TRICHOME BIREFRINGENCE-LIKE27 Affects Aluminum Sensitivity by Modulating the O-Acetylation of Xyloglucan and Aluminum-Binding Capacity in Arabidopsis$^{1[W]}$[OPEN]

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Xyloglucan (XyG) has been reported to contribute to the aluminum (Al)-binding capacity of the cell wall in Arabidopsis (Arabidopsis thaliana). However, the influence of O-acetylation of XyG, accomplished by the putative O-acetyltransferase TRICHOME BIREFRINGENCE-LIKE27 (TBL27 [AXY4]), on its Al-binding capacity is not known. In this study, we found that the two corresponding TBL27 mutants, axy4-1 and axy4-3, were more Al sensitive than wild-type Columbia-0 plants. TBL27 was expressed in roots as well as in leaves, stems, flowers, and siliques. Upon Al treatment, even within 30 min, TBL27 transcript accumulation was strongly down-regulated. The mutants axy4-1 and axy4-3 accumulated significantly more Al in the root and wall, which could not be correlated with pectin content or pectin methylesterase activity, as no difference in the mutants was observed compared with the wild type when exposed to Al stress. The increased Al accumulation in the wall of the mutants was found to be in the hemicellulose fraction. While the total sugar content of the hemicellulose fraction did not change, the O-acetylation level of XyG was reduced by Al treatment. Taken together, we conclude that modulation of the O-acetylation level of XyG influences the Al sensitivity in Arabidopsis by affecting the Al-binding capacity in the hemicellulose.

Aluminum (Al) is the most abundant metal in the earth’s crust. When soil pH drops below 5, Al is solubilized into toxic Al species (Al$^{3+}$) and becomes the single most important factor limiting crop production on 67% of the total acid soil area (von Uexküll and Mutert, 1995; Eswaran et al., 1997). Al ions at the micromolar level inhibit root elongation through structural and functional damage, presumably due to Al ion interaction with the wall. However, the underlying basis of Al toxicity is still not well understood (Kochian, 1995; Ma, 2007; Poschenrieder et al., 2008; Zhu et al., 2012).

Many plant species have developed strategies to cope with Al toxicity (Taylor, 1991; Kochian et al., 2004). One strategy is based on the exclusion of Al from the root symplasm, whereas another strategy relies on the ability of the plant to tolerate symptomatic Al by sequestering Al as an Al–organic acid complex into vacuoles (Shen et al., 2002). The secretion of organic acids from the root apex and the chelation of Al ions in the soil has been demonstrated to be the most efficient Al exclusion mechanism in more recent studies (Kochian, 1995; Ryan et al., 2001; Ma and Furukawa, 2003). Accumulating evidence also implies a pivotal role of the wall in plant Al resistance or sensitivity (Horst et al., 2010), as almost 90% of cellular Al is associated with the walls of cultured tobacco (Nicotiana tabacum) cells (Chang et al., 1999) or as up to 99.9% of total cellular Al accumulates in the wall of the cells of the alga Chara corallina (Taylor et al., 2000). Pectin polysaccharides have been proposed as the major Al-binding sites, since their negatively charged carboxylic groups have a high affinity for Al$^{3+}$ (Blamey et al., 1990; Chang et al., 1999). However, recent evidence suggests that the metabolism of hemicelluloses leads to higher Al stress susceptibility. For example, hemicellulose content is increased by Al in wheat (Triticum

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The hemicellulose xyloglucan (XyG) seems to be the major primary wall hemicellulose in non-poeaean monocotyledons and dicotyledons, such as Arabidopsis. It has been widely established that XyG can be a leading binding site for Al and impact its binding capacity (Hayashi, 1989; Pauly et al., 1999). Structurally, the backbone of XyG consists of (1\( \rightarrow \)4)-linked \( \beta \)-D-glucopyranosyl residues, which can be substituted in a regular pattern with \( \alpha \)-D-xylopyranosyl residues at O-6. These xylosyl residues can be further substituted. A one-letter code nomenclature abbreviates unsubstituted glucosyl residues with a G, while Glc residues substituted with \( \alpha \)-D-xylopyranosyl residues at O-6.

The biological function of the O-acetyl substituents on XyG is not known, as eliminating this substituent in a XyG mutant does not lead to any further decrease in XyG content in the T-DNA insertion mutant of Xyloglucan Endotransglucosylase-Hydrolase31 (axy1) or a loss of XyG in the xylosyltransferase1/xylosyltransferase2 mutant reduces Al accumulation in the wall, specifically in the hemicellulose fraction, and the mutants xth31 tolerate Al better (Zhu et al., 2012). However, the structural features of XyG that lead to the binding of Al and impact its binding capacity are still unclear.

XyG is the major primary wall hemicellulose in non-poeaean monocotyledons and dicotyledons, such as Arabidopsis. It has been widely established that XyG can attach to cellulose microfibrils through hydrogen bonds and cross-link them, forming a distinct network (Fry, 1989; Hayashi, 1989; Pauly et al., 1999). Structurally, the backbone of XyG consists of (1\( \rightarrow \)4)-linked \( \beta \)-D-glucopyranosyl residues, which can be substituted in a regular pattern with \( \alpha \)-D-xylopyranosyl residues at O-6. These xylosyl residues can be further substituted. A one-letter code nomenclature abbreviates unsubstituted glucosyl residues with a G, while Glc residues substituted with \( \alpha \)-D-xylopyranosyl residues at O-6.

The functional biology of the O-acetyl substituents on XyG is not known, as eliminating this substituent in a XyG mutant does not lead to any obvious plant growth and morphology phenotype (Gille et al., 2011). However, it is known that acetylases can protect polysaccharides from enzymatic digestion (Biely et al., 1986; Schols and Voragen, 1994; Chen and Mart, 1996; Benen et al., 1999; Bonnin et al., 2003).

As XyG is the major Al-binding site in Arabidopsis (Zhu et al., 2012), we studied if and to what extent the degree of O-acetylation in XyG affects Al binding and changes the Al sensitivity of the plant. For this purpose, mutants of TRICHOME BIREFRINGENCE-LIKE27 (TBL27/AXY4), a Golgi-localized putative XyG O-acetyltransferase, were utilized (Gille et al., 2011): the ethyl methansulfonate-mutagenized mutant axy4-1, with an exchange of the Pro-126Ser amino acid; a knockout T-DNA insertion line, axy4-3 (Salk_044972); and an overexpression line, 35S:AXY4. While in all three mutants no difference in overall wall acetylation was observed, the degree of O-acetylation of XyG in root tissues was reduced by 33% in axy4-1, was not detectable in axy4-3, and was increased 22% in the 35S:AXY4 line (Gille et al., 2011).

**RESULTS**

**Al Sensitivity Is Increased in the tbl27 Mutants**

When grown on agar medium containing 50 \( \mu \)M Al\(^{3+} \) for 7 d, the root growth of Arabidopsis wild-type Columbia-0 was inhibited by 34%, but more so in axy4-1 (59%) and axy4-3 (47%; Fig. 1, A and B), indicating that a reduced or lack of O-acetylation of XyG results in more Al-sensitive roots. The Al content of the roots of the axy4 mutants was higher than in the wild type (Fig. 1C). A higher degree of XyG O-acetylation found in the overexpression line was indistinguishable from the wild type in root growth, Al sensitivity, and root Al content, indicating that beyond the wild-type-level threshold, an increase in XyG O-acetylation does not lead to any further observable effects related to Al stress as tested here.

Besides AX4/BL27, there are other proteins that contribute to XyG O-acetylation. For example, Reduced Wall acetylation (RWA) proteins may function as Golgi transporters to move activated acetyl groups into the Golgi for wall polymer O-acetylation purposes. 

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**Figure 1.** Phenotypes, root elongation, and root Al content of the TBL27 mutants and overexpression line. A, Phenotypes. WT, Wild type CK, Control Check without Al. B, Root elongation. Root elongation was measured before and after treatment. Values are means ± so (n = 10). CK, Control Check without Al. C, Root Al content. Values are means ± so (n = 4). FW, Fresh weight. Seedlings with approximately 1-cm roots were grown in agar medium containing 0 or 50 \( \mu \)M Al for 24 h. Different letters above the bars represent significant differences at \( P < 0.05 \).
Reducing RWA transcripts thus affects multiple wall polysaccharides, including pectins and xylans, but also XyG (Manabe et al., 2011). In the rwa2 mutant, XyG O-acetylation was reduced by 24%. When tested for Al sensitivity, root growth, and Al content, no difference was observed compared with wild-type plants in any of these factors (Supplemental Fig. S1). Crossing rwa2 to axy4-1 mutants exhibited the effects seen in axy4-1 mutants alone; no further additive or synergistic effects were observed (Supplemental Fig. S1).

**Tissue-Specific Localization of TBL27 Expression**

The expression pattern of TBL27 mRNA measured by quantitative real-time RT-PCR (RT-qPCR) showed that the TBL27 transcripts were accumulated in roots as well as leaves, stems, flowers, and siliques, with the highest levels in leaves and stems and relatively lower levels in flowers and siliques (Fig. 2).

The in vivo tissue-specific localization of TBL27 was further investigated by a promoter:GUS staining approach. A 2.3-kb DNA fragment upstream of the TBL27 coding region was used to drive the expression of GUS reporter gene, and the promoter:GUS construct was stably transformed into wild-type Arabidopsis plants (Zhu et al., 2012). GUS staining was observed in both roots and shoots except the hypocotyl (Supplemental Fig. 2). In roots, pTBL27::GUS was constitutively expressed in the elongation and differentiation zone of the root but not in the root tip (Supplemental Fig. 2B).

**Dose and Time Responses of TBL27 Expression to Al**

To examine whether Al stress affects the transcriptional level of TBL27, dose-response and time-course experiments were conducted. RT-qPCR analysis revealed that the expression level of TBL27 in the wild type was substantially inhibited even at an Al concentration as low as 5 μM (Fig. 3A) and repressed by 50 μM Al³⁺ treatment even within 30 min (Fig. 3B), suggesting that TBL27 expression is very sensitive to Al stress. Moreover, Al treatment weakened GUS expression (Supplemental Fig. 2, C and D), further confirming its sensitivity to Al.

**TBL27 Contributes to the Al-Reduced Acetylation Level**

A lack of XyG O-acetylation in the axy4 mutant does not result in a significant reduction in overall cell wall O-acetylation probably because of the presence of other O-acetyl-substituents of pectic polysaccharides and other hemicelluloses. However, Al stress significantly decreased the overall wall O-acetylation level in the wild type, but no effect was observed in the axy4 mutant (Fig. 4).

In order to determine if and to what extent the reduced overall O-acetylation level of the wall under Al treatment was attributed to a decrease in XyG O-acetylation, we measured the O-acetylation level of XyG in the wild type after Al treatment for 6 and 24 h. XyG oligosaccharides were enzymatically released from cell wall preparations, and their oligosaccharide profile was analyzed by matrix-assisted laser-desorption ionization (MALDI)-time of flight (TOF) mass spectrometric analysis (Lerouxel et al., 2002). There was a reduction in total XyG acetate content between the wild type and the 6-h Al incubation (Fig. 5; 19%, P = 0.0054). No significant decrease was detected after 24 h of Al incubation (Fig. 5A; Supplemental Table S1). However, XXLG/XLXG + OAc (mass-to-charge ratio [m/z] = 1289) was significantly decreased by 35% after 24 h of Al treatment, while the O-acetylation of oligosaccharides XXFG (m/z = 1393) and XLFG (m/z = 1553) was significantly decreased by 16.5% and 14.9% after 6 h of Al treatment (Fig. 5B). These data suggested that XyG acetylation is decreased upon Al treatment at certain time intervals on different XyG substructures.
Increased Cell Wall Al Content in *axy4* Mutant Roots

Since XyG is the major Al-binding site in Arabidopsis (Zhu et al., 2012) and *axy4* mutants exhibited reduced levels of XyG O-acetylation (Gille et al., 2011), we measured Al content in the root walls and found that more Al was accumulated in the *axy4* mutants (Figs. 1C and 6A). The Al adsorption kinetics of whole crude wall preparations further demonstrated that the root walls of the *axy4* mutants adsorbed significantly more Al than the wild type (Fig. 6B).

Besides XyG, pectic polysaccharides also can bind Al in Arabidopsis (Yang et al., 2008; Zhu et al., 2012). In particular, the degree of methylesterification affects the binding capacity of pectin to Al (Eticha et al., 2005; Yang et al., 2008). Therefore, we also measured uronic acid content, pectin methylesterase (PME) activity, and Al retention in pectin. As expected, there was no difference in the hot water-extracted pectin between the wild type and *axy4* mutants, as measured by the uronic acid content of the pectin extract (Fig. 7A). However, Al treatment induced a significant increment in pectin content in the wild type but not in the *axy4* mutants (Fig. 7A). Despite this increase in the pectin content in the wild type, there was no difference in Al-binding capacity between the wild type and the *axy4* mutants in the extractable pectin (Fig. 7C), suggesting that the amount of Al per uronic acid is actually reduced in the pectic fraction of the wild type. Concerning PME activity, there was no difference between the *axy4* mutants and the wild type, as expected. However, differences became apparent upon Al treatment (Fig. 7B). The weak *axy4-1* allele did not lead to a reduction in PME activity, while the strong *axy4-3* allele exhibited a reduction upon Al treatment. In the Al-treated wild type, a strong increment in PME activity was detected (Fig. 7B). Hemicelluloses were extracted from depectinized root walls combining a mild alkali extract (4%, w/v) and a strong alkali extract (24% w/v KOH). While no difference in hemicellulose content was observed between the wild type and *axy4* mutants under normal growth conditions, upon Al treatment there was an increase in hemicellulose content in wild-type roots but not in *axy4* roots (Fig. 8). These data indicate that Al treatment under the conditions used here leads to an increased content of pectic polysaccharides and hemicellulose in the wild type but not in the *axy4* mutants. Therefore, the higher Al content in the hemicellulose in the *axy4* mutants compared with wild-type plants also is not attributed to the change of hemicellulose content (Fig. 8B).

DISCUSSION

The degree of methylesterification of pectin has been demonstrated to affect Al-binding capacity and has been related to Al sensitivity in maize (*Zea mays*; Eticha et al., 2005) and rice (Yang et al., 2008; Yang et al., 2013). In addition to methylesters, many wall polymers also contain acetylesters (Gille and Pauly, 2012), including the hemicellulose XyG. Recently, we found that XyG in root walls bind more Al in Arabidopsis (Zhu et al., 2012). Therefore, we investigated if and to what extent the acetylation of XyG will affect its Al-binding capacity and, if yes, what the consequences are for Al sensitivity. For this purpose, two XyG O-acetylation mutants (*axy4-1* and *axy4-3*) were further investigated and found to be more sensitive to Al in the medium than wild-type plants (Fig. 1). Upon addition of Al to the medium, more Al bound to roots and this in both cases: in *axy4-1* roots with only a 33% reduction in XyG acetylation and in *axy4-3* with a 100% reduction (lack of) XyG acetylation to root walls (Fig. 1). To our knowledge, this is the first report that...
gives any insights into the function of XyG O-acetylation, a role in the sensitivity to metal ion stresses.

Al-resistant plants have evolved two strategies to survive in an Al-toxic environment. One strategy entails restricting Al uptake (exclusion of Al from the root symplasm), and another strategy is by internalizing Al (tolerate symplastic Al; Taylor, 1991; Kochian et al., 2004). The exclusion mechanism prevents Al from entering root cells through the secretion of chelating organic acids from the root apex or fixation of Al into the apoplast. Al binding in the wall polymers negatively impacts wall function, resulting in a reduction in root elongation. Horst (1995) and Ma et al. (2004) reported that Al, when bound to wall components, increases wall rigidity, decreases wall viscosity and elasticity, and affects wall loosening; as a consequence, root elongation is inhibited. Blamey et al. (1990) as well as Grauer and Horst (1992) demonstrated that binding of Al to Al-sensitive binding sites determines Al-induced inhibition of root elongation. Therefore, higher Al sensitivity is correlated with higher Al accumulation in the wall, as demonstrated in maize suspension culture cells (Schmohl and Horst, 2000), maize intact root apex (Eticha et al., 2005), rice (Yang et al., 2008), triticale (Liu et al., 2008), rice bean (Zhou et al., 2012), and Arabidopsis (Zhu et al., 2012). Similarly, in this study, the more Al accumulation in the wall of axy4 mutants, the more Al sensitive they were.

TBL27 (AXY4) belongs to the large plant-specific family of TBL proteins with 46 members (TRICHOME BIREFRINGENCE and TBL1–TBL45; Bischoff et al., 2010). TBL27 represents a putative XyG O-acetyltransferase and when knocked out leads to a reduction of acetylation level in XyG in Arabidopsis (Gille et al., 2011). TBL27 is expressed constitutively in Arabidopsis, although it was preferentially expressed in roots, leaves, and stems (Fig. 2; Supplemental Fig. S2). The expression of TBL27 was reduced even at an Al concentration as low as 5 μM and an exposure duration as short as 30 min (Fig. 3).

When wild-type plants are stressed with Al, a reduction in overall wall O-acetylation can be observed (Fig. 4). It is noteworthy that the decrease in XyG acetylation is not visible at the total acetate level at 24 h (Fig. 5A), indicating that pectins and xylans may contribute to the overall acetylation, which is in accordance with the fact that XyG represents a negligible pool of wall acetates (Gille et al., 2011). Therefore, these data suggest that acetyl-esterases become activated to remove acetyl esters from other wall polymers, including but not restricted to XyG. However, since the only difference between the wild-type plants and the axy4 mutants is the degree of O-acetylation of XyG, and a 24-hr Al exposure causes a significant reduction (35%) of XXLG/XLXG + Ac (Fig. 5B), the degree of O-acetylation of XyG residues could possibly be attributed to Al accumulation in the wall because there is no difference in Al retention in cell wall pectins (Fig. 7C). Moreover, a significant reduction in acetate content of XyG after 6 h of Al incubation, especially in the oligosaccharides XXFG and XLFG, was detected (16.5% and 14.9%, respectively;
Fig. 5B), indicating that the reduced O-acetylation level of XyG at certain time intervals on certain XyG structures could be attributed to the increment of Al accumulation in the hemicellulose. Indeed, in many plant species, Al-inhibited elongation can be observed typically within hours or even minutes. Thus, it is possible that the rapid down-regulation of TBL27 expression by Al (within 30 min; Fig. 3B) may be relevant to Al-induced inhibition of root elongation.

Pectic polysaccharides and hemicelluloses, in particular XyG, in the walls are the two major components to bind Al in the wall. Pectin is first heavily methylesterified in the endomembrane system (Delmer and Stone, 1988; Vannier et al., 1992) and secreted into the wall (Micheli, 2001). Pectin undergoes partial apoplastic demethylesterification processes through the action of PME, resulting in the exposure of free pectic carboxylic groups, which could serve as binding sites for Al in the wall. It has been reported that Al resistance of both rice and maize cultivars is negatively related to pectin content and PME activity (Schmohl and Horst, 2000; Eticha et al., 2005; Yang et al., 2008). Overexpression of OsPME14 led to increased PME activity and Al retention in pectin and resulted in more Al sensitivity of the transgenic rice lines (Yang et al., 2013). As shown here, pectin content and PME activity are increased in wild-type roots when subjected to Al but not in the axy4-1 and axy4-3 mutants, suggesting that the plant’s response to Al is more complex than previously thought. In order to clarify whether the mutation of TBL27 results in the change of the extractability of pectin from the cell wall, we measured Rha content in the whole cell wall and hot water extracts, as Rha is specifically represented in the pectin domains rhamnogalacturonan I and II (Yang et al., 2011a). We found that there was no difference in the total Rha content (Supplemental Fig. S3A), and about 80% of Rha was extracted by hot water, with no difference detected between the wild type and mutants (Supplemental Fig. S3B). Moreover, around 80% of uronic acid was presented in the pectin fraction, with no difference between the wild type and mutants (Supplemental Fig. S3, C and D). All these data indicate that XyG O-acetylation does not change the extractability of the pectin but may impact pectin metabolism upon Al treatment through hitherto unknown pathways.

XyG has been demonstrated to be the major Al-binding site in the wall in Arabidopsis (Zhu et al., 2012). Moreover, decreasing XyG endotransglucosylase action in xth31 mutants modifies the incorporation of nascent XyG into the elongation zone walls, thus decreasing XyG content; as a result, Al retention in the wall is reduced and renders the mutant more Al resistant (Zhu et al., 2012). Neither the glycosyl structure nor the content of XyG is changed in the axy4 mutants (Gille et al., 2011), but a higher Al content was detected in the mutants (Figs. 1C and 8), further indicating the important role of the acetyl substituents in Al binding. The Al-binding capacity seems not to be dependent on the degree of XyG O-acetylation reduction, based on the mutants analyzed. A XyG O-acetylation reduction of 24% as in the rwa2 mutants did not lead to any effect (Supplemental Fig. S1). In contrast, a reduction of 33% (axy4-1) resulted in an Al-binding capacity increase that cannot be increased when all acetates are removed (axy4-3; Fig. 1). Furthermore, overexpression of TBL27 does lead to an increase in O-acetylation by 20% but no
effect on the Al attributes. It thus seems that there is a tight threshold of XyG O-acetylation that leads to the observed Al effects. If the XyG O-acetylation is above 76%, Al sensitivity cannot be further reduced. If XyG O-acetylation falls below 67%, Al sensitivity becomes more apparent but is not increased further, even if the degree of O-acetylation drops even lower (down to 0%). Once the threshold of 67% XyG O-acetylation is reached, Al-binding capacity is exhausted. Recently, Tahara et al. (2014) reported that a tannin compound in the roots of *Eucalyptus camaldulensis*, oenothein B, can efficiently bind Al by forming complexes with the adjacent phenolic hydroxyl groups. Therefore, it is reasonable to assume that the hydroxyl groups in XyG may bind Al, and the amount of the nonacetylated hydroxyl groups and their spatial distribution might be important to decide the amount of Al that XyG can bind. When the acetylated groups exceed a certain rate, the Al-binding capacity of XyG will no longer exist.

Since the Al treatment was carried out on 6-week-old seedlings when the majority of the wall structure is already formed and in place, the amount of cell wall being made in the next 24 h is next to nothing. Therefore, a 6- or 24-h Al treatment will not lead to any significant analytical changes in the overall wall structure by a synthesis mechanism, suggesting that apoplastic postdeposition changes through the action of acetylerases similar to PME would be more likely. Although there may not be a causal relationship between *TBL27* transcript reduction and a decrease in XyG O-acetylation, we cannot deduce the reduction extent of the XyG O-acetylation from the reduction extent of the *TBL27* transcript, as both the *oxy4* mutants that lack the *TBL27* transcript exhibit large differences in their root XyG O-acetylation.

In conclusion, we propose the following model to link the modification of pectin and hemicellulose to Al sensitivity (Fig. 9). Previous reports showed that pectin content and its methylesterification status through PME activity affect the Al-binding capacity and, thus, relate to Al sensitivity, as demonstrated in maize (Eticha et al., 2005), rice (Yang et al., 2008; Yang et al., 2013), and buckwheat (*Fagopyrum esculentum*; Yang et al., 2011b). XyG is the major component of hemicellulose, and decreasing XyG content confers increased Al resistance (Zhu et al., 2012). Here, we further demonstrated that Al stress decreased the degree of O-acetylation (Fig. 4), especially the XyG (Fig. 5); thus, more Al was bound to the cell wall hemicellulose (Fig. 8). In other words, plants with a lower O-acetyltransferase activity (lower O-acetylation level) might result in more Al accumulation in the cell wall, thus becoming more Al sensitive. However, there may be a pathway interaction of *TBL27* transcript, the action of acetylerases, and the O-acetylation of XyG that would require further study.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

All wild-type, mutant, and transgenic *Arabidopsis* (*Arabidopsis thaliana*) plants used were in the Columbia-0 ecotype background. Seeds were surface sterilized and germinated on an agar-solidified nutrient medium in petri dishes. The nutrient medium consisted of the following macronutrients in mM: KNO₃, 6; Ca(NO₃)₂, 4; MgSO₄, 1; and NH₄H₂PO₄, 0.1; and the following micronutrients in μM: Fe(II)-EDTA, 50; H₃BO₃, 12.5; MnSO₄, 1; CuSO₄, 0.5; ZnSO₄, 1; H₂MoO₄, 0.1; and NiSO₄, 0.1. The final pH was adjusted to 4.5. The seeds were vernalized at 4°C for 2 d. Petri dishes were placed into a growth chamber, positioned vertically, and kept under controlled environmental conditions at 24°C, 140 μmol m⁻² s⁻¹, and a 16/8-h day/night cycle.

For hydroponic culture, seedlings were first aseptically germinated on the above solid nutrient medium. After 2 weeks, the young plantlets were placed on vermiculite for an additional 3 weeks in an environmentally controlled growth chamber. Seedlings of similar rosette diameters were then transferred to the nutrient solution containing the above-mentioned nutrients for another 1 week. Then the plants were subjected to the following treatments: CK (0.5 mM CaCl₂, pH 4.5) and Al (50 μM Al in 0.5 mM CaCl₂, pH 4.5). After 24 h, the roots were excised for RNA extraction, cell wall extraction, or Al content analysis. For the Al dose-response experiment, hydroponic cultured seedlings were exposed to 0.5 mM CaCl₂ medium (pH 4.5) containing 0, 5, 10, 25, 50, 100, or 150 μM AlCl₃ for 24 h. For the time-response experiment, seedlings were exposed to 0.5 mM CaCl₂ medium (pH 4.5) containing 50 μM AlCl₃ for 0, 0.5, 1, 3, 6, or 24 h. For longer duration treatments (7 d), the nutrient solution (pH 4.5) was used as a control, while for short time treatments (24 h), the 0.5 mM CaCl₂ medium (pH 4.5) was used as a control, and 50 μM Al was added directly for the Al treatment.

For the root elongation experiments, vertical agar plates were used, and all other experiments were performed with hydroponically cultured seedlings.

**Effect of Al on Root Growth**

Seedlings with root length of 1 cm were selected and transferred to petri dishes containing agar-solidified CaCl₂ (0.5 mM) medium with different Al concentrations (0 and 50 μM total concentration of Al in the form of AlCl₃·6H₂O). Root length measurements were performed using a digital camera connected to a computer. Data were quantified and analyzed by Photoshop 7.0 (Adobe Systems). For long-term experiments, seedlings with a root length of 1 cm were transferred to petri dishes containing agar-solidified nutrient solution medium with different Al concentrations (0 and 50 μM total concentration of Al).

**Gene Expression Analysis**

Hydroponically cultured seedlings were harvested and divided into root, stem, leaf, flower, and silique. Total RNA was isolated using TRIzol (Invitrogen). Complementary DNA was prepared from 1 μg of total RNA using the PrimeScript RT reagent kit (Takara). For real-time PCR analysis, 1 μL of 10-fold diluted complementary DNA was used for the quantitative analysis of gene expression performed with SYBR Premix Ex Taq (Takara) with the following pairs of gene-specific primers (for *TBL27*, forward 5′-TTGCCCTACATCATTTCG-3′ and reverse 5′-AGCCGGCCGTTATATCCCT-3′); and for *TUBULIN*, forward 5′-AAGTCTGGCGAAGTGGTT-3′ and reverse 5′-CTCCCAATGACTGA-CCAA-3′). Each complementary DNA sample was run in triplicate. Expression data were normalized with the expression level of the *TUBULIN* gene.

**Al Content Measurement**

Hydroponically grown seedlings were washed three times with deionized water, and the fresh weight was recorded. The Al content in each fraction was extracted by 2 N HCl for at least 24 h with occasional shaking. For total Al determination, roots were harvested and treated with HNO₃/HClO₄ (4:1, v/v). Al concentrations in the extracts were determined by inductively coupled plasmaatomic emission spectrometry (IRIS/AP optical emission spectrometer).

**Root Cell Wall Extraction and Fractionation**

Extraction of root crude wall materials and subsequent fractionation of wall components were carried out according to Zhu et al. (2012). First, roots were ground with a mortar and pestle in liquid nitrogen and then suspended in 75% (v/v) ethanol for 20 min in an ice-cold water bath. The sample was then centrifuged at 8,000 rpm for 10 min, and the supernatant was removed. The pellet was suspended and washed with acetone, followed by methanol/chloroform at a ratio of 1:1 (v/v), then methanol. Each treatment lasted 20 min,
and each supernatant was removed after centrifugation. The remaining pellet (i.e., the wall material) was freeze dried and stored at 4°C for further use, including PME activity or adsorption kinetics analysis.

Pectic polysaccharides were extracted by combining three hot water extracts at 100°C for 1 h each. Subsequently, hemicellulose fraction 1 was extracted by treating the remaining pellet twice with 4% (w/v) KOH containing 0.02% (w/v) KBH₄ at room temperature for 12 h. Hemicellulose fraction 2 was extracted with a solution containing 24% (w/v) KOH and 0.02% (w/v) KBH₄ in a similar manner. Both hemicellulose fractions were combined to result in the hemicellulose material.

**Adsorption Kinetics**

To determine the ability of different cell wall components to adsorb Al, a total of 5 mg of wall material was placed in a 2-mL column equipped with a filter at the bottom as described by Zhu et al. (2012). The adsorption solution consisted of 20 μL AlCl₃ in 0.5 mL CaCl₂ at pH 4.5. The solution was passed through the bed of wall materials by a peristaltic pump at 12 mL h⁻¹. The eluate was collected in 4-mL aliquots, which were assayed for Al spectrophotometrically using Pyrocatechol Violet according to Kerven et al. (1989) with some modification (Zheng et al., 2004).

**Uronic Acid and Total Polysaccharide Measurements**

The uronic acid content in the pectin extract was assayed according to Blumenkrantz and Asboe-Hansen (1973) using galactaractone (Sigma) as a standard. Briefly, 200 μL of pectin extract was incubated with 1 mL of 98% H₂SO₄ (containing 0.0125 mM Na₂B₄O₇·10H₂O) at 100°C for 5 min. After cooling, 20 μL of M-hydroxy-diphenyl (0.15%) was added to the solution, and after 20 min at room temperature, the A₅₃₀ was measured spectrophotometrically.

The total polysaccharide content in the hemicellulose fraction was determined utilizing the phenol sulfuric acid method (Dubois et al., 1956) and expressed as Glc equivalents. Briefly, 200 μL of hemicellulose extracts was incubated with 1 mL of 98% H₂SO₄ and 10 μL of 80% phenol at room temperature for 15 min, then incubated at 100°C for 15 min. After cooling, the A₅₃₀ was measured with a spectrophotometer.

**PME Activity Assay**

PME was extracted by suspending 5 mg of cell wall material in a 1 mL NaCl solution (pH 6) at 4°C for 1 h with repeated vortexing (20 s for 10 min each) according to Yang et al. (2013). Extracts were centrifuged (13,200 rpm for 10 min), and the supernatant containing the PME enzyme was collected. The PME activity was assayed by a reaction containing 100 μL of 200 mM phosphate-buffered saline containing 0.64 mg mL⁻¹ pectin (Sigma; 90% methylsterification), 10 μL of alcohol oxidase, and 50 μL of the PME extract. After incubation for 10 min at 30°C, 200 μL of 0.5 mM NaOH solution containing 5 mg mL⁻¹ purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole; Sigma) was added and incubated for 30 min at 30°C to form the colored formaldehyde-purpald complex. Water (550 μL) was added to a final volume of 1 mL, and the A₅₃₀ was recorded.

**Determination of Wall-Bound Acetate Content**

The wall-bound acetate content of cell wall material was determined using the Megazyme Acetic Acid Kit. The assay was scaled down and adapted to a 96-well format as described previously (Gille et al., 2011).

**Determination of the Degree of Acetylation of XyG**

The cell wall material (four biological replicates each) was digested overnight at 37°C with 0.2 units of xylglucan-specific endoglucanase in 50 mM ammonium formate (50 μL), pH 4.5 (Paul et al., 1999). The samples were centrifuged, and the supernatant, containing the soluble xylglucan oligosaccharides, was transferred to new microtubes. To desalt samples for MALDI-TOF mass spectrometric analysis, the supernatant (10 μL) was incubated at 55°C with 10 conditional BioReX MSZ 501 cation-exchange beads. After spotting 2 μL of matrix (2,5-dihydroxybenzoic acid, 10 mg mL⁻¹ in water) onto the MALDI target plate, the plate was dried under vacuum, and 2 μL of desalted sample was added to the dried matrix spot. After 5 min of incubation, the MALDI target plate was dried under vacuum. Mass spectrometry was performed on a MALDI-TOF device (Shimadzu) in the positive linear mode with an acceleration voltage of 20,000 V (Günl et al., 2011).

**Generation of TBL27-GUS Transgenic Lines**

To assess the tissue-specific expression of Arabidopsis TBL27, transgenic plants expressing the GUS-encoding GUS reporter gene under the control of the TBL27 promoter were generated. Using gene-specific PCR primers (forward, 5'-CCCCAACCCTGTTGCGACAATTAAACA-3'; reverse, 5'-GATCCTTCCAGGAACATAGACG-3'), a DNA product corresponding to an approximately 2.3-kb region found upstream from the translational start site of TBL27 was produced. The PCR fragment was verified by sequencing and ligated upstream of the GUS reporter gene in the pBI101.3 vector capable of propagation in Agrobacterium tumefaciens and transformed into Arabidopsis (Jefferson et al., 1987; Harrison et al., 2006).

**Statistical Analysis**

Each experiment had at least three biological repeats. Data were analyzed by a one-way ANOVA procedure, and the means were compared by Duncan's multiple range test. Different letters on the histograms indicate that the means were statistically different at P < 0.05.

**Supplemental Data**

The following materials are available in the online version of this article.

- **Supplemental Figure S1.** Phenotypes of the wild type and the rwa2-3, rwa2-3xay4-1 mutants in the absence or presence of Al.
- **Supplemental Figure S2.** GUS analysis of TBL27 expression.
- **Supplemental Figure S3.** Purity verification of different cell wall components.
- **Supplemental Table S1.** Relative abundance of XyG oligosaccharides.

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