DEVELOPMENT OF THE ß-GLUCURONIDASE REPORTER GENE SYSTEM TO STUDY *ACREMONIUM* ENDOPHYTE INTERACTIONS WITH PERENNIAL RYEGRASS

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ABSTRACT

A transformant of the fungal endophyte *Acremonium lolii*, strain Lp19, containing the *gusA* gene under the control of the constitutive P*gpd* promoter was generated, and assigned the name KS1. Analytical digests and Southern hybridisation showed that this transformant contained a single chromosomally integrated copy of the *gusA* gene. The transformation frequency of Lp19 was found to be very low, and attempts to increase the transformation frequency were unsuccessful.

KS1 was used to artificially infect seedlings of several different genotypes of *Lolium perenne*, all of a single cultivar, 'Nui'. These seedlings were grown into mature plants, and the endophytically produced GUS enzyme was extracted from individual plant tissues. Assays were performed on the enzyme extracts, and the levels determined were used as a measure of endophyte metabolic activity. Alterations of the *gusA* gene in some plants was detected by Southern hybridisation. One alteration was found to result in loss of GUS activity, the other did not appear to alter *gusA* expression.

Levels of transformed endophyte GUS activity were initially compared between clonal plant material of a single genotype. Statistical analysis revealed that no significant differences were detectable for a particular tissue between the different plants. This showed that plant material of identical genotype could be pooled for analysis without the pooling of the individual plants having an affect on the outcome of the analysis.

Next, levels of the transformed endophyte GUS activity were compared between genetically diverse perennial ryegrass plants of cultivar 'Nui'. Significant differences in GUS activity were detected in most tissues tested between the different genotypes, with only the most mature tissue displaying no detectable differences.

Finally, a single plant of each of two individual genotypes was divided into several clonal plants, and the resulting mature plants were pooled in their genotypes for analysis of GUS, peramine, ergovaline and lolitrem B levels. The F test was not particularly sensitive in this experiment, and only one major difference between genotypes could be detected. Despite this, some trends emerged which were found to be consistent with those found in other studies. Metabolic activity and peramine levels were shown to be highest in the leaf sheath tissue, with levels generally decreasing with increasing tissue age. Lolitrem B was found to be highest in leaf sheath tissue also, but with levels increasing in general with tissue age. Ergovaline levels were very low in all tissues. The results presented show the potential of the use of the GUS reporter gene system to study endophyte gene expression *in planta*, and pooling of plants can be carried out to allow simultaneous study of toxin expression.

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CHAPTER 1. INTRODUCTION

1.1 THE BIOLOGICAL IMPORTANCE OF FUNGI

Fungi are highly versatile and adaptable organisms. They are extremely successful in competing and interacting with other organisms in a range of heterogeneous environments. Many fungi are well adapted to the niches they occupy; they are widely distributed and occur in many ecologically diverse and complex habitats. Fungi are heterotrophs and must obtain organic compounds from the surrounding substrate to support their growth. The various means by which fungi exploit their environment are diverse, making these organisms interesting and unusual. The very efficient breakdown of materials by fungi, from complex biological macromolecules into simpler molecules makes them an important component of the carbon and nitrogen cycles, as they make these compounds readily available for use by other organisms (Isaac, 1992). The use of fungi in large cultures on a commercial basis for industrial processes is widespread. For example fungi are used in baking and brewing industries (e.g. *Saccharomyces cerevisiae*), and are also used in the production of pharmaceutical compounds, for example antibiotics (Martin, 1991).

Fungi gain access to available nutrient substrates in different ways and are often grouped together accordingly. Three nutritional modes are recognised: biotrophy; living cells of an organism are used to obtain resources; saprotrophy, the use of non-living organic resources; and, necrotrophy, living tissues are first killed by the fungus then used saprotrophically. These modes are further subdivided to categorise fungi on the basis of their nutritional and ecological behavior in the natural environment. Fungi which interact with living organisms obtain nutrients through symbiosis; by parasitism in which nutrients are taken from, and to the detriment of the host, or mutualism where both partners derive benefit from the association (Isaac, 1992).

Research has revealed a widespread mutualistic association between grasses, our most familiar and important plant family, and endophytic fungi. Asymptomatic, systemic fungi that occur intercellularly within grass tissues have dramatic effects on the physiology, ecology, and reproductive biology of host plants (Clay, 1990). Two mutualistic symbioses, which have been the subject of great interest and documentation, are *Acremonium lolii* in perennial ryegrass (*Lolium perenne*) and *Acremonium coenophialum* in tall fescue (*Festuca arundinacea*).

1.2 *ACREMONIUM* ENDOPHYTES OF GRASSES

Acremonium endophytes are asexual filamentous fungi which form mutually symbiotic associations with grasses of the subfamily Pooideae (White, 1987). The symbioses of these endophytes in grasses has been known for decades, but their agricultural and economic importance was recognised only relatively recently. In 1977 Bacon (Bacon *et al.*, 1977) identified an association between *A. coenophialum* and fescue toxicosis, a toxicity syndrome affecting livestock consuming *A. coenophialum* infected tall fescue. Fletcher and Harvey made a similar type of association in 1981, between the endophyte in perennial ryegrass, *A. lolii*, and the syndrome known as ryegrass staggers (Fletcher and Harvey, 1981). In 1985, Stewart recognised the protective effect of a perennial ryegrass endophyte against Argentine stem weevil (*Listronotis bonariensis*) (Stewart, 1985). Since these discoveries, many other protective features have been found to be conferred on host grasses by these endophytes (reviewed by van Heeswijck and McDonald, 1992), and many studies have been carried out with the aim of gaining a better understanding of the components of these unique relationships. In this thesis introduction I will discuss aspects of the symbioses, focusing mainly on the relationships between *A. lolii* and perennial ryegrass, and *A. coenophialum* and tall fescue.

1.2.1 Taxonomy and Evolution

Acremonium endophytes are asexual and non-pathogenic, and are evolutionarily derived from the sexual (teleomorphic) fungi of genus *Epichloë* (Leuchtmann and Clay, 1990; Schardl *et al.*, 1991; Tsai *et al.*, 1994). The phylogenetic relationship between the asexual endophytes and the sexual endophytes is supported by their morphology, secondary product biochemistry, allozyme profiles, host range interactions, and DNA sequences (Schardl *et al.*, 1994; Scott and Schardl, 1993). This distinct and closely related group of endophytes are in the class Ascomycetes, the largest class of fungi, and belong to the tribe Balansiae of the Clavicipitaceae (Leuchtmann, 1992). The anamorphic state of *E. typhina*, and the symptomless endophytes related to it, are placed in the form genus *Acremonium* section Albo-lanosa, and are commonly referred to as the "*Acremonium* endophytes" (Schardl, 1995).

Species of this section of *Acremonium* are not considered to be naturally related to other *Acremonium* species (Leuchtmann, 1992). The taxonomy of this section is currently under review and Glenn *et al.* (1996) have recently proposed that the anamorphs of *Epichloë* and the closely related asexual grass endophytes be reclassified into the new form genus *Neotyphodium*. Molecular genetic studies have identified fundamental differences in the genome composition between the sexual and some of the asexual fungi, with the strong implication that interspecific hybridisation has played a significant role in the evolution of many of the asexual *Acremonium* endophytes (Schardl, 1995). An indication of hybrid origins is heteroploidy, observed as multiple loci for genes which are usually found as a single copy in the sexual species. This was first suggested by Leuchtmann and Clay (1990) when they found that multiband allozyme patterns were typical in the asexual *Acremonium* endophytes, but not for the sexual *Epichloë* isolates (Schardl, 1995). Furthermore, upon analysis and comparison of sequences of the noncoding segments of the β-tubulin (*tub2*) gene by Tsai *et al.* (1994), it was found that whereas each *Epichloë* isolate had a single *tub2* gene, most tall fescue endophytes had two or three distinct copies, suggesting that at least three hybridisation events had occurred. Comparisons of

the 5' noncoding regions of the *pyr4* gene, encoding orotidine-5'-monophosphate decarboxylase, in several fungal species by Collett *et al.* (1995) demonstrated that the *pyr* genes of two endophytes, *E. typhina* and *A. lolii* were the most likely ancestors of the two *pyr* genes found in an interspecific hybrid strain of *Acremonium*, known as strain Lp1 (Schardl *et al.*, 1994).

1.2.2 Life Cycle and Distribution

The *Acremonium* endophytes are identified in grasses by the presence of septate, intercellular, infrequently branching hyphae running longitudinally in plant tissue (Christensen *et al.*, 1991). *A. lolii* and *A. coenophialum* are true endophytes, unable to undergo an external infective stage, and thus spend their entire life cycle within organs of the host without external signs of infection (Siegel *et al.*, 1985). Spores of these two *Acremonium* species have never been found on or in plants, although conidia may be produced in artificial culture. These endophytes therefore must clonally propagate themselves through transmission of the hyphae present in the maternal plant to seeds produced on that plant as a result of the mycelium being present among terminal stem meristematic cells which give rise to the inflorescence (Fig. 1). The endophyte then spreads into the new tillers formed by the seedling (White *et al.*, 1993). Plants grown from seeds from infected plants are expected to contain the same endophytic individual present in the maternal plant, except in the development of endophyte free tillers. In a mature *A. lolii* infected perennial ryegrass plant, most of the mycelium present is found in leaf sheaths, flowering stems and seed heads, with less in leaf blades and very little or none in roots. Older sheath and blade tissue contains a higher concentration of mycelium than the less mature tissue. The amount of mature *A. lolii* mycelium has been observed to change seasonally, rising in spring and declining in the late autumn (Musgrave and Fletcher, 1984; Di Menna *et al.*, 1992; Keogh *et al.*, 1996).

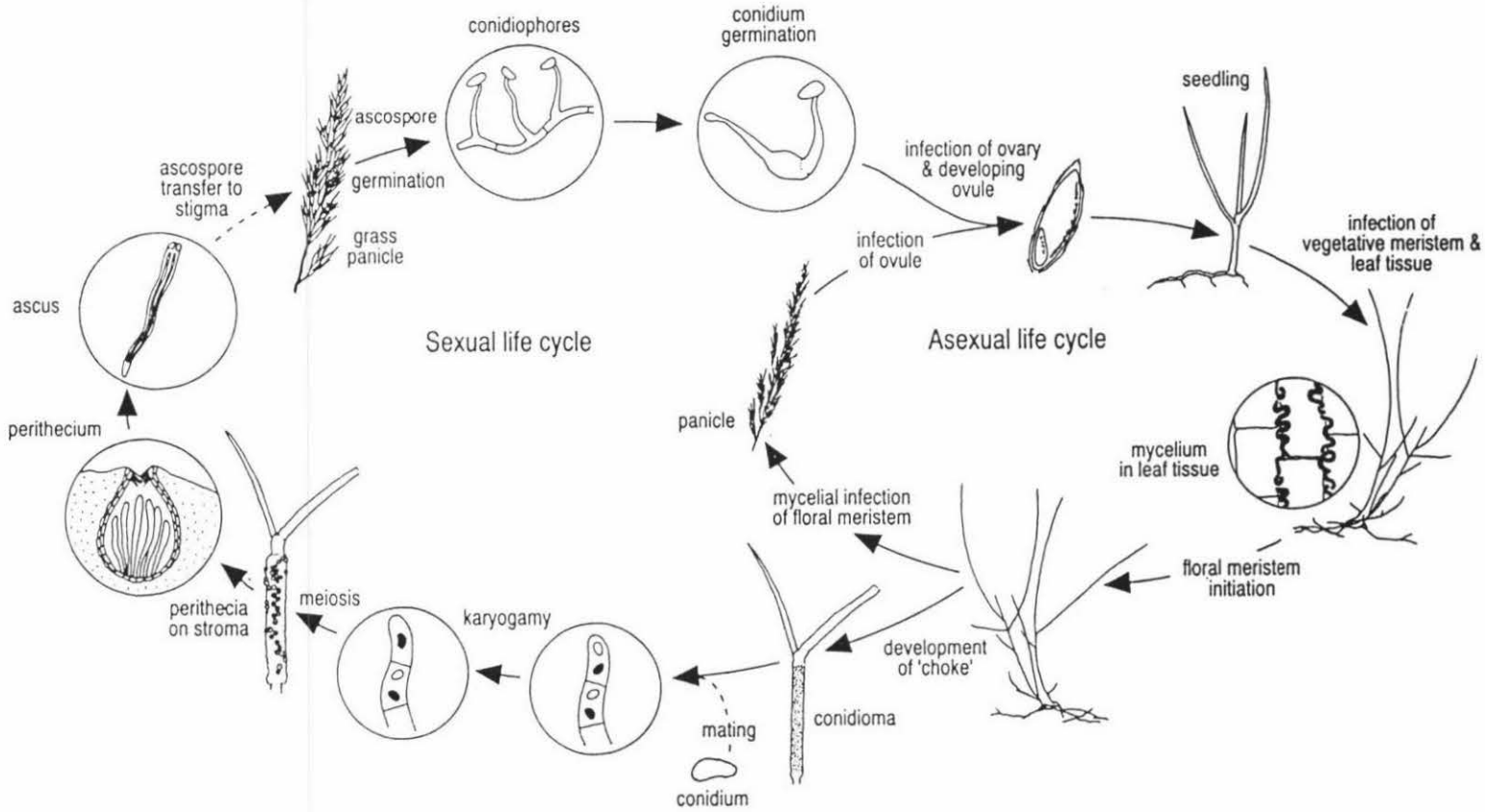
Perennial ryegrass and tall fescue are among the most important and widely grown pasture grasses in New Zealand and the United States respectively. Millions of hectares of

Fig. 1. Life cycle of *Epichloë typhina* (sexual) and an *Acremonium* endophyte (asexual) growing symbiotically with tall fescue (*Festuca rubra*).

The sexual cycle of *Epichloë* spp. (represented here by *E. festucae*) is initiated with the emergence of mycelia from the leaf sheath intercellular spaces, and the development of a conidioma on the leaf surface which causes 'choke' of the immature inflorescences, and gives rise following an insect-mediated cross, to sexual stromata. These stromata (ascostromata) have imbedded orange-tan perithecia bearing ascospores. The role of these spores in infection of the inflorescence has not been demonstrated but there is detailed description of microcyclic conidiation which usually follows germination of *E. typhina* ascospores in culture. The conidia form on short conidiophores, usually in a T-shaped configuration. The asexual life cycle of *E. typhina* involves intercellular growth and seed dissemination. If *E. typhina* was to lose the ability to form the conidioma, it would become an asexual endophyte, and follow the life cycle similar to that of an *Acremonium* endophyte (Scott and Schardl, 1993).

The endophytes are maintained in vegetative plants by mycelial growth in the apical meristems, which then spread through the intercellular spaces of the leaf sheaths. There is no evidence for invasion of host cells by mycelia, so nutrients must be obtained from the intercellular matrix and fluids of the tissue. Following floral meristem development, the mycelia invade the ovaries and ovules, and subsequently are present in the mature seed. Infected seed gives rise to infected plants, maternally transmitting the endophytes to new generations (Scott and Schardl, 1993).

Dashed lines indicate spore transfer. Diagram prepared by Liz Grant, Department of Ecology, Massey University, New Zealand.


conidiophores
conidium germination
ascospore
germination
grass panicle
ascospore transfer to stigma
ascus
perithecium
perithecia on stroma
meiosis
karyogamy
mating
conidium
conidioma
development of 'choke'
mycelial infection of floral meristem
panicle
infection of ovule
infection of ovary & developing ovule
seedling
infection of vegetative meristem & leaf tissue
mycelium in leaf tissue
floral meristem initiation
Sexual life cycle
Asexual life cycle

these grasses support many millions of sheep and cattle. As it has been estimated that over 90 percent of these pastures are infected with the *Acremonium* endophytes, (Siegel *et al.*, 1985) it is obvious that these unique symbiotic associations have a high impact on, and are of great importance to agriculture. The widespread association of *Acremonium* endophytes in pooid grass species suggests a coevolutionary relationship between the subfamily Pooideae and the endophytes (White *et al.*, 1993).

1.3 ENDOPHYTE - HOST COMPATIBILITY

All symbiotic associations between higher plants and fungi involve complex interactions between the host and endophyte, for example, recognition and communication events, metabolite transfer and in the case of the host, changes may result in the cell walls due to a physiological response to the interaction (Stone, 1989). The association between the *Acremonium* endophytes and their host grasses is very complex, resulting in the production of an array of protective alkaloids. The host range of endophytes may indicate under what circumstances the effects of the symbiosis is most beneficial to the host (Leuchtmann, 1992). Currently there is much interest in the identification of strains which produce the protective alkaloids effective against insect pests, but lack those which target livestock. Such studies have led to observations regarding host specificity and compatibility of *Acremonium* endophytes.

1.3.1 Variation within *Acremonium* endophyte isolates

Variation exists among isolates of the *Acremonium* endophytes of perennial ryegrass and tall fescue. Christensen and Latch (1991), studied endophytic fungi isolated from the tissue of tall fescue plants collected from New Zealand and other parts of the world and compared parameters such as growth rate, conidial length, antifungal activity and sensitivity to benomyl. Considerable cultural, morphological and physiological variations were observed. A great deal of variation was also observed within *Acremonium* endophytes isolated from perennial ryegrass worldwide (Christensen *et al.*, 1991). Similar

parameters to those used by Christensen and Latch (1991), were used for the comparisons, and in addition alkaloid (peramine and lolitrem) production was analysed. In both of these studies the range of variations observed show that there is significant variation not only between *Acremonium* endophytes isolated from different host grass species, but also between the endophytes found to occur naturally in a single host grass species.

1.3.2 Variation in the ability of *Acremonium* endophyte isolates to form associations with grasses

Host specificity is based on specific adaptations between fungus and host grasses, and determines the host range of an endophyte (Leuchtmann, 1992). The compatibility of an endophyte and a host can be studied within natural and novel associations by artificially inoculating grasses with a range of different endophytes (Latch and Christensen, 1985), and observing the outcomes of these associations.

Christensen (1995), reported on six distinctive *Acremonium* endophyte taxonomic groups identified from studies of perennial ryegrass, tall fescue and meadow fescue. Of these endophytes, three form a natural association with tall fescue, two with perennial ryegrass and one with meadow fescue When representative isolates of these six groups were used to inoculate all three grasses, in most cases compatible associations resulted showing that these strains are not host specific, although in nature they are confined to just one species (Christensen, 1995). This result is thought to reflect the close phylogenetic relationship between the three grass species used, and perhaps a similar result would not occur in less closely related species.

In the study by Christensen (1995), and in another by Koga *et al.* (1993), some endophyte-host grass combinations showed signs of incompatibility, which probably reflect a resistance of the grass towards that endophyte by way of a mechanism which is as yet unknown (Koga *et al.*, 1993). Two types of histological incompatibility reactions

were seen. One type was hyphal death, noted in *A. coenophialum*-perennial ryegrass associations. When perennial ryegrass plants were artificially inoculated with several *A. coenophialum* isolates and infected leaf sheaths were studied, it was found that most hyphae in the older sheath tissue were dead, with hyphae in the younger tissue sometimes exhibiting low vigour. Transmission electron microscopy revealed that the hyphae were distorted and collapsed, and contained structural abnormalities. Although no hypersensitive response was observed in the neighbouring host tissue, the intercellular matrix was found to be electron dense, signaling unusual changes to the matrix structure. This matrix sustains contact between host and endophyte tissue, and may be essential for nutrient supply to the endophyte (Christensen, 1995; Koga *et al.*, 1993). The second type of incompatibility reaction affected host tissue, resulting in stunted tillers with necrosis in the region of the apical meristem when tall fescue was inoculated with *A. uncinatum* or LpTG-2 (*Lolium perenne* taxonomic group 2; Christensen *et al.*, 1993). The hyphae in the mildly affected tillers appeared normal, but those in the dying tillers contained vacuoles and had low vigour (Christensen, 1995).

Evidence for the great complexity of the endophyte-host associations is provided by the considerable variability in endophyte characteristics, and in the ability for endophytes and host to form compatible associations. An understanding of this complexity is necessary before associations which could be used to improve pasture qualities can be determined. As the balance between compatibility and incompatibility appears to be fine, and no pattern for beneficial associations is clear, it is obvious that much work is required on these associations

1.4 BIOLOGICALLY ACTIVE SECONDARY METABOLITES AND THEIR EFFECTS

The relationship between host and endophyte results in the production of a range of secondary metabolites (alkaloids) (Fig. 2), which can be grouped into four classes, and

have been found to enhance the ecological fitness of both of the symbiotic partners. Not all associations result in the same array of responses; the compounds produced by endophytes differ both qualitatively and quantitatively in infected plants (Christensen *et al.*, 1993). The number and specific kind of responses depend not only on genetic factors, but also environmental factors, such as temperature and water stress. The measurement of endophyte incidence within cultivars or ecotypes is of importance as it indicates the potential impact of endophyte related effects on agronomic performance and animal production, but it does not enable an accurate prediction of the nature or scale of these effects (reviewed by van Heeswijck and McDonald, 1992). The regulation of alkaloid synthesis involves a complex interaction between the endophyte, the plant and their environment (Rowan, 1993).

The production of the tremorogenic neurotoxic lolitrems is a characteristic of the association between perennial ryegrass and *A. lolii*. Paxilline (Fig. 3), a biosynthetic precursor to the lolitrems is also a tremorogen (Rowan, 1993). Lolitrem B (Fig. 3) is believed to be responsible for the nervous disorder, perennial ryegrass staggers (Gallagher *et al.*, 1982), the most obvious sign of toxicity shown by livestock (e.g. sheep, cows and horses) grazing *A. lolii* infected perennial ryegrass pasture. This disorder is widespread in New Zealand, and occurs sporadically in south east Australia (Prestidge, 1993). As lolitrem B is concentrated at the base of the plant in the sheath material, this disorder usually occurs in dry conditions when feed is short and animals consume the basal sheath material (Prestidge, 1993). Clinical symptoms of ryegrass staggers include fine head tremors and trembling neck and limb muscles. These can increase in severity to uncoordinated and jerky movements causing collapse and severe muscular spasms lasting 5-10 seconds, after which the animal stands and appears unaffected (Prestidge, 1993). Although mortality due to staggers is generally low, major costs are incurred through lowered daily weight gains, reduced pasture suitable for feeding, and increased time and labour required to tend to the affected animals (Prestidge, 1993).

Ergovaline (Fig. 3) is the major ergopeptine alkaloid found in *A. coenophialum* infected tall fescue, and is responsible for fescue toxicosis (Garner *et al.*, 1993). Fescue toxicosis in cattle is often described by symptoms of stress, photosensitisation, excessive salivation, rough hair coats, increased respiratory rates and increased rectal temperatures with decreased body weight gains. Fat necrosis is shown as hard masses of mesenteric fat surrounding the intestinal tract. These lesions can disrupt normal movement of ingesta and interfere with both calving and renal function (Thompson *et al.*, 1993). Levels of ergovaline do not tend to correlate with levels of endophyte present, so it is thought that the plant genotype affects the levels. These alkaloids are believed to be produced by the fungus (Siegel and Schardl, 1991).

The saturated amino pyrrolizidines, or loline alkaloids (e.g. *N*-formyl loline and *N*-acetyl loline) (Fig. 3) have been identified in the associations between tall fescue and *A. coenophialum* (Bush *et al.*, 1993). Lolines are potent insecticides (Clay, 1991). Very high levels of these lolines have been detected in endophyte infected plant material, but not in fungal cultures or uninfected plants. They are possibly of plant origin, being synthesised as a consequence of the plant-endophyte interaction (Siegel and Schardl, 1991).

Another feature of endophyte infected grasses is the production of pyrrolopyrazine alkaloids, for example peramine (Fig. 3), which act as feeding deterrents to insects, thus reducing insect herbivory in endophyte infected pastures. Peramine, a fungal metabolite is a potent feeding deterrent. In particular peramine acts to protect the host from one of the most important pasture pests in New Zealand, the Argentine stem weevil which feeds on the foliage of perennial ryegrass (Latch, 1993). There is also evidence that several other insect pests are deterred from feeding on endophyte infected pasture, for example the black beetle (*Heteronychus arator* F.), and the grass grub (*Costelytra zealandica* Wh.).

1.5 TRANSFORMATION OF FILAMENTOUS FUNGI

Most *Acremonium* endophytes can be cultured, which lends these agriculturally and economically important fungi to molecular genetic analysis and manipulations (Schardl, 1995). The development of methods allowing transformation of fungi with DNA not only from other fungal species, but with sequences from prokaryotes, plants and animals has opened up many possibilities of engineering species suitable for various applications. Transformation is a method by which a vector carrying DNA sequences is taken up and integrated into the genome of the transformed cell. In the vector, the gene encoding the desired protein sequence is linked to a promoter and any other desired regulatory sequences capable of functioning in the transformed species so that the gene is transcribed and translated into a functional product (Fincham, 1989). The cells containing the transformed gene can then be selected by a visible change in the phenotype, or by using an indicator as necessary. Cotransformation is a technique which can be used when the gene used for the transformation is not selectable. In these cases the selectable marker can be simultaneously introduced on another plasmid to the cells. It appears that if recipient cells are exposed to two different kinds of DNA simultaneously, there is a high probability that a cell that takes up one will also take up the other (Fincham, 1989).

Two groups recently reported successful transformation methods for Acremonium endophytes. Murray *et al.* (1992) reported on conditions for transforming protoplasts of a perennial ryegrass endophyte, and Tsai *et al.* (1992) reported on an electroporative transformation method for *A. coenophialum.* In both cases, Southern blot analysis demonstrated that plasmid DNA randomly integrated into the endophyte genome. Murray *et al.* (1992) went one step further, and reintroduced the genetically modified endophyte into the natural host, successfully carrying out surrogate transformation of perennial ryegrass, thereby demonstrating the potential for powerful molecular analysis of the endophyte-host associations. An important component, thought to assist in DNA uptake, employed in the method used to transform *A. lolii*, is PEG (polyethylene glycol). PEG is added to the transformation mixture (Section 2.13), causing the treated cells to clump

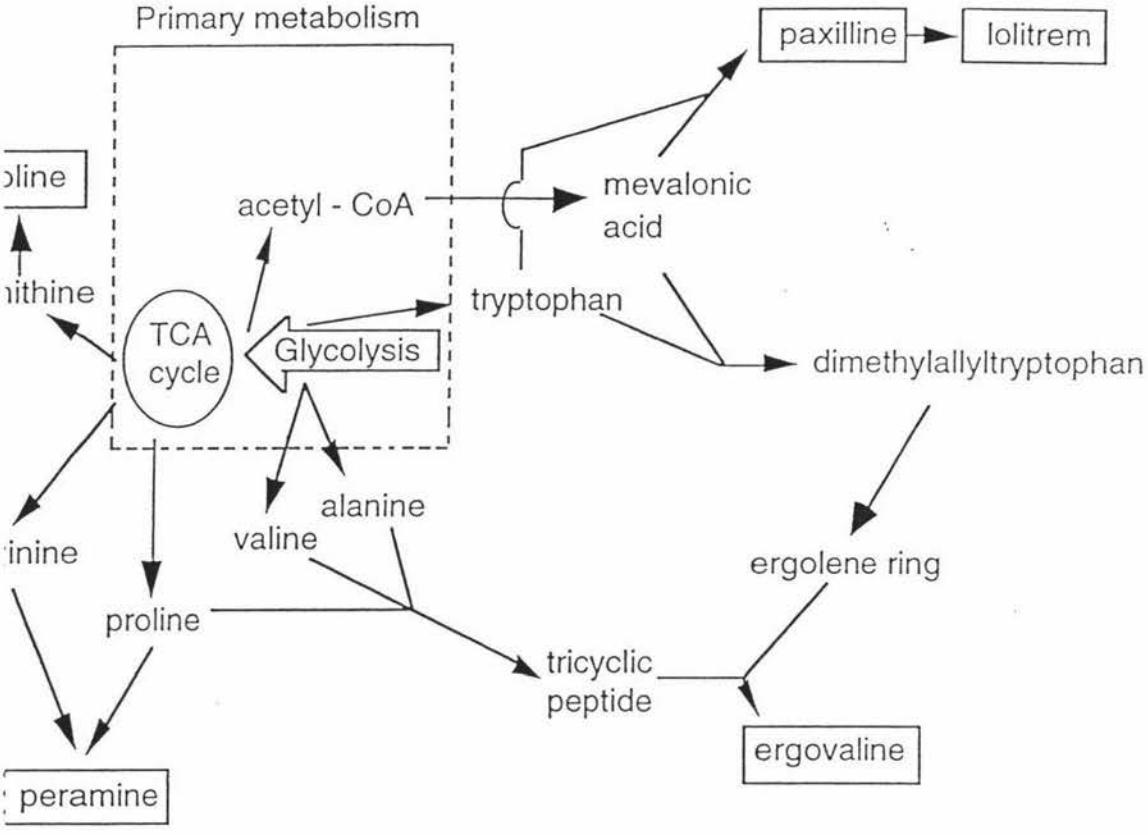


Figure 2. **Relationship of primary metabolism to the biologically active secondary metabolites (shown in boxes) believed to play an important role in grass-endophyte associations.**

Some probable precursors and intermediates are shown (Porter, 1994). Figure courtesy of Terri Sargent, Department of Microbiology and Genetics, Massey University, New Zealand.

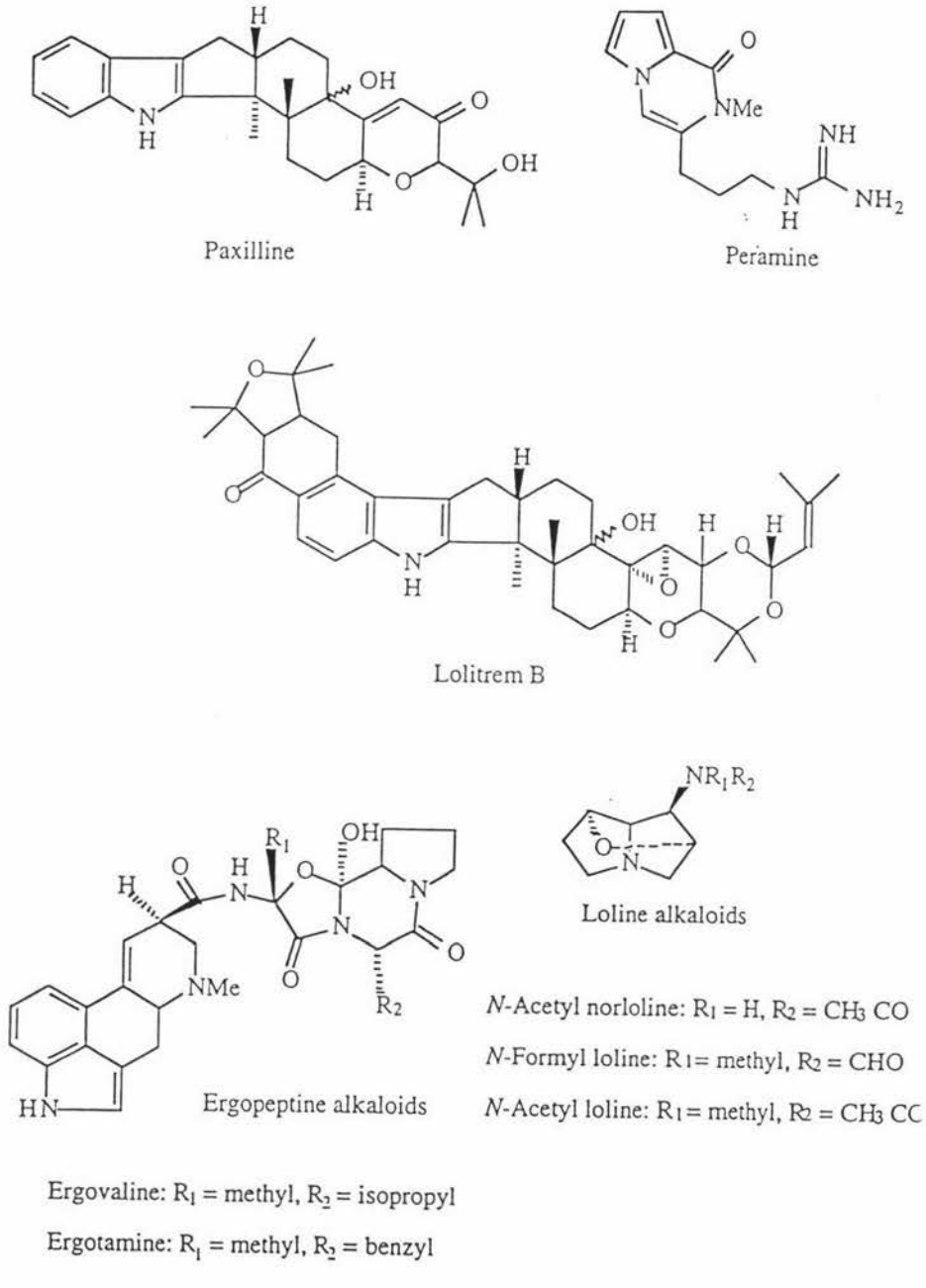


Figure 3. Structures of biologically active secondary metabolites believed to play and important role in grass-endophyte associations.

Figure courtesy of David McSweeney, Department of Biochemistry, Massey University, New Zealand

(Fincham, 1989) and is known to stimulate protoplast fusion (Murray *et al.*, 1992) This means that the formation of heterokaryons is possible, with transformant colonies potentially containing nuclei of different genotypes. The standard procedure to resolve this situation and obtain pure homokaryons is to plate out spores and isolate single colonies (Fincham, 1989). An alternative method for fungi which do not readily sporulate is to use centrifugation to concentrate mononucleate protoplasts for the preparation of homokaryons from heterokaryotic isolates, as has been carried out on the fungus *Rhizoctonia solani* (Phillips, 1993). This method could potentially be of use in purifying transformants of the asexual *Acremonium* endophytes which sporulate poorly in culture.

1.6 REPORTER GENE SYSTEMS TO STUDY GENE EXPRESSION

Gene regulation is a highly complex process, with the possibility of regulation occurring at many different steps, from transcription and translation through to mRNA and protein transport, processing and breakdown. The study of regulation can be further complicated by genes of interest belonging to multi-gene families, producing similar products to the gene of interest. The use of reporter genes, which are used to produce a protein not naturally occurring in the organism to be studied, has allowed advances in the study of temporal and spatial regulation of many genes.

1.6.1 Use and Characteristics of Reporter Gene Systems

A reporter gene is contained in a DNA construction and is processed under the direction of a controlling sequence of another gene, of which the regulation is to be studied. The DNA construct is transformed into cells, and as the reporter gene expression will reflect the expression characteristics of the controlling gene, the regulatory pathway can then be studied. A reporter gene requires several characteristics. The product has to be stable, easily detectable and able to be assayed rapidly and routinely, with high sensitivity. The reporter gene product must maintain activity when in a fusion construct with new genes, even if this results in processing and transport different to what would occur in the

endogenous system, and of course the protein must not interfere with normal cellular metabolism.

Several well known reporter genes exist, for example the *Escherichia coli* β-galactosidase (*lacZ*) gene (Silhavy and Beckwith, 1985) has been used to study the regulation of various promoters (Pérez-Esteban *et al.*, 1995). This gene has been used successfully in many systems, but has been of little use in plants due to high levels of endogenous β-galactosidase activity in plants. The luciferase genes (*luxAB*) have been cloned from a number of organisms, such as the firefly, and have been exploited in a number of molecular biology applications (Blouin *et al.*, 1996). A more recent development, the green fluorescent protein (GFP) was identified in the jellyfish *Aequoria victoria* and is responsible for a green bioluminescence. GFP retains its properties in many organisms and cell types, and the fluorescence of this protein can be used to label cells that can be followed in living systems (Haseloff and Amos, 1995; Prasher, 1995). The β-glucuronidase (GUS) reporter gene system, using the *gusA* gene from *Escherichia coli*, has been extensively used in many systems including plants, bacteria and fungi. This is a very useful reporter gene as it gives reliable and reproducible results, and can be used in both qualitative and quantitative assays (Jefferson, 1987, 1989; Mönke and Schäfer, 1993).

1.6.2 The GUS Reporter Gene System

The *E. coli gusA* reporter gene product is a hydrolase which catalyses the cleavage of a wide variety of β-glucuronides, many of which are commercially available. The advantages of the GUS system over others include the stability of β-glucuronidase, it tolerates a wide variety of conditions, the simplicity of the assay, and importantly there is little or no detectable endogenous GUS activity *in Saccharomyces cerevisiae*, *Drosophila melanogaster*, some strains of *Caenorhabditis elegans*, *Dictyostelium discoidum*, or in almost any higher plant (including perennial ryegrass, Section 3.4). This absence of endogenous GUS activity means that minute amounts of the reporter gene activity can be

measured, even in a single cell. GUS can tolerate large amino terminal substitutions, and unlike the *lacZ* encoded β-galactosidase, can be translocated across cell membranes with high efficiency (Jefferson, 1989). GUS can be fluorometrically detected, allowing two to four orders of magnitude greater sensitivity of detection than the spectrophotometric methods which are also available for GUS detection (Jefferson, 1987).

The substrate best suited to specifically detect GUS expression is 4-methyl umbelliferyl glucuronide (MUG). MUG is cleaved only by GUS, and very little spontaneous cleavage occurs. The cleavage product, methylumbellliferone (MU), is distinguishable from the substrate and can be measured quantitatively using fluorometry and spectrophotometry, or qualitatively with the use of ultra violet light (Jefferson, 1987).

Murray *et al.* (1992) demonstrated the potential for the GUS reporter gene to be used to analyse endophyte gene expression *in planta*, following the transformation of *Acremonium* endophytes with the GUS gene, and the artificial inoculation of grass plants with the transformed endophyte (Section 1.5). This approach could allow the level of GUS expression, along with the temporal and spatial pattern of expression to be determined *in planta* with the endophyte-host symbiosis intact. The regulation of genes of interest, for example those involved in toxin production, could potentially be studied using this system. Although this system allows the level and location of gene expression to be determined, it does not account for fungal biomass in the tissue analysed. The activity of the fungal hyphae does not necessarily reflect the total amount of hyphae present. There are however methods available for estimating the amount of filamentous fungi in plant tissue (Ride and Drysdale, 1972; Roberts *et al.*, 1988).

1.7 POTENTIAL APPROACHES TO MANIPULATING THE SYMBIOSES FOR AGRICULTURAL BENEFIT

Several options exist to control or minimise the effects of endophytes on animal performance, for example the use of endophyte free pasture species and toxin free endophytes (reviewed by van Heeswijck and McDonald, 1992; Scott and Schardl, 1993). Low endophyte and endophyte free cultivars have been developed to lessen the toxic effects on animals, (Siegel *et al.*, 1985) but evidence suggests that grasses which are endophyte free have a lower persistence than endophyte infected grass. Endophyte free grass is susceptible to drought, insect attack, and is more difficult to establish than endophyte infected grass. The use of endophyte free grasses, often at the expense of replanting entire pastures, has led to disappointing results overall (Hoveland, 1993).

Naturally occurring endophyte strains lacking animal toxicity (do Valle Ribeiro, 1993), may be able to be isolated and inoculated into pasture grass, but this system could be subject to similar problems as those found when using low endophyte and endophyte free cultivars. A consideration which must be kept in mind when using this approach is that endophytes with low or undetectable levels of toxins in one pasture species, or under specific conditions may not necessarily exhibit the same alkaloid spectrum in a different environmental situation. The array of alkaloids, and the levels of these compounds produced by an association are dependent on the genotype of the endophyte (Christensen *et al.*, 1993), and the environmental conditions (reviewed by van Heeswijck and McDonald, 1992; Di Menna *et al.*, 1992). The genotype of the host grass also alters the range of alkaloids produced, and in some combinations of endophyte and host incompatibility results in hyphal or host tissue death (Christensen, 1995).

Available evidence suggests that the symbiosis must be retained to ensure the survival of the pasture grass is at an optimum (Funk *et al.*, 1993). Ideally, the fungus needs to retain those properties seen to be beneficial to agriculture, and have none of the harmful properties present. Application of genetic manipulation would appear to have

considerable potential as this approach may allow precise changes to be made resulting in endophytes with expression of selected toxin genes altered. Murray *et al.* (1992) demonstrated that transformed endophytes introduced into perennial ryegrass (Latch and Christensen, 1985), were stably maintained and disseminated in the seed. Visualisation of expression of the GUS gene under the control of fungal promoters, in an assay using the substrate X-Gluc, demonstrated that the transformed endophyte can be used as a surrogate host to introduce foreign genes into grasses. The development of transformation and gene targeting techniques in the fungal endophytes may lead to isolation of the toxin genes and the targeted disruption of toxin pathways, with the objective of reducing or eliminating livestock toxicosis. Before full advantage can be made of genetically manipulating endophytes for improved pasture performance, a thorough understanding of the complex biological aspects of the association between endophyte and host must be achieved. The toxin genes must be isolated, and their regulation and pattern of expression in association with host grasses must be elucidated. When the symbiotic association between endophytes and pasture grasses is well understood, the transformation of endophytes may offer a powerful tool not only for manipulating natural outcomes of the association, but also for introducing new features to give grasses desirable attributes. The GUS (β-glucuronidase) reporter gene system will greatly facilitate the investigation of many economically and scientifically interesting fungi (Mönke and Schäfer, 1993).

1.8 BACKGROUND AND AIMS OF THIS PROJECT

While a considerable amount is known about the cultural, morphological and physiological characteristics of *Acremonium* endophytes in axenic culture, very little is known about gene expression in the grass host. The aim of this study was to use the GUS system to analyse gene expression of *Acremonium* endophytes *in planta*. While Lp1 had been previously transformed with GUS, the subsequent discovery that this strain was an interspecific hybrid makes it unsuitable for making gene knockouts. Also this strain does not produce lolitrem; a pathway of interest in this study. The aim here was to transform a haploid strain of *A. lolii*, Lp19, with the *gusA* gene under the control of the constitutive *A.*

nidulans glyceraldehyde-3-phosphate dehydrogenase promoter (P*gpd*), with the view of using GUS to measure fungal metabolic activity *in planta*. The aim was to assess whether there was any relationship between fungal metabolic activity, as measured by constitutive GUS expression, and the level of toxins produced in the association between the transformed strain and perennial ryegrass, cultivar 'Nui'.

CHAPTER 2. MATERIALS AND METHODS

2.1 FUNGAL STRAINS AND PLASMIDS

Fungal strains and plasmids used in this study are listed in Table 1.

2.2 GROWTH OF FUNGI

Acremonium cultures were grown at 22°C in potato dextrose (PD; Difco) broth (PDB), or on PD agar (PDA) (Section 2.3.1), or on complete media (CM) (Section 2.3.2). Hygromycin was supplemented as required at a concentration of 100 ug/ml. Plates were sealed with parafilm during incubation.

For the growth of fungal cultures from which protoplasts or DNA were to be prepared, 200 µl of a suspension containing mycelia homogenised in PDB (Section 2.3.1) was added to 150 ml capacity flasks containing 25-50 ml of PDB, and these cultures were incubated at 22°C with shaking, for 6 to 7 days.

A. lolii was reisolated from plant tissue by removing 1 mm transverse sections from sheath material which had been surface sterilised by first washing in 95 % ethanol followed by a 2 min wash in 10% bleach (v/v) (Janola), and finally washed in sterile H_2O. The tissue pieces were placed on PDA media and incubated at 22°C.

2.3 MEDIA

All media was prepared using MilliQ water. After preparation, media was sterilised by autoclaving at 121°C and 15 psi for 20 min. Liquid media was cooled to room temperature before inoculation. Solid media was cooled to 50°C prior to addition of antibiotic, and pouring. Uninoculated plates were stored at 4°C

2.3.1 Potato Dextrose Media

Potato dextrose broth (PDB) contained 24.0 g of dehydrated potato dextrose broth (Difco) rehydrated in 1 litre of MilliQ water. Potato dextrose agar (PDA) was prepared by adding 15.0 g/L of agar (Davis) to PDB

2.3.2 Complete Medium

Complete media (CM) contained 33.4 g Czapek Dox (Oxoid), 1.0 g yeast extract, 12.0 g potato dextrose broth, 1.0 g mycological peptone, 273.8 g sucrose, 1.0 g casein hydroysate, and 15 g agar per litre of MilliQ water.

2.4 COMMONLY USED BUFFERS AND SOLUTIONS

2.4.1 1 x TBE Electrophoresis Buffer contained 89 mM Tris, 2.5 mM Na_2EDTA, and 89 mM boric acid, pH 8.3.

2.4.2 1 x TAE Electrophoresis Buffer contained 40 mM Tris, 20 mM glacial acetic acid and 2 mM Na_2EDTA pH 8.2.

2.4.3 SDS Loading Buffer contained 1% (w/v) sodium dodecyl sulphate (SDS), 0.02% (w/v) bromophenol blue, 20% (w/v) sucrose, and 5 mM Na_2EDTA (pH 8.0).

2.4.4 OM Buffer contained 1.5 M $MgSO_4.7H_2O$, 10 mM Na_2HPO_4, and 100 mM $NaH_2PO_4.2H_2O$ added until pH was 5.8.

2.4.5 ST Buffer contained 0.6 M sorbitol and 100 mM Tris-HCl, pH 8.0.

2.4.6 STC Buffer contained 1 M sorbitol, 50 mM Tris, pH 8.0 and 50 mM $CaCl_2$.

Table 1. Fungal strains and plasmids

Strain or Plasmid	Relevant Characteristics	Source or Reference
Fungal strains		
Acremonium sp.		
Lp1	*Acremonium* sp. from *Lolium perenne* (=LpTG-2)	(Christensen *et al.*, 1993)
Acremonium lolii		
Lp19	*A . lolii* from *Lolium perenne* (=LpTG-1)	(Christensen *et al.*, 1993)
KS1	*A .lolii* cotransformed with pAN7-1 and pFG.gpd	This study
Plasmids		
pAN7-1	6.6 kb Hyg^R	(Punt *et al.*, 1987)
pNOM-102	7.5 kb *gusA*, Amp^R	(Roberts *et al.*, 1989)
pFG.gpd	7.7 kb *gusA*, Amp^R	(McGowan, 1996)

2.4.7 TE Buffer contained 10 mM Tris-HCl and 1 mM Na_2EDTA

2.4.8 10 x Hybridisation Buffer (Southern, 1975) contained (per litre): 50 ml 1 M Hepes (Sigma), pH 7.0, 150 ml 20xSSC (Section 2.4.9), 6 ml phenol extracted herring sperm DNA (3 mg/ml, Sigma), 2 ml 20% (w/v) sodium dodecyl sulphate, 2 g Ficoll (Sigma-70), 2 ml *Escherichia coli* transfer RNA (10 mg/ml, Sigma), 2 g bovine serum albumin, 2 g polyvinylpyrrolidone (Sigma PVP-10) and MilliQ water to 1 litre.

2.4.9 20 x SSC contained 3 M NaCl and 0.3 M sodium citrate.

2.4.10 2 x SSC was prepared by a 10-fold dilution of 20 x SSC (Section 2.4.9).

2.4.11 Tris Eqilibrated Phenol was purchased pre-prepared from United States Biochemical.

2.4.12 GUS Extraction Buffer contained 50 mM $NaPO_4$, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% Sodium Lauryl Sarcosine, and 0.1 % Triton X-100.

2.4.13 GUS Assay Buffer was prepared by adding 4-methylumbelliferyl β-D-glucuronide (MUG, Sigma) to GUS extraction buffer (Section 2.4.12) to a concentration of 1 mM.

2.5 DNA ISOLATION

2.5.1 Large Scale Total Fungal DNA Isolation

The method used for large scale fungal DNA isolation was based on that described by Byrd *et al.* (1990). Cultures to be used for DNA isolation were grown in PDB liquid culture as described in Section 2.2. After harvest by centrifugation, the mycelia was

washed in MilliQ water, freeze dried, then 0.5 - 1.0 g of freeze dried mycelia was ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was then resuspended in 10 ml of extraction buffer (150 mM EDTA, 50 mM Tris-HCl, and 1 % Sodium Lauryl Sarcosine) containing proteinase K (Boehringer Mannheim) added to a final concentration of 2 mg/ml. Following centrifugation at 2 000 x g for 10 min, the supernatant was incubated at 37°C for 20 min. Three extractions were carried out on the supernatant, the first with 2 volumes of phenol (Section 2.4.11), followed by 2 volumes of a 1:1 phenol:chloroform mix, and finally 2 volumes of chloroform. Each time the solution was mixed and the phases separated by centrifugation at 20 000 x g for 15 min, before the aqueous phase was removed and re-extracted. Following the final extraction, the polysaccharides were removed by centrifuging the aqueous phase at 25 000 x g for 20 mins, before one volume of isopropanol was added to the supernatant and the DNA pelleted at 16 000 x g for 30 min. The precipitated DNA was washed in 70 % (v/v) ethanol and resuspended in water.

2.5.2 Isolation of DNA Fragments from SeaPlaque Agarose Gels

DNA was recovered from SeaPlaque agarose gels, 0.7% to 1.5% (w/v) in TAE electrophoresis buffer (Section 2.4.2), by phenol freeze extraction (Thuring *et al.*, 1975). Following electrophoresis to separate the DNA fragments (Section 2.10), the appropriate fragments, determined by molecular weight (Section 2.11) were excised from the gel while visualising the fragments with long wave UV light. Each fragment was transferred to a 1.5 ml microcentrifuge tube, and the agarose melted in a 65°C heat block. An approximately equal volume of Tris-equilibrated phenol (Section 2.4.11) was added before mixing and freezing at -20°C for a minimum of 2 hours. The tube was centrifuged for 10 min in a microcentrifuge and the aqueous phase removed and extracted with phenol and chloroform (Section 2.6) before the DNA was precipitated (Section 2.7).

2.6 PURIFICATION OF DNA BY PHENOL/CHLOROFORM EXTRACTION

DNA was extracted with an equal volume of phenol (Section 2.4.11), centrifuged in a microcentrifuge for 5 min, and the aqueous phase re-extracted first with an equal volume of 50:50 phenol:chloroform, then with an equal volume of chloroform. The DNA was then precipitated by ethanol or isopropanol precipitation (Section 2.7).

2.7 PRECIPITATION OF DNA WITH ISOPROPANOL OR ETHANOL

A 1/10 volume of 3 M sodium acetate and 0.6 volumes of isopropanol or 2.5 volumes of 95 % ethanol (depending on the volumes present and capacity of the tube used) was added to the solution containing the DNA to be precipitated. The tube was inverted to mix, and stored at -20°C for at least 2 hours after which time the DNA was pelleted by micro-centrifugation for 15 min. The pellet was washed once with 70 % (v/v) ethanol and dried under vacuum before resuspension in MilliQ water.

2.8 DETERMINATION OF DNA CONCENTRATION

2.8.1 Fluorometric Determination of DNA Concentration

DNA was quantitated on a Hoefer Scientific TKO 100 Fluorometer using a dye solution containing 1 x TNE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA and 100mM NaCl, pH 7.4) and 0.1 µg/ml Hoechst 33258. The instrument was first set at a 0 reading with 2 ml of TNE buffer. The reading of the fluorometer was then standardised at 100 µg/ml after adding 2 µl of a 1µg/ml calf thymus DNA suspension to the 2 ml of TNE buffer. Standardisation was repeated until a steady reading was obtained twice. 2 µl of the DNA sample to be quantified (at a suitable dilution in water as necessary) was added to 2 ml of TNE buffer and the resulting reading recorded as a concentration in ng/µl.

2.8.2 Mini-gel Method for Determination of DNA Concentration

A sample of the DNA to be quantified was electrophoresed on an agarose gel (Section 2.10) together with a series of standard DNA solutions at known concentrations (λ DNA standards were used when quantifying genomic DNA, pUC118 DNA standards were used when quantifying plasmid DNA). After staining in ethidium bromide and photographing (Section 2.10), the intensity of the sample bands were compared to that of the bands of known concentration and their concentrations estimated accordingly.

2.9 RESTRICTION ENYME DIGESTION OF DNA

Restriction endonuclease digests were carried out in the appropriate salt restriction enzyme buffer supplied by the manufacturer. When a higher salt concentration was required upon addition of a second enzyme to a double enzyme digest, 1 M NaCl was used to adjust the salt concentration accordingly. Restriction enzymes were used to an excess of 2-3 times the amount recommended by the manufacturer when digesting DNA.

Digestions of plasmid DNA were carried out at 37°C for 1.5 to 3 hours, and stored on ice while an aliquot was checked for the extent of digestion on a 0.7% (w/v) agarose-TBE Buffer mini gel (Section 2.10). If incomplete digestion was observed, a second quantity of restriction enzyme was added, and the mixture incubated further at 37°C.

Digestion of fungal genomic DNA was carried out as above, with an increased digestion time of between 3 and 24 hours. Bovine serum albumin (BSA) was added at a rate of 1 μg/10 μl of digest mixture to increase restriction enzyme stability. To stop reactions 1/5 volume of SDS loading buffer (Section 2.4.3) was added to the mixture.

2.10 AGAROSE GEL ELECTROPHORESIS OF DNA

Horizontal agarose gel electrophoresis was carried out in a mini-gel apparatus for 1-2 hours at 80-100 V. Electrophoresis of large gels used for Southern blotting was carried out overnight at 30 V. The appropriate concentration of agarose was heated to dissolve in electrophoresis buffer. After cooling to 50°C, the gel was poured and allowed to polymerise for at least 30 min. DNA samples, with addition of sterile MilliQ water to a final volume of at least 10 µl and 1/5 volume of SDS loading buffer (Section 2.4.3), were loaded onto the gel and the fragments separated by electrophoresis. Gels were then stained in ethidium bromide (0.5 µg/ml in MilliQ H_2O) for 5 to 30 min, washed in MilliQ water and visualised on a UV transilluminator. Gels were photographed on Polaroid type 667 film, or using an Alpha Innotech gel documentation system.

2.11 DETERMINATION OF MOLECULAR SIZE

A 1 kb DNA ladder (Gibco BRL) or a *Hin*dIII digest of lambda DNA was loaded alongside the DNA samples on an agarose gel, and used as standard size markers to determine the molecular weight of DNA fragments. Relative mobilities of each fragment in the standard and the sample lanes were measured. Molecular size was determined by interpolation from a plot of the distance migrated against the logarithm of the molecular weight of the size markers.

2.12 PREPARARTION OF FUNGAL PROTOPLASTS

Protoplasts were prepared using a modification of the method described by Yelton *et al.* (1984). Mycelia from three 50 ml PDB (Section 2.2) cultures was distributed evenly between four 50 ml centrifuge tubes, and harvested by centrifugation at 27 000 x g then washed in sterile water. To each tube was added 5 ml of filter sterilised (Acrodisc syringe filters, 0.45 µm) novozyme 234 (InterSpex Products Inc.) (10 mg/ml) in OM buffer

(Section 2.4.4). This was incubated at 30°C with gentle shaking (80-100 rpm) for 4-5 hours, or until a large proportion of mycelia had released protoplasts. The protoplast solution was filtered through sterile Miracloth, placed inside a sterile glass funnel. The filtered solution was distributed evenly between 15 ml centrifuge tubes and overlayed carefully with 1 ml ST buffer (Section 2.4.5) so that the ST buffer formed a layer on top of the protoplast solution. The mixture was centrifuged at 3000 x g for 5 min and the protoplasts formed a white layer at the interface of the two solutions. The protoplasts were removed from the interface with a 1 ml Gilson pipette, pooled in a fresh sterile tube, and 5 ml of STC buffer (Section 2.4.6) was added. Protoplasts were pelleted by centrifugation at 7 700 x g for 10 min. The latter step was repeated to wash the protoplasts before they were quantified using a haemocytometer and diluted in STC buffer to a final concentration of 1.25 x 10^8/ml.

2.13 TRANSFORMATION OF FUNGAL PROTOPLASTS

Protoplasts were transformed using a modification of the method described by Vollmer and Yanofsky (1986). In a microcentrifuge tube 80 µl of protoplast suspension (1.25 x 10^8 protoplasts/ml), 20 µl of 40% (w/v) PEG 4000 solution (in STC buffer, Section 2.4.6), 2 µl 50 mM spermidine, 5 µl heparin (5 mg/ml in STC buffer) and 5 µl of DNA solution (or 5 µl of H_2O only for 'no DNA' controls) at a concentration of 1µg / µl were gently mixed then incubated on ice for 30 min. To this, a further 900 µl of the PEG solution was added, and the transformation mix further incubated for 15 min at room temperature after gentle mixing. Aliquots (100 µl) of the transformation mix were added to 5 ml of 0.8 % (w/v) molten CM (Section 2.3.2) (50°C) and spread on 1.5 % (w/v) CM plates. Plates were inverted and incubated at 22°C overnight, then overlayed with 5 ml of 0.8 % (w/v) molten CM (50°C) containing Hygromycin B to result in a final plate concentration of 100 µg/ml. Plates were sealed with parafilm, inverted and incubated at 22°C for 3-4 weeks. Transformants were then subcultured onto PDA (Section 2.3.1) plates containing Hygromycin B at a concentration of 100 µg/ml.

2.14 NUCLEAR STAINING OF FUNGAL PROTOPLASTS

Protoplasts were prepared as in section 2.5. To 5 - 10 µl of an appropriately diluted protoplast preparation on a microscope slide was added 10 µl of nuclear stain (100 µl of a 1 mg/ml solution of Hoechst 33258 dissolved in ethanol and added to 200 ml of 0.5 x SSC, pH 4.2) A coverslip was placed over the suspension, and after 30 min the nuclei of the protoplasts were examined by fluorescence microscopy using a Leitz fluorescence microscope using filter 3.

2.15 SOUTHERN BLOTTING AND HYBRIDISATION

2.15.1 Southern Blotting

The blotting method used is based on that of Southern (1975). DNA to be transferred to the nylon membrane (Hybond N, Amersham) was separated by overnight gel electrophoresis, stained in ethidium bromide for 30 min and destained for 30 min in MilliQ H_2O, before being visualised and photographed (alongside a ruler) as described in section 2.10.

The gel was placed in a plastic container and washed in several different solutions, a quantity suitable for the gel to be fully immersed was added each time, and the solution added prior was poured off before the next solution was added. The gel was first agitated gently for 15 min in 250 mM HCl, followed by a 30 min incubation in a solution of 500 mM NaOH and 500 mM NaCl, and then a 30 min incubation in a 500 mM Tris-HCl (pH 7.4) and 2 M NaCl. A final wash was carried out in 2 x SSC (Section 2.4.40) for 2 min.

The blotting apparatus was assembled on a plastic trough with wells at each end. Two sheets of Whatman 3MM chromatography paper, cut to the width of the trough, and greater than the length, were soaked in 20 x SSC (Section 2.4.9) then placed in the trough so that the ends of the paper sat in the wells. The wells were filled with 20 x SSC. A piece

of Gladwrap was placed over the entire, and a section 2 mm smaller all around than the gel was removed from the centre. The washed gel was placed over the hole in the Gladwrap. A piece of nylon membrane (Hybond-N, Amersham), was cut to 2 mm larger than the gel, presoaked in 2 x SSC, and placed over the gel. All air bubbles were eliminated from under the membrane. Four sheets of Whatman 3MM chromatography paper were cut to 2 mm smaller than the gel size. Two of these were presoaked in 2 x SSC and were placed over the membrane followed by two dry sheets. A stack of paper towels, approximately 50 mm high were placed on top of the 3MM paper, followed by a plastic tray containing a weight sufficient to keep the stack flat.

Following overnight DNA transfer, the apparatus was disassembled, and the membrane washed in 2 x SSC for 5 min, and then baked under vacuum for 2 hours at 80°C.

2.15.2 Preparation of Labelled Probe with the High Prime DNA Labelling Kit

DNA to be labelled (25 ng), in an 11 µl volume, was denatured in a boiling water bath for 10 min and then placed immediately onto ice. To the cooled denatured DNA was added 4 µl High Prime (Boehringer Mannheim) solution and 3-5 µl of [α-^{32}P] dCTP (3000 Ci/mmol, Amersham). The reaction was mixed gently, microcentrifuged briefly and incubated at 37°C for 1 hour. The reaction was stopped by the addition of 2 µl of 0.2 M EDTA (pH 8.0). Unincorporated nucleotides were removed from the mixture on a Sephadex G-50 column (ProbeQuant), after adjusting the total volume of the reaction mix to 50 µl with STE buffer (per 50 ml; 0.438 g NaCl dissolved in TE (Section 2.4.6), pH 8.0). The vortexed column was pre-spun for 1 min at 735 x g, with the lid loosened, then the sample was applied and the column centrifuged for 2 min at 735 x g.

2.15.3 DNA Hybridisation

The nylon membrane from a Southern blot (Section 2.15.1) was prehybridised for at least 2 hours in hybridisation buffer (Section 2.4.8) in a sealed glass tube at 65°C in a rotating

hybridisation oven. After prehybridisation, all but 5 ml of the hybridisation buffer was poured from the tube, and boiled [α-^{32}P]dCTP-labelled probe was added (Section 2.15.2). After overnight hybridisation at 65°C, the filter was removed from the tube and washed three times for 15 min, in 2 x SSC at room temperature. The filter was wrapped in Gladwrap while still damp, and exposed in the presence of a Cronex intensifying screen to a sheet of Fuji Medical X-ray film in an X-ray cassette. After exposure at -70°C for an appropriate period of time, the cassette was warmed to room temperature, and the film removed and developed in Kodak D19 developer for 2 min, rinsed in water and fixed in Kodak Rapid fixer for 2 min. The film was rinsed again in water and dried.

2.15.4 Stripping Filters for Re-Use

A boiling solution of 0.1 % (w/v) sodium dodecyl sulphate was poured over the filter to be stripped, and the filter was gently agitated in this solution while the solution cooled to room temperature. The filter was exposed to film overnight and the autoradiograph developed (Section 2.15.5) to check that no signal was present. If signal was present the stripping process was repeated.

2.16 GROWTH AND ARTIFICIAL INOCULATION OF *LOLIUM PERENNE* PLANTS

Artificial inoculation of *Lolium perenne* cv. 'Nui' was carried out as described in Latch and Christensen (1985). Mycelia was inserted into a longitudinal slit in 6 day old seedlings at the junction of the mesocotyl and coleoptile. Seedlings were grown in axenic culture for a further 10-15 days then potted in a sterilised sand-peat mixture and transferred to a glasshouse for further growth.

2.17 EXTRACTION OF THE GUS ENZYME FROM PLANT TISSUE

Plant tissue extracts were prepared using a modification of the method described by Jefferson, (1987). Plant tissue was harvested, weighed (20-50 mg) and placed in microcentrifuge tubes. If storage of tissue was required for assay at a later date, the tubes containing the material were submerged in liquid nitrogen, before storage at -70°C. Tissue samples were stored on ice as much as practicable, both after harvest and removal from storage. Immediately prior to extraction, the tissue was snap frozen by addition of liquid nitrogen, then ground as finely as possible with a plastic microcentrifuge tube pestle. 1 ml of extraction buffer (Section 2.4.12) was added to the tissue and the samples were microcentrifuged at 4°C for 5 min. The supernatant was removed to a fresh microcentrifuge tube.

2.18 β-GLUCURONIDASE ASSAYS

2.18.1 Quantitative Assay

Plant tissue extracts (Section 2.17) were quantitatively assayed for GUS activity using a modification of the method described by Jefferson, (1987). Aliquots of extracts were stored on ice until the substrate was added. Each tissue extract was assayed in duplicate. To 50 - 100 µl of plant extract in a microcentrifuge tube, (depending on the level of activity detected in a preliminary assay carried out on tissues from the plants to be tested) was added 900 - 950 µl (to a total volume of 1 ml of assay mix) of GUS assay buffer (Section 2.4.13) which had been prewarmed to 37°C. Assay buffer was added successively to the series of extract aliquots, typically every 30 sec. The tubes were immediately incubated at 37°C following addition of assay buffer. Aliquots (100 µl) were removed for assessment of the rate of reaction, from the tubes at pre-determined intervals, and immediately added to 2.9 ml of stop buffer (0.2 M sodium carbonate) typically every 10-15 min, (including 0 min) until 3 - 5 aliquots were taken.

The level of 4-methylumbelliferone (MU), indicative of the level of GUS activity in the stopped reactions, was measured using a Hoefer TKO 100 Mini Fluorometer. 2 ml of 0.2 M stop buffer was added to a cuvette, and the fluorometer scale set to zero. 100 µl of a 1 µM solution of MU (in distilled water) was added to the sodium carbonate buffer in the cuvette and mixed by pipetting up and down carefully, so as not to introduce air bubbles. The fluorometer scale was then set to 500 units. This was repeated until a 500 unit reading was reproducibly obtained. Samples were measured by pipetting 2 ml of the stopped reaction into the cuvette, and the relative fluorescence units were converted to nmoles MU mg^{-1} tissue. Values for nmoles MU mg^{-1} were graphed against time and the rate of MU production recorded as nmoles MU mg^{-1} min^{-1}.

2.18.2 Qualitative Assay

Fungal or plant material was qualitatively assessed for the presence of GUS expression by transferring a small quantity of material to be assessed to a microtitre plate well containing assay buffer (Section 2.4.13) The reactions were incubated in darkness at 37°C. After one hour the reactions were examined under ultra violet light for the presence of a blue fluorescent product.

2.19 ANALYSIS OF TOXINS IN PLANT MATERIAL

The method used to quantify toxins in grass material was as described by Barker *et al.* (1993). Material was harvested, freeze dried and then fine milled in a coffee grinder.

2.19.1 Peramine Analysis

A standard amount (about 1.5 µg of the free base in 50 µl of methanol) of the hydrogen nitrate salt of homoperamine was added to weighed samples of about 100 mg of dry ground herbage. Peramine and the internal standard were extracted into 3 ml of 30 %

(v/v) aqueous isopropanol at 90°C for 30 minutes. The cooled tubes were lightly centrifuged and 1 ml of the extract transferred to a cartridge column of weak cation exchanger on silica (100 mg absorbant, Analytichem Bond Elut CBA) preconditioned by washes with ammoniacal and aqueous methanol and 100 % methanol. After adding the samples the columns were re-washed with 1 ml of methanol. Peramine and the internal standard were eluted from the cartridge with 1 ml of 5 % (v/v) formic acid in 40 % (v/v) aqueous methanol. Chromatography was carried out on a silica HPLC column (Brownlee, 220 x 4.6 mm with silica NewGuard cartridges) with a buffer of 50 mM ammonium acetate, 5 mM guanidinium carbonate, and 0.2 % (v/v) acetic acid in about 18 % (v/v) aqueous methanol at 1 ml/minute. Detection was by UV absorption at 280 nm. Quantitative measurements were made by comparing integrated peak areas of peramine and the homoperamine internal standard. Peramine and homoperamine are approximately equally extracted.

2.19.2 Lolitrem B Analysis

Herbage samples (about 50 mg) were extracted for 1 hour with 1 ml of chloroform-methanol (2:1 v/v). The extraction solvent was separated by aspiration through and immersion filter and the plant residues rinsed with two further 0.5 ml portions of solvent. The solvent was evaporated without heating under reduced pressure and the residues were taken up in 1 ml of 1,1-dichloroethane:acetonitrile (4:1 v/v). The extract was transferred through a syringe filter into vials for HPLC sampling. Lolitrem B was measured following HPLC separation using a silica column (Brownlee, 220 x 4.6 mm) with a sovent of dichloromethane, acetonitrile and water (840:160:1 v/v) at 1 ml per minute. Fluoroscence detection (Shimadzu RF535) was with excitation at 268 nm and emission at 440 nm and the amount of lolitrem B estimated by comparison of the integrated peak area with those of reference standards measured in the same batch of samples. Reported values are corrected for a mean extraction efficiency of 90 %.

2.19.3 Ergovaline Analysis

About 50 mg of herbage with added ergotamine tartrate internal standard (less than 1 μg in 50 μl of methanol) was extracted with 1 ml of chloroform-methanol-ammonia (concentrated solution) (75:25:2 v/v) overnight at room temperature in darkness. The extraction solvent was separated by aspiration through an immersion filter and the plant residues rinsed with two further 0.5 ml portions of solvent. The combined extract was evaporated to dryness under vacuum without heating, redissolved and suspended in 0.5 ml carbon tetrachloride and 0.5 ml tartaric acid solution (25 mM in 50 % v/v aqueous methanol). The phases were allowed to separate on standing, and the aqueous phase transferred with filtration into a vial for HPLC sampling.

Ergovaline, ergovalinine, ergotamine and ergotaminine were separated by reversed phase HPLC (Brownlee RP-18 column, 5 μm, 100 x 4.6 mm with RP-18 NewGuard pre-column) at 30°C with a gradient solvent system of 0.1 M ammonium acetate in water and acetonitrile (25 % v/v acetonitrile to 50 - 60 % (v/v) acetonitrile with a concave 35 - 45 minute gradient; flow 1 ml/minute) and measured by fluorescence detection, with excitation of 310 nm and emission of 410 nm. Ergovaline in the sample was estimated as the sum of ergovaline plus ergovalinine concentrations compared to the sum of ergotamine plus ergotaminine internal standard. Pooling data for the isomers removed much of the variation in equilibration observed.

2.20 STATISTICAL ANALYSIS OF DATA

Values for nmoles MU min^{-1} were obtained by linear regression of the time course assay using Cricket Graph (Computer Associates Ltd). The values were transferred to Claris Works (Claris Works Corporation), and the spread sheet function was used to convert the values to nmoles MU min^{-1} mg^{-1}. This data, as well as the toxin analysis data were statistically analysed using the GLM (general linear model) procedure of SAS (SAS

Institute Inc. 1987). Analysis of variance (ANOVA) programmes used are as stated in Appendix 2.

CHAPTER 3. RESULTS

Previous work by Murray *et al.* (1992), using FM13, a GUS (β-glucuronidase) expressing transformant of *Acremonium* strain Lp1, demonstrated the potential of using GUS as a reporter gene to monitor endophyte gene expression *in planta*. The aim here was to use methods developed by Jefferson (1987), to quantitate *in planta* fungal GUS expression, regulated by fungal promoters, with the longer term goal of correlating GUS activity with metabolic activity of the fungus.

3.1 COTRANSFORMATION OF A HAPLOID STRAIN OF *A. LOLII*

A haploid isolate of *A. lolii*, Lp19, was chosen for transformation studies. This strain had been shown to be relatively fast growing, and a good producer of lolitrem, ergovaline and peramine *in planta* (Christensen *et al.*, 1993).

Lp19 was cotransformed with two plasmids using the PEG mediated DNA uptake transformation method described by Murray *et al.* (1992), to obtain a GUS expressing transformant which could be used for this study (see Appendix 1 for plasmid maps). The GUS reporter gene, under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (*gpd)* promoter was introduced into Lp19 on one of either two plasmids, pFunGus.gpd (pFG.gpd) (McGowan, 1996) or pNOM-102 (Roberts *et al.*, 1989). The dominant selectable *hph* gene, regulated by the *gpd* promoter, was introduced on the plasmid pAN7-1 (Punt *et al.*, 1987). Expression of the *hph* gene allowed selection of transformants on media containing hygromycin B.

3.1.1 Transformation of Lp19: Experiments 1 and 2

Transformation of protoplasts prepared from *A. lolii* (Section 2.12), strain Lp19 with *Hin*dIII linearised pAN7-1 resulted in 32 Hyg^R transformants per 1 μg of DNA, after one months growth (Table 2). The *gusA* gene was introduced into Lp19 by cotransforming

with *Hin*dIII linearised pFG.gpd. Three Hyg^R resistant colonies which arose from cotransformation were selected, and replated onto PDA containing 100 µg/ml hygromycin B. These Hyg^R resistant colonies were assessed for their ability to convert MUG to a fluorescent product (Section 2.18.2). One was found to express GUS, and this cotransformant was assigned the name KS1. Where water was used as a control in place of transforming DNA, no transformant colonies were observed.

A second attempt at transformation of protoplasts prepared from *A. lolii* strain Lp19, with linear pAN7-1 resulted in 5 Hyg^R transformants per 1 µg of DNA after one months growth (Table 2). The *gusA* gene was introduced into Lp19 by cotransforming with linear pFG.gpd. No Hyg^R transformants were present on the cotransformation plates, so no Gus^+ cotransformants were obtained. Where water was used as a control in place of transforming DNA, no transformant colonies were observed.

3.1.2 Transformation of Lp1 and Lp19: Experiment 3

Due to the low cotransformation rate observed when Lp19 protoplasts were transformed with linearised plasmids, an experiment was designed to test several parameters in an attempt to find better conditions for cotransformation of *Acremonium*. Lp1, an interspecific hybrid strain of *Acremonium* (Schardl *et al.*, 1994) was included in the experiment to allow comparison of Lp19 transformation with a strain of *Acremonium* which had been successfully transformed previously in the laboratory (Murray *et al.*, 1992). In particular, the rate of transformation with circular DNA was compared to that with linear DNA. Transformation of protoplasts prepared from *Acremonium* sp. strain Lp1 with linear pAN7-1 gave 3 transformants per 5 µg of DNA, in comparison to transformation with circular pAN7-1 which gave 24 transformants per 5 µg of DNA, after one months growth (Table 3). This corresponds to transformation frequencies of 0.003 and 0.023 % respectively of the original number of protoplasts, or 0.06 and 0.44 % if one takes into account that only 5.2 % of protoplasts regenerated. The *gusA* gene was

Table 2. Cotransformation of Lp19: Experiments 1 and 2

Experiment	Plasmid[a]	Viable protoplasts[b]	Viable protoplasts after Transformation[b]	Hyg^R transformants[c]	Hyg^R GUS^+ Cotransformants[d]
1	cells only	n/d[e]	n/d[e]	0	-
1	pAN7-1	n/d[e]	n/d[e]	32	-
1	pAN7-1 & pFG.gpd	n/d[e]	n/d[e]	3	1
2	cells only	$1.06x10^6$	$2.05x10^4$	0	-
2	pAN7-1			5	-
2	pAN7-1 & pFG.gpd			0	0

[a] plasmid DNA linearised with *Hin*dIII

[b] per ml

[c] per 1 µg DNA

[d] total number of cotransformants

[e] not determined

introduced into Lp1 by cotransforming linear or circular pFG.gpd or pNOM-102, with pAN7-1 in the corresponding conformational form. No Hyg^R resistant colonies resulted from cotransformation of linear forms of the plasmids. Cotransformation with circular pFG.gpd or pNOM-102 along with pAN7-1 resulted in 48 and 50 Hyg^R transformants respectively. When assessed for GUS expression (Section 2.18.2) 8% (4/48) of transformants carrying pFG.gpd and 10% (5/50) of transformants carrying pNOM-102 were found to be GUS positive. Where water was used as a control in place of transforming DNA, no transformant colonies were observed.

Transformation of *A. lolii* strain Lp19 protoplasts with linear pAN7-1 gave 1 transformant per 5 µg of DNA, while transformation with circular pAN7-1 gave 5 transformants per 5 µg of DNA after one months growth (Table 3). This corresponds to transformation frequencies of 0.001 and 0.007 % respectively of the original number of protoplasts, or 0.04 and 0.18 % if one takes into account that only 3.5 % of protoplasts regenerated. The *gusA* gene was introduced into Lp19 by cotransforming linear or circular pFG.gpd or pNOM-102, along with linear or circular pAN7-1 as appropriate. No hygromycin resistant colonies resulted from cotransformation with linear forms of either of the combinations of the plasmids. Where water was used as a control in place of transforming DNA, no transformant colonies were observed.

A problem experienced with Lp19 in this and other experiments was the failure of some Lp19 transformants to survive after being subcultured from CM to PDA plates. This was also experienced by other researchers in the laboratory. As this occurred here, only 14 of the 27 transformants resulting from cotransformation with circular pAN7-1 and pFG.gpd and none of the 29 transformants resulting from cotransformation with circular pAN7-1 and pNOM-102, could be tested for GUS expression. No Gus^+ transformants were detected.

While only one GUS^+ Lp19 cotransformant was obtained, no further time was allocated to optimisation of Lp19 cotransformation, or obtaining more Gus^+ cotransformants.

Table 3 Cotransformation of Lp1 and Lp19: Experiment 3

Strain	Plasmid[a]	Viable protoplasts[b]	Viable protoplasts after transformation[b]	Hyg^R transform-ants[c]	Hyg^R Gus^+ Cotransform-ants[c]
Lp1	cells only	1.05×10^5	5.48×10^3		
	linear pAN7-1			3 (0.055%[d])	
	linear pFG.gpd & pAN7-1			0	0
	circular pAN7-1			24 (0.438%[d])	
	circular pFG.gpd & pAN7-1			48	4
	linear pNOM-102 & pAN7-1			0	0
	circular pNOM-102 & pAN7-1			50	5
Lp19	cells only	7.68×10^4	2.72×10^3		
	linear pAN7-1			1 (0.037%[d])	
	linear pFG.gpd & pAN7-1			0	0
	circular pAN7-1			5 (0.184%[d])	
	circular pFG.gpd & pAN7-1			27[e]	0
	linear pNOM-102 & pAN7-1			0	0
	circular pNOM-102 & pAN7-1			29[e]	0

[a]Plasmids were linearised with HindIII

[b] Viable protoplasts per ml

[c]Colonies per 5 µg DNA

[d]Frequency of transformants based on protoplast viability after transformation

[e]Not all transformants were tested due to death of a number of subcultured transformants

3.2 NUCLEAR DISTRIBUTION

As Lp19 transformants can not be purified by plating for single spores, due to this isolate rarely producing asexual conidia, an experiment was carried out to determine the frequency of protoplasts with single nuclei. It was of interest to determine the nuclear distribution in protoplasts, to determine whether the transformant had regenerated from a protoplast with one nucleus, or whether it contained several nuclei. A protoplast with only one nucleus would form a genetically homogeneous culture. A protoplast with several nuclei could give rise to a heterokaryotic culture in which all cells did not contain the same genetic information. Distribution of nuclei was examined in protoplasts released by enzymic digestion of 7 day old mycelia, (Sections 2.2 and 2.12) (Fig. 4). The nuclei were observed by staining the DNA with Hoechst dye, as described in Section 2.14. As shown in Fig. 5 the majority of the protoplasts released from Lp19 mycelium were mononucleate.

3.2.1 Nuclear Distribution in Lp19

The frequency of mononucleate nuclei in protoplasts released from 7 day old Lp19 mycelia was 67.4 %, and anucleate protoplasts were detected at a frequency of 31.0 %. The frequency of protoplasts containing two or more nuclei was less than 2% (Fig. 5).

3.3 MOLECULAR CHARACTERISATION OF *A. LOLII* TRANSFORMANTS

Although only one Gus^+ transformant, KS1 was obtained from the transformation experiments described above in Section 3.1, the phenotype was stable, so further experiments were carried out using this transformant. Molecular analysis of this transformant was carried out to determine the number of sites and copies of pFG.gpd

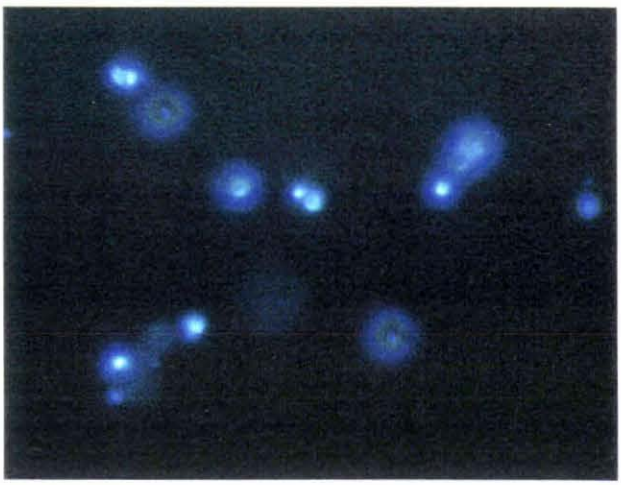


Figure 4. **Hoechst dye-stained Lp19 protoplast nuclei visualised by fluorescence microscopy.**

Magnification 400 x.

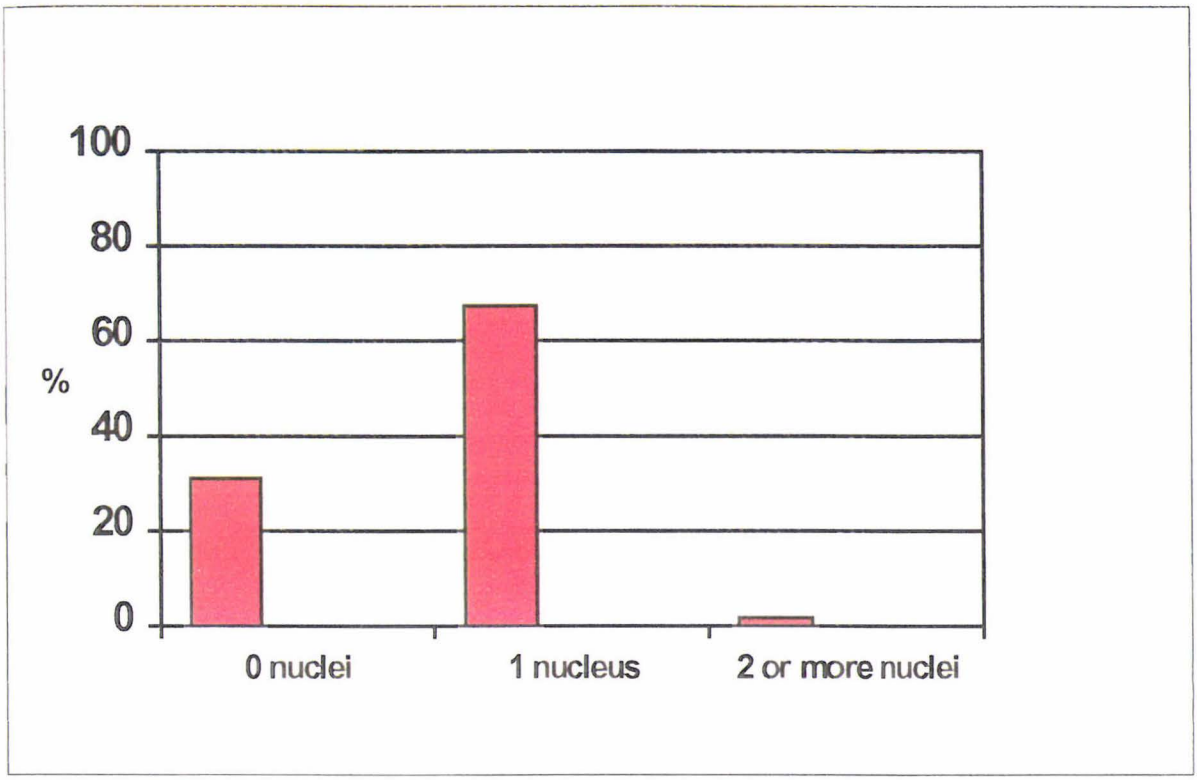


Figure 5. Distribution of nuclei number in protoplasts released from Lp19

A total of 800 protoplasts were scored for nuclei number. All were from a single protoplast preparation.

integrated in the KS1 genome. DNA was prepared as described in Section 2.5.1. The DNA was then analysed by Southern blotting and hybridisation (Section 2.15).

Genomic DNA prepared from KS1 (Section 2.5.1) was digested with *Eco*RI or *Nco*I (Section 2.9), both of which have a single recognition site in pFG.gpd outside of the *gusA* gene (See Appendix 1). A Southern blot of these digests was probed with a [^{32}P]-labelled 1.9 kb *gusA* gene fragment (Section 2.15) isolated by an *Nco*I digest of pNOM-102 (Roberts *et al.* 1989). The results of these hybridisations are shown in Fig. 6. One copy of pFG.gpd at a single site would generate one fragment with homology to the *gusA* gene probe when KS1 DNA was digested with either enzyme. This fragment would represent a portion of plasmid DNA attached to flanking fungal DNA. If several tandem copies are present, at least two bands will share homology to the probe per integration site, representing full length plasmid DNA, and plasmid DNA attached to flanking fungal DNA. Digestion of KS1 genomic DNA with either *Eco*RI (lane 2) or *Nco*I (lane 3) resulted in a single hybridisation signal in both of the digests, 6.7 kb and 3.2 kb respectively, indicating a single copy of the *gusA* gene was present in the KS1 genome. No hybridisation was observed to Lp19 DNA (lane 1).

3.4 ARTIFICIAL INFECTION OF PLANTS, AND STABILITY OF KS1 ENDOPHYTE *IN PLANTA*

The GUS reporter gene system is specific, reproducible and is also very sensitive (Jefferson 1987), requiring small amounts of grass material for the assay. In comparison to the sensitive GUS assay, the methods available for determining alkaloid levels in plant material are less sensitive, requiring tillers from several plants to be pooled in order to obtain sufficient material for assay (Barker *et al.*, 1993). As perennial ryegrass is outcrossing, this involves pooling material from plants of different genotypes. As the aim was to assess whether there was any relationship between fungal metabolic activity, as measured by constitutive GUS expression, and the levels of the various toxins produced by Lp19, it was first necessary to determine whether there were significant differences

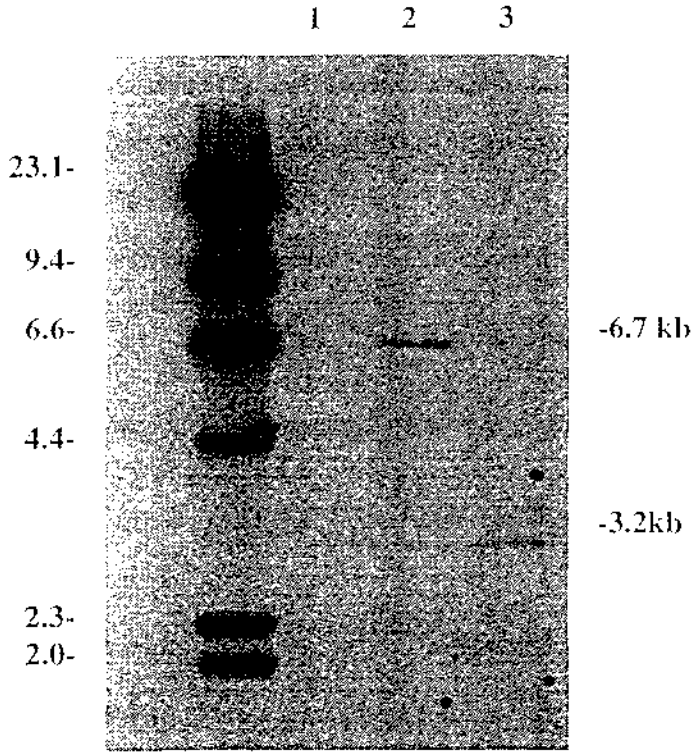


Figure 6. Southern hybridisation of KS1.

Hybridisation of a [α-^{32}P]dCTP-labelled 1.9 kb *Nco*I fragment (*gusA* gene) derived from pNOM-102 to KS1 transformant DNA. Autoradiograph of Southern blots of 2.5 µg of restriction endonuclease digested total *Acremonium* genomic DNA. Lane 1 *Eco*RI digested Lp19 DNA; Lane 2 *Eco*RI digested KS1 DNA; Lane 3 *Nco*I digested KS1 DNA.

between levels of fungal gene expression detected in endophyte infected plants of different genetic backgrounds, before carrying out experiments with pooled plants. In order to determine whether there was any relationship between fungal metabolic activity, (measured as constitutive GUS expression), and the levels of the various toxins produced by Lp19, an experiment using several different genotypes of perennial ryegrass plants was carried out. Perennial ryegrass, cultivar 'Nui' seedlings were artificially infected with transformant KS1, as described in Latch and Christensen (1985) (Section 2.16). The rate of infection was found to be approximately 25%, a typical result for strain Lp19 of *A. lolii* (M. Christensen, personal communication). The resulting plants, each grown from an individual seedling, and therefore of different genetic backgrounds were tested for GUS expression (Section 2.18.2). Five Gus^+ plants were divided up into separate tillers in November, and each tiller grown in a separate pot to generate several clones of each genotype. Before these lines were assayed approximately 3 weeks later, the presence or absence of the *gusA* gene was confirmed by hybridisation and by qualitative assay.

After several months growth (Section 2.16), KS1 was reisolated from plant tissue of the different genotypes as described in Section 2.2. Small portions of the reisolated cultures were tested for GUS expression (Section 2.18.2). All cultures were found to be Gus^+, except the reisolate from plant C which was GUS^-. Hybridisation patterns of the reisolated endophyte DNA were compared to those of the original inoculum strain (Fig. 7). Genotypes A (lane 2), B (lane 3), and D (lane 5) all showed hybridisation to a single 6.7 kb band, identical to that of the original inoculum strain (lane 7). In contrast genotype E showed hybridisation to a 5.2 kb band (lane 6), and no hybridising bands were detected in line C (lane 4). The latter is consistent with an absence of GUS activity in this line of plants (Section 3.6), and in the reisolated culture as described above.

To determine whether the P*gpd-gusA*- T*trpC* chimeric gene in pFG.gpd was complete, KS1 genomic DNA was digested with *Pst*I and *Eco*RI in a single reaction. Digestion with these enzymes should generate a single 4.88 kb fragment that will hybridise to the 1.9 kb *Nco*I fragment (*gusA* gene) derived from pNOM-102 (See Appendix 1 for plasmid map).

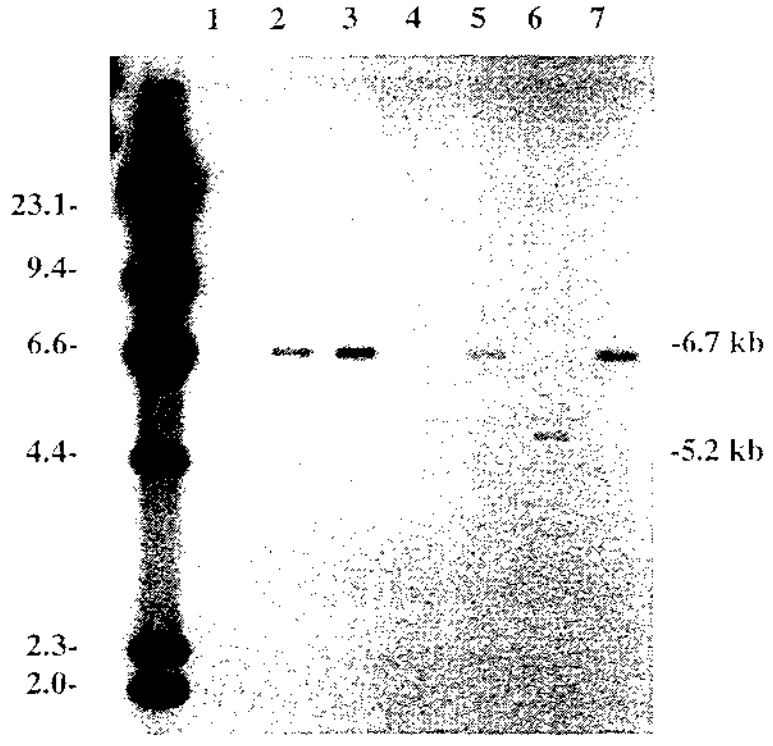


Figure 7. **Hybridisation of *gusA* to plant reisolates of KS1, and the original inoculum.**

Autoradiograph of Southern blots of 2.5 µg of *Eco*RI digested total *Acremonium* genomic DNA probed with labelled 1.9 kb *Nco*I fragment (*gusA* gene) derived from pNOM-102. Lp19 DNA (lane 1); reisolate from plant line: A (lane 2); B (lane 3); C (lane 4); D (lane 5); and E (lane 6); KS1 original inoculum (lane 7).

A Southern blot of these digests in shown in Figure 8. A single band of 4.88 kb was observed in lanes corresponding to reisolates from plants A (lane 1), B (lane 2), D (lane 4), E (lane 5) and the original KS1 inoculum (lane 6), indicating that the portion of the 3'components of the *gus* chimeric gene had remained intact upon integration into the Lp19 genome. These results showed that the rearrangement which had occured in KS1 from plant E, shown as a 5.2 kb band in Figure 6, had not occurred within the *Eco*RI/*Pst*I *gus* chimeric construct DNA. As expected, no signal was present in the lane corresponding to the isolate from plant C (lane 3). Although line C did not contain the *gusA* gene, it maintained the Hyg^R phenotype and therefore presumably contained pAN7-1.

3.5 DETERMINATION AND COMPARISON OF GUS ACTIVITY IN PLANTS OF IDENTICAL AND DIFFERENT GENOTYPES

The plants described in Section 3.4 were used to compare GUS activity in plants of different clonal lineages. To test whether GUS levels were significantly different in tissues derived from individual plants of either identical or different genotypes, split plot, repeated measures designs were chosen.

3.5.1 GUS Acitvity in Plants of Identical Genotype

The first aim was to determine if there were detectable significant differences in GUS activity levels among clonally derived plants of an identical genotype. If the outcome of this assessment showed tiller effects were not significant, tillers from plants of identical genotype could be pooled for analysis. This information was required before an investigation could be made as to whether there was any relationship between fungal metabolic activity, and the level of toxins produced in the association between the transformed strain and perennial ryegrass.

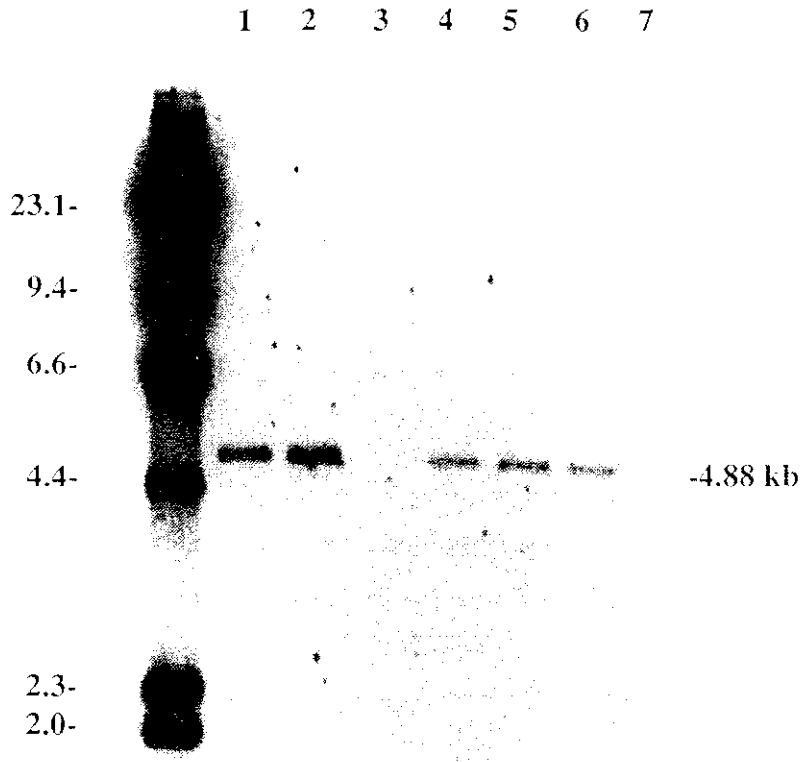


Figure 8. Hybridisation of *gusA* to plant reisolates of KS1, and the original inoculum.

Autoradiograph of Southern blots of 2.5 µg of *Eco*RI/*Pst*I digested total *Acremonium* genomic DNA probed with labelled 1.9 kb *Nco*I fragment (*gusA* gene) derived from pNOM-102. Reisolate from plant line: A (lane 1); B (lane 2); C (lane 3); D (lane 4); and E (lane 5). KS1 original inoculum (lane 6); Lp19 DNA (lane 7).

Four plants were propagated from Gus^+ tillers of KS1-infected genotype 'A'. Three tillers were selected at random from each of these four plants, and each tiller dissected and assayed quantitatively for GUS activity. In this experimental design the four plants of genotype 'A' constitute blocks, and the tillers are nested in blocks. Each tiller is a split plot from which two readings are taken. The rates of reaction calculated for each tissue analysed (Table 4) were analysed by SAS to determine whether any significant differences were detectable (See Appendix 2 for SAS program).

No significant differences were detected in the level of GUS determined for a particular tissue between the different plants (Table 5). The activity measured in the leaf sheaths was highest overall, with the leaf blades containing the lowest overall activity. The youngest tissue of each type, leaf sheath 3 and leaf blade 3 contained the highest activity for their respective tissue type.

3.5.2 GUS Activity in Plants of Several Different Genotypes

The next aim was to determine whether there were significant differences in GUS activity between plants derived from different genotypes. Sets of four plants were propagated from tillers from each of five KS1-infected plants, labelled genotypes 'A', 'B', 'C', 'D' and 'E'. Three tillers were selected at random from each of the 20 plants. As it was found that there were no significant differences in GUS activity between tissues of genetically identical tillers, (Section 3.5.1) the tillers from each individual plant were pooled, dissected, and a sample of the material assayed for GUS activity levels. A sample of material of the same weight used for assay was freeze dried to allow a dry weight determination to be made on material not required for the assay. Plant line 'C' was found to be Gus^- (Section 3.4), and was therefore not included in the final analysis. In the experimental design used in this experiment the genotypes are the blocks, the plants are nested in genotype for each genotype 'A', 'B', 'D', and 'E' and constitute 'split plots' from which two readings were taken. The three tillers from each of the 16 plants used for analysis do not form part of the statistical structure of the design, since the material from

Table 4. GUS activity measured in extracts from perennial ryegrass plants of genotype A.

Tissue	Plant	GUS activities[a]		
		Tiller i	Tiller ii	Tiller iii
Leaf sheath 1	1	0.007 / 0.007	0.002 / 0.007	0.016/ 0.016
Leaf sheath 2	1	0.020 / 0.020	0.011 / 0.012	0.006 / 0.006
Leaf sheath 3	1	0.018 / 0.012	0.024 / 0.024	0.024 / 0.023
Immature leaf	1	0.004 / 0.003	0.002 / 0.002	0.003 / 0.003
Leaf blade1	1	0.004 / 0.003	0.00 / 0.00	0.005 / 0.006
Leaf blade 2	1	0.002 / 0.004	0.002 / 0.001	0.006 / 0.006
Leaf blade 3	1	0.004 / 0.003	0.004 / 0.004	0.006 / 0.007
Leaf sheath 1	2	0.011 / 0.011	0.024 / 0.021	0.012 / 0.012
Leaf sheath 2	2	0.012 / 0.012	0.007 / 0.007	0.017 / 0.020
Leaf sheath 3	2	0.019 / 0.017	0.018 / 0.015	0.021 / 0.021
Immature leaf	2	0.002 / 0.003	0.002 / 0.003	0.005 / 0.004
Leaf blade1	2	0.001 / 0.000	0.005 / 0.003	0.005 / 0.004
Leaf blade 2	2	0.002 / 0.003	0.005 / 0.004	0.007 / 0.008
Leaf blade 3	2	0.002 / 0.002	0.004 / 0.004	0.004 / 0.004
Leaf sheath 1	3	0.029 / 0.033	0.016 / 0.016	0.015 / 0.011
Leaf sheath 2	3	0.030 / 0.027	0.011 / 0.011	0.026 / 0.026
Leaf sheath 3	3	0.043 / 0.040	0.019 / 0.019	0.007 / 0.000
Immature leaf	3	0.000 / 0.008	0.007 / 0.007	0.004 / 0.003
Leaf blade1	3	0.010 / 0.000	0.005 / 0.003	0.005 / 0.004
Leaf blade 2	3	0.002 / 0.003	0.005 / 0.004	0.007 / 0.008
Leaf blade 3	3	0.005 / 0.005	0.006 / 0.008	0.009 / 0.009
Leaf sheath 1	4	0.013 / 0.012	0.028 / 0.027	0.016 / 0.019
Leaf sheath 2	4	0.009 / 0.007	0.016 / 0.016	0.009 / 0.010
Leaf sheath 3	4	0.028 / 0.000	0.021 / 0.019	0.013 / 0.014
Immature leaf	4	0.008 / 0.009	0.024 / 0.026	0.002 / 0.002
Leaf blade1	4	0.009 / 0.011	0.022 / 0.023	0.005 / 0.005
Leaf blade 2	4	0.006 / 0.006	0.003 / 0.003	0.006 / 0.006
Leaf blade 3	4	0.007 / 0.008	0.017 / 0.016	0.003 / 0.003

[a] Rates of reaction (Rate 1 / Rate 2) calculated from duplicate GUS assays, recorded in nmoles MU min^{-1} mg^{-1}. The two reaction rates (nmoles MU min^{-1} mg^{-1} (wet tissue weight)) are noted for each tissue and are the result of individual GUS assays on two aliquots of each extract (calculated from reading 1 and reading 2, see Appendix 2). All readings are from extracts prepared from plants of genotype A.

Table 5. Analysis of variance of endophyte GUS activity within plants of genotype A.

Tissue	Mean nmoles MU[a] mg^{-1} min^{-1}	F-Statistic[b]	Pr>F
Leaf sheath 1[c]	0.01629	0.99	0.4433
Leaf sheath 2	0.01450	1.53	0.2801
Leaf sheath 3[d]	0.01913	0.19	0.9030
Immature leaf[e]	0.00567	1.45	0.2988
Leaf blade 1[c]	0.00575	2.65	0.1203
Leaf blade2	0.00454	0.28	0.8383
Leaf blade3[d]	0.00600	1.37	0.3200

[a] nmoles MU min^{-1} mg^{-1}. Activity was determined under standard assay conditions. These values were obtained from plant tissues derived from genotype A. Values are derived from analysis of duplicate assays. Rates of reaction are listed in Table 4.

[b]The F statistics for different tissues are correlated because the different tissues were derived from the same plants. This table does not consider plants within the genotype separately, since no significant differences were found.

[c] Oldest mature tissue of this type

[d] Youngest mature tissue of this type

[e] Has not yet formed a ligule, dividing leaf blade from leaf sheath

Table 6. **GUS activity measured in extracts from perennial ryegrass plant of different genotypes**

		GUS activities[a] in genotype:			
Tissue	Plant	A	B	D	E
Leaf sheath 1	1	0.008 / 0.009	0.007 / 0.006	0.011 / 0.012	0.006 / 0.006
Leaf sheath 2		0.007 / 0.008	0.006 / 0.005	0.007 / 0.007	0.003 / 0.002
Leaf sheath 3		0.006 / 0.007	0.003 / 0.003	0.014 / 0.013	0.002 / 0.003
Immature leaf		0.002 / 0.002	0.001 / 0.001	0.001 / 0.001	0.000 / 0.000
Leaf blade1		0.000 / 0.003	0.002 / 0.002	0.003 / 0.003	0.002 / 0.003
Leaf blade 2		0.002 / 0.003	0.005 / 0.005	0.001 / 0.001	0.000 / 0.001
Leaf blade 3		0.002 / 0.002	0.003 / 0.003	0.001 / 0.001	0.000 / 0.000
Leaf sheath 1	2	0.008 / 0.009	0.005 / 0.005	0.011 / 0.011	0.007 / 0.006
Leaf sheath 2		0.012 / 0.012	0.003 / 0.003	0.006 / 0.006	0.001 / 0.001
Leaf sheath 3		0.018 / 0.017	0.009 / 0.008	0.015 / 0.016	0.002 / 0.002
Immature leaf		0.002 / 0.003	0.000 / 0.000	0.000 / 0.000	0.000 / 0.000
Leaf blade1		0.002 / 0.003	0.004 / 0.003	0.001 / 0.001	0.003 / 0.003
Leaf blade 2		0.002 / 0.002	0.002 / 0.002	0.000 / 0.000	0.000 / 0.000
Leaf blade 3		0.002 / 0.002	0.002 / 0.002	0.003 / 0.003	0.000 / 0.000
Leaf sheath 1	3	0.008 / 0.008	0.013 / 0.013	0.004 / 0.004	0.007 / 0.007
Leaf sheath 2		0.016 / 0.015	0.009 / 0.008	0.005 / 0.005	0.003 / 0.003
Leaf sheath 3		0.018 / 0.018	0.008 / 0.009	0.007 / 0.007	0.002 / 0.002
Immature leaf		0.003 / 0.004	0.001 / 0.001	0.001 / 0.000	0.000 / 0.000
Leaf blade1		0.006 / 0.007	0.010 / 0.010	0.000 / 0.001	0.004 / 0.004
Leaf blade 2		0.001 / 0.001	0.004 / 0.005	0.002 / 0.001	0.001 / 0.001
Leaf blade 3		0.004 / 0.004	0.001 / 0.002	0.000 / 0.001	0.001 / 0.001
Leaf sheath 1	4	0.011 / 0.011	0.006 / 0.008	0.002 / 0.002	0.007 / 0.007
Leaf sheath 2		0.012 / 0.010	0.009 / 0.010	0.005 / 0.005	0.004 / 0.005
Leaf sheath 3		0.009 / 0.008	0.010 / 0.010	0.004 / 0.004	0.003 / 0.003
Immature leaf		0.006 / 0.005	0.002 / 0.001	0.000 / 0.000	0.001 / 0.000
Leaf blade1		0.005 / 0.005	0.003 / 0.003	0.002 / 0.001	0.005 / 0.006
Leaf blade 2		0.007 / 0.007	0.001 / 0.001	0.001 / 0.001	0.001 / 0.001
Leaf blade 3		0.002 / 0.002	0.001 / 0.001	0.001 / 0.000	0.001 / 0.001

[a] Rates of reaction (Rate 1 / Rate 2) calculated from duplicate GUS assays, recorded in nmoles MU min^{-1} mg^{-1}. The two reaction rates (nmoles MU min^{-1} mg^{-1} (dry tissue weight)) are noted for each tissue and are the result of individual GUS assays on two aliquots of each extract (calculated from reading 1 and reading 2, see Appendix 2).

Note that GUS activity was absent in all tissues of all plants of genotype C.

each set of three tillers is pooled. The rates of reaction calculated for each tissue analysed (Table 6) were assessed for significant differences in GUS activty using SAS (See Appendix 2 for SAS programme). Significant differences between GUS activity for a particular tissue type between different genotypes were detected (Table 7) in five of the tissue types. The tissue types displaying these signifiicant differences were leaf sheath 2, leaf sheath 3, immature leaf, leaf blade 2 and leaf blade 3. No significant differences were detected in the most mature plant tissues, leaf sheath 1 and leaf blade 1. Examination of the pairwise comparisons of the mean level of GUS detected for each tissue (See Appendix 2) reveals which genotypes were significantly different in each case. In leaf sheath 2 and immature blade, genotype A was found to be different to the other three genotypes. Genotype A was found to be different to both B and E in leaf sheath 3 tissue, and different to both D and to E in leaf blade 2 tissue. In leaf blade 3 tissue, genotype A was found to be significantly different to genotype E. In each case that genotype A was significantly different to another genotype, the mean GUS activity was found to be significantly greater to that found in the other plant. The GUS activity measured in the leaf sheaths was highest overall, with the leaf blades containing the lowest overall activity.

3.6 ANALYSIS OF TOXIN AND GUS ACTIVITY DISTRIBUTION

The final aim was to determine whether there was any relationship between fungal metabolic activity, as measured by GUS activity, and the level of toxins produced in the association between the transformed strain and perennial ryegrass. A single plant of each of two genotypes, A and D, corresponding to the genotypes described in Sections 3.4 and 3.5 above, was divided into six individual plants and repotted in August, and grown in the green house for two months. After this period the plant material was harvested, and analysed for GUS activity and for the toxins ergovaline, peramine and lolitrem B, (Sections 2.18 and 2.19). Material was pooled in order to obtain sufficient material for analysis. Based on the results found in Section 3.5, clonal material of a single genotype

Table 7. Analysis of variance of endophyte GUS activity between different genotypes of the same cultivar

Tissue	GUS Activity[a]				F-Statistic[b]	Pr>F
	A	B	D	E		
Leaf sheath 1[c]	0.058125	0.041125	0.031750	0.035375	1.98	0.1711
Leaf sheath 2	0.088500	0.039875	0.029250	0.018000	10.77	0.0010
Leaf sheath 3[d]	0.106750	0.049750	0.065625	0.018375	4.04	0.0337
Immature leaf[e]	0.029500	0.006375	0.003125	0.001625	9.73	0.0016
Leaf blade 1[c]	0.020875	0.017625	0.005375	0.019125	1.57	0.2490
Leaf blade2	0.018375	0.013375	0.003375	0.004125	3.49	0.0499
Leaf blade3[d]	0.014125	0.009500	0.007625	0.003750	3.78	0.0406

[a] nmoles MU min^{-1} mg^{-1}. Activity was determined under standard assay conditions. These values were obtained from plant tissues of four different genotypes, A, B, D, and E. All plant sets being derived from a different KS1 infected seedling. Values are means derived from analysis of duplicate assays, rates of reaction are listed in Table 6.

[b]The F statistics for each of the tissues are correlated because the tissues are derived from the same plants.

[c] Oldest mature tissue of this type

[d] Youngest mature tissue of this type

[e] Has not yet formed a ligule, dividing leaf blade from leaf sheath

was pooled. The six plants of each genotype were divided into two groups of three plants, labelled group i and group ii. The mean levels of GUS activity and toxins determined are shown in Table 8. These levels for the two genotypes studied were analysed by SAS. As there were only 2 degrees of freedom for the error mean square, an F value of >18.5 was required before a significant difference due to the genotypes could be detected in the SAS model (see Appendix 2 for raw GUS data and SAS program). The F test was not particularly sensitive and only one major difference between genotypes could be detected. This difference was found in the lolitrem B level detected for leaf sheath 1, which was found to have a significance level of 0.0006 (Table 9). As these significance levels generated by SAS were each examined at around the 5 % level for significance, then for the 28 sets of analyses carried out (3 toxins x 7 tissues, GUS x 7 tissues) one would expect to see one or two values appearing to be significant at 5 %, when they actually were not significant. The value of 0.0006 remains significant even adjusting for this effect; the probability of this value occuring over all 28 comparisons considered together is less than 5 %, ie the experiment-wise error rate is less than 5 %.

A significant difference was found overall between the two genotypes (A and D), for the toxin and GUS activity levels analysed, as the significance level was found to be 0.0236. The levels of the four parameters (the three toxins and GUS) measured were found to be different, depending on the tissue type, with a significance level of 0.0028. The effect of the genotype was found to be the same for each tissue, reflected by a level of significance of 0.2253. This experiment was a repeated measure design with the toxins or GUS, and the different tissues as the repeated measures, on the same plants. The genotype was found to be significant at $\alpha = 0.0357$ which probably reflects the 0.0006 level of significance found for lolitrem B in leaf sheath 1. If this value was treated as being falsely significant and subsequently omitted, the genotype would no longer be found to be significant.

Table 8. Mean toxin and endophyte GUS levels in two different genotypes of the same cultivar

Tissue	Mean Toxin Levels[a]			Mean GUS Activity[b]
	Ergovaline	Peramine	Lolitrem B	
GENOTYPE A				
Leaf sheath 1[c]	0.80	13.10	28.00	0.00700
Leaf sheath 2	1.25	35.70	25.80	0.00950
Leaf sheath 3[d]	1.30	52.10	13.65	0.01025
Immature leaf[e]	0.95	71.95	3.550	0.00625
Leaf blade 1[c]	0.10	24.95	3.05	0.00375
Leaf blade2	0.35	36.65	3.80	0.00375
Leaf blade3[d]	0.70	69.45	4.90	0.00300
GENOTYPE D				
Leaf sheath 1[c]	1.85	12.95	12.05	0.006500
Leaf sheath 2	1.95	26.25	11.40	0.006500
Leaf sheath 3[d]	2.20	39.25	7.05	0.006750
Immature leaf[e]	1.65	64.80	1.80	0.00450
Leaf blade 1[c]	0.35	16.30	2.55	0.00200
Leaf blade2	0.35	29.80	3.40	0.00325
Leaf blade3[d]	1.15	47.20	3.45	0.00350

a mg kg^{-1} (dry tissue weight)

b nmoles MU min^{-1} mg^{-1} (wet tissue weight). Activity was determined under standard assay conditions. These values were obtained from plant tissues of two different genotypes, A and D. Both plant sets were derived from a different KS1 infected seedling.

c Oldest mature tissue of this type

d Youngest mature tissue of this type

e Has not yet formed a ligule, dividing leaf blade from leaf sheath

Table 9. Analysis of variance of toxin and endophyte GUS levels in two different genotypes of the same cultivar

Tissue	Ergovaline		Peramine		Lolitrem B		GUS	
	F-Statistic	Pr>F	F-Statistic	Pr>F	F-Statistic	Pr>F	F-Statistic	Pr>F
.eaf sheath 1[a]	25.94	0.0365	0.00	0.9706	1565.55	0.0006*	0.20	0.6985
.eaf sheath 2	10.89	0.0809	6.32	0.1285	12.57	0.0711	1.00	0.4226
.eaf sheath 3[b]	4.50	0.1679	23.31	0.0403	12.72	0.0704	0.87	0.4500
mmature leaf[c]	5.76	0.1384	0.70	0.4898	20.08	0.0464	0.13	0.7566
.eaf blade 1[a]	5.00	0.1548	12.38	0.0721	10.00	0.0871	1.00	0.4226
.eaf blade2	0.00	1.0000	33.22	0.0288	0.62	0.5149	0.05	0.8378
.eaf blade3[b]	16.20	0.0565	10.37	0.0844	1.94	0.2981	1.00	0.4226

a Oldest mature tissue of this type

b Youngest mature tissue of this type

c Has not yet formed a ligule, dividing leaf blade from leaf sheath

* Lolitrem B level in leaf sheath 1 assessed as being significantly different between the two genotypes.

Some clear trends emerged in toxin and metabolic activity distributions (Figures 9a and 9b). Metabolic activity was highest in leaf sheath and lowest in leaf blade, generally decreasing as tissue age increased. Peramine reached the highest concentration in the immature leaf tissue, and was shown to decrease in concentration as sheath and blade tissue age increased, similarly to the metabolic activity distribution pattern. Lolitrem B was also highest in the leaf sheath tissue, but showed a different distribution pattern to the former two parameters, with the concentration increasing as the leaf sheath tissue age also increased. Lolitrem B concentration in the leaf blade tissue was low and almost constant. Ergovaline concentration in both genotypes was low in all tissues, and did not show a distinct localisation pattern.

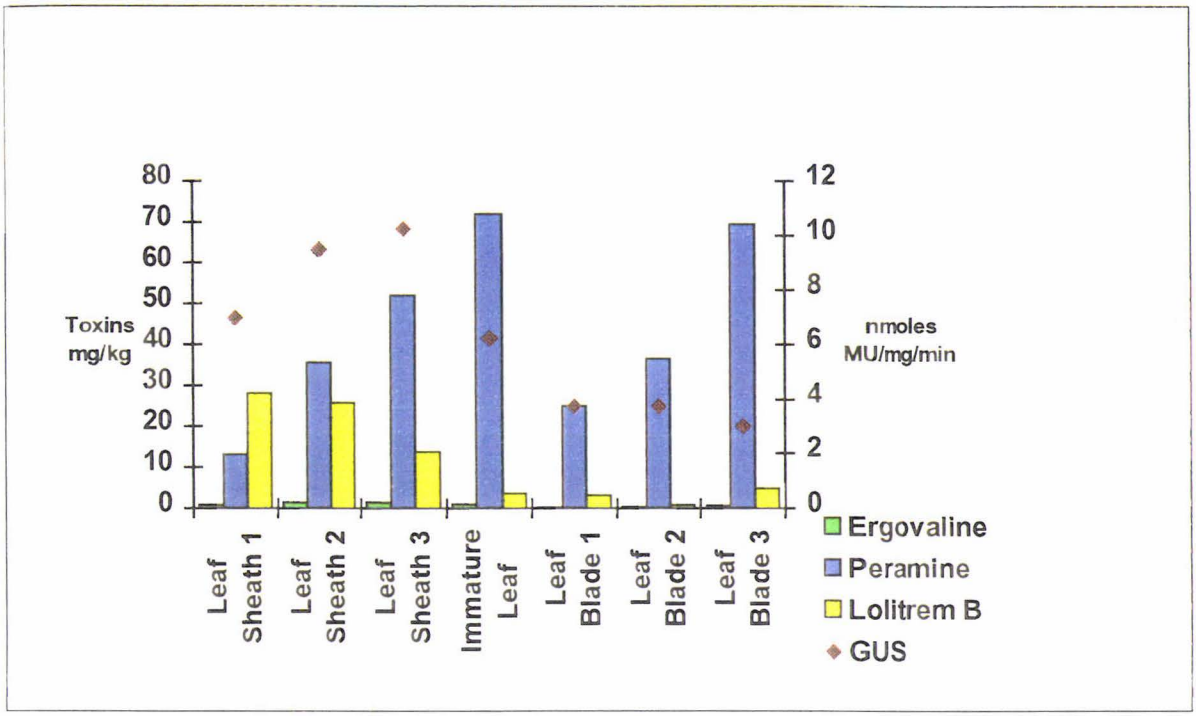


Figure 9a. **Toxin Level and GUS Activity in Various Tissues of Genotype A**

Mean levels of toxins and GUS measured in genotype A. Values are from Table 8. The age of the tissue decreases from leaf sheath/blade 1 to 3.

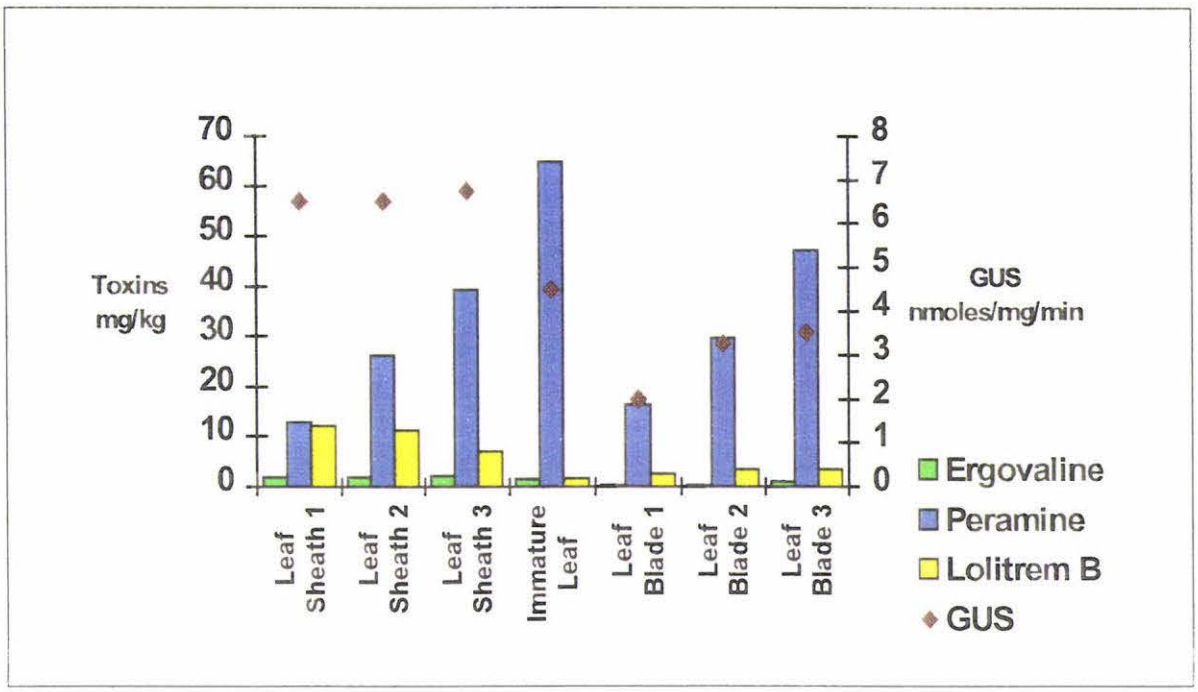


Figure 9b. **Toxin Level and GUS Activity in Various Tissues of Genotype D**

Mean levels of toxins and GUS measured in genotype D. Values are from Table 8. The age of the tissue decreases from leaf sheath/blade 1 to 3.

CHAPTER 4. DISCUSSION

4.1 COTRANSFORMATION OF *ACREMONIUM*

Several transformation systems for various filamentous fungi have been reported, as reviewed by Fincham (1989). Specific transformation methods for *Acremonium* endophytes were recently reported by Murray *et al.* (1992) and Tsai *et al.* (1992). Although the frequencies of fungal transformation reported are often very low, probably due to the low rate of protoplast regeneration, Murray *et al.* (1992) reported frequencies in the upper range for filamentous fungi. They proposed that this was due presumably to the use of a medium giving good protoplast regeneration, and also using sorbitol as an osmoticum during protoplast preparation, and also in the PEG solution used for transformation. In contrast to the results obtained by Murray *et al.* (1992), the frequency of cotransformation of Lp19 with pFG.gpd or pNOM-102 with pAN7-1 using the Murray *et al.* (1992) method was very low in this study, with only one Gus^+ Lp19 cotransformant being generated using pAN7-1 and pFG.gpd (Section 3.1.1). Protoplast regeneration was observed to occur at rates of <5%. Further work was carried out in an attempt to determine more optimal cotransformation conditions (Section 3.1.2) using Lp1; an endophyte strain of *Acremonium* which had been successfully cotransformed with pNOM-102 and pAN7-1 previously in the laboratory. Circular forms of the cotransforming plasmids were included in addition to the linear forms which had been used in previous experiments.

The use of linear versus circular plasmid DNA for transformations is a factor which could be used to increase transformation frequencies and stability of transformants in some fungal species. Judelson (1993) reported that in *Phytophthora infestans*, the use of two transforming plasmids linearised with the same restriction enzyme resulted in a high cotransformation frequency. A model used to explain this high frequency was provided, and supported by hybridisation studies. It was assumed that the selected and nonselected plasmids are ligated, and cointegrate into the DNA (ie a single integration event occurs),

in contrast to transformation using circular plasmids which resulted in the independent integration of the two plasmids (ie multiple integration events occur). This leads to the proposal that access to integration sites in the chromosome rather than the delivery of the DNA into the nucleus is the rate-limiting factor in cotransformation, and that this can be overcome by joining the cotransforming plasmids before integration. While the linearisation of plasmids can result in an increased rate of transformation in some species, in others it can result in no change in transformation success (Judelson, 1993). In this study, when the plasmids used to transform *Acremonium* were linearised with *Hin*dIII, the frequency of cotransformation was not improved.

It is common to see a high number of unstable transformants that initially express a selectable marker, but then fail to grow when replated onto selective media (Yelton *et al.* 1984). Murray *et al.* (1992) found that the use of a linearised plasmid to transform the particular *Acremonium* strain used in their study resulted in a comparable transformation rate, but that a greater proportion of the linear DNA transformants were stable after 3 weeks growth (60% stability) than when DNA was introduced in circular form (25% stability). As only one cotransformant was obtained from the first experiment carried out on Lp19 in this study, and this was obtained from transformation with linear plasmids pAN7-1 and pFG.gpd, both linearised with *Hin*dIII), no conclusions on stability of cotransformants resulting from different DNA conformations can be reached in this case. In later experiments with Lp19 and Lp1, a lower transformation frequency was observed when linearised plasmid DNA was used, and no Hyg^R colonies were observed when either *gusA* carrying plasmid was cotransformed in linearised form along with linear pAN7-1. Around 10% of the Hyg^R colonies resulting from cotransformation of Lp1 with circular DNA were subsequently found to be GUS^+. These cotransformants were stable when replated onto selective media.

As integration into the chromosome is the only way in which stable transformants can be obtained, the observation that Lp1 was able to be stably transformed and notably cotransformed at a higher frequency than Lp19, may be due to Lp1 having a more

efficient recombination system of the two strains. This could be explained by the difference in 'fitness' of the two strains. Lp1 is an interspecific hybrid strain of *Acremonium* (Section 1.2.1), whereas Lp19 is a haploid strain which may consequently make Lp1 'fitter' than Lp19.

In this study, a lower cotransformation rate was observed in both Lp1 and Lp19 when the plasmid pFG.gpd was used, as opposed to when pNOM-102 was used. The plasmid pFG.gpd was previously reported to also cotransform *A. nidulans* at a lower rate than the frequencies usually reported in the literature (McGowan, 1996), and at a lower rate than pNOM-102 which was used in comparison during transformation experiments. The reasons for this were not able to be determined in the course of that study (McGowan, 1996).

There are some methods which could possibly be used to assist in increasing cotransformation frequencies in this *Acremonium* system, and these are outlined below. The *hph* gene can be positively selected for by the addition of hygromycin B to the media used to regenerate transformed cells. Upon the addition of this antibiotic, it becomes an advantage for the cells to express the *hph* gene, as cells which do not express this gene become non-viable. The same selection pressure was not present for the expression of the *gusA* gene, as the product is of no positive benefit to the cell. This could result in the loss of the *gusA* gene from the fungal cells.

Mönke and Schäfer (1993) carried out experiments where they used a single plasmid to introduce both the *gusA* and *hph* genes into the fungal maize pathogen, *Cochlibolus heterostrophus*, as opposed to this study where each of these genes were introduced into *Acremonium* on a separate plasmid. Mönke and Schäfer used one of either two different plasmids to deliver the DNA into the fungal protoplasts, each with a different promoter to control *gusA* expression. The use of the two different promoters, the *C. heterostrophus P1* promoter, or the *C. heterostrophus gpd1* promoter, excluded the possibility of untoward effects of a particular promoter used in the transforming vector. It was found that GUS

gene expression was transient in nature under non-selective conditions after transformation. This was studied by transferring GUS expressing colonies onto fresh plates and testing them for the Hyg^R and Gus^+ phenotypes. A large number of Gus^+ transformants were seen after transformation, but at least 99 % of all transformants lost the introduced DNA, as Southern blot analysis of transformants that were initially Gus^+ lacked vector DNA. This demonstrates that the limiting step in transformation experiments is not the uptake of the introduced DNA, but the incorporation of this DNA into the fungal genome.

This work suggests that a single plasmid carrying a dominant selectable marker, as well as the *gusA* gene, along with the maintenance of selection pressure could lead to more successful cotransformation. Although selection pressure was maintained in the experiments carried out in this thesis, the *gusA* gene was not carried on the same DNA fragment as the *hph* gene. This would have decreased the chances of the *gusA* gene being integrated into the fungal genome and/or maintained in the genome after transformation and therefore may be at least part of the reason for the very low cotransformation rates observed.

The construction of strains with homologous DNA target sites may be a useful tool for improving the transformation frequency in filamentous fungi. It has been reported that transformation frequencies of filamentous fungi with positively selectable vectors that lack homology with the recipient genome were sometimes quite low, between 1-10 transformants per µg of DNA. Attempts to increase the transformation frequencies by incorporating chromosomal fragments into vectors resulted in limited success (Tilburn *et. al.*, 93). Farman *et al.* (1992) transformed *Leptosphaeria maculans*, a fungal pathogen of *Brassica* spp. with pAN8-1, a plasmid encoding phleomycin resistance. The Phl^R transformants were then retransformed with pAN7-1. Retransformation of the Phl^R strain to Hyg^R was observed to occur at frequencies consistently two-fold higher than with the original recipient strain. Linearised pAN7-1 transformed $Phleo^R$ protoplasts at higher frequencies than circular DNA. All the transformants that were tested retained a $Phleo^R$

phenotype. Molecular analysis of five transformants showed that in four cases the pAN7-1 vector DNA had integrated into pAN8-1 sequences. These results suggest that transformation frequencies in *L. maculans* are limited by the ability of vector DNA to integrate into the genome. This approach could also be taken with the *Acremonium* pFG.gpd/pAN7-1 transformation system, as these two plasmids share some homologous sequences.

4.1.1 Alteration of the *gusA* Gene in KS1

It was determined that a single copy of the *gusA* gene had integrated into the KS1 genome (Section 3.3). It was shown in Section 3.3 that although Lp19 transformants could not be single spore purified, the frequency of protoplasts released from Lp19 mycelia with 2 or more nuclei was less than 2 %. The greatest proportion of protoplasts were mononucleate, and would therefore give rise to a genetically homogeneous culture. Experiments were carried out where KS1 was grown in liquid culture, then a small piece of peripheral culture was selected and used to generate a new culture. After repeating this for several rounds, and using fluorescence microscopy to detect GUS activity with the aid of a specific dye (Imagene Green (Molecular Probes Inc.)), it was found that the staining was of uniform intensity, and no sectors of any culture were found which did not express GUS (M. Spiering, personal communication). These results suggest that the transformant generated, KS1, is a genetically homogeneous culture. A rearrangement of the fungal genome occurred in plant line E, resulting in a decrease in the length of the band hybridising to the *gusA* probe in an *Eco*RI digest. This rearrangement did not alter the length of the P*gpd-gusA*-T*trp*C chimeric fragment, and GUS expression was observed. A rearrangement also occured in plant line C, resulting in the loss of the *gusA* gene. The culture reisolated from plant line C retained the Hyg^R phenotype implying that the pAN7-1 was still present (this was not checked by hybridisation). As plant line C initially tested Gus^+, this alteration appears to have occurred after artificial inoculation of the plant lines. The rearrangements noted in these two plant lines may have occured due to recombination between the homologous sequences of the two transforming plasmids (See

Appendix 1 for plasmid maps), resulting in loss of pFG.gpd (and perhaps pAN7-1) DNA to various extents. The phenomenon of plants which are initially Gus^+, reverting to a Gus^- phenotype has been observed by other researchers (M. Spiering, personal communication) studying perennial ryegrass cultivar 'Nui', infected with KS1, and is currently being studied further.

4.2 EFFECT OF PLANT GENOTYPE ON ANALYSES

4.2.1 The Effect of Plant Genotype on GUS Activity

The analysis of GUS activity in plants derived from several different genotypes showed plants which are clonally related can be pooled for analysis. Three tillers from each of four plants, all of a single genotype were divided into 7 different tissues and the GUS activity in these tissues measured and analysed for significant differences. No significant differences were found within any of the tissue types for all of the 12 individual tillers. This outcome showed that pooling material from genetically identical plants would minimise genotype effects. A second experiment to investigate whether significant differences could be detected between plants of different genetic backgrounds made use of the information gained from the above experiment, and material from sixteen plants, of four different genotypes was harvested and pooled within genotypes. When the GUS activity measured was analysed, it was found genotype effects were present, with five out of the seven tissues tested displaying significant differences. The tissues that did not show significant differences in GUS activity between genotypes were the most mature plant tissues, leaf sheath 1 and leaf blade 1. Genotype A was shown to be significantly different to at least one another genotype in each case where a difference was detected.

It was already known that a great variation exists among isolates of the *Acremonium* endophytes of perennial ryegrass and tall fescue. Christensen and Latch (1991), studied endophytic fungi isolated from tall fescue plants and Christensen *et al.* (1991) studied *Acremonium* endophytes isolated from perennial ryegrass. When visible parameters such

as growth rate, conidial length, antifungal activity and sensitivity to benomyl were compared, considerable cultural, morphological and physiological variations were observed. The range of variations observed in the two studies showed that there is significant variation not only between *Acremonium* endophytes isolated from different host grass species, but also between the endophytes found to occur naturally in a single host grass species. The results presented in this thesis show that not only are there visible differences between different endophyte-grass associations, but that there are also non-visual differences between different genotypes of a single cultivar in association with a single *Acremonium* isolate. This indicates the high level of specificity and evolution of the grass - endophyte relationships. This work also highlights the sensitivity of the GUS reporter gene system, as it has been shown to allow detection of differences at the level of the cultivar in an endophyte - perennial ryegrass association.

4.2.2 The Effect of Plant Genotype on Toxin Production

It was found by Agee and Hill (1994) and Roylance *et al.* (1994), that endophyte production of toxins in tall fescue is controlled by the plant genotype as well as such factors as the environment of the plant, when they studied endophyte infected tall fescue. Tests were carried out by cross pollinating two genotypes, one which tested high in ergovaline concentration, the other low. The ranges of ergovaline concentration were similar among the progeny derived from the reciprocal cross, and were approximately double the mean value calculated from the two parents. Means of the progeny from each parent were not different from one another, or the parental mean. As the endophyte is passed on only through seed it behaves as a maternal trait, so if ergovaline was controlled exclusively by the endophyte, the progeny means would have been expected to be similar to that of their respective maternal parent (Agee and Hill, 1994). Results reflecting the same information were reported by Roylance *et al.* (1994), where peramine was also studied, and its production also found to be affected by the plant genotype. As no correlation was found between peramine and ergovaline concentration, it was suggested that the plant independently controls the production of each toxin by the endophyte. This

information suggests that as toxin production is controlled by the plant genotype, pooling plants of a single genotype in a study of toxins will minimise genotype effects.

4.3 METABOLIC ACTIVITY AND TOXIN DISTRIBUTION *IN PLANTA*

In a mature *A. lolii* infected perennial ryegrass plant, a high proportion of the mycelium present is found in leaf sheaths, with less in leaf blades, and with older sheath and blade tissue containing a higher concentration of mycelium than the less mature tissue (Musgrave and Fletcher, 1984). In this thesis, the highest level of GUS activity was found in the leaf sheaths, in particular in leaf sheath 3, the youngest tissue of this type. Blade tissue was found to contain the lowest activities. This distribution of GUS activity being higher in leaf sheaths than in leaf blades reflects the observation that there is a greater amount of fungal mycelia in the leaf sheath, compared to the amount present in the blade tissue. The observation that the youngest tissue contained the highest level of metabolically active endophyte, although immature tissue contains less endophytic mycelia, can perhaps be explained by the changes in the plant tissue structure which occur with age. In older plant tissue, the hyphal activity is known to decrease, probably due to their reduced area of contact with the surrounding host cells (Koga *et al.*, 1993). The matrix which sustains the contact between endophyte tissue and surrounding host cells is believed to be essential for nutrient supply to the endophyte (Christensen, 1995; Koga *et al.*, 1993). Thus, if the endophyte was receiving a reduction in the amount of nutrient supplied, it would follow that it would become less metabolically active.

Although the GUS reporter gene system allows the level and location of fungal metabolic activity to be determined, the activity of the fungal hyphae does not necessarily reflect the total amount of hyphae present. There are however methods available for estimating the amount of filamentous fungi in plant tissue (Ride and Drysdale, 1972; Roberts *et al.*, 1988), using measurements of the fungal chitin present. Oliver *et al.* (1993) have reported on a method using GUS activity of fungal transformants (of the species *Cladosporium fulvum* and *Leptosphaeria maculans*, both fungal plant pathogens), transformed with

pNOM-102 and pAN7-1, to measure hyphal biomass. As these plasmids were shown to be able to be used to transform *Acremonium*, this method could be applied to the *Acremonium*-perennial ryegrass interaction. A method where endophyte infected grass tissue is destained, then fungal hyphae are stained to assess the biovolume of the fungi in grass tissue has recently been developed (Y. Tan, personal communication).

The distribution of ergovaline, peramine and lolitrem B was also studied in the same plants at the same time as the GUS activity distribution was investigated. Ergovaline was found to be at a consistently low level in all tissues tested. Peramine was found to be at the highest levels in leaf sheath tissue, showing an inverse relationship with increasing sheath age, as did the metabolic activity distribution pattern described above. Lolitrem B was also highest in the leaf sheath tissue, but in contrast to the pattern displayed by peramine and metabolic activity, the concentration of lolitrem B increased as the leaf sheath tissue age also increased. Ball *et al.* (1995) reported that the mean concentrations of lolitrem B and peramine in individual plants were closely related to and probably largely determined by yearly mean concentrations of *A. lolii*.

The distributions of the three toxins studied in this thesis matched the distribution patterns for these toxins reported by others (Ball *et al.*, 1995; Keogh *et al.*, 1996). The levels of lolitrem B and peramine found in this study were also comparable with those found in perennial ryegrass reported by Rowan (1993), where lolitrem B levels greater than 25 mg kg^{-1} were detected in outer leaf sheaths, and peramine concentrations of between 10 and 30 mg kg^{-1} were found to be typical, although higher levels were detected, with the levels being dependent on the genotype of the host. Ergovaline was found at very low concentrations in tall fescue by Agee and Hill (1994), at up to 0.938 mg kg^{-1}.

Although trends in the levels of toxins and GUS activity determined were seen, the F test was not particularly sensitive in the experiment when GUS and toxin expression levels

were compared, and only one major difference between genotypes could be detected. This could be overcome in the future by including more plants of each genotype tested in future experiments.

4.4 SUMMARY AND FUTURE PROSPECTS

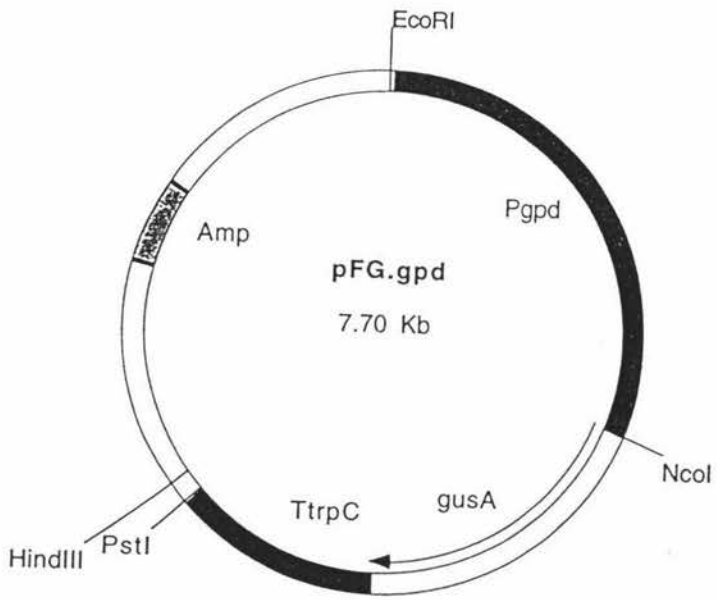
The positive effects of the endophyte on the host combined with deleterious effects on grazing animals present livestock producers with a biological dilemma. Without a full understanding of the regulation and expression of toxin genes, it is difficult to design meaningful experiments and novel or altered associations resulting in less or no potential threat to livestock while retaining the positive benefits to the pasture conferred by the endophyte. The results presented show the potential of the use of the GUS reporter gene system to study endophyte gene expression *in planta*, and that pooling plants of the same genotype can allow simultaneous study of the expression of toxins produced by the association. Genes involved in toxin metabolism could potentially be studied using their promoter regions inserted into a chimeric construct along with the *gusA* gene, which would allow the temporal and spatial expression of this gene to be determined *in planta*. Before these types of studies can progress, the cotransformation of *A. lolii* should be further optimised. An obvious experiment is the combination of the *hph* and *gusA* genes on a single plasmid. Alterations can then be made in the controlling sequences, and the gene expression re-examined along with any subsequent changes in toxin production or distribution to be simultaneously measured.

The use of an *in planta* system to monitor genetic alterations made to an endophyte is important and necessary, as a single endophyte strain does not necessarily produce the same array of toxins in different types of host plant. In addition, toxins are not produced in all plants (Christensen *et al.*, 1993). Another factor to be considered is the possibility that a knockout of a step in the pathway of toxin metabolism, may not result in an absence of that toxin. An alternative pathway may exist, or the precursors could also be redirected

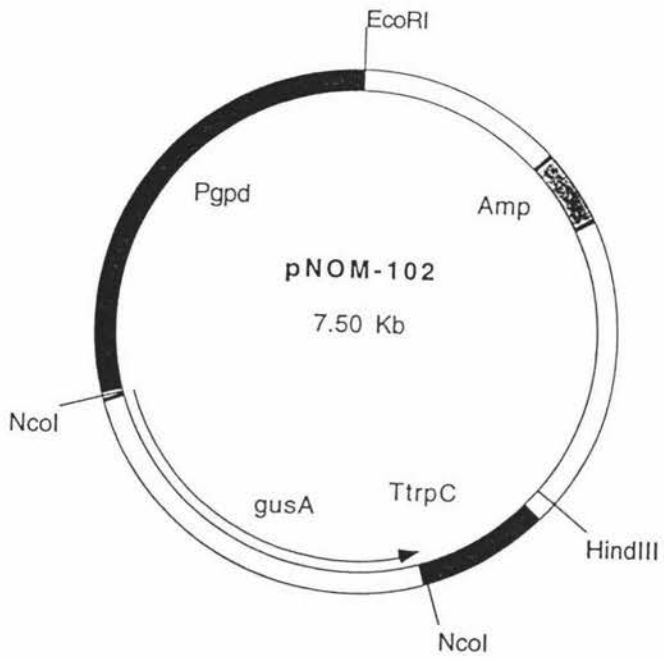
and used to produce higher amounts of another toxin. This situation could be monitored using the GUS reporter gene system.

The GUS reporter gene system, used as described in this thesis shows the potential for its use in the search of a better understanding of the fungal endophyte-host plant association, as it can be used to follow fungal gene expression which can in turn be compared and correlated to toxin production *in planta*. When a detailed understanding of these associations has been gained, technology may be used to alter the outcome of the associations in a desirable and beneficial manner.

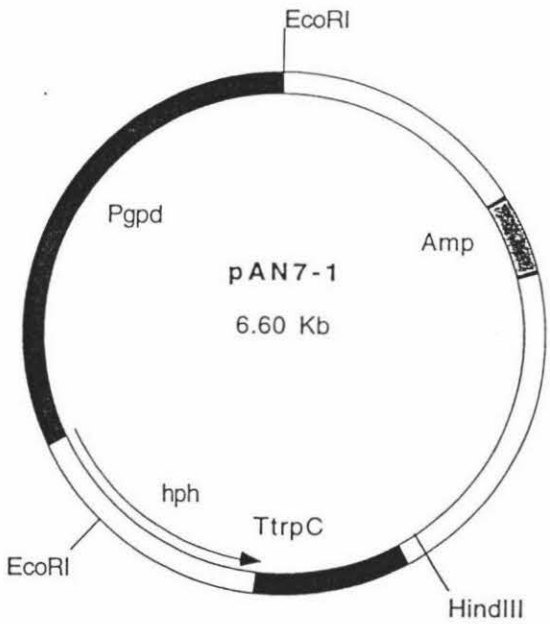
APPENDIX 1. PLASMID MAPS



Restriction map of pFG.gpd showing sites for: *Hin*dIII, *Eco*RI, *Pst*I and *Nco*I.



Restriction map of pNOM-102 showing sites for *Hin*dIII, *Eco*RI and *Nco*I.



Restriction map of pAN7-1 showing sites for *Hin*dIII and *Eco*RI.

APPENDIX 2. RAW DATA AND SAS PROGRAMS

Conversion of Raw Data - nmoles MU in 2 ml stopped reaction mix to nmoles MU min^{-1} mg^{-1}

Reading from fluorometer is converted to:

nmoles l^{-1}	÷ 10
nmoles per 3 ml stop buffer (or per 100 µl reaction mix added to stop buffer)	x 0.003
nmoles per ml of reaction mix (or per 50 µl extract)	x 10
nmoles per ml of crude extract	x 20
conversion factor	0.06

The figure obtained after multiplication by the conversion factor is the nmoles MU ml^{-1} per total tissue in the sample, so the figure is divided by the mg weight of the tissue in the sample and the resulting figure is the nmoles MU ml^{-1} mg^{-1}.

These figures were then entered into Cricket Graph (Computer Associates Ltd) (Section 2.20) and linear regression was used to obtain the nmoles MU ml^{-1} $mg^{-1}min^{-1}$.

Fluorometer Readings - GUS Assays of Extracts from *Lolium perenne* Plants Infected with Wildtype Endophyte

Plant	Tissue	Reading 1/Reading 2[a]		
		Minutes[b]		
		0	20	40
1	Leaf sheath 1	7/5	8/6	8/6
	Leaf sheath 2	5/3	7/8	6/7
	Leaf sheath 3	8/9	5/4	9/4
	Immature leaf	9/7	9/7	2/6
	Leaf blade 1	13/8	7/6	6/8
	Leaf blade 2	7/9	8/8	11/1
	Leaf blade 3	19/7	9/18	7/13
2	Leaf sheath 1	8/9	16/16	7/7
	Leaf sheath 2	19/5	8/5	11/7
	Leaf sheath 3	11/6	10/8	11/7
	Immature Leaf	11/13	14/13	15/10
	Leaf blade 1	12/10	13/15	13/15
	Leaf blade 2	19/15	25/14	14/14
	Leaf blade 3	12/18	18/14	7/4

[a]Two readings are determined for each time point[b] and are each the result of individual GUS assays on two aliquots of each extract.

A wet weight of 20 mg of each tissue was used for extraction.

Fluorometer Readings – GUS Assays of Extracts from *Lolium perenne* Plants of An Identical Genotype

Plant Number & Tiller	Tissue	Reading 1/Reading2[a]				
		Minutes[b]				
		0	15	30	45	60
1 i	Leaf sheath 1	17/16	69/66	*/108	137/161	187/155
	Leaf sheath 2	22/15	105/110	201/205	290/288	417/427
	Leaf sheath 3	19/16	112/100	232/123	231/168	421/281
	Immature leaf	15/15	36/33	59/62	65/55	96/85
	Leaf blade 1	14/18	39/37	64/63	*/82	90/*
	Leaf blade 2	13/12	45/33	55/55	87/69	82/89
	Leaf blade 3	12/12	30/32	49/49	70/75	89/74
1 ii	Leaf sheath 1	11/15	47/48	82/83	121/92	149/104
	Leaf sheath 2	20/18	76/75	130/130	183/189	244/241
	Leaf sheath 3	20/24	160/167	309/306	466/468	606/614
	Immature leaf	21/15	24/25	40/37	47/52	63/62
	Leaf blade 1	19/15	24/20	35/35	*/42	50/63
	Leaf blade 2	17/15	25/22	32/33	40/38	51/45
	Leaf blade 3	23/16	41/40	61/64	83/88	105/103
1 iii	Leaf sheath 1	33/21	85/79	147/147	218/219	288/282
	Leaf sheath 2	21/18	48/55	86/77	113/107	145/135
	Leaf sheath 3	15/29	139/137	260/257	383/381	506/491
	Immature leaf	21/16	34/32	79/52	73/68	88/85
	Leaf blade 1	31/17	55/55	79/86	114/115	144/148
	Leaf blade 2	24/18	54/44	73/75	113/106	138/133
	Leaf blade 3	25/18	48/49	79/81	118/105	145/156
2 i	Leaf sheath 1	16/22	78/87	148/153	209/210	274/275

Plant Number & Tiller	Tissue	0	15	30	45	60
2 i cont'd	Leaf sheath 2	21/16	79/80	134/145	195/197	254/250
	Leaf sheath 3	21/22	113/101	212/201	304/287	395/357
	Immature leaf	15/22	20/19	27/28	45/38	57/58
	Leaf blade 1	17/*	21/20	24/27	35/31	37/39
	Leaf blade 2	21/13	30/27	41/40	54/53	68/65
	Leaf blade 3	11/20	22/25	33/35	44/44	54/59
2 ii	Leaf sheath 1	25/26	104/102	184/183	282/267	373/328
	Leaf sheath 2	21/20	51/51	91/88	126/117	169/164
	Leaf sheath 3	18/24	139/113	240/219	353/347	446/353
	Immature leaf	21/17	31/32	44/48	59/58	71/74
	Leaf blade 1	18/18	43/35	55/59	81/74	103/92
	Leaf blade 2	18/18	40/40	65/58	77/88	114/103
	Leaf blade 3	16/*	53/35	63/57	91/73	112/97
2 iii	Leaf sheath 1	17/18	91/75	155/160	228/219	288/275
	Leaf sheath 2	17/19	103/102	198/207	289/287	369/435
	Leaf sheath 3	24/24	134/133	253/256	367/363	494/498
	Immature leaf	29/15	41/32	64/59	56/77	127/101
	Leaf blade 1	18/18	46/47	77/68	97/92	132/129
	Leaf blade 2	24/18	56/52	94/100	141/127	168/177
	Leaf blade 3	17/17	28/36	52/56	76/83	96/104
3 i	Leaf sheath 1	50/38	180/213	368/363	453/571	652/696
	Leaf sheath 2	40/33	197/180	319/320	492/469	652/572
	Leaf sheath 3	27/34	255/290	460/447	633/718	916/826

Plant Number & Tiller	Tissue	0	15	30	45	60
3 i cont'd	Immature leaf	32/63	63/59	97/68	130/1381	54/165
	Leaf blade 1	35/27	135/97	166/153	215/248	300/252
	Leaf blade 2	22/22	74/74	97/126	176/160	216/179
	Leaf blade 3	27/28	54/48	74/116	104/99	131/119
3 ii	Leaf sheath 1	22/24	75/76	129/129	190/184	210/215
	Leaf sheath 2	24/22	77/116	131/132	184/181	227/241
	Leaf sheath 3	23/28	122/103	220/210	330/301	410/403
	Immature leaf	28/26	56/54	92/89	131/113	157/165
	Leaf blade 1	29/35	71/78	121/119	168/170	210/206
	Leaf blade 2	25/26	73/77	127/135	178/164	206/242
	Leaf blade 3	28/*	60/61	95/91	133/129	151/172
3 iii	Leaf sheath 1	30/32	102/97	189/177	278/218	321/233
	Leaf sheath 2	27/36	162/160	290/293	412/431	570/580
	Leaf sheath 3	34/37	228/187	429/438	638/*	595/*
	Immature leaf	36/29	52/45	74/67	65/81	131/82
	Leaf blade 1	38/37	101/93	175/158	237/215	318/275
	Leaf blade 2	39/27	43/78	79/131	134/153	195/210
	Leaf blade 3	36/37	81/78	121/166	170/*	226/210
4 i	Leaf sheath 1	31/41	85/92	152/155	217/212	284/265
	Leaf sheath 2	34/64	70/68	120/115	165/156	208/203
	Leaf sheath 3	27/36	158/166	281/285	433/416	556/533
	Immature leaf	38/*	61/65	115/98	160/168	179/183
	Leaf blade 1	35/38	76/78	130/128	173/193	214/245
	Leaf blade 2	35/29	63/66	101/91	126/125	161/156
	Leaf blade 3	38/29	68/59	98/105	137/142	176/178
4 ii	Leaf sheath 1	31/34	119/111	198/195	291/287	375/371

Plant Number & Tiller	Tissue	0	15	30	45	60
4 ii cont'd	Leaf sheath 2	34/29	105/111	200/194	265/269	347/350
	Leaf sheath 3	30/47	127/135	228/230	340/341	426/419
	Immature leaf	33/35	154/157	286/286	417/416	516/557
	Leaf blade 1	36/34	142/147	249/283	361/384	478/509
	Leaf blade 2	32/27	41/*	63/63	79/77	102/102
	Leaf blade 3	22/31	103/103	190/184	274/263	347/346
4 iii	Leaf sheath 1	27/25	89/94	164/163	251/317	329/*
	Leaf sheath 2	72/23	65/83	123/132	193/181	233/229
	Leaf sheath 3	32/23	80/85	156/149	216/234	277/294
	Immature leaf	23/23	30/31	41/33	52/53	66/67
	Leaf blade 1	23/28	51/48	74/78	101/105	132/134
	Leaf blade 2	26/37	53/54	84/82	116/110	150/152
	Leaf blade 3	25/24	39/38	59/52	78/75	94/86

[a]Two readings are determined for each time point[b] and are each the result of individual GUS assays on two aliquots of each extract.

*No reading was available for this time point.

A wet weight of 20 mg of each tissue was used for extraction.

This table corresponds to Section 3.5.

Fluorometer Readings - GUS Assays of Extracts from *Lolium perenne* Plants of Different Genotypes

Genotype and Plant Number	Tissue	Reading 1/Reading[a]				
		Minutes[b]				
		0	15	30	45	60
A 1	Leaf sheath 1	20/17	54/51	121/167	139/142	185/190
	Leaf sheath 2	19/14	51/63	130/88	112/133	171/187
	Leaf sheath 3	32/27	53/54	91/89	122/125	160/168
	Immature leaf	17/21	26/24	45/42	54/49	65/62
	Leaf blade 1	24/11	32/35	50/47	57/61	78/76
	Leaf blade 2	11/10	18/21	24/31	57/39	*/64
	Leaf blade 3	29/23	41/31	44/41	61/56	68/66
A 2	Leaf sheath 1	24/22	68/59	100/104	153/165	177/190
	Leaf sheath 2	16/23	75/78	143/143	199/199	261/266
	Leaf sheath 3	22/25	108/102	196/191	284/271	375/357
	Immature leaf	21/12	26/26	37/35	50/49	64/69
	Leaf blade 1	23/11	38/35	39/47	56/58	66/74
	Leaf blade 2	12/10	22/24	37/31	46/43	51/53
	Leaf blade 3	14/7	17/20	27/34	37/38	44/49
A 3	Leaf sheath 1	20/19	58/55	96/95	138/132	181/178
	Leaf sheath 2	18/21	99/100	183/173	258/255	349/331
	Leaf sheath 3	23/24	113/109	201/221	292/291	381/390

Genotype and Plant Number	Tissue	0	15	30	45	60
A 3 cont'd	Immature leaf	22/16	33/35	52/54	69/74	87/89
	Leaf blade 1	19/19	49/52	81/84	114/118	147/150
	Leaf blade 2	25/17	24/43	38/*	45/45	51/52
	Leaf blade 3	16/17	33/35	57/58	75/66	93/93
A 4	Leaf sheath 1	20/18	59/69	123/141	173/183	230/239
	Leaf sheath 2	19/34	72/71	121/122	166/183	271/227
	Leaf sheath 3	19/20	61/56	103/105	149/143	193/184
	Immature leaf	19/29	49/54	86/77	115/107	136/136
	Leaf blade 1	19/18	42/44	65/68	89/92	117/119
	Leaf blade 2	17/18	53/52	93/86	124/129	148/*
	Leaf blade 3	19/15	28/27	38/41	48/54	*/55
B 1	Leaf sheath 1	22/17	52/46	107/82	129/102	157/137
	Leaf sheath 2	15/48	41/45	69/61	99/89	*/118
	Leaf sheath 3	11/16	30/29	57/46	61/59	83/78
	Immature leaf	19/11	13/15	17/17	22/22	26/26
	Leaf blade 1	7/8	15/17	28/*	32/32	38/40
	Leaf blade 2	11/9	36/35	56/56	80/81	105/105
	Leaf blade 3	18/8	22/22	37/36	51/52	67/66
B 2	Leaf sheath 1	23/19	44/43	68/70	92/93	120/122
	Leaf sheath 2	18/15	32/32	47/51	63/66	77/82
	Leaf sheath 3	22/20	61/62	105/102	150/146	197/186
	Immature leaf	21/16	21/19	21/20	22/21	23/23
	Leaf blade 1	19/19	39/39	53/71	72/71	91/89
	Leaf blade 2	21/19	26/29	42/39	46/51	57/58
	Leaf blade 3	18/20	26/25	35/37	42/44	50/52
B 3	Leaf sheath 1	15/14	74/79	137/*	204/199	275/285

Genotype and Plant Number	Tissue	0	15	30	45	60
B 3 cont'd	Leaf sheath 2	13/12	54/58	95/97	148/140	198/182
	Leaf sheath 3	12/11	46/54	98/100	124/140	164/185
	Immature leaf	15/11	14/16	19/20	24/28	27/30
	Leaf blade 1	12/14	61/58	108/116	160/163	203/216
	Leaf blade 2	19/11	33/37	58/58	83/82	106/104
	Leaf blade 3	11/9	16/19	26/28	33/36	40/43
B 4	Leaf sheath 1	10/12	35/35	65/72	95/102	127/176
	Leaf sheath 2	14/13	49/52	95/94	142/151	189/196
	Leaf sheath 3	15/17	52/49	108/110	159/162	216/220
	Immature leaf	10/15	15/17	24/25	33/32	44/41
	Leaf blade 1	10/11	20/22	37/33	49/54	65/69
	Leaf blade 2	9/9	15/19	15/24	26/31	35/35
	Leaf blade 3	7/10	11/12	19/18	26/27	24/33
C 1,2,3,4	NOTE: GUS activity was absent in all tissues of all plants of genotype C					
D 1	Leaf sheath 1	19/14	64/73	122/136	172/191	231/246
	Leaf sheath 2	17/14	47/48	81/85	125/*	150/153
	Leaf sheath 3	14/11	78/76	*/141	*/207	300/*
	Immature leaf	10/11	17/15	21/19	25/24	*/30
	Leaf blade 1	16/15	29/28	44/43	60/54	75/67
	Leaf blade 2	14/13	16/16	31/23	23/23	26/25
	Leaf blade 3	13/13	15/15	19/16	22/24	26/26
D 2	Leaf sheath 1	16/17	76/75	123/129	182/181	240/238
	Leaf sheath 2	17/16	43/42	72/73	105/104	134/133
	Leaf sheath 3	16/20	93/95	164/180	245/258	320/337
	Immature leaf	18/12	13/13	15/14	17/17	22/18

Genotype and Plant Number	Tissue	0	15	30	45	60
D2 cont'd	Leaf blade 1	12/14	16/15	19/20	23/22	25/25
	Leaf blade 2	14/14	16/15	15/16	20/19	19/18
	Leaf blade 3	15/18	31/37	51/49	69/68	83/81
D 3	Leaf sheath 1	15/17	32/28	51/49	76/68	101/89
	Leaf sheath 2	8/13	32/31	60/53	87/79	112/105
	Leaf sheath 3	10/11	41/41	72/79	107/104	162/146
	Immature leaf	7/7	8/11	11/11	14/12	21/15
	Leaf blade 1	13/11	11/10	15/13	20/18	21/22
	Leaf blade 2	8/8	14/14	22/23	29/30	39/37
	Leaf blade 3	6/4	13/8	12/15	16/23	16/14
D 4	Leaf sheath 1	7/2	17/14	36/22	*/27	42/35
	Leaf sheath 2	6/13	36/30	74/55	86/82	109/105
	Leaf sheath 3	10/11	31/25	53/50	70/74	95/93
	Immature leaf	7/6	8/7	9/12	13/14	13/14
	Leaf blade 1	9/8	14/13	20/18	26/25	59/30
	Leaf blade 2	9/11	14/12	18/16	25/23	30/26
	Leaf blade 3	5/9	9/8	13/12	15/14	21/18
E 1	Leaf sheath 1	13/15	44/41	75/73	110/104	131/140
	Leaf sheath 2	10/12	20/21	36/33	47/46	66/61
	Leaf sheath 3	9/10	*/20	35/34	47/50	55/64
	Immature leaf	7/5	7/9	9/9	12/12	14/14
	Leaf blade 1	22/9	26/22	37/35	50/52	64/65
	Leaf blade 2	6/3	7/8	13/12	13/13	15/16
	Leaf blade 3	15/13	13/16	*/17	20/19	23/21
E 2	Leaf sheath 1	12/14	45/44	*/71	112/102	145/137
	Leaf sheath 2	13/12	17/18	23/21	28/29	36/35
	Leaf sheath 3	12/8	21/21	30/32	41/40	53/54

Genotype and Plant Number	Tissue	0	15	30	45	60
E 2 cont'd	Immature leaf	11/18	13/10	12/12	12/11	13/12
	Leaf blade 1	12/12	24/25	37/37	50/51	64/64
	Leaf blade 2	16/12	13/14	15/15	17/16	18/18
	Leaf blade 3	14/10	15/11	14/14	16/15	16/16
E 3	Leaf sheath 1	16/14	44/46	79/78	118/117	148/146
	Leaf sheath 2	14/13	27/27	43/42	59/54	75/73
	Leaf sheath 3	10/8	20/18	28/27	39/36	46/54
	Immature leaf	10/8	22/12	10/12	*/16	15/14
	Leaf blade 1	13/13	29/29	46/48	63/79	86/82
	Leaf blade 2	13/12	23/18	22/24	37/28	40/32
	Leaf blade 3	11/10	*/15	15/17	21/19	22/*
E 4	Leaf sheath 1	11/15	44/45	79/89	114/118	148/*
	Leaf sheath 2	12/12	18/32	50/*	70/79	92/101
	Leaf sheath 3	9/10	23/25	41/41	58/55	68/72
	Immature leaf	12/15	12/12	15/15	21/19	22/21
	Leaf blade 1	12/23	36/40	60/69	88/124	115/153
	Leaf blade 2	18/10	17/17	24/25	29/31	37/36
	Leaf blade 3	12/12	18/18	23/25	29/30	36/36

[a]Two readings are determined for each time point[b] and are each the result of individual GUS assays on two aliquots of each extract.

*No reading was available for this time point.

See table below for dry weight of each tissue extracted.

This table corresponds to Section 3.5

Dry weights of tissues used for - GUS Assays of Extracts from *Lolium perenne* Plants of Different Genotypes

Tissue	Dry Weight (mg)			
	Genotype and Plant Number			
	A1	A2	A3	A4
Leaf sheath 1	2.5	3.0	4.2	5.5
Leaf sheath 2	2.5	2.8	3.1	3.0
Leaf sheath 3	2.4	2.4	3.2	3.1
Immature leaf	2.5	2.7	2.3	2.0
Leaf blade 1	4.3	4.0	5.1	4.4
Leaf blade 2	2.8	3.6	4.5	3.9
Leaf blade 3	3.1	3.2	3.5	3.7
	B1	B2	B3	B4
Leaf sheath 1	4.1	3.3	4.0	3.7
Leaf sheath 2	3.6	3.7	3.4	2.9
Leaf sheath 3	3.1	3.0	3.3	2.9
Immature leaf	2.9	2.8	2.4	2.9
Leaf blade 1	4.8	5.2	5.2	4.9
Leaf blade 2	5.5	3.7	4.6	3.9
Leaf blade 3	4.0	3.3	3.7	3.8

Tissue	Dry Weight (mg)			
	Genotype and Plant Number			
	C1	C2	C3	C4
Leaf sheath 1	3.9	4.1	3.7	4.1
Leaf sheath 2	3.3	3.1	2.7	2.3
Leaf sheath 3	3.2	2.8	2.4	2.8
Immature leaf	3.1	3.1	3.2	2.5
Leaf blade 1	3.8	3.8	4.0	4.5
Leaf blade 2	4.2	3.7	4.4	4.3
Leaf blade 3	3.2	2.9	2.8	3.7
	D1	D2	D3	D4
Leaf sheath 1	4.9	3.7	4.0	3.7
Leaf sheath 2	3.7	3.1	2.8	2.8
Leaf sheath 3	2.9	2.6	3.2	2.6
Immature leaf	2.8	2.8	3.0	3.0
Leaf blade 1	4.2	4.6	3.0	3.5
Leaf blade 2	4.0	4.0	3.5	3.8
Leaf blade 3	3.9	3.6	3.0	3.7
	E1	E2	E3	E4
Leaf sheath 1	3.5	3.9	4.2	5.5
Leaf sheath 2	4.2	3.3	3.1	3.0
Leaf sheath 3	2.7	2.0	3.2	3.1
Immature leaf	3.4	2.4	2.3	2.0
Leaf blade 1	5.5	5.3	5.1	4.4
Leaf blade 2	4.4	4.4	4.5	3.9
Leaf blade 3	3.4	3.4	3.5	3.7

Fluorometer Readings - GUS Assays of Extracts from *Lolium perenne* Plants of Two Different Genotypes To Correlate with Toxin Distribution

Genotype and Group	Tissue	Reading 1/Reading2[a]			
		Minutes[b]			
		0	15	30	45
A i	Leaf sheath 1	20/19	93/104	258/185	267/253
	Leaf sheath 2	19/31	171/174	327/315	558/449
	Leaf sheath 3	23/35	174/171	373/333	520/512
	Immature leaf	50/17	161/156	299/307	414/439
	Leaf blade 1	31/19	90/80	151/146	214/237
	Leaf blade 2	32/14	86/77	158/164	218/224
	Leaf blade 3	21/3	48/42	85/72	123/114
A ii	Leaf sheath 1	5/14	128/125	269/328	386/420
	Leaf sheath 2	17/8	66/65	140/141	216/212
	Leaf sheath 3	3/13	175/167	368/352	538/529
	Immature leaf	5/6	92/103	197/299	286/325
	Leaf blade 1	30/13	83/74	123/128	169/172
	Leaf blade 2	11/12	70/81	131/132	243/213
	Leaf blade 3	40/*	94/74	159/149	239/225
D i	Leaf sheath 1	28/38	89/94	159/167	238/241
	Leaf sheath 2	44/29	94/81	189/178	272/271
	Leaf sheath 3	23/18	88/117	186/219	276/292
	Immature leaf	33/28	96/114	156/231	280/313
	Leaf blade 1	50/46	58/60	85/91	125/137
	Leaf blade 2	25/25	85/84	142/147	231/186
	Leaf blade 3	20/35	71/69	128/138	162/187

Genotype and Group	Tissue	Reading 1/Reading2[a]			
		Minutes[b]			
		0	15	30	45
D ii	Leaf sheath 1	20/21	117/114	220/189	330/296
	Leaf sheath 2	19/27	104/81	205/198	234/276
	Leaf sheath 3	27/21	102/91	197/207	296/254
	Immature leaf	18/17	45/34	68/74	96/97
	Leaf blade 1	23/26	38/46	71/72	93/93
	Leaf blade 2	41/29	40/46	60/61	90/89
	Leaf blade 3	22/16	61/53	100/101	145/133

[a]Two readings are determined for each time point[b] and are each the result of individual GUS assays on two aliquots of each extract.

*No reading was available for this time point.

A wet weight of 50 mg of each tissue was used for extraction.

This table corresponds to Section 3.6.

SAS Program Used for ANOVA of Genetically Identical Plant Analysis

```
data D1;
infile 'tiller.txt';
input parent $ tiller tissue $ reading 1 reading 2;
cards;
;
run;

proc means;
run;
data D2 (keep = parent tiller tissue reading level);
        set D1;
        level = reading1; reading = 1; output;
        level = reading 2; reading = 2; output;

proc sort; by tissue;
 proc glm data = D2;
        class parent tiller reading;
        model level = parent tiller(parent) reading parent*reading;
        test H = parent E = tiller(parent);
        lsmeans parent/ E = tiller(parent) pdiff tdiff;
        lsmeans reading parent*reading / pdiff tdiff;
        by tissue;
```

SAS Program Used for ANOVA of Clonally Unrelated Plant Analysis

```
options ls=78 ps=66
data D1;
infile 'wetwtnoc.txt';
        input parent $ progeny tissue $ reading 1 reading 2;

cards;
;
run;
proc means;
run;
data D2 (keep = parent progeny tissue reading level);
        set D1;
        level = reading1; reading = 1; output;
        level = reading 2; reading = 2; output;

proc sort; by tissue;

proc glm data = D2;
        class parent progeny reading;
        model level = parent progeny(parent) reading parent*reading;
        test H = parent E = progeny(parent);
        lsmeans parent / E = progeny(parent)  pdiff tdiff;
        lsmeans reading parent*reading/ pdiff tdiff;
        by tissue;
```

SAS Program Used for ANOVA of Toxin and GUS Activity Analysis

```
options ls=78 ps=66;
data d1;
filename in1 'octa.txt';
infile in1;

input   #1 genotype $ gusls1  gusls2  gusls3  guseb   gusl1   gusl2   gusl3
        #2            ergols1 ergols2 ergols3 ergoeb  ergol1  ergol2  ergol3
        #3            perals1 perals2 perals3 peraeb  peral1  peral2  peral3
        #4            lolmls1         lolmls2         lolmls3         lolmeb lolml1  lolml2
        lolml3
;

proc glm;
        class genotype;
        model gusls1--lolml3 = genotype;

repeated toxin 4, location 7;
run;
proc sort; by genotype;
proc means n mean;
var gusls1--lolml3;

by genotype;
run;
```

APPENDIX 3. PAIRWISE COMAPARISONS

The following tables were generated by SAS when analysing GUS activity between different genotypes.

TISSUE=leaf sheath 1
General Linear Models Procedure
Least Squares Means

Standard Errors and Probabilities calculated using the Type III MS for PROGENY(PARENT) as an Error term

PARENT	LEVEL LSMEAN	T for H0: LSMEAN(i)=LSMEAN(j) / Pr > |T| i/j	1	2	3	4
KS1B	0.04112500	1	.	0.798498	0.489745	-1.44794
				0.4401	0.6331	0.1733
KS1D	0.03175000	2	-0.7985	.	-0.30875	-2.24644
			0.4401		0.7628	0.0443
KS1E	0.03537500	3	-0.48975	0.308752	.	-1.93769
			0.6331	0.7628		0.0766
KS1A	0.05812500	4	1.447942	2.24644	1.937688	.
			0.1733	0.0443	0.0766	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

TISSUE=leaf sheath 2

General Linear Models Procedure

Least Squares Means

Standard Errors and Probabilities calculated using the Type III MS for PROGENY(PARENT) as an Error term

PARENT	LEVEL LSMEAN	T for H0: LSMEAN(i)=LSMEAN(j) / Pr > |T| i/j	1	2	3	4
KS1B	0.03987500	1	.	0.794433	1.635597	-3.6357
				0.4424	0.1279	0.0034
KS1D	0.02925000	2	-0.79443	.	0.841164	-4.43013
			0.4424		0.4167	0.0008
KS1E	0.01800000	3	-1.6356	-0.84116	.	-5.2713
			0.1279	0.4167		0.0002
KS1A	0.08850000	4	3.635699	4.430132	5.271297	.
			0.0034	0.0008	0.0002	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

TISSUE=leaf sheath 3
General Linear Models Procedure
Least Squares Means

Standard Errors and Probabilities calculated using the Type III MS for PROGENY(PARENT) as an Error term

PARENT	LEVEL LSMEAN	T for H0: LSMEAN(i)=LSMEAN(j) / Pr > |T| i/j	1	2	3	4
KS1B	0.04975000	1	.	-0.61349	1.212485	-2.20276
				0.5510	0.2487	0.0479
KS1D	0.06562500	2	0.613488	.	1.825973	-1.58927
			0.5510		0.0928	0.1380
KS1E	0.01837500	3	-1.21248	-1.82597	.	-3.41525
			0.2487	0.0928		0.0051
KS1A	0.10675000	4	2.202761	1.589273	3.415246	.
			0.0479	0.1380	0.0051	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

TISSUE=immature blade

General Linear Models Procedure

Least Squares Means

Standard Errors and Probabilities calculated using the Type III MS for PROGENY(PARENT) as an Error term

PARENT	LEVEL	T for H0: LSMEAN(i)=LSMEAN(j) / Pr > |T|			
	LSMEAN	i/j	1	2	3	4
KS1B	0.00637500	1	.	0.549493	0.803106	-3.90986
			.	0.5927	0.4375	0.0021
KS1D	0.00312500	2	-0.54949	.	0.253612	-4.45935
			0.5927		0.8041	0.0008
KS1E	0.00162500	3	-0.80311	-0.25361	.	-4.71296
			0.4375	0.8041		0.0005
KS1A	0.02950000	4	3.909857	4.45935	4.712962	.
			0.0021	0.0008	0.0005	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

TISSUE=leaf blade 1

General Linear Models Procedure

Least Squares Means

Standard Errors and Probabilities calculated using the Type III MS for PROGENY(PARENT) as an Error term

PARENT	LEVEL	T for H0: LSMEAN(i)=LSMEAN(j) / Pr > |T|			
	LSMEAN	i/j	1	2	3	4
KS1B	0.01762500	1	.	1.538656	-0.18841	-0.40821
				0.1498	0.8537	0.6903
KS1D	0.00537500	2	-1.53866	.	-1.72706	-1.94687
			0.1498		0.1098	0.0753
KS1E	0.01912500	3	0.188407	1.727062	.	-0.21981
			0.8537	0.1098		0.8297
KS1A	0.02087500	4	0.408215	1.94687	0.219808	.
			0.6903	0.0753	0.8297	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

TISSUE=leaf blade 2

General Linear Models Procedure

Least Squares Means

Standard Errors and Probabilities calculated using the Type III MS for PROGENY(PARENT) as an Error term

PARENT	LEVEL	T for H0: LSMEAN(i)=LSMEAN(j) / Pr > |T|			
	LSMEAN	i/j	1	2	3	4
KS1B	0.01337500	1	.	1.811024	1.675197	-0.90551
				0.0952	0.1197	0.3830
KS1D	0.00337500	2	-1.81102	.	-0.13583	-2.71654
			0.0952		0.8942	0.0187
KS1E	0.00412500	3	-1.6752	0.135827	.	-2.58071
			0.1197	0.8942		0.0241
KS1A	0.01837500	4	0.905512	2.716536	2.580709	.
			0.3830	0.0187	0.0241	

TISSUE=leaf blade 3
General Linear Models Procedure
Least Squares Means

Standard Errors and Probabilities calculated using the Type III MS for PROGENY(PARENT) as an Error term

PARENT	LEVEL LSMEAN	T for H0: LSMEAN(i)=LSMEAN(j) / Pr > |T| i/j	1	2	3	4
KS1B	0.00950000	1	.	0.597772	1.833169	-1.47451
				0.5611	0.0917	0.1661
KS1D	0.00762500	2	-0.59777	.	1.235396	-2.07228
			0.5611		0.2403	0.0604
KS1E	0.00375000	3	-1.83317	-1.2354	.	-3.30767
			0.0917	0.2403		0.0063
KS1A	0.01412500	4	1.474505	2.072278	3.307674	.
			0.1661	0.0604	0.0063	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

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