Roles of Arabidopsis TBL34 and TBL35 in xylan acetylation and plant growth

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ABSTRACT

Xylan is one of the major polymers in lignocellulosic biomass and about 60% of its xylosyl residues are acetylated at *O*-2 and/or *O*-3. Because acetylation of cell wall polymers contributes to biomass recalcitrance for biofuel production, it is important to investigate the biochemical mechanism underlying xylan acetylation, the knowledge of which could be applied to custom-design biomass composition tailored for biofuel production. In this report, we investigated the functions of *Arabidopsis* TRICHOME BIREFRINGENCE-LIKE 34 (TBL34) and TBL35, two DUF231-containing proteins, in xylan acetylation. The *TBL34* gene was found to be specifically expressed in xylem cells in stems and root-hypocotyls, and both TBL34 and TBL35 were shown to be localized in the Golgi, where xylan biosynthesis occurs. Chemical analysis revealed that simultaneous mutations of *TBL34* and *TBL35* caused a mild decrease in xylan acetyl content and a specific reduction in xylan 3-*O*-monoacetylation and 2,3-di-*O*-acetylation. Furthermore, simultaneous mutations of *TBL34*, *TBL35* and *ESKIMO1* (*ESK1*) resulted in severely collapsed xylem vessels with altered secondary wall structure, and an extremely retarded plant growth. These findings indicate that TBL34 and TBL35 are putative acetyltransferases required for xylan 3-*O*-monoacetylation and 2,3-di-*O*-acetylation and that xylan acetylation is essential for normal secondary wall deposition and plant growth.

Keywords: Acetylation - *Arabidopsis* – DUF231 - secondary wall – xylan

1. Introduction

Xylan is one of the major polysaccharides in lignocellulosic biomass targeted for biofuel production. It is made of a linear chain of β -1,4-linked xylosyl residues, some of which are substituted with glucuronic acid (GlcA), 4-*O*-methylglucuronic acid (MeGlcA), or arabinose depending on plant species [1]. Xylosyl residues in xylan may also be acetylated at *O*-2 and/or *O*-3 [2]. The reducing end of xylan in gymnosperms and dicots contains a unique tetrasaccharide sequence, β -D-Xyl-(1 $\rightarrow$ 3)- α -L-Rha-(1 $\rightarrow$ 2)- α -D-GalA-(1 $\rightarrow$ 4)-D-Xyl [3-7]. Although xylan is essential for the normal biosynthesis and assembly of secondary walls, its presence contributes to biomass recalcitrance for biofuel production [8]. Xylan not only hinders enzymatic hydrolysis of lignocellulosic biomass for sugar release [9] but also releases acetate from the acetyl groups during biomass pretreatments, which is inhibitory to microorganisms used for fermentation of sugars [10]. Hence, it is important to have a thorough understanding of how xylan is synthesized, which may provide genetic tools for custom-designing lignocellulosic biomass tailored for biofuel production.

Xylan biosynthesis entails enzymatic activities of a number of glycosyltransferases, methyltransferases, and acetyltransferases that mediate xylan backbone elongation and substitutions [11]. In Arabidopsis, the elongation of the xylan backbone involves four GT43 family members [IRREGULAR XYLEM9 (IRX9), IRX9 HOMOLOG (I9H)/ IRX9-LIKE (IRX9-L), IRX14, and I14H/IRX14-L] and two GT47 family members (IRX10 and IRX10-L). Mutations of these glycosyltransferases cause severe reductions in xylosyltransferase activity, xylan backbone chain length, and xylan content [6,12-17]. Recombinant IRX10 and IRX10-L expressed in yeast or human cells display xylosyltransferase activity that is able to transfer xylosyl residues onto xylooligomer acceptors [18,19]. Although recombinant GT43 proteins

expressed in yeast do not exhibit xylosyltransferase activity, microsomes from tobacco BY2 cells co-expressing IRX9 and IRX14 exhibit a high level of xylosyltransferase activity that is capable of adding xylosyl residues onto xylooligomer acceptors [20]. GlcA substitution of xylan is mediated by three GT8 proteins, GlucUronic acid substitution of Xylan1 (GUX1), GUX2, and GUX3, as their simultaneous mutations result in a complete loss of GlcA side chains in xylan [21,22]. Methylation of the GlcA side chains in xylan is carried out by three DUF579-containing proteins, GLUCURONOXylan METHYLTRANSFERASE1 (GXM1), GXM2 and GXM3/GXMT1, and their simultaneous mutations lead to a complete loss of GlcA methylation in xylan [23-25]. Several other glycosyltransferases, including FRAGILE FIBER8 (FRA8), FRA8 HOMOLOG (F8H), IRX8 and PARVUS, have been shown to be involved in the biosynthesis of xylan reducing end tetrasaccharide sequence [6,12,26,27,28].

REDUCED WALL ACETYLATIONS (RWAs), ESK1/TBL29, and ALTERED XYLOGLUCAN9 (AXY9) have been implicated in xylan acetylation. RWAs are close homologs of the fungal capsule synthesis gene1 (Cas1p) that is essential for the acetylation of capsule polysaccharide glucuronoxylomannan [29]. The four Arabidopsis RWA genes, *RWA1/2/3/4*, are expressed in secondary wall-forming cells and their simultaneous mutations cause a 40% reduction in xylan acetyl content [30]. RWAs are proposed to be putative transporters of acetyl donors used for polysaccharide acetylation since they only have multiple transmembrane domains and lack a putative acetyltransferase domain [31]. Mutation of the *AXY9* gene results in a reduction in the acetylation of both xyloglucan and xylan and, therefore, *AXY9* is hypothesized to be involved in supplying acetyl donor substrates for acetylation of multiple polysaccharides [32]. The *ESK1* gene, which encodes a protein in the plant-specific DUF231 family [33], is specifically expressed in xylem vessels and fibers and its mutation causes a

specific reduction in xylan 2-*O*- and 3-*O*-monoacetylation and a decrease in xylan acetyltransferase activity [34,35]. In vitro activity assay has confirmed that recombinant ESK1 exhibits acetyltransferase activity capable of catalyzing 2-*O*- and 3-*O*-monoacetylation of xylooligomers [19]. Because the *esk1* mutation causes only a partial reduction in xylan 2-*O*- and 3-*O*-monoacetylation and has no effects on 2,3-di-*O*-acetylation or 3-*O*-acetylation of xylosyl residues substituted at *O*-2 with GlcA, it has been proposed that **close homologs of ESK1 are candidates to be acetyltransferases** involved in xylan acetylation [34]. In this report, we demonstrate the involvement of Arabidopsis TBL34 and TBL35, **two close homologs of ESK1**, in mediating 3-*O*-monoacetylation and 2,3-di-*O*-acetylation of xylan.

2. Materials and methods

2.1. Plant growth conditions

Wild type and mutant plants were grown in a greenhouse with 16-hr light/8-hr dark cycle. The plants were treated with fertilizer every two weeks. Both wild type and mutant plants were grown simultaneously for observation of their morphological phenotypes and their stems were collected for cell wall analysis.

2.2. Gene expression analysis

The following tissues were used for RNA isolation with a Qiagen RNA isolation kit (Qiagen). Wild-type leaves and roots were from 6-week-old plants. Wild-type stems from 6-week-old plants were divided into top, middle, and bottom parts, which represent the rapidly elongating segments, segments near cessation of elongation, and non-elongating segments, respectively. The top parts of stems contained only protoxylem as examined by toluidine blue

staining of stem cross sections, the middle parts developed both protoxylem xylem and metaxylem but lacked thick walls in interfascicular fibers, and the bottom parts had thick walls in interfascicular fibers. Only main inflorescence stems **with branches removed** were collected for RNA isolation. For the SECONDARY WALL NAC DOMAIN PROTEIN1 (SND1) overexpressors, leaves from 4-week-old plants were used. For the *snd1 nst1* mutant, stems from 6-week-old plants were used. First strand cDNAs were synthesized from total RNA and then used as a template for real-time quantitative PCR analysis with the QuantiTect SYBR Green PCR kit (Clontech). The PCR primers for *TBL34* **were** 5'-cattacgcaactatcagagtatcg-3' and 5'-tcaactagaatgatgtgcaagaat-3', and those for *TBL35* **were** 5'-agagattgggaccaaaggtctcag-3' and 5'-tcacaaaaatggaaaagcaattgattcc-3'. PCR threshold cycles for genes of interest were selected in the beginning of their exponential phases on PCR reaction curves. The expression level of each gene was calculated by normalizing their PCR threshold cycle numbers with those of the *UBQ10* (At4g05320), *GAPDH* (At1g13440), and *EF1 α* (□t□□□□□□□ reference genes and quantitated according to the instructions from the PCR cyclor manufacturer (BIO-RAD). The PCR primers for *UBQ10* **were** 5'-ggcctgtataatccctgatgaataag-3' and 5'-aaagagataacaggaacggaaacatagt-3', those for *GAPDH* **were** 5'-ttggtgacaacaggtcaagca-3' and 5'-aaactgtcgctcaatgcaatc-3', and those for *EF1 α* **were** 5'-tgagcacgctcttctgtttca-3' and 5'-ggtggtggcatccatctgttaca-3'. The data were the average of three biological replicates.

2.3. *β -Glucuronidase (GUS) reporter gene analysis*

For creating the GUS reporter gene constructs, the entire *TBL34* and *TBL35* gene sequences, including the 3-kb 5' upstream sequence, the entire coding region, and the 2-kb 3' downstream sequence were used. The whole gene sequence of *TBL34* was retrieved from the

1 BAC clone under GenBank accession number AC004683 and genomic locus number
2 T19C21.19, and that of TBL35 was retrieved from the BAC clone under GenBank accession
3 number AL161946 and genomic locus number F7A7_140. The GUS reporter gene was inserted
4 in frame just before the stop codon of the *TBL34* and *TBL35* genes, which were cloned in the
5 binary vector pBI101 (Clontech). The constructs were transformed into wild-type *Arabidopsis*
6 plants by Agrobacterium-mediated transformation [36]. The first generation of transgenic plants
7 was used to examine GUS activity as described previously [28]. At least 10 independent
8 transgenic plants were examined and a consistent GUS staining pattern was observed.

9

10 2.4. Subcellular localization

11 For subcellular localization, the full-length *TBL34* and *TBL35* cDNAs were ligated in
12 frame at the amino terminus of yellow fluorescent protein (YFP) in a high-copy pBI221 plasmid
13 vector (Clontech) to create YFP-tagged TBL34 and TBL35 expression constructs. The
14 expression of *TBL34-YFP* and *TBL35-YFP* was driven by the cauliflower mosaic virus 35S
15 promoter. They were co-expressed with cyan fluorescent protein (CFP)-tagged Golgi marker
16 FRA8 in *Arabidopsis* leaf protoplasts [37]. The FRA8-CFP construct was generated by ligating
17 the full-length *FRA8* cDNA in frame at the amino terminus of CFP in the pBI221 vector [28].
18 The fluorescence signals were visualized using a Leica TCS SP2 spectral confocal microscope
19 (Leica Microsystems). The TMHMM2.0 program (<http://www.cbs.dtu.dk/service/TMHMM-2.0>)
20 [38] and the Golgi Predictor program (http://ccb.imb.uq.edu.au/golgi/golgi_predictor.shtml) [39]
21 were used to predict putative subcellular localizations of TBL34 and TBL35.

22

23 2.5. Histology

1 Stems and root-hypocotyls of the wild type and mutants were fixed in 2% formaldehyde
2 and embedded in Low Viscosity (Spurr's) resin (Electron Microscopy Sciences) as described
3 [40]. One- μ m-thick sections were cut with a microtome and stained with 0.01% toluidine blue.
4 Stems and root-hypocotyls from at least 4 plants were sectioned and **representative data were**
5 shown. The bottom 2-cm segments of inflorescence stems of mature plants (8-week-old wild
6 type, *tbl34*, *tbl35* and *tbl34 tbl35*; 10-week-old *esk1*, *tbl34 esk1* and *tbl35 esk1*; and 12-week-old
7 *tbl34 tbl35 esk1*) were measured for their breaking force using a digital force/length tester
8 (Larson System) [41]. The breaking force was measured as the force needed to break apart a
9 stem segment.

10 11 *2.6. Cell wall sugar composition analysis*

12 Mature inflorescence stems from pools of at least 30 Arabidopsis plants for each mutant
13 line were ground in liquid nitrogen and isolated for cell wall residues [28]. Cell wall sugars (as
14 alditol acetates) were determined following the procedure as described [42]. The alditol acetates
15 of the hydrolyzed cell wall sugars were analyzed on a PerkinElmer Clarus 500 gas-liquid
16 chromatograph instrument equipped with a 30 m x 0.25 mm (i.d.) silica capillary column DB 225
17 (Alltech Assoc.). The sugar standards, including glucose, xylose, mannose, arabinose and
18 rhamnose, were purchased from Sigma-Aldrich.

19 20 *2.7. Quantitative measurement of acetyl groups*

21 Acetylated xylan from stem cell wall residues was extracted with dimethyl sulfoxide
22 (DMSO) following the procedure described [43]. The amount of acetyl groups in DMSO-
23 extracted xylan was determined by quantifying acetic acid released from xylan upon treatment

1 with NaOH [44]. The amount of acetic acid was determined using an acetic acid assay kit
2 (Megazyme), and acetic acid from the kit was used as a standard for quantitation according to the
3 protocol from Megazyme.

4 5 2.8. *¹H-nuclear magnetic resonance (NMR) spectroscopy*

6 DMSO-extracted xylans from various mutants were digested with β -endoxylanase M6
7 (Megazyme) and subsequently analyzed by NMR spectroscopy. NMR spectra were acquired at
8 20 °C on a 600 MHz spectrometer (599.7 MHz, ¹H) using a 3 mm cryogenic triple resonance
9 probe [45]. The samples were prepared with 100% D₂O, and 128 transients were collected for
10 each sample. The ¹H NMR assignments of resonances were established by comparing them to
11 the NMR spectral data for xylan structure [2,46]. For wild type and each mutant sample, xylan
12 was extracted from a pool of inflorescence stems of at least 30 individual plants. For the *tbl34*
13 *tbl35 esk1* triple mutant, inflorescence stems from a pool of 150 plants were used. NMR spectra
14 from three replicates were obtained for each sample and they showed nearly identical resonance
15 signals with less than 5% variation. The observed difference between the wild type and mutants
16 was statistically significant ($p < 0.001$) using the Student's *t* test program.

17 18 2.9. *Statistical analysis*

19 The data of quantitative PCR analysis, breaking force and cell wall chemical
20 analysis were examined using the Student's *t* test program
21 (<http://www.graphpad.com/quickcalcs/ttest1.cfm>).

22 23 2.10. *Accession numbers*

1 The *Arabidopsis* Genome Initiative locus identifiers for the *Arabidopsis* genes
2 investigated in this study are TBL34 (At2g38320), TBL35 (At5g01620), and ESK1
3 (At3g55990).

4 5 **3. Results**

6 *3.1. Xylem-specific expression of TBL34*

7 TBL34 and TBL35 are among the nine *Arabidopsis* DUF231-containing proteins that are
8 phylogenetically closely related to ESK1 [33] (Fig. 1A). For amino acid sequences, TBL34 and
9 TBL35 exhibit 36% and 39% identity and 62% and 66% similarity to ESK1, respectively. ESK1
10 is an acetyltransferase involved in xylan 2-*O*- and 3-*O*-monoacetylation [19,34]. To investigate
11 the functions of TBL34 and TBL35, we first studied their gene expression patterns. Quantitative
12 PCR analysis showed that although *TBL35* did not exhibit organ-specific expression, *TBL34* was
13 preferentially expressed in the middle and bottom parts of stems (Fig. 1B), in which secondary
14 walls were heavily deposited in xylem cells and interfascicular fibers. In addition, the expression
15 of *TBL34* was induced by overexpression of SND1, and the expression of both *TBL34* and
16 *TBL35* was downregulated in the stems of the *snd1 nst1* double mutant (Fig. 1C). SND1 was
17 previously discovered to be a fiber-specific NAC domain transcription factor and together with
18 its functionally-redundant paralog NST1, it controls the activation of secondary wall biosynthesis
19 genes, leading to the deposition of secondary wall components, including cellulose, xylan and
20 lignin [47]. Simultaneous mutations of *SND1* and *NST1* have previously been shown to cause a
21 loss of secondary walls in both xylary fibers and interfascicular fibers [48]. The finding that
22 *TBL34* expression is regulated by SND1 indicates the involvement of TBL34 in secondary wall
23 biosynthesis.

Examination of cell-type expression patterns of *TBL34* and *TBL35* using the GUS reporter gene revealed that while no GUS activity was detected in any tissues of *TBL35::GUS* transgenic plants probably due to its low expression level, *TBL34::GUS* plants displayed strong GUS expression in a tissue-specific manner. Intense GUS staining was observed in developing vessels in the rapidly elongating segments of stems (Fig. 1D), which had secondary wall thickening only in protoxylem as examined by toluidine blue staining of stem sections. In the segments near cessation of elongation, in which thin secondary walls were seen in interfascicular fibers, and the non-elongating segments, in which thick secondary wall thickening occurred in both metaxylem and interfascicular fibers as examined by toluidine blue staining of stem sections, GUS staining was seen in vessels and xylary fibers in the metaxylem (Fig. 1E and F). Heavy GUS staining was also observed in developing secondary xylem in root-hypocotyls (Fig. 1G). The observation that *TBL34* expression is xylem-specific provides further evidence suggesting its association with secondary wall biosynthesis.

3.2. Golgi localization of *TBL34* and *TBL35*

TBL34 and *TBL35* were predicted to be Golgi-localized type II membrane proteins by the TMHMM2.0 program (Fig. 2A) and by the Golgi Predictor program (data not shown). To verify the subcellular localization of *TBL34* and *TBL35*, we co-expressed yellow fluorescent protein (YFP)-tagged *TBL34* and *TBL35* together with cyan fluorescent protein (CFP)-tagged Golgi marker FRA8 [28] in *Arabidopsis* leaf protoplasts. The experiment showed that protoplasts expressing *TBL34*-YFP and *TBL35*-YFP exhibited punctate fluorescent signals that were superimposed with those of FRA8-CFP (Fig. 2C-J), indicating that *TBL34* and *TBL35* are

1 targeted to the Golgi, where xylan biosynthesis occurs. In contrast, protoplasts expressing YFP
2 alone had fluorescent signals throughout the cytoplasm (Fig. 2B).

3 4 3.3. Mutations of *TBL34* and *TBL35* in the *esk1* background exacerbated the defects in plant 5 growth and vessel morphology

6 The T-DNA insertion mutants of *TBL34* and *TBL35* (Fig. 3A) and the *esk1* mutant were
7 crossed to generate various double and triple mutants for examination of mutant phenotypes.
8 Although the single and double mutants of *TBL34* and *TBL35* did not show any alterations in
9 plant growth (Fig. 3B,C and E) and stem mechanical strength (Fig. 3F), their mutations in the
10 *esk1* background led to exacerbated defects in plant growth compared with the *esk1* mutant (Fig.
11 3B-F). In particular, the *tbl34 tbl35 esk1* triple mutant displayed severely retarded growth of
12 inflorescence stems, and only very tiny inflorescences developed after prolonged growth (12
13 weeks; inset in Fig. 3D). In addition, stem strength was decreased in *tbl34 esk1* and *tbl34 tbl35*
14 *esk1* compared with *esk1* (Fig. 3F).

15 Examination of xylem vessel morphology in stems of the single and double mutants of
16 *TBL34* and *TBL35* revealed no alterations compared with the wild type (data not shown). The
17 *esk1* mutant had a mild deformation of vessels in both stems and root-hypocotyls (Fig. 4B and
18 H). Examination of *tbl34 tbl35 esk1* plants showed that the vessels in stems and root-hypocotyls
19 were more severely deformed than those in *esk1* (Fig. 4), which was consistent with the
20 predominant expression of *TBL34* in xylem cells. Some of vessels were so severely collapsed
21 that they only had a small cavity (Fig. 4C and I). The exacerbated growth defect in *tbl34 tbl35*
22 *esk1* could be attributed to reduced water conducting capacity conferred by such drastically
23 collapsed vessels. Transmission electron microscopy further revealed that although three distinct

1 wall layers, namely S1, S2 and S3, were evident in the secondary walls of wild-type vessels (Fig.
2 5A), such layers were absent in the secondary walls of vessels of *esk1* (Fig. 5B) and *tbl34 tbl35*
3 *esk1* (Fig. 5C). We also noted that the secondary walls of vessels in these mutants appeared to be
4 stained unevenly and the inner surface of the secondary walls was wavy compared with the wild
5 type. The reduction in the wall thickness of interfascicular fibers in the stems of *tbl34 tbl35 esk1*
6 plants compared with the wild type appeared to be similar to that in *esk1* (Fig. 4D-F).
7 Furthermore, although the amount of xylose was not altered, that of glucose was further reduced
8 in the stem cell walls of *tbl34 tbl35 esk1* compared to those of *esk1* (Fig. 6A). This further
9 reduction in glucose was also observed in the stem cell walls of *tbl34 esk1* and *tbl35 esk1* (Fig.
10 6A). Because the majority of glucose in stem cell walls is from cellulose, this finding suggests a
11 defect in cellulose deposition in these mutants.

13 3.4. Involvement of TBL34 and TBL35 in xylan 3-O-monoacetylation and 2,3-di-O-acetylation

14 To determine whether TBL34 and TBL35 perform similar functions as ESK1 in xylan
15 acetylation, we measured the acetyl contents in DMSO-extracted xylans from the single, double,
16 and triple mutants. Although the amount of acetyl groups in *tbl35* xylan was not significantly
17 altered compared to that of the wild-type xylan, those in *tbl34* and *tbl34 tbl35* xylans were mildly
18 reduced (Fig. 6B). More severe reductions in acetyl groups were observed in *tbl34 esk1*, *tbl35*
19 *esk1* and *tbl34 tbl35 esk1* xylans, which contained 44%, 53% and 43%, respectively, of the wild-
20 type acetyl level (Fig. 6B).

21 We next investigated the patterns of acetyl substitutions in xylans from the double and
22 triple mutants of *TBL34*, *TBL35*, and *ESK1* using NMR spectroscopy. Examination of ¹H NMR
23 spectra showed that DMSO-extracted xylans from the wild type and various mutants exhibited

resonances typical for acetyl groups between 2.1 and 2.3 ppm and the sugar groups between 3.1 and 5.4 ppm (Fig. 7) [44]. Integration analysis showed that the relative amount of acetyl groups in these mutants was reduced by various degrees compared with that in the wild type (Table 1). Further examination of the fingerprint region of the ^1H NMR spectra of acetylated xylans revealed specific defects in acetyl substitutions in the mutant xylans (Fig. 8). The assignment of proton resonances for xylan structural fragments was based on published data [2,46]. Resonances corresponding to various acetyl substitutions on xylosyl residues, including 2-*O*- and 3-*O*-monoacetylated xylosyl residues, 2,3-di-*O*-acetylated xylosyl residues, and 3-*O*-monoacetylated xylosyl residues substituted at *O*-2 with GlcA/MeGlcA were readily observed in the wild-type and mutant xylans, albeit with different abundances (Fig. 8). Integration analysis showed that the signal abundance for 3-*O*-monoacetylated xylosyl residues and 2,3-di-*O*-acetylated xylosyl residues in *tbl34 tbl35* was reduced down to 67% and 65%, respectively, of that in the wild type (Table 2). In contrast, no reduction in the signal abundance for 2-*O*-monoacetylated xylosyl residues and 3-*O*-monoacetylated xylosyl residues substituted at *O*-2 with GlcA/MeGlcA was observed in *tbl34 tbl35*; instead, their signal abundance was relatively elevated. This finding indicates that TBL34 and TBL35 play a role in 3-*O*-monoacetylation and 2,3-di-*O*-acetylation of xylan. The *esk1* mutation also caused a reduction in the signal abundance for 3-*O*-monoacetylated xylosyl residues [34]. Consistently, simultaneous mutations of *TBL34*, *TBL35* and *ESK1* resulted in an exacerbated decrease in the signal abundance for 3-*O*-monoacetylated xylosyl residues compared with the *esk1* mutation alone (Table 2). However, a residual amount of 3-*O*-monoacetylated xylosyl residues remained in the xylan of *tbl34 tbl35 esk1*, indicating that additional acetyltransferase activities are involved. It was interesting to note that the elevation in the signal abundance for 2-*O*-monoacetylated xylosyl residues observed in *tbl34 tbl35* was

diminished in *tbl34 tbl35 esk1*, consistent with a role of ESK1 in 2-*O*-monoacetylation of xylan. Similar to the *esk1* mutant, the *tbl34 esk1*, *tbl35 esk1* and *tbl34 tbl35 esk1* mutants all had elevated abundance of GlcA/MeGlcA (Table 2), which was likely attributed to the reduction of 2-*O*-acetylation, leaving more *O*-2 positions available for GlcA substitutions [34].

4. Discussion

O-acetylation is one of the major substitutions of plant cell wall polymers, including hemicelluloses (xyloglucan, xylan and glucomannan), pectins (homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II), and lignin [31,49]. In lignocellulosic biomass targeted for biofuel production, xylan is one of the major polymers that is heavily acetylated. The degree of acetyl substitutions in xylan from various species, including *Arabidopsis*, aspen, *Paulownia elongate* x *fortune*, birch, beech, *Eucalyptus globulus*, *Agave sisalana*, ranges from 0.35 to 0.61 [2, 34,43, 44,50-53]. Acetyl substitutions in xylan can occur at *O*-2 or *O*-3 of different xylosyl residues, at both *O*-2 and *O*-3 of the same xylosyl residue, and at *O*-3 of xylosyl residues substituted at *O*-2 with GlcA/MeGlcA [2]. Recent studies have shown that ESK1 is an acetyltransferase specifically catalyzing 2-*O*- and 3-*O*-monoacetylation of xylan [19,34,34], suggesting that different acetyltransferase activities mediate the regiospecific acetyl substitutions of xylan. Our finding in this study that TBL34 and TBL35, two close homologs of ESK1, are putative acetyltransferases involved in 3-*O*-monoacetylation and 2,3-di-*O*-acetylation of xylan expands our understanding of the biochemical mechanism underlying xylan *O*-acetylation.

The fact that the *tbl34 tbl35 esk1* triple mutant has a more severe reduction in xylan 3-*O*-monoacetylation than either *tbl34 tbl35* or *esk1* alone indicates that TBL34, TBL35, and ESK1 function redundantly in xylan 3-*O*-monoacetylation. Despite this functional redundancy, TBL34

1 and TBL35 differ from ESK1 in several respects. In addition to the reduction in xylan 3-*O*-
2 monoacetylation, simultaneous mutations of *TBL34* and *TBL35* also led to a reduction in xylan
3 2,3-di-*O*-acetylation, whereas the *esk1* mutation also resulted in a reduction in xylan 2-*O*-
4 monoacetylation. Furthermore, *TBL34* is specifically expressed in xylem cells, whereas *ESK1* is
5 expressed in both xylem cells and interfascicular fibers. It is interesting to find that the 2-*O*-
6 monoacetylation of xylosyl residues is elevated in the *tbl34 tbl35* mutant and this elevation is
7 diminished in *tbl34 tbl35 esk1*. One plausible explanation is that xylan 2,3-di-*O*-acetylation may
8 be mediated by ESK1 and TBL34/35 in two steps: first, ESK1 catalyzes 2-*O*-acetylation of
9 xylosyl residues; second, TBL34/35 catalyze 3-*O*-acetylation of the already 2-*O*-acetylated
10 xylosyl residues. Therefore, mutations of *TBL34* and *TBL35* cause a reduction in the amount of
11 2,3-di-*O*-acetylation and a concomitant elevation in the amount of 2-*O*-monoacetylation. Since
12 mutation of ESK1 results in defective 2-*O*-acetylation, this elevation diminishes in *tbl34 tbl35*
13 *esk1*.

14 Although we were unable to detect *TBL35* expression using TBL35-GUS reporter gene
15 analysis, quantitative PCR analysis showed its expression in all organs (Fig. 1B). Chemical
16 analysis revealed that the *tbl35 esk1* double mutant had lower amounts of cell wall glucose (Fig.
17 6A), overall xylan acetyl groups (Fig. 6B), and 3-acetylated xylosyl residues compared with *esk1*
18 (Table 2), suggesting a role of TBL35 in xylan acetylation. It should also be pointed out that
19 although the *tbl34 tbl35 esk1* triple mutant had a significant reduction in 3-*O*-acetylation of
20 xylan, **there** remains a residual amount of 3-*O*-acetylated xylosyl residues, which implies that
21 additional acetyltransferase activities are involved in xylan 3-*O*-acetylation. There are six other
22 ESK1 close homologs in addition to TBL34 and TBL35 (Fig. 1A), all of which could be
23 involved in xylan acetylation. This possibility is consistent with our recent finding that two of

1 them, TBL3 and TBL31, are also involved in xylan 3-*O*-acetylation [51], which may explain
2 why a residual amount of 3-*O*-acetylated xylosyl residues remains in the *tbl34 tbl35 esk1* triple
3 mutant. More importantly, *TBL34* is specifically expressed in xylem cells, and therefore its
4 mutation does not affect 3-*O*-acetylation of xylan in interfascicular fibers.

5 It is of interest to note that simultaneous mutations of *TBL34*, *TBL35* and *ESK1* cause a
6 significant reduction in the glucose level but not the xylose level in the stem cell walls. This
7 finding indicates that such a reduction in the degree of acetyl substitutions has no major effect on
8 the biosynthesis of the xylan backbone but results in a significant negative impact on cellulose
9 deposition. Because acetylation affects the physical properties of xylan and its interaction with
10 cellulose [55,56], it is possible that xylan with a lower degree of acetyl substitutions alters its
11 normal interaction with cellulose and the subsequent assembly of cellulose-xylan polymers in
12 secondary walls. As a result, the mutant has a substantial reduction in cellulose content. The fact
13 that the *tbl34 tbl35 esk1* triple mutant has a much **greater** reduction in the amounts of both
14 cellulose and acetyl groups than the *esk1* mutant alone indicates a correlation between the
15 severity of defect in xylan acetylation and the reduction in cellulose content. The severe
16 deformation of vessels in *tbl34 tbl35 esk1* could be attributable to both the reduced cellulose
17 amount and the defective interaction between cellulose and mutant xylan. It is interesting to note
18 that the secondary walls of *tbl34 tbl35 esk1* vessels lack **distinct S1, S2 and S3 wall layers but**
19 **exhibit** uneven staining pattern and wavy inner surface compared with those of wild-type vessels.
20 Because the layered appearance of secondary walls is determined by the orientated deposition of
21 cellulose microfibrils [11], the abnormal secondary wall structure observed in the mutant vessels
22 is very likely due to an altered deposition of cellulose microfibrils, which further indicates the
23 importance of xylan acetylation in secondary wall assembly.

The *Arabidopsis* DUF231 family is a large, plant-specific family with 46 members [33]. So far, only a few of **family members** have been functionally characterized; these include PMR5/TBL44 whose mutation increases resistance to powdery mildew fungi [57], TBR involved in cellulose biosynthesis [33], AXY4/TBL27 and AXY4L/TBL22 required for xyloglucan acetylation [58], and ESK1/TBL29, TBL3 and TBL31 essential for xylan acetylation [34,35,51]. Our finding that TBL34 and TBL35 are involved in xylan acetylation expands our understanding of the functions of DUF231 members and lends further support to the hypothesis that members of the DUF231 family mediate acetylation of different wall polymers [58]. Deciphering the mechanisms underlying the acetylation of cell wall polymers will provide potential tools for genetic modification of biomass composition better suited for biofuel production.

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Figure legends

Fig. 1. Expression analyses of the *TBL34* and *TBL35* genes. (A) Phylogenetic relationship of *TBL34*, *TBL35*, and other ESK1 homologs in the ESK1 subgroup of the Arabidopsis DUF231 family. TBR is another DUF231 member involved in cellulose deposition in trichomes. The phylogenetic tree was constructed with the neighbor-joining algorithm. The 0.1 scale denotes 10% change, and the bootstrap values resulted from 1,000 replicates are presented in percentages at the nodes. (B) and (C) Quantitative PCR analysis of the expression of *TBL34* and *TBL35*. Shown are their expression levels in different Arabidopsis organs (B), in the *SND1* overexpressors (*SND1*-OE) (C, left panel), and in the *snd1 nst1* double mutant (C, right panel). The expression of each gene in the organ was presented as transcript copy number per ng total RNA (B). The change in the expression of *TBL34* and *TBL35* in *SND1* overexpressors (C, left panel) and the *snd1 nst1* double mutant (C, right panel) was compared with the wild type by setting the expression level in the wild type as 1 for *SND1* overexpressors and as 100 for *snd1 nst1*. Error bars denote SD of three biological replicates. Asterisks denote statistically significant differences compared with the wild type ($p < 0.001$). (D) to (G) Cell-type expression analysis of *TBL34* in Arabidopsis inflorescence stems and root-hypocotyls. The *TBL34* gene fused with the GUS reporter genes was introduced into Arabidopsis and the transgenic plants were examined for GUS activity (shown as blue). (D) Cross section of the top stem showing GUS staining in the vessels in the protoxylem. (E) and (F) Cross sections of the middle stem (E) and the bottom stem (F) showing GUS staining in the vessels and xylary fibers in the metaxylem. (G) Cross section of the root-hypocotyl showing GUS staining in the developing secondary xylem. co, cortex; if, interfascicular fiber; pi, pith; sp, secondary phloem; sx, secondary xylem; ve, vessel; xf, xylary fiber; xy, xylem. Bars = 266 μ m.

Fig. 2. Subcellular localization of fluorescent protein-tagged TBL34 and TBL35 in Arabidopsis protoplasts. TBL34 and TBL35 fused with YFP were co-expressed with the CFP-tagged Golgi marker FRA8 in Arabidopsis leaf protoplasts and their fluorescent signals were examined with a laser confocal microscope. (A) TBL34 and TBL35 are type II membrane proteins as predicted by the TMHMM2.0 program. Inside, the cytoplasmic side of the membrane; outside, the Golgi lumen side of the membrane. (B) Distribution of the fluorescent signals throughout the cytoplasm in an Arabidopsis protoplast expressing YFP alone. (C) to (F) Superimposition of the punctate fluorescent signals (F) in an Arabidopsis protoplast (C) co-expressing TBL34-YFP (D) and the Golgi-localized FRA8-CFP (E). (G) to (J) Superimposition of the punctate fluorescent signals (J) in an Arabidopsis protoplast (G) co-expressing TBL35-YFP (H) and FRA8-CFP (I). Bars in (B) to (J) = 8 μ m.

Fig. 3. Effects of mutations of *TBL34*, *TBL35* and *ESK1* on plant growth and stem mechanical strength. (A) Diagrams showing the sites of T-DNA insertions in the *TBL34* and *TBL35* genes. Filled boxes represent exons. (B) and (C) Morphology of 4-week-old (B) and 8-week-old (C) plants of the wild type and various mutants. (D) Morphology of 10-week-old mutant plants. Inset shows the image of a 12-week-old plant of *tbl34 tbl35 esk1*. (E) Measurement of inflorescence stem heights of the wild type and various mutant plants at different time points. (F) Measurement of the breaking force of stems of the wild type and various mutants. Error bars represent SD of 20 plants. Asterisks denote statistically significant differences compared with the wild type ($p < 0.001$).

Fig. 4. Simultaneous mutations of *TBL34*, *TBL35* and *ESK1* result in severely collapsed xylem vessels. The bottom segments of stems and the root-hypocotyls of 8-week-old wild-type, 10-week-old *esk1*, and 12-week-old *tbl34 tbl35 esk1* plants were sectioned and stained with toluidine blue for examination of anatomical structures. (A) to (C) Cross sections of xylem bundles of stems showing deformed vessels (arrows) in *esk1* (B) and *tbl34 tbl35 esk1* (C) compared with the wild type (A). (D) to (F) Cross sections of the interfascicular regions of stems showing a reduction in secondary wall thickness in *esk1* (E) and *tbl34 tbl35 esk1* (F) compared with the wild type (D). (G) to (I) Cross sections of root-hypocotyls showing deformed vessels (arrows) in *esk1* (H) and *tbl34 tbl35 esk1* (I) compared with the wild type (G). if, interfascicular fiber; sx, secondary xylem; xy, xylem. Bar in (A) = 72 μ m for (A) to (I).

Fig. 5. Transmission electron micrographs of vessel secondary walls of wild type (A), *esk1* (B) and *tbl34 tbl35 esk1* (C). Ultrathin sections from stems were stained with uranyl acetate and lead citrate, and observed using a transmission electron microscope. The numbers, 1, 2 and 3, marked in the secondary walls of wild-type vessels (A) indicate the three distinct layers, S1, S2 and S3, respectively. Note the lack of distinct layers, abnormal staining pattern and wavy inner surface of secondary walls of *esk1* (B) and *tbl34 tbl35 esk1* (C) vessels compared with the secondary walls of wild-type vessels (A). ve, vessel; xf, xylary fiber. Scale bar = 3 μ m.

Fig. 6. Cell wall sugar composition and xylan acetyl contents in the wild type and various mutants. Cell wall residues and xylan were extracted from the inflorescence stems of 8-week-old wild-type, 9-week-old *esk1*, and 12-week-old *tbl34 tbl35 esk1* plants. (A) Measurement of neutral sugar composition in the cell walls of the wild type and various mutants. (B)

Measurement of acetyl contents in the xylans of the wild type and various mutants. Error bars denote SD of three biological samples. Asterisks denote statistically significant differences compared with the wild type ($p < 0.001$).

Fig. 7. ^1H NMR spectra of DMSO-extracted xylans from the wild type, *tbl34 tbl35*, *esk1*, *tbl34 esk1*, *tbl35 esk1*, and *tbl34 tbl35 esk1*. The resonance regions for carbohydrate and acetyl groups are indicated. HDO, hydrogen deuterium oxide.

Fig. 8. Diagram of an acetylated xylooligomer from wild-type Arabidopsis xylan (A) and the fingerprint regions of the ^1H NMR spectra of DMSO-extracted xylans from the wild type, *tbl34 tbl35*, *esk1*, *tbl34 esk1*, *tbl35 esk1*, and *tbl34 tbl35 esk1* (B). The resonances for non-acetylated xylosyl residues (Xyl), 2-*O*-acetylated xylosyl residues (Xyl-2Ac), 3-*O*-acetylated xylosyl residues (Xyl-3Ac), 2,3-di-*O*-acetylated xylosyl residues (Xyl-2,3Ac), 3-*O*-acetylated xylosyl residues substituted at *O*-2 with GlcA/MeGlcA (Xyl-3Ac-2GlcA), and GlcA/MeGlcA are marked. H1, H2 and H3 indicate the protons attached to carbon at positions 1, 2 and 3, respectively, of the xylose ring.

Table 1. Relative integrated values of acetyl groups and carbohydrates in DMSO-extracted xylans from the wild type and various mutants

Sample	Carbohydrates ^a	Acetyl groups ^b	DS _{AC}	Relative amount of acetyl groups ^c
Wild type	1	0.19	0.60	100
<i>tbl34 tbl35</i>	1	0.18	0.57	95
<i>esk1</i>	1	0.14	0.44	73
<i>tbl34 esk1</i>	1	0.12	0.38	63
<i>tbl35 esk1</i>	1	0.13	0.41	68
<i>tbl34 tbl35 esk1</i>	1	0.12	0.38	63

^a The integration of carbohydrates was calculated from the NMR resonance between 3 and 5.5 ppm (Fig. 7) and taken as 1.

^b The integration of acetyl groups was determined from the ratio of the NMR resonance of acetyl groups to that of carbohydrates.

^c The amount of acetyl groups in the wild-type xylan is taken as 100, and the relative amount of acetyl groups in the mutants is calculated from the ratio of acetyl groups in the mutants over that of the wild type.

DS_{AC}, degree of substitution of xylosyl residues by acetyl groups. The DS_{AC} values were calculated by dividing the molar values of acetyl groups by those of carbohydrates (represented by xylose) according to Telemen et al. [44].

Table 2 Relative content of acetyl groups in xylans from the wild type and various mutants

Structural fragment	Relative abundance (mol%)					
	wild type	<i>tbl34 tbl35</i>	<i>esk1</i>	<i>tbl34 esk1</i>	<i>tbl35 esk1</i>	<i>tbl34 tbl35 esk1</i>
Non-acetylated xylose (Xyl)	40	43	56	62	59	62
2- <i>O</i> -Acetylated xylose (Xyl-2Ac)	18	23	9	9	10	10
3- <i>O</i> -Acetylated xylose (Xyl-3Ac)	18	12	10	7	7	7
2,3-di- <i>O</i> -Acetylated xylose (Xyl-2,3Ac)	17	11	15	11	12	10
3- <i>O</i> -Acetylated, 2- <i>O</i> -GlcA-substituted xylose (Xyl-3Ac-2GlcA)	7	11	9	11	12	11
GlcA/MeGlcA	8	9	14	17	17	17

Non-acetylated and 2-*O*-monoacetylated xylosyl residues are integrated based on their H1 anomeric proton resonances, and 3-*O*-monoacetylated, 2,3-di-*O*-acetylated and 3-*O*-acetylated 2-*O*-GlcA/MeGlcA-substituted xylosyl residues are integrated based on their H3 resonances (Fig. 8). The relative abundance (mol%) of each structural fragment was calculated by first dividing the integration value by its molecular mass and then by the molar values of total structural fragments according to Teleman et al. [44]. The total balance of acetyl groups was verified by integration of acetyl signals at 2.2 ppm (Fig. 7).

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Figure 1

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