CHARACTERIZATION OF THE FRAGILE FIBER1 (FRA1) PROTEIN AND NAC TRANSCRIPTION FACTORS IN THE REGULATION OF SECONDARY CELL WALL FORMATION

by

JIANLI ZHOU

(Under the Direction of Zheng-Hua Ye)

ABSTRACT

The deposition of cellulose microfibrils plays an important role in shaping plant cells. It is believed that cortical microtubules regulate the orientation of microfibrils, but the mechanism remains unknown. A previous study showed that mutation of the microtubule motor protein Fragile Fiber1 (FRA1) alters the organization of microfibrils in Arabidopsis thaliana. Here, we found that overexpression of FRA1 reduced the thickness of fiber secondary walls and caused deformation of vessels. Furthermore, we isolated three putative FRA1 interacting proteins by yeast 2-hybrid: the FRA1 interacting protein1 (FIP1), the gibberellin-regulated protein, and the actin-related protein.

Secondary cell wall biosynthesis is controlled by a transcriptional network, in which the secondary wall-associated NAC domain protein1 (SND1) and the NAC secondary wall thickening promoting factor1 (NST1) are master regulators and function redundantly. In this study, we demonstrated that expression of SND1 homologues in the snd1 nst1 mutant complemented the secondary wall defects in fiber cells, indicating that they are all functional homologues. Expression analysis revealed that one subgroup of SND1 homologues were
expressed in stem vessels, another subgroup of SND1 homologues were expressed in stem fiber cells, while some others were mainly expressed in root caps. Overexpression of some of these SND1 homologues caused ectopic secondary cell wall deposition-like phenotypes in the wild-type. However, dominant repression of their functions did not result in obvious phenotypes. Therefore, we suggest that these SND1 homologues are minor regulators of secondary cell wall biosynthesis.

INDEX WORDS: secondary cell wall, cellulose microfibril, cortical microtubule, FRA1, NAC transcription factor
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JIANGLI ZHOU

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by

JIANLI ZHOU

Major Professor: Zheng-Hua Ye
Committee: Jacek Gaertig
            Michelle Momany
            Gregory Schmidt
            XiaoYu Zhang

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

This dissertation is dedicated to my parents and my husband for their forever love and supports.
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Plants develop cell walls to hold their shapes and offer protection against turgor pressure (Sarkar et al., 2009). Further mechanical support is required in vascular plants, where water and mineral transport is an essential life need. As a result, the secondary cell wall is laid down between the primary cell wall and the plasma membrane in specialized cells, such as tracheary elements, fibers, and sclereids. Unlike the primary cell wall, which is produced during cell division, the secondary cell wall appears only after the size, shape, and fate of the cell are determined (Carpita and McCann, 2000). The scientific community pays much attention to the secondary cell wall because of its agronomic and industrial implications. First, the secondary cell wall offers support to crops. Reduction in the secondary cell wall content in the rice mutant brittle culm1 (Li et al., 2003) or the maize mutant brittle stalk2 (Ching et al., 2006) affects the mechanical strength of the crops, which is an important agronomic trait. Further, the secondary cell wall contains abundant cellulosic and hemicellulosic sugars, which can be used as energy sources. Finally, the secondary cell wall constitutes the majority of wood, which is of great importance in paper, timber, and furniture industries (Yuan et al., 2008; Zhong and Ye, 2009).

CHEMICAL COMPOSITION AND OCCURRENCE OF THE SECONDARY WALL

The secondary cell wall is mainly composed of cellulose, hemicelluloses, and lignin. Cellulose is composed of β-1, 4-linked glucan chains. These glucan chains pack parallelly into microfibrils and offer mechanical strength to cells (Somerville, 2004). Typically, the secondary cell wall has 3 distinct layers, namely S1, S2, and S3, in which the microfibrils are laid down in
different orientations. Layer S1 is most adjacent to the primary cell wall and has microfibrils running in a shallow helix. Layer S2, the thickest one, has microfibrils arranged in a steep angle helix. It is known that the microfibril angle (MFA) in the S2 layer is a key determinator of wood stiffness (Kretschmann, 2003; Barnett and Bonham, 2004). The innermost layer, S3, features parallely aligned microfibrils arranged in a flat helix (Cutter, 1978). In rare cases, such as bamboo fibers, the secondary cell wall has more than three layers (Gritsch and Murphy, 2005).

Xylan is the most abundant type of hemicelluloses in flowering plants (Zhong and Ye, 2009). Xylan has a β-1, 4-linked xylose backbone that is decorated with α-D-glucuronic acid (GA), 4-O-methyl-glucuronic acid (MeGA), arabinose, and/or acetyl residues. At the reducing end of a xylan chain, a short and unique sequence of glycosyl residues is always present (Pena et al., 2007; York and O’Neil, 2008). Xylan cross-links microfibrils through strong hydrogen bonding and further enhances the secondary cell wall strength (McCartney et al., 2005).

Lignin is a heteropolymer consisting of p-coumaryl, coniferyl, and sinapyl alcohol. It forms covalent bonds with cellulose, and thus strengthens the cell wall. Owing to its hydrophobic nature, lignin makes cells waterproof and thus allows water and minerals to be transported in the vascular tissue (Boerjan et al., 2003).

In different plant species and cell types, the types and proportions of the three secondary cell wall components vary. For example, dicotyledons normally contain 45%~50% cellulose, 20%~30% xylan (predominantly glucuronoxylan), and 7%~10% lignin (coniferyl alcohol and sinapyl alcohol are the major components). Monocotyledons contain 35~45% cellulose, 40%~50% xylan (mostly glucuronoarabinoxylan), and 20% lignin (includes all three types of lignin). As for cell types, some such as cotton fibers have a significantly high amount of cellulose. The percentage of lignin in tracheary elements is higher than that in fibers. In addition,
synapyl alcohol is often associated with fibers but rarely present in tracheary elements. The secondary cell wall composition may also adjust according to abiotic conditions (Boerjan et al., 2003; Vogel, 2008; Zhong and Ye, 2009).

As mentioned, tracheary elements, fibers, and sclereids are the three main secondary cell wall-containing cell types. Tracheary elements serve to conduct water and minerals from roots to aerial parts of plants. In non-flowering vascular plants, the tracheary elements are called tracheids, whereas vessel elements are the usual type of tracheary elements found in flowering plants (Ye, 2002). One of the main differences between tracheids and vessel elements is that tracheids have tapered ends whereas vessel elements develop opening ends at maturity and form tubular-like structures called vessels (Stern et al., 2002). Depending on developmental stages, vessel elements appear in the form of protoxylems, metaxylems, or secondary xylems. There are two ways to distinguish the three types of vessel elements. First, protoxylems and metaxylems are formed during primary growth, whereas secondary xylems are formed during secondary growth. Second, protoxylems normally deposit annual or helical secondary cell wall, whereas metaxylems and secondary xylems deposit reticulated or pitted walls (Ye, 2002; Kubo et al., 2005). Partial reduction in the secondary cell wall in vessels may lead to collapsed vessels and swollen tissues. In severe cases, a significantly reduced secondary cell wall causes lethality (Somerville, 2004).

In regards to fibers, two types occur: one is formed within vascular bundles (fascicular fiber), and the other is between vascular bundles (interfascicular fiber). Both types of fibers are much longer than they are wide and develop tapered ends at maturity. The secondary cell wall in fibers is laid down uniformly and offers great strength to plants (Zhong et al., 2001). Arabidopsis mutant plants that are deficient in fiber secondary cell wall thickening exhibit bending stems,
anther indehiscence, silique indehiscence, etc. (Zhong and Ye, 1999; Mitsuda et al., 2005; Zhong et al., 2006; Mitsuda et al., 2007; Mitsuda and Ohme-Takagi, 2008). Sclereids are more isodiametric than fibers but vary in shape. Sclereids are widely distributed in plant tissues, such as seed coats and the pits of stone fruits (Stern, 2002).

Vessel elements and fibers in Arabidopsis stems have been used as models to study secondary cell wall deposition (Zhong et al., 2001; Endo et al., 2009). Vessel elements originate from the procambium, which first occurs in the late embryogenesis stage. Upon germination, procambial cells propagate and give rise to xylem initials and phloem initials. Vessel elements and fascicular fibers, which are derived from xylem initials, and sieve elements and companion cells, which are differentiated from phloem initials, form a collateral pattern in which the xylem tissue is located on the adaxial side and the phloem tissue is located on the abaxial side. In other organs the vascular tissue can occur in an amphivasal (xylem surrounds phloem) or an amphicribral (phloem surrounds xylem) pattern. In plants that have secondary growth, procambial cells give rise to vascular cambial cells, which eventually produce secondary xylems and secondary phloems (Cutter, 1978; Ye, 2002). Once xylem cells reach their final destination, secondary cell walls are laid down. Interfascicular fibers are daughter cells of fiber cell initials that are developed from parenchyma cells. The secondary cell wall deposition in interfascicular fibers normally occurs simultaneously with the cessation of internode elongation.

After secondary cell wall deposition occurs, both vessel elements and fibers undergo programmed cell death (PCD). In vessel elements, PCD begins with vacuole rupture, and then the proteases and nucleases released help to digest the cellular content (Ye, 2002). PCD occurs in fibers in a different way, wherein autolysis of the cellular content happens before vacuoles break down (Courtois-Moreau et al., 2009).
SECONDARY CELL WALL BIOSYNTHESIS

• APPROACHES

Forward genetics has identified several genes involved in the biosynthesis of cellulose (Turner and Somerville, 1997), xylan (Zhong et al., 2005) and lignin (Chapple et al., 1992). Some of the phenotypes in the biosynthetic gene mutants include changes of the secondary cell wall thickness, deformed vessel elements, swollen roots, and alteration of lignin distribution, which can be easily detected using UV light. To facilitate large-scale screening, a Fourier Transform Infrared (FTIR) microspectroscopy system (Chen et al., 1998) and a pyrolysis molecular beam mass spectroscopy system (Sykes et al., 2009) have been developed. However, not all genes involved in secondary cell wall biosynthesis can be identified through mutant screening. To broaden our understanding of secondary cell wall biosynthesis, characterization of paralogues and orthologues of the confirmed enzyme genes is an effective tool (Lee et al. 2007&2009; Zhou et al., 2007). A summary of potential genes involved in secondary cell wall biosynthesis can be found at Carbohydrate Active Enzymes (CAZy, http://www.cazy.org/) (Cantarel et al., 2008). With the development of the microarray technique, the entire transcription profile can be generated from xylem tissues, which facilitates the identification of novel genes involved in secondary cell wall biosynthesis (Allona et al., 1998; Sterky et al., 1998; Israelsson et al., 2003; Paux et al., 2004; Brown et al., 2005). The in vitro zinna cell and Arabidopsis cell culture systems are noteworthy in that they provide a faster way to study vessel element differentiation and secondary cell wall biosynthesis. In these systems, mesophyll cells are extracted and cultured. Auxin, cytokinin, or brassinosteroid are added to the cell culture to induce transdifferentiation into vessel elements. By tracing the changes in transcripts during the different stages of secondary cell wall formation, candidate genes involved in vessel element
differentiation, cell elongation, secondary cell wall biosynthesis, and PCD can be identified (Fukuda, 1997; Yoshida et al., 2009).

Once identified, the mutant lines of candidate genes are generated and compared with the wild-type plants with regards to the secondary cell wall morphology (microscopy) and the chemical composition of the secondary cell wall (chromatography, mass spectroscopy, nuclear magnetic resonance). The expression pattern and enzyme activity are also studied. Another way to study the gene function is to transform the candidate gene into a well-characterized mutant background with known secondary cell wall defects. If the defects are complemented, the candidate gene may possess similar biochemical functions as the well-characterized gene. This approach can be carried out within the same plant species as well as cross species; thus it is especially useful for studying genes of tree species that have long life cycles and lack established transgenic techniques.

- **CELLULOSE BIOSYNTHESIS**

Cellulose is synthesized at the plasma membrane by a rosette like complex called the cellulose synthases complex (CSC). It is proposed that the CSC consists of 6 subunits, each of which is able to produce 6 glucan chains simultaneously. As the CSC moves through the plasma membrane, 36 glucan chains are made at the same time. Afterwards, the cellulose chains are crystallized into the form of cellulose microfibrils (Somerville, 2006). Based on the sequence similarity to a bacterial cellulose synthase gene, 10 *Cellulose Synthase A (CESA)* genes encoding the putative CSC subunits in Arabidopsis are identified (Pear et al., 1996). These CESA proteins contain a cystein-rich N-terminal domain followed by eight putative transmembrane domains. CESA1, CESA3, and CESA6 are suggested to produce cellulose in the primary cell wall (Desprez et al., 2007; Persson et al., 2007), whereas CESA4, CESA7, and CESA8 are thought to
be involved in cellulose synthesis in the secondary cell wall (Somerville et al., 2004; Atanassov et al., 2009).

Strong mutant alleles of *CESA1* result in embryo lethality. The *CESA1* weaker allele *radically swollen protein*1 (*rsw1*) has reduced cellulose content at the restrictive temperature, which in turn results in swollen roots. Moreover, CSCs disappear from the plasma membrane in the *rsw1* mutant, indicating that *CESA1* is an essential component of the CSC (Arioli, 1998; Williamson et al., 2001). The *CESA3* mutant allele, *isoxaben resistant 1* (*ixr1*), and the *CESA6* mutant allele, *isoxaben resistant 2* (*ixr2*), are resistant to the cellulose synthesis inhibitor *isoxaben* treatment (Desprez et al., 2002; Ellis et al., 2002). Reduction in the expression of *CESA1*, *CESA3*, or *CESA6* causes decreased cellulose production; although, the effect of *CESA6* is relatively mild. These three genes are expressed in the same tissue types in hypocotyls and can be co-immunoprecipitated. In addition, antibodies that are against any of the three CESAs pull down the other two proteins. Therefore, *CESA1*, *CESA3*, and *CESA6* are thought to form the CSC complex for the primary cell wall biosynthesis (Desprez et al., 2007; Persson et al., 2007; Wang et al., 2008). *The cesa4*, *cesa7*, and *cesa8* mutants are identified due to their irregular xylem phenotype and initially were named *irregular xylem 5* (*irx 5*), *irx 3/fra5*, and *irx 1* respectively. The cellulose content in xylems is reduced in the *irx 5*, *irx 3/fra5*, or *irx 1* mutant (Atanassov et al., 2009). Reduction of cellulose may lead to decreased support against turgor pressure, which explains the collapsed xylem phenotype. *CESA4*, *CESA7*, and *CESA8* have the same expression pattern in xylems and show interaction in the BiFc and co-immunoprecipitation assays, indicating that these 3 proteins form a complex to produce cellulose in xylems (Atanassov et al., 2009; Timmers et al., 2009).
The CESA proteins are the only identified components of the CSC, but there may be other proteins involved. KORRIGAN1 (KOR1) shares great sequence similarity to a tomato endo-β-1, 4-glucanase. Its mutant allele, abnormal cell wall1 (acw1), has the characteristic swollen root phenotype as does the rsw1 mutant. In addition, the acw1 mutant is defective in cellulose crystallization and cellulose microfibril organization, consequently inhibiting cell elongation in roots, leaf petioles, and hypocotyls (Sato et al., 2001). Somerville (2006) postulated that KOR1 may help to release the stress produced during microfibril assembly or to remove the microfibrils that are not crystallized. KOBITO1 (KOB1) is a plasma membrane-localized protein with a universal distribution pattern. Although overexpression of KOB1 doesn’t show any phenotype, the kob1 deletion mutant is dwarf with swollen roots (Pagant et al., 2002). The dwarf phenotype may be partially caused by reduction in cellulose synthesis. It was then found that cellulose microfibrils, which are supposed to orient in parallel in the wild-type plant, are randomly deposited in elongating cells in the kob1 root (Pagant et al., 2002). The Tracheary Element Differentiation-Related6 (TED6) and Tracheary Element Differentiation-Related7 (TED7) are expressed preferentially in vessel elements in Arabidopsis. TED6 interacts with CesA7 in vivo, suggesting its involvement in secondary cell wall biosynthesis. Simultaneous downregulation of the expression level of TED6 and TED7 disrupts the formation of secondary cell walls in xylems (Endo et al., 2009). However, the functions of TED6 and TED7 are yet to be determined.

- **DEPOSITION OF CELLULOSE MICROFIBRILS**

The arrangement of microfibrils is highly ordered. In elongation cells, microfibrils are oriented transversely to the long axis. In secondary cell walls, the different deposition patterns of microfibrils result in three distinct layers (Figure 1.1, Kretchmann, 2003). The question of how the deposition of microfibrils is regulated has long been studied. Green (1962) reported that
treating *Nitella* cells with the microtubule depolymerizing drug, colchine, interrupts their organization of microfibrils. Soon after, Ledbetter and Porter (1963) discovered cortical microtubules, which normally form aligned arrays beneath the plasma membrane and mirror the orientation of microfibrils. Since then, many studies showed that cortical microtubules control the orientation of microfibrils (Hepler and Newcomb, 1964; Giddings and Staehelin, 1991; Baskin, 2001; Hamant et al., 2008; Corson et al., 2009).

**Figure 1.1.** Schematic diagrams of secondary cell wall layers.

The leftmost diagram is a transverse view of a plant cell. From the primary cell wall to the cytoplasm, secondary cell walls are laid down successively as the S1 layer, the S2 layer, and the S3 layer. Note that the S2 layer is the thickest. The other diagrams are a longitudinal view. The black lines represent microfibrils. Note that the microfibrils in each layer are deposited in different angles to the long axis (white arrow). PM, primary cell wall. Redrawn and modified from Kretschmann (2003).

Microtubules are distributed both at the plasma membrane (thus named cortical microtubules) and deep inside the cell. The basic components of microtubule, α-tubulin and β-
tubulin, are polymerized into long strand-like structures called protofilaments. Protofilaments bundle together to form a microtubule (Alberts et al., 2002). One important feature of a microtubule is polarity. It has a plus end and a minus end, of which the plus end polymerizes and depolymerizes faster than the minus end. The majority of cortical microtubules have the same polarity (Chan et al., 2007). In animal cells, microtubules emerge from the microtubule organization center (MTOC). No MTOC in plant cells has been reported, but it is known that γ-tubulin is involved in microtubule nucleation (Alberts et al., 2002). Recently, several microtubule-associated proteins (MAPs) including γ-tubulin, MAP4, MAP65, end binding 1 (EB1) and katanin, have been used to label microtubule, which has allowed the study of microtubule activity. Studies found that most new cortical microtubules emerge from the extant ones at shallow angles, and the growing direction is towards the plus end of the extant ones (Murata et al., 2005; Chan et al., 2009). Dixit and Cyr (2004) reported that a growing cortical microtubule tends to co-align with the existing, old ones when interacting at narrow angles, whereas steep angles cause depolymerization of the newly formed cortical microtubule, explaining why cortical microtubule arrays are uniformly aligned. The dynamics and regulation of cortical microtubules play important roles in cell division, vesicle transport, orienting microfibrils, etc. (Alberts et al., 2002).

Several hypotheses attempt to explain how cortical microtubules regulate the orientation of microfibrils. Heath (1974) predicted that CSCs move along cortical microtubules and thus produce parallel cellulose microfibrils. Gidding and Staehelin (1991) postulated that cortical microtubules limit the path of CSCs so that the complexes can only move within the linear track defined by cortical microtubules. Baskin (2001) proposed a “template-incorporation” model in which a scaffold is involved in guiding nascent microfibrils. Taking advantage of immuno-
labeling and spinning disk confocal microscopy, Paredez et al. (2006) visualized the bi-directional movement of CSCs along cortical microtubules in hypocotyls cells in Arabidopsis. If cortical microtubules are slightly impaired and thus change their orientation, the CSCs adjust their tracks accordingly. However, the velocity of CSCs is not associated with that of cortical microtubules, indicating that cortical microtubules guide rather than power the movement of the CSCs. Similar findings are seen in the xylem secondary cell wall, where the distribution of the CSCs is dependent on cortical microtubules, but the CSCs seem to move unidirectionally (Wightman and Turner, 2008).

Crowell et al. (2009) and Gutierrez et al. (2009) found that cortical microtubules also participate in the delivery of CSCs. CSCs have two localizations, of which one is at the plasma membrane and the other so called microtubule-associated cellulose synthase compartment (MASC) is in the cytoplasm. CSCs tend to remain in the MASC of cells when treated with the cellulose synthesis inhibitor. Interestingly, the internalized CSCs interact with microtubules and the Golgi apparatus. When treated with microtubule depolymerization drugs, more internalized CSCs are observed, indicating that the delivery of CSCs is dependent on microtubule depolymerization (Crowell et al., 2009; Gutierrez et al., 2009).

A few putative regulators in the microtubule-regulated microfibril orientation have been identified in Arabidopsis. COBRA (COB) is a glycosylphosphatidylinositol-anchored protein. The cob mutant exhibits randomly oriented cellulose microfibrils. The COB protein is localized in a banding pattern perpendicularly to the elongation axis, coincident with the arrangements of cortical microtubules and microfibrils. Oryzalin (a microtubule depolymerizing drug) affects the localization of COB, suggesting that cortical microtubules have a role in regulating COB and in turn may regulate the orientation of microfibrils (Schindelman et al., 2001; Roudier et al., 2005;
A screening for reduced cell wall strength mutants identified the \textit{fragile fiber1} (\textit{fra1}) mutant. The \textit{fra1} mutant has a normal arrangement of cortical microtubules. The cell wall thickness and cell wall composition are not affected in the mutant, but microfibrils are deposited in a loose and random manner. The \textit{FRA1} gene encodes a kinesin-like protein. Kinesins are motor proteins of microtubules, normally consisting of a motor domain, a stalk domain and a tail domain. Kinesins move along microtubules using the motor domain and transport cargoes that are normally attached to the tail domain (Alberts et al., 2002). \textit{FRA1} is localized around the cell periphery where cortical microtubules are localized. Therefore, \textit{FRA1} is possibly involved in transporting cargoes, which may be involved in microfibril deposition, along cortical microtubules (Zhong et al., 2002). Fragile Fiber2 (\textit{FRA2}) is a katanin-like protein that severs microtubules. In the \textit{fra2} mutant, both the orientations of cortical microtubules and microfibrils are abnormal. In addition, the cell wall thickness and root cell elongation are reduced (Burk and Ye et al., 2002). The above cases further support the correlation between cortical microtubules and microfibrils.

- \textbf{XYLAN BIOSYNTHESIS}

The most common xylan in dicotyledons glucuronoxylan (GX) is produced in the cytoplasm. At least three types of glycosyltransferase activities are involved in GX synthesis: (1) synthesis of the $\beta$-1, 4 linked xylose backbone; (2) decoration of the backbone; (3) production of the unique short oligosaccharide sequence at the reducing end (York and O’Neil, 2008).

Glycosyl Transferases Family 43 (GT43) proteins are known to possess both $\beta$ glucuronyl-transferase activity and UDP-Xyl: xylan $\beta$-1, 4-xylosyltransferase activity, which makes them candidate enzymes for the production of the GX backbone (Cantarel et al., 2009). Irregular Xylem9 (IRX9) and Irregular Xylem14 (IRX14) are two of the four Arabidopsis GT43 proteins.
IRX9 and IRX14 are localized in the Golgi apparatus in secondary cell wall-containing cells. Mutation of either one results in a decrease in both the GX content and the length of the GX chain. Microsomes prepared from the *irx9* or *irx14* plants exhibit reduced xylosyltransferase activity. Therefore, Brown et al. (2007) and Lee et al. (2007) suggested that IRX9 and IRX14 are involved in GX backbone production. Additionally, Brown et al. (2008) proved that IRX10 and IRX10L, close homologues in the GT47 family, function redundantly in elongating the GX backbone.

However, another GT47 family protein, FRAGILE FIBER8 (FRA8), doesn’t seem to participate in the GX backbone extension. The *fra8* mutant shows reduced GX content, thinner secondary cell walls, and collapsed vessel elements. It was also found that *fra8* has decreased reducing end sequences but the GX chain length remains unaltered. Therefore, FRA8 may be involved in the reducing end production (Zhong et al. 2005; Pena et al., 2007). Double mutation of *fra8* and its homologue *fra8 homologue (f8h)* has more severe phenotypes than *fra8*. In addition, the *fra8* phenotype can be rescued by F8H, indicating that these two proteins are functional homologues (Lee et al., 2009). Additionally, IRX8 and PARVUS, belonging to the GT8 family, are confirmed to regulate the reducing end sequence production (Brown et al., 2007; Lee et al., 2007).

- **LIGNIN BIOSYNTHESIS**

  Lignin biosynthesis begins with several steps of modification on phenylalanine, including deamination by phenylalanine ammonia-lyase (PAL), aromatic ring hydroxylation by cinnamic acid 4-hydroxylase (C4H), and addition of Coenzyme A by CoA ligase (4CL). The resulting *p*-counmaryl CoA undergoes aromatic ring hydroxylation, phenolic O-methylation, substitution for the alcohol group, leading to the production of the three monolignols: *p*-counmaryl alcohol (H),
coniferyl alcohol (G), and sinapyl alcohol (S). The genes involved in the monolignol production pathway or phenylpropanoid pathway have been characterized (Boerjan et al., 2003; Rae et al., 2003).

Monolignols are transported through the plasma membrane to the secondary cell wall under unknown mechanisms. Although monolignols and glucosylated monolignols are both present in the cytoplasm, it is unclear whether monolignols storage and delivery requires glucosylation (Steeves et al., 2001). Seven Arabidopsis ATP binding cassette (ABC) transporter genes are expressed simultaneously with monolignol biosynthetic genes, indicating that the ABC transporters may be involved in the monolignol transport (Ehlting et al., 2005). Upon arrival at the secondary cell wall, monolignols are thought to be de-glucosylated, oxidized, and polymerized to form lignin polymers. Enzymes such as peroxidases and laccases are candidates in the regulation of monolignol oxidation, but no direct evidence has been found yet (Marjamaa et al. 2009).

Lignin reduces the digestibility of cell walls in chemical pulping and biomass processing (Vanholme et al., 2008). The amount and composition of lignin can be altered by regulating the expression levels of monolignol biosynthetic genes. Downregulation of *PAL* and *hydroxycinnamoyl CoA reductase (CCR)* reduces content of all the three monolignols (H, G, and S). Additionally, the S/G ratio is positively correlated with the expression of *F5H* (Vanholme et al., 2008). Overexpression of coniferyl aldehyde 5-hydroxylase (F5H) in poplar trees leads to an increased S/G ratio and easy processing of cell wall materials in chemical pulping (Huntley et al., 2003). Chen and Dixon (2007) found that reducing C4H, hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase (HCT), *p*-coumarate 3-hydroxylase (C3H), caffeoy-CoA O-methyltransferase (CCoAOMT), or caffeic acid O-methyltransferase
(COMT) decreases the lignin content in Medicago. In addition, reduced lignin increases sugar release rates during biomass digestion.

The phenylpropanoid pathway produces lignin as well as flavonoids, tannins, etc. (Weisshaar and Jenkins, 1998). Moreover, lignin is essential for cell waterproofing and cell support (Boerjan et al., 2003). Therefore, engineering the lignin content and composition is feasible but may cause side effects in plant growth. For example, downregulating of HCA in Medicago results in decreased lignin content as expected; however in severe lines the flowering time is delayed, and the total biomass is reduced (Shadle et al., 2007). Effort should be put into identifying genes that regulate lignin content and composition without influencing plant fitness.

TRANSCRIPTIONAL REGULATION OF SECONDARY CELL WALL BIOSYNTHESIS

• APPROACHES

Transcriptome analysis of xylem tissues in Arabidopsis, Eucalyptus, Pine, and Poplar reveals a coordinated expression of secondary cell wall biosynthetic genes during secondary cell wall formation (Allona et al., 1998; Sterky et al., 1998; Isaraelsson et al., 2003; Paux et al., 2004; Brown et al., 2005), indicating that these genes are under delicate transcriptional control. In fact, many transcription factors are identified in the genetic screenings for secondary cell wall deficient mutants or are largely expressed in cells developing secondary cell walls (Kubo et al., 2005; Demura and Fukuda, 2007). Transcription factors, normally consists of a DNA binding domain and an activation domain, bind to specific DNA sequences (cis-acting elements) and regulate gene transcription. Based on sequence homology of their binding domains, transcription factors are classified into more than 50 families (Grotewold, 2008). Some transcription factors contain a transcriptional repression domain instead of an activation domain. In Arabidopsis, an
ERF-associated amphiphilic repression (EAR) motif (Ohta et al., 2001) and a B3 repression domain (BRD) are confirmed to repress the target gene expression. Fusing the EAR repression domain or the BRD repression domain with a transcription activator would turn the activator into a repressor. The gene repressor may not only restrain its own functions, but also the functions of its homologues and thus acts as a dominant repressor (Ikeda and Ohme-Takagi, 2009).

Currently transcription factors involved in secondary cell wall biosynthesis are characterized in the following three aspects: (1) expression and mutant phenotype analysis, (2) transcriptional activity analysis, and (3) transcriptional network analysis.

The \( \beta \)-glucuronidase (GUS) gene has long been used as a reporter to study the pattern of gene expression. T-DNA insertional lines or RNAi lines are commonly used loss-of-function mutants. One obstacle with loss-of-function mutants is gene redundancy. Concurrently knocking out or knocking down the homologues is a strategy when the number of close functional homologues is not too large. Another strategy is to create gene repressor lines as mentioned above. To make gain-of-function mutants, the constitutive promoter cauliflower mosaic virus 35S (CaMV35S) is generally used to drive ubiquitous expression of the transcription factor of interest (Mitsuda and Ohme-Takagi, 2009). Some of the phenotypes caused by secondary cell wall-related transcription factor mutants include deformed xylems, changes of fiber strength, retarded growth, and abnormal organ morphology (Zhong et al., 2006).

To study the activity, a transcriptional activation assay can be conducted in a transient expression system. One construct, the effector, contains the fusion of a strong DNA binding domain and the transcription factor of interest. Another construct, the reporter, consists of a reporter gene downstream of several repeats of the cis-acting elements that can be recognized by the DNA binding domain in the effector. The effector and the reporter are co-transformed into a
transient expression system, such as yeast cells, protoplasts, or tobacco leaves. If the transcription factor is an activator, the reporter gene will be activated (Mitsuda and Ohme-Takagi, 2009). If the DNA binding domain in the effector is removed and the cis-acting elements in the reporter are replaced by the promoter sequences of a potential target gene, the assay can be used to study whether the transcription factor is an upstream regulator of the potential target gene. For example, several secondary cell wall-associated transcription factors activate reporter genes driven by the promoters of important secondary wall biosynthetic genes in the transcriptional activation assay (Zhong et al., 2008).

It has been proposed that secondary cell wall biosynthesis is under the control of a transcriptional cascade (Zhong and Ye, 2007). Deciphering the network can be achieved through the study of homologues, the traditional genetic repressor method, or the genome-wide screening method. As for the genome wide screening, one way is to search the transcriptional profiles of secondary cell wall containing tissue for highly expressed transcription factors. Another way is to compare the transcriptome of a known transcription factor mutant plant with that of the wild-type. Genes displaying dramatic changes may be potential targets of the known transcription factor. Finally, combining chromatin immunoprecipitation (ChIP), with microarray, or direct sequencing, can be employed to screen for direct targets on a large scale (Mitsuda and Ohme-Takagi, 2009).

• **NAC TRANSCRIPTION FACTORS INVOLVED IN SECONDARY CELL WALL BIOSYNTHESIS**

NAM, ATAF1, 2, CUC2 (NAC) transcription factors are only found in plants. NAC transcription factors have a conserved ~160 amino acid long DNA binding domain at the N-terminal (called the NAC domain) and a non-conserved activation domain at the C-terminal
(Olsen et al., 2005). More than 100 NAC genes are in the genome of Arabidopsis, rice, soybean, and tobacco (Ooka et al., 2003; Olsen et al., 2005; Rushton et al., 2008; Pinheiro et al., 2009; Jensen et al., 2010).

The expression of some NAC transcription factors could be regulated by miRNAs. Cup Shaped Cotyledon1 (CUC1), CUC2, NAC1, ATNAC4, ATNAC5, and ANAC092 are identified to be targets of miRNA164 (Guo et al., 2005; Mitsuda and Ohme-Takagi, 2009). In addition to miRNA164 control, NAC1 is also subjected to ubiquitin-mediated proteolysis (Xie et al., 2002), indicating that post-translational modification is also an important factor in controlling NACs.

NAC transcription factors play important roles in plant development and stress response. CUC1, the first identified NAC transcription factor, is involved in cotyledon separation and shoot apical meristem formation. Its closest homologues CUC2 in Arabidopsis, PhNAM in petunia, and ZmNAM1 and ZmNAM2 in maize possess similar functions (Zimmerman and Werr, 2005). NAC Regulated Seed Morphology1 (NARS1)/NAC2 and NARS2/NAM regulate seed integument development and thus influence embryogenesis (Kunieda et al., 2008). Overexpression of NAC1 promotes lateral root formation (Guo, 2005). ANAC019, ANAC055 (Jiang et al., 2009), NTM1-like Protein9 (NTL9) (Yoon et al., 2008), ATAF1 (Wang et al., 2009; Wu et al., 2009), and ANAC102 (Christianson et al., 2009) respond to biotic and/or abiotic stresses. NAC transcription factors are also involved in producing secondary metabolites. For example, the ANAC078 overexpressor induces the expression of flavonoid biosynthetic genes, which causes accumulated anthocyanin (Morishita et al., 2009).

The vascular-related NAC domain protein6 (VND 6) and VND7 are largely expressed when vessel elements begin to form in the in vitro Arabidopsis vessel element inducible system (Kubo et al., 2005). VND6 and VND7 were further characterized in Arabidopsis. VND6 is expressed
specifically in metaxylems, and VND7 is in both protoxylems and metaxylems (Zhong et al., 2008). Neither the vnd6 nor the vnd7 T-DNA insertional line shows any noticeable phenotype in the seedling stage. However overexpression of the VND6 repressor disrupts the secondary cell wall formation in metaxylems, while the unique helical cell wall deposition pattern in protoxylems is absent in the VND7 repressor. When overexpressed in Arabidopsis or poplar leaves, VND6 and VND7 activate metaxylem-like and protoxylem-like cell wall deposition in non-sclerified cells, respectively (Kubo et al., 2005).

**Figure 1.2.** Schematic diagram of a phylogenetic tree of SND1 homologues. The phylogenetic tree was constructed by the neighbor-joining method. The tree was determined and generated by MEGA4 (Kumar et al., 2008). Bootstrap values were calculated from 1000 replicates.

Secondary cell wall-associated NAC domain protein1 (SND1), NAC secondary wall thickening promoting factor1 (NST1) and NST2 are close homologues of VND6 and VND7 (Figure 1.2). SND1 shows strong expression in fibers in both stems and siliques (Zhong et al., 2006; Mitsuda et al., 2007; Mitsuda and Ohme-Takagi, 2008). NST1 and NST2 are present in
xylem tissues in stems and anther endothecia (Mitsuda et al., 2005). NST1 is also found in all the lignified cells in siliques. Knocking out of SND1, NST1, or NST2 alone doesn’t produce obvious phenotypes, except the valve margin lacks secondary cell walls in nst1. However, simultaneously reducing the expression of SND1 and NST1 dramatically blocks secondary cell wall formation in stem fibers, and thus the snd1 nst1 mutant loses the strength to support the entire plant body. Furthermore, cellulose, xylan, and lignin biosynthetic genes are downregulated in the double mutant, which leads to reduced fluorescence signals when the stem cross sections are probed with cellulose, xylan and lignin dyes or antibodies. In addition, as a consequence of a reduction in fiber walls in the enb layer in siliques, the snd1 nst1 seedpods are unable to generate enough strength to split open the siliques. In the nst1 nst2 double T-DNA lines, secondary cell wall deposition is inhibited in anther endothecia (Zhong et al., 2006&2007; Mitsuda et al., 2007; Mitsuda and Ohme-Takagi, 2008).

Not surprisingly, overexpression of VND6, VND7, SND1, NST1, or NST2 in the wild-type activates the expression of secondary wall biosynthetic genes and thus induces ectopic deposition of secondary cell walls in non-sclerified cells, such as in epidermal and mesophyll cells in cauliflower leaves (Kubo et al., 2005; Mitsuda et al., 2005; Zhong et al., 2006). Taken together, VND6 and VND7 are important regulators in vessel secondary wall biosynthesis; SND1 and NST1 are redundant key switches controlling secondary wall biosynthesis in fibers; whereas NST1 and NST2 work together in endothecia. (Demura and Fukuda, 2007; Zhong and Ye, 2007). Recently, Zhong et al. (2009) identified a group of SND1 homologues in poplar and proved that these poplar NACs are also functional homologues of SND1.
MYB TRANSCRIPTION FACTORS INVOLVED IN SECONDARY CELL WALL BIOSYNTHESIS

The DNA binding domain of MYB transcription factors is quite conserved among species. It contains up to three imperfect repeats, namely R1, R2, R3, with 50~53 amino acids each. In Arabidopsis, there are around 125 R2R3 MYB transcription factors, which regulate trichome formation, flavonoid biosynthesis, plant stress response, lignin biosynthesis, etc. (Stracke et al., 2001). One important feature of some MYB transcription factors is that they are able to activate AC cis-acting element-containing genes. AC elements were first identified in phenylalanine ammonia-lyase (PAL), one enzyme in the phenylpropanoid pathway. By a series deletion of the PAL promoter, Hatton et al. (1995) confirmed that three ~11bp fragments rich in adenosine and cytosine, namely AC-I, AC-II, and AC-III, direct the tissue specific expression of PAL and are the MYB protein binding sites. AC elements are also present in the promoters of the majority of lignin biosynthetic genes (Boerjan et al., 2003). Since then, several MYB transcription factors have been proved to regulate lignin production.

Overexpression of ZmMYB42 reduces lignin content and composition by down-regulating the expression levels of several lignin biosynthetic genes in maize (Sonbol et al., 2009). Overexpression of PtMYB1 leads to accumulated lignin deposition in tobacco leaves (Patzlaff et al., 2003). Overexpression of PtMYB8, another pine MYB transcription factor, results in ectopic lignin deposition in conifers (Bomal et al., 2008). EgMYB2 is capable of binding to the promoter regions of CCR and CAD, which contain AC elements. The EgMYB2 overexpressor induces the expression of some key lignin biosynthetic genes and consequently turns on lignin biosynthesis (Goicoechea et al., 2005). PtMYB1 and EgMYB2 possibly regulate cellulose and xylan biosynthesis as well, because their close Arabidopsis homologue MYB46 is capable of inducing
not only the lignin biosynthetic genes, but also the genes involved in cellulose and xylan biosynthesis (Zhong et al., 2007a). MYB46 is expressed in both vessel elements and fibers in stems. The MYB46 gene repressor line exhibits dramatic reduction in the secondary cell wall thickness in both stem vessel elements and fibers. The phenotype is mirrored when MYB46 and its homologue MYB83 are both knocked out. MYB46 and MYB83 are found to be the direct targets of SND1 and downstream targets of the SND1 homologues VND6, VND7, NST1, and NST2 (Zhong et al., 2007a; McCarthy et al., 2009). MYB103, MYB85, MYB52, and MYB54 are other downstream targets of SND1 that participate in secondary cell wall biosynthesis in stems (Zhong et al., 2008).

MYB58 and MYB63 are close homologues, whose expressions are strongly correlated with vessel elements and fibers in stems. The double myb58 myb63 RNAi mutant, the MYB58 repressor, or the MYB63 repressor exhibits reduced total lignin content. In the MYB58 or the MYB63 overexpressor, only the expression levels of lignin biosynthetic genes, but not those of cellulose or xylan biosynthetic genes are induced. As a consequence, the ectopically deposited secondary cell wall contains only lignin. In addition, MYB58 directly binds to the AC elements in the 4CL promoter and can turn on the expression of most monolignol biosynthetic genes as well as the laccase gene LAC4. It was also found that the expression of MYB58 and MYB63 is mediated by VND6, VND7, SND1, NST1, NST2, and MYB46, indicating that MYB58 and MYB63 are their downstream targets (Zhou et al., 2009).

- **TRANSCRIPTIONAL NETWORK**

Several cellular processes are regulated by transcriptional networks. For example, the AFL B3 and the VAL B3 transcription factors form a network, in which the two B3 subfamilies act antagonistically in regulating seed development (Suzuky and McCarty, 2008). MYB, Helix-
Loop-Helix (HLH) transcription factors, and WD40 proteins are known to control trichome formation and flavonoid biosynthesis coordinately (Ishida et al., 2008).

NAC and MYB transcription factors are currently the main regulators in secondary cell wall biosynthesis, although other transcription factors such as LIM and KNAT are also involved (Demura and Fukuda et al., 2006; Zhong et al., 2008). It has been proposed that a transcriptional network composed mainly of NAC and MYB transcription factors mentioned above regulates secondary cell wall biosynthesis. VND6, VND7, SND1, NST1, and NST2 are master regulators in vessel elements, fibers, or endothecia. Several levels of intermediate transcription factors, MYB46 for instance, transmit signals from the master regulators to specific secondary cell wall polymer activators, like MYB58 and MYB63, and finally reach the biosynthetic genes (Zhong and Ye, 2007; Zhong et al., 2008).

**SUMMARY OF THE STUDY**

FRA1 is involved in the cortical microtubule control of microfibril orientation (Zhong et al., 2002). We overexpressed FRA1 in the wild-type and found that the vessel elements were collapsed and the secondary cell wall thickness was reduced in fibers, which decreased the stem strength. Furthermore, microfibrils were deposited in multiple layers in both the vessel elements and fibers of overexpressors. These evidences further demonstrate that FRA1 is involved in the regulation of microfibril orientation. Since FRA1 is a kinesin-like protein, its cargoes may be related to microfibril deposition. In order to explore the functional mechanism of FRA1, we conducted a yeast two-hybrid screening for FRA1 interacting proteins. The FRA1 Interacting Protein1 (FIP1) was isolated and confirmed to interact with the first 2/3 of the FRA1 stalk domain by its C-terminal specifically in the yeast system. Besides FIP1, the gibberellin-regulated protein and the actin-related protein were identified as putative FRA1 interacting proteins.
In an effort to gain more insight into the transcriptional regulation of secondary cell wall biosynthesis in stems, we characterized the functions of SND1 close homologues in Arabidopsis. We found that VND3, VND4, and VND5 were expressed in vessel elements, while NST1 and NST2 were expressed in fibers. Under the control of the SND1 promoter, VND1~VND7, NST1, and NST2 were capable of complementing the secondary cell wall defect in the snd1 nst1 mutant, indicating that these genes are functional homologues. Overexpression of VND3 or VND4 resulted in ectopic secondary cell wall deposition-like phenotypes in cauliflower leaves. We also found that the VND1~VND5 repressors did not exhibit obvious phenotype. Therefore we suggest that VND1~VND5 are minor regulators of secondary cell wall biosynthesis. Although NAC15, NAC33, and NAC70 complemented the snd1 nst1 phenotype and induced ectopic secondary cell wall deposition-like phenotypes, they were mainly expressed in root caps and thus may function in a different cellular process.

REFERENCES


CHAPTER 2

ALTERATION IN SECONDARY WALL DEPOSITION BY OVEREXPRESSION OF THE FRAGILE FIBER1 KINESIN-LIKE PROTEIN IN ARABIDOPSIS


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ABSTRACT

Secondary walls in fibers and vessels are typically deposited in three distinct layers, which are formed by the successive re-orientation of cellulose microfibrils. Although cortical microtubules have been implicated in this process, the underlying mechanisms for the formation of three distinct wall layers are not known. The Fragile Fiber1 (FRA1) kinesin-like protein has been previously shown to be involved in the oriented deposition of cellulose microfibrils and important for cell wall strength in Arabidopsis thaliana. In the present report, we investigated the expression pattern of the FRA1 gene and studied the effects of FRA1 overexpression on secondary wall deposition. The FRA1 gene was found to be expressed not only in cells undergoing secondary wall deposition including developing interfascicular fibers and xylem cells, but also in dividing cells and expanding/elongating parenchyma cells. Overexpression of FRA1 caused a severe reduction in the thickness of secondary walls in interfascicular fibers and deformation of vessels, which are accompanied with a marked decrease in stem strength. Close examination of secondary walls revealed that unlike the wild-type walls having three typical layers with the middle layer being the thickest, the secondary walls in FRA1 overexpressors exhibited an increased number of layers, all of which had a similar width. Together, these results provide further evidence implicating an important role of the FRA1 kinesin-like protein in the ordered deposition of secondary walls, which determines the strength of fibers and vessels.
INTRODUCTION

Secondary walls, which are largely deposited in tracheary elements and fibers, are the most abundant component of biomass produced by plants. The major constituents of secondary walls are cellulose, hemicelluloses and lignin. Both cellulose and lignin provide mechanical strength to the walls, and lignin further renders the secondary walls waterproof by impregnating pores of secondary walls. Hemicelluloses, which are matrix polysaccharides mainly composed of xylan and mannan, are involved in cross-linking of cellulose microfibrils in secondary walls. The mechanical strength of secondary walls is determined by both the amount of wall components and their architecture. It has been known that the orientation of cellulose microfibrils in the secondary walls of tracheary elements and fibers is a key determinant of wood quality (Barnett and Bonham 2004). Understanding the mechanisms underlying the biosynthesis and the ordered deposition of secondary wall polymers will not only enrich our knowledge regarding wood formation but also have important implications in tree biotechnology.

Secondary walls are typically organized into three distinct layers, namely S1, S2 and S3, as visualized with a transmission electron microscope. The distinction of layers is due to the different orientations of cellulose microfibrils in secondary walls. In the S1 and S3 layers cellulose microfibrils are deposited in a flat helix, and in the S2 layer a steep helix (Harada and Coté 1985). The angle of cellulose microfibrils in the S2 layer, the thickest among the three layers, largely determines secondary wall strength and consequently wood quality (Barnett and Bonham 2004). Although the underlying mechanisms by which cellulose microfibrils are deposited into three distinct layers in secondary walls are still elusive, cortical microtubules (MTs) have been suggested to play an important role in this process. It has been shown that during secondary wall synthesis, cortical MTs undergo dynamic changes in their orientation and
they are aligned in parallel with cellulose microfibrils (Robards and Kidwai 1972; Seagull 1992; Abe et al. 1994, 1995; Prodhan et al. 1995). Consistent with the proposed role of cortical MTs in the oriented deposition of cellulose microfibrils, disruption of cortical MT organization by pharmacological drugs and mutation of the AtKTN1 MT-severing protein drastically alters the pattern of secondary wall thickening (Falconer and Seagull 1985; Burk and Ye 2002; Gardiner et al. 2003). Mutation of AtKTN1 in the fra2 mutant (Burk et al. 2001) results in an aberrant deposition of cellulose microfibrils in secondary walls, which leads to an absence of distinct layers (Burk and Ye 2002). Despite these lines of evidence indicating an involvement of cortical MTs in the ordered deposition of secondary walls, it is still not known how cortical MTs undergo dynamic re-orientation and thus direct the formation of three distinct wall layers during the process of secondary wall synthesis.

Kinesins are MT-binding motor proteins that are known to play important roles in many cellular processes, including mitosis, meiosis, and transport of vesicles and organelles (Lee and Liu 2004). In Arabidopsis, at least 61 kinesin genes have been identified and studies of several of these kinesin genes indicate their roles in spindle assembly and morphogenesis during mitosis and meiosis, cytokinesis, and cell morphogenesis (Yang et al. 2003; Lee and Liu 2004; Tanaka et al. 2004; Lu et al. 2005; Muller et al. 2006; Ambrose and Cyr 2007). One of them, the Fragile Fiber1 (FRA1) kinesin-like protein, has been shown to be involved in the oriented deposition of cellulose microfibrils in fibers (Zhong et al. 2002). The fra1 mutation causes an aberrant orientation of cellulose microfibrils in the innermost layer of secondary walls in fibers and concomitantly leads to a drastic reduction in stem strength. The fact that the fra1 mutation did not affect cortical microtubule organization or secondary wall synthesis indicates that FRA1 is required for the oriented deposition of cellulose microfibrils and cell wall strength.
To further understand the functions of FRA1, we investigated the expression pattern of the *FRA1* gene and studied the effects of FRA1 overexpression on secondary wall deposition. In this report, we show that the *FRA1* gene is ubiquitously expressed in cells undergoing expansion or elongation as well as in cells undergoing secondary wall deposition. We demonstrate that overexpression of FRA1 results in a drastic reduction in secondary wall thickness and an unusual increase in the number of layers in secondary walls. These findings provide further evidence supporting a role of FRA1 in the regulation of secondary wall deposition.

RESULTS

EXPRESSION PATTERN OF THE *FRA1* GENE

Mutation of the *FRA1* gene has been previously shown to cause a dramatic reduction in the mechanical strength of fibers and slightly affect plant growth (Zhong et al. 2002). To investigate whether the observed phenotypes are correlated with the expression pattern of *FRA1*, we used the β-glucuronidase (GUS) reporter gene to examine the tissue-level expression of the *FRA1* gene. To do so, we used a 10-kb genomic DNA fragment containing a 3-kb 5’ upstream sequence, the entire *FRA1* coding region including introns and exons, and a 2-kb 3’ downstream sequence. The GUS reporter gene was inserted in frame just before the stop codon of the *FRA1* gene in a binary vector to create the FRA1-GUS construct, which was used to transform wild-type *Arabidopsis* plants. Examination of the transgenic plants revealed ubiquitous GUS staining in young cotyledons (Figure 1A) and expanding leaves (Figure 1B). In primary roots, the GUS staining was intense in the apex and the elongating zone (Figure 1C) and gradually became concentrated in vascular tissues in the mature region (Figure 1D). In elongating internodes of inflorescence stems, the GUS staining was present in all tissues except epidermis (Figure 1E). In
non-elongating internodes in which fiber cells were undergoing secondary wall thickening, GUS staining was mainly seen in vascular bundles and interfascicular fiber cells (Figure 1F). These results demonstrate that the expression of the \textit{FRA1} gene is developmentally regulated and its expression in expanding or elongating parenchyma cells and in cells undergoing secondary wall thickening is consistent with the phenotypes conferred by the \textit{fra1} mutation.

\textbf{OVEREXPRESSION OF \textit{FRA1} CAUSES A REDUCTION IN SECONDARY WALL THICKNESS AND DEFORMATION OF VESSELS}

Our previous study on the \textit{fra1} mutant indicated a role of FRA1 in cellulose microfibril deposition and cell wall strength (Zhong et al. 2002). To further investigate the functions of FRA1 in secondary wall formation, we overexpressed FRA1 and studied its effects on secondary wall deposition. The full-length cDNA of \textit{FRA1} driven by the cauliflower mosaic virus 35S promoter was transformed into wild-type \textit{Arabidopsis} and overexpression of FRA1 in the transgenic plants was confirmed by reverse transcription-polymerase chain reaction (PCR) analysis (Figure 2A). Of 38 transgenic plants selected, 16 of them exhibited shorter inflorescence stems (Figure 2B) with reduced breaking strength (Figure 2C). The remaining transgenic plants did not exhibit obvious phenotypes probably due to a lower level of FRA1 overexpression. Because the stem strength is largely conferred by interfascicular fibers (Zhong et al. 1997), we examined the wall thickness of interfascicular fibers in the stems of one representative line of FRA1 overexpressors. Cross sections of stems revealed that FRA1 overexpression led to a drastic reduction in the wall thickness of interfascicular fibers compared with the wild-type (Figure 3A, B). Transmission electron microscopy of fibers allowed a quantitative measurement showing that the fiber wall thickness was reduced to 56% of that of the wild-type (Figure 4A, B; Table 1). In addition, the vessels in the FRA1 overexpressors exhibited a severely collapsed
phenotype (Figure 3C, D). Although on average the wall thickness of vessels in FRA1 overexpressors was reduced (Table 1), it was apparent that the thickness of some deformed vessels was comparable to that of the wild-type (Figure 4C, D). This observation indicates that the collapsed vessel phenotype is not always associated with reduced wall thickness.

Consistent with the decreased secondary wall thickness phenotype, monosaccharide composition analysis (Table 2) of cell walls isolated from inflorescence stems of FRA1 overexpressors revealed a 50% reduction in the level of glucose, the bulk of which is from cellulose. The amount of other cell wall monosaccharides, including xylose, mannose, galactose, arabinose and rhamnose, was not significantly affected.

**FRA1 OVEREXPRESSION RESULTS IN AN INCREASE IN THE NUMBER OF SECONDARY WALL LAYERS**

Although FRA1 overexpression caused a severe reduction in secondary wall thickness, it led to an apparent increase in the number of secondary wall layers. In the wild-type, the secondary walls of fibers and vessels are typically deposited in three layers, in which the middle layer S2 is the thickest (Figure 4A, C). By contrast, the secondary walls in FRA1 overexpressors (Figure 4B, D) had an increased number of layers, some up to eight, all of which had a similar width. Because the distinction of layers in secondary walls is due to the successive re-orientation of cellulose microfibrils, the formation of additional layers in the secondary walls of FRA1 overexpressors suggests that the regulated process of cellulose microfibril deposition is altered. This possibility is supported by the observation that in the FRA1 overexpressors, the cellulose microfibrils in the innermost layer of secondary walls of ~40% mature fibers are oriented in a nearly vertical direction along the elongation axis (Figure 5B), which is different from the
parallel flat helix as seen in the wild-type (Figure 5A). The remaining fiber cells of the FRA1 overexpressors had the typical parallel flat helix arrangement of cellulose microfibrils (data not shown).

DISCUSSION

Our expression analysis demonstrated that the FRA1 gene is expressed not only in cells undergoing secondary wall thickening but also in dividing cells and expanding or elongating parenchyma cells, suggesting that FRA1 might function in a common cellular process required for the deposition of both primary walls and secondary walls. The ubiquitous expression pattern of FRA1 is consistent with the genetic analysis showing that the fra1 mutation causes defects in both fiber strength and the overall plant growth (Zhong et al. 2002). Because the aberrant deposition of cellulose microfibrils was only observed in the secondary walls of fibers but not in the primary walls of parenchyma cells in the fra1 mutant (Zhong et al. 2002), it is likely that FRA1 plays a predominant role in the process of secondary wall deposition. In parenchyma cells, it is possible that the FRA1 homologue, At3g50240, can functionally compensate for the loss of FRA1. It will be interesting to investigate whether simultaneous mutations of both FRA1 and At3g50240 could lead to cell wall defects in parenchyma cells.

It is intriguing to find that overexpression of FRA1 led to reduced stem height and weakened stem strength, a phenotype similar to that of the fra1 mutant. However, a close examination of the secondary walls of fibers revealed distinct phenotypic differences between the FRA1 overexpressors and the fra1 mutant. The fra1 mutation causes an aberrant deposition of cellulose microfibrils but no alteration in fiber wall thickness (Zhong et al. 2002), whereas FRA1 overexpression results in a drastic reduction in secondary wall thickness and an increase in the
number of secondary wall layers. The observation of an increased number of secondary wall layers is of particular interest because it provides further evidence for a role of FRA1 in the regulation of secondary wall deposition. It is known that formation of distinct layers during secondary wall deposition is due to the successive re-orientation of cellulose microfibrils. Thus it is conceivable that excess FRA1 leads to an increase in the number of times of successive re-orientations of cellulose microfibrils, which results in a concomitant increase in the number of secondary wall layers. Because the thickness of the different wall layers does not exhibit much difference, it is apparent that excess FRA1 disrupts the normal cellular mechanisms leading to formation of the predominant S2 layer during secondary wall deposition as seen in the wild-type. Although secondary walls of fibers and vessels in plants typically have three layers, there are exceptions. For example, it has been found that in bamboo (*Dendrocalamus asper*) culms as many as six secondary wall layers can be seen in some fibers (Gritsch and Murphy 2005). It is interesting to note that although some vessels in the FRA1 overexpressors had walls as thick as those of the wild-type, they displayed a collapsed phenotype, suggesting that the ordered deposition of secondary walls in three typical layers is important for cell wall strength.

The fact that both the *fra1* mutation and FRA1 overexpression cause defects in the deposition of secondary walls, albeit in different ways, indicates that a tight regulation of FRA1 level is important for its normal cellular functions. There exist several precedents for this type of phenomenon. For example, a change in the level of the ubiquitous kinesin heavy chain (uKHC) kinesin in human cells by either overexpression or antisense suppression has been reported to result in a similar alteration in the cellular response to drugs (Axenovich et al. 1998). It was suggested that the observed alteration in drug response was due to an imbalance in the ratio of the uKHC kinesin to its interacting proteins or targets. In addition, it has been shown that either
overexpression or inhibition of the microtubule destabilizing kinesin xenopus kinesin catastrophe modulator-1 (XKCM1) causes the formation of aberrant spindles during mitosis and a mitotic delay (Kline-Smith and Walczak, 2002).

Our finding that FRA1 overexpression causes a reduction in secondary wall thickness and an increase in the number of secondary wall layers provides further evidence for the involvement of FRA1 in the regulation of secondary wall deposition. Because the fra1 mutation does not alter cortical MT organization (Zhong et al. 2002), it is likely that FRA1 participates in a cellular process between cortical MTs and deposition of cellulose microfibrils, which is consistent with the cell wall composition analysis showing that FRA1 overexpression causes a major reduction in cellulose content. FRA1 might directly or indirectly bind to cell wall synthesizing machinery and thus regulates cell wall deposition. Or alternatively, FRA1 might be involved in the transport of vesicles carrying cell wall materials and disruption of this process may lead to an alteration in the normal assembly of secondary walls. In either case, excess FRA1 could cause an alteration in the process of cell wall deposition, thus leading to defects in secondary wall formation. Since most kinesins are known to exert their cellular functions by carrying cargos along MTs, the next critical step is to identify the cargos that FRA1 binds to. Further functional characterization of FRA1 and its cargos will most certainly contribute to our understanding of the molecular mechanisms governing secondary wall deposition.

MATERIALS AND METHODS

GENE EXPRESSION ANALYSIS

The expression pattern of the FRA1 gene was studied using the GUS reporter gene. The FRA1 gene, including a 3-kb 5’ upstream sequence, the entire exon and intron region, and a 2-kb 3’
downstream sequence, was amplified from the wildtype *Arabidopsis* (ecotype Columbia) genomic DNA by PCR using gene-specific primers (5’-aacttgcgtcggtactgttcatgatg- 3’ and catgatcttagttagaggcc-3’; 5’-cttaaggctctacctaataagatcatg- 3’ and 5’
c tgacggaagcagttatatctctgc-3’) and a high-fidelity DNA polymerase (Elongase; Invitrogen, Carlsbad, CA, USA). The amplified DNA fragments were ligated into the SalI and EcoICRI sites of the pBI101 binary vector (Clontech, Mountain View, CA, USA). The GUS reporter gene was inserted in frame just before the stop codon of the *FRA1* gene to create the FRA1-GUS construct. The construct was transformed into wild-type *Arabidopsis* plants by the Agrobacterium-mediated transformation (Bechtold and Bouchez 1994). Transgenic plants were selected on kanamycin and the first generation of plants was used for expression analysis of the GUS reporter gene. Tissues were stained in GUS staining solution (100 mmol/L sodium phosphate, pH7.2, 10 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, and 1 mmol/L 5-bromo-4-chloro-3-indoly1 β-D-glucuronic acid) at 37°C for 4 h. Stems were cut into small segments before incubation with the GUS staining solution. After being cleared in 70% ethanol, the tissues were observed for GUS signals with a light microscope.

**GENERATION OF TRANSGENIC PLANTS OVEREXPRESSING FRA1**

The full-length *FRA1* cDNA was amplified with a high fidelity DNA polymerase (Elongase) by PCR (primers: 5’-ccaatggatatcctacaagccgcccagcagc-3’ and 5’ ttacatgatcattaggtccgaagtt-3’) and ligated into the pGEM T-Easy vector (Promega, Madison, WI, USA). The *FRA1* cDNA was confirmed by sequencing and ligated downstream of the cauliflower mosaic virus (CaMV) 35S promoter in a modified pBI121 binary vector (SalI site) to create the FRA1 overexpression (FRA1-OV) construct. The construct was introduced into wild-type *Arabidopsis* plants by the
Agrobacterium-mediated transformation. Transgenic plants were selected on kanamycin, and the T1 progeny were used for molecular and phenotypic characterizations.

**REVERSE TRANSCRIPTION-PCR ANALYSIS**

Total RNA was isolated from 2-week-old seedlings using the Qiagen RNeasy plant mini kit (Qiagen, Valencia, CA, USA). One microgram of the purified RNA was treated with DNase I to remove any potential genomic DNA contamination and then used for first strand cDNA synthesis. The first strand cDNA was used for PCR detection of the *FRA1* transcripts with gene-specific primers (5’-aagacaagtagatgagtcttcgagct-3’ and 5’- tgcagccttctctctatc-3’). The PCR was carried out for variable cycles to determine the logarithmic phase of amplifications for the samples. The reverse transcription (RT)-PCR reactions were repeated three times, and identical results were obtained. The expression of an ubiquitin gene was used as an internal control for determining the RT-PCR amplification efficiency among different samples.

**BREAK FORCE MEASUREMENT**

The main inflorescence stems of 10-week-old plants were used for breaking force measurement using a digital force/length tester (model DHT4-50; Larson System, Minneapolis, MN, USA). The breaking force (g) was calculated as the force needed to break apart a stem segment (Zhong et al. 1997).

**CELL WALL ANALYSIS**

The whole inflorescence stems of 10-week-old plants were collected for cell wall isolation. Stems were ground into fine powder in liquid nitrogen with a motar and pestle, homogenized with a polytron (PowerGen 700; Fisher Scientific, Pittsburgh, PA, USA), and extracted in 70% ethanol at 70°C. The remaining cell wall residues were dried in a vacuum oven at 60°C and used for analysis of cell wall monosaccharide composition. Cell wall monosaccharides (as alditol
acetates) were determined following the procedure described by Hoebler et al. (1989). All samples were run in duplicate.

**HISTOLOGY**

Stem samples were fixed in 2% (v/v) formaldehyde in PEMT buffer (50 mmol/L 1,4-piperazinediethanesulfonic acid [PIPES], 2 mmol/L ethylene glycol tetraacetic acid (EGTA), 2 mmol/L MgSO4, 0.05% [v/v] Triton X-100, pH 7.2) and further postfixed in 1% (v/v) OsO4. After dehydration through a gradient of ethanol and rinsed in propylene oxide, the samples were embedded in Araldite/Embed 812 resin (Electron Microscopy Sciences, Fort Washington, PA, USA) (Burk et al. 2006). One micrometer thick sections were cut, stained with toluidine blue, and viewed with a light microscope. For transmission electron microscopy, 90-nm ultra thin sections were cut, mounted onto formvar-coated gold slot grids, post-stained with uranyl acetate and lead citrate, and observed with a Zeiss EM 902A electron microscope (Carl Zeiss, Thornwood, NY, USA).

**VISUALIZATION OF CELLULOSE MICROFIBRILS**

The basal part of inflorescence stems was cut longitudinally through the interfascicular region and then fixed in PME buffer (25 mmol/L PIPES, 0.5 mmol/L MgSO4, 2.5 mmol/L EGTA, pH 7.2) containing 4% (v/v) formaldehyde (Sugimoto et al. 2000). After being rinsed with PME buffer, samples were treated with 0.1% (v/v) sodium hypochlorite for 10 min and then in cold 0.5% (v/v) OsO4 for 15 min. After dehydration through a gradient of ethanol series, samples were dried in a semi-dry critical point drier (Tousimis, Rockville, MD, USA) and mounted onto stubs with carbon paste. Samples were coated with platinum using an Edwards 306 vacuum evaporator (Edwards High Vacuum International, Wilmington, MA, USA), and then examined
for cellulose microfibrils using a LEO 982 FE scanning electron microscope (LEO, Thornwood, NY, USA).

ACKNOWLEDGEMENTS
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REFERENCES


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Table 2.1. Wall thickness of interfascicular fibers and vessels in the stems of the wild-type and Fragile Fiber1 (FRA1) overexpressors.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Interfascicular fiber</th>
<th>Vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRA1 overexpressor</td>
<td>1.43 ± 0.12</td>
<td>0.84 ± 0.41</td>
</tr>
<tr>
<td>Wild type</td>
<td>2.56 ± 0.38</td>
<td>1.25 ± 0.26</td>
</tr>
</tbody>
</table>

Wall thickness was measured from transmission electron micrographs of interfascicular fibers and vessels. Data are means (µm)±SE from 10 cells selected from different xylem bundles or interfascicular regions.
Table 2.2. Monosaccharide composition of cell walls from the stems of the wild-type and Fragile Fiber1 (FRA1) overexpressors.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>366 ± 45</td>
<td>141 ± 19</td>
<td>26.5 ± 3.5</td>
</tr>
<tr>
<td>FRA1 overexpressor</td>
<td>185 ± 56</td>
<td>115 ± 20</td>
<td>22.9 ± 2.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Galactose</th>
<th>Arabinose</th>
<th>Rhamnose</th>
<th>Fucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>9.6 ± 1.5</td>
<td>11.7 ± 0.6</td>
<td>6.5 ± 0.5</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>FRA1 overexpressor</td>
<td>11.2 ± 4.1</td>
<td>12.7 ± 1.3</td>
<td>7.8 ± 1.7</td>
<td>2.0 ± 0.1</td>
</tr>
</tbody>
</table>

Cell walls were prepared from stems of 10-week-old plants. Data are means (mg/g dry cell wall) ± variations of two independent assays.
**Figure 2.1** Expression pattern of the Fragile Fiber1 (*FRA1*) gene in *Arabidopsis* leaves, roots and stems.

Transgenic *Arabidopsis* plants expressing the β-glucuronidase (GUS) reporter gene fused with the *FRA1* gene were examined for GUS activity, which is shown as blue color. If, interfascicular fiber; pi, pith; xy, xylem. Bars, 135µm (E, F).

(A, B) Young cotyledon (A) from 3-day-old seedlings and expanding leaf (B) from 1-week-old seedlings showing GUS staining in all tissues with more prominent staining in vascular strands.

(C) Primary root of a 5-day-old seedling showing intensive GUS staining at the apex and elongating region.

(D) Mature region of the primary root of a 5-day-old seedling showing GUS staining mainly concentrated in the vascular cylinder.

(E) Section from a young elongating internode in which no interfascicular fibers were developed showing GUS staining in all tissues except epidermis.

(F) Section from a non-elongating internode in which interfascicular fibers were developed showing GUS staining predominantly in vascular bundles and interfascicular fibers. Note the prominent secondary walls in fiber cells.
**Figure 2.2** Effect of Fragile Fiber1 (FRA1) overexpression on plant morphology and stem strength.

**(A)** Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the *FRA1* mRNA level in the wild-type (WT) and six representative FRA1 overexpression lines. The expression of a ubiquitin gene was used as an internal control.

**(B)** Morphology of 8-week-old plants of the wild-type and one representative of 16 FRA1 overexpressor lines that exhibit shorter inflorescence stem phenotype.

**(C)** Breaking force measurement of stems showing the reduced stem strength in FRA1 overexpressors. The main inflorescence stems from eight independent transgenic lines were divided into three equal segments, and each segment was measured for breaking force. Data are means±SE of eight plants.
Figure 2.3. Fragile Fiber1 (FRA1) overexpression causes a reduction in fiber wall thickness and deformation of vessels.

The bottom internodes of inflorescence stems of 8-week-old plants were sectioned and stained with toluidine blue for examination of cell morphology. co, cortex; if, interfascicular fiber; ph, phloem; ve, vessel. Bars, 90μm.

(A) Cross section of an interfascicular region of wild-type showing interfascicular fibers with thick walls.

(B) Cross section of an interfascicular region of FRA1 overexpressors showing interfascicular fibers with thin walls.

(C) Cross section of a vascular bundle of wild-type showing vessels with regular shapes.

(D) Cross section of a vascular bundle of FRA1 overexpressors showing collapsed vessels (arrows).
Figure 2.4. Increased number of secondary wall layers in fibers and vessels of Fragile Fiber1 (FRA1) overexpressors.

Fibers and vessels from the bottom internodes of 8-week-old plants were examined for their wall morphology using a transmission electron microscope. Bars, 2.7µm.

(A) Secondary walls of wild-type interfascicular fiber cells showing three typical layers, S1, S2 and S3. The layer S2 is the thickest.

(B) Secondary walls of interfascicular fiber cells of FRA1 overexpressors showing an increased number of layers.

(C) Wild-type xylem cells showing vessel walls with three typical layers, S1, S2 and S3.

(D) Secondary walls of vessels of FRA1 overexpressors showing an increased number of layers.
Figure 2.5. Cellulose microfibrils in the innermost layer of fiber walls visualized with a field emission scanning electron microscope.

The vertical direction of the image corresponds to the elongation axis of the cell. Bars, 270 nm.

(A) Cellulose microfibrils in a wild-type fiber cell showing their parallel alignment in a nearly transverse orientation along the elongation axis.

(B) Cellulose microfibrils in a fiber cell of Fragile Fiber1 (FRA1) overexpressors showing their parallel alignment in a nearly vertical orientation along the elongation axis.
CHAPTER 3

ISOLATION OF PUTATIVE FRAGILE FIBER1 (FRA1) INTERACTING PROTEINS

ABSTRACT

The orientation of cellulose microfibrils is an important determinate of the direction of cell expansion. The deposition of microfibrils is believed to be regulated by cortical microtubules, but the mechanism remains unknown. Mutation or overexpression of the kinesin-like protein Fragile Fiber1 (FRA1) results in abnormal microfibril orientation, which makes FRA1 an important factor in studying the microtubule control of microfibril orientation. Here, we used a yeast 2-hybrid system to screen for putative FRA1 interacting proteins and isolated the FRA1 Interacting Protein1 (FIP1), the gibberellin-regulated protein1, and the actin-related protein5. We also found that the C-terminal of FIP1 interacted with the first two thirds of the stalk domain of FRA1.
INTRODUCTION

One of the most important features of plant growth is the establishment of polarity, which gives rise to various shapes and growth directions of plant organs. Many factors such as auxin gradients are of considerable importance to building plant polarity. At the cellular level, polarity is normally achieved by anisotropic cell expansion (Hepler et al., 2001). During anisotropic enlargement, the old cell wall rearranges, and the new cell wall materials are deposited in a way that accommodates the expansion. It is believed that cellulose microfibrils play important roles in the regulation of anisotropic expansion (Baskin et al., 2005).

Cellulose microfibrils, one of the major cell wall components, are assemblies of β-1, 4 glucan chains and are produced by a rosette-like enzyme complex called the cellulose synthase complex (CSC). As they move through the plasma membrane, CSCs produce glucan chains, which are then crystallized into parallel microfibrils (Somerville, 2006). The microfibrils in the primary cell wall normally form a disordered network but are aligned transversely to the elongating axis in growing cells. The secondary cell wall consists of three layers of microfibrils, denoted S1, S2, and S3. Layer S1 is the outmost, and layer S3 is most adjacent to the cytoplasm. The microfibrils in these two layers form a shallow helix. Layer S2, the thickest one, features a steep helix (Cutter, 1978). Disorganization of the microfibrils causes radical rather than longitudinal root growth in radical root swollen1 (rsw1), kobito1 (kob1), and corba (cob) (Martin et al., 2001; Sugimoto et al., 2001; Pagant et al., 2002).

Currently it is most widely accepted that microfibril deposition is regulated by cortical microtubules (Lloyd and Chan, 2008). Cortical microtubules are located underneath the plasma membrane and oriented perpendicular to the longitudinal axis during cell enlargement, just like microfibrils. When the orientation of cortical microtubules is disrupted, the cell tends to grow...
isotropically instead of anisotropically (Baskin et al., 2004). For example, at the restrictive
temperature, cortical microtubules of the temperature-sensitive mutant microtubule
organization1 (mor1) are disorganized. As a result, the mor1 mutant features isotropic growth
(Whittington et al., 2001). In many instances, abnormally aligned cortical microtubules cause
changes of the orientation of microfibrils. Treatment with the microtubule depolymerization or
stabilization drugs is known to affect the organization of microfibrils in algae and angiosperms
(Baskin et al., 2001). Mutation of the katanin-like protein Fragile Fiber2 (FRA2) alters the
organization of microtubules. In addition, the fra2 mutant causes microfibrils to run in different
directions rather than the usual transverse bands (Burk and Ye, 2002). These evidences confirm
that cortical microtubules have a role in regulating the orientation of microfibrils.

Heath (1974), and Gidding and Staehelin (1991) proposed that the movement of CSCs is
determined by cortical microtubules. Later, Paredez et al. (2006) proved the correlation between
the movement of CSCs and cortical microtubules, finding that the labeled CSCs travel along the
labeled cortical microtubules in Arabidopsis hypocotyls, and the trajectory of CSCs changes
accordingly if the cells are treated by low-dosage microtubule drugs. Recently, Wightman and
Turner (2008) discovered a similar consistency between the CSCs and the cortical microtubules
in xylems. Additionally, Guiterrez et al. (2009) and Crowell et al. (2009) demonstrated that
cortical microtubules participate in the delivery and internalization of CSCs.

Cortical microtubules can transport cargoes by their motor protein, kinesin. Most kinesins
consist of three domains: a motor domain, a stalk domain, and a tail domain. The motor domain,
normally located at the N-terminal, contains a highly conserved ~350 amino acid-long catalytic
core that is responsible for binding to microtubules and powering kinesin movement. The stalk
domain and tail domain are highly divergent even within the same kinesin family and are
involved in dimmer formation and cargo binding. Fragile Fiber1 (FRA1) is a kinesin-like protein localized beneath the plasma membrane, which coincides with the location of cortical microtubules. In the fra1 mutant, microfibrils in the innermost layer of fiber walls are no longer as densely packed and as orderly oriented as in the wild-type, consequently reducing the cell wall strength and somewhat inhibiting cell elongation. However, the cortical microtubule pattern appears normal (Zhong et al., 2002). When overexpressed, FRA1 results in a reduction of fiber secondary wall thickness and collapsed vessel elements, which in turn causes decreased stem strength and plant growth. Interestingly, the microfibrils form multiple layers, instead of three layers, in the secondary cell walls of vessel elements and fibers. A direct examination of the innermost wall layer in fibers in FRA1 overexpressors reveals that the microfibrils are orientated longitudinally instead of nearly transversely along the elongating axis, indicating that FRA1 is an important player in the regulation of microfibril orientation (Zhou et al., 2007). To gain more insight into the functional mechanism of FRA1, in this study, we conducted a yeast two-hybrid screening. We isolated the FRA1 Interacting Protein1 (FIP1), the gibberellin-regulated protein1, and the actin-related protein5 as putative FRA1 interacting proteins. We also demonstrated that FIP1 interacted with the first 2/3 of the FRA1 stalk domain through its C-terminal.

RESULTS

FIP1, THE GIBBERELLIN-REGULATED PROTEIN1, AND THE ACTIN-RELATED PROTEIN5 ARE PUTATIVE FRA1 INTERACTING PROTEINS

We used a yeast two-hybrid system to screen for FRA1 interacting proteins. The entire FRA1 cDNA sequence was used as the “bait”. An Arabidopsis seedling cDNA library consisting of cDNAs fused with the activation domain of Gal4 in the pACT vector was screened. To eliminate
the possibility that FRA1 alone activated the reporter genes, the FRA1 bait plus the empty pACT vector were transformed into yeast cells. Transformants were unable to grow on selection plate (synthetic complete minus Trp, Leu, His) nor activate the *lacZ* reporter gene (Figure 3.1). The results indicate that only in the presence of FRA1 and its interacting protein may the reporter genes be expressed.

A total of $4.9 \times 10^5$ transformants were plated on selection plates (synthetic complete minus Trp, Leu, His). Sixty-six colonies grew on these plates and were tested for the β-galactosidase activity using a filter lift assay. Most of the 38 colonies that exhibited the β-galactosidase activity were sequenced at the 5’end and then searched in the NCBI database (Table 3.1). The 16 colonies showing strong β-galactosidase activity contained partial sequences derived from the C-terminal of the same gene At3g15950. We named it FRA1 Interacting Protein1 (FIP1). Besides FIP1, a few other proteins were identified, among which the gibberellin-regulated protein1 (At1g75750) and the actin-related protein5 (At3g12380) were considered putative FRA1 interacting proteins. According to the AtGenExpress Visualization Tool, *FIP1* is predominantly expressed in roots, stems, leaves and shoot apex, the gibberellin-regulated gene is strongly expressed in all tissues, and the actin-related gene is ubiquitously present with a preference in shoot apex (Schmidt et al., 2005).

**SEQUENCE ANALYSIS OF THE FIP1 PROTEIN**

The *FIP1* gene is 4828bp long from the start codon to the stop codon with 28 exons and 27 introns. The FIP1 protein is 772 amino acid-long with a predicted isoelectric point of 4.3. Ten conserved glutamic acid-phenylalanine-glutamic acid (EFE) repeats, each of which is 40 amino acid-long, are present at the N-terminal of the FIP1 protein. The EFE repeats are known to bind to Ca$^{2+}$ (Suzuki et al., 2005). The sequencing results from the 16 colonies containing partial FIP1
sequences revealed that FIP1 interacted with FRA1 through its C-terminal. The longest sequence identified covered the amino acid 600-772, and the shortest one covered the amino acid 654-772. The pair coiled-coil prediction program (Lupas et al., 1991) indicates that two regions within FIP1 may form a coiled-coil structure (Figure 3.2). One region is inside the EFE repeats, and the other is located at the C-terminal.

Comparison of the FIP1 protein sequence with those protein sequences deposited in the GenBank database showed that its closest homologue in Arabidopsis is the TONSOKU (TSK)-Association Protein1 (TSA1). The function of TSA1 is unknown yet but is predicted to be involved in mitosis (Suzuki et al., 2005). No FIP1 homologue in animals or fungi has been identified.

**FIP1 INTERACTS WITH THE FIRST 2/3 OF THE STALK DOMAIN OF FRA1 THROUGH ITS C-TERMINAL**

To confirm that the FIP1-FRA1 interaction is specific, we cloned the last 474bp of the *FIP1* gene into the pACT vector and tested if this fragment could activate the reporter genes alone. The yeast cells carrying the pACT-FIP1-474 and the empty Bait vector failed to grow on the selection plate and induce the *lacZ* gene, indicating that the interaction between FIP1 and FRA1 is specific (Figure 3.1).

To further define the region within which FRA1 is responsible for the FIP1-FRA1 interaction, a series of deleted FRA1 sequences were cloned into the Bait vector: the FRA1 entire stalk domain plus the tail domain, the FRA1 stalk domain only, the last 1/3 of the FRA1 stalk at the C-terminal plus the tail domain, and the FRA1 tail domain. Each deletion construct was transformed into yeast cells together with pACT-FIP-474. We found that yeast cells containing the entire FRA1 protein, the stalk and tail domain, or the stalk domain survived under selection
and exhibited color response in the filter lift assay. In contrast, the last 1/3 of the FRA1 stalk domain at the C-terminal plus the tail domain, or the FRA1 tail domain failed to dimmerize with the C-terminal of FIP1 (Figure 3.3). Taken together, these results indicate that FRA1 interacts with FIP1 through the first 2/3 of its stalk domain.

DISCUSSION

FRA1 is demonstrated to be involved in the regulation of microfibril orientation (Zhong et al., 2002; Zhou et al., 2007), but its functional mechanisms remain unresolved. FRA1 belongs to the kinesin-4 subfamily (Lee and Liu, 2004). Its animal counterparts are involved in mitosis (Brouhard and Hunt, 2005) and vesicle trafficking (Martinez et al., 2008). Since microfibrils are produced at the plasma membrane, it is quite possible that FRA1 is responsible for transporting wall materials required for microfibril orientation.

To explore further the functional mechanisms of FRA1, we resorted to screening for FRA1 interacting proteins. The yeast 2-hybrid screen is one of the most effective approaches for studying protein interactions due to the fact that no protein purification or antibody preparation is required (Pandey and Mann, 2000). Since Heath (1974) proposed that cortical microtubules direct the movement of CSCs, we first tested if FRA1 interacted with CSCs. However, we didn’t detect any interaction between FRA1 and one of the secondary cell wall CSC subunit, CESA7, in a yeast 2-hybrid assay (data not shown). However, the possibility that FRA1 interacts with the other CESA subunits or the entire CESA complex as a whole cannot be excluded. Another concern is that post-translational modifications of FRA1 or CESA7 may not have occurred in the yeast expression system, which may affect protein interaction.
We isolated several proteins by yeast 2-hybrid. Since the structural protein, the endonuclease protein, and the chlorophyll binding proteins don’t seem to be directly related to microfibril orientation, we only consider FIP1, the gibberellin-regulated protein1, and the actin-related protein5 as putative FRA1 interacting proteins.

FIP1 shares 55.2% identity and 69% similarity with its closest homologue TSA1. It is conceivable that FIP1 follows a similar dynamic subcellular localization pattern as TSA1, which is around the cell periphery and nuclear envelop, associated with cytoplasmic vesicles during interphase, and has a close association with spindle microtubules during cell division (Suzuki et al., 2005).

Yamada et al. (2008&2009) demonstrated that down-regulation of FIP1/NAI2 changes the number and shape of the ER-body, a novel ER-derived structure that stores proteins. In addition, in the nai2/fip1 mutant, the ER-body-localized β-glucosidase protein PYK10 is spread out in the ER system instead of being concentrated in the ER-body, indicating that FIP1/NAI2 has a role in retaining proteins in the ER-body.

Typically, the interaction between kinesins and their cargoes occur in the kinesin tail region. However, the FRA1 interacting domain is mapped to the first 2/3 of the stalk domain. There are a few cases in which the kinesin domain is involved in protein binding. For example, the Rab3 GDP/GTP exchange protein, Rab3-GEP, binds to the stalk domain of KIF1Bbeta and KIF1A and then is transported toward the axon (Niwa et al., 2008). The human lymphocyte homologue of the Drosophila Discs Large tumor suppressor protein (hDlg) is distributed throughout the cytoplasm in resting cells. Upon activation, the cortical cytoskeleton reorganizes and hDlg is translocated towards the periphery. It is proposed that the interaction with the first half of the kinesin protein, GAKIN, is essential for hDlg translocation (Hanada et al., 2000). Perhaps FRA1
has a role in regulating the localization pattern of FIP1, which may then retain wall materials related to microfibril orientation in the cytosol. A test of this hypothesis would be to investigate the localization pattern of FIP1 in the \textit{fra1} mutant and the microfibril orientation in the \textit{fip1} mutant.

The second putative FRA1 interacting protein is the gibberellin-regulated protein1. Bayer et al. (2006) found that this protein is located in the cell wall, allowing it to possibly interact physically with proteins located around the cell periphery, such as FRA1. It is known that gibberellin treatment induces transverse microfibrils and thus promotes cell elongation (Shibaoka, 1994). Several gibberellin insensitive mutants exhibit a dwarf phenotype, which could be due to reduced cell expansion (Achard and Genschik, 2009). Funada et al. (2008) found that application of gibberellin to woody plants induces the formation of thicker cell walls in the inner cell layers in fibers. The expression of the gibberellin-regulated protein1 is regulated by gibberellin, indicating that it may have a role in aligning microfibrils.

The third putative FRA1 interacting protein is the actin-regulated protein5. In plants, actin cables normally run longitudinally along the long axis and are known to be involved in the regulation of cell expansion. Kobayashi et al. (1988) discovered that the actin bundles become denser as the cell wall is deposited. Wightman and Turner (2008) discovered that the CESA7 subunit containing-organelle moves along the actin cables in the cytoplasm. Additionally, disruption of actin polymerization prevents the organelle movement and affects the appearance of the CESA7 subunit at the cell wall place, indicating that actins also play a role in microfibril deposition (Wightman and Turner, 2008). The actin-related protein5 identified has been studied by a different group who found that the knockout mutant plant exhibits smaller cells and a dwarfish phenotype (Kandasamy et al., 2009), which may be caused by defects of cell expansion.
In summary, we identified three putative FRA1 interacting proteins. The various cellular processes in which these proteins may be involved all have a connection with microfibril deposition. The next step would be to validate their in vivo interaction with FRA1 and further characterize their functions.

Materials and Methods

Plasmid Construction

The Bait-FRA1-full was constructed by cloning the entire FRA1 sequence (amino acids 1~1035) into the pGEM-T vector. This plasmid was then cut with NcI1 and EcoRI, releasing a ~3kb fragment, which was ligated into the bait vector. FRA1 deletions were made by cloning the FRA1 entire stalk domain plus the tail domain (amino acids 384~1035), the last 1/3 of the FRA1 stalk at the C-terminal plus the tail domain (amino acids 730~1035), the FRA1 stalk domain only (amino acids 384~902), and the FRA1 tail domain (amino acids 902~1035) into the pBait vector, respectively. The pACT-FIP1-C was constructed by cloning the fragment (amino acids 614~772) of FIP1 into the pGEM-T vector. This plasmid was then cut, releasing a 474bp fragment, which was ligated into pACT.

YEAST TWO-HYBRID

To prepare yeast competent cells, 50ml yeast strain Y190 was cultured in the YPD liquid medium until the OD600 value reached 0.6. Yeast cells were centrifuged, washed in TE, and resuspended into 400µl TE-LiAc solution (10% TE, 10% LiAc). For transformation, 0.5µg pBait-FRA1 plasmid, 100µg salmon sperm carrier DNA, 600µl freshly made 40% PEG and 100µl competent cells were incubated at 30°C with shaking at 200rpm for 45 minutes. 70µl DMSO was added to the mixture, which was then heat shocked at 42 °C for 15 minutes. The
cells were centrifuged at 12,000rpm for 1 minute at room temperature, resuspended in TE, and plated out on synthetic medium (SD) lacking Trp plates. All the plates were incubated at 30 ºC. Next, competent cells were made from cultures of several colonies on the SD lacking Trp plates. 0.25mg Arabidopsis seedling cDNA library that is cloned in the pACT vector plus 1mg salmon sperm carrier DNA were transformed. Cells were diluted and plated out on SD lacking Trp-Leu and SD lacking Trp-Leu-His plates. The total number of transformants was calculated from the number of colonies grown on SD lacking Trp-Leu. For double transformation, two plasmids (0.5 µg each) and 100µg salmon sperm carrier DNA were transformed into competent cells prepared from Y190, plated on both selection and nonselection plates. Colonies growing on SD lacking Trp-Leu-His plates were patched to new plates for the filter lift assay.

FILTER LIFT ASSAY AND DNA SEQUENCING
Sterile filter papers were placed over the surface of colonies, lifted, and transferred into liquid nitrogen for 30 seconds, and then allowed to thaw at room temperature. Filter papers were then placed on top of another piece of filter paper that was pre-soaked in the Z buffer (see the CLONETECH manual), incubated at 30 ºC until blue color appeared. Colonies showing blue color were resuspended in SD lacking Trp-Leu-His medium and used as templates in PCR reactions to amplify the cDNA sequences inserted in pACT. Amplified sequences were purified using a Qiagen gel extraction kit (Invitrogen) and sequenced using the BidDye sequencing kit (Applied Biosystems).

REFERENCE


essential for oriented deposition of cellulose microfibrils and cell wall strength. Plant Cell 14: 3101-3117.


Table 3.1. Putative Fragile Fiber1 (FRA1) interacting proteins isolated by yeast 2-hybrid.

<table>
<thead>
<tr>
<th>Candidate Genes (annotation)</th>
<th># of Colonies</th>
<th>Interaction Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>At3g15950 (FIP1)</td>
<td>16</td>
<td>strong</td>
</tr>
<tr>
<td>At4g18730 (structural constituent of ribosome)</td>
<td>1</td>
<td>strong</td>
</tr>
<tr>
<td>At5g41150 (endodeoxyribonuclease)</td>
<td>1</td>
<td>strong</td>
</tr>
<tr>
<td>At5g54270 (chlorophyll binding protein3)</td>
<td>1</td>
<td>strong</td>
</tr>
<tr>
<td>At1g75750 (gibberellin-regulated protein1)</td>
<td>1</td>
<td>medium</td>
</tr>
<tr>
<td>At3g12380 (actin-related protein5)</td>
<td>1</td>
<td>medium</td>
</tr>
<tr>
<td>At1g64880 (ribosomal protein)</td>
<td>1</td>
<td>medium</td>
</tr>
<tr>
<td>At5g38420 (ribulose-biphosphate carboxylase)</td>
<td>1</td>
<td>medium</td>
</tr>
<tr>
<td>At1g04780 (ankyrin repeat family protein)</td>
<td>2</td>
<td>medium</td>
</tr>
<tr>
<td>At3g11590 (unknown protein)</td>
<td>1</td>
<td>weak</td>
</tr>
</tbody>
</table>

# of colonies refers to the number of colonies that grew on selection plates and exhibited color reaction in the filter lift assay. Interaction intensity was determined by the intensity of blue color in the filter lift assay. Twelve more colonies were either not sequenced or the sequences were not found in the Arabidopsis genome. Annotation (except FIP1) is based on the TAIR prediction (Swarbreck et al., 2008).
Figure 3.1. The C-terminal of FRA1 Interacting Protein1 (FIP1) interacts with Fragile Fiber1 (FRA1) specifically.

Note that only the cells containing both FRA1 and FIP1-C grew on the selection plate and showed blue color in the filter lift assay. BD: binding domain; AD: activation domain; FRA1, the FRA1 full length protein; FIP1-C: the C-terminal of FIP1 from the 614th to the 772nd amino acid.

Figure 3.2. FRA1 Interacting Protein1 (FIP1) has two putative coiled-coil domains.

The X axis indicates the entire FIP1 protein. Regions with peaks above the dash line are putative coiled-coil domains (Pair coiled-coil prediction program: Lupas et al., 1991).
Figure 3.3. Fragile Fiber1 (FRA1) interacts with the C-terminal of FRA1 Interacting Protein1 (FIP1) through the first 2/3 of its stalk domain. BD: binding domain; AD: activation domain; F1M: FRA1 motor domain; F1S: FRA1 stalk domain; F1T: FRA1 tail domain; FIP1-C: the C-terminal of FIP1 from the 614th amino acid to the 772nd amino acid.
CHAPTER 4

FUNCTIONAL CHARACTERIZATION OF NAC TRANSCRIPTION FACTORS INVOLVED
IN THE REGULATION OF SECONDARY CELL WALL BIOSYNTHESIS

Secondary cell wall biosynthesis is a complicated process that involves the cooperation of cellulose, hemicelluloses, and lignin biosynthetic genes. These secondary cell wall biosynthetic genes are regulated by a transcriptional network, in which the NAC transcription factors vascular related NAC domain protein6 (VND6) and VND7 are master regulators in vessel elements in primary roots, secondary wall-associated NAC domain protein1 (SND1) and NAC secondary wall thickening promoting factor1 (NST1) are main switches in fibers in stems, while NST1 and NST2 are key mediators in anther endothecia. To expand our understanding of the transcriptional regulation of secondary cell wall biosynthesis, SND1 homologues were functionally characterized in this study. We demonstrated that VND3, VND4, and VND5 were specifically expressed in stem vessel elements, NST1 and NST2 were expressed in stem fibers, while NAC15, NAC33, and NAC70 were mainly expressed in root caps. We also found that these NAC transcription factors were capable of complementing the secondary cell wall defects in fibers in the snd1 nst1 double mutant. In addition, overexpression of VND3, VND4, NAC15, NAC33, or NAC70 induced ectopic secondary cell wall deposition-like phenotypes in the wild-type Arabidopsis thaliana.
INTRODUCTION

Xylem is the primary water-conducting tissue that transports water and minerals from roots to all aerial organs in vascular plants. In flowering plants, the xylem tissue mainly consists of vessel elements, fibers, and parenchyma cells. Vessel elements can be further divided into three groups: protoxylem, metaxylem, and secondary xylem. At maturity, vessel elements join together through their openings at both ends to facilitate water and mineral transport. Fibers are normally much longer than they are broad and provide mechanical support to plants (Ye, 2002). Though functionally and morphologically different, both vessel elements and fibers deposit thick secondary cell walls after they cease enlargement. The secondary cell wall contains three types of polymers: cellulose, hemicelluloses, and lignin. These polymers strengthen and waterproof cells and are thus essential to plants. Moreover, the secondary cell wall is of considerable importance to humans for its applications in biofuel, paper, and timber production (Zhong and Ye, 2009). Therefore, the question of how the secondary cell wall components are synthesized has always been considered.

Formed of β-1, 4-linked glucan chains, cellulose is synthesized at the plasma membrane by the cellulose synthase complexes (CSCs). In Arabidopsis 10 genes encoding putative CSC subunits are identified, of which CESA4, CESA7 and CESA8 are shown to be secondary cell wall specific (Somerville et al., 2006; Mutwil et al., 2008). Knocking out CESA4, CESA7, or CESA8 causes reduced cellulose and thus collapsed xylems (Taylor et al., 2003; Zhong et al., 2003). The major type of hemicelluloses in dicotyledons is glucuronoxylan (GX), featuring a backbone chain of β-1, 4-linked xylose with side branches. Unlike cellulose, GXS are produced in the Golgi apparatus and transported to the plasma membrane. Recent genetic and biochemistry studies of collapsed xylem mutants revealed that glycosyltransferases Fragile Fiber8 (FRA8),
FRA8 Homologue (F8H), Irregular Xylem 8 (IRX8), PARVUS, IRX9, IRX14, IRX10, and IRX10L are involved in GX synthesis (Zhong et al., 2005, Brown et al., 2007; Lee et al., 2007; Pena et al., 2007; Brown et al., 2008; Wu et al., 2008; Lee et al, 2009). Lignins are heteropolymers consisting of three monolignols: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. A well-established monolignol biosynthetic pathway suggests that series steps of modification on phenylalanine through actions of around 10 enzymes leads to the production of the three monolignols in the cytoplasm. The resulting monolignols are then polymerized at the plasma membrane under unknown mechanisms (Boerjan et al., 2003).

The expression of secondary cell wall biosynthetic genes is highly coordinated and is proposed to be under the control of a transcriptional cascade (Zhong and Ye, 2007). In the cascade, a few NAC transcription factors are placed in the core position. Vascular-related NAC domain protein6 (VND6) and VND7 are expressed in root vessel elements, where they are the key switches in the formation of the secondary cell wall (Kubo et al., 2005). Secondary cell wall-associated NAC Domain protein1 (SND1), a fiber specific NAC transcription factor, not only induces the expression of several cellulose, xylan, and lignin biosynthetic genes, but also activates the majority of the transcription factors in the proposed transcriptional cascade. Overexpression of SND1 results in ectopic secondary cell wall deposition in nonsclerified cells in leaves and flowers, which in turn leads to curled leaves as well as smaller and sterile flowers. The loss-of-function snd1 mutant does not show obvious phenotype; however, the SND1 repressor, in which the entire SND1 coding region is fused with a transcriptional repression domain, dramatically turns down fiber secondary cell wall biosynthesis and decreases the fiber cell wall thickness. Due to the reduction in the fiber secondary cell wall, the SND1 repressor plant is unable to uphold its stems (Zhong et al., 2006). Simultaneously knocking down or
knocking out SND1 and its closest homologue NAC secondary wall thickening promoting factor1 (NST1) replicates the SND1 repressor phenotype and further causes defects of the fiber secondary cell wall thickening in siliques. SND1 and NST1 are thus considered functional homologues in controlling the secondary cell wall biosynthesis in fibers (Mitsuda et al., 2007; Zhong et al., 2007). Additionally, Mitsuda et al. (2005) confirmed that NST1 and its homologue NST2 redundantly regulate secondary cell wall thickening in anther endothecia.

VND6, VND7, SND1, NST1, and NST2 are grouped together with VND1, VND2, VND3, VND4, VND5, NAC15, NAC33, and NAC70 in the same NAC transcription factor subfamily (Figure 1.2, Mitsuda et al., 2005). Conceivably, all the other NAC genes are involved in the regulation of secondary cell wall biosynthesis. In the current study, we showed that VND3, VND4, and VND5 were expressed in vessel elements; while NST1 and NST2 were fiber-specific genes. We also demonstrated that all these NAC transcription factors were functional homologues of SND1. In addition, overexpression of VND3, VND4, NAC15, NAC33, or NAC70 in the wild-type resulted in curled leaves, which is an indication of ectopic secondary cell wall deposition.

RESULTS

EXPRESSION PATTERNS OF SND1 HOMOLOGUES

Previous studies showed that VND6 and VND7 are expressed in vessel elements in both stems and roots (Zhong et al., 2008), and SND1 is preferentially expressed in fibers in stems (Zhong et al., 2006). To characterize the NAC transcription factors, we first examined the expression patterns of the NAC genes using the β-glucuronidase (GUS) reporter system. For the GUS reporter gene analysis, a ~3kb 5’ sequence upstream of the translation start codon, the
entire exon and intron sequences, and a ~2 kb 3’ sequence downstream of the translation stop
codon were used. A comprehensive organ-level expression analysis showed that these NAC
genes displayed partially overlapping expression in seedlings, mature roots, stems, flowers, and
siliques (Table 4.1). NAC15, NAC33, and NAC70 were not expressed in stems but in root caps in
seedlings (Table 4.1), which is consistent with the finding of Bennett et al. (2010). NAC15 also
showed some expression in vascular tissues in seedlings (data not shown).

A closer examination of the stem cross sections revealed that VND3~VND5 were expressed
in vessels but not in fibers, while NST1 and NST2 were expressed in fibers but not in vessels. In
mature roots, VND3, VND4, VND5, NST1, and NST2 were all expressed in the secondary xylem
(Figure 4.1).

COMPLEMENTATION OF THE FIBER SECONDARY CELL WALL DEFECTS IN
THE SND1 NST1 DOUBLE MUTANT

The stems of the snd1 nst1 double T-DNA line fail to stand upright because of a almost
complete loss of secondary wall thickening in fibers (Mitsuda et al., 2007; Zhong et al., 2007).

To investigate the functions of the NAC genes, we adopted a mutant complementation approach.
These NAC genes were placed under the control of the SND1 promoter and transformed into the
snd1 nst1 double mutant plants, respectively. The stems of the complementation lines were as
erect as those in the wild-type (Figure 4.2A). As shown in Figure 4.3, the secondary cell wall in
fibers was almost completely lost in the snd nst1 mutant. However, the deposition of the
secondary cell wall was recovered largely by expressing VND1, VND2, VND3, VND4, VND5,
VND6, VND7, NST1, NST2, NAC15, NAC33, or NAC70 in the snd nst1 mutant, indicating that
these NACs are functional homologues of SND1.
It has been previously confirmed that overexpression of SND1, NST1, or NST2 induces ectopic secondary cell wall deposition in rosette leaves, and thus the overexpressors exhibit curled leaves (Mitsuda et al., 2005&2007; Zhong et al., 2007). We placed the coding sequences of \textit{VND1}, \textit{VND2}, \textit{VND3}, \textit{VND4}, \textit{VND5}, \textit{NAC15}, \textit{NAC33}, and \textit{NAC70} under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter and introduced the overexpression constructs into wild-type plants. Overexpression of VND1, VND2, or VND5 did not cause significant phenotypes, whereas VND3, VND4, NAC15, NAC33, and NAC70 resulted in small and curled leaves, an indication of ectopic secondary cell wall deposition.

Dominant repression of \textit{VND6}, \textit{VND7}, \textit{SND1}, \textit{NST1}, or \textit{NST2} represses secondary cell wall formation (Kudo et al., 2005; Mitsuda et al., 2005&2007; Zhong et al., 2007). Theoretically, the gene repressors could inhibit the functions of their own and their homologues by possibly suppressing the basal transcription factors or coactivators or recruiting a suppressor. The coding sequences of \textit{VND1}, \textit{VND2}, \textit{VND3}, \textit{VND4}, \textit{VND5}, \textit{NAC15}, \textit{NAC33}, and \textit{NAC70} were fused with the ERF associated amphiphilic repression (EAR) domain and placed under the control of its own promoter. However, the gene repressor lines did not show obvious phenotypes in stems (data not shown), indicating that these five NAC transcription factors may be minor regulators.

**TRANSCRIPTIONAL ACTIVITIES OF NAC15, NAC33, AND NAC70**

Since \textit{NAC15}, \textit{NAC33}, and \textit{NAC70} were expressed in roots, we examined 3-day old wild-types, NAC15 repressor, NAC33 repressor, and NAC70 repressor lines. Whereas 95\% of wild-type seeds germinated in 3 days, only 72.5\% of NAC15 repressors germinated. In addition, a great number of NAC33 repressor seedlings (16 out of 40) had roots less than 2 cm long at day 3 (Table 4.2). After a few days, some of the shorter NAC15 repressor, NAC33 repressor, and the NAC70 repressor lines were transferred to soil. The \textit{NAC15} repressor line had a very low
survival rate. In addition, the survived NAC15, NAC33, and NAC70 repressor plants were retarded in terms of overall plant growth (data not shown). The phenotypes in the adult stage may be due to the developmental defects in the younger stage. Since root caps normally do not develop secondary cell walls but contain abundant cellulose (Cutter, 1978), NAC15, NAC33, and NAC70 may regulate the production of cellulose or other primary cell wall materials, which is consistent with Bennett et al.’s (2010) findings that NAC15, NAC33, and NAC70 are able to regulate the expression of secondary cell wall biosynthetic genes and two cellulase genes involved in root cap maturation.

Next, the transcriptional activity of NAC15, NAC33, and NAC70 was studied in a yeast system. The full length cDNA sequence of each gene was fused with the Gal4 DNA Binding Domain (Gal4-DBD), which was transformed into the yeast strain that carries repeats of the Gal4 cis-acting element and reporter genes Histidine-3 (HIS3) and lacZ. The negative control Gal4-DBD failed to induce the expression of the His3 reporter gene and thus did not form colonies on the selection plate lacking histidine. The NAC15 transformant grew on nonselection plates but didn’t grow well on selection plates, suggesting that NAC15 is a very weak transcription activator. It is also possible that NAC15 requires facilitating proteins to turn on downstream genes. Similar cases have been reported. For example, the maize bHLH transcription factor B fails to activate the reporter genes in the transactivation assay, but by interacting with a MYB transcription factor C1, the B protein exhibits strong transactivation activity (Goff et al., 1992). The NAC33 transformant grew on the selection plate, and the lacZ reporter gene was slightly activated, indicating that it is a transcriptional activator (Figure 4.4). The NAC70 transformant didn’t grow on the non-selection plate, which could be due to the toxicity of NAC70 toward yeast cells.
DISCUSSION

To make the best use of secondary cell walls, it is important to regulate the amount or the proportions of secondary cell wall components (Yuan et al., 2008). Genetic engineering of the secondary cell wall can be achieved by manipulating the biosynthetic genes or the transcription factors that control the biosynthetic genes. For example, downregulating the lignin biosynthetic genes, such as 4CL, COMT, PAL and CCoAOMT in Arabidopsis, tobacco, and alfalfa, reduces the lignin content or alters the G/S ratio (Boerjan et al., 2003). NST1, a master transcription factor controlling secondary cell wall biosynthesis, has been proved to be effective in increasing the efficiency of secondary cell wall break-down in Arabidopsis when it is dominantly repressed (Iwase et al., 2009). Here, we demonstrate that some of the NST1 homologues are functionally exchangeable and expressed in distinct cell types. These findings provide us with more molecular tools to engineer the secondary cell wall in specific cell types.

In our experiment, VND1, VND2, VND3, VND4, VND5, VND6, VND7, NST1, NST2, NAC15, NAC33, and NAC70 are able to complement the secondary cell wall defects of fibers in the snd1 nst1 mutant. Since these transcription factors are within the same NAC subfamily, they are all likely to be functional homologues of SND1. It is interesting that there exists such an enormous functional redundancy.

To begin with, these NAC transcription factors perform similar functions but in different cell types. VND3~VND5 are expressed in vessel elements, NST1 and NST2 are expressed in fibers, while NAC15, NAC33, and NAC70 are found in root caps. Since vessel elements and fibers go through different developmental steps, the way how the secondary cell wall program in these cell types is turned on may be different. Conceivably, the expression of the vessel specific NACs and the fiber specific NACs are controlled by different upstream switches.
It is still mysterious that more than one NAC transcription factors is capable of turning on secondary cell wall biosynthesis even in the same cell type. Synergistic transcriptional regulation by transcription factors from the same transcription factor family has been reported in several developmental and metabolism pathways. For example, the B3 domain transcription factors work together in seed maturation (Suzuki and McCarty, 2008). Different sets of MYB-bHLH complexes regulate anthocyanin biosynthesis (Gonzalez et al., 2007). In fact, the NAC domains are capable of forming homodimers and heterodimers (Yamaguchi et al., 2008). Possibly, some of these NAC transcription factors form a complex.

Furthermore, the regulation of secondary cell wall biosynthetic genes may be contributed by the NACs in a dosage dependent manner. Some NACs such as SND1 and NST1 contribute more, whereas others including VND1~VND5 may contribute less. However, once the major NACs are absent, these minor ones could compensate, as shown in the mutant complementation lines (Figure 4.3). It is possible that the minor ones have weaker activation activity or need facilitating factors. The facilitating proteins may not be present in the leaf epidermis or mesophyll cells in the wild-type, possibly explaining why overexpression of VND1, VND2, or VND5 failed to cause obvious ectopic secondary cell wall deposition (Figure 4.2). In addition, since the major transcription factors may still be there, dominant repression of the minor ones, including VND1, VND2, VND3, VND4, or VND5, would not cause obvious phenotypes.

The secondary cell wall is vital to herbaceous Arabidopsis as to other vascular plants. Strong evidences show that the regulation of secondary cell wall biosynthesis may be quite conserved among species. Orthologues of Arabidopsis CESA genes, putative hemicelluloses biosynthetic genes, and lignin biosynthetic genes exist in the genome of many species (Holland et al., 2000; Boerjan et al., 2003; Yong et al., 2005; Vogel, 2008). Comparison of the CESA promoters in
Arabidopsis, Poplar and Eucalyptus reveals the presence of conserved cis-acting elements (Creux et al., 2008), indicating that the regulation of the CES\(A\) genes may be achieved by similar transcription factors.

Recently, Zhong et al. (2010) proved that SND1 orthologues in poplars are capable of rescuing the secondary cell defects in the Arabidopsis \textit{snd1 nst1} mutant and inducing the expression of cellulose, xylan, and lignin biosynthetic genes. Conceivably, a similar transcriptional cascade may exist in poplars and other plant species. SND1 orthologues have been reported in rice (Ooka et al., 2003), tobacco (Rushton et al., 2008), and soybean (Pinheiro et al., 2009). Although SND1 orthologues haven’t been well studied in gymnosperms, the orthologues of MYB46, a direct target of SND1, are confirmed to be involved in the regulation of secondary cell wall biosynthesis in pine trees (Patzlaff et al. 2003a & 2003b). High sequence similarity indicates that these orthologues may perform similar functions. Identification and characterization of SND1 orthologues would help us to understand better the regulation of secondary cell wall biosynthesis in these species and thus to utilize better the orthologues to modify cell wall composition according to need.

MATERIALS AND METHODS

PLANT MATERIALS

Seeds of \textit{Arabidopsis thaliana} (ecotype Columbia) were sowed in soil, cold stressed for 2 days, placed in a growth room for 4 weeks, and removed to a greenhouse under a long-day photoperiod. Transgenic seeds were first sowed on Murashige and Skoog plates plus appropriate antibiotic(s), cold stressed for 2 days, and placed in a growth chamber for 7 days. Transgenic plants were then transferred to soil and removed to a greenhouse.
CONSTRUCTS AND PLANT TRANSFORMATION

To study where these NAC genes are expressed, the *Escherichia coli uidA* gene, encoding β-glucuronidase (GUS), was used as a reporter. To ensure that all the regulatory elements are included, for each gene, an approximate 3-kb fragment before the translation start codon plus the entire coding region (including all the exons and introns) were amplified by PCR from the wild-type *Arabidopsis thaliana* genomic DNA. The promoter-gene fragment was then cloned into the BamHI site of pBI101 (Clontech) and thus was fused upstream in frame with the GUS reporter gene in the pBI101 vector. An approximate 2-kb terminator fragment downstream of the translation stop codon was amplified and inserted in the SacI site after the GUS reporter gene.

For the complementation lines, the 3-kb SND1 promoter region was amplified by PCR from the wild-type Arabidopsis genomic DNA and introduced into the multiple cloning sites of the pGPTV binary vector (ATCC). The full-length cDNA fragment of each NAC gene was amplified, digested by NheI, and placed between the SND1 promoter and the NOS-terminator in the pGPTV vector.

To make overexpression lines, the cDNA sequences of VND1~VND5, NAC15, NAC33 and NAC70 were amplified from the *Arabidopsis* stem 1st strand cDNA and inserted after the cauliflower mosaic virus 35S (CaMV35S) promoter in the pBI121 vector (Clontech) to replace the GUS reporter gene. The XbaI or BamHI restriction site was used. For dominant repressor lines, the ~3kb promoter and coding sequences of each gene were placed upstream of the EAR domain (Mistuda et al., 2005) in the modified pBI121 vector in which the GUS reporter gene had been pre-removed and the transcription repression domain (LDLDLELRLGFA) had been pre-inserted. The final constructs were transformed into wild-type plants by the standard *Agrobacterium tumefaciens*-mediated transformation procedure. For transcriptional activation
assay, the entire cDNA sequence of each gene was inserted in frame with the Gal4 DNA Binding Domain in the pAS2-1 vector (Clontech).

**GUS STAINING ASSAY**

Plant tissues were fixed in 90% aceton for 20 minutes on ice, washed, and immersed into the standard GUS staining solution (100 mmol/L sodium phosphate, pH7.2, 10 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, and 1 mmol/L 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid) at 37°C until blue color appeared. Stem cross-sections were cut with a razor blade by free hand and stained as above.

**TRANSCRIPTIONAL ACTIVATION ASSAY**

Vectors were transformed into yeast strain CG-1945, which contains the *His3* and *LacZ* reporter genes. The transformed cells were plated on non-selection synthetic medium (SD) lacking Trp plates and selection SD lacking Trp and His plates. Colonies grown on SD lacking Trp-His plates were patched to new plates for a filter lift assay. Sterile filter papers were placed over the surface of colonies, lifted, and transferred into liquid nitrogen for 30 seconds, and then allowed to thaw at room temperature. Filter papers were then placed on top of another piece of filter paper that was pre-soaked in the Z buffer (see the CLONETECH manual), incubated at 30°C until blue color appeared.

**HISTOLOGY**

Stems and leaf segments were fixed in formaldehyde at 4 °C overnight. The fixed segments were washed 3 times with PBS buffer and then dehydrated with a gradient of ethanol (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%, 100%, 100%). After dehydration, samples were embedded in the LR White resin (Electron Microscopy Sciences) and polymerized.
under UV light for one week. After polymerization, samples were sectioned (1 um) by a microtome, stained by toludine blue, and visualized under a light microscope.

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Zhong, R., Demura, T., and Ye, Z.-H. (2006). SND1, a NAC domain transcription factor, is a


Table 4.1. Summary of the expression patterns of SND1 homologues.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Seedling</th>
<th>Mature Root</th>
<th>Stem</th>
<th>Flower</th>
<th>Siliqke</th>
</tr>
</thead>
<tbody>
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**Table 4.2.** The three-day old NAC15 repressor has a lower germination rate than the wild-type.

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<td>5</td>
<td>16</td>
<td>8</td>
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Forty seeds of the wild-type, the NAC15, the NAC33, and the NAC70 repressors were sold on MS plates, respectively. Note that the germinated, 3-day old NAC33 repressor has a greater portion of seeds with root length less than 2cm.
Figure 4.1. Expression Patterns of VND3, VND4, VND5, NST1, and NST2 in Stems and Roots.
Cross sections of stems showing GUS staining in vessels in VND3p::VND3-GUS (A), VND4p::VND4-GUS (C), and VND5p::VND5-GUS (E). GUS staining was shown in fibers in NST1p::NST1-GUS (G) and NST2p::NST2-GUS (I). Cross sections of roots showing GUS staining in secondary xylems in VND3p::VND3-GUS (B), VND4p::VND4-GUS (D), VND5p::VND5-GUS (F), NST1p::NST1-GUS (H), and NST2p::NST2-GUS (J). Black arrow: interfascicular fibers; White arrows: vessels; Black arrowheads: secondary xylems in roots.

Figure 4.2. Expression of SND1 homologues in the snd1 nst1 mutant and the wild-type.
(A) The snd1 nst1 double T-DNA mutant (leftmost) showing the pendent stem phenotype due to lack of secondary cell walls in fibers. The double mutant transformed with the coding sequences of VND1~VND7, NST1, NST2, NAC15, NAC33, and NAC70 (from second left to right) under the control of the SND1 promoter showed wild-type-like stem phenotype.
(B) Overexpression of VND3, VND4, NAC15, NAC33, and NAC70 caused curled leaves.
if, interfascicular fiber.
**Figure 4.3.** Complementation of the fiber wall defect in the *snd1 nst1* mutant by expression of SND1 homologues.

The bottom parts of stems were sectioned transversely. Note the reduced secondary cell walls in interfascicular fiber cells in the *snd1 nst1* mutant (B) and the recovered walls in the complementation lines (C)~(N). if, interfascicular fiber.

**Figure 4.4.** NAC33 is a transcriptional activator. The binding domain (BD) alone did not activate the *Histidine-3* or *lacZ* gene. The clone containing the BD-NAC33 fusion grew on the selection plate and showed weak blue color in the filter lift assay.
CHAPTER 5

CONCLUSIONS

OVEREXPRESSION OF FRA1 AFFECTS THE DEPOSITION OF MICROFIBRILS

The secondary cell wall normally consists of three layers, each of which differs in the way microfibrils are laid down. We found that overexpression of FRA1 caused multiple secondary cell wall layers in vessel elements and fibers. Additionally, the microfibrils in those multiple layered-cells were oriented longitudinally instead of nearly transversely along the elongating axis. Our finding is consistent with the previous study in which the insertional or deletion fra1 mutant disrupts the organization of microfibrils (Zhong et al., 2002). Several studies have shown that the orientation of microfibrils is related to cell wall strength (Sato et al., 2001; Somerville, 2006). Consistently, in our study, as a result of the abnormal microfibril orientation in the overexpressor, the cell wall strength was reduced as indicated by collapsed vessels and reduced stem-breaking strength. Furthermore, FRA1 exhibited expression in cells undergoing substantial cell wall reconstruction including cells develop secondary cell walls and expanding cells. This is consistent with its functions in fibers and presumably in enlarging cells since the overall plant expansion was somewhat impaired. Taken together, we have demonstrated that FRA1 has a significant impact on the orientation of microfibrils.

POSSIBLE FUNCTIONAL MECHANISMS OF FRA1

FRA1 is a kinesin-like protein that binds to cortical microtubules (Zhong et al., 2002). Heath (1974) and Gidding & Staehelin (1991) proposed that cortical microtubules define the track of cellulose synthase complexes (CSCs) movement and thus regulates the deposition of
microfibrils, and thus it is FRA1 is very likely to be involved in the cortical microtubule regulation of microfibril orientation. We found that the cellulose content was reduced in the FRA1 overexpressor, which may be due to the aberrant CSC transport either at the plasma membrane or from the cytoplasm to the plasma membrane. However, we did not detect interaction between FRA1 and one of the CSC subunit CESA7. Therefore, if FRA1 transported CSCs, FRA1 would bind to the other CESA subunits or transport CSCs through intermediate proteins. Another possibility is that FRA1 may be responsible for transporting other cell wall materials that are involved in the microfibril deposition process (Zhou et al., 2007).

We identified three putative FRA1 interacting proteins using yeast two-hybrid although further confirmation of the interactions remains to be conducted. We found that the C-terminal of the FRA1 Interacting Protein1 (FIP1) specifically interacted with the stalk domain of FRA1. The other putative protein-protein interacting domain of FIP1 and the FRA1 tail domain may bind to other proteins. FRA1 and FIP1 possibly cooperate in delivering wall materials. The gibberrellin-regulated protein is localized at the plant cell wall and may be involved in cell elongation (Herzog et al., 1995; Bayer et al., 2005). It is therefore reasonable to predict that the gibberrellin-regulated protein identified has a role in ordering microfibrils upon gibberellin treatment, and its function may be assisted by FRA1. As an alternative, FRA1 may cooperate with the actin-related protein in delivering cell wall-associated materials since both cortical microtubules and actins are crucial in vesicle transport and cell wall organization (Baskin, 2001; Wightman and Turner, 2008).

**FUTURE DIRECTIONS IN STUDYING FUNCTIONAL MECHANISMS OF FRA1**

Our findings have furthered the understanding of the cortical microtubule regulation of microfibril orientation but have also raised several questions to be addressed in the future. First,
the *in vivo* interaction between FIP1 and FRA1 has not been confirmed, which can be achieved by co-immunoprecipitation. To study the potential functions of FIP1 in microfibril deposition, examining the arrangement of microfibrils in the *fip1* mutant would be helpful. Since there is another potential protein interaction domain other than the C-terminal of FIP1, it would be interesting to screen for other FIP1 interacting proteins. Second, further interaction analysis and functional characterization of the gibberellin-regulated protein and the actin-related protein are necessary. Third, the cDNA library we used for the yeast 2-hybrid screen is a seedling cDNA library in which only genes that are expressed in the seedling stage are included. However, *FRA1* is also expressed abundantly in stems and causes stem defects when mutated (Zhong et al., 2002). Using a stem cDNA library for a yeast 2-hybrid screen may help to identify other putative FRA1 interacting proteins. Finally, a breakthrough regarding the cortical microtubule regulation of microfibril orientation was made when the tubulin protein and the CESA subunit were dually labeled and visualized *in vivo* (Paredez et al., 2006). FRA1 is a kinesin-like protein that is supposed to move along cortical microtubules. We could investigate the movement patterns of CSCs in the *fra1* mutant background. As an alternative, we could label both the FRA1 protein and the CSC subunit and examine if there is a correlation between their movements.

**NAC TRANSCRIPTION FACTORS PLAY ROLES IN THE REGULATION OF SECONDARY CELL WALL BIOSYNTHESIS**

Vascular-related NAC domain protein6 (VND6), VND7, secondary wall-associated NAC domain protein1 (SND1), NAC secondary wall thickening promoting factor1 (NST1), and NST2 are master regulators in the proposed transcriptional network controlling secondary cell wall biosynthesis (Zhong and Ye, 2007). VND1, VND2, VND3, VND4, VND5, NAC15, NAC33, and NAC70 belong to the same NAC subfamily as the master regulators. We carried out a
detailed analysis of the expression patterns of these genes. Except for NAC15, NAC33, and NAC70, which were mainly expressed in root caps, the rest of the NAC genes showed expression in vascular tissues in seedlings, mature roots, and/or stems. VND3~VND5 were vessel element specific, while NST1 and NST2 were fiber specific like SND1.

We found that all these NAC transcription factors rescued the secondary cell wall defects in fibers in the snd1 nst1 double mutant, suggesting that they are all functional homologues of SND1. However, these NAC transcription factors may not be as important as the master regulators in terms of regulating secondary cell wall biosynthesis in stems. We also found that only VND3, VND4, NAC15, NAC33, and NAC70 induced ectopic secondary cell wall deposition-like phenotypes in the wild-type. The gene repressor lines of VND1~VND5 did not show obvious phenotypes. We then suggest that these NAC transcription factors, other than the master ones, may be minor regulators of secondary cell wall biosynthesis under normal conditions.

FUTURE DIRECTIONS REGARDING THE REGULATION OF SECONDARY CELL WALL BIOSYNTHESIS

An interesting question to consider is what decides the distinct expression patterns of these NAC genes, or what regulates these NAC genes. More than one possibility exists.

Secondary cell wall deposition occurs after the cell ceases differentiation and expansion (Carpita and McCann, 2000). It is possible that proteins controlling vessel or fiber cell differentiation or expansion act upstream of these NAC genes. Fruitful (FUL), a MADS transcription factor, controls the speciation of valve margin cells in siliques. The ful mutant induces ectopic secondary cell wall deposition in valves and prevents valve margin cells from obtaining their identity. It’s also noticed that NST1 is expressed in the cells with ectopic cell
walls. The ful snd1 or ful nst1 double mutant partially rescues the ful phenotype by eliminating ectopic secondary cell wall deposition, but the lost cell identity is not complemented (Mitsuda and Ohme-Takagi, 2008). Therefore, Mitsuda and Ohme-Takagi (2008) predicted that NST1 acts downstream of FUL and is turned on once the valve margin cell identity is established. No study has shown the correlation between anther endothecium identity genes and NST1 or NST2. However, MYB26, whose mutant lacks secondary cell walls in the anther endothelial layer, regulates the expression of NST1 and NST2 (Yang et al., 2007). The finding places MYB26 upstream of NST1 and NST2 in the transcriptional cascade (Zhong and Ye, 2007). Regulators of VND1 ~ VND7, and SND1 have not been reported yet. The candidate genes may be either the ones controlling the differentiation of xylems and interfascicular fibers, or other transcription factors that are in charge of secondary cell wall deposition.

Xylem differentiation is related to auxin, cytokinin, brassinosteroid (BR), and gibberellin (GA) signaling pathways. MONOPTEROS/Arabidopsis Response Factor5 (MP/ARF5) is mainly expressed in vascular tissues. Upon auxin treatment, MP/ARF5 dissociates from its repressor IAA12 and turns on a series of downstream targets. The mp/arf5 mutant fails to form continuous vascular tissues, which are derived from the procambium, as seen in the wild-type (Hardtke and Berleth, 1998), indicating that MP/ARF5 may control the procambium activity. Wooden Leg (WOL), Arabidopsis Histidine Kinase2 (AHK2), and AHK3 are cytokinin receptors and are shown to maintain the number of procambial cells (Fukuda, 2004; Hejátko et al., 2009). Mähönen et al. (2006) found that cytokinin has a negative role in the protoxylem formation in roots through the inhibitory pseudophototransfer protein AHP6. Inhibition of BR biosynthesis in cress plant cultures promotes phloem specification but prevents the formation of primary and secondary xylems (Nagata et al., 2001). BR also induces xylem cell differentiation in zinnia and
Arabidopsis cell cultures (Fukuda, 2004; Kubo et al., 2005). Overexpression of the GA receptor PttGID1.1 or PttGID1.3 in the hybrid aspen increases the number of xylems and promotes the length of xylary fibers, suggesting that the GA signaling pathway plays a positive role in xylem development (Mauriat and Moritz, 2009). Recently the Dof transcription factor Dof5.6/High Cambium Activity2 (HCA2) is found to control interfascicular cambium formation in Arabidopsis. The gain of function mutant hca2 exhibits ectopic interfascicular cambium, while the dominant negative mutant has dramatically reduced number of interfascicular cambial cells (Guo et al., 2009).

Interfascicular fibers are originally derived from interfascicular parenchyma cells (Ye, 2002). Interfascicular Fiberless1 (IFL1) is a homeodomain leucine zipper (HD-ZIP) transcription factor involved in auxin polar transport. The loss-of-function ifl1 mutant does not form interfascicular fibers, and the differentiation of xylem is also affected. IFL1 likely promotes interfascicular fiber differentiation by mediating auxin transport (Zhong and Ye, 1999).

Yeast cell wall biosynthesis is controlled by the cell wall integrity pathway, in which the wall damage is first sensed by membrane-associated receptors and then transducted gradually to downstream genes (Levin, 2005). However, it remains largely unknown what connects changes in the cell wall and its biosynthesis in vascular plants. THESEUS1 (THE1) is a membrane bound Ser/Thr kinase and distributed in vascular tissues and in elongating cells. Mutation of the THE1 gene partially enhanced ectopic lignin deposition in the cesa6 mutant where the cellulose content is reduced (Hematy et al., 2008). The receptor-like protein kinase2 (rpk2) mutant has reduced and abnormal lignification in anther endothecia, which results in male sterility. Moreover, microarray analysis of the rpk2 mutant reveals that many cell wall biosynthetic genes are
downregulated (Mizuno et al., 2007). These receptor-like kinases may directly or indirectly act upon cell wall biosynthesis.

Whether any of the above genes or their downstream targets have an actual role in initiating the expression of the master regulators in secondary cell wall biosynthesis remains to be determined. Ways to test the idea include: (1) examining the expression levels of the NAC transcription factors that are involved in secondary cell wall biosynthesis in the potential upstream regulator mutant background; (2) crossing the $nac$ mutant with, or overexpressing the $NAC$ genes in the potential upstream regulator mutant.

Another direction in studying the transcriptional regulation of secondary cell wall biosynthesis is to look for downstream targets of secondary wall-associated NAC transcription factors. One feasible approach would be to combine chromatin immunoprecipitation with the microarray technology.

Furthermore, Zhou et al. (2009) demonstrated that MYB58 specifically and directly regulates lignin biosynthetic genes. The specific regulators of cellulose or xylan biosynthesis are not known yet, and the identification of these regulators would provide us with more tools to modify secondary cell wall composition according to need.

Finally, the eventual goal of studying the regulation of secondary cell wall biosynthesis in Arabidopsis is to apply our knowledge regarding Arabidopsis into economically important species (such as poplar trees, maize, rice, and switchgrass) and benefit the agronomical, biofuel, and wood industry. The completion of genome sequencing in various plant species allows us to identify, characterize, and utilize the orthologues of the Arabidopsis transcription factors involved in secondary cell wall biosynthesis.
REFERENCES


Appendix A

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