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Stem transcriptome of cold stressed Eucalyptus globulus and E. urograndis

Pedro Araújo[1], Felipe Thadeu Tolentino[2], Adilson Pereira Domingues Junior[3], Flávia Camila Schimpl[4], Daniela Feltrim[2], Vanessa Tofanello[2], Nathália Volpi[2], Jullyana Cristina Magalhães Silva Moura Sobczak[5] and Paulo Mazzafera[2,3]

[1] Department of Genetic, Evolution, Microbiology and Immunology, Institute of Biology, State University of Campinas, São Paulo, Brasil

[2] Department of Plant Biology, Institute of Biology, State University of Campinas, São Paulo, Brasil

[3] Laboratório de Produção Vegetal, Escola Superior de Agricultura "Luiz de Queiroz", University of São Paulo, Piracicaba, São Paulo, Brasil

[4] Faculty of Agricultural Science, Federal University of Amazon, Manaus, AM, Brasil

[5] Department of Agronomy, Universidade da Integração Internacional da Lusofonia Afro-Brasileira, Redenção, Ceará, Brasil

ABSTRACT

Eucalyptus is an important source of cellulose and a widely cultivated plant. Biotechnology tools can save time spent in breeding and transcriptomic approaches generate a gene profile that allows the identification of candidates involved in processes of interest. RNA-seq is a commonly used technology for transcript analysis and it provides an overview of regulatory pathways. Here, we selected two contrasting Eucalyptus species for cold acclimatization and focused in responsive genes under cold condition aiming woody properties – lignin and cellulose. The number of differentially expressed genes identified in stem sections were 3.300 in Eucalyptus globulus and 1370 in Eucalyptus urograndis. We listed genes with expression higher than 10 times including NAC, MYB and DUF family members. The GO analysis indicates increased oxidative process for E. urograndis. This data can provide information for more detailed analyses for breeding, especially in perennial plants.

***Keywords*:** Eucalyptus; RNA-seq; Cold Stress; Cell Wall

1. Introduction

Eucalyptus is one of the most cultivated tropical trees for cellulose pulp production and it has been suggested for use as an alternative for biofuel production[1]. Species in this genus are widespread in the world as a crop due to their large range of adaptability on diverse climates. Eucalyptus species are grown all over the Australian continent and nearby islands, including Tasmania, in its coldest parts. Eucalyptus is mostly cultivated in tropical regions, although there is great interest to expand to subtropical and temperate climate[2]. Some species, such as E. globulus, E. viminalis, E. dunnii and E. gunnii can grow in low temperatures but so far, it has been shown that only E. gunnii seems to resist freezing temperature[3]. E. globulus wood has the highest S/G (syringyl/guaiacyl) ratio, a characteristic that confers low recalcitrance for lignin removal and, consequently high cellulose yield. Under high temperature the S/G ratio of E. globulus decreases, which is probably related with the worst growth performance of these trees compared with other species adapted to tropical temperature[4]. Other species exploited commercially such as E. saligna, E. urophylla, E. grandis, E cloezina and C. citriodora grow better in warmer places[5,6]. E. grandis, E. urophylla and the hybrid E. grandis x E. urophylla are the most planted Eucalypts in Brazil, showing rapid growth and good fiber quality[7].

The genetic mechanism behind cold tolerance is still unclear despite many genes that have been characterized. Several genes coordinate the response to cold, by altering lipid composition and activating Ca^{2+} channels, which is me-

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diated by reactive oxygen species and abscisic acid. Cold-regulated genes may range from 4% to 20% of the whole genome in Arabidopsis[8].

RNA-seq is a useful tool to study transcriptome profiling, providing a wide overview of different functional elements in the genome[9]. The quantification of these elements can provide specific information on the developmental stage and physiological conditions, thus enabling comparisons between samples under different treatments or conditions[9].

We sequenced a transcript profile of stems from seedlings of E. globulus and E. urograndis grown under low temperature. We detected differential expression of several genes focused in cold stress tolerance and economically important traits for Eucalyptus breeding. Our study provides an overview of two contrasting species of Eucalyptus and enumerates several differential expression genes.

2. Methods

2.1 Plant material, RNA extraction and sequencing

Plants of E. globulus and E. urograndis were obtained from seeds collected from clonal gardens (Caiçara Sementes - http://www.sementescaicara.com) and cultivated in a greenhouse for 180 days, without control of humidity and temperature. Then the plants were transferred to growth chambers set to 4 ºC or 25 ºC, at 300 μmol m^{-2} s^{-1} and an 8 h photoperiod for 30 days. Stems were collected starting at 5 cm from the soil and a pool for each species were used for RNA extraction.

2.2 RNA extraction

Trizol (Invitrogen) was used for RNA extraction following the manufacturer protocol. The RNA quantity and quality were verified using Nanodrop 2000 (Thermo scientific) and agarose gel.

2.3 RNA sequencing

Performed by Fasteris (DNA Sequencing Service - https://www.fasteris.com/dna/) using SN365 – HiSeq2000, number of cycling 1x100 + 7 (index). Sequencing was carried out on four samples: (i) E. globulus control (11.394.167 reads); (ii) E. globulus cold stress (19.973.014 reads); (iii) E. urograndis control (16.151.851 reads) and (iv) E. urograndis cold stress (15.779.681). Quality control was performed and had an average of 79.9% of Q30 score per base.

2.4 RNA-seq analysis

The software GeneSpring (Agilent) was used to analyze our dataset from Fasteris. Initially, the reads were aligned against Eucalyptus grandis database from Phytozome (www.phytozome.net) - 8X mapped E. grandis BRASUZ1 genome assembly and annotation were carried out with Avadis (version 1.1 within GeneSpring software, Agilent). We also excluded ribosomal and low quality reads. All genes aligned/identified (known-genes and predicted ones) were analyzed separately to evaluate the expression pattern in each treatment and only reads found in all four libraries were selected to calculate the fold change. We also performed scatter-plotting graphics to identify relationships in gene expression (fold change) between treatments on both species. The same was done for the predicted new genes (not annotated).

All genes identified using E. grandis genome as reference were functionally annotated using Blast2Go and Arabidopsis thaliana annotation. The expression levels of each gene were determined using the FPKM (fragments per kilobase of exon per million fragments) value. Data from the cold treatments were normalized using the data from the corresponding control plants of each Eucalyptus species and the normalized data was used to compare differentially expressed genes between species. A differentially expressed gene was defined using two cut-offs 1 and 10 ($\log_2$).

3. Results

3.1 mRNA distribution of two contrasting species of Eucalyptus

To evaluate the transcriptional response of E. globulus and E. urograndis to cold stress we performed a broad analysis of the sequenced transcripts. **Figure 1**A shows that 13.380 genes are shared between the two species and 3.306 and 1.376 genes are uniquely expressed in E. urograndis and E. globulus, respectively. **Figure 1**B shows control and cold treatments data for both species. It is evident that E. urograndis regulates more unique genes than E. globulus. In the former 2007 and 1299 annotated genes were down- and upregulated, respectively, while 741 and 625 were found in

E. globulus.

GeneSpring software aligns annotated reads and suggests “predicted new genes”, which were not annotated, using E. grandis genome as reference. The software identified as “new” 253 upregulated and 333 downregulated genes (**Figure 1**B). In contrast, 246 genes were upregulated in E. globulus but downregulated in E. urograndis and conversely 381 genes were downregulated in E. globulus and upregulated in E. urograndis.

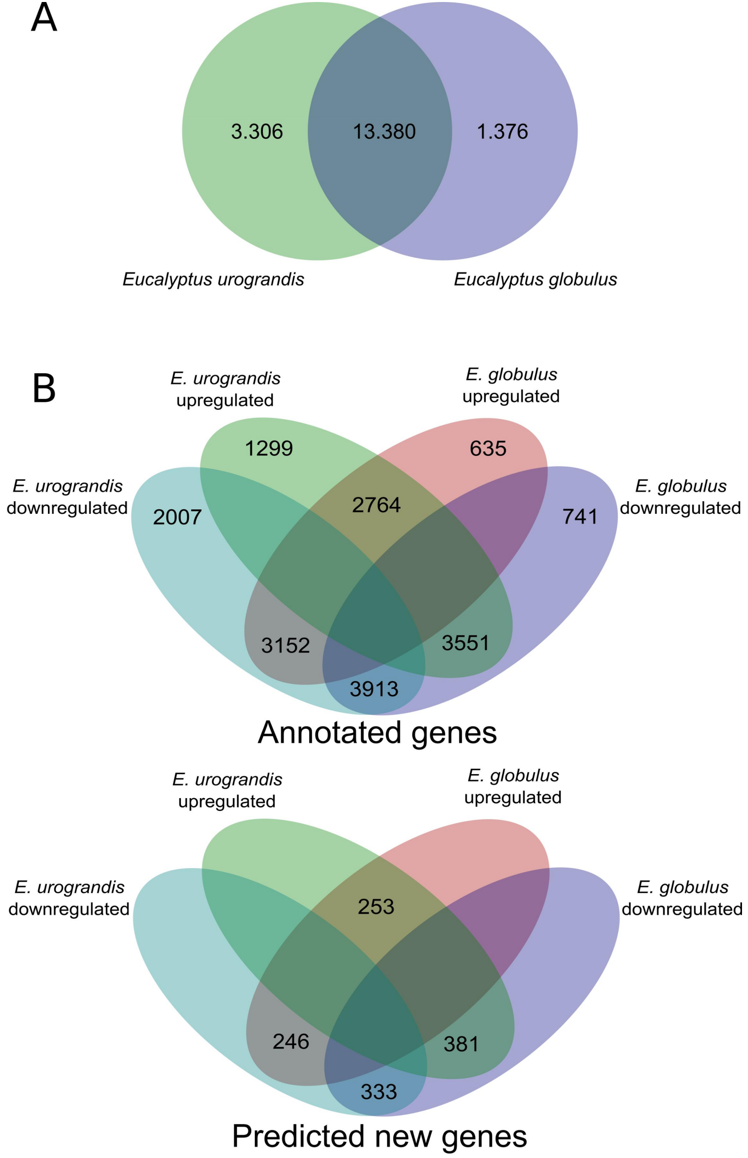

Figure 1; Overview of all annotated genes expressed under cold treatment for E. globulus (purple) and E. urograndis (green). They shared 13.380 genes for cold stress, being 3.306 genes differentially expressed for E. urograndis and 1.376 genes for E. globulus (A). Differentially expressed annotated genes and predicted new new genes by GeneSpring software (B).

A spatial distribution from **Figure 1** is shown in **Figure 2.** We explored the data to identify the most differentially expressed genes in Eucalyptus species. We used a cut-off of 10 to check the most differentially expressed genes found in both species. A cloud of genes (spots) tends to be aggregated in the middle close to line 0 for both species. E. globulus contributes for a more sparse distribution with higher levels of difference for up and down regulation. Two extreme sides were selected and a gene list was summarized on Tables 1 and 2. There is a brief description and function of each selected gene and Arabidopsis homolog. Several uncharacterized genes were present and some were without homologs (Eucgr.E03184). Interesting and well annotated homologs for these genes can be found: members of DUF genes: Eucgr.I02070, Eucgr.I02359, Eucgr.H03259 and Eucgr.L02157; NAC: Eucgr.C02105 and Eucgr.A02070 and MYB: Eucgr.A00996.

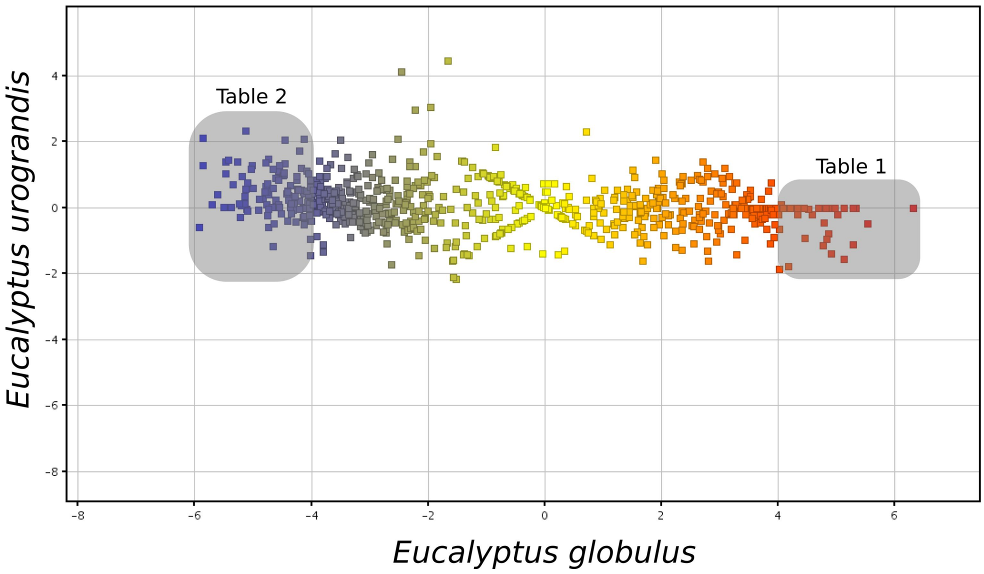

Figure 2; Distribution of gene expression profile between E. globulus and E. urograndis. We selected the outmost genes representing both species for detailed identification. Table 1 shows a list of genes upregulated for E. globulus cold and downregulated for E. urograndis cold and Table 2 shows a list of genes downregulated for E. globulus cold and upregulated for E. urograndis cold.

Gene ID	**Arabidopsis homolog***	**Description**	**Function***
Eucgr.F02784	AT1G75050	Pathogenesis-related thaumatin superfamily protein	Uncharacterized function and localized on endomembrane
Eucgr.E00767	AT1G01490	Heavy metal transport/detoxification superfamily protein	Metal ion transport
Eucgr.E01683	AT1G60320	Disease resistance protein (TIR-NBS-LRR class) family	Transmembrane receptor activity involved in signal transduction
Eucgr.J00988	AT4G27190	NB-ARC domain-containing disease resistance protein	NB-ARC domain-containing disease resistance protein, involved in apoptosis, defense response
Eucgr.H01219	AT3G44550	fatty acid reductase 4	Member of gene family encoding alcohol-forming fatty acyl-CoA reductases (FARs)
Eucgr.B00127	AT1G76650	calmodulin like 37	Calcium ion binding on plasma membrane
Eucgr.F02812	AT2G01300	-	Uncharacterized
Eucgr.L01926	-	-	-
Eucgr.B01326	AT5G26680	5\'-3\' exonuclease family protein	DNA binding and nuclease activity
Eucgr.C01418	AT2G42160	zinc finger domain-containing protein	Protein form a heteromeric complex required for the development
Eucgr.G01737	AT2G21250	NAD(P)-linked oxidoreductase superfamily protein	Oxidoreductase activity in response to cadmium ion
Eucgr.H03343	AT1G19640	S-adenosyl-L-methionine-dependent	Catalyzes the formation of methyljasmonate from

		methyltransferases	jasmonic acid
Eucgr.B00621	AT1G18610	Galactose oxidase/kelch repeat superfamily protein	Uncharacterized
Eucgr.H01117	AT1G09220	Pentatricopeptide repeat (PPR) superfamily protein	Uncharacterized function and localized on cytosolic ribosome
Eucgr.L02664	-	-	-
Eucgr.H04708	AT1G67730	beta-ketoacyl reductase 1	catalyzes the first reduction during very long chain fatty acids, >18 carbon elongation
Eucgr.K00822	AT3G21820	histone-lysine N-methyltransferase ATXR2	Zinc ion binding and unknown function

Table 1. Spatial gene distribution between E. globulus and E. urograndis. Genes upregulated in E. globulus and independently for E. urograndis (**Figure 2**). Cut-off fold change: 10. We also indicate the Arabidopsis homolog, description and function using TAIR database.

Gene ID	Arabidopsis homolog*	Description	Function*
Eucgr.G00235	AT4G24280	chloroplast heat shock protein 70-2	Involved in protein import into chloroplasts during early developmental stages
Eucgr.F03098	AT1G73040	Mannose-binding lectin superfamily protein	Mannose-binding lectin superfamily protein
Eucgr.C02554	AT5G23960	terpene synthase 21	Encodes a sesquiterpene synthase involved in generating all of the group. A sesquiterpenes found in the Arabidopsis floral volatile blend.
Eucgr.A01557	AT5G12100	pentatricopeptide (PPR) repeat-containing protein	pentatricopeptide (PPR) repeat-containing protein
Eucgr.C03056	AT2G26150	heat shock transcription factor A2	Heat Stress Transcription Factor (Hsf) family. Involved in response to misfolded protein accumulation in the cytosol.
Eucgr.H02642	AT1G05560	indole-3-acetate beta-D-glucosyltransferase	A UDP-glucose transferase localized in the phragmoplast. It has been co-purified with the callose synthase complex and may transfer UDP-glucose from sucrose synthase to the callose synthase and thus help form a substrate channel for the synthesis of callose at the forming cell plate. Induced by salicylic acid.
Eucgr.E00383	AT4G19050	NB-ARC domain-containing disease resistance protein	ATP binding. Involved in defense response and apoptosis
Eucgr.D02256	AT2G24190	NAD(P)-binding Rossmann-fold superfamily protein	Encodes an aldehyde reductase that catalyzes the reduction of the aldehyde carbonyl groups on saturated

			and alpha,beta-unsaturated aldehydes with more than 5 carbons in vitro. In addition, this enzyme can reduce methylglyoxal in vitro. It is believed that tThis enzyme is believed to be localizeds to the cytosol like such as the closely related protein encoded by AT3G61220
Eucgr.H03259	AT1G56230	Protein of unknown function (DUF1399)	Unknown function and expressed in plasma membrane
Eucgr.F00207	AT5G67090	Subtilisin-like serine endopeptidase family protein	Subtilisin-like serine endopeptidase family protein. Involved in proteolysis, negative regulation of catalytic activity on endomembrane system
Eucgr.A02555	AT5G06400	Pentatricopeptide repeat (PPR) superfamily protein	Pentatricopeptide repeat (PPR) superfamily protein
Eucgr.H01277	AT2G18360	alpha/beta-Hydrolases superfamily protein	alpha/beta-Hydrolases superfamily protein; hydrolase activity in endomembrane system; expressed in shoot apex, hypocotyl, root, leaf
Eucgr.C03936	AT2G19710	Regulator of Vps4 activity in the MVB pathway protein	Regulator of Vps4 activity in the MVB pathway protein. Unknown function.
Eucgr.F00173	AT4G37360	cytochrome P450	member of CYP81D
Eucgr.B03206	AT1G14140	Mitochondrial substrate carrier family protein	Transmembrane transporter activity. Mitochondrial substrate carrier family protein
Eucgr.A00876	AT3G18150	RNI-like superfamily protein	RNI-like superfamily protein
Eucgr.H01261	AT4G36670	Major facilitator superfamily protein	Major facilitator superfamily protein; carbohydrate transmembrane transporter activity, transmembrane transport
Eucgr.I00512	AT1G22360	UDP-glucosyl transferase 85A7	UDP-glucosyl transferase 85A2 (UGT85A2); UDP-glycosyltransferase activity, transferase activity, transferring glycosyl groups, glucuronosyltransferase activity
Eucgr.B03070	AT1G68740	EXS (ERD1/XPR1/SYG1) family protein	Involved in inorganic phosphate (Pi) transport and homeostasis
Eucgr.E00581	AT3G48990	AMP-dependent synthetase and ligase family protein	Encodes an oxalyl-CoA synthetase and is required for oxalate degradation
Eucgr.E03184	-	LRR and NB-ARC domains-containing disease resistance protein	-
Eucgr.F00195	AT5G42830	HXXXD-type acyl-transferase family protein	HXXXD-type acyl-transferase family protein, transferase activity, transferring acyl groups other than amino-acyl groups
Eucgr.L00154	AT5G47635	Pollen Ole e 1 allergen and extensin	Pollen Ole e 1 allergen and extensin family protein;

		family protein	located in endomembrane system.
Eucgr.C02105	AT2G24430	NAC domain containing protein 38	NAC Transcription factor (ANAC039)
Eucgr.H02515	AT1G10700	phosphoribosyl pyrophosphate (PRPP) synthase 3	Encodes a P-independent phosphoribosyl pyrophosphate (PRPP) synthase
Eucgr.G02674	AT1G79480	Carbohydrate-binding X8 domain superfamily protein	Carbohydrate-binding X8 domain superfamily protein; located in endomembrane system
Eucgr.I02302	AT4G35790	phospholipase D delta	Encodes a protein with phospholipase D activity. Involved in phospholipase metabolism. Mutants are affected in hydrogen peroxide- mediated cell death.
Eucgr.D00506	AT3G49340	senescence-associated gene 12	Cysteine proteinases superfamily protein involved in proteolysis
Eucgr.K01946	AT4G20910	double-stranded RNA binding protein-related / DsRBD protein-related	Encodes an enhancer of hua1 and hua2 that acts to specify reproductive organ identities and to repress A gene function.
Eucgr.I02070	AT2G21080	-	Unknown function. Similar to DUF3537
Eucgr.A01691	AT2G36970	UDP-Glycosyltransferase superfamily protein	transferase activity, transferring glycosyl groups localized on endomembrane
Eucgr.K02813	AT5G40390	Raffinose synthase family protein	Encodes a protein which might be involved in the formation of verbascose
Eucgr.F02756	AT1G17950	myb domain protein 52	R2R3-MYB transcription family
Eucgr.C01774	AT4G31980	-	Unknown protein and function
Eucgr.A00388	AT5G16990	Zinc-binding dehydrogenase family protein	Oxidative stress tolerance
Eucgr.H00964	AT1G75820	Leucine-rich receptor-like protein kinase family protein	Putative receptor kinase with an extracellular leucine-rich domain. Controls shoot and floral meristem size, and contributes to establish and maintain floral meristem identity
Eucgr.F03704	AT3G14810	mechanosensitive channel of small conductance-like 6	mechanosensitive channel of small conductance-like 5 (MSL5); INVOLVED IN: transmembrane transport
Eucgr.D02334	AT2G46240	BCL-2-associated athanogene 6	regulators of apoptosis
Eucgr.D00319	AT1G29930	chlorophyll A/B binding protein 1	Subunit of light-harvesting complex II (LHCII),which absorbs light and transfers energy to the photosynthetic reaction center
Eucgr.A00996	AT4G01680	myb domain protein 55	Encodes a putative transcription factor (MYB55)
Eucgr.H03111	AT1G06620	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase protein	similar to a 2-oxoglutarate-dependent dioxygenase

Eucgr.F03960	AT2G23060	Acyl-CoA N-acyltransferases (NAT) superfamily protein	Acyl-CoA N-acyltransferases (NAT) superfamily protein
Eucgr.A00632	AT2G38460	iron regulated 2	iron efflux transporter ferroportin
Eucgr.B02572	AT1G70140	Actin-binding FH2 (formin homology 2) family protein	Binds to F-actin barbed ends. Has severing actin filaments activity. InvovledInvolved in growth and cytoskeleton
Eucgr.E03270	AT4G20970	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	DNA binding - transcription factor activity. InvovledInvolved in defense response to fungus, regulation of transcription
Eucgr.G01747	AT4G08850	Leucine-rich repeat receptor-like protein kinase family protein	Leucine-rich repeat receptor-like protein kinase family protein; kinase activity; involved in protein amino acid phosphorylation on plasma membrane
Eucgr.K03566	AT1G09390	GDSL-like Lipase/Acylhydrolase superfamily protein	GDSL-like Lipase/Acylhydrolase superfamily protein; involved in glycerol biosynthetic process, lipid metabolic process.
Eucgr.G00487	AT2G23690	-	petal differentiation and expansion stage
Eucgr.F03623	AT5G41040	HXXXD-type acyl-transferase family protein	Encodes a feruloyl-CoA transferase required for suberin synthesis. It Hhas feruloyl-CoA-dependent feruloyl transferase activity towards substrates with a primary alcohol.
Eucgr.K01062	AT1G03840	C2H2 and C2HC zinc fingers superfamily protein	MGP is a nuclear-localized putative transcription factor with three zinc finger domains. MGP can interact with three proteins implicated in root patterning.
Eucgr.I02359	AT1G19835	Plant protein of unknown function (DUF869)	Unknown function (DUF869)
Eucgr.D00192	AT4G21120	amino acid transporter 1	Encodes a member of the cationic amino acid transporter (CAT) subfamily of amino acid polyamine choline transporters. Mediates efficient uptake of Lys, Arg and Glu in a yeast system.
Eucgr.F04109	AT4G10250	HSP20-like chaperones superfamily protein	endomembrane-localized small heat shock protein
Eucgr.A01605	AT3G53150	UDP-glucosyl transferase 73D1	transferase activity, transferring hexosyl groups localized on endomembrane
Eucgr.K00184	AT4G25420	gibberellin 20 oxidase 2	Encodes gibberellin 20-oxidase that is involved in the later steps of the gibberellin biosynthetic pathway. Regulated by a circadian clock. Weak expression response to far red light.
Eucgr.J01884	AT3G08510	Phosphoinositide-specific phospholipase C family protein	Phosphoinositide-specific phospholipase C (PI-PLC), catalyzes hydrolysis of phosphatidylinositol

			4,5-bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol.
Eucgr.K01503	AT3G03860	APR-like 5	Encodes a protein disulfide isomerase-like (PDIL) protein, a member of a multigene family within the thioredoxin (TRX) superfamily. This protein also belongs to the adenosine 5'-phosphosulfate reductase-like (APRL) group.
Eucgr.L00141	-	Ankyrin repeat family protein	-
Eucgr.C02224	-	-	-
Eucgr.A02746	ATCG00430	photosystem II reaction center protein G	Encodes a protein which was originally thought to be part of photosystem II but its wheat homolog was later shown to encode for subunit K of NADH dehydrogenase
Eucgr.K02809	AT5G63090	Lateral organ boundaries (LOB) domain family protein	Involved in lateral organ development
Eucgr.E00650	-	-	-
Eucgr.C03553	AT5G09380	RNA polymerase III RPC4	DNA-directed RNA polymerase activity. transcription from RNA polymerase III promoter
Eucgr.L01480	AT1G34420	leucine-rich repeat transmembrane protein kinase family protein	Leucine-rich repeat transmembrane protein kinase family protein; function in protein kinase activity, ATP binding and involved in protein amino acid phosphorylation
Eucgr.F01690	AT1G33790	Mannose-binding lectin superfamily protein	Jacalin lectin family protein. Uncharacterized function
Eucgr.E02849	AT4G18930	RNA ligase/cyclic nucleotide phosphodiesterase family protein	RNA ligase/cyclic nucleotide phosphodiesterase family protein
Eucgr.B00742	AT1G68390	Core-2/I-branching beta-1	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein
Eucgr.E00385	AT2G21840	Cysteine/Histidine-rich C1 domain family protein	Zinc ion binding and involved in intracellular signaling pathway
Eucgr.B03175	-	Remorin family protein	-
Eucgr.A02070	AT3G04070	NAC domain containing protein 47	multicellular organismal development, regulation of transcription, transcription factor (NAC047)
Eucgr.L02157	AT5G45470	Protein of unknown function (DUF594)	Unknown function (DUF594).
Eucgr.A02133	AT3G03750	SET domain protein 20	zinc ion binding, histone-lysine N-methyltransferase activity and chromatin modification
Eucgr.K01344	AT2G32270	zinc transporter 1 precursor	A member of Zrt- and Irt-related protein (ZIP) family.

			transcriptTranscript is induced in response to zinc deficiency in the root. Also, response to iron deficiency.
Eucgr.A00929	AT3G21760	UDP-Glycosyltransferase superfamily protein	inhibitor of cell expansion in vivo to form a bioactive glucoside
Eucgr.H03539	AT5G26340	Major facilitator superfamily protein	Encodes a protein with high affinity, hexose-specific/H+ symporter activity. The activity of the transporter appears to be negatively regulated by phosphorylationL5
Eucgr.F00591	AT1G75750	GAST1 protein homolog 1	GA-responsive GAST1 protein homolog regulated by BR and GA antagonistically. Possibly involved in cell elongation based on expression data
Eucgr.L01281	AT1G60420	DC1 domain-containing protein	Reduces transmission through pollen.
Eucgr.D00326	AT4G18210	purine permease 10	May be involved in the transport of purine and purine derivatives such as cytokinins, across the plasma membrane.
Eucgr.H01528	AT1G74310	heat shock protein 101	Encodes ClpB1, which belongs to the Casein lytic proteinase/heat shock protein 100 (Clp/Hsp100) family. Involved in refolding of proteins which form aggregates under heat stress
Eucgr.K01947	AT4G20900	Tetratricopeptide repeat (TPR)-like superfamily protein	ms5 mutants are male sterile, pollen tetrads undergo an extra round of division after meiosis II without chromosome replication, resulting in chromosome abnormalities.
Eucgr.L03554	AT3G07880	Immunoglobulin E-set superfamily protein	SUPERCENTIPEDE1 (SCN1), Rho GDP-dissociation inhibitor activity and involved in root epidermal cell differentiation, cell tip growth.
Eucgr.H00528	AT3G63380	ATPase E1-E2 type family protein / haloacid dehalogenase-like protein	ATPase E1-E2 type family protein; calcium-transporting ATPase activity; calcium ion transport located in membrane
Eucgr.L03371	AT2G30470	high-level expression of sugar-inducible gene 2	Member of B3 family. Active repressor of the Spo minimal promoter through the EAR motif.
Eucgr.G02802	AT2G16880	Pentatricopeptide repeat (PPR) superfamily protein	Pentatricopeptide repeat (PPR) superfamily protein
Eucgr.G01996	AT1G65810	P-loop containing nucleoside triphosphate hydrolases protein	P-loop containing nucleoside triphosphate hydrolases superfamily protein
Eucgr.C00666	AT2G26170	cytochrome P450	Encodes a protein with similarity to thromboxane-A synthase, member of the CYP711A cytochrome P450 family. Expressed in vascular traces in the rosette stem and axillary buds.
Eucgr.F01779	AT1G20640	Plant regulator RWP-RK family protein	Plant regulator RWP-RK family protein
Eucgr.I00729	AT5G43120	ARM-repeat/Tetratricopeptide repeat (TPR)-like protein	ARM-repeat/Tetratricopeptide repeat (TPR)-like protein; binding function
Eucgr.E03930	AT3G51970	acyl-CoA sterol acyl transferase 1	acyltransferase activity. Localized in endomembrane
Eucgr.G01935	AT5G53150	DNA heat shock N-terminal domain-containing protein	heat shock protein binding. Involved in protein folding
Eucgr.K01950	AT5G11720	Glycosyl hydrolases family 31 protein	Glycosyl hydrolases; involved in carbohydrate metabolic process; located in apoplast, vacuole, plant-type cell wall

Eucgr.J00165	AT2G40390	-	Unknow protein
Eucgr.J01979	AT5G59720	HSP20-like chaperones superfamily protein	Encodes a low molecular weight heat shock protein that contains the heat shock element in the promoter region. Expression is induced in response to heat shock.
Eucgr.K00177	AT1G65680	expansin B2	member of BETA-EXPANSINS
Eucgr.E01215	AT5G45380	solute:sodium symporters	Degradation of Urea (DUR3). Sodium/solute symporter
Eucgr.E03282	AT5G18360	disease resistance protein (TIR-NBS-LRR class)	Disease resistance protein family, transmembrane receptor activity, nucleoside-triphosphatase activity, nucleotide binding, ATP binding. Involved in signal transduction, defense response, apoptosis, innate immune response
Eucgr.G00561	AT1G77855	-	-
Eucgr.I01734	AT3G27550	RNA-binding CRS1 / YhbY (CRM) domain protein	RNA-binding CRS1 / YhbY (CRM) domain protein
Eucgr.A01170	-	-	-

Table 2. Spatial gene distribution between E. globulus and E. urograndis. Genes downregulated in E. globulus and independently for E. urograndis (**Figure 2**). Cut-off fold change: 10. We also indicate the Arabidopsis homolog, description and function using TAIR database.

3.2 Expression profile of selected genes focused in cold and economical traits for Eucalyptus

RNA-seq provided a broad gene expression overview and we explored the data from **Figure 1** to identify the most differentially expressed genes in Eucalyptus species. In addition, we selected based on literature cell wall related genes. Kinases are intrinsically related to cold response and affects the cell wall dynamics. Several proteins area activated by phosphorylation and kinases are the first step as a response to cold stress and they allow for the fluidity of the membrane to keep form, avoiding freezing and, consequently, cell damage[10]. Here, we selected, using GO annotation, all the kinases in both species and summarized and annotated them in **Figure 3.** A total of 62 kinases were identified and several showed high distinct patterns of expression. Two kinases are associated with cell wall and might be involved in response to cold stress: Eucgr.C03129 and Eucgr.F04287. Eucgr.C03129 is downregulated in E. globulus. Eucgr.F04287 is differently upregulated in E. globulus.

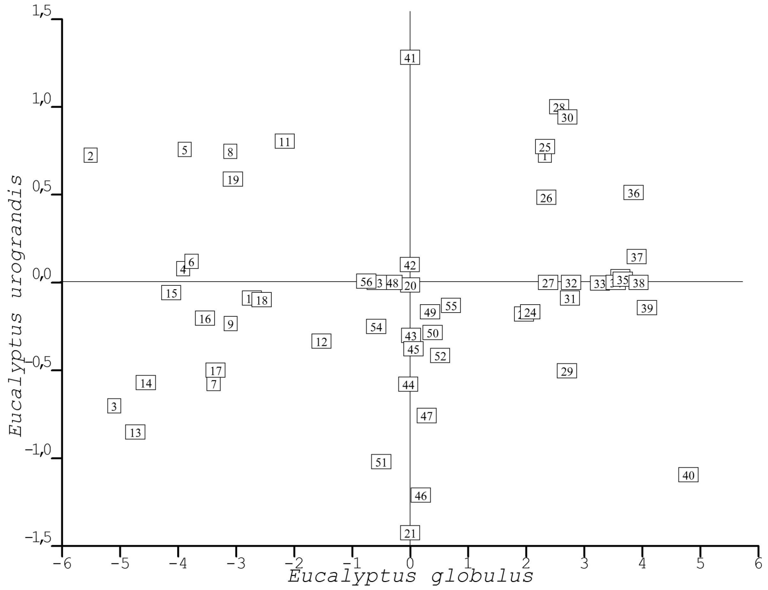

Figure 3; Spatial distribution of kinases between E. globulus and E. urograndis**.**

The x and y axes indicate fold change in cold response. List of genes: (1)Eucgr.A00282; (2)Eucgr.A01154;

(3)Eucgr.A01155; (4)Eucgr.A01482; (5)Eucgr.A01762; (6)Eucgr.A01765; (7)Eucgr.B00290; (8)Eucgr.B00556; (9)Eucgr.B00766; (10)Eucgr.B00782; (11)Eucgr.B00810; (12)Eucgr.B01034; (13)Eucgr.B02528; (14)Eucgr.B03649; (15)Eucgr.C01389; (16)Eucgr.C01451; (17)Eucgr.C02358; (18)Eucgr.C03129; (19)Eucgr.C03832; (20)Eucgr.C04151; (21)Eucgr.D00446; (22)Eucgr.D00914; (23)Eucgr.D00919; (24)Eucgr.E00581; (25)Eucgr.E02450; (26)Eucgr.E03457; (27)Eucgr.E04067; (28)Eucgr.F00850; (29)Eucgr.F02419; (30)Eucgr.F02500; (31)Eucgr.F02516; (32)Eucgr.F03308; (33)Eucgr.F03765; (34)Eucgr.F03981; (35)Eucgr.F04005; (36)Eucgr.F04287; (37)Eucgr.F04434; (38)Eucgr.G00775; (39)Eucgr.G01015; (40)Eucgr.G01747; (41)Eucgr.G01897; (42)Eucgr.G02274; (43)Eucgr.H00964; (44)Eucgr.H01556; (45)Eucgr.H02636; (46)Eucgr.I01659; (47)Eucgr.J01520; (48)Eucgr.J02873; (49)Eucgr.K01759; (50)Eucgr.K03047; (51)Eucgr.L00381; (52)Eucgr.L00480; (53)Eucgr.L01599; (54)Eucgr.L02004; (55)Eucgr.L02016; (56)Eucgr.J02803; (57)Eucgr.B03317; (58)Eucgr.E00442; (59)Eucgr.E03666; (60)Eucgr.E04192; (61)Eucgr.D00784; (62)Eucgr.B01186.

Few cold responsive genes were identified and special attention was given to CBF transcription factor genes, which triggers several responses to cold[11]. There are three homolog genes for E. urograndis and E. globulus. The expression pattern is different for Eucgr.A02831 and Eucgr.A02832 agreeing with the main characteristic of E. globulus – being adapted to cold places[12] (**Figure 4**). Both genes are upregulated, especially Eucgr.A02832, although this same gene is downregulated in E. grandis.

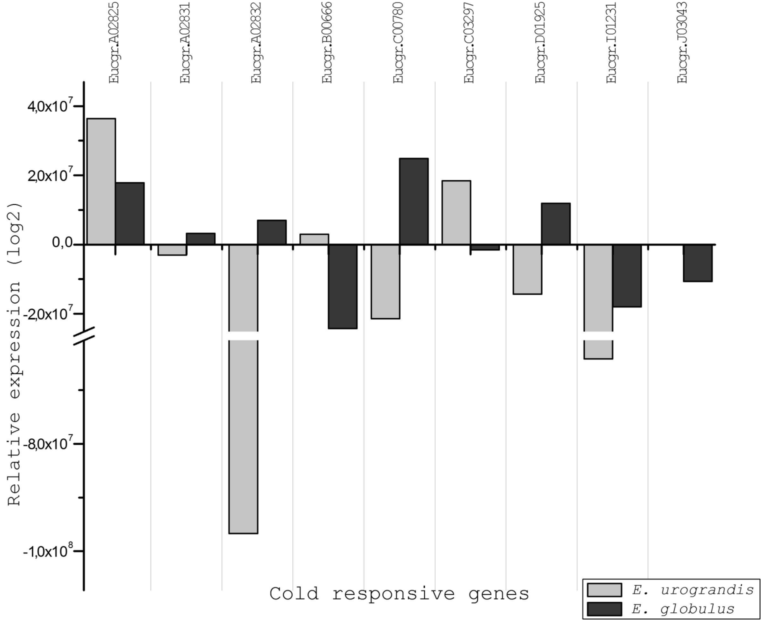

Figure 4; Expression profile of COR genes in Eucalyptus species. Annotated genes and homolog from Arabidopsis: Eucgr.A02825 (CBF1); Eucgr.A02831 (CBF1); Eucgr.A02832 (CBF1); Eucgr.B00666 (RAP2.11); Eucgr.C00780 (ERF017); Eucgr.C03297 (ERF017); Eucgr.D01925 (CBF2); Eucgr.I01231 (COR413); Eucgr.J03043 (LEA)**.**

The lignin pathway is coordinated by several and redundant genes[13]. In general, most of the genes in this pathway are: (i) upregulated in E. urograndis and (ii) downregulated in E. globulus. The genes F5H and CAD are consistently expressed following the characteristic behavior found in these species. Most CAD genes (Eucgr.E01103, Eucgr.E01104; Eucgr.E01105, Eucgr.F01677 and Eucgr.G01350) are upregulated and can be involved in the high levels of lignin in E. urograndis due to them being on the last step of monolignol synthesis. All the F5H are upregulated in E. urograndis and three other genes are more expressed in E. globulus. This step is important in synapyl alcohol synthesis and will increase the amount of S lignin units creating less C-C bonds leading to easier pulp extraction[13]. The S/G ratio is higher in E. globulus and the transcripts Eucgr.B00716, Eucgr.C00484 and Eucgr.K02211 might be regulating S lignin in this pathway (**Figure 5**).

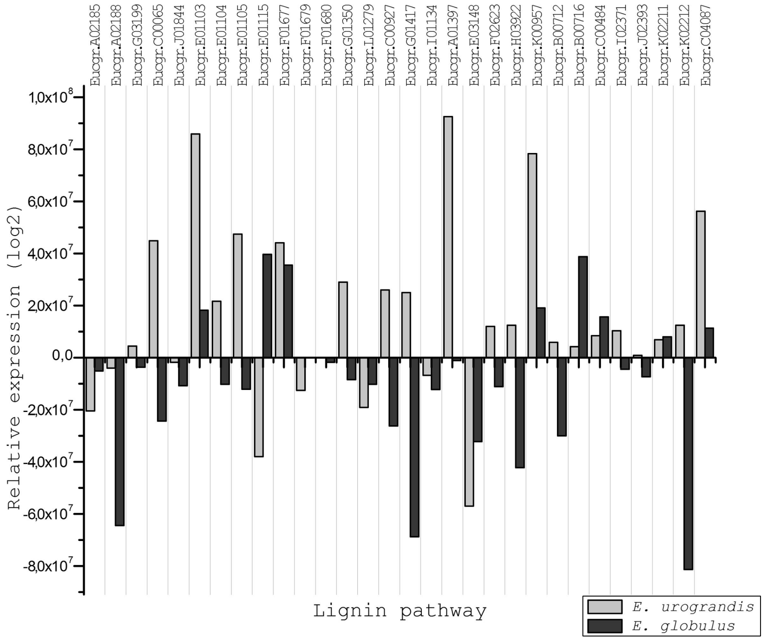

Figure 5. Expression profile of lignin pathway genes in Eucalyptus species.

Annotated genes and homolog from Arabidopsis: Eucgr.A02185 (C3H); Eucgr.A02188 (C3H); Eucgr.G03199 (C3H); Eucgr.C00065 (C4H); Eucgr.J01844 (C4H); Eucgr.E01103 (CAD); Eucgr.E01104 (CAD); Eucgr.E01105 (CAD); Eucgr.E01115 (CAD); Eucgr.F01677 (CAD); Eucgr.F01679 (CAD); Eucgr.F01680 (CAD); Eucgr.G01350 (CAD); Eucgr.L01279 (CAD); Eucgr.C00927 (CCoAOMT); Eucgr.G01417 (CCoAOMT); Eucgr.I01134 (CCoAOMT); Eucgr.A01397 (COMT); Eucgr.E03148 (COMT); Eucgr.F02623 (COMT); Eucgr.H03922 (COMT); Eucgr.K00957 (COMT); Eucgr.B00712 (F5H); Eucgr.B00716 (F5H); Eucgr.C00484 (F5H); Eucgr.I02371 (F5H); Eucgr.J02393 (F5H); Eucgr.K02211 (F5H); Eucgr.K02212 (F5H); Eucgr.C04087 (4CL).

Transcription factors reported in the literature to be related with wood formation were also selected for analysis[14] (**Figure 6**). In E. globulus, Eucgr.D00594 a homolog of SND2 was downregulated within auxin responsive genes. Eucgr.G00390 and Eucgr.G00392 are upregulated in E. globulus. The expression level of Eucgr.A01767 (MYB15) in E. urograndis was 8-fold as in E. globulus, possibly being be related to ICE1, which controls MYB15 negatively and promotes CBF3 expression[15]. In the same way, the cold treatment is stressful for E. urograndis and might regulate this differential expression of Eucgr.A01767. Eucgr.C03153 (MYB32) is upregulated in E. urograndis and is highly induced by MYB46. On the other hand, in E. globulus, Eucgr.C03153 is downregulated and may be related to lower lignin deposition, contrasting to E. urograndis phenotype. Eucgr.C03151 and Eucgr.J02817 are closely related to MYB4 from Arabidopsis, which inhibited the expression of hydroxycinnamate ester biosynthesis and affected UV-B tolerance (increased in myb4 by the absence of inhibition)[16]. C4H (cinnamate 4-hydroxylase) from lignin pathway is a second key enzyme coordinating the cinnamic acid production after PAL[13]. Thus, Eucgr.C03151 is upregulated in E. urograndis and Eucgr.J02817 is downregulated in both species.

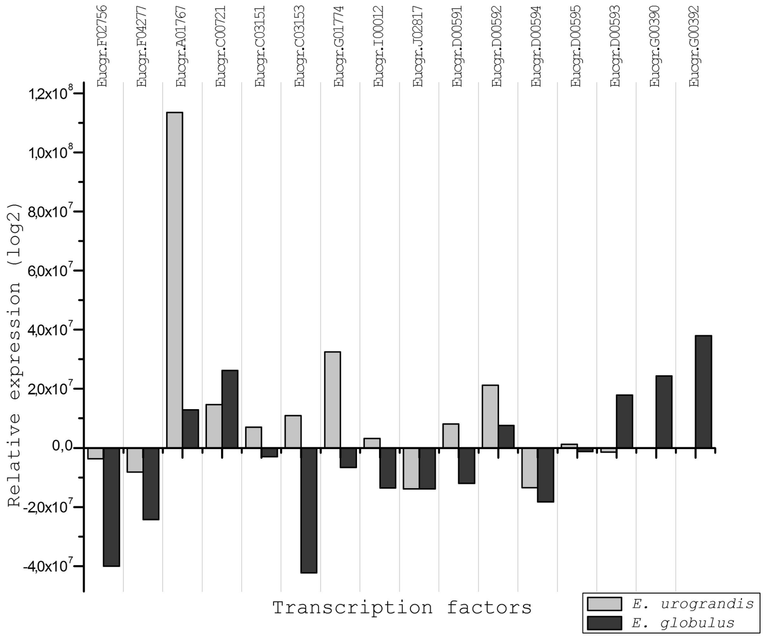

Figure 6. Expression profile of transcription factors related to lignin regulation in Eucalyptus species.

Annotated genes and homolog from Arabidopsis: Eucgr.F02756 (MYB52); Eucgr.F04277 (MYB52); Eucgr.A01767 (MYB15); Eucgr.C00721 (MYB7); Eucgr.C03151 (MYB4); Eucgr.C03153 (MYB32); Eucgr.G01774 (MYB4); Eucgr.I00012 (MYB7); Eucgr.J02817 (MYB4); Eucgr.D00591 (SND2); Eucgr.D00592 (SND2); Eucgr.D00594 (SND2); Eucgr.D00595 (SND2); Eucgr.D00593 (SND2); Eucgr.G00390 (ERF-13); Eucgr.G00392 (ERF-13).

Cellulose is the main economical product from Eucalyptus and it is used for paper production and synthesized by CELLULOSE SYNTHASE (CesA)[17]. Cellulose biosynthesis is intrinsically coordinated by NAC and MYB transcription factors which are responsible for primary and secondary cell wall deposition[18]. Only in E. globulus homologs CesA genes were upregulated, corresponding to CesA3, CesA5, CesA6 and possibly relating with better development of E. globulus under low temperature[12,17] (**Figure 7**).

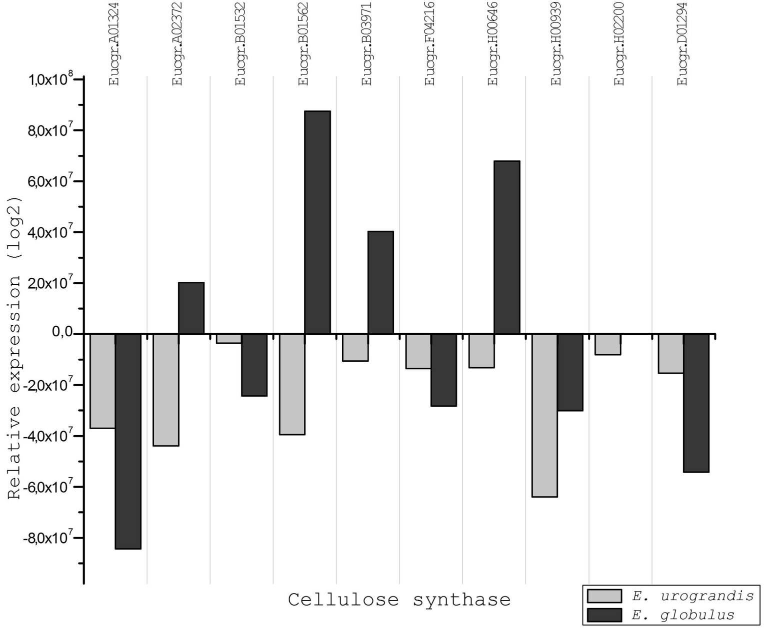

Figure 7; Expression profile of cellulose synthase genes in Eucalyptus species.

Annotated genes and homolog from Arabidopsis: Eucgr.A01324 (CesA4); Eucgr.A02372 (CesA3); Eucgr.B01532 (CesA6); Eucgr.B01562 (CesA6); Eucgr.B03971 (CesA5); Eucgr.F04216 (CesA6); Eucgr.H00646 (CesA6); Eucgr.H00939 (CesA1); Eucgr.H02200 (CesA9); Eucgr.D01294 (XTH8).

Expansin regulates relaxation of the cell wall allowing cell expansion and growth. In both Eucalyptus, Eucgr.A00988 and Eucgr.F03723 are downregulated in E. urograndis and upregulated in E. globulus. On the other hand, Eucgr.E01615 is upregulated in E. globulus and all the other transcripts keep the same pattern in both species (**Figure 8**).

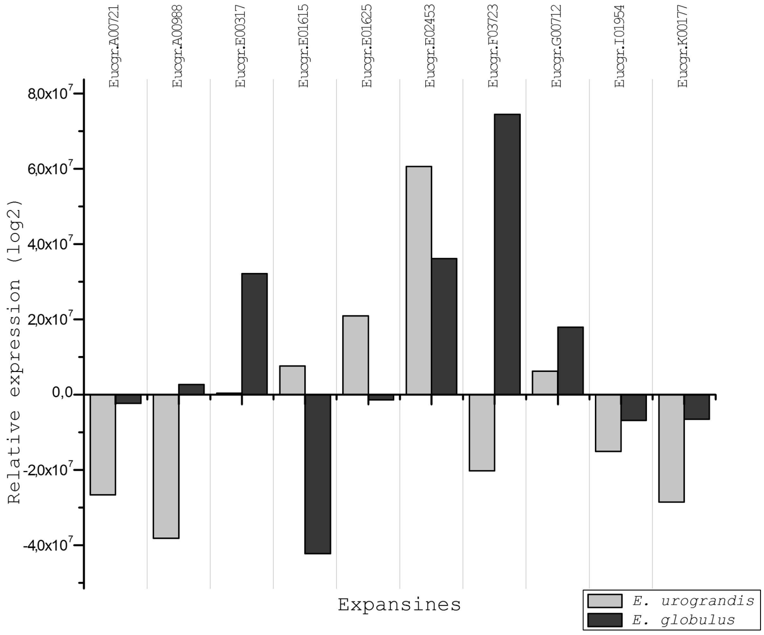

Figure 8; Expression profile of expansin genes in Eucalyptus species**.**

Annotated genes and homolog from Arabidopsis: Eucgr.A00721 (EXPA10); Eucgr.A00988 (EXPA17); Eucgr.E00317 (EXLB1); Eucgr.E01615 (EXPB2); Eucgr.E01625 (EXPB2); Eucgr.E02453 (uncharacterized expansin-like); Eucgr.F03723 (EXPA11); Eucgr.G00712 (EXPA4); Eucgr.I01954 (EXPA10); Eucgr.K00177 (EXPB2).

Flavonoid related genes have the same pattern in the two species. The only differentially expressed gene is a chalcone isomerase homolog Eucgr.J01153 (**Figure 9**). It is a key branch-point gene of the phenylpropanoid pathway after 4CL. Chalcone isomerase catalyzes the production of flavanones, which is an important skeletal backbone for further downstream metabolites.

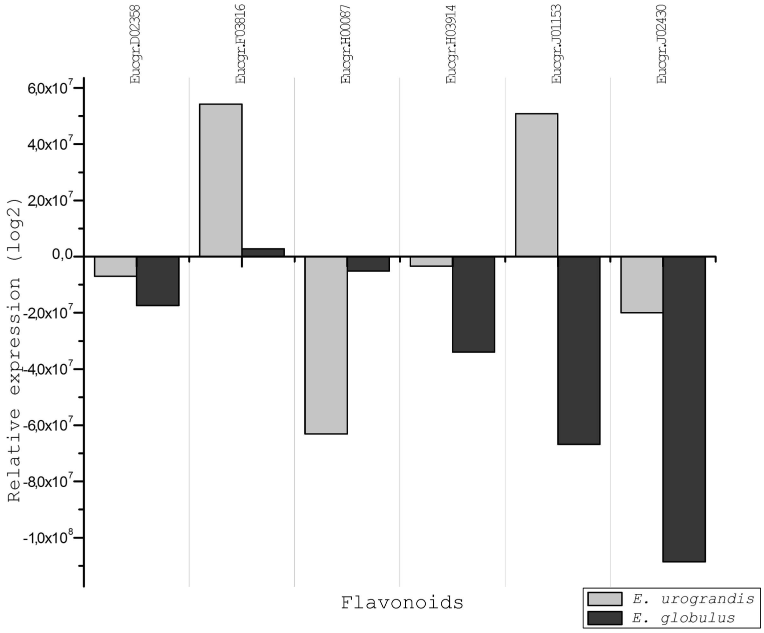

Figure 9; Expression profile of flavonoid genes in Eucalyptus species**.**

Annotated genes and homolog from Arabidopsis: Eucgr.D02358 (dihydroflavonol 4-reductase); Eucgr.F03816 (chalcone isomerase); Eucgr.H00087 (chalcone synthase); Eucgr.H03914 (chalcone synthase); Eucgr.J01153 (chalcone isomerase); Eucgr.J02430 (flavonone 3-hydroxylase).

Class III peroxidases are the last step in lignin incorporation into the secondary cell wall during development[19]. This class has more than a hundred genes in Eucalyptus and correlating one of them to lignin content is a challenge. Interestingly, with the exception of only two Eucgr.F03724 (PRX64) and Eucgr.H01218 (PRX03), all the other genes are downregulated in E. globulus and upregulated in E. urograndis (**Figure 10**).

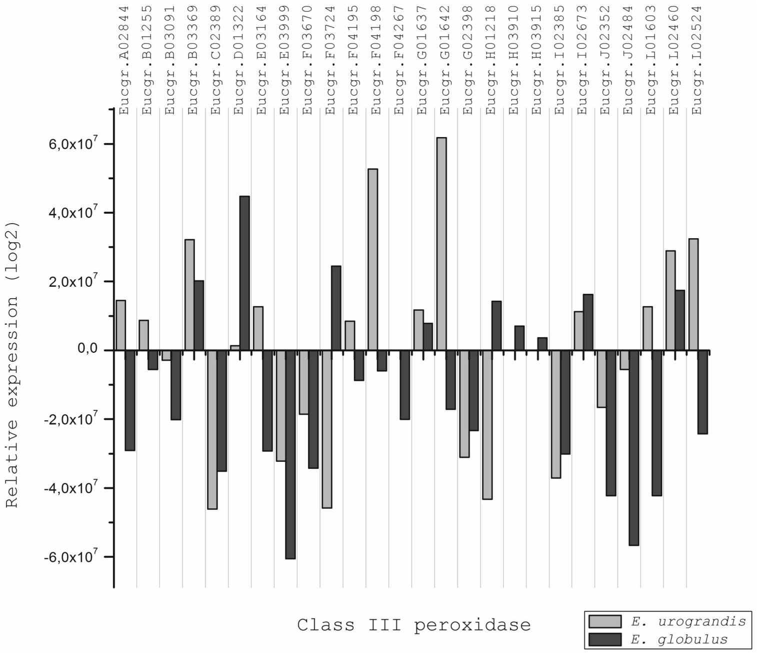

Figure 10; Expression profile of class III peroxidases genes in Eucalyptus species**.**

Annotated genes and homolog from Arabidopsis: Eucgr.A02844 (PRX66); Eucgr.B01255 (PRX03); Eucgr.B03091 (PRX11); Eucgr.B03369 (PRX06); Eucgr.C02389 (PRX16); Eucgr.D01322 (PRX42); Eucgr.E03164 (PRX19); Eucgr.E03999 (PRX29); Eucgr.F03670 (PRX07); Eucgr.F03724 (PRX64); Eucgr.F04195 (PRX12); Eucgr.F04198 (PRX12); Eucgr.F04267 (PRX43); Eucgr.G01637 (PRX66); Eucgr.G01642 (PRX11); Eucgr.G02398 (PRX11); Eucgr.H01218 (PRX03); Eucgr.H03910 (PRX47); Eucgr.H03915 (PRX47); Eucgr.I02385 (PRX41); Eucgr.I02673 (PRX71); Eucgr.J02352 (PRX51); Eucgr.J02484 (PRX64); Eucgr.L01603 (PRX30); Eucgr.L02460 (PRX71); Eucgr.L02524 (PRX64).

3.3 GO annotation and physiological overview of both species under cold treatment

The GO annotation provides a more comprehensive overview of the physiological events occurring in both species (**Figure 11**). The difference between E. globulus and E. urograndis is remarkable regarding ATP binding (GO:0005524) and oxidative reduction (GO:0055114). E. globulus seems to maintain the photosynthetic activity as a priority and have less oxidative stress derivate from the cold treatment. On the other hand, E. urograndis has the opposite behavior comparing both GO. The oxidative stress metabolism is a stress consequence, showing an inability during acclimatization under cold temperatures[20]. Another process pinned up in E. globulus is apoptosis (GO:0006915) which is intrinsically related to xylem differentiation and stressful condition[21].

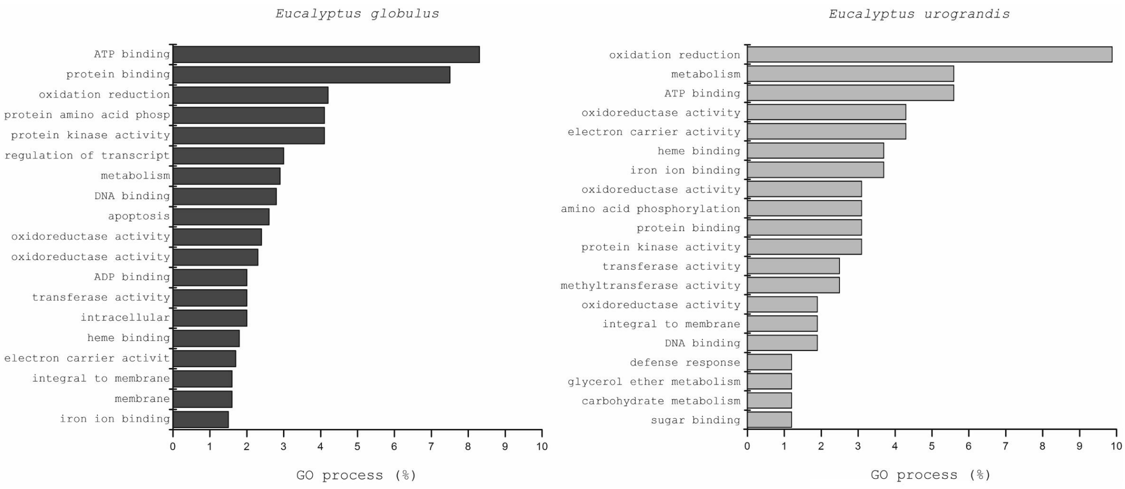

Figure 11; Most frequently observed GO processes in E. globulus and E. urograndis under cold stress.

4. Discussion

The RNA-seq approach reveals a large number of stress-regulated genes making it difficult to evaluate the results in a complex plant physiology context. E. urograndis has approximately 45% of its genes differentially expressed and inside this proportion, 36% of these genes are shared with E. globulus and 9% are uniquely expressed. On the other hand, E. globulus has 40% of the transcripts differentially expressed and only 3.8% are uniquely expressed. Arabidopsis transcriptome can share 30% of its genes among different abiotic stresses[22]. It is perfectly clear that there is a high number of shared genes (36%) during cold stress in both species on the Eucalyptus genus. It is important to realize that E. globulus has less expressed genes (about 5% less than E. urograndis) and this amount is enough to produce drastic differences among the occurring GO process. Under cold stress, the oxidation process is dominant for E. urograndis, but for E. globulus it is ATP binding. Insights about different genes related to cold tolerance still need to be validated using reverse genetics for comprehensive Eucalyptus response[23]. This methodology has been used for different non-model species and can aggregate value for further researches. The results among the species using different approaches can produce non-congruent overviews.

Eucalyptus is vulnerable to freezing injury and E. globulus is more tolerant to cold conditions than E. urograndis and, at the same time, it shows better wood quality for pulp production[4,12]. Therefore, we selected important groups of genes related to wood properties. The first step for tolerance is propitiated by kinases. We identified 64 genes and two of them are related to cell-wall and previously described here, number 40 is an interesting candidate because it is highly induced in E. globulus and strongly repressed in E. urograndis. Another interesting group of kinases formed by numbers: 22, 27, 32, 33, 34, 35 and 38. They are upregulated in E. globulus and equally regulated in E. urograndis. They may confer higher tolerance under cold treatment. Other kinases were already identified and characterized by cold induction and related to cold tolerance, indicating specificity in its activation[10]. A Camaldulin-like 38 (CML38 - AT1G76650) homolog was identified in Eucalyptus (Eucgr.B00127). It is strongly induced in E. globulus and downregulated in E. urograndis. This kinase is one of the calcium sensors for multiple cellular signals in response to environmental stresses and directly controls a downstream signaling pathways targeting different responses to stress induced responses[10].

CBF/DREB1 controls the transcription of various genes with important functions during cold acclimation and the development of freezing tolerance. ABA hormone, salinity and drought conditions induce significantly lower levels in the expression of CBF genes when comparing to cold induction[24]. CBFs interact with other cold-responsive genes that have cis elements[24]. We identified the homolog Eucgr.A02825 as induced by cold stress and could be intrinsically related to cold tolerance. The homolog of MYB15 is also induced and corresponds indirectly to up regulation of ICE in E. globulus as a response to cold induction and tolerance[15]. ICE1 binds to MYC cis-element of CBF3 and activates under cold stress. The ice1 reduces chilling tolerance and cold acclimation[25]. In fact, it is already expected due to the fact that E. globulus grows/develops better than E. urograndis in cold areas. The MYB15 is also a negative regulator of cold tolerance, suppressing CBF activity, binding in the promoter region of these genes. We also identified some other induced transcription factors belonging to the NAC family, MYB family and COR genes and they may contribute to increase the list of genes regulated by cold stress[11,22].

The lignin pathway genes are more prone to be downregulated in E. globulus agreeing with lower lignin content, higher levels of S lignin and consequently easier cellulose access for fermentation. Higher expression of CAD in E. urograndis could be involved in higher levels of monolignol production contributing for carbon fixation[13]. In the same way, switchgrass RNAi mutants for CAD correlates low levels of CAD with less lignin and improved sugar release. More than one CAD homologs were identified in Eucalyptus and further studies must be carried due to the fact that some of them are more related to lignin deposition and xylem differentiation as observed in Populus- a model woody plant[26]. In fact, three homologs of F5H higher expression in E. globulus and are correlated with higher levels of S lignin and better the delignification process[13]. Interestingly, Eucgr.J02393 homolog was characterized in Arabidopsis. A functional complementation of fah1-2 Arabidopsis mutant was performed by F5H from E. globulus (Eucgr.J02393 homolog)[27]. This gene was able to rescue syringyl units and sinapoylmalate. Thus, the high levels of syringyl lignin fit with E. globulus wood properties[4]. In our analysis, this gene was upregulated in E. globulus and downregulated in E. urograndis. These results strengthen this kind of genetic engineering approach for gene discovery and characterization aiming at better digestibility/saccharification.

Class III peroxidases are responsible for the radicalization and incorporation of monolignols into the lignin polymer (S, G and H units) randomly[19]. It is difficult to identify and correlate specific peroxidases with different biological processes[19]. Despite that, we observed that most PRXs are upregulated in E. urograndis and are related with oxidative stress under cold treatment – oxidative reduction is the first process on GO annotation. Plants exposed to stress are known to upregulate their overall peroxidases activity and this occurs equally for most abiotic stresses[19,20]. Therefore, this defensive response results in stronger cell wall or ROS production[20]. In fact, it can still be related with higher levels of lignin in E. urograndis, the same does not occur for E. globulus and the only gene Eucgr.F03724, related to Arabidopsis PRX, is more expressed. The characterization of PRX for lignin deposition is still laborious.

Expansins are proteins intrinsically responsible for cell wall loosening, cell enlargement and in a variety of cell wall modifications. Four families are identified in plants: α-expansin (EXPA), ß-expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB). Expansin-like A and B are uncharacterized and only the gene sequence is known. Here, we identified two differentially expressed expasins, upregulated, in E. globulus which has better development and grows under cold conditions. They are potential candidates for deeper analysis. The Arabidopsis EXPA17, a homolog of Eucgr.A0098, is involved in cell modification and strongly repressed under acidic conditions. The concomitant repression of EXPA17 and other expansin-like A indicate it may limit the duration of the growth phase induced by auxin acidification. EXPA17 is regulated by auxin (IAA) and brassinosteroid in Arabidopsis[28].

Cellulose content is an important factor for pulp production and is genetically regulated by Cellulose synthase genes (CesA). Cellulose is one of the main components in plant cell walls. It is structured into parallel unbranched ß-1, 4-glucan chains called microfibrils which consist of a well packed crystalline cellulose structure and an amorphous region. The Arabidopsis genome has 10 CesA genes and the first one characterized by the reduction of cellulose under restrictive temperatures[29]. We identified three CesA homologs upregulated in E. globulus (CesA3, 5 and 6). The cold condition does not appear to be prohibitive for CesA genes in E. globulus and can explain the expression profile observed. The Arabidopsis mutant for CesA6 has mild reduced root and elongated hypocotyl plants[17]. CesA2, CesA5 and CesA6 are partially functionally redundant and may indicate that the homologs of CesA5 and CesA6 from Eucalyptus have similar patterns of expression and function. It was observed that plants – Arabidopsis with reduced lignin content do not increase their cellulose content. However, reduced levels of CesA directly effects cell expansion and lignin synthesis, causing collapsed cell wall[17].

Flavonoids constitute a sub-group of the phenylpropanoids that accumulate in response to variety of factors. It is a derivate branch from the phenylpropanoid pathway and more precisely from p-coumaroyl-CoA[13]. Recently, tricin – a member of the flavonoid family – was characterized as incorporated into the lignin polymer due to specific moieties[30]. Eucgr.J01153 is upregulated in E. urograndis and flavonoid and lignin compete for precursors. Low temperatures can induce PAL and it is know that flavonoid production depends on PAL activity, and it triggers flavonoid accumulation[13].

5. Conclusion

We reported differentially expressed genes for cold tolerance using two contrasting Eucalyptus species. Our evaluation shows how they are expressed and can help to explain the physiological behavior for cold stress. We also indicate some interesting candidates for deeper analysis aiming to obtain transgenic plants. The genes somewhat focused in wood properties are able to elucidate cell wall properties – comparing with already available literature. This explanatory approach is useful to identify potential candidates for breeding.

Author Contributions

Experiments planning: JCMSMS, PA and PM; bioinformatic analysis: DF and PA; sample preparation, data organization, plot graphics: FTT, APFJ, FCS, DF, VT, NV; Manuscript writing: PA and PM.

Conflict of Interest

No conflict of interest was reported by the authors.

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