FUNCTIONAL ANALYSIS OF A THIAMINE BIOSYNTHETIC GENE IN THE INTERACTION OF *EPICHLOË TYPHINA* WITH PERENNIAL RYEGRASS

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Abstract

Epichloë/Neotyphodium endophytes are a group of clavicipitaceous fungi that form symbiotic associations with temperate grasses. The asexual *N. lolii* form asymptomatic mutualistic associations with ryegrass whereas the sexual *E. typhina* behaves similar to a mutualist during the vegetative phase of plant growth but switches to epiphytic growth and formation of an external stroma upon development of the floral inflorescence. The aim of this project was to study the metabolic interaction between these endophytes and their perennial ryegrass host. The role of endophyte thiamine biosynthesis in host colonisation and stroma development was chosen, because of the key role this coenzyme plays in primary cellular metabolism and because thiamine biosynthetic genes are induced in several fungal-plant interactions.

The orthologue (*thi1*) of *Saccharomyces cerevisiae THI4* was isolated from *N. lolii* and *E. typhina* by PCR using degenerate primers designed to conserved regions of known thiazole biosynthetic genes. This gene is expressed *in planta* and in culture, and is alternatively spliced, with distinct patterns of the isoforms expressed under different nutritional conditions. Mutant with a deletion in the *E. typhina thi1* gene was constructed and shown to have reduced hyphal density and branching compared to the wild-type on defined media lacking thiamine. Both thiamine and thiazole complemented this defect. Artificial inoculation of the mutants into plants showed that the *thi1* mutant retained the ability to colonise the perennial ryegrass host and form stromata. However, the mutant had some differences in host colonisation and growth, including reduced hyphal branching and reduced detrimental effects on the host. In addition, glycogen-like deposits, which were abundant in the wild-type hyphae, were not evident in the mutants.

Unexpectedly, both the *thi1* mutant and wild-type strains formed some stromata on vegetative tissue. Electron microscopic examination revealed that the cells of epiphytic

hyphae found on the vegetative tillers typically were enlarged, lacking in cytoplasm and highly vacuolated, an ultrastructure similar to that found for hyphae growing in reproductive tillers. The mutants retained the ability to form conidia on the outer layer of the stromata. Extensive vascular colonisation and hyphal ramification in the mesophyll were common characteristics of stromata bearing regions. Although the morphology and ultrastructure of stromata formed on vegetative tillers is very similar to those on reproductive tillers, one significant difference was the presence of abundant glycogen-like deposits in hyphae of vegetative tillers. Furthermore, there were dramatic differences in the levels of glycogen-like deposits in hyphae in different regions of the vegetative tillers, indicating that the energy demand changes during stroma development. This is the first report of *E. typhina* forming stromata on non-inflorescence tillers.

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Thanks

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Chapter one

Introduction

1.1. The importance of *Epichloë/Neotyphodium* endophytes

Epichloë/Neotyphodium endophytes are a group of Clavicipitaceous filamentous fungi that live in the intercellular space of temperate grasses (Pooideae grasses) including important forage grasses (Schardl, 1996a; Schardl and Moon, 2003). These endophytes comprise the sexual *Epichloë typhina*, other *Epichloë* species and their closely related asexual derivatives, *Neotyphodium spp.*, which were formerly classified in *Acremonium* sect. *Albo-lanosa* (Glenn *et al.*, 1996; Leuchtmann, 2003; Morgan-Jones and Gams, 1982; Schardl, 1996a).

These fungi live within the grass host, deriving nutrients from the host for growth. They confer benefits for the grass including protection from mammalian (Bacon *et al.*, 1977; Bacon *et al.*, 1991; Bush *et al.*, 1997) and insect herbivory (Siegel *et al.*, 1990), resistance to nematodes (Bacon and White, 2003; Kimmons *et al.*, 1990) and some fungal pathogens (Gwinn and Gavin, 1992), increased drought tolerance (Arachevaleta *et al.*, 1989; Malinowski and Belesky, 2000; Richardson, 1992; West, 1994) and greater field persistence (Hill *et al.*, 1990). Asexual *Neotyphodium* species are asymptomatic on their hosts, and are vertically transmitted through the seeds. The relationship of these endophytes with their host grasses is classified as mutualistic. The sexual *Epichloë* species may produce external stromata on host reproductive tillers. On the stromata, the sexual stage is initiated and infectious sexual spores are formed and horizontally transmitted. The production of stromata prevent emergence of the developing inflorescence, thus the relationship of these species with the host is described as antagonistic. *Epichloë* species vary widely in the degree of horizontal versus vertical seed transmission during their life cycles (White, 1988; White *et al.*, 1997). *Epichloë/Neotyphodium* endophytes thus form a continuum of symbiotic relationships with the plant host, spanning the spectrum from antagonistic to mutualistic, making them an ideal system for studying the evolution of fungal mutualists (Christensen *et*

al., 1993; Schardl *et al.*, 1994; Schardl and Moon, 2003; Tsai *et al.*, 1994; White, 1988; White *et al.*, 1997).

The major benefits of endophyte infection are largely attributed to a range of secondary metabolites (alkaloids) produced during the interactions of the endophyte and their host grasses (Bush *et al.*, 1997). While these alkaloids are highly beneficial to the endophyte-grass symbiosis, some of them are toxic to grazing mammals, resulting in syndromes such as ryegrass staggers and/or fescue toxicosis, which are responsible for large economic losses to agriculture in New Zealand and the United States. Consequently, 'scientists are studying the symbiotic interactions to exploit the potential of endophytes as biological control agents, and as a source of nematicides, insecticides and pharmaceuticals, as well as into the evolution of the mutualistic association' (Scott and Schardl, 1993; Yue *et al.*, 2000).

1.2. Life cycles and host interactions of *Epichloë/Neotyphodium* endophytes

1.2.1. Growth and life cycles of endophytes in grass host

A striking characteristic of *Epichloë/Neotyphodium* endophytes is their strictly controlled intercellular growth throughout the host plant (Christensen *et al.*, 2002; Schardl *et al.*, 2004; White *et al.*, 1997). The hyphae have never been found to enter the host cell or to form specialised feeding structures such as haustoria or arbuscules [Schardl, 2004 #945; Christensen, 2002 #391; White, 1997 #31. Microscopic examination showed that endophyte hyphae are unevenly distributed in the plant, with high concentrations in the leaf sheath, seeds and crowns, tissues which act as 'sinks' for accumulation of soluble nitrogen and carbohydrate substances (Christensen *et al.*, 1998; Siegel *et al.*, 1987). Different strains

of endophytes may vary in hyphal concentration and distribution within the plant (Christensen *et al.*, 2002). Hyphae of some strains may colonise vascular bundles, without apparent effects (Christensen *et al.*, 2001). Despite the differences, growth of all known endophytes in the host vegetative stage seems to be tightly controlled and cause little visible symptoms of infection (Christensen *et al.*, 2002; Tan *et al.*, 2001).

However, during the reproductive stage of the grass, endophytes may undergo either a sexual or asexual life cycle, resulting in dramatically different effects on the host (Leuchtmann and Clay, 1997; White, 1988). For the asexual endophytes, hyphae grow within the elongated inflorescence tiller, colonise the seed to be vertically transmitted (Majewska-Sawka and Nakashima, 2004; Philipson and Christey, 1986). Hyphae may directly invade the embryo sac soon after fertilisation (Philipson and Christey, 1986). Alternatively, hyphae may not colonise the embryo sac, but penetrate from the sporophytic tissue into the developing embryo during the development of the seed (Majewska-Sawka and Nakashima, 2004). The mode of vertical transmission is highly efficient in some associations, with nearly 100% of the seed from infected mother plants carrying the endophyte (Philipson and Christey, 1986).

For endophytes that have the potential to enter the sexual life cycle, once the grass host enters the reproductive stage, endophyte hyphae emerge from the flag leaf sheath and the inflorescence of flowering tillers, forming external stromata (White and Bultman, 1987). In this stage, the sexual life cycle can be initiated when spermatia (conidia) are deposited on a stroma of the opposite mating type. This heterothallic mating in nature is mediated by *Botanophila* flies (Leuchtmann, 2003). Following the mating and cross-fertilisation, ascospores are formed, ejected and may infect new hosts probably by way of the florets to ultimately give rise to infected embryos and seed (Chung and Schardl, 1997; White and Bultman, 1987). Thus the endophyte in a sexual life cycle is transmitted horizontally. As

Figure 1.1. The asexual and sexual life cycles of *Epichloë festucae* on *Festuca rubra*

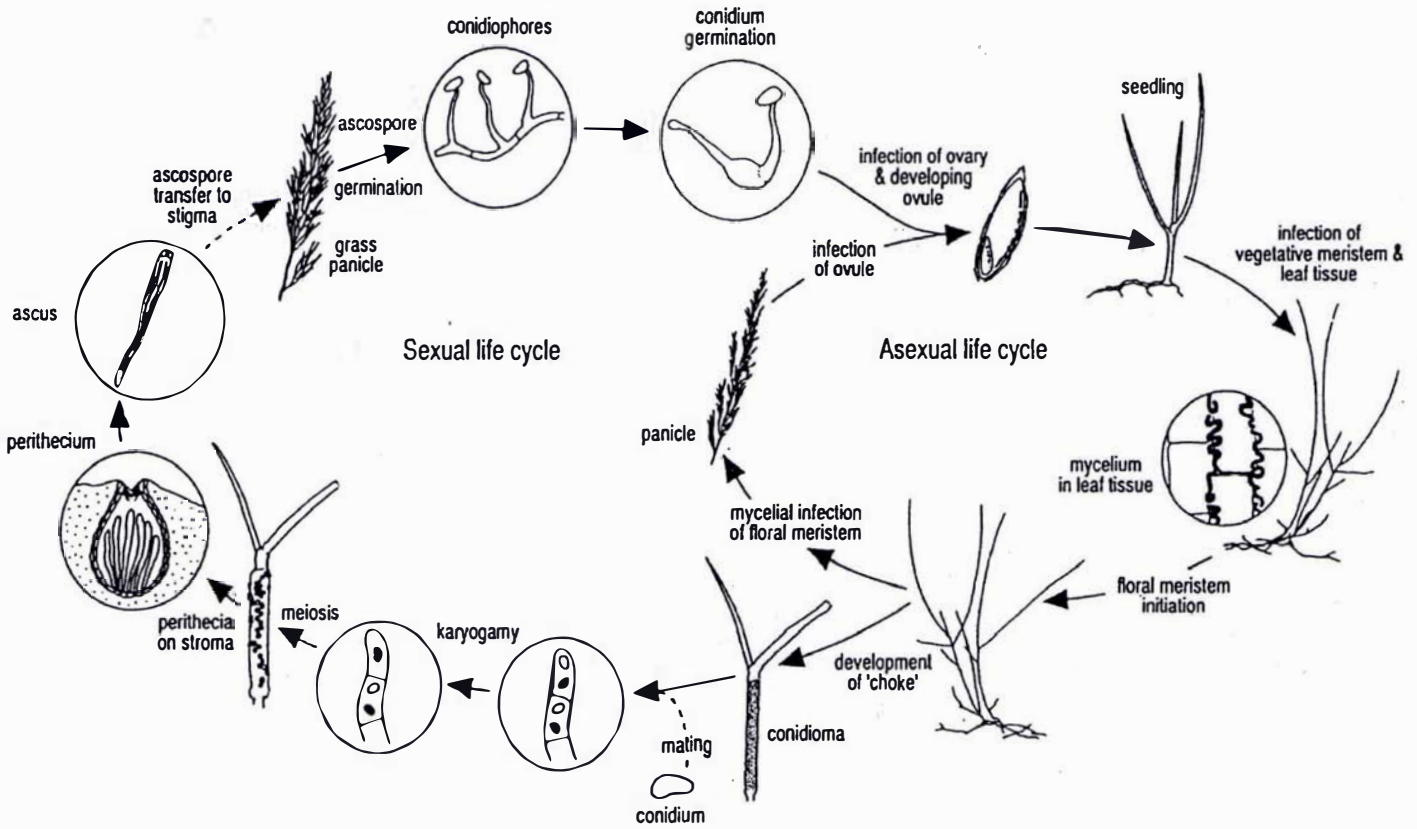


Diagram prepared by Liz Grant, department of ecology, massey university

development of stromata on a grass prevents emergence of the inflorescence, this horizontal mode of endophyte transmission is antagonistic to the host (Schardl, 1996a).

Some *Epichloë* species (for example *E. festucae*) are described as pleiotropic (Schardl, 1996a). For example, *E. festucae* in *F. rubra* can form stromata on some but not all flowers of a plant. In the asymptomatic tillers the endophyte is vertically transmitted (Schardl, 1996a).

As the expression of the endophyte life cycle depends on both the genotype of the endophyte and the host, White (1988) classified the endophyte-grass associations into three types, according to the life cycles they express on the grass host. Type-I include those associations where stromata form on all culms of endophyte-containing grasses. This type of association was described as the most pathogenic since the fungus effectively sterilises the host. Type-II associations include those that have stromata produce on tillers of some but not others of the infected individuals in a population. Type-III associations are those on which no stromata are formed and the endophyte remains completely asymptomatic in all infected individuals (White, 1988).

1.2.2. Host specificity and compatibility

Epichloë/Neotyphodium endophytes in nature are usually restricted to certain host species or genera, suggesting that these grass endophytes have host specificity (Leuchtmann and Clay, 1997). Evidence for this indirectly comes from studies of isozyme genotypes of endophytes from the host (Leuchtmann and Clay, 1990; Leuchtmann, 1992, 1994b). Isolates of *Epichloë* endophytes from the same host population are usually identical or very similar in isozyme genotypes, whereas those from different host populations are usually different from one other (Leuchtmann and Clay, 1990; Leuchtmann, 1992; Leuchtmann and Clay,

1997). The finding indicates that the endophyte and host plants have a history of coevolution and a broadly compatible genotype across host-species lines in fine fescues (Leuchtmann, 1994a).

Host specificity and compatibility was also studied by artificially inoculating endophytes into grasses that were the natural host or non-host and observing the outcome of these inoculations. *Epichloë* or *Neotyphodium* isolates often failed to form stable associations with grass species other than their original hosts (Leuchtmann, 1992). For some isolates, stable associations were obtained, but mostly with grasses closely related to their original hosts (Christensen, 1995). Some changes in host tissues and fungal hyphae were observed in the grasses artificially infected with endophytes (Christensen, 1995; Koga *et al.*, 1993) . These included death of host cells in the stem apex and premature death of hyphae (Christensen, 1995; Koga *et al.*, 1993). In other associations, changes in host wall or increased levels of peroxidase, which indicates reaction of the host to the endophyte infection, have also been detected (Christensen *et al.*, 2002; Naffa *et al.*, 1999). These incompatible responses presumably reflect a breakdown in compatibility between the host and endophyte and elicitation of a host defense response (Scott, 2001). Although the molecular basis for this remains unknown, host specificity may have been a major factor for the co-evolution of mutualistic endophyte-grass associations (Leuchtmann and Schardl, 1998).

1.2.3. Mutualistic relationship and alkaloid production

Epichloë/Neotyphodium endophytes are a group of biotrophic symbionts (Schardl *et al.*, 2004; Siegel *et al.*, 1987; White *et al.*, 1997). They complete their entire life cycle on or in host tissues, and obtain all their nutrients from the host-living tissue to sustain hyphal growth and form reproductive structures. It is clear that the endophyte benefits from the host by receiving nutrients, long-term protection and dissemination through the seed.

Although there is a cost to the plant host as endophytes absorb nutrients and may cause abortion of flower development and seed production, infection is generally advantageous to the host. Some studies showed that endophyte-infected grasses grow better (Belesky *et al.*, 1987; Cheplick *et al.*, 1989; Latch *et al.*, 1985), are more vigorous and aggressive than uninfected members of the same endophyte-free species (Clay, 1990) and have a greater field persistence (Hill *et al.*, 1990). Studies also showed that endophyte infection can increase host tolerance to drought (Arachevaleta *et al.*, 1989; Richardson, 1992; Richardson *et al.*, 1993; West, 1994) and resistance to fungal disease (Lewis *et al.*, 2003; Siegel and Bush, 1997) and nematodes (Joost, 1995; Kimmons *et al.*, 1990; Latch, 1993). However, the most striking characteristic of endophytes is that endophyte infection induces the synthesis of a wide range of secondary metabolite-alkaloids which confer host resistance to insect and animal herbivores (Bacon *et al.*, 1977; Bacon *et al.*, 1986; Siegel *et al.*, 1990; Siegel and Bush, 1997) (Spiering, 2000; Wilkinson *et al.*, 2000)

Four classes of alkaloids produced by the endophyte-grass associations have been intensively studied to date. These include lolines (pyrrolizidines), ergot alkaloids, indole diterpenes, and peramine (a pyrrolopyrazine) (Siegel and Bush, 1997). Lolines are potent insecticides, and peramine is an insect feeding deterrent which is important for protection of perennial ryegrass from Argentine stem weevil (ASW; *Listronotis bonariensis*) (Bush *et al.*, 1993; Bush *et al.*, 1997; Rowan and Latch, 1994; Wilkinson *et al.*, 2000). Ergot alkaloids are a group of anti-mammalian compounds with some insecticidal activity (Bush *et al.*, 1997). These compounds include clavines, ergolene acids and alcohols, simple lysergic acid derivatives and ergopeptines. They cause the classical symptoms of fescue toxicosis in grazing animals, for examples, elevated temperatures, vasoconstriction, reduced feeding, reduced fertility, stillbirth, gangrene and occasionally death (Bush *et al.*, 1997). Indole diterpenes include tremorgenic neurotoxins commonly known as lolitrems, of which the most abundant is lolitrem B. Lolitrem B is the main toxin implicated in ryegrass staggers

(Gallagher *et al.*, 1981; Raisbeck *et al.*, 1991). Ryegrass staggers is a neurotoxic disorder of livestock grazing perennial ryegrass infected with *N. lolii*. Indole diterpenes are potent inhibitors of high conductance potassium channels.

Although genes for alkaloid production are of fungal origin (Blankenship *et al.*, 2001; Panaccione *et al.*, 2001; Scott, 2003a; Spiering *et al.*, 2002; Wilkinson *et al.*, 2000), the type and levels of alkaloids synthesised are dependent on both fungal and host genotypes. Among the natural endophyte-grass associations analysed to date, peramine was detected in most associations, whereas ergot alkaloids were present in 50%, lolines in 35% and lolitrems in only 10% of the symbiotic associations (Siegel *et al.*, 1990). Among the important forage grasses, tall fescue grass in association with most *N. coenophialum* strains produces lolines, ergot alkaloids and peramine; perennial ryegrass in association with some *N. lolii* endophytes produces lolitrems, ergot alkaloids and peramine. Meadow fescue in association with *N. uncinatum* produces only lolines (Bush *et al.*, 1997). How the plant regulates the biosynthesis of the fungal alkaloids is unclear. Different plants may provide different types and/or levels of precursors, or exert different effects on the physiology of the fungi (Schardl, 1996a).

As the genes responsible for the alkaloid synthesis are of fungal origin, the distribution and concentration of the alkaloids in the plant may reflect the location and concentration of the endophyte hyphae (Keogh and Tapper, 1993; Keogh *et al.*, 1996; Siegel and Bush, 1997; Spiering, 2000). Plant tissues with abundant endophyte hyphae often contain high concentrations of alkaloids (Keogh and Tapper, 1993; Siegel and Bush, 1997; Spiering, 2000). However, translocation of the alkaloids may occur as indicated by the presence of alkaloids in tissues with no or little endophyte (Siegel and Bush, 1997). Environmental factors, such as nitrogen fertiliser, soil water deficit and temperature, can all affect alkaloid production (Lane *et al.*, 1997; Lyons *et al.*, 1986).

1.3. Genetic studies of endophyte-host interactions

1.3.1. Cloning and characterisation of genes for alkaloid biosynthesis

The cloning of alkaloid biosynthesis genes has been a challenging task as it is difficult to establish conditions for reliable expression of the metabolites in culture (Scott, 2003a). Genes involved in the biosynthesis of indole diterpenes and ergot alkaloids were initially cloned from other fungi, such as *Claviceps fusiformis, C. purpurea* or *Penicillium paxilli,* in which the intermediates for the alkaloid biosynthesis were detectable, and subsequently homologous genes were isolated from endophytes (Scott, 2003a). However, one advantage is that genes involved in secondary metabolite biosyntheses tend to be organised in large gene clusters, so that once one gene is cloned, the remainder of the genes in the pathway can be isolated by chromosome walking (Scott, 2003b).

Ergot alkaloids are metabolites produced by many members of Clavicipitaceae fungi including *Claviceps* species, *Epichloë/Neotyphodium* endophytes and other *Balansia* species (Panaccione and Schardl, 2003). The ergot alkaloid biosynthesis pathway and the enzymes involved have been intensively studied in *Claviceps* species (Tudzynski *et al.*, 2001). An enzyme (DMAT synthase) involved in the biosynthesis has been purified from *C. fusiformis* (Gebler and Poulter, 1992). The gene *dmaW* encoding for this enzyme has been isolated by a PCR approach using primers designed to the peptide sequence (Tsai *et al.*, 1995). Following this, several other genes including the lysergyl peptide synthetases (*cpps1* and *cpps2*) involved in synthesis of ergot alkaloids (ergopeptine) were isolated from *C. purpurea* isolates (Correia *et al.*, 2003; Panaccione *et al.*, 1999; Panaccione *et al.*, 2001; Panaccione and Schardl, 2003; Tudzynski, 1999). Based on this information, genes encoding lysergyl peptide synthetase (*lpsA*) and DMAT synthase (*dmaW*), were isolated from the

endophytes *N. lolii* (Panaccione *et al.*, 2001) and *Neotyphodium sp.* strain Lp1 (Wang *et al.*, 2004). Targeted deletion of *lpsA* or *dmaW* in Lp1 resulted in a strain that was unable to produce ergovaline in the grass host, confirming the function of the genes in production of ergovaline (Panaccione *et al.*, 2001; Wang *et al.*, 2004).

Cloning of genes responsible for lolitrem B biosynthesis started with the cloning of paxilline synthesis genes in the filamentous fungus *P. paxilli.* As the tremorgenic indole-diterpene paxilline is a common product of both *P. paxilli* and *N. lolii*, paxilline was predicted to be one of the precursors of lolitrem B (Young *et al.*, 1998). In order to clone the genes for paxilline biosynthesis, paxilline negative (Pax$^-$) mutants were generated using the technique of plasmid mutagenesis (Itoh *et al.*, 1994; Young *et al.*, 1998). The gene cluster responsible for paxilline biosynthesis was identified by mapping four Pax$^-$ deletion mutants and DNA sequence analysis of the region (Young *et al.*, 2001). The cluster contains a series of open reading frames (ORFs) including a geranylgeranyl pyrophosphate synthase (*paxG*), a FAD-dependent monooxygenase (*paxM*), a prenyl transferase (*paxC*), two cytochrome P450 monooxygenases (*paxP* and *paxQ*), and a DMAT synthase (*paxD*) (Scott *et al.*, 2004). Deletion of the *paxG* in this cluster gave rise to a paxilline negative mutant (Young *et al.*, 2001), and deletion of *paxP* or *paxQ* gave rise to mutants that accumulate proposed intermediates for paxilline synthesis (McMillan *et al.*, 2003), confirming that this region is indeed involved in paxilline biosynthesis (Scott *et al.*, 2004). The cloning and characterisation of the genes for the paxilline biosynthetic pathway from *P. paxilli* has opened the way for isolating the homologous genes from endophyte isolates. A gene *ltmG*, an orthologue of the *P. paxilli paxG*, was isolated from a *N. lolii* isolate by a PCR approach using degenerate primers designed to conserved regions of fungal GGPP synthases (Young *et al.*, 2004). Genes including *ltmM* and *ltmK*, homologues of *paxM* and *paxP*, clustered with *ltmG* in a region of 25-kb that was subsequently isolated from the *N. lolii* isolate. A similar gene cluster was isolated from *E. festucae* (Young *et al.*, 2004). Interestingly, both sides of the cluster in *N. lolii* are flanked by retro-elements which are abundant in the genome of

both *N. lolii* and *E. festucae* (Scott and Young, 2003). *E. festucae* transformants with the *ltmM* deleted have been constructed and introduced into perennial ryegrass. The lolitrem B negative phenotype of these associations confirmed that this gene is required for lolitrem B biosynthesis (Young *et al.*, 2004).

Cloning of genes for peramine synthesis has been achieved by a differential display-RT-PCR (DD-RT-PCR) technique combined with screening a cosmid genomic library (Aiko Tanaka and Barry Scott; unpublished results). Candidate genes were detected by DD-RT-PCR using primers designed to two conserved sequences within the putative modules of a non-ribosomal peptide synthase. One of the candidate gene fragments was used as a probe to screen an *E. festucae* cosmid genomic library. A gene of 8370 bp in size, designated as *perA* was isolated and confirmed to be required for peramine biosynthesis by gene deletion analysis (Aiko Tanaka and Barry Scott; unpublished results).

Genes for loline biosynthesis in *E. festucae* were shown to be clustered in a single genetic locus, which co-segregated with two AFLP polymorphic markers (Wilkinson *et al.*, 2000). This finding together with the observation that production of loline was induced in axenic culture *of N. uncinatum* growing in minimal media (Blankenship *et al.*, 2001) enabled two loline biosynthetic genes, *lolA* and *lolC*, to be cloned by suppression subtractive hybridisation, of which expression was strictly correlated with loline production in *N. uncinatum* (Kutil *et al.*, 2003; Spiering *et al.*, 2002). Subsequently, these two genes have been shown to be part of a cluster of 9 putative genes for loline biosynthesis (Kutil *et al.*, 2003; Spiering *et al.*, 2002).

The cloning and characterisation of these alkaloid biosynthetic genes now provides the opportunity to determine how these genes are regulated in endophyte-host interactions, determine the distribution of these genes, screen for toxin-negative strains for agricultural use or potentially modify endophyte isolates by genetic manipulation (Scott, 2003a).

1.3.2. Cloning and characterisation of other endophyte genes

A number of other endophyte genes have been isolated. Some of them have been used for endophyte phylogenetic studies. The *tub2* gene which encodes β-tubulin, was isolated and sequenced from *E. typhina* (Byrd *et al.*, 1990). The β-tubulin is a main component of the microtubule apparatus. The non-coding intronic region of this gene is informative and widely used for endophyte phylogenetic studies (Schardl *et al.*, 1994). Similarly, the non-coding sequences of *tef1* (elongation factor 1-α) and *act1* (actin), which were more recently isolated, have been used in phylogenetic studies to distinguish the endophyte species (Craven *et al.*, 2001; Moon *et al.*, 2002).

Other *Epichloë/Neotyphodium* endophyte genes that have been isolated and characterised include *pyr4*, *hmg* and *At1*. The *pyr4*, which encodes orotidine-5'-monophosphate decarboxylase, was isolated from an LpTG-2 strain Lp1 (Collett *et al.*, 1995). The *hmg* encoding 3-hydroxy-3-methylglutaryl coenzymeA reductase is a key enzyme required for the biosynthesis of mevalonic acid (Dobson, 1997). Mevalonic acid is a primary metabolite precursor required for the biosynthesis of cholesterol and isoprenoids, including the secondary metabolites lolitrem B and ergovaline. Endophyte transformants with the GUS reporter gene (*gusA*) under the control of the *hmg* promoter were used to detect metabolic activity, distribution, and propagation of endophytes *in planta* (Schmid *et al.*, 2000). Results showed that the gene is constitutively expressed in the host, but the growth of the transformants differed from the wild type. *At1* is a proteinase gene isolated from the endophyte *N. typhinum*. The product of this gene, a serine proteinase, is highly abundant in *N. typhinum/Poa ampla* associations, suggesting that it may be involved in host colonisation (Reddy *et al.*, 1996).

More recently, significant efforts have been directed towards the identification of genes that are differentially regulated in symbiosis. Techniques that have been applied include suppression subtractive hybridisation (Johnson *et al.*, 2004), EST (expressed sequence tags) sequence analysis (Spangenberg *et al.*, 2000), proteomics analysis (Zhang *et al.*, 2004) and plasmid mutagenesis (Tanaka, A. and Scott, B.; personal communication). Many genes that are activated in the endophyte-grass interactions have been identified, but functional analysis of these endophyte genes remains to be carried out.

1.4. Evolution of *Epichloë/Neotyphodium* endophytes

1.4.1. *Epichloë/Neotyphodium* endophyte species

To date, at least ten sexual stroma-forming *Epichloë* species have been formally identified (Table 1.1). Five of them form type I associations and the other five form type II associations with their host grasses (Leuchtmann, 2003; Schardl, 1996a; White *et al.*, 1993; White, 1994). Each of these 10 *Epichloë* species, with the exception of *E. clarkii*, constitutes a single mating population. *E. clarkii* belongs to MP-I mating type as does *E. typhina*, but it is in a different host species and is infertile in crosses with *E. typhina* (White *et al.*, 1993). All these species, except *E. typhina*, are host specific and restricted to one or a few grass species within the Pooideae. *E. typhina* has a broad host range forming associations with grasses in tribes Poeae, Aveneae and Brachypodieae (Schardl *et al.*, 1997). Host specialisation was suggested to drive the speciation of the sexual endophytes (Schardl and Moon, 2003).

Many asexual *Neotyphodium* species have been identified and shown to be derived from one or more of the *Epichloë* species (Table 1.1). Many of them are interspecific hybrids arising from two or even three ancestors (Schardl and Moon, 2003). The asexual *Neotyphodium*

Table 1.1. *Epichloë/Neotyphodium* species and their life cycles

Species	Mating type	Host genera	Life cycle[a]	Closest non-hybrid relatives	References
E. typhina (Per.: Fr.) Tul.	MP-I	*Anthoxanthum, Arrhenatherum, Brachypodium, Dactylis, Lolium, Phleum, Poa, Puccinellia*	S/P (I/II[e])		(Leuchtmann, 2003; White *et al.*, 1993)
E. clarkii	MP-I	*Holcus*	S (I)		(Leuchtmann, 2003; White *et al.*, 1993)
E. baconii	MP-V	*Calamagrostis, Agrostis*	S (I)		(Leuchtmann, 2003; White *et al.*, 1993)
E. bromicola	MP-VI	*Bromus, Hordelymus*	S/A (I/III)		(Leuchtmann and Schardl, 1998)
E. glyceriae	MP-VIII	*Glyceria*	S (I)		(Schardl and Leuchtmann, 1999)
E. festucae	MP-II	*Festuca, Koeleria*	P (II)		(Leuchtmann, 1994)
E. elymi	MP-III	*Elymus*	P (II)		(Schardl and Leuchtmann, 1999)
E. amarillans	MP-IV	*Agrostis, Calamagrostis, Sphenopholis*	P (II)		(Leuchtmann, 2003; White, 1994)
E. sylvatica	MP-VII	*Brachyopodium*	P (II)		(Leuchtmann and Schardl, 1998)
E. brachyelytri	MP-IX	*Brachyelytrum*	P (II)		(Schardl and Leuchtmann, 1999)
N. lolii	N/A	*L. perenne*	A (III)		(Latch *et al.*, 1984; Schardl and Moon, 2003)
N. typhinum	N/A	*Poa nemoralis*	A (III)		(Morgan-Jones and Gams, 1982; Schardl and Moon, 2003)
N. typhinum var. canariense	N/A	*Lolium canariensis*	A (III)		(Moon *et al.*, 2000)
N. aotearoae	N/A	*Echinopogon ovatus*	A (III)		(Moon *et al.*, 2002)
N. chilense	N/A	*Dactylis glomerata*	A (III)		(Morgan-Jones *et al.*, 1990; Schardl and Moon, 2003)
N. huerfanum	N/A	*F. arizonica*	A (III)		(Schardl and Moon, 2003; White and Bultman, 1987)

N. starrii	N/A	*Bromus anomalus*, *Festuca spp.*	A (III)		(Schardl and Moon, 2003; White and Morgan-Jones, 1987)
N. coenophialum	N/A	*Lolium arundina*	A (III)	*E. festucae*, *E. typhina*, *E. baconii*	(Morgan-Jones and Gams, 1982; Schardl and Moon, 2003)
LpTG-2	N/A	*L. perenne*	A (III)	*N. lolii* (or *E. festucae*), *E. typhina*	(Christensen *et al.*, 1993)
N. uncinatum	N/A	*F. pratensis* (Meadow fescue)	A (III)	*E. bromicola*, *E. typhina*	(Schardl and Moon, 2003)
N. occultans	N/A	*Lolium rigidum*, *L. multiflorum*, *Lolium spp.*	A (III)	*E. bromicola*, *E. baconii*	(Moon *et al.*, 2000)
FaTG-2	N/A	*F. arundinacea* (Tall fescue)	A (III)	*E. baconii* *E. festucae*	(Christensen *et al.*, 1993)
FaTG-3	N/A	*F. arundinacea* (Tall fescue)	A (III)	*E. baconii*, *E. typhina*	(Christensen *et al.*, 1993)
N. sieglii	N/A	*F. pratensis* (Meadow fescue)	A (III)	*E. bromicola*, *E. festucae*	(Schardl and Moon, 2003)
N. australiense	N/A	*Echinopogon ovatus*	A (III)	*E. typhina*, *E. festucae*	(Moon *et al.*, 2002)
N. melicicola	N/A	*Melica spp.*	A (III)	*N. aotearoae*, *E. festucae*	(Moon *et al.*, 2002)
N. tembladerae	N/A	*Poa hueca* and *Festuca spp.*	A (III)	*E. typhina*, *E. festucae*	(Cabral *et al.*, 1999)
N. chisosum	N/A	*Stipa eminens*	A (III)	*E. amarillans*, *E. typhina*, *E. bromicola*	(Schardl and Moon, 2003; White and Bultman, 1987)

[a] A = asexual only; S = sexual only; P = pleiotropic (sexual and seed-transmitted) (Leuchtmann, 2003)
[b] LpTG-2: L. perenne endophyte taxonomic grouping two (Schardl *et al.*,1994)
[c] FaTG-2: F. arundinacea endophyte taxonomic grouping two (Leuchtmann *et al.*,1994)
[d] FaTG-3: F. arundinacea endophyte taxonomic grouping three (Schardl and Moon 2003)
[e] Type I, II or III associations were defined according to White 1988

endophytes have significant diversity and are distributed widely in many host species (Schardl and Moon, 2003). As *Neotyphodium* endophytes show few if any symptoms on their hosts and some, such as *N. occultans*, are unculturable (Moon *et al.*, 2000), they are difficult to detect and identify. The *Neotyphodium* species listed in Table 1.1 probably represent only part of this genus. With the application of new detection methods such as PCR amplification of DNA from grass tissue using specific primers for microsatellite loci (Moon *et al.*, 2000) combined with systematic sampling of the grass, more species should be found.

1.4.2. Evolution of asexual *Neotyphodium* endophytes by interspecific hybridisation

Various lines of evidence indicate that asexual *Neotyphodium* species are close relatives of sexual *Epichloë* endophytes. The asexual *Neotyphodium* endophytes and sexual *Epichloë* endophytes have similar conidial morphology (White and Morgan-Jones, 1987), similar alkaloid production (Siegel *et al.*, 1990), and similar or related isozyme patterns (Leuchtmann and Clay, 1990). Recent molecular genetic analysis of *tub2* and *rrn* (rDNA) non-coding sequences also indicate that the asexual *Neotyphodium* and sexual *Epichloë* endophytes are closely related (Schardl *et al.*, 1991; Schardl *et al.*, 1994).

Many asexual endophytes appear to have evolved from sexual ones by interspecific hybridisation between two or more sexual and asexual strains (Moon *et al.*, 2002; Schardl *et al.*, 1994; Tsai *et al.*, 1994). Isozyme analysis showed that some asexual endophytes exhibit multiple bands for each of many enzymes, suggesting two or more loci for the enzymes (Christensen *et al.*, 1993; Schardl, 1994). In contrast, isolates of sexual *Epichloë* strains had a single band for each enzyme, which is typical of haploid species (Christensen *et al.*, 1993). Analysis of *tub2* and *rrn* non-coding sequences also showed that some asexual

isolates had two *tub2* copies. One (*tub2-1*) was closely related to that of sexual *Epichloë* strains. The other (*tub2-2*) had the same intron sequence as that of the *tub2* in isolates of *N. lolii* or *E. festucae* (Schardl *et al.*, 1994; Tsai *et al.*, 1994). The relationships were further confirmed by evidence from phylogenetic analysis of *pyr4* gene sequences (Collett *et al.*, 1995). To explain the origins of strain Lp1, an interspecific hybrid between *E. typhina* and *N. lolii*, Schardl proposed that a plant which had been infected with a *N. lolii* strain was co-infected with an *Epichloë typhina* strain by horizontal transmission, and the two strains subsequently underwent anastomosis and karyogamy to form an interspecific hybrid (Schardl, 1994; Tsai *et al.*, 1994). The recent finding that single plants were multiply infected in the natural population (Meijer and Leuchtmann, 2000) provides support for this hypothesis. Recent analysis of asexual endophytes has identified further interspecific hybrids (Moon *et al.*, 2002; Schardl and Moon, 2003). Interspecific hybridisation appears to be a relatively common event in the origin of the asexual *Neotyphodium* endophytes. Interspecific hybridisation has been proposed as a rapid evolution track of non-pathogenic fungi from the relative pathogenic ones, as the hybridisation event combines the potential of two genomes (Brasier, 2000).

1.4.3. Evolution of asexual *Neotyphodium* endophytes by accumulation of detrimental mutations

An alternative pathway by which the asexual *Neotyphodium* endophytes evolved from their sexual ancestors is that the endophyte lost the ability to complete the sexual life cycle and be transmitted horizontally through accumulation of gene mutations (Schardl and Moon, 2003). When sexual *Epichloë* endophytes lose their ability to produce stromata on which the sexual life cycle initiates, or lose the ability to complete sexual development (for example lose the ability to develop sexual fruiting bodies), they will lose the sexual life style (Schardl and Moon, 2003). *N. lolii* is an example of an endophyte that may have arisen from

its sexual ancestor, *E. festucae*, through loss of the sexual life cycle (Schardl and Moon, 2003). Molecular phylogenetic studies indicate that *N. lolii* endophytes are derivatives of pleiotropic *E. festucae* endophytes (Schardl and Moon, 2003). However, no interspecific hybridisation appears to have been involved as *N. lolii* is haploid (Kuldau *et al.*, 1999; Schardl *et al.*, 1994). *N. lolii* is commonly associated with *L. perenne* grasses and stromata have never been observed on its hosts. *E. festucae* endophytes have a pleiotropic life cycle when in association with *F. rubra*, but have not been observed to form stromata when in association with *L. perenne* (Moon *et al.*, 2000). *E. festucae* differs from the *N. lolii* endophyte in that it can produce abundant conidia in culture, and the conidia can act as spermatia when transferred onto a stroma of the opposite mating type. A hypothesis proposed for the origin of *N. lolii* is that *E. festucae* accumulated mutations which deleted or limited stroma development, leading to loss of the sexual life cycle (Schardl and Moon, 2003). Loss of mating-type genes would contribute to loss of the sexual life cycle, but artificial mating tests showed that many asexual endophytes still exhibit mating activity (Schardl, 1996b), suggesting that their asexual nature is not due to deletion or mutation of mating-type genes.

Mutations of genes in key metabolic pathways may contribute to the evolution of mutualistic endophytes, as these mutations may result in physiological dependence of the endophyte on the host, and reduced the ability of endophytes to form external stroma (Scott, D. B. personal communication). Recent analysis of genomic sequences of symbionts has given us an insight into genomic evolution of mutualistic microbes. For example, *Buchnera sp.* (a obligatory bacterial symbiont of aphids) is a close relative of *Escherichia coli*. Its genome size is only a seventh of that of *E. coli* (Shigenobu *et al.*, 2000). Many genes are missing from the genome of this symbiont, including genes for biosynthesis of amino acids and cell surface components, genes for DNA repair, recombination and regulation, and genes required for the tricarboxylic acid cycle (TCA cycle). The loss of these genes has been proposed to drive the co-evolution of each partner and the evolution of the

mutualistic association (Shigenobu *et al.*, 2000). Gene compaction and reduced metabolic capacity, such as lack of genes for some biosynthetic pathways and/or for the TCA cycle, appear to be traits associated with parasitic or symbiotic lifestyles in both bacteria and fungi (Henrissat *et al.*, 2002; Katinka *et al.*, 2001).

1.4.4. Evolution of asexual endophytes versus Muller's ratchet

Although the loss of the sexual life cycle reduces the detrimental impact of the endophyte on the host through loss of 'choking', loss of sex itself may be disadvantageous to the asexual endophytes (Muller, 1964). Muller pointed out that asexual clonal organisms should progressively accumulate deleterious mutations, which lead to decrease in fitness of the clonal organisms over successive generations or even extinction of the clonal organisms under selective conditions. This phenomenon known as Muller's ratchet can be overcome by recombination during the sexual cycle as a result of meiosis (Muller, 1964). Muller's ratchet as an important evolutionary phenomenon favouring sex has been supported by results of recent experiments (Lynch, 1997). For endophytes, selection acts on the association of endophyte and the grass host rather than the individuals. The sexual life cycle may be disadvantageous in nature as it results in sterilisation of the grass host, and horizontal transmission occurs at a very low rate in nature. The asexual life cycle, which benefits both the partners is favoured. In the long-term survival of the association, interspecific hybridisation was possibly a mechanism that evolved to reduce the impact of Muller's ratchet as it provides the opportunity for genome recombination (Schardl and Moon, 2003).

1.5. Variation in nutrition requirement and metabolism

1.5.1. Variation in nitrogen and carbon source utilisation

Endophytes can utilise a wide range of carbon and nitrogen sources in culture (Bacon and White, 2000). For example, the endophyte isolated from tall fescue grass, *N. coenophialum,* can utilise glucose, fructose, mannose, sucrose, trehalose, raffinose, mannitol and sorbitol (Kulkarni and Nielsen, 1986). Among nitrogen sources, ammonium and amino acids such as arginine, asparagine, cysteine, glutamine, proline and serine are effectively utilised, but nitrate is not utilised (Kulkarni and Nielsen, 1986). Further analysis of nutrient requirement showed that endophytes can grow well in complex media with a wide range of nitrogen sources, but growth can be variable in cultures supplemented with a single nitrogen source (Ferguson *et al.*, 1993; Naffaa *et al.*, 1998; White *et al.*, 1991). For example, leucine, arginine or glutamine supported good growth for some isolates of *N. coenophialum*, but not for others (Ferguson *et al.*, 1993). In contrast to the work of Kulkarni and Nielsen, experiments by two other groups showed that potassium nitrate was well utilised by most isolates of *N. coenophialum*, but poorly utilised *by N. lolii* isolates from *L. perenne* (Ferguson *et al.*, 1993; Naffaa *et al.*, 1998). As amino acids are important precursors for alkaloid biosynthesis, variation in nitrogen metabolism could impact on the types and levels of alkaloids in endophyte-grass associations and the dependence on hosts (Ferguson *et al.*, 1993).

Variations exist between asexual and sexual endophytes in their ability to utilise carbon and nitrogen sources (Bacon and White, 2000; Naffaa *et al.*, 1998; White *et al.*, 1991; White and Morrow, 1991). These studies showed that sexual endophytes are better adapted to growth in culture than asexual endophytes. Sexual endophytes can utilise a wider range of carbon and nitrogen sources, and grow faster on various media. In particular, sexual stroma-forming endophytes isolated from the grasses *Elymus, Hystrix* and *Agrostis* grew significantly faster

on low fructose, sucrose and xylose than asexual non-stroma formers. High levels of fructose, sucrose or xylose inhibit both stroma formers and non-stroma formers, however the former still grow more rapidly than the latter (White and Morrow, 1991). Similar results of the inhibitory effects of higher concentrations of glucose or fructose were also shown for several other *Epichloë* endophytes (Naffaa *et al.*, 1998; White *et al.*, 1991). The reduced ability of the asexual isolates to utilise common carbon sources may be responsible for the reduced hyphal growth *in planta*, and may be a contributing factor to reduced stroma development (Naffaa *et al.*, 1998; White *et al.*, 1991). Kirby (1961) showed that the growth rate of endophyte relative to that of the inflorescence is crucial for the development of an external stroma (Kirby, 1961). Reduced endophyte growth *in planta* may thus reduce the ability to form stromata.

1.5.2. Thiamine requirement for endophyte growth

Thiamine was first reported by Kulkarni and Nielsen (1986) as an obligate requirement for the growth of an asexual mutualistic *N. coenophialum* isolate. Thiamine or vitamin B1 is an essential cofactor for several important enzymes, including transketolase, pyruvate decarboxylase and α-ketoglutarate dehydrogenase, which play key roles in energy metabolism (Hohmann and Meacock, 1998). The requirement of thiamine for *N. coenophialum* growth in culture would suggest that this endophyte is dependant on the host for thiamine when growing *in planta* and is therefore physiologically adapted to mutualist growth – an endophytic rather than an epiphytic growth (Barry Scott; personal communication). Further studies of vitamin requirements showed that many asexual *Neotyphodium* endophytes require thiamine, nicotinic acid and pyridoxine for growth in culture (Ferguson *et al.*, 1993). Addition of thiamine can greatly increase the mycelial dry-weight of these asexual endophyte isolates *in vitro*.

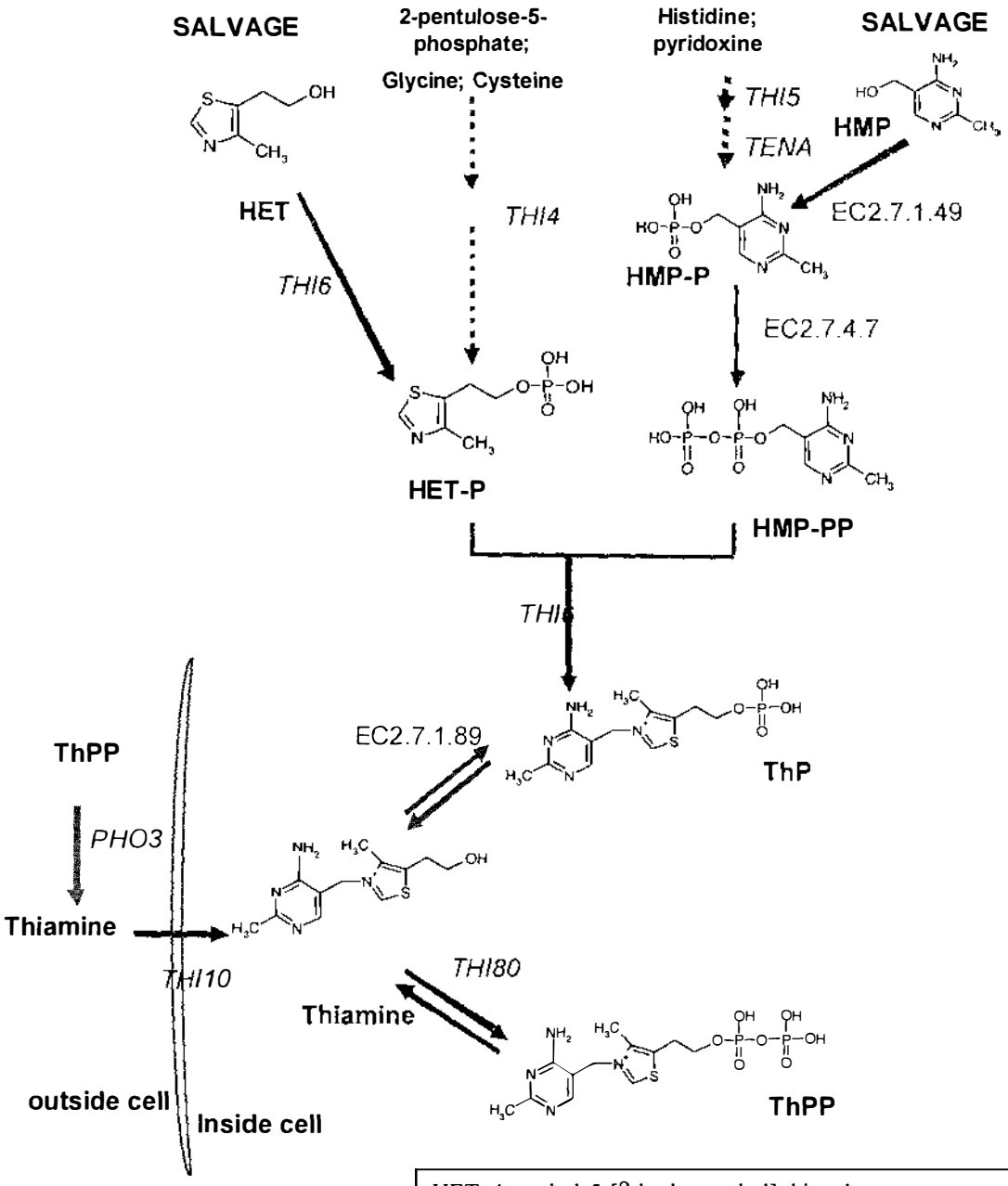
In a more comprehensive survey, 33 strains of *Neotyphodium, E. festucae, and E. typhina* were examined for thiamine auxotrophy. Four asexual mutualistic endophytes and two pleiotropic *E. festucae* isolates were found to be thiamine auxotrophs, while all the hybrid isolates and the one *E. typhina* examined were all prototrophic for thiamine (Xiuwen Zhang, and Barry Scott; unpublished results, Appendix 2), further indicating that many asexual *Neotyphodium* isolates have completely or partially lost the ability to synthesise thiamine. In a further survey of 28 isolates of *N. lolii*, *E. bromicola*, *E. festucae*, and *E. sylvatica* from different grass hosts (Adrian Leuchtmann and Barry Scott; unpublished results; Appendix 2), thiamine showed a tendency to promote growth of many *E. festucae* strains. In *E. bromicola*, thiamine supplementation had no significant effect on endophyte growth. Strains of *E. sylvatica* showed a range of responses to thiamine addition. While the growth of some strains of *E. sylvatica* were significantly promoted by thiamine, growth of others were not affected or repressed by supplementation with thiamine. It would be interesting to know the correlation of the thiamine response with the compatibility of these strains within the plant. Loss of the ability to synthesise thiamine and other key fungal metabolites such as amino acids may drive the co-evolution of mutualistic endophyte-grass associations, as these mutations may make the endophyte more dependent on the host and/or restrict external hyphal growth (Barry Scott; personal communication).

1.6. Thiamine biosynthetic pathway and genes

1.6.1. Thiamine biosynthesis pathways

Plants and many micro-organisms can synthesise thiamine *de novo* (Belanger *et al.*, 1995). While the genes and enzymes for thiamine biosynthesis have been studied for over 30 years

Figure 1.2. Thiamine biosynthesis pathway and genes in yeast



HET: 4-methyl-5-[β-hydroxyethyl] thiazole
HET-P: 4-methyl-5-[β-hydroxyethyl] thiazole monophosphate
HMP: 4-amino-5-hydroxymethyl-2-methylpyrimidine
HMP-P: 4-amino-5-hydroxymethyl-2-methylpyrimidine monophosphate
HMP-PP:4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate
ThP: thiamine monophosphate
ThPP: thiamine pyrophosphate

Diagram adapted from Hohmann, S. and Meacock, P. A. (1998) and Morett, E. *et al* (2003)

in a variety of organisms, including *Escherichia coli, Salmonella typhimurium, Bacillus subtilis, Saccharomyces cerevisiae* and *S. pombe*, the pathway has still not been completely characterised in any one organism (Morett *et al.*, 2003). Synthesis of thiamine requires two precursors: 4-methyl-5-[β-hydroxyethyl] thiazole monophosphate (HET-P) and 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate (HMP-PP) (Hohmann and Meacock, 1998). These two precursors condense to form thiamine monophosphate (ThP) by the action of thiamine phosphate pyrophosphorylase. In *S. cerevisiae*, thiamine monophosphate (ThP) is hydrolysed to form free thiamine, then transformed into thiamine pyrophosphate (ThPP) which is the active form of the enzyme cofactor (Fig 1.2). In *E. coli*, thiamine monophosphate is directly converted into thiamine pyrophosphate (Estramareix and David, 1996). Thiamine in the medium can be transported into the cell by the transporter protein which is encoded by *THI10* in yeast (Enjo *et al.*, 1997). The expression of *THI10* is regulated at the mRNA level by intracellular thiamine pyrophosphate and a positive regulatory factor encoded by THI3 gene. Thiamine monophosphate (ThP) and thiamine pyrophosphate (ThPP) may be hydrolysed into free thiamine by PHO3 before transport (Hohmann and Meacock, 1998).

The steps producing HET-P and HMP-PP appear to be different in different organisms (Young, 1986). The initial substrates for thiazole biosynthesis in yeast are glycine, cysteine and 2-pentulose-5-phosphate (probably D-ribulose-5-phosphate or D-xylulose-5-phosphate derived from the pentose phosphate pathway) (Hohmann and Meacock, 1998). In plants and bacteria, the initial substrates are tyrosine, cysteine and a 1-deoxy-2-pentulose-5-phosphate (Hohmann and Meacock, 1998). In *E. coli* at least six enzymes (encoded by *thiF, S, I, G, H* and *iscS*) are required for the formation of thiazole from tyrosine, cysteine and 1-deoxy-2-xylulose-5-phosphate (Leonardi and Roach, 2004). The pyrimidine precursor in yeast is proposed to be derived from histidine and pyridoxine which is derived from glutamine and 5-phosphoribosyl-1-amine) (Hohmann and Meacock,

1998), and in *E. coli* from 5-aminoimidazole ribonucleotide (AIR), a precursor shared between thiamine and *de novo* purine biosyntheses (Leonardi and Roach, 2004). The pyrimidine unit of thiamine in yeast originates from a C_5N fragment which is derived from C-2',2,N,C-6,5,5' of pyridoxol (Vitamin B6) and an N-C-N fragment derived from L-histidine (Zeidler *et al.* 2003). HET-P and HMP-P can be salvaged from HET and HMP respectively after degradation of thiamine or thiamine diphosphate (ThPP) (Hohmann and Meacock, 1998; Nosaka *et al.*, 1994).

1.6.2. Thiamine biosynthesis genes

Genetic studies and recent genome sequence analysis have identified many genes involved in the pathways in *E. coli, S. typhimurium, B. subtilis, S cerevisiae* and *S. pombe*, but not all the enzymes and the catalytic steps are understood (Hohmann and Meacock, 1998; Morett *et al.*, 2003). Table 1.2 lists the thiamine biosynthesis genes identified in *S. cerevisiae* and their proposed functions.

THI4 encodes a 35 kDa protein which possesses an adenine dinucleotide binding site and is required for the synthesis of the thiazole precursor HET-P (Praekelt *et al.*, 1994). This gene was originally isolated as a cDNA clone through a screen for genes expressed during entry into the stationary phase when yeast was grown in industry molasses medium and was thus named as *MOL1* (Praekelt and Meacock, 1992). Expression of this gene was later found to be induced under condition of thiamine depletion (Praekelt *et al.*, 1994). *THI4* of *S. cerevisiae* has dual roles in both DNA damage repair and thiazole biosynthesis (Machado *et al.*, 1996; Machado *et al.*, 1997). Analysis of the sequence of the THI4 protein revealed that This protein contain segments homologous to DNA binding motifs and to bacterial DNA polymerase (Machado *et al.*, 1996). The presence of a signal peptide suggests that it is localised to the mitochondria (Hohmann and Meacock, 1998).

Four genes, *THI11* (*YJR156c*), *THI12* (*YNL332w*), *THI13* (*YDL244w*) and *THI5* (*YFL058w*), are involved in the synthesis of hydroxymethylpyrimidine (HMP) (Wightman and Meacock, 2003). These four genes are highly conserved to each other and homologous to the *nmt1* gene from *S. pombe* (Hohmann and Meacock, 1998). A *nmt1*-negative mutant strain was reported to be a thiamine auxotroph that could be rescued by supplementation with thiamine or hydroxymethylpyrimidine (HMP) (Manetti *et al.*, 1994). Combinations of deletions in *THI11*, *THI12*, *THI13* and *THI5* were constructed and the phenotypes analysed (Wightman and Meacock, 2003). Only mutants with all four genes deleted showed severely reduced growth on medium lacking thiamine or HMP, suggesting redundant function of these genes in yeast. In addition, *TENA* has been identified to be involved in HMP-P biosynthesis in the yeast (Morett *et al.*, 2003).

THI6 is required for the condensation of HET-P and HMP-PP to form thiamine monophosphate (ThP) (Nosaka *et al.*, 1994). This gene may also play a key role in salvage of HET-P from thiazole (HET) (Hohmann and Meacock, 1998). *THI80* encoding the pyrophosphokinase (EC 2.7.4.16), is responsible for the phosphorylation of thiamine to thiamine diphosphate (ThDP) (Nosaka *et al.*, 1993). Other genes, including *SNZ2, SNZ3, SNO2* and *SNO3,* may also be required for thiamine biosynthesis. Transcripts of these genes accumulated in the absence of external thiamine (Rodriguez-Navarro *et al.*, 2002). in interaction with THI5 and THI11 and

Additional thiamine biosynthetic genes have been cloned and characterised from other fungi (Table 1.3). Most of these genes are either homologues of *Thi4* from *S. cerevisiae* or homologues of *nmt1* from *S. pombe*. *Nmt2* and *nmt1* were both isolated in a screen for thiamine-repressible genes in *S. pombe*. Disruption of *nmt2* resulted in thiamine auxotrophy (Manetti *et al.*, 1994). *Sti35* was isolated in a screen for genes induced under condition of heat shock and treatment with ethanol or phytoalexins (Choi *et al.*, 1990). This gene was shown to complement an *E. coli* thiamine biosynthesis minus mutant (Choi *et al.*, 1990).

Table 1.2. Genes and enzymes involved in thiamine biosynthesis in yeast

Enzyme	Gene	Function	Reference
EC 2.5.1.3 (EC 2.7.1.50)	*THI6*	Hydroxyethylthiazole kinase	(Nosaka *et al.*, 1994)
EC 2.7.1.49	THI20 (THI21)?	Hydroxymethylpyrimidine kinase.	www.yeastgenome.org
EC 2.7.4.16	*THI80*	Thiamine-phosphate kinase	(Nosaka *et al.*, 1993)
EC 2.7.1.89	?	Thiamine kinase	www.yeastgenome.org
EC 2.7.4.7	THI20 (THI21)?	Phosphomethylpyrimidine kinase	www.yeastgenome.org
	THI4	Thiazole biosynthesis	(Praekelt *et al.*, 1994)
	THI5	HMP biosynthesis	(Wightman and Meacock, 2003)
	THI11	HMP biosynthesis	(Wightman and Meacock, 2003)
	THI12	HMP biosynthesis	(Wightman and Meacock, 2003)
	THI13	HMP biosynthesis	(Wightman and Meacock, 2003)
	TENA	HMP biosynthesis	(Morett *et al.*, 2003)
	SNZ2	Interact with THI5	(Rodriguez-Navarro *et al.*, 2002)
	SNZ3	Interact with THI5	(Rodriguez-Navarro *et al.*, 2002)
	SNO2	Interact with THI protein	(Rodriguez-Navarro *et al.*, 2002)
	SNO3	Interact with THI protein	(Rodriguez-Navarro *et al.*, (2002)
	THI2 (*PHO6*)	Putative transcriptional activator	(Hohmann and Meacock, 1998)
	THI3	Regulator of gene expression	(Hohmann and Meacock, 1998)
	PDC2	Regulator of gene expression	(Hohmann and Meacock, 1998)
	RPI1	Multiple transcriptional activator	(Hohmann and Meacock, 1998)
	PHO3	Thiamine-repressible acid phosphatase	(Hohmann and Meacock, 1998)
	THI7 (*THI10*)	Thiamine transporter	(Hohmann and Meacock, 1998)

Table 1.3. Homologous genes of yeast THI4 and THI5 from other fungi

Gene	Fungi	Homology in yeast	Reference
nmt2	*S. pombe*	*THI4*	(Manetti *et al.*, 1994b)
thiA	*Aspergillus oryzae*		(Kubodera *et al.*, 2003)
thiF	*A. nidulans*		(Kubodera *et al.*, 2003)
Afu1	*A. fumigatus*		(Kubodera *et al.*, 2003)
Fgr1	*Fusarium graminearium*		(Kubodera *et al.*, 2003)
sti35	*F. oxysporum*		(Choi *et al.*, 1990)
sti35	*F. solani*		(Choi *et al.*, 1990)
nmt-1	*Neurospora crassa*		(Kubodera *et al.*, 2003)
mgr1	*Magnaporthe grisea*		(Kubodera *et al.*, 2003)
THI2	*Uromyces fabae*		(Sohn *et al.*, 2000)
TR21	*Puccinia triticina Eriks*		(Thara *et al.*, 2003)
nmt1	*S. pombe*	*THI5*	(Manetti *et al.*, 1994a)
nmt1	*A. parasiticus*		(Cary and Bhatnagar,
nmtA	*A. oryzae*		1995)
Afu2	*A. fumigatus*		(Kubodera *et al.*, 2003)
Fgr2	*F. graminearium*		(Kubodera *et al.*, 2003)
Ncr2	*N. crassa*		(Kubodera *et al.*, 2003)
THI1	*U. fabae*		(Kubodera *et al.*, 2003)
TR4	*P. triticina Eriks*		(Sohn *et al.*, 2000)
			(Thara *et al.*, 2003)

Disruption of *sti35* had no detectable effect on growth and development at various temperatures, nor on the ability to acquire thermotolerance in nutrient medium. But the *sti35* mutants showed increased thermotolerance compared to the wild-type strain when incubated in minimal medium after heat treatment (Thanonkeo *et al.*, 2000). In the rust pathogenic fungi, *Uromyces fabae* and *Puccinia triticina* Eriks, two thiamine biosynthesis genes (*THI1* and *THI2*) were isolated in a screen for genes that were differentially expressed during host colonisation (Hahn and Mendgen, 1997; Thara *et al.*, 2003). These two genes

were highly induced in the haustoria, a structure important for nutrient uptake and formed when the fungus invades host cells (Sohn *et al.*, 2000).

1.6.3. Regulation of thiamine biosynthesis

Thiamine biosynthesis in the cell is tightly regulated to prevent energy wastage (Hohmann and Meacock, 1998). In *S. cerevisiae*, several genes have been identified that are able to regulate thiamine biosynthesis. *THI2*, *THI3* and *PDC2* were identified as positive regulators of thiamine biosynthetic genes (Hohmann and Meacock, 1998). A mutation in *THI2* blocked thiamine production and reduced expression of many of the thiamine biosynthesis genes including *THI4*, *THI5*, *THI6* and *THI80* (Hohmann and Meacock, 1998). A mutation in *THI3* reduced both thiamine transport and thiamine biosynthesis (Nishimura *et al.*, 1992). The product of *PDC2* seems to be required for thiamine biosynthesis and may function as a transcription factor (Hohmann and Meacock, 1998).

Genes involved in thiamine biosynthesis (Hohmann and Meacock, 1998; Rodriguez-Navarro *et al.*, 2002; Wightman and Meacock, 2003) are repressed by the presence of thiamine and/or thiamine precursors. Thiamine may regulate the expression of thiamine biosynthesis genes through thiamine-dependent regulatory proteins that can sense the levels of thiamine and bind to the DNA or RNA to affect the transcription initiation (Hohmann and Meacock, 1998). However, transcription of *THI1* and *THI2* in *U. fabae* was not repressed by the presence of thiamine (Sohn *et al.*, 2000). Recent studies showed that thiamine or thiamine diphosphate could bind directly to the mRNA and subsequently lead to regulation of the gene (Mironov *et al.*, 2002; Winkler *et al.*, 2002). In *E coli*, a mRNA-thiamine complex is formed in the presence of thiamine and this complex adopts a distinct structure which inhibits translation of the mRNA (Winkler *et al.*, 2002). In *B. subtilis*, the

mRNA-thiamine complex adopts a structure that causes premature termination of transcription and thus represses gene expression (Mironov *et al.*, 2002).

The RNA sequence to which thiamine binds, designated a *thi*-box, is highly conserved in many thiamine biosynthesis genes in both bacteria and fungi, but the regulatory mechanism in fungi seems to be different from that in prokaryotes (Kubodera *et al.*, 2003). The *thi*-box of *thiA* from *Aspergillus oryzae*, contained two conserved motifs, regions A and B, in one of the two introns in the 5'-untranslated region. Deletion of either region A or B inhibits splicing of the introns and inhibits feed-back repression by thiamine or thiamine derivatives (Kubodera *et al.*, 2003).

1.6.4. Thiamine biosynthesis genes in microbe-plant interactions

Using suppressive subtractive hybridisation techniques to isolate and characterise genes induced in microbe-host interactions, high level expression of thiamine biosynthetic genes was detected in host tissues containing the fungal pathogens *U. fabae* (Hahn and Mendgen, 1997; Sohn *et al.*, 2000) and *P. triticina* (Thara *et al.*, 2003). The mRNA of thiamine biosynthetic genes *thi1* and *thi2* of *U. fabae* together represent about 5% of the total haustorial mRNA when the fungus switches from host plant invasion toward to parasitic growth (Hahn and Mendgen, 1997). *Thi1* and *thi2* are homologous to yeast *THI5* and *THI4* respectively which are involved in the biosynthesis of HMP and HET precursors (Hahn and Mendgen, 1997; Sohn *et al.*, 2000). The high level of expression of thiamine biosynthesis genes during host colonisation in different fungal pathogens further suggests an important role for thiamine biosynthesis in fungal colonisation (Thara *et al.*, 2003).

In some bacteria that colonise plants, thiamine biosynthetic genes have been mapped to large plasmids in *Rhizobium meliloti* (Finan *et al.*, 1986; Finan *et al.*, 2001) and *Erwinia herbicola* (Gantotti and Beer, 1982). *R. meliloti* is a mutualistic symbiotic bacterium that induces nodule formation of legumes. The megaplasmid, with a molecular mass of over 450 megadaltons, contains genes for nodulation (*nod*) and nitrogen fixation (*nif*) (Finan *et al.*, 1986; Finan *et al.* 2001). *E. herbicola* is an epiphytic bacterium that grows on various plant surfaces. Coincidentally, thiamine biosynthetic genes, together with genes for biosynthesis of biotin, nicotinate and genes required for nodule formation and nitrogen fixation were identified on a chromosomal symbiosis island of several *Mesorhizobium loti* strains (Sullivan *et al.*, 1995; Sullivan and Ronson, 1998; Sullivan *et al.*, 2002). This symbiosis island is a large chromosomally integrated element that can be transferred from symbiotic to non-symbiotic *Mesorhizobium* bacteria, converting the latter to symbiotic strains. Of the seven naturally occurring non-symbiont *Mesorhizobium* strains analysed, all were auxotrophic for thiamine. Presence of thiamine biosynthetic genes on plasmids of these symbiotic organisms was suggested to confer a selective advantage for the bacteria to colonise their hosts (McCallum, 2001).

1.7. Aims and objectives

Although considerable progress has been made in cloning and analysing genes involved in alkaloid secondary metabolites, little is known about the role of endophyte primary metabolic genes in the interaction of the endophyte with the grass host. The aim of this study was to determine the importance of endophyte thiamine biosynthesis in the endophyte-grass interaction, and in particular, the role in host colonisation and stromata development. Thiamine biosynthesis was chosen as thiamine is an important coenzyme in cell energy metabolism. In addition, thiamine is an essential growth factor for many asexual *Neotyphodium* endophytes and thiamine biosynthetic genes are up-regulated in a number of fungal-plant interactions.

The hypothesis to be tested was that thiamine biosynthesis in the sexual *E. typhina* endophyte is required for colonisation of the host, and for external growth and stroma development. Loss of endophyte thiamine biosynthesis might reduce the degree of host colonisation and/or stop stromata development on the host.

Chapter two

Materials and Methods

2.1. Biological materials

2.1.1. Fungal and bacterial strains, plasmids and λ clones

Fungal and bacterial strains, λ clones, plasmids and plants used throughout this study are listed in Table 2.1.

2.1.2. Growth and maintenance of organisms

Escherichia coli strains were grown at 37°C on LB agar plates (Appendix 4) or in LB broth with shaking at 200 rpm. The LB medium was supplemented with either tetracycline (10 mg/L) or ampicillin (100 mg/L) when necessary. For long-term storage, cultures were stored at -80°C in 15% (v/v) glycerol.

Epichloë and Neotyphodium strains were routinely grown on PD agar plates (Appendix 4) for several weeks or in PD broth with shaking at 200 rpm at 22°C for several days depending on the growth rate. For sub-culturing, a small piece of mycelial block was cut out from a colony edge and inoculated onto a fresh PD agar plate and grown at 22°C for several weeks. For short to medium-term storage, cultures were maintained on PD agar plates at 4°C. For long-term storage, fungal cultures were kept at -80°C in 30% (v/v) glycerol.

For cultures used for DNA extraction, or protoplast preparation, mycelia were collected by scraping the colony surface using a scalpel and were ground to a fine suspension in 500 µl of PD broth or CM broth (Appendix 4.4). The suspension was inoculated into 50 ml of PD broth in a 125 ml flask. This culture was incubated at 22°C with shaking at 200 rpm for several days (four to seven days) until a thick mycelial suspension was observed.

Table 2.1. Fungal and bacterial strains, λ clones, plasmids and plants

Strains	Relevant Characteristics	Other names	References
Fungi			
Neotyphodium lolii			
Lp19 (PN2191)	Wild-type isolate; haploid		(Christensen *et al.*, 1993)
Neotyphodium spp.			
Lp1 (PN2215)	Wild-type isolate; diploid		(Christensen *et al.*, 1993)
Epichloë typhina			
E8 (PN2238)	Wild type isolate, haploid, parent strain of the *thi1* deletion mutants		(Schardl *et al.*, 1997)
T1	[*thi1*::*hph*][a], derivative of E8 (PN2238)		This study
T120	[*thi1*::*hph*], derivative of E8 (PN2238)		This study
T48	[*thi1*::*hph*], derivative of E8 (PN2238)		This study
T56	[*thi1*::*hph*], derivative of E8 (PN2238)		This study
T63	[*thi1*::*hph*], derivative of E8 (PN2238)		This study
T97	*thi1*::*hph*, derivative of E8 (PN2238)		This study
T103	*thi1*::*hph*, derivative of E8 (PN2238)		This study
T140	*thi1*::*hph*, derivative of E8 (PN2238)		This study
T150	[*thi1*::*hph*], derivative of E8 (PN2238)		This study
T293	[*thi1*::*hph*], derivative of E8 (PN2238)		This study
T301	*thi1*::*hph*, derivative of E8 (PN2238)		This study
WT1 (PN2284)	E8 wild-type isolate sub-cultured from E8 (PN2238)	PN2284	This study
WT2 (PN2285)	E8 wild-type isolate regenerated from E8 (PN2238) protoplast	PN2285	This study
WT3 (PN2312)	E8 culture re-isolated from plant G1080 that was infected with E8 WT2 (PN2285).	PN2312	This study
WT4 (PN2313)	E8 culture re-isolated from E8 infected plant at AgResearch, Palmerston North (Christensen, M.).	PN2313	This study
EC1 (PN2286)	[*thi1*::*hph*]; single-spore isolate from T1	PN2286	This study
EC2 (PN2287)	[*thi1*::*hph*]; single spore isolate from T120	PN2287	This study
KO3 (PN2282)	*thi1*::*hph;* single spore isolate from T140	PN2282	This study
KO4 (PN2283)	*thi1*::*hph;* single spore isolate from T301	PN2283	This study
KO1	*thi1*::*hph;* single spore isolate from T97		This study
KO2	*thi1*::*hph;* single spore isolate from T103		This study
Escherichia coli			
KW251	F'*sup*E44 *gal*K *gal*T22 *met*B1 *hsd*R2 *mcr*B1		Promega Corp.

XL-1	*mcr*A [*arg*A81:Tn10]*rec*D1014 *sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac*⁻ F'[*pro*AB⁺ *lacI*^{q} *lacZ*ΔM15 Tn10(Tet^R)]		(Bullock *et al.*, 1987)
Lambda clone			
λXZ1	λGEM11 + Lp19 *thi1* fragment	λT1-1	This study
λXZ2	λGEM11 + Lp19 *thi1* fragment	λT2-1	This study
λXZ3	λGEM11 + Lp19 *thi1* fragment	λT3-1	This study
λXZ4	λGEM11 + Lp19 *thi1* fragment	λT4-1	This study
λXZ5	λGEM11 + Lp19 *thi1* fragment	λT5-1	This study
Plasmids			
pAN7-1	6.8 kb Hyg^R Amp^R		(Punt *et al.*, 1987)
pUC118	3.2 kb Amp^R		(Vieira and Messing, 1987)
pGEM-T	3.0 kb Amp^R		Promega Corp.
pGEM-T easy	3.0 kb Amp^R		Promega Corp.
PUChph (PN1687)	pUC118 + 2.37-kb *Xba*I fragment from pCHyg1	pPN1687	(McMillan *et al.*, 2003)
pXZ1	pUC118 + Lp19 *thi1* 3.2 kb *Hin*dIII fragment	pTH2	This study
pXZ2	pUC118 + Lp19 0.7 kb *Sst*I fragment	pT63-1	This study
pXZ3	pUC118 + Lp19 *thi1* 7.2 kb *Sst*I fragment	pT99-1	This study
pXZ4	pGEM-T + E8 *thi1* PCR product	pGEXZ105f	This study
pXZ5	pGEM-T + E8 *thi1* PCR product	pGEXZ75	This study
pXZ6	pGEM-T + E8 *thi1* TAIL-PCR product	pGXZ3T3	This study
pXZ7	pGEM-T + E8 *thi1* TAIL-PCR product	pGXZ3T2	This study
pXZ8	pGEM-T + E8 *thi1* TAIL-PCR product	pGXZ5T11	This study
pXZ9	pGEM-T + E8 *thi1* TAIL-PCR product	pTail1	This study
pXZ10	pGEM-T + E8 *thi1* TAIL-PCR product	pTail2	This study
pXZ11	pGEM-T + E8 *thi1* Inverse PCR product	pGXZ3120	This study
pXZ12	pGEM-T + E8 *thi1* Inverse PCR product	pGXZ2042	This study
pXZ13	pGEM-T + Lp19 *thi1* 5' RACE product	pXZ5RC-1	This study
pXZ14	pGEM-T + Lp19 *thi1* 5' RACE product	pXZ5RC-3	This study
pXZ15	pGEM-T + Lp19 *thi1* 5' RACE product	pXZ5RC-4	This study
pXZ16	pGEM-T + Lp19 *thi1* 5' RACE product	pXZ5RC-5	This study
pXZ17	pGEM-T + Lp19 *thi1* 5' RACE product	pXZ5RC-7	This study
pXZ18	pGEM-T + Lp19 *thi1* 5' RACE product	pXZ5RC-11	This study
pXZ19	pGEM-T + Lp19 *thi1* 5' RACE product	pXZ5RC12	This study
pXZ20	pGEM-T + Lp19 *thi1* 5' RACE product	pXZ5RC13	This study
pXZ21	pGEM-T + 3' RACE product	pG3RACE-1	This study
pXZ22	pGEM-T + 3' RACE product	pG3RACE-2	This study
pXZ23	pGEM-T + 3' RACE product	pG3RACE-3	This study

pXZ24	pGEM-T + 3' RACE product	pG3RACE-4	This study
pXZ50	E8 *thi1* disruption plasmid	pEXZ11	This study
pXZ51	E8 *thi1* disruption plasmid	pEXZ21	This study
Plants			
Nui	Perennial ryegrass		(Christensen *et al* 1997)
G1077	Nui grass infected with E8 (PN2284)		This study
G1078	Nui grass infected with E8 (PN2284)		This study
G1079	Nui grass infected with E8 (PN2284)		This study
G1080	Nui grass infected with E8 (PN2284)		This study
G1081	Nui grass infected with E8 (PN2284)		This study
G1082	Nui grass infected with E8 (PN2285)		This study
G1083	Nui grass infected with E8 (PN2285)		This study
G1084	Nui grass infected with E8 (PN2285)		This study
G1085	Nui grass infected with E8 (PN2285)		This study
G1086	Nui grass infected with E8 (PN2285)		This study
G1087	Nui grass infected with EC1 (PN2286)		This study
G1088	Nui grass infected with EC1 (PN2286)		This study
G1089	Nui grass infected with EC1 (PN2286)		This study
G1090	Nui grass infected with EC1 (PN2286)		This study
G1091	Nui grass infected with EC1 (PN2286)		This study
G1092	Nui grass infected with EC2 (PN2287)		This study
G1093	Nui grass infected with EC2 (PN2287)		This study
G1094	Nui grass infected with EC2 (PN2287)		This study
G1095	Nui grass infected with EC2 (PN2287)		This study
G1096	Nui grass infected with EC2 (PN2287)		This study
G1097	Nui grass infected with KO3 (PN2282)		This study
G1098	Nui grass infected with KO3 (PN2282)		This study
G1099	Nui grass infected with KO3 (PN2282)		This study
G1100	Nui grass infected with KO3 (PN2282)		This study
G1101	Nui grass infected with KO3 (PN2282)		This study
G1102	Nui grass infected with KO4 (PN2283)		This study
G1103	Nui grass infected with KO4 (PN2283)		This study
G1104	Nui grass infected with KO4 (PN2283)		This study
G1105	Nui grass infected with KO4 (PN2283)		This study
G1106	Nui grass infected with KO4 (PN2283)		This study
IMP566	Perennial ryegrass		AgResearch Grasslands
EGH314	IMP566 infected with EC2 (PN2287)		

EGH331	IMP566 infected with E8 (PN2285)
EGH332	IMP566 infected with E8 (PN2285)
EGH389	IMP566 infected with E8 (PN2284)
EGH396	IMP566 infected with E8 (PN2284)

[a] *hph* =P*glaA-hph*-T*trpC*; addition of [ ] indicates that the *thi1* replacement construct may have integrated into an ectopic location

2.2. DNA isolation, purification and quantification

2.2.1. Large-scale isolation of endophyte DNA

Endophyte genomic DNA was isolated using a modification of the Byrd *et al* (Byrd *et al.*, 1990) method. Freezed-dried mycelia (about 100 mg) were ground to a fine powder in liquid nitrogen using a pre-chilled mortar and pestle and re-suspended in 10 ml of extraction buffer (150 mM Na_2EDTA, 50 mM Tris-HCl (pH 8.0), 1% (w/v) sodium lauroyl sarcosine). To the suspension, proteinase K (Boehringer) was added to a final concentration of 2 mg/ml and the mixture incubated at 37°C for 20 min, followed by centrifugation at 20,200 x g at 4°C for 10 min to pellet the cellular debris. The supernatant was recovered and extracted with phenol/chloroform (Section 2.2.9.2). The DNA in the aqueous phase was precipitated with an equal volume of isopropanol at -20°C for at least 15 min and centrifuged at 8,000 x g for 30 min. In order to remove polysaccharides, the DNA pellet was re-suspended in 5 ml of 1 M NaCl, and centrifuged at 8,000 x g for 5 min. The DNA pellet was washed with 70% ethanol, air dried and re-suspended in 500 µl water.

2.2.2. Small scale isolation of endophyte DNA

Endophyte genomic DNA was isolated using the method described by Al-Samarrai and Schmid (2000). Freeze-dried mycelia (1 to 30 mg) were ground to a fine powder in liquid nitrogen, and re-suspended in 500 µl of freshly prepared lysis buffer (40 mM Tris-acetate; 20 mM sodium acetate; 1 mM EDTA and 1% (w/v) SDS; pH 7.8)). The suspension was mixed vigorously by pipetting about 50 times with a Gilson P1000 pipetmann until lots of froth formed indicating detachment of DNA from polysaccharides. Then 165 µl of 5 M NaCl solution was added, and the solution mixed by inverting 5 times and centrifuged at 13,000 x g for 20 min to precipitate polysaccharides, proteins and cellular debris. The supernatant was recovered and extracted with phenol/chloroform (Section 2.2.9.2). The DNA in the aqueous phase was precipitated with 2 volume of 95% ethanol and centrifuged at 13,000 x g for 10 min. The DNA pellet was washed with 70% ethanol three times, air dried then re-suspended in 50 µl water.

2.2.3. Bacteriophage λ DNA isolation

Bacteriophage λ DNA was isolated using the method based on that of Sambrook *et al* (Sambrook *et al.*, 1989). A mixture of 100 µl phage and 100 µl KW251 cells was incubated at 37°C for 30 min, mixed with 3 ml of top agarose (Appendix 4.1) then plated onto LB agarose (Appendix 4.1) plates and the mixture incubated at 37°C until there was confluent lysis (about 6 – 8 h). The plate was overlaid with 5 ml of SM buffer and left overnight at 4°C. The overlaying SM buffer (Appendix 4.3) was collected and the bacterial debris removed by centrifugation at 3,000 x g for 10 min (4°C). The supernatant was recovered and DNase and RNAse both added to a final concentration of 1 µg/ml, and incubated at 37°C for 30 min. PEG 6000 solution (20% (w/v) PEG 6000 in 2 M NaCl) was added (5 ml), and the solution mixed and incubated on ice for 1 h, then centrifuged at 5,800 x g for 30

min at 4°C. The pellet was re-suspended in 0.5 ml of SM buffer, containing 5 µl of 10% (w/v) SDS and 10 µl of 250 mM Na_2EDTA, then incubated at 68°C for 15 min. To remove the phage coat and protein, the supernatant was recovered and extracted with phenol/chloroform (Section 2.2.9.2). The DNA was precipitated by the addition of an equal volume of isopropanol and 50 µl of 3 M sodium acetate (pH 5.2), incubated at –20°C for 20 min and centrifuged at 13,000 x g for 10 min. The DNA pellet was washed with 70% ethanol, air dried and re-suspended in 50 µl of TE (10/0.1) (Appendix 4.3).

2.2.4. Plasmid DNA isolation using rapid boiling method

Plasmid DNA from *E. coli* was isolated using the method described by Holmes and Quigley (Holmes and Quigley, 1981). An overnight *E. coli* culture of 1.5 ml was centrifuged at 13,000 x g for 1 min and the pellet thoroughly re-suspended in 350 µl STET buffer (8% (w/v) sucrose; 5 % (v/v) Triton X-100; 50 mM Na_2EDTA (pH 8.0) and 50 mM Tris-HCl (pH 8.0)). An aliquot of 25 µl of lysozyme (10 mg/ml in 10 mM Tris-HCl (pH 8.0)) was added to the suspension and the mixture was boiled for 40 sec and centrifuged at 13,000 x g for 10 min. The gelatinous pellet was removed immediately. The DNA in the aqueous phase was precipitated by the addition of an equal volume of isopropanol, incubated at -20°C for at least 20 min, then centrifuged for 10 min at 13,000 x g. The DNA pellet was washed with 70 % ethanol, air dried and re-suspended in 50 µl of sterilised water.

2.2.5. Plasmid DNA isolation using an alkaline lysis method

Plasmid DNA was isolated using the alkaline lysis method described by Sambrook *et al* (Sambrook *et al.*, 1989). An overnight culture (1.5 ml *E. coli*) was centrifuged at 13,000 x g for 1 min and the pellet re-suspended in 100 µl of ice-cold solution I (50 mM glucose; 25

mM Tris-HCl and 10 mM Na_2EDTA; pH 8.0). To the suspension, 200 µl of freshly prepared solution II (0.2 M NaOH and 1 % (w/v) SDS) was added and mixed thoroughly by inversion. An aliquot of 150 µl of solution III (29.44% (w/v) potassium acetate and 11.5 ml glacial acetic acid per 100 ml) was added to the solution, and the solution was inverted several times to mix thoroughly. The mixture was incubated on ice for 3-5 min, and then centrifuged at 13,000 x g for 5 min. The supernatant was recovered and extracted with phenol/chloroform (Section 2.2.9.2). DNA in the supernatant was precipitated by the addition of two volumes of 95% ethanol, and centrifuged for 5 min. The DNA pellet was washed with 70 % ethanol, then air dried and re-suspended in 50 µl of sterilised water.

2.2.6. Plasmid DNA isolation using a Quantum Miniprep kit

Plasmid DNA was extracted using a Quantum Prep Plasmid Miniprep Kit (Bio-Rad) following the manufacturer's instructions. An overnight culture (1.5 ml *E. coli*) was centrifuged at 13,000 x g for 1 min and the pellet re-suspended in 200 µl of cell re-suspension solution. To the suspension, was added 250 µl of cell lysis solution, and 250 µl of neutralisation solution. The solution was mixed, then centrifuged at 13,000 x g for 5 min to remove cell debris. The supernatant was transferred into a spin filter which was inserted in an Eppendorf tube. To the supernatant, 200 µl matrix solution was added and mixed thoroughly, and centrifuged at 13,000 x g for 30 sec and the filtrate discarded. The matrix was washed twice with 500 µl wash solution and DNA in the matrix was eluted by the addition of 100 µl sterilised water to the matrix and centrifuged for 1 min.

2.2.7. Isolation of DNA from SeaPlaque agarose gel

DNA was recovered from SeaPlaque agarose gels using the method described by Thuring *et al* (Thuring *et al.*, 1975). After electrophoresis, fragments of interest were excised from the gel and melted at 65°C for 15 min, mixed thoroughly with an equal volume of Tris-equilibrated phenol (Appendix 4) and frozen at -20°C for at least 2 h. The mixture was centrifuged at 13,000 x g for 10 min. The aqueous phase was recovered and extracted with phenol/chloroform (Section 2.2.9.2). DNA in the supernatant was precipitated by the addition of 1/10 volume of 3 M sodium acetate (pH 5.2), 2.5 volume of 95% ethanol and chilled at -20°C for at least 2 h, and centrifuged at 13,000 x g for 15 min. The DNA pellet was washed in 95 % ethanol, air-dried and re-suspended in 10 to 20 µl sterilised water.

2.2.8. DNA isolation from plant material

Total genomic DNA was isolated from endophyte-infected or endophyte-free plant tissue using the method based on that of Moller (Möller *et al.*, 1992). Grass pseudostem tissue (0.5 to 1.0 g fresh weight) was ground to a fine power in liquid nitrogen, re-suspended in 10 ml TES buffer (100 mM Tris and 10 mM EDTA pH 8.0, 2% SDS). To this suspension, 2 mg of proteinase K was added and the mixture incubated at 65°C for 1 h with occasional gentle mixing. To the mixture, 2.8 ml of 5 M NaCl and 1.28 ml of 10% hexadecyltrimethylammonium bromide (CTAB) were added and incubated at 65°C for a further 10 min. Chloroform (14 ml) was then added and the solution mixed gently, placed on ice for 30 min then centrifuged at 1,600 x g for 10 min. The aqueous phase was recovered and 4.5 ml of 5 M ammonium acetate added. This was gently mixed and placed on ice for 30 min and then centrifuged at 1,600 x g for 10 min. DNA in the aqueous phase was precipitated by the addition of 10 ml cold isopropanol, centrifuged at 1,600 x g for 10 min.

The DNA pellet was washed in 70% (v/v) ethanol, air dried, then re-suspended in 500 µl sterilised water.

2.2.9. Purification and precipitation of DNA

2.2.9.1 Purification of PCR product by Concert™ Rapid PCR purification system

PCR products for molecular cloning or sequencing were purified using the Concert™ Rapid PCR purification system (GibcoBRL) following the manufacturer's instructions. To 100 µl of PCR product, 400 µl binding solution (H1) was added and mixed thoroughly, and the mixture loaded onto a spin cartridge and centrifuged at 13, 000 x g for 1 min. The DNA in the spin filter was washed by addition of 700 µl of washing solution and centrifuged for 1 min. The filtrate was discarded and the spin filter was centrifuged for a further min to remove trace ethanol. The DNA was then eluted by adding 50 µl of sterilised water at 65°C into the filter, incubated for 1 min, then centrifuged for 2 min.

2.2.9.2. Purification of DNA by phenol/chloroform extraction

DNA was purified by adding an equal volume of phenol/chloroform to the solution which was mixed by inversion, then centrifuged for 5 min at 13,000 x g. The aqueous phase was recovered and two volumes of chloroform added to the solution which was mixed and centrifuged at 13,000 x g for 5 min. The DNA in the aqueous phase was precipitated by adding 1/10 volume of 3 M sodium acetate, and either 2.5 volume of 95 % (v/v) ethanol or 0.6 to 1 volume of isopropanol and followed by incubation at -20°C for at least 30 min. DNA was pelleted by centrifugation for 10 min at 13,000 x g. The DNA pellet was washed with 70 % ethanol, air dried and re-suspended in sterilised water.

2.2.10. Detection, sizing and quantification of DNA

2.2.10.1. Fluorometric quantification of DNA

DNA was quantified using a Hoefer Scientific TKO 100 Fluorometer following the manufacturer's instructions. The fluorometer was calibrated by adjusting the zero dial to zero using 2 ml of dye solution (1 x TNE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, and 100 mM NaCl pH 7.4) and 0.1 μg/ml Hoechst 33258 dye (Sigma)). The scale dial was then set to 100 when 2 μl of 100 μg/ml calf thymus DNA was added to 2 ml of dye solution. With these settings, 2 μl of the sample DNA was added to 2 ml of dye solution and the resulting reading was recorded as the DNA concentration in μg/ml.

2.2.10.2. Agarose gel electrophoresis

DNA samples in SDS dye (Appendix 4.3) solutions were electrophoresed in an agarose gel alongside a series of DNA standards of known concentration. The concentration of agarose used varied from 0.7% to 2% (w/v) in either 1 x TBE buffer (Appendix 4.3) or 1 x TAE buffer (Appendix 4.3). Gels were run in TBE buffer or TAE buffer, at 80 to 120 V for 1-2 h, or at 30 V overnight for gels to be Southern blotted (Bio-Rad sub-Cell).

After electrophoresis, gels were stained in 1 mg/L ethidium bromide for 10 to 20 min for a small gel or 30 min for a large gel, then destained in sterilised water. Bands on the gel were visualised on a UV transilluminator and the image captured with an Alpha Innotech gel documentation system. DNA fragment sizes were determined by comparison to known standards using Alpha Innotech gel documentation system software. This program compares the distance the fragment has migrated from the well to the distance migrated by molecular markers of known size, such as λ *Hin*dIII digested DNA or 1 kb ladder, BRL.

2.3. Genomic library screening

The method of genomic library screening is based on that of Sambrook *et al* (Sambrook *et al.*, 1989).

2.3.1. Genomic library plating and filter lifts

An aliquot of 100 µl diluted phage (2 x 10^4 pfu/ml) in SM buffer (Appendix 4.3) was mixed with 100 µl of KW251 cells, incubated at 37°C for 30 min to allow phage to absorb, then the mixture added to 3 ml of top agarose (Appendix 4.2) at 50°C and poured onto LB plates. These plates were then incubated at 37°C for about 8 h until small plaques were visible.

Filters (Hybond-N+) were marked asymmetrically and placed onto the KW251/phage plates for 60 sec. The plates were labelled with the same marks for later alignment. The filters were removed from the plates and placed (DNA side up) successively onto 3 layers of 3 MM paper moistened with Southern blotting solution 1 (Appendix 4.5) for 2 min, Southern blotting solution 2 (Appendix 4.5) for 2 min, Southern blotting solution 3 (Appendix 4.5) for 5 min, and 2 x SSC (Appendix 4.5) for 2 min. The filters were then air dried and baked in a vacuum oven at 80°C for 2 h. Duplicate lifts were carried out, with the second filter left on the plate for 90 sec instead of 60 sec.

2.3.2. Probe labelling and plaque DNA hybridisation

The probe DNA was radio-labelled with [α-^{32}P]dCTP using the High Prime Labelling Kit (Boehringer Mannheim). DNA (25 ng) in a volume of 11 µl was denatured in a boiling water

bath for 10 min and immediately placed on ice. To this 4 µl of High Prime Solution and 5 µl (50 µCi) [α-^{32}P]dCTP (Amersham) was added. The reaction was mixed, spun briefly in a centrifuge and incubated at 37°C for 1 h. The reaction was stopped by addition of 2 µl of 0.2 M Na_2EDTA (pH 8) and 28 µl TES buffer (10 mM Tris-HCl, 0.1 mM EDTA, 100 mM NaCl). The mixture was run through a 0.9 ml (packed volume) Sephadex G-50 mini-spin column by centrifugation to remove the unincorporated nucleotides.

For hybridisation, the Nylon filters (Roche Mannheim) were pre-hybridised with 30 ml of 10 x Denhardts solution (Appendix 4.5) at 65°C for at least 2 h. After prehybridisation, boiled radio-labelled probe was added to the tube and hybridisation was carried out at 65°C overnight. The filter was removed and washed three times for 20 min each, in 3 x SSC (Section 2.2.5) at 50°C. The washed filter was wrapped in Gladwrap and exposed to Fuji X-ray film in the presence of Cronex intensifying screens at -70°C for an appropriate time. Once autoradiographs had been exposed for an appropriate time, they were developed in Kodak developer D-19 developing solution for 5 min, rinsed with water and fixed in Kodak Rapid Fixer solution A for 5 min. The autoradiographs were rinsed in water and dried before analysis.

2.3.3. Purification of positive lambda clones

Positively hybridising plaques were identified as signals on the autoradiographs in identical positions on duplicate filters. The positions of the positive plaques on the KW251 plates were determined by alignment of the asymmetrical markings on the plates and filters with the autoradiograph. Plaques corresponding to signals were picked with the pipette tip of a 1 ml Gilson autopipettor from which the end had been removed and were stored at 4°C in 500 µl SM buffer containing a drop of chloroform.

The phage from these plaques were purified twice more as outlined above except that 30-300 phage per plate were plated. DNA was extracted from the positive hybridising plaques for further analysis.

2.4. DNA digestion, ligation, cloning and subcloning

2.4.1. Restriction endonuclease digestion of genomic DNA

Digestion of endophyte genomic DNA was carried out in a reaction mixture (400 μl) containing 2 μg DNA, 1 x buffer, an excess of enzyme (about 50 to 60 units depending on the efficiency of the enzyme), and 20 μg BSA. The digestion was performed in a water bath at temperatures recommended by the manufacturer (usually 37°C) overnight. A small aliquot of digested DNA was checked by electrophoresis to ensure complete digestion. If digestion was incomplete more enzyme was added and the reaction re-incubated. If the digest failed to go to completion, the DNA was purified by phenol/chloroform extraction (Section 2.2.9.2) and re-digested. For Southern blotting, the digested DNA were precipitated by adding 1/10 volume of 3 M sodium acetate (pH5.2) and 2.5 volume of 95% (v/v) ethanol, incubated at -20 °C for at least 30 min, and centrifuged for 10 min at 13,000 x g. The DNA pellet was washed with 70% ethanol, air dried and re-suspended in 50 μl sterilised water.

2.4.2. Restriction endonuclease digestion of λ phage DNA, plasmid DNA and PCR products

Digestions of λ phage DNA, plasmid DNA or purified PCR products were carried out in a similar manner to that described for digestion of genomic DNA (Section 2.4.1), except that

the volume was smaller and incubation times were one to two hours. Usually, digestion of λ phage DNA was carried out in a reaction mixture (50 µl) containing 300 – 800 ng DNA, 1 x buffer and 20 units of enzyme. Digestions of plasmid DNA or purified PCR product were carried out in a reaction mixture (20 µl) containing 50 to 500 ng DNA, 1 x buffer and 10 units of enzyme.

2.4.3. CAP-treatment of vector DNA

Digested vector DNA was dephosphorylated using calf alkaline phosphatase (CAP, Boehringer) to prevent self ligation. Vector DNA (5 µg) was digested with the appropriate restriction enzyme (Section 2.4.2), then the restriction enzyme was inactivated by heating for 10 min at 65°C. To this digestion reaction, 0.5 units of calf alkaline phosphatase (CAP, Boehringer) was added, and the reaction mixture incubated at 37°C for 30 min. Na_2EDTA, SDS and proteinase K (Boehringer) were then added to final concentrations of 5 mM, 0.5% (w/v) and 50 µg/ml respectively, and the mixture was incubated at 56°C for 30 min. The DNA was purified by phenol/chloroform extraction (Section 2.2.9.2) and precipitated with ethanol (Section 2.2.9.2).

2.4.4. Ligation of DNA fragments

Ligation of DNA fragments into digested vectors (usually CAP-treated) was performed in a reaction mixture (15 to 20 µl) containing 30 to 60 ng DNA fragments, 20 ng vector DNA, 1 x ligation buffer and 10 units of T4 DNA ligase (NE Biolabs), and incubated at 4°C overnight.

Ligation of PCR products into pGEM-T vector was carried out using a pGEM-T ligation Kit (Promega). The reaction mixture (20 µl) contained 10 to 50 ng PCR products, 20 ng pGEM-T vector, 1 x ligation buffer supplied with the kit and 2 units of ligase, and incubated at 4°C overnight.

Ligations were checked by removing 2 µl samples before and after ligation and analysing the products by electrophoresis.

2.4.5. Preparation of competent cells and transformation by electroporation

One litre of XL-1 *E. coli* cell culture (A_{600} = 0.5-1.0) was chilled on ice for 20 min and the cells harvested by centrifugation at 4,000 x g for 10 min at 4°C. The cells were washed twice with 1 litre then 0.5 litre of ice-cold water by centrifuging (at 4,000 x g for 10 min at 4°C) and re-suspending, and finally washed with 20 ml ice-cold 10% (v/v) glycerol and centrifuged as above. The pellet was re-suspended in 4 ml ice-cold 10% (v/v) glycerol and the cells were stored at -80°C in 40 µl aliquots.

For transformation, 40 µl of competent *E.coli* cells were mixed with 2 µl ligated DNA and the mixture was transferred into a 0.2 cm cuvette then placed on ice for 1 min. After the Gene Pulser (Bio-Rad) was set at 25 µF, 2.5 kV and 200 ohms, the cuvette with the mixture in the bottom was pulsed at these setting. The cells were immediately re-suspended in 1 ml of SOC medium (Appendix 4), incubated at 37°C for 1 h and plated at suitable dilutions onto selective LB plates.

2.4.6. Screening for transformants

Transformants were usually screened by bacterial colony PCR (Section 2.7.7) with primers flanking the cloning site and primers within the insertion fragment to check the presence and orientation of the insert in the plasmid. The recombinant plasmids were also checked by restriction endonuclease digestion (Section 2.4.2).

2.5. DNA sequencing, sequence assembly and Bioinformatic analysis

2.5.1. DNA sequencing and sequence assembly

DNA sequencing was carried out by the DNA Analysis Service in the Allan Wilson Centre. DNA was sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), using Big-Dye chemistry (PE Applied Biosystems, Foster City, CA) and oligonucleotide primers synthesised by Invitrogen. The products were separated on an ABI Prism 377 sequencer (Perkin-Elmer) for detection. Sequences were assembled into contigs using Sequencher™ Version 3.1.1 (Gene Codes Corporation).

2.5.2. Bioinformatic analysis

Sequence data were analysed using the Wisconsin Package version 9.1 (Genetics Computer Group (GCG); Madison, WI). Homologues in the GenBank database were searched with the BLAST and FASTA programmes (Altschul *et al* 1997) through NETSCAPE NAVIGATOR version 4.7 at the National Centre for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov). Polypeptide sequences were aligned with the PILEUP

programme from the GCG package or CLUSTALW (http://www.ebi.ac.uk/clustalw). Conserved amino acids were shadowed using the SeqV1.1 program.

Motif searches, PI/MW calculations and predicted cellular location were made using PROSITE (http://www.expasy.ch/prosite). The promoter sequence was analysed using the TRANSFAC database (http://www.cbrc.jp/research/db/TFSEACH.html).

2.6. Southern blotting and hybridisation with Digoxigenin (DIG) labelled probe

2.6.1. Southern blotting

The blotting method used was based on that of Southern (Southern, 1975). DNA was separated by overnight gel electrophoresis and the ethidium bromide stained gel photographed. The gel was gently agitated in solution 1 (Appendix 4.5) for 15 min, solution 2 (Appendix 4.5) for 30 min, and solution 3 (Appendix 4.5) for 15 min, then finally washed in 2 x SSC (Appendix 4.5) for 2 min.

Two sheets of 3MM Whatman paper soaked in 20 x SSC were used as wicks. A film of gladwrap was used to cover the paper, and a rectangle was cut slightly smaller than the gel. The agarose gel with DNA was placed over the paper and overlapped the edge of the hole. A piece of positively charged Nylon membrane (Boehringer Mannhein GmbH) was cut slightly larger than the gel and placed on top of the gel. Anther two pieces of 3 MM paper were soaked in 2 x SSC, and placed on the membrane, followed by a stack of paper towels and a weight. After overnight transfer of DNA, the membrane was washed in 2 x SSC for 5 min and cross-linked by UV-ray (12,000 μjoules/cm^2).

2.6.2. Hybridisation using Digoxigenin (DIG) labelled probe

The membrane filters were hybridised using DIG-labelled probe according to the manufacturer's instructions (Boehringer Mannheim).

The DNA probe was labelled with digoxigenin using polymerase chain amplification (PCR). The PCR reaction (50 µl in 0.2 ml tube) consisted of 1x *Taq* DNA polymerase buffer (Roche), 250 µM each of dATP, dCTP and dGTP, 167 µM of dTTP, 100 µM of DIG-11-dUTP, 200 nM of each primer, 0.02 unit/µl of *Taq* DNA polymerase (Roche), and 0.5 ng/µl fungal genomic DNA or 0.4 to 1 ng/µl plasmid DNA as template. The amplification was performed using a program of 1 cycle of 5 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 50-60°C (depending on the Tm of the primers) and an extension of 1-2 min (depending on the size of the product) at 72°C, and 1 cycle of 5 min at 72°C. The DIG-labelled DNA showed a band of higher molecular weight than the non-labelled DNA on a gel. The efficiency of labelling of the probe was determined by autoradiography by comparing serial dilutions of DIG-labelled sample with standards spotted on a positively charged Nylon membrane.

The membranes were pre-hybridised in DIG standard hybridisation buffer (Appendix 4.5) at 39-42°C for 2 h. The probe was denatured by boiling for 10 min and placed on ice, then added to the hybridisation buffer at a concentration corresponding to 3,000 to 10,000 fold dilution of the PCR product, depending on the efficiency of the probe labelling. Hybridisations were performed at 39-42°C for 16 to 24 h. After hybridisation, the membranes were washed twice in 2 x SSC and 0.1% SDS for 5 min, twice in 0.5 x SSC and 0.1 % SDS at 68°C for 15 min, and signals detected by chemiluminescent detection as described in the following section.

2.6.3. Chemiluminescent detection of DIG-labelled probes

Chemiluminescent detection was carried out at room temperature with the membrane kept wet all time. The membrane was washed in DIG washing buffer (Appendix 4.5) for one min, then soaked in blocking buffer (Appendix 4.5) for 30 min. Anti-Digoxigenin–AP antibody (Boehringer Mannheim GmbH) was diluted 1:20,000 into the blocking buffer and incubated with the membrane for 30 min. The membrane was washed twice in washing buffer (Appendix 4.5) for 15 min, equilibrated in detection buffer (Appendix 4.5) for 2 min, drained and transferred to a plastic bag with the DNA face up. One millilitre of 1:200 diluted CDP-star (Boehringer Mannheim GmbH), in detection buffer (Appendix 4.5), was pipetted over the membrane. The bag was closed and bubbles on the membrane were removed. The membrane was then exposed to X-ray film (Fuji Photo Film Co.) for 5 to 30 min and the film developed.

2.7. Polymerase chain reaction (PCR) amplification

PCR reactions were carried out in a FTS-960 thermal sequencer, PC-960G gradient thermal cycler, a PC-960 thermal cycler (Corbett Research, Mortlake, Australia) or a GenAmp 2400 (Perkin-Elmer Corp.) model thermocycler. To optimise PCR reaction conditions, annealing temperatures were varied depending on the Tm of the primers. Extension times of 1 min/kb were used. Sometimes, Mg^{2+} concentration, primer and DNA template concentration were adjusted. PCR products were analysed by gel electrophoresis and visualised as in section 2.2.10.

2.7.1. Oligonucleotide primers

The primers were ordered from Gibco BRL, Sigma Corporation or Life Technologies. Each primer was re-suspended in water to a final concentration of 100 μM and stored at -20°C. The primers were diluted to a working stock concentration of 5 μM for PCR reactions and 3.2 μM for sequencing reactions. Table 2.2 lists the primers used in this study.

Table 2.2 Oligonucleotide primers used in this study

Names	Sequences (5'-3')	Applications
XZ 1	CAR TCI TGC ATG GAC CCI AA	Degenerate PCR, RT-PCR
XZ 3	CAT IGC ACC AAA IGT IGG ACC	Degenerate PCR
XZ5	AAG TAT CGG CGA GTC ATG	5'RACE, cloning and sequencing
XZ6	ATT GGA TGT CTG GAC TCG	Cloning and sequencing
XZ7	GCA ATC GCT ATC CAT ACC	Cloning and sequencing
XZ8	GAT GCA TAG AGA CCA AGG	5'RACE and cloning
XZ9	GTG TGC TAG GAT TGC TTC	Cloning and thi1 sequencing
XZ10	AGT TGA AGT CAG GTC TGG	Cloning
XZ11	TAC GAG GAT GAG GGT AAC	Cloning, 5'RACE
XZ12	GAC CAT GCA TCT AAC ACG	Cloning
XZ13	CCT GAC CTG AAG ATT TGC	Sequencing
XZ14	TCT TTC TGA CTG ACT GCC	Cloning
XZ15	GAA GCA ATC CTA GCA CAC	Sequencing
XZ16	TCC AGA CCT GAC TTC AAC	TAIL-PCR
XZ17	ATN AGN TCC CAA GCT T	TAIL-PCR
XZ18	ACT TGG AGG TTT GAA TCG	5'RACE
XZ19	CAG CAT ATC GAA AGC TTG C	TAIL-PCR
XZ20	ACG TGT GAC AAC TGG TTG AG	TAIL-PCR, Reverse PCR
XZ21	GAG GAC TTG ACG TTA TCC TC	TAIL-PCR
XZ22	CGT TGG TAC TGA TGA CAA TG	TAIL-PCR
XZ23	CAG CAA CCT CTC GAA TTG TC	TAIL-PCR
XZ24	GTC TTG ACA AAC CTC TCG	TAIL-PCR
XZ25	CCA TTG CAC CGA ATG TTG	RT-PCR
XZ26	TAC TCC AAT CCC GTC TTG	RT-PCR

XZ27	CAT CCA ATT TTC AAA CCG	TAIL-PCR
XZ28	TTG ACT CTC ACG AAT GGG	RT-PCR, 5'RACE
XZ29	TGA TCA CAA CTC TTC AGG	TAIL-PCR
XZ30	CAA CAT TCG GTG CAA TGG	TAIL-PCR
XZ31	GAT GAA GAT GTC GAC GTG	TAIL-PCR, Reverse PCR
XZ32	TTC TCG CTA TCT CGC ACC	Sequencing
XZ34	TGT GTT GCA ACG CTG GAA TC	Reverse PCR
XZ35	CGC CAA CGC ATC ATG CAT A	TAIL-PCR and sequencing
XZ36	TTT CAA GGA AGC ACA GAG	Reverse PCR
XZ40	TTG CAC CGA ATG TTG GAC	Cloning and sequencing
XZ41	CTT AGG AGC TTA CCT CTC	Cloning and sequencing
XZ42	GGT CTA GCT ATT AAA GTG C	Cloning
XZ43	GAA TCA AGC AAG CAT GGC	Sequencing
XZ44	GGA TTG TCG AAG TCG GAC	Sequencing
XZ45KpnI	GGG GTA ACC GGT GCA ATT GCA AAT CTC	Replacement vector construct
XZ46BamHI	CGG GAT CCC ATG CAG CAA ACA TTA CC	Replacement vector construct
XZ47HindIII	CCC AAG CTT TTG CGT GAA GCG TCT CG	Replacement vector construct
XZ48HindIII	CCC AAG CTT AGG GTA ACT ATC TTG TGC	Replacement vector construct
XZ49	GAG GTT TAG ATA AGG CAC	PCR screening for KO
XZ50	CGG GAA TTC GAT TCA TCG	PCR screening for KO
XZ51	AGC TAT TAC ACT CCC TAG	Sequencing
XZ54	GAG ATA GCG AGA AGT ACA TC	TAIL-PCR
XZ55	CTG CCA TGC TTG CTG ATT C	TAIL-PCR
XZ56	TGT ACT TCT CGC TAT CTC G	TAIL-PCR
XZ57	TGT AGG AAG CGA GAA CTC	PCR screening for KO
XZ58	GTC TGG TCC GAG GTT TTC	PCR screening for KO
XZ59	CTT CTG TTT CTC CTG GTG	PCR for probe
XZ60	GTC CAT GCA AGA CTG GTC	PCR for probe
XZ61	CCT ACT TGT CTA CTA GCA C	Sequencing
XZ62	CCT TAG ACT AGT AGC TTC C	Sequencing
AD1	TG(A/T) GNA G(A/T)A NCA (G/C)AG A	TAIL-PCR
AD2	AG(A/T) GNA G(A/T)A NCA (A/T)AG G	TAIL-PCR
AD3	CA(A/T) CGI CNG AIA (G/C)GA A	TAIL-PCR
AD4	TC(G/C) TIC GNA CIT (A/T)GG A	TAIL-PCR
M13F	GCC AGG GTT TTC CCA GTC ACG A	Sequencing
M13R	GAG CGG ATA ACA ATT TCA CAC AGG	Sequencing
T1.1	GAG AAA ATG CGT GAG ATT GT	RT-PCR
T1.2	TGG TCA ACC AGC TCA GCA CC	RT-PCR

hmg29	GAC GTG GTC AAA TCC GTG TTG	RT-PCR
hmg30	GTT GGA TGT GAT CCT CGC AC	RT-PCR
T7	TAA TAC GAC TCA CTA TAG GG	Sequencing
pUChph7	ATT CCT GCA GAG ACC ATC	PCR screening for KO
pUChph9	CTC CAC GCG ACT ATA TAT	PCR screening for KO
CYLp19-1	CAC CAT TTC GAG GTA GTC	Microsatellite PCR
CYLp19-6	CAT GAG GAA TCC CAC GAC	Microsatellite PCR
CYLp19-13	AAA GAT GTT GTG GGC GAC	Sequencing
UP	GAG AGA ATT CGG ATC CTC TAG AG	5' RACE
AAP	GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG	5'RACE
AUAP	GGC CAC GCG TCG ACT AGT AC	5'RACE

2.7.2. Routine PCR

Routine PCR reactions (25 μl in 0.2 ml tube) consisted of 1x *Taq* DNA polymerase buffer (Roche), 50 μM of each dNTP, 200 nM of each primer, 0.02 unit/μl of *Taq* DNA polymerase (Roche) or 0.04 unit/μl of Expand^{TM} High Fidelity polymerase (Roche). A concentraion of 0.6-1 ng/μl fungal genomic DNA or 0.4 to 1 ng/μl plasmid DNA was used as the template in the amplification. The thermocycle conditions were 1 cycle of 2 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 50–60°C (depending on the Tm of the primers) and 1-2 min (1 min/1 kb) at 72°C, and 1 cycle of 5 min at 72°C.

2.7.3. Degenerate PCR

Degenerate PCR was performed using the reaction and PCR cycle conditions described for routine PCR except that a higher primer concentration of 400 nM of each primer was used.

2.7.4. Thermal Asymmetric Interlaced PCR (TAIL-PCR)

TAIL-PCR was carried out based on the method of Liu (Liu and Whittier, 1995). Three nested sequence specific primers together with a shorter arbitrary degenerate (AD) primer were used in the TAIL-PCR to favour amplification of the desired specific product under controlled annealing temperatures. The TAIL-PCR consists of three consecutive PCR amplifications (primary, secondary and tertiary amplifications), which are summarised in Table 2.3.

The primary amplification was performed in a GenAmp 2400 (Perkin-Elmer Corp.) model thermocycler. The primary #2 procedure was run immediately after the #1 procedure. The secondary and tertiary amplifications were performed in either a GenAmp 2400 (Perkin-Elmer Corp.) model thermocycler or a PC-960G gradient thermal cycler.

The primary reaction (20 μl) contained 1 x *Taq* DNA polymerase buffer (Roche), 188 nM of each dNTP, 125 nM of gene specific primer, 125 nM to 2500 nM of AD primers (usually 500 to 2500 nM of AD3 or AD4 primers were suitable), 0.05 units/μl of *Taq* DNA polymerase (Roche), and 0.5 ng/μl of E8 genomic DNA as template.

The secondary reaction (20 μl) contained 1 x *Taq* DNA polymerase buffer (Roche), 125 nM of each dNTP, 125 nM of gene specific primer, 1250 nM of AD primer, 0.05 unit/μl of *Taq* DNA polymerase (Roche), and 2 μl of 1/100 diluted primary TAIL-PCR product as template.

The tertiary reaction (20 μl) contained 1 x *Taq* DNA polymerase buffer (Roche), 62.5 nM of each dNTP, 125 nM of both gene specific primer and AD primer, 0.05 unit/μl of *Taq* DNA polymerase (Roche), and 2 μl of 1/100 diluted secondary TAIL-PCR product as template.

Table 2.3. Cycling conditions used for TAIL PCR in this study

Amplifications	Thermal conditions	Cycle number.
Primary 1#	92°C (2 min), 95°C (1 min)	1
	94°C (20 sec), 65°C (1 min), 72°C (2 min)	10
	94°C (20 sec), 30°C (3 min), 72°C (3 min)	1
	Ramping slowly	
Primary 2#	94°C (10 sec), 50°C (1 min), 72°C (2 min)	6
	94°C (10 sec), 65°C (1 min), 72°C (2 min)	
	94°C (10 sec), 65°C (1 min), 72°C (2 min)	12
	94°C (10 sec), 50°C (1 min), 72°C (2 min)	
	72°C (5 min)	1
	4°C on	
Secondary	94°C (2 min)	1
	94°C (10 sec), 65°C (1 min), 72°C (2 min)	
	94°C (10 sec), 65°C (1 min), 72°C (2 min)	12
	94°C (10 sec), 50°C (1 min), 72°C (2 min)	
	72°C (5 min)	1
	4°C or 25°C on	
Tertiary	94°C (2 min)	1
	94°C (15 sec), 51°C (1 min), 72°C (2 min)	30
	72°C (5 min)	1
	4°C on	

2.7.5. Inverse PCR

Inverse PCR was applied to clone the AT rich region of E8 *thi11* locus using inverse primers based on the known sequence. A total of 500 ng of E8 genomic DNA was digested for 3 h at 37°C in a volume of 25 µl that contained 10 units of restriction enzyme *Hin*dIII, 20 µg RNase, and 1 x of restriction buffer. An aliquot of 10 µl of digestion mixture was self-ligated at 4°C overnight in 1 x T4 ligase buffer with 400 units of ligase in a total volume of

400 µl. The ligation mixture was precipitated with ethanol (Section 2.2.9.3), re-suspended in 100 µl water, and an aliquot of 30 µl of the DNA suspension was used as template for amplification in 50 µl using the reaction and PCR cycle conditions described for the routine PCR.

2.7.6. Long template PCR

Long template PCR reactions (50 µl in 0.2 ml tube) contained 1 x *Taq* DNA polymerase buffer (Roche), 350 µM of each dNTP, 150 nM of each primer, 0.525 unit/µl of Expand™ Long Template polymerase (Roche). A concentration of 0.1 – 0.5 ng/µl fungal genomic DNA or 0.05-0.5 ng/µl plasmid DNA was used as templates in the amplification. The thermocycle conditions were 1 cycle of 2 min at 93°C, 10 cycles of 10 sec at 93°C, 30 sec at 60°C and 4 min at 68°C, followed by 17 cycles of 10 sec at 93°C, 30 sec at 60°C and 4 min at 68°C with the extension time for each subsequent cycle increasing by 20 s, and 1 cycle of 7 min at 68°C.

2.7.7. Bacterial colony PCR

Bacterial colony PCR was used for identifying bacterial transformants using bacteria cells from the colony as template. A sterilised P10 pipette tip or toothpick was used to lightly touch the colony and the cells then suspended in the reaction mixture used for a routine PCR. The thermocycle conditions were 1 cycle of 3 min at 95°C, 35 cycles of 30 sec at 95°C, 30 sec at 60°C (depending on Tm of primers) and 1 to 2 min at 72°C, and 1 cycle of 5 min at 72°C.

2.7.8. Gel stab PCR

Gel stab PCR was used to amplify DNA fragments from agarose gels. The DNA band to be amplified was stained with ethidium bromide and visualised under UV light. A 20-200 µl pipette tip was used to stab the DNA band in the gel and re-suspended in 20 µl of sterilised water by pipetting. An aliquot of 5 µl of the suspension was used as template in a PCR reaction (25 µl) using reaction and PCR cycle conditions as described for the routine PCR (Section 2.7.2).

2.8. RT-PCR (reverse transcription PCR)

2.8.1. Isolation of total RNA with TRIzol® reagent

Fungal mycelia or plant tissue (about 1 g fresh weight) were ground to a fine powder in liquid nitrogen, and mixed with 10 ml of Trizol (BRL). After thawing, the mixture was centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was mixed vigorously with 2 ml chloroform, kept at room temperature for 3 min, then centrifuged at 12,000 x g for 15 min at 4°C. The RNA in the aqueous phase was precipitated by adding 5 ml isopropanol and recovered by centrifuging at 12,000 x g for 10 min at 4°C. The RNA pellet was washed with 10 ml of 75 % ethanol, air-dried, and re-suspended in 200 µl DEPC-treated water.

2.8.2. Quantification of RNA

RNA was quantified by measuring the absorbance at 260 and 280 nm using a Shimadzu UV spectrophotometer. Samples were usually diluted so that the absorbance reading was between 0.1 to 1.0. Good quality RNA samples should have a ratio of A260/A280 greater

than 1.8. The concentration of RNA was calculated by the following equation: [RNA] (μg/ml) = 40 x A260 x dilution factor.

2.8.3. DNase I treatment of RNA

RNA samples were treated with DNase I to remove residual DNA using the method described by Bradshaw & Piller (1992). Total RNA (1 μg) was incubated with 1 unit DNase I (Roche), 1 unit RNase inhibitor (Roche), 1 mM 1,4-dithiothreitol (DTT) (Roche) in 1 x DNase I buffer (Appendix 4.6) at 37°C for 30 min, then another 0.5 units DNase I was added and the mixture incubated for a further hour. The mixture was extracted with 2 volumes of chloroform and centrifuged at 13,000 x g for 10 min. The RNA was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of 95% (v/v) ethanol, and recovered by centrifuging at 13,000 x g for 10 min. The pellet was washed in 75% ethanol, air dried and re-suspended in DEPC-treated water and kept at -80°C.

2.8.4. Reverse transcription of RNA into cDNA and amplification

The RNA was reverse transcribed into cDNA using random hexamer primers and Expand Reverse transcriptase kits (Roche) based on the manufacture's instructions An aliquot of 1 μl of random hexamer primers (0.09 OD unit/μl) was mixed with DNase I treated RNA (1 μg), incubated at 90°C for 5 min, then cooled on ice immediately. Then a 'cocktail' of reagents was prepared and added to the RNA so that the final reaction mixture (20 μl) contained 1 x Expand RT buffer, 10 mM DTT, 50 μM dNTPs, 1 unit/μl RNase inhibitor and 2.5 units/μl Expand Reverse Transcriptase. The reaction was incubated at room temperature for 10 min and then reverse transcribed into cDNA at 45°C for 45 min.

An aliquot (5 µl) of each cDNA dilution (1/10, 1/100 and 1/1000) was PCR amplified as previously described (Section 2.7.2) in a reaction mixture of 25 µl using primers flanking the introns. The thermocycle conditions were 1 cycle of 2 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 60°C (depending on Tm of primers) and 1 min at 72°C, and 1 cycle of 5 min at 72°C.

In order to confirm removal of genomic DNA following DNase treatment, a duplicated reaction without addition of Reverse Transcriptase was set up, and PCR amplification was carried out as no-RT control. Absence of PCR product from the no-RT control was an indication of absence of genomic DNA.

2.9. RACE (rapid amplification of cDNA ends)

2.9.1. 3' RACE analysis

3' RACE was used to determine the 3'end of the mRNA. The method involved first strand cDNA synthesis, degradation of the RNA, synthesis of the second cDNA strand and nested amplification.

The first strand was synthesised from DNase I treated RNA using the method as described in section 2.8.4, except that oligo-dT primer and *C. therm* reverse transcriptase (Roche) were used instead of the random hexamer primer and Expand RT reverse transcriptase. DNase I treated RNA (1 µg) was mixed with 8 pmol of oligo-dT primers (BRL), incubated at 70°C for 10 min and then cooled on ice. A 'cocktail' of reagents was prepared and added to the RNA so that the final reaction mixture (20 µl) contained the reagents at final concentrations of 1 x *C. therm* Reverse Transcriptase buffer, 5 mM DTT, 800 µM dNTPs, 3 % (w/v) dimethyl sulfoxide (DMSO), 1 unit/µl RNAse inhibitor and 0.3 units/µl of *C.*

therm reverse transcriptase. The reaction mixture was incubated at 60°C for 30 min for reverse transcription, then heated at 95°C for 2 min to inactivate the reverse transcriptase.

To degrade the RNA, 1 µl of RNase mixture (Roche) containing both RNase H and RNase A, was added to the above reverse transcription reaction mixture, and incubated at 37°C for 30 min. The digestion mixture was purified using a CONCERT™ PCR clean-up (BRL) kit (Section 2.2.9) and the cDNA was re-suspended in 50 µl water.

An aliquot of 5 µl of the above-purified cDNA was used as template for the second cDNA strand amplification using a gene specific primer and an adapter UP primer that anneals to the oligo-dT region. This was followed by a nested amplification using UP primer and a nested gene specific primer to favour synthesis of the specific product. Both amplifications were performed using the reaction and PCR cycle conditions previously described (Section 2.7.2) except that the UP primer was added to a final concentration of 800 nM. The final PCR products were sub-cloned into pGEM-T Easy vector (Section 2.4) for sequencing.

2.9.2. 5' RACE analysis

5' RACE was used to determine the 5'end of the mRNA. The main difference between the 5'RACE and 3'RACE is that an extra step is required to add a poly-dC tail to the 5'end of cDNA so that an abridged adapter primer (AAP) can bind to it.

The first cDNA strand was synthesised as described above for 3'RACE in Section 2.9.1 except that a gene specific primer (at a final concentration of 1 µM) was used instead of the oligo-dT primer. RNA was degraded and the cDNA purified in the same way as described for that of 3'RACE.

To add a poly-dCTP tail to the 5' end of the first cDNA strand, an aliquot of 10 µl of purified cDNA was added to a reaction mixture (25 µl) containing 1 x Tailing buffer, 0.2 mM of dCTP and 0.6 units/µl of terminal transferase. The reaction was incubated at 37°C for 10 min and then heated at 65°C for 10 min to inactivate the enzyme.

The secondary cDNA strand was synthesised by amplification using a nested gene specific primer and an AAP primer that annealed to the poly-dC tail. Following that, a nested amplification was performed using primer AUAP and a secondary nested gene specific primer. Both amplifications were performed using the reaction and PCR cycle conditions previously described (Section 2.7.2). The final PCR products were sub-cloned into pGEM-T Easy vector (Section 2.4.4-2.4.6) for sequencing.

2.10. Endophyte transformation

2.10.1. Protoplast preparation

Protoplasts of endophyte strain E8 (PN2238) were prepared using modifications of the method described by Yelton *et al* (Yelton *et al.*, 1984). Mycelia collected by scraping the colony surface were ground into a fine suspension and inoculated in 200 ml of CM broth distributed into 4 flasks (125 ml) and grown at 22°C with gentle shaking at 100 rpm until clouds of mycelia formed (3-6 days). The mycelia were harvested by filtration through two layers of nappy liner on a funnel using gravity flow. The mycelia were washed with 300 ml H_2O then 100 ml OM buffer (Appendix 4.4). The mycelia were re-suspended in 20 ml of Glucanex cell wall lysis enzyme solution (Appendix 4.4), and cell walls digested by shaking at 100 rpm for 2-4 h at 28°C. During that time, the mixture was monitored for protoplast formation using microscopy. The digested hyphae were filtered through nappy liner and each of the 5 ml filtrates overlaid carefully with 2 ml of ST buffer (Appendix 4.4). The

protoplasts formed a white band at the interface between the enzyme solution and ST buffer after centrifugation at 3000 x g for 20 min. The protoplasts were recovered from the interface, washed 3 times with 5 ml of STC buffer with gentle shaking and centrifuging at 7,700 x g for 5 min. Protoplasts were re-suspended in STC buffer (Appendix 4.4) to a final concentration of 1.25 x 10^8 per ml.

2.10.2. Endophyte transformation

Protoplasts prepared as described above were transformed with the replacement DNA fragment by modifications of the method of Vollmer and Yanofsky (Vollmer and Yanofsky, 1986), and Young *et al.* (Young *et al.*, 1998). Each transformation used 80 µl protoplasts (1.25 x 10^8 protoplast/ml), 5 µl heparin (5 mg/ml), 2 µl spermidine (50 mM), 20 µl 40% PEG solution (Appendix 4.4) and 1.7-5 µg of DNA. The mixture was vortexed gently and left on ice for 30 min, then a further 900 µl of 40% PEG solution was added and mixed gently. The transformation mixture was incubated at room temperature for 15-20 min. An aliquot of 100 µl transformation mixture was mixed with 3 ml molten CM top agar (50°C) (Appendix 4.1) and spread onto a CM plate. The plates were incubated at 22°C overnight then overlaid as described above with hygromycin B (Boehringer) to a final concentration of 150 mg/L. Alternatively, the transformation mixture of 1 ml was incubated in 20 ml CM broth at 22°C for three days and then spread onto CM plate with 150 mg/L hygromycin B. In each transformation experiment, a sample of protoplasts without DNA was processed as a negative control. Aliquots (100 µl) of 1/100, 1/1000 and 1/10000 dilutions of the negative control protoplasts were grown on CM plates to determine the number of viable protoplasts after transformation, and an aliquot (100 µl) of the negative control protoplasts was grown on CM plates containing 150 mg/L hygromycin B to confirm sensitivity to hygromycin.

2.10.3. Single spore isolation

A drop of PD broth (50 µl) was added onto the colony margin, sucked up and down with a 200 µl pipetter several times, then spread onto a 3% PDA plate. The spores on the plate were incubated at 22°C for 24 h to germinate. Germinating spores that had one or two germ tubes extending from the spores were identified under a dissecting microscope and picked up and inoculated onto PDA plates containing hygromycin B (Boehringer) at a final concentration of 150 mg/L for further growth.

2.11. Examination of growth characteristics of E8 *thi1* deletion mutants in culture

2.11.1. Measurement of colony diameter

A block of mycelium (3 x 3 mm) was cut from the colony edge and placed in the center of a CD plate or CD plate supplemented with thiamine or thiazole for growth. At least two plates were prepared from each isolate and the plates were incubated at 22°C for three to five weeks depending on the growth rate of the endophyte. The diameter of each colony was measured along two axes intersecting at right angles. The average of these two measurements was taken as the diameter of the colony on each plate.

2.11.2. Examination of hyphal branching and morphology

Hyphal branching and morphology were observed using a stereo dissecting microscope (Leica MZ12) with a transmitted light beam passing upwards through the colony on

culture. The images were recorded using a JVC3-CCD Camera and an InDy silicon Graphics computer connected to the dissecting microscope.

2.11.3. Examination of conidia formation

Conidiospores were observed using a stereo dissecting microscope (Leica MZ12) with a transmitted light beam passing upwards through the colony. The conidia can usually be observed at the margins of a colony where they are attached to the tips of aerial hyphal branches. The length of the conidia were about 4 μm. The images were recorded using a JVC3-CCD Camera and an InDy silicon Graphics computer connecting to the dissecting microscope.

2.12. Examination of E8 *thi1* deletion mutants in the endophyte-plant interaction

2.12.1. Inoculation of endophyte into perennial ryegrass seedlings

Seedling inoculation was carried out by the method of Latch and Christensen (Latch and Christensen, 1985). The grass cultivar used was 'Nui' perennial ryegrass which were obtained from AgResearch Grasslands, Palmerston North, New Zealand. The seeds had been confirmed, by destructive examination of at least 50 seeds, to be free of fungal endophytes. Seeds were surface sterilised by soaking in 50% sulphuric acid for 30 min, rinsed with water three times, soaked in 50% commercial bleach for 20 min and rinsed in sterile water. The treated seeds were air dried then placed on 4% water agar for 1 week at 22°C to germinate. A slit of 2-3 mm was made longitudinally in the region between the

mesocotyl and coleoptile of each seedling using a scalpel under a dissecting microscope. Into the resulting slit a small piece of mycelia cut out from a colony edge was inserted. The inoculated seedlings were incubated at 22°C for 5-7 days in the dark, then 5-7 days under light and then transferred into potting mix (AgResearch Grasslands, Palmerston North) in root-trainers. For potting, the plants were transplanted into pots or bags containing potting mix into which Osmacote slow-release fertiliser was supplemented. The plants were randomly located in trays in the glasshouse (AgResearch Grasslands, Palmerston North) for growth and watered 2-3 times per week. The location of the plant was rotated regularly to minimise environment effects. Plants were maintained by regularly trimming several centimeters above the base. For measurement of fresh weight, the plants were cut at about 1 cm above the base.

2.12.2. Inoculation and treatment of the clonal plantlets

Perennial ryegrass clonal plantlets (IMP566) grown in MSO medium (Appendix 4.1) were obtained from AgResearch Grasslands. The plantlets were separated into individual tillers and with the mature leaf blade trimmed. A longitudinal slit was cut at the junction of the root and shoot using a scalpel. Into the resulting slit, a small piece of fungal mycelia cut from the endophyte colony was inserted. The inoculated plantlets were planted into MSO media (Appendix 4) for five days, then in transferred to MSO0.5 media (Appendix 4). After 13 days growth in the culture room at 25°C and 16 h of light, the plants were transferred into the greenhouse in which the plants were transplanted into pit pots and covered with a plastic container to maintain a high humidity for 11 days. These plantlets were then planted into potting mix into which Osmacote slow-release fertiliser was added and maintained as described above (Section 2.12.1).

2.12.3. Detection of endophyte in grass by tissue-print immuno blot

Tissue-print immunoblot was carried out using a modified method based on that of Gwinn 1991 (Gwinn *et al.*, 1991). The antibodies (AgResearch Grasslands, Palmerston North) were raised from New Zealand White rabbits that were injected with antigen fraction of *N. lolii* strain Lp5 (Christensen *et al.*, 1993). Grass tillers were cut transversely at the base and dotted onto a nitrocellulose membrane (NCM) for several seconds. The membrane was washed in BS solution (Appendix 4.7) at room temperature for 2 h, then transferred to 25 ml fresh BS solution with a dilution of antibody (1:1000) and incubated overnight at 4°C with gentle shaking. The membrane was rinsed in block solution (Appendix 4.7) 5 times, then submerged in BS solution (25 ml/membrane) with secondary antibody (1:2000) for 2 h at room temperature with shaking. After a final rinse with BS solution, the membrane was developed in freshly made Fast Red solution (Appendix 4.7) for about 15 min at room temperature with shaking. The prints of endophyte-infected tissues were deep red while the prints of the endophyte free tissues were brown or very light in colour. The membrane was then rinsed in water to stop the reaction and then air dried.

2.12.4. Examination of endophyte in grass tissue by aniline blue staining

Strips of epidermis were removed from the adaxial (upper) surface of the leaf sheath close to the base. The strips were mounted in a drop of aniline blue solution (Appendix 4.7) on a slide. The slide with the sample was heated over a flame until boiling to fix the stain. Hyphae within epidermal strips were examined using a compound microscope.

2.12.5. Re-isolation of endophyte from plant tissues

The endophytes were re-isolated from plant tissues using the method described in Christensen, Latch and Tapper (Christensen *et al.*, 1991). Plant tissue was surface sterilised by washing 1 min in 95% ethanol, 1 min in 10% bleach (v/v) (Janola), and finally submerged in sterile water. Transverse sections of about 2 mm in length were excised from the leaf sheath and blade and placed on PDA medium with 5 mg/L oxytetracycline and incubated at 22°C.

2.12.6. Extraction and assay of peramine in the symbiotic plants

Peramine levels were determined by the Chemical Analysis Service at AgResearch, Palmerston North, New Zealand using a modified method of Panaccione *et al* (2003) and Spiering *et al* (2002). Freeze dried pseudostem tissue of the plant was milled into a fine powder for extraction. Each sample (20 to 50 mg) of pseudostem powder was extracted with 1 ml of 50% isopropanol:water (w/v) and 1% lactic acid by shaking vigorously for 22 sec at speed 5 in a FastPrep FR120 machine, and then rotated with gentle agitation for 1 h at ambient temperature in darkness. The mixture was centrifuged at 450 rpm for 10 min in a Heraeus sepaTECH centrifuge and 0.5 ml of the supernatant was recovered. A standard amount of homoperamine nitrate (obtained from AgResearch Grasslands) was added into the supernatant as an internal standard and the supernatant in HPLC vials was put into HPLC rack for quantitation of peramine.

An aliquot of the extract (up to 50 µl) was injected into a solvent stream of isopropanol:water:ammonium hydroxide (25%); (60:40:1 v/v) pumped at a flow rate of 0.4 ml/min (M-6000A pump, Waters Associates, Milford, MA). The mixture was pumped onto a silica-based mixed-mode cation exchanger cartridge (RP-C8/cation guard column, 7.5 x

4.6 mm, 5 μm, Alltech Associates, Deefield, IL) for a two minute period. Peramine and homoperamine were retained in the cartridge while other compounds were washed away to waste. For separating peramine from homoperamine, the cartridge was washed with a solvent of 50 mM ammonium acetate, 5 mM guanidinium carbonate, 0.2% (v/v) acetic acid in water:methanol (4:1 v/v) at speed of 1 ml/min using a Perkin Elmer LC Pump 250 into a silica HPLC column (250 x 4.6 mm, Phenosphere 5 μm, Phenomenex, Torrance, CA) at 28°C. Peramine and homoperamine peaks were detected at 286 nm UV absorption (UV-970 detector, Jasco Corporation, Tokyo).

2.13. Statistical analysis

Statistical data analysis was carried out by the aid of programmes provided by Statistical Analysis System (SAS Institute Inc. 1987). Multiple-means comparisons were carried out with ANOVA (analysis of variance, $p = 0.05$) or the Tukey test (Tukey's test $\alpha = 0.05$). Two-means comparison was carried out with the t test. Comparison of infection frequencies and host survival frequencies were analysed by normal test for proportions.

2.14. Light and transmission electron microscopy

Both light and transmission electron microscopy were carried out in the Keith Williamson Electron Microscope Unit, Hort + Research, Palmerston North, New Zealand. Leaf blade, stem or pseudostem segments of about 1-2 mm long were excised from the tiller and fixed in 3% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer pH 7.2 for 2 h at room temperature, and then in 1% osmium tetroxide in 0.1 M phosphate buffer pH 7.2 for 0.5 h at room temperature. The tissues were washed 3 times in 0.1 M phosphate buffer pH 7.2, dehydrated in an acetone/water series and twice in 100% acetone, infiltrated with an acetone/polarbed 812 resin mixture (50/50, v/v), and then embedded in fresh resin mixture in silicone rubber moulds and cured for 48 h at 60°C. Transverse sections of about 1 μm thick

were prepared and stained with toluidine blue for compound microscopy. Sections for TEM were cut with a diamond knife and a Reichert ultracut E microtone, and collected on copper grids. These sections were stained with saturated uranyl acetate in 50% ethanol for 4 min followed by lead citrate for 4 min and examined with a Philips 201C TEM. For specific staining for 1, 2-glycol groups of mucosubstance and polysaccharides, the sections were stained in alkaline bismuth solution (0.05% sodium hydroxide, 0.02% potassium sodium tartrate, 0.01% bismuth subnitrate) for 30 min at 40°C (Koga *et al.*, 1993).

Chapter three

Results

3.1. Cloning and bioinformatic analysis of the *thi1* gene from *N. lolii* Lp19 and *E. typhina* E8

3.1.1. Cloning and sequencing of *thi1* gene from *N. lolii* strain Lp19

The *thi1* gene from *N. lolii* strain Lp19 was cloned by screening a genomic library (Section 2.3) with a probe amplified by PCR (Section 2.7.3) using degenerate primers XZ1 and XZ3 (Fig. 3.1). These primers were designed to conserved regions QSCMDPN and GPTFGAM, of thiamine biosynthetic polypeptide sequences from other organisms (Fig. 3.1). These primers amplified a product of 330 bp from genomic DNA of *S. pombe*, *N. lolii* Lp19, *Neotyphodium sp*. Lp1 and *E. typhina* E8 (Fig. 3.2 A). The PCR product amplified from Lp19 genomic DNA was purified (Section 2.2.9.1) and sequenced. Blast X search in website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) showed that the sequence was highly homologous to yeast THI4 and other putative thiamine biosynthetic genes, thus the PCR product from Lp19 genomic DNA was used as a probe to screen a Lp19 genomic library (Section 2.3). Five λ clones (λXZ1, λXZ2, λXZ3, λXZ4 and λXZ5) out of 10^5 plaques screened were confirmed as positive clones after three rounds of screening. Clones λXZ1 and λXZ3 were used to generate a physical map to the *thi1* locus (Fig. 3.2 B) and the *thi1* gene located by probing (Section 2.6) Southern digests of these clones with the PCR product. *Sst*I and *Hind*III fragments from λXZ1 were sub-cloned (Section 2.4) into pUC118. A total of 3.6-kb of double-stranded sequence was generated (Section 2.5) using these plasmids and λ clones λXZ1 and λXZ3 as templates.

The sequence data generated was assembled (Section 2.5) using the Sequencher^{TM}4.1 software and is shown in Appendix 5.

3.1.2. Analysis of the Lp19 *thi1* gene sequence

The nucleotide and deduced polypeptide sequence for Lp19 *thi1* is shown in Fig. 3.3. The translation start site for the Lp19 *thi1* gene was identified based on a comparison of the deduced amino acid sequence with the homologous peptide sequences from other organisms by a BLAST-X search (Section 2.5.2). The sequence around the predicted start codon, CCATC**ATG**AGTC, is similar to the fungal Kozak consensus sequence (CCACCATGGC) (Gurr *et al.*, 1987). The open reading frame was predicted to be disrupted by an intron of 59 bp at the C-terminus. This was later confirmed both by RT-PCR and by analysis of the sequences of 3'RACE products (Section 3.2.1.2).

The deduced polypeptide sequence of Lp19 *thi1* is 320 amino acids and has an unmodified molecular mass of 34.4 kD. This polypeptide shares greater than 48% identity with homologous sequences from plants and fungi (Table 3.1). An alignment of the sequences shows that significant differences are only seen in the N-terminal regions where in the plant these regions show features characteristic of transit peptides for chloroplast localisation, and in yeast these regions show features of mitochondrial targeting (Fig. 3.4).

Analysis of the Thi1 polypeptide with the PSORT programme (Section 2.5.2) indicates that it is probably a cytoplasmic located protein as it lacks signal sequences for any organelles.

The TFS search (Section 2.5.2) of the Lp19 *thi1* promoter region (-1575 to –1 bp) through Netscape Navigator version 4.7 identified many putative transcription factor-binding sites (Fig. 3.3). Of particular interest are CreA, NIT2 and StuAp binding sites. CreA is a negatively acting regulator of carbon catabolite repression (Dowzer and Keller, 1989; Felenbok and Kelly, 1996). NIT2 in *Neurospora* is responsible for inducing expression of a

Figure 3.1. Design of degenerate primers for cloning *thi1* gene

A: Alignment of partial deduced polypeptide sequences of thiamine biosynthetic genes. The genes include *Z. mays thi1-1* and *Z. mays thi1*-2 ((Belanger *et al.*, 1995), accession numbers U17350 and U17351), *A. thaliana thi1* ((Machado *et al.*, 1996), accession number U17589), *F. oxysporum* and *F. solani sti35* ((Choi *et al.*, 1990), accession numbers M33643 and M33642), *S. pombe nmt2* ((Manetti *et al.*, 1994), accession number X82363) and *S. cerevisiae THI4* (*MOL1*) ((Praekelt *et al.*, 1994), accession number X61669). The conserved sequences identified for designing primers are shown in purple.

B: Degenerate primers XZ1 and XZ3 were designed based on the two conserved polypeptide sequences (in purple) identified in **A**. Inosine was incorporated at highly degenerate positions (shown in blue) and T was used at the third position of the glycine (GGT) (shown in green). R (shown in yellow) is A or G.

A


201
XZ1
Z.maysthi1-1 GG.....RV GGVVTNWALV SMNHDTQSCM DPNVMEA... .....
Z.maysthi1-2 RG.....RV GGVVTNWALV SMNHDTQSCM DPNVMEA... .....
A.thaliana GN.....RV GGVVTNWALV AQNHHTQSCM DPNVMEA... .....
F.oxysporum .PSEEG.VRI AGVVTNWTLV SMHHDDQSCM DPNTINAP.. .....
F.solani .PSKEG.VRI SGVVTNWTLV SMHHDDQSCM DPNTINAP.. .....
S.pombe .EGKDGKQRI AGVVTNWTLV SLNHGLQSCM DPNTINAH.. .....
S.cerevisiae PPTEKGEVTV AGVVTNWTLV TQAHGTQCCM DPNVIELAGY KNDGTRDLSQ
251 300
Z.maysthi1-1 ..KVVVSSCG HDGPFGATGV KRLQDIGMIS AVPGMKALDM NTAEDEIVRL
Z.maysthi1-2 ..KVVVSSCG HDGPFGATGV KRLQDIGMIS AVPGMKALDM NAAEDEIVRL
A.thaliana ..KIVVSSCG HDGPFGATGV KRLKSIGMID HVPGMKALDM NTAEDAIVRL
F.oxysporum ...LIISTTG HDGPMGAFCV KRLVSMQRIE KLGGMRGLDM NLAEDAIV.
F.solani ...LVISTTG HDAPMGAFCV KRLVSMGRIE KLGGMRGLDM NVAEDAIV..
S.pombe ...LVVSATG HDGPFGAFCV KRLASAQLVS NLHDMRPLDM NRAEDLIV..
S.cerevisiae KHGVILSTTG HDGPFGAFCA KRIVDIDQNQ KLGGMKGLDM NHAEHDVVIH
301 350
Z.maysthi1-1 ..TREVVPGM IVTGMEVAEI DGAPRMGPTF GAMMISGQKA AHLALKALGR
Z.maysthi1-2 ..TREVVPGM IVTGMEVAEI DGAPRMGPTF GAMMISGQKA AHLALKALGR
A.thaliana ..TREVVPGM IVTGMEVAEI DGAPRMGPTF GAMMISGQKA GQLALKALGL
F.oxysporum KGTREIVPGL IVGGMELSEV DGANRMGPTF GAMALSGLKA AEEALKIFDT
F.solani KGTREIVPGL IVGGMELSEV DGANRMGPTF GAMVLSGLKA AEEALKVID
S.pombe KGTREVFPGM IVGGMELSEF DGANRMGPTS VV~~~~~~~~ ~~~~~~~~~~
S.cerevisiae SGAYAGVDNM YFAGMEVAEL DGLNRMGPTF GAMALSGVHA AEQILKHFAA
XZ3

B


Q S C M D P N
5'CAATCTTGTATGGATCCTAAT3'
G C C C C C
A A
G G
A
C
5'CA TCITGCATGGACCCIAA3'
primer XZ1
G P T F G A M
5'GGTCCTACTTTTGGTGCTATG3'
C C C C C C
A A A A A
G G G G G
Degenerate nucleotide sequences
Complement
3'CCAGGITGTAAACCACGITAC5'
primer XZ3

Figure 3.2. Cloning and sequencing of the Lp19 *thi1* gene

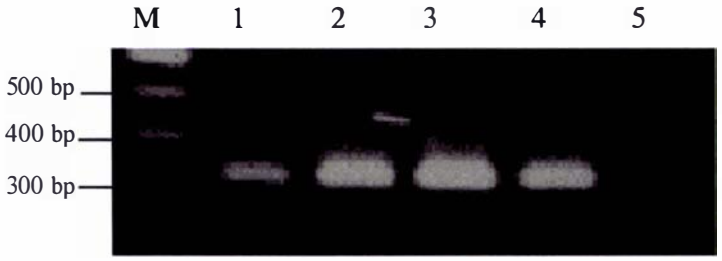
A: PCR products amplified from genomic DNA of *S. pombe* (lane 1), Lp19 (lane 2), Lp1 (lane 3) and E8 (lane 4) using the degenerate primers XZ1 & XZ3. Lane 5 was a DNA-free control. Lane M contained the 100 bp ladder (GibcoBRL).

B: Restriction map of the two overlapping λ clones λXZ1 and λXZ3.

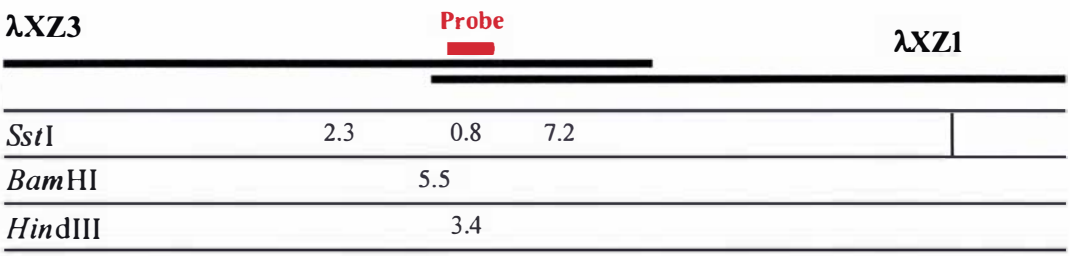
C: Primers used for sequencing *thi1*.

The black arrows indicate the position of primers used for sequencing *thi1*. The red arrows indicate the position of the degenerate primers XZ1 and XZ3 used for amplifying the probe.

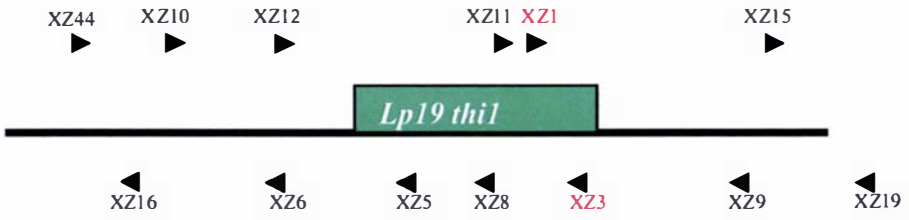
A


M
1
2
3
4
5
500 bp
400 bp
300 bp

B


λXZ3
Probe
λXZ1
SstI
2.3
0.8
7.2
BamHI
5.5
HindIII
3.4

C


XZ44
XZ10
XZ12
XZ11
XZ1
XZ15
Lp19 thi1
XZ16
XZ6
XZ5
XZ8
XZ3
XZ9
XZ19

large number of nitrogen catabolic genes (Fu and Marzluf, 1990). StuAp is an important regulator required for initiation of ascosporogenesis in sexual reproduction and mycelium morphological development of *Aspergillus nidulans* (Dutton *et al.*, 1997). A total of 10 CreA and 11 NIT2 binding sites were identified in the Lp19 promoter region, which may indicate the gene is regulated by carbon and nitrogen sources. The presence of 5 StuAp binding sites suggests that *thi1* may also be under the control of signals for morphological development.

The promoter region of *thi1* contains two motifs (region A and B) which are highly conserved among fungal thiamine biosynthesis genes (Kubodera *et al.*, 2003). These motifs are proposed to be required for efficient intron splicing and regulation of the gene expression (Kubodera *et al.*, 2003). Direct repeats of 58 bp and 52 bp were found in the promoter and 3' regions of the *thi1* respectively (Fig. 3.3). The function of these repeats is unclear.

3.1.3. Cloning and sequencing of *thi1* gene from *E. typhina* strain E8

The E8 *thi1* gene was cloned by PCR using sequence information from Lp19 *thi1*. The highly conserved coding region as well as the 5' and 3' flanking regions were cloned by PCR (Section 2.7.2) using primers designed to Lp19 *thi1* sequence. However, primers designed to Lp19 sequence outside these regions did not amplify E8 DNA. Alternative PCR approaches were therefore used to amplify these regions. TAIL-PCR (Section 2.7.4) was used to amplify regions from -1765 to –683 and +1153 to +1503. Inverse PCR (Section 2.7.5) was used to amplify a downstream AT-rich region from +1106 to +2636 to confirm the sequences obtained by TAIL-PCR. The combination of these methods allowed the cloning of a sequence of 4398 bp of the E8 *thi1* locus.

3.1.3.1. Amplification of E8 *thi1* by routine PCR

E8 *thi1* was cloned by routine PCR method (Section 2.7.2) using primers based on Lp19 *thi1* sequence (Fig. 3.5). Using most of the primer combinations shown in Fig. 3.5, PCR products amplified from E8 were identical in size to Lp19. However, PCR products amplified from E8 with primer pairs XZ10 and XZ5, and XZ10 and XZ6, were both smaller in size than those from Lp19. As there is no difference in size for products amplified with XZ7 and XZ5, we predicted that the region between XZ10 and XZ6 in the E8 *thi1* was smaller than the corresponding region in Lp19 *thi1*. Sequence analysis of these products confirmed this prediction.

No PCR product was amplified from E8 genomic DNA using primers XZ1 and XZ9, even at the lower annealing temperature of 50°C (results not shown), suggesting that the 3' region was divergent.

3.1.3.2. Amplification of E8 *thi1* 5' and 3' flanking regions by TAIL-PCR

The 5' and 3' regions flanking E8 *thi1* that could not be amplified by normal PCR were amplified by TAIL-PCR (Section 2.7.4).

Optimisation of the TAIL-PCR reaction conditions was necessary as annealing temperature, sequence of arbitrary primers and primer concentration are all critical to obtain specific products (Liu and Whittier, 1995). In amplification of the 3' flanking region using gene-specific primers XZ30, XZ20 and XZ22 in combination with arbitrary primer AD3, specific PCR products were obtained when the AD3 concentration was between 0.5 to 2.5 μM, which corresponds to a 4 and 20 fold difference in the concentration of specific primers (Fig. 3.6). A greater concentration of arbitrary primer may increase the probability

Figure 3.3. Nucleotide and predicted polypeptide sequence of the Lp19 *thi1* gene

The 3,675 bp nucleotide sequence from λXZ1 and λXZ3 contains the *thi1* open reading frame, starting at +1 (corresponding to the A of the translation start codon) and finishing at 1019 (*). The sequence (in dark green text) around the start codon is similar to the Kozak consensus sequence (Gurr *et al.*, 1987). The polypeptide sequence is shown below the nucleotide sequence. A putative TATA box (Gurr *et al.*, 1987) is outlined in green box.

The 5' and 3' ends of the mRNA, which were determined by RACE, are indicated by purple arrows. Four introns, determined by RACE and subsequently by RT-PCR in section 3.2, are shown in lower case letters. The potential 5' and 3' splice sites, and sequences for formation of the lariat (Gurr *et al.*, 1987), are outlined in green text. Two potential polyadenylation recognition sequences (Gurr *et al.*, 1987) are shown in blue text.

Two perfect 58 bp and two 52 bp direct repeats in the 5' and 3' region of *thi1* are shown in purple text. Motifs region A and B which are proposed to be required for efficient splicing (Kubodera *et al.*, 2003) are underlined.

Putative transcriptional factor binding sites for CreA (SYGGRG), NIT2 (eg. GCGATA) and StuAP (eg. TGTCGCGCAC), identified from the TRANSFAC database (http://www.cbrc.jp/research/db/TFSEACH.html) are highlighted in grey, turquoise and blue shading respectively.

Primers used for sequencing or analysing the gene are indicated in red text, with the accompanying arrow showing the direction of extension from the primer.


XZ32
-1575 CCCCGCGCAATCGCCGGCTCGTCGGCCTCTCTACAAAGATGTACTTCTCGCTATCTCGCA -1516
HindIII
-1515 ACCGCCAAGTCACGGAGGGCTTTCTGGAACGCCTCGGCAAGCTTGCGCCTTCATCCCTCG -1456
-1455 CCCACGTCGACATCTTCATCATTCCCGACTTTGTGTCCCTCGCCGAGACGGCAGAGCAGG -1396
XZ31
-1395 TCAAGACGTCCCCGGTGCCCGTCTGGCCGGGCGCGCAGGATTGCCACTGGGAAGACAGCG -1336
XZ44
-1335 GCGCGTTCACGGGCGAGGTCAGCCCCGCGGTGCTCAGCGAAGTGGGCGTCCGGATTGTCG -1276
-1275 AAGTCGGACACGCGGAGCGGAGGAGACTGTTTGGCGAGGACGATGCGGTCGTGGCGAAAA -1216
-1215 AGGCTGCAGCGGTGAGCAGGAACGGCATGGTCCCTCTGGTGTGCGTGGGGGAGCAGACGA -1156
-1155 GGGGGGGTATCGCCCTGGCGGTTGGTGAGTGTCGCGCAGAGGTCGAGGCAGTTCTGGCGG -1096
-1095 GCGTGTCTCCCGCCGCAGAGGTCGCGCTTGCGTATGAGCCGGTCTGGGCAATTGGTGCGA -1036
-1035 GCGAGCCTGCTGGTGAAGAGCATGTGCTGGGGGTGGTTGAGGGCATTCGGCAGCTCGGCT -976
-975 GTGTCAAGTCAAGGGAGGGCGTTACGAGGATTGTGTACGGTGGGAGCGCAGGACCCGGCT -916
XZ10
-915 TGTATGCGCAGTTGAAGTCAGGTCTGGATGGACTGTTTCTGGGACGCTTCGGACATGATC -856
XZ16
.XZ63
-855 CCGAGAGGTTTGTCAAGACAATTCAAGAGGTTGCTGAGGCGTAGGGGAGTCCAGGACCAC -796
-795 GGTCCACAAGGCAACAACGTGTTGCGATGCGCGCAGGGTGGGGCGATTATAACAACATGT -736
-735 TTGCCTGCTGCTTACTCCGTACTCCAGTGCTAGCTACACTTGCCAGTGCTGCTGCGTCGG -676
-675 AATATTTCACGCCCCGCCATGAGGCCCCATGGGCCATGAAGGAGCGATAATATAAAACCC -616
5' end of mRNA
-615 CTCATACAACTCACACAACGCGCACAACTCGACCCTTCTTGCAAAATACTTGCAAAATAC -556
XZ26.
-555 TCCAATCCCGTCTTGTCACTTTTGATTCTGTCAATCCTGCACTGCCTACCTGCATATTCG -496
Repeat
-495 CATCGAAAACATTGCAATCGCTATCCATACCACTTTGAGATGCCTACCTGCATATTCGCA -436
Repeat
.XZ7
-435 TCGAAAACATTGCAATCGCTATCCATACCACTTTGAGATACCATCTTgtttgataaaacc -376
Intron 1


XZ12
XZ6
Intron 2
Intron 3
Region B
Region A
SstI
XZ18
XZ28
XZ5
XZ13
XZ11


525 GTTGAGGATCTGATTACCCGTCCCTCTGAAGAAGGCGTCCGCATTTCCGGTGTCGTCACT 584
V E D L I T R P S E E G V R I S G V V T
XZ1
585 AACTGGCCTTGGTCTCTATGCATCAACGACGACCAGTCTTGCATGGACCCCAACACCATC 644
XZ8
N W T L V S M H H D D Q S C M D P N T I
645 AATGCTCCTCTTGTCATCTCCACCACTGGCCATGATGGTCCCATGGGTGCGTTCTGCGTG 704
N A P L V I S T T G H D G P M G A F C V
705 AAACGTCTCGTCAGCATGCAGCGCATTGAAAAGCTCGGTGGTATGCGCGGACTCGACATG 764
K R L V S M Q R I E K L G G M R G L D M
765 AACACCGCCGAAGATGCCATTGTCAAAAACACTCGTGAAATTGTTCCTGGTCTTATTGTC 824
N T A E D A I V K N T R E I V P G L I V
SstI
825 GGTGGCATGGAGCTCTCCGAGATTGACGGAGCCAACCGCATGGgtagggtgatctatcgt 884
Intron 4
G G M E L S E I D G A N R M G
XZ30
885 cgttctatgacattatacgcaactaacattctccctatcagGTCCAACATTCGGTGCAA 944
XZ3 (XZ25)
P T F G A M
945 TGGTCCTTAGCGGTCTCAAGGCTGCCGAGGAAGCGCTCAAGGTTTTTGACCTTCGCGCAA 1004
V L S G L K A A E E A L K V F D L R A K
XZ20
1005 AGCAGAATGCCATCTAAAGTATACGTGTGACAACTGGTTGAGAATGAAGACTTGACGTTA 1064
Q N A I *
1065 TCCTCGTCGTCCATCATTCTTTTACTTTGAATGGGATTTGACCGTAACCACGCTCACGTT 1124
repeat
1125 GATACTGATGGCAATGATGAATTAGTCGGCAGTCAGTCAGAAAGAAATACAATTGACCAT 1184
XZ14
mRNA 3'end I
1185 TCTCGCTCACGTTGATACTGATGGCAATGATGAATTAGTCGGCAGTCAGTCAGAATGAAA 1244
Repeat
mRNA 3'end II
1245 TACAATTGCCCTGACCTGTTGTACACTTATGTCGTGCTATTTGTGCTCATCCAACGTGAT 1304
1305 GTTTCAATGCTCACCGAGGTCATTTTCACTTTGTGGCAGCGACTCTTATTCTTCTTGCCC 1364


XZ15.
1365 AAATGAATACTGTTTGAAGCAATCCTAGCACACCGATTTACCGGACCAACAAATATGCTG 1424
XZ9
1425 GTGCCGTCAATTGCGTTGTGAGGATGGATAGAGCTTGATCGTGGTGGCTTGGCGAGATTT 1484
1485 GTGTTGCAACGCTGGAATGGGAAGGGCCTGCCTGCCTGGGCAAAAGAAACTCAAACAATA 1544
1545 GCAACAAGCAACATGGTGAGTGACCGCATGTTCAGCGCGTGGACCGTACCAGAACACCTT 1604
1605 GGGCCTACGTTGGTCACCAACGCATCATGGCTATTTCAAGGAAGCACAGAGTTCACTCGC 1664
1665 ATTCCTGGCGTGACACACGTTTTCGAAAGGGCGGTTCCGTGCAGCATACCGTTGGCCAAG 1724
1725 TGCAGCAGTACAATCTTGTGTTATTGACTAGGGGGACCTGCCAACCGCCACAAACCAACC 1784
1785 TCGACTAAGTCCTGCGACCAAGTTCACGAGGCTGGATTGGAGGCTTCAAGAACACAGATA 1844
1845 TGAATGCATTTGCATCCCTTTTGCATCTGGATTGCATGTGATGACATGATTTTTCTTCTC 1904
HindIII
1905 TTCCACGCTCAAAAGGGGTGGCTTGACCCCACCTTGCAAGCTTTCGATATGCTGGGCGGG 1964
XZ19
1965 AAAAGCCTGACACAAGCGGGTTGATCTCAATGTGAAATCGACGCTTGCCGTGTCCAC

Table 3.1. Blast X analysis of Thi1 homologous polypeptide sequences

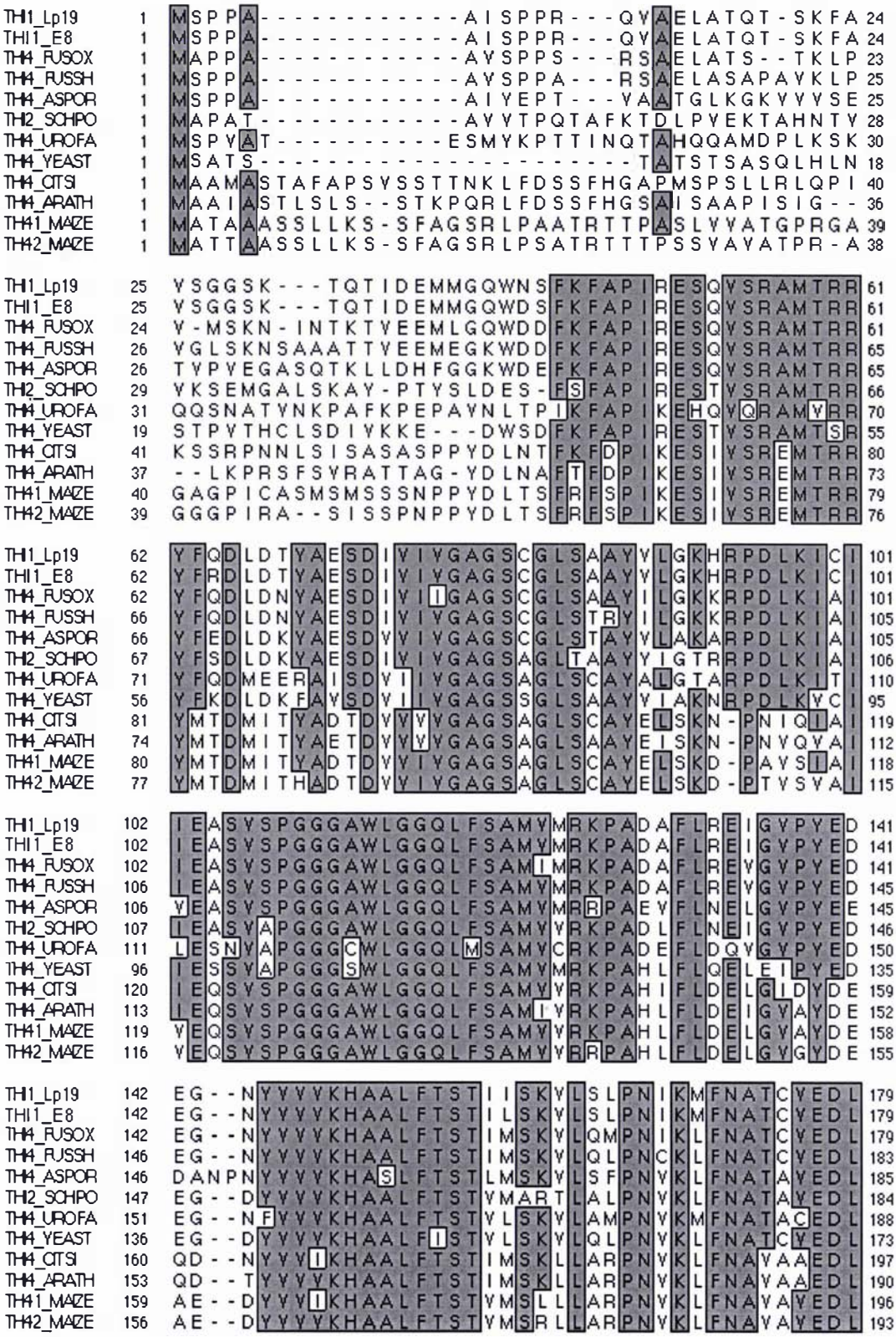
Polypeptide abbreviations (Original gene names)	Definitions (Organisms)	GeneBank accession number	Identity /similarity with Lp19 Thi1 (%)	References
THI4_FUSOX (*sti35*)	Thiazole biosynthetic enzyme, (*Fusarium sp*)	P23618	85.4/90.7	(Choi *et al.*, 1990)
THI4_FUSSH (*sti35*)	Thiazole biosynthetic enzyme, stress-inducible protein sti35 (*Fusarium sp*)	P23617	85.2/89.5	(Choi *et al.*, 1990)
THI4_ASPOR (*thiA*)	Thiazole biosynthetic enzyme, (*A. oryzae*)	Q9UUZ9	72.5/82.0	(Kubodera *et al.*, 2000)
THI2_SCHPO (*nmt2*)	Thiazole biosynthetic enzyme (*S. pombe*)	P40998	65.5/73.2	(Manetti *et al.*, 1994)
THI4_UROFA (*thi1*)	Thiazole biosynthetic enzyme (*U. viciae-fabae*)	Q9UVF8	56.9/66.7	(Sohn *et al.*, 2000)
THI4_YEAST (*MOL1*)	Thiazole biosynthetic enzyme (*S. cerevisiae*)	P32318	55.3/66.1	(Praekelt and Meacock, 1992)
THI4_CITSI (*C-thi1*)	Thiazole biosynthetic enzyme (*Citrus sinensis*)	O23787	48.8/60.7	(Jacob-Wilk *et al.*, 1997)
THI4_ARATH (*thi1*)	Thiazole biosynthetic enzyme (*A. thaliana)*	Q38814	48.6/61.5	(Machado *et al.*, 1996)
TH41_MAIZE (*thi1-1*)	Thiazole biosynthetic enzyme (*Zea mays*)	Q41738	50.1/61.3	(Belanger *et al.*, 1995)
TH42_MAIZE (*thi1-2*)	Thiazole biosynthetic enzyme (*Zea mays*)	Q41739	49.3/60.7	(Belanger *et al.*, 1995)

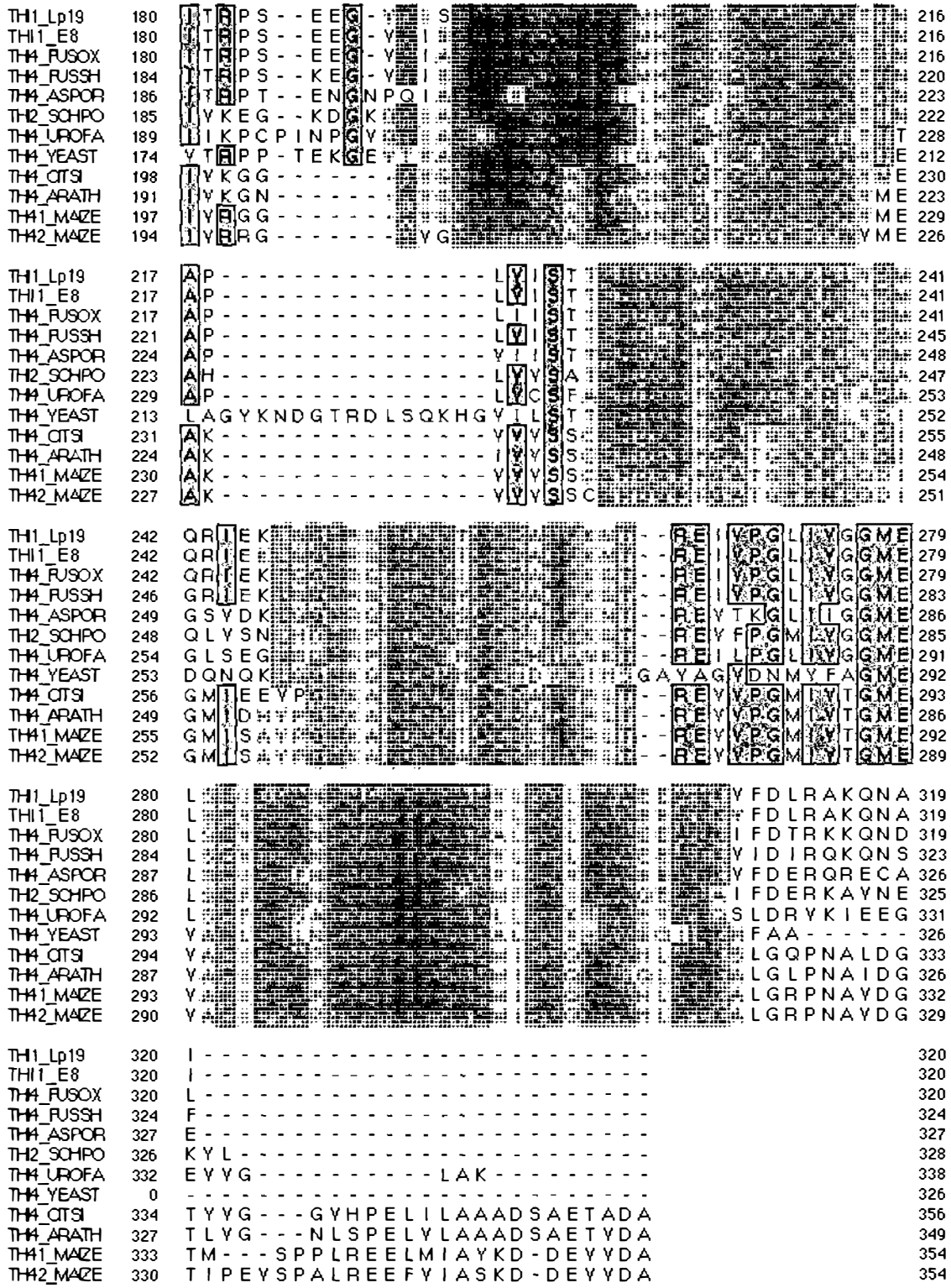
Homologues of Lp19 *thi1* polypeptide sequence in the GenBank were searched with the BlastX program (Altschul *et al.*, 1997) in website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Figure 3.4. Alignment of the deduced polypeptide sequences of Lp19 and E8 *thi1* genes with other homologous peptide sequences

Polypeptide sequences of Lp19 *thi1* and E8 *thi1* were aligned with the ten most similar polypeptides from other organisms identified by a BlastX (Altschul *et al.*, 1997) search of GenBank. These included two stress inducible proteins of *Fusarium oxysporum* and *F. solani* (THI4_FUSOX and THI4_FUSSH, accession numbers P23618 and P23617); a putative thiazole synthase of *Aspergillus oryzae* (THI4_ASPOR, accession number Q9UUZ9); a nmt2 protein of *Schizosaccharomyces pombe* (THI2_SCHPO, accession number P40998); a thiazole biosynthetic enzyme of *Uromyces fabae* (THI4_UROFA, accession number Q9UVF8); a thiazole biosynthetic enzyme of *Saccharomyces cerevisiae* (THI4_YEAST, accession number P32318), a thiazole biosynthetic enzyme of *Citrus sinensis* (THI4_CITSI, accession number O23787); THI4 of *Arabidopsis thaliana* (THI4_ARATH, accession number Q38814), THI1-1 and THI1-2 of *Zea mays* (TH41_MAIZE and TH42_MAIZE, accession numbers Q41738 and Q41739).

The sequences were formatted using FASTA, and aligned using CLUSTALW (http://www.ebi.ac.uk/clustalw) using the default settings. The alignment was shadowed using the SeqV1.1 programme. Grey shading indicates where over 60% of the sequence contain identical residues.


TH1_Lp19 1 MSPPA-----------AISPPR---QVAELATQT-SKFA 24
THI1_E8 1 MSPPA-----------AISPPR---QVAELATQT-SKFA 24
TH4_FUSOX 1 MAPPA-----------AVSPPS---RSAELATS--TKLP 23
TH4_FUSSH 1 MSPPA-----------AVSPPA---RSAELASAPAVKLP 25
TH4_ASPOR 1 MSPPA-----------AIYEPT---VAATGLKGKVVVSE 25
TH2_SCHPO 1 MAPAT-----------AVVTPQTAFKTDLPVEKTAHNTV 28
TH4_UROFA 1 MSPVAT----------ESMYKPTTINQTAHQQAMDPLKSK 30
TH4_YEAST 1 MSATS--------------------TATSTSASQLHLN 18
TH4_CITSI 1 MAAMASTAFAPSVSSTTNKLFDSSFHGAPMSPSLLRLQPI 40
TH4_ARATH 1 MAAIASTLSLS--STKPQRLFDSSFHGSAISAAPISIG-- 36
TH41_MAIZE 1 MATAAASSLLKS-SFAGSRLPAATRTTPASLVVATGPRGA 39
TH42_MAIZE 1 MATTAASSLLKS-SFAGSRLPSATRTTTPSSVAVATPR-A 38
TH1_Lp19 25 VSGGSK---TQTIDEMMGQWNSFKFAPIRESQVSRAMTRR 61
THI1_E8 25 VSGGSK---TQTIDEMMGQWDSFKFAPIRESQVSRAMTRR 61
TH4_FUSOX 24 V-MSKN-INTKTVEEMLGQWDDFKFAPIRESQVSRAMTRR 61
TH4_FUSSH 26 VGLSKNSAAATTVEEMEGKWDDFKFAPIRESQVSRAMTRR 65
TH4_ASPOR 26 TVPVEGASQTKLLDHFGGKWDEFKFAPIRESQVSRAMTRR 65
TH2_SCHPO 29 VKSEMGALSKAY-PTYSLDES-FSFAPIRESTVSRAMTRR 66
TH4_UROFA 31 QQSNATVNKPAFKPEPAVNLTPIKFAPIKEHQVQRAMVRR 70
TH4_YEAST 19 STPVTHCLSDIVKKE---DWSDFKFAPIRESTVSRAMTSR 55
TH4_CITSI 41 KSSRPNNLSISASASPPYDLNTFKFDPIKESIVSREMTRR 80
TH4_ARATH 37 --LKPRSFSVRATTAG-YDLNAFTFDPIKESIVSREMTRR 73
TH41_MAIZE 40 GAGPICASMSMSSSNPPYDLTSFRFSPIKESIVSREMTRR 79
TH42_MAIZE 39 GGGPIRA--SISSPNPPYDLTSFRFSPIKESIVSREMTRR 76
TH1_Lp19 62 YFQDLDTYAESDIVIVGAGSCGLSAAYVLGKHRPDLKICI 101
THI1_E8 62 YFRDLDTYAESDIVIVGAGSCGLSAAYVLGKHRPDLKICI 101
TH4_FUSOX 62 YFQDLDNYAESDIVIIGAGSCGLSAAYILGKKRPDLKIAI 101
TH4_FUSSH 66 YFQDLDNYAESDIVIVGAGSCGLSTRYILGKKRPDLKIAI 105
TH4_ASPOR 66 YFEDLDKYAESDVVIVGAGSCGLSTAYVLAKARPDLKIAI 105
TH2_SCHPO 67 YFSDLDKYAESDIVIVGAGSAGLTAAYYIGTRRPDLKIAI 106
TH4_UROFA 71 YFQDMEERAISDVIIVGAGSAGLSCAYALGTARPDLKITI 110
TH4_YEAST 56 YFKDLDKFAVSDVIIVGAGSSGLSAAYVIAKNRPDLKVCI 95
TH4_CITSI 81 YMTDMITYADTDVVYVGAGSAGLSCAYELSKN-PNIQIAI 119
TH4_ARATH 74 YMTDMITYAETDVVYVGAGSAGLSAAYEISKN-PNVQYAI 112
TH41_MAIZE 80 YMTDMITYADTDVVIVGAGSAGLSCAYELSKD-PAVSIAI 118
TH42_MAIZE 77 YMTDMITHADTDVVIVGAGSAGLSCAYELSKD-PTVSVAI 115
TH1_Lp19 102 IEASYSPGGGAWLGGQLFSAMYMRKPADAFLREIGVPYED 141
THI1_E8 102 IEASYSPGGGAWLGGQLFSAMYMRKPADAFLREIGVPYED 141
TH4_FUSOX 102 IEASYSPGGGAWLGGQLFSAMIMRKPADAFLREVGVPYED 141
TH4_FUSSH 106 IEASYSPGGGAWLGGQLFSAMYMRKPADAFLREVGVPYED 145
TH4_ASPOR 106 YEASYSPGGGAWLGGQLFSAMYMRRPAEVFLNELGVPYEE 145
TH2_SCHPO 107 IEASYAPGGGAWLGGQLFSAMYYRKPADLFLNEIGYPYED 146
TH4_UROFA 111 LESNYAPGGGCWLGGQLMSAMYCRKPADEFLDQVGVPYED 150
TH4_YEAST 96 IESSYAPGGGSWLGGQLFSAMYMRKPAHLFLQELEIPYED 135
TH4_CITSI 120 IEQSYSPGGGAWLGGQLFSAMYYRKPAHIFLDELGIDYDE 159
TH4_ARATH 113 IEQSYSPGGGAWLGGQLFSAMIYRKPAHLFLDEIGVAYDE 152
TH41_MAIZE 119 VEQSYSPGGGAWLGGQLFSAMYVRKPAHLFLDELGVAYDE 158
TH42_MAIZE 116 VEQSYSPGGGAWLGGQLFSAMYVRRPAHLFLDELGVGYDE 155
TH1_Lp19 142 EG--NYYVVKHAALFTSTIISKVLSLPNIKMFNATCVEDL 179
THI1_E8 142 EG--NYYVVKHAALFTSTILSKVLSLPNIKMFNATCVEDL 179
TH4_FUSOX 142 EG--NYYVVKHAALFTSTIMSKVLQMPNIKLFNATCVEDL 179
TH4_FUSSH 146 EG--NYYVVKHAALFTSTIMSKVLQLPNCKLFNATCVEDL 183
TH4_ASPOR 146 DANPNYYVVKHASLFTSTLMSKVLSFPNVKLFNATAVEDL 185
TH2_SCHPO 147 EG--DYYVVKHAALFTSTVMARTLALPNVKLFNATAVEDL 184
TH4_UROFA 151 EG--NFYVVKHAALFTSTVLSKVLAMPNVKMFNATACEDL 188
TH4_YEAST 136 EG--DYYVVKHAALFISTVLSKVLQLPNVKLFNATCVEDL 173
TH4_CITSI 160 QD--NYYVIKHAALFTSTIMSKLLARPNVKLFNAVAAEDL 197
TH4_ARATH 153 QD--TYYVVKHAALFTSTIMSKLLARPNVKLFNAVAAEDL 190
TH41_MAIZE 159 AE--DYYVIKHAALFTSTVMSLLLARPNVKLFNAVAVEDL 196
TH42_MAIZE 156 AE--DYYVVKHAALFTSTVMSRLLARPNVKLFNAVAVEDL 193


TH1_Lp19 180 ITRPS--EEG-SI 216
THI1_E8 180 ITRPS--EEG-VI 216
TH4_FUSOX 180 ITRPS--EEG-VI 216
TH4_FUSSH 184 ITRPS--KEG-VI 220
TH4_ASPOR 186 FTRPT--ENGNPQI 223
TH2_SCHPO 185 IYKEG--KDGK 222
TH4_UROFA 189 IIKPCPINPGY T 228
TH4_YEAST 174 YTRPP-TEKGE I E 212
TH4_CITSI 198 IYKGG------ E 230
TH4_ARATH 191 IYKGN------ ME 223
TH41_MAIZE 197 IYRGG------ ME 229
TH42_MAIZE 194 IYRRG------VG YME 226
TH1_Lp19 217 AP----------LYISST 241
THI1_E8 217 AP----------LYISST 241
TH4_FUSOX 217 AP----------LIISST 241
TH4_FUSSH 221 AP----------LYISST 245
TH4_ASPOR 224 AP----------VIISST 248
TH2_SCHPO 223 AH----------LYYSA 247
TH4_UROFA 229 AP----------LYCSF 253
TH4_YEAST 213 LAGYKNDGTRDLSQKHGYILSST 252
TH4_CITSI 231 AK----------VYYSS 255
TH4_ARATH 224 AK----------IYYSS 248
TH41_MAIZE 230 AK----------VYYSS 254
TH42_MAIZE 227 AK----------VYYSSC 251
TH1_Lp19 242 QRIEK--REIYPGLIYGGME 279
THI1_E8 242 QRIEE--REIYPGLIYGGME 279
TH4_FUSOX 242 QRIEK--REIYPGLIYGGME 279
TH4_FUSSH 246 GRIEK--REIYPGLIYGGME 283
TH4_ASPOR 249 GSYDK--REYTKGLIIGGME 286
TH2_SCHPO 248 QLYSN--REYFPGMLYGGME 285
TH4_UROFA 254 GLSEG--REILPGLIYGGME 291
TH4_YEAST 253 DQNQK--GAYAGYDNMYFAGME 292
TH4_CITSI 256 GMIEEYP--REYYPGMIYTGME 293
TH4_ARATH 249 GMIDHY--REYYPGMIYTGME 286
TH41_MAIZE 255 GMISAY--REYYPGMIYTGME 292
TH42_MAIZE 252 GMISAY--REYYPGMIYTGME 289
TH1_Lp19 280 LYFDLRAKQNA 319
THI1_E8 280 LYFDLRAKQNA 319
TH4_FUSOX 280 LIFDTRKKQND 319
TH4_FUSSH 284 LYIDIRQKQNS 323
TH4_ASPOR 287 LYFDERQRECA 326
TH2_SCHPO 286 LIFDERKAYNE 325
TH4_UROFA 292 LSLDRYKIEEG 331
TH4_YEAST 293 YFAA------ 326
TH4_CITSI 294 YLGQPNALDG 333
TH4_ARATH 287 YLGLPNAIDG 326
TH41_MAIZE 293 YLGRPNAYDG 332
TH42_MAIZE 290 YLGRPNAYDG 329
TH1_Lp19 320 I---------- 320
THI1_E8 320 I---------- 320
TH4_FUSOX 320 L---------- 320
TH4_FUSSH 324 F---------- 324
TH4_ASPOR 327 E---------- 327
TH2_SCHPO 326 KYL---------- 328
TH4_UROFA 332 EYYG--------LAK------ 338
TH4_YEAST 0 ---------- 326
TH4_CITSI 334 TYYG---GYHPELILAAADSAETADA 356
TH4_ARATH 327 TLYG---NLSPELYLAAADSAETYDA 349
TH41_MAIZE 333 TM---SPPLREELMIAYKD-DEYYDA 354
TH42_MAIZE 330 TIPEYSPALREEFYIASKD-DEYYDA 354

of annealing between the arbitrary primer and the target sequence. In order to determine whether the nucleotide sequence of the arbitrary primer affected the amplification, XZ17 was used instead of AD3. Amplification using the degenerate arbitrary primer XZ17 produced different product profiles as shown in Fig. 3.7, indicating that the nucleotide sequence of the arbitrary primer may affect the amplification. Under optimised reaction conditions, sequences of about 900 bp were amplified from the E8 *thi1* 5' flanking region in two independent TAIL-PCR reactions, and about 400 bp from the E8 *thi1* 3' flanking region was amplified (Fig. 3.8).

3.1.3.3. Amplification of an E8 *thi1* 3' AT-rich region by inverse PCR

In addition, inverse PCR (Section 2.7.5) was applied to generate further sequence data (Fig 3.9). A unique PCR product of 1.7 kb was amplified from self-ligated E8 genomic DNA using inverse primers XZ31 and XZ20, and a product of 1.3 kb was amplified using nested inverse primers XZ31 and XZ34 (Fig. 3.9). Sequence analysis of the 1.7-kb PCR product revealed that there is an AT-rich region in the downstream region. Blast N (X) analysis suggests that this AT rich sequence is highly corrupted retrotransposon DNA.

3.1.4. Analysis of the E8 *thi1* gene sequence

The nucleotide and deduced polypeptide sequences for E8 *thi1* are shown in Fig. 3.10. The sequence around the deduced E8 *thi1* translation start codon, CCATC**ATG**AGTC, is identical to that of Lp19 *thi1*. An intron of 59 bp was predicted to be at the corresponding location of the Lp19 *thi1*, and this intron was confirmed by analysis of the 3'RACE product from E8 *thi1* gene (Section 3.2.2).

The deduced polypeptide sequence of E8 *thi1* is identical to that of the Lp19 *thi1* (Fig. 3.11) with the exception of four conservative replacements. The polypeptide has 320 amino acid residues and a unmodified molecular mass was 34.5 kD.

An alignment of the E8 and Lp19 *thi1* sequences indicated significant sequence conservation in the coding regions. However, in the promoter region, the E8 *thi1* has several deletions compared to the Lp19 *thi1*, or alternatively, the Lp19 *thi1* has insertions (Fig. 3.12). Like Lp19 *thi1*, the E8 *thi1* promoter region contains many putative transcription factor-binding sites for CreA (Dowzer and Keller, 1989; Felenbok and Kelly, 1996), NIT2 (Fu and Marzluf, 1990) and StuAP (Dutton *et al.*, 1997). There were 9 CreA, 10 NIT2 and 4 StuAP binding sites (Fig. 3.10), suggesting that this gene may be regulated by carbon and nitrogen sources, as well as morphological development signals. The motifs (region A and B) which are required for intron splicing and thiamine-regulated gene expression are also present in the promoter region of E8 *thi1*. No significant conservation in the 3' flanking regions was observed between the Lp19 and E8 *thi1* genes.

3.1.5. Southern hybridisation analysis of *thi1* region in Lp19, Lp1 and E8 strains

Southern blot hybridisations (Section 2.6) were performed to determine *thi1* copy number in endophytes *N. lolii* Lp19, *Neotyphodium sp.* Lp1 and *E. typhina* E8 strains. Only a single band was observed in all the digestions from Lp19 or E8 genomic DNA (Fig. 3.13), demonstrating only a single *thi1* gene copy being in these two strains. Two bands were present in the Lp1 genomic DNA digested with *Hind*III, *Eco*RV, and *Pst*I (Fig. 3.13), indicating two *thi1* gene copies are present in the Lp1 genome. In Lp1 genomic DNA digested with *Bam*HI, one of the two bands was very faint probably due to size and concentration of the fragment. While it is known that Lp1 is an interspecific hybrid from *E.*

typhina and *N. lolii* strains (Schardl, et al. 1994), it was surprising that few of the bands in Lp1 matched in size to those from Lp19 or E8. These results suggest that the sequence has diverged in the region around the *thi1* locus following the interspecific hybridisation event.

3.1.6. Summary and discussion

This study utilised a combination of PCR methods to clone the *E. typhina* E8 *thi1* gene based on sequence information from the Lp19 *thi1* gene (Section 3.1.3). While application of PCR methods in molecular cloning does not require a genomic library, a weakness of this approach is the possible introduction of nucleotide mismatch during PCR amplifications. To overcome this limitation, both strands of the E8 *thi1* gene were sequenced from independent PCR reactions using genomic DNA as template. Whenever there was an ambiguity, additional PCR reactions were carried out and the PCR products sequenced to eliminate the ambiguity. In addition, the sequence obtained from inverse PCR (Section 3.1.3.3) overlapped with that from TAIL-PCR (Section 3.1.3.2), further verifying the consensus sequence. This approach resulted in a high quality consensus sequence for both the Lp19 and E8 *thi1* gene.

Although Bioinformatic analysis identified putative CreA and NIT2 binding sites in the promoter region of both E8 and Lp19 *thi1* gene (Sections 3.1.2 and 3.1.4), and some of these were conserved between the E8 and Lp19 *thi1* promoter (Fig. 3.12), whether these putative promoter elements are functional requires experimental analysis.

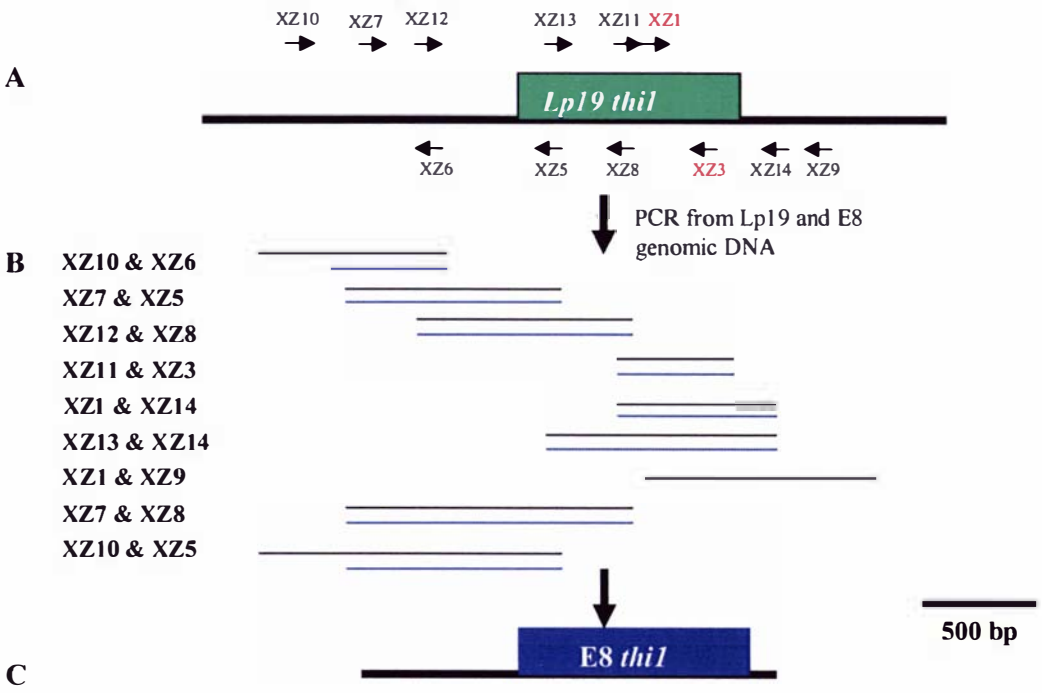
Figure 3.5. PCR amplification of E8 *thi1* gene

A: The Lp19 *thi1* gene and primer locations.

B: Comparison of the PCR products amplified from Lp19 (black) and E8 (blue) genomic DNA.

C: The E8 *thi1* gene.

D: Agarose gel analysis of the PCR products amplified from Lp19 (lanes 1, 3, 5, 7, 9, 11, 13, 15 and 17) and E8 (lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18) genomic DNA using Lp19 *thi1* primer sets XZ10 & XZ5 (lanes 1 and 2), XZ10 & XZ6 (lanes 3 and 4), XZ7 & XZ5 (lanes 5 and 6), XZ7 & XZ8 (lanes 7 and 8), XZ12 & XZ8 (lanes 9 and 10), XZ11 & XZ3 (lanes 11 and 12), XZ13 & XZ14 (lanes 13 and 14), XZ1 & XZ14 (lanes 15 and 16) and XZ1 & XZ9 (lanes 17 and 18). Lane M contained 1 kb + ladder (GibcoBRL).


A
XZ10
XZ7
XZ12
XZ13
XZ11
XZ1
Lp19 thi1
XZ6
XZ5
XZ8
XZ3
XZ14
XZ9
PCR from Lp19 and E8 genomic DNA
B
XZ10 & XZ6
XZ7 & XZ5
XZ12 & XZ8
XZ11 & XZ3
XZ1 & XZ14
XZ13 & XZ14
XZ1 & XZ9
XZ7 & XZ8
XZ10 & XZ5
500 bp
C
E8 thi1

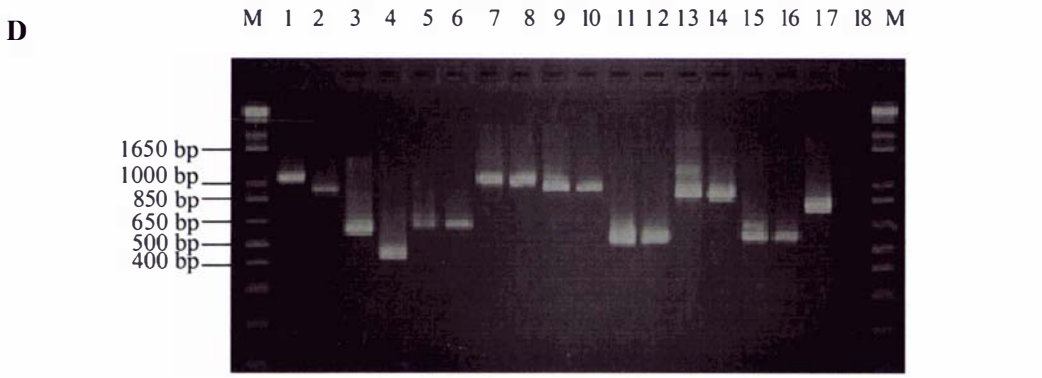

D
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp

Figure 3.6. TAIL-PCR amplification of the 3' region of E8 *thi1*

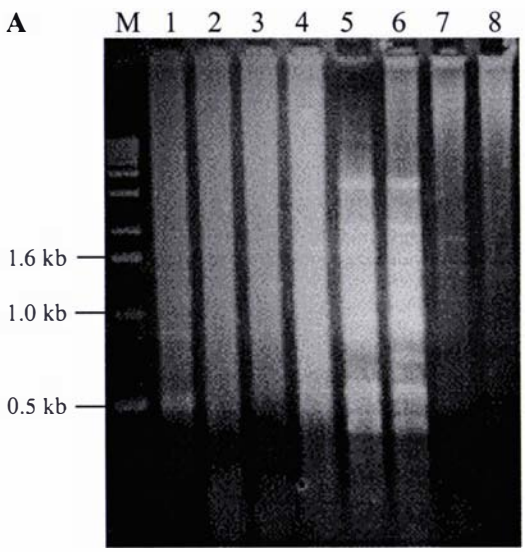
A: Profile of primary TAIL-PCR products amplified from E8 genomic DNA using a fixed concentration of primer XZ30 at 125 nM and variable concentrations of AD3 at 0 nM (lanel), 125 nM (lane 2), 250 nM (lane 3), 500 nM (lane 4), 1250 nM (lane 5) and 2500 nM (lane 6). Control amplifications were carried out using AD3 alone at 2500 nM (lane 7) and no primers (lane 8).

B: Profile of TAIL-PCR products amplified from a 1/50 dilution of primary PCR products shown in **A** using a nested primer XZ20 at 125 nM with AD3 at 1250 nM.

C: Profile of TAIL-PCR products amplified from a 1/50 dilution of secondary PCR products shown in **B** using a nested primer XZ22 with AD3 at 125 nM.

Lane M in **A** and **B** contained the 1 kb ladder and lane M in **C** the 1 kb + ladder (GibcoBRL).

The locations of primers XZ30, XZ20 and XZ22 are shown in Figure 3.10.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0.5 kb

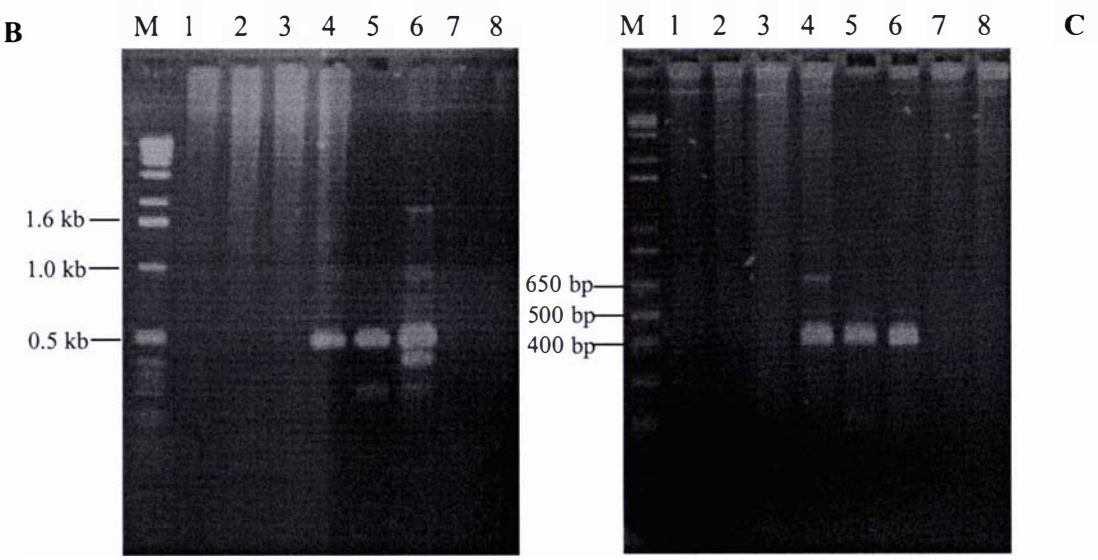

B
M 1 2 3 4 5 6 7 8
1.6 kb
1.0 kb
0.5 kb
M 1 2 3 4 5 6 7 8
C
650 bp
500 bp
400 bp

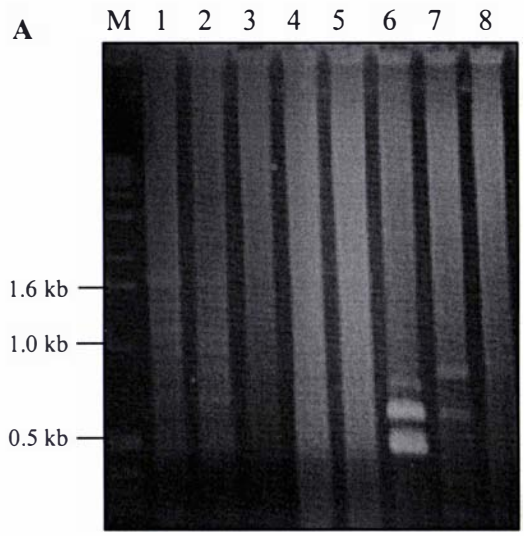
Figure 3.7. TAIL-PCR amplification of the 3' region of E8 *thi1* using arbitrary primer XZ17

A: Profile of primary TAIL-PCR products amplified from E8 genomic DNA using a fixed concentration of primer XZ30 at 125 nM and variable concentrations of XZ17 at 0 nM (lanel), 125 nM (lane 2), 250 nM (lane 3), 500 nM (lane 4), 1250 nM (lane 5) and 2500 nM (lane 6). Control amplifications were carried out using XZ17 alone at 2500 nM (lane 7) and no primers (lane 8).

B: Profile of TAIL-PCR products amplified from a 1/50 dilution of primary PCR products shown in **A** using a nested primer XZ20 at 125 nM with XZ17 at 1250 nM.

C: Profile of TAIL-PCR products amplified from a 1/50 dilution of secondary PCR products shown in **B** using a nested primer XZ22 with XZ17 at 125 nM.

Lane M in **A** contained the 1 kb ladder and M in **B** and **C** the 1 kb + ladder (GibcoBRL).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1.0 kb
0.5 kb

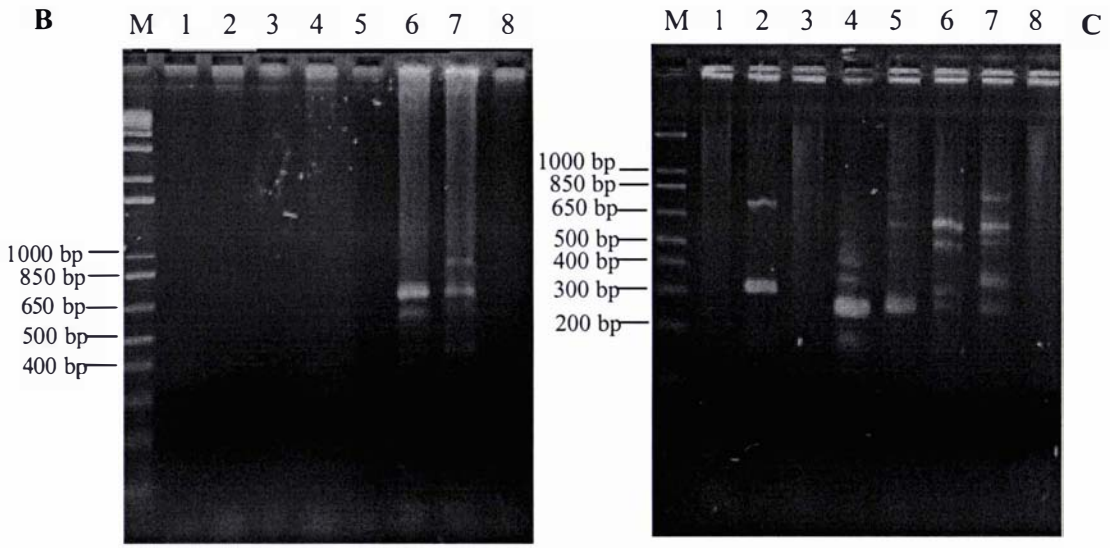

B
M 1 2 3 4 5 6 7 8
1000 bp
850 bp
650 bp
500 bp
400 bp
M 1 2 3 4 5 6 7 8
C
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp

Figure 3.8. Cloning the E8 *thi1* gene flanking regions by TAIL-PCR

A: The scheme for cloning the E8 *thi1* flanking regions by TAIL-PCR and the location of the primers used.

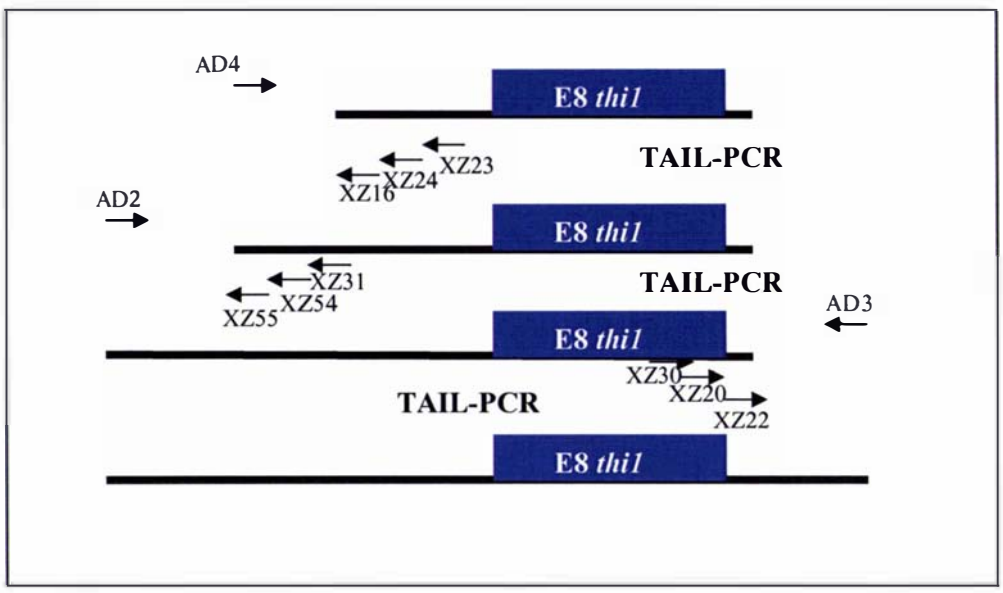
B: Primary, secondary and tertiary TAIL-PCR products amplified from E8 *thi1* 5' region using primers XZ23 & AD4 (lane 1), XZ24 & AD4 (lane 2), XZ16 & AD4 (lane 3) respectively.

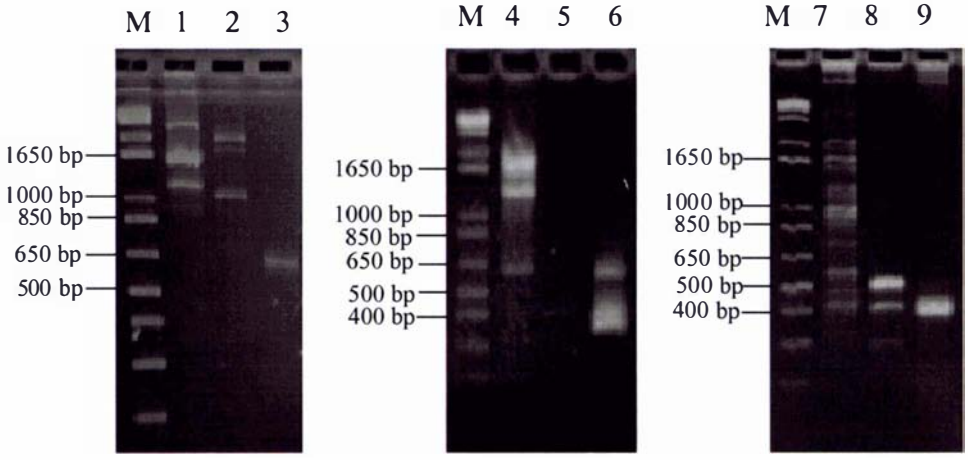
C: Primary, secondary and tertiary TAIL-PCR products amplified from E8 *thi1* 5' region using primers XZ31 & AD2 (lane 4), XZ54 & AD2 (lane 5), and XZ55 & AD2 (lane 6) respectively.

D: Primary, secondary and tertiary TAIL-PCR products amplified from E8 *thi1* 3' region using primers XZ30 & AD3 (lane 7), XZ20 & AD3 (lane 8), and XZ22 & AD3 (lane 9) respectively. A 1/50 dilution of primary and secondary products was used as template in the secondary and tertiary TAIL-PCR amplification.

Lane M contained the 1 kb + ladder (GibcoBRL).

A


AD4
E8 thi1
XZ23
XZ24
XZ16
TAIL-PCR
AD2
E8 thi1
XZ31
XZ54
XZ55
TAIL-PCR
AD3
E8 thi1
XZ30
XZ20
XZ22
TAIL-PCR
E8 thi1


M 1 2 3
1650 bp
1000 bp
850 bp
650 bp
500 bp
M 4 5 6
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
M 7 8 9
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp

B C D

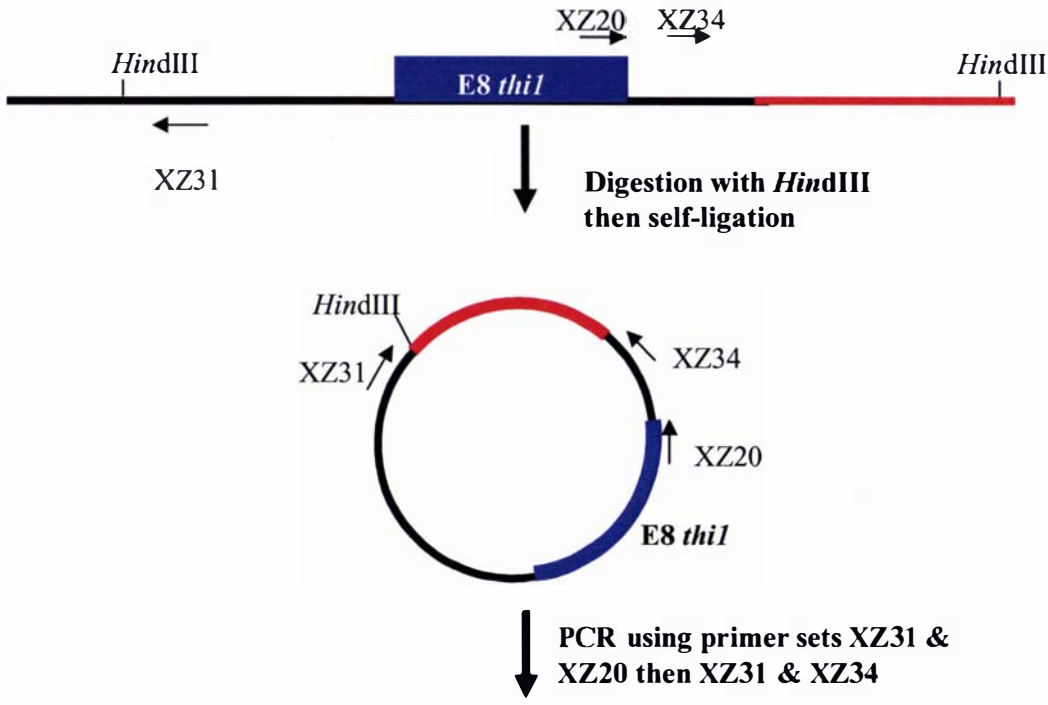
Figure 3.9. Cloning the E8 *thil* 3' AT rich region by inverse PCR

A: The location of primers used for amplifying the E8 3' flanking region

B: The inverse PCR products amplified by primers XZ31 & XZ20 (lane 1), and XZ31 & XZ34 (lane2) respectively from about 30 ng of E8 genomic DNA that had been *Hin*dIII digested and self-ligated.

Lane M contains the 1 kb + ladder (GibcoBRL). The black and the red lines indicate the known and unknown sequence regions respectively.

A


XZ20
XZ34
HindIII
E8 thi1
HindIII
XZ31
Digestion with HindIII
then self-ligation
HindIII
XZ31
XZ34
XZ20
E8 thi1
PCR using primer sets XZ31 &
XZ20 then XZ31 & XZ34

B

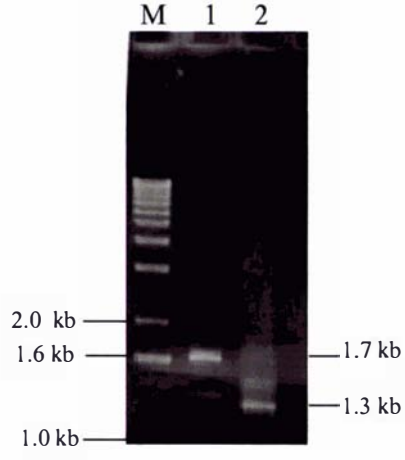

M
1
2
2.0 kb
1.6 kb
1.0 kb
1.7 kb
1.3 kb

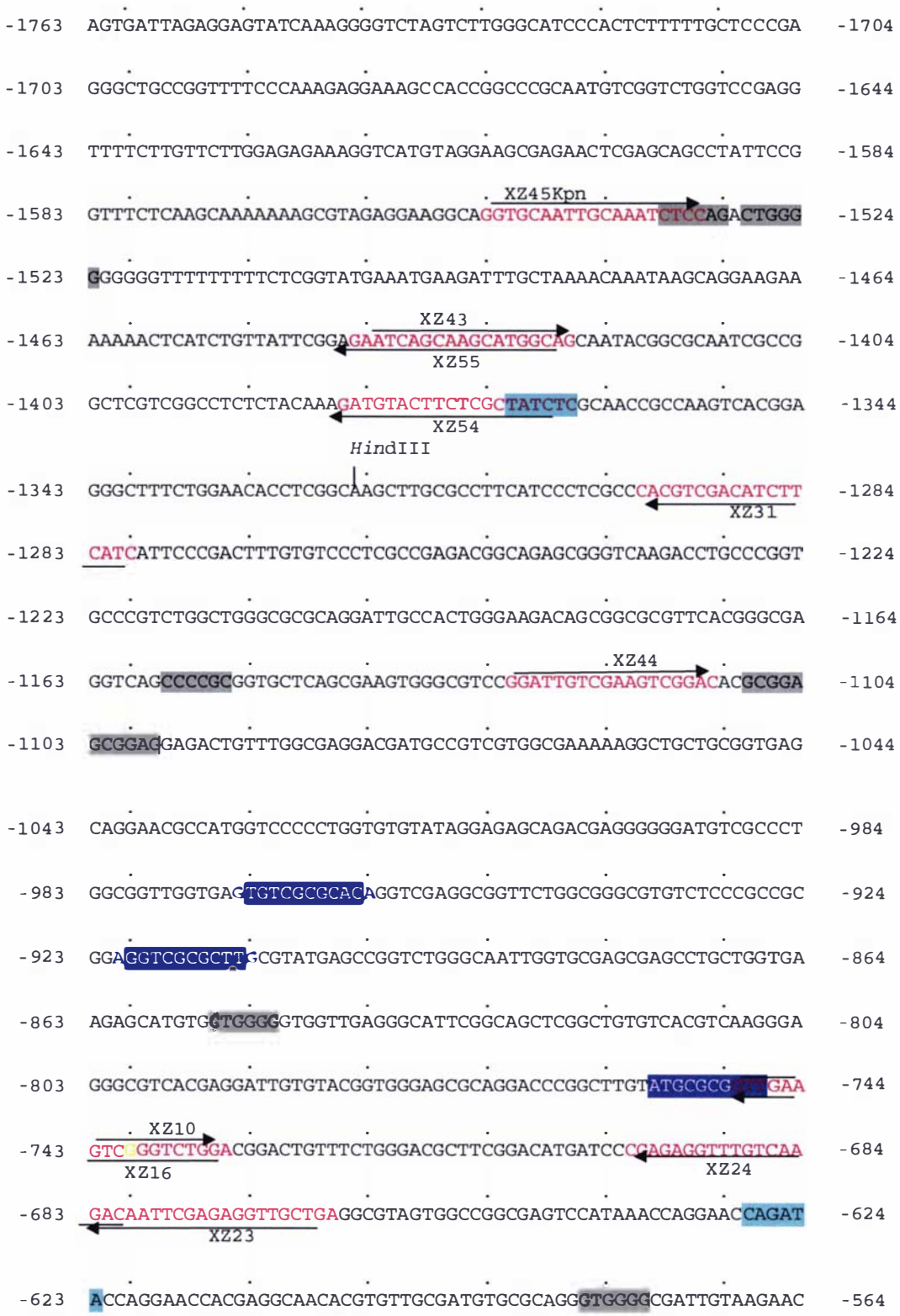
Figure 3.10. Nucleotide and predicted polypeptide sequence of the E8 *thi1* gene

The 4,398 bp nucleotide sequence from E8 contains the E8 *thi1* open reading frame, starting at +1 (corresponding to the A of the translation start codon) and finishing at 1019 (•). The sequence (in dark green text) around the start codon is similar to the Kozak consensus sequence (Gurr *et al.*, 1987). The polypeptide sequence is shown below the nucleotide sequence.

The 5' and 3' ends of the mRNA, which were determined by RACE are indicated by purple arrows. The introns, determined by RACE and RT-PCR, are shown in lower case letters. The potential 5' and 3' splice sites, and sequences for formation of the lariat (Gurr *et al.*, 1987), are outlined in green text. The potential polyadenylation recognition sequence (Gurr *et al.*, 1987) is shown in blue text.

Putative transcriptional factor binding sites for CreA (SYGGRG), NIT2 (eg. GCGATA) and StuAP (eg. TGTCGCGCAC), identified from the TRANSFAC database (http://www.cbrc.jp/research/db/TFSEACH.html) are highlighted in grey, turquoise and blue shading respectively. Motifs region A and B which are proposed to be required for efficient splicing (Kubodera *et al.*, 2003) are underlined.

Primers used for cloning, sequencing or analysing the gene are indicated in red text, with the accompanying red arrow showing the direction of extension from the primer.
An AT rich downstream region is highlighted in yellow.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-1703 GGGCTGCCGGTTTTCCCAAAGAGGAAAGCCACCGGCCCGCAATGTCGGTCTGGTCCGAGG -1644
-1643 TTTTCTTGTTCTTGGAGAGAAAGGTCATGTAGGAAGCGAGAACTCGAGCAGCCTATTCCG -1584
XZ45Kpn
-1583 GTTTCTCAAGCAAAAAAAGCGTAGAGGAAGGCAGGTGCAATTGCAAATCTCCAGACTGGG -1524
-1523 GGGGGGTTTTTTTTTCTCGGTATGAAATGAAGATTTGCTAAAACAAATAAGCAGGAAGAA -1464
XZ43
-1463 AAAAACTCATCTGTTATTCGGAGAATCAGCAAGCATGGCAGCAATACGGCGCAATCGCCG -1404
XZ55
-1403 GCTCGTCGGCCTCTCTACAAAGATGTACTTCTCGCTATCTCGCAACCGCCAAGTCACGGA -1344
XZ54
HindIII
-1343 GGGCTTTCTGGAACACCTCGGCAAGCTTGCGCCTTCATCCCTCGCCCACGTCGACATCTT -1284
XZ31
-1283 CATCATTCCCGACTTTGTGTCCCTCGCCGAGACGGCAGAGCGGGTCAAGACCTGCCCGGT -1224
-1223 GCCCGTCTGGCTGGGCGCGCAGGATTGCCACTGGGAAGACAGCGGCGCGTTCACGGGCGA -1164
XZ44
-1163 GGTCAGCCCCGCGGTGCTCAGCGAAGTGGGCGTCCGGATTGTCGAAGTCGGACACGCGGA -1104
-1103 GCGGAGGAGACTGTTTGGCGAGGACGATGCCGTCGTGGCGAAAAAGGCTGCTGCGGTGAG -1044
-1043 CAGGAACGCCATGGTCCCCCTGGTGTGTATAGGAGAGCAGACGAGGGGGGATGTCGCCCT -984
-983 GGCGGTTGGTGACTGTCGCGCACAGGTCGAGGCGGTTCTGGCGGGCGTGTCTCCCGCCGC -924
-923 GGAAGGTCGCGCTTGCGTATGAGCCGGTCTGGGCAATTGGTGCGAGCGAGCCTGCTGGTGA -864
-863 AGAGCATGTGGTGGGGGTGGTTGAGGGCATTCGGCAGCTCGGCTGTGTCACGTCAAGGGA -804
-803 GGGCGTCACGAGGATTGTGTACGGTGGGAGCGCAGGACCCGGCTTGTATGCGCGTTGGAA -744
XZ10
-743 GTCGGTCTGGACGGACTGTTTCTGGGACGCTTCGGACATGATCCCGAGAGGTTTGTCAA -684
XZ16
XZ24
-683 GACAATTCGAGAGGTTGCTGAGGCGTAGTGGCCGGCGAGTCCATAAACCAGGAACCAGAT -624
XZ23
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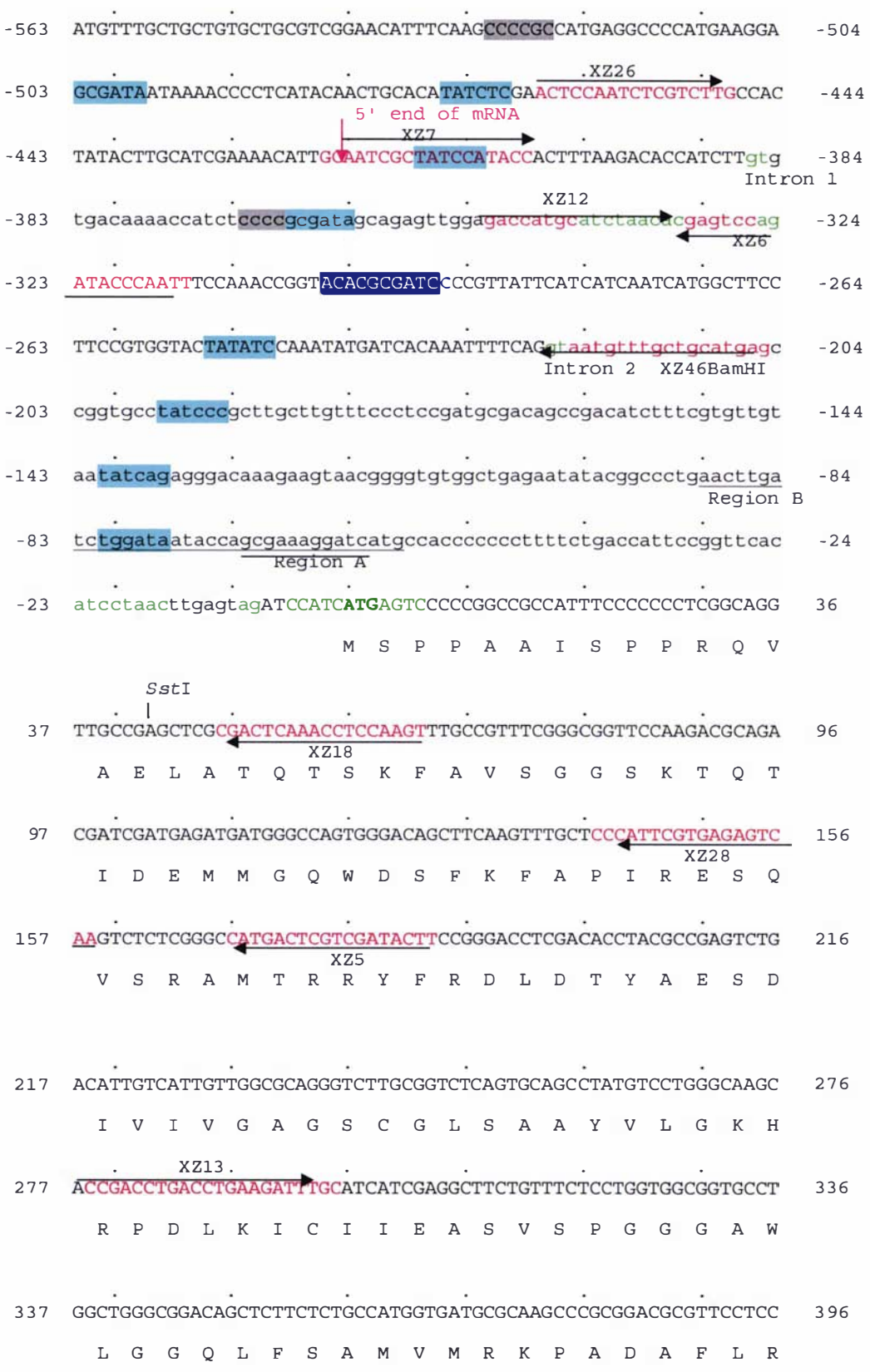
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-503 GCGATAATAAAACCCCTCATACAACTGCACATATCTCGAACTCCAATCTCGTCTTGCCAC -444
5' end of mRNA
XZ7
-443 TATACTTGCATCGAAAACATTGCAATCGCTATCCATACCACTTTAAGACACCATCTTgtg -384
Intron 1
XZ12
-383 tgacaaaaccatctccccgcgatagcagagttggagaccatgcatctaacacgagtccag -324
XZ6
-323 ATACCCAATTTCCAAACCGGTACACGCGATCCCCGTTATTCATCATCAATCATGGCTTCC -264
-263 TTCCGTGGTACTATATCCAAATATGATCACAAATTTTCAGgtaatgtttgctgcatgagc -204
Intron 2 XZ46BamHI
-203 cggtgcctatcccgcttgcttgtttccctccgatgcgacagccgacatctttcgtgttgt -144
-143 aatatcagagggacaaagaagtaacggggtgtggctgagaatatacggccctgaacttga -84
Region B
-83 tctggataataccagcgaaaggatcatgccacccccctttttctgaccattccggttcac -24
Region A
-23 atcctaacttgagtagATCCATCATGAGTCCCCCGGCCGCCATTTCCCCCCCTCGGCAGG 36
M S P P A A I S P P R Q V
SstI
37 TTGCCGAGCTCGCGACTCAAACCTCCAAGTTTGCCGTTTCGGGCGGTTCCAAGACGCAGA 96
XZ18
A E L A T Q T S K F A V S G G S K T Q T
97 CGATCGATGAGATGATGGGCCAGTGGGACAGCTTCAAGTTTGCTCCCATTCGTGAGAGTC 156
XZ28
I D E M M G Q W D S F K F A P I R E S Q
157 AAGTCTCTCGGGCCATGACTCGTCGATACTTCCGGGACCTCGACACCTACGCCGAGTCTG 216
XZ5
V S R A M T R R Y F R D L D T Y A E S D
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I V I V G A G S C G L S A A Y V L G K H
XZ13
277 ACCGACCTGACCTGAAGATTTGCATCATCGAGGCTTCTGTTTCTCCTGGTGGCGGTGCCT 336
R P D L K I C I I E A S V S P G G G A W
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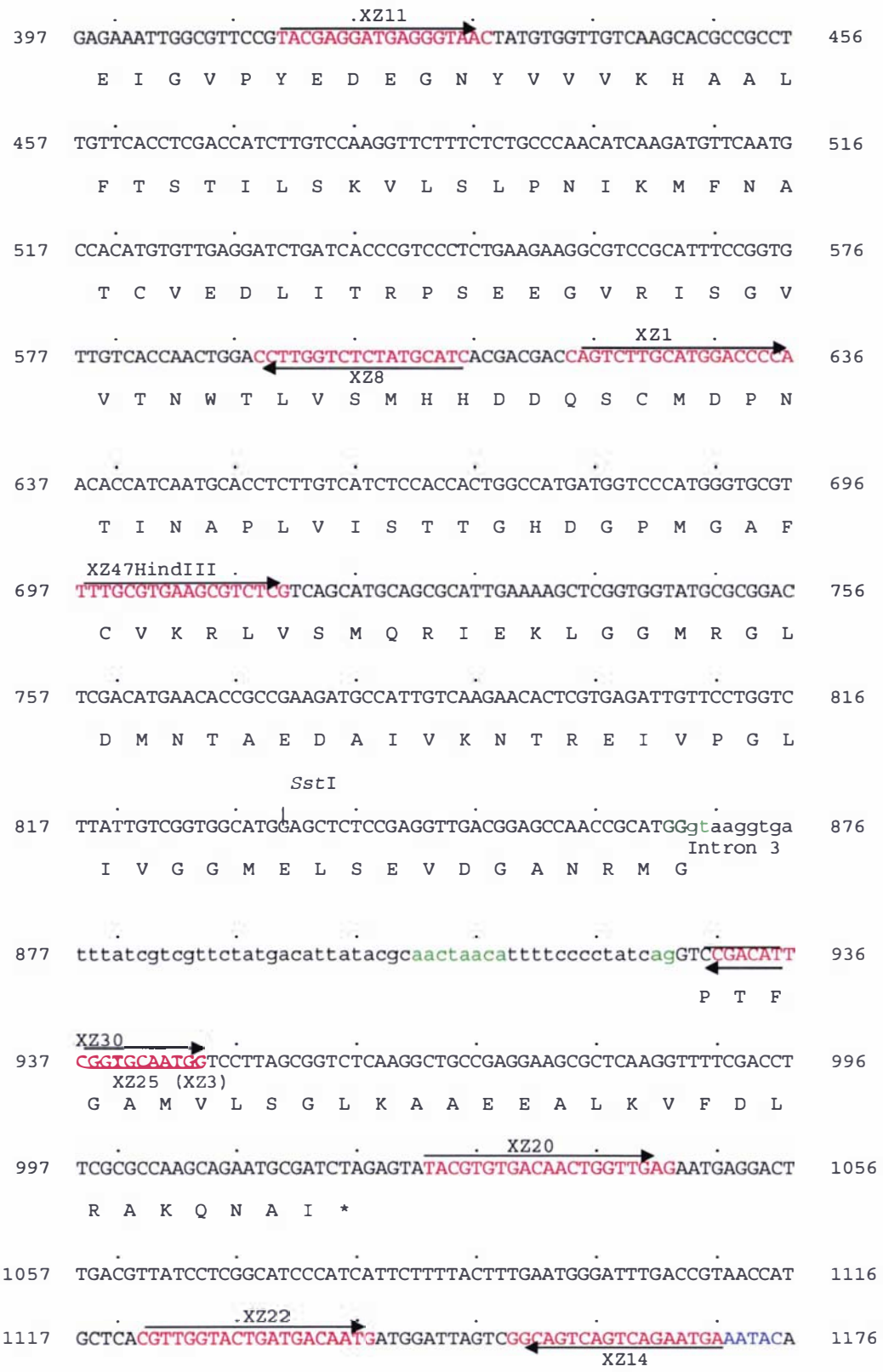
L G G Q L F S A M V M R K P A D A F L R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F T S T I L S K V L S L P N I K M F N A
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T C V E D L I T R P S E E G V R I S G V
XZ1
577 TTGTCACCAACTGGACCTTGGTCTCTATGCATCACGACGACCAGTCTTGCATGGACCCCA 636
XZ8
V T N W T L V S M H H D D Q S C M D P N
637 ACACCATCAATGCACCTCTTGTCATCTCCACCACTGGCCATGATGGTCCCATGGGTGCGT 696
T I N A P L V I S T T G H D G P M G A F
XZ47HindIII
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C V K R L V S M Q R I E K L G G M R G L
757 TCGACATGAACACCGCCGAAGATGCCATTGTCAAGAACACTCGTGAGATTGTTCCTGGTC 816
D M N T A E D A I V K N T R E I V P G L
SstI
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P T F
XZ30
937 CGGTGCAATGGTCCTTAGCGGTCTCAAGGCTGCCGAGGAAGCGCTCAAGGTTTTCGACCT 996
XZ25 (XZ3)
G A M V L S G L K A A E E A L K V F D L
XZ20
997 TCGCGCCAAGCAGAATGCGATCTAGAGTATACGTGTGACAACTGGTTGAGAATGAGGACT 1056
R A K Q N A I *
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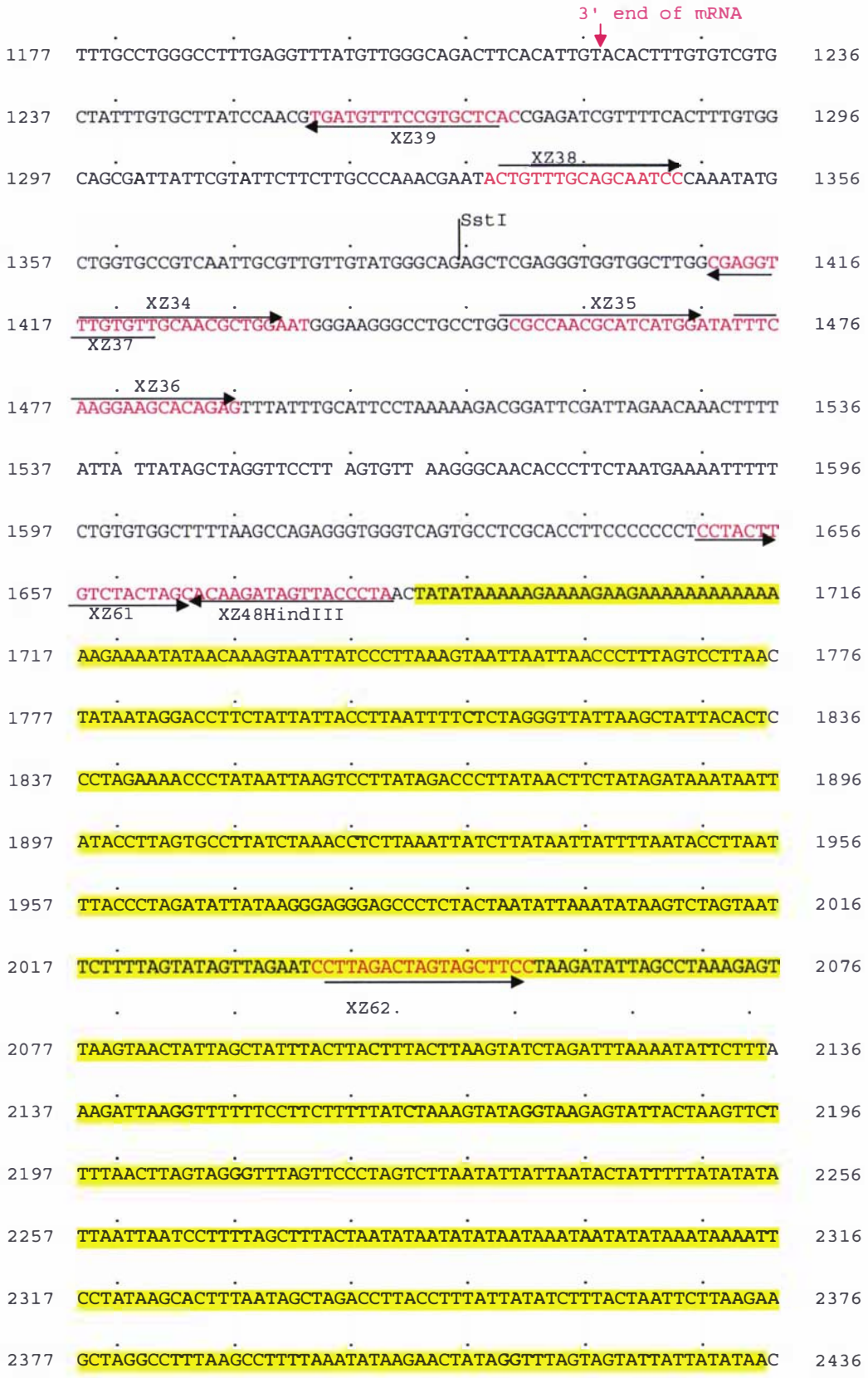
XZ14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1237 CTATTTGTGCTTATCCAACGTGATGTTTCCGTGCTCACCGAGATCGTTTTCACTTTGTGG 1296
XZ39
XZ38.
1297 CAGCGATTATTCGTATTCTTCTTGCCCAAACGAATACTGTTTGCAGCAATCCCAAATATG 1356
SstI
1357 CTGGTGCCGTCAATTGCGTTGTTGTATGGGCAGAGCTCGAGGGTGGTGGCTTGGCGAGGT 1416
XZ34
.XZ35
1417 TTGTGTTGCAACGCTGGAATGGGAAGGGCCTGCCTGGCGCCAACGCATCATGGATATTTC 1476
XZ37
XZ36
1477 AAGGAAGCACAGAGTTTATTTGCATTCCTAAAAAGACGGATTCGATTAGAACAAACTTTT 1536
1537 ATTA TTATAGCTAGGTTCCTT AGTGTT AAGGGCAACACCCTTCTAATGAAAATTTTT 1596
1597 CTGTGTGGCTTTTAAGCCAGAGGGTGGGTCAGTGCCTCGCACCTTCCCCCCCTCCTACTT 1656
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XZ61
XZ48HindIII
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1777 TATAATAGGACCTTCTATTATTACCTTAATTTTCTCTAGGGTTATTAAGCTATTACACTC 1836
1837 CCTAGAAAACCCTATAATTAAGTCCTTATAGACCCTTATAACTTCTATAGATAAATAATT 1896
1897 ATACCTTAGTGCCTTATCTAAACCTCTTAAATTATCTTATAATTATTTTAATACCTTAAT 1956
1957 TTACCCTAGATATTATAAGGGAGGGAGCCCTCTACTAATATTAAATATAAGTCTAGTAAT 2016
2017 TCTTTTAGTATAGTTAGAATCCTTAGACTAGTAGCTTCCTAAGATATTAGCCTAAAGAGT 2076
XZ62.
2077 TAAGTAACTATTAGCTATTTACTTACTTTACTTAAGTATCTAGATTTAAAATATTCTTTA 2136
2137 AAGATTAAGGTTTTTTCCTTCTTTTTATCTAAAGTATAGGTAAGAGTATTACTAAGTTCT 2196
2197 TTTAACTTAGTAGGGTTTAGTTCCCTAGTCTTAATATTATTAATACTATTTTTATATATA 2256
2257 TTAATTAATCCTTTTAGCTTTACTAATATAATATATAATAAATAATATATAAATAAAATT 2316
2317 CCTATAAGCACTTTAATAGCTAGACCTTACCTTTATTATATCTTTACTAATTCTTAAGAA 2376
2377 GCTAGGCCTTTAAGCCTTTTAAATATAAGAACTATAGGTTTAGTAGTATTATTATATAAC 2436

2437 TAGGAATAGGTTTCTCTAGAAGAAGTTTAAATTAGTTCTCTAATACCTTAACTCTAGGTA 2496

2497 GTAAGATAAGAGAGGTAAGCTCCTAAGAAGTAGTCTAGCCCTTAGCTTTAAGCCTAACTA 2556

2557 TAGCTACTATATTATTATATTCCTTAGGGCTTATATTTTAAACTTTAAATAACTTAGGTA 2616

2617 TGCCTTTTAGTATAAGCTT

Figure 3.11. Alignment of polypeptide sequences encoded by *thi1* from E8 and Lp19

The E8 (top line) and Lp19 (bottom line) Thi1 polypeptides were aligned using the GAP program from the Wisconsin Package version 9.1 (Genetics Computer Group). Vertical bars (|) represent identical amino acids; double dots (:) similar amino acids and single dot (·) non-similar amino acids. The non-identical amino acids are indicated by red letters.

```
E8Thi1      1 MSPPAAISPPRQVAELATQTSKFAVSGGSKTQTIDEMMGQWDSFKFAPIR 50
              |||||||||||||||||||||||||||||||||||||||.||||||||
Lp19Thi1    1 MSPPAAISPPRQVAELATQTSKFAVSGGSKTQTIDEMMGQWNSFKFAPIR 50

E8Thi1     51 ESQVSRAMTRRYFRDLDTYAESDIVIVGAGSCGLSAAYVLGKHRPDLKIC 100
              |||||||||||||.|||||||||||||||||||||||||||||||||||
Lp19Thi1   51 ESQVSRAMTRRYFQDLDTYAESDIVIVGAGSCGLSAAYVLGKHRPDLKIC 100

E8Thi1    101 IIEASVSPGGGAWLGGQLFSAMVMRKPADAFLREIGVPYEDEGNYVVVKH 150
              ||||||||||||||||||||||||||||||||||||||||||||||||||
Lp19Thi1  101 IIEASVSPGGGAWLGGQLFSAMVMRKPADAFLREIGVPYEDEGNYVVVKH 150

E8Thi1    151 AALFTSTILSKVLSLPNIKMFNATCVEDLITRPSEEGVRISGVVTNWTLV 200
              ||||||||:|||||||||||||||||||||||||||||||||||||||||
Lp19Thi1  151 AALFTSTIISKVLSLPNIKMFNATCVEDLITRPSEEGVRISGVVTNWTLV 200

E8Thi1    201 SMHHDDQSCMDPNTINAPLVISTTGHDGPMGAFCVKRLVSMQRIEKLGGM 250
              ||||||||||||||||||||||||||||||||||||||||||||||||||
Lp19Thi1  201 SMHHDDQSCMDPNTINAPLVISTTGHDGPMGAFCVKRLVSMQRIEKLGGM 250

E8Thi1    251 RGLDMNTAEDAIVKNTREIVPGLIVGGMELSEVDGANRMGPTFGAMVLSG 300
              |||||||||||||||||||||||||||||||:||||||||||||||||||
Lp19Thi1  251 RGLDMNTAEDAIVKNTREIVPGLIVGGMELSEIDGANRMGPTFGAMVLSG 300

E8Thi1    301 LKAAEEALKVFDLRAKQNAI 320
              ||||||||||||||||||||
Lp19Thi1  301 LKAAEEALKVFDLRAKQNAI 320
```

Figure 3.12. Alignment of the 5' upstream regions of *thi1* from Lp19 and E8

The 5' upstream regions of the E8 (top line) and Lp19 (bottom line) *thi1* genes were aligned using the GAP program from the Wisconsin Package version 9.1 (Genetics Computer Group). Vertical bars (|) represent identity. Mismatched nucleotides are indicated with red letters.

The proposed translation start site (in dark green bold text) was identified based on its similarity to the Kozak consensus sequence (Gurr *et al.*, 1987) (in dark green letters). The 5' ends of the mRNA, which were determined by 5'RACE are indicated by purple arrows. Two perfect 58 bp direct repeats in Lp19 *thi1* 5' region are shaded. Motifs region A and B which are proposed to be required for efficient splicing (Kubodera *et al.*, 2003) are underlined.

Putative transcriptional factor binding sites for CreA (SYGGRG), NIT2 (eg. GCGATA) and StuAP (eg. TGTCGCGCAC), identified from the TRANSFAC database (http://www.cbrc.jp/research/db/TFSEACH.html) and conserved between the Lp19 and E8 *thi1* promoter are highlighted in grey, turquoise and blue shading respectively.

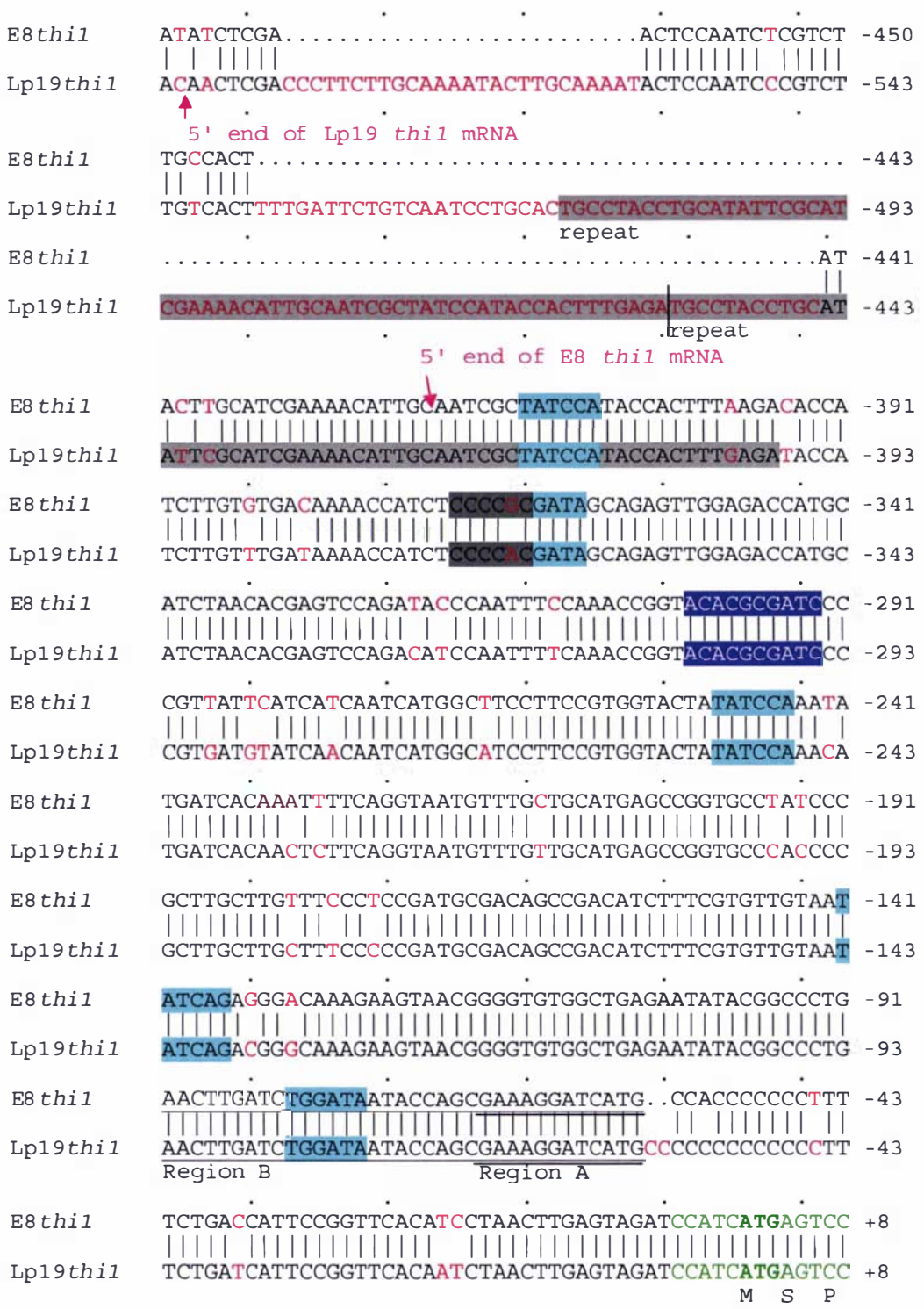

E8thi1
Lp19thi1
5' end of Lp19 thi1 mRNA
repeat
5' end of E8 thi1 mRNA
Region B
Region A
M S P

Figure 3.13. Southern blot analysis of the *thi1* gene in Lp19, Lp1 and E8 strains

A: The location of the Lp19 *thi1* sequence used as probe that was amplified using primer XZ59 & XZ60.

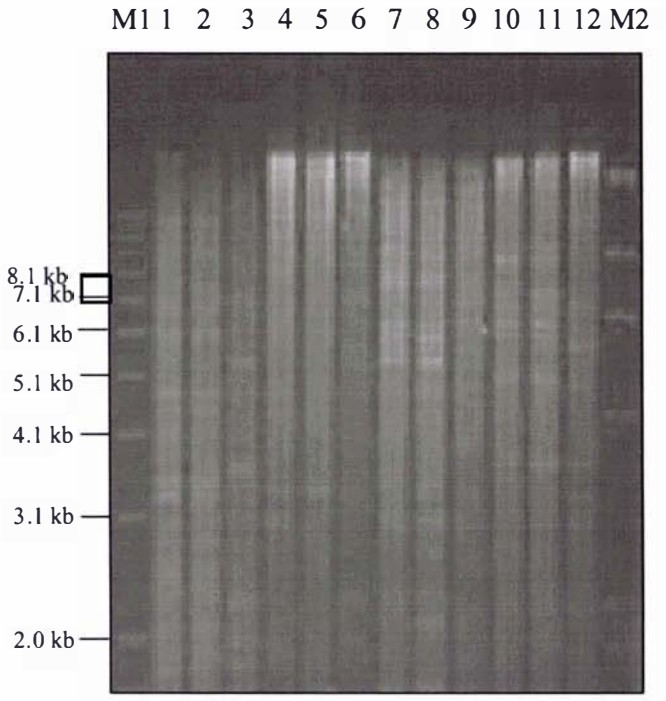
B: Agarose gel of 2 μg of Lp19 (lanes 1, 4, 7, 10), Lp1 (lanes 2, 5, 8, 11) and E8 (lanes 3, 6, 9, 12) genomic DNA digested with *Hin*dIII (lane 1, 2, 3), *Bam*HI (lane 4, 5, 6), *Eco*RV (lane 7, 8, 9), and *Pst*I (lane 10, 11, 12). Lane M1 and M2 contained 1 kb + ladder and λDNA/HindIII ladder (GibcoBRL).

C: Autoradiograph of the Southern blot of **B** probed with the digoxigenin-11-dUTP labeled probe shown in **A**.

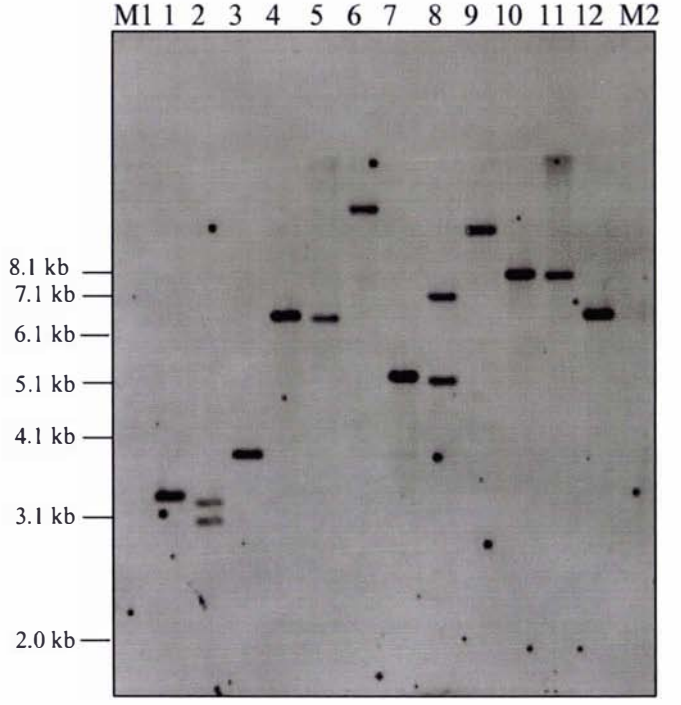
A


BamHIHindIII
Lp19 thil
HindIII
Probe

B


M1 1 2 3 4 5 6 7 8 9 10 11 12 M2
8.1 kb
7.1 kb
6.1 kb
5.1 kb
4.1 kb
3.1 kb
2.0 kb

C


M1 1 2 3 4 5 6 7 8 9 10 11 12 M2
8.1 kb
7.1 kb
6.1 kb
5.1 kb
4.1 kb
3.1 kb
2.0 kb

3.2. Characterisation and expression analysis of *thi1*

3.2.1. Characterisation of the Lp19 *thi1* mRNA

3.2.1.1. 5'RACE analysis of the Lp19 *thi1* mRNA

5'RACE (Section 2.9.2) was carried out to determine the 5'end of the Lp19 *thi1* mRNA as outlined in Fig. 3.14A. Multiple products were produced from the 5'RACE amplification (Fig. 3.14B), suggesting either multiple transcriptional start sites or alternative splicing in the 5' un-translated region. The final 5'RACE products were cloned into pGEM-T (Section 2.4) for sequence analysis. Among the recombinant plasmids analysed four different sized inserts were identified (Fig. 3.14C). A comparison of the sequences from pXZ15, pXZ16 and pXZ18 to the Lp19 *thi1* sequence showed that all started at 590 bp upstream of the putative translation start codon (Fig. 3.15). The sequence of pXZ15 was identical to that of pXZ16 except that it had a longer dC tail. Three introns were identified in the 5' un-translated region of the *thi1* transcript, at position -388 to –326, -306 to -226 and -225 to -8. Plasmids pXZ15 and pXZ16 had all three introns spliced, while pXZ18 had introns 1 and 3 spliced. Alignment of the sequence from pXZ20 with Lp19 *thi1* sequence suggested the presence of a second transcription start site at +28 bp (Fig. 3.15). However, this is most likely to be a truncated transcript rather than a true one.

The 5' un-translated region of *thi1* (-1 to –590) contains several potential translation initiation codons in addition to the Thi1 ATG (Fig 3.16). A short polypeptide (PEP18) of 18 amino acids could be translated from a mRNA in which introns 1, 2 and 3 are all spliced. Two polypeptides, PEP18 and PEP37 (37 amino acids), could be translated from mRNA with introns 1 and 3 spliced. For a mRNA with just intron 3 spliced, three polypeptides PEP18, PEP37 and PEP42 (42 amino acids) could be translated. All these polypeptides are

followed by a translation stop codon prior to the Thi1 translation start site, or are in a different reading frame to that of the Thi1 (Fig 3.16). These upstream open reading frames might inhibit initiation or re-initiation of translation of Thi1.

3.2.1.2. 3'RACE analysis of the Lp19 *thi1* mRNA

3'RACE (Section 2.9.1) was carried out to determine the 3' end of the Lp19 *thi1* mRNA as shown in Fig. 3.17A. Agarose gel analysis of the final PCR products showed that two bands were amplified (Fig. 3.17B). The final PCR products were cloned into pGEM-T and digestion (Section 2.4) of the recombinant plasmids identified fragments of two different sizes (Fig. 3.17C). Sequence analysis of these 3'RACE products identified two mRNA ends, one at +1187 and the other at +1257. Both were preceded by a consensus polyadenylation site (Fig. 3.18). In addition, an intron of 59 bp was identified in the 3' coding region of *thi1* (Fig. 3.18). This intron was previously predicted by comparison of the Lp19 *thi1* gene sequence with homologues from other organisms.

3.2.2. Characterisations of the E8 *thi1* mRNA

The 5' and 3' ends of the E8 *thi1* mRNA were also determined by RACE (Section 2.9). Sequence analysis of the 5' RACE product showed that the E8 *thi1* transcript starts at -420 bp (Fig. 3.19). Two introns at –386 to –324 and –223 to –8 were identified in the 5' un-translated region of the gene (Fig. 3.19). In addition to the major Thi1 polypeptide, a potential polypeptide (PEP43) of 43 amino acids could be translated in the E8 *thi1* 5' upstream region (Fig. 3.20). PEP43 is in a different frame from Thi1. In contrast to Lp19 *thi1*, 5' and 3' RACE analysis of E8 *thi1* identified single products. Sequence analysis of the 3' RACE product showed that transcription terminates at +1222 bp (Fig. 3.21). A consensus polyadenylation site precedes this termination site. An intron of 59 bp at +868

to +926 was identified in the 3' coding region of E8 *thi1*, at an identical position of Lp19 *thi1*. Although only one transcript was identified in our experiment, this does not necessarily mean that there is no alternative splicing present in E8 *thi1* gene.

3.2.3. Expression and alternative splicing of the Lp19 *thi1* gene in culture

As previous bioinformatic analysis showed that the Lp19 *thi1* gene promoter region contains many putative NIT2 and CreA factor binding sites, RT-PCR (Section 2.8) was employed to determine whether the pattern of alternative splicing of Lp19 *thi1* varied with the carbon and nitrogen source used for growth. Amplifications were carried out with primers that flank the introns present in the 5' UTR and the intron present in the 3' coding region of the gene (Fig. 3.22). To distinguish cDNA products from genomic products, Lp19 genomic DNA was amplified as a control. Lp19 β-tubulin cDNA was also amplified as a control for the different cDNAs amplified.

The RT-PCR products amplified using the β-tubulin gene primer pair (Fig. 3.22A) or the *thi1* primer pair XZ1 and XZ25 (Fig. 3.22B) were very similar and did not vary with the different growth conditions. When amplified with primer set XZ26 and XZ28, each of the RNA samples gave two to four products that differed in size (about 350 bp, 450 bp, 500 bp and 550 bp) and intensity (Fig. 3.19C), a reflection that *thi1* undergoes alternative splicing in different nutrient conditions. Furthermore, the levels and patterns of alternatively spliced transcripts varied in different nutrient conditions.

RT-PCR products amplified with primer set XZ26 and XZ28 were cloned into pGEM-T (Section 2.4) and recombinant plasmids containing 357 bp, 439 bp or 502 bp inserts, but not the 550 bp fragment, were obtained. Sequence analysis of these products showed that

three transcript isoforms were present as shown in Fig. 3. 22D. The intron boundaries of these products were identical to those identified in the 5'RACE analysis. The results suggested that carbon and nitrogen sources affect the expression of the Lp19 *thi1* gene by altering the splicing of the transcripts. These results might indicate that carbon and nitrogen sources may regulate Lp19 *thi1* gene expression by alternative splicing of transcripts.

Interestingly, addition of thiamine did not reduce the level of *thi1* (Fig. 3.22B lane 9) mRNA, nor change the alternatively spliced isoforms (Fig. 3.22C lane 9) when the hyphae were grown in basal salts without carbon or nitrogen source (Fig 3.22B and C lane 8).

3.2.4. Expression and alternative splicing of the Lp19 *thi1* gene *in planta*

RT-PCR (Section 2.8) was carried out to examine expression of the Lp19 *thi1* in the perennial ryegrass host. As shown in Fig. 3.23, the gene was expressed and more than one transcript isoform was produced in the host tissue. Cloning and sequence analysis of the RT-PCR products amplified from primers XZ26 and XZ28 identified two transcript isoforms, corresponding to the type I and type II alternatively spliced products as shown in Fig. 3.22D.

3.2.5. Summary and discussion

The transcription start site of both the Lp19 and E8 *thi1* genes in this study were determined by 5'RACE (Sections 3.2.1.1 and 3.2.2). It is interesting that the proposed transcription start site of the Lp19 *thi1* is at –590 bp (Section 3.2.1.1), and E8 at –420 (Section 3.2.1.1). As determination of the transcription start site relies on both the presence of a full-length 5' un-translated region and efficient reverse transcription, the 5'RACE

technique used in this study dose not necessarily generate full length cDNA products. The transcription sites detected by this method could be at truncated mRNA ends. Alternatively, the site detected could be one of several transcription start sites, since multiple transcription initiation sites are often detected in filamentous fungal genes (Gurr *et al.*, 1987).

The cloning and sequencing of the 5'RACE products and RT-PCR both demonstrated that the 5'UTR of the Lp19 *thi1* gene is alternatively spliced (Sections 3.2.1 and 3.2.2), while only one type of E8 *thi1* transcript was identified by 5'RACE (Section 3.2.2). This would be the result of limited number of clones containing 5'RACE products that were sequenced.

Although RT-PCR may not amplify each of the Lp19 *thi1* mRNA isoforms at the same efficiency, the different ratios of RT-PCR products would reflect the different ratios of the mRNA isoforms produced from alternative splicing of the Lp19 *thi1* gene under the different nutrient conditions. Alternative splicing has been found in some other fungal genes, including the transposable element *Restless* from *Neurospora crassa* (Kempken *et al.* 1996; 2004), a gene encoding a zinc finger protein in *S. pombe* (Okazaki and Niwa, 2000), the *rgb-1* gene encoding the B regulatory subunit of the type 2A phosphatase in *N. crassa* (Yatzkan and Yarden, 1999), and the *hex1* gene from *Trichoderma reesei* (Curach, *et al.* 2004). Mutations at the 5' and 3' splice sites can modify the alternative splicing of the transcripts of the transposon Restless (Kempken *et al.* 2004). The three transcripts of *hex1* gene involved in producing precursors of the Woronin body in the fungus could encode for two forms of the protein (Curach, *et al.* 2004). However, all transcripts of the Lp19 and E8 *thi1* genes appear to encode a single THI protein (Fig. 3.16 and Fig. 3.20).

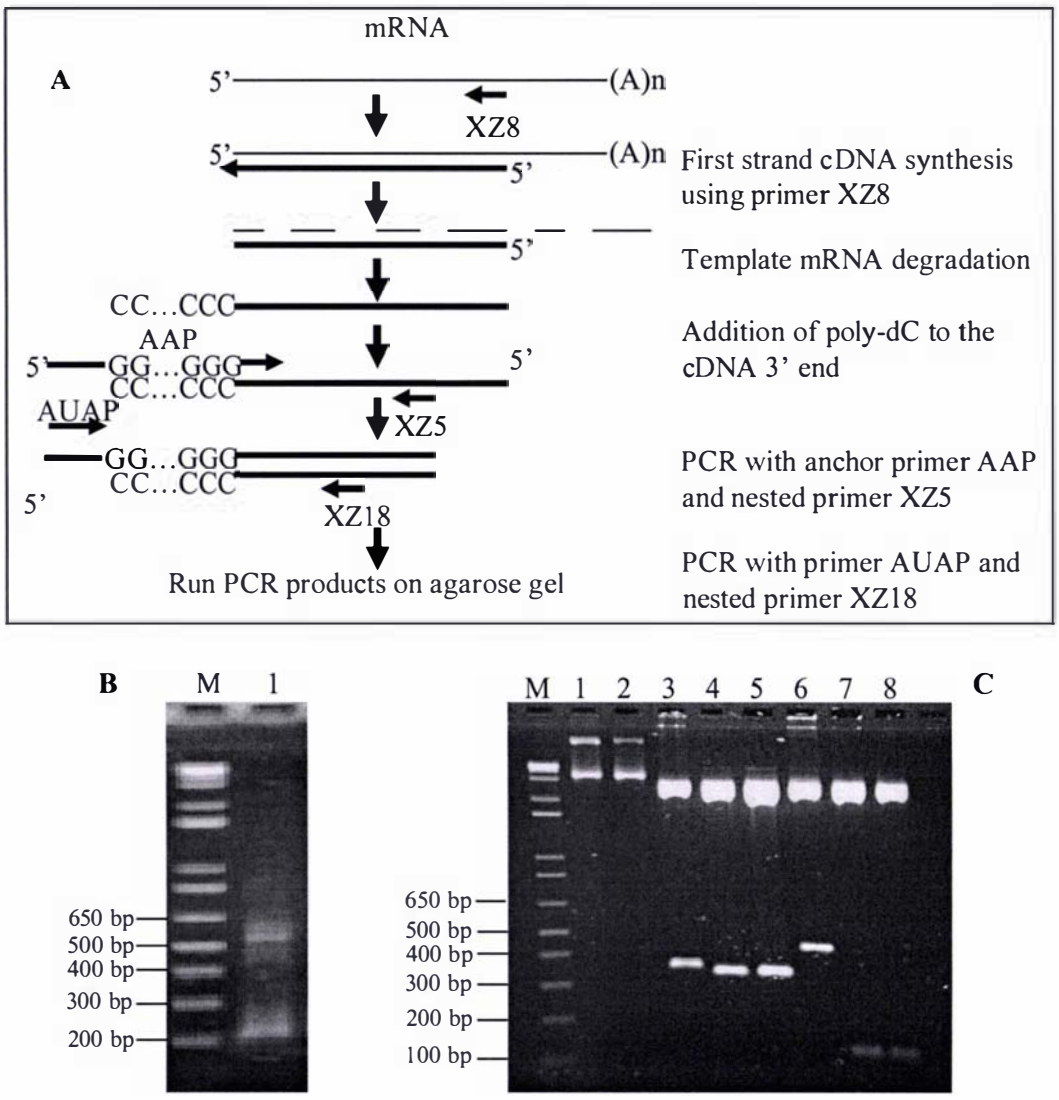


Figure 3.14. Determination 5' end of Lp19 *thi1* mRNA by RACE

A: Procedure used for 5'RACE analysis.

B: Agarose gel analysis of PCR products amplified using primers AUAP and XZ18.

C: *EcoR*I digests of plasmids pXZ13 (lane1), pXZ14 (lane 2), pXZ15 (lane 3), pXZ16 (lane 4), pXZ17 (lane 5), pXZ18 (lane 6), pXZ19 (lane 7) and pXZ20 (lane 8) containing 5'RACE products cloned into pGEM-T easy vector.

Lane M contains the 1 kb + ladder (GibcoBRL).

Figure 3.15. Alignment of sequences from the Lp19 *thi1* 5'RACE products

The sequences of Lp19 5'RACE products in plasmids pXZ15, pXZ18 and pXZ20 are shown in alignment with the Lp19 *thi1* gene sequence. The nucleotides are numbered (on the right side) with respect to the A (+1) of the proposed translation start site (in dark green text). Introns in the Lp19 *thi1* gene sequence are in lower case letters. The alternative spliced intron is highlighted in yellow letters. The 5' and 3' splice sites and proposed lariat sequences (Gurr *et al.*, 1987) are highlighted in green text. Two direct repeats of 58 bp are highlighted in purple text. The 5' end of Lp19 *thi1* mRNA is indicated by a purple arrow. Motifs region A and B which are proposed to be required for efficient splicing (Kubodera *et al.*, 2003) are underlined.

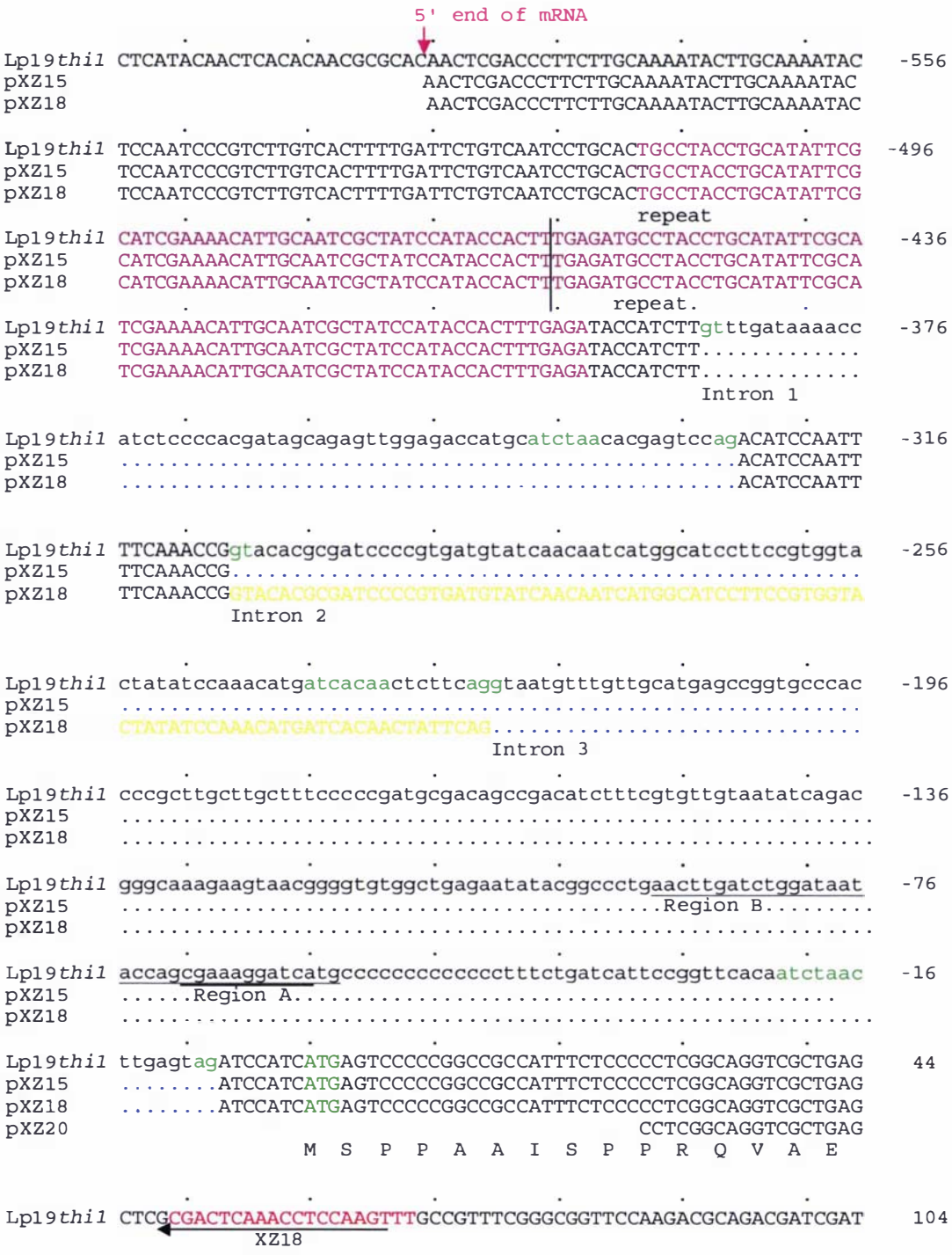
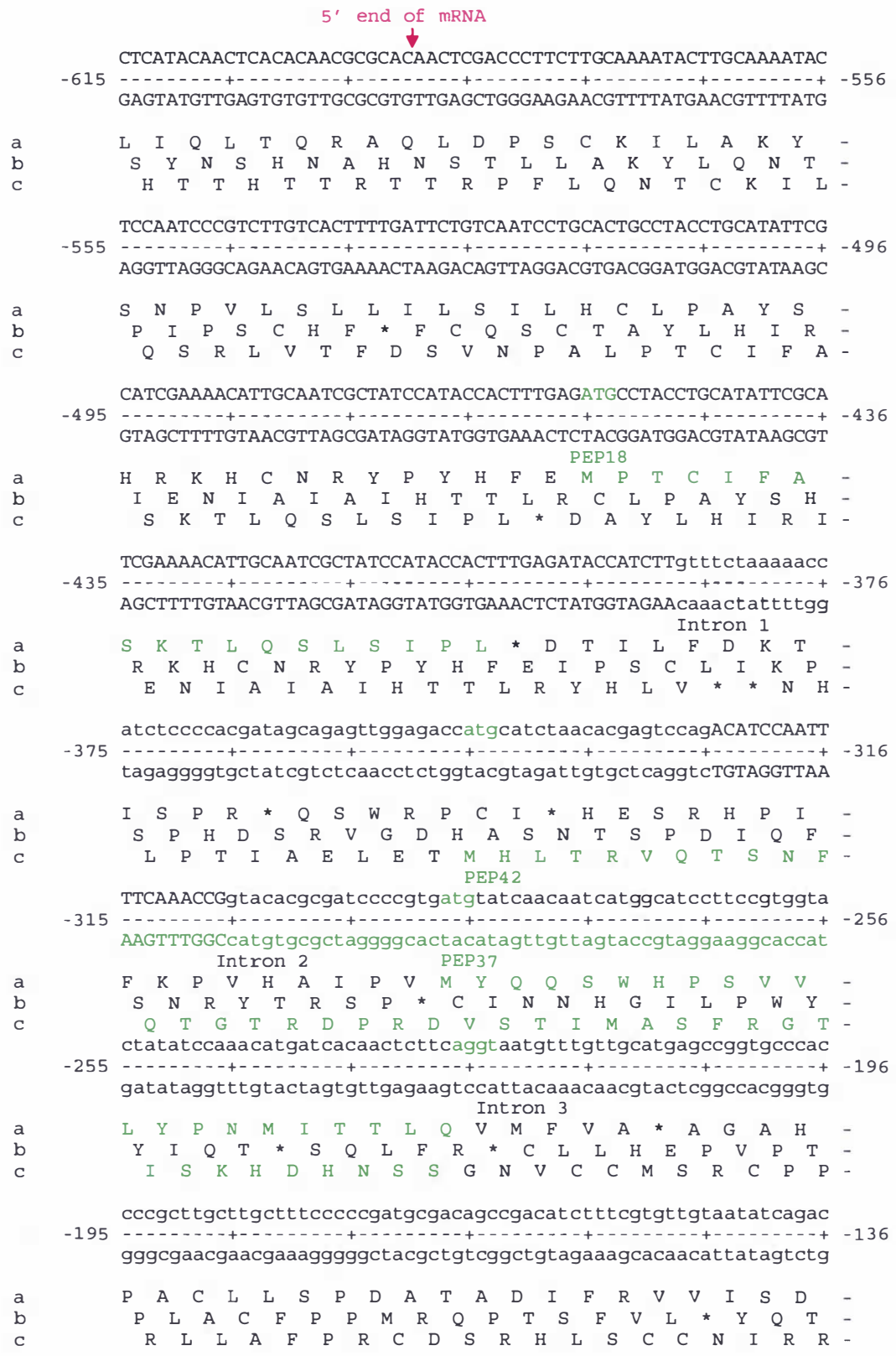
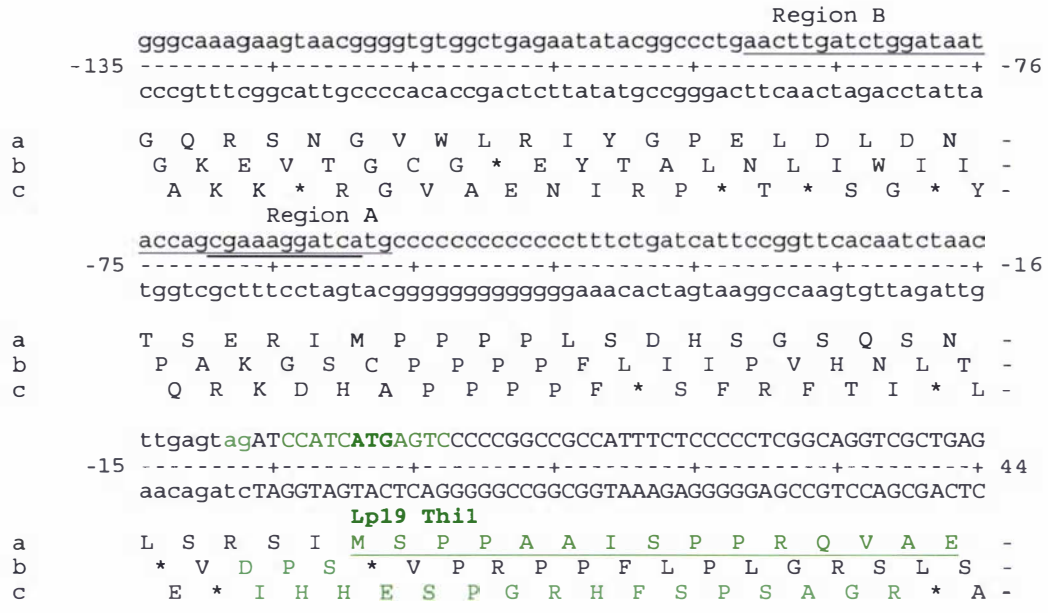

5' end of mRNA
Lp19thi1 CTCATACAACTCACACAACGCGCACAACTCGACCCTTCTTGCAAAATACTTGCAAAATAC -556
pXZ15 AACTCGACCCTTCTTGCAAAATACTTGCAAAATAC
pXZ18 AACTCGACCCTTCTTGCAAAATACTTGCAAAATAC
Lp19thi1 TCCAATCCCGTCTTGTCACTTTTGATTCTGTCAATCCTGCACTGCCTACCTGCATATTCG -496
pXZ15 TCCAATCCCGTCTTGTCACTTTTGATTCTGTCAATCCTGCACTGCCTACCTGCATATTCG
pXZ18 TCCAATCCCGTCTTGTCACTTTTGATTCTGTCAATCCTGCACTGCCTACCTGCATATTCG
repeat
Lp19thi1 CATCGAAAACATTGCAATCGCTATCCATACCACTTTGAGATGCCTACCTGCATATTCGCA -436
pXZ15 CATCGAAAACATTGCAATCGCTATCCATACCACTTTGAGATGCCTACCTGCATATTCGCA
pXZ18 CATCGAAAACATTGCAATCGCTATCCATACCACTTTGAGATGCCTACCTGCATATTCGCA
repeat.
Lp19thi1 TCGAAAACATTGCAATCGCTATCCATACCACTTTGAGATACCATCTTgtttgataaaacc -376
pXZ15 TCGAAAACATTGCAATCGCTATCCATACCACTTTGAGATACCATCTT.....
pXZ18 TCGAAAACATTGCAATCGCTATCCATACCACTTTGAGATACCATCTT.....
Intron 1
Lp19thi1 atctccccacgatagcagagttggagaccatgcatctaacacgagtccagACATCCAATT -316
pXZ15 .....ACATCCAATT
pXZ18 .....ACATCCAATT
Lp19thi1 TTCAAACCGgtacacgcgatccccgtgatgtatcaacaatcatggcatccttccgtggta -256
pXZ15 TTCAAACCG.....
pXZ18 TTCAAACCG
Intron 2
Lp19thi1 ctatatccaaacatgatcacaactcttcaggtaatgtttgttgcatgagccggtgcccac -196
pXZ15 .....
pXZ18 CTATATCCAAACATGATCACAACTATTCAG.....
Intron 3
Lp19thi1 cccgcttgcttgctttcccccgatgcgacagccgacatctttcgtgttgtaatatcagac -136
pXZ15 .....
pXZ18 .....
Lp19thi1 gggcaaagaagtaacggggtgtggctgagaatatacggccctgaacttgatctggataat -76
pXZ15 .....Region B.....
pXZ18 .....
Lp19thi1 accagcgaaaggatcatgccccccccccccctttctgatcattccggttcacaatctaac -16
pXZ15 .....Region A.....
pXZ18 .....
Lp19thi1 ttgagtagATCCATCATGAGTCCCCCGGCCGCCATTTCTCCCCCTCGGCAGGTCGCTGAG 44
pXZ15 .....ATCCATCATGAGTCCCCCGGCCGCCATTTCTCCCCCTCGGCAGGTCGCTGAG
pXZ18 .....ATCCATCATGAGTCCCCCGGCCGCCATTTCTCCCCCTCGGCAGGTCGCTGAG
pXZ20 CCTCGGCAGGTCGCTGAG
M S P P A A I S P P R Q V A E
Lp19thi1 CTCGCGACTCAAACCTCCAAGTTTGCCGTTTCGGGCGGTTCCAAGACGCAGACGATCGAT 104
XZ18

Figure 3.16. Analysis of potential peptide sequences in Lp19 *thi1* 5' upstream region

Potential polypeptide sequences from the Lp19 *thi1* 5' upstream region were translated using MAP program from the Wisconsin Package 9.1, GCG. The nucleotides are numbered with respect to the A (+1) of the proposed *thi1* translation start site (in dark bold green text), which is surrounded by a sequence with close similarity to the Kozak consensus sequence (Gurr *et al.*, 1987). Other potential translation start sites in the Lp19 *thi1* 5' upstream region are highlighted in green letters, stop codons with stars, and introns with lower case letters. Potential peptides are designated as PEP18, PEP37 and PEP42 respectively with the number after PEP indicating the number of amino acids in the peptide. Motifs region A and B which are proposed to be required for efficient splicing (Kubodera *et al.*, 2003) are underlined.


5′ end of mRNA
PEP18
Intron 1
PEP42
Intron 2
PEP37
Intron 3


Region B
gggcaaagaagtaacggggtgtggctgagaatatacggccctgaacttgatctggataat
-135 ---------+---------+---------+---------+---------+---------+ -76
cccgtttcggcattgccccacaccgactcttatatgccgggacttcaactagacctatta
a G Q R S N G V W L R I Y G P E L D L D N -
b G K E V T G C G * E Y T A L N L I W I I -
c A K K * R G V A E N I R P * T * S G * Y -
Region A
accagcgaaaggatcatgccccccccccccctttctgatcattccggttcacaatctaac
-75 ---------+---------+---------+---------+---------+---------+ -16
tggtcgctttcctagtacgggggggggggggaaacactagtaaggccaagtgttagattg
a T S E R I M P P P P L S D H S G S Q S N -
b P A K G S C P P P P F L I I P V H N L T -
c Q R K D H A P P P P F * S F R F T I * L -
ttgagtagATCCATCATGAGTCCCCCGGCCGCCATTTCTCCCCCTCGGCAGGTCGCTGAG
-15 ---------+---------+---------+---------+---------+---------+ 44
aacagatcTAGGTAGTACTCAGGGGGCCGGCGGTAAAGAGGGGGAGCCGTCCAGCGACTC
Lp19 Thi1
a L S R S I M S P P A A I S P P R Q V A E -
b * V D P S * V P R P P F L P L G R S L S -
c E * I H H E S P G R H F S P S A G R * A -

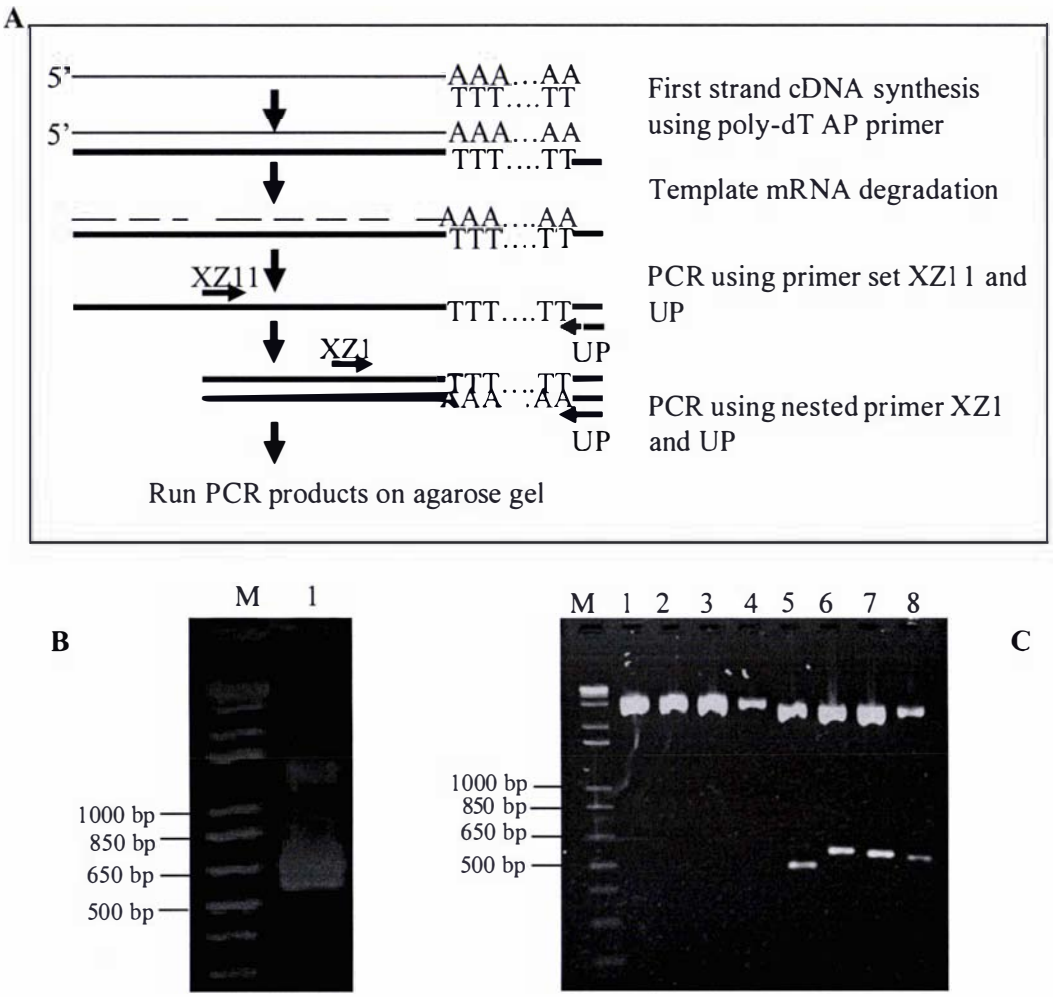


Figure 3.17. Determination of 3' end of Lp19 *thi1* mRNA by RACE

A: Procedure used for 3'RACE analysis.

B: Agarose gel analysis of PCR products amplified using primers XZ11 & UP (lane1).

C: *Pst*I digests (lanes 1-4) and *Pst*I/*Nco*I double digests (lanes 5-8) of plasmids pXZ21 (lanes 1 and 5), pXZ22 (lanes 2 and 6), pXZ23 (lanes 3 and 7) and pXZ24 (lanes 4 and 8) containing 3'RACE products cloned into vector pGEM-T.

Lane M contains the 1 kb + ladder (GibcoBRL).

Figure 3.18. Alignment of sequences from Lp19 *thi1* 3'RACE products

The sequences of Lp19 *thi1* 3'RACE products in plasmids pXZ21 and pXZ22 are shown in alignment with the Lp19 *thi1* gene sequence. The nucleotides are numbered (on the right side) with respect to the A (+1) of the proposed translation start site. The intron in the Lp19 *thi1* gene sequence is in lower case letters. The 5' and 3' splice sites and proposed lariat sequences (Gurr *et al.*, 1987) are outlined in green. Putative polyadenylation recognition sequences (Gurr *et al.*, 1987) are shown in blue text. Two perfect 52 bp repeat sequences are shown in purple. The 3' ends of Lp19 *thi1* mRNA is indicated by purple arrows. The translation stop codon is shown in red text with a star underneath.


Lp19thi1 AACACCGCCGAAGATGCCATTGTCAAAAACACTCGTGAAATTGTTCCTGGTCTTATTGTC 824
pXZ22 AACACCGCCGAAGATGCCATTGTCAAAAACACTCGTGAAATTGTTCCTGGTCTTATTGTC
pXZ21 AACACCGCCGAAGATGCCATTGTCAAAAACACTCGTGAAATTGTTCCTGGTCTTATTGTC
Lp19thi1 GGTGGCATGGAGCTCTCCGAGATTGACGGAGCCAACCGCATGGgtagggtgatctatcgt 884
pXZ22 GGTGGCATGGAGCTCTCCGAGATTGACGGAGCCAACCGCATGG.....
pXZ21 GGTGGCATGGAGCTCTCCGAGATTGACGGAGCCAACCGCATGG.....
Intron 4
Lp19thi1 cgttctatgacattatacgcaactaacattctcccctatcagGTCCAACATTCGGTGCAA 944
pXZ22 .....GTCCAACATTCGGTGCAA
pXZ21 .....GTCCAACATTCGGTGCAA
Lp19thi1 TGGTCCTTAGCGGTCTCAAGGCTGCCGAGGAAGCGCTCAAGGTTTTTGACCTTCGCGCAA 1004
pXZ22 TGGTCCTTAGCGGTCTCAAGGCTGCCGAGGAAGCGCTCAAGGTTTTTGACCTTCGCGCAA
pXZ21 TGGTCCTTAGCGGTCTCAAGGCTGCCGAGGAAGCGCTCAAGGTTTTTGACCTTCGCGCAA
Lp19thi1 AGCAGAATGCCATCTAAAGTATACGTGTGACAACTGGTTGAGAATGAAGACTTGACGTTA 1064
pXZ22 AGCAGAATGCCATCTAAAGTATACGTGTGACAACTGGTTGAGAATGAAGACTTGACGTTA
pXZ21 AGCAGAATGCCATCTAAAGTATACGTGTGACAACTGGTTGAGAATGAAGACTTGACGTTA
* Translation stop codon
Lp19thi1 TCCTCGTCGTCCATCATTCTTTTACTTTGAATGGGATTTGACCGTAACCACGCTCACGTT 1124
pXZ22 TCCTCGTCGTCCATCATTCTTTTACTTTGAATGGGATTTGACCGTAACCACGCTCACGTT
pXZ21 TCCTCGTCGTCCATCATTCTTTTACTTTGAATGGGATTTGACCGTAACCACGCTCACGTT
repeat
Lp19thi1 GATACTGATGGCAATGATGAATTAGTCGGCAGTCAGTCAGAAAGAAATACAATTGACCAT 1184
pXZ22 GATACTGATGGCAATGATGAATTAGTCGGCAGTCAGTCAGAAAGAAATACAATTGACCAT
pXZ21 GATACTGATGGCAATGATGAATTAGTCGGCAGTCAGTCAGAAAGAAATACAATTGACCAT
mRNA 3'end I
Lp19thi1 TCTCGCTCACGTTGATACTGATGGCAATGATGAATTAGTCGGCAGTCAGTCAGAATGAAA 1244
pXZ22 TCTCGCTCACGTTGATACTGATGGCAATGATGAATTAGTCGGCAGTCAGTCAGAATGAAA
pXZ21 TCT
repeat
mRNA 3'end II
Lp19thi1 TACAATTGCCCTG
pXZ22 TACAATTGCCCTG

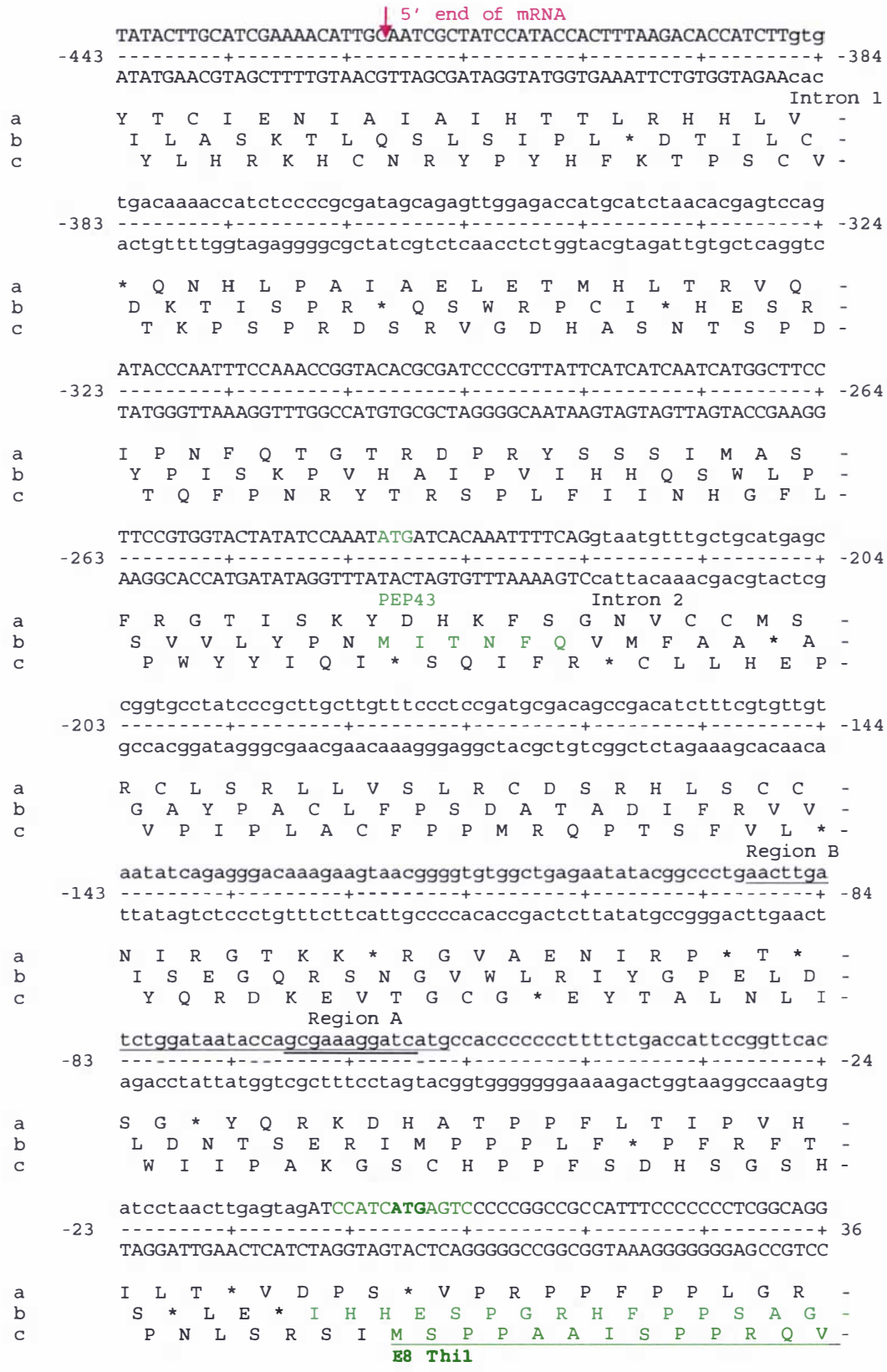
Figure 3.19. Alignment of 5' RACE sequence from E8 *thi1*

The sequence of E8 *thi1* 5'RACE product is shown in alignment with the E8 *thi1* gene sequence. The nucleotides are numbered (on the right side) with respect to the A (+1) of the proposed translation start site (in bold dark green). The intron is highlighted in lower case letters. The 5' and 3' splice sites and proposed lariat sequences (Gurr *et al.*, 1987) are highlighted in green text. The 5' end of E8 *thi1* mRNA is indicated by a purple arrow. Motifs region A and B which are proposed to be required for efficient splicing (Kubodera *et al.*, 2003) are underlined.


E8thi1 GCGATAATAAAACCCCTCATACAACTGCACATATCTCGAACTCCAATCTCGTCTTGCCAC -444
5' end of mRNA
E8thi1 TATACTTGCATCGAAAACATTGCAATCGCTATCCATACCACTTTAAGACACCATCTTgtg -384
5'RACE AATCGCTATCCATACCACTTTAAGACACCATCTT
E8thi1 tgacaaaaccatctccccgcgatagcagagttggagaccatgcatctaacacgagtccag -324
5'RACE
E8thi1 ATACCCAATTTCCAAACCGGTACACGCGATCCCCGTTATTCATCATCAATCATGGCTTCC -264
5'RACE ATACCCAATTTCCAAACCGGTACACGCGATCCCCGTTATTCATCATCAATCATGGCTTCC
E8thi1 TTCCGTGGTACTATATCCAAATATGATCACAAATTTTCAGgtaatgtttgctgcatgagc -204
5'RACE TTCCGTGGTACTATATCCAAATATGATCACAAATTTTCAG
E8thi1 cggtgcctatcccgcttgcttgtttccctccgatgcgacagccgacatctttcgtgttgt -144
5'RACE
E8thi1 aatatcagagggacaaagaagtaacggggtgtggctgagaatatacggccctgaacttga -84
5'RACE Region B
E8thi1 tctggataataccagcgaaaggatcatgccacccccccttttctgaccattccggttcac -24
5'RACE Region A
E8thi1 atcctaacttgagtagATCCATCATGAGTCCCCCGGCCGCCATTTCCCCCCCTCGGCAGG 36
5'RACE ATCCATCATGAGTCCCCCGGCCGCCATTTCCCCCCCTCGGCAGG
M S P P A A I S P P R Q V

Figure 3.20. Analysis of potential peptide sequences in E8 *thi1* 5' upstream region

Potential polypeptide sequences from the E8 *thi1* 5' upstream region were translated using MAP program from the Wisconsin Package 9.1, GCG. The nucleotides are numbered with respect to the A (+1) of the proposed *thi1* translation start site (in dark bold green text), which is surrounded by a sequence with close similarity to the Kozak consensus sequence (Gurr *et al.*, 1987). Other potential translation start sites in the E8 *thi1* 5' upstream region are highlighted in green letters, stop codons with stars, and introns with lower case letters. The potential peptide is designated as PEP43 with the number after PEP indicating the number of amino acids in the peptide. Motifs region A and B which are proposed to be required for efficient splicing (Kubodera *et al.*, 2003) are underlined.


5′ end of mRNA
TATACTTGCATCGAAAACATTGCAATCGCTATCCATACCACTTTAAGACACCATCTTgtg
-443
-384
ATATGAACGTAGCTTTTGTAACGTTAGCGATAGGTATGGTGAAATTCTGTGGTAGAAcac
Intron 1
a Y T C I E N I A I A I H T T L R H H L V -
b I L A S K T L Q S L S I P L * D T I L C -
c Y L H R K H C N R Y P Y H F K T P S C V -
tgacaaaaccatctccccgcgatagcagagttggagaccatgcatctaacacgagtccag
-383
-324
actgttttggtagaggggcgctatcgtctcaacctctggtacgtagattgtgctcaggtc
a * Q N H L P A I A E L E T M H L T R V Q -
b D K T I S P R * Q S W R P C I * H E S R -
c T K P S P R D S R V G D H A S N T S P D -
ATACCCAATTTCCAAACCGGTACACGCGATCCCCGTTATTCATCATCAATCATGGCTTCC
-323
-264
TATGGGTTAAAGGTTTGGCCATGTGCGCTAGGGGCAATAAGTAGTAGTTAGTACCGAAGG
a I P N F Q T G T R D P R Y S S S I M A S -
b Y P I S K P V H A I P V I H H Q S W L P -
c T Q F P N R Y T R S P L F I I N H G F L -
TTCCGTGGTACTATATCCAAATATGATCACAAATTTTCAGgtaatgtttgctgcatgagc
-263
-204
AAGGCACCATGATATAGGTTTATACTAGTGTTTAAAAGTCcattacaaacgacgtactcg
PEP43
Intron 2
a F R G T I S K Y D H K F S G N V C C M S -
b S V V L Y P N M I T N F Q V M F A A * A -
c P W Y Y I Q I * S Q I F R * C L L H E P -
cggtgcctatcccgcttgcttgtttccctccgatgcgacagccgacatctttcgtgttgt
-203
-144
gccacggatagggcgaacgaacaaagggaggctacgctgtcggctctagaaagcacaaca
a R C L S R L L V S L R C D S R H L S C C -
b G A Y P A C L F P S D A T A D I F R V V -
c V P I P L A C F P P M R Q P T S F V L * -
Region B
aatatcagagggacaaagaagtaacggggtgtggctgagaatatacggccctgaacttga
-143
-84
ttatagtctccctgtttcttcattgccccacaccgactcttatatgccgggacttgaact
a N I R G T K K * R G V A E N I R P * T * -
b I S E G Q R S N G V W L R I Y G P E L D -
c Y Q R D K E V T G C G * E Y T A L N L I -
Region A
tctggataataccagcgaaaggatcatgccaccccccctttctgaccattccggttcac
-83
-24
agacctattatggtcgctttcctagtacggtgggggggaaaagactggtaaggccaagtg
a S G * Y Q R K D H A T P P F L T I P V H -
b L D N T S E R I M P P P L F * P F R F T -
c W I I P A K G S C H P P F S D H S G S H -
atcctaacttgagtagATCCATCATGAGTCCCCGGCCGCCATTTCCCCCCCTCGGCAGG
-23
36
TAGGATTGAACTCATCTAGGTAGTACTCAGGGGGCCGGCGGTAAAGGGGGGGAGCCGTCC
a I L T * V D P S * V P R P P F P P L G R -
b S * L E * I H H E S P G R H F P P S A G -
c P N L S R S I M S P P A A I S P P R Q V -
E8 ThiI


TTGCCGAGCTCGCGACTCAAACCTCCAAGTTTGCCGTTTCGGGCGGTTCCAAGACGCAGA
37
96
AACGGCTCGAGCGCTGAGTTTGGAGGTTCAAACGGCAAAGCCCGCCAAGGTTCTGCGTCT
a L P S S R L K P P S L P F R A V P R R R -
b C R A R D S N L Q V C R F G R F Q D A D -
c A E L A T Q T S K F A V S G G S K T Q T -
CGATCGATGAGATGATGGGCCAGTGGGACAGCTTCAAGTTTGCTCCCATTCGTGAGAGTC
97
156
GCTAGCTACTCTACTACCCGGTCACCCTGTCGAAGTTCAAACGAGGGTAAGCACTCTCAG
a R S M R * W A S G T A S S L L P F V R V -
b D R * D D G P V G Q L Q V C S H S * E S -
c I D E M M G Q W D S F K F A P I R E S Q -

Figure 3.21. Alignment of 3'RACE sequence from E8 *thi1*

The sequence of E8 *thi1* 3'RACE product is shown in alignment with the E8 *thi1* gene sequence. The nucleotides are numbered (on the right side) with respect to the A (+1) of the proposed translation start site. The intron in the E8 *thi1* gene sequence is shown in lower case letters. The 5' and 3' splice sites and proposed lariat sequences (Gurr *et al.*, 1987) are shown in green. Putative polyadenylation recognition sequences (Gurr *et al.*, 1987) are shown in blue text. The translation stop codon is shown in red text with a star underneath. The 3' end of E8 *thi1* mRNA is indicated by a purple arrow. The translation stop codon is shown in red text.

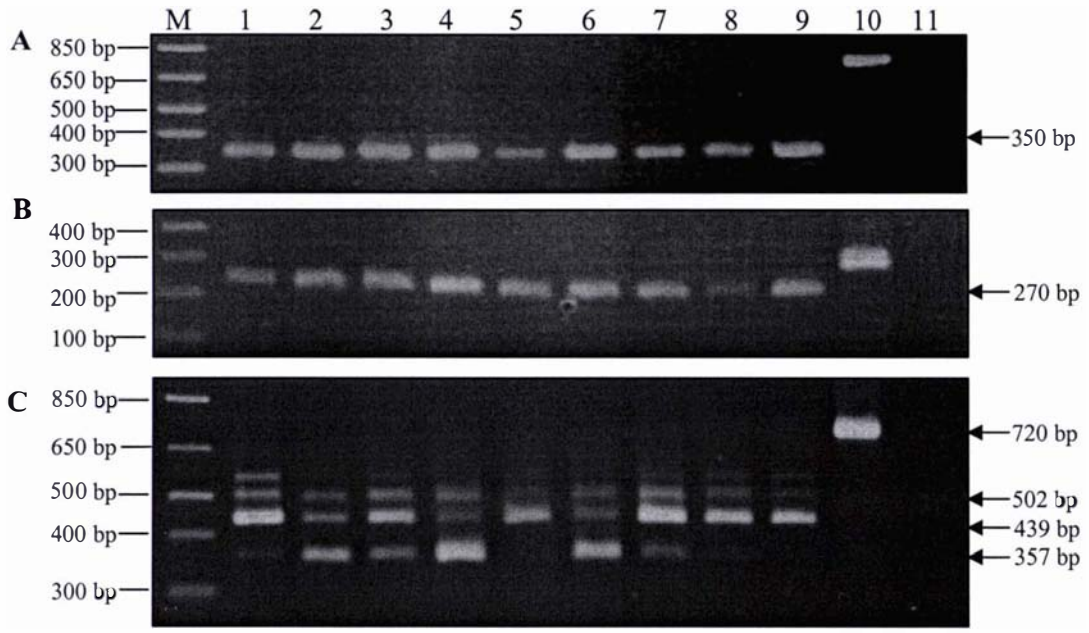
Figure 3.22. RT- PCR analysis of Lp19 *thi1* gene expression in culture

A: β-tubulin RT-PCR products amplified with primers T1.1 & T1.2 using RNA prepared from mycelium of Lp19 grown in PD for 9 days then washed and incubated for 2 days in CD basal salt medium (lane 8) or CD basal salt medium supplemented with sucrose and nitrate (lane 1), sucrose and glutamate (lane 2), mannitol and nitrate (lane 3), mannitol and glutamate (lane 4), nitrate (lane 5), glutamate (lane 6), sucrose (lane 7), or thiamine (lane 9). Lp19 genomic DNA (lane 10) and water only (lane 11) were amplified as controls. Lane M contained 1 kb + ladder (GibcoBRL).

B: RT-PCR products amplified from the same RNA samples in **A** using Lp19 *thi1* gene primers XZ1 and XZ25.

C: RT-PCR products amplified from the same RNA samples in **A** using Lp19 *thi1* gene primers XZ26 and XZ28.

D: Three types of Lp19 *thi1* transcript isoforms and the positions of primers (arrows) used for amplification. Grey blocks indicate exons and black lines indicate introns. The numbers are labeled with respect to the A (+1) of the translation start codon ATG.


M 1 2 3 4 5 6 7 8 9 10 11
A
850 bp
650 bp
500 bp
400 bp
300 bp
350 bp
B
400 bp
300 bp
200 bp
100 bp
270 bp
C
850 bp
650 bp
500 bp
400 bp
300 bp
720 bp
502 bp
439 bp
357 bp

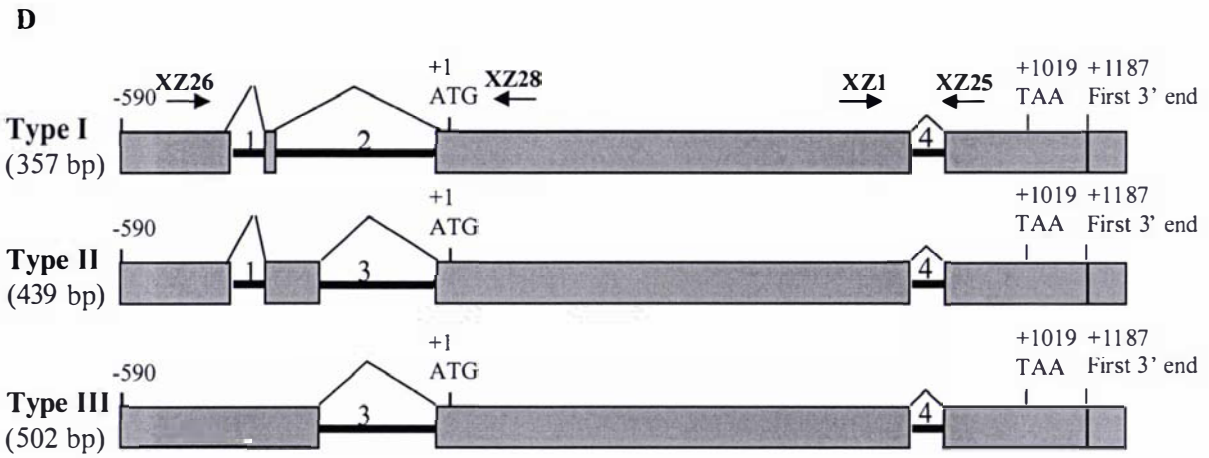

D
Type I
(357 bp)
-590
XZ26
+1
ATG
XZ28
XZ1
XZ25
+1019
TAA
+1187
First 3' end
1
2
4
Type II
(439 bp)
3
Type III
(502 bp)

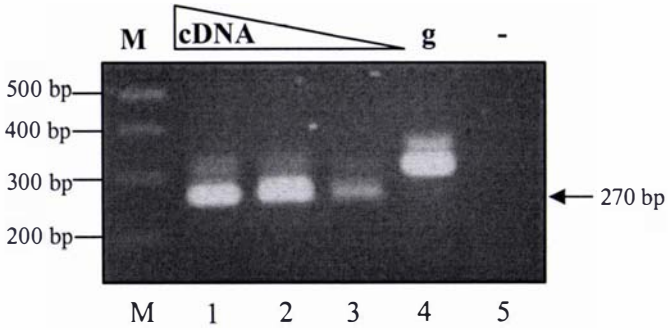
Figure 3.23. RT- PCR analysis of Lp19 *thi1* gene expression *in planta*

A: RT-PCR products amplified from 25, 2.5 and 0.25 ng of mRNA (lanes 1-3) isolated from perennial ryegrass pseudostem infected with Lp19 using primers XZ1 & XZ25.

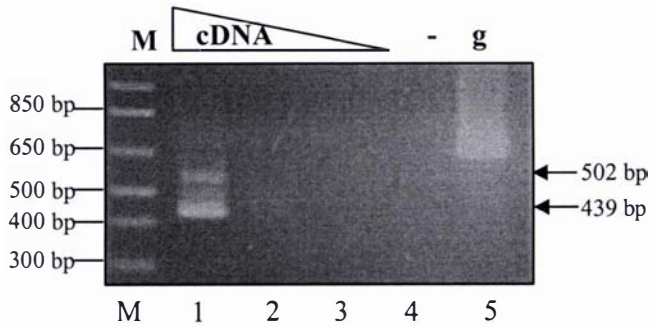
B: RT-PCR products amplified from the same samples (lanes 1-3) as in **A** using primers XZ26 & XZ28.

Lp19 genomic DNA (lane 4 in **A** and lane 5 in **B**) and water only (lane 5 in **A** and lane 4 in **B**) were amplified as controls. Lane M contained 1 kb plus ladder (Gibco BRL).

A


M
cDNA
g
-
500 bp
400 bp
300 bp
200 bp
270 bp
M 1 2 3 4 5

B


M
cDNA
-
g
850 bp
650 bp
500 bp
400 bp
300 bp
502 bp
439 bp
M 1 2 3 4 5

3.3. Targeted disruption of E8 *thi1* gene and growth characteristics of the *thi1* deletion mutant

3.3.1. Construction of the E8 *thi1* gene disruption plasmid

An E8 *thi1* gene disruption plasmid pXZ51 was constructed as shown in Fig. 3.24. Initially, a 1.0-kb fragment was amplified (Section 2.7.2) from the 3' portion of the E8 *thi1*. This PCR product was digested (Section 2.4.2) with *Hin*dIII and then ligated (Section 2.4.4) into the *Hin*dIII restriction site flanking a hygromycin resistance gene *hph* in the plasmid pPN1687. PCR (Section 2.7.7) using primers pUChph7 and M13F, and primers pUChph7 and XZ25 was carried out to determine the copy number and orientation of the inserted 1.0-kb fragment. One of the plasmids which contained a single 1.0-kb inserted fragment in the required orientation was designated as pXZ50 and used for further plasmid construction. A 1.4-kb fragment amplified from the 5' portion of the E8 *thi1* gene using primers XZ45*Kpn*I and XZ46*Bam*HI, was digested with *Kpn*I and *BamH*I and was ligated into pXZ50 digested with the same enzymes. This resulted in the E8 *thi1* disruption plasmid pXZ51 (Fig. 3.24), in which a 0.9 kb of the *thi1* gene (position –203 to 697) encompassing 0.7 kb of the ORF was replaced by an *hph* cassette.

3.3.2. Targeted deletion of E8 *thi1*

To generate a targeted deletion of *thi1*, E8 protoplasts were transformed (Section 2.10) with a 4.7-kb linear DNA fragment amplified from pXZ51 using primers XZ45*Kpn*I and XZ48*Hin*dIII by long template PCR (Section 2.7.6). A double crossover by homologous recombination would result in replacement of the E8 *thi1* gene with the *hph* cassette as shown in Fig. 3.25.

After two weeks incubation at 22°C, numerous tiny colonies (<2 mm) were observed on the hygromycin B containing CM plates (Appendix 4.1). However, only some of them continued growing into cottony white colonies to a size of approximately 4-10 mm about 4 weeks after the transformation. These colonies were sub-cultured onto PDA plates containing 100 mg/L hygromycin B. Stable colonies regenerated from sub-culturing were recorded as true transformants and the number used to calculate the transformation frequency. In contrast, protoplasts treated the same but without DNA gave no hygromycin resistant transformants.

The transformation frequencies ranged from 0.7×10^{-4} to 4.2×10^{-4} of the viable number of protoplasts per μg DNA (Table 3.2) in experiment 1 in which the treated protoplasts were grown for one day on CM plate then a hygromycin overlay was applied. In experiment 2 and 3 in which the protoplasts were incubated in liquid CM medium before spreading on hygromycin-containing CM, the transformation frequencies ranged from 3.4×10^{-4} to 5.9×10^{-4} (Table 3.3) and from 4.2×10^{-4} to 8.5×10^{-4} (Table 3.4) of the viable number of protoplasts per μg DNA.

3.3.3. Screening for E8 *thi1* deletion mutants

3.3.3.1. Screening for E8 *thi1* gene disrupted colonies on defined CD thiamine-free medium

In order to screen for E8 *thi1* deletion mutants, the hygromycin resistant colonies were sub-cultured respectively onto CD thiamine-free medium and CD medium supplemented with thiamine at a final concentration of 3 mg/L. Both the media contained 150 mg/L hygromycin B. As thiamine is an important cofactor of many enzymes in cell metabolism, it was

expected that *thi1* deletion mutants would show no or reduced growth on the CD thiamine-free medium in contrast to a normal growth on the thiamine-containing medium. A total of 33 out of 302 transformants screened had reduced colony size or reduced hyphal density on the CD thiamine-free medium. As a consequence, these isolates were screened by PCR to identify the type of DNA integration event that had occurred.

3.3.3.2. Screening for E8 *thi1* deletion mutants by PCR

Primers for PCR amplifications (Section 2.7.2) were designed to identify homologous recombination events and deletion of *thi1* as shown in Fig. 3.25. Primers XZ58 and pUChph9 were used to detect homologous recombination in the 5' region. A PCR product of 1.84 kb was an indication of homologous recombination in this region. Primers XZ49 and pUChph7 were used to detect homologous recombination in the 3' region. A product of 1.4 kb was an indication of homologous recombination in this region. Primers XZ7 and XZ25 were used to detect replacement of the *thi1* gene with the *hph* cassette. A single PCR product of 2.8 kb was indicative of replacement of the *thi1* gene by the *hph* cassette. An ectopic integration of the *hph* fragment into the genome would produce a 1.35 kb in addition to a 2.8-kb product with primers XZ7 and XZ25.

Out of the 33 transformants that had reduced growth on thiamine-free medium, only 4 (colonies T97, T103, T140 and T301) produced a 1.8-kb product with primers XZ58 and pUChph9, and a 1.4-kb product with primers XZ49 and pUChph7, indicating recombination in both the 5' and 3' regions (Fig. 3.26). Colonies T97, T103 and T140 showed that the *thi1* gene was deleted as indicated by the presence of only a 2.8-kb PCR product when genomic DNA was amplified with primers XZ7 and XZ25. However, colony T301 had both the 2.8-kb and 1.35-kb products with primers XZ7 and XZ25, indicating retention of the *thi1* gene. A likely explanation is that T301 was a heterokaryon containing both *thi1* deletion and wild-type nuclei.

Despite the above screening for thiamine auxotrophic mutants, all other transformants that showed no or little difference in growth on thiamine-free medium and thiamine-containing medium were subsequently screened by PCR to quantify homologous recombination events, as some of them might be heterokaryons. Out of 269 such transformants, 5 (colonies T48, T56, T63, T150 and T293) (Fig. 3.26) produced PCR products similar to T301, indicating homologous recombination in both the 5' and 3' regions but retention of the *thi1* gene, further suggesting heterokaryon colonies were present. All other colonies showed an ectopic integration of *hph* fragment into the genome. In order to obtain homokaryons, single-spore isolation (Section 2.10.3) was carried out on colonies T48, T56, T63, T150 and T293, and the other colonies (colonies T97, T103, T140 and T301) that showed reduced growth described above, as endophyte conidia are uninucleate (Schardl *et al.*, 1994). Two randomly selected ectopic transformants T1 and T120 were included in order to obtain uninucleate ectopic controls. From each of the transformant colonies, eight single spores were isolated and inoculated onto PDA (with hygromycin B at a final concentration of 150 mg/L) for growth. Four to eight single-spore colonies were generated from each of the transformants except T63 and T293, from which no single-spore colonies were obtained. PCR amplification were carried out on four single-spore colonies from each of the transformants to screen for *thi1* deletion. All single-spore colonies isolated from transformant colonies T97, T103, T140 and T301 produced PCR products indicating recombination in both the 5' and 3' regions and deletion of the *thi1*. All single-spore colonies from transformant colonies T1 and T120 produced PCR products indicating an ectopic integration of the *hph* fragment into the genome. However, single-spore colonies from transformants T48 or T56 produced two patterns of PCR products, one indicating an ectopic integration of *hph* fragment, the other indicating retention of the *thi1* with a homologous recombination in the 5' region but not in the 3' region. These results confirmed the heterokaryon nature of these two colonies before single-spore isolation. All four single-

spore colonies from T150 produced PCR products indicating retention of the *thi1* gene but with homologous recombination in the 5' region.

One single-spore colony from each of the transformants of T97, T103, T140, T301, T1 and T120 were selected for the following Southern blot hybridisation analysis. From T48, T56 and T150, a single-spore colony showing retention of the *thi1* gene with homologous recombination in the 5' region was selected.

3.3.3.3. Southern blot hybridisation analysis

Southern blot hybridisations (Section 2.6) were employed to confirm deletion of the *thi1* gene, to examine the copy number and the site of recombination of the replacement construct into the genome. As expected, a 4.0-kb *Hin*dIII fragment hybridized in wild-type (Fig. 3.27) but was absent in T97, T103, T140 and T301. This result confirms that the *thi1* gene was deleted in all four isolates. Two bands of 3.3-kb and 1.9-kb hybridized in T140 and T301. The 3.3-kb band corresponds to the *hph* fragment plus the 5' flanking portion of the *thi1* gene and the 1.9-kb band corresponds to the 3' flanking portion of the *thi1* gene. These results indicate that T140 and T301 have *thi1* replaced by a single-copy of the *hph* cassette (Fig. 3.28 B). In addition to the 3.3-kb and 1.9-kb bands, isolates T97 and T103 had an extra 1.1-kb band (Fig. 3.27 C lanes 4 and 5), characteristic of a head to tail tandem repeat. This band corresponded to the 3' and a small section of the 5' region of the *thi1* gene as shown in Fig. 3.28C. These two isolates are *thi1* deletions but contain multiple copies of the *hph* gene. Isolates T1 and T120 (Fig. 3.27C lanes 2 and 3) had a 4.0-kb band corresponding to the *thi1* *Hin*dIII restriction fragment, a 3.3-kb band corresponding to the *hph* *Hin*dIII restriction fragment and an extra band corresponding to *hph* 3' portion (Fig 3.28D), indicating integration of one *hph* cassette at an ectopic location. For isolates T48, T56 and T150, the presence of the 3.3 and 4.0-kb fragments are indicative of integration of the *hph* gene and retention of the *thi1* gene. The 1.1-kb band indicated a head-to-tail tandem

repeat of the *hph* fragment. These isolates are probably transformants with multiple *hph* copies organised as tandem repeats.

Further Southern hybridisation analysis was carried out by probing *Eco*RV digestion with the same probe. *Eco*RV was chosen as there is no site for this restriction enzyme within the *thi1* locus or the *hph* cassette. Isolates T140 and T301 (Fig. 3.29 lanes 5 and 6) had a single hybridizing band that was about 1.5 kb larger than that from the E8 wild-type (Figure 3.29 lane 1), indicating the *thi1* was replaced with a single copy of *hph* cassette. The extra large band in T97 and T103 (Fig. 3.29 lanes 3 and 4) is indicative of multiple copies of *hph* cassette at a single site. As expected, T120 (Fig. 3.29 lane 2) had two hybridisation bands, one corresponding to integration at both wild-type and ectopic sites. No hybridisation bands were produced from T48 and T56 (Fig. 3.29 lanes 7 and 8), probably due to too little DNA loaded. Only one band was produced from T150 (Fig. 3.29 lane 9), indicating integration of *hph* cassette into a single site.

The single-spore isolates from T97, T103, T140, T301, T1 and T120 were renamed as KO1, KO2, KO3, KO4, EC1 and EC2 respectively. KO3 (PN2282) and KO4 (PN2283) were selected for further analysis as they contain only a single copy of the *hph* cassette. The E8 wild-type WT1 (PN2284), a culture grown from E8 protoplasts WT2 (PN2285), EC1 (PN2286) and EC2 (PN2287), were used as controls in the following studies.

Figure 3. 24. Construction of E8 *thi1* gene replacement plasmid

The *thi1* gene replacement plasmid was made by sequentially cloning two PCR fragments, a 1.0 kb *Hin*dIII fragment (green) and a 1.4 kb *Kpn*I/ *Bam*HI fragment (red), into pPN1687.

B, H and K indicate restriction enzyme recognition sites for *Bam*HI, *Hin*dIII and *Kpn*I respectively. Primers used for PCR amplification of construct fragments and for screening orientation of fragments in plasmids are shown in arrows.

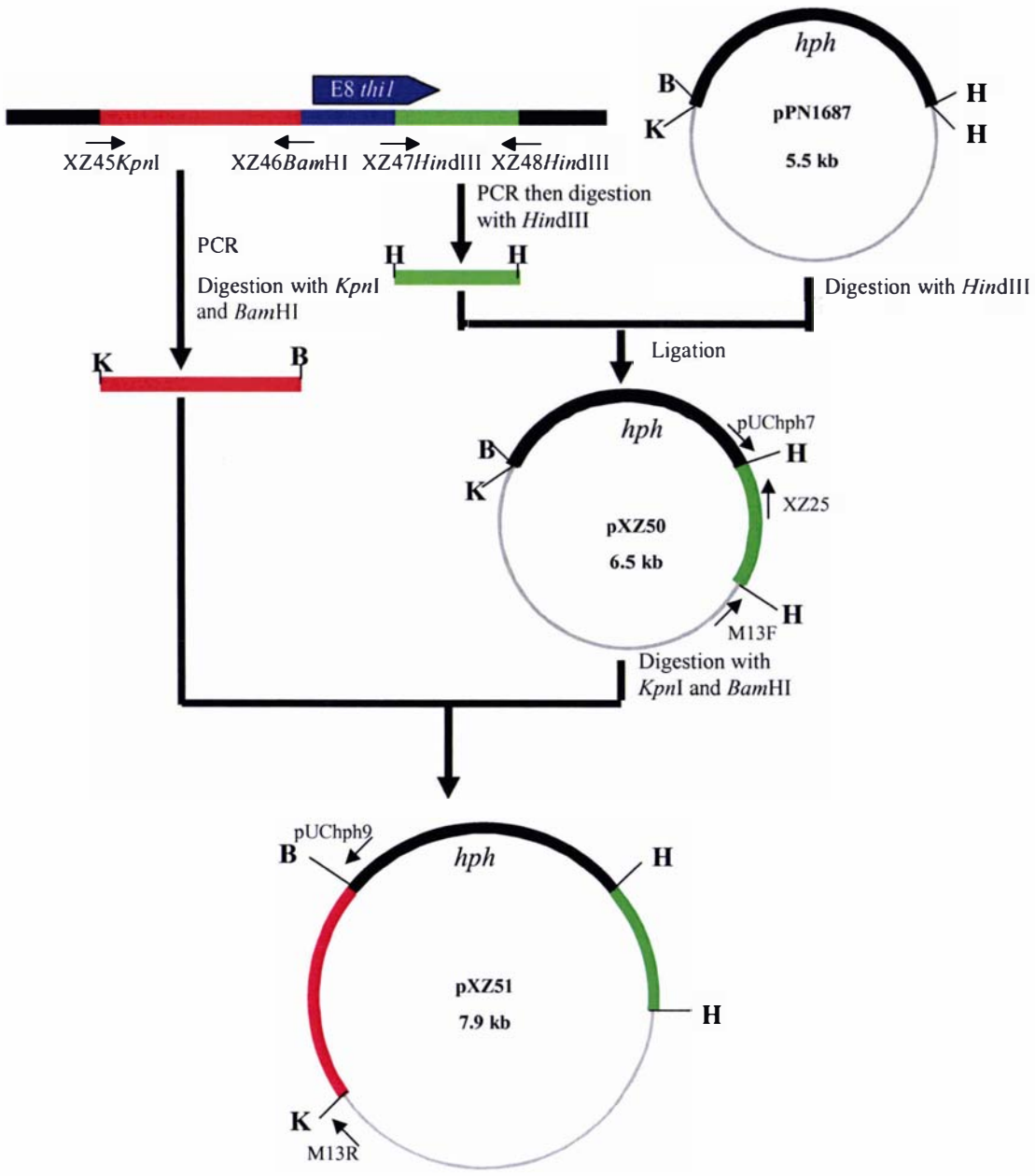

E8 thi1
XZ45KpnI
XZ46BamHI
XZ47HindIII
XZ48HindIII
PCR
Digestion with KpnI and BamHI
PCR then digestion with HindIII
hph
pPN1687
5.5 kb
Digestion with HindIII
Ligation
pUChph7
pXZ50
6.5 kb
XZ25
M13F
Digestion with KpnI and BamHI
pUChph9
pXZ51
7.9 kb
M13R

Figure 3.25. Strategy used for disrupting the E8 *thi1* gene

Physical maps of E8 wild-type *thi1* locus, *thi1* replacement fragment and E8 *thi1* disrupted locus showing primers used for PCR screening to identify double cross over events. The replacement fragment used to transform E8 protoplasts was amplified by PCR using primers XZ45*Kpn*I & XZ48*Hin*dIII and plasmid pXZ51 as template.

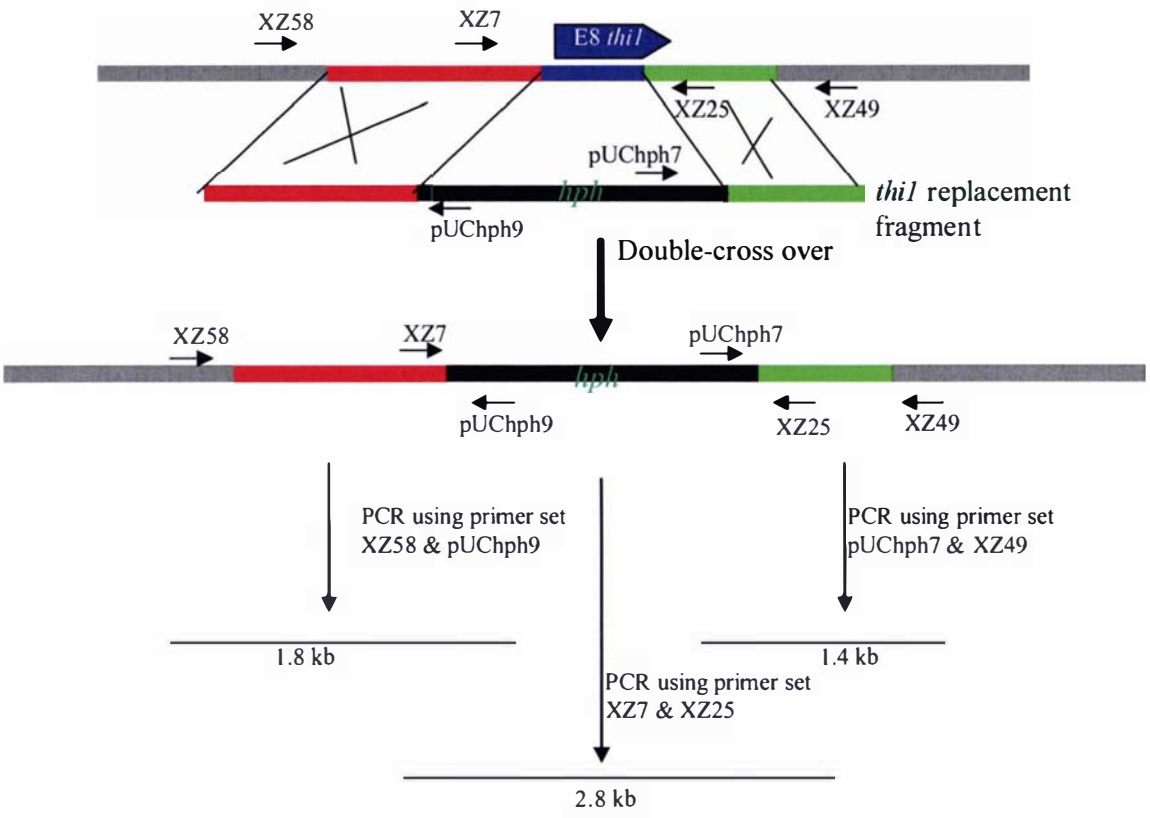

XZ58
XZ7
E8 thi1
XZ25
XZ49
pUChph7
hph
pUChph9
thi1 replacement fragment
Double-cross over
XZ58
XZ7
pUChph7
hph
pUChph9
XZ25
XZ49
PCR using primer set XZ58 & pUChph9
PCR using primer set pUChph7 & XZ49
1.8 kb
1.4 kb
PCR using primer set XZ7 & XZ25
2.8 kb

Table 3.2. E8 transformation frequencies (experiment 1[f])

DNA (μg)	Viable protoplasts before transformation (ml^{-1})	Viable protoplasts after transformation[c] (ml^{-1})	Hyg^R colonies[d] (ml^{-1})	Transformation frequency[e]
Cells only	1.1×10^6	1.9×10^4	0	
PCR product[a] (5.0 μg)			27	2.8×10^{-4}
PCR product[a] (3.3 μg)			24	3.8×10^{-4}
PCR product[a] (1.7 μg)			13	4.0×10^{-4}
Cells only	2.5×10^6	2.9×10^4	0	
pAN7-1 (5.0 μg)			24	1.7×10^{-4}
PCR product[a] (5.0 μg)			22	1.5×10^{-4}
PCR product[a] (5.0 μg)			15	1.0×10^{-4}
pXZ51 (5.0 μg)			19	1.3×10^{-4}
pXZ51 (5.0 μg)			15	1.0×10^{-4}
Linear pXZ51[b] (5.0 μg)			18	1.2×10^{-4}
Cells only	3.9×10^6	2.3×10^4	0	
pAN7-1 (5.0 μg)			20	1.7×10^{-4}
PCR product[a] (5.0 μg)			8	0.7×10^{-4}
PCR product[a] (5.0 μg)			12	1.0×10^{-4}
PCR product[a] (5.0 μg)			49	4.2×10^{-4}

[a] Linear PCR product was amplified from pXZ51 with primers XZ45*Kpn*I & XZ48*Hin*dIII.

[b] pXZ51 was linearised with *Bam*HI.

[c] Colonies were regenerated from 1 ml cell only mixture of which each 100 μl was mixed in 3 ml of CM top agar and spread on CM plate.

[d] Colonies were regenerated from 1 ml transformation reaction of which each 100 μl was mixed in 3 ml of CM top agar and spread on CM plate followed by an additional overlay next day to give a final concentration of hygromycin B of 150 mg/l.

[e] Transformation frequency = Hyg^R colonies/μg DNA/viable protoplast after transformation.

[f] Experiment 1 included three experiments in which the transformed protoplasts were treated in the same method.

Table 3.3. E8 transformation frequencies (experiment 2):

PCR product[a] (μg)	Viable protoplasts before transformation (ml^{-1})	Viable protoplasts after transformation[b] (ml^{-1})	Hyg^R colonies[c] (ml^{-1})	Transformation frequency[d]
Cell only	1.1×10^6	1.0×10^4	0	
5.0			17	3.4×10^{-4}
3.3			16	4.8×10^{-4}
1.7			10	5.9×10^{-4}

Table 3.4. E8 transformation frequencies (experiment 3)

PCR product[a] (μg)	Viable protoplasts before transformation (ml^{-1})	Viable protoplasts after transformation[b] (ml^{-1})	Hyg^R colonies[c] (ml^{-1})	Transformation frequency[d]
0	1.1×10^6	2.5×10^4	0	
5.0			77	6.2×10^{-4}
3.3			34	4.2×10^{-4}
1.7			36	8.5×10^{-4}

[a]Linear PCR product was amplified from pXZ51 with primers XZ45*Kpn*I & XZ48*Hin*dIII.

[b]Colonies were regenerated from 1 ml of cell only mixture that was incubated in 100 ml CM (Appendix 4.1) for 3 days, collected by centrifugation and spread on CM plates.

[c]Colonies were regenerated from 1 ml of transformation reaction that was incubated in 20 ml CM for 3 days then spread on CM plates supplemented with hygromycin B (150 mg/l).

[d]Transformation frequency = Hyg^R colonies /μg DNA/viable protoplast after transformation

Figure 3.26. PCR analysis of E8 *thi1* transformants

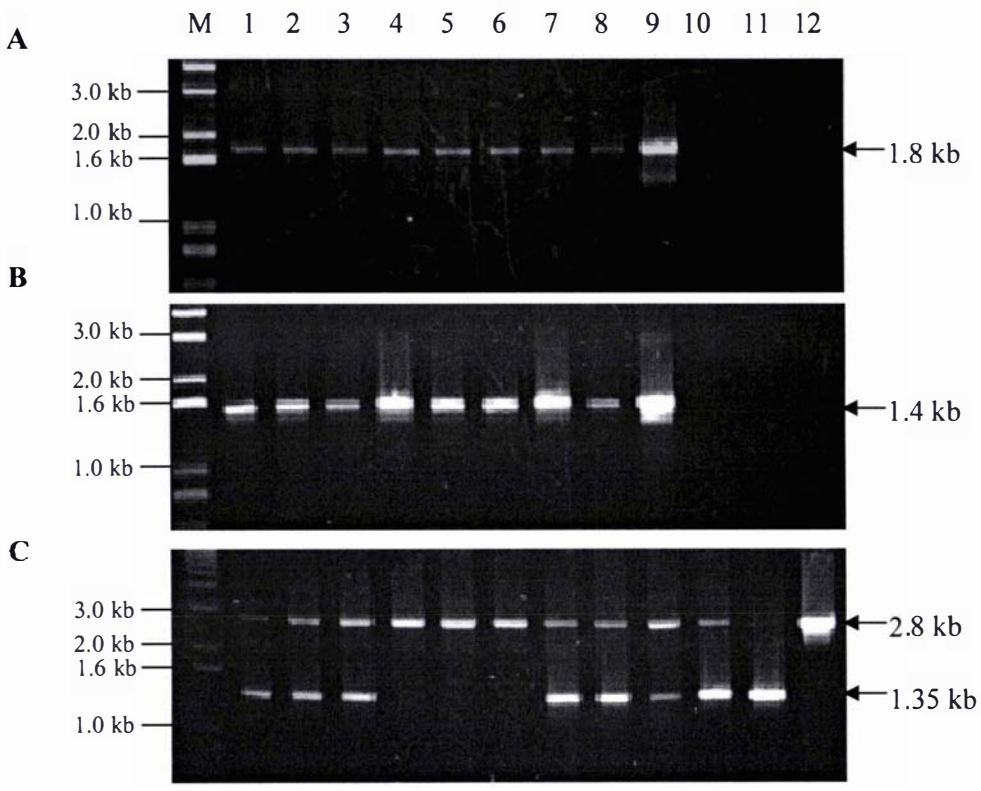
A: PCR products amplified with primers XZ58 & pUChph9 using genomic DNA from transformants T48 (lane 1), T56 (lane 2), T63 (lane 3), T97 (lane 4), T103 (lane 5), T140 (lane 6), T150 (lane 7), T293 (lane 8), T301 (lane 9) and T120 (lane 10) before single spore purification. E8 wild-type (lane 11) and pXZ51 (lane 12) were amplified as controls. A product of 1.8 kb indicates recombination in the 5' region of the *thi1* locus.

B: PCR products amplified with primers XZ49 & pUChph7 using the same samples as shown in **A**. A product of 1.4 kb indicates recombination in the 3' region of the *thi1* locus.

C: PCR products amplified with primers XZ7 & XZ25 using the same samples as shown in **A**. Products of 2.8 kb and 1.35 kb correspond to integration of *hph* and retention of wild-type *thi1*. A single 2.8 kb band is indicative of a gene replacement event at the *thi1* locus.

Lane M contains 1 kb + ladder (GibcoBRL).

For locations of the primers refer to Figure3.25.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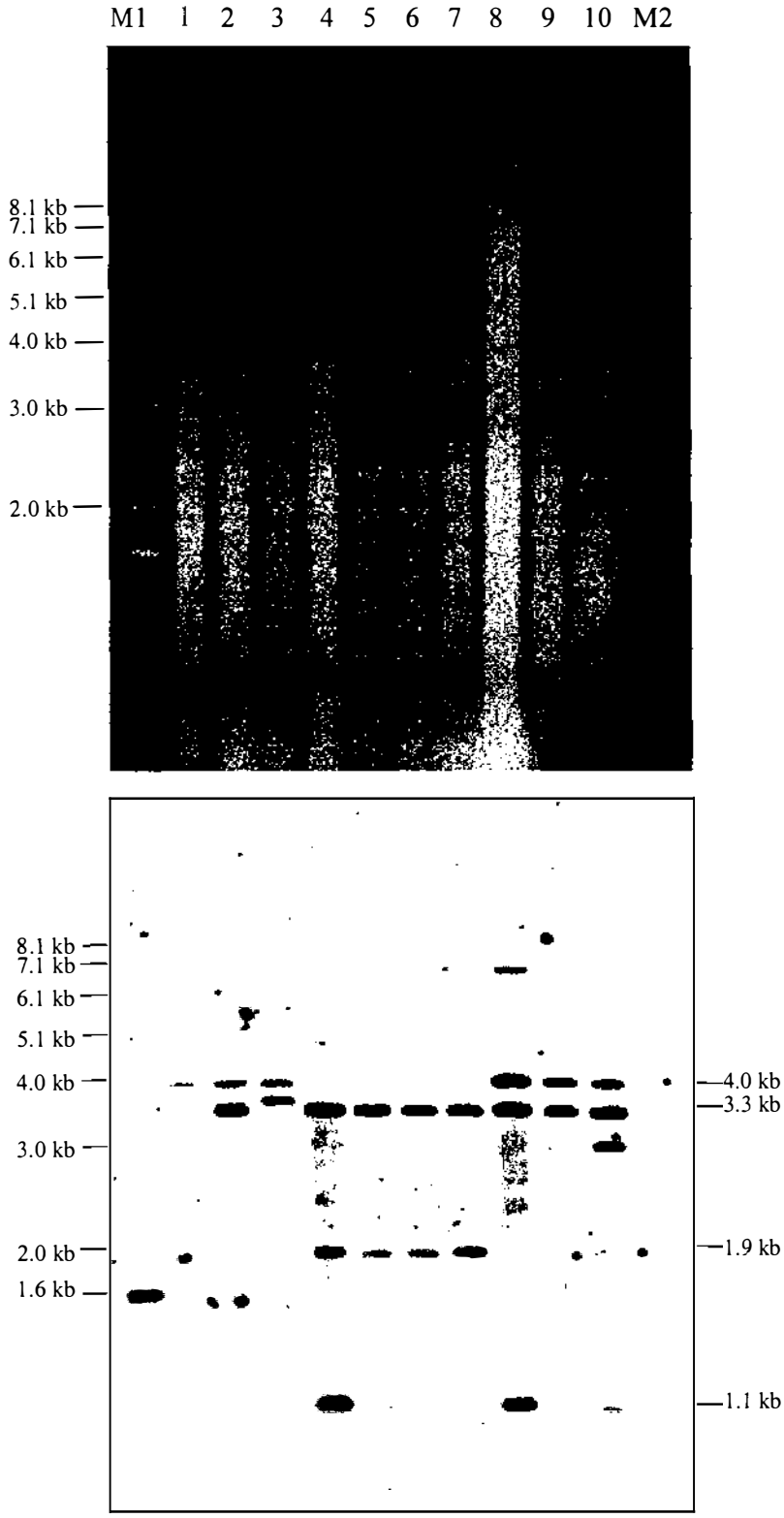
3.0 kb
2.0 kb
1.6 kb
1.0 kb
1.8 kb
B
3.0 kb
2.0 kb
1.6 kb
1.0 kb
1.4 kb
C
3.0 kb
2.0 kb
1.6 kb
1.0 kb
2.8 kb
1.35 kb

Figure 3.27. Southern blot analysis of E8 *thi1* disruptants

A. Agarose gel of genomic DNA of E8 wild-type control (PN2285) (lane 1), single spore isolates from T1 (lane 2), T120 (lane 3), T97 (lane 4), T103 (lane 5), T140 (lane 6), T301 (lane 7), T48 (lane 8), T56 (lane 9) and T150 (lane 10) digested with *Hin*dIII. Lane M1 shows a 1 kb plus ladder (GibcoBRL). Lane M2 shows λDNA digested with *Hin*dIII. E8 wild-type control (PN2285), and single spore purified isolates of T1, T120, T140 and T301 were renamed as WT2 (PN2285), EC1 (PN2286), EC2 (PN2287), KO3 (PN2282) and KO4 (PN2283) for subsequent experiments.

B. Autoradiograph of a Southern blot of **A** probed with Digoxigenin-11-dUTP labeled probe, Dig-hph-2 (see Figure3.28).


M1
1
2
3
4
5
6
7
8
9
10
M2
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7.1 kb
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6.1 kb
5.1 kb
4.0 kb
3.0 kb
2.0 kb
1.6 kb
4.0 kb
3.3 kb
1.9 kb
1.1 kb

Figure 3.28. Predicted outcomes for integration of replacement construct into E8 genome

A: Physical maps of *thi1* locus and *thi1* replacement fragment showing restriction enzyme recognition sites for *Bam*HI (H) and *Hin*dIII (H), and probe used for hybridisations in Figure3.27 and Figure3.29.

B: Physical map for a single copy replacement.

C: Physical map for a single site multiple copy replacement.

D: Physical map for a single copy single site ectopic integration.

E: Physical map for a wild-type E8 *thi1* locus.

The black lines indicate the *Hin*dIII restriction fragments. The numbers indicate the restriction fragment sizes. The red and green bars represent the regions flanking the *thi1* gene where homologous recombination might occur.

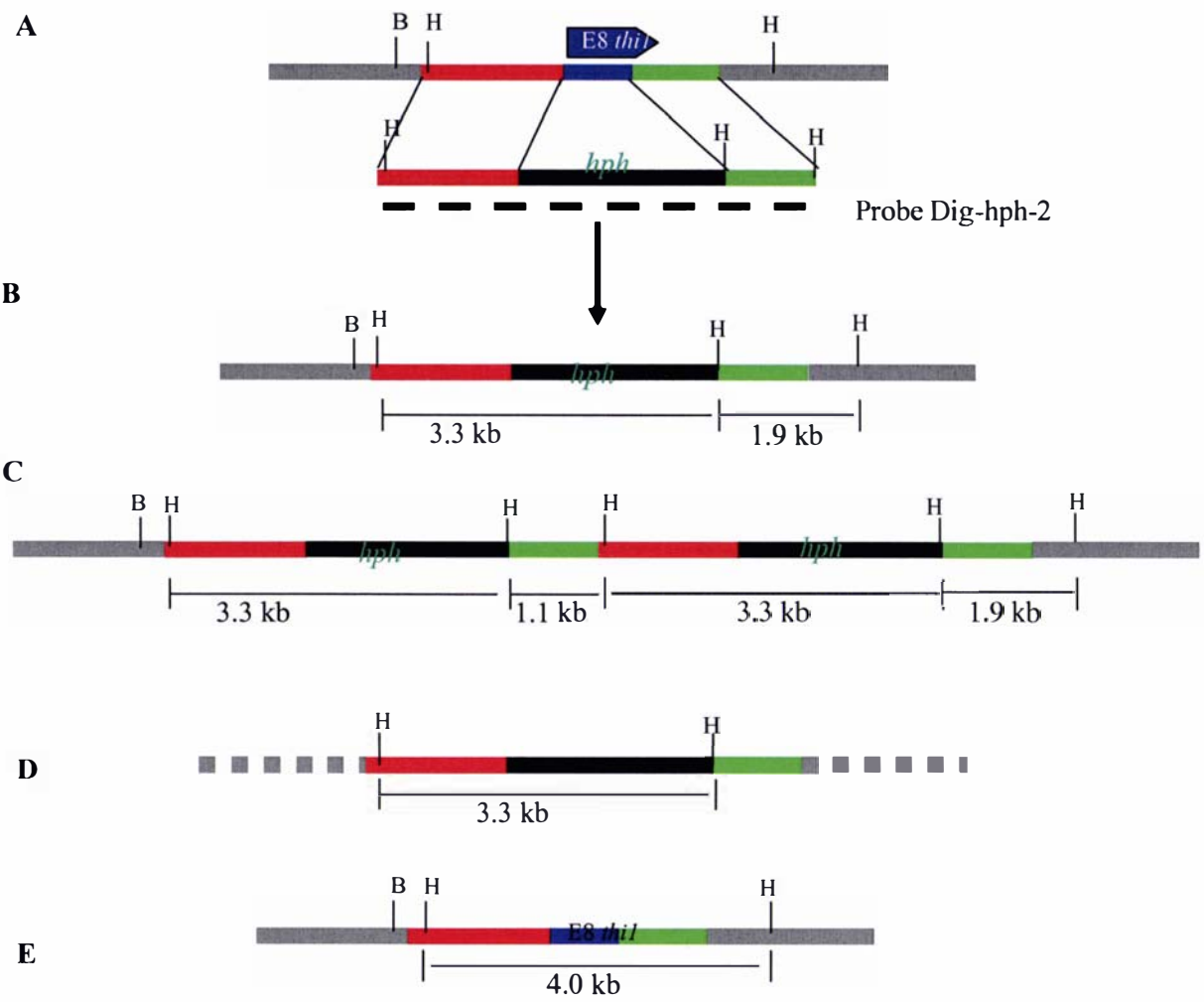
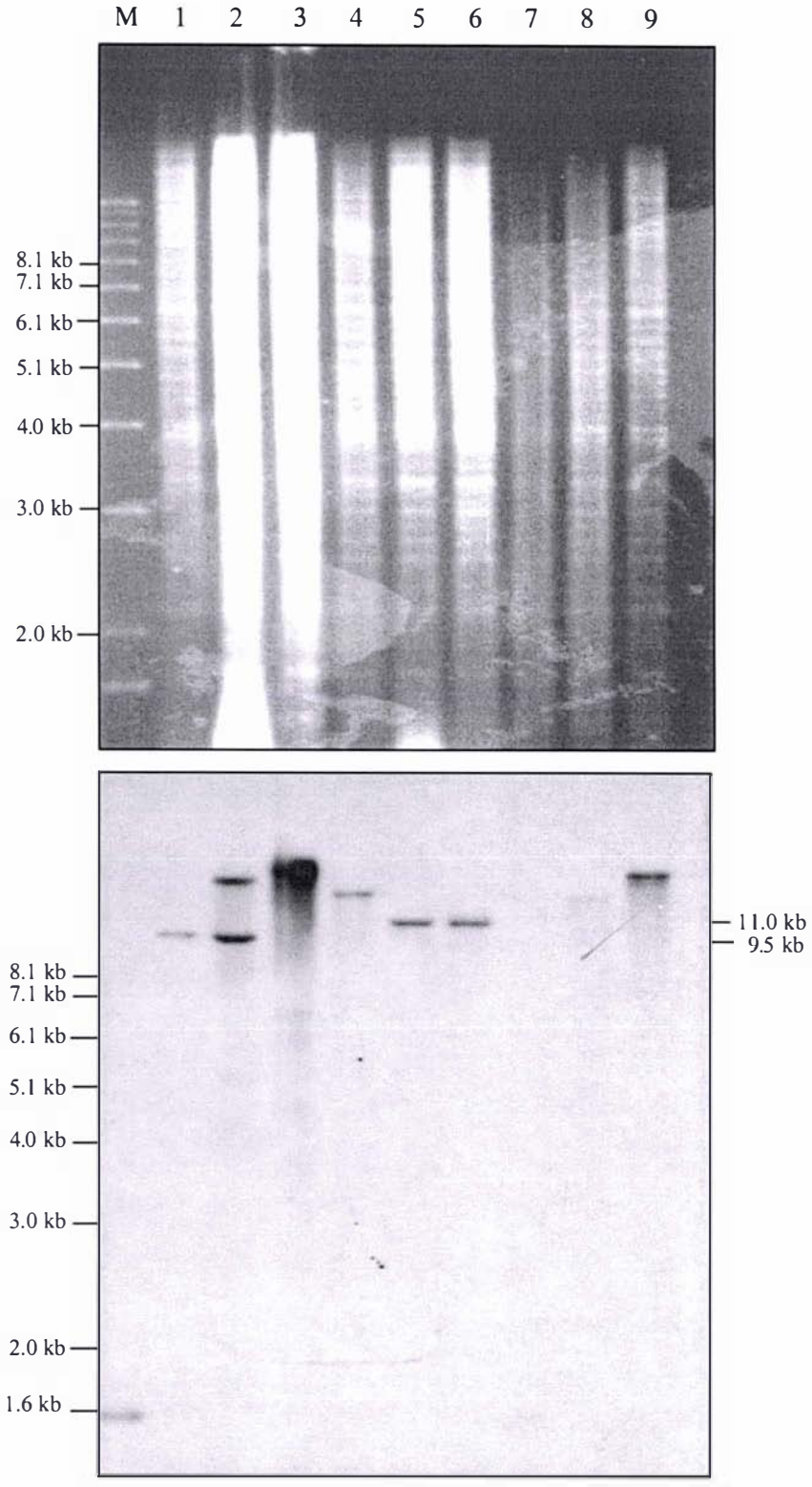

A
B H
E8 thi
H
H
H
H
hph
Probe Dig-hph-2
B
B H
H
H
hph
3.3 kb
1.9 kb
C
B H
H
H
H
H
hph
hph
3.3 kb
1.1 kb
3.3 kb
1.9 kb
D
H
H
3.3 kb
E
B H
H
E8 thi
4.0 kb

Figure 3.29. Southern blot analysis of E8 *thi1* disruptants

A. Agarose gel of 2 μg genomic DNA of E8 wild-type control (PN2285) (lane 1), single spore isolates from T120 (lane 2), T97 (lane3), T103 (lane 4), T140 (lane 5), T301 (lane 6), T48 (lane 7), T56 (lane 8) and T150 (lane 9) digested with *Eco*RV. Lane M1 shows a 1 kb plus ladder (GibcoBRL). Lane M2 shows λDNA digested with *Hin*dIII. E8 wild-type control (PN2285), and single spore purified isolates of T1, T120, T140 and T301 were renamed as WT2 (PN2285), EC1 (PN2286), EC2 (PN2287), KO3 (PN2282) and KO4 (PN2283) for subsequent experiments.

B. Autoradiograph of a Southern blot of **A** probed with Digoxigenin-11-dUTP labeled, probe Dig-hph-2 (see Figure3.28).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1.6 kb

3.3.4. Growth characteristics of the E8 *thi1* deletion mutants in culture

3.3.4.1. Effect of thiamine and thiazole on growth of E8 *thi1* deletion mutants

The phenotypes of the E8 *thi1* deletion mutants KO3 (PN2282) and KO4 (PN2283) were examined (Section 2.11) by growing them on CD thiamine–free medium and CD supplemented with thiamine or thiazole. Thiazole was used as it is a key precursor for thiamine biosynthesis, and *thi1* is homologous to a putative thiazole biosynthetic gene. E8 isolates WT1 (PN2284) and WT2 (PN2285) were used as wild-type controls. The ectopic transformant isolates EC1 (PN2286) and EC2 (PN2287) were included to evaluate the effect that ectopic integration of the replacement construct might have on the endophyte growth.

The *thi1* deletion mutants KO3 and KO4 had a low hyphal density (Fig 3.30) and branching frequency (Fig 3.31), and displayed convoluted hyphae (Fig 3.32) on CD thiamine-free medium in comparison to E8 wild-type controls. These growth deficiencies were overcome by supplementation with thiamine or thiazole (Fig. 3.30), demonstrating that *thi1* is involved in thiazole biosynthesis. However, colonies of the KO3 and KO4 appeared to be finer and more cottony than those of the wild-type, even on CD medium supplemented with thiamine.

Surprisingly, the ectopic transformant EC1 had reduced growth on CD medium compared to the wild-type (Fig 3.30). Supplementation with thiazole or thiamine overcame this defect. Ectopic transformant EC2 grew similarly to the wild-type. Both EC1 and EC2 had a hyphal branching frequency and hyphal morphology the same as the wild-type. Both the

KO strains retained the ability to produce conidia (Fig. 3.33) and had no significant changes in colony radial growth (Table 3.5) on thiamine-free CD medium.

3.3.4.2. Effects of thiamine concentration on the E8 *thi1* deletion mutants

The effect of thiamine concentration on the growth of the E8 *thi1* mutants was examined (Section 2.11) by growing them on CD thiamine–free medium and CD medium supplemented with various concentrations of thiamine (Fig. 3.34-3.36 and 3.38). Similar to the previous experiment, the *thi1* mutants KO3 and KO4 had a reduced hyphal density and branching frequency, and displayed convoluted hyphae on the CD thiamine-free medium. Supplementation of the medium with thiamine at a final concentration of 0.01 mg/L dramatically increased hyphal density and branching frequency, induced aerial hyphal growth and restored the hyphal morphology. These results indicate that a very low concentration of thiamine is sufficient to restore normal growth of the mutants. Higher concentrations of thiamine resulted in a slight increase in hyphal density. Again, colonies of KO3 and KO4 on thiamine-containing CD media appeared to be finer and more cottony than those of the wild-type, probably due to the abundant narrow aerial hyphae on these plates. The E8 *thi1* mutants KO3 and KO4 had a slightly smaller colony diameter (Table 3.6 and 3.7) than the wild-type, and this growth defect was not overcome by supplementation with thiamine.

Ectopic transformant EC1 had the smallest colony diameter on thiamine-free CD medium (Table 3.6 and 3.7) and the colony morphology differed from that of the wild-type (Fig. 3.34 and 3.38). The hyphal branching frequency on the edge of colonies of this strain was reduced but no change in the hyphal morphology was observed when grown on thiamine-free CD medium. Thiamine supplementation at a final concentration of 0.01 mg/L increased the branching frequency, induced aerial hyphal growth (Fig. 3.35) and increased the colony

size (Table 3.6 and 3.7). Supplementation with thiamine at a higher concentration had no further effect on the growth of this strain. Ectopic transformant EC2 had a denser mycelia and more cottony colony morphology than the wild-type. Addition of thiamine had no effect on either colony or hyphal morphology.

As ectopic control isolates EC1 and EC2 displayed colony morphologies different from the wild-type controls, a molecular examination of these isolates was carried out. As shown in Fig. 3.37, two bands of 2.8-kb (corresponding to an *hph* insertional replacement of *thi1*) and 1.4-kb (corresponding to the *thi1* wild-type locus) were amplified from genomic DNA of EC1 and EC2 using primers XZ7 and XZ25, indicating that these two isolates were indeed wild-type for *thi1* and had an ectopic integration of the construct. In contrast, only a single band of 1.4-kb was amplified from WT1 and WT2, and a 2.8-kb band from KO3 and KO4 by these two primers (Fig. 3.37 A). When amplifying with primer set XZ58 and pUChph9 that detect recombination on the left side of *thi1* locus (Fig. 3.37 B), or primers pUChph7 and XZ49 that detect recombination on the right side of *thi1* locus (Fig 3.37 C), only KO3 and KO4 gave products, further confirming homologous recombination at the *thi1* locus.

3.3.5. Summary and discussion

E8 *thi1* mutants in this study were obtained using the technique of targeted gene disruption (Section 3.3.2). When E8 protoplasts were transformed with the *thi1* replacement DNA carrying a selectable marker, integration occurred at homologous or ectopic sites. The transformation frequency was determined for varying concentrations of PCR generated replacement construct (Table 3.2), with different forms (PCR product, linear or circular plasmid) of DNA at the same concentration, and using different methods of treating the protoplasts after the transformation (Tables 3.3 and 3.4). The results obtained were significantly variable that it was not possible to determine whether the concentration or

topology of the DNA had any effect on the transformation frequencies. In general, the experiments in which the transformed protoplasts were incubated in CM liquid medium (Appendix 4.1) gave higher transformation frequencies (Tables 3.3 and 3.4) than the experiment (Table 3.2) in which the transformed protoplasts were mixed with CM top agar and spread on CM plates. While the increased transformation frequency may be attributed to that the transformed protoplasts recovering better in liquid CM medium than on plates, it is also possible that the protoplasts start to divide in the liquid culture and subsequently produced more colonies.

A concern of the targeted gene disruption technique is that homologous recombination could result in mutations in adjacent genes at the site of integration, with the frequency of mutations dependent on the genetic background of the host organism (Bell *et al.*, 1999; Yoshida *et al.*, 2003). A BlastX search showed that E8 *thi1* 5' flanking region overlaps with a putative open reading frame of triosephosphate isomerase 2 (TIM 2) (with 36% identity with TIM 2 from *Sinorhizobium meliloti*), which plays an important role in several metabolic pathways, including fatty acid biosynthesis, gluconeogenesis and glycolysis. In our study, two independent single-copy mutants KO3 and KO4 showed THI^- phenotype (Section 3.3.4), and both were complemented by free thiamine and thiazole (Section 3.3.4.2), indicating that the phenotype observed resulted from deletion of *thi1*. Therefore, it is very unlikely that the targeting integration had caused mutation in the TIM 2 gene. However, the possibility of mutations in the TIM2 gene could not be completely excluded.

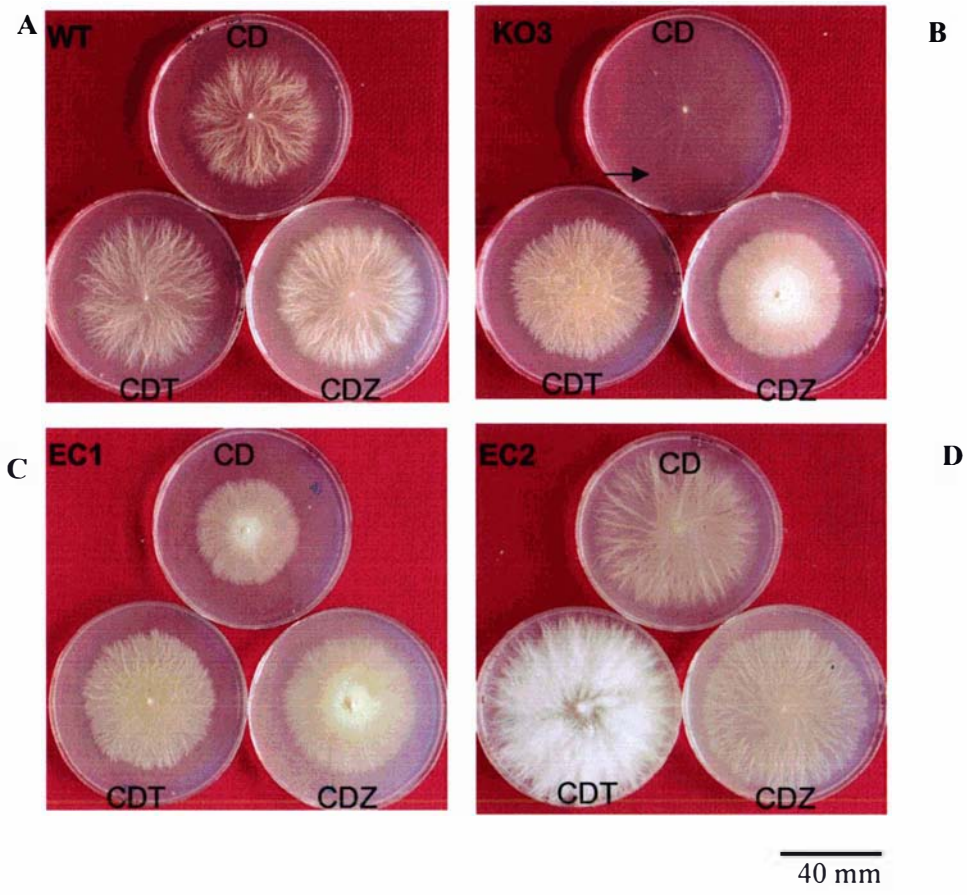


Figure 3.30. Effect of thiamine and thiazole on growth of E8 wild-type and *thi1* deletion mutants

Colonies of E8 wild-type WT2 (PN2285) **(A)**, *thi1* deletion mutants KO3 (PN2282) **(B)**, ectopic transformant EC1 (PN2286) **(C)** and EC2 (PN2287) **(D)** grown on CD defined medium (thiamine free), CDT medium (CD + 3mg/L thiamine) and CDZ medium (CD + 3 mg/L thiazole) at 22 °C for 32 days.

The arrow indicates the edge of the colony.

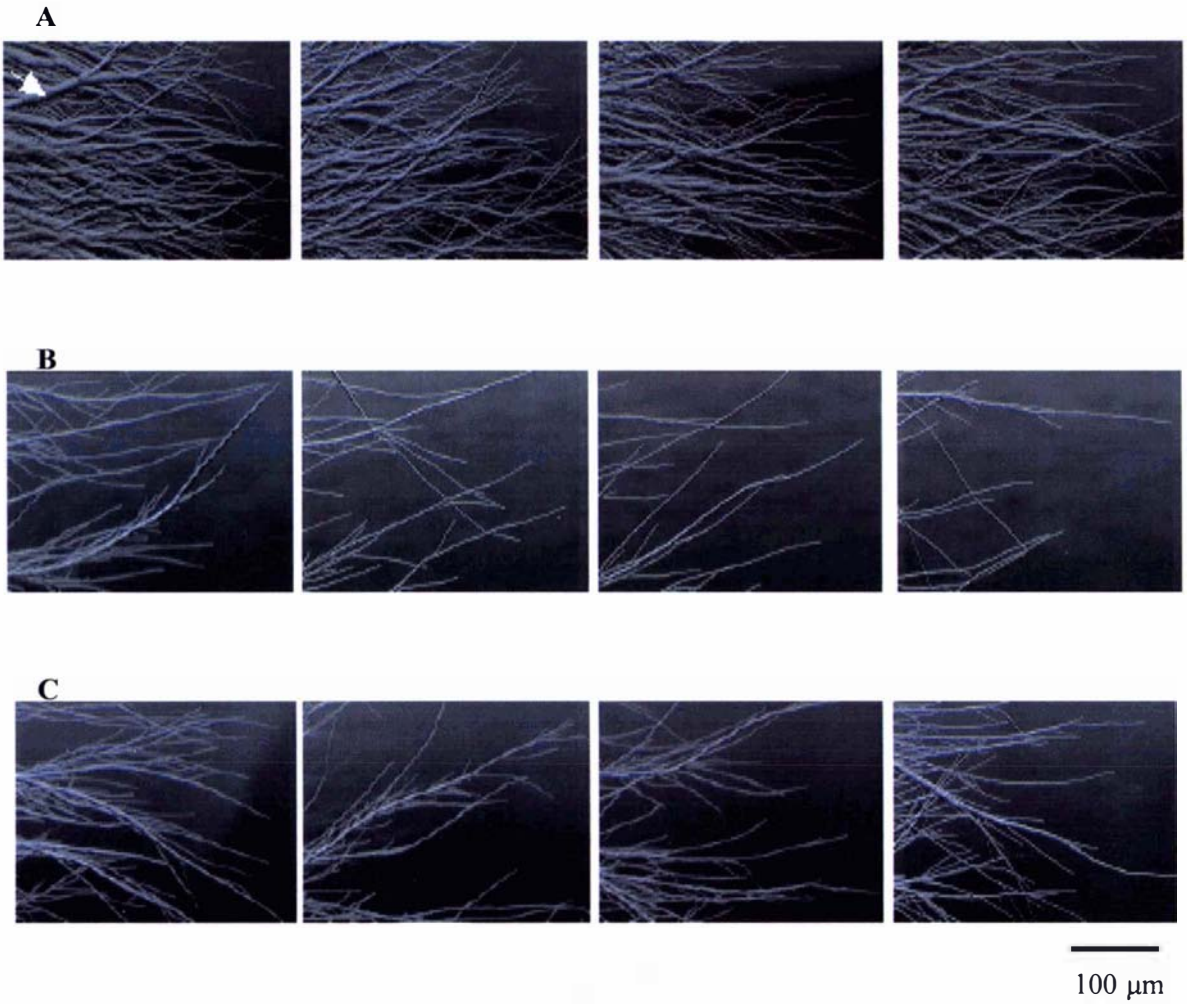


Figure 3.31. Frequency of hyphal branching of E8 wild-type and *thi1* deletion mutants

Frequency of hyphal branching on the edge of colonies of E8 wild-type (PN2285) **(A)**, *thi1* deletion mutants KO3 (PN2282) **(B)** and KO4 (PN2283) **(C)** grown on CD thiamine-free defined medium at 22°C for 27 days.

The arrow indicates aggregated hyphae.

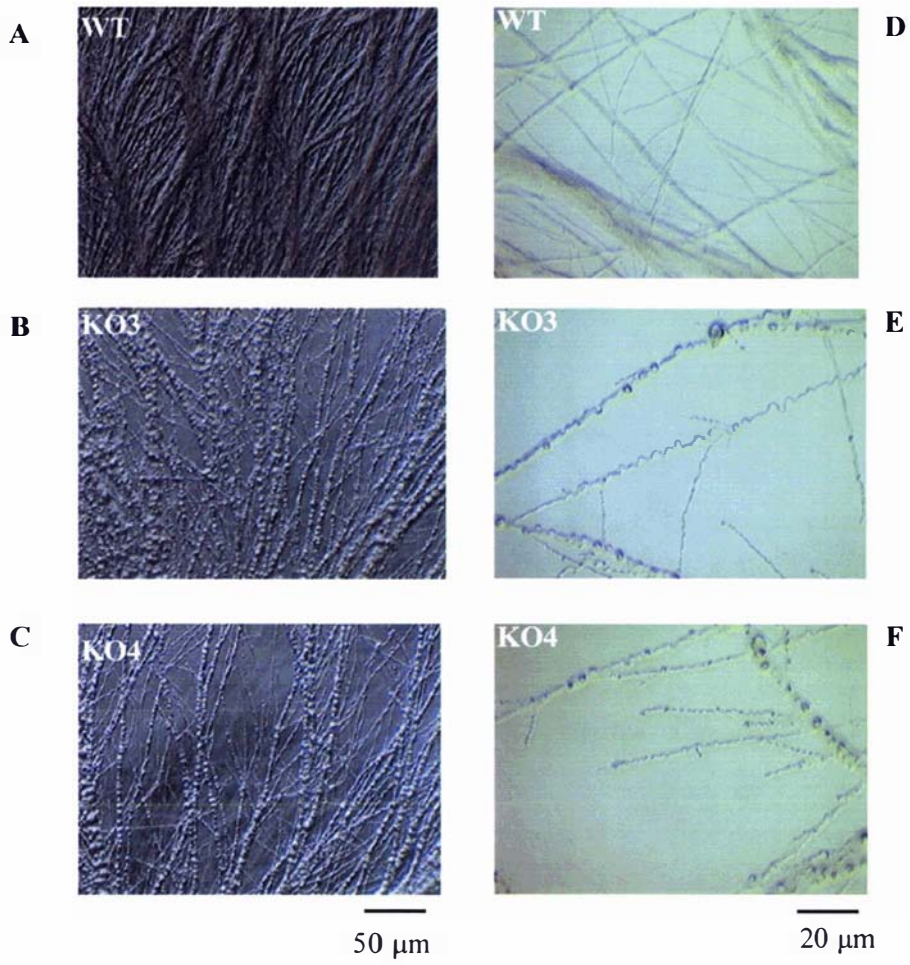


Figure 3.32. Hyphal morphology of E8 wild-type and *thi1* deletion mutants

Hyphal morphology of colonies of E8 wild-type (PN2285) (**A** and **D**), *thi1* deletion mutants KO3 (PN2282) (**B** and **E**) and KO4 (PN2283) (**C** and **F**) grown on CD thiamine-free defined medium at 22°C for 27 days.

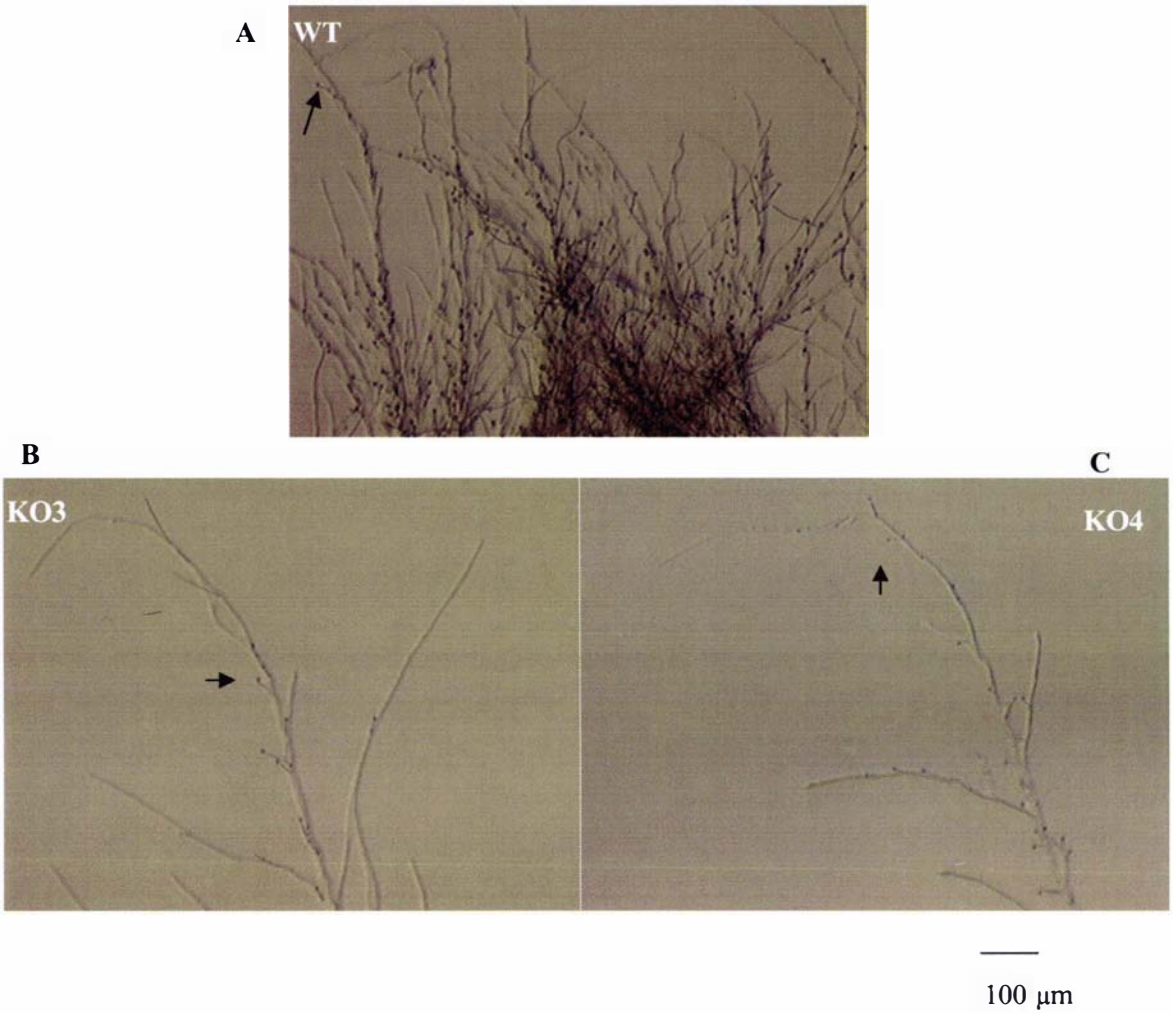


Figure 3.33. Conidia production of E8 wild-type and *thi1* deletion mutants

Conidia (arrow) on hyphal tips in colony margins of E8 wild-type (PN2285) (**A**), *thi1* deletion mutants KO3 (PN2282) (**B**) and KO4 (PN2283) (**C**) grown on CD thiamine-free defined medium at 22°C for 34 days followed by cold incubation (4°C) for a period of 31 days to induce conidiation.

Table 3.5. Colony diameter of the E8 *thi1* deletion mutants, E8 wild type and ectopic controls grown on defined medium supplemented with thiazole or thiamine

Isolates	Colony diameter[d] (mm)		
	CD[a]	CDZ[b]	CDT[c]
WT2	60 ± 2	67 ± 2	64 ± 3
EC1	51 ± 5	57 ± 2	59 ± 2
EC2	78 ± 3	76 ± 6	79 ± 1
KO3	64 ± 5	56 ± 3	66 ± 2
KO4	66 ± 2	56 ± 0	60 ± 1

[a]CD: CD thiamine-free defined medium.

[b]CDZ: CD supplemented with thiazole (3 mg/L).

[c]CDT: CD supplemented with thiamine (3 mg/L).

[d]Average colony diameter (± standard deviation) from duplicate colonies after growth at 22°C for 37 days from a tiny plug of each culture grown on PDA medium.

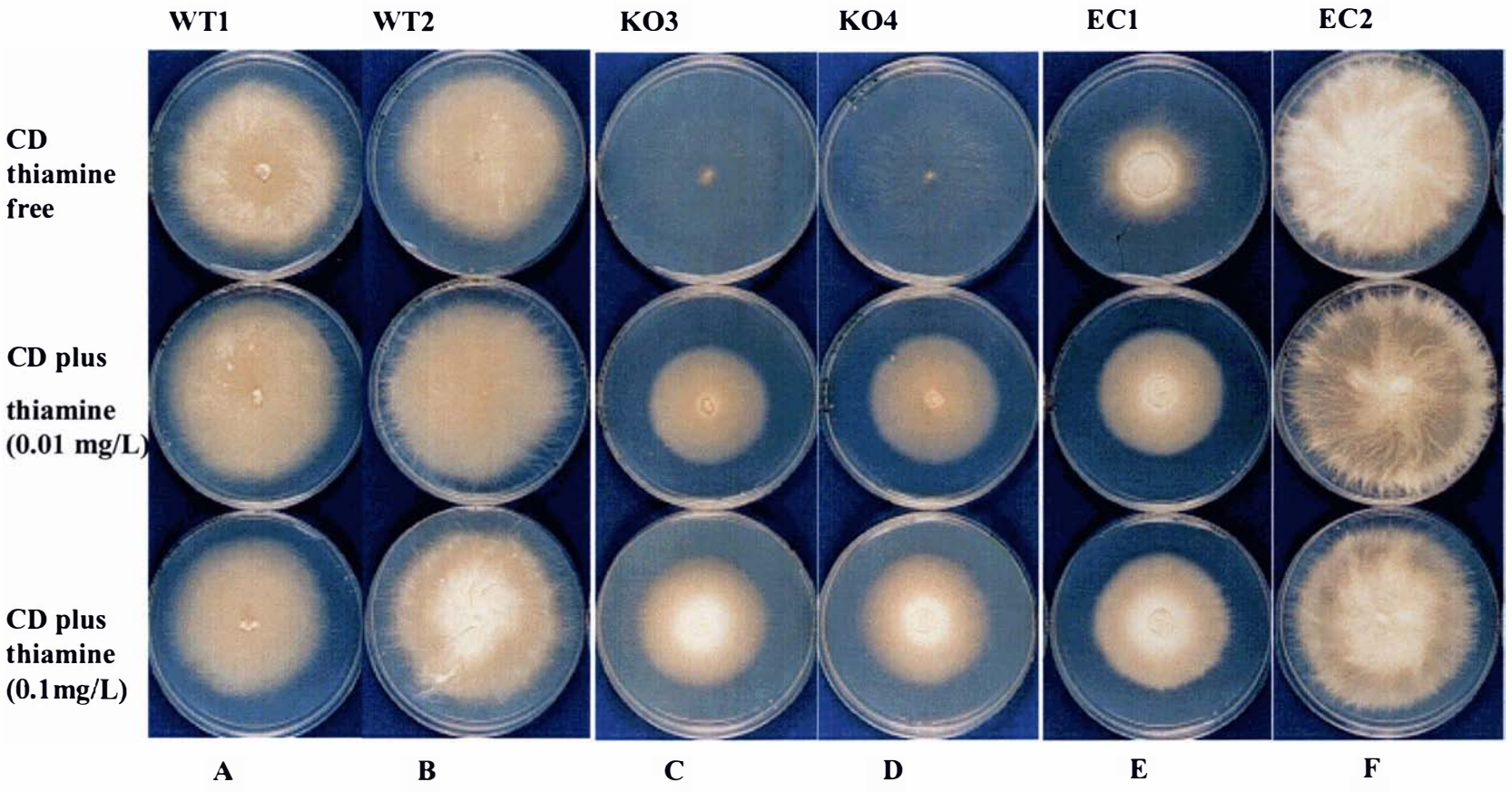


Figure 3.34. Effect of thiamine concentration on the growth of E8 wild-type, ectopic controls and *thi1* deletion mutants

Radial growth of E8 wild-types WT1 (PN2284) and WT2 (PN2285) (**A** and **B**), *thi1* deletion mutants KO3 (PN2282) and KO4 (PN2283) (**C** and **D**) and ectopic transformants EC1 (PN2286) and EC2 (PN2287) (**E** and **F**) grown on CD thiamine-free defined medium and CD medium supplemented with thiamine at final concentrations of 0.01 mg/L or 0.1 mg/L. Colonies were grown at 22°C for 28 days. Plates were inoculated with small blocks of PDA culture.

Figure 3.35. Effect of thiamine concentration on hyphal branching of E8 wild-type, ectopic controls and *thi1* deletion mutants

Hyphal branching on the edge of colonies of E8 wild-types WT1 (PN2284) and WT2 (PN2285) (**A** and **B**), *thi1* deletion mutants KO3 (PN2282) and KO4 (PN2283) (**C** and **D**), and ectopic transformants EC1 (PN2286) and EC2 (PN2287) (**E** and **F**) grown on CD thiamine-free defined medium and CD medium supplemented with thiamine at final concentrations of 0.01 mg/L or 0.1 mg/L. Colonies were grown at 22°C for 28 days. Plates were inoculated with small blocks of PDA culture.

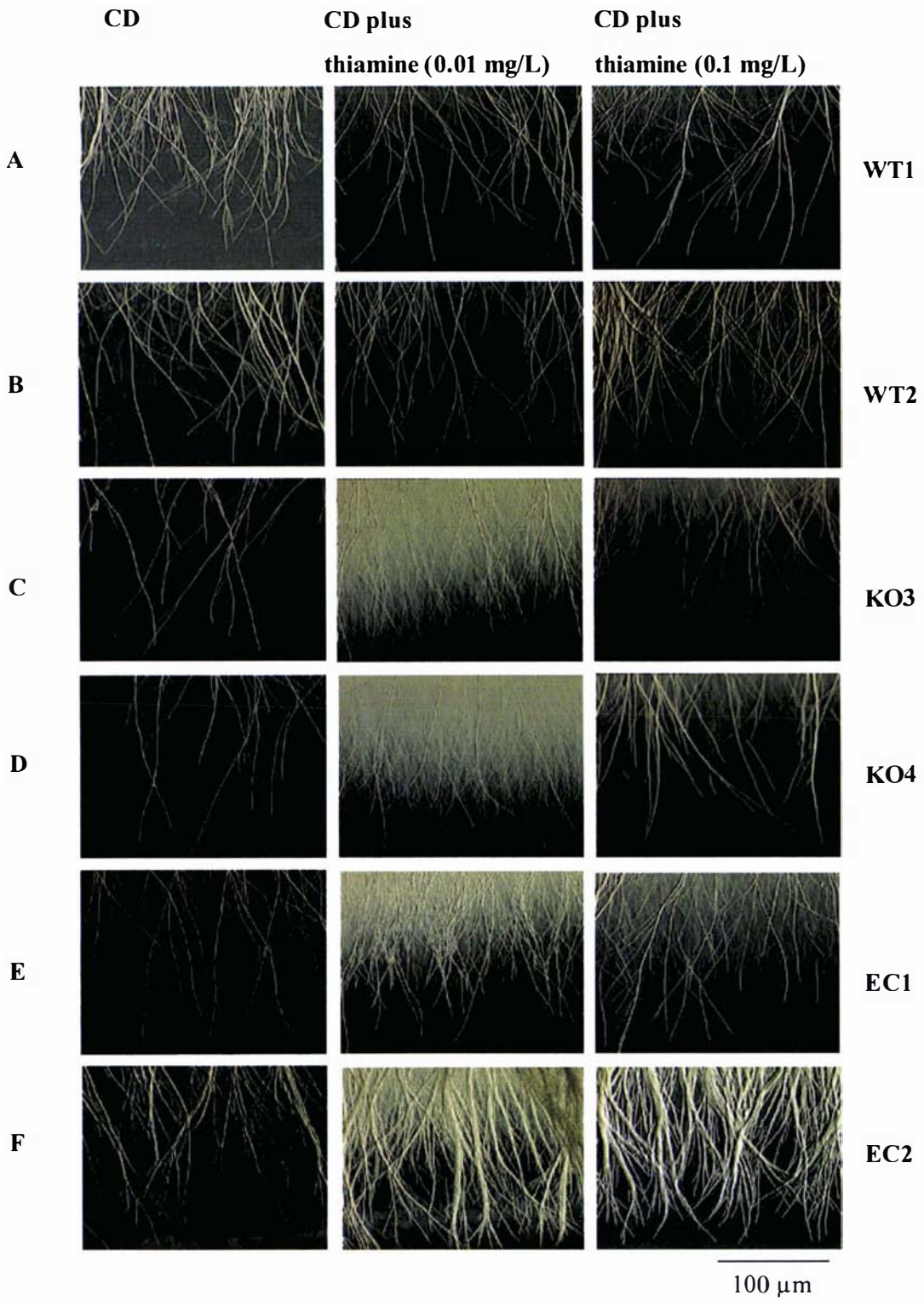

CD
CD plus
thiamine (0.01 mg/L)
CD plus
thiamine (0.1 mg/L)
A
WT1
B
WT2
C
KO3
D
KO4
E
EC1
F
EC2
100 μm

Figure 3.36. Effect of thiamine concentration on hyphal morphology of E8 wild-type, ectopic controls and *thi1* deletion mutants

Hyphal morphology of E8 wild-types WT1 (PN2284) and WT2 (PN2285) (**A** and **B**), *thi1* deletion mutants KO3 (PN2282) and KO4 (PN2283) (**C** and **D**) and ectopic transformants EC1 (PN2286) and EC2 (PN2287) (**E** and **F**) grown on CD thiamine-free defined medium and CD medium supplemented with thiamine at final concentrations of 0.01 mg/L or 0.1 mg/L. Colonies were grown at 22°C for 28 days. Plates were inoculated with small blocks of PDA culture.

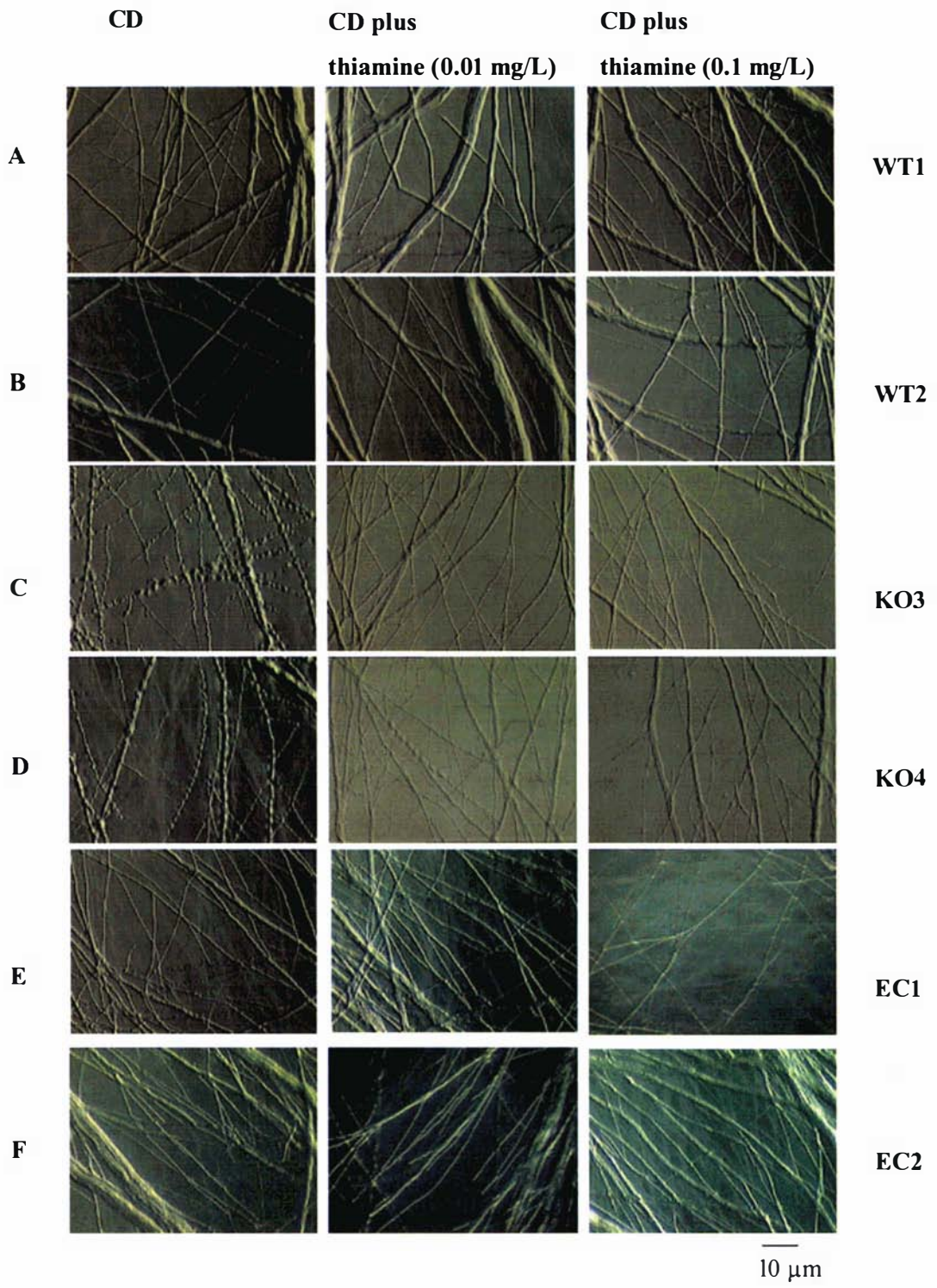
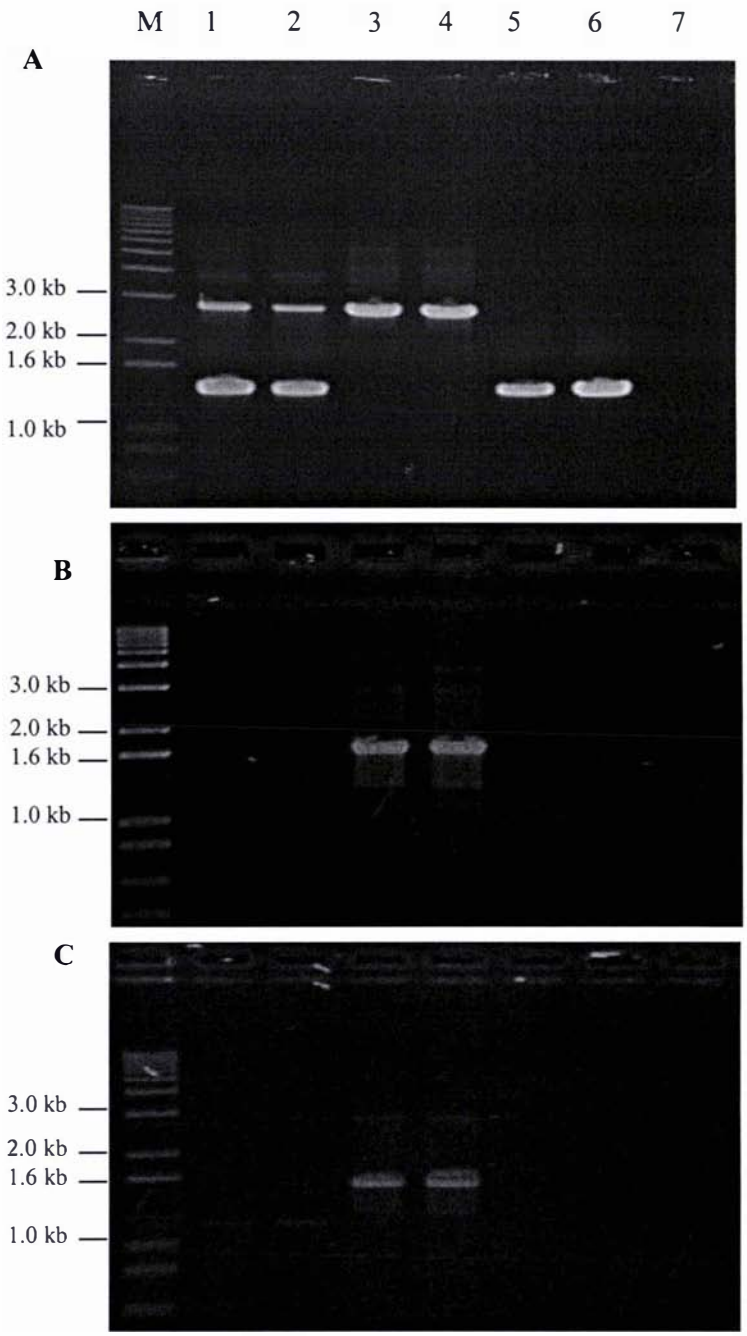

CD
CD plus
thiamine (0.01 mg/L)
CD plus
thiamine (0.1 mg/L)
A
B
C
D
E
F
WT1
WT2
KO3
KO4
EC1
EC2
10 μm

Figure 3.37. Molecular analysis of E8 wild-type, ectopic controls and *thi1* deletion mutants

A: PCR products amplified from genomic DNA of EC1 (PN2286) and EC2 (PN2287) (lanes 1 and 2), KO3 (PN2282) and KO4 (PN2283) (lanes 3 and 4) and E8 wild-types WT1 (PN2284) and WT2 (PN2285) (Lanes 5 and 6) using primers XZ7 & XZ25 that flank E8 *thi1* gene (Figure3.25).

B: PCR products amplified from the same samples as in **A** using primers XZ58 & pUChph9 that flank the recombination insertion in the 5' region of the *thi1* locus (Figure3.25).

C: PCR products amplified from the same samples as in **A** using primer pUChph7 & XZ49 that flank the recombination insertion in the 3' region of the *thi1* locus (Figure3.25).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1.0 kb

Figure 3.38. Colony morphology of the endophyte isolates sub-cultured from CD thiamine-free medium to CD media with varied concentrations of thiamine.

Radial growth of E8 wild-types WT1 (PN2284) and WT2 (PN2285) (**A** and **B**), *thi1* deletion mutants KO3 (PN2282) and KO4 (PN2283) (**C** and **D**) and ectopic transformants EC1 (PN2286) and EC2 (PN2287) (**E** and **F**) grown on CD thiamine-free defined medium (with Noble agar) and CD medium supplemented with thiamine at final concentration of 0.01 mg/l or 0.1 mg/l. Colonies were grown at 22°C for 40 days. Plates were inoculated with small blocks of each culture grown on CD thiamine-free medium (with Noble agar).

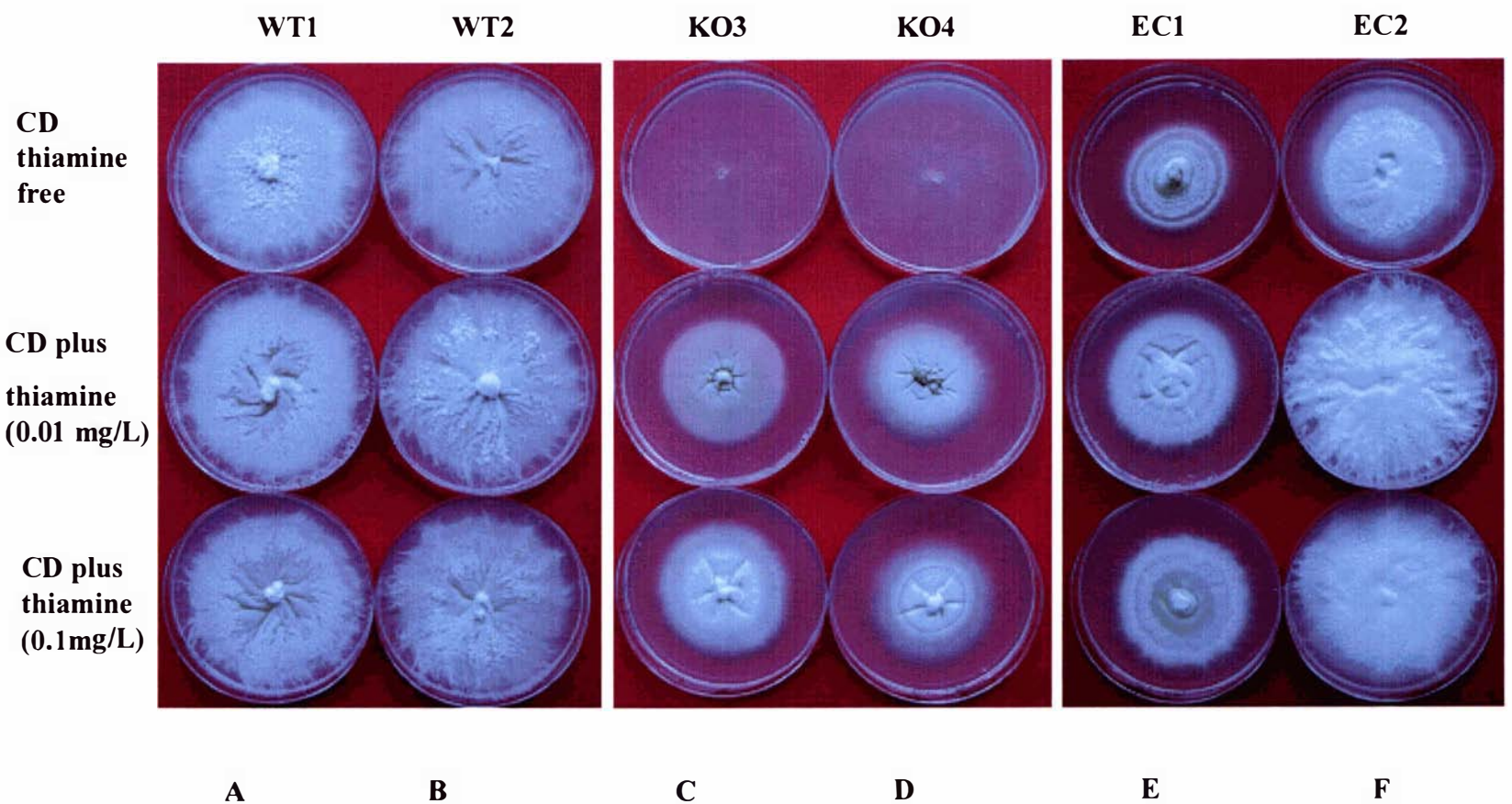

WT1
WT2
KO3
KO4
EC1
EC2
CD thiamine free
CD plus thiamine (0.01 mg/L)
CD plus thiamine (0.1mg/L)
A
B
C
D
E
F

Table 3.6. Effect of different thiamine concentrations on colony diameter of the E8 *thi1* deletion mutants, E8 wild type and ectopic controls (experiment 1)

Isolates	Colony diameter[a] (mm)			
	CD	CD + 0.01 mg/L thiamine	CD + 0.1 mg/L thiamine	CD + 1 mg/L thiamine
WT1 (PN2284)	63 ± 1	65 ± 1	55 ± 1	57 ± 1
WT2 (PN2285)	64 ± 3	65 ± 1	58 ± 3	59 ± 2
EC1 (PN2286)	37 ± 1	43 ± 1	47 ± 1	43 ± 1
EC2 (PN2287)	80 ± 0	80 ± 0	66 ± 2	67 ± 1
KO3 (PN2282)	49 ± 1	44 ± 1	46 ± 0	46 ± 0
KO4 (PN2283)	49 ± 1	45 ± 1	45 ± 1	47 ± 1

[a]Average colony diameter (± standard deviation) from duplicate colonies after growth at 22°C for 28 days from a tiny plug of each culture grown on PDA medium.

Table 3.7. Effect of different thiamine concentrations on colony diameter of the E8 *thi1* deletion mutants, E8 wild type and ectopic controls (experiment 2)

Isolates	Colony diameter[a] (mm)		
	CD	CD + 0.01 mg/L thiamine	CD + 0.1 mg/L thiamine
WT1 (PN2284)	81 ± 1	80 ± 0	81 ± 1
WT2 (PN2285)	81 ± 0	80 ± 0	80 ± 0
EC1 (PN2286)	53 ± 1	63 ± 2	63 ± 1
EC2 (PN2287)	78 ± 2	82 ± 1	81 ± 1
KO3 (PN2282)	61 ± 1	59 ± 1	61 ± 2
KO4 (PN2283)	61 ± 1	58 ± 1	60 ± 2

[a]Average colony diameter (± standard deviation) from duplicate colonies after growth at 22°C for 40 days on Noble agar from a tiny plug of each culture grown on CD thiamine free medium (with Noble agar).

3.4. Analysis of the symbiotic phenotype of E8 *thi1* deletion mutants in association with perennial ryegrass

3.4.1. Analysis of the symbiotic phenotype of E8 *thi1* deletion mutants in association with perennial ryegrass (experiments 1 and 2)

In order to investigate the symbiotic phenotype of E8 *thi1* deletion mutants in association with perennial ryegrass, the *thi1* deletion mutants KO3 (PN 2282) and KO4 (PN2283), ectopic transformant isolates EC1 (PN2286) and EC2 (PN2287), and E8 wild-type controls WT1 (E8, PN2286) and WT2 (E8, PN2287), were inoculated into perennial ryegrass seedlings (cultivar 'Nui' line A5322) using the insertion method described by Latch and Christensen 1985 (Section 2.12.1). The properties examined included host colonisation, host growth and stromata development.

3.4.1.1. Host survival and colonisation

The inoculated seedlings were observed at a regular period for the vigour of growth. Several weeks after transplantation into soil, many seedlings were green and healthy but others were stunted in growth and some of these eventually wilted and died. The death of these seedlings was probably due to a pathogenic interaction of the endophyte with the host and/or a low seedling vigour. As a result, host survival rates as well as the infection rates of these endophytes were examined. The presence of endophyte in plants was determined by tissue-print immuno blotting (Section 2.12.3) and/or by microscopic examination of aniline blue stained epidermal tissue (Section 2.12.4). A summary of endophyte infection rates and host survival rates are presented in Table 3.8 and Table 3.9.

The *thi1* mutants had higher average host survival rates than the wild-type in both experiment 1 and 2, but a meaningful statistical analysis requires more experimental data. The EC1 had a host survival rate similar to the mutants, and EC2 had a host survival rate similar to the wild-type.

The infection rates of strains based on surviving plants in experiment 1 ranged from 42% to 83%, with the average infection rate of the *thi1* mutants higher than that of the wild-type, but again a meaningful statistical analysis requires more experiment data. The relatively low infection rates for some isolates indicated that the endophyte failed to colonise some inoculated seedlings. The infection rates of strains based on surviving plants in experiment 2 ranged from 77% to 94%, with no significant difference between the wild-type and the *thi1* mutants. The infection rate of the wild-type in this experiment was higher than that obtained in experiment 1.

The infection rates as determined by the number of infected plants per number of inoculated seedlings ranged from 27% to 67% in experiment 1 and from 44% to 63% in experiment 2. More experiments are required to obtain a meaningful statistical analysis. These low infection rates were probably due to both the death of the host and the failure of *E. typhina* to colonise the host.

3.4.1.2. Endophyte growth phenotype

The hyphal concentration and morphology of the endophyte in leaf sheaths were examined by microscopy using aniline blue stained tissue (Section 2.12.4). The concentration of hyphae, hyphal branching frequency, hyphal septa spacing, and width of hyphae varied, depending on the endophyte isolate and the host grass. The phenotypes of hyphal growth were arbitrarily divided into four basic types (A, B, C and D, from low to high hyphal branching frequency and concentration) as shown in Fig. 3.39. Different plants infected

Table 3.8. Seedling survival and colonisation following inoculation with E8 *thi1* deletion mutants, E8 wild type and ectopic controls: experiment 1

Endophytes	Number of inoculated seedlings	Host survival rate[a] (% of inoculated seedlings)	Endophyte infection rate[b] (% of surviving seedlings)	Endophyte infection rate[c] (% of inoculated seedlings)
WT1 (PN2284)	30	63	42	27
WT2 (PN2285)	30	60	56	33
EC1 (PN2286)	30	77	83	63
EC2 (PN2287)	30	67	80	53
KO3 (PN2282)	30	83	80	67
KO4 (PN2283)	30	67	70	47

[a]Percentage of original seedlings surviving was determined as surviving seedlings/inoculated seedlings in January of 2002 (51 days after inoculation in November 2001).

[b]Percentage of surviving seedlings infected was determined as infected seedlings/surviving in January of 2002 (51 days after inoculation in November 2001).

[c]Percentage of original seedlings infected was determined as infected seedlings/inoculated seedlings in January of 2002 (51 days after inoculation in November 2001).

The seedlings were grown in root-trainers containing potting mix.

Table 3.9. Seedling survival and colonisation following inoculation with E8 *thi1* deletion mutants, E8 wild type and ectopic controls: experiment 2

Endophytes	Number of inoculated seedlings	Host survival rate[a] (% of inoculated seedlings)	Endophyte infection rate[b] (% of surviving seedlings)	Endophyte infection rate[c] (% of inoculated seedlings)
WT1 (PN2284)	25	68	82	56
WT2 (PN2285)	25	56	79	44
EC1 (PN2286)	25	76	79	60
EC2 (PN2287)	25	64	94	60
KO3 (PN2282)	35	74	77	57
KO4 (PN2283)	35	77	81	63

[a]Percentage of original seedlings surviving was determined as surviving seedlings/inoculated seedlings in January of 2002 (37 days after inoculation in December 2001).

[b]Percentage of surviving seedlings infected was determined as infected seedlings/surviving in January of 2002 (37 days after inoculation in December 2001).

[c]Percentage of original seedlings infected was determined as infected seedlings/inoculated seedlings in January of 2002 (37 days after inoculation in December 2001).

The seedlings were grown in root-trainers containing potting mix.

with the same isolate showed a range of different phenotypes. While the inoculation method and physiological conditions under which the plants were grown probably would affect the hyphal growth phenotypes within the hosts, the more likely explanation for the range of hyphal growth phenotypes is a grass genotype effect, given the highly heterozygous genetic background of the perennial ryegrass cultivar (Christensen *et al*,. 1997; Cheplick *et al*., 2000; Faeth and Sullivan, 2003). Among the ten grasses infected with KO3 or KO4, six had the type A phenotype (hyphae were straight, seldom branched and typically a single hypha was found in the intercellular space), and four had a branching growth phenotype (type B to C in Fig. 3.39) (Table 3.10). For the wild-type, four of ten infected plants had the type A phenotype. For the ectopic controls, three of the ten infected plants had the type A phenotype. For both the wild-type and ectopic control groups there were a greater number of the more severe hyphal growth phenotypes (C to D) compared to the mutants. The results might suggest that the *thi1* deletion mutants had a reduced hyphal branching and biomass in the plant. However, given the small sample size and the wide range of hyphal growth phenotypes, more observations are required for comparison of hyphal growth between the mutant and the controls.

3.4.1.3. Host morphology and growth phenotype

Following inoculation in December of 2001, the morphology and growth of the host was monitored on a regular (weekly) basis. What was striking was the range of plant phenotypes (Fig 3.40 A to G) observed for plants infected with the wild-type strain. Some plants grew vigorously whereas others showed a noticeable stunting, characterised by an increase in the number of tillers and reduced height of the plants (Fig 3.40 I and J). Again the wide range of host phenotypes probably highlight the very strong host genotype effect. For the limited number of associations analysed none of the plants infected with the *thi1* mutant showed the extreme phenotype of stunting observed with wild-type (Table 3.11). All 10 plants, randomly selected from root trainers and potted, had a normal growth

phenotype (Fig. 3.40 H and K). Correlation between the host stunting phenotype and the degree of hyphal branching was not evident (Table 3.12). The host stunting phenotypes were observed in grasses with an endophyte type A, B or C hyphal growth phenotype.

In order to more accurately described the host growth characteristics, tiller numbers of the plants were counted and the plant fresh weight determined. These plants showed a great variation in the tiller number and fresh weight. A comparison of the tiller number per plant, plant fresh weight, and tiller weight is summarised in Table 3.13. Statistical analysis by ANOVA (Least Significant Difference (LSD), P = 0.05) showed that plants infected with the wild-type had an average tiller number significantly greater than those infected with KO4, but not for the plants infected with KO3, EC1 or EC2. Significant differences were also observed in tiller weight among the different endophyte-grass associations. The tiller weight of the plants infected with wild-type were significantly less than that of plants infected with KO4 (ANOVA, LSD, P = 0.05). However, the tiller weights of plants infected with KO3, EC1 and EC2 were between those of the wild-type and of KO4. These results are consistent with the previous observations that stunted plants were predominantly present in the wild-type infected plants. No significant differences were detected in plant weight between the wild-type and KO infected plants.

3.4.1.4. Peramine concentration

Peramine concentration of the plants were examined (Section 2.12.6) to determine if there was any effect of the *thi1* deletion on alkaloid production. The average peramine concentrations ranged from 70 to 94 ppm, with the EC2 infected plants having the highest peramine concentration of 94 ppm (Table 3.13). Peramine concentrations varied considerably in different plants infected with the same isolate. No significant difference (P = 0.9181, unpaired T test in web site http://www.graphpad.com/quickcalcs/ttest2.cfm) was detected between plants infected with the KO compared to the wild-type.

3.4.1.5. Stromata development on vegetative tillers

An unexpected observation from experiment 2 was the development of stromata on vegetative tillers of infected plants. Previous to these experiments, stromata of *E. typhina* had been reported to be present only on reproductive tillers (White and Cole, 1985; White *et al.*, 1997). The presence of stromata on vegetative tillers of the infected plants was initially observed in March 2002. Additional stromata continued to form until the middle of June. The stromata on vegetative tillers were comprised of a conspicuous dense white layer of mycelium on leaves of tillers (Fig. 3.41). Numerous solitary, terminal small conidia on long slender conidiophores on epiphytic hyphae were observed on the leaf surfaces under a dissecting microscope. Often the stromata were confined to the blade, on both the abaxial (upper) and adaxial (lower) surfaces. Occasionally, external hyphae were present on the sheath, in close proximity to the ligular zone. The stromata typically covered just a part of the leaf blade immediately above the ligular zone or some distance above the ligular zone, and the edges of the blade were often fused together (Fig 3. 41Cand D). The stromata developed on only a few tillers on any one plant, and these plants were typically not stunted (Table 3.12). For one plant (G1080) with a few large and many small tillers, the stromata were formed only on the large tillers. The leaves on which stromata were formed initially appeared green and healthy. However, once the stromata were formed, the growth of immature leaves was stunted and mature leaves with stromata became wilted and brown within a short time. These observations indicated that the stromata may divert a large quantity of nutrients and water from the leaf tissues to the external hyphae and thus have a detrimental effect on leaf growth and survival. Among the 30 potted plants examined, 10 developed stromata on vegetative tillers. Of these, four were infected with the wild-type, four with ectopic controls and two with the *thi1* deletion mutants (Table 3.14). This is the first report of stroma development on vegetative tillers of a grass infected with *E. typhina* or any other *Epichloë* species.

3.4.1.6. Stromata development on reproductive tillers

In order to test whether the *thi1* deletion had any effect on stromata development on reproductive tillers, the 30 plants from experiment 2 were vernalized during the winter of 2001 to promote inflorescence development. During the period of October to December of 2002, a total of 15 of the 30 plants developed stromata on reproductive tillers (Fig. 3.42); six of these were infected with the wild-type, six with ectopic controls and three with KO3 or KO4 (Table 3.14). Examination of plants lacking stromata showed that reproductive tillers were absent or had not fully developed, suggesting that those plants lacking stromata either had no reproductive tillers or tillers that had not fully developed. These results demonstrate that the *thi1* deletion mutants retain the ability to form stromata. Most of the plants (although not all) that had stromata develop on vegetative tillers also had stromata on reproductive tillers. The exceptions were three plants (G1083, G1088 and G1106) for which the ability to produce stromata on reproductive tillers could not be determined due to the absence of such tillers. Other plants (G1081, G1082, G1085, G1087, G1095, G1096, G1100 and G1105) that had no stromata on vegetative tillers still had stromata develop on reproductive tillers.

There were a wide range of patterns of stromata development on reproductive tillers. The majority of the stromata-bearing plants had stromata on all the reproductive tillers. However, a few reproductive tillers had no stromata (Fig. 3.42 C) although they contained endophyte as indicated by microscopic examination. The flag leaf of the stromata-bearing tillers was typically shorter (ranging from 5 to 17 cm) than that of non-stromata reproductive tillers (typically over 20 cm). Elongation of the inflorescence and the internode below were typically reduced (Fig. 3.43). Stromata were not confined to the flag leaf. For one of the wild-type association, the second and the third leaf of some tillers was also

covered with mycelium. For all three *thi1* mutant associations with stromata, both the flag leaf and the second leaf were covered with mycelium and development of the inflorescence was nearly completely restricted (Fig. 3.43). With some tillers the mycelia covering was present on both the leaf sheath and blade.

3.4.1.7. Stability of the endophyte-grass associations

The 30 plants from experiment 2 were monitored regularly for the presence of endophyte and for changes in plant morphology over a period of more than one year. About 7 months after inoculation, one plant (G1091) infected with EC1 lost the endophyte as indicated by microscopic examination and tissue-printing immuno blotting. One other plant (G1084, infected with WT1) had dramatically less hyphae than previously detected. Hyphae in this plant were unevenly distributed, were highly branched and convoluted, were poorly stained, and the length of compartments was often short. In some tillers or leaves of plant G1084, no endophyte was detected. Plants G1091 and G1084 initially were classified as having type B and type C hyphal growth phenotypes respectively.

Changes in plant morphology and growth were also observed. One year after inoculation, three plants (G1077, G1098 and G1105 infected with WT1, EC2 and KO4 respectively) had died. No extreme stunting of these hosts was observed prior to host death. These observations suggest that the endophyte colonisation and/or host morphology can change over time as a consequence of the ongoing growth of the plant and the endophyte and the interaction between both. The loss of endophyte hyphae in the plant and loss of host viability probably reflect an incompatibility in the genotypes of the endophyte and the host during the interaction.

3.4.1.8. Analysis of the endophyte re-isolated from the plants

In order to confirm that the plants being analysed really carried the E8 *thi1* deletion mutants, E8 wild-type or ectopic controls, endophytes were re-isolated from these plants (Section 2.12.5) for molecular analysis. Fast-emerging and fast-growing hyphae developed from the surface sterilised excised leaf sheath and blade sections placed on PDA plates. The endophyte colonies were white and cottony, and conidia were present. These characteristics are typical of *E. typhina* endophytes. No morphological differences were observed among colonies of wild-type, *thi1* mutant and ectopic isolates (Fig. 3.44).

PCR amplifications of genomic DNA from these isolates was carried out using primers Lp19CY1 and Lp19CY6 which flank a highly polymorphic microsatellite located in the 5' coding region of a geranylgeranyl diphosphate (GGPP) synthase (C. Young personal communication). A PCR product of about 550 bp was amplified from all these isolates (Fig. 3.45). The PCR product from a representative number of these isolates from plants G1077 (WT1), G1082 (WT2), G1087 (1A), G1092 (120A), G1097 (KO3) and G1102 (KO4) was sequenced. All the sequences were identical to that of the original E8 wild-type isolate (Appendix 2), confirming that all isolates are either E8 or derivatives of E8.

Further PCR analysis was carried out to distinguish the individual isolates. PCR amplification (Section 2.7.2) using primers XZ7and XZ25 was carried out to confirm that these isolates were E8 *thi1* KOs, wild-type and ectopic controls. Primer set XZ7and XZ25 (flanking the *thi1* gene) should give a product of 1.4 kb in the wild-type, a product of 2.7 kb in the KOs, and two products of 1.4 kb and 2.7 kb in ectopic controls. As expected, a band of 1.4 kb was produced from the endophyte re-isolated from G1077-G1086 that were infected with the wild-type isolates. Two bands of 2.7 kb and 1.4 kb were produced from G1087-G1097 that were infected with EC1 and EC2. A single band of 2.7 kb was produced from G1097-G1106 that were infected with KO3 and KO4 (Fig. 3.46).

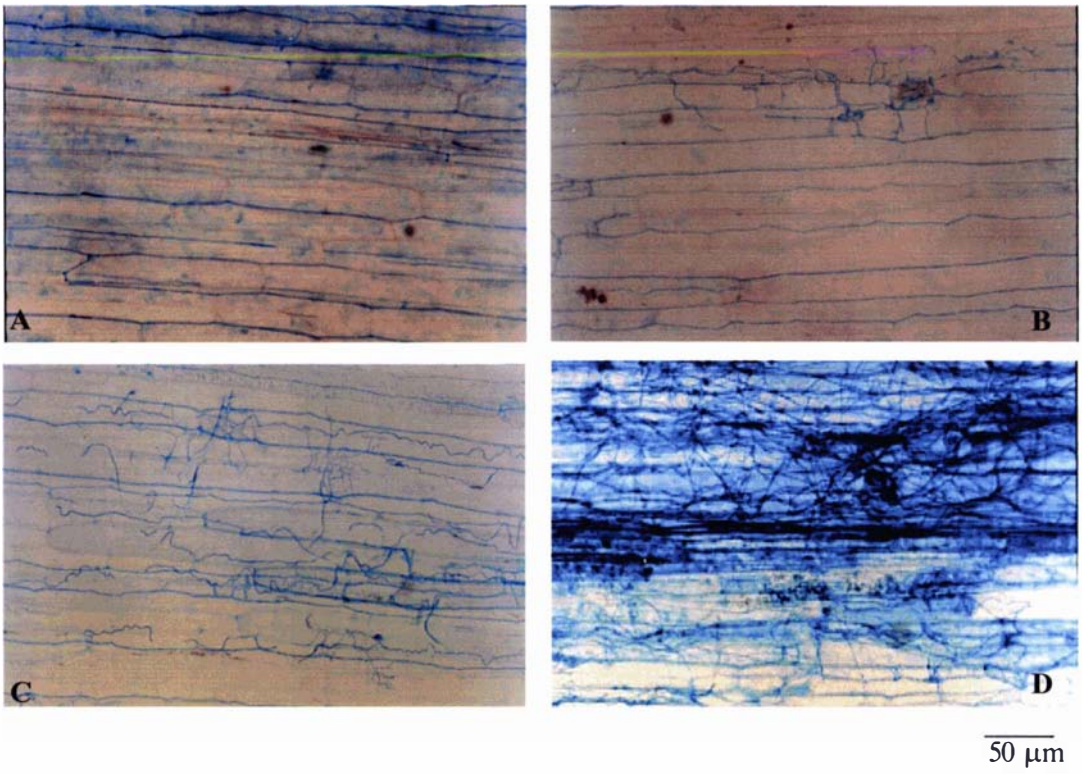


Figure 3.39. Light micrographs of aniline blue stained endophytes in leaf sheaths from perennial ryegrass

Thin epidermal strips from the inner leaf sheath were removed in January of 2002 (50 days after inoculation) and stained with aniline blue. Several different types of hyphal growth were observed:

A: Hyphae were straight, seldom branched and typically a single hypha was found in an intercellular space. The sample was taken from plant G1098 infected with E8 *thi1* deletion mutant KO3 (PN2282).

B: Hyphae were occasionally branched and convoluted. The sample was taken from plant G1099 infected with KO4 (PN2283).

C: Hyphae were typically branched, convoluted, uneven in diameter. The sample was taken from plant G1089 infected with ectopic control EC1 (PN2286).

D: Hyphae are highly branched and often aggregated together. The sample was taken from plant G1077 infected with E8 wild-type WT1 (PN2284).

Table 3.10. Number of different hyphal colonisation phenotypes

Endophytes	Number of plants[a]	Number of type A[b]	Number of type B[c]	Number of type C[d]	Number of type D[e]
WT1 (PN2284)	5	2	1	1	1
WT2 (PN2285)	5	2		3	
EC1 (PN2286)	5	1	1	3	
EC2 (PN2287)	5	2	1	2	
KO3 (PN2282)	5	3	1	1	
KO4 (PN2283)	5	3	1	1	

[a]The plants were from experiment 2 and examined in January of 2002 (50 days after inoculation in December of 2001) by light microscopy using aniline blue stained material.

[b]Hyphal colonisation phenotype as described in Figure 3.39 A

[c]Hyphal colonisation phenotype as described in Figure 3.39 B

[d]Hyphal colonisation phenotype as described in Figure 3.39 C

[e]Hyphal colonisation phenotype as described in Figure 3.39 D

The plants were grown in pots containing potting mix.

Figure 3.40. Host phenotype of plants infected with E8 wild-type and *thi1* mutants

Photograph of perennial ryegrass plants in February of 2002 (about 12 weeks post inoculation).

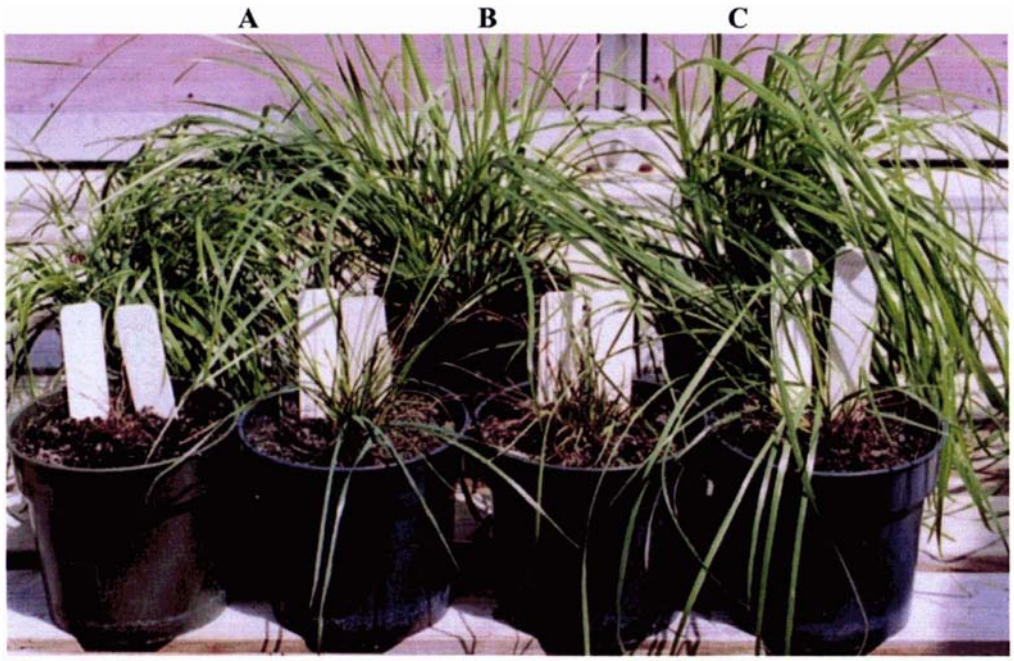
A - G: Seven plants infected with E8 wild-type.

H : A plant infected with E8 *thi1* mutant.

I: A plant infected with E8 wild-type.

J: A plant infected with E8 wild-type.

K: A plant infected with E8 *thi1* mutant.


A
B
C
D
E
F
G


H
I
J
K

Table 3.11. Number of stunted plants in experiment 2

Endophytes	Number of stunted plants/number of plants examined[a]
WT1 (PN2284)	2/5
WT2 (PN2285)	1/5
EC1 (PN2286)	1/5
EC2 (PN2287)	0/5
KO3 (PN2282)	0/5
KO4 (PN2283)	0/5

[a]The plants were examined during February to May of 2002 (about 12 to 25 weeks after inoculation in December of 2001). Stunted grasses typically have a turf-like appearance which is a consequence of reduced size of tillers as shown in Figure 3.40 I and J.

Table 3.12. Correlation of hyphal growth phenotypes, host stunting, and stromata development for experiment 2

Endophytes	Plant identification and phenotypes				
WT1 (PN2284)	G1077[a] **D[b] SV[c] SR[d]**	G1078 **B**	G1079 **C SV SR**	G1080 **A SV SR**	G1081 **A SR**
WT2 (PN2285)	G1082 **C SR**	G1083 **A SV**	G1084 **C**	G1085 **A SR**	G1086 **C**
EC1 (PN2286)	G1087 **C SR**	G1088 **C SV**	G1089 **C SV SR**	G1090 **A SV SR**	G1091 **B**
EC2 (PN2287)	G1092 **A**	G1093 **B SV SR**	G1094 **C**	G1095 **A SR**	G1096 **C SR**
KO3 (PN2282)	G1097 **A**	G1098 **A**	G1099 **B**	G1100 **A SR**	G1101 **C**
KO4 (PN2283)	G1102 **A**	G1103 **C SV SR**	G1104 **B**	G1105 **A SR**	G1106 **A SV**

[a]The G number indicates plant identification number.

[b]The letters A, B, C or D indicates the hyphal growth phenotypes shown in Fig. 3.39 examined in January of 2002 (about 7 weeks after inoculation in December of 2001).

[c]SV indicates stromata development on vegetative tillers during March to June of 2002 (about 13 to 27 weeks after inoculation in December of 2001).

[d]SR indicates stromata development on reproductive tillers during October to December of 2002 (about 46 to 53 weeks after inoculation in December of 2001).

The shaded Cell indicates plants that were stunted during February to May of 2002 (about 12 to 25 weeks after inoculation in December of 2001).

Table 3.13. Host tiller number, tiller weight, fresh weight and peramine concentration for experiment 2

Endophytes	Tiller number per plant[a]	Fresh weight per tiller[b] (g)	Fresh weight per plant[c] (g)	Peramine concentration[d] (ppm)
WT1 (PN2284)	102 ± 53 a[e]	0.15 ± 0.05 a	15.1 ± 2.8	82 ± 25
WT2 (PN2285)	115 ± 25 a	0.14 ± 0.04 a	15.5 ± 1.0	71 ± 10
EC1 (PN2286)	80 ± 43 ab	0.26 ± 0.11 bc	20.6 ± 4.6	70 ± 32
EC2 (PN2287)	78 ± 10 ab	0.24 ± 0.03 bc	18.3 ± 0.3	94 ± 43
KO3 (PN2282)	80 ± 20 ab	0.20 ± 0.02 ab	15.9 ± 0.3	82 ± 34
KO4 (PN2283)	54 ± 13 b	0.31 ± 0.14 c	17.0 ± 1.7	71 ± 33

[a]Average (n = 5) tiller number per plant (± standard deviation) examined in May of 2002 (about 22 weeks post inoculation in December 2001).

[b]Average (n = 5) tiller weight (± standard deviation) examined in May of 2002 (about 22 weeks post inoculation in December 2001).

[c]Average (n = 5) fresh weight per plant (± standard deviation) examined in May of 2002 (about 22 weeks post inoculation in December 2001).

[d]Average (n = 5) peramine concentration (± standard deviation) for plants examined in February of 2003 (about 64 weeks after inoculation in December of 2001). The plants were trimmed back and grown for three weeks before harvested.

[e]Average tiller number or tiller weights not followed by a common letter are significantly different ($P < 0.05$) according to ANOVA analysis.

Table 3.14. Number of plants with stromata on vegetative tillers or reproductive tiller for experiment 2

Endophytes	Stromata of vegetative tiller[a]	Stromata on reproductive tillers[b]
WT1 (PN2284)	3/5[c]	4/5
WT2 (PN2285)	1/5	2/5
EC1 (PN2286)	3/5	3/5
EC2 (PN2287)	1/5	3/5
KO3 (PN2282)	0/5	1/5
KO4 (PN2283)	2/5	2/5

[a]Stromata on vegetative tillers were observed on the plants from experiment 2 during March to June of 2002 (about 13 to 27 weeks after inoculation in December of 2001).

[b]Stromata on reproductive tillers were observed on the plants from experiment 2 during October to December of 2002 (about 46 to 53 weeks after inoculation in December of 2001).

[c]The numbers of plants with stromata/the number of plants observed.

Figure 3.41. Stromata on vegetative tillers

A and **B:** Stromata on vegetative tillers of perennial ryegrass infected with E8 wild-type.

C and **D:** Stromata on vegetative tillers of perennial ryegrass infected with E8 *thi1* deletion mutants.

E: Light micrograph of a transverse cross-section through the stroma-forming region of a vegetative tiller infected with E8 *thi1* deletion mutant, showing the distribution of epiphytic hyphae.

The arrow indicates stroma.

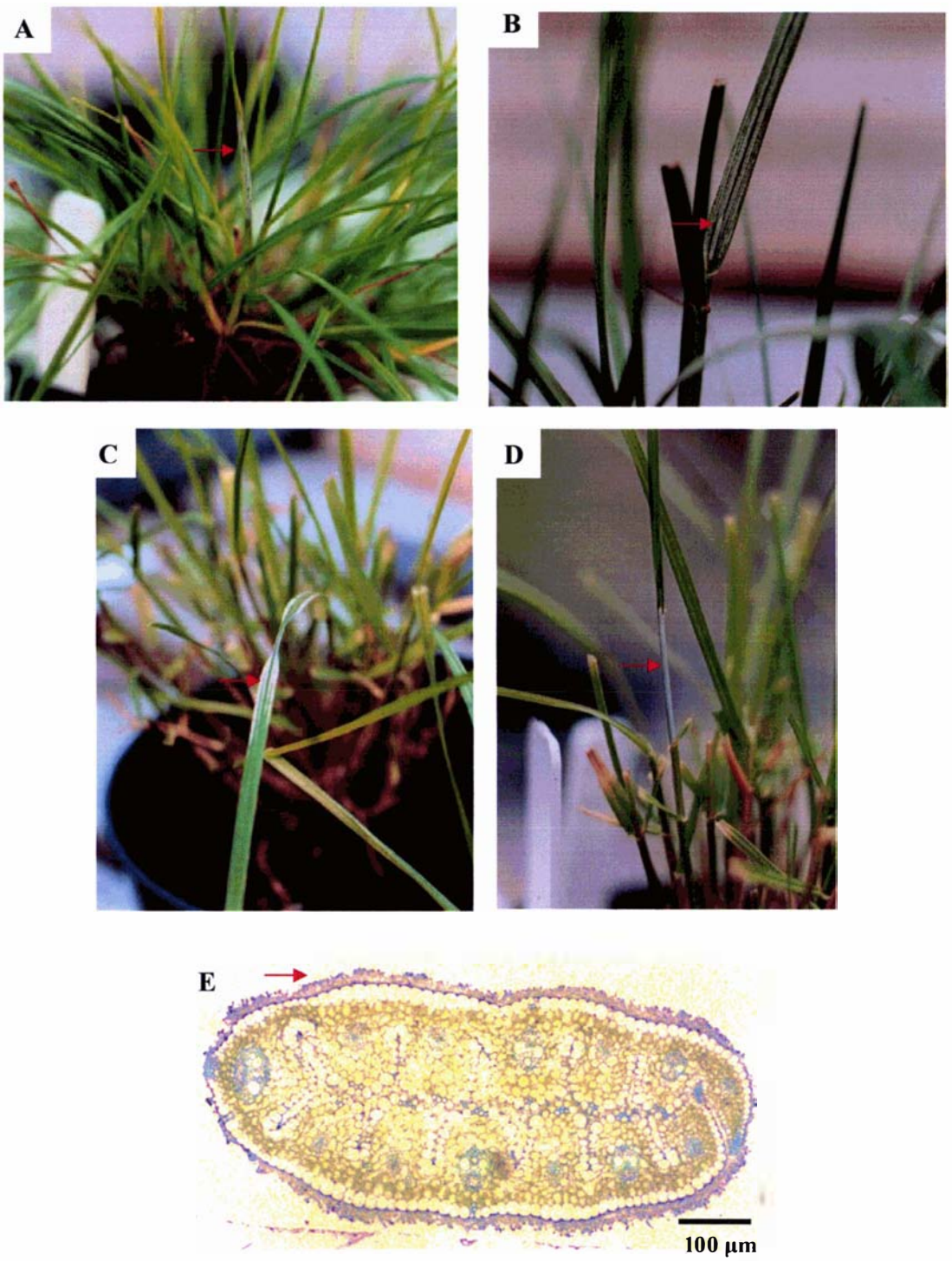

A
B
C
D
E
100 µm

Figure 3.42. Stromata on reproductive tillers

A - C: Stromata on reproductive tillers of perennial ryegrass infected with E8 isolate.

D: Stromata on reproductive tillers of perennial ryegrass infected with E8 *thi1* deletion mutant.

The red arrow indicates stroma. The black arrow indicates a reproductive tiller lacking stroma.

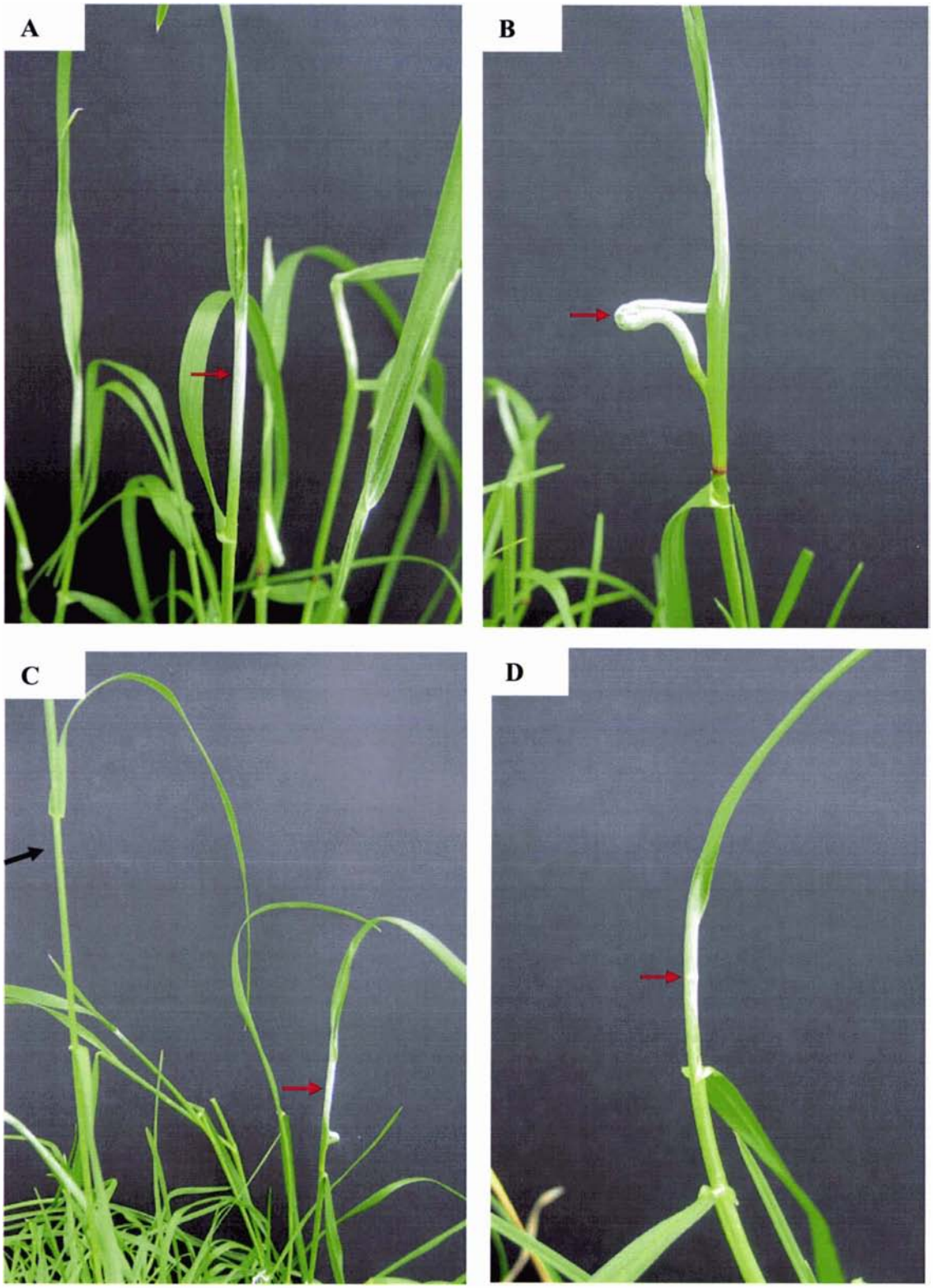

A
B
C
D

Figure 3.43. Stromata on reproductive tillers

A: Stromata on reproductive tillers of perennial ryegrass infected with E8 wild-type. Each tiller from an individual plant.

B: The reproductive tillers in **A** after removing the leaf with stroma showing the mycelium covered inflorescence.

C: Stromata on a reproductive tillers of perennial ryegrass infected with E8 *thi1* deletion mutants.

D: The reproductive tillers in **C** after removing the outer leaf with stromata.
The arrow indicates stroma.

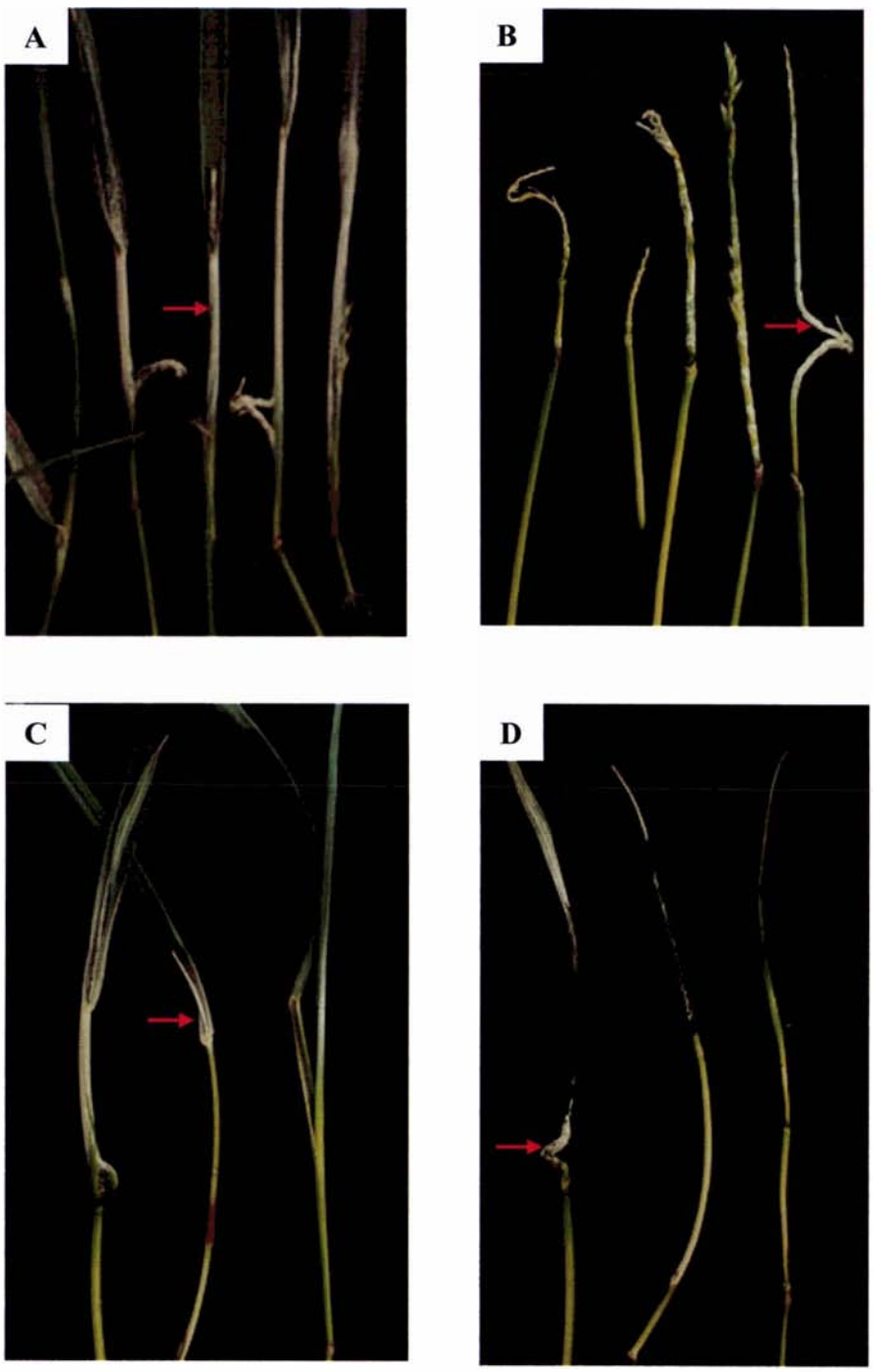

A
B
C
D

Figure 3.44. Growth of E8 *thi1* mutants, E8 wild-type and ectopic controls re-isolated from the host grass

Colony morphology of endophytes re-isolated from host grasses from experiment 2 at 7 weeks post inoculation, and grown for 13 days at 22°C on PDA containing oxytetracycline (5 mg/L).

A: Endophytes re-isolated from grasses G1077-G1081 infected with E8 wild-type WT1 (PN2284).

B: Endophytes re-isolated from grasses G1097-G1101 infected with E8 *thi1* mutant KO3 (PN2282).

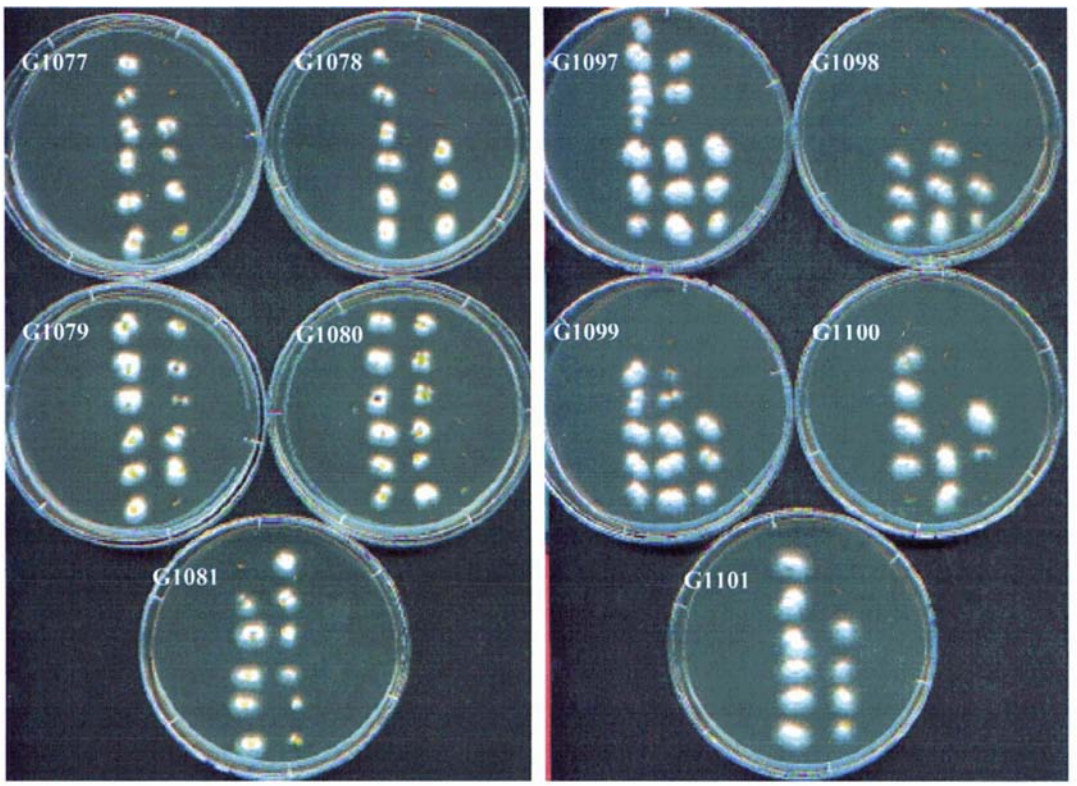

G1077
G1078
G1079
G1080
G1081
G1097
G1098
G1099
G1100
G1101
A
B
40 mm

Figure 3.45. Microsatellite PCR analysis of E8 *thi1* mutants, E8 wild-type and ectopic controls re-isolated from host grasses

A: PCR product amplified from genomic DNA of endophytes re-isolated from five different grasses (G1077-G1081) infected with wild-type E8 WT1 (PN2284) (lane 1 to 5) and five grasses (G1082-G1086) infected with wild-type E8 WT2 (PN2285) (lane 6-10). Primers used were CYLp19-1 & CYLp19-6.

B: PCR product amplified from genomic DNA of endophytes re-isolated from five different grasses (G1087-G1091) infected with ectopic transformant EC1 (PN2286) (lane 1-5) and five grasses (G1092-G1096) infected with EC2 (PN2287) (lane 6-10). Primers used were CYLp19-1 & CYLp19-6.

C: PCR product amplified from genomic DNA of endophytes re-isolated from five different grasses (G1097-G1101) infected with E8 *thi1* mutant (PN2282) (lane 1 to 5) and five grasses (G1102-G1106) infected with E8 *thi1* mutant (PN2283) (lane 6-10). Primers used were CYLp19-1 & CYLp19-6.

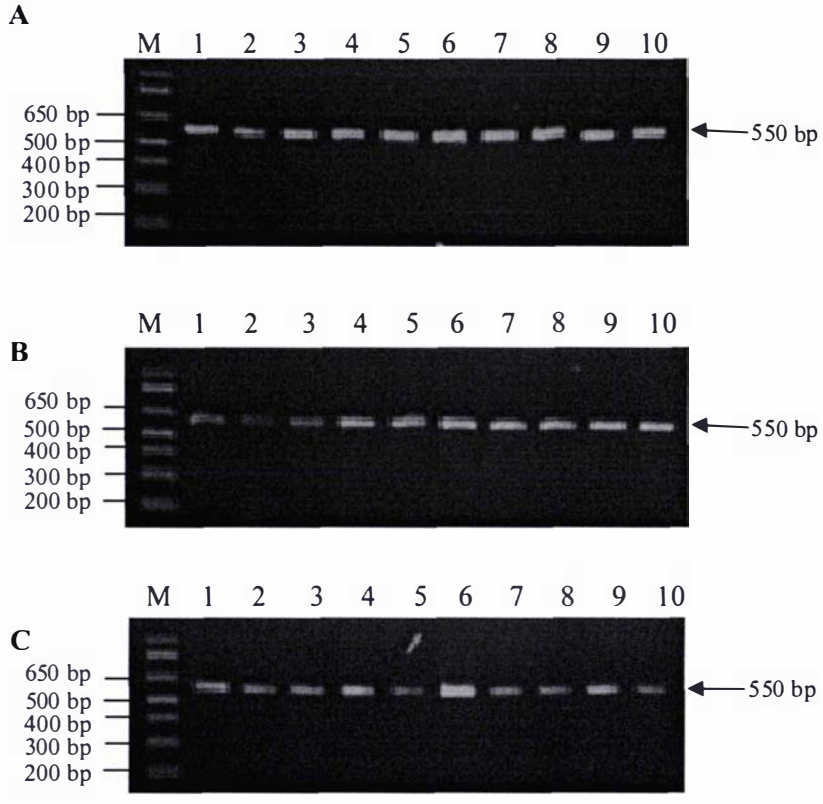

A
M 1 2 3 4 5 6 7 8 9 10
650 bp
500 bp
400 bp
300 bp
200 bp
550 bp
B
M 1 2 3 4 5 6 7 8 9 10
650 bp
500 bp
400 bp
300 bp
200 bp
550 bp
C
M 1 2 3 4 5 6 7 8 9 10
650 bp
500 bp
400 bp
300 bp
200 bp
550 bp

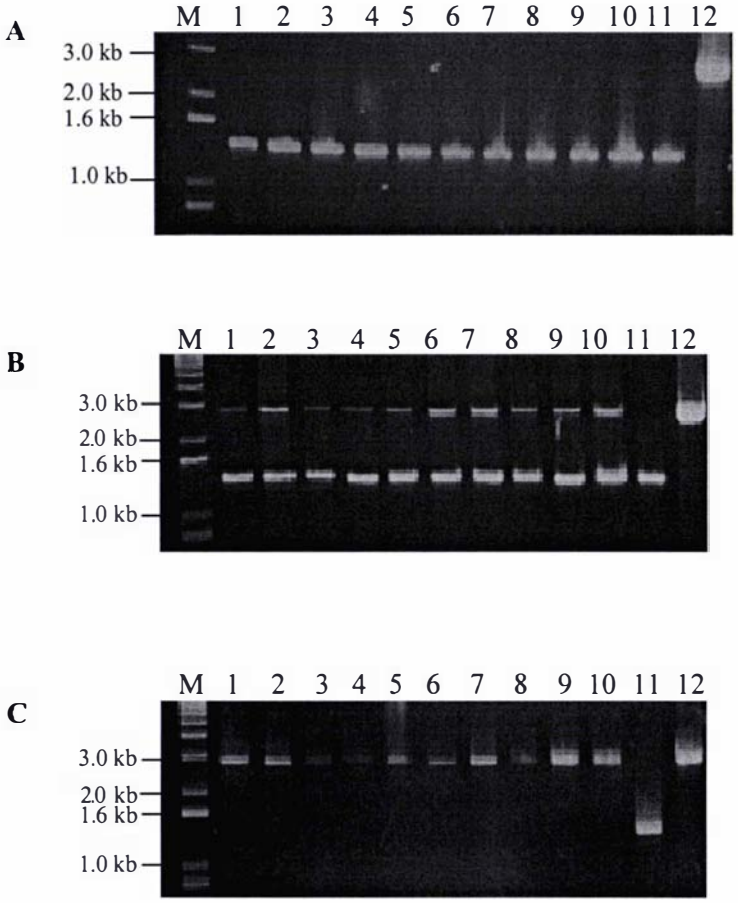
Figure 3.46. Molecular analysis of E8 *thi1* mutants, E8 wild-type and ectopic controls re-isolated from the host grasses

A: PCR product amplified from genomic DNA of endophytes re-isolated from five different grasses (G1077-G1081) infected with wild-type E8 WT1 (PN2284) (lane 1 to 5) and five grasses (G1082-G1086) infected with wild-type E8 WT2 (PN2285) (lane 6-10). Primers used were XZ7 & XZ25.

B: PCR product amplified from genomic DNA of endophytes re-isolated from five different grasses (G1087-G1091) infected with ectopic transformant EC1 (PN2286) (lane 1-5) and five grasses (G1092-G1096) infected with EC2 (PN2287) (lane 6-10). Primers used were XZ7 & XZ25.

C: PCR product amplified from genomic DNA of endophytes re-isolated from five different grasses (G1097-G1101) infected with E8 *thi1* mutant (PN2282) (lane 1 to 5) and five grasses (G1102-G1106) infected with E8 *thi1* mutant (PN2283) (lane 6-10). Primers used were XZ7 & XZ25.

Lanes 11 and 12 are PCR products amplified from genomic DNA of E8 (PN2238) and pXZ51 as controls. Primers used were XZ7 & XZ25.


A
M 1 2 3 4 5 6 7 8 9 10 11 12
3.0 kb
2.0 kb
1.6 kb
1.0 kb
B
M 1 2 3 4 5 6 7 8 9 10 11 12
3.0 kb
2.0 kb
1.6 kb
1.0 kb
C
M 1 2 3 4 5 6 7 8 9 10 11 12
3.0 kb
2.0 kb
1.6 kb
1.0 kb

3.4.2. Analysis of the symbiotic phenotype of E8 *thi1* deletion mutants in association with perennial ryegrass (experiment 3)

Observations from the previous experiments indicated that there was considerable variation in the symbiotic phenotype because of a strong endophyte-host genotype interaction. Consequently a larger population of plants was set up in September 2002 to test whether there were any differences in the symbiotic phenotype between *thi1* mutant and wild-type isolates. In order to test whether host death and the grass stunted phenotypes were results of endophyte infection, an endophyte-free group (EN) of which the seedlings were cut and inserted with endophyte-free PDA was included.

3.4.2.1. Host survival and colonisation

About six weeks after inoculation (Section 2.12.1), the number of surviving plants was recorded and the number of infected plants determined by tissue-printing immuno blotting (Section 2.12.3). The host survival rates and endophyte infection rates are summarized in Table 3.15. The group of seedlings inoculated with just PDA medium (EN) had a survival rate of 81%, indicating that seedling vigour and/or the method of inoculation has an impact on survival. This host survival rate were significantly higher than those groups inoculated with the six strains (isolates) indicating that the endophyte has a detrimental effect on seedling survival. KO3 had a host survival rate significantly higher than the wild-type. KO4 had a host survival rate higher than the wild-type, but this was not significantly different. However, when WT1 and WT2 are combined as a wild-type group, and KO3 and KO4 as a KO group, there was a significant difference ($P < 0.05$) in the survival rate between these two groups. EC1 had a host survival rate similar to that of KO3. EC2 had a host survival rate similar to those of the WT1 and WT2. These results are, in general, consistent with what was observed in experiment 2 (Table 3.9) in that KO3, KO4 and EC1 had higher host

Table 3.15. Seedling survival and colonisation following inoculation with E8 *thi1* deletion mutants, E8 wild type and ectopic controls: experiment 3

Endophytes	Number of inoculated seedlings	Host survival rate[b] (% of inoculated seedlings)	Endophyte infection rate[d] (% of surviving seedlings)	Endophyte infection rate[e] (% of inoculated seedlings)
WT1 (PN2284)	77	43 b[c]	52 e	22 h
WT2 (PN2285)	120	44 b	55 e	25 h
EC1 (PN2286)	122	59 c	61 e	36 i
EC2 (PN2287)	120	42 b	74 f	31 hi
KO3 (PN2282)	120	58 c	76 f	44 j
KO4 (PN2283)	122	51 bc	73 f	37 ij
EN[a]	78	81 a	0 d	0 g

[a]EN is endophyte free control inoculated with PDA.

[b]Percentage of original seedlings surviving was determined as surviving seedlings/inoculated seedlings in November of 2002 (45 days after inoculation in September 2002).

[c]Host survival rates or infection rates not followed by a common letter are significantly different ($P < 0.05$) according to normal test for proportion.

[d]Percentage of surviving seedlings infected was determined as infected seedlings/surviving in November of 2002 (45 days after inoculation in September 2002)

[e]Percentage of original seedlings infected was determined as infected seedlings/inoculated seedlings in November of 2002 (45 days after inoculation in September 2002).

The plants were grown in root-trainers containing potting mix.

survival rates than WT1, WT2 and EC2. However, the host survival rates in experiment 3 were all lower than the corresponding rates found in experiments 1 and 2, suggesting that the inoculation conditions or environmental conditions can vary, thereby impacting on host survival.

Infection rates of KO3 and KO4 were significant greater than those of WT1 and WT2 (Table 3.15). These results were consistent with the results obtained in experiment 1 (Table 3.8). EC1 had an infection rate similar to WT1 and WT2, while that of EC2 was similar to KO3 and KO4. The endophyte infection rates in this experiment ranged from 52% to 76%, which were lower than those detected in experiment 2 (77% to 94%); again emphasizing the variability associated with either the inoculation process or environmental conditions and the consequential impact on host infection rates. The considerably lower infection rates observed in this experiment and in experiment 1 (ranging from 42% to 80%) suggested that endophytes failed to colonise certain host genotypes.

The infection rates as determined by the number of infected plants per number of inoculated plants were also determined (Table 3.15), as a measure of the efficiency with which the endophyte can form an association with the host plant. WT1 and WT2 had significantly lower infection rates than KO3 and KO4. EC2 had a infection rate between the WT and the KO groups, and EC1 had an infection rate similar to the KO4. The low infection rate (22% to 44%) again indicated an incompatible interaction between E8 and some of the host plants.

3.4.2.2. Progressive host death

A feature of this and previous experiments was the high mortality of plants infected with E8 and E8 transformants. It was previously observed in experiment 2 that 3 among 30 infected plants died (Section 3.4.1.7). In experiment 3, some plants died during the period of

observation (Table 3.16). These observations suggest that the endophyte was detrimental to the host as 52 out of 63 (83%) endophyte-negative plants (EN) remained alive at 28 weeks after inoculation. However, all the endophyte infected groups, except EC1, had significantly greater rates of host death than the endophyte-free (EN) group, suggesting that infection with these endophytes had detrimental effects on the host. Plants infected with EC1 had a host death rate greater than the endophyte-free plants at three time points except 9 weeks post inoculation, but the difference was not significant. The KO groups had a lower percentage of host death than the wild-type, and the difference was significant at all time points, except for 28 weeks post inoculation. These results suggested that the *thi1* deletion mutants had reduced adverse effects on the host growth. EC2 had host death rates similar to those of the wild-type at 9 and 12 weeks post inoculation, but lower host death rates than the KO at 20 and 28 weeks post inoculation.

3.4.2.3. Host growth characteristics

As in experiment 2 some hosts showed a stunted phenotype. This phenotype predominated in plants infected with wild-type strains and was less frequently observed in mutants (Table 3.17). However, over the duration of this experiment some plants of all groups died (Section 3.2.2.2), leading to a reduced sampling size. This was particularly marked for plants infected with the wild-type. Given the relatively small population size, it was hard to have a meaningful statistical analysis when regarded WT1, WT2, EC1, EC2, KO3 or KO4 as individual groups. Thus WT1 and WT2 were grouped together as the WT group, KO3 and KO4 as the KO group, EC1 and EC2 as the EC group, and a Fisher's exact test (http://www.graphpad.com/quickcalcs/contingency2.cfm) was used to compare whether differences in the number of stunted plants versus non-stunted plants between the WT and the KO group were significant. Results showed that the WT group had a significantly higher number of stunted plants than the KO group at all the time points (P =

0.0056, 0.0040 and 0.0306 at 12, 20 and 28 weeks post inoculation respectively) (Appendix 6.2).

To further assess the symbiotic phenotypes of these associations, several other plant growth characteristics were determined. These included above ground fresh weight, tiller number and weight.

Measurement of fresh weight showed that at early development stages (before 20 weeks after inoculation) there were significant differences in host fresh weight between the endophyte-free (EN) and positive plants, and between the plants infected with the mutant and the wild-type (Table 3.18). At 6 weeks post inoculation, the endophyte-free plants (EN) had an average host fresh weight that was about 2 to 8-fold greater than the endophyte-infected groups. Plants infected with the *thi1* mutants had an average fresh weight about 2 to 4-fold that of plants infected with the wild-type. These differences were statistically significant. At 9 weeks post inoculation, the endophyte-free plants (EN) had an average host fresh weight that was significantly greater than that of the endophyte infected plants. The plants infected with the KO had a fresh weight greater than those infected with the wild-type, but the difference was not significant. At 12 weeks post inoculation, the endophyte-free plants and the KO infected plants had a similar fresh weight, which was significantly greater than plants infected with the wild-type. However at 20 weeks post inoculation, the endophyte-free plants had a significantly lower fresh weight than the endophyte-infected plants. The KO infected plants had a significantly greater host fresh weight than the wild-type. No significant difference was detected at 28 weeks post inoculation among these groups. The EC groups had fresh weights between that of the KO and WT groups, or were more often similar to the KO.

Differences in average tiller numbers were only detected at 20 and 28 weeks post inoculation (Table 3.19). The endophyte-free plants (EN) produced fewer tillers (17 at both

time points) than all the endophyte-infected plants (24-33 tillers per plant). The difference was significant at 20 weeks post inoculation. Plants infected with the mutant had fewer tillers than plants infected with the wild-type, and the ectopic controls, but these differences were not statistically significant.

The tiller weight of the endophyte-free group (EN) was consistently greater than that of the endophyte-infected groups. This difference was significant up to 9 weeks post inoculation (Table 3.20). These results indicated that endophyte infection reduced host tiller size, in particularly at early host development stages. Gradually, infection of endophyte can stimulate the host to produce more tillers and as a consequence increases host fresh weight. Tiller weights of the KO infected plants were greater than those of the wild-type up to 20 weeks post inoculation. This difference was significant at 12 weeks post inoculation. At later stages, the tiller weight of the KO infected plants were greater but not significantly different to the wild-type infected plants. Considering the high mortality for the plants infected with the wild-type and that smaller plants tend to die more easily, the remaining plants infected with the wild-type at later stage are more likely to have greater tiller weights.

3.4.2.4. Stromata development on vegetative tillers

Stromata on leaves of vegetative tillers were noted again in February of 2003 on a few plants from experiment 3. A total of 5 out of 150 plants examined had stromata develop on vegetative tillers; 2 of these were infected with the ectopic controls and 3 with the mutant. After trimming the plants to measure fresh weight no further stromata developed on vegetative tillers.

Table 3.16. Death rates of infected plants: experiment 3

Endophytes	Period post inoculation (weeks)			
	9	12	20	28
WT1 (PN2284)	59%[b] d[c]	71% d	ND[d]	ND
WT2 (PN2285)	28% c	41% c	52% c	69% c
EC1 (PN2286)	2% ab	14% a	14% a	30% a
EC2 (PN2287)	31% c	33% bc	33% b	44% b
KO3 (PN2282)	11% b	17% ab	17% a	58% c
KO4 (PN2283)	13% b	27% b	33% b	60% c
EN[a]	2% a	5% a	8% a	17% a

[a]EN is endophyte free control inoculated with PDA.

[b]Host death rates were determined as number of infected plants that died/number of infected plants examined at 6 weeks post inoculation.

[c]Host death rates not followed by a common letter are significantly different ($P < 0.05$) according to normal test for proportion.

[d]not determined due to small number of plants.

The plants were grown in root-trainers containing potting mix.

Table 3.17. Number of stunted plants: experiment 3

Endophytes	Period post inoculation (weeks)		
	12	20	28
WT1 (PN2284)	2/5[b] a[c]	1/1 d	0/0
WT2 (PN2285)	3/17 a	2/14 d	2/9 f
EC1 (PN2286)	2/38 ab	2/38 de	1/31 fg
EC2 (PN2287)	3/24 ab	1/24 de	2/20 fg
KO3 (PN2282)	1/44 b	0/44 e	0/22 g
KO4 (PN2283)	1/33 b	0/30 e	0/18 g
EN[a]	0/60 c	0/58 e	0/52 g

[a]EN is endophyte free control inoculated with PDA.

[b]Number of stunted plants/number of plants examined in root trainers.

[c]Number of stunted plants/number of plants not followed by a common letter are significantly different ($P< 0.05$) according to Fisher Exact Test (Daniel, 1995).

Table 3.18. Effects of E8 *thi1* deletion mutants, E8 wild type and ectopic controls on host fresh weight (mg): experiment 3

Endophytes	Period post inoculation (weeks)				
	6	9	12	20	28
WT1 (PN2284)	28 [b] b [c] (n [d] = 17)	137 e (n = 7)	478 g (n = 5)	ND [e] (n = 1)	ND (n = 1)
WT2 (PN2285)	56 b (n = 29)	328 e (n = 21)	1059 g (n = 17)	7576 i (n = 14)	8531 l (n = 9)
EC1 (PN2286)	104 c (n = 44)	567 f (n = 43)	1695 h (n = 38)	8145 j (n = 38)	8926 l (n = 31)
EC2 (PN2287)	90 bc (n = 36)	654 f (n = 25)	1576 h (n = 24)	9382 j (n = 24)	8578 l (n = 20)
KO3 (PN2282)	94 c (n = 53)	465 ef (n = 47)	1473 h (n = 44)	8561 j (n = 44)	7882 l (n = 22)
KO4 (PN2283)	105 c (n = 45)	536 ef (n = 39)	1467 h (n = 33)	10005 j (n = 30)	11326 l (n =18)
EN [a]	246 a (n = 63)	1067 d (n = 62)	1604 h (n = 60)	7051 k (n = 58)	8780 l (n = 52)

[a]EN is endophyte free control inoculated with PDA.

[b]Average plant fresh weight.

[c]Average fresh weights not followed by a common letter are significantly different (Tukey's test $\alpha = 0.05$) according to Tukey's test.

[d]n is the number of plants analysed in each group.

[e]Not determined.

Table 3.19. Effects of E8 *thi1* deletion mutants, E8 wild type and ectopic controls on host tiller number: experiment 3

Endophytes	Period post inoculation (weeks)				
	6	9	12	20	28
WT1 (PN2284)	2 [b] a [c] (n [d] = 17)	4 b (n = 7)	7 c (n = 5)	ND [e] (n = 1)	ND (n = 1)
WT2 (PN2285)	2 a (n = 29)	6 b (n = 21)	13 c (n = 17)	33 e (n = 14)	38 f (n = 9)
EC1 (PN2286)	2 a (n = 44)	7 b (n = 43)	16 c (n = 38)	31 e (n = 38)	39 f (n = 31)
EC2 (PN2287)	2 a (n = 36)	7 b (n = 25)	16 c (n = 24)	32 e (n = 24)	33 f (n = 20)
KO3 (PN2282)	2 a (n = 53)	6 b (n = 47)	14 c (n = 44)	24 de (n = 44)	33 f (n = 22)
KO4 (PN2283)	2 a (n = 45)	6 b (n = 39)	12 c (n = 33)	27 e (n = 30)	36 f (n = 18)
EN [a]	2 a (n = 63)	7 b (n = 62)	12 c (n = 60)	17 d (n = 58)	17 f (n = 52)

[a]EN is endophyte free control inoculated with PDA.

[b]Average plant tiller number.

[c]Average plant tiller numbers not followed by a common letter are significantly different (Tukey's test $\alpha = 0.05$) according to Tukey's test.

[d]n is the number of plants analysed in each group.

[e]Not determined.

Table 3.19. Effects of E8 *thi1* deletion mutants, E8 wild type and ectopic controls on host tiller number: experiment 3

Endophytes	Period post inoculation (weeks)				
	6	9	12	20	28
WT1 (PN2284)	2 [b] a [c] (n [d] = 17)	4 b (n = 7)	7 c (n = 5)	ND [e] (n = 1)	ND (n = 1)
WT2 (PN2285)	2 a (n = 29)	6 b (n = 21)	13 c (n = 17)	33 e (n = 14)	38 f (n = 9)
EC1 (PN2286)	2 a (n = 44)	7 b (n = 43)	16 c (n = 38)	31 e (n = 38)	39 f (n = 31)
EC2 (PN2287)	2 a (n = 36)	7 b (n = 25)	16 c (n = 24)	32 e (n = 24)	33 f (n = 20)
KO3 (PN2282)	2 a (n = 53)	6 b (n = 47)	14 c (n = 44)	24 de (n = 44)	33 f (n = 22)
KO4 (PN2283)	2 a (n = 45)	6 b (n = 39)	12 c (n = 33)	27 e (n = 30)	36 f (n = 18)
EN [a]	2 a (n = 63)	7 b (n = 62)	12 c (n = 60)	17 d (n = 58)	17 f (n = 52)

[a]EN is endophyte free control inoculated with PDA.

[b]Average plant tiller number.

[c]Average plant tiller numbers not followed by a common letter are significantly different (Tukey's test $\alpha = 0.05$) according to Tukey's test.

[d]n is the number of plants analysed in each group.

[e]Not determined.

3.4.3. Inoculation of E8 wild-type isolates into perennial ryegrass (experiment 4)

As host survival rates and infection rates of E8 wild-type in experiment 3 were unexpectedly low, and in general lower than those observed in experiment 1 and 2, an additional inoculation experiment (Section 2.12.1) was carried out to test whether there had been any change in the *E. typhina* E8 culture following growth in the laboratory that was influencing its impact on the host. For this experiment, several different E8 wild-type isolates were used for comparison: the relatively old E8 isolate WT2 (PN2285), a fresh E8 isolate (WT3 (PN2312)) from a plant infected with WT2 and a fresh E8 isolate (WT4 (PN2313)) from an E8 infected plant at AgResearch. The host survival rates and endophyte infection rates were examined and the results are summarised in Table 3.21. All three isolates gave very similar host survival rates and similar infection rates. The host survival rates and the endophyte infection rates in this experiment were even lower than those in experiment 1, 2 and 3. These results indicated that the low infectivity and high host mortality were due to a pathogenic interaction between *E. typhina* E8 and perennial ryegrass, rather than physiological or genetic changes to the endophyte strains. It is also possible that some environmental change was responsible for the increased mortality and decreased host colonisation. The presence of E8 may make the inoculated plants more susceptible to biotic and abiotic stresses.

3.4.4. Inoculation of the E8 *thi1* mutants and control endophytes into clonal plantlets (experiment 5)

Inoculation of the E8 *thi1* mutants and the control endophytes into clonal plantlets (IMP566) of perennial ryegrass was carried out (Section 2.12.2) to compare the endophyte-

host interaction in the same host genotype background. Table 3.22 summaries the results of host survival rates and endophyte infection rates from two inoculations.

Host survival rates for these experiments ranged from 40% to 70% in the first inoculation, and 45% to 53% in the second inoculation. The infection rates ranged from 10% to 70% in the first inoculation and 11% to 40% in the second inoculation. A major limitation with this experiment was the difficulty in locating the apical meristem during the inoculation. Consequently, it was difficult to compare the difference in host survival and colonisation rates between the *thi1* deletion mutants and wild-type. Some plants were partially infected, with some tillers infected while other tillers were endophyte-free. These endophyte-free tillers in infected plants could have resulted from the failure of hyphae to colonise the axillary buds from which the tillers developed. Alternatively, they could have arisen from infected tillers that lost endophyte hyphae over time.

The plants varied in size although they were of the same genotype. Stunted plants were observed in both inoculation 1 (plants EGH314, EGH331 and EGH332 infected with EC2, WT2 and WT2 respectively) and inoculation 2 (plants EGH389 and EGH396 both infected with WT1). The variations in host size may have been a consequence that the plantlets were at different growth stages when they were inoculated. This may have resulted in a difference in the physiological interaction between the host and endophyte. About 20 to 31 weeks post inoculation, plant fresh weight and tiller number were determined and the results are summarised in Table 3.23. Differences in plant fresh weight and tiller numbers were observed in individual plants infected with the same isolates, but these differences were smaller than those from non-clonal perennial grasses infected with the same isolate. This again suggests that plant development stage and physiological state may affect the endophyte-host interaction with respect to host growth. Although endophyte-free plants had slightly higher plant fresh weight than the endophyte-infected plants, the difference

was not significant. For plants infected with the *thi1* mutant and the wild-type, the differences in plant fresh weight and tiller number were not significant.

Peramine concentrations in the pseudostem of the clonal plants were also measured (Section 2.12.6) and the results are summarised in Table 3.24. The average peramine concentrations in inoculation 1 ranged from 47 ppm to 85 ppm, and overall were much higher than those from inoculation 2, which ranged from 44 to 53 ppm. The differences may be due to the fact that the plants from inoculation 2 were 11 weeks younger when sampled than those from inoculation 1. Plants infected with the KO3, KO4 or EC1 in inoculation 1 had higher average peramine concentrations than the plants infected with EC2 or WT2 group. In inoculation 2, plants infected with KO3 had peramine concentration similar to the WT2 group, and both were slightly lower than plants infected with KO4. The differences were not significant. It is hard to draw any meaningful conclusion from this experiment about whether the *thi1* deletion affects peramine production. However, in comparison to the peramine concentration found in the plants from experiment 2 (Table 3.13), variations among individual plants infected with the same endophyte were much smaller, which suggested that analysis of endophyte-host interactions using clonal plants has potential for other studies.

3. 4. 5. Summary and discussion

The effect of E8 *thi1* deletion on the *E. typhina* and ryegrass interaction, and comparison with endophyte-free plants were examined in this study by inoculating the ryegrass using the insertion method (Latch and Christensen 1985) and observing the symbiotic phenotypes including stromata development (Sections 3.4.1.5, 3.4.1.6 and 3.4.2.4), infection frequencies (Sections 3.4.1.1, 3.4.2.1 and 3.4.4), hyphal biomass within host (Section 3.4.1.2), host vigour (Sections 3.4.1.1, 3.4.2.1, 3.4.2.2 and 3.4.4) and morphology (Sections 3.4.1.3, 3.4.2.3 and 3.4.4).

The results showed that mutation in the E8 *thi1* did not prevent infection of the host (Sections 3.4.1.1, 3.4.2.1 and 3.4.4) and stromata development on host tissues (Sections 3.4.1.5, 3.4.1.6 and 3.4.2.4). Infection of the endophytes resulted in significantly lower host survival (Table 3.15) and significantly lower host fresh weight (Table 3.18) compared to the endophyte-free control group, demonstrating that *E. typhina* E8 is detrimental to the perennial ryegrass host under our experimental conditions. The E8 *thi1* mutants showed a reduced detrimental effect on the host grass at an early growth stage as indicated by the results that the KO group had a significant lower host death rate than the WT at 9 weeks post inoculation (Table 3.16), a significantly lower frequency of host stunting (Table 3.17), and a significantly higher host fresh weight at 6 and 12 weeks post inoculation compared to the WT group (Table 3.18).

Some discrepancies were observed between experiment 2 carried out with a small population and experiment 3 which included a larger populations of grasses. The E8 *thi1* mutants (KOs) displayed a significantly higher infection rate compared to the WT in experiment 3 (Table 3.15), but the difference was not detected in experiment 2 (Table 3.9). This variability could be due to: 1) effects of varied environmental conditions under which the plants were grown between experiment 2 and 3; 2) an earlier time of detecting endophyte infection in experiment 2 than experiment 3; 3) random errors caused by the limitation of the inoculation methods in that it is hard to insert a same amount of hyphae each time into the seedling, as well as a strong host genotype effect on the infection. Similarly, these factors may also contribute to the variaility in host survival rates between the experiment 2 and experiment 3.

A common feature of the symbiotic phenotypes observed in experiments 1 to 4 was the great variation of hyphal growth (Section 3.4.1.2) and host morphologies (Sections 3.4.1.3 and 3.4.2.3). These phenomena could be caused by the limitation of the inoculation method,

the varied microenvironments under which they were grown, but more likely were an effect of the host genotype on the endophyte-grass interactions (Christensen *et al*,. 1997; Hesse and Latch, 1999; Cheplick *et al*., 2000; Johnson-Cicalese *et al*., 2000; Faeth and Sullivan, 2003).

However, a similar wide range of symbiotic phenotypes were observed in experiment 5 in which clonal plants were used as hosts (Section 3.4.4.). While this data would suggest factors other than the plant genotype were responsible for the variability, it is much more difficult to synchronise the growth stage of the clonal plants compared to the seedlings, and it is much more difficult to identify the meristem to inoculate the mycelium at the same site.

Due to the limitations of the inoculation methods and effects of multiple factors on the endophyte-grass interactions, the results obtained from the large population of grasses would be more meaningful than experiments on a small population of grasses or the clonal plantlets.

Table 3.21. Comparison of E8 wild types

Endophytes	Number of inoculated seedlings	Host survival rate[e] (% of inoculated seedlings)	Endophyte infection rate[f] (% of inoculated seedlings)	Endophyte infection rate[g] (% of surviving seedlings)	Stunted plants/ plants observed
WT2 [a]	100	25	17	68	3/17
WT3 [b]	102	26	19	70	4/17
WT4 [c]	100	25	17	68	3/17
EN[d]	49	96	0	0	0/47

[a]E8 WT2 (PN2285) culture.

[b]WT3 (PN2312) was 12-day old E8 culture re-isolated from plant G1080 that was infected with E8 WT2 (PN2285).

[c]WT4 (PN2213) was 12-day old E8 culture re-isolated from E8 infected plant at AgResearch, Palmerston North (Christensen, M.).

[d]EN was endophyte free control inoculated with PDA.

[e]Percentage of original seedlings surviving was determined as surviving seedlings/inoculated seedlings in August of 2003 (72 days after inoculation in June of 2003).

[f]Percentage of original seedlings infected was determined as infected seedlings/inoculated seedlings in August of 2003 (72 days after inoculation in June of 2003).

[g]Percentage of surviving seedlings infected was determined as infected seedlings/surviving seedlings in August of 2003 (72 days after inoculation in June of 2003).

Table 3.22. Clonal plant survival and colonisation following inoculation with E8 *thi1* deletion mutants, E8 wild type and ectopic controls: experiments 5.

Inoculation	Endophyte	Number of plantlets inoculated	Number of surviving plants	Host survival rate[d](%)	Number of completely (partially) infected plants	Infection rate[e](%)
1[a]	WT1	10	5	50	0 (1)	10
	WT2	10	6	60	3 (1)	40
	EC1	10	4	40	4 (0)	40
	EC2	10	5	50	4 (0)	40
	KO3	10	7	70	6 (1)	70
	KO4	12	7	58	6 (0)	50
	EN[c]	3	3	100	0 (0)	0
2[b]	WT1	19	10	53	2 (0)	11
	WT2	20	9	45	6 (1)	35
	KO3	19	9	47	3 (0)	16
	KO4	15	7	47	5 (1)	40
	EN[c]	5	3	60	0 (0)	0

[a]Examination of the plants was carried out in October of 2002 (56 days after inoculation in August of 2002).

[b]Examination of the plants was carried out in January of 2003 (75 days after inoculation in October of 2002).

[c]EN is endophyte free control inoculated with PDA.

[d]Host survival rate was determined as number of plants survival/number of plants inoculated

[e]Infection rate was determined as number of completely and partially infected plants/number of plants inoculated

Table 3.23. Host tiller number, tiller weight and fresh weight for clonal plant experiment: experiment 5

Inoculation	Endophytes	Number of plants observed	Fresh weight per plant[d] (mg)	Tiller number per plant[d]	Fresh weight per tiller[d] (mg)
1[a]	WT2	1	1110	40	30
	EC1	3	2957 ± 863	82 ± 31	38 ± 8
	EC2	3	3367 ± 425	97 ± 4	35 ± 3
	KO3	5	2628 ± 1175	76 ± 10	35 ± 5
	KO4	4	2025 ± 591	65 ± 18	31 ± 3
2[b]	WT2	5	3266 ± 1762	57 ± 34	60 ± 14
	KO3	3	3586 ± 1375	58 ± 26	64 ± 10
	KO4	4	3660 ± 698	52 ± 12	72 ± 17
	EN[c]	3	4593 ± 980	58 ± 9	79 ± 7

[a]Examination of the plants was carried out in March of 2003 (about 31 weeks after inoculation in August of 2002).

[b]Examination of the plants was carried out in March of 2003 (about 20 weeks after inoculation in October of 2002).

[c]EN is endophyte free control.

[d]Average plant fresh weight, tiller number or tiller weight (± standard deviation).

The plants were trimmed back and grown in pots containing potting mix for three weeks before examination

Table 3.24. Peramine concentration for clonal plants

Inoculation	Endophytes	Number of plants observed	Peramine concentration[d] (ppm)
1 [a]	WT2 (PN2285)	1	47
	EC1 (PN2286)	3	82 ± 14
	EC2 (PN2287)	3	67 ± 27
	KO3 (PN2282)	5	83 ± 11
	KO4 (PN2283)	4	84 ± 11
2 [b]	WT2 (PN2285)	6	44 ± 6
	KO3 (PN2282)	3	45 ± 12
	KO4 (PN2283)	5	53 ± 5
	EN [c]	3	1 ± 2

[a]Examination of the plants was carried out in February of 2003 (about 28 weeks after inoculation in August of 2002).

[b]Examination of the plants was carried out in February of 2003 (about 17 weeks after inoculation in October of 2002).

[c]EN is endophyte free control.

[d]Average peramine concentration (± standard deviation) for plants that were trimmed back and grown in pots containing potting mix for three weeks before examination.

3.5. Microscopic examination of reproductive and vegetative tillers of perennial ryegrass with stromata

As E8 and E8 transformants developed stromata on vegetative tillers of perennial ryegrass (Section 3.4.2.4), and this was the first report of stromata development of *E. typhina* on the host vegetative tissue, both light and electron microscopic examinations (Section 2.14) were carried out to compare the morphology and ultrastructure of hyphae in the vegetative and reproductive tillers with stromata.

3.5.1. Microscopic examinations of reproductive tillers with stromata

Six stromata-forming reproductive tillers were chosen for microscopy, three from the wild-type associations and three from the *thi1* mutant associations. Microscopic examinations were carried out on transverse sections through both the stromata region and the stem region below each tiller.

3.5.1.1. Microscopic examinations of reproductive tillers with stromata of wild-type *E. typhina* E8

Transverse cross sections of the stroma-forming region showed that epiphytic hyphae were observed typically on the surface of the flag leaf, on the outer surface of the stem (Fig. 3.47A and C) and in the floret (Fig. 3.48A and F) in stroma-forming tillers of wild-type association. Distribution of the epiphytic hyphae within a single transverse cross section was often uneven (Fig. 3.47A), with abundant epiphytic hyphae in some regions (Fig. 3.47C) with few or even no epiphytic hyphae in other regions (Fig. 3.47B). In the region

without epiphytic hyphae, endophytic hyphae were evenly distributed in the mesophyll intercellular spaces but were sparse in the vascular bundle (Fig. 3.47B). The round shape of these hyphal sections indicates that they grow typically parallel to the longitudinal axis of the leaf blade in this region. Epidermal cells were rectangular in shape with evenly thickened cell wall and a cuticular layer (Fig. 3.47B). In the region with a mass of epiphytic hyphae, abundant endophytic hyphae were present in the mesophyll intercellular spaces (Fig. 3.47C) and within the vascular bundles (Fig. 3.47D). Hyphae penetrating through the epidermis were often observed (Fig. 3.47 C). These hyphae were poorly stained by toluidine blue (Fig. 3.47C), enlarged and highly vacuolated (Fig 3.48B). The plant epidermal cells in this region were ruptured (Fig. 3.47C). Hyphae in the mesophyll intercellular spaces typically were not strongly stained, indicating that the cytoplasm was not dense. These hyphae contained many mitochondria (Fig. 3.47E) and vesicles or small vacuoles were often present. Hyphae in the vascular bundle typically contained a denser cytoplasm than those in mesophyll intercellular space and had abundant lipid droplets (Fig. 3.47F).

The epiphytic hyphae close to the epidermis were typically enlarged and contained a light electron-dense cytoplasm (Fig. 3.48A and B). Vesicles or small vacuoles were often observed in these epiphytic hyphae. The outer epiphytic hyphae had a denser cytoplasm (Fig. 3.48A and C) and occasionally some glycogen-like deposits were observed in the hyphae distal to the epidermis (Fig. 3.48D). In some regions the epiphytic hyphae were sparse and in contrast to those regions where hyphae were profuse, they contained many lipid droplets (Fig. 3.48E), indicative of hyphal access to nutrient sources within the plant tissue. The vacuolated state of the hyphal cells from zones where there was intensive growth is indicative of depleted nutrients and of transfer of energy reserves towards the hyphal tips.

3.5.1.2. Microscopic examinations of reproductive tillers with stromata of E8 *thi1* deletion mutant

Hyphal morphology and ultra-structure of E8 *thi1* deletion mutants (Fig. 3.49) in reproductive tillers with stromata was in general similar to that of E8 wild-type. Hyphae were abundant in the intercellular spaces of the mesophyll of regions with stromata. Abundant hyphae were present in the vascular bundle (Fig. 3.49B). The epiphytic hyphae close to the epidermis were enlarged, poorly stained and contained many vacuoles (Fig. 3.49D). The outer epiphytic hyphae contained denser cytoplasm. No glycogen-like deposits were observed in hyphae of the E8 *thi1* deletion mutant. In contrast to the wild-type E8, the epiphytic hyphae of E8 *thi1* deletion mutant were often present on both the flag leaf blade and the second leaf sheath (Fig. 3.49A).

3.5.2. Microscopic examination of vegetative tillers with stromata

3.5.2.1. Distribution of epiphytic hyphae and hyphal ramification

Light microscopic examination showed that a mass of epiphytic hyphae was present on both the lower (Fig. 3.50A and E) and upper surface (Fig. 3.50C) of the mature leaf blade, on the surface of the immature leaf blade above the pseudostem (Fig. 3.50 B) and in the space between the immature leaf blade and the inner leaf sheath of the pseudostem region (Fig. 3.50D). Similar to that of reproductive tillers, host cells at this regions appeared to be ruptured.

In regions with no stromata, hyphae were evenly distributed in the mesophyll region with some colonisation of vascular bundles. The presence of circular hyphae in transverse

sections indicates that hyphae were parallel to the leaf axis and seldom branched (Fig. 3.51A, C and E). This hyphal distribution contrasts with the high concentration of hyphae in the mesophyll of the stroma-forming region. These hyphae were randomly orientated as indicated by their varied shape and size in transverse cross section. Several hyphae were present in each intercellular space of the mesophyll (Fig. 3.51B and D). Hyphae penetrating through the epidermis were often observed (Fig. 3.51F). These observations indicate that hyphae in the stroma region of a vegetative tiller were actively branching and growing, while hyphal growth appeared to be synchronised with the plant growth in the non-stroma region. A similar pattern of hyphal growth was observed for reproductive tillers.

3.5.2.2. Vascular bundle colonisation

Intensive vascular bundle colonisation is a common feature of hyphal growth in the stroma-forming region for both vegetative (Fig. 3.52) and reproductive tillers, and for both the E8 wild-type strain (Fig. 3.52 A, C and E) and the *thi1* deletion mutant (Fig. 3. 52 B, D and F). Hyphae were present in both the large and small vascular bundles of leaf sheaths and mature and immature leaf blades. Hyphae were present in intercellular spaces of both the phloem and xylem. In large vascular bundles, hyphae were most abundant in the large airspace (Fig. 3.52 C and F). For both the E8 wild-type and the *thi1* deletion mutant, the number of hyphae in vascular bundles in the stroma-forming region appeared to be greater than in vascular bundles in the non-stroma forming region. Hyphae in vascular bundles of both stroma-forming and non-stroma forming regions typically contained cytoplasm in which it was difficult to discern the organelles. Hyphae of the E8 wild-type typically contained abundant lipid droplets (Fig. 3.52 E) and glycogen-like deposits (Fig. 3.55). These energy storage substances appeared to be more abundant in hyphae in vascular bundles in non-stroma forming region than in the stroma-forming region. In comparison to the wild-type E8, hyphae of the *thi1* mutant contained abundant lipid droplets, but glycogen-like deposits

were not evident. In the vascular bundle in stroma-forming region, vacuoles were often observed in hyphae of the mutant but not of the wild-type E8.

3.5.2.3. Hyphal ultrastructure

Hyphae in non-stroma regions typically contained cytoplasm as dense as those in the vascular bundle, making it difficult to discern the organelles. In the immature blade hyphae were smaller in diameter, had electron-dense cytoplasm, and contained fewer lipid droplets (Fig. 3.51 E) than hyphae in the mature leaf blade or sheath. This indicates that hyphae tend to become larger, and accumulate some lipid droplets as they age. Hyphae of wild-type E8 in non-stroma regions typically contained abundant glycogen-like deposits (Fig 3.55) which were not observed in the *thi1* mutant.

In contrast to the non-stroma region, hyphae in stroma regions were highly vacuolated, contained fewer lipid droplets and had a less cytoplasm. Mitochondria were abundant in these hyphae. The epiphytic hyphae proximal to the epidermis almost always contained large vacuoles, which in some hyphae occupied almost the entire transverse cross section (Fig. 3.53 and 3.54). Some epiphytic hyphae in the distal region had electron-dense cytoplasm and were small in diameter (Fig. 3.53C and Fig. 3.54A). Some of these structures were probably conidia or hyphal tips. For some epiphytic hyphae (Fig. 3.53D) between the immature leaf blade and the inner sheath in the pseudostem region, cytoplasm with many lipid droplets were observed, indicating that these epiphytic hyphae had ready access to nutrients. The endophytic hyphae around the mesophyll cells in the stroma-forming region frequently contained large vacuoles and/or were rich in vesicles (Fig. 3. 53E and Fig. 3.54D). The vacuolation phenomena was more pronounced with hyphae close to the epidermis. Hyphae of the E8 wild-type and the *thi1* mutant were similar except that those of the E8 wild-type tended to form abundant vesicles while those of the *thi1* mutants tended to form large vacuoles surrounded by a relatively electron-dense cytoplasm.

3.5.2.4. Abundant glycogen deposits in wild-type E8 hyphae

Hyphae in the E8 infected tillers typically contained glycogen-like deposits (Christensen 1997). These glycogen-like deposits were distinguished using TEM as groups of small white granules at the periphery of the hyphae (Fig. 3.55). Glycogen-like deposits were observed within hyphae in sheaths, in both mature and immature leaf blades (Fig. 3.55A-D). In general, hyphae in the leaf blade contained more glycogen-like deposits than those in sheath. The concentration of glycogen deposits decreased in regions where there was an increase of epiphytic hyphae. Hyphae in vascular bundles (Fig. 3.55A, B and F) and close to vascular bundles contained more glycogen deposits than those more distant, and those that were epiphytic. For epiphytic hyphae, glycogen was sparsely present in a few hyphae distal to the epidermis (Fig. 3.55E). These observations indicated glycogen deposits tend to accumulate in hyphae in the region of the leaf where nutrients are abundant and readily accessible. No or few glycogen-like deposits were observed in hyphae of the E8 *thi1* deletion mutant in the vegetative tiller.

3.5.2.5. Alkaline bismuth staining of wild-type E8 hyphae in the vegetative tiller

In order to confirm the chemical presence of the glycogen-like deposits, electron microscopic examinations were carried out with hyphae of the E8 wild-type strain in vegetative tillers stained with alkaline bismuth, a procedure that is specific for 1,2-glycol groups of mucosubstance and polysaccharides (Koga *et al.* 1993). As expected, groups of small black granules stained with alkaline bismuth were observed in the periphery of the hyphal cell (Fig. 3.57), with the locations corresponding to the glycogen-like deposits. These results indicated that the groups of small non-staining granules were glycogen or related polysaccharides. As with the distribution of the glycogen-like deposits, the black

granules were most abundant in hyphae in the vascular bundle (Fig. 3.57A), and in hyphae around mesophyll cells which were close to the vascular bundle (Fig. 3.57B), but few in epiphytic hyphae. For epiphytic hyphae, the black granules were present occasionally in some hyphae in the epidermis (Fig. 3.57C) or some hyphae distal to the epidermis (Fig. 3.57D).

Figure 3.47. Hyphal morphology of *E. typhina* E8 in stroma-forming reproductive tillers

A: Light micrograph of a transverse cross-section through the stroma region of a reproductive tiller showing epiphytic hyphae (arrow) on the sheath of a flag leaf, the stem and in a floret of the inflorescence.

B: Light micrograph showing distribution of hyphae (arrow) in non-stroma region of the section shown in **A**.

C: Light micrograph showing hyphal distribution (arrow) and morphology in stroma region of the section shown in **A**. Hyphae that have penetrated the epidermis are indicated.

D: Light micrograph showing hyphal colonisation (arrow) of a vascular bundle in the stroma-forming region.

E: Electron micrograph of hyphae in mesophyll region of the flag leaf sheath with stroma.

F: Electron micrograph of a hyphal cross section within a large vascular bundle of the flag sheath with stroma.

The arrow or **H** indicates hyphae. **L** indicates lipid droplet. **M** indicates mitochondria. **Ch** indicates chloroplast.

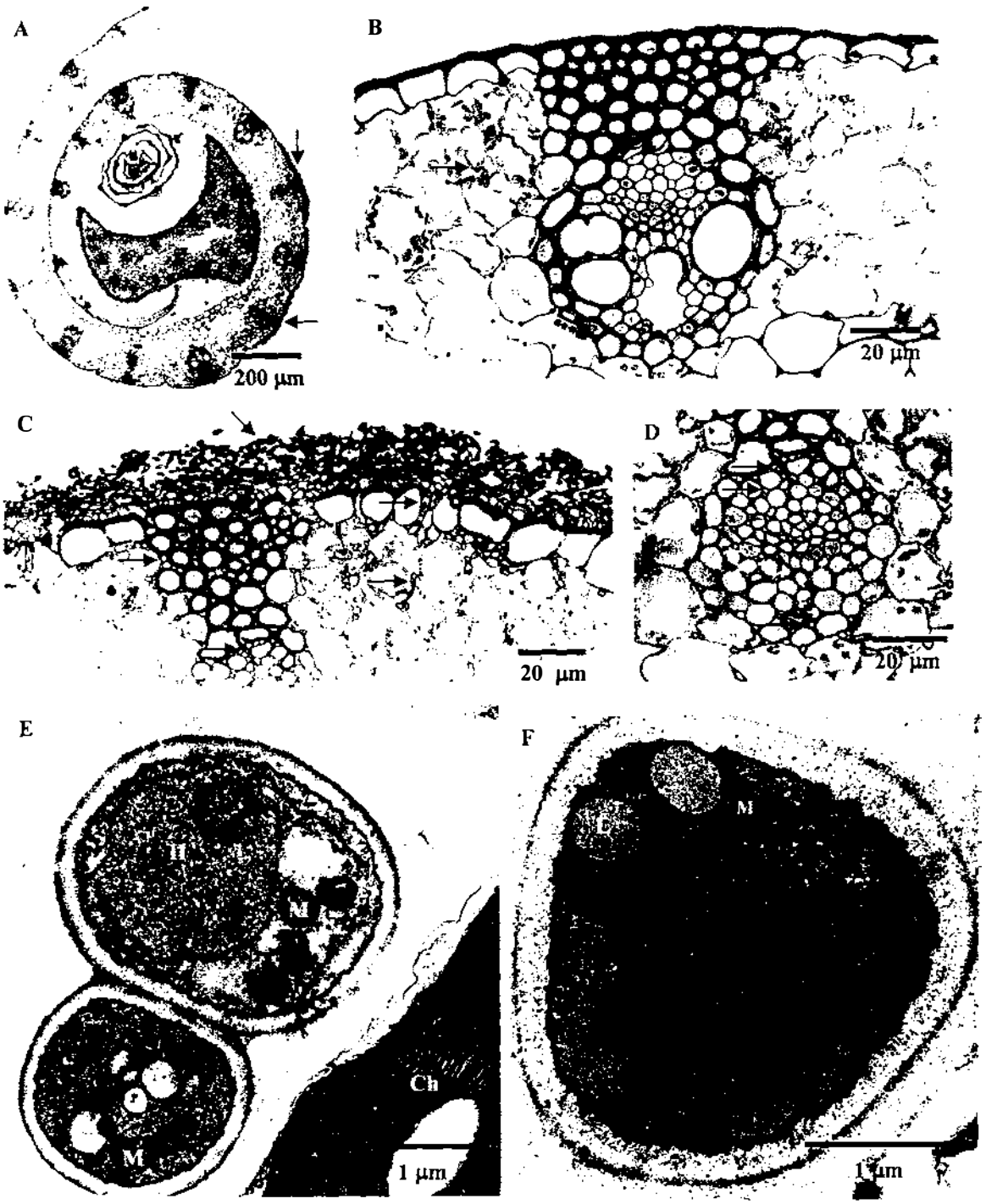

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Figure 3.48. Hyphal morphology of *E. typhina* E8 in stroma-forming reproductive tillers

A: Electron micrograph showing epiphytic hyphae on the lower epidermis of the sheath of a flag leaf.

B: Electron micrograph showing hyphae have penetrated through the epidermis of the leaf sheath.

C and **D:** Electron micrograph of a group of outer epiphytic hyphae on the sheath of a flag leaf.

E: Electron micrograph showing a group of epiphytic hyphae close to the upper epidermis.

F: Light micrograph showing hyphal colonisation (arrow) of a developing floret.

The arrow or **H** indicates hyphae. **L** indicates lipid droplet. **V** indicates vacuole. **G** indicates glycogen-like deposits. **M** indicates mitochondria. **Ch** indicates chloroplast. **Ep** indicates epidermis.

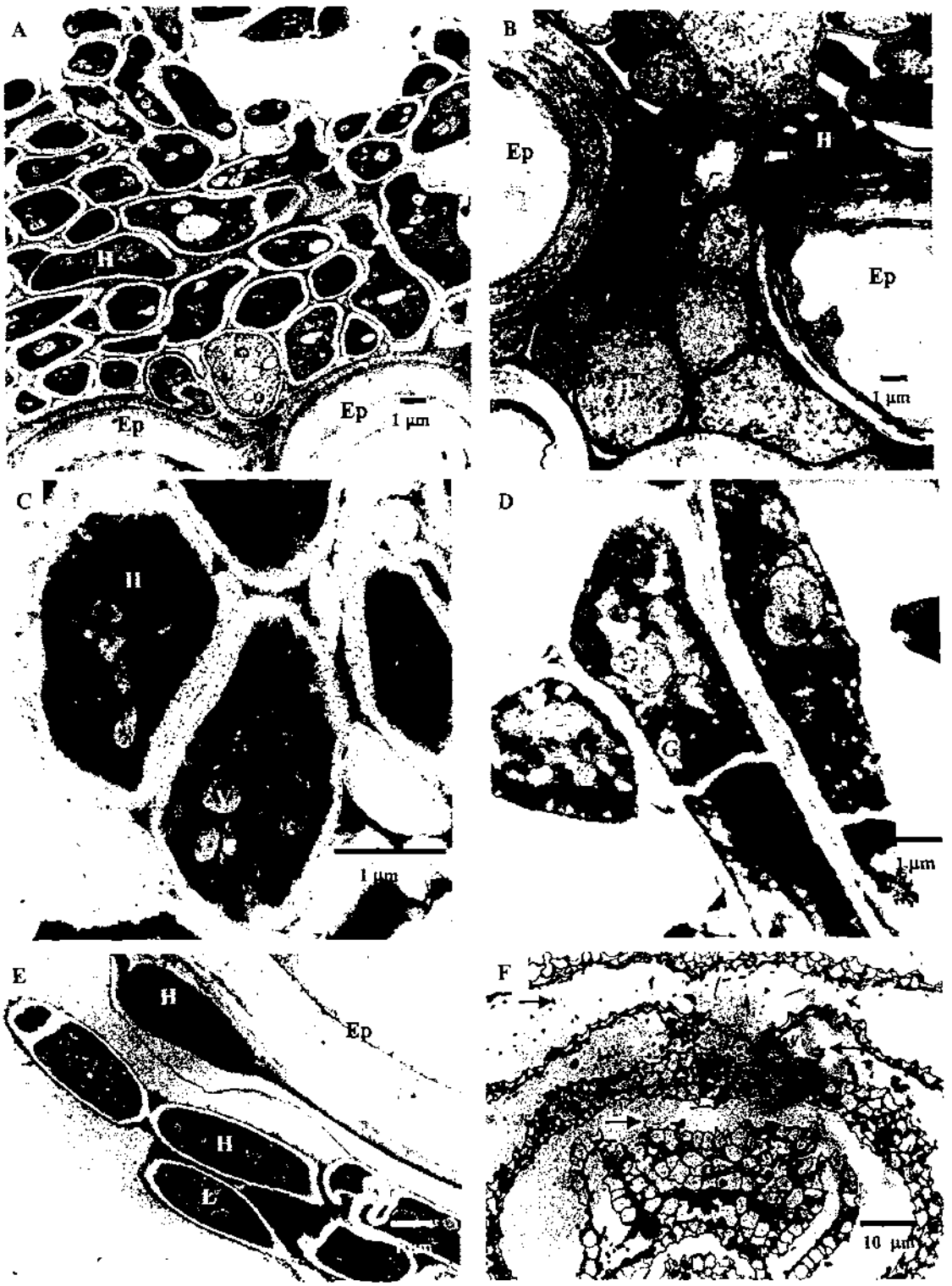
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Figure 3.49. Hyphal morphology of E8 *thi1* deletion mutants in stroma-forming reproductive tillers

A: Light micrograph of a transverse cross-section through the stroma-forming region of a reproductive tiller showing epiphytic hyphae (arrow) on both the sheath of the second leaf and the leaf blade of the flag leaf.

B: Electron micrograph showing hyphal colonisation of a vascular bundle in the stroma-forming region.

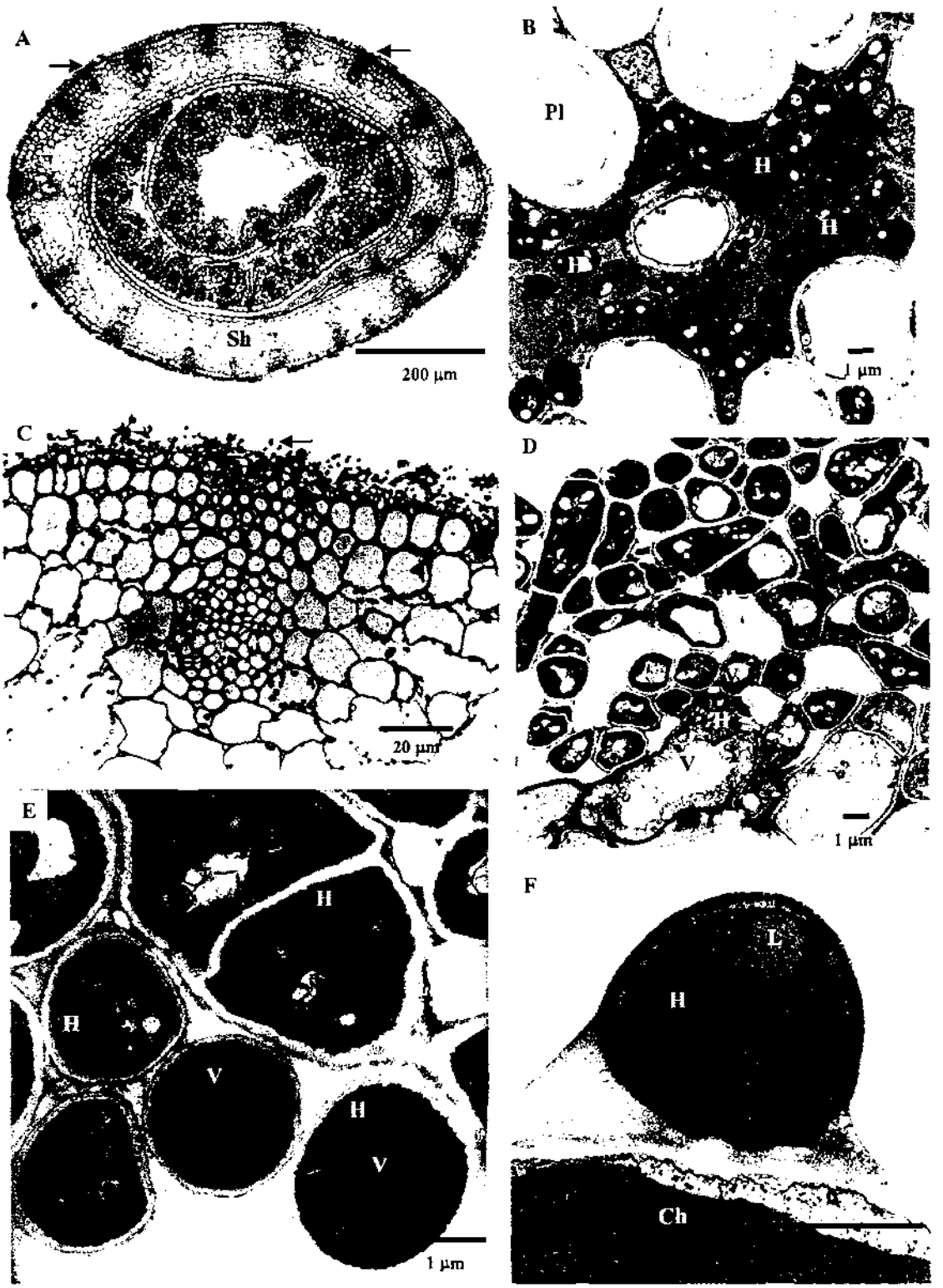
C: Light micrograph showing hyphal distribution (arrow) in the stroma region.

D: Electron micrograph showing epiphytic hyphae on the sheath of the second leaf.

E: Electron micrograph showing a group of outer epiphytic hyphae on the sheath of the second leaf.

F: Electron micrograph of a hypha in mesophyll region of the flag leaf sheath with stroma.

The arrow or **H** indicates hyphae. **L** indicates lipid droplet. **V** indicates vacuole. **Ch** indicates chloroplast. **Ep** indicates epidermis. **Bl** indicates leaf blade. **Sh** indicates leaf sheath. **Pl** indicates plant cell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L
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Figure 3.50. Epiphytic hyphae in stroma-forming vegetative tillers

A: Light micrograph showing epiphytic hyphae of E8 on abaxial (lower) surface of the mature leaf blade.

B: Light micrograph showing epiphytic hyphae of E8 on adaxial (upper) surface of the mature leaf blade and abaxial (lower) surface of immature leaf blade.

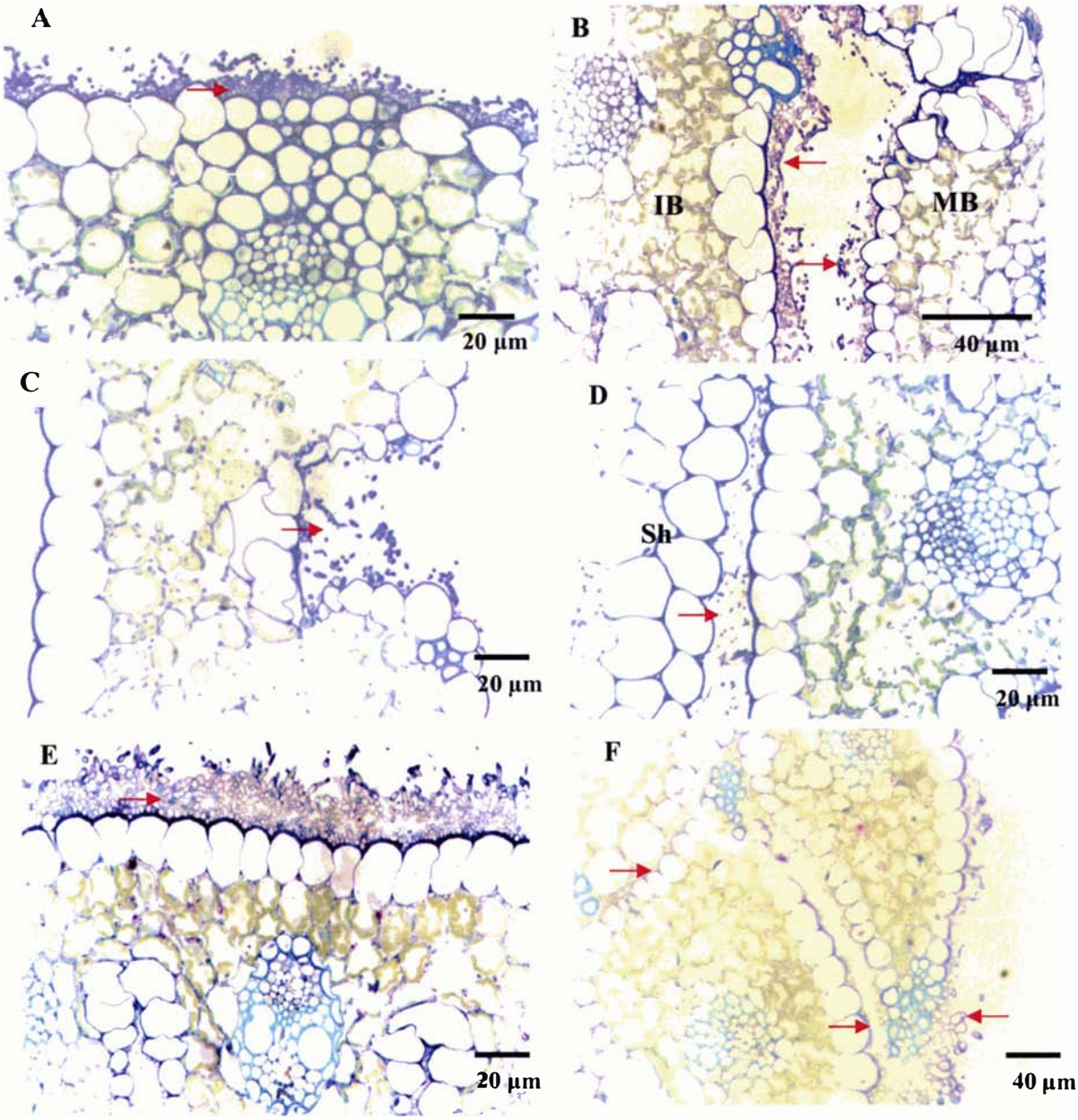
C:. Light micrograph showing epiphytic hyphae of E8 on adaxial (upper) surface of the mature leaf blade.

D: Light micrograph showing epiphytic hyphae of E8 in the space between immature leaf blade and inner leaf sheath of the pseudostem region of a stroma-forming tiller.

E: Light micrograph showing epiphytic hyphae of E8 *thi1* deletion mutant on abaxial (lower) surfaces of the mature leaf blade.

F: Light micrograph showing epiphytic hyphae of E8 *thi1* deletion mutant in the space between adaxial (upper) surfaces of the leaf blade, on abaxial (lower) surfaces of the leaf blade and at the edge of a fused leaf blade.

The arrow indicates epiphytic hyphae. **MB** indicates the youngest mature blade, and **IB** indicates the immature blade.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C
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E
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Figure 3.51. Hyphal ramification in the stroma-forming region of vegetative tillers

A: Light micrograph of a transverse cross-section through the non-stroma region showing distribution of hyphae (arrow) of E8 *thi1* deletion mutant.

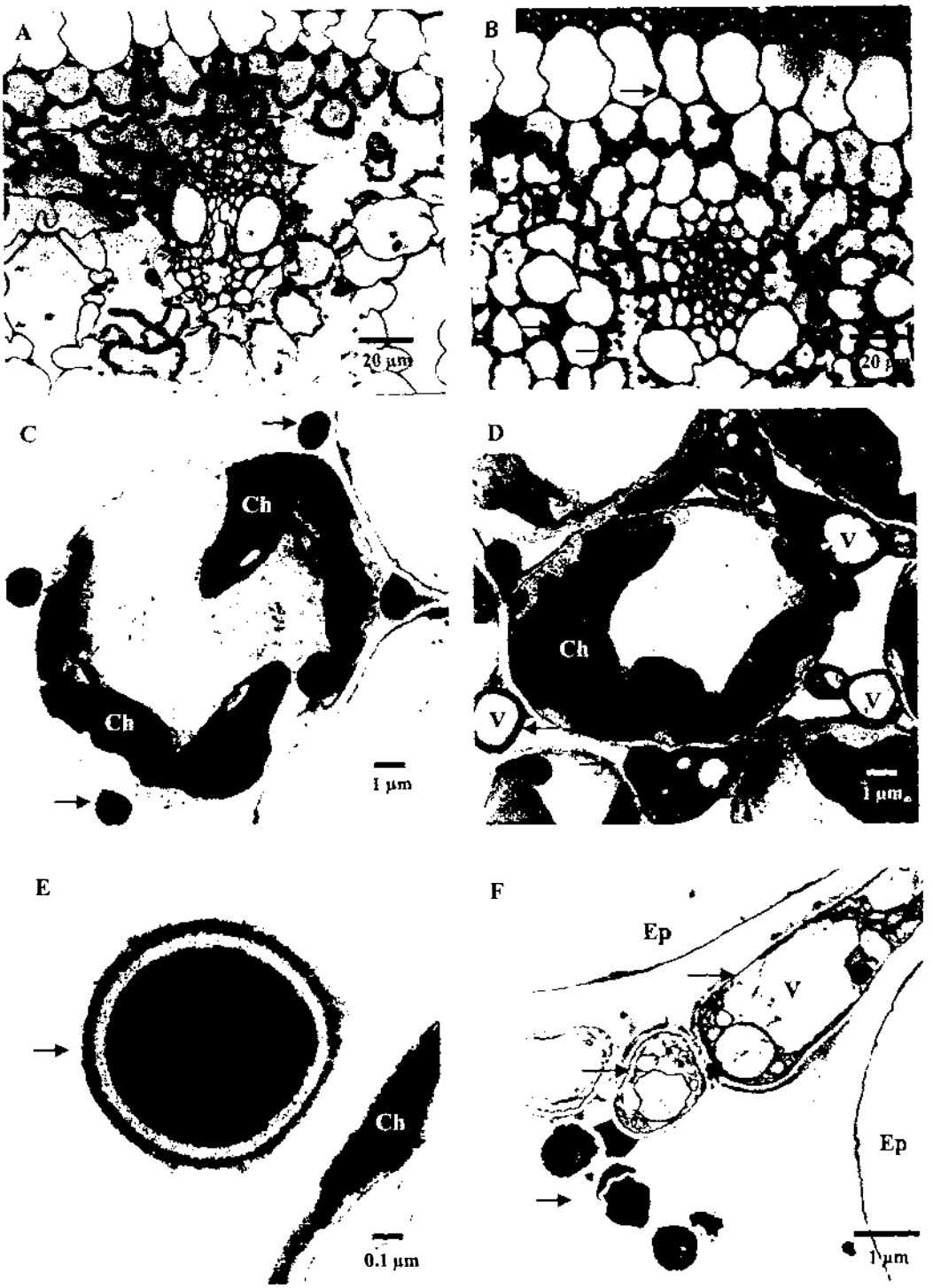
B: Light micrograph of a transverse cross-section through the stroma-forming region showing distribution of hyphae (arrow) of E8 *thi1* deletion mutant.

C and **E:** Electron micrographs of hyphae (arrow) of E8 *thi1* deletion mutant in transverse cross-section of the non-stroma region.

D: Electron micrograph of hyphae (arrow) of E8 *thi1* deletion mutant in transverse cross-section of the stroma-forming region.

F: Electron micrograph showing E8 hyphae (arrow) that have penetrated through epidermis in the stroma-forming region.

The arrow indicates hyphae. **V** indicates vacuole. **Ch** indicates chloroplast. **Ep** indicates epidermis.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V
Ep
1 µm

Figure 3.52. Vascular bundle colonisation of vegetative tillers with stromata

A: Light micrograph showing hyphae (arrow) of E8 in a large vascular bundle in the stroma-forming region.

B: Light micrograph showing hyphae (arrow) of an E8 *thi1* deletion in a large vascular bundle in the stroma-forming region.

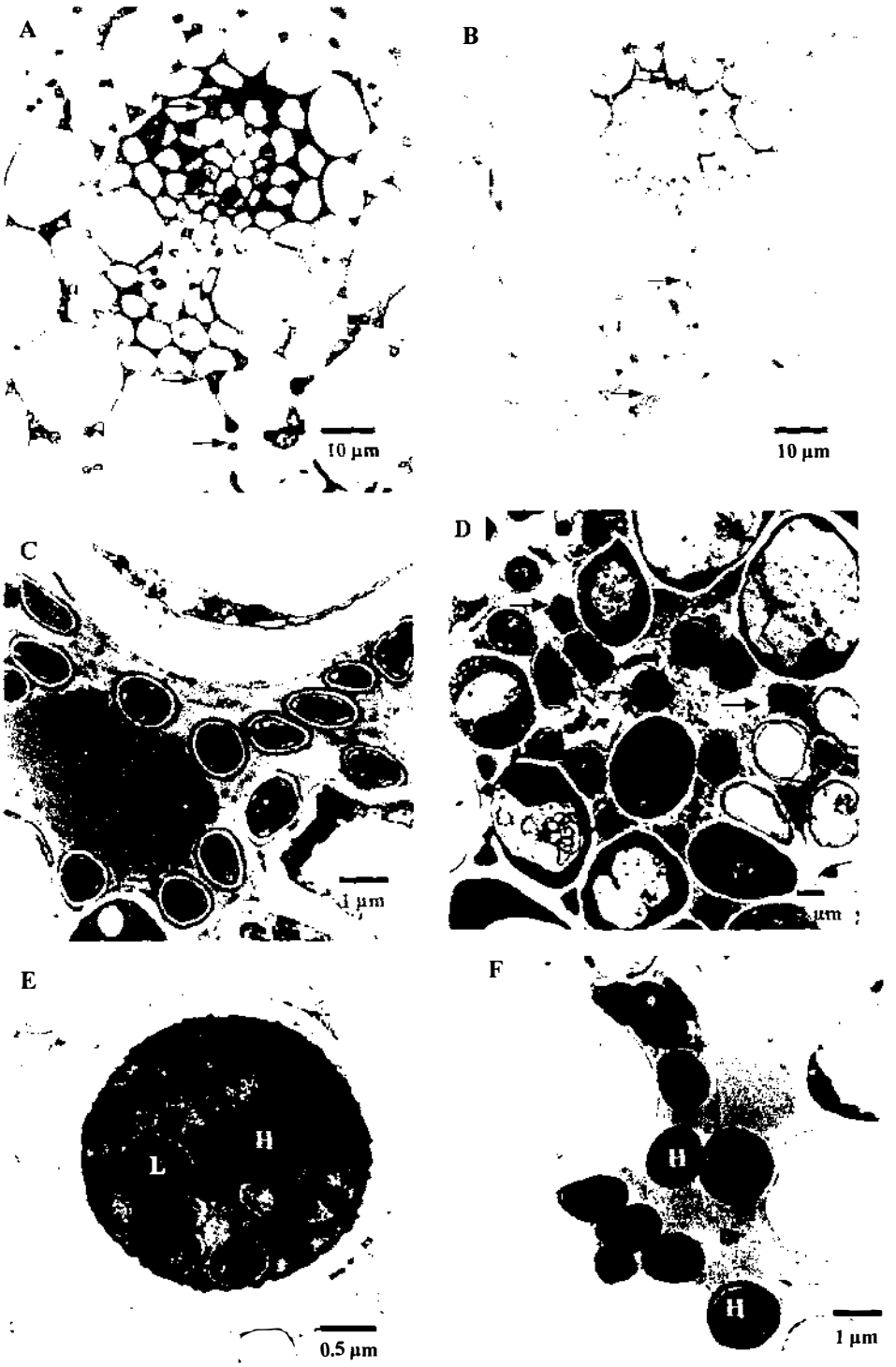
C: Electron micrograph showing E8 hyphae (arrow) in protophloem of a vascular bundle.

D: Electron micrograph of hyphae (arrow) of E8 *thi1* deletion mutant in phloem of a vascular bundle in the stroma-forming region.

E: Electron micrograph showing abundant lipid droplets in an E8 hypha in protophloem of a vascular bundle in non-stroma region.

F: Electron micrograph of hyphae of E8 *thi1* deletion mutant in protophloem of a vascular bundle in the non-stroma region.

The arrow or **H** indicates hyphae. **L** indicates lipid droplet.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H
H
1 µm

Figure 3.53. Ultrastructure of E8 wild-type hyphae on vegetative tillers.

A: Electron micrograph of E8 epiphytic hyphae on upper epidermis.

B: Electron micrograph of E8 epiphytic hyphae on lower epidermis.

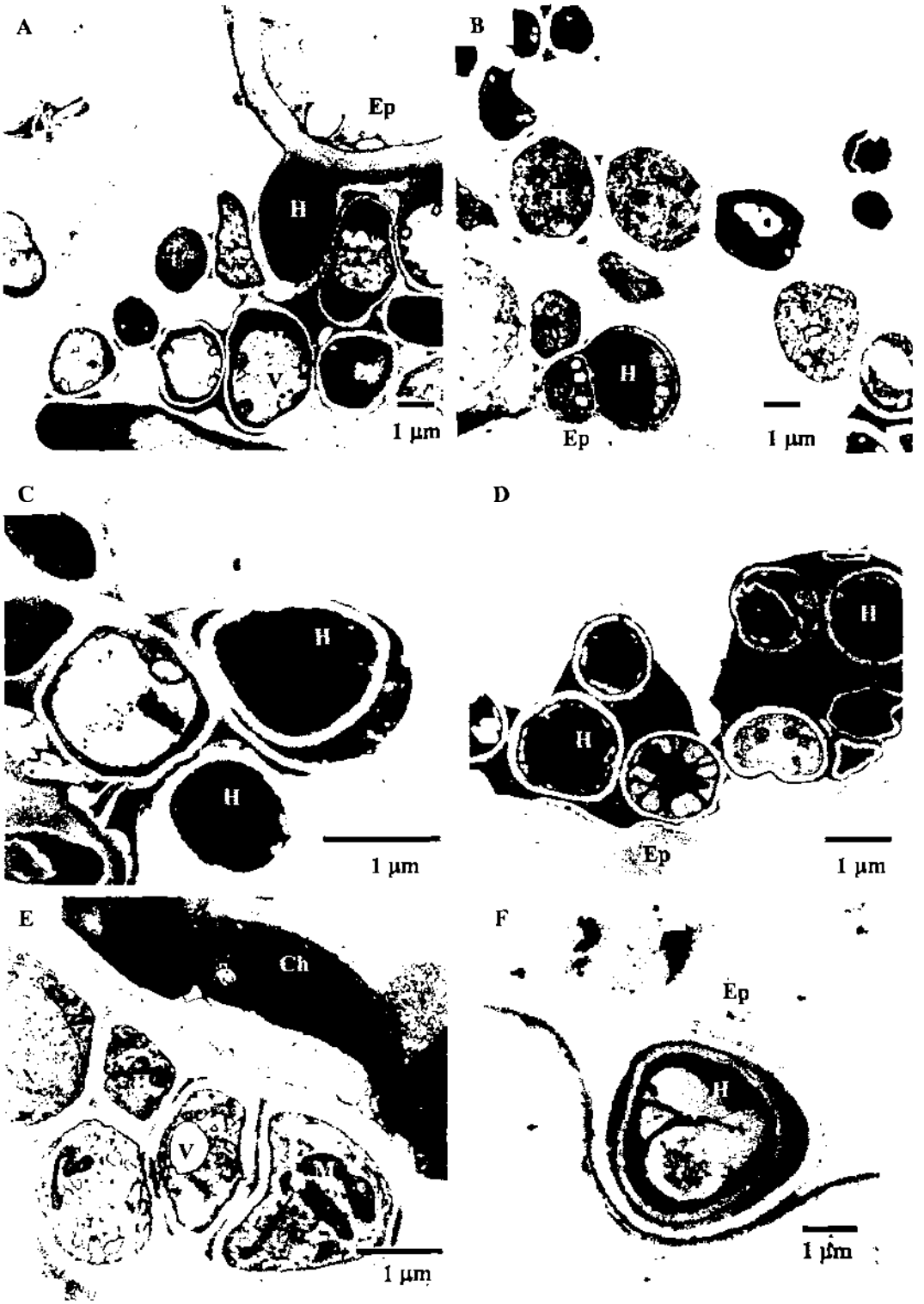
C: Electron micrograph of E8 epiphytic hyphae distal to upper epidermis.

D: Electron micrograph of a group of E8 epiphytic hyphae in the space between immature blade and inner sheath of pseudostem region.

E: Electron micrograph of E8 hyphae in mesophyll of stroma-forming region.

F: Electron micrograph of an E8 epiphytic hyphae close to epidermis.

The arrow or **H** indicates hyphae. **L** indicates lipid droplet. **V** indicates vacuole. **G** indicates glycogen-like deposits. **M** indicates mitochondria. **Ch** indicates chloroplast. **Ep** indicates epidermis.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Ep
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Figure 3.54. Ultrastructure of hyphae of E8 *thi1* deletion mutant on vegetative tillers.

A: Electron micrograph of epiphytic hyphae of E8 *thi1* deletion mutant on lower epidermis.

B: Electron micrograph of epiphytic hyphae of E8 *thi1* deletion mutant on upper epidermis.

C: Electron micrograph of epiphytic hyphae of E8 *thi1* deletion mutant close to the lower epidermis.

D: Electron micrograph of hyphae of E8 *thi1* deletion mutant in mesophyll of stroma-forming region.

Arrows indicate probably conidia. **H** indicates hyphae. **V** indicates vacuole. **Ch** indicates chloroplast. **Ep** indicates epidermis. **C** indicates conidia.

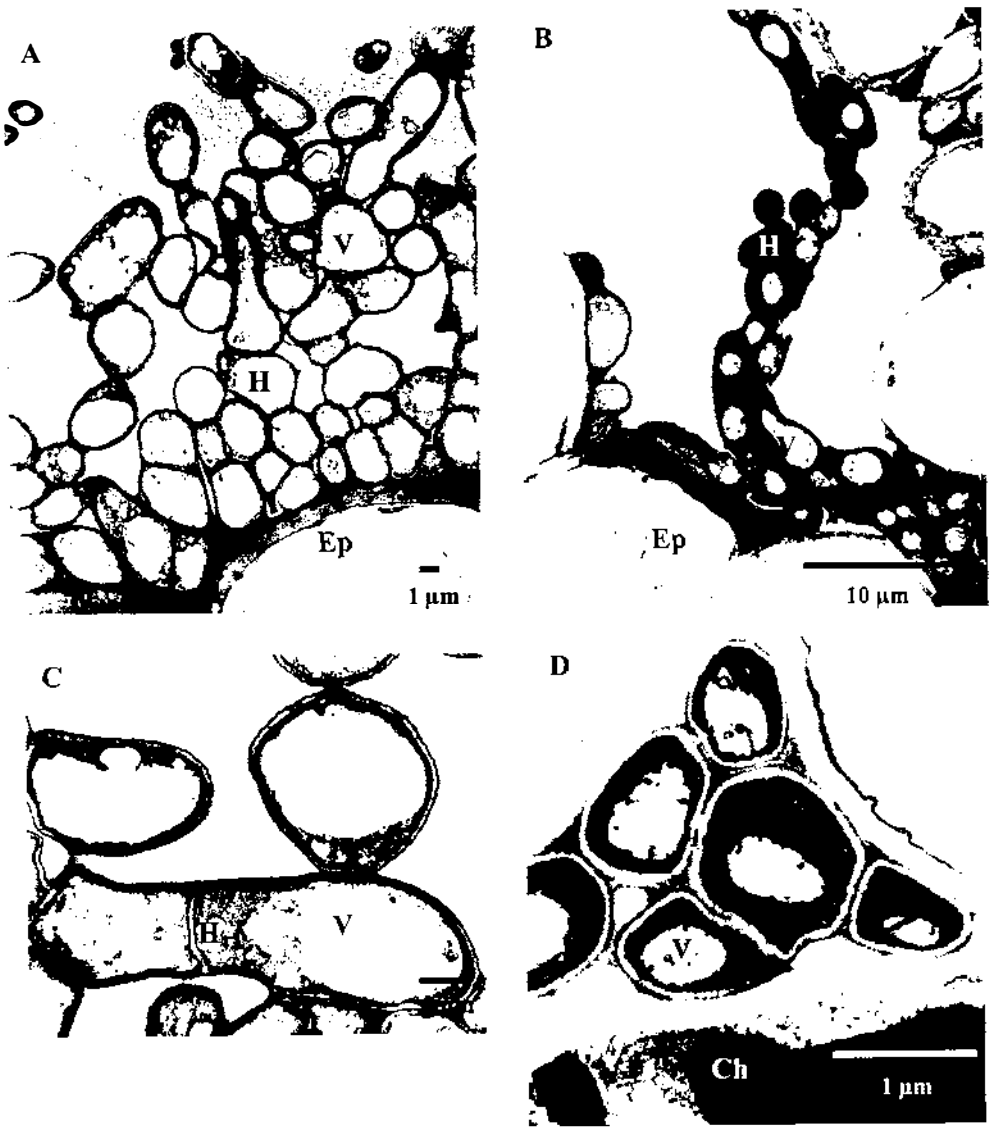

A
V
H
Ep
1 µm
B
H
Ep
10 µm
C
H
V
D
V
Ch
1 µm

Figure 3.55. Abundant glycogen-like deposits in wild-type E8 hyphae in vegetative tillers

A - B: Electron micrographs showing abundant glycogen-like deposits in E8 hyphae in a vascular bundle of a mature leaf blade in the non-stroma region.

C: Electron micrograph showing abundant glycogen-like deposits in E8 hyphae in a vascular bundle of an immature leaf blade in the pseudostem region.

D: Electron micrograph showing glycogen-like deposits in hyphae in mesophyll of an immature leaf in the pseudostem region.

E: Electron micrograph showing glycogen-like deposits in the outer epiphytic hyphae.

F: Electron micrograph showing glycogen-like deposits in a hyphae in a vascular bundle of leaf in the stroma-forming region.

H indicates hyphae. **L** indicates lipid droplet. **G** indicates glycogen-like deposits. **M** indicates mitochondria. **Ch** indicates chloroplast.

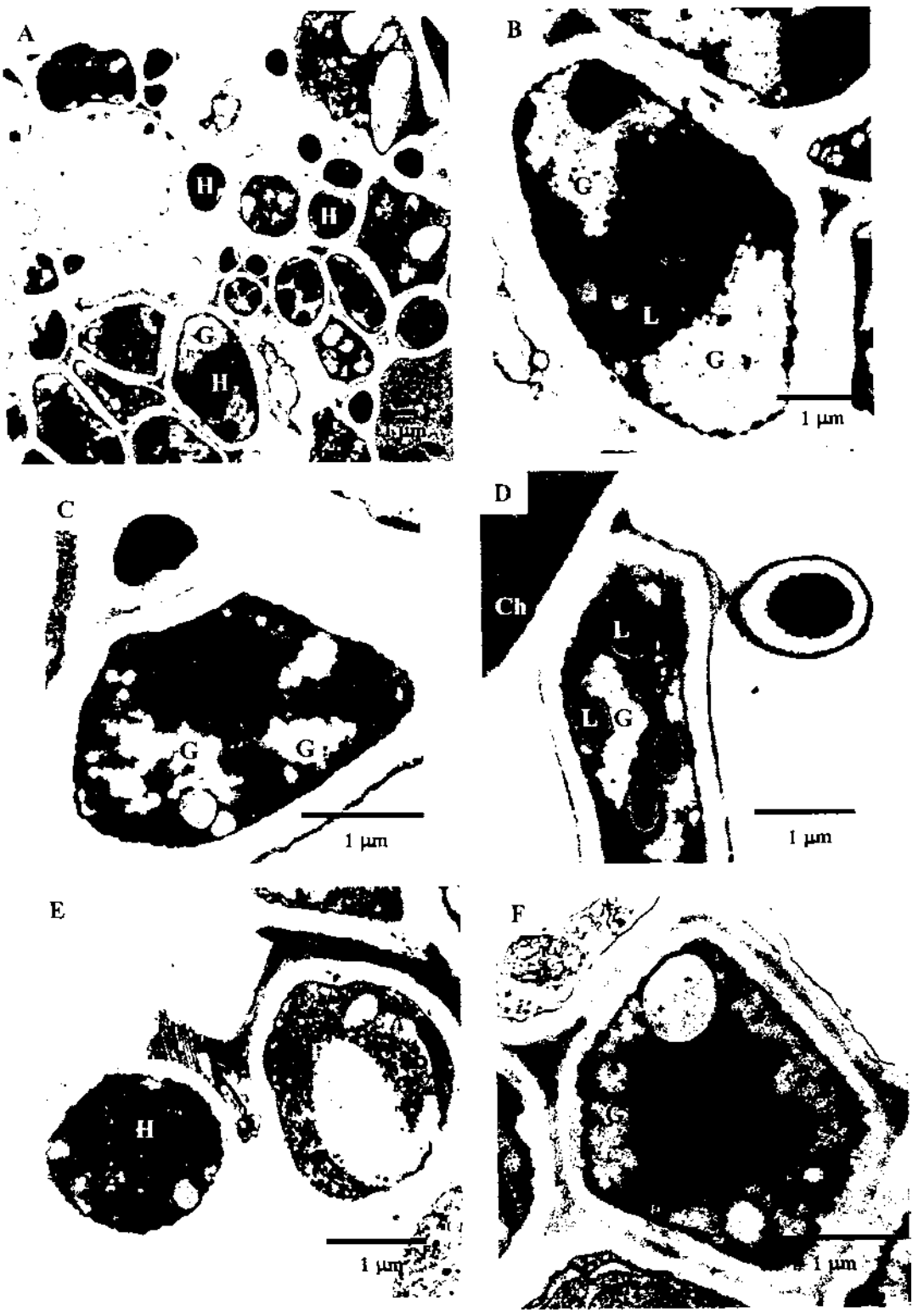

A
H
H
G
H
B
G
L
G
1 µm
C
G
G
1 µm
D
Ch
L
L
G
1 µm
E
H
1 µm
F
G
1 µm

Figure 3.56. Few glycogen-like deposits in hyphae of E8 *thi1* deletion mutant in vegetative tillers

A - B: Electron micrographs of hyphae of the E8 *thi1* deletion mutant in a vascular bundle of a mature leaf blade in the non-stroma region.

C - D: Electron micrograph of hyphae of the E8 *thi1* deletion mutant in the immature leaf blade in the pseudostem region.

E: Electron micrograph of the outer epiphytic hyphae of the E8 *thi1* deletion mutant.

F: Electron micrograph of hyphae of the E8 *thi1* deletion mutant in a vascular bundle of leaf in the stroma-forming region.

H or arrows indicate hyphae. **L** indicates lipid droplet. **Ch** indicates chloroplast. **V** indicates vacuole

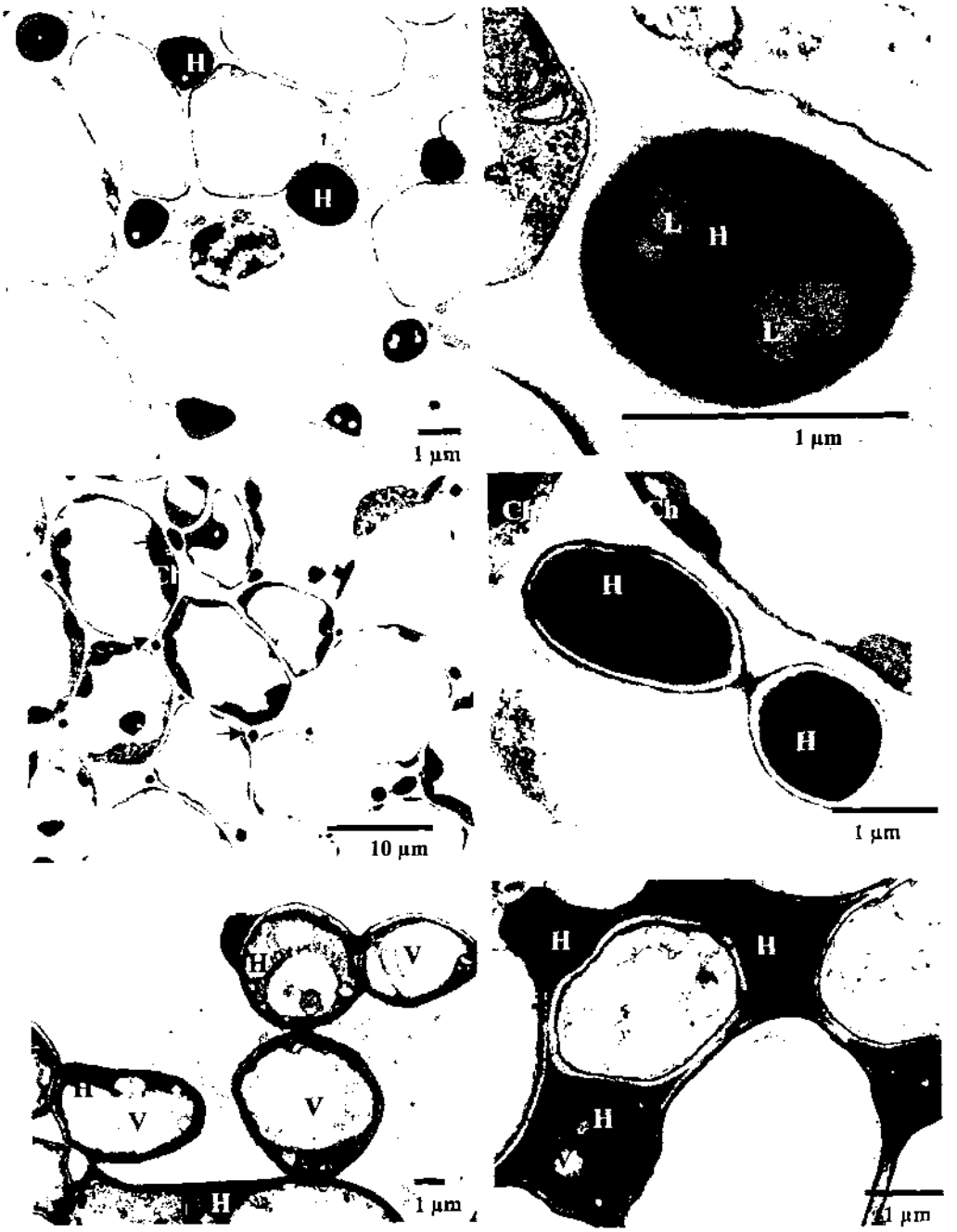

H
H
1 µm
L
H
L
1 µm
10 µm
Ch
H
H
1 µm
H
V
V
V
H
1 µm
H
H
H
1 µm

Figure 3.57. Alkaloid bismuth staining of wild-type E8 hyphae in vegetative tillers

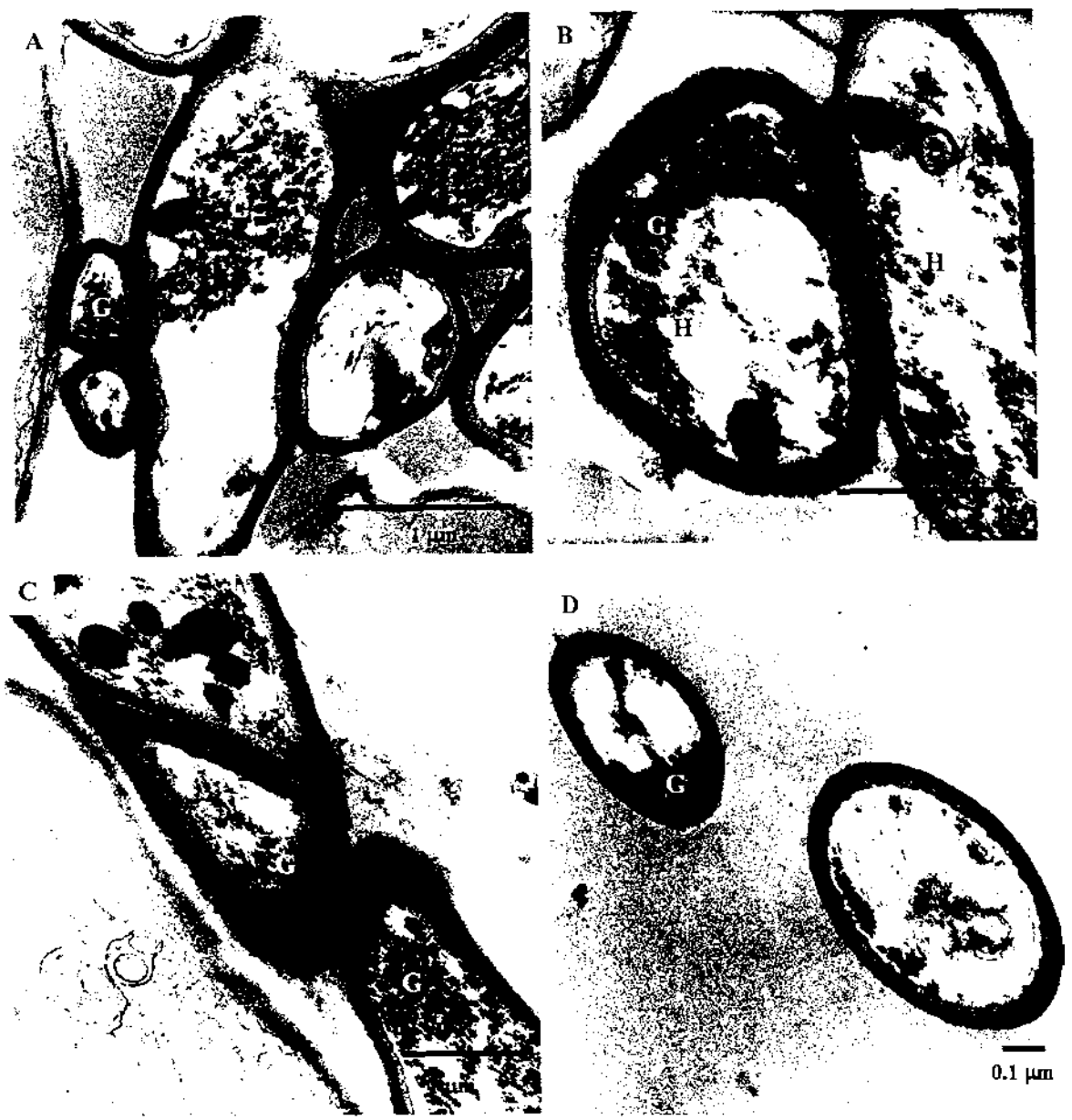
A: Electron micrograph showing densely stained glycogen-like deposits in hyphae in a vascular bundle of the leaf blade of a stroma-forming region.

B: Electron micrograph showing densely stained glycogen-like deposits in hyphae in mesophyll of the blade of a stroma-forming region.

C: Electron micrograph showing epiphytic hyphae with densely stained glycogen-like deposits.

D: Electron micrograph showing two outer epiphytic hyphae with densely stained glycogen-like deposits.

H indicates hyphae. **L** indicates lipid droplet. **G** indicates glycogen-like deposits. **Pl** indicates plant cell.


A
G
1 μm
B
G
H
H
C
G
G
D
G
0.1 μm

Chapter four

Discussion, conclusion and future work

This study employed the strategy of targeted gene disruption to test the hypothesis that endophyte thiamine biosynthesis is required for host colonisation and stromata development. The hypothesis was not supported by the results that the *E. typhina* E8 *thi1* mutants retained the ability to infect the host and form stromata on the host. Intriguingly, the *E. typhina* E8 *thi1* mutants appeared to have reduced detrimental effects on the host compared to the wild type isolates. In addition, stromata were observed on vegetative tillers of the host perennial ryegrasses, which may provided further insights into the mechanisms affecting endophyte-grass interactions.

4.1. Characteristics and expression of endophyte *thi1*

4.1.1. Endophyte *thi1* function

In this study, the *thi1* gene of *Neotyphodium lolii* strain Lp19 and of *Epichloë typhina* strain E8 was isolated based on its high homology with *THI4* of *Saccharomyces cerevisiae* and other organisms (Section 3.1.1). Disruption of the *thi1* gene in *E. typhina* E8 led to severely reduced growth on thiamine-free media. Restoration of wild-type growth occurred when the mutant was supplemented with thiamine or thiazole (Section 3.3). These results demonstrated that *thi1* isolated from *E. typhina* is indeed involved in thiamine biosynthesis, at a step prior to the formation of thiazole.

Genes homologous to *thi1* and its orthologue, *THI4* from *S. cerevisiae*, have been isolated from several pathogenic fungi (Section 1.6.2) on the basis of transcript abundance during host colonisation (Hahn and Mendgen, 1997; Thara *et al.*, 2003) and following treatment with copper(II) chloride, heat (37°C) or phaseollinisoflavan, an antifungal isoflavonoid produced by *Phaseolus vulgaris* (French bean) (Choi *et al.*, 1990). The up-regulation of these genes in the host suggests that they may be important in plant-fungal interactions. In

maize and *Alnus glutinosa*, the genes were isolated by differential cDNA cloning of genes induced in early embryo development and in actinorhizal nodules, respectively (Belanger *et al.*, 1995; Ribeiro *et al.*, 1996). Expression of these genes seems to be associated with rapidly dividing cells and/or high energy-demand in the cells (Belanger *et al.*, 1995; Ribeiro *et al.*, 1996). *Thi1* from *Arabidopsis thaliana* was isolated in the process of screening for genes capable of conferring resistance to methyl methane sulphonate (MMS) in an *E. coli* triple mutant (*xth*, *nfo*, *uvrA*), which is defective for exonuclease III, endonuclease IV and endonuclease III (Machado *et al.*, 1996). The *A. thaliana* gene is able to complement the yeast *thi14::URA3* disruption strain, confirming that it functions in the biosynthesis of thiamine (Machado *et al.*, 1996). Similarly, *THI4* from yeast encodes a protein with dual roles in mitochondrial DNA damage repair in addition to thiazole biosynthesis (Machado *et al.*, 1996; Machado *et al.*, 1997).

The predicted polypeptide sequences of the Lp19 and E8 *thi1* genes share greater than 48% identity with products of thiamine biosynthetic genes from other fungi and plants (Table 3.1), suggesting a conserved and important function of this protein in all organisms. The sequence differences mainly occur in the N-terminal region (Fig. 3.4), where there is a signal peptide in several fungi and plants for mitochondrial and chloroplast targeting respectively. A recent study showed that Thi1 from *A. thaliana* can be targeted to both the mitochondrion and the chloroplast (Chabregas *et al.*, 2001). No signal peptide was found in the predicted Thi1 polypeptide sequences of either E8 or Lp19.

Southern blotting hybridisation analysis showed that *N. lolii* strain Lp19 and *E. typhina* strain E8 contained a single copy of *thi1* while *Neotyphodium* sp. (LpTG-2) strain Lp1, a hybrid between *N. lolii* and *E. typhina* (Schardl *et al.*, 1994), contained two copies of this gene (Section 3.1.5). These results are consistent with the widely accepted hypothesis that Lp1 and many other asexual endophytes have evolved by interspecific hybridisation (Schardl *et al.*, 1994; Tsai *et al.*, 1994). While the restriction fragments that hybridised in

Lp19 were similar in size to those of Lp1, none of the restriction fragments of E8 matched the size of those from Lp1 (Section 3.1.5). The presence of an AT-rich sequence downstream of the E8 *thi1* (Section 3.1.4) may be a remnant of a retroelement. Retroelements might provide 'hot-spots' for DNA recombination, chromosome reorganisation or deletions (Scott and Young, 2003). The presence of these sequences at the *thi1* locus in E8 may account for the size differences observed between Lp1 and E8.

4.1.2. Endophyte *thi1* expression in culture

Like many metabolic pathways, the thiamine biosynthetic pathway is subject to feedback regulation (Section 1.6.3). Repression of thiamine biosynthetic gene expression by the presence of thiamine or thiamine precursors has been shown in a number of organisms (Hohmann and Meacock, 1998; Praekelt *et al.*, 1994; Wightman and Meacock, 2003). However, evidence is lacking showing that transcript levels and splicing of *thi1* was affected by addition of thiamine under carbon and nitrogen starvation conditions (Section 3.2.3). It has been previously reported that exogenous thiamine did not affect transcript levels of either *THI1* or *THI2* in *U. fabae*, homologues of *THI5* and *THI4* in yeast (Sohn *et al.*, 2000). The lack of repression of these genes by thiamine may be due to the absence of a repressive effect or the inability of the hyphae to take up thiamine (Sohn *et al.*, 2000). Alternatively, the regulation of these genes by thiamine may be at the post-transcriptional level. Recent work shows that in bacteria repression of the pathway may occur by a direct interaction of thiamine with thiamine biosynthetic transcripts, thereby blocking translation (Mironov *et al.*, 2002; Winkler *et al.*, 2002). In *Aspergillus oryzae*, two conserved motifs (regions A and B) in the 5' un-translated region (5'UTR) of *thi1* are required for thiamine mediated feed-back repression (Kubodera *et al.*, 2003). Sequence analysis of the Lp19 and E8 *thi1* sequences confirmed that both motifs are present in these genes (Section 3.1.2 and

3.1.4). This observation would suggest that both Lp19 and E8 *thi1* genes are also subject to a riboswitch mechanism of repression.

Expression of *thi1* was examined under conditions of varying carbon and/or nitrogen sources. The preliminary expression analysis by RT-PCR showed that carbon and/or nitrogen sources altered splicing of the introns in the 5' UTR region of *thi1*, resulting in three types of mRNA isoforms which differed in length of 5' UTR and number of upstream open reading frames (Section 3.2.3). These results are consistent with the 5' RACE analysis (Section 3.2.1). The relative levels of each isoform varied with the type and levels of carbon and nitrogen source present in the media (Section 3.2.4). Although alternative splicing does not appear to generate alternative Thi1 polypeptides, the differences in the length of the 5' UTR and uORFs can have a large influence on post transcriptional regulation of the gene (Rogers *et al.*, 2004; Vilela and McCarthy, 2003; Yaman *et al.*, 2003). Translation of the uORF may elicit a conformational change in the mRNA leading to translational control (Yaman *et al.*, 2003). Upstream ORFs have also been reported to be able to inhibit initiation or reinitiation of translation of the down stream major ORF, and the inhibition effect is particularly pronounced when the uORF is close to, or overlaps with, the down stream major open reading frame (Geballe, 1996; Hinnebusch, 1996). The Type I was the only isoform not to have an uORF overlapping with the Thi1 ORF. This type may therefore be translated at a higher efficiency than the others. Interestingly, under conditions where both utilisable carbon and nitrogen sources (glutamate) were present, the Type I isoform was abundant.

In filamentous fungi, global carbon and nitrogen regulation is mediated by two distinct types of regulatory proteins, CreA-like transcriptional repressor and NIT2/AreA transcriptional activators (Dowzer and Keller, 1989; Fu and Marzluf, 1990). The binding of CreA to the CreA binding site in the promoter of many carbon catabolic genes leads to repression of transcription of these genes in the presence of carbon sources such as glucose (Felenbok and

Kelly, 1996). Point mutations in the CreA binding sites resulted in derepression of transcription of genes in carbon catabolism (Mathieu *et al.*, 2000; Orejas *et al.*, 1999). However, CreA consensus sequences may be recognised by other regulatory proteins (de la Serna *et al.*, 1999). For example, recognition sequences for CreA were found to overlap transcriptional elements of a 40S rRNA gene and the *crp-2* ribosomal protein gene in *A. nidulans* (de la Serna *et al.*, 1999). The putative *CreA* homologue in *N. crassa, Cre-1*, has also been isolated and characterised (de la Serna *et al.*, 1999). Cre-1 protein produced in *E. coli* can bind to CreA sites in the promoters of the 40S rRNA and *crp-2* genes (de la Serna *et al.*, 1999). Base substitutions in the CreA binding sites of the *crp-2* promoter disrupted binding of Cre-1 *in vitro* but had no effect on transcription during steady state growth or carbon shifts (with or without sucrose), indicating that regulation of ribosomal genes by carbon source is not mediated by Cre-1, but via different proteins binding the Cre-1 sites (de la Serna *et al.*, 1999). NIT2 is a positive transcription factor which turns on many different nitrogen metabolism genes in response to nitrogen limitation in *Neurospora crassa* (Fu and Marzluf, 1990). The *nit2* homologues have been isolated from other filamentous fungi and the one in *A. nidulans* was designated as *areA* (Caddick *et al.*, 1986). NIT2/AreA proteins belong to the GATA zinc finger family (Marzluf, 1997). They possess a single DNA binding domain which binds to the core GATA sequence in the promoter region thereby regulating the transcription of the gene. The C-terminal region of this protein is required for interactions with other proteins, such as the regulatory proteins NIT4 and NMR, involved in regulation of nitrate assimilation in response to environmental stimuli (Tao and Marzluf, 1999). In *N. crassa*, expression of the *nit-3* gene, which encodes nitrate reductase, requires cooperation of both the NIT2 and NIT4 transcription factors and only occurs under conditions of nitrogen source derepression and nitrate induction (Mo and Marzluf, 2003). Nitrogen metabolic regulation appears to play significant roles in the pathogenicity of certain animal and plant fungal pathogens (Marzluf, 1997; Pellier *et al.*, 2003; Pérez-Garcia *et al.*, 2001). In *Colletotrichum lindemuthianum*, a fungal pathogen of common bean, targeted disruption of *clnr1*, the *areA/nit-2* orthologue, resulted in mutants

that were unable to use a wide array of nitrogen sources and were non-pathogenic (Pellier *et al.*, 2003). The *clnr1* mutants complemented with the *A. nidulans areA* gene are fully pathogenic (Pellier *et al.*, 2003).

Interestingly, potential CreA and NIT2 binding sites were identified in the *thi1* promoter. It is unknown whether CreA and/or NIT2-like proteins are required for *thi1* expression or whether they regulate alternative splicing. Expression of the endophyte *thi1* could be regulated at transcriptional and/or post transcriptional levels by thiamine, carbon and nitrogen. Understanding the regulation of *thi1* expression may require further determination of the transcript level, the profile of alternatively spliced isoforms and the protein level of *thi1* under different growth conditions with thiamine and/or other nutrients, through approaches such as Northern hybridisation, RT-PCR, Western hybridisation and/or analysis using reporter gene systems. Point mutations in the binding sites for CreA, NIT2 and the thi-box may also be required to determine whether CreA and NIT2 are involved and how thiamine regulates expression of this gene.

4.1.3. Endophyte *thi1* expression *in planta*

Analysis of *thi1* expression *in planta* by RT-PCR indicated that *thi1* transcript is abundant in endophyte hyphae growing within pseudostem tissue of the grass (Section 3.2.4). Cloning and sequence analysis of the *thi1* transcripts expressed *in planta* identified two *thi1* mRNA isoforms, type II and type III (Section 3.2.4).

Although the concentration of thiamine in the intercellular space is not known, the high level of *thi1* expression within endophyte hyphae *in planta* would suggest that the thiamine concentration is less than that required to repress gene expression. Similarly, the high level of expression of thiamine biosynthetic genes in the pathogenic fungi *U. fabae* and *P. triticina*

Eriks during host colonisation (Hahn and Mendgen, 1997; Sohn *et al.*, 2000; Thara *et al.*, 2003), would again suggest that thiamine levels in these plants are less than that required to repress *thi1* gene expression.

In higher plants, thiamine synthesis is developmentally and spatially regulated (Belanger *et al.*, 1995). In maize, *thi1* was expressed at a low level in developed embryos and early germinating seedlings, but at a high level in developing embryos and in photosynthetic organs (Belanger *et al.*, 1995). It is possible that expression of the endophyte *thi1* gene *in planta* is developmentally regulated and varies depending on the stages of either fungal and/or plant development. GUS or GFP reporter systems and/or immunofluorescence microscopy (Sohn *et al.*, 2000) are approaches that would allow these hypotheses to be tested.

4.2. Morphological change of the E8 *thi1* mutants

4.2.1. Morphological changes of the *thi1* mutants

Examination of the phenotype of the E8 *thi1* deletion mutants in culture showed that the two independently isolated *thi1* mutants (KO3 and KO4), when grown on thiamine-free medium, showed a reduced frequency of hyphal branching and the hyphae were convoluted (Section 3.3.4). Supplementation of the media with thiamine or thiazole restored normal hyphal growth and morphology to the mutants confirming that insufficient thiamine was responsible for both phenotypes.

To our knowledge, there are no reports indicating that thiamine or thiamine biosynthesis is involved in hyphal branching and morphology in filamentous fungi. However, it is

commonly known that poor nutrition can affect fungal growth and colony morphology (Olsson, 2001). The mechanisms controlling branch initiation and extension in filamentous fungi are still rather obscure. Several factors that can control the production and frequency of branching have been identified, including both physiological and genetic factors. Physiological factors that influence hyphal extension and branching include calcium, choline (Markham *et al.*, 1993; Pera and Callieri, 1997), calmodulin, phosphoinositides, cyclic nucleotides and pH (Trinci *et al.*, 1997). Mutations that affect colony morphology and hyphal branching have been identified in genes that encode components of the cytoskeleton (Robb and Wilson, 1995) and microtubule-associated motor proteins (Seiler *et al.*, 1997), in signal transduction pathways (Yarden *et al.*, 1992), and in transcription factors (Arnaise *et al.*, 2001). Proteins such as subunits of the cAMP-dependent protein kinase A (PKA), have also been reported to be involved in hyphal branching, growth and morphology in *N. crassa* (Lubbehusen *et al.*, 2004). There is good evidence that components of the cytoskeleton play a key role in controlling hyphal extension and growth, through vesicle transport and through control of the orderly deposition of wall and membrane components at the growing tips (Gooday, 1995). Deletion in the NhKIN1 gene, encoding a kinesin from *Nectria haematococca* resulted in mutants that had helical or wavy hyphae with reduced diameter and withdrawal of mitochondria from the growing hyphal apex and reduction in the size of the Spitzenkorper, an apical aggregate of secretary vesicles (Wu, 1998), suggesting that microtubule-based motor proteins such as kinesin are essential for normal positioning of the Spitzenkorper and hyphal morphology development (Wu, 1998).

It has been proposed that once the production of materials for cell wall extension exceed the quantity that can be used at the pre-existing apex, a signal is generated promoting hyphal branching and the concomitant formation of a new growing tip (Yarden *et al.*, 1992). Thiamine is an important cofactor for enzymes that play key roles in carbohydrate metabolism (Hohmann and Meacock, 1998). Lack of thiamine will therefore restrict these key biochemical pathways but how this is translated into an effect on growth of the

cytoskeleton is not clear. Thiamine deficiency may decrease the availability of materials for cell wall extension or cytoskeleton structure and thus affect hyphal branching and maintenance of extension.

As thiamine is an important coenzyme in cell metabolism, especially for energy metabolism (Hohmann and Meacock, 1998), it was anticipated that *thi1* mutants would not grow on thiamine-free medium. Although the mutants had a change in hyphal density and morphology, radial growth of the colonies was unchanged (Table 3.6 and 3.7). This limited growth was probably due to highly efficient salvage of trace amounts of thiamine and/or thiamine precursors from the mycelium and/or the medium (Hohmann and Meacock, 1998). Many fungi exhibit autolysis of older hyphae, especially when growing on nutrient-poor media. This material is likely to be translocated to actively growing hyphae especially the hyphal apices (Olsson, 2001). It is possible that there was a trace of thiamine in the agar used.

Typical convoluted hyphae of *Neotyphodium*-like endophytes have been observed in the leaf sheath in some genera of grasses including *Festuca* and *Lolium* spp. (Leyronas and Raynal, 2001). It has also been reported that many *Acremonium* (*Neotyphodium*) isolates produced convoluted hyphae from host pieces after transfer of the host pieces from nutrient-rich PDA medium to water agar plates (Kaiser, 1996). This form of hyphae was common in isolates that emerged rapidly from host tissues and sporulated poorly (Kaiser, 1996). The mechanisms underlining this phenomenon remains unclear. Hyphae of the rust fungus *Uromyces phaseoli* and *Puccinia recondita* often grow in twisted and convoluted form in susceptible plants treated with allopurinol – an inhibitor of xanthine oxidoreductase which is involved in oxidative catabolism (Marte and Montalbini, 1999). It is possible that an unbalance in metabolism resulted from inhibition of xanthine oxidoreductase caused the aberrant hyphal growth (Marte and Montalbini, 1999). Similarly, deficiency in thiamine may cause abnormal metabolism in *E. typhina* and thus result in hyphal convolution.

4.2.2. Morphological changes of the ectopic transformants

Surprisingly, one of the ectopic controls, strain EC1, showed reduced hyphal growth on thiamine-free medium (Section 3.3). Furthermore, this growth defect was overcome by the addition of thiamine to the media. The other ectopic control, strain EC2, had a slight change in colony morphology and a slight increase in frequency of hyphal branching, but otherwise was similar to the wild-type (Section 3.3). While a spontaneous mutation may be responsible for the Thi$^-$ phenotype shown by EC1, it is more likely due to ectopic integration of the *thi1* replacement construct, as 33 out of 302 of the ectopic transformants showed reduced growth on thiamine-free medium (Section 3.3.3.1). The phenotype of EC1 and other Thi$^-$ ectopic mutants may result from integration of the *thi1* replacement construct into other thiamine biosynthesis genes, other important metabolic genes, or alternatively could titrate out important transcription factors.

Schmid *et al.* (2000) reported that the growth of Lp19 endophyte transformants containing ectopic integrations of a plasmid with the GUS reporter gene (*gusA*) under the control of promoter of *hmg*, a gene encoding 3-hydroxy-3-methylglutaryl coenzymeA, grew differently to the wild-type *in planta* (Schmid *et al.*, 2000; Zhang *et al.*, 2001). Further, Northern analysis showed that the level of *hmg* transcripts in the transformant was lower than that of the wild-type (Ninxing Zhang and Jan Schmid; personal communication). The presence of additional copies of the *hmg* promoter in the genome may interfere with the transcription of *hmg* or reduce the stability of the *hmg* mRNA (Schmid *et al.*, 2000; Zhang *et al.*, 2001).

Similarly, ectopic integration of the *thi1* replacement vector, containing 1.3 kb of sequence upstream of the ATG may interfere with the expression of the endogenous *thi1* gene,

thereby changing the growth response of the transformants to thiamine. One possible mechanism for interference would be direct interaction between thiamine and the *thi1* transcript (Mironov *et al.*, 2002; Winkler *et al.*, 2002) leading to reduced availability of thiamine in these transformants. Alternatively, gene silencing may be involved (Agrawal *et al.*, 2003; Catalanotto *et al.*, 2004; Kadotani *et al.*, 2003). The transcripts of the ectopic *thi1* copies may co-suppress endogenous *thi1* transcription through a post-transcriptional RNAi-like mechanism that leads to degradation of the endogenous *thi1* transcript.

4.3. Effects of wild-type E8 and E8 *thi1* deletion mutants on the host

4.3.1. Host colonisation and incompatibility between *E. typhina* E8 and perennial ryegrass

4.3.1.1. Infectivity and hyphal growth in the host

Different endophytes have different abilities to colonise their hosts. This is reflected in the different infection rates and different hyphal concentrations in the host tissues (Christensen, 1995; Christensen *et al.*, 2002; Chung *et al.*, 1997). *E. festucae* strains typically have higher infection rates with *Festuca* and *Lolium* spp. than *Neotyphodium* spp. (Christensen, 1995). Hyphae of *Neotyphodium* spp are seldom branched, and seldom colonise vascular bundles or the leaf blade, while hyphae of *E. festucae* are more frequently branched, show some colonisation of vascular bundles (Christensen *et al.*, 1997; Christensen *et al.*, 2001, 2002; Tan *et al.*, 2001) and epiphyllous growth (Mike Christensen; personal communication). This study showed that *E. typhina* strain E8 had an infection rate and host colonisation ability very similar to *E. festucae* endophytes (Sections 3.4.1.1 and 3.4.1.2).

These differences in host colonisation may be related to the ability of the endophyte to obtain and utilise nutrients from the host tissues. As discussed in the Introduction (Section 1.5.1), *Epichloë* endophytes can utilise a greater range of carbon and nitrogen sources and grow much faster in culture than asexual *Neotyphodium* endophytes. Many *Neotyphodium* endophytes appear to have lost the ability to synthesise thiamine which is a key co-enzyme in cell metabolism (Section 1.5.1). The reduced metabolic ability of the *Neotyphodium* endophytes may contribute, at least partially, to their low infectivity and low hyphal concentration in the host tissues, compared to the *Epichloë* endophytes.

However, metabolic ability and growth rates in culture are unlikely to be the same as *in planta*, where the physiological growth conditions are different and the endophytes are subjected to host signalling. Endophyte hyphae are only observed in the host intercellular space, where a number of nutrients are thought to be low (Lohaus *et al.*, 1995). Consequently, the efficiency with which the endophyte can exploit the plant nutrients will be important for its growth and propagation (Knogge, 1996). Many obligate biotrophic fungi can form specialised infection structures, called haustoria, within infected host cells. These structures play a key role in nutrient acquisition from the host (Panstruga, 2003; Perfect and Green, 2001). Genes in haustoria involved in nutrient uptake and metabolism include those that encode transporters of amino acids, glucose and fructose and genes for thiamine biosynthesis (Hahn and Mendgen, 1997; Panstruga, 2003). Although no haustoria are formed by endophytes, the hyphae appear to possess specific mechanisms for the uptake of carbohydrates (Lam *et al.*, 1994). Some endophytes, for example *N. lolii*, are able to produce an abundant proteinase which may facilitate the degradation of apoplastic proteins for uptake and utilisation by the endophyte (Reddy *et al.*, 1996). The nutrients derived from the host may be converted into fungal storage substances, such as sugar alcohols and the diglucoside trehalose which is a key substrate for the pentose phosphate pathway (Bacon and White, 2000; Smith *et al.*, 1985). The ability of some endophytes to synthesise vitamins, such as thiamine, may increase the vigour and growth rate of these

endophyte strains *in planta*. Further analysis of the role and function of genes responsible for nutrient uptake and metabolism will be helpful in understanding the effects of these processes on host colonisation.

4.3.1.2. Host incompatibility

In some cases, E8 failed to infect particular hosts (Sections 3.4.1.1 and 3.4.2.1), and in others infection occurred but this was followed by severe stunting or death of the host (Sections 3.4.1.3, 3.4.1.7 and 3.4.2.2) and/or loss of the endophyte (Section 3.4.1.7). Usually, the percentage of seedlings that die after inoculation and transplantation vares dependent on the endophyte isolate and the host used. It has been reported that the percentage of host death rate of *E. typhina* inoculated into three different grasses Chewings fescue, perennial ryegrass and tall fescue were 13%, 33% and 76% respectively (Latch and Christensen, 1985). A stunting *E. festucae* isolate gave host death rates of 58%, 81% and 86% when inoculated into perennial ryegrass, Meadow fescue and tall fescue respectively, while a non-stunting *E. festucae* isolate gave host death rates of 36%, 39% and 53% when inoculated into perennial ryegrass, meadow fescue and tall fescue at the same time (Christensen *et al.,* 1997). These observations possibly reflect a breakdown in the balance of host resistance and anti-resistance during the endophyte-grass interaction.

Although most grass hosts seldom exhibit resistance to their natural endophyte, artificial infections by related endophytes can elicit a host response (Section 1.2.2), for example, enhanced peroxidase activity (Naffa *et al.*, 1999), necrosis in the region of the apical meristem (Christensen, 1995), ultrastructure changes in the host cells (Christensen, 1995; Christensen *et al.*, 2002; Koga *et al.*, 1993), and death of the hyphal cells (Koga *et al.*, 1993). These reactions may indicate that endophytes can trigger non-specialised or specialised host defence responses.

However, most host grasses in association with endophytes do not elicit a defense response at any stage of their life cycle (Christensen *et al.*, 2002). The lack of host response is possibly due to the ability of the endophyte to avoid or suppress host defence reactions. Recent studies have shown that micro-organisms have developed diverse strategies to undermine plant defence and target core components of plant immunity (Abramovitch and Martin, 2004; Panstruga, 2003; Schulze-Lefert, 2004). Defense suppression appears to play an important role in symbiotic plant-microbe interaction. In the interaction of nitrogen-fixing bacteria or mycorrhizal fungi with their hosts, infection can trigger a plant defense response (Jackson and Taylor, 1996), but the response seems to be modulated in the further development of the symbiosis (Bartsev *et al.*, 2004; Shaw and Long, 2003). For example, *Rhizobium* species that form symbiotic nitrogen-fixing associations with legumes can secrete a number of proteins (TTS and NopL) into host cells thereby repressing transcription of plant resistance genes (Bartsev *et al.*, 2004). The bacterium *Sinorhizobium meliloti,* a symbiont of the legume plant *Medicago truncatula*, can produce Nod factor molecules which can inhibit hydrogen peroxide efflux in legume hosts (Shaw and Long, 2003). For some obligate biotrophic fungal pathogens which form long-term compatible associations with host plants, the fungi may re-programme the host cell death response and/or suppress cell-wall-associated defense responses thereby forming compatible interactions with the host plant (Panstruga, 2003). The rust fungus *Melampsora lini* was reported to induce expression of the host gene *fis1* which may be responsible for degradation of proline (Ayliffe *et al.*, 2002). Proline may serve as a signal in plants to abiotic and biotic stresses. The activation of *fis1* might indirectly suppress the host defense (Ayliffe *et al.*, 2002).

Similarly, the ability of endophytes to suppress host defense responses may play a key role in the establishment and maintenance of the long-term compatible associations between

the endophyte and grasses. Identifying genes that are required for host defense suppression would enhance our understanding of symbiotic fungi-host compatibility.

4.3.2. Effects of wild type *E. typhina* E8 on growth and development of the plant host

It has been widely accepted that endophyte infection is advantageous to the growth and development of the host plant, in particular under the conditions of biotic and/or abiotic stress. Infection of endophyte has been reported to induce host tiller production, increase host biomass and seed germination (Belesky *et al.*, 1987; Cheplick *et al.*, 1989; Latch *et al.*, 1985). While the benefits that endophyte infection confers on the host have been well documented for the mutualistic asexual endophytes, such as tall fescue infected by *N. coenophialum* (Belesky *et al.*, 1987; Cheplick *et al.*, 1989) and perennial ryegrass infected by *Neotyphodium* endophytes (Latch *et al.*, 1985), there are very few report on the effects of *E. typhina* endophytes on the growth of their host plants.

Observations in this study indicated that infection by *E. typhina* E8 could have detrimental effects on the perennial ryegrass host, including reducing survival (Section 3.4.2.1), fresh weight and tiller weight (Section 3.4.2.3) and inducing stunting (Section 3.4.2.3). The detrimental effects were strong in seedlings or young plants, where the average fresh weight of the endophyte-free plants were over 4 times that of the E8-infected plants. Coincidentally, Faeth and Sullivan (2003) recently reported that infection of *Neotyphodium* endophyte can have detrimental effects on host plants (Faeth and Sullivan, 2003). *Festuca arizonica* plants infected with a *Neotyphodium* species have decreased host growth as measured by plant volume, number of tillers, and dry mass of shoots and roots compared to non-infected plants (Faeth and Sullivan, 2003). These negative effects of the symbiont were transferred to the next generation. *E. typhina* endophytes are considered to be the least

mutualistic of all grass endophytes as they routinely develop stromata on reproductive tillers of the host causing 'choke disease' (White and Bultman, 1987). It is not surprising that *E. typhina* is more detrimental than *N. lolii* on the perennial ryegrass host.

Competition for nutrients such as carbohydrates and amino acids may be a major factor responsible for the detrimental effects on the host (Faeth and Sullivan, 2003). Cheplick *et al* (1989) reported that compared to uninfected seedlings, tall fescue seedlings infected with *Neotyphodium* endophytes had a greater biomass at higher plant nutrient levels but a significantly lower biomass at lower nutrient levels, indicating a metabolic cost to the host due to competition for nutrients (Cheplick *et al.*, 1989). Endophytes are biotrophic organisms which must obtain all their basic nutrients from the host for their growth (Siegel *et al.*, 1987). These nutrients may include those produced by plant photosynthesis and those mobilised from plant cells, which would otherwise be delivered to regions of host active growth. The ability of some endophytes such as *E. typhina* to colonise the vascular bundles (Section 3.5.2.2) is even more detrimental to the host as this is a very effective mechanism for the fungus to gain access to nutrients (Christensen *et al.*, 1997). In addition, endophyte hyphae must be active in metabolism to produce secondary metabolites such as the nitrogen-rich alkaloids. This will also be a cost to host plants (Faeth and Sullivan, 2003). Production and storage of alkaloids require substances such as amino acids and energy sources. These alkaloids could be toxic to the host plant (Faeth and Sullivan, 2003). These may have a direct or indirect effect on the host nitrogen metabolism and growth.

A feature of E8 hyphae within host grasses is the presence of abundant lipid droplets (Section 3.5.2.3) and glycogen-like deposits (Sections 3.5.2.4 and 3.5.2.5). These storage products were previously observed in hyphae of *E. festucae* strains which can cause host stunting (Christensen *et al.*, 1997), but were not evident in non-stunting *E. festucae* strains. Glycogen-like deposits were not observed in hyphae of many mutualistic *Neotyphodium* isolates within grasses (Christensen *et al.*, 2001). In this study, hyphae with abundant

glycogen-like deposits could be observed even on hosts of non-stunting phenotypes. The presence of these abundant storage products may be indicative of greater access by these endophytes to host nutrients either directly or indirectly thereby adversely affecting host growth.

Although competition for nutrients seems to be linked with host stunting, other growth factors may be involved in host development and morphology. Endophytes are also able to produce phytohormones such as 3-indole acetic acid (IAA) in culture (De Battista *et al.*, 1990). The ability of endophytes to produce phytohormones or change the overall levels of phytohormones in the symbiosis will inevitably affect the host development and morphology and/or the endophyte metabolism.

The detrimental effect of endophyte infection on host growth could also be due to induction of host resistance responses as discussed above (Section 4.3.1.2). Although the mechanisms of resistance can vary, the cost of resistance in plants can lead to costs on host growth and fitness (Faeth and Sullivan, 2003; Purrington, 2000). Endophyte may suppress the host defense response as discussed above (Section 4.3.1.2). While this is essential for maintenance of the symbiotic associations, defense suppression may result in the host being more susceptible to abiotic or biotic stresses such as other pathogens, thereby resulting in reduced growth or even death of the infected hosts. This may explain the phenomenon that seedlings often died after transplantation (Sections 3.4.1.1 and 3.4.2.1).

Collectively, interactions between endophyte and grass, and influence of these interactions on the host development and growth are determined by combinations of factors mentioned above and many other factors as yet unknown. We do not know whether in natural environments infection of *E. typhina* has similar detrimental effects on the host growth. *E. typhina* is considered to be an antagonistic endophyte as it expresses the sexual life cycle on host reproductive tillers, resulting in host sterilisation (White and Chambless, 1991).

Results in this study showed that *E. typhina* also develop stromata on vegetative tillers which retard host leaf growth (Sections 3.4.1.5 and 3.4.2.4). This effect together with the other detrimental effects on growth of seedlings and young plants (Sections 3.4.1.3, 3.4.2.2 and 3.4.2.3), might explain why grasses species infected with *E. typhina* endophytes are only occasionally found in nature (White and Chambless, 1991). However, one potential benefit of *E. typhina* infection is the ability of this species to produce peramine, an insect feeding deterrent (Section 3.4.1.4).

4.3.3. Effect of *thi1* deletion in the endophyte-host interactions

In this study, two independent *thi1* deletion mutants of *E. typhina* strain E8 were generated and reintroduced into perennial ryegrasses to investigate the effects of the *thi1* mutation on the endophyte-host symbiotic phenotype. The *thi1* deletion mutants retained the ability to infect the host grass (Sections 3.4.1.1 and 3.4.2.1) and produce stromata (Sections 3.4.1.5 and 3.4.1.6). Consequently, our results gave no indications for a crucial role of endophyte thiamine biosynthesis in either host colonisation or external stromata production. These results confirmed that even if thiamine levels are low in the intercellular space, the mutants have the ability to uptake and/or salvage sufficient thiamine or thiamine precursors to grow well.

Despite these observations, deletion of the E8 *thi1* gene significantly: increased endophyte infection rate, increased host survival, and reduced detrimental effects on the host growth (Section 3.4). The above results and the fact that *thi1* is expressed by the endophyte within the plant (Section 4.2.3), may indicate that the endophyte can synthesis thiamine and promote an active endophyte metabolite and in return influence the endophyte-host interaction. Hyphae of the *thi1* mutants differ from those of the wild-type in that glycogen-like deposits, which were abundantly present in the wild-type E8 in both the leaf blade and

sheath were not as evident in the *thi1* mutants (Section 3.5.2). Taken together, these results suggest that disruption of *thi1* may reduce accumulation of nutrients as fungal storage products thereby reducing competition for nutrients from the host. This in turn could decrease hyphal growth, and as a consequence reduce the detrimental effects on host growth. Of interest is the observation that a mutation in glycogen synthase in the nitrogen-fixing bacterium *R. tropici* resulted in decreased levels of a high-molecular-weight exopolysaccharide, an enhanced respiration and increased host growth (Marroqui *et al.*, 2001). An analysis of the available fully sequenced bacterial genomes indicates that an inability to store glycogen is a trait associated with the parasitic or symbiotic lifestyle (Henrissat *et al.*, 2002). Loss of the ability to synthesise vitamin such as thiamine in endophytes may be an adaptation to a symbiotic lifecycle.

Results in this study also showed that the *thi1* mutants probably have an increased infection rate based on the number of surviving hosts (Section 3.4.2.1). The mechanism underlying this observation is unknown. It could be that the reduced hyphal growth of the *thi1* mutants within the host tissues induced a reduced host resistance response compared to the wild-type strain, thus favouring endophyte survival *in planta* and host growth.

The development of stromata in associations containing wild-type and *thi1* mutants may be due to an increase in permeability of host membrane in the stroma-forming region, resulting in leakage of host nutrients, including thiamine, into the intercellular space. In *Agrostis hiemalis* infected with the endophyte *E. amarillans*, changes have been observed in the host cell wall and membrane in the stroma-forming region (White *et al.*, 1997). Rupture of the host cells in the stroma-regions was also observed in this study (Sections 3.5.1.1 and 3.5.2.1). Alternatively, thiamine together with other nutrients may be mobilised through hyphae that have colonised the vascular bundles (Christensen *et al.*, 1997). Intensive vascular bundle colonisation was observed in stromata that developed in grasses infected

with either E8 wild-type or the *thi1* mutants (Section 3.5.2.2) and in other endophyte-grass associations (White *et al.*, 1997). Even so, hyphae of the *thi1* mutants in the stroma-forming region of vegetative tillers appeared to be more highly vacuolated and had reduced cellular contents compared to those of the wild-type (Section 3.5.2). These observations suggest that active metabolism and growth of hyphae of the mutant were thiamine limited. Since thiamine transport is mediated by the thiamine transporter protein (Enjo *et al.,* 1997), a mutant with mutations in both a thiamine biosynthetic gene and a gene encoding the thiamine transporter might affect the endophyte infection and stromata development.

Recent work suggests that the interaction between symbiotic micro-organisms and plant hosts is far more complex than hitherto realised, as the partners have evolved a metabolic dependence on one another (Lodwig *et al.*, 2003). For example, in the nitrogen-fixing symbiotic association between *Rhizobium* and legume plants, the plant provides a carbon energy source and amino acids to the bacteroids. In return, the bacteroids act like plant organelles to provide both ammonium and the amino acid aspartate for asparagine synthesis in the plant cytosol (Lodwig *et al.*, 2003). This cycling of amino acids between the bacteroids and plant host is essential for nitrogen fixation. Disruption of either amino-acid transporters or the principal aspartate amino transferase, an enzyme for production of aspartate in the bacteroids, results in a block in nitrogen fixation. Information on exchange of nutrients and primary metabolites between endophyte and the host is scarce. Future studies on these processes would allow us to identify the key metabolites exchanged between each partner, and the metabolite dependence of one partner on the other.

Mutation in an endophyte gene, for example the thiamine biosynthesis gene in this study, may reduce the detrimental effect of E8 infection on the host and therefore be beneficial to the host. A recent study showed that a single gene mutation may convert an *E. festucae* strain from mutualistic to antagonistic, causing death or severe stunting to the host (Aiko

Tanaka and Barry Scott, personal communication). This is an interesting observation as it has been reported that mutation in a single gene can convert *Colletotrichum magna* from a pathogen into a mutualistic endophyte (Redman *et al.*, 2001). While *C. coccodes* is pathogenic to its host tomato, a *C. coccodes* mutant which was deficient in extracellular protease activity became non-pathogenic (Redman *et al.*, 2002). Thus, single gene changes can have great effects on the virulence or avirulence of microbes on the host. This may be important in nature where selective forces are variable and can operate simultaneously on several traits of the endophyte, host or endophyte-host associations.

4.3.4. Effects of host genotypes and environmental factors on the symbiotic phenotypes

As discussed in Section 3.4.5, a wide range of endophyte-host symbiotic phenotypes were observed in this study which were probably caused by factors such as limitations of the inoculation methods, the effects of micro-environments and the host genotype effects. Effects of host genotype on symbiotic phenotype of endophyte-grass associations have been previously reported. For example, when *E. festucae* was inoculated into *Lolium perenne*, *Festuca arundinacea*, *F. longifolia*, *F. pratensis* and *F. rubra* subsp. *rubra*, growth of the host plants and colonisation of the endophyte hyphae within the host varied depending on the host species (Christensen *et al.*, 1997). In natural associations of Arizona fescue (*F. arizonica*) infected with a *Neotyphodium* endophyte, plant growth and reproduction, as well as the timing of germination of seeds were affected by the presence of the endophyte and the genotype of the host (Faeth and Sullivan, 2003). Agronomic grasses usually have less genetic diversity than native grasses because of restricted propagation from natural populations and subsequent inbreeding. However, even the limited genetic diversity between or within agronomic cultivars can alter host colonisation (Christensen *et al.*, 2001), host growth (Cheplick *et al.*, 2000; Meijer and Leuchtmann, 2000), stromata

development (Meijer and Leuchtmann, 2000), and alkaloid production (Spiering, 2000). The host genotype can affect alkaloid levels in agronomic cultivars by as much as 10-fold, and within cultivars by as much as 5-fold. Alkaloid levels can vary as much as 3 to 5-fold depending on the growth conditions (Brain Tapper, personal communication).

Effects of environmental factors on symbiotic phenotypes were also observed in this study. The host colonisation and the frequency of stromata formation varied depending on the growing condition, especially the season (Section 3.4). The exact factors responsible for these differences in *E. typhina*-ryegrass interactions are unclear. Water availability (soil moisture) was reported to be the main limiting factor to plant growth and reproduction in some endophyte-grass associations (Faeth and Sullivan, 2003; Purrington, 2000). Host growth as measured by average dry weight and tiller numbers is affected by environment factors such as shading, fertilisation and elevated CO_2 (Meijer and Leuchtmann, 2000). Fertilisation significantly stimulated plant growth in many genotypes, whereas the effects of shading and elevated CO_2 on plant growth were less pronounced and not always consistent (Meijer and Leuchtmann, 2000). A study on the effect of CO_2 on endophyte-infected grasses showed that elevated CO_2 increased plant reproductive growth of *Lolium perenne* but had no effect on vegetative growth (Groppe *et al.*, 1999). It appears that there is no uniform plant response and that effects of environmental factors on the interaction may depend on the genotype of both the endophyte and host involved (Meijer and Leuchtmann, 2000). Genetic factors of particular plant and endophyte genotype combinations appear to be more important for growth variation than environmental factors (Meijer and Leuchtmann, 2000).

Due to the great variation of symbiotic phenotypes caused by multiple factors, it was difficult to analyse the impact of the *thi1* mutation on the endophyte-grass interaction, particularly when the population of symbiotic associations analysed was small. For example, in experiment 1, although variations in symbiotic phenotypes (infection rate, host

survival rate and host stunting rate) were present between the *thi1* mutants and the wild-type, differences within groups were often greater than between groups, thus no meaningful conclusion could be drawn. The differences between the mutants and the wild-type observed could have resulted from either mutation of the *thi1* gene or random errors in the experiment. In the study of the effect of *Neotyphodium* infection on the native host, Faeth and Sullivan (2003) pointed out that host genotype is paramount in elucidating the interaction between host and endophyte, and investigating the endophyte-host interactions should be at the appropriate population scale or on the same host genetic background (Faeth and Sullivan, 2003). In an attempt to overcome the problem of genotype effects, a larger population of plants was tested (Section 3.4.2), and in this experiment significant differences were detected between the *thi1* mutant and wild-type strain in infection rates, host fresh weight and tiller weight (Section 3.4.2).

Variation in environmental factors also caused difficulties in analysing some symbiotic phenotypes. The host survival rates in experiment 3 were unexpectedly low and more infected grasses died in the period of observation than in earlier experiments (Sections 3.4.2.1, 3.4.2.2). These changes resulted in a much smaller population size for analysing phenotypes such as the host stunting rate. It was also difficult to analyse the effect of the *thi1* deletion on the frequency of stromata development on vegetative tillers as too few grasses developed stromata in experiment 3 (Section 3.4.2.4). A consideration of the effect of the host genotype and environmental factors on the symbiotic phenotype will be important in future studies on endophyte-grass interactions.

4.4. Stromata development on vegetative tillers

4.4.1. Stromata development on vegetative tillers

An unexpected observation in this study was the development of stromata on vegetative tillers of grasses infected with wild-type and mutant derivatives of E8. To our knowledge, this is the first report of an *Epichloë* endophyte forming stromata on vegetative tillers. Stromata development on vegetative tillers was similar to that observed on reproductive tillers. The hyphae were highly ramified (Sections 3.5.1.1 and 3.5.2.1). The cytoplasm of epiphytic hyphae were similar in stroma on reproductive and vegetative tillers (Section 3.5.2), suggesting a similar mechanism underlining the stromata development and nutrient conversion in these hyphae. However, slight differences were observed in that hyphae on vegetative tillers were more vacuolated and devoid of cellular contents than hyphae on reproductive tillers (Section 3.5), and the frequency of stroma development was lower (Sections 3.4.1.5 and 3.4.1.6). While it is widely accepted that stromata development of *Epichloë* endophytes is associated with development of reproductive tillers (White *et al.*, 1997), the observation here of stromata formation on vegetative tillers indicates that the factors that induce stromata development are not confined to reproductive tissues.

At present, little is known about the mechanisms underlying stromata development by *Epichloë* endophytes. *Epichloë* endophytes are characterised by growth in the intercellular space where nutrients are limiting (Lohaus *et al.*, 1995). Hyphae of endophytes are unevenly distributed with highest concentrations in regions (meristems, seeds and sheathes) which act as the 'sink' for nutrients (Section 1.2). The more limited growth of endophyte in the leaf blade possibly reflects that the endophyte growth is under nutrient limitation. However, formation of stromata by both E8 wild-type and *thi1* mutants on both vegetative

and reproductive tillers (Section 3.4) would indicate that vitamins such as thiamine are not limiting for initiation and development of stromata on perennial ryegrasses. Stromata development may be triggered by factor(s) which can induce an increased membrane permeability and nutrient efflux from the host cell to the hyphae (Aked and Hall, 1993).

Hyphae of many *Epichloë/Neotyphodium* endophytes are typically seldom branched in mature plant tissues although they show highly ramified growth in the basal meristem and reproductive tissues (Christensen *et al.*, 2002). This suggests that plant hormones, which play a key role in the tissues, may be important in triggering the stromata development. Supplementation of gibberellic acid, which is known to stimulate plant growth, has been reported to reduce stromata formation in *Dactylis glomerata* (Emecz and Jones, 1970). Conversely, other plant hormones such as cytokinins, which counteract the act of gibberellic acid might induce stromata development. Endophytes are able to produce phytohormone-like compounds (De Battista *et al.*, 1990), which could affect the growth of the host and/or the hyphae and stromata development.

Hyphal extension and branching in culture is regulated by pH, calcium concentration and a range of other factors (Shaw and Hoch, 2001; Trinci *et al.*, 1997). These factors, which are significantly influenced by the environment, may also be involved in stromata development. In symbiotic associations between rhizobia and legumes, localised changes of ion concentration are associated with the process of infection (Parniske, 2004).

An interesting observation in this study was that formation of stromata on vegetative leaf blades was often only midway along the blade with the hyphal growth above and below the stroma zone was still tightly regulated (Section 3.4.1.5). This observation suggests that factors that induce the stroma-formation are highly localised. While stromata development is always associated with profuse hyphal growth, this type of growth is not necessarily

sufficient for stromata development. For example, in some associations of perennial ryegrasses infected with an *E. festucae* FI1 mutant, hyphae were ramifying but no stroma developed (Mike Christensen and Aiko Tanaka, personal communications). This suggests that factors that induce stromata development must trigger both hyphal ramification and external growth.

As previously discussed (Section 4.3.1.2), the breakdown in the balance of host resistance and anti-resistance during the endophyte-grass interactions may cause death of the host or loss of endophyte in the host. Conversely, loss of host resistance may cause stroma development, which to some extent is similar to disease expression. Endophyte growth within the grass may be controlled by a mechanism similar to that of mycorrhizal symbiosis associations. Infection of mycorrhizal fungi can trigger a plant defense but this is modulated by the fungus. Further fungal colonisation appears to be controlled by the host plant (Jackson and Taylor, 1996; Parniske, 2004). Mutation or overexpression of some plant genes leads to increased fungal colonisation and greater arbuscule formation (Parniske, 2004; Solaiman *et al.*, 2000; Staehelin *et al.*, 2001). The activation of host responses toward endophytes (Christensen *et al.*, 2002; Naffa *et al.*, 1999; Roberts *et al.*, 1992), although these reactions are subtle and only observed in some interactions, indicates that the host grass is able to recognise the endophyte and trigger a host defense response that restricts endophyte growth (Section 4.3.1.2). For example, chitinase, which is enhanced in naturally infected grasses, might act to inhibit hyphal tip extension (Roberts *et al.*, 1992). The lack of an apparent host response indicates that endophytes are able to suppress or modulate the defense response as occurs in the mycorrhizal associations (Jackson and Taylor, 1996; Parniske, 2004) and many other plant pathogens (Abramovitch and Martin, 2004). The development of stromata, in which hyphae show unregulated growth, could be a result of suppression of the host defense response. In many cases, defense suppression is necessary for disease formation (Abramovitch and Martin, 2004).

Stromata development on both vegetative and reproductive tillers is strongly influenced by the host genotype and environmental factors (Section 3.4.1.5). Studies by Kirby (Kirby, 1961) indicated that environment conditions control the elongation rate of the flowering apex and thereby affect stroma formation. Rapid elongation of the flowering apex suppresses stroma formation (Kirby, 1961). High levels of nitrogen fertiliser diminished choke expression in infected *Festuca rubra* (Funk *et al.*, 1994). Nitrogen fertiliser might increase the elongation of the flowering apex and thereby suppress stroma development

While the host plant plays an important role in controlling endophyte growth, stromata formation is influenced by an interplay of factors from both the host and endophyte, and physiological changes that occur in response to variations in environment.

4.4.2. Nutrient translocation and conversion in hyphae in the stroma region

Stromata formation is characterised by extensive hyphal growth within and outside the plant tissues and the production of abundant conidia. Endophytes are biotrophic fungi which are dependent on the host for nutrients. To initiate and maintain rapid hyphal growth in stroma-forming regions, the endophyte must obtain sufficient nutrients from the host tissues and rapidly transfer these nutrients to the epiphytic hyphae. However, under normal conditions, the apoplasitic fluid is low in nutrients (Lohaus *et al.*, 1995). An intriguing question is how these fast-growing hyphae obtain their nutrients.

Many biotrophic fungi produce haustoria to extract nutrients from host plants, but endophytes do not develop this invasive structure, nor break the host cell for nutrient uptake (White *et al.*, 1997). However, alterations to the host parenchyma and epidermal

layers in the stroma-forming region were reported (White *et al.*, 1997). Changes in the shape of host epidermal cells, which indicates a softening of the cell wall, were observed in the stromata region but not in the non-stromata region (Section 3.5.2). These structural changes may lead to leakage of nutrients from host cells to the apoplast fluid, thereby allowing the endophyte to derive sufficient nutrients from the intercellular space of the stromata region without intra-cellular invasion. Specific signals or factors that can alter the host cell wall and membrane permeability might be produced in the stromata region thus facilitating the flow of nutrients into the apoplastic space and/or directly to the hyphal cell (Bacon and White, 2000). Stroma development is also characterised by intensive vascular bundle colonisation (White *et al.*, 1997). This is a feature of stromata formed on both vegetative and reproductive tillers by *E. typhina*. The association of hyphae with vascular tissues is likely to increase the availability of nutrients and water from these tissues. Changes in nutrient uptake mechanisms (Lam *et al.*, 1994; Reddy *et al.*, 1996) could also account for greater nutrient availability.

Another key question is how nutrients are transported to the epiphytic hyphae formed on the outside of the plant? A study of hyphal growth in the stroma region (White *et al.*, 1997) indicated that hyphae often penetrate through the epidermis and cuticle, which may ensure that nutrients pass from within the plant tissue to hyphae on the outside (White *et al.*, 1997). Hyphae penetrating through the epidermis are often observed in the stroma-forming region of leaves in both vegetative and reproductive tillers in this study. The transportation of nutrients from the inner region to the outer epiphytic hyphae is proposed by White to be facilitated by bulk water flow associated with evaporation (White *et al.*, 1997). Water evaporation from the surfaces of stromata was on average ten times greater than that from surfaces of uninfected leaf tissue (White and Camp, 1995). Nutrients are expected to flow to the epiphytic fungal fruiting structures with the water. In addition, Bacon suggested that an active transport of nutrients may be involved in stromata development (Bacon and White, 2000).

In this study, cytologically distinct changes were commonly observed with stroma development. The first was a high level of vacuolation of hyphae in the stromata region (Section 3.5). Hyphal cells close to the epidermis in the stroma-forming region were highly vacuolated and devoid of cytoplasm (Section 3.5), while endophytic hyphae close to vascular bundles and epiphytic hyphae distal to the epidermis were not. Vacuoles are thought to play an important role in the fungal life cycle (Weber, 2002). For example, vacuoles are the site for organelle degradation, resulting in recycling of macromolecules under conditions of nutrient starvation (Weber, 2002). Vacuoles can also transport nutrients over long distances through a network of fine channels that link adjacent vacuoles (Weber, 2002).

The second important feature is the change of levels of glycogen-like substance and lipid droplets in the cells (Section 3.5). These carbon storage compounds were abundant in hyphae in the non-stroma region, but decreased in hyphae in the stroma region (Section 3.5). In the stroma region, glycogen-like substance and lipid droplets were frequently observed in hyphae close to the vascular bundle, but progressively decreased in hyphae toward the epidermis (Section 3.5). Few glycogen-like substance and lipid droplets were observed in epiphytic hyphae close to the epidermis, but the amount increased in outer epiphytic hyphae. These changes in the concentration of carbon storage compounds reflect conversions and/or translocation of carbon sources. This process will require a number of polysaccharide and lipid metabolising enzymes. Few studies have been carried out on nutrient translocation and associated physiological changes in grasses infected with *Clavicipitaceous* fungi (Bacon and White, 2000). Studies of nutrient movement in *Myriogenospora atramentosa* Diehl following the incorporation of $^{14}CO_2$ and translocation of ^{14}C-sucrose in the plants revealed that there is a translocation and a conversion of sugars produced during photosynthesis into fungal carbohydrates (Smith *et al.*, 1985).

The *thi1* mutants in the stromata region differ from the wild-type in that the hyphae were more highly vacuolated, were largely devoid of cellular contents and lacked glycogen-like deposits (Section 3.5). These changes presumably reflect an inadequate supply of thiamine for nutrient metabolism. Pronounced vacuolisation was also observed in hyphae of the biotrophic pathogen-rust fungus *U. fabae* when growing in the plant intercellular space but not in haustoria. Interestingly, thiamine biosynthesis genes are highly up-regulated in the latter (Sohn *et al.*, 2000).

The production of stromata in vegetative tillers is very useful for identifying genes that play important roles in stromata production and nutrient conversion by technologies of subtractive screening. Genes that are involved should be induced in the stromata region in both vegetative and reproductive tillers but absent in non-stroma region.

4.5. Conclusion and future work

In this study, a thiamine biosynthesis gene *thi1*, the orthologue of *S. cerevisiae THI4*, was isolated and characterised from *N. lolii* strain Lp19 and *E. typhina* E8. Functional analysis employing the gene-targeting disruption approach confirmed that this gene is indeed involved in thiamine biosynthesis. Expression of the *thi1* gene was detected *in planta*. In culture, nitrogen and carbon nutrient sources were shown to affect the pattern of alternative splicing of the *thi1* transcript, which may alter the expression of the THI1 polypeptide. Conserved regions involved in the regulation of thiamine biosynthetic gene expression in both bacteria and fungi were identified in the endophyte *thi1*. How expression of the endophyte *thi1* gene is regulated by thiamine and/or carbon and nitrogen nutrients remains to be determined. Future studies will be required to determine whether the *thi1* gene is subject to a riboswitch type of mechanism of repression and whether *thi1* is differentially expressed in different tissues and at different developmental stages of the plant.

Deletion of the *thi1* gene resulted in some differences in the symbiotic phenotype, but did not disrupt the ability of *E. typhina* to infect the perennial ryegrass host and form stromata, suggesting that the mutants must have the ability for uptake and/or salvage of sufficient thiamine or thiamine precursors to grow well. Further investigation of the role that other thiamine biosynthetic genes, particularly genes such as *THI6*, *THI80* which are essential for late steps in thiamine biosynthesis, and/or the thiamine transporter gene (*THI10*), play in the endophyte-grass interaction will help us understand whether thiamine is derived *de novo*, by salvage or by uptake.

While it is widely accepted that stromata of *Epichloë* endophytes are confined to host reproductive tillers, this study showed that *E. typhina* develop stromata on vegetative tillers of plants. The stromatal hyphae on vegetative and reproductive tillers shared similar characteristics in hyphal morphology and ultrastructure, suggesting a similar mechanism for induction of stromata, and for nutrient translocation and conversion. Future studies on the biochemistry and physiology of nutrient uptake, and in particular the role of salvage pathways in endophyte growth *in planta* should increase our understanding of the metabolic exchange that occurs in these important associations. A comparative genome analysis, when the *E. festucae* genome sequence is available, would also be very useful in understanding the host metabolite dependence of the endophyte in the symbiotic association.

Appendix

Appendix 1. Abbreviations

ThP: Thiamine monophosphate

ThPP: Thiamine pyrophosphate

HET-P: Thiazole monophosphate

HMP-PP: 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate

DIG: Digoxigenin

GUS: β-D-glucuronidase

EDTA: Ethylenediamine tetraacetic acid

HPLC: High performance lipid chromatography

IAA: Indole acetic acid

SDS: Sodium dodecyl sulfate

PEG: Polyethylene glycol

BSA: Bovine serum albumin

PVP-10: Polyvinylpyrrolidone

CTAB: Hexadecyltrimethylammonium bromide

DTT: 1,4-dithiothreitol

CTAB: Hexadecyltrimethylammonium bromide

TCA cycle: Tricarboxylic acid cycle

uORF: Upstream open reading frame

5' UTR: 5' un-translated region

PCR: Polymerase chain reaction

TAIL-PCR: Thermal Asymmetric Interlaced PCR

RT-PCR: Reverse PCR

DD-RT-PCR: Differential display-RT-PCR

RACE: Rapid amplification of cDNA ends

Sec: Second(s)

h: Hour(s)

Appendix 2. Growth response of endophytes to thiamine

Appendix 2.1. Growth response of endophyte strains to thiamine (Experiment 1)

Endophytes	Characteristic	PDA	CD	CD+Thiamine (3 mg/L)
Neotyphodium lolii				
AR1	NS[a]	15.0 ± 0.00 [b]	2.5 ± 0.71[b]	10.0 ± 0.00[b]
Lp5	NS	40.0 ± 0.00	2.0 ± 0.00	12.0 ± 0.00
Lp7	NS	18.0 ± 0.00	10.0 ± 0.00	11.0 ± 0.00
Lp9	NS	8.0 ± 0.00	2.5 ± 0.71	4.0 ± 0.00
Lp13	NS	24.0 ± 0.00	3.5 ± 0.00	6.0 ± 0.00
Lp14	NS	8.5 ± 0.71	2.0 ± 0.35	3.5 ± 0.71
Lp19	NS	40.0 ± 0.00	2.0 ± 0.71	16.0 ± 0.00
Neotyphodium spp.				
Lp1 (LpTG-2)	NS	21.0 ± 1.41	16.0 ± 0.00	17.0 ± 1.41
Lp2 (LpTG-2)	NS	20.0 ± 0.00	17.0 ± 0.71	17.0 ± 0.00
Tf15 (FaTG-2)	NS	32.0 ± 0.00	3.0 ± 0.00	20.0 ± 0.00
Tf18 (FaTG-3)	NS	9.0 ± 0.71	5.5 ± 0.71	5.6 ± 0.71
Epichloë festucae				
Fl1	S	80.0 ± 0.00	2.3 ± 0.71	71.0 ± 1.41
Fg1	S	49.0 ± 4.24	40.0 ± 0.00	45.0 ± 4.24
Fr1	S	50.0 ± 0.00	43.0 ± 1.41	43.0 ± 1.41
Frc5	S	41.0 ± 1.41	8.0 ± 0.00	9.0 ± 1.41
Frr1	S	54.0 ± 5.66	45 ± 12.73	52.0 ± 2.82
Epichloë typhina				
E8	S	80.0 ±0.00	70.0 ± 0.00	70.0 ± 0.00

[a]NS indicates non-stroma forming endophytes and S indicates stroma forming endophytes

[b]Average colony diameter (± standard deviation) from duplicate colonies after growth at 22°C for 4 weeks.

Endophytes of which the growth is responsive to supplementation of thiamine are underlined.

Appendix 2.2. Growth response of endophyte strains to thiamine (Experiment 2)

Endophytes	Characteristic	PDA	CD	CD+Thiamine(3 mg/L)
E. bromicola				
Bb8918	NS[a]	58.0 ± 1.00	62.7 ± 0.58	60.7 ± 1.15
Bb8920	NS	54.0 ± 1.00	33.0 ± 0.00	35.0 ± 1.00
Bb9304	NS	59.7 ± 0.58	53.7 ± 0.58	54.0 ± 0.00
Br9035	NS	36.3 ± 0.58	30.7 ± 0.58	30.0 ± 0.00
Br9305	NS	35.3 ± 0.58	42.3 ± 0.58	40.3 ± 1.15
Br9708	NS	31.3 ± 0.58	24.7 ± 0.58	28.3 ± 4.16
Be9121	S	62.0 ± 3.46	57.7 ± 3.06	55.7 ± 8.74
Be9608	S	77.3 ± 17.9	77.3 ± 1.15	80.0 ± 3.00
Be9630	S	64.0 ± 1.00	83.0 ± 1.41	78.0 ± 2.00
E. festucae				
Fr9429	S	41.7 ± 1.15	34.0 ± 2.00	56.0 ± 0.00
Fr9431	S	71.0 ± 2.65	61.3 ± 2.31	70.0 ± 1.73
Fr9715	S	46.7 ± 1.15	42.0 ± 5.29	46.7 ± 1.53
Fr9721	S	60.7 ± 1.15	65.0 ± 2.65	64.0 ± 1.00
Fv9248	NS	75.0 ± 1.00	85.0 ± 0.00	85.0 ± 0.00
Fo9201	NS	77.7 ± 1.53	67.0 ± 3.61	79.3 ± 1.15
Fp9133	NS	37.7 ± 0.58	47.0 ± 1.41	45.7 ± 0.58
Fg9718	NS	64.7 ± 0.58	12.7 ± 0.58	69.7 ± 3.79
E. sylvatica				
Bs9816	S	46.3 ± 1.15	41.3 ± 3.21	48.7 ± 6.11
Bs9806	S	33.0 ± 1.00	41.7 ± 0.58	40.7 ±7 .51
Bs9703	S	37.7 ± 0.58	38.0 ± 0.00	21.7 ± 2.52
Bs9344	S	54.7 ± 0.58	63.3 ± 2.08	62.7 ± 2.08
Bs8928/2	S	44.7 ± 1.15	24.7 ± 2.08	25.7 ± 0.58
Bs9814	NS	57.3 ± 0.58	38.7 ± 5.51	68.0 ± 2.00
Bs9813	NS	41.0 ± 1.73	6.33 ± 1.53	32.7 ± 4.04
Bs9810	NS	42.7 ± 0.58	50.3 ± 0.58	49.7 ± 0.58
Bs9812	NS	60.3 ±0.58	36.3 ± 0.58	8.0 ± 2.65

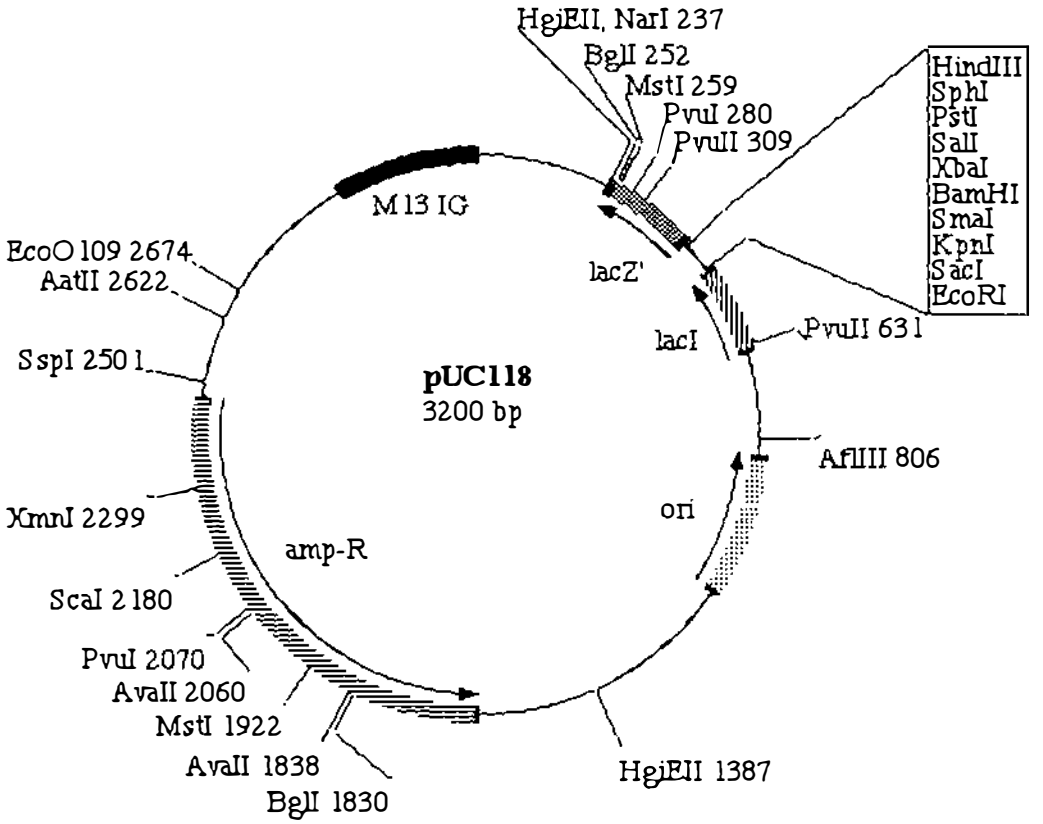
[a]NS indicates non-stroma forming endophytes and S indicate stroma forming endophytes

[b]Average colony diameter (± standard error) from duplicate colonies after growth at 22°C for 5 weeks.

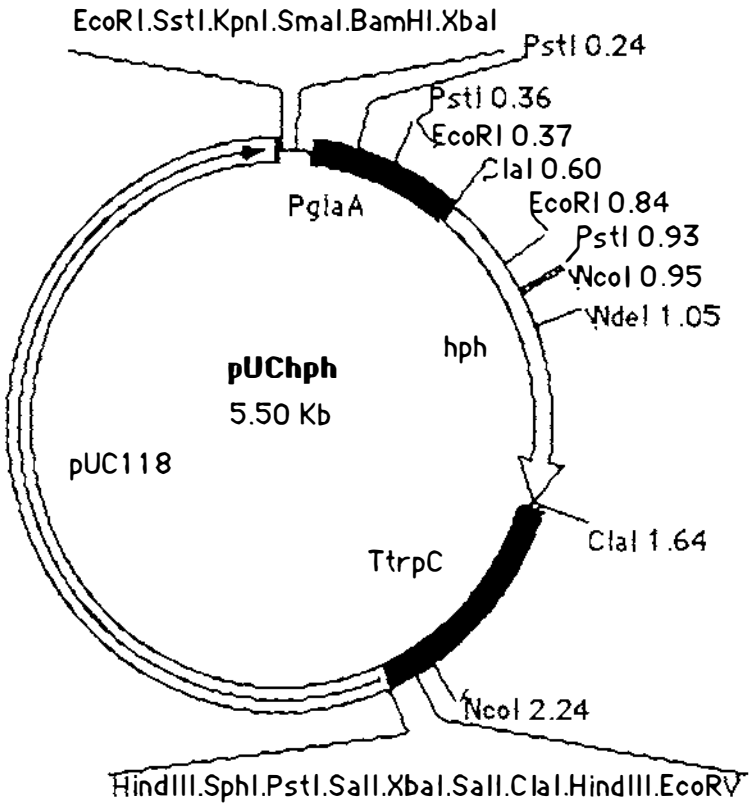
Endophytes of which the growth is responsive to supplementation of thiamine are underlined. Two endophytes, the growth of which is impeded by thiamine are shaded.

Appendix 3. Vector maps

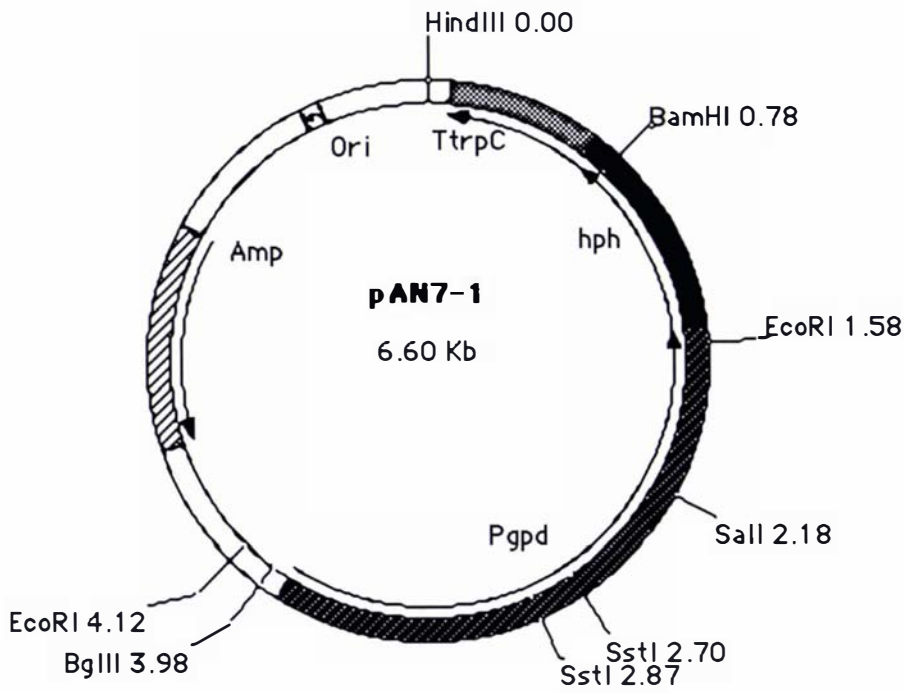
Appendix 3.1. pUC118



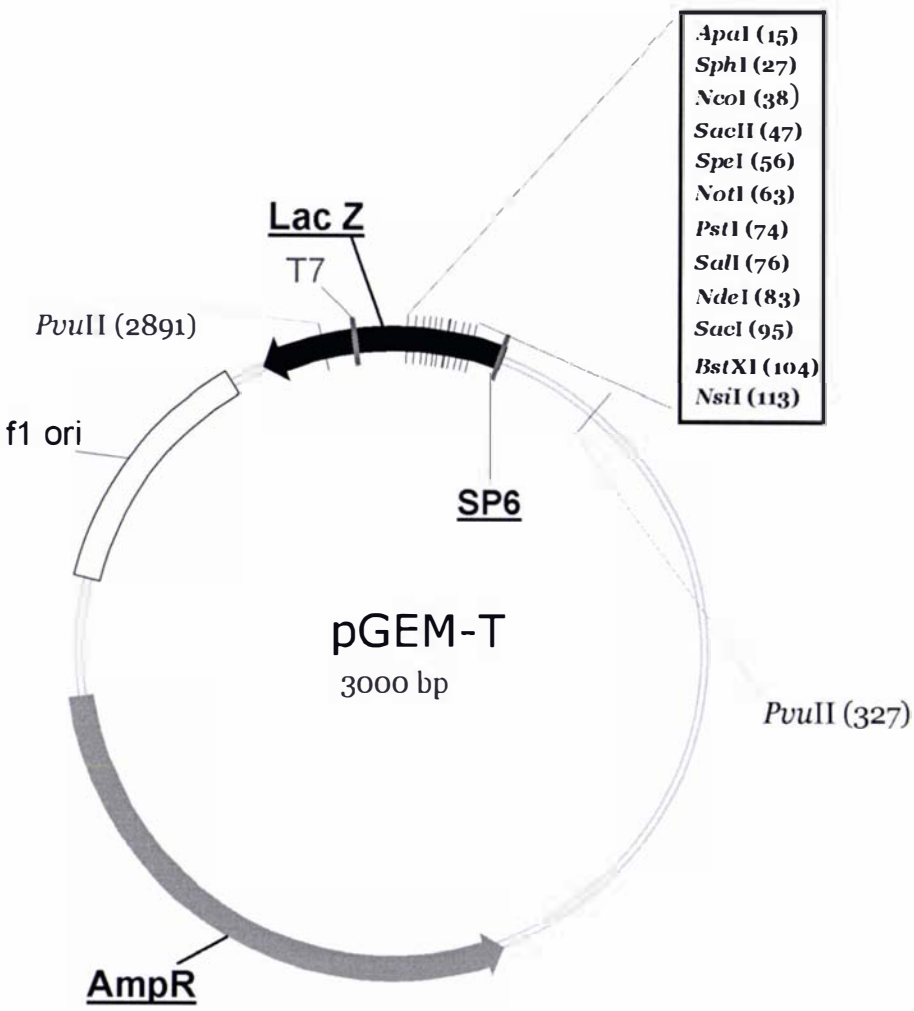
Appendix 3.2. pUChph



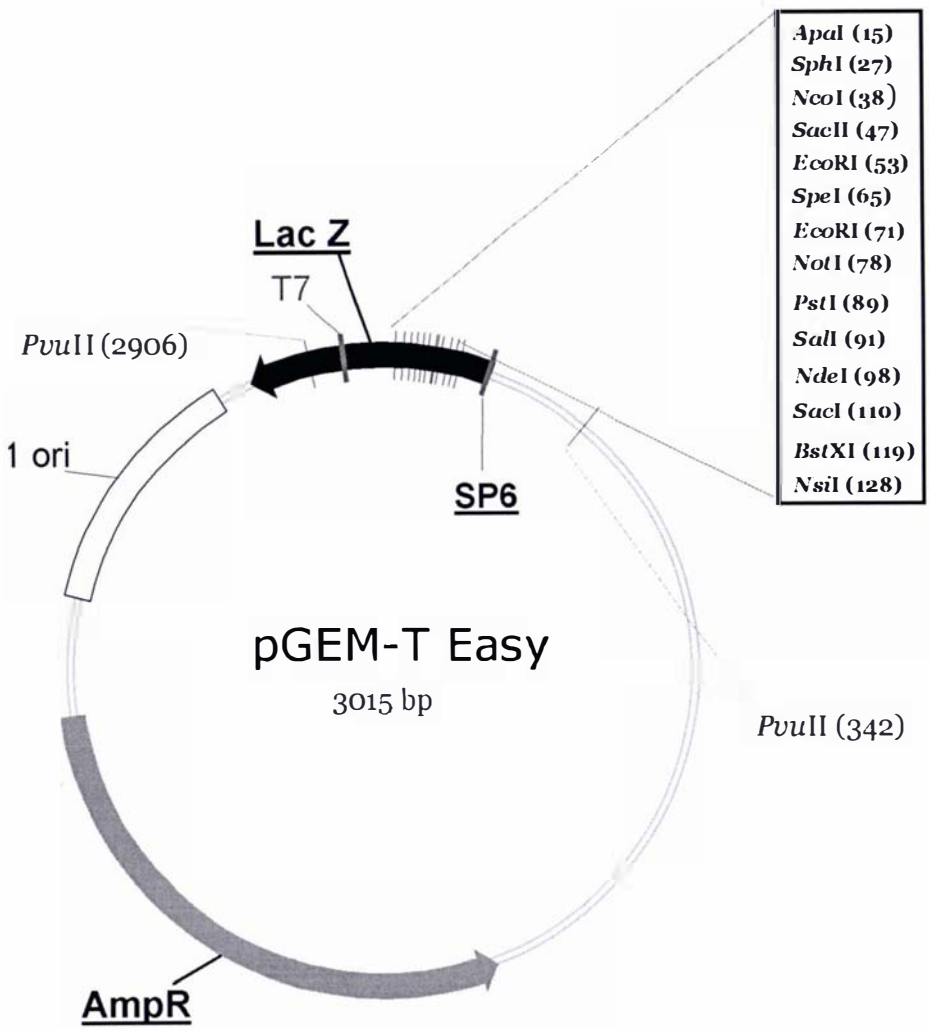
Appendix 3.3. pAN7-1



Appendix 3. 4. pGEM-T



Appendix 3.5. pGEM-Teasy



Appendix 4. Media, common solutions and buffers

Appendix 4.1. Media

All media were prepared with sterilised water and autoclaved at 121°C for 15 min unless stated otherwise. Solid media were prepared by adding 15 x g of agar (Life Technologies) per litre of liquid media before autoclaving.

CD (Czapek Dox; Oxoid) **medium** contains 0.2% (w/v) sodium nitrate; 0.05% (w/v) potassium chloride; 0.05% (w/v) magnesium glycerophosphate; 0.001% (w/v) ferrous sulphate; 0.035% (w/v) potassium sulphate and 3% (w/v) sucrose.

CD salt medium contained 0.1 % (w/v) K_2HPO_4, 0.05% (w/v) $MgSO_4$; 0.05% (w/v) KCl; 0.001% (w/v) Fe_2SO_4. The medium was supplemented with sucrose (30 g/L), sodium nitrate (2 g/L), sodium glutamate (4.4 g/L), mannitol (30 g/L) and thiamine (1 mg/L), singly or in cmbination. The pH was adjusted to 6.8 before autoclaving.

CM medium (Complete Medium) (Oliver *et al.*, 1987) contained 3.34% (w/v) Czapek Dox (CD, Oxoid); 0.1% (w/v) yeast extract; 1.2% (w/v) potato dextrose broth; 0.1% (w/v) tryptone; 0.1% (w/v) casamino acids (Difco); 0.4 M sucrose. The pH was adjusted to 6.5 with NaOH.

CM top agar contained CM medium with 0.8% agar.

LB medium (Miller, 1972) contained 0.5% (w/v) NaCl; 1% (w/v) tryptone (Difco); 0.5% (w/v) yeast extract (Difco). The pH was adjusted to 7-7.5.

LB top agarose contained LB with 1.5% agarose 15 (BDH). Before use, the LB agarose was supplemented with $MgSO_4.7H_2O$ to a final concentration of 10 mM.

PD (Potato Dextrose) **medium** contained 2.4% (w/v) dehydrated potato dextrose broth (Difco). The pH was adjusted to 6.5.

RG medium (Regeneration medium) contained 2.4% (w/v) dehydrated potato dextrose broth and 27.38% (w/v) sucrose. The pH was adjusted to 6.5.

SOC medium (Dower *et al.*, 1988) contained 0.36% (w/v) glucose; 0.02% (w/v) KCl; 0.095% (w/v) $MgCl_2$; 0.25% (w/v) $MgSO_4.7H_2O$; 0.06% (w/v) NaCl; 2% (w/v) tryptone (Difco) and 0.5% (w/v) yeast extract (Difco).

MSO medium (Murashige and Skoog complete medium) contained (per litre) NH_4NO_3 1.65 g, H_3BO_3 6.2 mg, $CaCl_2$ 332.2 mg, $CoCl_2$ 0.025 mg, $CuSO_4.6H_2O$ 0.025 mg, Na_2EDTA 37.26 mg, $FeSO_4.7H_2O$ 27.80 mg, $MgSO_4$ 180.70 mg, $MnSO_4.H_2O$ 16.90 mg, $Na_2MoO_4.2H_2O$ 0.25 mg, KI 0.83 mg, KNO_3 1.9 g, KH_2PO_4 170 mg $ZnSO_4.H_2O$ 8.6 mg, myo-inositol 100 mg, nicotine acid 0.50 mg, pyridoxine-HCl 0.50, thiamine-HCl 0.10 mg, glycine 2 mg, MES 500 mg and sucrose 30 g.

Appendix 4.2. Antibiotics and other supplement stocks

Antibiotics	Stock concentration	Final concentrations
Ampicillin	100 mg/ml	100 μg/ml
Hygromycin B	50 mg/ml	150 μg/ml, 100 μg/ml
Tetracycline	10 mg/ml	10 μg/ml, 15 μg/ml

Stocks	Concentration	pH
Ethidium bromide	10 mg/ml	
Glycerol	10%, 30%, 50%	
Maltose	20% (w/v)	
$MgSO_4.7H_2O$	1 M	
Na_2EDTA	250 mM	8.0
NaCl	5 M	
IPTG	24 mg/ml	
SDS (Sodium dodecyl sulfate)	10% (w/v)	
Sodium acetate	3 M	7.0, 4.5
Thiamine	1 mg/L, 10mg/ml	
Thiazole	10 mg/ml	
Tris-HCl	1 M	8.0, 7.6
Triton X-100	0.01% (v/v)	
X-gal	20 mg/ml	

Appendix 4.3. Buffers and solutions for DNA isolation and detection

Lysozyme was prepared at 10 mg/ml in 10 mM Tris-HCl (pH 8.0) buffer.

1 x TNE buffer contained 10 mM Tris-HCl (pH 7.4); 1 mM Na_2EDTA and 100 mM NaCl.

RNaseA (DNase free) (Sigma) was prepared at 10 mg/ml in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl. The solution was heated to 100°C for 15 min to inactivate DNase, cooled to room temperature and stored at -20 °C.

SM buffer contained 100 mM NaCl; 8 mM $MgSO_4.7H_2O$; 50 mM Tris-HCl (pH 7.5) and 0.01% (w/v) gelatin.

TE (10/1) buffer contained 10 mM Tris-HCl and 1 mM Na_2EDTA, pH 8.0.

TE (10/0.1) buffer contained 10 mM Tris-HCl and 0.1 mM Na_2EDTA, pH 8.0.

Tris-equilibrated phenol (Amersham) was added with 8-Hydroxyquinoline to a final concentration of 0.1% (w/v) and stored at 4 °C.

1 x TBE electrophoresis buffer contained 89 mM Tris; 2 mM Na_2EDTA and 89 mM boric acid. The pH was adjusted to 8.2.

1 x TAE electrophoresis buffer contained 20 mM Tris; 10 mM glacial acetic acid and 1 mM Na_2EDTA. The pH was adjusted to 8.2.

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).

Appendix 4.4. Buffers and solutions for endophyte protoplast preparation and transformation

OM buffer contained 1.2 M of $MgSO_4.7H_2O$; 10 mM Na_2HPO_4 and 100 mM $NaH_2PO_4.2H_2O$ added until pH is 5.8.

ST buffer contained 0.6 M sorbitol and 100 mM Tris-HCl pH 8.0.

STC buffer contained 1 M sorbitol; 50 mM Tris-HCl (pH 8.0) and 50 mM $CaCl_2$.

Cell wall lysis enzyme solution contained Glucanex (NZ chemcolour Industries) in OM buffer in a final concentration of 10 mg/ml. The solution was sterilised by Millipore filtration.

PEG solution contained PEG 4000 solved in STC buffer to a final concentration of 40% (w/v).

Spermidine solution was prepared at 50 mM in water and stored at 4 °C.

Heparin (Sigma) solution contained 5 mg/ml heparin in STC buffer.

Appendix 4.5. Buffers and solutions for Southern blotting, hybridisation and detection

Solution 1 (Southern, 1975) contained 0.25 M HCl.

Solution 2 contained 0.5 M NaOH and 0.5 M NaCl.

Solution 3 contained 0.5 M Tris-HCl (pH 7.4) and 2 M NaCl.

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

2 x SSC contained 0.3 M NaCl and 0.03 M sodium citrate.

TES (10/1/100) contained 10 mM Tris-HCl, 1 mM Na_2EDTA and 100 mM NaCl, pH 8.0.

10 x Denhardts (Southern, 1975) contained (per litre): 50 ml 1 M HEPES (Sigma), pH 7.0; 150 ml 20 x SSC; 6 ml phenol extracted herring sperm DNA (3 mg/ml, Sigma); 5 ml of 20%

(w/v) SDS; 2 g Ficoll (Sigma); 2 ml *E. coli* transfer RNA (10 mg/ml Sigma); 2 g bovine serum albumin (Sigma); 2 g polyvinylpyrrolidone (PVP-10, Sigma).

DIG standard hybridisation buffer contained 5 x SSC; 50% (v/v) formamide; 0.1% (w/v) sodium-lauroylsarcosine; 0.02% (w/v) SDS; 2% (w/v) blocking reagent (Roche).

Maleic acid buffer contained 100 mM maleic acid and 150 mM NaCl. The pH was adjusted to 7.5.

DIG washing buffer contained 100 mM maleic acid; 150 mM NaCl. The pH was adjusted to 7.5 and then Tween 20 was added to a final concentration of 0.3% (v/v).

Blocking solution contained 1% blocking reagent (Roche) in maleic acid buffer and heated to 60°C with stirring.

Detection buffer contained 100 mM Tris-HCl and100 mM NaCl. The pH was adjusted to 9.5.

Appendix 4.6. Buffers and solutions for RNA working

DNaseI buffer (Bradshaw and Pillar, 1992) contained 100 mM sodium acetate and 5 mM $MgSO_4$. The pH was adjusted to 5.0.

DNase (RNase free) contained deoxyribonuclease I at a concentration of 2 mg/ml in 0.15 M NaCl and 50% (v/v) glycerol.

RNA loading buffer contained 50% glycerol; 1 mM EDTA pH 8.0; 0.25% bromophenol blue; and 0.25% xylene cyanol FF.

1 x MOPs buffer contained 20 mM MOPs (3-[N-morpholino] propane-sulfonic acid); 3 mM sodium acetate and 1 mM EDTA. The pH was adjusted to 7.0.

DEPC treated H_2O was prepared by adding DEPC to H_2O at a final concentration of 0.01%, and the solution was stored at 37°C overnight before autoclaving twice.

Random hexamer primer (Roche) stock was prepared by diluting to a 0.09 OD units/µl.

Appendix 4.7. Buffers and solutions for artificial infection, isolation or microscopic analysis of endophytes *in planta*

Aniline blue stain was prepared with 0.05% (w/v) aniline blue (Michrone) dissolved in lactic acid, glycerol and water (1:2:1 v/v).

Chlorine bleach solution was a 1:10 dilution of commercially available sodium hypochlorite solution (Sophora Products Ltd.) in water.

Lacto-glycerol solution contained 20% lactic acid (BDH) and 50% (v/v) glycerol (BDH).

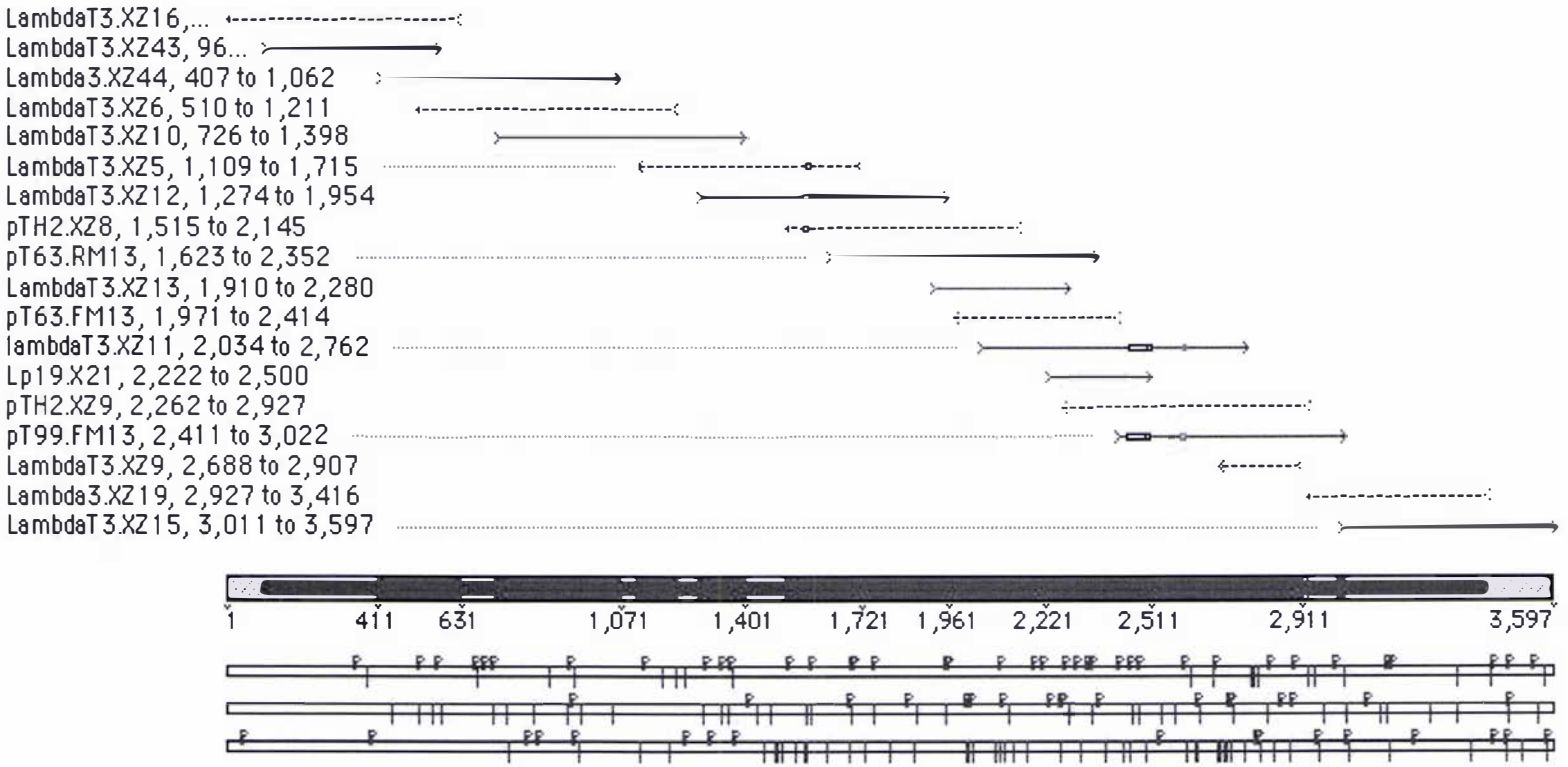
BS solution contained 0.242% (w/v) Tris(hydroxymethyl)methylamine, 0.292% (w/v) NaCl, 0.5 (w/v) non-fat milk powder and 10 mM HCl, pH 7.5.

Tris(hydroxymethyl)methylamine buffer was prepared by dissolving Tris(hydroxymethyl)methylamine in water to a final concentration of 0.242% (w/v), The pH was adjusted to 8.2.

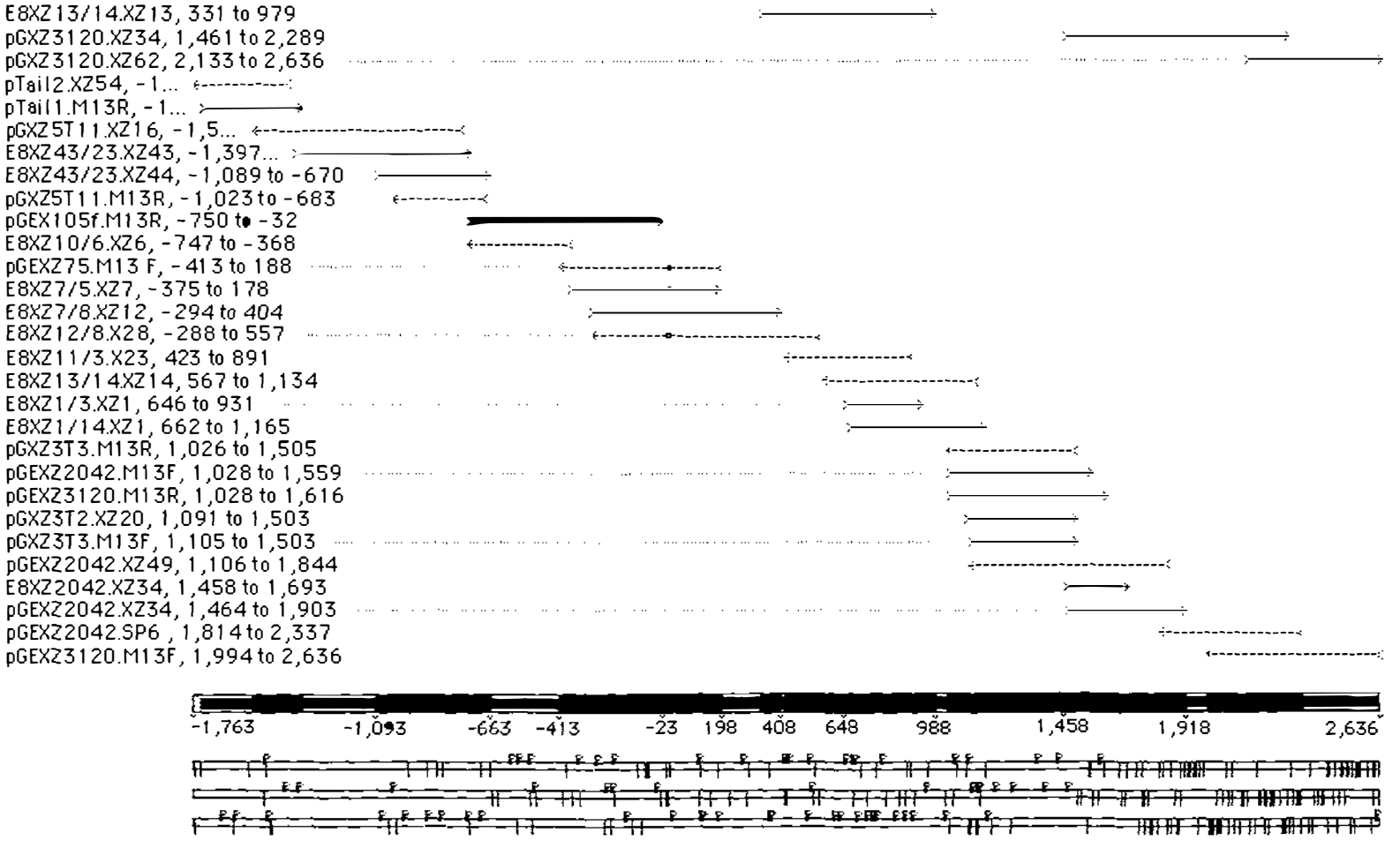
Fast Red solution was prepared by dissolving Fast Red in Tris(hydroxymethyl)methylamine buffer at a final concentration of 0.6% and Naphthol AS-MX phosphate in Tris(hydroxymethyl)methylamine buffer at a final concentration of 0.1%, then combining an equal volume of the two solutions.

Appendix 5. Sequence data

Appendix 5.1. Lp19 *thi1* contig



Appendix 5.2. E8 *thi1* contig



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Appendix 6. List of raw sequence data, plant data and statistical analysis on CD

Appendix 6.1. Sequences

SequenceLp19thi1

SequenceE8thi1

Lp19thi15RC

Lp19thi3RC

E8thi15RC

E8thi13RC

Lp19thi1RTPCR

PlantRTPCR

MicrosatellitePCR

Appendix 6.2. Plant data and statistical analysis results

(Date A: 6 weeks post inoculation; B: 9 weeks post inoculation; C: 12 weeks post inoculation; D: 20 weeks post inoculation; F: 28 weeks post inoculation;

FW: fresh weight; TN: tiller number: TW: tiller weight)

Experiment 1&2

Experiment3-FW

Experiment3-TW

Experiment3-TN

Experiment3-infectivity

SAS-code-for Tab3.13.doc

SAS-output-for-Tab 3.13.lst

Sta-for-Tab3.17

Sta-for-Tab3.23

Sta-for-Tab3.24

References

Abramovitch, R.B., and Martin, G.B. (2004) Strategies used by bacterial pathogens to suppress plant defenses. *Current Opinion in Plant Biology* **7**: 356-364.

Agrawal, N., Dasaradhi, P.V., Mohmmed, A., Malhotra, P., Bhatnagar, R.K., and Mukherjee, S.K. (2003) RNA interference: biology, mechanism, and applications. *Microbiology & Molecular Biology Reviews* **67**: 657-685.

Aked, J., and Hall, J.L. (1993) Effect of powdery mildew infection on concentrations of apoplastic sugars in pea leaves. *New Phytologist* **123**: 283-288.

Al-Samarrai, T.H., and Schmid, J. (2000) A simple method for extraction of fungal genomic DNA. *Letters in Applied Microbiology* **30**: 53-56.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Arachevaleta, M., Bacon, C.W., Hoveland, C.S., and Radcliffe, D.E. (1989) Effect of the tall fescue endophyte on plant response to environmental stress. *Agronomy Journal* **81**: 83-90.

Arnaise, S., Zickler, D., Poisier, C., and Debuchy, R. (2001) *pah1*: a homeobox gene involved in hyphal morphology and microconidiogenesis in the filamentous ascomycete *Podospora anserina*. *Molecular Microbiology* **39**: 54-64.

Ayliffe, M.A., Roberts, J.K., Mitchell, H.J., Zhang, R., Lawrence, G.J., Ellis, J.G., and Pryor, T.J. (2002) A plant gene up-regulated at rust infection sites. *Plant Physiology* **129**: 169-180.

Bacon, C.W., Porter, J.K., Robbins, J.D., and Luttrell, E.S. (1977) *Epichloë typhina* from toxic tall fescue grasses. *Applied and Environmental Microbiology* **34**: 576-581.

Bacon, C.W., Lyons, P.C., Porter, J.K., and Robbins, J.D. (1986) Ergot toxicity from endophyte-infected grasses: A review. *Agronomy Journal* **78**: 106-116.

Bacon, C.W., Battista, J.d., and De, B.J. (1991) Endophytic fungi of grasses. *Handbook of Applied Mycology* **1**: 231-256.

Bacon, C.W., and White, J.F., Jr. (2000) Physiological adaptations in the evolution of endophytism in the Clavicipitaceae. In *Microbial Endophytes*. Bacon, C.W. and White, J.F., Jr. (eds). New York: Marcel Dekker Inc, pp. 237-261.

Bacon, C.W., and White, J.F., Jr. (2003) Evidence for nematode defense in symbiotic grasses. In *Clavicipitalean fungi: Evolution biology, chemistry, biocontrol, and cultural impacts*. White, J.F., Jr., Bacon, C.W., Hywel-Jones, N.L. and Spatafora, J.W. (eds). New York: Marcel Dekker, Inc., pp. 549-569.

Bartsev, A.V., Deakin, W.J., Boukli, N.M., McAlvin, C.B., Stacey, G., Malnoe, P., Broughton, W.J., and Staehelin, C. (2004) *NopL*, an effector protein of *Rhizobium sp*. NGR234, thwarts activation of plant defense reactions. *Plant Physiology* **134**: 871-879.

Belanger, F.C., Leustek, T., Chu, B., and Kriz, A.L. (1995) Evidence for the thiamine biosynthetic pathway in higher-plant plastids and its developmental regulation. *Plant Molecular Biology* **29**: 809-821.

Belesky, D.P., Devine, O.J., Pallas, J.E., Jr., and Stringer, W.C. (1987) Photosynthetic activity of tall fescue as influenced by a fungal endophyte. *Photosynthetica* **21**: 82-87.

Bell, D.W., Wahrer, D.C., Kang, D.H., MacMahon, M.S., FitzGerald, M.G., Ishioka, C., Isselbacher, K.J., Krainer, M., and Haber, D.A. (1999) Common nonsense mutations in RAD52. *Cancer Research* **59**: 3883-3888.

Blankenship, J.D., Spiering, M.J., Wilkinson, H.H., Fannin, F.F., Bush, L.P., and Schardl, C.L. (2001) Production of loline alkaloids by the grass endophyte, *Neotyphodium uncinatum*, in defined media. *Phytochemistry* **58**: 395-401.

Bradshaw, R.E., and Pillar, T.M. (1992) Isolation and Northern blotting of RNA from *Aspergillus nidulans*. *Journal of Microbiological Methods* **15**: 1-5.

Brasier, C. (2000) The rise of the hybrid fungi. *Nature* **405**: 134-135.

Bullock, W.O., Fernandez, J.M., and Short, J.M. (1987) XL1-Blue: a high efficiency plasmid transforming *rec*A *Escherichia coli* strain with beta-galactosidase selection. *Biotechniques* **5**: 376-378.

Bush, L.P., Fannin, F.F., Siegel, M.R., Dahlman, D.L., and Burton, H.R. (1993) Chemistry, occurrence and biological effects of saturated pyrrolizidine alkaloids associated with endophyte-grass interactions. *Agriculture Ecosystems & Environment* **44**: 1-4.

Bush, L.P., Wilkinson, H.H., and Schardl, C.L. (1997) Bioprotective alkaloids of grass-fungal endophyte symbioses. *Plant Physiology* **114**: 1-7.

Byrd, A.D., Schardl, C.L., Songlin, P.J., Mogen, K.L., and Siegel, M.R. (1990) The β-tubulin gene of *Epichloë typhina* from perennial ryegrass (*Lolium perenne*). *Current Genetics* **18**: 347-354.

Cabral,, D., Cafaro, M.J., Saidman, B., Lugo, M., Reddy, P.V., and White, J.F.J. (1999) Evidence supporting the occurrence of a new species of endophyte in some South American grasses. *Mycologia* **91**: 315-325.

Caddick, M.X., Arst, H.N., Jr., Taylor, L.H., Johnson, R.I., and Brownlee, A.G. (1986) Cloning of the regulatory gene *areA* mediating nitrogen metabolite repression in *Aspergillus nidulans*. *The EMBO Journal* **5**: 1087-1090.

Cary, J.W., and Bhatnagar, D. (1995) Nucleotide sequence of a *Aspergillus parasiticus* gene strongly repressed by thiamine. *Biochimica et Biophysica Acta* **1261**: 319-320.

Catalanotto, C., Pallotta, M., ReFalo, P., Sachs, M.S., Vayssie, L., Macino, G., and Cogoni, C. (2004) Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in *Neurospora crassa*. *Molecular & Cellular Biology* **24**: 2536-2545.

Chabregas, S.M., Luche, D.D., Farias, L.P., Ribeiro, A.F., van Sluys, M.A., and Menck, C.F. (2001) Dual targeting properties of the N-terminal signal sequence of *Arabidopsis thaliana* THI1 protein to mitochondria and chloroplasts. *Plant Molecular Biology* **46**: 639-665.

Cheplick, G.P., Clay, K., and Marks, S. (1989) Interactions between infection by endophytic fungi and nutrient limitation in the grasses *Lolium perenne* and *Festuca arundinacea*. *New Phytologist* **111**: 89-97.

Cheplick, G.P., Perera, A., and Koulouris, K. (2000) Effect of drought on growth of *Lolium perenne* genotypes with and without fungal endophytes. *Functional Ecology* **14**: 657-667.

Choi, G.H., Marek, E.T., Schardl, C.L., Richey, M.G., Chang, S., and Smith, D.A. (1990) *Sti35*, a stress-responsive gene in *Fusarium spp*. *Journal of Bacteriology* **172**: 4522-4528.

Christensen, M.J., Latch, G.C.M., and Tapper, B.A. (1991) Variation within isolates of *Acremonium* endophytes from perennial rye-grasses. *Mycological Research* **95**: 918-923.

Christensen, M.J., Leuchtmann, A., Rowan, D.D., and Tapper, B.A. (1993) Taxonomy of *Acremonium* endophytes of tall fescue (*Festuca arundinacea*), meadow fescue (*F. pratensis*) and perennial ryegrass (*Lolium perenne*). *Mycological Research* **97**: 1083-1092.

Christensen, M.J. (1995) Variation in the ability of *Acremonium* endophytes of perennial rye-grass (*Lolium perenne*), tall fescue (*Festuca arundinacea*) and meadow fescue (*F. pratensis*) to form compatible associations in the three grasses. *Mycological Research* **99**: 466-470.

Christensen, M.J., Ball, O.J.-P., Bennett, R.J., and Schardl, C.L. (1997) Fungal and host genotype effects on compatibility and vascular colonisation by *Epichloë festucae*. *Mycological Research* **101**: 493-501.

Christensen, M.J., Easton, H.S., Simpson, W.R., and Tapper, B.A. (1998) Occurrence of the fungal endophyte *Neotyphodium coenophialum* in leaf blades of tall fescue and implications for stock health. *New Zealand Journal of Agricultural Research* **41**: 595-602.

Christensen, M.J., Bennett, R.J., and Schmid, J. (2001) Vascular bundle colonisation by *Neotyphodium* endophytes in natural and novel associations with grasses. *Mycological Research* **105**: 1239-1245.

Christensen, M.J., Bennett, R.J., and Schmid, J. (2002) Growth of *Epichloë/Neotyphodium* and p-endophytes in leaves of *Lolium* and *Festuca* grasses. *Mycological Research* **106**: 93-106.

Chung, K.-R., Hollin, W., Siegel, M.R., and Schardl, C.L. (1997) Genetics of host specificity in *Epichloë typhina*. *Phytopathology* **87**: 599-605.

Chung, K.-R., and Schardl, C.L. (1997) Sexual cycle and horizontal transmission of the grass symbiont, *Epichloë typhina*. *Mycological Research* **101**: 295-301.

Clay, K. (1990) Fungal endophytes of grasses. *Annual Review of Ecology and Systematics* **21**: 275-297.

Collett, M.A., Bradshaw, R.E., and Scott, D.B. (1995) A mutualistic fungal symbiont of perennial ryegrass contains two different *pyr4* genes, both expressing orotidine-5'-monophosphate decarboxylase. *Gene* **158**: 31-39.

Correia, T., Grammel, N., Ortel, I., Keller, U., and Tudzynski, P. (2003) Molecular cloning and analysis of the ergopeptine assembly system in the ergot fungus *Claviceps purpurea*. *Chemistry and Biology* **10**: 1281-1292.

Craven, K.D., Hsiau, P.T.W., Leuchtmann, A., Hollin, W., and Schardl, C.L. (2001) Multigene phylogeny of *Epichloë* species, fungal symbionts of grasses. *Annals of the Missouri Botanical Gardens* **88**: 14-34.

Curach, N.C., Te'o, V.S., Gibbs, M.D., Bergquist, P.L., and Nevalainen, K.M. (2004) Isolation, characterization and expression of the *hex1* gene from *Trichoderma reesei*. *Gene* **331**: 133-140.

De Battista, J.P., Bacon, C.W., Severson, R., Plattner, R.D., and Bouton, J.H. (1990) Indole acetic acid production by the fungal endophyte of tall fescue. *Agronomy Journal* **82**: 878-880.

de la Serna, I., Ng, D., and Tyler, B.M. (1999) Carbon regulation of ribosomal genes in *Neurospora crassa* occurs by a mechanism which does not require Cre-1, the homologue of the *Aspergillus* carbon catabolite repressor, CreA. *Fungal Genetics & Biology* **26**: 253-269.

Dobson, J.M. (1997) Molecular characterisation of the HMG CoA reductase gene from *Neotyphodium lolii*. Palmerston North, New Zealand: Massey University.

Dower, W.J., Miller, J.F., and Ragsdale, C.W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Research* **16**: 6127-6145.

Dowzer, C.E.A., and Keller, J.M. (1989) Cloning of *creA* from *Aspergillus nidulans*: a gene involved in carbon catabolite repression. *Current Genetics* **15**: 457-459.

Dutton, J.R., Johns, S., and Miller, B.L. (1997) StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. *The EMBO Journal* **16**: 5710-5721.

Easton, H.S., Latch, G.C.M., Tapper, B.A., and O.J.-P., B. (2002) Ryegrass host genotype control of concentrations of endophyte-derived alkaloids. *Crop Science* **42**.

Emecz, T.I., and Jones, D.G. (1970) Effect of gibberellic acid on inflorescence production in cocksfoot plants infected with choke (*Epichloë typhina*). *Transactions of The British Mycological Society* **55**: 77-82.

Enjo, F., Nosaka, K., Ogata, M., Iwashima, A., and Nishimura, H. (1997) Isolation and characterization of a thiamin transport gene, *THI10*, from *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **272**: 19165-19170.

Estramareix, B., and David, S. (1996) Biosynthesis of thiamine. *New Journal of Chemistry* **20**: 607-629.

Faeth, S.H., and Sullivan, T.J. (2003) Mutualistic asexual endophytes in a native grass are usually parasitic. *The American Naturalist* **161**: 310-325.

Felenbok, B., and Kelly, J.M. (1996) Regulation of carbon metabolism in mycelial fungi. In *The mycota*. Vol. III Biochemistry and molecular biology. Brambl and Marzluf, G.A. (eds). Berlin: Springer-Verlag, pp. 369-380.

Ferguson, N.H., Rice, J.S., and Allgood, N.G. (1993) Variation in nitrogen utilization in *Acremonium coenophialum* isolates. *Applied and Environmental Microbiology* **59**: 3602-3604.

Finan, T.M., Kunkel, B., Vos, G.F.D., and Signer, E.R. (1986) Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *Journal of Bacteriology*: 66-72.

Finan, T.M., Weidner, S., Wong, K., Buhrmester, J., Chain, P., Vorholter, F.J., Hernandez-Lucas, I., Becker, A., Cowie, A., Gouzy, J., Golding, B., and Puhler, A. (2001) The complete sequence of the 1,683-kb pSymB megaplasmid from the N_2-fixing endosymbiont *Sinorhizobium meliloti*. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 9889-9894.

Fu, Y.H., and Marzluf, G.A. (1990) *nit-2*, the major positive acting nitrogen regulatory gene of *Neurospora crassa*, encoding a sequence-specific DNA-binding protein. *Proceedings of the National Academy of Sciences (USA)* **87**: 5331-5335.

Funk, C.R., Belanger, F.C., and Murphy, J.A. (1994) Role of endophytes in grasses used for turf and soil conservation. In *Biotechnology of endophytic fungi of grasses*. Bacon, C.W. and White, W.F., Jr. (eds): CRC Press, pp. 201-209.

Gallagher, R.T., White, E.P., and Mortimer, P.H. (1981) Ryegrass staggers: Isolation of potent neurotoxins lolitrem A and lolitrem B from staggers-producing pastures. *New Zealand Veterinary Journal* **29**: 189-190.

Gantotti, B.V., and Beer, S.V. (1982) Plasmid-borne determinants of pigmentation and thiamine prototrophy in *Erwinia herbicola*. *Journal of Bacteriology* **151**: 1627-1629.

Geballe, A.P. (1996) Translational control mediated by upstream AUG codons. In *Translational control*. Hershey, J.W.B., Mathews, M.B. and Sonenberg, N. (eds). New York: CSHL Press, pp. 173-197.

Gebler, J.C., and Poulter, C.D. (1992) Purification and characterization of dimethylallyl tryptophan synthase from *Claviceps purpurea*. *Archives of Biochemistry and Biophysics* **296**: 308-313.

Glenn, A.E., Bacon, C.W., Price, R., and Hanlin, R.T. (1996) Molecular phylogeny of *Acremonium* and its taxonomic implications. *Mycologia* **88**: 369-383.

Gooday, G.W. (1995) The dynamics of hyphal growth. *Mycological Research* **99**: 385-394.

Groppe, K., Steinger, T., and Sanders, I. (1999) Interaction between endophytic fungus *Epichloë bromicola* and the grass *Bromus erectus*: effects of endophyte infection, fungal concentration and environment on grass growth and flowering. *Molecular Ecology* **8**: 1827-1835.

Gurr, S.J., Unkles, S.E., and Kinghorn, J.R. (1987) The structure and organization of nuclear genes of filamentous fungi. In *Gene structure in eukaryotic microbes*. Vol. 22. Kinghorn, J.R. (ed). Oxford: IRL press, pp. 93-139.

Gwinn, K.D., Collins-Shepard, M.H., and Reddick, B.B. (1991) Tissue print-immunoblot, an accurate method for the detection of *Acremonium coenophialum* in tall fescue. *Phytopathology* **81**: 747-748.

Gwinn, K.D., and Gavin, A.M. (1992) Relationship between endophyte infestation level of tall fescue seed lots and *Rhizoctonia zeae* seedling disease. *Plant Disease* **76**: 911-914.

Hahn, M., and Mendgen, K. (1997) Characterization of *in planta*-induced rust genes isolated from a haustorium-specific cDNA library. *Molecular Plant-Microbe Interactions* **10**: 427-437.

Henrissat, B., Deleury, E., and Coutinho, P.M. (2002) Glycogen metabolism loss: a common marker of parasitic behaviour in bacteria? *Trends in Genetics* **18**: 437-440.

Hesse, U., and Latch, G.C.M. (1999) Influence of *Neotyphodium lolii* and barley yellow dwarf virus, individually and combined, on the growth of *Lolium perenne*. *Australasian Plant Pathology* **28**: 240-247.

Hill, N.S., Stringer, W.C., Rottinghaus, G.E., Belesky, D.P., Parrott, W.A., and Pope, D.D. (1990) Growth, morphological and chemical component responses of tall fescue to *Acremonium coenophialum*. *Crop Science* **30**: 156-161.

Hinnebusch, A.G. (1996) Translational control of *GCN4*: gene-specific regulation by phosphorylation of elF2. In *Translational control*. Hershey, J.W.B., Mathews, M.B. and Sonenberg, N. (eds). New York: CSHL Press, pp. 199-244.

Hohmann, S., and Meacock, P.A. (1998) Thiamin metabolism and thiamin diphosphate-dependent enzymes in the yeast *Saccharomyces cerevisiae*: genetic regulation. *Biochimica et Biophysica Acta* **1385**: 201-219.

Holmes, D.S., and Quigley, M. (1981) A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry* **114**: 193-197.

Itoh, Y., Johnson, R., and Scott, B. (1994) Integrative transformation of the mycotoxin-producing fungus, *Penicillium paxilli*. *Current Genetics* **25**: 508-513.

Jackson, A.O., and Taylor, C.B. (1996) Plant-microbe interactions: life and death at the interface. *The Plant Cell* **8**: 1651-1668.

Jacob-Wilk, D., Goldschmidt, E.E., Riov, J., Sadka, A., and Holland, D. (1997) Induction of a Citrus gene highly homologous to plant and yeast *thi* genes involved in thiamine biosynthesis during natural and ethylene-induced fruit maturation. *Plant Molecular Biology* **35**: 661-666.

Joost, R.E. (1995) *Acremonium* in fescue and ryegrass: boon or bane? A review. *Journal of Animal Science* **73**: 881-888.

Kadotani, N., Nakayashiki, H., Tosa, Y., and Mayama, S. (2003) RNA silencing in the phytopathogenic fungus *Magnaporthe oryzae*. *Molecular Plant-Microbe Interactions* **16**: 769-776.

Kaiser, W.J., Bruehl, G.W., Davitt, C.M., and Klein, R.E. (1996) *Acremonium* isolates from *Stipa robusta*. *Mycologia* **88**: 539-547.

Katinka, M.D., Duprat, S., Cornillot, E., Metenier, G., Thomarat, F., Prensier, G., Barbe, V., Peyretaillade, E., Brottier, P., Wincker, P., Delbac, F., El Alaoui, H., Peyret, P., Saurin, W., Gouy, M., Weissenbach, J., and Vivares, C.P. (2001) Genome sequence and gene compaction of the eukaryote parasite *Encephalitozoon cuniculi*. *Nature* **414**: 450-453.

Kempken, F., and Kuck, U. (1996) restless, an active Ac-like transposon from the fungus *Tolypocladium inflatum*: structure, expression, and alternative RNA splicing. *Molecular & Cellular Biology* **16**: 6563-6572.

Keogh, R.G., and Tapper, B.A. (1993) *Acremonium lolii*, lolitrem B, and peramine concentrations within vegetative tillers of perennial ryegrass. In *The second international symposium on Acremonium/ Grass interactions*. Hume, D.E., Latch, G.C.M. and Easton, H.S. (eds.) Palmerston North, pp. 88-92.

Keogh, R.G., Tapper, B.A., and Fletcher, R.H. (1996) Distributions of the fungal endophyte *Acremonium lolii*, and of the alkaloids lolitrem B and peramine, within perennial ryegrass. *New Zealand Journal of Agricultural Research* **39**: 121-127.

Khan, S.G., Metin, A., Gozukara, E., Inui, H., Shahlavi, T., Muniz-Medina, V., Baker, C.C., Ueda, T., Aiken, J.R., Schneider, T.D., and Kraemer, K.H. (2004) Two essential splice lariat branchpoint sequences in one intron in a xeroderma pigmentosum DNA repair gene: mutations result in reduced XPC mRNA levels that correlate with cancer risk. *Human Molecular Genetics* **13**: 343-352.

Kimmons, C.A., Gwinn, K.D., and Bernard, E.C. (1990) Nematode reproduction on endophyte-infected and endophyte-free tall fescue. *Plant Disease* **74**: 757-761.

Kirby, E.J.M. (1961) Host-parasite relations in the choke disease of grasses. *Transactions of The British Mycological Society* **44**: 493-503.

Knogge, W. (1996) Molecular basis of specificity in host/fungus interactions. *European Journal of Plant Pathology* **102**: 807-816.

Koga, H., Christensen, M.J., and Bennett, R.J. (1993) Incompatibility of some grass/*Acremonium* endophyte associations. *Mycological Research* **97**: 1237-1244.

Kubodera, T., Yamashita, N., and Nishimura, A. (2000) Pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*: cloning, characterization and application as a dominant selectable marker for transformation. *Bioscience Biotechnology Biochemistry* **64**: 1416-1421.

Kubodera, T., Watanabe, M., Yoshiuchi, K., Yamashita, N., Nishimura, A., Nakai, S., Gomi, K., and Hanamoto, H. (2003) Thiamin-regulated gene expression of *Aspergillus oryzae thiA* requires splicing of the intron containing a riboswitch-like domain in the 5'-UTR. *FEBS Letters* **555**: 516-520.

Kuldau, G.A., Tsai, H.-F., and Schardl, C.L. (1999) Genome sizes of *Epichloë* species and anamorphic hybrids. *Mycologia* **91**: 776-782.

Kulkarni, R.K., and Nielsen, B.D. (1986) Nutritional requirements for growth of a fungus endophyte of tall fescue grass. *Mycologia* **78**: 781-786.

Kutil, B.L., Spiering, M.J., and Wilkinson, H.H. (2003) Evolution of a secondary metabolite gene cluster implicated in loline alkaloid biosynthesis of grass-endophytes (*Epichloë* and *Neotyphodium spp.*). *Fungal Genetics Newsletter* **50**: 41.

Lam, C.K., Belanger, F.C., White, J.F., Jr., and Daie, J. (1994) Mechanism and rate of sugar uptake by *Acremonium typhinum*, and endophytic fungus infecting *Festuca rubra*: evidence for presence of a cell wall invertase in endophytic fungi. *Mycologia* **8**: 408-415.

Lane, G.A., Tapper, B.A., Davies, E., Hume, D.E., Latch, G.C.M., Barker, D.J., Easton, H.S., and Rolston, M.P. (1997) Effect of growth conditions on alkaloid concentrations in perennial ryegrass naturally infected with endophyte. In *Neotyphodium/Grass interactions.* Bacon, C.W. and Hill, N.S. (eds). New York: Plenum press, pp. 179-182.

Latch, G.C.M., Christensen, M.J., and Samuels, G.J. (1984) Five endophytes of *Lolium* and *Festuca* in New Zealand. *Mycotaxon* **20**: 535-550.

Latch, G.C.M., and Christensen, M.J. (1985) Artificial infection of grasses with endophytes. *Annals of Applied Biology* **107**: 17-24.

Latch, G.C.M., Hunt, W.F., and Musgrave, D.R. (1985) Endophytic fungi affect growth of perennial ryegrass. *New Zealand Journal of Agricultural Research* **28**: 165-168.

Latch, G.C.M. (1993) Physiological interactions of endophytic fungi and their hosts. Biotic stress tolerance imparted to grasses by endophytes. *Agriculture, Ecosystems and Environment* **44**: 1-4.

Leonardi, R., and Roach, P.L. (2004) Thiamine biosynthesis in *Escherichia coli*: *in vitro* reconstitution of the thiazole synthase activity. *Journal of Biological Chemistry* **279**: 17054-17062.

Leuchtmann, A., and Clay, K. (1990) Isozyme variation in the *Acremonium/Epichloë* fungal endophyte complex. *Phytopathology* **80**: 1133-1139.

Leuchtmann, A. (1992) Systematics, distribution, and host specificity of grass endophytes. *Natural Toxins* **1**: 150-162.

Leuchtmann, A. (1994a) Isozyme relationships of *Acremonium* endophytes from twelve *Festuca* species. *Mycological Research* **98**: 25-33.

Leuchtmann, A. (1994b) Isozyme characterization, persistence, and compatibility of fungal and grass mutualists. In *Biotechnology of Endophytic Fungi of Grasses.* Bacon, C.W. and White, J.F.J. (eds). London: CRC Press, pp. 21-33.

Leuchtmann, A., and Clay, K. (1997) The population biology of grass endophytes. In *The mycota: Plant Relationships.* Vol. 5. Carroll, G.C. and Tudzynski, P. (eds). Berlin: Springer Verlag, pp. 185-202.

Leuchtmann, A., and Schardl, C.L. (1998) Mating compatibility and phylogenetic relationships among two new species of *Epichloë* and other congenetic European species. *Mycological Research* **102**: 1169-1182.

Leuchtmann, A. (2003) Taxonomy and diversity of *Epichloë* endophytes. In *Clavicipitalean fungi: Evolution biology, chemistry, biocontrol, and cultural impacts.* White, J.F., Jr, Bacon, C.W., Hywel-Jones, N.L. and Spatafora, J.W. (eds). New York: Marcel Dekker, Inc., pp. 169-194.

Leyronas, C., and Raynal, G. (2001) Presence of *Neotyphodium*-like endophytes in European grasses. *Annals of Applied Biology* **139**: 119-127.

Lewis, E.A., Sullivan, R.F., and White, J.F., Jr (2003) Distinguishing features of the tribe Balansieae. In *Clavicipitalean fungi: Evolution biology, chemistry, biocontrol, and cultural impacts.* White, J.F., Jr, Bacon, C.W., Hywel-Jones, N.L. and Spatafora, J.W. (eds). New York: Marcel Dekker, Inc., pp. 151-167.

Johnson, L.J., Johnson, R.D., Schardl, C.L., and Panaccione, D.G. (2004) Identification of differentially expressed genes in the mutualistic association of tall fescue with *Neotyphodium coenophialum. Physiological and Molecular Plant Pathology* **63**: 305-317.

Liu, Y.-G., and Whittier, R.F. (1995) Thermal Asymmetric Interlaced PCR: Automatable amplification and sequencing of insert end fragment from P1 and YAC clones for chromosome Walking. *Genomics* **25**: 674-681.

Lodwig, E.M., Hosie, A.H.F., Bourdes, A., Findlay, K., Allaway, D., Karunakaran, R., Downie, J.A., and Poole, P.S. (2003) Amino-acid cycling drives nitrogen fixation in the legume-*Rhizobium* symbiosis. *Nature* **422**: 722-726.

Lohaus, G., Winter, H., Riens, B., and Heldt, H.W. (1995) Further studies of the phloem loading process in leaves of barley and Spinach. The comparison of metabolite concentrations in the apoplastic compartment with those in the cytosolic compartment and in the sieve tubes. *Botanica Acta* **108**: 270-275.

Lubbehusen, T., Gonzalez Polo, V., Rossi, S., Nielsen, J., Moreno, S., McIntyre, M., and Arnau, J. (2004) Protein kinase A is involved in the control of morphology and branching during aerobic growth of *Mucor circinelloides*. *Microbiology* **150**: 143-150.

Lynch, M. (1997) Mutation accumulation in nuclear, organelle, and prokaryotic transfer RNA genes. *Molecular Biology & Evolution* **14**: 914-925.

Lyons, P.C., Plattner, R.D., and Bacon, C.W. (1986) Occurrence of peptide and clavine ergot alkaloids in tall fescue grass. *Science* **232**: 487-489.

Machado, C.R., de Oliverira, R.C., Bioteux, S., Praekelt, U.M., Meacock, R.A., and Menck, C.F. (1996) *Thi1*, a thiamine biosynthetic gene in *Arabidopsis thaliana,* complements bacterial defects in DNA repair. *Plant Molecular Biology* **31**: 585-593.

Machado, C.R., Praekelt, U.M., Oliveira, R.C.d., Barbosa, A.C.C., Byrne, K.L., Meacock, P.A., and Menck, C.F.M. (1997) Dual roles for the yeast *THI4* gene in thiamine biosynthesis and DNA damage tolerance. *Journal of Molecular Biology* **273**: 114-121.

Majewska-Sawka, A., and Nakashima, H. (2004) Endophyte transmission via seeds of *Lolium perenne* L.: immunodetection of fungal antigens. *Fungal Genetics & Biology* **41**: 534-541.

Malinowski, D.P., and Belesky, D.P. (2000) Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. *Crop Science* **40**: 923-940.

Manetti, A.G.O., Rossetto, M., and Maundrell, K.G. (1994) *nmt2* of fission yeast: A second thiamine-repressible gene coordinately regulated with *nmt1*. *Yeast* **10**: 1075-1082.

Markham, P., Robson, G.D., Bainbridge, B.W., and Trinci, A.P.J. (1993) Choline: its role in the growth of filamentous fungi and the regulation of mycelial morphology. *FEMS Microbiology Reviews* **104**: 287-300.

Marroqui, S., Zorreguieta, A., Santamaria, C., Temprano, F., Soberon, M., Megias, M., and Downie, J.A. (2001) Enhanced symbiotic performance by *Rhizobium tropici* glycogen synthase mutants. *Journal of Bacteriology* **183**: 854-864.

Marte, M., and Montalbini, P. (1999) Histological observations on *Uromyces phaseoli* and *Puccinia recondita* infection in Allopurinol-treated susceptible plants. *Journal of Phytopathology* **14**: 163-168.

Marzluf, G.A. (1997) Genetic regulation of nitrogen metabolism in the fungi. *Microbiology and Molecular Biology Reviews* **61**: 17-32.

Mathieu, M., Fillinger, S., and Felenbok, B. (2000) *In vivo* studies of upstream regulatory cis-acting elements of the alcR gene encoding the transactivator of the ethanol regulon in *Aspergillus nidulans*. *Molecular Microbiology* **36**: 123-131.

McCallum, N.G. (2001) Analysis of a 40-kb region of the *Mesorhizobium loti* symbiosis island that includes a thiamin operon. PhD thesis In *Department of Biochemistry* Dunedin: University of Otago.

McMillan, L.K., Carr, R.L., Young, C.A., Astin, J.W., Lowe, R.G., Parker, E.J., Jameson, G.B., Finch, S.C., Miles, C.O., McManus, O.B., Schmalhofer, W.A., Garcia, M.L., Kaczorowski, G.J., Goetz, M., Tkacz, J.S., and Scott, B. (2003) Molecular analysis of two cytochrome P450 monooxygenase genes required for paxilline biosynthesis in *Penicillium paxilli*, and effects of paxilline intermediates on mammalian maxi-K ion channels. *Molecular Genetics & Genomics* **270**: 9-23.

Meijer, G., and Leuchtmann, A. (2000) The effects of genetic and environmental factors on disease expression (stroma formation) and plant growth in *Brachypodium sylvaticum* infected by *Epichloë sylvatica*. *OIKOS* **91**: 446-458.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. New York: Cold Spring Harbor Laboratory Press.

Mironov, A.S., Gusarov, I., Rafikov, R., Lopez, L.E., Shatalin, K., Kreneva, R.A., Perumov, D.A., and Nudler, E. (2002) Sensing small molecules by nascent RNA: a mechanism to control transcription in bacteria. *Cell* **111**: 747-756.

Mo, X., and Marzluf, G.A. (2003) Cooperative action of the NIT2 and NIT4 transcription factors upon gene expression in *Neurospora crassa*. *Current Genetics* **42**: 260-267.

Möller, E.M., Bahnweg, G., Sandermann, H., and Geiger, H.H. (1992) A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Research* **20**: 6115-6116.

Moon, C.D., Scott, B., Schardl, C.L., and Christensen, M.J. (2000) The evolutionary origins of *Epichloë* endophytes from annual ryegrasses. *Mycologia* **92**: 1103-1118.

Moon, C.D., Miles, C.O., Järlfors, U., and Schardl, C. (2002) The evolutionary origins of three new *Neotyphodium* endophyte species from grasses indigenous to the Southern Hemisphere. *Mycologia* **94**: 694-711.

Morett, E., Korbel, J.O., Rajan, E., Saab-Rincon, G., Olvera, L., Olvera, M., Schmidt, S., Snel, B., and Bork, P. (2003) Systematic discovery of analogous enzymes in thiamin biosynthesis. *Nature Biotechnology* **21**: 790-795.

Morgan-Jones, G., and Gams, W. (1982) Notes on Hyphomycetes. XLI. An endophyte of *Festuca arundinacea* and the anamorph of *Epichloë typhina*, new taxa in one of two new sections of *Acremonium*. *Mycotaxon* **15**: 311-318.

Morgan-Jones, G., White, J.F.J., and Pointelli, E.L. (1990) Endophyte-host associations in forage grasses. 8. *Acremonium chilense*, an undescribed endophyte occurring in *Dactylis glomerata* in Chile. *Mycotaxon* **39**: 441-454.

Muller, H.J. (1964) The relation of recombination to mutational advance. *Mutation Research* **1**: 2-9.

Naffa, W., Ravel, C., Boyer, N., and Guillaumin, J.-J. (1999) Peroxidase activity of perennial ryegrass and tall fescue seedlings artificially infected with endophytes. *Agronomie* **19**: 611-619.

Naffaa, W., Ravel, C., and Guillaumin, J.-J. (1998) Nutritional requirements for growth of fungal endophytes of grasses. *Canadian Journal of Microbiology* **44**: 231-237.

Nishimura, H., Kawasaki, Y., Kaneko, Y., Nosaka, K., and Iwashima, A. (1992) A positive regulatory gene, *THI3*, is required for thiamine metabolism in *Saccharomyces cerevisiae*. *Journal of Bacteriology* **174**: 4701-4706.

Nosaka, K., Kaneko, Y., Nishimura, H., and Iwashima, A. (1993) Isolation and characterization of a thiamin pyrophosphokinase gene, *THI80*, from *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **268**: 17440-17447.

Nosaka, K., Nishimura, H., Kawasaki, Y., Tsujihara, T., and Iwashima, A. (1994) Isolation and characterization of the *THI6* gene encoding a bifunctional thiamin-phosphate pyrophosphorylase/hydroxyethylthiazole kinase from *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **269**: 30510-30516.

Okazaki, K., and Niwa, O. (2000) mRNAs encoding zinc finger protein isoforms are expressed by alternative splicing of an in-frame intron in fission yeast. *DNA Research* **7**: 27-30.

Oliver, R.P., Roberts, I.N., Harling, R., Kenyon, L., Punt, P.J., Dingemanse, M.A., and van den Hondel, C.A.M.J.J. (1987) Transformation of *Fulvia fulva*, a fungal pathogen of tomato, to hygromycin B resistance. *Current Genetics* **12**: 231-233.

Olsson, S. (2001) Colonial growth of fungi. In *The Mycota VIII: Biology of the Fungal Cell.* Howard, R.J. and Gow, N.A.R. (eds). Berlin Heidelberg: Springer-Verlag, pp. 125-141.

Orejas, M., MacCabe, A.P., Perez Gonzalez, J.A., Kumar, S., and Ramon, D. (1999) Carbon catabolite repression of the *Aspergillus nidulans xlnA* gene. *Molecular Microbiology* **31**: 177-184.

Panaccione, D.G., Wang, J., Young, C.A., Scott, D.B., Schardl, C.L., and Damrongkool, P. (1999) Cloning and characterization of a peptide synthetase gene associated with ergopeptine production. In *Joint Meeting of American Phytopathological Society and Canadian Phytopathological Society* Montreal.

Panaccione, D.G., Johnson, R.D., Wang, J., Young, C.A., Damrongkool, P., Scott, B., and Schardl, C.L. (2001) Elimination of ergovaline from a grass-*Neotyphodium* endophyte symbiosis by genetic modification of the endophyte. *Proceedings of the National Academy of Science (USA)* **98**: 12820-12825.

Panaccione, D.G., and Schardl, C. (2003) Molecular genetics of ergot alkaloid biosynthesis. In *Clavicipitalean fungi: Evolution biology, chemistry, biocontrol, and cultural impacts*. White, J.F., Jr, Bacon, C.W., Hywel-Jones, N.L. and Spatafora, J.W. (eds). New York: Marcel Dekker, Inc., pp. 399-424.

Panstruga, R. (2003) Establishing compatibility between plants and obligate biotrophic pathogens. *Current Opinion in Plant Biology* **6**: 320-326.

Parniske, M. (2004) Molecular genetics of the arbuscular mycorrhizal symbiosis. *Current Opinion in Plant Biology* **7**: 414-421.

Pellier, A.L., Lauge, R., Veneault-Fourrey, C., and Langin, T. (2003) CLNR1, the AREA/NIT2-like global nitrogen regulator of the plant fungal pathogen *Colletotrichum lindemuthianum* is required for the infection cycle. *Molecular Microbiology* **48**: 639-655.

Pera, L.M., and Callieri, D.A. (1997) Influence of calcium on fungal growth, hyphal morphology and citric acid production in *Aspergillus niger*. *Folia Microbiologica* **42**: 551-556.

Pérez-Garcia, A., Snoeijers, S.S., Joosten, M.H.A.J., Goosen, T., and De Wit, P.J.G.M. (2001) Expression of the avirulence gene *Avr9* of the fungal tomato pathogen *Cladosporium fulvum* is regulated by the global nitrogen response factor NRF1. *Molecular Plant-Microbe Interactions* **14**: 316-325.

Perfect, S.E., and Green, J.R. (2001) Infection structures of biotrophic and hemibiotrophic fungal plant pathogens. *Molecular Plant Pathology* **2**: 101-108.

Philipson, M.N., and Christey, M.C. (1986) The relationship of host and endophyte during flowering, seed formation, and germination on *Lolium perenne*. *New Zealand Journal of Botany* **24**: 125-134.

Praekelt, U.M., and Meacock, P.A. (1992) *MOL1*, a *Saccharomyces cerevisiae* gene that is highly expressed in early stationary phase during growth on molasses. *Yeast* **8**: 699-710.

Praekelt, U.M., Byrne, K.L., and Meacock, P.A. (1994) Regulation of *THI4* (*MOL1*), a thiamine-biosynthetic gene of *Saccharomyces cerevisiae*. *Yeast* **10**: 481-490.

Punt, P.J., Oliver, R.P., Dingemanse, M.A., Pouwels, P.H., and Van den Hondel, C.A.M.J.J. (1987) Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene* **56**: 117-124.

Purrington, C.B. (2000) Costs of resistance. *Current Opinion in Plant Biology* **3**: 305-308.

Raisbeck, M.F., Rottinghaus, G.E., and Kendall, J.D. (1991) Effects of naturally occurring mycotoxins on ruminants. In *Mycotoxins and Animal Foods*. Smith, J.E. and Henderson, R.S. (eds). Boca Raton: CRC Press, pp. 647-676.

Reddy, P.V., Lam, C.K., and Belanger, F.C. (1996) Mutualistic fungal endophytes express a proteinase that is homologous to proteases suspected to be important in fungal pathogenicity. *Plant Physiology* **111**: 1209-1218.

Redman, R.S., Dunigan, D.D., and Rodriguez, R.J. (2001) Fungal symbiosis from mutualism to parasitism: who controls the outcome, host or invader? *New Phytologist* **151**: 705-716.

Redman, R.S., and Rodrigues, R.J. (2002) Characterization and isolation of an extracellular serine protease from the tomato pathogen *Colletotrichum coccodes*, and it's role in pathogenicity. *Mycological Research* **106**: 1427-1434.

Ribeiro, A., Praekelt, U., Akkermans, A.D., Meacock, P.A., van kammen, A., Bisseling, T., and Pawlowski, K. (1996) Identification of *agthi1*, whose product is involved in biosynthesis of the thiamine precursor thiazole, in actinorhizal nodules of *Alnus glutinosa*. *The Plant Journal* **10**: 361-368.

Richardson, M.D. (1992) Morphological and physiological response of tall fescue to water stress and fungal endophytes. *Dissertation Abstracts International. B, Sciences and Engineering* **52**.

Richardson, M.D., Hoveland, C.S., and Bacon, C.W. (1993) Photosynthesis and stomatal conductance of symbiotic and nonsymbiotic tall fescue. *Crop Science* **33**: 145-149.

Robb, M.J., and Wilson, M.A. (1995) A fungal actin-related protein involved in nuclear migration. *Molecular General Genetics* **247**: 583-590.

Roberts, C.A., Marek, S.M., Niblack, T.L., and Karr, A.L. (1992) Parasitic *Meloidogyne* and mutualistic *Acremonium* increase chitinase in tall fescue. *Journal of Chemical Ecology* **18**: 1107-1116.

Rodriguez-Navarro, S., Llorente, B., Rodriguez-Manzaneque, M.T., A., R., Uber, G., Marchesan, D., Dujon, B., Herrero, E., Sunnerhagen, P., and Perez-Ortin, J.E. (2002) Functional analysis of yeast gene families involved in metabolism of vitamins B1 and B6. *Yeast* **19**: 1261-1276.

Rogers, G.W., Jr., Edelman, G.M., and Mauro, V.P. (2004) Differential utilization of upstream AUGs in the beta-secretase mRNA suggests that a shunting mechanism regulates translation. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 2794-2799.

Rowan, D.D., and Latch, G.C.M. (1994) Utilization of endophyte-infected perennial ryegrasses for increased insect resistance. In *Biotechnology of Endophytic Fungi of Grasses*. Bacon, C.W. and White, J.F., Jr. (eds). Boca Raton: CRC Press, pp. 169-183.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbour Laboratory Press.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences (USA)* **74**: 5463-5467.

Schardl, C.L., Liu, J.-S., White, J.F., Jr., Finkel, R.A., An, Z.-Q., and Siegel, M.R. (1991) Molecular phylogenetic relationships of nonpathogenic grass mycosymbionts and clavicipitaceous plant pathogens. *Plant Systematics and Evolution* **178**: 27-41.

Schardl, C.L. (1994) Molecular and genetic methodologies and transformation of grass endophytes. In *Biotechnology of Endophytic Fungi of Grasses*. Bacon, C.W. and White, J.F., Jr. (eds). Boca Raton: CRC Press, pp. 151-165.

Schardl, C.L., Leuchtmann, A., Tsai, H.F., Collett, M.A., Watt, D.M., and Scott, D.B. (1994) Origin of a fungal symbiont of perennial ryegrass by interspecific hybridization of a mutualist with the ryegrass choke pathogen, *Epichloë typhina*. *Genetics* **136**: 1307-1317.

Schardl, C.L. (1996a) *Epichloë* species: fungal symbionts of grasses. *Annual Review of Phytopathology* **34**: 109-130.

Schardl, C.L. (1996b) Interactions of grasses with endophytic *Epichloë* species and hybrids. In *Plant-Microbe Interactions*. Vol. 1. Stacey, G. and Keen, N. (eds). New York: Chapman Hall, pp. 107-140.

Schardl, C.L., Leuchtmann, A., Chung, K.-R., Penny, D., and Siegel, M.R. (1997) Coevolution by common descent of fungal symbionts (*Epichloë spp.*) and grass hosts. *Molecular Biology and Evolution* **14**: 133-143.

Schardl, C.l., and Leuchtmann, A. (1999) Three new species of *Epichloë* symbiotic with North American grasses. *Mycologia* **91**: 95-107.

Schardl, C.L., and Moon, C.D. (2003) Processes of species evolution in *Epichloë/Neotyphodium* endophytes of Grasses. In *Clavicipitalean fungi: Evolution biology, chemistry, biocontrol, and cultural impacts*. White, J.F., Jr, Bacon, C.W., Hywel-Jones, N.L. and Spatafora, J.W. (eds). New York: Marcel Dekker, Inc., pp. 273-309.

Schardl, C.L., Leuchtmann, A., and Spiering, M.J. (2004) Symbioses of grasses with seedborne fungal endophyte. *Annual Review of Plant Biology* **55**: 315-340.

Schmid, J., Spiering, M.J., and Christensen, M.J. (2000) Metabolic activity, distribution, and propagation of grass endophytes *in planta*: investigations using the GUS reporter gene system. In *Microbial Endophytes*. Bacon, C.W. and White, J.F.J. (eds). New York: Marcel Dekker, pp. 295-322.

Schulze-Lefert, P. (2004) Knocking on the heaven's wall: pathogenesis of and resistance to biotrophic fungi at the cell wall. *Current Opinion in Plant Biology* **7**: 377-383.

Scott, B., and Schardl, C.L. (1993) Fungal symbionts of grasses: evolutionary insights and agricultural potential. *Trends in Microbiology* **1**: 196-200.

Scott, B. (2001) Molecular interactions between *Lolium* grasses and their fungal symbionts. In *Molecular Breeding of Forage Crops*. Spangenberg, G. (ed). Netherlands: Kluwer Academic Publishers, pp. 261-274.

Scott, B. (2003a) Functional analysis of the perennial ryegrass-*Epichloë* endophyte interaction. In *Molecular Breeding of Forage and Turf*. Hopkins, A., Wang, Z.Y., Mian, R., Sledge, M. and Barker, R. (eds). Netherlands: Kluwer academic.

Scott, B. (2003b) The evolution of gene clusters in plant-associated filamentous fungi. *NZ BioScience* ***May***: 22-26.

Scott, B., and Young, C. (2003) Genetic manipulation of Clavicipitalean endophytes. In *Clavicipitalean fungi: Evolution biology, chemistry, biocontrol, and cultural impacts.* White, J.F., Jr, Bacon, C.W., Hywel-Jones, N.L. and Spatafora, J.W. (eds). New York: Marcel Dekker, Inc., pp. 425-443.

Scott, B., Jameson, G.B., and Parker, E.J. (2004) Isoprenoids: gene clusters and chemical puzzles. In *Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine*. Jane, S.T. and Lene, L. (eds): Kluwer Academic/Plenum Publishers.

Seiler, S., Nargand, F.E., Steinberg, G., and Schliwa, M. (1997) Kinesin is essential for cell morphologenesis and polarized secretion in *Neurospora crassa. The EMBO Journal* **16**: 3025-3034.

Shaw, B.D., and Hoch, H.C. (2001) Ions as regulators of growth and development. In *The Mycota VIII: Biology of the Fungal Cell.* Howard, R.J. and Gow, N.A.R. (eds). Berlin Heidelberg: Springer-Verlag, pp. 73-89.

Shaw, S.L., and Long, S.R. (2003) Nod factor inhibition of reactive oxygen efflux in a host legume. *Plant Physiology* **132**: 2196-2204.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., and Ishikawa, H. (2000) Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera sp. APS. Nature* **407**: 81-86.

Siegel, M.R., Latch, G.C.M., and Johnson, M.C. (1987) Fungal endophytes of grasses. *Annual Review of Phytopathology* **25**: 293-315.

Siegel, M.R., Latch, G.C.M., Bush, L.P., Fannin, F.F., Rowan, D.D., Tapper, B.A., Bacon, C.W., and Johnson, M.C. (1990) Fungal endophyte-infected grasses: alkaloid accumulation and aphid response. *Journal of Chemical Ecology* **16**: 3301-3315.

Siegel, M.R., and Bush, L.P. (1997) Toxin production in grass/endophyte associations. In *The mycota V part A Plant relationships.* Carroll, G.C. and Tudzynski, B. (eds). Berlin: Springer-Verlag, pp. 185-207.

Smith, K.T., Bacon, C.W., and Luttrell, E.S. (1985) Reciprocal translocation of carbohydrates between host and fungus in Bahia grass infected with *Myriogenospora atramentosa*. *Phytopathology* **75**: 407-411.

Sohn, J., Voegele, R.T., Mendgen, K., and Hahn, M. (2000) High level activation of vitamin B1 biosynthesis genes in haustoria of the rust fungus *Uromyces fabae*. *Molecular Plant-Microbe Interactions* **13**: 629-636.

Solaiman, M.Z., Senoo, K., Kawaguchi, M., Imaizumi-Anraku, H., Akao, S., Tanaka, A., and Obata, H. (2000) Characterization of mycorrhizas formed by *Glomus* sp. on root of hypernodulating mutants of *Lotus japonicus*. *Journal of Plant Research* **113**: 443-448.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**: 503-517.

Spangenberg, G., Kalla, R., Lidgett, A., Sawbridge, T., Ong, E.K., and John, U. (2000) Breeding forage plants in the genome Era. In *Molecular Breeding of Forage Crops*. Vol. 10. Spangenberg, G. (ed). London: Kluwer Academic Publishers.

Spiering, M.J. (2000) Distribution of *Neotyphodium Lolii*-endophyte metabolic activity in perennial ryegrass (*Lolium perenne*, L.) and its implications for alkaloid distribution and photosynthesis. PhD thesis In *Institute of Molecular BioScience* Palmerston North, New Zealand: Massey university.

Spiering, M.J., Wilkinson, H.H., Blankenship, J.D., and Schardl, C.L. (2002) Expressed sequence tags and genes associated with loline alkaloid expression by the fungal endophyte *Neotyphodium uncinatum*. *Fungal Genetics and Biology* **36**: 242-254.

Staehelin, C., Charon, C., Boller, T., Crespi, M., and Kondorosi, A. (2001) Medicago truncatula plants overexpressing the early nodulin gene *enod40* exhibit accelerated mycorrhizal colonization and enhanced formation of arbuscules. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 15366-15371.

Sullivan, J.T., Patrick, H.N., Lowther, W.L., Scott, D.B., and Ronson, C.W. (1995) Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. *Proceedings of the National Academy of Science (USA)* **92**: 8985-8989.

Sullivan, J.T., and Ronson, C.W. (1998) Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. *Proceedings of the National Academy of Science (USA)* **95**: 5145-5149.

Sullivan, J.T., Trzebiatowski, J.R., Cruickshank, R.W., Gouzy, J., Brown, S.D., Elliot, R.M., Fleetwood, D.J., McCallum, N.G., Rossbach, U., Stuart, G.S., Weaver, J., Webby, R.J., de Bruijn, F.J., and Ronson, C.W. (2002) Comparative sequence analysis of the symbiosis island of *Mesorhizobium loti* strain R7A. *Journal of Bacteriology* **184**: 3086-3095.

Tan, Y.Y., Spiering, M.J., Scott, V., Lane, G.A., Christensen, M.J., and Schmid, J. (2001) *In planta* regulation of extension of an endophytic fungus and maintenance of high metabolic rates in its mycelium in the absence of apical extension. *Applied and Environmental Microbiology* **67**: 5377-5383.

Tao, Y., and Marzluf, G.A. (1999) The NIT2 nitrogen regulatory protein of *Neurospora*: expression and stability of *nit-2* mRNA and protein. *Current Genetics* **36**: 153-158.

Thanonkeo, P., Akiyama, K., Jain, S., and Takata, R. (2000) Targeted disruption of *sti35*, a stress-responsive gene in phytopathogenic fungus *Fusarium oxysporum*. *Current Microbiology* **41**: 284-289.

Thara, V.K., Fellers, J.P., and Zhou, J.-M. (2003) *In planta* induced genes of *Puccinia triticina*. *Molecular Plant Pathology* **4**: 51-56.

Thuring, R.W., Sanders, P.M., and Borst, P. (1975) A freeze squeeze method for recovering DNA from agarose gels. *Analytical Biochemistry* **66**: 213-220.

Trinci, A.P.J., Wiebe, M.G., and Robson, G.D. (1997) The mycelium as an integrated entity. In *The Mycota*. Wessels, J.G.H. and Meinhardt, F. (eds). Berlin, Heidelberg: Springer-Verlag.

Tsai, H.-F., Liu, J.-S., Staben, C., Christensen, M.J., Latch, G.C.M., Siegel, M.R., and Schardl, C.L. (1994) Evolutionary diversification of fungal endophytes of tall fescue grass by hybridization with *Epichloë* species. *Proceedings of the National Academy of Sciences (USA)* **91**: 2542-2546.

Tsai, H.-F., Wang, H., Gebler, J.C., Poulter, C.D., and Schardl, C.L. (1995) The *Claviceps purpurea* gene encoding dimethylallyltryptophan synthase, the committed step for ergot alkaloid biosynthesis. *Biochemical and Biophysical Research Communications* **216**: 119-125.

Tudzynski, B. (1999) Biosynthesis of gibberellins in *Gibberella fujikuroi*: biomolecular aspects. *Applied and Environmental Microbiology* **52**: 298-310.

Tudzynski, B., Hedden, P., Carrera, E., and Gaskin, P. (2001) The *P450-4* gene of *Gibberella fujikuroi* encodes ent-kaurene oxidase in the gibberellin biosynthesis pathway. *Applied and Environmental Microbiology* **67**: 3514-3522.

Wu, Q., Sandrock, T.M., Turgeon, B.G., Yoder, O.C., Wirsel, S.G., and Aist, J.R. (1998) A fungal kinesin required for organelle motility, hyphal growth, and morphogenesis. *Molecular Biology of the Cell* **9**: 89-101.

Vieira, J., and Messing, J. (1987) Production of single-stranded plasmid DNA. *Methods in Enzymology* **153**: 3-11.

Vilela, C., and McCarthy, J.E. (2003) Regulation of fungal gene expression via short open reading frames in the mRNA 5'untranslated region. *Molecular Microbiology* **49**: 859-867.

Vollmer, S.J., and Yanofsky, C. (1986) Efficient cloning of genes of *Neurospora crassa*. *Proceedings of the National Academy of Sciences (USA)* **83**: 4869-4873.

Wang, J., Machado, C., Panaccione, D.G., Tsai, H.F., and Schardl, C.L. (2004) The determinant step in ergot alkaloid biosynthesis by an endophyte of perennial ryegrass. *Fungal Genetics & Biology* **41**: 189-198.

Weber, R.W.S. (2002) Vacuoles and the fungal lifestyle. *Mycologist* **16**: 10-20.

West, C.P. (1994) Physiology and drought tolerance of endophyte-infected grasses. In *Biotechnology of Endophytic Fungi of Grasses*. Bacon, C.W. and White, J.F., Jr (eds). Boca Raton: CRC Press, pp. 87-99.

White, J.F., Jr., and Cole, G.T. (1985) Endophyte-host associations in forage grasses. I. Distribution of fungal endophytes in some species of *Lolium* and *Festuca*. *Mycologia* **77**: 323-327.

White, J.F., Jr., and Bultman, T.L. (1987) Endophyte-host associations in forage grasses. VIII. Heterothallism in *Epichloë typhina*. *American Journal of Botany* **74**: 1716-1721.

White, J.F., Jr., and Morgan-Jones, G. (1987) Endophyte-host associations in forage grasses. X. Cultural studies on some species of *Acremonium* sect. Albo-lanosa, including a new species, *A. starrii*. *Mycotaxon* **30**: 87-95.

White, J.F., Jr. (1988) Endophyte-host associations in forage grasses. XI. A proposal concerning origin and evolution. *Mycologia* **80**: 442-446.

White, J.F., Jr., Breen, J.P., and Morgan, J.G. (1991) Substrate utilization in selected *Acremonium*, *Atkinsonella* and *Balansia* species. *Mycologia* **83**: 601-610.

White, J.F., Jr., and Chambless, D.A. (1991) Endophyte-host associations in forage grasses XV. Clustering of stromata-bearing individuals of *Agrostis hiemalis* infected by *Epichloë typhina*. *American Journal of Botany* **78**: 527-533.

White, J.F., Jr., and Morrow, A.C. (1991) Endophyte-host associations in forage grasses. XIV. Primary stromata formation and seed transmission in *Epichloë typhina*: developmental and regulatory aspects. *Mycologia* **83**: 72-81.

White, J.F., Jr., Morgan-Jones, G., and Morrow, A.C. (1993) Taxonomy, life cycle, reproduction and detection of *Acremonium* endophytes. *Agriculture Ecosystems & Environment* **44**: 1-4.

White, J.F., Jr. (1994) Endophyte-host associations in grasses. XX. Structural and reproductive studies of *Epichloë amarillans sp. nov.* and comparisons to *E. typhina*. *Mycologia* **86**: 571-580.

White, J.F., Jr., and Camp, C.R. (1995) A study of water relations of *Epichloë amarillans* White, an endophyte of the grass *Agrostis hiemalis* (Walt.) B. S. P. *Symbiosis* **18**: 15-25.

White, J.F., Jr., Bacon, C.W., and Hinton, D.M. (1997) Modifications of host cells and tissues by the biotrophic endophyte *Epichloë amarillans* (*Clavicipitaceae; Ascomycotina*). *Canadian Journal of Botany* **75**: 1061-1069.

Wightman, R., and Meacock, P.A. (2003) The *THI5* gene family of *Saccharomyces cerevisiae*: distribution of homologues among the hemiascomycetes and functional redundancy in the aerobic biosynthesis of thiamin from pyridoxine. *Microbiology* **149**: 1447-1460.

Wilkinson, H.H., Siegel, M.R., Blankenship, J.D., Mallory, A.C., Bush, L.P., and Schardl, C.L. (2000) Contribution of fungal loline alkaloids to protection from aphids in a grass-endophyte mutualism. *Molecular Plant-Microbe Interactions* **13**: 1027-1033.

Winkler, W., Nahvi, A., and Breaker, R.R. (2002) Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. *Nature* **419**: 952-956.

Yaman, I., Fernandez, J., Liu, H., Caprara, M., Komar, A.A., Koromilas, A.E., Zhou, L., Snider, M.D., Scheuner, D., Kaufman, R.J., and Hatzoglou, M. (2003) The zipper model of translational control: a small upstream ORF is the switch that controls structural remodeling of an mRNA leader. *Cell* **113**: 519-531.

Yarden, O., Plamann, M., Ebbole, D.J., and Yanofsky, C. (1992) *cot1*, a gene required for hyphal elongation in *Neurospora crassa*, encodes a protein kinase. *The EMBO Journal* **11**: 2159-2166.

Yelton, M.M., Hamer, J.E., and Timberlake, W.E. (1984) Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. *Proceedings of the National Academy of Sciences (USA)* **81**: 1470-1474.

Yatzkan, E., and Yarden, O. (1999) The B regulatory subunit of protein phosphatase 2A is required for completion of macroconidiation and other developmental processes in *Neurospora crassa*. *Molecular Microbiology* **31**: 197-209.

Yoshida, J., Umezu, K., and Maki, H. (2003) Positive and negative roles of homologous recombination in the maintenance of genome stability in *Saccharomyces cerevisiae*. *Genetics* **164**: 31-46

Young, C.A., Itoh, Y., Johnson, R., Garthwaite, I., O., M.C., C., M.-F.S., and Scott, B. (1998) Paxilline-negative mutants of *Penicillium paxilli* generated by heterologous and homologous plasmid integration. *Current Genetics* **33**: 368-377.

Young, C.A., McMillan, L., Telfer, E., and Scott, B. (2001) Molecular cloning and genetic analysis of an indole-diterpene gene cluster from *Penicillium paxilli*. *Molecular Microbiology* **39**: 754-764.

Young, C.A., Bryant, M.K., Christensen, M.J., Tapper, B.A., Bryan, G.T., and Scott, B. (2004) Molecular cloning and genetic analysis of a symbiosis-expressed gene cluster for lolitrem biosynthesis from a perennial ryegrass mutualistic endophyte. *Molecular Microbiology (submitted)*.

Young, D.W. (1986) The biosynthesis of the vitamins thiamin, riboflavin, and folic acid. *Natural Product Reports* **3**: 395-419.

Yue, Q., Miller, C.J., White, J.F., Jr., and Richardson, M.D. (2000) Isolation and characterization of fungal inhibitors from *Epichloë festucae*. *Journal of Agricultural Food Chemistry* **48**: 4687-4692.

Zhang, N., Scott, V., Al-Samarrai, T.H., Tan, Y., Spiering, M.J., McMillan, L., Scott, B., Christensen, M., and Schmid, J. (2001) Transformation of *Neotyphodium lolii* with plasmids containing a native promoter disturbs the symbiotic interaction with its host. In *Proceedings of the 4th Neotyphodium/ Grass Interactions Symposium*. Paul, V.H. and Dapprich, P.D. (eds.) Fachbereich Agrarwirtschaft, Soest, Germany, pp. 325-331.

Zhang, N., Liu, Z., Christensen, M.J., Richardson, K., Harding, D., and Schmid, J. (2004) Proteomic analysis of ryegrass-endophyte interactions. In *Proceedings of the 5th International Symposium on Neotyphodium/Grass Interactions*. Kallenbach, R., Rosenkrans, C. and Lock, T.R. (eds.) Fayetteville, Arkansas, USA.