



The activation of the NAC transcription factor by Poplar *PtrGATA9* facilitates the secondary cell wall deposition of interfascicular fiber cell in *Arabidopsis*

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Abstract

The secondary cell wall (SCW) in plant is vital for the plant growth, as it contributes to their physical resilience, aids in the movement of water and nutrients, and supports overall plant development. A transcriptional regulatory layer carefully governs the precise spatial and temporal deposition of SCW components within specific plant cells. Even though the regulatory mechanism of SCW biosynthesis in *Arabidopsis* is well comprehended, studies on woody perennials remain limited. In this study, we characterized Poplar *PtrGATA9* (Potri.001G188500), the poplar homolog of *Arabidopsis AtGATA9* (AT4G32890), in regulating SCW biosynthesis. Over-expression of *PtrGATA9* resulted in SCW thickening in interfascicular fiber cells, which was due to upregulated transcriptional expression of NAC-domain transcription factors and SCW biosynthetic genes downstream of NAC-domain transcription factors. Furthermore, the transfection assay of *Arabidopsis* protoplasts revealed that *PtrGATA9* acts as a transcriptional activator for NAC-domain transcription factors, which are recognized as key master regulators of SCW biosynthesis. This suggests that *PtrGATA9* functions as an upstream transcription factor for the NAC-domain transcription factors. Our findings offer valuable insights into the control of SCW biosynthesis in woody perennial plants and the potential significance of *PtrGATA9* in enhancing plant growth and development.

Keywords *Arabidopsis* · NAC-domain transcription factor · Poplar · *PtrGATA9* · Secondary cell wall (SCW)

Introduction

Plant cells meticulously regulate the synthesis, deposition, and remodeling of their surrounding cell wall throughout response to diverse environmental conditions. Plant cells possess two distinct types of cell walls: the primary cell wall (PCW) and the secondary cell wall (SCW). During cell expansion, the PCW is deposited, providing flexibility and

tensile strength to prevent cell bursting. After cellular expansion, particular cell types undergo the deposition of a SCW positioned between the PCW and the plasma membrane. This SCW structure comprises a rigid framework consisting of polysaccharides and a phenolic polymer. It imparts physical strength, enabling upright growth, and plays a crucial role in essential biological processes such as anther dehiscence, defense against pathogens, the transportation of nutrients, and silique shattering (Lucas et al. 2015; Kumar et al. 2016). Moreover, the SCW finds extensive applications as biomass (Furtado et al. 2014).

The SCW biosynthesis in specialized cells involves a complex transcriptional regulatory network (Wang and Dixon 2012; Hussey et al. 2013; Ko et al. 2014; Zhong and Ye 2015a, b). In *Arabidopsis* have confirmed the numerous NAC-domain and MYB-domain transcription factors were involved in SCW biosynthesis, leading to the establishment of a MYB-NAC-mediated transcriptional regulatory network (Nakano et al. 2015). NAC (NAM, ATAF, and CUC) transcription factor possess a conserved NAC-domain in their N-terminal region and have highly diverse C-terminal

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activation domain, playing a wide range of roles in plant defense, growth, and development (Olsen et al. 2005). Notably, the NAC sub-groups associated with secondary cell wall formation, ANAC012, NST1, NST2, VND6, and VND7, have been identified as key master regulators of this pathway (Ko et al. 2007; Mitsuda et al. 2007; Yamaguchi et al. 2010, 2011; Mousavi et al. 2014; Zhong and Ye 2015a, b). These master regulators directly influence MYB transcription factors (MYB46, MYB83, MYB103) (Kim et al. 2013; Chen et al. 2021), modulating gene expression through activation or repression mechanisms by interacting with specific promoter sequences of genes involved in SCW biosynthesis. However, the upstream signaling pathway leading to the activation of these master regulators remains unclear.

Throughout their entire life cycle, plants encounter diverse environmental stimuli, including light and temperature, which are vital factors for their growth and development. Plants demonstrate three fundamental responses or reactions to light, including photosynthesis, phototropism, and photoperiodism. Light also impacts various aspects of plant morphology, such as hypocotyl and root length, flowering, and leaf shape, as evidenced by studies (Kami et al. 2010; Lin et al. 2018; Kim et al. 2022, 2023). Furthermore, plants grown in shaded conditions exhibit reduced cell wall thickening and enhanced cell elongation, while exposure to UV light results in smaller leaves, thicker walls, and cuticles in epidermal cells (Gall et al. 2015; Wu et al. 2017). Additionally, the transcript of genes encoding enzymes involved in lignin biosynthesis are subject to significant fluctuations due to the circadian clock (Rogers et al. 2005). These findings indicate that light influences the formation of SCW. However, the precise mechanism by which light mediates SCW biosynthesis remains unknown.

Higher plants possess the ability to integrate signals from both their environment and internal factors to regulate gene expression, ensuring optimal development (Wang et al. 2016). Transcriptional control of external stimuli plays a crucial role in integrating these exogenous and endogenous signals, with the promoter region, an upstream regulatory DNA sequence of the transcriptional region, playing a significant role in determining whether and when transcription takes place. Key functional components of the promoter are short DNA sequence motifs that facilitate protein binding. Light-responsive elements (LREs) within the promoter mediate the regulation of gene expression by light. Consensus sequence elements (GATA, GT1, G, Z motif) are observed in the promoter of light-regulated genes. Furthermore, mutagenesis studies have demonstrated the essentiality of these elements for high promoter activity under light conditions (Arguello-Astorga and Herrera-Estrella 1996; Puente et al. 1996; Jiao et al. 2007; Luo et al. 2013).

The GATA motif, among these elements, serves as a binding site for GATA transcription factors. These transcriptional

regulators are found in plants, metazoans, and fungi, and they typically recognize the consensus sequence element (A/G)GATA(A/G). The GATA transcription factors consists of a type IV zinc-finger domain with the pattern $CX_2CX_{17-20}CX_2C$ (Lowry and Atchley 2000; Teakle et al. 2002; Reyes et al. 2004; Manfield et al. 2007). Moreover, GATA transcription factors are known to be influenced by light and regulated by the circadian clock (Naito et al. 2007). Previous research indicates that many GATA transcription factors exhibit light-dependent regulation and display rhythmic expression throughout the circadian cycle (Manfield et al. 2007). For instance, some of them regulate hypocotyl elongation, greening, GA biosynthesis, germination, flowering, senescence, or abscission in Arabidopsis (Behringer and Schwechheimer 2015).

Despite the significant discovery, there remains limited understanding regarding the role of GATA transcription factors in perennial woody plants. Previous studies have highlighted that *Populus trichocarpa*, a woody perennial plant, is an appropriate model system for investigating ecological interactions, perenniality, wood development, and phenology (Groover 2005; Jansson and Douglas 2007). With the growing interest in utilizing lignocellulose as a biomass source, understanding the control of SCW biosynthesis is crucial for guiding genetic modification strategies in energy crops like poplar (Demura and Ye 2010). While several GATA transcription factors have been identified in *Oryza* and *Arabidopsis*, their presence in poplar has been rarely reported. In this study, we present evidence demonstrating the functional role of the *PtrGATA9* from *Populus trichocarpa* in regulating NAC-domain transcription factors involved in the SCW biosynthesis pathway.

Materials and methods

Preparation of plant materials and growth condition

A growth room was utilized to cultivate *A. thaliana* ecotype Col-0 plants under long-day conditions, characterized by a 16-h light and 8-h dark condition at 23 °C for both wild type and transgenic experiments. Transgenic plants were generated using the *Agrobacterium tumefaciens* strain GV3101 via the floral dip method as described (Zhang et al. 2006). The collected T_0 seeds of the transgenic plants were surface sterilized and planted on agar plates (0.5 X Murashige and Skoog ($1/2$ MS) composition containing kanamycin (50 μ g/ml). Subsequently, the seeds were subjected to a 2-day cold treatment in the dark at 4 °C. Following the cold treatment, the seeds were transferred to a growth chamber where they were exposed to continuous light for 6 h, followed by 48 h of darkness at 23 °C. Subsequently, the seeds were incubated in continuous light for at least 12 h before surviving transgenic

plants were selected and their seedlings were transferred to soil (Harrison et al. 2006). Finally, selected T_1 generation transgenic plants derived from the T_0 generation transgenic plants was used for the further assessment.

Genomic DNA extraction of polymerase chain reaction (PCR)

In order to verify the presence of the introduced gene in the transgenic plants, genomic DNA was extracted from young rosette leaves and used in PCR-amplified reaction. The young rosette leaves from Arabidopsis were ground by using plastic pestle and were added 300 μ l of modified Edwards solution (400 mM LiCl, 200 mM Tris-HCl, 25 mM EDTA, 1% (w/v) SDS, pH 9.0) in 1.5 ml Eppendorf tube (referred to as E-tube). The samples were then subjected to centrifugation at 13,000 \times g for 10 min, and the resulting supernatant (250 μ l) was transferred to a new E-tube and mixed with the equal volume of isopropanol. Following centrifugation at 13,000 \times g for 20 min, the supernatants were discarded, and the resulting pellet was washed with 700 μ l of 70% (v/v) ethanol, followed by centrifugation at 13,000 \times g for 5 min. The pellet was then dissolved in 100 μ l of DNase-free distilled water.

The PCR reaction condition as follows: initial denaturation at 94 $^{\circ}$ C for 5 min; followed by 34 cycles of denaturation at 94 $^{\circ}$ C for 20 s, annealing at 55 $^{\circ}$ C for 30 s, and elongation at 72 $^{\circ}$ C for 1 min; then extension step at 72 $^{\circ}$ C for 5 min. The PCR products were displayed using DNA electrophoresis on 1% (w/v) agarose gel.

Phylogenetic analysis of GATA transcription factor and protein sequence alignment

The amino acid sequences were obtained from The Arabidopsis Information Resource (TAIR) and National Center for Biotechnology Information (NCBI). Subsequently, the obtained amino acid sequences were subjected to analysis using the ClustalW (BioEdit software). A phylogenetic tree was constructed utilizing the neighbor-joining method with 1000 bootstrap replicate in MEGA X software. Additionally, the deduced amino acid sequence similarity between *PtirGATA9* (Accession number: XP_052305966) and *AtGATA9* was assessed through Multiple Sequence Alignment using ClustalW.

cDNA synthesis and reverse-transcriptase PCR (RT-PCR) analysis

The segments located 0.5 cm from bottom of inflorescence stem of plant were ground in a E-tube to a fine power with a pestle under liquid nitrogen (LN₂). Total RNA was extracted from the collected inflorescence stem samples using TRIzol

reagent. Briefly, the rosette leaves crushed in E-tube and 1 mL of TRIzol reagent was added, followed by the addition of 200 μ l of a 24:1 (v/v) chloroform/isoamyl-alcohol mixture. The E-tubes were thoroughly vortexed and subsequently centrifuged at 13,000 \times g for 10 min. The resulting supernatant (300 μ l) was transferred to a fresh E-tube and mixed with a 300 μ l of isopropanol. After inversion, the E-tubes were centrifuged at 13,000 \times g for 20 min, and the resulting pellet was washed with 700 μ l of 70% (v/v) ethanol. The E-tubes were then centrifuged at 13,000 \times g for 5 min and the DNA precipitation was resuspended in 25 μ l of RNase-free distilled water. The concentration of total RNA was determined by measuring the A_{260}/A_{280} ratio using a NanoDrop ND-2000 spectrophotometer (ThermoFisher Scientific).

The total RNA extracted using the above method was used as a template for cDNA synthesis. For cDNA synthesis, 2 μ g of total RNA was reverse transcribed using SuperScript II working solution (4 μ M oligo (dT) primer, 0.5 mM dNTP, 50 mM Tris-HCl, 3 mM MgCl₂, 75 mM KCl, 10 mM dithiothreitol (DTT), pH 8.3). The synthesized cDNA was used as a template for gene expression analysis. For RT-PCR, 1 μ l of synthesized cDNA was used with Taq Smart mix (SolGent, Korea). All experiments were amplified in three parallel reaction as biological replicates, and the *ACTIN 8* gene was amplified as a control gene. The primers used for the expression analysis of SCW biosynthesis genes are listed in Supplementary Table 1.

For quantitative the bands, 10 μ l of the RT-PCR products was loaded onto a agarose gel containing final concentration of 1 μ g/ml ethidium bromide in TAE buffer (200 mM Tris-Acetate, 5 mM EDTA, pH 8.0), followed by electrophoresis to visualize the agarose gel. Pixel intensities of the bands were quantified using the Image J software, and all data were normalized based on the pixel intensities of *ACTIN8* gene bands.

Gene cloning and plasmid construct

The *PtirGATA9* genomic DNA fragment was amplified with specific primers using *PtirGATA9/pK2WG7* vector in *A. tumefaciens* strain GV3101. For effector construct of transcriptional activation analysis (TAA) experiment, the PCR products of *PtirGATA9* were inserted into cloning vector pTOP Blunt V2 vector using TOPcloner TA-Blunt kit (Enzymomics, Korea) and introduced into *Escherichia coli* (DH5 α). After the sequence was confirmed, the fragments were placed between the CaMV 35S promoter and NOS terminator in the pTrGUS vector.

For the reporter construct of TAA, genomic DNA was extracted from *A. thaliana* using modified Edwards solution, and the promoters of SCW biosynthesis genes (*ANAC012pro*, *NST1pro*, *NST2pro*) were amplified with

specific primers. The promoters were then inserted into cloning vector pTOP Blunt V2 vector and introduced into *E. coli* (DH5 α), and the sequence was confirmed. The promoter fragments were ligated in front of the GUS reporter gene after removing the CaMV 35S promoter of pTrGUS vector. The primers used for PCR amplification are listed in Supplementary Table 2.

Protoplast isolation and transcriptional activation analysis (TAA)

Transcriptional activation analysis was conducted by utilizing *Arabidopsis* mesophyll protoplasts. *Arabidopsis* mesophyll protoplast isolation and the subsequent transformation of effector and reporter constructs were carried out using polyethylene glycol (PEG), following the previously described methods (Yoo et al. 2007). Protoplasts were isolated from 4-week-old *A. thaliana* leaves by cutting 30 leaves into 0.5–1 mm strips and transferring them into 10 ml of enzyme solution. The leaves were vacuum infiltrated in a desiccator for 30 min and incubated for 2 h in dark to digest the cell wall. The resulting protoplasts were diluted with 10 ml of W5 solution, filtered through nylon mesh, and placed on ice for 30 min to allow protoplasts to subside. The supernatant was then removed, and the protoplasts were suspended in 2 ml of W5 solution. The protoplasts were resuspended in 2 ml of W5 solution. The number of protoplasts was counted using a hemacytometer under a microscope, and the protoplasts were resuspended at $2 \times 10^5 \text{ ml}^{-1}$ in MMG solution. For transfection, 100 μl of protoplasts ($2 \times 10^4 \text{ ml}^{-1}$) were added to each 5 μg of effector construct and reporter construct, followed by transfection with 110 μl of PEG solution for 15 min. The transfection process was stopped by adding 400 μl of W5 solution and gently mixing by inverting. The mixture was then centrifuged at $200 \times g$ for 2 min, and the supernatant was removed. The pellet was resuspended gently with 200 μl of WI solution and incubated in the dark for 12 h. In each experiment, protoplasts transfected with only the reporter construct were used as a control. After incubation, the sample was centrifuged, and the WI supernatant was removed. To lyse the protoplasts, 100 μl of protoplast lysis buffer was added, and the mixture was vortexed, incubated on ice for 5 min, and then centrifuged. The resulting protoplast lysate (20 μl) was added to 100 μl of MUG substrate mixes and incubated for 3 h at 37 $^\circ\text{C}$ for GUS reporter assay. The enzyme reaction was stopped by adding stop solution, and GUS activity was detected by measuring fluorescence using a microplate fluorometer (Spectramax Gemini XS).

Histochemical staining

The bottom of the inflorescence stems from 6-week-old plants were manually hand-sectioned using blades. To visualize lignin, staining was carried out using Phloroglucinol-HCl (1% (w/v) phloroglucinol, 66% (v/v) ethanol, 4N HCl). Each stained sections sample was subsequently rinsed with distilled water. Optical microscopy (Leica ICC50 HD) was utilized to examine the stem cross-sections.

Scanning electron microscopy (SEM) analysis

For scanning electron microscopy (SEM), the bottom of inflorescence stem was hand-cut into 0.5 cm segments and then incubated in a formaldehyde acetic acid solution (5% (v/v) acetic acid, 3.7% (v/v) formaldehyde, 50% (v/v) ethanol) for 16 h at 4 $^\circ\text{C}$. The segments were then rinsed three times in 25 mM sodium phosphate buffer (pH 7.0) and segments were dehydrated in an ethanol series for 30 min each. The segments were then stored at 4 $^\circ\text{C}$ for 16 h before critical point drying (CPD) the next day. After completely drying the segments using a critical point dryer, segments were mounted on the stub and coated with platinum (Bomblies et al. 2008; Talbot and White 2013). The thickness of cell wall was examined with a scanning electron microscope (Hitachi SU8220) and the SCW thickness was measured using ImageJ software. Statistical analysis was performed by collecting and analyzing data from 30 cells in each of three plants, employing Student's *t*-test.

SCW components analysis

The main stems of 7-week-old wild type and *PtrGATA9* over-expression transgenic plants that had grown to 35 cm were cut into 25 cm section and dried in the dry oven set to 55 $^\circ\text{C}$. The dried samples were treated then subjected to high-temperature treatment and finely ground to be decomposed into carbohydrates and lignin using the two-step sulfuric acid hydrolysis method. Gravimetric analysis was used to analyze acid-insoluble lignin, while spectrophotometry and HPLC were employed to analyze acid-soluble lignin and carbohydrates, respectively (Sluiter et al. 2008).

Results

Phylogenetic analysis of *PtrGATA9* and poplar homologue *Arabidopsis* GATA transcription factor, and expression pattern of *AtGATA9*

To identify the relationship between *PtrGATA9* and other GATA transcription factor of *P. trichocarpa* and the model plant *Arabidopsis thaliana*, a phylogenetic tree was

constructed. The *AtGATA9* was classified as a member of subfamily I within the GATA transcription factor family by constructing a phylogenetic tree based on the amino acid sequence of the GATA domain. The subfamily classification followed the established nomenclature for GATA transcription factors (Reyes et al. 2004; Manfield et al. 2007). As a result, it was confirmed that *PtrGATA9* has homology with *AtGATA9* (Fig. 1A). The amino acid sequence similarity between *PtrGATA9* and *AtGATA9* was similar (approximately 62.2%), suggesting that the two GATA transcription factors belong to subfamily I to identify the GATA zinc-finger domain (Fig. 1B). As a result, *PtrGATA9* and other GATA transcription factors have an acidic domain and GATA zinc-finger domain (CX₂C-X₁₈-CX₂C).

GATA transcription factors are known to bind to GATA motifs, which are elements that respond to light, and their transcriptional expression is known to be regulated by light (Manfield et al. 2007). Accordingly, it was investigated

whether the gene expression of *AtGATA9*, a GATA transcription factor, was actually affected by light. 3-week-old *A. thaliana* was grown in 12-h light/12-h dark period. Sampling was carried out at 4 h intervals to identify gene expression levels. The expression level showed a specific pattern that increase when exposed to light and decreased when exposed to dark, depending on the circadian clock (Supplementary Fig. 1A). In addition, the expression levels in constant light and constant dark also showed higher levels when exposed to light than in the dark condition (Supplementary Fig. 1B). Through these results, it was suggested that the transcriptional expression of *AtGATA9* is responded to light. Therefore, it can be inferred that *PtrGATA9* is also a light-responsive transcription factor.

SCW thickening of *PtrGATA9* over-expression transgenic plants

To evaluate the growth and development of transgenic plants overexpressing *PtrGATA9*, representative weak line L74 and strong line L87 were selected from a total of 89 transgenic plants analyzed (Supplementary Fig. 2A). No observable

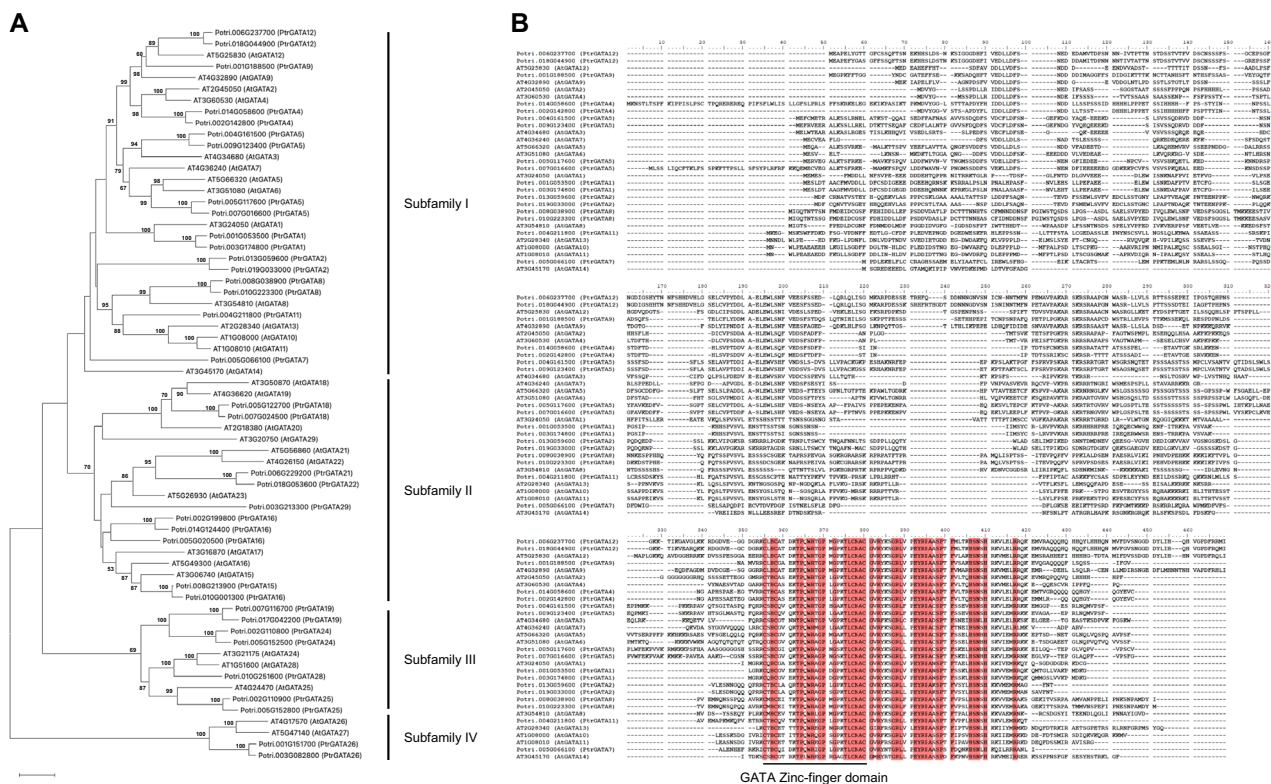


Fig. 1 Phylogenetic analysis and alignment of the deduced amino acid sequences of GATA transcription factor from *A. thaliana* and *P. trichocarpa*. **A** Phylogenetic tree of GATA transcription factor from *A. thaliana* and *P. trichocarpa*. The phylogenetic tree was generated using MEGA 11 by the neighbor-joining method. Bootstrap value

percentages of 1000 replicates are shown at the branching points. **B** Alignment of the GATA transcription factor amino acid sequence from *A. thaliana* and *P. trichocarpa* using ClustalW. Amino acid sequences conserved in all protein were scarlet shaded

differences were found in the outward appearance of *PtrGATA9* over-expression transgenic plant compared to wild type (Supplementary Fig. 2B). To explore the role of *PtrGATA9* in the regulation of SCW biosynthesis, the morphology of the SCW was observed in both wild type and *PtrGATA9* over-expression transgenic plants by sectioning the bottom part of the stem and staining the fragment with phloroglucinol-HCl. The staining displayed cinnamaldehydes in red color, which made lignin visible. Interfascicular fiber cells of the wild type exhibited less intense coloration than those of the *PtrGATA9* over-expression transgenic plants (Fig. 2). To quantify the thickness of the SCW, cross-sections of the stem specimens were observed using SEM. The average interfascicular fiber cells in SCW of the wild type was 1.0 μm , while that of the *PtrGATA9* over-expression transgenic plants was approximately 1.4 μm , indicating a 40% increase in thickness (Fig. 3A, B). The SCW of xylem vessel cells were observed not significantly changes in either the wild type or the *PtrGATA9* over-expression transgenic plants (Fig. 3C, D).

Measurement of components of SCW in Arabidopsis stem expressing *PtrGATA9*

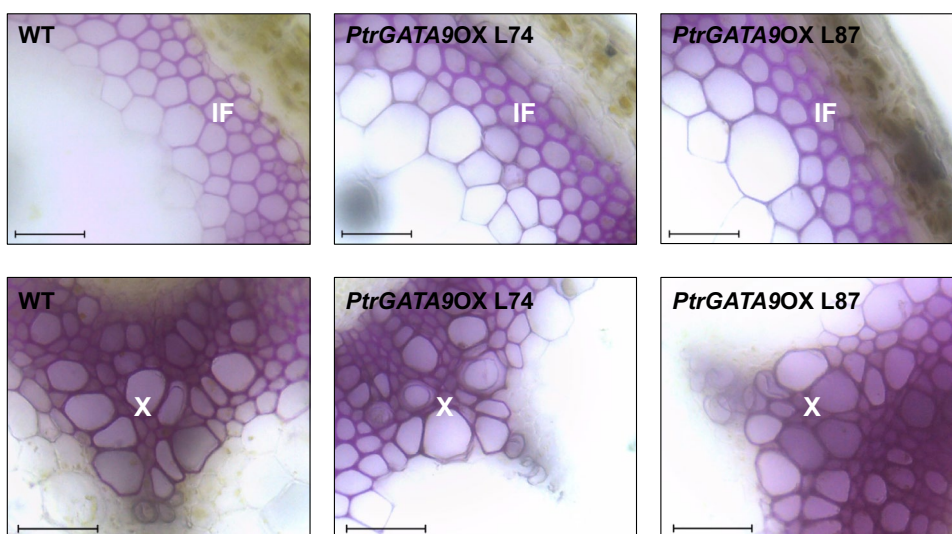
To measure the components of the SCW, 150 wild type and transgenic plants of each type were grown until the stems reached 35 cm, and the stems of the plants from the bottom to 25 cm were cut and dried to a weight of 2.0 g. The dried samples were ground and then decomposed into lignin and carbohydrates using two-step sulfuric acid hydrolysis method. The acid-insoluble lignin was quantified by gravimetric analysis and the amount of acid-soluble lignin, and carbohydrate was determined by spectrophotometer and HPLC, respectively. As a result, the amount of components that make up SCW has increased (Fig. 4). Specifically, the

amount of glucose comprising the cellulose that forms the backbone of SCW and the hemicellulose wrapping around cellulose increased by 13.6% compared to the wild type. Among the xylose, arabinose and galactose constituents of hemicellulose, the amount of xylose, which occupies the highest share, increased by 29.4%. Additionally, acid-insoluble lignin increased by 27.8%, resulting in a 13.6% increase in the overall SCW. Based on these results, it was verified that *PtrGATA9* induces SCW thickening in interfascicular fiber cell and increases the final SCW mass.

Regulation of SCW biosynthesis genes by *PtrGATA9*

The biosynthesis of the SCW is regulated by a precise spatial and temporal transcriptional regulatory network. It is essential to understand how *PtrGATA9* affects the transcriptional expression of SCW biosynthesis genes. We hypothesized that the SCW thickening is closely related to NAC-domain transcription factor (*ANAC012*, *NST1*, *NST2*), known as master regulators of the amount of deposited interfascicular fiber cells (Mitsuda et al. 2007; Zhong and Ye 2015a, b). To investigate that expression of NAC-domain transcription factors and their downstream regulatory genes, total RNA was extracted from the bottom of the stem of wild type and *PtrGATA9* over-expression transgenic plants. cDNA was synthesized to perform RT-PCR, and *ACTIN8*, a house-keeping gene, was used as an internal control to uniformly adjust the amount of template between samples, and the transcriptional expression level of *PtrGATA9* was confirmed (Fig. 5, Supplementary Fig. 3). As expected, the expression levels of NAC-domain transcription factor (*ANAC012*, *NST1*, *NST2*) involved in interfascicular cell regulation were increased by *PtrGATA9*. Next, SCW biosynthesis genes were identified, and the transcriptional expression levels of SCW biosynthesis genes were increased compared to wild

Fig. 2 Histochemical analysis using phloroglucinol-HCl staining of WT and transgenic plants of *PtrGATA9* in *A. thaliana*. Phloroglucinol-HCl staining for lignin in the basal part of 6-week-old inflorescence stems. Scale bars = 20 μm ; X, xylem vessel; IF, interfascicular fiber



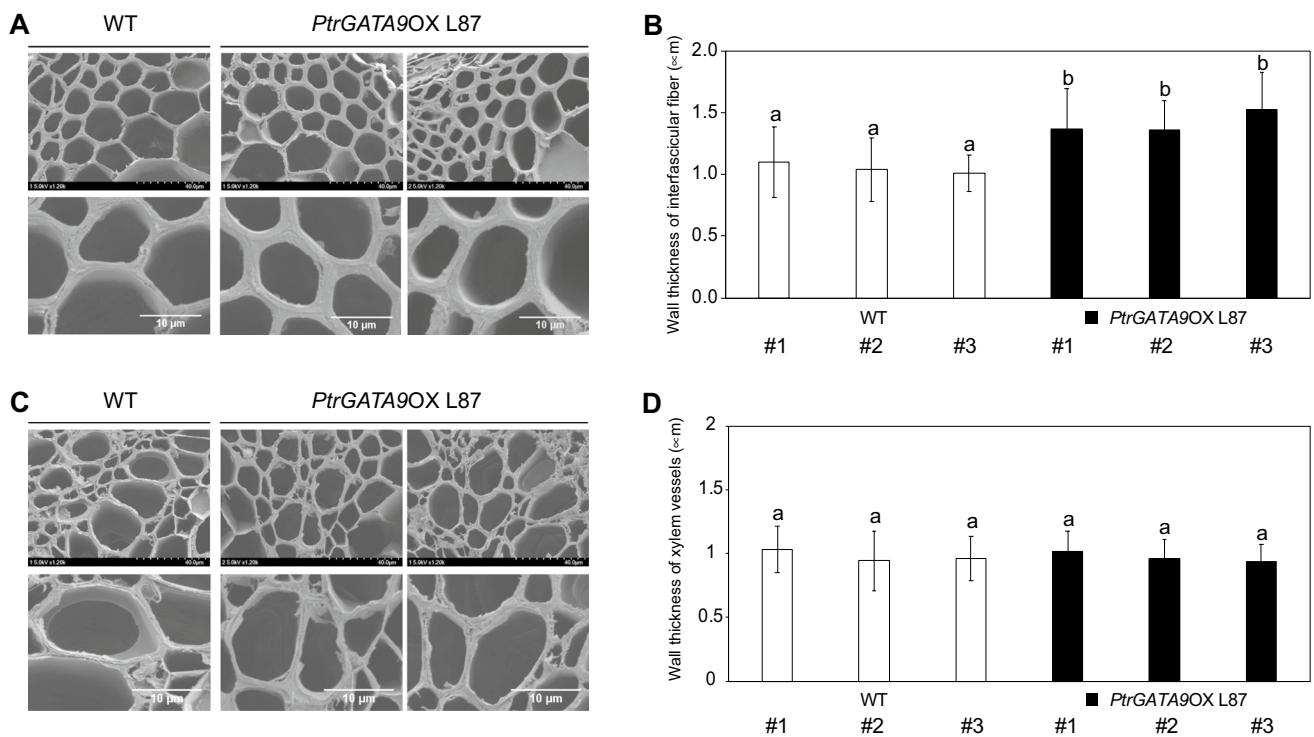
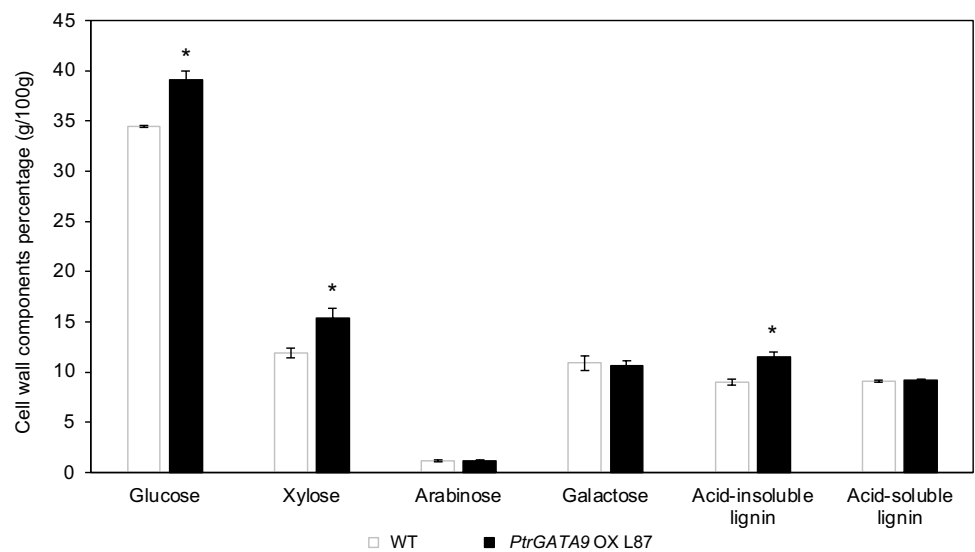


Fig. 3 Measurement of SCW thickness of using field emission scanning electron microscopy (SEM). **A** Field emission scanning electron micrographs of interfascicular fiber cell of inflorescence stem of WT, *PtrGATA9OX L87* transgenic lines. **B** Wall thickness of the vessel elements in interfascicular fiber cell of inflorescence stem of WT, *PtrGATA9OX L87* transgenic lines. **C** Field emission scanning electron micrographs of xylem vessel cell of inflorescence stems of WT, *PtrGATA9OX L87* transgenic lines. **D** Wall thickness of the vessel

elements in xylem vessel cell of inflorescence stem of WT, *PtrGATA9OX L87* transgenic lines. Scale bars of top panel=40 µm; bottom panel=10 µm. The error bars represent the mean ± SD ($n=30$ cells for 3 independent lines, labeled #1, #2 and #3). Statistical significance was assigned using a P-value cutoff of 0.05 by Student’s *t*-test. Lines that do not share any letter are significantly different from each other

Fig. 4 Analysis of SCW components in 25 cm of inflorescence stems from the bottom of 7-week-old WT and transgenic plants of *PtrGATA9* in *A. thaliana*. The error bars represent the mean ± SD ($n > 150$). Asterisks indicated statistically significant differences compared to WT as determined by Student’s *t*-test ($*p < 0.05$)



type. This suggests that *PtrGATA9* is located upstream of the NAC-domain transcription factors acting on the interfascicular fiber cells in the SCW. Correspondingly, the transcriptional expression levels of genes underlying the

NAC-domain transcription factors increased. The transcriptional expression levels of MYB-domain transcription factors (*MYB46*, *MYB58*, *MYB63*), and the underlying cellulose biosynthesis genes (*CesA4*, *CesA7*, *CesA8*), hemicellulose

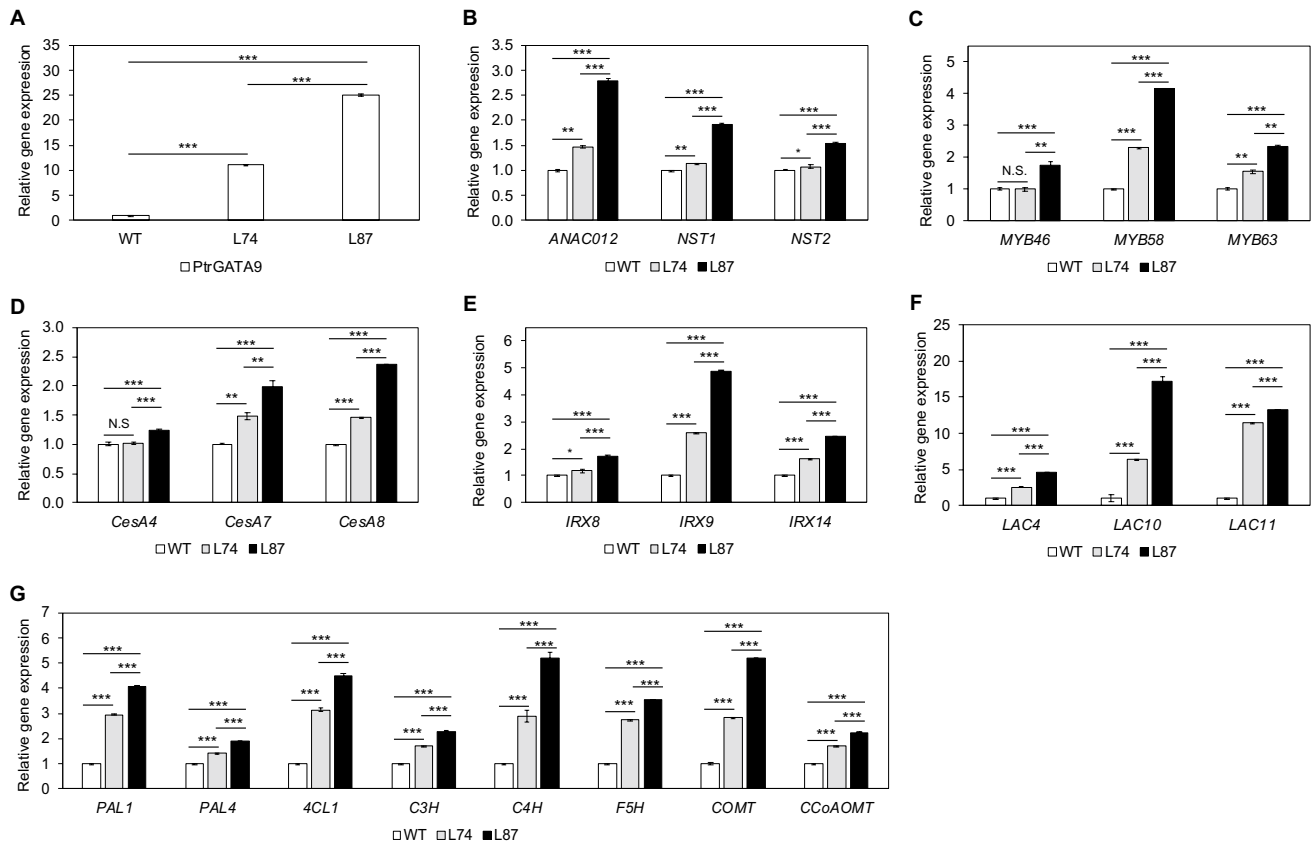


Fig. 5 Relative expression of NAC-domain transcription factor and SCW biosynthesis genes in stems of WT and transgenic plants of *PtrGATA9* in *A. thaliana*. **A** Analysis of the expression level of *PtrGATA9* in *A. thaliana*. **B–G** Analysis of the expression of levels of the NAC-domain transcription factors **B**, MYB-domain transcription factors **C**, cellulose biosynthesis **D**, hemicellulose biosynthesis

E, lignin polymerization **F**, and lignin biosynthesis **G**. The RT-PCR results were quantified by Image J software. The error bars represent the mean \pm SD. Asterisks indicated statistically significant differences compared to WT as determined by Student's *t*-test ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$)

biosynthesis genes (*IRX8*, *IRX9*, *IRX14*), lignin biosynthesis genes (*PAL1*, *PAL4*, *4CL1*, *C3H*, *C4H*, *F5H*, *COMT*, *CCoAOMT*), and polymerization genes (*LAC4*, *LAC10*, *LAC11*) were increased. Through this, it was confirmed that *PtrGATA9* functions as a transcription factor that regulates the transcriptional expression of genes involved in SCW biosynthesis.

***PtrGATA9* positively regulates NAC-domain transcription factors of SCW biosynthesis**

We hypothesized that *PtrGATA9* binds to the promoter of NAC-domain transcription factors involved in SCW biosynthesis. Therefore, we investigated whether *PtrGATA9* activates the transcriptional activation of NAC-domain transcription factors. The transient analysis of promoter activity using *Arabidopsis* mesophyll protoplasts, a widely employed approach in numerous studies (Kim et al. 2022, 2023). Our findings confirmed that *PtrGATA9* induces transcription of NAC-domain transcription factors involved in

the SCW biosynthesis, as demonstrated by performing the TAA method using *Arabidopsis* protoplasts. Specifically, we co-transfected an effector construct containing *PtrGATA9* driven by the CaMV 35S promoter, and a reporter construct containing the native target gene promoter-driven GUS expression in *Arabidopsis* protoplasts (Fig. 6A). A significant increase in GUS activity was observed in the reporter construct with the GUS gene driven by the NAC-domain transcription factors (*ANAC012*, *NST1*, *NST2*) compared to the control reporter construct (Fig. 6B).

To further explore whether *PtrGATA9* directly activates the transcriptional expression of NAC-domain transcription factors, we used the steroid receptor-based inducible activation system. For this purpose, the effector construct was designed to incorporate the *PtrGATA9* gene fused to the glucocorticoid receptor (GR) coding sequence (Fig. 6A). To validate the functionality of the inducible activation system, we performed co-transfection of the reporter and effector constructs mentioned earlier in *Arabidopsis* protoplasts. The GUS activities of the promoters of NAC-domain

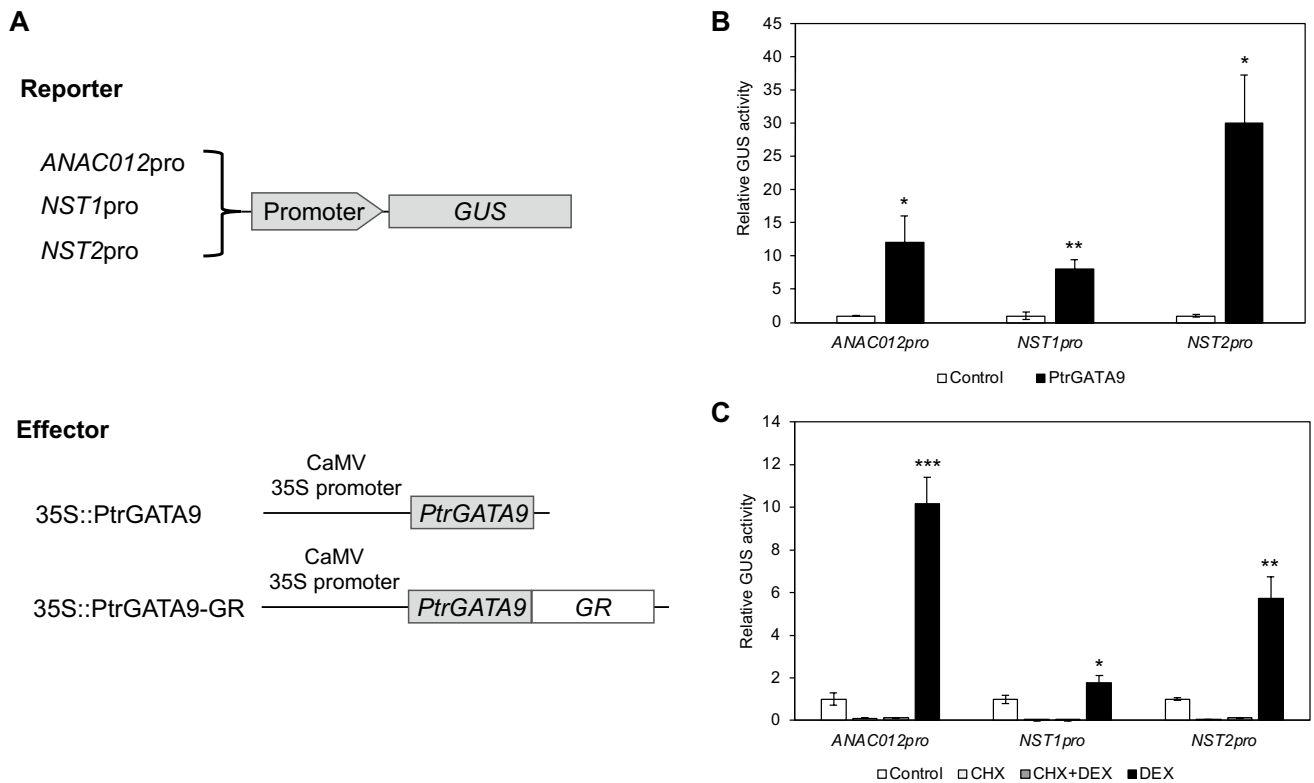


Fig. 6 Transcriptional activation analysis of master regulators of SCW biosynthesis by *PtrGATA9* transcription factor in *Arabidopsis* mesophyll protoplasts. **A** Schematic diagram of the effector and reporter constructs in the transcriptional activation analysis. **B** Transcriptional activation analysis showing that the promoters of *ANAC012*, *NST1*, and *NST2* were activated by expressing *PtrGATA9* transcription factor. **C** *PtrGATA9* transcription factor activates the promoters of *ANAC012*, *NST1*, and *NST2* in the presence of 10 μ M

dexamethasone, but GUS activity was inhibited in the presence of 2 μ M cycloheximide. The activity of the GUS in *Arabidopsis* protoplasts transfected with only reporter construct was used as a control. The error bars represent the mean \pm SD of three biological replicates. Asterisks indicated statistically significant different compared to control as determined by Student's *t*-test (* p < 0.05, ** p < 0.01, *** p < 0.001)

transcription factors, were induced upon the addition of dexamethasone. However, when we administered cycloheximide and dexamethasone simultaneously, the GUS activities of the reporters were observed not significantly differences (Fig. 6C). These findings suggest that *PtrGATA9* regulates the expression levels of NAC-domain transcription factors by activating their respective promoters.

Discussion

Higher plants possess specialized cells, such as vascular vessel cells and interfascicular fiber cells, which develop a SCW. This SCW is crucial for providing enabling long-distance transport and mechanical strength, supporting the plant's upright growth (Lucas et al. 2015). The components of the SCW, including polysaccharides and lignin, serve as valuable renewable biomass resources for human society (Kumar et al. 2016). The biosynthesis of the SCW in higher plants is intricately regulated by a complex network

of regulatory mechanisms (Wang and Dixon 2012; Hussey et al. 2013; Ko et al. 2014; Nakano et al. 2015; Zhong and Ye 2015a, b). Key enzymes, such as Cesa4, Cesa7, Cesa8, IRX8, IRX9, and IRX14, control the biosynthesis of cellulose and hemicellulose, which are essential polysaccharides in the SCW (Kumar et al. 2016). At the top of this biosynthetic pathway, five NAC-domain transcription factors, involved in *ANAC012*, *NST1*, *NST2*, *VND6*, and *VND7*, act as master regulators (Ko et al. 2007; Mitsuda et al. 2007; Yamaguchi et al. 2010; Yamaguchi et al. 2011; Zhong and Ye 2015a, b; Mousavi et al. 2014). Furthermore, the formation of the SCW is influenced not only by developmental signals but also by environmental cues (Rogers et al. 2005; Gall et al. 2015; Wu et al. 2017). However, the specific mechanisms by which physical factors affect SCW formation remain unknown. In our study, we propose that *PtrGATA9*, a GATA transcription factor that binds to the GATA motif responsive to light may play a role in modulating the formation of the SCW by acting upstream of the master regulator in this pathway.

Phylogenetic analysis confirmed that the *PtrGATA9* was homologous to *AtGATA9* (Fig. 1), and based on this, it was confirmed that *AtGATA9* specifically responds to light through expression profiling (Supplementary Fig. 1). Through this, it was indirectly confirmed that *PtrGATA9* responds to light based on the characteristics of the GATA transcription factor. Next, *PtrGATA9* was introduced and overexpressed in *A. thaliana*, a model plant, to identify its function. As a result, there was no change in appearance compared to the wild type (Supplementary Fig. 2). However, when the formation of the SCW was confirmed through cross-section and histochemical staining of the stem, SCW thickening of the interfascicular fiber cell was induced in *PtrGATA9* over-expression transgenic plants. Observation at high magnification using SEM showed that the SCW of the interfascicular fiber cell in *PtrGATA9* over-expression transgenic plants was about 40% thicker (Fig. 3A, B). Interestingly, the thickening of the SCW was only observed in the interfascicular fiber cell and not in the xylem vessel cell (Fig. 3C, D). Although we observed SCW thickening in the interfascicular fibers, we did not observe any outward appearance changes, indicating modifications in SCW components without evident morphological alterations in plants. This phenomenon has been elucidated in previous reports (Gille et al. 2009; Nakano et al. 2022), and even though there is no change in appearance, the increase in SCW thickness is considered of significant industrial importance.

To determine the cause of such SCW thickening, the transcriptional expression levels of genes involved in SCW biosynthesis were identified using RT-PCR (Fig. 5, Supplementary Fig. 3). As a result, the expression levels of *ANAC012*, *NST1*, and *NST2*, which are included in the master regulator, were increased, and the expression levels of most genes underlying them were also increased. In these RT-PCR results, the transcriptional expression levels of *ANAC012*, *NST1*, and *NST2*, which act in the interfascicular fiber cell, regulating SCW formation (Mitsuda et al. 2005; Zhong et al. 2006; Zhong and Ye 2015a, b), indicating that *PtrGATA9* acts upstream of the NAC-domain transcription factors to regulate their expression in the interfascicular fiber cell and subsequently regulate the expression of genes underlying it. Furthermore, a component analysis of SCW revealed an increase in the content of glucose, xylose, and acid-insoluble lignin (Fig. 4), providing evidence of a correlation between the enhanced expression of cellulose, hemicellulose, and lignin biosynthetic genes. However, an increase in laccase expression does not necessarily lead to a substantial change in acid-soluble lignin contents. Although increased laccase activity can lead to the breakdown of certain lignin molecules into acid-soluble forms, the plant may possess compensatory mechanisms that promote lignin synthesis to maintain the structural integrity and stability of the cell wall. As a consequence, the increase in laccase activity might be counteracted by elevated lignin synthesis or other

regulatory mechanisms, resulting in little to no net change in acid-soluble lignin contents. (Berthet et al. 2012; Bryan et al. 2016). Also, it was confirmed that *PtrGATA9* induces the transcription of which gene in performing such a function by using the TAA method and the dexamethasone-inducible transcription activation system. In TAA experiments, the activity of the NAC-domain transcription factors was high measured (Fig. 6). Eventually, it was confirmed that *PtrGATA9* induces SCW biosynthesis in the interfascicular fiber cell of the SCW and functions upstream of the NAC-domain transcription factors known as the master regulator in the pathway.

Conclusions

This study found that *PtrGATA9* induces SCW thickening of interfascicular fiber cell and functions at the upstream of the NAC-domain transcription factors to regulate SCW biosynthesis. These findings are of great value in discovering the upstream transcription factor rather than the previously reported NAC-domain transcription factors. Furthermore, the comprehension of their regulatory roles provides valuable insights for the improvement of plant cell wall properties.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11816-023-00856-3>.

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Data availability The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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