Neotyphodium lolii endophyte improves drought tolerance in perennial ryegrass (*Lolium perenne*. L) through broadly adjusting its metabolism

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Abstract

Perennial ryegrass (*Lolium perenne*) is a widely used pasture grass that is frequently infected by *Neotyphodium lolii* endophyte. The presence of *N. lolii* enhances grass resistance to several biotic and abiotic stresses such as insect, herbivory and drought. Recent studies suggest the effect of *N. lolii* on ryegrass drought tolerance varies between grass genotypes. However, little is known about the molecular basis of how endophytes improve grass drought tolerance, why this effect varies among grass genotypes, or how the endophytes themselves respond to drought stress. This knowledge will not only increase our knowledge of beneficial plant-microbe interactions, but will also guide better use of endophytes, such as selection of specific endophyte - cultivar combinations for growth in arid areas.

In this study, a real time PCR method that can accurately quantify *N. lolii* DNA concentration in grass tissue was developed for monitoring endophyte growth under drought. The effect of *N. lolii* on growth of 16 perennial ryegrass cultivars under drought was assessed, and a pair of endophyte-infected grasses showing distinct survival ability and performance under severe drought stress was selected. The transcriptome profiles of these two endophyte-infected grasses, as well as their clonal endophyte-free grasses, were analyzed using high-throughput RNA sequencing. The expression of endophyte and grass genes responsive to drought was analyzed simultaneously using different bioinformatic tools.

The results demonstrated that *N. lolii* enhanced the growth of perennial ryegrass under drought, but the effect varied between cultivars. On the molecular level, endophytes living in both drought-tolerant and drought-sensitive grasses responded to drought in similar ways, including increased expression of endophyte genes related to scavenging of reactive oxygen species (ROS), DNA replication and the cell cycle, and also reduced expression of genes involved in alkaloids biosynthesis. The presence of endophytes also led to enhanced grass tolerance that was associated with broad adjustments in the plant metabolism. This included up regulation of grass genes

involved in chloroplast maintenance and protection, osmotic adjustment and ROS scavenging capability. The extent of these endophyte-associated effects was greater in the drought tolerant grass genotype than in the drought sensitive genotype.

This work highlights the role of fungal endophytes in grass drought stress tolerance and provides a comprehensive insight into the mechanisms involved.

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1 Introduction

1.1 Plant-microbe symbioses

Plants often establish symbioses with microorganisms (microbes) that are important for survival in difficult environmental conditions (Barrow et al. 2008). The microbes can be either bacteria or fungi, and they can either live their entire or partial life in the plant. Plants benefit from their symbiotic microbes by enhanced growth and resistance to biotic and abiotic stresses. The microbes get a home, nutrients, and sometimes help with dissemination, from plants (Barrow et al. 2008).

Perhaps the most well studied plant-microbe symbioses so far are *Rhizobium* - legume symbioses and Mycorrhizal fungi that colonize roots of many land plants. *Rhizobium* bacteria play a very important role in agriculture by inducing the formation of nodules in which they fix nitrogen (Udvardi and Poole 2013). Some types of mycorrhizal fungi form arbuscules in plant root cells, which can improve host water and nutrient absorption, such as phosphate (Kohler et al. 2008; Smith et al. 2003; Tian et al. 2013). They can also modify nutrient availability to plants by changing soil chemical properties and soil microbial communities (Gamalero et al. 2009; Vivas et al. 2006). Both *Rhizobium* bacteria and mycorrhizal fungi live on carbohydrates produced by plants through photosynthesis.

Other well-studied symbioses are those between cool season grasses (Poaceae, subfamily Poöideae) and their fungal endophytes (family Clavicipitaceae). The relationship between endophyte and grass can be mutualistic, antagonistic or somewhere on the continuum between these two extremes. In a mutualistic symbiosis, the fungal endophyte has lost its sexuality and lives its entire life in intercellular spaces of grass. The grass is asymptomatic and the endophyte is only transmitted vertically through grass seed production (Christensen et al. 2002; Moon et al. 2004). In an antagonistic interaction, the endophyte can prevent plant flowering by causing “choke” disease and is transmitted horizontally between plants (Schardl and Siegel 1993; Western and Cavett 1959).

1.2 Perennial ryegrass - *Neotyphodium lolii* association

1.2.1 Effects of *Neotyphodium lolii* on its host

Perennial ryegrass (*Lolium perenne* L.) is a widely used pasture grass in New Zealand that is often infected with the endophytic fungus *Neotyphodium lolii*. The perennial ryegrass - *N. lolii* association is widely regarded as mutualistic (Latch et al. 1984; Schardl 2001). *N. lolii* confers several benefits to perennial ryegrass, such as enhancing growth and increasing resistance to several biotic and abiotic environmental stressors, such as insects, fungal pathogens and drought (Hahn et al. 2008; Thom et al. 2012; Tian et al. 2008). All these benefits make endophyte-infected perennial ryegrass more competitive than endophyte-free perennial grasses. *N. lolii* also produces several kinds of alkaloids, such as lolitrem B that causes ryegrass staggers – a livestock disease, and ergot alkaloids that cause toxic effects, including hyperthermia, convulsions, reduced fertility, and death (Bacon et al. 1986; Schardl et al. 2013). Both the benefit on grass and toxicity on livestock have a great impact on agricultural productivity (di Menna et al. 2012; Popay et al. 1999; Prestidge 1993). It has been recorded that ryegrass pastures with high endophyte infection rate produced 28% more yield than those with low incidence (Barker and Addison 1993). It was also reported that over 20,000 sheep deaths were due to the injury caused by ryegrass staggers in Australia in 2002 (Reed et al. 2008).

1.2.2 Taxonomy and life cycle of *Neotyphodium lolii*

Neotyphodium lolii, is an asexual type of *Epichloë festucae*, which is a member of fungal division Ascomycota, order Hypocreales, family Clavicipitaceae (Moon et al. 2004). It is likely that *N. lolii* will be re-named *Epichloë festucae* var. *lolii* (Leuchtmann et al. 2014) under the new 'one fungus-one name' system of nomenclature. But because this name change is not yet confirmed, the name *N. lolii* will continue to be used throughout this thesis.

Endophyte hyphae, 1 - 2 μm wide, grow asymptomatically in intercellular spaces of grass leaf sheaths and blades when perennial ryegrass is in the vegetative phase (di Menna et al. 2012). When perennial ryegrass is in a reproductive state, endophyte hyphae can grow into newly forming seed through reproductive tillers, then the endophyte is transmitted vertically into the next grass generation (Clay and Schardl 2002) (Figure 1.1). The *N. lolii* biomass is greater in grass sheath than in leaf blades (Spiering et al. 2005), and no endophyte is found in grass roots (di Menna et al. 2012)

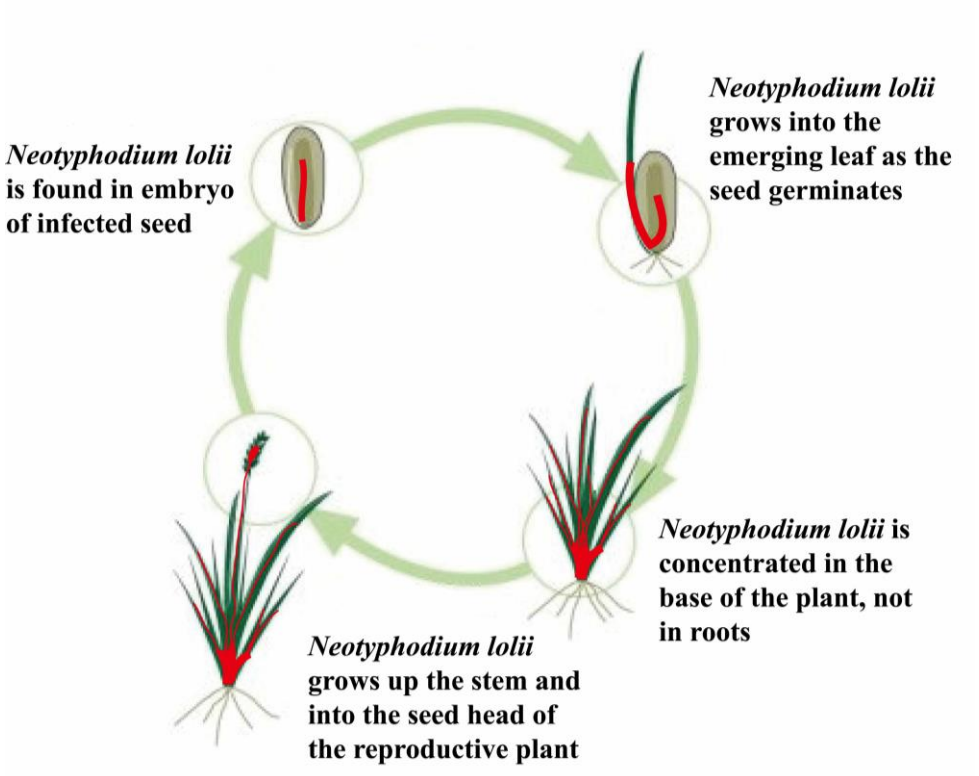

Figure 1.1 *Neotyphodium lolii* life cycle.

The red colour indicates the distribution of *N. lolii*. This figure was modified from a Grasslanz figure (http://www.grasslanz.com).

1.2.3 Secondary metabolites of *N. lolii*

Since the toxicity syndromes experienced by livestock were linked to endophyte infection of grass in the 1980s, there has been considerable progress in research of endophyte secondary metabolites (Gallagher et al. 1981). It was found that *Neotyphodium* and *Epichloë* endophytes produce a range of bioactive compounds: indole-diterpenes such as lolitrem B, peramine, loline alkaloids, ergot alkaloids, as well as other compounds whose bioactive potential has not yet been fully investigated (Cao et al. 2008; Lane et al. 2000; Riedell et al. 1991). These four main classes of alkaloids all provide the host grass with protection from a wide range of invertebrate pests, while the ergot alkaloids and indole diterpenes are also toxic to livestock (Siegel et al. 1990; Thom et al. 2012; Wilkinson et al. 2000).

At the molecular level, gene clusters for the biosynthesis of three major endophyte alkaloids, including ergovaline, indole-diterpenes and peramine, were well studied in the last decade (Fleetwood et al. 2007; Schardl et al. 2006; Schardl et al. 2013; Tanaka et al. 2005; Young et al. 2005; Young et al. 2006; Young et al. 2009).

Eleven *ltm* genes, distributed in three clusters at one locus, were found to be required for lolitrem B biosynthesis (Figure 1.2) (Schardl et al. 2013; Young et al. 2009). In all *Neotyphodium lolii* strains used in their study only one strain, Lp19 (also called AR42), contained the whole *LTM* locus and could produce lolitrem B. In contrast two genes, *ltmE* and *ltmJ*, were missing in non- lolitrem B producing *N. lolii* strains such as AR1, Lp14 (also called AR37) and Lp1 (also called AR6) (Young et al. 2009).

A single gene, *perA*, encoding a multifunctional non-ribosomal peptide synthetase, was sufficient for peramine biosynthesis (Tanaka et al. 2005). It was also noticed that the *perA* gene was highly variable among endophytes. Some strains (such as *Epichloë festucae* Fl1) contain the entire *perA* gene, whilst some (such as *Epichloë festucae*

E2368) only have a partial gene, and others (such as *Epichloë glyceriae* E277) have no *perA* gene at all (Schardl et al. 2013).

It has been found that 11 *lol* genes are required for loline biosynthesis (Figure 1.3) (Schardl et al. 2013). The presence of *lol* genes is variable among endophytes, even among different strains within the same species. For example, in the genome of a loline-producing *Epichloë festucae* strain (E2368) all 11 *lol* genes are present, while no *lol* genes are found in the genome of another strain (F11) that does not produce lolines (Schardl et al. 2013).

Ergovaline also requires 11 genes (*EAS* genes) distributed in three clusters (Figure 1.4) (Schardl et al. 2013). However not all endophytes that contain all 11 *EAS* genes can produce ergot alkaloids, for example *Epichloë festucae* E2368 (Schardl et al. 2013). Lack of ergot alkaloid production in this strain was suggested to be due to silencing of gene expression silence by insertion of miniature inverted-repeat transposable elements (MITEs) (Fleetwood et al. 2011).

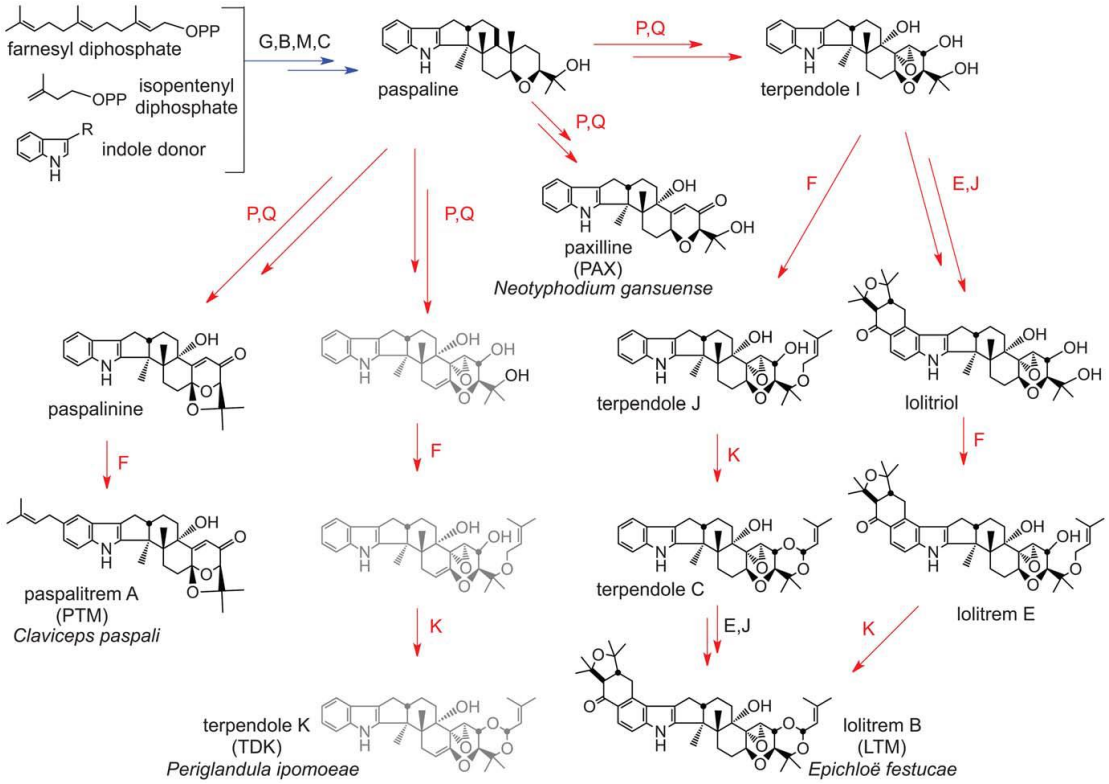

Figure 1.2 Summary of indole-diterpene biosynthesis pathways.

Arrows indicate one or more steps catalyzed by products of the genes indicated, where each *ltm* gene is designated by its final letter (*G* = *ltmG*, etc.). Arrows and genes in blue indicate steps in synthesis of the first fully cyclized intermediate (paspaline). Arrows and genes in red indicate steps in decoration of paspaline to give the variety of indole-diterpenes in the Clavicipitaceae. Structures shown in gray are not yet verified. Reproduced from Schardl *et al.* (2013). *PLoS Genetics* **9:** 1-26.

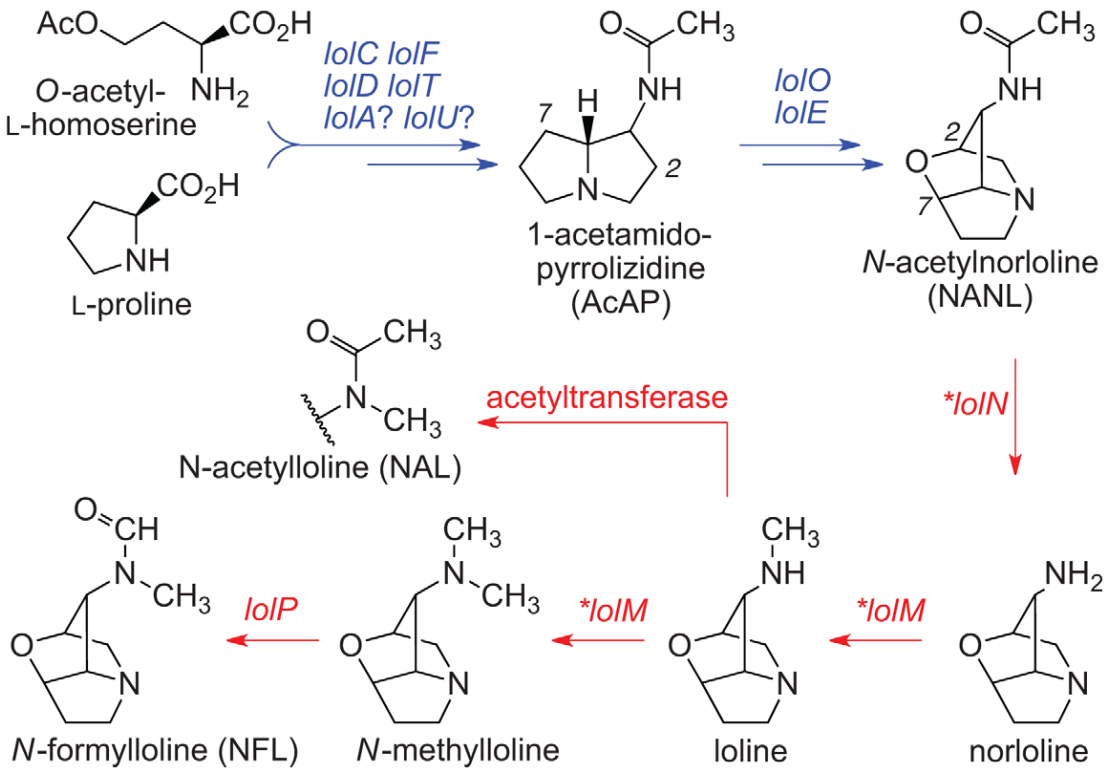

Figure 1.3 Summary of loline alkaloid-biosynthesis pathway.
Arrows indicate one or more steps catalyzed by products of the genes indicated. Arrows and genes in blue indicate steps in synthesis of the first fully cyclized intermediate (NANL). Arrows and genes in red indicate steps in modification of NANL to give the variety of lolines found in the *Epichloë*. Asterisks indicate *lol* genes that were newly discovered in the genome sequence of *E. festucae* E2368. Reproduced from Schardl *et al.* (2013). *PLoS Genetics* **9:** 1-26.

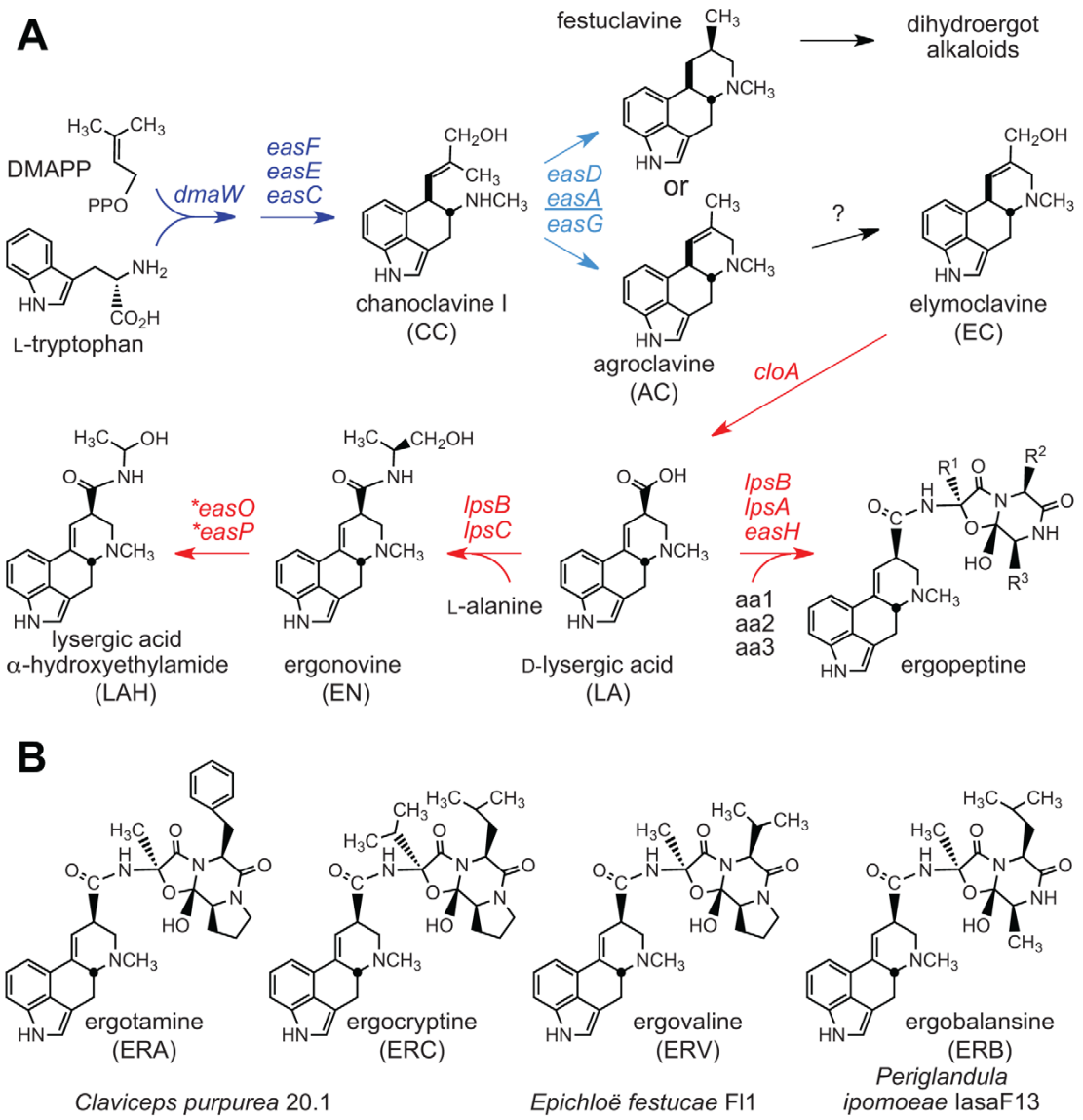

Figure 1.4 Summary of ergot alkaloids biosynthesis pathway.

(A) Ergoline alkaloid biosynthesis pathways in the Clavicipitaceae. Arrows indicate one or more steps catalyzed by products of genes indicated. Arrows and genes in blue indicate steps in synthesis of the first fully cyclized intermediate (skeleton). Variation in the *easA* gene (underlined) determines whether the ergoline skeleton is festuclavine or agroclavine. Arrows and genes in red indicate steps in decoration of the skeleton to give the variety of ergolines in the Clavicipitaceae. Asterisks indicate genes newly discovered in the genome sequences of *C. paspali*, *N. gansuense* var. *inebrians* and *P. ipomoeae*. (B) Ergopeptines produced by different endophytes. Reproduced from Schardl *et al*. (2013). *PLoS Genetics* **9:** 1-26.

1.2.4 Selected fungal endophytes

In order to utilize the benefits of endophytes, while excluding toxic effects on livestock, several selected endophyte strains which produce fewer, or no, animal toxic alkaloids have been selected and used in pasture grasses in the last decade. The AR1 strain, which produces peramine but not ergovaline or lolitrem B, has been widely used in New Zealand since 2001 (Thom et al. 2012). The effects of AR1 on perennial ryegrass agronomy and the health and productivity of grazing livestock have been intensively studied. It was shown that AR1 increases grass resistance to insects (e.g. Argentine stem weevil), enhances pasture herbage yield compared with endophyte-free, reduces fly strike, increases livestock weight gain and milk solids compared to NZCT (New Zealand common toxic *Neotyphodium lolii*), and does not cause ryegrass staggers (Fletcher 1999; Popay and Baltus 2001; Popay et al. 1999).

Another selected endophyte strain AR37, named Lp14 in scientific studies (Fleetwood et al. 2011; Schardl et al. 2012; Tanaka et al. 2005; Young et al. 2009), does not produce peramine, lolitrem B or ergovaline, but is known to produce epoxy-janthitrem, a pest deterrent chemical. Testing to date has shown that AR37 increases grass resistance to a wide range of pests including Argentine stem weevil, black beetle, root aphid, pasture mealybug and porina (Popay and Thom 2009). It is also more agronomically robust than NZCT and has similar animal performance levels to AR1, although it can cause ryegrass staggers, due to production of epoxy-janthitrems (Hume et al. 2007; Thom et al. 2013).

1.3 Endophyte detection and quantification

Nowadays, selected endophytes are widely used in pastures (Parish et al. 2013; Thom et al. 2012). However, relatively little is known about the survival and persistence of selected endophytes in pasture when in competition with common toxic strains (Hume and Barker 2005) and to what extent this may be influenced by stresses such as herbivory and climatic factors such as drought. Information on proportions of various endophyte strains in pastures could facilitate better use of the selected endophytes, for example, through the monitoring of toxic endophyte invasion in pastures. Until recently, the only reliable methods for assessing amounts of endophyte

biomass in infected tissues were hyphal counting in tissue cross sections under the microscope (Tan et al. 2001) and enzyme linked immunosorbent assays (ELISA) (Ball et al. 1995). Hyphal counting is an accurate method that determines endophyte biomass directly, however, it is time consuming and not suitable for bulk grass samples. Compared to hyphal counting, ELISA is more efficient, but is an indirect measure that depends on endophyte antigen concentration, which may vary between samples due to a multitude of factors such as differences in gene expression. Recently, quantitative PCR and real time PCR have been applied to endophyte quantification in several studies (Charlton et al. 2012; Hahn et al. 2008; Panaccione et al. 2001; Rasmussen et al. 2007; Young et al. 2005). Some of these studies used a ratio of endophyte DNA to plant DNA to reflect endophyte biomass. However, their method did not distinguish between different types of endophytes or verify the degree of correlation between their measurements and endophyte biomass.

Another problem facing seed suppliers and farmers is contamination of commercial selected endophyte-infected seed with common toxic strains (Dombrowski et al. 2006). In New Zealand no more than 5% of NZCT is permissible in traded commercial selected endophyte seed. The level of NZCT has been determined by HPLC and more recently by ELISA, which estimates the proportion of NZCT seeds on the basis of alkaloid content (lolitrem B). However, endophyte alkaloid concentrations are affected by many factors, such as temperature (Brosi et al. 2011), season (Moate et al. 2012), nutrients (Rasmussen et al. 2007) and drought (Hahn et al. 2008). This means the alkaloid content could under- or over-estimate endophyte contamination in grass seeds collected from different places with different environmental conditions. Another approach to detect and quantify strains in pastures and grass seeds is to use endophyte-SSR (simple sequence repeats), but this method is not as sensitive as real time PCR (Bluett et al. 2005; Briggs et al. 2007). A fast, sensitive, reliable and more versatile method that can directly determine different types of endophyte would assist in the deployment and management of selected endophytes.

1.4 Response of plants to drought stress

1.4.1 General considerations

Drought is one of the prime agricultural stresses all over the world (Katyal and Petrisor 2012). Pasture productivity losses caused by drought are considerable (Nandintsetseg and Shinoda 2013). Drought leads to plant cellular dehydration and causes osmotic stress, which decreases the rate of plant cell division and elongation and leads to growth arrest (Kantar et al. 2011). Plants have evolved a range of physiological and biochemical responses to cope with drought stress, such as reducing transpiration, increasing osmotic adjustment and enhancing antioxidant capability (Bhargava and Sawant 2013). The first step of plant acclimation to drought stress is stress perception (Bhargava and Sawant 2013; Shinozaki and Yamaguchi-Shinozaki 2007). An efficient perception system enables a plant to take prompt action in adapting to drought conditions, thereby increasing its chances of survival.

1.4.2 Stress perception and signalling

Plant stress perception is achieved by receptor-like kinases (RLKs) and histidine kinases (HKs), which regulate downstream signalling networks.

RLKs are a group of membrane localized transmembrane proteins with variable N-terminal extracellular domains and C-terminal intracellular serine/threonine kinase domains. They convey signals through phosphorylation of target proteins in the cytoplasm. (Shiu and Bleecker 2001). There are over 600 and 1000 predicted RLK encoding genes in *Arabidopsis* and rice, respectively (Marshall et al. 2012). It has been reported that some RLKs, including cysteine-rich RLK (CRK36), proline-rich-extensin-like-RLK4 (PERK4), and guard cell hydrogen peroxide-resistant1 (GHR1), are involved in water stress signalling in *Arabidopsis* (Abe et al. 2003; Bai et al. 2009; Busk and Pages 1998; Hua et al. 2012; Lee et al. 2011; Osakabe et al. 2005; Osakabe et al. 2010; Tanaka et al. 2012). In rice, over expression of some RLK encoding genes enhanced plant drought tolerance, such as OsSIK1 (*O. sativa* stress induced protein kinase gene 1), OsRLCK253 (*O. sativa* receptor-like cytosolic kinase 253) and PSTOL1 (phosphorus starvation tolerance 1) (Gamuyao et al. 2012; Giri et al. 2011; Ouyang et al. 2010).

HKs are members of histidine-to-aspartate (His-Asp) phosphorelays that regulate stress signals (Mizuno 2005). It has been found that several HKs, such as AHK2, AHK3, and AHK4 regulate stress signalling in *Arabidopsis*, and mutations of these genes showed enhanced drought tolerance (Jeon et al. 2010; Tran et al. 2007).

In response to stress perceived by RLKs and HKs, plants generate second messengers, such as inositol phosphates (InsP) and reactive oxygen species (ROS) (Mahajan and Tuteja 2005). InsP and ROS can modulate intracellular Ca^{2+} levels and activate protein phosphorylation cascades, including mitogen-activated protein kinase (MAPK), calcium-dependent protein kinase (CDPKs) and protein phosphatase, to initiate cellular defence responses by transcriptional regulation (Xiong et al. 2002).

1.4.3 Transcriptional responses to drought stress

The regulatory pathways involved in response to drought stress belong to two different groups: ABA (abscisic acid)-dependent and ABA-independent (Shinozaki et al. 2003). ABA is a phytohormone involved in regulating a broad range of processes, including stress signal transduction pathways, stomata closure, plant growth and development (Hubbard et al. 2010). Different TFs are involved in ABA-dependent or ABA-independent pathways and each TF can regulate the expression of many genes (Shinozaki and Yamaguchi-Shinozaki 2007).

An ABA-responsive element (ABRE) with a motif of (C/T) ACGTG(G/T)C is a key cis-acting element in ABA-responsive gene expression (Busk and Pages 1998). Two bZIP (basic leucine zipper) transcription factors can activate an ABA responsive gene, such as *RD29B*, by binding to ABRE (Uno et al. 2000). In Arabidopsis, TFs MYB2 and MYC2 were shown to bind on the promoter of the same ABA-responsive gene, *RD22*, and activate its expression co-operatively (Abe et al. 2003). Another ABA-dependent TF, RD26_NAC can activate the expression of downstream target gene- *Gly*, encoding a glyoxalase (a glutathione-dependent detoxification enzymes) (Dixon et al. 1998; Fujita et al. 2004).

The ABA-independent regulation pathway has also been intensively studied in *A. thaliana*. Important cis-acting regulatory elements associated with this pathway are those in the DRE (dehydration-responsive element)/CRT (C-RepeaT) class, with a

motif of G/ACCGAC (Lata and Prasad 2011). Transcription factors such as DREB2 (dehydration responsive element binding), which belongs to the ERF (ethylene response factor) /AP2 (APETALA2) family, can activate the expression of DRE/CRT element containing genes, such as *RD29A*, and improve drought tolerance in transgenic *Arabidopsis* (Sakuma et al. 2006). Similarly TF HD-ZIP_NAC (homeodomain-Leucine zipper) is involved in activating a nuclear gene - *ERD1* (early responsive to dehydration 1) in response of drought stress (Nakashima et al. 1997; Shinozaki and Yamaguchi-Shinozaki 2007).

1.4.4 Metabolic responses to drought stress

Plants produce a variety of molecules in response to drought stress. The function of some of these molecules is to achieve osmotic adjustment. Osmotic adjustment is defined as a lowering of osmotic potential due to net solute accumulation in response to water stress (Girma and Krieg 1992). It is an important trait for postponing dehydration under drought conditions because it maintains cell turgor and physiological processes as water deficits develop (Turner et al. 2007). The sooner and greater a plant can reduce its osmotic potential, the better it can adapt to drought (Turner et al. 2007). Sugars and proline are important types of molecules with a function of osmotic adjustment that help the plant cells to retain water under drought conditions. A strong correlation between sugar (sucrose and hexose) or sugar alcohols (mannitol, trehalose, galactinol, and raffinose) and osmotic stress in soybean and *Arabidopsis thaliana* has been reported (Streeter et al. 2001; Taji et al. 2002; Valliyodan and Nguyen 2006). Proline is an important osmolyte induced under drought stress (Chen and Jiang 2010; McCue and Hanson 1990).

Some drought induced proteins, including late embryogenesis abundant (LEA) proteins and heat shock proteins (Hsps), are chaperones which can protect other proteins under adverse stress. LEA proteins are widely distributed major stress-responsive proteins that protect other proteins from aggregation due to desiccation or osmotic stresses (Goyal et al. 2005). Hsps are usually not expressed under normal conditions, but under adverse stress they play important roles in protein folding, assembly, and protection (Feder and Hofmann 1999). The functions of LEA proteins and Hsps have been well studied, and genes encoding LEA proteins and

HSPs have been used to enhance drought tolerance in transgenic maize and rice (Amara et al. 2013; Liu et al. 2013; Zou et al. 2012).

As well as protecting certain proteins, plants under stress also produce proteolytic enzymes that play a very important role in degrading damaged proteins and thereby mobilizing nitrogen reserves. The increase of proteolysis under drought stress has been found in several studies (de Carvalho et al. 2001; Simova-Stoilova et al. 2010a).

Reactive oxygen species (ROS), including singlet oxygen, superoxide anion radicals, hydroxyl radicals and hydrogen peroxide, are natural byproducts of normal metabolism, such as photosynthesis (Asada 2006). They play an important role in signal transduction, and can be scavenged by antioxidant chemicals and enzymes under normal conditions. However, elevated levels of ROS that are produced under stress are toxic, can cause oxidative damage to proteins, lipids and DNA, and can lead to cell death. Thus plants increase the production of various antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and ascorbate peroxidase (APX), as well as non-enzymatic chemicals such as phenolic compounds, alkaloids, ascorbic acid (ASH), glutathione, (GSH) etc., to protect cells from oxidative damage through scavenging the over-produced ROS (Gill and Tuteja 2010).

1.5 The role of microbes in plant drought tolerance

1.5.1 Plant growth promoting rhizobacteria

Plant growth promoting rhizobacteria (PGPR) are a group of root-colonizing bacteria that enhance plant growth and resistance to diseases caused by plant pathogenic fungi, bacteria, viruses and nematodes (Kloepper et al. 2004). Furthermore PGPR also enhance plant tolerance to abiotic stresses, such as drought and salinity (Mayak et al. 2004a; Timmusk and Wagner 1999; Yang et al. 2009).

One example of a PGPR species is *Paenibacillus polymyxa*, a common endospore-forming soil bacterium that enhances drought tolerance of *A. thaliana.* Plants inoculated with *P. polymyxa* grew significantly better than non-treated plants under drought conditions (Timmusk and Wagner 1999). A set of *A. thaliana* genes that were induced in response to *P. polymyxa* were found; among these, a

drought-response gene ERD15 (early responsive to dehydration 15), was expressed at high levels only in inoculated plants under drought conditions (Timmusk and Wagner 1999).

Another PGPR, *Achromobacter piechaudii* (strain ARV8) promoted growth and also enhanced drought tolerance of tomato and pepper seedlings (Mayak et al. 2004b). The presence of the bacterium also improved recovery of tomato and pepper from drought after watering was resumed, and suppressed the production of ethylene, a plant growth inhibiting hormone (Burg 1973), by tomato under drought which might have enabled continued plant growth under water stress (Mayak et al. 2004b).

It is also reported that PGPR can interact with arbuscular mycorrhizal (AM) fungi to improve plant drought tolerance. Proline accumulation and peroxidase (POX) activity in *Lactuca sativa* L. cv. Tafalla leaves were increased by co-inoculation with both a PGPR, *Pseudomonas mendocina* Palleroni, and an arbuscular mycorrhizal fungus, *Glomus intraradices* (Schenk & Smith) or *Glomus mosseae* (Nicol & Gerd.) Gerd. & Trappe, under severe drought (Kohler et al. 2008). This suggests that PGPR might help play a role in alleviating oxidative damage to plant cells caused by drought.

1.5.2 Root colonizing fungi

Plants can be considered as a big habitat colonized by various microorganisms. Among these microorganisms, root colonizing fungi, such as the endophyte *Pirifomospora indica*, *Trichoderma* fungi and arbuscular mycorrhizal fungi (AMF), show beneficial interactions with their host plant by promoting plant growth and enhancing plant drought tolerance (Sherameti et al. 2008; Sun et al. 2010).

Recently, it was reported that *P. indica* confers fitness benefits to plants under water stressed conditions. *A. thaliana* seedlings co-cultivated with *P. indica* continued growing under mild drought stress, while the untreated control stopped growing (Sherameti et al. 2008). Seedlings colonized by this fungus had three-fold higher fresh biomass, two fold higher chlorophyll content and significantly higher seed production compared to fungus-free seedlings. *P. indica* colonization induced up regulated expression of various drought stress response genes in *A. thaliana*, including:

response to dehydration 29A (RD29A), early response to dehydration1 (ERD1), *Arabidopsis* NAC domain containing protein 72 (ANAC072), dehydration-response element binding protein 2A (DREB2A), salt and drought-induced ring finger1 (SDIR1), calcineurin b-like protein 1 (CBL1), cbl-interacting protein kinase 3 (CIPK3), and histone acetyltransferase (HAT) (Sherameti et al. 2008). In another study *P. indica* improved tolerance of Chinese cabbage (*Brassica campestris* L. ssp. *Chinensis*) under polyethylene glycol mimicked drought stress (Sun et al. 2010). The rates of decline of photosynthetic efficiency and degradation of chlorophylls and thylakoid proteins caused by drought were slowed down by the presence of *P. indica*. Compared with non-inoculated plants, activities of several antioxidant enzymes, such as superoxide dismutases (SOD), catalases (CAT) and peroxidases (POD) were significantly higher in *P. indica* inoculated plants after 24 hours under water stress. *P. indica* also induced expression of various drought responsive genes, such as orthologs of DREB2A, CBL1, ANAC072 and RD29A in leaves of Chinese cabbage under drought conditions (Sun et al. 2010).

Trichoderma spp. are widely distributed in soil and can form mutualistic relationships with many plant species (Harman et al., 2004). They have very important roles in increasing crop yields under field conditions and protecting crops against diseases (Harman et al., 2004). Strains of several species, such as *T. harzianum*, *T. viride* and *T. hamatum*, have been developed as biocontrol agents (Harman 2000). It has been reported recently that some *Trichoderma* fungi can also improve plant drought tolerance. For example the presence of *T. harzianum* significantly increased wheat (*Triticum aestivum* cv. Talhue´n) dry biomass and seed production under water deficit conditions compared to irrigated conditions (Donoso et al. 2008). A mixture of four *Trichoderma* isolates induced expression of several drought responsive genes in *Theobroma cacao* (cacao), including EST and P1 involved in polyamine biosynthesis, and P3 involved in aquaporin biosynthesis respectively (Bailey et al. 2006). Further work on a *Theobroma cacao*/*Trichoderma harzianum* association showed that the presence of the fungus (strain DIS 219b) delayed drought-induced changes in plant stomatal conductance and net photosynthesis (Bae et al. 2009).

Strains of *T. harzianum* have been associated with drought tolerance in other agricultural systems. Field trials showed that *T. harzianum* strain T-22 increased drought tolerance of sweet corn through improving plant root system development (Harman 2000). *T. harzianum* has also been shown to improve drought tolerance in rice (*Oryza sativa* L.) and tomato (Mastouri et al. 2012; Shukla et al. 2012). Rice seedlings colonized with *T. harzianum* showed a slower wilt, and lower proline, MDA and H_2O_2 contents, and a higher phenolics concentration and membrane stability index (MSI) in response to drought, compared with non-colonized seedlings (Shukla et al. 2012). *T. harzianum* strain T-22 significantly increased tomato dry weight and chlorophyll content under water deficit conditions (Mastouri et al. 2012). The benefit of *Trichoderma harzianum* T22 on tomato drought tolerance might be due to enhancing plant ROS scavenging capability through induction of activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Mastouri et al. 2012).

Arbuscular mycorrhizal fungi (AMF) are another important group of fungi associated with stress tolerance in plants. AMF colonize roots of many plant species and improve the absorption of water and nutrients from soil to plant. In return, the fungus establishes an internal structure to absorb carbohydrates synthesized by the plant through photosynthesis. The role of AMF in protection of plants from diseases caused by pathogenic fungi, bacteria and nematodes, and abiotic stresses, including salinity, low temperature, heavy metals and water deficit, has been well explored. It was found that inoculation of AMF (*Glomus versiforme* and *Paraglomus occultum*) stimulated growth and improved drought tolerance of Sacha Inchi (*Plukenetia volubilis* L.), an oilseed crop indigenous to the rain forests of the Andean region of South America (Tian et al. 2013). This was achieved through AMF-induced alterations in the plants' morphology (larger root volume), physiology (greater photosynthetic rate and water-use efficiency) and biochemistry (higher activity of antioxidant enzymes, including guaiacol peroxidase (GPX) and catalase (CAT)) (Tian et al. 2013). The phenomenon of AMF improving plant drought tolerance through enhancing antioxidant enzyme activity was also reported in other studies using *Chlorophytum borivilianum*, a medicinal herb, and *Poncirus trifoliate* (Trifoliate Orange), (Fan and Liu 2011; Sushma and Tarafdar 2012). Some drought responsive genes of AMF were also identified by their up-regulation under water deficit

conditions. In carrot (*Daucus carota* L.) grown under drought stress, the AMF *G. intraradices* showed induced expression of GiBiP, a molecular chaperone binding protein (BiP) gene (Porcel et al. 2007). BiP is an essential water stress response protein (Alvim et al. 2001) that can prevent protein misfolding, aggregation or loss of function under abiotic stresses (Hendershot et al. 1996). A 14-3-3 protein encoding gene, *Gi14-3-3*, was also found highly expressed in *G. intraradices* when the host plant (*Daucus carota* L) was subjected to drought stress (Porcel et al. 2006). 14-3-3 protein belongs to a family of conserved regulatory molecules, and plays an essential role in stress resistance (Chung et al. 1999; Roberts and de Bruxelles 2002). Besides these laboratory studies, it was reported that AM fungi *Glomus mosseae* or *G. etunicatum* also increased wheat (*T. Aestivum* L.) biomass and grain yields in the field under drought stress conditions (Al-Karaki et al. 2004).

1.5.3 *Epichloë* and *Neotyphodium* endophytes

Epichloë and *Neotyphodium* species are endophytes of cool season grasses that have been widely studied due to their importance in pastoral grass productivity. In spite of causing toxicity to livestock, these endophytes showed an ability to enhance grass resistance to several biotic and abiotic stresses (Briggs et al. 2013; Nagabhyru et al. 2013; Sabzalian and Mirlohi 2010; Tian et al. 2008). Many studies reported that plants infected with *Epichloë* and *Neotyphodium* endophytes performed better under drought conditions than endophyte-free plants (Elmi and West 1995; Hahn et al. 2008; Zhang and Nan 2007). Compared with endophyte-free plants, endophyte-infected plants had greater biomass and more tillers at the end of the water stress period (Hahn et al. 2008; Rudgers and Swafford 2009; Zhang and Nan 2007). These effects might have been caused by either morphological or physiological effects of endophyte infection. Several studies reported that, compared with endophyte-free plants, endophyte infected grass had a more extensive root system, which improved plant water acquisition (Malinowski et al. 1997; Malinowski et al. 1999). It was also reported that endophyte infection increased the rate of plant stomata closure under drought conditions so that reduced water loss would occur (Elmi and West 1995). In respect of physiological modifications, it was found that endophyte infected plants had more osmolytes, including water soluble sugars such as proline, mannitol and arabitol, and probably fungal alkaloids such as loline, compared to endophyte free

plants (Abernethy et al. 1998; Abernethy and McManus 1998; Bacon 1993; Richardson et al. 1992). It was also observed that endophyte infected plants had higher water use efficiency than endophyte-free plants under drought conditions, which makes them able to produce more biomass (Elmi and West 1995). Several other studies reported that endophytes enhanced plant drought tolerance by increasing the production of antioxidants, such as flavonoids, phenolic and antioxidative enzymes, which play a very important role in reducing cell damage caused by over produced reactive oxygen species (ROS) under water stress (Zhang and Nan 2007).

However, there are inconsistencies in the literature on the effect of *Epichloë* and *Neotyphodium* endophytes on grass drought tolerance. Several publications suggest that the effect of *Epichloë* and *Neotyphodium* endophytes on grass drought tolerance is variable and depends on host genotype and fungal haplotype (Cheplick 2008; Morse et al. 2007; Oberhofer et al. 2014). Some researchers also suggested that *Epichloë* and *Neotyphodium* endophytes had no, or even detrimental, effect on grass drought tolerance (Cheplick 2004; Cheplick 2007; Cheplick et al. 2000). These inconsistencies might be due to different growth conditions and drought scenarios that were used. In the field, but also in the glasshouse, many compounding factors such as plant pathogens, insect herbivory and changing environmental factors can affect the outcome of experiments. Furthermore the grass – endophyte association is a complex system, and it is difficult to determine whether responses measured in the plants, such as ROS levels, are due mainly to plant responses, endophyte responses, or both. Both partners need to be studied in detail and under tightly controlled conditions, preferably using technology that enables us to distinguish between the stress responses of the two organisms even though they are growing in close association with one another.

1.6 Transcriptome profiling of plant-microbe symbioses

Regardless of whether plant-microbe symbioses are beneficial, detrimental or neutral, the symbiotic partners have a complex relationship and affect each other on physiological and molecular levels. It is therefore essential to study plant and microbe simultaneously if we want to unravel their interactions. Transcriptome profiling, using high-throughput RNA-sequencing is an advanced technique that provides large-scale gene expression of both plant and microbe at the same time.

Although many transcriptomics studies have been conducted on plant and root colonizing endophytes, such as arbuscular mycorrhizal fungi and *Trichoderma* fungi (Bischof et al. 2013; Chacon et al. 2007; Franken and Krajinski 2006; Guimil et al. 2005; Salvioli et al. 2012; Siciliano et al. 2007; Tromas et al. 2012; Trushina et al. 2013), relatively few have been conducted on grass - endophyte symbioses (Ambrose and Belanger 2012; Eaton et al. 2010). Felitti, Shields, Ramsperger *et al*. (2006) generated two cDNA-based microarrays (NchipTM microarray and EndoChipTM microarray). They compared *N. lolii* gene expression under growth in solid and liquid culture, and explored different gene expression patterns between *N. coenophialum* and *N. lolii* grown in liquid culture (Felitti et al. 2006). Dinkins, Barnes and Waters (2010) used an Affymetrix Wheat Genome Array GeneChip® and a Barley1 Genome Array GeneChip® to identify genes differentially expressed between *N. coenophialum* infected and endophyte-free tall fescue (*Lolium arundinaceum*). Although transcriptomics data were obtained in the two studies above, only one partner of the symbiosis, either plant or fungus, was analyzed.

A few studies investigated the transcriptome of *Epichloë* or *Neotyphodium* endophytes and their host grasses simultaneously. Johnson, Johnson, Schardl et al. (2003) identified differentially expressed genes of both *Neotyphodium coenophialum* and tall fescue using suppression subtractive hybridization (SSH). There were 29 *N. coenophialum* infection induced grass genes found in this study (Johnson et al. 2003; Khan et al. 2010). Their results revealed that plant carbohydrate metabolism and photosynthesis were down-regulated by endophyte infection. Besides these, 24 differentially expressed endophyte transcripts, related to pathogenicity/virulence or fungal secondary metabolism, were found during symbiosis. Recently, high-throughput mRNA sequencing (RNA-seq) was used to explore the role of the fungal stress-activated protein kinase (*sakA*) in the establishment and maintenance of the mutualistic interaction of *Epichloë festucae* - perennial ryegrass (*L. perenne*) (Eaton et al. 2010). By comparative analysis of global gene expression of wild type and mutant (*sakA* deletion) *E. festucae* infected perennial ryegrass, these authors found differently expressed fungal genes related to proliferative growth *in planta* and also of plant genes relating to pathogen defence and transposon activation (Eaton et al. 2010). Another study used SOLiD-SAGE, a high-throughput adaptation of serial analysis of gene expression (SAGE), to compare the transcriptomes of endophyte-free

and *E. festucae*-infected strong creeping red fescue (*Festuca rubra* L. subsp. *rubra*) (Ambrose and Belanger 2012). Over 200 differently expressed plant genes induced by endophyte infection were found, and endophyte genes encoding secreted proteins were found highly expressed in the symbiosis (Ambrose and Belanger 2012).

Transcriptomics, especially high-throughput mRNA sequencing, is a very powerful approach for studying plant-microbe symbioses. It not only generates a large amount of information very quickly, but also provides the opportunity to study gene expression of multiple species simultaneously. So far, all transcriptomics studies on grass-fungal endophyte associations have been conducted under 'normal' un-stressed environmental conditions. Considering the special benefits of fungal endophytes on host resistance to biotic and abiotic stresses, transcriptome profiling studies conducted under stressed conditions would provide valuable clues for improving beneficial aspects of this important association and thereby increase agricultural productivity.

1.7 Aims and Objectives

There were two main aims in this research. The first aim was to develop a real time PCR method that can quantify *N. lolii* in perennial ryegrass accurately. The second was to determine the molecular basis of how *N. lolii* confers drought tolerance in perennial ryegrass.

Aim 1: Development of a real time PCR method of *Neotyphodium lolii in planta*

The primary purpose was to provide an accurate quantification tool for use in subsequent drought tolerance experiment. However because a reliable method to distinguish between ecotypes of *N. lolii* with different alkaloid profiles is needed to facilitate monitoring the persistence of selected non-toxic endophytes in pasture, this work was extended to provide such tools that will have practical applications. This aim was achieved by addressing the following objectives:

1. Select target genes and design primers and probes.
   In this objective, endophyte secondary metabolite genes (*ltmG, ltmJ, perA*) and a perennial ryegrass specific gene (*TB1*) were selected. Primers and Taqman probes were designed based on sequences of these genes.

2. Assess assay specificity, efficiency and reliability.
   Endophyte and grass primer/probe combinations were tested against other fungal or plant DNA. Standard curves were made for each primer/probe combination. The real time PCR method was validated using other quantitative methods.

3. Test the applicable value of the developed real time PCR method.
   The real time PCR method was used to test for endophyte contamination in grass seed and herbage samples.

Aim 2: Determine the molecular basis of drought tolerance in the *Neotyphodium lolii* - perennial ryegrass association.

This aim involved determining the influence of *N. lolii* on tolerance of perennial ryegrass in drought-tolerant and drought-sensitive grass genotypes and identification of both endophyte and plant genes involved in drought tolerance. This was achieved by addressing the following objectives:

1. Select grass materials for transcriptome profiling.

   To do this, grass genotypes of the same cultivar showing different levels of drought tolerance were selected from glasshouse trials and validated in tightly controlled experiments conducted in a growth chamber. Clonal endophyte-free grasses were generated and endophyte strains were identified.

2. Prepare samples for high throughput RNA sequencing.

   In this objective, grass samples were collected under drought and control conditions, and physiological measurements were taken. Endophyte DNA levels were determined using the real time PCR method developed in Aim 1 and total mRNA of grass samples isolated and sequenced.

3. Identify candidate endophyte genes, and endophyte-induced grass genes, involved in grass drought tolerance.

   Bioinformatic analysis was conducted on both grass and endophyte sequence reads to identify differentially expressed grass and fungal genes. Pathway analysis was undertaken on these differentially expressed genes and the findings related to physiological measurements from the samples.

2 Material and Methods

2.1 Biological material

NZCT (*N. lolii*), AR1 (*N. lolii*) and AR37 (*Neotyphodium sp.*) fungal cultures were supplied by AgResearch (Palmerston North, New Zealand) and maintained on 2.4% potato dextrose agar (PDA; Difco Laboratories, Detroit, Michigan, USA) at 22℃ as described previously (Moon et al. 1999). These three endophytes have different alkaloid production profiles: NZCT produces ergovaline, lolitrem B and peramine (Lane et al. 2000), AR1 only produces peramine but not ergovaline and lolitrem B (Thom et al. 2013), and AR37 produces none of these three kinds of alkaloids (Tanaka et al. 2005; Young et al. 2009).

Mature plants of NZCT-infected, AR1-infected, AR37 -infected or endophyte-free ryegrass (*Lolium perenne*) and clover (*Trifolium repens*) were provided by AgResearch, Palmerston North, New Zealand, and were grown in an unheated glasshouse without artificial illumination in September 2010. Nine clonal NuiD ryegrass plants (the same clones as those used by (Zhang et al. 2011) infected with Lp19 endophyte (French wild-type *N. lolii*) were used for endophyte biovolume measurements. These were grown from single tillers for 22 days in a controlled environment plant growth chamber (CAT630, Contherm Scientific Ltd, New Zealand), at 15ºC (13.7-17ºC) and with a light intensity of about 700 μmol/m^2/s for 12/24h with a relative humidity of 50%.

Grass seeds of 11 cultivars of *Lolium perenne* and 5 cultivars of *L. boucheanum* used in drought tolerance assays (as shown in Table 2.1) were provided by AgResearch (Palmerston North, New Zealand).

Three *L. perenne* ryegrass samples (each consisting of >50 tillers removed at ground level) used in multiplex real time PCR assays were collected from fields previously sown with seed of cultivar ‘Grasslands Samson’ infected with NZCT, AR1 or AR37, at AgResearch, Lincoln, New Zealand.

Table 2.1 Grasses used in real time PCR evaluation.

Cultivar or line ID	Ploidy and ryegrass species [a]	Accession number [b]			
		Endophyte -free	NZCT -infected	AR1 -infected	AR37 -infected
Commando	Diploid ryegrass	A14128	A15864	A14127	A15863
Grasslands Nui	Diploid ryegrass	A13509	A14129	A11678	A10533
Grasslands Samson	Diploid ryegrass	A16694	A16765	A16825	A16764
Bronsyn	Diploid ryegrass	A12337	A12339		
Grasslands Pacific	Diploid ryegrass	A13009	A13485		
Meridian	Diploid ryegrass	A13494	A13496		
Yatsyn	Diploid ryegrass	A11603	A11602		
Quartet	Tetraploid ryegrass	AT1115	AT1117		
Grasslands Coronet	Diploid ryegrass [c]	A12510	A5526		
Grasslands Trophy	Diploid ryegrass [c]	A12509	A5525		
Nine O One	Diploid ryegrass [c]	A14097	A14098		
Grasslands Marsden	Diploid long-term hybrid	N1912	N1502		
Grasslands Supreme	Diploid long-term hybrid	N2123	N1901		
GN139	Diploid long-term hybrid	N2115	N2112		
Grasslands Impact	Diploid long-term hybrid	N2198	N2200		
GNT83	Tetraploid long-term hybrid	NT1044	NT1014		

[a] Ryegrass (perennial) is *Lolium perenne* while ryegrass long-term hybrid is *L. boucheanum* (Syn. *L. hybridum)***.**

[b] Accession number indicates grass catalogue number in the Margot Forde Germplasm Centre, Palmerston North, New Zealand**.**

[c] Cultivars used for turf and not pasture

2.2 DNA isolation

Fresh fungal mycelium from six week-old fungal cultures grown on cellophane discs on PDA plates, or plant tissue, was ground into a fine powder in liquid nitrogen with a mortar and pestle. Genomic DNA was isolated from approximately 50 mg of ground tissue using a plant genomic DNA mini kit (Geneaid, Taipei, Taiwan) following the manufacturer's instructions.

DNA used for endophyte concentration determination for the RNA seq assay was isolated from the interface of the phase separation step during total RNA isolation extraction (see details in section 2.5.1). After the interface was transferred into a 14 mL BD™ Falcon™ Round-Bottom Polystyrene Tube (BD Biosciences, Franklin Lakes, USA), 3 mL of 100% ethanol was added, and then incubated for 3 min at room temperature (RT). DNA was pelleted by centrifugation at 2,000 rpm (Thermo Scientific Sorvall™ Evolution RC Superspeed Centrifuge with SLA-600TC rotor, Newtown, USA) at 4°C for 5 min, washed with 10 ml of sodium citrate/ ethanol solution (0.1 M sodium citrate in 10% ethanol, pH 8.5), then incubated for 30 minutes at RT with occasional mixing by gentle inversion. Following further centrifugation at 2,000 rpm for 5 minutes at 4°C, the DNA pellet was washed with 10 ml 75% ethanol, then air-dried and resuspended in water. Insoluble material was removed by centrifuging the sample at 12,000 g (Heraeus™ Pico 17 / 21 Centrifuge, Thermo Fisher Scientific Inc. Langenselbold, Germany) for 10 minutes at 4°C. The supernatant containing the DNA was transferred to a new tube and stored in a –20°C freezer.

2.3 DNA manipulation

2.3.1 Quantification

Genomic DNA used for standard PCR was quantified using a NanoPhotometer (Implen, München, Germany) according to the manufacturer's instructions. Genomic DNA used for real time PCR was quantified with a Dyna Quant DQ200 Single-wavelength Fluorometer (Amersham Biosciences Corp, CA, USA) according to its operating instructions, and calf thymus DNA (Invitrogen™, Catalog Number: 15633-019) was used as standard.

2.3.2 Agarose gel electrophoresis

Agarose (Invitrogen) was dissolved in TBE in a microwave with a suitable concentration according to the size of the DNA fragment. The agarose solution was poured after cooling down to 55℃ in a water bath. DNA samples were mixed with 0.1 volumes SDS dye prior to loading in the wells. A 1 kb DNA ladder (Invitrogen) was loaded as a standard. Gels were run at 80 to 100 V for about 45 minutes in TBE to separate DNA fragments. Gels were stained with ethidium bromide (EtBr) solution (1 μg/ml) for 10 to 15 minutes before being visualized on a UV transilluminator Gel Documentation system (Bio-Rad Universal Hood II).

2.3.3 Agarose gel purification of PCR product

EtBr stained gels were visualized on a Dark Reader ^{TM} Transilluminator DR-88M (400 to 500 nm). The target DNA bands were excised from the gel using a scalpel blade and transferred into a 1.7 ml microcentrifuge tube. DNA fragments were purified using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

2.3.4 PCR product purification

A High Pure PCR Product Purification Kit (Roche) was used to remove PCR reagents and excess primers according to the manufacturer's instructions.

2.4 PCR amplification

2.4.1 Standard PCR

The standard PCR reaction contained 5 to 10 ng genomic DNA, 2.5 μl 10× PCR buffer (-Mg, Solis Biodyne, Tartu, Estonia), 1.5 μl $MgCl_2$ (25 mM), 2 μl dNTP mixture (1.25 mM), 0.2 μl FIREPol® DNA Polymerase (5 U/μl, Solis Biodyne) and 0.5 μl primer mix (10 μM each) in a reaction volume of 25 μl. The cycling conditions used were 4 min at 94℃, 35 cycles of 10 s at 95℃, 30 s at 50 to 60℃ and 72℃ for 1 min per Kb, and 10 min at 72℃.

2.4.2 High fidelity PCR

PCR products required to be sequenced were amplified with Platinum® Taq DNA Polymerase, High Fidelity (Invitrogen). The PCR components included 5 to 10 ng genomic DNA, 2.5 µl 10× High Fidelity PCR buffer (-Mg), 1 µl $MgSO_4$ (50 mM), 0.2 µl dNTP mixture (25 mM), 0.1 µl Platinum® Taq High Fidelity (5 U/µl, Invitrogen) and 1 µl primer mix (10 µM each) in a reaction volume of 25 µl. The cycling conditions were the same as above.

2.4.3 Real time PCR

2.4.3.1 Primer and probe design

Beacon Designer™ software (version 7.0, Premier Biosoft, Palo Alto, California) was used in primer and probe design. Probes (Table 2) were custom synthesized by Biosearch Technologies (Novato, CA, USA) with fluorophores chosen according to the dye selection chart provided by the manufacturer (Biosearch-Technologies 2013).

2.4.3.2 Cycling conditions

Real time PCR was performed on a LightCycler® 480 (Roche, Germany). A LightCycler® 480 Probes Master mix kit (catalog number 04707494001, Roche) was used to perform the real time PCR. The simplex (PCR with only one probe/primer combination) reaction mix contained 5 µL of probe master mix, and a final concentration of 200 nM of each primer and probe. The duplex (PCR with two probe/primer combinations) reaction mix contained 7.5 µL of probe master mix, 0.05 mM additional dNTPs and a final concentration of 200 nM of each of *ltmG* and *TB1* primers and probes. In each case 1 µL (with a concentration in the range of 1 to 10 ng/µL) DNA template was used in a final volume of 10 µL. All real time PCR assays had the same cycling conditions: 5 min at 95°C, 45 cycles of 10 s at 95°C, 20 s at 60°C and 10s at 72°C, and 10s at 40°C.

2.4.3.3 Standard curves

For generating standard curves, a mix of ryegrass and NZCT DNA was used, with the highest concentrations of 100 ng/μL ryegrass and 1 ng/μL endophyte DNA. Ten-fold dilutions over six orders of magnitude of the template concentrations, each with three replicates, were used to generate all standard curves. The average crossing points (the point at which the fluorescence of a sample rises above background fluorescence) were calculated and plotted as a function of the Log_{10} of the template input amount in nanograms.

2.4.3.4 Colour compensation

As fluorophores have overlapping emission spectra, which can result in over- or under-estimation of data, colour compensation was applied to compare the emission spectra when the reporter dyes are used in separate reactions. LightCycler® 480 software uses those spectra to make adjustments when the reporter dyes are used together, and thus to eliminate errors associated with this spectrum overlap. To perform colour compensation, six replicates of simplex reactions containing the same amount of master mix and primer/probe as used in multiplex PCR, and 10 ng WT E+ genomic DNA template, were used for each reporter dye. Another six replicate reactions containing the same amount of master mix and template but no primer/probe were used as blank. The colour compensation was performed after 5 min pre incubation at 95℃ and 45 amplification cycles (10 s at 95℃, 20 s at 60℃ and 10s at 72℃) when reactions reached the plateau phase. The thermocycling profile consisted of 95℃for 1sec at 4.4℃/s, 40℃ for 30s at 2.5℃/s, and 80℃ at 0.1℃/s with continuous acquisition mode.

2.4.3.5 Calculation

Standard curves of each primer/probe were used to calculate the DNA biomass of endophyte or grass by using the advanced relative quantification method according to the LightCycler® 480 system manual, taking the colour compensation correction into account. The concentration was expressed as a ratio of copy number of endophyte gene (*ltmG*, *ltmJ* or *perA*) to ryegrass gene (*TB1*). One NZCT-infected ryegrass DNA sample was used as calibrator in all quantification experiments so that adjustments

could be made to compensate for between-experiment variation. For instance, the formula for calculating endophyte concentration using the *ltmG* gene is:

$$\text{Endophyte concentration} = \frac{\left(\frac{ltmG \text{ crossing point value}}{TB1 \text{ crossing point value}}\right)\text{Sample}}{\left(\frac{ltmG \text{ crossing point value}}{TB1 \text{ crossing point value}}\right)\text{Calibrator}}$$

2.4.3.6 Primer/probe specificity, efficiency and reliability evaluation

Primer/probe specificity was evaluated in a real time PCR assay using genomic DNA individually extracted from cultures of NZCT, AR1 and AR37 as well as from ryegrass plants that were endophyte-free or infected with NZCT. Clover DNA was used as a negative control. In addition to this, the specificity of the fungal primers was confirmed by lack of amplification of 16 fungal species from a range of classes and families (listed in Table 2.2).

To evaluate the reliability of the assays with other ryegrass and hybrid cultivars, DNA was extracted from 16 glasshouse-grown cultivars. The presence of endophyte in all endophyte-infected grasses was confirmed by immunoblotting (see section 2.6). Subsequently, real time PCR was done with DNA from the same samples and using all primer/probe combinations.

2.4.3.7 Evaluation of a dual *ltmG*/ *TB1* assay on quantification of endophyte concentration

To evaluate the correlation between endophyte nucleotide concentration and the ratio of endophyte biovolume/ryegrass biovolume, grass pseudostems (leaf sheaths and un-emerged leaf lamina) were collected from the base of the tiller upwards (approximately 1 cm long sections). The top and bottom parts (1-2 mm slices) of these pseudostem sections, including all leaf sheaths and emerging lamina, were cut and prepared for determination of the ratio of endophyte biovolume/ryegrass biovolume (see details in 2.8), whilst the central section was used for real time PCR analysis. Duplex real time PCR using *ltmG* and *TB1* primer/probe combinations was performed with DNA extracted from the central part of the grass pseudostem to quantify endophyte concentration.

Table 2.2 Fungi used in endophyte primer specificity evaluation.

Fungi	Division	Class	Family
Trichoderma atroviride	Ascomycota	Sordariomycetes	Hypocreaceae
Trichoderma viride	Ascomycota	Sordariomycetes	Hypocreaceae
Pestalotiopsis sp.	Ascomycota	Sordariomycetes	Amphisphaeriaceae
Glomerella cingulata	Ascomycota	Sordariomycetes	Glomerellaceae
Xylariaceae sp.	Ascomycota	Sordariomycetes	Xylariaceae
Eutypa lata	Ascomycota	Sordariomycetes	Diatrypaceae
Lophodermium conigenum	Ascomycota	Leotiomycetes	Rhytismataceae
Cyclaneusma minus	Ascomycota	Leotiomycetes	Rhytismataceae
Lophodermium pinastri	Ascomycota	Leotiomycetes	Rhytismataceae
Sclerotinia sp.	Ascomycota	Leotiomycetes	Sclerotiniaceae
Alternaria alternata	Ascomycota	Dothideomycetes	Pleosporaceae
Phoma glomerata	Ascomycota	Dothideomycetes	Didymellaceae
Passalora arachidicola	Ascomycota	Dothideomycetes	Mycosphaerellaceae
Aspergillus nidulans	Ascomycota	Eurotiomycetes	Trichocomaceae
Strasseria geniculata	Ascomycota	Not assigned	Not assigned
Sclerotium sp.	Basidiomycota	Agaricomycetes	Typhulaceae

2.4.3.8 Evaluation of *ltmJ and perA* primer/probes for detecting contaminating endophytes in seed

AR37-infected ryegrass seeds ('Grasslands Samson') were mixed with either NZCT-infected 'Grasslands Samson' or AR1-infected 'Grasslands Samson' at proportions by weight of 3%, 5%, 10%, 15%, 20% and 25% for the purpose of measuring each in the mixtures. Mixed seeds were ground into fine powder in liquid nitrogen, and 30 mg of the powder used for DNA extraction using a plant genomic DNA mini kit (Geneaid, Taipei, Taiwan) following the manufacturer's instructions. Three replicates were extracted separately for each mixed seed sample. Real time PCR analyses using *ltmJ* and *perA* primer/probe combinations were conducted to detect NZCT and AR1 contamination in AR37-infected ryegrass seed.

2.5 RNA isolation and manipulation

2.5.1 RNA isolation

Total RNA was isolated from grass tissue using Trizol reagent (Invitrogen). 1-1.25 g of tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle pre-treated with Ambion® RNase-away (Life Technologies). Each sample was suspended in 10 ml Trizol, transferred to a 14 ml BD™ Falcon™ Round-Bottom Polystyrene Tube (BD Biosciences, Franklin Lakes, USA), and centrifuged at 9,000 rpm (Thermo Scientific Sorvall™ Evolution RC Superspeed Centrifuge with SLA-600TC rotor, Newtown, USA) at 4℃ for 10 min. The supernatant was transferred to a new BD falcon tube, mixed thoroughly with 2 ml of chloroform for 15 s and incubated at RT for 10 min followed by centrifugation at 9,000 rpm for 15 min at 4℃. The upper aqueous phase and interface were each transferred to a new BD falcon tube separately for RNA and DNA isolation (see section 2.2). Then 2.5 ml isopropanol and 2.5 ml high salt buffer (0.8 M NaCi, 1.2 M NaCl) were added to the aqueous phase, mixed gently by pipetting and then incubated at RT for 10 min. The RNA was pelleted by centrifugation at 9,000 rpm at 4℃ for 10 min, washed with 75% ethanol, air-dried in a fume hood at RT, and finally dissolved in 200 μL of DEPC (diethylpyrocarbonate) -treated water. RNA samples were stored at -80℃ until use.

2.5.2 RNA quality check

2.5.2.1 Formaldehyde gel

An electrophoresis tank and comb were soaked in 2% H_2O_2 for 30 min to inactivate RNase, then rinsed with MQ water and air-dried. A 1.2% agarose gel was made by adding 1.8 g agarose (Invitrogen) to 110 ml DEPC-treated MQ water, melted and cooled to 60℃. Then 15 ml of 10× MOPS buffer (41.9 g MOPS, 6.8 g $NaAc{\cdot}3H_2O$ and 40 ml EDTA (250mM) in 1 litre DEPC treated water) and 25 ml of 37% formaldehyde were added to the melted agarose, which was then poured into the gel box. Approximately 1 μg of RNA was added to 16 μl of the following mixture: 2 μl 10× MOPS buffer, 10 μl formamide (Sigma), 3.2 μl formaldehyde (37%) and 0.08 μl EtBr (10 mg/ml), denatured by incubating at 65℃ for 15 min, then mixed with 2 μl Bromophenol Blue (BPB) loading dye (0.2% bromophenol blue dissolved in 1 × MOPS buffer). 1× MOPS buffer was poured into the gel box just reaching the surface of the gel. Denatured RNA samples were loaded into the wells. Buffer was filled up to the top surface of the gel after pre-running the samples into the gel for 5 min. The gel was run at 80 V for 45 min.

2.5.2.2 Bioanalyzer

The integrity and quantity of RNA samples for RNA sequencing were checked using an Agilent Bioanalyzer (Santa Clara, CA, USA) by the Massey Genome service, Palmerston North.

2.5.3 Reverse Transcription

5 μg of ryegrass total RNA was reverse transcribed using SuperScript® III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

2.6 Endophyte presence determination using Immunoblotting

Grass tillers (from 3-4 tiller stage) were cut about 5 mm from the base, and the cut end was pressed onto a nitrocellulose membrane (0.45 μm). The membrane was immersed into blocking solution (BS) (Tris (hydroxymethyl) methylamine 2.42 g,

NaCl 2.92 g, non-fat milk powder 5 g, 1 M HCl 10 ml, made up to 1 litre with RO water, adjusted to pH 7.5), shaken on an orbital shaker for 2 h, then rinsed twice with fresh blocking solution. After addition of 5 ml primary antibody (rabbit anti-endophyte produced at AgResearch in conjunction with Massey University's Small Animal Production Unit) and 25 ml BS (1:1000 dilution), the membrane was shaken for 15 min at RT and incubated overnight at 4℃. The membrane was then rinsed twice with BS solution before adding 6.25 ml secondary antibody (goat anti-rabbit IgG-AP, sc-2034, Santa Cruz Biotechnology, USA) mixed with 25 ml BS (1:4000 dilution). It was shaken for 15 min at RT, and then incubated at 4℃ for 5 h. Excess secondary antibody was poured off and the membrane was rinsed twice with BS before being immersed into chromogen solutions. Chromogens were prepared by dissolving separately 20 mg Fast Red TR (Sigma F-2768) in 12.5 ml Tris buffer (Tris (hydroxymethyl methylamine) 24.2 g in 1 litre RO water adjusted to pH 8.2) and 12.5 mg of naphtholAS-MXphosphate (SigmaN4875) in 12.5 ml Tris buffer per 10 cm^2 of membrane. The membrane was shaken for about 15 min at room temperature until red colour appeared on a positive control blot, then rinsed three times with tap water to stop the colour development. The presence of endophyte was scored by the red colour on each blot.

2.7 Endophyte detection using aniline blue

A piece of inner layer of grass sheath was peeled and stained with aniline blue (0.05% aniline blue in lactic acid/glycerol/water 1:2:1) and then observed under a light microscope at 400× for presence of endophyte. Endophyte hyphae that were stained blue could be observed in intercellular spaces of grass tissue.

2.8 Endophyte biovolume measurement

Samples were fixed with 2% (w/v) formaldehyde and 3% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. After three washes in the same buffer, the samples were fixed with 1% (w/v) OsO_4 in the same buffer for one hour at room temperature. The three buffer washes were repeated. The samples were then dehydrated by using an acetone/water series (25%, 50%, 75%, 95%, and 100%) and were kept in each gradient step for 10 min and the 100% for 2 h. The samples were first embedded with an acetone: resin (Procure 812 Embedding kit,

ProSciTech, Thuringowa, Australia) mixture (50%:50%) on a stirrer overnight, and then the acetone: resin was replaced with fresh 100% resin for another 8 hours on the stirrer. The samples were finally mounted in 100% fresh resin at 60°C for 48 hours. Sections 1 μm thick were cut from trimmed resin blocks using a glass knife and Ultramicrotome (Leica, Austria). They were heat-mounted onto a glass slide, stained with 0.05% Toluidine Blue and were viewed under a light microscope (Olympus BX51, Japan). Hyphae were counted at 400x magnification, and hyphal diameters from four tillers were also measured using imageJ software (http://rsb.info.nih.gov/ij/). The sections were photographed and the images printed on A4 paper. The entire paper was weighed on an analytical balance. Then the images of the separate tissues (emerging leaf, first, and second mature leaf sheaths) were cut out and weighed. This was repeated three times. From the weights, the hyphal numbers and diameters, and the percentage of biovolume contributed by the hyphae, were calculated.

2.9 Clonal endophyte-free ryegrass generation

Benlate (containing benomyl (methyl 1, 2-benimidzole carbamate), DuPont, Wilmington, DE, USA) was used to generate clonal endophyte-free ryegrass. Endophyte-infected grass was split into single tillers and rinsed thoroughly with tap water. Grass leaves and roots were cut into sections about 4 cm long and completely immersed in tap water containing 2 g L^{-1} Benlate, then incubated at RT for 6 h. Each single grass tiller was planted in a 150 ml container with 300 g of sand (with addition of Osmocote slow-release fertilizer) containing 40 ml of Benlate solution (200 mg g^{-1} benomyl (methyl 1, 2-benimidzole carbamate)), and then grown in a greenhouse. Grasses were watered every 2 to 3 days to maintain the initial pot weight. After 2-3 weeks, a small piece of leaf sheath was peeled and stained with aniline blue then observed under light microscopy (described in section 2.7) to examine the presence of endophyte. After confirming the absence of endophyte, a separated single tiller was re-potted into potting mix without fungicide for regeneration. The endophyte infection of newly emerged tillers was checked, and confirmed endophyte-free tillers were repotted again as before. Grasses were grown in the glasshouse for one year before being used in experiments. The absence of endophyte was finally verified using real time PCR before and after experiments.

2.10 Simple sequence repeats analysis

Simple Sequence Repeat (SSR) primers B10, B11 (Moon et al. 1999) and ans25 (Kirkby et al. 2011) were used for endophyte genotyping. 10 ng of genomic DNA isolated from endophyte-infected grass was mixed with 1 µl 10×PCR buffer (-$MgCl_2$), 0.5 µl $MgCl_2$ (50 mM), 0.4 µl dNTPs (5 mM each), 0.15 µl M13 primer + FAM (10 µM), 2 µl primer mix, 0.06 µl Platinum Taq (5U/µL) and 3.89 µl water. PCR was performed with the cycling conditions: 4 min at 94℃, 30 cycles of 30 s at 94℃, 30 s at 55℃ and 30s at 72℃, 8 cycles of 30 s at 94℃, 30 s at 53℃ and 30s at 72℃, and 30s at 72℃. 2 µl of PCR product was mixed with 10 µL of HI-DI formamide containing 0.12 µL of GeneScan™ 500 LIZ™ size standard (Applied Biosystems®), and then denatured in a thermocycler for 5 min at 94℃, followed by 5 min at 4℃. The denatured SSR products were run on an ABI3130xl capillary electrophoresis analyser, in a 22 cm capillary array with POP7 polymer (Applied Biosystems). Electropherograms were analysed using ABI Prism GeneScan (v 3.7, Applied Biosystems), and the sizes of the fragments were determined using GeneMarker v1.96 (SoftGenetics LLC, PA, USA).

2.11 Drought treatment

2.11.1 Glasshouse prescreen drought treatment

Grasses were geminated from seeds and grown in a heated glasshouse, with a temperature range from 12℃ to 25℃, the Plant Growth Unit of Massey University, Palmerston North. Two single grass tillers were separated from each plant (at three months old), trimmed into similar size (10 cm above ground part and 10 cm root), and potted into a black UV polythene bag (size: 120 mm ×120 mm × 230 mm) filled with potting mix (Daltons Premium potting mix, sieved through a 6 mm sieve). The remaining grasses were maintained for future use. Grasses were well watered for 4 weeks for recovery from re-potting, then trimmed down to 5 cm above ground. In each cultivar, 25 endophyte infected and 15 endophyte free plants were subjected to water deficit stress (no water supplied), whilst 5 of each type of grass were used as well watered controls. Grass total tiller number (TTN) and were recorded when soil moisture dropped to about 17% (v/v) (irrigation start point), and again when soil

moisture of the drought group grass reached about 4% (v/v) (permanent wilting point). Under the conditions used it took 7 days to reach 17% soil moisture from the point at which watering was stopped, then a further 17 days to reach 4% soil moisture, under the conditions used. Then water was supplied again to the drought group grass, and the total tiller number and dry biomass of all grasses were measured after two weeks of re-watering. The control group grasses were well watered throughout the experiment. Soil moisture was determined using a time-domain reflectometer (TDR, MiniTrase -6050X3, EM Giesbeek, Netherlands). Endophyte infection of all plants was checked on replicate grasses by immuno-blotting, and the presence of endophyte strains (WT, AR1 and AR37) in three cultivars (Samson, Nui and Commando) was checked by real time PCR using specific primers (*ltmG*, *ltmJ* and *perA*).

2.11.2 Outliers validation

Grasses that showed greatest and least biomass in each cultivar in the pre-screen experiment were selected as outliers. Four tillers of each outlier were split, cut into similar size (5 cm shoot and 5 cm root) and potted in polythene bags as described above. Grasses were well watered for 6 weeks for regeneration, then were trimmed into 5 cm above ground and no water was provided anymore. Grass survival was recorded after three weeks drought stress. The death of the grass was confirmed by no living tiller two weeks after re-watering. Outliers showing different survival characteristics were selected for further grass selection.

2.11.3 Drought treatment of time-course experiment in growth chamber

Six endophyte-infected ryegrasses were selected based on results of previous experiments and used in a time course drought tolerance experiment. Daltons premium potting mix (sieved as above) was sterilized by autoclaving at 121℃ for 45 min. Single tillers were split from each grass, cut into similar size (5 cm shoot and 5 cm root) and planted in a plastic pot (depth 15 cm; diameter 15cm) containing 1.1 kg (fresh weight) sterilized potting mix. Grasses were grown in an environmental control growth chamber (CAT 630, Contherm Scientific Limited, Lower Hutt, New Zealand). The environmental conditions were 22/15℃ (day/night), relative humidity of 60/40% (day/night), 14 hours photoperiod with a light intensity of 700 μmol/m^2/s. For each

grass type, 18 plants were used. Grasses were well watered for four weeks to recover from repotting before drought treatment. Then grasses were trimmed into 5 cm above ground and water supply to 15 plants of each grass was stopped until soil moisture dropped to 25% field capacity (FC) (see details in section 2.12), 1 day after which the plants were re-watered. Pots were weighed every day to track the soil moisture levels. Several physiological parameters, including Fv/Fm, (maximal photochemical yield), leaf osmotic potential, LRWC (leaf relative water content) (see details in Sections 2.13.1 to 2.13.3) and TTN (total tiller number), were determined on three drought treated plants of each grass when soil moisture dropped to 75%FC, 50%FC and 25%FC, and 1 day (1RW) and 3 days (3RW) after re-watering. The same physiological parameters were measured on three well-watered plants of each grass before drought treatment.

2.11.4 Growth chamber drought treatment for RNA seq assay

A single tiller was split from each grass (nine replicates for each type of grass), cut to 5 cm shoot and 5 cm root, and potted in plastic pots with sterilised potting mix as described above. Grasses were well watered and grown in an environmental control growth chamber under the same conditions as described in section 2.11.3, until week 9 when watering was stopped. Pots were weighed everyday to track soil moisture. Plants were kept under severe drought condition (soil moisture reached 20% FC) for one week before being re-watered. Several physiological parameters (Fv/Fm, LRWC, TTN, leaf osmotic potential) of three plants of each grass were determined at each of the following stages: when soil moisture dropped to 75%FC and stayed one week under 20% FC. Second mature leaf, second mature sheath and root of each grass was separated, immersed in liquid nitrogen immediately, and then stored in a -80℃ freezer. To account for variation, the same experiment was repeated three times. Thus, there were 9 biological replicates in total for each grass under each condition. All 9 samples were used for alkaloids analysis using LC-MS, as well as for endophyte DNA concentration quantification using real time PCR. Total RNA was extracted from sheaths of all grasses collected under control (75% FC) and severe drought (one week under 20% FC). This condition was chosen according to the results of the time course experiment described in section 2.11.4; plants were very wilted under this condition. Total RNA samples from one biological replicate from each repeat (same grass grown

under the same conditions) were pooled together in equal proportions (see Figure 2.1). In total, for each grass, there were three pooled RNA samples, of which two were sent for RNA sequencing and the remaining one was kept in a -80℃ freezer in case required for further validation.

2.12 Soil field capacity measurement

Soil field capacity (FC) was determined by the method described in (Klute 1965). The weight of an aluminium container (10 cm height and 10 cm diameter) was recorded before being filled with fresh soil, then covered using a piece of cheese cloth secured by a rubber band. The container and its contents were soaked in tap water overnight then placed on a wire rack (cheese cloth side down) for 48 hours to let the excess water drain away. The combined weight of container and saturated soil was recorded after this time. Soils were then dried in an oven at 105℃ for 48 hours and the total weight of container and dried soil determined. The soil field capacity was calculated according to the equation:

$$FC = [(\text{saturated soil weight} - \text{dry soil weight})/\text{dry soil weight}]*100$$

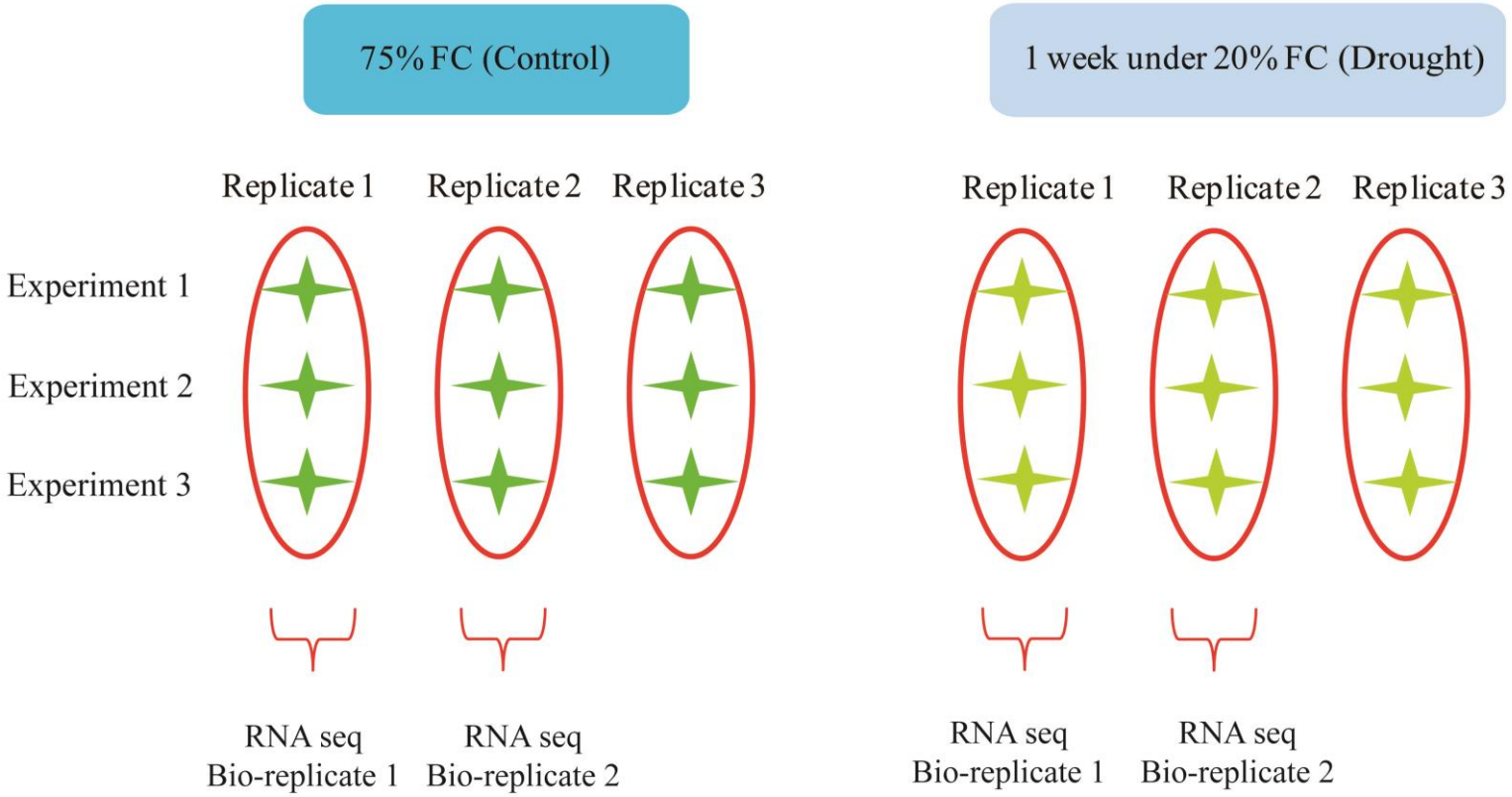

Figure 2.1. RNA samples pooling strategy.

FC' indicates field capacity. The three experiments (each with three biological replicates) were conducted at different times but under the same conditions. The figure displays the sample collection and RNA sample pooling strategy for one grass under two conditions. Nine replicates of this grass were collected under control and nine replicates under drought. RNA was extracted from each sample. Total RNA samples from one biological replicate from each experiment were pooled together in equal proportions. In total, there were three pooled RNA samples for each grass under each condition. Of these, two of them were sent for RNA sequencing and one retained for future validation work.

2.13 Physiological parameter measurements

2.13.1 Maximal photochemical yield

In green plants, the photosystem II maximum quantum yield is observed after dark adaption when all reaction centres are open and heat dissipation is minimal. A saturation light pulse given at this time induces maximal photochemical yield (Fv/Fm), which is a reliable indicator of the potential quantum yield of PS II. Fv/Fm is lowered by any effects (such as drought stress (Sherameti et al. 2008)) which cause inhibition of photosynthesis II reaction centres and increase heat dissipation. Thus, Fv/Fm was calculated to quantify photoinhibition.

Maximal photochemical yield was determined in randomly selected second grass mature grass leaves (Tan et al. 2001) using a portable modulated fluorometer (Mini-PAM, Walz, Eichenring, Germany). After 7 hours' light exposure (midday), leaves were dark adapted for 30 min using manufacturers (Mini-PAM) leaf clips (DLC-8). A standard instrument setting (saturating pulse of 12,000 mmol m^{-2} sec^{-1} for 0.8 sec) was applied. Maximal fluorescence yield (Fm) and maximal variable fluorescence (Fv) were determined after a saturation pulse was triggered.

2.13.2 Leaf relative water content and dry weight measurements

Leaf relative water content (LRWC) was determined by the method described in (Barrs and Weatherley 1962). The fresh weight (FW) of approximately ten leaves (second mature leaves) was measured. Leaves were then immersed in MilliQ water in a sealed plastic bag and incubated at RT for 24 h. The leaf surface was dried using tissue paper and its turgid weight (TW) determined. Leaf dry weight (DW) was measured after drying in an oven at 80℃ for 72 h. LRWC was calculated according to the equation: LRWC= (FW-DW)/ (TW-DW) ×100.

2.13.3 Leaf osmotic adjustment (LOA) measurements

Water potential is a measurement of the tendency of water to move from one area to another. Osmotic potential is a component of the water potential due to the presence of solutes. Osmotic adjustment is the lowering of osmotic potential that occurs due to solute accumulation in response to water stress (Girma and Krieg 1992).

A faster and greater osmotic adjustment can postpone dehydration under drought conditions as it facilitates plant maintains cell turgor and metabolic activities (Turner et al. 2007). Leaf osmotic potential (LOP) was determined using a HR-33T Dew Point Microvoltmeter (Wescor, Logan, USA) attached with C-52 chambers (Wescor, Logan, USA). To do this, grass tissue (about 3 mm long) from the middle part of a second mature leaf of each of 3 randomly selected tillers were pooled and put into an l.7 ml microcentrifuge tube then immediately immersed into liquid nitrogen. The frozen grass tissues were ground with a micro pestle then thawed at room temperature. Sap of the grass tissue was transferred into C-52 chambers attached to the microvoltmeter and incubated at room temperature for 1 hour. The microvolt reading of the sample was then determined according to the manufacturer's instructions. Standard curves for each chamber were determined using filter paper soaked with 200, 400, 600, 800, 1000 and 1500 mmol kg^{-1} NaCl. The osmotic potential of each sample was calculated according to the standard curve of each chamber. LOP value has a reversed linear relationship with LRWC (Morgan 1992). Leaf osmotic adjustment was calculated taking into account the LOP values and ratio of LRWC value measured under well-watered and drought conditions (nine replicate measurements for each condition) as described by (Babu et al. 1999):

$$LOA = LOP_d — (LOP_c * LRWC_c / LRWC_d)$$

Where LOP_c and LOP_d indicate leaf osmotic potential under well watered and drought conditions respectively; $LRWC_c$ and $LRWC_d$ represent leaf relative water content under well watered and drought condition respectively. The value of "$(LOP_c * LRWC_c / LRWC_d)$" represents the basal LOP value that would occur if no new solutes were produced under drought.

2.13.4 Alkaloids extraction and analyses

Approximately 50 mg freeze dried grass sample was ground in a 2 ml vial (Sarstedt) with a FastPrep FP120 Homogenizer (Thermo Savant, Carlsbad, USA). To each vial was added 1 ml of 80% methanol (containing Homoperamine (BDG Synthesis, Wellington, NZ) 2 ppm and Ergotamine (Sigma) 0.5 ppm as internal standards). Samples were mixed using a sample rotator for 1 h in the dark followed by centrifugation at 13,000 g for 5 min. The supernatant was filtered with a syringe filter

(0.22 µm) into a glass 2 ml HPLC vial. Samples were stored in a -20℃ freezer until analysis using methods described by (Rasmussen et al. 2012).

Alkaloids were analysed on a Thermo LTQ linear ion trap MS (mass spectrometer) equipped with an HPLC (High-performance liquid chromatography) system using a Jasco X-LC-3080DG degasser, two Jasco X-LC 3185PU high pressure LC pumps, a Jasco X-LC3180MX high pressure mixer and a HTS-Combi-PAL auto sampler.

To analyse indole diterpenes, the MS was set to operate in positive ESI mode with the capillary at 275°C, the probe voltage at 5 kV and with N_2 as carrier gas. Optimise the MS instrument tuning parameters while infusing the paxilline tuning standard solution (Acetonitrile, Baker Analyzed HPLC Solvent, J.T. Baker). HPLC was performed with a flow rate of 0.2 ml/min and the column oven set at 25°C. Five µl of the sample was injected. Two kinds of solvent, A (Water (MilliQ®) (40: 60 v/v) containing 0.1% acetic acid) and B (Acetonitrile containing 0.1% acetic acid (Analar, BDH)), were used. A gradient was applied from 80% A :20% B to 50% A:50% B over 20 min, then a further linear gradient to 100% B over another 20 min, and hold at 100% B for 10 min before returning to 80% A:20% B over 5 min, with a hold for re-equilibration of 5 min before injecting the next sample. Data were collected in both full scan and selective reaction monitoring modes, and Xcalibur software was used for quantification

To analyse ergot alkaloids and peramine, the MS was set to operate in positive ESI method with the capillary at 275°C, the probe voltage at 5 kV and N_2 as carrier gas. Optimise the MS instrument tuning parameters while infusing the paxilline tuning standard solution (Acetonitrile, Baker Analyzed HPLC Solvent, J.T. Baker). HPLC was performed with a flow rate of 0.2 ml/min with the column oven set at 25°C. Fifteen µl of the sample extract was extracted. Two kinds of solvent, C (5 mM ammonium acetate (Analar, BDH) in water (MilliQ®)) and D (Acetonitrile), were used. A linear gradient was applied from 95% C:5% D to 50% C:50% D for 38 min, then a further linear gradient to 100% D over another 7 min and hold at 100% D for 10 min before returning to 95% C:5% B for 3 min, with a hold for re-equilibration of 8 min before injecting the next sample. The column flow was diverted for the first 6 min and last 20 min of the run. Data were collected in both full scan and selective

reaction monitoring mode, three micro scans were averaged for every point collected, and then quantified as above.

2.14 RNA sequencing

mRNA focused library preparation and sequencing were performed by NZGL (New Zealand Genomics limited). RNA-Seq libraries were generated from total RNA and index-tagged using a TruSeq RNA Sample Preparation Kit v2 (Illumina). Multiplex sequencing was performed on a Hiseq 2000 sequencing platform (Illumina, San Diego, CA, USA) using a 100 bp paired end sequencing strategy. Two biological replicates were sequenced for each sample. Endophyte infected grass mRNA libraries were pooled and sequenced on 3 lanes. Libraries of endophyte free grass were mixed and sequenced on 2 lanes.

2.15 RNA seq analysis pipeline

The quality of RNA seq reads was checked using SolexaQA (http://solexaqa.sourceforge.net/) and FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), then the reads were trimmed and filtered using DynamicTrim (http://solexaqa.sourceforge.net/) (P>0.05). Reads with length shorter than 80 bp were discarded. The annotated *Epichloë festucae* gene models (EfM3; n = 9,350; http://csbio-l.csr.uky.edu/ef2011/) were used as reference mapping files for endophyte reads. A *de novo* assembled EST library (EST_28Mar13; n =50,194; URL: http://ryegrass.massey.ac.nz/), generated by Jan Schmid, Ningxin Zhang and Robert Day, using sequenced mRNA extracted from perennial ryegrass (NuiD genotype) grown under normal conditions as described in (Zhang et al. 2011), was used as reference mapping files for grass reads. To make sure that reads were only mapped once, EfM3 and ryegrass ESTs were merged as a combined reference. RNA seq reads were mapped against the combined reference with Bowtie2 (mapping stringency of no more than 1 base pair mismatch). Mapped reads were counted using an in-house software ‘map_count’. Paired end matches were counted as a single hit. Mapped endophyte and ryegrass reads were separated by their different headers. To make the read counts comparable among samples, RPKM (reads per kilobase per million) normalization was performed for each individual gene. The R package, DEGseq (version 1.14.0,

http://www.bioconductor.org/packages/2.6/bioc/html/DEGseq.html) was applied in statistical analysis of differently expressed genes. Fisher's exact test was applied with a significance cutoff of q = 0.05, which gave a whole false discovery rate of 0.05. Genes with ≥ 2 fold change and with significant difference in expression were selected as differently expressed genes. Genes of interest were annotated using Blast2go (http://www.blast2go.com/b2ghome). Gene ontology enrichment analysis was also done by Blast2go using a two-tailed test (the whole expressed gene set was used as reference) with a filter value of 0.05 and a filter mode of *P*-value (two sided). KO (KEGG (Kyoto Encyclopaedia of Genes and Genomes) Orthology ID was assigned to each endophyte gene of interest by BLAST of sequences against the manually curated KEGG GENES database, KAAS (KEGG Automatic Annotation Server: http://www.genome.jp/tools/kaas/). Then interesting endophyte genes with KO ID were mapped against the KEGG Pathways Database using KEGG Mapper (http://www.genome.jp/kegg/mapper.html) to look for altered pathways. The ryegrass gene pathway analysis was done using Mapman software according to (Usadel et al. 2009). Mapman "Bins" were assigned to all genes in ryegrass EST using the Mercator pipeline (http://mapman.gabipd.org/web/guest/app/mercator). Command lines used in RNA seq are summarized in Appendix 2.1

2.16 Statistical Analysis

SPSS 13.0 software was used for statistical analyses. The least significant difference (LSD) was calculated to determine the significance of treatment means at $P < 0.05$. One-way analysis of variance (ANOVA) was used to detect the significance of multifactor interaction at $P < 0.05$ (section 4.11).

3 Real time PCR method development for endophyte quantification

3.1 Results:

3.1.1 Selection of target genes for primers and probes

The primary purpose of these assays is to develop an accurate quantification method for use in drought tolerance experiments. However because a reliable method to distinguish between ecotypes of *N. lolii* (NZCT (New Zealand common toxic ecotype), AR1and AR37) with different alkaloid profiles is needed, this work was extended to provide such tools that will have practical applications. In order to do this, three endophyte primer/probe sets were developed, along with one primer/probe set targeted to a ryegrass host reference gene, as summarised in Table 3.1.

The three endophytes have different alkaloid profiles: of the alkaloids lolitrem B and peramine, NZCT produces both lolitrem B and peramine, AR1 produces peramine only and AR37 produces neither. Thus genes involved in biosynthesis of these alkaloids were used to develop a diagnostic test to distinguish between them. At the molecular level, 11 genes distributed in three clusters are required for lolitrem B biosynthesis (Schardl et al. 2013). All three endophyte strains have the *ltmG* lolitrem biosynthetic gene, thus *ltmG* (GenBank ID: AY742903.1) was selected as a positive control for amplification of NZCT, AR1 and AR37. The inability of AR1 and AR37 to produce lolitrems is partly due to lack of a *ltmJ* gene (Young et al. 2009), so *ltmJ* (GenBank ID: DQ443465.1) was selected for generation of an NZCT-specific product only. To further distinguish AR37 from NZCT as well as from AR1, a peramine biosynthetic gene, *perA* (Tanaka et al. 2005), was used. There is a 1,223 bp deletion in the 3' end of the *perA* gene in peramine non-producer AR37 (Fleetwood et al. 2011). Thus a primers/probe set was designed in this region to generate a product specific to NZCT and AR1, but not AR37, genomic DNA. Specific positions of all primers and probes within the *ltmG*, *ltmJ and perA* genes are shown in Figure 3.1.

For the plant host, perennial ryegrass-specific primers/probes were designed based on the perennial ryegrass teosinte branched1 (*TB1*) gene (GenBank ID: GU987123.1). This gene is absent from clover (*Trifolium repens*), a common forage plant used in pasture, but present in other grasses. To achieve specificity for perennial ryegrass, unique regions in an alignment of grass *TB1* genes were used for primer and probe design (Figure 3.2).

Table 3.1 Target genes and oligonucleotide primers and probes used for real time PCR

Target gene[a]	Primer or probe[b]	Sequence (5' - 3')	G+C content (%)	Melting temp (℃)	Amplicon size (bp)	Efficiency[c]	Target organism/strain
ltmG	F	GCGTAGTGAAGATGTTATGG	45.0	62.3	150	2.05	NZCT, AR1, AR37
	R	CCTCCACATGAACTGCTA	50.0	62.6			
	P	CAL610-TCTCTTGCTTGCTCCACCA-BHQ2	52.6	67.9			
ltmJ	F	CAAGGATTCCATGTTCAA	38.9	58.6	77	2.03	NZCT
	R	GCAAGTCATCCAAGTAG	47.1	58.1			
	P	CAL560-CGACCAACGATGCCGACT-BHQ1	61.1	69.0			
perA	F	GGAACACTCTTGCAGATG	50	61.9	158	2.02	NZCT, AR1
	R	GTAGACAACCCTCATGGA	50	61.8			
	P	Quasar670-CTCTGCTCTCCAGGCGG-BHQ2	70.6	68.3			
TB1	F	CTCGTCCTCCAGGAACA	58.8	64.2	113	2.09	Perennial ryegrass
	R	CGTATCCTCCATTGCTGA	50	62.7			
	P	FAM-CGACGAGCACCTCTCCTCAA-BHQ1	60.0	70.2			

[a] *ltmJ*, *ltmG* and *perA* are endophyte genes. *TB1* is a perennial ryegrass gene.
[b] 'F', 'R' and 'P' indicate forward primer, reverse primer and probe, respectively.
[c] PCR efficiency was calculated using the formula: Efficiency = $10^{-1/\text{slope of standard curve}}$.

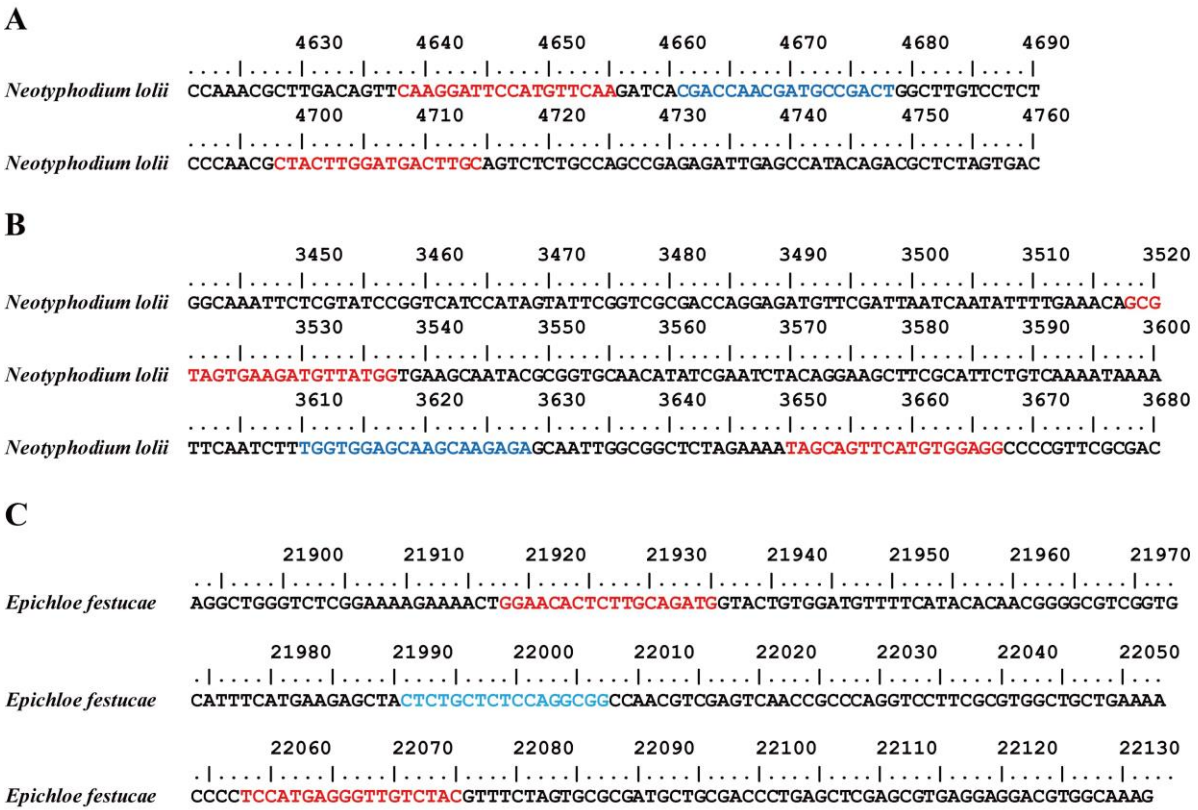

Figure 3.1 Alignment of primer and probe binding positions for endophyte genes.

Primer and probe binding positions are shown for *ltmJ* (A) *ltmG* (B) and *perA* (C) genes. Positions of primers are in red and the probe is in blue. The GenBank accession numbers of *Neotyphodium lolii* genes used for designing *ltmJ* and *ltmG* primer/probes are DQ443465.1 and AY742903.1 respectively. The numbers above the sequences are base numbers from the GenBank sequence accessions. The *perA* primer/probe combination was designed based on the *perA* gene sequence of *Epichloë festucae* Fl1 strain (GenBank accession no. AB205145.1), which is very closely related to *Neotyphodium lolii*(Moon et al. 2004). The *perA* primer/probe was designed in the region corresponding to the 1,223 bp deletion that is found in AR37, which commences from position 21600 bp, in the 3' end of the *perA* gene (Fleetwood et al. 2011).

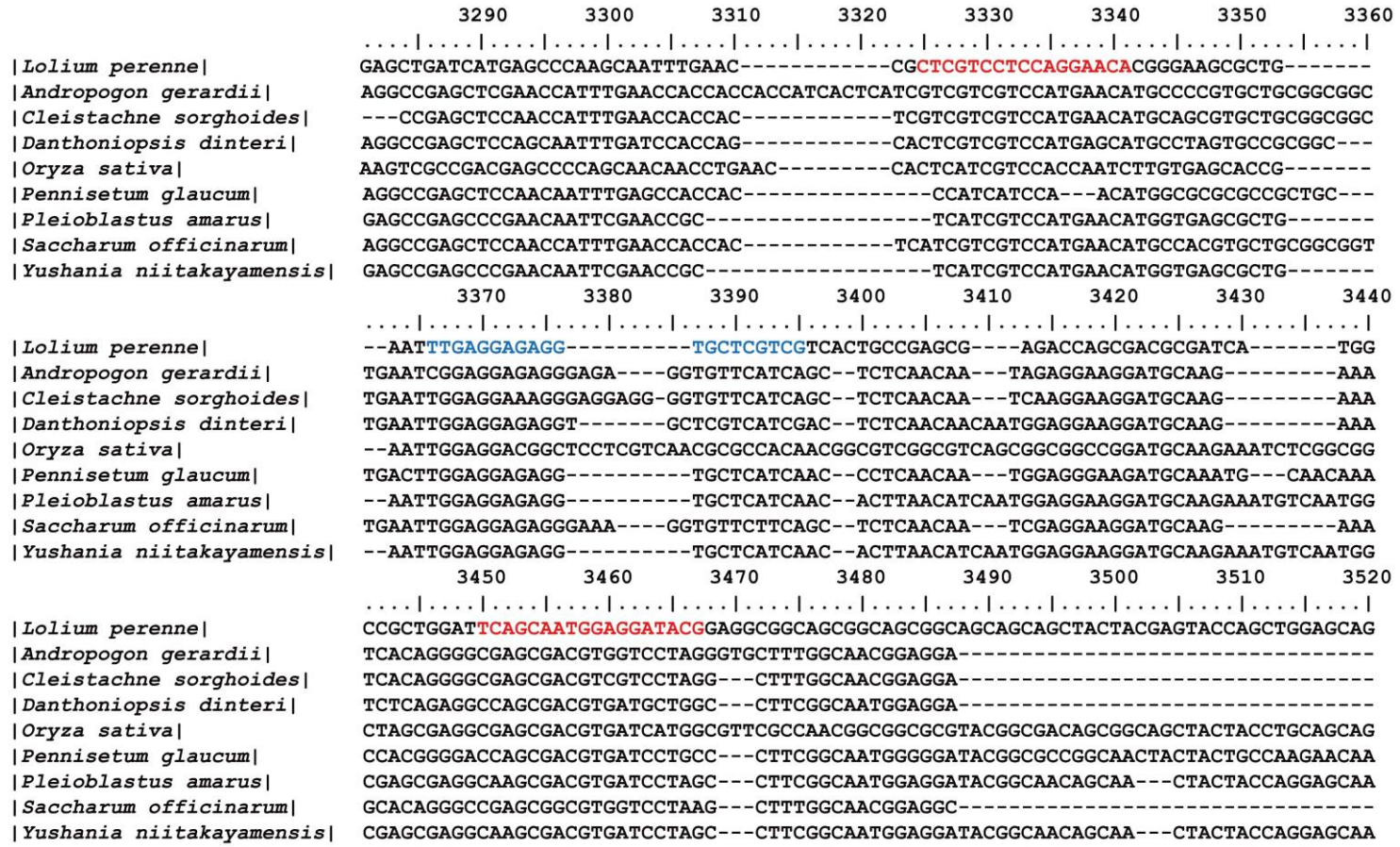

Figure 3.2 Alignment of primer and probe binding positions for the *TB1* gene.

Positions of primers are in red and the probe is in blue. Perennial ryegrass (*Lolium perenne) TB1* gene (GenBank accession no. GU987123.1) was aligned with the *TB1* gene of other monocotyledonous species: *Andropogon gerardii* (AF322119.1), *Cleistachne sorghoides* (AF322121.1), *Danthoniopsis dinteri* (AF322134.1), *Oryza sativa* (AY043215.1), *Pennisetum glaucum* (EF694162.2), *Pleioblastus amarus* (DQ910764.1), *Saccharum officinarum* (AF322127.1), *Yushania niitakayamensis* (DQ910763.1).

3.1.2 Assay specificity, efficiency and reliability

Primer/probe specificity was evaluated in a real time PCR assay using genomic DNA individually extracted from cultures of NZCT, AR1 and AR37 as well as from perennial ryegrass plants that were endophyte-free or infected with NZCT. Clover DNA was used as a negative control (Table 3.2). As expected, the NZCT-infected perennial ryegrass DNA was amplified by all four pairs of primers and endophyte-free perennial ryegrass DNA was only amplified by perennial ryegrass-specific *TB1* primers. The *ltmG* primers amplified all three endophyte strains whilst the strain-specific primers for *ltmJ* amplified only NZCT and those for and *perA* amplified only NZCT and AR1 DNA. AR37 infected perennial ryegrass DNA was only amplified by *ltmG* and *TB1* primers. AR1 infected perennial ryegrass DNA was amplified by all except *ltmJ* primers. To determine if these primers will amplify products from fungi other than *N. lolii*, we tested them on a broad range of fungal species (listed in Table 2.2). Some of these species, such as *Trichoderma atroviride* and *Trichoderma viride*, are quite closely related to *N. lolii*, being in the same order (Hypocreales), and can also be present in *L. perenne* roots (Humphreys Jones and Waid 1963). Others are very distantly related to *N. lolii*, such as the basidiomycete *Sclerotium sp*.. The specificity of the primers was confirmed by lack of amplification in all these fungal species.

To evaluate the reliability of the assays with other perennial ryegrass and hybrid cultivars, DNA was extracted from 16 glasshouse-grown cultivars (Table 2.1). These cultivars included diploid, tetraploid and long-term hybrid perennial ryegrass cultivars, representing almost all perennial ryegrass cultivars present in New Zealand pasture. Both endophyte-free and NZCT-infected perennial ryegrass lines of each of all 16 cultivars were tested. In addition, AR1-infected and AR37-infected perennial ryegrass lines of three of the cultivars (‘Commando’, ‘Grasslands Nui’ and ‘Grasslands Samson’) were included. The presence of endophyte in all endophyte-infected grasses was confirmed by immunoblotting. Subsequently, real time PCR was done with DNA from the same samples and using all primer/probe combinations. The *TB1* primer/probe amplified DNA from all these grasses, whilst the *ltmG* primer/probe amplified DNA from all endophyte-infected perennial ryegrass cultivars but not from

endophyte-free plants. The endophyte strain-specific probes *ltmJ* and *perA* amplified the expected targets (Table 3.1) from NZCT, AR1 and AR37 endophytes in *planta.*

To evaluate detection characteristics of the primer/probes, standard curves were generated by amplifying serial dilutions of target pure fungal or endophyte-free perennial ryegrass DNA (Figure 3.3). Amplification efficiencies were between 2.02 - 2.09 and there was a linear correlation between crossing point and log concentration (R^2> 0.995) over six orders of magnitude. Endophyte primer/probes (*ltmG*, *ltmJ* and *perA*) had a detection limit (with a crossing point less than 40 cycles) of 0.01 pg endophyte genomic DNA per reaction.

Table 3.2 TaqMan primer/probe specificity

Sample[a]	Primer/probe[b]			
	ltmG	***ltmJ***	***perA***	***TB1***
NZCT	+	+	+	−
AR1	+	−	+	−
AR37	+	−	−	−
NZCT infected perennial ryegrass	+	+	+	+
AR1 infected perennial ryegrass	+	−	+	+
AR37 infected perennial ryegrass	+	−	−	+
Endophyte-free perennial ryegrass	−	−	−	+
Clover	−	−	−	−

[a] NZCT, AR1 and AR37 represent pure fungal genomic DNA of each endophyte. Clover represents *Trifolium repens* genomic DNA.

[b] '+' and ' - ' indicate amplification or no amplification, respectively, within 40 cycles of real time PCR using each primer/probe indicated. Technical duplicates were used.

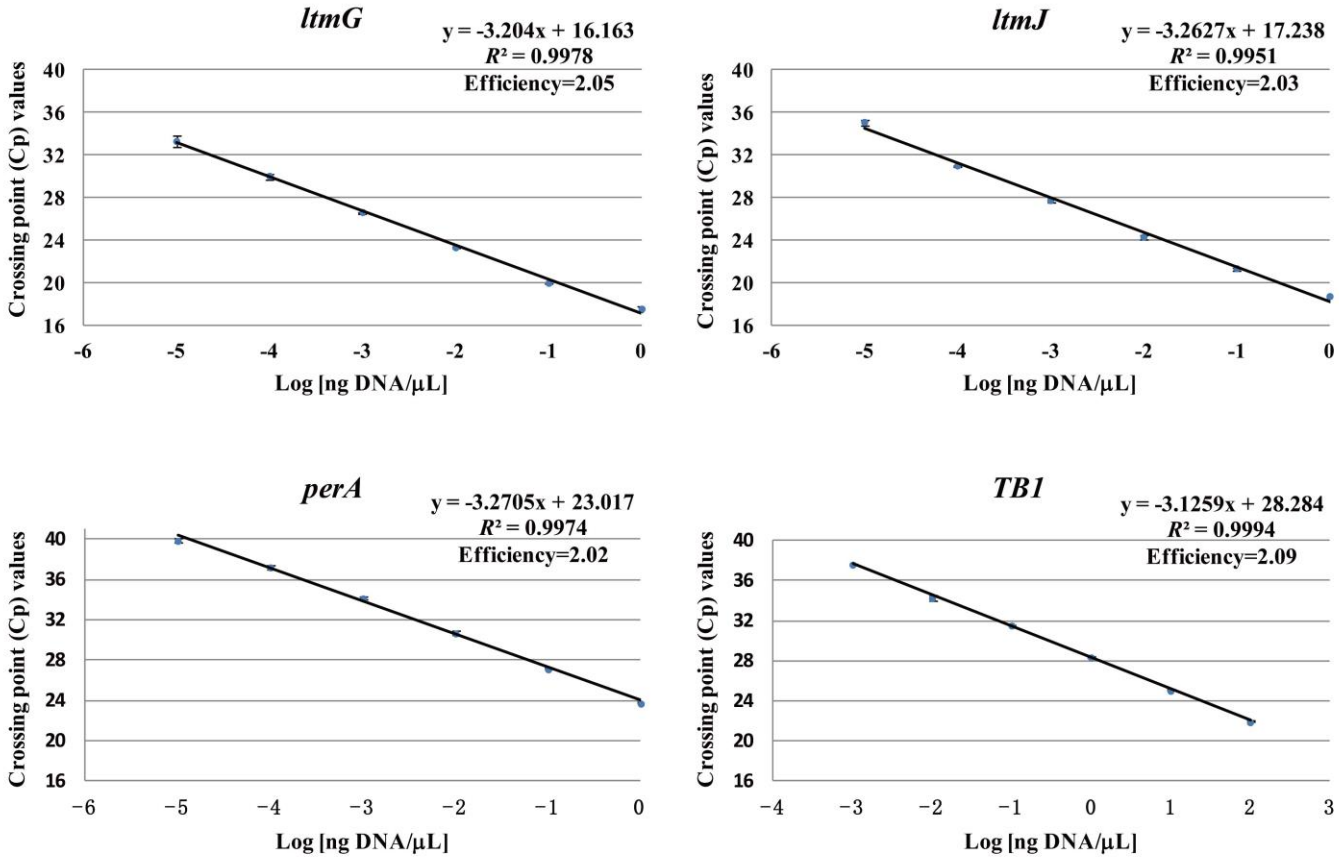

Figure 3.3 Standard curves derived from simplex real time PCR reactions.

Standard curves for fungal gene targets (*ltmG*, *ltmJ* and *perA*) were generated using a 10-fold series dilution of NZCT genomic DNA. Grass primer/probe (*TB1*) standard curve was generated using a 10-fold series dilution of endophyte-free perennial ryegrass genomic DNA. Error bars represent standard deviations.

3.1.3 A dual *ltmG*/ *TB1* assay can estimate relative endophyte levels

We compared the ratio of endophyte biovolume/grass biovolume obtained by hyphal counting (see methods section 2.8 for details) with endophyte to grass DNA ratios obtained with the quantitative duplex PCR assay (*TB1* and *ltmG* genes). The results showed a strong linear correlation (R^2>0.95; Figure 3.4) between the PCR-based estimate of hyphal biomass/ plant biomass ratio and endophyte biovolume/ plant biovolume ratio over a 7 fold endophyte concentration range.

3.1.4 Detection of contaminating endophytes in seed

To evaluate the ability of primer/probes on detecting endophyte contamination of selected endophyte-infected seed, we quantified NZCT and AR1 endophyte contamination in artificially mixed grass seeds (see methods section 2.4.3.8 for details). A good linear relationship (R^2 value > 0.98) was observed between the crossing point of *ltmJ* primer/probe and percent of NZCT-infected seed mixed with AR37-infected seed, and between crossing point value of *perA* primer/probe and percent of AR1-infected seeds mixed with AR37-infected seeds over the entire tested range (3-25%, Figure 3.5).

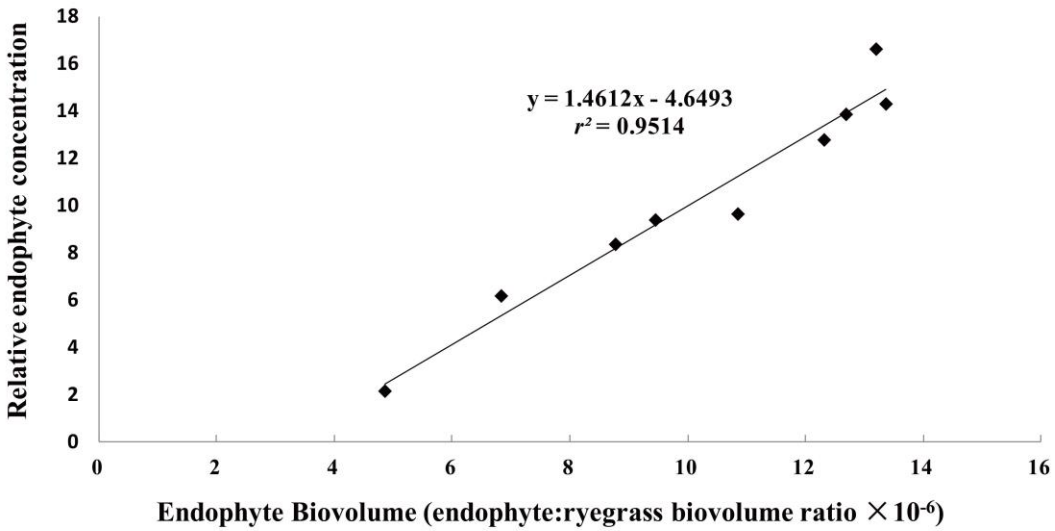

Figure 3.4 Correlation between relative endophyte concentration and fungal biovolume ratio.

Fungal biovolume ratio is expressed as a ratio of the total area of endophyte hyphae to the whole area of cross section of the grass tissue determined by microscopy. Relative endophyte concentration is the ratio of copy number of endophyte *ltmG* gene to perennial ryegrass*TB1* gene.

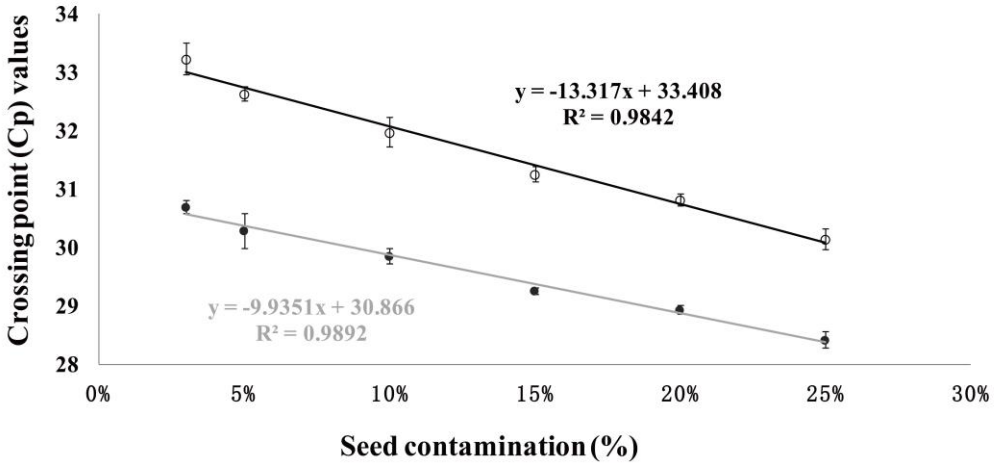

Figure 3.5 Grass seed contamination standard curves.

Gray line: Standard curve of % NZCT-infected perennial ryegrass seed contamination in AR37-infected perennial ryegrass seed, generated using *ltmJ* primer/probe. Black line: Standard curve of AR1-infected perennial ryegrass seed contamination in AR37-infected perennial ryegrass seed, generated using *perA* primer/probe. The percentage is the weight ratio of contaminating seeds (NZCT-infected or AR1-infected) in AR37-infected grass seeds. Error bars indicate standard error among three biological replicates.

3.2 Discussion

3.2.1 Assay specificity, efficiency and reliability

Primer/probe combinations only amplified their intended target endophyte/plant DNA. There was no amplification from DNA of 16 related fungal species and clover, a common forage plant in pasture, indicating that they have a high specificity.

It was important to evaluate the real time PCR method using other current methods. Here we compared the results of real time PCR using *ltmG* primer/probe and immunoblotting on 16 ryegrass cultivars. The good correlation found between results of the two methods suggests a good reliability of this real time PCR method for endophyte detection. Besides, the success of endophyte identification using *ltmJ* and *perA* primer/probes in three cultivars, which contain different endophytes, indicates a good reliability of this real time PCR method for endophyte identification.

The detection limit of 0.01 pg endophyte genomic DNA per reaction indicates that, in principle, real time PCR methods based on these primers should be able to quantify total endophyte for each of the three individual endophytes in a range of applications. This suggests that, theoretically, as little as 1 endophyte nucleus can be detected in a real time PCR reaction (based on an endophyte genome size of 30.6 Mb (Kuldau et al. 1999).

3.2.2 A dual *ltmG*/ *TB1* assay can estimate relative endophyte levels

Real time PCR estimates of endophyte infection levels depend on the copy number of plant and fungal genes, the DNA content of fungal and plant tissues, and the efficiency with which DNA is extracted from plant and fungal tissue. It is thus essential to verify that real time PCR estimates correlate with other estimates of endophyte biomass in plant tissue. Currently the only reliable methods for assessing amounts of endophyte biomass in infected tissues is hyphal counting in tissue cross sections under the microscope (Tan et al. 2001) and enzyme linked immunosorbent assays (ELISA) (Ball et al. 1995). Hyphal counting is an accurate method that determines endophyte biomass directly, however, it is time consuming and not suitable for bulk grass samples. Compared to hyphal counting, ELISA is more

efficient, but is an indirect measure that depends on endophyte antigen concentration, which may vary between samples due to a multitude of factors such as differences in gene expression. The ratio between PCR-amplifiable genes and endophyte biomass should be less changeable, although it could still be affected by factors such as spacing of nuclei along hyphae, hyphae thickness or differences between samples in the efficiency of DNA extraction. Although others have applied real time PCR to determine endophyte biomass based on a ratio of endophyte to plant DNA, they did not validate their real time PCR methods by comparing results with other measures of endophyte biomass (Charlton et al. 2012; Hahn et al. 2008; Young et al. 2005). Thus we validated our real time PCR (duplex PCR assay, *TB1* and *ltmG* genes) method with measurements of the ratio of endophyte biovolume/grass biovolume obtained by hyphal counting. The strong linear correlation between endophyte concentration determined by real time PCR results and ratio of endophyte biovolume/grass biovolume suggests real time PCR based estimates appears an excellent alternative to microscopy-based estimates of endophyte infection levels. We note however that, for reasons outlined above, the correlation between real time PCR based estimates of endophyte infection levels and actual infection levels could differ between plant-fungal associations and under different environmental conditions. Thus this correlation should probably be first assessed, by parallel microscopy and real time PCR in a small number of samples, if this real time PCR methodology is used on other fungal-plant associations.

3.2.3 Detection of contaminating endophytes in seed

A common problem facing seed suppliers and farmers is contamination of commercial seed with multiple endophytes, in particular contamination of selected endophyte-infected seed with common toxic strains (Dombrowski et al. 2006). This can be caused by impurity of the grass seeds initially sowed in the field, or invasion of grasses containing other endophytes. In New Zealand no more than 5% of NZCT is permissible in traded commercial selected endophyte seed. The level of NZCT has been determined by HPLC and more recently by ELISA, which estimates the proportion of NZCT seeds on the basis of alkaloid content (lolitrem B). However, endophyte alkaloid concentrations are affected by many factors, such as temperature (Brosi et al. 2011), season (Moate et al. 2012), nutrients (Rasmussen et al. 2007) and

drought (Hahn et al. 2008). This means the alkaloid content could under- or over-estimate endophyte contamination in grass seeds collected from different places with different environmental conditions. Unlike the HPLC and ELISA methods, real time PCR based on our primers measures the levels of DNA derived from different strains directly and should be a better indication of endophyte biomass. The linear correlations (R^2 value > 0.98) between crossing points of specific primer/probes (*ltmJ*, *perA*) and percent of targeted endophyte infected seeds over the entire tested range (3-25%) suggests a good capability of these primer/probe systems to detect contamination of commercial seed with certain endophytes.

3.3 General Conclusion

In conclusion our results suggest that this real time PCR method can sensitively detect and precisely quantify three distinct *N. lolii* endophytes in *L. perenne*. This technology could benefit management of pastures with selected endophytes through monitoring of toxic endophyte invasion of pastures. In addition, it could assist in assessing seed, since contamination in seed production stands can often lead to the presence of undesirable endophytes in commercially distributed seed. Also this method opens up many new opportunities for research, such as detailed investigations of endophyte survival in different grass genotypes, or under biotic or abiotic stress, as well as of endophyte distribution in different grass tissues.

4 Grasses selection and drought physiology

4.1 Results:

4.1.1 Preliminary screen of the effect of *Neotyphodium lolii* on perennial ryegrass drought tolerance

The impact of *N. lolii* on drought tolerance of 16 ryegrass cultivars (listed in Table 2.1) was assessed. These cultivars included diploid, tetraploid and long-term hybrid perennial ryegrass cultivars, representing almost all perennial ryegrass cultivars present in New Zealand pasture. For each cultivar, 30 wild-type *N. lolii*-infected (E+) and 25 endophyte-free (E-) plants were grown in a glasshouse (5 more E+ than E- were used in case endophyte infection was not 100%). Because it was unclear if endophytes mainly affect the drought stress or the recovery stage, grasses were grown without water for three weeks, followed by a well-watered 12 day recovery period. Control plants were watered throughout the whole period. The effects of endophyte were determined by measuring grass dry biomass (including roots) and tiller numbers after treatment.

Under drought conditions, the presence of endophyte increased average dry biomass and tiller number, compared to endophyte-free grasses, in 13 of 16 cultivars (Figure 4.1). In 5 of those 13 cultivars the effect of endophyte on biomass increase was statistically significant ($P<0.05$; *t*-test) (Figure 4.1). Under well-watered conditions, endophyte infection also significantly increased biomass in 8 cultivars and the average dry biomass and tiller number was also increased in 14 of 16 cultivars (Figure 4.2). The magnitude of endophyte-induced biomass increase did not differ significantly between drought and control conditions (average across cultivars of 22% and 41% increases in biomass, respectively). In 9 of the cultivars, significant endophyte × genotype × treatment (drought or control) interaction was observed (Figure 4.1; One-Way ANOVA, $P<0.05$). In one cultivar (Bronsyn), which responded to drought with inflorescence formation, the endophyte significantly increased the frequency of this response (83% of E+ with inflorescence versus 13% of E- with

inflorescence under drought; $P<0.001$; Fisher's exact test).

Our results suggested that *N. lolii* benefits ryegrass under drought conditions. But the extent of its effect varied between cultivars and significant endophyte × genotype × treatment interaction was observed, and the impact of endophyte seemed less pronounced in drought than when plants were watered.

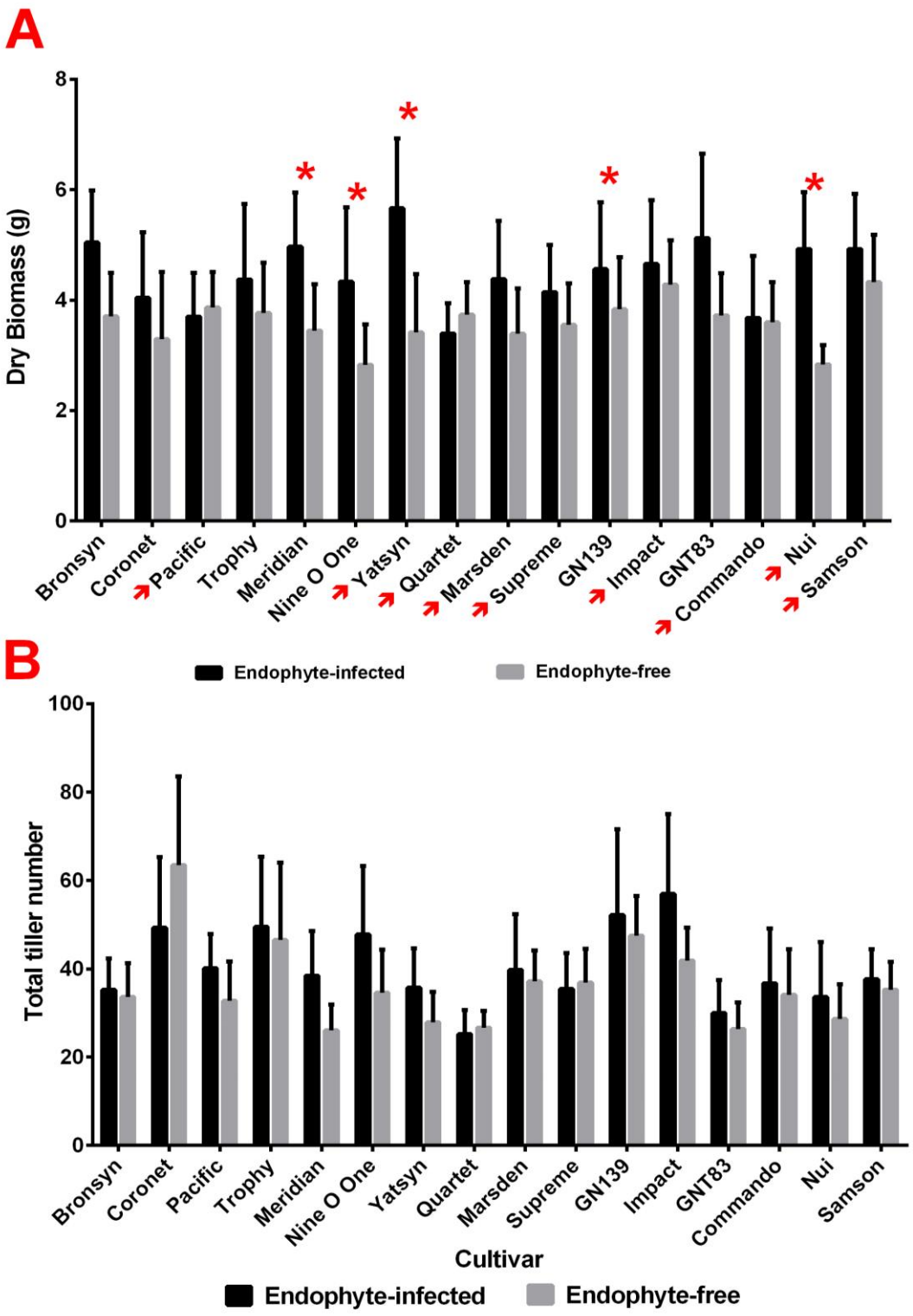

Figure 4.1 Grass dry biomass (A) and total tiller number (B) under drought treatment.

Red star above the column indicates significant difference between endophyte-infected and endophyte-free grass ($P<0.05$). No significant difference was seen in tiller number. Red arrows indicate cultivars with significant endophyte × genotype × treatment interaction ($P<0.05$). Error bars indicate standard error among three biological replicates.

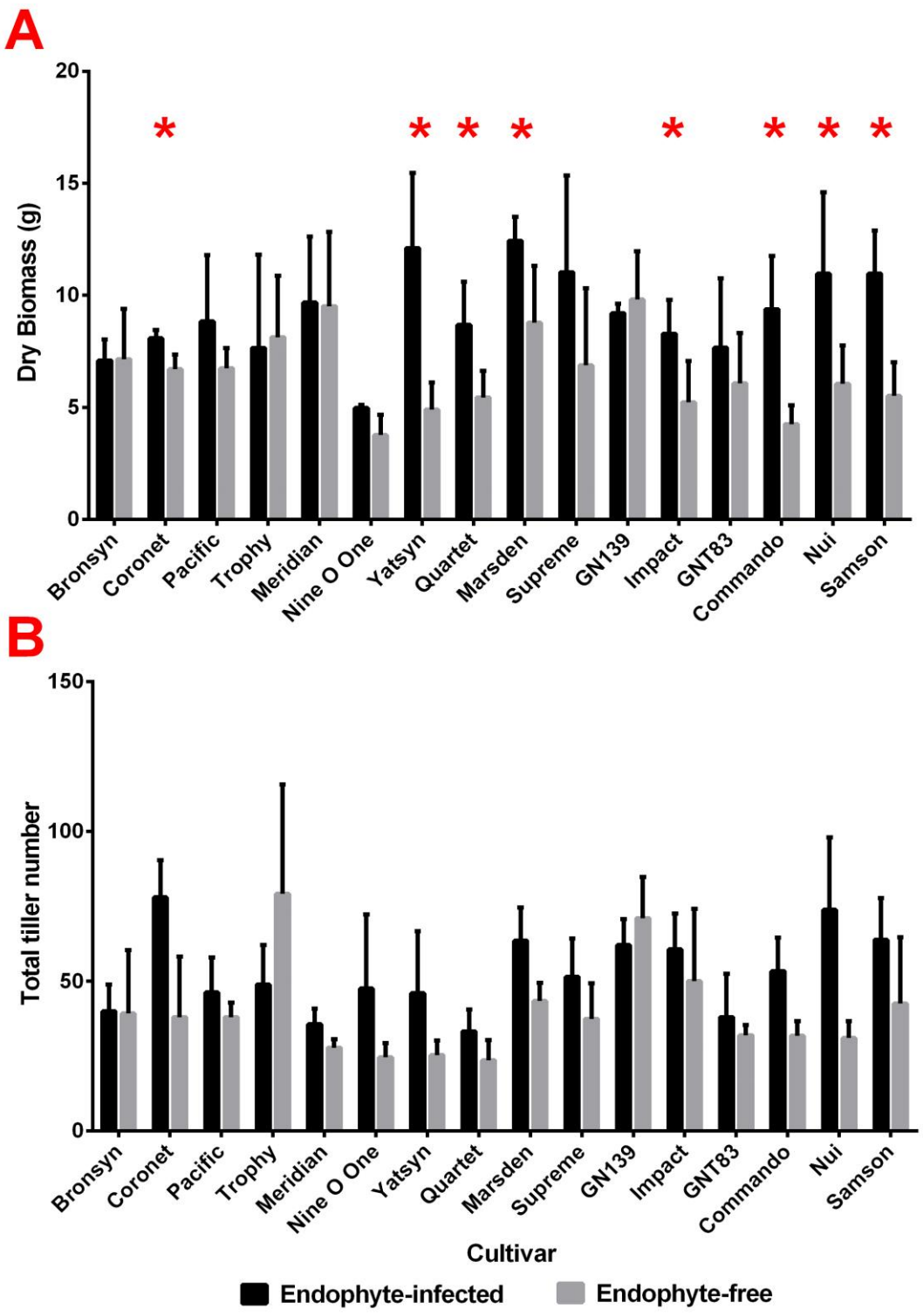

Figure 4.2 Grass dry biomass (A) and total tiller number (B) under well watered conditions.

Red star above the column indicates significant difference between endophyte-infected and endophyte-free grass ($P<0.05$). No significant difference was seen in tiller number. Error bars indicate standard error among three biological replicates.

4.1.2 Outlier selection and verification

The primary purpose of these assays was to select endophyte-infected grasses with distinct drought tolerance phenotypes for use in a transcriptomics study. A significant endophyte × genotype × drought treatment interaction (Figure 4.1; One-Way ANOVA, $P<0.05$) was found in section 4.1.1. This indicated that the benefit of endophyte on grass drought tolerance depends on grass genotype. Thus by comparing endophyte-infected grasses with extremely different drought tolerance levels it should be possible to identify plant and/or endophyte genes associated with drought tolerance. In order to do this, endophyte-containing outliers with the highest and lowest dry biomass levels (from the preliminary screen described in Section 4.1.1) were selected for further testing from each of the 16 cultivars (Figure 4.3) after drought and recovery. For each cultivar, the outliers had greater (outlier 1) or less (outlier 2) biomass than the median biomass of endophyte-free plants (Figure 4.3) and the biomass of outlier 1 was >2 fold more than that of outlier 2 (Figure 4.3).

So far only the effects of endophyte on grass growth had been determined under drought stress. Given the importance of grass survival under severe drought conditions, the ability of outliers to survive three weeks without watering was tested. For 13 of the 16 cultivars, both outliers (1 and 2) were dead at the end of the severe drought treatment. Of the remaining three cultivars (Nine O One, Trophy and Pacific) most of the outlier 2 (low biomass) plants survived, while the corresponding outlier 1 (high biomass) plants all died (Table 4.1).

These preliminary studies were carried out in a glasshouse. The unstable environmental conditions, as well as other environmental factors such as insect and plant pathogens, may have affected performance of the grasses Therefore another experiment was conducted in a controlled-environment plant growth chamber, using less extreme drought conditions (down to 25% FC). The aims of this were to (1) verify the drought tolerance of the selected outliers for the three cultivars that had shown the best ability to survive drought; (2) explore the best drought conditions to use in a transcriptomics study.

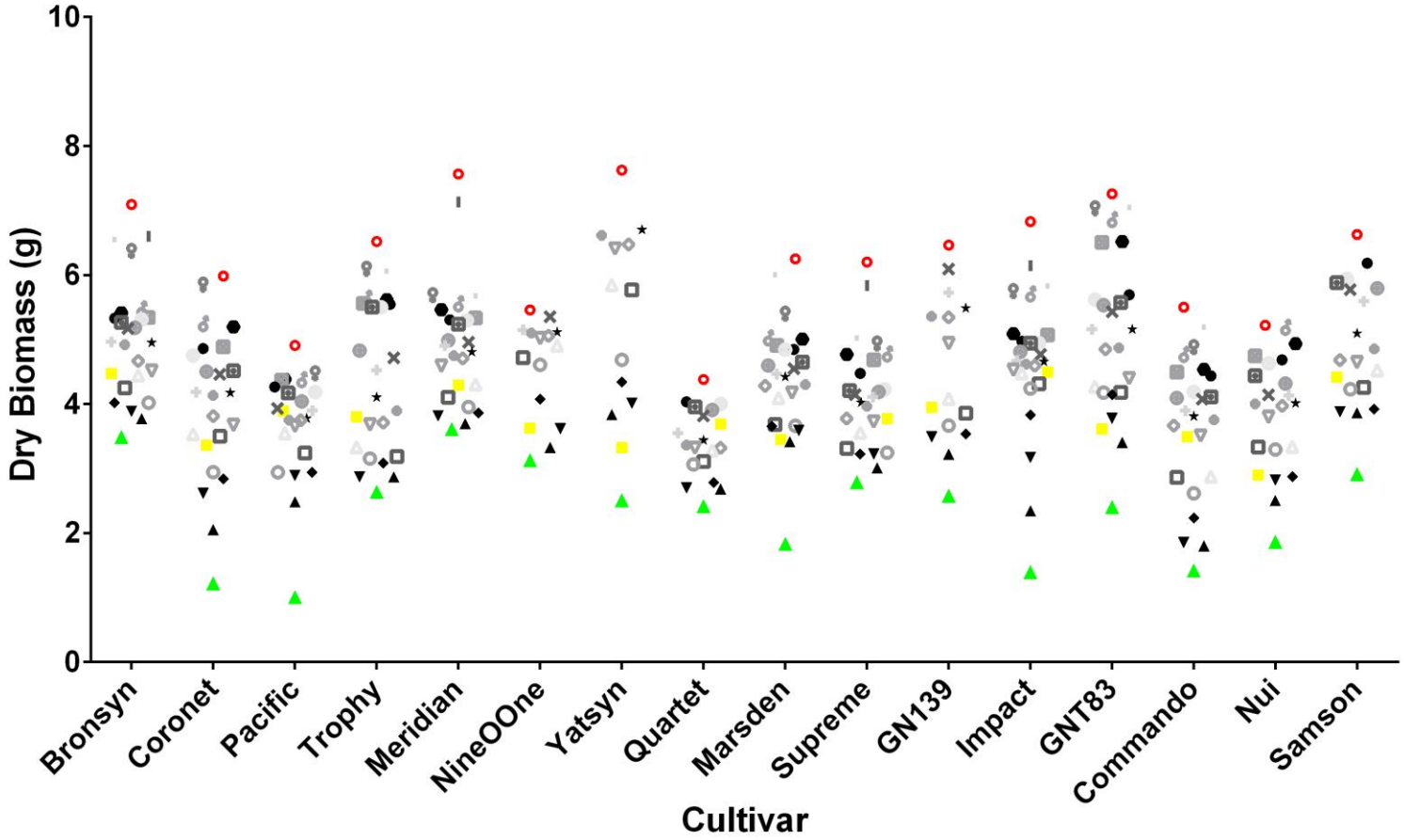

Figure 4.3 Dry biomass of endophyte-infected grass after drought and re-watering treatment.

The red circles and green triangles indicate grasses had greatest (outlier 1) or least (outlier 2) dry biomass in each cultivar respectively. The yellow squares show the median dry biomass of endophyte-free grasses in each cultivar.

Table 4.1 Ability of outliers to survive severe drought stress

Cultivar	Survival	
	Outlier 1[a]	Outlier 2[b]
Bronsyn	0	0
Coronet	0	0
Meridian	0	0
Yatsyn	0	0
Quartet	0	0
Marsden	0	0
Supreme	0	0
GN139	0	0
Impact	0	0
GNT83	0	0
Commando	0	0
Nui	0	0
Samson	0	0
Nine O One	0	**4**
Trophy	0	**4**
Pacific	0	**3**

[a]: Endophyte-infected grass had highest dry biomass in each cultivar in preliminary screen (Figure 4.3).
[b]: Endophyte-infected grass had lowest dry biomass in each cultivar in preliminary screen (Figure 4.3).
Four biological replicates of each grass were used to assess survival of three weeks without watering.

To track grass growth throughout the experiment in a non-destructive way, the grass total tiller number was determined. In Nine O One and Pacific cultivars, outlier 2 had significantly higher tiller numbers than outlier 1 at all time points. No significant difference was seen for the Trophy cultivar (Table 4.2). Whilst Nine O One grasses continued tillering under drought at 25% FC, outlier 1 of Pacific and Trophy cultivars appeared to have stopped tillering by this stage; however due to the short drought treatment period (one week) none of the differences in tiller numbers between time points were significant.

Leaf relative water content (LRWC) and photosynthesis Ⅱ maximum efficiency (Fv/Fm) was determined to assess the extent of drought stress. In all six grasses, the LRWC dropped below 47% when they were grown in 50% FC soil water content, but no significant difference between outliers was observed, in any of the three cultivars, under these conditions (Figure 4.4). However when the soil water content was reduced to 25% FC, outlier 2 of Nine O One and Trophy cultivars had significantly higher LRWC than their corresponding outlier 1 plants (*T*-test, $P<0.05$) (Figure 4.4 A and B). At the water recovery stage (1ARW) Nine O One outlier 2 also had significantly higher LRW than outlier 1 (*T*-test, $P<0.05$) (Figure 4.4 A). No significant difference in LRWC was observed between the two Pacific cultivar outliers throughout the experiment (Figure 4.4 C).

A similar pattern was also observed with Fv/Fm measurements. However the only significant difference between outliers was observed under severe drought stress (25% FC) in Nine O One and Trophy cultivars, where outlier 2 plants had higher Fv/Fm ratios (0.52 and 0.41 respectively) than outlier 1 plants (0.36 and 0.25 respectively) (*T*-test, $P= 0.026$ and 0.043 respectively) (Figure 4.5 A and B). No significant differences in Fv/Fm ratios were observed between the two Pacific outliers (Figure 4.5 C).

Table 4.2 Grass total tiller number

Water condition[a]	**Nine O One** (Mean ± SD)		**Trophy** (Mean ± SD)		**Pacific** (Mean ± SD)	
	Outlier 1	**Outlier 2**	**Outlier 1**	**Outlier 2**	**Outlier 1**	**Outlier 2**
Control	25 ± 1.7	34 ± 2	25.7 ± 0.6	24.7 ± 0.6	24.7 ± 1.5	36.7 ± 1.5
75% FC	27 ± 1.7	36.3 ± 2.1	28.3 ± 3.1	27 ± 1	27 ± 2	40.7 ± 2.1
50% FC	28.7 ± 1.5	38.7 ± 1.5	30.3 ± 1.5	29.7 ± 3.2	30.3 ± 1.5	47.3 ± 2.9
25% FC	29 ± 2.6	40.7 ± 1.5	30.7 ± 2.1	31 ± 2	30.7 ± 3.2	48.7 ± 2.1
3 ARW	29.7 ± 2.1	42.7 ± 1.5	31.7 ± 1.2	33.3 ± 1.5	31.7 ± 2.1	53.3 ± 1.5

Values are numbers of tillers per plant (mean ± SD of three biological replicates).

'FC' represents field capacity. '3RW' indicates three days after re-watering.

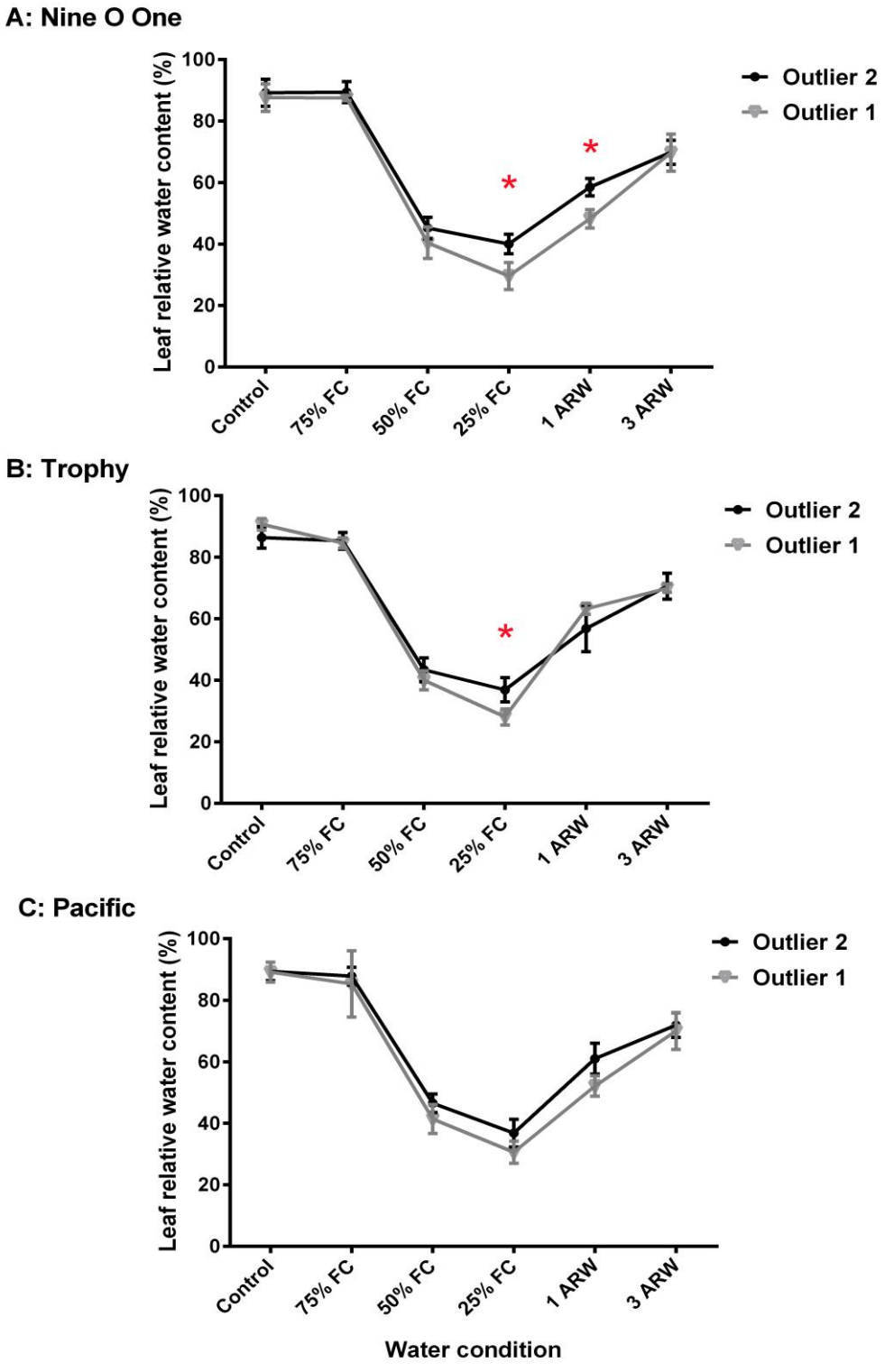

Figure 4.4 Grass leaf relative water content

A, B and C show leaf relative water content of Nine O One, Trophy and Pacific grasses respectively. 'FC' represents field capacity. '1ARW' and '3RW' indicate one day and three days after re-watering. Error bars indicate standard error among three biological replicates.

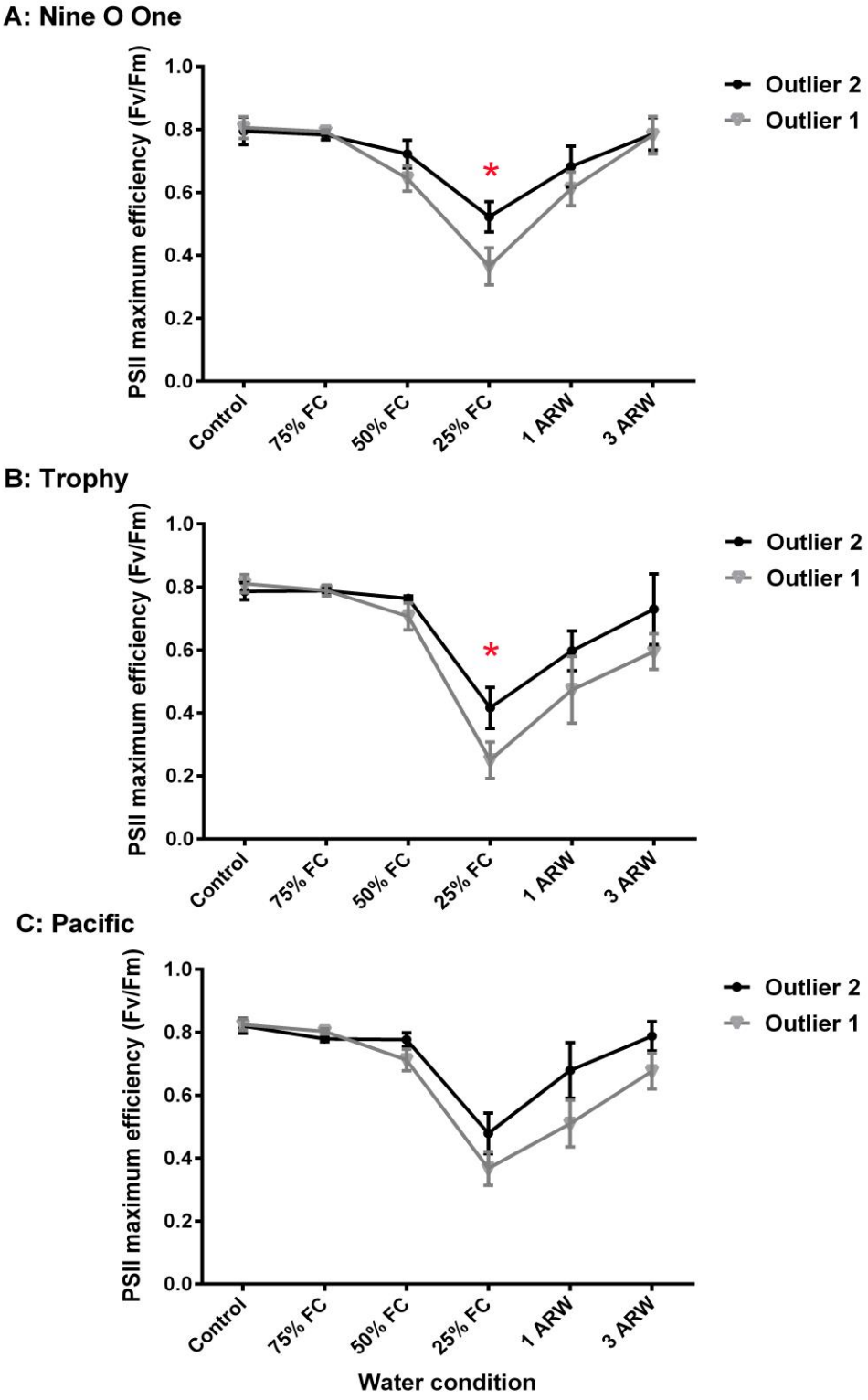

Figure 4.5 Grass photosynthesis (PS) Ⅱ maximum efficiency (Fv/Fm)

A, B and C show photosynthesis (PS) Ⅱ maximum efficiency of Nine O One, Trophy and Pacific grasses respectively. 'FC' represents field capacity. '1ARW' and '3RW' indicate one day and three days after re-watering. Error bars indicate standard error among three biological replicates.

Leaf osmotic adjustment (LOA) was measured to monitor the degree of osmolyte accumulation. All grasses lowered their osmotic potential under drought conditions, and, as expected, this occurred to a greater extent under severe drought stress (Figure 4.6). With 50% FC no significant difference in LOA was observed between the two outliers of any cultivar (Figure 4.6). But, at 25% FC, outlier 2 of Nine O One and Trophy had LOA values of -0.42 and -0.35 Mpa respectively, significantly different to their corresponding outlier 1 plants, (LOA values of -0.31 and -0.30 Mpa respectively; *T*-test, *P*= 0.017 and 0.044 respectively) (Figure 4.6 A and B). No significant differences in LOA were observed between the two Pacific outliers (Figure 4.6 C).

In summary, three pairs of outliers of Nine O One, Trophy and Pacific cultivars showed very different survival ability under drought. Among them, outlier 2 of Nine O One and Trophy cultivars had significantly higher LRWC, Fv/Fm and greater LOA than outlier 1 under severe drought conditions. The outlier 2 characteristics in these cultivars corresponded to their ability to survive in drought (Table 4.1) although they accumulated less biomass than outlier 1 plants under drought conditions (Figure 4.3).

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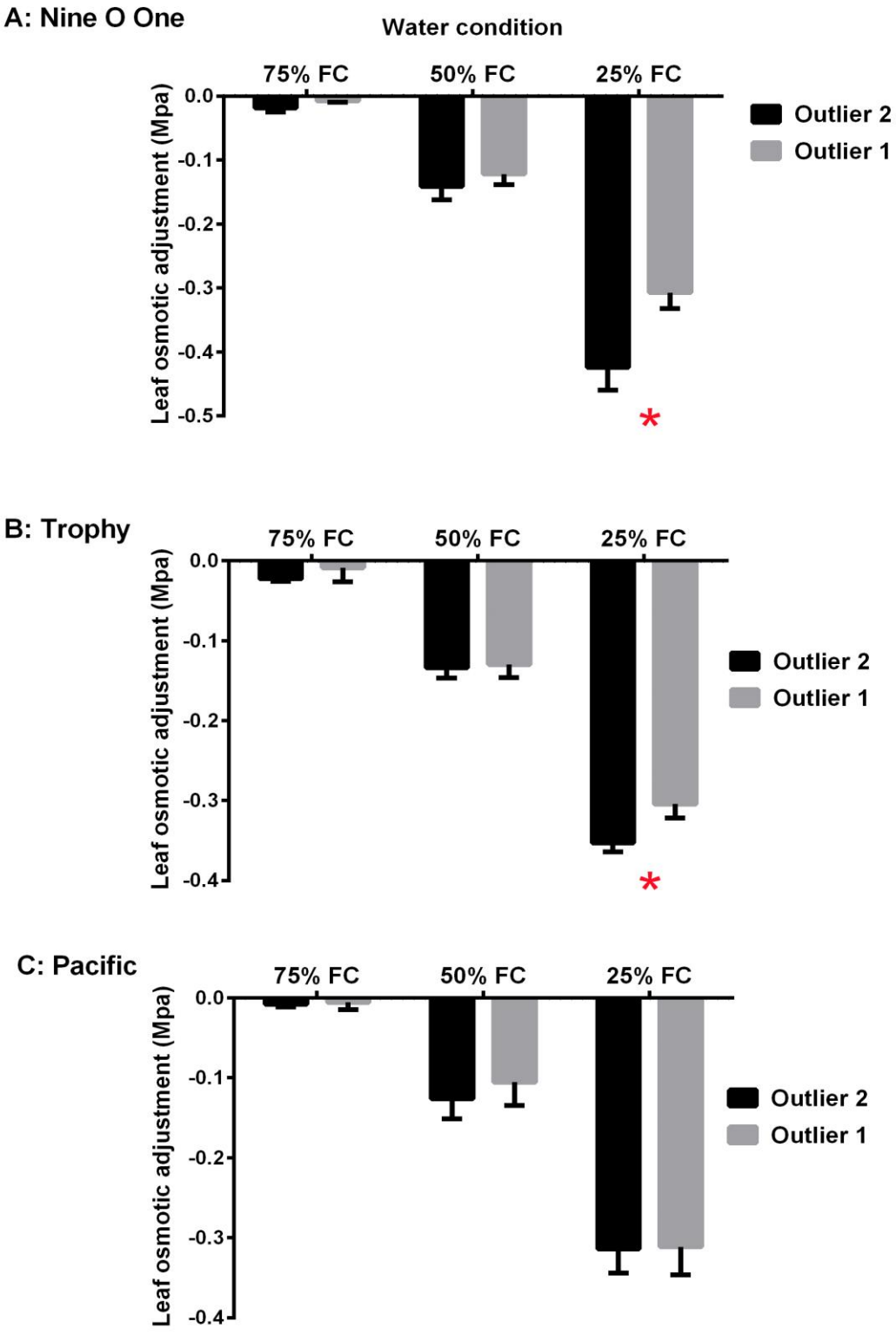


Figure 4.6 Grass leaf osmotic adjustment

A, B and C show leaf osmotic adjustment of Nine O One, Trophy and Pacific grasses respectively. 'FC' represents filed capacity. '1ARW' and '3RW' indicate one day and three days after re-watering. Error bars indicate standard error among three biological replicates.

4.1.3 Verification of endophyte status of outliers

Selected outliers were destined for a transcriptomics study, in which comparative analyses of endophyte impact on two grass hosts with distinct drought tolerance levels would be done. Thus, the same genetic background of endophyte was required. Before selecting the specific outliers for the transcriptomics study, the endophyte species and genotypes in the Nine O One, Trophy and Pacific cultivar outliers were tested using real time PCR and SSR (simple sequence repeats) respectively.

Two specific primer/probe combinations were used to test endophyte species. One was based on *ltmG*, a common gene found in all *N. lolii* strains; the other was based on *ltmJ* that is specific to wild type *N. lolii* (see details in Chapter 3). All six outliers showed positive results in real time PCR tests using both *ltmG* and *ltmJ* primer/probes, consistent with wild-type *N. lolii* (Table 4.3).

Because wild type *N. lolii* can have different genotypes, SSR tests were conducted. All six endophytes had the same B10 allele (178 nucleotides) as in standard wild type *N. lolii* (Table 4.4). Five of them also had the same B11 allele (177 nucleotides) as the wild type, but Trophy outlier 1 had a distinctly smaller size B11 allele (141 nucleotides). The five 'wild-type' endophytes (except Trophy outlier 1) were further tested using another locus marker, Ans25, and they all had the same allele (313 nucleotides) as standard wild type *N. lolii*.

In summary, all endophytes living in five of six outliers were verified as wild type *N. lolii* based on real time PCR and SSR tests. The endophyte in Trophy outlier 1 appeared to have a different genotype from standard wild type *N. lolii*.

Table 4.3 Endophyte strain test using specific TaqMan primer/probe sets

Sample[a]	Primer/probe[b]	
	ltmG	***ltmJ***
Nine O One outlier 1	+	+
Nine O One outlier 2	+	+
Trophy outlier 1	+	+
Trophy outlier 2	+	+
Pacific outlier 1	+	+
Pacific outlier 2	+	+
NZCT	+	+
AR1	+	−
AR37	+	−

[a] NZCT, AR1 and AR37 represent pure fungal genomic DNA of each endophyte.
[b] '+' and '—' indicate amplification or no amplification, respectively, within 40 cycles of real time PCR using each primer/probe indicated. Technical duplicates were used.

Table 4.4 SSR (simple sequence repeat) endophyte genotyping results

Host grass	Allele size of each locus (nucleotide units)		
	B10[a]	**B11[b]**	**Ans25[c]**
Nine O One outlier 1	178	177	313
Nine O One outlier 2	178	177	313
Trophy outlier 1	178	**141**	Not tested
Trophy outlier 2	178	177	313
Pacific outlier 1	178	177	313
Pacific outlier 2	178	177	313
Wild type *N. lolii* [a]	178	177	313

[a], [b] and [c] are SSR marker loci used in the test.

4.1.4 Endophyte influenced perennial ryegrass physiological response to drought

Outliers of Nine O One cultivar were selected for further study of the effects of endophyte on ryegrass response to drought. This work underpinned the subsequent transcriptomics study in which both endophyte and grass gene expression was studied in the same samples (Sections 5 and 6). Outlier 2 and outlier 1 are named as DTE+ (endophyte-infected drought tolerant genotype) and DSE+ (endophyte-infected drought sensitive genotype) in the rest of the thesis. Drought tolerant and drought sensitive labels here are not intended to describe complete drought tolerance or susceptibility but outliers with different responses to drought, including survival ability (Table 4.1), leaf relative water content (Figure 4.4), photosynthesis Ⅱ maximum efficiency (Figure 4.5) and leaf osmotic adjustment (Figure 4.6,).

To explore the endophyte effect on grass physiological response to drought, clonal endophyte free plants of DTE+ and DSE+ were generated using fungicide treatment (see section 2.9). These were named as DTE- (endophyte-free drought tolerant genotype) and DSE- (endophyte-free drought sensitive genotype), respectively. Both pairs of clonal grasses (DTE+ and DTE-, DSE+ and DSE-) were subjected to drought treatment as described in section 2.11.4. For each type, half of the grass samples were well watered, and the rest of the grasses were subjected to drought stress. The extent of drought stress was determined by measuring leaf relative water content (LRWC) and PS (photosynthesis) II maximum efficiency (Fv/Fm).

Under well-watered conditions, all four grasses had similar LRWC levels of about 82% (Figure 4.7), but under severe drought stress, their LRWC values dropped to less than 31%. In both genotypes, the endophyte-containing grasses had higher LRWC than their clonal endophyte-free controls under drought; in the case of the drought tolerant genotype this difference (13.8%) was significant (*T*-test, P=0.033).

A similar pattern was found with the measurements of Fv/Fm (photosynthesis II maximum efficiency). After 30 mins of dark adaption, the Fv/Fm ratio of all grasses was about 0.81 under well-watered conditions, but decreased below 0.54 under drought (Figure 4.8). Compared with endophyte-free plants, endophyte-infected plants had higher Fv/Fm ratios under drought; in case of the drought tolerant genotype this difference (23.2%) was significant (*T*-test, *P*=0.044).

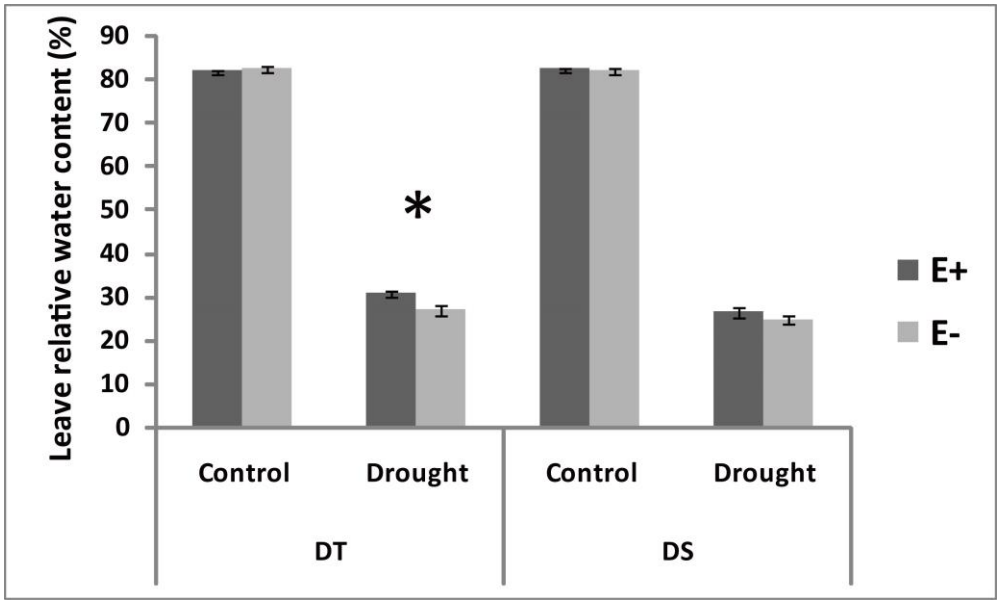


Figure 4.7 Grass leaf relative water content.

E+ and E- are endophyte-infected and endophyte-free perennial ryegrass respectively. Asterisk indicates significant difference between E+ and E- (*T*-test, P< 0.05).

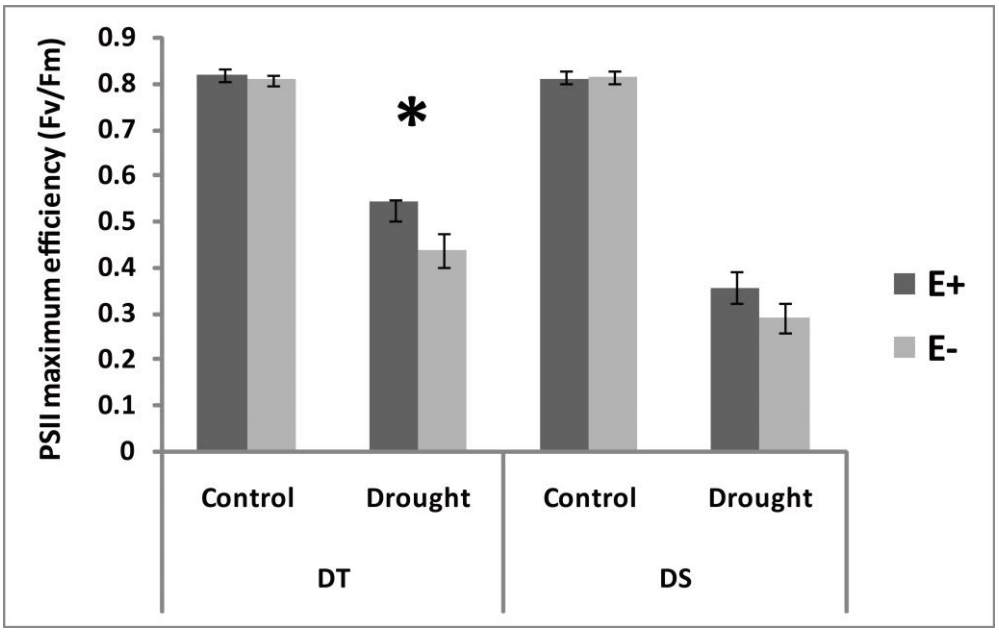


Figure 4.8 Grass photosynthesis II maximum efficiency (Fv/Fm).

E+ and E- are endophyte-infected and endophyte-free perennial ryegrass respectively. Asterisk indicates significant difference between E+ and E- (*T*-test, P< 0.05). Error bars indicate standard error among three biological replicates.

Leaf osmotic adjustment (LOA) was measured to monitor the degree of osmolyte accumulation. All grasses lowered their osmotic potential under drought conditions, but to different extents (Figure 4.9). DTE+ had the most substantial LOA, -0.43 Mpa, among all four grasses, which was significantly greater than DTE- with a LOA of -0.37 Mpa (*T*-test, *P*=0.022). Correspondingly, the LOA of DSE+, -0.35 Mpa, was slightly higher than that of DSE-, -0.33 Mpa, without significant difference (*T*-test, *P*=0.112).

To estimate the growth of the plants in the same experiment, total tiller numbers (TTN) were counted. As found in the preliminary trial, the drought tolerant genotype had more tillers (~110) than drought sensitive genotype (~92). However both endophyte-infected grasses had similar tiller numbers as their clonal endophyte-free plants under control conditions (Figure 4.10). Under drought, the presence of the endophyte was associated with a significant increase in tiller numbers in the tolerant genotype (DTE+ had 71 TTN; DTE- with 64 TTN; *T*-test, $P<0.05$). A similar trend was seen in the sensitive genotype but in this case the difference between TTN values of DSE+ (57) and DSE- (53) was not significant ($P>0.05$).

Together these analyses suggest that infection with *N. lolii* endophyte enhanced ryegrass drought tolerance but that the endophyte effects were more pronounced on the drought tolerant genotype than on the drought sensitive genotype.

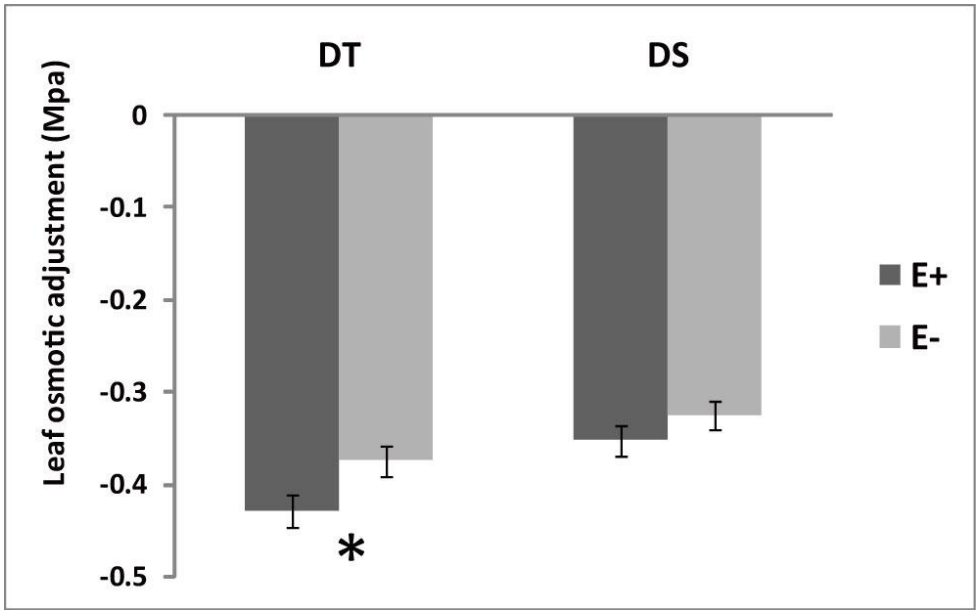


Figure 4.9 Grass leaf osmotic adjustment under drought

E+ and E- are endophyte-infected and endophyte-free perennial ryegrass respectively. Asterisk indicates significant difference between E+ and E- (*T*-test, P< 0.05). Error bars indicate standard error among three biological replicates.

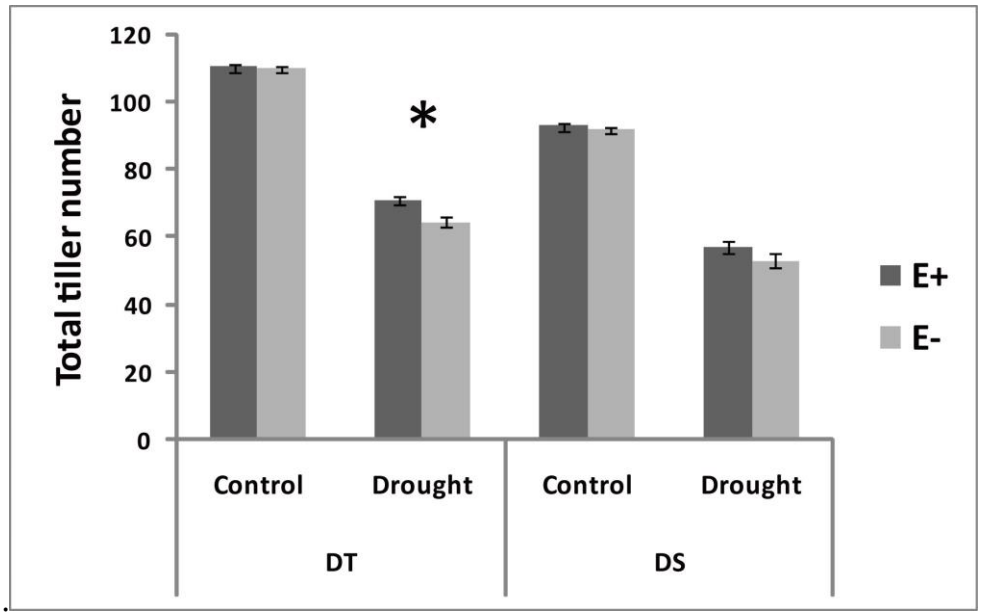


Figure 4.10 Grass total tiller number.

E+ and E- are endophyte-infected and endophyte-free perennial ryegrass respectively. Asterisk indicates significant difference between E+ and E- (*T*-test, P< 0.05). Error bars indicate standard error among three biological replicates.

4.2 Discussion

4.2.1 The impact of endophyte on improving grass growth under water deficit varies between cultivars

Many studies have been conducted on exploring the impact of *Epichloë* and *Neotyphodium* endophytes on host grass performance under water deficit; however, collective their results are inconsistent. Some studies reported that endophyte-infected plants had greater biomass and more tillers at the end of the water stress period compared with endophyte-free plants (Hahn et al. 2008; Rudgers and Swafford 2009; Zhang and Nan 2007). Other researchers suggested that *Epichloë* and *Neotyphodium* endophytes had no, or even detrimental, effects on grass drought tolerance (Cheplick 2004; Cheplick 2007; Cheplick et al. 2000). Thus we assessed the impact of *N. lolii* endophytes on growth of a range of perennial ryegrass cultivars.

Endophyte-infected plants had higher dry biomass and total tiller number in 13 out of 16 cultivars tested but the effect was only significant for 5 of these (Figure 4.1). This suggested that although *N. lolii* had a positive effect on grass growth under drought, the effect was variable between cultivars. This is consistent with another study in which *Neotyphodium* endophyte infection increased biomass of four of six grass populations under drought, only one of which was significant (Kane 2011). This author also found significant population × treatment × endophyte-infection interactions in 4 of 6 populations on grass total tiller number (Kane 2011).

The variation of endophyte effect on grass growth between cultivars may be due to variation in either grass and/or endophyte genotypes. In this study, we observed significant endophyte × genotype × treatment interactions in 9 of 16 cultivars (Figure 4.1). It has been reported that grass genotype affects endophyte influences on grass growth (Cheplick 1998, 2004; Hahn et al. 2008; Hesse et al. 2003). Also it has been suggested that endophyte genotype influences its interaction with host grass (Cheplick

and Faeth 2009). We found the endophyte in one of six outliers was distinctly different from the standard wild type *N. lolii* based on SSR genotyping (Table 4.4). This suggests there may have been other variation in endophyte genotypes in grasses tested in the preliminary screen. The fact that the Trophy outlier 1 grass harbouring the different endophyte had the greatest biomass in this cultivar under drought, whilst Trophy outlier 2 had a standard wild-type endophyte, is consistent with an influence of endophyte genotype on its effect on host growth under stress.

Another effect of endophyte infection was increased inflorescence formation of grasses in the Bronsyn cultivar. Whilst there was no significant effect of endophyte on grass growth in this cultivar (Figures 4.1 and 4.2), early flowering is a good strategy to escape from drought, because this enables a plant complete its life cycle before the most intense period of drought (Sherrard and Maherali 2006). The increased inflorescence formation indicates that endophytes can benefit grass in multiple ways.

4.2.2 Nine O One outliers are the best choice for a transcriptomics study

Even in the same cultivar, the growth of endophyte-infected plants under drought conditions was variable. Some endophyte-infected grasses had higher, and some had lower, levels of dry biomass than the median dry biomass of endophyte-free grass under drought (Figure 4.3). This suggests that effects of endophyte on grass performance under drought are quite different. Thus we selected outliers with the most different dry biomass as the preliminary set of candidates for a transcriptomics study.

Outliers with lower biomass (slower growth rates) showed higher survival ability than those with higher biomass (faster growth rates) (Table 4.1). This was possibly because outliers with greater biomass use more water and are more likely to have hydraulic failure (failure to pump sufficient water from roots to leaves) than outliers with lower biomass. Alternatively, compared to outliers with high biomass, outliers

with low biomass may have invested more carbohydrate on production of osmolytes rather than growth, thus they may have better osmotic adjustment to reduce water loss under severe drought stress (Burg and Ferraris 2008; McDowell et al. 2008).

Most studies on endophyte impact on grass performance under drought emphasize the enhancement of grass growth (Cheplick 2007; Cheplick et al. 2000; Hesse et al. 2003; Kane 2011). However, given climate change and extreme drought conditions such as those experienced in New Zealand in 2013 (APNZ-News-Service 2013), survival ability may be more important than growth rate. Only grasses that survive under severe drought conditions can continue to grow. Thus within the set of outliers a smaller set was selected according to their survival ability for future study.

The photosynthetic efficiency parameter Fv/Fm is often used as a sensitive indicator for many types of plant stress, including drought (Nogues and Baker 2000; Posch and Bennett 2009). A Fv/Fm ratio between 0.79 to 0.84 is recognized as an optimal value for many plants (Kitajima and Butler 1975). Normally, the Fv/Fm ratio decreases when plant is under stress, or when the stress becomes severe. The more stress the plants suffers, the lower the Fv/Fm ratio is (Kitajima and Butler 1975). The decreased Fv/Fm ratios and LRWC (leaf relative water content) under drought confirmed that the grasses suffered water deficit stress (Figures 4.5 and 4.6). However, outlier 2 in Nine O One and Trophy cultivars maintained a significantly higher Fv/Fm ratio and LRWC than corresponding outlier 1 of these cultivars under severe drought condition. This suggested that outliers for both Nine O One and Trophy cultivars had distinctly different drought tolerance levels, and were suitable to be used in a transcriptomics study.

An important way that plants cope with drought stress is to reduce water loss by decreasing osmotic potential in their cells. This can be achieved by accumulating osmolytes such as proline, glucose, fructose, sucrose, fructans, mannitol etc (Burg and Ferraris 2008). Outlier 1 had significantly greater LOA than outlier 2 in both Nine O One and Trophy cultivars, indicating that osmotic adjustment might be one reason for

the different survival ability under severe drought stress between these two pairs of outliers (Table 4.1).

As mentioned in section 4.2.1, endophyte genotype affects its influence on grass performance under drought. Thus the species and genotypes of endophytes living in outliers were tested. Whilst real-time PCR confirmed all outliers were *N. lolii* (Table 4.3), SSR analysis revealed that the endophyte in Trophy outlier 1 had a different genotype from the other endophytes tested (Table 4.3), thus Trophy outliers were not suitable for a transcriptomics study.

Outliers of Nine O One cultivar were selected for future study, as (1) outlier 2 showed the ability to survive under severe drought, (2) the two outliers showed significantly different drought tolerance under relatively severe drought stress, (3) endophytes living in the two outliers were determined to be the same genotype based on real-time PCR and SSR assays.

4.2.3 Endophyte improved drought tolerance of both selected ryegrass genotypes but to different extents

The endophyte effect on selected grasses in the same cultivar was studied by measuring several critical plant physiological parameters. The photosynthetic efficiency parameter Fv/Fm is often used as a sensitive indicator for many types of plant stress, including drought (Nogues and Baker 2000; Posch and Bennett 2009). Plants utilize light to produce ATP and NADPH (Baker 2008). Fv/Fm measurements compare the minimum fluorescence (dark-adapted leaf pre-photosynthetic fluorescent state) to maximum fluorescence (photosynthetic state in the presence of light). A Fv/Fm ratio between 0.79 to 0.84 is recognized as an optimal value for many plants (Kitajima and Butler 1975). Normally, the Fv/Fm ratio decreases when plant is under stress, or when the stress becomes severe (Kitajima and Butler 1975).

In the work described in this chapter, the Fv/Fm ratio of grasses grown under

control conditions was around 0.81, indicating all four types of grasses were healthy (Figure 4.8). The decreased Fv/Fm ratios and LRWC (leaf relative water content) under drought confirmed that the grasses suffered water deficit stress (Figures 4.7 and 4.8). However the endophyte-containing plants DTE+ and DSE+ had higher Fv/Fm ratios, LRWC and TTN (total tiller number) (Figure 4.9) than DTE- and DSE- respectively under drought stress, implying that harboring endophytes relieved grass suffering from water stress. These results are consistent with previous observations of a positive effect of endophyte on grass drought tolerance (Elmi and West 1995; Hahn et al. 2008; Zhang and Nan 2007). However, a significant difference on Fv/Fm, LRWC and TTN was only observed between E+ and E- of DT genotype but not in DS genotype indicating that the extent of positive effect of endophyte on grass drought tolerance was variable between two grass genotypes. The interaction of host genotype and endophyte on grass drought tolerance was also found in others' studies, where the authors found the effect of endophyte on grass drought tolerance can be either positive or negative depending on host genotype-endophyte combinations (Cheplick 2007; Kane 2011). However, no further comparison of positive genotype and negative genotype was reported in their papers.

4.3 Limitations and general conclusions:

There were several limitations in this study:

1. In the preliminary screen to identify drought-tolerant and -susceptible grasses, dry biomass was determined after a combined treatment (drought stress followed by water recovery). This represented an overall effect of endophyte on grass growth during the whole period and was considered relevant to the field situation. However, endophyte effects on grass growth might be different under drought stress than during the water recovery stage.

2. Unstable environmental conditions, as well as other factors such as insect and plant pathogens, might have affected preliminary experiments that were performed in the glasshouse. The Pacific outliers had distinctly different survival abilities in the glasshouse, but no significant differences in LRWC or Fv/Fm parameters were observed.

3. Three SSR markers were used in endophyte genotyping and successfully identified the different genotype of endophyte living in Trophy outlier 1. However, more SSR markers might be needed to confirm the same genotype of endophytes living in Nine O One outliers.

In summary, *N. lolii* benefits ryegrass under drought conditions. But the extent of its effect varies between cultivars and significant endophyte × genotype × treatment interaction was observed. Outliers of Nine O One showed distinct survival ability under severe drought stress. Their different drought tolerance was confirmed by physiological parameters, including LRWC and Fv/Fm, in a time-course experiment conducted in a growth chamber. The same samples described in section 4.1.4, with two Nine O One outlier plants (one with higher drought-tolerance, one relatively drought-susceptible), and endophyte-free clones of these plants, were studied further by transcriptome profiling.

5 Endophyte transcriptome profiling

5.1 Results:

5.1.1 General description of RNA-sequencing results

To explore the influence of endophyte on grass drought tolerance at the transcriptome level, total mRNA was extracted from the second sheath of four grass types (DTE+ (endophyte-infected drought tolerant grass genotype), DTE- (clonal endophyte-free grass of drought tolerant genotype), DSE+ (endophyte-infected drought sensitive grass genotype) and DSE- (clonal endophyte-free grass of drought sensitive genotype) that had been grown under well-watered conditions, or under drought stress conditions (see section 2.11.4 and 4.1.4).

RNA samples from all grass types under drought and control conditions were then submitted for Illumina sequencing. The sequencing results are summarized in Table 5.1. There were 960,407,898 reads in total and 61.6% of the reads were mapped to either grass EST or endophyte genome sequences. There were 6,054,743 fungal reads mapped to the endophyte genome. Only a minor variation between two replicates of the same sample was observed regarding to mapped reads number (see Appendix 5.1). RPKM (reads per kilobase per million) normalization was performed for each individual gene in order to make the read counts comparable among samples (see section 2.15). The proportion of fungal to plant reads in each sample ranged from 1.59% to 2.70%, and the average proportion in all E+ samples was 1.11% (Table 5.1, Table S5.1). Very few (less than 0.03%) cross-mapped fungal reads were found in E- samples (Table S5.1). This chapter focuses on analysis of the endophyte genes, whilst analysis of the plant genes is presented in chapter 6.

To understand the effect of drought on the endophyte transcriptome, statistical analyses were conducted to compare the expression of endophyte genes between drought and control conditions. As shown in Table 5.2, the numbers of differentially expressed genes were slightly higher in the 'DT endophyte' (endophyte living in drought tolerant grass, 2205 genes) than in the 'DS endophyte' (endophyte living in drought susceptible grass, 2054 genes). The abbreviated 'DT endophyte' and DS

endophyte' terminology will be used for the remainder of this thesis; note the ‘DT’ and ‘DS’ labels only indicate the host genotype; they do not describe any characteristics of the endophytes themselves.

A Venn diagram (Figure 5.1) showing the numbers of differentially expressed endophyte genes (when comparing drought to control) reveals further differences that depend on the grass host genotype of the endophytes. About 1/3 of differentially expressed genes are unique to the host genotype (807 and 656 endophyte genes in DT and DS hosts respectively), whilst 2/3 of differentially expressed endophyte genes (1398 genes) are shared by endophytes residing in either DT or DS hosts (Figure 5.1).

Table 5.1 General description of RNA-sequencing results

RNA-sequencing reads	Number or %
Read number of endophyte-free grass samples	422,063,343
Read number of endophyte-infected grass samples	540,344,555
Total read number	962,407,898
Number of total reads after quality trimming	894,729,479
Proportion of total reads after quality trimming	93%
Number of mapped reads	551,153,985
Proportion of mapped reads (% of trimmed reads)	61.6%
Number of grass reads mapped to perennial ryegrass EST[a]	545,099,242
Number of fungal reads mapped to endophyte gene models[b]	6,054,743
Proportion of mapped fungal reads to mapped grass reads	1.11%

[a]: The perennial ryegrass EST is a *de novo* assembled EST library (EST_28Mar13; n =50,194; URL: http://ryegrass.massey.ac.nz/), generated by Jan Schmid, Ningxin Zhang and Robert Day, using sequenced mRNA extracted from perennial ryegrass (NuiD genotype) grown under normal conditions as described in (Zhang et al. 2011).

[b]: The endophyte reference genome sequence used is *Epichloë festucae* gene models (EfM3; n = 9,350; http://csbio-l.csr.uky.edu/ef2011/).

Table 5.2 Number and proportion of differentially expressed endophyte genes

Endophyte[a]	DE gene [b] number	Proportion (%) [c]
Drought vs. Control (DT Endophyte)	2205	26.5
Drought vs. Control (DS Endophyte)	2054	24.7

[a]: "DT Endophyte" and "DS Endophyte" indicate *N. lolii* endophyte in drought tolerant and sensitive grass respectively.
[b]: "DE gene" indicates endophyte genes differently expressed under drought compared to control conditions, with a Qvalue (Storey, 2003) <0.05 and fold change >2.
[c]: "Proportion" indicates the percentage of differentially expressed genes in all expressed endophyte genes.

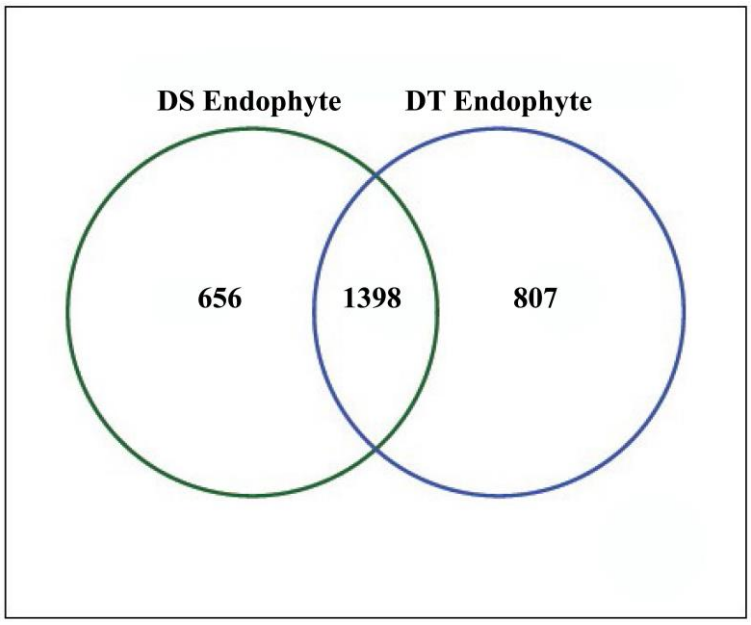


Figure 5.1 Distribution of differentially expressed endophyte genes under drought in two grass host genotypes.

"DT Endophyte" and "DS Endophyte" indicate *N. lolii* endophyte in drought tolerant and sensitive grass hosts respectively. Both endophytes are the same genotype. Numbers of differentially expressed genes that passed the cutoff (less than 0.05 q value and greater than 2-fold change) are shown.

5.1.2 Gene ontology enrichment analysis on differentially expressed endophyte genes under drought

To identify the overall pattern of endophyte transcriptome profiling in response to water deficit, GO (gene ontology) enrichment analysis (using Fisher's exact test, $p<0.01$) was undertaken on endophyte genes that were differentially-expressed under drought compared to control conditions, based on their biological process GO annotations.

Only three and two biological process GO categories were enriched in differentially expressed gene sets of endophytes living in DT and DS hosts respectively ('DT endophytes' and 'DS endophytes'). The enriched GO category of translation (GO0006412) was common to both DT and DS endophytes (Figure 5.2 A, B) and showed significant under-enrichment under drought conditions in both cases.

The other enriched GO categories that were host genotype specific all involved up regulation of endophyte genes under drought stress. GO categories corresponding to ribosomal export (ribosomal subunit export from nucleus (GO0000054)) and gene inactivation (gene silencing (GO0016458)) were enriched in the differentially expressed gene set of the DT endophyte (Figure 5.2 A). Closer examination of the genes in these two GO categories showed significant up regulation (with a greater than 2 fold increase) of them all in the DT endophyte under drought conditions, but only a few of them reached this threshold in the DS endophyte (Figure 5.3, 5.4).

In contrast, the GO category associated with fungal growth (DNA replication: GO0006260) was significantly over enriched in only the differentially expressed gene set of DS endophytes (Figure 5.2 B). Looking at the individual genes in this category (Figure 5.5) the expression of all of these genes was significantly up regulated in the DS endophyte under drought conditions. In the DT endophyte only 2/3 of the individual genes were significantly up-regulated and three genes were down-regulated under drought (Figure 5.5).

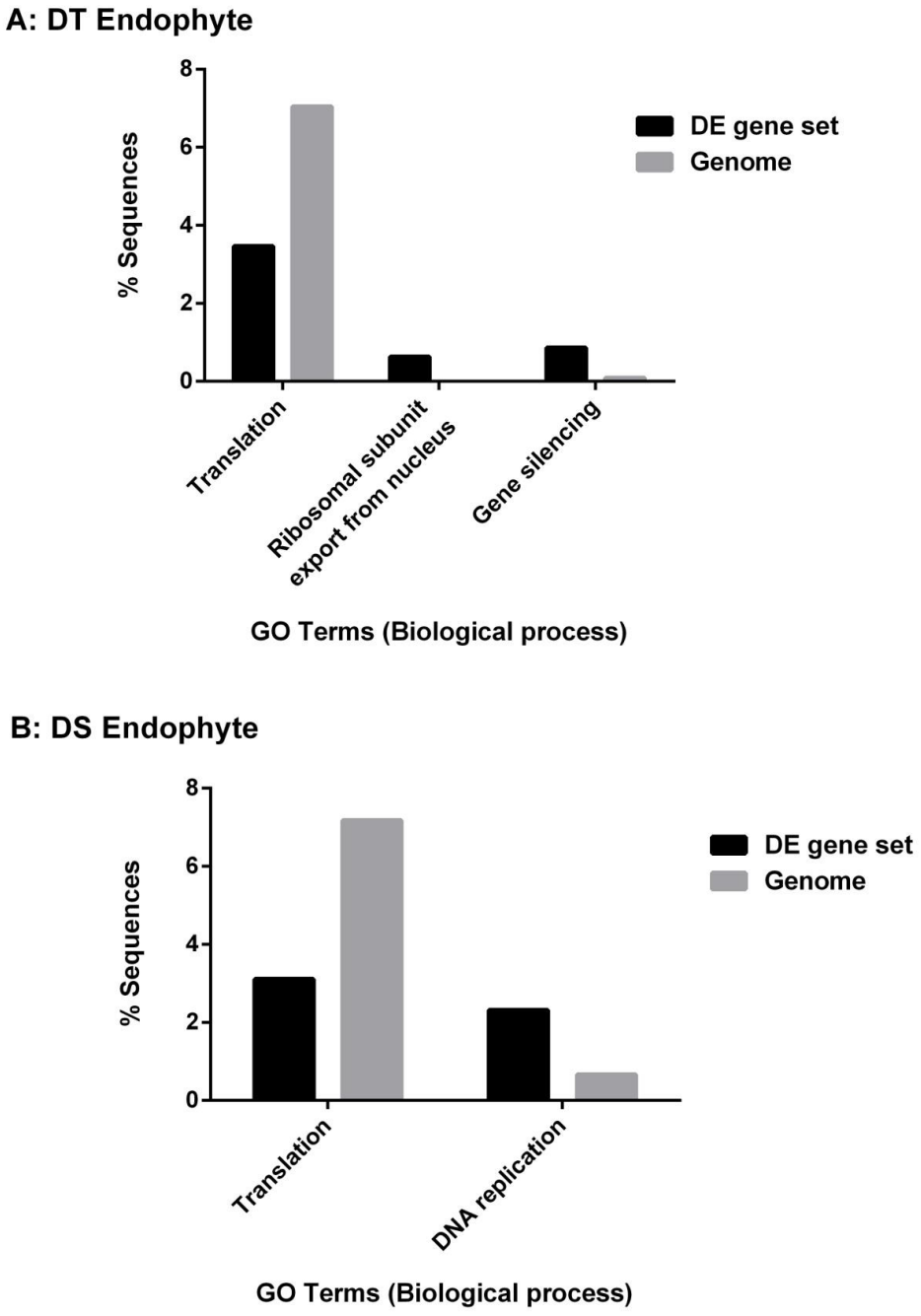


Figure 5.2 Biological process gene ontology (GO) terms enriched under drought stress in differentially expressed endophyte genes of DT endophyte (A) and DS endophyte (B)

"DT Endophyte" and "DS Endophyte" indicate *N. lolii* endophytes in drought tolerant and sensitive grass hosts respectively. "% sequences" indicates the proportion of genes with certain GO terms in the following gene sets: 'DE gene set' (all endophyte genes differentially expressed under drought) or 'Genome' (all *Epichloë festucae* gene models in the genome; EfM3; n = 9,350; http://csbio-l.csr.uky.edu/ef2011/). A *P* value cutoff of 0.01 was used to select enriched gene ontology categories.

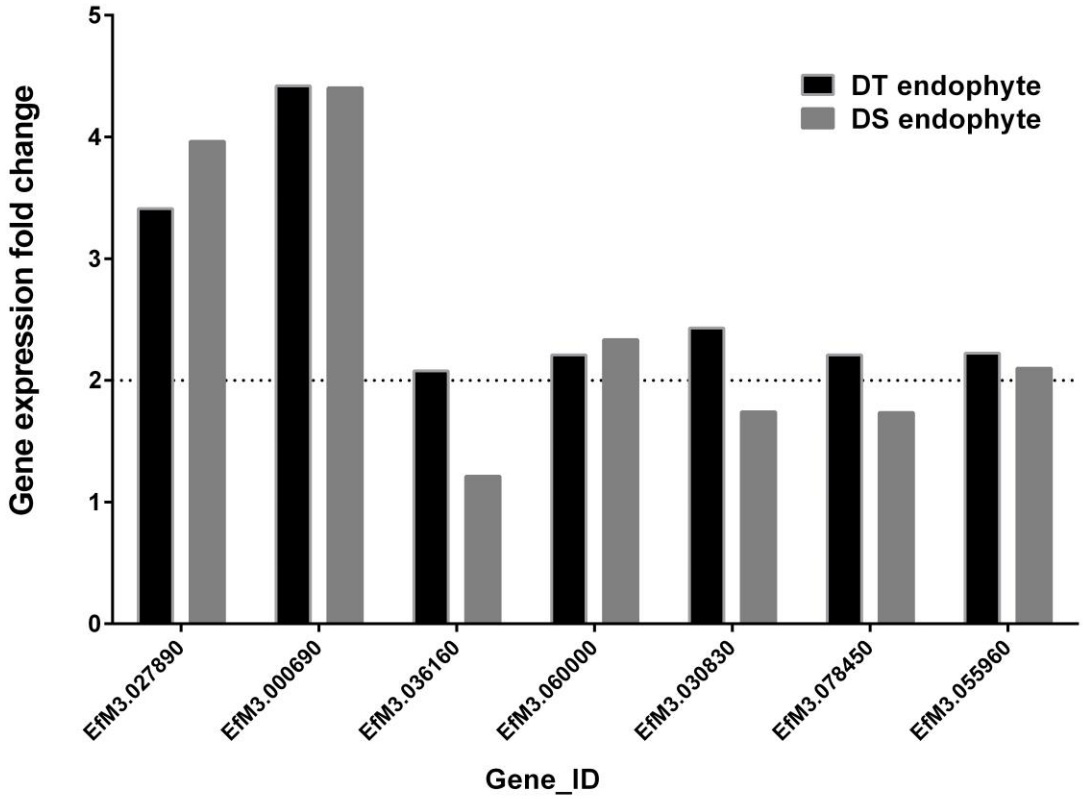


Figure 5.3 Expression of endophyte genes in gene ontology (GO0000054) category of ribosomal subunit export from nucleus under drought.

"DT Endophyte" and "DS Endophyte" indicate *N. lolii* endophyte in drought tolerant and sensitive grass respectively. Gene expression fold change indicates RPMK (reads per million per kilobase) of endophyte genes under drought compared to control conditions. All genes that passed the cutoff with greater than 2-fold change also had less than 0.05 q value for differential expression in DT endophyte. Gene annotations are listed in Appendix Table S5.1.

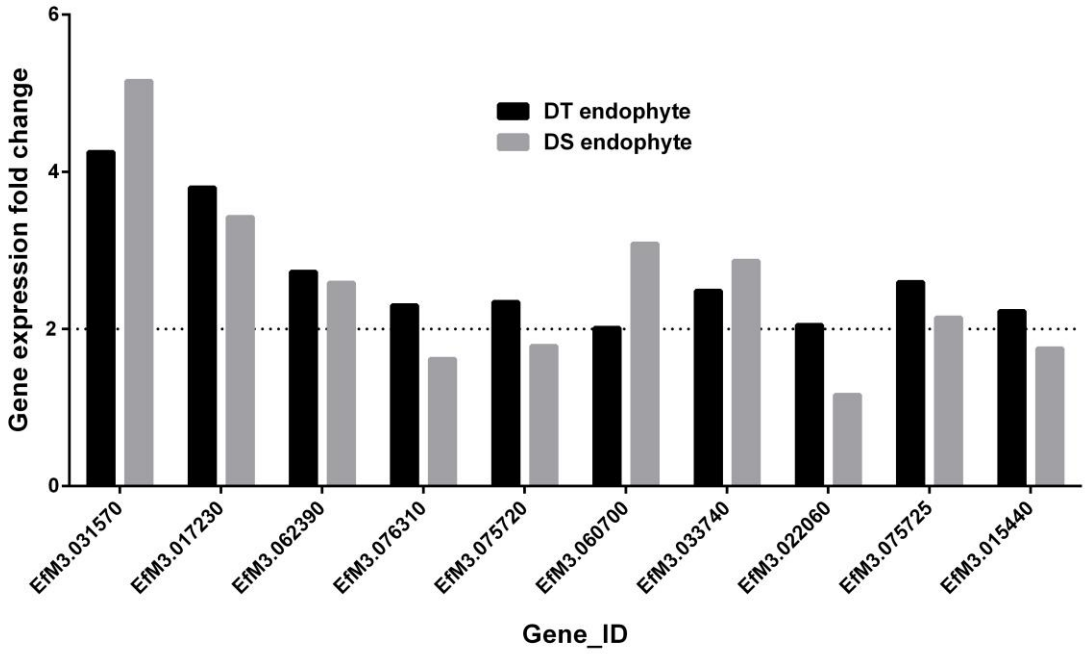


Figure 5.4 Expression of endophyte genes in gene ontology (GO0016458) category of gene silencing under drought.

"DT Endophyte" and "DS Endophyte" indicate *N. lolii* endophyte in drought tolerant and sensitive grass respectively. Gene expression fold change indicates RPMK (reads per million per kilobase) of endophyte genes under drought compared to control conditions. All genes that passed the cutoff with greater than 2-fold change also had less than 0.05 q value for differential expression in DT endophyte. Gene annotations are summarized in Appendix Table S5.2.

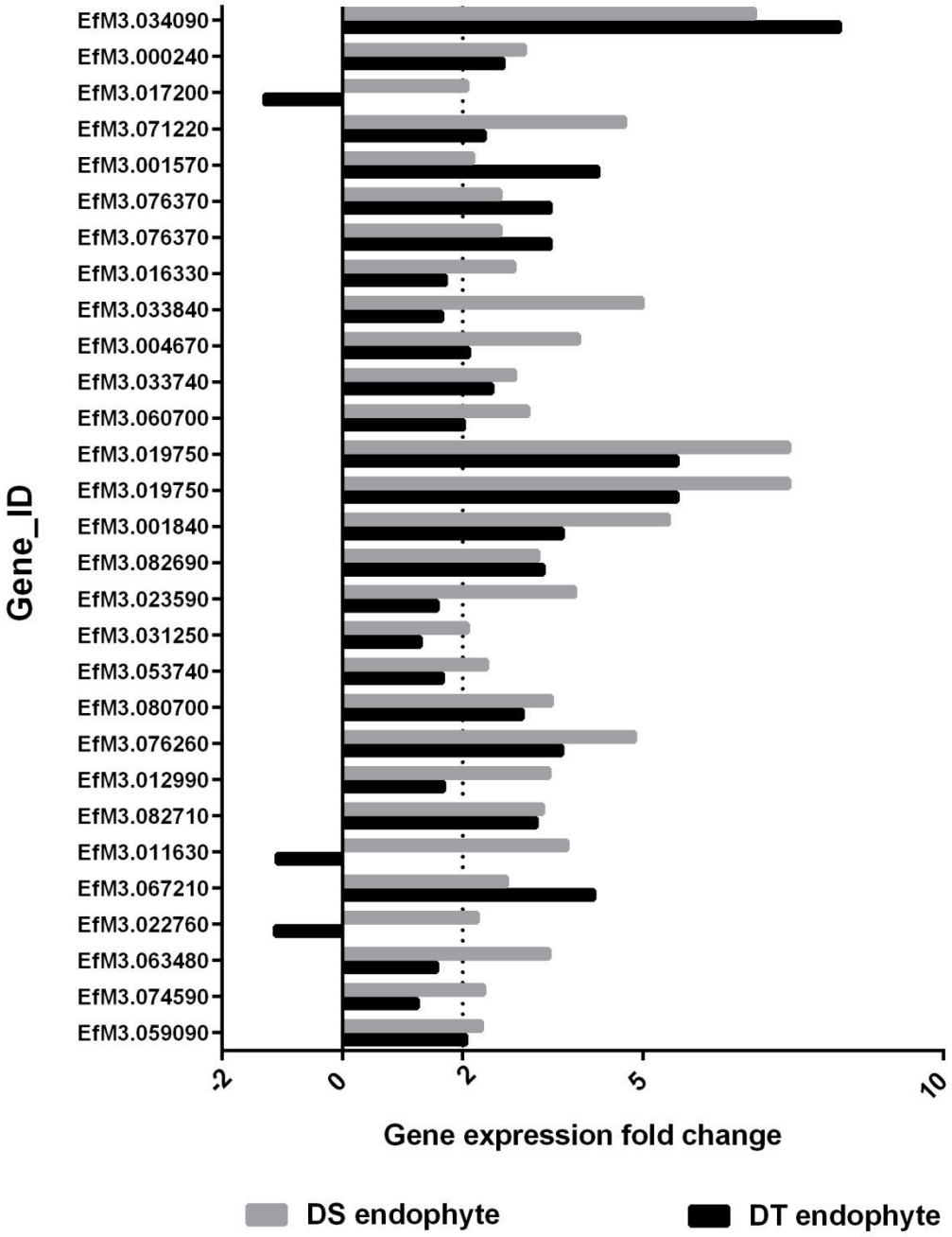


Figure 5.5 Expression of endophyte genes in gene ontology (GO0006260) category of DNA replication under drought.

"DT Endophyte" and "DS Endophyte" indicate *N. lolii* endophyte in drought tolerant and sensitive grass respectively. Gene expression fold change indicates RPMK (reads per million per kilobase) of endophyte genes under drought compared to control conditions. All genes that passed the cutoff with greater than 2-fold change also had less than 0.05 q value for differential expression in DS endophyte. Gene annotations are summarized in Appendix Table S5.3.

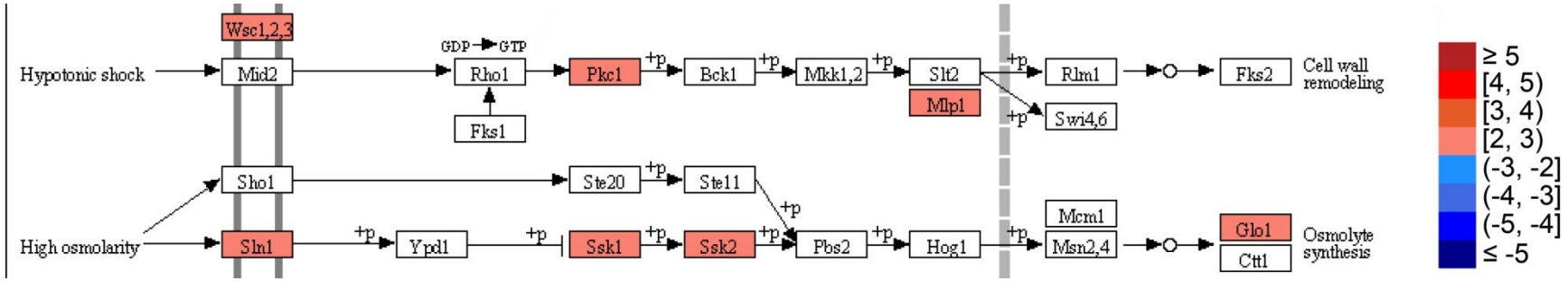
5.1.3 Functional annotations of differentially expressed endophyte genes

To further identify the response of endophyte to drought stress, differentially expressed fungal genes were assigned with KEGG (Kyoto Encyclopaedia of Genes and Genomes) ID and then mapped to KEGG pathways. The results showed similarities in the ways endophytes in DT and DS hosts responded to drought.

In both DT and DS endophytes, the expression of several genes involved in responses to hypotonic shock and high osmolarity were significantly up-regulated under drought stress (Figure 5.6). Two extra osmolarity responding genes, *Sln1* and *Glo1*, were only significantly up regulated in DT endophyte. A similar pattern was seen for peroxisome biogenesis (Figure 5.7), although more genes were differentially expressed, and with higher fold-changes, in the DT endophyte compared to the DS endophyte (Figure 5.7).

Looking in more detail at peroxisomal protein biosynthesis, two antioxidant enzyme biosynthesis genes, copper and zinc-containing superoxide dismutase (Cu/Zn SOD) and catalase A (CAT) were significantly up regulated in both DT and DS endophytes. The up regulation extent of CAT was greater in the DT endophyte (7.5-fold) than in the DS endophyte (4.2-fold) (Figure 5.8).

A: DT endophyte



B: DS endophyte

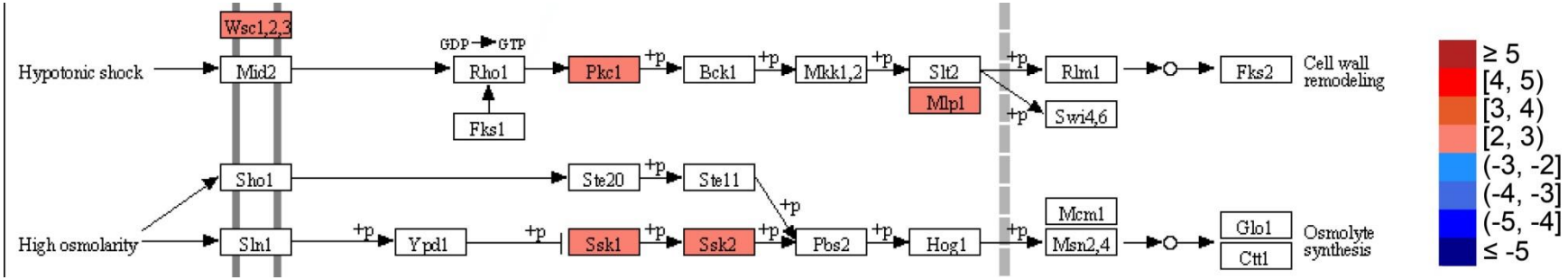
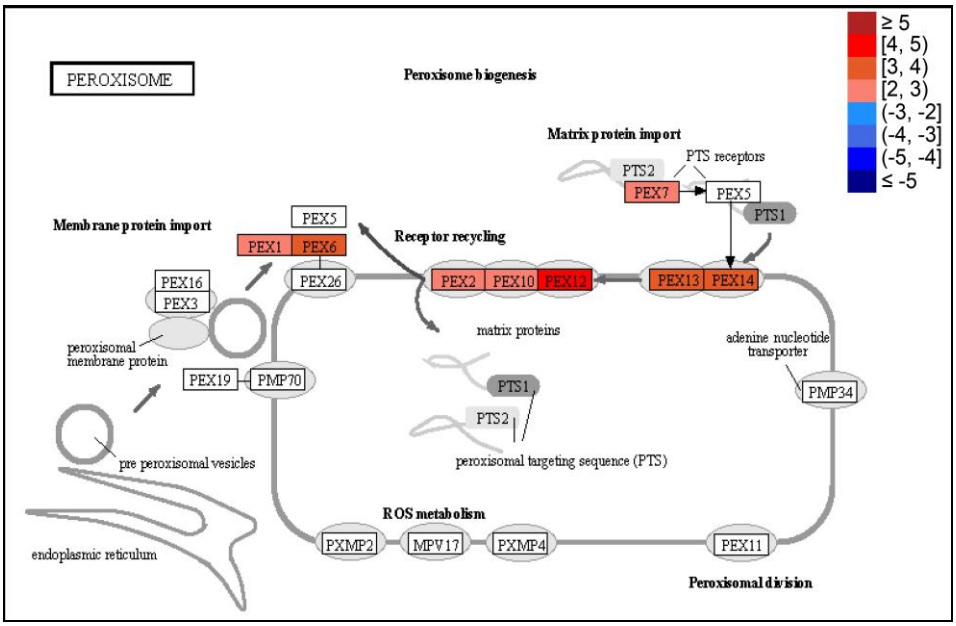


Figure 5.6 Effects of drought stress on the expression of endophyte genes responsive to hypotonic shock and high osmolarity.

A and B show the expression changes of genes in DT and DS endophytes respectively that are associated with responses to hypotonic shock and high osmolarity. The images were obtained using KEGG mapper (http://www.genome.jp/kegg/tool/map_pathway2.html). Each box represents an individual gene in that pathway, shaded according to the fold change in RPMK (reads per million per kilobase); red = up-regulated, blue = down-regulated under drought compared to control conditions.

A: DT endophyte



B: DS endophyte

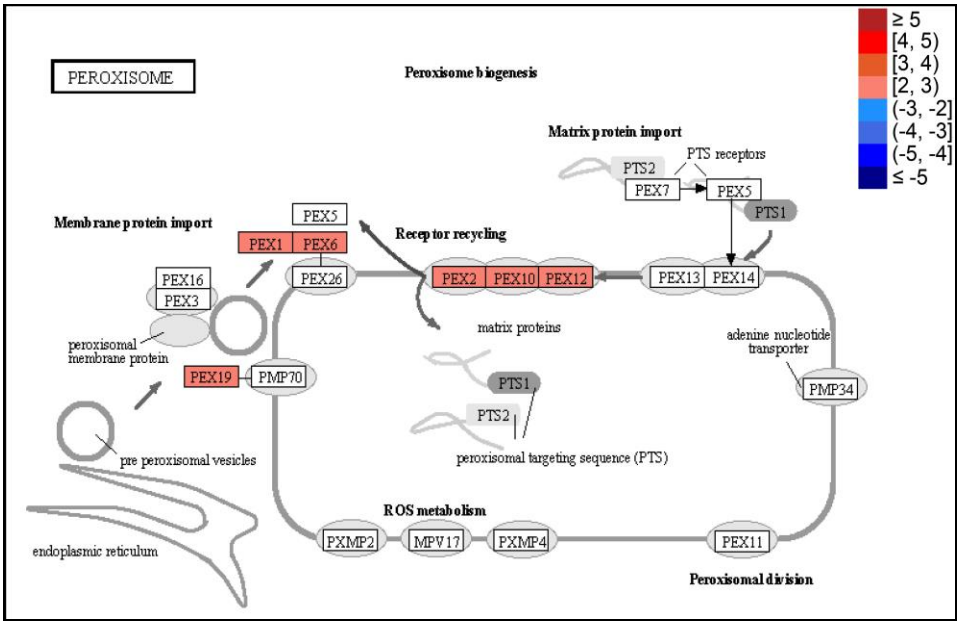
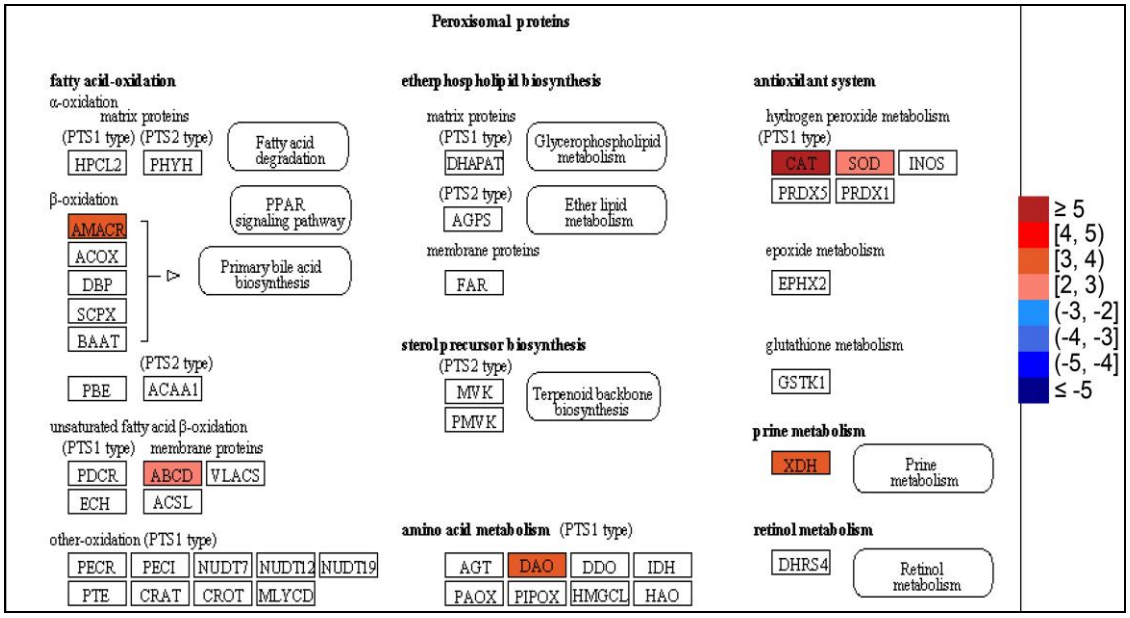


Figure 5.7 Effects of drought stress on the expression of endophyte genes associated with peroxisome biogenesis.

A and B show the expression changes of genes in DT and DS endophytes respectively that are associated with peroxisome biogenesis. The images were obtained using KEGG mapper (http://www.genome.jp/kegg/tool/map_pathway2.html). Each box represents an individual gene in that pathway, shaded according to the fold change in RPMK (reads per million per kilobase); red = up-regulated, blue = down-regulated under drought compared to control conditions.

A: DT endophyte



B: DS endophyte

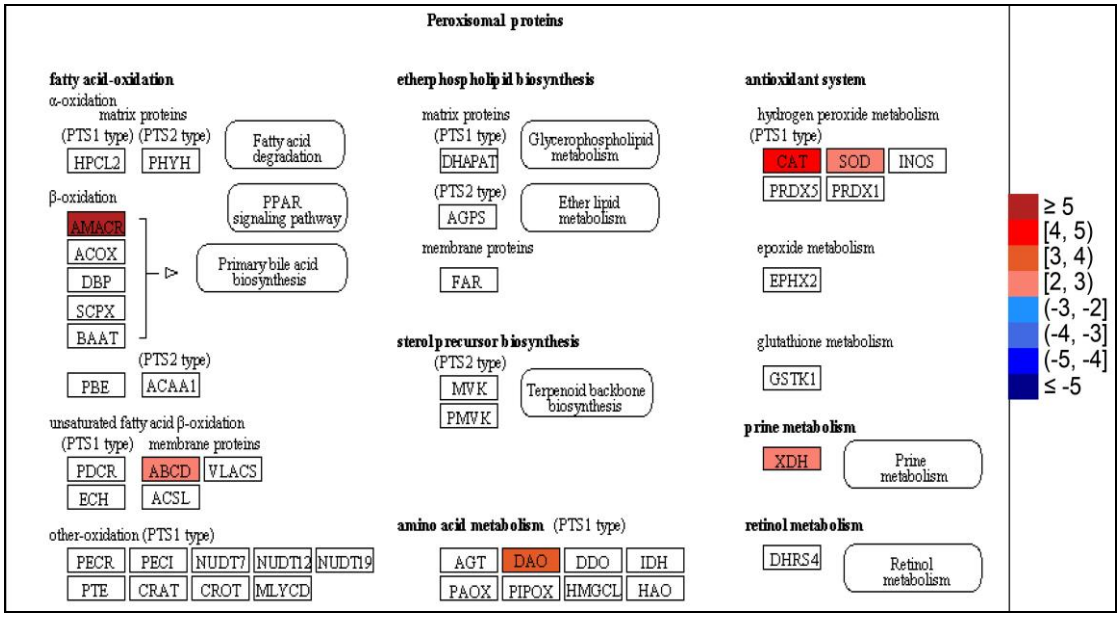


Figure 5.8 Effects of drought stress on the expression of endophyte genes associated with peroxisomal proteins biosynthesis.

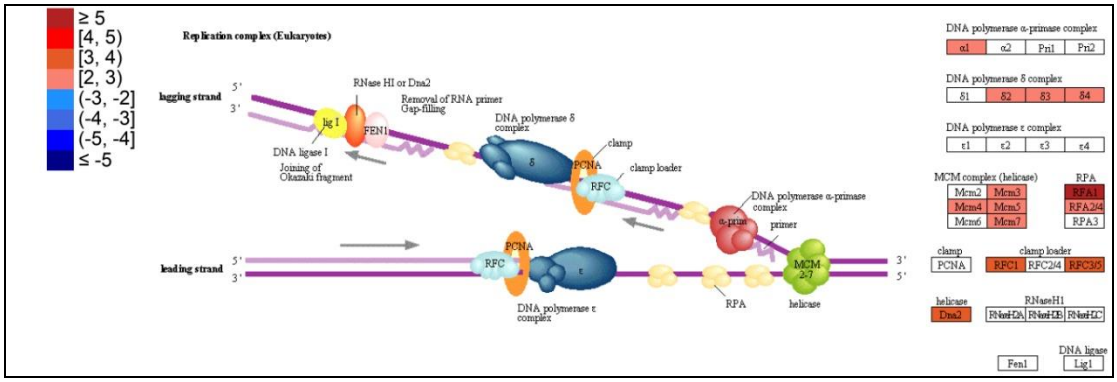
A and B show the expression changes of genes in DT and DS endophytes respectively that are associated with biosynthesis of peroxisome proteins. The images were obtained using KEGG mapper (http://www.genome.jp/kegg/tool/map_pathway2.html). Each box represents an individual gene in that pathway, shaded according to the fold change in RPMK (reads per million per kilobase); red = up-regulated, blue = down-regulated under drought compared to control conditions.

The expression of many genes associated with DNA replication and the cell cycle was significantly up regulated in both DT and DS endophyte under drought (Figure 5.9, Figure 5.10). The extent of this was more pronounced in the DS endophyte (Figure 5.9 B, Figure 5.10 B) with more up-regulated genes and generally higher fold-increases in expression compared to the DT endophyte (Figure 5.9 A, Figure 5.10 A).

To determine any changes in endophyte growth under drought stress, endophyte DNA concentrations were determined using the real time PCR method developed in Chapter 3. The relative DNA concentrations of both endophytes increased slightly under drought stress compared to well watered conditions, but the increases were not significant (Figure 5.11). The relative endophyte concentration in DS grass was 29.8% and 27.4% higher than in DT grass under control and drought respectively, although these differences were statistically non-significant (P = 0.126 and 0.114 under control and drought respectively) (Figure 5.11).

In summary, under drought stress, endophytes living in both DT and DS hosts showed up-regulated expression of genes involved in responses to hypotonic shock and high osmolarity, peroxisome biogenesis, Cu/Zn SOD and CAT biosynthesis, DNA replication and cell cycle control. The extent of up-regulation of peroxisome biogenesis and Cu/Zn SOD and CAT biosynthesis genes was greater in the DT endophyte compared with the DS endophyte. In contrast, the up-regulation of DNA replication and cell cycle genes was greater in DS endophyte compared with DT endophyte. Endophyte DNA concentrations were slightly higher in DS grass than in DT grass.

A: DT endophyte



B: DS endophyte

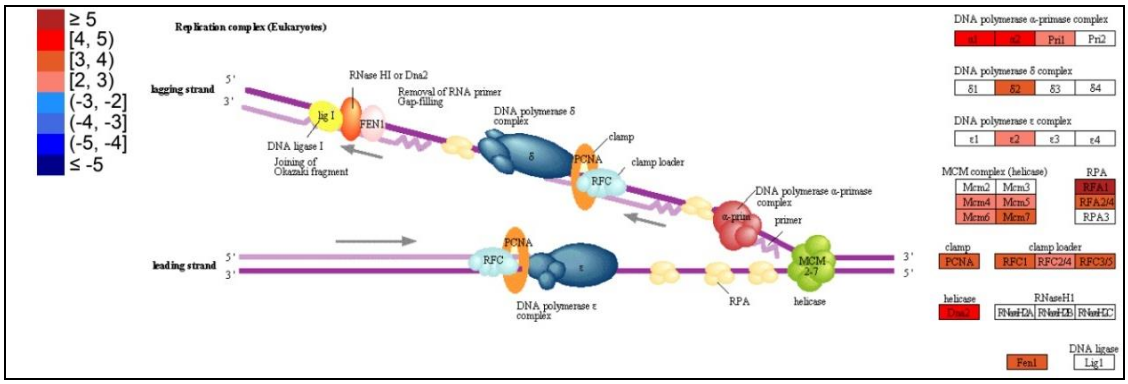
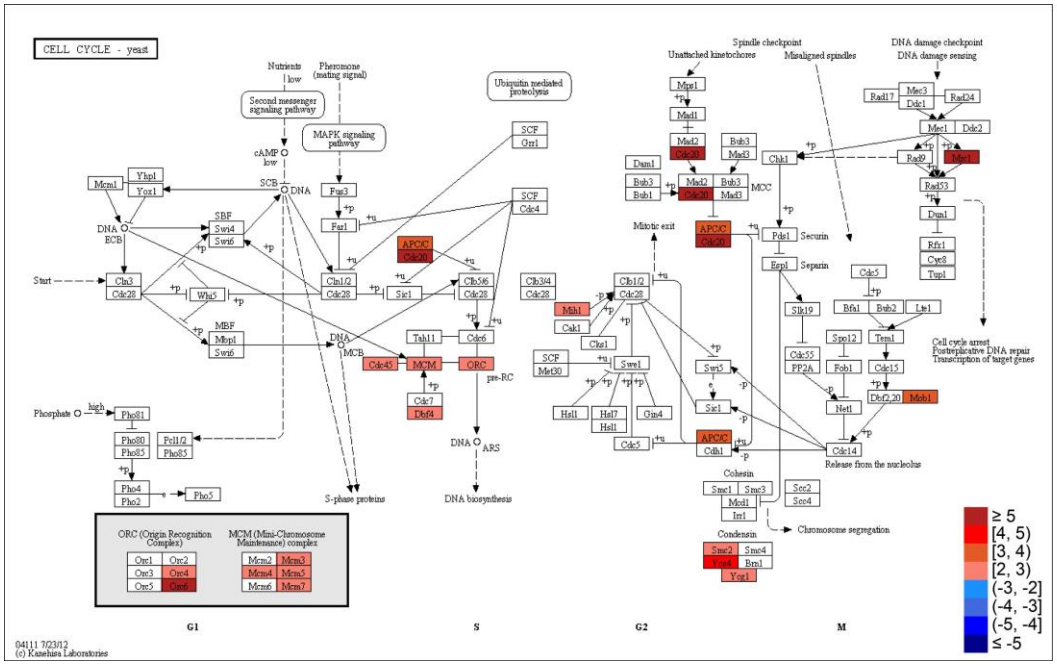


Figure 5.9 Effects of drought stress on the expression of endophyte genes associated with DNA replication.

A and B show expression changes of genes in DT and DS endophytes respectively that are associated with DNA replication. The images were obtained using KEGG mapper (http://www.genome.jp/kegg/tool/map_pathway2.html). Each box represents an individual gene in that pathway, shaded according to the fold change in RPMK (reads per million per kilobase); red = up-regulated, blue = down-regulated under drought compared to under control.

A: DT endophyte



B: DS endophyte

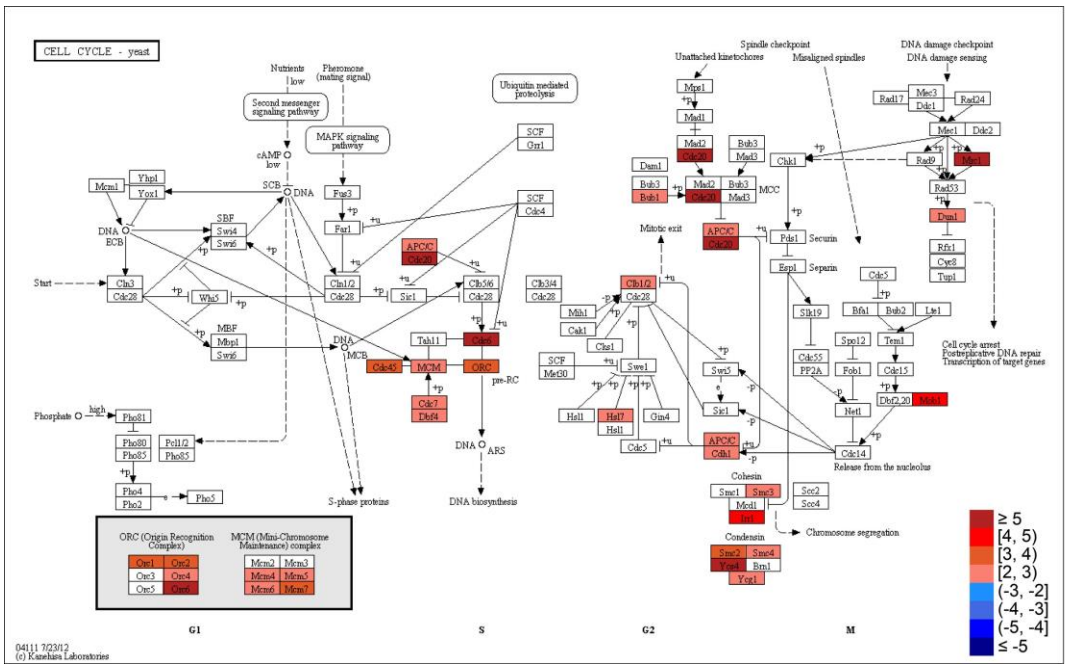


Figure 5.10 Effects of drought stress on the expression of endophyte genes associated with the cell cycle.

A and B show the expression changes of genes in DT and DS endophytes respectively that are associated with the cell cycle. The images were obtained using KEGG mapper (http://www.genome.jp/kegg/tool/map_pathway2.html). Each box represents an individual gene in that pathway, shaded according to the fold change in RPMK (reads per million per kilobase); red = up-regulated, blue = down-regulated under drought compared to under control.

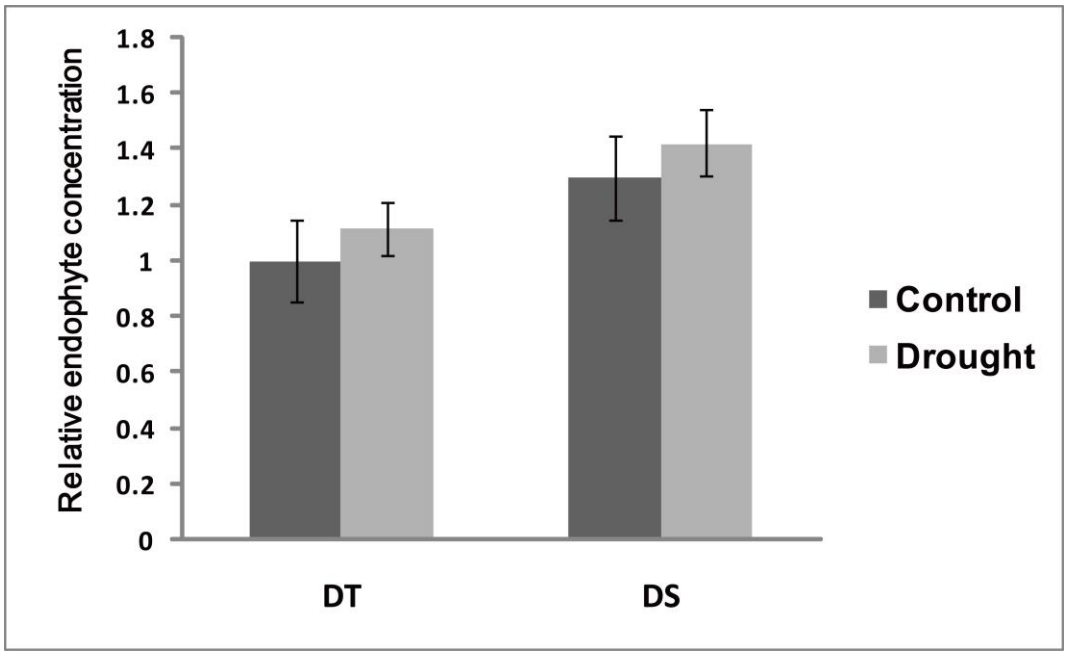


Figure 5.11 Relative endophyte concentrations in two genotypes.

'DT' and 'DS' indicate drought tolerant and sensitive grass respectively. Endophyte concentration is expressed as a ratio of endophyte level in the sample to that in the calibrator (a genomic DNA sample extracted from NZCT-infected perennial ryegrass sheath). *ltmG* and *TB1* genes were used as target genes for endophyte and grass respectively, as outlined in Chapter 3. Error bars indicate standard error among three biological replicates.

5.1.4 The effect of drought stress on alkaloids biosynthesis in endophytes.

Alkaloids are major secondary metabolites of endophyte. Although they are costly to produce, and may compete with other basic plant growth functions especially when resources are limited (Faeth 2002), they can have benefits as osmolytes (Malinowski et al. 1997). Thus we investigated changes in both endophyte alkaloids gene expression and alkaloid concentrations (ergot alkaloids, peramine and indole diterpenes) in grass tissue in response to drought stress.

Eleven genes are reported to be involved in ergot alkaloids biosynthesis by grass endophytes (Fleetwood et al. 2007; Schardl et al. 2013). In both DT and DS endophytes, two (*lpsA* and *easA*) of these 11 ergot alkaloid genes were down regulated (more than 2 fold) under drought conditions (Figure 5.12, Table 5.3). The expression of the remaining nine genes did not change significantly.

In the case of peramine, a single multifunctional nonribosomal peptide synthetase encoded by the *perA* gene, is involved in its synthesis (Tanaka et al. 2005). The *perA* gene was significantly (~4.2 fold) down regulated in both DT and DS endophyte under drought compared to control conditions (Figure 5.12, Table 5.3).

There are 11 genes found at the *IDT/LTM* locus of lolitrem B producers (Schardl et al. 2013); ten and eight of these were significantly down regulated in DT and DS endophytes under drought conditions respectively. The down regulation was more pronounced in the DT endophyte than in the DS endophyte (Figure 5.12, Table 5.3).

Given the changes in expression of alkaloids biosynthetic genes under drought stress, alkaloid concentrations were measured in the same batches of samples as those used for RNA profiling. The main alkaloids produced under all conditions were peramine and lolitrem B, along with lower levels of other indole diterpenes and ergot alkaloids (Table 5.4). In both DT (drought tolerant) and DS (drought sensitive)

grasses, ergot alkaloids concentrations did not show significant changes under drought compared to control conditions (*T* test, $P < 0.05$; Figure 5.13, Table 5.4). Peramine concentrations were significantly increased under drought stress in both grasses (*T* test, $P < 0.05$; Figure 5.13, Table 5.4). The concentrations of the indole diterpenes did not change significantly under drought (*T* test, $P < 0.05$), with the exception of lolitrem B and terpendole J levels that were considerably lower in DS grass under drought compared to control conditions (Figure 5.13, Table 5.4).

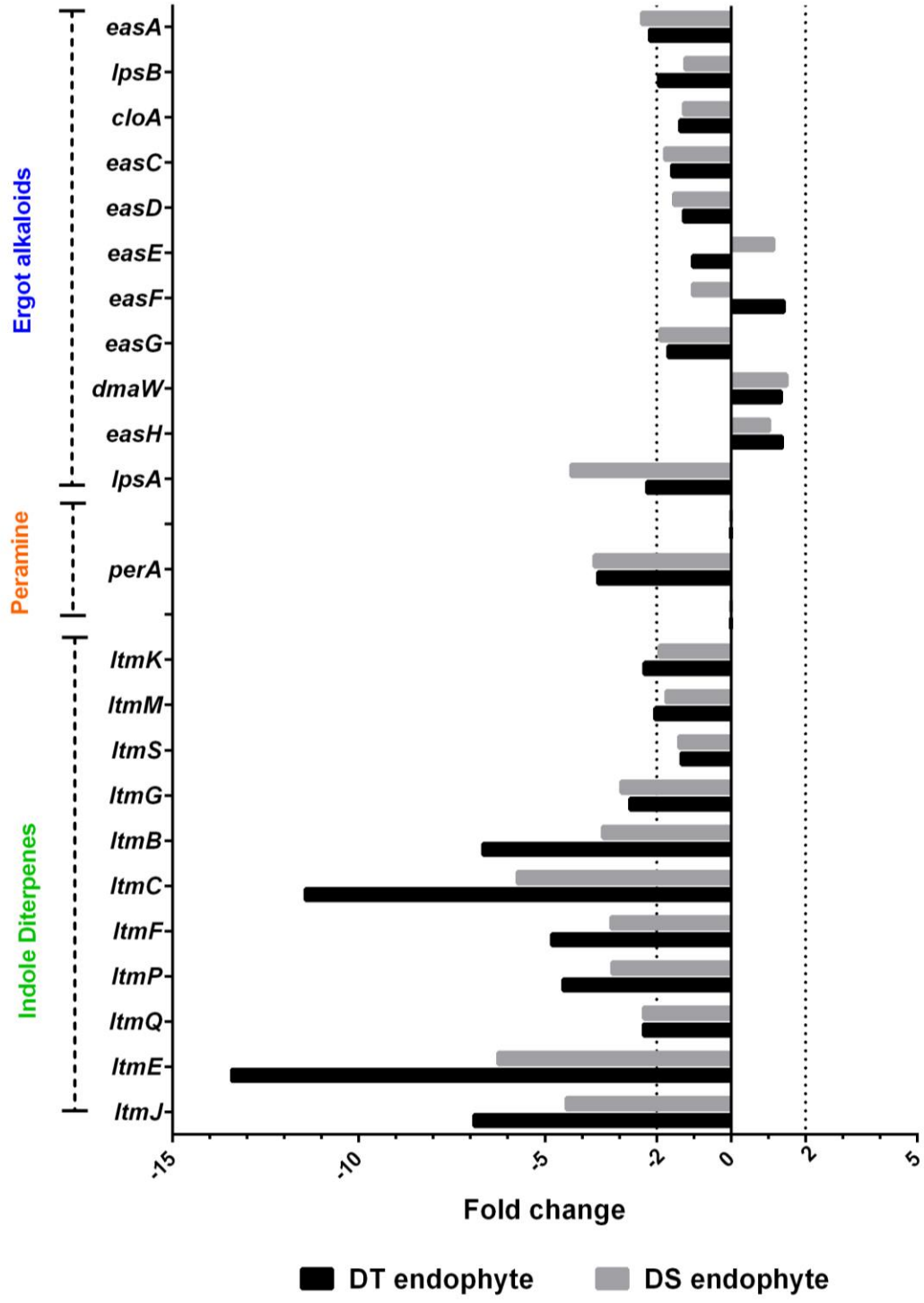


Figure 5.12 Expression changes of endophyte alkaloids biosynthesis genes in response to drought stress.

"DT Endophyte" and "DS Endophyte" indicate *N. lolii* endophyte in drought tolerant and sensitive grass hosts respectively. Fold change indicates the RPMK (read per million per kilo base) value of each gene under drought compared to control conditions.

Table 5.3 Expression level (RPMK value) of endophyte alkaloids biosynthesis genes under control and drought conditions

Alkaloids	Genes	DTC[a]	DTD[b]	DSC[c]	DSD[d]
Ergot alkaloids	*easA*	328.69	150.77	274.95	114.80
	lpsB	82.87	42.87	53.40	43.21
	cloA	134.33	98.09	124.01	97.62
	easC	267.48	168.53	258.82	145.81
	easD	90.12	70.86	88.04	57.39
	easE	172.35	168.19	134.27	152.13
	easF	186.32	262.95	222.27	217.14
	easG	1065.19	633.51	984.64	516.66
	dmaW	76.44	102.16	69.56	103.50
	easH	337.08	459.84	309.16	316.34
	lpsA	35.71	15.86	37.80	8.80
Peramine	*perA*	70.15	19.64	70.05	19.08
Indole diterpenes	*ltmK*	3315.71	1412.63	1103.78	471.93
	ltmM	2770.93	1361.73	2810.27	1614.51
	ltmS	3280.33	2456.36	3231.90	2313.23
	ltmG	7052.66	1467.99	2022.34	628.03
	ltmB	2408.20	347.61	2328.61	506.19
	ltmC	5436.57	458.10	6044.38	930.66
	ltmF	7323.94	1471.18	6478.34	1940.13
	ltmP	7221.56	1536.90	7194.71	2021.07
	ltmQ	3449.66	1411.40	3401.83	1708.82
	ltmE	5178.23	453.43	548.62	95.61
	ltmJ	1509.96	557.52	5921.09	2007.22

[a]: 'DTC' indicates drought tolerant grass grown under well watered conditions.
[b]: 'DTD' indicates drought tolerant grass grown under drought stress.
[c]: 'DSC' indicates drought sensitive grass grown under well watered conditions.
[d]: 'DSD' indicates drought sensitive grass grown under drought stress.
RPMK values are means of two biological replicates.

Table 5.4 Alkaloid concentrations of perennial ryegrass plants grown under control and drought conditions

Alkaloids (p.p.m.)		DTC[a] (Mean ± SD)	DTD[b] (Mean ± SD)	DSC[c] (Mean ± SD)	DSD[d] (Mean ± SD)
Ergot alkaloids	Chanoclavine	0.36 ± 0.09	0.59 ± 0.22	0.29 ± 0.10	0.42 ± 0.32
	Lysergyl Alanine	0.62 ± 0.25	0.55 ± 0.17	0.28 ± 0.08	0.24 ± 0.17
	Ergovaline	0.54 ± 0.17	0.78 ± 0.33	0.25 ± 0.13	0.47 ± 0.29
Peramine	Peramine	23.76 ± 2.73	31.74 ± 5.14	15.97 ± 3.48	26.10 ± 6.47
Indole diterpenes	Paspaline	0.78 ± 0.29	0.46 ± 0.24	0.63 ± 0.26	0.30 ± 0.16
	Terpendole J	0.16 ± 0.08	0.19 ± 0.10	0.12 ± 0.01	0
	Lolitriol	0.72 ± 0.26	0.86 ± 0.50	0.51 ± 0.21	0.59 ± 0.19
	Terpendole C	0.53 ± 0.12	0.56 ± 0.16	0.29 ± 0.09	0.39 ± 0.12
	HO-Lolitrem E	0.66 ± 0.61	0.71 ± 0.65	0.36 ± 0.29	0.14 ± 0.02
	Lolitrem_E	0.81 ± 0.39	1.19 ± 1.11	0.97 ± 0.61	0.30 ± 0.13
	Lolitrem B	19.11 ± 5.77	21.91 ± 12.24	18.25 ± 8.03	9.88 ± 4.00

[a]: 'DTC' indicates drought tolerant grass grown under well watered conditions.
[b]: 'DTD' indicates drought tolerant grass grown under drought stress.
[c]: 'DSC' indicates drought sensitive grass grown under well watered conditions.
[d]: 'DSD' indicates drought sensitive grass grown under drought stress.
Nine biological replicates (0.05 g freeze dried grass sheath) were used for each measurement.

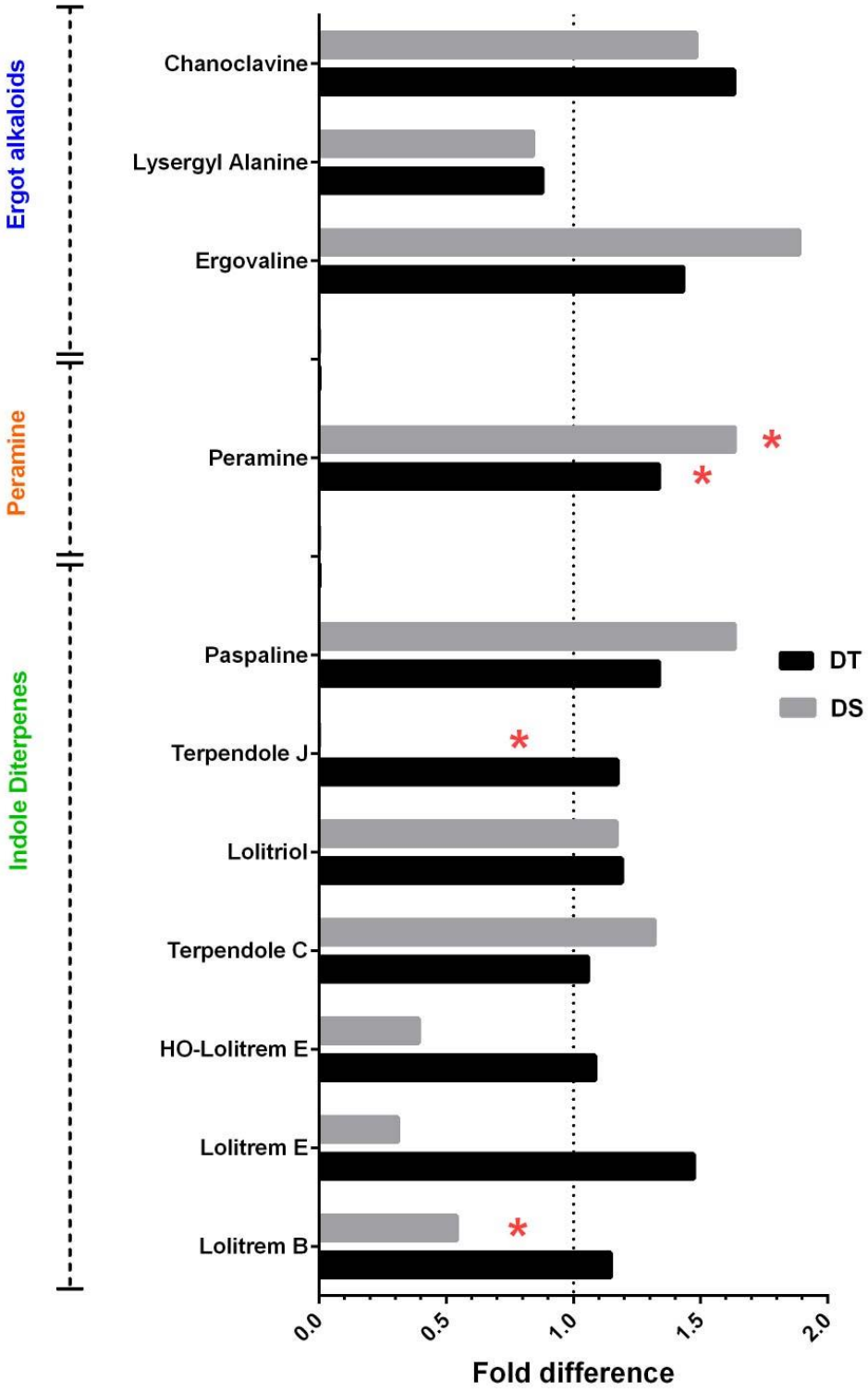


Figure 5.13 Endophyte alkaloids concentration changes in response to drought stress.

'DT' and 'DS' indicate drought tolerant and sensitive grass respectively. Fold difference indicates the difference of alkaloids concentrations in the same grass under drought compared to under control. Red asterisk represents significant difference between drought and control levels for each grass type (*T* test, $P < 0.05$).

Because endophyte alkaloid gene expression was affected by host genotype (DT or DS), especially for indole diterpenes, further comparisons were made between alkaloid concentrations of the two types of grass host (Figure 5.14, Table 5.4). The concentrations of two ergot alkaloids (chanoclavine and ergovaline), peramine, and one indole diterpene (terpendole C) were significantly higher in DT than in DS grass under both control and drought conditions (Figure 5.14, Table 5.4). The concentrations of four indole diterpenes (terpendole J, lolitriol, and lolitrem B and E) were significantly higher in DT than DS grass under drought but not under control conditions (*T* test, $P < 0.05$; Figure 5.14, Table 5.4).

In summary, the expression of most endophyte alkaloid biosynthetic genes was down regulated under drought, and the extent of down regulation was greater in endophytes living in drought tolerant (DT) than in susceptible (DS) hosts, particularly for indole diterpene genes. In keeping with this trend, endophytes in DS hosts generally showed reduced levels of indole diterpenes under drought stress. However endophytes in DT hosts appeared to maintain their levels of some indole diterpenes under drought stress, most notable the lolitrems. A disparity between gene expression and metabolite levels was also seen for peramine: despite reduced expression of the peramine biosynthetic gene, higher levels of peramine were found in both DT and DS grasses under drought stress compared to control conditions.

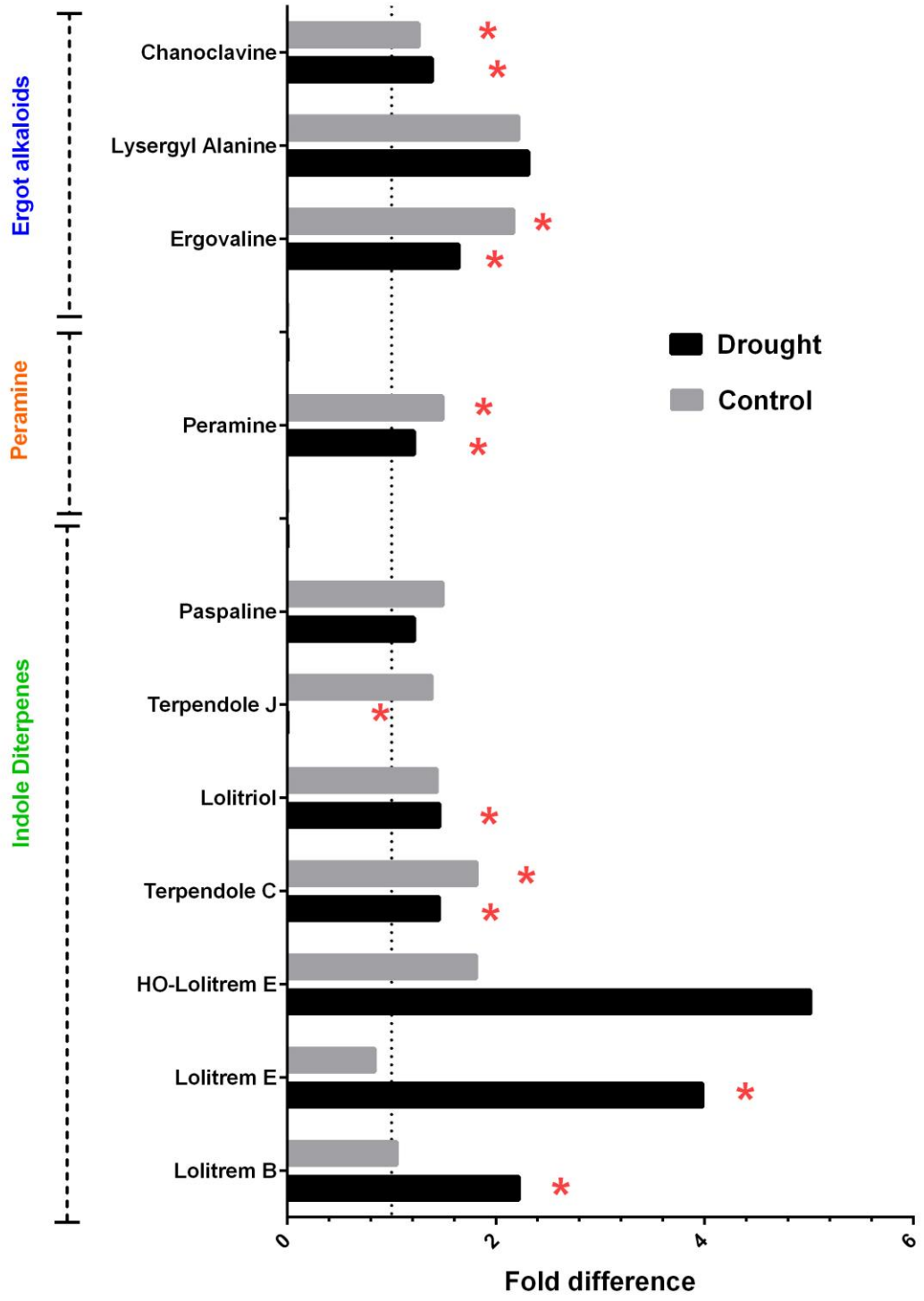


Figure 5.14 Comparison of endophyte alkaloids concentrations in different host genotypes.

Fold difference indicates the difference of alkaloids concentration in drought tolerant genotype versus drought sensitive genotype under either drought (black) or control (grey) conditions. Red asterisk represents significant difference (*T* test, $P < 0.05$).

5.2 Discussion:

5.2.1 Endophytes enhanced reactive oxygen species (ROS) scavenging capability under drought stress

ROS (reactive oxygen species) are important signal transduction chemicals that play an essential role in fungal development (Gessler et al. 2007). However, ROS production increases in fungi due to various stresses, including starvation, light and mechanical damage, and osmotic stress (Duran et al. 2010). Over-produced ROS under stress can damage cellular membranes and other cellular components (Cruz de Carvalho 2008). The up regulation of genes responsive to high osmolarity (Figure 5.6) suggests both endophytes were suffering osmotic stress under drought.

Peroxisomes are subcellular organelles that have an important role in balancing ROS levels in cells (Bonekamp et al. 2009). They have powerful ROS scavenging capability because they contain antioxidant enzymes, especially CAT (catalase) and SOD (superoxide dismutase) (del Río et al. 1992). CAT and SOD eliminate hydrogen peroxide (H_2O_2) and superoxide (O_2^-) respectively (Chelikani et al. 2004; Tainer et al. 1983). We found that the expression of several genes involved in peroxisome biogenesis and antioxidant biosynthesis (Cu/Zn SOD and CAT) were significantly up regulated under drought (Figure 5.7, 5.8). This indicates both endophytes probably enhanced their ROS scavenging capability under drought stress. It was previously reported that *Neotyphodium* spp. infected *Elymus dahuricus* had significantly higher antioxidant enzyme (SOD, CAT, POD (peroxidase) and APX (ascorbate peroxidase) activities than endophyte free plants under drought stress (Zhang and Nan 2007)

5.2.2 Endophytes elevated expression of growth genes under drought stress

It has been found that the relative *N. lolii* biomass in perennial ryegrass was between 0.3 and 1.9% (Young et al. 2005). Similar endophyte biomass (from 0.5% to

2.5%, roughly calculated based on real time PCR cycle number) was found in this study. Although this proportion is not high, given that nutrients are likely to be in extremely short supply when growing under severe drought stress, endophyte growth is still a burden to grass.

In both DT and DS endophytes, the expression of many genes involved in DNA replication and the cell cycle were significantly up regulated under drought conditions (Figure 5.9, 5.10). This indicates both endophytes were tuned to increase their growth under drought. However, the results of endophyte quantification showed that there was only an insignificantly small increase of endophyte DNA concentration (Figure 5.11). Possible reasons for this apparent discrepancy between the gene expression and biomass data are:

(1) The endophyte might not increase its growth rate until the grass suffers severe effects of drought. It has been reported that *Epichloë festucae*, the ancestor of *N. lolii* (Moon et al. 2004), grows by intercalary extension (Christensen et al. 2008) in which the fungal hyphae are attached to grass cells, and cumulative growth along the length of the filament enables the fungus to extend at the same rate as the host. Under mild drought stress, the endophyte might reduce its growth when the host slows down its growth because of lack of water. But under severe drought stress, when the plant cell wall starts shrinking because of losing water, the attached fungal hyphae will be stretched in a different way, which could trigger genes involved in accelerating endophyte growth. In support of this hypothesis, the observed up regulation of a stress receptor encoding gene (*Wsc*) (Figure 5.6) suggests both DT and DS endophytes were suffering cell wall perturbation under severe drought stress (Verna et al. 1997).

(2) There were not enough resources available for fungal DNA biosynthesis under severe drought conditions, even though gene expression had been up regulated. Because *N. lolii* is heterotrophic, it relies completely on nutrients produced by the grass host. Under severe drought stress, limited carbohydrate can be produced by the plant because of photosynthesis inhibition, and carbohydrate transport is constrained

because of water stress (McDowell 2011). Thus limited nutrients are available for endophytes under such drought stress.

(3) It is also possible that technical factors could have affected the results. The relative endophyte concentration determination relies on both plant and fungal genes. Plant and fungal materials are starting to degrade under severe drought. Thus the release of hydrolytic enzymes, such as nucleases, from the dying plant and fungal cells might have affected the accuracy of the real time PCR results. In contrast, RNA sequencing (RPMK) values were calculated from endophyte genes only.

5.2.3 Alkaloid biosynthetic gene expression was reduced under drought

Increased concentrations of peramine in drought over control are consistent with a possible role as an osmolyte. Peramine could be a good osmolyte, because it is water soluble and dispersed throughout the plant, including in roots (Ball et al. 1997). The accumulation of osmolytes is important for host resistance to drought stress (Burg and Ferraris 2008). Thus, an increase of alkaloids production under mild drought conditions could help the plant cell adjust osmotic potential and reduce water loss. In the current study the levels of some indole diterpenes (lolitrems) were maintained at control levels in DT grass under drought; lower levels of these compounds in DS grass might be the consequence of the more advanced state of drought symptoms in which lolitrem levels could no longer be maintained.

The down regulation of *perA* and most *ltm* genes (Figure 5.12) indicates both DS and DT endophytes started to decrease production of peramine and indole diterpenes under drought stress. This is a good way to reduce nutrient costs to the host under severe drought conditions. In a previous study it was found that two genes (*lpsA* and *ltmG*) required for the synthesis of ergovaline and lolitrem B were steadily expressed in perennial ryegrass / *N. lolii* symbiosis under drought stress (Hahn et al. 2008). However in the current study the expression of both *lpsA* and *ltmG* was significantly down regulated (more than 2 fold) under drought stress in both DT and DS grasses. The inconsistency between these results might be due to differences in levels of drought severity or host cultivars used, or to differences in the types of quantitative methods employed. It was suggested that ryegrass genotypes affect alkaloids production (Spiering et al. 2005). The perennial ryegrass cultivar, 'Grassland Nui', was used in the Hahn et al (2008) experiment and gene expression was determined immediately after the soil moisture arrived at 16% FC. In the current study another cultivar, 'Nine O One', was used and the grasses were treated with prolonged severe drought treatment (one week under 20% FC) prior to gene expression studies.

Changes in alkaloid concentrations in the grass sheath were not completely synchronized with expression of corresponding genes. Expression of the *perA* gene was significantly down regulated, but peramine concentration was significantly increased under drought stress (Figures 5.12 and 5.13). One reason for this may be because RNA sequencing only gives a snapshot of gene activity at one time point while alkaloid levels show the results of cumulative synthesis over time. Peramine is a potent insect feeding deterrent (Clay and Schardl 2002). It is possible that the endophyte increases peramine production when the grass senses drought stress, given the crosstalk between abiotic and biotic stress responses (Fujita et al. 2006). The impact of peramine on insect deterrence would benefit the plant if it accumulates under drought when the plants are weak and most likely to be eaten. But under severe drought conditions, when nutrients became very limited, expression of the *perA* gene, along with many of the other alkaloid biosynthetic genes, was shut down.

Ergot alkaloid genes with reduced expression under drought stress (Figures 5.12, 5.15) include *easA* that functions in converting chanoclavine to agroclavine, and *lpsA*, involved in converting D-lysergic acid to ergovaline. However, no significant accumulation of chanoclavine or decrease of ergovaline was seen in either DS or DT grass under drought (Figure 5.13, Table 5.5). It was noticed that *dmaW*, which functions in the first step of ergot alkaloids biosynthesis (Figure 1.4), was up regulated, whilst some of the later pathway genes (such as *lpsA*) were down regulated (Figure 5.13). It is possible that some kind of feedback inhibition could have occurred at either the substrate or gene expression levels. The fact of unchanged concentrations of chanoclavine and ergovaline also possibly reflects the involvement of many other gene products in these complex biosynthetic pathways, and suggests that EasA and LpsA proteins are not limiting factors in biosynthesis.

Similarly, the expression of almost all of the *ltm* genes involved in lolitrem B biosynthesis (Figure 1.2) was significantly down regulated (Figure 5.12). But the

concentrations of most of the indole diterpenes did not significantly change in either DS or DT grasses under drought compared to control conditions (Figure 5.13, Table 5.5). This might be due to a delayed response of lowered alkaloids production compared to corresponding gene expression, due to a longer half-life of the alkaloids themselves than of the mRNAs of the corresponding biosynthetic genes. Although the level of down-regulation of lolitrem genes was greater in DT than in DS endophytes, the actual lolitrem levels were higher in DT plants than in DS plants under drought (Table 5.4, Figure 5.14). It seems possible that the genotypes of the hosts and their different responses to drought stress could affect lolitrem levels. In the DS hosts that showed severe stress symptoms, synthesis of new lolitrems was likely to have been impaired and degradation of existing lolitrems and lolitrem biosynthetic enzymes was also likely to have occurred at a faster rate. For example proteolysis plays an essential role in the degradation of protein in cells (Vierstra 1996). It has been found that drought sensitive rice and wheat cultivars had higher levels of proteolysis than drought tolerant rice and wheat cultivars under similar levels of water deficit (Pyngrope et al. 2013; Simova-Stoilova et al. 2010b). Besides, leaf senescence is associated with increased proteolysis (Smart 1994). Given the water status of DT grass under drought (leaf relative water content = 30.7%) compared to DS grass under the same drought conditions (leaf relative water content = 26.4%), more severe leaf senescence occurred in DS than in DT grass.

5.3Limitations and general conclusions

There were several limitations in this study:

1. Due to the degradation of grass tissue under severe drought conditions, especially in the DS genotype, the accuracy of endophyte DNA level and alkaloids quantification might have been affected.

2. The two endophytes were associated with two different grass genotypes, thus the host genotype will have affected the response of endophyte to drought stress. For example, water status of DT (leaf relative water content = 30.7%) and DS (leaf relative water content = 26.4%) grasses were different under drought conditions (Figure 4.7), this means that the real living conditions of DT and DS endophytes were different, which might have affected the response of the endophyte to the same drought treatments. Besides this, even under control conditions, there were still host genotype effects on endophyte gene expression. We found 85 endophyte genes that showed significant differences in expression depending on whether they were in the DT or DS grass host, under control conditions.

3. Only two grass genotypes were used. The interaction between endophyte and ryegrass might be different in other grass genotypes. Thus some of the data might be averaged out if more host genotypes were tested.

4. The bioinformatic analyses did not capture all differentially expressed genes due to the limitations of the database and reference genome sequence. The reference genome used in this study (EfM3; n = 9,350; http://csbio-l.csr.uky.edu/ef2011/) is of an Only 46.6% of endophyte genes in the *Epichloë festucae* genome (EfM3; n = 9,350; http://csbio-l.csr.uky.edu/ef2011/) were annotated with biological GO terms. This low annotation rate decreased the accuracy of GO enrichment analysis. Similarly only 34.1% of the *Epichloë festucae* genes were annotated using the KAAS (KEGG automatic annotation server), suggesting that nearly two thirds of differentially expressed genes were missed due to lack of functional annotation.

Thus it is possible that other *N. lolii* genes additional to those discussed here are responsive to drought stress, and might be involved in enhancing grass drought tolerance.

In summary, endophytes living in DT and DS hosts responded to severe drought stress in similar ways: they enhanced ROS scavenging capability through increasing peroxisome biogenesis and antioxidant enzyme biosynthesis, increased expression of genes involved in DNA replication and the cell cycle, and suppressed expression of genes involved in alkaloids biosynthesis.

Compared to those in the DS host, endophytes in the DT host had greater increases in expression of peroxisome and antioxidant genes, and stronger decreases in expression of alkaloids genes under severe drought. In contrast, the DS endophyte emphasized more on accelerating DNA replication and cell cycle. However it needs to be recognized that the DS endophyte was in a plant showing more advanced drought symptoms than the DT endophyte.

6 Grass transcriptome profiling

6.1Results

6.1.1 General description of RNA-sequencing results

To explore the influence of endophyte on grass drought tolerance at the molecular level, the grass transcriptome in the same RNA samples as used in chapter 5 was profiled. In total there were 422,063,343 grass reads mapped to perennial ryegrass ESTs (Table 5.1). A detailed breakdown of reads in the different samples and replicates is given in Appendix 5.1.

To understand the effect of endophytes on the grass transcriptome, statistical analyses were conducted to compare the expression of grass genes between E+ and E- samples under the same conditions (control or drought stress). As shown in Table 6.1 there were more differentially expressed genes under drought than under control conditions (for example compare 2862 in drought to 799 in control for the drought tolerant plant, a 3.6-fold difference). The numbers of differentially expressed genes were generally higher in the drought sensitive than in the tolerant plants but the effect of drought was not so marked, with only a 2-fold difference (3359 in drought and 1626 in control) for the drought sensitive plants (Table 6.1).

A Venn diagram (Figure 6.1) showing the numbers of differentially expressed genes (when comparing E+ to E-) reveals distinct effects of plant genotype and treatment. There is little overlap in terms of differentially regulated genes shared by DT (drought), DT (control) DS (drought) and DS (control). Remarkably, only 14 grass genes were differentially expressed in response to presence of endophyte in all these four groups. Most genes are unique to one of these four classes, with the highest numbers of differentially expressed genes in either DT (1712) or DS (2130) under drought stress. However an additional 876 genes were differentially expressed in both DT and DS genotypes under drought (Figure 6.1). There was very little overlap in terms of differentially expressed genes shared by DT (drought) and DT (control) (less than 4.2%), and also shared by DS (drought) and DS (control) (less than 7.6%).

Table 6.1 Number and proportion of endophyte-induced differently expressed grass genes

Grasses comparison[a]	DE gene[b] number	Proportion (%)[c]
DT E+ vs. DT E- (Under control)	799	1.6
DT E+ vs. DT E- (Under drought)	2862	5.7
DS E+ vs. DS E- (Under control)	1626	3.2
DS E+ vs. DS E- (Under drought)	3359	6.7

[a] **"**DT" and "DS" indicate drought tolerant and drought sensitive genotype respectively**.**

[b] **"**DE gene" indicates differently expressed genes, with a Q value (Storey, 2003) <0.05 and fold change >2**.**

[c] "Proportion" indicates the percentage of DE genes of all expressed genes.

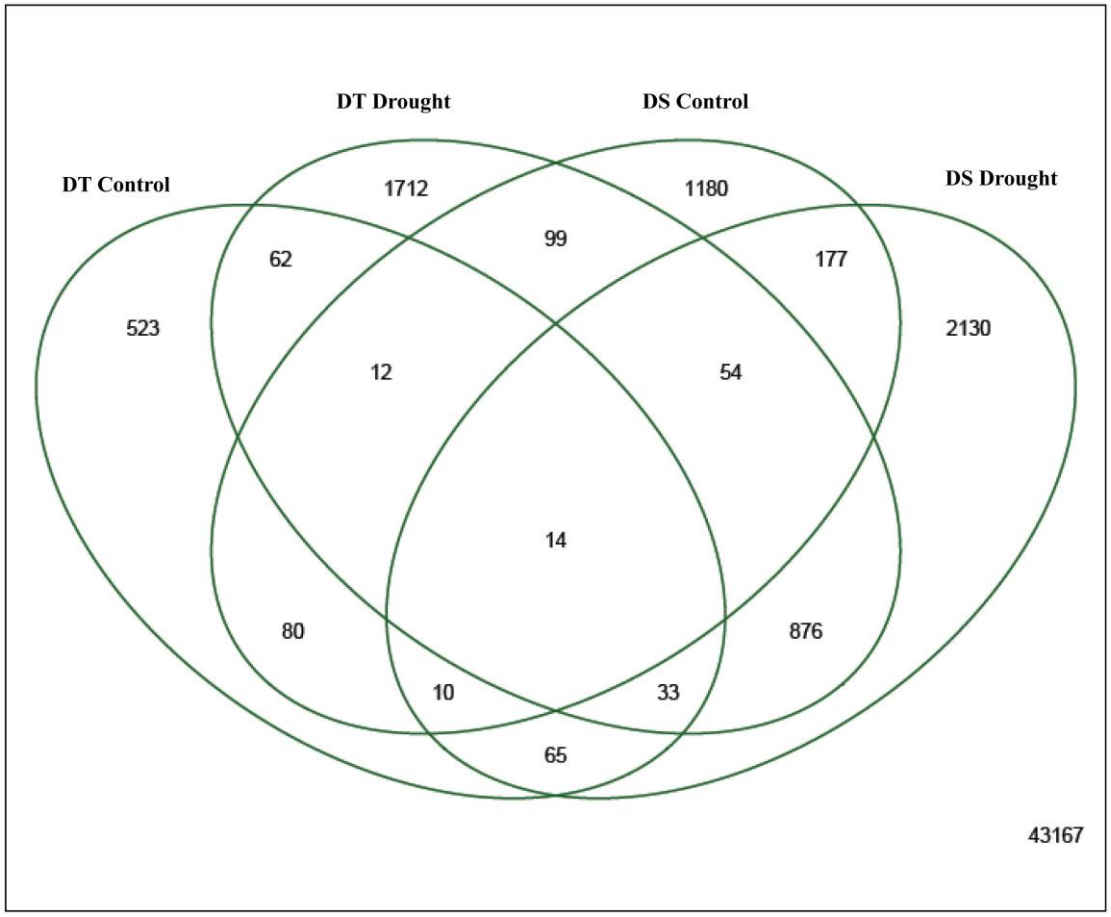


Figure 6.1 Distribution of differentially expressed grass genes caused by *N. lolii* infection.

"DT" and "DS" are drought tolerant and sensitive genotype respectively. All genes passed the cutoff (< 0.05 Q value and > 2-fold change) for differential expression in E+ compared to E- plants.

6.1.2 Gene ontology enrichment analysis on endophyte-regulated grass genes

To identify the overall pattern of endophyte impact on the transcriptome profile of perennial ryegrass in response to water deficit, GO (gene ontology) enrichment analysis (Fisher's exact test, $p<0.01$) was undertaken on grass differentially-expressed genes, those with significantly different expression in endophyte-infected plants compared to endophyte-free plants, under drought based on their biological process GO annotations.

There were 23 and 28 biological process GO categories over enriched in DT and DS genotypes respectively (Figures 6.2, 6.3). Thirteen of these GO categories, corresponding to photosynthesis (e.g. "light harvesting", "photosynthetic electron transport in photosystem I", "photosystem II assembly"), sugar metabolism (e.g. "starch biosynthetic process", "reductive pentose-phosphate cycle"), secondary metabolism (e.g. "lignin biosynthetic process", "stilbene biosynthetic process" and "coumarin biosynthetic process"), reactive oxygen species scavenging ("peroxidase reaction"), lipid metabolism ("fatty acid elongation") and oxidation-reduction processes ("electron transport"), were over-enriched in both DT and DS differentially-expressed gene sets under drought stress (Figure 6.2). Figure 6.3 shows the numbers of genes in each of the over-enriched GO categories (those shown in red in Figure 6.2, and in the red box in Figure 6.3). It shows that most differentially-expressed genes in the over enriched GO categories were significantly up-regulated, rather than down-regulated, by endophyte infection (except lignin, stilbene, peroxidase reaction and coumarin biosynthetic genes)

Gene enrichment of some GO categories was associated with just one grass genotype. There were ten GO categories that were only over-enriched in DT genotypes under drought (Figure 6.2, green box in Figure 6.3A). These included genes involved in sugar (starch/sucrose) degradation, chlorophyll biosynthesis, auxin

transport, wax biosynthesis, root growth and glutathione metabolism that were significantly up-regulated, and genes involved in mucilage metabolism that were significantly down-regulated under drought.

There were 15 GO categories that were only over-enriched in DS genotype under drought (Figure 6.2, blue box in Figure 6.3B). In these GO categories, genes associated with inositol and maltose metabolism, peptidyl-prolyl isomerization, pentose-phosphate shunt, rRNA processing, auxin signalling, chloroplast relocation, carbon utilization, mRNA modification, protein-chromophore linkage and regulation of protein dephosphorylation were significantly up-regulated, and genes involved in L-phenylalanine catabolism and alkaloid biosynthesis were significantly down-regulated.

In all, the results of GO enrichment analysis suggested that the presence of endophyte broadly adjusted grass metabolism. The endophyte accelerated some common biological processes (e.g. photosynthesis) of both DT and DS grasses but the effect of endophyte on several biological processes (e.g. wax biosynthesis) was genotype specific.

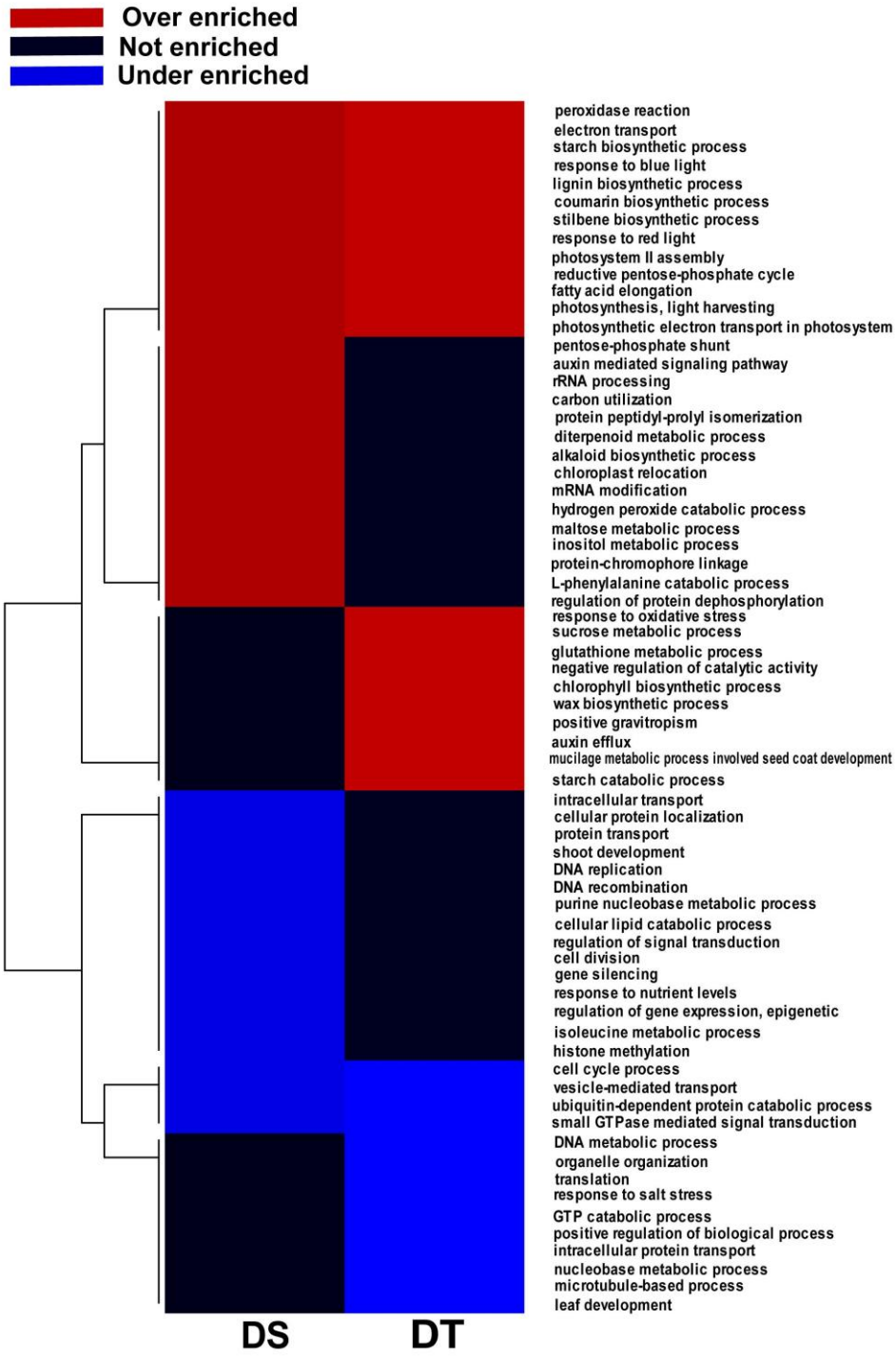


Figure 6.2 Figure showing biological process gene ontology (GO) terms enriched in differentially expressed grass genes caused by endophyte infection under drought stress.

'DS' and 'DT' indicate drought sensitive and tolerant perennial ryegrass genotypes respectively. A *P* value cutoff of 0.01 was used to select enriched gene ontology categories. The dendrogram on the left shows the result of a hierarchical clustering analysis. Gene ontology IDs for each category are listed in Appendix 6.1.

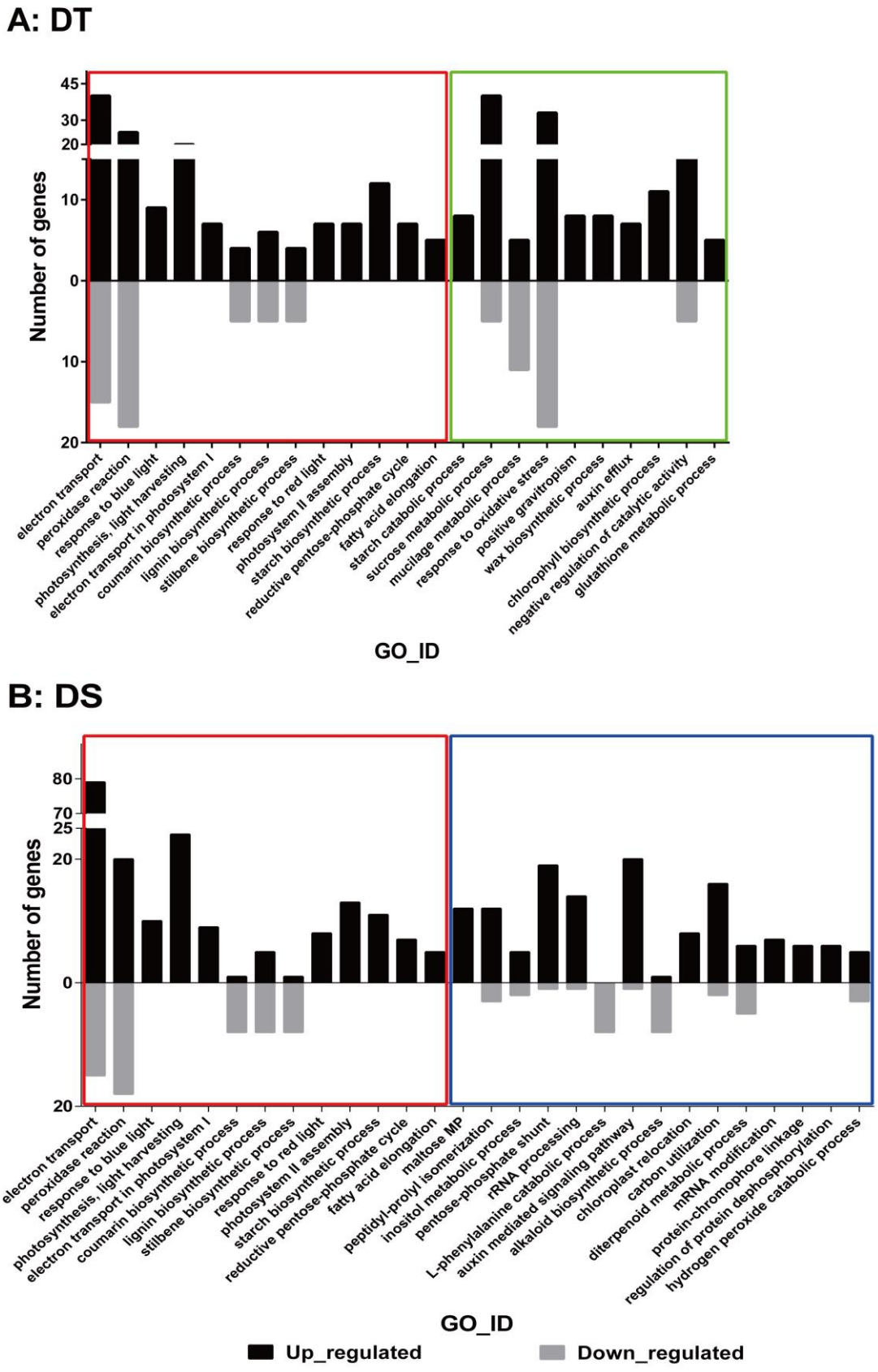


Figure 6.3 Differentially expressed grass genes caused by endophyte infection under drought, organized by over enriched biological process GO categories.

Organization of all endophyte regulated differentially-expressed genes under drought in drought tolerant genotype (A) and drought sensitive genotype (B) by biological process GO categories. Bars show the number of genes within each category that are up- or down-regulated in the endophyte-infected grass sample relative to the endophyte-free grass sample. GO categories framed with red blocks were over-enriched in differentially expressed gene sets of both DT and DS genotypes under drought. GO categories framed with green and blue blocks were only over-enriched in differentially expressed gene sets of DT and DS genotype respectively under drought. Gene ontology IDs for each category are listed in Appendix 6.1.

6.1.3 Functional annotations of endophyte-regulated grass genes

MapMan is a user-driven tool that can display large-scale gene expression datasets on diagrams of metabolic pathways. To further identify the influence of endophyte on grass metabolism under drought stress, Mapman metabolic pathway analysis software was used on grass genes that were differentially expressed in the presence or absence of endophyte. The results confirmed that endophyte infection adjusted perennial ryegrass metabolism on the transcriptome level, with overall similar responses in both DT and DS grass genotypes. There was significant up-regulation of genes associated with photosynthesis, chlorophyll biosynthesis, ascorbate and glutathione cycle, starch biosynthesis and degradation, sucrose degradation, terpenes (carotenoids and anthocyanins) biosynthesis in both DT and DS genotypes under drought stress (see green boxed regions A to F in Figure 6.4). The presence of endophyte also led to significant down-regulated expression of grass genes associated with mitochondrial electron transport (ATP synthesis) in the DT genotype under drought (see green box G in Figure 6.4). More down-regulation of genes involved in phenylpropanoid and phenolics metabolism was found in the DS genotype than in the DT genotype (Figure 6.4). A background mapman figure of Figure 6.4, showing all the genes assigned to the various pathways, is displayed in Appendix Figure S6.2.

Looking in more detail, expression of several genes associated with the glutathione-ascorbate cycle was up-regulated by endophyte infection in both DT and DS genotypes under drought conditions (green box C in Figure 6.4, Figure 6.5). Most of these up-regulated genes are predicted to encode antioxidant enzymes, such as ascorbate peroxidase (APX) and glutathione peroxidase (GPX) that are often associated with stress.

A more detailed look at other pathways in which grass genes were differentially expressed in response to endophyte is shown in Figures 6.6 - 6.10. Background mapman figures showing all the genes involved in corresponding pathways are

displayed in Appendix Figures S6.3 - S6.8.

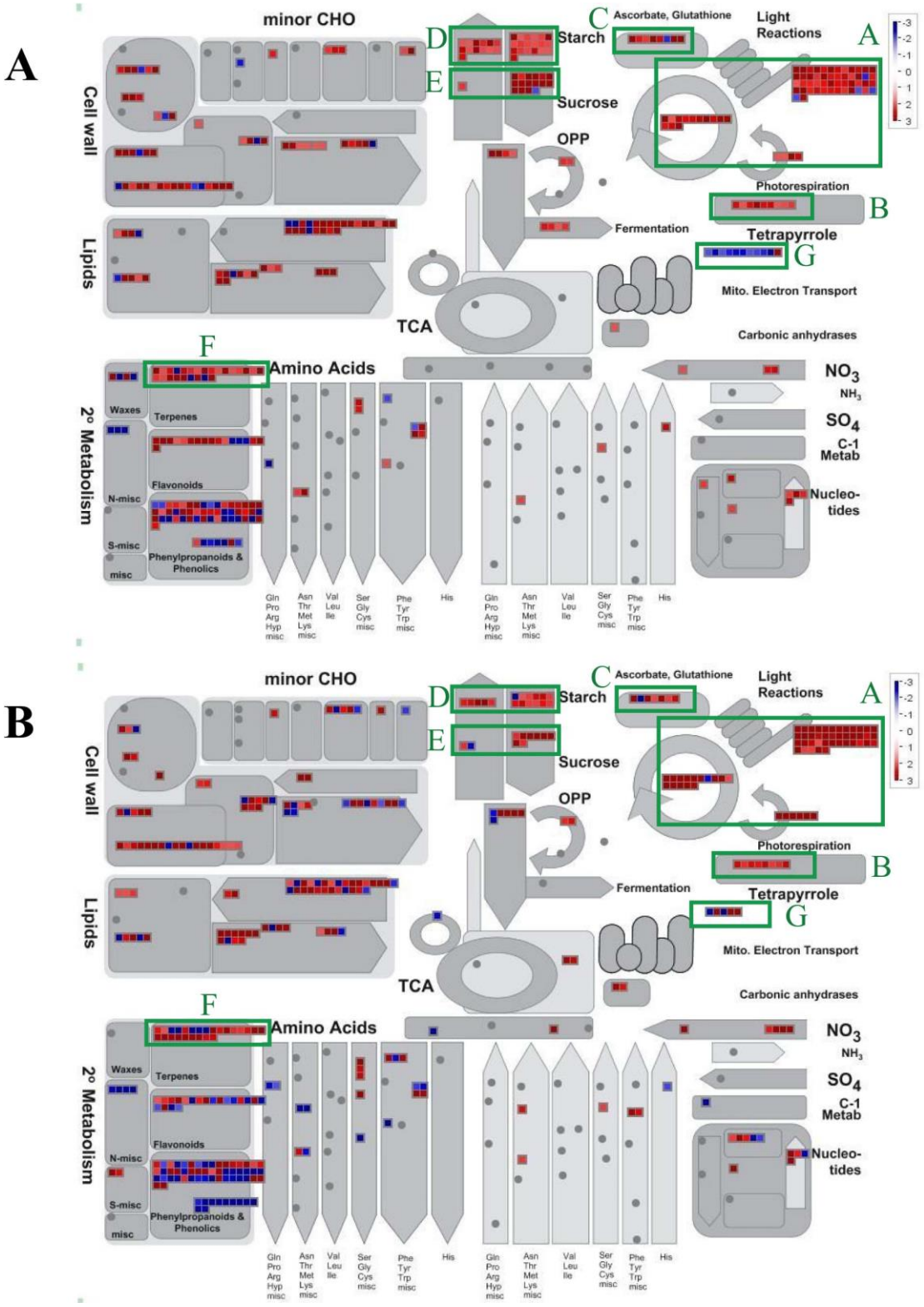


Figure 6.4 Overview of endophyte infection regulated differentially expressed grass transcripts involved in different metabolic processes under drought stress.

A and B show the effect of endophyte on expression of genes involved in different metabolic processes in drought tolerant and drought sensitive genotypes respectively. The images were obtained using MapMan, and show functional categories of genes that passed the cutoff (less than 0.05 q value and greater than 2-fold change) for differential expression. Letters in green in each graph indicate: “A” - photosynthesis, “B” - tetrapyrrole metabolism, “C” - ascorbate and glutathione cycle, “D” - starch metabolism, “E” - sucrose metabolism, “F” - terpenes metabolism, “G” - mitochondrial electron transport. Each small square represents an individual gene in that pathway, shaded according to the fold change in RPMK (read per million per kilo base); red = up-regulated, blue = down-regulated in endophyte-infected sample compared to endophyte-free sample. Genes with fold change ≥ 3 and ≤ -3 are shown in the same shades of red and blue respectively.

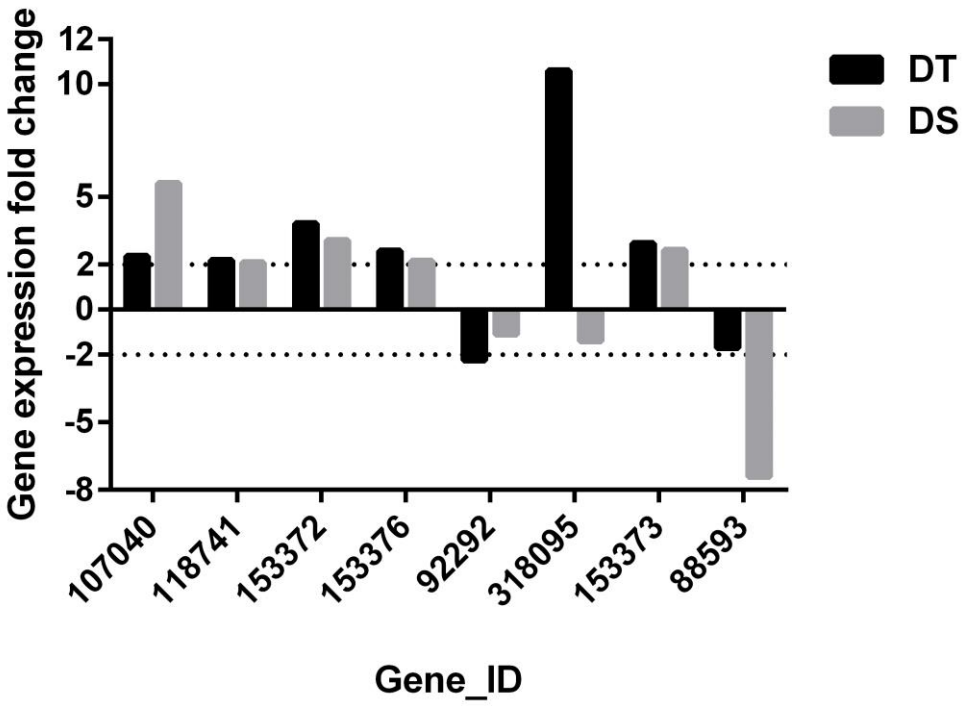


Figure 6.5 Effects of endophyte infection on the expression of grass genes associated with the glutathione-ascorbate cycle under drought stress.

DT and DS indicate drought tolerant genotype and drought sensitive genotype respectively. Annotations of genes are: 107040: l-ascorbate peroxidase, 118741: thylakoid-bound ascorbate peroxidase, 153372: gamma-glutamyl transpeptidase, 153376: gamma-glutamyltranspeptidase 1, 92292: phospholipid hydroperoxide glutathione peroxidase, 318095: glutathione peroxidase, 153373: gamma-glutamyl transpeptidase, 88593: l-ascorbate oxidase. All genes that passed the cutoff with greater than 2-fold change also had less than 0.05 q value for differential expression in DT genotype.

In both DT and DS genotypes, the expression of many genes involved in photosynthetic light reactions (particularly in photosystem II) and the Calvin cycle, and several genes involved in photorespiration, was significantly increased by *N. lolii* infection under drought stress (Figure 6.6, Table S6.1, and Table S6.2).

The expression of grass genes involved in chlorophyll biosynthesis was also up-regulated by endophyte infection in both DT and DS genotypes under drought (Figure 6.7, Table S6.3, and Table S6.4). More genes located in the chlorophyll branch (left arm in Figure 6.7), were up-regulated in the DT (6 genes) genotype than in the DS genotype (4 genes).

The pattern of ribosomal protein gene expression was consistent with the effects of endophyte on up-regulation of many photosynthetic genes. In both DT and DS genotypes, endophyte infection significantly up-regulated the expression of genes encoding chloroplast ribosomal proteins (both 50S and 30S ribosomal proteins), but down-regulated expression of genes associated with nuclear ribosomal protein biosynthesis (both 40S and 60S ribosomal proteins) under drought stress (Figure 6.8, Table S6.5, Table S6.6).

As predicted in plants with increased expression of photosynthetic genes, up-regulation of starch biosynthetic genes was also seen, particularly in the DT genotype (Figure 6.9). In addition, in both DT and DS genotypes, the expression of genes involved in sucrose degradation to glucose and fructose (Table S6.7 and Table S6.8), and starch degradation to maltose and glucose (Table S6.9 and Table S6.10) were up-regulated by endophyte infection under drought. More differentially expressed genes associated with these metabolic pathways were found in the DT than in the DS genotype (Figure 6.9).

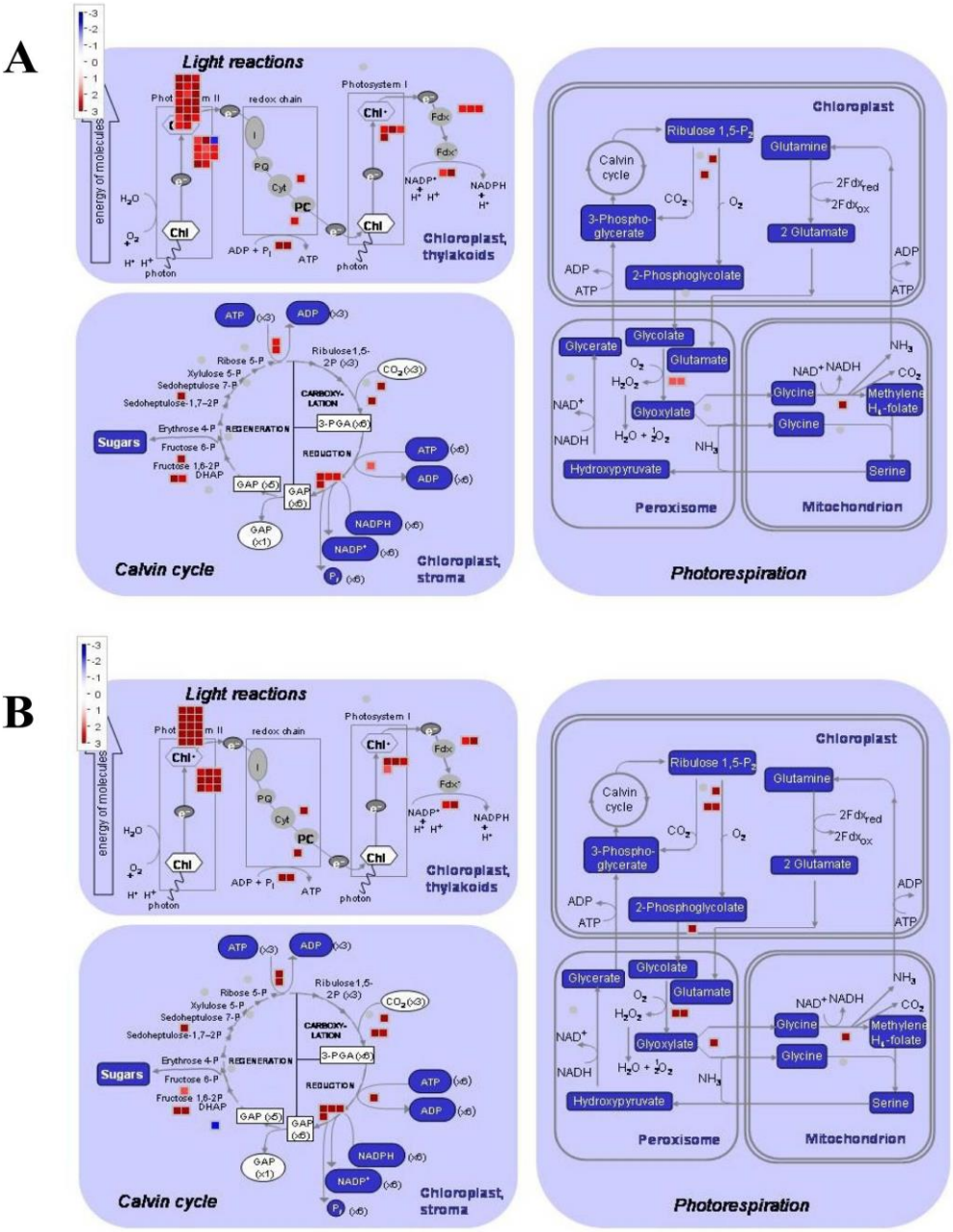


Figure 6.6 Effects of endophyte infection on the expression of grass genes associated with photosynthesis under drought stress.

A and B show the expression changes of grass genes in associated with photosynthesis caused by *N. lolii* infection in drought tolerant and drought sensitive genotypes respectively. The images were obtained using MapMan. Each small square represents an individual gene in that pathway, shaded according to the fold change in RPMK (read per million per kilo base); red = up-regulated, blue = down-regulated in endophyte-infected sample compared to endophyte-free sample.

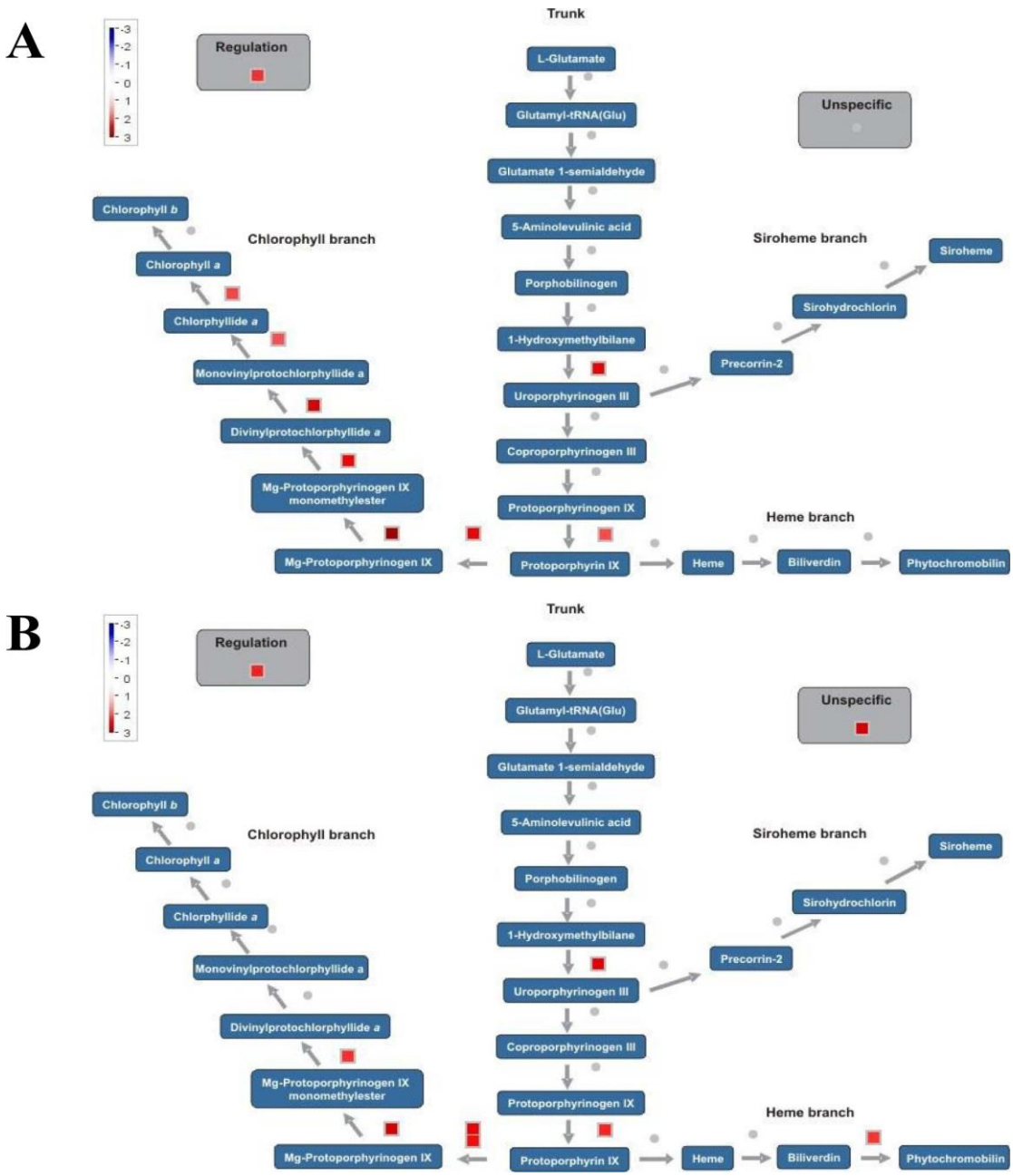


Figure 6.7 Effects of endophyte infection on the expression of grass genes associated with chlorophyll biosynthesis under drought stress.

A and B show expression changes of grass genes involved in chlorophyll biosynthesis caused by *N. lolii* infection in drought tolerant genotype and drought sensitive genotypes respectively. The images were obtained using MapMan. Each small square represents an individual gene in that pathway, shaded according to the fold change in RPMK (read per million per kilo base); red = up-regulated, blue = down-regulated in endophyte-infected sample compared to endophyte-free sample.

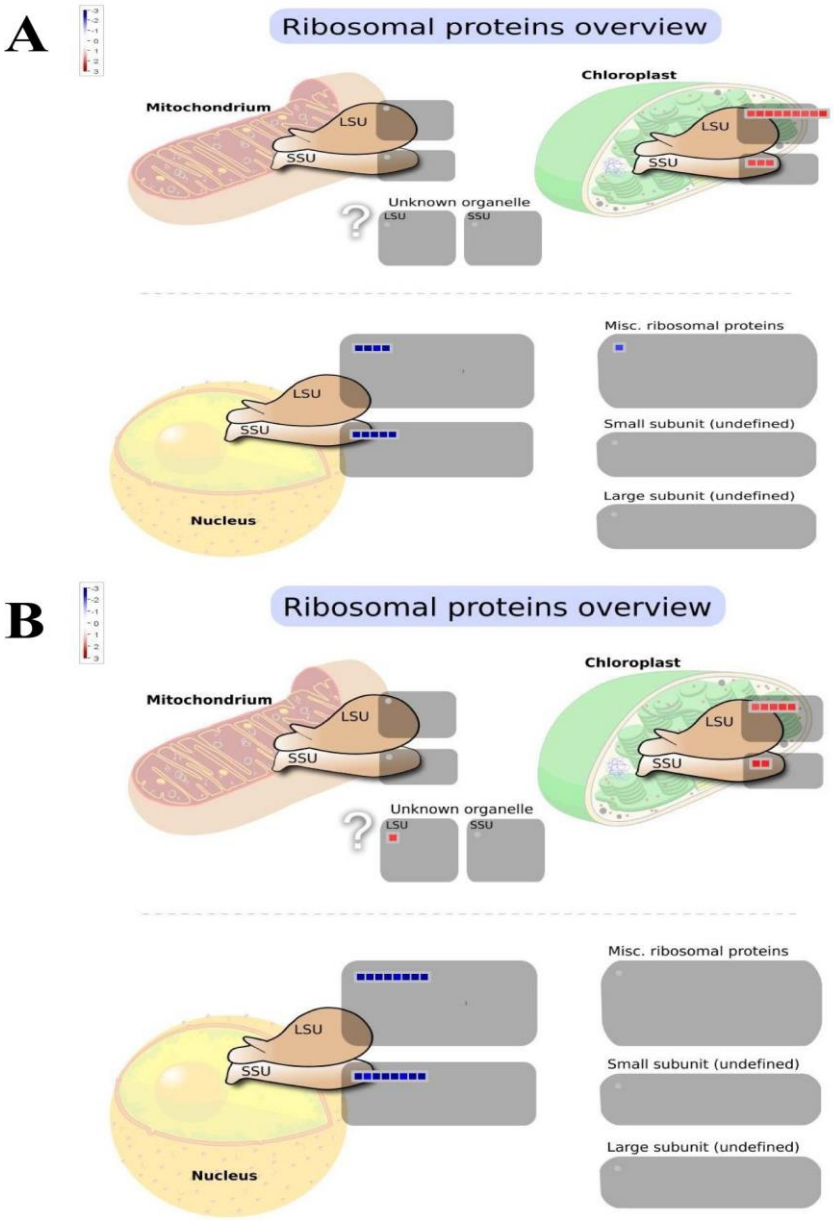


Figure 6.8 Overview of endophyte infection regulated differentially expressed grass genes involved in ribosomal protein biosynthesis under drought stress.

A and B show expression changes of grass genes involved in ribosomal protein biosynthesis caused by *N. lolii* infection in drought tolerant genotype and drought sensitive genotype respectively The images were obtained using MapMan. Each small square represents an individual gene in that pathway, shaded according to the fold change in RPMK (read per million per kilo base); red = up-regulated, blue = down-regulated in endophyte-infected sample compared to endophyte-free sample.

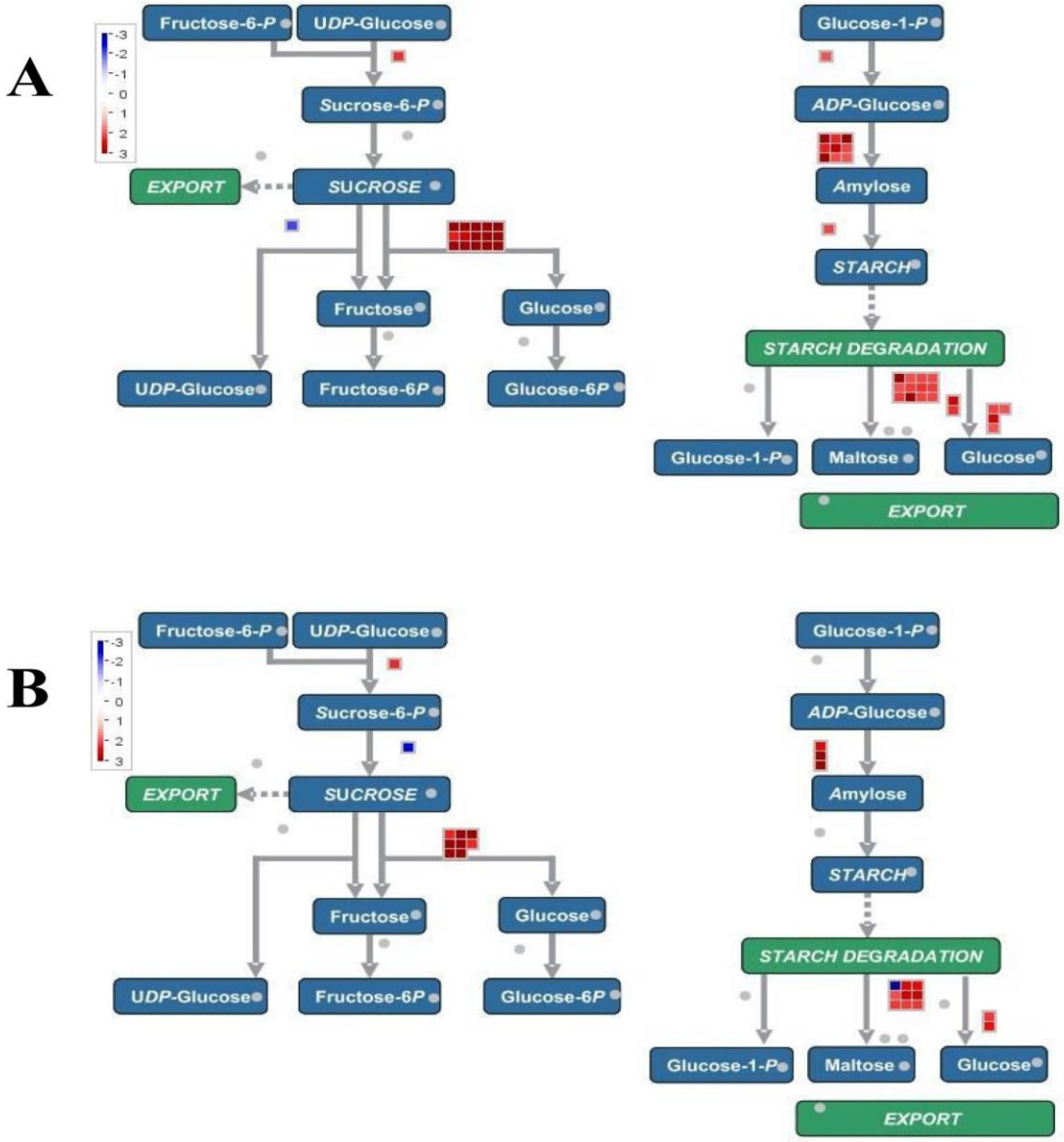


Figure 6.9 Effects of endophyte infection on the expression of grass genes associated with starch and sucrose metabolism under drought stress.

A and B show expression changes of grass genes involved in sucrose and starch metabolism caused by *N. lolii* infection in drought tolerant and drought sensitive genotypes respectively. The images were obtained using MapMan. Each small square represents an individual gene in that pathway, shaded according to the fold change in RPMK (read per million per kilo base); red = up-regulated, blue = down-regulated in endophyte-infected sample compared to endophyte-free sample.

The influence of endophyte on expression of secondary metabolism genes in grass was also investigated further as shown in Figure 6.10. Endophyte infection increased expression of genes involved in biosynthesis of carotenoids (Table S6.11 and S6.12) and anthocyanins (Table S6.13 and S6.14) in both DT and DS genotypes under drought stress (Figure 6.10). More differentially expressed genes associated with anthocyanin biosynthesis were found in the DT genotype (8) than the DS genotype (5) (Figure 6.10). Both carotenoids and anthocyanins are pigments with powerful antioxidant capability that play important roles in scavenging ROS. In general over all secondary metabolic pathways, there was less up-regulation and more down-regulation in the DS genotype compared to the DT genotype, except terpenoid metabolism which showed more up-regulation in the DS genotype (Figure 6.10).

The expression of several genes associated with mitochondrial electron transport (ATP synthesis) was significantly down-regulated by endophyte infection in the DT genotype under drought stress (Figure 6.11A, Table S6.15). However no overall trend of down regulation of ATP synthesis genes by endophyte infection was observed in the DS genotype (Figure 6.11B, Table S6.16).

The Mapman analysis supported the results obtained from the GO term enrichment analysis, but not all GO terms were represented in specific pathways in Mapman. For example, two GO terms, wax biosynthetic process (GO0010025) and positive gravitropism (GO0009958), were only over enriched in the endophyte regulated gene set in the DT genotype under drought (Figure 6.2). The expression of the individual differentially expressed genes was examined and significant up-regulation in DTE+ was shown (Figure 6.12, 6.13).

To sum up the main findings, pathway analyses showed that *N. lolii* infection significantly up-regulated the expression of genes associated with photosynthesis, chlorophyll biosynthesis, ascorbate and glutathione cycles, starch and sucrose metabolism, and carotenoid and anthocyanin biosynthesis, in both DT and DS genotypes under drought stress. For some of these pathways (chlorophyll, sucrose and

anthocyanin metabolism, wax biosynthesis and positive gravitropism) more differentially expressed genes were seen in the DT than in the DS genotype. *N. lolii* down-regulated the expression of genes involved in mitochondrial ATP synthesis in the DT, but not in the DS, genotype.

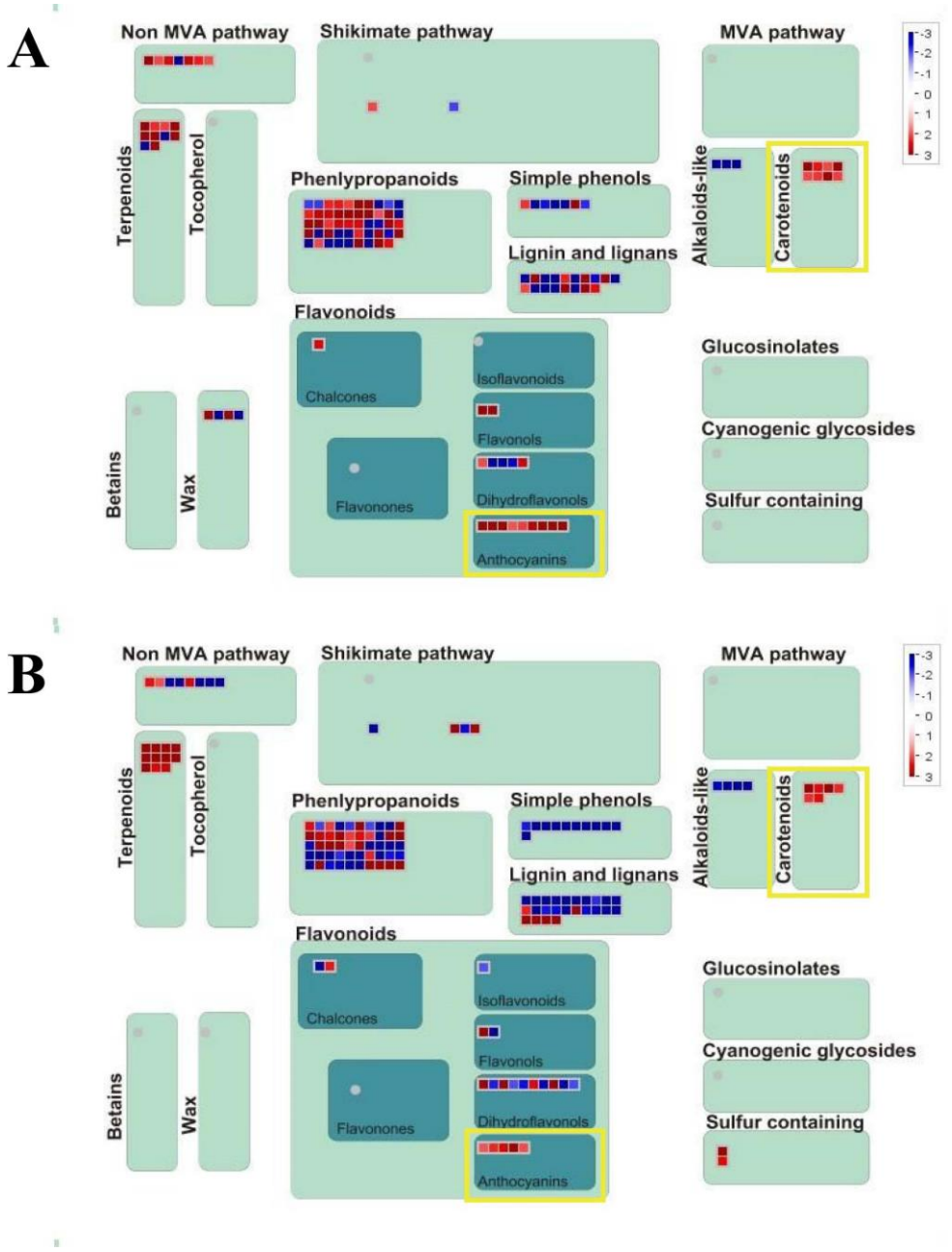


Figure 6.10 Overview of grass differentially expressed genes involved in secondary metabolism under drought stress.

A and B show expression changes of grass genes involved in secondary metabolism caused by *N. lolii* infection in drought tolerant and drought sensitive genotypes respectively. The images were obtained using MapMan. Each small square represents an individual gene in that pathway, shaded according to the fold change in RPMK (read per million per kilo base); red = up-regulated, blue = down-regulated in endophyte-infected sample compared to endophyte-free sample.

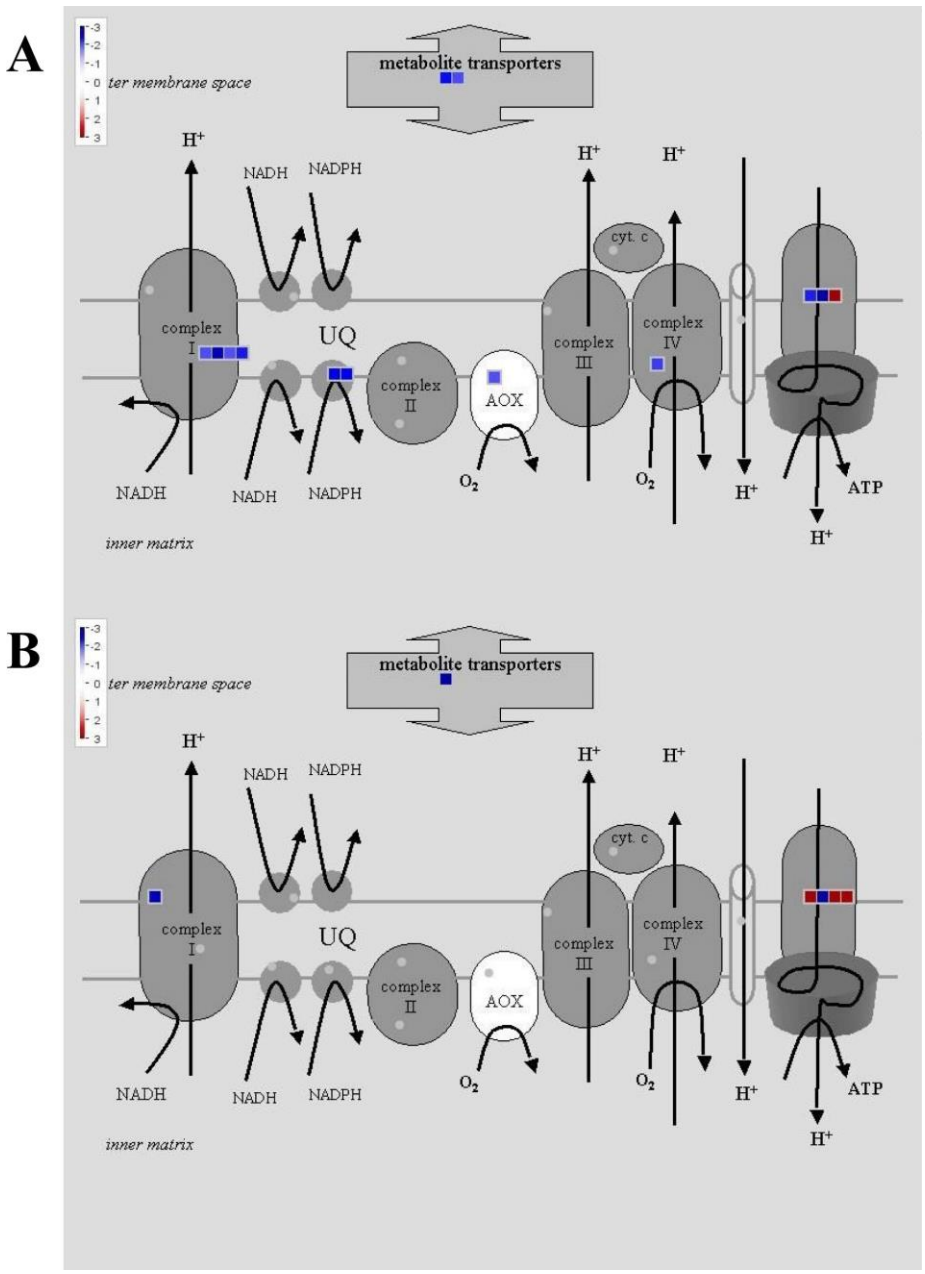


Figure 6.11 Effects of endophyte infection on the expression of grass genes associated with mitochondrial electron transport under drought stress.

A and B show expression changes of grass genes involved in mitochondrial electron transport caused by *N. lolii* infection in drought tolerant and drought sensitive genotypes respectively The images were obtained using MapMan. Each small square represents an individual gene in that pathway, shaded according to the fold change in RPMK (read per million per kilo base); red = up-regulated, blue = down-regulated in endophyte-infected sample compared to endophyte-free sample.

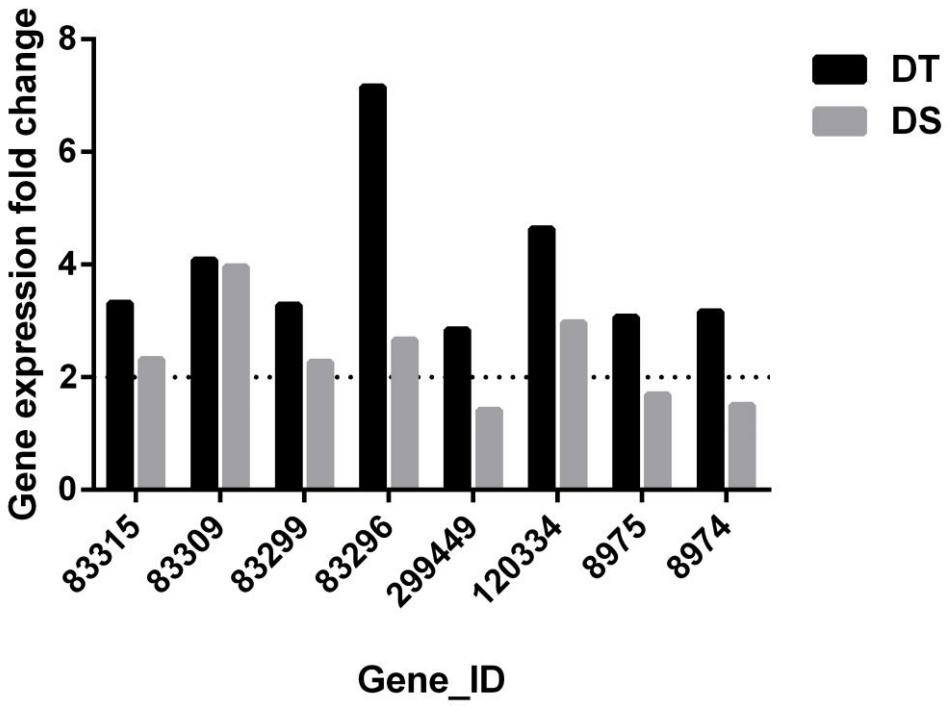


Figure 6.12 Effects of endophyte infection on the expression of grass genes associated with wax biosynthetic process under drought stress.

DT and DS indicate drought tolerant genotype and drought sensitive genotype respectively. Annotations of genes are: 83315: senescence-associated protein 15, 83309: senescence associated protein 15, 83299: fatty acid elongase, 83296: senescence associated protein 15, 299449: amp-binding enzyme family expressed, 120334: senescence-associated protein 15, 8975: protein wax2, 8974: cer1 expressed. All genes that passed the cutoff with greater than 2-fold change also had less than 0.05 q value for differential expression in DT genotype.

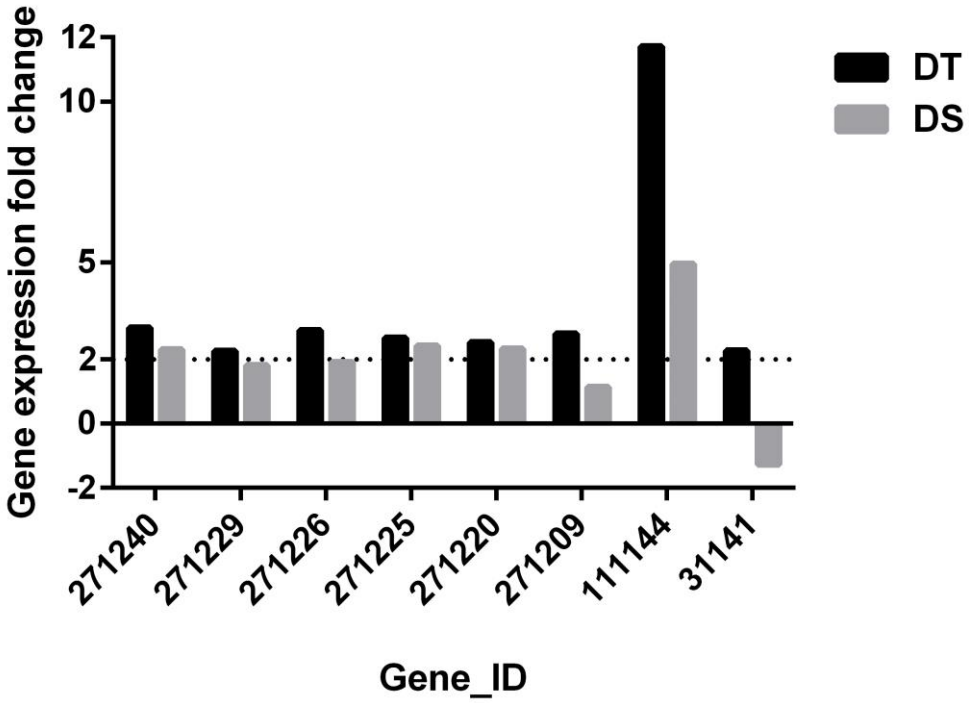


Figure 6.13 Effects of endophyte infection on the expression of grass genes associated with positive gravitropism under drought stress.

DT and DS indicate drought tolerant genotype and drought sensitive genotype respectively. Annotations of genes are: 271240: auxin efflux carrier, 271229: auxin efflux carrier, 271226: efflux carrier, 271225: efflux carrier, 271220: auxin efflux carrier, 271209: auxin efflux carrier, 111144: kinase domain containing protein, 31141: gpr1 family protein. All genes that passed the cutoff with greater than 2-fold change also had less than 0.05 q value for differential expression in DT genotype.

6.2 Discussion

6.2.1 Endophyte protected grass chloroplasts under drought stress through multiple approaches

The results of physiological measurements on grasses (Chapter 4) used in this RNA seq experiment indicate that *N. lolii* improved drought tolerance of both genotypes, although to different extents. The higher Fv/Fm ratio in endophyte-infected plants indicated their chloroplasts were probably less damaged under drought than those of endophyte-free grasses. Besides, the higher expression of grass genes involved in photosynthesis (Figure 6.6) also suggests that chloroplasts in endophyte-infected plants were more functional than those in endophyte-free plants. This might be due to endophyte enhanced grass chloroplast repair capability by increasing chlorophyll and chloroplast protein biosynthesis under drought stress. Analysis of endophyte regulated perennial ryegrass genes under drought showed that in both DT and DS genotypes, the expression of many genes involved in chlorophyll and chloroplast protein biosynthesis were significantly up-regulated by endophyte infection under drought stress (Figures 6.7 and 6.8). The effect of endophyte on grass chlorophyll a+b accumulation under drought condition has been observed by others (Zhang and Nan 2007). In their study, chlorophyll a+b concentrations in *Neotyphodium* endophyte-infected *Elymus dahuricus* plants was significantly higher ($P < 0.05$) than that in endophyte-free plants after an 8 week low water treatment.

Besides enhancing chlorophyll and chloroplast protein biosynthesis, endophyte infection might protect chloroplasts through enhancing plant ROS (reactive oxygen species) scavenging capability. ROS is an important signalling transduction chemical and under normal conditions plants keep ROS under a certain level (Gill and Tuteja 2010). However, ROS levels increase under drought stress. The over-produced ROS is extremely deleterious and can damage plant cellular membranes and other cellular components including chloroplasts (Cruz de Carvalho 2008). ROS can be scavenged

by several antioxidant compounds (Gill and Tuteja 2010). In the current study the expression of genes encoding ascorbate peroxidase (APX) and glutathione peroxidase (GPX) were significantly up-regulated by endophyte infection in both DT and DS genotypes under drought conditions. The glutathione-ascorbate cycle is a metabolic pathway that plays a very important role in detoxifying hydrogen peroxide (H_2O_2) in plant chloroplasts (Foyer and Noctor 2011). APX and GPX detoxify hydrogen peroxide to water using ascorbate and glutathione as substrates respectively. The significant up-regulation of expression of genes encoding APX and GPX synthesis indicates that endophytes might protect plant chloroplasts through enhancing ROS scavenging capability.

Increased levels of grass ROS scavenging enzyme activity by endophyte infection under drought has been found in other fungal-plant associations under drought stress. Zhang and Nan (2007) found that *Neotyphodium sp.* infection increased superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and APX activities, and decreased H_2O_2 levels in *Elymus dahuricus* under water deficit (Zhang and Nan 2007). Similarly Mastouri et al. (2012) reported that tomato seedlings (*Lycospersicum esculentum* L.) infected by *Trichoderma harzianum* had higher activities of ascorbate and glutathione-recycling enzymes, and of SOD, CAT, and APX, than uninfected tomato seedlings when subjected to drought stress. However, it was not clear from any of these studies if the increased antioxidant enzyme activity was of plant or fungal origin as both have genes encoding these enzymes. The transcriptome profiling carried out in this study enabled the expression of grass and fungal genes to be examined separately. We did not find extensive increased expression of grass genes encoding antioxidant enzymes, but only several APX and GPX genes in the glutathione-ascorbate cycle under drought stress (Figure S6.1). This indicates that the regulation of antioxidant enzyme activity by the endophyte itself might also play a very important role in endophyte-mediated enhancement of grass drought tolerance

Genes involved in carotenoid and anthocyanin biosynthesis were significantly

up-regulated by endophyte infection under drought in both DT and DS genotypes (Figure 6.10, Table S6.13 and Table S6.14). This suggested that endophyte-infected plants might produce more carotenoids and anthocyanins under drought conditions. The phenomenon of increased carotenoids and anthocyanins production under adverse environmental conditions, by host plants with fungal endophytes, has been reported in the literature. Li et al. found that carotenoid levels were significantly higher in rice (*Oryza sativa* L.) seedlings infected with *Sordariomycetes sp*., compared to uninfected, when the plants were subjected to lead (Pb) stress (Li et al. 2012). Likewise *Penicillium funiculosum* increased carotenoid concentrations in *Glycine max* L. under copper stress (Khan and Lee 2013). Shimizu et al. found that a bacterial endophyte, *Streptomyces sp.*, enhanced disease resistance of rhododendron plants to *Pestalotiopsis sydowiana* by inducing increased anthocyanin levels (Shimizu et al. 2006). Increased levels of carotenoids and anthocyanins can enhance plant resistant to either biotic or abiotic stresses by scavenging ROS, because both chemicals are pigments with powerful antioxidant capability (De Rosso et al. 2008; Paiva and Russell 1999). Since carotenoids are present in chloroplasts (Grumbach and Lichtenthaler 1982), they can be involved in detoxifying ROS in chloroplasts directly. Although anthocyanins are mainly located in vacuoles (Tanaka et al. 2008), they can also protect chloroplasts indirectly through decreasing ROS levels in the whole plant cell under drought stress.

6.2.2 Endophyte enhanced grass osmotic adjustment under drought by adjusting sugar metabolism

An important approach of plants in coping with drought stress is reducing water loss by decreasing osmotic potential in plant cells. This can be achieved by accumulating osmolytes such as proline, glucose, fructose, sucrose, fructans, mannitol etc (Burg and Ferraris 2008). We found that leaf osmotic adjustment (LOA) was greater in E+ than in E- plants under drought (Figure 4.10) suggesting that endophyte infection increased water-soluble osmolyte accumulation in the plant cells. This is

identical with other studies on the influence of *N. coenophialum* on tall fescue drought tolerance (Elmi and West 1995). In their study, it was found that osmotic adjustment was greater (to a similar extent as observed in our study) in E+ plants than in E- plants in both leaf blades and tiller basal zones under drought stress.

Analysis of ryegrass genes differentially expressed in response to endophyte showed that genes involved in sucrose degradation to fructose and glucose, and starch biosynthesis and degradation to maltose and glucose, were up-regulated in both DTE+ and DSE+. This indicates that endophyte infection might increase glucose, fructose and maltose accumulation in E+ plants under drought stress. Soluble carbohydrates, such as glucose, fructose and sucrose, can accumulate in plant leaves in response to drought stress (Valliyodan and Nguyen 2006). This suggests that increasing osmotic adjustment through adjusting grass sugar metabolism might be an important approach of enhancing grass drought tolerance by endophytes. This phenomenon was also found in studies of drought tolerance of tall fescue/ *N. coenophialum* endophyte associations (Nagabhyru et al. 2013; Richardson et al. 1992). In these studies, *N. coenophialum* infected plants had greater concentrations of fructose and glucose when grown under drought stress compared to uninfected plants.

6.2.3 Endophyte effect on grass drought tolerance was more pronounced in drought tolerant than in drought sensitive genotype

Only the DT genotype showed a significant difference (*T*-test, $P<0.05$) between the various measures of plant health (LRWC, TTN, Fv/Fm and LOA) between E+ and E- under drought conditions (Figures 4.7, 4.8, 4.9 and 4.10). This indicated that the effect of endophyte on grass tolerance was more pronounced in the drought tolerant genotype than in the drought sensitive genotype. This might be due to the endophyte having a stronger effect on grass chlorophyll biosynthesis, sucrose and starch metabolism, ascorbate and glutathione cycle, mitochondrial electron transport, wax

biosynthesis and root development.

GO enrichment analysis suggested that genes involved in chlorophyll and glutathione biosynthesis, and starch and sucrose metabolism, were only enriched in the endophyte infection regulated grass gene set of the DT genotype, but not in that of DS grass (Figure 6.2). More genes associated with these pathways, and with ascorbate and glutathione cycles, were found differentially expressed in the DT genotype (Tables S6.3 - S6.16).

Endophyte infection significantly down-regulated the expression of genes associated with mitochondrial electron transport (ATP synthesis) in the DT genotype, but not the DS genotype (Figure 6.11). ROS is a side product of ATP synthesis in mitochondria (Turrens 2003). Decreasing ATP synthesis can suppress ROS production and therefore reduce cell damage caused by ROS toxicity under drought stress.

In addition, the expression of several genes associated with wax biosynthesis and positive gravitropism were significantly up-regulated in DTE+ (Figure 6.12, 6.13). Wax plays a pivotal role in limiting water loss through transpiration across the plant surface (Meeks et al. 2012; Zhang et al. 2005). The up-regulated expression of genes associated with wax biosynthesis indicates that endophytes in drought tolerant plants might help reduce water loss through promoting host wax production. The GO term of "positive gravitropism" is also known as "downward growth of roots" (Muller et al. 1998). Up-regulation of genes associated with positive gravitropism can increase the water absorption capability by extending the root system deeper in the soil.

6.3 Limitations and general conclusions

Due to limitations in the bioinformatic analyses, it is possible that other genes additional to those discussed here are involved in endophyte regulation of drought tolerance. These limitations include:

1. Low mapped reads proportion (61.6%)

   The perennial ryegrass EST database that was used for mapping grass reads was generated from another cultivar (Nui) grown under normal conditions. The SNPs between the two cultivars may have reduced the proportion of mapped reads. Also, given the fact hat many genes are only expressed under drought stress, important drought responsive genes may not have been represented in the grass EST database.

2. Low GO annotation ratio

   Only 30.6% of grass genes in the EST database were annotated with biological GO terms. This low annotation rate decreased the accuracy of GO enrichment analysis.

3. Low gene annotation ratio in functional analysis

   Only 65% of grass genes in the EST database were annotated using the Mercator pipeline that was used to feed the ryegrass data into the Mapman program. This suggests that the functional analysis of about one third of differentially expressed genes has been missed.

Thus, although the GO and Mapman approaches provided complementary approaches to functional annotation of the functionally expressed genes, there is a lot more information in the transcriptome profiling data that is yet to be explored.

In summary, *N. lolii* endophyte enhanced both DT and DS grass drought tolerance by broadly adjusting the plant metabolism. The analysis of transcriptome profile data suggests the endophyte increases grass drought tolerance through: (1) reinforcing

grass chloroplast protection through increasing chlorophyll and chloroplast protein biosynthesis and ROS scavenging capability and (2) reducing water loss by enhancing osmotic adjustment through increased glucose, fructose and maltose accumulation. All these effects can help endophyte-infected grass maintain a relatively stable intracellular water balance, and then a higher rate of photosynthesis and greater biomass than endophyte-free plants. The more pronounced endophyte effect on drought tolerant grass than on drought sensitive grass might due to a greater increase in chlorophyll biosynthesis, osmolytes accumulation and ROS scavenging, but additional effects on promotion of wax biosynthesis and root growth are possible.

7 General conclusions

This PhD thesis focused on development of a method that can accurately quantify and differentiate three endophytes, exploring the effect of *Neotyphodium lolii* endophyte on perennial ryegrass drought tolerance, and investigating the response of *N. lolii* endophyte to drought stress. The major achievements of this research are summarized below.

A real time PCR method based on endophyte secondary metabolite genes, that can distinguish between three endophytes (AR1, AR37 and a New Zealand common toxic ecotype) and accurately quantify their concentration *in planta*, was developed. This method shows high sensitivity, specificity (lack of amplification from 16 other fungal species), and precision (correlation with endophyte biovolume concentration in tissue with R^2>0.95). Endophyte specific primer/probe combinations targeted to *ltmJ* and *perA* genes could detect contamination of AR37 seeds with either New Zealand common toxic ecotype or AR1 down to levels of 3-25%. This method has potential applications in management of endophytes in pastures and seed production as well as in fundamental research into this important plant-microbe symbiosis.

The impact of *N. lolii* on drought tolerance of 16 ryegrass cultivars (listed in Table 2.1) was assessed under glasshouse conditions. The results suggested that *N. lolii* benefits ryegrass growth under drought conditions. The extent of its effect varied between cultivars and significant endophyte × genotype × treatment interaction was observed.

Two *N. lolii* endophyte infected perennial ryegrass genotypes showing distinct survival and performance under severe drought condition were selected from one cultivar (Nine O One). The transcriptomes of these two endophyte-infected grasses, as well as endophyte-free clones of the same plants, were profiled under both control and drought conditions using high throughput RNA sequencing. Physiological parameters were also measured for these grasses under the same conditions.Physiological measurements showed that endophyte infection enhanced performance of both grasses under severe drought stress, including increasing leaf relative water content,

photosynthesis II maximum efficiency, leaf osmotic adjustment and grass total tiller number. However, endophyte effects on these parameters were more pronounced in the DT (drought tolerant genotype) grass compared to the DS (drought sensitive genotype) grass.

Bioinformatic analyses on fungal gene expression revealed that endophytes living in DT and DS hosts responded to severe drought stress in similar ways: they showed enhanced ROS scavenging capability through increasing peroxisome biogenesis and antioxidant enzyme biosynthesis, increased expression of genes involved in DNA replication and the cell cycle, and lower expression of genes involved in alkaloids biosynthesis. Compared to those in the DS host, endophytes in the DT host had greater increases in expression of peroxisome and antioxidant genes, and stronger decreases in expression of alkaloids genes under severe drought. In contrast, the DS endophyte emphasized more on accelerating DNA replication and the cell cycle.

Bioinformatic analyses on plant gene expression revealed that the presence of the *N. lolii* endophyte enhanced both DT and DS grass drought tolerance by broadly adjusting the plant metabolism. These effects included reinforcing grass chloroplast protection through increased chlorophyll and chloroplast protein biosynthesis and ROS scavenging capability, as well as reducing water loss by enhancing osmotic adjustment through increased glucose, fructose and maltose accumulation.

We also realize that limitations, such as low mapping and annotation rates, might have influenced the accuracy of the RNA sequencing results. This can be addressed by upgrading of the database in the future.

This work highlights the importance of fungal endophytes in resistance of grass to drought and provides a comprehensive insight into mechanisms by which endophytes improve grass drought tolerance. It also provides several gene sets that potentially could be developed as markers to be used in screening drought tolerance of grass/endophyte combinations in the field.

8 Future work

Following on from the work described in this thesis, several interesting studies could be done in the future:

1. **Real time PCR method extension and application.**

   The endophyte specific primers and probes developed in this work will enable survival and persistence of selected endophytes to be monitored in pasture when in competition with common toxic endophytes. Given the high similarity of *Neotyphodium lolii* and Epichloë endophytes, the real time PCR method developed in this thesis could be extended to measure endophyte concentrations in other *Epichloë* endophyte/grass symbioses.

2. **Further analysis of drought effect on metabolite production.**

   Genes involved in several metabolic pathways of both endophyte and grass were found to show differences in expression under drought stress based on RNA seq results. Given the fact that the final expression products can also be determined by post translational regulation, proteomics and metabolomics could be done to complement the RNA seq analysis results. This might help to provide some insight on why substantial decreases in expression of many endophyte alkaloids biosynthetic genes under drought did not lead to similar reductions in alkaloids levels.

3. **Analysis of different drought conditions.**

   Drought stress of less than 20% FC for a week was used in the RNA profiling experiment outlined in this thesis. It is possible the responses of the plant and endophyte would be different under different extents of drought severity. Thus it would be interesting to determine differences in expression of candidate genes found in this thesis under different drought conditions, such as mild drought stress or during water recovery.

4. **Analysis of combined stresses.**

   Candidate gene sets enhancing grass tolerance to drought in lab conditions were identified in this thesis. Given the fact that multiple stresses, such as drought, heat and insect, often happen simultaneously in field, it would be of considerable interest to measure differences in expression of these candidate genes under combined stresses.

5. **Generalize the gene expression results.**

   Only two perennial ryegrass genotypes from the same cultivar were used for RNA profiling in this thesis. It would be very interesting to test the expression of candidate genes found in this project on other perennial ryegrass cultivars, or even other endophyte/grass symbioses in both laboratory and field conditions. Once the general principles have been defined using a broader range of cultivars, a set of gene expression patterns and/or physiological measurements could be identified, and used to screen drought tolerance of grass/endophyte combinations to find those that are best suited for survival in very dry conditions.

9 Appendices

Appendix 2.1: Command lines used in RNA sequencing data analysis

SolexaQA

perl SolexaQA.pl FASTQ_input_files [-p|probcutoff 0.05] [-h|phredcutoff 41] [-v|variance] [-m|minmax] [-s|sample 10000] [-b|bwa] [-d|directory path] [-sanger -illumina -solexa]

DynamicTrim

perl DynamicTrim.pl FASTQ_input_files [-p|probcutoff 0.05] [-h|phredcutoff 41] [-b|bwa] [-d|directory path] [-sanger -illumina -solexa] [-454]

LengthSort

perl LengthSort.pl one single-end or two paired-end FASTQ files [-l|length 80] [-d|directory path]

Mapping and counting

```
export MAPPING=/ dir/mapping
export mapped=/dir/mapped
bowtie2-build RyegrassESTs_28Mar13.fasta ref
bowtie2 --phred33 --threads 20 -x $MAPPING/ref -U $file.fastq.trimmed.single    -S file.single.sam
bowtie2 --phred33 --threads 20 -x $MAPPING/ref -1 $file.fastq.trimmed.paired1 -2 $file.fastq.trimmed.paired2    -S E_R1.paired.sam
/home/m/mpcox/bin/map_count -f $MAPPING/refence.fasta -trim -s file.single.sam -o file.single.counts
/home/m/mpcox/bin/map_count -f $MAPPING/reference.fasta -trim -p -s file.paired.sam -o file.paired.counts
join $MAPPING/file.single.counts    $MAPPING/file.paired.counts    >> file.counts
wc -l *.counts >> linecounts.txt
```

Degseq

```
library(DEGseq)
input <- "/dir/ RawCounts"
output <- "/dir/data"
geneExpMatrix1 <- readGeneExp(file=input, geneCol=1, valCol=c(1, 2))
geneExpMatrix2 <- readGeneExp(file=input, geneCol=1, valCol=c(3, 4))
geneExpMatrix1[1:5,]
geneExpMatrix2[1:5,]
DEGexp(geneExpMatrix1=geneExpMatrix1, geneCol1=1, expCol1=c(2,3), groupLabel1="Epositive", geneExpMatrix2=geneExpMatrix2, geneCol2=1, expCol2=c(2,3), groupLabel2="Enegative", method="FET", thresholdKind=4, qValue=0.05, outputDir=outputDir)
```

Appendix 5.1 Mapping results of each sample used in RNA sequencing

	DTE+_Control		**DTE+_Drought**		**DTE-_Control**		**DTE-_Drought**	
	Rep 1	**Rep 2**	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**
Number of grass reads of each replicate mapped to perennial ryegrass EST[a]	36,469,249	32,319,404	42,060,430	40,446,429	40,314,521	41,276,250	26,000,269	22,626,006
Mean number of grass reads mapped to perennial ryegrass EST[a]	34,394,326.5		41,253,429.5		40,795,385.5		24,313,137.5	
Number of fungal reads of each replicate mapped to endophyte gene models[b]	980,051	876,355	729,688	898,051	4,763	5,074	4,764	3,621
Mean number of fungal reads mapped to endophyte gene models[b]	928,203		813,869.5		4,918.5		4,192.5	
Proportion of mapped fungal reads to mapped grass reads in each replicate (%)	2.69	2.71	1.73	2.22	0.01	0.01	0.02	0.02
Mean of proportion of mapped fungal reads to mapped grass reads (%)	2.7		2.0		0.01		0.02	

a: The perennial ryegrass EST is a de novo assembled EST library (EST_28Mar13; n =50,194; URL: http://ryegrass.massey.ac.nz/), generated by Jan Schmid, Ningxin Zhang and Robert Day, using sequenced mRNA extracted from perennial ryegrass (NuiD genotype) grown under normal conditions as described in (Zhang et al. 2011).
b: The endophyte reference genome sequence used is *Epichloë festucae* gene models (EfM3; n = 9,350; http://csbio-l.csr.uky.edu/ef2011/).
"DTE+" and "DTE-" indicate clonal endophyte-infected and endophyte-free grass of drought tolerant genotype respectively. "DSE+" and "DSE-" represent clonal endophyte-infected and endophyte-free grass of drought sensitive genotype respectively.
“Rep” indicates biological replicate.

Appendix 6.1: Gene ontology IDs for each category described in Figures 6.6 and 6.7

GO ID[a]	GO term[b]
GO0006118	Electron transport
GO0006804	peroxidase reaction
GO0009637	response to blue light
GO0009765	photosynthesis, light harvesting
GO0009773	photosynthetic electron transport in photosystem I
GO0009805	coumarin biosynthetic process
GO0009809	lignin biosynthetic process
GO0009811	stilbene biosynthetic process
GO0010114	response to red light
GO0010207	photosystem II assembly
GO0019252	starch biosynthetic process
GO0019253	reductive pentose-phosphate cycle
GO0030497	fatty acid elongation
GO0005983	starch catabolic process
GO0005985	sucrose metabolic process
GO0006749	glutathione metabolic process
GO0006979	response to oxidative stress
GO0009958	positive gravitropism
GO0010025	wax biosynthetic process
GO0010315	auxin efflux
GO0015995	chlorophyll biosynthetic process
GO0043086	negative regulation of catalytic activity
GO0048359	mucilage metabolic process involved seed coat development
GO0000023	maltose metabolic process
GO0000413	protein peptidyl-prolyl isomerization
GO0006020	inositol metabolic process
GO0006098	pentose-phosphate shunt
GO0006364	rRNA processing
GO0006559	L-phenylalanine catabolic process
GO0009734	auxin mediated signalling pathway
GO0009821	alkaloid biosynthetic process
GO0009902	chloroplast relocation
GO0015976	carbon utilization
GO0016101	diterpenoid metabolic process
GO0016556	mRNA modification
GO0018298	protein-chromophore linkage
GO0035304	regulation of protein dephosphorylation
GO0042744	hydrogen peroxide catabolic process

GO0022402	cell cycle process
GO0051301	cell division
GO0044242	cellular lipid catabolic process
GO0034613	cellular protein localization
GO0006259	DNA metabolic process
GO0006310	DNA recombination
GO0006260	DNA replication
GO0016458	gene silencing
GO0006184	GTP catabolic process
GO0016571	histone methylation
GO0006886	intracellular protein transport
GO0046907	intracellular transport
GO0006549	isoleucine metabolic process
GO0048366	leaf development
GO0007017	microtubule-based process
GO0009112	nucleobase metabolic process
GO0006996	organelle organization
GO0048518	positive regulation of biological process
GO0006144	purine nucleobase metabolic process
GO0040029	regulation of gene expression, epigenetic
GO0009966	regulation of signal transduction
GO0031667	response to nutrient levels
GO0009651	response to salt stress
GO0048367	shoot development
GO0007264	small GTPase mediated signal transduction
GO0006412	translation
GO0006511	ubiquitin-dependent protein catabolic process
GO0016192	vesicle-mediated transport
GO:0015031	protein transport

[a]**:** Gene ontology accession number (www.geneontology.org). [b]**:** Gene ontology name (www.geneontology.org).

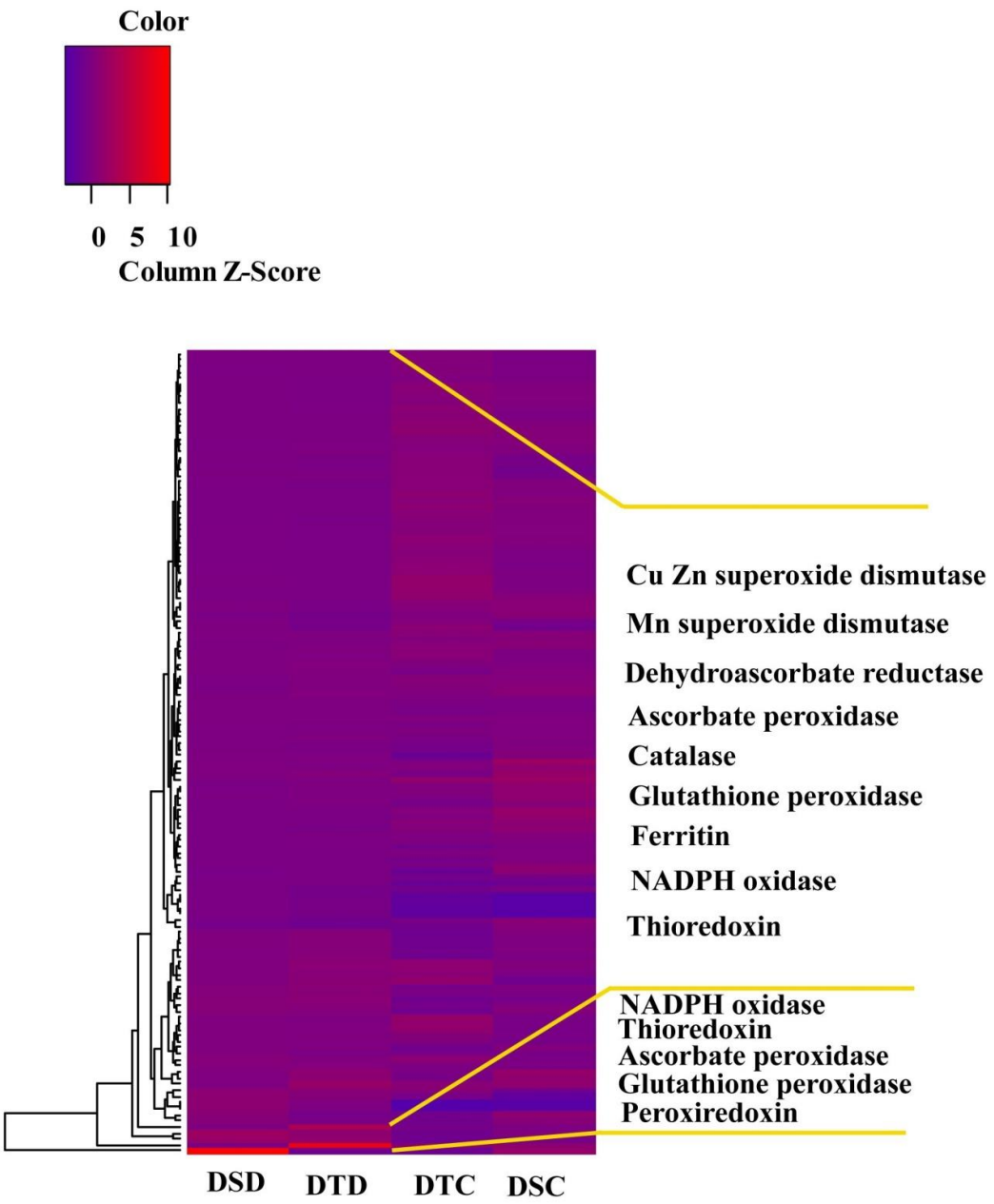


Figure S6.1. Heat map showing the fold change of grass antioxidant enzyme encoding gene expression caused by endophyte infection.

DSD and DTD indicate drought sensitive and tolerant genotypes under drought stress respectively. DSC and DTC indicate drought sensitive and tolerant genotypes under well watered conditions respectively. Genes that were up-regulated and down-regulated are shown in red and blue respectively. The heat map shows the Z score (standardized fold change for facilitating visualization of results) as obtained from the RPMK value ratio of endophyte-infected sample to endophyte-free sample. The dendrogram on the left shows the result of a hierarchical clustering calculation.

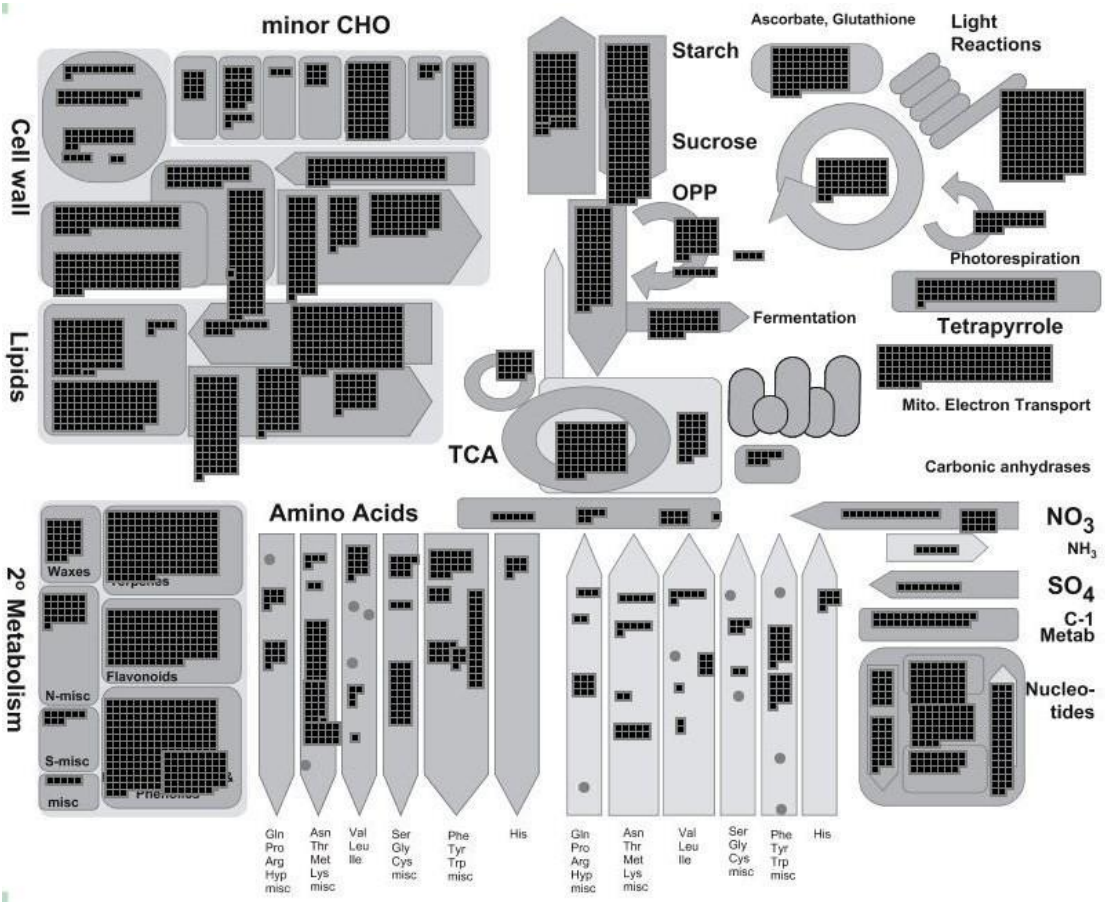


Figure S6.2 Background mapman figure showing all genes (displayed as small black squares) involved in various metabolic processes.

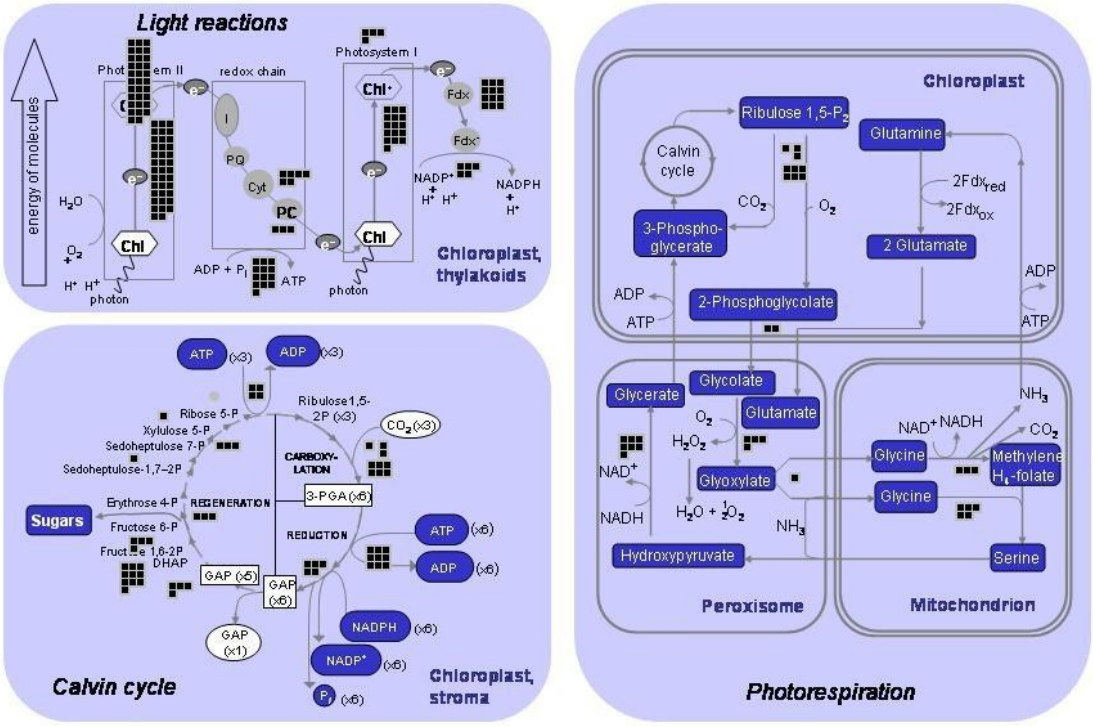


Figure S6.3 Background mapman figure showing all genes (displayed as small black squares) involved in photosynthesis.

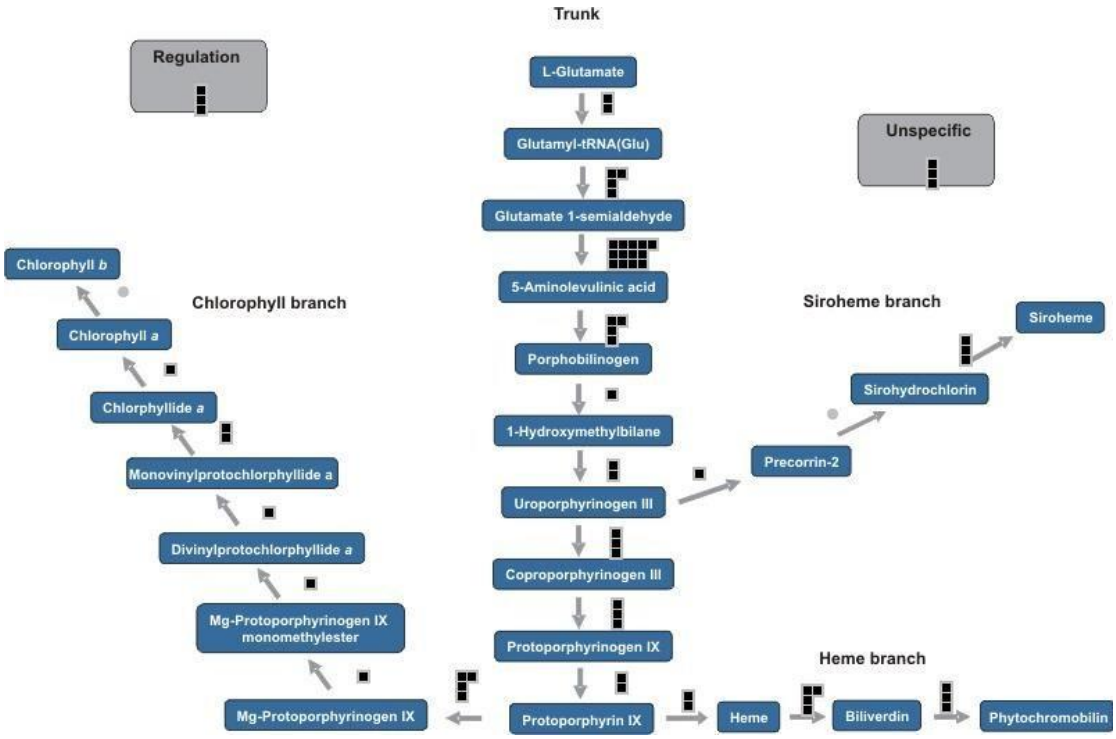


Figure S6.4 Background mapman figure showing all genes (displayed as small black squares) involved in tetrapyrrole biosynthesis.

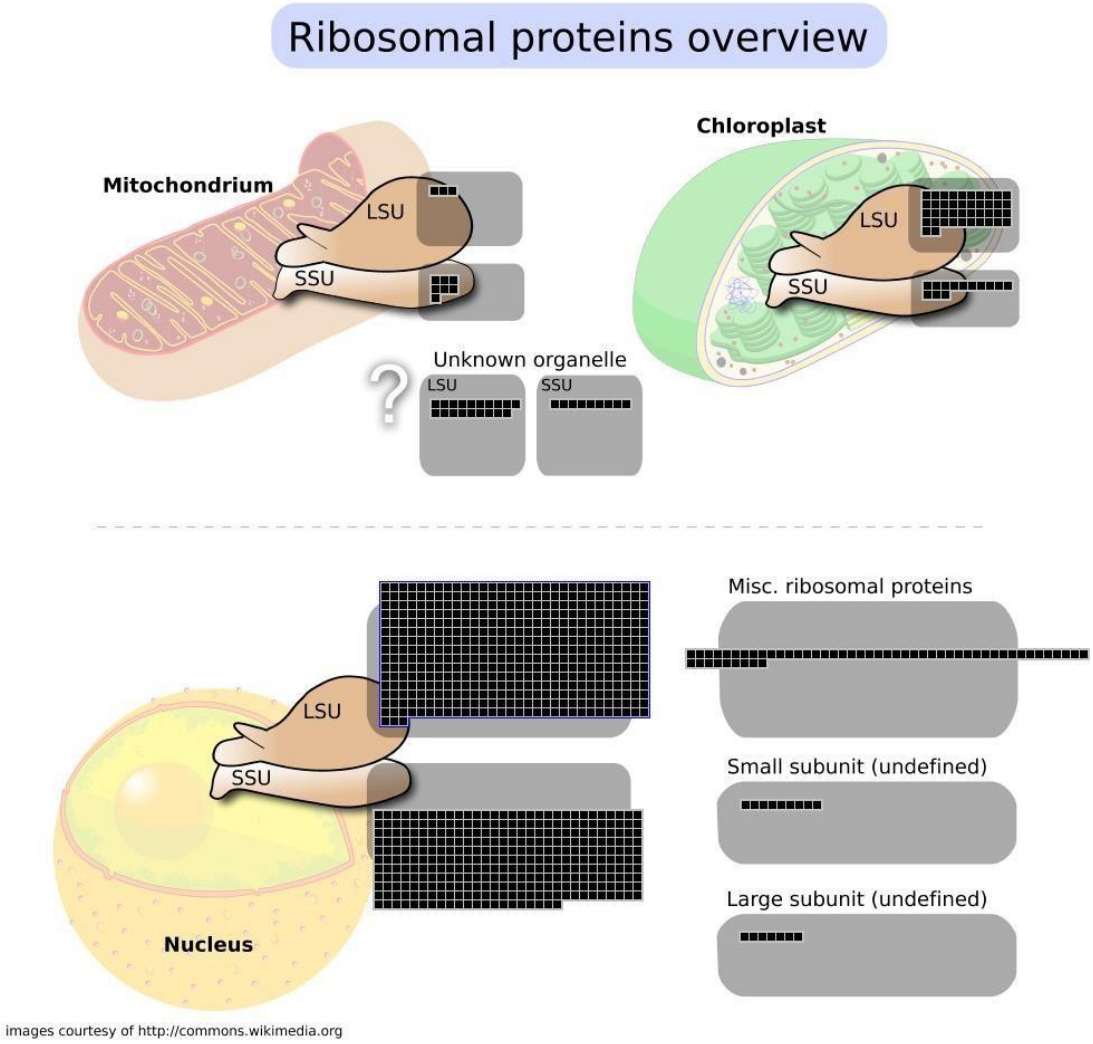


Figure S6.5 Background mapman figure showing all genes (displayed as small black squares) involved in ribosomal protein biosynthesis.

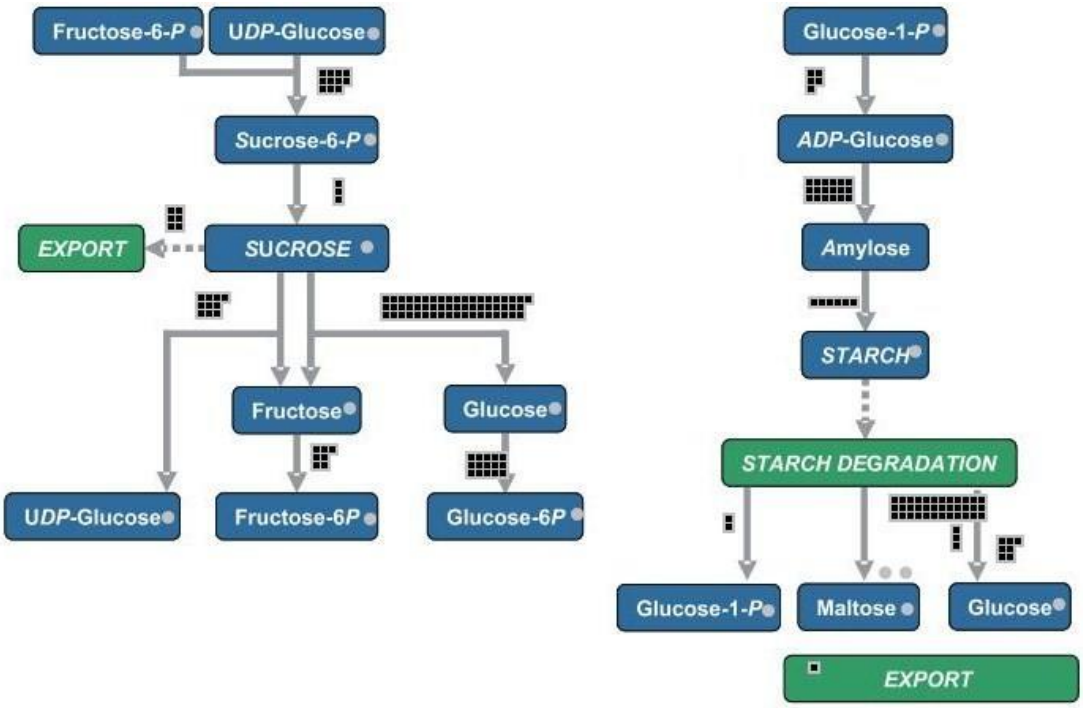


Figure S6.6 Background mapman figure showing all genes (displayed as small black squares) involved in starch and sucrose metabolism.

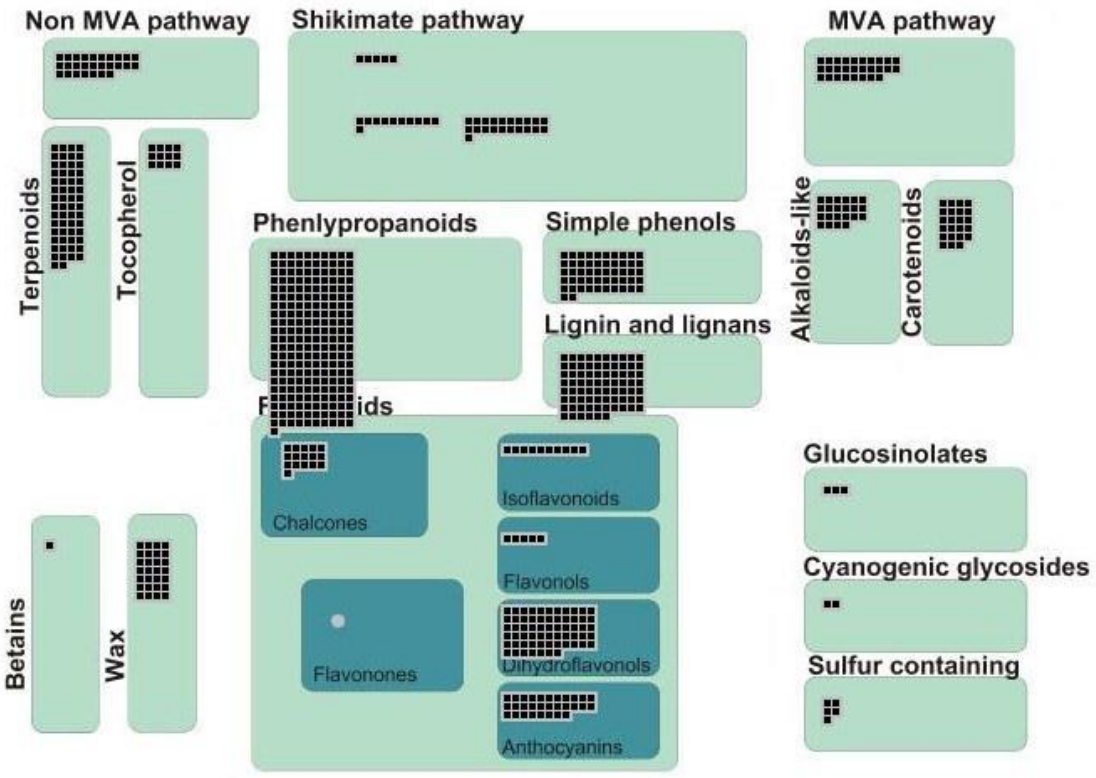


Figure S6.7 Background mapman figure showing all genes (displayed as small black squares) involved in secondary metabolism.

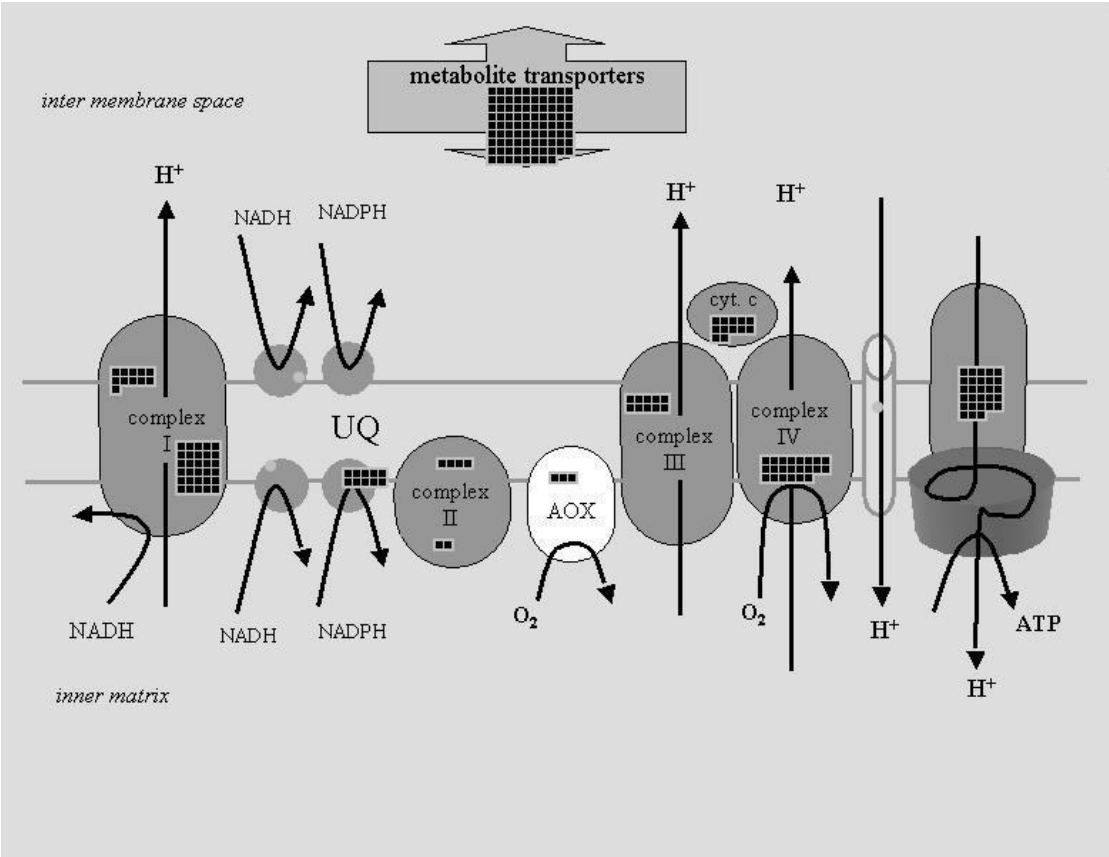


Figure S6.8 Background mapman figure showing all genes (displayed as small black squares) involved in mitochondrial electron transport.

10 Publication

Zhou Y, Bradshaw RE, Johnson RD, Hume DE, Simpson WR, Schmid J, 2014. Detection and quantification of three distinct *Neotyphodium lolii* endophytes in *Lolium perenne* by real time PCR of secondary metabolite genes. *Fungal Biology*. Accepted on 11 Jan, 2014.

This paper was based on content of Chapter 3 in this thesis.

11 References

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K, 2003. *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell* **15**: 63-78.

Abernethy GA, Fountain DW, McManus MT, 1998. Observations on the leaf anatomy of *Festuca novae-zelandiae* and biochemical responses to a water deficit. *New Zealand Journal of Botany* **36**: 113-123.

Abernethy GA, McManus MT, 1998. Biochemical responses to an imposed water deficit in mature leaf tissue of *Festuca arundinacea*. *Environmental and Experimental Botany* **40**: 17-28.

Al-Karaki G, McMichael B, Zak J, 2004. Field response of wheat to arbuscular mycorrhizal fungi and drought stress. *Mycorrhiza* **14**: 263-269.

Alvim FC, Carolino SMB, Cascardo JCM, Nunes CC, Martinez CA, Otoni WC, Fontes EPB, 2001. Enhanced accumulation of BiP in transgenic plants confers tolerance to water stress. *Plant Physiology* **126**: 1042-1054.

Amara I, Capellades M, Dolors Ludevid M, Pages M, Goday A, 2013. Enhanced water stress tolerance of transgenic maize plants over-expressing LEA Rab28 gene. *Journal of Plant Physiology* **170**: 864-873.

Ambrose KV, Belanger FC, 2012. SOLiD-SAGE of endophyte-infected red fescue reveals numerous effects on host transcriptome and an abundance of highly expressed fungal secreted proteins. *Plos One* **7**: e53214.

APNZ-News-Service, 2013. 2013 drought was worst since 1945 - study. http://www.nzherald.co.nz/business/news/article.cfm?c_id=3&objectid=10903236. Last updated on Jul 25, 2013. Acccessed on Jan 10, 2014.

Asada K, 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology* **141**: 391-396.

Babu RC, Pathan MS, Blum A, Nguyen HT, 1999. Comparison of measurement methods of osmotic adjustment in rice cultivars. *Crop Science* **39**: 150-158.

Bacon CW, 1993. Abiotic stress tolerances (moisture, nutrients) and photosynthesis in endophyte-infected tall fescue. *Agriculture Ecosystems & Environment* **44**: 123-141.

Bacon CW, Lyons PC, Porter JK, Robbins JD, 1986. Ergot toxicity from endophyte-infected grasses-a review. *Agronomy Journal* **78**: 106-116.

Bae H, Sicher RC, Kim MS, Kim S-H, Strem MD, Melnick RL, Bailey BA, 2009. The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in *Theobroma cacao*. *Journal of Experimental Botany* **60**: 3279-3295.

Bai L, Zhang G, Zhou Y, Zhang Z, Wang W, Du Y, Wu Z, Song C-P, 2009. Plasma membrane-associated proline-rich extensin-like receptor kinase 4, a novel regulator of Ca^{2+} signalling, is required for abscisic acid responses in *Arabidopsis thaliana*. *Plant Journal* **60**: 314-327.

Bailey BA, Bae H, Strem MD, Roberts DP, Thomas SE, Crozier J, Samuels GJ, Choi I-Y, Holmes KA, 2006. Fungal and plant gene expression during the colonization of cacao seedlings by endophytic isolates of four *Trichoderma* species. *Planta* **224**: 1449-1464.

Baker NR, 2008. Chlorophyll fluorescence: A probe of photosynthesis in vivo. *Annual Review of Plant Biology* **59**: 89-113.

Ball OJP, Barker GM, Prestidge RA, Lauren DR, 1997. Distribution and accumulation of the alkaloid peramine in *Neotyphodium lolii*-infected perennial ryegrass. *Journal of Chemical Ecology* **23**: 1419-1434.

Ball OJP, Prestidge RA, Sprosen JM, 1995. Interrelationships between *Acremonium lolii*, Peramine, and Lolitrem B in Perennial Ryegrass. *Applied and Environmental Microbiology* **61**: 1527-1533.

Barker GM, Addison PJ, 1993. Argentine stem weevil populations and damage in ryegrass swards of contrasting *Acremonium* infection, in: R.A. P (ed), *6th Australasian Conference on Grassland Invertebrate Ecology*, Hamilton, New Zealand, 161–168.

Barrow JR, Lucero ME, Reyes-Vera I, Havstad KM, 2008. Do symbiotic microbes have a role in plant evolution, performance and response to stress? *Communicative & Integrative Biology* **1**: 69-73.

Barrs HD, Weatherley PE, 1962. A re-examination of relative turgidity techique for estimating water deficits in leaves. *Australian Journal of Biological Sciences* **15**: 413-&.

Bhargava S, Sawant K, 2013. Drought stress adaptation: metabolic adjustment and regulation of gene expression. *Plant Breeding* **132**: 21-32.

Biosearch-Technologies, 2013. Fluorogenic probes and primers. https://www.biosearchtech.com/display.aspx?catid=224,171. Last updated on May 15. Acccessed on May 15, 2013.

Bischof R, Fourtis L, Limbeck A, Gamauf C, Seiboth B, Kubicek CP, 2013. Comparative analysis of the *Trichoderma reesei* transcriptome during growth on the cellulase inducing substrates wheat straw and lactose. *Biotechnology for Biofuels* **6**: 127.

Bluett SJ, Thom ER, Clark DA, Macdonald KA, Minnee EMK, 2005. Effects of perennial ryegrass infected with either AR1 or wild endophyte on dairy production in the Waikato. *New Zealand Journal of Agricultural Research* **48**: 197-212.

Bonekamp NA, Völkl A, Fahimi HD, Schrader M, 2009. Reactive oxygen species and peroxisomes: Struggling for balance. *BioFactors* **35**: 346-355.

Briggs L, Crush J, Ouyang L, Sprosen J, 2013. *Neotyphodium* endophyte strain and superoxide dismutase activity in perennial ryegrass plants under water deficit. *Acta Physiologiae Plantarum* **35**: 1513-1520.

Briggs LR, Sprosen JM, Tapper BA, Easton HS, 2007. AR1 quality assurance by ELISA, in: Popay AJ, Thom ER (eds), *Proceedings of the 6th International Symposium on Fungal Endophytes of Grasses*. Grassland Research and Practice Series, 289-291.

Brosi GB, McCulley RL, Bush LP, Nelson JA, Classen AT, Norby RJ, 2011. Effects of multiple climate change factors on the tall fescue-fungal endophyte symbiosis: infection frequency and tissue chemistry. *New Phytologist* **189**: 797-805.

Burg MB, Ferraris JD, 2008. Intracellular organic osmolytes: function and regulation. *Journal of Biological Chemistry* **283**: 7309-7313.

Burg SP, 1973. Ethylene in plant-growth. *Proceedings of the National Academy of Sciences of the United States of America* **70**: 591-597.

Busk PK, Pages M, 1998. Regulation of abscisic acid-induced transcription. *Plant Molecular Biology* **37**: 425-435.

Cao M, Koulman A, Johnson LJ, Lane GA, Rasmussen S, 2008. Advanced data-mining strategies for the analysis of direct-infusion ion trap mass spectrometry data from the association of perennial ryegrass with its endophytic fungus, *Neotyphodium lolii*. *Plant Physiology* **146**: 1501-1514.

Chacon MR, Rodriguez-Galan O, Benitez T, Sousa S, Rey M, Llobell A, Delgado-Jarana J, 2007. Microscopic and transcriptome analyses of early colonization of tomato roots by *Trichoderma harzianum*. *International Microbiology* **10**: 19-27.

Charlton ND, Shoji J-Y, Ghimire SR, Nakashima J, Craven KD, 2012. Deletion of the fungal gene soft

disrupts mutualistic symbiosis between the grass endophyte *Epichloe festucae* and the host plant. *Eukaryotic Cell* **11**: 1463-1471.

Chelikani P, Fita I, Loewen PC, 2004. Diversity of structures and properties among catalases. *Cellular and Molecular Life Sciences* **61**: 192-208.

Chen H, Jiang J-G, 2010. Osmotic adjustment and plant adaptation to environmental changes related to drought and salinity. *Environmental Reviews* **18**: 309-319.

Cheplick GP, 1998. Genotypic variation in the regrowth of *Lolium perenne* following clipping: effects of nutrients and endophytic fungi. *Functional Ecology* **12**: 176-184.

Cheplick GP, 2004. Recovery from drought stress in *Lolium perenne* (Poaceae): Are fungal endophytes detrimental? *American Journal of Botany* **91**: 1960-1968.

Cheplick GP, 2007. Costs of fungal endophyte infection in *Lolium perenne* genotypes from Eurasia and North Africa under extreme resource limitation. *Environmental and Experimental Botany* **60**: 202-210.

Cheplick GP, 2008. Host genotype overrides fungal endophyte infection in influencing tiller and spike production of *Lolium perenne* (Poaceae) in a common garden experiment. *American Journal of Botany* **95**: 1063-1071.

Cheplick GP, Faeth HS, 2009. Genotypic specificity of grass-endophyte interactions, in: Cheplick GP, Faeth HS (eds), *Ecology and Evolution of the Grass-Endophyte Symbiosis*. Oxford University Press, New York.

Cheplick GP, Perera A, Koulouris K, 2000. Effect of drought on the growth of *Lolium perenne* genotypes with and without fungal endophytes. *Functional Ecology* **14**: 657-667.

Christensen MJ, Bennett RJ, Ansari HA, Koga H, Johnson RD, Bryan GT, Simpson WR, Koolaard JP, Nickless EM, Voisey CR, 2008. Epichloe endophytes grow by intercalary hyphal extension in elongating grass leaves. *Fungal Genetics and Biology* **45**: 84-93.

Christensen MJ, Bennett RJ, Schmid J, 2002. Growth of *Epichloe*/*Neotyphodium* and p-endophytes in leaves of *Lolium* and *Festuca* grasses. *Mycological Research* **106**: 93-106.

Chung HJ, Sehnke PC, Ferl RJ, 1999. The 14-3-3 proteins: cellular regulators of plant metabolism. *Trends in Plant Science* **4**: 367-371.

Clay K, Schardl C, 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *American Naturalist* **160**: S99-S127.

Cruz de Carvalho MH, 2008. Drought stress and reactive oxygen species: production, scavenging and signaling. *Plant Signaling & Behavior* **3**: 156-165.

de Carvalho MHC, d'Arcy-Lameta A, Roy-Macauley H, Gareil M, El Maarouf H, Pham-Thi AT, Zuily-Fodil Y, 2001. Aspartic protease in leaves of common bean (*Phaseolus vulgaris* L.) and cowpea (*Vigna unguiculata* L. Walp): enzymatic activity, gene expression and relation to drought susceptibility. *FEBS Letters* **492**: 242-246.

De Rosso VV, Moran Vieyra FE, Mercadante AZ, Borsarelli CD, 2008. Singlet oxygen quenching by anthocyanin's flavylium cations. *Free Radical Research* **42**: 885-891.

del Río LA, Sandalio LM, Palma J, Bueno P, Corpas FJ, 1992. Metabolism of oxygen radicals in peroxisomes and cellular implications. *Free Radical Biology and Medicine* **13**: 557-580.

di Menna ME, Finch SC, Popay AJ, Smith BL, 2012. A review of the *Neotyphodium lolii*/*Lolium perenne* symbiosis and its associated effects on animal and plant health, with particular emphasis on ryegrass staggers. *New Zealand Veterinary Journal* **60**: 315-328.

Dixon DP, Cummins I, Cole DJ, Edwards R, 1998. Glutathione-mediated detoxification systems in plants. *Current Opinion in Plant Biology* **1**: 258-266.

Dombrowski JE, Baldwin JC, Azevedo MD, Banowetz GM, 2006. A sensitive PCR-based assay to detect *Neotyphodium* fungi in seed and plant tissue of tall fescue and ryegrass species. *Crop Science* **46**: 1064-1070.

Donoso EP, Bustamante RO, Caru M, Niemeyer HM, 2008. Water deficit as a driver of the mutualistic relationship between the fungus *Trichoderma harzianum* and two wheat genotypes. *Applied and Environmental Microbiology* **74**: 1412-1417.

Duran R, Cary JW, Calvo AM, 2010. Role of the Osmotic Stress Regulatory Pathway in Morphogenesis and Secondary Metabolism in Filamentous Fungi. *Toxins* **2**: 367-381.

Eaton CJ, Cox MP, Ambrose B, Becker M, Hesse U, Schardl CL, Scott B, 2010. Disruption of signaling in a fungal-grass symbiosis leads to pathogenesis. *Plant Physiology* **153**: 1780-1794.

Elmi AA, West CP, 1995. Endophyte infection effects on stomatal conductance, osmotic adjustment and drought recovery of tall fescue. *New Phytologist* **131**: 61-67.

Faeth SH, 2002. Are endophytic fungi defensive plant mutualists? *Oikos* **98**: 25-36.

Fan Q-J, Liu J-H, 2011. Colonization with arbuscular mycorrhizal fungus affects growth, drought tolerance and expression of stress-responsive genes in *Poncirus trifoliata*. *Acta Physiologiae*

Plantarum **33**: 1533-1542.

Feder ME, Hofmann GE, 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annual Review of Physiology* **61**: 243-282.

Felitti S, Shields K, Ramsperger M, Tian P, Sawbridge T, Webster T, Logan E, Erwin T, Forster J, Edwards D, Spangenberg G, 2006. Transcriptome analysis of *Neotyphodium* and *Epichloe* grass endophytes. *Fungal Genetics and Biology* **43**: 465-475.

Fleetwood DJ, Khan AK, Johnson RD, Young CA, Mittal S, Wrenn RE, Hesse U, Foster SJ, Schardl CL, Scott B, 2011. Abundant degenerate miniature inverted-repeat transposable elements in genomes of *Epichloe* fungal endophytes of grasses. *Genome Biology and Evolution* **3**: 1253-1264.

Fleetwood DJ, Scott B, Lane GA, Tanaka A, Johnson RD, 2007. A complex ergovaline gene cluster in *Epichloe* endophytes of grasses. *Applied and Environmental Microbiology* **73**: 2571-2579.

Fletcher LR, 1999. "Non-toxic" endophytes in ryegrass and their effect on livestock health and production. *Grassland Research and Practice Series - New Zealand Grassland Association*: 133-139.

Foyer CH, Noctor G, 2011. Ascorbate and glutathione: the heart of the redox hub. *Plant Physiology* **155**: 2-18.

Franken P, Krajinski F, 2006. Transcriptomics for determining gene expression in symbiotic root-fungus interactions, in: Cooper JE, Rao JR (eds), *Molecular approaches to soil, rhizosphere and plant microorganism analysis*, 213-235.

Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran LSP, Yamaguchi-Shinozaki K, Shinozaki K, 2004. A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. *Plant Journal* **39**: 863-876.

Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K, 2006. Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Current Opinion in Plant Biology* **9**: 436-442.

Gallagher RT, White EP, Mortimer PH, 1981. Ryegrass staggers - isolation of potent neurotoxins lolitrem A and lolitrem B from staggers-producing pastures. *New Zealand Veterinary Journal* **29**: 189-190.

Gamalero E, Lingua G, Berta G, Glick BR, 2009. Beneficial role of plant growth promoting bacteria and arbuscular mycorrhizal fungi on plant responses to heavy metal stress. *Canadian Journal of*

Microbiology **55**: 501-514.

Gamuyao R, Chin JH, Pariasca-Tanaka J, Pesaresi P, Catausan S, Dalid C, Slamet-Loedin I, Tecson-Mendoza EM, Wissuwa M, Heuer S, 2012. The protein kinase Pstol1 from traditional rice confers tolerance of phosphorus deficiency. *Nature* **488**: 535-539.

Gessler NN, Aver'yanov AA, Belozerskaya TA, 2007. Reactive oxygen species in regulation of fungal development. *Biochemistry* **72**: 1091-1109.

Gill SS, Tuteja N, 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* **48**: 909-930.

Giri J, Vij S, Dansana PK, Tyagi AK, 2011. Rice A20/AN1 zinc-finger containing stress-associated proteins (SAP1/11) and a receptor-like cytoplasmic kinase (OsRLCK253) interact via A20 zinc-finger and confer abiotic stress tolerance in transgenic *Arabidopsis* plants. *New Phytologist* **191**: 721-732.

Girma FS, Krieg DR, 1992. Osmotic adjustment in sorghum .1. Mechanisms of diurnal osmotic potential changes. *Plant Physiology* **99**: 577-582.

Goyal K, Walton LJ, Tunnacliffe A, 2005. LEA proteins prevent protein aggregation due to water stress. *Biochemical Journal* **388**: 151-157.

Grumbach KH, Lichtenthaler HK, 1982. Chloroplast pigments and their biosynthesis in relation to light intensity. *Photochemistry and Photobiology* **35**: 209-212.

Guimil S, Chang HS, Zhu T, Sesma A, Osbourn A, Roux C, Ionnidis V, Oakeley EJ, Docquier M, Descombes P, Briggs SP, Paszkowski U, 2005. Comparative transcriptomics of rice reveals an ancient pattern of response to microbial colonization. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 8066-8070.

Hahn H, McManus MT, Warnstorff K, Monahan BJ, Young CA, Davies E, Tapper BA, Scott B, 2008. *Neotyphodium* fungal endophytes confer physiological protection to perennial ryegrass (*Lolium perenne* L.) subjected to a water deficit. *Environmental and Experimental Botany* **63**: 183-199.

Harman GE, 2000. Myths and dogmas of biocontrol - Changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Disease* **84**: 377-393.

Hendershot L, Wei JY, Gaut J, Melnick J, Aviel S, Argon Y, 1996. Inhibition of immunoglobulin folding and secretion by dominant negative BiP ATPase mutants. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 5269-5274.

Hesse U, Schoberlein W, Wittenmayer L, Forster K, Warnstorff K, Diepenbrock W, Merbach W, 2003. Effects of *Neotyphodium* endophytes on growth, reproduction and drought-stress tolerance of three *Lolium perenne* L. genotypes. *Grass and Forage Science* **58**: 407-415.

Hua D, Wang C, He J, Liao H, Duan Y, Zhu Z, Guo Y, Chen Z, Gong Z, 2012. A plasma membrane receptor kinase, *GHR1*, mediates abscisic acid- and hydrogen peroxide-regulated stomatal movement in *Arabidopsis*. *Plant Cell* **24**: 2546-2561.

Hubbard KE, Nishimura N, Hitomi K, Getzoff ED, Schroeder JI, 2010. Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. *Genes & Development* **24**: 1695-1708.

Hume DE, Barker DJ, 2005. Growth and management of endophytic grasses in pastoral agriculture, in: Roberts C.A., West C.P., D.E. S (eds), *Neotyphodium in Cool-Season Grasses*. Blackwell Publishing, Ames, IA, 201-226.

Hume DE, Ryan DL, Cooper BM, Popay AJ, 2007. Agronomic performance of AR37-infected ryegrass in northern New Zealand. *Proceedings of the New Zealand Grassland Association* **69**: 201-205.

Humphreys Jones DR, Waid JS, 1963. Influence of fungal isolates on germination and growth of perennial ryegrass. *Plant and Soil* **19**: 139-150.

Jeon J, Kim NY, Kim S, Kang NY, Novak O, Ku S-J, Cho C, Lee DJ, Lee E-J, Strnad M, Kim J, 2010. A subset of cytokinin two-component signaling system plays a role in cold temperature stress response in *Arabidopsis*. *Journal of Biological Chemistry* **285**: 23369-23384.

Johnson LJ, Johnson RD, Schardl CL, Panaccione DG, 2003. Identification of differentially expressed genes in the mutualistic association of tall fescue with *Neotyphodium coenophialum*. *Physiological and Molecular Plant Pathology* **63**: 305-317.

Kane KH, 2011. Effects of endophyte infection on drought stress tolerance of *Lolium perenne* accessions from the Mediterranean region. *Environmental and Experimental Botany* **71**: 337-344.

Kantar M, Lucas SJ, Budaki H, 2011. Drought stress: molecular genetics and genomics approaches, in: Türkan I (ed), *Plant Responses to Drought and Salinity Stress: Developments in a Post-Genomic Era*, London, 445 - 493.

Katyal AK, Petrisor IG, 2012. Innovative sustainable drought management strategy incorporating forensic techniques and policy framework. *Environmental Forensics* **13**: 122-139.

Khan A, Bassett S, Voisey C, Gaborit C, Johnson L, Christensen M, McCulloch A, Bryan G, Johnson R,

2010. Gene expression profiling of the endophytic fungus *Neotyphodium lolii* in association with its host plant perennial ryegrass. *Australasian Plant Pathology* **39**: 467-476.

Khan AL, Lee I-J, 2013. Endophytic *Penicillium funiculosum* LHL06 secretes gibberellin that reprograms *Glycine max* L. growth during copper stress. *BMC Plant Biology* **13**: 86.

Kirkby KA, Pratley JE, Hume DE, Faville MJ, An M, Wu H, 2011. Incidence of endophyte Neotyphodium occultans in Lolium rigidum from Australia. *Weed Research* **51**: 261-272.

Kitajima M, Butler WL, 1975. Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. *Biochimica Et Biophysica Acta* **376**: 105-115.

Kloepper JW, Ryu CM, Zhang SA, 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* **94**: 1259-1266.

Klute A, 1965. Water Capacity, in: Black CA (ed), *Methods of Soil Analysis. Part 1. Physical and Mineralogical Properties, Including Statistics of Measurement and Sampling*. American Society of Agronomy, Soil Science Society of America, Madison, Wisconsin, 273-278.

Kohler J, Hernandez JA, Caravaca F, Roldan A, 2008. Plant-growth-promoting rhizobacteria and arbuscular mycorrhizal fungi modify alleviation biochemical mechanisms in water-stressed plants. *Functional Plant Biology* **35**: 141-151.

Kuldau GA, Tsai HF, Schardl CL, 1999. Genome sizes of *Epichloe* species and anamorphic hybrids. *Mycologia* **91**: 776-782.

Lane GA, Christensen MJ, Miles CO, 2000. Coevolution of fungal endophytes with grasses: The significance of secondary metabolites, in: Bacon CW, White JF (eds), *Microbial Endophytes*. Marcel Dekker, New York, 341-388.

Lata C, Prasad M, 2011. Role of DREBs in regulation of abiotic stress responses in plants. *Journal of Experimental Botany* **62**: 4731-4748.

Latch GCM, Christensen MJ, Samuels GJ, 1984. Five endophytes of *Lolium* and *Festuca* in New Zealand. *Mycotaxon* **20**: 535-550.

Lee IC, Hong SW, Whang SS, Lim PO, Nam HG, Koo JC, 2011. Age-dependent action of an ABA-inducible receptor kinase, rpk1, as a positive regulator of senescence in *Arabidopsis* leaves. *Plant and Cell Physiology* **52**: 651-662.

Leuchtmann A, bacon cW, Schardl CL, White JF, Tadych M, 2014. Nomenclatural realignment of Neotyphodium species with genus Epichloë. *Mycologia*.

Li X, Bu N, Li Y, Ma L, Xin S, Zhang L, 2012. Growth, photosynthesis and antioxidant responses of endophyte infected and non-infected rice under lead stress conditions. *Journal of Hazardous Materials* **213**: 55-61.

Liu A-L, Zou J, Liu C-F, Zhou X-Y, Zhang X-W, Luo G-Y, Chen X-B, 2013. Over-expression of OsHsfA7 enhanced salt and drought tolerance in transgenic rice. *BMB Reports* **46**: 31-36.

Mahajan S, Tuteja N, 2005. Cold, salinity and drought stresses: An overview. *Archives of Biochemistry and Biophysics* **444**: 139-158.

Malinowski D, Leuchtmann A, Schmidt D, Nosberger J, 1997. Symbiosis with *Neotyphodium uncinatum* endophyte may increase the competitive ability of meadow fescue. *Agronomy Journal* **89**: 833-839.

Malinowski DP, Brauer DK, Belesky DP, 1999. The endophyte *Neotyphodium coenophialum* affects root morphology of tall fescue grown under phosphorus deficiency. *Journal of Agronomy and Crop Science* **183**: 53-60.

Marshall A, Aalen RB, Audenaert D, Beeckman T, Broadley MR, Butenko MA, Cano-Delgado AI, de Vries S, Dresselhaus T, Felix G, Graham NS, Foulkes J, Granier C, Greb T, Grossniklaus U, Hammond JP, Heidstra R, Hodgman C, Hothorn M, Inze D, Ostergaard L, Russinova E, Simon R, Skirycz A, Stahl Y, Zipfel C, De Smete I, 2012. Tackling drought stress: receptor-like kinases present new approaches. *Plant Cell* **24**: 2262-2278.

Mastouri F, Bjoerkman T, Harman GE, 2012. *Trichoderma harzianum* enhances antioxidant defense of tomato seedlings and resistance to water deficit. *Molecular Plant-Microbe Interactions* **25**: 1264-1271.

Mayak S, Tirosh T, Glick BR, 2004a. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant Physiology and Biochemistry* **42**: 565-572.

Mayak S, Tirosh T, Glick BR, 2004b. Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. *Plant Science* **166**: 525-530.

McCue KF, Hanson AD, 1990. Drought and salt tolerance - towards understanding and application. *Trends in Biotechnology* **8**: 358-362.

McDowell N, Pockman WT, Allen CD, Breshears DD, Cobb N, Kolb T, Plaut J, Sperry J, West A, Williams DG, Yepez EA, 2008. Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? *New Phytologist* **178**: 719-739.

McDowell NG, 2011. Mechanisms linking drought, hydraulics, carbon metabolism, and vegetation mortality. *Plant Physiology* **155**: 1051-1059.

Meeks M, Murray SC, Hague S, Hays D, Ibrahim AMH, 2012. Genetic variation for maize epicuticular wax response to drought stress at flowering. *Journal of Agronomy and Crop Science* **198**: 161-172.

Mizuno T, 2005. Two-component phosphorelay signal transduction systems in plants: from hormone responses to circadian rhythms. *Bioscience Biotechnology and Biochemistry* **69**: 2263-2276.

Moate PJ, Williams SRO, Grainger C, Hannah MC, Mapleson D, Auldist MJ, Greenwood JS, Popay AJ, Hume DE, Mace WJ, Wales WJ, 2012. Effects of wild-type, AR1 and AR37 endophyte-infected perennial ryegrass on dairy production in Victoria, Australia. *Animal Production Science* **52**: 1117-1130.

Moon CD, Craven KD, Leuchtmann A, Clement SL, Schardl CL, 2004. Prevalence of interspecific hybrids amongst asexual fungal endophytes of grasses. *Molecular Ecology* **13**: 1455-1467.

Moon CD, Tapper BA, Scott B, 1999. Identification of *Epichloe* endophytes in planta by a microsatellite-based PCR fingerprinting assay with automated analysis. *Applied and Environmental Microbiology* **65**: 1268-1279.

Morgan J, 1992. Osmotic components and properties associated with genotypic differences in osmoregulation in wheat. *Functional Plant Biology* **19**: 67-76.

Morse LJ, Faeth SH, Day TA, 2007. *Neotyphodium* interactions with a wild grass are driven mainly by endophyte haplotype. *Functional Ecology* **21**: 813-822.

Muller A, Guan C, Galweiler L, Tanzler P, Huijser P, Marchant A, Parry G, Bennett M, Wisman E, Palme K, 1998. AtPIN2 defines a locus of *Arabidopsis* for root gravitropism control. *EMBO J* **17**: 6903-6911.

Nagabhyru P, Dinkins RD, Wood CL, Bacon CW, Schardl CL, 2013. Tall fescue endophyte effects on tolerance to water-deficit stress. *BMC Plant Biology* **13**.

Nakashima K, Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K, 1997. A nuclear gene, erd1, encoding a chloroplast-targeted Clp protease regulatory subunit homolog is not only induced by water stress but also developmentally up-regulated during senescence in *Arabidopsis thaliana*. *The Plant Journal* **12**: 851-861.

Nandintsetseg B, Shinoda M, 2013. Assessment of drought frequency, duration, and severity and its

impact on pasture production in Mongolia. *Natural Hazards* **66**: 995-1008.

Nogues S, Baker NR, 2000. Effects of drought on photosynthesis in Mediterranean plants grown under enhanced UV-B radiation. *Journal of Experimental Botany* **51**: 1309-1317.

Oberhofer M, Güsewell S, Leuchtmann A, 2014. Effects of natural hybrid and non-hybrid *Epichloë* endophytes on the response of *Hordelymus europaeus* to drought stress. *New Phytologist* **201**: 242-253.

Osakabe Y, Maruyama K, Seki M, Satou M, Shinozaki K, Yamaguchi-Shinozaki K, 2005. Leucine-rich repeat receptor-like kinase1 is a key membrane-bound regulator of abscisic acid early signaling in *Arabidopsis*. *Plant Cell* **17**: 1105-1119.

Osakabe Y, Mizuno S, Tanaka H, Maruyama K, Osakabe K, Todaka D, Fujita Y, Kobayashi M, Shinozaki K, Yamaguchi-Shinozaki K, 2010. Overproduction of the membrane-bound receptor-like protein kinase 1, *RPK1*, enhances abiotic stress tolerance in *Arabidopsis*. *Journal of Biological Chemistry* **285**: 9190-9201.

Ouyang S-Q, Liu Y-F, Liu P, Lei G, He S-J, Ma B, Zhang W-K, Zhang J-S, Chen S-Y, 2010. Receptor-like kinase OsSIK1 improves drought and salt stress tolerance in rice (*Oryza sativa*) plants. *Plant Journal* **62**: 316-329.

Paiva SAR, Russell RM, 1999. Beta-carotene and other carotenoids as antioxidants. *Journal of the American College of Nutrition* **18**: 426-433.

Panaccione DG, Johnson RD, Wang JH, Young CA, Damrongkool P, Scott B, Schardl CL, 2001. Elimination of ergovaline from a grass-*Neotyphodium* endophyte symbiosis by genetic modification of the endophyte. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 12820-12825.

Parish JA, Parish JR, Best TF, Boland HT, Young CA, 2013. Effects of selected endophyte and tall fescue cultivar combinations on steer grazing performance, indicators of fescue toxicosis, feedlot performance, and carcass traits. *Journal of Animal Science* **91**: 342-355.

Popay AJ, Baltus JG, 2001. Black beetle damage to perennial ryegrass infected with AR1 endophyte. *Proceedings of the New Zealand Grassland Association* **63**: 267-271.

Popay AJ, Hume DE, Baltus JG, Latch GCM, Tapper BA, Lyons TB, Cooper BM, Pennell CG, Eerens JPJ, Marshall SL, 1999. Field performance of perennial ryegrass (*Lolium perenne*) infected with toxin-free fungal endophytes (*Neotyphodium spp*.), in: Woodfield DR, Matthew C (eds), *Ryegrass*

endophyte: an essential New Zealand symbiosis. New Zealand Grassland Association, Napier, 113-122.

Popay AJ, Thom ER, 2009. Endophyte effects on major insect pests in Waikato dairy pasture, *Proceedings of the New Zealand Grassland Association*, 121-126.

Porcel R, Aroca R, Cano C, Bago A, Ruiz-Lozano J, 2006. Identification of a gene from the arbuscular mycorrhizal fungus *Glomus intraradices* encoding for a 14-3-3 protein that is up-regulated by drought stress during the am symbiosis. *Microbial Ecology* **52**: 575-582.

Porcel R, Aroca R, Cano C, Bago A, Ruiz-Lozano JM, 2007. A gene from the arbuscular mycorrhizal fungus *Glomus intraradices* encoding a binding protein is up-regulated by drought stress in some mycorrhizal plants. *Environmental and Experimental Botany* **60**: 251-256.

Posch S, Bennett LT, 2009. Photosynthesis, photochemistry and antioxidative defence in response to two drought severities and with re-watering in *Allocasuarina luehmannii*. *Plant Biology* **11**: 83-93.

Prestidge RA, 1993. Causes and control of perennial ryegrass staggers in New Zealand. *Agriculture, Ecosystems & Environment* **44**: 283-300.

Pyngrope S, Bhoomika K, Dubey R, 2013. Oxidative stress, protein carbonylation, proteolysis and antioxidative defense system as a model for depicting water deficit tolerance in Indica rice seedlings. *Plant Growth Regulation* **69**: 149-165.

Rasmussen S, Lane GA, Mace W, Parsons AJ, Fraser K, Xue H, 2012. The use of genomics and metabolomics methods to quantify fungal endosymbionts and alkaloids in grasses. *Methods in molecular biology (Clifton, N.J.)* **860**: 213-226.

Rasmussen S, Parsons AJ, Bassett S, Christensen MJ, Hume DE, Johnson LJ, Johnson RD, Simpson WR, Stacke C, Voisey CR, Xue H, Newman JA, 2007. High nitrogen supply and carbohydrate content reduce fungal endophyte and alkaloid concentration in *Lolium perenne*. *New Phytologist* **173**: 787-797.

Reed KFM, Scrivener CJ, Rainsford KA, Walker LV, 2008. *Neotyphodium* Research and Application in Australia, *Neotyphodium in Cool-Season Grasses*. Blackwell Publishing Ltd, 43-54.

Richardson MD, Chapman GW, Hoveland CS, Bacon CW, 1992. Sugar alcohols in endophyte-infected tall fescue under drought. *Crop Science* **32**: 1060-1061.

Riedell WE, Kieckhefer RE, Petroski RJ, Powell RG, 1991. Naturally-occurring and synthetic loline alkaloid derivatives - insect feeding-behavior modification and toxicity. *Journal of Entomological*

Science **26**: 122-129.

Roberts MR, de Bruxelles GL, 2002. Plant 14-3-3 protein families: evidence for isoform-specific functions? *Biochemical Society Transactions* **30**: 373-378.

Rudgers JA, Swafford AL, 2009. Benefits of a fungal endophyte in *Elymus virginicus* decline under drought stress. *Basic and Applied Ecology* **10**: 43-51.

Sabzalian MR, Mirlohi A, 2010. *Neotyphodium* endophytes trigger salt resistance in tall and meadow fescues. *Journal of Plant Nutrition and Soil Science* **173**: 952-957.

Sakuma Y, Maruyama K, Osakabe Y, Qin F, Seki M, Shinozaki K, Yamaguchi-Shinozaki K, 2006. Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression. *Plant Cell* **18**: 1292-1309.

Salvioli A, Zouari I, Chalot M, Bonfante P, 2012. The arbuscular mycorrhizal status has an impact on the transcriptome profile and amino acid composition of tomato fruit. *BMC Plant Biology* **12**: 1-12.

Schardl CL, 2001. *Epichloe festucae* and related mutualistic symbionts of grasses. *Fungal Genetics and Biology* **33**: 69-82.

Schardl CL, Panaccione DG, Tudzynski P, 2006. Ergot alkaloids--biology and molecular biology. *The Alkaloids. Chemistry and biology* **63**: 45-86.

Schardl CL, Siegel MR, 1993. Molecular genetics of *Epichloe typhina* and *Acremonium coenophialum*. *Agriculture Ecosystems & Environment* **44**: 169-185.

Schardl CL, Young CA, Faulkner JR, Florea S, Pan J, 2012. Chemotypic diversity of *epichloae*, fungal symbionts of grasses. *Fungal Ecology* **5**: 331-344.

Schardl CL, Young CA, Hesse U, Amyotte SG, Andreeva K, Calie PJ, Fleetwood DJ, Haws DC, Moore N, Oeser B, Panaccione DG, Schweri KK, Voisey CR, Farman ML, Jaromczyk JW, Roe BA, O'Sullivan DM, Scott B, Tudzynski P, An Z, Arnaoudova EG, Bullock CT, Charlton ND, Chen L, Cox M, Dinkins RD, Florea S, Glenn AE, Gordon A, Gueldener U, Harris DR, Hollin W, Jaromczyk J, Johnson RD, Khan AK, Leistner E, Leuchtmann A, Li C, Liu J, Liu J, Liu M, Mace W, Machado C, Nagabhyru P, Pan J, Schmid J, Sugawara K, Steiner U, Takach JE, Tanaka E, Webb JS, Wilson EV, Wiseman JL, Yoshida R, Zeng Z, 2013. Plant-Symbiotic Fungi as Chemical Engineers: Multi-Genome Analysis of the *Clavicipitaceae* Reveals Dynamics of Alkaloid Loci. *PLoS Genetics* **9**: e1003323.

Sherameti I, Tripathi S, Varma A, Oelmuller R, 2008. The root-colonizing endophyte *Piriformospora indica* confers drought tolerance in *Arabidopsis* by stimulating the expression of drought stress-related genes in leaves. *Molecular Plant-Microbe Interactions* **21**: 799–807.

Sherrard ME, Maherali H, 2006. The adaptive significance of drought escape in *Avena barbata*, an annual grass. *Evolution* **60**: 2478-2489.

Shimizu M, Meguro A, Hasegawa S, Nishimura T, Kunoh H, 2006. Disease resistance induced by nonantagonistic endophytic *Streptomyces* spp. on tissue-cultured seedlings of rhododendron. *Journal of General Plant Pathology* **72**: 351-354.

Shinozaki K, Yamaguchi-Shinozaki K, 2007. Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany* **58**: 221-227.

Shinozaki K, Yamaguchi-Shinozaki K, Seki M, 2003. Regulatory network of gene expression in the drought and cold stress responses. *Current Opinion in Plant Biology* **6**: 410-417.

Shiu SH, Bleecker AB, 2001. Plant receptor-like kinase gene family: diversity, function, and signaling. *Signal Transduction Knowledge Environment* **2001**: re22.

Shukla N, Awasthi RP, Rawat L, Kumar J, 2012. Biochemical and physiological responses of rice (*Oryza sativa* L.) as influenced by *Trichoderma harzianum* under drought stress. *Plant Physiology and Biochemistry* **54**: 78-88.

Siciliano V, Genre A, Balestrini R, Cappellazzo G, deWit PJGM, Bonfante P, 2007. Transcriptome analysis of arbuscular mycorrhizal roots during development of the prepenetration apparatus. *Plant Physiology* **144**: 1455-1466.

Siegel MR, Latch GCM, Bush LP, Fannin FF, Rowan DD, Tapper BA, Bacon CW, Johnson MC, 1990. Fungal endophyte-infected grasses: Alkaloid accumulation and aphid response. *Journal of Chemical Ecology* **16**: 3301-3315.

Simova-Stoilova L, Vaseva I, Grigorova B, Demirevska K, Feller U, 2010a. Proteolytic activity and cysteine protease expression in wheat leaves under severe soil drought and recovery. *Plant Physiology and Biochemistry* **48**: 200-206.

Simova-Stoilova L, Vaseva I, Grigorova B, Demirevska K, Feller U, 2010b. Proteolytic activity and cysteine protease expression in wheat leaves under severe soil drought and recovery. *Plant Physiology and Biochemistry* **48**: 200-206.

Smart CM, 1994. Gene expression during leaf senescence. *New Phytologist* **126**: 419-448.

Smith SE, Smith FA, Jakobsen I, 2003. Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. *Plant Physiology* **133**: 16-20.

Spiering MJ, Lane GA, Christensen MJ, Schmid J, 2005. Distribution of the fungal endophyte *Neotyphodium lolii* is not a major determinant of the distribution of fungal alkaloids in *Lolium perenne* plants. *Phytochemistry* **66**: 195-202.

Streeter JG, Lohnes DG, Fioritto RJ, 2001. Patterns of pinitol accumulation in soybean plants and relationships to drought tolerance. *Plant Cell and Environment* **24**: 429-438.

Sun CA, Johnson J, Cai DG, Sherameti I, Oelmuller R, Lou BG, 2010. *Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. *Journal of Plant Physiology* **167**: 1009-1017.

Sushma D, Tarafdar JC, 2012. Arbuscular mycorrhizal fungi encourage drought tolerance of *Chlorophytum borivilianum* by enhancing antioxidant enzyme system. *Applied Biological Research* **14**: 60-70.

Tainer JA, Getzoff ED, Richardson JS, Richardson DC, 1983. Structure and mechanism of copper, zinc superoxide dismutase. *Nature* **306**: 284-287.

Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K, 2002. Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant Journal* **29**: 417-426.

Tan YY, Spiering MJ, Scott V, Lane GA, Christensen MJ, 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.

Tanaka A, Tapper BA, Popay A, Parker EJ, Scott B, 2005. A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. *Molecular Microbiology* **57**: 1036-1050.

Tanaka H, Osakabe Y, Katsura S, Mizuno S, Maruyama K, Kusakabe K, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K, 2012. Abiotic stress-inducible receptor-like kinases negatively control ABA signaling in *Arabidopsis*. *Plant Journal* **70**: 599-613.

Tanaka Y, Sasaki N, Ohmiya A, 2008. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *The Plant Journal* **54**: 733-749.

Thom ER, Popay AJ, Hume DE, Fletcher LR, 2012. Evaluating the performance of endophytes in farm systems to improve farmer outcomes - a review. *Crop and Pasture Science* **63**: 927-943.

Thom ER, Popay AJ, Waugh CD, Minneé EMK, 2013. Impact of novel endophytes in perennial ryegrass on herbage production and insect pests from pastures under dairy cow grazing in northern New Zealand. *Crop and Pasture Science*: In press: DOI: 10.1111/gfs.12040.

Tian P, Nan ZB, Li CJ, Spangenberg G, 2008. Effect of the endophyte *Neotyphodium lolii* on susceptibility and host physiological response of perennial ryegrass to fungal pathogens. *European Journal of Plant Pathology* **122**: 593-602.

Tian Y-h, Lei Y-b, Zheng Y-l, Cai Z-q, 2013. Synergistic effect of colonization with arbuscular mycorrhizal fungi improves growth and drought tolerance of *Plukenetia volubilis* seedlings. *Acta Physiologiae Plantarum* **35**: 687-696.

Timmusk S, Wagner EGH, 1999. The plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: A possible connection between biotic and abiotic stress responses. *Molecular Plant-Microbe Interactions* **12**: 951-959.

Tran L-SP, Urao T, Qin F, Maruyama K, Kakimoto T, Shinozaki K, Yamaguchi-Shinozaki K, 2007. Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 20623-20628.

Tromas A, Parizot B, Diagne N, Champion A, Hocher V, Cissoko M, Crabos A, Prodjinoto H, Lahouze B, Bogusz D, Laplaze L, Svistoonoff S, 2012. Heart of endosymbioses: transcriptomics reveals a conserved genetic program among arbuscular mycorrhizal, actinorhizal and legume-rhizobial symbioses. *Plos One* **7**: e44742.

Trushina N, Levin M, Mukherjee PK, Horwitz BA, 2013. PacC and pH-dependent transcriptome of the mycotrophic fungus *Trichoderma virens*. *BMC Genomics* **14**: 138.

Turner NC, Abbo S, Berger JD, Chaturvedi SK, French RJ, Ludwig C, Mannur DM, Singh SJ, Yadava HS, 2007. Osmotic adjustment in chickpea (*Cicer arietinum* L.) results in no yield benefit under terminal drought. *Journal of Experimental Botany* **58**: 187-194.

Turrens JF, 2003. Mitochondrial formation of reactive oxygen species. *Journal of Physiology-London* **552**: 335-344.

Udvardi M, Poole PS, 2013. Transport and metabolism in legume-rhizobia symbioses. *Annual Review*

of Plant Biology **64**: 781-805.

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K, 2000. *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 11632-11637.

Usadel B, Poree F, Nagel A, Lohse M, Czedik-Eysenberg A, Stitt M, 2009. A guide to using MapMan to visualize and compare Omics data in plants: a case study in the crop species, Maize. *Plant Cell and Environment* **32**: 1211-1229.

Valliyodan B, Nguyen HT, 2006. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. *Current Opinion in Plant Biology* **9**: 189-195.

Verna J, Lodder A, Lee K, Vagts A, Ballester R, 1997. A family of genes required for maintenance of cell wall integrity and for the stress response in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 13804-13809.

Vierstra R, 1996. Proteolysis in plants: mechanisms and functions. *Plant Molecular Biology* **32**: 275-302.

Vivas A, Biro B, Ruiz-Lozano JM, Barea JM, Azcon R, 2006. Two bacterial strains isolated from a Zn-polluted soil enhance plant growth and mycorrhizal efficiency under Zn-toxicity. *Chemosphere* **62**: 1523-1533.

Western JH, Cavett JJ, 1959. The choke disease of cocksfoot (*Dactylis glomerata*) caused by *Epichloe typhina* (Fr.) Tul. *Transactions of the British Mycological Society* **42**: 298-IN296.

Wilkinson HH, Siegel MR, Blankenship JD, Mallory AC, Bush LP, Schardl CL, 2000. Contribution of fungal loline alkaloids to protection from aphids in a grass-endophyte mutualism. *Molecular Plant-Microbe Interactions* **13**: 1027-1033.

Xiong LM, Schumaker KS, Zhu JK, 2002. Cell signaling during cold, drought, and salt stress. *Plant Cell* **14**: S165-S183.

Yang J, Kloepper JW, Ryu C-M, 2009. Rhizosphere bacteria help plants tolerate abiotic stress. *Trends in Plant Science* **14**: 1-4.

Young CA, Bryant MK, Christensen MJ, Tapper BA, Bryan GT, Scott B, 2005. Molecular cloning and genetic analysis of a symbiosis-expressed gene cluster for lolitrem biosynthesis from a mutualistic endophyte of perennial ryegrass. *Molecular Genetics and Genomics* **274**: 13-29.

Young CA, Felitti S, Shields K, Spangenberg G, Johnson RD, Bryan GT, Saikia S, Scott B, 2006. A complex gene cluster for indole-diterpene biosynthesis in the grass endophyte *Neotyphodium lolii*. *Fungal Genetics and Biology* **43**: 679-693.

Young CA, Tapper BA, May K, Moon CD, Schardl CL, Scott B, 2009. Indole-diterpene biosynthetic capability of *Epichloe* endophytes as predicted by *ltm* gene analysis. *Applied and Environmental Microbiology* **75**: 2200-2211.

Zhang JY, Broeckling CD, Blancaflor EB, Sledge MK, Sumner LW, Wang ZY, 2005. Overexpression of WXP1, a putative Medicago truncatula AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*). *The Plant Journal* **42**: 689-707.

Zhang N, Zhang S, Borchert S, Richardson K, Schmid J, 2011. High levels of a fungal superoxide dismutase and increased concentration of a PR-10 plant protein in associations between the endophytic fungus *Neotyphodium lolii* and ryegrass. *Molecular Plant-Microbe Interactions* **24**: 984-992.

Zhang YP, Nan ZB, 2007. Growth and anti-oxidative systems changes in *Elymus dahuricus* is affected by *Neotyphodium* endophyte under contrasting water availability. *Journal of Agronomy and Crop Science* **193**: 377-386.

Zou J, Liu C, Liu A, Zou D, Chen X, 2012. Overexpression of OsHsp17.0 and OsHsp23.7 enhances drought and salt tolerance in rice. *Journal of Plant Physiology* **169**: 628-635.