Foliar terpenoid levels and corresponding gene expression are systemically and differentially induced in *Eucalyptus grandis* clonal genotypes in response to *Chrysoporthe austroafricana* challenge

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Abstract

Induction of systemic defences to fungal pathogens is well-described in model plant systems, but not extensively studied in large woody perennials. Systemic induced defences in long-lived tree species, such as *Eucalyptus grandis,* could provide resistance against subsequent biotic challenges. Terpenoids are a class of plant specialised metabolites implicated in defence against herbivores and pathogens. The aim of this study was to characterize the systemic induction of terpenoids in *E. grandis* clones challenged with the fungal pathogen, *Chrysoporthe austroafricana.* Ramets of *E. grandis* clones previously classified as moderately resistant (TAG5) and susceptible (ZG14) were inoculated with *C. austroafricana* on the main stem with mock-inoculated ramets to serve as controls. Leaf tissue was harvested three days post inoculation and terpenoid levels were measured using gas chromatography coupled with mass spectrometry. Foliar mRNA abundance profiles of four candidate terpenoid biosynthesis pathway genes were compared in mock and inoculated ramets of each genotype. Monoterpene levels differed between clonal genotypes and *p*-cymene was

induced systemically in TAG5. Expression profiling of geranyl-pyrophosphate synthase (*EgrGPPS*), farnesyl-pyrophosphate synthase (*EgrFPPS2*), myrcene synthase (*EgrTPS084*) and β-caryophylene synthase (*EgrTPS038*) show induction of transcripts involved in terpenoid biosynthesis in leaves following pathogen challenge on the main stem. Together these results suggest that resistant and susceptible *Eucalyptus* genotypes may have different constitutive and induced terpenoid profiles. The significance of the systemic induction of terpenoids levels in TAG5 and terpenoid biosynthesis transcripts in both genotypes, in response to *C. austroafricana* challenge in the stem, remains to be determined in defence against secondary biotic attack.

Keywords: Terpenoids, *Chrysoporthe austroafricana*, *Eucalyptus grandis*

Introduction

Eucalyptus species, hybrids and clones have been adopted as wood fibre crops worldwide. These fast-growing hardwood trees are sourced primarily for timber, paper and pulp production as well as essential oils, utilized in the cosmetics and pharmaceutical industry, and have been recognised as a potential renewable source for biofuel production (Silva *et al.* 2003; Batish *et al.* 2008; Hinchee *et al.* 2009; Shepherd *et al.* 2011). The importance of the genus as a future biomass crop has motivated the sequencing the genome of *Eucalyptus grandis*, a widely grown eucalypt species often used as a parent for interspecific hybrid breeding (Grattapaglia *et al.* 2012; Myburg *et al.* 2014). The genome sequence provides a resource to elucidate the molecular underpinnings that make *Eucalyptus* spp. such a successful plantation species world-wide and provides clues to immunity mechanisms compromised by pests or pathogens.

It has been well documented that, after an initial period of relatively low pathogen challenge in areas where eucalypts were grown as exotic crops, many introduced and native pathogens now threaten *Eucalyptus* production (reviewed in Wingfield *et al.* 2008). One example is *Chrysoporthe*

stem disease caused by *Chrysoporthe austroafricana* in Africa (Gryzenhout *et al.* 2004). The pathogen attacks the cambium and the sapwood of *Eucalyptus* (Gibson 1981; Nakabonge 2006) causing brown staining at the initial site of infection. Cracks appear across the length of the infected bark followed by canker formation characterised by dead tissue in the centre surrounded by callus formation. Symptoms of the disease are discoloration of the root collar, leading to stem girdling and root rot, resulting in wilting and death in younger trees. Symptoms in older trees are the formation of "skirts" as the base of the tree swells and the bark cracks (Nakabonge 2006). Consequently, the weakened stem increases the chances of breakage under stresses such as strong winds (Gibson 1981). *Eucalyptus grandis* clonal genotypes TAG5 and ZG14 (Mondi Tree Improvement Research, South Africa) have been shown to be moderately resistant and susceptible to *C. austroafricana*, respectively (Van Heerden *et al.* 2005). These two clonal genotypes provide a valuable comparative pathosystem to study defence mechanisms in *Eucalyptus.*

Systemic defences, such as the induction of defence responses in tissues distal to the site of infection, play an important role in priming of defences in plant tissues for secondary attack (Eyles *et al.* 2010; Spoel and Dong 2012). Terpenoids, which form the bulk of specialized metabolites produced by plants, are well known for their role in systemic defence against insect pests and herbivores (Croteau *et al.* 2010; Eyles *et al.* 2010). *Eucalyptus* trees are known to produce large amounts and a large variety of terpenoids (the *Eucalyptus* genome encodes the largest family of terpene synthase genes of sequenced plant genomes, Myburg et al. 2014), which have been implicated in undergrowth suppression (Setia *et al.* 2007) and in defence against insect and vertebrate herbivores (Keszei *et al.* 2008, Padovan *et al.* 2013). Terpenoids have also been implicated in defence against fungi at various levels in conifers (Hammerschmidt 2009; Spoel and Dong 2012) and have been reported to have direct anti-fungal activity (Morcia *et al.* 2012). While the antifungal role of *Eucalyptus* essential oils has been documented (Batish *et al.* 2008), information regarding the role of terpenoids in induced defence against fungi in *Eucalyptus* host

plants is lacking. The aim of this study was to analyse foliar terpenoid profiles of *E. grandis* clonal genotypes TAG5 and ZG14 challenged with the stem pathogen *C. austroafricana* under the hypothesis that terpenoid biosynthesis is induced in leaves by systemic defence responses.

Materials and Methods

Plant material

Ramets of *E. grandis* clones TAG5(moderately resistant) and ZG14 (susceptible) were provided by Mondi Tree Improvement Research Department, South Africa and maintained in a greenhouse at 25-28°C under approximately long day conditions (16 h light/ 8 h dark), using supplemental lighting (140 µmol/m^2/s), and watered every two days. The plants were approximately one year old with a stem diameter of ~1 cm prior to inoculation.

Inoculations with *C. austroafricana*

Ramets of TAG5 and ZG14 were inoculated on the main stem, ~30 cm from the root collar, with *C. austroafricana* CMW2113 as described by Roux *et al.* (2003). Similarly, control plants were mock inoculated using an agar plug devoid of fungus. Lesion lengths were recorded from at least six trees per group for each time point, at 3, 7, 21 and 42 days post inoculation (dpi) by scraping away bark around the inoculation site to reveal the lesions. Due to the destructive nature of this sampling each tree was sampled once. Significance of differences in mean lesion lengths between inoculated and mock-inoculated plants was determined using a Kruskal-Wallis test (Analyse-it version 2.30 for Microsoft® Office Excel 2010) for each time point ($p \leq 0.05$). *Chrysoporthe austroafricana* infection was confirmed based on culture morphology on 2% Malt Extract Agar by re-isolation using tissue harvested from inoculated plants five days after inoculation. Leaf tissue was harvested at three days post inoculation for three biological replicates per group, frozen using liquid nitrogen

and stored at -80°C until use. Each biological replicate consisted of leaf tissue harvested to represent different developmental stages, from each of three plants.

Chemical profiling

Ethanol extracts of leaf tissue were separated by gas chromatography and detected by mass spectroscopy as described by Padovan *et al.* (2012) for the detection of mono- and sesquiterpenes. Peaks were identified by comparison of mass spectra to reference spectra in the National Institute of Standards and Technology library (Agilent Technologies, Deerfield, IL) and major peaks were verified by reference to authentic standards. The area under each peak was measured manually with the help of MSD Chemstation Data Analysis (Agilent Technologies) and converted to a relative concentration by comparison to the internal standard (dodecane). Differences in compound abundance were tested by two-way analysis (genotype and treatment) of variance (ANOVA) using Analyse-it (version 2.30) for Microsoft® Office Excel 2010 ($p \leq 0.05$).

Expression profiling

RNA was isolated from leaf tissue; collected from infected and uninfected TAG5 and ZG14 ramets 3 dpi, using a modified cetyl-trimethyl-ammonium-bromide (CTAB) method (Naidoo *et al.* 2013). DNA contamination was removed by treating extracted total RNA samples with RNase-free DNaseI enzyme (Qiagen Inc, Valencia, CA). Total RNA samples were then purified using the RNeasy® MinElute Kit (Qiagen Inc.) and subsequently analysed using a Bio-Rad Experion automated electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA), to determine RNA integrity. The Improm-II^{TM} Reverse Transcription System (Promega, Wisconsin, USA) was used to synthesize first strand cDNA from purified RNA samples. Four terpenoid biosynthesis marker genes, geranyl- (*EgrGPPS*) and farnesyl-pyrophosphate synthase (*EgrFPPS2*) putative orthologs, representing early terpenoid biosynthesis, as well as myrcene (*EgrTPS084*) and β-caryophyllene

synthase (*EgrTPS038*), representing terminal steps, were chosen from the *E. grandis* v1.1 genome sequence (www.phytozome.net v8.0) as targets for expression profiling. Target specific primers (Table 1) were designed using Primer Designer 4 v4.20 (Sci Ed Central, Cary, North Carolina, USA) and synthesised by Inqaba biotec (Inqaba Biotechnical Industries, Pretoria, South Africa). A BLASTn analysis against the *E. grandis* v1.1 genome sequence was performed to test *in silico* primer specificity.

Table 1: Primer sequences for *E. grandis* terpene synthesis-related and reference genes. Gene names and phytozome IDs are indicated in brackets.

Gene	Forward Primer Sequence	Reverse Primer Sequence	Amplicon Size (bp)	Tm (°C)
EgrGPPS (Geranyl pyrophosphate synthase; Eucgr.E02451)	CAGGCGTCTAGCAGAGATAA	AGTACCGCTATCCTTGTTCC	132	60
EgrFPPS2 (Farnesyl pyrophosphate synthase; Eucgr.E03835)	GTCTTCGCAGAGTATGAGAG	CCGACAACTTGGAGTAGTAG	194	60
EgrTPS084 (Myrcene Synthase; Eucgr.K00881)	TTCGCGCTGATCCTTGTCCT	GCCATCTCGTTCACGCTGTT	170	65
EgrTPS038 (β-caryophyllene synthase; Eucgr.J01451)	ACGGAACAAGAAGCGGAGAA	TGAGCACCGAGCGTATGTAA	214	63
EgrARF (ADP-Ribosylation factor; Eucgr.I01780)	TGCGTACCGAGTTGTTGAGG	GTTGCACAGGTGCTCTGGAT	195	64
EgrFBA (Fructose bisphosphate aldolase; Eucgr.B02864)	TGAAGACATGGCAAGGAAGG	GTACCGAAGTTGCTCCGAAT	190	64

Quantitative Real-Time PCR was conducted according to the Minimum Information for Publication of Quantitiative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.* 2009) using a LightCycler® 480 Real-Time PCR system (Roche Diagnostics, GmBh, Basa, Switzerland) following parameters described in Naidoo *et al.* (2013). The *qBASE*plus v1.0 (Biogazelle NV, Belgium) software package was used to perform normalization and relative quantification. Difference in mean relative transcript abundance between genotypes was compared by two-way ANOVA (genotype and treatment) using Analyse-it (version 2.30) for Microsoft® Office Excel 2010 ($p \leq 0.05$). Differences in mean relative transcript abundance between inoculated and mock-inoculated samples for each genotype were compared by a Student's *t*-test ($p \leq 0.05$).

Results

Confirmation of differential susceptibility of *E. grandis* genotypes to *C. austroafricana*

Lesion length at the site of inoculation was measured to quantify differences in susceptibility between the *E. grandis* clonal genotypes TAG5 and ZG14. No difference was observed between TAG5 and ZG14 ramets in early symptom development (Figure 1). At 42 days post inoculation (dpi) there was a significant difference in average lesion length on stems of TAG5 (48.4 ± 2.1 mm) and ZG14 (82.4 ± 3.8 mm) ramets inoculated with *C. austroafricana* (Kruskal-Wallis test; $p < 0.05$; Figure 1).

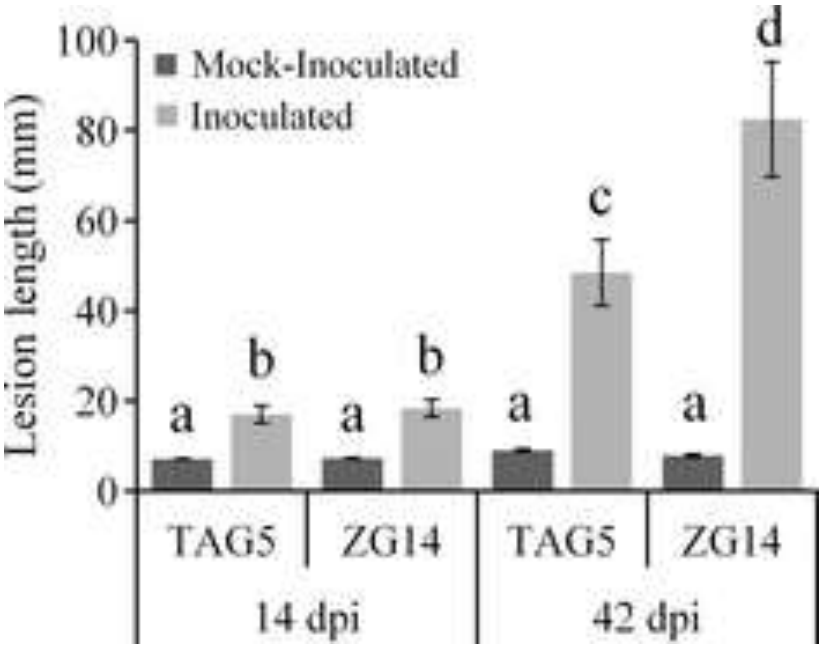

Figure 1: Difference in lesion lengths between *Eucalyptus grandis* clonal genotypes TAG5 and ZG14 following stem inoculation with *Chrysoporthe austroafricana*. No significant difference in average lesion length was observed between TAG5 and ZG14 at 14 days post-inoculation (dpi). Lowercase letters above bars indicate significant differences (Kruskal–Wallis test; $P < 0.05$). Average lesion lengths differed significantly between inoculated and mock-inoculated plants for both genotypes as well as between inoculated TAG5 and ZG14 ramets at 42 dpi. Error bars show standard error of the mean for biological replicates (n = 3).

Difference in monoterpenoid concentrations between genotypes

Chemical profiling of leaf tissue was conducted to investigate the hypothesis that there is a difference in constitutive and induced terpenoid levels in tissues distal to the site of infection in the

TAG5 and ZG14 genotypes. Nine monoterpenes could be reliably identified. Of these, α-pinene had the highest concentration, between 5 and 95 times higher than that of the other monoterpenes identified. Sesquiterpene levels were below detectable range. α-Phellandrene, β-phellandrene, p-cymene and pinocarvone were detected in TAG5 but not in ZG14 (Figure 2), indicating that a

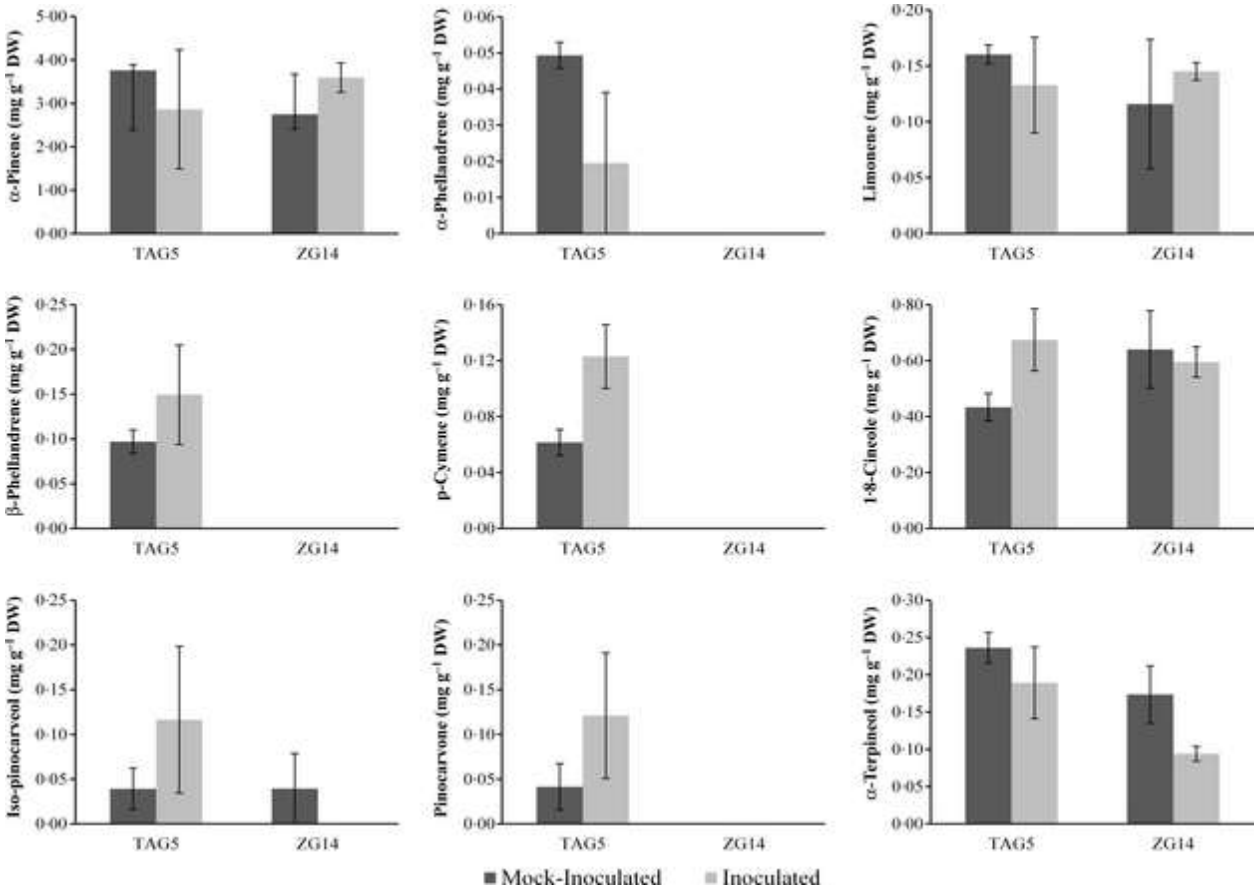

Figure 2: Comparison of foliar monoterpenoid concentrations between TAG5 and ZG14 *Eucalyptus grandis* clonal genotypes 3 days after stem inoculation with *Chrysoporthe austroafricana*. The y-axis represents compound abundance in mg.g^{-1} dry weight (DW) relative to the internal standard (dodecane). Error bars show standard error of the mean for biological replicates (n = 3)

difference in constitutive terpenoid levels exists between these genotypes. A genotype effect was observed for α-phellandrene, β-phellandrene, p-cymene and α-terpineol (Table S1). A treatment effect could only be observed for p-cymene (Table S1), which showed significantly higher levels in inoculated TAG5 plants compared to mock-inoculated. This indicates that foliar terpenoid levels can be altered systemically during *C. austroafricana* inoculation. A genotype-treatment interaction

was also observed for p-cymene (Table S1), indicating that terpenoid levels are altered differently between the genotypes.

Difference in systemic terpenoid biosynthesis gene induction between genotypes

Relative transcript abundance profiles of selected terpene biosynthetic pathway genes were investigated to determine if leaf terpene synthase transcription differed between genotypes during systemic response to stem infection. Treatment effects were observed for *EgrGPPS* (geranyl pyrophosphate synthase) and *EgrFPPS2* (farnesyl pyrophosphate synthase), indicating that these genes undergo systemic induction following *C. austroafricana* inoculation (Table S2). Genotype effects were observed for *EgrGPPS* and *EgrTPS084* (myrcene synthase), indicating that these genes undergo differential regulation between genotypes (Table S2). No genotype by treatment interactions were observed. Despite showing no genotype or treatment effects, *EgrTPS038* (β-caryophylene synthase) showed a significant transcript abundance increase in inoculated TAG5 plants compared to mock-inoculated (Figure 3).

EgrGPPS also showed a significant increase in relative transcript abundance in TAG5 alone, while a significant increase in *EgrTPS084* transcript abundance was observed in ZG14 only (Figure 3). *EgrFPPS2* transcript abundance increased in both genotypes post inoculation, but this increase was greater in ZG14 compared to TAG5, despite similar constitutive abundances, indicating that a difference between the genotypes exists in how this gene is induced following *C. austroafricana* inoculation (Figure 3).

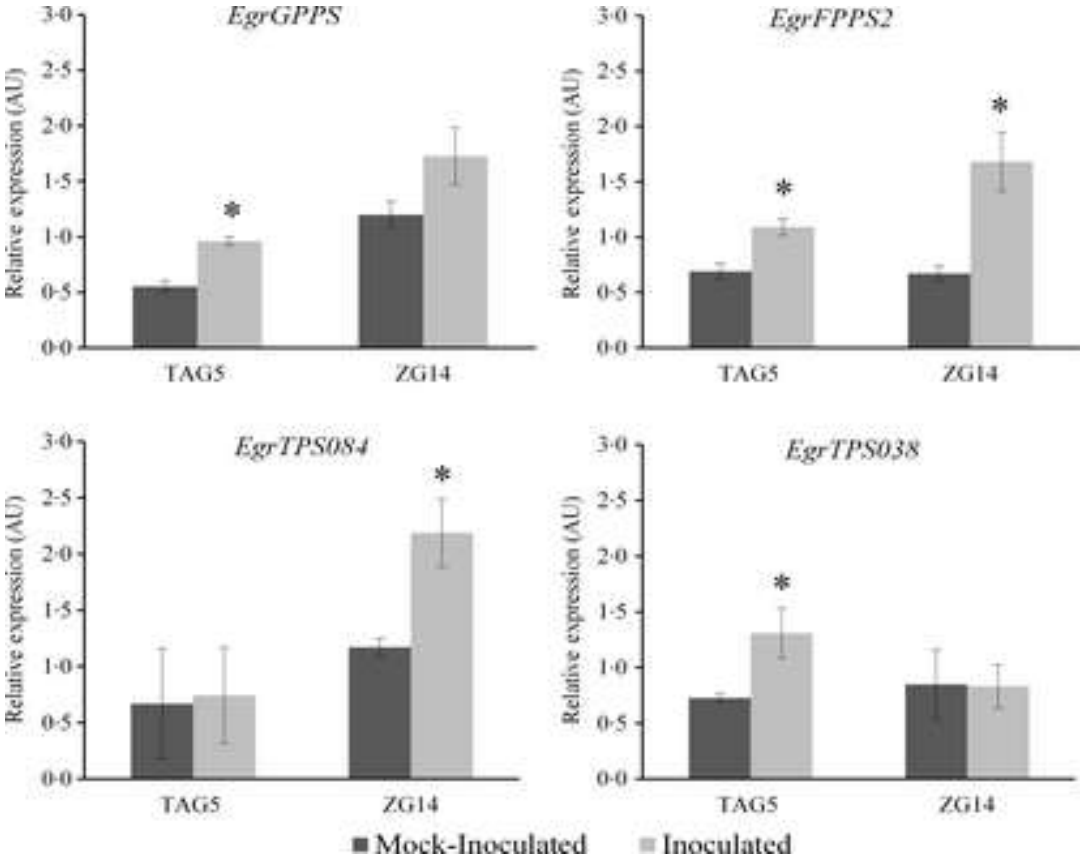

Figure 3: Relative transcript abundance for candidate terpenoid biosynthesis related genes in *Eucalyptus grandis* leaves 3 days after stem inoculation with C*hrysoporthe austroafricana*. Relative abundances of candidate genes were normalized using EgrARF and EgrFBA. *indicates significance relative to mock-inoculated controls calculated using a Student's t-test ($P < 0.05$). Error bars show the standard error of the mean for biological replicates (n = 3).

Discussion

Eucalyptus grandis clonal genotypes TAG5 and ZG14 display consistent differences in tolerance to a range of pests and pathogens, with TAG5 being moderately resistant and ZG14 mostly susceptible. This includes the fungal pathogens *C. austroafricana* (Roux *et al.* 2003), *Botryosphaeria dothidea* and *Coniothyrium zuluense* (Chimwamurombe 2001; Rodas *et al.* 2008) as well as various pests such as termites (Van Zyl 1995). These observations suggest that there are differences in defence responses that may contribute to the general resistance of TAG5 against a range of pests and pathogens as compared to ZG14, which may be constitutive, induced or a combination of both mechanisms.

Here we investigated the distal (leaf) induction of terpenoids and terpenoid biosynthesis genes in TAG5 and ZG14 subsequent to stem inoculation with *C. austroafricana.* Although the difference in susceptibility between the genotypes at 42 dpi was not large (Figure 1) the moderately resistant phenotype observed by Van Heerden *et al.* (2005) for TAG5 has previously been validated (Naidoo *et al.* 2013). Thus, a genetic difference exists between TAG5 and ZG14 that consistently gives rise to moderate resistance and susceptibility, respectively.

Terpene profiles in mature leaves of *Eucalyptus* are stable over a wide range of temporal scales - from diurnal to seasonal and over years. This is due to the relatively static nature of the secretory cavities that contain terpenes and other plant specialized metabolites. These cavities are filled during the ontogeny of the leaf (Carr *et al.* 1970) and the terpenes remain there for the lifetime of the leaf or until physical damage (e.g. chewing or penetration by fungal hyphae) releases the terpenes. So far there has been little evidence of inducibility of terpenes in eucalypts (Henery *et al.* 2008) as observed in many other species (Martin *et al.* 2003). However, Eyles *et al.* (2004) found significant concentrations of terpenes in new phloem of wound tissue of *E. globulus* and observed traumatic secretory cavities in the wood of *E. globulus* after pruning and fungal (*Cytonaema*) attack (Eyles *et al.* 2003), suggesting inducibility of terpenes in *Eucalyptus*.

Terpenoid profiles in leaves of *E. grandis* clones TAG5 and ZG14 were quantified to determine whether systemic induction of terpenes occurs. Large differences in foliar constitutive monoterpenoid concentrations were observed, mainly due to a larger variety of monoterpenoids in TAG5 (Figure 2). Significantly higher levels of p-cymene were observed for inoculated TAG5 ramets compared to mock-inoculated ramets (Table S1). Although this terpenoid was not detected in ZG14, the increased concentration in *C. austroafricana* challenged TAG5 ramets suggests inducibility of terpenes during systemic defence responses in *E. grandis*.

Changes in foliar terpenoid levels could arise due to systemic induction of terpenoid biosynthesis or transport of terpenoids through the plant's vascular system. Gene expression was used as a measure

of increased terpenoid biosynthesis in leaf tissue, following on previous studies which have indicated that this pathway is regulated mainly at the level of transcription (Nagegodwa 2010). Lack of functional knowledge regarding *Eucalyptus* terpene synthases, however, prevents direct comparison of terpene levels with gene expression. *EgrGPPS* and *EgrTPS038* showed increased relative transcript abundance in TAG5 ramets alone while *EgrTPS084* relative abundance only increased significantly in ZG14 ramets (Figure 3). A significant difference in relative transcript abundance between genotypes was also observed for *EgrGPPS* and *EgrTPS084* (Table S2).This indicates that differential systemic elicitation of terpenoid biosynthesis occurs between the two genotypes. The increase in *EgrGPPS* expression, which synthesizes the main precursor for monoterpene biosynthesis, indicates an increased investment in monoterpene production by the TAG5 genotype during pathogen challenge (Maffei *et al.* 2011).

EgrFPPS2 showed increased relative transcript abundance following *C. austroafricana* challenge in ramets of both genotypes (Table S1; Figure 3). Farnesyl pyrophosphate is the main precursor for sesquiterpene biosynthesis (Maffei *et al.* 2011). Thus, the increased *EgrFPPS2* expression observed for both genotypes could indicate increased general sesquiterpenoid investment in *E. grandis* during *C. austroafricana* infection, although sesquiterpenoid levels in the leaves were below detectable levels. This is further supported by stem RNA-seq expression data for the same infection trial (unpublished data), which shows increased *EgrFPPS2* expression as well as increased expression of genes involved in the cytosolic mevalonate synthesis pathway (reviewed in Nagegowda 2010 and Maffei *et al.* 2011) in both genotypes.

Although this study was limited to expression profiling of only four of the 113 *Eucalyptus* terpenoid biosynthesis pathway genes, differences were observed in both expression profiles and monoterpene levels between *E. grandis* genotypes following *C. austroafricana* inoculation. Therefore, our data suggests that the differences in defence mechanisms between genotypes extend to monoterpenoid levels and expression of terpenoid biosynthesis genes in systemic tissues. Monoterpenes have

previously been shown to be involved in defence against various pathogens and *Eucalyptus* spp. essential oils containing major monoterpene constituents have antimicrobial and insecticidal activity (Batish *et al.* 2008). Still, the significance of the systemic induction of terpenoid biosynthesis in TAG5 would have to be determined by subjecting the leaves to a secondary biotic stress, e.g. an insect or fungal pathogen.

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Supplemental Material

Table S1: Two-way ANOVA results comparing terpenoid concentrations in leaves of TAG5 and ZG14 (Genotype) plants under mock-inoculated and inoculated (Treatment) conditions ($\alpha = 0.05$).

Monoterpene	Factor	*df*	SS	MS	*F*	*p*
α-Pinene	Genotype	1	0.002	0.002	0.028	0.871
	Treatment	1	0.0001	0.0001	0.001	0.978
	Genotype x Treatment	1	0.077	0.077	1.061	0.333
	Residual	8	0.582	0.072		
α-Phellandrene	Genotype	1	1.034	1.034	12.010	**0.008**
	Treatment	1	0.193	0.193	2.246	0.172
	Genotype x Treatment	1	0.193	0.193	2.246	0.172
	Residual	8	0.689	0.086		
Limonene	Genotype	1	0.017	0.017	0.194	0.671
	Treatment	1	0.0001	0.0001	0.001	0.981
	Genotype x Treatment	1	0.053	0.053	0.603	0.460
	Residual	8	0.711	0.088		
β-Phellandrene	Genotype	1	0.672	0.672	18.655	**0.002**
	Treatment	1	0.030	0.030	0.841	0.386
	Genotype x Treatment	1	0.030	0.030	0.841	0.386
	Residual	8	0.288	0.036		
p-Cymene	Genotype	1	1.075	1.075	55.850	**0.0001**
	Treatment	1	0.119	0.119	6.210	**0.037**
	Genotype x Treatment	1	0.119	0.119	6.210	**0.037**
	Residual	8	0.154	0.019		
1,8-Cineole	Genotype	1	0.015	0.015	0.439	0.526
	Treatment	1	0.036	0.036	1.048	0.336
	Genotype x Treatment	1	0.078	0.078	2.219	0.175
	Residual	8	0.281	0.035		
Iso-pinocarveol	Genotype	1	0.133	0.133	1.527	0.252
	Treatment	1	0.014	0.014	0.163	0.697
	Genotype x Treatment	1	0.134	0.134	1.538	0.250
	Residual	8	0.700	0.087		
Pinocarvone	Genotype	1	0.268	0.268	3.831	0.086
	Treatment	1	0.118	0.118	1.685	0.230
	Genotype x Treatment	1	0.118	0.118	1.685	0.230
	Residual	8	0.560	0.070		
α-Terpineol	Genotype	1	0.230	0.230	5.772	**0.043**
	Treatment	1	0.148	0.148	3.707	0.090
	Genotype x Treatment	1	0.009	0.009	0.244	0.634
	Residual	8	0.319	0.039		

Table S2: Two-way ANOVA results comparing relative transcript abundance in leaves of TAG5 and ZG14 (Genotype) plants under mock-inoculated and inoculated (Treatment) conditions ($\alpha = 0.05$).

Gene	Factor	*df*	SS	MS	*F*	*P*
EgrGPPS	Genotype	1	1.494	1.494	23.634	**0.001**
	Treatment	1	0.658	0.659	10.418	**0.012**
	Genotype x Treatment	1	0.010	0.011	0.168	0.692
	Residuals	8	0.505	0.063		
EgrFPPS2	Genotype	1	0.245	0.245	3.868	0.084
	Treatment	1	1.492	1.493	23.553	**0.001**
	Genotype x Treatment	1	0.278	0.278	4.387	0.069
	Residuals	8	0.507	0.063		
EgrTPS084	Genotype	1	2.846	2.847	7.274	**0.027**
	Treatment	1	0.890	0.891	2.276	0.169
	Genotype x Treatment	1	0.664	0.665	1.698	0.228
	Residuals	8	3.131	0.391		
EgrTPS038	Genotype	1	0.095	0.095	0.679	0.434
	Treatment	1	0.237	0.238	1.696	0.229
	Genotype x Treatment	1	0.269	0.270	1.922	0.203
	Residuals	8	1.121	0.140		