Characterisation of *Eucalyptus grandis* SWEET and SWI/SNF proteins during symbiosis with *Pisolithus microcarpus*

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution. All experimental work reported in this thesis was performed by the author, unless stated otherwise.

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List of abbreviations

Δd13C	Change in amount of ^{13}C
^{13}C	Carbon isotope 13
^{13}CO$_2$	Carbon (isotope 13) dioxide
ABA	Abscisic acid
AM	Arbuscular mycorrhizal
Avr	Avirulence
AzA	Azelaic acid
BiFC	Bimolecular fluorescence complementation
BR	Brassinosteroids
BzSA	Benzoylsalicylic acid
cDNA	Copy deoxyribonucleic acid
CK	Cytokinins
CO$_2$	Carbon dioxide
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DA	Dehydroabietinal
DNA	Deoxyribonucleic acid
dsiRNA	Double stranded interfering ribonucleic acid
ECM	Ectomycorrhizal
ESL	ERD six-like transporters
ET	Ethylene
ETI	Effector-triggered immunity
Fru	Fructose
GA	Gibberellin

GFP	Green fluorescent protein
Glu	Glucose
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
IAA	Indole-3-acetic acid
INT	Inositol transporter
JA	Jasmonic acid
LiAC	Lithium acetate
MiSSP	Mycorrhizal induced small secreted protein
MMN	Modified Mylin Norkin
MS medium	Murashige and Skoog medium
MST	Monosaccharide transporters
N	Nitrogen
NLP	Nep1-like proteins
P	Phosphorus
PAMPs/MAMPs	Pathogen/Microbe associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pGlcT	Plastidic glucose transporter
pH	Salinity
PI	Propidium Iodide
PiP	Pipecolic acid
PMT	Polyol monosaccharide transporter
PR	Pathogenesis-related

PRR	Pattern recognition receptors
PTI/MTI	PAMP/MAMP-triggered immunity
QPCR	Quantitative polymerase chain reaction
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
RT-QPCR	Real time-quantitative polymerase chain reaction
SA	Salicyclic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulphate
SLs	Strigolactones
STPs	Sugar transporter proteins
Suc	Sucrose
SUT	Sucrose transporters
SWEET	Sugars Will Eventually be Exported Transported
SWI/SNF	Switch/Sucrose Non-Fermenting
TAL	Transcription activator-like
TMT	Tonoplast membrane transporter
VGT	Vacuolar glucose transporter
WT	Wild-type
YFP	Yellow fluorescent protein
YIIH	Yeast two-hybrid
YPM	Yeast extract peptone with maltose (growth medium)
WGA-FITC	Wheat germ agglutinin-Lectin

Abstract

Eucalyptus grandis, an economically important bioenergy tree, is constantly bombarded by different fungal lifestyles seeking to acquire photosynthetically fixed sugar. How the plant immune system filters beneficial fungi from pathogenic is poorly understood. This thesis investigates two aspects of plant immunity: shuttling of sugar and interference by fungal effectors. Plant sugars are known to play a dual role in plant-microbe interactions: they can either feed the microbe with growth-limiting carbon or they can act as fuel for plant secondary metabolism and, subsequently, plant defence. In my first study I consider how hexose SWEET transporters respond at the transcriptomic level in *E. grandis* roots during challenge by different microbes covering the fungal lifestyles from pathogenic through mutualistic. Further, I characterise four *E. grandis* SWEET proteins that share sequence homology to previously identified SWEET proteins and determine their cellular localization, their sugar transport capabilities and their role in shuttling carbon during plant-microbe interactions. In the second part of my thesis, I investigate how a mutualistic fungus attempts to manipulate plant defences through the use of effector like proteins. Specifically, I characterise the role of *Pisolithus albus* MiSSP9.7, a highly induced secreted fungal protein of unknown function. I demonstrate that it interacts with a member of the SWI/SNF protein complex previously identified as being responsible for the regulation of plant hormone signalling pathways used in immune responses against microbes. Increased expression of *SWI3D* in *E. grandis* roots is tied to the colonisation process and may regulate a key aspect of plant immunity towards mutualistic fungi. Taken together, my work provides a better

understanding of the controls used by plants to modulate plant-microbe

interactions and the counter-measures utilized by fungi to overcome host

immunity.

Chapter 1 Introduction

1.1 Mutualistic and Pathogenic Microbe Interactions

In their natural environment, plants are constantly confronted with many different types of soil-borne microbes. Microbes is a broad term that describes all microscopic organisms, such as bacteria, fungi, nematodes, oomycetes, archaea, protists, microscopic animals, and microscopic plants (Genetic Science Learning Center 2017). Plant interactions with these microbes can be classified in three main categories: parasitic, mutualistic or commensal. These classifications are an oversimplification as these plant-microbe associations are dynamic and can range from mutualistic to parasitic depending on the abiotic factors affecting the ecosystem (Francis and Read 1995). Parasitic plant-microbe interactions involve microbes colonising host plants and hindering plant growth by feeding on plant tissues and or sugar storages. Thus, one organism benefits at the others expense. In contrast, mutualistic pant-microbe interactions involve microbes providing host plants with scarce nutrients (such as nitrogen (N) and phosphorus (P)) in exchange for (up to 30% of) the plant's photosynthetically-derived sugars (Pellegrin *et al.* 2015). Thus, both the plant and microbe benefit from this association. Commensal plant-microbe interactions describe microbes who do not harm or benefit plants, instead commensal microbes only decompose dead plant matter (for example (e.g.) decomposing plant litter fall).

1.1.1 Ectomycorrhizal symbiosis

One major type of mutualistic plant-microbe interaction found in forest ecosystems is the relationship between soilborne ectomycorrhizal (ECM)

fungi and trees. This symbiosis involves the transfer of growth limiting soil nutrients from the fungus to the host plant and photosynthetically derived sugars from the host to the fungus. ECM fungi play a further role in plant survival as they support host adaptation to changing environmental conditions such as climate extremes, drought and soil pollution (Redman et al. 2009). ECM fungi are commonly used in nurseries to inoculate trees used for re-forestation because the presence of ECM fungi increases the establishment success of trees by enhancing tree growth (Brundrett *et al.* 2005).

To establish mutualistic associations with plants and begin nutrient exchange, ECM fungi must first form two essential ectomycorrhizal structures: the Hartig net (formed within the root) and fungal sheath/mantle (formed outside the root by surrounding the entire root tip with extrametrical hyphae). Upon initial contact with host roots, ECM fungi attach to the root surface and hyphae surrounds the outside of the root, forming the fungal mantle (Fig. 1). During this contact, ECM fungi secrete effectors (i.e. proteins and signaling molecules), metabolites and phytohormones (e.g. auxin) that cause physiological changes within host roots cells to allow fungal hyphae to penetrate into the root apoplast (i.e. spaces in between root cells) (Fig. 1). Within the apoplast, the extensive network of fungal hyphae form the Hartig net completing establishment of the mutualism. The Hartig net is the interface in which nutrient exchange between the fungus and plant occurs and the colonised root is called a mycorrhizal root tip (Smith and Read 1997).

Although ECM fungi are free-living they are inefficient decomposers when compared to saprotrophs, thus they form mutualisms with the roots of trees to gain access to sugars as a carbon source, improving their survivability (Smith and Read 2008). ECM fungi are free-living because they originate from saprotrophic ancestors (Hibbett *et al.* 2000), but have evolved multiple times to be mutualistic with many plants (Hibbet and Matheny 2009). Phylogenically, ECM fungi belong to the phyla Ascomycota and Basidiomycota together with saprotrophic fungi (Plett and Martin 2011, 2015). Ascomycota is a division of fungi that whose spores are contained in sac-like structures called an ascus (Plett and Martin 2011). Basidiomycota is a division of fungi who produce spores using a specialised spore producing organ (called basidium) (Plett and Martin 2011).
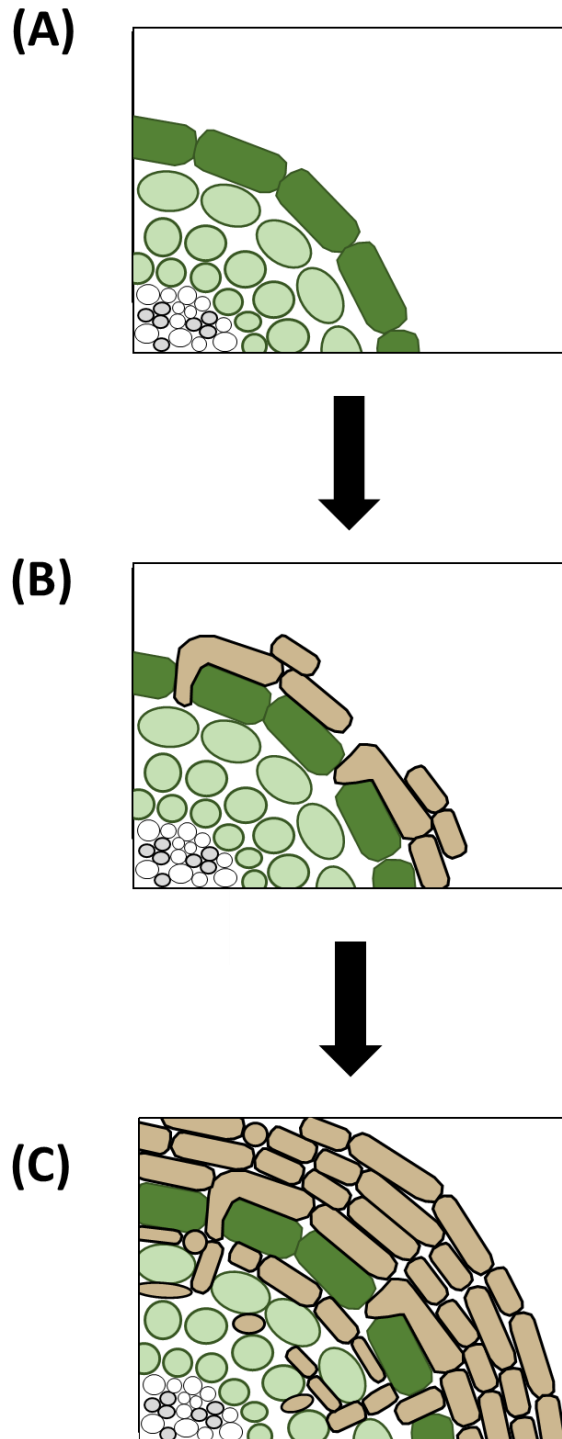
Figure 1. Representational diagram of a transverse cross-section of a root undergoing colonisation by an ECM fungus (adapted from Plett and Martin 2011). **(A)** Representation of a transverse cross-section of a plant lateral root before ECM fungal colonisation. **(B)** Initial contact between host plant root (green cells) and ECM fungal hyphae (brown cells). The fungus attaches to root surface and secretes effectors that cause physiological changes within root cells, which then allow fungal hyphae to penetrate into the root apoplast. **(C)** Representation of a transverse cross-section of a mature ectomycorrhizal root tip. At this stage of colonisation fungal hyphae has covered the entire root surface forming a thick fungal mantle. Other hyphae have penetrated into the apoplastic space, forming the Hartig net where nutrient exchange occurs.

Saprotrophic fungi produce enzymes (e.g. cellulases and hemicellulases) that deconstruct and hydrolyse plant cell wall materials (Plett and Martin 2011). Biotrophic, hemi-biotrophic and nectrophic fungal pathogens (e.g. *Armillaria* and *Phytophthora*) produce toxins, harmful effectors and carbohydrate-cleaving enzymes that digest or rot plant tissues (Lo Presti *et al.* 2015). These features make it hard, if not impossible, for saprotrophic and pathogenic fungi to form a mutualistic relationship with plants because these enzymes would damage the host and elicit plant defence responses (Plett and Martin 2011). However, over the course of their evolution, ECM fungi have lost a large majority of genes encoding plant cell wall degrading enzymes (Martin *et al.* 2008). While the genomes of ECM fungi (e.g. *Laccaria bicolor*) still encode a small group of plant cell wall degrading enzymes, these genes are only expressed when the fungus acts as a weak decomposer in soil litter and is not in symbiosis with a plant (Martin *et al.* 2008; Plett and Martin 2011). The loss of plant cell wall degrading enzymes makes ECM fungi more dependent on utilising photosynthetically derived sugars as carbon source received from host plants, but in turn allows ECM fungi to colonise roots without threatening the integrity of the plant root cells (Plett and Martin 2011).

1.1.2 Pathogenic microbes

Pathogenic microbes syphon plant nutrients and or directly feed on plant tissues (or plant sugar storages) for their own growth and development. Pathogenic fungi are subdivided into three groups based on the way they parasitise plants, these are: necrotrophs, biotrophs and hemi-biotrophs.

Nectrophic pathogens (such as *Phytophthora cinnamomi* (Eshraghi *et al.* 2011) and *Armillaria luteobubalina* (Coetzee *et al.* 2001)) actively kill host plant cells and feed on the contents of dead or dying tissues. In general, these pathogens infect plant tissues and kill host cells by secreting toxic metabolites, effectors and proteins, and plant cell wall degrading enzymes (Lo Presti *et al.* 2015). *A. luteobubalina* is the most prevalent and widespread *Armillaria* species in Australia (Kile and Watling 1981, 1983; Shearer *et al.* 1997). *A. luteobubalina* causes root rot and wood decay in many native and introduced plant species (Royal Botanic Garden 2017a). *P. cinnamomi* is a widespread plant pathogen that causes death of roots in many different plant species (Royal Botanic Garden 2017b).

In contrast to nectrophic pathogens, biotrophic pathogens establish a long-term feeding relationship with the livings cells of their hosts, instead of killing host cells upon infection (Deacon 1997). In general, these fungi grow in the apoplastic space of host tissues and produce nutrient-absorbing structures called haustoria. By absorbing significant amounts of host nutrients using haustoria, the pathogen creates a nutrient sink at the site of infection, causing the host to be disadvantaged but not killed (Deacon 1997). By keeping the host plant alive, biotrophic pathogens gain access to a long-term food source. Many biotrophic pathogens (such as the rust fungi (*Uromyces viciae-fabae*) and powdery mildew (*Blumeria graminis*)) use turgor pressure and plant cell wall degrading enzymes to breach the cell wall without

affecting host viability, after which they develop haustoria (O'Connell and

Panstruga 2006; Lo Presti *et al.* 2015).

Hemi-biotrophs use both biotrophic and necrotrophic methods of acquiring

host plant nutrients depending on the stages of their life cycle (Lee and Rose

2010). During initial infection, hemi-biotrophs establish a biotrophic

relationship with the host, but as they develop, they then later kill host cells

and feed on the contents of dead or dying tissues. Hemibiotrophic fungi such

as *Phytophthora infestans* and *Magnaporthe oryzae* initially develop bulged

biotrophic invasive hyphae that later change into thin necrotrophic hyphae

(O'Connell and Panstruga 2006).

1.1.3 Host plant

Eucalyptus grandis is an important forest tree that interacts with a variety of

microbes, including both mutualistic ECM and parasitic fungi. *E. grandis* is

the most widely planted hardwood forest tree because of its many industrial

and environmental uses (Myburg *et al.* 2014). The tree's easy maintenance,

high adaptability and rapid growth has led to the adoption of Eucalypt

plantations in over 100 countries worldwide (Myburg *et al.* 2014). Planted

Eucalypts provide key renewable sources for the production of solid timber,

pulp, paper, bioenergy and biomaterials, while reducing human impacts on

native forests (Bauhus *et al.* 2010). Furthermore, Eucalypts provide many

environmental services including sequestering atmospheric CO_2 to reduce

global warming, providing habitats for native Australian animals and soil,

water and forest conservation. Eucalypts also have a large diversity and high

concentration of essential oils that are key ingredients in commercial products, as well as having medicinal and ecological functions (Myburg *et al.* 2014).

1.2 Plant defences against microbes

To survive, plants must effectively defend against pathogenic microbes. Unlike animals, plants do not have mobile immune cells, a somatic adaptive immune system, or circulatory system, instead plants rely on multifaceted innate immune defences (Jones and Dangl 2006). The first line of defence against most microbes consists of physical barriers, such as the waxy cuticle on the surface of leaves, the cell wall and plasma membrane (Fu and Dong 2013). Furthermore, some plants produce chemicals, such as glycosylated triterpenoids, saponins and reactive oxygen species (ROS), that can disrupt the plasma membranes of most fungal pathogens (Bednarek and Osbourn 2009; Hemetsberger *et al.* 2012). Plant cells express pattern recognition receptors (PRRs) on their surface that recognise invariant molecular patterns found on invading microbes, called pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) (Jones and Dangl 2006; Fu and Dong 2013). PAMPs/MAMPs are essential components found on microbes including, short peptides, peptidoglycans, chitin, bacterial flagellin and lipopolysaccharides. For example, the receptor kinase FLS2 acts as a PRR by detecting bacterial flagellin (Yoon et al. 2012). When PAMPs/MAMPs are recognised by PRRs, it leads to PAMP/MAMP-triggered immunity (PTI/MTI) (Boller & Felix, 2009).

In addition, initial pathogen attacks induce the production and transfer of signalling molecules (such as salicylic acid (SA), glycerol-3-phosphate (G3P), diterpenoid dehydroabietinal (DA), benzoylsalicylic acid (BzSA), pipecolic acid (Pip) and azelaic acid (AzA)) all throughout the plant (Anand *et al.* 2008; Chanda *et al.* 2011; Chaturvedi *et al.* 2012; Kamatham *et al.* 2016; Reimer-Michalski and Conrath 2016; Jung *et al.* 2009). These molecules stimulate the expression of antimicrobial genes resulting in broad-spectrum resistance against future infections in distal, uninfected plant tissues (Conrath 2006; Durrant and Dong 2004; Fu and Dong 2013). This phenomenon, called systemic acquired resistance (SAR), is conserved throughout many plant species. SAR results in extended periods of resistance (ranging from several weeks to months) against many different types of pathogens (Gao *et al.* 2015; Kuc 1987). Further, SAR establishes transgenerational immune memory within plants (a process referred to as priming) (Mauch-Mani and Mauch 2005; Luna *et al.* 2012; Rasmann *et al.* 2012; Slaughter *et al.* 2012). This immune memory enables stronger and faster defence responses against future pathogen attacks. Although some plant immune responses are associated with cell death at the site of infection, SAR promotes cell survival in uninfected plant tissues. However, immune responses and systemic synthesis of SA are known to trigger SAR. SAR can also be induced by invading fungi, bacteria, fungi, oomycetes and viruses (Conrath 2006; Durrant and Dong 2004; Fu and Dong 2013; Ryals *et al.* 1996).

Plants produce a wide range of hormones, including salicylic acid (SA), jasmonic acid (JA), ethylene (ET), auxin, abscisic acid (ABA), gibberellin

(GA), brassinosteroids (BR), cytokinins (CK), and strigolactones (SLs) (Bari and Jones 2009; Gomez-Roldan et al. 2008; Umehara et al. 2008). These hormones play essential roles in growth, responses to biotic and abiotic stresses and in immune responses against pathogens (Robert-Seilaniantz et al. 2007; Adie et al. 2007). SA is involved in the activation of defence responses against biotrophic and hemi-biotrophic pathogens, and is a key contributor to SAR (Grant and Lamb 2006). SA levels increase in infected plant tissues and high levels activate PR genes, enhancing resistance to a wide spectrum of pathogens (Denance *et al.* 2013). JA and ET are generally involved in defence against necrotrophic pathogens and herbivorous insects (Bari and Jones 2009). Auxin and ABA contribute to plant play important roles as signaling molecules in plant defence responses (Zhang *et al.* 2007; Mauch-Mani and Mauch 2005; Navarro *et al* 2008;). GA and BR enhances resistance to biotrophic pathogens (Bari and Jones 2009). Cytokinins act as signaling molecules regulating plant defence responses against some pathogens (Bari and Jones 2009). SLs are involved in establishing resistance to specific bacterial and fungal pathogens (Marzec 2016).

1.3 Microbe Responses: Effectors

To overcome plant defences and establish either infection or mutualistic associations, microbes have evolved various mechanisms. One mechanism utilised by both pathogenic and mutualistic fungi is the use of small secreted molecules called effectors. Effectors suppress the host plant's immune responses and modulates host cell physiology (Giraldo and Valent 2013; Plett *et al.* 2011, 2014a, b). Thus, pathogenic fungi secrete effectors to

establish infection, whereas mutualistic fungi secrete effectors to form mutualisms and mycorrhizal structures (e.g. Hartig net of ectomycorrhizal (ECM) fungi and arbuscules of arbuscular mycorrhizal (AM) fungi). Effectors secreted by fungi are classified as either apoplastic or cytoplasmic effectors (Kamoun 2006). Apoplastic effectors target surface receptors within the apoplast and cytoplasmic effectors directly enter inside the plant cell (Dong *et al.* 2011, Djamei *et al.* 2011; Park *et al. 2012*).

Effectors can be used to avoid or suppress PTI/MTI and successfully establish infection. For instance, *Pseudomonas syringae* establishes infection by secreting an effector called AvrPtoB that promotes the degradation of FLS2 in Arabidopsis (Göhre et al. 2008). Effectors that supress PTI/MTI are commonly used by pathogens to infect plants, and thus have been termed avirulence (avr) factors. Each respective gene that encodes avr proteins is called an *avr* genes. However, it has recently been discovered that mutualistic microbes also produce effectors to suppress host immunity and form mutualistic associations (Klopphoiz *et al.* 2011; Plett *et al.* 2011; 2014a, b). Thus, the term effectors is not specific to pathogens, but denotes a broad range of secreted molecules that suppress plant immune responses to allow either pathogens to infect plants, or allow beneficial microbes to colonise plants and form mutualistic associations.

However, plants have evolved to recognise effectors and respond using intracellular immune receptors, such as resistance proteins (R proteins), that directly detect effectors, or indirectly detect their activity (Fu and Dong 2013).

Detected effectors then lead to effector triggered immunity (ETI) (Jones and Dangl 2006). For example, tomato plants (*Lycopersicon esculentum* 76R lines) produce an R protein called Prf, that detects the AvrPtoB effector, thus triggering ETI (Salmeron *et al.* 1996). ETI prevents further pathogen growth and spread, and normally causes apoptosis (programmed cell death) at the site of infection, a phenomenon known as the hypersensitive response (HR) (Caplan *et al.* 2008; Holliday *et al.* 1981).

Pathogenic fungi have evolved different lifestyles and each promote virulence via effectors in different ways. Biotrophic and hemibiotrophic fungal pathogens feed on living host cells, and secrete many effectors to suppress immune responses. The fungal pathogen *U. maydis* secretes the Pep1 (protein essential for penetration 1) effector that accumulates in the host's apoplast (Doehlemann *et al.* 2009). Pep1 binds and inhibits the activity of the plant peroxidase protein 12 (POX12) (Hemetsberger *et al.* 2012). POX12 activity is essential for producing ROS (such as H_2O_2) that are key components of PTI (Jermy 2012). This suppression of PTI components allows *U. maydis* to grow and feed on host cells within the apoplast. Further, *U. maydis* also secretes the enzyme chorismate mutase (Cmu1) during infection to reduce the levels of chorismate within host cells. Chorismate serves as a precursor for the production of SA, thus virulence is enhanced.

Unlike biotrophs and hemibiotrophs, nectrophic fungi feed on dead plant tissues and secrete effectors to induce host plant death. These include polyketide toxins, secondary metabolites, non-ribosomal peptide toxins and

necrosis-inducing proteins (Lo Presti *et al.* 2015; Stergiopoulos *et al.* 2013; Qutob *et al.* 2006). For example, the pathogen *Phytophthora* produces Nep1-like proteins (NLPs) that directly cause plant cell death in many NLP sensitive dicotyledonous plants (Feng *et al.* 2014; Glazebrook 2005; Bailey *et al.* 2005). Also the wheat pathogens *P. tritici-repentis* and *S. nodorum* produce ToxA effectors that targets host chloroplasts and binds to ToxABP1 (Lo Presti *et al.* 2015). ToxABP1 is a protein involves in thylakoid formation and thus ToxA-ToxABP1 binding hinders photosynthesis resulting in cell death (Lo Presti *et al.* 2015; Manning *et al.* 2007).

Although effectors are commonly used by pathogens to induce virulence, beneficial microbes use their own unique effectors to form mutualistic associations with plants. For instance, the SP7 effector secreted by the AM fungus *Glomus intraradices*, binds with the transcription factor ERF19 in *Medicago truncatula* (Kloppholz *et al.* 2011). ERF19 regulates the expression of several defence genes in *M. truncatula* (Kloppholz *et al.* 2011). When constitutively expressed in roots, SP7 results in increased mycorrhization while decreasing the levels of defence responses within the host plant (Kloppholz *et al.* 2011). Klopphoiz (*et al.* 2011) further showed that overexpressing *ERF19* within *M. truncatula* significantly impaired mycorrhizal colonisation, whereas repressing ERF*19* accelerated mycorrhizal colonisation (Kloppholz *et al.* 2011). These findings indicate that the SP7 effector modulates the activity of the ERF19 transcription factor to suppress PTI and allow formation of mutualistic associations.

Like SP7, the MiSSP7 effector of *Laccaria bicolor* is essential for the establishment of mycorrhizal root tips with *Populus trichocarpa* (Plett *et al.* 2011). MiSSP7 is secreted upon receiving diffusible signals from *P. trichocarpa* roots (Plett *et al.* 2011). Repression of MiSSP7 in *L. bicolor* mutants (via RNAi knockdown) were unable to form mycorrhizal structures and enter into symbiosis with host plant roots (Plett *et al.* 2011). Plett *et al.* (2014a, b) later discovered that MiSSP7 enters the plant cell nucleus and interacts with PtJAZ6, a negative regulator of JA–induced gene regulation (Plett *et al.* 2014a). MiSSP7 reduces JA–induced degradation of PtJAZ6, resulting in the repression of JA–induced genes (Plett *et al.* 2014a). Most these repressed JA-induced genes have functions relating to cell wall modification (Plett *et al.* 2014a). Thus repression of these genes enables hyphal penetration into the root and formation of the Hartig net (Plett *et al.* 2014a, b). Interestingly, ECM fungi secrete effectors to repress the expression of JA-induced genes in their host, in contrast to AM fungi and biotrophic pathogens that induce jasmonic acid responses during host colonization (Doehlemann *et al.* 2008, Lopez-Raez *et al.* 2010). Thus, ECM fungi are thought to have evolved unique colonisation strategies (Lo Presti *et al.* 2015).

In addition, to promote virulence pathogenic microbes induce the expression of sugar transporter proteins (STPs) in host plants. The rice pathogen *Xanthomonas oryzae* secretes the effector PthXo1, a transcriptional activator-like (TAL) protein that binds directly to the *OsSWEET11* promoter to increase its expression (Yang *et al.* 2006). Reducing the levels of

OsSWEET11 via RNA interference (or when mutations are present in the *OsSWEET11* promoter) slows the growth of the pathogen (Yang *et al.* 2006). Further, studies in *Arabidopsis* have shown that bacterial and fungal pathogens (e.g. *Golovinomyces cichoraceacam* and *Botrytis cinerea*) induce the expression of host cell *SWEET* genes to successfully obtain sugars (Chen et al. 2010).

1.4 Sugar Transport in Plants

1.4.1 Sugar transporters

In terrestrial ecosystems, plants and other photosynthetic organisms fix atmospheric CO_2 via photosynthesis to produce sugars for energy, as well as the organic compounds of which they are composed of (Raven *et al.* 2011). The coordination of these photosynthetically produced sugars is essential for plant development, adapting to environmental stresses and cell to cell communication (Doidy *et al.* 2012). Not only do sugars provide the energy to drive cellular machinery, they also serve as key signalling molecules that can travel all throughout the plant (Rolland *et al.* 2006). In plants, transport of sugars from photosynthetic source leaves to sink organs (or sink organisms, e.g. associating mycorrhizal fungi) comprises several different steps depending on plant species and organ type (Doidy *et al.* 2012). Sugars produced in source tissues (e.g. mesophyll) are transported throughout plants via phloem (vascular tube-like tissues that run throughout plants) in conjunction with sugar transporter proteins. Currently, there are three known major families of sugar transporter proteins: sucrose transporters (SUTs),

monosaccharide transporters (MSTs) and SWEETs (Sugars Will Eventually be Exported Transported) (Fig. 2) (Doidy *et al.* 2012).
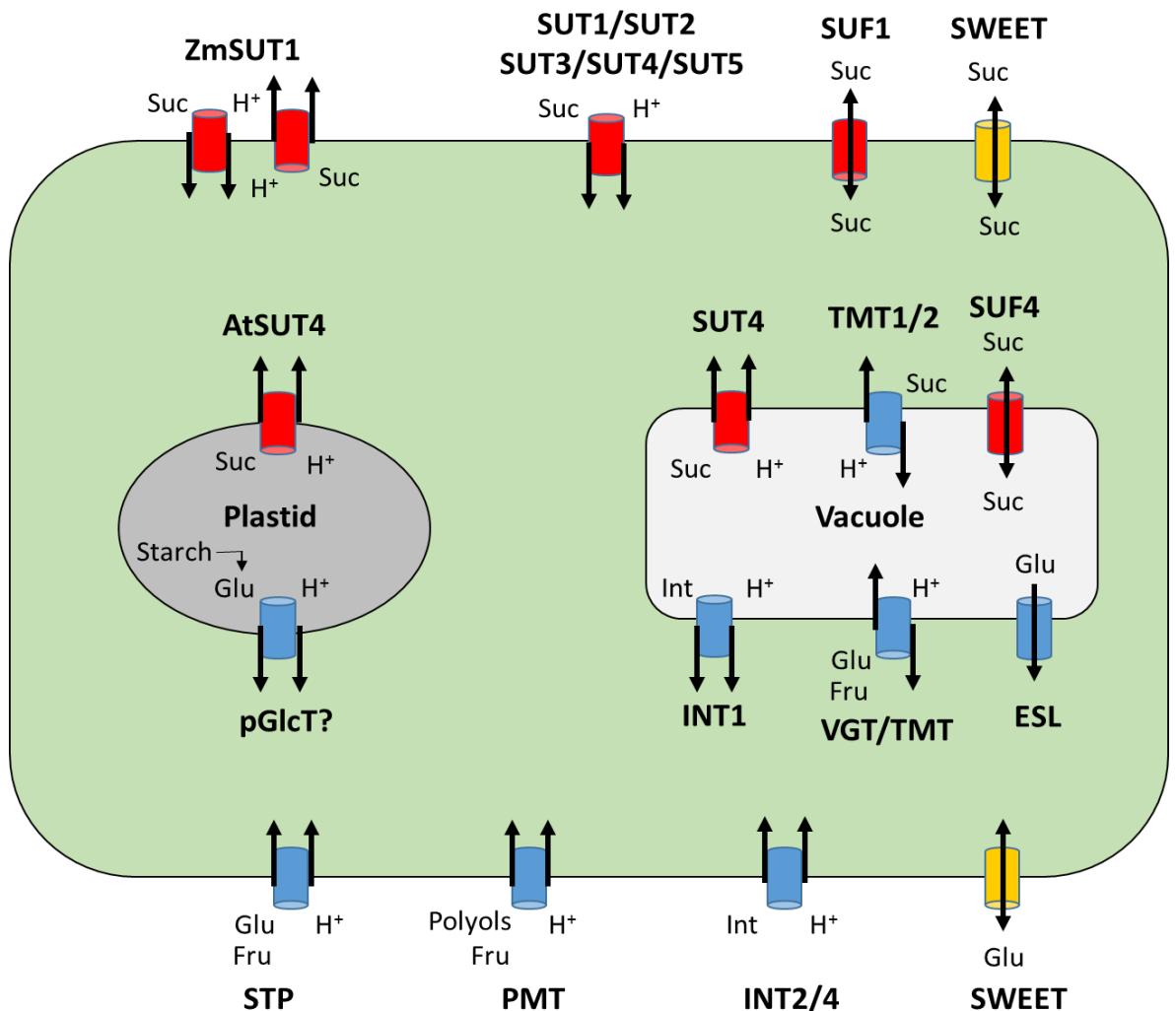
Figure 2. Representational diagram of the intracellular distribution of plant sugar transporter proteins (adapted from Doidy *et al.* 2012). Three families of transporters (sucrose in the upper half and monosaccharides in the lower half) within the plant cell: SUTs (in red), MSTs (in blue) and SWEETs (in yellow). Most transporters that localise to the plasma membrane have been characterised as H^+/sugar importers, although ZmSUT1 was shown to mediate active efflux of sucrose (Carpaneto *et al.* 2005, 2010). In contrast, SWEETs and SUFs function as energy-independent uniporters that mediate sugar influx and or efflux (Chen *et al.* 2010, Zhou *et al.* 2007). At the vacuolar membrane, the MST subfamilies, VGT (vacuolar glucose transporter) and TMT (tonoplast membrane transporter) act as sugar/H^+ antiporters loading sugars into the vacuole (Schulz *et al.* 2011, Aluri and Buttner 2007). At the plastid two SUT4 and the SMT subfamily pGlcTs serve as sugar efflux pumps (Ferro *et al.* 2003). ESLs (ERD six-like transporters) are involved in energy-independent sugar efflux from the vacuole (Doidy *et al.* 2012).

Sucrose is the main form of sugar used in long distance transport, and all plants possess a family of SUTs (Doidy *et al.* 2012). There are five classes of SUTs: SUT1-5 (Kuhn *et al.* 2010; Braun and Slewinski *et al.* 2009). SUT1 transporters are only found in dicot plants and are responsible for sucrose phloem loading (Zhang *et al.* 2016) and sucrose partitioning in sink organs (Buttner 2007). SUT2 act as sugar sensors as well as transporters (Barth *et al.* 2003). SUT 3 transporters function identically to SUT1 transporters, but are only found in monocot plants (Doidy *et al.* 2012). SUT4 regulate intracellular sucrose partitioning, sucrose efflux from source leaves and the utilisation of sucrose in lateral and terminal sink organs (Payyavula *et al.* 2011; Eom *et al.* 2011). Finally, SUT5 is the least studied transporter, but is thought to play a role in sucrose phloem loading in source tissues (Zhang *et al.* 2016)

In addition to long-distance transport, sugars (such as monosaccharides) are also distributed within cells, i.e. partitioned into different organelles depending on requirements, as well as between cells (Buttner 2007). For example, during the day many plant species temporarily store sugars in the form of starch in the chloroplasts of source leaves (Weise *et al.* 2006). At night, that starch is catalysed to release monosaccharides (such as glucose) (Weise *et al.* 2006; Weber *et al.* 2000), which is then exported from the chloroplast (Buttner 2007). Furthermore, in sink tissues sucrose is hydrolysed by invertases which yields glucose and fructose which are transported via sugar transporter proteins (STPs) (Fig. 2) (Doidy *et al.* 2012). The STPs responsible for monosaccharide transport are MSTs and SWEETs (Doidy *et*

al. 2012). The plant MST gene family is large, containing 53 MSTs in *Arabidopsis*, 65 in rice, 58 in *Medicago truncatula*, and 59 in grapevine (*Vitrus Vinifera*) (Doidy *et al.* 2012). Monosaccharides are further subdivided into several subfamilies based on their substrate specificity, these are: polyol monosaccharide transporter (PMT), inositol transporter (INT), vacuolar glucose transporter (VGT), tonoplast membrane transporter (TMT), and plastidic glucose transporter (pGlcT) (Doidy *et al.* 2012).

SWEETs belong to a distinct transporter family that contain a novel structure consisting of a tandem repeat of three transmembrane domains connected by a linker-inversion transmembrane domain (Chen *et al.* 2010). There are 17 *SWEET* genes in *Arabidopsis*, 21 in rice, 15 in *M. truncatula* and approximately 47 in *Eucalyptus grandis* (Chen *et al.* 2010; Eom *et al.* 2015). In *Arabidopsis*, SWEETs are divided into four phylogenetic clades, clade I (*AtSWEET1-3* homologues, typically monosaccharide transporters), clade II (*AtSWEET4-8* homologues, typically monosaccharide transporters), clade III (*AtSWEET9-15*, sucrose transporters) and clade IV (AtSWEET16, 17, fructose transporters) (Eom et al, 2015). Of note, SWEET clades do not determine which physiological process the protein is involved in, for example AtSWEET5, AtSWEET8 and AtSWEET13 are involved in pollen nutrition, yet they are found in either clades II or III (Eom *et al.* 2015).

SWEETs play important roles in many plant processes, including nectar secretion, phloem loading, sugar filling in seeds, regulating pollen nutrition, vacuolar hexose transport, carbon reallocation in leaves during stress or

senescence and during plant-microbe interactions (both pathogenic and mutualistic) (Eom *et al.* 2015; Guo *et al.* 2014). Pathogens are known to use effectors to manipulate host plant *SWEET* expression to increase the amount of sugars at the site of infection (Chen *et al.* 2010; Streubel *et al.* 2013). For example, the rice pathogen *Xanthomonas oryzae* grows in the apoplasm and xylem of the host and secretes the transcription activator-like (TAL) effectors PthXo1 and AvrXa7 to induce the expression of host Os*SWEET11* and *OsSWEET14* respectively, which increases the amount of sugar released into the apoplasm for the pathogen to utilise (Chen *et al.* 2010). Mutant *X. oryzae* lacking the PthXo1 effector was less virulent and repressing the expression of *OsSWEET11* (via RNA interference) resulted in decreases in pathogen growth (Chen *et al.* 2010). In addition, adding mutations in the promoter of Os*SWEET11* provided protection from *X. oryzae* infection (Chen *et al.* 2010). Bacterial and fungal pathogens induce the expression of different sets of SWEETs (Chen *et al.* 2010). For example, the bacterial pathogen *Pseudomonas syringae* highly induces the expression of *AtSWEET4, AtSWEET5, AtSWEET7, AtSWEET8, AtSWEET10, AtSWEET12 and AtSWEET15* in *Arabidopsis* (Chen *et al.* 2010). However, infection with the fungal pathogen *Golovinomyces cichoracearum* induces *AtSWEET12*. Infection with a different fungal pathogen, *Botrytis cinereal*, induced the expression of *AtSWEET4, AtSWEET15* and *AtSWEET17* (Ferrari *et al.* 2007). Interestingly almost all SWEETs targeted by pathogen effectors are clade III SWEETs and have been shown to export sucrose (Eom *et al.* 2015). Furthermore, in grapevine (*Vinus Vinifera*) the glucose transporter *VvSWEET4* is highly induced by necrotrophic pathogens, but not biotrophic

pathogens (Chong et al 2014). *VvSWEET4* is upregulated ROS production and necrotrophic pathogen virulence factors (Chong et al 2014). Additionally, *AtSWEET4 Arabidopsis* mutants were less susceptible to *B. cinereal* (necrotrophic pathogen) infection (Chong et al 2014).

While the role of SWEETs in plant-pathogen interactions has been (and is still being) widely researched (Chen *et al.* 2010, 2012, 2013, 2015a, 2015b; Chong *et al.* 2014; Cohn *et al.* 2014; Liu *et al.* 2011; Perotto *et al.* 2014), the role of SWEETs in mutualistic plant-microbe interactions is mostly unknown (Casieri *et al.* 2013; Tarkka *et al.* 2013). An early study found that the MtN3 SWEET in *Medicago truncatula* is highly upregulated after exposure to *Rhizobium meliloti* (Gamas et al, 1996). Therefore, MtN3 SWEET was thought to play a role in nodulation (Gamas *et al.* 1996), perhaps by providing the associating bacteria with hexoses in exchange for nitrogen thus stabilising the mutualism.

Much of what is known about the role of SWEETs in parasitic interactions and plant development has been studied in Arabidopsis and rice plants (Chen *et al.* 2012; Zhou *et al.* 2014). Although SWEET genes have been identified in most sequenced plant genomes, their individual roles in plant development and pathogen nutrition has yet to be explored. In addition, since Arabidopsis cannot form mutualistic interactions with fungi, the role of each individual SWEET protein in mutualistic plant-microbe interactions has yet to be determined.

1.5 Transcriptional control and the SWI/SNF complex

Extensive gene regulation occurs within plant root cells during plant-fungal interactions. Eucalypts can form mutualistic relationships with ECM fungi, but are also the target of many soil borne pathogens (e.g. *Armillaria* spp.). However very little is known about the mechanisms that control gene activation or repression in plants during interactions between long lived perennial trees and their mycorrhizal associates. Chromatin modifications are thought to be one way in which these gene activation or repression pathways are controlled. While many different nuclear protein complexes regulate this process, the most studied class of Chromatin Remodelling Complexes (CRCs) is the SWI/SNF (Switch/Sucrose Non-Fermenting) complex (Sarnowska *et al.* 2016).

SWI/SNF genes were first identified in the yeast *Saccharomyces cerevisiae* (Abrams *et al.* 1986) and later in *Arabidopsis thaliana* (Brzeski *et al.* 1999), *Drosophila* and mammals (Mohrmann and Verrijzer 2005). The original yeast SWI/SNF complex consists of 12 subunits. The core of the complex is made up of one SWI2/SNF-2type ATPase, one SNF5, and two copies of SWI3 subunits (Narlikar *et al.* 2002). This core is adequate for nucleosome sliding but it is normally associated with other subunits, which act as receptors for the SWI/SNF complex to interact with other proteins that affect chromatin remodelling (Phelan *et al.* 1999). The core subunits of yeast SWI/SNF complexes are similar to the SWI/SNF complex found in *Arabidopsis thaliana*. In *A. thaliana* there are four putative SWI/SNF-type SNF2-ATPases (SYD, BRM, CHR12/MINU1, CHR23/MINU2), and one SNF5 (BSH) subunit

(Farrona *et al.* 2004). Because of their sequence similarity, SYD, BRM and MINU1/2 are thought to have chromatin remodelling activity, but to date, only MINU2 has been shown to do this (Han *et al.* 2015). In addition, there are four homologs of *SWI3* genes in *A. thaliana* (*SWI3A, B, C* and *D*) (Sarnowski *et al.* 2002). The remaining subunits of the *Arabidopsis* SWI/SNF are LUH, SWP73(A,B), ARP(4,7), GIF, BRD, GLTSCR, G248101, LFR, BCL7B (Fig. 3).
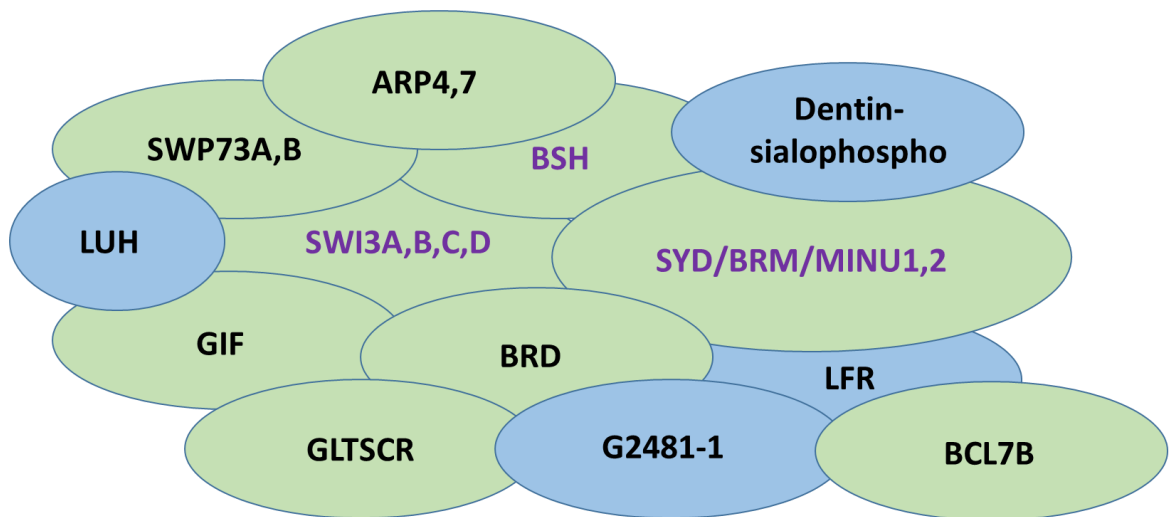
Figure 3. The subunits of *Arabidopsis thaliana* SWI/SNF complex (adapted from Sarnowska *et al.* 2016). Subunit names labelled with purple text represent the core subunits. Green subunits represent the homologous subunits to the human SWI/SNF complex and blue subunits represent *Arabidopsis* specific SWI/SNF subunits. Figure adapted from: Sarnowska, E, Gratkowska, D.M., Sacharowski, S.P., Cwiek, P, Tohge, T, Fernie, A.R., Siedlecki, J.A., Koncz, C, Sarnowski, T.J., (2016) The Role of SWI/SNF Chromatin Remodeling Complexes in Hormone Crosstalk, *Trends in Plant Science*, vol. 21, no. 7, pp. 594-608.

In *A. thaliana* the SWI/SNF complex plays an important role in the regulation of jasmonate (JA), abscisic acid (ABA), gibberellin (GA), ethylene (ET) and cytokinin signalling pathways (Archacki *et al.* 2013; Saez *et al.* 2008; Walley *et al.* 2008; Efroni 2013). Studies on the SYD subunit shows it regulates the expression of genes within the ethylene and jasmonate pathways, thus

contributing to the plant's immune response against fungal pathogens (Walley *et al.* 2008). In addition, studies have shown that the *syd* and *brm* mutations change the expression of genes controlled by the ABA and GA hormone signalling pathways (Bezhani *et al.* 2007). Numerous experiments have indicated a link between the germination of seedlings on exogenous sugar and ABA/ethylene activity (Gazzarrini and McCourt 2001). Gazzarrini and McCourt (2001) found that low sugar levels interfere with the inhibitory effects of ABA on germination, whereas prevention of seedling development post-germination by high sugar concentrations is dependent on ABA synthesis.

A series of signalling events are involved in the interaction between fungal and root cells, necessary for forming functional symbiotic structure. This appears to be caused by activating and deactivating of genes in both fungus and host plant. Certain elicitors are produced by the root cells that regulate the expression of fungal genes to establish symbioses (Burgess et al. 1995). Certain genes are activated that are responsible for the development of a Hartig net and hyphal mantle (Salzer et al. 1997) and the deactivating of certain fungal genes encoding factors for host plant defence reactions. Certain elicitors present in ECM fungi are deactivated by chitinases of the root cortex without harming the fungus, thus establishing the formation of ectomycorrhizas (Salzer and Boller 2000). Plants must regulate their defence pathways (e.g. regulate hormone pathways ABA, JA, SA, auxin, CK) and activate sugar-related genes to form (or stabilise) mutualistic associations, but still defend against pathogens who attempt to avoid plant defences by

secreting effectors that suppress plant immunity (Lo Presti *et al.* 2015) or upregulate the transcription of sugar-related genes in order to syphon sugar (Chen *et al.* 2010). We hypothesize that the SWI/SNF complex regulates the expression of sugar-related genes and hormone pathways within the plant during these interactions.

While the role of the SWI/SNF complex during plant-ECM fungal relationships is largely unknown, production of defence and growth hormones (such as auxin (IAA), ABA, JA, ET, SA and SK) are regulated by the SWI/SNF complex, and these hormones are also produced by ECM fungi (Ma *et al.* 2009). For example, Auxin regulates the development of embryo and fruit, vascular bundle and root growth (Parvaiz 2011). It is synthesized in the stem tip and young leaf and is then translocated to the required location. There are different soil microbes that are able to produce auxin. Ectomycorrhizal fungi produce cytokinin and indole acetic acid (IAA part of the auxin class of plant hormones) to stimulate host plant root growth. In addition, plant produce auxins and the expression of auxin genes are controlled by the SWI/SNF complex.

Extensive gene regulation occurs within plant root cells during plant-fungal interactions. Eucalypts can form mutualistic relationships with ECM fungi, but are also the target of many soil borne pathogens (e.g. *Armilarria* spp.). However very little is known about the mechanisms that control gene activation or repression in plants during interactions between long lived perennial trees and their mycorrhizal associates. Chromatin modifications are

thought to be one way in which these gene activation or repression pathways are controlled. While many different nuclear protein complexes regulate this process, one of the main complexes identified is the 'SWI/SNF' protein complex. The SWI/SNF complex controls many plant hormone signalling pathways. The role of the SWI/SNF complex in interacting with fungal effectors, in perennial trees during their interaction with mycorrhizal associates has yet to be explored.

1.6 Overview of Research

To determine the role of SWEET proteins in plant-microbe interactions, we identified and categorised the *SWEET*-like genes of *E. grandis.* Further, we determined if there was tissue-specific expression profiles of the identified *E. grandis SWEET*-like genes throughout seedling tissues. We also compared and contrasted the expression of these genes in roots when in contact with a pathogenic, saprotrophic and mutualistic fungi. Finally we determined the effects of overexpressing and repressing 4 eucalypt SWEET genes.

To understand transcriptional regulation in mutualistic plant-microbe mutualisms, we performed qPCR over a time course of 2 weeks on four *E. grandis* SWI/SNF complex subunits (i.e. SWI3A, B, C, and D). A *Pisolithus microcarpus* MiSSP9.7 effector was found to interact with the SWI3D subunit using yeast two-hybrid experiments between MiSSP9.7 and nuclear proteins. Bimolecular fluorescence complementation (BiFC) experiments further proved those protein interactions. Further, we identified where MiSSP9.7

localises within host plant cells. Lastly, we determined whether overexpressing or repressing *MiSSP9.7* and *SWI3D* affects mycorrhization.

The information gleaned from this study will further our understanding about the controls and mechanisms involved in different types of plant-microbe interactions. The belowground microbes play a significant role in plants' growth and health (Artursson *et al.* 2006; Richardson *et al.* 2009). One of the major constraints in eucalypts is soilborne pathogens. Thus, this study could potentially find ways to improve Eucalyptus growth and health that will result in economic and environmental benefits.

Chapter 2 Characterisation of *Eucalyptus grandis* hexose transporters implicated in symbioses with fungi

2.1 Introduction

Carbon, in the form of simple sugars, is essential for the development of all living organisms. In terrestrial ecosystems plants, animals and microbes interact with one another and the environment to obtain, utilize and eventually recycle carbon. Fixation of light energy and atmospheric carbon dioxide (CO_2) by plant photosynthesis produce organic compounds such as sugars utilized by plants for maintenance and growth. Sucrose is the main product of photosynthesis and is transported from source to sink tissues via the phloem (Koch 2004). Sucrose plays a key role in many regulatory mechanisms, including growth and development, differential gene expression, stress-related responses and plant innate immunity (Gomez-Ariza *et al.* 2007; Tognetti *et al.* 2013; Tauzin and Giardina 2014). Sucrose cleavage products, glucose and fructose, also act as signaling molecules. Of the two hexoses, glucose has been better described in relation with the hexokinase signaling pathway (Moore *et al.* 2003; Cho *et al.* 2009) while for fructose a specific pathway has been proposed involving the ABA- and ethylene-signaling pathway (Cho and Yoo 2011; Li *et al.* 2011).

Herbivorous animals and insects obtain energy from these plant organic compounds through ingestion while microbes obtain photosynthate from plants by three main mechanisms: parasitism, mutualism or via decomposition. While these mechanism classifications are an oversimplification, with plant-microbe associations being able to dynamically change from mutualism to parasitism depending on both biotic and abiotic factors (Francis and Read 1995; Johnson *et al.* 1997; Saikkonen *et al.* 1998; Jones and Smith 2004), they serve as a useful framework for understanding how plant immune system response differs based on the benefit of the microbe to the plant. Pathogenic microbes may exploit photosynthetically derived sugars through manipulation of host plants sugar transporter proteins (STPs or SWEETs) (Chen *et al.* 2010; Cohn *et al.* 2014). Chen *et al.* (2010) first identified and characterised SWEETs in Arabidopsis, highlighting the fact that pathogens hijack sugars by sending TAL effectors to induce expression of specific SWEETs. Similar to Chen *et al.* (2010), Cohn *et al.* (2014) found that the bacterial pathogen *Xanothomonas axonopodis* syphons sugar from cassava plants by using TAL effectors to induce *MeSWEET10a*. Similar to pathogens, virus infection can lead to increases in sugar levels within plant tissues, although the benefit of this to virus replication is unknown (Shalitin and Wold 2000). As a means to combat sugar leakage, plants interacting with pathogenic microbes have been found to uptake/retrieve sugars from the apoplast through the increased expression of specific STPs (Chen *et al.* 2015b). This SWEET2 limits the amount of sugar obtained by the pathogen; thus restricting the pathogen's spread and growth throughout the rest of the plant (Chen *et al.* 2015b).

In contrast to pathogens, beneficial microbes obtain sugars from plants by forming mutualistic symbioses. Plants form mutualistic symbioses to improve acquisition of growth limiting nutrients, and mutualistic microbes associate with plants to gain carbon (Smith and Read 2008). Over 80% of trees associate with ectomycorrhizal fungi (Pellegrin *et al.* 2015; Wang and Qui 2006; Smith and Read 2008). Ectomycorrhizal fungi (e.g. *Pisolithus*) utilise hyphal networks to efficiently explore soil, acquiring nutrients (such as N and P) to provide to the host plant in exchange for carbon (in the form of sugars) (Nehls 2008; Smith and Read 2008). Therefore, this mutualistic symbiosis is constituted by a constant exchange in nutrients between the two partners, resulting in better growth for both symbionts. ECM fungi further aid plant survival by supporting host adaptation to changing environmental conditions such as climate extremes, drought and soil pollution (Redman *et al.* 2011; Kipfer *et al.* 2012).

One aspect of sugar transport and accumulation in plant tissues during microbial challenge that is often overlooked is the use of certain sugars as substrates for the synthesis of defensive metabolites and as priming agents. It is thought that plants modulate their sugar pools to act either as a source of carbon and energy, or to use as signals and priming molecules to enhance defence responses (Gomez-Ariza *et al.* 2007). These conclusions come based on observations that sugars are able to regulate pattern recognition genes used in plant innate immunity (Jones and Dangl 2006; Mohammad *et*

al. 2012) and because increases in sucrose and myo-inositol concentrations are often observed under biotic stresses (Valluru and Van den Ende 2011). Gomez-Ariza *et al.* (2007) demonstrated that the external application of sucrose in *Maize* plants increased plant expression of pathogenesis-related (PR) genes and overall resistance to a wide range of microbial pathogens. Morkunas *et al.* (2014) showed that soluble sugars contribute to immune responses against pathogens by stimulating isoflavone production in plants. Phloem mobile oligosaccharides have also been found to induce defence responses within plants. These include the: 1-ketose (a fructosyl oligosaccharide), raffinose (a galactosyl oligosaccharide:), trehalose (a disaccharide of glucose) and galactinol (galactosyl-myo-inositol; Hofmann *et al.* 2015; Mohammad *et al.* 2012). Kim *et al.* 2008 showed galactinol activates defence genes (*NtACS1*, *PR1a* and *PR1b*) in response to fungal pathogen attacks. Trehelose, meanwhile, can partly induce resistance against powdery mildew (*Blumeria graminis*) in wheat by activation of phenylalanine ammonia-lyase and peroxidase genes (Reignault *et al.* 2001; Muchembled *et al.* 2006) while the *Trehalose Phosphate Synthase11 (TPS11)* gene regulates defence responses in Arabidopsis against aphids (Singh *et al.* 2011). Therefore, sugars function as priming molecules and as signalling molecules, that lead to effective immune responses (Morkunas and Ratajczak 2014).

Given the complex roles of sugars in plant-microbe interactions, it is important that we characterise the mechanism by which these compounds are transported in plant tissues during microbial challenge. Compared to

annual model plants (Büttner 2010; Yamada *et al.* 2016; Chen *et al.* 2010,

2012, 2013, 2015a, 2015b), less is known about how sugar transport

systems activated during interactions between long lived perennial trees and

various types of fungi. The interaction between *Eucalyptus grandis* with

different types of fungi (i.e. pathogenic, saprotrophic and mutualistic fungi)

offers a good model for studying this topic as the *E. grandis* genome has

been sequenced (Myburg *et al.* 2014), because *E. grandis* is a tractable

system for genetic modification and because a number of pathogens and

symbionts of *E. grandis* are culturable. The aims of this study were to identify

the *SWEET*-like genes of *E. grandis* and characterize a number of these

proteins that are differentially regulated during plant-microbe interaction.

2.2 Materials and methods

2.2.1 Plant and fungal growth conditions

Growth of *E. grandis* seedlings was performed following the methods outlined in Plett *et al.* (2014a). *E. grandis* seeds were obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO, Clayton, Vic., Australia) tree seed centre (Seedlot 21068) and sterilised in 30% hydrogen peroxide for 10 min followed by five washes with sterile water for 5 min each. Seeds were then transferred onto 1% agar water medium and allowed to germinate at 25°C with a 16/8 hour light/dark cycle. Germinated seedlings were then transferred to ½ Modified Mylin Norkin (MMN) medium on top of a sterile cellophane membrane to prevent root growth into the medium.

One oomycete eucalypt pathogen (*Phytophthora cinnamomi*) and 3 genera of fungi were used in this study: *Suillus granulatus* (non-*Eucalyptus* colonising ECM which acts parasitically in our experimental set-up), *Fusarium oxysporum* (non-*Eucalyptus* specific parasite), *Suillus luteus* (non-*Eucalyptus* colonising ECM), and *Pisolithus microcarpus* isolate SI12 (*Eucalyptus* colonising ECM). All fungal cultures used in this study were propagated 1 month on 1x MMN before subculturing hyphae from the growing edge of the colony onto ½ MMN medium covered in a sterile cellophane membrane and grown in the dark at 25°C for 2 weeks.

For plant colonisation experiments, plant seedlings were transferred directly onto each fungal colony. The contact plates were then placed in a growth cabinet under a 16/8 h light/dark cycle at 25°C for 2 weeks after which they were harvested and frozen directly in liquid nitrogen. *E. grandis* control plants

were grown axenically and treated identically for the same length of time and under the same conditions.

2.2.2 Generation of *SWEET* constructs and mutant eucalypts, and ^{13}C transfer tests

35S:Eucgr.K02678, *35S:Eucgr.B00363*, RNAi:*Eucgr.L02615* and RNAi:*Eucgr.K02688* were amplified from cDNA synthesized using KAPA HiFi polymerase (KAPA Biosystems) following the manufacturer's instructions (see Table 1. for primers used). The amplified fragments were gel purified and ligated into pDONR222, PCR verified and sequence verified. Positive inserts were then ligated into pH2GW7 (*35S:*) or to pH7GWIWG2(II) (RNAi) plasmids using Gateway Gene Cloning (Life Technologies) and transformed into *Rhizobium rhizogenes* (formerly known as *Agrobacteria rhizogenes*) isolate K599. *E. grandis* seedlings were grown from seed to one month old on 1% agar media.

To generate mutant roots, *E. grandis* seedling roots were severed from the stem using sterile scalpels. The remaining wounded part of the stem was dipped into growing colonies of mutant *R. rhizogenes* containing SWEET constructs, and grown upside down on ½ MS media for 1 week in a growth cabinet with a constant temperature of 25°C and a 16 hour photoperiod. To prevent *R. rhizogenes* from killing the seedlings, *E. grandis* stems were then transferred to ½ MS Timentin (conc. 150 µg/mL) media. Once per week the stems were transferred to new ½ MS Timentin media and grown under the same conditions. Mutant roots usually emerged within 1-2 weeks, but took a

total of 3-4 weeks to grow long enough for fungal contact. After 4 weeks of growth, transgenic plants were transferred onto ½ MMN media covered with a sterile cellophane membrane and colonies of 2-week-old *Pisolithus microcarpus* isolates (SI-12) placed on top, making direct contact with the roots of the plant. These contacts were left for a total of 14 days in a cabinet with a daytime high temperature of 30°C and low of 22°C with a 16-hour photoperiod. To prepare for ^{13}C transfer tests, on the 8th day two holes were burnt into the lids of each plate (using a soldering iron), and covered with micropore tape. On the morning 9th day all contacts were placed into a plastic tank (which had a rubber septum on one side and a fan for circulation on the inside) and the lid was sealed down using clamps so no air could escape. That same morning 12ml of $^{13}CO_2$ gas (99% atom enrichment) was injected into the tank by using a syringe that penetrated through the rubber septum on the side of the tank, and left for 5 hours to allow for gas uptake. Afterwards, the lid of the tank was opened and aerated. Contacts were then placed back into the tank and left in the cabinet above. This $^{13}CO_2$ pulse was repeated on the 12th day. On the 14th day extra-radical fungal hyphae harvested for ^{13}C analysis. ^{13}C labelling of eucalypts in contact with different fungal types was carried out identically.

2.2.3 RNA extraction and Quantitative PCR analysis

RNA was extracted from four tissue types: photosynthate source tissues (mature leaves), transport tissues (stem) and photosynthate sink tissues (shoot apex including the 2 youngest developing leaves and roots) using the Qiagen RNeasy Plant Kit with the RLC lysis buffer supplemented with 25 mg

ml^{-1} PEG 8000 and following the manufacturer's instructions thereafter. The cDNA was synthetized using the iScript Select cDNA Synthesis Kit (BioRAD). The Quantitative PCR (QPCR) reactions were performed using Sensifast Sybr Low-ROX Mastermix (Bioline) and QPCR machine (C1000 Touch TC, CFX96 RTsystem (BioRAD)), where the cycle parameters were as follows: 1. 95°C 3 mins, 2. 95°C 30 seconds, 3. 55°C 30 seconds, 4. 72°C 30 seconds (steps 2-4 was repeated x39), 5. Melt curve analysis. Tissue wide SWEET-like gene expression was normalized to the expression of the housekeeping genes *Eucgr.C00350.1* and *Eucgr.K02046.1* (Plett *et al.* 2014a). The primers used in this study have been tested for their efficiency and their specificity. To visualize tissue wide and root-fungi SWEET-like expression profiles, we used two programs: 'Cluster 3.0' (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm last accessed 16/3/17) and 'Java TreeView' (http://jtreeview.sourceforge.net/ last accessed 16/3/17).

2.2.4 Identification of SWEET genes in *E. grandis* and other plant species

Using the Phytozome database, we identified SWEET-like genes by examining the *E. grandis* 2.0 genome (*Eucalyptus grandis* v2.0; http://www.phytozome.net/ last accessed: 2/7/2016) and identifying sequences that share homology (based on the 3-transmembrane-helix-domain polypeptide) to previously identified SWEETs. Sequences were aligned to *E. grandis* transcripts taken from Phytozome v9.1 and corresponding to the *E. grandis* genome v2.0 using the Phytozome database

(https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Egrandis last

accessed 16/3/17). This led to the identification of 52 genes with high

homology (Fig. 4). The SWEET genes identified in our study were designated

as *AtSWEET1-like* to *AtSWEET17-like* based on their homology to each

Arabidopsis SWEET gene respectively (Fig. 4). Likewise SWEET genes in

other species were obtained by identifying sequences in their genome that

share homology to previously identified SWEETs, using Phytozome

(https://phytozome.jgi.doe.gov/pz/portal.html last accessed: 16/3/17). All

species used: *Arabidopsis thaliana (At) TAIR10, Amborella trichopoda v1.0,*

Citrus clementina (Cc) v1.0, Eucalyptus grandis (Eg) v2.0, Metacargo

truncatula (Mt) Mt4.0v1, Populus trichocarpa (Pt) v3.0.

2.2.5 Construction of the phylogenetic tree

To determine the phylogenetic relationships of *E. grandis* SWEET-like

proteins, we constructed a phylogenetic tree using the online tool

'Phylogeny.fr' (Dereeper *et al.* 2008). SWEET-like gene sequences of plants

(*Arabidopsis thaliana (26), Amborella trichopoda (9), Citrus clementina (18),*

Eucalyptus grandis (52), Medicago truncatula (25), Populus trichocarpa (28))

were downloaded from the Phytozome database. "One click" phylogenetic

analysis was used, with a concatenated MUSCLE alignment adjusted by

Gblocks. The tree was rooted with Human SWEET Transporter 1 as the

outgroup.

2.2.6 Yeast complementation

To test whether four putative eucalypt SWEET proteins (*AtSWEET11, 12, 13, 14-like* [Eucgr.K02694], *AtSWEET4, 5-like* [Eucgr.K02688, Eucgr.B00363, Eucgr.L02615]) could transport sugars we performed yeast complementation assays. We used *S. cerevisiae* (strain EBY.VW4000) as a model system to test the sugar capabilities of these 4 putative SWEETs. EBY.VW4000 was chosen because the sugar transporter genes in this strain has been knocked-out (i.e. has mutations in its sugar transporter genes), except for the genes that encode maltose transporters (Wieczorke *et al.* 1999). Therefore EBY.VW4000 cannot grow on media with a carbon source other than maltose. However, when EBY.VW4000 is transformed with the vector containing the putative *SWEET* genes, the yeast will begin to transcribe and express those *SWEET* genes. Thus growth on other types of sugar media will be restored if the SWEET protein product is able to transport sugars.

The open reading frames (ORF) of *Eucgr.K02678*, *Eucgr.B00363*, *Eucgr.L02615* and *Eucgr.K02688* were cloned into pYES2 vector using In-Fusion ligation kit (Clonetech) using BamH1 and HindiIII ligases, and transformed into *E. coli* (strain Top10). Constructs were selected for using antibiotic resistance and PCR verification followed by sequencing. These constructs were then transformed into *Saccharomyces cerevisiae* strain EBY.VW4000 following Easy TRAFO protocol (Gietz and Woods 2002). In brief, EBY.VW4000 was grown in YPM liquid media at 30^0C overnight with shaking (at 255 rpm) (i.e. pre-grown to the log phase). The next morning these yeast were used for transformation of four SWEET constructs (i.e.

Eucgr.K02678, *Eucgr.B00363*, *Eucgr.L02615* and *Eucgr.K02688* all in pYES-GFP vector). 1.5 ml of Yeast were harvested into 1.5ml Eppendorf tubes via centrifugation (3000 x g for 30 seconds) and media removed, re-suspended in sterile water, centrifuged again and sterile water removed. The pellets were re-suspended while adding each of the following chemicals: 240 ml of PEG (50% w/v), 36 ml of LiAC (pH7), 50 µl of pre-boiled Salmon Sperm DNA, 29 µl of sterile water, and 5 µl of each construct (concentration 0.1 – 1 µg of DNA). Yeast transformants were then incubated at 42^0C for 3 hours. During the 3 hour incubation, the cells were re-suspended every 15 mins by vigorously shaking the tube until the transformation solution looked homogenous. Afterwards yeast transformants were centrifuged at max speed for 30 seconds and resuspended in 1 ml of sterile water. Cells were then plated on SC media lacking uracil where maltose was the carbon source, incubated at 30^0C for 3-4 days and the resulting colonies PCR screened. Verified colonies were plated on media supplemented with different sugar sources (fructose, galactose, glucose, sucrose and maltose) at dilutions 10^{-1}, 10^{-2} and 10^{-3} and were incubated at 30^oC for 3 days and photographed. Control yeast were transformed with pYES-GFP vector without *SWEET* gene inserts and treated identically for the same length of time and under the same conditions.

2.2.7 Glucose efflux test

Glucose efflux tests were performed following methods described in Jansen *et al.* (2002) study, with minor adjustments. *Eucgr.K02678*, *Eucgr.B00363*, *Eucgr.L02615* and *Eucgr.K02688* cloned into pYES2 vector were tested. All

yeast strains (i.e. *S. cerevisiae* mutants) used for testing glucose efflux were

grown to the stationary phase (i.e. 3-4 days incubation at 30°C) in liquid SC

medium lacking uracil with a maltose concentration of 7.5 grams per litre.

Samples were then harvested by centrifugation (5,000 x g, 3 mins), media

removed and samples weighed (to obtain wet weight). Yeast samples were

then resuspended in five-fold liquid SC medium lacking both uracil and a

sugar source. After 10 mins of incubation, maltose solution (100 grams per

litre) equivalent to 1/5 of total volume was added, and samples (150 μl per

sample) were taken at 15 min time intervals for 90 mins. Sugar

concentrations were determined using the Sigma-Aldrich Glucose Assay Kit

and a CLARIOstar® spectrophotometer.

2.2.8 GFP localisation

All 4 SWEET constructs were made as previously described above (see

Materials and methods, yeast complementation). The ORF of *Eucgr.K02678*,

Eucgr.B00363, *Eucgr.L02615* and *Eucgr.K02688* were cloned into pYES2

vector using In-Fusion ligation kit (Clonetech) using BamH1 and HindiIII

ligases, and transformed into *E. coli* (strain Top10). Constructs were selected

for using antibiotic resistance, PCR verified and sequenced. These

constructs were then transformed into *Saccharomyces cerevisiae* strain

EBY.VW4000 following Easy TRAFO protocol (Gietz and Woods 2002). *S.

cerevisiae* (strain EBY.VW4000) were transformed with each of the 4

constructs following the quick and easy TRAFO protocol (Gietz and Woods

2002). After 3 days, colonies were observed using confocal scanning

microscopy. The GFP was excited at 488nm excitation and emission

captured between 520-540nm.

2.3 Results

2.3.1 Phylogenetic relationships of *E. grandis* SWEET-like transporters

A total of 52 SWEET-like genes were identified in the *E. grandis* genome using the Phytozome database (available at http://phytozome.jgi.doe.gov/pz/portal.html last accessed: 16/3/17) based on homology to *Arabidopsis SWEET* transporters with proven hexose transfer capability (Chen *et al.* 2010) as a template. Compared to annual plants and other trees genomes (*A. thaliana (17* SWEETs*), A. trichopoda (9), C. clementina (18), E. grandis (52), M. truncatula (25), P. trichocarpa* (*28*)), a significantly larger number of *SWEET-like* genes were found in the *E. grandis* genome (52 potential *SWEET*-like genes; Fig. 4). This large number of SWEET-like genes was the result of expansions and duplications that have occurred within the *E. grandis* genome rather than the occurrence of novel SWEET-like gene families (Fig. 4). The genes *SWEET1-14, 16 and 17* show expansion within the *E. grandis* genome (Fig. 4). These same SWEET genes show expansion in *A. trichopoda, C. clementina, M. truncatula* and *P. trichocarpa* genomes.
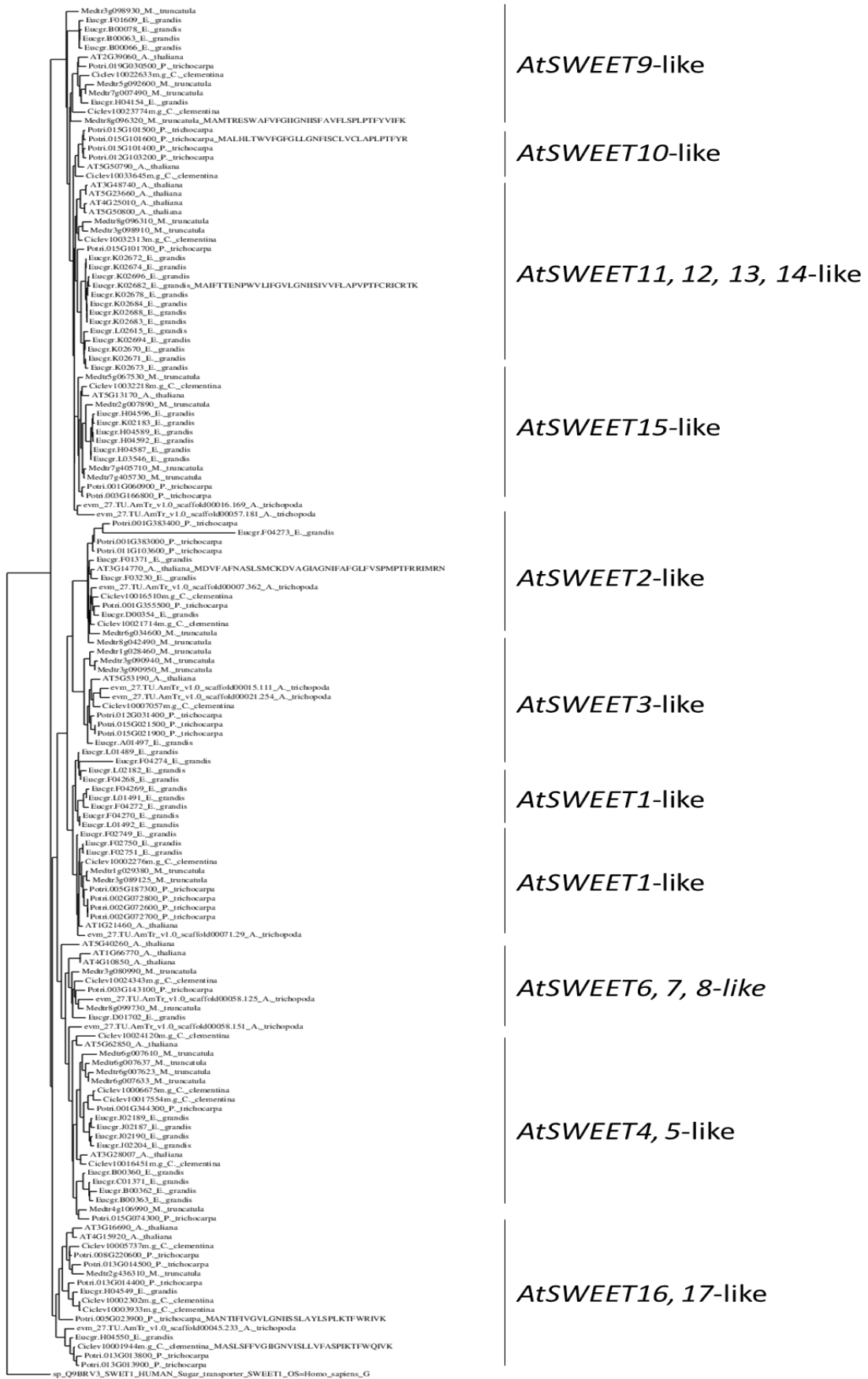
Figure 4. Phylogenetic relationships between SWEET-like proteins collected from different species. *A. thaliana, A. trichopoda, C. clementina, E. grandis, M. truncatula, P. trichocarpa*. The tree was rooted with Human SWEET Transporter 1 as outgroup.

2.3.2 SWEET gene expression in *E. grandis* tissues

Quantitative PCR of 52 *SWEET*-like genes in 4 major tissue types of *E. grandis* seedlings showed a variety of expression patterns (Fig. 5B). We were able to identify three groupings of genes based on expression patterns (Fig. 5B). Group 1 was highly expressed throughout the all tissues tested; with Eucgr.C01371 being the most highly expressed gene at 12 fold above the reference genes. Group 2 genes expression profiles were consistently similar to the reference genes and Group 3 genes exhibited lower expression levels as compared to the reference genes with Eucgr.F01609 being the lowest expressed gene across all four tissues (Fig. 5B). As with Groups 1 and 2, group 3 genes showed nearly identical expression levels in all tissues tested.
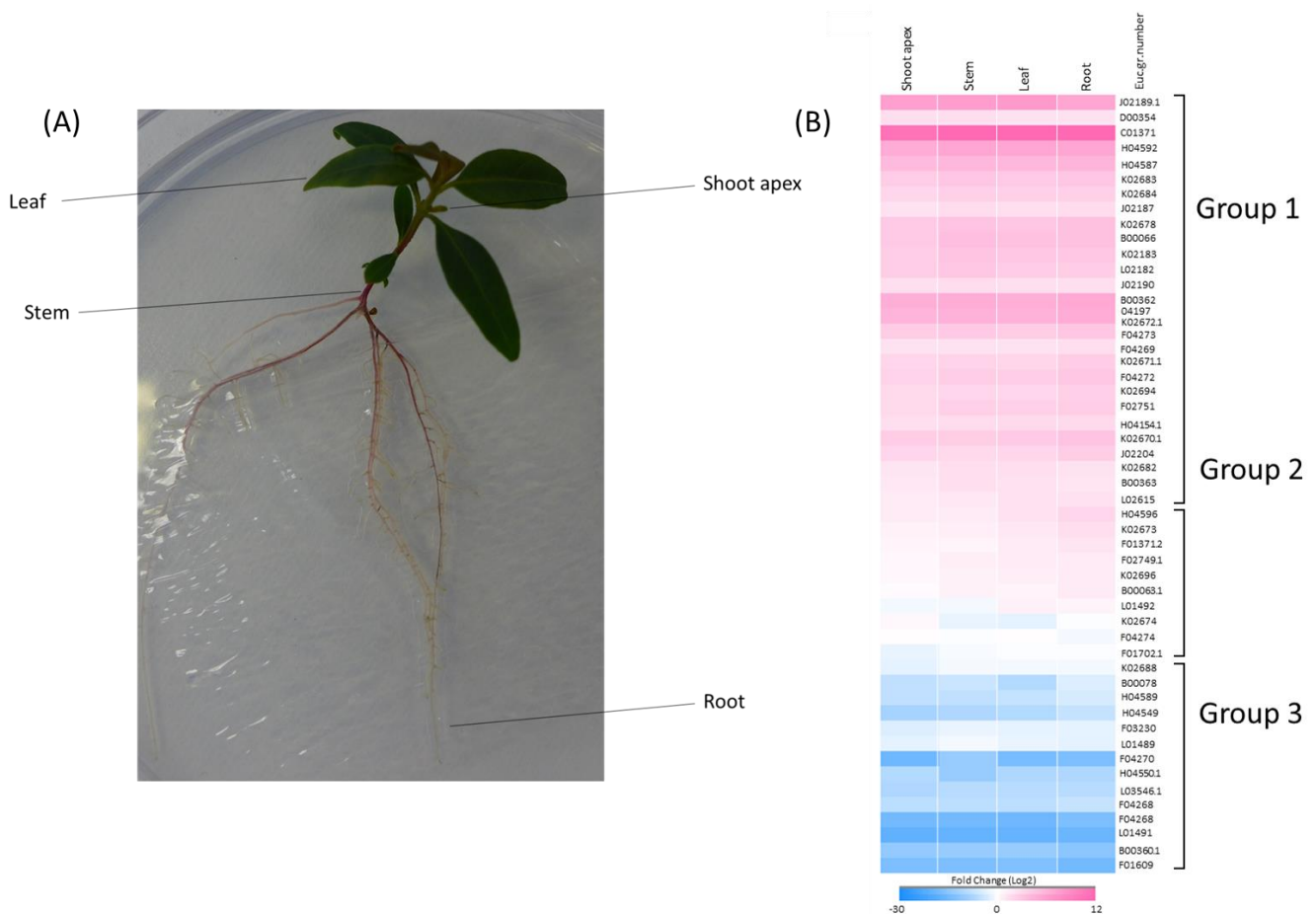
Figure 5. Expression profile of 52 SWEET-like gene throughout different tissue in *E. grandis*: Shoot apex, stem, leaves and root. **(A)** Image of *E. grandis* control plant grown axenically; labels show the different tissue and where RNA was extracted. **(B)** A total of 52 *E. grandis* SWEET-like genes show similar levels of expression throughout the four tissues. The heat map is annotated on the right-hand side, with genes grouped based on amount of expression: highly expressed genes (group 1), averagely expressed (group 2), and lowly expressed (group 3).

2.3.3 *E. grandis* roots exhibit different morphologies when in contact with different fungal lifestyles

The effect of different fungi and oomycetes on *E. grandis* root morphology was lifestyle dependent. In all cases roots were surrounded by fungal hyphae (excluding control plant which were grown axenically) (Fig. 6). *S. granulatus*, *Phytophthora cinnamomi*, and *Armillaria luteobubalina* led to hallmarks of parasitic interactions, i.e. discoloration of leaves (reddish appearance compared to controls) and blackening of roots tips. *A. luteobubalina* is a well-known eucalypt pathogen that causes death of eucalypt roots (Kile 1981, 1983) and *P. cinnamomi* is a common plant pathogen that causes death of roots in many different plant species (Royal Botanic Garden 2017a, b). *S. granulatus* is mycorrhizal on pines, however in our experimental set-up the fungus acted parasitically on the plant as the interaction resulted in short and unhealthy roots, discoloration of leaves and root death (blackened roots) (Fig. 6B and G), which was a similar result to *P. cinnamomi* and *A. luteobubalina* contacts (Fig. 6F and H). When *E. grandis* was grown with commensal fungi (i.e. *Suillus luteus*) plants exhibited no evidence of parasitism or of pathogenesis (Fig. 6C and I). *E. grandis* was grown in contact with the mutualistic ECM fungal isolates of *P. microcarpus*, we observed significant hyphal growth around the roots and successful colonisation of roots as denoted by shortening of lateral roots and the formation of a thick fungal mantle around the root surface (Fig. 6D and J).
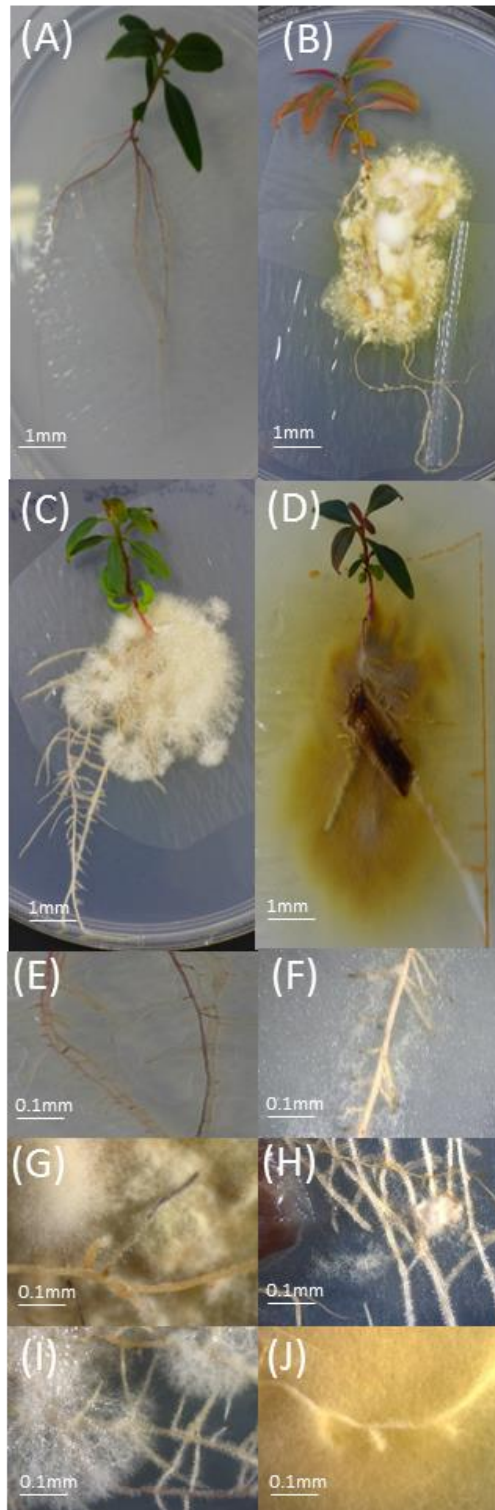
Figure 6. Images taken of 2 weeks old and *E. grandis* under different conditions. **(A)** Two weeks old control *E. grandis* grown in isolation. **(B)** *E. grandis* in contact with *Suillus granulatus* (parasite). **(C)** *E. grandis* in contact with *Suillus leuteus* (ECM non-Eucalyptus colonising). **(D)** *E. grandis* in contact with *Pisolithus microcarpus* (ECM Eucalyptus colonising). **(E)** Close up of control *E. grandis* roots grown in isolation. **(F)** Roots of *E. grandis* in contact with *Phytophthora cinnamomi* (parasite) where death of root is visible (i.e. blackening of root tips). **(G)** Roots of *E. grandis* in contact with *S. granulatus* (parasite) **(H)** Close up of *E. grandis* roots in contact with *Armillaria luteobubalina* (parasite). **(I)** Close up of *E. grandis* roots in contact with *S. leuteus*. **(J)** Close up of *E. grandis* roots in contact with *P. microcarpus*.

2.3.4 *SWEET*-like genes exhibit differential expression in *E. grandis* roots during challenge by different fungi

SWEET genes in *E. grandis* roots showed differential regulation when exposed to the presence of a fungus (Fig. 7). We found 51 *SWEET-like* genes were significantly upregulated during *E. grandis- P. cinnamomi* interaction, 36 *SWEET-like* genes were significantly upregulated during *E. grandis-A. luteobubalina* interaction, 36 *SWEET-like* genes were significantly upregulated during *E. grandis-S. granulatus* interaction, 37 genes in *E.grandis-F.* oxysporum condition, 30 genes in *E. grandis- S. luteus* interaction, 30 genes in *E.grandis- P. microcarpus* isolate (*SI-12*), 40 genes for *E.grandis-P. microcarpus* isolate *SI-9*, 37 genes for *E.grandis- P. microcarpus* isolate *R4*, and 35 genes for *E.grandis- P. microcarpus* isolate *R10*. Induction of a large number of *SWEET-like* genes did not show different expression profiles based on the lifestyle of the fungus interacting with the root system (i.e. groups 1; Fig. 7). Conversely, group 2 genes were found to have more varied expression that was dependent upon the identity or lifestyle of the fungus in contact with the plant. Examples include *Eucgr.B00360, Eucgr.H04550, Eucgr.H04154, Eucgr.F01371* that are highly expressed when in contact with the isolate of highly aggressive, incompatible *S. granulatus* as opposed to the compatible mutualist *P. microcarpus* isolate SI-12. In contrast, the genes *Eucgr.K02672.1, Eucgr.F01609, Eucgr.L01489, Eucgr.F01371.2, Eucgr.K02673, Eucgr.L01492, Eucgr.B00360.1, Eucgr.H04550.1, and Eucgr.H04154.1* are specific to parasitic and non-colonising fungal associations.
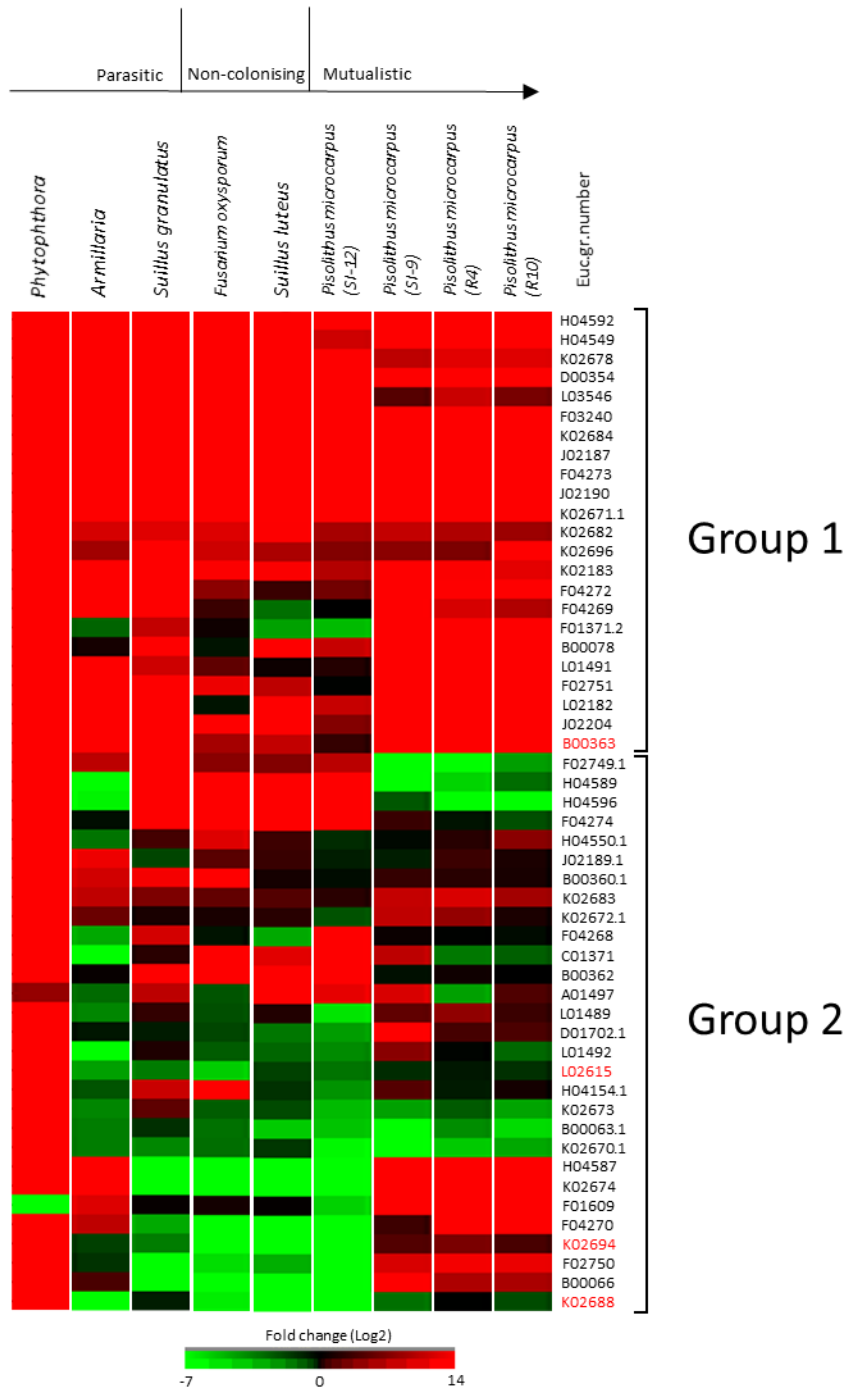
Figure 7. Regulation of 52 *E. grandis* SWEET genes when *E. grandis* associates with: parasites (Phytophthora, Armillaria and S*. granulatus)*, a non-Eucalyptus coloniser (*F. oxysporum* and *S. luteus*), or a mutualistic fungus (*P. microcarpus* strains). The heat map is annotated on the right-hand side, groups formed based on amount of expression within the roots during these associations: highly regulated in all conditions (group 1) and isolate dependent expression (group 2). The position of *Eucgr.B000363, Eucgr.L02615, Eucgr.K02694* and *Eucgr.K02688* were highlighted using red text for the Eucgr.number.

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2.3.5 *E. grandis SWEET-like* genes encodes STPs that localise to the plasma membrane of plant cells

If EgSWEET-like proteins facilitate the uptake and or export of sugars between plant cells and the apoplast, then these proteins must localise to the plasma membrane of plant cells. To test this, we used *S. cerevisiae* (EBY.VW4000) as a model organism. *S. cerevisiae* (EBY.VW4000) was transformed with four SWEET constructs (*Eucgr.K02694, Eucgr.K02688, Eucgr.B00363, Eucgr.L02615*-GFP tagged genes), including a positive control (*AtSWEET1*). We examined GFP fluorescence within those cells using confocal microscopy (Fig. 8). As expected AtSWEET1 localised to the plasma membrane (Fig. 8). Interestingly, only Eucgr.K02694 localised to the plasma membrane, whereas the rest of the EgSWEETs localised to the cytoplasm (Fig. 8)
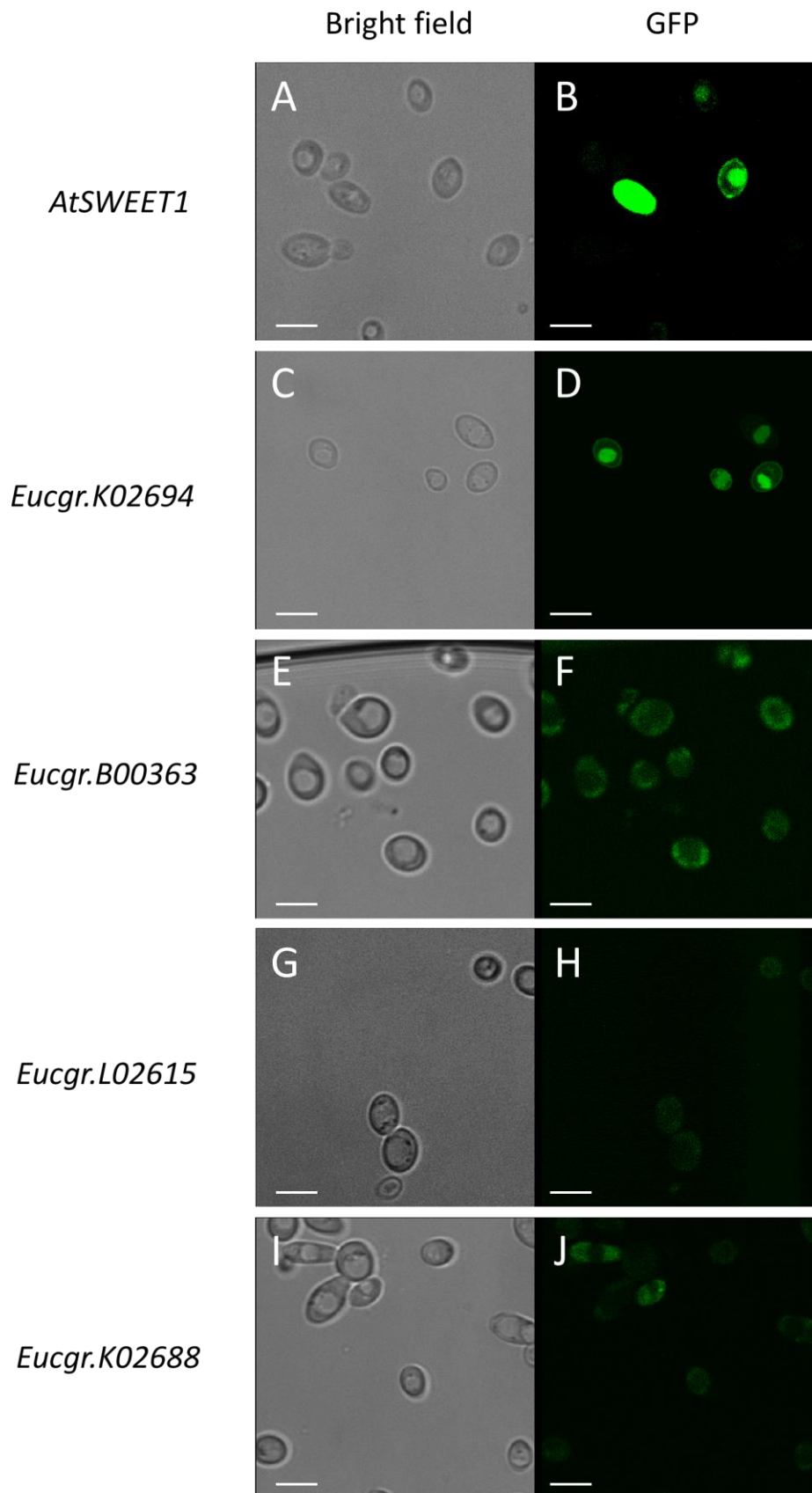
Figure 8. Localisation of *SWEET-like* proteins Eucgr.K02694, Eucgr.K02688, Eucgr.B00363, Eucgr.L02615-GFP fusions in yeast (VW4000). *AtSWEET1* was used as a positive control. Bright field and GFP fluorescence images were taken by confocal microscopy. Scale bar: 4*µm*

2.3.6 *E. grandis SWEET*-like proteins can act as sugar symporters

To determine the sugar transport capability of 4 putative *E. grandis* SWEET-like proteins *in vivo,* we tested the ability of the 4 *EgSWEET*-like genes to complement VW4000 *S. cerevisiae* (Fig. 9). As explained previously, EBY.VW4000 cannot grow on media with a carbon source other than maltose. The negative control (VW4000 only) grew on maltose media and was unable to grow on other sugar media. All other yeast heterologously expressing *EgSWEET* genes complemented the VW4000 strain when grown on galactose. *AtSWEET1* constructs (i.e. positive control) complemented VW4000 allowing growth and utilisation of all four sugar sources. Most notably *Eucgr.*K02694 had stronger growth compared to the other SWEETs. Both *Eucgr.L02615* and *Eucgr.K02694* were able to grow on all sugar media, while *Eucgr.B00363* and *Eucgr.K02688* had weak growth on fructose, sucrose and glucose. Therefore, the 4 *EgSWEET* genes tested had different affinities and abilities to transport sugar.

To determine if these same SWEET-like proteins were able to export glucose, we performed glucose efflux tests as per (Jansen *et al.* 2002). A maltose solution was added to mutant EBY.VW4000 colonies containing the 4 EgSWEET constructs (*Eucgr.K02678*, *Eucgr.B00363*, *Eucgr.L02615* and *Eucgr.K02688* in pYES2) and extracellular glucose concentration was measured over a total time period of 90 mins. All EgSWEETs exhibit glucose exporting capabilities at varying levels (Fig. 10). Most notably Eucgr.K02694 exhibited the highest rate of glucose export, even higher than *AtSWEET1*.
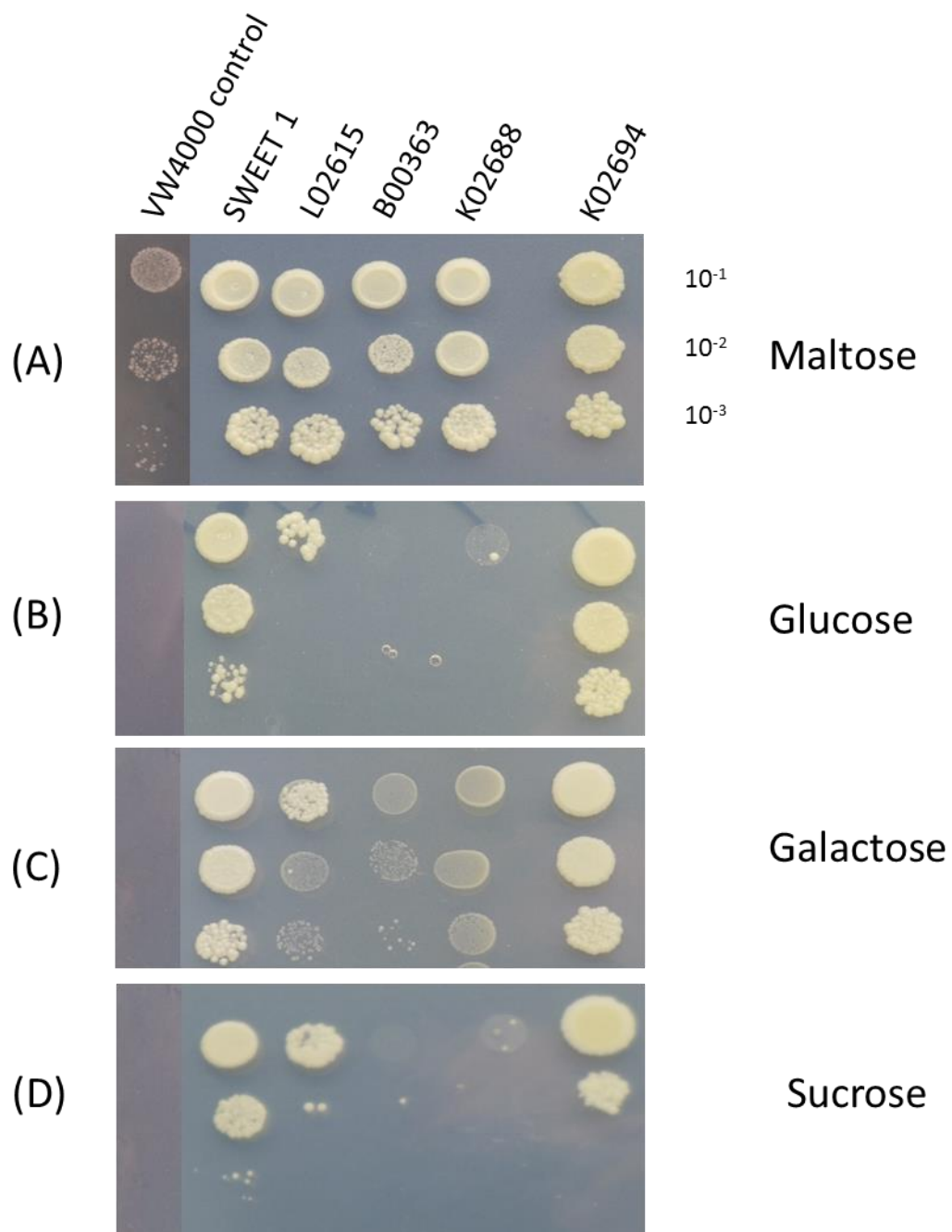
Figure 9. Transport activity of *EgSWEET* K02694, K02688, B00363, L02615 in yeast. These SWEET proteins complemented VW4000 *S. cerevisiae* mutants (which lacked 18 hexose transporter genes). Yeast VW4000 was transformed with pYES-GFP vector which each had *EgSWEET* gene inserts respectively. Ten-fold serial dilutions of the transformants were transferred to plates with minimal media lacking uracil and containing either **(A)** fructose, **(B)** sucrose, **(C)** glucose, **(D)** galactose or **(E)** maltose. SWEET1 was used as a positive control. VW4000 *S. cerevisiae* negative control was transformed with an empty pYES. Ten-fold serial dilutions of the transformants were transferred to plates with minimal media lacking uracil and containing either maltose, glucose, sucrose, fructose and galactose. All plates were incubated for 3 days at 30ºC and then photographed.
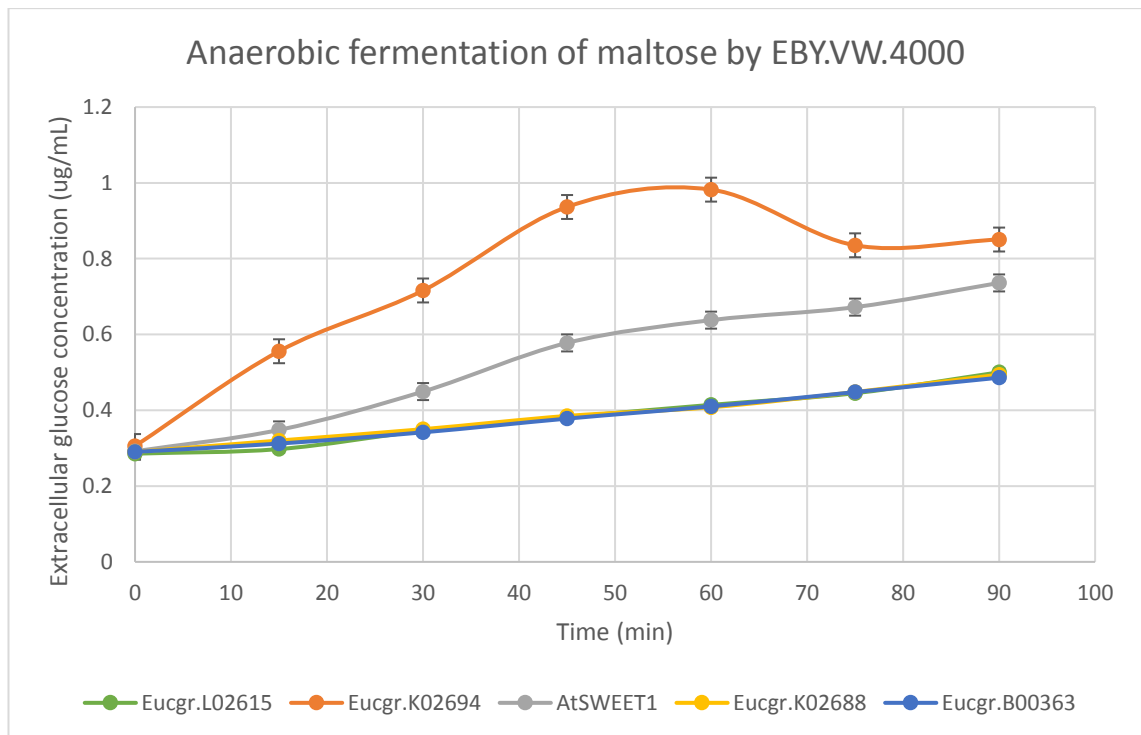
Figure 10. Glucose export activity of heterologously expressed *Eucgr.K02694,*

Eucgr.K02688, Eucgr.B00363, Eucgr.L02615 SWEET-like sugar transporter

proteins in EBY.VW4000 yeast.

2.3.7 Altered expression of *EgSWEET*-like genes in *E. grandis* roots affects carbon export from *E. grandis* roots

We determined whether the amount of carbon transferred from eucalypt to fungi differs depending on fungal lifestyle. $^{13}CO_2$ carbon transfer experiments were performed on *E. grandis* replicates in contact with different fungi (ranging from parasitic, saprotrophic and mutualistic) over a time period of 2 weeks (Fig. 11). Overall the amount of ^{13}C obtained by the fungi showed little difference relative to control fungi that had been grown axenically, with the exception of *P. cinnamomi*. *P. cinnamomi* obtained large amounts of ^{13}C relative to control *P. cinnamomi*. Apart from the eucalypt-*Phytophthora* contact, changes in ^{13}C levels for all fungi were not statistically significant. However there was insufficient time to run all eucalypt-fungal controls, thus these are preliminary results only.

We used stable isotope carbon tracing to determine whether overexpression or repression of *EgSWEET* genes affects carbon transfer from host *E. grandis* to fungal symbionts (Fig. 12). Overexpression of *Eucgr.L02615* showed little effect on carbon transfer, however overexpression of *Eucgr.K02688* greatly increased the amount of ^{13}C transferred from *E. grandis* roots to *P. microcarpus SI-12* relative to empty-vector control (Fig. 12). Repression of *Eucgr.K02694* and *Eucgr.B00363* had no significant effect of carbon transfer. Our results show that increased expression of certain EgSWEETs in the roots of *E. grandis* affects the amount of carbon transferred from the host eucalypt to the associating *Pisolithus*. Although no

changes were significant, these results suggest that the efficiency of sugar transport may differ between families and individual EgSWEETs.

Δd13C (from free living mycelium) of different fungi in contact with *E. grandis*
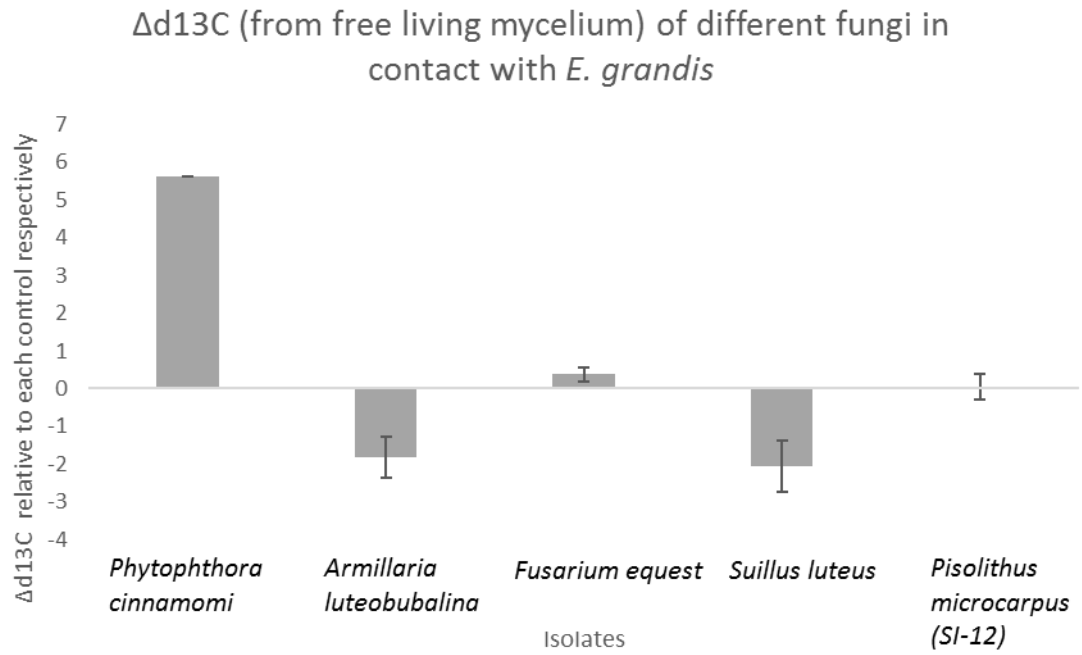
Figure 11. Amount of ^{13}C obtained by different types of fungi when associating with wildtype *E. grandis* relative to each respective control over a time period of 2 weeks. No changes were statistically significant.

Δd13C (from free living mycelium) of SI-12 contacts with mutant eucalypts
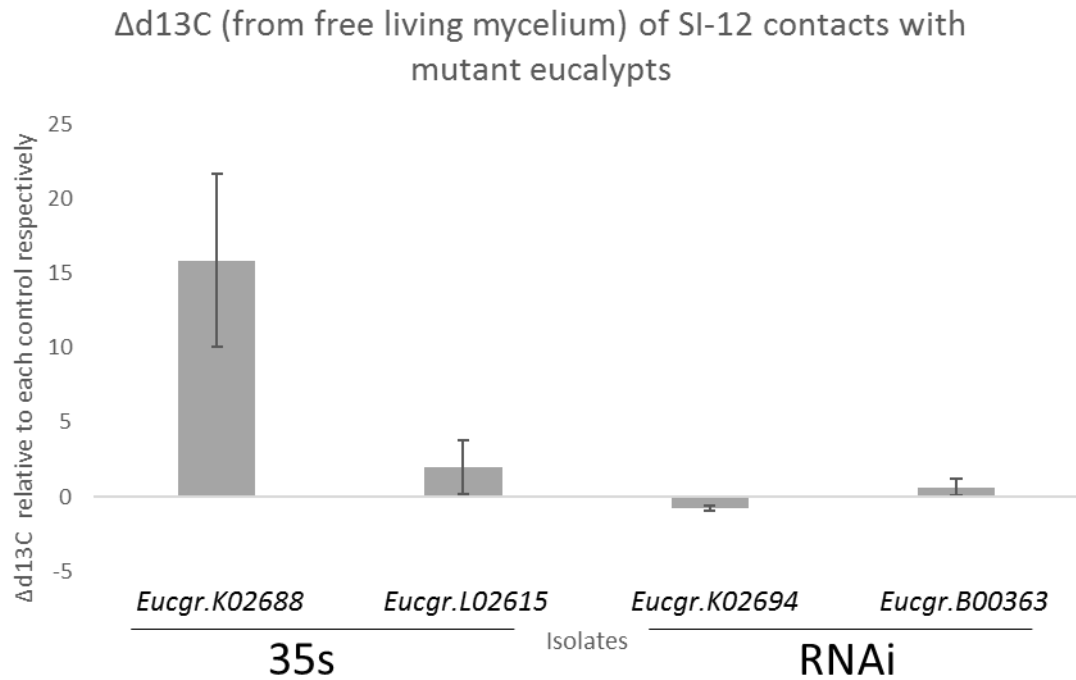
Figure 12. Amount of ^{13}C obtained by SI-12 when associating with mutant eucalypt relative to control SI-12 over a time period of 2 weeks. *EgSWEET K02694 and B00363* were repressed using RNAi knockdown, whereas *EgSWEET K02688* and *L02615* were overexpressed using 35s promoter. No changes were statistically significant.

Table 1. Forward (F) and Reverse (R) primers used for overexpression of

Eucgr.K02694 and *Eucgr.B00363* using 35s promoter, and repression of

Eucgr.K02688 and *Eucgr.L02615* using RNAi knockdown.

Primer name	Primer sequence
35S_EgK02688_F	GGACAAGTTTGTACAAAAAAGCAGGCTCGATGCTTTGGCTGTACTATGCATCC
35S_EgK02688_R	GGACCACTTTGTACAAGAAAGCTGGGTCTTAAACTTCAGATAGAATGGC
35S_EgL02615_F	GGACAAGTTTGTACAAAAAAGCAGGCTCGATGGTCTTTGCTATTGGCCTTC
35S_EgL02615_R	GGACCACTTTGTACAAGAAAGCTGGGTCTCAAACTTCACATGAACTATT
Ri_EgK02694_F	GGACAAGTTTGTACAAAAAAGCAGGCTCGCTCTTCAGCGCGATGCTTTG
Ri_EgK02694_R	GGACCACTTTGTACAAGAAAGCTGGGTCCACGTGTGGTTCCTTTTGCC
Ri_EgB00363_F	GGACAAGTTTGTACAAAAAAGCAGGCTCGGGGCTATCTATGCGCTCCTC
Ri_EgB00363_R	GGACCACTTTGTACAAGAAAGCTGGGTCGGACATCGGTTTCTCTGGCT

2.4 Discussion

Eucalyptus trees have provided human society with many environmental and economic benefits throughout the world. Like all trees, the growth and health of eucalypts depend upon the types of soil microbes (e.g. parasitic, saprotrophic and mutualistic microbes) present within the rhizosphere (i.e. soil area surrounding the root) (Aggangan *et al.* 2013). Despite society's heavy reliance on these trees' growth (e.g. for industrial and medicinal oils, renewable energy source, paper and pulp production, CO_2 sequestration), little is known about the underlying controls and mechanisms used by these trees when interacting with soil microbes. In this study we annotated 52 *E. grandis SWEET*-like genes, we determine the tissue-specific profiles of their expression and compared and contrasted the expression of these genes in roots when in contact with a parasitic, saprotrophic and mutualistic fungi.

We found that there is no tissue specificity of SWEET-like gene expression between the different tissues of *E. grandis*, with the exception of *Eucgr.L01492* (Fig. 5B). Similar to our findings, studies on *G. max* found similar, non-specific expression of *SWEET* genes throughout the plants' tissue (Patil *et al.* 2015). In contrast, one study on *Arabidopsis* found that *AtSWEET11* and *AtSWEET12* are specific and highly expressed in phloem tissue (Chen *et al.* 2014). However, this difference in specificity may be due to the greater number of *SWEET* genes found in *E. grandis* and *G. max* compared to *Arabidopsis.*

While interaction with fungi induced differential expression of *SWEET*-like genes in *E. grandis,* very few of these genes showed expression patterns specific to a particular plant-fungal combination. It was interesting to note that the fewest number of significantly up-regulated *SWEET*-like genes were found in the mutualistic plant-fungal interaction and the largest number of significantly up-regulated *SWEET*-like was in the parasitic plant-fungal interaction. Bacterial and fungal pathogens are known to modulate the expression of plant *SWEET* genes (Chen *et al.* 2010; Chen *et al.* 2014; Liu *et al.* 2011), i.e. *SWEETs* are differentially expressed in root cells colonised by pathogenic bacteria or fungi. Previous studies on annual plants (e.g. *Arabidopsis*) found high expression of *AtSWEET12, AtSWEET4, AtSWEET15* and *AtSWEET17* during pathogen infection (Ferrari *et al.* 2007; Chen *et al.* 2010; Chen *et al.* 2014; Xuan *et al.* 2013). We found that the following genes are up-regulated only in plant-parasitic interactions: *Eucgr.B00360 (AtSWEET4 homologue), H04550 (AtSWEET17 homologue), H04154, K02672 and K02673 (AtSWEET12 homologue), L01492 (AtSWEET1 homologue)*; in addition to other SWEET homologues *F01371 (AtSWEET2 homologue), L01489 (AtSWEET3 homologue), F01609 (AtSWEET9 homologue).* Other similar findings are the up-regulation of *AtSWEET12*, *AtSWEET4*, *AtSWEET15*, and *AtSWEET17* homologues in *E. grandis* roots under all fungal conditions. Also studies on *M. truncatula* shows up-regulation of STP genes, containing a conserved MtN3/saliva (*SWEET-like*) domain, in roots when associating with AM fungi and *Rhizobia* bacteria (Liu *et al.* 2011; Perotto *et al.* 2014). *Arabidopsis AtSWEET11 and AtSWEET12*, both have two MtN3/saliva domains, and function as sucrose

exporters which efflux sucrose from phloem parenchyma cells out into the apoplast (Chen *et al.* 2012). *atsweet11:atsweet12* double mutants showed slower growth, chlorosis, and large amounts levels of sugar and starch accumulation in leaves relative to wild-type (WT) plants (Chen *et al.* 2012).

With large reserves of sugars, plants are commonly targeted by microbes. Bacteria, fungi and oomycetes have all evolved various strategies to proliferate by tapping into the nutritional reserves of plants. Previous research showed that pathogens increase the flow of sugars (such as sucrose and glucose) towards the apoplast, where they grow, by manipulating plant plasma membrane sugar transporters (Chen *et al.* 2010; Cohn *et al.* 2014). However our results suggest that some SWEET proteins (for e.g. *Eucgr.K02694*) may be used by cells to uptake sugars from the external environment (Fig. 9). Interestingly, *Eucgr.K02694* is repressed in mutualistic interactions (at least for *P. microcarpus SI-12*) but highly upregulated during parasitic interactions (at least for *P. cinnamomi*), suggesting that eucalypts may upregulate *Eucgr.K02694* to restrict sugar uptake by pathogens. This result has also been described by Yamada *et al.* (2016) who found that plants retaliate to pathogen infections by reabsorbing the sugars inside their cells, using a select few STPs (i.e. STP13), which results in less sugar in the apoplast and ultimately starves the pathogen.

Our study lays the foundation to further our understanding of the controls surrounding sugar transport in perennial trees during plant-microbe

interactions. In terms of perennial trees, the induction of SWEET-like genes by TAL effectors used by pathogenic, saprotrophic and mutualistic fungi has yet to be explored. Furthermore the effects of elevated CO_2 on the expression of SWEET-like genes under different plant-microbe interactions, the efficiency of sugar transport of each SWEET-like protein and characterisation of these SWEET-like sugar transporter proteins still needs to be done. Understanding these mechanisms could prove useful for increasing *E. grandis* growth and promote stress tolerance, and this warrants further investigation.

Chapter 3 Characterisation of the *Pisolithus albus* effector MiSSP9.7 and its interactant, *Eucalyptus grandis SWI3D*

3.1 Introduction

Plants constantly interact with a wide range of soilborne microbes. Microbes associate with plants to obtain carbon (in the form of sugars) for their own growth and development. These microbes do this by either: forced syphoning host plant sugars (i.e. parasitic/pathogenic interactions), or by exchanging growth limiting nutrients (such as N and P) for the plant's sugars (i.e. mutualistic interactions). Plants are constantly attacked by pathogens, but they commonly associate with mutualistic microbes (Partida-Martinez and Heil 2011; Smith and Read 1995, 1997, 2008). Over 80% of terrestrial plants form mutualistic associations with mycorrhizal fungi (Pellegrin *et al.* 2015) as well as associating with many different species of beneficial bacteria (Franche *et al.* 2009). Thus, plants have evolved signaling pathways and an adaptable immune system that is thought to distinguish and facilitate the establishment of microbes within roots (Jayaraman *et al.* 2012). Exactly how signaling pathways and the plant immune system distinguish between pathogens and mutualists is not fully understood.

To form intimate symbioses with plants, fungi must first overcome or manipulate their hosts' immune system. The plant innate immune system consists of physical barriers (such as the plant cell wall and waxy cuticle

layer protecting the epidermis of plant organs) and chemical defence

mechanisms (such as the hypersensitive cell death response (HR)). To deal

with host immune responses, pathogenic microbes have evolved a plethora

of mechanisms to detoxify host metabolites, hijack host sugar transporters,

hide their presence from host immune receptors or to kill host tissues (Lo

Presti *et al.* 2015; Chen *et al.* 2010; Yang *et al.* 2006; Brown and Tellier

2011; Schmidt and Panstruga 2011; Ciuffetti *et al.* 1997).

Another means by which microbes, in particular pathogenic fungi, directly

manipulate host immunity is through the production of small secreted

proteins called effectors. Effectors broadly describe secreted proteins that

either: kill the host plant (in necrotrophic and hemibiotrophic fungi),

manipulate host cell physiology or suppress the host's immune response (de

Jonge *et al.* 2011; Rafiqi *et al.* 2012; Stassen *et al.* 2011; Bozkurt *et al.* 2012;

Chuma *et al.* 2011; Angot *et al.* 2006; Giraldo *et al.* 2013a, 2013b; Okmen *et

al.* 2014; Stergiopoulos *et al.* 2009, 2013; Zuccaro *et al.* 2014). For example,

necrotrophic fungi (e.g. wheat pathogens *Stagonospora nodorum* and

Pyrenophora tritici-repentis) produce nectrophic effectors, such as secondary

metabolites, toxic polyketides and non-ribosomal peptides, to kill host plant

cells (Horbach *et al.* 2011; Qutob *et al.* 2006; Stergiopoulos *et al.* 2009, 2013;

Oliver *et al.* 2012). Other pathogens initially secrete effectors that suppress

host immune response and allow the pathogen to thrive and eventually kill

the host. This is seen in the plant pathogen *Magnaporthe oryzae* which

secretes SLp1 and AvrPiz-t effectors which suppresses chitin-activated

pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and

cytoplasmic plant immunity (Lo Presti *et al.* 2015). In addition, some small

RNAs have been described as pathogen effectors. *B. cinerea* produce small

RNAs that disrupt *A. thaliana* RNA interference machinery by binding to

AtARGONAUTE1 which selectively silences immunity related genes that are

complementary to these small RNAs (Weiberg *et al.* 2013, 2014).

While effectors have been studied extensively in plant pathogens, mutualistic

fungi also use effectors to suppress the host's immune response to form

mycorrhizal associations and structures (e.g. Hartig net and arbuscules).

Ectomycorrhizal (ECM) fungi secrete many different effectors (Pellegrin *et al.*

2015), called Mycorrhizae induced Small Secreted Proteins (MiSSPs), which

are thought to play a key role for establishing plant-ECM mutualisms (Plett

and Martin 2015; Plett *et al.* 2017). The ECM fungus *L. bicolor* produces the

effector MiSSP7 that interacts with the jasmonic acid co-receptor PtJAZ6 (a

negative regulator of jasmonic acid-induced gene transcription) (Plett *et al.*

2011, 2014a, b). This interaction results in the suppression of jasmonic acid-

induced genes, allowing *L. bicolor* to develop the apoplastic Hartig net and

establish symbiosis (Plett *et al.* 2014a, b). In compatible *Suillus*-plant

pairings, SSP genes were found to be significantly upregulated when

compared to controls (Liao *et al.* 2016). Similarly, to ECM fungi, the use of

effectors to establish mutualisms have been identified in arbuscular

mycorrhizal (AM) fungi. *Rhizofagus irregularis* (formerly *Glomus intaradices*)

secretes the SP7 effector that targets the pathogenesis-related transcription

factor ERF19 in the plant nucleus, which temporarily suppresses the plants

immune response (Klopphoiz *et al.* 2011). *ERF19* is highly upregulated

during infection by the fungal pathogen *Colletotrichum trifolii*, but only temporarily during AM colonisation (Klopphoiz *et al.* 2011). Taken together, these findings suggest that effectors are essential components for the establishment of plant-fungal mutualisms.

The recent release of new ECM fungal genomes has highlighted the presence of dozens more effector like proteins encoded by a range of mycorrhizal fungi (Kohler *et al.* 2015). One of these fungi, *Pisolithus albus,* is of special interest to Australia as it makes up a critical component of forest biota through its symbiotic interaction with the roots of eucalypt trees. The aims of this study were to characterise one of the effector-like genes of the closely related *Pisolithus albus*, called *PaMiSSP9.7* (homologous to Pismi*.*63008) and the potential role it plays during host-fungal interaction. We show that it interacts with SWI3D, a subunit of the SWI/SNF complex implicated in the transcriptional regulation of sugar and hormone related genes.

3.2 Materials and methods

3.2.1 MiSSP9.7-GFP production and absorption by eucalypt root cells

The open reading frame for *PaMiSSP9.7* was amplified from cDNA synthesized using iScript (BioRad) following the manufacturer's instructions. The amplified fragments were gel purified and ligated into a pET22(b)-GFP plasmid using In-Fusion clonase (Clontech), and plasmids transformed in *Escherichia coli* (strain BL21) where *PaMiSSP9.7* was expressed. *PaMiSSP9.7* protein expression and purification was performed following methods described by Shen *et al.* (2009) using GE Healthcare His SpinTrap columns. The eluted protein was purified via dialysis using 1x phosphate buffered saline (PBS pH 7.4) and running on SDS-Page gel to validate the purity of *PaMiSSP9.7*.

To test the up-take of *PaMiSSP9.7* into *E. grandis* root cells, roots of *E. grandis* seedlings were submerged into a MiSSP9.7-GFP solution, and incubated in a growth chamber at 25°C with light for 4 hours. Negative controls were made by submerging roots in either pure GFP without MiSSP9.7 diluted in PBS or in only PBS solution, and treated identically from there onwards. Roots treated with MiSSP9.7-GFP or GFP alone were then rinsed in PBS, severed from the stem of the seedling, fixed in 4% paraformaldehyde and left at 4°C overnight. Afterwards, roots were washed with new PBS and then stained with 1% propidium iodide for 10 mins. Roots were then examined under a Leica SP6 confocal microscope.

3.2.2 Yeast One- and Yeast Two-Hybrid analyses

Yeast I and II hybrid screens were carried out as per Plett *et al.* (2011).

PaMiSSP9.7 was amplified from cDNA synthesized using iScript (BioRad)

following the manufacturer's instructions. The amplified fragments were gel

purified and ligated into pDONR222 and PCR and sequence verified.

Positive inserts were then ligated into pDEST22 plasmids using Gateway

Gene Cloning (Life Technologies) and transformed into *S. cerevisiae* strain

MAV203 using a library scale transformation procedure as per the ProQuest

yeast two-hybrid system protocol (ProQuest catalog number PQ10001-01

and PQ10002-01). One yeast II hybrid analysis was performed and plated

on selective medium (-L-W-H + 25 mM 3-amino-triazol) which tests for

putative interacting protein(s). A further test of protein-protein interaction was

performed using a β-Gal activity assay as described by Walout and Vidal

(2001). After the blue colour was developed for 24 hours at 37°Celsius,

photos were taken of each colony using a Zeiss stereomicroscope with

attached colour camera.

For Yeast I Hybrid assay, bait sequences were constructed by cloning

MiSSP9.7 into pDEST32 plasmid in frame with Gal4-DBD and transformed

into MaV103 cells (mating type a). Self-activation tests for the DBD bait strain

were then performed using the interaction controls described above (Yeast I

and II analyses done by J. Plett).

3.2.3 BiFC testing in *E. grandis*

SWI3D (i.e. *Eucgr.I01261*) and *MiSSP9.7* (i.e. *Pa683008)* genes used in BiFC cloning were inserted directly into the N-labelled version of the pBiBCt-2in1 vector using GATEWAY cloning techniques (Grefen and Blatt, 2012). Firstly, SWI3D and MiSSP9.7 were amplified from *E. grandis* cDNA via PCR and inserted into pDONR221 using BP ligation. These vectors with inserts were then transformed into *E. coli* for cloning. *E. coli* colonies containing the correct vector and insert were identified by growing transformants on LB media supplemented with appropriate antibiotics and then PCR screening colonies to verify colonies containing the correct gene insert based on size. Verified *E. coli* colonies were grown at 37°C overnight and purified using Zippy plasmid purification kit (Zymo). Purified SWI3D and MiSSP9.7 genes in pDONR221 were then ligated into the N-labelled version of the pBiBCt-2in1 vector using LR ligase. The pBiBCt-2in1 vector produced a red fluorescent protein (RFP) signal that enabled identification of successfully transformed cells based on the presence of RFP. Genes inserts were sequence verified and the final construct was transformed into *Agrobacterium tumefaciense* clone GV3101. One positive colony was grown overnight at 28°C in LB supplemented with appropriate antibiotics and then used for transformation. After 48 hours, leaf discs were excised and observed using confocal scanning microscopy. The settings used to observe the yellow fluorescent protein (YFP) was 20% argon power to excite at 488nm excitation followed by emission capture between 520-540nm. For red fluorescent protein (RFP) internal transformation control, we excited the samples at 561nm followed by emission capture between 580-650 nm.

3.2.4 Construction of the phylogenetic tree

To determine the phylogenetic relationships of *E. grandis* SWI3 proteins, we constructed a phylogenetic tree using the online tool 'Phylogeny.fr' (Dereeper *et al.* 2008). *SWI3* gene sequences of plants (*Arabidopsis thaliana (4), Citrus clementina (4), Eucalyptus grandis (4), Medicago truncatula (4), Populus trichocarpa (6)*) were downloaded from the Phytozome database. "One click" phylogenetic analysis was used, with a concatenated MUSCLE alignment adjusted by Gblocks. The tree was rooted with Yeast SWI3 as the outgroup.

3.2.5 Plant and fungal growth conditions

Growth of *E. grandis* seedlings was performed similarly to chapter 2, following the methods outlined in Plett *et al.* (2014a). *E. grandis* seeds obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO, Clayton, Vic., Australia) tree seed centre (Seedlot 21068) were sterilised in 30% hydrogen peroxide for 10 mins followed by 5 washes with sterile water for 5 mins each. Seeds were then transferred onto 1% agar water medium and allowed to germinate at 25°C with a 16/8 hour light/dark cycle. Once germinated, seedlings were transferred to ½ Modified Mylin Norkin (MMN) medium on top of a sterile cellophane membrane to prevent root growth into the medium. *Pisolithus albus* cultures used in this study were propagated at least 4 weeks on 1x MMN before subculturing the outer growing hyphae onto ½ MMN medium covered in a sterile cellophane membrane and grown under a 16/8 h light/dark cycle at 25°C for 2 weeks.

All plant colonisation experiments were carried out by placing eucalypt seedlings directly onto each fungal colony. The contact plates were grouped and labelled as 0 hour (pre-symbiosis contact), 24 hour contacts, 48 hour contacts and 2 week contacts (at least 3 replicates per time group), then all placed in a growth cabinet under a 16/8 h light/dark cycle at 25°C. Eucalypt roots were harvested and frozen directly in liquid nitrogen at each respective time period (i.e. 0 hour, 24 hour, 48 hour and 2 week contact periods).

3.2.6 RNA extraction and Real Time Quantitative PCR (RT-QPCR)

RNA extraction was performed on all contact groups (minimum of 3 replicates per group) using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. A total of 1 μg of RNA for each sample was used to synthesize cDNA using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's instructions. This cDNA was used as a template for RT-QPCR using the Bio-Line Sensifast™ reaction mix and the Bio Rad CFX96 Touch Real-Time PCR Detection System. The reference genes *Eucgr.C00350.1* and *Eucgr.K02046.1*, were utilized to normalize the results.

3.2.7 Generation of SWI3D constructs and *transgenic* eucalypts

Generation of EgSWI3D (i.e. *Eucgr.I01261*) constructs was performed following methods described in chapter 2. *35S:EgSWI3D* and RNAi:*EgSWI3D* were amplified from cDNA synthesized using iScript (BioRad) following the manufacturer's instructions. The amplified DNA fragments were gel purified, ligated into pDONR222 and PCR verified. Positive inserts were then ligated into pH2GW7 (*35S:*) or pH7GWIWG2(II)

(RNAi) vectors using Gateway Gene Cloning (Life Technologies) forming the SWI3D constructs. These SWI3D constructs were then transformed into *Rhizobia rhizogenes* (formerly known as *Agrobacteria rhizogenes*) isolate K599. *E. grandis* seedlings were grown from seed to one month old on 1% agar media. These seedlings were used to generate transgenic roots that either overexpressed or repressed *Eucgr.I01261*.

To generate mutant roots, *E. grandis* seedling roots were cut and removed from the stem using sterile scalpels. The remaining wounded part of the stem was dipped into growing colonies of mutant *R. rhizogenes* containing SWI3D, and grown upside down on ½ MS media for 1 week in a growth cabinet with a constant temperature of 25°C and a 16 hour photoperiod. To prevent death of seedlings by *R. rhizogenes*, *E. grandis* stems were then transferred to ½ MS Timentin (conc. 150 µg/mL) media. Once per week the stems were transferred to new ½ MS Timentin media and grown under the same conditions. Mutant roots became visible usually within 1-2 weeks, but took a total of 3-4 weeks to grow long enough for fungal contact. After 4 weeks of growth, transgenic eucalypts were transferred onto ½ MMN media on top a sterile cellophane membrane and colonies of 2-week-old *Pisolithus albus* isolates (SI-12) placed on top, making direct contact with the roots. These contacts were left for 2 weeks at 25°C with a 16/8 hour light/dark cycle.

3.2.8 Preparation of double stranded interfering RNAs (dsiRNAs) and treatment of roots undergoing colonization by *P. albus*

Using the method of Wang et al. (2016), we targeted *Pisalb.683008* for inhibition of translation using double stranded interfering RNA (dsiRNA). In short, *Pisalb.683008* and *LbMiSSP7* (used as a negative control) were PCR amplified using pairs of gene-specific primers modified to include T7 promoter sequences. The resulted PCR products with T7 promoter flanking at both ends were then purified and used as templates for *in vitro* transcription following manufacturer's instructions of the Riboprobe in vitro Transcription Systems (Promega). The synthesized dsiRNAs were then purified with RNA PowerClean Pro Cleanup Kit (Mo Bio) and eluted in nuclease-free water (dsiRNA production done by J. Wong).

Two-month old *E. grandis* seedlings were put into contact with 12-d-old *P. albus* isolate SI12. For each plant treated, four spots of fungal mycelia in close contact with lateral roots of *E. grandis* seedlings were dosed with 2 µl of purified dsiRNAs (5 ng/µl) every second day for two weeks. Plants were either treated with dsiRNA *Pisalb.683008* (to knock down production of Pisalb.683008 during colonization) or with dsiRNA *LbMiSSP7* (a negative control). DsiRNAs targeting MiSSP7, an unrelated MiSSP gene originated from *Laccaria bicolor* (Martin et al., 2008) was determined to be a proper negative control of the experiment as BLAST results against the *E. grandis* and *P. albus* genomes using the gene sequence of MiSSP7 found no sequence similarity to protein-coding regions of either genomes. After two

weeks of treatment, percent root colonization was assessed and samples were taken for microscopy and performed RNA extraction.

3.2.9 Microscopy of transgenic/dsiRNA treated eucalypt roots and Hartig net measurements

Transgenic or dsiRNA treated *E. grandis-P. albus contacts* described above were assessed for percent mycorrhization before severing the roots from stems, where they were fixed in 4% paraformaldehyde and left at 4°C overnight. Roots were then embedded in 6% agarose and mycorrhizal root tips were sectioned on a Campden Instruments vibratome into 30μm cross sections. Cross sections were stained with 1% propidium iodide for 10 mins, washed with PBS and stained again with WGA-FITC and rinsed in PBS (Embedding and cross sectioning done by K. Plett). Roots were then examined under a Leica SP6 confocal microscope and photos taken. Hartig net depths were calculated using ImageJ software. Controls were fixed, stained and examined identically to the test samples.

3.3 Results

3.3.1 *MiSSP9.7* encodes an effector protein that enters plant root cells

We tested the up-take of Pa683008-GFP (MiSSP9.7-GFP) by *E. grandis* roots in the absence of *P. albus*. We observed fluorescence within the nucleus of host cells, indicating MiSSP9.7-GFP localised to the nucleus (Fig. 13B). The merge of the two signals with bright-field confirms this. The negative control (i.e. GFP with no attached protein) was not visibly taken up into host cells under our experimental conditions (Fig. 13F). Since PI localised to the nucleus and the GFP signal co-localized with the PI signal (Fig. 13E), we can conclude that the MiSSP9.7 enters the nucleus.

MiSSP9.7 entry into *E. grandis* roots
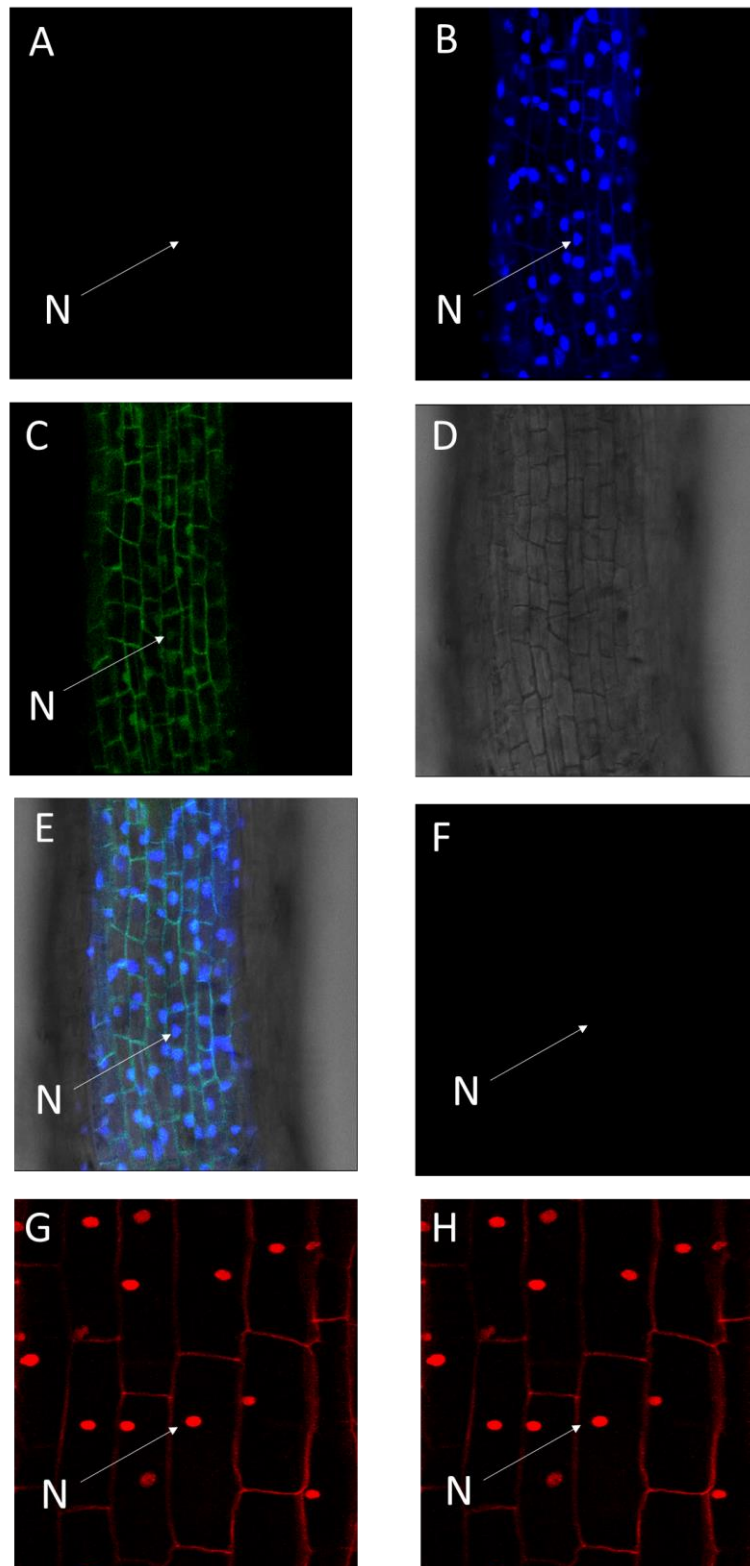
Figure 13. Images of MiSSP9.7-GFP (i.e. Pa683008-GFP) and Propidium Iodide (PI) stain taken using confocal microscopy. The first panel shows auto-fluorescence (Fig. 13A) and the nucleus (N) is shown in each panel. PI (blue fluorescence) (Fig. 13B) and MiSSP9.7-GFP (green fluorescence) (Fig. 13C) co-localised within the nucleus of cells (Fig. 13E). Negative controls show GFP only without any attached protein (Fig. 13F), PI only (Fig. 13G) and a merge of the two negative control images (Fig. 1H). GFP alone (i.e. without any attached protein) was not able to enter into root cells (Fig. 13F, H).

3.3.2 MiSSP9.7 interacts with a chromatin remodelling complex (CRC)

subunit of *E. grandis*

Because the MiSSP9.7 localises to the nucleus in host plant cells, we

determined if there were any nuclear proteins of *E. grandis* that interact with

this effector (Fig. 14). Using a yeast II hybrid we determined that MiSSP9.7

interacts with SWI3D (Eucgr.I01261), a subunit of the SWI/SNF CRC. This

interaction was compared to three controls: a strong positive interaction

(Krev1/RalGDS-*wt*), a weak positive interaction (Krev1/RalGDS-*m1*) and two

proteins that do not interact (Krev1/RalGDS-*m2*). Yeast colonies under 3AT

selection and X-Gal tests showed that MiSSP7.9: Eucgr.I01261 closely

resembled a weak positive interaction (Fig. 14).

This interaction was further confirmed using Bi-Fluorescent Complementation

(BiFC) (Fig. 15). *Nicotiana benthamiana* leaf cells were transformed with

pBiFCt-2in1-NN (containing MiSSP9.7 and SWI3D) in which positive

transformants are identified by constitutive expression of red fluorescent

protein (RFP) (Fig. 15 third pane from left). Examination of these cells using

confocal microscopy showed a strong YFP signal, showing that 683008 and

SWI3D interacted causing the two halves of YFP to join and fluoresce (Fig.

15). A merge of the two signals with bright-field shows that the interaction is

occurring in the nucleus of the cell (right most pane).
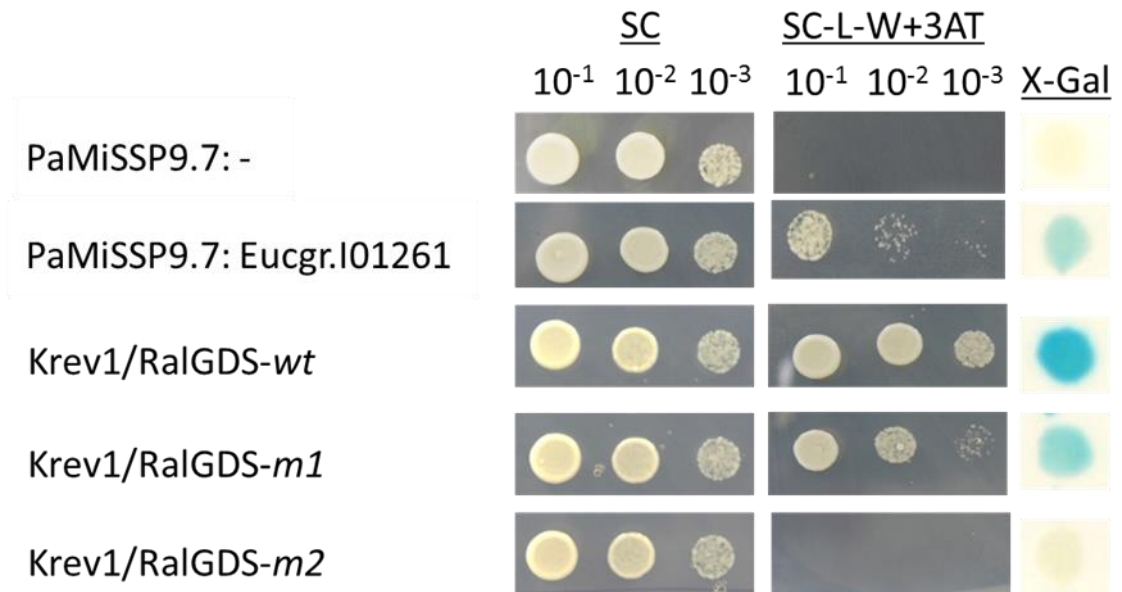
Figure 14. MiSSP9.7 (i.e. Pa683008) interacts with *E. grandis* nuclear protein
SWI3D (*Eucgr.I01260*). The interaction is compared to three control interactions
where Krev1/RalGDS-*wt* is a strong positive interaction, Krev1/RalGDS-*m1* is a
weak interacting pair, and Krev1/RalGDS-*m2* are two proteins that show no
detectable interaction.

MiSSP9.7 + SWI3D
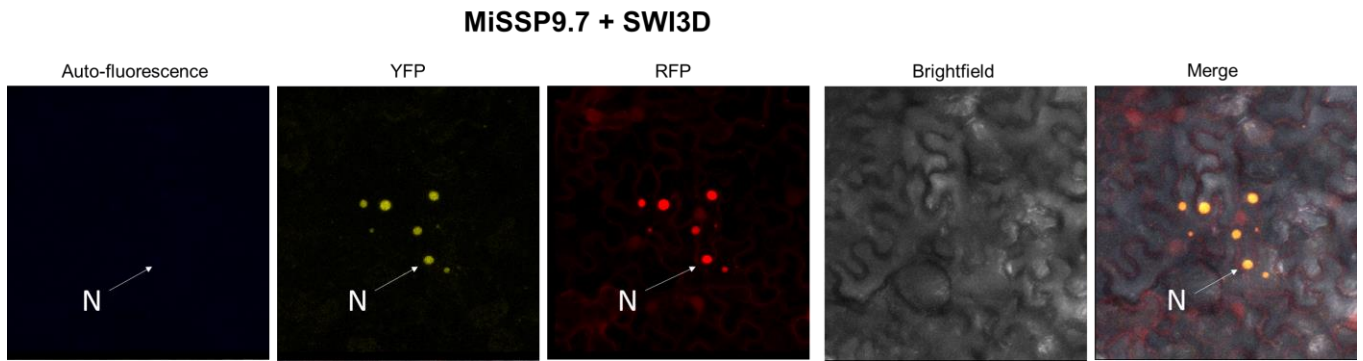
Figure 15. *In vivo* BiFC proof of the interaction between 683008 and SWI3D. *N. benthamiana* leaf cells transformed with vector containing both genes, where transformed cells are denoted by constitutive expression of RFP (third panel), interaction between 683008 and SWI3D is shown by the reconstitution of the YFP signal (second panel) and the merge of the two signals with bright-field (right most panel).

3.3.3 Phylogenetic relationships of *E. grandis* SWI3 proteins

A total of 4 *SWI3* genes were identified in the *E. grandis* genome using the Phytozome database (available at http://phytozome.jgi.doe.gov/pz/portal.html last accessed: 28/3/17) based on homology to *Arabidopsis* SWI3 subunits, which have been proven form part of the SWI/SNF complex (Sarnowska *et al.* 2016), as a template, in addition to keyword searches (Fig. 16). Similar numbers of *SWI3* genes were found to be encoded by the *E. grandis* genome when compared to annual plants and other trees genomes (*Eucalyptus grandis (4), Arabidopsis thaliana (4), Citrus clementina (4), Medicago truncatula (4), Populus trichocarpa (6)*) (Fig. 16).
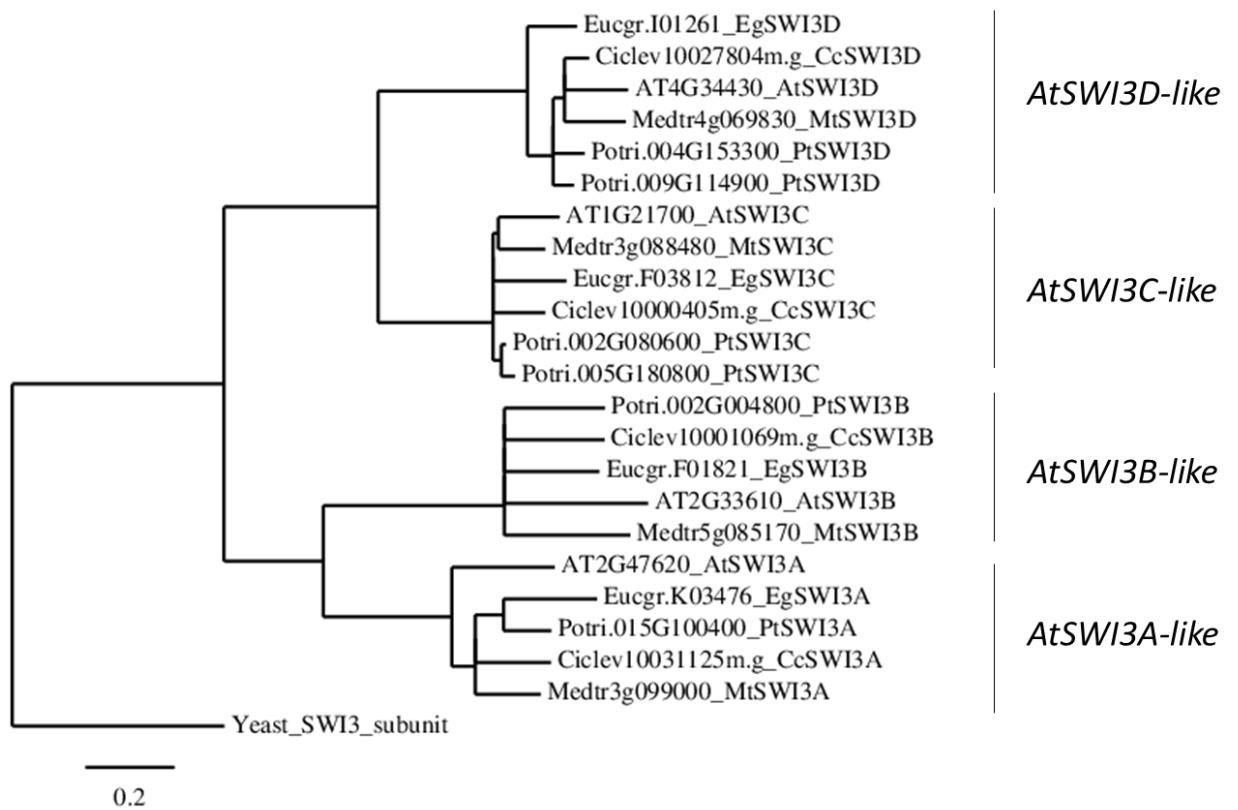
Figure 16. Phylogenetic relationships between SWI3 proteins collected from different species. *A. thaliana, C. clementina, E. grandis, M. truncatula, P. trichocarpa*. The tree was rooted with *S. cerevisiae* Yeast SWI3 subunit as the outgroup.

3.3.4 SWI3 gene expression in *E. grandis* roots over a two-week time course of colonization by *P. albus*

Since all four SWI3 subunits play a role in the SWI/SNF complex, we determined the gene expression of all four eucalypt *SWI3* genes (*SWI3A, B, C and D*) using quantitative PCR (QPCR), during pre- and post-colonisation of *E. grandis* roots by *P. albus* over a 2 week time period. The time points were 24 hour in-direct contact (i.e. pre-symbiosis signaling), 24 hour direct contact, 48 hour direct contact and 2 week colonized root tissues. We found that *SWI3D* was significantly upregulated in roots during pre-symbiosis signaling and 48 hours after contact (Fig. 17). *SWI3A* was significantly up-regulated during pre-symbiotic signaling and then later was significantly repressed. *SWI3B* was significantly repressed at during the early stages of physical interaction between *E. grandis* and *P. albus*. *SWI3C* transcription was not significantly altered.
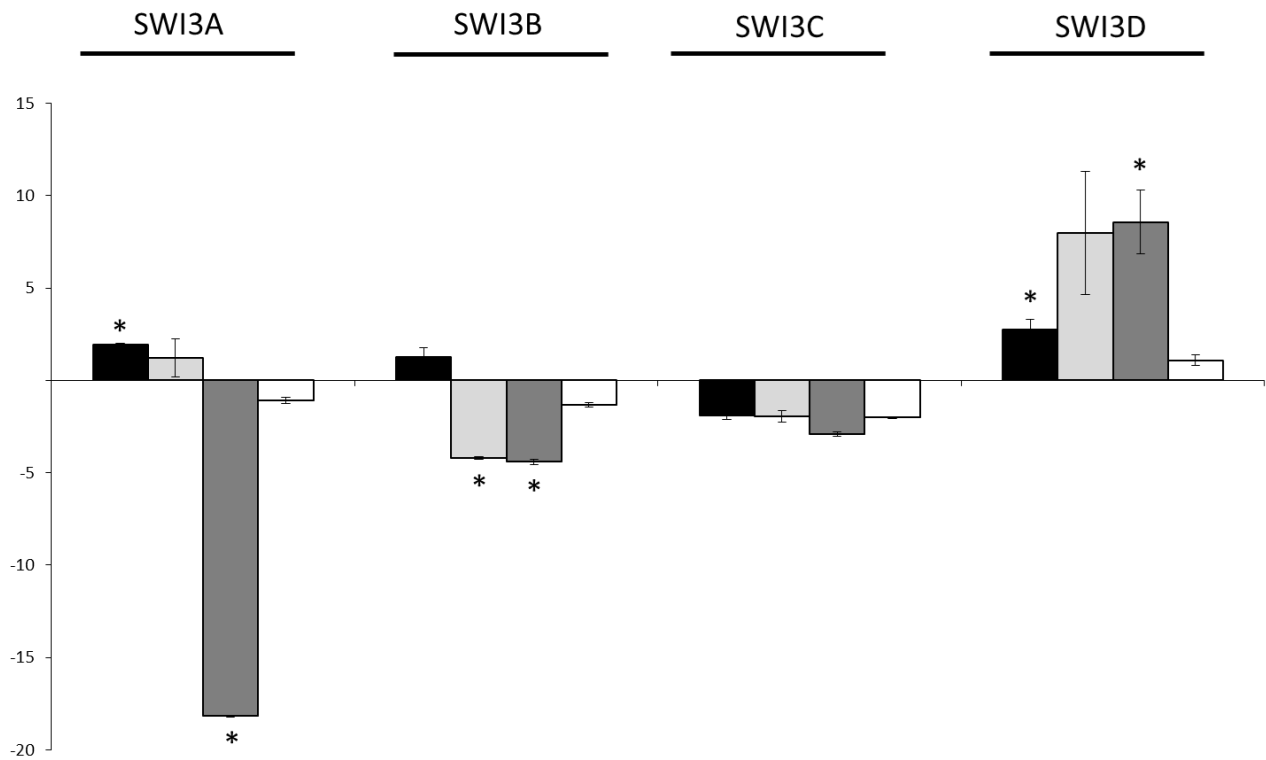
Figure 17. RT-QPCR of *SWI3* genes in *E. grandis* over a time course of 2 weeks. Black bars indicate 24 hour pre-symbiotic contact, light grey bars represent expression at 24 hours post physical contact between *E. grandis* roots and *P. albus;* dark grey bars are expression values after 48 hours of direct contact and white bars are expression values in mature mycorrhizal root tips. All values are reported as fold change from gene expression levels in axenically grown *E. grandis* roots. $\pm$ SE; * indicate statistically significant findings (p value < 0.05).

3.3.5 Altered expression of *MiSSP9.7* significantly affects *P. albus* Hartig net development during colonisation

We transgenically altered the expression of *SWI3D* in the roots of *E. grandis*, but this had little effect on Hartig net development (Fig. 18A). The percentage of mycorrhizal roots formed were lower in *SWI3D* overexpressed mutants compared to wildtype eucalypts and *SWI3D* repressed mutants (Fig. 19). However, wildtype and *SWI3D* repressed values were not statistically significant.

We repressed the expression of *MiSSP9.7* in *P. albus* (using dsiRNA) when in contact with *E. grandis* and found that Hartig net depth significantly increased compared to the control eucalypt-*P. albus* contacts (treated with *Lb*MiSSP7 dsiRNA; Fig. 18B). This result suggests repression of *MISSP9.7* may be beneficial to mycorrhization during eucalypt-*Pisolithus* symbioses.
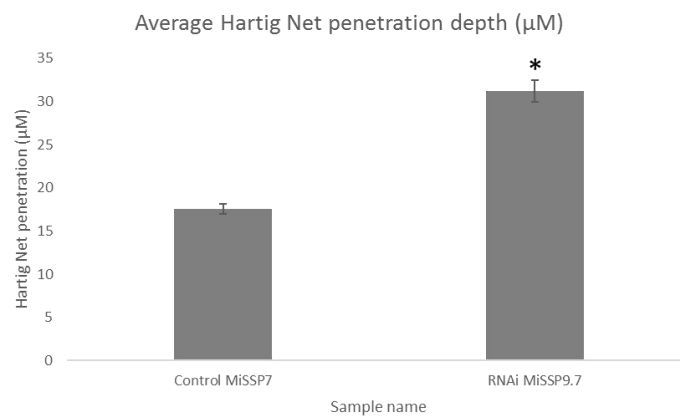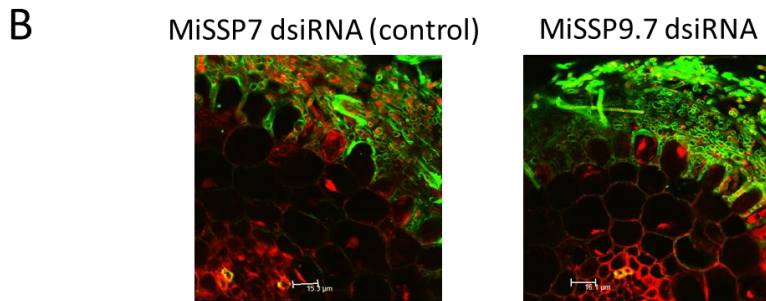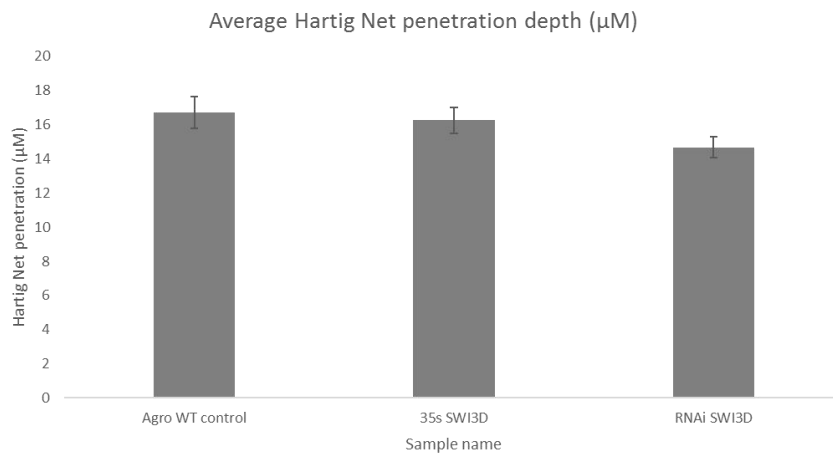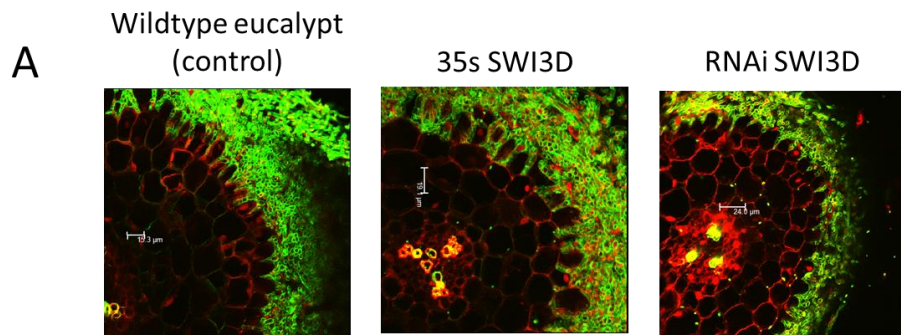
Figure 18. Altered expression of *SWI3D* (A) or repression of MiSSP9.7 (Pa683008) by dsiRNA (B) and its effect on Hartig net development. * indicates statistically significant findings (p value < 0.05).

% Mycorrhization of SWI3D mutant eucalypts
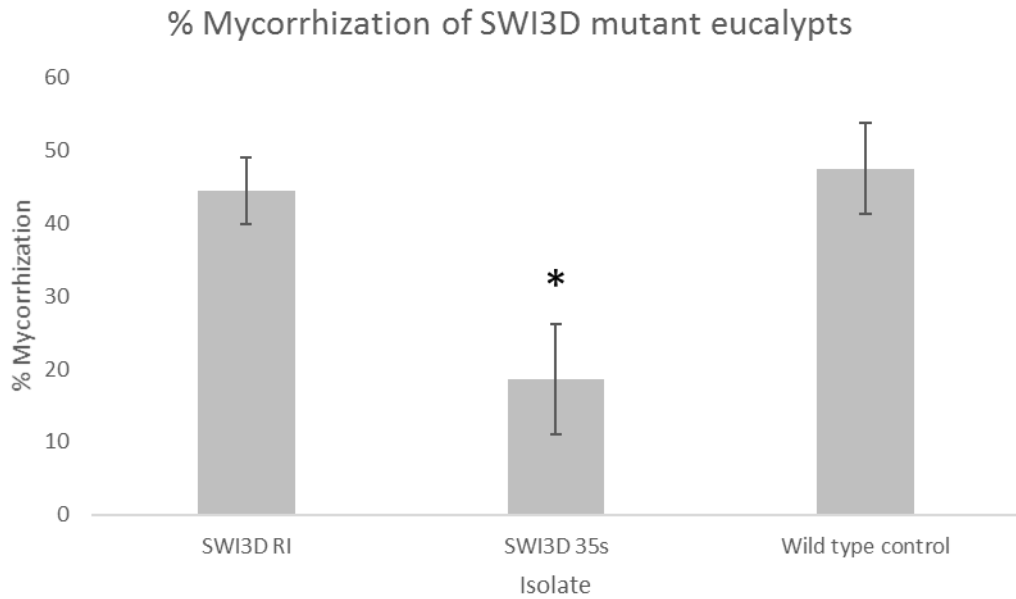
Figure 19. Percent mycorrhization of *SWI3D* (i.e. *Eucgr.I01261*) transgenic

eucalypts. *Eucgr.I01261* was overexpressed (using 35s promoter) in 3 *E. grandis*

replicates and repressed (using RNAi knockdown) in another 3 replicates.

Percentage of mycorrhized roots were counted after 2 weeks of contact with

Pisolithus albus cultures. Wild type control eucalypts were treated identically to the

mutants, except for altering *SWI3D* expression. * indicates statistically significant

findings (p value < 0.05).

3.4 Discussion

Plants are constantly confronted by a range of different soil microbes (in particular parasitic and mutualistic microbes) present within the rhizosphere. Plants must associate with mutualistic fungi to improve their survivability, while defending against pathogens. The mechanisms used by plants to do this is still currently being researched (Rey *et al.* 2015; Plett *et al.* 2015). Effectors are signaling molecules secreted by fungi that are used to communicate to plants (Lo Presti *et al.* 2015; Kloppholz *et al.* 2011; Plett *et al.* 2011, 2014a, 2014b, 2015). Plants in turn, respond in many different ways depending on the types of effectors secreted. Indeed, plants respond differently to effectors secreted by parasitic fungi, compared to plants responding to effectors secreted by mutualistic fungi (Lo Presti *et al.* 2015; Rafiqi *et al.* 2012; Plett *et al.* 2011, 2014a, 2014b, 2015; Klopphoiz *et al.* 2011). Our research describes how the MiSSP9.7 effector modulates ECM-plant fungal interactions and formation of mycorrhizal structures. We determined that MiSSP9.7 enters host root cells and localizes to the nucleus. We further show that MiSSP9.7 interacts with a CRC subunit (SWI3D) and we characterise the expression of *SWI3* genes in eucalypt roots in contact with *P. albus*. Finally, we show that differential expression of either SWI3D or MiSSP9.7 can alter the outcome of mycorrhization.

The SWI/SNF complex has been characterized in other plant systems where it directly regulates multiple plant hormone pathways, including auxin (IAA), Abscisic Acid (ABA), Jasmonic Acid (JA), Ethylene (ET), Salicylic acid (SA) and cytokinins (Sarnowska *et al.* 2016). Plants hormones such as SA, JA,

and ethylene (ET) play major roles in regulating plant defence responses. SA is involved in the reaction against biotrophic and hemi-biotrophic pathogens, while JA and ET are associated with defence against necrotrophic pathogens and herbivorous insects. Increased (SA) accumulation and signaling repress biotrophic pathogens but encourage the colonization of plant tissues by ECM fungi (Plett *et al.* 2014a, b; Tschaplinski *et al.* 2014; Lebeis *et al.* 2015). Therefore, *P. albus* may be able to indirectly modulate host immune responses by using certain effectors that bind to essential DNA regulating complexes, such as the SWI/SNF CRC.

We found that there is no difference in Hartig net formation when transgenically over-expressing or repressing *SWI3D* in *E. grandis* roots (Fig. 18). Overexpression of SWI3D, however, lowers the percentage of root tips colonized. Reduced expression of MiSSP9.7 however caused significant increases in the Hartig net root penetration depth into host root cells (Fig. 18). This result is counter-intuitive as all previously characterized mutualistic effectors (Plett *et al.* 2011; Kloppholz *et al.* 2011; Plett *et al.* 2014a, b) have been found to repress host immunity and increase Hartig net formation. These results would suggest that mycorrhizal fungi also encode effector proteins that curb the colonization success of the fungus. This may be a mechanism by which the ECM fungus is able to remain non-pathogenic and still benefit the host. This result, however, will require further experimentation to substantiate.

This study provides further insight for understanding the controls used during plant-fungal interactions. Our results suggest that pre-symbiotic signaling ECM fungi, such as *Pisolithus*, release effectors from hyphae that bind to nuclear proteins within plant root cells, which then regulate transcription Understanding this mechanism could prove useful for increasing *E. grandis* colonisation by beneficial microbes and prevent disease and deserves further investigation.

Chapter 4 Conclusion and Future Perspective

In this study, we identified and categorised 52 *SWEET*-like genes of *E. grandis.* We further determined that there was no tissue-specific expression profiles of the identified *E. grandis SWEET*-like genes throughout seedling tissues. Additionally, we also characterised the expression of these genes in roots when in contact with pathogenic, saprotrophic and mutualistic fungi. We identified 9 eucalypt *SWEET-like* genes that were only upregulated in plant-parasitic interactions. Finally, we characterised further 4 SWEET-like proteins.

This study expanded our understanding of the role of SWEETs in plant-microbe interactions. In contrast with previous SWEET studies, this study determined the role of SWEETs in a large, long-lived perennial tree species (i.e. eucalypts) that has the largest number of *SWEET* genes known to date. Previous studies have only studied SWEETs in small annual plants, such as *Arabidopsis*, rice and legumes. While other studies have provided evidence for SWEET involvements in parasitic plant-microbe interactions, the role of SWEETs in mutualistic plant-microbe interactions was largely unknown. This study revealed that only a few *SWEET* genes were upregulated in mutualistic plant-microbe interactions compared to parasitic plant-microbe interactions.

In addition to eucalypt SWEET characterisation, we also characterised MiSSP9.7 and the SWI3D subunit of the eucalypt SWI/SNF complex. We identified that MiSSP9.7 localises within the nucleus of host plant cells. We determined that the MiSSP9.7 effector interacts with the eucalypt SWI3D

subunit belonging to the SWI/SNF CRC complex. We further performed qPCR over a time course of 2 weeks on four *E. grandis* SWI/SNF complex subunits (i.e. SWI3A, B, C, and D). Lastly, we found that repressing *MiSSP9.7* significantly affects Hartig net penetration depth during mycorrhization. Although a number of studies have shown that the SWI/SNF complex plays a role in plant defence and plant-microbe interactions, to our knowledge no other study has determined the role of SWI3 subunits in plant-microbe interactions, let alone mutualistic plant-microbe interactions. Moreover, to our knowledge no other study has determined the role the MiSSP9.7 effector during plant-microbe interactions. This study expanded our understanding of the effectors ECM fungi use to communicate with plants, as well as transcriptional regulation in mutualistic plant-microbe interactions.

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