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Functional co-expression of a fungal ferulic acid esterase and a β-1,4 endoxylanase in *Festuca arundinacea* (tall fescue) modifies post harvest cell wall deconstruction.

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Abbreviations

AIR: alcohol insoluble residues; AX: Arabinoxylan; XYN2: β-1,4 endo-xylanase; FA: Ferulic acid; FAEA: ferulic acid esterase; HCAs: Hydroxycinnamic acids; HPAEC: Anion exchange chromatography; HPLC: High performance liquid chromatography; IVDMD*: in vitro* dry matter digestibility; LmSee1: *Lolium multiflorum* senescence promoter; PAHBAH: ρ-hydroxybenzoic acid hydrazide; *p*CA: *p*-coumaric acid; TFA: trifluoroacetic acid.

Abstract

Tall fescue plants (*Festuca arundinacea*) constitutively expressing vacuole or apoplast targeted ferulic acid esterase from *Aspergillus niger* were retransformed with a senescence induced and apoplast targeted β-1,4 endo-xylanase from *Trichoderma reesei.* Enzyme activities in co-expressing plants stabilized after repeated vegetative propagation, with xylanase activity in senescent leaves increasing and ferulic acid esterase activity decreasing after tillering. Plants co-expressing both enzymes in the apoplast, with the lowest levels of ferulate monomers and dimers and the lowest levels of cell wall arabinoxylans, released ten times more cell wall hydroxycinnamic acids and five times more arabinoxylan from the cell wall on autodigestion compared to expression of ferulic acid esterase or xylanase alone. These plants also showed a 31% increase in cellulase-mediated release of reducing sugars, a 5% point increase in in-vitro-dry-matter-digestibility and a 23% increase in acetyl bromide soluble lignin. However, plant growth was adversely affected by expressing FAE in the apoplast, giving plants with narrower shorted leaves, and a 71% decrease in biomass.

Keywords

Ferulic acid esterase, Xylanase, Cell wall structure, Digestibility, Ferulates

Introduction

The potential of grass lignocellulose for the production of fermentable sugars to ethanol arises from their high yield, low cost, high sustainability on marginal land and low impact on food supply and the environment compared to current grain-based ethanol (Rogner 2000). However, due to the complex structure of grass cell walls and their recalcitrance to enzymatic attack, the conversion of lignocellulosic biomass to ethanol is still an inefficient process (Saha 2003),requiring expensive pre-treatments, high enzyme inputs, and long digestion times (Mosier et al. 2005).

In relation to the structure of plant cell walls, some hemicelluloses, in contrast to cellulose, are branched xylan-rich polysaccharides and as such require a spectrum of hydrolytic enzymes for hydrolysis (Biely 1985). In grasses, arabinose constitutes the main side chain connected to the xylan backbone which is further esterified by phenolic acids where ferulic acid is the most common. The importance of ferulic acid stems from its ability to undergo oxidative coupling reactions to form ferulate dimers cross-linking xylan (Hatfield et al. 1999) and in linking lignin to the xylan/cellulose network via a lignin-ferulate-xylan complex (Buanafina 2009).

Xylanases (i.e. β-1,4 endo-xylanase), are the major enzymes required for arabinoxylan (AX) breakdown as reviewed by Collins *et al*., (2005) but a second important class of enzymes, which plays a key role in the degradation of the complex AX cell wall structure, are the hydroxycinnamoyl hydrolases such as ferulic acid esterases (FAE) (Faulds and Williamson 1993) . These enzymes have

the ability to hydrolyse the esterified feruloylated groups involved in cross-linking between AX and between AX and lignin (Williamson et al. 1998) and have been classified according to their substrate specificity and amino acid sequence into 4 types A, B, C and D (Crepin et al. 2004).

Ferulic acid esterase A from *Aspergillus niger* is one of the most widely studied of the feruloyl esterases and is known to act on ferulic acid residues attached to the *O*-5 of arabinose in arabinoxylan (Ralet et al. 1994). FAEA releases 5-5' and 8-0-4' ferulate dimers from plant cell walls when incubated with xylanase or when pre-treated with xylanase (Kroon et al. 1999). This positive synergism of FAEA with other catalytic or non-catalytic proteins is shown by the release of ferulates from different substrates by different ferulic acid esterases alone or in combinations with xylanases. For example, the efficiency of ferulate release by *A. niger* FAEA from wheat straw increases when combined with xylanase or with *T. reesei* cellulase during saccharification (Tabka et al. 2006), or when fused to the non-catalytic *T. reesei* swollenin protein (Levasseur et al. 2006). Generally, the glycoside hydrolase family 11 xylanases favours the release of ferulic acid while family 10 xylanases are more efficient at releasing ferulate dimers (Faulds et al. 2003).

Strategies for producing the large amounts of exogenous cell wall degrading enzymes required to degrade lignocellulosic biomass into its constituent sugars include the production of transgenic plants with high levels of enzyme activity. To this end, the production of an active insoluble aggregate of xylanase in tobacco where a Zera-xylanase chimeric protein accumulated within ER-derived protein bodies has been demonstrated (Llop-Tous et al. 2011), as well as the targeting of constitutive expression of the *xyn2* gene from *Trichoderma reesei* or the alkali-

thermostable xylanase gene from *Bacillus* sp. to the chloroplast or peroxisome (Leelavathi et al. 2003; Bae et al. 2006; Verma et al. 2010). In addition, an elegant new way of expressing xylanase in plants without inducing problems with plant development has recently been demonstrated by engineering thermo-regulated intein splicing to control induced xylanase catalytic activity (Shen et al. 2012).

An alternative approach to reducing the requirements for large amounts of cell wall degrading enzyme may be the use of genetically improved biomass with modified cell wall structures produced by induced targeting of *in-planta* expression of these enzymes. There have been only a few reports of the effects of *in-planta* expression of ferulic acid esterase. When FAEA, under the control of an endosperm-specific promoter was expressed in wheat, the water-unextractable arabinoxylan of grain cell walls increased by 15%–40% and monomeric ferulic acid decreased by 13%-34% irrespective of the addition of a 3' KDEL, but seeds were shrivelled with a 20–50% decrease in weight (Harholt et al. 2010). However, transgenic plants of *Arabidopsis thaliana* expressing *Aspergillus nidulans* feruloyl esterase in the apoplast showed no visible phenotype and the induced compositional changes increased biomass degradability (Pogorelko et al. 2011) . A type B ferulic acid esterase (*faeB*) from *Aspergillus niger* when expressed in alfalfa targeted to the apoplast, endoplasmic reticulum or vacuole, modified cell wall composition with a reduction in ester linkages but with elevated lignin, resulting in recalcitrance to digestion by mixed ruminal microorganisms but with no visible plant phenotype (Badhan et al. (2014). However grass cell wall architecture is much more dependent upon the incorporation of ferulates than in alfalfa, which contain relatively low levels of ferulate.

When constitutively expressed in leaves of *Lolium multiflorum* (Buanafina et al. 2006) or *Festuca arundinacea* (Buanafina et al. 2008), *Aspergillus niger* ferulic acid esterase (*faeA*) targeted to the vacuole had little effect on plant growth or biomass yield but resulted in reduced levels of cell wall esterified monomeric and dimeric ferulates and increased rates of *in vitro* dry-matter digestibility. Constitutive intracellular targeted expression of FAEA to the apoplast, endoplasmic reticulum or Golgi in *Festuca* also disrupted feruloylation of the growing cell wall with significant reductions in the levels of monomeric and dimeric ferulates resulting in increased biodegradability in terms of cell wall digestibility and increased rates of cellulase-mediated release of fermentable sugars (Buanafina et al. 2010). In addition, the release of monomeric and dimeric ferulic acids from cell walls on autodigestion of leaves expressing FAEA was enhanced several fold by the addition of exogenous β-1,4 endoxylanase (Buanafina et al. 2008; 2010). Expression of beta endo-xylanases in plants has however received considerable attention, although reports of the effects on plant growth and development of constitutive expression of either microbial or fungal endo-xylanases in different cellular compartments in both monocot or dicot species are highly inconsistent (reviewed by Taylor et al. 2008; Bae et al. 2008; and Sainz 2009). Few reports however have described the effects of these *in-planta* expressed cell wall degrading enzymes on cell wall structure. Expression of *Trichoderma reesii xyn2* controlled by a senescence promoter in the apoplast of *Festuca arundinacea,* had no effect on the level of monomeric hydroxycinnamic acids or lignin in the cell walls, but resulted in increased levels of ferulate dimers, decreased levels of xylose and increased levels of arabinose in the cell walls, compared with non transformed plants (Buanafina et al. 2012). High-level

xylanase expression in the apoplast of *Festuca arundinacea* leaves also resulted in ethylene and H_2O_2 accumulation and necrotic lesions on the leaves indicative of plant defence responses analogous to foliar pathogen attack (Buanafina et al. 2012). These changes in cell wall composition resulted in decreases in both tissue digestibility and cellulase mediated sugar release (Buanafina et al. 2012).

In order to test if *in-planta* co-expression of FAEA and XYN2 could improve the digestion of polysaccharides and increase post harvest cell wall deconstruction in grasses more effectively than expression of xylanase or FAE alone, FAEA expressing plants were re-transformed with XYN2. To date *in-planta* co-expression of these two cell wall degrading enzymes has not been previously tested and our experience of expressing FAE and xylanase in *Festuca* indicated that constitutive co-expression might be detrimental to cell growth. We specifically chose therefore to separate the activities of the two enzymes either spatially or temporally using the *Lolium* See1 senescence enhanced promoter for xylanase expression as this is activated at the end of plant growth prior to the start of leaf senescence (Li et al. 2004). Our aim was to determine the feasibility of exploiting synergy between FAE and xylanase to achieve higher levels of cell wall arabinoxylan deferuloylation both at the end of active leaf growth during senescence, and subsequently following cell death. The effects of constitutive co-expression of FAEA in the vacuole or apoplast combined with senescence inducible expression of XYN2 in the apoplast, on plant growth, cell wall ferulates and cell wall sugar composition, lignification, and cell wall degradability are reported here.

Materials and Methods

Plasmid construction

The plasmids used in this study (Figure 1a), were based on expression vectors containing the *faeA* and *xyn2* genes that have been described previously (Buanafina et al. 2008, 2010, 2012). The *Trichoderma reesei* β-1,4 endo-xylanase gene (*xyn*2) gene was placed under the control of a *Lolium multiflorum* senescence promoter (LmSee1) targeted to the apoplast (pIOM6). The *faeA* gene was placed under the rice actin promoter and targeted to the apoplast (pIGB6). In addition, a new construct with constitutive FAE targeted to the vacuole (pINH1Δ) was made by modifying the original pTP3 vector where the Knp1-ECORI fragment (Del in Figure 1a) was removed from the actin promoter in order test if this modification would increase FAEA activity.

Plant transformation

In order to test re-transformation strategies new transformants were produced by retransformation of plants previously transformed with pINH1Δ or with pIGB6 [plant T27 (Buanafina et al. 2008)] (Figure 1a), with a xylanase containing vector pIOM6 (Buanafina et al. 2012). Several plants expressing each FAE construct were selected and the shoot tips cultured *in vitro* to produce *in vitro* stock plants. Callus and then cell suspensions were produced (as in Buanafina et al. 2006) and cell suspensions cultures were re-transformed by bombardment with plasmid pIOM6, containing the XYN2 gene, and as the FAE expressing plants were hygromycin resistant, they were co-transformed with pBKS containing the *npt*II gene under the maize ubiquitin promoter and transformants selected with paromomycin (50-100 mg L^{-1}).

Plant growth and harvesting

Regenerated plants were transferred to soil in 8” pots containing a 5:1 mixture of Miracle-Gro Potting Mix (The Scotts Company, Marrysville, OH 43041) and vermiculite, and grown in a controlled environment chamber at 22/16°C (day/night) temperature, 16 h photoperiod, and 180 µmol photons m^{-2} s^{-1} photosynthetically active radiation. When established, plants were screened for FAE activity. As plants reached maturity they were screened for xylanase activity using green or naturally senescent leaves. Plants showing the highest xylanase activities were harvested to 5 cm above soil level and the plants allowed to regrow. Sub samples of harvested leaves from regrown plants were frozen for FAE and xylanase activity and for Southern and self-digestion analysis. The remaining tissue was freeze-dried and powdered for cell wall chemistry, digestibility and lignin determinations.

Southern analysis of transgenic plants

Total genomic DNA for Southern analysis was isolated from leaves frozen in liquid nitrogen. Each DNA sample was digested overnight at 37ºC with HindIII restriction enzyme (Roche) to liberate the *fae2* gene. For the *xyn2* gene samples were digested with EcoRI and NotI. Ten micrograms of digested genomic DNA were separated by agarose electrophoresis, transferred onto Hybond N^+ membrane by capillary blotting according to the manufacturer’s instructions (Roche) and DNA was fixed to the membrane by UV cross-linking and probed using a digoxigenin-labelled *fae2* or *xyn2* probe, prepared by PCR of plasmid DNA as described in Buanafina *et al*. (2010, 2012).

Determination of XYN2 and FAEA activities

For enzyme activities, fresh leaves were ground to a fine powder in liquid nitrogen and total protein was extracted with 0.1 M Na acetate, pH 5.5 buffer. FAEA activities were determined in soluble protein extracts incubated with 24 mM ethyl ferulate as substrate, at 28ºC for 24hrs, and the released ferulic acid was determined by high-performance liquid chromatography (HPLC) as described in Buanafina *et al.* (2012).

One unit of FAE activity equals 1 µg ferulic acid released from ethyl ferulate in 24 h at 28°C. Xylanase activities were determined by mixing crude protein extracts (190 µl) and 120 µl of 2% Oat Azo-xylan (Megazyme) as substrate. As a control known amounts of IRG 40 xylanase (Genencor Inc) were also incubated with buffer and Azo-xylan. After incubation for 23h at 37 ºC, the reaction was stopped with 800 µl of 98% ethanol (v/v) and the water-soluble blue products released from Azo-xylan measured at 590 nm by spectrophotometry as in Buanafina et al. (2012). Xylanase IRG 40 from *T. reesei* (Genencor Inc) of known specific activity determined with Birchwood xylan substrate using the 3,5,dinitrosalicylic acid method was used as a standard. One unit of plant derived xylanase activity was equivalent to 1 unit of IRG 40 xylanase, defined as 1 µmole xylose released from Azoxylan min^{-1} at 37^{o}C.

Preparation of isolated cell walls

Cell walls were prepared as follows: 150 mg of freeze-dried milled samples were boiled in 90% ethanol, centrifuged and the supernatant removed. Pellets were incubated in 90% ethanol, with shaking at room temperature for 10 min, centrifuged to remove the supernatant and the procedure repeated. Pellets were

then washed with 100 % methanol, shaken for 10 min, centrifuged and the supernatant discarded. The procedure was repeated with distilled water and shaken for 1 h, centrifuged and the pellets of purified cell walls (AIR) freeze dried.

Determination of cell wall hydroxycinnamic acid and arabinoxylan composition

Quantitative analysis of ester-bound HCAs was determined in isolated cell walls by High Performance Liquid Chromatography (HPLC) carried out as previously described (Buanafina et al. 2012). The levels of the monosaccharides xylose, and arabinose in isolated cell walls were determined by High Performance Anion Exchange Chromatography (HPAEC) of hydrolysed samples based on the method of Øbro et al. (2004) with modifications as described in Buanafina et al.(2012) .

Lignin Determinations

Acetyl bromide soluble lignin: Cell wall material for analysis was prepared according to Dean (1977). Approximately 50 mg of freeze-dried ground tissue was sequentially washed with ethanol (95%) / toluene for 4 h, followed by 95% ethanol for 4 h and then water for 2 h. Following washes, pellets were lyophilized overnight and lignin content of dried material determined using the acetyl bromide method (Chang et al. 2008). Briefly, 5 mg of cell wall tissue was digested in 1 ml 25 % acetyl bromide in acetic acid and incubated in a 70^{o}C water bath for 30 min and vortexed every 10 min. Following digestion and cooling; 5 ml acetic acid was added to each sample. To 300 µl of the mixture, 400 µl of 1.5 M NaOH and 300 µl of 0.5 M hydroxylamine hydrochloride were added. The solution was mixed, diluted with 1.5 ml acetic acid and the absorbance of the solution read

at 280 nm using a Multiskan Spectrum high performance spectrophotometer (Thermo Scientific). A blank was used to correct the background absorbance of the reagents. Lignin concentrations were calculated using the extinction coefficient (17.75 cm^2/g) derived from purified HCL-dioxane lignin isolated from corn stems (Fukushima and Hatfield 2004).

Klason Lignin: Determinations were based on the Tappi T 222 OM-11 method (2011) using 0.5 g of freeze dried powdered sample, hydrolysed with 5 ml of 72 % H_2SO_4 for 2 h at ~ 20 ^oC, with stirring every 15 minutes. Deionised water (140 ml) was added and samples refluxed for 4 h. Filtered sample were washed with deionised water, dried and weighed and then ashed and reweighed. Based on in-house QC material for Klason lignin RSD = 0.03487.

Digestibility Determinations

Auto-digestion: The loss of cell wall HCAs and sugars mediated by the action in *in-planta* expressed FAEA and XYN2 was calculated as the difference between amounts in purified cell wall AIR and the amounts found in AIR recovered from digested cell wall pellets. Briefly, leaves (1.0 g fresh wt) were first ground in liquid nitrogen and then incubated in 2 ml 0.1 M Na acetate extraction buffer pH 5.5 at 37 ºC for 48 h. Following centrifugation, cell walls were extracted from the self-digested pellets (as described above) and incubated with either 1M NaOH for 24h at 25 °C under nitrogen and the released ferulates quantified by HPLC, or were hydrolysed with TFA and the levels of xylose and arabinose quantified as described above.

Cellulase mediated sugar release from autodigested cell walls: Freeze-dried powdered leaf material (10 ± 0.2 mg), was incubated with shaking in

extraction buffer (0.1 M sodium acetate, pH 5.5), for 12 h, and the supernatant containing soluble sugars removed following centrifugation. Washed, autodigested cell wall pellets were incubated with 63 units/ml of *T. reesei* cellulase (Sigma) at 37 ºC for 24 h. Reducing sugars in the medium were determined by the ρ-hydroxybenzoic acid hydrazide (PAHBAH) method as in Buanafina et al. (2010). All assays were performed in duplicate.

In vitro dry matter digestibility (IVDMD): The digestibility of leaves was estimated by a two-stage *in vitro* pepsin-cellulase solubility technique (Jones and Howard, 1975), which determines the amount of biomass remaining at the end point of 48h digestion of 1.0g dry weight of powdered whole tissue. Based on in-house reference samples included in each analysis, the RSD of the method was 0.1904.

Statistical analysis

All statistical analyses were performed with Statistical Analysis System (SAS) (2010) software. Values in the text are means ± standard error (sem). Bars with different letters are significantly different (Tukey's, $\alpha = 0.05$). Linear correlations between HCAs and soluble acetyl bromide lignin were calculated using the Pearson product-moment correlation coefficient. Before ANOVA some of the data was log transformed to stabilize variance and a permutation test was applied using GenStat for Windows 16th Edition, VSN International Ltd., Hemel Hempstead, UK.

Results

Transformation and molecular analysis of plants co-expressing FAEA and xylanase

Previously produced plants expressing FAEA targeted to either the vacuole (line 397) (Buanafina et al. 2008) or apoplast (line 320) (Buanafina et al. 2010) were re-transformed with apoplast targeted XYN2 regulated by a senescence promoter in the combinations shown in Figure 1a&b. Three T0 plants from each of the 320 and 397 lines were selected at random without reference to their expressed enzyme activities for genomic DNA extraction and Southern hybridization analysis using *xyn2* and *faeA* genes as probes in order to confirm integration of the transgenes. Following DNA extraction samples were digested with HindIII, (releasing a 1.2-Kb fragment containing the *faeA* sequence) or with EcoR1/NotI, (releasing a 0.595 Kb fragment containing the *xyn2* sequence). The hybridization patterns observed were absent in controls, and confirm the integration of the *faeA* and *xyn2* genes in the plant genome (Figure 1c).

FAEA and xylanase activities of leaf extracts of senescing T0 and T5 plants.

Five 320line plants produced by re-transforming a single parent plant constitutively expressing apoplast targeted FAEA (plant T27 [Buanafina et al. 2008]), (Figure 2 a&c), and 15 plants of the 397line produced by re-transforming three independently transformed parent plants constitutively expressing vacuole targeted FAEA, (Figure 2b&d), showed FAEA and XYN2 activities similar to those reported previously in *Festuca* (Buanafina et al. 2006, 2008, 2012).

Three plants from line 320 and four plants from line 397 were selected for further analysis. Based on previous results where the levels of XYN2 expression in

Festuca plants transformed with XYN2, increased following cycles of vegetative propagation (Buanafina et al. 2012) , plants expressing FAEA and XYN2 were propagated by *in vivo* tillering and re-growth for five cycles to give T5 generation plants before further analysis. As previously found, the levels of XYN2 activity were significantly higher in T5 generation plants compared to activities in T0 plants, in most of the selected lines (Figures 2e&f), with an average 24 fold increase in xylanase activity in T5 plants of the 320 line and an average 6 fold increase in T5 plants of the 397 line. However in some lines such as 320 BN2, 4 and, 5 and 397 BN5, 6,110,120,127, and 198 XYN2 activity was silenced in T5 plants and these lines were not studied further. As previously reported apoplastic XYN2 activity driven by the See1 promoter in T0 *Festuca* plants was not expressed until the early stages of leaf senescence and in the selected 397 and 320 re-transformed lines XYN2 activity remained inducible on leaf senescence in T5 plants after repeated tillering (Figure 3).

In contrast to XYN2 activities, the levels of FAEA activity in T5 plants of the 320 and 397 lines decreased an average 2.7 and 2.5 fold respectively compared with T0 plants (Figure 2a, b, e). This may be a result of variation in transgene expression in meristems during the formation of new tillers and/or biased selection of the more vigorous tillers during propagation.

Effect of XYN2 and FAEA co- expression on growth and development of T5 plants.

Measurements of growth and biomass of co-expressing T5 plants showed that at harvest both parameters were adversely affected by constitutive FAEA and senescence induced XYN2 expression. Co-expression of both FAEA and XYN2 in

the apoplast (line 320BN) resulted in T5 plants with narrower leaves and a 71 % decrease in mean biomass accumulation and a 31% decrease in mean plant height (Figure 4). The phenotype of plant 320 BN 3, compared to a non-transformed control plant at harvest is shown in Figure 4a. T5 plants co-expressing FAEA targeted to the vacuole, and XYN2 to the apoplast (397BN plants) were less affected and showed a 32% decrease in mean biomass levels and a 13 % decrease in mean leaf length compared to controls. Similar results were obtained previously in T5 plants with XYN2 constitutively expressed in the apoplast, but no effects on growth were found when XYN2 was expressed under a senescence promoter (Buanafina et al. 2012). FAEA targeted to the vacuole (Buanafina et al. 2008) or apoplast (Buanafina et al. 2010), at similar levels of expression, resulted in morphologically and developmentally normal T0 plants with little or no effect on fresh weight yield or plant height at harvest, contrary to the growth inhibition of constitutive FAEA expression in the T5 plants reported here.

Effect of FAEA and XYN2 co-expression on cell wall composition of senescing leaves of T5 plants at harvest

The effect of co-expressing FAEA and XYN2 on the levels of cell wall esterified hydroxycinnamic acids (HCAs), sugars and lignin was determined in leaves of selected T5 FAEA+XYN2 expressing plants and compared to clonal control non transformed plants.

Cell wall phenolics: Co-expression of constitutively expressed FAEA with senescence induced XYN2 both targeted to the apoplast (320 lines) had a small but not statistically significant effect on mean levels of cell wall *p*-coumaric acid

(Figure 5a), but resulted in highly significant reductions in the mean levels of esterified cell wall ferulate monomers (31%) (Figure 5a), and dimers (36%) (Figure 5c), compared to control means. However when vacuolar targeted FAEA was combined with senescence induced apoplast targeted XYN2, (397 lines), this resulted in smaller non-significant reductions in the level of cell wall esterified ferulate monomers (4.6%) (Figure 5b), and dimers (2.6%) (Figure 5c).

Cell wall sugars: Constitutive expression of FAEA and senescence induced apoplast expression of XYN2 also resulted in significant reductions in the mean levels of cell wall arabinose and xylose in both 397 and 320 lines compared with controls (Figure 5d). This reduction ranged from 10% for xylose and 19% for arabinose in 397 lines with vacuole targeted FAE to 18% for xylose and 34% for arabinose in 320 lines with apoplast targeted FAE compared to control plants (Figure 5d-e). As a consequence of these changes the arabinose:xylose ratio of the cell wall sugars decreased in both the 397 and 320 lines compared to control plants (Figure 5e).

Lignification: Considering the potential of FAEA and XYN2 co-expression to release ferulates and diferulates as well as arabinose and xylose from the arabinoxylan backbone, and consequently to potentially decrease the cross-linking between arabinoxylan and lignin, the level of acetyl bromide soluble lignin was determined and compared to levels of total Klason lignin.

No significant changes in Klason lignin levels of whole leaf biomass were found by co-expression of either apoplast or vacuole targeted FAEA with apoplast targeted xylanase (Figure 5f). However, the levels of acetyl bromide soluble lignin

extractable from partially purified cell walls (AIR) increased by 18% in plants co-expressing vacuole targeted FAEA with apoplast targeted xylanase (397 lines) and by 31% in plants co-expressing apoplast targeted FAEA and xylanase (320 lines) (Figure 5f). Levels of ferulate monomers, dimers and total ferulates were negatively correlated (P=0.0028, r^2= 0.755; P=0.0008 r^2= 0.807 and P= 0.0006 r^2= 0.8197, respectively) to acetyl bromide lignin, but no significant correlation was found between *p*-coumaric acid and levels of acetyl bromide soluble lignin

Effects of FAEA and XYN2 co-expression on cell wall digestion of senescing leaves of selected T5 plants at harvest

Three methods were used to assess the effects of FAEA and XYN2 co-expression on different aspects of post harvest cell wall biodegradability. The loss of cell wall esterified monomeric and dimeric ferulates and cell wall sugars on autodigestion of leaf samples was used to assess the effectiveness of *in-planta* expressed enzymes to degrade cell walls following cell disruption. Cellulase mediated sugar release from residual cell walls after autodigestion was used to determine the effects these changes had on subsequent sugar release, and *in vitro* dry matter digestibility (IVDMD) was used to determine the direct effect FAEA and XYN2 co-expression may have had on cell wall composition on the loss of biomass following direct treatment of whole tissues.

Auto-digestion: The effects of transgene-expressed FAEA and XYN2 activities were determined by the release of cell wall esterified HCAs and sugars from senescing leaves using an auto-digestion procedure, which involved incubating macerated leaves in buffer for 48h and determining the released esterified ferulates diferulates and arabinoxylan sugars from the cell walls.

Significant amounts of the cell wall HCAs (p=0.05) were removed on auto-digestion irrespective of whether FAE was expressed in the vacuole or apoplast. The released HCAs consisted of *p*-coumaric acid and both ferulate monomers and dimers, with mean losses of total HCAs for apoplast located FAE amounting to 16%, and for vacuole located FAE, 9% of the total HCA content of the leaf cell walls compared with 3.6% for control plants (Figure 6a). ANOVA showed a significant contrast between control and the two transgenic lines for *p*CA (p=0.06), and ferulate dimers (p=0.048). The total amount of HCAs released on autodigestion was 6 to 10 times higher than previously found with plants expressing either vacuolar or apoplast FAEA, or apoplastic XYN2 alone, and 1.3 and 2.4 times greater than where FAEA expressing tissues were digested in the presence of external applied XYN2, or where XYN2 expressing tissues were digested in the presence of external applied FAEA (Table 1).

Auto-digestion also resulted in the release of significant amounts arabinose and xylose from cell walls of senescing leaves compared to controls (Figure 6b), with means losses of the total arabinoxylan content of the leaf cell walls amounting to 16% for apoplast located FAE and 19% for vacuole located FAE compared with 3% for control plants. In addition, the mean arabinose to xylose ratio decreased significantly in the cell wall residue following auto-digestion from 0.54 in control plants to 0.3 in the 397 lines and to 0.38 in the 320 plants. However the release of ferulate crosslinked arabinoxylan from co-expressing FAEA and XYN2 tissue on autodigestion could not be established.

Cellulase mediated release of glucose from autodigested cell walls: The ability of plant expressed FAEA and xylanase to render the cell walls more susceptible to further enzymatic degradation was assessed by determining the

release of glucose from autodigested cell walls treated with *T. ressei* cellulase. Plants of line 320, which showed reduced levels of ferulate monomers and dimers (Figure 5b&c) and the lowest levels of cell wall arabinoxylans (Figure 5d) showed a significant 31 % increase in the mean levels of glucose released from autodigested cell walls (Figure 6c). Plants from line 397 showed a 12 % increase in the mean levels of glucose released from autodigested cell walls treated with *T. ressei* cellulase (Figure 6c), and although this was not statistically significant, glucose released by two of the individual lines were significantly different from controls.

***In vitro* dry matter digestibility** (IVDMD*).* The direct effect of reduced levels of cell wall HCAs and AX on tissue digestibility was also determined by the extent of biomass loss following the two-stage *in vitro* pepsin-cellulase solubility technique of Jones and Hayward (1975). Mean levels of IVDMD were not significantly different from controls in 397 plants and while mean levels of IVDMD increased by 5 percentage units in 320 plants this was not statistically significantly different from control values (Figure 6d).

Discussion

Following re-transformation of fescue plants constitutively expressing apoplast or vacuole targeted FAE, with a senescence induced apoplast targeted xylanase, a set of plants with both FAE and XYN2 enzyme activity, and with significantly reduced levels of esterified cell wall ferulates, diferulates and arabinoxylans was obtained. The expression of both constitutive and senescence induced FAE or XYN2 alone has been reported previously in forage grasses (Buanafina et al.

2004, 2006, 2010; 2012), but this is the first report of *in-planta* co-expression of both enzymes.

FAE and XYN2 activities in co-expressing plants were initially found to be unstable on repeated tillering, with the level of xylanase activity increasing and FAE activity decreasing following five cycles of vegetative propagation. This could possibly be as a result of biased selection of the more vigorous tillers during propagation, as tillers were not screened for FAE activity, and some of the T5 plants, which originally expressed both FAEA and XYN2 in T0 plants, were silenced for either FAE or xylanase expression. Increased levels of xylanase activity (Buanafina et al. 2012), and both increased and decreased GUS activity, as well as transgene silencing following tillering in tall fescue has been reported previously (Bettany et al. 1998) and re-emphasises the need for expression analysis when choosing tillers for vegetative plant propagation. As xylanase was not constitutively expressed in tillers but only at leaf senescence, any subsequent effects of xylanase expression on the plant phenotype would not have influenced the visual selection of tillers at the time of propagation. It is therefore possible that any deleterious effects of constitutive FAE activity on plant development could have been selected against by this procedure. Constitutive apoplastic expression of FAE in *Festuca* was previously found to have little effect on the growth and development of T0 plants (Buanafina et al. 2010), but on repeated vegetative propagation by tillering these plants developed a new and stable phenotype with narrow leaves and with modified leaf growth kinetics. The FAE and xylanase co-expressing plants reported in this work also developed this narrow leaf phenotype following repeated tillering, and is suspected to be responsible for the reduction in

biomass. To overcome this in the future it may be advisable to target both xylanase and FAE expression to the apoplast under senescence promoters.

The initial instability of FAE and XYN2 activities in co-expressing plants on repeated tillering might indicate that transgene expression is unpredictably modified during the formation of new meristems from somatic stem cells. This may suggest evidence for the mitotic modification of epigenetic effects similar to the transcriptional silencing of transgene expression in plants, associated with meiotically heritable epigenetic modifications (Matzke and Matzke, 1988). A similar effect was reported with lateral buds in plants regenerated from cultured leaf discs, which showed that “once established, competence for silencing can persist in dormant, actively growing and de-novo established shoot meristems” (Kunz et al. 1996).

In contrast to previous reports where high levels of apoplast located XYN2 activity was found to induce pathogen defence responses (Buanafina et al. 2012), plants expressing similar levels of XYN2 activity, but with low FAEA activities, showed no signs of xylanase induced pathogen responses, such as high levels of cell wall ferulate dimers or necrotic lesions on the leaves. Comparing the mean levels of HCAs and AX of cell walls of senescing leaves of co-expressing plants with the original parent plants expressing apoplast FAE only, and with plants expressing senescence induced xylanase only, it is possible to conclude that constitutive expression of FAE in the apoplast may have been sufficient to overcome the ferulate dimerisation triggered by apoplast located xylanase (Figure 7). Possible reasons for this may be that either there is a continual cycling of dimers with FAE removing them faster than they can be added by the action of xylanase induction of ethylene and H_2O_2, or that constitutive FAE expression in the apoplast results in

a reduction in the ferulate levels such that even with an increase in cell wall esterified diferulates due to xylanase induction of crosslinking via ethylene induced H_2O_2 production in the cell wall, the levels of diferulates remain below control levels.

The finding that co-expression of FAEA and XYN2 significantly increased the amount of acetyl bromide soluble lignin, with a significant negative correlation between ferulates and acetyl lignin levels suggests that the ester linkages in some ferulate dimers may be reduced by expressed FAEA, resulting in an increase in acetyl bromide lignin solubility. Furthermore, with reduced levels of ferulate monomers the rate of ferulate dimerisation may be reduced and consequently the level of ether linkages to lignin. Similar increases in lignin solubility were also found in alfalfa expressing vacuole or apoplast FAE, but with increased residual lignin content following digestion (Badhan et al. 2014).

The limited effects of co-expression of FAEA and XYN on IVDMD and cellulase mediated sugar release of the 397 plants may be partially due to the much lower mean levels of vacuolar FAE activity of T5 plants (75 units g fresh wt $^{-1}$) than the mean levels of vacuolar FAE activity previously reported (200-300 units g fresh wt $^{-1}$) in T0 plants which showed increases in IVDMD of 6 to 10 percentage units (Buanafina et al. 2008). In addition much higher levels of apoplastic xylanase activities (7500 mU g fresh wt $^{-1}$) were found in these co-expressing T5 plants than in T0 plants expressing only senescence induced apoplastic xylanase (400 mU g fresh wt $^{-1}$). These lower activities previously resulted in both a 12 percentage units decrease in IVDMD and a 30% decrease in cellulase mediated sugar release due to induced pathogen defense responses resulting in enhanced ferulate dimerization (Buanafina et al. 2012).

It is difficult therefore to reconcile the observed changes in cell wall structure and enhanced post harvest deferuloylation of cell wall arabinoxylans in plants expressing both FAE and xylanase with the reduced levels of cellulase-mediated digestion. However, this may be partially explained by recent studies showing that arabinoxylan adsorption onto cellulose is controlled by the fine structure of the arabinoxylan. In particular where a reduction in the arabinose content of arabinoxylan leads to stronger adhesion between xylan and cellulose (Köhnke et al. 2011) and where a lower cell wall arabinose:xylose ratio lead to reduced enzymatic digestibility of *Miscanthus* lignocellulose (Li et al. 2013).

In contrast to first and second generation biofuels we anticipate that future third generation biofuels may well be produced by combined saccharification and fermentation of lignocellulose biomass under mild processing conditions In this respect expressing cell wall degrading enzymes such as xylanases (Bae et al. 2006; Shen et al. 2012; Borkhardt et al. 2010; Kim et al. 2011; Chou et al. 2011) , cellulases (Klose et al. 2013; Kawazu et al. 1999; Ransom et al. 2007; Oraby et al. 2007; Hood et al. 2007; Klose et al. 2012), esterases (Harholt et al. 2010; Buanafina et al. 2006, 2008, 2010) and glycosidases (Montalvo-Rodriguez et al. 2000) have been shown to improve post harvest cell wall degradation and provide examples of how progress is being made towards generating novel biomass for these third generation biofuels.

Conclusions

The present study demonstrates the principle that the synergic effect of xylanase on FAE mediated release of ferulates from cell wall arabinoxylans can be achieved by *in-planta* co-expression of these two cell well degrading enzymes.

Targeted co-expression of a fungal FAE and xylanase to the apoplast was shown to significantly reduced the levels of esterified ferulates, diferulates and arabinoxylans of the cell wall and increase the extent of post-harvest cell wall deconstruction of the relatively low digestible temperate forage grass *Festuca arundinacea*. Higher levels of HCAs and arabinoxylan were removed from the cell wall following autodigestion, and significantly increased cellulase mediated sugar release and lignin solubility were found compared to plants expressing FAE or xylanase alone. It is clear that for future application the effect of constitutive FAE expression in the apoplast on biomass yield needs to be mitigated, perhaps by senescence induced apoplast expression of both FAE and xylanase. We consider that this technology may have potential to contribute to more efficient and environmentally benign third generation biofuel production, characterized by mild enzyme based post-harvest treatment of genetically modified lignocellulose biomass in a combined saccharification and fermentation system.

Acknowledgments

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Figure Legends

Figure 1. **Molecular aspects.**

(a) Vector components and **(b**) vector combinations used for re-transformation of plants expressing an *Aspergillus niger* gene (*fae*A) targeted to the vacuole (pINH1Δ) or apoplast (pIGB6), under the rice actin promoter with a *Trichoderma reesei* ß-1,4 endo-xylanase gene (*xyn*2) targeted to the apoplast under a senescence promoter (LmSee1) (pIOM6) See1 is a *Lolium multiflorum* senescence enhanced gene promoter and intron. Aleurain is a barley aleurain signal sequence with a vacuolar targeting motif. PPI is a potato protease inhibitor conferring apoplast targeting. **(c)** Southern blot analysis of digested genomic DNA hybridised with *faeA* probe (a) or with *xyn2* probe (b). C+1, C+1* and C1+1# = control plant DNA with one genome equivalent of plasmids pINHIΔ; or pIGB6 or pIOM6 respectively. DNA digested with HindIII which excises a 1.2-Kb *faeA* fragment (a), or EcoR1/NotI, which cuts out a 0.595 Kb *xyn2* fragment (b). Each lane contains 10 µg DNA. The expected fragments [1.2 Kb in (a) and 595 bp in (b)] for intact transgenes are indicated by arrow. Sizes of molecular weight markers on the left.

Figure 2. FAEA and xylanase activities of leaf extracts of senescing T0 and T5 plants.

(a) Levels of FAEA enzyme activity with ethyl ferulate in T0 plants of line 320Bn **(b)** Levels of FAEA enzyme activities in T0 plants of line 397Bn Activities are compared with the FAE activities in the parent FAE expressing plants. **(c)** Levels of XYN2 enzyme activities with Oat Azo-xylan in T0 plants of line 320BN. **(d)**

Levels of XYN2 enzyme activities in T0 plants of line 397BN . **(e)** Levels of FAEA enzyme activity with ethyl ferulate in T5 plants of lines 320BN and 397BN. **(f)** Levels of XYN2 enzyme activities in T5 plants of lines 320BN and 397BN. T5 plants had undergone 5 cycles of vegetative propagation by *in vivo* tillering. Plants were constitutively expressing vacuole (Line 397) or apoplast (Line 320) targeted FAEA, and senescence induced apoplast targeted XYN2. Control plants were non transformed regenerates of the same *Festuca* genotype. Single determinations from independently transformed or control plants. ***** indicates plants which were subsequently characterized in detail. One unit of FAE activity equals 1 µg ferulic acid released from ethyl ferulate in 24 h at 28°C. One unit of xylanase activity equals 1 µmole xylose released from Azoxylan / min at 37 ^{o}C.

Figure 3. Senescence induced xylanase activity in leaves of two T5 plants, 320 BN5 and 320 BN3 with FAE under a constitutive promoter and senescence induced xylanase, both targeted to the apoplast after repeated vegetative propagation by *in vivo* tillering. Single determinations from pooled leaves of individual plants

Figure 4. Effects on plant growth.

(a) Control *Festuca* plant (a) and a T5 plant 320 BN3 (b) at harvest. Plant 320 BN3 was constitutively expressing FAEA and senescence induced apoplast targeted XYN2. **(b)** Effect of FAEA and XYN2 expression on leaf growth and **(c)** biomass accumulation of T5 plants at harvest. Plants were constitutively expressing vacuole (Line 397) or apoplast (Line 320) targeted FAEA, and senescence induced apoplast targeted XYN2. Plants harvested after 8-10 months when the

leaves began to senesce. Mean ± SEM of single determinations from (n) independently transformed or control plants. * indicates significant differences from the control (Tukey's, α = 0.05).

Figure 5. Effects on cell wall structure of senescing leaves of selected T5 plants at harvest.

(a) *p*-coumaric acid, **(b)** ferulate monomers, **(c)** ferulate dimers, **(d)** arabinose and xylose, **(e)** arabinose:xylose ratio, **(f)** Klason lignin and acetyl bromide lignin of isolated cell walls (AIR). Plants were constitutively expressing vacuole (Line 397) or apoplast (Line 320) targeted FAEA, and senescence induced apoplast targeted XYN2. Ferulate monomers: trans-ferulic + cis-ferulic acid. Dimers = 8-0-4'-diferulic; 5-5'-diferulic; 8-5cyc-diferulic benzofuran; and an unknown ferulate acid dimer quantified as ferulic acid. Mean ± SEM of triplicate determinations from (n) independently transformed or control plants. Based on in-house QC material RSD and %CV for Klason lignin were, STM 0.50 RSD = 0.03487 %CV= 3.487. * indicates significant differences from the control (Tukey's, α = 0.05).

Figure 6. Effects on cell wall digestion of senescing leaves of selected T5 plants at harvest. (a) Loss of hydroxycinnamic acids and **(b)** arabinose and xylose on auto-digestion. **(c)** Cellulase mediated release of glucose from autodigested cell walls on further digestion with 63 units/ml *T. reesei* cellulase. **(d)** *In vitro* dry matter digestibility Plants were constitutively expressing vacuole (Line 397) or apoplast (Line 320) targeted FAEA, and senescence induced apoplast targeted XYN2. Mean ± SEM of triplicate determinations from (n) independently transformed or control plants as the % of corresponding undigested

cell walls (A, B). Mean ± SEM of triplicate (C) or single (D) determinations from (n) independently transformed or control plants. Based on in-house QC material RSD and %CV were STM 0.26 RSD = 0.1904 %CV= 1.904 for IVDMD.

Figure 7. Comparison of the mean levels of HCAs (**a**) and AX (**b**) of cell walls of senescing leaves of T5 plants constitutively expressing FAE and xylanase targeted to the apoplast with the original parent plant expressing apoplast FAE only (T27), and with plants expressing senescence induced xylanase only (X1-X9). Mean ± SEM (n=3) determination from n plants. Corresponding control plants for each vector =100%. For further details on the characteristics of the parent plant 266Bn10 see plant T27 in Buanafina et al 2010 and for plants X1-X9 see Buanafina et al. (2012).

Table 1

Levels of total HCAs released from leaf cell walls of transformed plants on autodigestion. Values were corrected for the soluble HCAs release from corresponding control plants, which varied from 20.0 ± 7.3 (n=11) to 34.4 ± 7.1 (n=11) µg/g fresh wt. in the absence or presence of applied xylanase or FAE respectively. (* based on 80% water content of leaves)

Plant	- Xylanase	+ Xylanase (1000U)	+FAE (1U)	Reference
Constitutive vacuole FAE	27 ± 9 (n= 12)	152 ± 23 (n=12)	-	Buanafina et al. 2008
Constitutive apoplast FAE	21 ± 2 (n= 10)	155 ± 25 (n= 10)	-	Buanafina et al. 2010
Constitutive golgi FAE	26 ± 2 (n= 5)	135 ± 20 (n= 5)	-	Buanafina et al. 2010
Constitutive ER FAE	22 ± 2 (n= 6)	140 ± 35 (n= 6)	-	Buanafina et al. 2010
Senescence apoplast xylanase	30 ± 10 (n= 3)	-	72 ± 50 (n= 3)	Buanafina et al. 2012
* Constitutive apoplast FAE + senescence apoplast xylanase	297 ± 46 (n= 2)	-	-	This paper Fig 5
* Constitutive vacuole FAE + senescence vacuole xylanase	213 ± 52 (n= 4)	-	-	This paper Fig 5

Figure

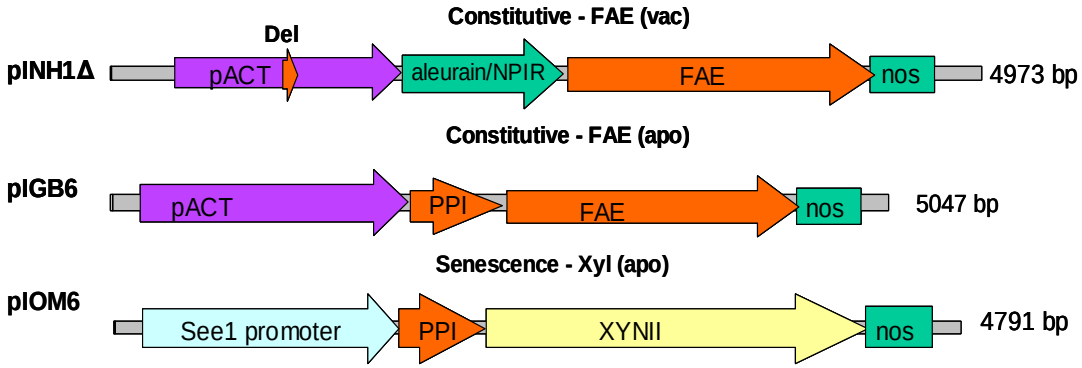
Functional co-expression of a fungal ferulic acid esterase and a β-1,4 endoxylanase in *Festuca arundinacea* (tall fescue) modifies post harvest cell wall deconstruction.

Marcia M. de O. Buanafina[1 3 *], Sue Dalton[2 3], Tim Langdon[2 3], Emma-Timms-Taravella[2 3], Erica A. Shearer[1] and Phillip Morris[3]

Figures

Figure 1:

a



b

Vector combinations	Targeting	Plants	N° Plants
pIGB6 then pIOM6	FAE (apo) + See1 xyl (apo)	320 BN	7
pINH1Δ then pIOM6	FAE (vac) + See1 xyl (apo)	397 BN	15

c

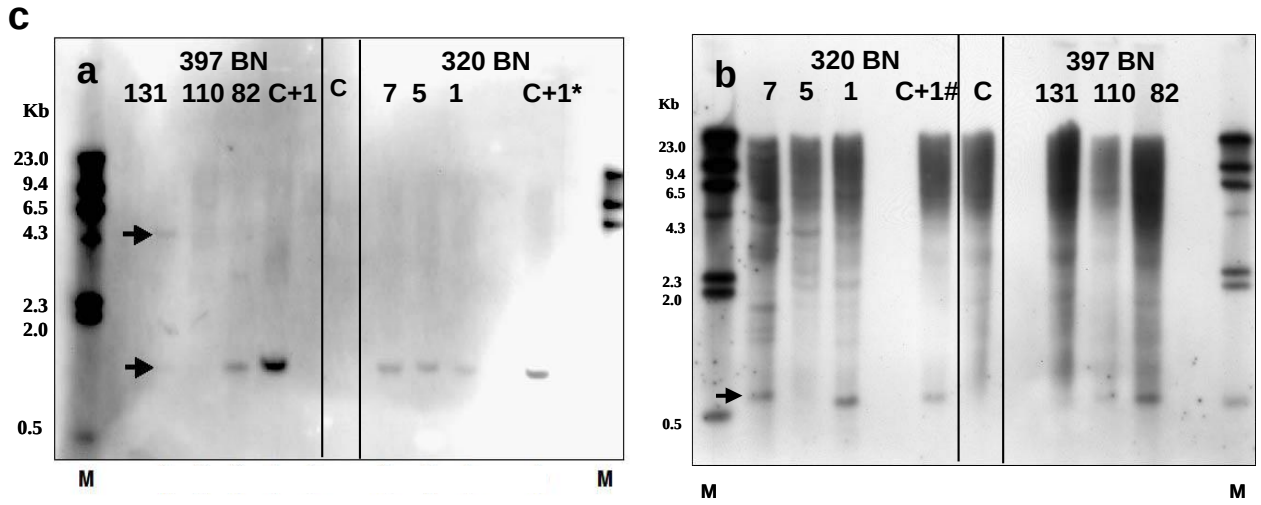


Figure 2:

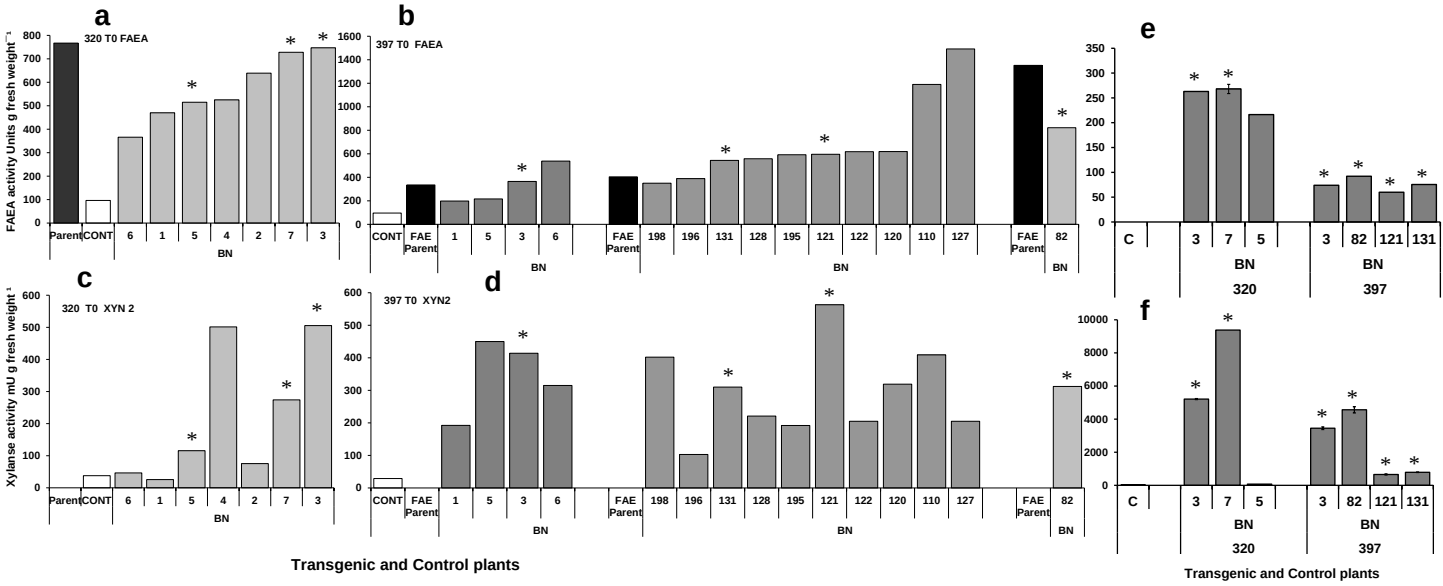

a
320 T0 FAEA
FAEA activity Units g fresh weight⁻¹
Parent CONT 6 1 5 4 2 7 3
BN
b
397 T0 FAEA
CONT FAE Parent 1 5 3 6 FAE Parent 198 196 131 128 195 121 122 120 110 127 FAE Parent 82
BN
e
C 3 7 5 3 82 121 131
BN 320
BN 397
c
320 T0 XYN 2
Xylanse activity mU g fresh weight⁻¹
Parent CONT 6 1 5 4 2 7 3
BN
d
397 T0 XYN2
CONT FAE Parent 1 5 3 6 FAE Parent 198 196 131 128 195 121 122 120 110 127 FAE Parent 82
BN
f
C 3 7 5 3 82 121 131
BN 320
BN 397
Transgenic and Control plants
Transgenic and Control plants

Figure 3:

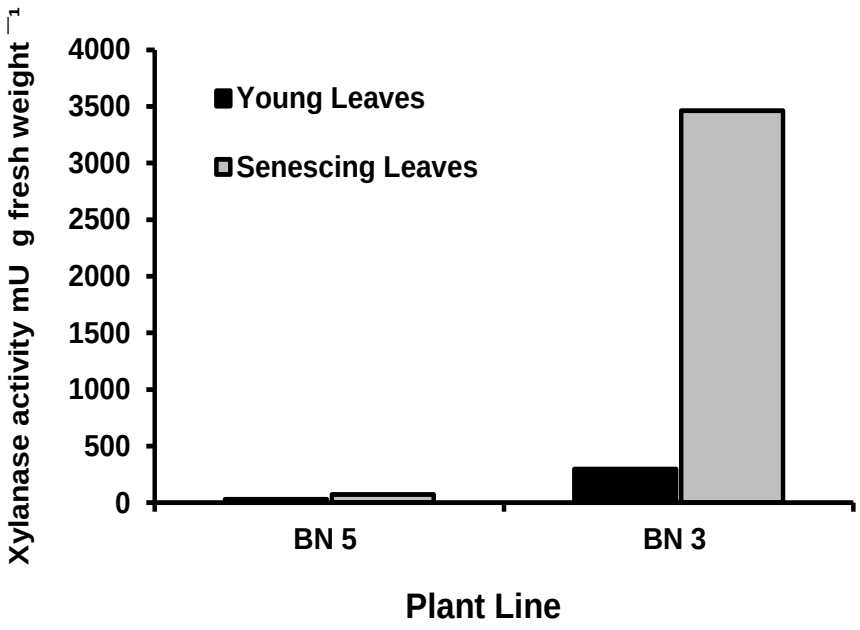

Xylanase activity mU g fresh weight ⁻¹
4000
3500
3000
2500
2000
1500
1000
500
0
Young Leaves
Senescing Leaves
BN 5
BN 3
Plant Line

Figure 4:

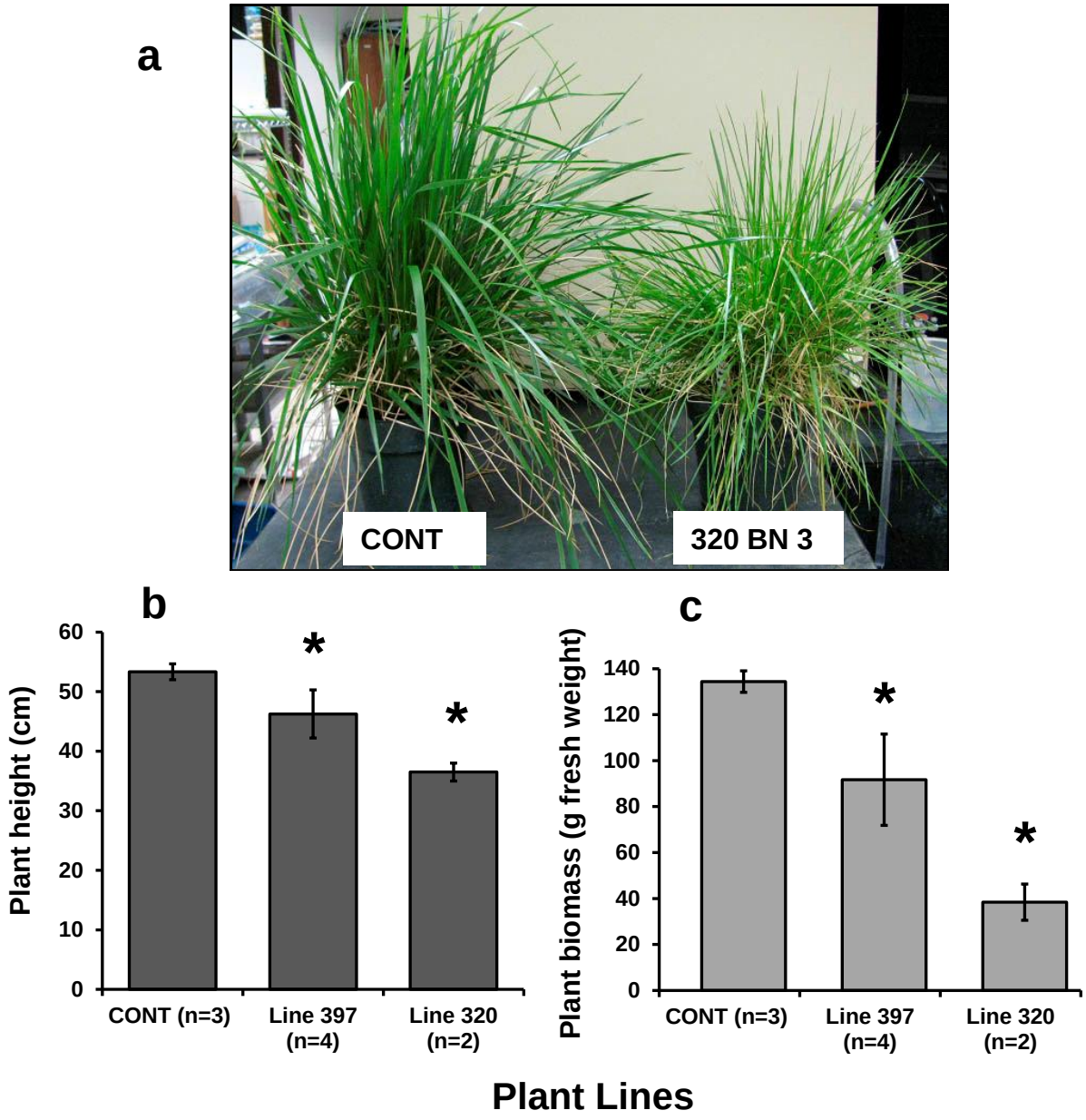

a
CONT
320 BN 3
b
Plant height (cm)
60
50
40
30
20
10
0
*
*
CONT (n=3)
Line 397 (n=4)
Line 320 (n=2)
c
Plant biomass (g fresh weight)
140
120
100
80
60
40
20
0
*
*
CONT (n=3)
Line 397 (n=4)
Line 320 (n=2)
Plant Lines

Figure 5:

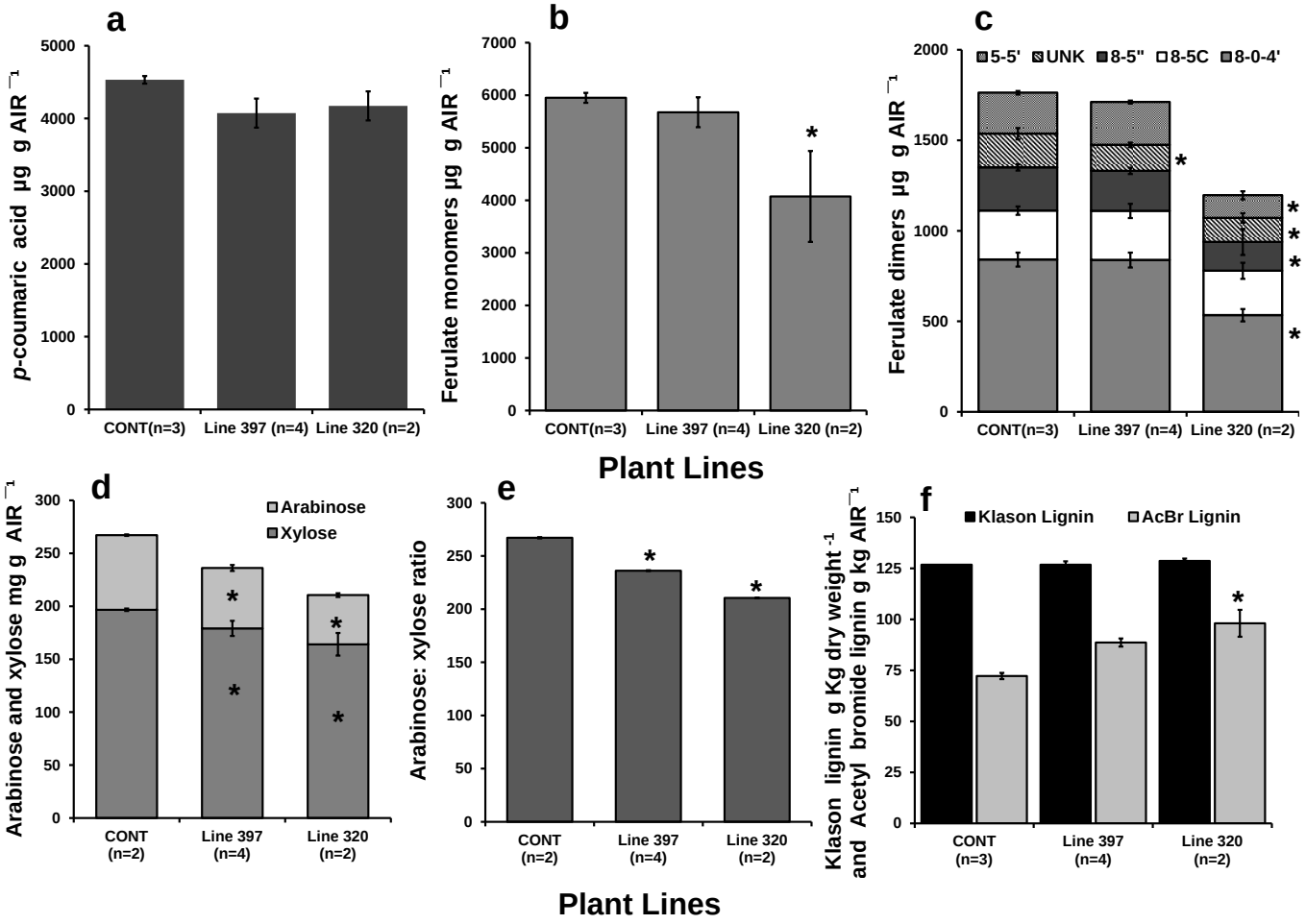

a
p-coumaric acid µg g AIR⁻¹
5000
4000
3000
2000
1000
0
CONT(n=3)
Line 397 (n=4)
Line 320 (n=2)
b
Ferulate monomers µg g AIR⁻¹
7000
6000
5000
4000
3000
2000
1000
0
CONT(n=3)
Line 397 (n=4)
Line 320 (n=2)
c
Ferulate dimers µg g AIR⁻¹
5-5'
UNK
8-5"
8-5C
8-0-4'
2000
1500
1000
500
0
CONT(n=3)
Line 397 (n=4)
Line 320 (n=2)
Plant Lines
d
Arabinose and xylose mg g AIR⁻¹
Arabinose
Xylose
300
250
200
150
100
50
0
CONT (n=2)
Line 397 (n=4)
Line 320 (n=2)
e
Arabinose: xylose ratio
300
250
200
150
100
50
0
CONT (n=2)
Line 397 (n=4)
Line 320 (n=2)
f
Klason lignin g Kg dry weight⁻¹ and Acetyl bromide lignin g kg AIR⁻¹
Klason Lignin
AcBr Lignin
150
125
100
75
50
25
0
CONT (n=3)
Line 397 (n=4)
Line 320 (n=2)
Plant Lines

Figure 6:

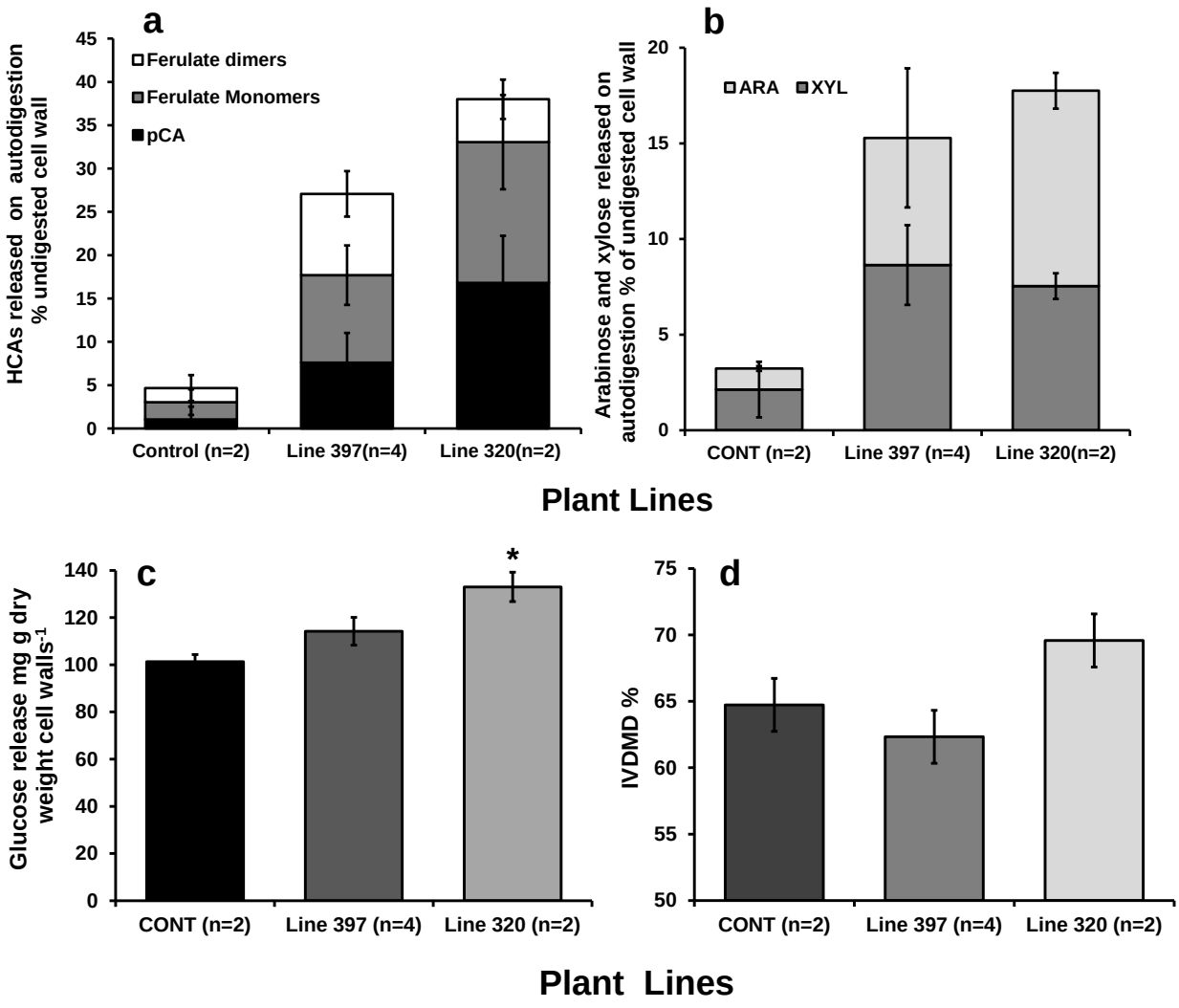
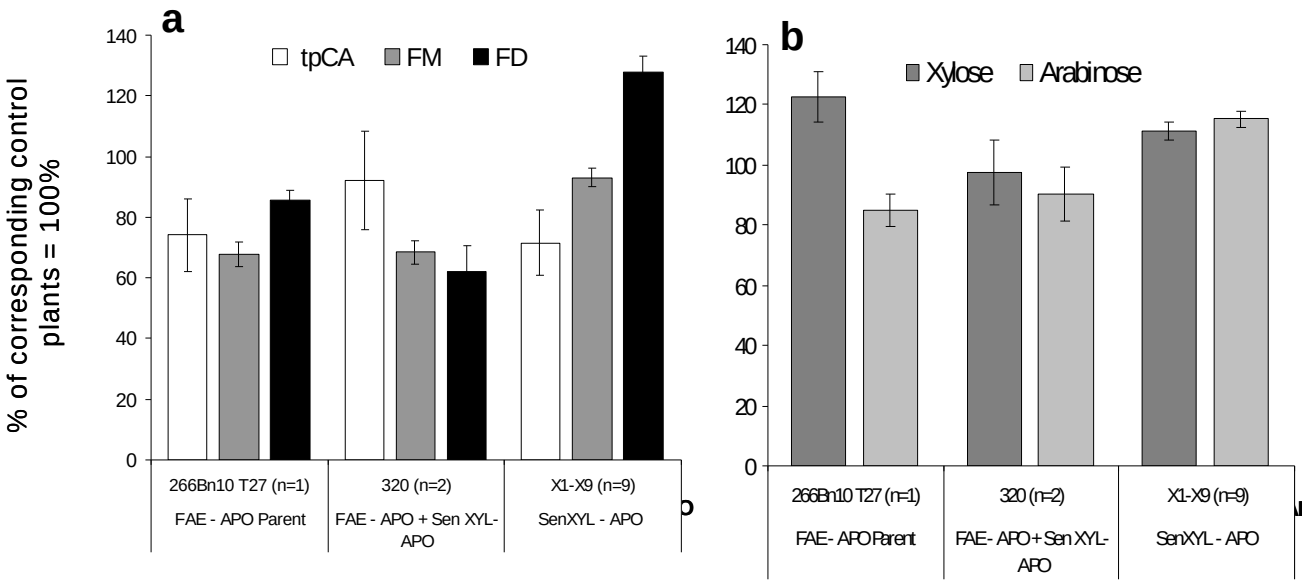

a
Ferulate dimers
Ferulate Monomers
pCA
HCAs released on autodigestion % undigested cell wall
Control (n=2)
Line 397(n=4)
Line 320(n=2)
b
ARA
XYL
Arabinose and xylose released on autodigestion % of undigested cell wall
CONT (n=2)
Line 397 (n=4)
Line 320(n=2)
Plant Lines
c
*
Glucose release mg g dry weight cell walls-1
CONT (n=2)
Line 397 (n=4)
Line 320 (n=2)
d
IVDMD %
CONT (n=2)
Line 397 (n=4)
Line 320 (n=2)
Plant Lines

Figure 7:


a
tpCA
FM
FD
% of corresponding control plants = 100%
140
120
100
80
60
40
20
0
266Bn10 T27 (n=1)
FAE - APO Parent
320 (n=2)
FAE - APO + Sen XYL-APO
X1-X9 (n=9)
SenXYL - APO
b
Xylose
Arabinose
140
120
100
80
60
40
20
0
266Bn10 T27 (n=1)
FAE- APO Parent
320 (n=2)
FAE- APO + Sen XYL-APO
X1-X9 (n=9)
SenXYL - APO