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Manipulating the Phenolic Acid Content and Digestibility of Italian Ryegrass (*Lolium multiflorum*) by Vacuolar-Targeted Expression of a Fungal Ferulic Acid Esterase

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Abstract

In grass cell walls, ferulic acid esters linked to arabinosyl residues in arabinoxylans play a key role in crosslinking hemicellulose. Although such crosslinks have a number of important roles in the cell wall, they also hinder the rate and extent of cell wall degradation by ruminant microbes and by fungal glycohydrolyase enzymes. Ferulic acid esterase (FAE) can release both monomeric and dimeric ferulic acids from arabinoxylans making the cell wall more susceptible to further enzymatic attack. Transgenic plants of *Lolium multiflorum* expressing a ferulic acid esterase gene from *Aspergillus niger*, targeted to the vacuole under a constitutive rice actin promoter, have been produced following microprojectile bombardment of embryogenic cell cultures. The level of FAE activity was found to vary with leaf age and was highest in young leaves. FAE expression resulted in the release of monomeric and dimeric ferulic acids from cell walls on cell death and this was enhanced severalfold by the addition of exogenous β-1,4-endoxylanase. We also show that a number of plants expressing FAE had reduced levels of cell wall esterified monomeric and dimeric ferulates and increased in vitro dry-matter digestibility compared with nontransformed plants.

Index Entries: Constitutive vacuolar-targeted expression; digestibility; ferulic acid esterase; *L. multiflorum*; transgenic grasses.

Introduction

Cellulose and hemicellulose make up the bulk of plant material providing a large and renewable source of biomass with a high potential for degradation for generating fermentable sugars as feedstock for industry or for the production of biofuels. They also provide the major feed for the world's livestock, especially for ruminants, and could in fact meet their

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energy requirements. However, in grasses this potential is not usually achieved because of cell wall lignification and the crosslinking of cell wall polysaccharides with phenolic residues *(1)* resulting in a low rate of cell wall digestion. For industrial uses, the degradation of the cell wall components require a combination of degrading enzymes and the cost of enzymatic hydrolysis of biomass is one of the major factors limiting the economic feasibility of the process.

Ferulic acid (4-hydroxy-3-methoxy-cinnamic acid) is the major hydroxycinnamic acid (HCA) identified in grass cell walls and is attached to the cell wall polymers via an ester linkage to the arabinose side chain of arabinoxylan *(2)* and can also be ether-linked to lignin monomers *(3)*. These HCAs are important components of the cell because they can be oxidatively coupled to form a variety of dehydrodiferulate dimers, crosslinking hemicellulose polysaccharides chains *(4)*; however, they also limit plant cell wall hydrolysis, hindering the rate and extent of cell wall degradation by ruminant microbes *(5)* or by fungal glycohydrolyases *(6)*.

We are interested in producing transgenic plants that can efficiently synthesize hydrolytic cell wall degrading enzymes so the plant cell wall can be broken down during biomass conversion or during processing of plants for foodstuffs and so will be more efficiently used not only for ruminant nutrition but also as a biomass resource.

In the present study, we test the targeted expression of a ferulic acid esterase in order to control the level of phenolic acids in *Lolium multiflorum* cell walls available for crosslink cell wall polysaccharides. A ferulic acid esterase (FAE) gene has been cloned from *Aspergillus niger (7)* and it has been shown that the recombinant enzyme releases ferulic acid and diferulate dimers from grass cell walls. Here, we test the vacuole targeting of FAE expressed using a constitutive actin promoter and delivered by microprojectile bombardment. We expect reduced levels of phenolic acids in preexisting cell walls by release of FAE on cell death, which will increase the rates of cell wall digestion and carbohydrate availability. The efficiency of the gene targeting strategies, the levels of enzyme activity and the effects on cell wall composition and the release of esterified ferulates on cell death following release of vacuolar-targeted FAE of transgenic *L. multiflorum* is reported together with effects on end point digestibility.

Material and Methods

Vector Construction and Targeting

The pUA1K3 vector (Fig. 1) was constructed in a pUC plasmid with FAE from *A. niger (7)* provided as a genomic clone by Dr. Ben Bower (Genencor Inc., Palo Alto, CA). Modifications to the core open reading frame (ORF) of FAE were made to enhance expression in plants with a codon choice based on published barley preferences. The N-terminus of the protein contained an intact barley aleurain vacuolar targeting sequence *(8)* and was

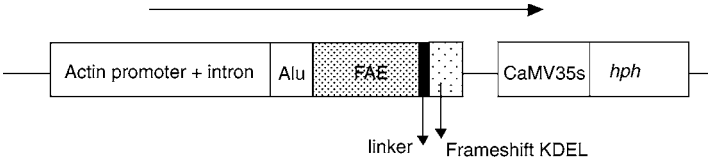

Fig. 1. Vector plasmid (pUA1-K3) used for plant transformation to target FAE to the vacuole under control of a rice actin promoter and intron.

driven by a rice actin promoter and first intron derived from pCOR105 *(9)*. Transient expression of FAE activity following microprojectile bombardment of grass cell cultures was found to be lower with the native *Aspergillus* C-terminal end (CTW) than when a linker sequence was added (CTW-PVAAA), suggesting that the native *Aspergillus* C-terminal end probably has deleterious effects in grasses. This linker and a mutated ER KDEL retention signal (ETTEG) formed the final C-terminal sequence. In order to verify that the targeting sequences were effective in delivering FAE to the vacuole, parallel vectors containing a monocot optimized green fluorescent protein (sgfp) *(10)* were constructed and the cellular distribution of gfp fluorescence was determined by confocal microscopy of single cells in transient expression studies of embryogenic cell cultures. The cointegrative vector pUA1K3 also contained a Hind*III* casset containing the hygromycin resistance gene *(hph)* under the control of a CaMV 35S promoter *(11)*. Further details of vector construction can be found in Dunn-Coleman et al. *(12)*.

Tissue Culture and Plant Transformation

Eight- to ten-week-old embryogenic suspension cultures of a responsive *L. multiflorum* genotype were bombarded with pUA1K3 using a Particle Inflow Gun as described by Dalton et al. *(13)*. Transformants were selected with hygromycin (25–50 mg/L) over a 10- to 12-wk period at 25°C under continuous white fluoresvcent light (60 µmol/m^2/s) and plants were regenerated via somatic embryogenesis. Regenerated plants were screened for FAE activity on transfer to soil and expressing plants were grown to maturity in a containment growth room at 18°C under 16 h fluorescent lights (350 µmol/m^2/s). Mature plants (6- to 8-wk old) were reassayed for FAE activity and fresh tissue harvested for self-digestion analysis. The remaining tissue was freeze-dried and powdered for cell wall phenolic analysis, and for in vitro dry-matter digestibility (IVDMD) determinations.

Determination of FAE Activity

The FAE activity was determined in soluble extracts of fresh (or frozen at –70°C) leaves (0.5 g fresh wt extracted with 0.1 *M* sodium acetated, pH 5.0 buffer). Extracts were incubated with 24 m*M* ethyl ferulate (EF ethyl-4-hydroxy-3-methoxy cinnamate) as substrate, at 28°C for 24 h and FAE activity calculated as the amount of ferulic acid released in which 1 unit FAE activity equals 1 µg ferulic acid released from EF in 24 h

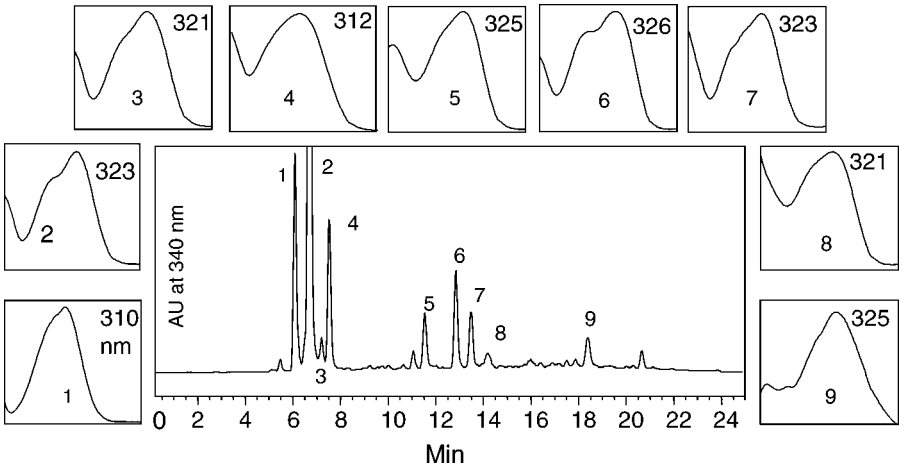

Fig. 2. HPLC and UV/visible spectra of esterified monomeric and dimeric ferulates of cell walls of mature *Lolium* leaves. (1) Trans *p*-coumaric acid. (2) Trans-ferulic acid, (3) 8-5′ diferulic acid, (4) *cis*-ferulic acid, (5) 5-5′ diferulic acid, (6) 8-0-4′ diferulic acid, (7) 8-5′ diferulic acid benzofuran form, (8) N, (9) K-unknown ferulate dimers.

at 28°C. FAE activity was also determined by measuring the release of monomeric and dimeric ferulates from "self-digested" leaf samples. Leaves (0.5 g fresh wt) were ground in 0.1 *M* sodium acetate extraction buffer pH 5.0, in the presence and absence of GC140 β-1,4-endoxylanase (1000 U/sample Genencor Inc.), without additional substrate, and incubated at 28°C for 72 h. Following centrifugation, soluble extracts were loaded onto a reverse phase C_{18} µNova Sep-Pak column (Waters Inc.), eluted with 100% MeOH and analyzed by high-performance liquid chromatography (HPLC).

Cell Wall Phenolic Analysis

Following exhaustive extraction of soluble phenolics and chlorophyll pigments with aqueous methanol, ester-bound compounds were extracted from the cell walls of freeze-dried powdered leaf material (50 mg), with 1 *M* NaOH (5 mL) followed by incubation at 25°C for 23 h, in the dark under N_2. After centrifugation, acidification and precipitation of solubilized carbohydrates with MeOH at 4°C, the extracted phenolics in the aqueous phase were loaded onto an activated reverse phase C_{18} µNova Sep-Pak column and eluted with 100% MeOH, and analyzed by HPLC.

HPLC analysis was carried out on a µNova Pak C_{18} 8 × 10 RCM (Waters Inc.) in 100% methanol: 5% acetic acid, either with a linear 35–65% MeOH gradient in 15 min (FAE assay) or with a 20–70% MeOH gradient in 25 min (for monomer and dimer cell wall components) (Fig. 2), at a flow rate of 2 mL/min. Hydroxycinnamic acids were monitored and quantified at 340 nm with a Waters 996 photo-diode array detector with UV/visible spectra collected at 240–400 nm, and analyzed with Millennium software (Waters Inc.) against authentic monomer standards,

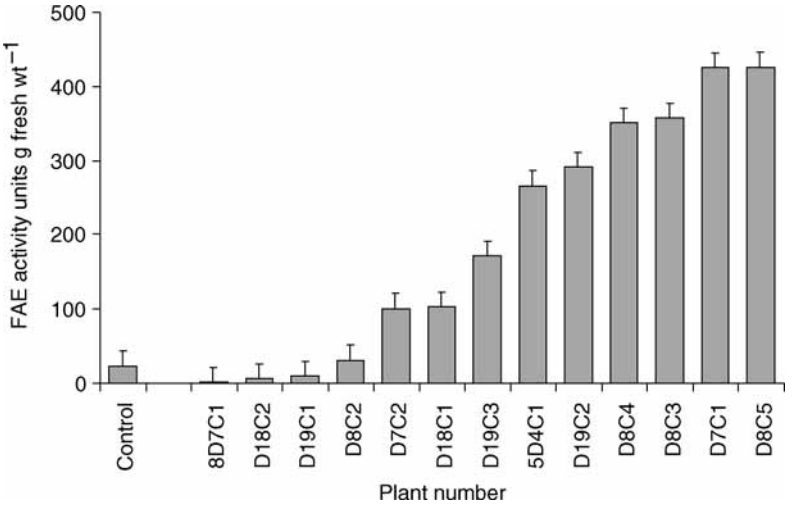

Fig. 3. Levels of FAE activity of mature leaves of 13 *L. multiflorum* plants transformed with pUA1-K3. One unit FAE activity equals 1 µg ferulic acid released from ethyl ferulate in 24 h at 28°C.

or using response factors for the various dehydrodiferulate dimers reported by Waldron et al. *(14)*.

End Point Digestion

IVDMD was estimated on 1 g dry weight of powdered leaf tissue of FAE expressing and control plants, using the pepsin/cellulase method of Jones and Hayward *(15)*.

Results

FAE Expression in Transgenic Plants

Mean FAE activity in the leaves of 13 transgenic plants was found to vary nearly 10-fold, between independent transgenic events (e.g., plants D8C2 and D7C1, Fig. 3). However, FAE activity was also found to vary with leaf age, with the highest activities in young leaves (Fig. 4) and FAE activity was also higher in young plants when directly removed from tissue culture and fell to a lower level as the plants matured (data not shown). This may partially explain some, but not all, of the observed variation in FAE activity shown in Fig. 3.

Cell Wall Composition of Transgenic Plants

The levels of esterified cell wall monomeric and dimeric hydroxycinnamic acid constituents of leaves of transformed plants expressing vacuolar-targeted FAE were also compared with tissue-cultured, nontransformed control plants. In some plants (e.g., D19C2, D8C4, D8C3, D7C1, and D8C5),

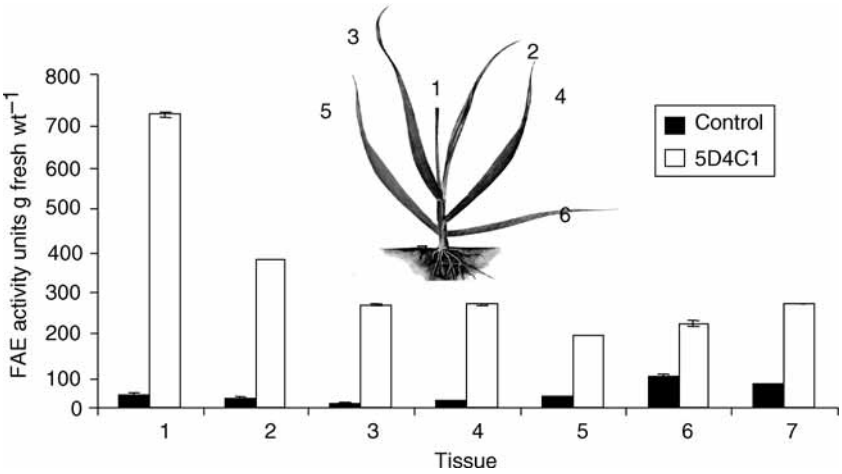

Fig. 4. FAE activities in leaves of different ages and leaf bases of plant 5D4C1 transformed with pUA1-K3, compared with a nontransformed control plant. One unit FAE activity equals 1 µg ferulic acid released from ethyl ferulate in 24 h at 28°C. Mean ± sem ($n = 3$).

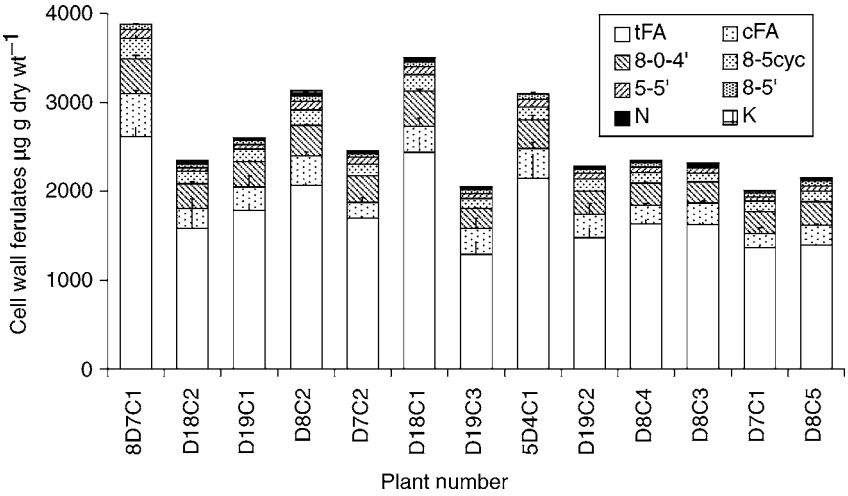

Fig. 5. Levels of ester-bound monomeric and dimeric ferulates in leaves of mature *L. multiflorum* plants transformed with pUA1-K3. tFA = trans-ferulic acid; cFA = cis-ferulic acid; 8-5cyc = diferulic acid benzofuran form, 8-0-4′ diferulic acid, 5-5′ diferulic acid, 8-5 diferulic acid, N and K = unknown ferulate dimers quantified as for ferulic acid. Mean ± sem ($n = 3$).

levels of both monomeric and dimeric ferulates were significantly lower than in control plants (Fig. 5) and this correlated with the highest levels of FAE expression (Fig. 3). However, other plants (e.g., 5D4C1) showed similar levels of FAE activity but with no significant changes in wall phenolics, whereas some plants with low measurable FAE activity (e.g., D18C2, and D19C1) showed significantly reduced levels of wall phenolics. Such

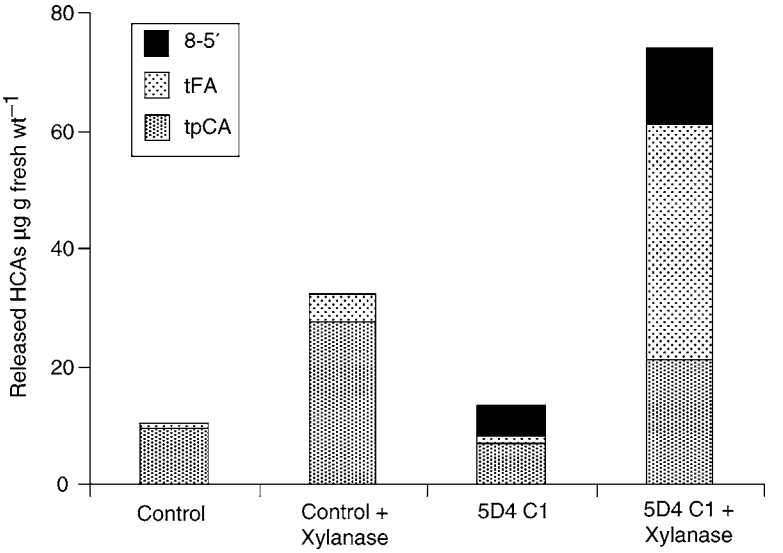

Fig. 6. Release of monomeric and dimeric hydroxycinnamic acids (HCAs) and effect of exogenous β-1,4-endo-xylanase following homogenization of leaves of plant 5D4C1 transformed with pUA1-K3 expressing FAE. tpCA = trans *p*-coumaric acid; tFA = trans ferulic acid; 8-5′ = diferulic acid.

anomalies may have arisen as a result of multiple gene insertions (evident in some plants following Southern blot analysis, data not shown) resulting in disrupted vacuolar targeting in which low levels of FAE resulted in disproportionate effects on cell wall feruoylation.

Effect of FAE Expression on Cell Death

We have demonstrated the potential of vacuole targeted FAE to aid "self-digestion" of the hemicellulose component of the cell wall after death in FAE-expressing plants. This "self-digestion" resulted in the release of small but significant quantities of esterified monomeric and dimeric ferulic acids as well as substantial amounts of *p*-coumaric acid (Fig. 6). In all cases this release was greatly stimulated by the exogenous addition of recombinant β-1,4-endo-xylanase (Fig. 6), which is known to act synergically with FAE in cell wall digestion by rumen microorganisms. This xylanase-enhanced release of HCAs was specific for FAE-expressing plants and was significant even in plants with very low levels of FAE activity. The kinetics of FAE-induced, xylanase-enhanced release of HCAs from constitutively expressed vacuolar-targeted FAE showed maximal release after 24 h incubation at 28°C, and after 8 h incubation at rumen temperatures of 40°C, amounting to some 70–80% release of the cell wall monomers and dimers (data not shown). It would appear therefore that, as with exogenously supplied FAE, xylanase activity is required in order to release arabinoxylans and to allow substrate access for FAE.

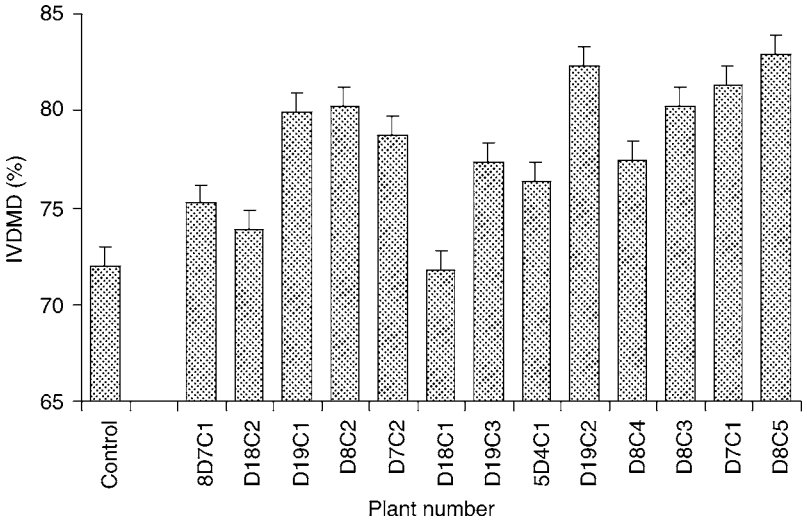

Fig. 7. In vitro dry-matter digestibility (IVDMD) of mature leaves of 13 *L. multiflorum* plants transformed with pUA1-K3.

Effects of FAE Expression on Cell Wall Digestion

End point digestibility, as determined by IVDMD using the pepsin/cellulase method of Jones and Hayward *(15)*, was in some cases found to be higher in leaf tissues of some transformed plants expressing FAE compared to untransformed control plant (Fig. 7). However, this was not consistent, and control levels in other experiments were more variable and were much higher than in clonal plants of the parental genotype, suggesting some effects of in vitro-induced somaclonal variation. Lack of effects on end point digestion, but significant increases in the initial rates of fermentation and reductions in the time to reach maximal rates of fermentation under rumen-like conditions were found with *Festuca arundinacea* plants expressing FAE using in vitro gas production measurements with data fitted to the model of France et al. *(16)*.

Discussion

Recent advances in plant biotechnology have given plant breeders new opportunities, not available using conventional plant breeding techniques, to transfer foreign genes from different genera, and kingdoms and to produce plants expressing these novel genes. Increasing the digestibility index of grasses has been a plant-breeding objective for many years, but owing to difficulties in fixing natural variation in the synthetic varieties derived from these outbreeding species, progress has been slow.

Grass cell walls, in particular, are characterized by the presence of a large amount of esterified ferulates. Removing labile phenolics by chemical treatment with alkali is known to increase biodegradability and the nutritional value of low-quality feed. Reducing the level of crosslinking

of cell wall carbohydrate is therefore a predictable way of improving the rate of digestion of grass.

A ferulic acid esterase gene from *A. niger* has been cloned and sequenced *(7)* and the recombinant enzyme shown to release monomeric and dimeric ferulates from plant cell walls *(17)*. In the present investigation this gene has been modified to make it more compatible with monocot codon usage, and we have produced *L. multiflorum* plants expressing this gene driven by a constitutive promoter with the enzyme targeted to the vacuole. The level of FAE enzyme activity was found to vary between independent transformants. This variability may simply reflect the differing proportions of young and old leaves in different plants growing at different rates (as FAE activity was found to decline on leaf maturation) or may be because of typical differences in transgene expression found in different transformation events resulting from different copy numbers *(18)* and/or integration sites *(19)*.

It was expected that there would be no significant changes in wall phenolics with FAE targeted to the vacuole until cell death, yet some plants with the highest level of FAE expression, showed reduced levels of cell wall monomeric and dimeric ferulates. This may be due either to disrupted vacuole targeting in some lines or alternatively to reduced levels of feruoylation of the arabinoxylan in the ER/Golgi intramembrane system, prior to incorporation into the cell wall, during transit, and processing of the enzyme in the secretory pathway to the vacuole. Multiple gene insertions with sequence deletions and rearrangements of some copies are well known to occur following transformation by microprojectile bombardment, whereas disrupted feruoylation was only considered likely with FAE specifically targeted to the ER or Golgi.

We have also demonstrated that expression of FAE in *Lolium* leads to an increase in in vitro dry-matter digestibility. Whether this is because of the reduced levels of esterified cell wall phenolics or as a result of the release of vacuolar localized FAE on cell death, has yet to be determined. However, increased IVDMD could lead to significant financial benefits to the agricultural community, as Casler and Vogel *(20)* have estimated that for beef cattle, 1% unit increase in dry-matter digestibility can lead to a 3.2% increase in average daily live weight gain.

Although there are a number of reports of the expression of cell wall degrading enzymes in dicot plants, mainly cellulases and endo-xylanases, aimed at the possibility of using plant systems for the large-scale, cost-efficient production of heterologous enzymes, little attention has been given to alterations in cell wall chemistry of monocots aimed at increasing their digestibility. For example Ziegelhoffer et al. *(21)* reported the expression of thermostable cellulases from *Thermomonospora fusca* in Medicago, potato, and tobacco at levels as high as 0.1% soluble protein with no effects on plant phenotypes. However, Armstrong et al. *(22)* looked at the expression of a *Fibrobacter succinogenes* 1,3-1,4-β-glucanase in potato with the glucanase

mature peptide-coding region, inserted in the sense orientation relative to a constitutive promoter (CaMV 35S). Analysis of the transformed plants revealed low levels of expression and defects in cell wall morphology within the transgenic potato lines, with the greatest effects detected in those plants exhibiting the highest levels of expression. Herbers et al. *(23)* also reported that expression of a thermostable xylanase from *Clostridium thermocellum* expressed at high levels in the apoplast of transgenic tobacco had no detrimental effects on plant development. Recently Kimura et al. *(24)* reported the constitutive expression of a xylanase domain from *C. thermocellum* in rice, and no phenotypic effects were noted.

In conclusion, we have been able to demonstrate for the first time that it is possible to genetically modify the phenolic composition and digestibility of monocot cell walls by vacuolar targeting of a fungal ferulic acid esterase in plants of *L. multiflorum*. To our knowledge this is the first report of genetically engineering plants to express a gene encoding a fungal esterase, and indicates the potential of using the vacuole as a reservoir of esterase, which can be released on cell death to aid cell wall degradation. This strategy could prove to be useful in other species in which FAE activity is desired to increase the rate of breakdown of plant cell walls more effectively and inexpensively than exogenous application of these enzymes, such as during biomass conversion to fermentable sugars, or during processing of plants for foodstuffs. In this respect we have also targeted FAE to the apoplast, ER, Golgi, and vacuole under both constitutive and inducible promoters in *Festuca arundiancea* which is less digestible, but more well adapted than *Lolium*. The results of this work will be reported elsewhere.

Acknowledgments

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