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Life cycle expression analysis of three cell wall degradation-related genes in ethylene-treated grass

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Abstract Ethylene regulates multiple developmental processes during a plant life cycle, but the effect of ethylene on the upregulation of senescence-, stress-, and post-harvest-related genes in forage grasses is poorly understood. In this work, we used quantitative PCR to determine whether ethylene application affected the expression of selected cell-wall degradation related genes that are typically upregulated post-harvest. The expression levels of beta-D-glucan exohydrolase isoenzyme, alpha glucosidase, and arabinoxylan arabinofuranohydrolase isoenzyme, all putative cell wall degrading enzymes, were quantified at six points in the life cycle of the model grass species Darnel ryegrass (*Lolium temulentum* L.). We also quantified the expression of ACC oxidase and ACC synthase in response to ethylene application to determine if endogenous upregulation of ethylene biosynthesis occurred. Grass developmental stage had a significant impact on gene expression response to ethylene-treatment, indicating that discrete life cycle stages present different ethylene-responsive windows for treatment. Under our experimental conditions, ACC oxidase and ACC synthase expression were downregulated in response to ethylene-treatment, suggesting that exogenous ethylene served an auto-inhibitory role. Transcripts corresponding to the three cell wall degradation related genes increased significantly in response to ethylene treatment, suggesting that ethylene may have future utility in the pretreatment of lignocellulosic biomass. To our knowledge, this is the first report of a life cycle analysis of ethylene-induced genes in forage grasses.

Keywords Cell wall · Darnel ryegrass · Ethylene · *Lolium temulentum* · RT-qPCR

Introduction

Second-generation biofuels that utilize non-food cellulosic biomass as feedstock are critical to the development of renewable bioenergy. Recent attention has been directed toward post-harvest residues of forage grasses as sources for bioenergy and bioproducts. One major drawback, however, is the high concentration of indigestible matter due to lignified cell walls commonly found in forage plants, a factor that also limits digestibility by ruminants (Grabber et al. 2004). The same mechanical properties that limit forage grass digestibility also constrain their cost-effective use for biofuels production. Pretreatment and enzyme costs continue to limit the economic implementation of cellulosic ethanol technologies. In contrast, by altering the normal biological control of cell wall modification, it might be possible to maximize access to valuable plant polysaccharides, while minimizing costly inputs.

Lolium temulentum L. (Darnel ryegrass) has been utilized as a model forage grass for molecular genetic analyses of stress-response genes (Baldwin and Dombrowski 2006; Dombrowski et al. 2008; Dombrowski and Martin 2009; Wang et al. 2005) and is closely related to other ryegrasses that may provide biomass for bioenergy

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production. A recent study (Baldwin et al. 2007) utilized this grass to generate a suppression subtractive hybridization library based on differential expression of genes expressed between preharvest and postharvest. Based on sequence orthologies, genes that were upregulated in response to post-harvest conditions represented multiple functional categories, including detoxification, energy transfer, general metabolism, and stress responses. Several of these upregulated post-harvest genes have been characterized in other plant systems as senescence-related, suggesting an overlap between post-harvest and senescence-associated genes. In fact, several studies indicate an overlap between transcription of genes induced by stress and senescence (Nam 1997; Quirino et al. 2000).

Senescence is a well-documented ethylene-regulated process, and multiple experimental observations have suggested a role for ethylene and its substrates in promoting expression of senescence-associated genes (Nam 1997; Weaver et al. 1998). In addition to mediating plant responses in several plant developmental, cell death and senescence pathways, ethylene also regulates certain biotic and abiotic stress responses (Mattoo and Aharoni 1988). We hypothesized that some of these post-harvest genes might also be upregulated in response to exogenous ethylene treatment. Many pretreatments have been evaluated for their utility in altering the physical and chemical composition of lignocellulose (Hendriks and Zeeman 2009), but they often proved too expensive compared to the value of the glucose ultimately harvested from the cell walls. Ethylene may have utility as an inexpensive grass pretreatment to enhance the digestibility of lignocellulosic biomass.

In this study, we established trials to determine whether the use of ethylene as an exogenous stimulus affected the expression levels of three enzymes related to biomass modification. *L. temulentum* genes specifically selected from the Baldwin et al. (2007) study were beta-D-glucan exohydrolase isoenzyme, alpha glucosidase, and arabinoxylan arabinofuranohydrolase isoenzyme. Beta-D-glucan exohydrolases (EC 3.2.1.58) release glucose as the primary degradation product from the nonreducing termini of the oligomeric substrates. Beta-D-glucan exohydrolases have been implicated in cell wall loosening in elongating maize coleoptiles (Hoson and Nevins 1989). Two beta-D-glucan exohydrolases have been implicated in depolymerizing the endosperm cell wall in barley (Hrmova et al. 1996). Alpha glucosidases (EC 3.2.1.20) degrade cellulose by catalyzing the hydrolysis of glycosidic linkages to generate two smaller sugars. These enzymes appear to act on starch during germination of barley seeds (Frandsen et al. 2000). Arabinoxylan arabinofuranohydrolases (EC 3.2.1.55) catalyze the removal of the arabinofuranosyl residues from arabinoxylans. Xylans are heteropolysaccharides with homopolymeric backbones consisting of 1,4-linked β-D-xylopyranose units. Xylans are the dominant component of hemicellulose from grasses (Saha 2003), and represent a barrier to the bioconversion of hemicellulose to value-added fermentation products. The barley homolog of the arabinoxylan arabinofuranohydrolase may have a central role in cell wall metabolism during wall expansion and maturation, though it might also be involved in wall turnover and biotic or abiotic stress response (Lee et al. 2001).

Plants may decrease (autoinhibition) or increase (autocatalysis) endogenous ethylene synthesis in response to exogenous ethylene application (Kende 1993). To determine whether either of these pathways functioned in *L. temulentum*, we monitored the expression of two genes associated with ethylene biosynthesis. The first was 1-aminocyclopropane-1-carboxylate (ACC) oxidase, which was upregulated post-harvest in the Baldwin et al. (2007) study. The other gene, ACC synthase, was cloned from *L. temulentum*. ACC synthase catalyzes the formation of ACC from S-adenosyl-L-Met, whereas ACC oxidase catalyzes the conversion of this intermediate to ethylene. Both ACC oxidase and ACC synthase catalyze rate-limiting steps in ethylene biosynthesis (Kende 1993), and their expression patterns in this study served as internal controls.

The possibility of controlling the senescence processes in forage grasses could present an economic asset for the biofuels industry, provided expression of the appropriate genes is well-coordinated. To explore this possibility, the goals of this study were (1) to determine if ethylene treatment influenced expression of three cell wall degradation-related genes in a model forage grass, and (2) to determine whether developmental stage and leaf position impacted the expression or upregulation of these genes.

Materials and methods

Plant material and growth conditions

Darnel ryegrass (*Lolium temulentum* L., cv. Ceres) seeds were sterilized as previously described (Banowetz et al. 2008). Seed was germinated in sterile dishes on absorbent filter paper saturated with distilled water. Once established, seedlings were transferred to pots containing vermiculite, moved to a growth chamber under a cycle of 14 h illumination at 25°C (300 μE m^{-2} s^{-1}) and 10 h dark at 20°C. Plants were watered daily and fertilized twice per week using Technigro 20-18-20 All Purpose (Sun Gro Horticulture, Vancouver, B.C.). The first true leaf to emerge was tagged with a small piece of twine.

Plant treatments and leaf collection

When quantifying treatment-induced changes in gene expression, it is critical that leaves not be pooled across developmental stages. An annual plant can be viewed as a colony of leaves undergoing a temporally staged pathway to senescence, so gene expression can vary according to specific developmental stages (Weaver et al. 1998). To determine whether a discrete correlation existed between gene expression, developmental stage and leaf position, we monitored expression of these genes at six different stages in the life cycle of *L. temulentum*, in both the presence and absence of ethylene. We used the Feekes growth scale (Large 1954) (Fig. 1), a rating system originally designed for describing stages of wheat growth, but increasingly used to describe the growth of other grasses and small grains. Plants were treated with ethylene at six different growth stages, as classified by the Feekes growth scale (Large 1954), including Feekes stage 2.1 (1 Tiller), 2.2 (2 Tiller), 2.4 (4 Tiller), 8.0 (Flag Leaf Visible), 10.5.1 (Beginning Flowering), and 11.2 (Mealy Ripe). No reproductive tillers (spikes) were present for the first 3 collection stages, so only the first true leaf was collected. After spikes emerged (for the last 3 collection stages), basal leaves including the first true leaf died. Thus, a leaf was collected from each available position along one representative spike. At Flag Leaf Visible and Beginning Flowering stages, this encompassed 5 leaves/spike (the uppermost flag leaf, followed by leaf 1, leaf 2, leaf 3, and leaf 4). At the last collection stage, Mealy Ripe, leaf 4 was necrotic, so only the 4 upper leaves were collected. Three true biological replicates were used for each time point and treatment.

Potted *L. temulentum* plants were placed in 3L sealed chambers. The chambers were injected with 3 mL of 10,000 ppm ethylene, to create a final ethylene concentration of 10 ppm. Control plants were placed in identical chambers under the same conditions but without ethylene. Ethylene concentration was monitored with an ethylene detector tube (QA Supplies, Norfolk, VA). After 24 h of exposure, leaf samples from both treated and control plants were harvested. For each sample, the entire leaf blade from tip to ligule was collected. In the case of larger leaves, a blade was cut down the middle, and half of the blade was collected. For each treatment, three plants (biological replicates) representative of each developmental stage and leaf position were pooled, and three pooled replicates were collected. Leaves were submerged into 1 mL RNALater (Ambion, Austin, TX) immediately after they were cut into $\sim$3 mm pieces. Samples were maintained at 4°C overnight, and then at -20°C until all samples had been collected.

Preparation of RNA

Total RNA was isolated from the treated and control leaf tissues using RNAzol (Molecular Research Center, Cincinnati, OH). Leaf tissue was initially blotted to remove residual RNALater, then pulverized in Lysing Matrix D tubes (MP Biomedicals, Solon, OH) using a Bead Beater (MP Biomedicals). Samples were treated with DNase using the Turbo DNA-free kit (Ambion), with 1 h incubation and using 2 μL Turbo DNase. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA integrity was assessed by gel-red stained 1.0% agarose gel

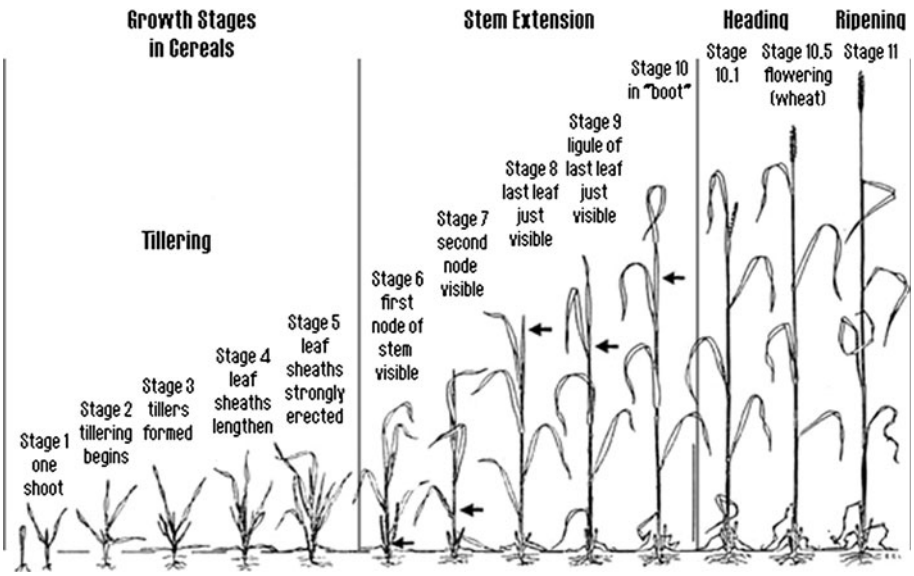


Fig. 1 Feekes growth scale for wheat (Large 1954). This rating system was originally designed for describing stages of wheat growth, but is increasingly used to describe the growth of other grasses and small grains

electrophoresis. Intact rRNA subunits of 18S and 28S were observed on the gel, as was the absence of smears indicating minimal degradation of the RNA. RNA was converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's protocol included with the First-Strand Synthesis System for RT-PCR Kit. Reverse transcription of 2.5 μg of the RNA was completed using random hexamers (50 ng/μL) provided in the kit in a total volume of 65 μL. A subset of samples was carried through the experiment with no RT to assess the absence of DNA.

Cloning of *L. temulentum* cDNA and primer design

Primers and PCR conditions previously used to isolate maize ACC synthase genes (Young et al. 2004) were used to isolate ACC synthase from *L. temulentum* DNA. The 1,824 bp product, homologous to the near entirety of the maize gene, was gel purified, cloned, and sequenced, and then used as a template for the design of real-time quantitative RT-PCR (RT-qPCR) primers. Primers for all RT-qPCR genes of interest and candidate reference genes were designed using Primer3Plus software (Untergasser et al. 2007) (Table 1) and manufactured by Sigma-Aldrich (St. Louis, MO).

Primer choice and optimization

Reference genes have been identified for use in *L. temulentum* (Dombrowski and Martin 2009), and in the closely related *Lolium perenne* (Martin et al. 2008; Lee et al. 2010), but none of these genes had been validated within the parameters of the current study. To identify appropriate reference genes for this study, nine candidate genes were tested both for their expression stability and the proximity of expression level to that of the genes of interest. We tested previously designed primers used to amplify the following genes from *L. perenne*: *Eukaryotic elongation factor 1α* (eEf-1α), *Eukaryotic initiation factor 4a* (eIF-4a), *Glyceraldehyde-3-phosphate dehydrogenase* (GADPH), *β-tubulin* (β-TUB), and *Ubiquitin conjugating enzyme E2* (UBC) (Martin et al. 2008). We also tested primers designed to amplify the following genes from *L. temulentum*: *ACTIN 11* (ACT11), *Cap Binding Protein 20* (CAP), *Eukaryotic elongation factor 1α* (eEf-1α-Lt), *Eukaryotic initiation factor 4a* (eIF-4a-Lt), *Ubiquitin 5* (UBQ5), and *25S rRNA* (25S) (Dombrowski and Martin 2009). We also tested an alternative set of primers designed to amplify a moderately but stably expressed copy of *Eukaryotic elongation factor 1α* (eEf-1α-s) in *L. perenne*, as well as primers described for *YT521-B-like protein family protein* (YT521B) (Lee et al. 2010).

Prior to validating reference genes and performing expression studies, each primer set (for reference genes and genes of interest) was tested for efficiency against a standard curve, consisting of five fold serial dilutions, covering a range from 15.4 ng to 4.9 pg using leaf tissue from mid-life cycle of the control treatment. For each primer pair, amplification efficiency was calculated as $E = -1 + 10^{(-1/\text{slope})}$.

Validation of reference genes

Each primer set for the candidate reference genes was tested against an entire 32-sample cDNA experimental set, representing tissue collected from the 1 Tiller stage through Mealy Ripe stage. Both ethylene-treated and non-treated control samples comprised this set. At least one sample from each primer pair was analyzed via agarose gel electrophoresis to verify the product was a single band of the correct size. Additionally, one sample from each primer pair was sequenced to verify its identity. For these validation experiments, triplicate sets of each cDNA were amplified using the conditions described below. The threshold cycle, Cq (i.e., the amplification cycle in which product formation exceeds background fluorescence) was averaged for each triplicate and transformed to quantities relative to the sample with the highest expression. These values were imported into geNorm v3.5 software (Vandesompele et al. 2002) for analysis of gene expression stability and subsequent selection of optimal reference genes.

RT-qPCR

Three biological replicates for each treatment were used for RT-qPCR analysis, and two technical replicates were analyzed for the cDNA of each biological replicate. In addition, each 96-well plate contained two replicates of no-template controls to screen for possible contamination and dimer formation, and two replicates of calibrator samples (untreated leaf sample from 1 Tiller stage) for each primer set to normalize readings between plates. Real-time PCR was performed using a Multicolor Real-Time PCR Detection system (Model iQ5; Bio-Rad Laboratories, Hercules, CA). Each cDNA extract was diluted 1:5, and 2 μL were added to amplification reaction mixtures (20 μL) containing 200 nmol of each primer and 10 μL of iQ SYBR green Supermix (Bio-Rad Laboratories). Cycling conditions were 95°C for 3 min and 40 cycles of 95°C for 10 s and 57°C for 30 s. Fluorescence readings were recorded after each cycle. A final melting analysis was obtained by slow heating, with 10 s increments of 0.5°C from 57 to 95°C, and fluorescence collection at intervals of 0.5°C. The Cq of each sample was determined during the exponential phase of amplification. Each dissociation curve

Table 1 Primer sequences, amplicon sizes, and real-time quantitative polymerase chain reaction (RT-qPCR) amplification efficiency for the candidate reference genes and target genes

Gene	Primer sequence (5′–3′)	Product Size (bp)	Efficiency (%)	Reference
UBC-F*	GAGGTGCAGCGAGAAAAGTC	98	99.4	Martin et al. 2008
UBC-R*	TCTGATGGGCCCTTGATTAG			
eEf-1α-F*	GGCTGATTGTGCTGTGCTTA	114	105	Dombrowski and Martin 2009
eEf-1α-R*	CTCACTCCAAGGGTGAAAGC			
eIF-4a-Lt-F*	TGCTTTCACGTGGTTTCAAG	95	97	
eIF-4a-Lt-F*	AGGAGGCATGGTAGCAGAGA			
Exohydrolase-F*	CTCCACCGTCATGATCTCCT	123	100.5	This study
Exohydrolase-R*	GTCAATGCCCTCCCAGTC			
Glucosidase-F*	TGAAGTCCGGGAAGTAGACG	135	101	This study
Glucosidase-R*	TAGACCCAGGGATCAGCATC			
Xylanase-F*	TCCAAACCACTTGACCATCC	94	95	This study
Xylanase-R*	TTCGATCAAATGTGCCCTTC			
ACC Oxidase-F*	GGAGCTGCAGAAGAGTTTGG	101	103.5	This study
ACC Oxidase-R*	CCTTGTAAGGGCAGCTCAAG			
ACC Synthase-F*	CCGGAGTTCGTGAGCATC	83	99.6	This study
ACC Synthase-R*	AGGCTGTAGGCGATGTGG			
eIF-4a-F	GGTCGTGTGTTTGACATGCT	103	102.7	Martin et al. 2008
eIF-4a-R	CCTTGAAACCACGAGAAAGC			
GADPH-F	CATCACCATTGTCTCCAACG	92	156.7	
GADPH-R	AACCTTCAACGATGCCAAAC			
β-TUB-F	CGTGGAAAGATGAGCACAAA	106	104.4	
β-TUB-R	CGCTGGACTTCACATTGTTG			
ACT11-F	CCTTTTCCAGCCATCTTTCA	100	95.8	Dombrowski and Martin 2009
ACT11-R	GAGGTCCTTCCTGATGTCCA			
CAP-F	CTCCAGGGAAGATGCTGAAG	95	105	
CAP-R	CTTGAAAGCCCCAATCAAAA			
eEf-1α-Lt-F	CCTTGCTTGAGGCTCTTGAC	103	89.2	
eEf-1α-Lt-R	GTTCCAATGCCACCAATCTT			
UBQ5-F	AAGGAGTCAACCCTCCACCT	109	103.3	
UBQ5-R	TCACCTTCTTGTGCTTGTGC			
25S-F	CCCAGTGCTCTGAATGTCAA	98	107.4	
25S-R	ATGACGAGGCATTTGGCTAC			
eEf-1α-s-F	CCGTTTTGTCGAGTTTGGT	113	98.5	Lee et al. 2010
eEf-1α-s-R	AGCAACTGTAACCGAACATAGC			
YT521B-F	TGTAGCTTGATCGCATACCC	122	88.5	
YT521B-R	ACTCCCTGGTAGCCACCTT			

* Primer used either for a final reference gene or for a target gene. Remaining primers represent candidate reference genes that were not chosen

trace was checked for specificity to ensure that no non-specific products were present in the amplification.

Data analysis

All postrun data analyses were performed using iQ5 Optical System software, version 2.0 (Bio-Rad). For data analysis, the Cq values of the genes were converted to relative quantities and normalized using the geometric mean of three reference genes, followed by inter-run calibration (IRC) using the included standards as inter-run calibrators.

Statistical analysis

Two-way analysis of variance (ANOVA) was used to assess the statistical significance of gene expression between the ethylene-treated and non-treated samples, as well as the significance of the interaction between treatment and Feekes growth stage/leaf position. Two-sample

t tests (assuming unequal variances) were used to analyze the means of the three replicates for each parameter, as well as to compare the normalized expression means of vegetative and reproductive phase groups. Both tests utilized Microsoft Excel version 11.3.5. Statistical significance was defined as $P < 0.05$.

Results

Amplification efficiencies and expression levels of reference gene candidates

All amplification efficiencies were between 95 and 105% (Table 1), with the exception of GADPH (156.7%), eEf-1α-Lt (89.2%) and YT521B (88.5%). Because recorded efficiency for GADPH was consistently high, GADPH was dropped from the contender reference genes.

Average Cq values of the candidate reference genes except for 25S ranged from 23.36 (eEf-1α) to 34.02 (ACT11). 25S had exceptionally high expression levels, with an average Cq value of 10.71. Specificity of products was verified by representative dissociation curves, all of which displayed comparable single peak patterns.

Expression stability of the reference gene candidates

To evaluate the stability of expression of reference genes, RNA transcription levels were measured for a full sample set, representing both treated and non-treated grass across its life cycle. The input for geNorm analysis consisted of results from twelve primer pairs representing nine candidate reference genes (eIF-4a was represented by two primer pairs, and eEF-1α was represented by three primer pairs). The two most stable primer pairs were both representing eIF-4a, with an *M* value of 0.391 (Fig. 2a). However, the *V* value was 0.235 (Fig. 2b), suggesting that including more reference genes would be optimal. When using geNorm, a lower *M* value denotes a more stable gene expression, whereas the pairwise variation (*V*) acts as a proxy for the number of reference genes to include for normalization (Vandesompele et al. 2002). The proposed value of 0.15 for *V* is used as the cutoff value, below which the inclusion of an additional gene is not required. The *V* value decreased with the addition of the primer pairs for amplification of eEf-1α (based on the *L. perenne* design) and eEf-1α-Lt (based on the *L. temulentum* design), but it was not until a fifth primer pair was added (representing the gene UBC) that the *V* value dropped below the proposed cutoff value, to 0.140. Because these five primer pairs represent the three most stable reference genes, the three reference genes chosen for this study were UBC, eEf-1α, and eIF-4a, with the most optimal primer pair selected for each of the latter two genes (eEf-1α derived from *L. perenne*, and eIF-4a-Lt, derived from *L. temulentum*).

Quantitative real-time RT-PCR analysis

To determine whether beta-D-glucan exohydrolase isoenzyme (exohydrolase), alpha glucosidase (glucosidase), arabinoxylan arabinofuranohydrolase isoenzyme (xylanase), ACC oxidase, and ACC synthase were differentially expressed in *L. temulentum* relative to Feekes stage, as well as in response to ethylene treatment, expression of these genes was quantified by RT-qPCR. The relative expression profiles, normalized to UBC, eEf-1α, and eIF-4a-Lt, are shown in Fig. 3a–e. Glucosidase transcripts (Fig. 3a) peaked among the leaves treated with ethylene at the 4 Tiller Stage where the mean normalized expression level of ethylene-treated samples was significantly higher (13.5-fold) than the non-treated controls using the two-sample *t* test for unequal variances (Online Resource 1). In contrast, the only other evidence of a stage-specific significant difference in glucosidase expression was at the Flower Stage Leaf 1 where the non-treated control showed 3.5-fold greater expression than the ethylene-treated samples (Online Resource 1). ANOVA indicated that (1) there is a significant effect from the Feekes stage/leaf position on the expression of glucosidase; (2) there is a significant effect from ethylene treatment on glucosidase expression; and (3) the interaction between treatment and Feekes stage/leaf position is a significant factor contributing to the response of glucosidase expression (Online Resource 2).

Similarly, expression levels of exohydrolase were greater after ethylene treatment at the 4 Tiller Stage (Fig. 3b, Online Resource 1). At this stage, ethylene treatment promoted exohydrolase expression 61-fold over the non-treated control. ANOVA indicated that both treatment and Feekes stage/leaf position affected exohydrolase gene expression. However, the interaction between treatment and Feekes stage/leaf position did not affect the level of response (Online Resource 2).

Ethylene treatment was associated with a significant, though small (1.3-fold) increase in xylanase gene expression compared to that measured in the non-treated control at Mealy Ripe Stage in the flag leaf sample (Fig. 3C, Online Resource 1). ANOVA demonstrated that both the Feekes stage/leaf position and the sample treatment had a significant effect on xylanase gene expression. The interaction between Feekes stage/leaf position and treatment also had a significant effect on xylanase gene expression (Online Resource 2).

ACC synthase expression levels were quite low for both treated and non-treated samples, with the exception of single non-treated outlier at the 4 Tiller Stage (Fig. 3d). Flag Stage Leaf 4 was the only developmental stage at

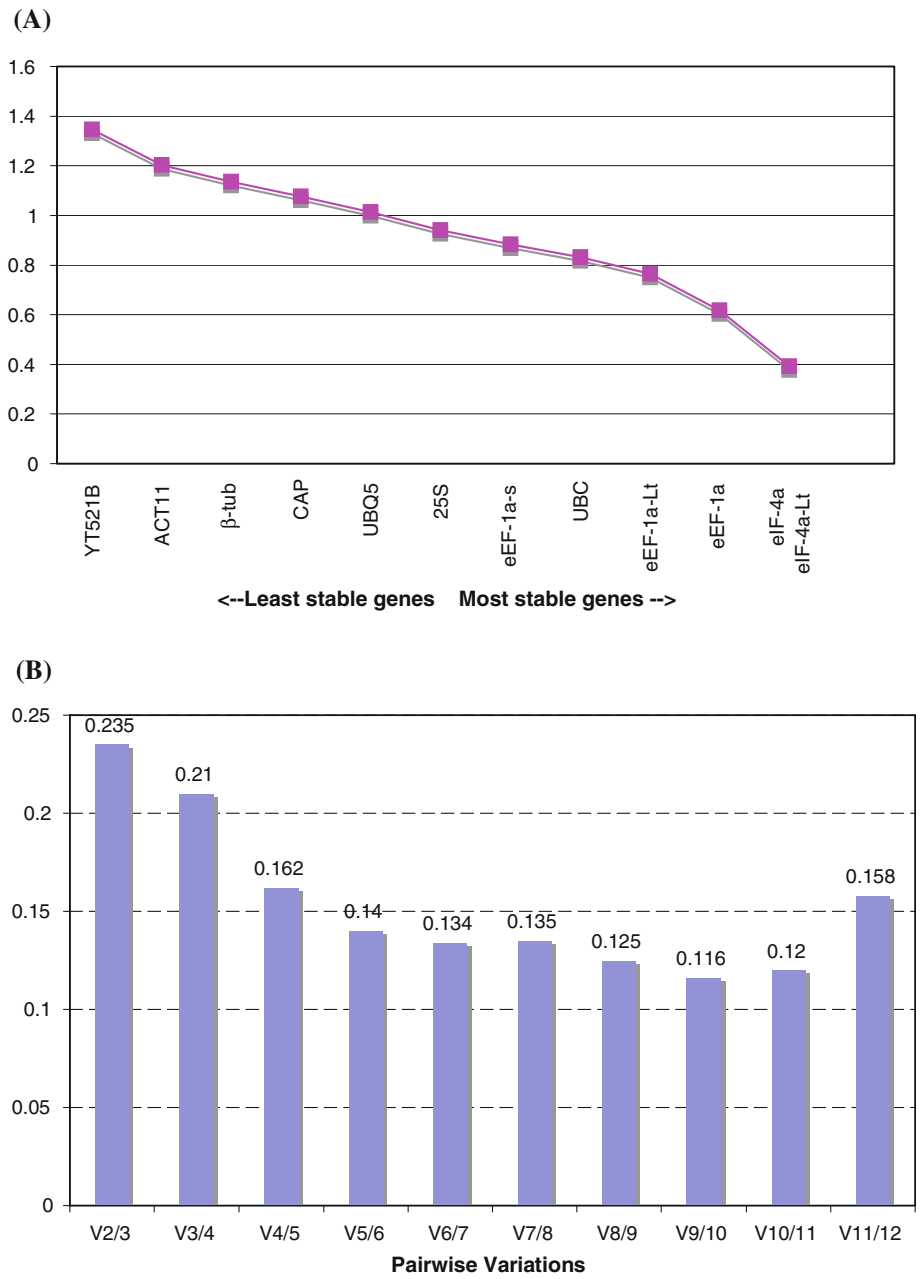
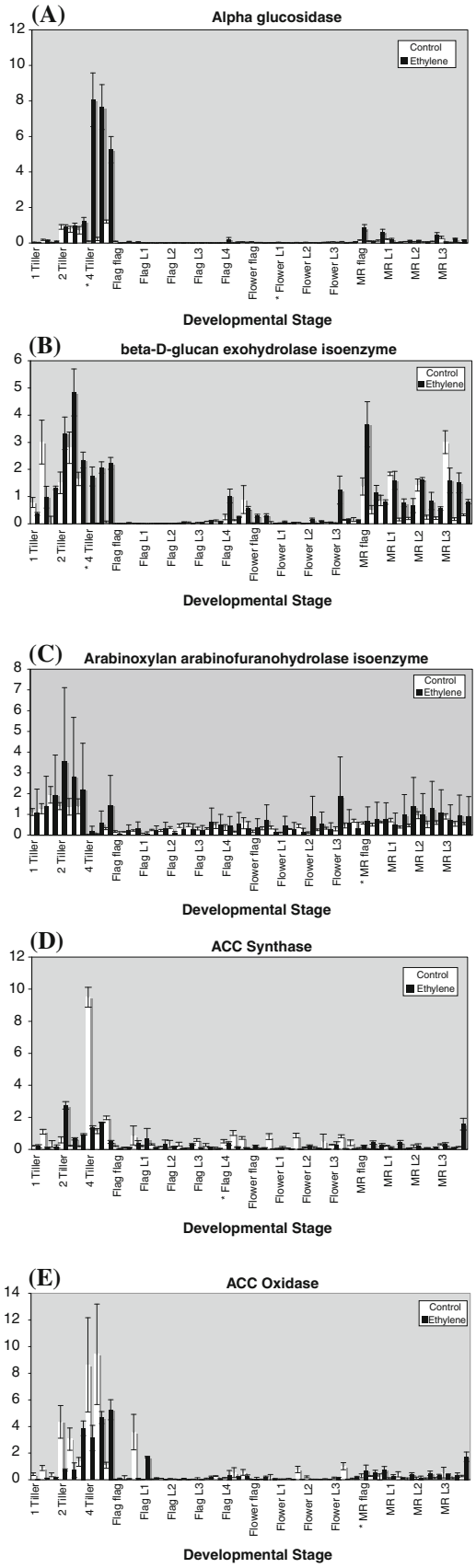


Fig. 2 **a** Expression stability and reference gene ranking based on geNorm calculations. **b** Pairwise variation analysis to determine the optimal number of reference genes needed for accurate normalization. *V* values less than 0.15 indicate no further genes are needed for calculation of a reliable normalization factor

which there was a significant difference in ACC synthase gene expression between treated and non-treated samples where non-treated samples had significantly higher expression (Online Resource 1). In 11 of the 16 stages and leaf positions surveyed, ACC synthase expression in the non-treated control was higher than expression in the ethylene-treated counterpart. The four leaves sampled at Mealy Ripe Stage were among the sole five samples in which mean expression levels were higher among ethylene-treated samples. Though the differences in means were not statistically significant, this trend suggested that leaves at this stage may have a different perception of ethylene. ANOVA indicated no significant impact on ACC synthase expression from the Feekes stage/leaf position. There was a significant effect from treatment on gene expression, though unlike the expression of the other genes analyzed, this effect associated higher gene expression levels with the non-treated samples. The interaction between Feekes stage/leaf position and treatment did not affect the level of ACC synthase expression (Online Resource 2).

With the exception of the 2 Tiller and 4 Tiller stages, ACC oxidase expression levels, like those for ACC synthase, were low for both treated and non-treated samples (Fig. 3e). These samples showed the greatest variability in levels of gene expression. Three of the biological replicates had expression levels that were so low that the Cq did not



◄ **Fig. 3** Gene expression analysis by quantitative RT-PCR. Leaves from plants under control conditions (*white*) or ethylene treatment (*black*) were collected at 1 Tiller, 2 Tiller, 4 Tiller, Flag Stage (Flag), Beginning Flowering Stage (Flower), and Mealy Ripe Stage (MR). Each biological replicate is shown individually ± standard deviation. For the first three developmental stages, only the first true leaf of each plant was sampled. For the latter three stages, the flag leaf (flag), leaf 1 (*L1*), leaf 2 (*L2*), leaf 3 (*L3*) and leaf 4 (*L4*), if available, were sampled. Expression levels were calculated relative to the geometric mean of *Eukaryotic initiation factor 4a (*eIF-4a-Lt), *Eukaryotic elongation factor 1α (*eEf-1α), and *Ubiquitin conjugating enzyme E2* (UBC) expression. Units of expression are values linearized with the $2^{(-\Delta\Delta Cq)}$ method, where Cq is the threshold cycle. Expression levels of alpha glucosidase [GenBank:EL738578] **a** beta-D-glucan exohydrolase [GenBank:EL738351] **b** arabinoxylan arabinofuranohydrolase isoenzyme [GenBank:EL738538] **c** ACC synthase [GenBank:JN587114] **d** and ACC oxidase [GenBank:EL738567] **e**. Significant differences in expression between the means of the three biological replicates for developmental stage/leaf position are shown by one *asterisk* ($P < 0.05$)

reach the threshold in either of the technical replicates. For each of the following samples (ethylene-treated 1 Tiller sample A and Flag Stage Leaf 3 Sample A; non-treated Flag Stage Leaf 2 sample B), a value of 0 was assigned for the relative expression level. The only sample in which a significant difference in mean ACC oxidase expression level occurred was Mealy Ripe Stage flag leaf, where the mean of the ethylene-treated samples, while still very low, was 2.5-fold greater than that of the non-treated counterpart (Online Resource 1). In nine of the 16 sample means, however, the non-treated control mean was greater than the ethylene-treated counterpart. Similar to the pattern observed with ACC oxidase expression, the final four samples (all leaves belonging to the Mealy Ripe Stage) had greater ACC expression levels in ethylene-treated samples relative to non-treated counterparts. ANOVA conclusions were identical to those of ACC synthase: Feekes stage/leaf position had no significant impact on ACC oxidase expression. Treatment had a significant effect on gene expression, though this effect associated higher gene expression levels with the non-treated samples. The interaction between Feekes stage/leaf position and treatment did not affect the level of gene expression (Online Resource 2).

Regardless of treatment, most samples had higher expression levels of the respective genes during the vegetative phase (1 Tiller, 2 Tiller, 4 Tiller) as compared to the reproductive phase (all stages sampled thereafter). To test the significance of this observation, we conducted another set of two-sample *t* tests to compare the mean expression level of the vegetative phase to that of the reproductive phase. These means were compared within each non-treated control group and within each ethylene-treated group for each gene. Except for the ACC synthase non-treated groups and the exohydrolase non-treated groups, mean

expression levels of each vegetative phase group were significantly higher ($P < 0.05$) than the mean expression levels of each reproductive phase group, regardless of treatment (Online Resource 3).

Discussion

The Energy Independence and Security Act of 2007 calls for a Renewable Fuels Standard that increases the amount of renewable biofuel that is blended into transportation fuels from the current nine billion gallons up to 36 billion gallons by 2022 (Gelhar et al. 2010). Two crucial factors in reaching this goal will be the net productivity of the plants used as source material, and the cost of harvesting the cellulosic biomass. Toward this goal, we established trials to determine whether the use of an inexpensive exogenous stimulus, ethylene, could affect the expression levels of three enzymes related to biomass modification, thereby increasing the energy efficiency of biomass production. We observed temporal expression differences among our genes of interest, suggesting that some genes may respond directly to ethylene application at discrete developmental stages.

The remarkable expression increase (in 8 of the 10 comparisons) of the five genes in the 1, 2, and 4 tiller phases relative to later phases may be correlated to juvenility. Plants first pass through a juvenile (early vegetative) phase before they are able to move into an adult (late vegetative) and then subsequent reproductive phase (Poethig 1990). The juvenile to adult transition can be difficult to distinguish within the vegetative phase. However, the extreme changes from vegetative to reproductive phase are obvious (Asai et al. 2002), as the vegetative structures are suppressed to allow the development of elaborate inflorescence structures. After plants have passed through their juvenile and adult phases, floral initiation can occur whenever conditions are favorable.

Considering the observed temporal shifts in basal gene expression levels, it seems logical that exogenous factors such as ethylene treatment could have profoundly different effects depending on plant developmental stage. Our data support this idea by demonstrating that Feekes developmental stage and leaf position impacted the expression of the three cell wall-degradation related genes. Additionally, the interaction between the developmental stage/leaf position and ethylene treatment contributed significantly to upregulation of genes encoding arabinoxylan arabinofuranohydrolase and the alpha glucosidase. Several other studies support this concept, suggesting that the capacity of plant organs to respond to exogenous applications of ethylene is developmentally regulated. Weaver et al. (1998) demonstrated that the enhanced expression of senescence-associated genes in ethylene-treated *Arabidopsis* leaves was greatest in older leaves, and moderate in younger green leaves. Likewise, immature tomato fruits were insensitive to exogenous ethylene, and did not respond to ethylene applications with subsequent fruit ripening (Lincoln et al. 1987). The differences in temporal expression of the genes targeted in our study indicate that some genes respond directly to ethylene application, at discrete developmental stages, while other genes are regulated by other stress- or senescence-related signals. Cell wall degradation-related genes likely depend on developmental signals related to growth stage, as well as other factors, so it is possible that ethylene may have an effect only in coordination with multiple other developmental signals. The complex interplay between ethylene as a signal and the consequent stress response suggests that multiple signal transduction pathways and hormones are involved (Morgan and Drew 1997). At certain developmental stages, other factors such as abscisic acid, jasmonic acid, brassinosteroids, dehydration, and darkness may play a greater role than ethylene in promoting the expression of senescence-related genes (He et al. 2001).

For each of the cell wall degradation-related genes, there was one discrete developmental stage in which the mean gene expression in ethylene-treated samples was significantly higher than that of the non-treated controls. However, there were many developmental stages for each of these genes in which the differences of the means were very close to being significant. Thus, when ANOVA was conducted on the pooled set of all samples for each individual cell wall degradation-related gene, ethylene treatment overall had a significant impact on gene expression. For instance, although the 61-fold difference in expression of exohydrolase at the 4 Tiller stage was the single developmental stage increase that was significantly different, ethylene treatment significantly enhanced greater expression levels of this gene overall.

Our measurements of ethylene biosynthesis enzyme activity suggest that autoinhibitory ethylene production may occur following ethylene treatment. ANOVA results demonstrated a significant impact of ethylene treatment on ACC synthase and ACC oxidase gene expression, though unlike the expression of exohydrolase, glucosidase, and xylanase, the higher gene expression levels occurred in the non-treated samples. This effect on ACC synthase and ACC oxidase expression suggested that exogenous ethylene alone is not the only source of measurable differences in gene expression between the ethylene-treated samples and the controls. In fact, endogenous signals may be present which attempt to counter the exogenous ethylene application. This attempt to suppress ethylene formation during a physiologically inopportune time may be a survival response on the part of the plant. However, if ethylene concentrations were greater, or treatments were of

longer duration, it is possible that exogenous ethylene may have had more influence upon the regulation of endogenous ethylene. Although ACC synthase and ACC oxidase both belong to multigene families, it appears the ACC oxidase allele that was upregulated post-harvest (Baldwin et al. 2007) and the ACC synthase allele we cloned from *L. temulentum,* are among those alleles that specifically respond to exogenous ethylene. Differential regulation of individual alleles can vary by cultivar, tissue, and developmental stage (Morgan and Drew 1997), and can be impacted by environmental, developmental, and hormonal signals (Kende 1993). Potentially, the expression and response of alternate, uncloned alleles would provide a different temporal signature, though differentiating which allele regulates the onset of senescence, and which alleles control other aspects of leaf development can be difficult (Young et al. 2004).

In this investigation, we quantified stage-specific gene expression involved in cell wall degradation in response to ethylene treatment. We also present three fully validated reference genes that will prove useful in future studies encompassing comparable life-cycle analyses in this model system. This study portrays three genes identified from a post-harvest expression study, and demonstrates that *L. temulentum* plants treated with ethylene significantly upregulate these genes as compared to non-treated controls. However, significant upregulation occurs during discrete developmental stages, suggesting that a larger genetic and hormonal network underlies these responses and the accompanying morphological changes. It also appears that under the experimental conditions, *L. temulentum* responds to exogenous ethylene application with a downregulation of endogenous ethylene production. Unraveling the signaling mechanisms behind these expression differences will lend greater understanding to the means by which ethylene affects grass development. Furthermore, knowledge related to these genetic mechanisms and the temporal expression of cell wall degradation in grasses will prove valuable as agricultural residues play a greater role in the production of fermentable sugars.

This research opens up opportunity for future work that may address research questions outside the scope of this study. Such work may examine biochemical issues, such as the cell wall composition or ACC content after ethylene treatment. Future work might also utilize synthetic plant growth regulators, such as 1-Methylcyclopropene (1-MCP), which blocks ethylene receptors in plants, or 4-aminoethoxyvinylglycine (AVG), which inhibits ethylene synthesis. Use of such a regulators would help strengthen the understanding of ethylene's role in gene regulation and promote better understanding of the feedback mechanisms triggered between exogenous ethylene application and endogenous ethylene regulation.

In an effort to improve the efficiency of conversion of grass biomass into biofuels, future experiments will identify additional ethylene responsive, cell wall degradation-related genes. Redirecting the expression of these genes via fusion to promoters of genes identified in the current study will enable the development of new gene constructs that potentially regulate the expression of cell wall degradation genes in a very specific temporal manner. These genes may be drawn from the Baldwin et al. (2007) post-harvest expression study, from a cell wall-related gene database, or from a novel suppression subtractive hybridization library identifying differential expression of genes that are upregulated in response to ethylene treatment. Such a study might ultimately enable a way to improve grass that will respond to post-harvest or ethylene treatment with cell wall degradation. Alternately, this knowledge could herald the potential of manipulating specific promoters, enabling the activation and acceleration of cell wall degradation in the absence of ethylene. The application of metagenomic approaches to identify new enzymes in lignocelluloytic microbial communities will provide additional candidate genes for use in such an approach. The ultimate goal will be to develop molecular tools that lead to high levels of cell wall degradation-related gene expression in targeted tissues.

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