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**MOLECULAR CHARACTERISATION OF THE  
HMG CoA REDUCTASE GENE FROM  
*NEOTYPHODIUM LOLII***

A Thesis presented in partial fulfilment of  
the requirements for the degree of  
Master of Science in Molecular Genetics  
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New Zealand.

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## ABSTRACT

3-Hydroxy-3-methylglutaryl Coenzyme A reductase (Hmg) catalyses the conversion of HMG CoA to mevalonic acid; the first step of the isoprenoid biosynthetic pathway. This pathway produces a wide variety of primary metabolites which are involved in many different cellular processes. *Neotyphodium* endophytes in association with the grass host are known to produce a range of secondary metabolites including the indole diterpenoids (eg paxilline and lolitrem) and the ergopeptine alkaloids (eg ergovaline). Given that these pathways are upregulated *in planta* the availability of mevalonic acid, be it from fungal or plant source, will be important in controlling the levels of the different toxins synthesised. The aim of this work was to clone the fungal endophyte *hmg* and characterise the promoter to enable study of its regulation *in planta* via reporter gene studies.

Using degenerate primers designed against conserved regions of other *hmg* genes a 359 bp fragment was amplified from the *Neotyphodium lolii* isolate Lp19, which grows in perennial ryegrass (*Lolium perenne*). DNA sequencing confirmed that the sequence amplified was part of a unique *hmg* gene. Southern hybridisations suggest that there is a single copy of *hmg* in strain Lp19 (a haploid) but two copies in strain Lp1 (an interspecific hybrid; Schardl *et al.* 1994). The fragment of *N. lolii hmg* was used to screen a  $\lambda$ GEM-12 genomic library of Lp19 and four positive overlapping clones were isolated.

Fragments of one clone,  $\lambda$ JD12, were subcloned, sequenced and a physical map of this region of the genome was constructed. The entire sequence of *hmg* was determined using primer walking and was found to encode a 1188 amino acid polypeptide. From comparison to other Hmg proteins the catalytic domain has been shown to be highly conserved while the amino-terminal domain, containing transmembrane regions is divergent with very little sequence similarity near the translation start site and promoter region.

Using RT-PCR analysis the *hmg* gene was shown to consist of two open reading frames separated by a 73 bp intron. RT-PCR was also used to determine the location of the transcriptional start site and this is supported by the presence of putative CAAT and TATA consensus sequences. With the promoter region identified and characterised further analysis of the regulation of *hmg in planta* can be undertaken.

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## Chapter 1

### INTRODUCTION

#### 1.1 GRASS ENDOPHYTES

The filamentous fungi of the Ascomycete family Clavicipitaceae (tribe Balansieae) form symbiotic associations with grasses of the Pooideae subfamily. *Epichloë* endophytes are natural agents of biological protection, and infection can be important for fitness, competitiveness and persistence of their grass hosts under conditions of biotic or abiotic stress. These symbionts inhabit reproductive and aerial host tissue, growing as sparsely branched hyphae in intercellular spaces. Nutrients are absorbed by these organisms from freely available materials (Clay 1990).

##### 1.1.1 Life Cycle

*Neotyphodium* (formerly *Acremonium*) endophytes are anamorphic (asexual) forms of the ascomycetous *Epichloë* spp. and share many features with them. *Epichloë* spp. are the causative agents of "choke" disease of grasses and can be seed transmitted when the "choke" stage is not exhibited. Chokes are sporogenous stromata produced in association with host inflorescences, preventing seed production by arresting development of the florets. At this stage, *E. typhina* can undergo sexual crosses, producing ascospores which are, presumably, capable of infecting new host plants. Following fertilisation of *E. typhina* stromata by conidia of the opposite mating type, perithecia develop on this structure and release haploid ascospores when mature. The ascospores germinate to produce phialides and conidia, which in turn germinate to produce hyphae and more conidiophores. After the last cycle of this conidiation, the mycelia are thought to infect the host via the stigmata, leaf stem stomata and/or ancillary buds. In addition, meristematic tissue must become infected for survival of the fungus (Siegel and Schardl 1991). It is this spore forming stage which the asexual endophytes lack. The life cycle of the *Neotyphodium* endophytes occurs within organs of the host grass without eliciting any symptoms of disease and the maternal-line transmission due to seed dissemination of the endophytes is a highly efficient process, removing the need for spore dissemination (Schardl and An 1993).

Genetic analysis based on isozyme profiles and DNA sequence comparisons supports the theory of being multiple evolutionary origins of the asexual endophytes from *Epichloë* species, and even isolates from the same grass species can show considerable genetic diversity. Perennial ryegrass is a known host of three species or taxa in the *Epichloë* group. The two known taxa of asexual, seed-borne endophytes are *Neotyphodium lolii* (Latch *et al.* 1984) also classified as taxonomic grouping one from *Lolium perenne* (LpTG-1) and LpTG-2 (Christensen *et al.* 1993). LpTG-2 is a heteroploid derived from an interspecific hybridisation whose most likely ancestors were *N. lolii* and *E. typhina* (Schardl *et al.* 1994).

Extensively studied grass-endophyte interactions include the tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*) with the endophytes *Neotyphodium coenophialum* and *Neotyphodium lolii* respectively. These two grass species are major forage crops in grazing agro-ecosystems, with endophyte infected ryegrass and tall fescue being widespread in New Zealand and the United States, respectively. The importance of the endophytes to these grasses has recently led to their intense investigation.

### **1.1.2 Biological Benefits of Endophyte/Plant Interaction**

Endophytic fungi have dramatic effects on the physiology, ecology and reproductive biology of host plants. The presence of the endophyte increases drought tolerance and leads to changes of grass morphology, growth rate and growth yield. The production of toxic alkaloids enable endophytic fungi to defend their host against mammalian and insect herbivory, and offers resistance to fungal pathogens and nematodes. This contributes to the excellent field persistence of these grasses with little metabolic cost to the plant (Clay 1990).

Endophyte infection alters the host plant physiology, providing greater vegetative vigour. More inflorescences and seeds are produced and the seeds are able to germinate more rapidly, with faster growing seedlings. Infected seeds have a higher survival rate as they contain high concentrations of alkaloids and so are less likely to be eaten. Endophyte infected tall fescue has been found to maintain a higher productivity than uninfected plants under drought conditions. This is due to the infected plants maintaining a higher net photosynthetic rate, as well as having greater stomatal resistance (Clay 1990).

A number of alkaloids are produced by endophytes within plant tissues and these are active against insects, fungal pathogens, nematodes and mammals. These include peramine, ergovaline, lolines and the indole diterpenoids, paxilline and the lolitrems.

The pyrrolizidine alkaloids (*N*-formyl loline and *N*-acetyl loline), detected in *A. coenophialum*-infected tall fescue, are the most abundant secondary metabolites that have been identified and the most important bioprotective alkaloids known to be involved in many grass-endophyte associations. These compounds have been detected in very high levels in infected plants (> 0.8% plant dry weight) and have been shown in culture to be fungal metabolites (Wilkensen *et al.* 1997). The production of these alkaloids is dependent on the fungal strain (Siegel *et al.* 1990) and are known to be potent insecticides. Their contribution to host fitness may be due to their ability to disrupt physiological processes controlled by biogenic amines (Scott and Schardl 1993).

Peramine, a pyrrolopyrazine alkaloid, is produced by a number of endophytes including *N. lolii*, *N. coenophialum* and *E. typhina* strains. This is a potent insect feeding deterrent which is thought to be derived from proline and arginine. This alkaloid has been found to provide resistance to the Argentine stem weevil (*Listronotus bonariensis*), a major pest of pastures in New Zealand. While the mechanism by which this is achieved remains unclear, the alkaloid has not been shown to be a mammalian toxin. Peramine has not been detected in fungal cultures or in uninfected plants, and therefore may be unique to the mutualistic association (Rowan and Gaynor 1986).

Endophyte infection has also been shown to negatively influence populations of certain nematodes, thus offering the plant protection against nematode infection (Kimmons *et al.* 1990). There are contrasting reports regarding the effects of endophytes on plant parasitic nematodes but this variation in effect may reflect the relative sensitivities of different species to the toxins produced. Anti-fungal activity has also been reported in endophyte infected grasses. Resistance to pathogens has not been widely demonstrated, however isolation of endophytes which confer resistance to important fungal diseases would provide significant potential for improving the productivity of perennial ryegrass pastures (van Heeswijck and McDonald 1992).

*A. coenophialum* infection of tall fescue produces ergopeptine alkaloids, in particular ergovaline, which is thought to be responsible for fescue toxicosis. Cattle grazing on infected tall fescue present a number of symptoms which are clinically identical with ergot poisoning. This condition is usually observed in summer, with cattle showing a range of symptoms which include, reduced weight gain, decreased milk production, lower feed intake, rough hair-coat, excessive salivation, increased respiration and high

temperatures. Fescue foot is another syndrome observed in cattle that have been feeding on infected tall fescue. This occurs most frequently in winter causing lameness and dry gangrene of extremities which can, in severe cases, lead to loss of hooves (van Heeswijck and McDonald 1992). The biochemical mechanisms responsible for these syndromes are not known, but the ergot alkaloids are known to interact with the  $\alpha$ -adreno and dopamine receptors in the brain, and these compounds have vasoconstrictive effects. Ergovaline is thought to be composed of a tricyclic peptide and an ergolene ring, synthesised from the precursors mevalonic acid and tryptophan. Ergopeptines are found in a number of grass-endophyte interactions, including perennial ryegrass associations (Christensen *et al.* 1993).

Cattle grazing on *N. lolii* infected perennial ryegrass suffer from the neurological disorder 'ryegrass staggers'. The potent neurotoxin lolitrem A, and other indole diterpenoid lolitrems, have been isolated from perennial ryegrass (*Lolium perenne*) pastures on which this livestock disease occurs. These compounds have been shown to produce tremors in livestock and therefore may be the causative agents of the disease (Gallagher *et al.* 1982; Gallagher *et al.* 1981). These compounds are tremorgenic mycotoxins which are thought to bind to the GABA<sub>A</sub> receptor in the brain and inhibit its function (Gant *et al.* 1987), thus causing affected animals to display muscle spasms and a hypersensitivity to external stimuli. Little is known about the biochemical pathways leading to the synthesis of these compounds, although putative intermediates paxilline, paspaline and lolitriol have been identified and the outlines of a biosynthetic pathway suggested (Miles *et al.* 1992; Munday-Finch *et al.* 1996). Paxilline, a known tremorgen, is thought to be derived from tryptophan and mevalonic acid, and produced by a series of modifications of geranyl geranyl pyrophosphate. Cultures of *N. lolii* produce a reduced amount of lolitrem compared to amounts detected *in planta*, suggesting that the host plant may have a role in biosynthesis.

Endophyte concentrations vary throughout the host plant, with higher concentrations of hyphae and metabolic activity in lower parts. Distribution is also high in the leaf sheath in comparison to the leaf blade. There are also concentration differences between leaves of different ages: younger leaves contain lower *N. lolii* concentrations, perhaps reflecting the pattern and stage of development of fungal and plant tissue (Herd *et al.* 1997; Keogh *et al.* 1996). The distribution of alkaloids also varies throughout the host plant, with lolitrem B being present in higher concentrations in the leaf sheath than the blade and in particular, the outer leaf sheath. High lolitrem B concentrations have also been found in the older leaves, mostly in the upper portion of the leaf sheath, where the concentration of *N. lolii* was the highest. This distribution pattern correlates with livestock grazing patterns, the acquisition of the neurotoxin and development of

ryegrass staggers. Peramine was found in concentrations greater than the 10 ppm threshold which deters feeding by adult and larval Argentine stem weevil, in all portions of the grass except the tip of the older blades. As peramine is produced by *N. lolii*, it is apparent that this fungal metabolite may be mobile within the ryegrass tiller. Argentine stem weevil have been noted to often feed only on the top portion of ryegrass leaves, but it is not clear whether this behaviour is affected by the presence of *N. lolii* (Keogh *et al.* 1996).

As the above compounds are not often detected in fungal culture or in endophyte free grass, it seems likely that molecular interactions occur between the grass host and endophytic fungus which result in the increased expression of these compounds within the symbiotic association. Thus these alkaloid production pathways provide an area for investigation into the symbiotic relationship between grasses and endophytes. The carbon skeleton for both indole diterpenoids, paxilline and lolitrem B, and the ergot alkaloid ergovaline, is derived from long chain isoprenoid units which are in turn derived from mevalonic acid. High level synthesis of these compounds with therefore be dependant on an abundant supply of mevalonic acid. The synthesis of mevalonic acid is catalysed by 3-hydroxy-3-methylglutaryl Coenzyme A reductase (Hmg); a key primary metabolic step in isoprenoid biosynthesis and therefore toxin production (Figure 1).

## 1.2 HMG-CoA REDUCTASE

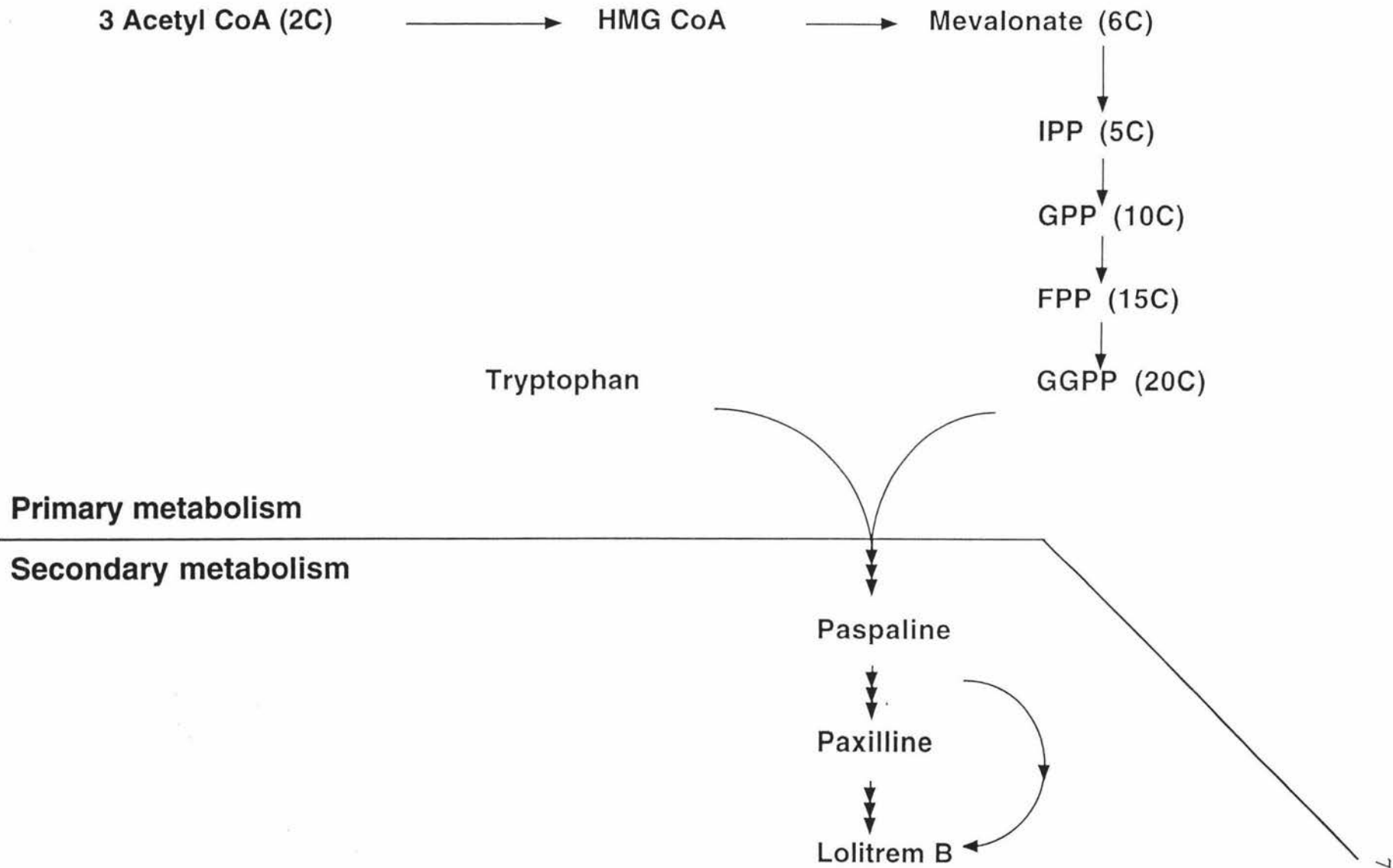
3-Hydroxy-3-methylglutaryl Coenzyme A reductase (Hmg) catalyses the synthesis of the primary metabolite, mevalonic acid. This is the first step in the isoprenoid biosynthetic pathway which leads to intermediates that serve key roles in protein synthesis, protein glycosylation, electron transport, cell cycle control and maintenance of membrane fluidity.

### 1.2.1 Structure and Function

Eukaryotic Hmg is an integral membrane protein of the endoplasmic reticulum (ER). It is bound to the ER via an amino-terminal membrane domain which contains a number (1-8) of hydrophobic regions or potential membrane spanning domains. A linker region

Figure 1            Biosynthesis of lolitrem B from acetyl CoA.

The isoprenoid biosynthetic pathway produces essential primary metabolites which act as precursors for synthesis of secondary metabolites, not essential for basic function of the organism. Hmg converts Hmg CoA to mevalonate, a precursor for the diverse range of secondary metabolites produced via this pathway. The pathway for production of lolitrem B from geranyl geranyl pyrophosphate has not been elucidated, except for the two metabolites, paspaline and paxilline.



separates this domain from the carboxy-terminal domain, which contains the hydrophilic catalytic site which projects into the cytoplasm (Basson *et al.* 1988; Liscum *et al.* 1985). Mammalian Hmg is encoded by a single gene, resulting in a single glycoprotein localised to the ER. In contrast, plants possess two or more isozymes of Hmg and these are encoded by small gene families. The activity of these enzymes is associated with plastid, mitochondrial and ER membranes.

The amino-terminal domain shows a low level of sequence similarity between phylogenetic kingdoms. This membrane situated domain is complex and appears to consist of 7 or 8 transmembrane domains in yeast, animals and fungi (Basson *et al.* 1988; Croxen *et al.* 1994; Liscum *et al.* 1985; Roitelman *et al.* 1992) in comparison with two domains in plants (Denbow *et al.* 1996; Enjuto *et al.* 1994). Despite the lack of sequence conservation between organisms, this domain mediates important regulatory controls such as the stability of the protein (Gil *et al.* 1985; Kumagai *et al.* 1995).

In comparison, the amino acid sequence of the catalytic domain is conserved across eukaryotes, archaeobacteria and eubacteria, suggesting it is under strong selective pressure (Lum *et al.* 1996). This domain catalyses the conversion of HMG CoA to mevalonate, the first committed step of the isoprenoid biosynthetic pathway. The crystal structure of Hmg from *Pseudomonas mevalonii* has been determined, showing Hmg to be a tightly bound dimer that brings together at the subunit interface the conserved residues implicated in substrate binding and catalysis. Regions have been identified that might be involved in the binding of Hmg-CoA as well as the reduced and oxidised forms of NAD(P) (Lawrence *et al.* 1995). A number of specific amino acid residues within the conserved catalytic domain are known to be required for catalysis and production of mevalonate; these include histidine, aspartate and glutamate residues (Bischoff and Rodwell 1997; Darnay *et al.* 1992; Frimpong and Rodwell 1994). Mevalonate provides a five-carbon building block for the synthesis of a wide and diverse range of products produced via this pathway (Goldstein and Brown 1990). Many essential primary metabolites are produced as well as secondary metabolites which are not essential for the functioning of the organism.

### 1.2.2 Fungal Hmg

The complete *hmg* gene has been isolated and characterised from the fungi *Gibberella fujikuroi* (Woitek *et al.* 1997) and *Ustilago maydis* (Croxen *et al.* 1994). *G. fujikuroi* is a rice pathogen which produces high levels of gibberellins, a family of diterpenoid plant hormones, while *U. maydis* is a pathogenic basidiomycete which induces tumours in

maize. The gene has also been studied in the yeasts *Schizosaccharomyces pombe* (Lum *et al.* 1996) and *Saccharomyces cerevisiae* (Basson *et al.* 1986).

*G. fujikuroi* contains only one *hmg* gene, encoding a predicted protein of 976 amino acids. This is somewhat smaller than other fungal Hmg polypeptides and Woitek *et al.* (1997) determined this to be due to a shortened N-terminal domain. This truncated domain has been determined to still contain seven putative transmembrane regions by the Kyte and Doolittle (1982) method. The *G. fujikuroi hmg* gene consists of a 2928 bp open reading frame which is interrupted by a putative 47 bp long intron. *U. maydis* appears to contain two structural genes encoding Hmg. Only one of these has been isolated and sequenced, and this was found to encode a 1165 amino acid polypeptide (Senstag *et al.* 1990). The N-terminal domain in *U. maydis* has been shown to contain eight putative hydrophobic domains, as determined by the Kyte and Doolittle (1982) method. Although, of these eight, only seven of these have been recognised as possible transmembrane regions. The *U. maydis* sequence does not possess any intronic regions and the position and presence of introns does not appear to be conserved throughout eukaryotes. The catalytic domain of Hmg from another plant pathogenic fungus, *Sphaceloma manihoticola* has also been isolated and sequenced. It has been determined that this fungus also contains only one *hmg* gene (Woitek *et al.* 1997).

*S. pombe hmg* encodes a predicted protein of 1053 amino acids and is considered to be intron free, as no intron consensus sequences have been located within the open reading frame. It is also predicted to contain eight transmembrane domains. *S. cerevisiae* has been found to possess two Hmg isozymes, each encoded by genes with single continuous open reading frames, 1054 amino acids for HMG1 and 1045 amino acids for HMG2.

Each of the Hmgs contain the recognised catalytically important histidine, aspartate and glutamate amino acid residues (Bischoff and Rodwell 1997; Darnay *et al.* 1992; Frimpong and Rodwell 1994) as well as the residues implicated in substrate binding (Lawrence *et al.* 1995). Of importance with respect to the regulation of Hmg activity is a serine located 6 residues downstream from the catalytically important histidine. This residue is present in each of the fungal Hmgs cloned to date and is known to be phosphorylated by an AMP-activated protein kinase, resulting in a loss of catalytic activity. The yeast Hmg peptides lack this regulatory serine residue and are therefore not regulated by phosphorylation at this position.

### 1.2.3 Regulation

The compounds and enzymes of primary metabolism have essential roles in the survival of an organism. Intermediates of primary metabolic pathways often supply precursors of secondary metabolism, which give rise to compounds whose function is not essential. Therefore factors that influence primary metabolism will have some effect on secondary metabolism (Drew and Demain 1977). Primary metabolism is controlled by a number of mechanisms, including substrate induction, feedback repression and inhibition, catabolite repression and inhibition, and ATP regulation. In each case, regulation is a dynamic process dependent on the local concentration of effector molecules (Drew and Demain 1977).

#### 1.2.3.1 Regulation of Hmg

In higher eukaryotes Hmg is considered to be the key regulatory enzyme of the isoprenoid biosynthetic pathway and as it is a typical housekeeping gene it is usually transcriptionally active. In mammals, Hmg activity can be regulated by feedback inhibition of steady state levels of Hmg mRNA, as well as at the molecular level to control the activity of the enzyme. This occurs by a coordinate modulation of synthesis of the protein when the pathway flux is low and degradation when the level of the pathway products are high. Regulation is therefore dependent on the concentration of an end-product. For regulation at both levels, two products of the terpenoid pathway are necessary: a sterol (low density lipoprotein) and a non-sterol product whose structural identity is yet to be determined. The amino-terminal anchor has been found to be necessary and sufficient to mediate the regulated degradation of Hmg in the endoplasmic reticulum (Hampton *et al.* 1996; Stermer *et al.* 1994).

Hmg activity is also regulated by processes independent of the mevalonate pathway such as reversible phosphorylation. Enzyme activity is down-modulated due to phosphorylation by an AMP-activated protein kinase, which has no effect on the stability of the enzyme but instead alters the kinetics of the enzyme (Hampton *et al.* 1996; Stermer *et al.* 1994). Phosphorylation occurs at a serine located 6 residues from the catalytic histidine, a spacing that is conserved in the *hmg* of most eukaryotes (Friesen and Rodwell 1997).

In plants, the number of genes encoding Hmg varies, depending on the species. Differential expression of the multiple *hmg* genes could play an important role in the regulation of enzyme activity. These *hmg* genes are also under a number of developmental and environmental regulatory mechanisms unique to plants, such as

light, phytohormones, pathogen attack, wounding, feedback mechanisms and endogenous protein factors. Much of the isoprenoid biosynthesis in plants is localised in specific compartments within a cell, which allows for the independent regulation of parallel pathways that produce different end products (Stermer *et al.* 1994).

Regulation of Hmg activity in turn determines mevalonate production and thus the amount of end-products produced.

### 1.3 AIMS AND BACKGROUND OF THIS STUDY

Although filamentous fungal endophytes confer many beneficial properties onto their host, perennial ryegrass, the production of animal toxins is agriculturally undesirable for livestock. Genetic strategies are therefore being undertaken to manipulate fungal endophytes and their hosts for agricultural benefit. Mevalonic acid is a precursor of both paxilline and ergovaline and synthesis of this compound by Hmg is considered an important regulatory step. Therefore, disruption of the paxilline pathway could increase the pool of mevalonic acid available for ergovaline synthesis, due to continued expression of *hmg*. It is also possible that in the symbiotic association, the endophyte draws on mevalonic acid produced by the plant, and so does not use its own enzyme at all. This may still lead to an increased pool of mevalonic acid within the endophyte even if Hmg is tightly regulated. It is therefore important to understand how this gene is regulated *in planta*. The aim of this project was to clone the *hmg* gene from the haploid *Neotyphodium* isolate Lp19 (LpTG-1) (Christensen *et al.* 1993) and to identify the upstream regulatory region of this gene. The availability of these sequences will allow *hmg* expression to be determined *in planta* using a *gus* reporter gene construct.