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Mohan-Anupama Pawar, Prashant

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Expression of fungal acetyl xylan esterase in Arabidopsis thaliana improves saccharification of stem lignocellulose

Prashant Mohan-Anupama Pawar1, Marta Derba-Maceluch1, Sun-Li Chong2, Leonardo D. Gómez3, Eva Miedes4, Alicja Banasiak5, Christine Ratke1, Cyril Gaertner6, Grégory Mouill6, Simon J. McQueen-Mason3, Antonio Molina3, Anita Sellstedt7, Maija Tenkanen2 and Ewa J. Mellerowicz1,*

1Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, Umeå Plant Science Centre, Umeå, Sweden
2Department of Food and Environmental Sciences, University of Helsinki, Helsinki, Finland
3Center for Novel Agricultural Products Department of Biology, University of York, York, UK
4Centro de Biotecnología y Genómica de Plantas (UPM-INIA), Universidad Politécnica de Madrid, Pozuelo de Alarcón, Madrid, Spain
5Institute of Experimental Biology, University of Wroclaw, Wroclaw, Poland
6Institut Jean-Pierre Bourguin UMR 1318 INRA/AgroParisTech, Saclay Plant Sciences, Centre de Versailles-Grignon, Versailles Cedex, France
7Department of Plant Physiology, Umeå University, Umeå Plant Science Centre, Umeå, Sweden

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 AnAXE1 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 xyans 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 xylglucan 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.

Keywords: acetyl xylan esterase, biofuels, saccharification, O-acetylation, glucuronoxylan, secondary cell wall.

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). Xylglucan is acetylated at the O-6 position on side chain galactosyl residues in Arabidopsis (Pauly and Scheller, 2000) and on O-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 (Telemann et al., 2000), whereas in (galacto)glucomannan, mannosyl residues are acetylated at O-2 and/or O-3 (Capek et al., 2002; Telemann 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 ACETYLYATION (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 acylate xylo-oligosaccharides (XOS) in a cell-free system using acetyl-CoA as a donor (Urbanowicz et al., 2014). Postsynthetic deacetylation 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.
Botrytis cinerea pathogen (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 glucuronan esterases, which deacylate polymeric xylan, xylo-oligosaccharides, RG-I and glucuronan, 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 plants.

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 (AnAXE1) from family CE1 in Arabidopsis to deacetylate xylan. This enzyme is active in the acidic pH range typical of cell walls, and deacylates 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 AnAXE1 in cell walls

Sequence analysis shows that AnAXE1 (An12 g05010) is most similar to CE1 enzymes from A. ficuum, 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 AnAXE1 with its own signal peptide to direct secretion was expressed in Arabidopsis using a 3SS promoter. To verify that the fungal signal peptide targets the protein to the plant cell wall, we expressed the fusion protein AnAXE1: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 AnAXE1 is therefore sufficient to target the protein to the apoplast in Arabidopsis.

Four independent homozygous Arabidopsis lines (A–D) carrying 3SS:AnAXE1 were analysed in the T3 generation. AnAXE1 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- to 3-fold higher in lines B and D, respectively, than in wild type (WT) (Figure 2c). Relatively smaller increases were evident in soluble protein fractions. Thus, AnAXE1 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 AnAXE1 but xylan acetyl content was reduced

To test whether overexpression of AnAXE1 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 $Q^2$ (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, (CH2) 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 AnAXE1, 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.
in acetic acid content for all transgenic lines taken together as compared to WT ($P \leq 0.017$).

To investigate further whether AnAXE1-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

![Figure 2](image)

**Figure 2** Expression of AnAXE1 in Arabidopsis and morphology of transgenic plants. (a) Immunolocalization of AnAXE1:eGFP stably expressed in Arabidopsis in plasmolyzed root cells. Top: AnAXE1:eGFP signal (red channel); middle: the same image with superimposed transmitted light signals (grey channel) showing anatomy. Arrowhead: shrunken protoplast; arrow: AnAXE1: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](image)

**Figure 3** Expression of AnAXE1 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.
evident in acidic fractions from transgenic lines similar to the 
neutral fractions. These results indicate that AnAXE1 reduced 
xylan acetylation because shorter and less acetylated XOS were 
formed by xylanase hydrolysis of the transgenic lines expressing 
this enzyme.

**AnAXE1-expressing plants exhibited increased saccharification**

To test the way in which AnAXE1 expression affected enzymatic 
saccharification of lignocellulose, dry stems were pretreated with 
ether 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 AnAXE1 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 monosaccha-
dride composition in the pretreatment liquids. Each pretreatment 
apparently extracted different sets of polysaccharides, but the 
effects of AnAXE1 expression were consistent in all the pretreat-
ments, resulting in the release of more Xyl and less GaIA (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 AnAXE1 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 
(Johnsson 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.

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 
ed with WT hydrolysates (Figure 6b). After day 7, the 
acetic acid concentration decreased in all cultures. Deacet-
ylation of xylan in planta can therefore decrease the inhibitory 
effects of acetic acid in the fermentation medium, substantially 
increasing ethanol yields.

**Plants expressing AnAXE1 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
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 was confirmed by histochemical analysis of reporter hydrolysates (b).

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 35S:AnAXE1 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 AnAXE1 expression by adjusting their cell wall acetylation machinery.

Responses of transgenic plants expressing AnAXE1 to different pathogens

As cell wall acetylation can affect plant susceptibility to different pathogens (Manabe et al., 2011; Pogorelko et al., 2013), we
investigated whether AnAXE1 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 cell wall deacetylation were comparable, in both cases, to the necrotrophic fungal pathogen *Plectosphaerella cucumerina*.

To check whether activation of basal immunity might be responsible for increased resistance to *H. arabidopsidis*, we assessed the basal expression levels, in the absence of pathogen, of four defence-related genes, PATHOGEN RELATED 5 (PR5, At1G80840), WRKY40 (At1G80840), W-box-containing transcription factor 40 (Gille 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).

*Figure 7* Changes in acetylation of different cell wall polymers in transgenic lines expressing AnAXE1 (D-E) compared with WT. (a) Xyloglucan acetylation was determined as the ratio of signals from all xyloglucan-oligosaccharides having an acetyl group to all oligosaccharides that could potentially accommodate acetylation (having a Gal). 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 \(\leq 0.01\)).

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 AnAXE1 has been reported to deacetylate xylan in *vivo* (Kormelink et al., 1993; Koutaniemi et al., 2013). As expression strategies were similar, and levels of expression of RWA genes in *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.
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 AnAXE1 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 AnAXE1 was expressed from the GT43B promoter (Figure 7). 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 AnAXE1 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 andABA 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 H₂O₂ and callose. Similarly, Arabidopsis plants expressing AnRAE had higher levels of PAD3 transcripts, and accumulated H₂O₂ and callose (Pogorelko et al., 2013), suggesting a link between pectin acetylation and the PAD3 pathway. The compensatory increase in pectin acetylation seen in AnAXE1-expressing plants might therefore trigger additional pathways in these plants that are not directly related to xylan deacetylation-triggered defences.

Plants expressing AnAXE1 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 nva1 nva2 nva3 nva4 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 AnAXE1 was expressed in Arabidopsis, reducing xylan acetylation and the overall cell wall acetyl content by 0 to ~30% (Figures 3 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 AnAXE1 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 xyans might increase interaction between xyans 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 AnRAE (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 xyans 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 AnAXE1 in planta improves the extractability of xyans 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 AnAXE1 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 XCI 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 AnAXE1 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 (CAAA01634), obtained from the Centre for Structural and Functional Genomics at Concordia University, Canada (Sembola 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). Arabidopis plants were grown under 16-h light/8-h dark cycle, 150 µE/m²/s, at 22 °C and 70% humidity for 8 weeks.

Cellular localization of AnAXE1: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 MgSO₄, pH 7) supplemented with 0.1% w/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. CATTIP041, 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 (Gandia et al., 2015). Stem powder was analysed by pyrolysis GC-MS (Gandia 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 (Gandia 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% 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 microwells 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 H2SO4, or 0.4 N NaOH, washed with 25 mM Na acetate buffer at pH 4.5 and subjected to 8 h of saccharification using 7 FPUL 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 HPAC 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% H2SO4 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 hydrolysate plant material. Three cultures were established for each hydrolysate, each using 3 gF 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 × 106/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. cactorum 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 × 106/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.

References
Expression of AnAXE1 in Arabidopsis


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 pectin fractions from transgenic and WT plants.

Table S3 Primer sequences used for cloning and qPCR analysis.