Patterns of Expression of a Lolitrem Biosynthetic Gene in the *Epichloë festucae*–Perennial Ryegrass Symbiosis

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Lolitrems B is synthesized by *Epichloë festucae* in associations with Pooid grasses. A complex cluster of at least 10 genes (*ltm* genes) is required for its synthesis. An early step in this pathway is catalyzed by *ltmM*, a symbiosis-expressed gene. *PltmM-gusA* reporter gene analysis was used to monitor *ltmM* gene expression patterns in planta. The minimum promoter length required for high-level *gusA* expression in infected seedlings is in the range of 480 to 782 bp. *gusA* was expressed by the endophyte in all infected vegetative plant tissues and in epiphyllous hyphae. Spikelets from reproductive tillers were analyzed at different developmental stages. During pre-anthesis, *gusA* expression was observed in all infected floral organs except the immature gynoecium. In post-anthesis florets, gene expression occurred almost exclusively in the gynoecium. Expression of *gusA* by the endophyte was observed in germinating seeds 24 h postimbibition and seedlings older than 6 days postimbibition in hyphae from the mesocotyl to the tip of the emerging first leaf. This work provides a detailed analysis of the spatial and temporal expression patterns of a symbiosis-expressed gene in planta.

Additional keywords: *Epichloë festucae*, perennial ryegrass, secondary metabolites.

Many fungal species of the family Clavicipitaceae form symbiotic associations with grasses of the Poaceae family (Schardl et al. 2004). The endophyte *Epichloë festucae* systemically colonizes perennial ryegrass (*Lolium perenne*) growing within the intercellular spaces of vegetative and reproductive tillers (Fig. 1). This is a mutually beneficial interaction between the fungus and host plant, where the fungus gains shelter, nutrients, and a means of dissemination, while infected hosts appear to have increased persistence in pastoral ecosystems compared to uninfected plants (Clay 1990; Malinowski and Belesky 2000). In vegetative tissues (Fig. 1A), hyphae colonize the leaf sheath and leaf blade of all tillers (Fig. 1, A2), with greater hyphal abundance observed in the sheath compared with the blade (Christensen 1995; Christensen et al. 1997, 2002). *E. festucae* hyphae colonize all meristematic tissues (Fig. 1, A3) (Christensen et al. 2002), including the stem apex, leaf primordia, axillary buds, and even the root tips (Christensen and Voisey 2007).

A comprehensive analysis of hyphal distribution in *E. festucae* in reproductive tissues has not been performed; however, the growth of several closely related *Neotyphodium* spp. in grass inflorescences has been examined (Majewska-Sawka and Nakashima 2004; Philipson and Christey 1986; Sugawara et al. 2006). During reproductive development of the plant (Fig. 1B), hyphae grow from the vegetative apex into the inflorescence primordium and floral apices, infecting the ovary and ovule (Philipson and Christey 1986). Soon after fertilization, the fungus gains entry into the embryo sac. During early embryogenesis, hyphae can be found on the surface of the embryo (Fig. 1C). As the seed matures, hyphae penetrate and become widespread throughout the embryo, with hyphae observed in the plumule apex, aleurone layer, between the scutellum and the endosperm, and in the nucellus (Majewska-Sawka and Nakashima 2004; Philipson and Christey 1986). During seed germination (Fig. 1C), hyphae in the shoot apex (coleoptile, plumule, and mesocotyl) have a significant role in further colonization of the seedling (Philipson and Christey 1986).

E. festucae in association with its host produces a range of secondary metabolites, including the indole-diterpenes, which provide protection against insect and small animal herbivory (Clay 1990; Clay and Schardl 2002; Parker and Scott 2004; Tanaka et al. 2005). The predominant indole-diterpene found in *E. festucae*–grass associations is lolitrems B, a potent mammalian tremorgen that causes the condition known as ryegrass staggers syndrome (Gallagher et al. 1981; Miles et al. 1994). Investigations have revealed that a gene cluster of at least 10 genes was required for lolitrems B biosynthesis in *Neotyphodium lolii* and *E. festucae* (Young et al. 2005, 2006). Functions have been predicted for nine of the genes, and experiments have shown *ltmM* to be necessary for lolitrems B production (Young et al. 2005, 2006). The *ltmM* gene encodes an FAD-dependent monooxygenase that is essential in an early step of the biosynthetic pathway. Because *ltmM* has an important role in lolitrems B biosynthesis, it was used to examine the expression patterns of *ltm* genes in planta.

Previous investigations into *ltm* gene expression have used reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of pseudostem tissue from vegetative tillers of perennial ryegrass and of hyphae grown in artificial media, which showed that all 10 genes were preferentially expressed in planta (Young et al. 2005, 2006). The temporal and spatial expression patterns of the *ltm* genes in planta have not been examined and it is not known whether *ltm* gene expression is influenced by the developmental stage of the plant or the tissue type. The objective of this study was to examine the expression patterns of the *ltm* genes in planta using *PltmM-gusA* reporter gene analysis to monitor expression. This study involved a detailed analysis of in planta expression patterns of the symbiosis-expressed gene, *ltmM*.

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*The e-Xtra logo stands for “electronic extra” and indicates additional information is available online. Nine supplemental figures and two supplemental tables are published online.

A **B** **C**

Fig. 1. *Epichloë festucae*–*Lolium perenne* association. **A**, *E. festucae* colonization of vegetative tillers. 1, Drawing of the vegetative morphology of *L. perenne* showing the aboveground tillers (T) and belowground roots (R). Leaves are composed of blade (B) and sheath tissue (Sh). Stylized longitudinal sections through 2, colonized leaf tissue and 3, colonized apical meristem. Colonization by *E. festucae* is found between cell walls and is indicated by dark lines. **B**, Morphology of *L. perenne* reproductive structures. 1, Reproductive morphology of *L. perenne* showing the elongated reproductive axis, the inflorescence (I), which bears the spikelets (S). 2, Stylized close-up of an individual spikelet that has one subtending glume (G) that encloses approximately 10 florets (F). 3, Stylized close-up of a floret at the pre-anthesis reproductive stage with each floret bound by a lemma and palea (P) that enclose three anthers (A), two lodicules (Lo), and the gynoecium (Gy); and 4, a floret shown at the post-anthesis reproductive stage illustrating the developing gynoecium or seed. **C**, Morphology of *L. perenne* seed. 1, Mature *L. perenne* seed with attached palea (P). 2, Stylized longitudinal section through the basal portion of *L. perenne* seed showing the scutellum (Sc), coleoptile (C), shoot apex (Sa), mesocotyl (M), aleurone layer (Al), testa (Te), endosperm (E), radical (Ra), and coleorhiza (Cz); and 3, a seedling of *L. perenne* showing the cotyledons (Co) and mesocotyl (M). 4, Longitudinal section through the leaf tissue of young leaf seen in 3. Colonization of seedlings by *E. festucae* is illustrated by bold black lines shown between plant cell walls.

RESULTS

Analysis of *PltmM-gusA* transformants and *ltmM* transcription start site.

Previous work established that all 10 genes that compose the *ltm* gene cluster are preferentially expressed in planta with only weak or no expression in culture (Young et al. 2005, 2006). Qualitative RT-PCR was used to determine if there were any culture conditions that mimicked in planta levels of *ltm* mRNA abundance. A comprehensive analysis using different physiological conditions, including plant extracts, was performed; however, none of the culture conditions tested resulted in a level of *ltm* transcript abundance that would be observed in plants (data not shown). The focus of this work has been a detailed analysis of *ltmM* gene expression patterns in planta. To this end, five different *ltmM* promoter deletion fragments of approximately 1.6 kb, 1.3 kb, 782 bp, 480 bp, and 280 bp were generated and translationally fused to *gusA* to generate the constructs pXZ55, pXZ56, pXZ57, pKM8, and pKM9, respectively (Fig. 2A). Each construct was used to transform *E. festucae* protoplasts, and transformants were obtained with the con-

struct ectopically integrated into the *E. festucae* genome. PCR analysis of these transformants, using the primers T7 and GUSR, confirmed that each deletion construct had integrated into the genome with an intact promoter fragment of the expected size (data not shown). Southern analysis of *Bam*HI digests of these transformants using a *gusA*-specific probe was used to determine the integration pattern and copy number of the construct within each transformant. Several transformants exhibited complex banding patterns, suggesting multiple site and multiple copy integrations (copy number >10) (Table 1). However, most transformants had relatively simple banding patterns, indicative of low copy number (copy number ≤4) (Table 1; Supplemental Figure S1). The banding pattern for each transformant was unique, indicating that the construct was inserted into the genome at different positions for each transformant. The positive controls KM1.1 and KM1.5 were generated by transforming *E. festucae* protoplasts with the plasmid pLM1, which integrated ectopically into the genome. The pLM1 plasmid has the *gusA* gene translationally fused to the *Aspergillus nidulans gpd* promoter.

Because *ltmM* is part of a gene cluster that exhibits co-regulated gene expression, expression patterns of the ectopically integrated *PltmM-gusA* constructs may differ from the wild type. To test whether ectopic integration or construct copy number had any effect on *PltmM-gusA* expression, transfor-

Fig. 2. In planta expression analysis of *PltmM-gusA* deletion constructs in *Epichloë festucae*. **A**, Schematic of the five *PltmM-gusA* deletion constructs representing sequential deletions of the *ltmM* promoter region translationally fused to *gusA*. **B**, Whole-mount β-glucuronidase (GUS) staining of perennial ryegrass seedlings inoculated with *E. festucae* transformed with the promoter deletion constructs pKM9 (KM8.7), pKM8 (KM7.9), pMM57 (MM17.3), pMM56 (MM16.3), and pMM55 (MM15.3). The GUS staining is indicated by the blue precipitate.

Table 1. Analysis of *PltmM-gusA* transformants^a

Transformant	Construct	Southern analysis ^b	Fluorescence assay ^c	In planta assay ^d
KM1.1	pLM1	ND	High	S
MM15.1	pXZ55	High	High	S
MM15.2	pXZ55	Low	Weak	S
MM15.3	pXZ55	Low	Weak	S
MM15.4	pXZ55	High	High	S
MM16.1	pXZ56	High	High	S
MM16.2	pXZ56	Low	Weak	S
MM16.3	pXZ56	Low	Weak	S
MM16.4	pXZ56	High	High	S
MM17.1	pXZ57	Low	Weak	S
MM17.2	pXZ57	High	High	S
MM17.3	pXZ57	Low	Weak	S
MM17.4	pXZ57	Low	Weak	S
MM17.5	pXZ57	High	High	S
KM7.1	pKM8	Low	None	ND
KM7.2	pKM8	Low	Weak	NS
KM7.3	pKM8	Low	None	NS
KM7.4	pKM8	Low	None	ND
KM7.5	pKM8	Low	None	NS
KM7.6	pKM8	Low	None	NS
KM7.7	pKM8	Low	None	NS
KM7.8	pKM8	High	None	NS
KM7.9	pKM8	Low	Weak	S
KM7.10	pKM8	Low	Weak	WS
KM8.1	pKM9	Low	Weak	NS
KM8.2	pKM9	Low	None	NS
KM8.3	pKM9	Low	Weak	ND
KM8.4	pKM9	Low	None	ND
KM8.5	pKM9	Low	Weak	ND
KM8.6	pKM9	Low	None	ND
KM8.7	pKM9	Low	None	NS
KM8.8	pKM9	Low	None	NS
KM8.9	pKM9	Low	Weak	ND
KM8.10	pKM9	Low	None	ND

^a ND = not done.

^b Indicates level of copy number: high ≥ 10 copies and low ≤ 4 copies.

^c Indicates level of fluorescence observed in culture: high = high level of fluorescence, weak = weak level of fluorescence, and none = no fluorescence detected

^d Indicates whether or not histochemical staining of seedlings was observed: S = staining observed, WS = weak staining observed, and NS = no staining observed.

plants were analyzed for *gusA* expression in culture using a fluorescence (MUG [methylumbelliferyl- β -glucuronide]) assay. For most of the transformants, fluorescence was either not detected or very weak (Table 1; Supplemental Figure S2). However, the high copy number transformants showed consistently higher levels of fluorescence compared with the positive control, KM1.1 (Table 1). Wild-type *E. festucae* consistently did not fluoresce. To determine the minimum promoter length required for *gusA* expression in planta, *L. perenne* seedlings were inoculated with a subset of the transformants (Table 1). A histochemical assay was used to analyze *gusA* expression. Much lower β -glucuronidase (GUS) activity was observed for the transformants containing the 280- and 480-bp promoter fragments in planta compared with those with the larger promoter fragments (Fig. 2B; Table 1). This suggests that an element between 480 and 782 bp upstream of the *ltmM* translational start site was required for increased levels of *ltmM* expression in planta.

A 5' rapid amplification of cDNA ends (RACE) analysis was used to determine where the transcription start sites of the *ltmM* gene were located using mRNA isolated from *L. perenne* infected with wild-type *E. festucae*. Multiple transcription start sites were identified that clustered at two different locations in the 5' untranslated region of the promoter (Supplemental Figure S3). Approximately 30 bases upstream of region 1, a putative TATA-like element (TLE) was found in the *ltmM* promoter; however, a TLE was not found near the other region. Sequences that matched the mammalian initiator (Inr) sequence were found near both regions (Javahery et al. 1994; Lo and Smale

1996). Upstream of the TLE, the eukaryotic TFIIB recognition sequence, CGCC (Lagrange et al. 1998), was found, suggesting that the putative core *ltmM* promoter consists of the first 167 bases upstream of the *ltmM* translational start site.

Based on the Southern and *gusA* expression analysis, the transformants containing the 782-bp (MM15.2 and MM15.3), 1.3-kb (MM16.2 and MM16.3), and 1.6-kb (MM17.1, MM17.3 and MM17.4) promoter fusions (Table 1) were used for further analysis of *gusA* expression patterns in mature perennial ryegrass plants.

Analysis of *ltmM* gene expression patterns in vegetative tillers.

Preliminary analysis using fluorescence assays was performed to examine the patterns of *PltmM-gusA* activity in hyphae present in infected vegetative tillers. Fluorescence assays using the leaf sheath and leaf blade of each individual leaf of the tiller suggested that there was GUS activity in all these tissues (data not shown). Histochemical assays indicated that GUS activity occurred in the leaf sheath and blade of all the infected tillers (Fig. 3A through C and D through F). GUS activity also was detected in epiphyllous hyphae on the surface of the leaf sheath (Fig. 3B and E). Faint histochemical staining indicative of low GUS activity was observed in the true stem and meristematic tissues, including the stem apex, intercalary meristem, and axillary buds of vegetative tillers (Supplemental Figure S4). These results indicate the *ltmM* promoter is expressed in all aerial parts of the infected vegetative tillers.

Fig. 3. Analysis of β -glucuronidase (GUS) activity patterns of *Epichloë festucae* hyphae in vegetative tillers of perennial ryegrass using bright field microscopy ($\times 400$ magnification). These photographs are of whole-mount GUS-stained perennial ryegrass leaf tissue infected with *E. festucae* hyphae. The GUS staining is indicated by the blue precipitate in the hyphae. **A through C**, In planta GUS activity patterns conferred by the $-1,307$ to $+1$ *PltmM* sequence (transformant MM16.3). **D through F**, In planta reporter gene activity patterns of the -782 to $+1$ *PltmM* sequence (transformant MM17.1). **A and D**, GUS activity in hyphae growing in the leaf sheath of vegetative tillers. **B and E**, GUS activity in epiphyllous hyphae (Eh) growing on the leaf sheath surface. **C and F**, GUS activity in hyphae growing in the leaf blade of vegetative tillers. Arrows point to where epiphyllous hyphae emerge from leaf surface. Scale bars are 500 μ m in length.

The *gusA* expression patterns of the positive control, KM1.1, were similar to the *PltmM-gusA* transformants, confirming that *ltmM* is expressed at all times in infected vegetative tillers (Supplemental Figure S5). However, the levels of

staining in the true stem and meristematic tissues were more intense in the positive control than in the *PltmM-gusA* transformants. No GUS activity was observed in tissues containing wild-type *E. festucae*.

Fig. 4. β -Glucuronidase (GUS) activity patterns in perennial ryegrass spikelets at different developmental stages. Plants were infected with *Epichloë festucae* transformants containing the $-1,307$ to $+1$ *PltmM-gusA* construct (transformant MM16.3). **A**, GUS activity in whole-mount GUS-stained spikelets. 1 and 2, Activity patterns in pre-anthesis spikelets with staining observed in the glume (G), lemma (L), palea (P), anthers (A), and rachilla (R) but not in the gynoecium (Gy). 3 and 4, Activity patterns in post-anthesis spikelets with staining observed in the gynoecium (Gy) and the stigma (St) but not in the lemma (L) or palea (P). 5 and 6, Activity patterns in post-anthesis spikelets just prior to seed drop, with staining observed in the disintegrating stigma (St) and at the base of the seed (Sd). 1, 3, and 5, *gusA* expression patterns in spikelets; and 2, 4, and 6, *gusA* expression patterns in individual florets using dark-field microscopy. **B**, GUS activity patterns in longitudinal sections of individual florets using bright-field microscopy ($\times 200$ magnification). 1, Longitudinal sections through a pre-anthesis floret showing GUS staining in the ovary (O), the ovule (Ov), the outer and inner integuments (Olt and Ilt, respectively), the nucellus (N), the embryo sac (EmS), and the anthers (A). 2, Longitudinal sections through a post-anthesis spikelets showing GUS staining in the ovary (O), the ovule (Ov), the integuments (It), the embryo (Em), and the lodicules (Lo). Scale bars are $50\ \mu\text{m}$ in length.

Analysis of *ltmM* gene expression patterns in reproductive tillers.

Histochemical staining of hyphae was used to examine GUS activity patterns of the transformants in reproductive tillers of perennial ryegrass. Expression patterns were analyzed first by examining *gusA* expression in intact spikelets and second by generating sections of pre-anthesis and post-anthesis spikelets and florets.

At the pre-anthesis stage of reproductive development, GUS activity was observed largely in hyphae infecting the rachis, rachilla, glumes, lodicules, lemma, palea, and epiphyllous hyphae (Fig. 4, A1 and 2 and B1; Supplemental Figure S6). Occasionally, hyphae in anthers and filaments were observed to exhibit GUS activity (Fig. 4, A1 and 2 and B1). Infection of anthers by

E. festucae occasionally has been observed (Christensen and Voisey 2007), explaining the irregular GUS staining of anthers. No GUS activity was observed in the ovary, ovule, or stigma during pre-anthesis. Similar GUS activity patterns were observed in control plants infected with KM1.5, except the base of the ovary was colonized (Supplemental Figure S7).

In early post-anthesis spikelets, GUS activity was observed mainly in the gynoeceum. Intense staining was observed in the stigma, with sections showing densely packed hyphae where the stigma joins to the ovary (Fig. 4, A3 to 6, B2; Supplemental Figure S8). Hyphae expressing *gusA* were observed in the ovary and in the developing embryo (Fig. 4, B2). During the later stages of post-anthesis, GUS activity was observed in the disintegrating stigma and in the mature embryo (Fig. 4, A5 and

Fig. 5. β -Glucuronidase (GUS) activity patterns in germinating seed and seedlings colonized by the *Epichloë festucae* transformants. **A**, 1 and 2, GUS activity in hand-sectioned transverse sections of the embryo in germinating seed infected with the transformants containing the $-1,601$ to $+1$ *PltmM* sequence (transformant MM15.3). GUS staining observed in the embryo (Em) and the coleoptile (C) but not the endosperm (En). **B**, GUS activity in whole-mount GUS-stained germinating seed and seedlings. 1 and 4, In planta GUS activity of transformants containing the $-1,307$ to $+1$ *PltmM* sequence (transformant MM16.3); and 5 through 8, in planta GUS activity of transformants containing the -782 to $+1$ *PltmM* sequence (transformants MM17.1). 1 and 5, GUS activity in germinating seed 24 h postimbibition with stained hyphae observed in the coleoptile (C). 2 and 6, No GUS activity observed in seedlings 4 days postimbibition in any of the hyphae colonizing the mesocotyl (M) or the coleoptile (C). 3 and 7, GUS activity 6 days postimbibition with stained hyphae observed from the mesocotyl (M) to the tip of the first leaf (L). B4 and 8, GUS activity in the emerging first leaf (L).

6). In control plants, GUS activity was observed in all tissues of the spikelet (Supplemental Figure S9). No staining was observed in wild-type *E. festucae* for any tissues in either pre-anthesis or post-anthesis spikelets.

Analysis of *ltmM* gene expression in germinating seed and seedlings.

Seed was collected from endophyte-infected plants for the analysis of GUS activity patterns in seedlings. Germinating seed and seedlings were analyzed for GUS activity every 24 h over a period of 1 to 8 days postimbibition using a histochemical assay. Activity first was observed in endophyte-infected germinating seed approximately 24 h postimbibition, where staining was observed in the embryo, but not in the endosperm (Fig. 5A and B1 and 5). GUS activity also was observed between the embryo and the seed coat but not in the layer between the aleurone layer and the seed coat (Fig. 5, A1 and 2). *gusA* expression was not observed again in germinating seedlings until day 6 postgermination (Fig. 5, B2 and 6). From day 7 postgermination, GUS activity was observed consistently in infected seedlings from the mesocotyl to the tip of the emerging first leaf (Fig. 5, B3 and 4 and 7 and 8).

Based on GUS activity patterns, RNA was isolated from infected seed and seedlings 1 h, 24 h, 4 days, and 7 days postimbibition and RT-PCR was performed to analyze *ltm* transcript abundance. No *ltm* or *tubB* transcript was observed for any of the time points tested except for RNA isolated from 7-day-old seedlings (data not shown). In this case, this may be due to the very low levels of fungal RNA compared with the amount of plant RNA. Thus, there was a significant benefit in using the GUS reporter gene system in this case.

DISCUSSION

Analysis of *PltmM-gusA* transformants and *ltmM* transcription start site.

Low levels of *ltmM* expression were observed in *E. festucae* grown in a defined media. In addition, many of the *PltmM-gusA* transformants, when grown in axenic culture, showed some fluorescence in MUG assays, confirming that *ltmM* was expressed at low levels. The high copy number transformants exhibited brighter fluorescence, presumably due to higher levels of *gusA* transcript. This suggests that ectopic integration of the *PltmM-gusA* constructs into the genome does not affect expression patterns; however, increasing the number of copies of the construct does affect expression levels.

Histochemical staining of plant tissue infected with the different *PltmM-gusA* transformants showed that low levels of staining occurred with the 480- and 280-bp promoter fragments. The transformants with the larger promoter fragments exhibited higher and more intense levels of staining. These results suggest that there are promoter elements upstream of the 480-bp promoter fragment that significantly increase *ltmM* transcript abundance when *E. festucae* infects its host grass. There was always some level of *gusA* expression, either in culture or in planta, regardless of the length of the promoter deletion; therefore, the *ltmM* transcription initiation site was determined by 5' RACE. Two potential regions of initiation were identified at approximately -51 and -113 bp upstream of the *ltmM* translational start site, with a putative core promoter at -167 bp.

Analysis of *ltmM* gene expression patterns in vegetative tillers.

GUS activity patterns indicated that *gusA* was expressed in all aerial leaf tissues of *E. festucae*-infected perennial ryegrass. GUS staining was more intense in the leaf sheath and blade compared with the meristematic tissues and the true stem, where

histochemical staining was very faint. The ratio of lolitrem B to endophyte increases with leaf age, suggesting that lolitrem B is constitutively produced and has low mobility (Spiering et al. 2005). However, stem tissues, which are considered to be one of the oldest plant tissues, showed much lower lolitrem B–endophyte ratios (Spiering et al. 2005). This suggests that lolitrem B is not constitutively produced in all tissues. The observations of Spiering and associates (2005) agree with our observations of higher levels of GUS activity in leaf sheath and blade, and lower levels of expression in meristematic tissues and the true stem.

GUS activity detected in epiphyllous hyphae suggests that the fungus does not need to be inside the plant to express the *ltm* genes. It is possible that cytoplasmic streaming could move the GUS protein into epiphyllous hyphae. The fact that the hyphae were stained evenly, even though they showed extensive branching, suggests that the GUS protein is produced in these cells and not moved into the cells by cytoplasmic streaming.

Analysis of *ltmM* gene-expression patterns in reproductive tillers.

Previous work showed that *N. lolii* hyphae colonize the rachilla, glumes, lodicules, lemma, palea, filaments, ovule, and base of the ovary of pre-anthesis spikelets of perennial ryegrass (Majewska-Sawka and Nakashima 2004; Philipson and Christey 1986). Hyphae were not observed in the upper ovary, stigma, anthers, or integuments (Majewska-Sawka and Nakashima 2004). The immature female gametophyte was not colonized; however, hyphae were concentrated in the nucellus closest to the antipodal cells (Philipson and Christey 1986). Similar colonization patterns were observed for *E. festucae*, except hyphae were observed in anthers but not in the ovule. A positive correlation between hyphal distribution and *gusA* expression was observed, with the exception that no *gusA* expression was observed in the ovary in pre-anthesis florets. In these previous studies, hyphae were not observed in anthers; however, the anthers of the *E. festucae*-perennial ryegrass interaction are colonized occasionally (Christensen and Voisey 2007) and exhibited GUS activity. Although *N. lolii* is an asexual derivative of *E. festucae*, there may be some small differences in hyphal distribution between the two species in planta.

At anthesis, the lodicules swell and the floret opens, releasing the anthers and exposing the stigmas to the external environment and pollination from other ryegrass genotypes. Because hyphae have been observed at the base of the ovary, hyphae probably enter into the embryo sac via the antipodal cells soon after fertilization (Philipson and Christey 1986). Our control plants were used to determine the colonization patterns of *E. festucae* in perennial ryegrass. GUS activity was observed in all the tissues of the post-anthesis spikelets, indicating colonization of the whole spikelet. In contrast, the *PltmM-gusA* transformants exhibited *gusA* expression almost exclusively in the gynoecium after fertilization, with staining observed in the ovary, ovule, stigma, embryo, and lodicules.

These results show that there are different spatial and temporal *ltmM* expression patterns during the reproductive development of the plant. The expression of *ltmM* throughout the spikelet during pre-anthesis and in the gynoecium after fertilization would result in lolitrem B synthesis in these structures. Thus, the endophyte has developed a means for protecting the developing seed from herbivory, because the seed is its most important means for dispersal.

Analysis of *ltmM* gene expression in germinating seed and seedlings.

Hyphae in the mature embryo have been observed in the scutellum, embryonic axis, and shoot apex, but rarely in the root apex (Philipson and Christey 1986). Extensive hyphal rami-

fication occurs in the layer between the embryo and the seed coat and between the aleurone layer and the seed coat. The hyphae in this layer produce large amounts of lolitrem B prior to seed drop (Ball et al. 1997). The endosperm generally remains endophyte free. During germination, the rudimentary coleoptile is infected and retains the hyphae as it grows. The shoot apex within the coleoptile also is colonized with hyphae, which invade the leaf primordia as they arise on the apex (Philipson and Christey 1986). Progressive degradation of hyphae between the scutellum and endosperm and between the seed coat and the aleurone layer has been observed, suggesting that hyphae in these layers do not contribute to infection (Philipson and Christey 1986).

These observations provide an explanation for the spatial *gusA* expression patterns we have seen during seed germination. GUS activity in hyphae only was observed either during the very early stages of germination or after approximately 6 days postimbibition from the mesocotyl to the tip of the first leaf. Although the embryo appears to be heavily colonized by the endophyte, it appears that only hyphae found in the coleoptile and the shoot apex from the mesocotyl to the plumule contribute to seedling infection. It is difficult to explain the lack of GUS activity from 2 to 4 days postimbibition. However, it is possible that either the promoter activity at that time is too low to detect expression, or hyphae are too young to have accumulated enough GUS to observe any staining. Hyphae between the seed coat and the aleurone layer are known to contain high levels of lolitrem B produced during seed development (Ball et al. 1997), and these hyphae also may produce lolitrem B during germination. However, because no GUS activity was observed in this layer, it is possible that either hyphae in this region are not metabolically active or lolitrem B is not produced by these hyphae at germination.

Conclusion.

A detailed analysis of the expression patterns of a symbiosis-expressed, metabolite biosynthesis gene has been presented in this article. The initial premise was that the *ltm* genes were constitutively expressed in all the plant tissues where the endophyte was present. Basal expression was observed for most of the *ltm* genes in culture, and differential GUS expression patterns were observed at each stage of plant development. It is very likely that *ltm* gene expression is controlled by some plant signal. Efforts to identify endophyte genes that regulate *ltm* gene expression are currently underway.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The *Escherichia coli* strains and plasmids used in this study are listed in Supplemental Table S1. *E. coli* strains were grown on Luria-Bertani agar plates, supplemented with ampicillin (100 µg/ml) where necessary.

Fungal strains and growth conditions.

Cultures of *Epichloë festucae* F11 (ex cv. SR3000) and its derivatives were grown and maintained as described previously (Moon et al. 1999, 2000). Cultures of *E. festucae* used for RNA analysis were grown in potato dextrose media for 7 days, then washed and transferred to a defined medium containing Czapek-Dox salts (Young et al. 2005) amended with plant extracts (apoplastic fluids or a crude preparation) at a final concentration of 20%.

Plant growth conditions and inoculations.

Endophyte-free seedlings of perennial ryegrass (*L. perenne* cv. Samson or cv. Nui) were inoculated with fungal mycelia

and grown as described previously (Tanaka et al. 2005). Mature plants were tested for endophyte infection by tissue-print immunoblotting (Gwinn et al. 1991) using polyclonal rabbit antibodies raised against homogenized mycelium of *N. lolii* Lp5 (Christensen et al. 1993), in conjunction with a goat anti-rabbit secondary antibody linked to an alkaline phosphatase conjugate (Sigma, St. Louis). Endophyte infection of grasses was confirmed by light microscopic examination of epidermal strips from the outermost leaf sheath stained with aniline blue. Hyphae within epidermal strips were examined using a compound microscope at ×400 magnification.

Apoplastic fluid was isolated from both uninfected *L. perenne* cv. Nui and *E. festucae* F11-infected *L. perenne* cv. Nui. Leaf tissue was cut into approximately 0.5-cm pieces and the intercellular spaces were vacuum infiltrated with sterile water for several hours, then blotted dry. The intercellular fluid was isolated from the plant tissue via centrifugation at 3,000 × *g* for 30 min using 10-µm VectaSpin columns (Whatman International Ltd., Maidstone, England). A crude preparation was made by freezing the plant material overnight at −20°C to freeze fracture the plant cells and the cellular fragments isolated via centrifugation at 3,000 × *g* for 30 min using 10-µm VectaSpin columns. Plant extracts were stored at −20°C.

Molecular biology techniques.

Fungal genomic DNA was isolated from freeze-dried mycelia using previously described methods (Byrd et al. 1990; Möller et al. 1992). Plasmid DNA was isolated and purified by alkaline lysis using either the Quantum Prep miniprep or midiprep kits (Bio-Rad, Hercules, CA, U.S.A.) or the High Pure plasmid isolation kit (Roche, Branchburg, NJ, U.S.A.). Procedures for Southern blotting have been described previously (Young et al. 2005). Probe preparation and filter hybridization and washing have been described previously (Young et al. 2005). Standard PCR amplifications of genomic and plasmid DNA templates were performed using *Taq* DNA polymerase and carried out as previously described (Young et al. 2005). The sequences of the primers used are listed in Supplemental Table S2.

DNA sequencing.

DNA fragments were sequenced by the dideoxynucleotide chain-termination method (Sanger et al. 1977) using Big Dye (version 3) chemistry with oligonucleotide primers (Sigma Genosys, Castle Hill, Australia) with primers specific for phFunGus or genomic sequences from *E. festucae*. Products were separated on either an ABI Prism 377 sequencer (Perkin Elmer, Scoresby, Victoria, Australia) or an ABI 3730 analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

RNA isolation, cDNA synthesis, and expression analysis.

Total RNA was isolated from snap-frozen fungal mycelium, pseudostem tissue, seed, or seedlings of perennial ryegrass as described previously (Young et al. 2005). The GenElute mRNA miniprep kit (Sigma) was used to isolate poly(A) RNA from total RNA isolated from plant tissues. The poly(A) RNA then was used to determine the transcription start site or sites of *ltmM* using the GeneRacer kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, U.S.A.).

DNA was removed by incubating 1-µg samples of either total RNA or mRNA with 1 unit of DNase I (amplification grade; Invitrogen) for 15 min at room temperature. The DNase I enzyme was inactivated by adding EDTA to a final concentration of 2.5 mM and incubating at 65°C for 1 min. Samples of denatured RNA were reverse transcribed at 42°C for 45 min in a 20-µl reaction volume containing 1× Expand RT reaction buffer (Roche), 10 mM dithiothreitol, 1 mM dNTPs, 8 U of RNaseOUT RNase inhibitor (Invitrogen), and 50 U of Expand

reverse transcriptase (Roche). PCR amplifications were carried out in a 25- μ l reaction containing 10 mM Tris-HCl, 1.5 mM MgCl₂ and 50 mM KCl (pH 8.3), 50 μ M each dNTP, 200 nM each primer, and 0.5 U of *Taq* DNA polymerase (Roche). The thermocycler conditions used were one cycle at 94°C for 2 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; followed by a final step at 72°C for 5 min. For expression analysis, cDNA was amplified with the primer pairs.

Preparation of promoter deletion reporter constructs.

Five *ltmM* promoter fragments were prepared by amplifying *E. festucae* Fl1 genomic DNA with the primer ltmMNcoI and either lol68, lol57BamHI, lol92, XZlol1, or lol107BamHI primers. The amplified fragments were digested with *Bam*HI and *Nco*I and ligated into the pHFunGus vector to generate pXZ55, pXZ56, pXZ57, pKM8, and pKM9, respectively (Fig 2A). The ltmMNcoI primer contained a mismatch relative to genomic DNA to introduce an *Nco*I recognition site.

PCR amplification was done using either Expand Hi Fidelity kit (Roche) or the TripleMaster PCR System (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions. PCR amplification was performed for Expand Hi Fidelity in a 25- μ l volume containing 50 ng of *E. festucae* Fl1 genomic DNA, 1 $\times$ reaction buffer (Roche), each primer at 200 nM, each dNTP at 125 mM, and 0.875 U of Expand Hi Fidelity polymerase (Roche). For the TripleMaster system, amplification was performed in a 50- μ l volume containing 200 ng of *E. festucae* Fl1 genomic DNA. The thermocycler conditions used were one cycle at 94°C for 2 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; followed by incubation at 72°C for 5 min.

Transformation of *E. festucae* and molecular analysis of transformants.

Protoplasts of *E. festucae* were prepared as described previously (Young et al. 2005). Protoplasts were transformed with either 5 μ g of linear (pKM8 and pKM9) or circular (pLM1, pXZ55, pXZ56, and pXZ57) DNA using the method of Vollmer and Yanofsky (1986) as modified by Itoh and associates (1994). Transformants were selected on RG medium containing hygromycin (150 μ g/ml) (McMillan et al. 2003). The resulting transformants were nuclear purified by two rounds of subculturing mycelia (Young et al. 2005).

The transformants initially were screened by PCR using primers T7 and GUSR to determine that a band of the correct size occurred in all the transformants. Southern analysis of the transformants also was done to determine copy number and integration patterns.

GUS staining and microscopy.

Plants were inoculated with *E. festucae* isolates transformed with the *PltmM-gusA* reporter constructs as described. Expression of *gusA* in pseudostems from vegetative tillers was tested using a fluorometric assay (Blázquez et al. 1997). Leaf material (5 mm in length) was placed in 200 μ l of GUS assay buffer (1 mM MUG [Sigma]; 50 mM Na₂HPO₄, pH 7.0; 10 mM β -mercaptoethanol; 10 mM Na₂EDTA; 0.1% sodium lauryl sarcosine; and 0.1% Triton X-100) in a 96-well microtiter plate. The leaf material was incubated at 22°C for 16 h in the dark. Plants infected with *E. festucae* transformed with pLM1 were used as a positive control and plants infected with untransformed *E. festucae* were used as a negative control. Fluorescence was visualized with UV light using a Gel Doc EQ System (Bio-Rad).

A histochemical assay was used to examine the expression patterns of *gusA* in *E. festucae* in both vegetative and reproductive tillers and germinating seed and young seedlings

(Blázquez et al. 1997). Seed were surface sterilized and germinated on 3% water agar, and seedlings were collected every 24 h for 8 days. All plant material was prefixed in cold 90% acetone for 20 min at room temperature and then washed in GUS-staining buffer (50 mM Na₂HPO₄, pH 7.0; 2 mM K₃Fe(CN)₆; 2 mM K₄Fe(CN)₆; and 0.2% Triton X-100). The buffer was replaced with GUS-staining solution (2 mM X-GLUC [Progen Biosciences, Brisbane, Australia] in GUS-staining buffer) and the plant material was vacuum infiltrated in the solution for 20 min in the dark. The plant material then was incubated at 37°C for 16 h in the dark and later stored in 70% ethanol at -4°C. Stained leaf sheath, leaf blade, and meristem from vegetative tillers were examined using an Olympus BX51 light microscope and images were captured using the Optronics MagnaFire 2.1 Digital Camera System. Whole-mount images of inflorescences at different stages and seedlings were obtained using a Leica MZ12 Stereomicroscope and photographed using a Leica DFC320 camera and IM50 (version 4.0) software.

Sections of GUS-stained inflorescences.

GUS-stained inflorescences were fixed in 50% ethanol, 10% acetic acid, and 5% formaldehyde, then dehydrated in ethanol solutions of increasing concentrations. The inflorescences were infiltrated with Rotoclear (Carl Roth, Karlsruhe, Germany) and Rotoclear/Paraplast Extra and embedded in pure Paraplast. Tissues were cut into 10- μ m-thick sections with a Leica RM2145 microtome and serial sections were collected on to HistoBond slides (Marienfeld, Lauda-Königshofen, Germany). Sections were either stained in 0.2% eosin Y or left without any additional staining. Sections were viewed and photographed using the Olympus system described above.

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