Uncovering the defence responses of *Eucalyptus* to pests and pathogens in the genomics age

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Abstract

Long-lived tree species are subject to attack by various pests and pathogens during their lifetime. This problem is exacerbated by climate change which may increase the host range for pathogens and extend the period of infestation by pests. Plant defences may involve pre-formed barriers or induced resistance mechanisms based on recognition of the invader, complex signalling cascades, hormone signalling, the activation of transcription factors and the production of pathogenesis-related (PR) proteins with direct antimicrobial or anti-insect activity. Trees have evolved some unique defence mechanisms compared to well-studied model plants. The genome sequence of *Eucalyptus grandis* has recently become available and provides a resource to extend our understanding of defence in large woody perennials. This review synthesises existing knowledge of defence mechanisms in model systems and other tree species and features mechanisms that may be important for defence in *Eucalyptus*, such as anatomical variants and the role of chemicals and proteins. Based on the *E. grandis* genome sequence, we identified putative PR proteins based on sequence identity to the previously described plant PR proteins. Putative orthologs for PR-1, PR-2, PR-4, PR-5, PR-6, PR-7, PR-8, PR-9, PR-10, PR-12, PR-14, PR-15 and PR-17 were identified and compared to the number of PR genes in *Populus trichocarpa* and *Arabidopsis thaliana*. Genomic resources available for *Eucalyptus* are discussed and approaches for improving resistance in these hardwood trees, earmarked as a bioenergy source in future, are considered.

Keywords: *plant immunity; anatomical defences; terpenoid; phytohormone; genomic resources; transcriptomics; breeding; biotechnology*

Introduction

Eucalyptus species and hybrids form the basis of the hardwood forestry industry worldwide. These species are grown in tropical, subtropical and temperate regions, and are the largest hardwood plantation crop in the world, estimated to occupy over 20 million hectares (www.git-forestry.com). *Eucalyptus* trees are sourced for wood, paper and pulp products and have also been recognised as a potential source of biofuels (Hinchee et al. 2011). The use of forest trees as a biofuel source is an attractive alternative to biofuel crops, since it would circumvent competition with food production. Another advantage is that trees would potentially have a higher biomass production capacity than biofuel crops (Rathmann et al. 2010).

Eucalyptus species are endemic to Australia and neighbouring islands, but are planted as exotics in various other countries due to their fast growth rate, short rotation time, high productivity and adaptability to a broad range of environments (Eldridge et al. 1993). Despite the success eucalypts have shown as a plantation species, these trees do succumb to pests and pathogens that may originate from their native or introduced environments. Host shifts from native Myrtacea to *Eucalyptus* plantations have previously been reported (Slippers et al. 2005). Examples of pests and pathogens currently posing a threat to *Eucalyptus* include the myrtle rust pathogen *Puccinia psidii*, the stem canker pathogen *Chrysoporthe austroafricana*, the root rot pathogen *Phytophthora cinnamomi*, and the insect pest *Leptocybe invasa* (reviewed in Wingfield et al. 2008). These threats are managed by planting tolerant *Eucalyptus* genotypes (hybrids) or, in the case of *L. invasa*, the use of biological control as part of an integrated management system to curb losses (Prof. Bernard Slippers, FABI, personal communication). While resistance against pests and pathogens in forest trees can be ascribed to a combination of stochastic genetic variation (Yanchuk et al. 1988), evolved immunity (Liu and Ekramoddoullah 2004), plasticity and environmental conditions (Cruickshank et al. 2010), climate change is predicted to make environments more favourable for pests and pathogens in future (reviewed in Sturrock et al. 2011). A single insect or pathogen threat could have devastating consequences for *Eucalyptus* plantations, especially since *Eucalyptus* is increasingly being clonally propagated. An entire clone could be lost due to a single pathogen or pest. Forest disease and pest management have recognised the need for the development of resistant plant varieties as part of an integrative approach to curb disease incidence (reviewed in Wingfield et al. 2013). This will require an understanding of the defence responses to these pests and pathogens in *Eucalyptus*. The increasing availability of genomic tools is expected to accelerate this type of research and this goal may be achieved in the near future. This review describes our current knowledge of plant defence, garnered from model systems, and explores the recent advances in this area within the *Eucalyptus* genus.

Eucalyptus ***genomic resources***

One of the most significant milestones in *Eucalyptus* genomics is the recent sequencing of the *E. grandis* genome (www.phytozome.net) by the United States (US) Department of Energy (DOE) Joint Genome Institute (JGI, Myburg et al. 2011). Additionally, the *Eucalyptus* research community has produced extensive genomic and transcriptomic data from various sources that provide opportunities for genome mining. Most of these studies focused on understanding and improving wood properties for commercial applications (reviewed in Grattapaglia et al. 2012).

Rengel et al. (2009) developed the EUCAWOOD database as a resource for functional genomics studies investigating wood formation and molecular breeding. In 2010, Rosa et al. used the *Eucalyptus* expressed sequence tags (ESTs) Genome Project (FORESTs) transcriptome data to identify *Eucalyptus* ESTs that were either induced or not induced by various stress agents. A number of known defence-associated genes were identified and various mechanisms of defence against abiotic and biotic factors were described (Rosa et al. 2010). Much of the data produced from these studies is publicly available and has been used to describe various aspects of *Eucalyptus* biology. In addition, a number of studies have used expression-based data from Suppressive Subtractive Hybridisation (SSH) studies, EST libraries and microarrays to infer defence responses in *Eucalyptus* (Duplessis et al. 2005; Feng et al. 2012; Rosa et al. 2010). The *Eucalyptus* Genome Network (EUCAGEN, web.up.ac.za/eucagen/) is a consortium of researchers aimed at the development of *Eucalyptus* genomic resources and provides links to various useful sites and tools for this field. For example, the *Eucalyptus* Genome Integrative Explorer (EucGenIE, www.eucgenie.org) is an online resource for *Eucalyptus* genomics and transcriptomics that provides access to several RNA sequencing (RNA-Seq) datasets, including those described by Mizrachi et al. (2010), that were produced from a range of tissues.

Analysis of the *Eucalyptus* transcriptome during pest or pathogen challenge is a more direct approach to obtaining a genome-wide view of defence responses (Padovan et al. 2013) and gene discovery within important *Eucalyptus* defence signalling pathways in these studies is facilitated by novel, high-throughput technologies such as Illumina® RNA-Seq.

These resources may be instrumental in obtaining a better understanding of the defensive capabilities of these economically important trees, which will certainly be of key interest in the future (Wingfield et al. 2013). Although a number of studies have been conducted in this field, much of the understanding of the defensive capabilities is dependent on inferences from other, better-studied organisms such as *Arabidopsis thaliana* (thale cress).

Plant defence systems: lessons from model species

Plants have to defend themselves against various pathogens and pests during their life-time. Pathogens may be viral, bacterial, oomycete or fungal and can adopt a biotrophic, hemibiotrophic, or necrotrophic lifestyle. Biotrophs feed on living cells, maintaining host cell viability, while necrotrophs rely on dead tissues as a source of nutrients. Hemibiotrophs have an early, transient biotrophic phase followed by a necrotrophic phase. Pests may be specialists (small host range) or generalists (broad host range), and include chewing, piercing and sucking, mining, boring and galling insects (Wylie and Speight 2012). In addition to the virulence determinants of the invading agent and environmental factors, the outcome of the host-pest or -pathogen interaction also depends on the plant's constitutive and induced defences.

The first line of defence against biotic invaders in plants is preformed. Plants can possess anatomical variants correlated with levels of disease resistance (Fahn 1988). Some of these anatomical features include mechanical barriers to pest or pathogen invasion, such as the bark, the pectin and lignin components of plant cell walls and the leaf cuticle. Other anatomical features associated with defence include secretory cells, glands and ducts that produce and transport defensive substances. These anatomical characteristics can be constitutive or induced by injury or exposure to invading agents (Eyles et al. 2004; Fahn 1988; Franceschi et al. 2005; Kovalchuk et al. 2013). Other preformed defences include the production of antimicrobial peptides and toxic secondary metabolites that are released upon insect or pathogen attack (recently reviewed in Kovalchuk et al. 2013).

In addition to being a passive barrier to pathogen invasion, plant cell walls are actively modified at sites of interaction with fungi and bacteria, and become reinforced by the deposition of cell wall appositions, referred to as papillae. In the event of successful fungal penetration, cell wall-associated structures such as haustorial encasements, collars or neck bands, are formed to halt pathogen spread (reviewed in Micali et al. 2011; Underwood 2012). In the event of oviposition, some host plants are able to produce neoplasms (tumour-like growth of undifferentiated cells) beneath the egg, halting larval entry (Doss et al. 2000). Other preformed defences may involve stored chemicals that are released upon attack.

Vascular plants can have single cells, cavities, ducts or glandular trichomes that secrete substances such as terpenes, waxes and flavonoids. In ducts and cavities, the innermost epidermal cell layer secretes most of these substances, but the other layers may also become involved. The outermost cell layer may become thickened and act as a protective sheath surrounding the cavity (Fahn 1988). In *Eucalyptus* leaves, the essential oils are produced and stored in sub-dermal secretory cavities. These oil glands can also occur in the bark, pith, phloem, roots, petiole and midrib, and the number and location of secretory cavities and ducts, as well as the age at which they are most abundant, differs widely between eucalypt species (Carr and Carr

1970). It was recently shown that the secretory cavities of several species of *Eucalyptus* do not only contain essential oils, but also a resinous substance of unknown function (Goodger et al. 2009).

An interesting non-specific response to wounding or infection in *Eucalyptus* and many other woody plants is the formation of barrier zones or reaction zones in the new tissue produced by the vascular cambium (Tippett and Shigo 1981; Wilkes 1986). These zones appear to protect the healthy sapwood from damage by separating it from the adjacent damaged tissue. Reaction zones formed after inoculation of *E. nitens* with decay fungi are visually distinct from healthy sapwood. They also have a lower pH and moisture content than the adjacent sapwood and are enriched with phenolics, hydrolysable tannins and tyloses (Barry et al. 2001; Barry et al. 2000). In *E. globulus* and *E. nitens*, barrier zones that form after wounding can contain dark extractives and occasionally kino (gum) veins (Eyles and Mohammed 2002b). In another study, it was shown that the degree of kino vein formation in response to treatment with the ethylene-releasing compound 2-chloroethyl phosphonic acid (CEPA) differs between species, and that kino veins may occur in different tissues depending on the age of the tree (Eyles and Mohammed 2002a). The new tissue formed at the wound site, referred to as wound wood, contains both callus and altered wood and is morphologically variable. The presence of dark extractives in this tissue, which consist of various secondary metabolites, could be unique to eucalypts (Eyles et al. 2003c). This suggests the involvement of reaction zones in chemical and mechanical defence against damage and invading agents in *Eucalyptus*. Similarly, the bark wounding response usually involves the formation of a ligno-suberised boundary zone directly adjacent to the wound site (Biggs 1985; Woodward and Pearce 1988; Woodward and Pocock 1996). The cells adjacent to the boundary zone de-differentiate, forming a wound periderm, and phenolic and terpene compounds have been found in the lesion margin. A wound periderm layer was formed in *E. globulus* naturally infected with stem canker fungi in the genus *Cytonaema*. This wound periderm appears to separate the necrotic and healthy tissues, possibly preventing the spread of infection. A complete or a partial wound periderm layer was formed in superficially infected plants, while traumatic oil glands with suberised cell walls were present in the newly synthesised phloem following more severe infection resulting in destruction of the vascular cambium. The lesion margin contained a number of compounds that were absent from healthy phloem, and several constituents of the essential oil also differed in relative abundance between these tissues (Eyles et al. 2003a). Mechanical wounding also results in the formation of traumatic oil glands and changes in oil composition within the new phloem (Eyles et al. 2004). Very few studies have focused on identifying the anatomical variants associated with disease resistance in eucalypts.

If preformed defences are breached, a pathogen or pest would encounter inducible defence responses. Induced responses rely on the plant's ability to distinguish self from non-self, which is analogous to that seen in animal immunity (Jones and Dangl 2006). However, in contrast to animals, plants lack an adaptive immune system involving somatic recombination of genes, and have no circulating immune cells. Therefore, they rely on the innate defences of each cell to respond to microbial or pest attack. This requires recognition, subsequent signalling, and the production of defensive products. Figure 1 depicts the recognition and induced defence events that follow pest or pathogen challenge.

Recognition of non-self relies on the perception of general elicitors called pathogen-associated molecular patterns, microbe-associated molecular patterns (PAMPs or MAMPs; Figure 1), or in the case of insect pests, damage-associated molecular patterns (DAMPs; Heil 2009) or herbivore-associated molecular patterns (HAMPs; Mithöfer and Boland 2008). These molecular patterns are perceived by pattern recognition receptors (PRRs; Figure 1; Dardick and Ronald 2006), and recognition leads to the relatively weak, non-specific immune response termed pattern-triggered immunity (PTI). These general elicitors are usually molecules that are essential for the invader's life cycle (reviewed in Nurnberger and Lipka 2005;

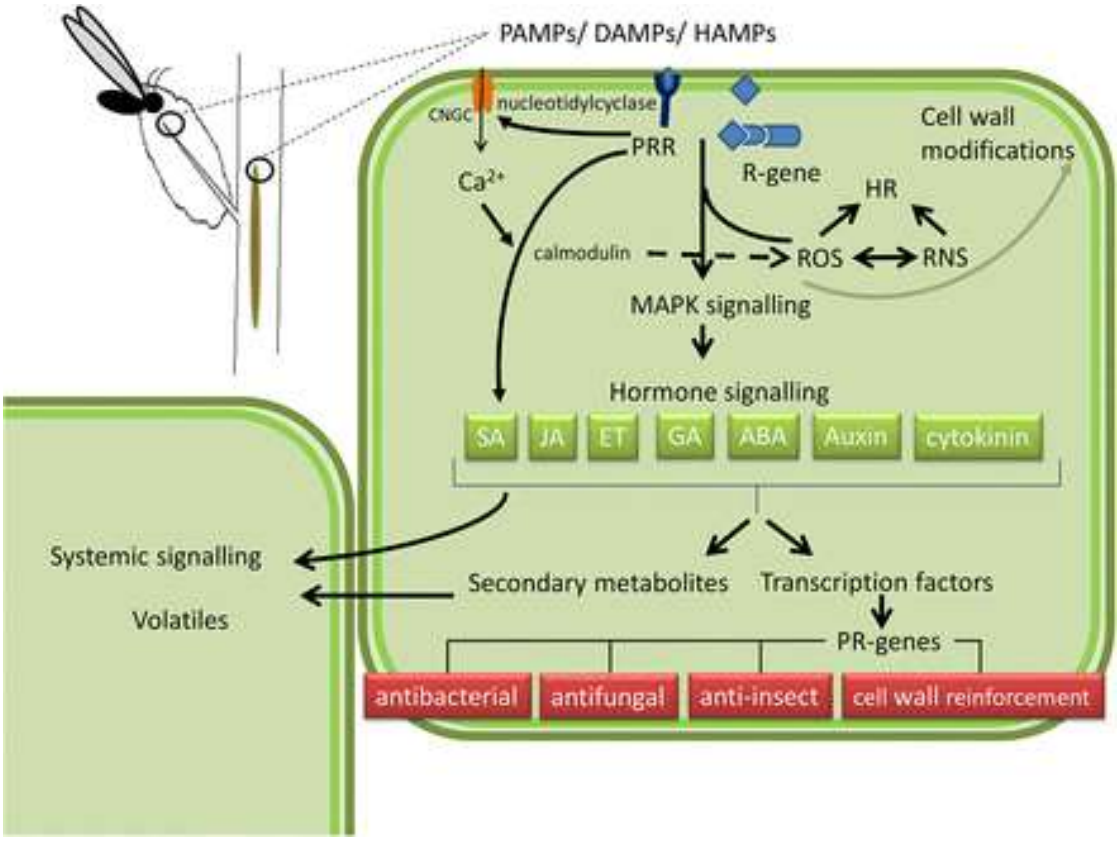

Figure 1. Simplified schematic representing induced responses in host cells to pest and pathogens. PAMPs, HAMPs or DAMPs from pest or pathogen attack are perceived by PRRs in the plasma membrane. Recognition may alternatively involve the detection of avirulence (*Avr)* genes by *R* genes. This results in the production of ROS and reactive nitrogen species (RNS) which contributes to the formation of the HR. Following recognition, a MAPK signalling cascade is initiated and various hormones are also involved in amplifying the defence signal. Secondary metabolites are produced that may result in volatile production, alerting neighbouring cells to the threat. Various TFs are produced that activate *PR* genes which may have direct antibacterial, antifungal or anti-insect activity. Systemic signals prime neighbouring cells and distal tissue for subsequent attack.

van Loon 2009). Interestingly, the expression of the *Arabidopsis* PRR known as EF-Tu receptor (EFR) that recognizes the bacterial elongation factor Tu, or EF-Tu in *Nicotiana benthamiana* (tobacco) and *Solanum lycopersicum* (tomato) enhanced broad-spectrum resistance to various phytopathogenic bacteria (Lacombe et al. 2010). This is evidence that cross-species PRRs could potentially be used to improve resistance and could be attractive targets for manipulation in *Eucalyptus.*

Some pathogens are able to suppress PTI by delivering specific effector proteins to the plant cells. This is known as effector-triggered susceptibility (ETS, Jones and Dangl 2006). Recent evidence suggests that diverse pathogens have shared or similar effector targets within the host (Dou and Zhou 2012). Various levels of plant defence are targeted by effectors, including penetration resistance, recognition by PRRs, phytohormone levels and signalling pathways, host secretory pathways, plant cell death (Dou and Zhou 2012), and suppression of cell wall modifications (Truman et al. 2006). Downstream of PTI, effectors can directly target aspects of the mitogen-activated protein kinase (MAPK) cascade (Wang et al. 2010; Zhang et al. 2010; Zhang et al. 2007). In another example, an RXLR effector from the potato blight fungus *Phytophthora infestans* can target the host immune secretory pathway by inhibiting secretion of C14, a papain cysteine protease (Bozkurt et al. 2011). Apart from effectors, it has also recently been demonstrated that small fungal RNAs are able to hijack the host's RNA interference pathway by binding to Argonaute 1 and silencing genes involved in plant defence (Weiberg et al. 2013). Based on these examples, it seems highly likely that host targets are actively suppressed in *Eucalyptus* pest or pathogen interactions as well. As a first step towards testing this hypothesis, the genome sequence data that are available for pests or

pathogens of *Eucalyptus* can be used to identify orthologs of the above-mentioned effectors alongside potential targets in the host.

The second type of pest or pathogen perception involves the recognition of effectors by plant resistance (R) proteins (Dodds and Rathjen 2010; Jones and Dangl 2006); Figure 1). Several classes of *R* genes have been identified and the majority contain nucleotide binding site (NBS) and leucine-rich repeat (LRR) domains (Dodds and Rathjen 2010). R protein-mediated recognition leads to a more specific, rapid and usually effective defence response that is termed effector-triggered immunity (ETI). This response involves mechanisms such as the oxidative burst and the hypersensitive response (HR, a type of programmed cell death which serves to restrict the spread of the pathogen) and is associated with extensive changes in gene expression (Dodds and Rathjen 2010; Jones and Dangl 2006). *R* genes have been exploited in a number of crop species as an apparently effective part of strategies to control various pests and pathogens (Dodds and Rathjen 2010; Stuart et al. 2012). An *R* gene for resistance against *Puccinia psidii* (*Ppr1*) has been discovered in *E. grandis* (Junghans et al. 2003) and identified as a target for marker-assisted introgression into susceptible backgrounds. This approach may be implemented in the future. Gene expression analysis suggested that a HR may also be involved in conferring resistance to this pathogen (Moon et al. 2007).

Defence signals following pathogen and pest recognition

The oxidative burst is one of the most immediate pathogen-induced defence responses and is characterized by the rapid and transient production of large amounts of reactive oxygen species (ROS) at the site of attempted pathogen invasion (Wojtaszek 1997 ; Figure 1). ROS act as signals for the activation of plant defence responses and are able to diffuse across membranes, reaching locations distal to the initial site of production (Wojtaszek 1997). The generation of ROS promotes the accumulation of salicylic acid (SA) and pathogenesis-related (*PR*) gene transcripts (Chen et al. 1995; Maleck and Dietrich 1999). The oxidative burst is directly harmful to invading pathogens and also contributes to cell death, since ROS generated via the oxidative burst play a central role in the development of a HR (Grant and Loake 2000; Lamb and Dixon 1997; Lecourieux et al. 2002). Nitric oxide (NO) is also a key player in the development of HR (Figure 1; Delledonne et al. 2001). Whilst the HR is efficient in curbing the spread of biotrophic pathogens, it contributes to the virulence of necrotrophic pathogens because it involves cell death (Mengiste 2012). Therefore, it is crucial for the host to be able to adjust its responses to the type of invading pathogen.

Calcium (Ca^{2+}) is another important secondary messenger in plant defence. Specific changes in spatial and temporal cytosolic calcium concentrations brought about by various signalling pathways are referred to as 'calcium signatures,' which are thought to encode stimulus-specific information (reviewed in Lecourieux et al. 2006). However, elicitors (substances that stimulate plant defence) are able to induce increases in both cytosolic and nuclear Ca^{2+} concentrations, which suggests that the levels of nuclear Ca^{2+} are also important during defence (Lecourieux et al. 2006). Interestingly, it has been suggested that these prolonged increases in cytosolic Ca^{2+} concentration induce similar defence responses irrespective of the elicitor (Ma and Berkowitz 2007); Figure 1). Although it is not yet clear exactly how changes in calcium concentration enhance defence responses, some potential mechanisms have been proposed. For example, changes in cytosolic calcium levels also affect the expression of *PR* genes, phytoalexin accumulation (Blume et al. 2000; Mithöfer et al. 1999) and HR-related cell death (Grant and Loake 2000; Lecourieux et al. 2002). The importance of calcium signalling during morphogenesis has been demonstrated in *E. urophylla* callus tissue (Arruda et al. 2000), and Ramos et al. (2009) showed that colonisation of *E. globulus* roots by an ectomycorrhizal fungus alters calcium ion flux. However, the role of calcium signalling during defence in *Eucalyptus* has not, to our knowledge, been studied.

Once a pathogen has been detected, MAPKs translate the extracellular signal, which is perceived by a plasma membrane receptor *via* a phospho-relay of three types of reversibly phosphorylated kinases: MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). This leads to the phosphorylation of substrate proteins, effecting a range of responses involving subcellular modelling, which occurs shortly after signal transduction, and gene expression, which occurs at later stages of the defence response (reviewed in Rodriguez et al. 2010; Samajova et al. 2013); Figure 1). MAPKs may target various effector proteins in the cytoplasm or nucleus, such as other kinases, enzymes, and transcription factors (TFs, Rodriguez et al. 2010).

Various phytohormones are involved in amplifying the initial defence signal, including jasmonic acid (JA), ethylene (ET) and SA (Figure 1). A large number of studies conducted in various hosts have shown that an antagonistic relationship exists between SA and JA (Pieterse et al. 2012). In *Arabidopsis*, this antagonism is partially controlled by ET, and is dependent on the concentration of each of the hormones (Leon-Reyes et al. 2010; Pre et al. 2008). The antagonistic relationship between SA and JA also been observed in *Eucalyptus* (Naidoo et al. 2013), but the role of ET in this antagonism remains to be investigated. It has also been shown that treatment with an ethylene-releasing compound results in defence-related anatomical and chemical responses in *E. globulus, E. nitens* and *E. obliqua* (Eyles and Mohammed 2002a). Interestingly, in a recent study where the foliar defences of different *Eucalyptus* clones were compared, treatment with methyl jasmonate (MeJA) did not induce a significant change in either terpene and formylated phloroglucinol compound (FPC) production or herbivore performance. Instead, differences between clones suggested that constitutive expression of secondary metabolites may be more important in herbivore defences (Henery et al. 2008).

In *Arabidopsis*, SA is required for hemi- and biotrophic disease resistance whilst JA and ET are essential for defences against necrotrophs (Glazebrook 2005; Mengiste 2012; Pieterse et al. 2012). While this holds true for the majority of plant-pathogen interactions, some exceptions have been identified in tree species. In *Populus* (poplar), JA and ET are required for defence against biotrophic rust fungi in the genus *Melampsora*. (Azaiez et al. 2009). Similarly, SA is required for defence against the suspected fungal necrotroph *C. austroafricana* in *Eucalyptus* (Naidoo et al. 2013). It is plausible that the relationships between the phytohormones and the balance required for maintaining an effective defence response may be more complex in woody tree species than in the non-woody model plant *Arabidopsis*. Furthermore, evidence that suggests the involvement of other phytohormones, such as brassinosteroids, auxins, cytokinins, gibberelins (GAs) and abscisic acid in maintaining homeostasis between signalling pathways during defence is emerging. Further exploration will be required to define the defence-related functions of these hormones more precisely (Bari and Jones 2009; Denancé et al. 2013). Pathogens are also able to target components of the host signalling pathways in order to evade host defences. For example, an ectomycorrhizal fungus antagonises the activity of *Eucalyptus* signalling molecules such as indole-3-acetic acid (IAA) and ET (Ditengou and Lapeyrie 2000), possibly by inhibiting the IAA-dependent superoxide production of plant peroxidases (Kawano et al. 2001).

Apart from local responses to threats and pathogens, plants are able to activate three main signalling pathways that induce resistance in tissues distal to the primary site of attack (Eyles et al. 2010; Spoel and Dong 2012). The pathways that lead to induced resistance in plants are systemic acquired resistance (SAR), induced systemic resistance (ISR) and systemic induced resistance (SIR; Eyles et al. 2010). SAR arises mainly from SA signalling pathways during the HR (Mishina and Zeier 2007; Ryals et al. 1996). SAR and SA-related defence responses are traditionally associated with defence against biotrophic pathogens. Even so, a recent study by Naidoo et al. (2013) showed the induction of SA-related *PR* genes in response to *C.*

austroafricana infection, indicating that infection by this suspected necrotroph could also induce SAR in *E. grandis*. ISR is established and maintained through JA/ET signalling pathways that are induced by non-pathogenic rhizobacteria and fungi that promote plant growth or certain necrotizing pathogens (Thatcher et al. 2005; van Loon 2007; van Wees et al. 2008). The siderophore pseudobactin from fluorescent *Pseudomonas* bacteria induces ISR against the bacterial wilt pathogen, *Ralstonia solanacearum* in *E. urophylla* (Ran et al. 2005). SIR differs from ISR mainly because it is induced by both biotic wounding (for example, by herbivores) and abiotic (mechanical) wounding, whilst ISR is not induced by abiotic wounding (Gurr and Rushton 2005; van Loon 2007). SAR, ISR and SIR all result in priming, the phenomenon where appropriate stimuli induce cellular defence responses in tissues distal to the point of stimulation as well as in neighbouring plants (Pozo et al. 2008). Since this priming effect could be exploited for disease protection, the effects of various biotic and abiotic inducers have been investigated in a wide range of crop species (reviewed in Walters et al. 2013). Many of these inducers remain to be tested on long-lived species such as eucalypts.

PR proteins are another important part of the plant immune system (Figure 1). There are 17 currently known PR protein families (PR-1 through -17) in plants (reviewed in van Loon 2009) and a possible PR-18 family in *Helianthus annuus* (sunflower) and *Amaranthus caudatus* (foxtail amaranth) (Custers et al. 2004; van Loon et al. 2006). The expression of genes encoding the PR families PR-1, PR-2, PR-3, PR-5, PR-9, PR-10 and PR-12 is induced by pathogens in different forest tree species (Veluthakkal and Dasgupta 2010). Rosa et al. (2010) also identified *PR-1* through *PR-10* based on EST sequences from *Eucalyptus*. We determined the repertoire of *PR* genes in the *E. grandis* genome. Genes were either annotated as *PR* genes in the *E. grandis* v1.1 genome sequence available on Phytozome, or the putative orthologs were determined based on sequence identity to the type sequences listed by van Loon (2009); BLASTP; $e < 10^{-50}$). Figure 2 shows the number of genes within the PR-gene family in the *E. grandis* genome alongside the number identified in *Populus trichocarpa* and *A. thaliana* (Duplessis et al. 2009). Our recent analyses of the *E. grandis* transcriptome under biotic stress (Naidoo et al. unpublished, Oates et al. unpublished) provides evidence for differential expression of a number of the PR gene family members (Figure 2). Except for *PR-5* and *PR-9*, the *PR* gene family sizes in *E. grandis* and *P. trichocarpa* are similar. The gene families in these species are closer to each other in size than to those of *A. thaliana.* There are twice as many *PR-5* genes in *E. grandis* compared to *P. trichocarpa* and almost three times more compared to *A. thaliana*. PR-5 proteins, which are part of the large thaumatin-like protein (TLP) family, have previously been shown to have activity against fungal and oomycete pathogens. For example, over-expression of a thaumatin-like protein from *Camellia sinensis* (tea plant) provided enhanced tolerance to *Phytophthora infestans* in potato (Acharya et al. 2013), and expression of *PdPR5* in *Prunus domestica* (European plum) facilitated resistance to brown rot caused by the necrotrophic fungus *Monilinia fructicola* (El-kereamy et al. 2011). There are twice as many PR-9 genes in *E. grandis* compared to *A. thaliana*. PR-9 proteins are peroxidases, which are involved in the cross-linking of polysaccharides and extension of phenylpropanoid monomers during cell wall reinforcement (Passardi et al. 2004). Some members of this group, such as AtPRX33, are also involved in PTI. While no PR-7, PR-8, PR-15 and PR-17 orthologs have been identified in *P. trichocarpa* or *A. thaliana*, putative homologs of their type members (Sels et al. 2008) were identified in the *E. grandis* genome. PR-7 proteins are similar to those within the PR-6 family and are considered proteinase inhibitors, which are important for defence against insects (Ryan 1990). PR-8 proteins (like PR-3, PR-4 and PR-11 proteins) are chitinases that hydrolyse the β-1,4-linkage between *N*-acetylglucosamine residues of fungal chitin (van Loon 2009). PR-15 proteins are involved in the production of hydrogen peroxide (H_2O_2), which is toxic to pests and pathogens (van Loon 2009). PR-17 proteins from *Hordeum vulgare* (barley) have antifungal activity against the powdery mildew pathogen *Blumeria graminis* (Christensen et al. 2002). Similarly, the PR-17 protein NtPRp27 from tobacco accumulates in response to viral infection (Okushima et al. 2000), whilst WCI-5

from wheat accumulates in response to fungal infection (Görlach et al. 1996). While the putative *PR* genes identified in *E. grandis* remain to be functionally characterised, an exciting next step would be to uncover *PR* genes unique to *Eucalyptus* that could serve as novel targets to improve defence in this woody host.

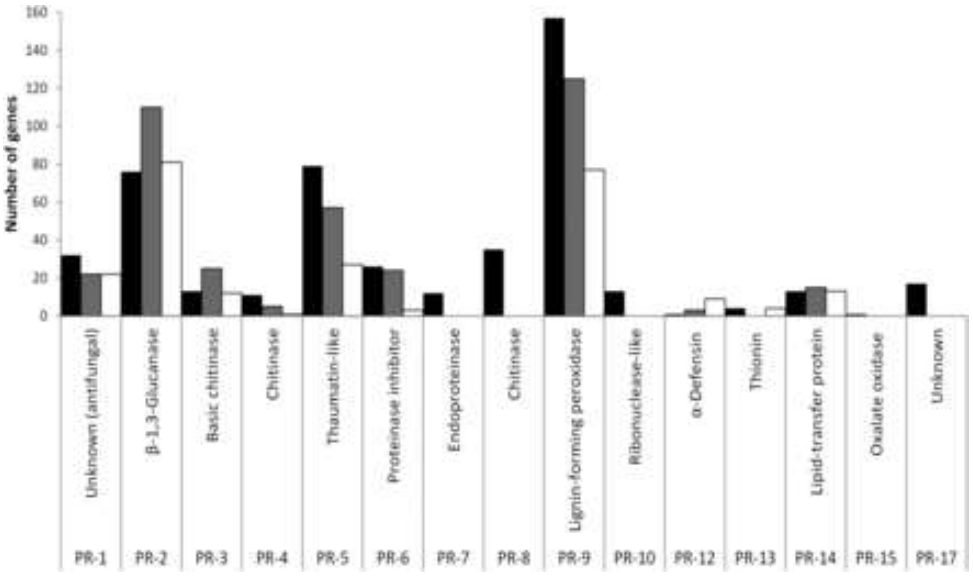

Figure 2. Number of genes in each PR gene family in the *Eucalyptus grandis* (dark blue), *Populus trichocarpa* (red) and *Arabidopsis thaliana* (green) genomes. The number of differentially expressed genes based on transcriptome sequencing in *E. grandis* is indicated in light blue.

Regulation of gene expression during defence responses

Plant defence is costly to the host and therefore the plant invests in mechanisms to fine-tune its responses to effectively control the spread of pests and pathogens whilst conserving cellular resources. TFs play an important role in these mechanisms by coordinating the expression of defence-related genes in response to invasion (Figure 1). Functional studies on TFs that mediate defence responses in *Eucalyptus* are limited, but future research could be based on studies of TF function during wood formation (Botha et al. 2011; Creux et al. 2013; Hussey et al. 2011). Epigenetic modifications add another level of complexity to the regulation of host defences (Berr et al. 2012). Epigenetic regulation of gene expression during various tree physiological processes has been reported in pine (Boyko and Kovalchuk 2011) as well as poplar (Conde et al. 2013) and was recently reviewed (Bräutigam et al. 2013). However, the majority of studies pertaining to epigenetic gene regulation in plants have focused on model organisms, herbaceous plants (Holeski et al. 2012) or abiotic stress responses. The role of epigenetics in *Eucalyptus* defence responses has not been studied. Understanding the regulation of gene expression during defence responses could lead to the identification of powerful targets for coordinate manipulation of entire cascades of defence events.

Eucalyptus *oils in defence*

Eucalypts are well known for their essential oils, which are typically stored in the sub-dermal secretory cavities of mature leaves. These oils are a complex mixture of monoterpenes, sesquiterpenes and FPCs (reviewed in Keszei et al. 2008). Significant quantitative and qualitative variation of essential oil components has been observed within and between species, but also within populations and even within individuals, with strong implications for plant-herbivore and plant-pathogen interactions (Padovan et al. 2013). Stored oils function as preformed chemical defences against defoliating animals such as *Trichosurus vulpecula* (common brush-tailed possum), *Phascolarctos cinereus* (koala) as well as leaf-chewing insects (Chen et al. 2002; Lawler et al. 1999). The defensive action of essential oils can be direct through toxicity

(McLean et al. 1993), but we know from other plant species that some volatile organic compounds (VOC) that are released upon chewing act as specific cues to attract parasitoids (Giamakis et al. 2001). While many individual compounds are toxic to herbivores, others act as antifungal, antibacterial, or allelopathic agents, or for priming of systemic defences in both the host and neighbouring plants (Alves et al. 2004; Eyles et al. 2003b; Zulak and Bohlmann 2010). Other VOCs include three important phytohormones involved in plant defence: SA, JA and ET. These phytohormones form part of the phenylpropanoid, fatty acid degradation product and amino acid derivative classes, respectively. Synthesis of certain *A. thaliana* sesquiterpenes, including β-caryophyllene, is induced by both GA and JA and involves the TF MYC2, which is repressed by DELLA proteins. This indicates that the production of these sesquiterpenes is integrated with the GA and JA signalling pathways (Hong et al. 2012). The biosynthesis and functions of plant VOCs have been reviewed (Maffei et al. 2011; Nagegowda 2010).

It has been shown that leaves of individual eucalypts contain over 100 different terpenes and more than a dozen FPCs. While this is only a small fraction of the more than 20,000 characterized terpene structures in plants (Degenhardt and Gershenzon 2003), it allows for a very large number of combinations. Because of this large number of combinations and dosage effects of individual compounds, the combined effects of different terpenes have hardly been evaluated. Most published work on *Eucalyptus* essential oils has focused on the constitutively stored compounds in the leaves, and their exact function is still unknown. Külheim et al. (unpublished) showed that of the 113 terpene synthases (TPSs) in the *E. grandis* genome - the largest number of *TPS* genes found in any plant to date - about one third is expressed in the roots and a smaller fraction in the phloem and xylem. The role of terpenes in these tissues is also unknown, but could involve defence. A recent study tentatively identified more than 40 *Eucalyptus* VOCs that can potentially be used as disease biomarkers once proper models have been established, allowing diagnosis within hours of sample collection that is robust against age differences in the harvested tissue (Hantao et al. 2013).

Converting defence gene discovery to application in **Eucalyptus** *production*

The characterization of a gene discovered through genomic studies is no easy task. A survey of databases and comparative transcriptome analyses involving other plant-pathogen interactions can provide information about the priority of candidate genes for functional characterisation. For example, over-representation of Gene Ontology (GO) terms in a particular dataset, which can be analysed with tools such as BiNGO (Biological Networks Gene Ontology), may indicate which defence signalling pathways are important for a particular host-pathogen interaction (Maere et al. 2005). Genevestigator V3 is a functional annotation tool that enables the integrative analysis of transcriptome data from different organisms and treatments (Hruz et al. 2008). Systems biology approaches are increasingly being used to uncover candidate genes for enhanced resistance in plant-pathogen interaction studies (Pritchard and Birch 2011; Windram et al. 2014). In *Eucalyptus*, functional characterisation may involve interaction studies that make use of techniques such as yeast one-hybrid and yeast two-hybrid assays, which can identify interactions between two proteins or between a protein and a DNA sequence (Botha et al. 2011; Creux et al. 2011).

Researchers also rely on *in planta* methods for functional studies, which were initially conducted with *Arabidopsis* or tobacco as models (Unver et al. 2013), and more recently in poplar and *Eucalyptus*. Different methods have been developed for introducing DNA into eucalypts, with varying degrees of success in different genotypes (reviewed in Girijashankar 2011). In an attempt to produce trees for enhanced biofuel production, the biotechnology company FuturaGene© successfully generated transgenic *Eucalyptus* trees (Abramson et al. 2011). An alternate approach to modification was also demonstrated in poplar (Wilkerson et al. 2014). Gene stacking is an interesting approach which has the potential to produce trees with enhanced resistance to various pests and pathogens (Chan et al. 2005) whilst retaining their valuable wood properties.

A novel approach to gene manipulation *in planta* involves the use of transcription activator–like effectors (TALEs) combined with nucleases (TALENs) to knock out a gene or modify its sequence (Pennisi 2013). This technology has been applied to rice, where TALEs were used to activate genes targeted by the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* (Li et al. 2013). This approach to genome editing is expected to be more acceptable to regulatory bodies and society than conventional genetic modification, since the TALENs are considered mutagens and are comparable to radiation-induced mutagens in plants. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system from bacteria can also be used to edit host-specific targets and requires the attachment of nucleases to target-specific RNAs (Belhaj et al. 2013; Jinek et al. 2012). The CRISPR technology is being refined for various applications, including host-pathogen interaction studies, and could therefore be applied to *Eucalyptus* in the future.

Translating information about the defence responses in *Eucalyptus* to field applications is not limited to the production of transgenic material. The knowledge garnered by studying hormone responses in *Eucalyptus* could be applied by treating plants with a suitable phytohormone prior to pathogen challenge to enhance resistance. For example, Naidoo et al. (2013) showed that SA treatment of more susceptible *Eucalyptus* plants prior to inoculation with *C. austroafricana* increases their disease tolerance to levels comparable to those of the more tolerant genotype.

Marker assisted breeding is an important application of *Eucalyptus* genomic variation data, since markers linked to resistance can be identified in segregating populations. For example, Freeman et al. (2008) described important Quantitative Trait Loci (QTLs) for resistance against the leaf blight fungus *Mycosphaerella cryptica* in *E. globulus*, and Junghans et al. (2003) identified QTLs in *E. grandis* that are associated with resistance to the fungal rust pathogen *P. psidii*. The sequencing of various eucalypt genomes (Grattapaglia et al. 2012) facilitates the discovery of single nucleotide polymorphisms (SNPs), which can be genotyped in segregating populations. This results in denser marker coverage of the genome and more efficient identification of loci linked to disease resistance. In addition, expression abundance QTLs (eQTLs) can be determined based on transcriptomic changes in gene expression during pest or pathogen challenge, and groups of co-regulated genes linked to defence mechanisms can be identified. This is a powerful approach, termed systems genetics, to dissecting complex traits such as resistance. Not only does it yield markers linked to disease resistance, it also provides more insight into the molecular mechanisms underlying the biology. This approach has been proposed for studying wood properties in *Eucalyptus* (Mizrachi et al. 2012), but has not yet been applied to studies of defence responses in these organisms.

Conclusion and future prospects

In order to understand the defence mechanisms underlying various pest and pathogen interactions in *Eucalyptus*, reliable pest or pathosystems have to be developed so that the molecular mechanisms involved in these interactions can be interrogated. The genome sequences of different pests and pathogens are also becoming available, and this provides a unique opportunity to formulate hypotheses about defence response mechanisms at the molecular level. Comparative transcriptome analyses of *Eucalyptus* under various biotic challenges will enable the identification of specific and tailored defence mechanisms which will reveal potential targets for enhancing defence. In summary, the genomic resources that are available for *Eucalyptus* and the pests and pathogens that threaten it provide a platform to improve our understanding of the plant defence responses in these long-lived tree species and to test the established dogmas based on studies involving model organisms directly in the *Eucalyptus* host. The discovery of candidate genes for disease resistance based on these studies, coupled with advancements in breeding and transgenic technology, is expected to enhance defence responses in commercially propagated *Eucalyptus* in the future. These tree

improvement strategies form an essential part of a multi-disciplinary approach to circumvent losses brought about by existing pests and pathogens, and may inform strategies to prevent new diseases that *Eucalyptus* has yet to encounter.

Funding

The authors gratefully acknowledge funding from Mondi and Sappi South Africa. This work is based on the research supported in part by the National Research Foundation of South Africa, Thuthuka funding instrument provided to Dr Sanushka Naidoo (Defence responses in forest trees). Any opinion, finding and conclusion or recommendation expressed in this material is that of the author(s) and the NRF does not accept any liability in this regard.

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