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Original Paper

ATL54, a RING-H2 domain protein selected by a gene co-expression network analysis, is associated with secondary cell wall formation in *Arabidopsis*

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Abstract Biosynthesis of plant secondary cell walls is controlled by several master transcription factors. Ubiquitin ligases, which mediate ubiquitination of proteins, including transcription factors in the protein degradation pathway, are also believed to regulate secondary wall biosynthesis; however, the exact ubiquitin ligases involved in secondary wall formation have not yet been identified. We conducted a gene co-expression network analysis and found that *ATL54*, annotated as a RING-finger protein, was highly co-expressed with several transcription factor and enzyme genes involved in secondary wall formation. A recombinant ATL54 protein showed ubiquitin ligase activity. The expression of several biosynthetic genes of cellulose, lignin, and xylan in apical portions of inflorescence stems was up-regulated by *ATL54* knock-out. The expression of *Xylem Cysteine Peptidase1* (*XCP1*), which participates in the programmed cell death process of xylem tracheary elements, was down-regulated in middle stem portions of both *ATL54*-knock-out and *ATL54*-overexpressed mutants. Alteration of *ATL54* expression levels did not, however, affect lignin and polysaccharide content and composition in whole mature stems. Our results suggest that ATL54 is an E3 ubiquitin ligase involved in secondary wall biosynthesis and programmed cell death during xylogenesis.

Key words: *Arabidopsis thaliana*, E3 ubiquitin ligase, gene co-expression network analysis, secondary cell wall formation.

In vascular plants, secondary cell walls exist in sclerenchymatous cells such as vessel elements, tracheids, and fibers. Secondary walls give these cells rigidity and confer water impermeability required for mechanical support and water transportation. Secondary cell walls are major components of wood, an essential feedstock for pulp and paper manufacturing, construction, and potential biofuel production. Understanding the mechanism of secondary cell wall formation would therefore not only shed light on metabolic events specific to vascular plants, but would also assist efforts to improve the utility of wood through biotechnological manipulation.

Recent progress on studies of transcription factors involved in secondary cell wall formation has revealed that a number of transcription factors play important roles. For example, in *Arabidopsis*, VASCULAR-RELATED NAC-DOMAIN6 (VND6) and VND7 regulate the development of metaxylem and protoxylem vessel elements, respectively (Kubo et al. 2005;

Abbreviations: 4CL, 4-coumarate CoA-ligase; ANAC, Arabidopsis NAC domain containing protein; ATL, Arabidopsis Tóxicos en Levadura; CCoAOMT, caffeoyl-CoA 3-*O*-methyltransferase; CesA, cellulose synthase; COR, correlation coefficient; F5H1, Ferulate 5-Hydroxylase1; FRA8, fragile fiber8; GUX2, glucuronic acid substitution of xylan2; HRP, horseradish peroxidase; IRX9, irregular xylem9; KNAT7, Knotted1-like homeodomain protein7; MBP, maltose binding protein; NST, NAC secondary wall thickening promoting factor; RAFL clones, RIKEN *Arabidopsis* full-length cDNA clones; RING, really interesting new gene; S/V ratio, syringaldehyde/vanillin ratio; SND, secondary wall-associated NAC domain; VND, vascular-related NAC domain; XCP1, xylem cysteine peptidase1; YFP, yellow fluorescent protein.

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Yamaguchi et al. 2008), while NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1) and NST3 [also called SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1) or ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN012 (ANAC012)] redundantly act as master switches that turn on secondary wall formation in fibers (Mitsuda et al. 2007; Zhong et al. 2006). MYB46 and MYB83 are direct downstream transcription factors of VND6, VND7, NST1, and NST3/SND1/ANAC012 (McCarthy et al. 2009; Ohashi-Ito et al. 2010; Yamaguchi et al. 2011). MYB46 and MYB83 can activate biosynthetic pathways for cellulose, xylan, and lignin, the three major secondary wall components (Ko et al. 2009; McCarthy et al. 2009; Nakano et al. 2010; Zhong et al. 2007). In contrast, MYB58 and MYB63 specifically activate lignin biosynthetic genes (Zhou et al. 2009). *MYB58* and *MYB63* expression is regulated by NST1, VND6, VND7, and MYB46, with the expression of *MYB58* additionally regulated by NST3/SND1/ANAC012 (Zhou et al. 2009). In addition, several other NAC and MYB transcription factors regulated by NST3/SND1/ANAC012 have been found to affect secondary wall formation (Zhong et al. 2008). Among NST3-regulated transcription factors, MYB103 has been shown to be responsible for *Ferulate 5-Hydroxylase1* (*F5H1*) expression and syringyl lignin biosynthesis (Öhman et al. 2012).

In addition to transcriptional regulation by transcription factors, it is presumed that protein degradation also plays a regulatory role in secondary wall formation, which is exemplified by the regulation of VND7 protein by proteasome-mediated degradation (Yamaguchi et al. 2008). Protein degradation via the ubiquitin-26S proteasome pathway is mediated by ubiquitination of target proteins (Moon et al. 2004). The process of ubiquitination requires three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). E3 ubiquitin ligases are thought to determine the substrate specificity of ubiquitination. More than 1,300 members of E3 ligase families have been predicted in *Arabidopsis* (Mazzucotelli et al. 2006), but no E3 ligases related to secondary wall formation have yet been reported.

In this study, we conducted a gene co-expression network analysis and uncovered *Arabidopsis Tóxicos en Levadura54* (*ATL54*, At1g72220), a putative ubiquitin ligase gene co-expressed with genes involved in secondary wall formation. We characterized ATL54 through a ubiquitination assay, a subcellular localization analysis, and analyses of transgenic plants in which *ATL54* gene expression was up- or down-regulated.

Materials and methods

Gene co-expression network analysis

Gene co-expression data were obtained from the ATTED-II ver. c4.1 database (Obayashi et al. 2008), which includes expression data from 1,388 *Arabidopsis thaliana* samples derived from 58 experimental studies related to developmental stages and responses to biotic stress, abiotic stress, and hormones. A co-expression module was constructed using Microsoft Office PowerPoint 2007.

Phylogenetic analysis

The amino acid sequences of ATLs were obtained from The Arabidopsis Information Resource [TAIR; the Arabidopsis Biological Resource Center (ABRC), Ohio State University]. Alignment of the amino acid sequences and the construction of a phylogenetic tree by neighbor-joining method (1,000 bootstrap trials) were performed using ClustalX ver 2.0.11 (Larkin et al. 2007).

Vector construction

The open reading frame of the *ATL54* gene was PCR-amplified from a RIKEN *Arabidopsis* full-length cDNA (RAFL) clone (RIKEN BioResource Center) and subcloned into a pENTR/D-TOPO vector (Invitrogen Japan KK, Tokyo, Japan). The subcloned DNA was then transferred by recombination using LR Clonase II (Invitrogen) into several vectors: pH35GS for generation of overexpressors, pH35YG and pH35GY for a subcellular localization analysis (Kubo et al. 2005), and pMAL-DC-6myc for a ubiquitination assay (Zhang et al. 2005). The resulting plasmids were named pH35GS-*ATL54*, pH35YG-*ATL54*, pH35GY-*ATL54*, and pMAL-*ATL54*-6myc, respectively. For construction of a control vector for the subcellular localization analysis, the yellow fluorescent protein (YFP) gene cloned from pH35GY was transferred into pH35GS to obtain pH35GS-*YFP*. All constructs described above were verified by DNA sequencing. Primers used for vector construction and DNA sequencing are listed in online Supplemental Table S2.

Plant materials and growth conditions

Seeds of *A. thaliana* (ecotype Col-0) were sterilized with 70% ethanol and 2% Plant Preservative Mixture (Plant Cell Technology Inc., Washington DC, USA). The seeds were then sown on germination medium (Kubo et al. 2005), vernalized at 4°C overnight, and incubated at 23°C under long-day conditions (16 h light, 8 h dark). Two-week-old seedlings were transferred to Jiffy-7 peat plugs (Jiffy Products International AS, Stange, Norway) and grown at 23–25°C under long-day conditions (16 h light, 8 h dark). A 1,000-fold dilution of Hyponex liquid concentrate (Hyponex Japan, Osaka, Japan) was given twice a month, and water was supplied twice weekly.

A. thaliana suspension-cultured T87 cells (Axelos et al. 1992) were obtained from RIKEN BioResource Center. T87 cells were maintained in mJPL3 medium (Ogawa et al. 2008) at 23°C under long-day conditions (16 h light, 8 h dark) with rotary

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shaking at 120 rpm.

Plant transformation

To obtain *ATL54*-overexpressed mutants, wild-type *A. thaliana* plants were transformed with pH35GS-*ATL54* and selected according to the method described by Kubo et al. (2005).

Screening for T-DNA insertional mutants

T3 seeds of a T-DNA insertional mutant for *ATL54* (SALK_072859; Alonso et al. 2003) were obtained from the Arabidopsis Biological Resource Center. The leaves of 10-day-old seedlings were immersed in PrepMan Ultra Sample Preparation Reagent (Applied Biosystems Japan Inc, Tokyo, Japan), and heated at 100°C for 10 min to extract genomic DNA. The supernatant containing the DNA was used as a template for PCR to select a homozygous mutant line (primers are listed in online Supplemental Table S2).

Ubiquitination assay

pMAL-*ATL54*-6myc was introduced into *Escherichia coli* strain BL21 Star (DE3) (Invitrogen) to produce a recombinant ATL54 protein fused with the maltose binding protein (MBP) at its N-terminus and the 6×myc tag at its C-terminus. Growth of bacteria and induction of protein expression were performed according to the manufacturer's protocol. The bacteria were lysed by sonication in lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM $MgCl_2$, 10 mM β-mercaptoethanol, and Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics KK, Tokyo, Japan)], and centrifuged at 10,000×*g* for 10 min at 4°C. The recombinant protein in the supernatant was purified using amylose resin (New England Biolabs Japan Inc, Tokyo, Japan) according to the manufacturer's protocol.

A ubiquitination assay using the recombinant protein (MBP-ATL54-6myc) was performed following the method of Zhang et al. (2005). Reaction products were separated by electrophoresis on a 5% polyacrylamide gel. Ubiquitin was detected by Western blotting using anti-ubiquitin antibodies [Ubiquitin (P4D1) Mouse mAb; Cell Signaling Technology Japan KK, Tokyo, Japan] and horseradish peroxidase (HRP)-labeled secondary antibodies (Anti-mouse IgG, HRP-linked Antibody; Cell Signaling Technology).

Subcellular localization analysis

Protoplast preparation and transformation was performed as described by Fujimoto et al. (2005) with modified concentrations of protoplasts and plasmids. The protoplast concentration was adjusted to 4×10^6 cells ml^{-1} before transformation, and 15 μg of pH35YG-*ATL54*, pH35GY-*ATL54*, or pH35GS-*YFP* was introduced. After transformation and washing, protoplasts were incubated at 23°C overnight in mJPL3 medium containing 0.4 M mannitol and 0.4 M glucose instead of sucrose. After incubation, protoplasts were examined under an epifluorescence microscope (BX51; Olympus Co, Tokyo, Japan) using a YFP filter set: a BP490-500HQ excitation filter, a DM505 dichroic mirror, and a BA515-560HQ emission filter.

Quantitative RT-PCR analysis

Approximately 20-cm-high inflorescence stems of wild-type plants, *ATL54*-knocked-out mutants, and *ATL54*-overexpressed mutants (T3 homozygous lines transformed with pH35GS-*ATL54*) were harvested. After removal of flowers and peduncles, 3-cm segments from the top of stems, middle of stems, and internodes just above the rosette leaves (hereafter referred to as the apical, middle, and basal portions, respectively) were cut off and immediately frozen in liquid nitrogen. Each sample was pulverized three times with zirconia beads (5 mm diameter) in 2-ml tubes using a TissueLyser (Qiagen KK, Tokyo, Japan) for 30 s at 25 Hz. Total RNAs were isolated using an RNeasy Plant Mini Kit (Qiagen). RNAs extracted from three individuals of each line were combined and used as one sample. RNAs were treated with RQ1 RNase-Free DNase (Promega KK, Tokyo, Japan) to remove genomic DNA contamination, and reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen) to obtain first-strand cDNAs. Quantitative PCR was conducted on a 7300 Real Time PCR System (Applied Biosystems) using the cDNAs, gene-specific primers (listed in online Supplemental Table S2), and *Power* SYBR Green PCR Master Mix (Applied Biosystems) as previously described (Suzuki et al. 2006). A standard curve for each gene was prepared using a cDNA dilution series. The relative mRNA level of each sample was determined by Ct value using the standard curve, and normalized against *ACTIN2* (At3g18780) mRNA levels to obtain the relative expression level.

Chemical analyses of lignin and polysaccharides

Mature (over 3 months old) inflorescence stems of wild-type plants, *ATL54*-knock-out mutants, and *ATL54*-overexpressed mutants were harvested. The stems were dried and powdered with stainless steel balls in stainless steel grinding jars using a TissueLyser for 2 min at 25 Hz. The powdered samples were treated with an ethanol/toluene (2 : 1, v/v) solution for 6 h and with methanol for 2 h using a Soxhlet apparatus. The residual powder was dried under high vacuum at room temperature to obtain organic solvent insoluble residues.

Lignin content in the residues was determined using the acetyl bromide method (Umezawa et al. 2007; Yamamura et al. 2011). A microscale nitrobenzene oxidation experiment (Yamamura et al. 2010) was performed for determination of relative amounts of guaiacyl and syringyl units in lignin.

Starch in the organic solvent insoluble residues was removed using an amylase treatment (Hattori et al. 2012), and the residues were subjected to sulfuric acid hydrolysis and alditol acetate derivatization (Hoebler et al. 1989). The carbohydrate content of the sulfuric acid hydrolysate was determined by the phenol-sulfuric acid method using 5% phenol aqueous solution (Dubois et al. 1956). Alditol acetates were analyzed by gas chromatography-mass spectrometry on a GCMS-2010 Plus system (Shimadzu, Kyoto, Japan) under the following conditions: electron impact mode (70V); column, Supelco SP-

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2330 (30 m×0.25 mm); initial temperature 170°C for 2 min, then increased to 235°C by 4°C min^{-1}; carrier gas, He; splitless injection.

Results

Gene co-expression network analysis

To find a ubiquitin ligase involved in secondary wall formation, we conducted a gene co-expression network analysis using the ATTED-II database. We first investigated genes co-expressed with transcription factor and enzyme genes involved in secondary wall formation. Among E3 ubiquitin ligase families, RING (Really Interesting New Gene)-finger proteins are the most frequent catalytic subunits of E3 ligases in *Arabidopsis* (Mazzucotelli et al. 2006). We therefore looked for genes annotated as "zinc finger (C3HC4-type RING finger) family protein" in the co-expressed gene list and found that At1g72220 (*ATL54*), At2g20650, At1g72200 (*ATL11*), and At3g10910 (*ATL72*) appeared frequently (Supplemental Table S1). Among these genes, *ATL54* showed the highest correlation coefficients (CORs) for several transcription factor genes such as *MYB58* (Atlgl6490) (COR=0.67), *NST3*/*SND1*/*ANAC012* (Atlg32770) (COR=0.68), and *Knotted1-like Homeodomain Protein7* (*KNAT7*, At1g62990; Zhong et al. 2008) (COR=0.65). Xylan biosynthetic genes, including *GlucUronic Acid Substitution of Xylan2* (*GUX2*, At4g33330; Mortimer et al. 2010) (COR=0.71) and *Irregular Xylem9* (*IRX9*, At2g37090; Lee et al. 2007) (COR=0.69), were also highly co-expressed with *ATL54*. *ATL11* showed modest CORs for *KNAT7* (COR=0.56), *IRX9* (COR=0.55) and another xylan biosynthetic gene *Fragile Fiber8* (*FRA8*)/*IRX7* (At2g28110; Peña et al. 2007) (COR=0.61). *ATL72* showed a modest COR for *FRA8*/*IRX7* (COR=0.62). Because of the highest CORs for several secondary wall-associated genes, we focused on *ATL54* in further analyses.

We then conducted a reciprocal search for co-expressed genes in the *ATL54* co-expression network and created a co-expression module (Figure 1). In this module, several genes were highly co-expressed with *ATL54*: *Caffeoyl-CoA 3*-O-*MethylTransferase7* (*CCoAOMT7*, At4g26220) encoding a putative CCoAOMT potentially involved in lignin biosynthesis (Raes et al. 2003), *Lateral Organ Boundaries Domain30* (*LBD30*, also called *Asymmetric Leaves2-like19* (*ASL19*) or *Jagged Lateral Organ* (*JLO*), At4g00220) (COR=0.65) encoding a transcription factor regulating tracheary element differentiation (Soyano et al. 2008), and At2g47670 (COR=0.67) encoding an invertase/pectin methylesterase inhibitor superfamily protein (Raiola et al. 2004; Scognamiglio et al. 2003). Several signaling-related genes, such as *Rho GTPase-activating Protein5*

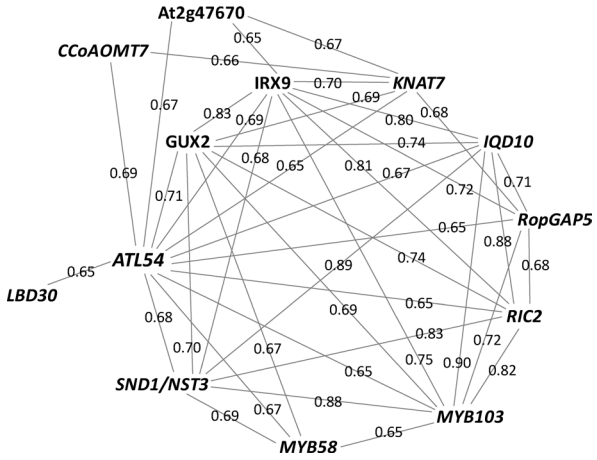

Figure 1. Co-expression relationship between *ATL54* and other genes. A co-expressed pair of genes is connected with a line, with the correlation coefficient (COR) between them on the line. Only annotated genes for which CORs with *ATL54* are greater than 0.64 are shown.

(*RopGAP5*, At1g08340; Wu et al. 2000) (COR=0.65), *Rop-interactive CRIB motif–containing Protein2* (*RIC2*, At1g27380; Wu et al. 2001) (COR=0.65), and *IQ-Domain10* (*IQD10*, At3g15150; Abel et al. 2005) (COR=0.67), also showed relatively high CORs for *ATL54*.

In silico and biochemical analysis of ATL54

Alignment of amino acid sequences of ATL54 and several ATLs showed that ATL54 has typical characteristics of ATL proteins; ATL54 contains a RING-H2 domain, one hydrophobic region near the N-terminus, and a conserved motif named GLD (Supplemental Figure S1). Our phylogenetic analysis showed that ATL54 was far from both ATL11 and ATL72 and close to ATL55/RING1 (At5g10380; Lin et al. 2008) (Supplemental Figure S2). However, ATL54 and ATL55/RING1 shared only 23% identity and 43% similarity (see Supplemental Figure S1).

A RING-H2 domain is a type of RING domain frequently found in *Arabidopsis* (Kosarev et al. 2002). It has been shown that a RING domain protein alone can promote E1- and E2-dependent polyubiquitin chain formation, often referred to as auto-ubiquitination (Joazeiro et al. 1999; Leverson et al. 2000). We conducted an auto-ubiquitination assay to test ubiquitin ligase activity of ATL54. A recombinant ATL54 protein was expressed in *E. coli* and purified. The protein (MBP-ATL54-6myc) was then subjected to a ubiquitination reaction, and the reaction products were analyzed by Western blotting. Ubiquitinated proteins were detected in the presence of ubiquitin, ATP, E1 (UBE1), E2 (UbcH5b), and MBP-ATL54-6myc (Figure 2). When the reaction mixture did not contain E1, E2, or MBP-ATL54-6myc, ubiquitinated proteins were not detected (Figure 2). These results indicate that MBP-ATL54-6myc can function as an E3 ubiquitin ligase.

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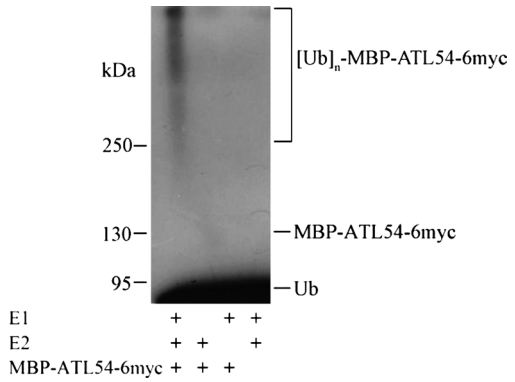

Figure 2. Auto-ubiquitination assay of a recombinant ATL54 protein. The recombinant MBP-ATL54-6myc protein was mixed with rabbit E1, human E2 (UbcH5b), ubiquitin, and ATP, and incubated at 30°C for 2 h. The reaction products were subjected to a Western blot analysis using anti-ubiquitin antibodies. Ub, ubiquitin; $[Ub]_n$, polyubiquitin chain.

Subcellular localization of ATL54

The ATL54 sequence was analyzed using the TMHMM 2.0 program (http://www.cbs.dtu.dk/services/TMHMM-2.0/), which predicted with high probability that amino acid residues 82–104 of ATL54 correspond to a transmembrane region (Figure 3A), suggesting that ATL54 is a membrane protein. To determine subcellular localization of ATL54, YFP-tagged ATL54 or YFP alone was transiently expressed in protoplasts of *A. thaliana* T87 suspension-cultured cells. When only YFP was expressed, its signal was distributed throughout the cytoplasm and the nucleus (Figure 3B). When the N-terminus of ATL54 was fused with YFP, results were similar to that of YFP alone (Figure 3C). In contrast, when the C-terminus of ATL54 was tagged with YFP, the YFP signal was detected on plasma membranes (Figure 3D).

Effect of ATL54 *knock-out and overexpression*

To investigate whether ATL54 is involved in secondary wall biosynthesis, we examined mutants with altered *ATL54* expression. We prepared a homozygous T-DNA-tagged line of *ATL54* (SALK_072859) as an *ATL54*-knock-out mutant (Figure 4B). Transgenic plants overexpressing *ATL54* were generated by *Agrobacterium*-mediated transformation. When these plants were grown under long-day conditions, there was no apparent difference in stem growth among wild-type plants and *ATL54*-knock-out and *ATL54*-overexpressed mutants.

According to the Arabidopsis eFP browser (Winter et al. 2007), *ATL54* is highly expressed at the base of inflorescence stems. We therefore used inflorescence stems for a gene expression analysis. We obtained 3-cm segments from the apical, middle, and basal portions of 20-cm-high inflorescence stems, and subjected them to quantitative RT-PCR. The correct transcripts of *ATL54* were not detected in the *ATL54*-knock-out mutants

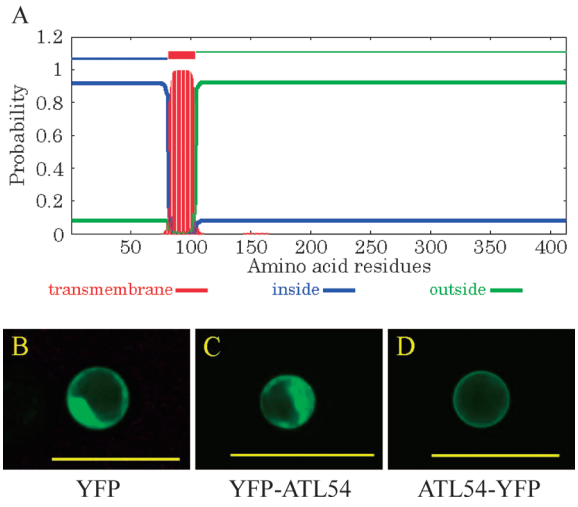

Figure 3. Subcellular localization of the YFP-fused ATL54 protein. (A) A diagram of a transmembrane region predicted by the TMHMM 2.0 program. (B–D) Fluorescent images of transformed protoplasts. *A. thaliana* T87 cells in which YFP (B), ATL54 with its N-terminus YFP-tagged (C), or ATL54 with its C-terminus YFP-tagged (D) was expressed were detected under an epifluorescence microscope. Bars = 100 μm.

(Figure 4C), and *ATL54* expression was increased in three lines of *ATL54*-overexpressed mutants, i.e., OX1, OX9, and OX10 (Figure 4D). Because the OX1 line showed the highest increase in *ATL54* expression in all the three stem portions (Figure 4D), we used this line for further analyses.

Expression of secondary wall-specific cellulose synthase genes *Cellulose Synthase8* (*CesA8*)/*IRX1* (At4g18780), *CesA7*/*IRX3* (At5g17420), and *CesA4*/*IRX5* (At5g44030) (Taylor et al. 2003), and xylan biosynthetic genes *IRX9*, *GUX1* (At3g18660), and *GUX2* (Lee et al. 2007, Lee et al. 2012; Mortimer et al. 2010; Peña et al. 2007) was elevated two- to four-fold in apical stem portions of the *ATL54*-knock-out mutants (Figure 4E). Expression of a xylan biosynthetic gene *IRX14* (At4g36890; Brown et al. 2007) and a lignin biosynthetic gene *CCoAOMT1* (At4g34050; Do et al. 2007) was also up-regulated. The expression level of another lignin biosynthetic gene, *4-coumarate CoA-ligase1* (*4CL1*, At1g51680; Ehlting et al. 1999; Lee et al. 1995), was not changed significantly, and expression of a programmed cell death-associated gene, *Xylem Cysteine Peptidase1* (*XCP1*; Funk et al. 2002; Zhao et al. 2000), was not affected. On the other hand, in middle stem portions, tested secondary wall biosynthetic genes were virtually unchanged by *ATL54* knock-out and *ATL54* overexpression, whereas *XCP1* expression was significantly repressed in both mutants (Figure 4F). There were no significant gene expression changes in basal stem portions of either mutant (Figure 4G).

To investigate whether alteration of *ATL54* expression affects cell wall composition, we performed chemical

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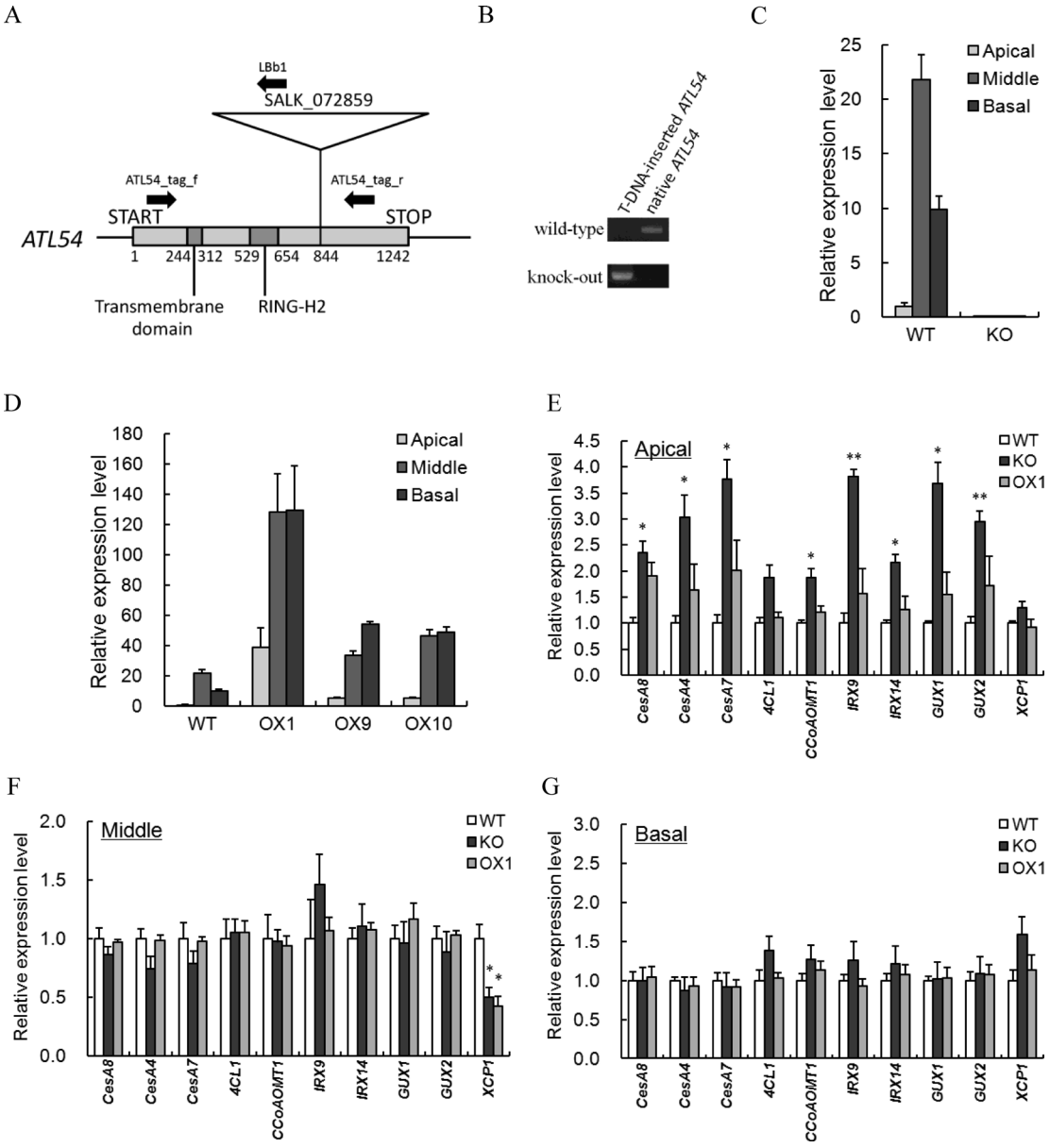

Figure 4. Effect of *ATL54* knock-out and *ATL54* overexpression on expression levels of secondary wall- and programmed cell death-related genes. (A) Schematic diagram of the T-DNA insertion site (SALK_072859) in the *ATL54*-knock-out mutant. The box represents the coding region. Arrowheads indicate annealing sites of primers used in confirmation of the insert (see Supplemental Table S2). (B) Results of PCR from genomic DNA. The T-DNA-inserted sequence was amplified from the genomic DNA of *ATL54*-knock-out mutants, whereas the native *ATL54* was not amplified, confirming that the knock-out line was homozygous. (C–D) *ATL54* expression level. Expression level of *ATL54* in the apical, middle and basal stem portions of wild-type (WT), *ATL54*-knock-out (KO), and *ATL54*-overexpressed (OX) plants was measured by quantitative RT-PCR. Three lines of the overexpressors (OX1, OX9, and OX10) were examined. The expression level in the apical portions of stems of the wild-type plants is set to 1. (E–G) Relative gene expression levels in apical (E), middle (F), and basal (G) stem portions. Expression levels of genes involved in biosynthesis of cellulose (*CesA8*, *CesA4*, and *CesA7*), lignin (*4CL1* and *CCoAOMT1*), and xylan (*IRX9*, *IRX14*, *GUX1*, and *GUX2*), and a gene involved in programmed cell death (*XCP1*) were measured. Among the overexpressors, only OX1 is shown in (E–G) (see Results). Gene expression levels in wild-type plants are set to 1. Error bars indicate standard errors (n=3). Asterisks indicate statistically significant differences (* $p<0.05$; ** $p<0.01$) from the expression levels of wild-type plants.

analyses of organic solvent insoluble residues from whole mature stems of the *ATL54*-knock-out and *ATL54*-overexpressed mutants. No drastic changes in lignin content and syringyl/guaiacyl ratio were observed in either (Supplemental Figure S3A, B). Furthermore, total carbohydrate content and neutral sugar composition were not statistically different between wild-type and mutant plants (Supplemental Figure S4A, B).

Discussion

Gene co-expression network analyses have been successfully used to identify novel genes participating in a particular metabolism (e.g., Ozaki et al. 2010).

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Consequently, we employed a gene co-expression network analysis to look for a ubiquitin ligase gene involved in secondary wall formation. We found that *ATL54* was co-expressed with several genes involved in secondary wall formation with high correlation coefficients (Supplemental Table S1) and that the genes formed a co-expression module (Figure 1). *ATL11* and *ATL72* were also co-expressed with several secondary wall-associated genes with weaker correlation (Supplemental Table S1). A phylogenetic analysis showed that ATL54, ATL11, and ATL72 belong to distinct clades in the phylogenetic tree of ATLs (Supplemental Figure S2). This result is consistent with the classification of ATLs by Aguilar-Hernández et al. (2011), where ATL54, ATL11, and ATL72 were classified into distinct groups. ATL55, which is also named RING1 and triggers fungal toxin-induced programmed cell death (Lin et al. 2008), is the most similar protein to ATL54 (Aguilar-Hernández et al. 2011; Serrano et al. 2006). However, ATL55 and ATL54 share only 23% identity and 43% similarity (Supplemental Figure S1).

In our experiment using YFP-fused proteins, the fluorescence of N-terminus-tagged ATL54 was observed in cytoplasm and nuclei, whereas the fluorescence of C-terminus-tagged ATL54 was localized on plasma membranes. In the case of N-terminus-tagged ATL54, YFP might be released from YFP-tagged ATL54 by the cleavage of the N-terminus of ATL54. Another possible explanation is that anchorage of N-terminus YFP-tagged ATL54 to plasma membranes might be interrupted by YFP, resulting in its incorrect localization.

We demonstrated that the recombinant ATL54 protein catalyzed E1- and E2-dependent auto-ubiquitination (Figure 2), indicating that ATL54 is an E3 ubiquitin ligase. The best characterized function of E3 ubiquitin ligases is labeling of target proteins for degradation via the ubiquitin-26S proteasome pathway. ATL31, an ATL family member, was recently shown to regulate response to the carbohydrate-nitrogen ratio via 14-3-3 protein degradation (Sato et al. 2009; Sato et al. 2011). ATL54 may also target such regulatory proteins.

Gene expression analysis of the mutants revealed that knock-out of *ATL54* activated expression of three cellulose biosynthetic genes, four xylan biosynthetic genes, and one lignin biosynthetic gene in apical portions of stems (Figure 4E). These genes are all associated with secondary wall biosynthesis (Do et al. 2007; Mortimer et al. 2010; Peña et al. 2007; Taylor et al. 2003). Our results thus indicate that *ATL54* affects the expression of a number of secondary wall biosynthetic genes in elongating portions of stems. On the other hand, in middle stem portions, *ATL54* knock-out and overexpression repressed expression of *XCP1*, a gene involved in programmed cell death in tracheary elements (Funk et al. 2002). ATL54 may have a role in the regulation of programmed cell death as well as secondary wall biosynthesis during xylogenesis.

Overexpression of *ATL54* did not affect the expression of secondary wall biosynthetic genes in any tested inflorescence stem portions (Figure 4E–G). One possible reason for this result is that accumulation of the target protein of ATL54 might not be significantly affected by *ATL54* overexpression. Knock-out of *ATL54* also did not change expression of secondary wall biosynthetic genes in middle and basal stem portions (Figure 4F, G). These results might explain why quantity and composition of cell wall polysaccharides and lignin in whole mature stems were not substantially altered by either knock-out or overexpression of *ATL54* (Supplemental Figure S3, 4). The fact that knock-out of *ATL54* did not affect secondary wall biosynthesis in middle and basal stem portions suggests that functionally redundant proteins of ATL54 are present.

In conclusion, we found that *ATL54* is co-expressed with secondary wall-associated genes. We demonstrated that ATL54 has E3 ubiquitin ligase activity and appears to be involved in secondary wall formation and programmed cell death in inflorescence stems. The target of ATL54, necessary for understanding detailed function of ATL54, remains to be elucidated.

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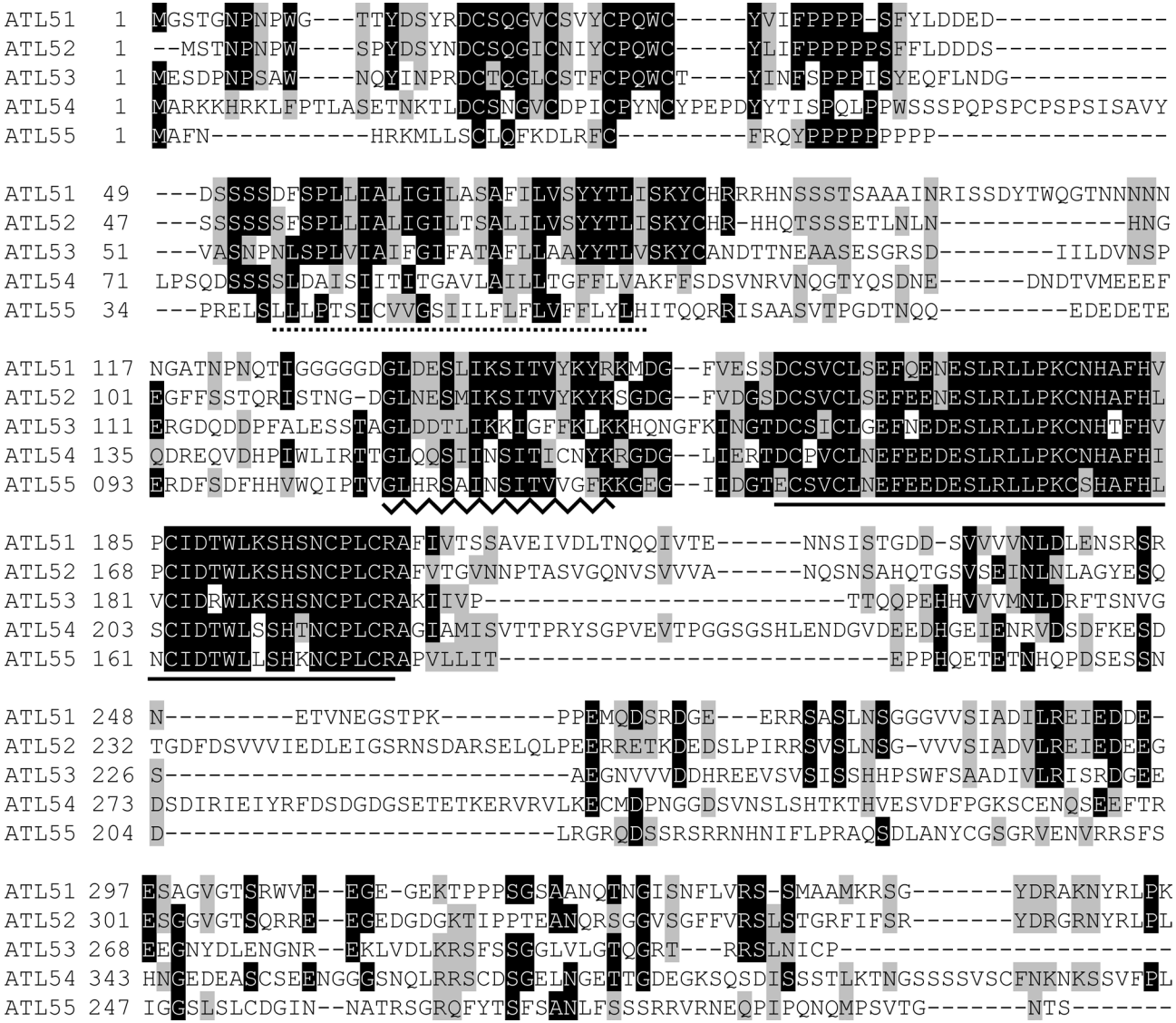
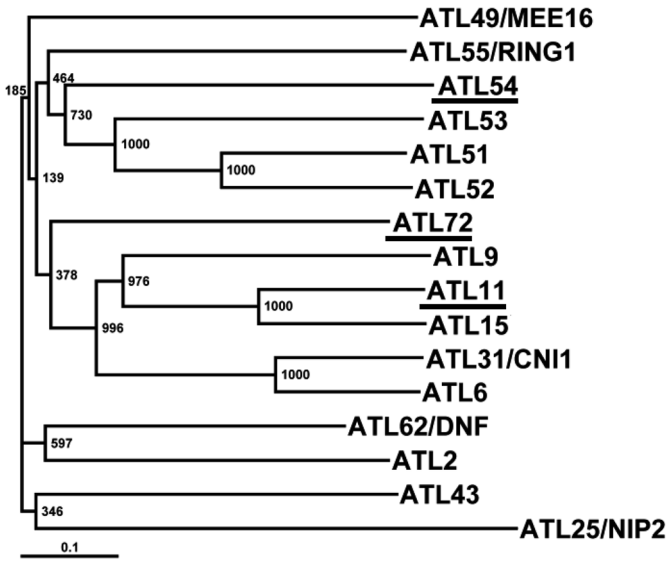


Supplemental Figure S1. Alignment of the amino acid sequences of ATLs. ATL51, ATL52, ATL53 and ATL55 were phylogenetically close to ATL54 according to the phylogenetic analysis by Aguilar-Hernández et al. (2011). The black and gray shades designate identical and similar amino acid residues, respectively. The solid, dotted, and wavy lines indicate RING-H2 domains, hydrophobic regions, and GLD motifs, respectively.

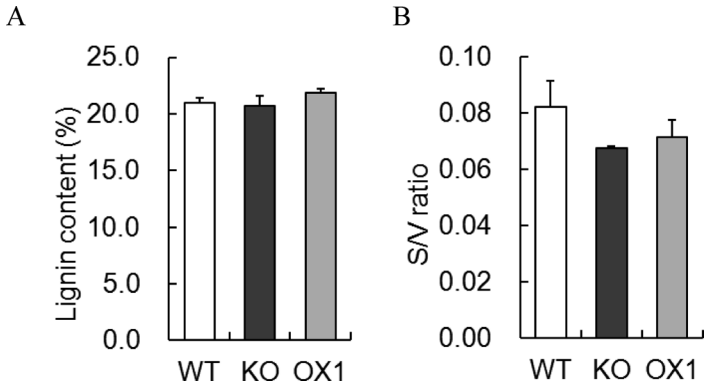
A



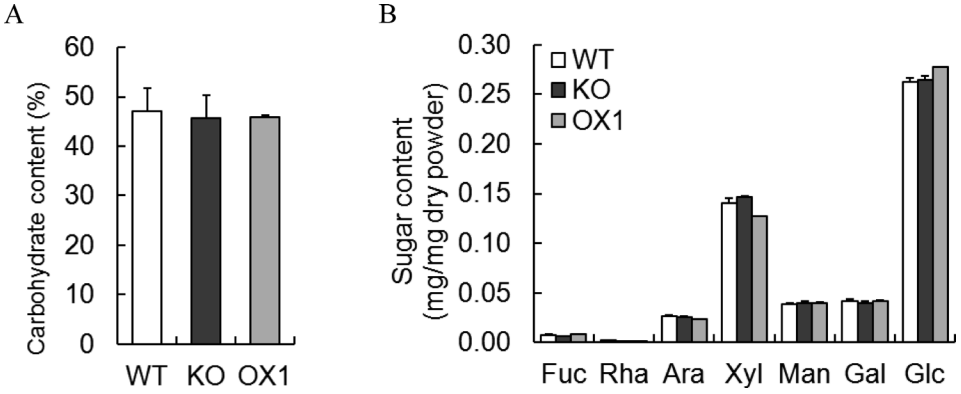
B

Name	Corresponding gene ID	Function	Reference
ATL2	At3g16720	early elicitor response	Salinas-Mondragón et al. (1999) *Plant Mol Biol* 40: 579-590
ATL6	At3g05200	carbon/nitrogen response defense response	Sato et al. (2009) *Plant J* 60: 852-864 Maekawa et al. (2012) *Plant Mol Biol* 79: 217-227
ATL9	At2g35000	chitin-induced defense response	Berrocal-Lobo et al. (2010) *PLoS ONE* 5: e14426
ATL11	At1g72200	(unknown)	-
ATL15	At1g22500	ascorbic acid response	Gao et al. (2011) *J Exp Bot* 62: 3647-3657
ATL25/NIP2	At2g17730	fixing RPOTmp on thylakoid membrane	Azevedo et al. (2008) *Proc Natl Acad Sci USA* 105: 9123-9128
ATL31/CNI1	At5g27420	carbon/nitrogen response defense response	Sato et al. (2009) *Plant J* 60: 852-864 Maekawa et al. (2012) *Plant Mol Biol* 79: 217-227
ATL43	At5g05810	abscisic acid response	Serrano et al. (2006) *J Mol Biol* 62: 434-445
ATL49/MEE16	At2g18650	embryo development	Pagnussat et al. (2005) *Development* 132: 603-614
ATL51	At3g03550	(unknown)	-
ATL52	At5g17600	(unknown)	-
ATL53	At4g17905	(unknown)	-
ATL54	At1g72220	(investigated in this study)	-
ATL55/RING1	At5g10380	fungal toxin-induced programmed cell death	Lin et al. (2008) *Plant J* 56: 550-561
ATL62/DNF	At3g19140	photoperiodic response	Morris et al. (2010) *Plant Cell* 22: 1118-1128
ATL72	At3g10910	(unknown)	-

Supplemental Figure S2. Phylogeny of ATL proteins. (A) Phylogenetic tree of previously reported ATLs and the ATLs remarked in this study. The tree was constructed by the neighbor-joining method with a bootstrap of 1,000 replicates. Bootstrap values are indicated near nodes. ATLs found by the gene co-expression network analysis were underlined. (B) Description of ATLs shown in (A).



Supplemental Figure S3. Lignin content and composition of *ATL54*-knock-out and *ATL54*-overexpressed mutants. (A) Lignin content in organic solvent insoluble residues of mature inflorescence stems determined by the acetyl bromide procedure. Error bars indicate standard errors (n=3–5). (B) Syringaldehyde/vanillin (S/V) ratio obtained by a nitrobenzene oxidation analysis. S/V ratio corresponds to syringyl/guaiacyl ratio in lignin. Error bars indicate standard errors (n=3–4).



Supplemental Figure S4. Carbohydrate content and neutral sugar composition of *ATL54*-knock-out and *ATL54*-overexpressed mutants. (A) Total carbohydrate content in organic solvent insoluble residues of mature inflorescence stems determined by the phenol-sulfuric acid method. Error bars indicate standard errors (n=3). (B) Neutral sugar composition determined as alditol acetates. Fuc, fucose; Rha, rhamnose; Ara, Arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose. Error bars indicate standard errors (n=3).

Supplemental Table S1. RING-finger protein genes co-expressed with secondary wall-associated genes.

	Gene ID	Gene alias	Co-expressed genes*					
			COR≧0.65	0.6≦COR<0.65	0.55≦COR<0.6	0.5≦COR<0.55	0.45≦COR<0.5	0.4≦COR<0.45
Transcription factor	At1g32770	*NST3/SND1*	*ATL54*					At2g20650
	At2g46770	*NST1*	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	At3g61910	*NST2*				*ATL54*		
	At1g71930	*VND7*						
	At5g62380	*VND6*						
	At5g12870	*MYB46*					*ATL54*	At3g47180
	At3g08500	*MYB83*						
	At1g62990	*KNAT7*	*ATL54*		*ATL11*		*ATL72*, At5g55970	
	At1g16490	*MYB58*	*ATL54*					
	At1g79180	*MYB63*			*ATL54*			*ATL11*
Cellulose synthesis	At5g44030	*CesA4*				At2g20650	*ATL54*	
	At5g17420	*CesA7*			*ATL54*	At2g20650	*ATL11*	
	At4g18780	*CesA8*					*ATL54*, At2g20650	
Xylan biosynthesis	At2g37090	*IRX9*	*ATL54*		*ATL11*	At2g20650		
	At4g36890	*IRX14*					At2g20650	
	At4g33330	*GUX2*	*ATL54*			*ATL11*	At2g20650	
	At2g28110	*FRA8/IRX7*		*ATL72*, *ATL11*	At4g23450, At5g01520, At3g53410	At5g55970		
Lignin biosynthesis	At2g37040	*PAL1*						
	At2g30490	*C4H*						
	At1g51680	*4CL1*						
	At5g48930	*HCT*						
	At2g40890	*C3H1*						
	At4g34050	*CCoAOMT1*						At2g20650
	At1g15950	*CCR1*						*ATL72*
	At4g36220	*F5H1*						
	At5g54160	*ATOMT1*						
	At4g34230	*CAD6*			At1g63840			

Lists of genes co-expressed with secondary wall-associated transcription factor and enzyme genes involved in cellulose, xylan, and lignin biosynthesis were obtained from the ATTED-II ver c4.1 database. Genes annotated as "zinc finger (C3HC4-type RING finger) family protein" are shown. The co-expressed gene list of *NST1* was not available in the ATTED-II database N.A., not available. * *ATL54*, At1g72220; *ATL11*, At1g72200; *ATL72*, At3g10910.

Supplemental Table S2. Oligonucleotides used in this study.

Name	Sequence	Purpose
ENTR-ATL54_f	CACCATGGCGAGGAAGAAGCATCGAAAG	For subcloning ATL54 cDNA to insert into pH35GS, pH35YG, pH35GY and pMAL-DC-6myc
ATL54_r	CAGCGGAAAAACCGAACTCTTG	For subcloning ATL54 cDNA to insert into pH35GS, pH35YG, pH35GY and pMAL-DC-6myc
M13 forward (-20)	GTAAAACGACGGCCAG	For DNA sequencing of pENTR/D-TOPO and pMAL-DC-6myc
M13 reverse	CAGGAAACAGCTATGAC	For DNA sequencing of pENTR/D-TOPO
p35Sf3	AGGAAGGTGGCTCCTACAAATGCCATC	For DNA sequencing of pH35GS and pH35GY
Tnosr1	TGATAATCATCGCAAGACCGGCAAC	For DNA sequencing of pH35GS, pH35YG and pH35GY
malE primer	GGTCGTCAGACTGTCGATGAAGCC	For DNA sequencing of pMAL-DC-6myc
ATL54_tag_f	GACTGCTCGAACGGAGTCTGC	For PCR to detect native *ATL54* gene and T-DNA-inserted *ATL54* gene
LBb1	GCGTGGACCGCTTGCTGCAACT	For PCR to detect T-DNA-inserted *ATL54* gene
ATL54_tag_r	CCGTAATTGGTTGCTGCCACC	For PCR to detect native *ATL54* gene
qRT ACT2_f	TCCTCTCCGCTTTGAATTGTCT	For quantitative RT-PCR analysis of the transcript of *ACT2*
qRT ACT2_r	CTGTCAAGTCGCCGGAGATT	For quantitative RT-PCR analysis of the transcript of *ACT2*
qRT ATL54_f	CGTCTCTGTTTCAGATCCATCACCG	For quantitative RT-PCR analysis of the transcript of *ATL54*
qRT ATL54_r	ACTCCCGGAGGATCAGGTTCAC	For quantitative RT-PCR analysis of the transcript of *ATL54*
qRT CesA8_f	CTTCACGCACGGCTTTGCTATTGT	For quantitative RT-PCR analysis of the transcript of *CesA8*
qRT CesA8_r	ATATAGCGGTGCTCGCGACATTGA	For quantitative RT-PCR analysis of the transcript of *CesA8*
qRT CesA4_f	CCAAACACCATGGCCAGCTTCG	For quantitative RT-PCR analysis of the transcript of *CesA4*
qRT CesA4_r	ACCGGGTAAACGCACACGTGAC	For quantitative RT-PCR analysis of the transcript of *CesA4*
qRT CesA7_f	TGAGGGAGGATGGCGGGAAAGA	For quantitative RT-PCR analysis of the transcript of *CesA7*
qRT CesA7_r	AACTTTCCGCGAGAGTGGCTGC	For quantitative RT-PCR analysis of the transcript of *CesA7*
qRT 4CL1_f	TGCTGTAGCTGCCGGATTGTT	For quantitative RT-PCR analysis of the transcript of *4CL1*
qRT 4CL1_r	TGCCAAACTCGGTCAGGGATA	For quantitative RT-PCR analysis of the transcript of *4CL1*
qRT CCoAOMT1_f	TCGTTGATGCTGACAAAGACA	For quantitative RT-PCR analysis of the transcript of *CCoAOMT1*
qRT CCoAOMT1_r	ACTGATCCGACGGCAGATAG	For quantitative RT-PCR analysis of the transcript of *CCoAOMT1*
qRT IRX9_f	AATCTTGTGCCGGAAGTCCCTTCA	For quantitative RT-PCR analysis of the transcript of *IRX9*
qRT IRX9_r	GTGGTAGTAGAAGGGCCTGTTTGT	For quantitative RT-PCR analysis of the transcript of *IRX9*
qRT IRX14_f	ACCACGACGGTGTTGCCTTG	For quantitative RT-PCR analysis of the transcript of *IRX14*
qRT IRX14_r	GCGCTGATAGCAAATTCCCTCCCG	For quantitative RT-PCR analysis of the transcript of *IRX14*
qRT GUX1_f	ATTTGCGGCCTCTGCTTGCC	For quantitative RT-PCR analysis of the transcript of *GUX1*
qRT GUX1_r	CCTTGGGATGAAGCCGTGGTTATGT	For quantitative RT-PCR analysis of the transcript of *GUX1*
qRT GUX2_f	GCGTTTGGTGTTTGTCTGAAGGAGG	For quantitative RT-PCR analysis of the transcript of *GUX2*
qRT GUX2_r	TCGCCCTGAGGTGGTTAGGT	For quantitative RT-PCR analysis of the transcript of *GUX2*
qRT XCP1_f	TGTGACAATCAGCGGCTACGAAGA	For quantitative RT-PCR analysis of the transcript of *XCP1*
qRT XCP1_r	ATGAACCATATCCAACCTCTGCCA	For quantitative RT-PCR analysis of the transcript of *XCP1*