Identification of genes regulating the plant-specific expression of the *ltmM* gene in *Epichloë festucae*

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Abstract

The fungal endophyte *Epichloë festucae* forms a largely mutualistic association with the ryegrass species *Lolium perenne*. *E. festucae* produces a range of bio-protective alkaloids that protect the host grass from herbivory by both mammals and insects. One such alkaloid, Lolitrem B, is a potent mycotoxin and the causative agent of ryegrass staggers in livestock.

Ten genes required for biosynthesis of lolitrem B are encoded in the *ltm* gene cluster. The *ltm* genes are expressed in a plant-specific manner, with high levels of expression *in planta* and very low levels of expression in culture. The mechanism regulating *ltm* gene expression is unknown but it is predicted to involve signalling from the host plant.

The *ltmM* gene was chosen for use in the investigation of *ltm* gene regulation because the flanking regions do not contain retrotransposon sequence, which surrounds much of the *ltm* gene cluster. To identify fungal genes involved in the plant-induced expression of *ltmM*, a mutagenesis and screening system was developed using a P*ltmM-gusA* 'knock-in' construct to detect expression from the *ltmM* promoter. *Agrobacterium tumefaciens*-mediated T-DNA mutagenesis was used to create a set of mutants with random insertions in the genome. Mutants were then screened for altered P*ltmM-gusA* expression, both in culture and *in planta*. Three mutants were identified with increased P*ltmM-gusA* expression in culture, however, no mutants were identified with loss of P*ltmM-gusA* expression *in planta*. This indicates that a mechanism of repression is involved in the plant-induced expression of *ltmM*, either directly or indirectly.

TM mutants of interest were also observed for altered symbiosis phenotypes. Mutants were identified with reduced colonisation rates and altered hyphal growth *in planta*. Integration sites were identified for two colonisation mutants and the disrupted genes are predicted to be the CTP:cholinephosphate cytidylyltransferase (CCT) gene *PCT1* and the mitogen-activated protein kinsase kinase (MAPKK) gene *mkk2*.

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Abbreviations

bp	base pairs
kb	kilo bases
Mb	mega bases
AM	Arbuscular mycorrhiza
ASW	Argentine stem weevil
ATMT	*Agrobacterium tumefaciens*-mediated T-DNA (mutagenesis)
CCT	CTP:cholinephosphate cytidylyltransferase
CD	Czapek Dox
DNA	Deoxyribonucleic acid
EZ	Expansion zone
GABA	Gamma aminobutyric acid
GGPP	Geranyl geranyl diphosphate
GUS	β-glucuronidase
IM	Induction media
LB (media)	Luria-Bertaini
LB	Left border
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinse
MAPKKK	MAPKK kinse
MUG	4-methylumbelliferyl β-D-glucuronide
NRPS	Non-ribosomal peptide synthase
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PD	Potato Dextrose
RB	Right border
REMI	Restrction enzyme-mediated integration
RIP	Repeat-Induced Point (mutation)
SAM	Shoot Apical Meristem
ST	Sterigmatocystin
TAIL-PCR	Thermal Assymetric Interlaced Polymerse Chain Reaction
T-DNA	Transferred DNA
WA	Water agar
X-Gluc	5-bromo-4-chloro-3-indolyl-β–D-glucuronic acid

Chapter One

Introduction

1.1 Plant-fungal symbioses

Plant-fungal associations are essential for the establishment and stability of many plant communities. Fungal symbionts can enhance nutrient uptake in their host plants and some increase host resistance to biotic and abiotic stresses. Fungi also rely on plants for survival as they obtain nutrition from organic matter and are grouped as saprophytes, parasites, or mutualists depending on their method of obtaining nutrients. Parasites and mutualists live symbiotically with their host and harvest carbon and nitrogen from the living organism, while saprophytes absorb carbon and nitrogen from dead organic matter.

Symbiosis was defined by de Bary in the nineteenth century as 'a common life' and refers to any organism living on or within another organism (Schardl et al., 2008). Symbioses can be either parasitic, mutualistic, or a combination of both. Parasitic symbioses are associations in which only the symbiont benefits and can also be pathogenic in that they cause perceptible damage to the host. Mutualism is defined as symbiosis in which the benefits outweigh the costs for both the host and the symbiont (Schardl et al., 2004). Mycorrhizae are a widespread example of mutualism, present in 70 to 90 % of land plants (Parniske, 2008). Mycorrhizal fungi infect the plant root, facilitating nutrient absorption from the soil. Aerial endophytes of the *Epichloë* and *Neotyphodium* species provide another example. In symbioses with Pooid grasses, these species produce secondary metabolites that protect the plant against herbivory and parasitism (Fletcher and Harvey, 1981; Tanaka et al., 2005) and may also increase drought tolerance and field persistence of the host (Clay and Schardl, 2002). There is a long history of symbioses and co-evolution of plants and fungi, indicating that these associations may pose a selective advantage.

1.2 Epichloë endophytes

Fungi of the family Clavicipitaceae are aerial endophytes that form symbioses with cool season grasses (family Poaceae). The most wide spread of the Clavicipitaceae fungi are the *Epichloë* species and their asexual derivatives, *Neotyphodium*, which associate

with grasses of the subfamily Pooideae (Schardl et al., 2004). For the purposes of this study, the term 'epichloë endophytes' is used to encompass both *Epichloë* and *Neotyphodium* species and the Latin names are used when referring to one genus or the other. These endophytes have been extensively studied as model systems for understanding endophyte-host interactions and signalling.

The endophyte-host interactions vary between epichloë endophytes, ranging from mutualistic to antagonistic. This depends largely on the life cycle of the endophyte as fungal reproduction strongly influences host reproduction (Schardl et al., 2004). Species that reproduce asexually (by vertical transmission) are mutualistic and are classified separately in the genus *Neotyphodium* (previously *Acremonium*) (Glenn et al., 1996). These species infect the intercellular spaces of the host embryo and are dispersed within the seed (Philipson and Christey, 1986). Upon germination, hyphae from the intercellular spaces colonise leaf primordia and axial buds in preparation to colonise new tillers as they arise (Schmid et al., 2000; Tan et al., 2001; Christensen et al., 2002). Conversely, species that reproduce sexually (by horizontal transmission) repress seed formation on the infected grass tiller. Fungal mycelia proliferate in the inflorescence primordia and the leaf sheath surrounding the inflorescence, thus preventing the inflorescence from emerging (Bultman et al., 1995). The mycelia form a fungal stroma, which produces conidia (asexual spores). This is often termed 'choke disease', as stroma-forming *Epichloë* species effectively choke the inflorescence. The fungus then relies on flies of the genus *Botanophila* to disseminate conidia of the opposite mating type to the stroma (Bultman et al., 1995). The sexual species are classified in the genus *Epichloë* (Glenn et al., 1996) but these species, to varying degrees, are also capable of asexual reproduction (Schardl et al., 2004). Therefore, the nature of individual symbioses depends on how dominant the sexual cycle is in the lifecycle of the fungus.

1.2.1 *Epichloë festucae*

Epichloë festucae was first observed, but not defined, in the 1930s (Sampson, 1933). An unknown endophyte (now known to be *Epichloë festucae*) was compared with the choke disease fungus, *Epichloë typhina*. It was established that *E. festucae* could exist separately in its sexual or asexual cycle on different tillers of the same plant. This was novel at the time, but later appeared to be true for most *Epichloë* species (Schardl et al.,

2004). The pleiotrophic nature of *E. festucae* provided an evolutionary link previously missing between sexual and asexual epichloë endophytes.

Until 1993, all *Epichloë* species colonising Pooid grasses remained characterised as *Epichloë typhina*, an antagonistic species of *Epichloë* that infects a broad range of hosts. Since then, nine other *Epichloë* species have been described that show more strict host specificity than *E. typhina* (Schardl et al., 2004). *Epichloë festucae* was characterised when a distinct mating population was identified in *Festuca* host grasses (Leuchtmann et al., 1994). Results of mating tests were correlated with morphological and cultural characteristics to define the novel species, which the authors named *Epichloë festucae* after the *Festuca* host plant.

1.2.2 The *E. festucae* genome

E. festucae is a haploid organism with a genome size of approximately 30 Mb, comprised of four chromosomes (Kuldau et al., 1999). The *E. festucae* genome (strain E2368) was sequenced in 2007 (Schardl et al., 2008), making it the first epichloë endophyte genome to be sequenced. *Neotyphodium* endophytes are interspecific hybrids of up to three epichloë endophytes, including other *Neotyphodium* species. Thus, *Neotyphodium* genomes tend to be larger (up to 63 Mb) and are generally heteroploid (Kuldau et al., 1999). *Neotyphodium lolii* is an exception, having a haploid genome, and is the closest relative of *E. festucae*.

1.2.3 *E. festucae* growth *in planta*

E. festucae exhibits strictly controlled cellular growth throughout the host. For most of its lifecycle, the endophyte inhabits the intercellular spaces of the plant without breaching host cells or developing feeding structures. There is no evidence that hyphae secrete pectic enzymes to loosen the middle lamella during penetration of dense tissues, instead it appears that the hyphae physically push between cells (Christensen et al., 2002). This is possibly an explanation as to why the fungus does not trigger a host defence response. The growth of sexual *Epichloë* species only differs from asexual *Neotyphodium* in reproductive plant tissue, however, the sexual cycle was seldom observed in experiments by Christensen et al. (2002), indicating vertical transmission is dominant in the *E. festucae* lifecycle.

Hyphae of the fungus originate from dormant mycelia in the shoot apical meristem (SAM), the part of the plant that forms new leaves, tillers, and inflorescences (Schmid et al., 2000). Upon seed germination, hyphae grow from the SAM into leaf primordia and axial buds forming a multi-branched mycelial mass between the newly dividing plant cells (Philipson and Christey, 1986). The plant cells then move into the expansion zone (EZ) of the leaf where they expand dramatically while being displaced by newer cells developing below. Accordingly, hyphal growth is coordinated with the growth of the leaf, with hyphae extending as the leaf extends and stopping when the leaf ceases elongation (Christensen, 1995; Tan et al., 2001). The hyphae grow in parallel with the axis of the leaf and are largely unbranched in the leaf sheaths and blades of tillers. Christensen et al. (2002) observed that the hyphae at the apical end of the leaf are older than those found at the base and suggested intercalary growth, rarely seen in filamentous fungi, as a mechanism for hyphal extension. Furthermore, Christensen et al. (2008) observed that hyphae are attached to plant cell walls and that hyphal branches are maintained throughout the EZ, which is inconsistent with a mechanism of apical growth. When the host leaf reaches maturity (extension ceases) the hyphae maintain a non-intrusive existence with no further hyphal growth. Conversely, metabolic activity is maintained at a high level in mature leaves and throughout the life of the plant (Tan et al., 2001). This is contrary to results from epichloë endophytes grown in liquid culture, which exhibit reduced reporter gene activity when hyphal elongation has stopped (Tan et al., 2001). This indicates an uncoupling of hyphal growth and metabolism *in planta*, which may be important for the symbiosis, particularly in the switch to production of secondary metabolites.

There is no detectable host response to endophyte colonisation in host-specific symbioses but when transferred to alternative hosts epichloë endophytes display genotype-specific incompatibility (Koga et al., 1993; Christensen et al., 2002). These responses result in a range of phenotypes in the host, including increased seedling mortality, stunted growth, and cell death at the stem apex (Koga et al., 1993; Christensen et al., 2002). Premature death of fungal hyphae is also observed. The equilibrium between a genotype-specific compatible and incompatible relationship illustrates the delicate balance between host and endophyte metabolic and cellular processes (Scott, 2001).

1.2.4 The benefits of mutualsim

The association of *E. festucae* with perennial ryegrass provides multiple benefits for both host and symbiont. The fungus gains nutrients and a means of dissemination via the host seed. The plant acquires drought tolerance and greater field persistence, but the most prominent advantage is the protection provided by alkaloids produced by the fungus. These alkaloids protect the host plant from herbivory by mammals and insects, and from parasitism by some bacteria and fungi. Consequently, these alkaloids are termed bio-protective molecules (Scott, 2001).

1.3 Bio-protective molecules

Bio-protective molecules increase host plant fitness by protecting against biotic stresses. Collectively, epichloë endophytes produce bio-protective molecules classified into four alkaloid groups: peramine, lolines, ergot alkaloids, and indole-diterpenes (reviewed in Fleetwood et al., 2008). Alkaloid profiles vary between individual *Epichloë* species, but many make at least one of these bio-protective molecules. *Neotyphodium* species generally have a larger alkaloid profile with many producing more than one alkaloid. *E. festucae* is unique amongst the *Epichloë* in that it can potentially produce all four groups of alkaloids (Siegel et al., 1990; Clay and Schardl, 2002).

1.3.1 Peramine

Peramine is an insect feeding deterrent produced by epichloë endophytes in a symbiosis-specific manner (Tanaka et al., 2005). This alkaloid is particularly beneficial in an agricultural sense, because it protects pastures from herbivory by the Argentine Stem Weevil (ASW), a potentially devastating pest (Rowan and Gaynor, 1986; Tanaka et al., 2005). Tanaka et al., (2005) identified *perA*, a non-ribosomal peptide synthetase (NRPS), required for peramine synthesis in *N. lolii*. The *perA* gene is preferentially expressed *in planta,* however, it is not known what regulates expression.

1.3.2 Lolines

Lolines are potent insecticides and feeding deterrents, which accumulate to high levels *in planta* (Spiering et al., 2002). These alkaloids are generally produced in a plant-

specific manner and are not detected in complex medium cultures. However, the fungal origin of lolines was confirmed by detection of the alkaloids in axenic cultures of *Neotyphodium uncinatum* grown in defined minimal medium (Blankenship et al., 2001). A single locus that segregated for loline production, designated *LOL*, was identified in *E. festucae* (Wilkinson et al., 2000). Subsequently, the genes potentially involved in loline biosynthesis were found in *N. uncinatum* (Spiering et al., 2002; Spiering et al., 2005). The genes at the *LOL* locus are organised in two almost identical clusters, each containing nine genes. RNA interference was used to confirm that one of the genes, *lolC*, is required for loline synthesis (Spiering et al., 2005). It is not known what mechanism regulates plant-induced expression of these alkaloids.

1.3.3 Ergot alkaloids

The ergot alkaloid group consists of clavines, lysergic acid amides, and ergopeptines. Ergovaline is a prominent ergopeptine implicated in the potentially fatal condition fescue toxicosis in livestock (Bacon, 1995) and in feeding deterrence of the African black beetle (Ball et al., 1997). The first gene required in the ergovaline biosythesis pathway, *dmaW*, was identified in the pathogenic fungi *Claviceps fusiformis* and *Claviceps purpurea* (Wang et al., 2004). The final precursor to ergovaline, lysergyl peptide lactam, is formed in a reaction catalysed by two NRPSs. One of these NRPSs, *lpsA*, was identified by Panaccione et al., (2001) in the *N. lolii* strain Lp1. The second NRPS was found to include one of the NRPS fragments cloned in the *perA* study by Tanaka et al. (2005) (Fleetwood et al., 2007). Deletion confirmed the role of this gene, designated *lpsB*, in ergovaline synthesis. Deletion mutants did not produce ergovaline or ergot alkaloids of the lysergic acid amide group, instead there was accumulation of the ergovaline precursor lysergic acid and of other intermediates of the clavine group (Fleetwood et al., 2007). Surprisingly, infection of grasses with the Δ*lpsB* mutant still caused a feeding deterrent effect on the African black beetle. This suggests that other intermediates, or other unidentified alkaloids, are responsible for black beetle feeding deterrence (Fleetwood et al., 2007). Ergolvaline is only detectable *in planta*, not in culture, but it has not yet been confirmed whether the *dmaW*, *lpsA*, and *lpsB* genes are expressed in a plant-specific manner (Fleetwood et al., 2007).

1.3.4 Indole-diterpenes

Indole-diterpenes are a large group of secondary metabolites found in filamentous fungi of the genera *Penicillium*, *Aspergillus*, *Claviceps*, and *Epichloë/Neotyphodium*. Indole-diterpenes are grouped into four structural classes, penitrems, janthitrems, paspalitrems and lolitrems. The compounds share a common core structure comprised of an indole-diterpene ring, derived from a geranylgeranyl diphosphate (GGPP) and an indole moiety (Parker and Scott, 2004). Diversity among indole-diterpenes is due to different modifications and ring stereochemistry. Four fungal gene clusters for cyclic diterpene biosynthesis have been identified and all contain a gene for GGPP synthase (Young et al., 2005). In addition, the organisms each have a second GGPP synthase gene indicating that two GGPP synthase genes may be a molecular signature for indole-diterpene biosynthesis (Young et al., 2005). Examples of indole-diterpenes include lolitrems, paspaline and paxilline, all of which are potent mycotoxins (mammalian toxins of fungal origin). Their toxic properties arise from their ability to inhibit high-conductance Ca^{2+}-activated K^{+} (maxi-K) channels and modulate the γ-aminobutyric acid-gated ion channel (Young et al., 2001).

1.3.4.1 Paxilline

Paxilline is constitutively produced by *Penicillium paxilli* and is a proposed intermediate of other indole-diterpenes (Young et al., 2001). This means that the biochemistry of paxilline biosynthesis can be applied to other indole-diterpenes to gain insight into uncharacterised pathways. There are seven genes in the *pax* gene cluster required for paxilline production including: a GGPP synthase (*paxG*), a prenyl transferase (*paxC*), an FAD-depedent monooxygenases (*paxM*), two cytochrome P450 monooxygenases (*paxP and paxQ*), and two putative membrane-associated proteins (*paxA* and *paxB*) (Young et al., 2001). The second GGPP synthase, *ggs1*, is involved in primary metabolism. Paspaline is key intermediate for the synthesis of indole-diterpenes and is a precursor to paxilline. Four *pax* genes, *paxG, paxM, paxB* and *paxC,* are needed for paspaline production (Saikia et al., 2006). Deletion of *paxP* and *paxQ* confirmed that they act later in the paxilline biosynthetic pathway (McMillan et al., 2003).

1.3.4.2 Lolitrem B

Lolitrems are synthesised by *E. festucae* and its asexual derivatives in association with grasses (Young et al., 2005). However, the sequenced *E. festucae* strain, E2368, does not produce lolitrems (Schardl et al., 2008). Lolitrem B is a mammalian mycotoxin and the causative agent of ryegrass staggers in livestock. There are ten *ltm* genes required for lolitrem B biosynthesis, which are organised in three mini-clusters that make up the *ltm* gene cluster. Cluster 1 contains three genes *ltmG, ltmM,* and *ltmK* (Young et al., 2005), cluster 2 contains five genes, *ltmP, ltmQ, ltmF, ltmC* and *ltmB,* and cluster 3 contains two genes, *ltmE* and *ltmJ* (Young et al., 2006). Four genes, *ltmE, ltmF, ltmJ,* and *ltmK,* are unique to lolitrem B biosynthesis, while the remaining six genes appear to be orthologs of *pax* genes. The *ltm* genes are preferentially expressed *in planta*, indicating co-regulation of gene expression, although the mechanism of regulation is not known (Young et al., 2006). Complementation analyses provided evidence that paxilline and lolitrem B arise from similar pathways. Deletion of *ltmM* results in loss of detectable lolitrem expression, and its role in lolitrem synthesis was confirmed by complementation with wildtype *ltmM* and the *P. paxilli* homologue, *paxM* (Young et al., 2005). In addition, *paxC*- and *paxM*- deficient *P. paxilli* strains were successfully complemented with the *ltmC* and *ltmM* genes, respectively, under the control of the *paxM* promoter (Young et al., 2006).

The LTM locus is located in the sub-telomeric region of the chromosome (Young et al., 2006), a rapidly evolving region that has been shown to contain multiple secondary metabolite gene clusters in the pathogenic fungi *Aspergillus fumigatus* and *Aspergillus oryzae* (Machida et al., 2005; Nierman et al., 2005). The locus is surrounded by AT-rich retrotransposon DNA, indicating it has undergone extensive repeat induced point (RIP) mutation (Young et al., 2005). Retro elements are present adjacent to cluster 1 and 2, and two imperfect direct repeats are present between cluster 2 and 3 (Young et al., 2006). The LTM locus is very similar in *N. lolii* and *E. festucae* except that *E. festucae* lacks the retrotransposon platform on the right flank of cluster 1. The locus appears to be shaped by recombination, RIP mutation, and mutations associated with type I transposon elements. Remnants of other transposable elements are present at the locus, including elements in the 5' upstream regions of *ltmG, ltmK, ltmM, ltmE, ltmP,* and *ltmF*. Retrotransposon sequence promotes heterochromatin formation, which raises

the possibility of chromatin remodelling as a mechanism for *ltm* gene regulation (Young et al., 2006).

1.3.4.3 The *ltmM* gene

The *ltmM* gene is known to be expressed in all vegetative tissues of infected plants and in epiphyllous hyphae (May et al., 2008). May et al. (2008) observed the spatial and temporal expression of the *ltmM* promoter in reproductive tissue using the *gusA* reporter gene. In the pre-anthesis floret, P*ltmM-gusA* is expressed in all reproductive tissues except the gynoecium. Conversely, in the post-anthesis floret, expression is solely in the gynoecium. Promoter analysis revealed that elements further than 480 bp upstream of *ltmM* are required for the high levels of expression observed *in planta* (May et al., 2008).

1.4 Regulation of plant-induced genes

In order to maintain mutualistic symbioses, or to inhibit antagonistic ones, communication or signalling occurs between the partners in symbiotic associations. There is currently much research into the role of plant signals in the regulation of fungal genes. No one symbiosis has been completely characterised but comparisons can be drawn between findings on many different symbiotic relationships, both mutualistic and pathogenic.

In the maize pathogen, *Ustilago maydis*, motifs required for plant-induced expression were identified in promoters of some of the *mig2* genes. The *mig2* gene cluster contains five highly homologous genes, denoted *mig2-1* to *mig2-5* (Basse et al., 2002), while a sixth *mig2* gene, *mig2-6*, exists at another location (Farfsing et al., 2005). These genes are not essential for pathogenicity. The *mig2* genes are differentially expressed *in planta* with the *mig2-6* gene having the strongest differential expression profile. Several 5'-CCA-3' motifs are present in a 350 bp section of the *mig2-5* promoter and are required for gene induction *in planta* (Farfsing et al., 2005). The *mig2-6* gene also has an over-representation of the consensus sequence 5'-MNMNWNCCAMM-3'. Substitutions and deletions in the *mig2-5* promoter do not induce activity in culture but

have varying effects on induction *in planta*, suggesting a mechanism of gene activation *in planta* over a mechanism of repression in culture (Farfsing et al., 2005).

The symbiosis between the fungal pathogen, *Cladosporium fulvum*, and the tomato plant, *Lycopersicon esculentum* is a well studied pathogenic association. The *Avr9* gene is an avirulence gene that is specifically expressed *in planta* (van Kan et al., 1991), but can also be induced in culture under nitrogen-starved conditions (van den Ackerveken et al., 1994). However, this requires the presence of the *NFR1* gene, a homolog of *AreA* (*Aspergillus nidulans*) and *Nit2* (*Neurospora crassa*) (Pérez-Garcia et al., 2001). AREA has previously been shown to regulate plant-induced expression of *Avr9* in *A. nidulans* (Snoeijers et al., 1999). AREA and NIT2 are members of the GATA family of DNA-binding proteins and are required for the uptake and utilisation of many nitrogen sources when primary nitrogen sources are limiting (Caddick et al., 1994; Marzluf, 1997). These proteins bind to specific motifs in the promoters of nitrogen-regulated genes. Each motif contains a TAGATA consensus sequence, a GATA core sequence, or their complement. Altering the number, orientation, or location of the (TA)GATA motifs allows for differential expression of the target genes. The *C. fulvum Avr9* promoter contains 12 (TA)GATA sequences in the 0.6 kb region upstream of the transcriptional start site (van den Ackerveken et al., 1994; Snoeijers et al., 1999). Mutational analysis of the 0.6 kb sequence identified two regions required for *Avr9* promoter activity, each region contained two TAGATA boxes in inverted orientation with a two base pair overlap (Snoeijers et al., 2003). Other regions of the promoter had little effect on inducibility. These results suggest that the nitrogen-control circuit is conserved in *A. nidulans* and *C. fulvum* and exemplifies the role of nutritional regulators in plant-specific gene expression.

The *C. fulvum* gene *Gat1* is also up-regulated during symbiosis. *Gat1* encodes a GABA transaminase and is required for the metabolism of the non-protein amino acid γ–aminobutyric acid (GABA) (Solomon and Oliver, 2002). GABA is present at a high concentration in the apoplast of the host plant during compatible symbiotic associations (Solomon and Oliver, 2001). *Gat1* is also induced in culture by addition of GABA or glutamate to growth media (Solomon and Oliver, 2002). This suggests a mechanism whereby the fungus induces GABA production in the host plant, which in turn triggers up-regulation of *Gat1* in the fungus.

Appressoria are formed by a large proportion of pathogenic fungi and are necessary as the mode of infection for these species. Regulation of the process is linked to physiology of both the fungus and the host plant and is often triggered by plant-produced factors. In *Magnaporthe grisea*, a pathogen of the rice plant, two genes involved in nitrogen metabolism are required for the expression of the plant-induced gene *MPG1* (Lau and Hamer, 1996). The *MPG1* gene is up-regulated 60-fold at a very early stage in plant infection, coinciding with appressoria formation (Talbot et al., 1993). In addition, Mpg1 mutants have a reduced ability to form appressoria, resulting in reduced pathogenicity. The gene is also induced in culture under carbon or nitrogen starved conditions (Talbot et al., 1993). The two regulatory genes, *NPR1* and *NPR2*, are *trans*-acting effectors of genes involved in pathogenicity and nitrogen utilisation (Lau and Hamer, 1996), providing a further link between nutritional regulators and plant-specific gene expression.

Puccinia graminis is another appressoria-forming symbiont that causes the pathogenic association known as wheat stem rust. Appressoria formation in *P. graminis* can be induced in culture by addition of two plant-produced alcohols, *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol (Grambow and Riedel, 1977). This indicates that expression of genes involved in appressoria formation is induced in response to perception of these alcohols. However, the greatest number of appresoria were formed when a combination of chemical (*trans*-2-hexen-1-ol) and topographical (stomata-shaped structure) signals were used (Collins et al., 2001). This suggests that there is cross-talk between the two signal transduction pathways to mediate pathogenicity.

Nectria haematococca is a pathogen of the pea plant and one of two *pel* genes required for pathogenicity, *pelD*, is only expressed *in planta*. Homoserine and asparagine were identified as the *pelD*-inducing signals (Yang et al., 2005). These amino acids are found at high levels in pea seedlings, thus it appears that the fungus has evolved a mechanism to sense the host environment by recognition of these compounds. Again this exemplifies a role for nutrition in plant-induced gene expression.

There are also signalling mechanisms to maintain beneficial associations. Arbuscular mycorrhizae (AM) are the most widespread of all mycorrhizae. AM exist between

fungi of the phylum Glomeromycota and, what is thought to be, 70 to 90 % of land plant species (Parniske, 2008). Recent studies in the AM fungus *Gigaspora margarita* have identified root factors that induce the 'presymbiotic stage' of the fungus. Plant hormones called strigolactones induce hyphal branching (Akiyama et al., 2005) and changes in physiology and metabolism (Besserer et al., 2006). This is consistent with induction of the 'presymbiotic stage' of the fungus, which is characterised by hyper-branching of hyphae and increase physiological activity (Parniske, 2008). Strigolactones are sesquiterpene lactones and have been identified as novel plant hormones in many angiosperms, including *Arabidopsis thaliana*, pea and rice (Gomez-Roldan et al., 2008; Parniske, 2008; Umehara et al., 2008).

There are also fungal signalling molecules, collectively called Myc factors, which have a symbiosis-specific effect on the host plant. The existence of Myc factors was apparent from experiments by Kosuta et al., (2003). The AM fungi *Gigaspora rosea*, *Gigaspora gigantea*, *Gigaspora margarita*, and *Glomus intraradices* induced activity of the *gusA* reporter gene under the control of the *Medicago truncatula* symbiosis-responsive promoter *ENODII* without direct contact with the host. The diffusable Myc factor was identified as a transcriptional activator of symbiosis-related genes (Kosuta et al., 2003).

Based on current knowledge it appears that most regulatory mechanisms for plant-induced gene expression involve the activation of expression *in planta*, not repression of expression in culture. Furthermore, many fungal regulators involved in plant-induced gene expression are also involved in nutritional functions, particularly nitrogen utilisation. These studies provide a comprehensive background in host plant signalling and mechanisms of plant-specific regulation of fungal genes.

1.5 *Agrobacterium*-mediated T-DNA mutagenesis

Agrobacterium tumefaciens-mediated T-DNA mutagenesis (ATMT mutagenesis) involves insertion of a DNA fragment (transferred DNA or T-DNA) into the genome of a target organism. *A. tumefaciens* is naturally a pathogen of plants and has traditionally been used for mutagenesis in species such as *Arabidopsis*, however, the technique has been adapted as a mutagenesis tool for fungi (Bundock et al., 1995; de Groot et al., 1998). T-DNA transfer is induced following perception of the plant wound signal acetosyringone, a phenolic compound that triggers activity of the *Agrobacterium* virulence genes on the Ti plasmid. Vir proteins excise the T-DNA fragment from the binary vector and facilitate its transfer to the target organism. The T-DNA fragment then integrates randomly into the genome (Fig. 1.1).

Prior to the development of ATMT mutagenesis in fungi there were two other methods of insertional mutagenesis, restriction enzyme-mediated integration (REMI), and transposon mutagenesis. REMI (Schiestl and Petes, 1991) involves the transformation of fungal protoplasts with linearised plasmid together with a small quantity of the restriction enzyme used for linearisation. The restriction enzyme generates breaks in the genome, which are subsequently repaired with random incorporation of the plasmid. REMI often produces multiple-copy or multiple-site integrations as well as untagged deletions, all of which add to the complexity of linking a phenotype to the causative mutation. It can also cause large deletions of genomic sequence, which makes it more difficult to rescue flanking sequence and confirm the location of insertion. The second method refers to the utilisation of class II (DNA) transposons already present in the fungal genome that excise and reinsert at new sites (Kempken, 1999; Villalba et al., 2001). Large chromosomal rearrangement can result from transposon mutagenesis and integration appears to be biased for non-coding regions.

ATMT mutagenesis overcomes many of the issues associated with these methods. The previous methods can only be performed on protoplasts, whereas ATMT mutagenesis can be performed on protoplasts, mycelium, spores, and fruiting body tissue (Michielse et al., 2005). Numerous studies have shown a high proportion of single copy insertions

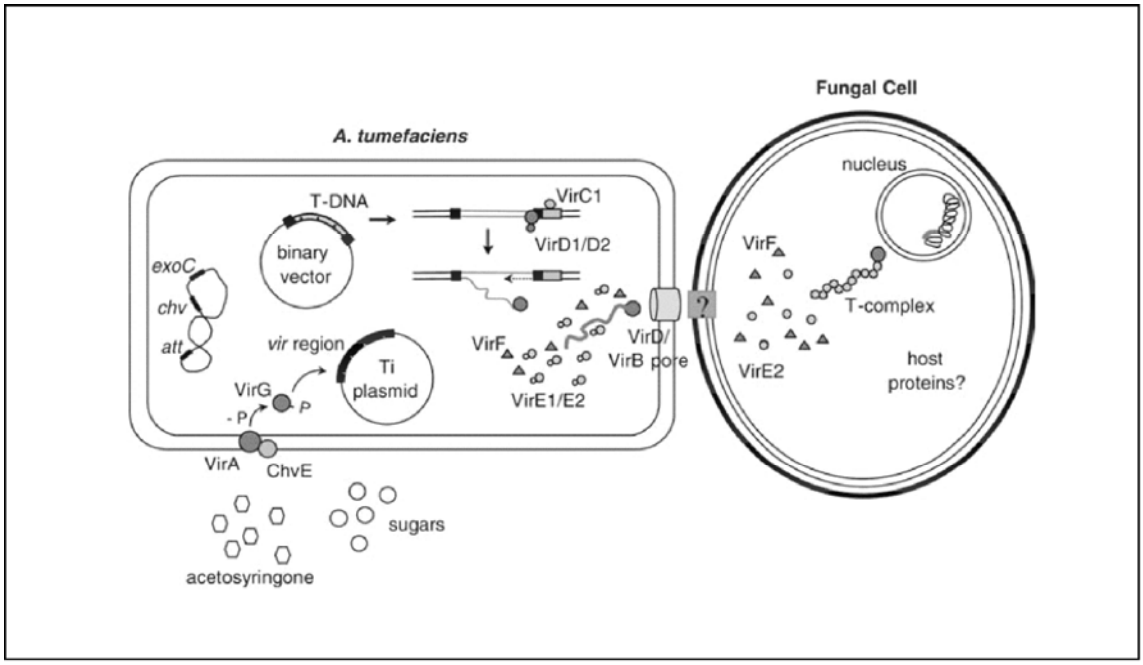

Figure 1.1 Schematic diagram of *Agrobacterium*-mediated T-DNA mutagenesis (from Michielse *et al*., 2005).

The presence of acetosyringone triggers expression of the *A. tumefacians* vir genes on the Ti plasmid. Vir proteins excise the T-DNA fragment from the binary vector and facilitate the transfer of the T-DNA to the fungal cell. Vir proteins then associate with the T-DNA to form the T-complex and the T-DNA is inserted into the host genome.

resulting from ATMT mutagenesis in filamentous fungi (Michielse et al., 2005; Betts et al., 2007; Blaise et al., 2007; Choi et al., 2007; Meng et al., 2007). Tanaka et al. (2007) reported 63 % single copy integration for ATMT mutagenesis in *E. festucae*. T-DNA also integrates relatively randomly. However, recent studies indicate that insertion of T-DNA is not completely random, having a bias towards intergenic sequence of gene-rich regions, particularly promoters (Michielse et al., 2005; Walton et al., 2005; Blaise et al., 2007; Choi et al., 2007; Meng et al., 2007). T-DNA borders are generally well preserved in ATMT transformants and deletions of flanking genomic sequence are usually small (Blaise et al., 2007; Meng et al., 2007). The major limitation of this method is that the proportion of tagged deletions appears to be only approximately 50 % (Walton et al., 2005; Blaise et al., 2007). Choi et al., (2007) reported 1110 T-DNA tagged loci out of a subset of 2026 flanking sequences in *M. oryzae*.

1.6 P*ltmM-gusA* transformants

The *ltmM* gene was chosen for use in the investigation of *ltm* gene regulation because the flanking regions do not contain retrotransposon sequence, which surrounds much of the *ltm* gene cluster (Young et al., 2006). Due to the difficulty of detecting the *ltmM* gene product, a reporter gene was used to study *ltmM* expression. A construct was made containing the *gusA* reporter gene under the control of the endogenous *ltmM* promoter (Appendix 5.2; May, unpublished data). The *gusA* gene flanked by 5′ and 3′ flanking sequence from the native *ltmM* gene was cloned into the previously constructed 'knock-out' vector pCY39 (Young et al., 2005) to produce the vector pKM6 (May, unpublished data). pKM6 was used to transform *E. festucae* wildtype strain, Fl1. Transformant colonies were purified and screened by PCR. A set of transformants was obtained including a combination of 'ectopics' (non-homologously integrated P*ltmM-gusA*) KM2.1, KM2.60, and KM2.79, and 'knock-ins' (homologous replacement of the native *ltmM* gene with P*ltmM-gusA*) KM2.34, KM2.41, KM2.47, KM2.49, and KM2.83. Another *E. festucae* strain, KM1.1, was produced as a positive control for *gusA* expression using the constitutively active glyceraldehyde-3-phosphate dehydrogenase promoter (P*gpd*) from *Aspergillus nidulans*. The P*gpd-gusA* construct

was ectopically integrated into Fl1 to produce KM1.1. These strains were the starting point for this study.

1.7 Aim

The aim of this research is to identify fungal genes involved in the plant-induced expression of the gene *ltmM*, one of ten genes in the biosynthetic gene cluster for production of the secondary metabolite lolitrem B in *E. festucae*. It is proposed that plant-specific regulation of *ltm* genes involves signalling from the host plant, *L. perenne* (Perennial Ryegrass). No plant-produced factors have been specifically linked to *ltmM* gene regulation thus far, however, identification of fungal genes involved in *ltmM* gene regulation will give an indication of what plant-produced factors could be involved. The focus of this research is to test the hypothesis that disruption of a fungal gene involved in the regulation of *ltmM* will result in loss of the plant-specific expression pattern of the P*ltmM-gusA* reporter gene. It is unknown whether the *ltmM* gene is regulated by repression or activation, therefore, an altered P*ltmM-gusA* phenotype could be either a decrease in expression *in planta* or an increase in expression in culture.

Four objectives were devised based on the hypothesis stated above:
(1) To select a P*ltmM-gusA* 'knock-in' transformant of *E. festucae* that is suitable for analysing plant-specific gene expression.
(2) To perform *Agrobacterium tumefaciens*-mediated T-DNA mutagenesis on the 'knock-in' transformant to generate a set of mutants with random insertions in the genome.
(3) To screen the mutants for altered expression of the P*ltmM-gusA* reporter gene, both in culture and *in planta*.
(4) To observe infected plants and identify any mutants of interest with altered symbiotic phenotypes.
(5) To identify the T-DNA integration site in mutants of interest.

In objective one, the expression pattern of P*ltmM-gusA* was assessed in a set of transformants using fluorescent and histochemical assays for the *gusA* gene product, β-

glucuronidase (GUS). A 'knock-in' transformant was selected that expressed P*ltmM-gusA in planta* but not in culture, a pattern similar to the plant-induced expression of the native *ltmM* gene in wildtype *E. festucae*.

In objective two, *A. tumefaciens*-mediated T-DNA mutagenesis was performed on the selected transformant, generating 698 T-DNA mutants (TM mutants).

Objectives three and four involved the analysis of the TM mutants in culture and *in planta*. Firstly, the TM mutants were screened for P*ltmM-gusA* expression in culture and *in planta* using a histochemical assay for GUS. Mutants with altered P*ltmM-gusA* expression were then further characterised in mature plants. Infection analysis and aniline blue staining for endophytic hyphae were performed on all mutants of interest to identify any TM mutants with altered symbiotic interactions. Potential GUS^- mutants were then assayed for P*ltmM-gusA* expression *in planta* using a fluorescent assay for GUS. The copy number of T-DNA insertions in each mutant of interest was determined by Southern blot.

In objective five, *TAIL*-PCR was performed on TM mutants of interest to rescue the genomic sequence flanking the T-DNA insert. Rescued fragments were purified, sequenced, and analysed to identify the T-DNA integration site in the *E. festucae* genome.

Chapter Two

Materials and Methods

Table 2.1 Biological material

Biological material	Strain	Relevant characteristics	Reference
Fungal strains			
Epichloë festucae			
PN2278	Fl1	Host grass *Festuca longifora*, wildtype	Leuchtmann et al., (1994)
PN2610	KM1.1	PN2278/pLM-1	May et al., (2008)
PN2595	KM2.1	PN2278/pKM6; Hyg^R	May, unpublished.
PN2600	KM2.60	PN2278/pKM6; Hyg^R	May, unpublished.
PN2691	KM2.79	PN2278/pKM6; Hyg^R	May, unpublished.
PN2596	KM2.34	PN2278/Δ*ltmM*::P*ltmM-gusA*-P*trpC-hph*; Hyg^R	May, unpublished.
PN2597	KM2.41	PN2278/Δ*ltmM*::P*ltmM-gusA*-P*trpC-hph*; Hyg^R	May, unpublished.
PN2598	KM2.47	PN2278/Δ*ltmM*::P*ltmM-gusA*-P*trpC-hph*; Hyg^R	May, unpublished.
PN2599	KM2.49	PN2278/Δ*ltmM*::P*ltmM-gusA*-P*trpC-hph*; Hyg^R	May, unpublished.
PN2603	KM2.83	PN2278/Δ*ltmM*::P*ltmM-gusA*-P*trpC-hph*; Hyg^R	May, unpublished.
-	TM373	PN2599/T-DNA, pBSYT7; Hyg^R, Gen^R	This study
-	TM888	PN2599/T-DNA, pBSYT7; Hyg^R, Gen^R	This study
-	TM944	PN2599/T-DNA, pBSYT7; Hyg^R, Gen^R	This study
-	TM953	PN2599/T-DNA, pBSYT7; Hyg^R, Gen^R	This study
-	TM984	PN2599/T-DNA, pBSYT7; Hyg^R, Gen^R	This study
-	TM1093	PN2599/T-DNA, pBSYT7; Hyg^R, Gen^R	This study
PN2684	TM1066	PN2599/T-DNA, pBSYT7; Hyg^R, Gen^R	This study
-	TM1197	PN2599/T-DNA, pBSYT7; Hyg^R, Gen^R	This study
-	TM1382	PN2599/T-DNA, pBSYT7; Hyg^R, Gen^R	This study
Bacterial strains			
Agrobacterium tumefaciens			
PN1828	EHA105	contains disarmed agropine plasmid pTiBo542; Rif^R	Hood et al., (1993)
PN4016	EHA105/pBSYT7	EHA105/pBSYT7; Rif^R, Amp^R, Kan^R, Gen^R	This study
Escherichia coli			
PN4080	pBSYT7	DH5α/pBSYT7; Amp^R, Kan^R, Gen^R	This study
DH5α	MAX Efficiency® DH5α	Competent cells	Invitrogen
TOP10	One Shot® TOP10	Competent cells	Invitrogen
Plasmids			
pBSYT7		pBSYT6/Δ*hph*::*nptII*, Amp^R, Kan^R, Gen^R	Tanaka et al., (2007)
pCR4®-TOPO®		Empty vector, linearised.	Invitrogen
pCY39		pUC118 containing *NlltmM*5′-P*trpC-hph-ltmM*3′; Amp^R, Hyg^R	Young et al., (2005)

Biological material	Strain	Relevant characteristics	Reference
pFunGus		Promotorless *gusA*; Amp^R, Hyg^R	McGowan, unpublished.
phFunGus		pFunGus containing 1.4kb *Hind*III *hph* gene from pCYhphJ (*Nco*I site removed); Amp^R, Hyg^R	Bryant et al., (2007)
pKM6		pCY39/*Xma*I 2.6 kb fragment containing *gusA*-T*rpC* from phFunGus; Amp^R, Hyg^R	May, unpublished.
pLM1		P*gpd-gusA*-TtrpC; Amp^R	McMillan, unpublished.
Plant material			
Lolium perenne	cv. Samson	Used for all plant work in this study.	

2.1 Biological material

All Biological materials used for this study are listed in Table 2.1

2.2 Media

All media were prepared with milli-Q water and sterilised at 121°C for a minimum of 15 min, unless otherwise stated.

2.2.1 Luria-Bertaini (LB) media

LB media (Miller, 1972) contained 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl. The pH was adjusted to 7.0–7.5 prior to autoclaving. LB agar was made by the addition of agar to a final concentration of 1.5 % (w/v).

2.2.2 SOC medium

SOC medium (Dower et al., 1988) contained 20 mM glucose, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4.7H_2O$, 10 mM NaCl, 2 % (w/v) tryptone and 0.5 % (w/v) yeast extract.

2.2.3 Potato Dextrose (PD) media

PD broth contained 2.4 % (w/v) potato dextrose broth (Difco). PD agar was prepared by addition of agar to a final concentration of 1.5 % (w/v)

2.2.4 Modified Czapek Dox (CD) media

Modified CD media contained 4.4 mM $K_2HPO_4.3H_2O$, 2 mM $MgSO_4.7H_2O$, 6.7 mM KCl, 36 µM $FeSO_4.\ 7H_2O$, and 1.5 % agar.

2.2.5 Water agar (WA) media

Water agar contained 3 % (w/v) agar.

2.2.6 Induction media (IM)

IM contained 20 % (v/v) 5 % minimal salts, 10 % (v/v) 0.4 M MES pH 5.3, 1 % (v/v) 1 M glucose, 0.07 % (v/v) $CaCl_2$, 0.2 % (v/v) $MgSO_4$, 0.5 % (v/v) glycerol. IM agar included 1.5 % (w/v) agar. Immediately before use, acetosyringone was added to broth (200 μM) and plates (spread with 10 μL of 400 μM stock).

2.2.6.1 5 % Minimal salts

5 % minimal salts contained 0.05 M $K_2HPO_4.3H_2O$, 0.05 M KH_2PO_4, 0.01 M NaCl, 0.2 mM $FeSO_4.7H_2O$, 94.5 mM $(NH_4)_2SO_4$, made up with milli-Q water and autoclaved.

2.2.7 Media supplements

2.2.7.1 Antibiotics

Where appropriate, the media were supplemented as follows: cefotaxime 200 μg/mL, geneticin 200 μg/mL, kanomycin 50 μg/mL, rifampicin 50 μg/mL, tetracycline 5 μg/mL

2.2.7.2 Nutritional supplements

Where appropriate, the media were supplemented as follows: glucose 100 mM, glutamate 20 or 100 mM, glycerol 100 mM, mannitol 100 mM, $NaNO_3$ 20 mM, NH_4 20 mM, proline 20 or 100 mM, serine 20 mM, sucrose 100 mM.

2.3 Bacterial growth conditions

E. coli cultures were grown at 37 °C in LB broth or on LB agar medium and, where appropriate, media was supplemented with ampicillin or kanamycin. *A. tumefaciens* was grown at either 22 °C or 28 °C, using either IM or LB media. Where appropriate, media was supplemented with rifampicin or kanamycin.

Cultures were stored at 4 °C and maintained by streaking from a single colony once a month.

2.4 Fungal growth conditions

Fungal strains were grown at 22 ºC in PD broth or on PD agar medium until a suitable level of growth was attained. Cultures were then maintained at 4 ºC.

2.5 DNA isolation

2.5.1 Plasmid DNA

Plasmid DNA was isolated and purified using a Roche High Pure Plasmid Isolation Kit as per the manufacturer's instructions.

2.5.2 Cracking

Cracking was used as a crude plasmid isolation method to check transformants for the presence of insert. Transformants were subcultured using sterile toothpicks and inoculated into 20 µL of cracking buffer (20 mM NaOH, 0.5 % SDS, 5 mM N_2EDTA). Samples were incubated at 70 ºC for 5 min, vortexed, and spun down. Samples were separated on a minigel (Section 2.6.3) and compared with empty vector to identify desired transformants.

2.5.3 Genomic DNA

Genomic DNA was isolated from freeze-dried mycelia using the method of Byrd et al. (1990). Approximately 15–20 mg of mycelia was ground to a powder in liquid nitrogen, resuspended 800 µL of Byrd extraction buffer (150 mM Na_2EDTA, 50 mM Tris-Hcl, 1 % SLS; pH 8.0) with 2 mg/mL proteinase K (Roche) and incubated at 37 °C for 20 min. The samples were then centrifuged for 10 min at 13 000 rpm. Three phenol/chloroform extractions were performed followed by a single extraction with chloroform only (Section 2.6.2.2). DNA was then precipitated by isopropanol precipitation (Section 2.6.2.4) and resuspended in 50 µL sterile milli-Q water.

2.6 DNA manipulation

2.6.1 DNA quantification

Plasmid DNA was quantified using a Nanophotometer (Implen), as per the manufacturer's instructions. Genomic DNA was quantified by fluorometric assay using a DyNA Quant (Hoefer), according to the manufacturer's instructions. Low concentration DNA samples were concentrated by ethanol precipitation (Section 2.6.2.3) and resuspended in a smaller volume.

2.6.2 DNA purification and precipitation

2.6.2.1 Column purification

PCR fragments were column purified using the Wizard® SV Gel and PCR Clean-up System (Promega) as per the manufacturers instructions.

2.6.2.2 Phenol/chloroform purification

Phenol/chloroform purification was performed to remove impurities from genomic DNA preparations. Equal volumes of phenol and chloroform were added to DNA solutions and mixed gently. Samples were centrifuged for 10 min at 13 000 rpm and the aqueous phase removed to a fresh 2 mL Eppendorf tube. The phenol/chloroform extraction step was repeated multiple times, as required. A final extraction was performed with 1 volume of chloroform and samples centrifuged for 10 min at 13 000 rpm. The aqueous phase was removed to a fresh tube and the DNA precipitated by either ethanol or isopropanol precipitation.

2.6.2.3 Ethanol precipitation

2.5 volumes of 95 % ethanol and 0.1 volume of 3 M sodium acetate were added to solutions of DNA. Samples were mixed gently by inversion. DNA was precipitated at –20 °C for 2 h then pelleted at 13 000 rpm for 10 min. The pellet was washed with 70 % ethanol, air dried, and resuspended in sterile milli-Q water.

2.6.2.4 Isopropanol precipitation

1 volume of isopropanol was added to solutions of DNA. Samples were mixed gently by inversion. DNA was precipitated for a minimum of 2 h to overnight then pelleted at 13 000 rpm for 10 min. The pellet was washed with 70 % ethanol, air dried, and resuspended in sterile milli-Q water

2.6.3 Agarose gel electrophoresis

Agarose (Roche) was melted in TBE (89 mM Tris, 89 mM boric acid, 2.5 mM Na_2EDTA, pH 8.2) to concentration of 0.7 %. Gels were run in TBE, with mini gels run at 100 volts and overnight horizontal gels run at 30 volts. DNA samples were loaded with 1/5 volume of SDS dye (1 % SDS, 0.02 % bromophemol blue, 20 % sucrose, 5 mM Na_2EDTA). Gels were stained in 1 μg/mL ethidium bromide solution and visualised using a UV transilluminator and Gel documentation system (Bio-Rad).

2.6.4 Restriction endonuclease digest

SstI was purchased from Invitrogen, all other restriction enzymes used in this study were purchased from Roche.

Plasmid DNA was digested for 1 h at 37 °C. The total reaction volume was 10 μL consisting of 100 ng of DNA, 0.3 μL (10 U/μL) of enzyme, and commercial buffer according to the manufacturer's instructions. Digests were checked for completion on a mini gel (Section 2.6.3). Fungal genomic DNA was digested overnight at 37 °C in a total volume of 50 μL containing 1.2 μg DNA, 3 μL (10 U/μL) of enzyme, and commercial buffer according to the manufacturer's instructions. 100 ng of digested DNA was run on a mini gel (Section 2.6.3) to check for complete digestion.

2.6.5 DNA cloning

2.6.5.1 Cloning into pCR4®-TOPO®

Purified PCR product was diluted by adding 3.5 μL to 1.5 μL H_2O. 1 μL of TOPO vector (Invitrogen) and 1 μL of salt solution (1.2 M NaCl, 0.06 M $MgCl_2$; Invitrogen) was then added and the mixture incubated at room temperature for 20 min.

2.6.5.2 Transformation of TOP10 *E.coli* cells

One Shot® TOP10 cells (Invitrogen) were combined with pCR4®-TOPO® vector containing the fragment of interest (Section 2.6.5.1), mixed gently, and incubated for 20 min on ice. To start the transformation, the cells were heat shocked at 42 °C for 30 sec then incubated for 2 min on ice. 250 μL of SOC medium was added and the mixture was incubated at 37 °C for 1 h. 40 μL was spread on LB agar containing ampicillin and incubated overnight at 37 °C.

2.6.6 Plasmid rescue

2.6.6.1 Ligation

Fungal genomic DNA was digested as in Section 2.6.4. 1 μg of genomic DNA was ligated in a total volume of 200 μL with 1 μL (3 U/μL) of T4 DNA ligase and commercial ligase buffer according to the manufacturer's instructions. Samples were ligated at room temperature for a minimum of 2 h. The reaction was stopped with the addition of 250 mM Na_2EDTA. *E. coli* DH5α cells were transformed with the ligation mixture, as below in Section 2.6.6.2.

2.6.6.2 Transformation of DH5α *E.coli* cells

Max Efficiency® DH5α cells (Invitrogen) were combined with ligation mixture (Section 2.6.6.1), mixed gently, and incubated for 20 min on ice. To start the transformation, the cells were heat shocked at 42 °C for 1 min then incubated for 2 min on ice. 900 μL of SOC medium was added and the mixture was incubated at 37 °C for 1 h. The cells were then pelleted at 13 000 rpm for 2 min and resuspended in a volume appropriate for spreading (100-200 μL). Samples were spread on LB agar containing ampicillin and incubated overnight at 37 °C.

2.6.7 Thermal assymetric interlaced PCR (*TAIL*-PCR)

Oligonucleotide primers cited within this thesis are listed in Table 2.2. Primers were synthesised by Invitrogen. All primers were stored at –20 °C.

Table 2.2 PCR primers cited within this thesis

Primer name	Sequence 5′-3′	Used for
pBSYT7 TAIL LB-1	GGAGAACCTGCGTGCAATCCAT	*TAIL*-PCR
pBSYT7 TAIL LB-2	AGGGCGAACTTAAGAAGGTATGACCG	*TAIL*-PCR
pBSYT7 TAIL LB-3	CGCTACTGCTACAAGTGGGGCTG	*TAIL*-PCR
pBSYT7 TAIL LB-4	GCCAAGCCCAAAAAGTGCTCCTTC	*TAIL*-PCR
pBSYT7 TAIL LB-5	CCTATAGGGTTTCGCTCATGTGTTG	*TAIL*-PCR
pBSYT7 TAIL RB-1	TGCGTTATCCCCTGATTCTGTGGATAA	*TAIL*-PCR
pBSYT7 TAIL RB-2	CATTAATGCAGCTGGCACGACAG	*TAIL*-PCR
pBSYT7 TAIL RB-3	CAGCCCGGGGGTTAACGCTA	*TAIL*-PCR
pBSYT7 TAIL RB-4	TTGCCGGTCTTGCGATGATTA	*TAIL*-PCR
pBSYT7 TAIL RB-5	ACAAAATATAGCGCGCAAACTAGG	*TAIL*-PCR
RANDOM-T1	GGTGCGGGAA	*TAIL*-PCR
RANDOM-T2	CCAGATGCAC	*TAIL*-PCR
RANDOM-T3	GTGACATGCC	*TAIL*-PCR
RANDOM-T4	AGATGCAGCC	*TAIL*-PCR
RANDOM-T5	TGCGGCTGAG	*TAIL*-PCR
RANDOM-T6	GGACCCAACC	*TAIL*-PCR
RANDOM-T7	CACCGTATCC	*TAIL*-PCR
RANDOM-T8	ACCCGGTCAC	*TAIL*-PCR
RANDOM-T9	GGAGCCCAC	*TAIL*-PCR
RANDOM-T10	ACGATCGCGG	*TAIL*-PCR

pBSYT7 TAIL LB and RB binding sites are indicated in Appendix 5.1.

Method by Liu and Whittier (1995), with modifications described by Terauchi and Kahl (2000). *E. festucae* genomic DNA was amplified using three consecutive PCR programs (Appendix 5.6). For the first reaction, TAIL1, primer pBSYT7 TAIL LB-1 was paired with each of ten random primers (RANDOM-T1 to RANDOM-T10), in ten separate reactions, to amplify 1 μL (50-100ng) of genomic DNA. For the second reaction, TAIL2, 1 μL of the ten TAIL1 products was used as a template for the primer pBSYT7 TAIL LB-2 paired with the same ten random primers. The last reaction, TAIL3, involved thirty individual reactions, ten reactions each for primers pBSYT7 TAIL LB-3, LB-4, and LB-5. Each of the nested primers was paired in separate reactions with each of the ten random primers, with 1 μL of the ten corresponding TAIL2 products as the template. For TAIL1 and TAIL2, primers were used at concentrations of 0.2 M for LB/RB primers and 2 M for random T primers. For TAIL3, a concentration of 0.2 M was used for all primers. Taq DNA polymerase (Roche) and the corresponding 10x PCR reaction buffer were used for all reactions, as per the manufacturers instructions. dNTPs (Sigma) were added to a final concentration of 0.2 mM. The PCR products from TAIL3 were analysed by mini gel electrophoresis (Section 2.6.3). A successful TAIL-PCR produces a fragment for each of LB3, LB4

and LB5, decreasing in size by ~ 100 bp for each. If TAIL-PCR was unsuccessful in identifying the LB flanking sequence, the method was repeated using the RB primers pBSYT7 TAIL RB1 to RB-5.

2.6.8 Southern blotting

DNA was run on a 300mL overnight horizontal agarose gel (Section 2.6.3). The gel was agitated in the following solutions: 15 min in solution 1 (0.25 M HCl), 45 min in solution 2 (0.5 M NaOH, 0.5 M NaCl), and 45 min in solution 3 (2.0 M NaCl, 0.5 M Tris, pH 7.4). The gel was then washed for 2 min in 2x SSC (0.3 M NaCl, 0.03 M trisodium citrate), and assembled on the blotting stand as follows: wicks were made from two sheets of 3MM paper (Whatman) and soaked in 20x SSC (3.0 M NaCl, 0.3 M trisodium citrate), plastic wrap was laid over the blotting apparatus and a hole cut slightly smaller than the size of the gel. The gel was then laid over the hole, followed by a positively charged nylon membrane (Roche) presoaked in 2x SSC. On top of the membrane were two sheets of 3MM wetted with 2x SSC, two sheets of dry 3MM, and finally paper towels were stacked on top with a small weight. The DNA was allowed to transfer overnight. The membrane was then washed briefly in 2x SSC, air dried, and the DNA was cross-linked by UV irradiation of 120 000 μJoules/cm^2 using a Cex-800 UV-crosslinker (Ultra-Lum Inc.)

2.6.9 Radioactive hybridisation

30 ng of DNA was denatured by boiling for 3 min and was then placed on ice. 4 μL of High Prime solution and 3-5 μL [α-^{32}P]dCTP were added then samples were mixed, centrifuged, and incubated at 37 °C for 1 h. 35 μL TES buffer was added and samples were mixed again. The samples were transferred to pre-spun G-50 columns and centrifuged at 2 050 rpm for 2 min. Samples were then transferred into fresh Eppendorf tubes with an additional 50 μL of TES buffer and stored at –20 °C.

2.6.10 Southern hybridisation

Filters were placed in hybridisation tubes with approximately 30 mL of 10x Denhardt's solution, the tubes were then pre-hybridised at 65 °C for a minimum of 2 h. Most of the Denhradt's solution was then drained, leaving approximately 5 mL to cover the membrane. Probe solution was added and allowed to hybridise overnight. The next day

the solutions were drained off, and the membrane washed 3 times in 2x SSC containing 0.1 % SDS (equilibrated at approximately 50 °C). The membranes were blotted with blotting paper to remove excess moisture, wrapped in plastic wrap, and exposed to X-ray film at –80 °C. Films were developed using a 100Plus Automatic X-ray Film Processor (All-Pro Imaging).

2.6.11 Stripping of membrane

Membranes were stripped of their radioactive signal by washing in boiling 0.1 % (w/v) SDS. The membrane was then exposed to X-ray film for 2 days to ensure there was no detectable signal.

2.7 Plant growth conditions

2.7.1 Sterilisation of seeds

Endophyte-free *L. perenne* seeds of Samson cultivar were surface sterilised by soaking in 50 % sulfuric acid for 30 min, rinsing three times with water, soaking 20 min in 50 % chlorine bleach (Janola), rinsing three times with sterile milli-Q water, and finally air drying in axenic conditions.

2.7.2 Seedling germination and inoculation

Sterile seeds were plated onto 3 % water agar plates and germinated in darkness at 22 ºC for 7 days. The Seedlings were then inoculated using the method of Latch and Christensen (1985). A 1–2 mm incision was made at the basal meristem and small block of mycelia was placed onto the incision to inoculate the endophyte. Multiple seedlings were inoculated per endophyte strain, with separate strains confined to separate plates. Seedlings were then maintained on water agar at 22 ºC for a further 7 days in the dark then 7 days in the light.

2.7.3 Mature plant maintenance

Inoculated seedlings were transferred to the greenhouse and planted in root trainers with potting mix. Plants were transferred from root trainers to bags at approximately 10 weeks post-inoculation and maintained with regular watering and trimming.

2.8 *in planta* analysis

2.8.1 Immunoblot for presence of endophyte

Tillers were cut close to the base, the outermost leaf sheath was removed, and the base was pressed onto a nitrocellulose membrane. The membrane was soaked in blocking solution for 2 h at room temperature, then transferred to fresh blocking solution containing primary antibody (1:1000 dilution) and incubated overnight at 4°C. Unbound primary antibody was removed with several washes in fresh blocking solution, then the membrane was transferred to blocking solution containing secondary antibody (1:2000 dilution) and incubated for 2 h at room temperature. Unbound secondary antibody was removed with several washes in fresh blocking solution. The membrane was then transferred to chromogen solution (a combination of 75 mg Fast Red in 12.5 mL Tris buffer and 12.5 mg naphthol asmx phosphate in 12.5 mL Tris buffer) and incubated at RT for 15 min. Finally, the membrane was rinsed in RO water and air dried.

2.8.2 Aniline blue staining

Epidermal peels were taken from the leaf sheaths of infected plants and placed on slides with aniline blue stain (88 % lactic acid, 50 % glycerol, 0.1 % (w/v) aniline blue). Slides were heated over a flame to fix the samples. Blue staining of fungal hyphae was then observed using a compound microscope at magnifications up to 400×.

2.8.3 Isolation of endophyte from seedlings

Seedlings were germinated and inoculated (Section 2.7.2). At 7 days post-inoculation, the seedlings were transferred to PD agar plates and moved to into the light. Hyphae were then allowed to grow from the inoculation site onto the PD agar.

2.9 Fluorescent (MUG) assay for β-glucuronidase

2.9.1 In culture MUG assay

Cultures of *Epichloë festucae* were grown for 7 days, at which point plugs of a uniform size were taken from the edge of fungal colonies. The plugs of mycelia were placed in

a 96-well microtitre plate containing 200 µL of 4-methylumbelliferyl-β-D-glucuronide (MUG) assay buffer per well (GUS extraction buffer (50 mM $NaPO_4$ pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1 % SLS, 0.1 % Triton X-100) containing 1 mM MUG). Samples were incubated overnight at 22 ºC and visualised under UV light using a UV transilluminator and Gel documentation system (Bio-Rad).

2.9.2 *in planta* MUG assay

Endophyte colonisation was confirmed by immunoblot (Section 2.8.1) then a MUG assay was performed on infected plants. Tillers were cut close to the base and the outer sheath was removed. Sections of approximately 5 mm were taken from the base end of each tiller and placed in a 96-well microtitre plate containing 200 µL of MUG assay buffer per well. The plate was incubated overnight at 22 ºC and visualised under UV light using a UV transilluminator and Gel documentation system (Bio-Rad).

2.10 Histochemical (X-Gluc) assay for β-glucuronidase

2.10.1 In culture X-Gluc assay

Endophyte was grown at 22ºC for 7 days on PD agar (Section 2.2.3) containing 40 µg/mL 5-bromo-4-chloro-3indolyl-β-D-glucuronc acid (X-Gluc; Quantum Scientific). Plates were incubated in the dark to minimise break down of X-Gluc.

2.10.2 *in planta* X-Gluc assay

Seedlings were germinated and inoculated as in Section 2.7.2. At 2 weeks post-inoculation, aerial tissues were collected by cutting below the inoculation point. The seed and root portion was discarded. The aerial tissue was put into 2 mL Eppendorf tubes, with seedlings inoculated with the same fungal strains put into the same tube. The seedlings were fixed for 20 minutes in cold 90 % acetone, washed with GUS staining buffer (500 mM sodium phosphate, 100 mM ferrocyanide, 100 mM ferricyanide, 0.2 % (v/v) Triton X-100), then left overnight in GUS staining solution (GUS staining buffer containing 2 mM X-gluc) at 37 ºC. Samples were incubated in the dark due to the light sensitivity of the GUS staining solution. After a minimum of 16

hours incubation, seedlings were examined for blue staining by eye or under a light microscope. Samples were stored in 70 % ethanol at 4ºC.

2.11 *Agrobacterium*-mediated T-DNA mutagenesis

Method adapted from Bundock et al., (1995) and Mullins et al., (2001). *Agrobacterium tumefaciens* strains from Hood et al., (1993).

2.11.1 Growth conditions

Mycelial cultures of *E. festucae* KM2.49 (PN2599) were grown in 4 mL PD broth for 6 days at 22 °C. *A. tumefaciens* strains EHA105 (PN1828) and EHA105/pBSYT7 (PN4016) were grown overnight in LB medium at 28 °C, harvested by centrifugation, and resuspended in IM medium to A_{600} of 0.15. The culture was then grown for a further 6 h at 28 °C in the presence of 200 μM acetosyringone. Where appropriate, media was supplemented with rifampacin and kanamycin.

2.11.2 Transformation conditions

E. festucae cultures were washed twice in 5 mL of IM medium by centrifugation and washing of the pellet, then 0.5-1 mL of mycelia was resuspended in 100 μL of the *A. tumefaciens* culture (Section 2.11.1) per 1 mL of mycelia. The mixture was spread on a nitrocellulose membrane (Millipore 4.0 μm HA, 47 mm) on IM agar medium spread with 10 μL of 400 μM acetosyringone, a phenolic plant hormone that induces T-DNA transfer. The membrane was incubated at 22 °C for 2 days to allow the transformation to take place then transferred to a PD agar plate containing geneticin and cefotaxime. The membrane was incubated for a further 14-21 days, until colonies appeared.

2.11.3 Nuclear purification

Transformants were subcultured with sterile toothpicks onto fresh PD agar medium containing geneticin to select for transformants. This process was repeated a further three times to ensure nuclear purification.

Chapter Three

Results

3.1 Analysis of *E. festucae* P*ltmM-gusA* transformants

3.1.1 Copy number of P*ltmM-gusA* insertion affects GUS levels in culture

A set of *E. festucae* P*ltmM-gusA* transformants (Section 1.6) was obtained from Dr. Kimberley May. Included were transformants KM2.1, KM2.60, and KM2.79 with 'ectopic' integrations, and transformants KM2.34, KM2.41, KM2.47, KM2.49, and KM2.83 with 'knock-in' integrations in which the native *ltmM* gene was replaced with a construct containing the P*ltmM-gusA* reporter gene (Appendix 5.2). The native *ltmM* gene is only expressed *in planta*, hence the transformants were screened to assess the expression of the P*ltmM-gusA* reporter gene. A qualitative fluorescent assay was performed (Section 2.9.1) to detect the presence of β-glucuronidase (GUS), the protein product of the *gusA* gene, in fungal colonies grown on PD agar plates. The results of this analysis are summarised in Table 3.1. Mycelial plugs of 7 day-old mycelia were incubated in buffer containing MUG as a substrate for GUS (Fig. 3.1). No fluorescence was detected in the negative control Fl1 (wildtype), whereas strong fluorescence was observed in the positive control KM1.1. KM2.60 and KM2.41 showed strong fluorescence, KM2.1 showed weak fluorescence, while KM2.79, KM2.34, KM2.47, KM2.49, and KM2.83 did not show detectable levels of fluorescence.

Independently, a Southern analysis was performed on the transformants by Dr. Kimberley May (unpublished data) to confirm the location and copy number of the P*ltmM-gusA* insertion (Appendix 5.3). The results of the analysis are summarised in Table 3.1.

Table 3.1 Copy number and in culture expression of P*ltmM-gusA* in KM transformants

KM transformant	In culture expression	Copy number and location*
KM2.1	weak	Double copy, ectopic integrations
KM2.60	strong	Multiple copy, ectopic integrations
KM2.79	not detectable	Single copy, ectopic integration
KM2.34	not detectable	Single copy, knock-in integration
KM2.41	strong	Multiple copy, knock-in and ectopic integrations
KM2.47	not detectable	Single copy, knock-in integration
KM2.49	not detectable	Single copy, knock-in integration
KM2.83	not detectable	Single copy, knock-in integration

* Copy number and location results are from analyses performed by Dr. Kimberley May

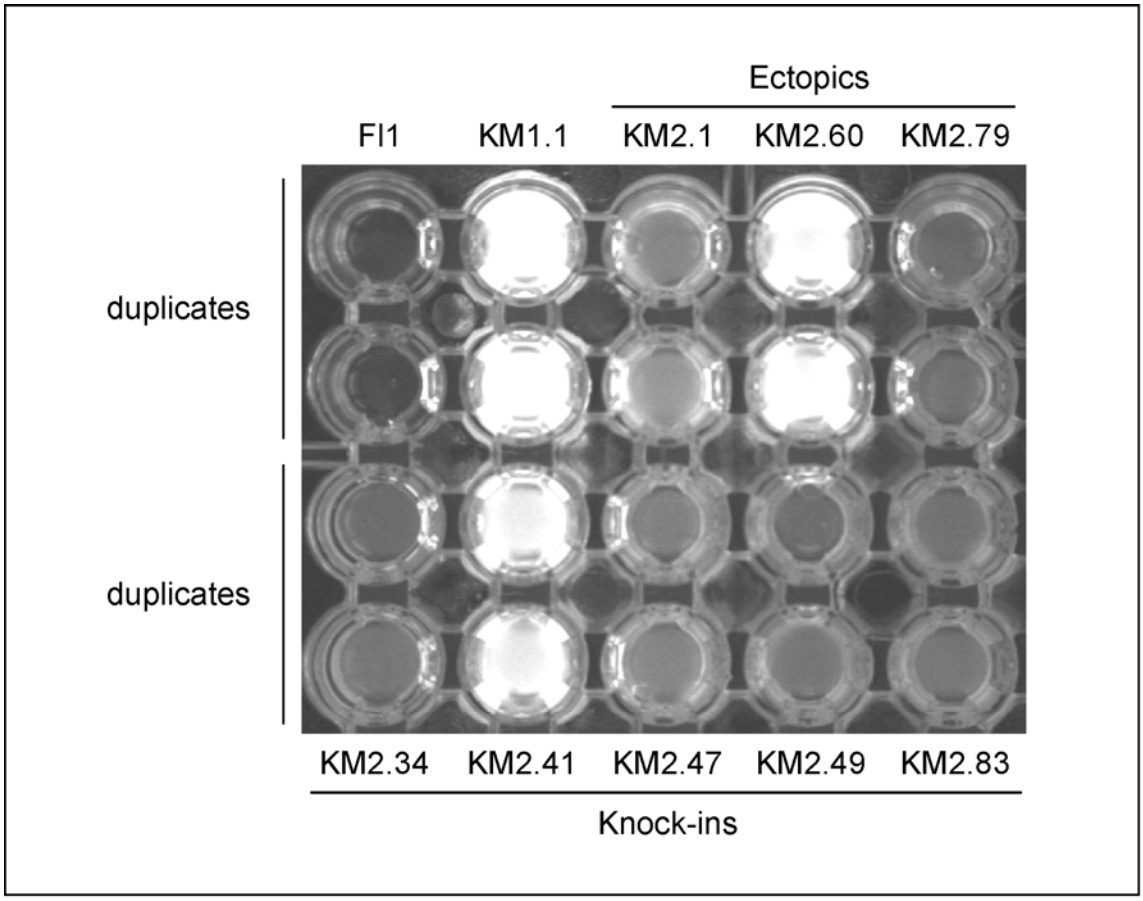

Figure 3.1 Fluorescent assay for β-glucuronidase activity in *E. festucae* transformants

Mycelial plugs (4-5 mm) of 7 day old mycelia from *E. festucae* wildtype FI1 (negative control), KM1.1 (positive control), KM2.1, KM2.60, KM2.79 (ectopic transformants), KM2.34, KM2.41, KM2.47, KM2.49, and KM2.83 (knock-in transformants) were assayed for β-glucuronidase activity by UV fluorescence using MUG as a substrate.

A comparison of results from the Southern analysis with the GUS expression data revealed a direct relationship between copy number and GUS expression. Strains KM2.60 and KM2.41, which had high levels of GUS expression, had multiple copies of P*ltmM-gusA*. KM2.1 had two ectopic integrations of the vector, corresponding to the weak GUS expression seen in the previous assay. Ectopic KM2.79 and knock-ins KM2.34, KM2.47, KM2.49, and KM2.83 had no detectable GUS expression and all contained only a single copy integration at a single site. These results indicate, firstly, that the fluorescent assay is sensitive enough to distinguish between levels of GUS produced by single and double copy P*ltmM-gusA* integrations. Secondly, it shows that any basal GUS expression by a transformant with a single copy integration is not detectable by this assay, demonstrating that single copy integrants are suitable as reporters to monitor increases in GUS expression in culture.

3.1.2 P*ltmM-gusA* expression is unaltered in KM2.49

To assess P*ltmM-gusA* expression *in planta*, a histochemical assay (Section 2.10.2) was performed to detect GUS expression *in planta*. Each transformant was inoculated into five *L. perenne* seedlings. At 2 weeks post-inoculation, seedlings were stained with a solution containing X-Gluc as a substrate for GUS. As expected, no staining was present in Fl1-infected (negative control) seedlings. However, blue precipitate was observed in seedlings infected with KM1.1 (positive control) and the single copy knock-in transformant KM2.49 (Fig. 3.2). All of the additional KM transformants were also found to express GUS *in planta* (data not shown). Based on the results of the expression analyses, both in culture and *in planta,* KM2.49 was chosen for use in subsequent work on P*ltmM-gusA* expression. Collation of these results indicates that the pattern of P*ltmM-gusA* expression in KM2.49 is the same as that previously reported for *ltmM* in Fl1, with a high expression level *in planta* and a very low expression level in culture (Young et al., 2005).

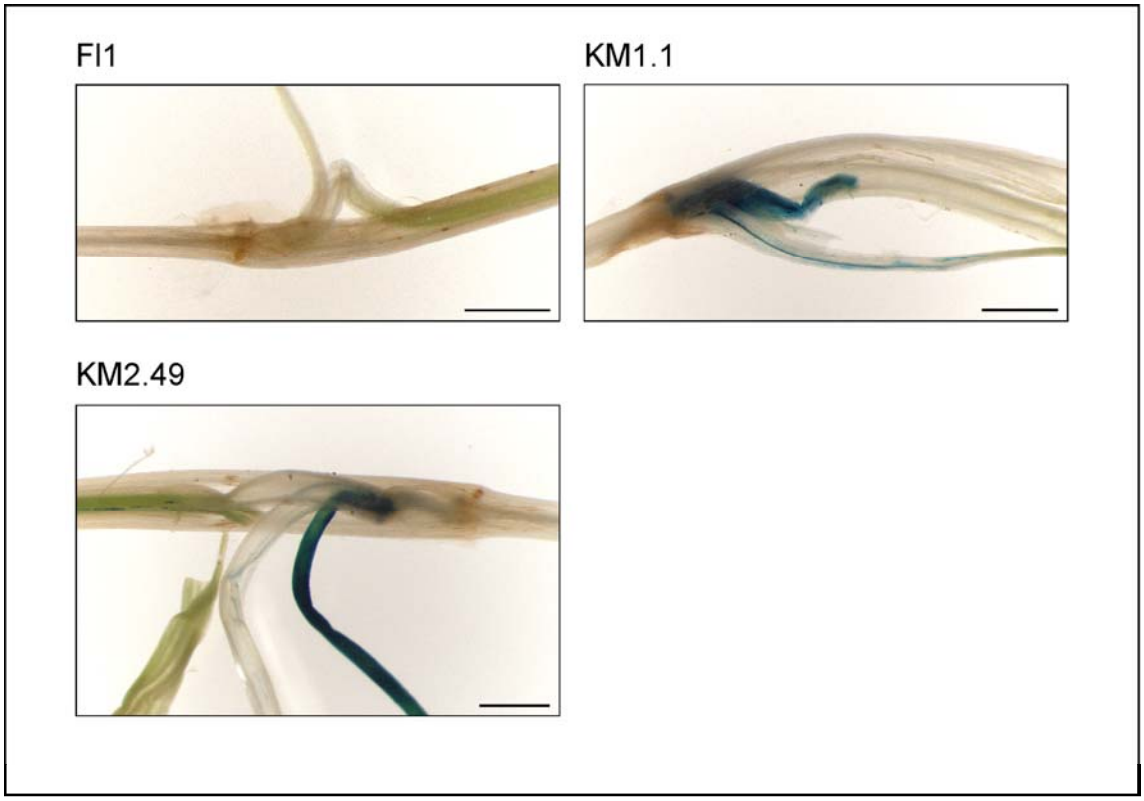

Figure 3.2 Histochemical assay for β-glucuronidase activity in *L. perenne* seedlings

L. perenne seedlings at 14 days post-inoculation infected with *E. festucae* wildtype Fl1 (negative control), KM1.1 (positive control), and KM2.49 (single copy knock-in transformant) were assayed for β-glucuronidase activity by histochemical dye reaction using X-Gluc as a substrate. GUS activity is indicated by blue precipitate. Bar = 1 mm.

3.1.3 P*ltmM-gusA* does not affect hyphal growth of KM2.49 *in planta*

E. festucae Fl1 has a distinctive, tightly regulated, pattern of hyphal growth *in planta* that is crucial for the stability of the symbiosis (Tan et al., 2001; Christensen et al., 2002). Therefore, hyphal growth of Fl1 and KM2.49 was compared to determine whether insertion of the P*ltmM-gusA* construct had any effect on the growth of KM2.49 *in planta*. Epidermal peels were taken from tillers of endophyte-infected, mature *L. perenne* plants and stained with the aniline blue (Section 2.8.2), which stains the fungal cytoplasm (Fig. 3.3). At least six epidermal peels were examined for each strain. As previously observed (Tan et al., 2001; Christensen et al., 2002; Tanaka et al., 2006; Christensen et al., 2008), Fl1 grows as individual, predominantly unbranched hyphae within the intercellular spaces of the leaf sheath. When compared to Fl1, KM2.49 was found to have unaltered hyphal growth *in planta*. This result once again confirms that KM2.49 behaves similarly to wildtype.

3.1.4 P*ltmM-gusA* expression is not induced under altered physiological conditions

In pathogenic fungi such as *C. fulvum* and *M. grisea*, genes that are normally expressed in a symbiosis-specific manner have been shown to be inducible in culture under carbon- or nitrogen-limited conditions (Talbot et al., 1993; van den Ackerveken et al., 1994). This suggests that physiological or genetic changes related to metabolic state may also affect genes involved in the symbiosis. One possible explanation for the plant-specific expression of *ltmM* is that the regulation is linked to the metabolic state of the fungus. Hence, a series of in culture growth experiments were performed in an effort to mimic the *in planta* conditions inducing P*ltmM-gusA* expression.

The apoplast of plants, where *E. festucae* resides, is a nutrient poor environment. To test whether P*ltmM-gusA* is affected by nutrient source, a series of fluorescent assays were performed on fungal colonies grown on media with various carbon and nitrogen compositions and concentrations. Nutrient-based repression mechanisms have to date not been investigated in *E. festucae*, however, it is likely that they are similar to those commonly observed in other filamentous fungi. The nutrient sources and levels used were based on those of widely accepted carbon and nitrogen repressed and derepressed states of other filamentous fungi. Fl1, KM1.1, KM2.49, and the single copy ectopic strain KM2.79, were grown for 5 days on PD agar plates layered with cellophane membranes to obtain suitable mycelial mass.

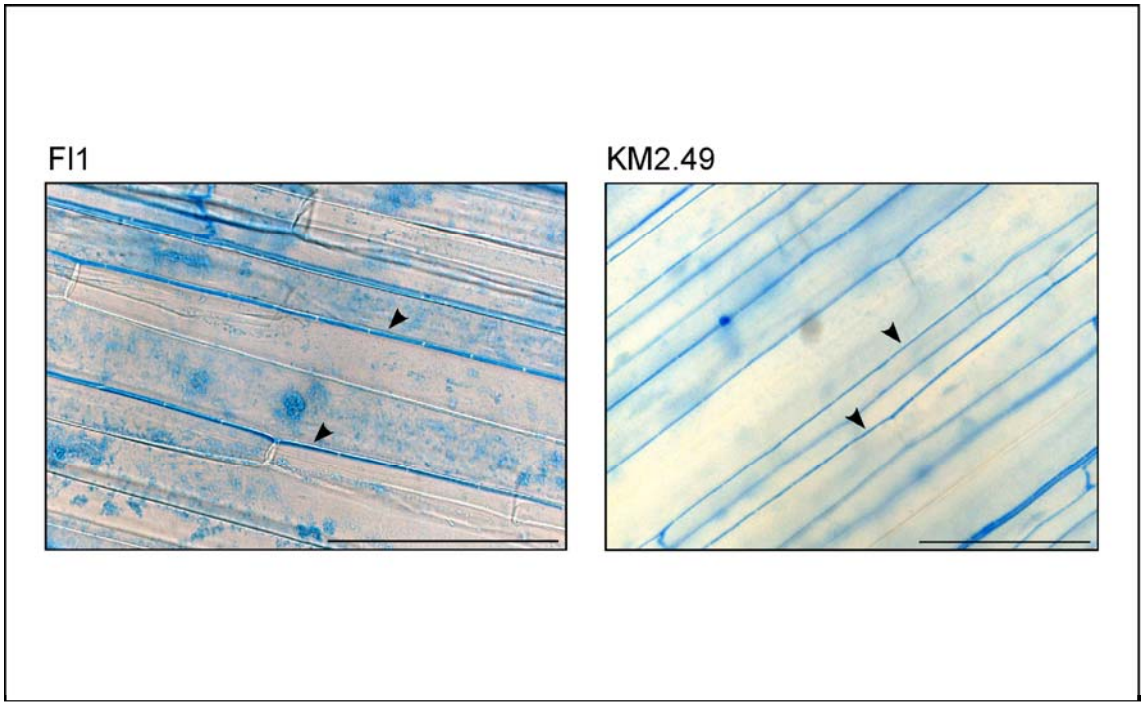

Figure 3.3 Aniline blue stain for detection of hyphae in mature plant tissue

Light micrographs of epidermal peels from endophyte-infected *L. perenne* plants stained with aniline blue. Endophytic hyphae of Fl1 (wildtype) and KM2.49 (single copy knock-in transformant) are observed growing in the inter-cellular spaces as indicated by arrowheads. Bar = 100 μm.

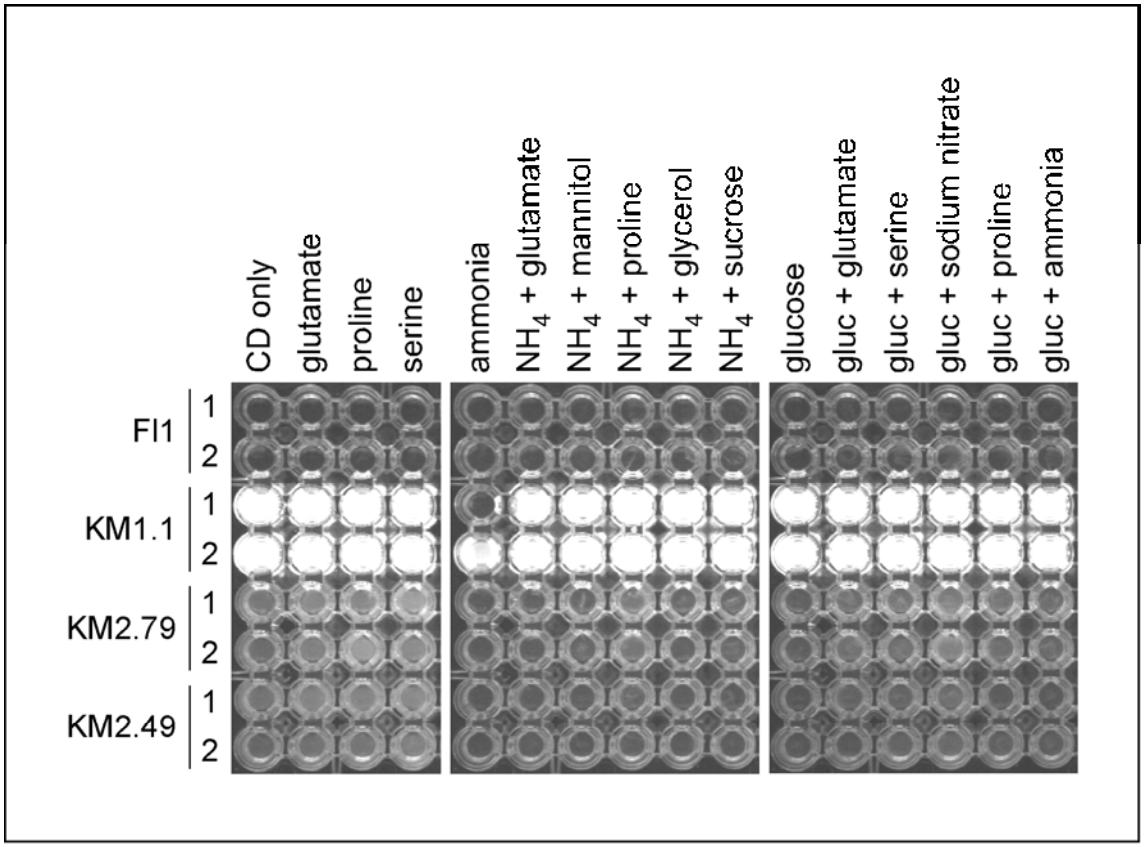

Figure 3.4 Fluorescent assay for β-glucuronidase activity in *E. festucae* grown in various carbon & nitrogen conditions

E. festucae wildtype FI1 (negative control), KM1.1 (positive control), KM2.79 (single copy ectopic transformant), and KM2.49 (single copy knock-in transformant) were grown for 5 days on PD agar then 2 days on modified CD agar with carbon and nitrogen supplements as indicated. Mycelial plugs (4-5 mm) were assayed for β-glucuronidase activity by UV fluorescence using MUG as the substrate. Carbon and nitrogen derepressed conditions include glutamate, proline, and serine; carbon and nitrogen repressed conditions include ammonia + sucrose and glucose + ammonia; nitrogen repressed/carbon derepressed conditions include ammonia + glutamate, + mannitol, + proline, + glycerol; carbon repressed/nitrogen derepressed conditions include glucose + glutamate, + serine, + sodium nitrate, + proline.

The membranes were then transferred to Modified CD media (Section 2.2.4) supplemented with the various carbon and nitrogen sources (Section 2.2.7.2) and incubated for 2 days to allow any physiological changes to take place. Plugs of mycelia were taken from duplicate colonies and incubated with solution containing MUG as a substrate for β-glucuronidase (Section 2.9.1). None of the fifteen conditions tested induced fluorescence in KM2.49 or KM2.79 (Fig. 3.4). Strong fluorescence was observed in the positive control, KM1.1, while the negative control, Fl1, did not fluoresce. KM2.79 was included to compare positional effects on the P*ltmM-gusA* insertion.

ltmM gene expression is not only seen in hyphae growing completely within the host plant, but also in epiphyllous hyphae growing on the plant surface (May et al., 2008). To examine whether hyphae growing further from the host plant produce GUS, a grow-out experiment was performed (Section 2.8.3). Mycelia were allowed to grow from the inoculation site of *L. perenne* seedlings for 7 days then a fluorescent assay was used to detect GUS (Section 2.9.1). The seedling was removed from the plate for sampling of the fungal colony. Mycelial samples were taken from the centre and from both edges of the colony (Fig. 3.5 A) then incubated with solution containing MUG as a substrate for β-glucuronidase. KM2.49 did not show detectable levels of GUS at the colony edges, however the centre sample did show weak fluorescence (Fig. 3.5 B). The centre samples likely contained some epiphyllous hyphae that were left behind when the seedling was removed, which may account for the fluorescence.

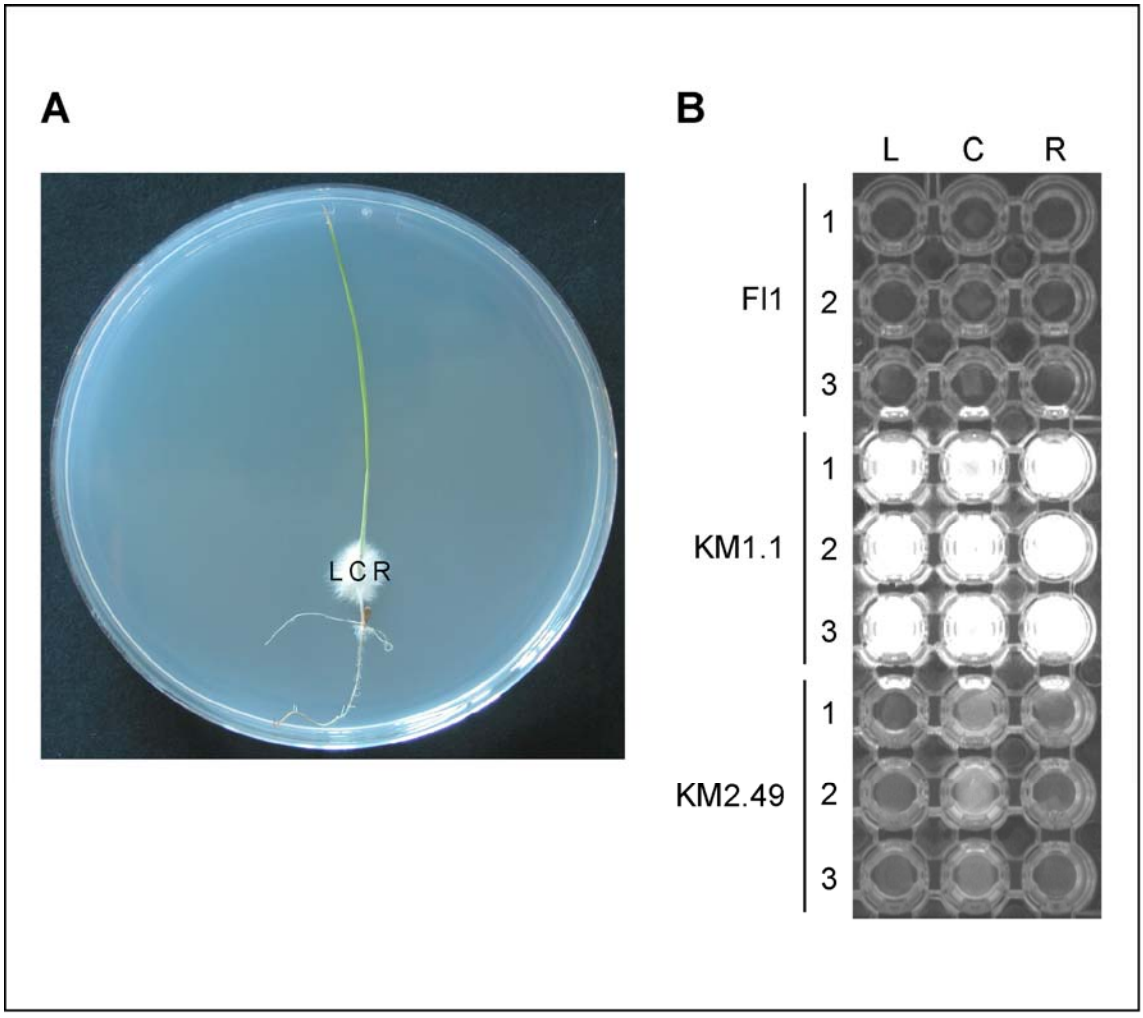

Figure 3.5 Grow-out analysis of endophyte-infected *L. perenne* seedlings

A. 7 days growth of Fl1 endophyte from inoculation point on *L. perenne* seedling. Position of mycelial samples is indicated **L**, left; C, centre; R, right. **B.** Mycelial plugs of *E. festucae* wildtype Fl1 (negative control), KM1.1 (positive control), and KM2.49 (single copy knock-in transformant) were assayed for β–glucuronidase activity by UV fluorescence using MUG as a substrate. Mycelial samples are denoted L, C, and R. Separate seedlings are indicated by numbering 1-3.

3.2 *Agrobacterium*-mediated T-DNA mutagenesis of KM2.49

Agrobacterium-mediated T-DNA mutagenesis was performed on KM2.49 to generate a set of mutants (TM mutants) with random insertions of a construct containing the geneticin resistance gene, *nptII*, as a selectable marker (from pBSYT7, see Appendix 5.1). The aim of the experiment was to produce mutants with disruptions in genes involved in regulation of P*ltmM-gusA*. A total of 1,414 mutants were obtained in collaboration with Dr. Kimberley May, 698 of which were analysed as a part of this study. The T-DNA mutagenesis rounds and the transformants yielded are listed in Table 3.2. *E. festucae* KM2.49 and *Agrobacterium* cultures were grown as described (Section 2.11.1) then *E. festucae* was transformed (Section 2.11.2) in the presence of the plant wound signal acetosyringone. The amount of mycelia used was varied between rounds to optimise the number of transformants generated. The best results were obtained with 0.5-1 mL of mycelia and 100 μL of *Agrobacterium* culture per 1 mL of mycelia. Transformants were nuclear purified (Section 2.11.3).

Table 3.2 TM transformants obtained from T-DNA mutagenesis

Round	TM transformants obtained	TM numbers
1	53	TM1-53
2	86	TM331-416
3	23	TM438-460
4	0	-
5	0	-
6*	190	TM879-1068
7	0	-
8	0	-
9	71	TM1069-1139
10	0	-
11	54	TM1140-1193
12	20	TM1194-1213
13	201	TM1214-1414

* Round 6 performed by Dr. Kimberley May.

3.2.1 Some TM mutants displayed altered growth and morphology phenotypes

During nuclear purification, some TM mutants were observed to have altered colony phenotypes. TM mutants, TM373, TM984, TM1093, and TM1382 showed a reduced growth rate and altered colony morphology (Fig. 3.6). These four mutants were retained for further analysis as they may be of interest in relation to the symbiosis. Many other mutants showed small variations in colony morphology without a reduced growth rate.

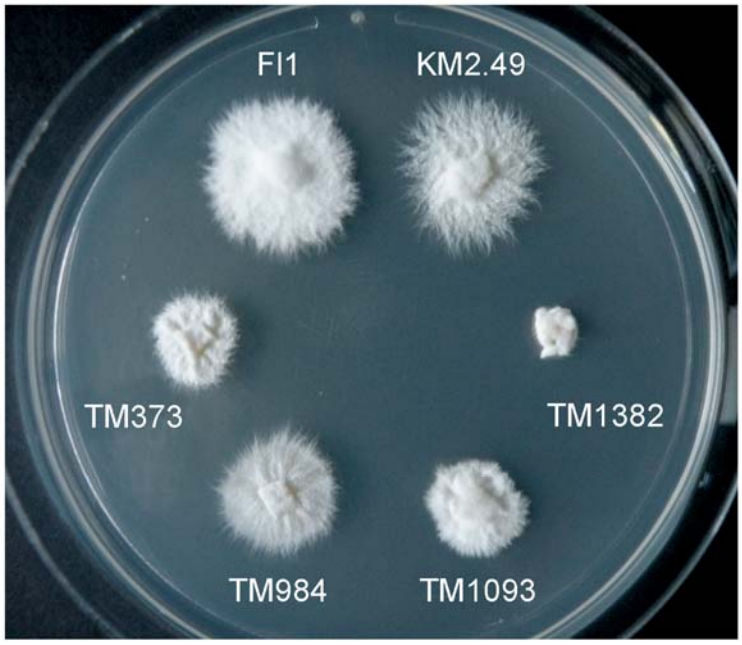

Figure 3.6 TM transformants with altered growth phenotype in culture

E. festucae FI1 (wildtype), KM2.49 (single copy knock-in transformant), and TM transformants TM373, TM984, TM1093, and TM1382 were grown for 8 days on PD agar.

3.2.2 Copy number of T-DNA insert in TM mutants

To determine the proportion of single copy integrations in the set of TM mutants obtained, a random number generator was used to pick 27 TM mutants between TM331 to TM1414. TM1 to TM53 were subcultured separately from the same membranes as TM331 to 416 and therefore were excluded to avoid repeat selection. DNA from the selected TM mutants was digested with *Sst*I, which cuts the middle of the T-DNA insert (Appendix 5.1). Southern blots were performed and probed with ^{32}P-labelled pBSYT7, which hybridises to two bands for a single copy insertion (Fig. 3.7). It was found that 11/27 (41%) of the selected TM mutants contained a single copy of the T-DNA insert (Table 3.3). Two background bands of 11.5 kb and 4.3 kb were observed in KM2.49, these were common to all TM mutants. The P*ltmM-gusA* construct contains the *trpC* promoter and terminator, which are also present in the probe, thus accounting for the common bands. Blots were over-exposed to confirm the number of bands for weakly hybridised samples (data not shown). Gel photographs of the Southern Blots are shown in Appendix 5.4.

Table 3.3 Copy number of T-DNA insert in TM mutants

TM mutant	Number of bands*	Copy number
339	Multiple	Multiple
368	2	Single
381	1 (dark)	Single (?)
388	Multiple	Multiple
403	2	Single
886	2	Single
894	2	Single
905	Multiple	Multiple
908	Multiple	Multiple
973	Multiple	Multiple
379	Multiple	Multiple
995	Multiple	Multiple
1043	2	Single
1058	1-2 (very dark)	Multiple (?)
1066	2	Single
1078	2	Single
1099	Multiple	Multiple
1105	2	Single
1161	2	Single
1067	2	Single
1230	Multiple	Multiple
1265	Multiple	Multiple
1311	Multiple	Multiple
1330	Multiple	Multiple
1340	Multiple	Multiple
1357	Multiple	Multiple
1412	Multiple	Multiple

* Excluding common bands.

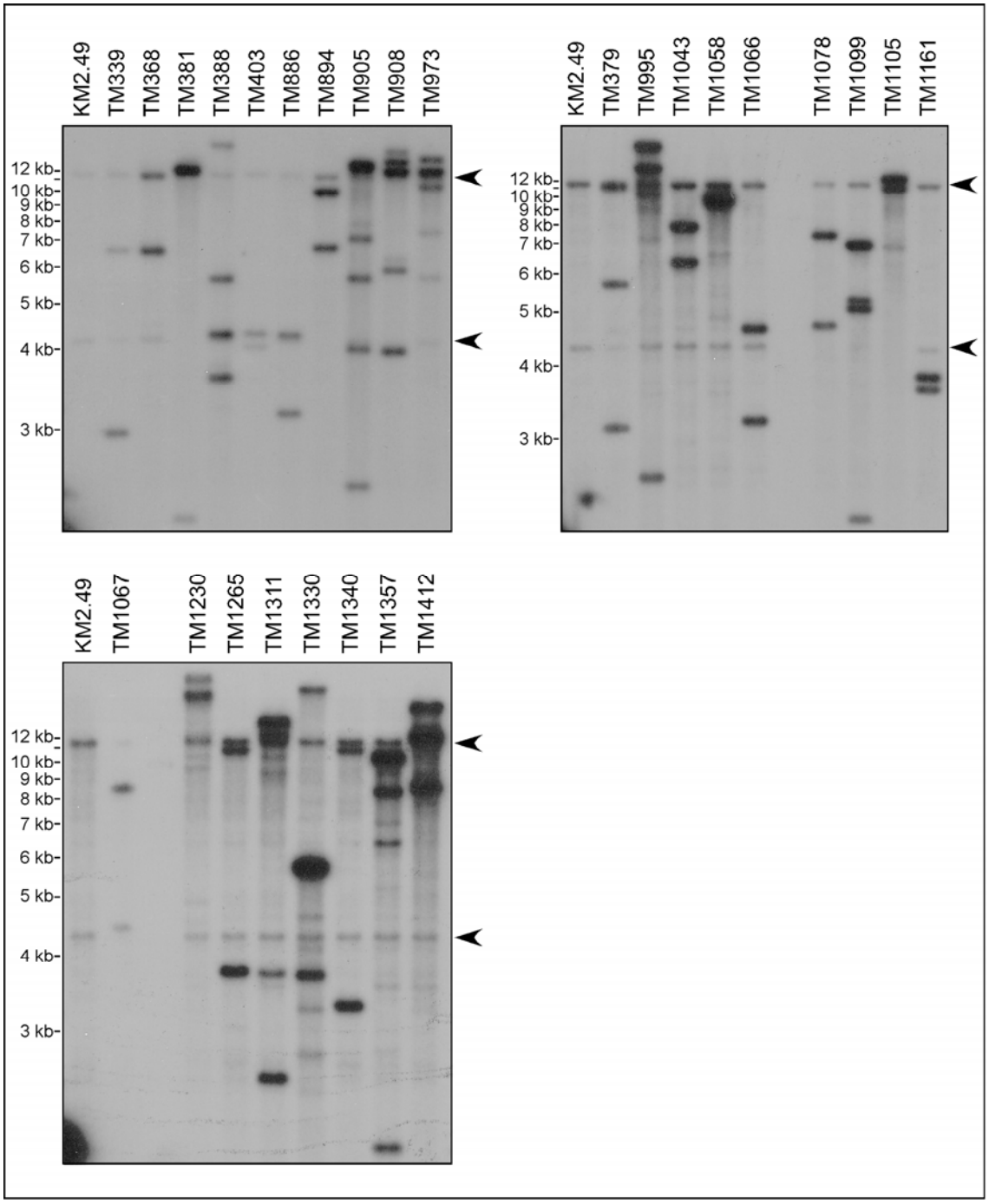

Figure 3.7 Southern analysis of TM mutants

Autoradiographs of Southern blots of *Sst*I digested genomic DNA (1μg) from randomly selected TM mutants probed with with ^{32}P-labelled pBSYT7. A single copy insertion is indicated by 2 bands. Bands common to KM2.49 and TM mutants are denoted by arrowheads. 1 kb+ standards are as marked.

3.3 TM mutant screening

The TM mutants were screened for acquisition of P*ltmM-gusA* expression in culture or loss of P*ltmM-gusA* expression *in planta*.

3.3.1 Identification of GUS$^+$ TM mutants in culture

The TM mutants were screened for the presence of GUS in culture by histochemical assay using X-Gluc as the substrate (Section 2.10.1). Mutants were allowed to grow for 7 days on PD agar containing X-Gluc then were compared to KM2.49. KM2.49 did not show any staining but three TM mutants, TM373, TM944, and TM953, produced markedly stained mycelia (Fig. 3.8). The GUS$^+$ phenotype was confirmed by screening in triplicate.

3.3.2 Identification of GUS$^-$ TM mutants *in planta*

The TM mutants were screened for loss of GUS expression *in planta* by histochemical assay using X-Gluc as the substrate (Section 2.10.2). Mutants were inoculated (Section 2.7.2) into *L. perenne* seedlings (five per mutant) with each mutant confined to a separate Petri dish. Seedlings were incubated as described (Section 2.7.2) then stained with solution containing X-Gluc. GUS activity is indicated by blue staining of the plant tissue around the inoculation site and sometimes in the emerging leaf. TM mutants were screened up to three times to confirm the GUS phenotype, the results of which are displayed in Table 3.4. Any seemingly GUS negative seedlings were examined closely under a light microscope to look for small areas of staining. Five TM mutants, TM888, TM984, TM1066, TM1197, and TM1382, did not produce any GUS staining after three separate screens. It is important to note that there are two possible reasons for GUS negative results in this assay. The mutants may have altered GUS expression due to disruption of a gene involved in regulation of P*ltmM-gusA*, including the P*ltmM-gusA* insertion itself. Alternatively, the mutant may have reduced ability to colonise the host.

Table 3.4 TM mutants with GUS negative expression *in planta*

Screen number	GUS$^+$	GUS$^-$
1	640/698	58/698
2	48/58	10/58
3	5/10	5/10

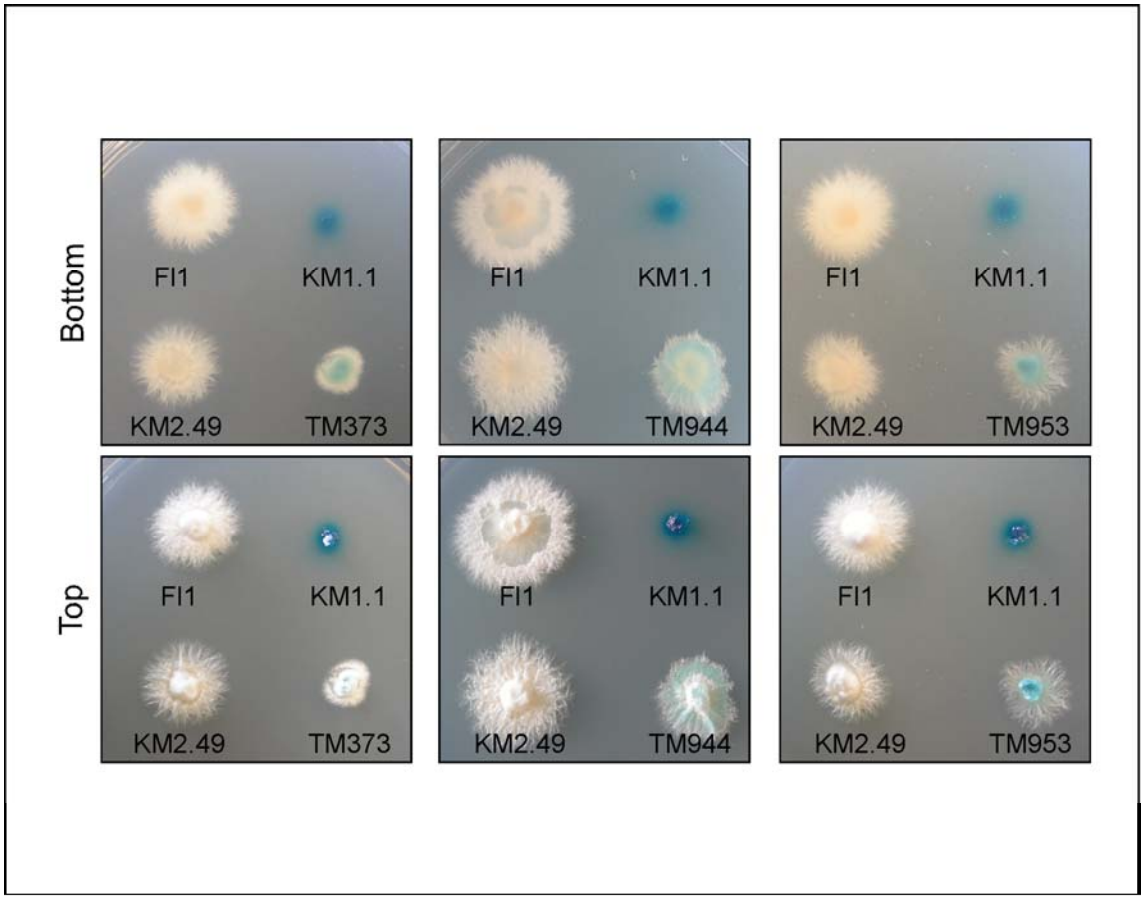

Figure 3.8 Histochemical assay for β-glucuronidase activity in TM mutants

E. festucae wildtype FI1 (negative control), KM1.1 (positive control), KM2.49 (single copy knock-in transformant), TM373, TM944, and TM953 (TM mutants) were screened for β-glucuronidase activity by histochemical assay using X-Gluc as the substrate. Colonies were grown for 7 days on PD agar containing X-Gluc. GUS expression is indicated by blue precipitate.

3.4 Analysis of T-DNA mutants of interest

Based on P*ltmM-gusA* expression analyses (Sections 3.3.1 and 3.3.2) and colony morphology (Section 3.2.1), nine TM mutants, TM373, TM888, TM944, TM953, TM984, TM1066, TM1093, TM1197, and TM1382, were selected for further analysis.

3.4.1 Copy number of T-DNA insert in TM mutants of interest

To determine the copy number of the T-DNA insertion in the TM mutants of interest, Southern blots were performed. Two separate analyses were done with *Sst*I (Fig. 3.9 A) and *Hind*III (Fig. 3.9 B). *Sst*I cuts in the middle of the T-DNA insert, producing two bands for a single copy integration (Appendix 5.1). *Hind*III cuts ~ 400 bp from the right border (RB) of the insert, producing one strong band and one very weak band (not always visible) for a single copy integration. Deletion of sequence at the RB would eliminate the weak band altogether. KM2.49 produced two bands of 11.5 kb and 4.3 kb in the *Sst*I digest and two bands of 3.1 kb and 2.7 kb in the *Hind*III digest, these were common to all TM mutants. The number of bands observed for each TM mutant and the corresponding copy numbers are recorded in Table 3.5. Based on these results mutants TM373, TM984, TM1066, and TM1382 appear to be single copy transformants, while TM888, TM944, and TM1197 were found to have two or more copies at different sites. However, the two restriction enzymes produced conflicting results for TM953 and TM1093. These TM mutants showed two bands for the *Sst*I Southern blot, suggesting a single copy integration, but also showed two strong bands for the *Hind*III Southern blot, suggesting a double copy integration. It is more likely that TM953 and TM1093 are double copy because, in both cases, the larger band in the *Sst*I Southern blot is more intense than the smaller band, suggesting that there are multiple fragments of the same size. The blots were over-exposed to confirm the number of bands for TM1066, which hybridised weakly (data not shown). Gel photographs of the Southern Blots are shown in Appendix 5.5.

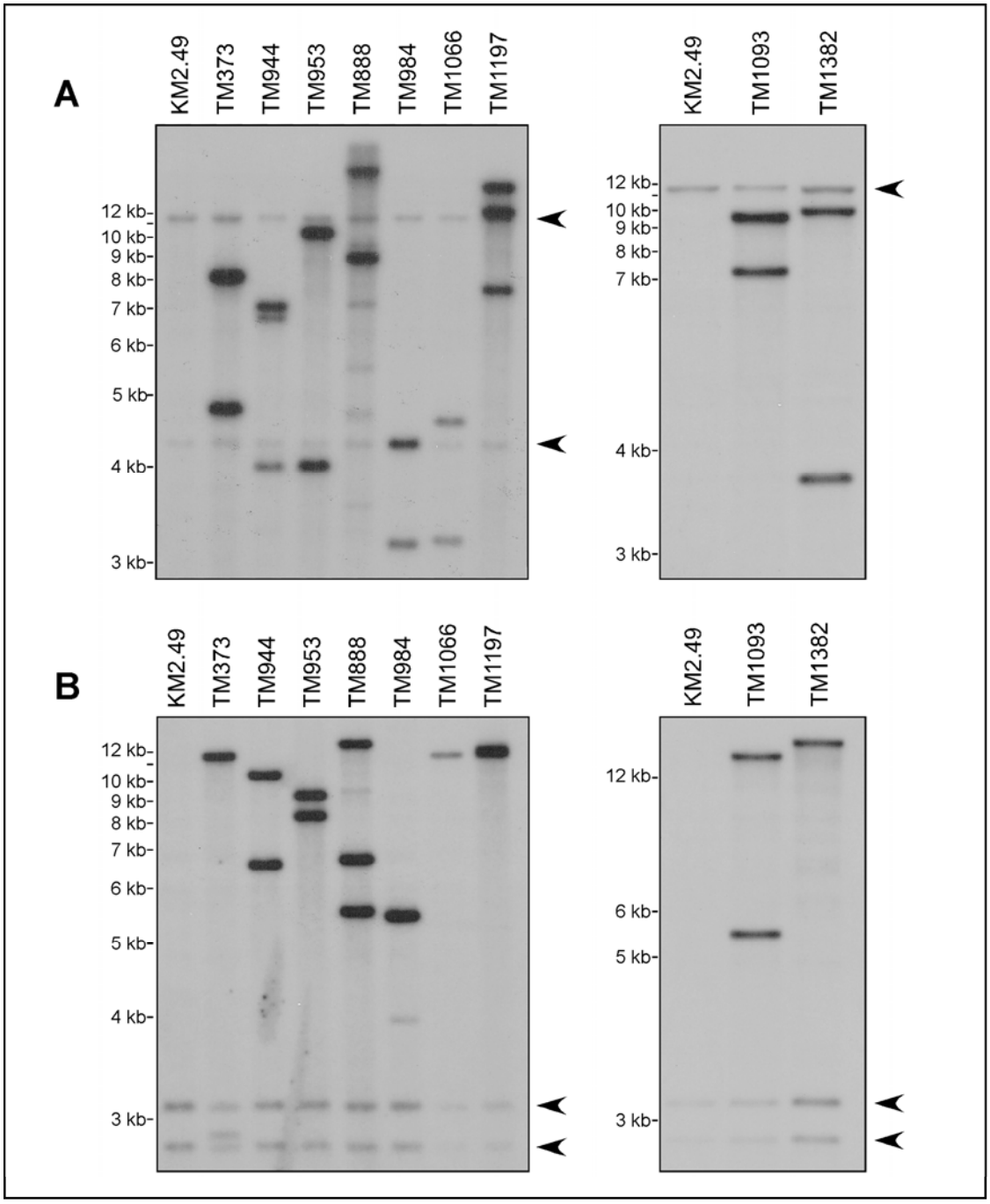

Figure 3.9 Southern analysis of TM mutants of interest

Autoradiographs of Southern blots of *E. festucae* TM mutants of interest probed with ^{32}P-labelled pBSYT7. Bands common to KM2.49 and TM mutants are denoted by arrowheads. 1 kb+ standards are as marked. **A.** Genomic DNA (1 μg) was digested with the restriction enzyme *Sst*I. A single copy insertion is indicated by 2 bands. **B.** Genomic DNA (1 μg) was digested with the restriction enzyme *Hind*III. A single copy insertion is indicated by 1 band.

Table 3.5 Copy number of T-DNA insert in TM mutants of interest

TM mutant	Number of strong bands	Copy number
***Sst*I digest**		
TM373	2	Single
TM944	3*	Double
TM953	2	Single**
TM888	Multiple	Multiple
TM984	2	Single
TM1066	2	Single
TM1197	3*	Double
TM1093	2	Single**
TM1382	2	Single
***Hind*III digest**		
TM373	1	Single
TM944	2	Double
TM953	2	Double**
TM888	3	Multiple
TM984	1	Single
TM1066	1	Single
TM1197	2	Double (close together)
TM1093	2	Double**
TM1382	1	Single

* 3 bands with one of greater intensity are assumed to be 4 fragments.

** These mutants produced inconsistent results in the two restriction enzyme analyses.

3.4.2 TM mutants of interest display varied rates of colonisation

As previously stated, there are two possible explanations for absence of P*ltmM-gusA* activity *in planta*, disruption of a gene required for GUS expression, or reduced ability to colonise the host. Therefore, infection of the host plant was investigated. GUS positive TM mutants were also included to observe any effects on the symbiosis.

Fungal infection cannot be easily determined in young seedlings due to the small size of the plant and the small amount of mycelia present, hence further analyses were carried out in older plants. *L. perenne* seedlings were inoculated and grown as described (Sections 2.7.2 and 2.7.3). Initially, five seedlings were inoculated per mutant, a sufficient number to get seedlings infected with KM2.49. Seedlings were allowed to grow until at least two tillers were present on each plant then were immunoblotted for the presence of endophyte (Section 2.8.1). Infected seedlings were found for TM373, TM944, TM953, and TM1093, indicating these mutants have a colonsation rate similar to KM2.49. The remaining potential GUS^- mutants, TM888, TM984, TM1066, TM1197, and TM1382, were inoculated again into a larger number of seedlings (minimum 50 seedlings per mutant) to test the colonisation rate. KM2.49 was inoculated at the same time as each TM mutant as a control for survival and colonisation rate. Results of survival counts and of the immunoblots to test for colonisation rate are shown in Table 3.6. No colonisation was observed for TM888, TM984, and TM1382 compared to 79–95 % colonisation of KM2.49 controls. This indicates little, or no, ability of these mutants to colonise the host. Consequently, these mutants could not be included in any further analyses of P*ltmM-gusA* expression *in planta*. TM1066 and TM1197 displayed reduced colonisation rates of 29 % and 8 % respectively, compared to 95 % colonisation in KM2.49 control plants.

It was also observed that TM1382-infected seedlings had a survival rate 20 % lower than the control, however, the total number of plants tested for each fungal strain is too low to provide conclusive evidence for reduced survival rate. It is worthwhile to note the possibility that TM1382 may produce a low colonisation rate due to death of infected seedlings, as opposed to an inability to colonise. In either circumstance, it does not allow further investigation of P*ltmM-gusA* expression in TM1382.

Table 3.6 Survival and colonisation of endophyte-infected *L. perenne* seedlings

TM mutant	Survival rate*		Colonisation rate	
	TM mutant	KM2.49	TM mutant	KM2.49
TM888	30/52 = 58%	19/42 = 45%	0/30 = 0	18/19 = 95%
TM984	44/66 = 67%	35/54 = 65%	0/44 = 0	26/35 = 74%
TM1066	14/54 = 26%	19/60 = 32%	4/14 = 29%	18/19 = 95%
TM1197	24/60 = 40%	19/60 = 32%	2/24 = 8%	18/19 = 95%
TM1382	35/60 = 58%	28/36 = 78%	0/35 = 0	25/28 = 89%

* Variation in seedling survival between separate experiments can be attributed to maintenance of plants, seasonal variation, and biotic stresses such as pest infestation.

3.4.3 TM1066 and TM1197 express GUS in mature plants

Tillers of TM1066 and TM1197 infected plants were harvested for a fluorescent assay (Section 2.9.2) to test for the presence of GUS *in planta*. Sections of pseudostem were cut 4–5 mm in length then stained in solution containing MUG as a substrate for GUS. Three separate tillers were sampled per strain. Two sections of pseudostem were pooled as one sample for TM1066 because the pseudostems were much thinner than those of the plants infected with the other endophyte strains. Both TM1066 and TM1197 were found to express GUS in two out of the three samples (Fig. 3.10) indicating that P*ltmM-gusA* expression is not affected by the T-DNA insertion. The previous GUS negative results (Section 3.3.2) can be attributed to the reduced colonisation rate of the two mutants.

3.4.4 TM1066 causes stunting of the host plant

In addition to a reduced colonisation rate, the TM1066 infected plants displayed a stunted phenotype (Fig. 3.11). The stunted plants and KM2.49 infected control plants were repotted at nine and a half weeks post-inoculation (Section 2.7.3). The plants were allowed to grow for five weeks, during which, leaves of some stunted plants displayed premature senescence and one stunted plant died. When compared to KM2.49, leaf blades of TM1066-infected plants were observed to be shorter and thinner, and pseudostems were so short that they were not visible above the soil.

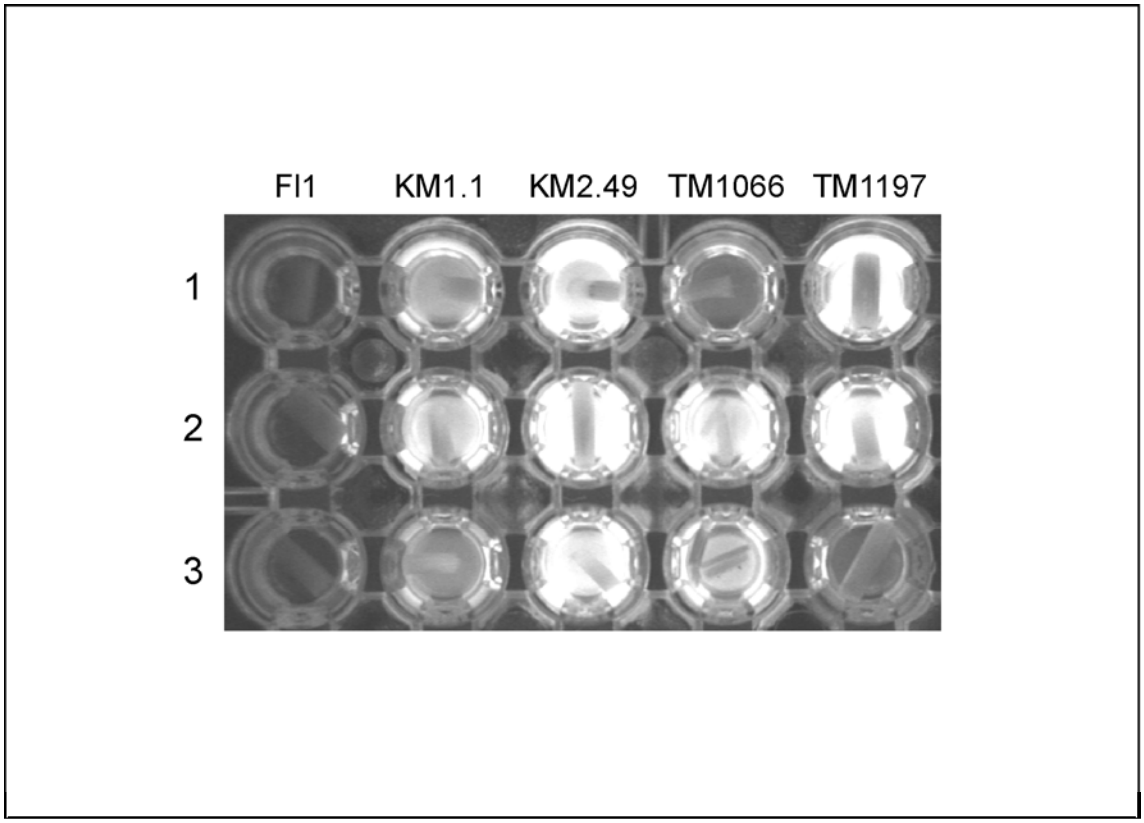

Figure 3.10 Fluorescent assay for β-glucuronidase activity in endophyte-infected mature plant tissue

Pseudostem tissue from mature *L. perenne* plants infected with wildtype Fl1 (negative control), KM1.1 (positive control), KM2.79 (single copy ectopic transformant), and KM2.49 (single copy knock-in transformant) were assayed for β-glucuronidase activity by UV fluorescence using MUG as the substrate. Separate tillers are indicated by numbering 1-3.

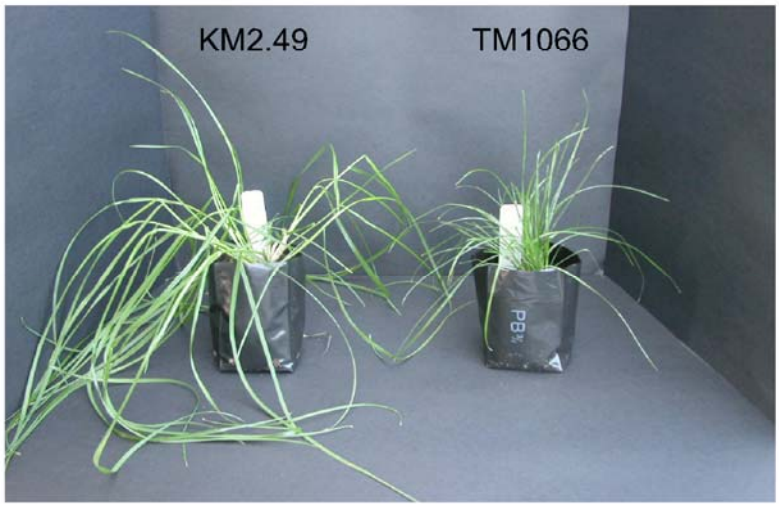

Figure 3.11 *L. perenne* plants infected with *E. festucae*

L. perenne plants infected with KM2.49 (single copy knock-in transformant) and TM1066 (single copy insertion T-DNA mutant) at 15 weeks post-inoculation.

3.4.5 TM944 and TM1066 have altered hyphal growth *in planta*

Epidermal peels from endophyte-infected *L. perenne* plants were stained with aniline blue (Section 2.8.2) to examine whether the TM mutants had altered hyphal growth *in planta*. TM1093 (data not shown), TM373, TM953, and TM1197 were found to have growth similar to that of KM2.49 (Fig. 3.12 A). TM1093 was excluded from any further analyses because it did not show a phenotype of interest for use in this study. Conversely, TM944 and TM1066 did display altered growth characteristics. Both TM944 and TM1066 displayed increased branching *in planta* (Fig. 3.12 B), and TM944 showed instances of anastomosis (Fig. 3.12 C). In older tillers, TM1066 also showed increased fungal biomass, with many intercellular spaces containing multiple hyphae (Fig. 3.12 D). This phenotype suggests uncoupling of the coordination of growth between the endophyte and the leaf of the host.

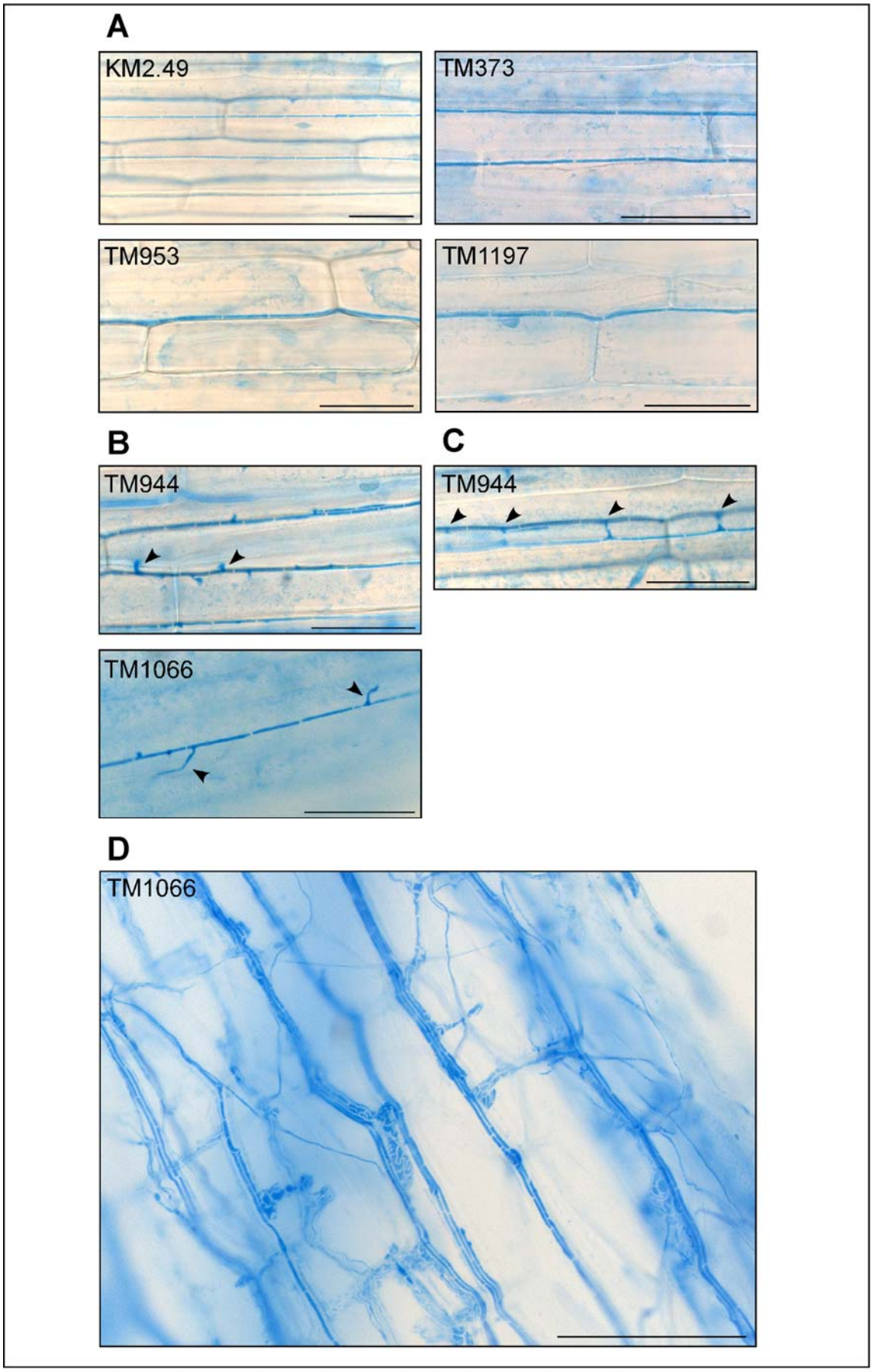

Figure 3.12 Aniline blue stain of TM mutants of interest in *L. perenne*

Figure 3.12 Aniline blue stain of TM mutants of interest in *L. perenne*

Light micrographs of epidermal peels from endophyte-infected, mature *L. perenne* plants stained with aniline blue. **A.** KM2.49 (single copy knock-in transformant) and TM mutants TM373, TM953, and TM1197. Bar = 50μm **B.** TM944 and TM1066. Branching indicated by arrowheads. Bar = 50μm. **C.** TM944. Anastomosis indicated by arrowheads. Bar = 50μm **D.** TM1066 in older tiller. Bar = 100μm.

3.5 Identification of disrupted genes in TM mutants of interest

All three mutants with altered P*ltmM-gusA* expression, TM373, TM944, and TM953, were GUS positive in culture. TM888, TM984, TM1066, TM1197, and TM1382 did not display altered P*ltmM-gusA* expression, however, these mutants displayed altered colonisation, suggesting the disrupted genes are involved in establishment or maintenance of the symbiosis. TM944 and TM1066 also showed altered hyphal growth in culture. Further analysis of TM1066 was carried out by Dr. Yvonne Rolke, while the remaining seven mutants were retained for further analysis as part of this study.

Initially, plasmid rescue (Section 2.6.6) was used in an attempt to rescue sequence flanking the T-DNA inserts in the TM mutants of interest. Despite multiple attempts, no ampicillin resistant transformants were obtained using this method.

Due to the low concentration of target DNA in the genomic digests, *TAIL*-PCR (Section 2.6.7) was used in another attempt to identify sites of T-DNA integration. *TAIL*-PCR involves three rounds of amplification using nested primers designed to the LB (left border) and RB of the T-DNA insert (see pBSYT7, Appendix 5.1) and a set of ten random, low stringency primers that, potentially, bind within the genome. Each PCR program involves a combination of low and high stringency annealing conditions designed to maximise the yield of desired product (Appendix 5.6). The LB of TM984 was successfully amplified using random primer T10. Successful *TAIL*-PCR produces three products per random primer, in this case, one product each for T10 paired with nested primers LB3, LB4, and LB5. Each product is approximately 100 bp smaller than the previous, this appears on an agarose gel as three 'steps' (Fig. 3.13 A). The largest of the three products, LB3-T10 (~ 450 bp), was cloned and isolated from the vector (Section 2.6.2.1; Section 2.6.5; Section 2.5.1). The insert was excised using *Eco*RI, which excises the LB3-T10 *TAIL*-PCR product with an additional 16 bp from the vector. The digested DNA was visualised on a mini gel (Section 2.6.3) and the fragment was found to be ~ 500 bp (Fig. 3.13 B), including the 16 bp of vector sequence.

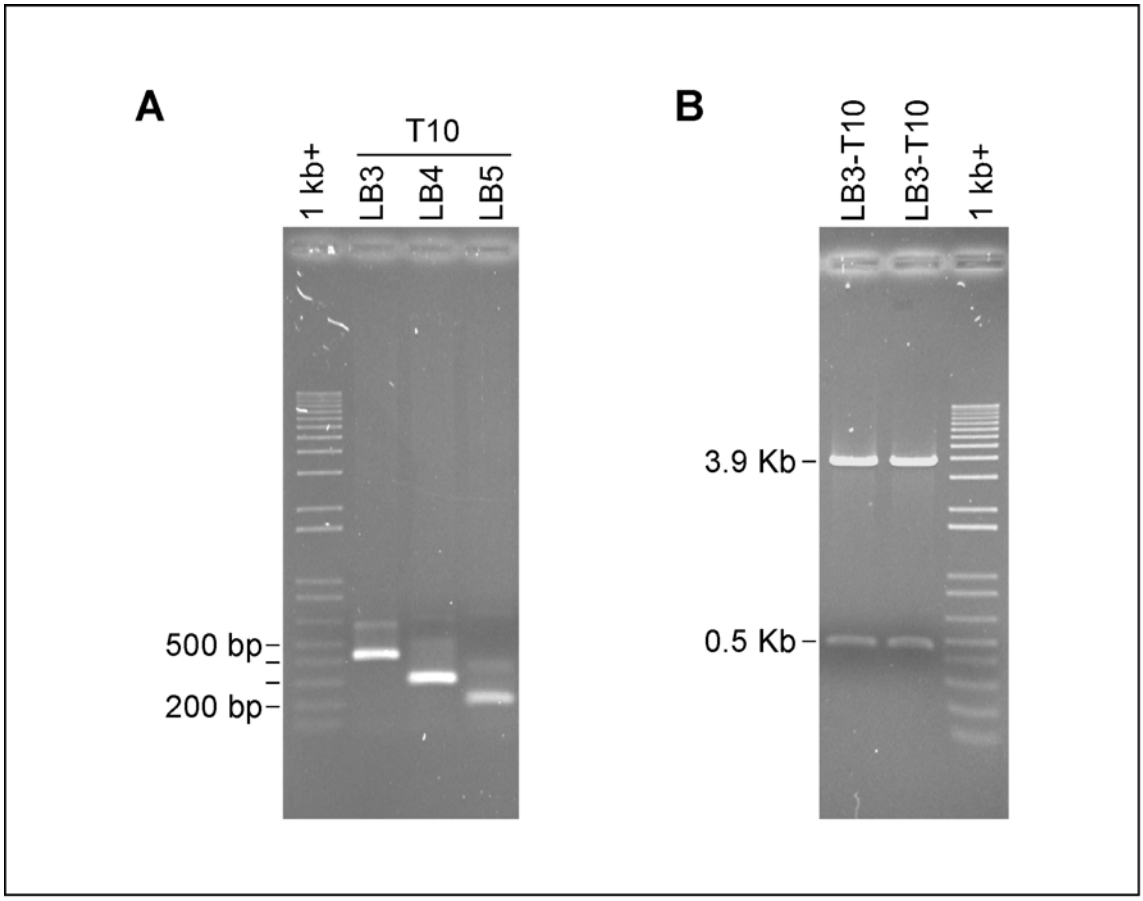

Figure 3.13 Gel photographs of TM984 *TAIL*-PCR products

TAIL-PCR was performed on TM984 genomic DNA (66 μg) using the LB primers and random primer T10. **A.** Gel photograph of LB products of random primer T10. 1 kb+ markers are as labelled. **B.** Gel photograph of product LB3-T10 excised from the pCR4-TOPO vector by digestion with *Eco*RI. The linearised vector (3.9 kb) and the LB3-T10 insert (0.5 kb) are indicated.

3.5.1 TM984 contains a disrupted CTP:cholinephosphate cytidylyltransferase gene

The LB3-T10 fragment was sequenced using M13 forward and reverse primers. The resulting sequence included 518 bp of overlapping sequence (Appendix 5.7). By manual sequence alignment using MacVector, it was found that bp 22 to 73 did not match sequence from the vector or pBSYT7, therefore, it was assumed that this was *E. festucae* genomic sequence (Fig. 3.14 A). The proposed genomic sequence was aligned with the *E. festucae* genome using Blastall. The 45 bp region from 29 to 73 was found to be a match for bp 5026 to 5070 of contig 356 in the gene A.1.7942 (Fig. 3.14 B). The gene is proposed to encode a CTP:cholinephosphate cytidylyltransferase (CCT). Six bp from 22 to 28 of the sequenced fragment aligned with part of *TAIL*-PCR primer T10 but not with the *E.festucae* genome, indicating a possible sequence variation between the strain used in this study, Fl1, and the sequenced strain, E2368. The *TAIL*-PCR primer site for LB3 was identified at bp 428 to 450 but the LB site could not be identified at the opposite end of the pBSYT7 sequence. Further analysis revealed that 85 bp were missing from the LB end of the T-DNA insert (Appendix 5.8), this probably occurred during T-DNA transfer.

The translated sequence of gene A.1.7942 was compared to the CCT genes of related filamentous fungi (Fig. 3.15). The sequence showed 74% identity to *Fusarium*, 52% identity to *Neurospora*, 56% identity to *Podospora*, 59% identity to *Magnaporthe*, and 55% identity to *Botrytis*. These results strongly support the annotation of gene A.1.7942 as a CCT gene.

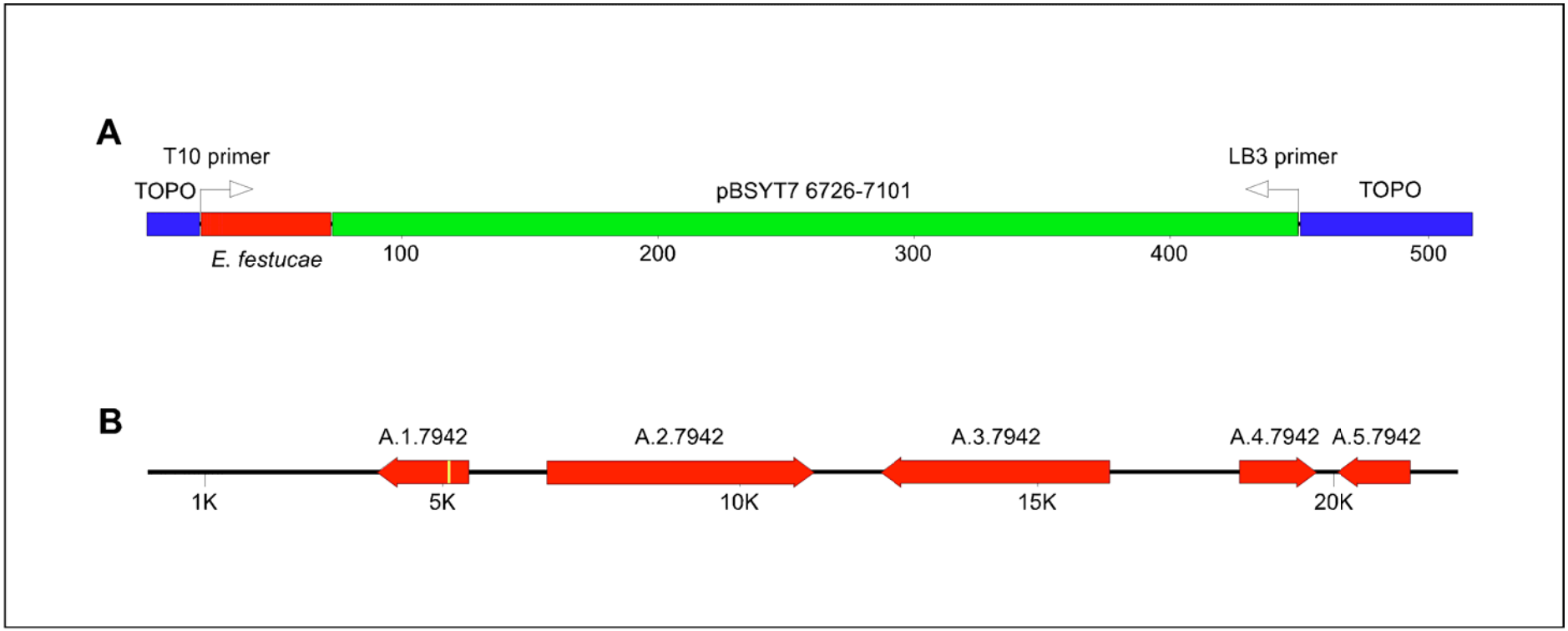

Figure 3.14 Schematic diagrams of TM984 LB flanking sequence

A. Schematic diagram of the *TAIL*-PCR product LB3-T10. *E. festucae* genome fragment (red), pBSYT7 sequence corresponding to bp 6726-7101 of the plasmid (green), Flanking TOPO vector sequence (blue). The T10 primer site (T10 primer) marks the 5′ end of the *TAIL*-PCR product, the LB3 primer binding site (LB3 primer) marks the 3′ end. Primer direction indicated by arrows. **B**. Schematic diagram of contig 356 from the *E. festucae* genome. Genome fragment from LB3-T10 (yellow).

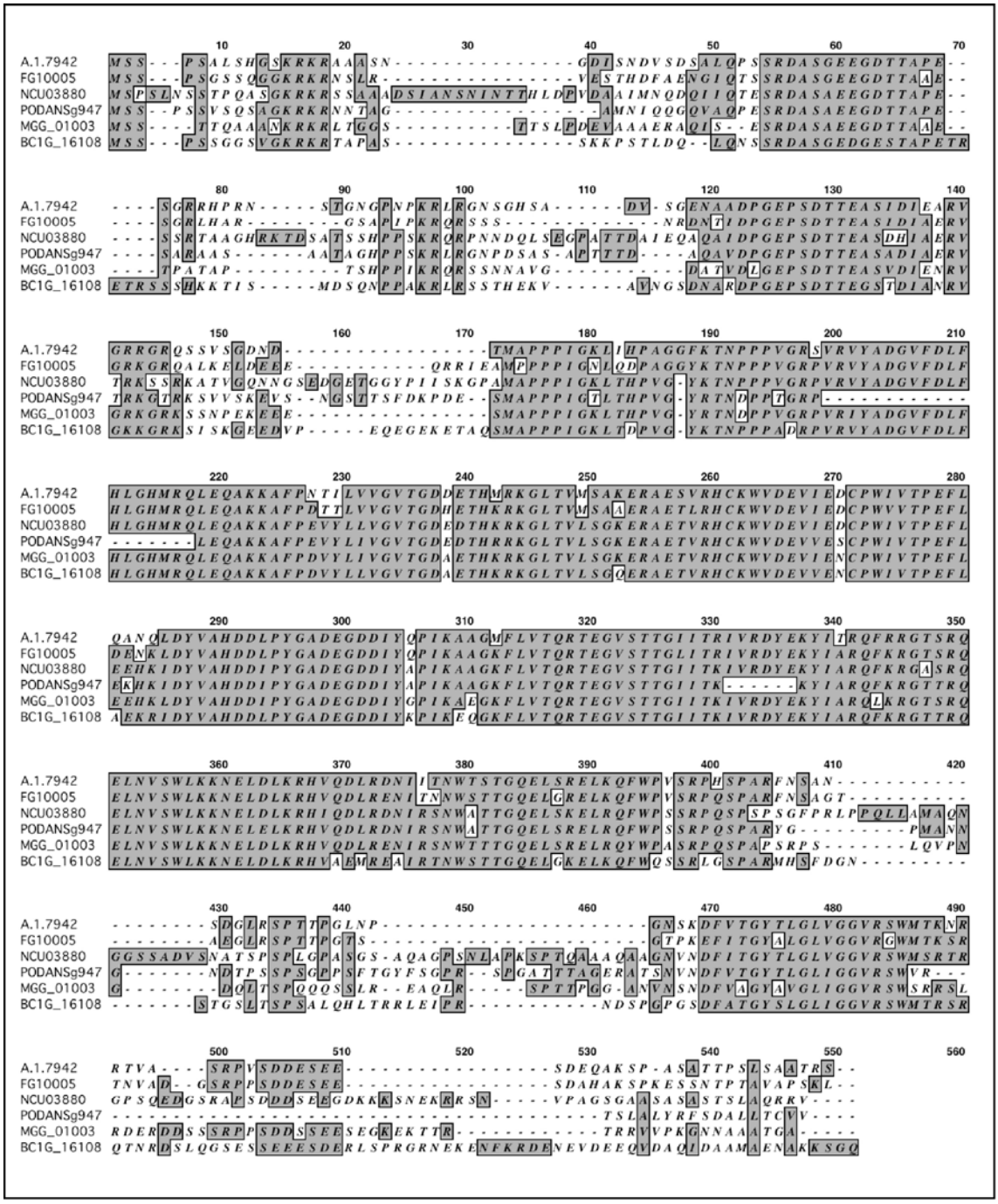

Figure 3.15 ClustlW alignment of *E. festucae* gene A.1.7942

The *E.festucae* gene A.1.7942 was aligned with CTP:cholinephosphate cytidylyltransferase genes from *Fusarium* (FG10005), *Neurospora* (NCU03880), *Podospora* (PODANSg947), *Magnaporthe* (MGG_01003), and *Botrytis* (BC1G_16108).

Chapter Four

Discussion

4.1 Development of a mutagenesis and screening system in *E. festucae*

Levels of *E. festucae ltmM* gene expression are high *in planta* but very low in culture. It is not known whether a mechanism of repression in culture or activation *in planta* regulates this pattern of expression. Therefore, a forward genetics approach (mutant phenotype to gene) was employed.

Agrobacterium tumefaciens-mediated T-DNA (ATMT) mutagenesis using P*ltmM-gusA* transformant KM2.49 provided a powerful system for studying gene expression in *E. festucae*. ATMT mutagenesis has previously been used to generate mutants screened for certain phenotypes, such as the altered symbiotic phenotypes reported by Tanaka et al., (2007) in *E. festucae*, or for genome wide mutagenesis (Betts et al., 2007). This is the first time in filamentous fungi that ATMT mutagenesis has been used with a reporter system targeted at identifying mutations affecting a specific gene activity. Reported benefits of ATMT mutagenesis over other mutagenesis methods include, increased single copy integration, more random integration, better preservation of borders, and the use of a greater variety of starting materials (Michielse et al., 2005; Betts et al., 2007; Blaise et al., 2007; Choi et al., 2007; Meng et al., 2007; Tanaka et al., 2007). ATMT mutagenesis of KM2.49 produced 41% single copy integrations. This was lower than previously reported percentages, including 63% single copy integrations reported in *E. festucae* (Tanaka et al., 2007). Choi et al., (2007) performed a comprehensive analysis of T-DNA transformants generated in *M. oryzae* and reported 82 % single copy integrations from a subset of 2026 T-DNA flanking sequences. The most likely reason for the low percentage observed in this study is the small sample number. Also, in most other studies the flanking sequence was rescued before determination of copy number. This study is limited in that there is no accounting for possible deletion of restriction enzyme sites in the T-DNA insert.

The P*ltmM-gusA* construct in KM2.49 was a reliable reporter system that mimicked the plant-induced expression pattern of the native *ltmM* gene. A single copy integration of P*ltmM-gusA* was shown to express GUS *in planta* at levels easily detected using fluorescent or histochemical assays. Furthermore, basal P*ltmM-gusA* expression was not observed in culture, allowing detection of mutants with induced P*ltmM-gusA*

expression. A 'knock-in' transformant was selected because ectopically integrated reporter genes are susceptible to positional effects. The location of the P*ltmM-gusA* reporter at the native *ltmM* gene site provided a chromatin environment comparable to that of the endogenous *ltmM* gene. Chromatin remodelling is a possible mechanism for regulation of the *ltm* genes based on the presence of AT-rich retrotransposon sequence at the *LTM* locus that is known to promote heterochromatin formation (Martienssen and Colot, 2001; Volpe et al., 2002; Young et al., 2006). The use of a knock-in was further supported by reported location specific regulation of secondary metabolite gene clusters in *Aspergillus* species. For example, *LaeA*, a regulator of secondary metabolite clusters in *A. nidulans*, is a location specific regulator (Bok et al., 2006). Genes of the sterigmatocystin (ST) gene cluster are up-regulated by LaeA only when present in the ST cluster, not when integrated ectopically into the genome. Furthermore, placement of the primary metabolic gene, *argB*, into the ST gene cluster of a Δ*laeA* mutant strain results in gene silencing (Bok et al., 2006). These results indicate that the crucial factor dictating regulation by LaeA is placement within the ST cluster chromosome environment, not the presence of upstream regulatory elements. This effect was also observed for the *nor-1* gene in the aflatoxin gene cluster of *Aspergillus parasiticus* (Chiou et al., 2002). The reporter construct P*nor-1-gusA* was only inducible when integrated at the native *nor-1* site, not when integrated ectopically.

4.2 Regulation of plant-induced P*ltmM-gusA* expression

As a result of mutagenesis and screening, three mutants were identified which showed increased expression of P*ltmM-gusA* in culture. No mutants were identified with loss of P*ltmM-gusA* expression *in planta*. These results support a mechanism of repression of P*ltmM-gusA* in culture. This is contrary to many other mechanisms of plant-induced gene expression that typically require the presence of an inducing factor for increased activity (Lau and Hamer, 1996; Collins et al., 2001; Pérez-Garcia et al., 2001; Akiyama et al., 2005; Yang et al., 2005).

The affected genes have not been identified and fully characterised in the GUS^+ mutants, therefore, when discussing these results the assumption must be made that the

effect of T-DNA integration is to decrease activity of the affected genes, not increase activity. Elliot and Howlett (2006) reported an instance of increased gene expression of two genes flanking the T-DNA integration site in a transformant of *Leptosphaeria maculans*. In this case, the T-DNA fragment had inserted in the intergenic sequence between two divergently transcribed genes and altered the promoter sequence such that both genes were over-expressed in the mutant. Hence, there is a possibility the GUS^+ phenotype is due to over-expression of an activating factor.

Generation of GUS^+ in culture mutants without any GUS^- *in planta* mutants supports a mechanism of repression of P*ltmM-gusA* in culture. Alternatively, if GUS^- *in planta* mutants were obtained as well as GUS^+ in culture mutants it implies a more complex mechanism in which an activator and a repressor work together. Possible scenarios include a repressor blocking an activation mechanism in culture or an activator inducing a repression mechanism in culture. However, screening of a greater number of mutants is required to confirm whether GUS^- mutants can be generated.

There are various explanations for why GUS^- mutants were not identified *in planta*, the simplest being that a greater number of mutants needed to be screened. Conversely, the pathway or specific activator inducing P*ltmM-gusA* expression *in planta* could have an alternative role that is essential to the symbiosis, hence, GUS^- mutants could not colonise the host. Furthermore, disruption of the pathway or specific activator may be lethal, in which case GUS^- mutants would never have been obtained.

Genes involved in utilisation of carbon or nitrogen have been linked to plant-induced genes of various symbiotic fungi (Talbot et al., 1993; Pérez-Garcia et al., 2001; Solomon and Oliver, 2002; Yang et al., 2005). This highlights the fact that plant-specific regulators can have multiple and, potentially, important roles. However, GUS was not detected in culture under the combinations of carbon or nitrogen, repressed or derepressed conditions tested (Section 3.1.4). Furthermore, *ltmM* mRNA levels do not increase under carbon- or nitrogen-limited conditions (Young et al., 2005). Together, these experiments test transcriptional and some post-transcriptional mechanisms for increased expression levels *in planta*. In addition, P*ltmM-gusA* is not induced when grown on medium with perennial ryegrass extract (May et al., 2008). Another *E. festucae* gene, *lpsB*, required for ergovaline biosynthesis, is not induced on carbon-,

nitrogen-, or phosphate- limited media, nor is it induced on medium containing perennial ryegrass extract (Fleetwood et al., 2007). Hence, there is little evidence for a nutritional link to the regulation of secondary metabolites in *E. festucae* thus far.

Results of the grow-out analysis of KM2.49 (Section 3.1.4) showed that P*ltmM-gusA* expression is not induced in mycelium at the edge of fungal colonies. This result confirms that the signal that induces P*ltmM-gusA* expression is not diffusible. Samples taken from the centre of the fungal colony, containing mycelia closest to the seedling, showed slightly elevated levels of GUS. The most likely explanation is that epiphyllous hyphae were left behind when the seedling was removed, thus producing a small amount of fluorescence.

It is worthwhile to mention that three of the four mutants that displayed altered colony morphology (Section 3.2.1) also had other phenotypes of interest. TM373 expressed P*ltmM-gusA* both in culture and *in planta*, while TM984 and TM1382 had a reduced ability to colonise the host. These results suggest altered colony morphology is a good indicator of symbiotically defective mutations.

4.3 Identification of genes involved in establishment or maintenance of the symbiosis

Investigation into potential GUS$^-$ mutants *in planta* led to the identification of five mutants with mutations affecting establishment or maintenance of the symbiosis. Mutant phenotypes included reduced colonisation rate, altered hyphal growth, and stunting of the host plant. One of the disrupted genes was identified as a CTP:cholinephosphate cytidylyltransferase (CCT).

The TM mutant TM984 did not infect any plants in the colonisation test (Section 3.4.2). Rescue of the LB of the T-DNA insert by *TAIL*-PCR allowed identification of the integration site in gene A.1.7942 on contig 356 of the *E. festucae* genome, however, rescue of the RB is required to confirm the integration site and check for deletions. The gene was annotated as encoding a CCT. Alignment of A.1.7942 with CCT genes from

other filamentous fungi strongly supported this annotation. CCT catalyses the conversion of cholinephosphate to CDP-choline, this is the rate-limiting step in the CDP-choline pathway for the production of phosphatidylcholine (PC) from free choline (Kent, 1990). The CDP-choline pathway is the main pathway for PC production in mammals but in yeast it is secondary to the phosphatidylethanolamine-methylation pathway (Friesen et al., 2001). Phosphatidylcholine is an important component of eukaryotic membranes and a source of secondary messengers in signal transduction (Billah and Anthes, 1990; Exton, 1990). CCT is active at low levels in its soluble form but is activated in response to lipids, often resulting in translocation to the membrane (Watkins and Kent, 1992). Activation also coincides with a decrease in phosphorylation of the enzyme (Watkins and Kent, 1991). CCT is known to have a regulatory function in mammals but a regulatory role in yeast has yet to be identified (Johnson et al., 1992). However, the activation of CCT by lipids is thought to be essential for regulatory function and is a common property of mammalian and yeast CCT. In *Aspergillus fumigatus*, the CCT gene is called PCT1 (Do et al., 2005). Little is known about CCT in filamentous fungi but the CDP-choline pathway of *A. fumigatus* is very similar to that of *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Candida albicans,* and *Neurospora crassa* (Do et al., 2005).

Two TM mutants were also observed to have altered hyphal growth *in planta*. Staining of TM1066 hyphae *in planta* showed increased fungal biomass and hyper-branching, indicative of a change in the regulation of hyphal growth (Tanaka et al., 2006). In addition to a reduced colonisation rate, these phenotypes suggest disruption of a gene involved in the establishment or maintenance of the symbiosis. This was supported by stunting of all plants infected with TM1066, implying a response by the host plant to a normally asymptomatic association. A similar phenotype characterises the *noxA* and *noxR* mutants of *E. festucae*. Plants infected with the *noxA* mutant are stunted, exhibit premature senescence, and eventually die. The mutant shows increased hyphal biomass, hyper-branching, and vacuolation *in planta* (Tanaka et al., 2006). Plants infected with the *noxR* mutant show stunting and premature senescence, with increased fungal biomass and some hyper-branching early in the colonisation of the tiller (Takemoto et al., 2006). The *noxA* and *noxR* genes regulate hyphal growth through a mechanism involving reactive oxygen species and *noxA* is essential for establishment of the symbiosis (Takemoto et al., 2006; Tanaka et al., 2006). Analysis of TM1066 by Dr.

Yvonne Rolke revealed that the single copy T-DNA insert had disrupted two genes. The RB of the insert was deleted, as well as genomic sequence containing an intron and part of the disrupted genes. The genes were identified as a pseudouridine synthase gene and an *mkk2* (a mitogen-activated protein kinase kinase or MAPKK) gene. The *mkk2* gene is a strong candidate for this phenotype as it is part of a MAPK cascade called the Slt2 pathway. This pathway is generally important for pathogenesis and cell wall integrity in fungal pathogens of plants and humans (Zhao et al., 2007). The pathway consists of Bck1 (MAPKKK), Mkk1 and Mkk2 (MAPKK), and Slt2 (MAPK) (Jimenez-Sanchez et al., 2007). The two MAPKKs are functionally redundant as either Mkk1 or Mkk2 alone can maintain signal transduction through the pathway. Mkk1 and Mkk2 are phosphorylated concomitantly with activation of the Slt2 pathway. However, Slt2, the end product of the pathway, is required for the phosphorylation of both MAPKKs suggesting a feedback mechanism of regulation (Jimenez-Sanchez et al., 2007). MAPKs are involved in a variety of extracellular signals and in regulation of developmental processes. Activation of a MAPK cascade results in the activation of transcription factors that regulate expression of specific genes in response to external stimuli (Zhao et al., 2007). Pseudouridine synthases are involved in changing the structure of non-coding RNAs (Hellmuth et al., 2000), which is less likely to be involved in the symbiosis.

The GUS^{+} mutant, TM944, showed increased hyphal branching and instances of anastomosis *in planta*. However, TM944 is a double copy T-DNA mutant so the *in planta* growth phenotype may be unrelated to the mutation inducing P*ltmM-gusA* in culture. Rescue of sequences flanking both T-DNA integrations is needed then individual knock-outs can be made to assign the phenotypes to the causative genes.

4.5 Conclusion

In collaboration with Dr. Kimberley May, a mutagenesis and screening system was developed for identifying genes involved in the regulation of plant-induced expression of *ltmM*. While only 1,414 mutants have been screened to date, many interesting mutants have been obtained with altered *ltmM* expression and symbiosis phenotypes. Thus far, only GUS^+ in culture mutants have been isolated, although characterisation of colonisation mutants is still in progress and could lead to identification of GUS^- phenotypes. It can be concluded that a mechanism of repression is involved in the plant-induced expression of *ltmM*, either directly or indirectly.

4.6 Future work

Further analyses will be carried out on all mutants of interest. Rescue of flanking sequences from the three GUS^+ mutants is a high priority, while screening of a greater number of mutants is required to confirm whether GUS^- mutants can be generated. Part of this analysis would involve confirmation of colonisation rates of mutants that, as yet, have not been successfully inoculated into plants. Any mutants that achieve infection can then be assayed for GUS.

Detailed analysis of in culture and *in planta* phenotypes of TM984 and TM1066 will be carried out. The phenotype of TM1066-infected plants will be fully characterised including examination of the roots, measurement of leaf dimensions, and observation of senescence patterns. The pseudouridine synthetase and *mkk2* genes will be knocked out to confirm which gene is causing the phenotype. The RB of TM984 needs to be rescued to check for deletions and confirm the insertion site. Identification of the affected genes in the TM mutants of interest will allow more targeted investigations into potential pathways or mechanisms associated with those genes.

Lastly, analysis of more integration sites is required to improve the mutagenesis system, including investigation into average copy number, proportion of tagged deletions, preservation of borders, and bias of integration. This would provide a better estimate of the number of transformants required to cover the genome.

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Appendix

5.1 Physical map of pBSYT7 vector

5.2 P*ltmM-gusA* knock-in construct

5.3 Southern blot of P*ltmM-gusA* transformants

5.4 Gel photographs of Southern blots of TM mutants

5.5 Gel photographs of Southern blots of TM mutants of interest

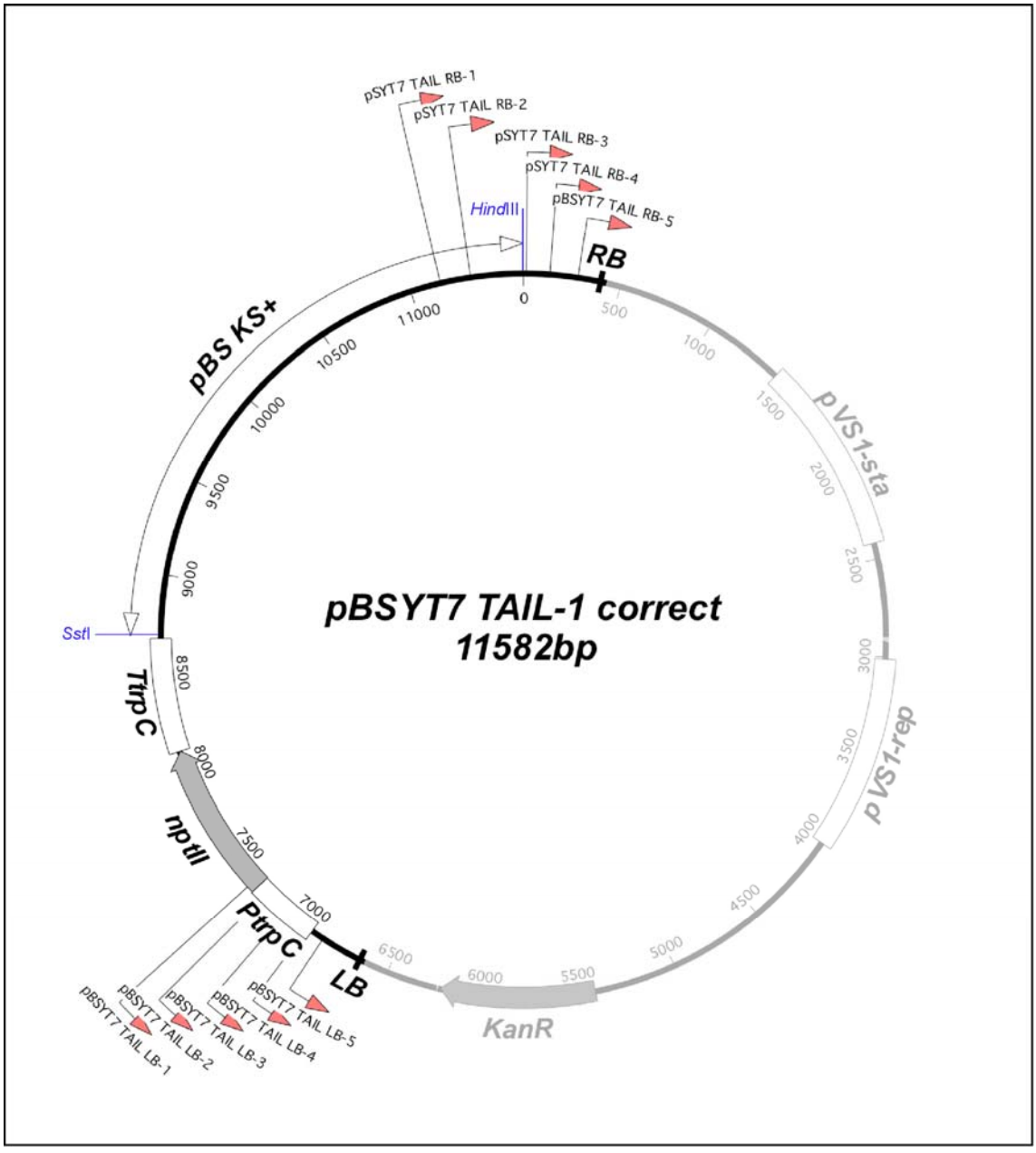
5.6 *TAIL*-PCR conditions

5.7 Sequence data for TM984 LB3-T10

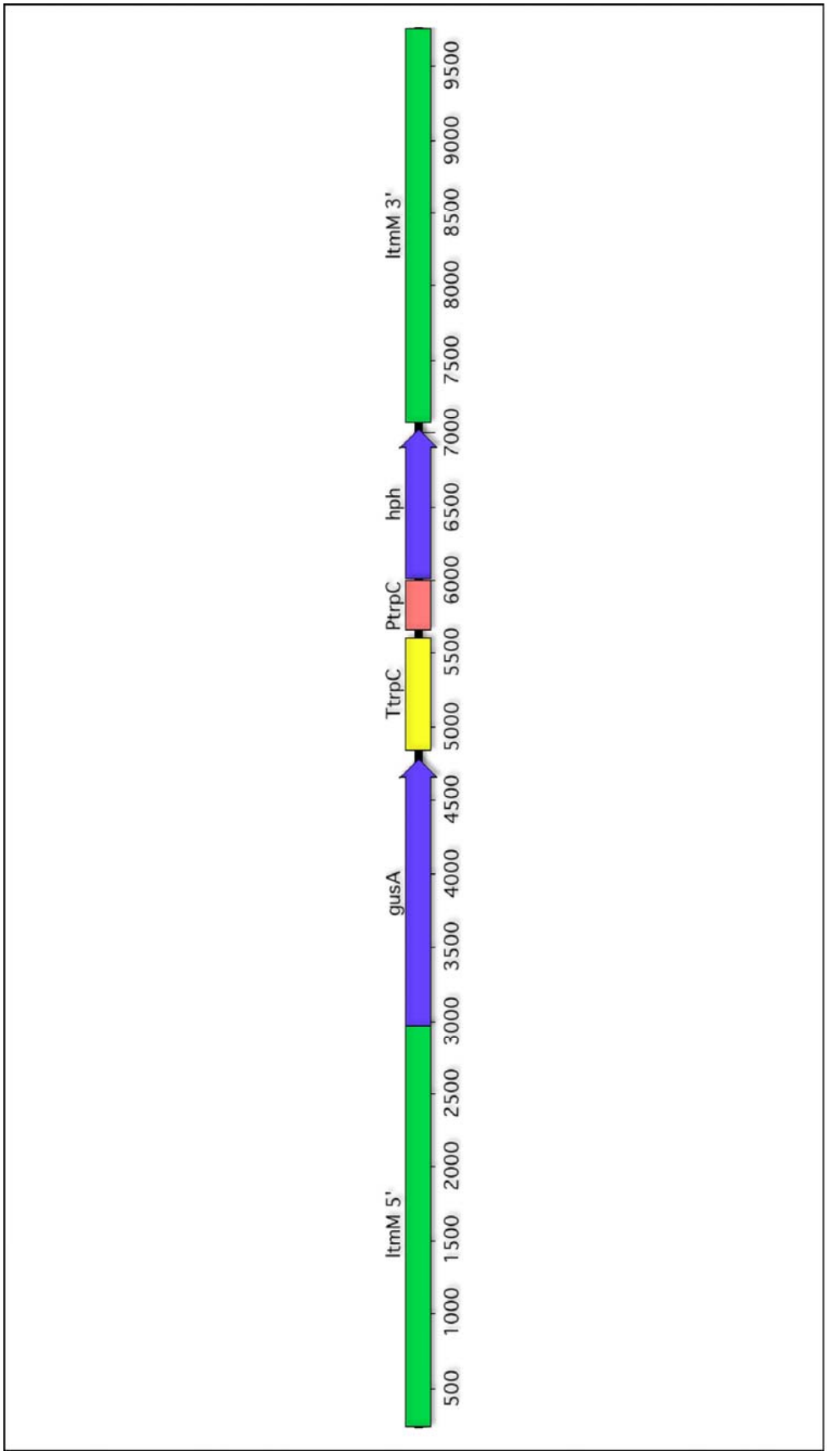
5.8 pBSYT7 LB deletion in TM984

Appendix 5.1 Physical map of pBSYT7 vector

The solid sequence represents the T-DNA insert, which is excised at LB and RB during T-DNA transfer. *TAIL*-PCR nested primers are labelled at the LB (pBSYT7 TAIL LB-1 to LB-5) and RB (pBSYT7 TAIL RB-1 to RB-5). Primer direction is indicated by arrows.



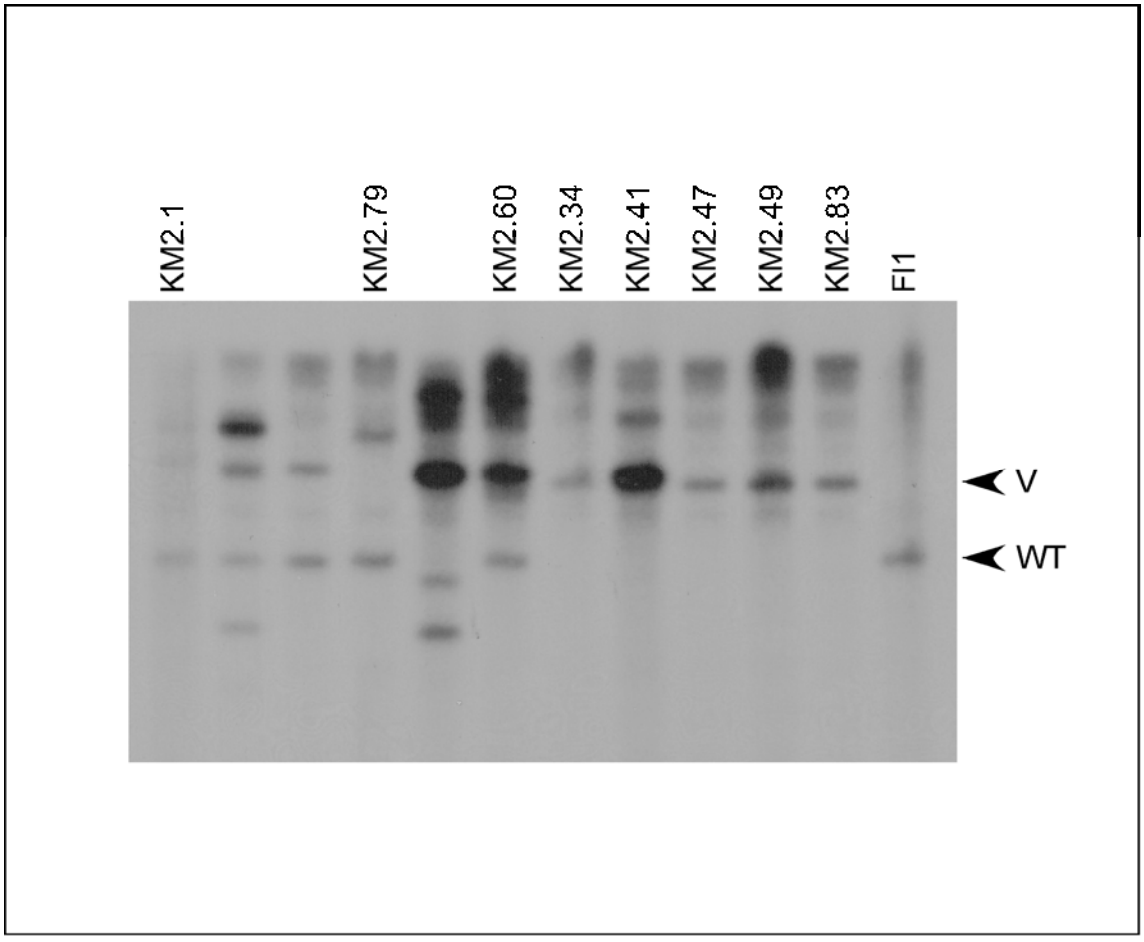
Appendix 5.2 P*ltmM-gusA* knock-in construct



P*ltmM-gusA* reporter construct. Genes are depicted as blue arrows and ltmM flanking sequence is represented by green boxes. The *trpC* promoter (pink) and terminator (yellow) are also shown as boxes.

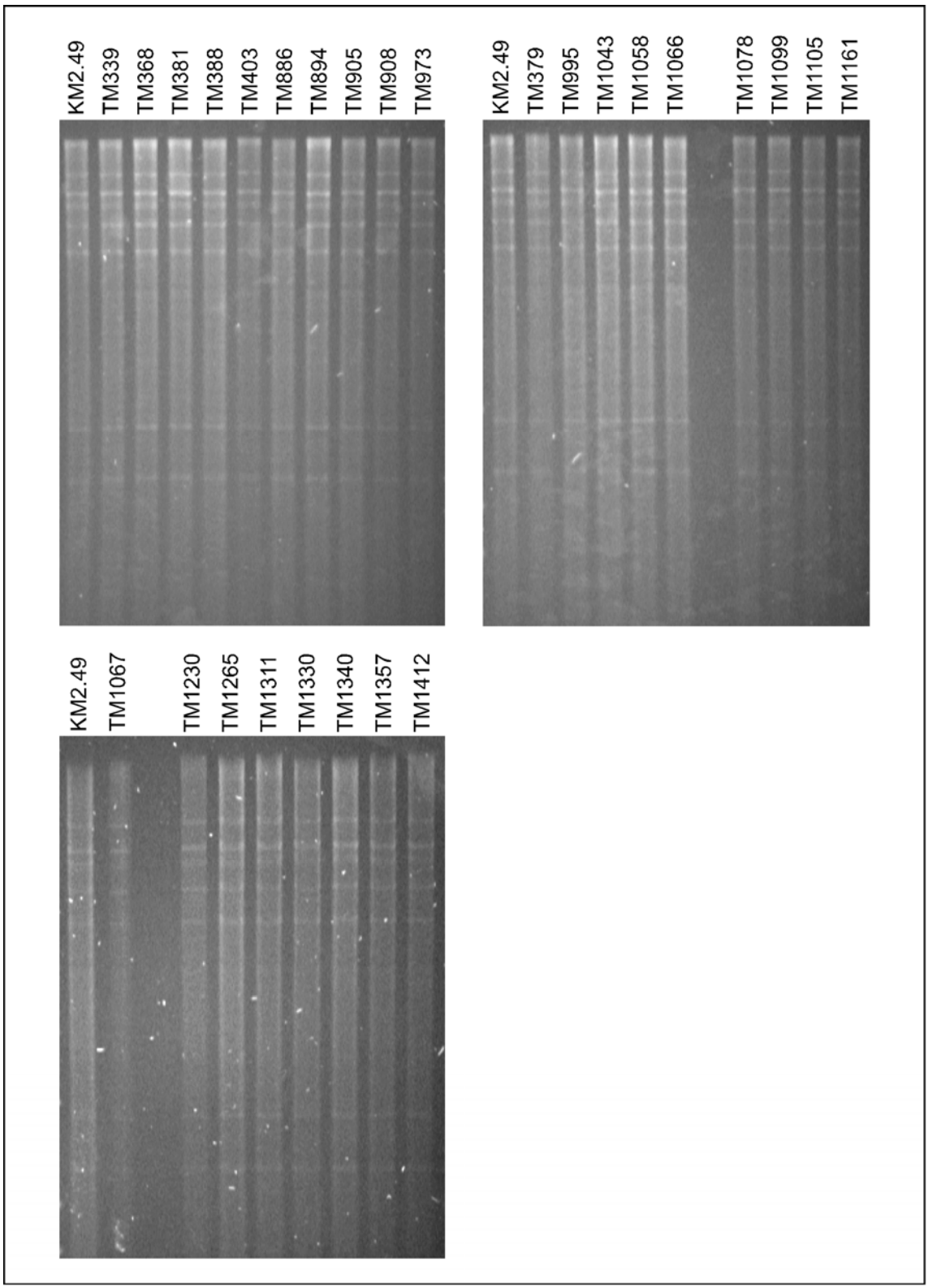
Appendix 5.3 Southern blot of P*ltmM-gusA* transformants

Autoradiograph of Southern blot of *Xho*I digested genomic DNA from *E. festucae* P*ltmM-gusA* transformants probed with ^{32}P-labelled *ltmM* 3' flanking sequence. (V) 9.6 kb vector insert containing the P*ltmM-gusA* construct. (WT) 7 kb wildtype *ltmM* gene. Tandem repeats of vector insertion (9.9 kb) are not distinguishable from single vector homologous integration. (May, unpublished data).



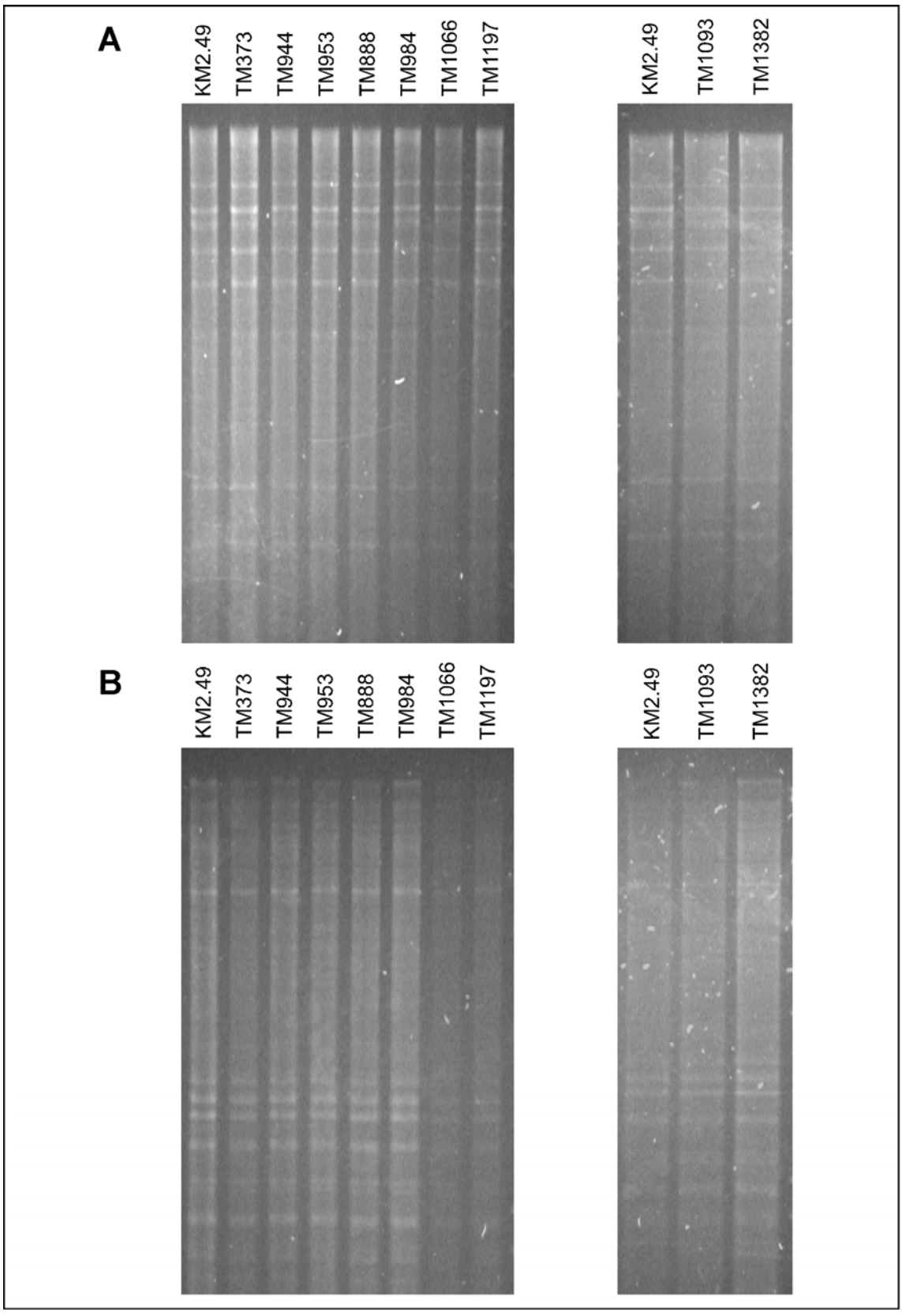
Appendix 5.4 Gel photographs of Southern blots of TM mutants

Gel photographs of Southern blots from Fig. 3.7.



Appendix 5.5 Gel photographs of Southern blots of TM mutants of interest

Gel photographs from Southern blots from Fig. 3.9. **A**. *Sst*I digested DNA. **B**. *Hind*III digested DNA.



Appendix 5.6 *TAIL*-PCR conditions

TAIL1 Program (4.5 h) (using the Eppendorf gradient cycler):

93 °C	1 min	
95 °C	1 min	
94 °C	30 s	5x
59 °C	1 min	
70 °C	2.5 min	
94 °C	30 s	
25 °C	3 min	
ramping to 70 °C	Over 3 min	
70 °C	2.5 min	
94 °C	10 s	15x
66 °C	1 min	
70 °C	2.5 min	
94 °C	10 s	
66 °C	1 min	
70 °C	2.5 min	
94 °C	10 s	
29 °C	1 min	
70 °C	2.5 min	
70 °C	5 min	

TAIL2 Program (~3 h):

94 °C	1 min	
94 °C	10 s	12x
59 °C	1 min	
70 °C	2.5 min	
94 °C	10 s	
59 °C	1 min	
70 °C	2.5 min	
94 °C	10 s	
29 °C	1 min	
70 °C	2.5 min	
70 °C	5 min	

TAIL3 Program (~1.5 h):

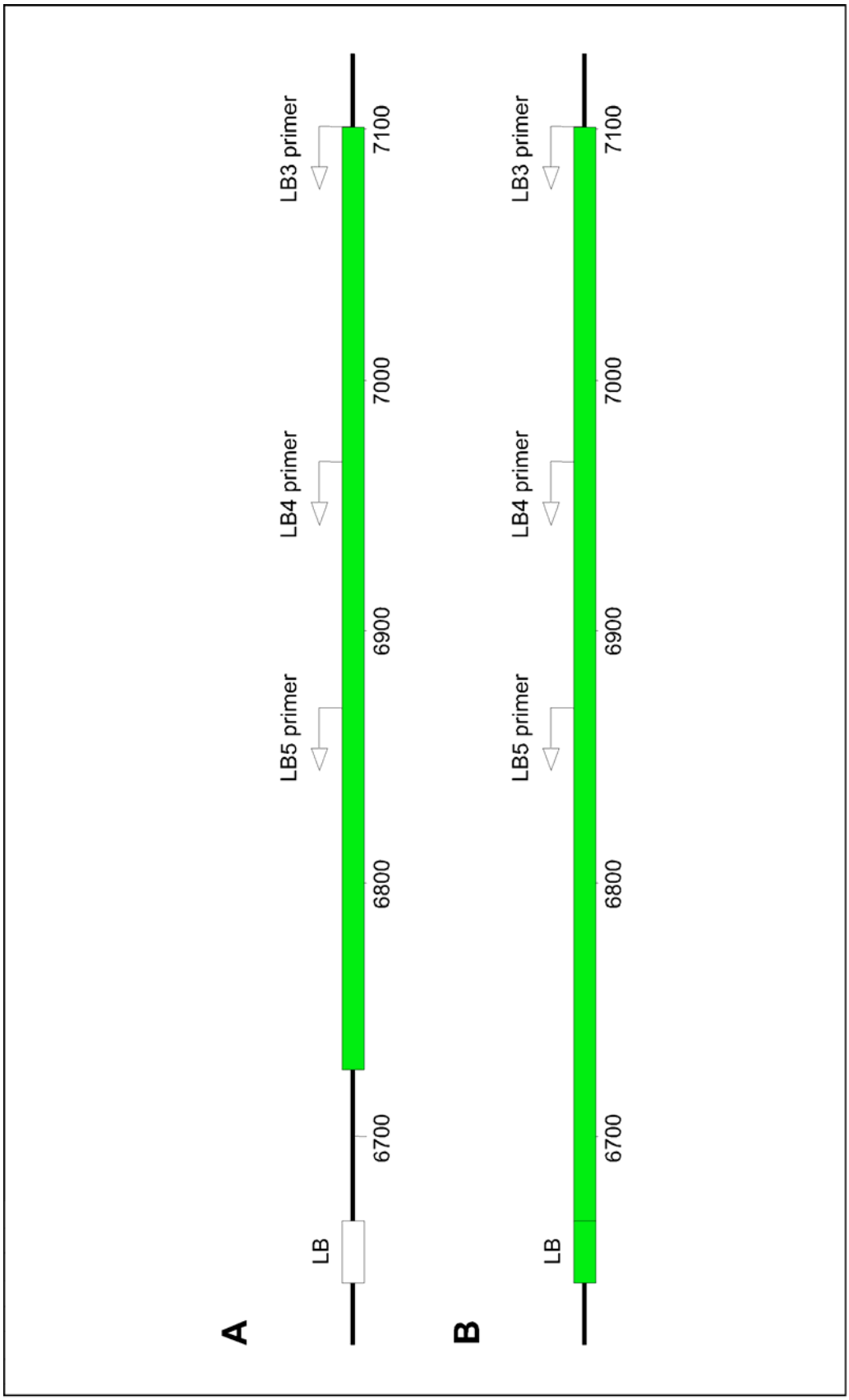
94 °C	1 min	
94 °C	15 s	20x
29 °C	30 s	
70 °C	2 min	
70 °C	5 min	

Appendix 5.7 Sequence data for TM984 LB3-T10

bp 29–73 aligned with part of contig 356 of the *E. festucae* genome. The sequence corresponds to part of a CTP:choline phosphate cytidylyltransferase gene.

```
Sequence Range: 1 to 518

         10         20         30         40         50
AGCGGCCGCGAATTCGCCCTTACGATCGCGGCCAACAGGCGGAGGATTGG
TCGCCGGCGCTTAAGCGGGAATGCTAGCGCCGGTTGTCCGCCTCCTAACC

         60         70         80         90        100
TCTTGAAGCCTCCAGCAGGATGACGCCGAATTAATTCGGGGGATCTGGAT
AGAACTTCGGAGGTCGTCCTACTGCGGCTTAATTAAGCCCCCTAGACCTA

        110        120        130        140        150
TTTAGTACTGGATTTTGGTTTTAGGAATTAGAAATTTTATTGATAGAAGT
AAATCATGACCTAAAACCAAAATCCTTAATCTTTAAAATAACTATCTTCA

        160        170        180        190        200
ATTTTACAAATACAAATACATACTAAGGGTTTCTTATATGCTCAACACAT
TAAAATGTTTATGTTTATGTATGATTCCCAAAGAATATACGAGTTGTGTA

        210        220        230        240        250
GAGCGAAACCCTATAGGAACCCTAATTCCCTTATCTGGGAACTACTCACA
CTCGCTTTGGGATATCCTTGGGATTAAGGGAATAGACCCTTGATGAGTGT

        260        270        280        290        300
CATTATTATGGAGAAACTCGACTCTAGAGGATCCCCGATATTGAAGGAGC
GTAATAATACCTCTTTGAGCTGAGATCTCCTAGGGGCTATAACTTCCTCG

        310        320        330        340        350
ACTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACAATGAATGCCTATT
TGAAAAACCCGAACCGACCTCGATCACCTCCAGTTGTTACTTACGGATAA

        360        370        380        390        400
TTGGTTTAGTCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAG
AACCAAATCAGCAGGTCCGCCACTCGTGTTTTAAACACAGCAAACTGTTC

        410        420        430        440        450
ATGGTTCATTTAGGCAACTGGTCAGATCAGCCCCACTTGTAGCAGTAGCG
TACCAAGTAAATCCGTTGACCAGTCTAGTCGGGGTGAACATCGTCATCGC

        460        470        480        490        500
AAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTA
TTCCCGCTTAAGCAAATTTGGACGTCCTGATCAGGGAAATCACTCCCAAT

        510
ATTCTGAGCTTGGCGTAA
TAAGACTCGAACCGCATT
```

Appendix 5.8 Deletion of LB of T-DNA insert in TM984



A. Fragment of LB of T-DNA insert rescued from TM984 (green). **B**. Intact LB of T-DNA insert (green).