Transcriptional responses of *Eucalyptus* clones to the gall wasp, *Leptocybe invasa*

by

Caryn Nicole Oates

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Supervisor: Dr Sanushka Naidoo

Co-supervisors: Prof AA Myburg and Prof B Slippers

Declaration

I, Caryn Nicole Oates, declare that the thesis, which I hereby submit for the degree *Magister Scientiae* at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Caryn Nicole Oates

December 2013

This thesis is dedicated to my parents

Arthur and Mairin Oates

who taught my sisters and I the importance of

hard work, perseverance, discipline and humility.

“If you can fill the unforgiving minute

with sixty seconds worth of distance run

yours is the earth and everything that’s in it”

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Preface

Eucalyptus species constitute some of the most widely grown and economically important hardwood trees in global plantation forestry. This is due primarily to their remarkable growth and adaptability. Much of the initial success of exotic *Eucalyptus* plantations was attributed to the separation from natural enemies. However, there has been a recent increase in the number of introductions of *Eucalyptus* pests and pathogens in these exotic plantations. One such scenario is the spread of *Leptocybe invasa* that is currently described as one of the most devastating pests of global *Eucalyptus* plantations.

Leptocybe invasa (Hymenoptera:Eulophidae) is an Australian gall-inducing wasp that oviposits along immature midribs, petioles and stems. The larvae are endophytic herbivores that cause the development of coalescing galls (abnormal plant growths) and lead to a wide range of symptoms such as stunted growth, die-back and death. In their native environment, populations of *L. invasa* are maintained to almost below observational level; however, once removed from this environment, the pest causes extensive damage in young, susceptible trees. Pesticides are ineffectual against the gall wasp and biological control is considered the key tool in controlling this pest. The molecular interaction between *Eucalyptus* and *L. invasa* is poorly understood and limits the design of biotechnological control measures aimed at reducing losses.

Plants have evolved a complex, multi-layered system of constitutive and inducible defences that protect against pests and pathogens. Results from numerous studies have shown that there is extensive overlap in the response of plants to a wide variety of stresses. This means that it is possible to develop a hypothetical model of the response of *Eucalyptus* to *L. invasa* by incorporating results from studies investigating the response of other plant species to insect pests. This model can then be refined as evidence for the target system is obtained. Transcriptomic analyses are commonly used to investigate the plant response to biotic stress and allow for the identification of genes that may be manipulated to improve plant resistance through genetic engineering.

The **aim of this MSc** study was to investigate the transcriptional responses that dictate the defences employed by a resistant *Eucalyptus grandis* clonal genotype against an infestation by *L. invasa*.

Chapter 1 is a review of the literature describing the *Eucalyptus-Leptocybe invasa* interaction. Initially, the importance of *Eucalyptus* and the availability of genomic resources to help describe the interaction are addressed. The current understanding on *L. invasa* biology as well as a motivation for the selection of this system for investigation is also discussed. In this chapter, a model of the interaction is developed through a literary investigation that focuses on plant defence responses to herbivorous insects.

Chapter 2 is a research chapter which addresses the transcriptional changes upon *L. invasa* infestation. The results of the RNA-sequencing analyses are reported and the putative functions of the differentially expressed genes in cellular processes are discussed. Validation of the RNA-sequencing expression values was demonstrated using five target genes. Finally, the aforementioned model of the hypothetical defence mechanisms employed by *E. grandis* is expanded using the biological process categorization of the differentially expressed genes, determined in this study.

The **Concluding Remarks** are included at the end of the thesis and put the results into context of their value in published literature. Implications, limitations in the field and future prospects are also outlined and discussed.

The research findings that encompass this study represent the outcomes undertaken from March 2010 until December 2013 in the Department of Genetics, University of Pretoria under the supervision of Dr S Naidoo and co-supervision of Prof AA Myburg and Prof B Slippers. The following conference presentations were generated based on the results obtained from this study:

Oates C, Myburg AA, Slippers B and Naidoo S. 2011. RNA-Sequencing as a tool to investigate host responses of *Eucalyptus grandis* to *Leptocybe invasa*. Entomological Society of Southern Africa (ESSA) Congress. Bloemfontein, Free State, South Africa (Poster Presentation).

Naidoo S, Naidoo R, Oates C, Wilken F and Myburg AA. 2011. Investigating *Eucalyptus*-pathogen and pest interactions to dissect broad spectrum defence mechanisms. International Union of Forest Research Organizations (IUFRO) Tree Biotechnology Conference. Arraial d'Ajuda, Bahia, Brazil (Poster Presentation by Dr S Naidoo, published online in BMC Proceedings).

Oates C, Myburg AA, Slippers B and Naidoo S. 2011. A hypothetical *Eucalyptus grandis* defence model against *Leptocybe invasa* based on transcriptome sequencing. IUFRO Forest Protection and Entomology Conference. Colonia del Sacramento, Uruguay (Poster Presentation by Prof B Slippers).

Oates C, Myburg AA, Slippers B and Naidoo S. 2012. A hypothetical *Eucalyptus grandis* defence model against *Leptocybe invasa*. South African Association of Botanists (SAAB) Conference. Pretoria, Gauteng, South Africa (Oral Presentation).

Oates C, Myburg AA, Slippers B and Naidoo S. 2012. A transcriptomic point of view: Insights into the incompatible interaction between a gall wasp and its *Eucalyptus* host. South African Genetics Society (SAGS) Conference. Stellenbosch, Western Cape, South Africa (Oral Presentation).

Oates C, Myburg AA, Slippers B and Naidoo S. 2013. The *Eucalyptus grandis-Leptocybe invasa* interaction. IUFRO Tree Biotechnology Conference. Asheville, North Carolina, United States of America (Oral Presentation).

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CHAPTER 1

LITERATURE REVIEW

Host-insect interactions: a *Eucalyptus*-*Leptocybe invasa* perspective

1.1. Introduction

Exotic pests and pathogens are the cause of major devastation in conservation, agriculture and forestry areas around the world. For example, Pimentel *et al.* (2005) estimated that alien species caused damages and losses in excess of $100 billion annually in the United States of America alone. Pests and pathogens that are introduced into new environments often cause more serious damage than in their natural environment due to the lack of natural enemies and other population controlling factors (Nasim and Shabbir 2012). In agriculture and forestry, this effect is exacerbated by the fact that these industries frequently deploy limited genotypes or clones with desirable characteristics in order to maximise productivity. One example of this is the worldwide devastation caused by *Leptocybe invasa*, an Australian gall wasp, in *Eucalyptus* plantations.

Various control measures are available to limit the spread and damages caused by invasive species, for example biological control (Clewley *et al*. 2012), pesticides (Hagenbucher *et al*. 2013) and breeding for resistance (Bux *et al*. 2012, Cock *et al*. 2009). However, pests and pathogens are capable of developing resistance to these control strategies (Guedes and Siqueira 2012). Improving the understanding of the molecular interaction between the organisms will highlight manipulable targets that can be used in concert with other control strategies in an effort to curb losses. Furthermore, this approach can also identify similarities and differences in responses of numerous host species to various pests and pathogens thus allowing hypotheses to be drawn regarding plant defences across species. This review introduces the *Eucalyptus* gall wasp, *L. invasa*, and discusses hypothetical defence mechanisms that may be employed by a resistant *Eucalyptus grandis* host through comparisons with other plant-insect interactions.

1.2. *Eucalyptus* genomic resources as a platform for describing host-pest interactions

The Myrtaceae is a family of angiosperm plants comprising numerous ecologically and economically important species (Hanegraaf *et al*. 1998). *Eucalyptus* is a member of the myrtales and constitutes some of the most widely grown plantation trees in the world, due primarily to their

extraordinary growth and adaptability (www.git-forestry.com). These hardwood trees are mainly used in the pulp, paper and timber industries (www.git-forestry.com). Furthermore, *Eucalyptus* has been identified as a genus that shows potential for the production of biofuels and for this reason the United States (US) Department of Energy (DOE) Joint Genome Institute (JGI) has recently sequenced the *E. grandis* genome (www.jgi.doe.gov/, Myburg *et al.* unpublished). The sequencing and release of the *Eucalyptus grandis* genome (www.phytozome.com) provides one of the most valuable resources for understanding *Eucalyptus* and tree biology.

The expansion of the *Eucalyptus* forestry industry has led to the development of several genomic resources and breeding technologies (Grattapaglia *et al.* 2012). The *Eucalyptus* community has collected extensive omics data; including genomics, transcriptomics, proteomics and metabolomics; and developed novel tools that provide an unprecedented opportunity to identify and exploit desirable traits in plantation forestry. Although, the primary aim of the current genomic research is to improve characteristics such as wood density, these tools may also be used as a means to understand and improve the plant's resistance to various biotic and abiotic factors (Alves *et al*. 2010, Rosa *et al*. 2010).

In terms of plant-pest and plant-pathogen interactions, transcriptomics are commonly used to elucidate the responses of each participant (Zhu *et al*. 2013, Rawat *et al*. 2012, Xu *et al*. 2011, Liu *et al*. 2007). Studies such as these highlight candidate genes that can be used to genetically modify plant species in order to improve resistance, for example Santamaria *et al*. (2012) demonstrated that pyramiding genes encoding peptidase inhibitors in *A. thaliana* improved plant resistance against *Tetranychus urticae* (spider mite). These applications have yet to be deployed in *Eucalyptus*; however, as omics data accumulates our understanding of the defensive capability of these trees will expand. This information, in conjunction with the improvement of other techniques, such as genetic transformation (Deepika *et al*. 2011), will provide a robust means for improving resistance traits of *Eucalyptus*.

1.3. *Leptocybe invasa*

Leptocybe invasa Fisher and La Salle (Hymenoptera: Eulophidae) is a gall-forming pest of numerous *Eucalyptus* species (Dittrich-Schröder *et al.* 2012, Nyeko *et al.* 2009). This pest was first described in Israel in 2000 following the extensive damage it caused to *Eucalyptus* trees in this country (Mendel *et al.* 2004). This pest has since spread to several countries in Africa, the Mediterranean Basin, South East Asia, Europe and South America (Mendel *et al.* 2004, Kim *et al.* 2009, Nyeko *et al.* 2009, Thu *et al.* 2009, Kumari *et al.* 2010). The insect's hosts appear to be restricted to three sections of the *Eucalyptus* genus, namely the Maidenaria, Transversaria and Latoangulatae (Mendel *et al.* 2004). This is of particular concern because these sections comprise the dominant species in global *Eucalyptus* forestry (Grattapaglia and Kirst 2008). This, coupled with the speed with which the wasp has spread and the extent of the damage it can cause, clearly demonstrate the need to understand this pest in order to develop preventative measures against it.

1.3.1. Biology of *Leptocybe invasa*

Leptocybe invasa is a small (1.1-1.4 mm in length) black or brown wasp that is capable of surviving a wide range of environmental conditions (Mendel *et al.* 2004, Nyeko *et al.* 2009). It has a thelytokous reproductive system (produces females) and mulivotonous development (multiple generations per season), characteristics that may have contributed to its successful dispersal and rapid population growth (Mendel *et al.* 2004, Nyeko *et al.* 2009). Kumari *et. al.* (2010) noted the occurrence of a small number of male wasps in the population; however, males are only found in certain environmental conditions and their role in the population is not known. The wasps show a distinct preference for oviposition in immature leaf and stem tissue (Mendel *et al.* 2004). The larvae are endophytic herbivores whose presence leads to the development of coalescing galls in susceptible trees (Mendel *et al.* 2004). These galls develop through stages while the larvae grow and mature into adults over a period of 5 months (Figure 1) (Mendel *et al.* 2004). Galling by *L. invasa* may present a broad range of symptoms in susceptible hosts, including stunted growth, die-

back and death in severe cases (Nyeko *et al.* 2009, Thu *et al.* 2009, Kumari *et al.* 2010). However, the exact mechanism of gall development remains poorly understood.

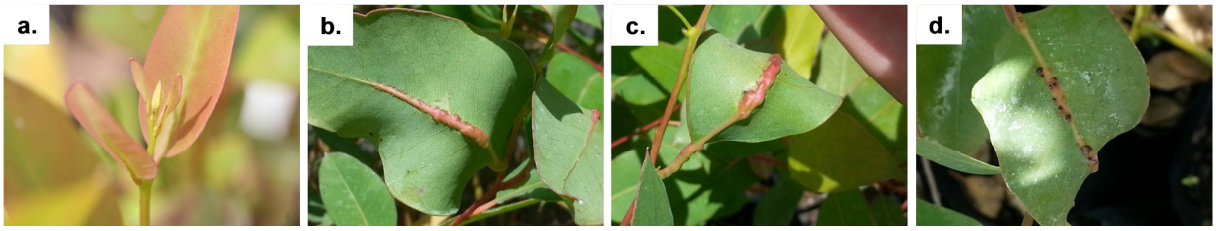

Figure 1. Progression of *Leptocybe invasa* gall development from oviposition to emergence. (a) A cork scar develops at the site of oviposition within 14 days. (b) The typical bump shape of the gall develops. The size of the gall is determined by the number of *L. invasa* larvae developing within (Thu *et al.* 2009). (c) The galls coalesce as they mature. (d) Emergence holes are seen where adult wasps have emerged.

A study by Nyeko *et al.* (2009) showed that hot, dry areas show a marked increase in the frequency and severity of *L. invasa* attacks. Pupae and larvae inhabiting mature galls are capable of undergoing diapause (suspended development during unfavourable environmental conditions) during winter months and emerge the following spring, when average temperatures have reached 20℃ or higher (Mendel *et al.* 2004, Dittrich-Schröder *et al.* 2012). Nyeko *et* al. (2009) also found that *L. invasa* infestations were more prevalent in hot, dry areas and that there was a negative correlation between infestation prevalence and altitude.

1.3.1.1. Gall Development

The interactions between herbivorous insects and plants may be described as generalised or specialised. Generalists make use of a wide variety of plant species, whereas specialists utilise a single species or a few, closely related species (Ali and Agrawal 2012). The ability to induce galls is a specialised feeding behaviour amongst arthropods that requires a tight evolutionary relationship between the gall-inducer and its host plant and has been adopted by species of

aphids, coleopterans, mites, midges, wasps, flies, lepidopterans, and thrips (Raman 2007, Fernandes *et al.* 2008, Inbar *et al.* 2010, Oliveira and Isaias 2010).

Galls are abnormal plant growths that provide a favourable environment for the development of the galling insect (Oliveira and Isaias 2010, Inbar *et al.* 2010, Raman 2007). These endophytic herbivorous insects induce gall development, presumably through salivary or mandibular stimuli that allows the insect to assume control of the host's cellular machinery (Pitino and Hogenhout 2013). The insect is able to manipulate the plant to such an extent that the plant's physiology, morphology, anatomy, development and chemistry are altered in favour of the pest (Tooker *et al.* 2008, Oliveira and Isaias 2010, Inbar *et al.* 2010). Furthermore, the insect is capable of avoiding or actively suppressing the host's immune system, thus reducing its exposure to toxic chemicals and preventing the release of volatile compounds that may trigger indirect defences (Tooker *et al*. 2008). The galls act as incubators for the developing arthropods, providing them with high-quality nutrients and protection from biotic and abiotic factors (Raman 2007, Fernandes *et al.* 2008, Tooker *et al.* 2008, Oliveira and Isaias 2010, Inbar *et al.* 2010).

The majority of our knowledge on the molecular mechanism of gall development has been collected through studies on the Hessian fly-wheat interaction, which is considered to be the model for galling insects and their host plants (Stuart *et al.* 2012). It has been shown that the Hessian fly and wheat share a gene-for-gene interaction, a situation in which the host may show resistance or susceptibility to the pest depending on whether plant resistance (R) proteins recognise insect effectors (Jones and Dangl 2006). A transcriptomic study of the Hessian fly salivary gland by Chen *et al*. (2004) has revealed numerous secreted salivary gland proteins that show no sequence similarity to any known proteins and are alleged to be effector molecules. An effector is a molecule with specific host targets that may allow the attacker to undermine the host's immune system and modulate the cellular processes (Deslandes and Rivas 2012). This hypothesis about the Hessian fly–wheat interaction is supported by studies regarding effector biology of various aphid species and the interactions with their hosts (Rodriguez and Bos 2013).

A study by Oliveira and Isaias (2010) describes the histological development of midrib galls through tissue redifferentiation, a process by which novel cell types with specialised functions are formed following a change in cell identity (Figure 2). This interaction involved an undescribed species of Cecidomyiidae (Diptera) on the diesel tree, *Copaifera langsdorffii*, and provides an example of the complexity of the interactions between the gall-inducer and its host plant.

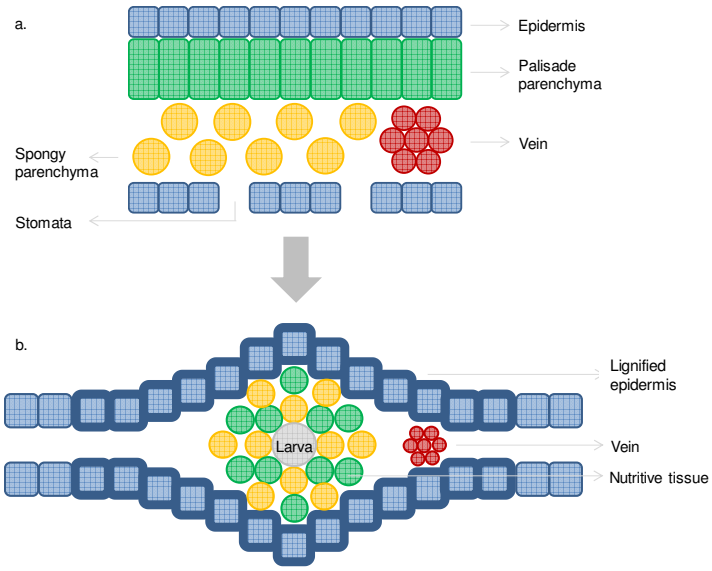

Figure 2. Simplified diagram of the process of tissue redifferentiation in a susceptible plant-galling insect interaction. (a.) The basic anatomy of a leaf. (b.) The larva assumes control of the host cellular machinery and induces the formation of gall-specific tissues. These include a heavily lignified epidermis that provides protection and a stable microenvironment as well as a nutritive tissue upon which the larva feeds.

In general, the relationship between gall-inducers and their host plants has received little attention and remains a poorly understood concept. The mechanisms behind gall initiation and development by the *Eucalyptus* gall wasp, *L. invasa*, have not been investigated in detail since its discovery. Hypotheses regarding the mechanisms of *L. invasa* gall formation and identification of manipulable targets for improving host resistance can be developed from studies investigating other galler-plant interactions such as those described above.

1.3.2. Control Strategies

Chemical control is commonly used to control populations of invasive arthropods; however, Kulkarni (2010) showed that the spread of *L. invasa* galls was not affected by applications of insecticides. Classical biological control is considered a key tool to manage foreign insect pests due to the feasibility of using it over large areas and its lower environmental impact (compared to chemicals) (Wingfield *et al.* 2008, Kim *et al.* 2009, Kulkarni *et al.* 2010). In addition, identifying and breeding resistant *Eucalyptus* genotypes may significantly improve pest and disease avoidance by the vegetative propagation of desirable hybrid trees (Wingfield *et al.* 2008). In the case of *L. invasa*, the lack of information regarding the molecular interaction between the gall wasp and its host has limited the development of biotechnological and breeding strategies aimed at reducing losses caused by this pest.

The severity of the damage caused by the *Eucalyptus* gall wasp warranted a search for its natural enemies (Kim *et al.* 2009). In Australia, the population density of *L. invasa* is presumably controlled by the wasps' natural enemies; to almost below observation level (Rocha *et al*. 2013). Two species of Tetrastichinae (Eulophidae), namely *Quadrastichus mendeli* and *Selitrichodes kryceri* were reported to be native parasitoids of *L. invasa* (Kim *et al.* 2009). These hymenopterans were introduced into Israel as the primary biological control agents against the destructive gall wasp (Kim *et al.* 2009). Kelly *et al*. (2012) identified and described an additional parasitoid of *L. invasa*, named *Selitrichodes neseri*. This wasp has been released in South Africa in an effort to curb the losses inflicted by *L. invasa* (Dittrich-Schröder *et al.* 2012). Biological control of a *Eucalyptus* gall wasp has been successful in the past. *Closterocerus chamaeleon* was used against the *Eucalyptus* gall wasp, *Ophelimus maskelli*, and appears to be effectively managing the spread of this pest (Protasov *et al*. 2008). It appears as though the use of biological control agents is a viable management option for *L. invasa*.

Identifying and breeding resistant *Eucalyptus* genotypes may be used as in addition to biological control in the effort to control *L. invasa* (Basavanagoud *et al.* 2010). Variations in susceptibility and

resistance have been observed between and within species suggesting that resistant germplasms may be a feasible option for diminishing the effect of *L. invasa* (Nyeko *et al.* 2009, Thu *et al.* 2009, Dittrich-Schröder *et al.* 2012). Vegetative propagation of desirable genotypes means that new plantations may be established containing only trees with the selected genotype (Wingfield *et al.* 2012). By combining information from the wealth of data that has been produced for *Eucalyptus* with new recombinant DNA techniques, the identification of resistant planting stock and the development of new resistant lines are becoming plausible options for future plantation forestry.

1.4. Plant Defence Responses

In this section, a hypothetical model of the *Eucalyptus* inducible defence response against *L. invasa* is developed through studies spanning numerous plant species. It must be noted that not all plant lineages possess all described defence mechanisms. Relatively little is known about the resistance mechanisms employed by *Eucalyptus* species to overcome pests and pathogens. However, literary evidence for a number of general defences have been described for this genus. This section aims to highlight broad defence mechanisms that have been described in a variety of plant species that are likely to be used by *Eucalyptus* during this interaction although the details are likely to be different between species. For example, secondary metabolites are ubiquitous within plants but their use, type and composition differ between species (Moore *et al*. 2013).

Plants have evolved a complex, multi-layered system of direct and indirect defences against destructive pests and pathogens. The direct responses include physical or chemical barriers possessing toxic, anti-xenotic (repellant) or anti-nutritive properties (Fürstenberg-Hägg *et al*. 2013). The indirect responses offer protection by interacting with the pest's natural enemies as well as priming defences to defend against future herbivory (Unsicker *et al*. 2009, Troncoso *et al*. 2012). Furthermore, plant defences can be divided as constitutive and inducible, both of which can be further divided into mechanical and chemical components. These mechanisms or a combination thereof, determine the resistance or susceptibility of a plant species to a particular attacker.

The constitutive defence forms the plant's first line of resistance and provides generalised protection against most potential attackers (Fürstenberg-Hägg *et al*. 2013). Mechanical constitutive defences are associated with the plant's normal anatomical features, such as waxes covering *Eucalyptus* leaves have been shown to reduce insect herbivory (Jones *et al*. 2002). The chemical constitutive defences include pre-existing toxic or antixenotic compounds, for example a phytoanticipin such as nicotine (Kohler *et al.* 2011). Once these preluding defences have been compromised, the inducible responses can be activated. The inducible plant defence response is a multifaceted, broad-spectrum system that is sequentially activated following invader recognition (Figure 3) (Zhao *et* al. 2005, Fürstenberg-Hägg *et al*. 2013). The induced defence signal can be transmitted throughout the plant through systemic acquired resistance (SAR) to protect against future attack (Fu and Dong 2013). A plant is able to differentiate between different modes of attack, allowing it to select the most appropriate response to the stress (Major and Constabel 2006, Troncoso *et al.* 2012). The selected response is regulated by an intricate cross-talk between signalling pathways. The induced defence leads to the activation of downstream responses including phytoalexin production, defence-related protein synthesis and barrier reinforcement (Zhao *et* al. 2005, Fürstenberg-Hägg *et al*. 2013).

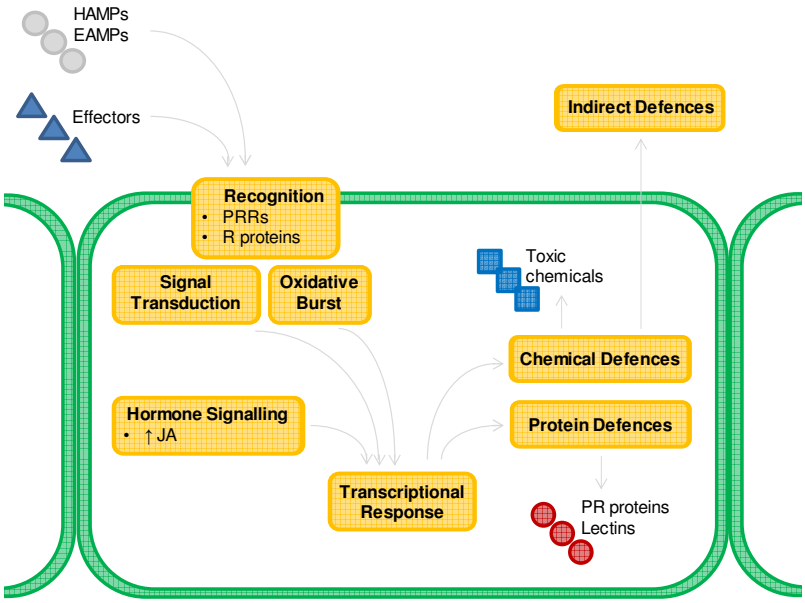

Figure 3. Hypothetical model of the putative induced defences that could be employed by a resistant *E. grandis* against *L. invasa* oviposition based on literary evidence. The induced defences are activated following recognition of insect effectors, herbivore- or egg-associated molecular patterns (HAMPs, EAMPs). The recognition signal is transferred through the cell by a number of signalling pathways resulting in transcriptional modifications that activate defence mechanisms. The response is modulated by hormone-signalling pathways, particularly jasmonic acid (JA). These defences include the production of toxic chemicals and defence-associated proteins such as pathogenesis-related (PR) proteins.

This system of broad-spectrum and specific resistance provides a durable means of protection to plants against an extensive number of pests and pathogens. These mechanisms can be combined into an intricate and tightly-regulated network to ensure that the most appropriate response is elicited. It is imperative that the plant responds effectively and efficiently, particularly in a situation where the pest can manipulate its host as extensively as a galling insect can.

1.4.1. Pest Perception

In order for a plant to elicit defence responses, the plant must first recognise the presence of an attacker. This recognition may be specific or non-specific, resulting in effector-triggered immunity (ETI) or pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), respectively (Jones and Dangl 2006). The elicitation of plant defences is dependent on the surveillance capabilities of each individual plant cell. Pest perception might be considered the most important step in the activation of the inducible defence mechanisms.

Non-specific recognition is initiated through the interaction of PAMPs with plant transmembrane, pattern recognition receptors (PRRs) and results in PTI (Jones and Dangl 2006). A number of studies have identified herbivore- and egg-associated molecular patterns (HAMPs and EAMPs) from the salivary and oviposition fluids of different insect species, including fatty acid-amino acid conjugates (FACs), inceptins, caeliferins and bruchins (Alborn *et al.* 1997, Doss *et* al. 2000, Alborn *et al.* 2007, Yoshinaga *et al.* 2010). These molecules are similar to PAMPs in that they play important roles in insect metabolism and thus evolve relatively slowly, making them good markers for general herbivore recognition by plant cells (Alborn *et al.* 2007). Plant perception of HAMPs has shown similar defence responses to PTI. Gouhier-Darimont *et al.* (2013) showed that the application of *Pieris brassicae* (large white butterfly) egg extract to *Arabidopsis thaliana* leaves shows a similar, but not identical, response to PTI elicited by *Pseudomonas syringae* infection.

PTI may in turn be suppressed by the pathogen through the release of specific effectors thus resulting in effector-triggered susceptibility (ETS) (Jones and Dangl 2006). Microbial effectors suppress PTI by targeting PRRs or other proteins that play important roles in various defence mechanisms (Deslandes and Rivas 2012). A number of studies have shown that insects may use effectors in a similar manner (Pitino and Hogenhout 2013). For example, and as previously discussed, the Hessian fly has been shown to produce numerous secreted salivary gland proteins that are hypothesized to be effectors (Chen *et al*. 2004). The use of effectors in a galling insect-plant interaction is plausible considering that the insect would suppress its host's immune system

and assume control of the cellular machinery in order to create the gall. To counter defend ETS, the plant immune system then relies on resistance (R) proteins to detect specific effectors and activate effector-triggered immunity (ETI) (Jones and Dangl 2006).

Specific recognition involves the interaction of attacker effectors with plant R proteins resulting in ETI (Jones and Dangl 2006). R proteins can identify the presence of an attacker either directly or indirectly. The indirect recognition, also known as the guard interaction, involves a R protein that identifies effector-modified "self" molecules, as has been demonstrated during the interaction between *A. thaliana* and *P. syringae* (Mackey *et al*. 2003). The gene-for-gene interaction involves the direct interaction between the R protein and an effector. The Hessian fly was the first insect hypothesized to have a gene-for-gene interaction with its host (Hatchett and Gallun 1970). Supporting evidence for this hypothesis was later described when the tomato *Mi R* gene was shown to confer resistance to whiteflies and potato aphids (Rossi *et al*. 1998, Nombela *et al*. 2003). Subsequently, numerous plant species were shown to have a gene-for-gene interaction with certain insect pests, including wheat and the Hessian fly (Gururani *et al*. 2012).

1.4.2. Signal Transduction

In order for a plant to launch a defence response, the recognition of the attacker must be linked to the downstream responses. This is achieved through a signal transduction network that includes calcium ion flux and protein kinase cascades (Tena *et al*. 2011). These early signalling events are not well understood but have been shown to be involved in plant-insect interactions.

A cytosolic increase in calcium ion concentration is an important early event in signal transduction (Tena *et al*. 2011). Feeding by *Spodoptera littoralis* (Egyptian cotton worm) on *Phaseolus lunatus* (lima bean) caused a temporary increase in cytoplasmic Ca^{2+} in cells adjacent to the insect bite (Maffei *et al*. 2004). This temporary increase in Ca^{2+} levels activate calmodulin and other calcium-sensing proteins that subsequently promote downstream signalling events, including

phosphorylation and transcriptional responses (Howe and Jander 2008, Ma and Berkowitz 2011, Tena *et al*. 2011).

MAPK cascades function through a phosphorelay system that links upstream receptors to downstream targets (Pitzschke *et al*. 2009). Activated (phosphorylated) MAPKs can phosphorylate downstream targets that mainly include transcription factors, such as WRKYs, that in turn regulate various defence mechanisms (Pitzschke *et al*. 2009). For example, a study by Kandoth *et al*. (2007) showed that co-silencing of the tomato MPK1 and MPK2 weakens the proteinase inhibitor-associated defence against the specialist herbivore, *Manduca sexta* (tobacco hornworm). These signalling cascades, along with various other cellular signalling networks such as the phytohormones, induce feedback mechanisms that allow fine regulation during defence responses (Arimura *et al*. 2011).

1.4.3. Oxidative Burst

The oxidative burst is an early plant defence response against pathogens and pests (Gayoso *et al*. 2010). It involves the accumulation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-) and the hydroxyl radical (OH^-). The role of ROS in plant defence has been extensively reviewed in literature, for example Sharma *et al*. (2012), O'Brien *et al*. (2012) and Kerchev *et al*. (2012). ROS are highly reactive compounds that cause cellular oxidative stress (Sharma *et al*. 2012). The oxidative burst can cause damage through reactions with important biomolecules such as lipids (Singh *et al*. 2009). During the oxidative burst, ROS can be as detrimental to the plant cell structures as to the attacker's. Up-regulation of plant genes encoding anti-oxidant enzymes, such superoxide dismutase and catalase, is frequently observed during this response (Liu *et al*. 2010). Additionally, ROS are also involved in other defence responses including signal transduction and elicitation of the hypersensitive response amongst others (Sewelam *et al*. 2013, Radville *et al*. 2011).

The oxidative burst has been described in plant-insect interactions. Resistant wheat was shown to produce ROS in response to Hessian fly infestation (Mittapalli *et* al. 2007, Liu *et al*. 2010). Anti-oxidant gene transcripts increase in the insect midgut in order to reduce the oxidative stress produced by plant ROS (Zhang *et al*. 2010). Feeding of larvae on resistant plants can have dramatic effects, for example Shukle *et al*. (2010) showed complete destruction of the intestinal villi in Hessian fly feeding on resistant wheat, owing to ROS and other toxic compounds and leading to antibiosis. This and other studies highlight the importance of the oxidative burst in plant defence against insects.

1.4.4. Phytohormone-Mediated Signalling Pathways

Plant hormones play a central role in the regulation of developmental and immunological processes (Pieterse *et al*. 2012). Plants produce a number of hormones including the main defence-associated hormones jasmonic acid (JA) and salicylic acid (SA). The phytohormones are connected in an intricate communication network that allows the plant to modulate responses to different biotic and abiotic stresses (Pieterse *et al*. 2012).

1.4.4.1. Jasmonic Acid

A recent review by Wasternack and Hause (2013) discusses the biology of JA and its role in various cellular pathways. JA has been shown to be the dominant hormone regulating resistance against necrotrophic pathogens and phytophagous insects (Erb *et al*. 2012). Schmelz *et al*. (2009) showed that a variety of HAMPs were able to activate the JA-mediated signalling pathway. This result highlights the importance of JA in regulating general defence against phytophagous insects. Activation of the JA pathway affects a number of downstream defence mechanisms. For example, the function of JA in defence against insects was originally proposed by Farmer and Ryan (1992). This study provided evidence that JA and several precursors induce the expression of proteinase inhibitors upon wounding. Other examples include a study by Bruinsma *et al*. (2009) that described how feeding by *Pieris rapae* (cabbage white butterfly) and *Plutella xylostella* (diamondback moth)

led to the induction of the JA pathway as well as JA-induced volatile release that attracted parasitoids of the herbivores.

1.4.4.2. Salicylic Acid

Boatwright and Pajerowska-Mukhtar (2013) have recently reviewed the biology of SA and its role in various cellular pathways. SA is synthesized by the shikimate-phenylpropanoid pathway via two routes, one requires cinnamic acid and the other isochorismate production. SA synthesized from isochorismate has recently been shown to be essential for the elicitation of systemic acquired resistance (SAR). SA is considered to be the dominant hormone regulating defence against biotrophic pathogens and is crucial for the initiation and maintenance of SAR (Pieterse *et al*. 2012). SAR generates a mobile signal that boosts broad-spectrum defence in distal parts of the plant to protect against secondary infection (Fu and Dong 2013). SA can be present in several conjugated forms, for example conjugations with certain amino acids have been shown to activate defence responses. A number of receptors have been identified that are important for SA signalling including NPR1 (non-repressor of pathogenesis-related 1), NPR3, NPR4 and NPR1-like proteins (Boatwright and Pajerowska-Mukhtar 2013). While JA has been described as the dominant hormone mediating plant resistance against insect herbivores, this role can also be taken by SA. For example, Ollerstam and Larsson (2003) described a rapid, salicylic acid (SA)-mediated HR in *Salix viminalis* (basket willow) that is resistant to the gall midge, *Dasineura marginemtorquens*.

1.4.4.3. JA-SA Interactions in Defence Against Insects

The antagonistic relationship between JA and SA has been demonstrated in many plant species including *Eucalyptus* (Naidoo *et al*. 2013). This relationship is thought to provide the plant with a means to prioritise one pathway over another in order to elicit the most appropriate response (Pieterse *et al*. 2012). However, this antagonism also provides the opportunity for some attackers to manipulate the hormonal responses. For example, Bruessow *et al*. (2010) showed that *P.*

brassicae eggs on *A. thaliana* hijacked the SA signalling pathway in order to inhibit the JA pathway and JA-mediated defences.

Evidence is now accumulating that shows that the relationship between SA and JA pathways is more complex than previously described. Numerous studies have described the induction of both the SA and JA signalling pathways in response to insect herbivory. For example, Abe *et al*. (2008) showed that *Frankliniella occidentalis* (Western flower thrips) feeding on *A. thaliana* induced the expression of marker genes of the JA, JA-ethylene and SA pathways. Furthermore, results from investigations into compatible interactions between plants and some insect species show that both the SA and JA pathways are suppressed. This was observed during Hessian fly feeding on susceptible wheat and the Asian rice gall midge feeding on susceptible rice (Rawat *et al* 2012, Tooker and De Moraes 2011).

1.4.4.4. Crosstalk between hormone pathways

It is becoming increasingly evident that crosstalk between the hormone-mediated signalling pathways is crucial for fine-tuning responses to various stresses. Ethylene is an important modulator of plant defence and has been shown to act synergistically and antagonistically with SA and JA in many plant-pathogen and plant-insect interactions (Pieterse *et al*. 2012). Plants produce a number of hormones that have also been shown to be important components of eliciting defences. For example, Coppola *et al*. (2013) described an interaction between *Solanum lycopersicum* (tomato) and *Macrosyphum euphorbiae* (potato aphid). SA was shown to be the dominant hormone regulating defence; however, the response was dependent on communication between the SA, JA, ethylene and brassinosteroid signalling. Abscisic acid is known to be an important hormone in regulating plant defence against abiotic stress and has recently been described as an important component of plant defence against insects (Ton *et al*. 2009). For example, Dinh *et al*. (2013) described how the interaction between ABA and the JA pathway allows *Nicotiana attenuata* to mount a complete response against *M. sexta*. The intricate communication

between the various phytohormone pathways provides the plant with powerful regulatory potential that allows it to respond to a wide range of stresses.

1.4.5. Defence-related Chemical Production

Plants are able to produce a wide range of secondary metabolites to defend themselves against insect herbivory. These compounds are divided into numerous groups that include cyanogenic glucosides, phenolics and terpenoids amongst others (Fürstenberg-Hägg *et al*. 2013). *Eucalyptus* species also produce formylated phloroglucinols that are unique to the genus (Eschler *et al*. 2000). Secondary metabolites have a number of uses in defence including direct entomotoxicity, barrier reinforcement and indirect defences, the latter being discussed in further detail in the subsequent section.

Secondary metabolites that show insecticidal activity generally target specific insect biological systems such as the nervous and digestive systems (Fürstenberg-Hägg *et al*. 2013). These compounds are normally stored in an inactive state to prevent self-toxicity (Mithöfer and Boland 2012). Herbivore damage exposes the inactive compounds to hydrolyzing enzymes that release the active derivatives. For example, cyanogenic glucosides are hydrolysed by β-glucosidases upon feeding thereby releasing hydrogen cyanide (HCN) from the molecule which in turn inhibits the mitochondrial respiratory pathway of the insect (Bond 1961).

Secondary metabolites also participate in other defence mechanisms such as barrier reinforcement that reduce the herbivore's ability to penetrate the plant. Plants may impede insect herbivory through cell wall reinforcement, which is predominantly achieved through lignification of the cell walls as well as deposition of other compounds such as suberin and callose (Fürstenberg-Hägg *et al*. 2013). This defence mechanism has been recorded as a response to Hessian fly larval feeding on wheat (Liu *et al*. 2007). Some studies have described the ability of galling insects to suppress the expression genes encoding proteins that contribute to lignification thus highlighting the importance of this response (Rawat *et al*. 2012). It must be noted that plants may employ a variety

of different mechanisms to induce this defence response, for example *Pisum sativum* (pea) creates a mound of undifferentiated cells beneath eggs of *Bruchus pisorum* (pea weevil) that prevent entry into the pod (Doss *et al*. 2000).

1.4.6. Indirect Defences

As with the direct defences, the indirect defence response may be divided into constitutive and inducible defences. This response is controlled by the release of substances that allow the plant to interact with the pest or its predators and parasitoids (Kessler and Heil 2011). The constitutive indirect responses may involve a distinct blend of secreted compounds that have an antixenotic effect or provide some resource to predators of the herbivores. For example, *Acacia collinsii* invests in extrafloral nectaries to attract *Pseudomyrmex spinicola* ants that in turn protect the plant from herbivores and competitors (Fiala and Maschwitz 1994). The inducible indirect defences provide information to the herbivores' natural enemies about the location and identity of their prey in the form of altered visual and olfactory cues (Kessler and Heil 2011). For example, Bruinsma *et al*. (2009) showed that feeding by *P. rapae* and *P. xylostella* on *Brassica oleracea* resulted in JA-induced volatile release that attracted parasitoids of the herbivores. Furthermore, induced indirect defences have been shown to cause defence priming in neighbouring, unaffected plants. Troncoso *et al*. (2012) described this phenomenon in *E. globulus* following attack by *Ctenarytaina eucalypti* (bluegum psyllid) that lead to the production of secondary metabolites in neighbouring plants.

The indirect response has also been shown to be an important defence mechanism in plant-galling insect interactions. Damasceno *et al*. (2010) showed that feeding by unidentified galling psyllids caused a change in the volatile profile of *Schinus polygamous* (peppertree) and *Baccharis spicata*. Furthermore, galling insects have been shown to actively suppress the indirect responses of their hosts in susceptible interactions (Tooker and De Moraes 2007, Tooker and De Moraes 2008). Improved control measures can be developed by utilising information that involves all three trophic levels as well as inter- and intra-plant communication.

1.4.7. Defence-Related Protein Production

The production of defence-associated proteins is an important inducible response in plant resistance against insect herbivores. These proteins promote resistance through anti-feedant activity, either reducing nutrient quality or blocking nutrient uptake, in the insect digestive system (Fürstenberg-Hägg *et al*. 2013). Numerous classes of proteins have been shown to be involved in plant defence against insects including a number of pathogenesis-related (PR) proteins and others such as lectins. This section will only describe the role of three protein classes in plant resistance to insects namely proteinase inhibitors, chitinases and lectins.

The insect gut contains a variety of proteases that break down proteins present in their diet and thus provide amino acids for insect growth and development. Proteinase inhibitors act by blocking these digestive enzymes thereby reducing the ability of the insect to digest plant material (Bode *et al*. 2013). The up-regulation of proteinase inhibitor-encoding genes and the synthesis of proteinase inhibitors in order to defend against insect herbivory have been described in numerous plant-insect interactions (Sinha *et al*. 2011, Hartl *et al*. 2010, Bode *et al*. 2013). Descriptions of this response have also been noted for resistance against galling insects, for example Wu *et al*. (2008) showed up-regulation of inhibitor-like genes during an incompatible interaction between wheat and the Hessian fly.

Chitinases are another class of proteins that are known to promote plant resistance against insect herbivores. These proteins act by binding to chitin that is present in the insect midguts thereby blocking nutrient absorption (Major and Constabel 2006, Büchel *et al*. 2012). The expression of chitinase-encoding genes in response to insect feeding has been described in numerous studies. For example, Major and Constabel (2006) observed an up-regulation in the expression of a chitinase-encoding gene in a poplar hybrid (*Populus trichocarpa* x *P. deltoides*) upon feeding by *Malacosoma disstria* (forest tent caterpillar).

Finally, lectins are carbohydrate-binding proteins that appear to act in a similar manner to chitinases by binding to unknown targets in the insect midgut, thus blocking nutrient absorption (Büchel *et al*. 2012). The up-regulation of lectin-encoding genes has been described in numerous plant-insect interactions including a number of galling insects (Liu *et al*. 2007). Furthermore, a number of lectins have been demonstrated to possess broad-spectrum activity, making them good targets for improving resistance across a variety of species of insects (Das *et al*. 2013).

All three of the aforementioned protein classes have been shown to improve plant resistance against a variety of insect species through transgenic studies. Furthermore, these protein classes possess demonstrable broad-spectrum activity, making them suitable candidates for transgenic breeding programs. The importance of these proteins in plant defence has been further highlighted by the fact that a number of studies have shown that insects selectively down-regulate the expression of genes encoding these proteins in compatible interactions.

1.5. Conclusion

Invasive pests and pathogens cause severe losses in global agricultural and forestry industries. The widespread damage to *Eucalyptus* plantations caused by *L. invasa* has highlighted this pest as one of the leading concerns in the *Eucalyptus* industry (Dittrich-Schröder *et al.* 2012, Nyeko *et al*. 2009, MJ Wingfield, personal communication). Many farmers and industries depend on eucalypt plantations for revenue, thus the reduced productivity caused by *L. invasa* will have both social and economic implications (Nyeko *et al*. 2007). In its native habitat, *L. invasa* populations are naturally controlled (Rocha *et al*. 2013). However, once removed from this background, the wasp is capable of causing extensive damage. The speed at which the wasp has spread and the extent of the damage it can cause has created an urgent need to understand this pest and improve preventative measures against it.

To date, biological control and the use of resistant clones or species have been used to control *L. invasa*. However, very little information is available regarding the interaction between *Eucalyptus*

and *L. invasa*. This limits the capacity to further improve these control measures, especially exploiting resistance that is present in *Eucalyptus*. Novel technologies such as RNA sequencing (RNA-Seq) provide a powerful means to address this lack of understanding, by investigating the responses of these plants to *L. invasa* and make inferences regarding defence mechanisms. This thesis aims to investigate the transcriptional responses of a *E. grandis* clone to *L. invasa*. Combined with information gathered from other plant-pest interactions, this information is used to define the key resistance mechanisms employed by the host. Information gathered from this study will be used to identify putative targets that may be used to improve the current control strategies.

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CHAPTER 2

The transcriptome of *Eucalyptus grandis* suggests mechanisms of defence against the insect pest, *Leptocybe invasa*

Caryn N Oates, Alexander A Myburg, Bernard Slippers and Sanushka Naidoo

Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), Genomics Research Institute (GRI), University of Pretoria, Private bag x20, Pretoria, 0028, South Africa

This chapter has been prepared as a manuscript for the research journal Plant New Phytologist. I extracted total RNA from infested and uninfested *E. grandis* and *E. grandis* x *E. camaldulensis* ramets for RNA-sequencing and RT-qPCR. I validated the expression profiles of five target genes. I performed all the data analysis for this study and prepared this manuscript. Dr S Naidoo, Prof AA Myburg and Prof B Slippers provided advice, direction and supervision in the planning of the project. They also provided direction in the interpretation of the results and provided critical revision of the manuscript. Other technical assistance is acknowledged at the end of the Chapter.

2.1. Abstract

Eucalyptus species constitute some of the most widely planted and economically valuable hardwood fibre crops in the world. The Australian blue gum chalcid wasp, *Leptocybe invasa* (Hymenoptera: Eulophidae), is a specialist pest of *Eucalyptus* and is currently one of the most serious threats to plantation forestry. The larvae are endophytic herbivores whose feeding leads to gall formation within host tissues resulting in a range of symptoms including stunted growth and death of severely infested trees. Variation in resistance and susceptibility has been noted across different *Eucalyptus* genotypes and species. To date, there is limited information regarding the mechanism underlying the defence response of these trees to insect pests such as *L. invasa*. We investigated an incompatible interaction between a resistant *Eucalyptus grandis* genotype and *L. invasa*. Transcript profiling using RNA-Seq of midrib tissue from challenged and unchallenged trees revealed 2117 genes with significantly altered expression patterns. Functional annotation of the differentially expressed genes provides support for specific resistance mechanisms being employed by the host. This includes the production of entomotoxic chemicals and proteins, cell wall reinforcement as well as an apparent suppression of host targets that can be manipulated by the insect. This study represents the first transcriptome-wide characterisation of defence responses in *Eucalyptus* to destructive plantation pests such as *L. invasa*.

Keywords: *Leptocybe invasa*, *Eucalyptus*, plant defence, transcriptome, RNA-Seq

2.2. Introduction

Plants have evolved a complex, multi-layered system of direct and indirect defences to protect themselves against phytophagous insects. These include physical or chemical barriers with toxic, repellant or anti-nutritive properties (Mithöfer and Boland 2012). These defences may be further divided into constitutive or inducible types. Constitutive defences form the plant's first line of defence and provide generalised protection against most potential attackers (Fürstenberg-Hägg *et al*. 2013). Once this barrier is compromised, inducible responses have to be activated. The

inducible defences comprise a multifaceted, broad-spectrum system that is sequentially activated following invader recognition (Jones and Dangl 2006, Pieterse *et al*. 2012). The recognition signal is transferred to downstream defence pathways through a sophisticated network that ensures the most appropriate response is elicited (Tena *et al*. 2011). Inducible defences include the oxidative burst, synthesis of secondary metabolites and defence-associated proteins, barrier reinforcement and indirect defences (Fürstenberg-Hägg *et al*. 2013). Suppression of the plant's primary metabolism and cell cycle are additional responses that have been described as responses to various pests (Rawat *et al*. 2012).

Gall-inducing insects include some of the most devastating pests in agriculture and forestry, for example phylloxera on grapes (Nabity *et al*. 2013) and the Hessian fly on wheat (Stuart *et al*. 2012). Galls are abnormal plant tissue structures that are induced and maintained by the insect and provide both nourishment and protection (Inbar *et al.* 2010). Gall development occurs through a process of tissue redifferentiation, defined as the formation of novel cell types with specialised functions following a change in host cell identity (Oliviera and Isaias 2010). The ability of a galler to assume control of its host's cellular machinery is such that the physiology, morphology, anatomy, development and chemistry are altered in favour of the pest (Oliveira and Isaias 2010, Inbar *et al.* 2010, Compson *et al*. 2011). Furthermore, the insect is capable of avoiding or actively suppressing the host's immune system, thus reducing its exposure to toxic chemicals and preventing the release of volatile compounds that may trigger indirect defences (Tooker and De Moraes 2008). The mechanism of gall development remains poorly understood. Work in model systems such as the interaction between *Mayetiola destructor* (Hessian fly) and wheat are starting to unravel this process (Stuart *et al*. 2012). For example, a study of the Hessian fly salivary gland detected numerous secreted salivary gland protein transcripts that allegedly encode effectors and may provide the means for the insect to manipulate its host (Chen *et al*. 2004).

A relatively recently described galling insect, *Leptocybe invasa* Fisher and La Salle (Hymenoptera: Eulophidae), has emerged as one of the most damaging pests of global *Eucalyptus* forestry resulting in the complete failure of some industrially important clones (Dittrich-Schröder *et al.* 2012,

Nyeko *et al*. 2010, Nyeko *et al*. 2009, MJ Wingfield personal communication). *Eucalyptus* species constitute some of the most widely grown and economically valuable plantation trees in the world (Grattapaglia *et al*. 2012). *L. invasa* was first described in Israel in 2000 following the extensive damage it caused in in the region and has since spread to Africa, the Mediterranean Basin, South East Asia, Europe and South America (Mendel *et al*. 2004, Kim *et al.* 2009, Nyeko *et al.* 2009, Thu *et al.* 2009, Kumari *et al.* 2010). *L. invasa* is an Australian, gall-forming wasp that preferentially oviposits on immature leaf and stem tissue (Mendel *et al.* 2004). The larvae are endophytic herbivores that cause the development of coalescing galls on leaves, petioles and twigs of susceptible trees. An infestation can have devastating effects on a plantation as it may result in stunted growth, die-back and death of infested trees (Nyeko *et al.* 2009, Thu *et al.* 2009, Kumari *et al.* 2010).

Biological control is currently considered to be the key tool in controlling this pest (Wingfield *et al.* 2008, Kim *et al.* 2009, Kulkarni *et al.* 2010). A number of parasitoid wasp species have been introduced as biological control agents (Kim *et* al. 2009, Kelly *et* al. 2012). Furthermore, varying degrees of tolerance and resistance are observed among species and genotypes of *Eucalyptus* indicating the potential for using resistant planting stock in affected areas (Nyeko *et* al. 2009, Dittrich-Schröder *et al.* 2012). Defence mechanisms providing resistance or tolerance to *L. invasa* are essentially unknown and, therefore, restrict the design of biotechnological strategies that can be used against the pest.

This study aims to describe the transcriptional responses that govern the interaction between *L. invasa* and a resistant *E. grandis* clonal genotype. RNA-Seq is a next-generation sequencing technique that allows rapid and accurate profiling of total RNA (Mortazavi *et al.* 2008). We show that RNA-Seq of *Eucalyptus* mRNA provides a robust means for investigating induced transcriptional responses in *Leptocybe*-challenged plants. This study identified genes that showed differential expression profiles in response to *L. invasa* oviposition. Categorisation and enrichment analysis of these genes identified putative defence mechanisms that are employed by *Eucalyptus* in response to the gall wasp. This information was used to propose a model of the defence

response of the host. Improving the current knowledge of the induced transcriptional responses between *E. grandis* and the invasive gall wasp will lead to improved and integrated control strategies by illuminating key defence mechanisms that may be manipulated to improve resistance. This information, in combination with biological control and silvicultural practices will help minimise the losses caused by *L. invasa*.

2.3. Materials and Methods

2.3.1. *Leptocybe invasa* Infestation Trial

Two-year-old ramets of an *E. grandis* clone (Tag 5, Mondi, South Africa) and an *E. grandis* x *E. camaldulensis* hybrid clone (GC 540, Mondi) were coppiced and subsequently grown in a field cage insectarium enclosed by a mesh that excluded *L. invasa*. Tag 5 was selected as a *L. invasa*-resistant clone and GC 540 as a susceptible clone (Dittrich-Schröder personal communication). After four months, ramets of each clone were divided into two groups, each composed of three replicates of six plants. The control group remained in the insectarium while the test group was exposed to a natural infestation by *L. invasa* in an unwalled nursery for seven days.

2.3.2. RNA Isolation and Sequencing

Infested and uninfested leaves were collected from the test (Tag 5 Infested 1, 2, 3 and GC 540 Infested 1, 2, 3) and control (Tag 5 Control 1, 2, 3 and GC 540 Control 1, 2, 3) groups and frozen in liquid nitrogen. Midribs were then excised and total RNA was extracted using the protocol described by Naidoo *et al*. (2013). Samples were treated using Qiagen RNase-free DNase I enzyme (Qiagen Inc, Valencia, California, USA) and purified using the Qiagen RNeasy Mini Kit following the manufacturer's instructions. The concentration and quality of the RNA samples was tested using the Bio-Rad Experion analyser (Bio-Rad, Hercules, California, USA). Tag 5 Total RNA was submitted to the Beijing Genomics Institute (BGI) for RNA-Seq analysis (50 bp paired-end (PE) reads, 20 million reads per sample).

2.3.3. Mapping and Analysis of Reads against the *E. grandis* v1.0 Genome Assembly (Phytozome 9.0)

RNA-Seq data was analysed using the Galaxy workspace (Goecks *et al.* 2010, Blankenberg *et al.* 2010, Giardine *et al.* 2005). FASTQ (Blankenberg *et al.* 2010) was used to verify RNA-Seq data quality. Reads were mapped to the *E. grandis* v1.0 genome assembly on Bowtie (Langmead *et al.* 2009), Tophat v1.3.1 (Trapnell *et al.* 2010) and Cufflinks v1.0.3 (Trapnell *et al.* 2010). Bowtie is an ultra-high-throughput short read mapper that is used in conjunction with Tophat, a splice junction mapper. Recommended, default settings were used in both cases. Cufflinks assembled the accepted mapped reads to predicted *E. grandis* transcripts and calculated the expression value as fragments per kilobase of transcript per million fragments mapped (FPKM). Cufflinks was set to use a maximum intron length of 30,000 bp, a minimum isoform fraction of 0.05 and perform quartile normalisation and bias correction for each sample. Thereafter, test and control samples were analysed with Cuffdiff v1.0.3 (Trapnell *et al.* 2010) to determine significant differential expression. Cuffdiff was set to use a minimum alignment count of 1000, a false discovery rate (FDR) of 0.05 and perform quartile normalisation and bias correction.

2.3.4. Gene Ontology Enrichment Analyses

Differentially expressed genes were assigned an *Arabidopsis thaliana* (TAIR 10) annotation based on a reciprocal BLAST search (www.eucgenie.org) where possible. Annotated genes were analysed for Gene Ontology (GO) term over-representation using the Cytoscape v2.8.2 (Shannon *et al.* 2003) plugin, BinGO v2.44 (Maere *et al.* 2005). BinGO was set to use a hypergeometric test and a Benjamini and Hochberg FDR of 0.05. Mapman v3.5.1R2 (Thimm *et al.* 2004) was used to display the data on maps of biological processes to visualise which genes are involved in various described defences and aid in the selection of target genes for RT-qPCR validation and comparison between Tag 5 and GC 540 expression profiles.

2.3.5. RT-qPCR Validative and Comparative Analyses

Total RNA from Tag 5 and GC 540 was used for RT-qPCR validative and comparative analyses, respectively. First strand cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Five target genes were selected based on the fold change calculated by Cuffdiff and the defence response category, as determined by the GO enrichment and Mapman analysis. The target genes encoded an auxin responsive protein (*EgrARP*, Eucgr.A01790), a cinnamyl-alcohol dehydrogenase (*EgrCAD*, Eucgr.E01115), a disease resistance-responsive dirigent-like protein (*EgrDIR*, Eucgr.A01114), ethylene-forming enzyme (*EgrEFE*, Eucgr.K00739) and O-methyltransferase (*EgrOMT*, Eucgr.L01145). Two reference genes, encoding an ADP ribosylation factor (*EgrARF*, Eucgr.I01779) and a fructose bisphosphate aldolase (*EgrFBA*, Eucgr.B02864), were used for normalisation. Gene-specific primers (Table 1) were designed for each target using Primer Designer 4 v4.20 (Sci Ed Central, Cary, North Carolina, USA) and were synthesised by Whitehead Scientific (Cape Town, Western Cape, South Africa). Primer sequences were verified in Phytozome v9.0 using a BLASTN similarity search against the *E. grandis* v1.0 genome to ensure that the gene of interest was targeted by the primer pair.

Quantitative PCR was performed according to the Minimum Information for Publication of Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al*. 2009) using the LightCycler 480 Real-Time PCR system (Roche Diagnostics, GmBH, Basa, Switzerland). Each reaction contained 1 µl of 1:10 diluted cDNA template, 5 µl of LightCycler SYBR Green Master Mix 2X concentration (Roche, Mannheim, Germany), 4 µl of 50 ng/µl yeast tRNA and 0.5 µl of each 1:10 diluted primer. The PCR involved a 95 ℃ hold for 5 minutes to activate the FastStart SYBR Green Mix. Quantification involved 45 cycles of 95 ℃ for 10 seconds, 60 ℃ for 10 seconds and acquisition at 72 ℃ for 15 seconds. Melting curve analysis involved one cycle of 95 ℃ for 5 seconds, 65 ℃ for 1 minute and a continuous signal acquisition at 95 ℃ with 10 acquisitions per 1 ℃. The reaction was completed with a cooling cycle of 40 ℃ for 5 seconds. Samples of each amplified target were also used for DNA sequencing to verify that the gene of interest was acquired.

Selected concentrations from a serial dilution set of pooled cDNA samples were used to determine the amplification efficiency of each primer pair. The experiment was set up using the sample maximisation method, which analyses all samples of a particular gene in a single run and different genes in separate runs. Normalisation of the target genes was based on stable transcript abundance of the reference gene set. Normalisation and relative quantification was performed using *qBase*plus v1.0 (Hellemans *et al*. 2007) and statistical significance of differences in transcript abundance was calculated using a Student's T-test in Microsoft® Excel 2010.

Table 1. Oligonucleotide primer sequences of selected defence-associated genes used for RT-qPCR analysis of transcript abundance.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Amplicon size (bp)	Tm (°C)
EgrARF	TGCGTACCGAGTTGTTGAGG	GTTGCACAGGTGCTCTGGAT	195	60
EgrFBA	TGAAGACATGGCAAGGAAGG	GTACCGAAGTTGCTCCGAAT	190	60
EgrARP	CCATAGTTCGCAGACTACAC	GTGAAGGACTTGGTCTTCTC	213	60
EgrCAD	GCCACTGCTCTGTGCTGGAA	CCGGCTGCCGTGTCGATTAT	285	60
EgrDIR	ACCACCGACATGGTGTTGTA	AGAACATGGCACGAGTGCTT	232	60
EgrEFE	CTTGAAGCACCTTCCTGTCT	GAGGTTGACGACGATGGAAT	379	60
EgrOMT	AGCTCGGCATCCTCAAGCTC	GCCTTCCTCGCTCACGTAGT	233	60

2.4. Results

2.4.1. Infestation of Eucalyptus grandis

Infested and uninfested leaves were collected from Tag 5 and GC 540 after seven days. All of the challenged groups showed *L. invasa* oviposition, particularly along the midribs (Figure 1). To confirm the resistant and susceptible identity of Tag 5 and GC 540, ramets were further observed for gall development. GC 540 showed extensive gall development, whereas Tag 5 showed signs of oviposition but no subsequent galling. Total RNA obtained from the midrib material had high RNA

quality scores following Experion analyses (Table S1). In addition, clear 18S and 28S bands are observed on the Experion RNA gel (Figure S1) indicating non-degraded RNA. This material was considered suitable for submission to BGI for RNA-Seq analysis.

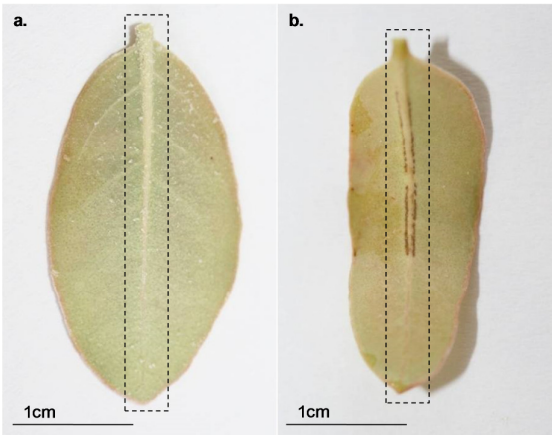

Figure 1. Example of the leaf material collected for the study (a) Non-infested leaf and (b) infested leaf showing *Leptocybe invasa* oviposition. The leaves were frozen in liquid nitrogen. Dotted lines indicate leaf area that was then excised for total RNA extraction.

2.4.2. RNA-Seq Data Analysis

Sequencing of the Tag 5 infested and non-infested samples yielded 21-25 million read pairs (Table 2). Good quality scores were obtained for all samples (Figures S4-S15). Cufflinks produced two outputs containing transcript abundance data for all genes and transcripts annotated to the genome. Between 30,713 and 31,846 genes showed an FPKM greater than 0 and were considered as expressed (Table 2, File S1). Similarly, between 35,761 and 39,009 transcripts were expressed (Table 2, File S1). These results indicated no anomalies between the samples and were deemed acceptable for further analysis.

Table 2. Statistics of the Tag 5 reads obtained from Illumina RNA-Seq and mapping to the *Eucalyptus grandis* version 1.0 genome.

Sample Name	Total PE Reads	Mapped Reads (%)			Expressed Genes	Expressed Transcripts
		Paired	Singletons	Total		
Infested 1	21,250,222	62.48	9.29	71.77	30,713	37,111
Infested 2	21,290,809	74.98	6.99	81.97	31,674	38,065
Infested 3	21,060,999	67.79	6.74	74.53	30,846	35,761
Control 1	21,482,571	66.45	6.63	73.08	31,314	37,159
Control 2	21,340,672	45.23	33.67	78.90	31,234	38,619
Control 3	25,355,724	38.52	41.93	80.45	31,300	39,009

Cuffdiff identified 1381 significantly differentially expressed (DE) gene models out of 44,974 annotated to the version 1.0 genome, hereafter referred to as Group A (Figure 2, File S2). Group A showed 769 genes as up-regulated and 612 genes as down-regulated. Furthermore, 2507 transcripts out of 55,935 were identified as significantly DE, showing 1275 transcripts as up-regulated and 1232 as down-regulated (Figure 2, File S2). The transcripts were derived from 2098 gene models, hereafter referred to as Group B. There were 1362 gene models common to Group A and B. Gene models from Group A and Group B were combined to form a single dataset including 2117 genes. This combined group was used in all subsequent analyses.

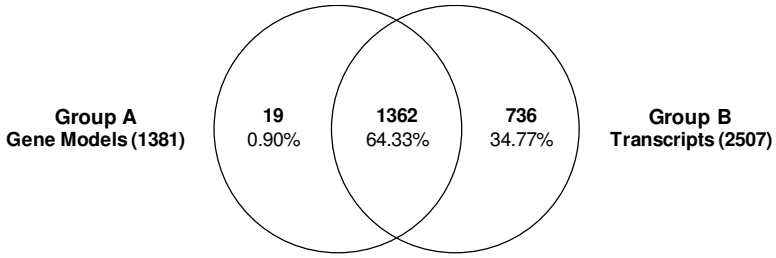

Figure 2. Gene Model (Group A) and Transcript (Group B) datasets showing unique and shared differentially expressed genes. Cuffdiff identified 1381 differentially expressed gene models and 2507 differentially expressed transcripts encoded by 2098 gene models. Upon comparison, 1362 gene models were common between the two groups, 19 were unique to the gene model set and 736 were unique to the transcript set.

Of the 2117 genes that were determined to be differentially expressed, 47.38% were genes possessing a single transcript and 52.62% were genes possessing multiple transcripts (File S2). Further analysis of the multi-transcript genes showed that 11.49% showed significant differential expression in all transcripts, whereas the rest showed significant differential expression in a subset of the transcripts (File S2). Certain cases showed opposite transcript abundance profiles where one transcript would be significantly up-regulated and another would be significantly down-regulated (Figure 3). Figure 3 provides an example of alternative transcripts showing selective expression of one and suppression of the other. The difference in the transcripts is very small in this case and the majority of reads will map to both gene models, however, the Cufflinks suit of tools is sufficiently accurate to identify these differences. The impact of alternative splicing remains relatively undescribed in plants although recent studies have demonstrated its importance in modulating biological processes in plants (Syed *et al*. 2012).

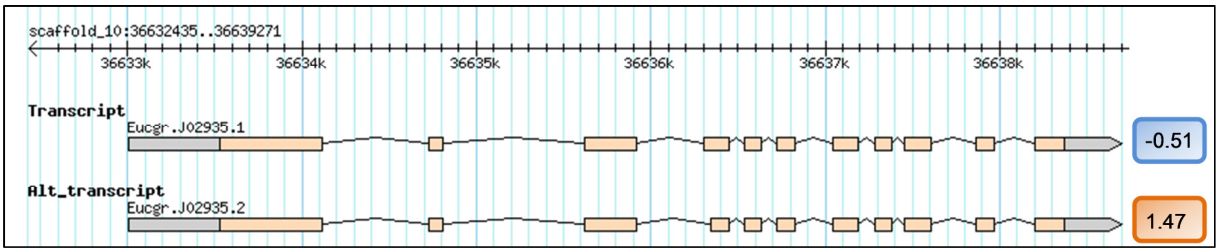

Figure 3. *Eucalyptus grandis* genome representation of a gene, Eucgr.J02935, showing selective differential expression between transcripts. The alternative splicing event involves exon 4 in this example. The grey regions on the transcript represent the 5` and 3` UTR. The orange region represents coding sequence. The black connectors represent introns. The numbers, highlighted by the blue and orange boxes, indicate the ln(fold_change) value as calculated by Cuffdiff.

The range of FPKM values for the differentially expressed genes was between ln(fold_change) of 5.92 and -4,69 (Table 3, 4). The genes showing the largest changes in expression levels were considered to shed light on probable cellular pathways that are important in the interaction. In the up-regulated group, a cytochrome P450-encoding gene showed the highest fold change and a UDP-glucosyl transferase showed the lowest. The up-regulated group identified genes with known functions in plant defence including two pathogenesis-related proteins, a protease inhibitor- and basic chitinase-encoding gene, and two genes involved in hormone signalling, an ethylene forming enzyme- and jasmonic acid carboxyl methyltransferase-encoding gene. This group of top differentially expressed genes included others with less apparent roles in defence such as a number of GDSL-like lipase-, a pectin lyase-like protein- and a cinnamate-4-hydroxylase-encoding gene.

Table 3. Top 15 up-regulated genes showing the highest fold changes identified.

Gene ID	TAIR10 Description	ln(fold_change)	q-value
Eucgr.H04897	Cytochrome P450, family 79, subfamily B, polypeptide 2	5.92	<0.001
Eucgr.J00826	Unknown protein	3.46	<0.001
Eucgr.K00739	Ethylene-forming enzyme	2.70	<0.001
Eucgr.H03441	Bifunctional inhibitor	2.61	<0.001
Eucgr.J01279	Pectin lyase-like superfamily protein	2.52	<0.001
Eucgr.K02477	GDSL-like Lipase	2.40	<0.001
Eucgr.C00136	GDSL-like Lipase	2.38	<0.001
Eucgr.I01017	GDSL-like Lipase	2.30	<0.001
Eucgr.C00065	Cinnamate-4-hydroxylase	2.22	<0.001
Eucgr.I02624	Hydroxyproline-rich glycoprotein family protein	2.10	<0.001
Eucgr.I02271	Basic chitinase	2.10	<0.001
Eucgr.H03340	Jasmonic acid carboxyl methyltransferase	2.08	<0.001
Eucgr.G01304	Cysteine/Histidine-rich C1 domain family protein	2.08	<0.001
Eucgr.G01352	N/A	2.04	<0.001
Eucgr.L01565	N/A	2.04	<0.001

The down-regulated group identified one gene involved in the gibberellin biochemical pathway, gibberellin 2 oxidase 8-encoding gene. Interestingly, AGD2-like defence response protein-encoding gene, a positive regulator of the salicylic acid pathway in *A. thaliana* (Nie *et al*. 2011), was also highly down-regulated. A defence-associated protein-encoding gene, D-mannose binding lectin, was also observed in this group. Furthermore, there were a number of genes in this group with less clear roles in the interaction such as a UDP-glucosyl transferase- and a major facilitator protein-encoding gene as well as genes with no known function or annotation.

Table 4. Top 15 down-regulated genes showing the highest fold changes identified.

Gene ID	TAIR10 Description	ln(fold_change)	q-value
Eucgr.H01247	UDP-glucosyl transferase 72E1	-4.69	<0.001
Eucgr.G01934	CCR-like	-4.65	<0.001
Eucgr.A02803	N/A	-4.47	<0.001
Eucgr.A02802	N/A	-4.27	<0.001
Eucgr.F01234	D-mannose binding lectin protein	-4.24	<0.001
Eucgr.C04369	Receptor-like protein kinase 1	-3.89	<0.001
Eucgr.H03662	Major facilitator superfamily protein	-3.75	<0.001
Eucgr.E01669	Ankyrin repeat family protein	-3.60	<0.001
Eucgr.J01950	N/A	-3.18	<0.001
Eucgr.I02123	N/A	-2.87	<0.001
Eucgr.I02127	Protein of unknown function	-2.79	<0.001
Eucgr.G02581	N/A	-2.51	<0.001
Eucgr.I02128	Gibberellin 2-oxidase 8	-2.42	<0.001
Eucgr.J02231	DNAJ heat shock N-terminal domain-containing protein	-2.37	<0.001
Eucgr.F03710	AGD2-like defence response protein 1	-2.37	0.004

2.4.3. Gene Ontology Analysis

2.4.3.1. GOSlim Analysis

To clarify the interaction between *E. grandis* and *L. invasa*, the GO functional categorisation of the annotated genes was investigated. Of the 2117 genes that were identified as DE, 1177 could be annotated through BLAST analysis against *A. thaliana* (File S3). The annotated genes were initially analysed for GO term over-representation using the BinGO GOSlim_Plants ontology file in the three main categories of cellular component (CC), molecular function (MF) and biological process (BP) (Figure 4, File S4). In all cases, more GO terms were identified as significantly (p-value<0)

enriched in the down-regulated dataset than in the up-regulated dataset. Furthermore, a number of GO terms were shared between the two groups in all three categories. The most significant over-represented GO terms in the up-regulated dataset included catalytic activity, secondary metabolic process and response to stress. The GO terms showing the highest significance in the down-regulated dataset included cell, catalytic activity and DNA metabolic process. The CC class highlights various internal and external organelles as sites of gene activity in both datasets. The MF class showed a variety of protein activities in the up-regulated group whereas the down-regulated group showed prolific binding activities. Finally, the up-regulated dataset shows a number of BP GO terms pertaining to defence that might reflect the responses of *E. grandis* to *L. invasa*. These include secondary and lipid metabolic processes as well as responses to stress and various stimuli. The down-regulated dataset shares a number of GO terms with the up-regulated dataset; however, it included numerous GO terms pertaining to the cell cycle and development.

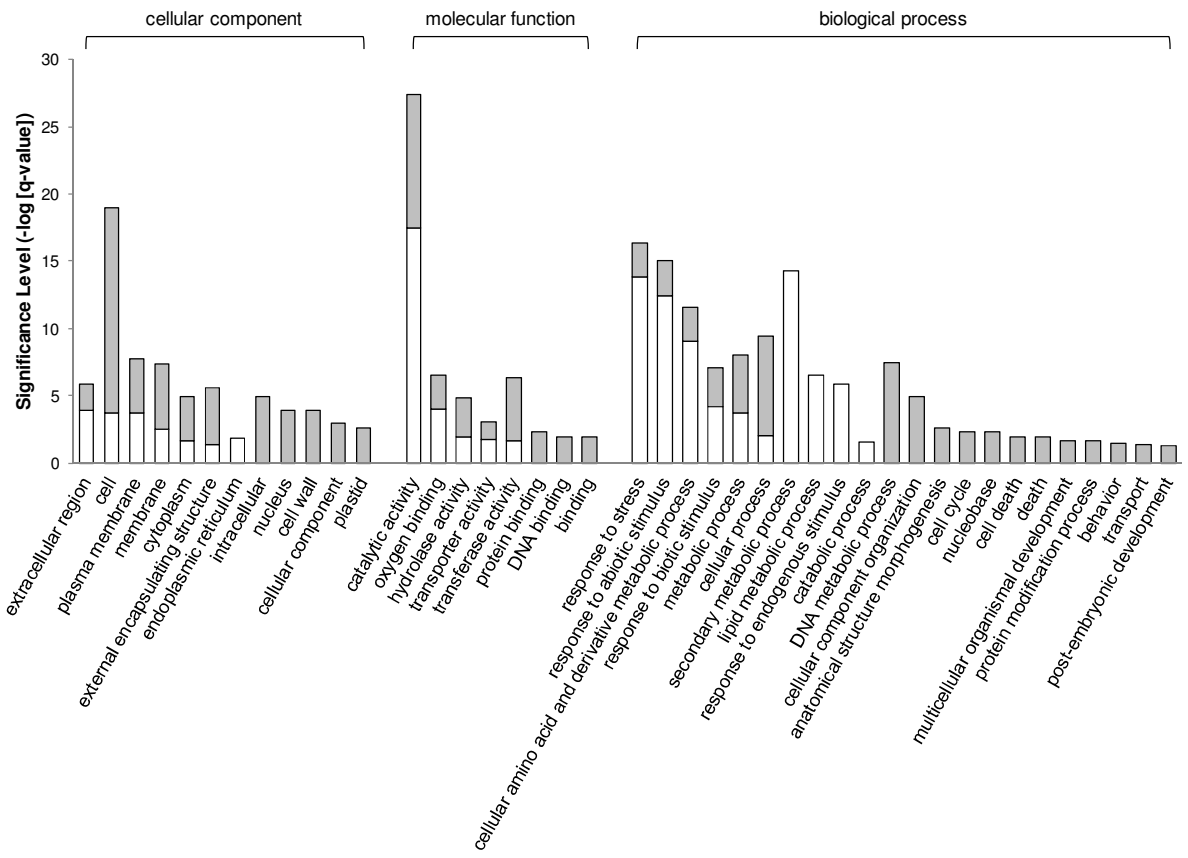

Figure 4. GOSlim functional categorisation of annotated genes in the up- and down-regulated dataset in the three main categories of cellular component, molecular function and biological process. White bars represent enriched GO terms in the up-regulated dataset. Grey bars represent enriched GO terms in the down-regulated dataset.

2.4.3.2. GO Biological Processes Analysis

In order to better elucidate the defence mechanisms employed by *Eucalyptus* upon attack by *L. invasa*, the over-representation analysis of the up- and down-regulated datasets were expanded in the BP category. The terminal nodes of the GO hierarchy were particularly used to understand the interaction because they describe more specific associated activity than their parent terms and thus provide a greater level of detail regarding the putative defence mechanisms. The enriched, terminal GO terms included 29 categories for the up-regulated dataset and 14 for the down-regulated dataset (File S4). These groups were manually assigned to putative defence

mechanisms (Figure 5) based on literary evidence and hypotheses regarding plant resistance against galling insects. The putative defence mechanisms included the oxidative burst, hormone-mediated signalling pathways, secondary metabolite production, cell wall reinforcement and indirect defences in the up-regulated dataset and cell cycle suppression and tissue redifferentiation in the down-regulated dataset. Additionally, various responses to abiotic stimuli, transport of cellular compounds and developmental processes are also over-represented.

2.4.4. Mapman Gene Categorisation

The annotated genes were further analysed with Mapman to visualise the genes on maps of cellular processes. These categorisations provided an additional line of evidence for the defences involved in the interaction between *E. grandis* and *L. invasa*. The majority of the defence categories displayed different genes in both the up- and down-regulated datasets. The categorisation of the DE genes generally supported the responses calculated by BinGO. A number of genes were assigned to additional categories thus creating a more complete defence pathway (Figure 5, File S5). These included *R* gene-mediated recognition, hormone-independent signalling pathways, various transcription factors (TFs), heat shock proteins and pathogenesis-related (PR) proteins. Additionally, the GO enrichment analysis and Mapman categorisations did not assign certain genes to any defence mechanism although they were annotated as *R* genes, transcription factors and *PR* protein-encoding genes. These were manually recorded in File S3. Putative defence responses determined by Mapman analyses were summarised in Figure 5 along with the enriched GO terms.

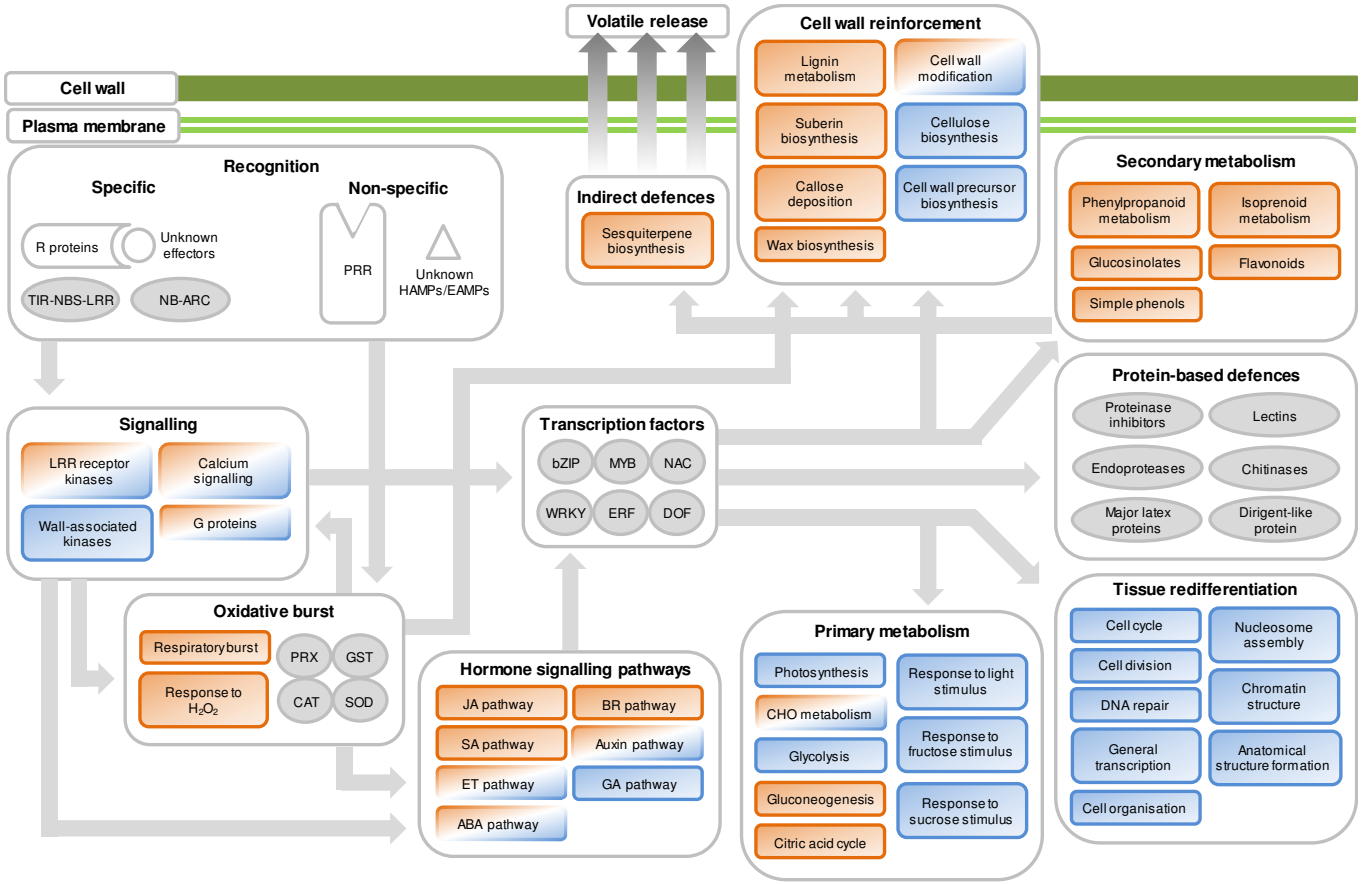

Figure 5. Hypothetical model of the progression of defences employed by *E. grandis* against *L. invasa* oviposition based on alterations in the transcriptomic landscape after infestation. This model was created by combining information from the GO enrichment analyses, Mapman categorisations and Cuffdiff differential expression analysis. The broad defence mechanisms were established from literary evidence. Orange blocks represent processes that show up-regulated genes. Blue blocks represent processes that showed down-regulated genes. Orange and blue blocks represent processes where a subset of genes showed up-regulation and others down-regulation. Grey ovals represent differentially expressed genes as calculated by Cuffdiff. Grey arrows indicate the event in the defence response, some of which take place concurrently.

2.4.5. RT-qPCR Validation and Comparative Analysis

Five differentially expressed genes were selected for RT-qPCR expression profiling in Tag 5 and GC 540 including an auxin-responsive protein (*EgrARP*, fold change of -2.3), cinnamyl-alcohol dehydrogenase (*EgrCAD*, fold change of 2.1), disease resistance-responsive dirigent-like protein (*EgrDIR*, fold change of 2.2), ethylene-forming enzyme (*EgrEFE*, fold change of 14.9) and O-methyltransferase (*EgrOMT*, fold change of -3.0) (Figure 6). These single-transcript genes were selected based on their RNA-Seq transcript abundance profiles and the defence category into which they were categorised. In all cases the transcript abundance pattern of the infested and control Tag 5 samples was the same as that calculated by Cufflinks with all except *EgrOMT* showing significant differential expression. In the case of GC 540, all genes except *EgrOMT* showed significant differential expression. *EgrCAD* and *EgrOMT* showed different transcript abundance profiles between the resistant and susceptible interactions, whereas *EgrARP*, *EgrDIR* and *EgrEFE* were shown to follow the same pattern in both interactions. Quality control was performed using melting curve analysis to verify target specificity (Figure S21, S22) and *qBase*plus to verify reaction efficiency (Table S3).

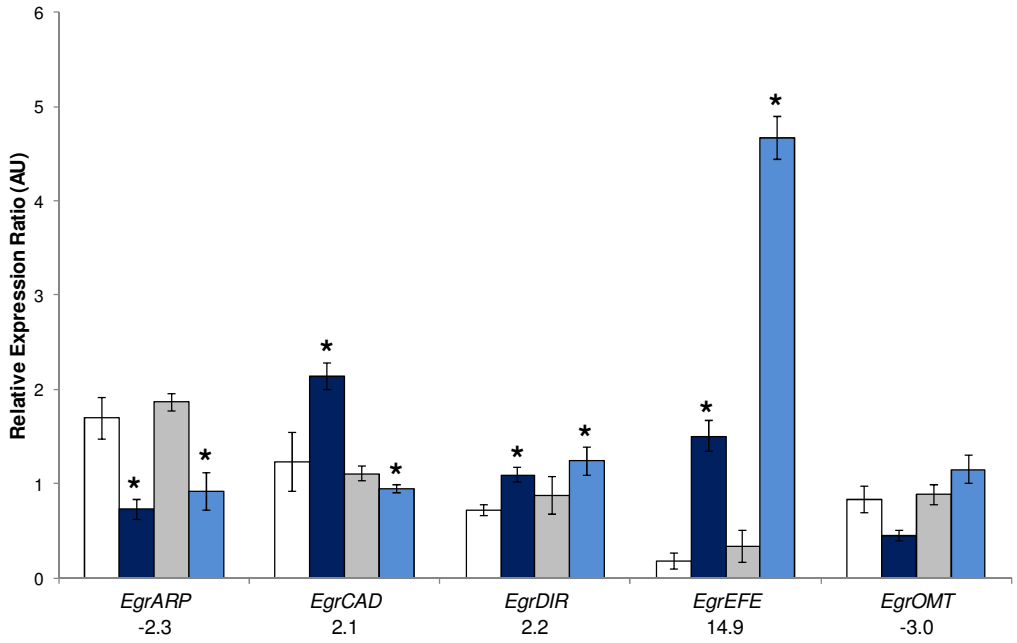

Figure 6. RT-qPCR validation and comparison of RNA-Seq transcript abundance values in Tag 5 and GC 540, respectively. Target gene transcript abundance was normalised to two reference genes, *EgrARF* and *EgrFBA*. White bars represent Tag 5 Control, dark blue bars represent Tag 5 Infested, grey bars represent GC 540 Control and light blue bars represent GC 540 Infested. Asterisks indicate significant differential expression at p-value<0.05. Numbers below the gene names indicate the RNA-Seq-derived fold change of each sample as calculated by Cuffdiff.

2.5. Discussion

The purpose of this study was to elucidate the molecular basis of the incompatible interaction between *L. invasa* and *E. grandis*. *L. invasa* has become an important global plantation pest since its discovery (Dittrich-Schröder *et al.* 2012, Nyeko *et al*. 2010, Nyeko *et al*. 2009, Bernard Slippers personal communication). An analysis of the changes in the host transcriptome following oviposition in leaf tissue has provided a glimpse of the defence mechanisms that may contribute to resistance. It must be noted that changes in the transcriptome do not necessarily correspond to changes in protein level. In addition, it is difficult to determine which responses are primary (caused by oviposition) and which are secondary (caused by changes in primary and secondary

metabolism). Determination of changes in the transcriptome, proteome and metabolome over a time course is needed in order to improve our inference of which defence responses are employed by *E. grandis* during the interaction with *L. invasa* in a primary or secondary manner.

There is relatively little information regarding the *Eucalyptus* defensome and inferences from other plant species may not accurately reflect the defences employed by this genus. This study has produced evidence for broad defence mechanisms employed by *Eucalyptus.* Transcriptomic studies have previously been used to clarify the interaction between many organisms (Liu *et al*. 2007, Zhang *et al*. 2010). For example, Santamaria *et al*. (2012) demonstrated that pyramiding genes encoding peptidase inhibitors in *A. thaliana* improved plant resistance against *Tetranychus urticae* (spider mite). The possibility of identifying and using such candidates in *Eucalyptus*, along with biocontrol agents, may provide a robust means of controlling the pest. RNA-Seq revealed differential expression of defence-related genes known to be important for incompatibility.

2.5.1. Resistance Gene-Mediated Recognition and Signal Transduction

Putative TIR-NBS-LRR and NB-ARC *R* genes (File S3) were shown to have altered expression profiles, both up- and down-regulated, in response to *L. invasa* oviposition. This is the first, albeit indirect, evidence of a possible gene-for-gene interaction between *Eucalyptus* and *L. invasa*. A number of other insects, including gall-inducers, have been shown to display a gene-for-gene interaction with their hosts (Nombela *et al*. 2003, Rossi *et al*. 1998). *R* gene-mediated recognition is a specific recognition of a particular strain or genotype of the pest and involves the interaction of plant R proteins with pest effector molecules (Jones and Dangl 2006). Following recognition, signalling cascades transfer the signal into the host cell in order for an appropriate defence response to be elicited (Fürstenberg-Hägg *et al*. 2013). The mechanism is incompletely understood, although mitogen-activated and calcium-dependent protein kinase (MAPK and CDPK) cascades have been shown to be important in signal transduction (Tena *et al*. 2011). This signalling process was evident in our study through the Mapman categorisation of a number of genes as signalling-related, for example calcium-binding and calmodulin-related genes. Feeding by

insects causes a temporary increase in cytosolic calcium levels (Maffei *et al*. 2004). The increased calcium levels activate calcium-sensing proteins such as calmodulin and promote signal transduction events that link pest perception to the downstream responses (Ma and Berkowitz 2011, Tena *et al*. 2011).

R genes are considered as highly effective tools for minimising damage by pests and pathogens (Gururani *et al*. 2012). The potential for using *R* genes in transgenic crops to boost resistance to various stresses has been shown in numerous studies. For example the tomato *Mi* gene was introgressed into susceptible tomato cultivars and shown to provide broad-spectrum resistance against *Bemisia tabaci* (Nombela *et al*. 2003), *Meloidgyne incognita* and *Macrosiphum euphorbiae* (Rossi *et al* 1998, Vos *et al*. 1998). Junghans *et al*. (2003) identified and mapped the first *Eucalyptus* resistance locus,built around *Ppr 1* (*Puccinia psidii* resistance gene 1), thereby introducing the possibility of developing resistant breeding lines in these plantation trees. The identification of *R* genes that may play a role in *E. grandis* resistance to *L. invasa* may prove to be an important feature of future pest control through marker-assisted breeding programs or deployment of transgenic lines. However, segregating populations of trees will be required to map the location of putative *R* genes involved in resistance to *L. invasa*.

2.5.2. Oxidative Burst

The oxidative burst is a defence mechanism involving the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (O'Brien *et al*. 2012). In this study, the oxidative burst was implicated in resistance to *L. invasa* by genes categorised as response to hydrogen peroxide through the GO term enrichment analysis as well as by the assignment of genes to the respiratory burst-related Mapman bins. Additionally, known antioxidant genes, such as catalase and superoxide dismutase, were shown to be up-regulated. Numerous plant species are known to transcribe antioxidant genes following an oxidative burst to protect native cellular structures (O'Brien *et al*. 2012). For example, in the wheat-Hessian fly interaction, Mittapalli *et al*. (2007) observed an up-regulation of Hessian fly antioxidant genes during a resistant interaction and *vice*

versa during a susceptible interaction, thereby linking the oxidative burst to the defence response. This association was confirmed by Liu *et al*. (2010) following the observation that resistant wheat showed a sustained accumulation of hydrogen peroxide as well as enhanced expression profiles of ROS-producing genes in response to Hessian fly feeding, whereas the susceptible interaction did not. The *E. grandis-L. invasa* interaction appears to show a similar trend, although confirmation through subsequent studies, such as enzyme activity assays, is required.

2.5.3. Phytohormone-Mediated Signal Transduction

Differentially expressed genes related to jasmonic acid (JA), salicylic acid (SA), ethylene (ET), abscisic acid (ABA), brassinosteroid, gibberellin and auxin signalling pathways were identified through GO and Mapman categorisation (Files S4, S5). The phytohormones are connected in an intricate communication network that allows the plant to modulate responses to different biotic and abiotic stresses (Pieterse *et al*. 2012).

The JA pathway is described as the dominant hormone-mediated pathway that regulates plant resistance against insects (Erb *et al*. 2012). In this study, the JA and SA pathways were linked to the *Eucalyptus* defence response through the GO enrichment analysis and Mapman categorisations of the up-regulated genes. Furthermore, some of the most highly up- and down-regulated genes were involved in these signalling pathways. Jasmonic acid carboxyl methyltransferase (*JMT*) was identified as highly up-regulated. JMT is responsible for the formation of methyl jasmonate, an important signalling molecule that regulates local and distal JA-dependent responses in plant defence (Seo *et al*. 2011). An ethylene-forming enzyme-encoding gene (also called 1-aminocyclopropane-1-carboxylate oxidase, *ACO*) was also highly up-regulated. ACO catalyses the final step in ethylene biosynthesis (Yu *et al*. 2011) and up-regulation of this gene is associated with defence responses (Díaz *et al*. 2002). Ethylene is an important modulator of plant defence and has been shown to act synergistically and antagonistically with SA and JA in many plant-pathogen and plant-insect interactions (Pieterse *et al*. 2012). GO terms and Mapman bins from the up-regulated group revealed that the SA pathway was also putatively involved. Although

JA is considered the key hormone regulating plant-insect interactions, cases have been described that show SA plays an important role in certain instances (Coppola *et al*. 2013). An AGD2-like defence response protein-encoding gene (*ALD*) was down-regulated. ALD acts as a positive regulator of the salicylic acid pathway in *A. thaliana* (Nie *et al*. 2011). The results from this study support a scenario that suggests all three defence-associated phytohormones may be important in eliciting a resistant response.

In addition to the three key defence-associated phytohormones, genes related to ABA, auxin, gibberellin and brassinosteroid pathways are differentially expressed (Figure 5). ABA is known to possess a wide range of biological functions including a role in defence (Ton *et al*. 2009). Furthermore, this hormone is also known to crosstalk with various other phytohormones including SA, JA and ET (Yasuda *et al*. 2008, Dinh *et al*. 2013, Anderson *et al*. 2004). There is evidence to suggest that ABA is an important component in plant resistance to insects, for example Dinh *et al*. (2013) described how the interaction between ABA and the JA pathway allows *Nicotiana attenuata* to mount a complete response against *Manduca sexta* (tobacco hornwood). ABA pathway-related genes were identified through the GO enrichment and Mapman categorisation analyses and supports a role for ABA as a putative regulator of plant defence against insects.

A number of genes related to auxin metabolism showed differential expression upon oviposition (Figure 5). Auxin has previously been hypothesised to be involved in gall development. Tooker and De Moraes (2011) described altered levels of auxins in a susceptible wheat-Hessian fly interaction and suggested that auxins play a role in the development of nutritive tissue at the larval feeding site. Therefore, suppression of auxin signalling is likely an important defence mechanism. In this study, genes that were categorised under the Auxin signalling pathway were both up- and down-regulated. It is possible that this is an example of attempted infestation by the pest and important for gall development. However, further study is required for clarification.

Oviposition resulted in the differential expression of a number of genes related to brassinosteroid metabolism (Figure 5). Campos *et al*. (2009) discussed how brassinosteroids inhibited the

development of anti-herbivory traits in *Solanum lycopersicum* through a negative interaction with JA, although the opposite relationship has been observed in *A. thaliana*. Further study is required to more clearly elucidate the role of this hormone in the interaction.

Finally, a number of genes relating to gibberellin (GA) signalling were shown to be down-regulated. A gibberellin 2 oxidase-encoding gene was highly down-regulated. The enzyme catalyses the metabolism of GA_4 and is thought to be involved in plant-microbe interactions (Lee *et al*. 2012). Gibberellins are known to negatively interact with the JA signalling pathway via DELLA proteins (Fürstenberg-Hägg *et al*. 2013). Due to the apparent importance of JA in mediating this interaction, it would be expected that the gibberellin pathway would be down-regulated. The identification of genes related to JA, SA, ET, ABA, brassinosteroid, gibberellin and auxin pathways provides additional evidence for what has become evident through numerous studies on the topic; plants require the integration of multiple hormone signalling pathways in order to elicit a complete, effective and well-coordinated defence response.

2.5.4. Chemical-Based Defences

Plants possess a wide variety of secondary metabolites that may act directly or indirectly during a defence response (Mithöfer and Boland 2012). These compounds can be divided into various classes such as phenylpropanoids, alkaloids and isoprenoids and are used for a wide array of defence mechanisms such as antibiosis, antixenosis and cell wall reinforcement (Fürstenberg-Hägg *et al*. 2013). In this study, genes were assigned to a number of secondary metabolic defences (Figure 5) that are known to be entomotoxic. These compounds include terpenoids, phenylpropanoids, flavonoids, glucosinolates and simple phenols, all of which have been shown to be involved in plant defence against insects (Mithöfer and Boland 2012). *Eucalyptus* essential oils are known to possess a wide variety of biological activities, including antixenotic and entomotoxic activity and synthesis of these molecules was demonstrated in *E. globulus* in response to an insect pests (Troncoso *et al*. 2012). The deployment of the secondary metabolic defences has been

described in numerous plant resistance responses, including the incompatible wheat-Hessian fly interaction (Liu *et al* 2007).

Sesquiterpenes have been shown to possess insecticidal activity (Alarcon *et al*. 2013) and play an important role in establishing the volatile profile of the plant (Copolovici *et al*. 2011). Furthermore, Troncoso *et al*. (2012) showed that a number of terpenoids were important in inducing resistance mechanisms by functioning in the indirect defences. The plant indirect defence mechanism involves the production of volatile signalling compounds in response to various stresses, including insects (Damasceno *et al*. 2010, Unsicker *et al*. 2009). These chemicals serve as messengers that alert distal parts of the same plant and neighbouring plants of the threat as well as attract natural enemies of the pest (Bruinsma *et al*. 2009, Troncoso *et al*. 2012). Tooker *et al*. (2008) demonstrated that the Hessian fly was capable of selectively down-regulating the production of certain volatile compounds in susceptible wheat biotypes. Parasitoids of *L. invasa* have been shown to maintain the population to almost below observation level in the natural environment indicating the importance of the natural enemies in the ecosystem (Dittrich-Schröder *et al*. 2012, Rocha *et al*. 2013). This study shows that *E. grandis* up-regulates the expression of genes involved in terpenoid metabolism. It is possible that the host employs these chemicals not only as direct toxic metabolites but also as important messengers in indirect defence. Terpenoids contain a wide range of molecules, with more than 20,000 characterised (Mithöfer and Boland 2012). They are involved in numerous biological pathways and their function in this study is not clear. Additional research, such as that described in Troncoso *et al*. (2012) may help clarify their role in this interaction.

2.5.5. Cell Wall Reinforcement

In this study, numerous genes were assigned to enriched GO terms and Mapman bins related to the biosynthesis of cell wall components (Figure 5, File S4, S5). These categorisations may describe reinforcement of the cell wall by lignification and the deposition of other structural compounds such as suberin in an effort to prevent the larvae from establishing a feeding site. The

strengthening of cell walls is a frequently described defensive mechanism employed by plants against a wide range of attackers including pests and pathogens (Fossdal *et* al. 2012). For example, Liu *et al*. (2007) described a similar expression pattern in lignin biosynthetic genes in the incompatible wheat-Hessian fly interaction as observed from this study. Evidence for the importance of this response is highlighted in reciprocal studies that observed how insects selectively suppress cell wall reinforcement during successful attacks, for example in interactions between susceptible wheat and Hessian fly (Liu *et* al. 2007) as well as *Gossypium hirsutum* and *Aphis gossypii* (Dubey *et al*. 2013).

Additionally, galls are known to be composed of heavily lignified tissue that creates a protective environment for the insect (Oliviera and Isaias 2010). Therefore, a number of the genes that were categorised within the lignin biosynthesis GO term may be induced by the wasp. A comparative transcriptomic study of the susceptible interaction may help to clarify those genes that are involved

2.5.6. Protein-Based Defences

Significant DE was observed for a number of defence-related protein-encoding genes (Figure 5, File S3). Numerous studies have highlighted the importance of various classes of proteins in plant resistance against insects. These proteins commonly possess anti-feedant activities and contribute to larval antibiosis by disrupting the digestive process (Bode *et* al. 2013, Liu *et* al. 2007).

In this study, proteinase inhibitors were found to be the dominant class of defence-related protein-encoding genes that were up-regulated. These proteins have been shown to be a commonly observed plant resistance mechanism against a wide range of insect species, including the Hessian fly and Asian rice gall midge (Sinha *et al*. 2011, Liu *et al*. 2007, Hartl *et al*. 2010, Bode *et al*. 2013, Wu *et al*. 2008). These proteins act by inhibiting insect digestive proteins thus leading to poor nutrient availability and starvation (Zhang *et al*. 2010).

A number of chitinases were up-regulated during the interaction, including one that was one of the most highly up-regulated genes identified. This protein class has also been shown to promote plant resistance against insects by binding with chitin present in insect midguts thus blocking nutrient absorption (Major and Constabel 2006, Büchel *et al*. 2012). For example, Major and Constabel (2006) observed an up-regulation in the expression of a chitinase-encoding gene in a poplar hybrid (*Populus trichocarpa* x *P. deltoides*) upon feeding by *Malacosoma disstria* (forest tent caterpillar).

A number of lectins were also identified in this study. One D-mannose binding lectin-encoding gene was highly down-regulated. It is possible that *L. invasa* is capable of suppressing the expression of genes encoding defence-related proteins. Lectins have been shown to play an important role in wheat defence against the Hessian fly (Liu *et al*. 2007, Subramanyam *et al*. 2013, Das *et al*. 2013, Williams *et al*. 2002, Büchel *et al*. 2012). These proteins act in a similar manner to chitinases by binding to targets in the insect midgut and blocking nutrient absorption (Büchel *et al*. 2012).

A single disease resistance-responsive dirigent-like (DIR) protein-encoding gene (Eucgr.A01114) was shown to be up-regulated. Subramanyam *et al*. (2013) reported a similar result during an incompatible wheat-Hessian fly interaction. DIR are involved in the synthesis of lignan monomers and may be involved in cell wall reinforcement. The fifth group of defence-associated proteins that were identified were peptidase inhibitors. These proteins have been shown to be induced upon insect feeding and Santamaria *et al*. (2012) showed that pyramiding of peptidase inhibitor-encoding genes in *A. thaliana* improved plant resistance against *Tetranychus urticae* (two-spotted spider mite).

Finally, a single major latex protein (MLP)-encoding gene (Eucgr.A02983) was up-regulated during the response. These proteins have previously been shown to respond to insect herbivory (Corrado *et al*. 2013, van de Ven *et al*. 2000). A variety of defence-related proteins have been shown to be involved in the resistant interaction between *E. grandis* and *L. invasa*. A number of studies, such as the aforementioned Santamaria *et al*. (2012), have shown success in using genes encoding

these proteins in transgenic approaches to improve plant resistance. This possibility adds an additional approach in improving *Eucalyptus* resistance to this invasive pest and highlights the potential for developing more robust means of controlling it in the future.

2.5.7. Suppression of the Cell Cycle

The GO terms and Mapman bins identified in this study revealed a marked suppression of the cell cycle (Figure 5). This may be explained as suppression of cellular development allowing resource allocation to defence-associated pathways (Schultz *et al*. 2013). However, the opposite was observed during a compatible interaction between rice and the Asian rice gall midge (Rawat *et al*. 2012). Numerous studies have described the process of tissue redifferentiation in gall formation. Tissue redifferentiation may be defined as the process whereby novel cell types with specialised functions are formed following a change in native cell identity (Chapter 1 Figure 2) (Oliviera and Isaias 2010). The up-regulation of genes involved in the cell cycle described by Rawat *et al*. (2012) was hypothesised to lead to vegetative tissue production. In this study, suppression of the cell cycle may reduce the availability of manipulable targets for *L. invasa* gall development through tissue redifferentiation. Further histological study is required to describe the formation or cessation of *L. invasa* galls from native *Eucalyptus* tissues and link the observed gene expression patterns to resistance to the pest.

2.5.8. Suppression of Primary Metabolism

A number of studies have described the suppression of primary metabolic processes during a defence response, for example the incompatible interaction between wheat and the Hessian fly (Liu *et al*. 2007). This response may be of particular importance during a galling insect-plant interaction due to the fact the gall serves as a nutrient sink for the larvae during development (Inbar *et al*. 2010). This is illustrated by the fact that the compatible interactions between wheat and the Hessian fly (Liu *et al*. 2007) and rice and the Asian rice gall midge (Rawat *et al*. 2012) showed enhancement of primary metabolism presumably to aid in the creation of the nutritive

tissue. In this study, suppression of a number of genes relating to various primary metabolic pathways was observed such as photosynthesis and carbohydrate metabolism in accordance with these other studies (Figure 5). However, genes functioning in other pathways, such as the citric acid cycle, were shown to be up-regulated. A recent review by Kangasjärvi *et al.* (2012) discusses the ability of plants to maintain balance between basic metabolism and immunity as well as the role of photosynthesis-related processes in defence. The results from this study show that the majority, but not all, primary metabolic processes are suppressed, possibly indicating the ability of the plant to balance defensive and primary pathways.

2.5.9. RT-qPCR Validation and Comparison

The expression profiles of all five genes were validated in Tag 5 and compared in GC 540. All Tag 5 and GC 540 analyses showed significant differential expression between the control and the infested except *EgrOMT*. The discrepancy between the Tag 5 expression profile for *EgrOMT* between the RNA-Seq and RT-qPCR results may be clarified by redesigning the primer pair. *EgrARP* fell within the Mapman category “Hormone Signalling, Auxins” (Figure 5). Auxin has previously been associated with Hessian fly gall development (Tooker and De Moraes 2011) and was thus hypothesised to show different expression profiles between the resistant and susceptible interactions. *EgrCAD* and *EgrOMT* were categorised in the “Secondary Metabolites” (Figure 5) and were shown to be enzymatically involved in the synthesis of lignin and were thus hypothesised to inhibit the establishment of the larval feeding site through cell wall reinforcement. Liu *et* al. (2007) described this situation during an incompatible wheat-Hessian fly interaction and the opposite during a compatible interaction. It was, therefore, expected that these genes would show differing expression profiles between the resistant and susceptible interactions as shown (Figure 6). The *EgrEFE*, alternatively named 1-aminocyclopropane-1-aminocarboxylate oxidase (*ACO*), is involved in the formation of ET (Pieterse *et al*. 2012). Galling insects are known to suppress their host’s immune response during a susceptible interaction (Tooker and De Moraes 2008). It was hypothesised that different expression profiles would be observed for this gene as *L. invasa* might similarly manipulate its host. Finally, *EgrDIR* was categorised as a “PR protein” (Figure 5). A single

DIR was also shown to be up-regulated during an incompatible wheat-Hessian fly interaction (Subramanyam *et al*. 2013). This protein plays a role in the formation of lignan monomers and was thus anticipated to be similarly involved as *EgrCAD* and *EgrOMT* in the interaction.

2.6. Conclusion

In this study, we observed the categorisation of *E. grandis* genes into various defence mechanisms putatively deployed in response to *L. invasa* oviposition (Figure 6). Resistance appears to be achieved through the synthesis of insecticidal chemicals and proteins, cell wall reinforcement and the suppression of the primary metabolism and cell cycle. It is interesting to note that, although the *L. invasa* larvae had not yet hatched, the defence mechanisms that are apparently employed at this stage might be induced to prevent establishment of the feeding site as well as in anticipation of galling and herbivory. Furthermore, the resistance mechanisms identified in this study bear a striking resemblance to other transcriptome-based studies that have investigated galling insect-plant interactions, such as Liu *et al*. (2007). The use of RNA-Seq in combination with the available *Eucalyptus* genomic resources has provided the means to propose a model of the incompatible interaction between *E. grandis* and *L. invasa* upon which we can build forthcoming investigations. The integration of future studies aimed at understanding interactions between *L. invasa* and *Eucalyptus*, the transcriptional landscape across time-points and the integration of additional levels of omics data will allow the identification of the key players modulating the interaction. This study has provided some of the earliest molecular information regarding the *E. grandis*-*L. invasa* interaction which will allow for the deployment of integrated control strategies in an effort to minimise losses caused by this destructive pest.

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CHAPTER 3

Concluding Remarks

From a review of host-insect pest interactions (Chapter 1) it is clear that there is very little understanding of the plant responses to galling insects at the molecular level. For wheat, the interaction with the Hessian fly has been well characterised, whereas, knowledge of the interaction between *Eucalyptus* and the devastating insect pest, *L. invasa*, is lacking at the molecular level. The aim of Chapter 2 was to determine the transcriptomic responses of a resistant *E. grandis* clone following oviposition by the gall wasp, *L. invasa*. This is one of the earliest studies to have explored the *Eucalyptus* "defensome" using RNA-sequencing. Furthermore, this study provides one of the earliest models of the *Eucalyptus* resistance response against insects and specifically, galling insects. Future studies will have a platform upon which improvements and novel information can be added. This information will play an important role in allowing suitable targets to be selected for biotechnological applications.

Interestingly, the results from this study were supported by evidence from transcriptomic studies investigating other plant-insect interactions indicating a strong similarity in the manner in which plants generally respond to insects, even amongst groups, such as angiosperms and gymnosperms, which diverged millions of years ago. Specificity may be achieved at various stages of the interaction; however, this was beyond the design of the project and was not observed. Finally, this study produced the first comprehensive list of differentially expressed genes that may be mined for potential targets for future biotechnological applications aimed at improving resistance against *L. invasa*. A critical assessment of this study follows highlighting the difficulty associated with a host-insect interaction. The opportunity for further bioinformatics analysis to improve characterisation of splice variants as role players in defence is discussed.

Insect infestation strategy

Studies investigating plant-insect interactions use a number of different strategies to apply the herbivores to their hosts. These may include application of insect eggs, larvae or adults directly to the plant surface and spraying of insect macerate onto the plant. This study used a natural infestation of *Eucalyptus* by *L. invasa* over seven days. There are both positive and negative

consequences of this approach. This approach was used because of the difficulties involved in artificial infestations with *L. invasa* (M. Harney personal communication). The negative consequences of a natural infestation include limited control over the timing of insect oviposition thus the seven day time point that was selected would include eggs that were layed on day one through until day seven. Furthermore, other species of insects would also be free to interact with the *E. grandis* ramets, although none were observed. However, by taking this approach, the study more closely reflected the normal interaction between the gall wasp and its host.

The choice to select a single time point at seven days also has positive and negative consequences. Numerous studies have reported plant transcriptomic responses to stress within a matter of minutes to hours meaning that this study would not have observed these early responses. This time point was selected because the egg developmental phase of *L. invasa* is 14 days (Mendel *et al*. 2004) thus ensuring the same lifestage throughout the experiment and this was the minimum period of time in which sufficient material could be collected to perform RNA-sequencing. Furthermore, as the first study to explore the defensome employed by *Eucalyptus* against the gall wasp, a single time point would provide the platform from which to build a model of the interaction. Future studies will refine and improve the predictive value of the model in order to select suitable target genes for engineering resistance.

Bioinformatic opportunities to investigate unknown genes and splice variants

The sequencing and release of the *Eucalyptus grandis* genome (www.phytozome.com) provides one of the most valuable resources for understanding *Eucalyptus* and tree biology. Additionally, the *Eucalyptus* community has generated extensive “omics” data that allow identification and characterisation of large gene sets. Although the focus of most *Eucalyptus* research has been on improving wood properties (Grattapaglia *et al* 2012), the same resources may be applied to understanding and improving resistance to various stresses. This study is one of the earliest examples of this application in *Eucalyptus*. This study used the *A. thaliana* orthologs of the 2117 differentially expressed genes in order to define the putative defence mechanisms that dictated the

interaction between *E. grandis* and *L. invasa*. However, at the time of this study only 56% of the genes that showed differential expression possessed an *A. thaliana* annotation. Therefore, the role of 44% of the genes determined to respond to *L. invasa* oviposition remains unknown. This limitation in the project will be reduced in the future as the genome annotation improves.

The suit of bioinformatics tools developed by Trapnell *et al*. (2010) that were used to map the RNA-sequencing reads to the *E. grandis* genome and calculate significant gene differential expression provides a robust means of analyzing transcriptome data. The Cuffdiff program produces two outputs, one for differential expression of genes and one for transcripts. In this study, 1381 out of 44974 genes and 2507 out of 55935 transcripts were determined to be significantly differentially expressed. The transcripts were derived from 2098 genes. Of these 1362 were included in the initial 1381 genes. Differentially expressed transcripts derived from the same gene showed either that all were up-regulated, all were down-regulated or some were up- and others down-regulated. Alternative splicing events and their role in cellular biology are not well understood in plants (Syed *et al*. 2012). The majority of studies that report RNA-sequencing results refer only to differentially expressed genes and not transcripts. In this study, the datasets were combined in an attempt to include as much of the information as possible but the biological importance was beyond the scope of this investigation. It seems prudent that effort be made to improve the current understanding of alternative splicing in plants and in so doing, improve the understanding of plant biology as a whole.

Future prospects

This study reported the results of a transcriptomic response of a resistant *E. grandis* clone to oviposition by *L. invasa* at a single time point of seven days. The results highlighted a number of putative defence mechanisms employed by the host to boost resistance. However, this study does not provide a complete view of the interaction between *Eucalyptus* and the gall wasp; instead it has provided a broad outline of the defensome of *Eucalyptus* in this interaction upon which future studies can build. With the increasing availability of information, technologies and tools produced

by the plant community, it is possible to start building an "interactome" of this plant-insect relationship using a system's biology approach. For example, the sequencing of the *L. invasa* genome (B Slippers personal communication) will provide insight into the biology of the pest. The development of a model in this way will significantly improve the accuracy of predicting important genes or biological pathways that can be targeted for engineering resistance.

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SUMMARY

Summary: Transcriptional responses of *Eucalyptus* clones to the gall wasp, *Leptocybe invasa*

Caryn N Oates

Supervised by ***Dr S Naidoo, Prof AA Myburg*** *and* ***Prof B Slippers***

Submitted in partial fulfilment of the degree Magister Scientiae

Department of Genetics

University of Pretoria

Eucalyptus species constitute some of the most widely planted and economically valuable hardwood fibre crops in the world. *Leptocybe invasa* (Hymenoptera: Eulophidae), a specialist pest of *Eucalyptus*, is currently one of the most serious threats to plantation forestry. There is limited information regarding the mechanism underlying the defence response of these trees to insect pests such as *L. invasa* that limits the development of biotechnological applications aimed at reducing losses to this pest.

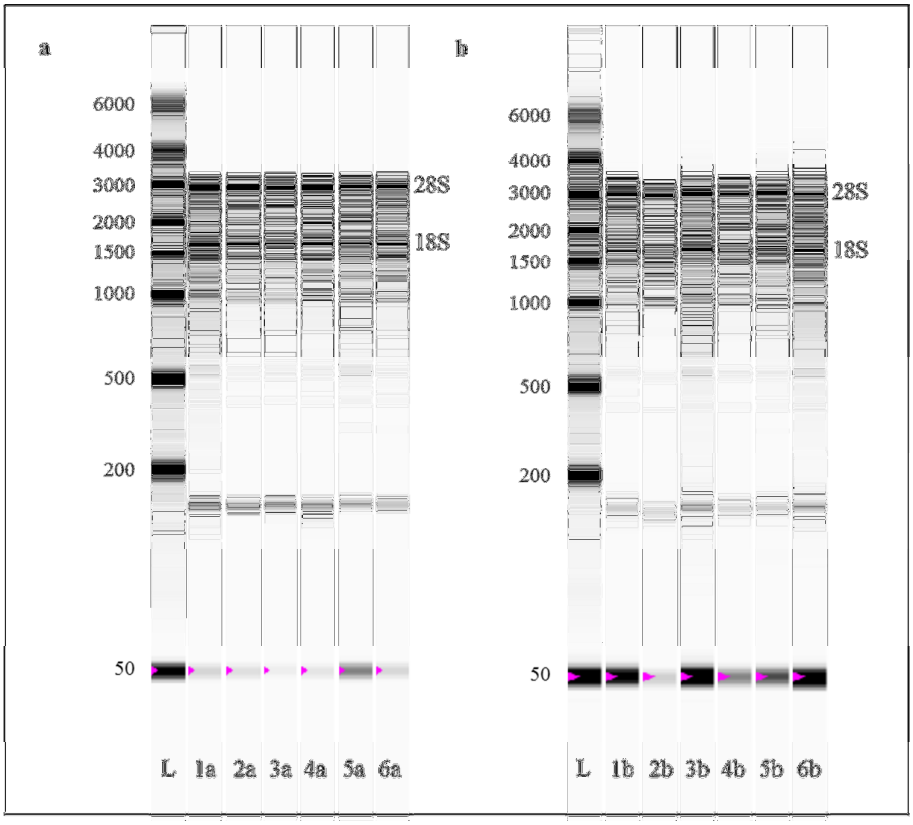
In this study we developed a hypothetical model of the *Eucalyptus* defence response through literary evidence of various plant-insect interactions. Plants have evolved a complex, multi-layered system of constitutive and inducible defences to protect themselves against phytophagous insects. These include physical or chemical barriers with toxic, repellant or anti-nutritive properties. Constitutive defences form the plant's first line of defence and provide generalised protection against most potential attackers. Once this barrier is compromised, inducible responses are activated. These comprise a multifaceted, broad-spectrum system that is sequentially activated following invader recognition. This system activates an array of defence mechanisms against the insect that is tightly regulated through hormonal and other signaling pathways.

We investigated an incompatible interaction between a resistant *Eucalyptus grandis* genotype and *L. invasa*. Transcript profiling using RNA-Seq of midrib tissue from challenged and unchallenged trees revealed 2117 genes with significantly altered expression patterns. Functional annotation of

the differentially expressed genes provides support for specific resistance mechanisms being employed by the host. This information was used to fine-tune the literary model that was initially hypothesised.

This study found differentially expressed genes that were categorized into recognition, regulatory and defence mechanisms. These included *R* genes which encode proteins that allow a specific recognition of the pest. The recognition signal is transferred to the cell through signalling pathways that allow an appropriate response to be elicited. We identified genes encoding calcium-responsive proteins that are known signal transducers. Other responses include redox-related whose products aid the oxidative burst, an accumulation of toxic reactive oxygen species. The phytohormones mediate signalling pathways that allow a fine regulation of the induced defence responses. We identified genes related to the JA, SA, ET, GA, BR and auxin metabolic pathways. JA is described as the dominant hormone regulating plant defence against insects and crosstalk between the hormone pathways allow fine-tuning of the defence response. Other differentially expressed genes encoded proteins involved in larval antibiosis, cell wall reinforcement to impede the establishment of the feeding site and volatile release to attract natural enemies of the pest. Furthermore, we observed apparent suppression of the primary metabolism to reduce nutrient availability and suppression of the cycle to reduce the insect's ability to manipulate its host. The results were used to improve the hypothetical defence model and provide a first glimpse of the *Eucalyptus* defensome.

Supplementary Information



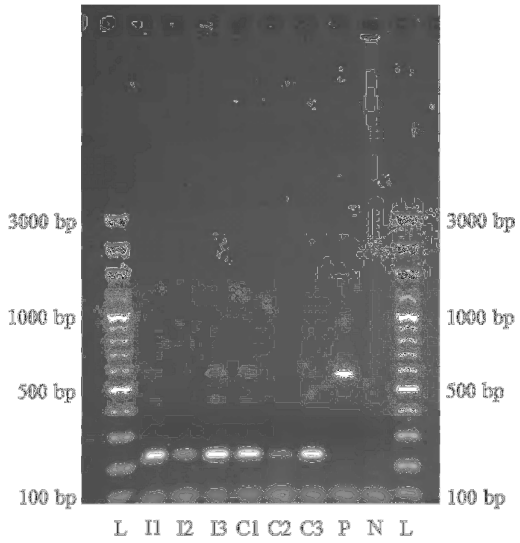
FigureS1. Experion RNA pseudo-gel of total RNA isolated from (a) Tag 5 and (b) GC 540. A prominent band was observed for both the 18S and 28S ribosomal units. These results indicate high-quality, non-degraded RNA. The wells are labelled as follows (L) Molecular Weight Standard, (1) Infested 1, (2) Infested 2, (3) Infested 3, (4) Control 1, (5) Control 2 and (6) Control 3.

TableS1. Concentration and quality control of total Tag 5 and GC 540 RNA material as calculated using the Experion RNA analysis system.

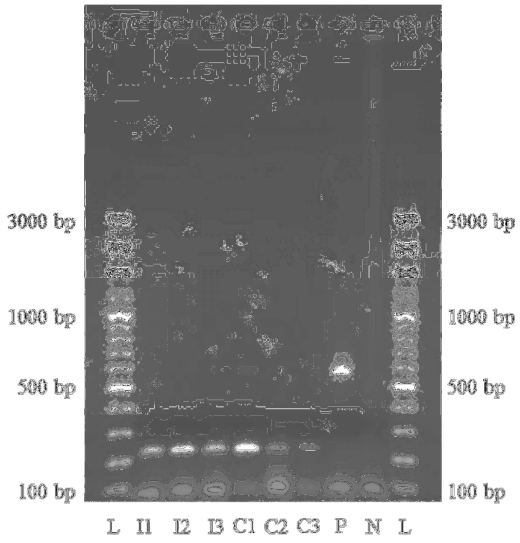
Well	Sample Name	RNA Concentration (ng/µl)	RQI
L	Ladder	160.0	-
1a	Tag 5 Infested 1	67.5	9.7
2a	Tag 5 Infested 2	347.1	9.8
3a	Tag 5 Infested 3	58.6	9.1
4a	Tag 5 Control 1	140.4	9.6
5a	Tag 5 Control 2	108.7	9.5
6a	Tag 5 Control 3	64.9	9.0
1b	GC 540 Infested 1	539.6	8.9
2b	GC 540 Infested 2	468.2	9.7
3b	GC 540 Infested 3	866.1	9.7
4b	GC 540 Control 1	601.1	9.7
5b	GC 540 Control 2	130.2	9.1
6b	GC 540 Control 3	382.2	9.4

Well names correspond to lane labels in Figure S1.

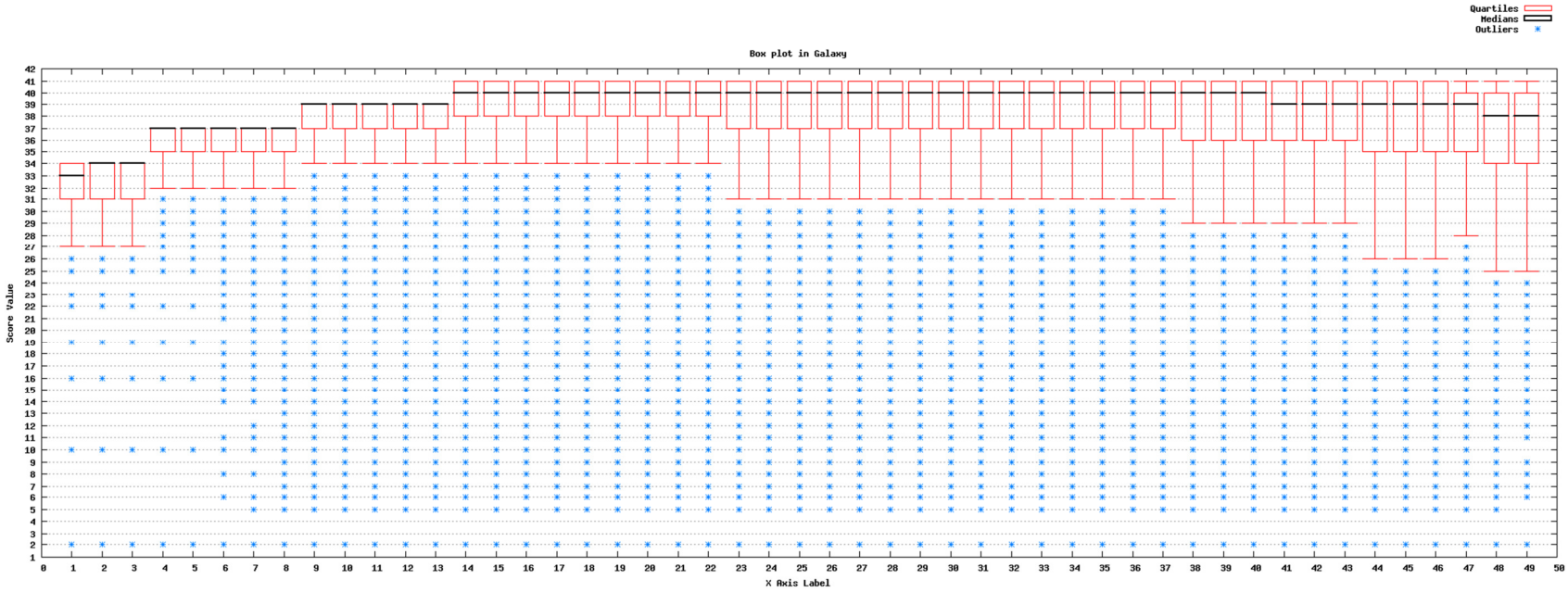
RNA Quality Index (RQI) value is an indication of the quality of the RNA. Values of 8.0 and higher indicate high-quality, non-degraded RNA.



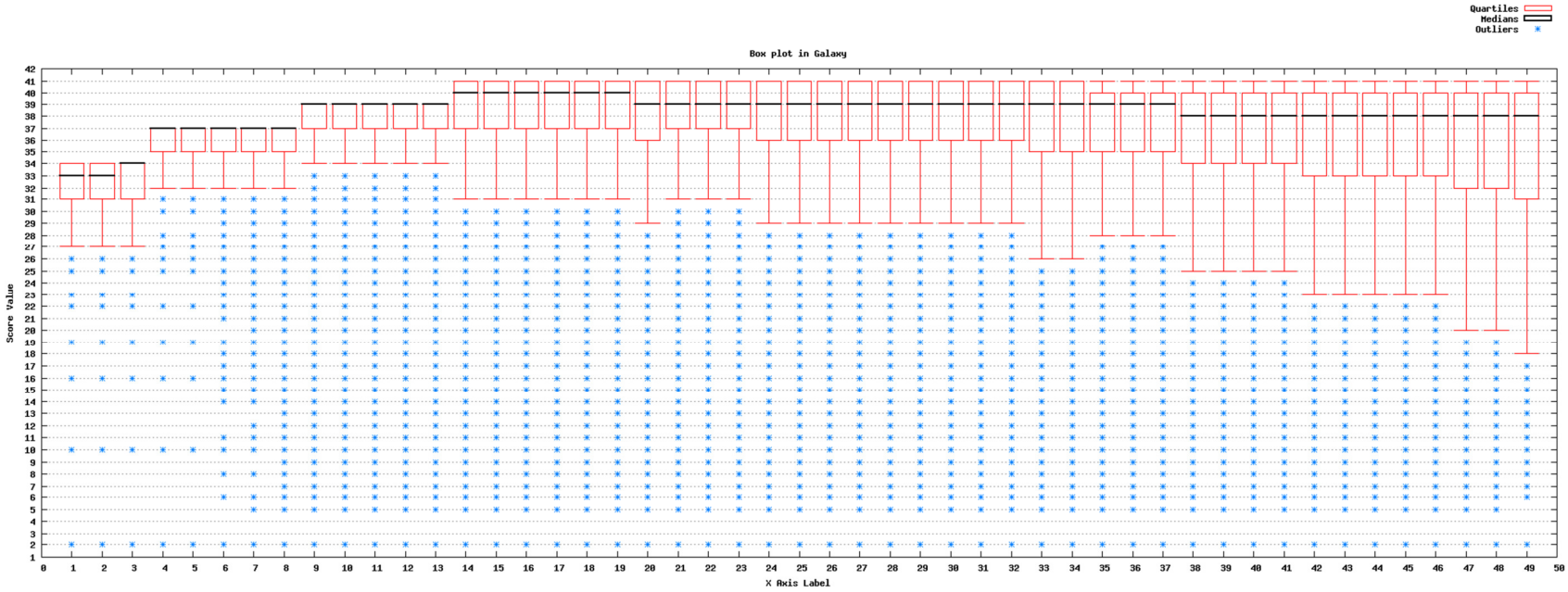
FigureS2. Gel electrophoresis results testing for the presence of contaminant genomic DNA in cDNA synthesised from Tag 5 material. cDNA was PCR amplified using the intron-spanning primer pair *EgrPIPB*. The presence of genomic DNA contamination would be identified by a 590 bp band, as observed in the positive control (P) lane. Uncontaminated cDNA was identified by a single 217 bp band. The lanes are identified as follows: (L) Molecular Weight Standard, (I1) Infested 1, (I2) Infested 2, (I3) Infested 3, (C1) Control 1, (C2) Control 2, (C3) Control 3, (P) positive control and (N) negative control. PCR amplicons were analysed on a 1% (w/v) agarose gel in 1X TAE buffer.



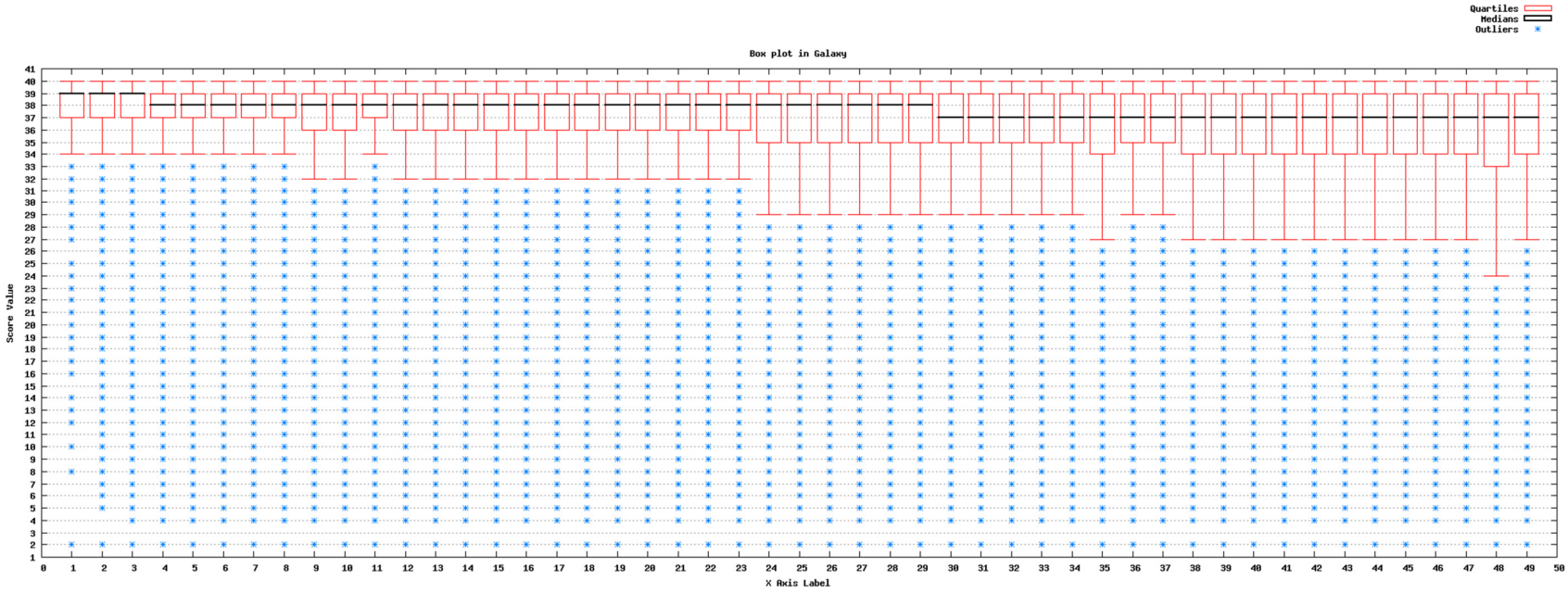
FigureS3. Gel electrophoresis results testing for the presence of genomic DNA in cDNA synthesised from GC 540 material. cDNA was PCR amplified using the intron-spanning primer pair *EgrPIPB*. The presence of genomic DNA contamination would be identified by a 590 bp band, as observed in the positive control (P) lane. Uncontaminated cDNA was identified by a single 217 bp band. The lanes are identified as follows: (L) Molecular Weight Standard, (I1) Infested 1, (I2) Infested 2, (I3) Infested 3, (C1) Control 1, (C2) Control 2, (C3) Control 3, (P) positive control and (N) negative control. PCR amplicons were analysed on a 1% (w/v) agarose gel in 1X TAE buffer.



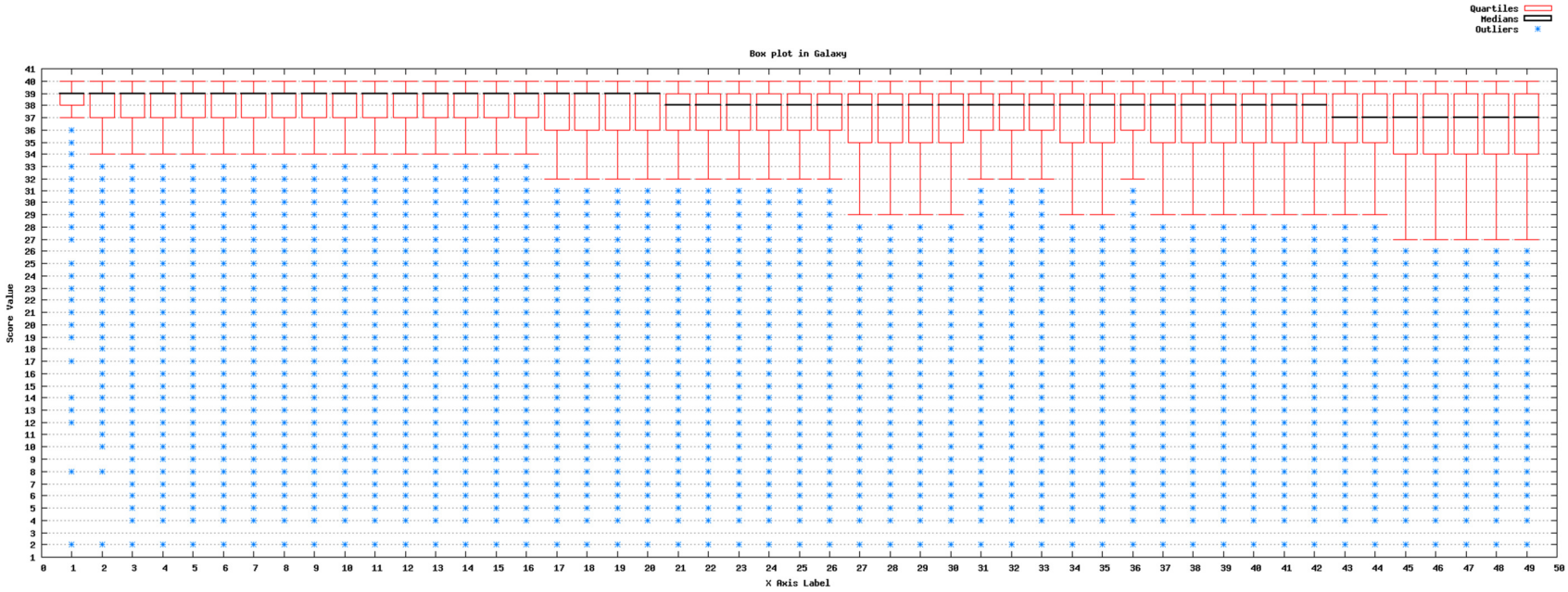
FigureS4. Boxplot of quality values of Tag 5 Infested 1 forward reads as determined by FASTQ. The y-axis represents the Phred score. The x-axis represents the base position in the read. Black horizontal lines are medians. Red boxes show the inter-quartile range (IQR) (top value is Q3, bottom value is Q1). Whiskers show outliers at max 1.5 x IQR. Blue asterisks indicate outliers.



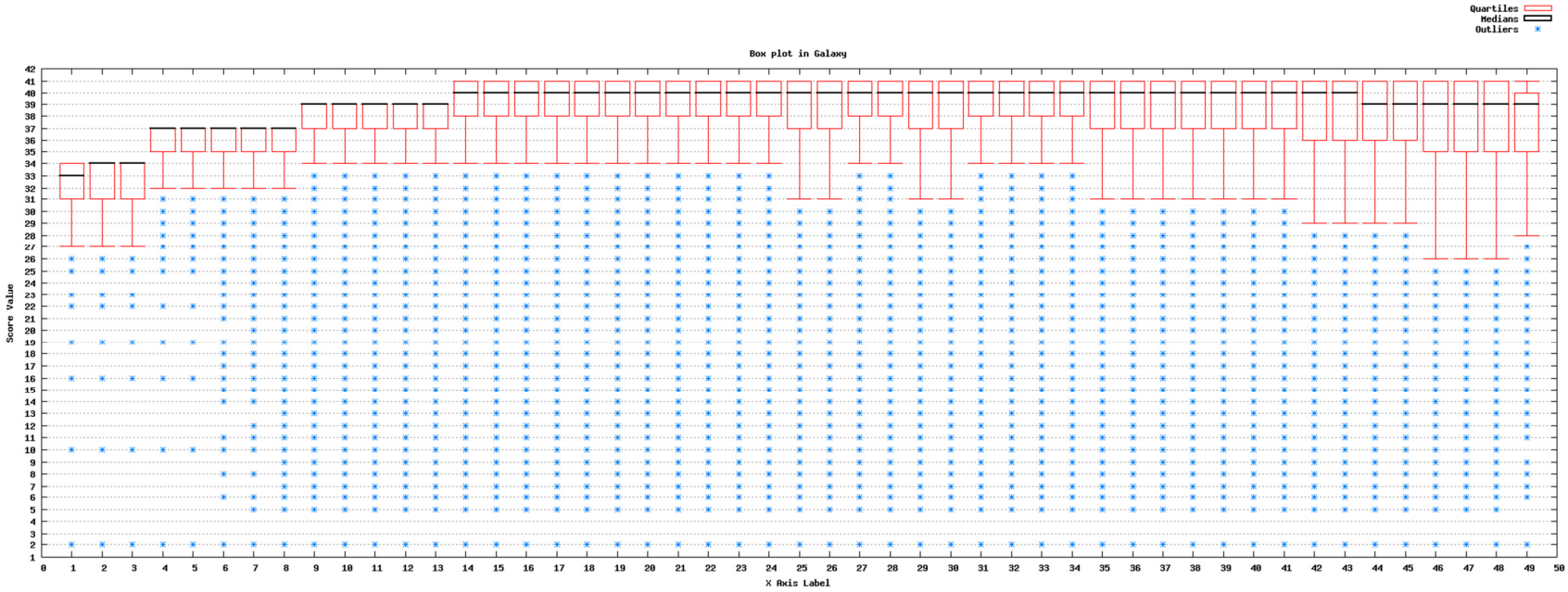
FigureS5. Boxplot of quality values of Tag 5 Infested 1 reverse reads as determined by FASTQ. The y-axis represents the Phred score. The x-axis represents the base position in the read. Black horizontal lines are medians. Red boxes show the inter-quartile range (IQR) (top value is Q3, bottom value is Q1). Whiskers show outliers at max 1.5 x IQR. Blue asterisks indicate outliers.



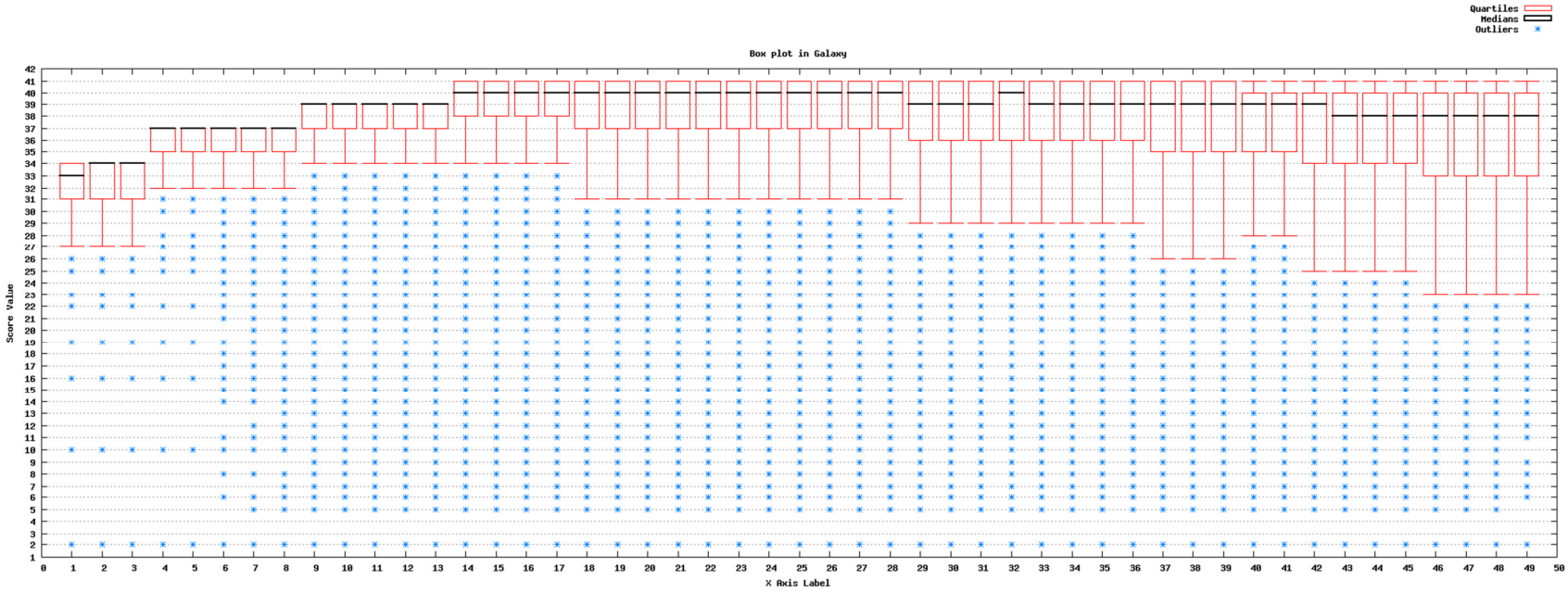
FigureS6. Boxplot of quality values of Tag 5 Infested 2 forward reads as determined by FASTQ. The y-axis represents the Phred score. The x-axis represents the base position in the read. Black horizontal lines are medians. Red boxes show the inter-quartile range (IQR) (top value is Q3, bottom value is Q1). Whiskers show outliers at max 1.5 x IQR. Blue asterisks indicate outliers.



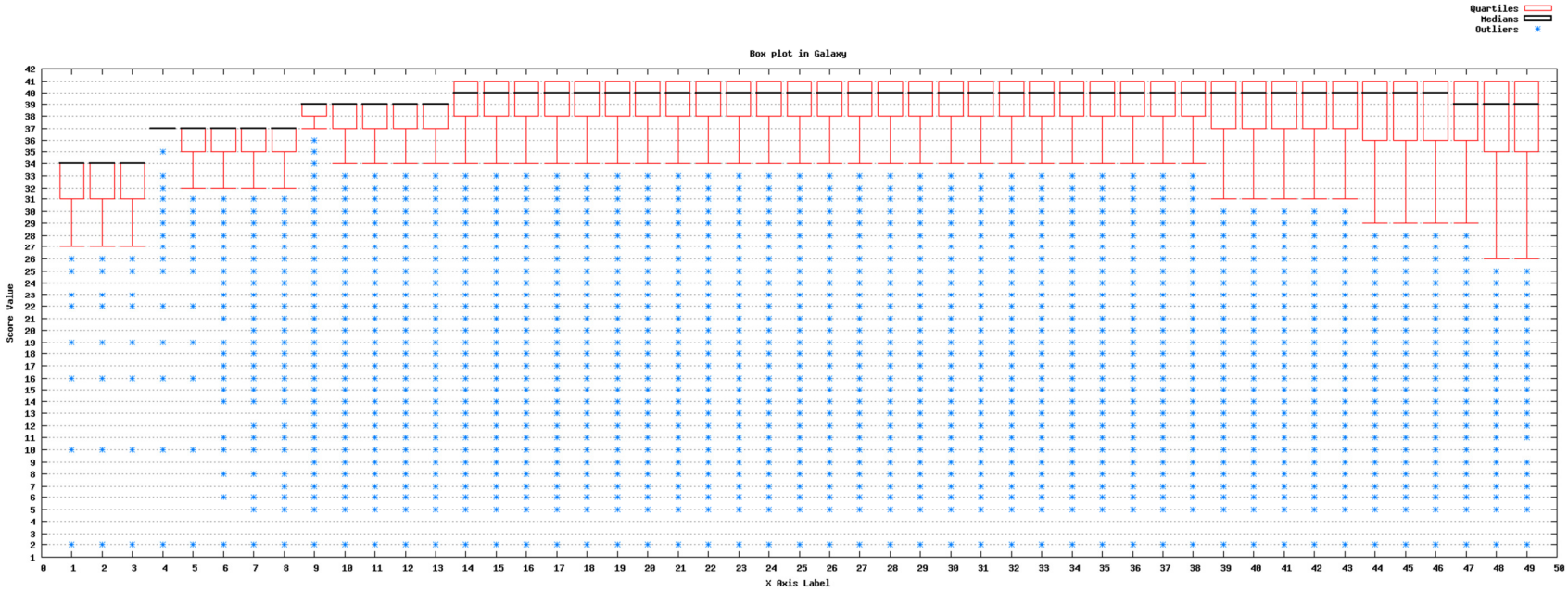
FigureS7. Boxplot of quality values of Tag 5 Infested 2 reverse reads as determined by FASTQ. The y-axis represents the Phred score. The x-axis represents the base position in the read. Black horizontal lines are medians. Red boxes show the inter-quartile range (IQR) (top value is Q3, bottom value is Q1). Whiskers show outliers at max 1.5 x IQR. Blue asterisks indicate outliers.



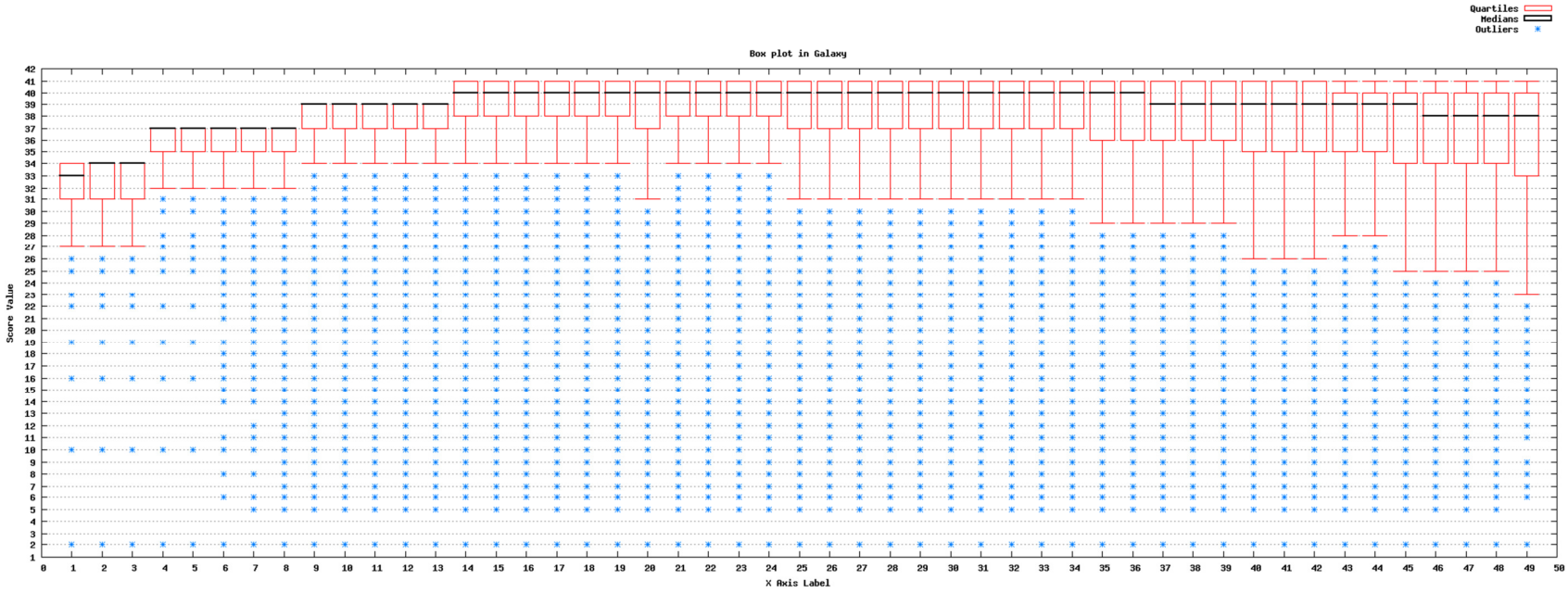
FigureS8. Boxplot of quality values of Tag 5 Infested 3 forward reads as determined by FASTQ. The y-axis represents the Phred score. The x-axis represents the base position in the read. Black horizontal lines are medians. Red boxes show the inter-quartile range (IQR) (top value is Q3, bottom value is Q1). Whiskers show outliers at max 1.5 x IQR. Blue asterisks indicate outliers.



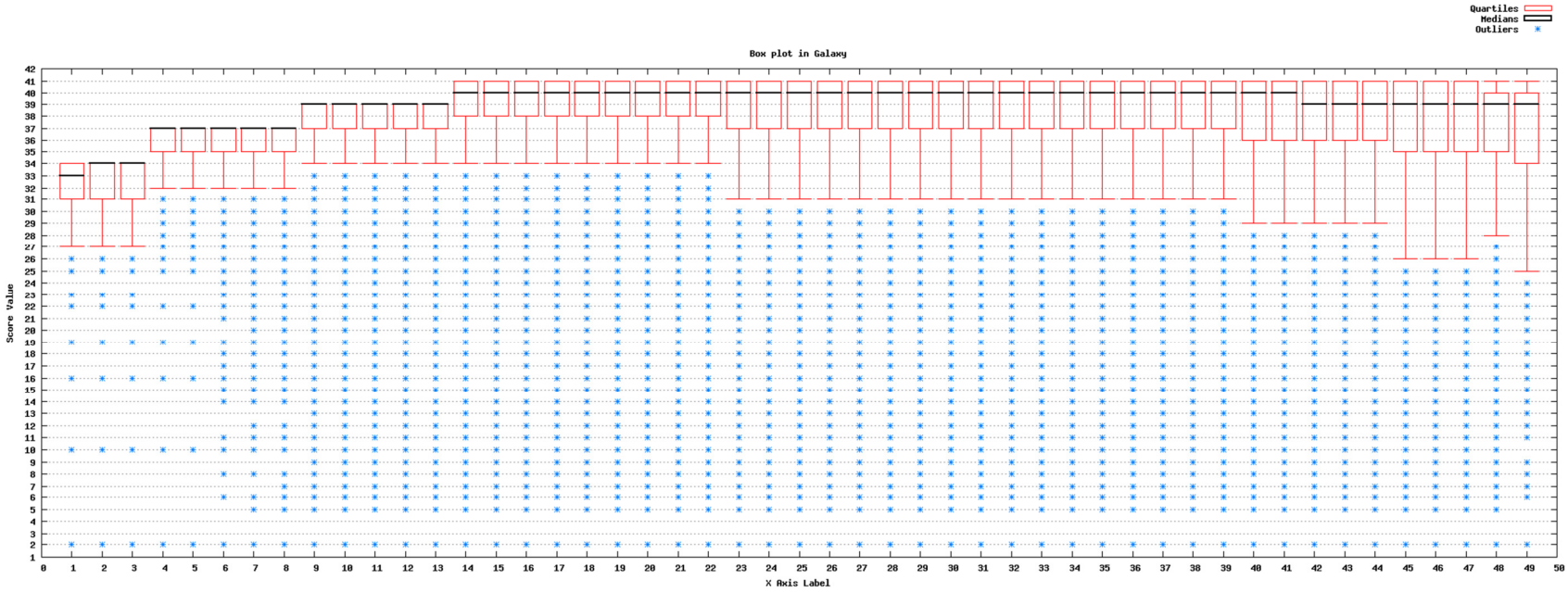
FigureS9. Boxplot of quality values of Tag 5 Infested 3 reverse reads as determined by FASTQ. The y-axis represents the Phred score. The x-axis represents the base position in the read. Black horizontal lines are medians. Red boxes show the inter-quartile range (IQR) (top value is Q3, bottom value is Q1). Whiskers show outliers at max 1.5 x IQR. Blue asterisks indicate outliers.



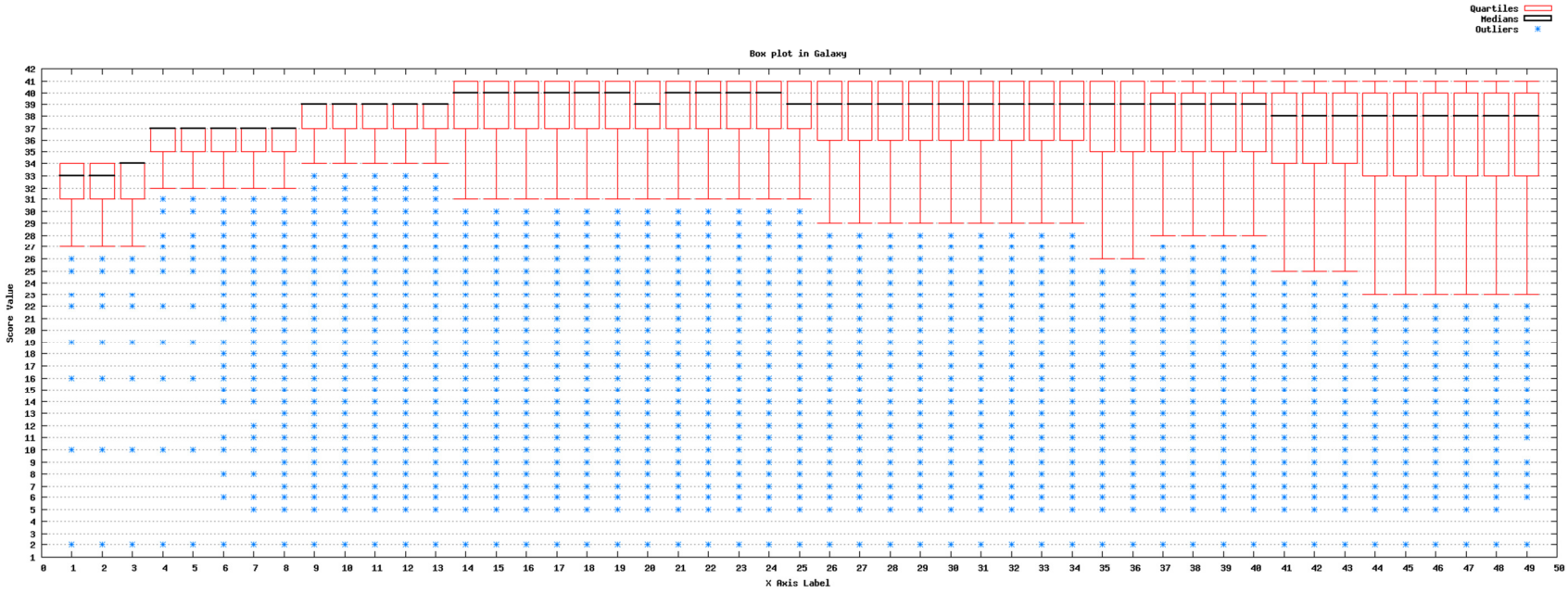
FigureS10. Boxplot of quality values of Tag 5 Control 1 forward reads as determined by FASTQ. The y-axis represents the Phred score. The x-axis represents the base position in the read. Black horizontal lines are medians. Red boxes show the inter-quartile range (IQR) (top value is Q3, bottom value is Q1). Whiskers show outliers at max 1.5 x IQR. Blue asterisks indicate outliers.



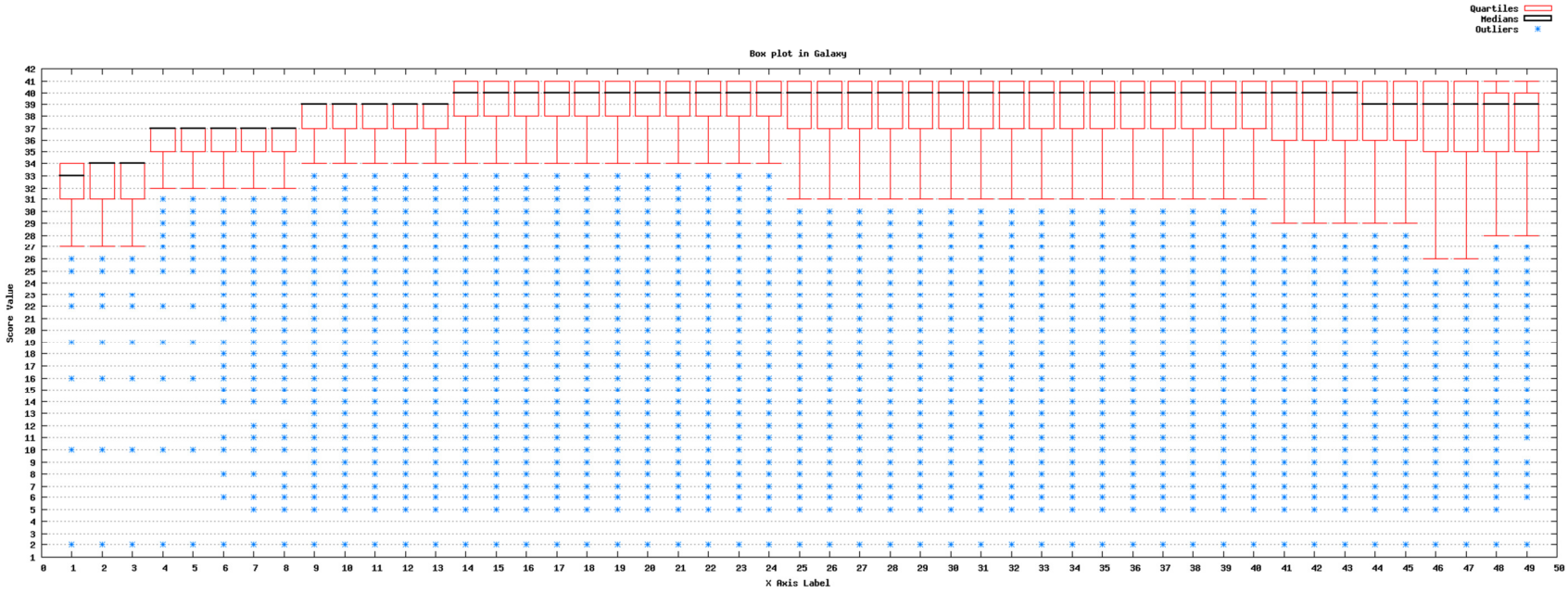
FigureS11. Boxplot of quality values of Tag 5 Control 1 reverse reads as determined by FASTQ. The y-axis represents the Phred score. The x-axis represents the base position in the read. Black horizontal lines are medians. Red boxes show the inter-quartile range (IQR) (top value is Q3, bottom value is Q1). Whiskers show outliers at max 1.5 x IQR. Blue asterisks indicate outliers.



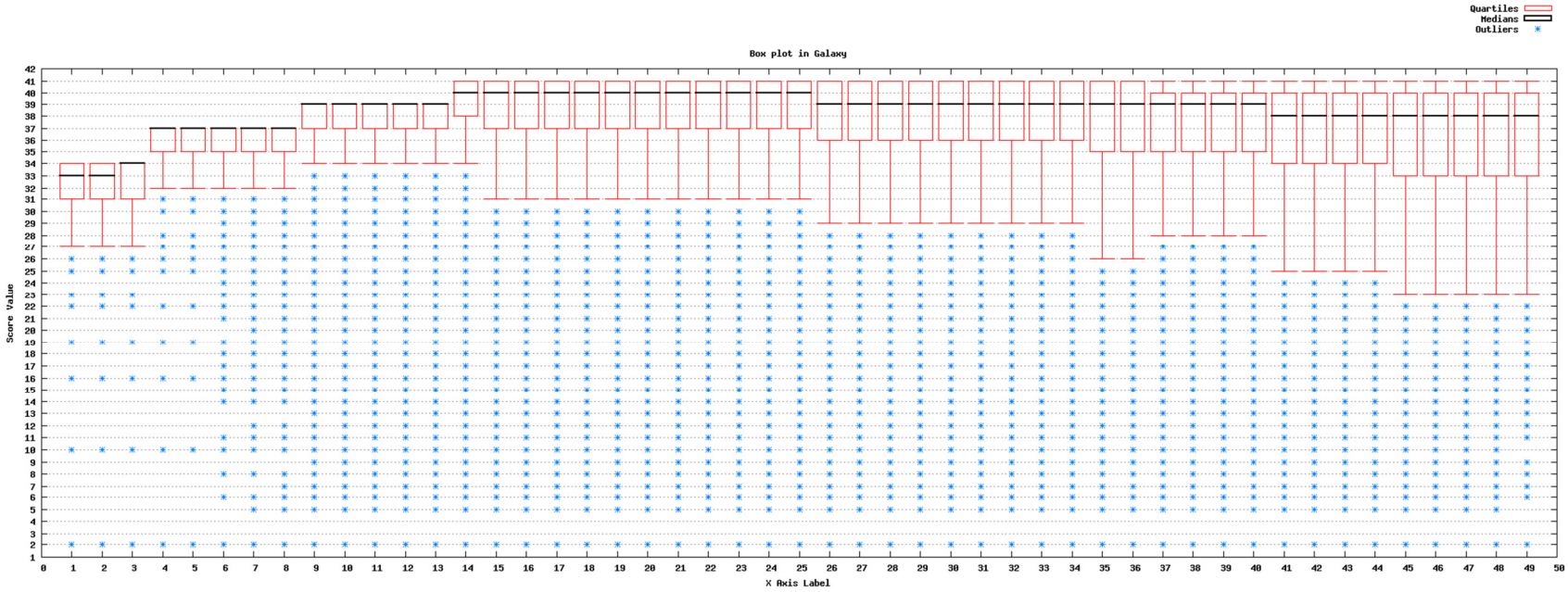
FigureS12. Boxplot of quality values of Tag 5 Control 2 forward reads as determined by FASTQ. The y-axis represents the Phred score. The x-axis represents the base position in the read. Black horizontal lines are medians. Red boxes show the inter-quartile range (IQR) (top value is Q3, bottom value is Q1). Whiskers show outliers at max 1.5 x IQR. Blue asterisks indicate outliers.



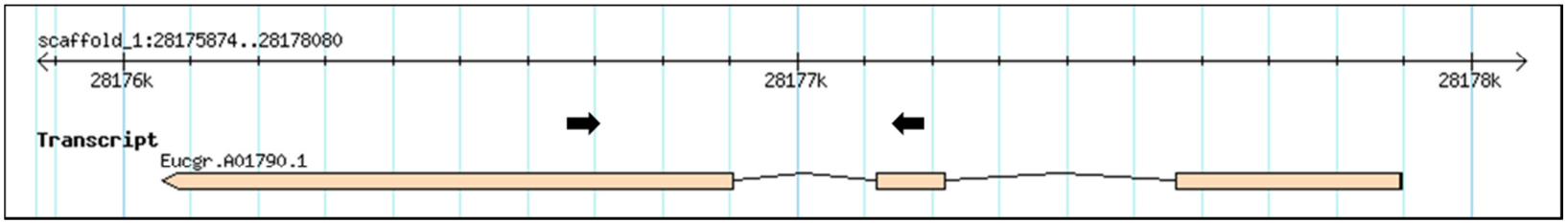
FigureS13. Boxplot of quality values of Tag 5 Control 2 reverse reads as determined by FASTQ. The y-axis represents the Phred score. The x-axis represents the base position in the read. Black horizontal lines are medians. Red boxes show the inter-quartile range (IQR) (top value is Q3, bottom value is Q1). Whiskers show outliers at max 1.5 x IQR. Blue asterisks indicate outliers.



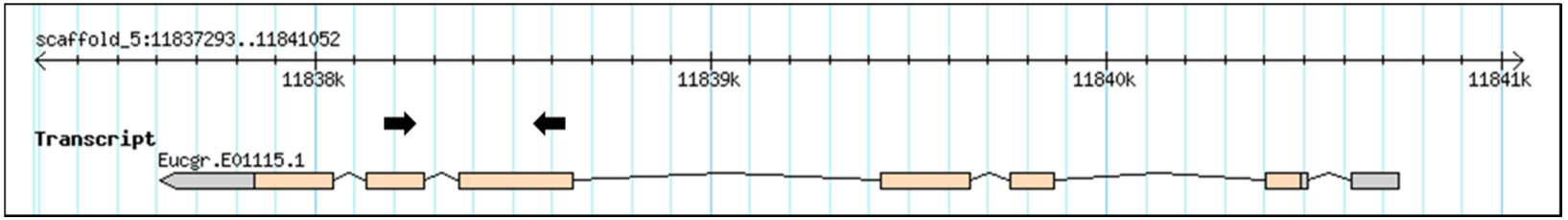
FigureS14. Boxplot of quality values of Tag 5 Control 3 forward reads as determined by FASTQ. The y-axis represents the Phred score. The x-axis represents the base position in the read. Black horizontal lines are medians. Red boxes show the inter-quartile range (IQR) (top value is Q3, bottom value is Q1). Whiskers show outliers at max 1.5 x IQR. Blue asterisks indicate outliers.



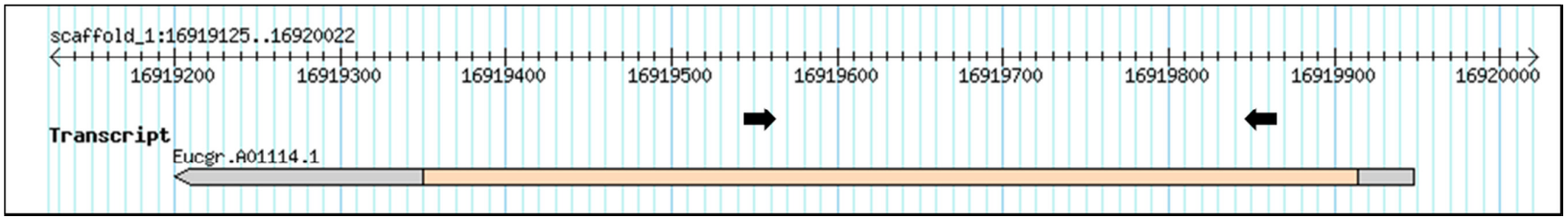
FigureS15. Boxplot of quality values of Tag 5 Control 3 reverse reads as determined by FASTQ. The y-axis represents the Phred score. The x-axis represents the base position in the read. Black horizontal lines are medians. Red boxes show the inter-quartile range (IQR) (top value is Q3, bottom value is Q1). Whiskers show outliers at max 1.5 x IQR. Blue asterisks indicate outliers.



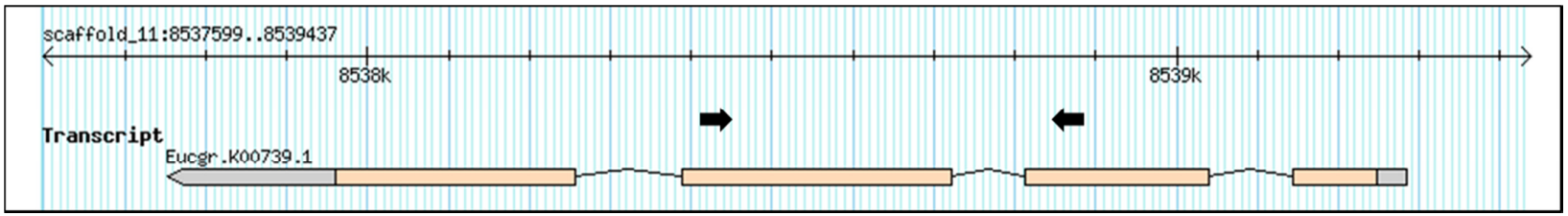
FigureS16. *EgrARP* amplicon map. Genome location of the auxin-responsive protein gene that was amplified in this study. The orange region represents coding sequence. The black connectors represent introns. The black arrows show the region of the transcript that was amplified (213 bp).



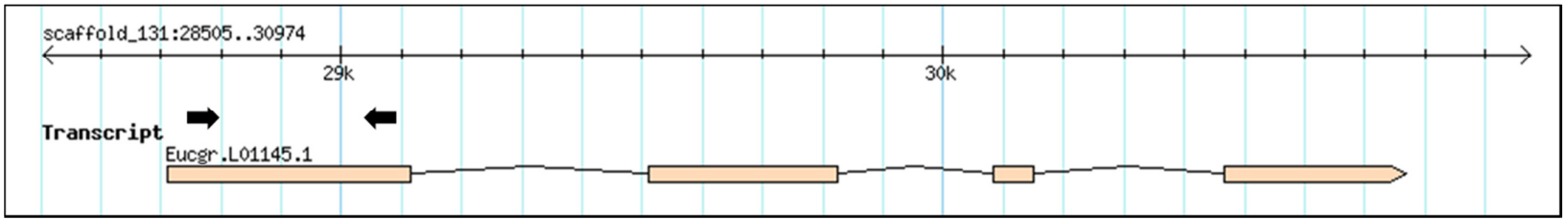
FigureS17. *EgrCAD* amplicon map. Genome location of the cinnamyl-alcohol dehydrogenase gene that was amplified in this study. The grey regions on the transcript represent the 5` and 3` UTR. The orange region represents coding sequence. The black connectors represent introns. The black arrows show the region of the transcript that was amplified (285 bp).



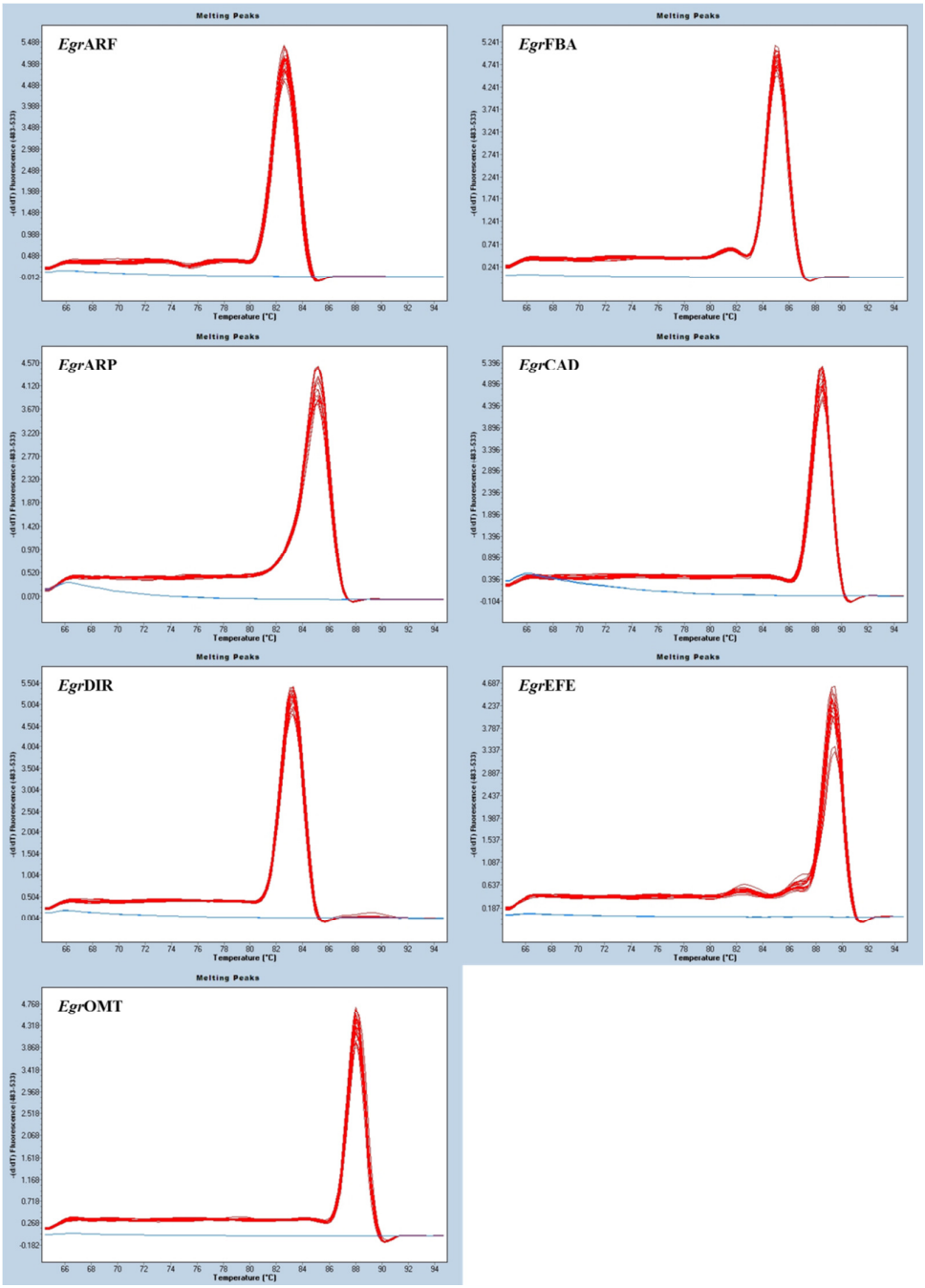
FigureS18. *EgrDIR* amplicon map. Genome location of the disease resistance-responsive dirigent-like protein gene that was amplified in this study. The grey regions on the transcript represent the 5` and 3` UTR. The orange region represents coding sequence. The black connectors represent introns. The black arrows show the region of the transcript that was amplified (232 bp).



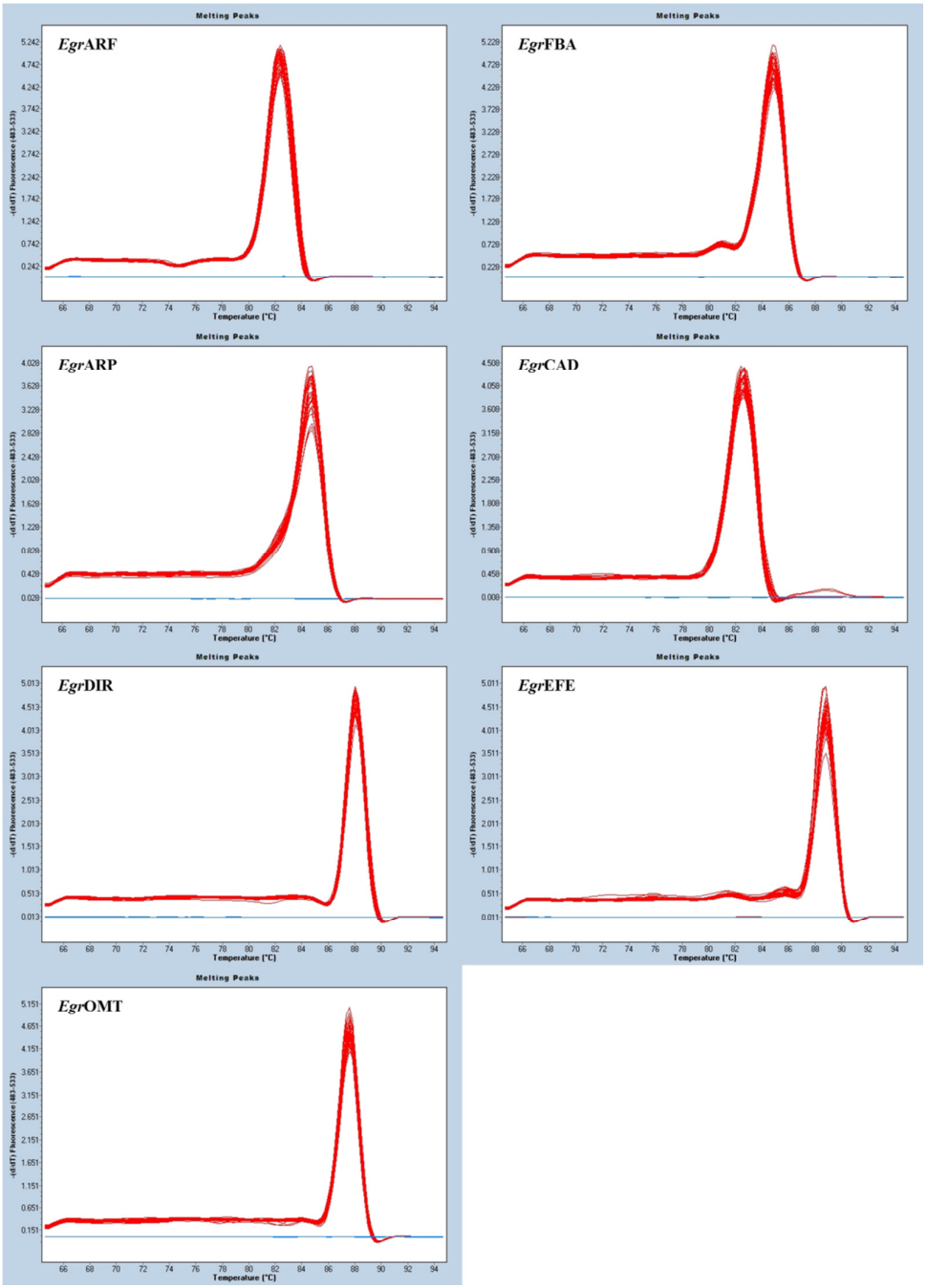
FigureS19. *EgrEFE* amplicon map. Genome location of the ethylene-forming enzyme gene that was amplified in this study. The grey regions on the transcript represent the 5` and 3` UTR. The orange region represents coding sequence. The black connectors represent introns. The black arrows show the region of the transcript that was amplified (379 bp).



FigureS20. *EgrOMT* amplicon map. Genome location of the O-methyltransferase gene that was amplified in this study. The orange region represents coding sequence. The black connectors represent introns. The black arrows show the region of the transcript that was amplified (233 bp).



FigureS21. Melting curves of the target and reference genes used in the Tag 5 expression validation analyses. Melting curves were generated from the Roche LightCycler® 480 Real-Time PCR system. The x-axis depicts the temperature range. The y-axis depicts the -(d/dT) fluorescence (483-533) measurement.



FigureS22. Melting curves of the target and reference genes used in the GC 540 comparative transcript abundance analyses. Melting curves were generated from the Roche LightCycler® 480 Real-Time PCR system. The x-axis depicts the temperature range. The y-axis depicts the -(d/dT) fluorescence (483-533) measurement.

TableS2. Amplification efficiencies of the primer pairs for the selected target and reference genes used for RT-qPCR validation and comparison in Tag 5 and GC 540, respectively.

	Resistant Interaction: Tag 5				Susceptible Interaction: GC 540			
Gene	**E [(a)]**	**r^2 [(b)]**	**M [(c)]**	**CV [(d)]**	**E [(a)]**	**r^2 [(b)]**	**M [(c)]**	**CV [(d)]**
EgrARF	1.934	0.99	0.186	0.064	1.975	0.915	0.276	0.094
EgrFBA	1.835	0.967	0.186	0.064	2.023	0.96	0.276	0.097
EgrARP	1.835	0.91	-	-	2.008	0.907	-	-
EgrCAD	1.994	0.945	-	-	1.831	0.95	-	-
EgrDIR	1.95	0.955	-	-	1.92	0.906	-	-
EgrEFE	1.807	0.931	-	-	2.111	0.898	-	-
EgrOMT	2.052	0.974	-	-	1.838	0.971	-	-

[(a)] Efficiency (E) indicates amplification efficiency of the primer pair.

[(b)] Coefficient of determination (r^2) indicates a linear standard curve and high reaction efficiency. The r^2 value should be close to 1.000.

[(c)] M value indicates the stability of a gene when tested in combination with all other reference genes.

[(d)] Coefficient of variance (CV) indicates the stability of the reference gene across all samples in dataset.