cassette and exposed to X-ray film (Kodak) for over night. Radioactive fragments were visualized by autoradiography.

Transcriptional activation analysis

Preparation of *Arabidopsis* leaf protoplasts and transient transfection of reporter and effector constructs were carried out as described previously (Ko et al. 2009). Briefly, transfected protoplasts were lysed, and the soluble extracts were used for analysis of GUS, after 16 h of incubation. For the time-course experiment, each transfected protoplasts were harvested at the indicated time for GUS activity measurement. In each experiment, the expression level of

the GUS reporter gene in the protoplasts transfected with the reporter construct alone was used as control. The data were the average of three biological replications with S.E. pTrGUS vector was used to produce both effector and reporter constructs. pTrGUS is a home-made vector designed to reduce the vector size around 4 kb to improve transfection efficiency and was provided by Dr. Soo-Un Kim (Seoul National University, Korea). For effector constructs, the full-length cDNAs of transcription factors were ligated between CaMV 35S promoter and NOS terminator after removing GUS of the pTrGUS vector. The reporter constructs were created by placing promoter fragments in front of the GUS reporter gene after removing 35S promoter region of the pTrGUS. High quality plasmids were prepared by PureHeix™ Fast-n-Pure plasmid kit (Nanohelix, South Korea). Transfection and transient expression by using this vector system was successfully demonstrated (Ko et al. 2009). Primers used for PCR amplification of full-length genes and promoter fragments were summarized in Table S1.

Chromatin immunoprecipitation analysis

The full-length cDNA of *MYB46* was fused in frame with GFP cDNA and inserted behind the GAL4 upstream activation sequence of the pTA7002 binary vector (Aoyama and Chua 1997). The construct was introduced into wild-type *Arabidopsis thaliana* (Col-0) by *Agrobacterium*-mediated transformation and the resulting transgenic plants were used for chromatin immunoprecipitation analysis as described in Zhong et al. (2007) with slight modification. Briefly, the *MYB46*-GFP/pTA7002 transgenic plants were grown on soil for 3 weeks and treated 10 μ M DEX with 0.02% silwet surfactant (Lehle Seeds) for 8 hours. The seedlings were then harvested and cross-linked in 1% formaldehyde for 10 min under vacuum and quenched by adding 0.125 M of glycine for 5 min. The seedlings were washed twice with deionized water and then ground in liquid nitrogen. To extract chromatin, 2 g of ground powder was resuspended in 30 mL of extraction buffer 1 (10 mM Tris-HCl, pH 8.0, 0.4 M sucrose, 5 mM 2-mercaptoethanol, 1 mM PMSF, 1X protease inhibitor cocktail, and 4 μ g/mL pepstain A) and filtered through two layers of miracloth before centrifugation at 2,500 g for 20 min at 4°C. The pellet was resuspended in 1 mL of extraction buffer 2 (10 mM Tris-HCl, pH 8.0, 0.25 M sucrose, 10 mM MgCl₂, 1% Triton X-100, 5 mM 2-mercaptoethanol, 1 mM PMSF, 1X protease inhibitor cocktail, and 4 μ g/mL pepstain A) and centrifuged at 14,000 g for 10 min at 4°C. The pellet was resuspended in 300 μ L of extraction buffer 3 (10 mM Tris-HCl, pH 8.0, 1.7 M sucrose, 0.15% Triton X-100, 2 mM MgCl₂, 5 mM 2-mercaptoethanol, 1 mM PMSF, 1X protease inhibitor cocktail, and 4 μ g/mL pepstain A) and layered on top of

300 μ L of extraction buffer before centrifugation at 14,000 g for 1 h at 4°C. The chromatin pellet was resuspended in 500 μ L of ice-cold nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, 1 mM PMSF, 1X protease inhibitor cocktail, and 4 μ g/mL pepstain A) and sonicated to small fragments with an average size of 600–800 bp. Sonication was done by Omni-Ruptor 250 (OMNI International Inc., USA) with four cycles of 12 s pulse (40% duty) and 1 min pause. The sonicated chromatin was diluted 1:10 in ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.0, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 1 mM PMSF, 1X protease inhibitor cocktail, and 4 μ g/mL pepstain A) and pre-cleared by incubation with Protein A agarose beads (Roch) for 1 h. The pre-cleared chromatin was then incubated with 2 μ g of GFP antibody (Abcam) overnight at 4°C. The immune complexes were collected by incubation with Protein A agarose beads for 1 h and subsequent washing for 5 min with 1 mL of each in low-salt wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% SDS, 0.5% Triton X-100 and 2 mM EDTA), high-salt wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.2% SDS, 0.5% Triton X-100 and 2 mM EDTA), LiCl wash buffer (10 mM Tris-HCl, pH 8.0, 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate and 1 mM EDTA) and TE buffer. The immune complexes were then eluted with 500 μ L elution buffer (1% SDS and 0.1 M sodium bicarbonate) at 65°C for 15 min with agitation. To reverse protein-DNA cross-linking, the eluted chromatin was incubated in 0.2 M NaCl at 65°C without agitation for overnight. Chromatin DNA was further treated with proteinase K (0.2 mg/mL) for 1 h to remove proteins before used for quantitative PCR analysis. The enrichment of the AtC3H14 promoter sequence was examined by real-time quantitative PCR analysis of chromatin samples before (input) and after (bound) immunoprecipitation. The promoter sequence of the *MYB46* gene, which has been shown not to be a downstream target of MYB46 (Ko et al. 2009), was used as an internal control for nonspecific binding of genomic DNA. The values of bound over input for AtC3H14 promoter fragment was normalized against that of the control DNA (*MYB46* promoter, –206 to –440 bp). A promoter sequence (–264 to –529 bp) of *MYB54* (At1g73410) was used as a negative control. The experiments were repeated three times and obtained similar results.

Results

Identification of MYB46 binding site in the promoter region of AtC3H14

In order to identify a *cis*-acting regulatory motif recognized by MYB46, we characterized the promoter sequence of

AtC3H14 gene, a known direct target of MYB46 (Ko et al. 2009; Fig. S1). MYB46 activates *AtC3H14* transcription in vivo (Fig. S1a and b) as shown previously (Ko et al. 2009). We then confirmed that the recombinant MYB46 protein binds to the *AtC3H14* promoter fragment evidenced by the mobility shift in the EMSA (Fig. S1c). This interaction was sequence-specific because the addition of a cold (i.e., unlabeled) *AtC3H14* promoter fragment (Pro_AtC3H14-S2; -250 to -500 bp upstream of the start codon) effectively competed with the binding in a highly dose-dependent manner, and the mobility shift was not observed when the *AtC3H14* promoter fragment was incubated with GST alone (Fig. S1c).

To narrow down MYB46 binding region in the *AtC3H14* promoter, pro_AtC3H14-S2 was divided into three fragments (P1, P2, and P3) with 25-bp overlap (Fig. 1a) and the fragments were used as competitors against pro_AtC3H14-S2 probe in the EMSA experiments. Cold pro_AtC3H14-S2 was used as a positive control in the experiments (Fig. 1b). The result showed that one region, pro_AtC3H14-P3, exhibited very strong binding by MYB46 (Fig. 1b). We

next divided the pro_AtC3H14-P3 fragment into seven 25-bp oligonucleotides (P3-1 to P3-7) with 13-bp overlap to further narrow down the sequence that was recognized by MYB46 (Fig. 1c, d). Following EMSA analysis identified the pro_AtC3H14-P3-2 as the binding region recognized by MYB46 (Fig. 1d).

Defining MYB46-responsive cis-regulatory element (M46RE)

To identify the specific MYB46 binding sequence, we created a series of point mutations in the pro_AtC3H14-P3-2 sequence (Fig. 2a). Further competition analysis using these mutated oligonucleotides as competitor revealed that mutations in m13, m15, m16, m17, m18, m19 or m20 resulted in loss of binding ability to MYB46 (Fig. 2b), identifying the 8-bp AGTAGGTG as a putative core sequence.

The identified 8-bp core sequence is present in the overlapping region of P3-1 and P3-2 fragments (Fig. S2a). Interestingly, the P3-1 sequence, which starts with the core

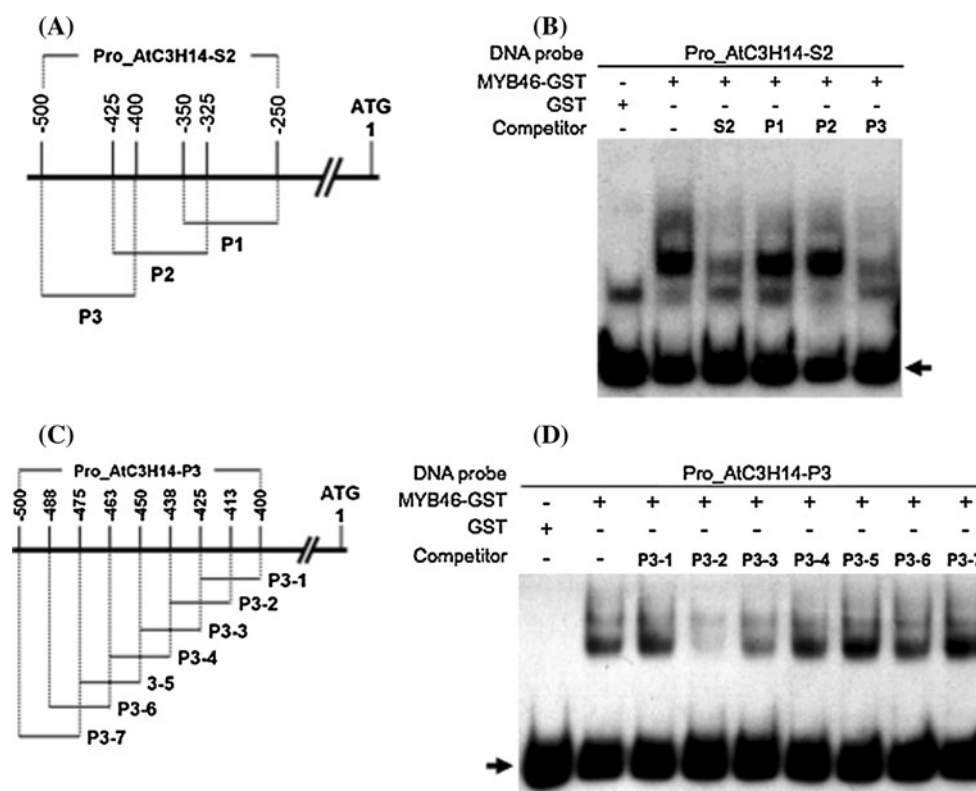
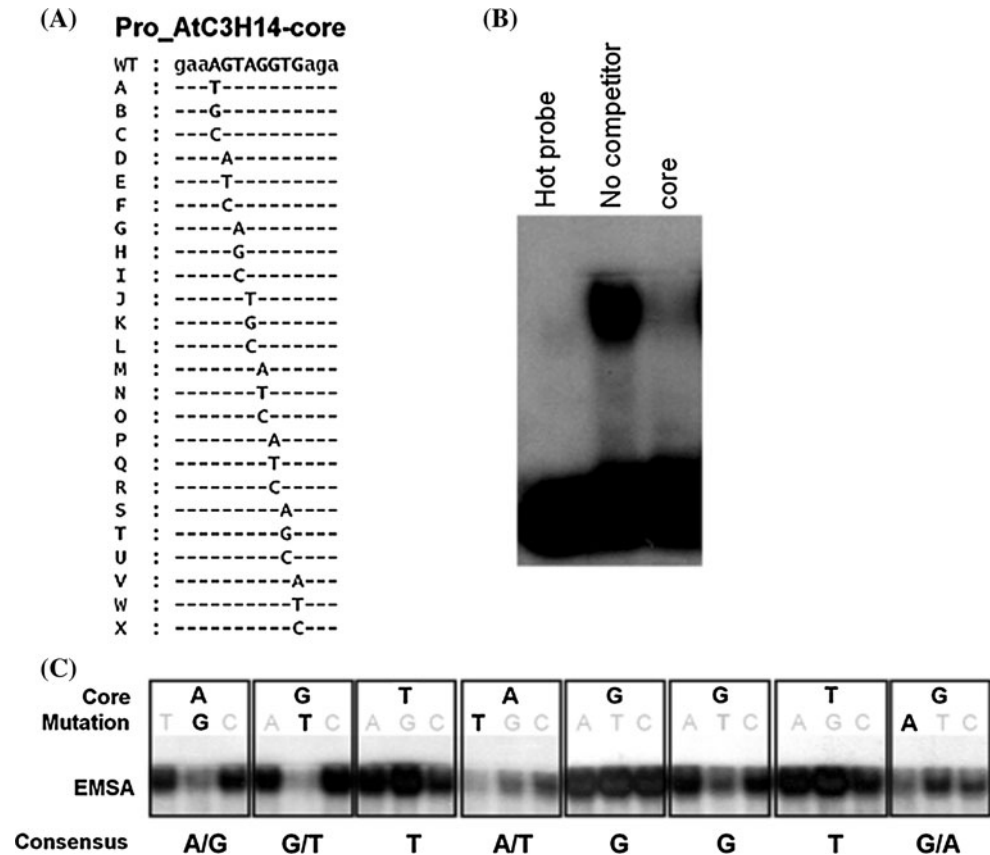


Fig. 1 Determination of MYB46 binding region in *AtC3H14* promoter. **a** Diagram of the *AtC3H14* promoter region used in the EMSA experiment in **b**. **b** MYB46 binds to the 100-bp fragment (P3) of *AtC3H14* promoter, which is located between -400 and -500 relative to the start codon. GST was used as control. For competition analysis, competitors (unlabeled DNA fragments) were added to the reaction mixture with 30-fold molar excess relative to the labeled probes. S2 indicates Pro_AtC3H14-S2 in **a**. **c** Diagram of overlapping

25-bp DNA oligonucleotides in the *AtC3H14* promoter fragment (pro_AtC3H14-P3) used in the EMSA experiment in **d**. **d** Competition analysis of MYB46 binding to Pro_AtC3H14-P3 by the overlapping 25-bp DNA oligonucleotides shown in **c**. Competitors (25-bp unlabeled DNA oligonucleotides) in 50-fold molar excess were used in the EMSA. A 25-bp oligonucleotide, P3-2, competed with Pro_AtC3H14-P3 for binding to MYB46. GST was used as control. The free DNA probes are indicated by *arrow*

Fig. 3 Identification of a consensus MYB46-responsive *cis*-regulatory element (M46RE) in *AtC3H14* promoter.

a Wild-type (WT) Pro_AtC3H14-core sequence was mutated by substituting one nucleotide at a time with all other three nucleotides (A–X). Dashes indicate no change. **b** MYB46 binds to 14-bp fragment (core) of the *AtC3H14* promoter. **c** Competition analysis of MYB46 binding to Pro_AtC3H14-core by mutated Pro_AtC3H14-core oligonucleotides shown in **a**. Competitors (unlabeled WT and mutated Pro_AtC3H14-core oligonucleotides) in 50-fold molar excess were used in the EMSA



of *AtC3H14* promoter (pro_AtC3H14-WT) showed high level of GUS activity by MYB46, all of the mutant versions showed very little GUS activity (Fig. 4c). This result shows that the M46RE is necessary for MYB46-dependent *AtC3H14* promoter activity in vivo (Fig. 4c).

Is M6RE sequence alone sufficient for MYB46 binding? To address this question, we carried out TAA experiments with GUS reporter gene driven by either 500-bp region of MYB46 promoter as negative control or the MYB46 promoter with three tandem repeats of M46RE fused to the upstream and 500-bp region of *AtC3H14* promoter as positive control (Fig. 5a). We found that *AtC3H14* promoter and *MYB46* promoter with the M46REs were able to increase the GUS activity while the native MYB46 promoter did not activate the reporter gene (Fig. 5b), indicating that M46RE sequence alone is sufficient for MYB46 binding.

MYB46 bind promoter of *AtC3H14* in planta

To further corroborate the results from the EMSA and TAA, we used a chromatin immunoprecipitation (ChIP) assay to examine whether MYB46 binds to the *AtC3H14* promoter in planta. For this experiment, we overexpressed GFP-tagged

MYB46 protein in *Arabidopsis* under the control of a dexamethason (DEX)-inducible promoter (Fig. 6a). Inducible overexpression of GFP-tagged MYB46 resulted ectopic secondary wall thickenings in curled leaves (Fig. 6c), as shown in the transgenic *Arabidopsis* overexpressing *MYB46* (Ko et al. 2009), indicating that the MYB46-GFP fusion protein is fully functional in the activation of secondary wall biosynthesis. After the induction of MYB46-GFP by DEX treatment, formaldehyde cross-linked chromatin was isolated, fragmented and immunoprecipitated with GFP antibodies for enrichment of MYB46-GFP bound DNA fragments. Quantitative real-time PCR was performed to detect *AtC3H14* promoter sequence using the immunoprecipitated DNA fragments as templates. The results showed that the pro_AtC3H14-P3 fragments, which were strongly bound by MYB46 in the EMSA, were enriched more than three-fold compared to the control DNA (Fig. 6b). For a negative control, we used the promoter sequence of *MYB54* (At1g73410), which is highly up-regulated by MYB46 overexpression (Ko et al. 2009) but has no M46REs in its promoter sequence. As expected, no enrichment of MYB54 promoter was detected (Fig. 6b). The results from the in vitro and in vivo binding analyses demonstrate that MYB46 directly binds to the promoter of *AtC3H14*.

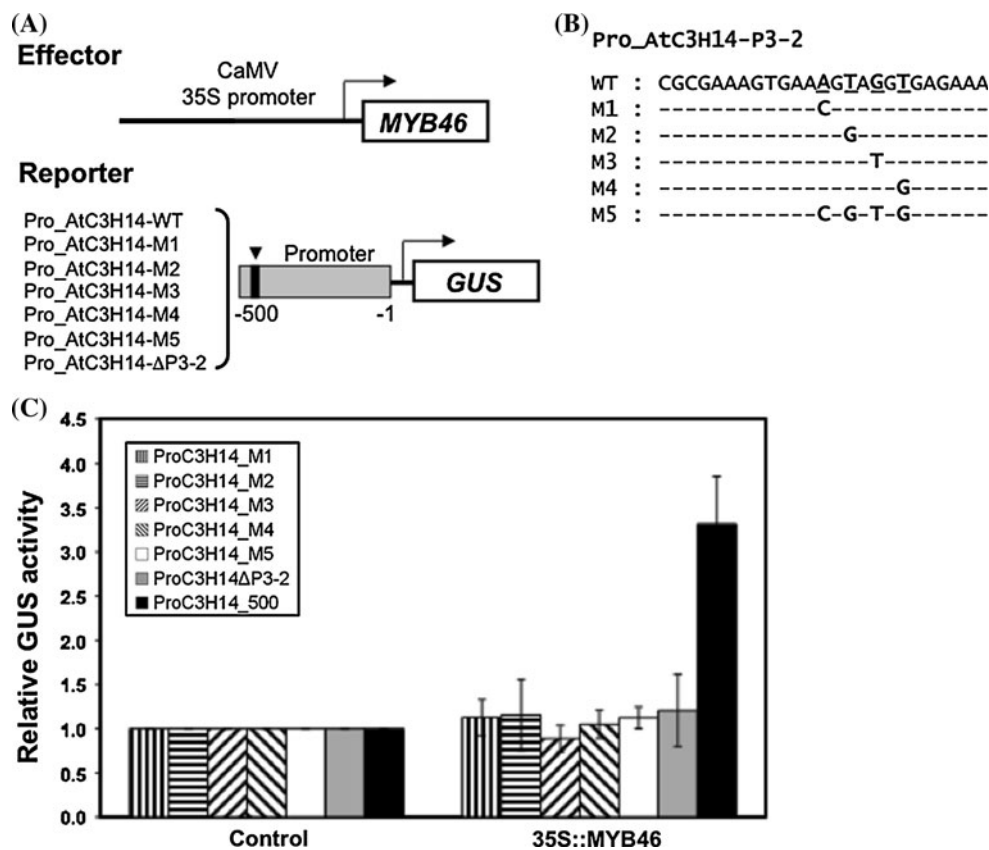


Fig. 4 M46RE is necessary for MYB46 activation of *AtC3H14* transcription in vivo. **a** Diagram of the effector and reporter constructs used in the transcriptional activation assay. The effector construct contained MYB46 gene driven by CaMV 35S promoter. The reporter constructs contained GUS reporter gene driven by the indicated wild type (WT) or mutated *AtC3H14* promoters (M1 to M5 and DP3-2). Mutated region (P3-2, red box) is indicated by arrowhead. DP3-2 means the deletion of P3-2 sequence from *AtC3H14* promoter. **b** Base substitutions of M46RE in the mutated region of *AtC3H14* promoter

in **a**. Dashes indicate no change. **c** MYB46 could not activate *AtC3H14* transcription driven by the mutated promoters in vivo. MYB46 was co-expressed in *Arabidopsis* leaf protoplasts with GUS reporter gene driven by wild-type (WT) or mutated *AtC3H14* promoters (M1 to M5 and DP3-2) shown in **a**. Activation of the promoter by MYB46 was measured by assaying GUS activity after 15-h of incubation. The expression level of the GUS reporter gene in the protoplasts transfected with no effector was used as control and was set to 1. Error bar indicates SE of three biological replications

Significance of M46RE as a cis-element in secondary wall biosynthesis

In order to survey genome-wide distribution of M46RE in the promoter regions (within 1 kb upstream sequence from start codon ATG), we used a ‘Patmatch’ program (<http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl>) with RKTWGGTR as a query. This analysis resulted in identification of 9,836 genes, which is 43.5% out of 22,591 genes included in the ATH1 Genome Array (Affymetrix), that have M46REs in their promoters (Table 1). The frequency of the promoters having M46REs increases up to 70% among secondary wall biosynthesis-related genes, compared to the genome-wide average of 43.5% (Table 1). The frequency goes even higher among the 414 genes that are upregulated three-fold or higher within 6-h of MYB46 induction (i.e., induction of ectopic secondary wall biosynthesis) (Ko et al. 2009). For examples, all of the five

MYB46-induced cellulose synthase (CesA) and cellulose synthase-like (CSL) genes had M46RE motifs in their promoter regions while only 48.7% (19 of 39) of CesA/CSL genes in the genome had the motif. Furthermore, 91.3% (21 of 23) of the MYB46-upregulated lignin biosynthesis genes had the motifs while only 53% (187 out of 353) such genes in the genome had the motif (Table 1). Taken together, it appears that M46RE is highly enriched in the promoters of the genes that are involved in MYB46-regulated secondary wall biosynthesis.

Utility of M46RE in the identification of MYB46 target genes

In order to assess the utility of M46RE motif, we surveyed the promoter regions of the 52 genes that we identified as not only co-expressed with but also up-regulated by MYB46. Over 71% (37 out of 52) of the genes contain

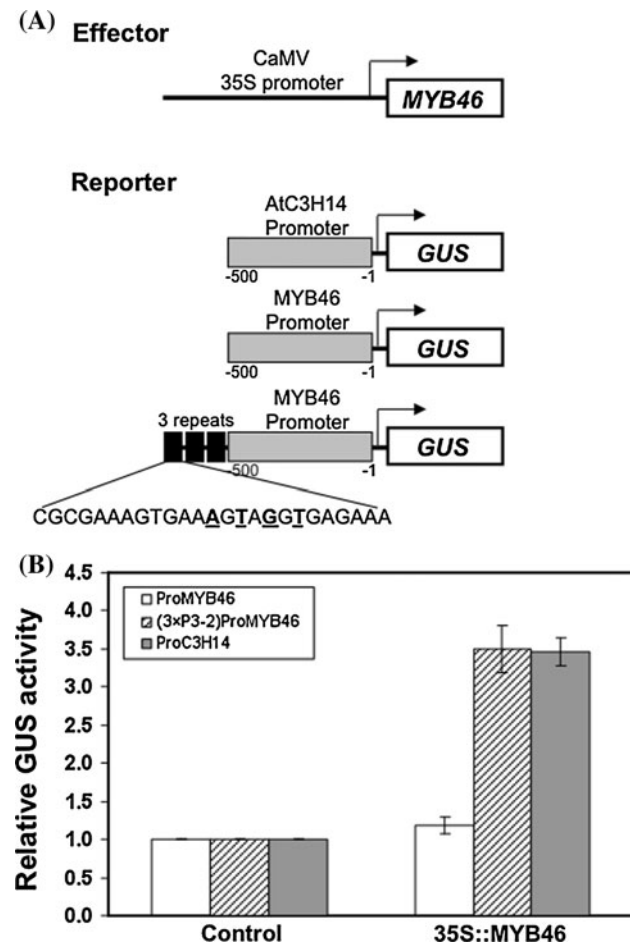


Fig. 5 M46RE is sufficient for MYB46-mediated activation of *AtC3H14* transcription in vivo. **a** Diagram of the effector and reporter constructs used in the transcriptional activation assay. The effector construct contained MYB46 gene driven by CaMV 35S promoter. The reporter constructs contained GUS reporter gene driven by the indicated *AtC3H14* promoter, MYB46 or MYB46 promoter plus three tandem repeats of M46RE. **b** MYB46 could activate *GUS* gene transcription when the three tandem repeats of M46RE were added to the promoter. MYB46 was co-expressed in *Arabidopsis* leaf protoplasts with *GUS* reporter gene driven by wild-type (WT) or MYB46 promoter plus three tandem repeats of M46RE as shown in **a**. Activation of the promoter by MYB46 was measured by assaying *GUS* activity after 15-h of incubation. The expression level of the *GUS* reporter gene in the protoplasts transfected with no effector was used as control and was set to 1. Error bar indicates SE of three biological replications

multiple M46REs in their promoter regions (Tables 1 and 2), suggesting that these genes are likely directly regulated by MYB46. Three (*AtC3H14*, *KNAT7* and *MYB63*) of the five transcription factors on the list have been shown to be activated by MYB46 in vivo (Ko et al. 2009). EMSA results show that MYB46 specifically bind to the promoter of *KNAT7* (Kim et al. unpublished data). Interestingly, many of cell wall modification, secondary wall biosynthesis genes (e.g., cellulose, xylan and lignin) or cellular processes related to cell wall biosynthesis, such as cytoskeletal

organization and signal transduction, were present on this list. Specifically, the genes on the list include three secondary wall-associated cellulose synthase (*IRX1/AtCesA08*, *IRX3/AtCesA07* and *IRX5/AtCesA04*) (Taylor et al. 2003), xylan biosynthesis genes (*IRX8*, *IRX9*, *IRX14*, and *IRX15-L*) (Peña et al. 2007; Wu et al. 2010; Jensen et al. 2010; Brown et al. 2011), three laccase (*IRX12/laccase4*, *laccase10* and *laccase11*) involved in lignin biosynthesis (Brown et al. 2005), two cytoskeleton-related genes (*Myosin5*, microtubule-associated protein) (Kaneda et al. 2010; Pesquet et al. 2010), and two protein kinases (*At1g24030* and *At1g03920*). In addition, the list includes two DUF579 (*At1g33800* and *At4g09990*), which are homologous to two xylan biosynthesis genes *IRX15* and *IRX15-L* (Jensen et al. 2010; Brown et al. 2011). This result suggests that MYB46 may directly regulate not only transcription factors but also structural genes of secondary wall biosynthesis as Zhong et al. (2010) have shown with *ANAC012/SND1*. Furthermore, most of the putative direct targets of MYB46 were co-expressed with secondary wall-associated cellulose synthases (*AtCesA4*, *AtCesA7* and *AtCesA8*), and up-regulated by as well as co-expressed with *ANAC012*, a direct upstream regulator of MYB46 (Table 2).

Discussion

Secondary wall biosynthesis is one of the most important biological processes on Earth because secondary walls constitute the majority of lignocellulosic biomass, which is of primary importance to humans as feedstock for biomaterials and as an environmentally cost-effective renewable source of energy. Recent studies have demonstrated that MYB46, one of the direct targets of *ANAC012*, functions as a master regulator of secondary wall biosynthesis (Zhong et al. 2007; Ko et al. 2009). However, the mechanism by which MYB46 activates the transcription of downstream target genes is largely unknown. Identification of MYB46 target sequences in the promoter of downstream genes will facilitate our efforts to understand the mechanism.

A recent report described that MYB46 strongly binds to the promoter region of *AtC3H14*, a CCCH-type zinc finger protein (Ko et al. 2009). To identify the *cis*-acting regulatory motif recognized by MYB46, we used the promoter sequence of *AtC3H14* gene. First, we successfully confirmed the strong interaction between MYB46 and the promoter sequence (pro_AtC3H14-S2; Fig. S1). And then, a series of competitive EMSAs with various deletions of the promoter were carried out to narrow down the binding region to a 14-nucleotide motif (Pro_AtC3H14-core; Figs. 1, 2, 3). Subsequent EMSAs with a series of point mutations within the sequence identified an eight-nucleotide motif, RKTWGGTR, as a MYB46-responsive

Fig. 6 Chromatin immunoprecipitation (ChIP) analysis of MYB46 binding to the *AtC3H14* promoter sequence in planta. **a** Diagram of the vector construct used for inducible expression of MYB46-GFP in the ChIP analysis. **b** Real-time PCR analysis shows the enrichment of the *AtC3H14* promoter sequence after chromatin immunoprecipitation. The values were normalized against that of the control DNA (promoter of MYB46). MYB54 promoter was used as a negative control which does not have M46RE. Error bars represent SE from three biological replicates. **c** Leaf-curling phenotype was observed after 24 h of DEX treatment. **d** Primer sequences used for the ChIP experiments

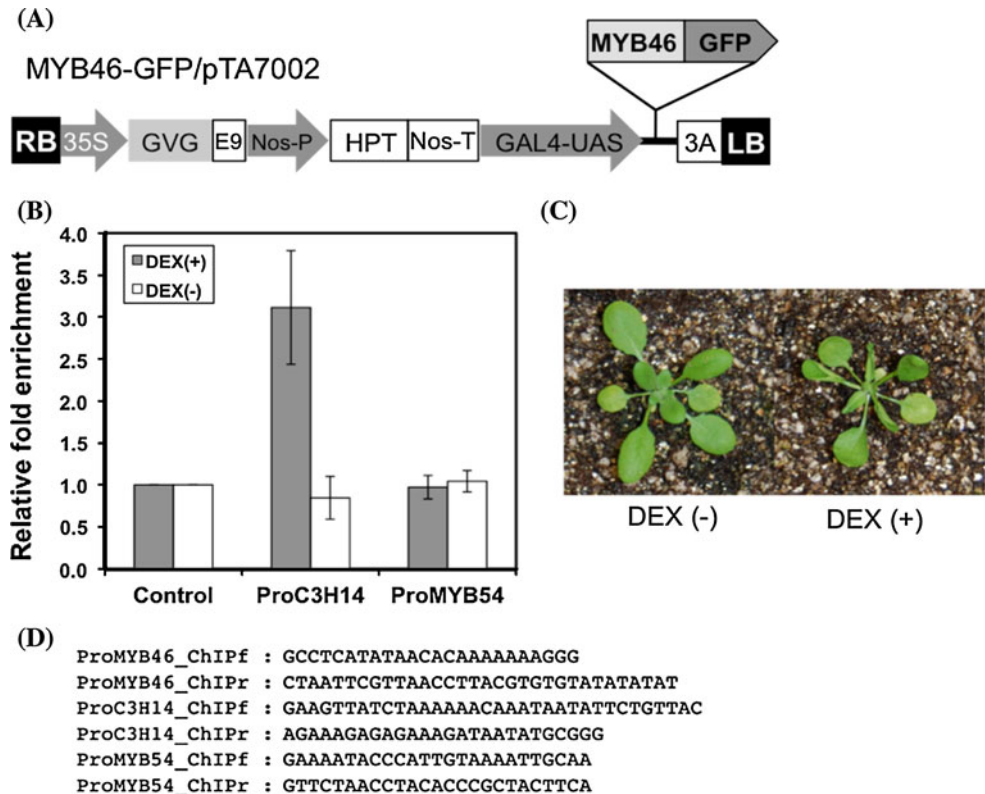


Table 1 Global analysis of genes having M46RE in the promoter region

	Whole transcriptome ^a			Upregulated by MYB46 ^b		
	No. of genes	No. of genes with M46RE	Frequency (%)	No. of genes	No. of genes with M46RE	Frequency (%)
Total	22,591	9,836	43.5	414	250	60.4
Stem abundant genes ^c	283	162	57.2	61	44	72.1
Co-expressed genes ^d with Cellulose synthases	123	81	65.9	36	29	80.6
CesA and CSL genes ^e	39	19	48.7	5	5	100.0
Xylan biosynthesis ^f	10	7	70.0	9	7	77.8
Lignin biosynthesis ^g	353	187	53.0	23	21	91.3
Co-expressed genes ^h with MYB46	299	182	60.9	52	37	71.2

Motif search performed using Patmatch(<http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl>) with TAIR10 Loci Upstream Sequences (1 kb)

^a ATH1 genome array (Affymetrix) was Used

^b Upregulated (>3X) genes within 6 h of MYB46 induction (Ko et al. 2009)

^c Genes abundantly expressed in stem (Ko et al. 2006)

^d Top 100 co-expressed genes with secondary wall specific cellulose synthases (either AtCesA4 or AtCesA7 or AtCesA8) identified from ATTED-II (<http://atted.jp/>)

^e Cellulose synthase A (CesA) and cellulose synthase like (CSL) gene family (listed in supplemental Table S2)

^f Genes involved in xylan biosynthesis (listed in supplemental Table S3)

^g Genes involved in lignin biosynthesis including putative transporters and polymerization-related (Shi et al. 2010; listed in supplemental Table S4)

^h Top 300 co-expressed genes with MYB46 identified from ATTED-II (<http://atted.jp/>)

cis-regulatory element (M46RE) (Fig. 3). The *in vivo* activation analyses indicated that M46RE was both necessary and sufficient for MYB46-responsive transcription

(Figs. 4, 5). Furthermore, we demonstrated that MYB46 directly binds to the *AtC3H14* promoter in planta using a chromatin immunoprecipitation analysis (Fig. 6). We also

Table 2 Putative direct targets of MYB46

AGI ^a	Gene description	Function ^b	TAA ^c	M46RE (1 kb) ^d	UP by ^e ANAC012	Co-ex with ANAC012 ^f	Co-ex with CesA ^g
At4g18780	IRX1/AtCesA08	SCW	YES	2	YES	YES	YES
At5g17420	IRX3/AtCesA07	SCW	YES	2	YES	YES	YES
At5g44030	IRX5/AtCesA04	SCW	YES	3	YES	YES	YES
At5g15630	IRX6/COBRA-LIKE (COBL4)	SCW		1	YES	YES	YES
At5g54690	IRX8/Glycosyltransferase family 8	SCW	YES	2	YES	YES	YES
At2g37090	IRX9/Glycosyltransferase family 43	SCW	YES	1	YES	YES	YES
At2g38080	IRX12/Laccase4	SCW		1	YES	YES	YES
At4g36890	IRX14/Glycosyltransferase 43	SCW		1		YES	YES
At5g67210	IRX15-L/unknown function DUF579	SCW		3	YES	YES	YES
At3g16920	ArCTL2, Glycoside hydrolase family 19	SCW		1	YES	YES	YES
At5g03170	FLA11(fasciclin-like arabinogalactan-protein)	SCW		1	YES	YES	YES
At4g33330	Glycosyltransferase family 8/PGSIP3	SCW		2	YES	YES	YES
At3g03690	Glycosyltransferase family 14	SCW		2			
At5g01190	Laccase 10 (LAC10)	SCW	YES	3	YES	YES	YES
At5g03260	Laccase 11 (LAC11)	SCW		1	YES		YES
At1g66810	AtC3H14; CCCH-type zinc finger	TR	YES	2		YES	YES
At5g16600	AtMYB43	TR		2		YES	YES
At1g16490	AtMYB58	TR		2		YES	
At1g79180	AtMYB63	TR	YES	3	YES	YES	
At1g62990	KNAT7	TR	YES	2		YES	
At2g20650	C3HC4-type RING finger	UNK		1		YES	YES
At3g62020	Germin-like protein (GLP10)	UNK		1	YES	YES	YES
At5g57070	Hydroxyproline-rich glycoprotein	UNK		1			
At4g39320	Microtubule-associated protein-related;	UNK		2	YES		
At2g31900	MYOSIN 5 (ATMYO5)	UNK		2	YES	YES	YES
At5g46340	O-acetyltransferase-related;	UNK		2		YES	YES
At2g38060	PHOSPHATE TRANSPORTER4;2	UNK		2	YES		
At1g24030	Protein kinase family protein	UNK		2	YES	YES	YES
At5g06930	Similar to nucleolar protein gar2-related	UNK		1		YES	YES
At1g03920	Similar to protein kinase family protein	UNK		2	YES		
At5g55950	Transporter related	UNK		1	YES	YES	
At5g60720	Unknown function DUF547	UNK		1	YES	YES	YES
At1g33800	Unknown function DUF579	UNK		1	YES	YES	YES
At4g09990	Unknown function DUF579	UNK		4		YES	YES
At2g27740	Unknown function DUF662	UNK		2	YES		YES
At1g63520	Unknown protein	UNK		1		YES	YES
At5g46115	Unknown protein	UNK		2		YES	

^a Arabidopsis Gene Index^b SCW, secondary wall biosynthesis; TR, transcriptional regulation; UNK, unknown function^c Activated by in vivo transient activation analysis (Ko et al. 2009)^d Number of M46RE in the promoter region^e Upregulated (>3X) genes by ANAC012 over-expression (Ko et al. 2007)^f Top 300 co-expressed genes with ANAC012 identified from ATTED-II (<http://atted.jp/>)^g Top 100 co-expressed genes with secondary wall specific cellulose synthases (either AtCesA4 or AtCesA7 or AtCesA8) identified from ATTED-II (<http://atted.jp/>)

examined the expression level of AtC3H14 in a T-DNA knockout mutant *myb46* (SALK_100993C) and found no changes in its expression (Fig. S3). This was expected because, in addition to MYB46, there are other upstream regulators (e.g., MYB83 and ANAC012 of AtC3H14 (Ko et al. 2009; Kim, Ko, and Han, unpublished result).

It appears that M46RE is highly enriched in the promoters of MYB46-regulated secondary wall biosynthesis genes (Table 1). A promoter sequence analysis using Patmatch (TAIR) revealed that 43.5% of the 22,591 on the ATH1 genome array have M46RE in their promoters. However, the frequency dramatically increases among the genes up-regulated by MYB46, especially the genes involved in secondary wall biosynthesis (Table 1). For example, all of the five Cesa/CSL genes (*AtCesA4*, *AtCesA7*, *AtCesA8*, *AtCslA9*, and *AtCslB2*) upregulated by MYB46 have multiple M46REs in their promoter regions. Seven of the nine MYB46-induced xylan biosynthesis genes have M46REs in their promoter region. Furthermore, 21 of the 23 lignin biosynthesis genes that are upregulated by MYB46 have multiple M46REs in their promoter regions.

Romero et al. (1998) divided MYB transcription factors into three groups (Group A, B, and C) based on sequence similarity and found that several MYB proteins in Group C, to which MYB46 belongs, bind to a consensus sequence GKTWGGTR, named MBSIIG. Rammírez et al. (2011) reported a similar cis-element (GTTAGGT) from the promoter region of a fungal pathogen-induced type III cell wall-bound peroxidase gene, Ep5C. This cis-element has the consensus sequence of both MBSIIG and M46RE and is recognized by MYB46 (Rammírez et al. 2011). A *myb46* mutant showed enhanced induction of Ep5C gene following fungal pathogen infection and subsequent resistance to the pathogen. This observation is interesting because there appears to be no functional redundancy between MYB46 and its close homolog MYB83, which also binds to the M46RE. Whether the GTTAGGT motif is specific to MYB46 and, therefore, not recognized by MYB83 remains to be tested. Moreover, M46RE is complementary to a class of CA-rich regulatory elements named ACI and ACII (i.e., AC element), which are known to mediate transcriptional activation of lignin biosynthetic genes (Hatton et al. 1995; Raes et al. 2003). For example, PtMYB4 and EgMYB2 bind these elements to activate AC element-dependent transcription of lignin biosynthesis genes (Patzlaff et al. 2003; Rahantamalala et al. 2010). It appears that M46RE serve as a cis-acting element responsible for MYB46-mediated regulation of not only lignin biosynthesis but also cellulose and xylan biosynthesis (Tables 1, 2). Recently, Winzell et al. (2010) proposed that a conserved CA-rich motif (CCACCAAC, almost identical to ACII) is responsible for PtxtMYB021 (MYB46-like gene from

hybrid aspen)-mediated transcriptional regulation of xylan-active CAZymes GT43A, GT43B and Xyn10A. This fact further supports our hypothesis that M46RE may function as a cis-acting element in MYB46-mediated transcriptional regulation of secondary wall biosynthesis.

One possible utility of M46RE is in the identification of potential direct target genes of MYB46 in a genome-wide promoter sequence analysis. We identified 37 putative direct target genes of MYB46 based their spatio- and temporal expression pattern and the presence of M46RE motif in their promoters (Table 2). This list includes not only downstream transcription factors but also a number of structural genes involved in secondary wall biosynthesis, cell wall modification, and unknown function (Table 2). Secondary wall-associated cellulose synthases (*AtCesA4*, *AtCesA7* and *AtCesA8*; Taylor et al. 2003) on the list contain M46REs in their promoters, which serve as a binding site for MYB46 (Kim, Ko, and Han, unpublished results). This finding suggests that MYB46 may directly regulate some of the structural genes involved in secondary wall biosynthesis. Similarly, ANAC012/SND1, a direct upstream regulator of MYB46, has been suggested to regulate not only downstream transcription factors but also the structural genes involved in secondary wall biosynthesis (Zhong et al. 2010). However, the direct targets of ANAC012/SND1 that Zhong et al. (2010) identified do not overlap with those of MYB46 except *laccase11* (At5g03260), although most of the putative direct targets of MYB46 are up regulated and co-expressed with ANAC-12/SND1 (Table 2). This finding suggests that ANAC012- and MYB46-mediated transcriptional regulation of secondary wall biosynthesis is closely associated but separated, further confirming the multifaceted and complex nature of the transcriptional regulation.

Lignocellulosic biomass, consisting mainly of secondary wall, is gaining research interest as a feedstock for production of bio-based materials and biofuel. However, our understanding of the molecular mechanisms underlying its biosynthesis is limited. In this study, we identified a cis-acting element, M46RE, which plays a critical role in the interaction of MYB46, a master regulator of secondary wall biosynthesis, and its downstream target genes. The findings in this study will facilitate our efforts to better understand the transcriptional regulatory network that controls secondary wall biosynthesis.

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