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Short communications

# Transcription factor MYB46 is an obligate component of the transcriptional regulatory complex for functional expression of secondary wall-associated cellulose synthases in *Arabidopsis thaliana*

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ABSTRACT

Cellulose, the most abundant biopolymer on Earth, is a central component in plant cell walls and highly abundant (up to 50%) in the secondary walls. In *Arabidopsis thaliana*, the cellulose biosynthesis in the secondary walls is catalyzed by three cellulose synthases *CESA4*, *CESA7* and *CESA8*. The transcription factor MYB46 and its close homolog MYB83 directly regulate the expression of the three secondary wall cellulose synthases (CESAs). However, it is not known whether MYB46 is the necessary regulator for functional expression of the secondary wall *CESA*s or one of the multiple transcriptional factors involved in the transcriptional regulatory program. To address this question, we used a series of genetic complementation experiments of the *cesa* knock-out mutants with the *CESA* coding sequence driven by either native- or mutated promoter of the genes. The mutant promoters have two nucleotide point mutations in the MYB46 binding *cis* element (M46RE) such that MYB46 cannot bind to the promoter, while the binding of other known secondary wall transcription factors is not affected. The mutant complementation results showed that MYB46 is essential to restore normal phenotype from the *cesa* mutants. We conclude that MYB46 is an obligate component of the transcriptional regulatory complex toward the commitment of secondary wall cellulose synthesis in *Arabidopsis*.

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## Introduction

Cellulose is a vital component of the load-bearing structure (e.g., xylem fibers) that allows the plant to grow tall. In properly functioning xylem vessels, a thick secondary wall is critical to resist the forces of tension associated with the transpirational pull on a column of water. Economically, cellulose is of primary importance to humans as feedstock for fiber, solid wood products, bioenergy, and industrial compounds.

Cellulose is synthesized by multi-meric cellulose synthase (CESA) complexes at the plasma membrane (Somerville, 2006). In the secondary cell walls of *Arabidopsis*, cellulose is synthesized by only three CESAs (CESA4, CESA7, and CESA8) as catalytic subunits of the complex, although the *Arabidopsis* genome contains 10 *CESA* genes (Turner and Somerville, 1997; Doblin et al., 2002; Williamson et al., 2002; Taylor et al., 2003). The three *CESA*s are specifically expressed in the secondary wall forming tissues (Taylor et al., 1999, 2003; Doblin et al., 2002; Williamson et al., 2002; Ko et al., 2004), suggesting that they may be under strict transcriptional control.

Transcription factor (TF) MYB46 and its close homolog MYB83 have been reported as a master regulator of secondary wall formation in *Arabidopsis* (Zhong et al., 2007; Ko et al., 2009, 2012). Two MYB46-responsive *cis*-regulatory elements, M46RE (Kim et al., 2012) and SMRE (Zhong and Ye, 2012), have been reported as responsible for MYB46/MYB83 binding. The two regulatory motifs are almost identical, except for the 2-bp difference in length (i.e., M46RE is shorter than SMRE); despite they were identified in different promoters. The promoter sequences of the three secondary wall *CESA*s (*CESA4*, *CESA7*, and *CESA8*) contain multiple copies of M46RE, suggesting that they might be directly regulated by MYB46. Subsequent investigation provided experimental evidences in

*Abbreviations:* CDS, coding sequence; CESA, cellulose synthase A; TF, transcription factor.

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support of the hypothesis that MYB46/MYB83 binds to the M46RE and directly regulates the expression of the three secondary wall *CESA* genes in *Arabidopsis* (Kim et al., 2013). However, it is not known how critical this MYB46-mediated regulation of the *CESA*s is in the transcriptional control of secondary wall cellulose biosynthesis. Is MYB46 necessary for the functional-level expression of the three *CESA*s *in planta*? It has been suggested that the transcriptional network regulating secondary wall biosynthesis involves a multi-layered feed-forward loop regulatory structure (Zhong et al., 2010). For example, secondary wall-associated NAC transcriptional factors (e.g., ANAC0123/SND1, VND6, VND7) and their direct targets MYB46/MYB83 can each independently and directly activate a set of common downstream targets in the secondary wall biosynthesis pathway, including secondary wall CESAs (Ohashi-Ito et al., 2010; Zhong et al., 2010; Yamaguchi et al., 2011; Ko et al., 2012). However, it is unknown whether these TFs alone (i.e., without MYB46) can activate the expression of the secondary wall CESAs *in planta*.

To address this question, we carried out genetic complementation of T-DNA insertion mutants of the *CESA*s with the *CESA* coding sequence (CDS) driven by either native- or mutated promoter of the genes. The mutated promoters were created by introducing point mutations in the M46RE such that MYB46 can no longer recognize and bind to the promoter. Here, we report the mutant complementation results showing that MYB46/MYB83 is required for functional expression of the three secondary wall *CESA*s.

## Materials and methods

### Plant materials and growth conditions

*Arabidopsis thaliana* (L.) Heynh ecotype Columbia (Col-0) and three T-DNA insertional mutants of *cesa* [*cesa4* (SALK_084627), *cesa7* (SALK_029940) and *cesa8* (SALK_026812)] (Fig. S1) were used in the experiments. Plants were grown on soil in a growth chamber (16 h light/8 h dark) at 23 °C. All experiments were performed in triplicates and repeated at least three times.

### Plasmids construction and plant transformation

All of the constructs used in this study were verified by DNA sequencing. The CDSs of *CESA4* (At5g44030), *CESA7* (At5g17420) and *CESA8* (At4g18780) were PCR-amplified from stem cDNAs of *Arabidopsis*. For the genetic complementation, the PCR-amplified CDS was fused with either native or mutated promoter of the *CESA*s (Fig. 1). The mutated promoter was created by PCR-based point mutations of the two base pairs critical in the M46RE (Kim et al., 2012) as shown in Fig. 1. The primers used in this

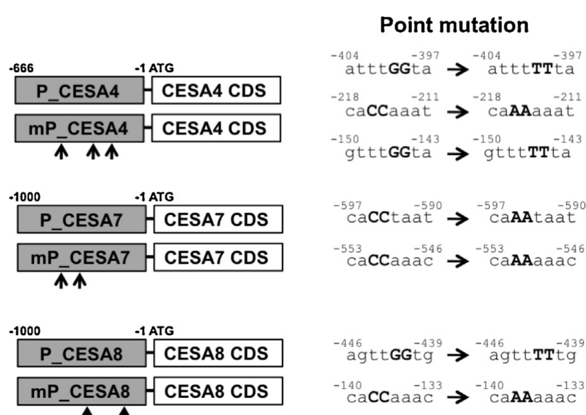


**Fig. 1.** Point mutations in the promoters of *CESA4*, *CESA7* and *CESA8*. The vertical arrows indicate the locations of the mutation points.

experiment are listed in Table S1. The resulting fragment was introduced into a binary vector pCB308 (Xiang et al., 1999) and used in the *Agrobacterium*-mediated transformation of both wild-type *Arabidopsis* (Col-0) plants and *cesa* T-DNA insertion mutants. Homozygocity of these *cesa* mutants and their genetic complementation were confirmed by genomic DNA PCR (Fig. 2).

### RNA extraction and RT-PCR

Total RNAs were extracted using Plant RNeasy extraction kit (Qiagen) according to the manufacturer's protocol. For RT-PCR analysis, total RNAs were first treated with DNaseI before the first-strand cDNA synthesis by SuperScript II Reverse Transcriptase (Invitrogen). RT-PCR was carried out using 1 μL of the reaction products as a template. Amplified DNA fragments were separated on 1% agarose gel and stained with ethidium bromide. The primers used for RT-PCR are shown in Table S1.

### Histological analysis

The stem area located immediately above the rosette leaves (basal level) was harvested, embedded and cross-sectioned using Microtome (Leica RM2025) into thin sections (5 μm thick) and paraffin embedded as described previously (Ko et al., 2004, 2007). The sections were then stained with 0.05% toluidine blue *O* for 1 min to visualize secondary xylem.

## Results and discussion

We obtained the T-DNA insertional mutants of three secondary wall *cesa* (*cesa4*, *cesa7*, and *cesa8*) from ABRC (http://abrc.osu.edu/) (Fig. S1). We transformed the *cesa* mutants with its corresponding *CESA's* CDS driven by either its native- or mutated promoter (Fig. 1). The genomic complementation was confirmed using genomic PCR (Fig. 2). The mutated promoters have two point mutations in the M46RE, which effectively eliminate MYB46 binding (Kim et al., 2012), resulting in failure of *CESA* expression (Fig. 3). All of the mutants used in the study displayed previously described phenotypes such as collapsed/irregular xylem and pendent stem (Turner and Somerville, 1997; Taylor et al., 1999, 2004; Zhong et al., 2003). The three *CESAs* (*CESA4*, *CESA7* and *CESA8*) are required for cellulose synthesis in the secondary walls of *Arabidopsis* plants, each of them appears to be equally important in the function of the complex and they cannot substitute each other (Gardiner et al., 2003). Therefore, even a single T-DNA insertion mutant of one of the three *CESA* genes results in a severe phenotype (Figs. 3 and 4).

Both wild-type and vector control plants grew upright and appeared to be normal. On the other hand, the *cesa* mutants showed retarded growth and the characteristic 'pendent stem' phenotype (Fig. 3) and collapsed xylem (Fig. 4). As expected, transgenic plants expressing native promoter-driven *CESAs* restored wild-type phenotype. However, the genetic complementations with the mutant promoters, which cannot be recognized by MYB46, showed mutant phenotypes (i.e., pendent stem and collapsed xylem phenotype) (Figs. 3 and 4). These results suggest that MYB46 binding to the M46RE is required for functional expression of the secondary wall *CESAs in planta*.

TF MYB46 and its orthologs have been shown to be master switches for the biosynthesis of the three major components of secondary walls (e.g., cellulose, hemicellulose, and lignin) in *Arabidopsis*, poplar, rice and maize (Zhong et al., 2007, 2011; Ko et al., 2009, 2012; McCarthy et al., 2010; Zhong and Ye, 2012). Furthermore, MYB46 has been recently shown to be a direct regulator of all three secondary wall *CESA* genes (*CESA4*, *CESA7* and *CESA8*) (Kim et al., 2013). TF MYB83, a close homolog of MYB46, is functionally redundant with MYB46 and also requires M46RE (Zhong and

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