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ORIGINAL ARTICLE

Defense response-like reaction associated with an anomaly in *Eucalyptus grandis*

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Abstract

Key message **Based on transcriptome and qPCR analysis, a phenotypic abnormality resulting from a Eucalyptus crossing is associated with overexpression of metabolic pathways related to defense responses against biotic and abiotic stress.**

Abstract Genetic recombination by outcrossing, a strategy used in improvement programs, can present a low frequency of individuals within progeny with morphological and physiological abnormalities. An abnormality was identified in progeny from a controlled cross of *Eucalyptus grandis*. Applying RNA-Seq technology to leaf, stem, and root tissue of normal and abnormal seedlings grown under the same greenhouse conditions, we examined global gene expression changes and compared the transcriptional profiles of the two contrasting phenotypes. We also conducted functional enrichment and metabolic pathway analyses. Among the identified differentially expressed genes, most were upregulated in leaf and stem tissues of abnormal plants, with gene ontology (GO) terms associated with 'response to stress', 'response to stimulus', and 'immune system process' categories. Further, in differential gene expression and functional enrichment analyses, we observed overexpression of metabolic pathways related to defense response processes in abnormal plants. Our results suggest that the anomaly is associated with changes in the defense response to biotic and abiotic stresses. As all progeny were grown in a controlled environment and there was no evidence of pathogenic attacks, the defense response in abnormal plants may be due to an autoimmune mechanism caused by genetic incompatibility.

Keywords Autoimmune syndrome · Defense response · Deleterious phenotype · Differential gene expression · Genetic incompatibility

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Introduction

The majority of species of *Eucalyptus*, a genus of woody perennials, are native to Australia with some endemic species found in New Guinea (*Eucalyptus deglupta*) and Timor (*Eucalyptus urophylla*) (Pryor 1985; Ladiges et al. 2003; Brooker and Kleinig 2004). Superior growth, high productivity, adaptability, and multipurpose end uses (i.e., paper, furniture, flooring, framing, and bioenergy) make *Eucalyptus* the most widely planted hardwood tree genus in the world (Grattapaglia and Kirst 2008; Rockwood 2012; Gonçalves et al. 2013). Brazil is considered a leader in the forestry sector, with approximately 73% of its total area of forest plantations (5.26 million ha) made up of eucalypts (ABRAF 2016).

Forest tree improvement programs are conducted to increase productivity, introduce desirable traits, and reduce environmental impacts (Rockwood 2012; Gonçalves et al. 2013). Genetic recombination of an established base population through outcrossing and progeny tests are strategies used to select superior genotypes in improvement programs (Golle et al. 2009). Nevertheless, outcrossed progeny can exhibit a low frequency of individuals with morphological and physiological abnormalities that can develop due to recessive deleterious mutations in homozygous individuals (Zobel and Talbert 1984; Klekowski Jr 1988; Quero-García et al. 2009). The negative effects of deleterious recessive alleles appear in the embryonic and seedling stages resulting in seedlessness, a lack of germination, the presence of abnormal, smaller, or weaker seedlings that die prematurely, and, rarely, improved seedling quality (Zobel and Talbert 1984). Preferential outcrossing in *Eucalyptus* populations contributes to their high heterozygosity and, consequently, hides the recessive deleterious alleles (Griffin et al. 1987; Golle et al. 2009). Nevertheless, genetic incompatibility can lead to strong autoimmune responses in plants as a form of hybrid necrosis (Wan et al. 2021).

In a full-sib offspring from a controlled cross of *Eucalyptus grandis* performed at Suzano Papel and Celulose SA company, we observed an anomaly with a Mendelian segregation ratio of 3 normal: 1 abnormal seedling, indicating deleterious recessive alleles in homozygosis as the cause of the anomaly, as described in a previous study (Fuchs et al. 2015). Our previous work showed that the abnormal seedlings died within a few months and presented some peculiar characteristics, including branching, reduced height, reduced individual leaf area, and leaf shape modifications. We subsequently repeated the crossings to confirm the results obtained in Fuchs et al. (2015) and included a Sequence Characterized Amplified Region (SCAR), a PCR-based marker associated with these morphological effects. In the present study, we evaluated plants at 4 months of age, a period at which the symptoms are more evident, enabling us to correctly distinguish phenotypes. From this, we conducted an RNA-Seq analysis with the same basis for reproducibility of symptoms and evaluation of gene expression. Our main objective was to identify the possible genes associated with the abnormal phenotype and obtain additional insights into the molecular mechanisms underlying this anomaly by comparing the transcriptome profiles of normal and abnormal phenotypes using RNA-Seq, Real-Time Quantitative Reverse Transcription PCR (qRT-PCR), and bioinformatics enrichment tools.

Material and methods

Plant material

Eucalyptus grandis seedlings from a controlled cross between G026 and G07 genotypes of Suzano Papel and Celulose SA company, were planted and grown under greenhouse conditions. Leaf, stem, and root tissue of normal and abnormal seedlings were harvested 4 months after germination, frozen in liquid nitrogen, and stored at − 80 °C until nucleic acid extraction. The phenotypes under study were described in Fuchs et al. (2015), where abnormal plants presented reduced growth, leaf malformation, shoot branching, and reduced root volume, in addition to mortality after 4 months.

RNA extraction

Total RNA was extracted for anomalous and normal phenotypes (a total of 10 samples, with five biological replicates for each phenotype) according to the protocol described by Chang et al. (1993) with modifications. About 200 mg of plant material were ground into a fine powder in liquid nitrogen. The powder was then homogenized in 1 mL of CTAB extraction buffer (2% CTAB; 2% PVP; 100 mM Tris–HCl pH 8.0; 25 mM EDTA; 2 M NaCl) and heated for 15 min at 65 ºC. One mL of chloroform-isoamyl alcohol (CIA 24:1) was then added and the mixture was centrifuged for 10 min at 10,000 rpm. The upper phase was transferred to a new tube, and a new extraction with CIA was carried out. The upper phase was transferred to a new tube, one volume of lithium chloride was added, and the mixture was incubated overnight at 4 ºC. The samples were again centrifuged at 12,000 rpm for 30 min at 4 °C, the supernatant discarded, and the pellet washed with 70% ethanol. Total RNA was eluted with 30 µl DEPC-treated water and stored at − 80 °C.

To remove any trace of contaminating genomic DNA, RNA elutes were subjected to a DNase treatment. Briefly, 1 µg of total RNA was treated using the DNase I

Amplification Grade (Invitrogen), following the manufacturer's guidelines. RNA quantity was measured by spectrophotometer (Thermo Scientific NanoDrop™ 1000), and its integrity was verified by capillary electrophoresis (Agilent Bioanalyzer 2100) using RNA 6000 Nano kit (Agilent Technologies).

Library preparation for Illumina sequencing platform

Sequencing cDNA libraries were prepared using the TruSeqRNA kit (Illumina) starting with an RNA pool containing an equal quantity of total RNA of five plants of the same phenotype (normal and abnormal). We performed a pool of individuals for each indexed libraries (leaf, stem, and root) for each phenotype. For MiSeq sequencing, the indexed libraries were mixed in equimolar amounts (12.5 pM). Single-end sequencing was performed with 51 cycles. The Illumina reads generated in this study are available at NCBI Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra) accessions SRR1481484-SRR1481488 and SRR1481490-SRR1481491.

RNA-Seq data analysis

Single-end reads were mapped with the Bowtie software v. 1.1 (Langmead et al. 2009) using the *E. grandis* genome reference obtained from Phytozome 12 (*Eucalyptus grandis* v2.0 Genome Project). The mapped reads were converted to binary sorted BAM files, using SAMtools software v. 1.4 (Li et al. 2009), and the number of reads were counted with BEDtools v. 2.22 (Quinlan and Hall 2010) and the *E. grandis* transcriptome as a reference (*Eucalyptus grandis* Genome Project, http://www.phytozome.net/eucalyptus). The table of read counts were used as input for normalization and in the prediction of differentially expressed genes with the DESeq package, an R package from Bioconductor (http://www.bioconductor.org/; Anders and Huber 2010).

Functional enrichment and metabolic pathways analysis

Gene ontology (GO) annotations and enzyme commission numbers (EC numbers) were assigned to differentially expressed genes according to the functional annotation data retrieved from Phytozome (http://www.phytozome.net/eucalyptus) through Blast2GO software (Conesa and Gotz, 2008).

GO-based functional enrichment analysis was carried out for the identified differentially expressed genes. For each type of GO term (i.e., biological process, molecular function, or cellular component), we compared the frequencies of GO terms mapped to the differentially expressed genes with those of entire set of *Eucalyptus* genes. To this end, we used the Biological Networks Gene Ontology tool v. 3.0.3 (BiNGO; Maere et al. 2005), an open-source Java tool to determine which GO terms are significantly overrepresented in a set of genes. The statistical test (hypergeometric test) used multiple testing corrections (Benjamini and Hochberg 1995) and a confidence level of $p < 0.05$. The reference set, ontology, and organism/annotation files were all prepared particularly for this study. For each type of ontology, the test set consisted of the set of differentially expressed transcripts and the reference set consisted of the whole set of *Eucalyptus* genes.

To determine which metabolic pathways were differentially expressed between abnormal and normal plants, we first identified the genes coding for enzymes according to Phytozome, that is, genes associated with EC numbers. The retrieved EC numbers were then mapped to the plant metabolic pathways collected from the Gramene database (Gupta et al. 2016), an integrated data resource for comparative functional genomics in crops and model plant species. We kept only pathways with at least five mapped EC numbers for further analysis.

We then mapped the EC numbers back to their coding genes and calculated the fold change in gene expression in abnormal plants compared to normal plants. The fold change is given as $\log_2$(expA/expB), where expA and expB are expression values for genes in abnormal and normal plants, respectively. For each pathway containing k enzymes, the average fold change was calculated and statistically compared with the average fold change obtained by 100 sets of k enzymes randomly taken from the pathways. From this average fold change and its corresponding standard deviation, a Z-score and a *p-value* were calculated for each metabolic pathway. Pathways were considered differentially expressed when the p-value < 0.05. Positive and negative Z-scores indicate upregulated and downregulated pathways, respectively.

Real-time quantitative reverse transcription PCR analysis (qRT-PCR)

Considering the association of the previous marker identified in Fuchs et al. (2015) with BET v1-like genes, as well as the many other genes associated with pathogenesis-related proteins identified in the present study, we used the SCAR marker as a reference to identify genes associated with the phenotype and plant defense pathways. We selected the genes with the most differential expression in terms of defense pathways, as has been demonstrated in the literature for the selected genes in other species: Eucgr.F00644 (GRP), Eucgr.L00937 (CHI I), Eucgr.B02124 (BARWIN), and Eucgr.E01382 (THAU) for stem and leaf tissue; Eucgr.H04017 (BET v1) for leaves; Eucgr.H04012 (BET v1 b) exclusively for stems; and finally Eucgr.I01236 (GLOB), Eucgr.H00329 (CHI I b), Eucgr.A01578 (CUP), and Eucgr.

L00605 (PROT) for roots (Bocca et al. 2005; O'Leary et al. 2007; Tobias et al. 2008; Wanderley-Nogueira et al. 2012; Filipenko et al. 2013; Wang et al. 2014; Peri et al. 2017; Lakhssassi et al. 2020; Figueiredo et al. 2021). Ubiquitin was used as an endogenous control (Boava et al. 2010).

Total RNA was reverse transcribed to make cDNAs using oligo dT primer and the Omniscript RT Kit (Qiagen). The qRT–PCR reaction was performed in a 15-µl reaction containing 0.1 µM cDNA template, 1 µM of forward and reverse primers (Table 1), and 7.5 ml of FastStart Universal SYBR Green Master mix (Roche) in a ABI7500 Real-Time PCR system (Applied Biosystems). All reactions were performed with three biological and three technical replications using a dissociation curve as a control for the absence of primer dimers in the reactions. Normalization of Ct values of each gene and determination of relative expression levels (fold changes) were calculated according to the $2^{-\Delta\Delta Ct}$ method by Schmittgen and Livak (2008).

Results

RNA-Seq results and differentially expressed genes

To identify the genes and biological processes associated with the anomaly observed in *E. grandis* as previously described (Fuchs et al. 2015), we first generated transcriptome profiles of leaves, stems, and roots of normal and abnormal seedlings using RNA-Seq.

We constructed six cDNA libraries derived from leaves, stems, and roots from normal and abnormal plants that were then sequenced in an Illumina MiSeq platform. A total of 4.87 million (0.63–1.09 million reads per cDNA library) 50-bp single-end reads, with 4.32 million passing filter reads (98% PF reads) and 94.75% with Q30 quality, were obtained.

The sequence reads were mapped to the *E. grandis* reference genome, which revealed the expression of the following: 21,793 and 22,321 genes in leaves of normal and abnormal plants, respectively; 23,959 and 21,338 genes in roots of normal and abnormal plants; and 22,083 and 22,073 genes in stems of normal and abnormal plants.

The analysis of RNA-Seq expression data between the two distinct phenotypes showed differentially expressed genes ($p < 0.05$; FDR < 0.05) in all tissues: 26 in leaf; 59 in stem; and 11 in root (Table 2; Supplementary data S1). From these, eight genes are common to leaf and stem tissues. Most of differentially expressed genes were upregulated in abnormal plants (Fig. 11; Supplementary data S3-S5).

Table 1 Primer sequences used for RT-PCR analyses

Primer ID	Gene product	Primer sequences forward/reverse	Plant material used	Study
UBQ[a]	Ubiquitin	GAGGGACATCTATCTCTATGAC/CAACAGTAAGCACACGAG	Endogenous control—leaf, stem and root	Boava et al. (2010)
GRP	Glycine rich protein	GCTAACTACGGCCAAGGATATG/GTGCTCGCCACCTTCATAC	Leaf and stem	This study
CHI I	Chitinase class I	TCATTGCTCATGTACTGGTGAT/GCTTCTGCAAACTTCATTGAGAT	Leaf and stem	This study
BARWIN	Barwin family proteins	GCTTGTGCCCAGAAAGAATAAG/CGTAACGACAACTTCTATTACATAACG	Leaf and stem	This study
THAU	Thaumatin-like proteins	TCTGAACTTGGATGGACCATTT/CCAGTGACGAGGGTACAAC	Leaf and stem	This study
BET vI	Pathogenesis-related protein Bet v I family	CCACCGTGGAGTTGTTTAGT/TACACACCCGACCATCAAATC	Leaf	This study
BET vI b	Pathogenesis-related protein Bet v I family	CGCTGGTAGTGGATGTGTT/CCTTCTTAATGTCCTCCTCCTTC	Stem	This study
GLOB	Globin	TTCAGATTTGATAGCAGCAACAAG/GAGATGTGGTGCCCAGAAA	Root	This study
PROT	Aspartyl protease/proteolysis and peptidolysis	ATCGGCCTCAACAGCATATC/AACAGTGTACGGAAGCACAG	Root	This study
CUP	Cupin	GAGGTAGTCAACCACCTTCTTG/CCATTGCTAATGCTGTCTTTGG	Root	This study
CHI I b	Chitinase class I	GCAACCCGAACGTAGGATATTA/GGGAGTTTAAGCCGTCGAAT	Root	This study

[a]Endogenous control gene

Table 2 Differentially expressed genes in comparative transcriptome analysis of normal and abnormal phenotypes from *E. grandis*

Gene ID	Gene annotation	Expression in abnormal plants	Tissues
Eucgr.A01080	Glycine rich protein family	Upregulated	Leaf
Eucgr.B00946	Methyltransferase domain	Upregulated	Leaf
Eucgr.B03013	Ribulose-1,5-bisphosphate carboxylase small subunit/Ribulose bisphosphate carboxylase, small chain	Upregulated	Leaf
Eucgr.B03328	BURP domain	Downregulated	Leaf
Eucgr.D02043	Protease inhibitor/seed storage/LTP family	Upregulated	Leaf
Eucgr.D02045	Protease inhibitor/seed storage/LTP family	Upregulated	Leaf
Eucgr.E00814	Dirigent-like protein	Upregulated	Leaf
Eucgr.F00639	Glycine rich protein family	Upregulated	Leaf
Eucgr.F01234	D-mannose binding lectin/PAN-like domain	Upregulated	Leaf
Eucgr.H00206	Mediator complex subunit 28	Upregulated	Leaf
Eucgr.H04017	Pathogenesis-related protein Bet v I family	Upregulated	Leaf
Eucgr.I01144	Ferric reductase NAD binding domain/FAD-binding domain/Ferric reductase like transmembrane component	Downregulated	Leaf
Eucgr.J00051	Major intrinsic protein	Upregulated	Leaf
Eucgr.J00362	Transferase family	Upregulated	Leaf
Eucgr.J00363	Transferase family	Upregulated	Leaf
Eucgr.K02125	Alcohol dehydrogenase GroES-like domain/Zinc-binding dehydrogenase	Upregulated	Leaf
Eucgr.K02129	Cytochrome P450	Upregulated	Leaf
Eucgr.L03158	Trypsin and protease inhibitor	Upregulated	Leaf
Eucgr.B02124	Barwin family	Upregulated	Leaf and stem
Eucgr.L00937	Chitinase class I	Upregulated	Leaf and stem
Eucgr.F00644	Glycine rich protein family	Upregulated	Leaf and stem
Eucgr.B03654	Glycosyl hydrolase family 1	Upregulated	Leaf and stem
Eucgr.L02460	Peroxidase	Upregulated	Leaf and stem
Eucgr.E00560	Thaumatin family	Upregulated	Leaf and stem
Eucgr.E01382	Thaumatin family	Upregulated	Leaf and stem
Eucgr.H03865	Thaumatin family	Upregulated	Leaf and stem
Eucgr.B01106	Aspartyl protease/proteolysis and peptidolysis	Upregulated	Root
Eucgr.L00605	Aspartyl protease/proteolysis and peptidolysis	Upregulated	Root
Eucgr.H00329	Chitinase class I	Upregulated	Root
Eucgr.A01578	Cupin	Upregulated	Root
Eucgr.A01579	Cupin	Upregulated	Root
Eucgr.B01353	Cupin	Upregulated	Root
Eucgr.L03367	Cupin	Upregulated	Root
Eucgr.L02577	EXS family	Upregulated	Root
Eucgr.I01236	Globin	Upregulated	Root
Eucgr.K00308	Glycosyl hydrolases family 18	Upregulated	Root
Eucgr.H03072	There are no functional annotations	Upregulated	Root
Eucgr.B02637	Aldo/keto reductase family	Upregulated	Stem
Eucgr.F02347	Alpha amylase, catalytic domain/Alpha-amylase C-terminal beta-sheet domain	Upregulated	Stem
Eucgr.H00325	Chitinase class I	Upregulated	Stem
Eucgr.I01495	Chitinase class I	Upregulated	Stem
Eucgr.J02518	Chitinase class I	Upregulated	Stem
Eucgr.J02519	Chitinase class I	Upregulated	Stem
Eucgr.K02166	Chitinase class I	Upregulated	Stem
Eucgr.L00941	Chitinase class I	Upregulated	Stem
Eucgr.B01008	Chlorophyll A-B binding protein	Upregulated	Stem

Table 2 (continued)

Gene ID	Gene annotation	Expression in abnormal plants	Tissues
Eucgr.H04194	Cupin	Upregulated	Stem
Eucgr.H04196	Cupin	Upregulated	Stem
Eucgr.B03596	Cytochrome P450	Upregulated	Stem
Eucgr.J02209	D-isomer specific 2-hydroxyacid dehydrogenase, NAD binding domain/D-isomer specific 2-hydroxyacid dehydrogenase, catalytic domain	Upregulated	Stem
Eucgr.C04179	Early nodulin 93 ENOD93 protein	Upregulated	Stem
Eucgr.F02743	Fatty acid desaturase	Upregulated	Stem
Eucgr.I01326	Fructose-bisphosphate aldolase class-I	Upregulated	Stem
Eucgr.I01237	Globin	Upregulated	Stem
Eucgr.F02915	Glutathione S-transferase, C-terminal domain/Glutathione S-transferase, N-terminal domain	Upregulated	Stem
Eucgr.B03657	Glycosyl hydrolase family 1	Upregulated	Stem
Eucgr.J00124	Iron/manganese superoxide dismutases, alpha-hairpin domain/Iron/manganese superoxide dismutases, C-terminal domain	Upregulated	Stem
Eucgr.D01485	MATH domain	Upregulated	Stem
Eucgr.H00199	Mediator complex subunit 28	Upregulated	Stem
Eucgr.H00201	Mediator complex subunit 28	Upregulated	Stem
Eucgr.L00852	Mediator complex subunit 28	Upregulated	Stem
Eucgr.I01148	Nitrite/Sulfite reductase ferredoxin-like half domain/Nitrite and sulphite reductase 4Fe-4S domain	Upregulated	Stem
Eucgr.G02175	Pathogenesis-related protein Bet v I family	Upregulated	Stem
Eucgr.H04003	Pathogenesis-related protein Bet v I family	Upregulated	Stem
Eucgr.H04010	Pathogenesis-related protein Bet v I family	Upregulated	Stem
Eucgr.H04012	Pathogenesis-related protein Bet v I family	Upregulated	Stem
Eucgr.H04013	Pathogenesis-related protein Bet v I family	Upregulated	Stem
Eucgr.K02208	PLAC8 family	Upregulated	Stem
Eucgr.E01625	Pollen allergen/Rare lipoprotein A (RlpA)-like double-psi beta-barrel	Upregulated	Stem
Eucgr.G03134	Pollen allergen/Rare lipoprotein A (RlpA)-like double-psi beta-barrel	Upregulated	Stem
Eucgr.B00305	RCD1-SRO-TAF4 (RST) plant domain/Poly(ADP-ribose) polymerase catalytic domain	Upregulated	Stem
Eucgr.H03539	Sugar (and other) transporter	Upregulated	Stem
Eucgr.E01384	Thaumatin family	Upregulated	Stem
Eucgr.E01389	Thaumatin family	Upregulated	Stem
Eucgr.B02525	There are no functional annotations	Upregulated	Stem
Eucgr.G00423	There are no functional annotations	Upregulated	Stem
Eucgr.H00205	There are no functional annotations	Upregulated	Stem
Eucgr.I02127	There are no functional annotations	Upregulated	Stem
Eucgr.I01671	Transmembrane amino acid transporter protein	Upregulated	Stem
Eucgr.K02326	Trypsin and protease inhibitor	Upregulated	Stem
Eucgr.F01943	Wall-associated kinase/Protein tyrosine kinase/Calcium-binding EGF domain	Upregulated	Stem
Eucgr.B01107	There are no functional annotations	Downregulated	Stem
Eucgr.C00179	Glycosyl hydrolases family 16	Downregulated	Stem
Eucgr.C00180	Glycosyl hydrolases family 16	Downregulated	Stem
Eucgr.C00181	Glycosyl hydrolases family 16	Downregulated	Stem
Eucgr.E00357	Pollen allergen/Rare lipoprotein A (RlpA)-like double-psi beta-barrel	Downregulated	Stem
Eucgr.F02706	EamA-like transporter family	Downregulated	Stem
Eucgr.J01066	Transmembrane amino acid transporter protein	Downregulated	Stem

Validation of RNA-Seq results with qRT-PCR

Despite the low coverage of sequencing and possible underestimation of the expressed genes, the validation of differentially expressed genes by qRT-PCR confirmed the results obtained by RNA-Seq, with upregulated expression found in both analyses. We verified the expression pattern of four to five genes selected from the most differentially expressed genes for each tissue with three biological replicates and three technical replicates. The target genes chosen were Eucgr.F00644 (GRP), Eucgr.L00937 (CHI I), Eucgr.B02124 (BARWIN), and Eucgr.E01382 (THAU) for stem and leaf tissue; Eucgr.H04017 (BET v1) for leaf tissue; Eucgr.H04012 (BET v1 b) exclusively for stems; and Eucgr.I01236 (GLOB), Eucgr.H00329 (CHI I b), Eucgr.A01578 (CUP), and Eucgr.L00605 (PROT) for roots. In addition to these target genes, the gene UBQ was chosen as an endogenous control as it is one of the most stably expressed genes tested by Boava et al. (2010). Similar to RNA-Seq data, the qRT-PCR analysis showed a statistically significant difference between the contrasting phenotypes ($P < 0.001$) (Fig. 2), thus confirming the accuracy of the RNA-Seq results.

GO-based general functional analysis of differentially expressed genes

In general, the extraction of biological insights from differentially expressed genes is performed via single-gene analysis. However, conclusions based on a group of relevant genes rather than individual genes can increase the probability of identifying the correct biological processes that are most prominent in the biological phenomena under study (Huang et al 2009) since single-gene analysis may miss important effects on pathways. Based on this strategy, we decided to analyze the functional annotation of differentially expressed genes as a group to find the molecular triggers of the anomaly under investigation.

We analyzed the distribution of GO terms encompassing the three ontologies (biological process, molecular function, and cellular component) associated with the differentially expressed genes. Through this analysis, we verified the most representative GO terms associated with the up- and downregulated genes of abnormal plants in all analyzed tissue types. The most representative biological process terms associated with the abnormal phenotype in all tissues are 'metabolic process', 'response to stimulus', and 'response to stress'. In leaf and stem tissue, the major groups are related to 'signaling', 'cell communication', and 'immune system process'. In roots, GOs are related to 'catabolic process' and 'cell wall organization or biogenesis' (Supplementary data S3–S5). When considering the common genes between leaf and stem tissues, the representative GOs are 'response to stress', 'response to stimulus', 'immune system process' and others (Fig. 1).

GO-based functional enrichment analysis of differentially expressed genes

To gain further insights into the biological processes related to the pathogenesis of the genetic anomaly, we conducted a deeper investigation to identify which specific GO terms are associated with the differentially expressed genes. This analysis can reveal the specific metabolic or non-metabolic pathways related to response to stimulus that are associated with the anomaly and their binding and catalytic activity events.

Although the simple counting of differentially expressed genes associated with certain GO terms can offer some insights, as demonstrated in the previous section, the enrichment analysis of GO terms assigned to these genes in each tissue is more informative since the analysis can reveal the statistically significant occurrence of GO terms in the set of differentially expressed genes.

Using BiNGO, we compared the frequencies of GO terms mapped to the differentially expressed genes for each tissue with those of entire set of *E. grandis* genes. We determined which GO term was significantly enriched (corrected $p < 0.05$) in the differentially expressed genes compared to all genes as shown below. In leaves of abnormal plants, 13 biological process terms and 16 cellular component terms are significantly enriched in the set of upregulated genes (Table 3). The significant biological process terms can be grouped in four general categories: defense response; choline metabolism; pollen tube growth; and lipid transport (Table 3; Supplementary data S2a). In leaves of normal plants, five biological process terms are significantly enriched in the set of upregulated genes and all these biological process terms are related to photosynthesis. No molecular function and cellular component terms are significantly enriched in upregulated genes in leaves of normal plants.

In roots of abnormal plants (Supplementary data S5), while the biological process terms are all related to response to abiotic stimulus (i.e., response to temperature stimulus and response to cold), the single molecular function term is related to the storage of nutritious substrates (nutrient reservoir activity). In roots of normal plants, no GO terms are significantly enriched in the set of upregulated genes (Table 3; Supplementary data S2b).

In stems of abnormal plants, 29 biological process terms and five molecular function terms are significantly enriched in the set of upregulated genes. The biological process terms can be grouped in five general categories: response to stimulus; pollen tube growth; cell wall metabolism; chitin metabolism; and reactive oxygen species metabolic process. Interestingly, the five significantly

Table 3 Gene ontology annotations significantly enriched in sets of differentially expressed genes

	GO ID	GO terms	Corrected *p*-value	Tissue
Enriched in abnormal phenotype				
BP	GO:0006032	Chitin catabolic process	3.0310E–12	Stem
	GO:0006030	Chitin metabolic process	3.0310E–12	Stem
	GO:0006026	Aminoglycan catabolic process	3.0310E–12	Stem
	GO:0046348	Amino sugar catabolic process	3.0310E–12	Stem
	GO: 1901071	Glucosamine-containing compound metabolic process	3.0310E–12	Stem
	GO: 1901072	Glucosamine-containing compound catabolic process	3.0310E–12	Stem
	GO:0006022	Aminoglycan metabolic process	3.2677E–12	Stem
	GO:0006040	Amino sugar metabolic process	5.4529E–12	Stem
	GO:0016998	Cell wall macromolecule catabolic process	1.4223E–9	Stem
	GO:0044036	Cell wall macromolecule metabolic process	1.0181E–8	Stem
	GO:1901136	Carbohydrate derivative catabolic process	1.0551E–8	Stem
	GO:0071554	Cell wall organization or biogenesis	2.4819E–7	Stem
	GO:1901565	Organonitrogen compound catabolic process	2.9763E–7	Stem
	GO:0009057	Macromolecule catabolic process	1.4755E–5	Stem
	GO:0009056	Catabolic process	5.2603E–5	Stem
	GO: 1901135	Carbohydrate derivative metabolic process	7.4656E–5	Stem
	GO: 0044248	Cellular catabolic process	9.8903E–5	Stem
	GO: 1901575	Organic substance catabolic process	9.8965E–5	Stem
	GO:0006950	Response to stress	9.6463E–4	Stem
	GO:0009607	Response to biotic stimulus	1.5703E–3	Stem
	GO:0006952	Defense response	1.6078E–3	Stem
			4.7718E–2	Leaf
	GO:1901564	Organonitrogen compound metabolic process	4.4227E–3	Stem
	GO:0009409	Response to cold	8.8863E–3	Root
	GO:0009266	Response to temperature stimulus	1.1102E–2	Root
	GO:0009628	Response to abiotic stimulus	1.5461E–2	Root
	GO:0050896	Response to stimulus	1.6915E–2	Stem
	GO:0072593	Reactive oxygen species metabolic process	2.3389E–2	Stem
	GO:0009826	Unidimensional cell growth	2.6904E–2	Stem
	GO:0006869	Lipid transport	3.3839E–2	Leaf
	GO:0010876	Lipid localization	3.3839E–2	Leaf
	GO:0042425	Choline biosynthetic process	3.3839E–2	Leaf
	GO:0019695	Choline metabolic process	3.3839E–2	Leaf
	GO:0009932	Cell tip growth	3.3839E–2	Leaf
	GO:0006656	Phosphatidylcholine biosynthetic process	3.3839E–2	Leaf
	GO:0048868	Pollen tube development	3.3839E–2	Leaf
	GO:0009860	Pollen tube growth	3.3839E–2	Leaf
			4.3386E–2	Stem
	GO:0046470	Phosphatidylcholine metabolic process	3.6107E–2	Leaf
	GO:0042439	Ethanolamine-containing compound metabolic process	3.6107E–2	Leaf
	GO:0048588	Developmental cell growth	3.6107E–2	Leaf
	GO:0060560	Developmental growth involved in morphogenesis	4.0044E–2	Stem
	GO:0048589	Developmental growth	4.4257E–2	Stem
	GO:0000904	Cell morphogenesis involved in differentiation	4.7718E–2	Leaf
	GO:0000902	Cell morphogenesis	4.9684E–2	Stem
MF	GO:0004568	Chitinase activity	1.9014E–12	Stem
	GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds	1.6354E–8	Stem
	GO:0016798	Hydrolase activity, acting on glycosyl bonds	1.6855E–8	Stem
	GO:0045735	Nutrient reservoir activity	2.2795E–6	Root

Table 3 (continued)

	GO ID	GO terms	Corrected *p*-value	Tissue
	GO:0008061	Chitin binding	2.0628E–5	Stem
	GO:0016787	Hydrolase activity	3.7401E–2	Stem
CC	GO:0016020	Membrane	3.5733E–4	Leaf
			2.2790E–3	Root
	GO:0009535	Cloroplast thylakoid membrane	3.5733E–4	Leaf
	GO:0055035	Plastid thylakoid membrane	3.5733E–4	Leaf
	GO:0042651	Thylakoid membrane	3.5733E–4	Leaf
	GO:0034357	Photosynthetic membrane	3.5733E–4	Leaf
	GO:0031976	Plastid thylakoid	3.5733E–4	Leaf
	GO:0009534	Chloroplast thylakoid	3.5733E–4	Leaf
	GO:0031984	Organelle subcompartment	3.5733E–4	Leaf
	GO:0044436	Thylakoid part	4.3592E–4	Leaf
	GO:0009579	Thylakoid	4.6321E–4	Leaf
	GO:0044434	Chloroplast part	1.4505E–3	Leaf
	GO:0044435	Plastid part	2.1580E–3	Leaf
	GO:0048492	Ribulose bisphosphate carboxylase complex	2.2077E–3	Leaf
	GO:0009573	Chloroplast ribulose bisphosphate carboxylase complex	2.2077E–3	Leaf
	GO:0031012	Extracellular matrix	2.2790E–3	Root
	GO:0009507	Chloroplast	8.8065E–3	Leaf
	GO:0005618	Cell wall	4.9039E–2	Leaf
Enriched in normal phenotype				
BP	GO:0009664	Plant-type cell wall organization	3.1853E–5	Stem
	GO:0071669	Plant-type cell wall organization or biogenesis	9.3380E–5	Stem
	GO:0071555	Cell wall organization	9.7157E–5	Stem
	GO:0045229	External encapsulating structure organization	1.2317E–4	Stem
	GO:0009612	Response to mechanical stimulus	2.6505E–4	Stem
	GO:0071554	Wall organization or biogenesis	4.3798E–4	Stem
	GO:0009741	Response to brassinosteroid	1.0265E–3	Stem
	GO:0044042	Glucan metabolic process	4.5930E–3	Stem
	GO:0006073	Cellular glucan metabolic process	4.5930E–3	Stem
	GO:0009408	Response to heat	4.8655E–3	Stem
	GO:0044264	Cellular polysaccharide metabolic process	4.8655E–3	Stem
	GO:0005976	Polysaccharide metabolic process	5.4831E–3	Stem
	GO:0005975	Carbohydrate metabolic process	5.5450E–3	Stem
	GO:000 9733	Response to auxin	7.3217E–3	Stem
	GO:0044262	Cellular carbohydrate metabolic process	7.3217E–3	Stem
	GO:0016043	Cellular component organization	7.3217E–3	Stem
	GO:0071840	Cellular component organization or biogenesis	1.0786E–2	Stem
	GO:0009409	Response to cold	1.1604E–2	Stem
	GO:0014070	Response to organic cyclic compound	1.2419E–2	Stem
	GO:0097305	Response to alcohol	1.6715E–2	Stem
	GO:0009266	Response to temperature stimulus	1.8228E–2	Stem
	GO:0009828	Plant-type cell wall loosening	1.8785E–2	Stem
	GO:0033993	Response to lipid	2.0373E–2	Stem
	GO:0009767	Photosynthetic electron transport chain	2.0691E–2	Leaf
	GO:0022900	Electron transport chain	2.0691E–2	Leaf
	GO:0019684	Photosynthesis, light reaction	2.0691E–2	Leaf
	GO:0009827	Plant-type cell wall modification	2.5447E–2	Stem
	GO:0015979	Photosynthesis	2.8017E–2	Leaf
	GO:0006091	Generation of precursor metabolites and energy	2.8017E–2	Leaf

Table 3 (continued)

	GO ID	GO terms	Corrected *p*-value	Tissue
MF	GO:0016762	Xyloglucan:xyloglucosyl transferase activity	9.4158E–5	Stem
	GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl	9.4158E–5	Stem
	GO:0016798	Hydrolase activity, acting on glycosyl bonds	9.4158E–5	Stem
	GO:0016758	Transferase activity, transferring hexosyl groups	1.6159E–3	Stem
	GO:0016757	Transferase activity, transferring glycosyl groups	2.3843E–3	Stem
	GO:0016787	Hydrolase activity	7.3544E–3	Stem
CC	GO:0048046	Apoplast	3.7771E–3	Stem
	GO:0005576	Extracellular region	9.1960E–3	Stem
	GO:0005618	Cell wall	1.7939E–2	Stem
	GO:0030312	External encapsulating structure	1.7939E–2	Stem
	GO:0016020	Membrane	2.7535E–2	Stem

BP: biological processes; MF: molecular function; CC: cellular component

enriched molecular function terms are also related to chitin metabolism (chitinase activity, hydrolase activity hydrolyzing *O*-glycosyl compounds, hydrolase activity acting on glycosyl bonds, chitin binding, hydrolase activity) (Table 3; Supplementary data S2c). In stems of normal plants, 24 biological process terms, six molecular function terms, and five cellular component terms are significantly enriched in the set of upregulated genes. The significant biological process terms can be grouped in three major categories: response to stimulus (response to heat, cold, temperature, mechanical stimulus, auxin, alcohol, organic cyclic compound, lipid, and brassinosteroid), cell wall organization, and glucan metabolism. To a certain extent, the significant molecular function terms are also related to glucan metabolism since all of these terms are associated with xyloglucan metabolism. Finally, the five significant cellular component terms are related to communication between cells and their environment (membrane, extracellular region, apoplast, external encapsulating structure, and cell wall) (Table 3; Supplementary data S2d).

These results suggest that abnormal plants are responding defensively to a fungal attack; besides the enrichment of 'defense response' and 'response to biotic stimulus' GO terms, those related to chitin metabolism—in both biological process and molecular function ontologies —are also significantly enriched in upregulated genes in stems. Moreover, in relation to defense response to pathogens, we found that the GO term 'metabolism of reactive oxygen species' is significantly enriched in the upregulated genes in stems of abnormal plants. Furthermore, responses to abiotic stimuli and organic compounds such as hormones (specially auxin and brassinosteroid) are likely to influence the pathogenesis of the anomaly since GO terms are significantly enriched in differentially expressed genes. Finally, GO terms related to cell wall metabolism and organization are significantly enriched in the differentially expressed genes in stems, thus indicating that these processes play important roles in the pathogenesis of the anomaly.

Differentially expressed metabolic pathways

In addition to identifying the biological processes likely to be altered in the anomaly under investigation, we also sought to determine which metabolic pathways were differentially expressed in their entirety when comparing normal and abnormal plants. Based on pathways retrieved from the Gramene database, we found that 35 metabolic pathways are differentially expressed between abnormal and normal plants, of which 18 are upregulated (positive *Z*-scores) and 17 are downregulated (negative *Z*-scores) in abnormal plants (Supplementary data Table S6). Of these, four are differentially expressed both in leaf and stem tissue (arginine biosynthesis I, fatty acid oxidation pathway I, folate transformations, and 4-hydroxybenzoate biosynthesis V), nine are differentially expressed in both leaf and root tissue (chorismate biosynthesis I, glycine cleavage complex, lisine degradation II, vitamin E biosynthesis, lipid-independent phytate biosynthesis, tetrapyrrole biosynthesis I, flavin biosynthesis, thiamine biosynthesis II, and thiamine biosynthesis), and four are differentially expressed in all three tissues (lisine degradation II, urea cycle, superpathway of citrulline metabolism, and starch biosynthesis II from UDP-D-Glucose). Leaf tissue by far shows the greatest amount of differentially expressed metabolic pathways, with 33 being differentially expressed; in abnormal plants 17 are upregulated and 14 are downregulated compared to normal plants. In roots, seven and nine pathways are up- and downregulated, respectively, and in stems four pathways are up- and down-regulated. Compared with normal plants, the most upregulated pathway in the leaves of abnormal plants is light-dependent, aerobic biosynthesis of chlorophyllide *a* (*Z*-score = 4.1) and the most downregulated is degradation

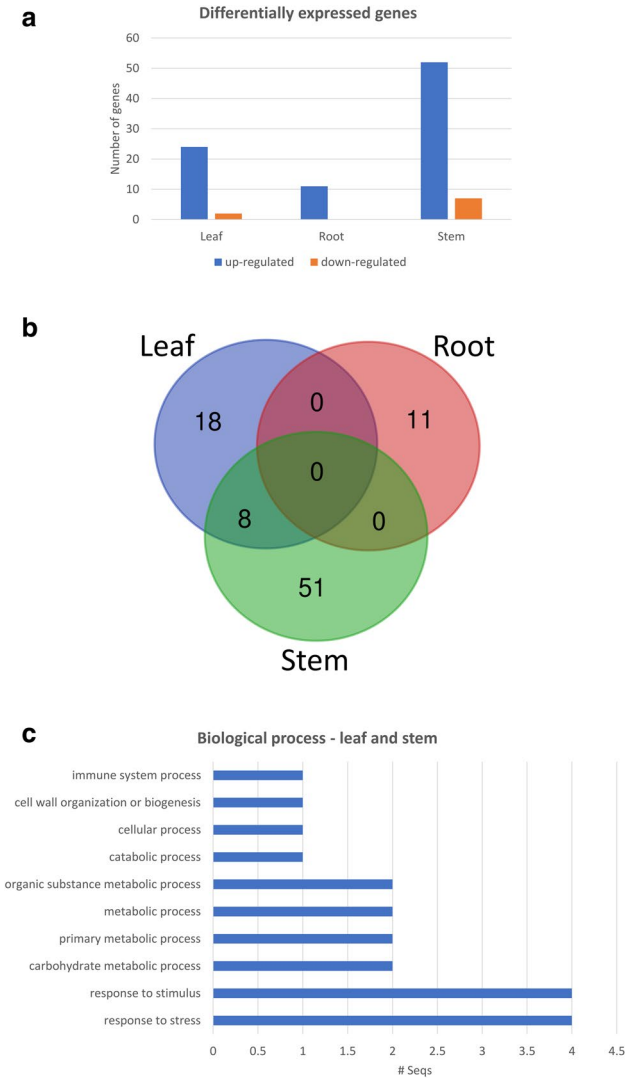

Fig. 1 *Eucalyptus* gene expression profile related to the abnormal phenotype: **a** total number of upregulated and downregulated genes in the different tissue types; (**b**) Venn diagram of differentially expressed genes in all tissue types; (**c**) Gene ontology of biological process category for common genes in leaf and stem tissue

of tyrosine (*Z*-score = – 4.1). In roots, the most upregulated and downregulated pathways are thiamine biosynthesis (*Z*-score = 2.1) and vitamin E biosynthesis (*Z*-score = – 2.8), respectively. Finally, in stems, the most upregulated is biosynthesis of folate (Z-score = 2.1) and downregulated is biosynthesis of 4-hydroxybenzoate (Z-score = – 2.6). As with differential gene expression and GO enrichment analysis, we observed overexpression of metabolic pathways related to defense response processes in the abnormal phenotype (thiamine diphosphate biosynthesis—THISYNARA-PWY and THISYN-PWY; glucosinolate biosynthesis from homomethionine—PWY-1187; glucosinolate biosynthesis from phenylalanine—PWY-2821; and glucosinolate biosynthesis from dihomomethionine—PWYQT-4471).

Discussion

Comparing transcriptome profiles of different phenotypes can offer insights into the underlying biological mechanisms associated with an anomaly. In our analysis, we observed that in all tissue types of the abnormal phenotype most of the upregulated genes, GO-terms, and upregulated pathways are involved in defense responses. The three gene families most representative in abnormal upregulated genes (class I chitinase, thaumatin family, and pathogenesis-related protein BET v1) play an important role in resistance against pathogens. Chitinases are enzymes that degrade chitin, a cell wall constituent of pathogenic fungi, thus playing a crucial role in plant defense against biotic stress (Flach et al. 1992; Kasprzewska 2003; Kumar et al. 2018; Malik 2019). In addition to gene upregulation in all tissues, abnormal plants showed a statistically significant presence of GO terms related to chitinase activity in the set of upregulated genes. Genes of the thaumatin family are present in several organisms (i.e., plants, fungi, and animals). They are associated with defense and developmental processes and act on plasma membrane permeabilization of pathogens (Ruiz-Medrano et al. 1992; Kitajima and Sato 1999; Liu et al. 2010; Jesús-Pires et al. 2020). Meanwhile, BET v1-like genes comprise the pathogenesis-related protein 10 (PR10) family which is associated with plant defense functions in response to biotic and abiotic stresses and allergens (Hoffmann-Sommergruber et al. 1997; Liu and Ekramoddoullah 2006; Wen et al. 1997; Žiarovská and Zeleňáková 2018; Santoni et al. 2019).

In addition, some abnormal upregulated genes may also be involved in defense processes, such as cytochrome P450 (Eucgr.K02129, Eucgr.B03596) which converts benzoic acid (BA) to salicylic acid (SA), an important phytohormone that induces a wide spectra of antistress reactions, including responses to pathogen infection (Hammond-Kosack and Jones 1996; Taiz and Zeiger 2013; Lefevere et al. 2020). SA is synthesized from chorismate in two different ways: (1) chorismate is converted into isochorismate and then into SA; (2) chorismate is first converted to phenylalanine, which is converted into BA and then into SA by cytochrome P450 monoxygenase (Hammond-Kosack and Jones 1996; Shah 2003; Mustafa and Verpoorte 2005; Chen et al. 2009; Lefevere et al. 2020). Chorismate biosynthesis is upregulated in abnormal plants; it is synthesized by the shikimate pathway that starts with phosphoenolpyruvate and D-erythrose-4-phosphate (Coruzzi and Last 2000; Malkin and Niyogi 2000; Mustafa and Verpoorte 2005). Glyceraldehyde-3-phosphate is a precursor of D-erythrose-4-phosphate, and it is involved in several metabolic pathways, such as glucose biosynthesis which is necessary for complex carbohydrate synthesis (Taiz and

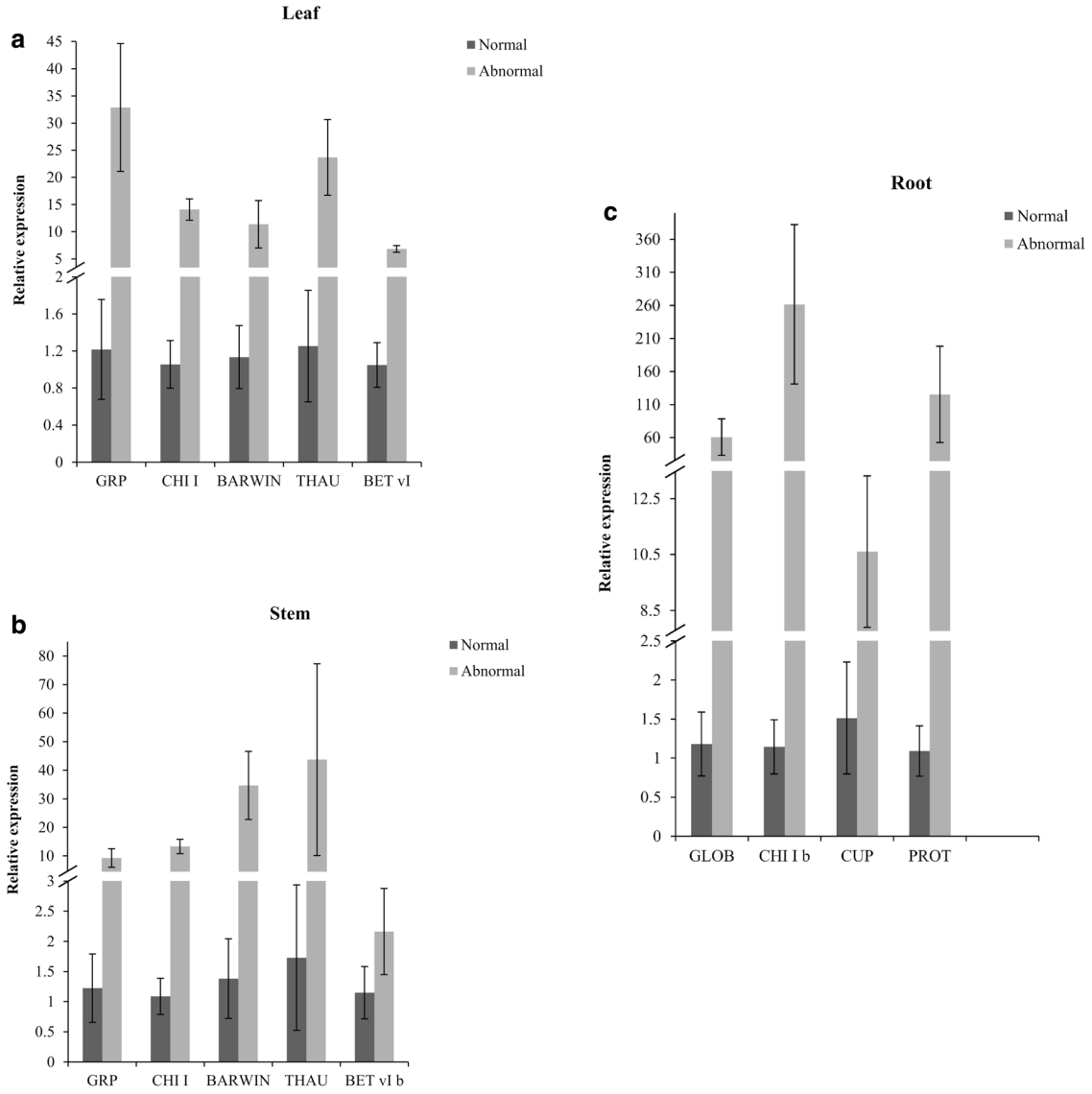

Fig. 2 Expression analysis of selected differentially expressed genes in abnormal and normal phenotypes by quantitative RT-PCR of leaf (**a**), stem (**b**), and root (**c**) tissues

Zeiger 2013). Since reserve carbohydrate biosynthesis (PWY-5067—Starch biosynthesis II from UDP-D-Glucose) is downregulated in abnormal plants, glyceraldehyde-3-phosphate is possibly used in chorismate biosynthesis, thus generating D-erythrose-4-phosphate.

Other defense responses can be attributed to the superoxide dismutase gene that is upregulated in abnormal plants, as well as GO terms related to reactive oxygen species metabolic processes which are significantly enriched in the set of upregulated genes. Levels of hydrogen peroxide are increased by superoxide dismutase activity, which are important antioxidant enzymes during physiological defense responses (Hammond-Kosack and Jones 1996; Heath 2000; Durrant and Dong 2004; Stephenie et al. 2020). Other substances like nitric oxide and hydrogen peroxide were also found to be upregulated in abnormal plants (L-arginine to L-citrulline conversion of citrulline metabolism and urea cycle pathways—PWY-5004). Nitric oxide and hydrogen peroxide are important components in the process of hypersensitive response and systemic acquired resistance, where nitric oxide acts a trigger for the hypersensitive disease resistance response (Hammond-Kosack and Jones 1996; Delledonne et al. 1998; Heath 2000; Durrant and Dong 2004; Zeier et al. 2004; Chen et al. 2014).

Our results also demonstrate differential gene expression, metabolic pathways, and GO terms related to the photosynthesis process that are significantly enriched in the set of differentially expressed genes. Reductions or increases in

photosynthetic activity have been described in plants under biotic and abiotic stress (Alam 1999; Scharte et al. 2005; Chaves et al. 2009; Bilgin et al. 2010; Pérez-Bueno et al. 2019). According to Ayres (1984, 1991) and Ney et al. (2013), increases in photosynthetic activity can occur as a compensation mechanism; a reduced photosynthetic rate at the infection site can induce photosynthetic activity in other uninfected tissues. Although the plants in this study were not subjected to stress, the results indicate that they are exhibiting a defense against stress, as demonstrated by the metabolic pathways. Plants under stress not only adjust their photosynthetic activity, but also increase their potential to absorb nutrients (Alam 1999; Pérez-Bueno et al. 2019), which could explain the significant enrichment of the GO term 'nutrient reservoir activity' in the set of upregulated genes in abnormal plant roots. These results suggest that abnormal plants may be responding to abiotic stimulus via roots through the accumulation of nutrients.

Glycine betaine is a representative member of the osmolyte group that is synthesized in the chloroplast from choline (Wang et al. 2003). The biosynthesis process of choline seems to be highly active in the leaves of abnormal plants as demonstrated by the significant enrichment of GO terms related to choline biosynthesis in the set of upregulated genes in abnormal plant leaves. Choline and glycine betaine levels are increased when plants are subjected to abiotic stress (Storey and Jones 1975; Russel et al. 1998; Annunziata et al. 2019), again indicating a defense-like reaction in the development of the *E. grandis* abnormality. Similar to choline, thiamine and glucosinolate biosynthesis are elevated in plants experiencing environmental stress, and thus play a fundamental role in inducing the expression of defense-related proteins (PRs) in salicylic acid-dependent pathways (Wang et al. 2006; Clay et al. 2009; Goyer 2010). Glucosinolate components are required for the deposition of callose between the membrane and the cell wall (Clay et al. 2009), preventing cell-to-cell spread of pathogenic microorganisms (Hammond-Kosack and Jones 1996; Singh 2017). Glucosinolate biosynthesis (PWY-1187, PWY-2821, and PWYQT-4471) is upregulated in leaves of abnormal plants, while thiamine (THISYNARA-PWY and THISYN-PWY) biosynthesis is upregulated in leaves and roots of abnormal plants. In roots, we found significant enrichment of terms related to cell communication—'membrane' and 'extracellular matrix'. We would expect such enrichment since responses to stimuli are usually performed through communication between cells and the environment.

When considering the development and maintenance of seedlings, normal plants show upregulated metabolic pathways related to lipid biosynthesis (PWY-5080) and degradation (FAO-PWY and PWY-5136) and lisine degradation (LYSINE-DEG1-PWY and LYSINE-DEG2-PWY). As with lipid degradation, lisine degradation pathways generate acetyl-CoA as a final product, indicating a lower level of adenosine triphosphate (ATP) production in abnormal plants. This can affect the plant's ability to obtain the energy needed for growth and maintenance of life, which can result in the anomalous phenotype and stunted development, leading to seedling death within a few months. Other substances, such as phenylpropanoid compounds (upregulated Eucgr.L02460 gene in abnormal plants), are associated with lignin biosynthesis, the structural components of the secondary cell wall of plants (Sato et al. 1993; Croteau et al. 2000; Taiz and Zeiger 2013; Carocha et al. 2015). Cell wall lignification is a defense strategy that allows plants to become more resistant to pathogen attacks (Hammond-Kosack and Jones 1996; Durner et al. 1997). Nevertheless, early cell wall lignification blocks cell growth and, consequently, restricts plant growth, as found in the anomalous phenotype (Huang et al. 2010; Taiz and Zeiger 2013). On the other hand, the GO term 'xyloglucan xyloglucosyl transferase activity' (GO:0016762) and the GO terms related to cell wall organization in normal plants indicate a cell wall loosening process, enabling cell growth. This can explain the difference in height between normal and abnormal plants (Fry et al. 1992; Stratilová et al. 2020). In terms of hormones, GO terms related to response to auxin and brassinosteroid, which are plant hormones with growth-promoting effects (Taiz and Zeiger 2013), are significantly enriched in the set of upregulated genes in stems of normal plants.

Curiously, GO terms related to pollen tube development are significantly enriched in the set of upregulated genes in abnormal plants. Among the genes associated with these terms is Eucgr.E01625, a pollen allergen with rare lipoprotein A (RlpA) DPBB (double-psi beta-barrel) domain. The biological function of pollen allergens is unknown, but they are structurally related to expansins, proteins able to induce extension (creep) of plant cell walls by acid growth (Cosgrove et al. 1997; Chen et al. 2016). Plant growth by cell-wall acidification can be induced in shoots, roots, and leaves as a response to water deficits, salt stress, and the fungal toxin fusicoccin. If the pH of the cell wall is not between 4.5 and 6, expansins are not activated regardless of their overexpression in the tissues (Cosgrove 2005). Thus, cell wall acidification in abnormal plants is insufficient to activate expansins. In addition to a possible early cell wall lignification, this could impede the regular growth of abnormal plants. An alternative explanation is that these genes may be involved in other unknown biological processes. Taken together, our results suggest that the characteristics observed in the abnormal plants are associated with alterations of pathways related to response to stimulus in which both metabolic and cellular processes are participating in the response through catalytic activity and binding of proteins and enzymes. Thus, the identified differential expression of genes and the enrichment analysis of pathways and GO

terms between abnormal and normal seedlings, suggest that the anomaly is associated with changes in defense responses to biotic and abiotic stress. Since progeny showed no evidence of pathogenic attacks and all were grown in a controlled environment, the pathway changes in abnormal plants are likely not related to environmental conditions. As such, the defense response activation in abnormal plants may be due to some autoimmune process caused by genetic incompatibility. Known as hybrid necrosis (or weakness hybrid), genetic incompatibility is associated with the inappropriate activation of the plant's immune system (autoimmune system) (Bomblies and Weigel 2007; Bomblies et al. 2007; Mizuno et al. 2010). This scenario was demonstrated by Wan et al. (2021), where hybrid necrosis is strongly linked with autoimmunity in plants.

Hybrid necrosis can be seen in interspecific crosses (Burkart-Waco et al. 2012; Jeuken et al. 2009; Masuda et al. 2007; Mizuno et al. 2010), intraspecific crosses between accessions (Bomblies et al. 2007; Smith et al. 2011; Xue et al. 2015; Świadek et al. 2017), cultivars (Khanna-Chopra et al. 1998; Reiber and Neuman 1999), and varieties (Knight 1947; Yamamoto et al. 2010; Vikas et al. 2013), or between individuals of the same species from different populations (Singh and Gutiérrez 1984; Galloway and Etterson 2005). Progeny with hybrid necrosis show phenotypic characteristics similar to those associated with responses to environmental stress (Bomblies and Weigel 2007; Bomblies et al. 2007; Jeuken et al. 2009; Mizuno et al. 2010; Yamamoto et al. 2010; Smith et al. 2011). Mizuno et al. (2010) found hybrid necrosis in part of a triploid hybrid progeny from crosses between *Triticum turgidum* (tetraploid wheat) and *Aegilops tauschii* (wild diploid wheat). Transcriptional analysis of these triploid hybrids experiencing hybrid necrosis showed overexpression of genes related to defense response and metabolism, similar to the results discussed herein. The similarity between our results and those obtained by Mizuno et al. (2010) supports the hypothesis of autoimmune mechanism activation in the eucalypt anomaly. Similar phenomena have been described in lettuce (Jeuken et al. 2009), *Nicotiana* spp. (Masuda et al. 2007), and rice (Yamamoto et al. 2010).

In general, genetic incompatibility involves negative epistatic interactions, as explained by the Bateson-Dobzhansky-Muller (BDM) model (Orr 1996; Bomblies and Weigel 2007; Bikard et al. 2009; Yamamoto et al. 2010). However, the 3:1 Mendelian segregation observed in the anomalous phenotype by Fuchs et al. (2015) indicates that the anomaly is not caused by epistatic interactions, but rather by allelic interactions in which the homozygosity of recessive alleles determines the anomalous characteristics. Similarly, Mizuno et al. (2010) observed that hybrid necrosis type III in triploid wheat was associated with one locus from the diploid parent that results in the anomaly when homozygous. Studies on *Arabidopsis thaliana* (Smith et al. 2011) and rice (Li et al. 1997; Heuer and Miézan 2003; Chen et al. 2008) have also shown allelic incompatibility in heterozygous loci.

Several genes related to defense responses (R genes) have characteristics that may predicate negative epistatic interactions, i.e., rapid sequence evolution and high copy number in the genome (Bomblies and Weigel 2007). Rapid changes in R gene sequences can be attributed to the host–pathogen coevolution process, in which the mutual, continuous, and intense selective pressure between pathogen and host induce genetic variability (McHale et al. 2006; Ting et al. 2008; Ridley 2009; Ispolatov and Doebeli 2009; Wan et al. 2021). Myburg et al. (2014) have suggested that the *Eucalyptus* genome has been shaped by genome duplication events and a subsequent high rate of tandem gene duplication. Duplications increase the copy number of R genes in the genome as well as the tendency of genetic incompatibility (Galloway and Etterson 2005; Paterson et al. 2010).

Conclusions

Differentially expressed genes and metabolic pathways between the contrasting phenotypes show high activation of defense response-related processes in abnormal plants. These results suggest that the anomaly is caused by the inappropriate activation of the plant's immune system (autoimmune response) associated with genetic incompatibility, a mechanism identified in numerous hybrids (Bomblies et al. 2007; Bomblies and Weigel 2007; Bomblies 2009; Wan et al. 2021). It is important to note that normal and abnormal plants were grown under the same greenhouse conditions, with no evidence of pathogen infection. Thus, the differences cannot be attributed to environmental conditions. We would like to highlight that, to the best of our knowledge, this is the first study to explore the genetic basis of a *Eucalyptus* phenotype that exhibits the same autoimmune response characteristics that have been described in the literature and attributed to hybrid necrosis. The in-depth resequencing of parental and offspring genomes is an area of future research that can help to better elucidate the mechanisms involved, such as their interactions and genetic compatibility. Moreover, this study offers some perspectives on the possibility of inappropriate immune system activation in plants, particularly in tree species.

Author contribution statement MCPF conducted the experiment and data collection. MCPF, MLA, NL, and BCR conducted data analysis. MCPF, MLA, and BCR wrote the final manuscript. ERG, SO, and CLM designed the experiment and study concept. MCPF, BCR, SM, TP, ELF and CLM revised the final manuscript.

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Data availability All sequence data generated in this study are available at NCBI Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra) accessions SRR1481484-SRR1481488 and SRR1481490-SRR1481491.

Declarations

Conflict of interest None declared.

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