Expression profiling marker genes of the salicylic acid and methyl jasmonate signalling pathways in *Eucalyptus grandis*

by

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This thesis is dedicated to

my late grandfather:

Mr. P. Govender

We miss you

May you rest in peace

TABLE OF CONTENTS

Appendices

DECLARATION

I, Ronishree Naidoo, hereby declare that the dissertation submitted herewith for the degree *Magister Scientiae* at the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other university.

Ronishree Naidoo

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PREFACE

Eucalyptus spp. constitute a significant portion of the pulp and paper production sector within the South African forestry industry. The wood and fibre property of these trees is the reason they are so widely planted. Due to short fibres and thinner fibre walls, the pulp generated from *Eucalyptus* produces paper that has a smooth surface which is ideal for writing and printing. Additionally, the oils and other secreted compounds produced by these trees are used in the manufacturing of numerous cosmetic products. However, these trees are affected by various pathogens and pests throughout their lifetime that reduce the quality of the pulp that is produced. Due to the fact that over 40% of the paper and pulp generated by the South African forestry industry relies primarily on *Eucalyptus* spp. production, a means to reduce the losses caused by pathogens is needed. One such possibility lies in understanding the defence architecture of eucalypts.

The mechanisms that are employed by plants to deter pathogens have been mainly investigated in model organisms such as *Arabidopsis thaliana*. It has been established that a pathogen can be detected by the host either through receptors located in the cell membrane or through proteins within the cell. Once the pathogen has been perceived by the host, a cascade of defences are activated which includes amongst other processes the increase of reactive oxygen species, the initiation of defence signalling pathways and the expression of proteins that inhibit migration of the pathogen. In particular the phytohormones salicylic acid (SA) and jasmonic acid (JA) have been extensively investigated in various pathosystems. This is because the activation of these pathways creates environments that are unfavourable to the proliferation of biotrophic and necrotrophic pathogens respectively. The activation of these pathways has also been shown to be associated with an increase in expression of certain pathogenesis-related proteins.

These proteins have hence been established as markers of the induction of the signalling pathways and could be targets for improving plant defence. A stepping stone for improving our understanding of the *Eucalyptus* defence would be to identify genes associated with the salicylic acid and jasmonic acid signalling pathways. Using the information from *Arabidopsis* as well the genome sequence of *E. grandis* one can begin to decipher how well the information from model organisms transcends to *Eucalyptus.*

Therefore the **aim of this MSc** study was to identify putative orthologs in *E. grandis* for known marker genes of the SA, and JA signalling pathways based on information from model organisms. Additionally the expression pattern of these putative orthologs in *E. grandis* under various treatments was investigated.

Chapter 1 encompasses literature pertaining to the knowledge of plant defence as known from model species. This chapter begins with information on how the pathogen is perceived by the host and then progresses to the processes that are activated in an attempt to confine the pathogen. The biosynthesis and interaction of the plant phytohormones associated with plant defence will be highlighted. Furthermore, an explanation and motivation for employing *Chrysoporthe austroafricana* as the pathogen of interest is provided in this chapter.

Chapter 2 of this dissertation encompasses the approach taken to identify and characterize the expression pattern of putative defence orthologs for the SA and MeJA signalling pathways in *E. grandis*. Upon commencement of this study, only the 4x draft genome sequence was available and therefore these sequences were used as the basis for the initial phylogenetic identification of putative orthologs. When the latest version of the genome was released in 2011, the selected orthologs were verified on Phytozome v7.0. Additionally, the expression pattern of the putative markers was assessed under

mock induction of the signalling pathways at various concentrations of a phytohormone and over a period of time. The hypothesis that SA and MeJA pathways exhibit an antagonistic relationship was also examined in this study in terms of suppression or induction of the putative markers. A canker pathogen, *C. austroafricana* was employed to elucidate the difference in response of the orthologous markers to pathogen attack in a tolerant and susceptible host.

Final conclusions, implications and future prospects are outlined and discussed at the end of this dissertation in the **Concluding Remarks** section.

The research findings that encompass this study represent the outcomes undertaken from March 2009 to January 2011 in the Department of Genetics, University of Pretoria under the supervision of Dr. S. Naidoo and co-supervision of Prof. D.K. Berger and Prof A.A. Myburg. The following congress presentations were generated based on the results obtained from this study:

Naidoo R., Berger D.K., Myburg A.A and Naidoo S. 2011. Profiling candidate defence marker gene orthologs expressed in response to treatment with MeJA and SA in *Eucalyptus grandis* trees. Southern African Society for Plant Pathology (SASPP). January 23-26. Berg-en-Dal, Kruger Park, South Africa. (Oral Presentation).

Naidoo R., Berger D.K., Myburg A.A and Naidoo S. 2011. Expression profiling of putative *Eucalyptus grandis* defence marker genes in response to treatment with methyl jasmonate and salicylic acid. IUFRO Tree Biotechnology Conference S5 P18. June 26 – July 2. Arraial d'Ajuda, Bahia, Brazil. (Awarded 2nd place for best poster presentation) (Published online in BMC proceedings)

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ABBREVIATIONS

bp	Base pair
cDNA	Complementary DNA
Egr	*E. grandis*
Egu	*E. grandis x E. urophylla*
ETI	Effector Triggered Immunity
hrs	Hours
hpt	Hours post treatment
HR	Hypersensitive response
JA	Jasmonic acid
LOX	Lipoxygenase
ML	Maximum Likelihood
MeJA	Methyl jasmonate
MAMP/PAMP	Microbial-Pathogen associated molecular patterns
NJ	Neighbour Joining
PTI	PAMP Triggered Immunity
PR	Pathogenesis-related
PRR	Pattern Recognition Receptors
RT-qPCR	Real-Time Quantitative PCR
SA	Salicylic acid
TTSS	Type III Secretion System
wpi	Weeks post inoculation

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

LIST OF TABLES

CHAPTER 1

LITERATURE REVIEW

PLANT DEFENCE:

A SURVEY FROM *ARABIDOPSIS* TO FOREST TREES

1.1 Introduction

Eucalyptus species, hybrids and clones are increasingly planted in various regions of the world because of their commercially valuable wood and paper properties. Due to this reason, much research is being invested in the successful propagation of these essential trees. An obstacle that needs to be overcome in this endeavour is the understanding of the environment in which these trees are found. Within this environment, there exists various fungal and bacterial pathogens that pose a threat to *Eucalyptus* species. These pathogens include the causal agents of stem canker disease namely *Cytospora* spp. (Burgess and Wingfield 2002), *Endothiella* spp. (Burgess and Wingfield 2002) and *Chrysoporthe* spp. (Nakabonge *et al.* 2006) as well as the fungal leaf pathogen *Mycosphaerella* spp. (Hunter *et al.* 2006), the bacterial root pathogen, *Ralstonia solanacearum* (Coutinho *et al.* 2000) and the oomycete *Phytophthora* spp. (Maseko *et al.* 2007). Elucidating the interaction of these pathogens with *Eucalyptus* will be of great importance to the forestry industry as it will allow for the production of resistant or tolerant plantations in future.

Many of these pathogens have been characterized and studied in the model organism, *Arabidopsis thaliana*, which has in many aspects provided a glimpse into the complex relationship that exists between pathogens and their respective hosts. In association with these model organisms, advances in genomic tools have allowed researchers to enhance our knowledge of complex plant-pathogen interactions by identifying key defence genes required during resistance. However, despite the vast knowledge in model organisms very little information exists pertaining to defence in *Eucalyptus*. Part of this research will look at how the information from model organisms transcends to trees. The complete genome sequence of *Eucalyptus* is currently available and this indispensable resource can be exploited to further assist in understanding tree-pathogen

interactions. Using the information known in *Arabidopsis* as well as the *Eucalyptus* genome sequence, the study at hand aims to elucidate part of the complex defence network in this valuable tree. In particular this study will focus on the putative orthologs of marker genes associated with induction of the salicylic acid and methyl jasmonate signalling pathways. The long-term contribution of this project, in combination with future studies, will be to provide the Forestry industry with the means to enhance tree-breeding programs and improve *Eucalyptus via* genetic modification.

1.2 Plant defence

Plants are sessile organisms but they are challenged with a wide variety of biotic and abiotic factors. Over the course of evolution, they have developed complex mechanisms to defend themselves hence ensuring their survival. Plant defence against an invading organism is highly intricate and multifaceted but with the use of model organisms such as *Arabidopsis thaliana,* researchers have been able to improve our understanding of what occurs within the host.

1.2.1 *Arabidopsis thaliana*

A. thaliana has widely been used to study various aspects of plant development. This angiosperm is commonly known as thale cress or mouse-ear cress and forms part of the *Brassicaceae* family. Due to its importance in plant research, a project to sequence the genome of *Arabidopsis* was initiated in 1990 and completed in December 2000 (www.*arabidopsis*.org). The genome consists of 125MB encoding approximately 30 000 genes (TAIR, version 10). This diploid plant is ideal as a model organism due its numerous advantages amongst which includes its small size, short generation time and the relative ease of transforming. Other than its use to study plant development, this organism is also widely used to study numerous plant-pathogen interactions. This is

because many pathogens are able to infect *A. thaliana* and cause disease as it would a natural host. Examples of these include *Pseudomonas syringae, Ralstonia solanacearum, Alternaria brassicicola* and *Xanthamonas campestris* (Simpson and Johnson 1990; Schenk *et al.* 2000; Hirsch 2002; Kim *et al.* 2008). A significant portion of our knowledge regarding plant defence has stemmed from research using this model organism.

Plant defence can be broadly divided into two types of resistance namely vertical resistance and horizontal resistance. Basal defence, also known as horizontal resistance is comprised of a substantial number of defence genes involved in various biochemical pathways which are effective against a broad range of pathogens (Agrios 2005). With horizontal resistance, a host is neither completely susceptible nor completely resistant. This type of resistance is weaker than vertical resistance however, it is more challenging for the pathogen to overcome because of the numerous aspects involved in horizontal resistance. On the other hand vertical resistance, also known as the *R-Avr* interaction, involves the interaction of a few specific resistance (*R*) genes that counteract the effect of a pathogen's avirulence (*Avr*) genes. Vertical resistance enables the plant to induce a strong defence response against certain pathogens due to the high specificity of the *R-Avr* interaction (Agrios 2005). However, this specialized aspect of vertical resistance is inadequate for the survival of the host in terms of evolution because new virulent pathogens constantly arise which are able to avoid recognition by the R proteins. Both horizontal and vertical resistance activate various signalling pathways that alter the host in order to prevent pathogen proliferation. These different aspects of resistance will be discussed in the following sections.

1.2.2 Horizontal resistance – Broad spectrum defence

The first response activated when the presence of a pathogen is detected is known as the basal or innate defence system. This primary line of defence is based on the recognition of a pathogen by receptors located in the transmembrane region of the cell, known as Pattern Recognition Receptors (PRRs) (Jones and Dangl 2006). Exudes of pathogens such as flagellin, lipopolysaccharides (LPS) and peptidoglycans are perceived by these receptors (Figure 1.1a) (Jones and Dangl 2006). These exudates/molecular structures are collectively known as Microbial – or Pathogen – Associated Molecular Patterns (MAMP/PAMPs). These MAMP/PAMPs are structurally and molecularly different from the receptors encoded by the host and, as a result, the host is able to distinguish between self and non-self molecules (Kim *et al.* 2008). This distinction is crucial for the activation of defences specific to pathogen invasion (Kim *et al.* 2008).

The responses triggered by MAMP/PAMPs can be divided into the early, intermediate and late phases. Early stages which occur during the first few seconds or minutes, involves an oxidative burst as well as changes in ion fluxes across the plasma membrane (Zipfel and Robatzek 2010). The intermediate stage occurs during the next few hours and involves responses such as stomatal closure, ethylene production and transcriptional response. Lastly, the final stage which occurs over the days following invasion by the pathogen, involves processes such as callose deposition and SA accumulation (Zipfel and Robatzek 2010). PAMP-triggered immunity (PTI) which occurs as result of the interaction between the MAMPs/PAMPs and cell PRRs activate various defence related genes in a signalling cascade that attempts to confer resistance against the pathogen (Figure 1.1b). The signalling cascade consists of the salicylic acid, jasmonic acid and ethylene pathways as well as other plant hormones.

Various pathogenesis-related (PR) genes are up-regulated in response to this interaction in an attempt to confine pathogen propagation (Kim *et al.* 2008). Examples of *PR* genes include β-1,3-glucanase and antifungal isozymes of chitanase which can induce a hypersensitive response (Section 1.2.4.1) (Agrios 2005). As illustrated in figure 1.1, a PAMP/MAMP receptor located in the cell wall perceives the pathogen by recognizing the bacterial flagellin exudes (Figure 1.1b). This recognition triggers MAMP/PAMP - induced plant defences such as signalling cascades and the up-regulation of PR genes. Although PTI is generally weaker than ETI, it is more effective against a diverse array of pathogens because it encompasses various plant physiological processes. Initially it may be challenging for a pathogen to induce disease on the host due to the collective involvement of the defence processes, however over the course of evolution pathogens adapt to overcome these barriers. This is known as the host-pathogen evolutionary arms race (Stavrinides *et al.* 2008). It is called the arms race because each attempt by the host to detect and eliminate an invading pathogen is counteracted by a pathogen that has evolved mechanisms to avoid this detection system and cause disease in the host.

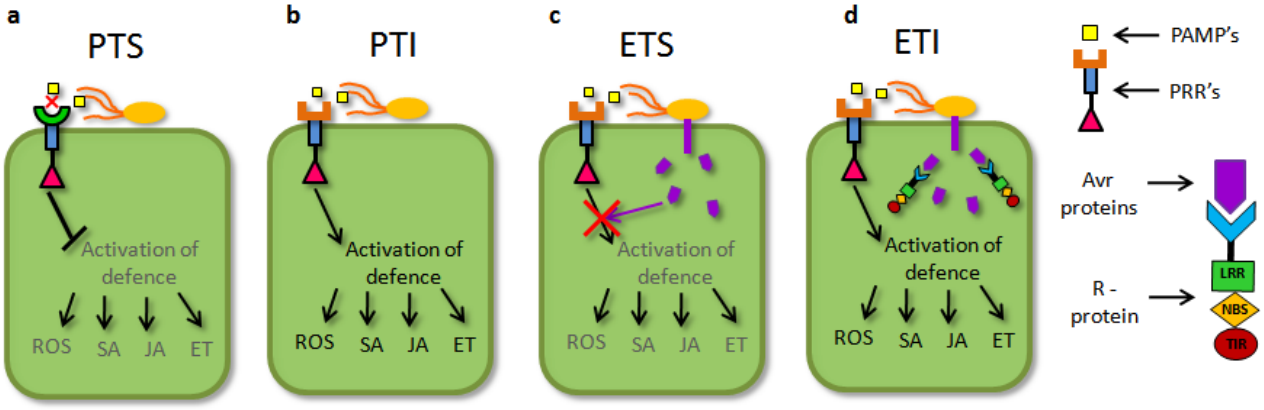

Figure 1.1: Evolution of the plant-pathogen arms race – (a) Pathogen Triggered Susceptibility (PTS) - Prior to the evolution of receptors, pathogens were able to colonize the host without inducing a defence response. (b) Pathogen Triggered Immunity (PTI) - Receptors located in the transmembrane region react to MAMP/PAMPs such as flagellin secreted by the pathogen. This interaction initiates the defence cascade and resistance ensues. (c) Effector Triggered Susceptibility (ETS) - In response to these receptors, pathogens devised a method to avoid detection and this is achieved by the secretion of effector molecules *via* TTSS. This bypass tactic allows the pathogen to initiate disease on its host and confer susceptibility. (d) Effector Triggered Immunity (ETI) - As a countermeasure to this line of attack, plants evolved *R* genes which the proteins thereof subdue the effector molecules (Adapted from Pieterse *et al.* 2009).

1.2.3 Vertical resistance - *R* – *Avr* interaction

An example of the perpetuating arms race mentioned above is the evolution of the type III secretion system used by pathogens to directly secrete effector molecules into the host cell. Effectors are used as a means to bypass or disrupt the defence system and cause disease (Stavrinides *et al.* 2008). One of these strategies is the development and utilization of the type III secretion system (TTSS). These TTSS effectors are encoded by *Avr* genes and target various aspects of the defence network such as blocking cell wall strengthening *via* inhibiting callose deposition, manipulation of signalling pathways to promote pathogen promulgation and interfering with programmed cell death initiated by

defence response genes (Figure 1.1c) (Grant *et al.* 2006; Kim *et al.* 2008; Pieterse *et al.* 2009). The pathogen may also use the effectors to manipulate the host secretion of extracellular proteins thereby allowing infiltration of the host's resources (Kaffarnik *et al.* 2009). A recent study has revealed that these effectors also inhibit the expression of PAMP-responsive miRNA's or miRNA biogenesis and stability (Navarro *et al.* 2008b). By suppressing or interfering with RNA silencing, the pathogen is able to propagate within the host and cause disease (Navarro *et al.* 2008b).

The tactics utilized by pathogens need to be counteracted by the host and therefore novel defence mechanisms need to be devised to prevent colonization by pathogens. In response to the evolution of Avr effector proteins emitted *via* TTSS, plants have evolved *R* genes to inhibit the effect these molecules have on the defence network (Jones and Dangl 2006). The latter is known as Effector-Triggered Immunity and is mediated by R proteins (ETI) (Figure 1.1d) (Jones and Dangl 2006). When an *R* gene is able to positively recognize a particular effector molecule as non-self, activation of defence genes and signalling pathways ensue (Dangl 2001; Kim *et al.* 2008). This type of reaction is referred to as an incompatible interaction. However, when an *R* gene is unable to identify an effector molecule, the plant perceives no signal to induce defence responses and as a result the invading pathogen is able to colonize the host (Dangl 2001). This type of reaction is referred to as a compatible interaction. In an interaction between poplar and *Melampsora larici-populina*, there was a strong induction of defence responses within 48 hours post inoculation in the incompatible interaction. Concomitantly, in a compatible interaction, there was only a low level of defence gene expression (Rinaldi *et al.* 2007; Boyle *et al.* 2010). This denotes the importance of timing of the activation of defence genes and the level of gene expression as it has a pivotal role in determining the outcome of an interaction.

The interaction between R proteins and their corresponding effector molecules may either be direct or indirect. Many R proteins are structurally related and the signalling pathways that they activate are in many cases highly similar (Kim *et al.* 2008). Various R proteins consist of a leucine - rich repeat (LRR) as well as a nucleotide binding site (NBS) domain (Tameling and Takken 2008). The NBS-LRR family can be subdivided based on the presence or absence of a Toll-interleukin-1 receptor-like domain (TIR) and are known as TIR-NBS-LRRs (TNL). The TNL group represents the largest gene family in *Arabidopsis* consisting of 94 genes, whereas in *Populus* only 91 were identified (Kohler *et al.* 2008). If the TIR domain is absent, a coiled coil motif is usually present in its place and this group of R proteins are referred to as the CC-NBS-LRRs (CNL) or non-TNL's (Tameling and Takken 2008). The non TNL group represents the largest gene family in *Populus* comprising of approximately 225 genes (Kohler *et al.* 2008). It is interesting to note that *Populus* contains 34 predicted R proteins that have been shown to contain a zinc finger DNA-binding domain (Kohler *et al.* 2008). However to date, there has been no report of a DNA-binding domain present in *Arabidopsis R* genes (Veluthakkal and Dasgupta 2010). This could be evident to the evolution of *R* genes within tree species that derived through sequence divergence for adaptability in different environments.

The direct interaction, first described by Flor in 1971 and termed the "gene-for-gene" model, was based on a single R protein altering the effect of a single effector molecule (Agrios 2005). *R* genes have been identified in various tree species such as *Populus trichocarpa, E. grandis*, *Pinus taeda, Pinus lambertiana* and hybrid poplar (Veluthakkal and Dasgupta 2010). An example of this is found to occur between *E. grandis* and *Puccina psidii.* Resistance to this causal agent of rust disease is hypothesized to be mediated by the *Ppr1* gene (*P. psidii* resistance gene 1) (Junghans *et al.* 2003;

Veluthakkal and Dasgupta 2010). Another poplar *R* gene, *PtDrl01* (*P. tomentosa* disease resistance-like 01) was over-expressed in tobacco and conferred long-term resistance to tobacco mosaic virus (TMV). This long-term resistance in tobacco was mediated by the activation of various PR proteins (Zheng *et al.* 2011). Although evidence for the "gene-for-gene" model is limited, theoretical modelling of R proteins suggests that direct recognition can lead to the rapid evolution of new virulent phenotypes (Dodds *et al.* 2006).

Conversely, the most prominent type of relationship found to occur between the R proteins and the effectors is an indirect interaction. The R proteins monitor the influence of the effectors on various other plant proteins that the pathogen would manipulate in order to facilitate its propagation (Grant *et al.* 2006). This phenomenon is known as the "Guard hypothesis" because the R proteins oversee various proteins against the effect of virulence factors and observe any alteration induced by the effectors (Grant *et al.* 2006; Jones and Dangl 2006). A notable example of this is found in the interaction between *Arabidopsis thaliana* and *Pseudomonas syringae*. In this association, a protein known as RIN4 interacts with the R protein, RPM1 as well as with the Avr proteins, AvrRPM1 and AvrB (Kim *et al.* 2008). Phosphorylation of RIN4 by the Avr proteins is detected by RPM1 and as a result defence responses are induced to confer resistance against the invading pathogen (Kim *et al.* 2008). Following perception of a pathogen, the host activates a suite of defences including phytohormones and PR genes in an attempt to confine the host. These defences will be reviewed in the subsequent sections.

1.2.4 Induced Defence

When a pathogen is detected by the host, either through PAMP receptors or *via* R proteins, various aspects of defence are activated to confine the pathogen and resist infection. It has been shown that although the signalling pathways activated downstream of PTI and ETI are similar, distinct differences do exist. MAMP/PAMPs are also highly conserved among various microbial groups including those that are pathogenic and non-pathogenic. It would not be beneficial to the plant to activate a strong prolonged defence response to non-pathogenic organisms as this would lower the overall fitness of the host (Tsuda and Katagiri 2010). Instead, PTI has evolved to allow the plant to activate defence signals at a low level in order to assess if the invading organism poses a threat or not. Conversely, ETI is a highly specific interaction between the pathogen and the host and, due to this specificity, it allows the host to fine-tune its defences (Figure 1.2). The defences activated during ETI are more robust and persist for longer periods of time than those activated during PTI (Figure 1.2) (Tsuda and Katagiri 2010).

The signalling pathways are activated synergistically and at a low level during PTI whereas a more compensatory or “sector switching” mechanism is employed during ETI (Sato *et al.* 2010; Tsuda and Katagiri 2010) (Figure 1.2). This is because, should the pathogen overcome one of the pathways e.g. the SA pathway during ETI, the host can then switch to another pathway such as JA or ET without severely affecting its overall fitness (Figure 1.3). Signalling pathways such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are induced during pathogen invasion and as a result of this induction other responses such as programmed cell death and systemic acquired resistance follow (Berrocal-Lobo and Molina 2008).

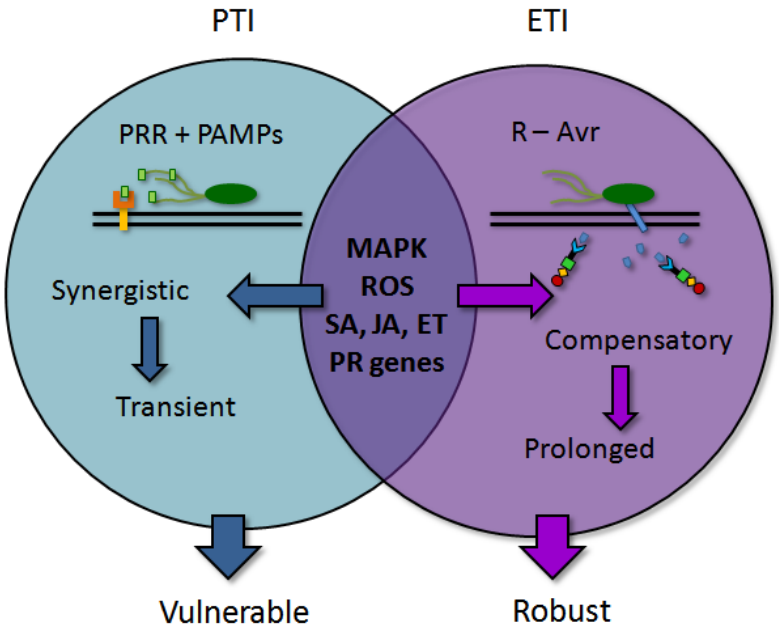

Figure 1.2: Differences between PTI and ETI – This diagram illustrates the differences between PTI and ETI with respect to how the various defence signalling mechanism are utilized. PTI has a transient period of activated defences whereas ETI has a more prolonged period (Adapted from Tsuda and Katagiri 2010).

As seen in figure 1.3, the defence network activated in response to a pathogen can be complicated, however the host is able to fine tune this network and induce specific responses required for particular pathogens. Biotrophic pathogens are vulnerable to the affects of the SA signalling pathway whereas necrotrophic pathogens and herbivorous insects are more susceptible to the JA/ET pathway (Pieterse *et al.* 2009; Verhage *et al.* 2010). In addition to the well studied pathways namely SA, JA and ET, other role players such as abscisic acid (ABA), gibberellin signalling (GA) and auxin signalling, cytokinins (CK) and brassinosteriods (BR) have all been recently shown to also have an effect on the outcome of resistance or susceptibility (Figure 1.3) (Pieterse *et al.* 2009). These signalling pathways and the interactions between them will be discussed in more detail in the subsequent sections.

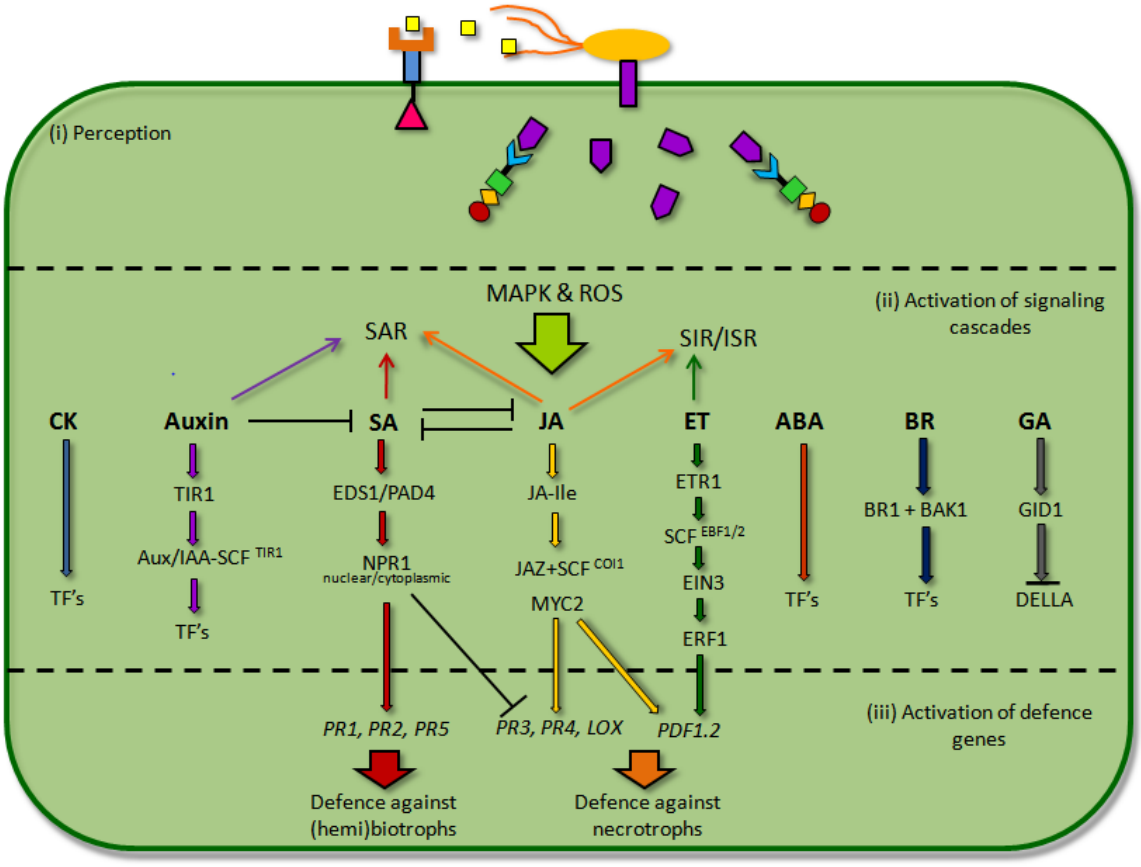

Figure 1.3: Signalling pathways activated during plant defence in *Arabidopsis thaliana* in response to a pathogen – (i) Perception – The invading pathogen may be perceived by the host either through receptors in the cell wall or through R proteins. (ii) Activation of signalling cascades – Once the pathogen is perceived various signalling pathways are activated. SA, JA and ET comprise the backbone of phytohormone signalling with the various other pathways (ABA, GA, BR, CK, Auxin) feeding into and interacting with these pathways. (iii) Activation of defence genes - The signalling pathways activate the expression of various PR proteins. These pathways are also fine-tuned according to the type of pathogen that is perceived (Adapted from Bari and Jones 2009 and Pieterse *et al.* 2009).

1.2.4.1 Hypersensitive response and Reactive oxygen species

During early defence responses, an increase in the levels of reactive oxygen species (ROS) such as superoxide ions (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH) produced from the reduction of H_2O_2 *via* electron transfer occurs. An increase in the level of these products is observed within the host cell undergoing pathogen invasion and thus is known as the oxidative burst (Lamb and Dixon 1997; Yoshioka *et al.* 2008). In resistant plants, a second more prolonged oxidative burst is perceived shortly after invasion by a pathogen (Lamb and Dixon 1997). Therefore the first oxidative burst occurs in non-specific responses whereas the second oxidative burst is dependent on exclusive interactions such as those caused by the production of effector molecules (Lamb and Dixon 1997). These reactive oxygen species have been shown to be involved in multiple roles during defence and these include: microcidal effects, cell wall strengthening *via* lignification and the induction of other defence - associated genes (Thatcher 2005). Callose deposition can also occur at the site of infection due to the production of ROS such as singlet oxygen ($^1O_2$) and/or O_2^- (Vellosillo *et al.* 2010).

Different cellular compartments are involved in the utilization of various ROS products including the apoplast, chloroplast, peroxisomes and mitochondria (Vellosillo *et al.* 2010). The chloroplast in particular has been shown to have a pivotal role by mediating ROS production and utilization during plant defence (Belhaj *et al.* 2009). In an interaction between *Phytophtora brassicae* and *Arabidopsis,* a chloroplast encoding gene *RPH1* (Resistance to Phytophtora 1) was shown to have a vital role in enhancing resistance. This was done by increasing the levels of H_2O_2 as well as having an effect on the expression of defence-related genes (Belhaj *et al.* 2009). Mutants of this gene showed enhanced susceptibility to infection by the pathogen. In tobacco, ROS generated from the chloroplast is crucial for the progression of localized cell death but it is not required

for defence gene induction when the host is challenged with *Xanthomonas campestris* pv. *vesicatoria (*Zurbriggen *et al. 2009)*.

Pathogens have devised tactics to subdue the effects of ROS as seen with the causal agent of maize smut disease, *Ustilago maydis*. This pathogen uses a gene called Yap1 (Yeast AP-1 like) to subdue the effects of ROS in particular by detoxifying H_2O_2 (Molina and Kahmann 2007). As a result, the pathogen is able to cope with the early defence strategy of the host thereby causing infection. Although the oxidative burst is employed by the host as a defence strategy, it has been shown to be used by some pathogens to induce infection. The causal agent of rice blast disease, *Magnaporthe grisea* has been shown to undergo a specialized oxidative burst of its own during plant infection (Egan *et al.* 2007). Initiation of the disease requires the production of superoxide which results in the development of infection structures known as appressoria (Egan *et al.* 2007). Since ROS production can occur in the mitochondria, this plant organelle may be targeted by pathogens to suppress the defence mechanisms (Vellosillo *et al.* 2010).

An increase in ROS has also been shown to be the early phase of the hypersensitive response (HR). HR is usually associated with an increase in plant cell death following pathogen invasion and is activated by the host to confine pathogen proliferation and limit nutrient uptake (Thatcher 2005). This can be illustrated in the interaction between *Fagus sylvatica* (European Beech) and the gall forming insects *Mikiola fagi* and *Hartigiola annulipes*. In this insect-host system, the HR response was pivotal in hindering the development of the galling lavae resulting in their eventual death. The HR response reduced the formation of galling larvae by 77% and the remainder of the galls were destroyed by natural enemies (Fernandes *et al.* 2003). Although HR is costly to the plant, in this scenario the HR is more beneficial and would have a lower impact on the fitness of the host as opposed to festering galls.

Other than the levels of ROS, calcium influx and the level of glutathione (GSH) have both been shown to orchestrate HR. The *Arabidopsis* CYCLIC NUCLEOTIDE GATED CHANNEL 2 (CNGC2) is involved in the movement of Ca^{2+} into cells and the bacterial PAMP elicitor LPS has been shown to activate this channel (Ali *et al.* 2007). A mutation in this channel abolished HR and therefore it was concluded that calcium has an integral role in HR (Ali *et al.* 2007; Mur *et al.* 2008). In addition to ROS having a role in the induction of HR, nitric oxide (NO) has also been shown to have some involvement. The balance between NO and H_2O_2 is critical for the induction and control of plant cell death caused as a result of the HR response (Yoshioka *et al.* 2011). NO contributes to plant immunity by activating the mitogen activated protein kinase (MAPK) cascade which subsequently increases the expression of numerous defence related genes (Yoshioka *et al.* 2011). In tobacco leaves inoculated with *Botrytis cinerea*, both ROS and NO were shown to have accumulated in the area surrounding the infection site. However, in this interaction ROS was beneficial for the spread of the lesion whereas NO was central in curbing the spread of the disease (Asai and Yoshioka 2009).

Initially it was thought that HR is a process activated in response to the pathogen, however recent evidence suggests that HR may be the result of other processes occurring during defence. Processes such as the oxidative burst, nitric oxide and the signalling pathway salicylic acid have been shown to be sufficient to confer resistance (Mur *et al.* 2008). Yet these processes cause extensive damage to cellular organelles such as the mitochondria and therefore it has been hypothesized that HR may be a secondary effect due to the other processes (Mur *et al.* 2008). Further investigation into this theory is still required. Nonetheless the HR response does have a vital role in confining the pathogen and it is also involved in the activation of another important defence response known as Systemic Acquired Resistance (SAR).

1.2.4.2 Systemic Acquired Resistance and Salicylic Acid

1.2.4.2.1 Biosynthesis and metabolism of Salicylic Acid

SA forms part of the benzoic acid phytohormone group that is synthesized by the plant and is involved in a diverse range of physiological processes such as flowering, thermogenesis and disease resistance (Wildermuth 2006; Vlot *et al.* 2009). The role of this hormone in pathogen resistance has been extensively studied and it has been shown that SA plays has an integral part in the activation and sustenance of SAR. Additionally SA also influences the activation of other signalling pathways such as ET and JA e.g. SA and JA are known to exhibit an antagonistic relationship (Loake and Grant 2007). Within the plant, SA can be synthesized by one of two pathways involving either phenylalanine ammonia lyase (PAL) or isochorismate synthase (ICS). Both these pathways begin with the common metabolite chorismate which is obtained *via* the shikimate pathway (Figure 1.4) (Shah 2003; Chen *et al.* 2009; Vlot *et al.* 2009). The majority of the SA generated during a plant defence response is synthesized *via* the ICS pathway (Wildermuth *et al.* 2001). Following synthesis, SA can be converted to SA *O*-β-glucoside (SAG) which is the storage form of the hormone. Additionally SA can be conjugated to amino acids or be converted to the less common derivative salicyloyl glucose ester (SGE). During an SAR response, SA can be converted to either methyl salicylate (MeSA) or its glucosylated derivative methyl salicylate *O*-β-glucoside (MeSAG) (Figure 1.4) (Vlot *et al.* 2009).

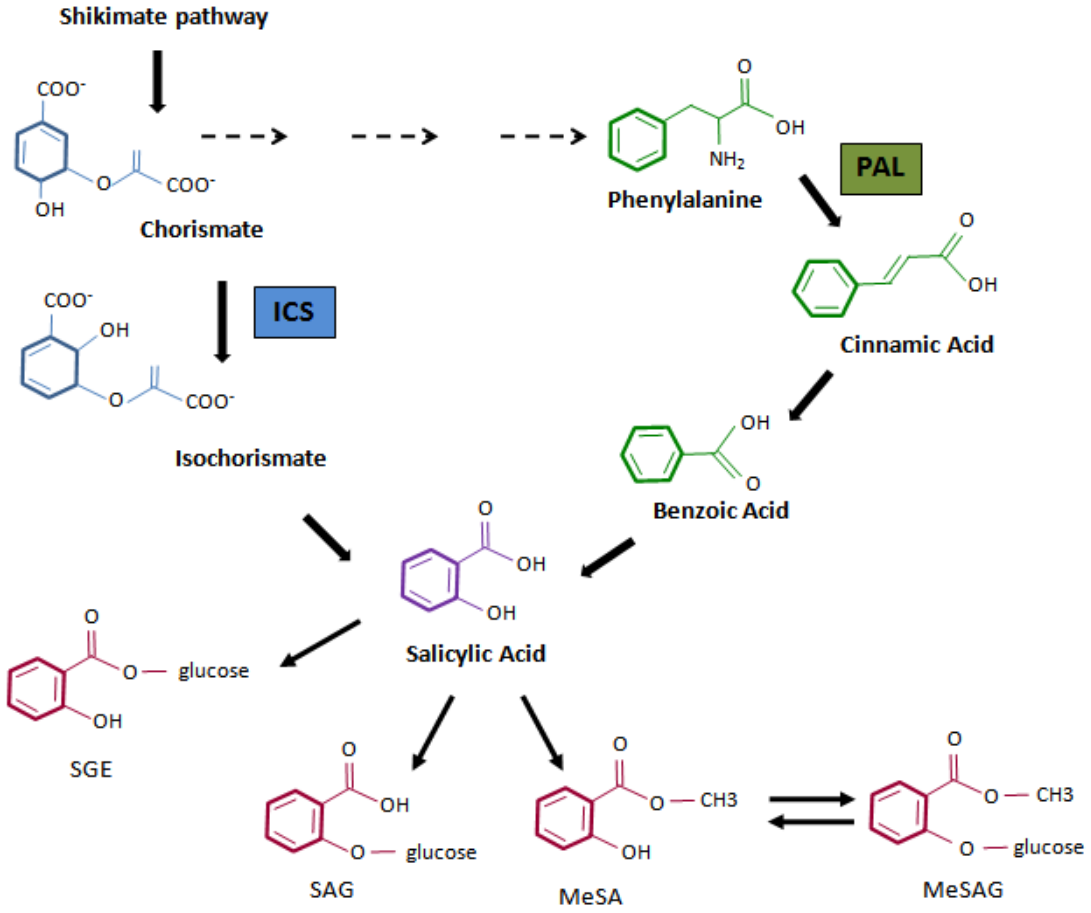

Figure 1.4: Biosynthesis and metabolism pathway of SA – SA can be synthesized either *via* the ICS pathway which is the predominant pathway for defence responses or *via* the PAL pathway. Once synthesized, SA can be metabolized to form on the following conjugates: salicyloyl glucose ester (SGE), SA *O*-β-glucoside (SAG), methyl salicylate (MeSA), methyl salicylate *O*-β-glucoside (MeSAG) (Adapted from Shah 2003, Chen *et al.* 2009 and Vlot *et al.* 2009).

The chorismate pathway has previously been shown to be localized to the chloroplasts and therefore it has be proposed that the synthesis of SA may also occur within these organelles (Garcion *et al.* 2008). This hypothesis was investigated by using transgenic *Arabidopsis* lines over-expressing the sodium hydroxylate (*NahG)* gene in combination with a chloroplast targeting sequence. The *NahG* gene prevents an accumulation of SA by converting it to catecol and therefore in transgenic plants that harbour this gene, only

low SA levels are detected (Gaffney *et al.* 1993). Under abiotic stress e.g. *P. syringae*, transgenic plants expressing chloroplast-localized NahG exhibited a decrease in the levels of SA compared to the wild type (Fragnière *et al.* 2011). Taken together with the premise that ICS is localized to the chloroplast, it can be inferred that SA biosynthesis could be targeted to the chloroplastic region (Fragnière *et al.* 2011). Further investigation into this hypothesis however is still required as it is not known how SA moves from the chloroplast to the cytoplasm where it is presumed to be active.

1.2.4.2.2 Induction and maintenance of SAR

Subsequent to the formation of necrotic lesions associated with HR, a distinct signalling pathway is activated in healthy areas of the plant that have not been infected. This long distance signalling is referred to as SAR (Ryals 1996). SAR results in the durable resistance of systemic regions of the plant to a wide range of bacteria, fungi and viruses (Ryals 1996). Therefore this type of resistance is also known as broad-spectrum resistance. SAR is usually associated with an increase in the levels of salicylic acid however this is not the signal that is transported to systemic tissue. Instead, the SA derivative methyl salicylate (MeSA) is the signal transmitted to uninfected regions of the plant where an enzyme known as salicylic acid-binding protein 2 (SABP2) converts MeSA to SA (Figure 1.5) (Park *et al.* 2007).

This conversion triggers SAR in systemic tissue by initiating the SA positive feedback loop (Vlot *et al.* 2008). Due to the fact that SA inhibits SABP2 in the infected tissue, the latter feedback loop ensures that there is sufficient accumulation of the signal (Forouhar *et al.* 2005). Vasculature-associated SAR is thought to be mediated by nitric oxide, in particular the levels of a protein known as *S*-nitrosothiols (SNOs) (Vlot *et al.* 2008). Over-expression of *S*-nitrosoglutathione reductase (GSNOR), which controls the levels

of *S*-nitrosylated proteins, leads to reduced levels of SNOs and, as a result, suppression of SAR ensues (Rusterucci *et al.* 2007). Expectedly, in GSNOR antisense plants, SAR is enhanced. Due to the fact that GSNOR is situated in the phloem, SAR signalling can be regulated through the vascular structure (Rusterucci *et al.* 2007). Induction of SAR can additionally be activated through volatile signals emitted through the wounding responses of neighbouring plants *via* interplant communication.

In hybrid poplar (*P. simonii x P. pyramidalis*), MeSA and MeJA have been found to elicit a defence response in plants adjacent to ones that have been damaged through cutting or wounding (Hu *et al.* 2009). These volatile compounds may enter the neighbouring plant *via* the stomata and be hydrolyzed into SA and JA in the cytoplasm (Hu *et al.* 2009). The receptors for these inducers have not been identified in poplar as yet, however it has been demonstrated that MeSA and MeJA do play a significant role in interplant communication (Hu *et al.* 2009).

HR does contribute to the induction of the SAR signalling mechanism, however it is not indispensable nor is it the only signal required for this process to occur. In the much exploited model pathosystem between *Arabidopsis-Pseudomonas*, it was demonstrated that PAMPs such as flagellin and LPS can elicit SAR without the formation of a necrotic lesion. These PAMPs, known to activate PTI, increased the level of SA in the localized tissue as well as distant leaves by enhancing the expression of SA markers such as *PR-2* and *PR-5* (Mishina and Zeier 2007). SAR signals can also be directly generated by ETI without the induction of HR. This was revealed in the interaction between potato and Potato Virus X whereby the host has the *R*-gene *Rx* which corresponds to the pathogen's Avr proteins. The *Rx* gene is a typical coiled-coil NB-LRR protein that is able to induce SAR by increasing SA levels in distant cells without prior cell death caused by HR (Liu *et al.* 2010).

Other than SA, lipid signalling and vasculature-associate signalling have also been identified as key players in the induction of SAR. A mutation in the apoplastic lipid transfer protein *DIR1* inhibits the activation of SAR in *Arabidopsis* leaves challenged with *P. syringae* without affecting local resistance (Maldonado *et al.* 2002). Mutations in other plastid glycerolipid biosynthesis such as *FAD7, SFD1* and *SFD2* also affect the induction of SAR because basal resistance remains unaffected (Grant *et al.* 2006; Chaturvedi *et al.* 2008). This suggests the involvement of lipid protein in the long distance signalling of SAR. Another potential inducer of SAR may be jasmonic acid, which could possibly be required for the early establishment of systemic resistance. JA mutants, *sgt1b, opr3* and *jin1* involved in perception, biosynthesis and signalling respectively all lead to attenuation of SAR (Truman *et al.* 2007). Due to the fact that the SA and JA pathways are antagonistic it is hypothesized that JA may be the signal required to induce the early phase of SAR, whereas SA is required in the later phase (Truman *et al.* 2007).

Recently, ABA has been shown to have a substantial influence in determining the outcome of plant-pathogen interactions. In terms of SAR, research in tobacco has revealed that ABA suppresses SA levels thereby haltering the SAR signalling pathway when plants are inoculated with *Tobacco mosaic virus* and *Pseudomonas syringae* pv. *tabaci* (Kusajima *et al.* 2010). Pre-treatment of plants with ABA reduced the expression of SA marker genes such as acidic *PR-1*, *PR-2* and *PR-5* in the infected tobacco plants (Kusajima *et al.* 2010). Auxin is another hormone known to play a role in the establishment and maintenance of SAR. However it is not auxin directly that is involved in SAR, instead this hormone is necessary for maintaining the homeostasis of various other indolic compounds required for systemic resistance (Truman *et al.* 2010). Figure 1.5 illustrates what is currently known about the different phytohormones involved in the induction of SAR and their hypothesized regulation and timing.

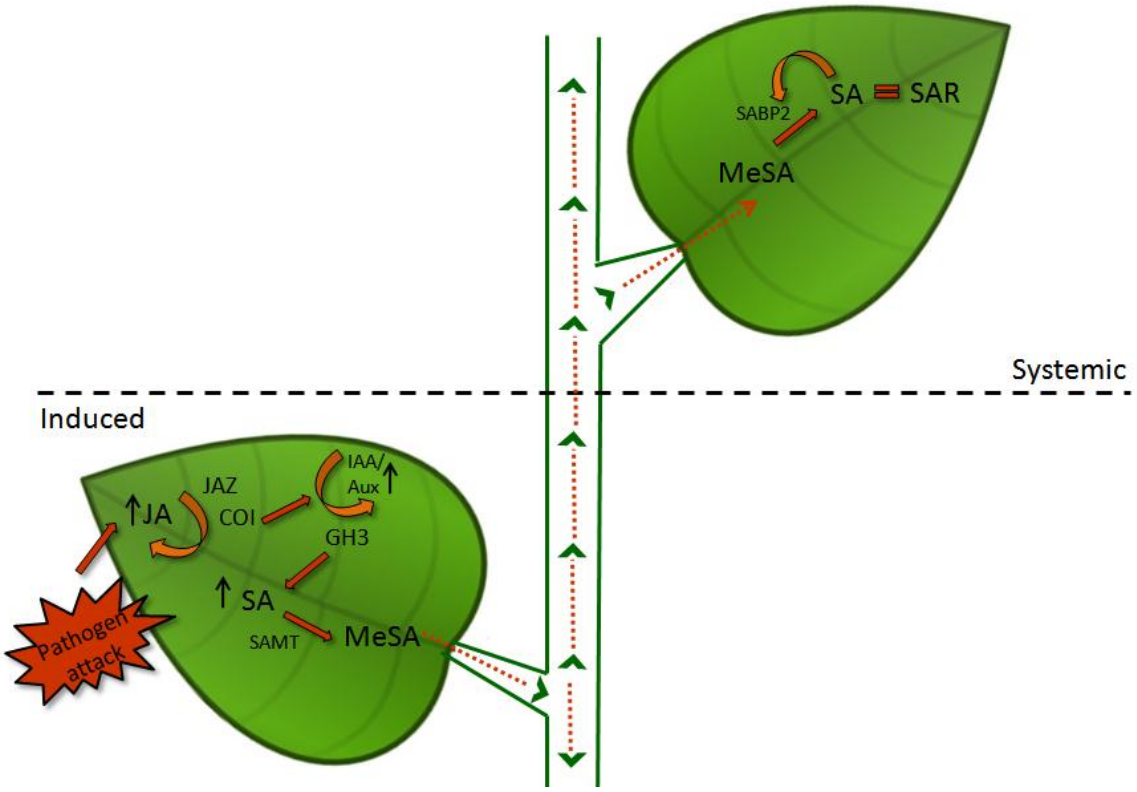

Figure 1.5: Phytohormones involved in the induction of SAR. Initially SAR was thought to only involve SA however research has shown that other hormones such as JA, ABA and auxin all contribute to systemic resistance (Based on Truman *et al.* 2010).

Once the pathogen is perceived by the host, an increase in JA occurs during the first few hours post infection. JA is subsequently regulated through a negative feedback loop involving JASMONATE ZIM DOMAIN (JAZ*)* transcripts and the CORONATINE INSENSITIVE1 (COI1) jasmonate receptor (Figure 1.5) (Truman *et al.* 2010). The COI1 receptor then facilitates the induction of auxin and other indolic compounds (Truman *et al.* 2010). Transition from the auxin phase into the SA mediated signalling phase is mediated by an auxin inducible *GH3* gene which encodes auxin-conjugating enzymes. In particular *GH3-8* encodes an indole acetic acid (IAA) synthetase which catalyzes the synthesis of IAA, a major form of auxin, thereby inhibiting the increase of auxin (Ding *et al.* 2008). This decrease in auxin levels allows SA to accumulate and be converted to

MeSA by salicylic acid carboxyl methyltransferase (SAMT) (Park *et al.* 2007). MeSA is then transported to distal areas of the plant for priming of defence responses. In the systemic tissue, MeSA is converted back to SA *via* SABP2 which is subsequently controlled by the levels of SA (Park *et al.* 2007).

Other than the activation of SAR *via* a pathogen, the pathway may also be artificially induced through the exogenous application of SA and by compounds that mimic the mode of action of SA. Known elicitors of the SA pathway that have been used commercially include 2,6-Dichloroisonicotinic acid (INA) and a benzothiadiazol, acibenzolar-S-methyl (ASM), also known as BION (Figure 1.6) (Kessmann *et al.* 1994; Durner *et al.* 1997). These elicitors have been shown to enhance the expression of the same suite of PR genes that are known to be responsive to the SA pathway such as *PR1a* and *PR2* (Ward *et al*. 1991).

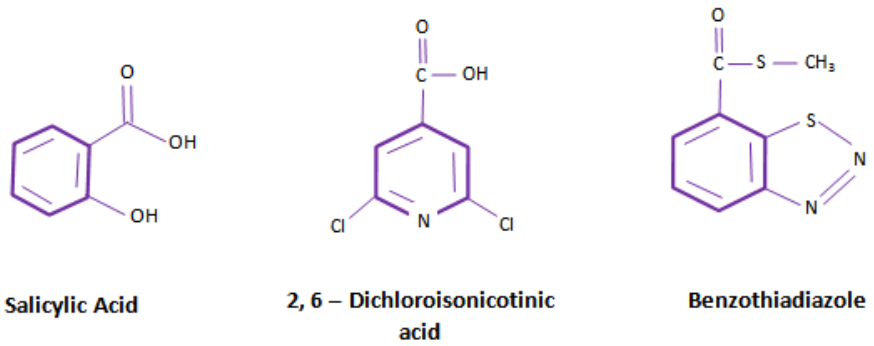

Figure 1.6: Functional analogs of SA and inducers of SAR – Biochemical structures of 2, 6-Dichloroisonicotinic acid (INA) and benzothiadiazol. These compounds have been identified as functional analogs of SA and are used commercially to induce SAR in various crops (Adapted from Durner *et al.* 2007).

Furthermore, the application of these elicitors to a host prior to infection by a pathogen confers resistance *via* SAR mechanisms (Walters *et al.* 2005). Tobacco plants, under greenhouse conditions and field trials, demonstrated enhanced resistance against *Pseudomonas syringae, Thanatephorus cucumeris* and *Cercospora nicotianae* when treated with ASM (Cole 1999). The use of SA directly as an exogenous activator of SAR is not regarded as the preferred option as it has been demonstrated in tobacco that such an application results in the conversion of the SA to SAG. Unlike free SA, SAG does not have the same phloem mobility therefore limiting the spread of this compound to distal tissues to induce SAR (Enyedi and Raskin 1993).

1.2.4.3 Induced systemic resistance, Jasmonic acid and ethylene

Priming is a phenomenon where the plant is able to induce cellular defence responses following suitable pathogen stimulation (Pozo *et al.* 2008). In this section the involvement of JA and ET in priming distal plant tissue and activating induced systemic resistance will be reviewed. The transcription factors JAMYC/AtMYC2 are potential regulators of priming and therefore play a key role in the induction of JA-dependent defence genes (Boter *et al.* 2004; Pozo *et al.* 2008). Jasmonic acid is a phytohormone activated in response to necrotrophic pathogens and wounding caused by herbivorous insects (Figure 1.3). Wounding of the cell membrane which occurs as a result of chewing herbivores allows for the release of linolenic acid (Figure 1.7, no. 1) (Creelman and Mullet 1997; Bonaventure and Baldwin 2010; Svoboda and Boland 2010). This substrate is subsequently converted to jasmonic acid *via* various enzymatic conversations, reductions and oxidations (Figure 1.7, no. 2 - 4) (Vick and Zimmerman 1984; Creelman and Mullet 1997; Svoboda and Boland 2010).

However it is not JA or methyl JA (MeJA) that is perceived by the cell, instead an amino-acid conjugate jasmonoyl-isoleucine (JA-Ile) is recognized by an F-Box protein known as COI1 in association with JAZ proteins (Figure 1.7, no. 5) (Yan *et al.* 2009). The COI1 protein associates with the *Arabidopsis* Skp1-like1, *Arabidopsis* Skp1-like2, cullin 1 and a ring box protein 1 to form the SCF^{COI1} complex (Moon *et al.* 2004; Yan *et al.* 2009). This complex forms an E3 ubiquitin ligase which is responsible for the ubiquitination and proteosomal degradation of target proteins such as JAZ proteins (Moon *et al.* 2004; Ballare 2011). The complex formed between COI1 and JAZ is regarded as the true receptor for JA-Ile (Figure 1.7, no. 6). This is because COI1 contains the binding domain that directly interacts with JA-Ile, whereas the JAZ proteins assist in securing the JA-Ile within the binding domain (Yan *et al.* 2009; Ballare 2011). Modifications, by the host, of the JAZ proteins allow for the expression of JA responsive genes such as *PR3, PR4, LOX*, *VSP* and *JR1 via* the JAMYC/AtMYC2 transcription factors (Figure 1.7, no. 7) (Dong 1998; Boter *et al.* 2004).

Exogenous application of MeJA on *P. deltoides* has been shown to influence the growth and development of *Lymantria dispar* larvae. MeJA increased the expression of various defence genes which in turn stimulated the synthesis of secondary metabolites such as terpenoids and phenolics (Hu *et al.* 2006). In leaves treated with MeJA, the phenolic contents comprising of caffeic acid, benzoic acid and ferulic acid were significantly higher than in control plants. This increase in phenolic composition decreased the nutritional value and increased the toxicity of the leaves thereby decreasing the food consumption and weight of the invading larvae (Hu *et al.* 2006).

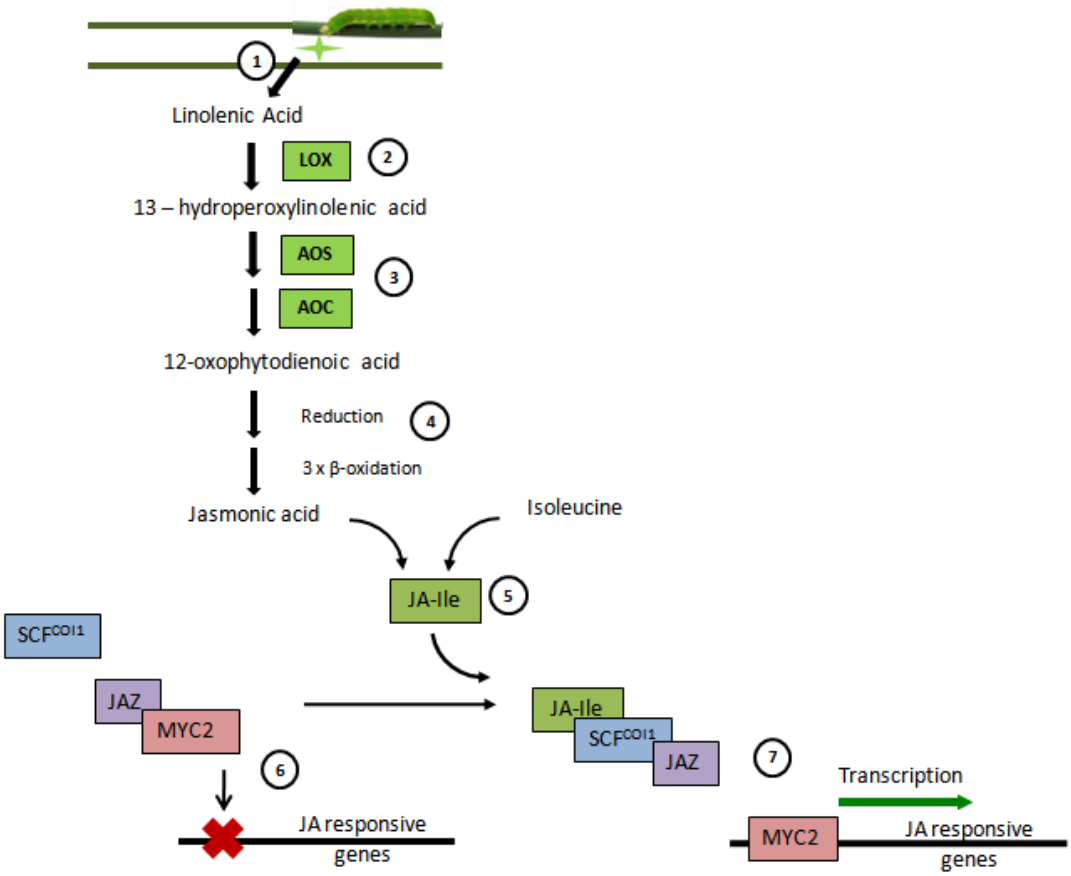

Figure 1.7: Biosynthesis and metabolism of Jasmonic acid – 1. Wounding caused by, for example,, an insect chewing on the leaf results in the release of linolenic acid from the membrane and initiates the cascade. **2.** Linolenic acid is then converted to 13-hydroperoxylinolenic acid by lipoxygenase (LOX). **3.** 13-hydroperoxylinolenic is then converted *via* allene oxide synthase (AOS) and allene oxide cyclise (AOC) to produce 12-oxophytodienoic acid. **4.** The latter substrate is then converted to jasmonic acid *via* reduction and β-oxidation steps. **5.** Jasmonic acid is then conjugated with isoleucine to produce JA-Ile which is perceived by the cell. **6.** In the absence of JA-Ile, JAZ proteins remain bound to the MYC2 transcription factor which prevents the initiation of transcription of JA responsive genes. **7.** Once JA-Ile is present, it binds to SCF^{COI1} which then associates with the JAZ proteins thereby releasing the MYC2 transcription factor to initiate transcription of JA responsive genes (Adapted from Creelman and Mullet 1997, Svoboda and Boland 2010, Vick and Zimmerman 1984).

Ethylene is a plant hormone involved in numerous developmental signalling aspects such as germination, flowering and leaf senescence (Figure 1.3) (Dong 1998). Due to the involvement of this hormone in various facets of the plant, multiple receptors been identified in *Arabidopsis* and these include ETR1, ETR2, EIN4 and ERS2 (Hua and Meyerowitz 1998). Receptors ETR2, EIN4 and ERS2 have been shown to display high sequence similarity to the amino terminal domain of ETR1 indicating that they may interact with ET in the same manner as ETR1. The SCF complex utilized in the JA signalling pathway has also been shown to have a role in ethylene signalling. The $SCF^{EBF1/EBF2}$ complex is similar to SCF^{COI1} however this complex is responsible for the ubiquitination and proteosomal degradation of ETHYLENE INSENSTIVE (EIN3) in the absence of ethylene, whereas SCF^{COI1} degrades repressors of the JA responsive genes (Moon *et al.* 2004). EIN3 is constitutively expressed in the cell however the protein is unable to accumulate due to the proteolysis activity of the $SCF^{EBF1/EBF2}$ complex (Potuschak *et al.* 2003). In the presence of ethylene, EIN3 is not affected by the $SCF^{EBF1/EBF2}$ complex and is therefore allowed to enhance expression of ET responsive genes (Potuschak *et al.* 2003).

The role of this gaseous phytohormone in plant defence is obscure as it may have a role in susceptibility or resistance depending on the type of plant-pathogen interaction (Hirsch 2002). An ethylene responsive factor *ERF1* can bind to the GCC box situated in the promoter region of various pathogen-responsive defence genes and thereby induce their expression (Thatcher 2005). The expression of *ERF1* can be induced by both the ET and JA pathways, thereby indicating a downstream convergence point of these two pathways (Thatcher 2005). Recently, ET has been implicated in ROS production in response to flagellin *via* the FLS2 receptor. Mutants of ET, *etr1* and *ein2* were shown to accumulate reduced amounts of FLS2 transcripts and therefore produced significantly

lower levels of flg22-induced ROS (Mersmann *et al.* 2010). In addition to this, mutant plants displayed hindered callose deposition as well as defects in stomatal closure thereby increasing susceptibility (Mersmann *et al.* 2010). Olive trees exhibited increased resistance to *Prays oleae* when treated with Ethrel®, a chemical that converts to ethylene upon the induction of fruit formation. Treated trees showed a 50% reduction in fruit loss as well as a decrease in the population of *P. oleae* compared to control plants (Rosales *et al.* 2006).

As mentioned, JA may have a role in the early induction of SAR however this pathway and the ethylene pathway are more associated with induced systemic resistance (ISR). The latter is similar to SAR in that both responses enhance the resistance of systemic tissue against a broad-spectrum of invading pathogens (Bakker *et al.* 2007). However, SAR confers resistance to pathogens normally evaded by the SA pathway, whereas ISR confers resistance to pathogens normally evaded by the JA/ET pathway (Bakker *et al.* 2007). ISR usually occurs in response to root colonization by non-pathogenic rhizobacteria (Thatcher 2005). When plants were treated with STS or DIECA, chemicals that inhibit ethylene and jasmonic acid respectively, ISR was abolished thereby substantiating the role of JA and ET in ISR (Shoresh *et al.* 2004). Additionally, JA and ET also facilitate in the induction of systemic induced resistance (SIR) which is similar to ISR however SIR is induced upon mechanical wounding or as a results of chewing caused by herbivorous insects (Gurr and Rushton 2005).

Induction of ISR *via* rhizobacteria can be illustrated in the example of *Arabidopsis* and a non-pathogenic basidomycete, *Piriformospora indica*. Root colonization by *P. indica* enhances resistance against the causal agent of powdery mildew *Golovinomyces orontii via* JA and ET. Mutants of the JA signalling pathway, *jasmonate resistant 1-1 (jar 1-1)* and *jasmonate insensitive 1(jin1)* were fully susceptible to infection by *G. orontii* despite

the presence of *P. indica* (Stein *et al*. 2008). In addition to this, marker genes of the JA pathway such as *LOX2* and *vegetative storage protein (VSP)* were found to be up-regulated in the leaves of *A. thaliana* plants colonized with *P. indica* 3 dpi as opposed to non-colonized plants (Stein *et al.* 2008). In this interaction, ET is imperative for maintaining the beneficial symbiotic relationship between *Arabidopsis* and *P. indica.* Absence of ET resulted in uncontrolled hyphal growth whereas over-expression of ET resulted in the restriction of fungal growth on the roots (Camehl *et al.* 2010). Therefore, taken together, JA and ET both contribute to and are essential for the establishment and maintenance of ISR.

1.2.4.4 Pathogenesis-related genes involved in defence

Various PR proteins are expressed in response to the induction of a particular signalling pathway and these proteins assist with curbing further spread of the disease. Due to the fact that these *PR* genes are activated upon induction of a particular pathway, they are said to be diagnostic of that pathway and can be targeted for enhancing resistance. In this study these genes that have been found to be diagnostic to a pathway will be referred to as marker genes. This nomenclature should not be misconstrued with the genetic markers obtained from RFLPs or RAPDs which are used in quantitative trait locus (QTL) studies (Strauss *et al.* 1992). Studies conducted in model organisms such as tobacco and *Arabidopsis* have revealed that the over-expression of these proteins can result in increased resistance to numerous pathogens.

The SA pathway has the following marker genes which have been shown to be associated with the induction of this signalling cascade: *PR1, PR2* and *PR5.* In tobacco, over-expression of the acidic *PR1* isoform was shown to have no effect on tobacco mosaic virus or potato virus Y however the transgenic lines had a considerable reduction

in disease severity caused by the oomycetes *Peronospora tabacina* and *Phytophthora parasitica* (Alexander *et al.* 1993). Additionally, transgenic tobacco plants over-expressing *PR2* and *PR5* were shown to have enhanced resistance to *A. alternata* and *Rhizoctonia solani* respectively (Jach *et al.* 1995; Velazhahan and Muthukrishnan 2004).

Like the SA pathway, the JA pathway also has marker genes that are associated with the induction of this signalling cascade namely *PR3, PR4* and *LOX*. The over-expression of wheat *PR4* in tobacco enhanced resistance to infection by *Phytophthora nicotianae* whereas the over-expression of barley *PR3* enhanced resistance to *R. solani* (Jach *et al.* 1995; Fiocchetti et al. 2008). Interestingly the combinatorial over-expression of barley *PR2* and *PR3* in tobacco enhanced resistance to the fungal pathogens *A. alternata* and *B. cinerea* (Jach *et al.* 1995). Transgenic tobacco lines constitutively over-expressing *LOX1* were also found to have reduced susceptibility to *Phytophthora parasitica* var. *nicotianae* (Mène-Saffrané et al. 2003).

In this section only a few core genes relevant to the signalling pathways that will be investigated have been explained, however there exists transcriptional data for various others in the form of northern blot analysis and microarrays (Ward *et al.* 1991; Schenk *et al.* 2000; Veluthakkal and Dasgupta 2010). This information on the different *PR* families that are found in plant species has been summarized in Table 1.1.

Table 1.1: PR proteins and families involved in plant defence

Gene Name	Molecular Function	Pathogens*/Elicitors	References
PR-1	Unknown	SA, *Phytophthora infestans, Erysiphe graminis*	(Niderman *et al.* 1995)
PR-2	β-1,3-Glucanase	SA, *Rhizoctonia solani, Aspergillus fumigates*	(Jung and Hwang 2000)
PR-3	Chitinase	JA, *Alternaria brassicola, Botrytis cinerea, Fusarium oxysporum*	(Melchers *et al.* 1994)
PR-4	Hevein-Like protein	JA, *Botrytis cinerea, Trichoderma harzianum*	(Hejgaard *et al.* 1992)
PR-5	Thaumatin	SA, *Candida albicus*	(Coca *et al.* 2000)
PR-6	Protease Inhibitors	*Aspergillus niger, Thielaviopsis paradoxa*	(Lopes *et al.* 2009)
PR-7	Proteinase	Unknown	(Fritig *et al.* 1998)
PR-8	Endochitinase	Unknown	(Fritig *et al.* 1998)
PR-9	Peroxidase	SA, *Heterobasidion annosum, Ceratobasidium bicorne*	(Asiegbu *et al.* 1994)
PR-10	Antifungal properties	*Cronartium ribicola, Phellinus sulphurascens*	(Liu and Ekramoddoullah 2006)
PR-11	Chitinase	Unknown	(Fritig *et al.* 1998)
PR-12	Defensin	*Cylindrocladium floridanum, Fusarium oxysporum and Nectria galligena.*	(Kovalyova and Gout 2008)

*Refers to pathogens that induce expression of a specific PR gene family

1.2.5 Involvement of additional plant hormones

1.2.5.1 Abscisic Acid (ABA)

This plant hormone is associated with several abiotic stresses such as drought, salinity and cold stress but it is also has a role in plant development such as root elongation, seed germination and embryo maturation (Figure 1.3) (Thatcher 2005). ABA is perceived by and binds to a membrane GDP-bound G protein coupled receptor which activates the signalling cascade (Pandey *et al.* 2009). The exact involvement of this hormone in pathogen defence varies depending on the different pathosystems (Wasilewska *et al.* 2008). ABA is involved in defence responses by influencing callose deposition, the production of ROS intermediates and by altering the expression of defence response genes (Bari and Jones 2009). In the *Pseudomonas syringae* DC300 interaction with *Arabidopsis*, exogenous application of ABA enhanced susceptibility towards the pathogen, whereas an ABA biosynthetic mutant reduced bacterial colonization (Torres-Zabala *et al.* 2007). This suggests that the bacterial effectors may manipulate this signalling pathway for their proliferation and as a result are able to colonize the host (Torres-Zabala *et al.* 2007). Concomitantly it was found that following inoculation with *Alternaria brassicicola*, there was a significant decrease in the accumulation of ABA levels in *Arabidopsis* within the first 24hpi (Flors *et al.* 2008). This suggests that the ABA pathway may be targeted by this fungus in order to increase its own virulence.

Resistance against necrotrophic pathogens could require the beneficial involvement of ABA as this signalling pathway can strengthen cell walls by promoting callose deposition (Mauch-Mani and Mauch 2005). In the interaction of *Leptosphaeria maculans,* the causal agent of blackleg found on Brassica crops, resistance is conferred by the involvement of the ABA pathway. Exogenous application of ABA increased resistance to *L. maculans*

and pathogen inoculation induced the ABA signalling pathway (Kaliff *et al.* 2007). Although the most prominent cause of resistance against *L. maculans* was callose deposition, ABA was found to induce other resistance responses (Kaliff *et al.* 2007). On the contrary, ABA deficient tomato mutants were found to have enhanced resistance against *Botrytis cinerea*. This was due to increased cuticle permeability and pectin methylesterification in the mutants which allowed for earlier perception of the pathogen thereby resulting in a more rapid induction of defence responses (Curvers *et al.* 2010).

ABA may also facilitate resistance in an indirect manner through the formation of a secondary messenger hydrogen peroxidase (Wasilewska *et al.* 2008). The closure of stomatal openings is triggered by the detection of PAMPs by receptors within the cell wall, however pathogens have evolved effectors to manipulate and re-open stomatal pores (Melotto *et al.* 2006; Maksimov 2009). This secondary messenger in association with ABA assists in the opening and closure of stomatal apertures which has been shown to be involved in innate pathogen defence (Melotto *et al.* 2006). Therefore the outcome of defence with respect to ABA strongly depends on the specific plant-pathogen interaction and the interplay of this phytohormone with the other phytohormones.

1.2.5.2 Auxin

Auxin is generally known to be involved in plant growth and development, however recent studies have revealed a role for this plant growth regulator in determining the outcome of pathogen interactions. Auxin/Indole-3-acetic acid (Aux/IAA) is perceived in the cell by a transport inhibitor response 1 (TIR1) protein (Figure 1.3). This protein forms part of an AUX/IAA-SCF^{TIR1} complex that is responsible for the ubiquitination and proteosomal degradation of AUX/IAA repressor proteins in the presence of auxin thereby

facilitating the expression of auxin responsive genes (Bari and Jones 2009). For example the *GH3* gene family is a faction of proteins that respond to auxin and are responsible for maintaining Aux/IAA homeostasis. As illustrated in figure 1.5, auxin has been elucidated to participate in the establishment and maintenance of SAR. However due to the fact that this hormone is involved in the expression of various expansin genes that encode for cell-wall loosening proteins, pathogens may hijack this pathway for increasing their own virulence (Kazan and Manners 2009).

P. syringae is an example of a pathogen that manipulates the auxin pathway to assist in its colonization of *Arabidopsis.* This pathogen uses the avirulence protein AvrRpt2 to alter the levels of auxin within the host (Chen *et al.* 2007b). Increased levels of auxin may promote pathogen invasion by promoting stomatal opening, influencing the loosening of cell walls and changing the permeability of cell membranes (Chen *et al.* 2007b). In Rice, the bacterial pathogens *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas oryzae* pv. *oryzicola* and the fungal pathogen *Magnaporthe grisea* have all been shown to utilize the auxin pathway to enhance the expression of cell wall expansin genes, thereby facilitating their own proliferation (Ding *et al.* 2008; Fu *et al.* 2011). In response to this the host can induce the expression of *GH3-2* and *GH3-8*. These proteins form part of the GH3 family which is responsible for suppressing the effects of Aux/IAA by conjugating the hormone to amino acids, sugars or peptides hence inactivating it. Over-expression of these proteins resulted in enhanced resistance to the invading organisms (Ding *et al.* 2008; Fu *et al.* 2011).

On the contrary, auxin has been illustrated to have a pivotal role in maintaining resistance against necrotrophic pathogens such as *Plectosphaerella cucumerina* and *B. cinerea. Arabidopsis* auxin mutants *axr1, axr2* and *axr6* which are hindered in the ability to ubiquinate and degrade the proteosome of auxin repressor proteins *via* the AUX/IAA-

SCF^{TIR1} complex were more susceptible to infection by the *P. cucumerina* and *B. cinerea* than the wild type (Llorente *et al.* 2008). The opposing roles of auxin with respect to biotrophic and necrotrophic pathogens illustrates that the host is able to fine tune this complex and integrated signalling pathway depending on the invading organism.

1.2.5.3 Brassinosteriods

Brassinosteriods (BR) are plant hormones that facilitate processes such as cell division and elongation, seed germination, flowering and reproductive development however its role in pathogen defence is still elusive (Bari and Jones 2009; Pieterse *et al.* 2009). In tobacco and rice a well known BR, brassinolide (BL) was shown to induce broad spectrum resistance to a range of pathogens. In tobacco plants treated with BL resistance was enhanced against the viral pathogen tobacco mosaic virus, a bacterial pathogen *Pseudomonas syringae* as well as a fungal pathogen *Oidium* sp (Nakashita *et al.* 2003). Additionally in rice, BL induced resistance against *Magnaporthe grisea* and *Xanthomonas oryzae*, the causal agents of rice blast and bacterial blight disease respectively (Nakashita *et al.* 2003).

BR is perceived by the cell by a receptor known as Brassinosteriod-insensitive1 (BR1) (Bari and Jones 2009; Pieterse *et al.* 2009; Tang *et al.* 2010). In the absence of BR, BR1 remains unphosphorylated and therefore unable to interact with any other proteins (Figure 1.3). The presence of BR then stimulates the phosophorylation of BR1, hence allowing it to bind to other co-receptors such as, a receptor-like kinase, BR1-associated kinase 1 (BAK1) (Tang *et al.* 2010). This complex allows for the initiation of the signalling cascade that regulates the various growth and developmental processes.

However the BAK1 receptor has also been linked with other MAMP/PAMP receptors such as FLS2 which recognizes flagellin secreted by bacterial pathogens independently

BR (Bari and Jones 2009). Due to the multi-functionality role of the BAK1 receptor, it has been shown that it is a target for avirulence proteins released by pathogens. The effector molecules AvrPto and AvrPtoB found in *P. syringae*, physically interact with BAK1 and prevent further association with other receptors such as BR1 and FLS2 thereby promoting virulence (Shan *et al.* 2008). Because BAK1 can function in plant defence in the absence of BR, the role of this receptor and the BR signalling pathway still needs to be clarified.

1.2.5.4 Gibberellin

Gibberellin (GA) is a hormone that mediates the degradation of regulators that negatively affect growth within a plant known as the DELLA proteins (Figure 1.3) (Bari and Jones 2009). The receptor that perceives GA was identified in rice as gibberellin insensitive dwarf 1 (GID1). This receptor in conjunction with GA facilitates the degradation of DELLA proteins *via* the SCF^{SLY1} complex (Navarro *et al.* 2008a). DELLA mutants were shown to have increased resistance to biotrophic pathogens such as *P. syringae* Pto DC3000 but conversely displayed enhanced susceptibility to nectrotrophic pathogens such as *A. brassicicola* (Navarro *et al.* 2008a). This exemplifies the role for GA in augmenting the virulence of biotrophic pathogens whereas it is has a significant role in curbing necrotrophic pathogens. In addition to the role of GA in plant defence, it also has a role in maintaining the homeostatic and symbiotic relationship between barley and *P. indica*. Mutants of GA exhibited reduced root colonization by *P. indica* due to an increase in the expression of plant defence genes such as *PR1* and *PR5* (Schafer et al. 2009). Hence the mutualistic relationship between barely and *P. indica* relies on the ability of GA to modify signalling pathways thereby allowing *P. indica* to colonize the roots.

1.2.5.5 Cytokinins

Cytokinins (CK) are a group of plant hormones that work in conjunction with auxin to facilitate process such as stem cell differentiation in the roots and shoots, vascular differentiation, seed development, nutrient balance and stress tolerance (Figure 1.3) (Müller and Sheen 2007; Bari and Jones 2009; Pieterse *et al.* 2009). The role of CK in mediating plant-pathogen interactions has however not been well-characterized. It has been shown to impede disease progression of clubroot disease caused by *Plasmodiophora brassicae* in *Arabidopsis*. In a susceptible interaction, *P. brassicae* down-regulates CK to allow infection to progress whereas over-expression lines of genes involved in CK synthesis displayed enhanced resistance to the pathogen (Siemens *et al.* 2006). CK has also been implicated in priming host plants against wounding and herbivory. In Poplar, CK activated induced resistance when the plants were wounded and inoculated with Gypsy moth eggs (*Lymantria dispar*) (Dervinis *et al.* 2010). Poplar plants that were treated with CK also exhibited a reduction in larval weight in distal parts of the plant (Dervinis *et al.* 2010). Despite this, the molecular mechanisms of CK signalling and its role in defence induction remains unclear and further research is required.

1.2.5.6 Emerging plant hormones

As the field of plant defence continuously progresses, plant hormones that were previously overlooked have been recently shown to contribute to defence responses. For example, stringolactones interact with the auxin pathway to facilitate the development of auxillary shoot buds and although their role in plant defence has not be fully elucidated they have been shown to influence mycorrhizal symbioses (Bari and Jones 2009; Bednarek and Osbourn 2009; Hayward *et al.* 2009). Additionally, peptide hormones

which are derived from larger polypeptide precursors e.g. systemins, have also been implicated in plant defence (Bari and Jones 2009).

1.2.6 Interaction and cross-talk between signalling pathways

1.2.6.1 SA and JA

The antagonistic relationship between SA and JA has been well-documented in various plant-pathogen interactions and involves a diverse array of proteins. Among these is the role of *NPR1* which is required both for the expression of SA-responsive genes as well as for the repression of JA-responsive genes by SA (Dong 2003; Spoel *et al.* 2003; Koornneef *et al.* 2008; Pieterse *et al.* 2009). When a pathogen such as *P. syringae* induces expression of SA within the plant upon infection, the activation of NPR1 ensues. NPR1 is subsequently localized in the nucleus whereby it promotes activation of SA-responsive gene expression. Concurrently, activated NPR1 in the cytosol mediates the repression of JA-responsive genes by inhibiting the binding of positive regulators or mediating the binding of negative regulators (Dong 2003; Spoel *et al.* 2003).

Although these pathways predominantly have an antagonistic relationship, there have been situations whereby these pathways act synergistically. The outcome of the interaction between SA and JA seems to be largely dependent of the timing of activation and the concentration of the phytohormones. At low concentrations these hormones act synergistically to facilitate processes such as programmed cell death (Mur *et al.* 2006; Łazniewska *et al.* 2010). The antagonistic relationship becomes apparent at high concentrations of the hormones (Mur *et al.* 2006; Łazniewska *et al.* 2010). Concurrent application of the hormones has also been demonstrated to enhance resistance in certain plant species. The application of JA followed by SA after 24hrs was shown to improve resistance in *Arabidopsis*, tobacco and tomato against *cucumber mosaic virus*

(CMV), *tobacco mosaic virus* (TMV) and *turnip crinkle virus* (TCV) respectively (Shang *et al.* 2010)

1.2.6.2 JA and ET

These pathways can act in synergy or independently in response to necrotrophic pathogens. Convergence and integration of these two signalling pathways involves transcription factors such as ERF1 and ORA59 (Lorenzo *et al.* 2003; Pre *et al.* 2008). Both these transcription factors are required for the expression of defence genes such as *PDF1.2* (Lorenzo *et al*. 2003; Pre *et al*. 2008). Conversely, only JA is required for the expression of genes such as *VSP2* and *LOX2* (Pieterse *et al.* 2009). ET is also responsible for the inhibition of SA-mediated suppression on JA responsive genes. Plants that were treated with pathogens that elicit the JA/ET pathway, such as *B. cinerea* and *A. brassicicola*, were insensitive to future suppression of JA genes by SA (Leon-Reyes *et al.* 2010). This inability of SA to suppress JA responsive genes following induction of ET is mediated by the transcription factor ORA59 (Leon-Reyes *et al.* 2010). Therefore, if the SA pathway is induced prior to induction of the JA pathway, or if JA is induced with ET, the antagonistic relationship between these pathways persists (Leon-Reyes *et al.* 2010). Concomitantly, if ET is present and the JA pathway is subsequently induced, it becomes immune to future suppression by SA (Leon-Reyes *et al.* 2010).

Phytohormones, their interaction with each other and level of their induction are all facets that have a crucial role in determining the outcome of a plant-pathogen interaction. Model species have provided researchers with the platform to further our comprehension of plant defence mechanisms however one needs to also understand these processes in various tree species. The importance and significance of gaining this knowledge in *Eucalyptus* will be reviewed in the next section.

1.3 The host, *Eucalyptus*

1.3.1 Introduction

Eucalypts belong to the family Myrtaceae (Myrtles) and were introduced into South Africa from Australia in the late nineteenth century. Presently, eucalypts are the second most commonly planted forest trees in the world, falling shortly behind pine trees. In South Africa this genus accounts for approximately 40.4% of the forestry industry whilst pine trees account for 51% (FSA 2009). The majority of the hardwood (*Eucalyptus*) plantations occurs in two provinces, namely Kwa-Zulu Natal (Midlands) and Mpumalanga south with 39.6% and 40.8% of total land area respectively (FSA 2010). Three main species and clones of *Eucalyptus* are commercially planted in the forestry industry: *E. saligna*, *E. camaldulensis* and *E. grandis*.

Among the numerous beneficial uses for this tree, its use for pulpwood and mining timber in South Africa are among the main reasons this invasive tree is planted. Eucalypt trees have shorter fibers with thinner walls as compared to other Northern hemisphere hardwood trees. This property of eucalypts allows for a larger number of fibres per unit mass or volume (Clarke *et al.* 2008). For example, 14 million fibres can be acquired from one gram of eucalypt pulp. These characteristics propel eucalypts to be planted for their advantage in producing paper because the short fibres create a smooth opaque matrix (Clarke *et al.* 2008). Wood-pulp and paper production dominated the industry with an overall sales value of R9 831 million in the year 2006/2007 (FSA 2008). Other species such as *E. smithii* are commercially planted for their use in *Eucalyptus* oil production. South Africa accounts for 5% of the world's eucalypt oil supply, manufactured by Busby Oils situated in Pietermaritzburg Kwa-Zulu Natal (Chapman 2009).

The residue from the paper production process can furthermore be used for second generation biofuel production by converting lignocellulosics to ethanol (Rakshit 2010). In 2008, a project to sequence the *E. grandis* genome was undertaken by the DOE joint genome institute. A selfed (S_1) tree of an *E. grandis* breeding population in Brazil known as *BRASUZ1* was used as the material to sequence the genome *via* whole-genome shotgun sequencing. The first version of the complete genome sequence was released in January 2011 on Phytzome version 7.0 (www.phytozome.net) and consists of 691MB arranged in 4952 scaffolds on 11 main linkage groups/chromosomal assemblies (Phytozome 2010). Gene models were predicted using homology based software (FgenesH and GenomeScan) and integrated with the ~260,000 previously available *Eucalyptus* EST assemblies, thereby generating approximately ~9700 putative transcripts (Phytozome 2010). This genome sequence and predicted gene models provide a reservoir of information that can be further mined to elucidate the evolution and function of various genes.

1.3.2 Comparative genomics and Gene orthology

Comparative genomics allows researchers to assign putative functions to unknown genes based on synteny, the conservation of linkage, collinearity and gene order (Schmidt 2000). Numerous genes have been well characterized in the model organism *Arabidopsis* and the relationship of this host with other plant species provides a resource for identifying orthologous genes (Brunner *et al.* 2004). By applying a comparative genomics approach, synteny and collinearity has been found to occur between *Arabidopsis* and various other distantly related taxa. Low but detectable synteny and micro-collinearity has been identified between rice (a monocot) and *Arabidopsis* (a eudicot).The largest syntenic group was found to occur between the *Arabidopsis* chromosome 5 and the rice chromosome 4. In terms of defence related genes, rice

homologs were identified for *NDR1*, *NPR1*, *EDS1*, COI1 and *PAD4* (Goff *et al.* 2002). Comparative sequence analysis between tomato and *Arabidopsis* also revealed synteny and collinearity between these two taxa. A conservation of gene order was found to occur between a 105kb BAC clone representing a region on the tomato chromosome 2 and *Arabidopsis*. The gene order and content of the BAC clone aligned with four segments in *Arabidopsis* distributed between chromosomes 2 – 5 (Ku *et al.* 2000). Additionally using a similar approach with *Populus* BAC clones a high degree of synteny and micro-collinearity was found to occur with *Arabidopsis*. A total number of 33 *Populus* orthologs were identified within *Arabidopsis*. By analyzing the gene order of these orthologs an estimated probability of synteny was found to be approximately 58% between these two taxa (Stirling *et al.* 2003).

Based on these studies one can conclude that synteny does occur between *Arabidopsis* and other higher plant species despite the large phylogenetic distance. Extrapolating from this, one can use the information in *Arabidopsis* to confer putative functions in hosts such as *Eucalyptus* and identify putative orthologs. A high degree of synteny occurs between *Populus* and *Arabidopsis* even though they form part of two different clades namely Eurosid I and Eurosid II respectively (Brunner *et al.* 2004). Therefore one can predict a theoretically higher degree of synteny between *Arabidopsis* and *Eucalyptus* based on the fact that both of these plant species occur within the Eurosid II clade.

Parallel to synteny, BLAST-based and phylogeny-based approaches provide additional tools for the identification of putative orthologs (Chen *et al.* 2007a; Paterson *et al.* 2010). For example, the TBLASTX algorithm can be used to identify orthologs for the gene of interest between species that are distantly related (Fulton *et al.* 2002). BLAST-based methods elucidate candidates that can be further investigated, however in order to narrow and add confidence in selecting a putative ortholog one needs to incorporate

phylogeny based methods such as Maximum Likelihood (Conte *et al.* 2008). This information can then be applied in a comparative phylogenomics approach to determine the degree of synteny between various species and elucidate candidate orthologs (Dubcovsky *et al.* 2001; Causier *et al.* 2010). A similar phylogenomics approach will be executed in this study for identifying candidates of putative orthologous defence genes. Elucidating the suite of defence genes in *Eucalyptus* will provide a mode of action to enhance the resistance of the hosts against pathogens and to limit the impact these pathogens have on the forestry industry.

1.3.3 *Eucalyptus* pathogens and their impact on the Forestry industry

Eucalyptus spp. are exotic to South Africa and as a result are separated from most of their natural enemies found in their native ecosystems (Darrow 1996; Wingfield *et al.* 2001). In the new ecosystem, these exotic species become vulnerable to novel pathogens that reside in that environment or that are subsequently introduced. This is because the introduced host would not have developed the adaptive mechanisms necessary to curb infection by a pathogen native to the new environment (Keane and Crawley 2002). Such pathogens that cause severe disease outbreaks have a critical role in influencing which species of eucalypts are planted by the forestry industry. For example the planting of pure *E. globulus* has been restricted due to its high level of susceptibility to *Mycosphaerella* spp. (Wingfield 2003).

Due to the demand for this hardwood and availability of land space, certain eucalypt species are planted in high altitude areas. These plantations are exposed to harsh winters with severe frost and therefore need to be able to adapt as well as thrive in such conditions. Species such as *E. globulus, E. smithii* and *E. viminalis* are all highly susceptible at high altitudes to attack by the eucalypt snout beetle *Gonipterus scutellatus*

and therefore the planting of these cold-tolerant species is limited (Swain 1995; Darrow 1996). Cold tolerant eucalypts such as *E. smithii, E. fastigata* and *E. fraxinoides* have important commercial value to the forestry industry as they are high quality pulp producers (Linde *et al.* 1999). However, these species are especially susceptible to *P. cinnamomi* and as a result are not widely planted (Swain 1995; Darrow 1996). In recent years a quince borer cossid moth known as *Coryphodema tristis* has also been shown to cause immense damage to *E. nitens* plantations in the Mpumalanga highveld area and therefore restricts the plantation of this species (Boreham 2006).

With the prospect of elucidating the marker genes associated with defence signalling pathways in *Eucalyptus*, one can utilize the information currently known in *Arabidopsis* to further understand plant defence in this tree species. By amalgamating the information obtained from this study one can also equate how well the information from *Arabidopsis* transcends to *Eucalyptus*. This study will furthermore attempt to clarify the response of the orthologous markers identified in *Eucalyptus* to a fungal pathogen *Chrysoporthe austroafricana.* The impact of this pathogen on *Eucalyptus* will be reviewed in the next section.

1.4 The pathogen, *Chrysoporthe austroafricana*

This fungus is the causal agent of stem cankers found on the *Myrtaceae* and *Melastomataceae* family, for example on *Eucalyptus* spp. and *Tibouchina* spp. respectively. It was first isolated in northern Natal on *E. grandis* during the late 1980's and was originally classified as *Chrysoporthe cubensis* (previously known as *Cryphonectria cubensis*) (Wingfield *et al.* 1989; Gryzenhout *et al.* 2004). Extensive DNA and phylogenetic studies revealed that *C. cubensis* occurs natively in South East Asia,

Australia, Hawaii and Zanzibar whereas *C. austroafricana* is located exclusively to South Africa (Gryzenhout *et al.* 2004).

DNA sequence comparisons using histone *H3* and β-tubulin have shown that the isolates from South Africa form a distinct phylogenetic group separate from the isolates of South East Asia, Australia and South America (Myburg *et al.* 2002). *C. austroafricana* also has characteristics that are unique to this isolate. It has been shown that the South African isolate causes cankers mainly at the base of the tree whereas the isolates from South America and South East Asia cause cankers at various heights of the tree. Additionally only asexual structures of *C. austroafricana* have been found to occur on the cankers whereas the cankers caused by the isolates from South America and South East Asia have predominantly sexual structures (Myburg *et al.* 2002).

It is hypothesized that *C. austroafricana*, which has been shown to occur on native *Syzygium* spp. throughout South Africa, could possibly have undergone a host shift to *Eucalyptus* spp. (Heath *et al.* 2006; Wingfield *et al.* 2008). This theory was substantiated by pathogenicity trials conducted on *Syzygium cordatum* and a susceptible *E. grandis* clone, ZG14. The experiment revealed significantly smaller lesions on *S. cordatum* spp., whereas the ZG14 plants had severely larger lesions (Heath *et al.* 2006). A native host would have developed mechanisms to subdue the effects of the pathogen as seen with *S. cordatum* hence corroborating the theory that *C. austroafricana* has undergone a host shift. Although this pathogen has caused severe losses to the forestry industry during the late 1990's, further spread of this pathogen has been effectively managed through controlled breeding programs with tolerant species (Wingfield *et al.* 2001). This involved vegetative propagation of a natural hybrid between *E. grandis x E. urophylla* (GU21) (Wingfield 2003; Wingfield *et al.* 2008).

Another method of screening various eucalyptus clones for resistance against *C. austroafricana* is to artificially inoculate new hybrids to assess their ability to withstand infection. However, it is crucial that such experiments be conducted in the areas where the clones will be commercially propagated due to genotype x environmental interaction (Van Heerden and Wingfield 2002). Despite a significant decrease in the occurrence of this pathogen, it can still occasionally be found in particular seedling stands when conditions permit (Prof Jolanda Roux, Personal communication). Currently in terms of plant defence against this pathogen, it has been shown that tolerant species have an increase in callose deposition and are able to close the wound significantly faster than susceptible clones (Van Zyl and Wingfield 1999).

A study by Lezar *et al.* also revealed the association of selected restriction polymorphisms with tolerance in a segregating population of *E. grandis*. The segregating population consisted of the progeny of a controlled cross between the susceptible *E. grandis* clone ZG14 and the tolerant *E. grandis* clone TAG5. By applying a combination of bulk segregation analysis and diversity array technology (DArT), two cleaved amplified polymorphic sequence (CAPS) markers were identified. These two CAPS markers were able to distinguish between susceptible and tolerant individuals in the F1 progeny (Lezar 2005).

Although this pathogen will not be the focus of this study, it will be used as an example to illustrate the validity of the putative orthologs in determining the importance of a particular signalling pathway. Pathogens that cause and proliferate on extensively damaged plant tissue are classified as necrotrophs (Glazebrook 2005). It was hypothesized that *C. austroafricana* may fall into this category because the pathogen feeds off the nutrients of the host thereby resulting in the death of the plant tissue during infection. This death of the plant tissue can clearly be seen as a distinct necrotic lesion

surrounding the infection site. Research conducted in *Arabidopsis* with the fungal necrotrophs *B. cinerea* and *A. brassicola* has revealed a role for the JA/ET signalling pathway in facilitating resistance (Glazebrook 2005). The JA mutant *coi1* exhibited a higher level of susceptibility compared to the wild-type against infection with both *B. cinerea* and *A. brassicola* hence substantiating a role for JA (Thomma *et al.* 1998; Glazebrook 2005). Based on experiments such as this, we hypothesize that the JA pathway may also facilitate tolerance against *C. austroafricana*. The expression profile of the putative orthologs may assist in elucidating the role of the SA and JA signalling pathways in the interaction with *C. austroafricana* and *Eucalyptus.*

1.5 Conclusion

Model organisms such as *Arabidopsis* have for many years provided a glimpse into elucidating the complex interactions that exists between plants and various pathogens. Although it provides a starting point into understanding these interactions, it may not be truly representative of the actual responses in a natural host. This has prompted research to move towards clarifying pathogen interactions involving more pertinent and natural hosts. Information regarding defence responses within trees is currently limited to hosts such as *Populus* and *F. sylvatica,* and while this information provides a stepping-stone there are numerous aspects that still require further investigation. *Eucalyptus* species are highly valuable to the South African forestry industry and are affected by a plethora of biotic stresses such as fungi, bacteria, viruses and insects, however there is very little information pertaining to defence in this host. The release of the *Populus* genome has allowed researchers to further investigate host-pathogen responses and elucidate candidate defence genes. The fact that *Populus* can be transformed with relative ease also facilitates in clarifying the role of genes of interest. It is envisaged that the release of the *Eucalyptus* genome will provide the same platform, allowing

researchers to further understand various physiological and genetic characteristics of this host. Transformation of *Eucalyptus* is also being investigated and established in various research labs around the world. At the Forestry and Agricultural Biotechnology Institute (FABI), located at the University of Pretoria, progress has been made in the transformation and generation of transgenic *Eucalyptus* plantlets.

By utilizing the genome sequence, this project will assist in elucidating the complex and intricate mechanisms of the eucalyptus defence system by identifying possible putative marker genes. Furthermore, it will allow us to comprehend how well the information transcends from model organisms. It is hypothesized that the putative marker genes elucidated in *Eucalyptus* would respond in a similar manner as in *A. thaliana*. The fungus *C. austroafricana* provides a suitable example of pathogen that poses a threat to *E. grandis.* This pathogen will be used as a case in point to illustrate the validity of the designated putative marker genes. In future, these defence-related genes could provide the key to improving resistance of *Eucalyptus via* genetic engineering as well as improving tree-breeding programs and thereby provide the South African Forestry with the tools to manage key pathogens.

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CHAPTER 2

Expression profiling of *E. grandis* defence marker gene orthologs of the salicylic acid and methyl jasmonate signalling pathways

2.1 Abstract

Eucalyptus species form an integral part of the South African forestry industry and their uses extend from paper and pulp production to fuelwood, mining timber and the synthesis of essential oils. Throughout their lifetime they are naturally challenged with various pests and pathogens, some of which cause devastating diseases. An approach to control the spread of pathogens is to enhance the defence response of the host. Most of the information pertaining to defence against pathogens stems from studies conducted in model organisms such as *Arabidopsis*, however such information is scarce in woody perennials such as *Eucalyptus*. In model systems it has been shown that the salicylic acid (SA) and jasmonic acid (JA) signalling pathways enhance resistance against biotrophic and necrotrophic pathogens, respectively. These pathways in turn activate genes involved in limiting the spread of the pathogen such as pathogenesis-related (PR) genes, which have also been shown to be diagnostic of the induction of specific pathways e.g. *PR2* is a diagnostic marker for the SA pathway. The aim of this study was to identify *Eucalyptus* orthologs of defence marker genes specific for the SA (*PR1, PR2* and *PR5*) and JA (*PR3, PR4* and *LOX*) signalling pathways using sequence information from other plant species and the *E. grandis* genome sequence. A co-phylogenetic approach using neighbour joining analysis and maximum likelihood was applied to identify and add confidence in the selection of putative orthologs. Following the selection of orthologous genes, their expression profiles were assessed using reverse transcriptase quantitative PCR (RT-qPCR). Transcript profiling was conducted under mock induction of the signalling pathways at various doses of SA and MeJA as well as at different time points. This revealed dose-dependent induction of the orthologous marker genes, as well as key time points for their expression. Exogenous application of SA and MeJA revealed that a concentration of 5 mM and 100 µM

respectively, elicited the most significant differential expression in transcript expression of the putative marker genes. Additionally, the expression profiles of these putative orthologs were analyzed in response to the causal agent of *Eucalyptus* stem canker, *Chrysoporthe austroafricana*. In the interaction with *C. austroafricana,* it was observed that *EgrPR2* transcripts were up-regulated in the tolerant *E. grandis* genotype, TAG5 at an earlier time point as opposed to the susceptible genotype (ZG14). Therefore it was inferred from this SA marker gene, that this signalling pathway could have a role in the tolerance of TAG5, possibly by limiting the spread of the pathogen as was evident from the smaller lesions observed in the tolerant genotype. These putative marker genes could provide diagnostic tools for the screening of pathogen challenged eucalypt plants to determine which signalling pathway(s) are activated against various pathogens. In addition, this research adds to our knowledge of defence responses in *E. grandis* and provides a stepping stone for understanding mechanisms to curb future tree diseases.

2.2 Introduction

Eucalyptus species and hybrid clones are commercially planted because of their valuable wood properties which have been exploited by the pulp and paper industry. In South Africa, the main *Eucalyptus* species planted include *E. grandis, E. camaldulensis, E. urophylla* and hybrids thereof (FSA 2010). Although primarily planted for their use in paper production and wood timber, the prospective of these trees extends to the generation of biofuels and the manufacturing of cosmetic products derived from the essential oils of species such as *E. smithii* (Bignell *et al.* 1998). Due to the importance and value associated with this genus of hardwood trees, the initiative to sequence the genome of *E. grandis* was undertaken by the US Department of Energy (DOE - Joint Genome Institute) in 2008. Currently, the first annotated version of the genome, released in January 2011, is available through Phytozome v7.0 and consists of 4952 scaffolds arranged on 11 linkage groups/chromosomal assemblies (Phytozome 2010).

Putative transcripts as well as *ab initio* protein coding gene models have been generated in the genome using homology-based tools as well as *Eucalyptus* ESTs thus creating a resource for data mining and gene investigation. In addition to the availability of the genome sequence, transcriptome data for an *E. grandis* x *E. urophylla* hybrid is at present accessible *via* a database known as EucGenIE (http://eucgenie.bi.up.ac.za) (Mizrachi *et al.* 2010). The transcriptome data for pure *E. grandis* clones will be added to this database in the near future (Hefer *et al.* 2011). These resources provide a useful platform for elucidating various physiological aspects of *Eucalyptus*, such as their responses to biotic and abiotic factors. Although *Eucalyptus* species are durable trees, they can and do succumb to diseases caused by a wide range of fungal and bacterial pathogens such as the oomycete *Phythophthora cinnamomi*, the causal agent of root rot and *Chrysoporthe* spp., the causal agents of stem cankers (Wingfield *et al.* 2008).

The vulnerability of eucalypts, particularly young trees, to pathogen invasion is of major concern to the South African forestry industry. In many instances, the invasion of a pathogen on the host not only reduces the quality of paper produced due to damaged timber, but also results in the death of the trees. For example, *Cryphonectria* stem canker has restricted the plantation of pure *E. grandis* and *E. saligna.* In the case of the *C. austroafricana* canker, a naturally occurring *E. grandis x E. urophylla* hybrid (GU21) was found to be resistant to this pathogen. This hybrid as well as other GU clones have since been propagated through controlled crosses by the commercial forestry industry which has limited the spread of the pathogen (Wingfield 2003). However in cases where no enhanced resistance to pathogens *via* the deployment of interspecific hybrids has been identified e.g. *P. cinnamomi*, one needs to investigate other aspects to improve tree health.

Understanding and enhancing the defence mechanisms of eucalypts is one avenue that can be explored to improve their tolerance to invading pathogens. Currently, most of the information pertaining to host responses against pathogens stems from research conducted in model organisms such as *Arabidopsis* and tobacco (Pieterse *et al.* 2009). These model systems have created a foundation for comprehending general host responses to pathogens. Although there have been significant advances in the understanding of plant defences in model systems, this niche has not been thoroughly investigated in woody plants such as *Eucalyptus*. In order to know which facets of defence can be improved through tree breeding or genetic manipulation one needs to have insight into the defence responses within eucalypts (Rosa *et al.* 2010). A starting point in this endeavour would be to identify and characterize putative orthologs of defence response genes in *E. grandis* based on information from *Arabidopsis* and other model species (Rosa *et al.* 2010).

From the studies conducted in model species, we know that the pathogen can be perceived either by receptors located in the cell membrane region or *via* R proteins within the cell (Jones and Dangl 2006). Transmembrane receptors interact with Pathogen-Associated Molecular Patterns (PAMPs) exuded from the pathogen such as flagellin or present on the pathogen surface, while the R proteins interact with effector molecules which are secreted directly into the cell by the pathogen in an attempt to bypass the outer receptors (Jones and Dangl 2006; Kim *et al.* 2008). Following perception, the host activates signalling pathways such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) which assist in curbing infection by promoting the expression of pathogenesis-related genes. SA has been shown to be effective in suppressing the proliferation of biotrophic or hemibiotrophic pathogens e.g. *P. syringae* whereas JA is essential for defence against necrotrophic pathogens e.g. *B. cinerea* (Pieterse *et al.* 2009).

Each of these signalling cascades has been shown to involve certain marker genes e.g. *PR* genes, which can be representative of the induction of a pathway. In *Arabidopsis* stimulation of the SA pathway can be represented by an increase in the expression levels of *PR1, PR2* and *PR5*. Transgenic plants over-expressing these SA markers resulted in increased resistance against pathogens such as *Phytophthora parasitica* and *Alternaria alternata* (Alexander *et al.* 1993*;* Jach *et al.* 1995). Induction of a derivative of JA, MeJA, can be represented in *Arabidopsis* by an increase in the expression levels of *PR3*, *PR4* and *LOX* and over-expression of these proteins has been shown to confer resistance to *Phytophthora nicotianae* and *Rhizoctonia solani* (Boter *et al.* 2004; Mishina and Zeier 2007; Kusajima *et al.* 2010). The aim of this study was to begin to elucidate the defence network of *E. grandis* by identifying and characterizing putative orthologs of marker genes associated with the SA and MeJA signalling pathways. To elucidate these

orthologous marker genes in *E. grandis*, a co-phylogenetic (Neighbour Joining and Maximum Likelihood) approach was implemented. Expression profiles generated from RT-qPCR experiments were compiled for each of the putative orthologs (*EgrPR1_like, EgrPR2, EgrPR3, EgrPR4, EgrPR5, EgrLOX*). The response of these marker genes to various concentrations of the applied phytohormone and their change in expression over a time course was investigated. It was found that from the range of concentrations explored in this study, 100 µM (Student's *t*-test, $p < 0.001$) elicited the most considerable change in transcript expression for the MeJA marker genes. For SA, the marker genes revealed that 5 mM (Student's *t*-test, $p < 0.01$) was the optimal concentration for this signalling pathway. Furthermore, the orthologous genes were found to corroborate the antagonistic relationship observed between SA and JA in *Arabidopsis*.

The ability of these putative markers to respond to fungal infection by *Chrysoporthe austroafricana* was examined in a tolerant (TAG5) and susceptible (ZG14) *E. grandis* genotype (Van Heerden *et al.* 2005). Although *C. austroafricana* is currently being controlled by the propagation of *E. grandis* hybrids e.g. GU21, outbreaks of this pathogen occasionally occur when conditions permit. Therefore *C. austroafricana* was selected in this study as a model for examining the diagnostic potential of putative marker gene orthologs. It was hypothesized that MeJA would confer tolerance against *C. austroafricana* as this pathogen is presumed to be a necrotroph. However, based on the comparison between the susceptible and tolerant genotypes, the SA signalling pathway was found to restrict pathogen proliferation in TAG5. Although only a limited number of genes (*EgrPR1_like, EgrPR2, EgrPR3, EgrPR4, EgrPR5, EgrLOX*) were profiled, this study was able to demonstrate how defence response genes are influenced in *Eucalyptus* and how the information transcends from known model systems to this woody plant.

2.3 Materials and Methods

2.3.1 Plant material

2.3.1.1 Propagation of *E. grandis* clone A tissue culture plantlets

Disease free *E. grandis* (Clone A, Mondi Forestry) plantlets were propagated *in vitro* at the FABI tissue culture facility. *E. grandis* clone A explants were cultured on Murashige & Skoog media containing vitamins and growth hormones until shoots developed. These shoots were then transferred to multiplication media and allowed to grow for at least 3-6 months at 25^{o}C-28^{o}C with a 16hr photoperiod, after which the shoots were transferred to rooting media until significant root development had occurred (four - six weeks). The exact hormone composition of the multiplication and rooting media is subject to an IP agreement with Mondi and therefore cannot be disclosed at present. Following the rooting period, the plantlets were rinsed in distilled water to remove excess rooting medium and transferred to Jiffy® pots (Jiffy products International AS, Norway). Trays containing the rooted plantlets were covered with cling-wrap for two weeks to maintain high humidity. After the two week adjustment period slits were made in the cling-wrap to allow the plantlets to slowly adapt to the change in humidity. Plantlets were grown at 25^{o}C-28^{o}C under long day (16h) conditions with a light intensity of 300-500 lum/sqf. Growth conditions were monitored using a HOBO® data logger (Onset Computer Corporation, Bourne, USA). Plants were watered every second day and artificial fertilization in the form of Multifeed® (Plaaskem, Witfield) was administered weekly as per the manufacturer's instructions.

2.3.1.2 *Eucalyptus* clones for the infection trial with *C. austroafricana*

E. grandis clonal genotypes, ZG14 and TAG5 (MONDI Forestry) were obtained from the experimental farm at the University of Pretoria. Potted cuttings with a stem diameter of 1 cm were selected for experimental use and kept under the same conditions mentioned above.

2.3.2 Identification of putative defence marker orthologs in *E. grandis*

2.3.2.1 Preliminary identification of *E. grandis* defence orthologs

At the time this study commenced, only the 4.4X coverage assembly of the *E. grandis* genome sequence was available and therefore the following methodology was executed. The *Arabidopsis thaliana* coding sequences (CDS) of the genes of interest (Table 2.1) were obtained from **T**he ***A****rabidopsis* **I**nformation **R**esource (TAIR, version 10) (https://www.arabidopsis.org). A local TBLASTX analysis was conducted against the 4.4X assembly sequence of *E. grandis* (http://eucalyptusdb.bi.up.ac.za/) using the CDS as the query sequence. *E. grandis* scaffolds ($e < 10^{-50}$) were examined for the specific region within the scaffold that showed the highest similarity to the CDS sequence.

The coding sequence plus 500 – 1000 bp upstream and downstream of the specified homologous region was retrieved. Gene prediction tools, GeneMark.hmm (http://exon.gatech.edu/eukhmm.cgi) and GenScan (http://genes.mit.edu/GENSCAN.html) were used to predict the CDS and peptide sequence contained in the *E. grandis* gene loci. To verify that the correct target gene had been obtained through the gene prediction tools, the predicted peptides were used as the query sequence for a local BLASTP similarity search on TAIR v10.

Co-phylogenetic analysis was performed to determine which *E. grandis* gene loci would group closest with the *A. thaliana* gene, thereby suggesting a putative ortholog. For each gene of interest a file was assembled containing the *A. thaliana* amino acid sequence, as well as of several predicted peptide sequences of the *E. grandis* scaffolds. These sequences were aligned using CLUSTALX and the alignment file was imported into MEGA v4.1 (Tamura *et al.* 2007) for phylogenetic tree construction. A neighbour joining tree was subsequently constructed with the following parameters: Pairwise deletion option and homogenous pattern among lineages. Included in the tree were the sequences of any closely related *A. thaliana* family members ($e < 10^{-50}$). Confidence in the clades was substantiated with the addition of a bootstrap value calculated after 10 000 permutations.

2.3.2.2 Verification of selected *E. grandis* defence genes against predicted proteome

Upon release of the 8X genome assembly in January 2011, the results obtained from the preliminary ortholog identification were verified against the updated genome sequence (Phytozome v7.0). The *Arabidopsis thaliana* amino acid sequences of the genes of interest (Table 2.1) were obtained from **T**he ***A****rabidopsis* **I**nformation **R**esource (TAIR, version 10) (https://www.arabidopsis.org). A BLASTP similarity search was conducted against the predicted *E. grandis* proteome (first *ab initio* and homology-based annotation) using the amino acid sequence as a query. This analysis was performed in Phytozome v7.0 (www.phytozome.net) and predicted *E. grandis* transcripts with e-values $< 10^{-50}$ were downloaded. Putative *Populus trichocarpa* orthologs of the gene of interest were retrieved from NCBI and added to the analysis using the same BLAST parameters. Alignment of the sequences (*A. thaliana* target gene, *A. thaliana* gene family members, *E. grandis* predicted peptides and *P. trichocarpa* putative orthologs) was performed

using the online version of MAFFT with E-INS-I setting (http://mafft.cbrc.jp/alignment/server/). The E-INS-I setting is used for a more accurate alignment of sequences that contain conserved domains with substantial regions of lower conservation in between (Nuin *et al.* 2006). The parameters for the alignment were as follows: BLOSUM62 scoring matrix for amino acid sequences; Gap penalty = 1.53 and an offset value = 0. Sequence files were converted to Phylip format (Felsenstein 1989) using Readseq for further analysis. Aligned sequences were imported into MEGA v5.01 (Tamura *et al.* 2011) for the construction of a neighbour joining (NJ) tree. Parameters were as follows: Pairwise deletion option and homogenous pattern among lineages. Confidence in the clades was substantiated by a bootstrap value calculated after 10000 permutations. In addition to the NJ tree, a maximum likelihood (ML) analysis was conducted. The aligned sequences were assessed using Prottest 3.0 (Abascal *et al.* 2005) to determine the best-fit model of amino acid substitution. PhyML 3.0 (Guindon and Gascuel 2003) was used to perform the ML analysis using the parameters of the best model obtained from the Prottest results. Confidence in the clades was substantiated by a bootstrap value calculated after 1000 permutations.

Furthermore the expression pattern of the selected gene model across different tissues was assessed on the ***Euc****alyptus* **Gen**ome **I**ntegrative **E**xplorer (EucGenIE, http://eucgenie.bi.up.ac.za). The data present on EucGenIE was obtained from an Illumina mRNA-Seq experiment of different tissues (xylem, immature xylem, phloem, shoot tips, young leaves and mature leaves) from a *E. grandis* x *E. urophylla* hybrid clone (Mizrachi *et al.* 2010).

2.3.3 Primer design and cloning

Putative orthologs in *E. grandis* were determined based on the NJ and ML trees and primers were designed for the defence genes using Primer Designer 4 v4.20 (Sci Ed Central, Cary, North Carolina, USA). Primer sequences were verified in Phytozome v7.0 using a BLASTN similarity search against the *E. grandis* genome to ensure that the scaffold of interest and corresponding predicted transcript was targeted by the primer pair. The average amplicon size expected when employing the primers ranged between 150 bp and 300 bp and primers were synthesized by Whitehead Scientific (Cape Town, South Africa, Table 2.1).

E. grandis cDNA (section 2.3.8), diluted 1:10 with distilled H_2O, was used as template for the amplification of the putative orthologs *via* polymerase chain reaction (PCR) with 0.4 U Excel High Fidelity Taq (Southern Cross Biotechnologies, Cape Town, South Africa), 1x PCR buffer, 0.20 mM dNTPs and gene specific primers (0.4 µM) in a final reaction volume of 20µl. The PCR reaction was performed using the following conditions: initial denaturation for 1 minute at 94^{o}C followed by 30 cycles of 30 seconds denaturation at 94^{o}C, 30 seconds annealing at Tm of primer pair (Table 2.2), 30 seconds elongation at 72^{o}C, 5 minutes additional elongation at 72^{o}C and a final hold at 4^{o}C. Upon optimization of PCR conditions, the amplicon of interest was gel purified from a 0.8% agarose gel using the MinElute® Gel Extraction Kit (Qiagen Inc, Valencia, CA), cloned using the InsTAclone™ PCR cloning kit (Fermentas, Ontario, Canada) and introduced into *Escherichia coli* DH5α competent cells that were generated *via* the $CaCl_2$ protocol (Cohen *et al.* 1972). The transformed cells (100 µl) were then plated out on LB media containing 0.5 mM IPTG, 80 µg/ml X-gal and 50 µg/ml carbenicillin and incubated for 16 -18hrs at 37^{o}C. This cloning step was done to increase the sequence coverage of the amplified target.

Colonies (5 – 10) were selected based on blue/white screening and colony PCR was performed using the universal M13 sequencing primer set: M13_Foward 5' CACGACGTTGTAAAACGAC 3' and M13_Reverse 5' GGAAACAGCTATGACCATG 3'. The PCR reaction was performed using the selected colony as a template and the same reagents stated above, at the following conditions: initial denaturation for 1 minute at 95^{o}C followed by 30 cycles of 30 seconds denaturation at 94^{o}C, 30 seconds annealing at 53^{o}C, 2 minute elongation at 72^{o}C, 10 minutes additional elongation at 72^{o}C and a final hold at 4^{o}C. Positive colonies, selected based on the approximate size of the amplicon, were grown overnight at 37^{o}C and plasmid DNA was isolated according to the manufacturer's protocol using the GeneJET™ Plasmid Miniprep Kit (Fermentas, Ontario, Canada).

Isolated plasmids were sequenced bi-directionally using the universal M13 primer set. A consensus sequence was constructed based on the forward and reverse sequence data and was used as the query sequence for the BLASTN function on Phytozome v7.0 against the *E. grandis* genome to ensure that the correct scaffold was amplified. However, because this genome is currently not fully annotated in terms of gene function, the consensus sequences were also used as the query sequence in a local TBLASTX similarity search on the TAIR website to confirm that the correct target gene had been obtained.

Table 2.1: Oligonucleotide primer sequences for putative defence gene orthologs in *E. grandis* as representative markers of the SA and MeJA signalling pathways. This list represents all the genes for which primers were designed in this study however only selected genes were profiled using RT-qPCR (see Table 2.2).

[(a)]Primer Name	ATG Number	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5' – 3')	Amplicon size (bp)
SA marker genes				
*EgrPR1*_like	AT2G14610	AGCCCGCTCCCAGGTCGGTGT	CGGCTGGTCCATTCAGGCGGA	118
EgrPR2	AT3G57260	GCTCTACAACCAGCGCAATATC	GCCAACTGCTATGTACCTGAAC	214
EgrPR5	AT1G75040	CCTGTTGGACGTCAACGCC	GTCGTCGTACTCGAAGATT	167
EgrNPR1	AT1G64280	CTGTGATGCGAAGACCTTGAC	CTTCCTCGGTAGACTCCATGT	239
EgrEDS1	AT3G48090	TGCGGTCCTTCGACTGTT	AAGGCACAGCCTCACCTT	286
EgrEDS5	AT4G39030	GCGCGAACATTGCTGAAG	TGCCAGTCCAACTGCTCTA	243
EgrPAD4	AT3G52430	GTACGCATGATGGACAGGAAC	GTCCAATGGCTCCACAAGAAG	102
EgrFMO	AT1G19250	TCCGAACATGGCATTCCT	GGTCGTCCTCTTCATCATCT	169
EgrDIOX	AT3G01420	TATTCGAGCCTTGCGAGAAGTC	CCTTCAATGTCTCCGTCCTGTT	236
JA marker genes				
EgrPR3	AT3G12500	CGGCCGCGAAGTCGTTCCC	AACTATAACTACGGGCAAT	277
EgrPR4	AT3G04720	ATGCCGTGAGCGCATACTG	GCGTGTTGGTCCTGGTGTT	156
EgrLOX2	AT3G45140	ATGAACACTTGCTTCCATT	TCCTACCATACGTGAACAA	165
EgrLOX3	AT1G17420	CATATGCGACTCGCACCATC	CCAGCATCGTTGGAGTTGAC	187
EgrLOX5	AT3G22400	AGCCGTCGGATAGGATCTCAAT	CTCATCCAGTCATGCACCATCA	250
EgrAOS	AT5G42650	CGGCTCTACGACTTCTTCCA	GTAGACGGCCGACTTCATCA	288

(a) No Tm's are given for these genes as not all of the PCR conditions were optimized.

2.3.4 Dose response of putative orthologous markers

SA and MeJA were administered to *E. grandis* clone A plantlets (Section 2.3.1.1) by spraying the aerial portions of the plants with varying concentrations of the inducers until run-off. The following inducer concentrations were assessed: 25 µM, 50 µM, 100 µM, 250 µM, 500 µM, 1 mM and 5 mM. Sodium salicylate (Riedel-de Haen, Seelze, Germany, Cat no: 31493) was used to prepare the SA solutions (adjusted to pH 7.0 with NaOH solution) with the addition of 0.1% Tween® 20 (Sigma-Aldrich, Missouri, USA). Control plants were sprayed with distilled water containing 0.1% Tween® 20. MeJA (methyl jasmonate 95%, Sigma-Aldrich, Missouri, USA, Cat no: 392707) was prepared with the addition of 0.1% Ethanol (100%) as well as 0.1% Tween® 20 (Sigma-Aldrich, Missouri, USA). Control plants were sprayed with distilled water containing 0.1% Tween® 20 and 0.1 Ethanol.The plants were kept in separate rooms for a minimum of 30 minutes following the application of inducers to allow for the evaporation of excess vapours. Three biological replicates consisting of five plantlets each was harvested for the each concentration and controls. Aerial parts of the plantlets were sampled 24 hours post treatment (hpt), flash frozen in liquid nitrogen and stored at -80^{o}C. RNA extraction of these samples was performed within two weeks following sampling (Section 2.7). Expression profiling samples was conducted *via* RT-qPCR using the primer set listed in Table 2.2.

Table 2.2: Oligonucleotide sequence of *Eucalyptus* reference genes and target genes assessed using RT-qPCR. The target genes represent putative marker gene orthologs for the SA and MeJA signalling pathways in *E. grandis.*

Primer Name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Amplicon size (bp)	Tm (°C)
SA marker genes				
[1] *EgrPR1_* like	AGCCCGCTCCCAGGTCGGTGT	CGGCTGGTCCATTCAGGCGGA	118	67
EgrPR2_1	TCGCCGTGAACAGAGCATAG	CTGTGGACACGCGCATCTTA	195	60
EgrPR2_2	GCTCTACAACCAGCGCAATATC	GCCAACTGCTATGTACCTGAAC	214	60
EgrPR5	CCTGTTGGACGTCAACGCC	GTCGTCGTACTCGAAGATT	167	66
EgrNPR1	TGTGCAGTAATGCGTGTGAG	CTGGCCTATTCAAGCCAAGT	153	64
JA marker genes				
EgrPR3	CGGCCGCGAAGTCGTTCCC	AACTATAACTACGGGCAAT	277	60
EgrPR4	ATGCCGTGAGCGCATACTG	GCGTGTTGGTCCTGGTGTT	156	60
EgrLOX2	ATGAACACTTGCTTCCATT	TCCTACCATACGTGAACAA	165	60
EgrLOX3	CATATGCGACTCGCACCATC	CCAGCATCGTTGGAGTTGAC	187	64
Reference Genes				
[2] *EguIDH*	TGGAACTGTTGAGTCTGG	TTAGGACCATGAATGAGGAG	59	64
EguF4B	CCCAAATATGAACCGTCCA	GTTCGATCCATAGCGTCC	146	60
EguAPRT	TTTCTCTTCGTGTCGCTG	ACGCCATGTGTTGATCTC	85	60
EguActin	TAAGCATGACAAGGAACCAG	TCAGGTCCAAGAAATCGT	110	60
EguTUB	GTGACATTCCTCCGACTG	GCAAGAAAGCCTTCCTG	123	60
EguUBQ	GAGGGACATCTATCTCTATGAC	CAACAGTAAGCACACGAG	131	60
EgrARF	TGCGTACCGAGTTGTTGAGG	GTTGCACAGGTGCTCTGGAT	195	64
EgrFBA	TGAAGACATGGCAAGGAAGG	GTACCGAAGTTGCTCCGAAT	190	64
EgrTUB	TGAGGTCTTCTCGCGCATTG	AGAGATCATGGCGCAGACAC	222	64

[1] *Egr = Eucalyptus grandis*
[2] *Egu = Eucalyptus grandis x E. urophylla*

2.3.5 Investigation of the expression profiles of putative *E. grandis* orthologs over a time course

Inducers, SA and MeJA, were administered to *E. grandis* clone A plantlets as described in the previous section. A single concentration selected from the dose response experiment for SA and MeJA was assessed at the following time points: 6 hpt, 12 hpt, 24 hpt and 48 hpt. Controls were harvested at each individual time point as well as at time zero which refers to the time prior to the application of inducers. Three biological replicates consisting of five plantlets each were harvested for each time point and control. Aerial parts of the plantlets were sampled at the specified time points, flash frozen in liquid nitrogen and stored at -80^{o}C. RNA extraction of these samples was performed within two weeks following sampling and subsequently analyzed *via* RT-qPCR using the primer set listed in Table 2.2.

2.3.6 Infection trial with *Chrysoporthe austroafricana*

Ramets of two *E. grandis* clones, TAG5 and ZG14 trees, with an approximate stem diameter of 1 cm were inoculated with the fungus *C. austroafricana* CMW2113 (Van Heerden *et al.* 2005). These two clones were selected as it was previously shown by Van Heerden *et al.* (2005) that TAG5 is tolerant to the pathogen whereas ZG14 is susceptible. The response of *E. grandis* clone A to *C. austroafricana* has not been documented thus far and therefore was not included in this experimental section. Potted ramets were allowed to acclimatize to glasshouse conditions (Section 2.3.1) for two weeks prior to inoculation. Each tree was inoculated 30 cm above the soil by removing the bark and vascular cambium with a 5 mm cork borer. *C. austroafricana* was grown on 2% MEA plates (20 g malt extract agar in 1 L distilled water) for 5 days at 25^{o}C and an agar plug of the fungus, corresponding to the wound size, was placed on the opening

with the mycelium facing inward. Control trees were mock inoculated with sterile 2% MEA agar plugs which contained no fungus.

The wounds were subsequently sealed with Parafilm® (Plastic packaging, Labretoria) and the trees monitored for a period of six weeks. A daily watering regime was implemented and the glasshouse temperature was maintained between 25^{o}C and 28^{o}C. Plant material (stem tissue, 1 cm above and below the lesion) was harvested at 48 hours post inoculation, 2 weeks and 6 weeks post inoculation (wpi). Three biological replicates consisting of three trees per replicate were harvested at each time point. In addition to collecting plant material, the stem lesion lengths of the trees were recorded at the three time points. Samples were flash frozen in liquid nitrogen and stored at -80^{o}C. RNA was extracted within one week of the individual time points. Re-isolation of the fungus was performed by excising a piece off the periphery of the lesion after six weeks and placing the block on 2% MEA. At least five pieces per lesion were excised and placed on an agar plate. Confirmation of infection by *C. austroafricana* was done by observing the culture morphology after five days.

2.3.7 RNA extraction and quality control

Frozen plant material was ground using liquid nitrogen and a high-speed grinder (IKA-Werke, Staufen, Germany) to produce a fine powder. Total RNA was extracted from the plant powder using a modified cetyl-trimethyl-ammonium-bromide (CTAB) extraction protocol (Zeng and Yang 2002). The following adaptations were made to the original protocol: 5ml of extraction buffer was used per 1g of plant tissue and RNA was recovered from the samples by centrifugation for 1hr after overnight precipitation. All centrifugation steps were performed at 10 000rpm. Total RNA samples were treated with RNase-free DNaseI enzyme (Qiagen Inc, Valencia, CA) and subsequently purified using

the RNeasy® MinElute Kit (Qiagen Inc, Valencia, CA) as per the manufacturer's instructions. Purified RNA was analyzed on a 1% formaldehyde agarose gel (RNeasy® Mini Handbook) and the sample preparation was executed as follows: ethidium bromide (1:10) was added to the sample in addition to RNA loading buffer (made up according to the RNeasy® Mini Handbook); samples were heated at 65^{o}C for 10 minutes and subsequently cooled on ice for 2 minutes and loaded onto the gel (5-7 V/cm). The concentration of the samples and the purity ratio's were obtained using a Nanodrop spectrophotometer (Nanodrop technologies, ND1000, DE, USA) and a subset of the samples was analyzed using a Bio-Rad Experion Spectrophotometer (Bio-Rad Laboratories, South Africa).

2.3.8 First strand cDNA synthesis and quality control

Purified RNA was used as the template for reverse transcription using Improm II reverse transcriptase enzyme (Promega). The reaction was set up as follows: 1 µg purified RNA template, 1 µl Oligo dT primer (0.5 µg/µl) and 0.5 µl RNase Inhibitor was incubated at 70^{o}C for 10 minutes and chilled on ice for 1 minute. The following reagents were subsequently added to the reaction: 4 µl 5X buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs and 1 µl Improm II enzyme. A reaction cycle of 25^{o}C for 10 minutes, 42^{o}C for 60 minutes and 70^{o}C for 15 minutes followed by incubation at 4^{o}C for infinity was performed. Samples were stored at - 20^{o}C in aliquots.

Samples of cDNA were assayed to detect the presence of genomic DNA contamination using an amplicon from the *Eucalyptus grandis* ADP ribosylation factor (*EgrADP*). The *EgrADP* gene was amplified from genomic DNA and cDNA to produce a band that was ~1.5kb and ~250bp respectively using the following intron spanning primer set: *EgrADP*_Forward 5' TTCTGGTGCCATGCTGAGAA 3' and *EgrADP*_Reverse 5'

GATGCTGTGTTGCTCGTCTT 3'. The PCR reaction was performed using 10 ng of 1:10 diluted cDNA at the following conditions: initial denaturation for 1 minute at 94°C followed by 30 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 60°C, 1 minute elongation at 72°C and a final hold at 4°C. Samples were analyzed on a 1% (w/v) agarose gel to confirm presence or absence of genomic DNA.

2.3.9 Reverse transcriptase quantitative PCR (RT-qPCR) analysis

Quantitative real-time PCR was performed according to the **M**inimum **I**nformation for Publication of **Q**uantitative Real-Time PCR **E**xperiments guidelines (MIQE) using the LightCycler® 480 Real-Time PCR system (Roche Diagnostics, GmBH, Basa, Switzerland) (Bustin *et al.* 2009). This system was used for the expression profiling of selected targets from Table 2.2, as not all the primer pairs shown in Table 2.1 produced the correct amplicon. For each target, three biological replicates and three technical replicates per biological replicated were performed. First strand cDNA was synthesized as mentioned in section 2.3.8 and 1 µl (1:10 diluted cDNA template) of each sample was used in the RT-qPCR reaction. In addition to the template, the following reagents were added: 5 µl of LightCycler ® 480 SYBR Green I Master Mix 2x concentration (Roche, Mannheim, Germany); 0.5 µl of each primer and water to a reaction volume of 11 µl.

Activation of the FastStart SYBR Green Mix required a pre-incubation step which was performed at 95°C for 5 minutes. For quantification, the amplification phase was set up to execute 45 cycles of 95°C for 10 seconds, the Tm of the primer pair (Table 2.2) for 10 seconds and a single acquisition at the end of each elongation step at 72°C for 15 seconds. Melting curve analysis was performed as follows: one cycle of 95°C for 5 seconds, 65°C for 1 minute and a continuous signal acquisition at 95°C with 10 acquisitions per 1°C. The reaction was completed with a cooling phase of one cycle at

40^{o}C for 5 seconds. Products were analyzed on 1% (w/v) agarose gel to verify that the correct size fragment was amplified and that a single product was obtained. Samples of each amplified target were also sent for DNA sequencing to further verify that the specific gene of interest was acquired.

Selected concentrations from a serial dilution set (1:1; 1:10; 1:20; 1:50; 1:100; 1:1000) made from a pool of cDNA samples was used to determine the amplification efficiency of each primer pair. The experiment was set up using the sample maximization method which analyzes all the samples of a particular gene in a single run and different genes in separate runs. The sample maximization method negates the need for an inter run calibrator, hence none were included in this study (Hellemans *et al.* 2007). Nine putative reference genes were analyzed, three were designed previously from *E. grandis* [*ADP ribosylation factor (EgrARF), Fructose bisphosphate aldolase (EgrFBA) and α-Tubulin (EgrTUB)*] and six were obtained from a study conducted by Boava *et al* (2010), which were designed from an *E. grandis x E. urophylla* hybrid [*NADP-isocitrate dehydrogenase (EguIDH), Eukaryotic translation initiation factor 4B (EguF4B), Adenine phosphoribosyltransferas (EguAPRT), Actin (EguActin), Tubulin (EguTUB) and Ubiquitin (EguUBQ)*] (Boava *et al.* 2010).

Normalization of the target genes was based on the reference gene set that had the most stable expression across the range of samples. Table 2.2 comprises the primer sequence of the reference genes and targets that were analyzed in this study. Normalization and relative quantification was performed using *qBASE*plus v1.0 (Hellemans *et al.* 2007) and significance was determined using the two-tailed Students *t*-test in Microsoft® Office Excel 2007.

2.4 Results:

2.4.1 Identification of putative defence marker orthologs in *E. grandis*

Putative defence orthologs for known marker genes of the SA and MeJA signalling pathways were identified in *E. grandis* using BLAST algorithms and phylogenetic analysis. The BLAST analysis provided a suite of candidates that could be incorporated into the phylogenetic tree to elucidate a potential ortholog. With the initial 4.4X genome assembly, a TBLASTX similarity search was used to probe for *E. grandis* candidates. This was done as the predicted proteome was not available and therefore the CDS sequence was used as the query for a TBLASTX analysis, an algorithm used for cross-species gene predictions (Korf *et al.* 2003). However, with the release of the 8X genome assembly, the predicted *E. grandis* proteome was available and therefore a BLASTP similarity search was conducted. The top three results of the BLASTP analysis against the 8X genome assembly of *E. grandis* are shown in Appendix A.

Phylogenetic analysis was performed to determine which predicted gene of *E. grandis* would group closest with the *A. thaliana* and *P. trichocarpa* orthologs thereby suggesting a putative ortholog for further investigation. Initially, primers were designed using the 4.4X assembly of the *E. grandis* genome as the 8X sequence was unavailable at the time this study commenced. The preliminary NJ phylogenetic trees that were constructed with the 4.4X assembly sequence are shown in Appendix B (Figure S1 – S6). Upon release of the 8X assembly, all of the genes were re-analyzed based on this updated version of the genome sequence to ensure that the primers previously designed would correspond to the updated genome version. Only the phylogenetic analysis of the SA and MeJA target genes that were used for further gene expression experiments are shown.

The results of the ML phylogenetic analysis are displayed in the figures (Figure 2.1 – 2.6) below with the Prottest results for each gene given in the figure legends. The Prottest results are presented as follows: the best fit substitution model which includes the Jones, Taylor and Thornton (JTT) model (Jones *et al.* 1992), the Le and Gascuel (LG) model (Chaturvedi *et al.* 2008) and the Whelan and Goldman (WAG) model (Whelan and Goldman 2001). For each model the following parameters are given: (a) the proportion of invariable sites [I] which refer to the expected frequency of sites that do not evolve (b) the gamma distribution [G] parameter and (c) the amino acid frequencies [F]. If “F” is not stated in the equation then the amino acid frequencies were determined by the substitution model else “F” is calculated by selecting the empirical function in the software which counts the amino-acid differences in the alignment. In each tree the target gene and the corresponding putative ortholog are indicated by the arrows.

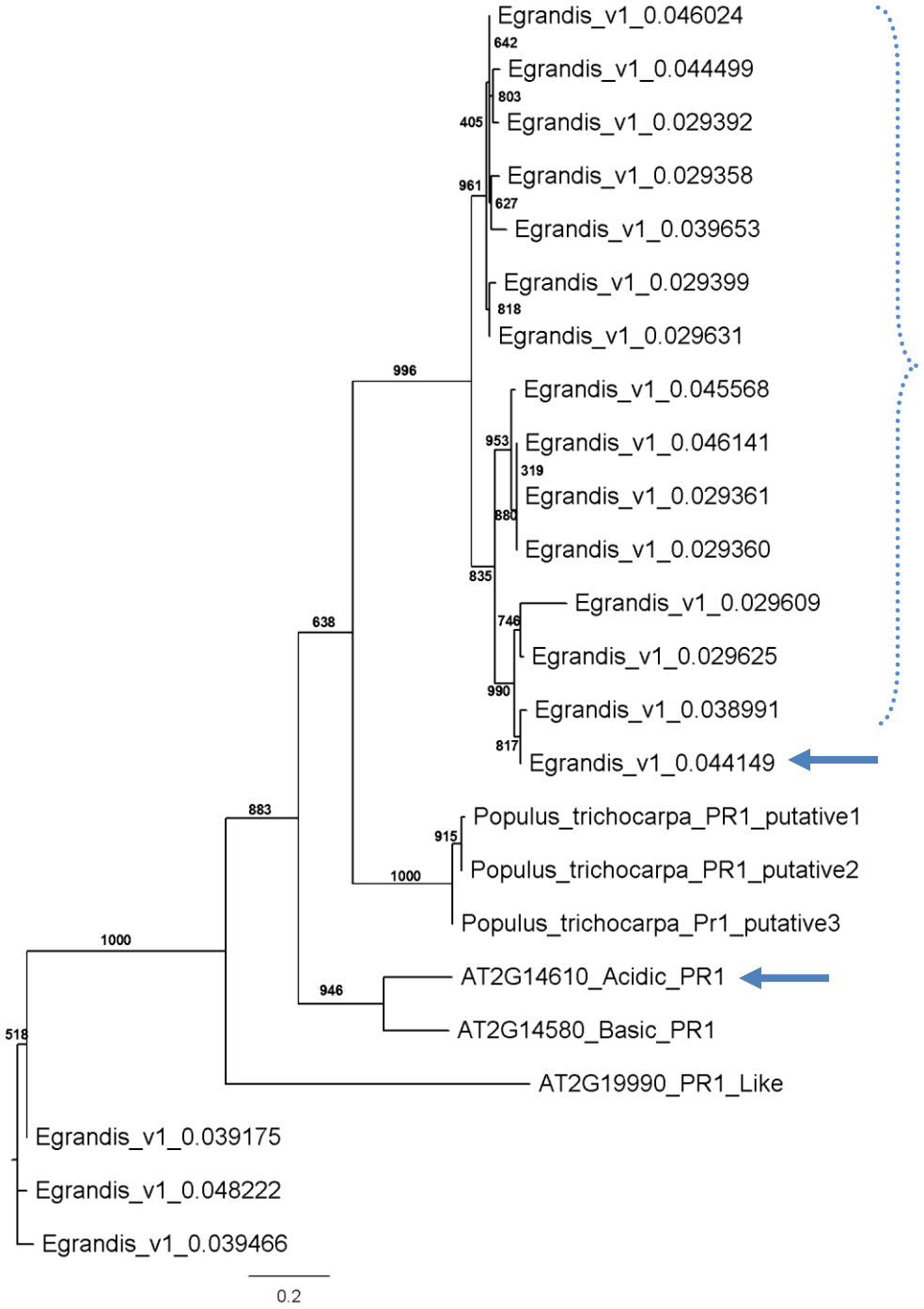

Figure 2.1: Maximum likelihood tree of *EgrPR1-like* (Pathogenesis-related gene 1) proteins. Bootstrap values are representative of 1000 permutations. Included in the tree are closely related family members, *ATPRB1* (AT2G14580) and *PR1-Like* (AT2G19990). Amino acid sequences for all the *Eucalyptus* candidates were obtained from Phytozome v7.0. The bracket indicates other putative orthologous candidates in *Eucalyptus*. Prottest best fit model = JTT + I + G (I = 0.155; G = 2.779). The target gene and the corresponding putative ortholog are indicated by the arrows.

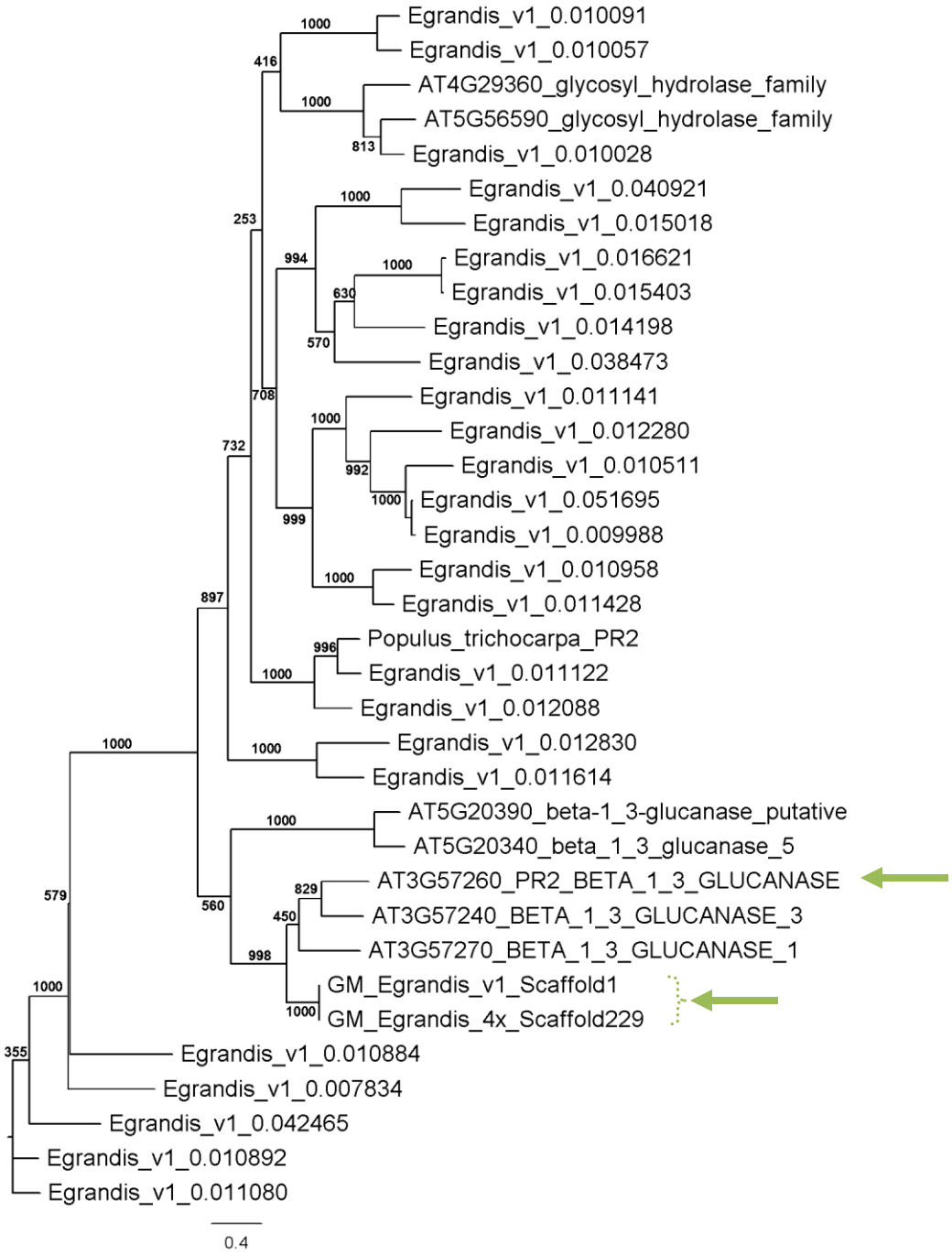

Figure 2.2: Maximum likelihood tree of *EgrPR2* (Beta - 1, 3 - glucanase) and related proteins. Bootstrap values are representative of 1000 permutations. Included in the tree are other closely related *Arabidopsis* family members that contain the glycosyl hydrolase domain. Amino acid sequences for all the *Eucalyptus* candidates were obtained from Phytozome v7.0. Prottest best fit model = LG + I + G + F (I = 0.023; G = 1.419; F = Empirical amino acid frequencies). The target gene and the corresponding putative ortholog are indicated by the arrows.

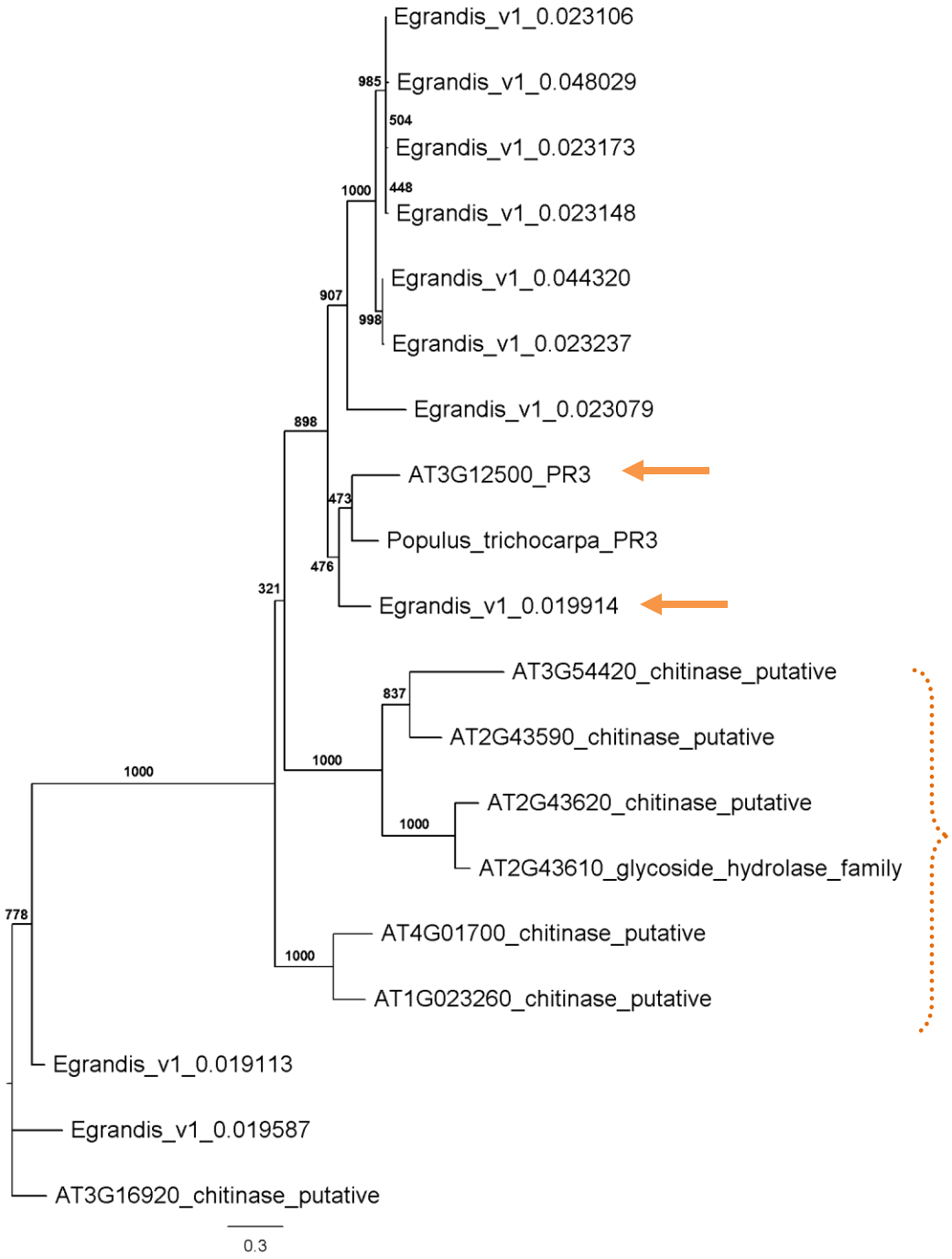

Figure 2.3: Maximum likelihood tree of *EgrPR3* (Chitinase) and related proteins. Bootstrap values are representative of 1000 permutations. Included in the tree are other *Arabidopsis* chitinase family members indicated by the bracket. Amino acid sequences for all the *Eucalyptus* candidates were obtained from Phytozome v7.0. Prottest best fit model = WAG + I + G + F (I = 0.058; G = 2.124; F = Empirical amino acid frequencies). The target gene and the corresponding putative ortholog are indicated by the arrows.

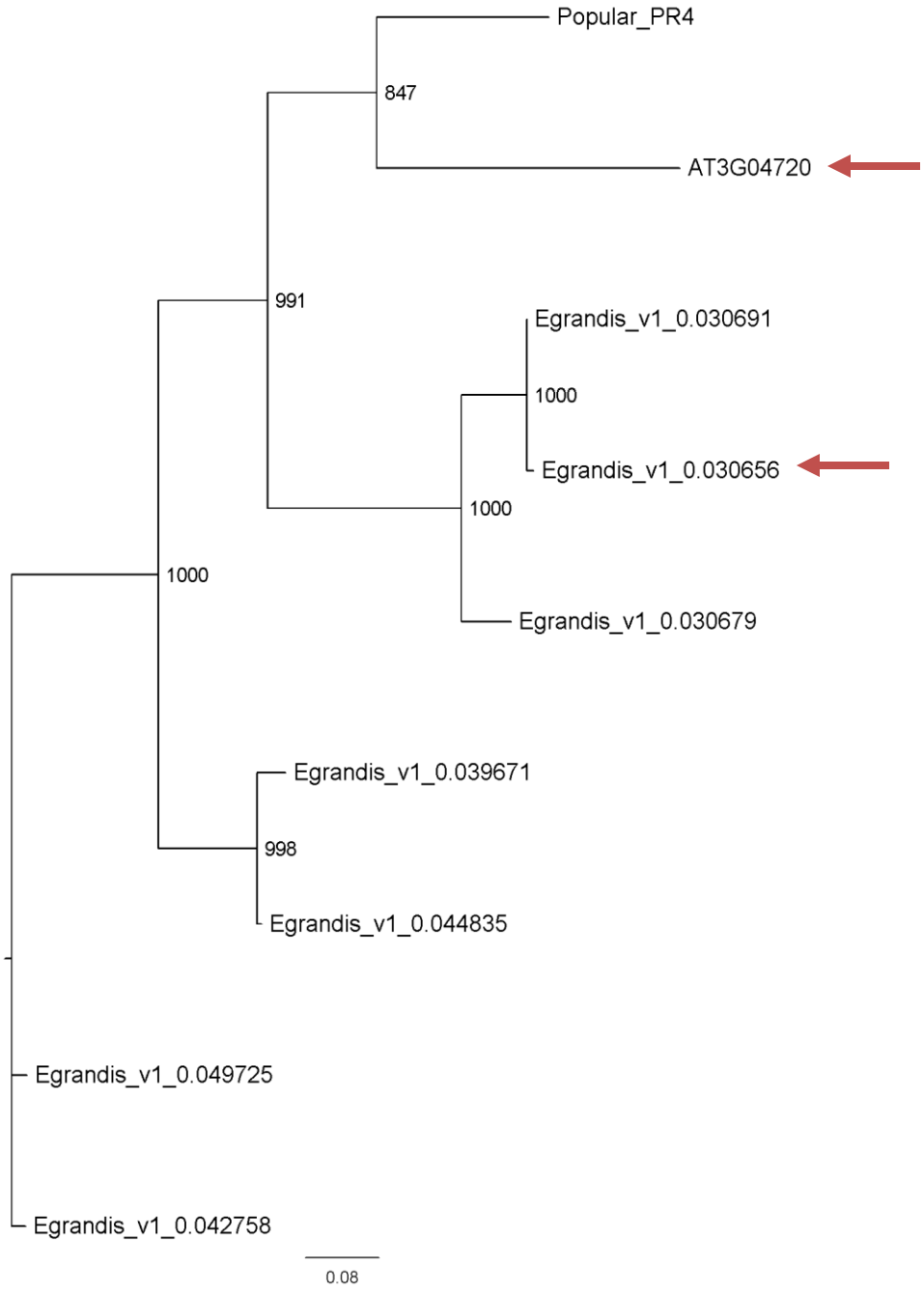

Figure 2.4: Maximum likelihood tree of *EgrPR4* (Hevein-like protein). Bootstrap values are representative of 1000 permutations. There were no family member with significant e-values (e < -50) hence only the *P. trichocarpa* sequence is included in the tree. Amino acid sequences for all the *Eucalyptus* candidates were obtained from Phytozome v7.0. Prottest best fit model = WAG + G + F (G = 0.936; F = Empirical amino acid frequencies). The target gene and the corresponding putative ortholog are indicated by the arrows.

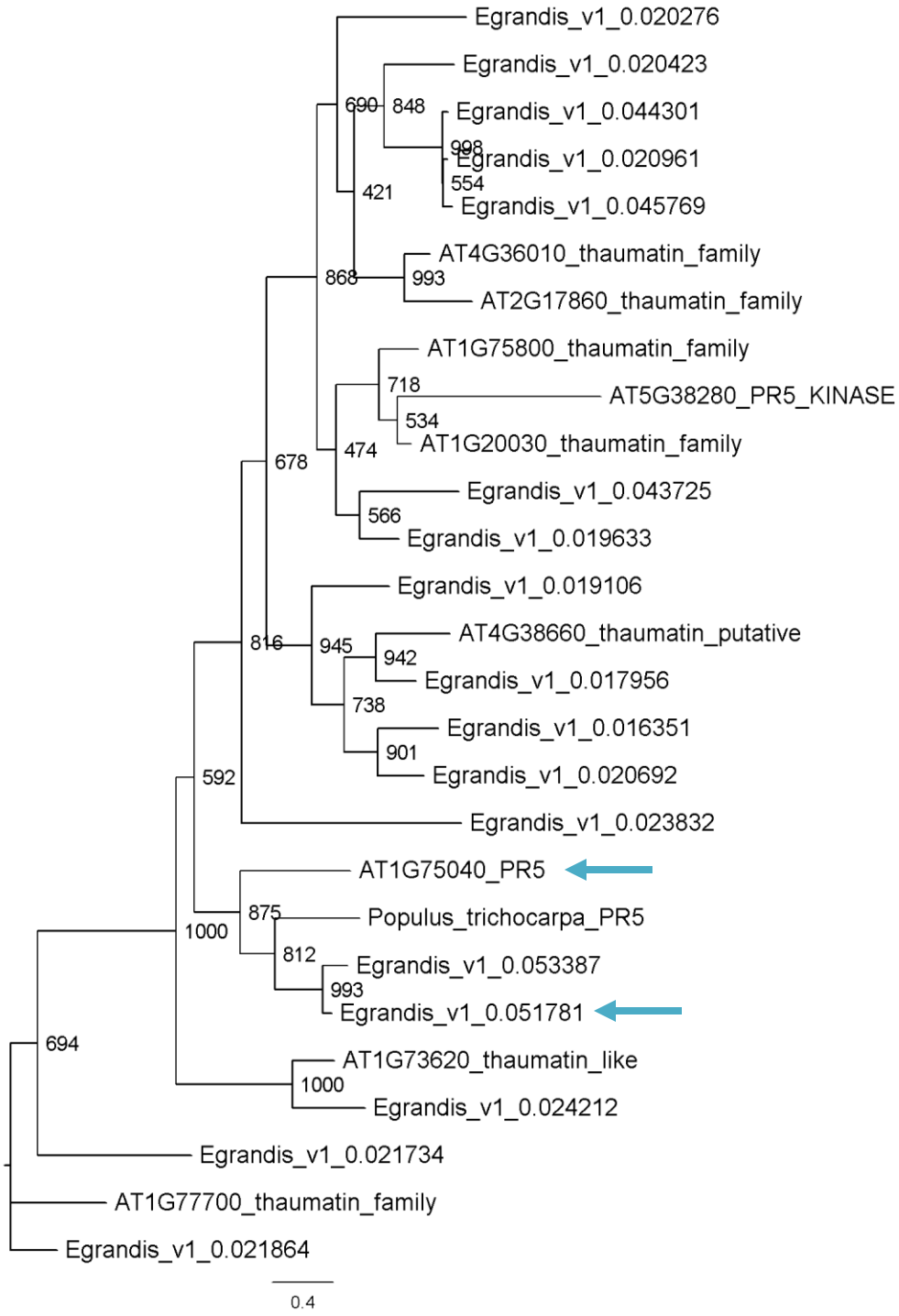

Figure 2.5: Maximum likelihood tree of *EgrPR5* (Thaumatin) proteins. Bootstrap values are representative of 1000 permutations. Included in the tree are other *Arabidopsis* thaumatin family members. Amino acid sequences for all the *Eucalyptus* candidates were obtained from Phytozome v7.0. Prottest best fit model = WAG + I + G (I = 0.105; G = 1.195). The target gene and the corresponding putative ortholog are indicated by the arrows.

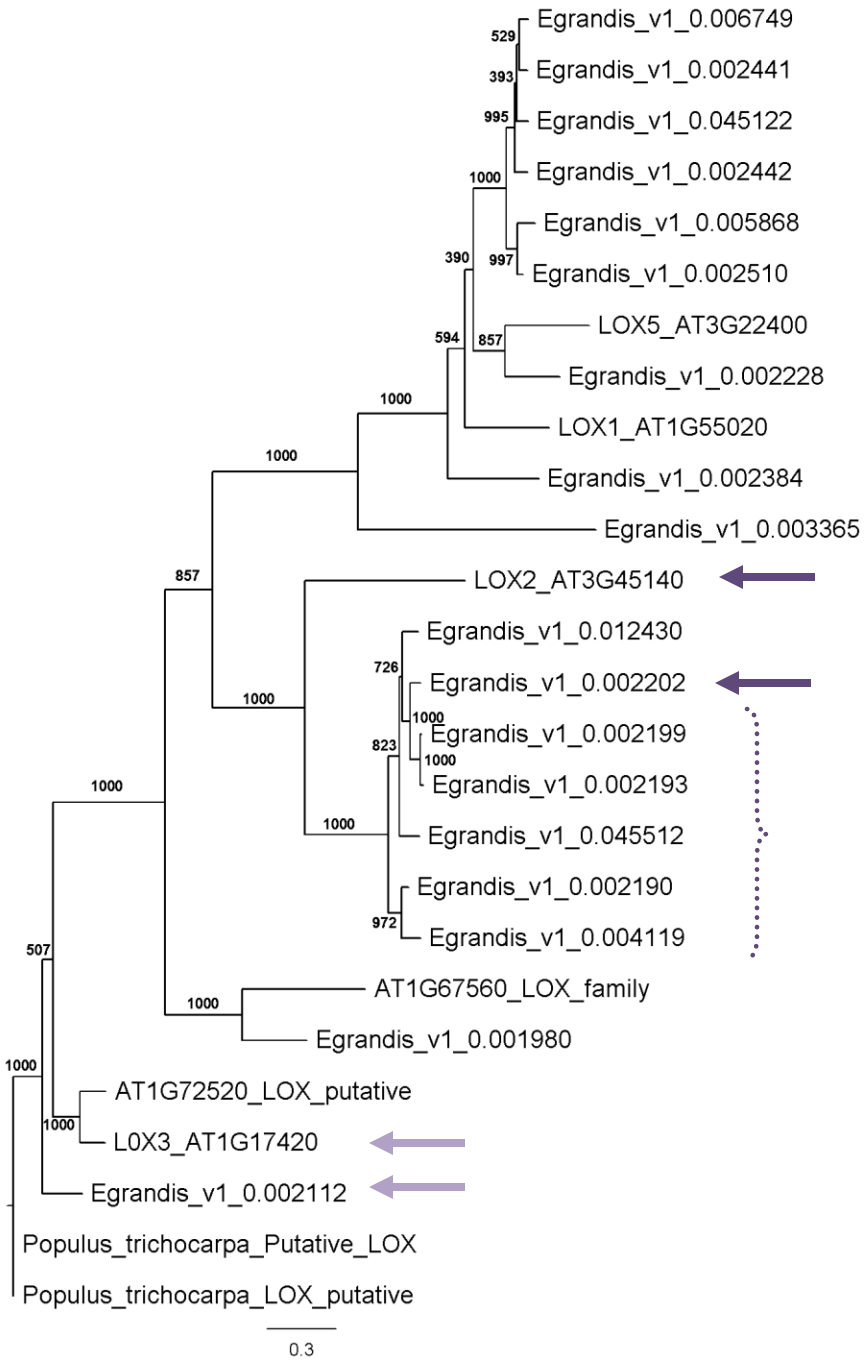

Figure 2.6: Maximum likelihood tree of *EgrLOX* (Lipoxygenase) proteins. Bootstrap values are representative of 1000 permutations. Included in the tree are *LOX1, LOX2, LOX3, LOX5* and other closely related *Arabidopsis* family members. Amino acid sequences for all the *Eucalyptus* candidates were obtained from Phytozome v7.0. The bracket indicates other putative orthologous candidates in *Eucalyptus*. Prottest best fit model = LG + I + G (I = 0.082; G = 1.233). The target gene and the corresponding putative ortholog are indicated by the arrows.

The primer set for *EgrNPR1* was obtained from a study conducted by Lourens *et al* (unpublished) and therefore no phylogenetic tree is shown for this gene family. The result of the NJ analysis for these genes can be found in the supplementary data (Appendix C, Figure S7 – S12). Table 2.3 summarizes the predicted gene models and corresponding genomic scaffold regions that were selected for each of the targets. The position of the primers and the region of the transcript that was amplified for each putative ortholog are represented as amplicon maps in Appendix E.

From the initial NJ phylogenetic tree that was constructed for the *PR1* ortholog (Appendix B, Figure S1), scaffold_98:122000_122500 (4.4X assembly) was selected as a representative from the *E. grandis* clade. This scaffold region corresponded to the genomic region scaffold_7:19000976 – 19001631 from the 8X genome assembly, which encoded for the predicted gene model *Egrandis*_v1_0.044149 (Appendix A). The expression pattern of this predicted gene model across different tissues indicated that it is primarily expressed in shoot tips, young leaves and mature leaves (Appendix F). However, because there was also equivalent expression data for the other gene models within the bracket indicated in Figure 2.1, there is a high probability that there could be more than one putative ortholog for the *PR1* protein in *Eucalyptus.* Due to limitations in further resolving this gene family, *Egrandis*_v1_0.044149 was selected as a representative of this group of potential orthologs for this study and will hereafter be referred to as *EgrPR1_like*.

Table 2.3: Predicted gene models and corresponding genomic scaffold regions selected as putative orthologs for the SA and MeJA marker genes in *E. grandis.*

Gene	AT number	Predicted gene model	Genomic scaffold region	E- value	% Identity
EgrPR1_like	AT2G14610	*Egrandis*_v1_0.044149	Scaffold_7: 19000976 - 19001631	$5.7e^{-55}$	63.9
EgrPR2	AT3G57260	*GM_*Egrandis*_V1_Scaffold1	Scaffold_1: 33791675 – 33792649	e^{-116}	68
EgrPR3	AT3G12500	*Egrandis*_v1_0.019914	Scaffold_9: 25149898 – 25151718	$4.3e^{-119}$	69.2
EgrPR4	AT3G04720	*Egrandis*_v1_0.030656	Scaffold_2: 42319519 - 42320281	$1.9e^{-50}$	74.8
EgrPR5	AT1G75040	*Egrandis*_v1_0.051781	Scaffold_1: 7623283 – 7624480	$5.6e^{-66}$	63.4
EgrLOX2	AT3G45140	*Egrandis*_v1_0.002202	Scaffold_10: 8809509 – 8814780	0	57.4
EgrLOX3	AT1G17420	*Egrandis*_v1_0.002112	Scaffold_10: 35104347 – 35114175	0	72.4

*No predicted transcript on Phytozome v7.0 for the selected scaffold region

All the genes, except for *EgrPR2*, had predicted transcripts that were congruent with the sequence of *E. grandis* located on Phytozome v7.0 (http://www.phytozome.net). In the case of the *EgrPR2* gene family, a TBLASTX analysis against the 4.4X assembly (http://eucalyptusdb.bi.up.ac.za) revealed high similarity with a region on scaffold 229 (Appendix B, Figure S2). This region corresponded with a region on scaffold 1 in the *E. grandis* genome sequence (8X assembly) on Phytozome v7.0. However, the predicted transcript for the region on scaffold 1 was not identified in Phytozome v7.0, which is based on FgenesH and GenomeScan gene predictions. Therefore an *ab initio* prediction of this region on scaffold 1:7623283_7624480 was performed using GeneMark and the result of this was included in the phylogenetic tree designated as GM_Egrandis_v1_Scaffold1 (Figure 2.2).

The GeneMark prediction of the scaffold 229 region from the 4.4X *E. grandis* coverage sequence was also included in the tree and designated as GM_Egrandis_4x_Scaffold229. Additionally, to confirm that the sequence was not an artefact or a pseudogene, scaffold 1:7623283_7624480 was assessed using the genome browser on Phytozome v7.0. This revealed that the region is in fact expressed as confirmed by the Eucspresso read track (Appendix D). Additionally, this region was found to have homology in other plant species and when the sequences of these reads (Appendix D, purple arrow) were used as a query sequence for a local TBLASTX in TAIR, it was found to correspond to *A. thaliana PR2* and not other family members e.g. POPTR_0016s05780 had an value of e^{-113} against AT3G57260 (β-1,3-glucanase). The *Arabidopsis PR2* gene formed a clade with GM_*Egrandis*_V1_Scaffold1 that was accompanied by strong bootstrap statistical support in both the NJ and ML phylogenetic trees and therefore was selected as the putative ortholog (Figure 2.2, Appendix C Figure S8).

Confidence in the *EgrPR3* predicted transcript, *Egrandis*_v1_0.019914, was substantiated by its grouping with the *Arabidopsis* and *Populus* orthologs in both the NJ and ML trees (Figure 2.3, S9). Although the bootstrap statistic in the ML tree is relatively low, there was higher support in the NJ tree which produced the same clade thus corroborating this candidate (Appendix C, Figure S9). Additionally the primers that were designed based on scaffold 9498:500_2000 from the 4x genome sequence (Appendix B, Figure S3) bind to and amplify the region encoding the *Egrandis*_v1_0.019914 gene model (Appendix A). This gene model has also been shown to be expressed in xylem, immature xylem, phloem, young leaf, shoot tips and mature leaf tissue (Appendix F) thereby providing additional evidence for further investigating this ortholog.

In the case of *EgrPR4*, *Egrandis*_v1_0.030656 was selected as the putative ortholog because the scaffold selected from the initial NJ phylogenetic tree (Appendix B, Figure S4) aligned with this gene model. From the NJ and ML phylogenetic trees shown in Figure 2.4 and Figure S10 (Appendix C) respectively, *Egrandis*_v1_0.030691 and *Egrandis*_v1_0.030679 could also potentially be putative orthologs of *PR4* as they group with a strong bootstrap support with *Egrandis*_v1_0.030656. The RNA-Seq expression data revealed that *Egrandis*_v1_0.030656 is only expressed in the shoot tips (Appendix F), whereas *Egrandis*_v1_0.030691 and *Egrandis*_v1_0.030679 are both expressed in the shoot tips, young leaves and mature leaves (not shown). From this data it can be extrapolated with a high possibility that *Egrandis*_v1_0.030691 and *Egrandis*_v1_0.030679 could be additional putative orthologs for the PR4 protein. However in this study *Egrandis*_v1_0.030656 was investigated as a representative ortholog for *PR4* as the primers designed from the 4.4X assembly target this gene model.

The initial NJ phylogenetic tree for *PR5* (Appendix B, Figure S5) constructed from the 4.4X *E. grandis* genome assembly was not able to resolve a putative candidate as the bootstrap support within this tree was too low to have any confidence in the groupings. Therefore for *EgrPR5*, the predicted transcript *Egrandis*_v1_0.051781 was selected as the putative ortholog based on its grouping with *Arabidopsis* and *Populus* in the subsequent NJ and ML trees (Figure 2.5 and Appendix C, Figure S11). In addition to *Egrandis*_v1_0.051781, *Egrandis*_v1_0.053387 was also found to group with the *Arabidopsis* and *Populus* orthologs (Figure 2.5 and Appendix C, Figure S11). Further investigation into the expression patterns of these gene models revealed that *Egrandis*_v1_0.051781 was expressed in phloem tissue and shoot tips (Appendix F) whereas no expression was detected for *Egrandis*_v1_0.053387. Thus *Egrandis*_v1_0.051781 was selected as the candidate of interest for *PR5.*

The NJ phylogenetic trees (Appendix B Figure S6 and Appendix C Figure S12) for the *LOX* family provided the basis for the selection of predicted gene models. With *EgrLOX2*, the NJ phylogenetic tree (Appendix C, Figure S12) revealed a grouping between the *Arabidopsis LOX2* ortholog and several *E. grandis* gene models, indicated by the bracket in Figure 2.6. This grouping was corroborated in the ML phylogenetic tree (Figure 2.6). All of these candidates are potential orthologs however scaffold 25:1533000_153400 (4.4X assembly) selected from the initial NJ phylogenetic tree (Appendix B, Figure S6) corresponded with the predicted gene model, *Egrandis*_v1_0.002202 (8X assembly). Expression data exists for all of the gene models within the bracket including *Egrandis*_v1_0.02202 which was shown to be expressed in immature xylem, shoot tips and young leaves (Appendix F). Thus *Egrandis*_v1_0.002202 was selected as a representative of the larger group and designated as the putative ortholog *EgrLOX2*.

The predicted gene model for *EgrLOX3*, *Egrandis*_v1_0.002112, formed a clade with the *Arabidopsis* and *Populus* orthologs in the NJ phylogenetic tree and was supported by a strong bootstrap statistic (Appendix C, Figure S12). Even though this grouping did not occur in the ML phylogenetic tree (Figure 2.6), this gene model correlated with scaffold 430:240000_250000 (4.4X assembly) from the initial NJ phylogenetic tree (Appendix B, Figure S6). The RNA-Seq data for this gene model revealed that it was expressed in all of the tissues examined, thereby validating this candidate for further investigation (Appendix F).

2.4.2 Dose response profiling of putative defence marker orthologs

To investigate which concentration would elicit the most prevalent and significant induction in gene expression of the putative defence marker orthologs, *E. grandis* clone A tissue culture plantlets were sprayed with various concentrations of SA and MeJA. This clone was selected for the experiment as it was routinely propagated at the FABI tissue culture facility, grown under sterile conditions, displayed no known disease symptoms and thus considered to be disease-free.

RNA harvested from the plant material was analyzed on a denaturing gel to determine the integrity and quality of the samples. All samples showed prominent bands for the 28S and 18S ribosomal subunits which is indicative of intact RNA (Figure 2.7). Only selected samples are shown in the figure, however the same pattern was observed for all other biological replicates of MeJA and SA treated tissues.

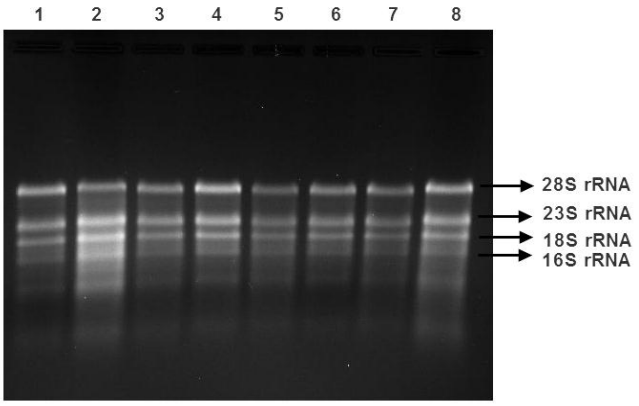

Figure 2.7: Total RNA extracted from *E. grandis* clone A leaf tissue treated with various concentrations of MeJA: Samples were analyzed on a 1% (w/v) formaldehyde agarose gel and are representative of total RNA (~500 ng) extracted from one biological replicate. Lane 1: T=24 control; Lane 2: 25 µM; Lane 3: 50 µM; Lane 4: 100 µM; Lane 5: 250 µM; Lane 6: 500 µM; Lane 7: 1 mM and Lane 8: 5 mM.

Following quality control, first strand cDNA was synthesized and the *EgrADP* intron spanning primer set was used to confirm the presence or absence of any residual genomic DNA. No genomic DNA, indicated by the ~1.5 kb band in the positive control, was present in the samples (Figure 2.8). All samples shown in the figure contained the intronless cDNA fragment of ~250 bp and this was observed for the other biological replicates of MeJA and SA treated tissue. Expression profiling of the candidate marker genes was performed *via* RT-qPCR and primary verification of amplification was assessed using melting curve analysis. A distinct single peak was observed for each target thereby indicating that only a single product had been amplified (Appendix G, Figure S21). In addition to the melt curve analysis, the three technical replicates of the products were pooled and analyzed on 1% (w/v) agarose gel (Figure 2.9). Samples were also sequenced to ensure that the correct amplicon had been obtained (Appendix J, Table S3).

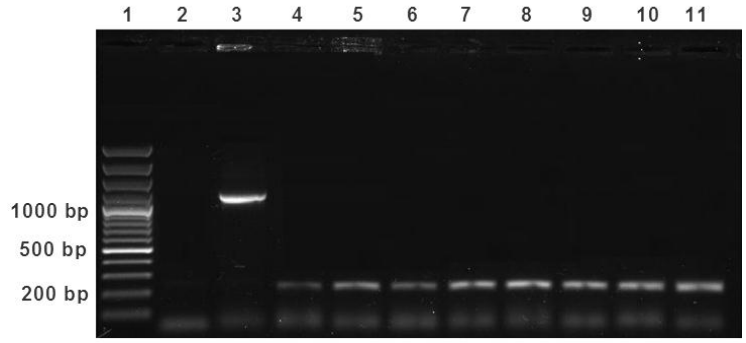

Figure 2.8: Gel electrophoresis illustrating results of a PCR analysis using the *EgrADP* intron spanning primer set to test for genomic DNA contamination: Samples are representative of cDNA synthesized from the purified RNA material of one biological replicate of MeJA treated *E. grandis* clone A leaf tissue. PCR products were analyzed on a 1% (w/v) agarose gel in 1 x TAE buffer. Lane 1: 100 bp DNA ladder plus molecular marker (Fermentas); Lane 2: Non-template control; Lane 3: Positive control of *E. grandis* genomic DNA; Lane 4: T=24 control; Lane 5: 25 µM; Lane 6: 50 µM; Lane 7: 100 µM; Lane 8: 250 µM; Lane 9: 500 µM; Lane 10: 1 mM and Lane 11: 5 mM.

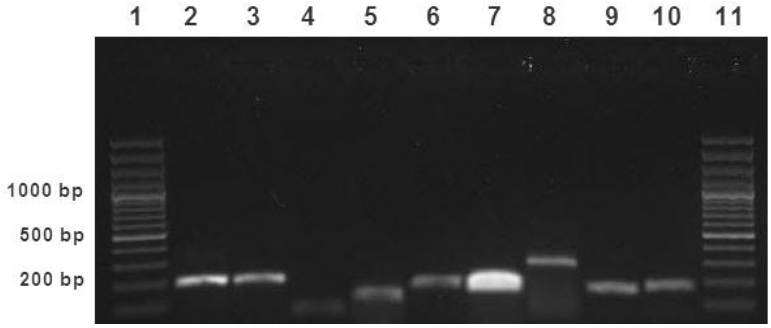

Figure 2.9: Gel electrophoresis of RT-qPCR products for the putative marker genes of the SA and MeJA pathways assessed during the dose response trial: Each sample is a pool of three technical replicates for a randomly selected concentration of one biological replicate. Products of the reference genes (Lane 2 - 4) and target-specific primers (Putative SA markers – Lane 5-7; Putative MeJA markers – Lane 8-10) were analyzed on 1% (w/v) agarose gel in 1 x TAE buffer. Lane 1 and 11: 100 bp DNA ladder plus molecular marker (Fermentas); Lane 2: *EgrARF*; Lane 3: *EgrFBA*; Lane 4: *Egu*IDH; Lane 5: *EgrPR1_like*; Lane 6: *EgrPR5*; Lane 7: *EgrNPR1*; Lane 8: *EgrPR3*; Lane 9: *EgrPR4* and Lane 10: *EgrLOX2*.

EgrPR2 was not included in the gel (Figure 2.9) as it produced a smear with a single band, however the product was sequenced to ensure the correct target had been amplified. Primers for this gene pair were re-designed from the sequenced product for the subsequent trials. Following the melting curve analysis, the quantification cycle (Cq) values were calculated and exported to *qBASE* for relative quantification. In order to control for the biological and technical variation among samples, normalization using stable reference genes is required. A reference set is regarded as stable when it has an average *M* and *CV* value that corresponds to the maximum threshold in *qBASE.* The default threshold of the *M* and *CV* values in *qBASE* is set at 0.5 and 25% respectively with lower values indicative of higher stability (Hellemans *et al.* 2007). The *M* value refers to the stability of a particular gene when tested in combination with all other candidate reference genes and assuming constant cDNA input (Vandesompele *et al.* 2009). The coefficient of variance (*CV*) refers to the stability of the reference gene across all the samples in the data set (Vandesompele *et al.* 2009). In addition to being stable according to the criteria mentioned above, a candidate reference gene should also not respond to the treatment in question.

For the SA treatment, it was found that *EgrARF* and *EgrFBA* were the most stable reference set having a mean *M* and *CV* value of 0.52 and 18.2% respectively (Appendix H, Figure S22). Although the *M* value is slightly higher than the threshold, this set of reference genes had the lowest mean value compared to all the other combinations of reference genes. For the JA treatment, it was found that *EguIDH* and *EgrARF* were the most stable reference set, with an *M* and *CV* value of 0.29 and 10.3% respectively (Appendix H, Figure S23). In *qBASE* the relative quantification of the targets of interest is performed by first determining the standard deviation of the technical replicates.

Any of the technical replicates within a biological replicate that had a Cq value difference greater than 0.5 were excluded from further analysis. This exclusion of outlying technical replicate resulted in a technical reproducibility of 91.46% and 93.65% for SA and JA treatments respectively. The software then converts the Cq values obtained from the experiment into relative quantities based on the standard curve of the specific gene pair (Appendix I, Table S2). An efficient gene pair will produce a coefficient of determination (R^2) close to 1, which is indicative of a linear standard curve and high reaction efficiency (Taylor *et al.* 2010). Relative quantities of the unknown samples are then normalized to the normalization factor obtained from the geometric mean of the reference genes and expressed as a relative expression ratio in arbitrary units (AU) (Hellemans *et al.* 2007; Bustin *et al.* 2009). These values which incorporate the three technical replicates for each biological replicate, were exported to Microsoft® Excel to further test for statistical significance. The relative expression values of the biological replicates for each concentration were compared to the biological replicates of the control using the Student's *t*-test.

2.4.2.1 Differential gene expression of putative marker genes during a dose response

The expression profiles of candidate marker genes were assessed after 24 hrs following treatment with various concentrations of SA (*EgrPR1_like, EgrPR2, EgrPR5 and EgrNPR1*) and MeJA (*EgrPR3, EgrPR4* and *EgrLOX2)* on *E. grandis* clone A plantlets. This was done to elucidate which concentration would elicit the most considerable change in gene expression for use in subsequent experiments. *EgrPR2* transcript abundance was significantly increased at 25 µM and 5 mM whereas transcript abundance of *EgrPR5* was significantly higher at 25 µM and 500 µM SA (Figure 2.10B and C). On the contrary *EgrPR1_like* transcripts were suppressed by various concentrations (50 µM, 100 µM, 250 µM, 1 mM and 5 mM) (Figure 2.10A). *EgrNPR1* transcripts were also statistically significantly repressed at 100 µM (Figure 2.10D). Although 25 µM was significantly up-regulated in both *EgrPR2* and *EgrPR5* (Figure 2.10B & C), the level to which these transcripts were differentially expressed, namely 3-fold and 4-fold respectively, was considerably lower than the transcript levels of *EgrPR2* at 5 mM.

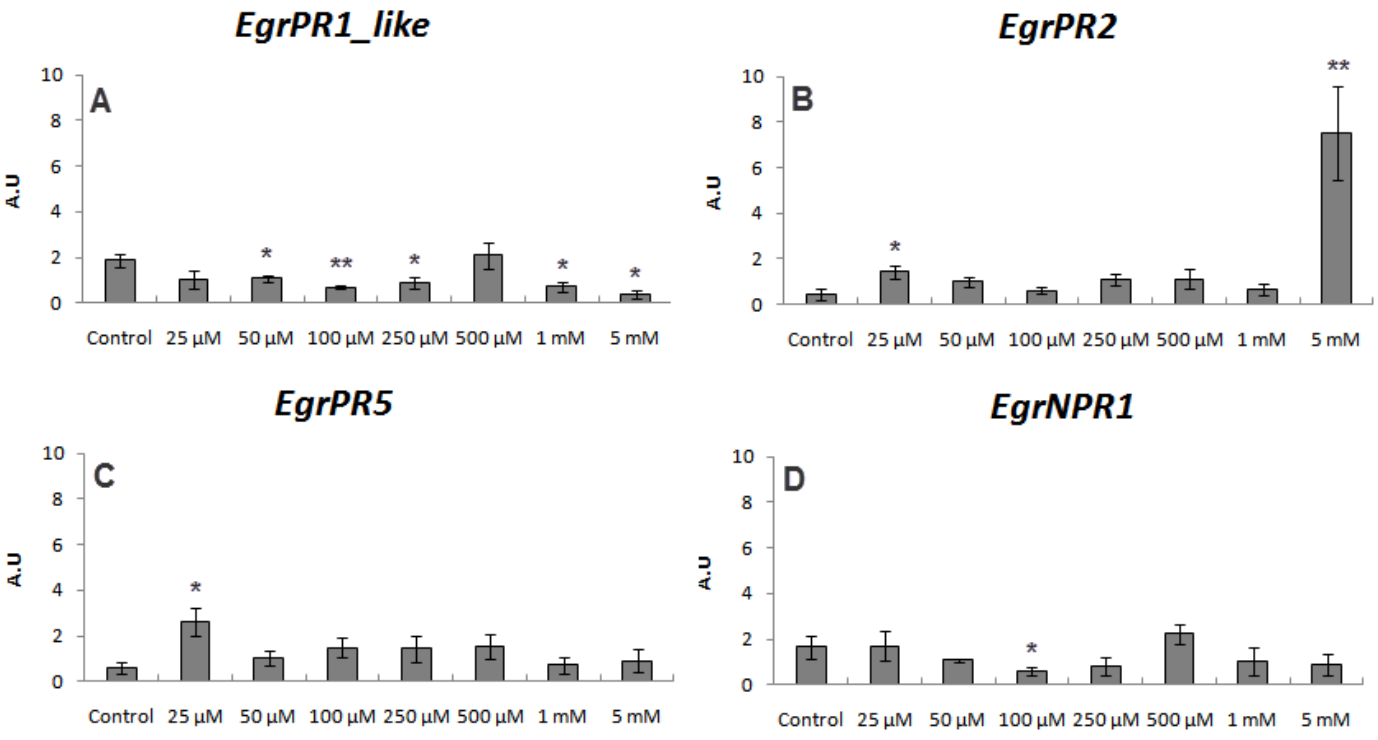

Figure 2.10: Relative dose response expression profiles of putative SA marker genes following normalization with *EgrARF* and *EgrFBA*: The y-axis represents the relative expression ratios expressed in arbitrary units (A.U). The x-axis represents the SA concentration range that was applied to the aerial parts of *E. grandis* clone A plants. Error bars show the standard error of the mean of the biological replicates (n=3). Significance is relative to the control in each graph and was calculated by the Student's *t*-test which is indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

From this data it was concluded that 5 mM of SA would be the most suitable to proceed with for further experiments as this concentration induced a change in the *EgrPR2* transcript levels that was elevated by approximately 16-fold compared to the control.

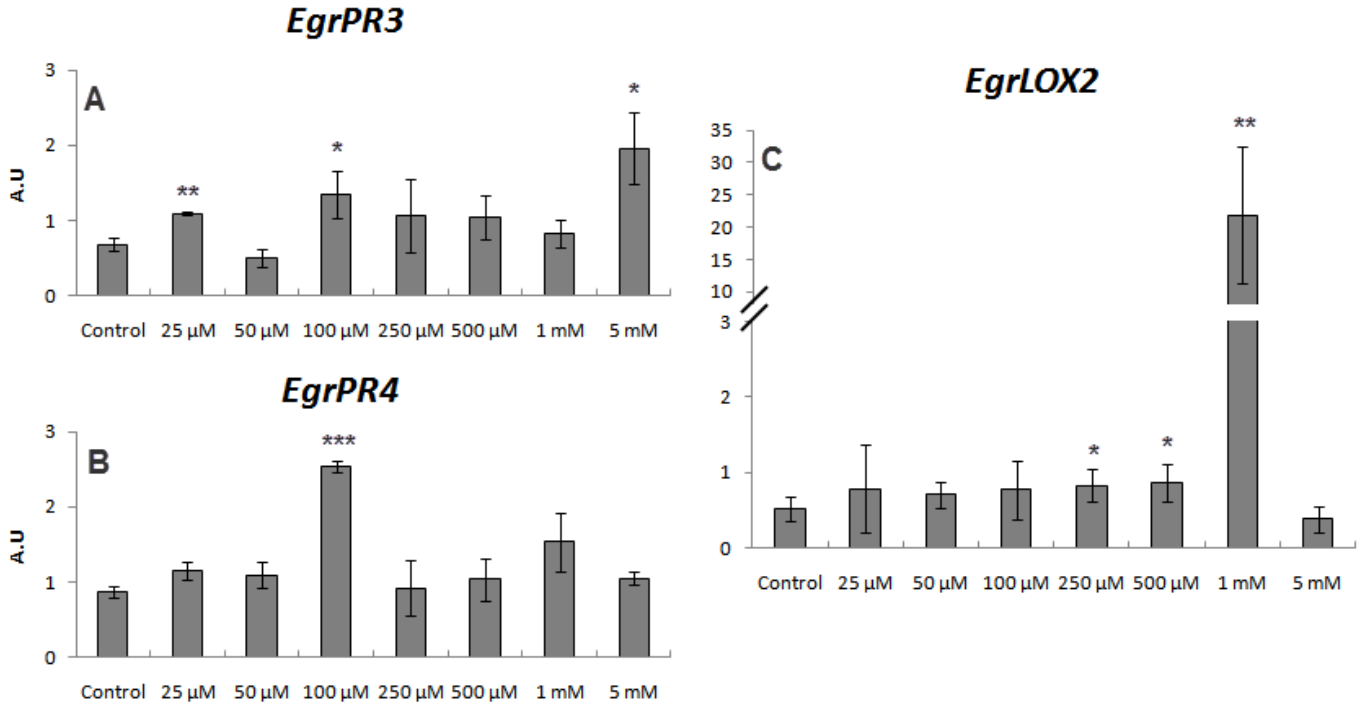

Figure 2.11: Relative dose response expression profiles of putative MeJA marker genes following normalization with *EguIDH* and *EgrARF*: The y-axis represents the relative expression ratios expressed in arbitrary units (A.U). The x-axis represents the MeJA concentration range that was applied to the aerial parts of *E. grandis* clone A plants. Error bars show the standard error of the mean of the biological replicates (n=3). Significance is relative to the control in each graph and was calculated by the Student's *t*-test which is indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

In response to MeJA treatment, significant *EgrPR3* transcript expression was observed for 25 µM, 100 µM and 5 mM (Figure 2.11A). *EgrPR4* transcripts were significantly elevated at 100 µM whereas *EgrLOX2* transcripts were significantly elevated at 250 µM, 500 µM and 1 mM, respectively (Figure 2.11B & C). It was observed that at 100 µM both *EgrPR3* and *EgrPR4* exhibited enhanced transcript expression and therefore this concentration was selected for further profiling experiments of putative MeJA markers.

2.4.2.2 Specificity of putative marker genes

To test the hypothesis that the putative expression markers are specific to the pathways for which they were designed and to validate the antagonistic relationship of SA and JA, the candidates were assessed for their ability to respond to the opposite pathway. The profile of putative SA responsive genes was examined in MeJA induced tissue and the MeJA responsive genes were investigated in SA induced tissue. In *Arabidopsis*, it is well documented that SA and JA have an antagonistic relationship thus it was hypothesized that MeJA responsive genes would be suppressed by SA treatment and *vice versa* (Pieterse *et al.* 2009). This suppression can be seen as a decrease in the transcript levels of a marker gene due to an increase in the concentration of the applied phytohormone (Koornneef *et al.* 2008). The antagonistic relationship between SA and MeJA was clearly validated with *EgrPR2, EgrPR3*, *EgrNPR1* and *EgrLOX2* as these genes were significantly suppressed at selected concentrations when analyzed with the induced tissue of the opposite pathway (Figure 2.12).

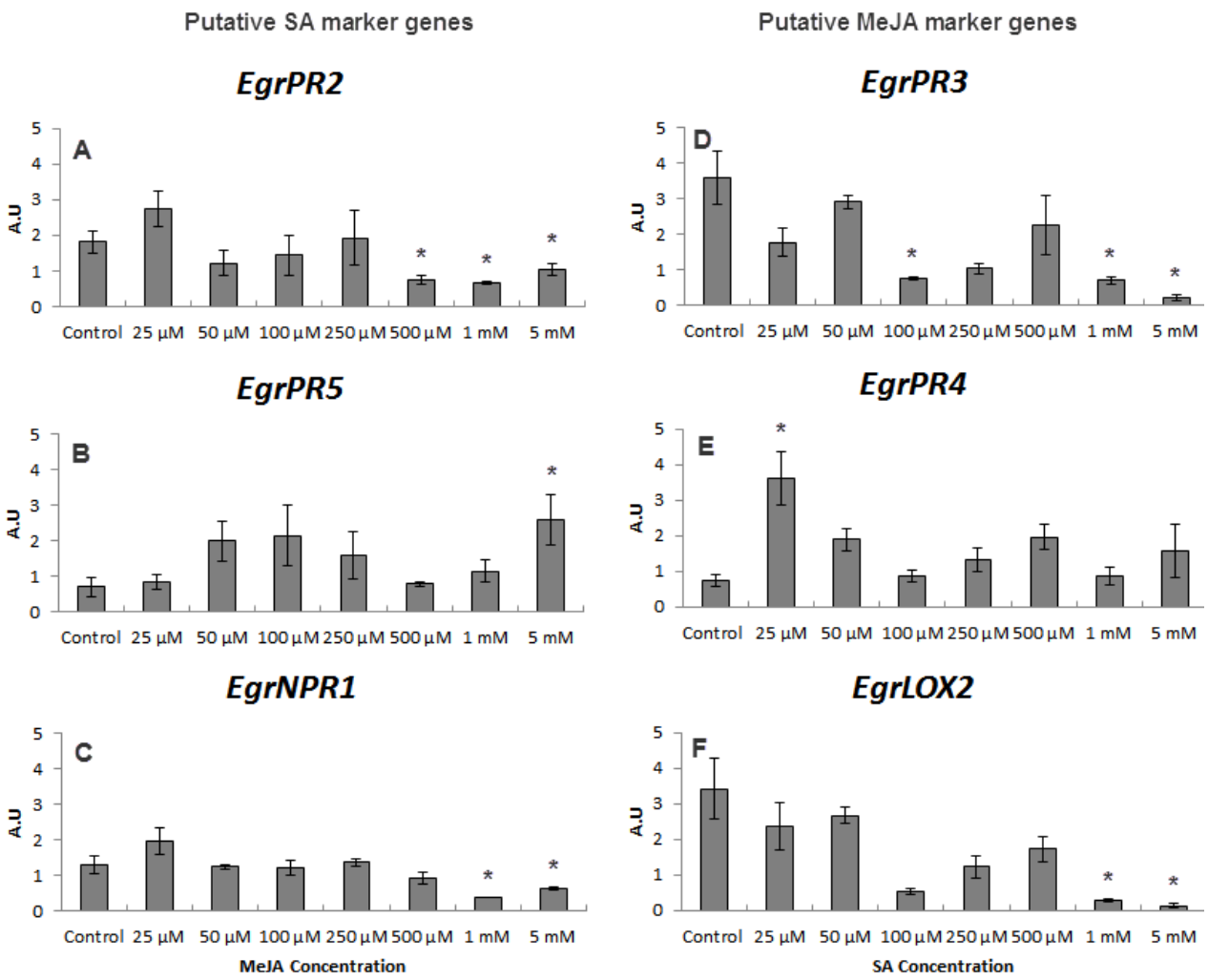

Figure 2.12: Relative expression profiles of the putative SA and MeJA marker genes in tissue treated with the opposite phytohormone: The y-axis represents the relative expression ratios expressed in arbitrary units (A.U). Putative SA markers (A, B and C) were normalized with *EguIDH* and *EgrARF* whereas the putative MeJA markers (D, E and F) were normalized with *EgrARF* and *EgrFBA*. The x-axis represents the concentration range for the applied inducer. Error bars show the standard error of the mean of the biological replicates (n=3). Significance is relative to the control in each graph and was calculated by the Student's *t*-test which is indicated by * $p < 0.05$.

EgrPR2 expression was significantly lower at 500 µM, 1 mM and 5 mM MeJA whereas *EgrNPR1* transcript levels were suppressed by 1 mM and 5 mM MeJA (Figure 2.12A & C). *EgrPR3* expression was suppressed by 100 µM, 1 mM and 5 mM SA whereas *EgrLOX2* was significantly lower at 1 mM and 5 mM SA (Figure 2.12D & F). *EgrPR4* had higher transcript abundance at 25 µM SA, but not repressed at any of the concentrations whereas *EgrPR5* transcript levels were up-regulated at the highest concentration of MeJA, 5 mM (Figure 2.12B & E). The basic isoform of *EgrPR1_like* was not significantly altered by any of the concentrations of MeJA (data not shown).

2.4.3 Time dependent expression of putative marker genes

To investigate how the expression profile of the candidates change over time, the selected concentration from the dose experiment was applied to *E. grandis* clone A tissue culture plantlets and material was harvested at various time points. Once again this clone was selected for this experiment as it was routinely propagated at the FABI tissue culture facility, known to be completely disease free and was the same clone used for the dose response experiment. RNA harvested from the plant material was analyzed on a denaturing gel to determine the integrity and quality of the samples. All samples showed a prominent band for the 28S and 18S ribosomal subunits which is indicative of intact, high-quality RNA (Figure 2.13). Only selected samples are shown in the figure, however the same pattern was observed for all other biological replicates of MeJA and SA treated tissue. Following quality control, first strand cDNA was synthesized and the *EgrADP* intron spanning primer set was used to confirm the presence or absence or any residual genomic DNA (Figure 2.14). No genomic DNA, determined by the absence of a ~1.5kb fragment, was observed in the samples shown in the figure or in any of the remaining biological replicates for MeJA and SA.

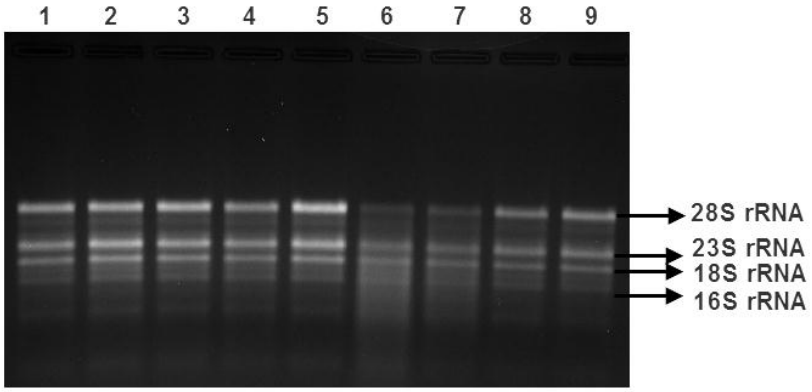

Figure 2.13: Gel electrophoresis illustrating total RNA extracted over a time course from *E. grandis* clone A leaf tissue treated with 100 µM MeJA: Samples were analyzed on a 1% (w/v) formaldehyde agarose gel and are representative of the total RNA (~500 ng) extracted from one biological replicate. Lane 1: T=0 control; Lane 2: T=6 hpt; Lane 3: T=6 hpt control; Lane 4: 12 hpt; Lane 5: 12 hpt control; Lane 6: 24 hpt; Lane 7: 24 hpt control; Lane 8: 48 hpt and Lane 9: 48 hpt control.

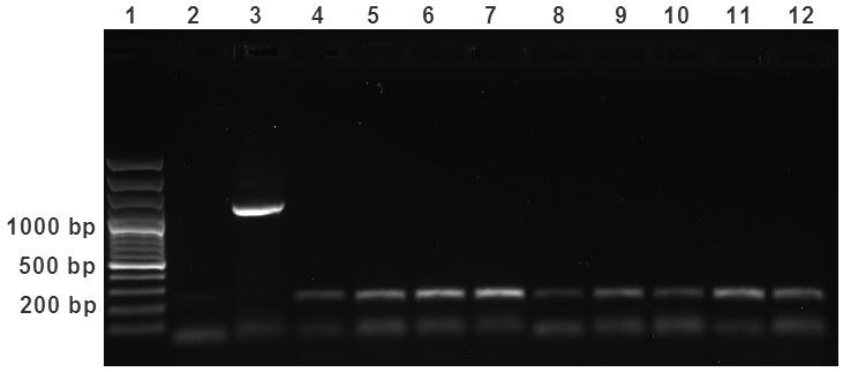

Figure 2.14: Gel electrophoresis illustrating the results of a PCR analysis to test for genomic contamination in cDNA synthesized from the time course sample set: Samples are representative of cDNA synthesized from the purified RNA material of one biological replicate of MeJA treated *E. grandis* clone A leaf tissue at various time points. PCR products were analyzed on a 1% (w/v) agarose gel in 1 x TAE buffer. Lane 1: 100 bp DNA ladder plus molecular marker; Lane 2: Non-template control; Lane 3: Positive control of *E. grandis* genomic DNA; Lane 4: T=0 control; Lane 5: T=6 hpt; Lane 6: T=6 hpt control; Lane 7: 12 hpt; Lane 8: 12 hpt control; Lane 9: 24 hpt; Lane 10: 24 hpt control; Lane 11: 48 hpt and Lane 12: 48 hpt control.

Analysis of expression profiling *via* RT-qPCR was executed in the same manner as for the dose response experiment. For the melt curve analysis, distinct single peaks were observed for all the target genes profiled (Appendix G, Figure S21). Figure 2.15 illustrates that single products of the correct size were obtained when the samples were analyzed on an agarose gel. These samples were sequenced to further verify that the correct amplicon had been amplified (Appendix J, Table S3).

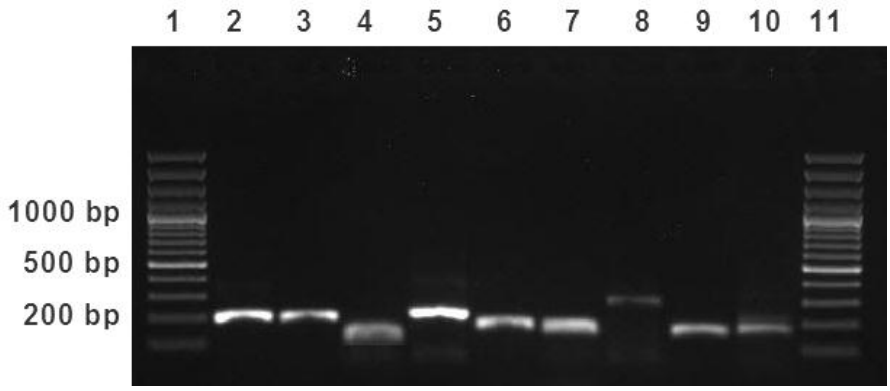

Figure 2.15: Gel electrophoresis of RT-qPCR products for the putative marker genes of the SA and MeJA pathways assessed during a time course: Each sample is a pool of the technical replicates for a randomly selected time point of one biological replicate. Products of the reference genes (Lane 2 and 3) and target specific primers (Putative SA markers – Lane 4-7; Putative MeJA markers – Lane 8-10) were analyzed on a 1% (w/v) agarose gel in 1 x TAE buffer. Lane 1 and 11: 100 bp DNA ladder plus molecular marker (Fermentas); Lane 2: *EgrARF*; Lane 3: *EgrFBA*; Lane 4: *EgrPR1_like*; Lane 5: *EgrPR2*; Lane 6: *EgrPR5*; Lane 7: *EgrNPR1*; Lane 8: *EgrPR3*; Lane 9: *EgrPR4* and Lane 10: *EgrLOX2*.

The reference genes *EgrARF* and *EgrFBA* were found to be stable for both SA and MeJA treatments. For the SA treatment, the M and *CV* values were 0.371 and 12.9% respectively whilst for the MeJA treatment the M and *CV* values were 0.361 and 12.5% respectively (Appendix H, Figure S24 & S25). The technical reproducibility was 90.48% for the SA treatment and 90.7% for the MeJA treatment. Efficiency parameters for the primer specific standard curves are given in Appendix I (Table S2). The relative expression values of the biological replicates for each time point was compared to the biological replicates of the T=0 control as well as the time specific control using the Student's *t*-test. However the significance indicated on the graphs is only in relation to the time specific control.

EgrPR1_like was significantly induced only at 6 hpt, but not at any of the other time points (Figure 2.16A). *EgrPR2* was significantly increased at 12 hpt, 24 hpt and 48 hpt with a drastic peak at 24 hpt followed by a decline at 48 hpt (Figure 2.16B). It should be noted that the level of expression of *EgrPR2* at 24 hpt was approximately the same as in the dose response experiment, indicating reproducibility of the results. *EgrPR5* displayed a gradual increase in expression from 6 hpt to 48 hpt, with all the time points except 12 hpt showing statistical significance (Figure 2.16C). Conversely, *EgrNPR1* expression decreased over time with the highest expression observed at 6 hrs and subsequently declined steadily to 48 hpt (Figure 2.16D).

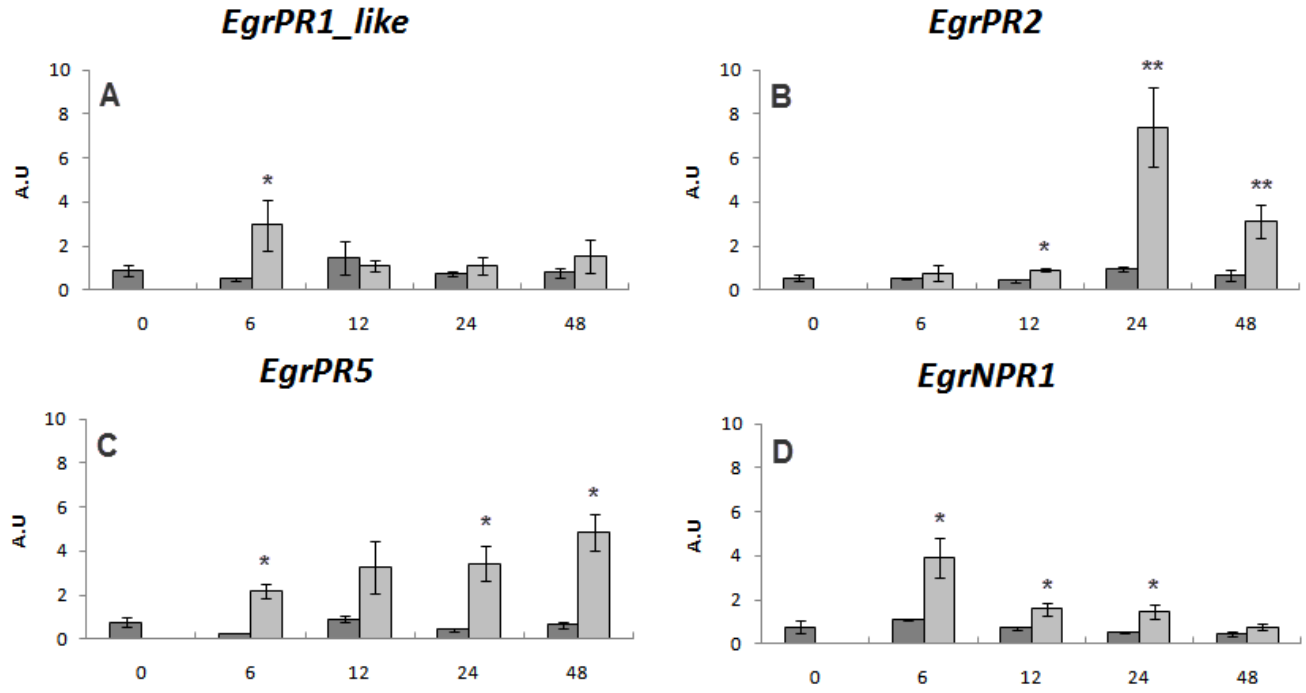

Figure 2.16: Relative expression profiles of putative SA marker genes assessed during the time course trial after normalization with *EgrARF* and *EgrFBA*: The y-axis represents the relative expression ratios expressed in arbitrary units (A.U). The x-axis represents the time course (hrs) post treatment with 5 mM SA (pH 7.0). Error bars are show the standard error of the mean of the biological replicates (n=3). Dark grey boxes represent the control samples whereas the light grey boxes represent the treated samples. Significance between the control and treated samples at a specific time point was calculated by the Student's *t*-test is indicated by * $p < 0.05$ and ** $p < 0.01$.

Markers genes for MeJA, *EgrPR3*, *EgrPR4* and *EgrLOX2* all displayed altered levels of expression at the various time points. *EgrPR4* transcript levels increased progressively from 6 hpt to 48 hpt, with all the time points being statistically significant (Figure 2.17B). Notably the level at which *EgrPR4* is expressed at 24 hpt was approximately the same as in the dose response experiment, thereby signifying the reproducibility of the results.

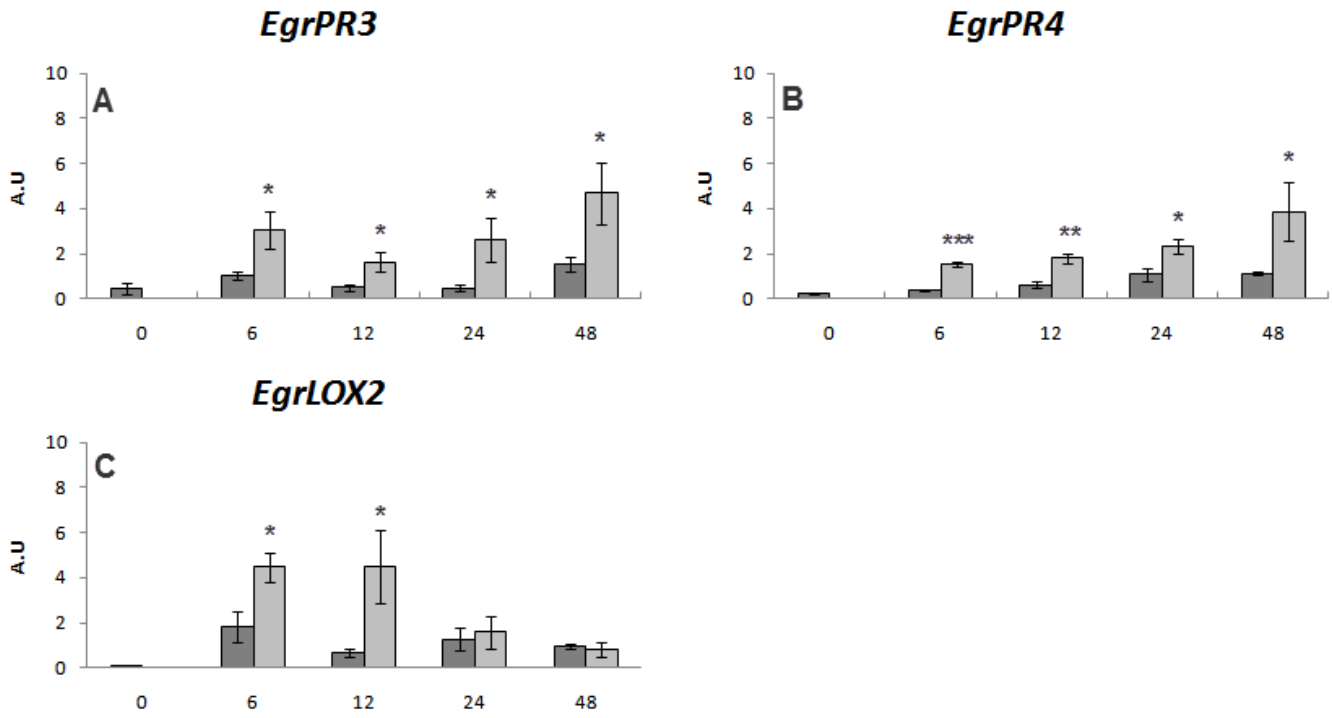

Figure 2.17: Relative expression profiles of putative MeJA marker genes during the time course after normalization with *EgrARF* and *EgrFBA*: The y-axis represents the relative expression ratios expressed in arbitrary units (A.U). The x-axis represents the time course (hrs) post treatment with 100 µM MeJA. Error bars are representative of the standard error of the mean of the biological replicates (n=3). Dark grey boxes represent the control samples whereas the light grey boxes represent the treated samples. Significance between the control and treated samples at a specific time point was calculated by the Student's *t*-test is indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The expression profile of *EgrPR3* was slightly ambiguous as it was found to increase at 6 hpt, followed by a decrease in transcript expression at 12 hpt and then subsequently increasing from 24 hpt to 48 hpt (Figure 2.17A). All of the time points for *EgrPR3* were found to be statistically significant. *EgrLOX2* was found to be significant only at 6 hpt and 12 hpt therefore suggesting a possible role for this gene in early defence responses (Figure 2.17C).

2.4.4 Infection trial with *C. austroafricana*

To investigate if the putative expression markers would be diagnostic under pathogen stress, ZG14 and TAG5 trees were infected with the fungus *C. austroafricana* and the expression pattern of the SA marker genes (*EgrPR2, EgrPR5*) and the MeJA marker genes (*EgrPR4, EgrLOX3*) were assessed. TAG5 is known to be moderately tolerant to the pathogen whereas ZG14 is known to be susceptible (Van Heerden *et al.* 2005). *EgrLOX3* was used as a marker for MeJA for the infection trial instead of *EgrLOX2* which was used in the previous experiments. This is because *EgrLOX2* did not produce a single melting peak with the TAG5 and ZG14 plant material, suggesting that the primers are targeting multiple regions. Lesion length was measured at each time point with no significant difference between TAG5 and ZG14 at 48 hrs and at 2 weeks. At 6 weeks a clear difference in the lesion length was observed between the two clones which substantiated the susceptibility of ZG14 and the tolerance of TAG5 (Figure 2.18).

RNA harvested from the plant material was analyzed on a formaldehyde denaturing gel to determine the integrity and quality of the samples. All samples showed a prominent band for the 28S and 18S ribosomal subunits which is indicative of intact RNA (Figure 2.19). Only samples from one biological replicate are shown in the figure however the same was observed for the other two biological replicates. Following quality control, first strand cDNA was synthesized and the *EgrADP* intron spanning primer set was used to confirm the presence or absence or any residual genomic DNA (Figure 2.20). No genomic DNA, determined by the absence of a ~1.5kb fragment, was observed in the samples shown in the figure or in any of the remaining biological replicates

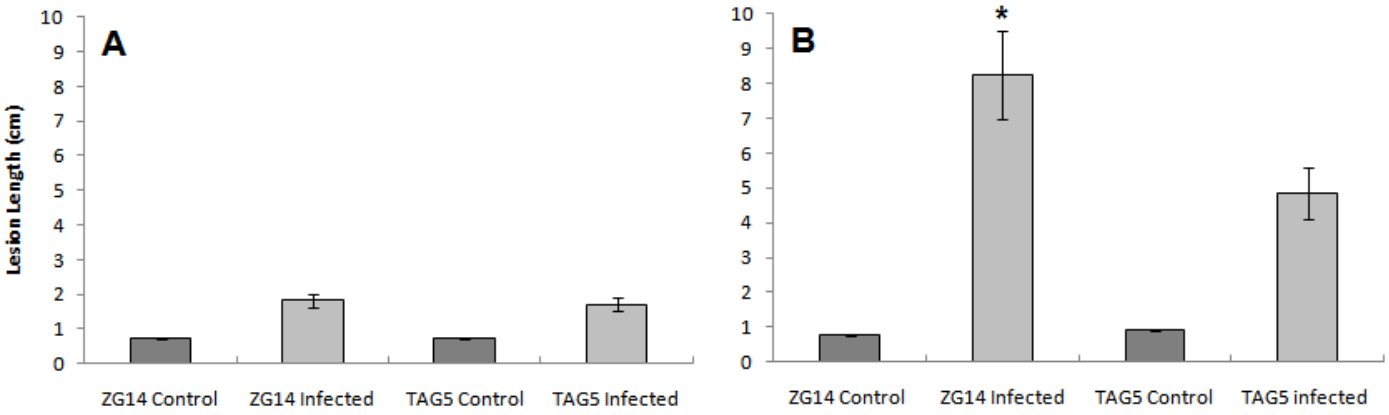

Figure 2.18: Lesion lengths on TAG5 and ZG14 following infection with *C. austroafricana*. **A:** 2 weeks post inoculation. There was no significant difference observed between TAG5 and ZG14. **B:** 6 weeks post inoculation there is a significant difference in lesion length between TAG5 and ZG14. Error bars show the standard error of the mean of the biological replicates (n=3). Significance was calculated based on the comparison of lesion lengths between ZG14 infected and TAG5 infected using the Kruskal-Wallis test, as indicated by * $p<0.05$.

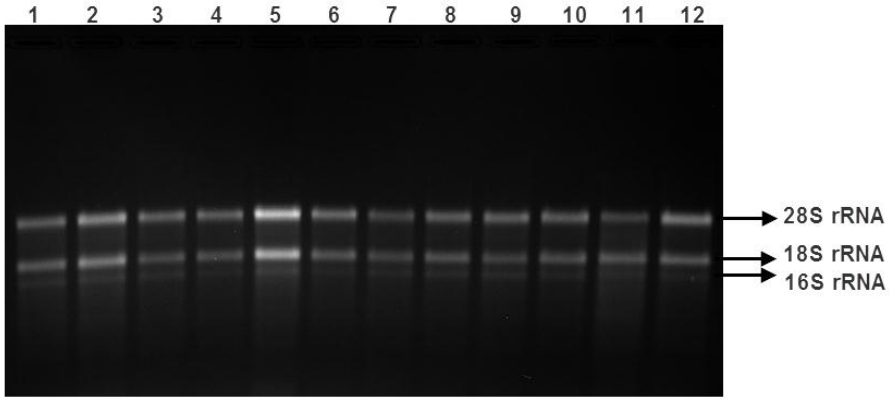

Figure 2.19: Total RNA extracted from the *C. austroafricana* infection trial with *E. grandis* genotypes ZG14 and TAG5: Samples were analyzed on a 1% (w/v) formaldehyde agarose gel and are representative of the total RNA (~500 ng) extracted from one biological replicate. Lane 1 - 3: TAG5 control (48 hours, 2 wpi, 6 wpi); Lane 4 - 6: TAG5 infected (48 hours, 2 wpi, 6 wpi); Lane 7 – 9: ZG14 control (48 hours, 2 wpi, 6 wpi); Lane 10 - 12: ZG14 infected (48 hours, 2 wpi, 6 wpi).

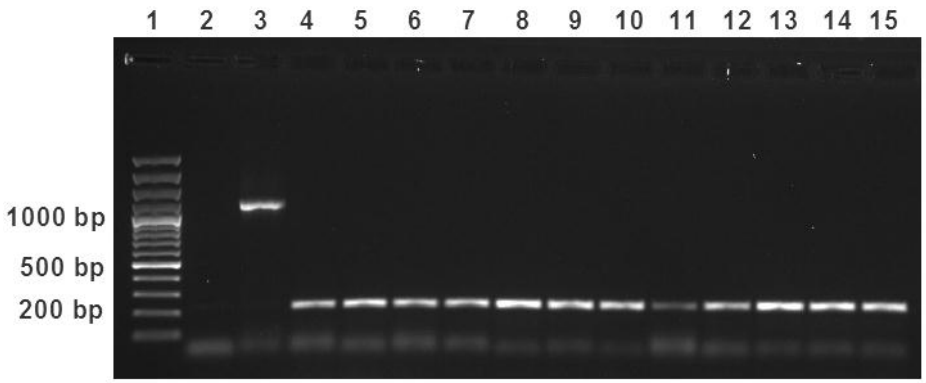

Figure 2.20: Gel electrophoresis illustrating the results of a PCR analysis to test for genomic contamination in cDNA synthesized from material harvested during the *C. austroafricana* infection trial: Samples are representative of cDNA synthesized from the purified RNA material of one biological replicate. PCR products were analyzed on a 1% (w/v) agarose gel in 1 x TAE buffer. Lane 1: 100 bp DNA ladder plus molecular marker (Fermentas); Lane 2: Non-template control; Lane 3: Positive control of *E. grandis* genomic DNA; Lane 4 - 6: TAG5 control (48 hours, 2 wpi, 6 wpi); Lane 7 - 9: TAG5 infected (48 hours, 2 wpi, 6 wpi); Lane 10 – 12: ZG14 control (48 hours, 2 wpi, 6 wpi); Lane 13 - 15: ZG14 infected (48 hours, 2 wpi, 6 wpi).

Expression profiling was performed on all three time points to determine if either SA or MeJA could play a role in facilitating tolerance against *C. austroafricana*. Analysis of expression profiling *via* RT-qPCR was executed in the same manner as for the dose response experiment and the time trial. For the melt curve analysis, distinct single peaks were observed for all the target genes profiled (Appendix G, Figure S21). Single products of the correct size were obtained when the samples were analyzed on an agarose gel (Figure 2.21).

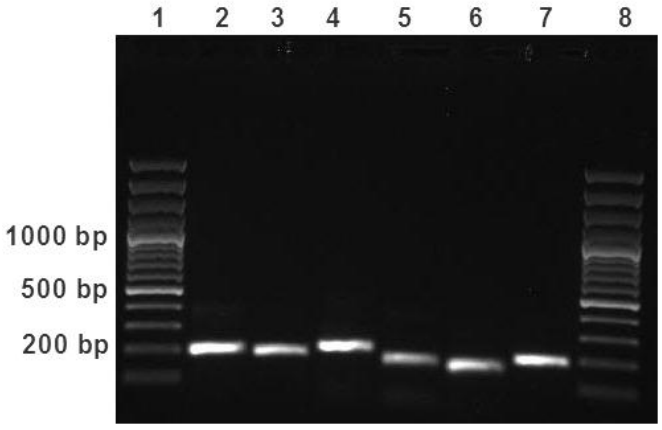

Figure 2.21: Gel electrophoresis of RT-qPCR products for the putative marker genes of the SA and MeJA pathways assessed during the infection trial with *C. austroafricana*. Products of the reference genes (Lane 2 and 3) and target specific primers (Putative SA markers – Lane 4 and 5; Putative MeJA markers – Lane 6 and 7) were analyzed on 1% (w/v) agarose gel in 1 x TAE buffer. Each sample is a pool of the technical replicates for a randomly selected representative of one biological replicate. Lane 1 and 8: 100bp DNA ladder plus molecular marker (Fermentas); Lane 2: *EgrARF*; Lane 3: *EgrFBA*; Lane 4: *EgrPR2*; Lane 5: *EgrPR5*; Lane 6: *EgrPR4*; Lane 7: *EgrLOX3*.

These samples were sequenced to further verify that the correct amplicon had been amplified (Appendix J, Table S3). The following reference genes were analyzed across the sample set of all three time points for both TAG5 and ZG14: *Egu*APRT, *EguACT*, *EgrARF* and *EgrFBA*. It was found that a combination of *EgrARF* and *EgrFBA* had the most stable parameters with an M and *CV* value of 0.53 and 18.4% respectively (Appendix H, Figure S26). The technical reproducibility for the infection trial was 91.69% and the efficiencies of the gene pairs are noted in Appendix I (Table S2). Analysis of the expression data was performed using the Student's *t*-test by comparing the mean of three biological replicates of the infected material to the mean of three biological replicates of the control material for each time point investigated.

In TAG5, *EgrPR2* transcript levels were significantly increased at 2 wpi and at 6 wpi whereas *EgrPR5* was significantly differentially expressed at 48 hours post inoculation and 6 wpi (Figure 2.22). With the MeJA markers in TAG5, *EgrLOX3* was significant up-regulated at 48 hours post inoculation whereas *EgrPR4* transcripts significantly decreased at 2 wpi and increased once again at 6 wpi. Despite significant up-regulation in *EgrPR4* transcripts at 6 wpi compared to its control, the level to which it was induced was lower than *EgrPR2* transcript levels. This could indicate an increased level of SA in TAG5.

In ZG14, the level of expression of *EgrPR2* was only significantly up-regulated at 6 wpi and *EgrPR5* was significantly induced at 48 hours post inoculation and 6 wpi. With respect to the MeJA markers, *EgrLOX3* was not found to be significantly differentially expressed compared to the control at any of the investigated time points. Expression of *EgrPR4* transcripts was found to be significantly up-regulated at 2 wpi and 6 wpi.

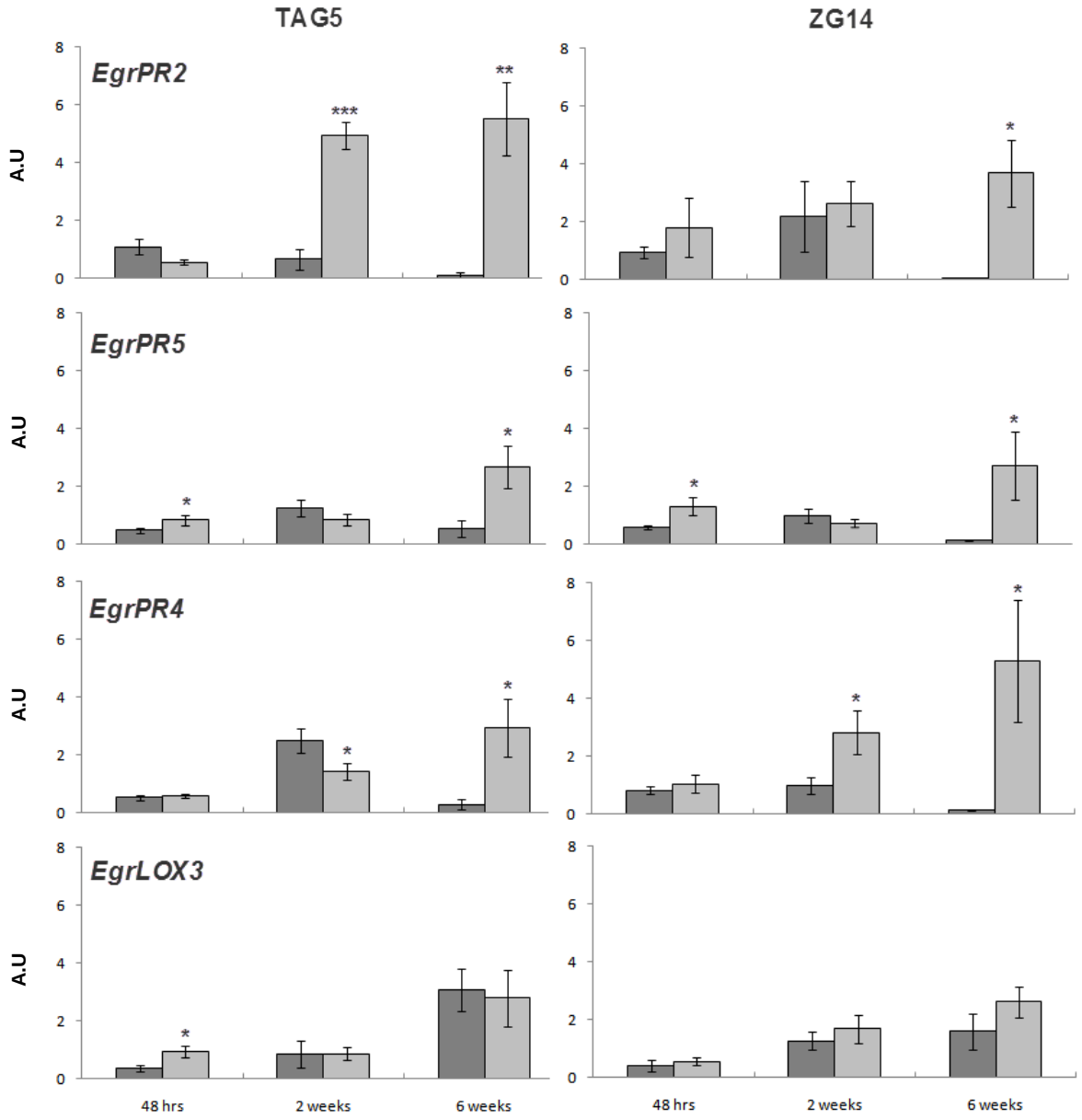

Figure 2.22: Relative expression profile of putative SA and MeJA marker genes during infection with *C. austroafricana* after normalization with *EgrARF* and *EgrFBA*: The y-axis represents the relative expression ratios expressed in arbitrary units (A.U). The x-axis represents the time points at which samples were analyzed. Error bars show the standard error of the mean of the biological replicates (n=3). Dark grey boxes represent the control samples whereas the light grey boxes represent the samples infected with *C. austroafricana*. Significance is relative to the control and was calculated by the Student's *t*-test as indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

2.5 Discussion

Eucalyptus species are predominantly planted in South Africa for their wood properties and use in the production of mining timber as well as for pulp and paper production. Due to their valuable properties, eucalypts constitute a significant portion within the South African Forestry industry. These trees are exposed to various pathogens throughout their lifetime, however information pertaining to the defence system within this host is limited (Wingfield *et al.* 2008). Hence there is a need to further investigate the role of these potential threats and to elucidate the *Eucalyptus* defence network. In *A. thaliana,* the involvement of signalling pathways such as ET, JA and SA in plant defence has been documented in various studies. It has also been demonstrated that each of the pathways elicit the expression of a particular suite of genes such as *PR* genes which are involved in defence e.g. *PR2* is a widely used as an expression marker for SA in *Arabidopsis* (Mishina and Zeier 2007). Under pathogen invasion, these *PR* genes can be diagnostic of a particular pathway and thereby shed light on how resistance could possibly be enhanced.

This study aimed to identify orthologs of marker genes for the SA and MeJA signalling pathways from *A. thaliana* in *E. grandis* using sequence similarity and phylogenetic methodology in conjunction with the first annotated version of the *E. grandis* genome sequence that has recently been made available on Phytozome v7.0. NJ and ML analysis was conducted on each of the target gene data sets to add confidence in selecting a putative ortholog to further investigate. Subsequent to optimizing the primer sets of the designated orthologs, the putative marker gene fragments were cloned and sequenced to verify that the correct target was obtained. Following verification of the putative orthologous markers, an assessment of the transcript abundance of the putative genes was conducted using RT-qPCR. The expression profile of potential markers was

investigated under mock induction of the signalling pathways and under pathogen inoculation. The fungus *C. austroafricana* was used in this study as a model to determine if the putative marker genes would be diagnostic for the responses caused by this pathogen. Additionally, it was investigated if the marker genes identified in this study would be congruent in terms of transcription profiles and responses with what is currently known in *A. thaliana*.

2.5.1 Phylogenetic identification of putative defence marker genes

This study employed both NJ and ML algorithms for phylogenetic analyses because each method has its own advantages and disadvantages. NJ is a distance based algorithm in which the end topology represents the least total branch length and due to this, a sub-optimal tree may be produced (Saitou and Nei 1987). On the other hand, ML is a computationally intensive algorithm however because is it based on an evolutionary model, this algorithm is able to generate a tree that is statistically solid (Yang 1994). By applying a co-phylogenetic based approach (NJ and ML), putative orthologous marker genes for the SA and MeJA signalling pathways were identified in *E. grandis* with added confidence. These putative markers were assessed under various experimental conditions to determine whether the response of these genes in *E. grandis* was similar to what is known from model species. The results of the latter experiments will be discussed in subsequent sections.

Phylogenetics provides a solid starting point for selecting candidates to investigate, however it does not provide definitive evidence that the selected gene is the true functional ortholog (Chen *et al.* 2007). Firstly, the gene of interest may have evolved as multiple copies within the genome of *E. grandis* as compared to *Arabidopsis* (Paterson *et al.* 2010). *Eucalyptus*, *Populus* and *Arabidopsis* share an ancient hexaploidization

event and therefore on average there should be three genes in each species relative to the ancestor (Jaillon *et al.* 2007). However, these genes have undergone various gene loss and/or duplication events which have changed this number for many genes and gene families thereby possibly creating multiple functional orthologs. This was observed for example in the phylogenetic tree of *PR1*, whereby many gene models were predicted for this gene in *E. grandis* (Figure 2.1). In order to clarify which of the predicted gene models could be the true ortholog/s, one would need to examine several candidate genes in a complementation experiment.

Such cases, where more than one gene model could be the putative ortholog, a representative of the clade was selected and further assessed in gene expression experiments. Therefore a limitation of this study was that only one ortholog was selected for each marker gene whereas there could potentially be multiple functional orthologs in *Eucalyptus*. Additionally, no alternative transcripts or different splice variants were investigated in this study which could influence the expression profiles of the targets. However the orthologous marker genes identified in this study does provide a stepping-stone for the elucidation and understanding of the role these defence genes in *E. grandis.*

2.5.2 Putative markers exhibit dose-specific induction and pathway specificity

The concentrations used in this study were based on experiments conducted in *A. thaliana* and other model organisms as there was no prior knowledge in *Eucalyptus* (Li *et al.* 2004; Jung *et al.* 2007). Additionally, the concentrations were also based on the level of the phytohormone following pathogen challenge. For example, in cucumber plants infiltrated with *P. syringae,* at 24 hrs post inoculation the concentration of SA in the cell ranged between 200 µM – 550 µM (Rasmussen *et al.* 1991). These

concentrations are within the range investigated in this study. In the dose response experiment, it was hypothesized that the expression of the markers would increase proportionally with the dosage, reaching a plateau at an optimal concentration and subsequently decline thereafter resulting in a bell-shaped graph. However this was not observed for the markers assessed in this study and could be attributed to several reasons. Firstly, a thicker cuticle in the *Eucalyptus* plantlets could have restricted the permeability of the inducer, thereby resulting in a lower concentration within the cell than that on the surface of the leaf (Calas *et al.* 2009; Peachey *et al.* 2009). Chemically measuring basal levels of the inducer within the cell and after application of the inducer would be necessary to further investigate this hypothesis. Secondly, the range of concentrations tested may have been too broad for *Eucalyptus* and possibly a finer scale would be required as this could result in the expected bell-shaped graph. Nonetheless, the concentrations that were investigated sufficed to address the null hypothesis that the putative orthologs could serve as diagnostic marker genes.

The expression levels of two putative SA marker genes, *EgrPR2* and *EgrPR5* were enhanced by application of the inducer which was consistent with literature in *Arabidopsis*. Furthermore, when these markers were evaluated for their specificity to the SA pathway, it was clearly demonstrated that expression levels of *EgrPR2* was suppressed by the higher concentrations of MeJA (Figure 2.12). The results suggest that in *E. grandis EgrPR2* could be a diagnostic marker for the SA pathway as has been reported in *Arabidopsis* literature (Loon and Strien 1999). In addition to being induced by SA, *EgrPR5* was found to be induced at high concentrations of MeJA (Figure 2.12). Although *PR5* is classified as a marker for the SA pathway, it has been shown to be up-regulated by the application of MeJA in *Arabidopsis*. A microarray profiling experiment in which *Arabidopsis* Col-0 plants were treated with MeJA, *PR5* transcript levels were

found to be 2.5 fold up-regulated in response to this inducer compared to a 2.3 fold up-regulation in tissue treated with SA (Schenk *et al.* 2000). In accordance with Schenk *et al.* (2000) this study also revealed that the *EgrPR5* identified in *E. grandis* was induced by MeJA, thereby suggesting that this gene would not be a suitable diagnostic marker for the SA pathway. Additionally the predicted transcript *Egrandis*_v1_0.051781, which was selected as the putative ortholog for *EgrPR5* was shown to be primarily expressed in the phloem and shoot tips (Mizrachi *et al.* 2010). Therefore, because the tissue sampled for this experiment consisted of mainly young leaf tissue, the ambiguous expression pattern observed for this putative ortholog may be associated with the type of tissue profiled instead of the function.

Due to the fact that *EgrPR1_like* was found to be repressed by exogenously applied SA (Figure 2.10), we postulated that the basic form of *EgrPR1_like* was being profiled instead of the acidic form of *EgrPR1_like.* In *Arabidopsis,* the acidic isoform of *PR1* is known to be induced by SA whereas the basic isoform is suppressed (Selitrennikoff 2001). We hypothesized the basic *EgrPR1_like* isoform could be enhanced by application of MeJA (Santamaria *et al.* 2001). However, there was no significant change in the levels of *EgrPR1_like* transcript expression following MeJA treatment. High amino acid and CDS sequence similarity was found to exist between the acidic and basic isoforms in *Arabidopsis* and therefore we presume that the primers designed in this study could amplify both of these isoforms. The promoter of the basic *PR1* isoform in *Arabidopsis* has been shown to be responsible for the organ-specific expression of this gene and to contain specific regions that mediate the response of this gene to MeJA. Within a specific region of this promoter lies *cis* elements such as a GCC box which is found to occur in various ethylene-inducible promoters thus allowing this isoform to respond to MeJA and ET (Santamaria *et al.* 2001). These specific promoter regions

distinguish the expression patterns of the acidic and basic PR1 isoforms in *Arabidopsis*. The significance of the upstream elements involved in *EgrPR1_like* expression remains to be elucidated in *E. grandis*. In the NJ and ML phylogenetic trees, it was found that there were multiple possible gene models that could encode for *PR1* and further investigation into these other candidates may elucidate a functional ortholog for the acidic *PR1* in *E. grandis.*

On the other hand, *EgrNPR1* transcript levels were found to not be positively influenced by the exogenous application of SA. However these transcripts were repressed by high concentrations of MeJA treatment (Figure 2.12). In *A. thaliana,* when the plant is infected by a pathogen, the levels of SA are allowed to accumulate and the NPR1 protein which usually exists in an oligomeric formation in the cytoplasm, is then converted to a monomeric form and translocated to the nucleus. In the nucleus NPR1 interacts with various other transcription factors to enhance expression of PR proteins (Pieterse and Loon 2004; Mukhtar *et al.* 2009). In *Eucalyptus*, the NPR1 protein may have evolved multiple copies that execute a similar role to that of the *Arabidopsis* NPR1 or a more complex role. Functional experiments such as complementation studies are needed to further clarify the identity and functions of this gene in *Eucalyptus*.

In terms of expression of the MeJA markers, *EgrPR3* and *EgrLOX2* transcripts were significantly differentially regulated at varying concentrations of this phytohormone (Figure 2.11). These markers were additionally repressed at high concentrations of SA treatment confirming the suppressive effect of SA on MeJA responses (Figure 2.12) (Niki *et al.* 1998). Transcripts of *EgrPR4* were found to be up-regulated by the application of MeJA, but showed no differential expression under SA treatment. Nonetheless, *EgrPR4* can be utilized as a marker for MeJA as transcript levels of this gene are only significantly altered upon application of that inducer. The data suggests that the known

antagonistic relationship between MeJA and SA in *Arabidopsis* is also present in *Eucalyptus* (Niki *et al.* 1998; Koornneef *et al.* 2008; Pieterse *et al.* 2009). All of the MeJA responsive markers profiled in this study were found to be diagnostic to the MeJA pathway in *E. grandis* and could be suitable markers for the pathway. Additionally WRKY70 could be another gene that can be used as a marker to investigate the antagonistic relationship between SA and MeJA however the selection of a putative candidate was confounded by the presence of the WRKY domain in multiple orthologs.

2.5.3 Time dependent expression profiles revealed different key phases for various putative markers

The time at which a host's defences are activated has a crucial role in determining the outcome of a pathogen interaction. Susceptibility may not only be due to the lack of required artillery, but also to the delayed activation of the genes required to curb the pathogen (Loon 2009). Elucidating the time dependent expression profiles of the putative orthologous markers under mock induction of the signalling pathways would provide a glimpse into how the genes would respond under pathogen conditions. In addition, a time course expression profile would allow us to understand how the putative markers of *E. grandis* respond in comparison to what is known in *Arabidopsis* and other host species. The SA marker, *EgrPR2* had a distinctive profile with a drastic peak at 24 hours post treatment (hpt) followed by a sudden decrease at 48 hpt (Figure 2.16). Tobacco plants infected with tobacco mosaic virus (TMV) exhibited a SA profile that was similar to that observed for *EgrPR2* under mock induction of SA in *E. grandis*. The levels of SA in tobacco infected with TMV began to rise after 12 hpt, peaking at 36 hpt followed by a drop at 48 hpt and then a continuous rise up to 72 hpt (Malamy *et al.* 1990; Malamy *et al.* 1996). This suggests that the marker genes identified in this study respond to mock

induction of the signalling pathway in a similar manner as they would under pathogen incursion.

The time points investigated here only extended to 48 hpt and more time points will need to be investigated to further understand how the levels of SA fluctuate in *E. grandis* thereafter. In addition, only a few markers were assessed for this study and although these markers have been shown to be responsive to the exogenous application of SA, they are not indicative of the entire signalling pathway or the final level of SA within the cell. Furthermore, in tobacco two forms of SA exist namely, the free endogenous form of SA and the sugar conjugate known as salicylic β-glucoside (SAG) (Enyedi and Raskin 1993; Hennig *et al.* 1993). SAG may serve as a storage form of active SA, which could occur when the levels of SA increase to toxic levels within a cell. This reserve SAG may then be hydrolyzed when a second pathogen invades the host thereby leading to a rapid activation of defence related genes (Hennig *et al.* 1993). The storage form of SA could also exist in *E. grandis* and to account for both of these, the free and bound levels of SA should be directly measured within the cell over a period of time. This would need to be done using chemical techniques such as HPLC and will be a subsidiary experiment to be conducted in future. *EgrPR2* is known only to be induced in response to the levels of free SA and the drop in expression seen after 24 hrs could be attributed to an increase in SAG level thereby resulting in a reduction of free SA.

The SA signalling pathway has also been shown to have a different response curve based on the composition of the inducer that is applied. For example in tobacco, the application of polyacrylic acid (PAA) and thiamine-HCI, which are inducers of SAR and result in the activation of *PR* genes, displayed different peaks in levels of SA at different time points. The application of PAA showed the highest peak of SA levels in tobacco plants 10 hours after treatment, whereas thiamine-HCI induced the most prominent

induction in SA levels as early as 6 hours following treatment (Malamy *et al.* 1996). This could partially explain the difference in expression profiles observed for the putative orthologs. In contrast to *EgrPR2*, *EgrPR5* was shown to gradually increase over the time points with a maximum expression level detected at 48 hpt (Figure 2.16). Notably the level to which *EgrPR5* is expressed at 48 hpt is approximately the same as *EgrPR2* at this time point. The profiles of *EgrPR2* and *EgrPR5* are also consistent with the expression profiles of tobacco plants treated with SA (Ward *et al.* 1991). Unexpectedly, *EgrPR1_like* was shown to be significantly up-regulated at 6 hpt (Figure 2.16). Based on the results obtained from the dose response experiment, in which the *EgrPR1_like* target was suppressed by SA, it was expected that this gene would be suppressed at all the time points. This result was elusive and it is hypothesized that we could have amplified the gene that encodes for basic isoform of the protein instead of the acidic isoform.

In terms of the MeJA markers, the pattern of the expression profiles observed in *E. grandis* was in accordance to what is known to occur in other plant species. Tobacco plants that have been treated with exogenous MeJA displayed time course patterns similar to that found in *E. grandis* for *LOX* and *PR3* (Bell and Mullet 1993; Avdiushko *et al.* 1995; Rickauer *et al.* 1997). It was shown that in tobacco expression of *LOX2* was enhanced as early as 2 hours following MeJA treatment with a decrease in expression by 24 hours. A similar pattern was found in *E. grandis* where *EgrLOX2* transcript levels were significantly up-regulated at 6 hpt and 12 hpt followed by a drop at 24 hpt (Figure 2.17). This could indicate a possible role for *EgrLOX2* in the early stages of defence activation in a host as this gene is involved in jasmonate biosynthesis.

EgrPR3 displayed a similar profile with the level of transcripts increasing from 6 hpt to 48 hpt in *E. grandis* (Figure 2.17) compared to increasing levels from 8 hours to 24 hours post MeJA treatment in tobacco (Rickauer *et al.* 1997). In pepper, CACBP1, which has

approximately 83% homology to the tobacco chitinase protein, was also shown to be induced from 12 hours to 24 hours after treatment with MeJA (Lee *et al.* 2001). This adds confidence that the data obtained for *EgrPR3* aligns with the information currently known from other plant species in terms of its response over time. A microarray time course study in which *Arabidopsis* plants were treated with MeJA revealed that *EgrPR4* transcripts began to increase at 1 hour, peaking at 8 hours then slowly declined by 24 hours (Jung *et al.* 2007). Conversely in *E. grandis*, *EgrPR4* increased from 6 hpt with the maximum expression level detected at 48 hpt (Figure 2.17). Although the time points differ between the two organisms, the general trend of expression remains the same. The observed increase in the transcript levels of *EgrPR3* and *EgrPR4* over time could also be due to the role of these proteins in the host during defence. Both of these genes encode for products that target and alter the cell wall composition of a fungal pathogen and during infection an increase in expression would be beneficial in preventing the spread of the pathogen (Selitrennikoff 2001). Once again only a few markers were assessed for MeJA and more markers as well as a chemical measurement of the levels of this substance within the cell would be needed to further elucidate this signalling pathway. Nonetheless, based on the results obtained here, we can conclude that the time dependent expression profiles of the putative markers does in fact correspond to the information known from model species.

2.5.4 Pathogenicity experiments conducted with C. austroafricana establishes the diagnostic potential of putative orthologous markers and the possible involvement of SA in defence

In *Arabidopsis*, the involvement of a specific signalling pathway during an interaction with a pathogen can be elucidated by the diagnostic ability of the assigned marker genes. This study examined the diagnostic potential of the putative orthologous markers

for SA and MeJA found in *Eucalyptus* upon infection with *C. austroafricana.* It was found that at 2 wpi there was no substantial difference in lesion length between the susceptible ZG14 and the tolerant TAG5, whereas at 6 wpi there was a significant difference (Figure 2.18). This could indicate that during the initial two weeks following infection, TAG5 was able to initiate a certain response to curb the spread of the disease whereas ZG14 was not able to elicit such a response. Interestingly, the putative marker gene expression profiles that were observed in the two hosts suggest a probable role of SA in the tolerance mechanism of TAG5.

In the TAG5 and *C. austroafricana* interaction, at 2 wpi, the expression level of *EgrPR2* transcripts were considerably up-regulated compared to the control, whereas no such differential expression was observed in ZG14 (Figure 2.22). In addition, the level of MeJA at 2 wpi was lower in TAG5 than in ZG14 as indicated by the expression levels of *EgrPR4* (Figure 2.22). The antagonistic relationship between SA and MeJA evidently occurs within these hosts at this time point and could possibly have a key role in determining the outcome of the interaction with *C. austroafricana*. In TAG5, MeJA levels were presumably low enough to allow sufficient accumulation of SA at 2 wpi, whereas in ZG14 the level of SA was possibly unable to increase due to an elevated level of MeJA. Therefore 2 wpi appeared to be a crucial time point in determining if the host would be susceptible or tolerant.

At 6 wpi, ZG14 exhibited a significant increase in *EgrPR2* levels however this attempt to confine the pathogen possibly occured too late and could be one of the reasons this host is susceptible. From other plant species, *PR2* is known to encode for the β-1, 3-glucanase enzyme which facilitates the enzymatic degradation of the glucan component of fungal cell wall (Theis and Stah 2004). In TAG5, the elevated level of *EgrPR2* could contribute to confining the spread of *C. austroafricana* by hydrolyzing the β-1, 3-glucan

component of the cell wall. This particular glucan component is abundant in the hypal apex of a growing fungus and degradation of the β-1, 3-glucan leads to a loss in rigidity of the cell wall thereby resulting in cell lysis and eventual cell death (Selitrennikoff 2001; Theis and Stah 2004). *EgrPR2* was only found to be significantly up-regulated at 2 wpi and not at 48 hrs hence suggesting that this later induction could be a partial reason as to why TAG5 is tolerant but not fully resistant against *C. austroafricana*.

In addition to *EgrPR2* having a possible role in tolerance, *EgrPR5* which encodes a thaumatin-like protein could also influence the outcome of the interaction. PR5 acts as an antifungal protein by altering the permeability of the fungal cell wall. This protein has also been found to have the ability to bind to the β-1, 3-glucan component of the fungal cell wall (Selitrennikoff 2001; Theis and Stah 2004; Loon *et al.* 2006). Although *PR5* has weak β-1, 3-glucanase activity, it may act in conjunction with *PR2* to hydrolyze and degrade the cell wall. *EgrPR5* is up-regulated as early as 48 hrs in both TAG5 and ZG14 (Figure 2.22), however in TAG5 an increase in *EgrPR2* transcripts could reinforce the host's defences. In ZG14 this increase in *EgrPR2* did not occur until 6 wpi and as a result may be in part responsible for susceptibility of this host. Likewise in TAG5, this joint role of *EgrPR2* and *EgrPR5* may also be a contributing factor for tolerance.

EgrPR4 encodes a hevein-like protein which acts like a chitin binding protein by targeting the β-chitin component of the cell wall. These proteins migrate to the cell walls of an invading fungus and disrupt the formation of the septa and hyphal tips (Selitrennikoff 2001; Theis and Stah 2004). In ZG14, *EgrPR4* was elevated at 2 wpi, however the host was still susceptible to *C. austroafricana* (Figure 2.22). A possible explanation for this is that the level to which this gene is expressed was not high enough to curb the pathogen. Timing of defence gene expression is crucial in a pathogen interaction and the lack of significant *EgrPR4* expression at 48hrs in TAG5 or in ZG14,

may attribute to the reason *C. austroafricana* is able to proliferate within these hosts during the initial 2 weeks of infection. However with TAG5, an increase in the levels of *EgrPR2* could assist with preventing the spread of the pathogen, but this increase does not occur in ZG14 until 6 wpi. At 6 wpi, both TAG5 and ZG14 had significant up-regulation of *EgrPR2*, *EgrPR4* and *EgrPR5* (Figure 2.22) however this time point is very late and responses observed here may be insignificant in terms of curbing the disease.

Since only a few genes representative of each pathway was profiled in this study we cannot definitively predict whether SA or MeJA would enhance resistance against *C. austroafricana* in *Eucalyptus*. One could artificially induce these pathways by spraying ZG14 with the inducer of interest to determine if it would confer resistance to *C. austroafricana*. In addition, the profile of a resistant host could be examined to further elucidate the role of SA and MeJA. Furthermore no markers for the ET pathway were investigated in this study and therefore we cannot exclude the possibility that this pathway and other signalling molecules (Figure 1.3) may have a role in facilitating resistance. Numerous studies in *Arabidopsis* as well as other species such as tobacco, grape and tomato have indicated that transgenic plants over-expressing PR proteins exhibited increased resistance to various pathogens (Loon *et al.* 2006). For example in *Medicago sativa* over-expression of *PR2* provided resistance to *Phytophthora megasperma* whereas in potato over-expression of *PR5* conferred resistance to *Phytophthora infestans* (Grover and Gowthaman 2003). The generation of transgenic *Eucalyptus* plantlets is an ongoing effort and is currently being optimized in various laboratories worldwide. Once that platform has been established one can investigate the role of particular genes involved in pathogen resistance by creating transgenic lines that over-express the gene of interest. Concomitantly one can knock-down or knock-out a gene of interest to elucidate the importance of a specific target.

Overall the results obtained from this study reveal that the putative markers do have the potential to be diagnostic for a specific signalling pathway under pathogen attack. The markers assessed indicate that in *Eucalyptus* the SA pathway could facilitate resistance against the fungal pathogen *C. austroafricana*. This notion was substantiated by evidence of the antagonistic relationship between SA and MeJA as well as the high level of *EgrPR2* induction in the tolerant interaction. The involvement of SA in facilitating a defence response to a necrotrophic pathogen is in contrast to the published literature from *Arabidopsis* which implicates the involvement of the MeJA pathway (Pieterse *et al.* 2009). In spite of this, there have been studies that have shown that SA could also assist in impeding necrotrophic pathogens. Ferrari *et al* (2003) showed that *Arabidopsis* mutants of the SA signalling pathway were more susceptible to infection by the necrotrophic fungal pathogen *B. cinerea* (Ferrari *et al.* 2003). Additionally, a study conducted with *Populus* and the biotrophic rust fungi *Melampsora* spp. revealed a role for JA/ET in an incompatible interaction which is also in contrast to *Arabidopsis* literature (Azaiez *et al.* 2009).

Based on this, it may well be possible that in tree species the roles of SA and MeJA in pathogen defence could differ from what is known in *Arabidopsis.* The genes identified in this study are known in *Arabidopsis* to be markers for the induction of the signalling pathways and have been shown to be promising targets for enhancing resistance against pathogens (Jach *et al.* 1995; Mène-Saffrané *et al.* 2003; Velazhahan and Muthukrishnan 2004; Fiocchetti *et al.* 2008; Kusajima *et al.* 2010). It is envisaged that the identification of putative orthologs of these marker genes in *Eucalyptus*, will provide researchers with similar targets to improve resistance of this woody species to various pathogens. Although a limitation from this study was that only one putative ortholog was profiled for each marker gene, it does elucidate candidates for further functional studies.

2.6 Conclusion

In this study, putative orthologous marker genes for the SA and JA signalling cascades were identified in *E. grandis.* An antagonistic relationship between SA and JA is known from *Arabidopsis* literature and this premise appears to transcend to *E. grandis*. This was shown by the repression of the markers from the opposing pathway in the dose response experiment. Investigating the orthologous markers over a window period of 48 hrs revealed how the expression of the genes changes during this period of time, which is crucial in determining the outcome of a host-pathogen interaction. Furthermore, the ability of these markers to respond to pathogen infection implies that these genes could potentially be used as a screening tool to identify the pathways involved in various responses. In the example of *C. austroafricana*, the expression profile of the putative markers suggests that SA could have a role in tolerance against this pathogen.

Prior to confirming the diagnostic potential of these markers, one would need to investigate how the genes respond to different pathogens in various species of *Eucalyptus.* Additionally, more genes need to be explored to determine if eucalypts have their own suite of defence related markers. Expanding the number of genes to investigate would allow one to also elucidate which of the signalling pathways play a major role in the various interactions. Concomitantly one could investigate the genes that are found to overlap between the various *Eucalyptus* - pathogen interactions to identify candidates for improving broad spectrum resistance. Whole genome microarray profiling and transcriptome sequencing of *E. grandis* following treatment of an inducer/pathogen would reveal novel candidates specific to this host. Chemical measurements of the inducer levels *in vivo* using techniques such as HPLC (Huckelhoven *et al.* 1999) and liquid chromatography-tandem mass spectrometry (Kojima *et al.* 2009) will also aid in deciphering the various signalling cascades in *Eucalyptus*.

Transformation procedures and the subsequent generation of transgenic eucalypts are currently underway in various laboratories worldwide. Once these platforms are established one can further investigate the role of specific marker genes by either creating over-expression lines or knock-out lines. In the interim, one can use *Arabidopsis* as a host in a complementation study to determine if a correct functional ortholog has been identified. Despite characterizing only a few marker genes in *E. grandis*, this study does provide a step forward in understanding the defence responses of this host. It allows us to comprehend what could possibly be occurring at the genetic level within these hosts and to align this information with the currently used model systems.

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3. Concluding remarks

Plant defence is activated when a pathogen is perceived through receptors located on the surface of the cell wall or through R proteins that interact with avirulence proteins exuded from the pathogen into the cell. This results in the activation of PTI and ETI respectively. Downstream of this perception, various signalling pathways such as SA, JA and ET are activated and these pathways in turn activate the expression of various PR proteins. These proteins can be markers for the induction of the pathway and be used as targets for enhancing defence. This study can be contextualized at this level of plant defence, which focuses on elucidating PR proteins to improve broad spectrum resistance.

Plant-pathogen interactions have been extensively studied in model systems, however little is known about *Eucalyptus* – pathogen interactions. This study embarked on deciphering the *Eucalyptus* defence network in particular the SA and JA signalling pathway by firstly identifying putative orthologs of genes involved in these pathways. A phylogenetic approach was used in combination with sequence similarity and gene expression data. ML and NJ algorithms were employed to provide evidence and confidence in selecting potential orthologous genes. Secondly these putative orthologous genes were characterized in their response to the exogenous application of these inducers. Using RT-qPCR we were able to elucidate the expression profile of these markers under mock induction of the signalling pathway. Thirdly the putative markers were assessed for their ability to respond to pathogen stress. Intriguingly it was found that the information known in *Arabidopsis* is congruent with what occurs in *E. grandis* (Pieterse *et al.* 2009).

This study focused on a few marker genes that have been well defined and characterized in *Arabidopsis*. However there could be novel genes within eucalypts that could be more appropriate for use as markers. Whole genome microarrays or transcriptome profiling can be used to identify such genes. Nonetheless the putative orthologous markers identified in this study are one of the first suites of defence genes to be profiled in *E. grandis* under SA and JA treatment. These markers can be further analyzed under various biotic and abiotic stresses to explore and clarify the role of these genes within the host. In addition to profiling the genes, it would be interesting to investigate the role of upstream control elements such as promoters and transcription factors. This would allow us to generate models, identify key elements within the signalling cascades and to elucidate convergence points between pathways.

Research in *Arabidopsis* has revealed that there are specific transcription factors involved in mediating the convergence of signalling pathways as well as mediating a defence response to invading organisms (Li *et al.* 2004). Identifying and characterizing such transcription factors would assist in assembling a systems biology model for pathogen defence in *Eucalyptus.* Although this study investigated SA and JA as separate entities, there have been experiments conducted in *Arabidopsis* that suggest that there could be an additive effect of these inducers when applied simultaneously (Shang *et al.* 2010). This additive effect is strongly dependent on various factors such as the concentration, timing and the sequence in which the inducers are applied.

From this study we can extrapolate, based on the profile of the putative marker genes, which concentration and time point would be an appropriate starting point. The novelty of this study lies in the prospect that the information gained can be used to begin piecing together the puzzle of the *Eucalyptus* defence architecture. Additionally this work provides a stepping stone upon which other projects can be built and hence add to our

understanding of *Eucalyptus* – pathogen interactions. One such example can stem from the experiments conducted with *C. austroafricana*. In this study it was shown that SA could facilitate resistance against this pathogen. It would be intriguing to examine if the exogenous application of SA could in fact confer resistance to a susceptible host.

The release of the *E. grandis* genome sequence provides an indispensible resource for the identification and characterization of a large number of genes. Using this resource we were able to identify putative orthologs of the SA and JA pathway and profile the expression of these orthologous markers under different conditions. Despite investigating a subset of genes, this study provides some preliminary yet essential information in terms of *Eucalyptus* defence. Additional information such as functionality of the genes, chemical analyses, mutant analyses and protein analyses is required to fully comprehend the complexity of the defence network in *Eucalyptus*. Further exploration into the various aspects of plant defence and the genes associated with resistance could in future assist with tree breeding programs and thereby work towards achieving the ultimate goal of improving tree health.

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Appendices

Appendix A

Table S1: Results of BLASTP similarity search conducted to identify putative orthologs in *E. grandis* for genes of the SA and MeJA signalling pathways. The table below indicates the top three results that were obtained from the BLASTP similarity search used to identify potential candidate orthologs in *E. grandis* on Phytozome v7.0 for marker genes of the SA and MeJA signalling pathway.

Arabidopsis target gene	AT number	Gene Model	Score Bit	E-value	% Identity
PR1	AT2G14610	Egrandis_v1_0.044149	209.9	$5.7e^{-55}$	63.9
		Egrandis_v1_0.029631	209.1	$1.0e^{-54}$	64.5
		Egrandis_v1_0.029399	207.6	$2.9e^{-54}$	63.9
***PR2**	AT3G57260	Scaffold 229_328000..329400	226	e^{-116}	68
		Scaffold 96_592600..593500	222	e^{-102}	55
		Scaffold 149_369200..370100	245	$5e^{-96}$	53
PR3	AT3G12500	Egrandis_v1_0.019914	424.9	$4.3e^{-119}$	69.2
		Egrandis_v1_0.023106	364.0	$8.7e^{-101}$	69.8
		Egrandis_v1_0.023237	363.6	$1.1e^{-100}$	68.6
PR4	AT3G04720	Egrandis_v1_0.030679	195.7	$1.9e^{-50}$	74.8
		Egrandis_v1_0.030656	194.1	$6.8e^{-50}$	74.8
		Egrandis_v1_0.030691	192.6	$1.6e^{-49}$	74.8
PR5	AT1G75040	Egrandis_v1_0.051781	247.7	$5.6e^{-66}$	63.4
		Egrandis_v1_0.020692	234.2	$7.2e^{-62}$	56.0
		Egrandis_v1_0.017956	233.4	$1.2e^{-61}$	55.1
LOX2	AT3G45140	Egrandis_v1_0.002199	1015.4	0	58.2
		Egrandis_v1_0.002193	1013.1	0	58.9
		Egrandis_v1_0.002202	1009.6	0	57.7
LOX3	AT1G17420	Egrandis_v1_0.002112	1372	0	72.4
		Egrandis_v1_0.003846	1042	0	71.7
		Egrandis_v1_0.001980	964.9	0	56.3

*Refers to the scaffolds obtained with the 4.4X *E. grandis* genome assembly

Appendix B

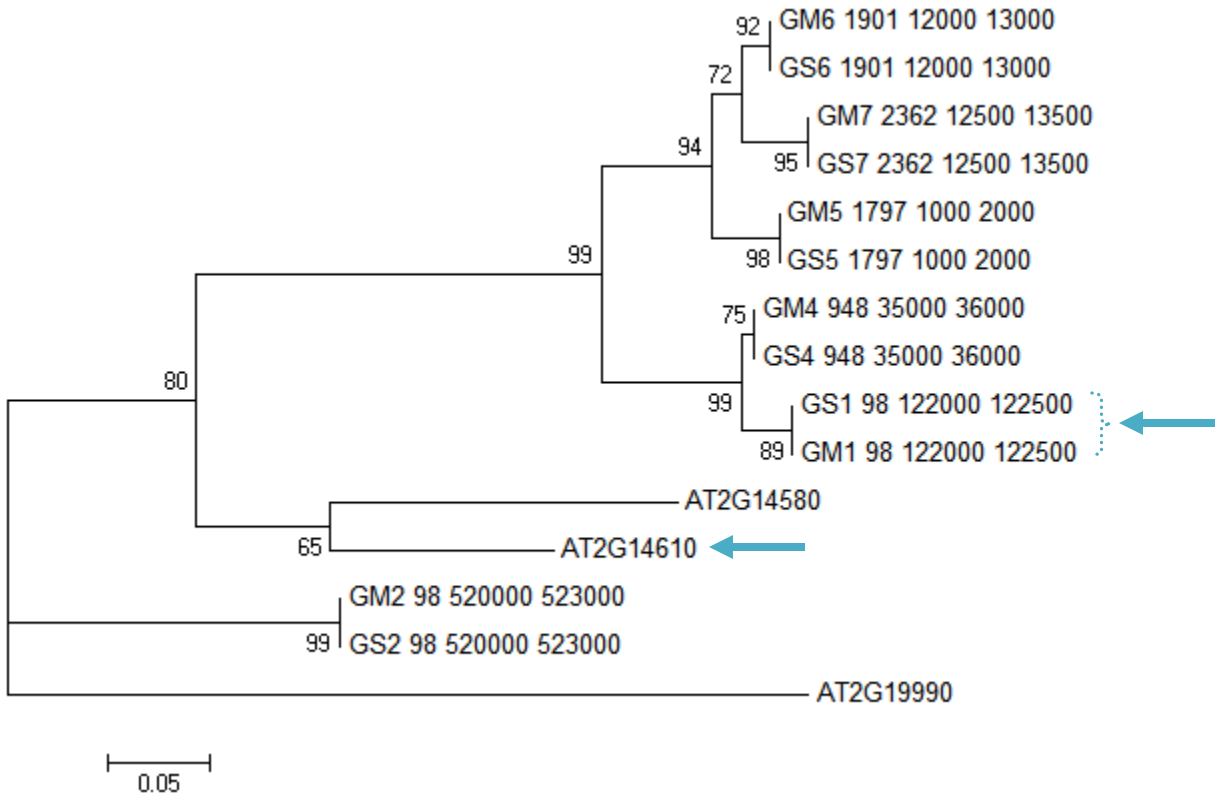

Figure S1: Neighbour joining tree of *EgrPR1_like* (Pathogenesis-related gene 1) constructed with the 4X *E. grandis* genome draft assembly using MEGA v4.1. Bootstrap values are representative of 10 000 permutations. Included in the tree are family members, ATPRB1 (AT2G14580) and *PR1*-Like (AT2G19990). GM refers to the predicted peptide obtained through GeneMark whereas GS refers to the predicted peptide obtained through GenScan. E.g. GM1_98_122000_122500 = GeneMark prediction of scaffold 98, genomic base pair region 122000-122500. The arrows represent the target gene (*PR1*) and the scaffold that was selected as the putative ortholog.

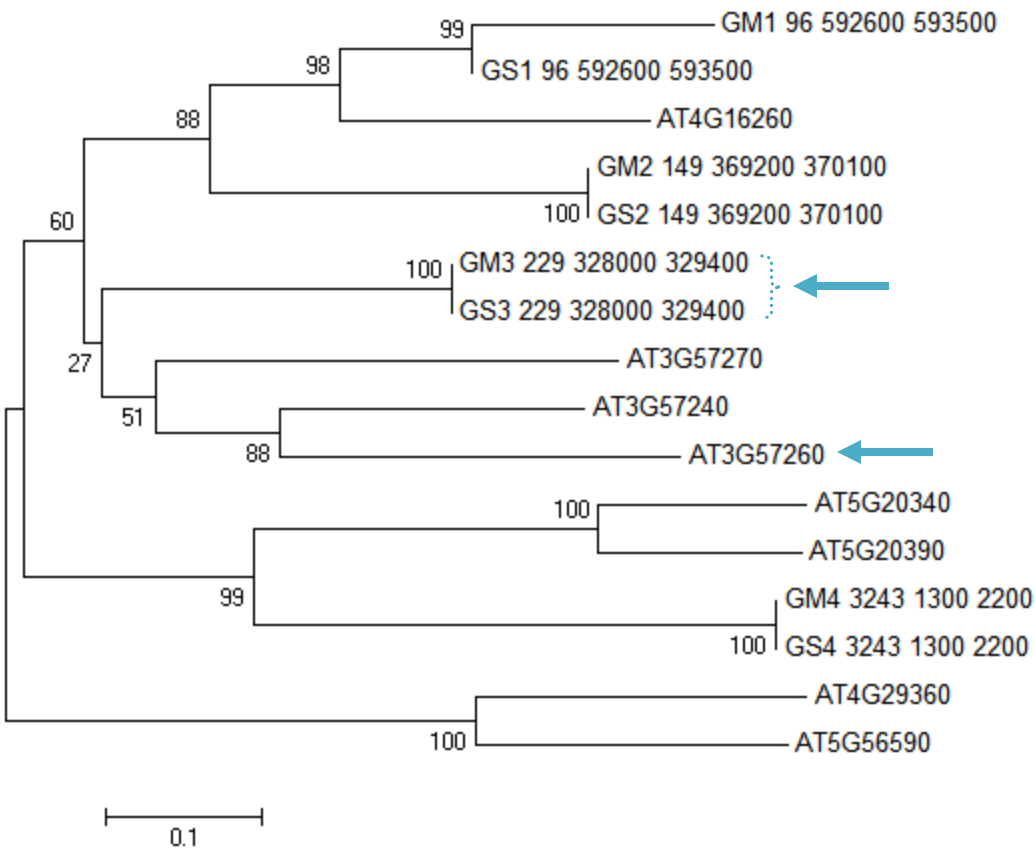

Figure S2: Neighbour joining tree of *EgrPR2* (*Beta - 1, 3 - Glucanase*) constructed with the 4X *E. grandis* genome draft assembly using MEGA v4.1. Bootstrap values are representative of 10 000 permutations. Included in the tree are glucanase family members. GM refers to the predicted peptide obtained through GeneMark whereas GS refers to the predicted peptide obtained through GenScan. E.g. GM3_229_328000_329400 = GeneMark prediction of scaffold 229, genomic base pair region 328000-329400. The arrows represent the target gene (*PR2*) and the scaffold that was selected as the putative ortholog.

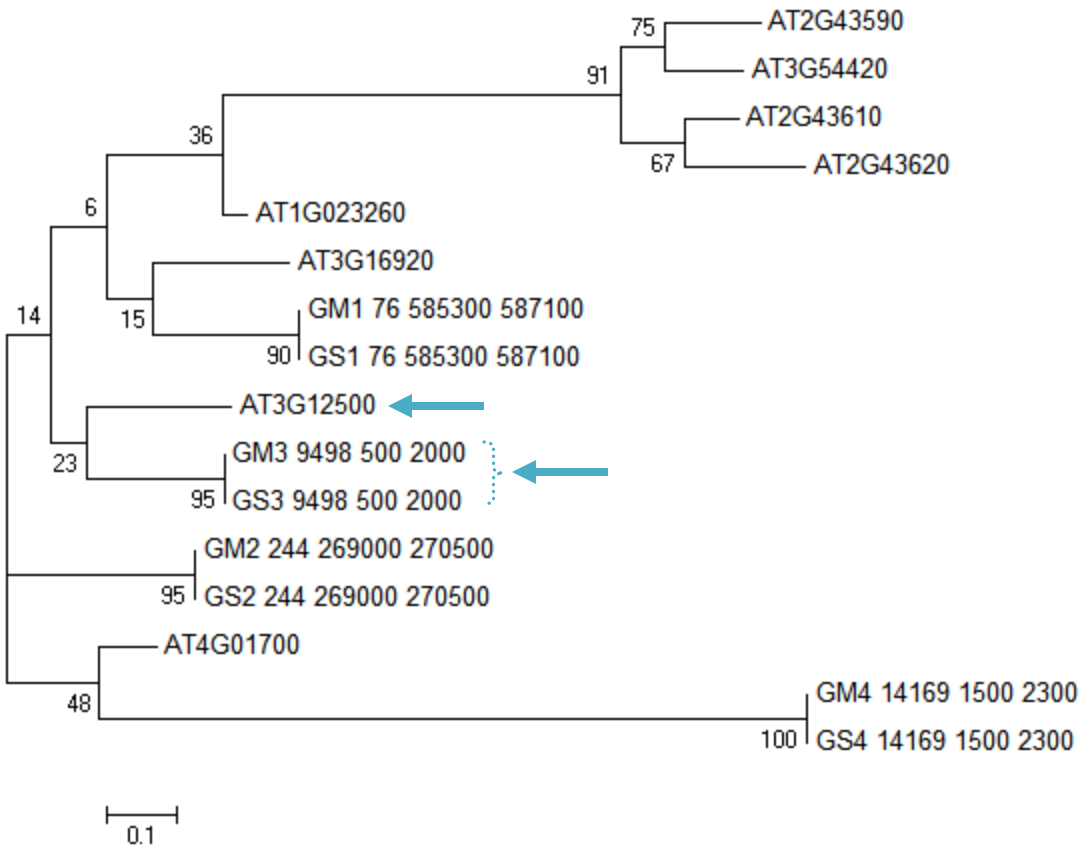

Figure S3: Neighbour joining tree of *EgrPR3* (Chitinase) constructed with the 4X *E. grandis* genome draft assembly using MEGA v4.1. Bootstrap values are representative of 10 000 permutations. Included in the tree are chitinase family members. GM refers to the predicted peptide obtained through GeneMark whereas GS refers to the predicted peptide obtained through GenScan. E.g. GM3_9498_500_2000 = GeneMark prediction of scaffold 9498, genomic base pair region 500_2000. The arrows represent the target gene (*PR3*) and the scaffold that was selected as the putative ortholog.

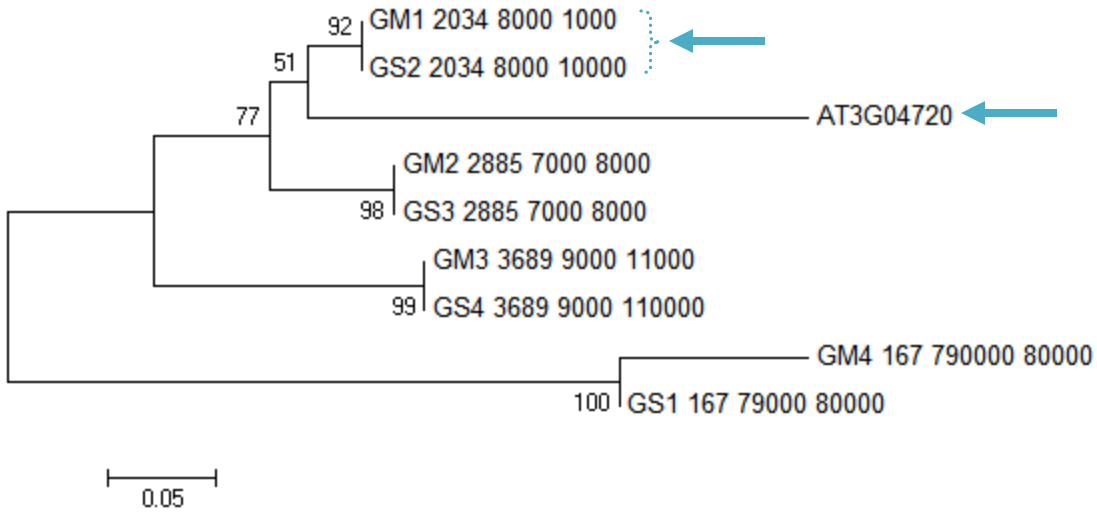

Figure S4: Neighbour joining tree of *EgrPR4* (Hevein-like protein) constructed with the 4X *E. grandis* genome draft assembly using MEGA v4.1. Bootstrap values are representative of 10 000 permutations. Included in the tree are chitinase family members. GM refers to the predicted peptide obtained through GeneMark whereas GS refers to the predicted peptide obtained through GenScan. E.g. GM1_2034_8000_10000 = GeneMark prediction of scaffold 2034, genomic base pair region 8000_10000. The arrows represent the target gene (*PR4*) and the scaffold that was selected as the putative ortholog.

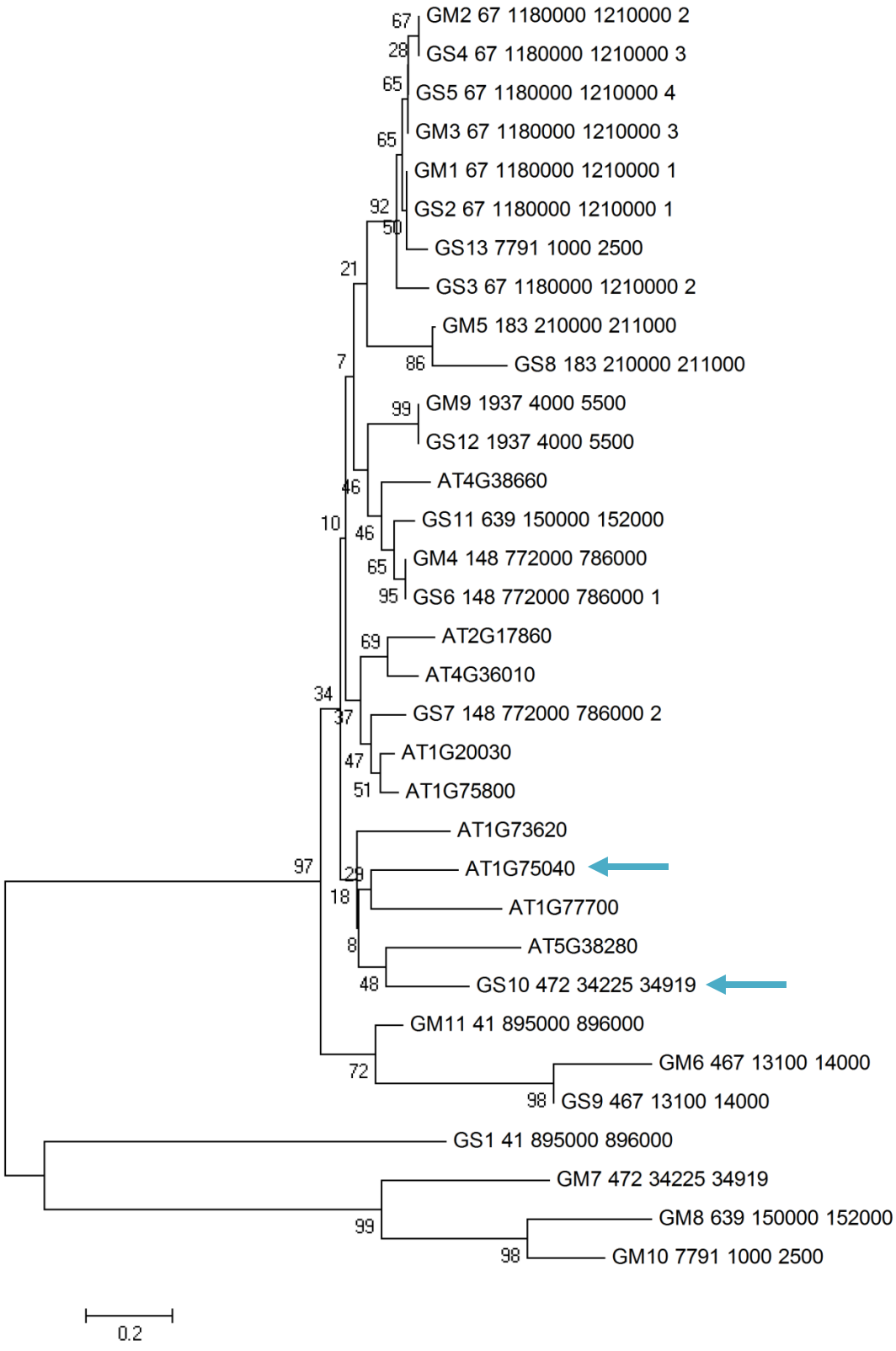

Figure S5: Neighbour joining tree of *EgrPR5* (Thaumatin) constructed with the 4X *E. grandis* genome draft assembly using MEGA v4.1. Bootstrap values are representative of 10 000 permutations. Included in the tree are thaumatin family members. GM refers to the predicted peptide obtained through GeneMark whereas GS refers to the predicted peptide obtained through GenScan. E.g. GS10_472_34225_34919 = GenScan prediction of scaffold 472, genomic base pair region 34225_34919. The arrows represent the target gene (*PR5*) and the scaffold that was selected as the putative ortholog.

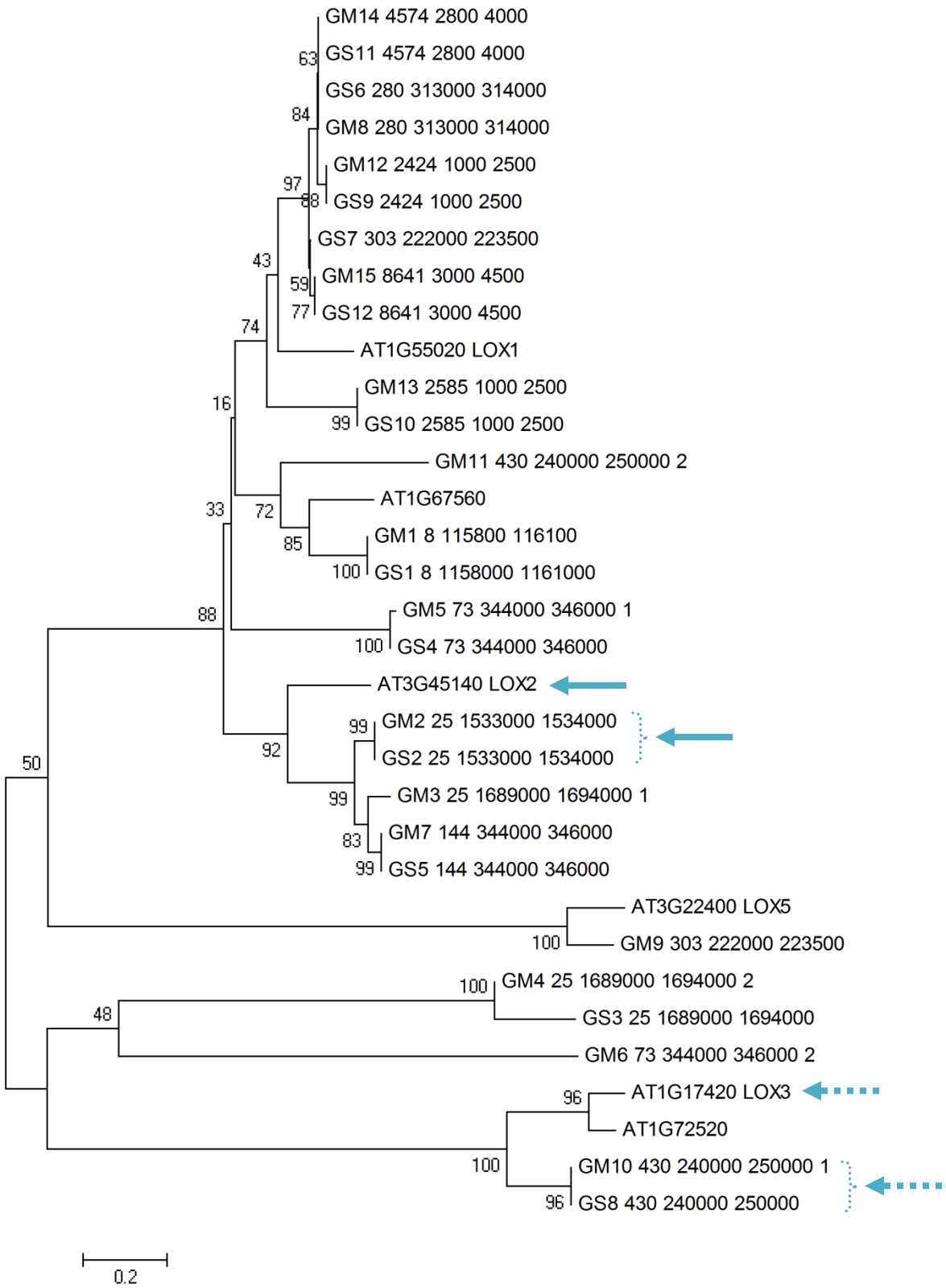

Figure S6: Neighbour joining tree of *EgrLOX* (Lipoxygenase) constructed with the 4X *E. grandis* genome draft assembly using MEGA v4.1. Bootstrap values are representative of 10 000 permutations. Included in the tree are lipoxygenase family members. GM refers to the predicted peptide obtained through GeneMark whereas GS refers to the predicted peptide obtained through GenScan. E.g. GM10_430_240000_250000 = GeneMark prediction of scaffold 430, genomic base pair region 240000_250000. The arrows represent the target gene (*LOX2* and *LOX3*) and the scaffold that was selected as the putative ortholog.

Appendix C

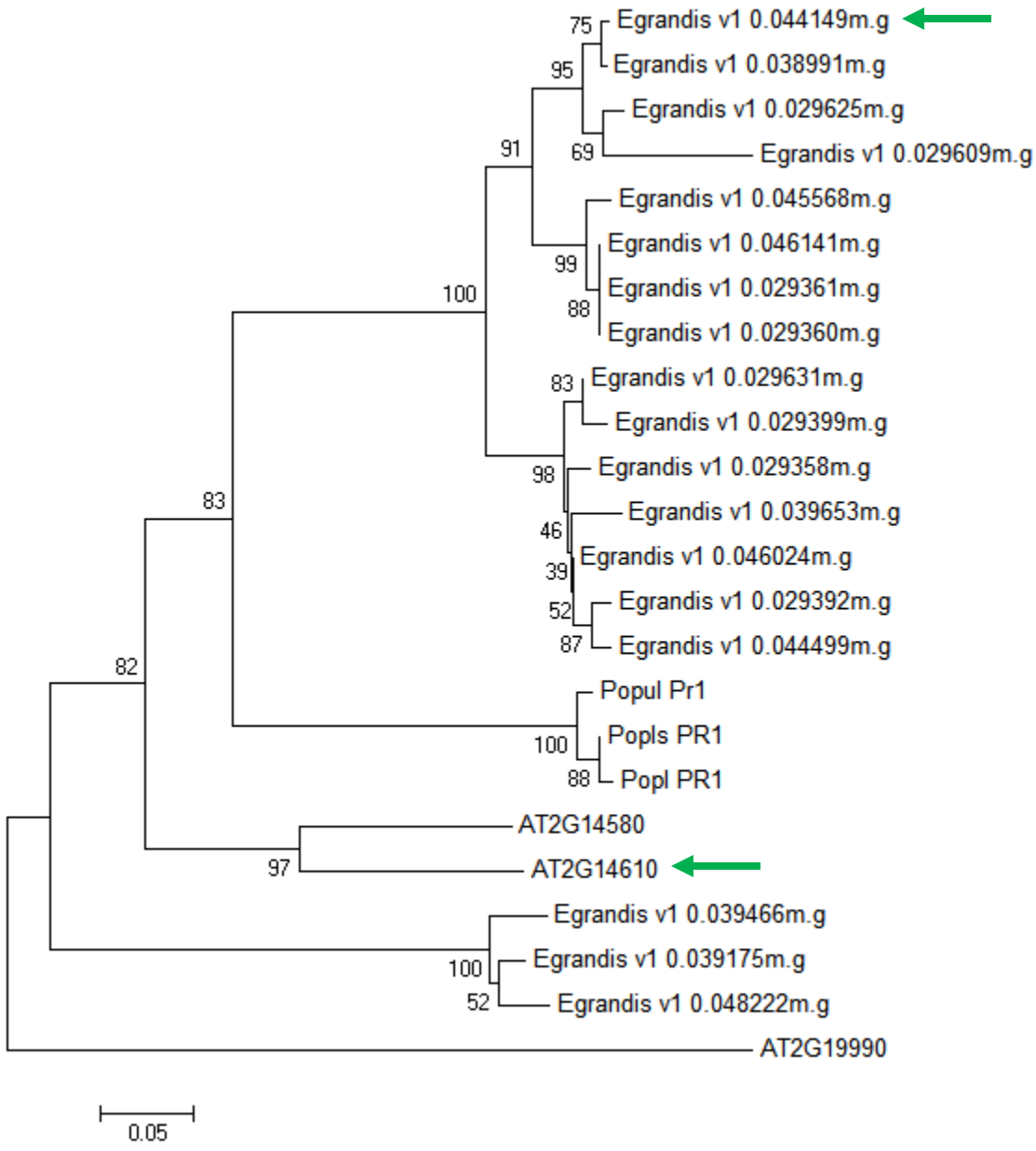

Figure S7: Neighbour joining tree of *EgrPR1_like* (Pathogenesis-related gene 1) constructed with the *E. grandis* predicted proteome available on Phytozome v7.0 using MEGA v5.01. Bootstrap values are representative of 10 000 permutations. Included in the tree are ATPRB1 (AT2G14580) and *PR1*-Like (AT2G19990). The arrows represent the target gene (*PR1*) and the gene model that was selected as the putative ortholog.

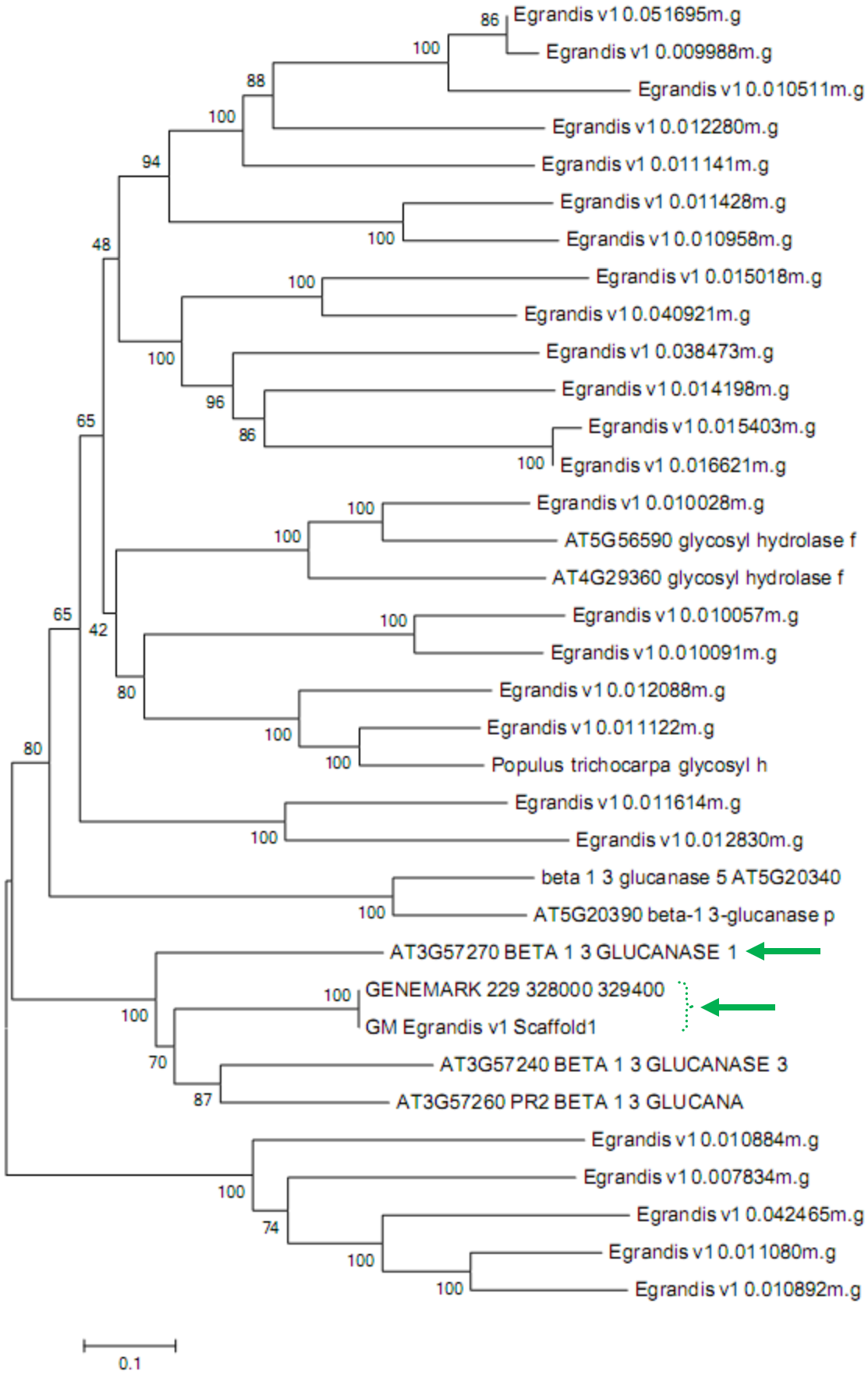

Figure S8: Neighbour joining tree of *EgrPR2* (Beta - 1, 3 - glucanase) constructed with the *E. grandis* predicted proteome available on Phytozome v7.0 using MEGA v5.01. Bootstrap values are representative of 10 000 permutations. Included in the tree are other closely related family members that contain the glycosyl hydrolase domain. The arrows represent the target gene (*PR2*) and the gene model that was selected as the putative ortholog. Since there was no predicted gene model for this target on Phytozome v7.0, a peptide was predicted on GeneMark using the corresponding genomic sequence.

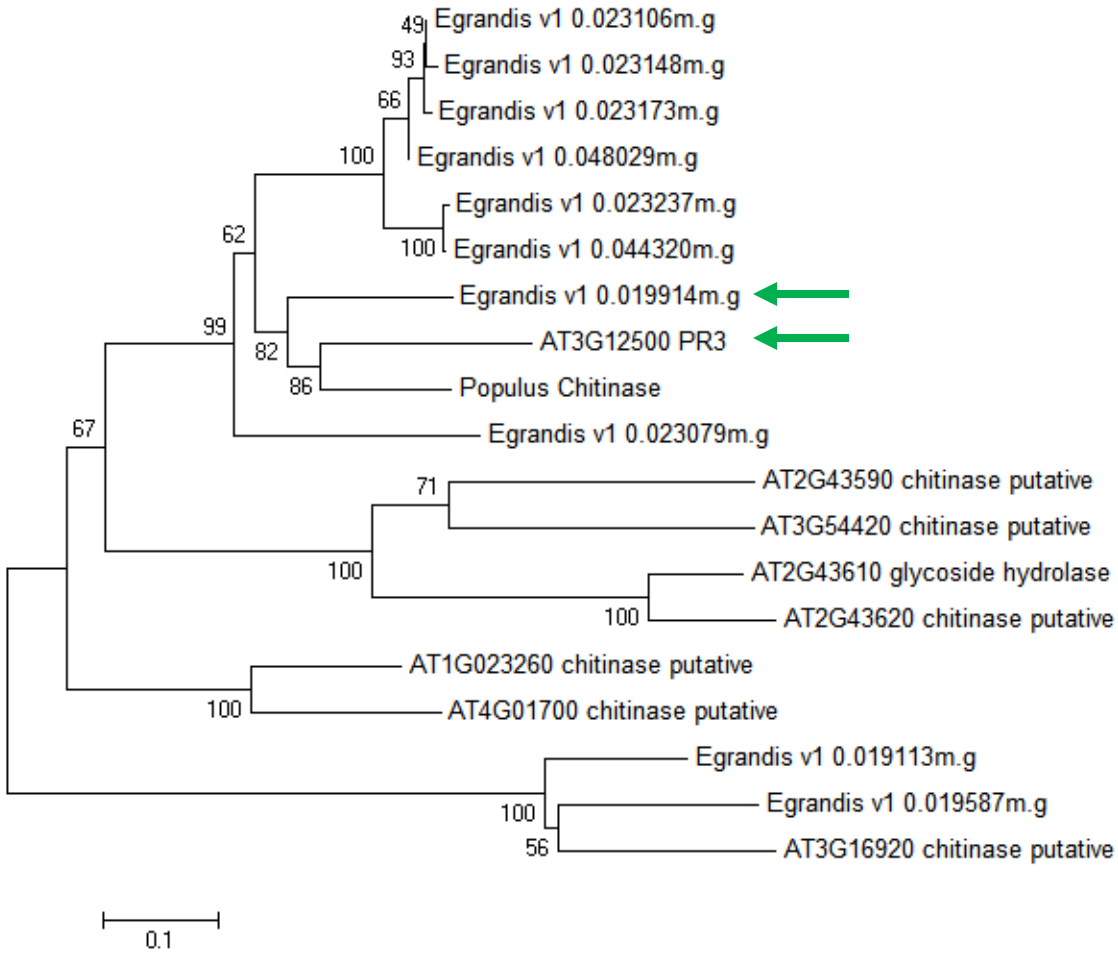

Figure S9: Neighbour joining tree of *EgrPR3* (Chitinase) constructed with the *E. grandis* predicted proteome available on Phytozome v7.0 using MEGA v5.01. Bootstrap values are representative of 10000 permutations. Included in the tree are other chitinase family members. The arrows represent the target gene (*PR3*) and the gene model that was selected as the putative ortholog.

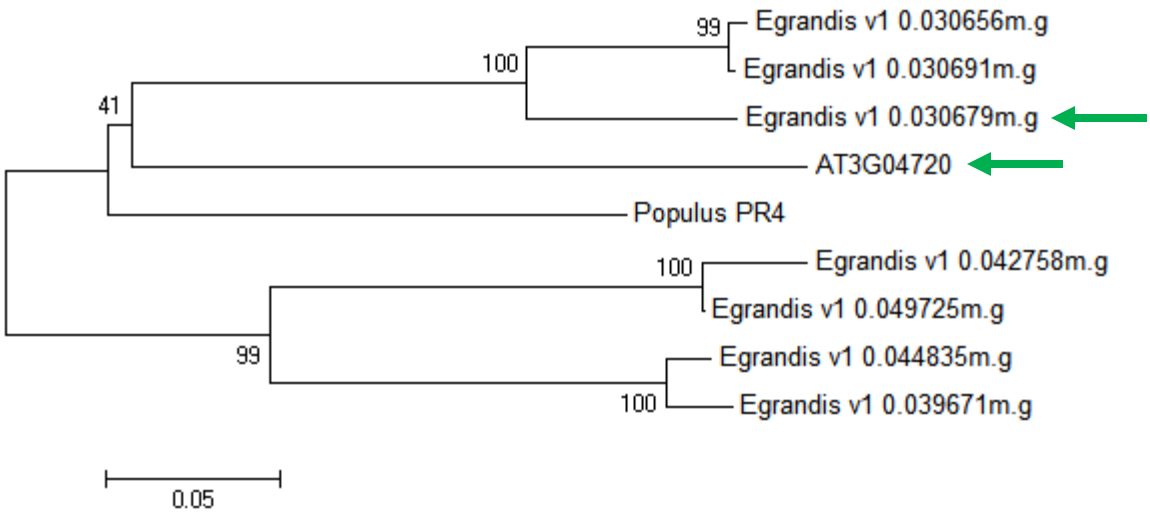

Figure S10: Neighbour joining tree of *EgrPR4* (Hevein-like protein) constructed with the *E. grandis* predicted proteome available on Phytozome v7.0 using MEGA v5.01. Bootstrap values are representative of 10000 permutations. There were no *Arabidopsis* family member with significant e-values (e < -50) hence only the *P. trichocarpa* sequence is included in the trees. The arrows represent the target gene (*PR4*) and the gene model that was selected as the putative ortholog.

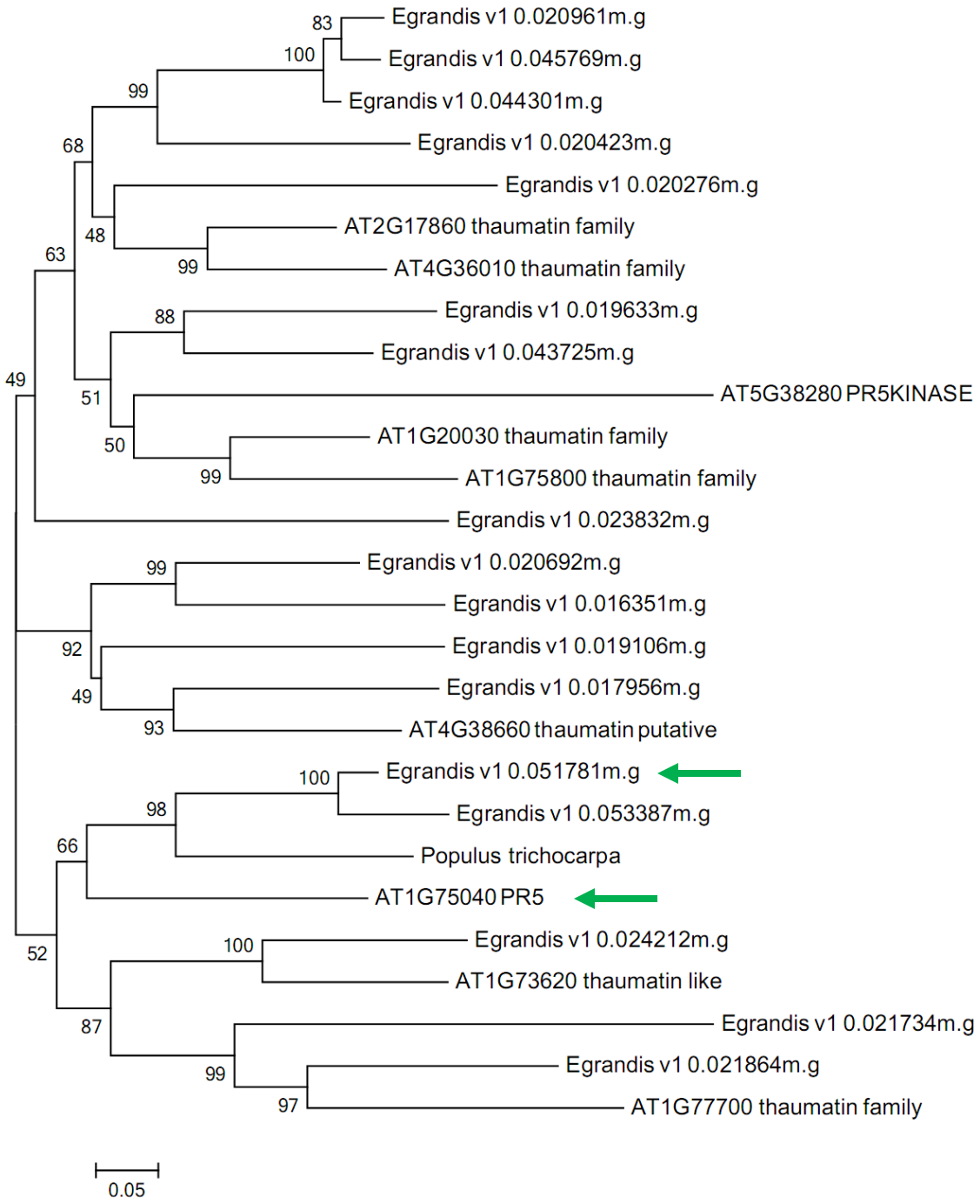

Figure S11: Neighbour joining tree of *EgrPR5* (Thaumatin) constructed with the *E. grandis* predicted proteome available on Phytozome v7.0 using MEGA v5.01. Bootstrap values are representative of 10000 permutations. Included in the tree are other thaumatin family members. The arrows represent the target gene (*PR5*) and the gene model that was selected as the putative ortholog.

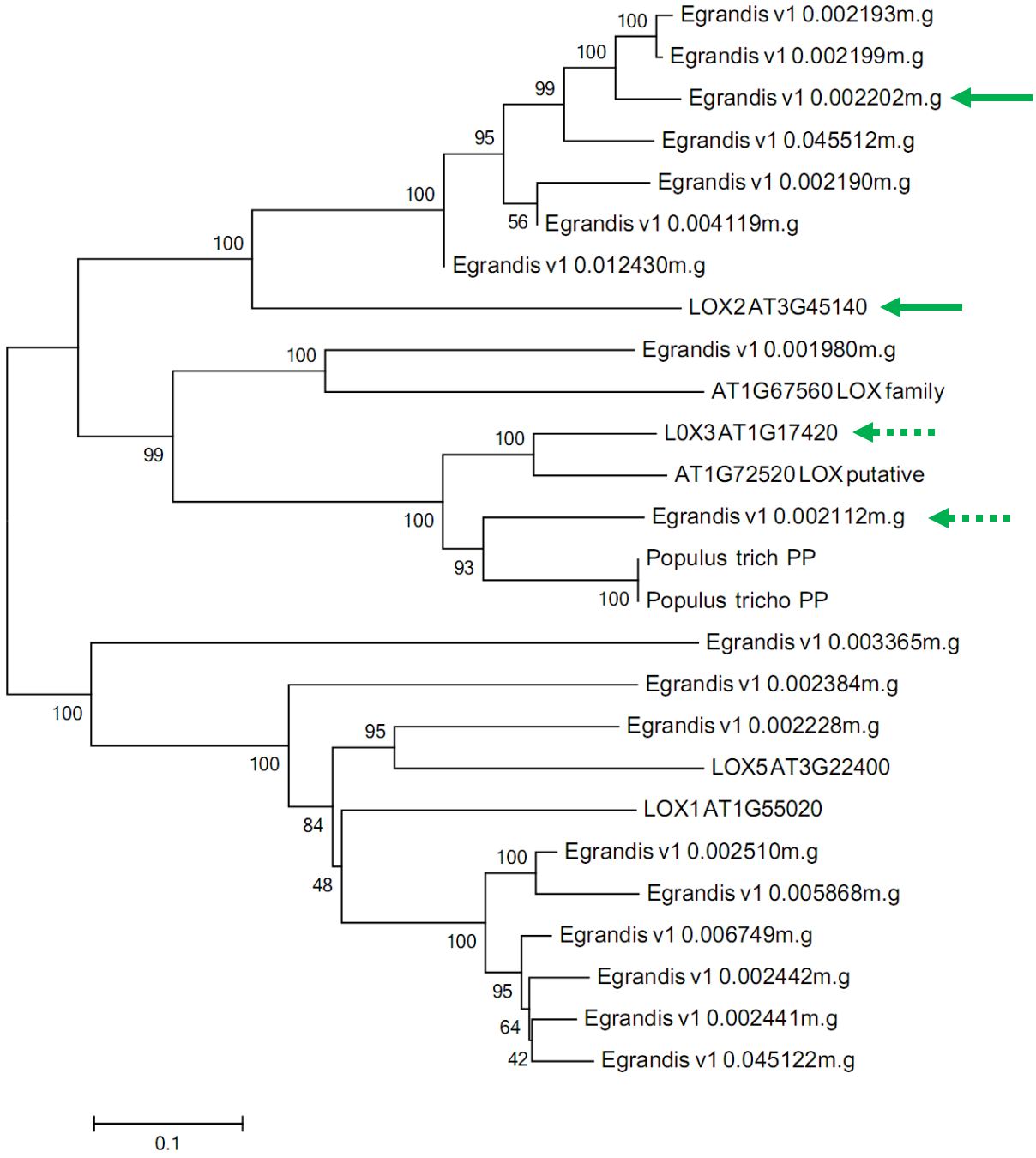

Figure S12: Neighbour joining tree of *EgrLOX* (Lipoxygenase) constructed with the *E. grandis* predicted proteome available on Phytozome v7.0 using MEGA v5.01. Bootstrap values are representative of 10000 permutations. Included in the tree are *LOX1, LOX2, LOX3, LOX5* and other closely related family members. The boxes represent the target gene (*LOX*) and the gene model that was selected as the putative ortholog.

Appendix D

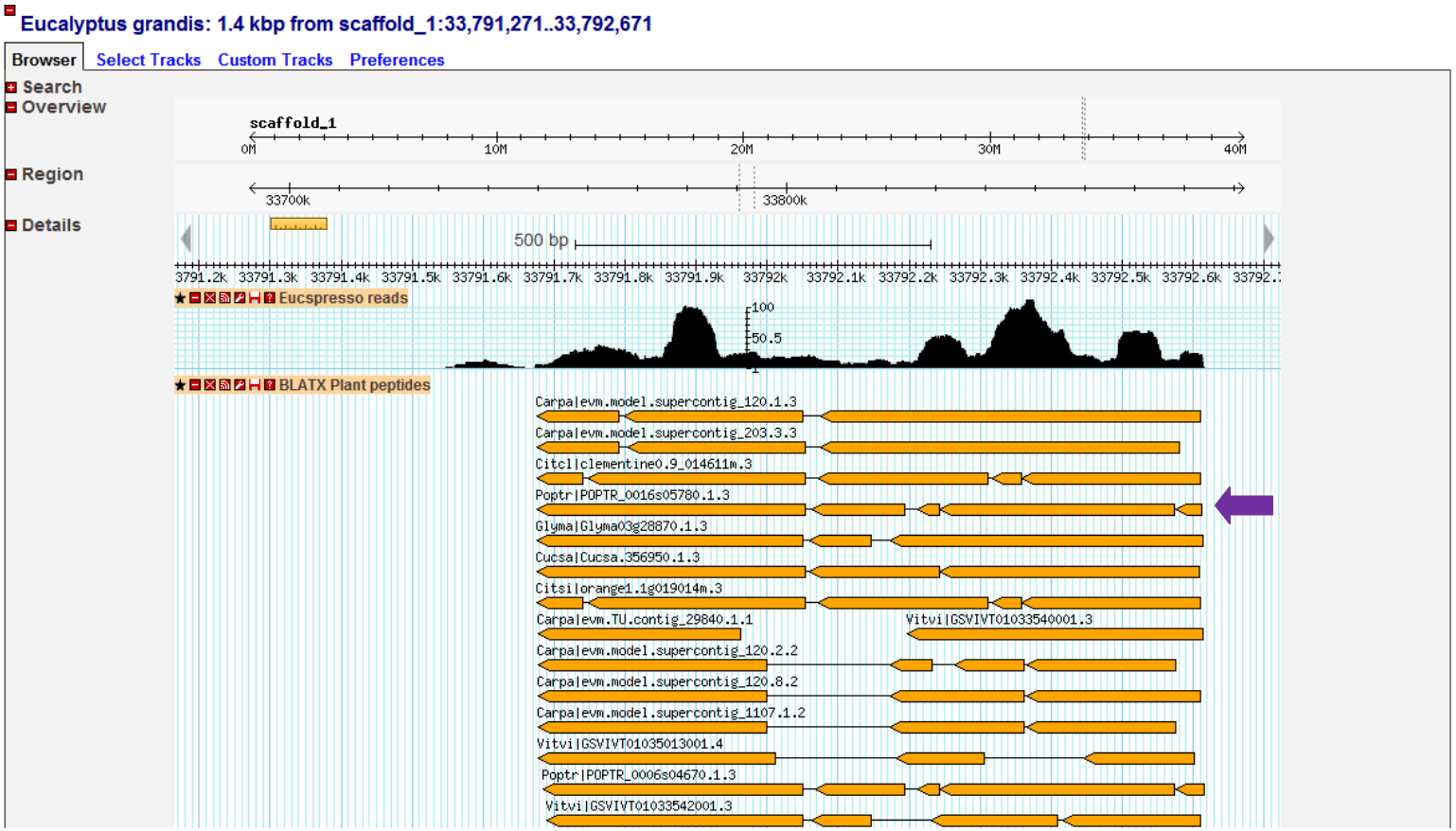

Figure S13: Phytozome v7.0 screen capture illustrating expression data: This data pertains to the genomic scaffold region of the *PR2* target gene that was selected as the *E. grandis* putative ortholog. The purple arrow indicates the plant peptide for which the amino acid sequence was used as a query in a TBLASTX against TAIR to verify that this sequence encodes for *PR2.*

Appendix E

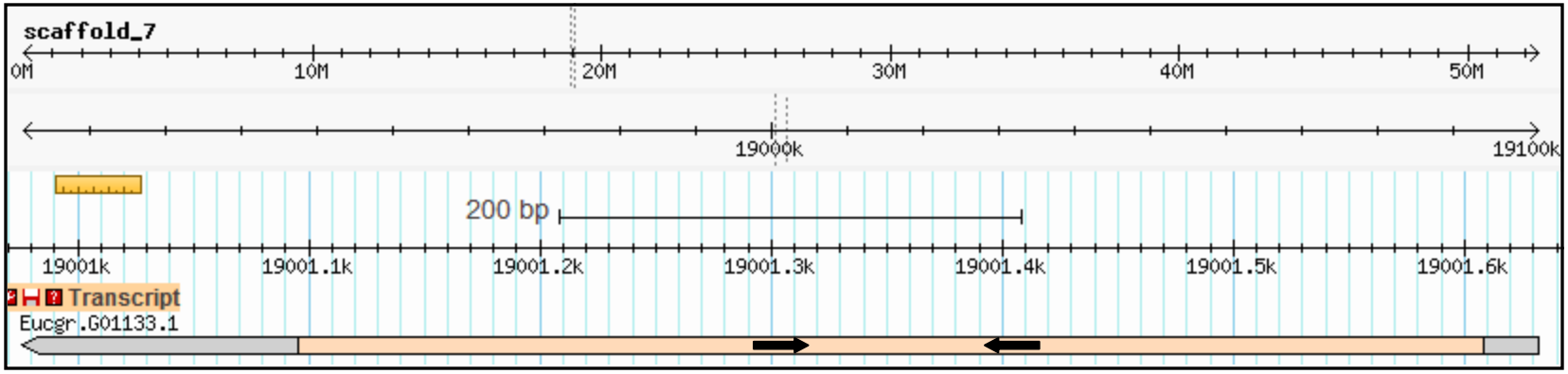

Figure S14: *EgrPR1_like* amplicon map. Genome location of the *EgrPR1_like* transcript that was amplified in this study. The grey regions on the transcript (Eucgr.G01133.1 = Egrandis_v1_0.044149) represent the 5' and 3' UTR whereas the black arrows indicate the region of the transcript that was amplified.

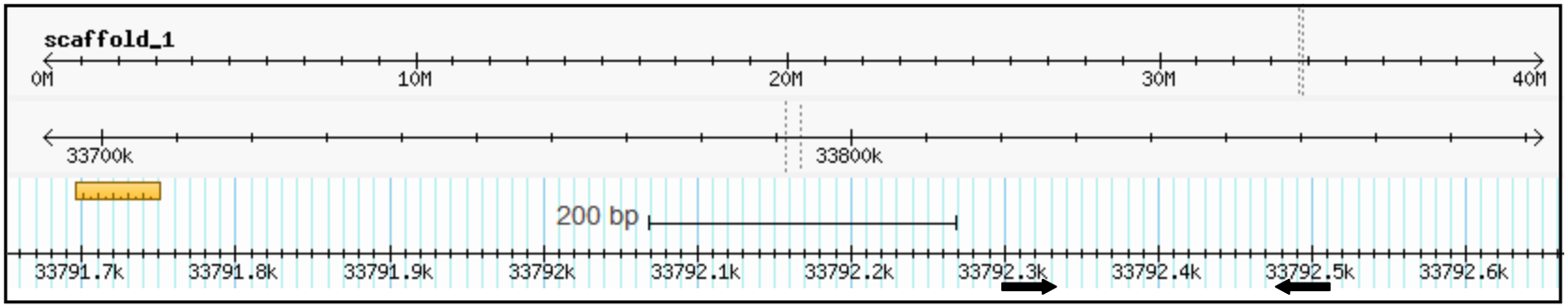

Figure S15: *EgrPR2* amplicon map. Genome location of the *EgrPR2* transcript that was amplified in this study. The black arrows indicate the region of the scaffold that was amplified.

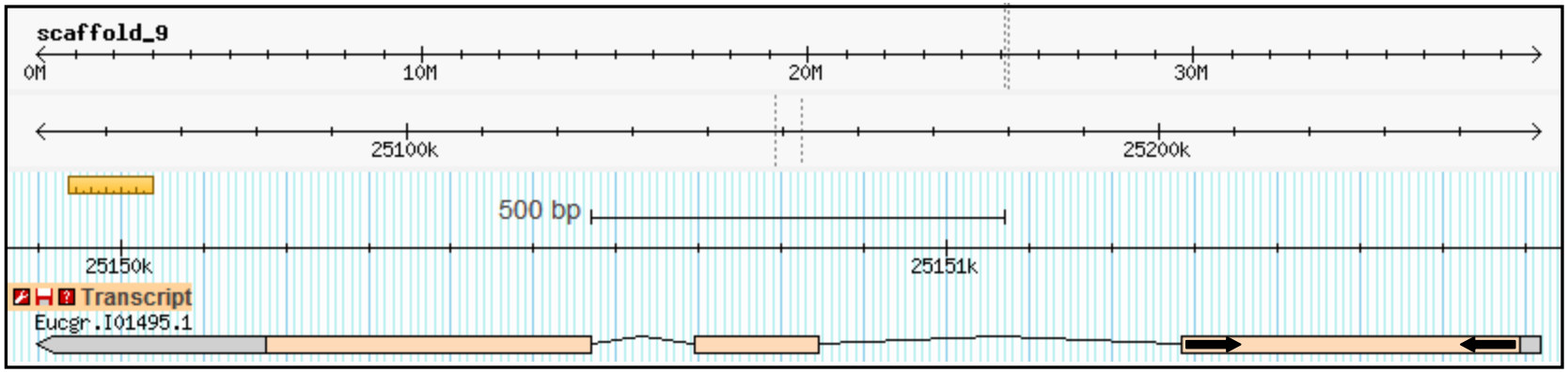

Figure S16: *EgrPR3* amplicon map. Genome location of the *EgrPR3* transcript that was amplified in this study. The grey regions on the transcript (Eucgr.I01495.1 = Egrandis_v1_0.019914) represent the 5' and 3' UTR, the arched lines represent the position of putative introns whereas the black arrows indicate the region of the transcript that was amplified.

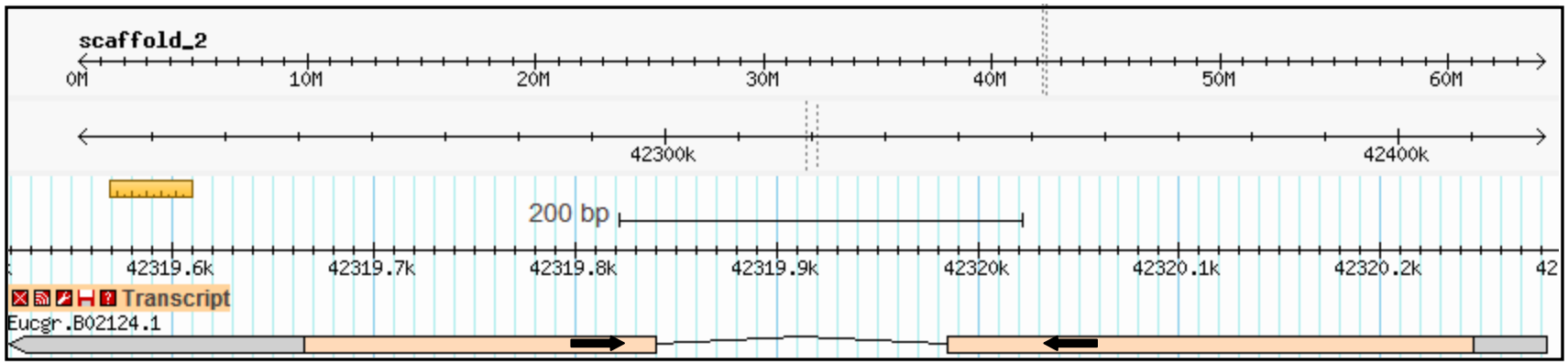

Figure S17: *EgrPR4* amplicon map. Genome location of the *EgrPR4* transcript that was amplified in this study. The grey regions on the transcript (Eucgr.B02124.1 = Egrandis_v1_0.030656) represent the 5' and 3' UTR whereas the arched line represents the position of a putative intron. The black arrows indicate the region of the transcript that was amplified.

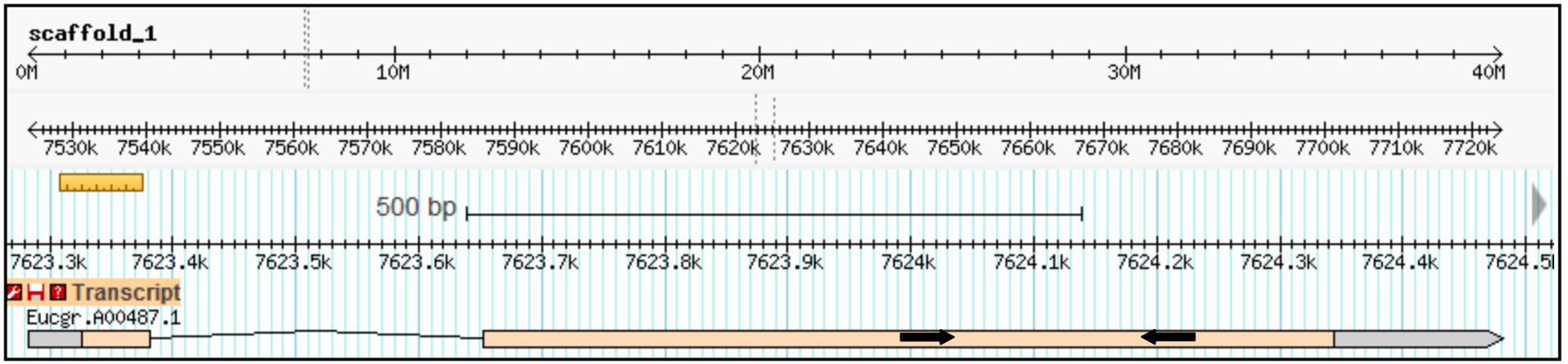

Figure S18: *EgrPR5* amplicon map. Genome location of the *EgrPR5* transcript that was amplified in this study. The grey regions on the transcript (Eucgr.A00487.1 = Egrandis_v1_0.051781) represent the 5' and 3' UTR whereas the arched line represents the position of a putative intron. The black arrows indicate the region of the transcript that was amplified.

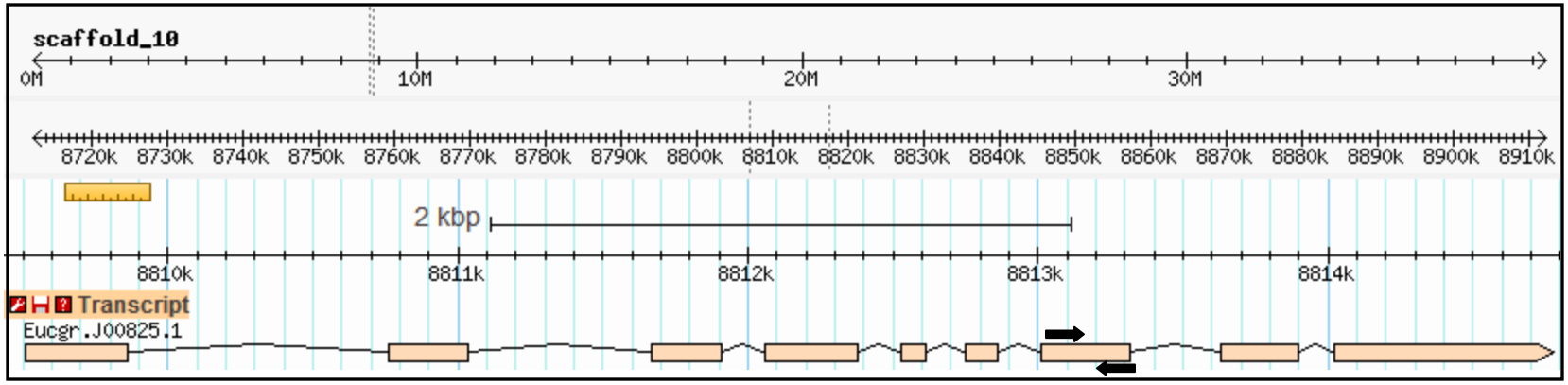

Figure S19: *EgrLOX2* amplicon map. Genome location of the *EgrLOX2* transcript that was amplified in this study. The grey regions on the transcript (Eucgr.J00825.1 = Egrandis_v1_0.002202) represent the 5' and 3' UTR whereas the arched lines represent the position of putative introns. The black arrows indicate the region of the transcript that was amplified.

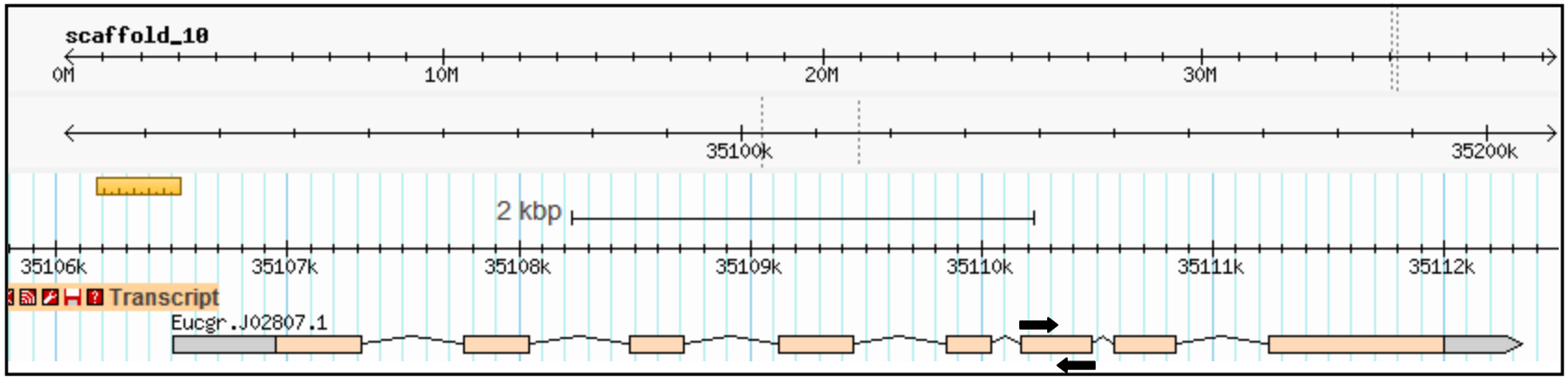

Figure S19: *EgrLOX3* amplicon map. Genome location of the *EgrLOX3* transcript that was amplified in this study. The grey regions on the transcript (Eucgr.J02807.1 = Egrandis_v1_0.002112) represent the 5' and 3' UTR whereas the arched lines represent the position of putative introns. The black arrows indicate the region of the transcript that was amplified.

Appendix F

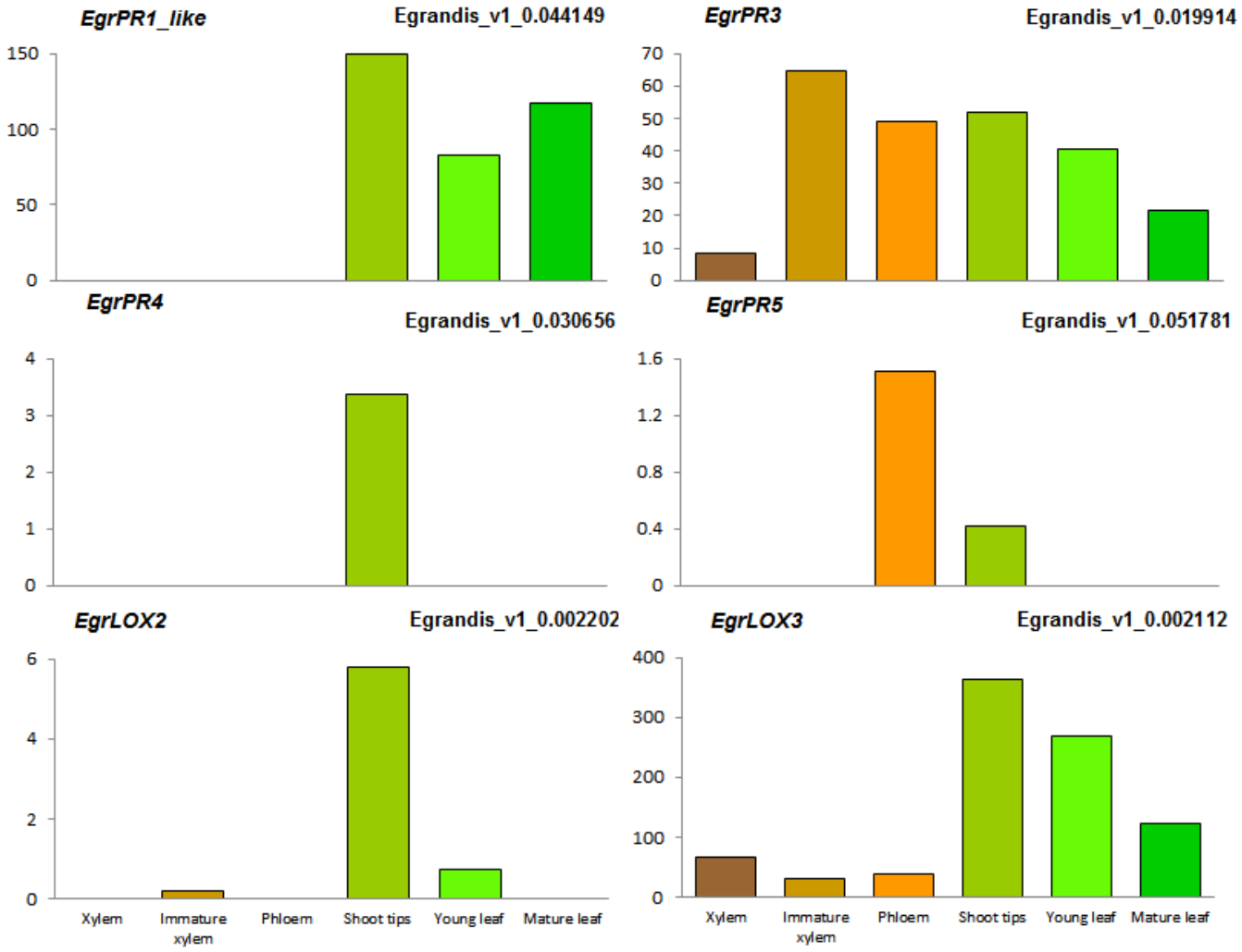

Figure S20: Expression data for the predicted gene models selected as putative orthologs for the SA (*EgrPR1, EgrPR2, EgrPR5*) and MeJA (*EgrPR3, EgrPR4, EgrLOX2, EgrLOX3*) target genes obtained from EucGenIE: The x-axis represents the digital expression of the transcripts in the various tissues of a *E. grandis x E. urophylla* hybrid clone (Left to right: Xylem, Immature xylem, Phloem, Shoot tips, Young leaf and Mature leaf) (Mizrachi *et al.* 2010) and the y-axiz represents the relative abundance values obtained by calculating the Fragment Per Kilobase of exon per Million fragments mapped (FPKM). No expression data is given for *EgPR2* as no predicted gene model was obtained for this gene on Phytozome v7.0.

Appendix G

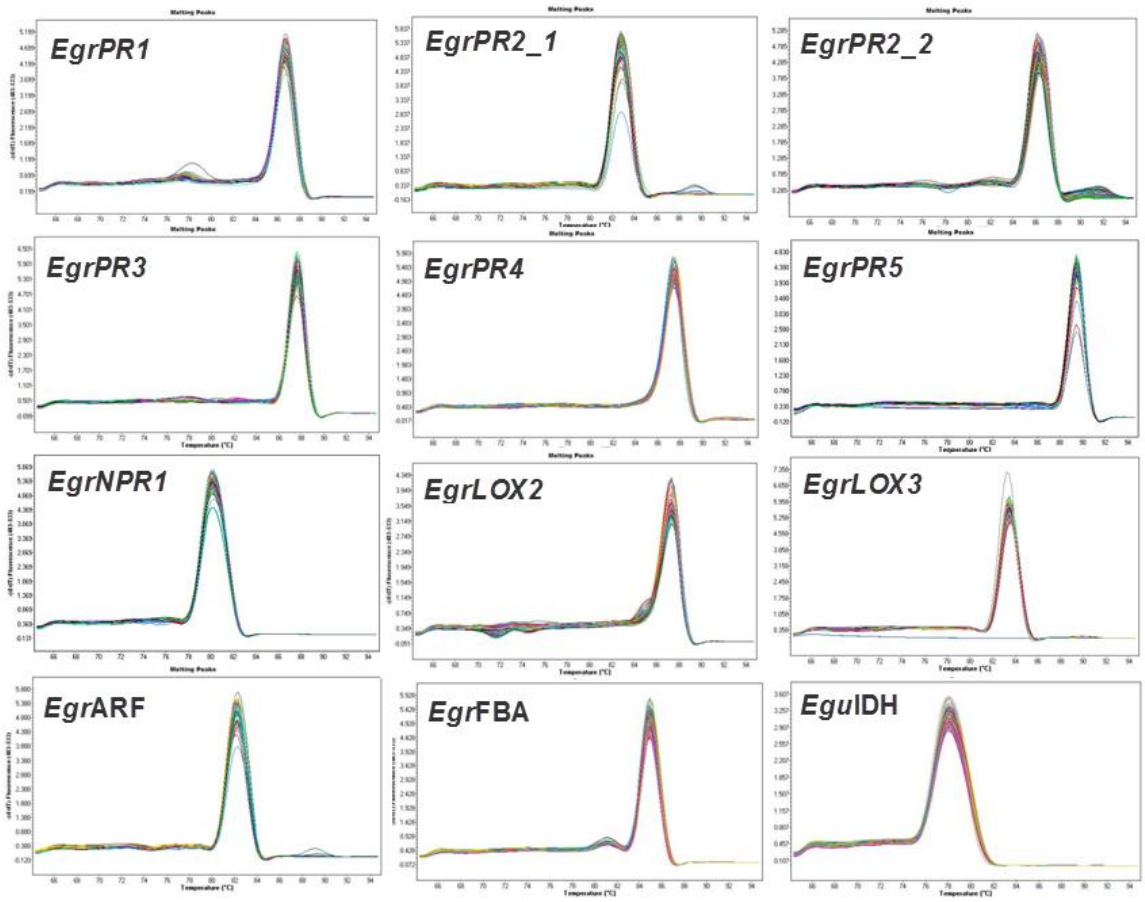

Figure S21: Melting curves of all the SA (*EgrPR1, EgrPR2, EgrPR5, EgrNPR1*) and MeJA (*EgrPR3, EgrPR4, EgrLOX2, EgrLOX3*) target genes and reference genes used for the expression profiling experiments: Melting curves were generated from the LightCycler® 480 Real-Time PCR system. The x-axis depicts the temperature range and the y-axiz depicts the –(d/dT) Fluorescence (483-533) measurement. *EgPR2_1* refers to the primer pair used in the concentration trial whereas *EgPR2_2* refers to the primer pair used for the time trial and the infection trial. *EgrLOX2* illustrates the melting curves obtained for the concentration trial and the time trial whereas *EgrLOX3* illustrates the melting curves obtained for the infection trial.

Appendix H

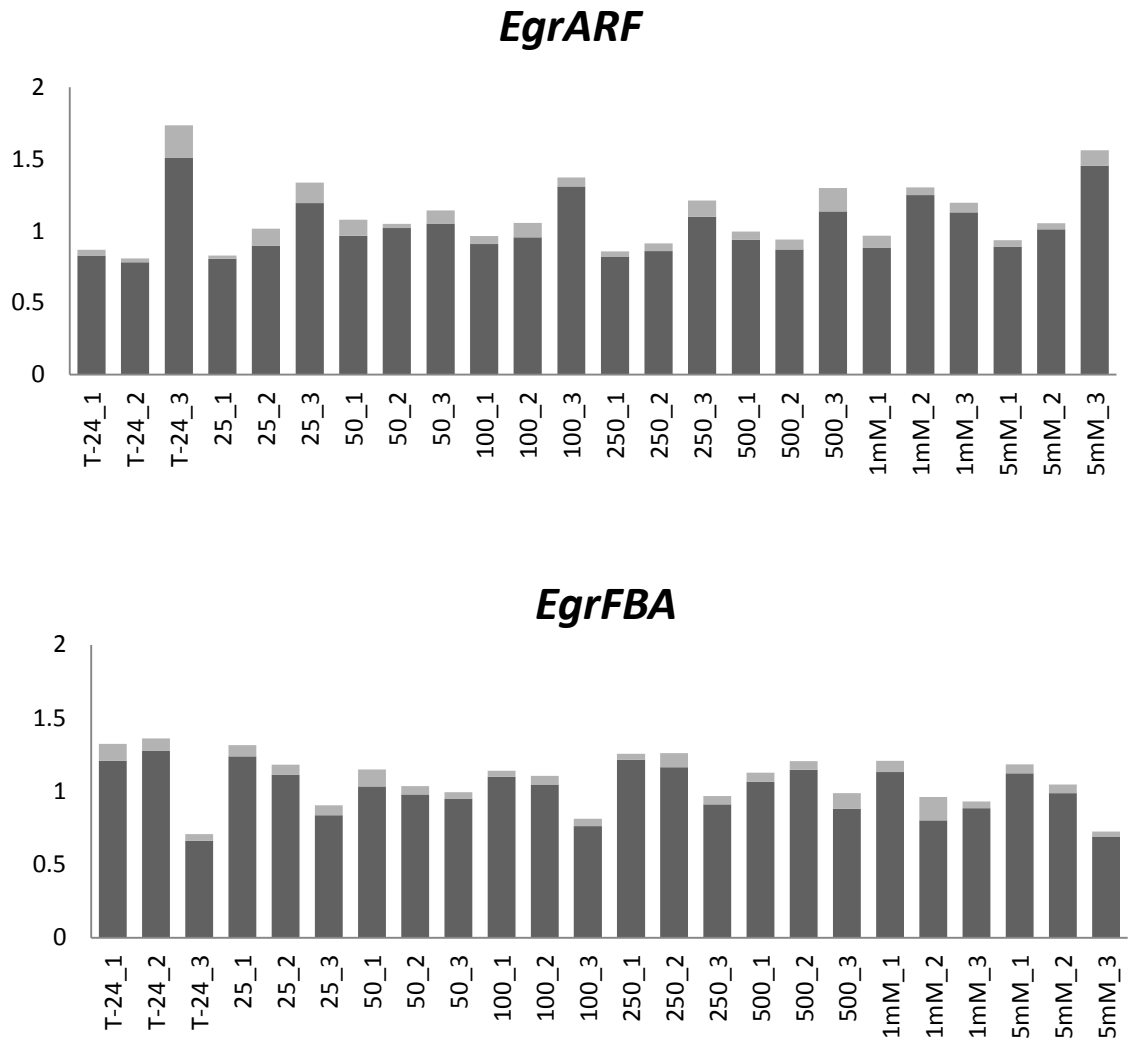

Figure S22: Expression profiles of reference genes (*ADP ribosylation factor* [*EgrARF*] and *Fructose biphosphate aldolase* [*EgrFBA*]) for the SA dose response experiment. The y-axis represents the relative expression ratios in arbitrary units. The x-axis represents the samples that were analyzed. The light grey segments of the graphs represent the standard error of the mean of the technical replicates (n=3) for each sample. Stability criteria: *EgARF* (M = 0.524; CV = 19.2%) and *EgFBA* (M = 0.52; CV = 17.2%)

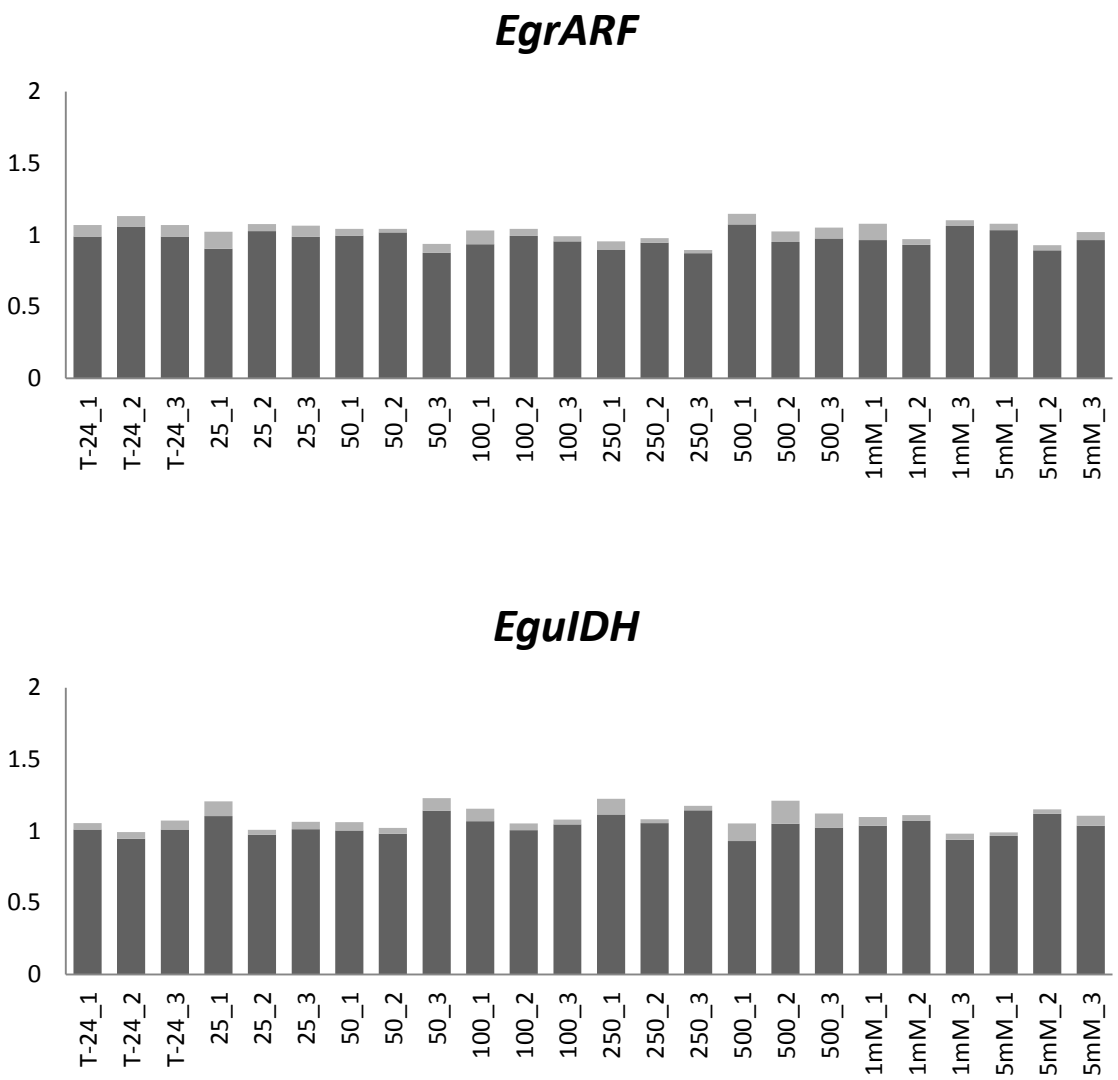

Figure S23: Expression profiles of reference genes genes (*ADP ribosylation factor* [*EgrARF*] and *NADP isocitrate dehydrogenase* [*EguIDH*]) for the MeJA dose response experiment. The y-axis represents the relative expression ratios in arbitrary units. The x-axis represents the samples that were analyzed. The light grey segments of the graphs represent the standard error of the mean of the technical replicates (n=3) for each sample. Stability criteria: *EgARF* (M = 0.295; CV = 10.9%) and *EguIDH* (M = 0.295; CV = 9.6%)

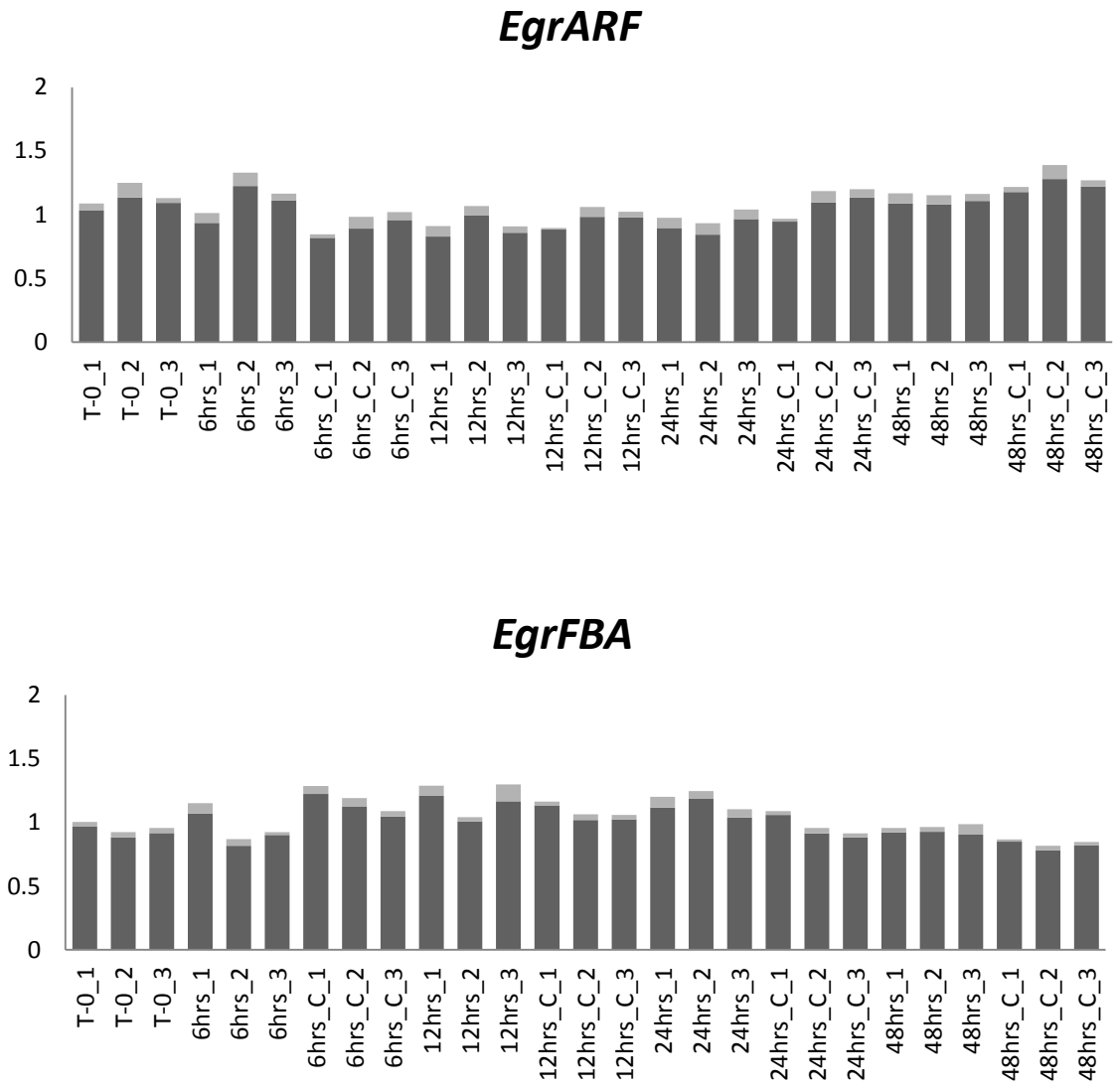

Figure S24: Expression profiles of reference genes (*ADP ribosylation factor* [*EgrARF*] and *Fructose biphosphate aldolase* [*EgrFBA*]) for the SA time course experiment. The y-axis represents the relative expression ratios in arbitrary units. The x-axis represents the samples that were analyzed. The light grey segments of the graphs represent the standard error of the mean of the technical replicates (n=3) for each sample. Samples abbreviated as e.g. 6hrs_C refers to control samples. Stability criteria: *EgARF* (M = 0.371; CV = 12.8%) and *EguIDH* (M = 0.371; CV = 12.9%)

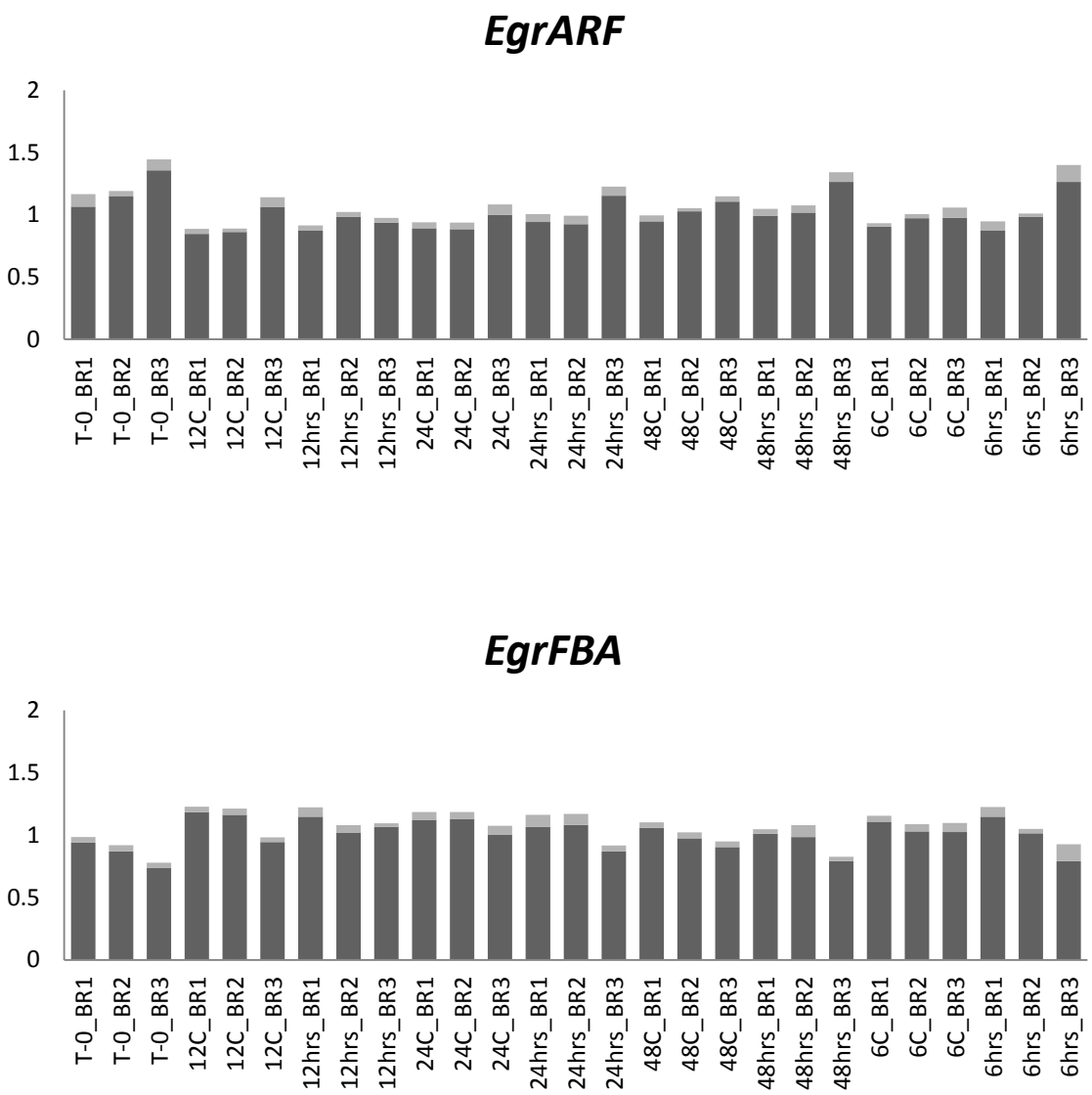

Figure S25: Expression profiles of reference genes (*ADP ribosylation factor* [*EgrARF*] and *Fructose biphosphate aldolase* [*EgrFBA*]) for the MeJA time course experiment. The y-axis represents the relative expression ratios in arbitrary units. The x-axis represents the samples that were analyzed. The light grey segments of the graphs represent the standard error of the mean of the technical replicates (n=3) for each sample. Samples abbreviated as e.g. 6hrs_C refers to control samples. Stability criteria: *EgARF* (M = 0.361; CV = 13.2%) and *EguIDH* (M = 0.361; CV = 11.9%)

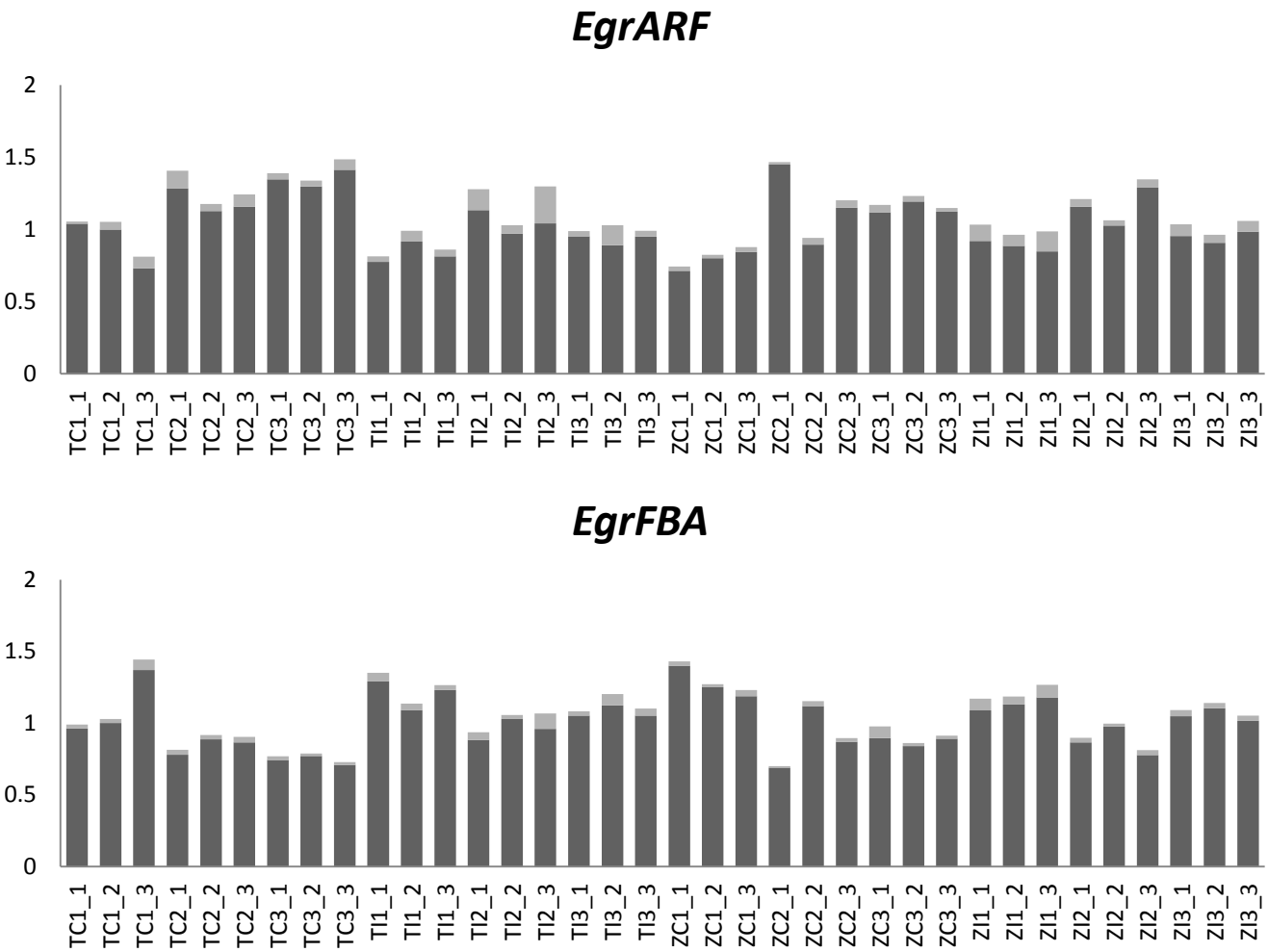

Figure S26: Expression profiles of reference genes (*ADP ribosylation factor* [*EgrARF*] and *Fructose biphosphate aldolase* [*EgrFBA*]) for the infection trial with *C. austroafricana*. The y-axis represents the relative expression ratios in arbitrary units. The x-axis represents the samples that were analyzed. The light grey segments of the graphs represent the standard error of the mean of the technical replicates (n=3) for each sample. Samples are labelled as follows e.g. TC1_1 = TAG5 control time point 1 biological replicate 1. Stability criteria: *EgARF* (M = 0.53; CV = 18.6%) and *EguIDH* (M = 0.53; CV = 18.3%)

Appendix I

Table S2: Amplification efficiencies of the primer pairs designed for the SA and MeJA target genes and reference genes used in RT-qPCR expression profiling experiments.

	Dose Response Trial			Time Course Experiment			*C. austroafricana* infection trial		
	Efficiency (E) [a]	SE [b]	R^2 value [c]	Efficiency (E)	SE	R^2 value	Efficiency (E)	SE	R^2 value
EgrPR1_like	2.5	0.09	0.952	2.5	0.09	0.952	-	-	-
EgrPR2	1.95	0.008	0.993	1.8	0.02	0.974	2.1	0.01	0.969
EgrPR3	2.2	0.05	0.947	2.4	0.05	0.976	-	-	-
EgrPR4	1.9	0.02	0.964	1.8	0.006	0.99	2.07	0.02	0.901
EgrPR5	2.04	0.04	0.92	2.04	0.04	0.92	2.4	0.1	0.863
EgrNPR1	2.1	0.03	0.95	1.7	0.018	0.974	-	-	-
EgrLOX2	2.4	0.03	0.993	2.2	0.05	0.976	-	-	-
EgrLOX3	-	-	-	-	-	-	2.3	0.02	0.964
EgrARF	2.05	0.01	0.992				1.9	0.006	0.996
EgrFBA	2.0	0.04	0.947	1.9	0.006	0.996	1.8	0.009	0.993
EguIDH	1.8	0.005	0.998	-	-	-	-	-	-

[a] Efficiency (E) – PCR efficiency/quality of the primer pair

[b] Standard Error (SE) – Standard error across all samples analyzed in the experiment to obtain E

[c] R^2 value - coefficient of determination (R^2) which is indicative of a linear standard curve and high reaction efficiency. The value R^2 should be close to 1.

Appendix J

Table S3: TBLASTX results of RT-qPCR targets against the TAIR database: Bulked products of each target from each experiment were sequenced and were used as a query for a local BLASTN against the *E. grandis* genome to ensure that the correct scaffold region and corresponding predicted transcript was amplified. Additionally sequences were used as the query for a local TBLASTX against the TAIR v10 database to ensure that the correct target had been amplified. This was done because the *E. grandis* genome is currently not fully annotated in terms of gene function. The results of the TBLASTX analysis are given in the table below.

	AT number	Predicted transcript name	Dose Response experiment	Time Course experiment	*C. austroafricana* infection trial
EgrPR1_like	AT2G14610	*Egrandis*_v1_0.044149	5e-04	5e-04	-
EgrPR2	AT3G57260	GM_*Egrandis*_V1_Scaffold1	7e-14	2e-17	2e-08
EgrPR3	AT3G12500	*Egrandis*_v1_0.019914	2e-32	2e-31	-
EgrPR4	AT3G04720	*Egrandis*_v1_0.030679	4e-11	4e-11	7e-15
EgrPR5	AT1G75040	*Egrandis*_v1_0.051781	4e-08	4e-08	3e-05
EgrNPR1	AT1G64280	N/A*	2e-05	2e-05	-
EgrLOX2	AT1G17420	*Egrandis*_v1_0.012430	6e-12	5e-09	-
EgrLOX3	AT3G22400	*Egrandis*_v1_0.002112	-	-	1e-19

*Primers for *EgNPR1* were obtained from a study conducted by Lourens *et al.* (Unpublished)