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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

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

85 Ferulic acid esterase A from *Aspergillus niger* is one of the most widely studied of
86 the feruloyl esterases and is known to act on ferulic acid residues attached to the
87 *O*-5 of arabinose in arabinoxylan (Ralet et al. 1994). FAEA releases 5-5' and 8-0-
88 4' ferulate dimers from plant cell walls when incubated with xylanase or when pre-
89 treated with xylanase (Kroon et al. 1999). This positive synergism of FAEA with
90 other catalytic or non-catalytic proteins is shown by the release of ferulates from
91 different substrates by different ferulic acid esterases alone or in combinations
92 with xylanases. For example, the efficiency of ferulate release by *A. niger* FAEA
93 from wheat straw increases when combined with xylanase or with *T. reesei*
94 cellulase during saccharification (Tabka et al. 2006), or when fused to the non-
95 catalytic *T. reesei* swollenin protein (Levasseur et al. 2006). Generally, the
96 glycoside hydrolase family 11 xylanases favours the release of ferulic acid while
97 family 10 xylanases are more efficient at releasing ferulate dimers (Faulds et al.
98 2003).

99 Strategies for producing the large amounts of exogenous cell wall degrading
100 enzymes required to degrade lignocellulosic biomass into its constituent sugars
101 include the production of transgenic plants with high levels of enzyme activity. To
102 this end, the production of an active insoluble aggregate of xylanase in tobacco
103 where a Zera-xylanase chimeric protein accumulated within ER-derived protein
104 bodies has been demonstrated (Llop-Tous et al. 2011), as well as the targeting of
105 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.

131 When constitutively expressed in leaves of *Lolium multiflorum* (Buanafina et al.
132 2006) or *Festuca arundinacea* (Buanafina et al. 2008), *Aspergillus niger* ferulic
133 acid esterase (*faeA*) targeted to the vacuole had little effect on plant growth or
134 biomass yield but resulted in reduced levels of cell wall esterified monomeric and
135 dimeric ferulates and increased rates of *in vitro* dry-matter digestibility.
136 Constitutive intracellular targeted expression of FAEA to the apoplast,
137 endoplasmic reticulum or Golgi in *Festuca* also disrupted feruloylation of the
138 growing cell wall with significant reductions in the levels of monomeric and dimeric
139 ferulates resulting in increased biodegradability in terms of cell wall digestibility
140 and increased rates of cellulase-mediated release of fermentable sugars
141 (Buanafina et al. 2010). In addition, the release of monomeric and dimeric ferulic
142 acids from cell walls on autodigestion of leaves expressing FAEA was enhanced
143 several fold by the addition of exogenous β-1,4 endoxylanase (Buanafina et al.
144 2008; 2010). Expression of beta endo-xylanases in plants has however received
145 considerable attention, although reports of the effects on plant growth and
146 development of constitutive expression of either microbial or fungal endo-
147 xylanases in different cellular compartments in both monocot or dicot species are
148 highly inconsistent (reviewed by Taylor et al. 2008; Bae et al. 2008; and Sainz
149 2009). Few reports however have described the effects of these *in-planta*
150 expressed cell wall degrading enzymes on cell wall structure. Expression of
151 *Trichoderma reesii xyn2* controlled by a senescence promoter in the apoplast of
152 *Festuca arundinacea,* had no effect on the level of monomeric hydroxycinnamic
153 acids or lignin in the cell walls, but resulted in increased levels of ferulate dimers,
154 decreased levels of xylose and increased levels of arabinose in the cell walls,
155 compared with non transformed plants (Buanafina et al. 2012). High-level

156 xylanase expression in the apoplast of *Festuca arundinacea* leaves also resulted
157 in ethylene and H_2O_2 accumulation and necrotic lesions on the leaves indicative of
158 plant defence responses analogous to foliar pathogen attack (Buanafina et al.
159 2012). These changes in cell wall composition resulted in decreases in both tissue
160 digestibility and cellulase mediated sugar release (Buanafina et al. 2012).
161 In order to test if *in-planta* co-expression of FAEA and XYN2 could improve the
162 digestion of polysaccharides and increase post harvest cell wall deconstruction in
163 grasses more effectively than expression of xylanase or FAE alone, FAEA
164 expressing plants were re-transformed with XYN2. To date *in-planta* co-
165 expression of these two cell wall degrading enzymes has not been previously
166 tested and our experience of expressing FAE and xylanase in *Festuca* indicated
167 that constitutive co-expression might be detrimental to cell growth. We specifically
168 chose therefore to separate the activities of the two enzymes either spatially or
169 temporally using the *Lolium* See1 senescence enhanced promoter for xylanase
170 expression as this is activated at the end of plant growth prior to the start of leaf
171 senescence (Li et al. 2004). Our aim was to determine the feasibility of exploiting
172 synergy between FAE and xylanase to achieve higher levels of cell wall
173 arabinoxylan deferuloylation both at the end of active leaf growth during
174 senescence, and subsequently following cell death. The effects of constitutive co-
175 expression of FAEA in the vacuole or apoplast combined with senescence
176 inducible expression of XYN2 in the apoplast, on plant growth, cell wall ferulates
177 and cell wall sugar composition, lignification, and cell wall degradability are
178 reported here.

179

180 **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°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 °C, 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

399 (Figure 5a), but resulted in highly significant reductions in the mean levels of
400 esterified cell wall ferulate monomers (31%) (Figure 5a), and dimers (36%) (Figure
401 5c), compared to control means. However when vacuolar targeted FAEA was
402 combined with senescence induced apoplast targeted XYN2, (397 lines), this
403 resulted in smaller non-significant reductions in the level of cell wall esterified
404 ferulate monomers (4.6%) (Figure 5b), and dimers (2.6%) (Figure 5c).
405
406 ***Cell wall sugars:*** Constitutive expression of FAEA and senescence
407 induced apoplast expression of XYN2 also resulted in significant reductions in the
408 mean levels of cell wall arabinose and xylose in both 397 and 320 lines compared
409 with controls (Figure 5d). This reduction ranged from 10% for xylose and 19% for
410 arabinose in 397 lines with vacuole targeted FAE to 18% for xylose and 34% for
411 arabinose in 320 lines with apoplast targeted FAE compared to control plants
412 (Figure 5d-e). As a consequence of these changes the arabinose:xylose ratio of
413 the cell wall sugars decreased in both the 397 and 320 lines compared to control
414 plants (Figure 5e).
415
416 ***Lignification:*** Considering the potential of FAEA and XYN2 co-expression
417 to release ferulates and diferulates as well as arabinose and xylose from the
418 arabinoxylan backbone, and consequently to potentially decrease the cross-
419 linking between arabinoxylan and lignin, the level of acetyl bromide soluble lignin
420 was determined and compared to levels of total Klason lignin.
421 No significant changes in Klason lignin levels of whole leaf biomass were found by
422 co-expression of either apoplast or vacuole targeted FAEA with apoplast targeted
423 xylanase (Figure 5f). However, the levels of acetyl bromide soluble lignin

424 extractable from partially purified cell walls (AIR) increased by 18% in plants co-
425 expressing vacuole targeted FAEA with apoplast targeted xylanase (397 lines)
426 and by 31% in plants co-expressing apoplast targeted FAEA and xylanase (320
427 lines) (Figure 5f). Levels of ferulate monomers, dimers and total ferulates were
428 negatively correlated (P=0.0028, r^2= 0.755; P=0.0008 r^2= 0.807 and P= 0.0006 r^2=
429 0.8197, respectively) to acetyl bromide lignin, but no significant correlation was
430 found between *p*-coumaric acid and levels of acetyl bromide soluble lignin
431

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

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

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

449 Significant amounts of the cell wall HCAs (p=0.05) were removed on auto-
450 digestion irrespective of whether FAE was expressed in the vacuole or apoplast.
451 The released HCAs consisted of *p*-coumaric acid and both ferulate monomers and
452 dimers, with mean losses of total HCAs for apoplast located FAE amounting to
453 16%, and for vacuole located FAE, 9% of the total HCA content of the leaf cell
454 walls compared with 3.6% for control plants (Figure 6a). ANOVA showed a
455 significant contrast between control and the two transgenic lines for *p*CA (p=0.06),
456 and ferulate dimers (p=0.048). The total amount of HCAs released on
457 autodigestion was 6 to 10 times higher than previously found with plants
458 expressing either vacuolar or apoplast FAEA, or apoplastic XYN2 alone, and 1.3
459 and 2.4 times greater than where FAEA expressing tissues were digested in the
460 presence of external applied XYN2, or where XYN2 expressing tissues were
461 digested in the presence of external applied FAEA (Table 1).
462 Auto-digestion also resulted in the release of significant amounts arabinose and
463 xylose from cell walls of senescing leaves compared to controls (Figure 6b), with
464 means losses of the total arabinoxylan content of the leaf cell walls amounting to
465 16% for apoplast located FAE and 19% for vacuole located FAE compared with
466 3% for control plants. In addition, the mean arabinose to xylose ratio decreased
467 significantly in the cell wall residue following auto-digestion from 0.54 in control
468 plants to 0.3 in the 397 lines and to 0.38 in the 320 plants. However the release of
469 ferulate crosslinked arabinoxylan from co-expressing FAEA and XYN2 tissue on
470 autodigestion could not be established.
471 **Cellulase mediated release of glucose from autodigested cell walls:**
472 The ability of plant expressed FAEA and xylanase to render the cell walls more
473 susceptible to further enzymatic degradation was assessed by determining the

474 release of glucose from autodigested cell walls treated with *T. ressei* cellulase. 475 Plants of line 320, which showed reduced levels of ferulate monomers and dimers 476 (Figure 5b&c) and the lowest levels of cell wall arabinoxylans (Figure 5d) showed 477 a significant 31 % increase in the mean levels of glucose released from 478 autodigested cell walls (Figure 6c). Plants from line 397 showed a 12 % increase 479 in the mean levels of glucose released from autodigested cell walls treated with *T.* 480 *ressei* cellulase (Figure 6c), and although this was not statistically significant, 481 glucose released by two of the individual lines were significantly different from 482 controls.

483 ***In vitro* dry matter digestibility** (IVDMD*).* The direct effect of reduced 484 levels of cell wall HCAs and AX on tissue digestibility was also determined by the 485 extent of biomass loss following the two-stage *in vitro* pepsin-cellulase solubility 486 technique of Jones and Hayward (1975). Mean levels of IVDMD were not 487 significantly different from controls in 397 plants and while mean levels of IVDMD 488 increased by 5 percentage units in 320 plants this was not statistically significantly 489 different from control values (Figure 6d).

490

491 Discussion

492 Following re-transformation of fescue plants constitutively expressing apoplast or 493 vacuole targeted FAE, with a senescence induced apoplast targeted xylanase, a 494 set of plants with both FAE and XYN2 enzyme activity, and with significantly 495 reduced levels of esterified cell wall ferulates, diferulates and arabinoxylans was 496 obtained. The expression of both constitutive and senescence induced FAE or 497 XYN2 alone has been reported previously in forage grasses (Buanafina et al.

498 2004, 2006, 2010; 2012), but this is the first report of *in-planta* co-expression of
499 both enzymes.

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

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

524 The initial instability of FAE and XYN2 activities in co-expressing plants on
525 repeated tillering might indicate that transgene expression is unpredictably
526 modified during the formation of new meristems from somatic stem cells. This may
527 suggest evidence for the mitotic modification of epigenetic effects similar to the
528 transcriptional silencing of transgene expression in plants, associated with
529 meiotically heritable epigenetic modifications (Matzke and Matzke, 1988). A similar
530 effect was reported with lateral buds in plants regenerated from cultured leaf discs,
531 which showed that "once established, competence for silencing can persist in
532 dormant, actively growing and de-novo established shoot meristems" (Kunz et al.
533 1996).

534 In contrast to previous reports where high levels of apoplast located XYN2 activity
535 was found to induce pathogen defence responses (Buanafina et al. 2012), plants
536 expressing similar levels of XYN2 activity, but with low FAEA activities, showed no
537 signs of xylanase induced pathogen responses, such as high levels of cell wall
538 ferulate dimers or necrotic lesions on the leaves. Comparing the mean levels of
539 HCAs and AX of cell walls of senescing leaves of co-expressing plants with the
540 original parent plants expressing apoplast FAE only, and with plants expressing
541 senescence induced xylanase only, it is possible to conclude that constitutive
542 expression of FAE in the apoplast may have been sufficient to overcome the
543 ferulate dimerisation triggered by apoplast located xylanase (Figure 7). Possible
544 reasons for this may be that either there is a continual cycling of dimers with FAE
545 removing them faster than they can be added by the action of xylanase induction
546 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.

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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)**

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

871

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

877

878 **Figure 4. Effects on plant growth**.

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

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

888

889 **Figure 5. Effects on cell wall structure of senescing leaves of selected T5**
890 **plants at harvest.**
891 **(a)** *p*-coumaric acid, **(b)** ferulate monomers, **(c)** ferulate dimers, **(d)** arabinose and
892 xylose, **(e)** arabinose:xylose ratio, **(f)** Klason lignin and acetyl bromide lignin of
893 isolated cell walls (AIR). Plants were constitutively expressing vacuole (Line 397)
894 or apoplast (Line 320) targeted FAEA, and senescence induced apoplast targeted
895 XYN2. Ferulate monomers: trans-ferulic + cis-ferulic acid. Dimers = 8-0-4'-
896 diferulic; 5-5'-diferulic; 8-5cyc-diferulic benzofuran; and an unknown ferulate acid
897 dimer quantified as ferulic acid. Mean ± SEM of triplicate determinations from (n)
898 independently transformed or control plants. Based on in-house QC material RSD
899 and %CV for Klason lignin were, STM 0.50 RSD = 0.03487 %CV= 3.487. *
900 indicates significant differences from the control (Tukey's, α = 0.05).

901

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

910 cell walls (A, B). Mean ± SEM of triplicate (C) or single (D) determinations from (n)
911 independently transformed or control plants. Based on in-house QC material RSD
912 and %CV were STM 0.26 RSD = 0.1904 %CV= 1.904 for IVDMD.
913
914 **Figure 7.** Comparison of the mean levels of HCAs (**a**) and AX (**b**) of cell walls of
915 senescing leaves of T5 plants constitutively expressing FAE and xylanase
916 targeted to the apoplast with the original parent plant expressing apoplast FAE
917 only (T27), and with plants expressing senescence induced xylanase only (X1-
918 X9). Mean ± SEM (n=3) determination from n plants. Corresponding control plants
919 for each vector =100%. For further details on the characteristics of the parent plant
920 266Bn10 see plant T27 in Buanafina et al 2010 and for plants X1-X9 see
921 Buanafina et al. (2012).
922
923

924 **Table 1**

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

930

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

931

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.

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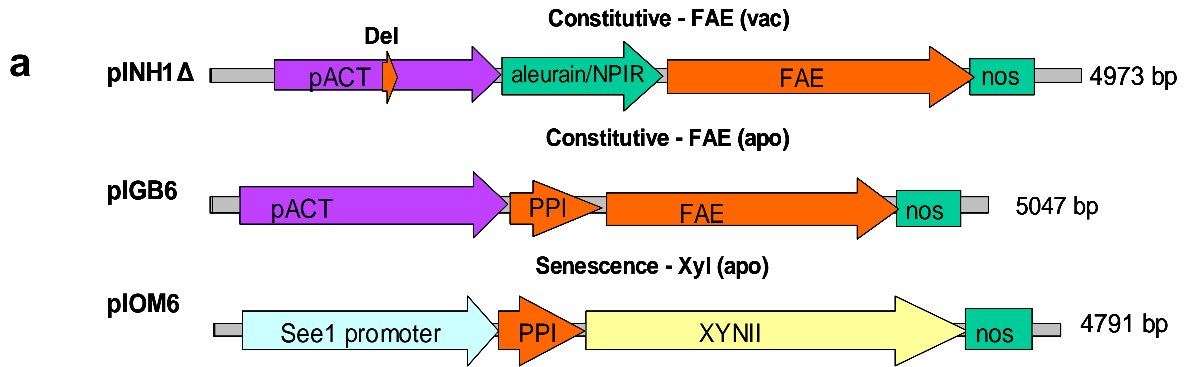
Figures

Figure 1:



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

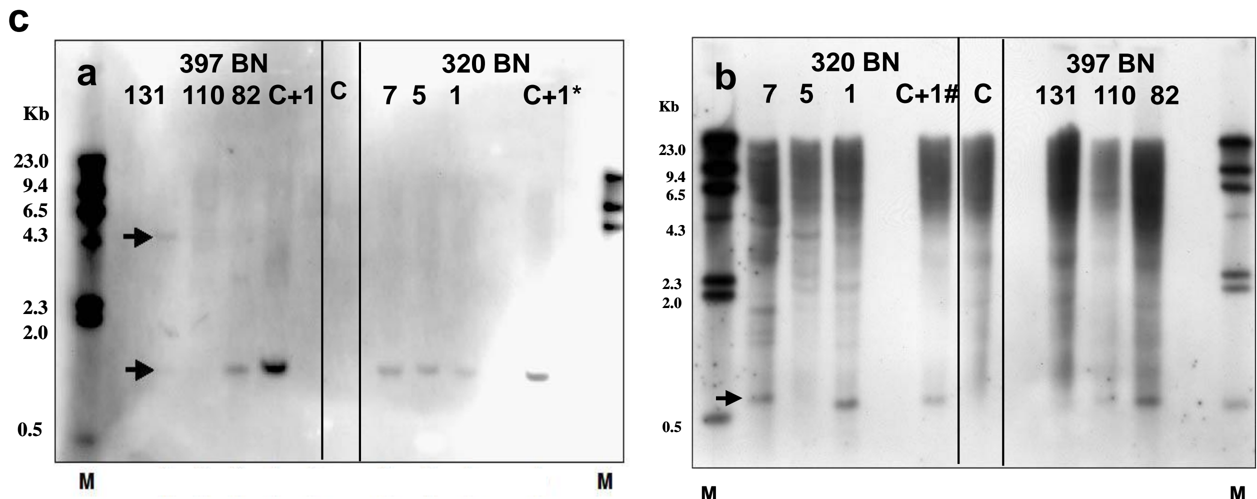


Figure 2:

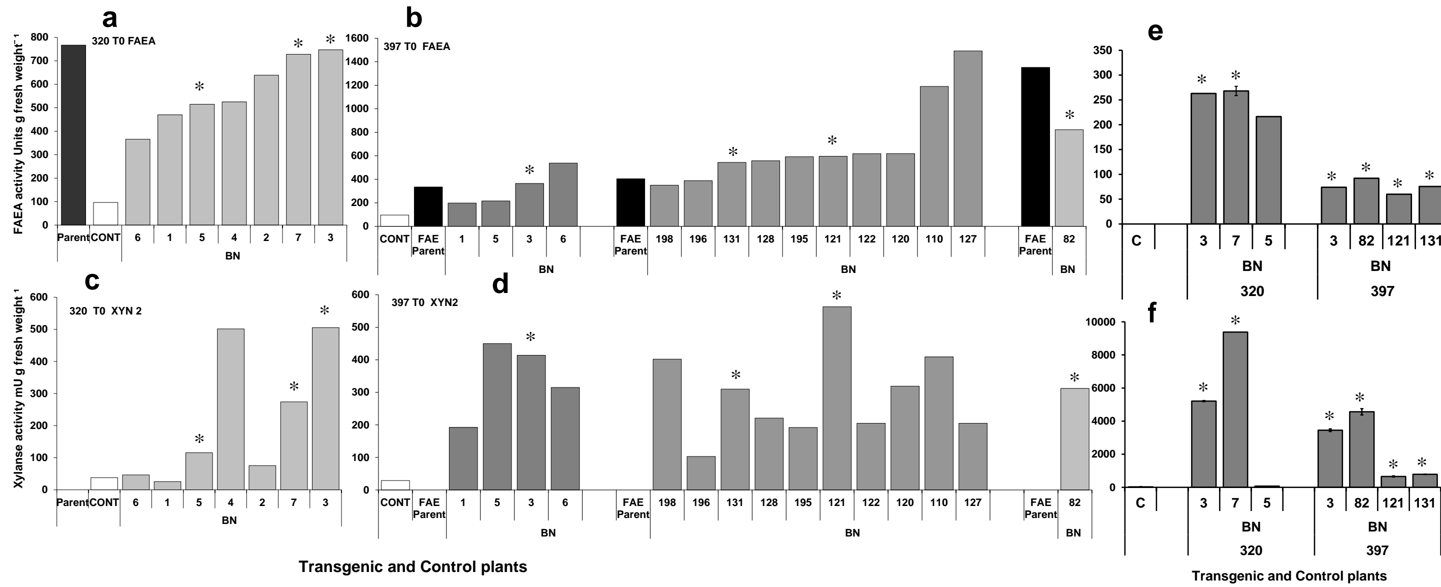


Figure 3:

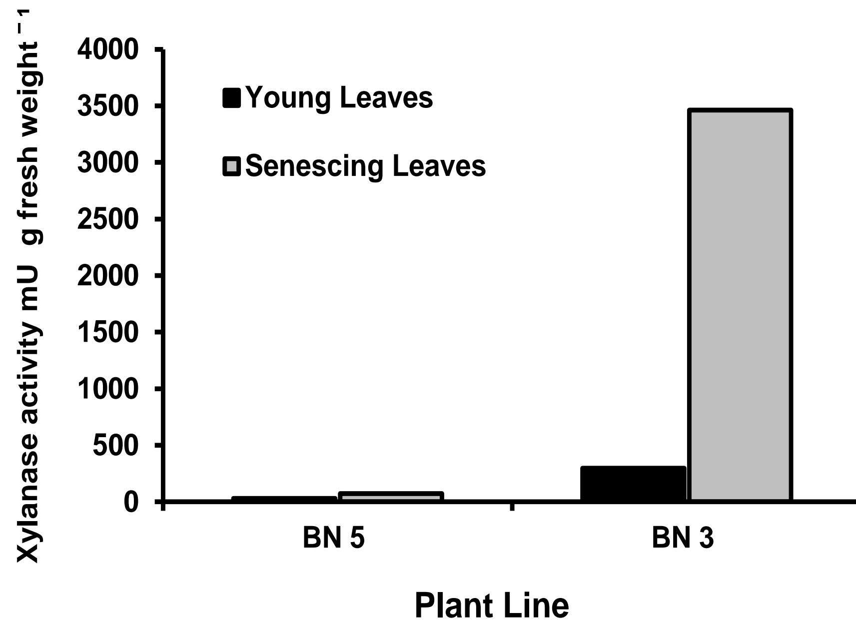


Figure 4:

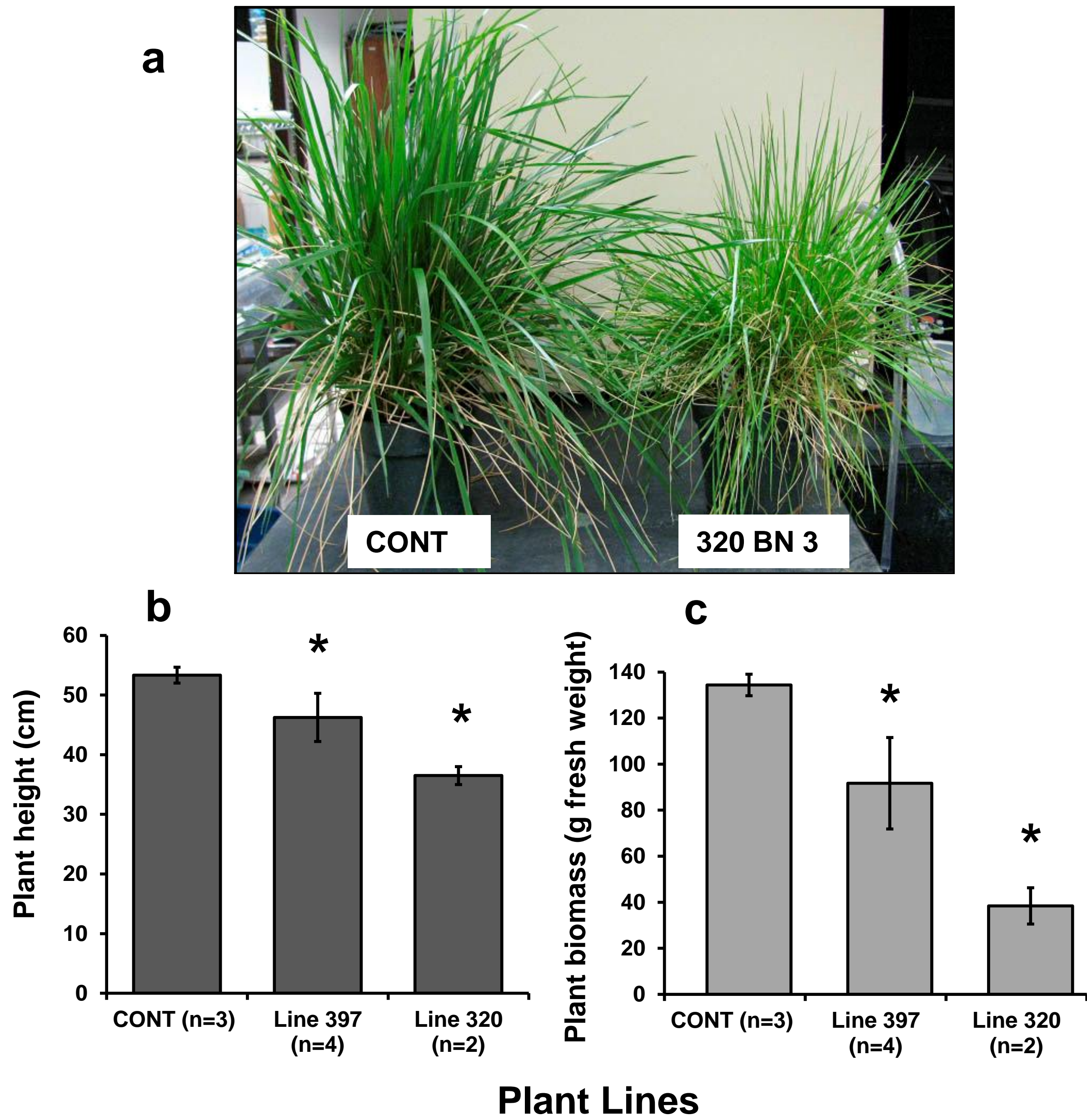


Figure 5:

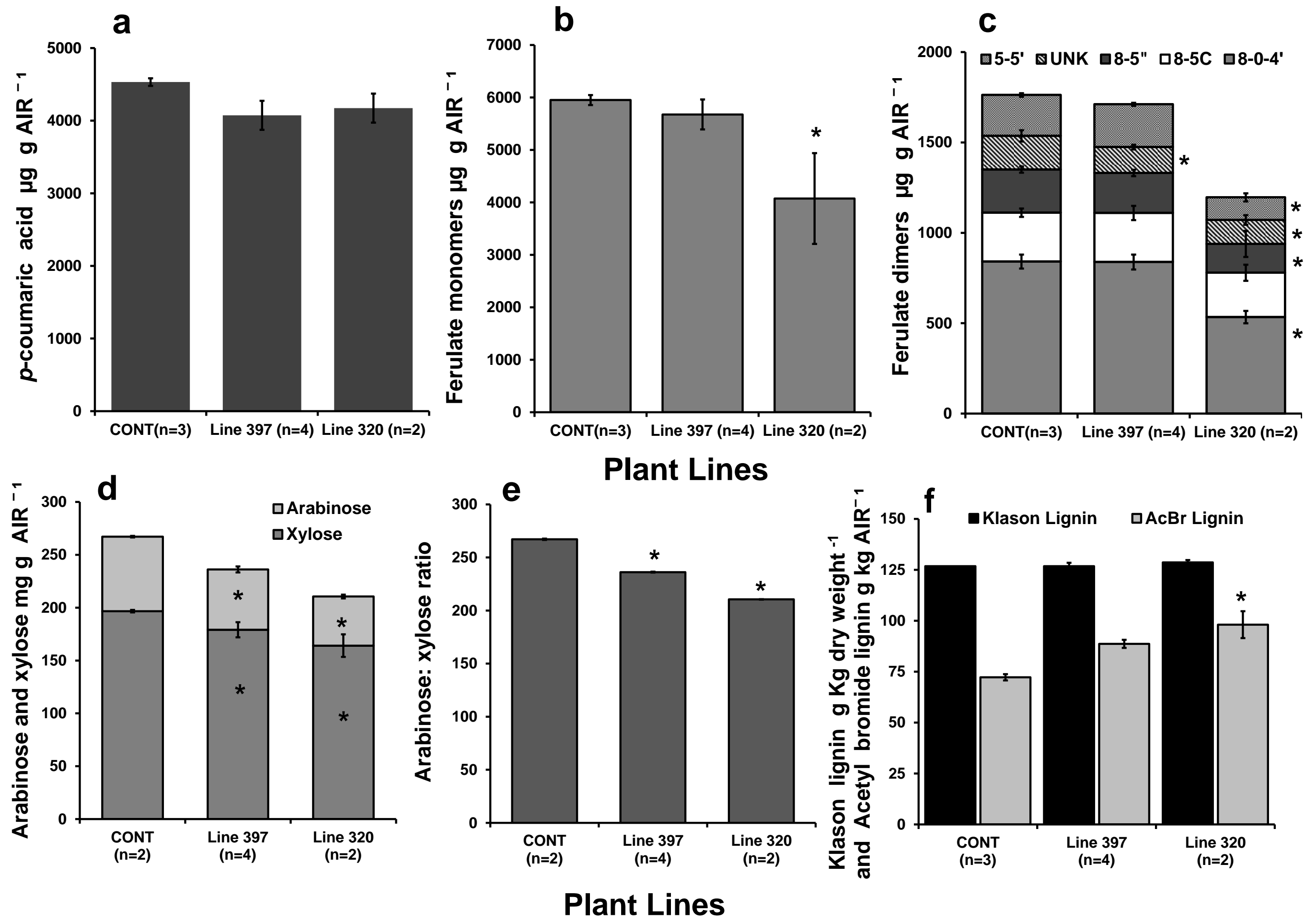


Figure 6:

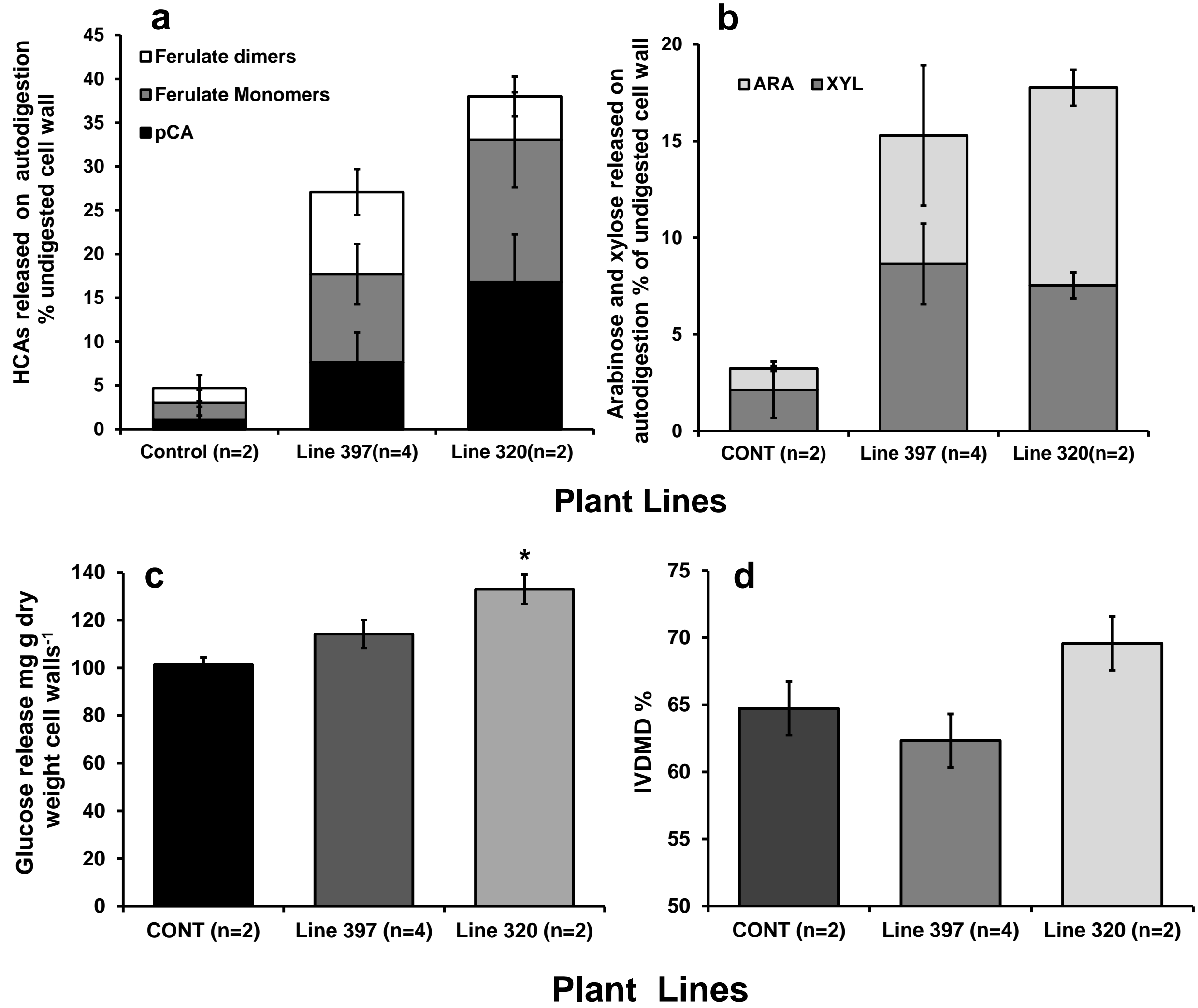


Figure 7: