

Arabidopsis and *Brachypodium distachyon* Transgenic Plants Expressing *Aspergillus nidulans* Acetyltransferases Have Decreased Degree of Polysaccharide Acetylation and Increased Resistance to Pathogens^{1[C][W][OA]}

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The plant cell wall has many significant structural and physiological roles, but the contributions of the various components to these roles remain unclear. Modification of cell wall properties can affect key agronomic traits such as disease resistance and plant growth. The plant cell wall is composed of diverse polysaccharides often decorated with methyl, acetyl, and feruloyl groups linked to the sugar subunits. In this study, we examined the effect of perturbing cell wall acetylation by making transgenic *Arabidopsis* (*Arabidopsis thaliana*) and *Brachypodium* (*Brachypodium distachyon*) plants expressing hemicellulose- and pectin-specific fungal acetyltransferases. All transgenic plants carried highly expressed active *Aspergillus nidulans* acetyltransferases localized to the apoplast and had significant reduction of cell wall acetylation compared with wild-type plants. Partial deacetylation of polysaccharides caused compensatory up-regulation of three known acetyltransferases and increased polysaccharide accessibility to glycosyl hydrolases. Transgenic plants showed increased resistance to the fungal pathogens *Botrytis cinerea* and *Bipolaris sorokiniana* but not to the bacterial pathogens *Pseudomonas syringae* and *Xanthomonas oryzae*. These results demonstrate a role, in both monocot and dicot plants, of hemicellulose and pectin acetylation in plant defense against fungal pathogens.

The cell wall is one of the most important compartments of the plant cell. This external cell skeleton plays an important role in cell and tissue shape determination. Besides its structural role, this extracellular complex is involved in the control of important functions such as cell-cell interactions, whole-plant growth, development, and interaction with the environment. The plant cell wall is mainly composed of highly dynamic heteropolysaccharides assembled in macromolecular

networks (Albersheim et al., 2010). Cellulose is the major component of most plant cell walls, and it consists of unsubstituted (1,4)- β -D-glucan chains, which form microfibrils. Hemicelluloses include xyloglucan, arabinoglucuronoxylans, different mannans, and β -(1-3,1-4)-glucan (Scheller and Ulvskov, 2010). The pectic polysaccharides are a class of α -1,4-linked galacturonic acid (GalA)-containing polysaccharides and include homogalacturonan, xylogalacturonan, apiogalacturonan, rhamnogalacturonan II, and rhamnogalacturonan I (Caffall and Mohnen, 2009). Different plants have somewhat different cell wall compositions; dicotyledonous and non-Poales monocotyledonous plants (type I cell walls) have cellulose, pectin, and hemicelluloses, whereas grasses have cellulose with relatively small amounts of pectin and commensurately higher levels of hemicelluloses (type II cell walls), and their endosperm often contains high levels of mannans, galactomannans, galactoglucomannans, and β -(1-3,1-4)-glucans (Burton et al., 2010).

Besides the diversity of monosaccharide composition, cell wall polysaccharides are also modified with methyl, acetyl, and feruloyl groups, which are mainly O-linked to sugars. These functional groups are believed to protect polysaccharides from the action of specific glycosyl hydrolases and to cross link cell wall constituents controlling cell extensibility (Perrone et al., 2002; Gou et al., 2012). For example, methylesterification/

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deesterification acts in the control of cell growth, fruit ripening, and plant protection against biotic stresses (Koch and Nevins, 1989; Wolf et al., 2009; Lionetti et al., 2012). Demethylesterification of homogalacturonan allows the aggregation of polyuronides into calcium-linked structures, resulting in less porous, stiffer cell walls (Willats et al., 2001).

The complexity of cell wall polysaccharides and the formation of polysaccharide networks determine the strength, flexibility, and functionality of the primary cell wall. Recently, plant cell wall polysaccharides have attracted significant attention because they are a major source of reduced carbon for bioethanol production, which has the potential to supply part of our fossil fuel demand (Jordan et al., 2012). Direct modification of cell wall polysaccharides can increase the efficiency of biomass digestibility and improve saccharification during ethanol production (Lionetti et al., 2010; Pogorelko et al., 2011; Fursova et al., 2012; Jung et al., 2012; Song et al., 2012). However, modification of cell wall polysaccharides may also affect important cell wall functions, and the plant cell must maintain the functional integrity of the wall during morphogenesis and exposure to environmental cues (Hamman, 2012). Indeed, the existence of a dedicated cell wall integrity maintenance mechanism has been proposed (Nühse, 2012; Wolf et al., 2012a).

The cell wall acts as a mechanical barrier and also participates in signal transduction (Somerville et al., 2004; Underwood, 2012; Wolf et al., 2012a) that allows plants to sense and respond to changes in environmental conditions. Therefore, plants are very sensitive and responsive to changes in their cell walls. The mechanism of signal transduction through the cell wall during pathogen invasion is not well understood, but some evidence indicates that the signal originates from cytoplasmic membrane deformation caused by changes in wall structure and internal cell turgor pressure mediated by mechanosensitive ion channels (Haswell et al., 2008). Considering this, specific cell wall modifications could induce plant defense reactions even prior to pathogen attack, and these defenses may be able to reduce pathogen penetration and growth (Underwood, 2012). Indeed, increasing evidence demonstrates that changes in cell wall composition can induce reactions similar to the stress response in plants (Hamann, 2012; Nühse, 2012). For example, suppression of cellulose synthesis causes the activation of stress-related gene expression (Ellis et al., 2002; Hamann et al., 2009). The expression of fungal polygalacturonase (PG) and consequent reduction of homogalacturonan content in the cell wall increases resistance to pathogens in both tobacco (*Nicotiana tabacum*) and Arabidopsis (*Arabidopsis thaliana*; Ferrari et al., 2007).

Genetic and molecular evidence indicates the critical role of pectin methylesterification in plant defense; for example, in Arabidopsis and wheat (*Triticum aestivum*), high methylesterification of pectins decreased plant susceptibility to fungal and bacterial necrotrophs (Raiola et al., 2011; Volpi et al., 2011), and in wild

strawberry plants (*Fragaria* spp.), overexpression of a fruit-specific pectin methylesterase (PME) increased plant resistance to *Botrytis cinerea* (Osorio et al., 2008). Demethylesterification makes homogalacturonans more susceptible to degradation by fungal pectic enzymes like PG and pectate lyase (Limberg et al., 2000). Also, deesterification can be induced by pathogen action and is required for colonization by fungal and bacterial cells (Raiola et al., 2011). Similarly, the establishment of ferulic acid cross linking results in cell wall stiffening and growth deceleration (Buanafina, 2009), and negative correlations between the level of cell wall feruloyl esterification and pathogen invasion were observed for some plant species (Bily et al., 2003).

In contrast to other modifications, the biological function of *O*-acetyl substituents is not known, although there is some evidence that the degree of acetylation is modified during plant growth and development (Pauly et al., 2001; Obel et al., 2009). For example, it was proposed that the acetylation of cell wall polysaccharides impacts their noncovalent interactions, affecting their accessibility to invading pathogens (Gille and Pauly, 2012). Also, adding a small acetyl group instead of a bigger sugar residue seems to be an energetically favorable strategy that is broadly used in grass xyloglucan, where one xylosyl residue is regularly replaced with an acetyl group (Gibeaut et al., 2005; Gille and Pauly, 2012). The Arabidopsis *reduced wall acetylation2* (*rwa2*) mutant, which exhibits a 20% reduction in cell wall acetylation, has increased resistance to the fungal pathogen *B. cinerea* (Manabe et al., 2011), although the authors did not consider a direct relationship between a decreased degree of cell wall acetylation and increased resistance. Mutations in other pectin-related proteins, powdery mildew-resistant PMR5 and PMR6, caused pectin alterations and increased resistance to powdery mildew (Vogel et al., 2002, 2004).

To investigate the effect of the deacetylation of different polysaccharides on type I and type II cell wall integrity and, consequently, on plant responses to biotic stresses, we generated transgenic Arabidopsis and Brachypodium (*Brachypodium distachyon*) plants expressing fungal acetyl esterases, which are proposed to act specifically on hemicellulose or pectin polysaccharides. The results of this study provide new insights into the relationship between cell wall acetylation, polysaccharide digestibility, and plant resistance to pathogens and demonstrate that postsynthetic polysaccharide modification is a powerful approach to investigate the contribution of the cell wall to plant fitness.

RESULTS

Generation and Characterization of Arabidopsis and Brachypodium Transgenic Plants Expressing Fungal Acetyl Esterases

To alter the acetylation of cell wall polysaccharides, we used two different enzymes from *Aspergillus nidulans*, xylan acetyl esterase (AnAXE; AN6093.2)

and rhamnogalacturonan acetylase (AnRAE; AN2528.2). AnAXE and AnRAE are annotated as specific for xylan deacetylation and pectin deacetylation, respectively. To target the enzymes to the plant apoplast, the 5' end of the full-length complementary DNA (cDNA) of each gene was fused with the expansin-B signal peptide coding sequence from Arabidopsis (for expression in Arabidopsis) or from maize (*Zea mays*; for expression in Brachypodium), and the 3' end of each gene was fused with the GFP coding sequence. DNA constructs harboring hydrolase-encoding genes were created to produce a single transcription unit containing the three fused parts (signal peptide, hydrolase cDNA, and GFP) in the same open reading frame (Fig. 1A).

After transformation, three independent transgenic lines were selected for each construct, and homozygous plants were obtained using herbicide resistance and confirmed to carry the complete construct by PCR. No visible phenotypes were observed during the development of all transgenic

plants, which were grown side by side with wild-type plants.

Expression of Fungal Acetylases Increased Acetylase Activity in Arabidopsis and Brachypodium Apoplastic Fluids

Transgenic plants were first tested for the production of the fungal enzymes in the extracellular matrix. Apoplastic fluids were isolated from leaves of 4-week-old transgenic plants and analyzed by western blot using anti-GFP antibodies. Bands at molecular masses of 65 and 58 kDa, corresponding to the expressed *A. nidulans* xylan acetylase and rhamnogalacturonan acetylase fused with GFP, respectively, were found (Fig. 1B).

The apoplastic fluids extracted from three independent homozygous lines for each gene were tested for acetylase activity using 4-nitrophenyl (pNP)-acetyl as a substrate. Apoplast extracts from the Arabidopsis

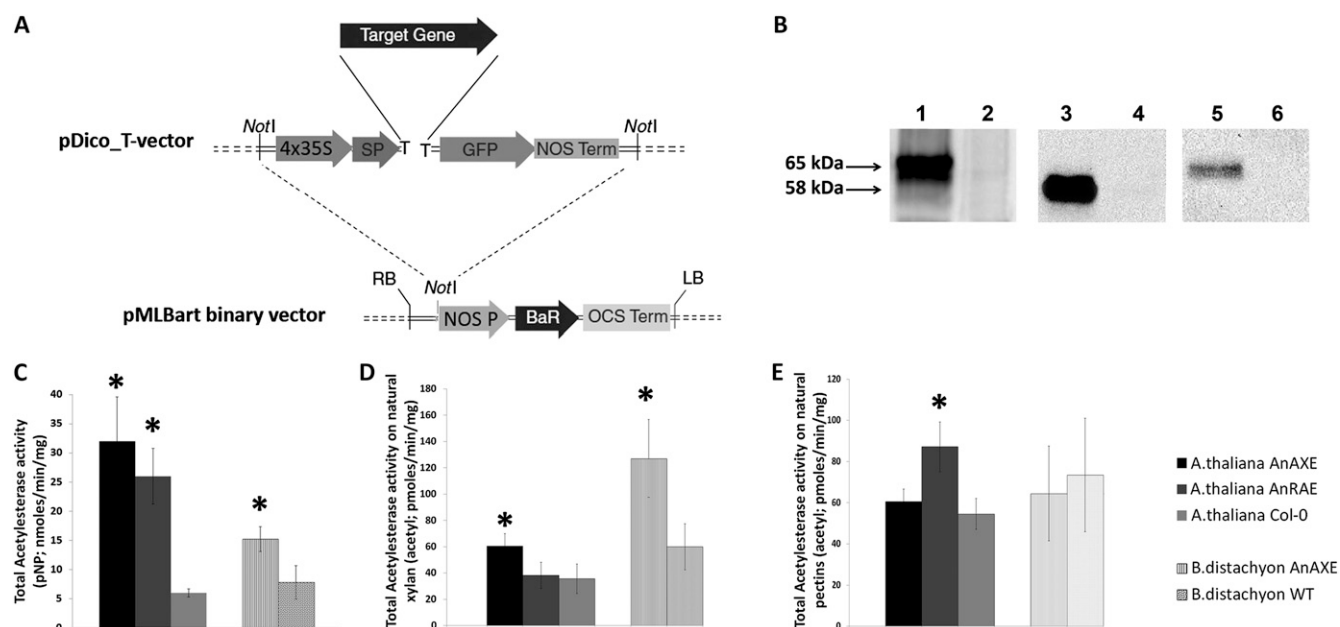


Figure 1. A, The expression cassette of the vector developed for Arabidopsis transformation. BaR, Gene for herbicide resistance (Basta; DL-phosphinothricin); LB, left border; NOS Prom, promoter from nopaline synthase; NOS Term, terminator from nopaline synthase; OCS Term, octopine synthase terminator; RB, right border; SP, Arabidopsis expansin-B signal peptide; Target Gene, gene of interest; 4x35S, tetramer of the CaMV 35S RNA promoter. B, Western-blot analysis of total proteins from apoplast of transgenic and wild-type plants. On the left, the arrows show the size of proteins in kDa. Lane 1, Arabidopsis plant expressing AnAXE fused with GFP (65 kDa); lane 3, Arabidopsis plant expressing AnRAE fused with GFP (58 kDa); lane 5, Brachypodium plant expressing AnAXE fused with GFP (65 kDa); lanes 2, 4, and 6 represent total protein from wild-type plants prepared in parallel with transgenic plant protein extracts. Equal amounts of total protein were loaded for each line, and expressed proteins were detected using GFP monoclonal antibodies (1:5,000 dilution). C, Esterase activities in apoplast from transgenic and wild-type plants (pNP-acetyl was used as a substrate). D, Esterase activities in apoplast from transgenic and wild-type plants estimated using birch wood xylan. E, Esterase activities in apoplast from transgenic and wild-type plants determined using citrus pectin. In all assays, apoplast fluids were prepared from the combined three plants for each of three independent transgenic homozygous lines, of three lines expressing empty vector, and of three Col-0 plants, all of the same age. Bars represent mean \pm SD of total acetylase activity averaged for three independent transgenic lines. Data represent average values obtained for three independent transgenic lines. Asterisks indicate data sets significantly different between transgenic plants and wild-type plants (Student's *t* test, $P < 0.05$; $n = 3$).

plants expressing AnAXE and AnRAE showed 4.3- and 5.3-fold higher activity compared with apoplast from wild-type Columbia (Col-0) plants, whereas *Brachypodium* AnAXE lines showed 2-fold higher activity compared with wild-type plants (Fig. 1C). To confirm the annotated substrate specificity of AnAXE and AnRAE, the acetyltransferase activity in the apoplast from transgenic plants was assayed using commercially available pure acetylated polysaccharides. The apoplastic fluids obtained from both *Arabidopsis* and *Brachypodium* transgenic plants expressing AnAXE have 1.7- to 2.1-fold higher activity deacetylating beech wood (*Fagus* spp.) xylan compared with wild-type plants, whereas there was no difference between apoplastic extracts from AnAXE and wild-type plants in the ability to deacetylate pectin (Fig. 1, D and E). By contrast, apoplastic fluid prepared from *Arabidopsis* AnRAE plants showed higher activity only when pectin was used as a substrate (1.6-fold), and no differences with wild-type plants were observed when beech xylan was used as a substrate (Fig. 1, D and 1E). *Brachypodium* plants expressing AnRAE (Fursova et al., 2012) did not show a difference in cell wall acetylation in comparison with wild-type plants, most likely due to the low amounts of pectin present in the *Brachypodium* cell wall; therefore, we did not extend our studies on these plants in this work.

Fungal Acetyltransferases Deacetylate Specific Polysaccharides and Affect Their Accessibility to Glycosyl Hydrolases

The total contents of acetyl groups of the cell walls extracted from aerial parts of transgenic and wild-type plants were quantified. *Brachypodium* wild-type plants showed five times more acetyls in their cell wall in comparison with *Arabidopsis* wild-type plants (Fig. 2, A and B). AnAXE *Arabidopsis* and *Brachypodium* plants showed 1.8- and 1.3-fold reductions of acetyl levels in their cell walls, respectively, consistent with the different levels of AnAXE activity observed in this study. Expression of AnRAE in *Arabidopsis* plants reduced cell wall acetylation by 1.3-fold. Analysis of monosaccharide composition did not reveal significant differences between transgenic and wild-type plants (Supplemental Table S1).

To verify the substrate specificity of the expressed enzymes *in vivo*, we treated cell walls of transgenic and wild-type plants with xylanase that hydrolyzes xylan backbone between two consecutive unsubstituted xylose residues or with a mixture of PG and PME. If xylan is the substrate of AnAXE *in vivo*, treatment of AnAXE plants with xylanase should not affect the total polysaccharide acetyl content, as the degraded xylooligosaccharides would not have substantial acetyl content. By contrast, treatment of AnAXE plants with pectinase should substantially reduce the polysaccharide acetyl content. Conversely, treatment of AnRAE plants with xylanase should have the opposite effect, due to a substantial decrease in polysaccharide acetyl content. Treatments of cell walls with either xylanase or a mixture of PME

and PG reduced the amount of acetylated polysaccharides to approximately the same level (1.25-fold) in the wild-type plants (Fig. 2A). In AnAXE plants, PME+PG treatment reduced the amount of acetylated polysaccharides, but no significant differences were observed after xylanase treatment. By contrast, in AnRAE plants, treatment with xylanase significantly reduced the amount of acetylated polysaccharides and treatment with PME+PG did not affect the amount of acetylated polysaccharides. In *Brachypodium* cell walls, due to the low amount of pectin, treatment with PME+PG did not change the amount of acetylated polysaccharides in wild-type plants or in AnAXE-expressing plants (Fig. 2B). However, treatment with xylanase reduced the amount of acetylated polysaccharides in wild-type *Brachypodium* but not in AnAXE plants (Fig. 2B), which is similar to results obtained for *Arabidopsis* AnAXE plants.

Oligo Mass Profiling analysis was used to examine whether expressed acetyltransferases affect xyloglucan acetylation in *Arabidopsis* plants (Fig. 2D). Cell walls prepared from AnAXE, AnRAE, and Col-0 plants were treated with xyloglucan endoglucanase, and acetylated and nonacetylated xyloglucan subunits were measured using matrix-assisted laser-desorption ionization time of flight (MALDI-TOF). The nonacetylated:XXLG-acetylated:XXLG ratio was significantly higher in AnRAE in comparison with AnAXE and Col-0 plants, indicating a reduction of xyloglucan acetylation in AnRAE-expressing plants.

All these results, together with the activity assays using natural substrates, suggest that AnRAE deesterifies pectins and to some extent xyloglucan, but AnAXE is more specific toward xylan but not xyloglucan and pectin.

To detect whether deacetylation can alter polysaccharide digestibility, the amount of total sugars released during xylanase and PME+PG treatment of total plant cell walls was measured. Significantly higher amounts of sugars were released from the cell walls of AnRAE plants when they were treated with PME+PG (Fig. 2C), indicating their increased digestibility.

The monosaccharide composition of the cell wall residue left after xylanase and PME+PG treatments was analyzed. After xylanase treatment, higher amounts of pectin-related monosaccharides (Fuc, Rha, Ara, Gal, and a slight but not significant increase of GalA) were observed in *Arabidopsis* AnAXE cell walls in comparison with the wild type (Table I). Additionally, significant reduction of GlcA but not of Xyl was observed in cell wall residue that remained after xylanase treatment of AnAXE plants. On the contrary, cell wall residue from *Arabidopsis* AnRAE plants after treatment with xylanase contained higher amounts of Xyl, mainly present in hemicellulosic polysaccharides. These results indicate that the partial deacetylation of xylan does not significantly affect its xylanase digestibility, but pectin deacetylation seems to decrease xylan accessibility to xylanase.

After PME+PG treatment, the cell wall residue of AnAXE *Arabidopsis* plants showed higher amounts of Gal but AnRAE plants showed a reduced amount of GalA. Interestingly, Fuc and GlcA were completely eliminated in all plants after treatment with PME+PG (Table II). This

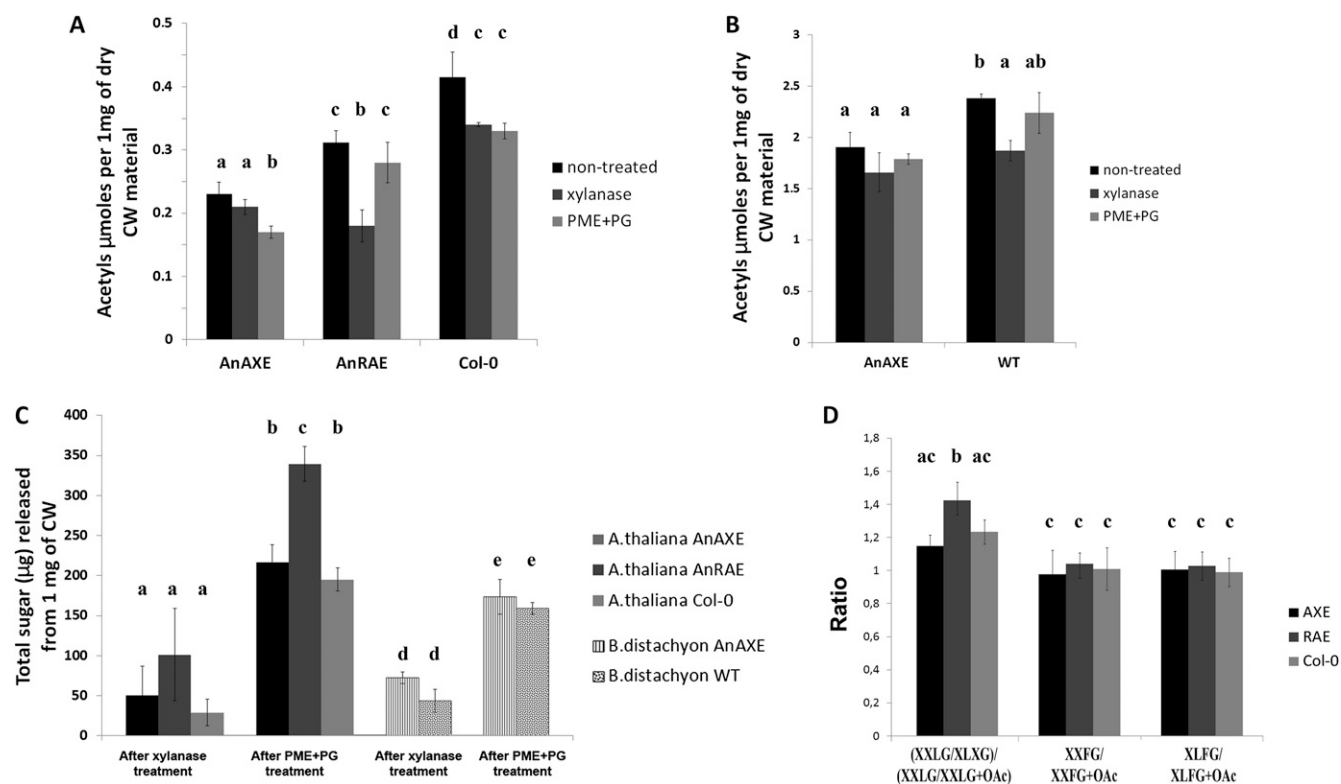


Figure 2. A and B, Cell wall (CW) acetylation of transgenic Arabidopsis (A) and Brachypodium (B) lines and wild-type plants (WT). Shown mean values are averages for three independent transgenic lines for each construct and three wild-type plants grown simultaneously. Acetyl content is shown in $\mu\text{mol mg}^{-1}$ dry cell wall material. Bars show means \pm SD averaged for three independent transgenic lines. C, Amount of reducing sugars released after incubation of dry cell wall material from transgenic and wild-type control plants with xylanase and PME+PG during 24 h. D, Ratios of nonacetylated to acetylated xyloglucan oligosaccharides obtained after xyloglucan endoglucanase digestion of cell walls from Arabidopsis AnAXE, AnRAE, and wild-type plants analyzed by MALDI-TOF. Nomenclature of subunits is according to Fry et al. (1993). Data represent average values obtained for three independent transgenic lines. Letters (a–e) indicate significant differences among the combinations of genotypes and treatments (ANOVA, $P \leq 0.05$).

suggests that partial deacetylation of both pectin and xyloglucan increases pectin digestibility with PG.

Deacetylation of Different Cell Wall Polysaccharides Causes the Up-Regulation of Different Acetyltransferases

We next investigated the possible intensification of biosynthetic acetylation of polysaccharides as a plant compensatory reaction to postsynthetic deacetylation

by overexpressed fungal acetyltransferases. Quantitative reverse transcription (RT)-PCR was used to measure the transcript levels of four previously discovered acetyltransferase-encoding genes. Arabidopsis *RWA1*, *RWA2*, *RWA3*, and *RWA4* were demonstrated to control xylan acetylation during secondary cell wall formation and to have high identity with each other (Lee et al., 2011). RT-quantitative PCR (qPCR) analysis showed higher expression of *RWA2* and *RWA4* (1.9- and 3.4-fold,

Table 1. Monosaccharide composition (mol %) of cell walls from transgenic and wild-type plants after xylanase treatment

Asterisks indicate data sets significantly different between transgenic plants and wild-type plants, according to Student's *t* test ($P < 0.05$; $n = 3$ plants from independent transformant progeny).

Monosaccharide	Arabidopsis AnAXE	Arabidopsis AnRAE	Arabidopsis Col-0	Brachypodium AnAXE	Brachypodium Wild Type
Fuc	2.23 \pm 0.16*	1.78 \pm 0.08	1.87 \pm 0.25	0.24 \pm 0.03	0.39 \pm 0.01
Rha	8.32 \pm 0.85*	5.68 \pm 1.34	7.20 \pm 0.39	5.78 \pm 0.27	5.56 \pm 0.17
Ara	9.01 \pm 0.97*	6.16 \pm 1.13	7.81 \pm 0.39	6.27 \pm 0.29	6.02 \pm 0.15
Gal	18.4 \pm 1.33*	14.53 \pm 0.42	15.77 \pm 0.07	2.51 \pm 0.12	2.69 \pm 0.06
Glc	11.88 \pm 1.49	14.48 \pm 0.41	16.05 \pm 3.14	27.75 \pm 1.24	30.69 \pm 0.76
Xyl	39.20 \pm 2.28	46.38 \pm 1.16*	41.44 \pm 1.51	47.96 \pm 1.11	45.04 \pm 4.34
Man	8.03 \pm 0.89	9.58 \pm 0.76	8.49 \pm 0.91	9.62 \pm 0.46	9.23 \pm 0.59
GalA	2.81 \pm 0.82	1.18 \pm 0.63	0.9 \pm 0.11	0.14 \pm 0.02	0.12 \pm 0.01
GlcA	0.15 \pm 0.06*	0.32 \pm 0.08*	0.54 \pm 0.07	0.76 \pm 0.09	0.32 \pm 0.19

Table II. Monosaccharide composition (mol %) of cell walls from transgenic and wild-type plants after PME+PG treatment

Asterisks indicate data sets significantly different between transgenic plants and wild-type plants, according to Student's *t* test ($P < 0.05$; $n = 3$ plants from independent transformant progeny).

Monosaccharide	Arabidopsis AnAXE	Arabidopsis AnRAE	Arabidopsis Col-0	Brachypodium AnAXE	Brachypodium Wild Type
Rha	8.11 ± 1.02	5.44 ± 2.56	7.03 ± 0.34	1.91 ± 0.16	1.63 ± 0.32
Ara	8.79 ± 0.78	5.84 ± 2.14	7.55 ± 0.23	2.07 ± 0.11	1.72 ± 0.23
Gal	21.01 ± 0.42*	18.52 ± 2.26	19.63 ± 0.58	6.25 ± 0.60	6.73 ± 0.27
Glc	15.64 ± 1.27	21.94 ± 2.9	17.98 ± 0.87	42.68 ± 4.61	39.38 ± 2.85
Xyl	44.39 ± 1.16	46.91 ± 3.03	45.68 ± 0.26	46.85 ± 4.32	50.35 ± 2.52
Man	7.99 ± 0.91	8.44 ± 1.31	8.22 ± 0.38	8.43 ± 0.29	9.06 ± 0.76
GalA	2.05 ± 0.72	1.4 ± 0.25*	2.19 ± 0.06	0.24 ± 0.15	0.25 ± 0.05

respectively) in Arabidopsis AnAXE plants in comparison with wild-type and AnRAE plants and higher expression of *RWA3* and *RWA4* (3.3- and 6.6-fold, respectively) in AnRAE plants (Fig. 3). Using in silico analysis of primary DNA and protein sequences, two Brachypodium genes were identified and found to be 70% identical to the Arabidopsis *RWA* genes and to their protein products (Supplemental Fig. S1). No difference in expression of the two *RWA*-like genes was detected in Brachypodium AnAXE plants in comparison with wild-type plants.

Acetyltransferase-Expressing Plants Have Increased Resistance to Fungal Pathogens

To examine whether the expression of acetyltransferases in the plant cell wall causes any changes in cell morphology, thick cross sections were prepared from 5-week-old transgenic plant stems and stained with toluidine blue (Fig. 4). Examination of stained sections by light microscopy did not reveal differences in cell morphology or cell wall thickness between transgenic lines and wild-type plants.

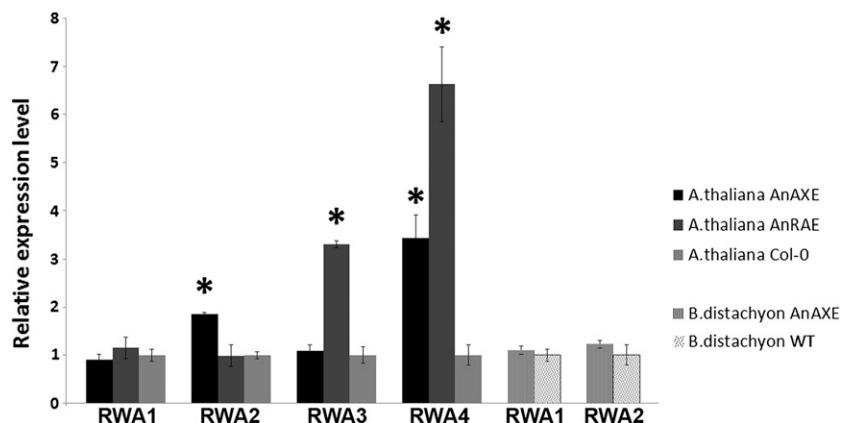
Since modification of plant cell wall structure may be critical for plant-pathogen interactions (Vorwerk et al., 2004; Lionetti et al., 2007; Ferrari et al., 2008), transgenic plants obtained in this study were challenged with pathogenic microorganisms. The susceptibility of Arabidopsis plants ectopically expressing AnAXE or AnRAE was

assessed by inoculating leaves with *B. cinerea* conidia and subsequently evaluating symptoms 48 h post inoculation (hpi). AnAXE- and AnRAE-expressing plants displayed reduced lesion areas compared with Col-0 (Fig. 5, A and B), but no significant differences were observed in the number of expanding lesions per inoculated area between transformed lines and wild-type plants. The reduced symptoms detected in transgenic plants were related to reduced fungal growth, as determined by RT-qPCR analysis of transcript accumulation of the *B. cinerea* housekeeping gene *Actin* after 48 hpi in transgenic plants in comparison with wild-type plants (Fig. 5C).

In contrast to fungal infection, when Arabidopsis transgenic and wild-type plants were treated with the bacterial pathogen *Pseudomonas syringae* pv *tomato* strain DC3000, no difference in bacterial growth was observed in the infected leaves (Supplemental Fig. S2A). However, in both AnAXE and AnRAE plants, the appearance of yellowish spots on the leaves, typical of bacterial infection, was delayed in comparison with the wild type (Supplemental Fig. S2B).

To verify whether the expression of AnAXE in grass species, which contain high levels of xylans in their cell walls, can affect resistance to fungal pathogens, Brachypodium plants expressing AnAXE were infected with *Bipolaris sorokiniana*. Water-soaked necrotic lesions developed between 48 and 72 hpi with *B. sorokiniana* on wild-type leaves and were followed at later stages by the development of a surrounding chlorotic

Figure 3. Real-time RT-PCR analysis of *RWA* genes in transgenic lines and wild-type plants (WT). Relative expression levels were calculated in comparison with an appropriate control gene from wild-type plants. *ACTIN2* (At3g18780; for Arabidopsis) and *GADPH* (for Brachypodium), whose expression levels were not affected, were used as reference genes. The comparative threshold cycle method was used for determining the difference (asterisks indicate significant differences) between transcript copy numbers in wild-type and transgenic plants. Data represent average values ± SE obtained for three independent transgenic lines.



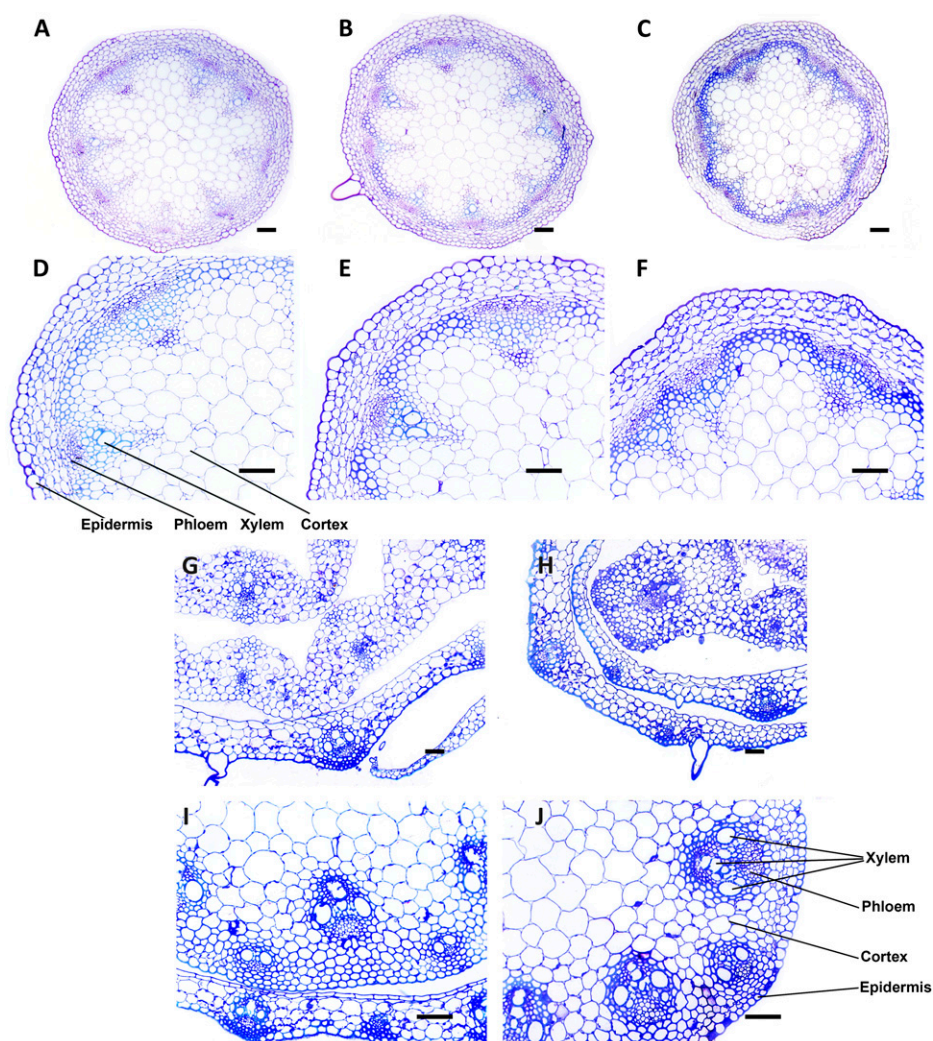


Figure 4. Toluidine blue-stained cross sections and enlargements of specific areas of stem from transgenic and wild-type plants. A to F, Arabidopsis AnAXE (A and D), AnRAE (B and E), and Col-0 (C and F) plants. G to J, Brachypodium AnAXE (G and I) and wild-type (H and J) plants. Bars = 1 mm in cross sections and 25 μm in enlargements. [See online article for color version of this figure.]

area and the appearance of more widespread necrosis. After 48 hpi, the lesion areas on AnAXE plants were significantly reduced compared with wild-type plants (Fig. 5E). Also in this case, quantitative RT-PCR analysis of transcripts of the *B. sorokiniana* housekeeping gene *GLYCERALDEYDE-3-PHOSPHATE DEHYDROGENASE-LIKE* indicated that the reduced symptoms detected in transgenic plants were related to reduced *B. sorokiniana* growth (Fig. 5F). In addition, at a later stage of infection (at 170 hpi), leaves from transformed plants exhibited reduced chlorosis in comparison with wild-type plants (Fig. 5D).

No differences were observed between Brachypodium AnAXE and wild-type plants after treatment with *Xanthomonas oryzae*, a pathogenic bacterium of rice (*Oryza sativa*) and some weedy grasses (Supplemental Fig. S2C).

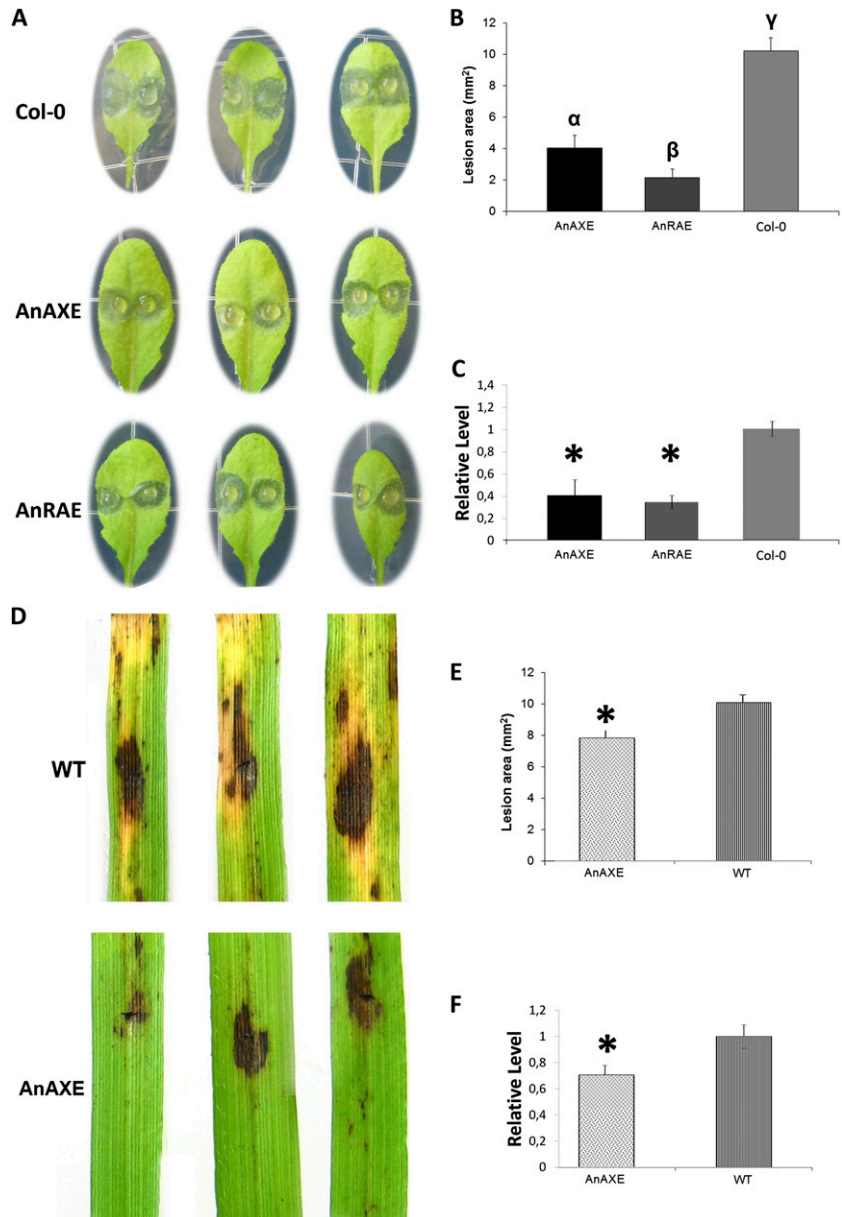
Expression of Acetylsterases in the Plant Cell Wall Leads to Changes in Pathogen-Responsive Gene Expression

Increased resistance to pathogens can be caused by the release of molecules that are toxic to microbial cells from the modified plant cell wall or by plant-specific

responses to cell wall modification. Measurement of acetlys in apoplastic fluids of transgenic plants did not reveal any differences in comparison with wild-type plants (data not shown). In addition, incubation of *B. cinerea* cells with apoplastic fluids prepared from Arabidopsis AnRAE, AnAXE, or wild-type plants did not reveal differences in fungal growth (Supplemental Fig. S3), suggesting that any apoplastic compositional changes due to the presence of fungal acetylsterases do not have a direct effect on fungal growth.

To investigate whether the expression of fungal acetylsterases and the consequent decrease of cell wall acetylation affect the pathways involved in plant defense responses to pathogens, the transcript levels of several defense genes were measured using RT-qPCR in healthy Arabidopsis and Brachypodium plants and upon fungal infection (Fig. 6). In Arabidopsis plants, the expression levels of genes encoding defense proteins (*PR1* and *PR5*), β -glucanase2 (*bG2*), plant defensin1.2 (*PDF1.2*), phytoalexin deficient3 (*PAD3*), wound responsive3 (*WR3*), jasmonic acid responsive gene1 (*JR1*), pathogen-induced W box-containing transcription factor (*WRKY40*), and polygalacturonase-inhibiting protein1 (*PGI1*) involved in

Figure 5. Analysis of the susceptibility of Arabidopsis and Brachypodium transgenic plants to fungal pathogens. A and B, *B. cinerea* symptoms on Arabidopsis transgenic and wild-type leaves. α , β , and γ indicate data sets significantly different according to one-way ANOVA followed by Tukey's tests ($n = 44$; $P < 0.001$). D and E, *B. sorokiniana* symptoms on Brachypodium transgenic and wild-type plants (WT). The asterisk indicates a significant difference between transgenic plants and wild-type plants, according to Student's *t* test ($n = 24$; $P < 0.001$). All lesion areas were measured 48 h after inoculation. Data points represent average lesion areas \pm se. Similar results were obtained from three independent experiments. C and F, Real-time RT-PCR analysis of housekeeping pathogen gene transcript accumulation in infected transgenic lines and wild-type plants. Relative expression levels of *B. cinerea* *ACTIN* (C) and *B. sorokiniana* *GDP* (F) genes were calculated as a comparison with the appropriate reference plant housekeeping gene *ACTIN2* (for Arabidopsis) or *GADPH* (for Brachypodium), expression of which was not affected. The comparative threshold cycle method was used for determining differences between transcript copy numbers in wild-type and transgenic plants. Data represent average values obtained for three independent transgenic lines. Asterisks indicate significant differences between transgenic plants and wild-type plants (Student's *t* test, $P < 0.05$; $n = 3$).



the defense response, *CYP81F2* involved in the response to bacterium, fungus, and insects by initiating callose deposition in the cell wall, and oxidoreductase (*RetOx*) were analyzed. In noninfected AnAXE plants, the expression of *PR5* (2-fold) and *WRKY40* (27-fold) was higher than in wild-type plants. Increased levels of expression were also observed in *PAD3* (29-fold), *WRKY40* (11-fold), and *CYP81F2* (5-fold) in Arabidopsis AnRAE plants in comparison with wild-type plants (Fig. 6A). Using BLAST searches of the Brachypodium genome, pathogen-related genes with high identity to Arabidopsis genes were identified. In noninfected Brachypodium AnAXE plants, the expression of *PR5* (3.3-fold), *bG2* (6.4-fold), and *WRKY40* (2.6-fold) was higher in comparison with wild-type plants (Fig. 6B).

After pathogen inoculation (48 hpi), expression levels of all analyzed defense-related genes were approximately 100-fold higher in comparison with noninfected plants (data not shown). While the difference in expression of *PAD3*, *WRKY40*, and *CYP81F2* between transgenic and wild-type plants was lower than that in uninfected plants, the expression of *RetOx* was 2-fold higher in both Arabidopsis transgenic lines, AnAXE and AnRAE, in comparison with wild-type plants (Fig. 6C). In Brachypodium, the difference in defense gene expression was lowered to 1.6- and 2-fold for *PR5* and *bG2*, respectively. No difference in *WRKY40* between transgenic and wild-type plants after 48 hpi was detected (Fig. 6D). At 72 hpi for Arabidopsis and 170 hpi for Brachypodium plants, the difference in the level of defense gene expression disappeared completely,

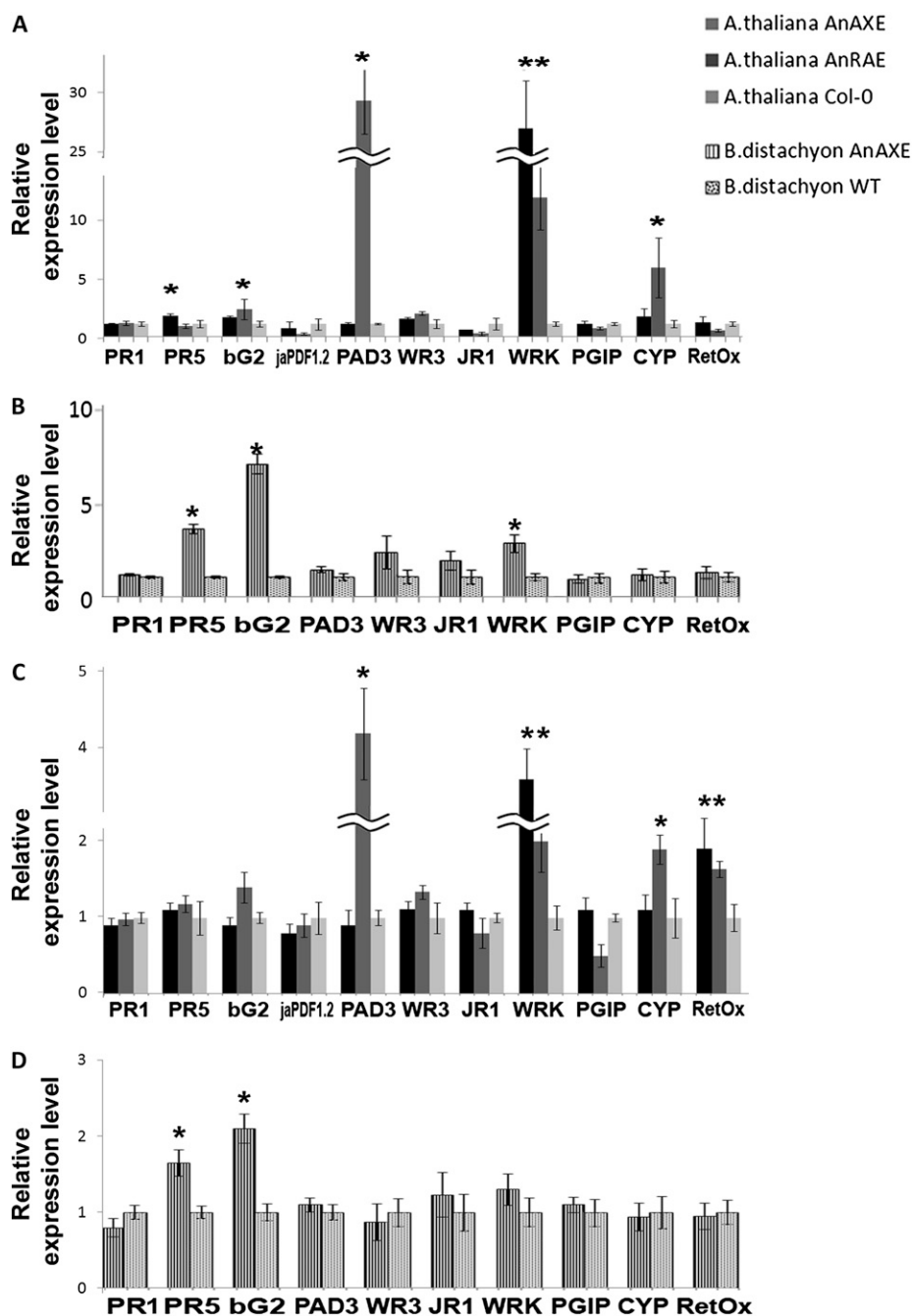


Figure 6. Real-time RT-PCR analysis of selected pathogen-related genes in uninfected and infected transgenic lines and wild-type plants (WT). A and B, Expression levels of defense-related genes in Arabidopsis (A) and Brachypodium (B) transgenic plants. C and D, Gene expression in Arabidopsis (C) and Brachypodium (D) transgenic plants upon 48 h of infection with *B. cinerea* and *B. sorokiniana*, respectively. Gene expression levels in transgenic plants normalized to the expression of the same gene detected in wild-type plants (for which gene expression was set to 1), *ACTIN2* (for Arabidopsis) or *GADPH* (for Brachypodium), were used as reference genes. The comparative threshold cycle method was used for determining differences between transcript copy numbers in wild-type and transgenic plants. Data represent average obtained for three independent transgenic lines. Asterisks indicate significant differences between transgenic plants and wild-type plants (Student's *t* test, $P < 0.05$; $n = 3$).

except for *RetOx*, which showed a 2- to 2.5-fold up-regulation in both Arabidopsis lines (Supplemental Fig. S4).

Arabidopsis AnAXE and AnRAE Plants Have Enhanced Defense Responses

To verify if the reduced susceptibility to pathogens observed in Arabidopsis and Brachypodium transgenic plants is due to the constitutive activation of defense responses, we quantified callose deposition and hydrogen

peroxide (H_2O_2) accumulation in leaves. Arabidopsis AnAXE and wild-type plants did not show callose deposition, but nontreated AnRAE plants showed a significant number of callose deposits (Fig. 7, A–C). This result is consistent with the induction of *CYP81F2* detected in these plants. Brachypodium AnAXE and wild-type plants did not show any callose deposition (Fig. 7, D and E).

Increased accumulation of H_2O_2 was observed in AnRAE and AnAXE Arabidopsis plants in comparison with wild-type plants (Fig. 7, F–H). No difference in the accumulation of H_2O_2 was observed between Brachypodium transgenic and wild-type leaves (Fig. 7, I and J).

DISCUSSION

Postsynthetic modification of the plant cell wall by polysaccharide-modifying enzymes overexpressed in the apoplast represents a promising strategy to examine the contributions of structural polysaccharides to plant fitness. Transgenic expression of microbial hydrolases with well-characterized enzymatic activities provides a useful approach for a targeted modification of the plant cell wall to study their physiological effects in planta.

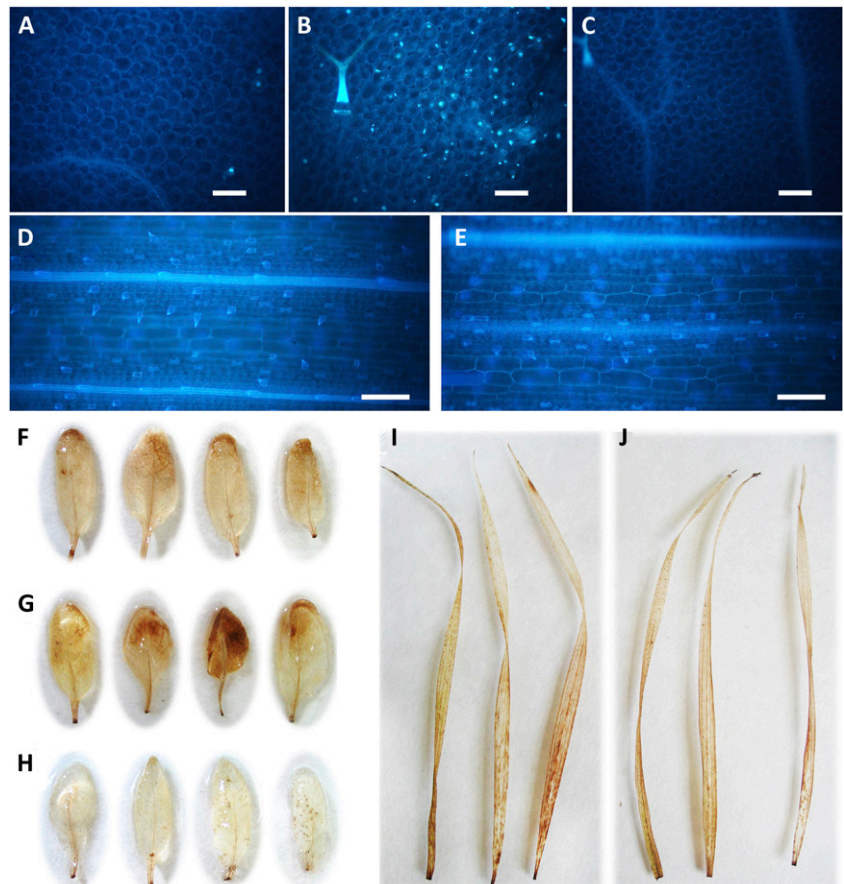
Earlier, we reported the preparation of Arabidopsis plants expressing AnAXE and demonstrated that a signal peptide fused to the N terminus of the hydrolase ensures its delivery to the apoplast, and a fluorescent protein fused to the C terminus of the hydrolase does not affect its activity (Pogorelko et al., 2011). In this study, we constructed new versatile transformation vectors and expressed AnAXE and AnRAE esterases in the apoplast of Arabidopsis and Brachypodium plants characterized by type I and type II cell walls, respectively. Using Arabidopsis AnAXE created earlier and new Arabidopsis AnRAE and Brachypodium AnAXE transgenic plants, we have investigated the effect of deacetylation on plant cell wall integrity and obtained new insights into plant responses to cell wall modifications, specifically the potential role

of polysaccharide acetylation in the defense against fungal infection.

Up-regulation of three putative acetyltransferases (RWA2, RWA3, and RWA4; Lee et al., 2011; Manabe et al., 2011) in Arabidopsis AnAXE and AnRAE plants suggested that plants respond to these postsynthetic modifications using compensatory mechanisms. Moreover, deacetylation of xylan in AnAXE plants promotes different acetyltransferases, most likely with different polysaccharide specificity, than deacetylation of pectin and xyloglucan in AnRAE plants. Similarly, a compensatory effect was observed when increased methylesterification of pectins achieved by the inhibition of PME in Arabidopsis plants caused the up-regulation of several PME-encoding genes via an unknown feedback mechanism for the BRI1 receptor (Wolf et al., 2012b). The lack of up-regulation of two RWA-like genes in Brachypodium AnAXE plants can be due to the involvement of other acetyltransferases in such compensatory reactions in Brachypodium. It is also possible that the low level of Brachypodium cell wall deacetylation in the transgenic plants is insufficient to induce compensatory responses.

Deacetylation of polysaccharides can lead to changes in cell wall integrity and, hence, can affect plant defense against environmental stresses. There is substantial evidence that esterification/deesterification and subsequent changes in the polysaccharide cross-linking network

Figure 7. A to E, Determination of callose deposition. Leaves from Arabidopsis AnAXE (A), AnRAE (B), and Col-0 (C) and Brachypodium AnAXE (D) and wild-type (E) plants were stained with aniline blue for callose visualization. Calculation of callose deposit number in three microscopic fields of 0.5 mm² for each leaf (six leaves were analyzed for each transgenic line) resulted in 89 ± 7 deposits in AnRAE against 9 ± 1 and 6 ± 1 deposits in Arabidopsis AnAXE and Col-0, respectively. The experiment was repeated twice with similar results. Bars = 100 μ m. F to J, Accumulation of H₂O₂ in transgenic and wild-type plants. Staining of detached leaves with 3,3'-diaminobenzidine revealed an accumulation of H₂O₂ in leaves from Arabidopsis AnAXE (F) and AnRAE (G) plants and no accumulation in leaves from Col-0 plants (H). No difference between Brachypodium AnAXE (I) and wild-type plants (J) was observed.



affect plant resistance to pathogenic microorganisms (Vogel et al., 2002, 2004; Lionetti et al., 2007; Manabe et al., 2011; Gille and Pauly, 2012). Here, we demonstrate that the reduction of polysaccharide acetylation in both *Arabidopsis* and *Brachypodium* transgenic plants significantly increases plant resistance to necrotrophic fungi, *B. cinerea*, and *B. sorokiniana* but not to bacterial pathogens, which suggests the importance of polysaccharide acetylation for the cell wall's protective function against fungal invasion.

The observed lack of *B. cinerea* growth inhibition in the presence of apoplastic fluids prepared from either *Arabidopsis* transgenic or wild-type plants (Supplemental Fig. S3) suggests that the inhibition of fungal growth in the leaves of transgenic plants is not due to compositional changes in the apoplast of transgenic plants. Transcript analysis of the known plant defense-related genes *PR1*, *PR5*, *bG2*, *jaPDF1.2*, *PAD3*, *WR3*, *JR1*, *WRKY40*, *PGIP1*, *CYP81F2*, and *RetOx* (Galletti et al., 2008; Raiola et al., 2011) demonstrated that particular plant defense responses are constitutively initiated in our transgenic plants. Thus, overexpression of AnRAE in *Arabidopsis* and consequent deacetylation of pectins and xyloglucan in this plant induce the expression of *PAD3*, involved in camalexin biosynthesis and known to mainly affect plant defense against fungal pathogens (Schuhegger et al., 2006), and of *CYP81F2*, involved in glucosinolate metabolism and callose deposition in the cell wall (Po-Wen et al., 2013). However, we were unable to detect camalexin in *Arabidopsis* AnRAE plants (data not shown), most likely because other rate-limiting proteins involved in its biosynthesis were not induced in uninfected plants. Higher levels of *PAD3* and *CYP81F2* expression, together with callose accumulation, suggest a possible contribution of these specific defense pathways to the reduced susceptibility of AnRAE plants to *B. cinerea*. In addition, *WRKY40* is known to act as a transcriptional suppressor in the mitogen-activated protein kinase cascade during pathogen invasion (Chen et al., 2010). The fact that this gene is up-regulated in all our transgenic lines, *Arabidopsis* AnAXE, AnRAE, and *Brachypodium* AnAXE, suggests that this regulatory pathway also responds to cell wall deacetylation.

Overexpression of AnAXE and deacetylation of xylan in both *Arabidopsis* and *Brachypodium* plants increased transcript levels of *PR5* and *bG2*, two other defense-related genes. *PR5* is normally induced by salicylic acid and secreted into the extracellular spaces (Linthorst, 1991). Its expression was associated with drought (Jakab et al., 2005) and salt stress responses and seed germination (Seo et al., 2008). *bG2* is a β -glucanase-encoding gene that was shown to be up-regulated during the *Arabidopsis* response to biotic stress (Dong et al., 1991). β -Glucanase purified from pea (*Pisum sativum*) protein extract was shown to inhibit, together with chitinase, the growth of *Fusarium solani* f. sp. *phaseoli* (Mauch et al., 1988), *Sclerotinia homoeocarpa*, and *Rhizoctonia solani* (Wang et al., 2003).

Normally, upon infection with necrotrophic fungi, wild-type plants respond by the regulation of a large

subset of signaling pathways involving a broad spectrum of defense-related genes (Denby et al., 2004). In our study, all analyzed defense-related genes, including those that had higher transcript levels in uninfected transgenic plants, *PAD3*, *WRK*, *CYP*, *PR5*, and *bG2* in *Arabidopsis* and *PR5* and *bG2* in *Brachypodium*, were up-regulated in wild-type plants after infection. qPCR analysis performed for different postinfection time points (Supplemental Fig. S4) demonstrated that the expression of these genes in wild-type plants gradually became even with their expression levels in transgenic plants, suggesting that in uninfected transgenic plants, some defense-related pathways, which are induced in the wild type only in the response to fungal treatment, are induced prior to infection. Significantly higher up-regulation of the oxidoreductase-encoding gene *RetOx* (Carter and Thornburg, 2004) and the higher level of H_2O_2 observed in *Arabidopsis* AnAXE and AnRAE plants, but not in *Brachypodium* AnAXE plants, in comparison with wild-type plants indicate that dicots and monocots have different mechanisms to respond to pathogens, although both are sensitive to cell wall deacetylation. Our results also suggest that different polysaccharides and their acetylation contribute differently to plant defense reactions against fungal pathogens, most likely through the involvement in different specific signaling pathways.

We demonstrated that deacetylation of pectin and xyloglucan in the cell wall in transgenic plants makes pectic polysaccharides more accessible to pectinases. This can cause the production of small oligosaccharides and monosaccharides, which can act as elicitors of plant defense through signal transduction and transcription activation (Gille and Pauly, 2012). Ferrari et al. (2007, 2008) showed that transgenic plants expressing microbial PG were less susceptible to *B. cinerea* and had constitutively activated defense responses, possibly due to an increased accumulation of bioactive oligogalacturonides (OGs), elicitors perceived by the plant cell as danger-associated molecular patterns (DAMPs). It was demonstrated that OG-induced activation of basal resistance against fungal pathogens is mediated by molecular patterns independently of jasmonate-, salicylic acid-, and ethylene-mediated signaling but dependent on the expression of *PAD3* (Ferrari et al., 2007). OGs also increase the levels of H_2O_2 and callose deposition. Considering that our *Arabidopsis* AnRAE plants have higher levels of *PAD3* expression, H_2O_2 accumulation, and callose deposition, we hypothesize that the deacetylation of pectic polysaccharides increases their susceptibility to plant PG. This leads to the accumulation of elicitor OGs and the initiation of defense-related pathways in noninfected plants, which reduces their susceptibility to pathogen. Similarly, deacetylation of xylan might result in the production of elicitor-like oligosaccharides, which would act as DAMP-inducing defense reactions in the plant. It was reported that the expression of xylanase can increase resistance in rice (Weng et al., 2012); thus, it is possible that in AnAXE-expressing plants, xylan deacetylation results in the liberation of

yet-unknown DAMPs. It is worth noting that some up-regulated genes were the same in both *Brachypodium* and *Arabidopsis* AnAXE-expressing plants, indicating that xylan deacetylation induces similar responses in dicot and monocot plants. It was reported earlier that reduction of polysaccharide acetylation by knockout of the acetyltransferase *RWA2* results in *Arabidopsis* with increased resistance to *B. cinerea* (Manabe et al., 2011). Although the authors did not observe significant constitutive activation of *PDF1.2* and *PAD3*, they proposed that *rwa2* mutant plants have either other defense-related genes up-regulated or faster activation of defenses upon infection. It is also possible that *rwa2* plants and our transgenic plants have different patterns of deacetylated polysaccharides in their cell walls, which might differently affect DAMP formation. Nevertheless, the results of Manabe et al. (2011) support our notion that it is polysaccharide deacetylation that affects the plant's susceptibility to pathogens and not the presence of fungal acetyltransferases in the apoplast. Further investigations will be needed to confirm the accumulation of oligosaccharide DAMP in the apoplast of transgenic plants.

In conclusion, the results obtained in this study point to a potential utility of the postsynthetic modification of cell wall properties. First, specific modifications of cell wall polysaccharides by the overexpression of polysaccharide-modifying enzymes can initiate a plant compensatory response including the up-regulation of biosynthetic genes. This may help to reveal key genes involved in polysaccharide biosynthesis and to elucidate the pathways involved in cell wall integrity signaling. Second, specific polysaccharide modifications can change plant fitness or induce responses to environmental stresses, which can be beneficial to plants. These results have key implications for strategies that seek to modify the plant cell wall for biofuels or other applications. Finally, we also propose that deacetylation of the plant cell wall activates plant defense mechanisms against pathogens and can be used in the future as a tool to increase the resistance of valuable crops to biotic stresses.

MATERIALS AND METHODS

Generation of Transgenic Plants

The pDico_T-vector was constructed using the pXcmkn12 (AB107950-1) vector obtained from Dr. S. Yasuda (National Institute of Genetics, Shizuoka, Japan). The cauliflower mosaic virus (CaMV) promoter of the 35S RNA (CaMV 35S promoter) was amplified using Encyclo DNA polymerase (Evrogen; PK001) from the pEnLox vector (Pogorelko et al., 2008) with the use of primers CaMV_F and CaMV_R harboring *EcoRI*+*NotI* and *EcoRI* restriction sites, respectively. The CaMV 35S promoter PCR fragment was cloned into pGEM-T Easy vector (Promega; A1360) and moved by *NotI* and *EcoRI* restriction enzymes into *NotI*-*EcoRI*-pretreated pXcmkn12 plasmid, resulting in the pXcmkn12-CaMV vector. The *Arabidopsis* (*Arabidopsis thaliana*) expansin gene signal peptide-coding sequence was amplified by PCR with primers AtSP_F and AtSP_R containing *EcoRI* and *KpnI* restriction sites, respectively (for primer sequences, see Supplemental Data S1), using Encyclo DNA polymerase from the pBAt plasmid (obtained from Dr. M.G. Hahn, The Complex Carbohydrate Research Center), and the product was cloned into the pGEM-T Easy vector. After the digestion of recombinant pGEM-T_Easy_AtSP vector, the *EcoRI*-*KpnI* fragment was moved into the pXcmkn12-CaMV plasmid treated with the same enzymes

to obtain the pXcmkn12-CaMV-AtSP vector. The GFP coding sequence, together with the transcription terminator, was amplified with Encyclo DNA polymerase from the psmGFP plasmid (www.arabidopsis.org; CD3-326) using GFP_F and GFP_R primers containing *XbaI* and *PstI* restriction sites, respectively. The product was cloned into a pGEM-T Easy vector. After digestion of the recombinant pGEM-T_Easy_GFP vector, the *XbaI*-*PstI* fragment was moved into the pXcmkn12-CaMV-AtSP plasmid cleaved with the same enzymes. To obtain the final pDico_T-vector, a PCR fragment amplified with CaMV_F and GFP_R primers from the pXcmkn12-AtSP-GFP plasmid was cloned into the pGEM-T Easy vector containing two *NotI* restriction sites flanking the cloning site (for complete sequence, see Supplemental Data S2).

Aspergillus nidulans AN2528.2 (AnRAE) and AN6093.2 (AnAXE) cDNAs extracted from *Pichia pastoris* recombinant strains (Bauer et al., 2006) obtained from the Fungal Genetics Stock Center (www.fgsc.net) were amplified with Encyclo DNA polymerase with appropriate forward and reverse primers (Supplemental Data S1). All forward primers started from the second nucleotide of the ATG start codon to fit into the same open reading frame with the signal peptide and GFP sequences from pDico_T-vector pretreated with *XcmI* (New England Biolabs) restriction enzyme to provide single T-overhanging ends. The AnRAE-coding sequence was cloned into the pDico_T-vector and treated with *NotI* to move the CaMV-AtSP-AnRAE-GFP cassette into the dicot-specific pMLBart binary vector (Gleave, 1992) treated with the same enzyme. For *Brachypodium* (*Brachypodium distachyon*) transformation to express AnAXE, the pMono_T/pUbiP2 vector system described previously (Fursova et al., 2012) was used. The binary vectors were transformed into *Agrobacterium tumefaciens* EHA101 cells by electroporation, and *Arabidopsis* plants were transformed with these cells by the floral-dip method (Clough and Bent, 1998). *Brachypodium* transgenic plants were created via *A. tumefaciens*-mediated transformation of calluses (Vogel and Hill, 2008) at the Plant Transformation Facility of Iowa State University (<http://www.agron.iastate.edu/ptf/>).

Apoplastic Fluid Preparation and Western-Blot Analysis

Apoplastic fluids from aerial plant parts were extracted as described previously (Pogorelko et al., 2011). Immunoblot analyses were performed according to Magi and Liberatori (2005). Apoplastic proteins after precipitation with 10% (v/v) TCA were resuspended in 1× Laemmli's buffer (125 mM Tris-HCl [pH 6.8], 1% SDS, 37.7% glycerol, 1% β-mercaptoethanol, and 0.01% bromophenol blue), boiled for 5 min, and used immediately for SDS-PAGE. Fifteen micrograms of total protein was loaded per well for 10% SDS-PAGE. After separation, the proteins were electrophoretically transferred to a Trans-Blot Transfer Medium membrane (Bio-Rad; 162-0112) using the Trans-Blot Cell (Bio-Rad) with standard transfer buffer. Primary anti-GFP monoclonal antibody (Covance; MMS-118P) at a dilution of 1:5,000 and secondary anti-mouse IgG (whole molecule) peroxidase antibody (Sigma; A9044) at a dilution of 1:40,000 were used for the detection of GFP fusion proteins. Membranes were treated with the HyGlo Quick Spray Reagent B for peroxidase activity (Denville; E2400) and immediately visualized by the ChemiDoc XRS+ System (Bio-Rad; 170-8265).

Assays for Acetyl Esterase Activity

Acetylxylan esterase and rhamnogalacturonan acetyltransferase activities were measured in 100 μL of reaction mixture containing 2 mM pNP-acetyl (Sigma; N8130) in 0.1 M assay buffer (25 mM Tris-HCl, 50 mM EDTA, and 150 mM MgCl₂, pH 7.4). Apoplastic fluids were added to obtain 1.28 × 10⁻¹ mg of protein in each reaction mixture. As a negative control, reaction mixtures with boiled apoplastic extract were used. The reactions were carried out at 37°C and terminated at different time points. Absorbance was measured at 460 nm on a microplate reader. Specific activity was expressed as the amount of pNP (mmol) released per 1 min by 1 mg of protein.

Acetylxylan esterase and rhamnogalacturonan acetyltransferase activities in apoplastic fluids were also assayed using xylan from beech wood (*Fagus* spp.; Sigma; X4252) and pectin from citrus (*Citrus aurantium*) fruit (Sigma; P9436). Apoplastic fluid (1.28 × 10⁻¹ mg of total protein) was incubated with 20 mg of xylan or pectin in 1 mL of 0.1 M sodium phosphate buffer (pH 7.0) at 37°C for 24 h. At each time point, 100 μL of sample was boiled for 15 min at 100°C to terminate the reaction, dried, and used for acetyl amount determination according to the protocol of McComb and McCready (1957; see below).

Cell Wall Composition Analysis and Treatments

Cell walls were isolated from the plant tissue residues after apoplastic fluid extraction as described by Zabolina et al. (2008). To determine monosaccharide

composition, 1 mg of dry cell wall was hydrolyzed with 2 N trifluoroacetic acid at 120°C for 2 h. After the acid was evaporated, the hydrolysate was redissolved in water and analyzed by high-performance anion-exchange chromatography-pulsed-amperometric detection using a CarboPac PA-20 column (Dionex) as described earlier (Zabotina et al., 2008). Monosaccharide standards included L-Fuc, L-Rha, L-Ara, D-Gal, D-Glc, D-Xyl, D-Man, D-GalA, and D-GlcA (all from Sigma). To determine response factors, standard curves were created using mixtures of all standard monosaccharides at different concentrations.

Acetyl content in cell walls was estimated using an assay adapted from McComb and McCready (1957). Briefly, 10 mg of dry cell wall material was mixed with 2.5 mL of 0.5 M hydroxylamine hydrochloride solution, followed by the gradual addition of 2.5 mL of 2 N sodium hydroxide solution. A total of 0.5 mL of the resulting mixture was mixed with 0.2 mL of water and 0.5 mL of acid methanol solution (35.2 mL of 70% perchloric acid in 500 mL of absolute methanol). A total of 1.3 mL of ferric perchlorate solution was added in small increments, mixing after each addition. After 15 min of reaction, the absorbance of the formed ferric acetoxyhydroxamic complex was determined at 510 nm. The amount of acetyls was calculated using a standard curve created using Glc pentaacetate as a standard.

Partial removal of hemicelluloses was done using 30 mg of dry cell wall material incubated with a mixture of 150 units of endo-1,4- β -xyylanase M6 (rumen microorganism; Megazyme) and 15 units of endo-1,4- β -xyylanase M1 (*Trichoderma viride*; Megazyme) in a 1-mL total volume of sodium phosphate buffer (pH 6.0) for 24 h at 37°C. For the partial removal of pectins, 30 mg of dry cell wall material was incubated with a mixture of 150 units of endo-polygalacturonase (Megazyme) and 50 units of PME (PROZOMIX) in a 1-mL total volume of sodium phosphate buffer (pH 6.0) for 24 h at 37°C.

Reducing sugars were measured using the PAHBAH assay (Lever, 1972) with minor modifications. Briefly, 15 μ L of supernatant was mixed with 135 μ L of freshly prepared PAHBAH reagent (1 volume of 5% *p*-hydroxybenzoic acid hydrazide in 5% HCl mixed with 9 volumes of 1.25% trisodium citrate, 0.11% calcium chloride, and 2% sodium hydroxide) and heated at 95°C for exactly 6 min. Absorbance was measured at 410 nm. Calculations were done using a standard curve prepared using different concentrations of Glc.

The cell walls were analyzed by Oligo Mass Profiling according to previously published methods (Lerouxel et al., 2002; Obel et al., 2009). The dry cell wall (2 mg) was suspended in 50 mL of 100 mM ammonium formate buffer, pH 4.5, containing 0.02 units of purified recombinant xyloglucan endoglucanase (EC 3.2.1.151; Pauly et al., 1999) and incubated at 37°C for 18 h. The reactions were centrifuged to pellet undigested material, and the supernatant containing soluble xyloglucan oligosaccharides was removed and dried in a Speed-Vac. The released xyloglucan oligosaccharides were dissolved in 10 μ L of water containing approximately 10 beads of Bio-Rex MSZ 501(D) resin (Bio-Rad) to remove buffer salts. One microliter of the oligosaccharide solution was mixed with 2,5-dihydroxybenzoic acid, spotted onto a MALDI-TOF sample plate, and dried under vacuum. Spectra from five independent randomly distributed spots for each sample were collected and analyzed on a Voyager DE-Pro MALDI-TOF-mass spectrometry instrument in positive reflection mode with an acceleration voltage of 20 kV and an extraction delay time of 350 ns. Ratios of corresponding peak intensities for each xyloglucan subunit and acetylated subunit were determined, and Student's *t* test was performed to compare transgenic and wild-type plants.

Sample Fixation, Sectioning, and Light Microscopy

Four-week-old Arabidopsis and 6-week-old Brachypodium stems were fixed as described previously (Pattathil et al., 2010) with minor modifications. Stem segments 3 to 5 mm long were incubated for 2.5 h in 1.6% (w/v) paraformaldehyde and 0.2% (w/v) glutaraldehyde in 25 mM sodium phosphate buffer, pH 7.1, washed with water twice for 15 min, and dehydrated using a graded ethanol series (35%, 50%, 75%, 95%, 100%, 100%, and 100% [v/v] ethanol) for 30 min at each step. The dehydrated samples were moved to 4°C and then gradually infiltrated with cold LR White embedding resin (Ted Pella) using 33% (v/v) and 66% (v/v) resin in 100% ethanol for 24 h for each concentration, followed by 100% resin for 24 h, three times. The infiltrated samples were transferred to gelatin capsules containing 100% resin for embedding, and resin was polymerized by incubation at 65°C for 24 h.

Sections of 300 nm were prepared using a Leica VT1000S vibratome, stained with 0.05% toluidine blue, and visualized with a Zeiss Axioskop 2 plus (Carl Zeiss) microscope. Images were captured with a Nikon DXM 1200 digital camera.

RNA Extraction, cDNA Synthesis, and Real-Time qPCR

Total RNA was extracted from the uninfected and infected leaves of 5-week-old plants using the SV Total RNA Isolation kit (Promega), and cDNA synthesis

was performed with the SuperScript III First Strand Synthesis system (Invitrogen) following the manufacturer's recommendations. The Maxima SYBR Green qPCR Master Mix (2X; Fermentas) with appropriate primers (Supplemental Data S1) and the Roche LightCycler 480 qPCR apparatus (Plant Science Institute at Iowa State University) were used to determine relative expression of the genes. Relative expression levels were calculated in comparison with an appropriate control gene (for primer sequences, see Supplemental Data S1) from wild-type plants, and the *ACTIN2* gene (At3g18780; for Arabidopsis) and the *GADPH* gene (for Brachypodium), whose expression levels were not affected, were used as reference genes. Gene expression levels in transgenic plants are presented relative to the expression of the same gene detected in wild-type plants (for which gene expression was set to 1). The comparative threshold cycle method (Schmittgen and Livak, 2008) was used for determining differences between transcript copy numbers in wild-type and transgenic plants.

Arabidopsis Infection with *Botrytis cinerea*

B. cinerea strain SF1 (Lionetti et al., 2007) was grown for 15 d on potato dextrose agar at 39 g L⁻¹ at 23°C with a 12-h photoperiod before spore collection. The spores were harvested by washing the culture surface of the agar and suspended in 5 mL of sterile distilled water. Spore suspensions were filtered through glass wool to remove residual mycelia, and the concentration was determined using a Thoma chamber. To synchronize germination, the conidia, at the concentration of 5 × 10⁵ conidia mL⁻¹, were germinated in potato dextrose broth at 24 g L⁻¹ at room temperature for 3 h. The infection of Arabidopsis leaves was performed as described previously (Ferrari et al., 2008; Manabe et al., 2011). Fully developed leaves were detached from four 6-week-old Arabidopsis plants (three leaves per plant) and grown in a growth chamber maintained at 22°C and 70% relative humidity with a 12-h/12-h light/dark photoperiod. The detached leaves were placed in square petri dishes with petioles embedded in 0.8% agar. Two droplets of spore suspension (5 μ L each) were placed on the surface of each leaf. Mock inoculation was performed using potato dextrose broth. Leaves were incubated at 24°C with a 12-h photoperiod, and lesion diameter was measured 48 hpi. Lesion sizes were measured using ImageJ software (Abramoff and Magalhães, 2004).

Brachypodium Infection with *Bipolaris sorokiniana*

Macroconidia of *B. sorokiniana* strain DSMZ 62608 (kindly provided by Prof. F. Favaron, University of Padua) were produced by culturing the fungus on potato dextrose agar medium before spore collection. The conidia were collected by washing the culture surface with 3 mL of sterile water, and conidia concentrations were estimated by using a Thoma chamber. The infection of Brachypodium leaves was performed as described previously (Peraldi et al., 2011). Twenty-four fully expanded leaves were detached from 60-d-old Brachypodium plants (three leaves per plant) grown under a 16-h/8-h light/dark cycle in a climatically controlled chamber with a relative humidity of 70% at 22°C. The leaves were cut to 5-cm lengths and placed in square petri dishes containing 0.8% agar. Two droplets (10 μ L) of conidial suspension (1 × 10⁶ conidia mL⁻¹), with 0.05% Tween 20, were deposited onto each leaf at a distance of about 2 cm from each other. Mock inoculation was performed using sterile distilled water with 0.05% Tween 20. The plates were incubated at 22°C under a 16-h/8-h light/dark cycle. Disease symptoms were recorded every 24 h, and lesion sizes were measured using ImageJ software (Abramoff and Magalhães, 2004).

Determination of H₂O₂ Accumulation and Callose Deposition

For H₂O₂ visualization, leaves were cut from adult plants and dipped for 16 h in a solution containing 1 mg mL⁻¹ 3,3'-diaminobenzidine, pH 5.0. Chlorophyll was extracted with hot ethanol, and the leaves were photographed. The two more expanded leaves from six independent 4-week-old plants were cleared with 100% ethanol. Leaves were fixed in an acetic acid: ethanol (1:3) solution for 2 h, sequentially incubated for 15 min in 75% ethanol, in 50% ethanol, and in 150 mM phosphate buffer, pH 8.0, and then stained for 2 h at 25°C in 150 mM phosphate buffer, pH 8.0, containing 0.01% (w/v) aniline blue. After staining, leaves were mounted in 50% glycerol and examined by UV2A filter (excitation wavelength = 330–380) using the epifluorescence microscope Nikon Eclipse E200. Photographs were taken with a Nikon Digital Sight DS-Fi1c camera.

Supplemental Data

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

Supplemental Figure S1. In silico identification of Brachypodium genes similar to Arabidopsis *RWA* genes.

Supplemental Figure S2. Infection of transgenic Arabidopsis lines with *P. syringae* and Brachypodium transgenic lines with *X. oryzae*.

Supplemental Figure S3. Analysis of apoplast from Arabidopsis transgenic and wild-type plant inhibition of *B. cinerea* growth.

Supplemental Figure S4. Real-time RT-PCR analysis of selected pathogen-related genes in infected transgenic lines and wild-type plants.

Supplemental Table S1. Monosaccharide composition (mol %) of total cell wall extracted from aerial parts of transgenic plants expressing AnAXE and AnRAE and wild-type plants.

Supplemental Data S1. Primer sequences used in this work.

Supplemental Data S2. pDico_T-vector sequence.

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