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Summary

Cell wall hemicelluloses and pectins are *O*-acetylated at specific positions, but the significance of these substitutions is poorly understood. Using a transgenic approach, we investigated how reducing the extent of *O*-acetylation in xylan affects cell wall chemistry, plant performance and the recalcitrance of lignocellulose to saccharification. The *Aspergillus niger* acetyl xylan esterase *An*AXE1 was expressed in Arabidopsis under the control of either the constitutively expressed 35S CAMV promoter or a woody-tissue-specific GT43B aspen promoter, and the protein was targeted to the apoplast by its native signal peptide, resulting in elevated acetyl esterase activity in soluble and wall-bound protein extracts and reduced xylan acetylation. No significant alterations in cell wall composition were observed in the transgenic lines, but their xylans were more easily digested by a β-1,4-endoxylanase, and more readily extracted by hot water, acids or alkali. Enzymatic saccharification of lignocellulose after hot water and alkali pretreatments produced up to 20% more reducing sugars in several lines. Fermentation by *Trametes versicolor* of tissue hydrolysates from the line with a 30% reduction in acetyl content yielded ~70% more ethanol compared with wild type. Plants expressing *35S:AnAXE1* and *pGT43B:AnAXE1* developed normally and showed increased resistance to the biotrophic pathogen *Hyaloperonospora arabidopsidis*, probably due to constitutive activation of defence pathways. However, unintended changes in xyloglucan and pectin acetylation were only observed in *35S:AnAXE1*-expressing plants. This study demonstrates that postsynthetic xylan deacetylation in woody tissues is a promising strategy for optimizing lignocellulosic biomass for biofuel production.

Introduction

Plant cell wall matrix polysaccharides are typically *O*-acetylated (Gille and Pauly, 2012; Pawar *et al.*, 2013). Homogalacturonan (HG) and rhamnogalacturonan I (RG-I) are acetylated at the *O*-2 and/or *O*-3 positions of galacturonic acid (Ishii, 1997; MacKinnon *et al.*, 2002), and RG-I is also acetylated at the *O*-3 sites of rhamnosyl residues (Voragen *et al.*, 2009). Rhamnogalacturonan II (RG-II) has an acetylation at 2-*O*-Me-Fuc side chain (Gille and Pauly, 2012; Glushka *et al.*, 2003). Xyloglucan is acetylated at the *O*-6 position on side chain galactosyl residues in Arabidopsis (Pauly and Scheller, 2000) and on *O*-6 positions in backbone glucosyl residues in the Solanaceae and Poaceae (Jia *et al.*, 2005). Glucuronoxylan is acetylated at the *O*-2 and/or *O*-3 of xylosyl residues in hardwoods but not in softwoods (Teleman *et al.*, 2000), whereas in (galacto)glucomannan, mannosyl residues are acetylated at *O*-2 and/or *O*-3 (Capek *et al.*, 2002; Teleman *et al.*, 2002; Willfor *et al.*, 2003).

Acetyl groups are transferred from acetyl-CoA to polysaccharides during their biosynthesis in the Golgi, a process in which proteins of the REDUCED WALL ACETYLATION (RWA) and TRICHOME BIREFRINGENCY–LIKE (TBL) families participate. It is thought that RWA proteins are responsible for translocating acetyl-CoA to the Golgi (Gille and Pauly, 2012; Manabe *et al.*, 2011, 2013), and it has recently been demonstrated that different TBL proteins transfer acetyl groups to different specific cell wall polysaccharides (Gille and Pauly, 2012; Gille *et al.*, 2011; Xiong *et al.*, 2013; Yuan *et al.*, 2013). The TBL29 (ESKIMO1) protein can acetylate xylo-oligosaccharides (XOS) in a cell-free system using acetyl-CoA as a donor (Urbanowicz *et al.*, 2014). Postsynthetic deacetylation *in muro* has been reported only for HG and RG-I (Gou *et al.*, 2012); this was catalysed by pectin acetyl esterase (PAE), but two putative acetyl xylan esterases (AXEs) from family CE6 have been also identified in Arabidopsis (http://www.cazy.org/Carbohydrate-Esterases.html).

Acetylation of polysaccharides substantially affects their properties *in vitro* (Pawar *et al.*, 2013), including those relevant to lignocellulose saccharification and biomass decomposition. Deacetylation of xylan, mannan and RGI is a prerequisite for enzymatic degradation by microbial pathogens and saprophytes

(reviewed in (Biely, 2012; Pawar *et al.*, 2013)). To digest plant cell walls, these organisms secrete AXEs, acetyl esterases, rhamnogalacturonan acetyl esterases (RAEs) and acetyl glucomannan esterases, which deacetylate polymeric xylan, xylo-oligosaccharides, RG-I and glucomannan, respectively. Chemical acetylation of wood increases its resistance to microbial attack (Behbood, 2003). It is therefore believed that cell wall acetylation protects against pathogens. However, Arabidopsis plants with reduced acetylation of some cell wall polymers (Manabe *et al.*, 2011; Pogorelko *et al.*, 2013) showed increased resistance to the fungal pathogen *Botrytis cinerea* but not to the bacterial pathogen *Pseudomonas syringae*. These observations reveal the complexity of the relationship between cell wall acetylation and biotic stress resistance *in planta*.

Consistent with acetylation having a protective role against enzymatic hydrolysis *in vitro*, chemical or enzymatic deacetylation of lignocellulose increases sugar yields from enzymatic hydrolysis (Kong *et al.*, 1992; Zhang *et al.*, 2011). Moreover, the presence of acetyl groups inhibits microbial activities during alcoholic fermentation, reducing ethanol yields (Helle *et al.*, 2003; Jönsson *et al.*, 2013; Ranatunga *et al.*, 1997). There is therefore interest in reducing the acetylation of lignocellulose to increase its saccharification and fermentation potential. This has recently been attempted by Pogorelko *et al.* (2011, 2013), who expressed *Aspergillus nidulans* AXE in plants. However, surprisingly, no change in lignocellulose saccharification was observed in the transgenic lines, despite a 50% reduction in cell wall acetylation. Other AXEs from eight different carbohydrate esterase (CE) families, representing a wide spectrum of specificities, enzymatic properties and different catalytic mechanisms (Biely, 2012; Biely *et al.*, 2014; Pawar *et al.*, 2013), have potential for delivering desirable biotechnological effects. In this work, we expressed an *Aspergillus niger* AXE (*An*AXE1) from family CE1 in Arabidopsis to deacetylate xylan. This enzyme is active in the acidic pH range typical of cell walls, and deacetylates polymeric xylan but not pectin (Kormelink *et al.*, 1993; Koutaniemi *et al.*, 2013). We demonstrate here its effects on cell wall chemistry, plant growth and biotic stress resistance. Importantly, we report for the first time that *in planta* deacetylation of xylan using heterologous AXE improves sugar yields in saccharification and substantially increases ethanol yields during fermentation.

Results

Generation of Arabidopsis transgenic lines accumulating *An*AXE1 in cell walls

Sequence analysis shows that *An*AXE1 (An12 g05010) is most similar to CE1 enzymes from *A. ficcum*, *A. awamori* and *Penicillium purpurogenum*, which form a separate clade from the acetyl xylan esterase of *A. nidulans*, an enzyme previously used by Pogorelko *et al.* (2011, 2013), and the AXE of *A. oryzae* (Figure 1).

A cDNA encoding *An*AXE1 with its own signal peptide to direct secretion was expressed in Arabidopsis using a 35S promoter. To verify that the fungal signal peptide targets the protein to the plant cell wall, we expressed the fusion protein *An*AXE1:eGFP in Arabidopsis. After plasmolysis, the GFP signal was detected in the cell walls of transgenic plants (Figure 2a). The native *A. niger* signal peptide of *An*AXE1 is therefore sufficient to target the protein to the apoplast in Arabidopsis.

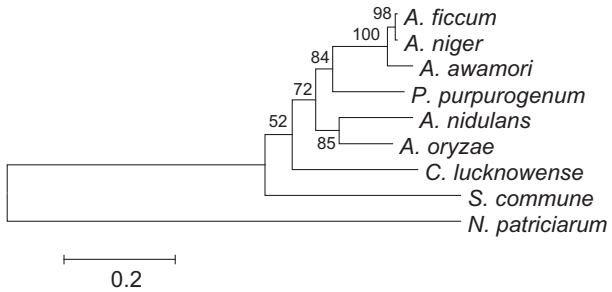

Figure 1 Phylogenetic analysis of AXE from *Aspergillus niger An*AXE1 and its closest CE1 homologs. Evolutionary history was inferred using the neighbour-joining method with MEGA4. ENA/GenBank accession numbers: *A. ficcum*: AAK60128, *A. niger*: CAA01634, *A. awamori*: BAA13434, *P. purpurogenum*: AAM93261, *A. nidulans*: ABF50875, *A. oryzae*: BAD12626, *C. lucknowense*: ADZ98864, *S. commune*: EFJ02032, *N. patriciarum*: ACL68347.

Four independent homozygous Arabidopsis lines (A-D) carrying *35S::An*AXE1 were analysed in the T3 generation. *An*AXE1 transcripts were detected in all lines and were most abundant in line D (Figure 2b). Specific esterase activity in the wall-bound protein fraction was 2.5-, 3-, and 20-fold higher in lines B, C and D, respectively, than in wild type (WT) (Figure 2c). Relatively smaller increases were evident in soluble protein fractions. Thus, *An*AXE1 was expressed in an active form and targeted mainly to cell walls. This did not affect overall plant development (Figure 2d), nor did it induce secondary wall thinning or an 'irregular xylem' phenotype (Figure S1).

Cell wall polymer composition remained unaltered in lines expressing *An*AXE1 but xylan acetyl content was reduced

To test whether overexpression of *An*AXE1 had any impact on lignin composition and relative lignin and carbohydrate content, we analysed inflorescence stems by pyrolysis GC-MS (Gerber *et al.*, 2012). No significant differences were detected between the transgenic lines and the WT (Table S1).

Diffuse reflectance Fourier transform infrared (FTIR) spectroscopy of stem tissues was used to detect differences in the abundance of different bonds in transgenic and WT plants. An OPLS-DA model showed clear separation between the two genotypes with 1 + 1 components having $Q2_{(cum)} = 0.688$ (Figure 3a). Of the main bands that contributed to the separation (Figure 3b), the intensities of those at 1240, 1370 and 1740/cm were more prominent in WT than in transgenic lines. These correspond, respectively, to the C-O stretch, (CH_2) bending and C = O stretch that are found in acetyl esters (Gorzsás *et al.*, 2011). Conversely, the 1620 per cm peak, which corresponds to absorbed water (Gorzsás *et al.*, 2011), was more prominent in transgenic lines than in WT (Figure 3b). These data are consistent with reduced acetyl ester content and an increase in adsorbed cell wall water in transgenic lines.

To quantify the total acetyl content in cell walls, cell wall powder from inflorescence stems was saponified and the acetic acid released was quantified. Line D, the line most strongly expressing *An*AXE1, showed an ~30% decrease in acetic acid content compared with WT, whereas other lines, with weaker transgene expression, showed no significant difference (Figure 3c). Nonetheless, there was a significant overall decrease

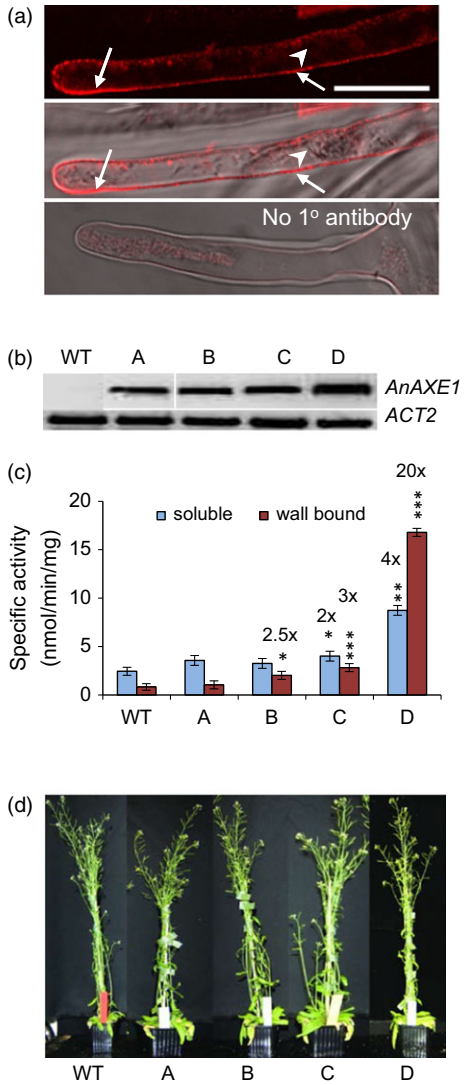

Figure 2 Expression of *An*AXE1 in Arabidopsis and morphology of transgenic plants. (a) Immunolocalization of *An*AXE1:eGFP stably expressed in *Arabidopsis* in plasmolysed root cells. Top: *An*AXE1:eGFP signal (red channel); middle: the same image with superimposed transmitted light signals (grey channel) showing anatomy. Arrowhead: shrunken protoplast; arrow: *An*AXE1:eGFP signal in the cell wall. Bottom: negative control without primary antibody, imaged using the same settings as for the experimental sample above. Bar = 20 μm. (b) Expression of *AnAXE1* gene in inflorescence stems of independent transgenic lines A-D. *ACTIN2* is the loading control. (c) Esterase activity in soluble and wall-bound protein fractions of transgenic and WT plants. Asterisks: lines significantly different from WT (Student's *t*-test; * - $P \leq 0.1$, ** - $P \leq 0.05$ and *** - $P \leq 0.01$) with approx. fold increases shown above the bars. Means ± SD, $n = 2$ biol. replicates. (d) Morphology of transgenic and WT plants.

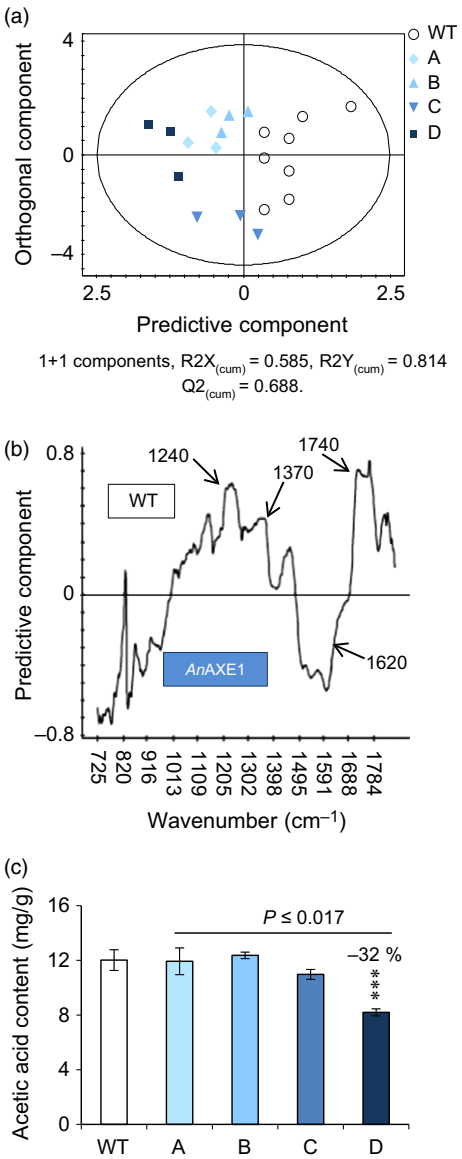

Figure 3 Expression of *An*AXE1 in transgenic plants reduces cell wall *O*-acetylation in inflorescence stems. (a) OPLS-DA scatter plot obtained from FTIR spectral analysis showing separation between WT and transgenic plants. (b) OPLS-DA loading plot representing wavenumbers contributing to the separation. Arrows show ester (1240, 1370 and 1740/cm) and absorbed water (1620/cm) signals. (c) Acetic acid content in cell walls in WT and transgenic lines. Means ± SE, $n = 5$ (WT) or 3 (transgenic lines) biol. replicates. Asterisks: significant differences between individual lines and WT (Student's *t*-test; ***$P \leq 0.01$). *P*: probability in a post-ANOVA contrast analysis between WT and all transgenic lines.

in acetic acid content for all transgenic lines taken together as compared to WT ($P \leq 0.017$).

To investigate further whether *An*AXE1-expressing plants had reduced xylan acetylation, alcohol-insoluble residue of inflorescence stems was subjected to xylan oligosaccharide mass profiling (OLIMP) analysis (Chong *et al.*, 2011, 2014). XOS released by GH10 endo-1,4-β-D xylanase were separated into neutral and acidic fractions. The neutral fraction contained XOS of DP 2-4 having different degrees of acetylation, xylobiose being the most abundant (Figure 4a). A clear decrease in xylobiose acetylation could be observed in the transgenic lines compared with WT, consistent with the AXE activity in these lines. Simultaneously, there was a shift from longer to shorter XOS in the transgenic lines compared with WT. Acidic fraction XOS ranged in DP from 3 to 7, with xylotetraose the most abundant (Figure 4b). Shifts from longer to shorter XOS were

evident in acidic fractions from transgenic lines similar to the neutral fractions. These results indicate that *An*AXE1 reduced xylan acetylation because shorter and less acetylated XOS were formed by xylanase hydrolysis of the transgenic lines expressing this enzyme.

*An*AXE1-expressing plants exhibited increased saccharification

To test the way in which *An*AXE1 expression affected enzymatic saccharification of lignocellulose, dry stems were pretreated with either hot water, acid or alkali, and the reducing sugars released following incubation in a mixture of Celluclast and Novozyme 188 were determined (Gomez *et al.*, 2010). Transgenic lines exhibited higher rates of sugar production than WT after both hot water and alkali pretreatments, showing increases in the amounts of reducing sugars of up to ~20% (Figure 5). Unexpectedly, the improvement in saccharification did not differ among lines having very different levels of *An*AXE1 activity and xylan deacetylation (Figure 5; compare to Figures 2–4).

To understand the effects of *AnAXE1* expression on the efficiency of different pretreatments, we analysed monosaccharide composition in the pretreatment liquids. Each pretreatment apparently extracted different sets of polysaccharides, but the effects of *An*AXE1 expression were consistent in all the pretreatments, resulting in the release of more Xyl and less GalA (Table 1). As in the case of sugar production rates (see above), the extent to which sugars released from transgenic lines during pretreatment differed from those released from WT plants was apparently unrelated to the levels of *An*AXE1 activity in those lines.

In planta deacetylation of the cell wall increases ethanol yields

As it is known that acetic acid released from lignocellulose during saccharification inhibits ethanol production by microorganisms (Jönsson *et al.*, 2013), we carried out an experiment to determine the impact of the ~30% reduction in *in planta* acetylation in line D on the production of ethanol by the fungus *Trametes versicolor*. Fungal cultures were grown for 15 days on hydrolysates of stem lignocellulose from either transgenic or WT plants. Ethanol was detected after 5 days of culture, reaching a plateau between 11 and 15 days (Figure 6a). The ethanol yields at days 5–15 were ~70% higher ($P \leq 0.0001$) when hydrolysates of transgenic plants were used compared to the amount produced from WT material.

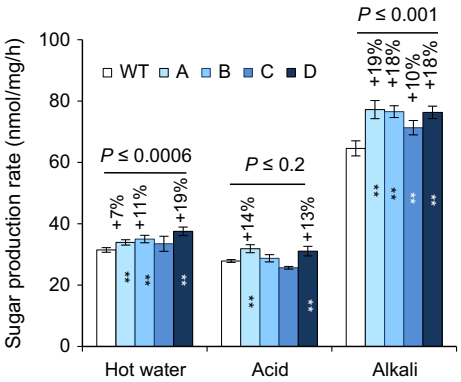

Figure 5 Saccharification of lignocellulose from plants expressing *An*AXE1 and WT plants after pretreatments with hot water, acid and alkali. Means ± SE, $n = 4$ technical replicates representing pool of 30 plants. Asterisks: means significantly different from WT (Student's t-test, ** – $P \leq 0.05$). P: probability in the contrasts between WT and all transgenic lines.

Media from cultures fed with hydrolysates of transgenic plants of line D contained 24% less acetic acid ($P \leq 0.0001$) during the initial phase of fermentation (days 0–7) than cultures fed with WT hydrolysates (Figure 6b). After day 7, the acetic acid concentration decreased in all cultures. Deacetylation of xylan *in planta* can therefore decrease the inhibitory effects of acetic acid in the fermentation medium, substantially increasing ethanol yields.

Plants expressing *An*AXE1 under control of a 35S promoter showed changes in xyloglucan and pectin acetylation

To determine whether AnAXE1 induced any unintended changes in the acetylation of pectin and xyloglucan, we selected the most highly expressing line, D. We hypothesized that unintended

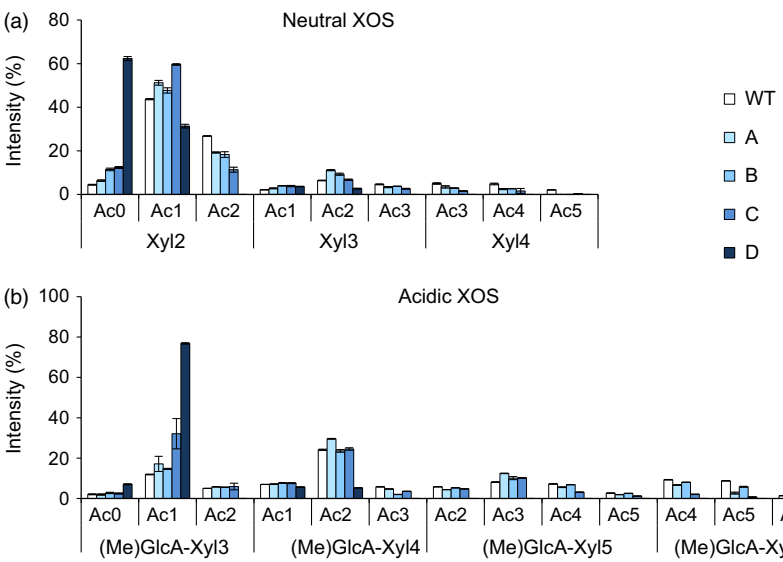

Figure 4 Reduction in xylan acetylation in transgenic lines expressing *An*AXE1 (A-D) compared with WT revealed by xylan OLIMP analysis. Neutral (a) and acidic (b) XOS. The distribution of different XOS species in each sample is shown as a percentage (sum of signals from all XOS in a sample is 100%). Neutral XOS ranged from DP 2 to 4 (Xyl2 – Xyl4) and had 0-5 acetyl groups (Ac0 – Ac5). Acidic XOS ranged from DP 3 to 6 ((Me)GlcA-Xyl3 – (Me)GlcA-Xyl6) and had 0-6 acetyl groups (Ac0 – Ac6). Means ± SE, $n = 3$ biol. replicates representing pools of individual plants.

Table 1 Monosaccharide composition (mol %) of pretreatment liquids from hot water, acid, and alkali pretreatments of lignocellulose from stems of lines expressing *An*AXE1 (A-D) and WT

	Hot water pretreatment					Acid pretreatment					Alkali pretreatment				
Sugar	WT	A	B	C	D	WT	A	B	C	D	WT	A	B	C	D
Fuc	0.3	nd	nd	0.3	0.3	0.7	nd	nd	nd	0.5	0.9**	0.6**	0.6**	0.9	0.7*
Ara	7.7**	5.9***	7.0	6.7*	6.8	11.7**	9.9***	8.2**	10.8	11.0	6.9	6.3**	5.4***	8.2***	7.0
Rha	7.5	7.0	7.4	6.9	7.7	3.9	5.3	1.9**	4.8	2.5	4.6	4.0	3.6*	5.3	3.8
Gal	21.5**	17.4*	16.0**	19.8	16.9*	19.4*	20.2	27.5***	22.4	21.4	17.7***	13.8***	11.9***	14.5***	16.0**
Glc	39.4**	43.8**	45.0**	41.7	42.5	40.0*	46.1***	43.5*	41.0	41.9	23.7***	22.2**	17.8***	23.9	23.6
Xyl	6.6**	11.2**	9.2	10.5*	11**	4.4**	6.9**	7.1**	6.1	6.8*	38.3***	47.8***	55.7***	41.2*	42.5**
Man	12.2	10.6	10.7	11.3	10.8	5.9*	3.9*	3.0**	4.3	5.2	5.8***	4.4***	3.8***	5.3	5.7
GalA	6.7**	4.4*	5.2	3.3**	4.0*	13.9**	7.8***	8.9**	10.3*	10.8	2.2***	0.9***	1.1***	0.7***	0.7***

Values are means from 3 technical replicates representing pool of 30 plants. Asterisks beside means for transgenic lines**:** values significantly different from WT. Asterisks next to WT means: significance of contrast between WT and all lines. Contrasts significant at $P \leq 0.1$ are shaded. *$P \leq 0.1$, **$P \leq 0.05$, ***$P \leq 0.01$, Student's *t*-test. nd, not detected.

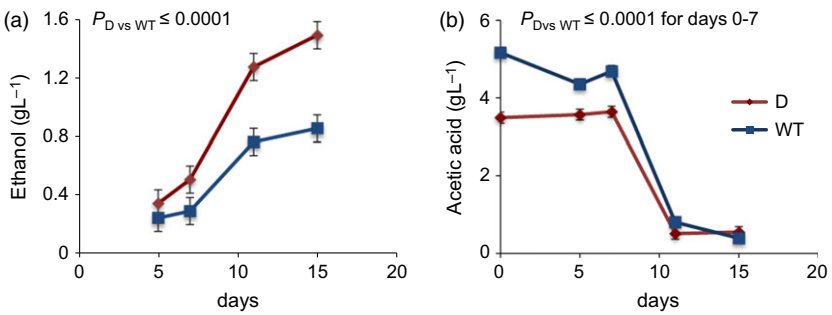

Figure 6 Ethanol production by *Trametes versicolor* from hydrolysates obtained from either transgenic (line D) or WT plants (a) and acetic acid concentration in culture media based on these hydrolysates (b). *P* values correspond to the significance of the genotype effect (F-test in ANOVA), as described in Materials and Methods. Means ± SE, $n = 9$ biol. replicates representing fungal cultures.

changes might be minimized by limiting *AnAXE1* expression to secondary-walled cells. To test this, we created plants expressing *AnAXE1* from the aspen *GT43B* promoter (Ratke *et al.*, 2015). The specificity of this promoter for secondary-walled cells in Arabidopsis was confirmed by histochemical analysis of reporter lines (Figure S2). Of five independent transgenic lines carrying p*GT43B:AnAXE1*, we selected line E, which displayed a significant increase in acetyl esterase activity in the soluble and wall-bound fractions (Figure S3a) and exhibited similar characteristics – reduced cell wall acetylation (Figure S3b), reduced xylan acetylation (Figure S3b), increased saccharification (Figure S4) and unaltered plant morphology (Figure S2d) – as those observed in line D. Thus, the intended effects of *AXE1* expression were similar in lines D and E, regardless of the promoter used.

To investigate changes in xyloglucan acetylation, stems of lines D and E and of WT plants were analysed by xyloglucan OLIMP (Gille *et al.*, 2011; Lerouxel *et al.*, 2002). A clear decrease relative to WT in the signals from acetylated XGOs was seen in line D but not in line E, indicating that xyloglucan acetylation was substantially decreased only when *AnAXE1* was expressed from the constitutive promoter (Figure 7a). To analyse changes in pectin acetylation, alcohol-insoluble residues of rosette leaves were pre-extracted with ammonium formate then digested with pectate lyase, and acetyl contents were determined in all fractions. Acetyl contents were unchanged in the ammonium formate fraction; they were increased in line D but unchanged in line E in the pectate lyase fraction, whereas in the pellet, they were reduced in both transgenic lines compared with WT (Figure 7b). The pectate lyase fraction was rich in GalA, consistent with the expected enrichment for HG oligosaccharides, whereas the pellet was rich in Glc, GalA, Man and Xyl, indicating enrichment for hemicelluloses (Table S2). Thus, plants expressing *35S:AnAXE1*, but not those expressing *GT43B:AnAXE1*, exhibit a compensatory increase in pectin acetylation, and both types of transgenic plants show reduced acetylation of noncellulosic polymers that are resistant to pectate lyase. These results are consistent with our expectation that using a tissue-specific promoter can limit the extent of unintended changes.

Changes in xyloglucan and pectin acetylation suggested the possibility of compensatory mechanisms affecting acetylation in plants expressing *35S:AnAXE1*, similar to those identified in plants expressing 35S: *A. nidulans AXE* (Pogorelko *et al.*, 2013). We therefore investigated expression of the RWA genes in plants expressing *35SAnAXE1* and *pGT43B:AnAXE1*. RT-qPCR analyses showed that *RWA1* was up-regulated in the stems of line D and down-regulated in line E, and *RWA3* was up-regulated in the leaves of line E, whereas *RWA4* was up-regulated in the stems of line D and down-regulated in the leaves of both transgenic lines compared with WT (Figure 8). Thus, both plants with the *35S* promoter and those with the *GT43B* promoter compensate for the effect of *An*AXE1 expression by adjusting their cell wall acetylation machinery.

Responses of transgenic plants expressing *An*AXE1 to different pathogens

As cell wall acetylation can affect plant susceptibility to different pathogens (Manabe *et al.*, 2011; Pogorelko *et al.*, 2013), we

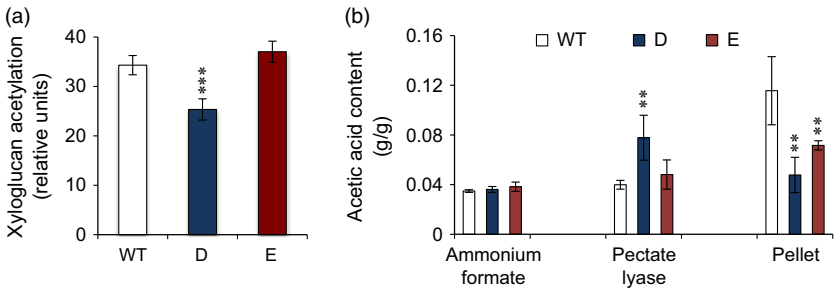

Figure 7 Changes in acetylation of different cell wall polymers in transgenic lines expressing *An*AXE1 (D-E) compared with WT. (a) Xyloglucan OLIMP analysis. n = 5-6 biol. replicates. Xyloglucan acetylation was determined as the ratio of signals from all xylogluco-oligosaccharides having an acetyl group to all oligosaccharides that could potentially accommodate acetylation (having a Gal). (b) Acetic acid content in ammonium formate-soluble polymers, in pectate lyase-released oligosaccharides and in the residual pellet, expressed per unit sugar content. n = 3 biol. replicates. (a–b) Means ± SE; asterisks: means significantly different from WT (Student's *t*-test,** – $P \leq 0.05$; *** – $P \leq 0.01$).

investigated whether *An*AXE1 expression altered the response to the biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* and to the necrotrophic fungal pathogen *Plectosphaerella cucumerina*. The highly expressing lines D and E both exhibited increased resistance to *H. arabidopsidis* (Figure 9a) but not to *P. cucumerina* (data not shown). For comparison, we also analysed disease susceptibilities in a mutant having reduced acetylation across a broad range of cell wall polymers, *rwa2-3* ((Manabe *et al.*, 2011), and in a mutant lacking acetylation specifically in xyloglucan, *axy4-3* (Gille *et al.*, 2011). The *rwa2-3* mutant showed an increase in resistance to *H. arabidopsidis* similar to that of *An*AXE1-expressing plants (lines D and E), whereas the susceptibility of *axy4-3* was similar to that of WT (Figure 9a). These results suggest that the decrease in xylan, but not xyloglucan, acetylation probably plays a role in susceptibility to *H. arabidopsidis*.

To check whether activation of basal immunity might be responsible for increased resistance to *H. arabidopsidis*, we analysed the basal expression levels, in the absence of pathogen, of four defence-related genes, *PATHOGEN RELATED 5* (*PR5,* At1G75040), β-*1,3-GLUCANASE 2* (*bG2,* At3G57260), W-box-containing transcription factor 40 (*WRKY40,* At1G80840) and *PHYTOALEXIN DEFICIENT3* (*PAD3*, At3G26830), in rosette leaves of transgenic and WT plants. The only substantial difference was that *WRKY40* was up-regulated in line E, but we also observed a tendency for *WRKY40* to be up-regulated in other acetylation-deficient mutants that were tested (Figure 9b) and up-regulation of *PR5* in the *rwa2-3* mutant. In contrast, *PAD3* was slightly down-regulated, but only in *35S:AnAXE1*-expressing plants.

Discussion

*An*AXE1 expressed in Arabidopsis reduces acetylation in xylan and xyloglucan and triggers compensatory acetylation of pectins

AXEs from the CE1 family belong to a group of serine esterases with relatively broad substrate specificities which include acetylated hexosides at positions 2 and 3 (Biely, 2012). Purified *An*AXE1 has been reported to deacetylate xylan *in vitro* (Kormelink *et al.*, 1993; Koutaniemi *et al.*, 2013). When constitutively expressed in Arabidopsis, the enzyme not only deacetylated xylan but also triggered xyloglucan deacetylation (Figures 4 and 7). Xyloglucan acetyl esterase activity has not previously been reported for any CE family member, but given that CE1 enzymes can deacetylate cellulose acetate, it is possible that these enzymes could deacetylate Gal at position 3 in xyloglucan (Biely *et al.*, 2014). However, we cannot exclude that xyloglucan deacetylation have occurred by an indirect mechanism.

Deacetylation of xyloglucan in *AnAXE1*-expressing plants contrasts with results obtained with *A. nidulans* AXE (Pogorelko *et al.*, 2013). As expression strategies were similar, and levels of cell wall deacetylation were comparable, in both cases, *A. nidulans* AXE and *A. niger* AXE1 may have different properties despite their overall 80% identity at the amino acid levels.

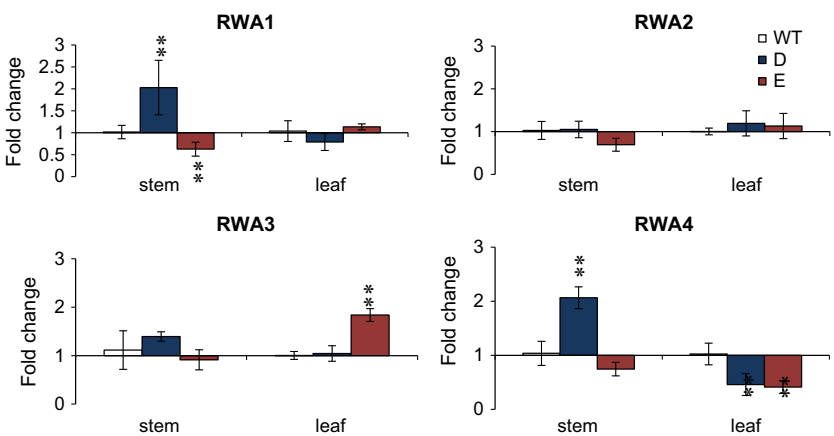

Figure 8 Expression of RWA genes in *AnAXE1*-expressing plants (Lines D and E) compared with WT. Means ± SE, n = 3 biol. replicates. Values significantly different from WT are marked by asterisks, ** – $P \leq 0.05$, Student's *t*-test.

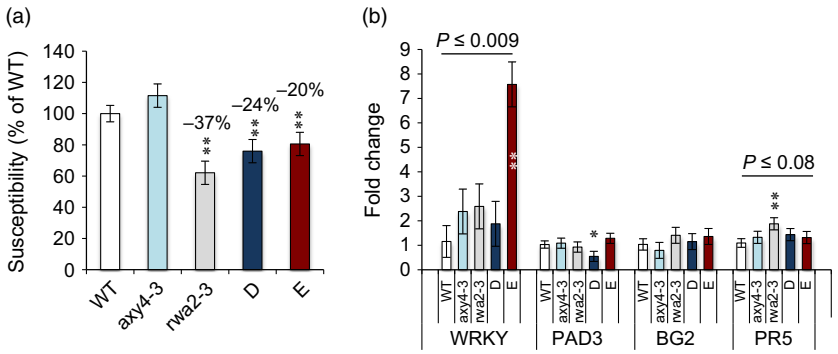

Figure 9 Resistance of acetylation-challenged lines expressing *AnAXE1* (D and E) and acetylation mutants to biotic stress. (a) Susceptibility to *Hyaloperonospora arabidopsidis* monitored by the number of conidiospores per plant fresh weight, expressed as % of WT. Means ± SE; $n = 10$ biological replicates. (b) Changes in the constitutive levels of transcripts of defence-related genes in leaves, determined by RT-qPCR. Means ± SE; $n = 3$ biol. replicates. (a, b) Asterisks: means significantly different from WT (Student's *t*-test; ** – $P \leq 0.05$; * – $P \leq 0.1$). P: probability in the contrast analysis between WT and transgenic lines and mutants.

The compensatory increase in pectin acetylation observed in the leaves of plants ectopically expressing *AnAXE1* suggests 'acetylation homeostasis' and mirrors changes in acetylation patterns observed when poplar *PAE1* was overexpressed in tobacco (Gou *et al.*, 2012). We saw no increase in pectin acetylation or decrease in xyloglucan acetylation when *An*AXE1 was expressed from the aspen *GT43B* promoter (Figure 7), probably because this promoter is active in secondary-walled tissues where pectins and xyloglucan are of low abundance. However, changes in expression of *RWA1* (in stems), and *RWA3* and *RWA4* (in leaves) were observed when *An*AXE1 was expressed from the *GT43B* promoter (Figure 8). Similarly, *A. nidulans* AXE and RAE induced expression of different RWA genes (Pogorelko *et al.*, 2013). This suggests that acetylation homeostasis may be maintained for different polymers and tissues using a number of pathways. Compensatory acetylation could also involve regulation via an alteration in the acetyl pool in the Golgi and/or in cell walls.

Plant growth is not affected by *An*AXE1 expression

AnAXE1-expressing plants exhibited no growth defects or abnormalities of the xylem cells, despite a one-third reduction in the total acetyl content. This indicates that plants can tolerate quite large postsynthetic reductions in xylan acetylation. However, we cannot rule out potential problems that may affect the performance of plants grown under field conditions. Results from other studies (Gille *et al.*, 2011; Gou *et al.*, 2012; Manabe *et al.*, 2013; Pogorelko *et al.*, 2013; Xiong *et al.*, 2013; Yuan *et al.*, 2013) suggest that the maintenance of acetyl groups is essential in some polymers, such as HG, but not in others such as xyloglucan, and that xylan deacetylation is tolerated better by plants when acetylation is reduced postsynthetically than when the reduction takes place during biosynthesis. Thus, postsynthetic xylan deacetylation, especially combined with tissue-specific expression, is attractive as a potential route for attaining low xylan acetylation levels and maintaining good plant productivity.

Plants expressing *AnAXE1* exhibited increased resistance to the biotrophic pathogen *H. arabidopsidis*

Plants expressing *AnAXE1*, from either constitutive or woody tissue-specific promoters, exhibited enhanced resistance to the biotrophic oomycete *H. arabidopsidis* (previously known as *Peronospora parasitica)* (Figure 9a), whereas their resistance to the necrotrophic fungus *P. cucumerina* was unaffected. These results confirm the relevance of polysaccharide acetylation to plant resistance to pathogens (Manabe *et al.*, 2011; Pogorelko *et al.*, 2013). As examples, an Arabidopsis *rwa2* mutant, affected in pectin/xyloglucan/xylan acetylation (Manabe *et al.*, 2011), and a *pmr5* (*tbl44*) mutant, affected in pectin esterification (Manabe *et al.*, 2011; Vogel *et al.*, 2004), show increased resistance to the necrotrophic fungal pathogen *B. cinerea* and to powdery mildew, respectively. However, the resistance of *pmr5* to *H. arabidopsidis* is not affected. Remarkably, we found that the *rwa2-3* mutant also showed enhanced resistance to *H. arabidopsidis*, whereas *axy4* plants, which are defective in xyloglucan acetylation (Gille *et al.*, 2011), had similar susceptibility to that of the WT (Figure 9a). Thus, the increased resistance to *H. arabidopsidis* observed in *rwa2-3* mutants and *AnAXE*-expressing plants is probably related to xylan deacetylation, the only change common to both these lines.

Our findings (Figure 9b) and previous results (Pogorelko *et al.*, 2013) indicate that xylan deacetylation increases the basal expression of *WRKY40,* a transcription factor involved in the pathogen attack-related mitogen response and ABA and SA signalling (Chen *et al.*, 2010; Xu *et al.*, 2006) and of *PR5*, which participates in SA signalling (El-Kereamy *et al.*, 2011). As previously reported for the *rwa2* mutant (Manabe *et al.*, 2011), we did not observe up-regulation of *PAD3*, which encodes an enzyme involved in camalexin biosynthesis (Ferrari *et al.*, 2007; Glawischnig *et al.*, 2004); this gene was down-regulated in constitutively expressing *AnAXE1* plants (Figure 9b). These results are consistent with several scenarios that could explain the reduced susceptibility to *H. arabidopsidis* of plants with reduced xylan acetylation. The resistance could be related to deacetylation-related changes in the cell wall or cuticle that make them more resistant to pathogen attack. Deacetylated xylan could be hydrolysed more easily by cell-wall-residing endohydrolases or endotransglucosylases to release oligosaccharides, which are recognized by the plant as damage-associated molecular patterns (DAMPs) and thus trigger defence responses. Finally, the bioactivity of oligosaccharides may vary according to the extent of acetylation, as was observed in wheat (Randoux *et al.*, 2010). Ferrari *et al.* (2007, 2008) showed that transgenic plants expressing microbial polygalacturonase (PG), which were less

susceptible to *B. cinerea*, had constitutively activated defence responses. These responses included an increase in oligogalacturonides (OGs)-induced basal resistance, which was independent of the JA, SA and ethylene pathways but required *PAD3* and was correlated with increased levels of H_2O_2 and callose. Similarly, Arabidopsis plants expressing *AnRAE* had higher levels of *PAD3* transcripts, and accumulated H_2O_2 and callose (Pogorelko *et al.*, 2013), suggesting a link between pectin acetylation and the *PAD3* pathway. The compensatory increase in pectin acetylation seen in *35S:AnAXE1*-expressing plants might therefore trigger additional pathways in these plants that are not directly related to xylan deacetylation-triggered defences.

Plants expressing *An*AXE1 produce lignocellulose with improved potential for saccharification and bioethanol production

Although chemical deacetylation of lignocellulose is known to increase yields of xylose and glucose (Kong *et al.*, 1992), recent reports have shown that mutants and transgenic plants impaired in hemicellulose acetylation do not produce the expected effect. For example, quadruple *rwa1 rwa2 rwa3 rwa4* mutants with an ~40% reduction in cell wall *O*-acetylation (Lee *et al.*, 2011), *tbl29* mutants with a 50% decrease in xylan and mannan *O*-acetylation (Xiong *et al.*, 2013), and plants expressing *A. nidulans* AXE resulting in a 50% reduction in acetylation (Pogorelko *et al.*, 2011; 2013) showed no improvement in enzymatic sugar release. These results contrast with our findings that when *An*AXE1 was expressed in Arabidopsis, reducing xylan acetylation and the overall cell wall acetyl content by 0 to ~30% (Figures 3 and 4 and S3), the lignocellulose enzymatic saccharification after hot water, acid and alkali pretreatments was increased (Figures 5 and S4). Surprisingly, even transgenic lines with relatively low levels of *An*AXE1 expression and cell wall deacetylation showed substantial improvements in rates of sugar production after alkali pretreatments. Although the reason for this is unknown, we presume that extensive deacetylation of xylans might increase interaction between xylans and cellulose microfibrils, resulting in recalcitrance to saccharification. This would not apply to HG or RG-I backbones, which are not predicted to interact with cellulose. Consistent with this reasoning, no positive effect on xylan hydrolysis was seen in lines with high levels of xylan deacetylation, whereas pectin hydrolysis was improved when RG-I was extensively deacetylated by *An*RAE (Pogorelko *et al.*, 2013).

By what mechanism could a decrease in xylan acetylation improve saccharification after alkali pretreatment, even though acetyl ester linkages would be broken during this pretreatment so that it should no longer matter how many of them there were on the xylan backbone? From the composition of the pretreatment liquids (Table 1), it appears that deacetylation of xylans facilitates their extraction by all the pretreatments tested here, while inhibiting pectin extraction. The effects on pectin might result from a compensatory increase in HG acetylation (Figure 7). These observations suggest that altering polymer acetylation induces changes, of an as yet unknown nature, in cell walls. For example, it could affect the formation of lignin–carbohydrate complexes, making xylan more extractable and HG more cross-linked, consistent with the altered acetylation levels of the two polymers. It could also affect ester linkages in other polymers not tested here. Although the exact mechanism needs to be established, our work demonstrates that expressing *An*AXE1 *in planta* improves the extractability of xylans under a variety of conditions, a finding which has positive implications for cellulose conversion efficiency.

We further confirmed the predicted positive effect of xylan deacetylation, and overall cell wall deacetylation, on ethanol yields (Figure 6a). *Trametes versicolor*, which can ferment both glucose and xylose to ethanol (Kudahettige *et al.*, 2012), produced almost 70% more ethanol when provided with cell wall hydrolysates from *An*AXE1 plants compared with WT. Moreover, a substantial reduction in acetic acid levels was observed in cultures grown on transgenic lignocellulose compared with WT (Figure 6b). These results support the hypotheses that XOS with fewer acetyl groups would be more accessible to enzymes secreted by fungi, resulting in better hydrolysis, and/or that they would yield less acetic acid, a potent inhibitor of microbial fermentation (Jönsson *et al.*, 2013). This is the first report showing that AXE overexpression in plants can increase saccharification and fermentation potential.

In conclusion, we have shown that overexpression of *An*AXE1 in Arabidopsis increases the saccharification efficiency after different pretreatments by increasing xylan solubility, and substantially increases ethanol production by *Trametes versicolor*; however, the level of xylan acetylation should ideally be optimized to avoid it precipitating and interacting with cellulose. We suggest that postsynthetic modification of cell wall polymers has promise in the field of cellulose-based biofuel production.

Experimental procedures

Generation of Arabidopsis lines expressing *AnAXE1*

A cDNA clone encoding AnAXE1 (CAA01634), obtained from the Centre for Structural and Functional Genomics at Concordia University, Canada (Semova *et al.*, 2006), was cloned into the vector pENTR/D-TOPO using primers Fc2f1 and Fc2r1s (Table S3), and then into the binary vectors pK7FGW2.0 and pK2GW7 for GFP localization and overexpression in Arabidopsis (Karimi *et al.*, 2002), respectively, using a Gateway® System (Life Technologies™, Stockholm, Sweden). The *pGT43B:AnAXE1* vector was obtained by subcloning the same pEntry clone into the destination vector pK-pGT43B-GW7 (Ratke *et al.*, 2015). Arabidopsis thaliana (Col0) was transformed by Agrobacterium (GV3101) infiltration using the floral dip method (Clough and Bent, 1998), and homozygous transgenic plants were selected on half-strength MS plates containing kanamycin (50 μg/mL). Arabidopsis plants were grown under 16-h light/8-h dark cycle, 150 $\mu E/m^2/s$, at 22 °C and 70% humidity for 8 weeks.

Cellular localization of *An*AXE1:GFP

Seven-day-old seedlings were plasmolysed in 20% mannitol, fixed in 2% w/v paraformaldehyde in microtubule-stabilizing buffer (MTSB) (50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$, pH 7) supplemented with 0.1% v/v Triton X-100 for 1 h, washed in water, treated with 100% methanol for 20 min and gradually rehydrated to a final methanol concentration of ~20% (v/v). Cell walls were digested with 0.2% w/v driselase and 0.15% w/v macerozyme in 2 mM MES pH 5.5 for 30 min at 37 °C, washed in water and permeabilized with 3% w/v IGEPAL® CA-630 and 10% w/v DMSO in MTSB for 30 min at 37 °C. Seedlings were washed in MTSB, blocked with 3% w/v bovine serum albumin (BSA) in MTSB for 20 min, incubated for 2 h at 37 °C in rabbit anti-GFP antibody (Torrey Pines Biolabs Inc. CATTP401, Secaucus, NJ) diluted 1:1000 in MTBS containing 1% w/v BSA, washed in MTSB and incubated for 1 h at 37 °C in goat anti-rabbit IgG-DyLight™ 549 conjugate (Agrisera, A509 642, Vännäs, Sweden), diluted 1: 2000 in MTBS and 1% w/v BSA. After washing in MTSB,

seedlings were mounted in Citifluor AF1 (Agar Scientific, Stansted, UK) and observed with a Leica TCS SP2 AOBS confocal laser scanning system (Leica Microsystems, Mannheim, Germany) at an excitation wavelength of 561 nm with detection by sequential line scanning between 540 and 600 nm.

Enzyme activity

Soluble and wall-bound proteins were extracted from stems as described by Biswal *et al.* (2014). Acetyl esterase activity was tested at 37°C using 0.1 M 4-nitrophenyl acetate in 25 mM sodium phosphate buffer, pH 7.0, as substrate (Chung *et al.*, 2002). Total protein concentration was determined using a Bradford assay. Specific activity was expressed as nmol of 4-nitrophenol released per min/mg protein.

RNA extraction and RT-PCR

Total RNA was extracted using TRIzol® Reagent (Ambion 15596-026, Foster City, CA) and treated with a DNA-free™ Kit (Ambion), and cDNA was synthesized using iScript™ (BIO-RAD Laboratories AB, Sundbyberg, Sweden). qPCR was performed using master mix from an IQ™ SYBR® Green Supermix (BIO-RAD) in a Light Cycler 480 II (Roche, Rotkreuz, Switzerland). Two reference genes, EF1 (At5 g60390) and ubiquitin (At3 g62250), were chosen for normalization using geNorm (Vandesompele *et al.*, 2002). Relative expression was calculated using the ΔΔCt method with a fixed efficiency value of 2 (Hellemans *et al.*, 2007; Pfaffl, 2001). Primers used for RT-PCR, and efficiencies, are given in Table S3. For semi-quantitative PCR, loading was adjusted according to *ACTIN2* (At3 g18780).

Cell wall analysis

The basal 10 cm of inflorescence stems and rosette leaves were freeze-dried and milled as previously described (Gandla *et al.*, 2015). Stem powder was analysed by pyrolysis GC-MS (Gandla *et al.*, 2015). The alcohol-insoluble residue (AIR) was prepared according to Biswal *et al.* (2014), and monosaccharide composition was analysed by the trimethylsilyl method (Gandla *et al.*, 2015). The cell wall acetyl content was determined according to Gille *et al.* (2011) using an acetic acid analysis kit (Megazyme, K-ACET, Wicklow, Ireland). FTIR spectroscopy of milled stems was carried out according to Ratke *et al.* (2015).

Determination of xylan and xyloglucan acetylation using OLIMP

AIR from stem tissues was digested with GH10 endo-1,4-β-D xylanase from *Aspergillus aculeatus* (a gift from Novozyme). The resulting hydrolysate was desalted and separated into neutral and acidic fractions on Hypersep Hypercarb Porous Graphitized Carbon (PGC) columns (Thermo Scientific, Waltham, MA), using 50% acetonitrile and 50% acetonitrile containing 0.05% of trifluoroacetic acid, respectively. The two fractions were analysed by AP-MALDI-ITMS as described by Chong *et al.* (2011).

Basal stem tissues were ground in 96% ethanol using ceramic beads, left for 10 min at 80 °C, and the pellet was washed with ethanol and dried overnight at room temperature. One hundred micrograms of the pellet was digested with an endoglucanase and the released oligosaccharides were analysed using MALDI-TOF-MS as described by Lerouxel *et al.* (2002).

Determination of pectin acetylation

Destarched AIR from leaves was extracted in 50 mM ammonium formate buffer, pH 4.5, at 37°C overnight, with shaking at 250 rpm. The extract was separated from the pellet by centrifugation (20 min, 18620 × g) as described by Gille *et al.* (2011). The pellet was digested with *Cellvibrio japonicus* pectate lyase (Megazyme, Wicklow, Ireland) in CAPS buffer (50 mM *N*-cyclohexyl-3-aminopropanesulfonic acid, pH 10) at 40°C for 6 h. The enzyme was deactivated at 100°C for 15 min. The supernatant was separated from the pellet by centrifugation (18620 × g, 15 min) and pooled with two subsequent water washes (PL fraction). The supernatants and remaining pellet were freeze-dried. The acetyl content and sugar composition of all fractions were determined using an acetic acid kit (Megazyme) and the trimethylsilyl method, respectively.

Saccharification efficiency and monosaccharide composition of pretreatment liquors

Saccharification assays were performed after three types of pretreatment using 4 technical replicates consisting of 30 basal stems of 8-week-old plants. The stems were pretreated at 90 °C for 30 min in either water, 0.4 N H_2SO_4 or 0.4 N NaOH, washed ×5 with 25 mM Na acetate buffer at pH 4.5 and subjected to 8 h of saccharification using 7 FPU/g of a 4:1 mixture of Celluclast and Novozyme 188 (Novozymes, Denmark). After hydrolysis, reducing sugars were determined using MBTH (Gomez *et al.*, 2010). The pretreatment liquid was collected, neutralized and subjected to hydrolysis with 2 M trifluoroacetic acid. The monosaccharide composition was determined by HPAEC using Dionex ICS-3000 chromatography as described in Jones *et al.* (2003).

Fermentation assay using *Trametes versicolor*

Three portions, three grams each, of ground stems from approx. 60 plants for each genotype (line D or WT) were hydrolysed with 28 mL of 1% H_2SO_4 at 120 °C and 1.01 atm. The hydrolysates were centrifuged (3100 × g, 15 min), the pH of the supernatant was adjusted to 10.0, and material precipitating overnight at 4°C was removed by centrifugation as above. The pH was adjusted to 6.0, followed by centrifugation as above. The three supernatants thus obtained were used as substrates for fermentation. A liquid culture of *Trametes versicolor* maintained as described by Kudahettige *et al.* (2012) was washed in sterile water before being added to the hydrolysed plant material. Three cultures were established for each hydrolysate, each using 3 g/L of *T. versicolor*, and samples of the medium were collected after 0, 5, 7 and 15 days of fermentation to analyse ethanol (Holmgren and Sellstedt, 2008; Kudahettige *et al.*, 2012) and acetic acid content, as described above. Data were analysed by four-way nested ANOVA, with *genotype*, *hydrolysate* (nested in *genotype)*, *culture* (nested in *hydrolysate)* and *day* (nested in *culture)* as factors.

Pathogen inoculation assays

Fifteen-day-old Arabidopsis plants grown under 10-h light + 14-h dark cycles at 22 °C were inoculated with *H. arabidopsidis Noco* spores (5×10^4/mL) as described by Hernandez-Blanco *et al.* (2007). Conidiospores were counted 7 days postinoculation (dpi) using a Neubauer improved cell counting chamber with 10× objective magnification. Three experiments were performed; they gave similar results. Susceptibility to the *P. cucumerina* isolate, BMM was tested according to Delgado-Cerezo *et al.* (2012): 18-day-old plants grown as above were sprayed with a fungal spore suspension (6×10^6/mL), and the disease rating (DR), ranging from 0 (no symptoms) to 5 (dead plant), was determined for each genotype at 3, 5 and 7 dpi. This experiment was repeated twice with similar results.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Anatomy of the stem in WT and transgenic lines.
Figure S2 GUS expression driven by the aspen *pGT43B* promoter in Arabidopsis.
Figure S3 Characterization of transgenic line E expressing *pGT43B:AnAXE1*.
Figure S4 Saccharification of lignocellulose from plants expressing *AnAXE1* and WT plants after pretreatments with hot water and acid.
Table S1 Relative carbohydrate and lignin contents in transgenic and WT plants determined by pyrolysis GC-MS.
Table S2 Monosaccharide composition of pectate lyase and pellet fractions from transgenic and WT plants.
Table S3 Primer sequences used for cloning and qPCR analysis.