PEDRO ARAÚJO

"DESENVOLVIMENTO DE SISTEMA *IN VITRO* E IDENTIFICAÇÃO DE GENES PREFERENCIALMENTE EXPRESSOS NO PROCESSO DE LIGNIFICAÇÃO EM *Eucalyptus globulus*"

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“Eu Sou o que Sou”

Êxodo 3:14

Índice

Resumo

Eucalyptus globulus é amplamente cultivado devido à sua alta capacidade na produção de celulose, fibras pouco densas e flexíveis, tornando-se industrialmente vantajoso. A lignina é fundamental para o desenvolvimento das plantas superiores, pois confere resistência mecânica, hidrofobicidade do xilema e facilita o transporte de água ao longo do vegetal. A biossíntese da lignina ocorre a partir de três precursores formados na via dos fenilpropanóides e, posteriormente incorporados: *p*-hidroxifenil (H), guaiacil (G) e siringil (S). Na última etapa da biossíntese de lignina temos as enzimas peroxidases de classe III que exercem papel fundamental. Esta tese foi divida em quatro capítulos: o Capítulo I aborda a caracterização de genes de referência que podem ser utilizados para a normalização de dados de PCR em tempo real para *Eucalyptus* sob diferentes tipos de estresses abióticos. Duas abordagens foram utilizadas para o cálculo do gene mais estável e, dentre as análises realizadas, três genes se destacaram aos demais, independentemente do tratamento proposto: *IDH*, *SAND* e *ACT*. No Capítulo II, é descrita uma condição *in vitro* que propicia acúmulo diferencial de lignina em *Eucalyptus globulus*. Desse modo, identificamos duas peroxidases de classe III preferencialmente induzidas com o acúmulo de lignina – *EglPrx121* e *EglPrx122*. O Capítulo III contém dados de caracterização de uma peroxidase de classe III induzida no escuro. Experimentos de microdissecção e hibridização *in situ* suportam a hipótese de que esse gene pode estar relacionado com a deposição de lignina em *E. globulus*. Por fim , o Capítulo IV descreve uma abordagem inédita na utilização de espectrometria de massas acoplada a um sistema de imagem na caracterização de precursores de lignina - unidades S e G. Esses trabalhos podem servir de base para estudos posteriores envolvendo biologia molecular básica para arbóreas e caracterização de madeira e suas propriedades.

Abstract

Eucalyptus globulus is widely cultivated due to its high cellulose content, low flexible and density fibers, becoming industrially advantageous. Lignin is essential for the development of higher plants, conferring mechanical strength, xylem hydrophobicity and facilitating water transport through the plant. The biosynthetic lignin pathway occurs from three precursors and subsequently incorporated as: *p*- hydroxyphenyl (H), guaiacyl (G), and syringyl (S). The last step has class III peroxidases playing a key role. The thesis is divided into four chapters. Chapter I, characterization of reference genes which may be used for qPCR normalization for *Eucalyptus* under different treatments - abiotic stresses. Two approaches were used to calculate the most stable gene and we selected three genes, regardless of the proposed treatment: *IDH*, *SAND* and *ACT*. In Chapter II, is described *in vitro* condition that promotes differential accumulation of lignin in *Eucalyptus globulus*. We identified two class III peroxidases that were preferentially induced with the accumulation of lignin - *EglPrx121* and *EglPrx122*. Chapter III describes one class III peroxidase induced in the dark condition. Microdissection and *in situ* hybridization support the hypothesis that this gene might be related to the deposition of lignin in *Eucalyptus globulus*. Finally, Chapter IV describes a novel approach for mass spectrometry coupled to an imaging system for the characterization lignin precursors - S and G units which are widely used to correlate plants and their recalcitrance. This thesis can be used for further studies involving basic molecular biology to characterize tree and wood properties.

Introdução geral

Eucalyptus e a produção de celulose e papel

O Eucalipto foi introduzido no Brasil na segunda metade do século XIX oriundo da Oceania. Foi originalmente utilizado nas linhas férreas e atualmente tem importância econômica na produção de celulose e papel, representando 4% do PIB e 8% das exportações brasileiras (Ministério da Ciência e Tecnologia, 2003). Atualmente investimentos são realizados na expansão de produção de Eucalipto: (i) R$ 210 milhões na geração de energia alternativa a partir dos cavacos de madeira para a ERB Arating S/A; (ii) R$ 2,7 bilhões em financiamento para a maior fábrica de celulose do mundo – Eldorado Celulose e Papel em Três Lagoas, Mato Grosso do Sul (Portal Brasil, 2011, 2012). Além disso, a utilização de eucalipto como fonte de energia renovável está mais perto da viabilidade, devido à possível redução de custo com a introdução de novas plantas lenhosas modificadas geneticamente (Wilkerson *et al*., 2014). Concomitantemente, o aumento da população requer cada vez mais recursos florestais. Em 2010 estes recursos foram utilizados basicamente em iguais proporções pela indústria e para a produção de combustível (FAO, 2010). Essa nova fonte de recurso tem levado a considerações importantes quanto o cultivo e manejo do eucalipto (Häggman *et al*., 2013).

Na produção de celulose e papel, dois métodos podem ser utilizados na polpação da celulose dependendo da qualidade a ser obtida. A polpação mecânica é utilizada basicamente para jornais ou papel é de baixa qualidade, enquanto a química utilizada em papel de alta qualidade que acaba por exigir um laborioso processo de produção (Baucher *et al.*, 1999). O método clássico de polpação química é o Kraft, que consisti em: (i) tratar os cavacos de madeira em altas temperaturas; (ii) tratar com hidróxido de sódio e (iii) tratar com sulfeto de sódio (Lisboa *et al.*, 2005). Métodos são propostas para aumento de eficiência (Khristova *et*

al., 2006; Akgül *et al.*, 2007; Huang *et al.*, 2007) e compostos ecologicamente corretos (Nascimento *et al.*, 1998). Alguns exemplos são a utilização de reagentes sem cloro (Ibarra *et al.*, 2005). Outras estratégias como o uso de fungos e bactérias vêem sendo estudados como alternativas na polpação (Raj *et al.*, 2007).

Diante da importância econômica e das dificuldades encontradas no manejo de Eucalipto para a produção de papel e celulose, diferentes abordagens são desenvolvidas com o intuito de aumentar a eficiência de produção e diminuir o uso de compostos tóxicos. Para isso, ferramentas moleculares podem ser uma alternativa compreender e modificar propriedades da madeira. Um dos maiores gargalos é a presença do polímero lignina que limita e dificulta a extração de celulose pela indústria. A principal característica é a recalcitrância da lignina na extração, que por ser um polímero heterogêneo complexo dificulta a identificação de enzimas específicas para sua degradação (Weng *et al.*, 2008).

Lignina

Ao longo da evolução, durante o desenvolvimento de processos de seleção adaptativos, as plantas obtiveram, por meio do metabolismo secundário, uma série de subprodutos que conferiram vantagens para sobrevivência no ambiente que habitam. Dentre uma série de vias metabólicas encontradas nas plantas, a dos fenilpropanoides desempenha vital importância (Vogt, 2010). Com a deposição de lignina nas paredes celulares das plantas, adquirindo: (i) rigidez na parede celular; (ii) suporte mecânica; (iii) desenvolvimento de vasos hidrofóbicos para condução de água pelo corpo da planta e (iv) defesa contra microrganismos (Belgacem & Gandini, 2008; Weng & Chapple, 2010). Todas essas características permitiram a conquista da vida terrestre (Belgacem & Gandini, 2008). A deposição de lignina é um processo irreversível, pois não existem mecanismos conhecidos de degradação desse polímero em plantas e, sendo

assim, deve existir um controle de deposição de lignina na parede celular que não prejudique e/ou demande a disponibilidade de carbono pelo organismo vegetal (Rogers *et al.*, 2005).

A lignina é um polímero aromático abundante, inferior somente à celulose em termos de quantidade, e que representa 30% do estoque de carbono do planeta; heterogênea por ter basicamente três álcoois hidroxicinamílicos na composição e alguns outros poucos compostos relacionados. A biossíntese da lignina (Figura 1) inicia-se com a desaminação do aminoácido fenilalanina pela enzima fenilalanina amônia-liase (PAL) que, após uma série de reações, origina os três álcoois hidroxicinamílicos, que diferem entre si pelo padrão de hidroxilação e metoxilação, e que serão incorporados ao polímero de lignina (Boerjan *et al.*, 2003; Liu *et al.*, 2010).

Os três álcoois, *p*-cumarílico, coniferílico e sinapílico, comumente chamados de monolignóis (Figura 2), são sintetizados no citoplasma e transportados para o apoplasto, onde ocorre a radicalização por desidrogenação, gerando as subunidades constituintes da lignina, sendo esta etapa intermediada por enzimas específicas: peroxidases de classe III e lacases [Figura 3, (Onnerud *et al.*, 2002)]. A polimerização ocorre de forma combinatória e aleatória no polímero de lignina. Os monômeros de lignina, após serem incorporados, são chamados, respectivamente, de *p*-hidroxifenil (**H**), guaiacil (**G**) e siringil (**S**) (Boerjan *et al.*, 2003; Vanholme *et al.*, 2008). A alteração na composição da lignina pode diminuir a recalcitrância das moléculas e facilitar a extração (Simmons *et al.*, 2010).

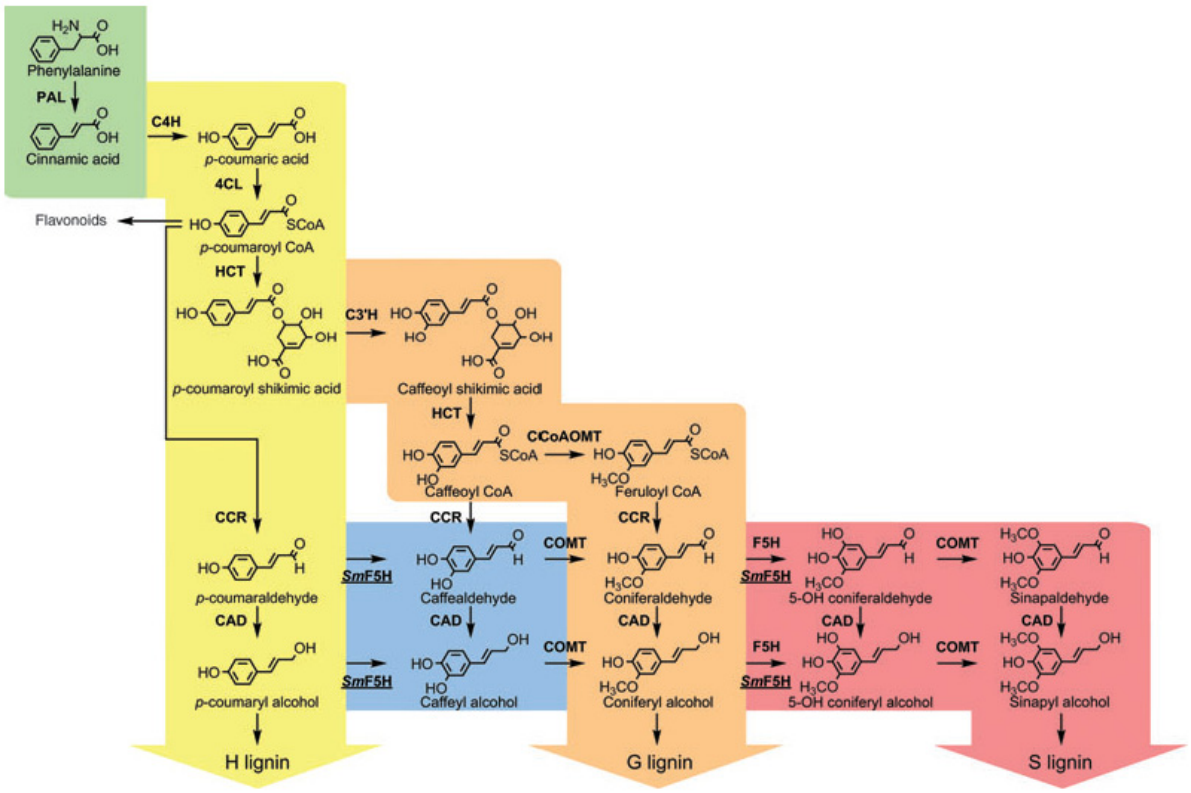

Figura 1: Via dos fenilpropanoides. A deposição de lignina inicia-se com o aminoácido fenilalanina e por diversas reações originam os precursores álcoois: *p*-coumarílico, sinapílico e coniferílico. Estes serão, posteriormente, oxidados por peroxidases classe III e lacases, e depositados na parede celular como unidades **H**, **S** e **G**. Legenda: PAL, fenilalanina amônia-liase; C4H, cinamato 4-hidroxilase; 4CL, 4-hidroxicinamoil CoA ligase; HCT, *p*-hidroxicinamoil transferase; C3H, *p*-cumaroilchiquimato 3-hidroxilase; CCoAOMT, cafeoil CoA *O*-metiltransferase; CCR, hidroxicinamoil CoA redutase; F5H, coniferaldeído/ferulato 5-hidroxilase; COMT, cafeato/5-hidroxiferulato 3/5-*O*-metiltransferase e CAD, (hidroxi)cinamil álcool (Weng & Chapple, 2010).

As diferentes proporções dos monômeros afetam a estrutura física e a degradação da lignina. Lignina rica em subunidade **S** é mais facilmente degradada em comparação com G, pois são menos condensadas por serem mais metoxiladas e, portanto, terem ligações menos estáveis e mais facilmente clivadas que outras subunidades (Ziebell *et al.*, 2010). Nas dicotiledôneas, as subunidades **G** e **S** são abundantes, enquanto **H** é encontrada em pouca quantidade. Em gimnospermas, existe o domínio da subunidade **G** e um pouco de **H**. Essas subunidades são ligadas por diferentes padrões, sendo β-*O*-4 o mais comum (Figura 3) (Boerjan *et al.*, 2003). Outros compostos que não os tradicionais álcoois hidroxicinamílicos

podem compor o polímero de lignina e estão descritos na literatura (Figura 2) (Del Rio *et al.*, 2007; Ralph *et al.*, 2008).

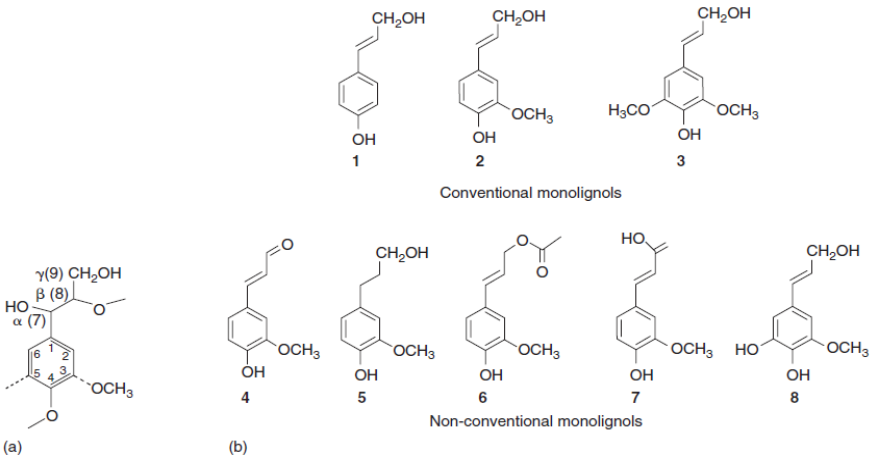

Figura 2: (a) Demonstração da numeração dos carbonos na estrutura dos monolignóis. (b) Números 1 ao 8, exemplificando os possíveis estados de oxidação dos monolignóis e posteriormente incorporados no polímero heterogêneo de lignina. Os números 1, 2 e 3 representam os comumente encontrados e descritos na Figura 1. Dos números 4 ao 8 estão outros não convencionais que também podem fazer parte da estrutura de lignina, após oxidação por peroxidases de classe III e lacases. Legenda: (1) álcool *p*-cumarílico; (2) álcool coniferílico; (3) álcool sinapílico; (4) álcool coniferaldeído; (5) álcool desidroconiferil; (6) coniferil-9-acetato; (7) ácido ferúlico e (8) 5-hidroxyconiferil (Belgacem & Gandini, 2008).

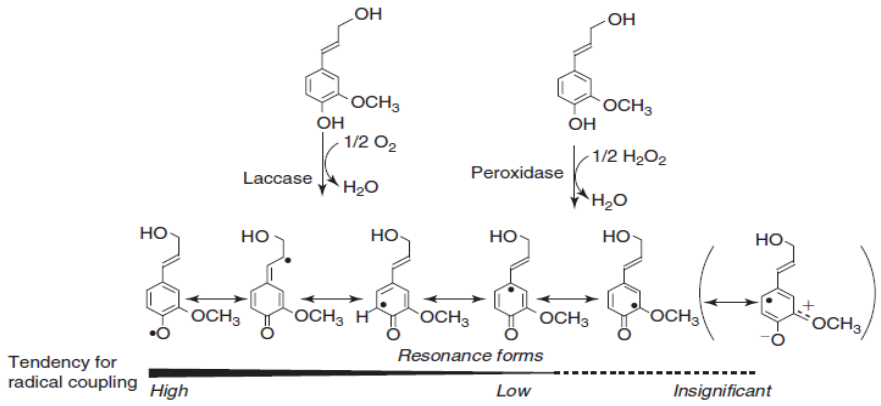

Figura 3: Tendência de acoplamento/ligação dos monômeros após oxidação realizada por peroxidase de classe III e lacase. A ligação β -O-4 é a mais comum e com maior tendência a ligação dentre as quimicamente possíveis (Belgacem & Gandini, 2008).

Peroxidases de classe III

Estudos laboriosos têm foco nas peroxidases de classe III, responsáveis pela radicalização dos precursores de lignina e na busca por fenótipos que não fossem prejudiciais ao desenvolvimento da planta. A família das enzimas peroxidases é classificada em três classes distintas (I, II e III) e todas possuem um grupo heme de protoporfirina IX de ferro em sua estrutura básica e estruturas tridimensionais similares, mas diferem entre si: (i) na sequência de aminoácidos; (ii) na localização celular e (iii) na função fisiológica (Mathé *et al.*, 2010). Dentro dessa classificação, a classe III é encontrada em plantas terrestres e ausente nas algas verdes unicelulares, são glicoproteínas contendo Ca^{2+} ligado a quatro/cinco pontes de dissulfeto, além de possuírem peptídeo sinal para secreção (Passardi *et al.*, 2004, 2005; Mathé *et al.*, 2010).

As peroxidases de classe III são codificadas por uma família multigênica que sofreu diversos eventos de duplicação, neo- e sub-funcionalização. Devido à necessidade das plantas em se adaptar a vida terrestre e as altas concentrações de oxigênio. Entre as funções descritas estão: (i) biossíntese de lignina e suberina (Christensen *et al.*, 1998, 2001); (ii) alongamento celular (Passardi *et al.*, 2006); (iii) degradação de auxina (Cosio & Dunand, 2009); (iv) defesa contra patógenos (Almagro *et al.*, 2009). Na via de lignina, as peroxidases de classe III oxidam os monolignóis com o uso de H_2O_2 que serão, posteriormente, incorporados ao polímero de forma aleatória na parede celular (Passardi *et al.*, 2004; Ros-Barceló, 2005). Sendo uma família numerosa em diversos organismos, foram encontrados 73 genes no genoma de *Arabidopsis thaliana*, 138 em *Oryza sativa* e 105 em *Populus trichocarpa* (Tognolli *et al.*, 2002; Bakalovic *et al.*, 2006; Passardi *et al.*, 2007). Além disso, isoformas das enzimas podem ser formadas seja por modificações pós-transcricionais ou pós-traducionais (Mika *et al.*, 2010).

Entretanto, apesar das peroxidases de classe III poderem ter suas atividades facilmente detectadas, tem sido um problema relacioná-las como uma função específica, pois: (i) mutantes (superexpressão ou silenciamento) não geram fenótipos facilmente detectáveis devido à redundância genética e (ii) por serem enzimas sem substrato específico *in vitro*, o que dificulta a identificação dos verdadeiros substratos *in planta*. Com isso, os estudos acabam por envolver diversas técnicas de biologia molecular a fim de, mesmo que indiretamente, relacionar a atividade de peroxidases classe III com lignificação.

Mesmo assim, a caracterização dessas enzimas foi realizada em diferentes organismos: *Arabidopsis thaliana* (Valerio *et al.*, 2004; Cosio & Dunand, 2010), *Medicago sativa* (Watson *et al.*, 2004), *Picea abies* (Marjamaa *et al.*, 2006), *Eucalyptus viminalis* (Aoyama *et al.*, 2001), cultura de tecidos (Karkonen & Koutaniemi, 2010; Cesarino *et al*., 2013) e *Eeucalyptus globulus* (Rencoret *et al.*, 2011). Alguns exemplos bem sucedidos na identificação e caracterização podem ser encontrados: silenciamento de *NtPrx60* em *Nicotiana tabacum* resultou na redução de 50% nos níveis de lignina (Blee *et al.*, 2003). De forma semelhante, o silenciamento de *PkPrx03* reduziu em 20% lignina em álamo (Li *et al.*, 2003), identificação de *AtPrx2*, *AtPrx25* e *AtPrx71* foram relacionados com os níveis de lignina (Shigeto *et al.*, 2013) e *AtPrx37* foi associado ao desenvolvimento vascular (Pedreira *et al.*, 2011).

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Objetivos

- Identificar peroxidases de classe III que estejam relacionadas com lignificação em *Eucalyptus globulus*.

Objetivos específicos

- Identificar genes de referência para serem utilizados como normalizadores em experimento de PCR em tempo real.

- Caracterização de uma peroxidase de classe III – *EglPRX17* – selecionada por estar potencialmente envolvida com lignificação.

- Análise dos tipos de monolignóis presentes em diferentes espécies utilizando-se de técnica inédita para sua identificação e caracterização.

Capítulos da tese

A tese foi dividida em quatro capítulos nos quais são abordados aspectos moleculares e caracterização de *Eucalyptus* com foco particular em peroxidases de classe III.

No capítulo I, realizou-se a identificação de genes de referência que fossem estáveis para a quantificação de transcritos nas espécies de *Eucalyptus* sob diferentes estresses. Esse trabalho ajudou posteriormente a realização dos demais por ter caráter básico, mas de suma importância nos experimentos moleculares. O capítulo II tem a proposta de um sistema *in vitro* de cultivo de *E. globulus* para o estudo de lignina. Neste sistema houve deposição diferencial de lignina quando diferentes tratamentos foram aplicados e, com isso, permitiu a identificação possíveis genes relacionados à formação da madeira. O capítulo III aborda a caracterização parcial de uma peroxidase de classe III – *EglPrx17* - que apresentou um padrão de expressão atípico. Neste capítulo, experimentos ainda estão sendo realizados a fim de uma caracterização correlacionando a expressão e atividade da EglPrx17 com lignificação. Por fim, o capítulo IV mostra uma alternativa rápida e simples para a identificação e quantificação de monolignóis (em especial S e G), além de oligômeros que formam o polímero de lignina, usando espectrometria de massas acoplada a um sistema de imagens. O trabalho pretende substituir o uso de reagentes químicos tóxicos e reduzir o tempo gasto.

Todos esses capítulos foram desenvolvidos exclusivamente durante o doutorado, apesar de abordarem temas diversos relacionados sempre com lignina. O estabelecimento de genes de referência para estudos de expressão que servem como base e monitoramento na caracterização de genes-alvo (de interesse científico ou comercial) são importantes em futuros estudos realizados envolvendo *Eucalyptus*. O sistema de cultura *in vitro* viabiliza a caracterização precoce de plantas quanto à deposição de lignina e na seleção de genes que possam alterar satisfatoriamente a quantidade e qualidade da lignina. Sendo assim, fornecem

uma metodologia rápida para estudos em plantas lenhosas. Novas técnicas abrem possibilidades para novas interpretações/abordagens, dessa forma os experimentos realizados no capítulo IV fornecem um enfoque prático na caracterização de lignina. Além do mais, contribuem para a redução de reagentes químicos, tempo de trabalho e possibilita análises *a posteriori* por moléculas utilizando o mesmo espectro obtido inicialmente. Deseja-se que esses trabalhos sejam suporte para outros estudos com plantas lenhosas.

Capítulo I

"Validation of reference genes from *Eucalyptus* spp. under different stress conditions"

Artigo publicado

Referência: Moura, J.C.M.S., Araújo, P., Brito, M.S., Souza, U.R., Viana, J.O. & Mazzafera, P., 2012. Validation of reference genes from Eucalyptus spp. under different stress conditions. BMC Research Notes 5: 634.

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Abstract

Background: The genus *Eucalyptus* consists of approximately 600 species and subspecies and has a physiological plasticity that allows some species to propagate in different regions of the world. Eucalyptus is a major source of cellulose for paper manufacturing, and its cultivation is limited by weather conditions, particularly water stress and low temperatures. Gene expression studies using quantitative reverse transcription polymerase chain reaction (qPCR) require reference genes, which must have stable expression to facilitate the comparison of the results from analyses using different species, tissues, and treatments. Such studies have been limited in eucalyptus.

Results: *Eucalyptus globulus* Labill, *Eucalyptus urograndis* (hybrid from *Eucalyptus urophylla* S.T. Blake X *Eucalyptus grandis* Hill ex-Maiden) and *E. uroglobulus* (hybrid from *E. urograndis* X *E. globulus*) were subjected to different treatments, including water deficiency and stress recovery, low temperatures, presence or absence of light, and their respective controls. Except for treatment with light, which examined the seedling hypocotyl or apical portion of the stem, the expression analyses were conducted in the apical and basal parts of the stem. To select the best pair of genes, the bioinformatics tools *GeNorm* and *NormFinder* were compared. Comprehensive analyses that did not differentiate between species, treatments, or tissue types, showed that *IDH* (isocitrate dehydrogenase), *SAND* (SAND protein), *ACT* (actin), and *A-Tub* (α-tubulin) genes were the most stable. *IDH* was the most stable gene in all of the treatments.

Conclusion: Comparing these results with those of other studies on eucalyptus, we concluded that five genes are stable in different species and experimental conditions: *IDH*, *SAND*, *ACT*, *A-Tub*, and *UBQ* (ubiquitin). It is usually recommended a minimum of two reference genes is expression analysis; therefore, we propose that *IDH* and two others genes among the five

identified genes in this study should be used as reference genes for a wide range of conditions in eucalyptus.

Key words: *Eucalyptus*, drought, cold, light, reference genes.

Background

The genus *Eucalyptus* belongs to the Myrtaceae family, which consists of approximately 600 species and subspecies, exhibits physiological plasticity with worldwide dispersion and successfully grows in different regions. Eucalyptus is a major source of cellulose for manufacturing paper [1] and globally, Brazil ranks first in the production of short fibre cellulose [2]. Due to its economic importance, the genome sequencing of the species *Eucalyptus grandis* was recently completed [3].

E. globulus and *E. grandis* are the two main species cultivated in the world [4]. Among the existing species, *E. globulus* is known for its resistance to cold temperatures and its lower content and type (greater amount of S units) of lignin, which results in a higher extraction yield of cellulose [5-7]. *E. globulus* also exhibits tolerance to cold weather, which allows it to be cultivated in areas where other species do not grow well. In Brazil, the hybrid *E. urograndis* (hybrid of *E. urophylla* x *E. grandis*) has been widely cultivated for its unique features, compared with other cultivated species [8, 9]. It has been reported that more than 600,000 acres are cultivated with this hybrid species in Brazil, being the basis of the Brazilian silviculture for the production of cellulose and paper [10]. *E. uroglobulus* is a hybrid derived from *E. urograndis x E. globulus*, with a high cellulose yield [11].

One of the most widely used approaches for molecular characterisation is gene expression analysis, which aims to identify genes that can be used as molecular markers or for

genetic manipulations. The most common technique for analysing gene expression is quantitative reverse transcription polymerase chain reaction (qPCR), which is advantageous because of its speed, sensitivity and specificity [12].

One of the most important aspects in gene expression analysis by qPCR is the determination of genes that have constitutive expression, which will serve as references in the analysis of plant material from different origins. Thus, the expression of these genes must be constant, regardless of the tissue under analysis comes from different parts of the same plant or plants from different origins. Such genes are called normalisers because it is possible to compare gene expression in various types of biological materials [13-15] based on the individual expression of these genes. However, genes that have previously been described as adequate normalisers for some plant species and/or experimental conditions are not always valid for other species or working conditions [16]. Typically, the selected endogenous genes are related to cellular maintenance processes that are common among different cell types [17]. The use of the appropriate normaliser genes is a limitation to the use of qPCR, and this justifies the considerable number of published studies aimed at identifying useful endogenous genes for the study of gene expression in different species, including rice [18], sugarcane [19], *A. thaliana* [20], potato [21], *Brachypodium* spp. [22], tomato [23], coffee [24], wheat [25], tobacco [26] and *Eucalyptus* spp. [27-30].

de Oliveira [27] performed an extensive search of 21,432 eucalyptus genes in *E. grandis* leaves and xylem and *E. globulus* xylem. The genes were evaluated using microarray hybridisation analysis, and 50 candidate genes were identified that showed little variation in their expression. These genes were evaluated in gene expression studies associated with the flower, leaf, and xylem of six species of eucalyptus. de Almeida et al. [30] aimed to identify endogenous genes for gene expression studies associated with adventitious rooting in *E.*

globulus microcuttings because low rooting efficiency is a problem for the cloning and multiplication of this species. Fernández et al. [28] attempted to identify normaliser genes in *E. globulus* for gene expression studies related to cold acclimation. Similarly, Boava et al. [29] sought to identify normaliser genes for studies of Urograndis hybrid resistance to leaf rust caused by *Puccinia psidii*.

None of these studies identified normaliser genes in eucalyptus that could be widely used in gene expression studies related to cold and drought stress in species of significant economic importance, such as *E. urograndis*, *E. uroglobulus*, and *E. globulus*. Low temperature and limited water are stresses that limit the growth of eucalyptus [11, 31-35]. In this study, we evaluated normaliser genes in *E. urograndis*, *E. uroglobulus*, and *E. globulus* plants exposed to low temperature and stressed with water shortage. The recovery of the water stressed plants after re-watering was also evaluated. Additionally, we also evaluated normaliser genes in the seedlings and plants of *E. globulus* grown in the presence and absence of light, which have been used in lignification studies in this species (Araújo and Mazzafera, unpublished). The validation of endogenous genes under different growth conditions could enable their use in a broad range of studies of gene expression in eucalyptus. In our evaluations we compared the bioinformatics programs *NormFinder* and *GeNorm*.

Results

Several comparisons were performed combining species, treatments, tissues and all data using both *GeNorm* and *NormFinder*. The results of these analyses are shown in Table 3, which was split into four sections to improve the presentation of the data.

A							
Species	**Software**	**CTRL**	**DRT**	**REC**	**CTRL+DRT+REC Apex**	**CTRL+DRT+REC Base**	**All treatments**
E. globulus	GeNorm	A-Tub/UBQ	A-Tub/H2B	IDH/SAND	UBQ/H2B	H2B/A-Tub	H2B/UBQ
	NormFinder	IDH/ACT	ACT/A-Tub	A-Tub/IDH	IDH/A-Tub	A-Tub/ACT	A-Tub/ACT
E. urograndis	GeNorm	SAND/A-Tub	H2B/UBQ	A-Tub/SAND	UBQ/A-Tub	IDH/ACT	IDH/ACT
	NormFinder	ACT/SAND	UBQ/H2B	SAND/A-Tub	ACT/SAND	A-Tub/SAND	ACT/SAND
E. uroglobulus	GeNorm	H2B/UBQ	EF1/IDH	IDH/ACT	SAND/IDH	IDH/18S	A-Tub/ACT
	NormFinder	A-Tub/EF1	SAND/ACT	18S/H2B	H2B/A-Tub	A-Tub/ACT	A-Tub/H2B
All species	GeNorm	UBQ/H2B	IDH/SAND	IDH/ACT	IDH/ACT	IDH/ACT	IDH/ACT
	NormFinder	IDH/SAND	ACT/IDH	SAND/IDH	SAND/ACT	SAND/IDH	SAND/IDH
B							
Species	**Software**	**CTRL**	**COLD**	**CTRL+COLD Apex**	**CTRL+COLD Base**	**All treatments**	
E. globulus	GeNorm	A-Tub/UBQ	EF1/IDH	EF1/IDH	SAND/EF1	EF1/IDH	
	NormFinder	ACT/UBQ	SAND/UBQ	ACT/A-Tub	ACT/SAND	ACT/A-Tub	
E. urograndis	GeNorm	IDH/UBQ	A-Tub/UBQ	UBQ/A-Tub	UBQ/18S	A-Tub/UBQ	
	NormFinder	IDH/UBQ	SAND/UBQ	SAND/IDH	ACT/IDH	IDH/SAND	
E. uroglobulus	GeNorm	IDH/ACT	UBQ/SAND	SAND/UBQ	B-tub/SAND	UBQ/SAND	
	NormFinder	A-Tub/H2B	ACT/IDH	H2B/UBQ	A-Tub/ACT	H2B/A-Tub	
All species	GeNorm	SAND/UBQ	ACT/SAND	UBQ/IDH	IDH/SAND	IDH/SAND	
	NormFinder	A-Tub/EF1	IDH/ACT	H2B/UBQ	IDH/SAND	IDH/SAND	
C							
Species	**Software**	**DRT+COLD Apex**	**DRT+COLD Base**	**All treatments**			
E. globulus	GeNorm	IDH/A-Tub	IDH/SAND	IDH/ACT			
	NormFinder	UBQ/IDH	UBQ/A-Tub	UBQ/A-Tub			
E. urograndis	GeNorm	H2B/UBQ	SAND/A-Tub	IDH/ACT			
	NormFinder	ACT/A-Tub	SAND/A-Tub	ACT/IDH			
E. uroglobulus	GeNorm	IDH/ACT	EF1/IDH	IDH/ACT			
	NormFinder	H2B/A-Tub	A-Tub/ACT	A-Tub/H2B			
All species	GeNorm	ACT/IDH	IDH/SAND	IDH/ACT			
	NormFinder	ACT/IDH	SAND/IDH	IDH/ACT			
D							
Species	**Software**	***in vitro* (dark and light)**	**Greenhouse (dark and night)**	**All treatments**			
E. globulus	GeNorm	H2B/IDH	EF1/ACT	UBQ/IDH			
	NormFinder	UBQ/ACT	IDH/EF1	ACT/IDH			

Table 3 Identification of the best pairs of genes for different treatments, species, and tissues. A – Water stress experiment; B – Low-temperature experiment; C – Water stress + low-temperature experiments in tissues (apex and base), D – light experiments. CTRL = control; DRT – water stress; REC – recovery from water stress; COLD – low temperature.

***GeNorm*:** In the water stress trial, the analyses using *GeNorm* showed a wide range of genes, depending on the combination of treatments, species, and types of tissues analysed (Table 3A). For *E. globulus,* the most stable pair of genes, regardless of treatment or stem position, was *H2B*/*UBQ*. *IDH*/*ACT* and *A-Tub*/*ACT* were the best pairs for *E. urograndis* and *E. uroglobus*, respectively. When analysing the best pair of genes among all of the situations (treatments, species, and types of tissues), the most stable pair was *IDH*/*ACT*. Among the nine genes

analysed, only five were observed in the combinations using GeNorm: *IDH*, *UBQ*, *ACT*, *A-Tub* and *H2B*.

In the low-temperature experiment, a large variation between the gene pairs was observed, depending on the type of treatment, species and tissue combination (Table 3B). In the general analyses within each species *IDH*, *UBQ*, *A-Tub*, *SAND* and *EF1* emerged as the most stable. From the general analysis, disregarding treatments, species, and types of tissues, the best gene pair was *IDH/SAND*.

Using *GeNorm*, the comparison of tissue type (Table 3C) without distinction between treatments, tissue types and species showed the gene pair *IDH*/*ACT* as the most stable. *GeNorm* indicated that *UBQ*/*IDH* was the best gene pair from the analyses with seedlings and plants of *E. globulus* with or without exposure to light (Table 3D).

***NormFinder*:** When *NormFinder* was used, several pairs of genes were identified and like in the *GeNorm* analysis, they varied depending on the treatment, species, and plant tissue. In the water stress treatments (Table 3A), at least one of the genes identified was consistently different from those suggested using *GeNorm*. For example, in the evaluation of *E. urograndis*, the general analysis of the treatments and tissues resulted in the identification of *IDH*/*ACT* by *GeNorm* and *ACT*/*SAND* by *NormFinder*. There were cases that both genes of the pair were different, such as for *E. globulus,* where *H2B*/*UBQ* and *A-Tub*/*ACT* were identified by *GeNorm* and *NormFinder*, respectively. In the analysis grouping the data from the water stress experiments, which included all species, treatments (control, water stress and recovery) and tissue types (apex and base), the observed most stable gene pair was *SAND*/*IDH*. When the low-temperature data were analysed altogether (Table 3B), the gene pair *IDH*/*SAND* was identified as the best combination for *NormFinder*. Among the nine

genes analysed, five genes were present among all of the possible pairs during the water stress and low-temperature treatments when the analysis was carried out with *NormFinder*: *A-Tub*, *ACT*, *SAND*, *H2B* and *IDH*.

When comparing the tissue types (Table 3C), the best combination of primers for all species was *IDH*/*ACT*, which was also the same pair of genes identified in the analysis of plants/seedlings of *E. globulus* subjected to light and dark treatments (Table 3D).

In the general analysis, i.e., all treatments, species and tissues types, *SAND*, *IDH* and *ACT* presented the lower stability values, indicating they were the most stable genes (Figure 2).

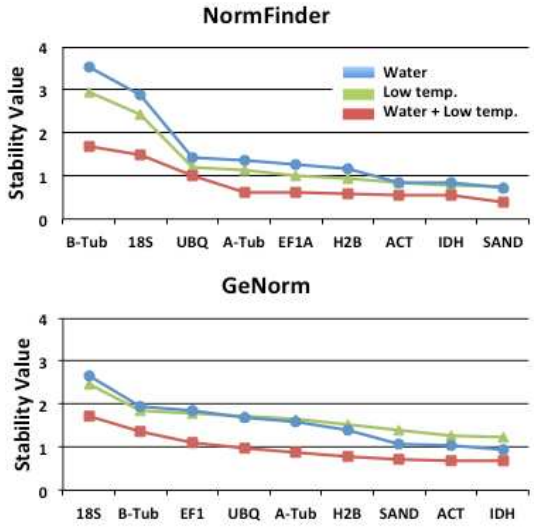

Figure 2. The stability values of the genes calculated according to the two software programmes used in the analyses of the most stable genes. The graph shows the analyses differentiating the water stress treatments (control + water stress + water stress recovery), the temperature treatments (control + low temperature), and both treatments together, regardless of species or tissue types (apex and base).

Discussion

In this study, we used three *Eucalyptus* species in experiments where plants were exposed to different water regimes (sufficient, stressed, and recovery) and low temperature. For the analysis of plants under water stress, plant tissues were harvested for gene expression

analysis only when they displayed the same wilting characteristics, i.e., lack of turgor recovery, in the youngest leaves. Consequently, the time required for wilting varied among plants. We previously used this same strategy to induce drought stress in coffee plants and observed that the water potential in the leaves, even among different species, was homogeneous, resulting in reduced variation in the gene expression analysis [36]. For the low-temperature treatments, the plants were placed in a cold chamber for 12 h daily for at least 6 days. We chose water stress as a treatment because it is the field condition that most limits plant growth [37]. The low-temperature treatment was selected because *E. globulus* is adapted and cultivated in cold regions [5-7].

We also used in our assays seedlings/plants of *E. globulus* subjected to light treatment. For these experiments, the seedlings were grown in either the light or the dark. In addition, the stem apex of six-month-old plants were covered with aluminium foil and grown under greenhouse conditions. The reason to include such treatments was because we have been using light in our studies on modulation of lignin biosynthesis in *E. globulus* (Araújo and Mazzafera, unpublished data).

The qPCR technique facilitated the discrimination of genes expressed during different treatments among plants of the same or different species [38, 39]. However, to validate these comparisons, the analysis must consider genes that have constitutive expression to serve as references for the analysis of plant material from different sources [14, 15]. The selection of these genes is used to evaluate the stability of gene expression in different tissues, using various bioinformatics tools. In the present study, two bioinformatics tools were used that differ in terms of data analysis. The software *GeNorm* analyses the data comprehensively, establishing a reference value or M-value (stability value). The candidates with the highest values are eliminated until only two candidates remain [40]. The tool *NormFinder* only

analyses variations between samples and variations within each sample group [41]. Subsequently, the variances between and within groups are used to determine the stability value, for which smaller values are considered most stable. Although *NormFinder* provides a more detailed analysis as compared with *GeNorm*, both tools are widely used for the validation of normalisers.

In the evaluations performed in the present study using *GeNorm* and *NormFinder*, few consistencies were observed among the data generated using both methodologies, as also observed in other studies with eucalyptus [27]. This was mainly observed when data were compared separately. Some few exception were observed: for example, the water stress treatment (DRT column in Table 3A) showed that for both methodologies, the best endogenous gene pair in *E. urograndis* was *H2B* /*UBQ*. However, consistency appeared when more comprehensive analyses involving different species and treatments were carried out, what then highlighted the *IDH* gene. In the analyses related to the low-temperature treatment, the genes *IDH* and *SAND* were common in the analyses using *GeNorm* and *NormFinder*. However, in addition to *IDH*, *ACT* appeared to be stable in the analyses distinguishing the tissue type (apex or base) in the samples from for the stress and low-temperature treatments.

IDH was also the most common gene in the *GeNorm* and *NormFinder* analyses for the material subjected to different light regimens. It is interesting to observe that the gene *UBQ* was also identified in the *E. globulus* greenhouse material using *GeNorm*, while *ACT* was identified using the *in vitro* material with *NormFinder*. The results reflect differences in the experimental conditions and the developmental stages of the plants.

IDH, *SAND* and *ACT* were the genes identified here as the most frequent in the gene pairs indicated in the analyses using *GeNorm* and *NormFinder*. *IDH*, *SAND* and *ACT* perform different roles related to housekeeping functions in plant cells. SAND is a member of a large

family of proteins that are found in many organisms, which was identified initially in *Saccharomyces cerevisiae* and subsequently in *Schizosaccharomyces pombe, Caenorhabditis elegans*, *Drosophila melanogaster*, mouse, human, and *A. thaliana* [42]. SAND protein is involved in membrane trafficking, enabling the fusion of the vesicle to the vacuole [43]. IDH is a mitochondrial enzyme involved in the oxidative decarboxylation of isocitrate in the Krebs cycle to produce α-ketoglutarate and CO_2, a reaction coupled with the conversion of NAD^+ to NADH. Five isoforms of IDH are found in *A. thaliana*. Four of these enzymes are expressed in all tissues, and only one is specific to the pollen grain [44]. ACT is an important plant cytoskeleton protein that has a pivotal role in several cellular processes involved in the regulation of cell growth and morphology [45].

de Oliveira et al. [27] evaluated the microarray data from 21,432 genes and selected the 50 most stable genes expressed in the leaves of *E. grandis* and xylem from *E. grandis* and *E. globulus*. Of these 50 genes, eight were chosen, together with seven other genes that were previously tested in the literature, for studies on the stability of gene expression in the tissues of six *Eucalyptus* species (leaf and xylem of *E. grandis*, *E. dunnii*, *E. pellita*, *E. saligna* and *E. urophylla*; xylem of *E. globulus*; and flowers of *E. grandis*). Overall, these 15 genes showed adequate expression stability in the tissues analysed, but the best results were obtained with the genes *Eucons04*, *Eucons08*, and *Eucons21*, which were selected from the microarray analyses. *Eucons04* putatively encodes a protein similar to cyclin-dependent protein kinases (CDKs). *Eucons08* encodes a transcription elongation factor SII (TFIIS). *Eucons21* encodes a protein similar to aminoacyl-tRNA synthetases. All three genes showed similarities with genes from *Ricinus communis*. Among the genes used in our study, only H2B was included in the study of de Oliveira et al. [27], and it was moderately expressed. In our results, the *H2B/A-Tub* pair was consistently observed in the general analyses of drought and cold treatments in *E.*

uroglobulus using the *NormFinder* software. Our data showed that the stability value places *H2B* as the fourth most stable gene, after *ACT*, *IDH*, and *SAND*. The differences observed between the data obtained in this study and that of de Oliveira et al. [27] could reflect differences in the species used and the treatments applied.

Other authors evaluated the stability of the genes used herein in eucalyptus. Almeida et al. [30] used the genes coding for 18S ribosomal RNA (18S), actin 2/7, histone H2B, NADP-isocitrate dehydrogenase, polyubiquitin, SAND protein, α-tubulin, TIP41-like protein, translation elongation factor 2, a expressed protein without determined function (33380), and a putative RNA-binding protein (EUC12) to study the formation of adventitious roots in *E. globulus*, of which the first eight genes were the same genes used in our study. de Almeida et al. [30] identified the best gene pairs as *NADP-Isocitrate Dehydrogenase/SAND* using *GeNorm* software, and *Histone H2B/α-Tubulin* using *NormFinder*. However, among the 13 genes, *UBQ* and *IDH* displayed the highest stability in two eucalyptus clones (rust-resistant and susceptible) exposed to biotic (*P. psidii*) and abiotic (acibenzolar-S-methyl) stresses [29]. In choosing the reference genes in studies on the acclimatisation of *E. globulus* to the cold, Fernández et al. [28] used the genes for elongation factor 1-a, actin, α-tubulin, protein phosphatase 1A, SAND, and ubiquitin C, concluding that the genes for ubiquitin, elongation factor 1-a and α-tubulin were the most stable.

Conclusions

The data of Boava et al. [29], Fernández et al. [28], de Almeida et al. [30], and the current study indicate that the most frequent stable genes in the various analyses performed in different eucalyptus species and under various conditions are *ACT*, *UBQ*, *SAND*, *IDH*, and *A-Tub*, with *IDH* as the most stable. Therefore, apparently these five genes could be used as

reference genes in a wide range of treatments in eucalyptus research where gene expression is evaluated. Given that the use of more than one reference gene [46] or even several genes [47] have been recommended to minimise errors in the estimation of gene expression, we propose that *IDH* and at least two more of the five genes listed could be used in a wide range of studies of biotic and abiotic stresses in eucalyptus.

Methods

Plant Material: The low-temperature and water stress experiments were performed with *E. uroglobulus* (*E. urograndis* x *E. globulus* Labill), *E. globulus* Labill and *E. urograndis* (*E. urophylla* S.T. Blake x *E. grandis* Hill ex-Maiden), which were kindly provided by the Fibria Cellulose SA (São Paulo, Brazil). Upon receipt, the seedlings were maintained in a greenhouse at room temperature until the low-temperature and drought trials were performed. The plants for the low-temperature trials were grown in 10 L pots containing a mixture of soil and sand (1:1, v/v). The plants for the water stress experiments were transplanted into 10 L pots containing a soil-sand mixture (2:1, v/v) and were maintained at three plants per pot. At the time of the experiments, the plants were 90 days old in the cold experiment and 120 days old in the water stress experiment.

Low temperature experiments: All the plants were maintained in a greenhouse at room temperature during the day. At the end of the day, half of the plants were transferred to cold and dark chambers, and the other half were transferred to chambers that were only dark and without temperature control. The plants were kept in these chambers for 12 h. The average values of the minimum and maximum temperature were, respectively, 3.2 and 5.7°C for the trial with *E. urograndis,* 6.1 and 9.1°C for *E. globulus*, and 5.7 and 8.8°C for *E. uroglobulus*. The cold experiments lasted seven (*E. urograndis*), eight (*E. globulus*), and ten (*E.*

uroglobulus) days. In the control dark chamber, the average temperature ranged from 20 to 26°C. In the greenhouse, the temperature ranged from 22 to 30°C during the day.

Water stress experiments: In these trials, 120-day-old plants grown in a greenhouse with no temperature control were divided into three groups (control, water stress, and water stress recovery). The control plants were watered daily. For the plants subjected to water stress, the irrigation was interrupted. When each individual plant showed symptoms of wilting in the younger leaves at 12:00 pm on a given day, without recovery on the morning (8:00 am) next day, the plant tissues were collected. This strategy indicated that the plant had lost the capacity to recover water turgor during the night [36]. Because the plants were only collected when showing constant signs of wilting, the development of stress in each plant occurred at different times. In general, the experiments lasted an average of 13 (*E. uroglobulus*), 19 (*E. globulus*), and 21 (*E. urograndis*) days. In the third group, the plants that did not recover their water turgor in the morning were watered, and after recovery, which occurred at approximately 16 h after irrigation, the tissues were collected.

Light treatment: The seedling of *E. globulus* originating from seeds germinated *in vitro* were subjected to light treatments. The seeds were sterilised with 70% alcohol for 2 min, followed by four washes with 2% hypochlorite solution and five washes with sterile water. The seeds were subsequently placed in vials containing 2.5% (w/v) phytagel, prepared with 0.5X Murashige and Skoog media (MS; [48]). The vials were placed either under light (25°C, 12 h photoperiod, 120 µmols photons.m^{-2}.s^{-1}) or in the dark (25°C) for 15 days (Figure 1). After this period hypocotyls were collected for analysis. To compare the results obtained with seedlings hypocotyls with plants at a more advanced stage of development, the apexes of six-month-old *E. globulus* plants were either covered with aluminium foil or uncovered for 30 days. The plants were maintained in the greenhouse without temperature control.

Tissue collection: At the end of the water stress and low-temperature trials, the leaves were removed, and stem collection was performed. The first 5 cm from the apex (denominated apex) was collected, the following 15 cm were discarded, and the following 5 cm were collected (denominated base) (Figure 1). The apex represented the region where longitudinal cell growth is predominant, and the base represented the region where radial growth increases in intensity. The collected tissues were immediately frozen in liquid N_2 and stored in a biofreezer (-80ºC). Hypocotyls segments from seedlings grown *in vitro* were cut from the middle of the hypocotyl, frozen in liquid N_2, and stored in a biofreezer (-80ºC) (Figure 1).

In all experiments three biological replicates were used, each one composed by five plants (water and low temperature experiments) or ten seedlings (light experiment). For each replicate three technical replicates were made in the qPCR analysis.

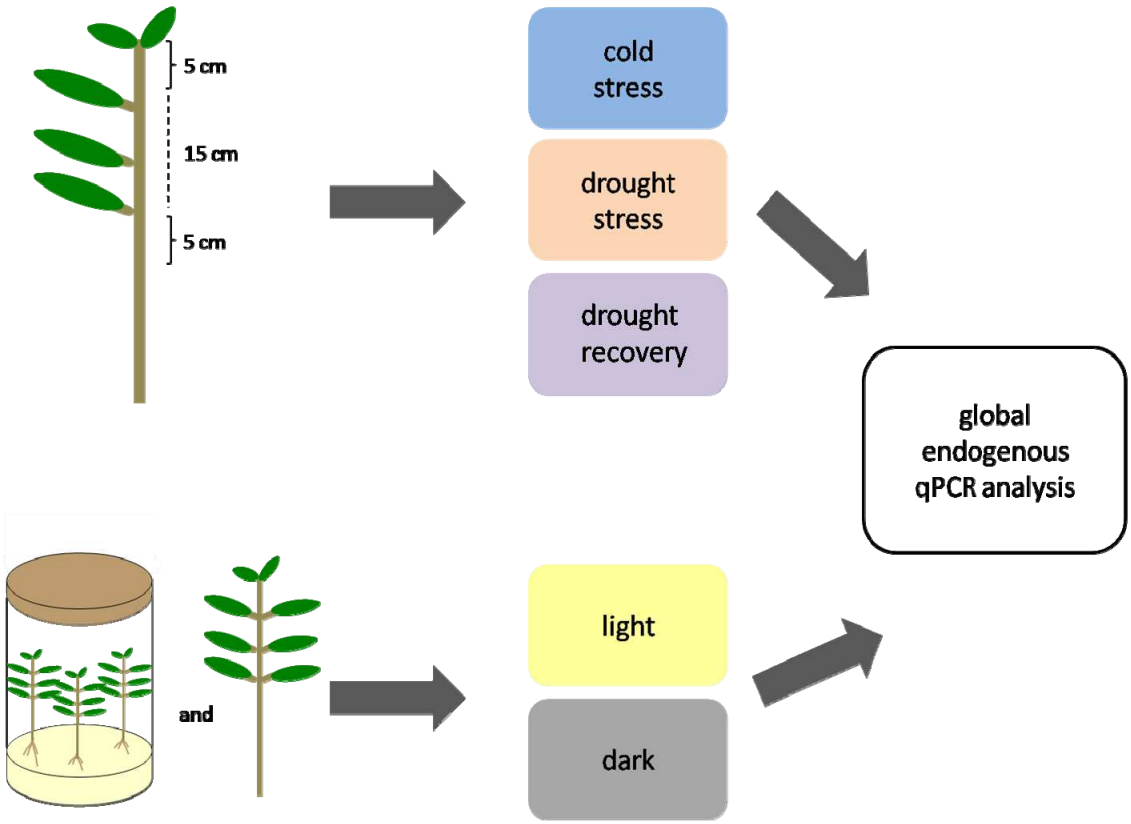

Figure 1 A schematic diagram of the treatments used in the characterisation of the pattern of endogenous gene expression in eucalyptus. Gene expression under low temperatures, drought, and recovery from drought treatments was analysed in *E. globulus*, *E. uroglobulus*, and *E. Urograndis* plants. Only *E. globulus* was used in the light and dark trial. The seedlings were cultivated *in vitro*, and the six-month plants were grown in the greenhouse.

Nucleic acid manipulation: Total RNA extraction from the stem segments of the plants under stress was performed according to Chang et al. [49], with slight modifications. Briefly, 750 µL of extraction buffer was heated to 65°C in 2 mL Eppendorf tubes (1 tube per extracted sample), and 7.5 µL of 2-mercaptoethanol was added to each tube. Approximately 100 mg of sample was macerated in N_2 and added to each tube followed by intense agitation in a vortex and cooling at room temperature. Subsequently, the RNA was extracted once with 750 µL of chloroform:isoamyl alcohol (24:1), mixed for 2 min, and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected, and after the addition of 188 µL of a solution of 10 M LiCl, the RNA was precipitated for 12 h at 4°C. Then, the samples were centrifuged at 10,000 rpm for 30 min at room temperature. The supernatant was then discarded, and the pellet was dissolved in 200 µL of SSTE (37°C). A total of 200 µL of chloroform:isoamyl alcohol (24:1, v/v) was added. After manual agitation by tube inversion, the mixture was centrifuged at 10,000 rpm for 15 min, and the supernatant was collected. Subsequently, 1 mL of absolute ethanol was added to the supernatant and incubated at -20°C for 2 h. The samples were centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatants were discarded. The pellet was washed with 75% ethanol and centrifuged at 14,000 rpm for 20 min at 4°C. The final supernatant was discarded, and the pellet was dried at room temperature. The dried RNA pellet was dissolved in 20 µL of water treated with diethylpyrocarbonate. Total RNA was extracted from the seedling hypocotyls using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). All of the extracted RNA was treated with DNAse (turbo DNA-*free*, Ambion), and the cDNA synthesis was performed using the SuperScript^{R} VILO^{TM} cDNA synthesis kit

(Invitrogen). The quantification of the RNA concentration from each sample was performed in a Qubit fluorometer (Invitrogen), and the RNA quality was assessed on a 1% (m/v) agarose gel using ethidium bromide with subsequent visualisation under UV light.

Primer design: The sequences of the genes used were obtained from four public banks related to *Eucalyptus* (http://bioinformatics.psb.ugent.be/webtools/bogas, http://www.phytopkmdzome.net/eucalyptus.php, http://eucalyptusdb.bi.up.ac.za, and http://www.polebio.scsv.ups-tlse.fr/eucatoul/). The primers were designed using the internet software Primer3 [50] according to the following parameters: 18 and 30 bp in length, 60°C melting temperature, 40-60% GC content, and a 50-150 bp amplified fragments length.

Nine genes were selected for the stability study (Table 1): elongation factor 1-alpha (*EF1*), ß-tubulin (*B-Tub*), actin (*ACT*), ubiquitin (*UBQ*), SAND protein (*SAND*), isocitrate dehydrogenase (*IDH*), histone (*H2B*), α-tubulin (*A-Tub*), and 18S ribosomal RNA (*18S*). The choice of these genes was based on previous studies with *Eucalyptus* [28, 30]. A pair of primers was designed for each gene, and for the UBQ gene, one pair of primers was designed based on the sequence of *E. globulus* and another based on the sequence of *E. urograndis* (Table 2). For each primer pair, the amplification efficiency was determined based on the slope of the standard curve for each of the genes.

Table 1 The gene identification numbers and names of *Eucalyptus* genes and their orthologous genes and functions in *A. thaliana*.

Gene ID	Gene name	*A. thaliana* gene	Function	Reference
EF1a	Translation elongation factor	At1g18070	Translation factor activity, nucleic acid binding, GTP binding, GTPase activity;	[51]
B-Tub	β-Tubulin	At5g62690	GTPase activity, protein binding, structural molecule activity	[52]
ACT	Actin	At5g09810	Structural constituent of cytoskeleton	[53]
UBQ	Ubiquitin	At4g05050	Signalling complexes for protein degradation, translation control, DNA repair, endocytosis regulation, protein traffic	[54]
SAND	SAND protein	At2g28390	Intracellular vesicular transport, biogenesis and vacuole signalling	[17]
IDH	Isocitrate dehydrogenase	At1g54340	Isocitrate dehydrogenase (NADP+) activity, oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	[55]
H2B	Histone H2B	At5g22880	Structural constituent of the eukaryotic nucleosome core	[51]
A-Tub	α-Tubulin	At5g19780	Structural constituent of cytoskeleton, microtubule-based processes	[56]
18S	RNA ribosomal 18S	At3g41768	Cytosolic small ribosomal subunit, translation	[57]

Table 2 Gene identification and primers sequences used in the qPCR analyses.

Gene	Primer	Primer sequence (5'-3')	Amplificon (bp)*	Amplification efficiency (%)**
Elongation factor-1α	Forward	CCTGTCCTTGATTGTCACACTTCC	130	110
	Reverse	CCATTCCAGCATCACCGTTCTTC		
Ubiquitin (*E. gobulus*)	Forward	TCCGTCAAAAGCGAACAGA	173	97
	Reverse	CATTTCCCTCCAGATTACCC		
Ubiquitin (*E. urograndis*)	Forward	GGACTTTCGTTCGTTTTGGT	107	97
	Reverse	GTGATTTGGGGAGGGTTTG		
Actin	Forward	AGATGACCCAGATTATGTTTGAGACCTTC	122	97
	Reverse	ACCATCACCAGAATCCAACACAATACC		
SAND protein	Forward	TGGGTCACACAGGATTTTGA	130	100
	Reverse	CTCCCAGCAAAAAGATCTCG		
Isocitrate dehydrogenase-NADP	Forward	AGTTTGAGGCTGCTGGAATC	100	103
	Reverse	CTTGCATGCCCACACATAAC		
Histone H2B	Forward	AACAAGAAGCCCACCATCAC	142	96
	Reverse	ACAACTTCCTCCTCGCTCAC		
α-Tubulin	Forward	CCAGTGAACAAATGCCCTCT	92	107
	Reverse	TGATCAGCAACAACACAGCA		
Ribossomal 18S	Forward	CATGGCCGTTCTTAGTTGGT	71	95
	Reverse	TAGCAGGCTGAGGTCTCGTT		
β-Tubulin	Forward	GATGGGGACGCTATTGATTT	225	100
	Reverse	CTTGGGTTGATGAGTTTCAGG		

* Melting temperature = 60°C for all primers. **E=10^(-1/slope)-1

Real time quantitative RT-PCR (qPCR): The qPCR reactions were performed using a iCycler iQ5 (Bio-Rad). The reactions contained a final volume of 10 µL and included 5 µl of QuantiFast™ SYBR Green – PCR Mix (Qiagen), 0.4 µM of primers, 3 µL of cDNA, and 1.6 µL of autoclaved MilliQ water. The thermocycling conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec and 71 cycles of 60°C for 30 seconds. The reactions were performed in triplicate, and for each biological replicate, technical triplicates were performed. Control reactions, which contained no DNA template, were conducted for all of the experiments (NTC – Non Template Control).

Data analysis: Determination of the best normaliser gene or best gene pair was performed using two software programmes: *NormFinder* [41] and *GeNorm* [40]. Given that *NormFinder* allows the selection of up to two genes and *GeNorm* allows the selection of more than two, we chose to work with the best combination of two genes. Expression levels were determined as

the necessary number of cycles for the amplifications reach a threshold fixed in the exponential phase of PCR reaction (CT) [58]. The CTs were transformed into quantities using PCR efficiencies [40] in order to use *GeNorm* and *NormFinder*.

List of abbreviations

EF1: Elongation factor 1-alpha; B-Tub: ß-tubulin; ACT: Actin; UBQ: Ubiquitin; SAND: SAND protein; IDH: isocitrate dehydrogenase; H2B: histone; A-Tub: α-tubulin; 18S: 18S RNA ribosomal; qPCR: reverse-transcription followed by quantitative real- time Polymerase Chain Reaction; RNA: Ribonucleic Acid; PCR: Polymerase Chain Reaction; cDNA: complementary Deoxyribonucleic Acid.

Competing interests

The authors declare no competing interest.

Authors' contributions

JCMSM and PA contributed equally to this work, as they performed the experiments, analysed the data and helped to write the manuscript draft. URS helped to perform RNA extraction and some of the experiments. MSB analysed the data and helped to write the manuscript draft. JOFV produced the plants and gave information about their agronomical characteristics. PM designed and supervised the study, wrote the final version of the paper. All authors read and approved the last version of the manuscript.

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Capítulo II

"A model system to study the lignification process in *Eucalyptus globulus*"

Artigo aceito para publicação

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Abstract

Recalcitrance of plant biomass is closely related to the presence of the phenolic heteropolymer lignin in secondary cell walls, which has a negative effect on forage digestibility, biomass-to-biofuels conversion and chemical pulping. The genus *Eucalyptus* is the main source of wood for pulp and paper industry. However, when compared to model plants such as *Arabidopsis thaliana* and poplar, relatively little is known about lignin biosynthesis in *Eucalyptus* and only a few genes were functionally characterized. An efficient, fast and inexpensive *in vitro* system was developed to study lignification in *Eucalyptus globulus* and to evaluate the potential role of candidate genes in this biological process. Seedlings were grown in four different conditions, in the presence/absence of light and with or without sucrose in the growth medium, and several aspects of lignin metabolism were evaluated. Our results showed that light and, to a lesser extent sucrose, induced lignin biosynthesis, which was followed by changes in S/G ratio, lignin oligomers accumulation and gene expression. In addition, higher total peroxidase activity and differential isoperoxidase profile were observed when seedlings were grown in the presence of light and sucrose. Peptide sequencing allowed the identification of differentially expressed peroxidases which can be considered potential candidate class III peroxidases involved in lignin polymerization in *Eucalyptus globulus*.

Introduction

Lignin is a phenolic heteropolymer deposited in the secondary thickened walls of specialized cells to confer mechanical support and strength for the plant to stand upright and hydrophobicity for the xylem elements to enable long-distance water transport (Weng and Chapple 2010). Moreover, due to its distinctive polymeric structure and consequent recalcitrance to chemical and enzymatic degradation, lignin is an effective defensive barrier against herbivores and pathogens (Cesarino et al. 2012a). The lignin polymer is produced by the oxidative combinatorial coupling of mainly three *p*-hydroxycinnamyl alcohols, *p*-coumaryl, coniferyl and sinapyl alcohols, differing in their degree of methoxylation (Boerjan et al. 2003). These monolignols are synthesized in the cytoplasm and further transported to the apoplast, where they are oxidized by peroxidases and laccases prior to the incorporation into the polymer (Wang et al. 2013). After their incorporation, these monolignols are called *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively, and their monomeric contribution to lignin composition significantly varies among cell types, tissues and plant species (Bonawitz and Chapple 2010). Nevertheless, cell wall lignification shows a remarkable metabolic pliability since phenylpropanoid derivatives other than the three canonical monolignols can also be incorporated at varying levels, such as hydroxycinnamates, hydroxycinnamyl acetates and aldehydes and diferulate xylan esters (Grabber et al. 2010). In addition, lignin deposition in plant cell walls follows a tightly regulated developmental program but can also be affected by environmental conditions such as biotic and abiotic stresses (Moura et al. 2010).

Recently, interest in lignin research has increased significantly because of its negative effect on forage digestibility, biomass-to-biofuels conversion and chemical pulping (Vanholme et al. 2008). Indeed, lignin has been recognized as the major plant cell wall

component responsible for biomass recalcitrance due to its ability to resist degradation (Chen and Dixon 2007, Van Acker et al. 2013). The fact that lignin is a non-linear polymer built with chemically diverse subunits linked by low reactive bonds hampers its proper recognition by any single degrading enzyme (Li et al. 2008). Importantly, the S/G ratio in lignin is an important feature for the prediction of the degree and nature of polymeric cross-linking. Accordingly, the additional methoxy group at the C-5 position of S-units precludes the formation of degradation-resistant carbon-carbon bond at this position, leading to a less condensed lignin that is more easily depolymerized (Ziebell et al. 2010).

The genus *Eucalyptus* represents the main source of wood for pulp and paper industry worldwide and includes the most widely tree species used for industrial plantations (Legay et al. 2010). Wood in trees is mainly composed of fibers and tracheary elements whose secondary cell walls deposit high amounts of lignin (Demura and Fukuda 2007). During kraft pulping procedure, the wood is treated with harsh and toxic chemicals to ensure proper delignification for further production of paper. Moreover, the fibers derived from kraft pulping are further bleached to produce white fiber for papermaking. Engineering lignin in poplar resulted in significant savings in pulping process and chemicals (Huntley et al. 2003). Although most *Eucalyptus* and poplar species share similar characteristics such as relatively small genome size, diploid inheritance, easy clone propagation and fast growth, but genetic transformation of *Eucalyptus* is still a major bottleneck, which makes functional genomics studies a difficult task (Foucart et al. 2006). Nevertheless, due to the remarkable economical potential of *Eucalyptus*, an array of other technical advances has been reported in recent years, including the release of the *E. grandis* (http://www.phytozome.net/eucalyptus.php) and *E. camaldulensis* genome sequence (Hirakawa et al. 2011), which might contribute to the understanding of wood formation in these tree species. Still, when compared to model plants

such as *Arabidopsis thaliana* and poplar, relatively little is known about lignin biosynthesis in *Eucalyptus* and only a few genes has been functionally characterized, mainly transcriptional factors that regulate lignin deposition (Goicoechea et al. 2005, Legay et al. 2010).

In order to facilitate the study of the lignification process in *Eucalyptus*, an efficient, fast and inexpensive *in vitro* system was developed. *Eucalyptus globulus* seedlings were grown in the presence or absence of sucrose in two distinct light conditions, long-day photoperiod or continuous darkness. Accordingly, several aspects of lignin metabolism were evaluated such as lignin content and composition, lignin oligomers accumulation and gene expression. Our results showed that sucrose and especially light have a positive impact on lignin deposition in the cell walls of developing *E. globulus* seedlings. Moreover, after its initial characterization, the *in vitro* system was used in an attempt to identify class III peroxidases potentially involved in lignin polymerization. For this purpose, determination of enzymatic activity of class III peroxidases and a proteomic approach were performed for the most contrasting conditions regarding lignin deposition. These analyses helped in the identification and initial characterization of two peroxidases potentially involved in the constitutive lignification of *E. globulus*.

Material and Methods

Plant material and growth conditions

Eucalyptus globulus Labill seeds were sterilized once with 70 % ethanol for 2 min, four times with 1.4 % hypochlorite for 2 min and washed five times with sterile water. Seeds were germinated in 0.5 x MS (Murashige and Skoog 1962) containing 2.5 % phytagel, supplemented or not with 5 g l^{-1} sucrose for 14 days at 25 °C in the dark or long-day

photoperiod, 16 h of light and 8 h of dark (50 ±5 μmol m^{-2} s^{-1} – OSRAM 40W special daylight). Accordingly, four different conditions were established: dark (D), dark with sucrose (DS), long-day photoperiod (L) and long-day photoperiod with sucrose (LS). Hypocotyls were harvested, immediately frozen in liquid nitrogen and kept at -80 °C until use. Hypocotyl material was used in all the analyses carried out in this study.

Lignin histochemical analysis

The hypocotyls were fixed in 4 % paraformaldehyde (w/v) for 16 h, subsequently dehydrated in ethanol series and embedded in HistoResin (Leica), following the manufacturer's instructions. Sections of 5 μm thickness were obtained with a rotary microtome and stained for lignin with toluidine blue (O'Brien et al. 1964), phloroglucinol-HCl and Mäule reagent (Guo et al. 2001). Photomicrographs were taken with an Olympus BX 51 photomicroscope equipped with an Olympus DP71 camera.

Determination of soluble and insoluble lignin, and S/G ratio

Insoluble and soluble lignin contents were determined by the Klason method (Hatfield and Fukushima 2005) using 10 mg of lyophilized hypocotyls. Thioacidolysis was carried out for S/G ratio determination and the reaction products were analyzed by gas chromatography mass spectrometry using a Shimadzu GCMS-QP Plus 2010 (Lapierre et al. 1991). Lignin analyses were performed in triplicates.

LC-MS analysis of lignin oligomers

Soluble phenolic extracts were prepared from 25 mg of lyophilized hypocotyls and LC-MS analysis was performed as described previously (Kiyota et al. 2012). Acquity UPLC coupled to a TQD triple-quadrupole mass spectrometer (Micromass-Waters) was used and the chromatographic separation was carried out on a Waters Acquity C18-BEH (2.1 mm × 50 mm, 1.7 μm) column. The identity of lignin oligomers was confirmed by accurate mass measurements and fragmentation patterns as described (Morreel et al. 2010).

Identification of Eucalyptus lignin genes homologs and sequence analyses

A transcriptome database made from stems of seedlings of *E. globulus* and *E. urograndis* (P. Mazzafera, unpublished) was surveyed to identify lignin biosynthesis genes. All the genes selected were analyzed using Phytozome and NCBI database as background, and their classification and annotation name was made according the *E. globulus* annotation in the Peroxibase (Passardi et al. 2007). The obtained EST sequences were assembled using the CAP3 algorithm and the resulting consensus sequences were aligned with homolog sequences using the BioEdit software package (Hall 1999). Evolutionary relationships were inferred with the neighbour-joining method and bootstrap test for 1000 replicates with MEGA 5 software (Tamura et al. 2011). The presence of typical S peroxidise motifs was manually investigated based on a previous report (Gómez Ros et al. 2007).

Total RNA isolation, cDNA synthesis and quantitative RT-PCR

Hypocotyls material from three independent seedling pools was ground in liquid nitrogen to a fine powder and stored at –80 °C until use. Total RNA isolation, cDNA synthesis and quantitative RT-PCR were performed as previously described (Moura et al. 2012).

Sequences of gene specific primers used in this study are shown in Table S1. The expression of the housekeeping gene *isocitrate dehydrogenase* (*IDH*) was used for normalization (Moura et al. 2012).

Protein extraction and in vitro peroxidase activity

Fresh hypocotyl material was ground in liquid nitrogen and proteins were extracted for 2 h at 4 °C using 100 mM Tris-HCl buffer pH 7.5, containing 2.5 % polyvinylpolypyrrolidone (PVPP), 3 mM dithiothreitol (DTT) and 10 mM ascorbic acid. After centrifugation at 12000 g and 4°C for 15 min, the supernatant was transferred to a new tube and the pellet was further extracted twice with the same method. The supernatants from each extraction were pooled, filtered through a 0.22 µm filter (GSWP04700, Millipore), lyophilized overnight and frozen at -80^{o}C. The resulting pellet was further extracted 100 mM Tris-HCl buffer pH 7.5, containing 2.5 % polyvinylpolypyrrolidone (PVPP), 3 mM DTT and 10 mM ascorbic acid, 1 M NaCl, 1 % TRITON X-100 for 6 h to remove cell wall bound proteins. The extract was centrifuged at 12000 g, 4 °C for 15 min, filtered and lyophilized before storage at -80 °C (Barcelo et al. 1987). The protein pellets were solubilized in 50 mM Na acetate buffer pH 5.0 and passed through an affinity chromatography Concanavalin A column, for trapping of N-glycosylated proteins. The proteins were eluted with 1 M sucrose in the same buffer, and subsequently buffer exchanged in mini-PD10 columns (Sephadex G25, GE) with 50 mM Na acetate buffer pH 5.0. The extracts were treated with Pectinase/Macerozyme R-10 (Sigma – 232-885-6) at 37 °C for 2 h to remove pectin (Barcelo and Aznar-Asensio 1999) and once again buffer exchanged to 50 mM Tris-HCl buffer pH 7.5 in PD10 columns. After freeze-drying the pellets were stored at -80 °C until use. The freeze-dried proteins were solubilized in 50 mM Na acetate buffer pH 5.0 and a total of 10 µg of total proteins were used to determine *in vitro*

peroxidase activity as described (Cesarino et al. 2012b). Reactions were performed in triplicates. Protein quantification was carried out with Qubit 2.0 Fluorometer (Invitrogen).

In gel peroxidase activity and peptide sequencing

Determination of the translational profile of class III peroxidases in hypocotyls total protein extracts and peptide sequencing were performed according to Cesarino et al. (2013). The freeze-dried proteins were solubilized in 50 mM Na acetate buffer pH 5.0 and a total of 30 μg protein were loaded in each well. In parallel, denaturing SDS-PAGE (Laemmli 1970) was performed for the visualization of total proteins in the extracts.

Results

Induction and initial characterization of the in vitro system

E. globulus seedlings were cultivated *in vitro* to develop contrasting conditions for the promotion of differential deposition of lignin. Based on previous reports, two parameters that influence lignin deposition were chosen: light stimulus and the presence of sucrose in the growth medium (Rogers et al. 2005). Therefore, four different treatments were applied, seedlings grown in continuous darkness without sucrose (D), in continuous darkness with sucrose (DS), under long-day photoperiod without sucrose (L), and under long-day photoperiod with sucrose (LS). Obvious phenotypic differences could be observed between plants grown in the dark/light treatments, but not for the addition to sucrose. Light-grown seedlings showed shorter and thicker pigmented hypocotyls and expanded green cotyledons, while dark-grown seedlings were etiolated and showed minimum pigmentation (Fig. 1; left panel).

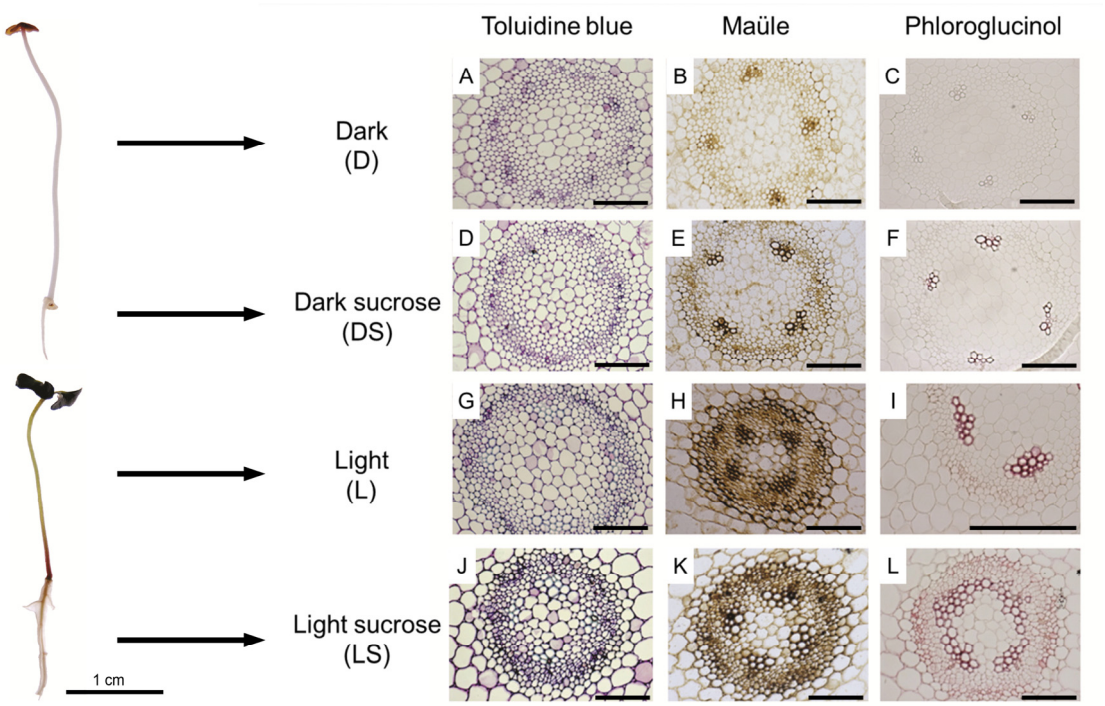

Fig. 1. *Eucalyptus globulus* seedlings were germinated in the dark or under light in medium supplemented or not with sucrose. Lignin deposition was evaluated *in situ* with different staining methods: toluidine blue (left; A, D, G, J), Mäule reagent (middle; B, E, H, K) and phloroglucinol-HCl (right; C, F, I, L). Bars: 75 µm.

Lignin deposition was first evaluated by histochemical analysis of hypocotyls cross-sections using three distinct methods: toluidine blue staining, which stains phenolic compounds in green; Mäule staining, which stains S-lignin red and G-lignin brown, and phloroglucinol staining, which stains lignin red. In all treatments, cross sections of the hypocotyl intermediate region showed typical stem structures, consisting of four collateral vascular bundles (Fig. 1). Seedlings grown in the dark and without sucrose showed the lowest degree of vascular system development, with less conductive xylem cells (Fig. 1A-C). Moreover, these cells were weakly stained with both lignin-specific Mäule and phloroglucinol, which suggests a lower overall lignin deposition (Fig. 1B-C). Interestingly, the presence of sucrose in the growth medium remarkably induced lignin deposition in dark-grown seedlings, as well as the differentiation of more conductive xylem cells (Fig. 1D-F). In contrast to dark-

grown seedlings, vascular tissue differentiation and especially lignin deposition were largely induced by the presence of light (Fig. 1G-I), which presented an additive effect in combination with sucrose (Fig. 1J-L). In seedlings growing in the light with sucrose, it was already possible to visualize installed cambium (Fig. 1J) and the differentiation of secondary xylem fibers in addition to tracheary elements (Fig. 1L).

Lignin content and composition are largely affected by light

To investigate lignin deposition more precisely, lignin content in differentially treated hypocotyls was quantified by the Klason method. This analysis showed that accumulation of both insoluble and soluble lignin was induced by light, while sucrose showed a positive effect in the accumulation of soluble lignin only in the presence of light (Fig. 2A-B). Insoluble lignin levels were approximately 30 % higher in light-grown seedlings, while the difference in soluble lignin contents was approximately 40 %. Moreover, the presence of sucrose in the medium of light-grown seedlings led to an additional increase of around 16 % in soluble lignin when compared to light-grown seedlings without sucrose (Fig. 2B). In addition the presence of light showed a remarkable effect on lignin composition, increasing the S/G ratio from 0.125 (±0.001) to 0.526 (±0.008) when compared to dark conditions (Fig. 2C). Interestingly, the S/G ratio was not changed in the presence of sucrose in the growth medium (Fig. 2C).

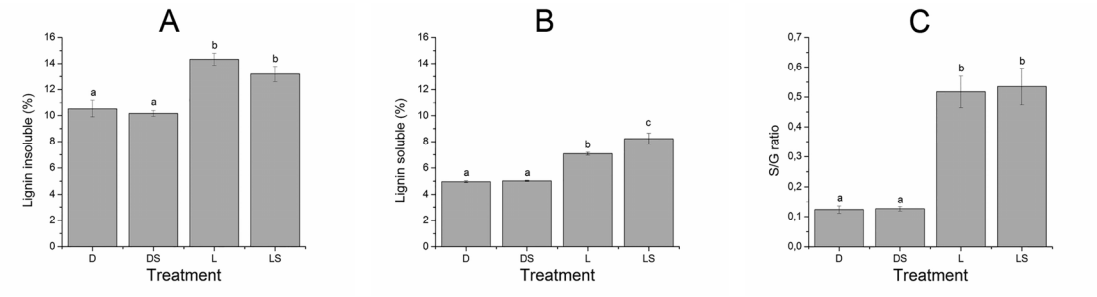

Fig. 2. Quantification of insoluble (A) and soluble (B) lignin contents using the Klason method. (C) Changes in S/G ratio induced by light were assessed by thioacidolysis. Legend: D, dark; DS, dark with sucrose; L, light; LS, light with sucrose. Bars indicate standard deviation and letters significantly different values (Tukey´s test, $p < 0.05$, n=3).

Lignin oligomers profiling

Because lignin analyses showed that light and, to a lesser extent sucrose, altered lignin content and composition in *E. globulus* seedlings, a shift in the oligomers formed during lignin deposition was anticipated (Boerjan et al. 2003, Kiyota et al. 2012). To further confirm this hypothesis, methanol extracts of seedlings grown under all four different conditions were evaluated by ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) following Kiyota et al (2012). A sequencing strategy was used for the identification of the monomeric subunits and the linkage types between the units within an individual lignin oligomer (Morreel et al. 2010). However, only a few lignin oligomers could be detected with this approach, regardless of the treatment. A possible explanation is that *Eucalyptus* hypocotyls deposit only limited amounts of lignin, mainly in the secondary cell walls of xylem vessels and fibers found in the four collateral vascular bundles (Fig 1), which also results in limited amounts of soluble lignin oligomers that are lower than the detection limit of our mass spectrometer. Nevertheless, the detection of higher levels of common lignin oligomers S(8-5)G or S(8-8)G; S(8-O-4)G; G(8-O-4)G(8-O-4)S and S(8-O-4)S(8-O-4)S in L and LS extracts (Table 1) suggests a positive effect of light in the accumulation of these

compounds *in planta*, in agreement with the histochemical and Klason lignin analyses. Furthermore, many lignin oligomers were detected exclusively in the extracts of light-grown seedlings, such as G(8-O-4)G, S(8-5)G/S(8-8)G, S(8-8)S, S(8-O-4)G, G(8-5)G(8-5)G, G(8-O-4)G(8-O-4)S, S(8-O-4)S(8-8)S and S(8-O-4)S(8-O-4)S. Interestingly, most of these oligolignols present structures enriched with S moieties, which is in agreement with the light-induced increase in the S/G ratio.

Table 1. Identification of lignin oligomers in *E. globulus* seedlings by UPLC-MS/MS. Oligolignols were named according to Morreel et al. (2004). Legend: t_r, retention time; D, dark; DS, dark with sucrose; L, light; LS, light with sucrose; MS^2, fragmentation pattern (Tukey´s test, $p < 0.05$, n=3).

t_r	Relative abundance				*m/z*	MS^2	Compound
(min)	D	DS	L	LS			
2,14	$^{a}2{,}1x10^{4}$	$^{b}1{,}4x10^{6}$	$^{c}2{,}9x10^{5}$	$^{a}1{,}6x10^{4}$	179	135, 151, 133	coniferil alcohol (G)
3,42	$^{a}1{,}2x10^{5}$	nd	nd	$^{a}1{,}2x10^{5}$	209	165, 194, 207, 139, 181	sinapyl alcohol (S)
3,76	nd	nd	nd	$8{,}3x10^{3}$	375	347, 357, 331, 249, 303, 237	G(8-O-4)G
3,99	nd	nd	$^{a}4{,}4Ex10^{5}$	$^{b}1{,}6x10^{5}$	387	359, 343, 369, 250, 208, 317, 331	S(8-5)G; S(8-8)G
3,2	nd	$6{,}75x10^{4}$	nd	nd	405	377, 361, 387, 275, 349, 333, 390	S(8-O-4)G
3,51	nd	nd	nd	$5{,}5x10^{4}$	417	389, 237, 399, 373, 249, 181, 345	S(8-8)S
3,88	nd	nd	$^{a}4x10^{5}$	$^{b}7{,}4x10^{4}$	435	407, 417, 391, 393, 379, 363	S(8-O-4)G
3,62	$^{a}6{,}5x10^{4}$	nd	$^{b}1{,}4x10^{5}$	nd	493	475, 465, 449, 254, 249, 407	H(8-5)H(8-O-4)G
4,58	nd	nd	$8{,}2x10^{4}$	nd	535	517, 507, 491, 465	G(8-5)G(8-5)G
3,89	nd	nd	nd	$1x10^{5}$	553	535, 525, 509, 497	G(8-O-4)G(8-5)G
3,54	nd	$2{,}2x10^{5}$	nd	nd	571	543, 553, 527, 483	G(8-O-4)G(8-O-4)G
3,65	$^{a}4{,}6x10^{4}$	$^{b}1{,}2x10^{5}$	$^{c}2{,}8x10^{5}$	$^{a}5{,}2x10^{4}$	583	565, 555, 539, 541, 511, 451	G(8-0-4)S(8-5)G; S(8-O-4)G(8-5)G
2,84	nd	nd	$^{a}1{,}3x10^{5}$	$^{a}7x10^{4}$	601	583, 573, 557	G(8-O-4)G(8-O-4)S
3,19	$^{a}3{,}2x10^{4}$	$^{a}5{,}1x10^{4}$	$^{b}1{,}4x10^{5}$	$^{a}5{,}3x10^{4}$	613	595, 585, 569, 598, 537	S(8-O-4)S(8-5)G; G(8-O-4)S(8-8)S
3,6	$^{a}2{,}3x10^{4}$	$^{a}1{,}4x10^{5}$	$^{b}9{,}8x10^{5}$	$^{a}1{,}4x10^{5}$	631	613, 603, 587, 575, 559	S(8-O-4)G(8-O-4)S
3,38	nd	nd	$1{,}3x10^{5}$	nd	643	625, 615, 599, 543	S(8-O-4)S(8-8)S
3,9	nd	nd	$^{a}9{,}8x10^{4}$	$^{b}6{,}7x10^{4}$	661	643, 633, 617, 533	S(8-O-4)S(8-O-4)S

Expression of lignin genes is affected by light and the presence of sucrose

In order to evaluate how the expression of lignin biosynthetic genes is affected by the different experimental conditions used in the *in vitro* system, quantitative RT-PCR experiments were conducted. *E. globulus* sequences were retrieved from the NCBI EST

sequences based on their similarity with annotated *Arabidopsis* lignin genes and used for specific primers design. The *Arabidopsis* genome was used as reference because the members of each gene family specifically involved in lignin biosynthesis are known. A total of 17 unigenes were identified, which were: 3 *EglPALs* (EC 4.3.1.5), 1 *EglC4H* (EC 1.14.13.11), 2 *Egl4CLs* (EC 6.2.1.12), 1 *EglHCT* (EC 2.3.1.133), 1 *EglC3H* (EC 1.14.13.36), 3 *EglCCoAOMTs* (EC 2.1.1.104), 2 *EglCCRs* (EC 1.2.1.44), 1 *EglCOMT* (EC 2.1.1.68), 1 *EglF5H* (EC 1.14.13.-), and 2 *EglCADs* (EC 1.1.1.195). The *E. globulus* consensus number, the respective gene names and the primer sequences designed for qRT-PCR are shown in Table S1. Confirmation of sequence identities and classification within each gene family for the putative *Eucalyptus* orthologs were performed by identification of conserved protein motifs followed by phylogenetic analysis using *Arabidopsis* amino acid sequences, as well as previously characterized enzymes from other plant species (data not shown).

A snapshot of expression levels for all the available lignin homologs in *E. globulus* was generated for each treatment, allowing a comprehensive comparison between them (Fig. 3). Surprisingly, most of the analyzed lignin genes were down-regulated by light, while the effect of sucrose on gene expression showed no obvious trend and varied significantly from gene to gene. In addition, paralogs within the same gene family were also differentially regulated by the presence of light and sucrose, suggesting that they might be implicated in different biological processes. Nevertheless, some of the genes were significantly up-regulated by light (i.e. *EglPAL3*, *Egl4CL2*, and *EglF5H*). In agreement with the remarkable increase in the S/G ratio induced by light, *EglF5H*, which is involved in the S-specific branch of lignin biosynthesis, was up-regulated by light. Moreover, different combinatorial effects were observed between sucrose and light conditions. For example, *Egl4CL1* was up-regulated by sucrose both in the dark and in continuous light, while *EglPAL1*, *EglHCT*, *EglCCoAOMT2*

and *EglCCR1* were up-regulated only in the dark. Despite the fact that the presence of sucrose led to an up-regulation of some genes in light-grown seedlings (i.e. *Egl4CL1* and *EglC3H*), no additive effect between light and sucrose was observed when compared to dark-grown seedlings.

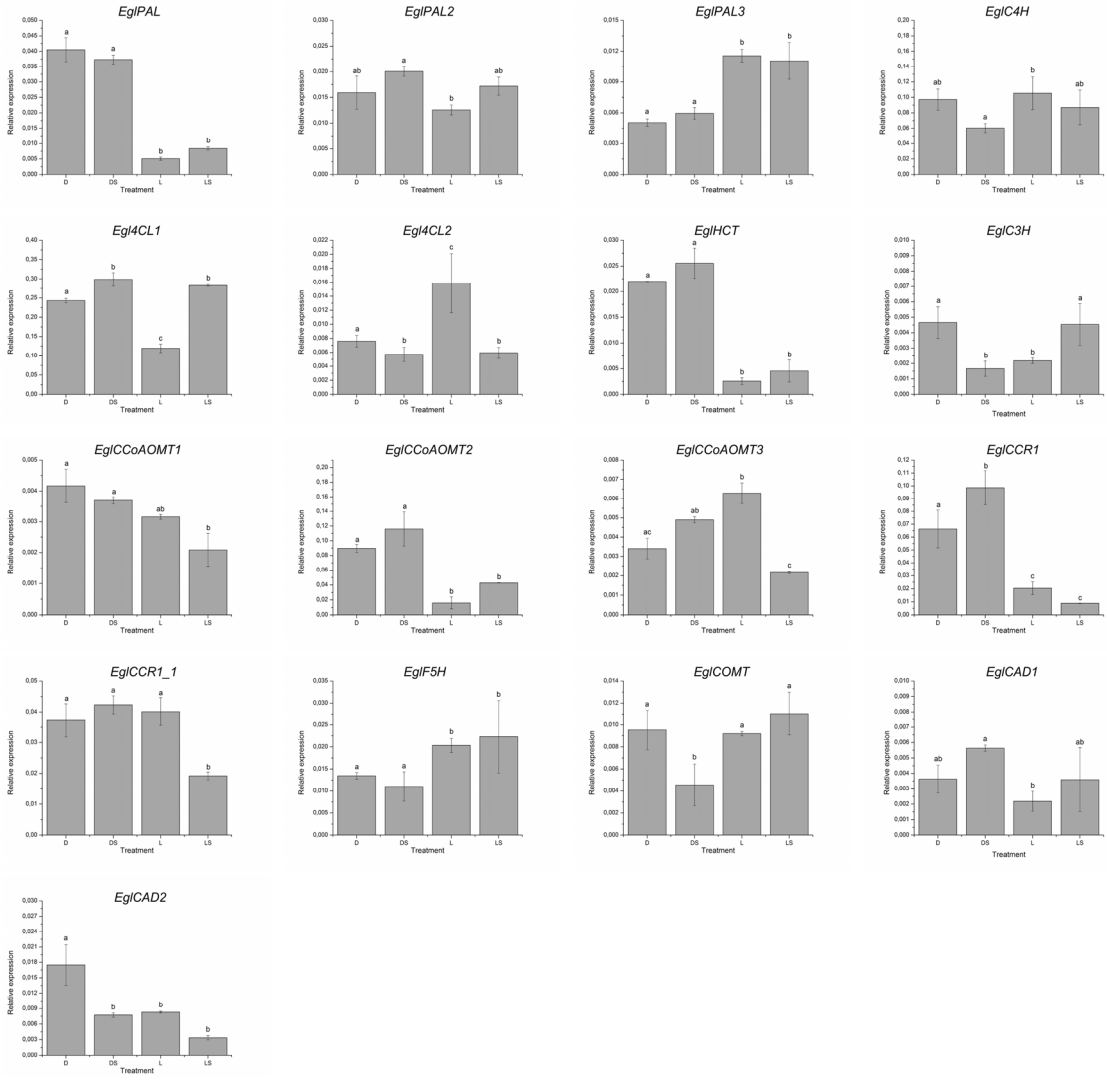

Fig. 3. Expression analysis of lignin biosynthesis genes in *E. globulus* seedlings subjected to different treatments by quantitative RT-PCR. *IDH* was used as reference gene. Legend: D, dark; DS, dark with sucrose; L, light; LS, light with sucrose. Bars indicate standard deviation and letters significantly different values (Tukey´s test, $p < 0.05$, n=3)

Characterization of potential lignin-related class III peroxidases

Although peroxidases are highly active during the whole lifespan of plants, playing a role in a broad range of physiological processes, it is difficult to precisely determine a specific function to a particular isoform (Passardi et al. 2005). Moreover, only a very limited number of isoperoxidases have been actually correlated with monolignol oxidation and mostly in an indirect manner. Therefore, we anticipated that the characterized *in vitro* system could be a valuable tool to identify potential class III peroxidases involved in the constitutive lignification of *E. globulus*. To reduce the complexity of the experimentation, the two most contrasting conditions for lignin deposition (i.e. D versus LS) were chosen for further analyses.

The first step was to compare the *in vitro* peroxidase activities of protein extracts from hypocotyls of seedlings grown under the selected contrasting conditions. Since class III peroxidases involved in lignification are in principle located in the apoplast, proteins were fractionated into soluble and cell wall-bound proteins. Three peroxidase substrates were used: the more general substrate guaiacol (GPrx) and two lignin-related substrates, coniferyl alcohol (CAPrx) and syringaldazine (SYRPrx). The results showed that the combination of light and sucrose led to significant increase in GPrx and CAPrx activities in both soluble and cell wall protein fractions (Fig. 4A-B). Conversely, total SYRPrx activity in cell wall protein fractions was reduced in these conditions, despite the induction of the same activity in soluble protein fractions. Nevertheless, it seems that the same conditions that induce lignin deposition in *E. globulus* hypocotyls have also a positive effect on total peroxidase activity, at least for two out of three tested substrates.

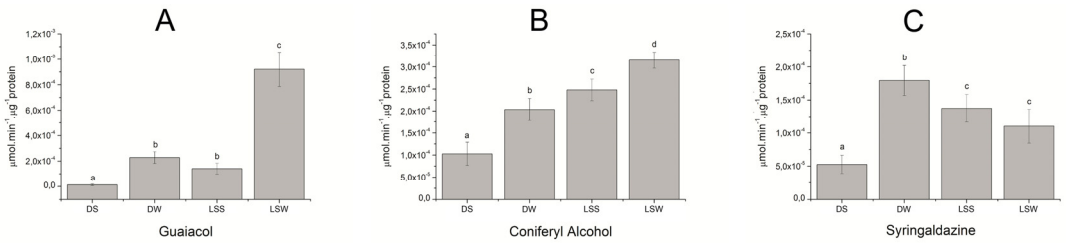

Fig. 4. *In vitro* activity of guaiacol peroxidase (A), coniferyl alcohol peroxidase (B), and syringaldazine peroxidase (C) in protein extracts from *E. globulus* seedlings subjected to different treatments. Proteins were fractionated as soluble and cell wall-bound proteins. Bars indicate standard deviation and letters significantly different values (Tukey´s test, $p < 0.05$, n=8). Legend: DS, dark soluble proteins; DW, dark cell wall-bound proteins; LSS, light with sucrose soluble proteins; LSW, light with sucrose cell wall-bound proteins.

The increased total peroxidase activity of LS protein extracts suggested a differential expression of isoperoxidases. To evaluate this hypothesis, class III peroxidases in D and LS protein extracts, fractionated into soluble and cell wall-bound proteins, were analyzed using in-gel activity after protein separation by semi-denaturing SDS-PAGE (Fig. 5).

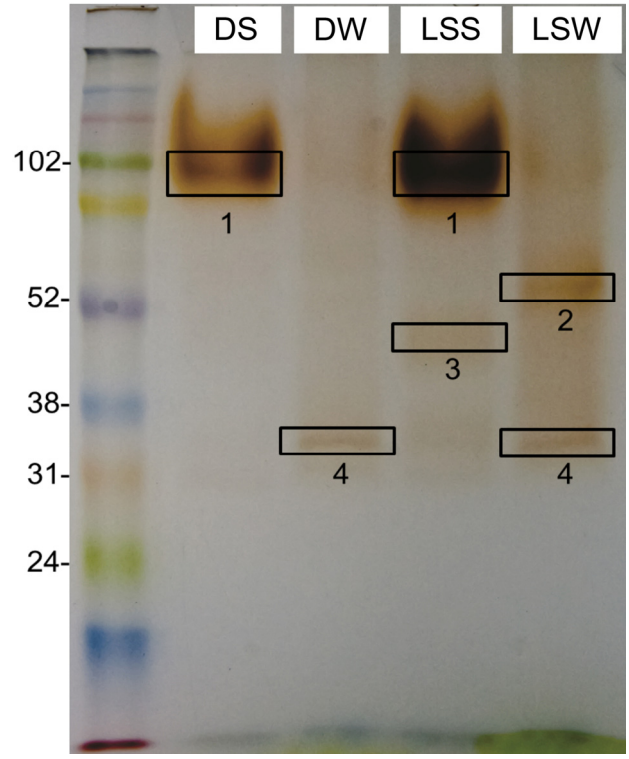

Fig. 5. Activity staining after semi-denaturing SDS-PAGE showing distinct isoperoxidase profiles among soluble and cell wall-bound protein fractions. Each well was loaded with 30 µg of protein. Numbered bands were excised from the gel for further mass spectrometry analysis. Legend: DS, dark soluble proteins; DW, dark cell wall-bound proteins; LSS, light with sucrose soluble proteins; LSW, light with sucrose cell wall-bound proteins.

Few activity bands were detected but a SDS-PAGE gel showed that protein extraction was efficient (see Figure S1 in Supporting Information). Different patterns of isoenzymes were obtained for soluble and cell wall-bound fractions, with a predominance of higher molecular weight (MW) isoforms in the former and lower MW isoforms in the later. The remarkably high MW found for some isoforms is in contrast with the relatively conserved MW of typical class III peroxidases, ranging from 28 to 60 kDa (Hiraga et al. 2001). This discrepancy might be due to the semi-denaturing conditions that do not completely solubilize protein aggregates. In addition, highly cationic proteins are not very well separated in semi-denaturing conditions. Nevertheless, a previous study reported the characterization of plasma membrane-bound peroxidases with MW of 70 and 155 kDa isolated from corn roots (Mika

and Lüthje, 2003). As expected, the LS treatment led to increased in-gel activity of isoforms common to both LS and D protein profiles (i.e. bands 1 and 4; Fig. 5) and induced the expression of additional isoforms (i.e. bands 2 and 3; Fig. 5) in both soluble and cell wall-bound fractions. The cell wall peroxidases induced by LS, which was previously shown to also induce lignin deposition, are good candidates to play a role in the constitutive lignification of *E. globulus* but certainly this need confirmation in further studies. In order to identify the induced peroxidases, especially the cell wall specific isoforms, their corresponding bands were excised from the gel, digested with trypsin and the resulting peptides were sequenced using LC-ESI-QTOF. All analyzed bands generated peptides that matched peroxidases contigs recorded in Peroxibase (Passardi et al. 2007) - see Table 2. The identifications were normally based on single peptide match, with the exception of EglPrx55 and EglPrx112/EglPrx113, but their scores were still higher than the accepted threshold. In addition, two or more peroxidases were identified in bands 2 and 4, while the other 2 bands yielded a single identification. Indeed, since protein separation by mono-dimensional electrophoresis is based exclusively on the differences of molecular weight, peroxidases with similar masses but distinct isoelectric points might co-migrate in the gel. The identified cell wall peroxidases induced by LS were EglPrx121 and EglPrx122 (band 2, Table 2). Interestingly, phylogenetic analysis showed that these two *Eucalyptus* peroxidases clustered in the same clade as AtPrx47, AtPrx64 and AtPrx66 from *Arabidopsis thaliana* (Fig. 6A), which were previously suggested to be involved with lignification (Tokunaga et al. 2009). Multiple amino acid sequence alignment with typical G, which can only oxidize guaiacyl moieties, and S peroxidases, capable to oxidize both guaiacyl and syringyl moieties (Gómez Ros et al. 2007), allowed the identification of structural determinants of S peroxidases in the protein sequence of both identified *Eucalyptus*

peroxidases (see Figure S2 in Supporting Information). These results suggest that these peroxidases can presumably oxidize syringyl moieties.

Table 2. Identification of *E. globulus* peroxidases after in-gel digestion with trypsin using LC-ESI-QTOF and homology-based search against the NCBI database.

Band Number	Tryptic peptide	Mascot score (Significance level)	Contig
1	ITASLLR	17 (>14)	EglPrx55
	GFDVVDNIK	20 (>14)	
2	AMVKMGSIDVLTGK + 2 Oxidation (M)	18 (>14)	EglPrx121
	GLSVDEMVTLSGAHSIGISHCSSFSSR	17 (>14)	EglPrx122
3	GFEVIDR	27 (>14)	EglPrx112/EglPrx113
	LDIQTPTFFDNLYYHNLLQK	18 (>14)	
4	DSTVLTGGPSWVVPLGR	59 (>14)	EglPrx137
	IGVLTGSAGEIR	53 (>14)	EglPrx43/EglPrx45
	IGVLTGTAGEVR	32 (>14)	EglPrx87
	NAMPNQNSLR + Oxidation (M)	16 (>14)	EglPrx114/EglPrx115
	IVISFLSLGLSF	15 (>14)	EglPrx95

In order to validate the MS identifications and further confirm the induction of these peroxidases by the LS treatment, gene expression analysis was performed as described for the *Eucalyptus* lignin genes. The expression of *EglPrx121* was clearly induced by the presence of sucrose in the medium, as observed by the comparison of the pairs D vs DS and L vs LS (Fig. 6B), while a positive effect of light was only observed in the absence of sucrose. A different scenario was found for *EglPrx122*, whose expression was mainly induced by the combination of light and sucrose. Importantly, the comparison of the expression levels in the contrasting conditions used for the proteomics approach (i.e. D *vs* LS) showed a remarkable increase for both peroxidase genes, confirming the induction of these enzymes in the *in vitro* system. Importantly, the comparison of the expression levels in the contrasting conditions used for the proteomics approach (i.e. D vs LS) showed a remarkable increase of both peroxidases genes, confirming the induction of these enzymes in the *in vitro* system. We also checked the spatial

expression of *EglPrx121* and *EglPrx122* in different tissues of seedlings used in this study. Both genes were more expressed in roots and hypocotyls of seedlings grown in the light and with sucrose (Fig. 6C). Curiously, expression of both genes was absent in the cotyledons of seedlings grown in the light.

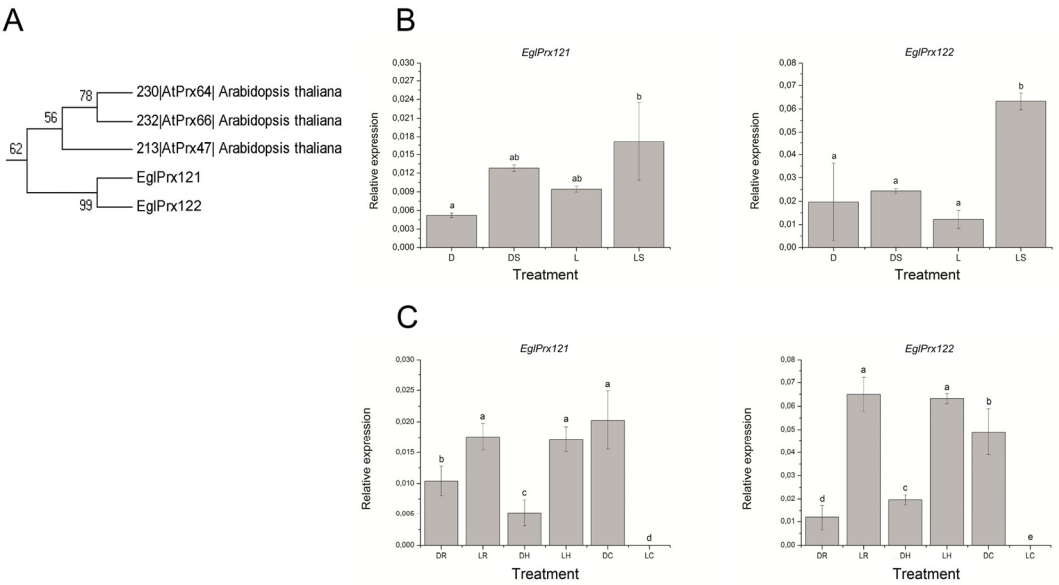

Fig. 6. Two cell wall-bound peroxidases whose expression was induced by LS (light with sucrose) treatment were identified by peptide sequencing. (A) Phylogenetic analysis showing the relationship between the two *Eucayptus* peroxidases and their closest homologs in *Arabidopsis thaliana*. (B) Expression analysis of *EglPrx121* and *EglPrx122* in hypocotyls of *E. globulus* seedlings subjected to different treatments. *IDH* was used as reference gene. Legend: D, dark; L, light; DS, dark with sucrose; LS, light with sucrose; DR and LR, roots of seedlings grown in the dark or in the light with sucrose, respectively; DH and LH, hypocotyls of seedlings grown in the dark or in the light with sucrose, respectively; DC and LC, cotyledons of seedlings grown in the dark or in the light with sucrose, respectively. Tukey´s test, $p < 0.05$, n=3.

Discussion

The hardwood of *Eucalyptus* species is among the world's leading sources of woody biomass for pulp and paper, energy wood and timber production (Gion et al. 2011). Therefore, there is an increasing interest in the understanding of the genetic control and biochemistry of wood formation in these woody species. Here we aimed to develop a fast and efficient *in vitro*

system to study the lignification process in *E. globulus*. Although the genome sequences of *E. grandis* and *E. camadulensis* have been recently released, there is a significant interest in studying the lignification process in other species, particularly *E. globulus* which has superior wood properties This Eucalyptus species is known for its relatively low recalcitrance due to lower amounts of lignin compared to other Eucalyptus species, higher S/G ratio and higher total carbohydrate content (Rodrigues et al. 1999, Cetinkol et al. 2012).

An important advantage of *in vitro* systems is that they allow studies to be performed in controlled conditions, regardless of the season (Kärkönen and Koutaniemi 2010). Moreover, because they are considered a closed system, compounds of interest (e.g. growth regulators, elicitors and signaling molecules) can be simply added to the culture medium. Accordingly, *E. globulus* seedlings were grown in the presence or absence of sucrose in long-day photoperiod or continuous darkness, after which several aspects of lignin deposition were evaluated. Several studies have shown that light induces the phenylpropanoid pathway and promotes the biosynthesis of lignin in distinct tissues of many plant species (Chen et al. 2002, Hemm et al. 2004, Su et al. 2005, Thomas et al. 2005). Light is known to play a role in a wide range of plant developmental processes, including hypocotyl elongation, and affects the accumulation of primary and secondary metabolites (Nemhauser and Chory 2002). Rapid growth of the hypocotyls in the dark is a strategy for the plant to reach the light before the seed reserves are exhausted. During this etiolated growth, hypocotyl cells undergo fast cell expansion with minimal cell differentiation. In the presence of light, hypocotyl elongation is immediately suppressed and photomorphogenetic responses are triggered by the downstream activity of photoreceptors (Nemhauser and Chory 2002). One of the targets of these photoreceptors is the plant hormone auxin, a key regulator of vascular tissue development (Lucas et al. 2013). Therefore, it seems likely that light-dependent induction of lignin biosynthesis is connected

with auxin, that promotes the differentiation of vascular tissue, whose vessels are reinforced with lignin (Friedrich and Peter 1992, Cosio et al. 2009). In agreement, *E. globulus* seedlings grown in light not only showed higher levels of insoluble and soluble lignin but also presented a more developed vascular tissue, with the cambium already installed and more advanced differentiation of secondary xylem fibers.

Lignin content was not the only parameter affected by light in growing *E. globulus* seedlings. A remarkable increase in the S/G ratio was also observed, which corresponded well with the light-induced up-regulation of the S branch-specific genes *EglF5H* and *EglCOMT*. Accordingly, recent microarray analysis comparing 7-days light- and dark-grown *Arabidopsis* seedlings showed significant up-regulation of both *F5H1* and *COMT* by light (Cheng et al. 2012). Moreover, expression analysis of *Arabidopsis* plants grown under different light/dark periods suggested that *F5H* expression might be differentially regulated than other lignin biosynthetic genes (Ruegger et al. 1999). Indeed, while all G branch lignin genes in *Arabidopsis* present at least one AC element (i.e. *cis*-elements that determine xylem-specific expression), these regulatory sequences were not found in the promoter region of S branch-specific *F5H* and *COMT* (Raes et al. 2003). The differential regulation of *F5H* gene expression is further supported by the recent discovery that *F5H* is the only lignin biosynthetic gene directly regulated by the secondary cell wall master switch *SND1* in *Medicago truncatula* (Zhao et al. 2010). Additionally, it has been shown in *Arabidopsis* that secondary cell wall master switches from the NAC family exhibit fiber- (i.e. *SND1* and *NST1*) or vessel-specific expression (i.e. *VND6/7*), xylem cell types that are enriched in S- and G-lignin respectively (Kubo et al. 2005, Zhong et al. 2006, Mitsuda et al. 2007). However, the poplar orthologs *PtrWNDs* are expressed in both vessels and fibers, which suggest that a more complex redundancy among regulatory genes had evolved to ensure wood formation in trees, when

compared to herbaceous plants with limited secondary cell wall deposition (Zhong et al. 2010).

In addition to their role as carbon/energy sources and structural components, sugars act as physiological signaling molecules whose signal transduction pathways regulate a variety of genes involved in distinct biological processes in the plant (Solfanelli et al. 2006). For example, the activation of anthocyanin biosynthesis, a group of compounds belonging to the phenylpropanoid pathway, was often observed in plants grown on medium containing sugars (Solfanelli et al. 2006, Ferri et al. 2011). Increased sucrose concentrations in the medium of *Vitis vinifera* cell suspensions led to an accumulation of specific polyphenols such as anthocyanins, catechins and stilbenes (Ferri et al. 2011). This effect might be simply due to the higher availability of carbon skeletons for such metabolic processes. However, sucrose was able to specifically modulate some key biosynthetic enzymes for each branch pathway, which suggest an additional role for this sugar as a signaling molecule. Similarly, exogenously supplied sucrose elicited the accumulation of lignin in dark-grown *Arabidopsis* seedlings (Rogers et al. 2005). The possibility of an osmotic effect was ruled out by the fact that only metabolizable sugars induced lignin deposition. Moreover, comparison of transcript abundance between wild-type plants grown in the dark and in the presence of sucrose with plants grown without sucrose showed that sucrose may indeed function as a signal to induce the expression of lignin biosynthetic genes. In our study, sucrose supply resulted in a slight increase in soluble lignin contents of *E. globulus* seedlings only in the presence of light, which suggest either that this signaling pathway works differently in *Eucalyptus* than in *Arabidopsis* or that the amount of sucrose in the medium was not enough to trigger more pronounced responses.

Qualitative changes in lignin deposition induced by light and sucrose in *E. globulus* seedlings were also evaluated by the analysis of the soluble lignin oligomers. In-depth analyses of lignin structure in *E. globulus* have been previously performed by advanced techniques such as two-dimensional nuclear magnetic resonance (2D-NMR) and analytical pyrolysis-gas chromatography-mass spectrometry (Rencoret et al. 2011). The data revealed the main changes in lignin monomeric composition, substructures and linkage types, as well as hemicellulosic polysaccharides, during plant growth. However, to our knowledge, a lignomics approach as described by Morreel et al. (2010) has never been reported for *Eucalyptus*. In agreement with the higher degree of vascular tissue differentiation and consequent higher amounts of lignin, seedlings from the treatments L and LS showed higher levels of individual lignin oligomers. Moreover, their lignin oligomer profiles were enriched with S moiety-containing olignolignols, in line with the up-regulation of S-branch specific genes and increased S/G ratio. In summary, it seems that light responses, and possibly sucrose to a lesser extent, channel the pathway towards the production of S units, most probably by inducing the differentiation of a robust vascular tissue with developed xylary fibers and by ultimately upregulating S-branch specific lignin genes. Expression of *EglF5H* seems to be closely related with such alterations in lignin composition.

The established *in vitro* system was further used in an attempt to identify class III peroxidases potentially involved in the constitutive lignification process in *E. globulus*. These enzymes belong to large multigene families and are believed to be involved in a wide range of physiological processes in plants (Cosio and Dunand 2010). However, to precisely determine a specific function to an individual peroxidase gene is a major bottleneck due to gene redundancy within the family, which results in mutants with no visible phenotype, and to the low substrate specificity *in vitro*, hampering the estimation of real *in planta* substrates (Cosio

and Dunand 2009). Transcriptome analysis may therefore be a powerful tool for the screening of peroxidases functionally related to a specific physiological process. However, the large number of genes encoding class III peroxidases would make the screen laborious and expensive. Therefore, a proteomic approach based on the determination of isoperoxidase profiles by semi-denaturing mono-dimensional electrophoresis followed by peptide sequencing was employed. Two potential candidates were identified and phylogenetic analysis showed that they might be the *Eucalyptus* orthologs of *Arabidopsis* AtPrx47, AtPrx64 and AtPrx66. These peroxidases have been associated with lignin deposition in different cell types, with AtPrx64 playing a role in lignification of sclerenchyma while AtPrx47 and AtPrx66 are likely associated with lignification of vessels (Tokunaga et al. 2009).

Determination of timing and tissue specificity of gene expression is important during functional characterization of individual peroxidases (Cosio and Dunand 2009). Hence, a peroxidase gene whose expression pattern is correlated with the spatio-temporal deposition of lignin is likely to be involved in lignin polymerization. It was therefore demonstrated that both peroxidases identified with the proteomic approach showed increased expression in *E. globulus* seedlings with higher lignin contents (i.e. from the LS treatment). Moreover, structural motifs of typical S peroxidases were found in the primary protein sequences of these peroxidases, suggesting that they are able to oxidize syringyl alcohol, an activity exclusively observed in lignifying tissues (Harkin and Obst 1973). Interestingly, the induction of S peroxidases is also in agreement with the increased S/G ratio and the preferential accumulation of S moiety-containing oligno lignols in the LS treatment. Altogether, the phylogenetic, gene expression and primary sequence analyses suggest that these peroxidases might play a role in the lignification process in *E. globulus*.

Although lignin is deposited in secondary cell walls of all vascular plants, the mechanisms underlying monolignol biosynthesis, transport and polymerization are not necessarily conserved (Li et al. 2008). While Arabidopsis showed only limited secondary growth, wood lignification requires an enormous amount of resources, which highlights the differential regulation of lignin deposition between herbaceous and woody species. Despite of their rapid growth rate, the development of alternative, fast and reproducible systems to study the lignification process in woody species can help to enhance our knowledge of lignin biosynthesis in these species. Here, our *in vitro* system allowed in *Eucalyptus* the study of several aspects related with lignin biosynthesis, including biochemical and gene expression analysis. The system was also suitable for the identification and initial characterization of candidate class III peroxidases potentially involved in lignin polymerization in *E. globulus*. This system can be further used for the characterization of other lignin-related genes such as laccases, as well as to evaluate other compounds or conditions that might influence lignin deposition in *Eucalyptus*

Author contributions

P.A., I.C. and P.M. designed the research; P.A., I.C., J.L.M., I.F.F, E.K., A.C.H.F.S., and A.F.P.L. performed the experiments and analyses; P.A., I.C. and P.M. wrote the manuscript.

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Table S1. List of primers used for qRT-PCR.

Lignin gene	Forward 5´-3´	Reverse 5´-3´	Size (bp)
EglPAL1	GAACGCGAGAGCTCAGTTC	CATGGCGGAGAACACCAG	172
EglPAL2	GGCGTTCGAGTCCAGTACG	GGGATATCACGCCCTCG	136
EglPAL3	TAATTGCCGGAGTGCCTG	CTCCGGTTAAGAGCTCGGTT	122
EglC4H	CAGGCTGCAGATGATGATGTA	TCGCCGTAGTTGTAGTCGAAG	150
Egl4CL1	GCTGATTGCACACCCAAGTAT	TTGCTTGATTTCGTCCTCAGT	130
Egl4CL2	TAGTAAGCCACCCATCCATTG	ACTGCTTCTTCCGTCAGTTCA	121
EglHCT	GTTCGCATGGACAACGACTAT	CCACCAGGCCCCATAAAT	188
EglC3H	AGGACTCCTCTGGGACATGAT	CACTGGAGGTAAGGGAGGTTC	186
EglCCoAOMT1	ATCGCGGATGTAAGTATATGCA	CTTGTTGGCTCTACATCAGTCAA	142
EglCCoAOMT2	CAATAGGGAGAACTTCGAGATCG	GCATCCACGAATATGAAGTCG	156
EglCCoAOMT3	TTACCCATTATCAAGAAAGCCG	ACAACCTCTCGTGATAATTCCAA	169
EglCCR1	GTGAAGGAGCAGACGGATCT	AGGACCTTGTCGGAGTCGT	197
EglCCR1_1	TGCCAAGTTCTTCCCTGAGT	TCGTACAGGCACTGCTTCAC	141
EglF5H	GAAGCGAAGGTGAACGAGTC	CGAGTTCTTGCTGGACCTTC	184
EglCOMT	GTTCGAGTATCATGGCACCG	CGTGCTTGACACCAGGAAG	257
EglCAD1	AGTACAGAGTTGGTGACCGAGTG	CTGGAATTTTCACCACAAACC	201
EglCAD2	TACGATCTCAGGAGCACGG	GGTCCCCAACTCTCAGACC	190
EglIDH	AGTTTGAGGCTGCTGGAATC	CTTGCATGCCCACACATAAC	100
EglPrx121	CCGAACTCGTCCCTAAAGGT	CCTAGAGACGGCCTCAAACA	93
EglPrx122	GCCTCTCTCTGGCTACATGG	CCATTGCAGAAACCGATACA	83

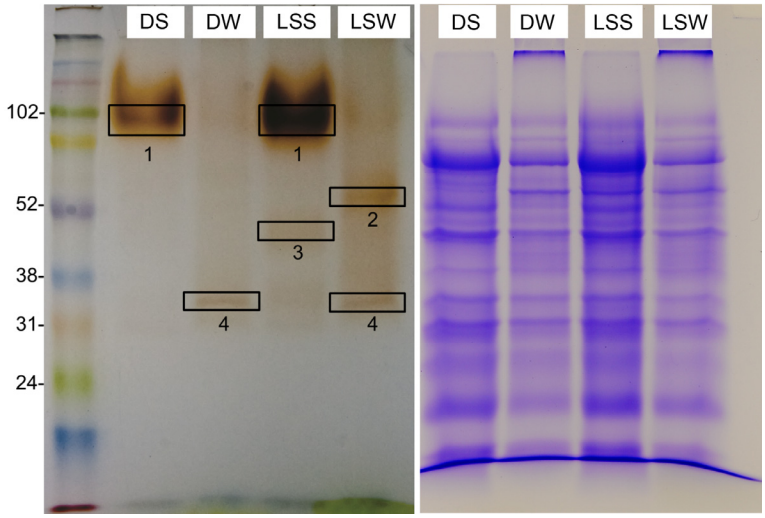

Figure S1. Activity staining after semi-denaturing SDS-PAGE (shown in Figure 5) and denaturing SDS-PAGE of the same hypocotyl protein fraction.

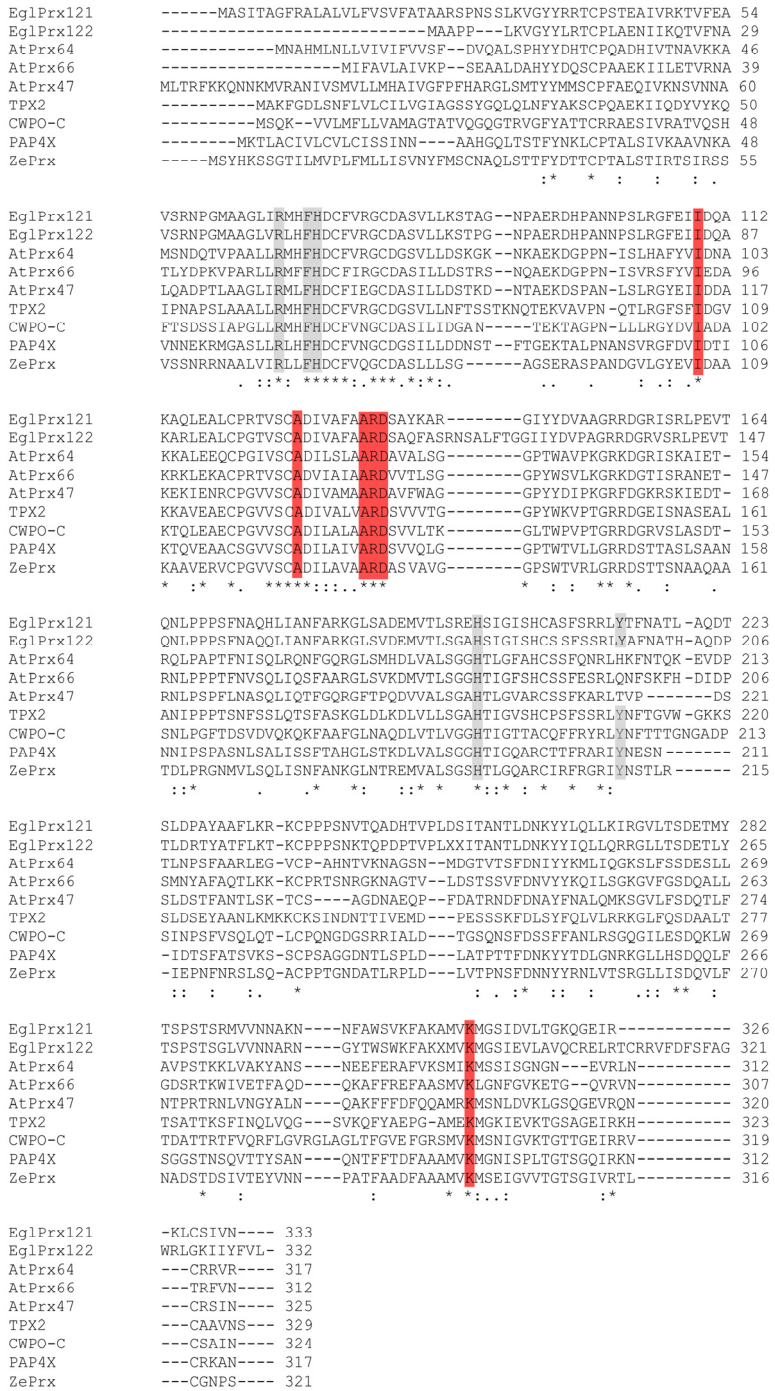

Figure S2. Multiple amino acid alignment of EglPrx121 and EglPrx122, they closest homologs of *Arabidopsis thaliana*: AtPrx47, AtPrx64 and AtPrx66 and typical S peroxidases: PAP4X (*Picea abies* - CAH10839), ZePrx (*Zinnia elegans* - CAI54302), TPX2 (*Solanum lycopersicum* - AAA65636) and CWPO-C (*Populus alba* - BAE16616). Structural motifs characteristics of S peroxidases are shaded in red, while the conserved residues involved in the catalytic mechanism are highlighted in gray (Gómez Ros et al. 2007, Martínez-Cortés et al. 2012). "*" indicates identical amino acid residues in all sequences and "." indicates when at least half of the sequences share the same amino acid in a specific position.

Capítulo III

"Caracterização da *EglPRX17* relacionada com lignificação"

Resultados parciais obtidos a partir de experimentos realizados no Capítulo II

Introdução

As peroxidases de classe III são enzimas amplamente relacionadas com processos de polimerização de lignina, oxidando os monolignóis com o uso de H_2O_2 (Passardi *et al.*, 2004; Ros-Barceló, 2005). Ainda assim possuem atividade promíscua devido à inespecificidade de substrato oxidáveis, além de outras funções em diversas plantas (Boerjan *et al.*, 2003; Passardi *et al.*, 2005; Cosio & Dunand, 2009; Marjamaa *et al.*, 2009). A caracterização de peroxidases no processo de lignificação é amplamente estudada em diversas plantas: *Arabidopsis thaliana* (Valerio *et al.*, 2004; Cosio & Dunand, 2010), *Medicago sativa* (Watson *et al.*, 2004); árvores: *Picea abies* (Marjamaa *et al.*, 2006), *Eucalyptus viminalis* (Aoyama *et al.*, 2001); além de estudo utilizando cultura de tecidos (Karkonen & Koutaniemi, 2010) e de estrutura/composição de lignina em *Eucalyptus globulus* (Rencoret *et al.*, 2011). Foram identificados 73 genes em *Arabidopsis thaliana*, 138 em *Oryza sativa* e 105 em *Populus trichocarpa* (Tognolli *et al.*, 2002; Bakalovic *et al.*, 2006; Passardi *et al.*, 2007).

Entretanto, ensaios simples permitem a verificação da atividade dessas enzimas, mas relacioná-las individualmente como uma função específica é difícil. Isso se deve a: (i) mutantes (superexpressão ou silenciamento) não gerarem fenótipos facilmente detectável devido à redundância genética e (ii) por serem enzimas sem substrato específico *in vitro*, o que dificulta a identificação dos verdadeiros substratos *in planta* (Cosio & Dunand, 2010). Dessa forma, os estudos conduzidos envolveram diferentes experimentos como PCR em tempo real e microarranjos a fim de selecionar e identificar genes que fossem específicos para a função de radicalização e polimerização dos monolignóis (Cosio & Dunand, 2009). Portanto, abordagens que envolvam diversas técnicas como transcriptômica e proteômica são interessantes na caracterização de genes dessa família multigênica redundante.

Resultados Parciais e Discussão

Bancos de EST de *Eucalyptus* foram analisados para a obtenção de sequências de peroxidases de classe III e o seu perfil de expressão *in silico* em diferentes tecidos (caule, raiz, floema, folha e etc). Foram inicialmente selecionadas peroxidases de classe III que fossem restritas às bibliotecas feitas a partir de plântula, casca e caule.. Essa abordagem foi utilizada para reduzir o número inicial de transcritos a serem analisados. Dentre as sequências obtidas a *EglPrx17* foi exclusivamente encontrada em plântula e caule (Figura 1).

Tecido	plântula	casca	caule	floema	folha	flores	raízes	calos
EglPrx 17								

Figura 1: Análise de transcritos presente no banco EST para *Eucalyptus*. O northern eletrônico mostrou que a *EglPrx17* é preferencialmente expressa em plântula e caule (cores mais intensas – vermelho – indicam maior quantidade relativa do transcrito).

Ensaios de PCR em tempo real dentro do sistema descrito no capítulo II mostraram que *EglPrx17* era induzida no escuro em comparação com as demais enzimas no hipocótilo. Dessa forma, para microdisecção do xilema foram utilizadas plantas cultivadas em luz, com células do xilema diferencialmente diferenciadas quando comparadas com plantas crescidas no escuro. Usando essa técnica, retiramos somente o xilema diferenciado (Figura 2) do qual extraímos o RNA para análises de PCR em tempo real (Figuras 3). A fim de verificar a expressão de demais peroxidases de classe III obtidas do banco de EST de Eucalyptus, o mesmo cDNA obtido do material de microdissecção foi utilizado. Entretanto, somente a *EglPrx17* obteve expressão relativa relevante.

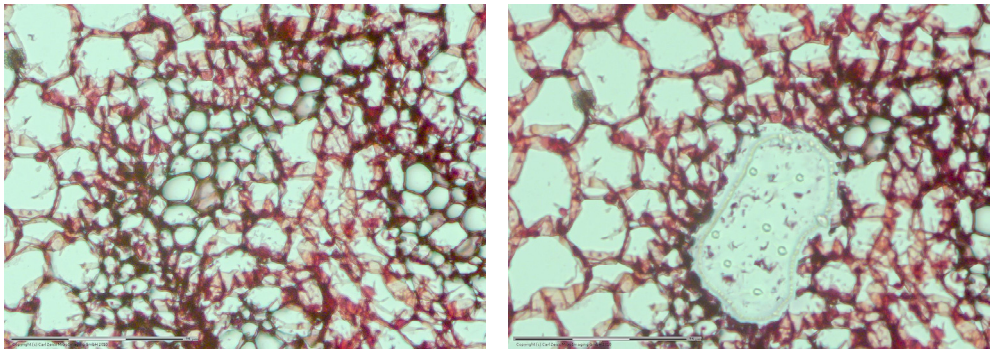

Figura 2: Secção transversal do caule de *Eucalyptus globulus*. Por meio da microdisecção, foi selecionado somente o xilema. A partir desse material foi realizada PRC em tempo real para verificar a expressão de genes codificadores de peroxidases de classe III previamente selecionadas pelo banco de EST de *Eucalyptus*.

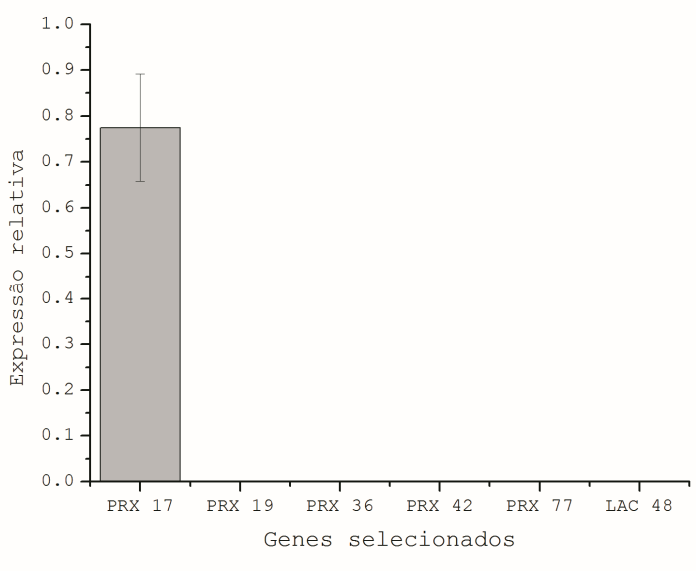

Figura 3: Expressão relativa de genes codificadores de peroxidases de classe III, previamente selecionados em xilema de *E. globulus* por meio de microdissecção (Figura 2). Somente transcritos de *EglPrx17* foram detectados. Foi utilizado o *ICDH* como gene de referência (n=3).

Complementarmente, sondas específicas foram sintetizadas para o gene *EglPrx17*. Obteve-se hibridização em células do xilema, novamente confirmando os resultados obtidos com a microdisecção (Figura 4). Devido ao padrão interessante e a intensidade de expressão desse gene com os demais analisados, o uso de transgenia seria interessante para verificar a função fisiológica e, por fim, afirmar sobre a sua relação com a lignificação.

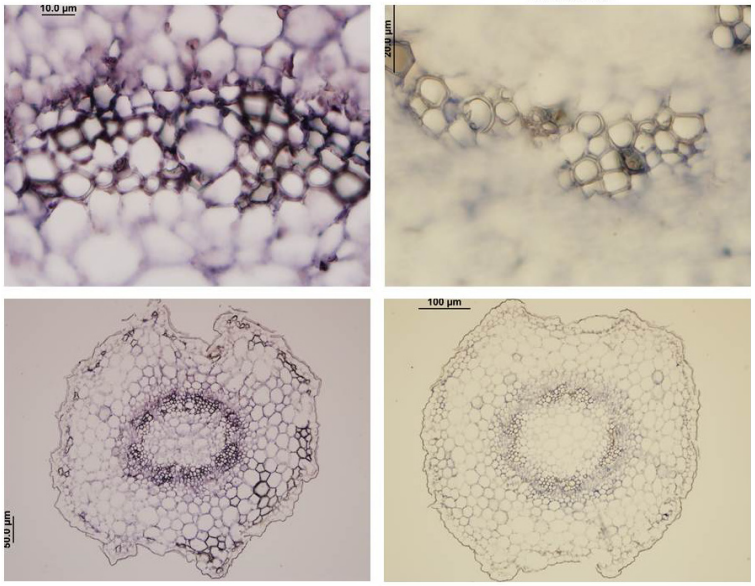

Figura 4: Hibridização *in situ* de *EglPrx17* no caule de *Eucalyptus globulus*. Transcritos foram detectados através de sonda específica no xilema na coluna da esquerda. O controle negativo, através de uma sonda sense, pode ser visualizado na coluna da direita.

Devido à dificuldade em se obter plantas de *Eucalyptus* transformadas geneticamente, principalmente em função de problemas de regeneração de plantas, a caracterização do gene selecionado para validar a função foi realizada com a utilização de *Arabidopsis thaliana*. As construções utilizadas foram: (i) promotor constitutivo com o gene *EglPrx17* (35S::EglPrx17) e (ii) promotor constitutivo com o gene *EglPrx17* sem "*stop codon*" com GFP, para verificar a localização celular (35S::EglPrx17::GFP).

Como perspectivas, têm-se a caracterização funcional dessa enzima e a associação dela com o aumento na deposição de lignina, assim como modificação na proporção de monômeros incorporados (S/G). Ensaios para transformação de *Eucalyptus* são conduzidos a fim de se estabelecer um protocolo no Departamento de Biologia Vegetal, e futuramente, utilizar esse gene/construção para caracterização propriamente na planta alvo.

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Capítulo IV

"Mass spectrometry imaging: an expeditious and powerful technique to study *lignomics* in *Eucalyptus*"

Artigo aceito para publicação

Referência: Araújo, P., Ferreira, M.S., de Oliveira, D.N., Pereira, L., Sawaya, A.C.H.F., Catharino, R.R. & Mazzafera, P., 2014. Mass spectrometry imaging: an expeditious and powerful technique to study *lignomics* in *Eucalyptus*. Analytical Chemistry XX:XX-XX

Abstract

Plant biomass has been suggested as an alternative to produce bioethanol. In part the recalcitrance to convert cellulose in simpler carbohydrates to be used in the fermentation process is due to lignin, but the standard methods used to analyze lignin composition frequently use toxic solvents, are laborious and time consuming. MS imaging was used to study lignin in *Eucalyptus*, since this genus is the main source of cellulose in the world. Hand-cut sections of stems of two *Eucalyptus* species were covered with silica and directly analysed by MALDI-imaging MS. Information available in the literature about soluble lignin sub units and structures were used to trace their distribution in the sections and using a software image a relative quantification could be made. Matrices routinely used in MALDI-imaging analysis are not satisfactory to analyze plant material and were efficiently substituted by silica TLC grade. Twenty two compounds were detected and relatively quantified. It was also possible to establish a proportion between syringyl and guaiacyl monolignols, characteristic for each species. Because the simple way that samples are prepared the MALDI-imaging approach presented here can replace, in routine analysis, complex and laborious MS methods in the study of lignin composition.

Keywords: *Eucalyptus globulus*, *Eucalyptus grandis*, lignin, MALDI, wood

Introduction

Lignin is the second major component of plants, only behind cellulose in plant mass. Lignin is studied due to the recalcitrance it confers to the cell wall, with a consequent economic impact on pulp and cellulose industry as well as second-generation biofuel production from biomass [1]. The demand for safe and clean energy has driven the research on

biomass as a renewable source [2] at the same time that cellulose industry uses massive amounts of chemicals to remove lignin from the wood pulp during cellulose production [3].

Lignin is basically composed of three phenylpropanoid compounds known as monolignols, which once incorporated in the lignin backbone are conventionally designated as S (syringyl), G (guaiacyl) and H (hydroxyphenyl) units [4] . In angiosperms, lignin is mainly composed of S and G units. S-rich lignin is less cross-linked than G-lignin because it is methoxylated at C5 of the aromatic ring, preventing the establishment of the resistant C-C bond at this position. Consequently, degradability or digestibility tends to be higher in S-rich lignin [5] . Significant efforts have been made to decrease the amount of lignin in plants and also to design a "new lignin" by introducing new/artificial monolignol molecules or changing the S/G ratio [6]. The study of the lignin composition coined a new term in plant research known as *lignomics* [7, 8].

All methods currently used to quantify lignin have pros and cons and none of them are accurate [9] and expeditious enough to be used with large numbers of samples. The S/G ratio, which is used in both science and industry as an indicator of potential cellulose extractability, is usually obtained by GC-MS analysis, after nitrobenzene oxidation of the biomass, which is a laborious process employing toxic chemicals [10]. As an alternative method to study the lignin composition, soluble oligomers still not linked to the rigid lignin structure can be analyzed by LC-MS (liquid chromatography mass spectrometry) as long as a reference library is provided [7, 8].

Imaging mass spectrometry has been applied the study of lignin: TOF-SIMS (time-of-flight secondary ion mass spectrometry) was used for mapping heartwood of *Cryptomeria* and *Chamaecyparis* [11, 12] and recently for cell wall metabolites of *Acer micranthum* [13]. However, mass spectrometry fragmentation (MS^n) was not performed in these studies, which

is decisive for the unambiguous identification of molecules. Furthermore, the presence of contaminants from sample preparation and interference of non-lignin cell components are still obstacles for this technique. Herein, we used MALDI-imaging to identify structures of soluble lignin oligomers in stem sections of *Eucalyptus grandis* and *Eucalyptus globulus*. Globally, *Eucalyptus* is the main source of cellulose for paper production [14, 15].

Material and Methods

Plant material and sample preparation

Five month old seedlings of *E. globulus* and *E. grandis* were used in the study. They were produced from seeds in greenhouse and grown in 1 L pots containing a mixture of soil and sand. The seedlings were taken to the laboratory and a section of the stem was manually (~1.5 mm thick) obtained from the first internode using a sharp razor blade and fixed on glass slides with double-faced tape. The cuts had on average 0.3 to 0.5 cm diameter. A layer of silica TLC powder (Silica gel 60 0.2 mm, Merck, Darmstadt, Germany) was uniformly applied on the exposed surface. All the experiments were performed using this strategy, avoiding the usual interference in the range below *m/z* 600 from routinely used matrix materials [16]. Three replicates were made for each *Eucalyptus* species.

MS/MS and Image acquisition

Images and mass spectra were acquired in a MALDI-LTQ-XL (matrix-assisted laser desorption ionization-linear trap quadrupole-XL) linked with imaging feature (Thermo Scientific, California, USA). The instrument was equipped with an ultraviolet laser (Nd:YAG, 355 nm) and a quadrupole-ion-trap analyzing system. All data were acquired in the negative ion mode. For image acquisition, a 50 μm raster width was selected. Mass spectrometry

fragmentation data (MS/MS) were acquired by collision-induced dissociation (CID) with energy set to 40 eV. Helium was used as the collision gas. All analyses were performed in triplicate and all imaging data were then processed in triplicate using ImageQuest software v.1.0.1 (Thermo Scientific, California, USA), by setting the equipment to acquire MS/MS fragmentation of specific ions. In general each hand-cut took 8 minutes to be analysed.

Compounds relative quantification

The image obtained for each compound identified in the stem cuts was analysed using the software ImageJ (National Institutes of Health, USA – Open Source) on gray scale images. The software assigned a value for each image (corresponding to a single compound) in each stem cut, which resulted from the sum of the intensity of each pixel. This was then divided by the sum of the all values (corresponding to all compounds) obtained in the same cut, providing a relative quantitation of each compound in each cut. Other stem cuts, not used for MALDI-imaging, were stained with phloroglucinol [17]. They were immersed in phloroglucinol solution (2.0 g phloroglucinol in aqueous solution containing 20% ethanol and 20% HCl) for 10 minutes and immediately photographed using an Olympus BX 51 microscope with an Olympus DP 71 camera.

Results

It was possible to identify by MALDI-imaging 22 out of the 24 compounds described by Kiyota et al. [7] which represent the main soluble lignin monomers and oligomers, as well as to determine their localization in the sections (Fig. 1, Table 1). Coniferyl and sinapyl aldehydes were not detected. It was observed 8-O-4 (or β-O-4) linkages were predominant in lignin structure (Table 1). The presence of a higher amount of S (*m/z* 209) units over G (*m/z*

179) and H (*m/z* 149) is easily observed through the visual inspection of the images of the *Eucalyptus* stem sections (Fig. 1). H unit image was less intense than S and G, although curiously the highest relative intensity was observed for the trimer H(8-5)H(8-O-4)G (Table 1). The calculated S/G ratio obtained with the normalized data was higher ($p<0.05$) in *E. globulus* (1.37 ± 0.05) than *E. grandis* (1.16 ± 0.13).

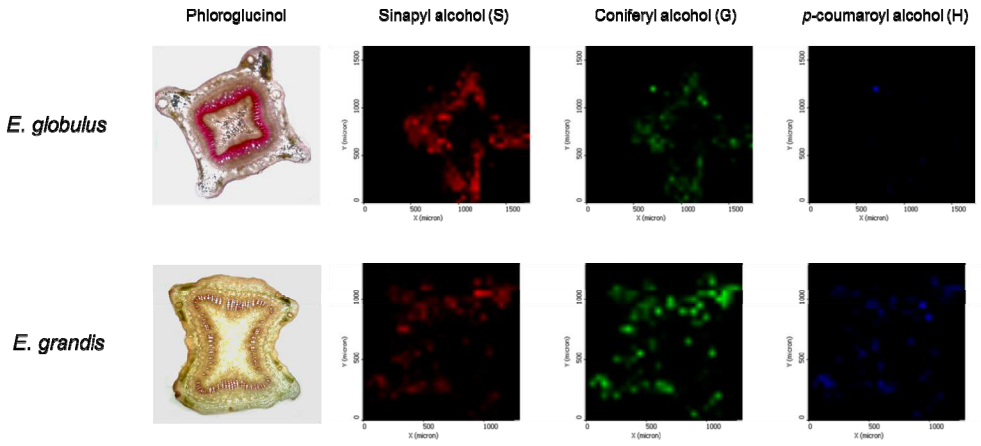

Figure 1: Phloroglucinol staining and mapping of sinapyl (*m/z* 209), coniferyl (*m/z* 179) and *p*-coumaroyl (*m/z* 149) alcohols in freshly hand-cut sections of *Eucalyptus* stems. Different colors were selected for each unit for illustrative reasons. For relative quantification the figures were converted to gray images.

Table 1: Relative pixel quantification of oligomers in *E. globulus* and *E. grandis* using the ImageJ software. The MS/MS data for each ion is available on Kiyota et al. [7]. The structures are listed in order of the *m/z* of their deprotonated molecules (negative ion mode). Data are means of three replicates ± standard deviation.

m/z	**Structure**	***E. globulus***			***E. grandis***		
149	*p*-coumaroyl alcohol (H)	0.024	±	0,01	0,010	±	0.00
179	coniferyl alcohol (G)	0.068	±	0,01	0,079	±	0.01
209	sinapyl alcohol (S)	0.088	±	0,02	0,064	±	0.01
297	H(8-5)H H(8-8)H	0.066	±	0,02	0,029	±	0.00
327	G(8-5)H	0.056	±	0,02	0,042	±	0.00
357	G(8-5)G G(8-8)G	0.048	±	0,01	0,075	±	0.01
375	G(8-O-4)G	0.042	±	0,01	0,050	±	0.01
387	S(8-5)G S(8-8)G	0.065	±	0.01	0.098	±	0.01
405	S(8-O-4)G	0.048	±	0.02	0.046	±	0.00
417	S(8-8)S	0.064	±	0.00	0.100	±	0.01
435	S(8-O-4)G	0.060	±	0.00	0.037	±	0.01
493	H(8-5)H(8-O-4)G	0.090	±	0.02	0.106	±	0.01
535	G(8-5)G(8-5)G	0.034	±	0.00	0.054	±	0.00
553	G(8-O-4)G(8-5)G	0.046	±	0.01	0.060	±	0.00
571	G(8-O-4)G(8-O-4)G	0.025	±	0.01	0.032	±	0.01
583	G(8-0-4)S(8-5)G S(8-O-4)G(8-5)G	0.044	±	0.03	0.054	±	0.01
601	G(8-O-4)G(8-O-4)S	0.028	±	0.00	0.033	±	0.00
613	S(8-O-4)S(8-5)G G(8-O-4)S(8-8)S	0.025	±	0.00	0.035	±	0.,00
631	S(8-O-4)G(8-O-4)S	0.025	±	0.00	0.033	±	0.01
643	S(8-O-4)S(8-8)S	0.015	±	0.00	0.030	±	0.00
661	S(8-O-4)S(8-O-4)S	0.026	±	0.01	0.042	±	0.00
851	S(8-O-4)S(8-8)S(8-O-4)S	0.014	±	0.02	0.045	±	0.00

Discussion

Sinapinic acid, alpha-CHCA and 2,5-DHB, three matrices normally used for MALDI-imaging were initially tested for lignin oligomers detection on the *Eucalyptus* stem hand-cuts, but ionization efficiency was very low. As an alternative, and focusing on the purpose to create an expeditious method, silica TLC grade was used, based on the fact that

desorption/ionization on silicon (DIOS) has been proposed to map small molecules on biological tissues [18].

Aiming to have the simplest sample preparation our choice to use hand-cut sections was based on technical and practical reasons. Here we used ~1.5 mm thick tissue cuts, a dimension that is far from the thickness used for animal samples, which varies from 10 to 100 μm [19]. There are two ways to obtain plant cuts with this thickness: a) alcoholic dehydration and further resin imbibition and microtome sectioning, b) microtome sectioning at cryogenic temperatures. In the first case, lignin oligomers are soluble and can be washed out during sample preparation. In the second case, the frozen cut may thaw during handling, leading to sample structure degradation and causing distortion in the subsequent mass spectra [20]. To overcome the thickness problem and considering that only the surface of the cut is analyzed, we chose to take samples from the first internode from the top of the stem, which is soft enough to allow uniform and regular cuts. Additionally we used ImageJ software to normalize the data of each compound in relation to the data of all the compounds detected in the same cut. Normalization allowed obtaining small standard deviation values of the measurements (Table 1).

This simple and expeditious experiment shows that MALDI-imaging is an efficient tool which may be applied to studies of the "normal and conventional" lignin deposition in fibers and xylem tissues as well as studies where lignin composition is altered by addition of new phenylpropanoids, as observed is plants exposed to biotic and abiotic stress [21], particularly diseases [22]. However, this is possible as long as a library is available with MS/MS data of lignin oligomers. Additionally, quantification of each compound may be also possible by setting a calibration curve with available pure standards. We suggest that due to

the simplicity of the method, the approach used by Kiyota et al. [7] may be useful in this regard.

Rencoret et al. [23] used two-dimensional nuclear magnetic resonance (2D-NMR) to study lignin in *E. globulus* and showed that S was predominant over G and H in the wood of 1 month-, 18 month- and 9 year-old plants. They also observed that 8-O-4 (or β-O-4) linkages were predominant in lignin structure, as also observed herein (Table 1). Rencoret et al. [23] suggested that H and G units in the *E. globulus* wood are deposited in the lignin structure at the earlier stages of plant growth, and higher amounts of S are deposited later in the lignification process. [13].

The presence of a higher amount of S (*m/z* 209) units over G (*m/z* 179) and H (*m/z* 149) is easily observed through the visual inspection of the images of the *Eucalyptus* stem sections (Fig. 1). In agreement with biochemical data, H unit image was less intense than S and G, although curiously the highest relative intensity was observed for the trimer H(8-5)H(8-O-4)G (Table 1). The calculated S/G ratio obtained with the normalized data was higher ($p<0.05$) in *E. globulus* (1.37 ± 0.05) than *E. grandis* (1.16 ± 0.13), which is in agreement with previous reports indicating that *E. globulus* has higher S/G ratio than other *Eucalyptus* species [24, 25]. A comparison of the Py-GC/MS and nitrobenzene oxidation methods to determine S/G ratios in 18 *Eucalyptus* spp. hybrids indicated overestimation when the second method was used [26]. In general, when *E. globulus* was one of the parental of the hybrids the S/G ratio was higher by both methods. Despite the divergence among the results of these techniques, it was consistent that the S/G ratio was always higher in *E. globulus* than in other *Eucalyptus*. Since the amount of each soluble monolignol is expected to have a correlation with its total content in the polymerized lignin our data suggest that it is possible to estimate the S/G ratio using

MALDI-imaging in fresh hand-cut samples by determining non-polymerized soluble monolignols.

As conclusions we described herein a simple method to study lignomics in plants using MALDI-imaging system. Hand-made sections of fresh material are dusted with silica powder and are ready to be inserted in the equipment. If standards are not available, ion fragmentation data from the literature may be used. The method can be potentially applied for any plant material. This approach can replace the time-consuming and complex available techniques, avoiding toxic solvents used for sample preparation. Because of its simplicity and short analysis time, large numbers of samples may easily be processed. Additionally, silica can ionize soluble lignin structures and replaces more expensive matrixes, without causing chemical noise in the spectrum. We anticipate that this technique will be extensively applied to study several plant metabolites and the development of equipment with finer laser resolution will allow studies on a cellular level. As several aspects of lignin deposition in the cell wall remain unclear, MS-imaging allied to single single-cell mass spectrometry technology [27] will allow obtaining a precise *in vivo* picture of lignomic during plant tissue development, as recently obtained employing fluorogenic monolignols [28], but probably in a easiest manner.

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Conclusões gerais

Esta tese é composta por quatro capítulos. No primeiro, foram feitas análises para a identificação de genes de referência estáveis em plantas submetidas a diferentes estresses abióticos, em duas espécies de eucalipto. Tais dados referentes à expressão gênica com PCR em tempo real serviram de base para os estudos apresentados nos Capítulos II e III.

No método desenvolvido *in vitro* e apresentado no Capítulo 2, utilizaram-se diferentes abordagens na avaliação de plântulas cultivadas em escuro e luz. Novamente algumas peroxidases classe III foram induzidas na luz, o que é corroborado com a literatura e permitiu selecionar de um banco inicial de 83 peroxidases classe III, aquelas que poderiam estar estreitamente relacionadas com lignificação. Dessa forma, estudos posteriores podem ser utilizados por essa metodologia, sendo sua grande vantagem a praticidade e o tempo gasto na implantação do cultivo *in vitro* e as respostas satisfatórias obtidas. Duas peroxidases de classe III: *EglPrx121* e *EglPrx122*, podem ser alvo de estudos mais aprofundados como transgenia na elucidação das mesmas no processo de polimerização. A *EglPrx17* obteve um padrão de expressão diferente por ser induzida basicamente no escuro, entretanto resultados de hibridização *in situ* identificaram os transcritos na parede celular. Complementarmente, linhagens homozigotas de *A. thaliana* superexpressando *EglPrx17* sob o promotor constitutivo CaMV-35S estão sendo cultivadas e serão avaliadas posteriormente.

Por fim, o uso espectrometria de massas acoplada a um sistema de imagens abre novas possibilidades de análise de *Eucalyptus* na caracterização da qualidade de madeira. Os resultados obtidos para *E. grandis* e *E. globulus* estão de acordo com os resultados encontrados na literatura e acabam por validar esse nova metodologia.

Todos esses dados gerados são de grande valia, pois o *Eucalyptus* é uma planta lenhosa e de ciclo longo, e poucos estudos têm sido desenvolvidos apesar do genoma de *E. grandis* estar

disponível em bancos públicos de EST. Os genes identificados podem servir de alvo para a melhoria do cultivo, pois podem alterar a quantidade de lignina e sua relação S/G, melhorando a extração de celulose e a redução de compostos tóxicos emitidos no ambiente.

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