Xyloglucan Endotransglucosylase-Hydrolase17 Interacts with Xyloglucan Endotransglucosylase-Hydrolase31 to Confer Xyloglucan Endotransglucosylase Action and Affect Aluminum Sensitivity in Arabidopsis1

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Previously, we reported that although the Arabidopsis (Arabidopsis thaliana) Xyloglucan Endotransglucosylase-Hydrolase31 (XTH31) has predominately xyloglucan endohydrolase activity in vitro, loss of XTH31 results in remarkably reduced in vivo xyloglucan endotransglucosylase (XET) activity and enhanced Al resistance. Here, we report that XTH17, predicted to have XET activity, binds XTH31 in yeast (Saccharomyces cerevisiae) two-hybrid and coimmunoprecipitations assays and that this interaction may be required for XTH17 XET activity in planta. XTH17 and XTH31 may be colocalized in plant cells because tagged XTH17 fusion proteins, like XTH31 fusion proteins, appear to target to the plasma membrane. XTH17 expression, like that of XTH31, was substantially reduced in the presence of aluminum (Al), even at concentrations as low as 10 μM for 24 h or 25 μM for just 30 min. Agrobacterium tumefaciens-mediated transfer DNA insertion mutant of XTH17, xth17, showed low XET activity and had moderately shorter roots than the wild type but was more Al resistant than the wild type. Similar to xth31, xth17 had low hemicellulose content and retained less Al in the cell wall. These data suggest a model whereby XTH17 and XTH31 may exist as a dimer at the plasma membrane to confer in vivo XET activity, which modulates cell wall Al-binding capacity and thereby affects Al sensitivity in Arabidopsis.

Soil acidity (pH < 5.5) affects about 40% of the world’s arable land (von Uexküll and Mutert, 1995) and more than 50% of land that is potentially arable, particularly in the tropics and subtropics (Kochian et al., 2004; Ethica et al., 2010). Al is the most growth-limiting factor for crop production on acid soils worldwide (Foy, 1988; Kochian, 1995), especially when the pH drops below 5 (Eswaran et al., 1997).

To survive in an Al-toxic environment, Al-resistant plants have evolved two mechanisms to cope with Al toxicity. One is to restrict Al uptake from the root, while the other is to cope with internalized Al (Taylor, 1991; Kochian et al., 2004). The latter is usually employed by Al-accumulating species such as Hydrangea macrophylla (Ma et al., 1997a) and buckwheat (Fagopyrum esculentum; Ma et al., 1997b). In most cases, secretion of the organic acid anions is the most important Al exclusion mechanism (Kochian, 1995; Ryan et al., 2001; Ma and Furukawa, 2003), although it does not explain all the Al resistance in some plants such as signalgrass (Brachiaria decumbens Stapf cv Basilisk; Wenzl et al., 2001), maize (Zea mays; Piñeros et al., 2005), buckwheat (Zheng et al., 2005), rice (Oryza sativa; Ma et al., 2005; Yang et al., 2008), or Fagopyrum tataricum (Yang et al., 2011a). Therefore, it is possible that for some plant species (such as buckwheat), Al tolerance is a combination of mechanisms including organic anion efflux.

Recently, evidence has accumulated that the cell wall, especially the hemicellulose component, may impact Al resistance. For example, Al induces significant changes in the hemicellulose fraction of wheat (Triticum aestivum; Tabuchi and Matsumoto, 2001), triticale (× Triticosecale Wittmack; Liu et al., 2008), rice (Yang et al., 2008), and Arabidopsis (Arabidopsis thaliana; Zhu et al., 2012), especially the Al-sensitive cultivars. Moreover, we found that Arabidopsis hemicellulose is not only very sensitive to Al stress (the content of hemicellulose increased quickly under Al stress), but is also the principal binding site for Al (Yang et al., 2011b). Furthermore, loss of Xyloglucan Endotransglucosylase-Hydrolase31 (XTH31) function resulted in lower xyloglucan content and cell wall Al-binding capacity in Arabidopsis (Zhu et al., 2012).

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Thus, xyloglucan may be a major Al-binding site in Arabidopsis, and any effects leading to xyloglucan modifications may regulate Al sensitivity.

XTHs are a family of enzymes that play principal roles in the construction and restructuring of the load-bearing cross links among cellulose microfibrils (Osato et al., 2006) through catalyzing the molecular grafting or hydrolyzing of the xyloglucans to form the framework (Fry et al., 1992; Nishitani and Tominaga, 1992; Okazawa et al., 1993; Rose et al., 2002). There are 33 identified XTH genes in the Arabidopsis genome, and one-third occur as clusters resulting from genome duplication (Blanc et al., 2000; Yokoyama and Nishitani, 2001); XTH1-11 are classified in subgroup 1, XTH12-26 are in subgroup 2, and XTH27-33 are in subgroup 3 (Rose et al., 2002). Each member of the XTH gene family is likely regulated by specific cues and committed to cell wall dynamics specific to certain tissues or cell types (Nishitani, 2002; Becnel et al., 2006; Osato et al., 2006). For example, XTH27 is involved in the cell wall modification of tracheary elements at a specific stage of rosette leaf development and is essential for tertiary vein development (Matsui et al., 2005), whereas XTH31 is involved in cell wall modification and cell elongation through modulating xyloglucan endotransglycosylase (XET) action under Al stress (Zhu et al., 2012). However, XTH31 is an XTH for which xyloglucan endohydrolase (XEH) activity has been predicted (Baumann et al., 2007), and in our previous report, we demonstrated that XTH31 produces heterologously in Pichia pastoris has high XEH activity but low XET activity in vitro (Zhu et al., 2012), which is in accordance with Kaewthai et al. (2013), who reported that XTH31 is a predominant hydrolase using the in vitro activity assays and enzyme product analysis, as well as the use of a fluorogenic substrate in vivo. Unexpectedly, however, the xth31 mutant has very low XET action and activity (Zhu et al., 2012). One possible explanation for this result is that XTH31 may interact with and be required for activity of XET-active XTHs.

In this study, we demonstrate that XTH17 can bind to XTH31 in vitro and in vivo and that a transfer DNA (T-DNA) insertion mutant of to XTH31 in vitro and in vivo and that a transfer DNA and be required for activity of XET-active XTHs. An explanation for this result is that XTH31 may interact with XET action and activity (Zhu et al., 2012). One possible interpretation that XTH17 and XTH31 may interact with xth31 Al resistance and exhibits a phenotype very similar to xth31 mutant has very low XET action and activity (Zhu et al., 2012). As 11 XTH genes are predominantly expressed in the roots (Yang et al., 2011b), we used the yeast (Saccharomyces cerevisiae) two-hybrid system to screen for XTHs that can bind XTH31 and found that only XTH17 showed strong interaction with XTH31 (Fig. 1A). XTH17 (At1g65310) is classified, together with XTH12 to XTH16 and XTH18 to XTH26 in subgroup 2, while XTH31 belongs to the XTH subgroup 3 (Rose et al., 2002). To confirm the interaction between XTH17 and XTH31 in plant cells, we performed coimmunoprecipitation assays. Transient expression of XTH31-GFP proteins in tobacco (Nicotiana benthamiana) was detected after coimmunoprecipitation experiments with 3XFlag-XTH17 using anti-Flag M2 affinity gel (Fig. 1B). Lack of 3XFlag-tagged XTH17 detected when assayed in the presence of control protein (35S-driven GFP) indicates that interaction requires XTH31 (Fig. 1B). These results are strong evidence that 3XFlag-XTH17 can bind with XTH31-GFP in plant cells.

The Cellular Localization of the XTH17 Protein

Similar to XTH31, XTH17 harbors a potential signal peptide at the N terminus. To elucidate the subcellular localization of XTH17, we produced three different constructs fused to GFP (XTH17 Full-GFP: the full XTH17 coding sequence fused in the 3' region with the GFP, XTH17 sp-GFP: the XTH17 signal peptide fused in the 3' region with the GFP, and XTH17 Wint-GFP: only the XTH17 signal peptide fused in the 3' region with the GFP; Fig. 2A) and used these to transform onion (Allium cepa) cells. The full-length XTH17 fusion protein (XTH17 Full-GFP) was found around the cell periphery but proximal to and distinct from the cell wall and colocalized with a plasma membrane marker, suggesting plasma membrane localization (Fig. 2, B, C, and D). A XTH17 Wint-GFP fusion protein that lacks the N-terminal 26 amino acids

RESULTS

XTH17 Can Bind XTH31 in Vitro and in Vivo

In our previous study, we found that xth31 mutant is compromised in in situ XET action and in extractable XET activity. Because XTH31 produced in Pichia spp. presents predominantly XEH activity, we proposed that XTH31 in the Arabidopsis root may undergo synergistic or direct protein-protein interactions with other XTHs and that loss of XTH31 function diminishes the action and activity of other XTH proteins with XET activity (Zhu et al., 2012). As 11 XTH genes are predominantly expressed in the roots (Yang et al., 2011b), we used the yeast (Saccharomyces cerevisiae) two-hybrid system to screen for XTHs that can bind XTH31 and found that only XTH17 showed strong interaction with XTH31 (Fig. 1A). XTH17 (At1g65310) is classified, together with XTH12 to XTH16 and XTH18 to XTH26 in subgroup 2, while XTH31 belongs to the XTH subgroup 3 (Rose et al., 2002). To confirm the interaction between XTH17 and XTH31 in plant cells, we performed coimmunoprecipitation assays. Transient expression of XTH31-GFP proteins in tobacco (Nicotiana benthamiana) was detected after coimmunoprecipitation experiments with 3XFlag-XTH17 using anti-Flag M2 affinity gel (Fig. 1B). Lack of 3XFlag-tagged XTH17 detected when assayed in the presence of control protein (35S-driven GFP) indicates that interaction requires XTH31 (Fig. 1B). These results are strong evidence that 3XFlag-XTH17 can bind with XTH31-GFP in plant cells.

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predicted to compose the signal peptide showed similar localization to that of GFP alone (Fig. 2, E and F). We also attempted to assay the putative signal peptide function by fusing only the first 26 amino acids of XTH17 to GFP (XTH17 sp-GFP; Fig. 2A), but we were unable to determine the subcellular localization, as we could not detect the XTH17 sp-GFP protein, which may be due to the reason that these amino-terminal amino acids are not sufficient to confer localization of GFP.

XTH17 Contributes to XET Action

In our previous study, we demonstrated that in situ XET action was reduced remarkably in xth31 (Zhu et al., 2012). However, as Pichia spp.-derived XTH31 has strong XEH activity and poor XET activity, the reduction of XET action in the xth31 mutant remained unexplained. Using endogenous xyloglucan as the donor substrate, we found that XET action was also sharply decreased in the xth17 mutant under control growth conditions (Fig. 3, A and C). Furthermore, in contrast to ecotype Columbia (Col-0; wild type; Fig. 3, A and B; Yang et al., 2011b), xth17 failed to show a dramatic reduction in XET action upon Al stress (Fig. 3, C and D), indicating that XTH17 is required for a major proportion of the assayable XET action in the root tip.

The Dose and Time Response of XTH17 Expression to Al

In our previous report, time course and dose response experiments indicated that XTH31 transcript accumulation is suppressed by 50 μM Al within 30 min of Al treatment, and a significant change in XTH31 RNA abundance can be detected even at an Al concentration as low as 5 μM (Yang et al., 2011b; Zhu et al., 2012). To examine whether XTH17 transcript accumulation is also sensitive to Al stress, we conducted both the time course and dose response experiments and found that XTH17 transcript levels was significantly reduced at Al concentrations higher than 10 μM for 24 h and the largest inhibition of RNA accumulation occurred at 25 μM (Fig. 4A). We then used this Al concentration to conduct a time course analysis and found that a significant down-regulation of XTH17 expression could be detected even within 30 min (Fig. 4B). These data indicate that XTH17 expression, like that of XTH31, is sensitive to Al stress.

An XTH17 T-DNA Insertional Mutant Has Increased Al Resistance

In our previous study, we found that XTH31 is required to confer Al sensitivity in Arabidopsis by controlling the xyloglucan incorporated into the cell wall and Al-binding capacity of the cell wall (Zhu et al., 2012). To determine whether XTH17 function is also related to Al stress responses, we characterized a T-DNA insertional line of XTH17 (CS16535; Fig. 5A). The XTH17 transcripts were down-regulated in the homozygous line (Fig. 5B), indicating that the T-DNA insertion leads to the knockdown of the XTH17 function. The roots of xth17 were shorter than the wild type under normal growth conditions (Fig. 5C), suggesting that XTH17 function is
required for normal elongation; a similar phenotype was found for \textit{xth31} (Zhu et al., 2012). Moreover, although the root elongation of the wild type was decreased from an average of 0.22 cm when grown on agar medium without Al$^{3+}$ to an average of 0.16 cm when grown with 50 mM Al$^{3+}$ for 24 h, the same Al treatment had no significant effect on \textit{xth17} seedling root growth (Fig. 5, C and D). Strikingly, Al-exposed \textit{xth17} roots accumulated significantly less Al than that in wild-type roots grown under similar conditions (Fig. 5E). These data strongly suggest that \textit{XTH17} impacts Al sensitivity by affecting Al accumulation in the roots.

**DISCUSSION**

In our previous study, we found that the \textit{xth31} heterologously produced in \textit{Pichia} spp. showed high XEH but low XET activity in vitro, which agrees with sequence-based predictions that \textit{XTH31} and \textit{XTH32} are the only two \textit{XTH} members with XEH activity (Baumann et al., 2007). However, the \textit{xth31} mutant root has much reduced in situ XET action and extractable XET activity compared with the wild-type Col-0. Thus, we proposed that \textit{XTH31} in the root may undergo synergistic or direct

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**Lower Hemicellulose Content in \textit{xth17} Roots**

As hemicellulose is a major Al-binding site in Arabidopsis cell wall and \textit{XTH} genes are involved in the modulation of hemicellulose (Fry et al., 1992; Zhu et al., 2012), we measured hemicellulose content in \textit{xth17} and the wild type and found that the total sugar and Al content in hemicellulose were both significantly lower in \textit{xth17} than in the wild type (Fig. 6, A and B). Furthermore, less Al was found in the cell wall of \textit{xth17} than in the wild type (Fig. 7A). Finally, the in vitro Al adsorption kinetics also showed that the extracted root cell walls of \textit{xth17} adsorbed significantly less Al than that of the wild type (Fig. 7B). These results indicate that the loss of \textit{XTH17} affects Al response association with cell wall components.
protein-protein interactions with other XTHs to confer XET activity (Zhu et al., 2012). Here, by yeast two-hybrid and transient expression assays with Agrobacterium tumefaciens infiltration of tobacco leaves, we found that among the XTH genes expressed in roots, XTH17 could directly interact with XTH31 in vitro and in vivo (Fig. 1B). Moreover, using a XTH17-GFP fusion protein, we confirmed that XTH17 is localized at the plasma membrane (Fig. 2), which is in accordance with XTH31 (Zhu et al., 2012). Because xyloglucan is synthesized in the Golgi and undergoes transglycosylation immediately after release into the wall (Thompson and Fry, 2001), this membrane-localized XTH17 is well positioned for catalyzing this process. As XTH31 is also localized at the plasma membrane (Zhu et al., 2012), it is reasonable to hypothesize that XTH17 and XTH31 may form a dimer at the plasma membrane surface, where they could potentially function together to modify the newly secreted xyloglucans.

Xyloglucan is an important hemicellulose component of dicotyledonous plant cell walls, accounting for up to 20% of cell wall content (Fry, 1989; Hayashi, 1989). Thus, it is not surprising that plant genomes contain a large number of XET/XEHs to modify xyloglucans, with up to 33 genes in Arabidopsis (Campbell and Braam, 1999; Yokoyama and Nishitani, 2001), 29 in rice (Yokoyama et al., 2004), 25 in tomato (Solanum lycopersicum; Saladié et al., 2006), 41 in Populus spp. (Geisler-Lee et al., 2006), 22 in barley (Hordeum vulgare; Strohmeier et al., 2004), and 57 in wheat (Liu et al., 2007). All XTH enzymes studied to date display XET, XEH (Tabuchi et al., 2001; Rose et al., 2002), or both activities (de Silva et al., 1993; Fanutti et al., 1993), and their activity can loosen plant cell walls (Van Sandt et al., 2007). Recently, expression profiles of several XTH genes have provided evidence that distinct XTHs may have particular physiological roles in cell wall dynamics (Vissenberg et al., 2005; Becnel et al., 2006). Some XTH genes are prominently expressed in rapidly expanding tissues such as the elongation zone of roots, sites of future root hair initiation, which can be induced by Al in maize (Doncheva et al., 2005), growing root hairs (Vissenberg et al., 2000, 2001, 2003).
Moreover, it was previously reported that XTH17 wall undergoes thickening (Matsui et al., 2005). Furthermore, the development of tracheary elements, when the secondary wall undergoes thickening (Matsui et al., 2005). Further, it was previously reported that XTH17 is expressed in all tissue types in the elongation and differentiation zone (Vissenberg et al., 2005), while XTH31 is predominantly expressed in the root tips, including the elongation zone (Zhu et al., 2012). The protein encoded by XTH17 belongs to the class II subfamily in the XTH protein family; members of this subfamily have been shown to exhibit exclusively XET activity (Maris et al., 2011). Thus, it is not unexpected that the xth17 mutant exhibited lower XET action (Fig. 3). However, as the xth31 mutant also harbors very low XET action/activity (Zhu et al., 2012), based on the data presented here, we now propose that XTH17 can confer its XET function only when both XTH17 and XTH31 coexist.

XET action/activity has been proposed to play important roles in the process of cell expansion (Smith and Fry, 1991; Fry et al., 1992; Van Sandt et al., 2007). For instance, extractable XET activity correlates well with growth rate (Fry et al., 1992; Hetherington and Fry, 1993; Potter and Fry, 1993, 1994; Pritchard et al., 1993; Zhu et al., 2012). Here, we found that the roots of xth17 grow shorter than the wild type (Fig. 4C), which is in accordance with the remarkable reduction of its XET action (Fig. 3), while the root growth of xth17 was less inhibited under Al stress (Fig. 4, C and D), being consistent with its almost unchanged XET action under Al (Fig. 3). As the in vivo XET action of XTH17 at or near the plasma membrane may modify the newly secreted xyloglucan to enhance further xyloglucan incorporation, the lower XET action may lead to the reduced cell wall polysaccharides (Fig. 6), which also result in the lower Al-binding capacity (Figs. 5 and 6B).

XTH genes have been shown to be responsive to diverse stimuli. As reported by Xu et al. (1995, 1996), expression of XTH genes is regulated by abiotic factors, such as touch, darkness, cold shock, heat shock, and others (Wu et al., 1994). Zurek and Clouse (1994) suggested that several hormones, e.g. abscisic acid, brassinosteroids, and GAs, can also regulate the XTH expression. Recently, Divol et al. (2007) and Maldonado-Mendoza et al. (2005) demonstrated that expression of XTH genes can be regulated by parasites or induced during arbuscular mycorrhizal symbiosis, respectively. In this study, we found that similar to XTH31, the expression of XTH17 was also very responsive to Al (Fig. 7).

Modification of the root cell wall’s binding properties has been taken as an important Al-resistant/-sensitive mechanism. Pectin content and degree of methylation have been implicated in cell wall Al-binding capacity due to pectin carboxylate groups (Horst et al., 2010). For example, Yang et al. (2008) found that in sensitive rice cultivar root tips, both the higher pectin content and the higher degree of demethylesterification in pectin result in the greater Al-binding capacity (Yang et al., 2008). However, we have recently found that hemicellulose binds much more Al than pectin, and XET action, along with the expression of XTH31, is significantly inhibited by Al stress; thus, we proposed that XTH function is a factor that predisposes plants to Al toxicity (Yang et al., 2011b; Zhu et al., 2012). In this study, we further investigated the function of XTH17 in its relationship with Al resistance and propose that XTH17 works with XTH31 through the formation of an XTH17-XTH31 dimer and that dimer formation is required for XTH17 XET action (Fig. 3) and, therefore, loss of XTH17 function results in lower Al accumulation in the cell wall (Fig. 5). As a consequence, xth17 is more Al resistant (Fig. 4). Altogether, these data indicate that XTH17 plays an important role in conferring Al sensitivity in Arabidopsis.

In conclusion, our results indicate that XTH17, together with XTH31, can form a complex required for XET action, thus modulating the cell wall Al-binding capacity in Arabidopsis.
MATERIALS AND METHODS

Plant Material and Growth Conditions

Both wild-type and xth17 (Arabidopsis thaliana) plants used were in the Col-0 wild-type background. Seeds were surface sterilized and germinated on an agar-solidified nutrient medium in petri dishes. The nutrient medium was based on Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) containing the macronutrients KNO₃, 6.0 mM; Ca(NO₃)₂, 4.0 mM; MgSO₄·7 H₂O, 1 mM; and NH₄H₂PO₄, 0.1 mM; and the micronutrients Fe(III)-EDTA, 50 μM; H₃BO₃, 1 μM; MnSO₄·H₂O, 0.5 μM; ZnSO₄·7 H₂O, 1 μM; H₂MoO₄, 0.1 μM; and NiSO₄·7 H₂O, 0.1 μM. The final pH was adjusted to 4.5. The seeds were vernalized at 4°C for 1 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-h/8-h day/night rhythm.

For hydroporic culture, seedlings were first aseptically germinated on the above solid nutrient medium. After 2 weeks, the young plantlets were placed on vermiculite for 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: control check (0.5 mM CaCl₂, pH 4.5) and Al (50 μM Al in the 0.5 mM CaCl₂, pH 4.5). After 24 h, the roots were excised for RNA extraction or for Al content analysis. For Al content analysis, the roots were washed three times with deionized water, and the fresh weight was recorded. For the dose response experiment, 6-week-old 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, 6-week-old seedlings were exposed to 0.5 mM CaCl₂ medium (pH 4.5) containing 50 μM AlCl₃ for 0.5, 1.5, 6, 12, and 24 h.

Effect of Al on Root Growth

Seedlings with a root length about 1 cm were selected and transferred to petri dishes containing agar-solidified CaCl₂ (0.5 mM) medium with or without 50 μM Al in the form of AlCl₃ for 24 h. Root morphology was recorded using a digital camera connected to a computer. Data were quantified and analyzed by Photoshop 7.0 (Adobe Systems). For the long-term treatment, seedlings about 1 cm long were selected and transferred to petri dishes containing agar-solidified MS medium with or without 50 μM Al in the form of AlCl₃. Plants were grown vertically for an additional 7 d, at which point photographs were taken.

Cytochemical Assay

The XET action was determined according to Visserenbergen et al. (2000). In brief, roots were incubated in a 6.5 μM sulphorhodamine-labelled oligosaccharides of xyloglucan (XG-5SR) mixture according to Zhu et al. (2012). The assay was followed by a 10-min wash in ethanol/formic acid/water (15:1:4, v/v/v) to remove any remaining unreacted XG-5SR. A further incubation overnight in 5% (v/v) formic acid removed apoplastic, non-wall-bound xyloglucan-

Gene Expression Analysis

Total RNA was isolated from root using TRIzol (Invitrogen). Complementary DNA was prepared from 1 μg of total RNA using the PrimeScript RT reagent kit (Takara). For real-time reverse transcription (RT)-PCR analysis, 1 μl of 10-fold diluted complementary DNA was used for the quantitative analysis of gene expression performed with SYBR Premix ExTaq (Takara) with the following pairs of gene-specific primers (for tubulin: forward, 5′-AAAGTTCTGGGAAAGTCTTGC-3′; reverse, 5′-CTCCCAAATGAGTACAACTTTA-3′ and for XTH17: forward, 5′-AGTTCAAGAAAGCCCGAGCGGCT-3′; reverse, 5′-TGCCAAAATGGCTTCGTCCTC-3′). Each complementary DNA sample was run in triplicate. Expression data were normalized with the expression level of the tubulin gene.

Cell Wall Extraction and Fractionation

Extraction of crude cell wall materials and subsequent fractionation of cell wall components were carried out according to Zhong and Lauchli (1993) with minor modifications. Roots were ground with a mortar and pestle in liquid nitrogen and then homogenized with 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 pellets were homogenized and washed with acetone, methanol:chloroform at a ratio of 1:1, and methanol for 20 min each, with each supernatant removed after centrifugation before the washes. The remaining pellet, i.e. the cell wall material, was dried for further use.

Pectin was triple extracted by hot water for 1 h each and pooling the supernatants (pectin). The pellet was subjected to twice extraction with 24% (v/v) KOH for a total time of 24 h and pooling the supernatants (hemicelluloses).

Determination of Total Polysaccharide

The total polysaccharide contents in the hemicellulosic fractions were determined by the phenol-sulfuric acid method (Dubois et al., 1956) and expressed as Glc equivalents according to Zhu et al. (2012). Briefly, 200 μL of hemicellulosic extracts was incubated with 1 mL of 98% (v/v) H₂SO₄ and 10 μL of 80% (v/v) phenol at room temperature for 15 min and then incubated at 100°C for 15 min. After cooling, the A₅₄₀ was measured spectrophotometrically.

Al Content Measurement

Al content in each cell wall pellet was extracted by 2 x HCl for 24 h with occasional shaking. Al concentrations in the hemicellulosic fraction were determined by inductively coupled plasma-atomic emission spectrometry (IRIS/ AP optical emission spectrometer).

Adsorption Kinetics

To determine the ability of xth17 and Col-0 to adsorb Al, a total of 5 mg of cell wall materials was placed in a 2-mL column equipped with a filter at the bottom. The adsorption solution consisted of 20 μM AlCl₃ in 0.5 mM CaCl₂ at pH 4.5. The solution was passed through the bed of cell walls by a peristaltic pump at 12 mL h⁻¹. The eluate was collected in 5-mL aliquots, which were assayed for Al spectrophotometrically with pyrocatechol violet according to Kerven et al. (1989) with some modification (Zhu et al., 2012). The kinetics study was carried out twice independently, and one set of adsorption curves is presented in “Results.”

Yeast Two-Hybrid Analysis

Yeast (Saccharomyces cerevisiae) two-hybrid analysis was performed using MatchMaker GAL4 Two-Hybrid System 3 (Clontech, http://www.clontech.com/) according to the manufacturer’s instructions. A yeast strain (AH109) was transformed with pairs of pGBK7 vectors (Clontech) harboring XTH31 and pGADT7 vectors (Clontech) harboring XTH17. The transformants were tested on the screening medium.

Agrobacterium tumefaciens-Mediated Infiltration of Tobacco Leaves

The A. tumefaciens-mediated transient expression in tobacco (Nicotiana benthamiana) leaves was conducted as described by Liu et al. (2012). A preculture of the A. tumefaciens EHA105 strain harboring the constructs of 3XFlag-XTH17 and XTH31-GFP was prepared in Luria-Bertani medium with the proper antibiotics and incubated overnight with shaking at 28°C. A 1-mL aliquot of preculture was used to inoculate 50 mL of Luria-Bertani medium with the appropriate antibiotics, 10 mM MES, 20 μM acetylsyringone, and the bacteria were allowed to grow overnight. After centrifugation at 5,000 rpm for 10 min at 4°C, the cell pellet was resuspended in the infiltration medium (10 mM MgCl₂, 10 mM MES, and 100 μM acetylsyringone) to an optical density at 600 nm OD₆₀₀ of 1.0. The cell suspension was then left standing at room temperature for 2 to 3 h before infiltration of tobacco leaves. A mix of cells containing the 3XFlag-XTH17 and XTH31-GFP was then prepared to infiltrate the second or third true leaves of 5-week-old tobacco plants. Infiltrated tobacco was grown for another 3 d before sample collection.

Protein Extraction and Protein Gel-Blot Analysis

To prepare total protein extracts, leaves of 5-week-old tobacco were ground into fine powder in liquid nitrogen and thawed in cold lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.05%-0.1% [v/v] Tween 20, and a protease inhibitor mixture [Roche Applied Science, Plant Physiol. Vol. 165, 2014]
Transformation, and Plasmolysis


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Dark. Samples were mounted on glass slides and inspected under a laser-scanning control using a biolistic PSD-1000/He particle delivery system (Bio-Rad). After using polyclonal anti-GFP antibody, washing. After being centrifuged at 3,000 rpm, pellets were washed twice with 1 mL of lysis buffer containing 250 mM NaCl. Finally, the pellets were resuspended in SDS protein loading buffer. The XTH31-GFP protein was detected using polyonal anti-GFP antibody.

35S: XTH17–GFP Expression Constructs, Transient Onion Transformation, and Plasmolysis

The full XTH17 coding sequence (XTH17 Full), the same without the signal peptide (XTH17 Int), and only the XTH17 signal peptide (XTH17 sp) were cloned in pBl221 vector under the control of a CaMV 35S promoter and fused in the 3’ region with the GFP according to Zhu et al. (2012). Onion cells were bombarded at 900 psi with 5 g of DNA plasmids for expression of the fusion with or without plasma membrane marker pm-rk CD-1007, or GFP alone as a control using a bioistolic PDS-1000/He particle delivery system (Bio-Rad). After particle bombardment, the samples were incubated for 24–48 h at 25°C in the dark. Samples were mounted on glass slides and inspected under a laser-scanning confocal microscope (LSM 510; Zeiss). When indicated, cells were plasmolysed in saturated Suc for 15 min.

Statistical Analysis

Each experiment was repeated independently at least two times, and one set of representative data are shown in the results. Data were analyzed by one-way ANOVA, and the means were compared by Student’s t test. Different letters and asterisks on the histograms indicate statistical differences at the P < 0.05 level.

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