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Combination of Pretreatment with White Rot Fungi and Modification of Primary and Secondary Cell Walls Improves Saccharification

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Abstract Plant cell walls have protective and structural functions conferring resistance to degradation. The lignin and hemicellulose network surrounding the cellulose microfibrils is insoluble unless subjected to harsh treatments. As lignin, pectin and xylan are effective barriers to cellulose extraction and hydrolysis, reducing their presence in cell walls improves saccharification. Microorganisms that can depolymerise lignin are of extreme interest to the biofuel industry. White rot fungi can be effective in pretreatment of lignocellulosic biomass prior to saccharification. Here, we show the cumulative effects of pretreating biomass with two white rot fungi, *Phanerochaete chrysosporium* and *Trametes cingulata*, on tobacco lines with reduced lignin or xylan, caused by suppression of the *CINNAMOYL-CoA REDUCTASE*, *CINNAMATE-4-HYDROXYLASE*, *TOBACCO PEROXIDASE 60* or *UDP-GLUCURONATE DECARBOXYLASE* and on *Arabidopsis thaliana* with reduced de-esterified homogalacturonan content, obtained by overexpressing a pectin methyl esterase inhibitor or constitutively expressing the *Aspergillus nigerPOLYGALACTURONASE II* gene. Tests were extended to fresh material from an *Arabidopsis* mutant for a cell wall peroxidase. We demonstrate that fungal pretreatment is a reliable method of improving cellulose accessibility in biofuel feedstocks, fresh material and cell wall residues from different plants. These results contribute to the understanding of the consequences of primary and secondary cell wall perturbations on lignocellulosic biomass accessibility to white rot fungi and on saccharification yield. A comparison of the effects of *P. chrysosporium* and *T. cingulata* on tobacco saccharification also highlights the limitation of current knowledge in this research field and the necessity to systematically test culture conditions to avoid generalisations.

Keywords Cell wall · Lignin · Pectin · White rot fungi · *Phanerochaete chrysosporium* · *Trametes cingulata*

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Background

Due to dwindling fossil fuel resources and global climate change, it is becoming clear that mankind's reliance on fossil energy must draw to an end at some point in the future. Bioethanol is an alternative energy source which provides liquid fuel, significant as the majority of fossil energy is used

as transport fuel. So-called first-generation biofuels require feedstock rich in starch or sucrose, which are easily converted into ethanol, but plants that are rich in these sugars, such as wheat, maize and sugar cane, are important food crops. Converting natural landscapes such as grasslands or rainforest to farmland for bioenergy crops releases a 'carbon debt' of 17 to 240 times more greenhouse gas into the atmosphere than simply continuing with fossil fuels. However, fermenting biomass from waste or non-food crops grown on disused farmland would not result in the same 'biofuel carbon debt' [1].

All plants contain glucose in the cellulose microfibrils that, together with hemicelluloses, pectins and lignin, make up cell walls. Many waste products are rich in lignocelluloses, for example wood chips, sawdust, food waste and corn stover. Utilisation of local feedstocks and resources is key to maximising the benefits of bioethanol, as this reduces fossil fuel input at the cultivation and transportation stages of the life cycle [2]. However, in spite of the ubiquity of 'second-generation' biofuel feedstock, cell walls have protective and structural functions and are therefore resistant to degradation: the lignin and hemicellulose network surrounding the glucose-rich cellulose microfibrils is soluble only in harsh conditions (strong acids or alkalis, high temperature and pressure). Cellulose microfibrils form the cell wall framework of the cell [3]. Hemicellulose, for example xylan, cross-links the cellulose microfibrils via hydrogen bonds, increasing the strength of the cellulose frame [4]. Pectins, such as homogalacturonan (HG), are the third main component of the primary cell walls of leaves and fruits. In contrast, secondary cell walls, making up wood and hardy grasses, contain low levels of pectin and are enriched in the phenolic macromolecule lignin. These three main constituents form the structured, protective network of the cell. Lignin is waterproof, which serves the double function of allowing the transport of water through xylem cells and providing resistance to water soluble enzymes.

Many industrial pretreatments have been developed to physically remove lignin from cell walls and to expose cellulose to hydrolytic enzymes. Common pretreatments are thermal (for example steam pretreatment), in which high temperatures dissolve first hemicelluloses and eventually lignin; acid or alkali, which again work by dissolving hemicelluloses; and irradiation [5–8]. These pretreatments are effective but have side effects. Solubilising the cell wall matrix can result in phenolic compounds that re-condense and inhibit fermentation of the released sugars. This problem is accentuated in acidic conditions [5, 8, 9]. The biomass can be washed before fermentation, but soluble sugars can be lost in this way [10]. Pretreatments such as pyrolysis and thermal pretreatments also require energy, commonly sourced from fossil fuel, to work. Using microorganisms that naturally degrade or metabolise lignin, leaving cellulose exposed, is therefore a valid alternative to aggressive and expensive pretreatments [11–13].

White Rot Fungi as a Pretreatment

Microorganisms that can depolymerise lignin are of extreme interest to the biofuel industry because lignin is the main barrier to cellulose hydrolysis [11, 13–15]. Fungi and bacteria depolymerise lignin by secreting extracellular enzymes such as lignin peroxidase (LiPs; reviewed by [16]). LiPs, such as those produced by white rot fungi, generate free radical species which attack aromatic rings in the lignin polymer to effectively degrade it. Consistently, white rot fungi have proved to be effective in the pretreatment of lignocellulosic biomass prior to saccharification [17–21]. However, only in recent times, genome-wide analysis of polysaccharide-degrading enzymes in *Polyporales* has provided insight into wood decay mechanisms [22]. *Phanerochaete chrysosporium* is a white rot fungus from the order *Polyporales* in the class *Basidiomycetes*. It metabolises monosaccharides and polysaccharides as well as lignin. The ability of *P. chrysosporium* to produce both LiPs and manganese peroxidases (MnPs) makes it a model for lignin-degrading enzyme production [16]. When this fungus is grown with cellulose as the sole carbon source, a fully competent ligninolytic system is expressed. *P. chrysosporium* and other white rot fungi use a wide range of glycoside hydrolases that act on cellulose and xylan: endoglucanases that break glycosidic bonds in the middle of a glucan, cellobiohydrolases that act on chain ends of cellulose and β-glucosidases that clean up stray cello-oligosaccharides that are left over [23]. Comparative secretomic analysis to examine the effects of xylan and starch on the expression level of proteins secreted by *P. chrysosporium* showed that adding starch to the system decreased the production of total extracellular enzymes, whilst the addition of xylan had the opposite effect [24]. *P. chrysosporium* has been proven to improve glucose extractability from lignocellulosic biomass on a range of starting materials, including rice and wheat straw [17, 20, 21], cotton stalks [19] and corn fibres [18].

P. chrysosporium synthesises veratryl alcohol (VA) from phenylalanine during secondary metabolism. LiP synthesis is induced by VA, which also stabilises it against H_2O_2 and free radical species. VA is then oxidised by LiP, generating veratryl alcohol free radicals [25]. These radicals and others, including •OH, attack aromatic rings in the lignin polymer [26] to start a series of reactions. This may result in the production of a lignin phenoxy radical or the release of a hydroperoxyl radical. Both of these can further depolymerise the lignin molecule.

Trametes cingulata is another white rot fungus that depolymerises lignin, but there is no available evidence that it expresses any kind of detectable LiP activity [27]. Recent studies have focused on the use of various strains of *Trametes*, such as *Trametes versicolor* and *Trametes pubescens*, to improve fermentation of rapeseed meal [28] or laccase production on agro-industrial waste substrate [29].

Modification of Plant Cell Walls for Improvement of Cellulose Hydrolysis

As lignin, pectin and xylan are effective barriers to cellulose extraction and hydrolysis, reducing their presence in cell walls improves saccharification [8, 30–37]. We previously showed that in the tobacco (*Nicotiana tabacum*) *prx* line, in which the *TOBACCO PEROXIDASE 60* gene is down-regulated and polymerisation of lignin monomers is prevented, lignin content is reduced by 25 % [38]. This line showed a 30 % improvement in sugar released per milligram of cell wall material compared to the wild type (WT) [39]. A xylan down-regulated tobacco line (*uxs*) also showed a significant improvement in saccharification efficiency [39]. An independent study showed that the expression of a fungal polygalacturonase (PG) in *Arabidopsis thaliana* caused a decrease in levels of de-esterified HG, with a consequent improvement of saccharification efficiency [35]. Similarly, the overexpression of a pectin methyl esterase inhibitor (*AtPMEI-2*) in *Arabidopsis* resulted in a 16 % decrease of de-esterified HG with respect to the WT [40]. In PMEI transgenic plants, the saccharification efficiency also increased up to 50 % above WT levels [35]. Significantly, the ectopic expression of another PMEI (AcPMEI) member in wheat plants also improved saccharification of leaves and stems [35].

In this study, we investigated the cumulative effects, on saccharification efficiency, of pretreating lignocellulosic biomasses obtained from the above-mentioned tobacco and *Arabidopsis* lines with altered cell wall composition with *P. chrysosporium* or *T. cingulata*.

Results

Selection of Pretreatment Method

Initial pretreatment trials were carried out on tobacco WT plants belonging to the K326 cultivar [39, 41] according to a method adapted from Keller and colleagues [42]. Acetone-insoluble cell wall material (AIM) pretreated with this technique did not result in improved sugar release after enzymatic saccharification (data not shown). Conversely, *P. chrysosporium* pretreatment using the method described by Tien and Ma [43] successfully improved saccharification. Whilst both protocols use a culture medium containing glucose, potassium dihydrogen orthophosphate (KH_2PO_4), magnesium sulphate ($MgSO_4$) and $FeSO_4$, the second protocol also included a trace element solution in the culture medium that contained $MnSO_4$, NaCl and $CuSO_4$, thiamine and 0.4 mM veratryl alcohol. Tien and Ma [43] also compared the treatment in static and agitated conditions. In our hands, both methods proved to be effective, and the static method was therefore used. Though not significant, a slight increase in sugar release from AIM agitated in culture medium seemed to be associated with increased variability of the results obtained from untreated control samples. This may be due to the constant agitation of AIM within the culture medium, which increased exposure to solvent and ions. These data are consistent with previous observations showing that results obtained in agitated cultures have lower reproducibility [43].

Pretreatment with *P. chrysosporium* Improves Sugar Release from Tobacco Plants with Modified Secondary Cell Walls

In order to verify the origin of the reducing sugars released from pretreated AIM by saccharification, we subjected comparable amounts of agar-grown fungal mycelium to enzymatic saccharification and used the results to control for sugars of fungal origin. As shown in Fig. 1a, the concentration of the sugars released from the fungus in the absence of plant material, using the total hydrolysis method [44], does not change from the control where only the enzymes were present. We therefore concluded that the sugars analysed throughout this work are derived from the plant tissues and not from the fungus. Similar results were obtained when *T. cingulata* was tested in our study (d.n.s.). We then pretreated AIM from tobacco v. Samsun (NVS) transgenic *prx* plants and two additional lines with modified lignin, caused by antisense suppression of *CINNAMOYL-CoA REDUCTASE* (*ccr*) and *CINNAMATE-4-HYDROXYLASE* (*c4h*) [45, 46, 38]. We also included in the experiment line *uxs*, which was obtained in the K326 background [46]. Improved saccharification of *uxs* and *prx* material in the absence of pretreatments is already described [39].

For all tested genotypes, pretreatment with *P. chrysosporium* reduced both carbohydrate and lignin content in the cell wall biomass (Table 1). Lignin content was reduced by at least 45 % in all tobacco lines, with the biggest reduction in *ccr* plants, whilst the WT NVS was the least affected. Total carbohydrate content was also less affected in NVS than in the other lines. We concluded that fungal pretreatments increase exposure of sugars for saccharification by cellulolytic enzymes. The pretreatment released about 87 % of the sugars from *prx* cell walls compared to 46 % of the total sugars released from untreated plant material (Table 1). A significant improvement was also observed for the *c4h* line where the proportion of total sugars released from the AIM almost tripled after pretreatment (Fig. 1b). In comparison to the corresponding WT (NVS), the saccharification profile of the lignin down-regulated tobacco lines (*ccr* and *c4h*) did not differ after pretreatment. Although more sugar was released from all pretreated genotypes, *prx* was the line with the highest release of sugar per milligram of AIM both in the absence and presence of the fungus. This is consistent with previously published data [39]. Conversely, the increase in sugar released from *uxs* as compared to the WT K326 after

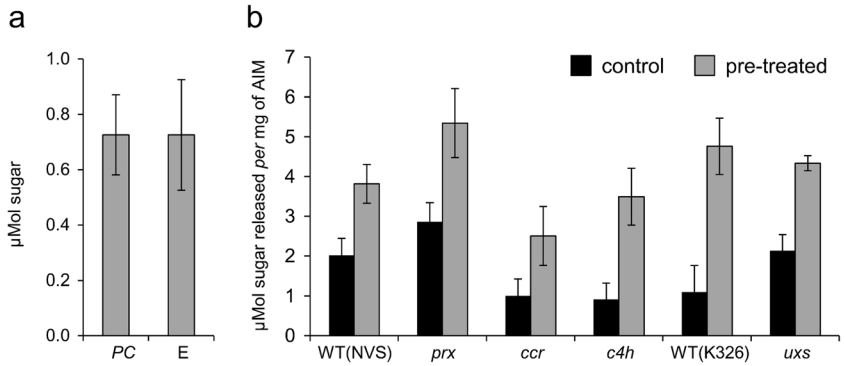

Fig. 1 Improvement in sugar release from cell walls after pretreatment with *Phanerochaete chrysosporium*. **a** Sugars present in the supernatant after saccharification of fungal mycelium (PC) and after incubating the enzymes (*E*) with no substrate under the same conditions. **b** Sugars released by enzymatic saccharification of AIM after 10 days of incubation with *P. chrysosporium* (pretreated) and the culture medium with no fungus (control). The absorbance of the enzyme background was subtracted prior to conversion to micromolar. Data presented are the average of three biological replicates±SE, each containing three technical replicates

fungal pretreatment was not striking. Surprisingly, the latter shows the biggest percentage increase in sugar release without pretreatment (Fig. 1b).

Notably, improvement in sugar release per milligram of AIM was not proportional to the percentage of lignin depolymerised by *P. chrysosporium* during the 10 days of pretreatment (Table 1). The *prx* line showed the same high reduction in lignin content after pretreatment as *ccr* and *c4h* (about 80 %) but had the lowest improvement in sugar release. In addition, the xylan down-regulated *uxs* line showed a degree of lignin depolymerisation comparable to K326 but a lower fold increase in sugar release.

Quantification of *P. chrysosporium* Growth in Tobacco Lines

The plant tissues and the fungal mycelium become densely intertwined during the infection process, and it is impossible to separate them after pretreatment. Therefore, we assessed the growth of *P. chrysosporium* on AIM substrates from the different tobacco lines using marker genes specific for the fungus. Genomic DNA was extracted from NVS, *prx*, *ccr* and *c4h* samples after 4 and 10 days of incubation with *P. chrysosporium*, and the levels of the fungal 18S ribosomal DNA were evaluated by PCR. Total RNA was also extracted from K326 or *uxs* biomass at the same points during pretreatment, and the corresponding cDNA was prepared. The transcript levels of *P. chrysosporium* 18S, of a *-tubulin* (which has previously been used to measure mycelial growth rate [47]) and a *LiP*, were compared by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) (Table 2). Genomic levels of 18S indicated the quantity of fungus grown on different AIMs was similar; therefore, the experimental conditions were comparable (Fig. 2a). The expression of the 18S gene was also similar in *P. chrysosporium* grown on K326 and *uxs* (Fig. 2b). The expression of *-tubulin* was lower comparatively to 18S but showed a similar trend that was consistent between the two tobacco lines. A similar expression pattern can be observed for *LiP*. These results also confirmed that during the 10-day incubation period, the fungus had grown similarly on the different substrates (Fig. 2b).

P. chrysosporium Pretreatment Improves Sugar Release from *Arabidopsis* Plants Modified for Primary Cell Wall

The effectiveness of the fungal pretreatment was tested also on *Arabidopsis* transgenic plants in which primary cell wall

Table 1 Comparison of consequences of *P. chrysosporium* pretreatment on saccharification efficiency and reduction in carbohydrate and lignin content

	% of theoretical yield released by enzymes		% change		Fold improvement in sugar release	*p* value
Line	Control	*P. chrysosporium* pretreatment	Carbohydrates	Lignin		
NVS	29.9	56.7	1.7±0.9	46.41±4.5	1.9	1.29E-01
prx	46.8	87.6	3.6±0.7	80.85±3.6	1.9	1.89E-09
ccr	22.5	56.1	10.7±0.6	82.15±2.3	2.5	8.15E-04
c4h	11.8	44.9	12.1±0.5	74.82±6.6	3.8	3.24E-01
K326	9.5	41.2	7.3±1.1	70.11±6.2	4.3	8.69E-12
uxs	22.6	45.8	9.0±0.6	65.03±10.5	2.0	3.31E-08

p value refers to significance of improvement in sugar release per milligram of AIM

Table 2 Primers used for PCR

Gene	Accession number	Forward (5′ to 3′)	Reverse (3′ to 5′)	Amplicon size (bp)
-tubulin1	AADS01000121	GCTTGGACTTCTTGCCATAG	TGGCTTCAGCACTTTCTTCT	411
18S	GU966518	TGGCTCATCCACTCTTCAAC	AAGCGATCCGTTACACTCAC	540
Lignin peroxidase (*LiP*)	gb|EF644559.1|	CGATGCTATTGCCAT	GAAAGCATCCAGACA	415

components had been targeted. The PG line (PG57) expresses an attenuated version of *Aspergillus niger POLYGALACTURONASE II* gene, which resulted in a decreased content of de-esterified HG with respect to the WT [35]. The PMEI2 line overexpresses an endogenous PMEI, causing a higher degree of HG methyl esterification. Both lines showed improved sugar release from fresh tissue after treatment with a commercial cellulase [35]. Here, we showed that without any pretreatment, PG and PMEI AIM is significantly more amenable to saccharification with respect to the untransformed control ($p=1.9\times10^{-3}$ PG and $p=3.7\times10^{-4}$ PMEI; Fig. 3). Moreover, AIM obtained from WT *Arabidopsis* stems pretreated with *P. chrysosporium* was more prone to saccharification (about 80 % increase), whereas modification of pectin content and methyl esterification appear to have different effects on AIM saccharification after fungal pretreatment (Fig. 3): Saccharification of lines in which pectin is degraded by the polygalacturonase (PG57) was improved by pretreatment of about 45 % ($p=5.9\times10^{-4}$), whilst plants with reduced pectin methyl esterase activity (PMEI2) showed the greatest improvement in saccharification (about 100 %) after pretreatment with *P. chrysosporium* with respect to WT ($p=5.1\times10^{-7}$).

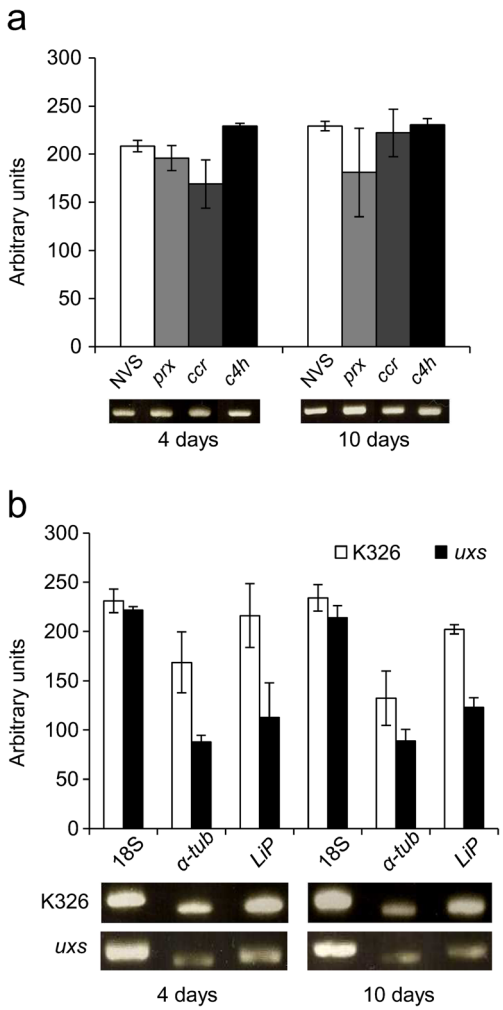

Fig. 2 Quantification of *Phanerochaete chrysosporium* by PCR and semiquantitative RT-PCR. **a** Genomic levels of 18S ribosomal RNA (18S) in NVS, *prx*, *ccr* and *c4h* lines. **b** Transcript levels of *18S*, *-tubulin* and *LiP* in K326 or *uxs*. The size of the amplicons is indicated in Table 2. *Bars* indicate the pixel intensity of an average of three replicates (±SD) expressed in arbitrary units, calculated by MatLab. The images shown are representative of the replicates

P. chrysosporium Pretreatment Improves Sugar Release from *Arabidopsis* Fresh Stem Material

P. chrysosporium pretreatments were effective on *Arabidopsis* fresh stem material (Fig. 4). Here, we used the *Arabidopsis* knock-out line for the cell wall localised *PEROXIDASE 34* (*prx34*), known to be involved in cell elongation [48] and hydrogen peroxide generation as part of the defence response [49]. This gene has not been shown so far to be directly

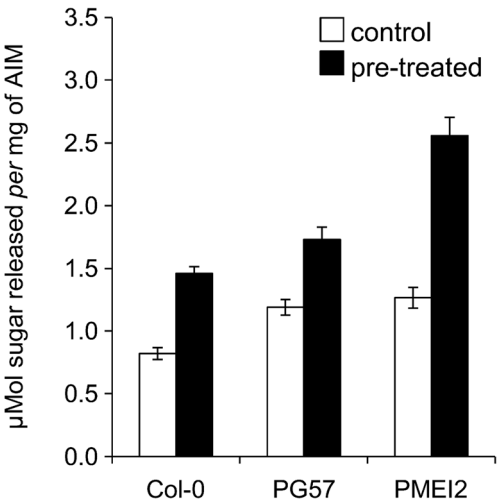

Fig. 3 Sugar release from cell walls of Col-0, PG and PMEI *Arabidopsis* stems after pretreatment with *Phanerochaete chrysosporium*. Saccharification of AIM was performed 10 days after the incubation with the fungus (pretreated) or with water (control). Data presented are the average of three biological replicates±SE, each containing three technical replicates

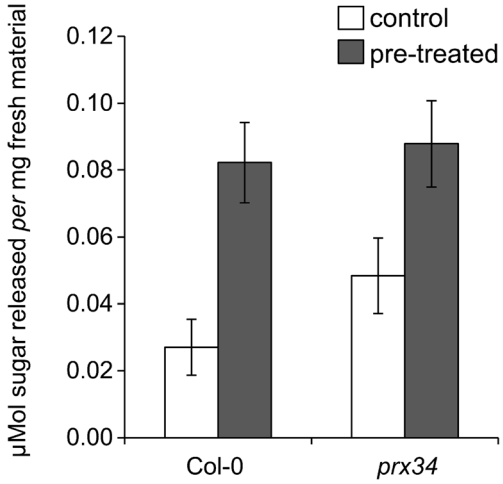

Fig. 4 Sugar release from *Arabidopsis* stem material with and without pretreatment with *Phanerochaete chrysosporium*. Pretreatment improved sugar release from fresh stem material of cell wall localised *PEROXIDASE 34* (*prx34*) as well as Col-0. Data presented are the average of three biological replicates±SE, each containing three technical replicates

involved in cell wall synthesis. Sugar release from fresh *Arabidopsis* stem material increased threefold after pretreatment. *Arabidopsis prx34* showed only a slightly improvement in sugar release from unpretreated fresh stem material compared to the WT, and no significant difference in sugar release was observed between the two lines following pretreatment with the fungus (Fig. 4).

T. cingulata, a Second White Rot Fungus, Improves Sugar Release from Tobacco Cell Wall Material

We analysed the effect of *T. cingulata* on the saccharification of AIM from the same tobacco transgenic lines tested with *P. chrysosporium*. WT NVS and *prx* lines responded similarly to both fungal pretreatments (Fig. 5 and Supplemental Table 1). *P. chrysosporium* and *T. cingulata* pretreatment significantly increased sugar release in the two lignin-modified lines *ccr* and *c4h*. The xylan down-regulated line *uxs* showed the smallest, though still significant (about 1.4-fold; $p=0.0436$), effect after *T. cingulata* pretreatment.

Discussion

Saccharification Is Sensitive to Different Experimental Conditions: the Need to Optimise Treatments and Strains

Investigating optimal conditions for LiP production by *P. chrysosporium* has been the topic of several studies (reviewed by [16]). The correct culture medium composition and culture conditions are required to induce this fungus to produce large amount of these enzymes.

Here, we have significantly improved a pretreatment method that consistently and reproducibly produces enhanced sugar release after enzymatic saccharification in static conditions, in agreement with previous studies [43]. The pretreatment was generally effective in both tobacco and *Arabidopsis* lines with modified cell walls (Figs. 1b and 3). In tobacco, the effect was particularly positive on the *prx* line, highlighting the importance of *TP60* in the biosynthesis of lignin [39, 50]. The increment observed in the industrial variety line K326 was surprising in comparison to the other WT background used in this work (NVS) and the antisense *uxs* line. The reduced effect of the fungus on the latter is in agreement with previous results [41, 24], arguing that in a background of xylan reduction, lignin availability for removal could be less crucial.

We also observed that in *Arabidopsis*, pectin modifications appear to improve cell wall saccharification, in particular after pretreatment with *P. chrysosporium*. Notably, the highest level of sugars released after enzymatic hydrolysis was exhibited by

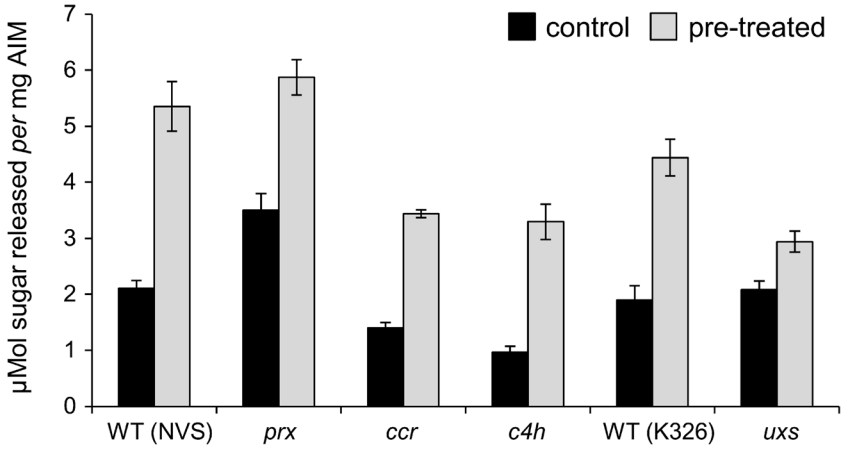

Fig. 5 Sugar release from tobacco cell wall material with and without pretreatment with *Trametes cingulata*. Sugars released by enzymatic saccharification of AIM after 10 days of incubation with *T. cingulata* (pretreated) and the culture medium with no fungus (control). The absorbance of the enzyme background was subtracted prior to conversion to micromolar. Data presented are the average of three biological replicates±SE, each containing three technical replicates

the AIM fraction isolated from PMEI-expressing plants (Fig. 3). It has been shown that pectin de-esterification affects the assembly of the load-bearing cellulose network [51] and can influence the formation of benzyl-uronate cross-links in lignin [52]. It is possible that higher saccharification, observed after *P. chrysosporium* pretreatment of higher methyl esterified cell walls of PMEI plants [40], is due to greater accessibility of lignin to the fungal lignolytic enzymes. Conversely, reducing pectin content by over-expressing PG does not appear to significantly improve the effect of the fungal lignin-degrading enzymes on the saccharification of AIM with respect to the control.

The data obtained in tobacco show that the improvement in sugar release per milligram of AIM was not proportional to the percentage of lignin depolymerized by the fungus (Table 1). These findings are consistent also with other studies [53] which showed, using *Arabidopsis* lignin mutants, saccharification models and Pearson correlation, that lignin content is the main factor in determining the saccharification yield. The same work suggested that lignin composition and matrix polysaccharide content and composition also affect the saccharification yield. Here, we specifically highlight the role of pectin in altering the accessibility of lignin by a white rot fungus for saccharification pretreatments.

The pretreatment methodology used in this work was optimised to enhance fungal degradation activity [43]. However, in control experiments (in the absence of the fungus), it lead to results that differ from those obtained in a previous work [39]. We tested the same batch of AIM used previously [39] alongside new AIM preparations, with no significant differences in saccharification efficiencies (d.n.s.). The differences observed, in particular for the lines *ccr*, *ch4* and NVS, are therefore due to the methods used. Control and pretreated samples were processed simultaneously: during the 10 days of pretreatment period, they were incubated with non-inoculated culture medium of the same composition as the pretreated samples. This treatment included extended incubation at 37 °C with culture medium containing glucose and metal salts and multiple washes with water and 80 % acetone, followed by air drying. Air drying in particular affects the physical properties of the cell wall [54]. It is therefore possible that the effect of physical cell wall degradation in these conditions, which differs according to the genetic background, negatively affects the saccharification of lignin-modified lines with respect to their wild-type NVS. As the methodology chosen here is optimised for the activity of the fungus according to previous literature, it is clear that future investigation will need to focus on improving the synergy between pretreatments and cell wall modifications obtained by genetic engineering or other means under different experimental conditions.

We investigated the expression of marker genes to monitor fungal growth during incubation with different tobacco AIMs. The analysis showed consistent genomic and expression levels of *18S* grown on different substrates (Fig. 2). The transcript levels of *-tubulin* and *LiP* appeared however, on average, about two times lower in the *uxs* than the K326 line. Whilst this was not reflected in significant differences in saccharification between the two lines after pretreatment, it is useful to note that further comparisons between the expression of different genes may reveal alterations in the molecular mechanisms involved to help fine tune future experimental conditions with different microorganisms.

Differences from Pretreatments with *T. cingulata* and *P. chrysosporium* Highlight the Necessity to Identify Targets for Genetic Improvement

Our results show that pretreating AIM with *P. chrysosporium* improves saccharification of all the tobacco lines tested (Fig. 1b). This effect, likely due to the reduction in lignin content seen in pretreated AIM (Table 1), supports the evidence that this cell wall component is the main barrier to efficient sugar release from cell walls. Remarkably, the pretreatment of AIM with *T. cingulata* was generally more effective at improving sugar release than *P. chrysosporium* (Fig. 5 and Supplemental Table 1). We observed that the greatest increase in sugar release caused by *P. chrysosporium* treatment was about fourfold for K326, but the same line, as well as *ccr* and *c4h*, yielded at least twofold increase after pretreatment with *T. cingulata*. These three lines also showed the greatest improvement in sugar release after incubation with *P. chrysosporium*. The fact that pretreatment with two species of white rot fungi had the largest effects on the same three plant lines reflects inherent lower saccharification properties of these lines in the experimental conditions tested. It is plausible the differences observed are due to different modes of action in *Trametes* and *Phanerochaete*. As *P. chrysosporium* has been used in several studies into lignin degradation of different plant species, the knowledge of its lignolytic complex is currently more advanced than other white rot fungi [17, 19–21]. Conversely, the lignolytic properties of *T. cingulata* are not yet fully understood. It is known that peroxidase or MnPs are crucial for lignin degradation by *P. chrysosporium* [17]. *P. chrysosporium*'s genome contains ten LiPs and five MnPs with abundant other related genes [55]. However, Nutsubidze and colleagues [27] conducted a study on the lignin depolymerising capabilities of *T. cingulata* and concluded that such enzyme activity was not detectable in the medium where the fungus had successfully depolymerised kraft lignin. Our study highlights the current need for further characterisation, at genome, transcriptome and biochemical levels of different fungi, as well as of their careful and systematic comparison at evolutionary level, to identify targets for genetic improvement. Nevertheless, our results demonstrate that, despite the limited understanding of the delignification properties of white rots such as *T. cingulata*, these are very valuable tools for

improving access to industrially important sugars as well as for increasing the volume usable plant biomass.

Fungal Pretreatment Is Effective at Improving Sugar Release from Different Substrates

We show here that fungal pretreatment is a reliable method of improving cellulose accessibility in biofuel feedstocks from different plants, whether fresh material or cell wall residue is used. The results are consistent: in every material, whether dried or fresh stem was used as a substrate, saccharification increased after pretreatment. Pretreatment of agricultural or wood waste would make the prospect of using such material as a feedstock for bioethanol production more promising, as it eases the difficult process of extracting fermentable sugars. The scale of improvement observed in our work is equivalent to that seen in published research, which used fresh material as the substrate and ranges from 2.5- to 3.5-fold improvement in pretreated rice [17] or wheat [20], respectively. Our data show that pretreatment of fresh biomass was even more effective than pretreatment of dry cell wall material (AIM) when it comes to improving sugar release. In addition, the data we present argue for the existence of a differential role of tobacco and *Arabidopsis* peroxidase genes in cell wall synthesis affecting the accessibility of cellulose for saccharification. In fact, whilst modification of the expression of *TOBACCO PEROXIDASE 60* proved most effective in enhancing saccharification both in the presence and in the absence of fungal pretreatment (this work and [39]), knocking out the *Arabidopsis PRX34* gene did not have the same success. In *Arabidopsis*, other peroxidases have been proposed as candidates involved in lignin biosynthesis [56, 57]. Future work focusing of the comparative functional analysis of these genes will provide further insights on this controversy to evaluate direct involvement of this class of enzymes in cell wall biosynthesis.

It remains to be demonstrated whether the presence of nutrients originating from underlaying live tissues contributes to the improved saccharification observed on fresh material, as in nature *P. chrysosporium* grows on the trunks of trees. Experimental conditions need to be optimised, on a case specific base to start with, but combined with manipulation of the cell wall, fungal pretreatment is even more effective as a mean of improving sugar extractability from cell walls. For example, the difference between tobacco line *prx* after pretreatment and the wild-type NVS with no pretreatment becomes enormous when considered on a large scale: before pretreatment, it would require 252.96 g of wild-type NVS cell wall material to extract 100 g of glucose, and after pretreatment, 145.52 g would be necessary, but only 103.93 g of pretreated *prx* cell wall material is required for 100 g glucose in the same conditions. Similarly, the wild-type line K326 requires only 116 g cell wall material to yield 100 g glucose after fungal pretreatment, but with no pretreatment, it requires 302 g.

Conclusions

Taken together, our results contribute to a better understanding of the consequences of primary and secondary cell wall perturbations on the accessibility of lignocellulosic biomass by white rot fungi and on saccharification yield. The similarities, as well as the differences, identified between *Arabidopsis* and tobacco lines provide potential targets for genetic improvement of lignin, xylans and pectins. The comparison of the effect of *P. chrysosporium* and *T. cingulata* on the saccharification yield of tobacco also highlights the limitation of the current knowledge in this research field and the necessity to systematically test culture conditions to avoid generalisations and to further study the regulatory cascade in the lignin-degrading process, which may contribute to developing improved strains, leading to stable enzyme production.

Methods

Plant and Fungal Materials

P. chrysosporium and *T. cingulata* (kind gifts from Prof. Norman Lewis and Dr. Laurence Davin, Washington State University) were characterised by Dr. Alan Buddie (CABI, Egham, UK). They were propagated on malt agar plates and subcultured every 4 days.

Wild-type tobacco line *N. tabacum v. Samsun* (NVS) and lines down-regulated in lignin synthesis genes *TP60*, *CCR* and *C4H* (*prx*, *ccr* and *c4h*, respectively) along with *UXS*-down-regulated line (*uxs*) with its wild-type *N. tabacum* K326 (K326) were propagated from cuttings and grown on Levington M3 Pot and Bedding Compost High Nutrient (The Scotts Company LLC) in a glasshouse. Supplementary lighting allowed a photoperiod of 16 h. The temperature was between 20 and 25 °C, as the temperature fell at night. Lines *prx*, *ccr*, *c4h* and *uxs* have been described previously [58, 41]. Line *prx* contains 77 % acetyl bromide, *ccr* contains 58 % Klason lignin content and an increased syringyl/guaiacyl ratio as compared to NVS and *c4h* has an acetyl bromide lignin content of 86 % [46]

A. thaliana ecotype Columbia (Col-0) and transgenic *Arabidopsis* plants constitutively expressing *An*PGII (line 57) and overexpressing the *At*PMEI-II (line 7) were previously described [35, 40]. Seeds were germinated aseptically on 0.8 % agar Murashige and Skoog medium supplemented with 0.5 % sucrose and 0.25 % 2-(*N*-morpholino)ethanesulfonic acid, following 24 h stratification at 4 °C. The seedlings were transferred to soil after 15 days and grown in a climate-controlled environment at 23 °C. Supplementary lighting allowed a photoperiod of 16 h.

Preparation of AIM

AIM of stems was isolated as previously described [39, 58]. Primary walls were isolated according to [40]. The method was modified to include de-proteinisation with phenol following 2 days of destarching with 5 U α-amylase/mg cell wall material to eliminate any remaining enzymes. Prior to fungal pretreatment or treatment with control culture medium, the AIM was sterilised by heating for 15 min on a heat block at 100 °C.

Fungal Pretreatment

The same method was used for pretreating plant biomass with *P. chrysosporium* and *T. cingulata*. The culture medium was adapted from [43]: KH_2PO_4 14.7 mM; $MgSO_4$ 8.75 mM; calcium chloride ($CaCl_2$) 0.901 mM; manganese sulphate ($MnSO_4(H_2O)$ 0.527 mM; sodium chloride (NaCl) 2.73 mM; iron sulphate $Fe_2(SO_4)_3$ 0.105 mM; zinc sulphate ($ZnSO_4$) 0.099 mM; glucose ($C_6H_{12}O_6$) 55.6 mM; thiamin ($C_{12}H_{17}ClN_4OS$) 0.00332 mM; and veratryl alcohol ($C_9H_{12}O_3$) 0.4 mM. The culture medium was filter sterilised. Prior to use, fungi were grown for 48 h on malt agar plates. One agar square (5×5 mm) with the grown fungus was cut out of a plate and homogenised with 50 ml of culture medium and diluted to have a concentration of 100 spores/ml. Fungus (2 ml) inoculated medium was added to 10 mg of sterile AIM in a 15-ml tube which was incubated on the long side. As primary cell wall AIM from *Arabidopsis* was less abundant than secondary cell wall AIM, these assays were performed in 0.5 ml inoculated medium in the presence of 2 mg of sterile AIM. The substrate control in all cases was AIM incubated with water or uninoculated medium. Inoculated medium incubated with no AIM was the non-substrate control. Incubations took place at 37 °C. Prior to fungal pretreatment or treatment with control medium, the AIM was sterilised for 15 min on a heat block at 100 °C. Prior to use in the assays, fresh material was surface-sterilised with 5 % HClO (*v*/*v*) for 5 min and then washed four times with sterile water.

After 10 days, the pretreated AIM samples were centrifuged at ~2,200×*g* (3,500 rpm) for 5 min. The supernatant was removed and the pellet, consisting of AIM and mycelium, was washed twice with 80 % acetone. The pellet was transferred to a 2-ml tube, dried at 40 °C and then heated to 100 °C for 15 min. The fresh material was carefully separated from the mycelia and washed as described above.

Saccharification Assays

After pretreatment or control incubation, the AIM was heated at 100 °C for 10 min and subsequently cooled on ice. The saccharification assay was performed according to [39] and adapting a method from [54]. Briefly, each reaction tube consisted of 10 mg AIM soaked in 2 units each of Driselase, cellulase from *Trichoderma reesei*, cellulase from *Aspergillus* spp. (all from Sigma-Aldrich, UK) and Macerase (EMD Chemicals, Germany) in the presence of kanamycin (100 μg/ml), tetracycline (15 μg/ml), and gentamicin (25 μg/ml).

The fresh biomass samples (*Arabidopsis* stems of Col-0 and *prx34*) were subjected to saccharification using a method adapted from [35]. The enzyme solution was 1 % Celluclast and 0.4 % antibiotic cocktail in 50 mM sodium acetate buffer (pH 5.5). The stem sections were incubated at 37 °C in an incubator shaker at 180 rpm for 24 h.

After saccharification, the samples were centrifuged and the supernatant analysed to determine the total sugar content using the phenol sulphuric acid assay protocol described by [44].

Carbohydrate Determination

Total carbohydrates were determined according to the Analytical Procedure of the National Renewable Energy Laboratory (http://www.nrel.gov/biomass/analytical_procedures.html). AIMs were treated in a screw cap tube with 72 % (vol/vol) sulphuric acid at 30 °C for 90 min. The concentration of sulphuric acid was diluted until 4 % (vol/vol) and tubes were heated at 120 °C for 90 min. Tubes were centrifuged and the supernatant was analysed to determine the total carbohydrates amount by using the phenol-sulphuric acid assay [44].

The carbon loss after fungal treatment was expressed as percentage of total carbohydrate ratio (% change) between control and treated AIM.

Lignin Quantification

The acetyl bromide method to quantify lignin content was adapted from [59]. Briefly, 100 μl of freshly made acetyl bromide solution (25 % *v*/*v* acetyl bromide in glacial acetic acid) was gently added to 1–1.5 mg of AIM, and the tubes were heated at 50 °C for 3 h. The tubes were transferred to reach room temperature. Four hundred microliters of 2 M sodium hydroxide and 70 μl of freshly prepared 0.5 M hydroxylamine hydrochloride were added to the sample, and the tube was gently resuspended by vortex. The assay volume was made up to 2 ml total volume with glacial acetic acid and mixed. The absorbance of the sample was detected at A280.

Molecular Biology Techniques

Genomic DNA (gDNA) was extracted from tobacco and *P. chrysosporium* materials using a method adapted from [60].

Purification of total RNA from plant and fungus was performed using the RNeasy plant Mini Kit (Qiagen), and cDNA was synthesised using the QuantiTect Reverse Transcription kit (Qiagen) that includes a gDNA wipeout step. Semiquantitative RT-PCR was performed to analyse mRNA transcript levels of 18S ribosomal RNA (*18S*), α-tubulin and *LiP*.

Oligonucleotide primers were designed using Primer3 (http://primer3.source-forge.net/webif.php), and their specificity was verified using Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/). The primers were synthesised by Eurofins MWG Operon (London). The primer sequences are listed in Table 2.

PCR was carried out using GoTaq PCR polymerase (Promega) with purified gDNA or cDNA as the templates using standard PCR reaction protocols. The products were analysed by gel electrophoresis on a 1 % agarose gel containing 1× SYBR Safe DNA gel stain (Invitrogen, USA) and were visualised under UV light. Expression values were obtained calculating the average light intensity of gel images on Matlab (MathWorks, http://www.mathworks.com/).

Statistical Analysis

For all experiments described, at least three independent biological replicates were tested, unless otherwise stated. The standard error (SE) is shown as ± of the mean. All graphs and associated statistics were performed using Microsoft Office Excel 2003.

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Conflict of Interest The authors declare no competing interests.

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