Eucalyptus grandis THIOREDOXINS, DIVERSITY AND GENE EXPRESSION

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ABSTRACT – Tree genomes have been sequenced in recent years providing a source of basic information on multigenic family characterization. Comparative genomics based on those complete genome sequences available in public database is an important tool providing useful information to progress on functional gene characterization. In this work, we focus on gene encoding for Thioredoxins (Trxs) in *Eucalyptus grandis* genome, which are oxidoreductase enzymes, involved in significant biochemical processes, above all the maintenance of cellular homeostasis. Here we investigate the diversity, structure and expression of these genes in eucalyptus. For this purpose, bioinformatics tools were employed, using public platforms data, to identify coding sequences and validate gene expression. Specific softwares were employed to characterize gene structure and expression. RT-PCR assays were carried out to specifically verify the expression of 4 cytoplasmic thioredoxin genes, observed *in silico* from leaf, phloem, xylem and apical meristem tissues. Twenty-two Trxs with characteristic and canonic active sites were identified, confirming the presence of all types of the three main groups already defined as plastidial (m, f, x, y, z) cytoplasmatic (h) and mitochondrial (o). However, differences in the number of genes per group were observed when compared with other tree genomes. The expression of these thioredoxin genes compared to some homologous genes presented divergent expression patterns compared to *Arabidopis thaliana* suggesting a functional specificity in eucalyptus, such as in the case of Eucgr.F01604 gene encoding an h1 cytoplasmic Trx, which presents a strong expression in conductor tissues.

Keywords: Thioredoxin; Comparative genomics; RNA-seq

TIORREDOXINAS DE Eucalyptus grandis, DIVERSIDADE E EXPRESSÃO GÊNICA

*RESUMO – Genomas de árvores tem sido sequenciados nos últimos anos e constituem uma fonte de informação de base para o estudo de famílias multigências em genômica comparativa. Neste trabalho, o interesse se concentra nos genes que codificam Tiorredoxinas (Trxs) em **Eucalyptus grandis**, enzimas oxidoredutases envolvidas em significativos processos bioquímicos, sobretudo na manutenção da homeostase celular. Aqui se considera a diversidade, estrutura e expressão desses genes no eucalipto. Para tanto, ferramentas de bioinformática foram empregadas para identificar sequências codantes e validar dados. Programas específicos foram empregados para caracterizar a estrutura e expressão dos genes. Ensaios de RT-PCR foram realizados para 4 genes de tiorredoxinas citoplasmáticas a partir de tecidos de folha, floema, xilema e meristema apical para confirmar a expressão observada in silico. Foram identificadas 22 Trxs com sítio ativo característico confirmando a existência de todos os representantes dos três principais grupos já definidos em plantas, plastidiais (m, f, x, y, z) citoplasmáticas (h), e mitocondriais (o) no genoma do eucalipto. Observaram-se, no entanto, diferenças quanto ao número de genes por grupos em comparação com outros genomas de árvores. A expressão desses genes de tiorredoxina mostrou-se, para alguns genes homólogos, divergente do que fora observado em Arabidopis thaliana sugerindo uma especificidade de função em eucalipto a exemplo do gene Eucgr.F01604 que codifica uma Trx citoplasmática h1 e que apresenta forte expressão em tecidos condutores.*

Palavras-Chave: Tiorredoxinas; Genômica comparativa; RNA-seq

1. INTRODUCTION

Thioredoxins (Trxs) are small ubiquitous enzymes – approximately 14kDa - present in all organisms, and responsible for maintaining the redox state in cells (Geigenberger et al., 2017). They were first discovered in *Escherichia coli* (Laurent et al., 1964) as hydrogen donors for nucleotide reductase (RNR), and present a protruding and conserved active site, frequently earing WCGPC amino acids. Between the two cysteine, on the canonic active center, a dithiol is formed, and responsible for their enzymatic activity by reducing and opening disulfide bridges of other proteins (Holmgren et al., 1985).

In plants, thioredoxins present a surprising complexity compared to animals in terms of number of genes per genome, and functional diversity. Initially, they were identified as responsible for the activation of two important Calvin-Benson cycle enzymes, FBPase-Fructose 1,6-bisphosphatase - Malate dehydrogenase - MDH (Jacquot and Buchanan, 1981). In this system, Trxs f and m are reduced by Fdx-dependent FTR-Ferredoxin thioredoxin reductase (Wolosiuk and Buchanan, 1977; Buchanan et al., 2002). The complexity and abundance of Trxs in plants was confirmed by the *Arabidopsis thaliana* genome-sequencing project (Arabidopsis Genome Initiative, 2000). They were then classified according to their cell compartimentalization, as chloroplastic, cytoplasmic (Reichheld et al., 2002), mitochondrial (Laloi et al., 2001), and, more recently, nuclear (Reichheld et al., 2007). For these last three Trx types, their reducing agents are NADPH-dependent thioredoxins reductases (NTR), giving rise to the second complete system described (Jacquot et al., 1994). In chloroplasts, other Trxs named x (Bernal-Bayard et al., 2014) y and z (Arsova et al., 2010; Chibani et al., 2011) were identified. In addition, Rivera-Madrid et al. (1995) clearly characterized cytoplasmic h Trxs (heterotrophic).

Systematic sequencing of plant genomes (Michael and Jackson, 2013) has allowed a better characterization of gene function (Rhee and Mutwil, 2014), mostly by the implementation of NGS (Next-Generation Sequencing) platforms, allowing high-throughput DNA sequencing, comprising RNA-seq strategies. These efforts, allied to comparative genomics supported by increasingly robust Bioinformatics tools, have contributed to a better understanding, and analysis, of functional characterization of multigene families, such as Trxs in vegetables.

In this paper, we analyze sequences of Trxs genes obtained from the *Eucalyptus grandis* genome sequencing project, published by Myburg et al. (2014). This is an approach to the genetic diversity of Trxs in eucalyptus, which intends to validate data obtained during the FORESTs initiative (Eucalyptus Genome Sequencing Project Consortium) carried out by Brazilian groups (Vicentini et al., 2005), and focusing on a comparative genomic strategy based on available tree genomes. Our interest is to advance the functional characterization of these enzymes in trees, considering differential, and specific, gene expression in different plant tissues. Gene expression from RNA-seq data is analyzed as well as protein interaction based on a comparative approach with the *Populus trichocarpa* genome (Tuskan et al., 2006), the first tree genome sequenced. Special attention is paid to semi-quantitative RT-PCR assays, with genes encoding for 4 Trxs h. The practical outcome of this analysis is the identification of genes that may be good candidates for obtaining commercial transgenic plants overexpressing interesting transcripts that could enhance plant productivity related to different aspects, such as growth or stress tolerance to biotic or abiotic agents.

2. MATERIAL AND METHODS

Trxs sequences were identified within three databases. Firstly, we had access to the eucalyptus genome directly by the AUSX00000000 GenBank link provided in the article (www.ncbi.nlm.nih.gov/nuccore/AUSX00000000). The other two sources were the plant genome database, Phytozome (https://www.phytozome.jgi.doe.gov/), and the eucalyptus-specific database, Eucgenie.org (https: // eucgenie.org /). In GenBank searches, we obtained sequences via NCBI-National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), performing BLAST alignments using *Arabidopsis thaliana* thioredoxin nucleotide sequences. In the other specific databases, a textual search, with the word "thioredoxin", was used.

Multiple sequence alignments were initially carried out with the Clustal Omega platform (Larkin et

al., 2007) hosted at EMBL - European Bioinformatic Institute website (https://www.ebi.ac.uk/Tools/msa/clustalo/). In these experiments, sequences of *Populus trichocarpa*, *Vitis vinfera* and *Arabidopsis thaliana* were also obtained from the *Populus trichocarpa* genome supplementary material, available in the article. Phylogenetic trees were then developed using BEAST2 program (http://www.beast2.org/), and Trxs domains were deduced from CDD alignments (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) employing MAFFT software (https: //mafft. cbrc. jp / alignment / server /). Cellular localization was performed using PREDOTAR software (Small et al., 2004) by submitting all protein sequences found directly on the online platform.

RT-PCR studies were performed in 4 steps, using total RNA extraction from *E. grandis* tissues (adult leaves, young leaves, xylem, phloem and apical meristem), cDNA preparation using 1μg of total RNA, PCR amplifications with specific primers for h Trxs, and the analysis of PCR products on agarose gel under electrophoresis.

Total RNA extraction was made with Trizol (Invitrogen 15596026) following the manufacturer's standard protocol. RT-PCR reactions were performed with the Promega AccessQuick RT-PCR system (A1701) kit, which ensures reverse transcription, and PCR reaction in a single tube. In the kit, standard PCR conditions, performed on a Techne TC-512 thermal cycler, were recommended. The PCR reactions consisted the following basic cycling: initial denaturation at 95 °, 2 min, followed by 30 PCR cycles containing denaturation at 95 °, 1 min, annealing at 55 ° , 30 sec, extension at 72 °, 1 min, followed by final extension at 72 °, 4 min. Primers for PCR amplification of 4 Trx h genes (I01913.1, AGT TCA GTG TGC AGG CAA TG1, AGC AAA AGG GAT CGA ACT CA r; F01854.1, ACA CCG TCG ATT CTT GGA AC 1, ACA CCG TCG ATT CTT GGA AC R; F01604.1, AGA AGA TGC CCC ATG TTT TG 1, ATC GGA GCC GGT TTA TTT TT; I02383.1AGC TGG TGG TTG TCG ATT TC 1, GAT CCT TCT GGG CTC CTA CC r; I00241. 1 (actin) AGG ATA TTC AGC CCC TCG TT 1, TGG GCT TCA TCA CCA ACA TA r) were added to the reactions (50μM), and their annealing temperatures considered individually in each reaction.

PCR products were analyzed in 1.5% agarose gel on a runVIEW Cleaver electrophoresis apparatus. Samples were loaded within Blue / Orange Loading Dye 6X Promega Buffer, added with 2μl of GelRed Biotium (10.000 dilution) for DNA labeling. Gels were then visualized in Hoefer transilumidor and registered in Cleaver photocumentation equipment.

3. RESULTS

In this work, weclassified plant trxs according to Chibani et al. (2009), who recognizes 4 groups. Typical CXXC / S Trxs possessing a classic active site, atypical Trxs with single or multi-domain protein domain, TDX, and the thioredoxin reductases, which are the reducing agents in the different thiorredoxin systems described. Data mining investigation has shown that the *Eucalyptus grandis* genome encodes more than 50 Trxs among typical, atypical, single domain and those from multiple domains.

Here we are especially interested in typical Trxs. Our results in table 1 show that eucalyptus has representatives for all seven types of Trxs described (m, f, h, x, y, z, o) in the genome, and that in terms of gene numbers, they are of 4, 2, 9, 1, 2, 1, 1 respectively, plus 2 genes presenting a CxxS site.

Phylogenetic trees obtained in approaches as described here aimed to allow information about structural and functional characterization of genes by rooting analysis and do not perform classical evolution analysis. In this context, we sought to confirm the identification of genes and also to group different types of thioredoxins. In this comparative analysis we used sequences homologous to Trxs from 4 different plant species, *A. thaliana*, *V. vinifera*, *P. thicocarpa*, and *J. curcas*. Results of this analysis, presented in figure 1, allowed for the grouping and identification of all thioredoxin sequences with their potential orthologs in the other species.

The expression study was initially based on RNA-Seq data, available from the *E. grandis* genome. This raw FPKM data is available on public domain platforms (https://eucgenie.org/), and after standardization was used to generate the heatmap shown in figure 2. It comprises, then, the 22 Trx coding sequences for m, f, h, o, x, y, and z.

The heatmap obtained allowed for the identification of those Trxs h with gene expression

Table 1 – Typical *Eucalyptus grandis* thioredoxins identified on the Phytozome platform from direct search by keyword "thioredoxin" and classified according to conserved active site and cell location.
Tabela 1 – *Tiorredoxinas típicas de Eucalyptus grandis identificadas na plataforma Phytozome a partir de pesquisa direta com palavra-chave "tiorredoxina" e classificadas de acordo com o sítio ativo conservado e a localização celular.*

Identification	Type	Locus	Chromosome	Aa	Location	Active site
Eucgr.A00783.2.p	h2	Eucgr.A00783	Chr01	148	P/M/C	WCGPC
Eucgr.A01813.1.p	m2	Eucgr.A01813	Chr01	193	P/M/C	WCGPC
Eucgr.B01424.1.p	f2	Eucgr.B01424	Chr02	332	P	WCGPC
Eucgr.B02586.4.p	h2	Eucgr.B02586	Chr02	165	C	WCGPC
Eucgr.D00028.1.p	o	Eucgr.D00028	Chr04	184	M	WCGPC
Eucgr.D00853.1.p	CxxS1	Eucgr.D00853	Chr04	207	C	WCMPS
Eucgr.F01604.2.p	h1	Eucgr.F01604	Chr06	150	C	WCGPC
Eucgr.F01854.1.p	h(5Pt)	Eucgr.F01854	Chr06	118	C	WCGPC
Eucgr.F02754.1.p	m(5Pt)	Eucgr.F02754	Chr06	188	P/M/C	WCGPC
Eucgr.F03319.1.p	m(4Pt)	Eucgr.F03319	Chr06	187	P	WCGPC
Eucgr.F04223.1.p	y	Eucgr.F04223	Chr06	109	P	WCGPC
Eucgr.F04229.1.p	y	Eucgr.F04229	Chr06	152	P	WCGPC
Eucgr.G03224.1.p	z	Eucgr.G03224	Chr07	186	P	WCGPC
Eucgr.H01629.1.p	f1	Eucgr.H01629	Chr08	185	P	WCGPC
Eucgr.I01912.1.p	h2	Eucgr.I01912	Chr09	158	C	WCGPC
Eucgr.I01913.1.p	h(2Pt)	Eucgr.I01913	Chr09	159	C	WCGPC
Eucgr.I02383.1.p	h1	Eucgr.I02383	Chr09	117	C	WCGPC
Eucgr.J00880.2.p	x	Eucgr.J00880	Chr10	182	P	WCGPC
Eucgr.J02387.1.p	h(2Pt)	Eucgr.J02387	Chr10	119	C	WCGPC
Eucgr.K01294.1.p	h9	Eucgr.K01294	Chr11	137	P/M/C	WCGPC
Eucgr.L01943.1.p	cxxs	Eucgr.L01943	scaffold_464	81	C	WCGPC
Eucgr.L03049.1.p	m3	Eucgr.L03049	scaffold_1873	116	P	WCGPC

PMC, Plastid, Mitochondria, Cytoplasm; Pt, Populus trichocarpa.
PMC, Plastídio, Mitocondria, Citoplasma; Pt, Populus trichocarpa.

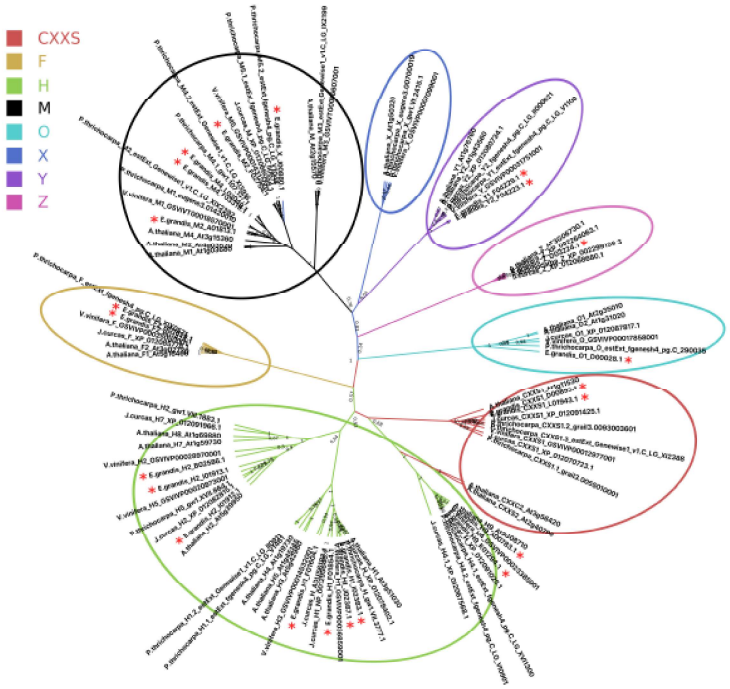

Figure 1 – Phylogenetic tree generated with BEAST2 showing different types (letters on the left) of *E. grandis* thioredoxins grouped with their potential orthologs in *P. trichocaprpa*, *V. vinifera*, *J. curcas* and *A. thaliana*.
Figura 1 – *Árvore filogenética gerada com BEAST2 mostrando diferentes tipos (letras à esquerda) de tiorredoxinas de E. grandis agrupadas com seus potenciais ortólogos em P. trichocaprpa, V. vinifera, J. curcas e A. thaliana.*

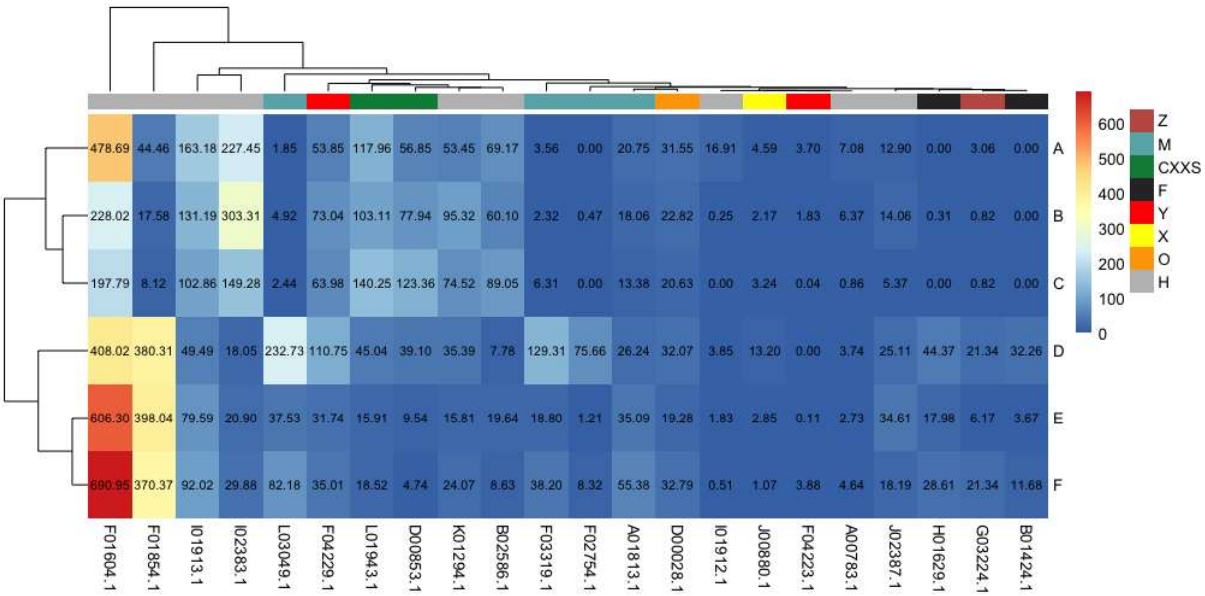

Figure 2 – Heatmap of typical thioredoxin transcripts of *E. grandis* in different tissues (letters on the top right). A, Phloem. B, Immature Xylem. C, Xylem. D, Adult leaves. E, Apical meristema. F, Young leaves).

Figura 2 – *Mapa de expressão dos transcritos tiorredoxinas típicas de E. grandis em diferentes tecidos (legenda à direita). A, Floema. B, Xilema Imaturo. C, Xilema. D, folhas adultas. E, meristema apical. F, folhas jovens).*

that apperar to be more abundant in certain tissues. This is particularly the case for Eucgr.I02383, Eucgr. I01913, Eucgr.F01854 and Eucgr.F01604 genes. This result has driven preliminary RT-PCR assays to study the expression of these genes more precisely in the laboratory. Results (Figure 3) of these tests suggest a confirmation of what is observed *in silico* analyses.

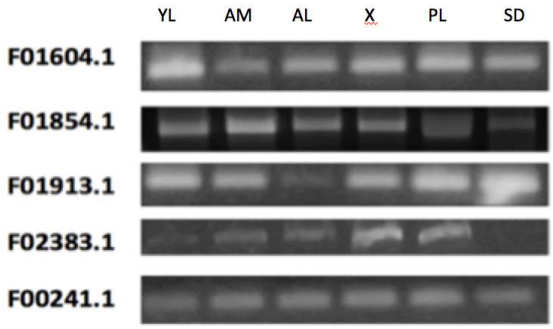

Figure 3 – Semi-quantitative RT-PCR gene expression of thioredoxin genes in different E. grandis tissues: YL, young leaves. AM, apical meristem. AL, adult leaves. X, xylem. PL, Phloem. SD, seedlings. F00241.1, actin

Figura 3 – *Expressão genica de tiorredoxinas de E. grandis por RT-PCR semi-quantitativa em diferentes tecidos : YL, folhas jovens. AM, meristema apical. AL, folhas adultas. X, xilema. PL,Floema. SD, mudas. F00241.1, actina.*

4. DISCUSSION

Barbosa and Marinho (2005) when analyzing the first eucalyptus transcriptome, found 7 Trxs h, one of them with CXXS site, 1 Trx x, but they were unable to identify Trxs of groups z and o. In the same work, 4 m and 2 f Trxs were identified. The absence of transcripts for Trxs o and z in that first approach may be explained by the lack of those transcripts for these genes in the libraries employed in that transcriptome that can currently be found in the E. grandis genome. These genes are known to be transcribed at low expression levels because they are involved in more specific situations, such as oxidative stress response (Balsera and Buchanan, 2019), and, therefore, not constitutively (Laloi et al., 2001; Chibani et al., 2011). Regarding Trxs m and f, involved in the Calvin-Benson cycle reactions (Buchanan, 2002), then abundant in terms of transcripts, the results are repeated among those we observe here for the number of genes, 4 and 2 respectively. The presence of 9 Trxs h and not 7, as seen by Barbosa and Marinho (2005), is also explained by the transcript levels, but also by the redundancy of these genes. However, it is interesting to note that even in a transcriptome performed with major technical limitations, and only with the analysis

of the 5' transcripts (ESTs), 7 of them were already identified at the time. Most likely, this shows the plasticity of this group of Trxs, preferentially acting on the cytoplasm. With respect to the CXXS site Trxs h, the genome presents two of them while only one was found in the previous data mining approach.

Here we can also observe that the basic set of Trxs genes, found in *A. thaliana* during the genome analysis, in the green lineage as defined by Meyer et al. (2009), remains in the *E. grandis* genome as in the other plant genomes studied. However, the number of genes, and, consequently, of proteins, change what can be verified in comparative genomics analyses. It is likely that, as Meyer et al. (2009) points out, plant gene duplication throughout evolution has occurred, which could be explained here by the location of some genes on the same chromosomes.

If we still take these numerical data for Trxs genes in *E. grandis*, we can see that gene diversity within typical Trxs subfamily also appears in other plant tree sequenced genomes. *P. trichocarpa*. For example, Chibani et al. (2009) presents a similar situation to E. grandis, but differs when poplar presents 8 Trxs m genes, which is an unusual situation. This analysis, however, will be improved by the sequencing of other tree genomes being performed around the world, in a fast and high quality way, and will allow further hypotheses about the numerical diversity of Trxs genes in these genomes.

The general expression profile in eucalyptus, considering all genes, is consistent with what is found in the literature (Meyer et al., 2009), and justifies their classification by cell compartimentalization and function. The expression profile in conductive tissues, for example, is more discrete than in leaf or meristem tissues in most genes, except in the case of the thioredoxin group h, which has a more varied profile. Trxs f acts clearly on adult leaves, which is already expected due to their well-characterized performance in photosynthetic tissues. The same can be said for Trxs m, which also acts, on photosynthetic green tissues.

Gene expression of eucalyptus thioredoxins by RNA-seq transcripts had never been reported until now, as this is the first work with this approach. Vining et al. (2015) have analyzed a eucalyptus floral transcriptome, but these tissues are not present in this work. However, a large study was performed by Belin et al. (2015) in *A. thaliana* analyzing RNA-seq data for all plant Trx genes. From this work, some relevant considerations can be made in relation to the results obtained here.

If we consider individually different thioredoxin groups in our study, we can say that the 4 plastid Trxs m are predominantly expressed in adult leaf tissues, such as in *A. thaliana*. In this plant, however, Trx m3 has low expression, and is constitutive in the tissues analyzed. The Eucgr.L03049 gene that corresponds to the Trxm3 in eucalyptus has a strong expression in adult leaves compared to the other 3 Trxs m, which may indicate a possible functional specificity in tree genomes. The same expression pattern can be observed for the two eucalyptus Trx f, which are significantly present in photosynthetic adult leaf tissues. The other eucalyptus plastidial Trxs x, y and z are more discreetly expressed than m or f Trxs in the tissues studied. There is a predominance of transcripts for these plastidial Trxs in mature or young leaf tissues, nevertheless, the lack of values for Trx z in adult leaves may be noticed. This thioredoxin is, in fact, particular, presenting more specific functions in the target proteins, FLN1, and FLN2 (Arsova et al., 2010; Meng et al., 2010). This is the likely explanations for the absence of transcripts in the library studied. With respect to *A. thaliana*, Belin et al. (2015) mentioned a strong expression of Trx z in ovarian tissues, which reinforces its more specific character.

Eucalyptus Trx o has a discreet and constant expression in the six libraries analyzed – the same as observed in *A. thaliana*, which indicates its recently described more generalist character (Geigenberger et al., 2017).

Eucalyptus CXXS Trxs have an interesting expression that is more relevant in conductive tissues, although also present in young or adult leaf tissues. They differ from the other Trxs regarding this specificity by conductive tissues, also suggesting specific functions. In *A. thaliana*, CXXS2 is strongly detected in pollen grains, according to Belin et al. (2015).

The Trxs h of eucalyptus, represented here by 9 genes, present gene expression in all tissues studied, and can be characterized in two groups. There are some with more discrete and uniform discresion expression in the six libraries: genes

Eucgr.J02387, Eucgr.A00783, Eucgr.I01912, Eucgr.B02586, and Eucgr.K01294. There is also a second group, consisting of 4 Trxs, Eucgr.I02383, Eucgr.I01913, Eucgr.F01854, and Eucgr.F01604, with an expression that is clearly more abundant than all the others, and even larger than all Trxs studied here. This possible functional role, characterized by this division of labor in a tree genome, may be of great interest. For example, the genes that are more abundantly expressed are Eucgr.F01854 and Eucgr.F01604. The firsthas the lowest expression in conductive tissues, but with a high number of reads in young or adult leaf tissues. The second is the Eucgr.F01604 gene that has a strong expression in all six libraries, suggesting a possible and important role in plant growth, and its vertical expansion, because it is supposedly involved in transport in conductive tissues. This differential expression profile of Trxs, considering tissues or cell partitioning, is a recurrent approach in the study of the functional characterization of these enzymes (Meyer et al., 2012). The central point of the study of the characterization of these genes is the simplicity of the biochemical redox system, represented by the reduction of disulfide bridges of target proteins via electron transfer by Trxs, on the one hand, and the absence of mutants for Trxs h allowing a specific function determination for each one on the other. In this sense, data presented here, based on expression profile analysis, and the simple presence of transcripts in different tissues and their intensity, could be of great interest. The presence of strong differential expression, observed in some genes, justifies this assumption, and reinforces the idea of not substituting one Trx h for another in specific situations. Those assumptions, however, must be confirmed by obtaining knockout mutants, for instance, which are not available in eucalyptus.

Our semi-quantitative RT-PCR results are relevant because they suggest a possible division of labor among the Trxs h genes in eucalyptus. This aspect is not negligible in the functional study of thioredoxins, of which its functional gene redundancy has already been widely verified with the use of insertion mutants (Meyer et al., 2012).

5. CONCLUSIONS

The results presented here confirm the numerical and group complexity for Trx genes already observed in the first Eucalyptus transcriptome. Genes have been identified for all described Trxs groups. Eucalyptus possesses at least 22 typical thioredoxin genes identified by comparative phylogenetic reconstruction. The numerical distribution of genes by groups in eucalyptus is similar to that reported for *P. trichocarpa*, showing the maintenance of 4 Trx m genes as in *A. thaliana*, instead of 8 in *P. trichocarpa*. The most abundant group in genes is represented by Trx h, with 9 genes, and the expression profile of these genes revealed unique expression patterns not yet reported in eucalyptus. The Eucgr.F01604 gene, encoding a Trx h1, could possibly play a somewhat significant role in conductive tissues, as well as relevant expression in young leaf tissues. This specific tissue expression was verified in semi-quantitative RT-PCR experiments. The expression of the Eucgr.I02383, Eucgr.I01913, Eucgr.F01854, and Eucgr.F01604 genes by RT-PCR confirms what is observed *in silico*. This genetic characterization, via differential expression of transcripts, indicates that future studies to obtain commercial transgenic plants with biotechnological potential using those genes are of interest. However, the need remains to carry out more experiments on the functional characterization of eucalyptus thioredoxin h *in plantae*.

"This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001"[Coordination of Personnel Development in Higher Education – Brazil; finance code 001]

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