An investigation into the effects of environmental stresses on gene family expression in *Eucalyptus*

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Abstract

Many RNA-seq studies have analyzed the effects of individual biotic and abiotic stresses on gene expression in *Eucalyptus*. Until now, RNA-seq data has not been used to investigate the effect of several environmental stresses on the expression of gene families in a single study on *Eucalyptus*. We organized an analysis of 18 studies that investigated the effects of various biotic and abiotic stresses on gene expression in *Eucalyptus*. In this study, six stresses including fungus, high temperature and elevated CO_2, drought, *potassium* deficiency, nitrogen deficiency, and *phosphor* deficiency were used. These stresses fall into four categories including drought, climate change, nutrient deficiency, and fungus. We found that the expression of over 341 gene families (protein kinase gene superfamily, Glycoside hydrolase (GH) gene family, ABC transporter gene family, etc.) in a range of *Eucalyptus* species and hybrids changed after exposure to biotic and abiotic stresses. Among these gene families, only glycoside hydrolase gene family was found to be differentially expressed in all six stresses. The proportion of differentially expressed genes (DEG) that were down relative to up-regulation was significantly higher in the main biological processes. Many of the gene families that responded to biotic and abiotic stresses encoded products involving in response to stimulus and metabolic process, developmental process, localization, and cellular component organization or biogenesis. Results of this study will be used to further characterize the gene regulatory networks underlying stress responsive genes in *Eucalyptus*.

Keyword: drought, gene family expression, gene ontology, reactive oxygen species, stress

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Introduction

There are more than 700 species and hybrids of *Eucalyptus*, genus of *Myrtaceae* (Boland et al., 2006). Eucalyptuses grow in different environmental conditions (Mugunga et al., 2015). They are tolerant to a wide range of ecological conditions (Mugunga et al., 2015, Sadeghi et al., 2018) and are relatively resistant to high temperatures and drought stress (Teulieres and Marque, 2007). Unfortunately, human activities have introduced a wide range of stresses around the world. These stresses threaten natural diversity and spread to all biological levels from genes to biospheres (Anderson et al., 2015). Environmental stresses such as drought, high temperatures and elevated CO_2, fungus, and nutrient deficiencies cause large losses to plants. Abiotic stresses are challenges for tree breeding in the coming decades (Fritsche-Neto and Borém, 2012). It is usually not possible to change the

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stress conditions, but plant improvement is possible to deal with all kinds of stresses. Therefore, the development of cultivars compatible with environmental stresses is the most sustainable way to deal with the current and future stresses, although the development of genotypes with high yield and adaptability is a major challenge. The prerequisite for the application of molecular breeding or biotechnological approaches is the identification of stress-tolerant genes whose expression enables plants to adapt to or tolerate such stresses and the dissection of the biological mechanisms in which these genes are involved (Baldoni et al., 2021). The development of forests in new areas, especially those with limited water resources, requires the search for genotypes that tolerate environmental stresses. A deep understanding of the mechanisms of tolerance to environmental stresses is essential for the reforestation of forest trees. Forest tree improvement programs have been done more on the growth, quality traits of wood, and tolerance to environmental stresses and drought (Fritsche-Neto and Borém, 2012). By analyzing the gene family expression dataset, we can identify genes that are differentially expressed in different tissues and environmental conditions.

RNA-seq technique is a modern, efficient, and inexpensive method compared to the old methods used to study the pattern of gene family expression. The RNA-seq method consists of two parts: laboratory and bioinformatics. The laboratory section includes RNA extraction, RNA fragmentation, double-stranded cDNA synthesis, adapter binding, selection of appropriate length, and amplification by PCR and sequencing. The bioinformatiphase includes quality control of readings, trim, placement of readings, analysis of differential expression of genes, and gene ontology (Upasani et al., 2017). Today, a large volume of RNA-seq data is available to researchers in public resources, which has made it possible to retrieve and reanalyze these data to extract more information. Gene expression analysis was performed for some species using RNA-seq data (Akter, 2017; Fan et al., 2021). Many studies have examined the effect of biotic and abiotic stresses on gene expression in *Eucalyptus* (Table 1). Until now, RNA-seq data has not been used to investigate the effect of several environmental stresses on the expression of gene families in a single study on *Eucalyptus*. The aim of this study was to identify stress responsive gene families under different biotic and abiotic stresses in *Eucalyptus* using RNA- seq data.

Material and Methods

In this study, a complete search was performed on the NCBI (http://www.ncbi.nlm.nih.gov), Array Express (http://www.ebi.ac.uk/arrayexpress), Science Direct, Web of Science, Scopus, and Google Scholar by May 2022 using the keywords RNA-seq, RNA sequencing, biotic stress, abiotic stress, and *Eucalyptus* (Table 1). These databases were searched with the aim of investigating the presence of published articles related to the effect of environmental stress on gene family expression in the *Eucalyptus* genus. In this analysis, six stresses including fungus (*Chrysoporthe austroafrican*a), high temperature and elevated CO_2, drought, K deficiency, N deficiency, and P deficiency were included. These stresses fall into four environmental stress categories including drought, climate change, nutrient deficiency, and fungus (Table 1). This study was performed independently of species and tissue type (Anderson et al., 2015). Also, in these studies RNAs has been extracted from different tissues, namely leaves, roots, stems, xylems, and shoot apex of *Eucalyptus alba*, *E. urophyllax, E. grandis*, *E. camaldulensis, E. grandis*, and *E. globulus.*

We analyzed the raw data with one bioinformatics pipeline; however, for some studies this did not give any DEGs, and for some other studies, more than 5,000 DEGs were obtained. Therefore, following Yang et al. (2021) who used the list of DEGs available in the published articles, appropriate DEGs lists were also used in this research from the published articles, where all genome references were *Eucalyptus grandis* v2 – Phytozome.

Individual genes were classified according to family genes (https://www. mandadilab. com/family/) (Bedre and Mandadi, 2019). Individual biological processes were assigned into the most important categories of biological

Table 1
Studies included in this research

Stresses	Stress Categories	Species & Hybrids	Tissues	Accession Number	Reference
drought	drought	*E. alba*	Shoot apex	SRA012867	Villar et al., 2011
drought	drought	*E. urophylla * E. grandis*	shoot apex	SRA012867	Villar et al., 2011
drought	drought	*E. camaldulensis* Mt.Isa= semi-arid tropics	Leaf	GSE39369	Thumma et al., 2012
drought	drought	E. *camaldulensis* Petford= humid tropics	Leaf	GSE39369	Thumma et al., 2012
drought	drought	E. *camaldulensis* Katherine = dry tropics	Leaf	GSE39369	Thumma et al., 2012
Chrysoporthe austroafricana	Fungus	*E. grandis*	Stem	GSE67554	Mangwanda et al., 2015
drought	drought	*E. grandis*	xylem	PRJNA514408	Ployet et al., 2019
K deficiency	nutrient deficiency	*E. grandis*	xylem	PRJNA514408	Ployet et al., 2019
drought	drought	*E. globulus*	Root	PRJNA486291	Ulloa et al., 2021
drought	drought	*E. globulus*	leaf	PRJNA486291	Ulloa et al., 2021
N deficiency	nutrient deficiency	*E. grandis*	leaf	PRJNA627686	Rossini et al., 2022
N deficiency	nutrient deficiency	*E. grandis*	xylem	PRJNA627686	Rossini et al., 2022
P deficiency	nutrient deficiency	*E. grandis*	leaf	PRJNA627686	Rossini et al., 202
P deficiency	nutrient deficiency	*E. grandis*	xylem	PRJNA627686	Rossini et al., 2022
K deficiency	nutrient deficiency	*E. grandis*	leaf	PRJNA627686	Rossini et al., 2022
K deficiency	nutrient deficiency	*E. grandis*	xylem	PRJNA627686	Rossini et al., 2022
high temperature and elevated CO_2	climate change	*E. grandis*	Stem	GSE165931	Feltrim et al., 2022
high temperature & elevated CO_2	climate change	*E. globulus*	Stem	GSE165931	Feltrim et al., 2022

process (http://www.informatics.jax.org/ vocab/ gene_ontology/). Variability in the responses to various stresses was tested with χ^2 test, df = 27 ($P \leq 0.05$), and difference between up- and down-regulation for all categories was tested with t-test ($P \leq 0.05$).

Result

Structure of the differentially-expressed genes (DEGs)

DEGs (N=26156) were identified in *Eucalyptus* that had been exposed to biotic and abiotic stresses. We found 341 gene families. DEGs were identified in 18 studies that used RNA-seq to test the effects of stress on *Eucalyptus* (Table 2).

Seven out of 18 studies tested gene expression in the leaf of *Eucalyptus*, five used xylem tissue, three used stem, two used shoot apex, and one used root as the source of RNA. The environmental stresses to which *Eucalyptus* was exposed included, fungus (*Chrysoporthe austroafricana*), high temperatures and elevated CO_2, drought, K deficiency, N deficiency, and P deficiency.

Ontology of DEG

The functional analysis of the DEG showed that 41 discrete biological processes were affected by stress. Genes associated with response to stimulus were the most frequently-identified ones (13.92%) as shown in Fig. I.

Genes associated with metabolic and developmental processes were the next most abundant category, comprising 20.62% of the differential transcriptome. Localization genes accounted for 2.81% of the differential transcripts. Cellular component organization or biogenesis, cell wall organization or biogenesis, biological regulation, cell communication, and biological process involved in interspecies interaction between organisms represented approximately 9.57% of the differential transcriptome. The genes involved in cell division accounted for 0.98% of the

differentially expressed transcripts. The most frequently-identified genes in each biological process are presented in Table 2. The direction of change of genes (up-regulation or down-regulation) comprising the different biological processes is shown in Fig. II.

The proportion of genes that were down-, relative to up-regulation, was significantly higher (t, $P \leq 0.05$) in the main biological process. The gene families most frequently affected by stresses are involved in the response to stimulus, metabolic process, developmental process, etc.

Table 2
The top 5 most commonly identified types of gene families in the main biological process affected by biotic and abiotic stresses

Biological Process	Gene Families				
metabolic process	Protein kinase (PK) gene superfamily (143)	Cytochrome P450 gene family (133)	Glycoside hydrolase (GH) gene family (118)	Acyl Lipid Metabolism (ALP) superfamily (113)	UDP-glycosyltransferases (UGT) gene family (95)
response to stimulus	UDP-glycosyltransferases (UGT) gene family (124)	Cytochrome P450 gene family (113)	Protein kinase (PK) gene superfamily (107)	MYB gene family (93)	Short-chain dehydrogenases/reductases (SDR) gene superfamily (79)
developmental process	Acyl Lipid Metabolism (ALP) superfamily (81)	Glycoside hydrolase (GH) gene family (75)	MYB gene family (68)	ABC transporter gene family (63)	Cytochrome P450 gene family (60)
Localization	Amino acid transporters (AAT) gene family (64)	ABC transporter gene family (58)	Inorganic solute cotransporter (ISCT) gene family (57)	Aquaporins (AQP) gene family (48)	Mitochondrial carrier (MCF) gene family (33)
cellular component organization or biogenesis	HSP20 gene family (71)	Kinesins gene family (30)	Tubulin gene family (23)	ABC transporter gene family (21)	Acyl Lipid Metabolism (ALP) superfamily (19)
cell communication	Protein kinase (PK) gene superfamily (92)	NB-LRR Gene Family (53)	Glycoside hydrolase (GH) gene family (24)	Rab gene family (16)	Short-chain dehydrogenases/reductases (SDR) gene superfamily (14)
cell wall organization or biogenesis	Glycoside hydrolase (GH) gene family (66)	Carbohydrate esterase (CE) gene family (34)	Acyl Lipid Metabolism (ALP) superfamily (31)	Expansin gene family (30)	Cellulose synthase (CS) gene family (29)
biological regulation	Protein kinase (PK) gene superfamily (35)	Cytochrome P450 gene family (29)	Inorganic solute cotransporter (ISCT) gene family (20)	Pectin methylesterase inhibitor (PMEI) gene family (19)	Acyl Lipid Metabolism (ALP) superfamily (18)
biological process involved in interspecies interaction between organisms	Protein kinase (PK) gene superfamily (54)	Acyl Lipid Metabolism (ALP) superfamily (32)	Terpene synthase (TPS) gene family (25)	UDP-glycosyltransferases (UGT) gene family (21)	2OG-Fe(II) oxygenase (Fe2OG) gene family (18)
cell division	Cellulose synthase (CS) gene family (49)	Kinesins gene family (16)	IQD gene family (12)	Microtubule-associated protein (MAP65) gene family (12)	Protein kinase (PK) gene superfamily (11)

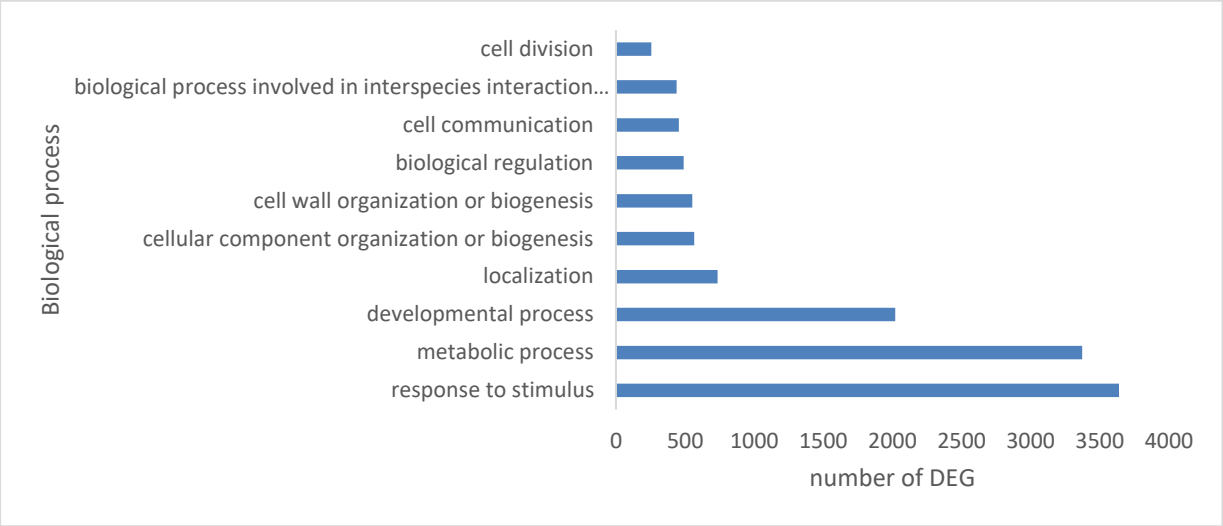

Fig. I. The number of DEG from all biotic and abiotic stresses that fall into the categories of biological processes

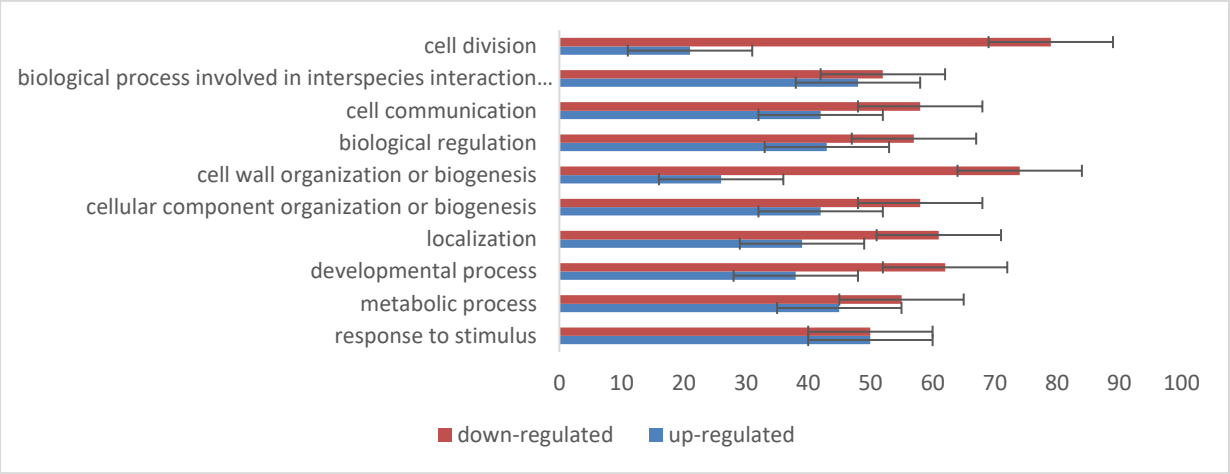

Fig. II. The percentage of genes within biological process that were introduced as either up-regulated or down-regulated; Bars = SEM; A significant difference was observed between up- and down-regulation for all categories (t, P≤ 0.05).

Among 341 gene families identified in this analysis, only glycoside hydrolase (GH) gene family was found to be DEG in all six stressors (*Chrysoporthe austroafricana* fungus, high temperature and elevated CO_2, drought, K deficiency, N deficiency, and P deficiency) (Table 3).

Protein kinase (PK) gene superfamily was the most frequently affected family. It was DEG in all treatments except P deficiency, and high temperature and elevated CO_2. ABC transporter gene family expression was effected by four out of six stresses. Commonly affected genes such as kinesins gene family were relatively rare. Only 2% of the 341 gene families in this analysis were DEG in more than four of the stresses, and the expression of 81% of genes was affected by just one of the stresses. Figure (III) shows the 10 most common differentially-expressed gene families across all stresses. Protein kinase (PK) gene superfamily was by far the most commonly identified differential transcript. This superfamily accounted for 17% of the differential transcriptome. Their transcript abundance was significantly affected by four stresses.

Among the top 10 gene families, those associated with the metabolic process (protein kinase (PK) gene superfamily, cytochrome P450 gene family, glycoside hydrolase (GH) gene family, acyl lipid metabolism (ALP) superfamily, UDP-glycosyltransferases (UGT) gene family, 2OG-Fe(II) oxygenase (Fe2OG) gene family, MYB gene family,

Table 3
The top 10 gene families whose expression was affected by the greatest number of biotic and abiotic stresses

Gene	Drought	P Deficiency	N deficiency	K deficiency	high temperature and elevated CO_2	Fungus (*Chrysoporthe austroafricana*)
Protein kinase (PK) gene superfamily	+	-	+	+	-	+
Glycoside hydrolase (GH) gene family	+	+	+	+	+	+
ABC transporter gene family	+	-	+	+	-	+
Acyl Lipid Metabolism (ALP) superfamily	+	-	+	+	-	+
Cytochrome P450 gene family	+	-	+	+	-	+
MYB gene family	+	-	+	+	-	+
UDP-glycosyltransferases (UGT) gene family	+	-	+	+	-	+
Kinesins gene family	+	-		+	-	+
Ring finger domain gene family	+	-	+	+	-	+
HSP20 gene family	+	-	+	+	-	+

+: present; -: absence

serine carboxypeptidase (SCP) gene family, glutathione S-transferase (GST) gene family, and ring finger domain gene family) represented 11.9% of the entire differential transcriptome, whilst response to stimulus (UDP-glycosyltransferases (UGT) gene family, cytochrome P450 gene family, protein kinase (PK) gene superfamily, MYB gene family, short-chain dehydrogenases/reductases (SDR) gene superfamily, HSP20 gene family, chlorophyll a/b-binding (LHC) gene family, acyl lipid metabolism (ALP) superfamily, ABC transporter gene family, and glycoside hydrolase (GH) gene family) each comprised 13.3% of differential transcripts.

Effects of different groups of stress on transcription

We compared just the effect of four different types of stress (drought, climate change, nutrient deficiency, and fungus) on gene expression. The percentages of differentially-expressed genes comprising the different biological process categories were broadly similar among all four general classes of stress (Fig. IV).

Genes in the metabolic process and response to stimulus were the most frequently affected by all stresses. There was significant variability in the responses to various stresses ($\chi 2 = 137.21$, $df = 27$, $P \leq 0.05$). Adaptive cellular responses to stress lead to increased protein kinase (PK) family and reactive oxygen species (ROS) (Fig. V).

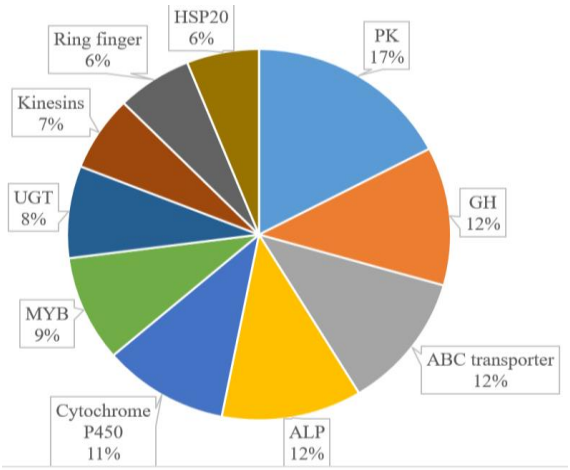

Fig. III. The 10 most common DEG families identified in *Eucalyptus* exposed to six different types of biotic and abiotic stresses; PK: Protein kinase (PK) gene superfamily, GH: Glycoside hydrolase (GH) gene family, ABC transporter: ABC transporter gene family, ALP: Acyl Lipid Metabolism (ALP) superfamily, Cytochrome P450: Cytochrome P450 gene family, MYB: MYB gene family, UGT: UDP-glycosyltransferases (UGT) gene family, Kinesins: Kinesins gene family, Ring finger: Ring finger domain gene family, and HSP20: HSP20 gene family

Discussion

Of the 26156 DEG identified, 42.27% were up-regulated and 57.73% were down-regulated in response to environmental stress. It is noted that

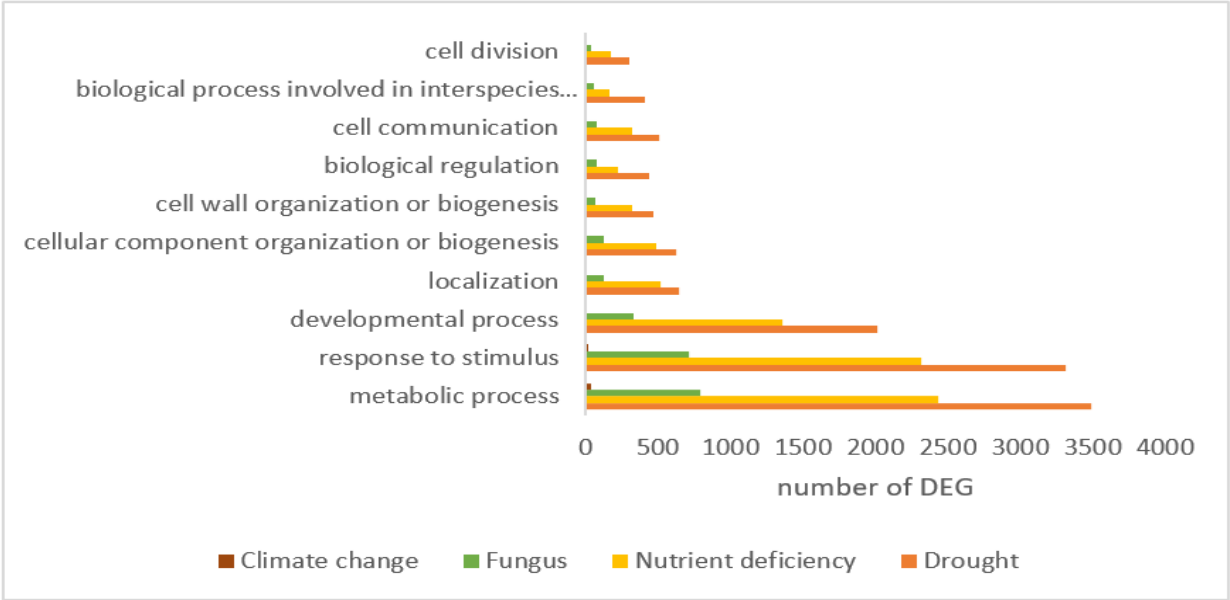

Fig. IV. Biological processes (BP) of genes that were differentially expressed in response to 4 types of stress: drought, nutrient deficiency, fungus, and climate change

individual genes could be either up- regulated or down-regulated depending on the duration of stress exposure (Anderson et al., 2015). We compared just the effect of different types of stresses (drought, climate change, nutrient deficiency, and fungus) on gene family expression. Genes in the metabolic process (1038 unique biological processes were observed, and top 10 most biological process were translation, protein phosphorylation, translational elongation, cellular catabolic process, secondary metabolic process, ubiquitin-dependent protein catabolic process, carbohydrate metabolic process, oxoacid metabolic process, phosphorylation, and carboxylic acid metabolic process) and response to stimulus (379 unique biological processes were observed, and top 10 most biological process were response to light stimulus, defense response to bacterium, response to water deprivation, response to abscisic acid, response to wounding, defense response to fungus, defense response to other organisms, response to inorganic substances, response to salt stress, and regulation of defense response) were the most frequently affected by all stresses.

Drought extremes have affected the transcription of many more gene families involved in the metabolic process than the other kinds of stress. Research advances have elucidated the role of

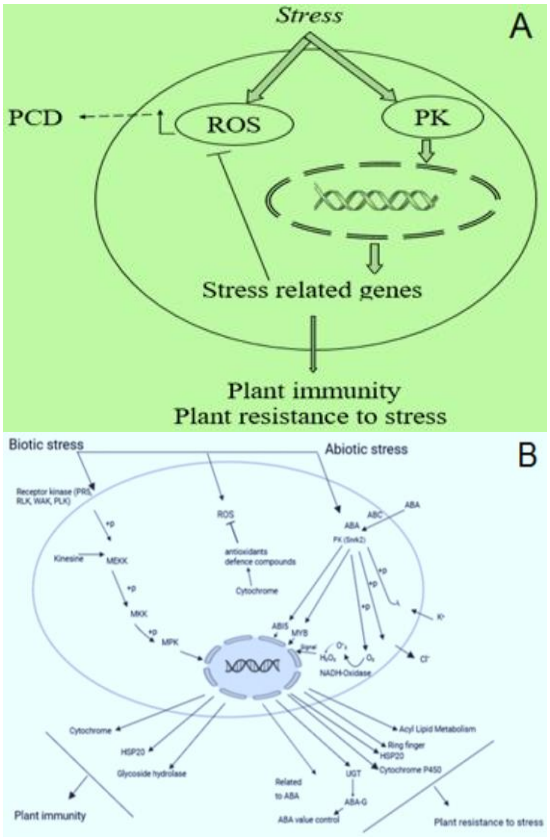

Fig. V. Adaptive cellular responses to stress lead to increased protein kinase (PK). Reactive oxygen species (ROS) are produced and controlled by stress-related genes (molecular chaperones, anti-oxidant gene, etc.). Excessive ROS can disrupt the cell leading to programmed cell death (PCD). A: detail view and B: general view

signaling networks in drought stress response due to a powerful interconnection of signals from hub transcription factors, MAPK pathways, ROS, and lipid derived pathways (Golldack et al., 2014). Drought and nutrient deficiency had a proportionally higher effect on gene families involved in the metabolic process as compared to fungus and climate change. Despite these minor variations, the gene expression profiles of *Eucalyptus* responding to various stresses were similar. This suggests that there is a core set of stress response genes within *Eucalyptus* cells, primarily involved in metabolic process, and that the response to stimulus of these genes is responsive to a wide range of biotic and abiotic stresses. Many studies have found differential responses to biotic and abiotic stresses in many of the same cellular pathways identified in the analysis. Leonardi Gde et al., (2015) showed that *E. urograndis* stem proteome responds quickly to cold stress, mostly by down-regulating specific proteins involved in energy metabolism, protein synthesis, and signaling. This study has compiled data from 18 different gene expression studies of *Eucalyptus* exposed to a wide range of environmental conditions. The results of this research can shed some light on how *Eucalyptus* responds to environmental stress which needs further studies. Biotic and abiotic stresses activate protein kinases, some of which act as receptors and others play a role in stress transduction (the attack of pathogens is sensed by pr5-RLK and WAK kinase receptors). This causes the expression of many genes related to stress, such as genes related to defense against pathogens and glycoside hydrolases. Glycoside hydrolases are enzymes with roles in nature including degradation of cellulose, in anti-bacterial defense strategies, and in pathogenesis mechanisms. The expression of kinesins is increased. The number of kinesins is closely associated with the MAPK cascade (Liang and Yang, 2019). Increased expression of genes related to stress resistance such as MYB gene family and UDP-glycosyltransferase (UGT) gene family (in drought stress), Acyl lipid metabolism (ALP) superfamily (in heat stress), Ring finger domain gene family (protein modification related to stress), HSP20 (chaperone), and cytochrome P450, which causes the biosynthesis of antioxidants and defensive compounds, reduction of free radicals, plant immunity, and stress resistance. Interestingly, there is a central protein kinase in the central part of ABA signal transduction called SNRK2, which phosphorylates its downstream targets and ultimately leads to the expression of genes related to abscisic acid and drought stress. Abscisic acid activates many channels that release potassium and chlorine and eventually causes water loss and closing of the stomata. A number of ABC transporters such as ABCG25 and ABCG4 are abscisic acid transporters and their expression increases in response to drought stress. A number of ABC transporters are involved in the detoxification of toxic compounds. Enzymes such as chitinases and *β* -1, 3-glucanse (GH) which are glycoside hydrolases are involved in plant defense (Minic, 2008). Abscisic acid induces the expression of MYB transcription factors and drought-related genes. Also, the UDP-glycosyltransferase (UGT) gene family causes the conjugate of abscisic acid with glucose (glucosyl ester) and thus controls the amount of abscisic acid and response to stress (Chen et al., 2020). HSP20 is a kind of chaperone and protects biological molecules. In response to abiotic stresses, the ring finger domain gene family generally acts as modification of stress-related proteins. It performs the degradation of proteins by ubiquitination (Han et al., 2022). Many enzymes involved in lipid metabolism are cytochrome P450. As a result of high temperature stress, the metabolism of saturated lipids increases, which can improve the resistance of tissues and organs to heat stress. Cytochrome P450 enzymes are involved in many defense-related processes in plants such as xenobiotic metabolism, hormone regulation, antioxidant biosynthesis, stress signaling, and biosynthesis of secondary metabolites that ultimately lead to plant defense and resistance to stress. Almost all of these effects have been shown in numerous studies of cellular function in *Eucalyptus* responding to environmental stress (Feltrim et al., 2022; Rossini et al., 2022; Ployet et al., 2019). Many gene families (341) were identified in this analysis. Many of them, for example DREB gene family, which are related to various types of biotic and abiotic stresses, were not mentioned here.

It is worth mentioning that investigation of transcriptomic data alone is not sufficient to understand the plant response to stresses. Because, some gene expressions may increase, it may increase to proteome (central dogma), but at the metabolome level, the level of metabolites may not be changed. Because the level of metabolites may be high, but it is consumed in other reactions at the level of the biological system. In this case, by examining the level of metabolites, we find that despite the increase in some gene expressions, the phenotype was not affected. This indicates that there are different regulatory relationships between genotype and phenotype. In quantitative traits such as drought tolerance, there are many interactions between system components that are not yet well understood. So, the use of a system biology approach is necessary to understanding the plant response to environmental stresses, a prerequisite for *Eucalyptus* breeding for biotic and abiotic stresses. Also, based on the DEG, primers and single nucleotide polymorphism (SNP) markers can be designed to be used in natural population to screen resistance genotypes. These molecular markers after validation in future studies are used as informative markers which can be used in marker assisted selection (MAS) in *Eucalyptus* breeding. It can be anticipated by using this technique the breeding cycle of forest trees would decrease dramatically. RNA-Seq technique produces large amounts of raw data and, in general, the reads are short and have sequencing errors. Reference genome sequences are of great importance for RNA-Seq data analysis because they provide the necessary templates for mapping reads. Most of the important forest tree species are without an up-to-date reference genome file (description of the genome sequence), which should be considered. The challenges of this study was the ambiguity of some sample names, lack of the details of materials and methods, unavailability of all data and the difference between the article and the related data in the database. So, we suggest that future researchers pay more attention to these important points. Also, the analysis of the cis-elements of promoters of the gene families should be done in order to understand the molecular mechanisms that respond to stress.

Conclusion

Many gene families (341) which are related to various types of biotic and abiotic stresses, were identified in this analysis. Of the 26156 DEGs identified in this study, 42.27% were up-regulated and 57.73% were down-regulated in response to environmental stresses. The results from this study will be used to further characterize the gene regulatory networks underlying stress responsive genes in *Eucalyptus*. Also, based on the DEG, informative markers can be designed to be used in natural population to screen resistant genotypes.

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