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MÁRCIA RODRIGUES DE ALMEIDA

Bases moleculares da recalcitrância ao enraizamento adventício em *Eucalyptus globulus* Labill

Porto Alegre

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Tese apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular como requisito parcial para obtenção do título de Doutor em Ciências.

Orientador: Prof. Dr. Arthur Germano Fett-Neto

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"Somewhere, something incredible is waiting to be known."

— Carl Sagan

Aos meus pais,
por acreditarem em mim,
mais do que eu mesma...

Resumo

O eucalipto é uma das espécies arbóreas mais plantadas no mundo atualmente, principalmente devido ao seu uso como matéria prima para as indústrias de celulose, papel e madeireira. *Eucalyptus globulus* e seus híbridos possuem baixos teores de lignina e despertam grande interesse da indústria, já que essa característica facilita e diminui o custo do processo de extração da celulose. Entretanto, essa espécie é recalcitrante ao enraizamento adventício, o que dificulta a propagação vegetativa de suas mudas. Com o objetivo de melhor entender os mecanismos moleculares envolvidos na recalcitrância ao enraizamento em *E. globulus*, o presente estudo envolveu análises de parâmetros morfológicos, anatômicos e moleculares durante a rizogênese adventícia nesta espécie. A exposição à auxina exógena reverteu o fenótipo recalcitrante em *E. globulus*, aumentando significativamente a porcentagem de enraizamento. O câmbio vascular foi identificado como uma região de acúmulo de auxina e local de origem das raízes adventícias. Através de análises da expressão gênica em células do câmbio, observou-se que *TOPLESS* e *IAA12*, repressores da sinalização de auxina, e *ARR1*, envolvido na rota de sinalização de citocininas, parecem atuar como reguladores negativos do enraizamento adventício. A alta expressão destes genes em plantas controle foi significativamente diminuída com aplicação de auxina exógena. Comparativamente, em espécie de fácil enraizamento, *E. grandis*, a expressão destes genes se manteve em níveis mais baixos em ambas as condições de tratamento, e a concentração de ácido indol-3-acético endógeno em plantas controle mostrou-se mais elevada. Análises do padrão proteico durante o enraizamento em plantas de *E. globulus* tratadas ou não com auxina exógena identificaram proteínas envolvidas em diversos processos biológicos, principalmente estresse oxidativo e metabolismo energético. Diferenças interessantes foram identificadas ao comparar as diferentes condições e fases do enraizamento. Várias proteínas foram claramente relacionadas com o respectivo fenótipo apresentado pela planta em cada situação, principalmente considerando plantas controle. Os resultados aqui apresentados representam avanços relevantes no conhecimento sobre a rizogênese adventícia em plantas lenhosas, podendo ser utilizados como ferramentas no desenho de estratégias visando melhorar o enraizamento em genótipos recalcitrantes de valor para a indústria.

Palavras chave: auxina, expressão gênica, captura por microdissecção laser, proteômica, espécies lenhosas, enraizamento, *Eucalyptus*

Abstract

Eucalyptus is one of the most planted tree species in the world today, mainly due to its use as raw material for paper, cellulose and wood industries. *Eucalyptus globulus* and its hybrids have low lignin contents and are of great interest to industry, as this feature facilitates and reduces costs of the cellulose extraction process. However, this species is recalcitrant to adventitious rooting, making vegetative propagation by cuttings difficult. Aiming at a better understanding of the molecular mechanisms involved in rooting recalcitrance in *E. globulus*, this study analyzed changes in morphological, anatomical and molecular patterns during adventitious rooting in this species. Exogenous auxin exposure reversed the recalcitrant phenotype in *E. globulus*, significantly increasing rooting percentage. The vascular cambium was identified as a region of auxin accumulation and also the site from where adventitious roots originated. Gene expression analysis in cambium cells indicated that *TOPLESS* and *IAA12*, auxin signaling repressors, and *ARR1*, involved in cytokinin signaling pathway, appear to act as negative regulators of adventitious rooting. The high expression of those genes in control plants was significantly decreased by exogenous auxin treatment. Comparatively, in an easy-to-root species, *E. grandis*, the expression of these genes was significantlylower in both treatment conditions, and the concentration of endogenous indole-3-acetic acid in control plants was higher. Analysis of the protein pattern during rooting in *E. globulus* plants treated or not with exogenous auxin allowed the identification of proteins involved in diverse biological processes, mainly oxidative stress and energy metabolism. Interesting differences were identified when comparing different rooting conditions or phases. Several proteins were clearly associated with the respective plant phenotype in each situation, particularly considering control plants. These results represent relevant advances in the knowledge about adventitious rooting in woody plants and can be used as tools in the design of strategies aiming at improving adventitious rooting in recalcitrant genotypes of industrial value.

Key words: auxin, gene expression, laser capture microdissection, proteomics, woody plants, rooting, *Eucalyptus*

Lista de abreviaturas

ABA	Ácido abscísico
ABRAF	Associação Brasileira dos Produtores de Florestas Plantadas
AR	do inglês Adventitious rooting
AS	Ácido salicílico
Aux/IAA	do inglês *Auxin/Indole-3-Acetic Acid protein*
GH3	do inglês *Gretchen Hagen 3*
Ha	hectare
IAA	do inglês *Indole-3-acetic-acid*
IAM	Indol-3-acetamida
IBA	do inglês *Indole butyric acid*
IPA	do inglês *Indole piruvic acid*
LCM	do inglês *Laser Capture Microdissection*
MS	formulação de sais desenvolvida por Murashige & Skoog (1962)
MS/MS	do inglês *Tandem Mass spectrometry*
PIB	Produto Interno Bruto
RNAi	RNA de interferência
SCR	do inglês *Scarecrow*
SDS-PAGE	do inglês *Sodium Dodecyl Sulfate Poliacrylamide Gel Electrophoresis*
SHR	do inglês *Short Root*
TMK1	do inglês *Transmembrane Kinase 1*
TPL	do inglês *Topless*

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Sumário

1. Introdução Geral

1.1 O Eucalipto

O eucalipto é uma planta arbórea originária da Austrália que possui grande importância econômica, sendo o seu uso industrial uma das atividades que mais se expande no setor florestal mundial. Espécies de eucalipto e seus híbridos estão entre as principais fontes de biomassa lenhosa e possuem alto potencial de utilização como matéria-prima para diversos setores, como o de papel e celulose, madeireiro e energético, sendo uma alternativa às florestas nativas (Grattapaglia & Kirst, 2008). Características como o rápido crescimento aliado à adaptabilidade a diferentes tipos de solo e fibras de alta qualidade contribuem para o crescente interesse nesta árvore (Paiva et al, 2011). Estima-se que as plantações de eucalipto ocupem mais de 20 milhões de hectares (ha) ao redor do mundo (Iglesias-Trabado & Wilstermann, 2008).

No Brasil, as florestas plantadas de eucalipto ocupam 5,1 milhões de ha, o que corresponde a 70,8% da área total de florestas plantadas no país, a qual é também composta por plantações de Pinus (22%) bem como de Teca, Acácia, Araucária, Populus, Seringueira e Paricá as quais, juntas, correspondem a 7,2% (ABRAF, 2013). O Brasil é atualmente o quarto maior produtor mundial de polpa para celulose e o nono maior produtor de papel, sendo que os produtos de base florestal representam 1,2% do Produto Interno Bruto (PIB) brasileiro, movimentando cerca de 56 bilhões de reais em 2012 (ABRAF, 2013). A eucaliptocultura brasileira é baseada principalmente em florestas clonais formadas a partir de materiais elite, que apresentam características interessantes à indústria (Mora & Garcia, 2000).

As espécies *Eucalyptus globulus* e *E. grandis* estão entre as mais amplamente utilizadas para fins industriais em zonas temperadas e áreas tropicais ou subtropicais, respectivamente. Além disso, o interesse nessas espécies tem aumentado devido ao seu potencial para uso em setores emergentes, como o de biocombustíveis e biomateriais (Bozell et al, 2007), além dos usos comuns citados anteriormente. Tanto no Brasil como mundialmente, há um grande interesse em *E. globulus* e seus híbridos devido à alta qualidade da sua madeira e ao baixo teor de lignina característico da espécie. A lignina interfere negativamente no processo de extração de celulose, aumentando significativamente os custos deste processo, além de interferir na produção de bioetanol (Chiang, 2002; Bozell et al, 2007). No entanto, *E. globulus* é recalcitrante ao enraizamento, dificultando a sua propagação. *E.*

grandis, por sua vez, é a espécie mais comumente plantada no Brasil (Canettieri et al, 2007), possuindo madeira macia e menos densa que outras espécies de eucalipto, sendo também utilizada no setor de móveis e construção (Eldridge et al, 1997). Diferentemente de *E. globulus*, é considerada de fácil enraizamento.

1.2 Enraizamento adventício

O enraizamento adventício é baseado nos princípios da regeneração a qual, em plantas, corresponde à formação de uma nova parte aérea, raiz ou embrião a partir de tecidos sem o respectivo meristema pré-existente (De Klerk, 2002). No caso de raízes, estas podem ser formadas a partir de órgãos como caules, hipocótilos ou folhas (Esau, 1977). Este processo pode ser dividido em três fases principais, as quais possuem diferentes requerimentos hormonais: (1) indução, compreendendo os primeiros eventos moleculares e bioquímicos, sem mudança morfológica visível, (2) iniciação, durante a qual ocorrem as primeiras divisões celulares, formação dos meristemas de raiz e estabelecimento dos primórdios radiculares, e (3) expressão, em que ocorrem o crescimento, alongamento e emergência das raízes (Kevers et al, 1997). As duas últimas fases são geralmente agrupadas e referidas como fase de formação (Fogaça & Fett-Neto, 2005).

A propagação vegetativa é o método mais comumente empregado para a multiplicação de materiais-elite e é particularmente importante para a produção de florestas clonais. No caso de eucalipto e outras plantas lenhosas de interesse econômico, as quais são multiplicadas através de estaquia, o enraizamento adventício é uma etapa determinante para que a propagação seja bem sucedida (De Klerk et al, 1999; Fett-Neto et al, 2001, Welander et al, 2014). Além disso, a qualidade do sistema radicular formado também é bastante importante, já que plantas propagadas por estacas podem ser mais sujeitas ao tombamento, uma vez que raízes adventícias não penetram tão profundamente no solo como as raízes pivotantes (Assis et al, 2004).

Raízes adventícias compartilham algumas similaridades com raízes laterais, porém descobertas recentes tem elucidado importantes diferenças entre estes tipos radiculares, bem como diferenças entre raízes adventícias formadas em diferentes órgãos da planta (Bellini et al, 2014; Verstraeten et al, 2014) (Figura 1). Raízes laterais geralmente se originam a partir de células do periciclo de raízes existentes, como raízes primárias e raízes laterais mais velhas. Raízes adventícias, por sua vez, podem se originar de células do periciclo do hipocótilo, células do parênquima do xilema ou floema, células jovens do floema secundário e células do

câmbio interfascicular próximas ao floema, dependendo da espécie (Bellini et al, 2014). Além disso, raízes adventícias podem se originar direta ou indiretamente, através da formação de calos (Verstraeten et al, 2014). Quanto ao tempo médio de enraizamento, raízes adventícias originadas do hipocótilo tendem a se desenvolver mais rápido do que as formadas a partir de caules (Welander et al, 2014).
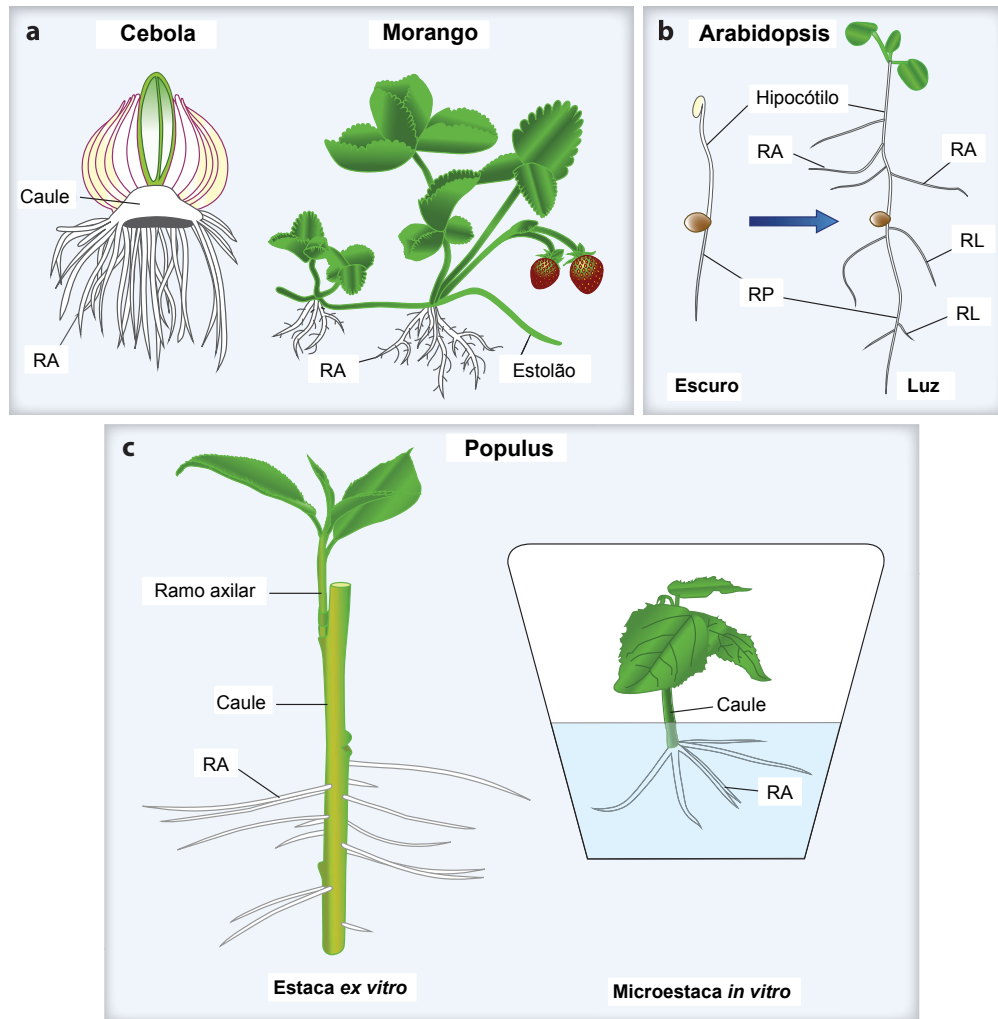
Figura 1. Esquema representando diferentes tipos de raízes adventícias. (a) Propagação vegetativa natural através de raízes formadas a partir do bulbo (cebola) ou estolão (morango). (b) Indução de raízes adventícias no hipocótilo de Arabidopsis após transição da planta do escuro para a luz. (c) Indução de raízes adventícias em Populus após ferimento (separação da estaca da respectiva planta mãe). RA, raiz adventícia; RL, raiz lateral; RP, raiz primária. Modificado de Bellini et al (2014).

O enraizamento adventício é um processo extremamente complexo, sendo influenciado por inúmeros fatores, como fitormônios, luz, temperatura, disponibilidade de água e nutrientes, ferimentos e estresse (Correa & Fett-Neto, 2004; Da Costa et al, 2013). Uma correta modulação destes fatores poderia melhorar a qualidade do sistema radical, permitindo o estabelecimento da planta até a idade adulta. Devido a essa complexidade, a

utilização do cultivo *in vitro* é uma alternativa válida, principalmente ao se estudar plantas com crescimento lento, como espécies arbóreas. Nessa técnica é possível conduzir os experimentos com explantes menores, como segmentos de caule, além de garantir a adição controlada de componentes inorgânicos e carboidratos, bem como o fornecimento de fitormônios e vitaminas e demais controles ambientais de luz e temperatura. Além disso, esta técnica diminui significativamente a chance de degradação microbiana de componentes testados (Van der Krieken et al, 1993). No entanto, obviamente é preciso cautela na extrapolação dos resultados obtidos *in vitro* para situações *ex vitro*.

1.3 Fitormônios e enraizamento adventício

Dentre os inúmeros fatores que afetam o enraizamento adventício, os fitormônios têm papel fundamental, pois além de desempenharem efeitos diretos, também medeiam respostas derivadas do efeito de outros fatores. Vários fitormônios estão envolvidos no enraizamento adventício, porém, na maioria dos casos, o efeito positivo ou negativo no enraizamento depende da espécie, da origem das raízes e das condições de cultivo (Figura 2).

Estudos com feijão mungo (*mung bean – Phaseolus radiatus*) mostraram que ácido salicílico (AS) tem efeito positivo no enraizamento adventício dessa espécie, atuando via acúmulo de peróxido de hidrogênio (Yang et al, 2013). Mutantes de Arabidopsis com alteração na biossíntese de AS desenvolveram menos raízes do que o tipo selvagem, indicando um efeito promotor do enraizamento para este fitormônio (Gutierrez et al, 2012).

Em experimentos com tabaco, Niu et al (2013) verificaram que giberelinas tem efeito inibitório na formação de raízes adventícias, porém estimulam o seu alongamento, apresentando um modo de ação contrário às auxinas nesta espécie. O efeito inibitório de giberelinas no enraizamento também foi verificado em estacas de Populus e tomate (Busov et al, 2006; Lombardi-Crestana et al, 2012, respectivamente).

O etileno era frequentemente indicado com tendo ação inibitória no enraizamento ou sem efeito nenhum. No entanto, estudos recentes têm verificado efeito positivo de etileno tanto no enraizamento adventício em plantas intactas de tomate e arroz como em estacas de diferentes espécies, incluindo girassol, maçã, feijão mungo e petúnia (revisado em Pacurar et al, 2014). Possivelmente esse efeito positivo seja derivado da interação com auxina. Em Populus, um fator de transcrição do tipo *AP2/ERF* foi indicado como regulador positivo dos enraizamentos adventício e lateral, e os autores enfatizaram a provável ligação deste fator de transcrição na via de sinalização de auxina (Trupiano et al, 2013).
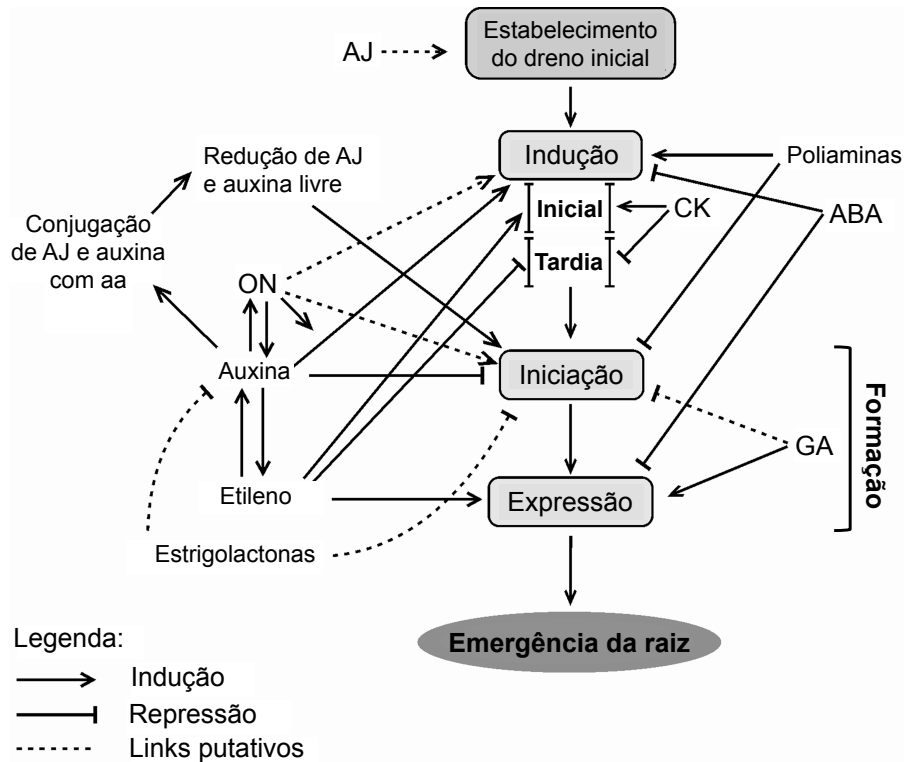
Figura 2. Esquema representando as possíveis interações entre fitormônios durante as diferentes fases do enraizamento adventício. As fases de Iniciação e Expressão são comumente agrupadas como fase de Formação. AJ, ácido jasmônico; CK, citocinina; ABA, ácido abscísico; ON, óxido nítrico; GA, giberelina; aa, aminoácidos. Modificado de Da Costa et al (2013).

O Ácido jasmônico, por sua vez, foi descrito como indutor de enraizamento adventício em estacas de petúnia (Ahkami et al, 2009). Já em hipocótilos de Arabidopsis, o mesmo fitormônio inibiu a formação dessas raízes (Gutierrez et al, 2012).

Ácido abscísico (ABA), strigolactonas e citocininas parecem ter efeito primariamente negativo no controle do enraizamento adventício. Avaliações do efeito de ABA em plantações de arroz em alagado o indicaram como altamente inibitório, sendo que o tratamento com ABA diminuiu em 50% a taxa de produção de raízes adventícias (Steffens et al. 2006). As strigolactonas compõem uma classe relativamente nova de fitormônios cujo efeito inibitório na formação de raízes adventícias foi recentemente verificado em Arabidopsis e ervilha (Rasmussen et al, 2012a; Rasmussen et al, 2012b). Os reguladores de resposta à citocinina do tipo B são sabidamente responsáveis pela regulação de milhares de genes de resposta à citocinina. Em estacas de *Populus*, *PtRR13*, membro da classe de reguladores do tipo B, foi indicado como um regulador negativo do enraizamento adventício, confirmando o efeito já sabido de citocinina no enraizamento (Ramirez-Carvajal et al, 2009). Em Arabidopsis, Zhu et al, (2015) verificaram que *ARR1* e *ARR12*, também membros da classe de reguladores de

citocinina do tipo B, estão envolvidos na inibição do crescimento de raízes em plantas submetidas a condições de baixa temperatura, reduzindo o acúmulo de auxina.

As auxinas, por sua vez, desempenham papel central na determinação da capacidade de enraizamento, além de serem essenciais para praticamente todas as etapas de desenvolvimento dos vegetais. Porém, concentrações que são favoráveis para a indução do enraizamento bloqueiam o alongamento das raízes, de acordo com o consenso que as diferentes fases da rizogênese possuem requerimentos hormonais diferentes ou mesmo opostos (De Klerk et al, 1999). As respostas ao enraizamento também são fortemente afetadas pelo conteúdo endógeno de auxina e sua taxa de transporte (Fogaça & Fett-Neto, 2005). O ápice da parte aérea é a principal fonte de auxina endógena (Normanly, 2010), porém ferimentos resultantes da excisão de estacas da planta mãe ou remoção da raiz principal sinalizam para o rápido acúmulo de auxina na base do segmento, estimulando a divisão celular nesta região (da Costa et al, 2013).

Até o momento, a única rota biossintética de auxina completamente estabelecida envolve Indol-3-acetamida (IAM) como intermediário entre o precursor triptofano e ácido indol-3-acético (IAA). Embora esta rota seja mais utilizada por bactérias e algas, o composto IAM já foi encontrado em alguns extratos de plantas. Além disso, enzimas do tipo amidases, capazes de converterem IAM em IAA, foram detectadas em Arabidopsis, indicando que algumas plantas podem utilizar IAM como intermediário para produzir IAA (revisado em Zhao, 2010). No entanto, estudos recentes têm demostrado que a rota do Indol-3-Piruvato (IPA) é a mais comum em plantas. Nesse caso, membros da família de aminotransferases *Tryptophan Aminotransferase of Arabidopsis* (TAA) fazem a conversão de triptofano em IPA e membros da família de flavina monooxigenases YUCCA (YUC) participam da conversão de IPA em IAA (Stepanova et al, 2011; Won et al, 2011).

O transporte e a sinalização de auxina são tópicos relativamente bem elucidados atualmente. Caules apresentam um característico transporte ativo basípeto através de células parenquimáticas dos tecidos vasculares, onde a auxina é conduzida por transportadores de influxo e efluxo, chamados AUX1 (*Auxin Resistant 1*) e PIN (*PIN Formed 1*), respectivamente (Muday & Delong, 2001) (Figura 3). A atividade de IAA envolve a ligação à proteína do tipo F-box TIR1, desencadeando a degradação via proteasoma de proteínas repressoras Aux/IAA, que se encontram ligadas aos fatores de transcrição de resposta à auxina (ARFs), inibindo a ação destes. Uma vez que Aux/IAA são degradados, ocorre transcrição de genes ativados por auxina através da ação dos ARFs (Dharmasiri et al, 2005; Woodward & Bartel, 2005). O co-repressor transcricional TOPLESS (TPL) está envolvido na

sinalização de auxina através da sua interação física com a proteína do tipo Aux/IAA denominada IAA12/BODENLOS (IAA12/BDL). Este complexo atua reprimindo genes de resposta à auxina e TPL é necessário para a ação repressiva de IAA12/BDL (Szemenyei et al, 2008) (Figura 4). A proteína ABP1 (*Auxin Binding Protein*) também atua como um receptor de auxina, estando mais relacionada com respostas de turgor e crescimento celular, sem necessariamente envolver expressão gênica. Recentemente foi demostrado que ABP1 atua através da ligação à proteína quinase do tipo transmembrana TMK1 na superfície celular, de forma auxina-dependente (Xu et al, 2014) (Figura 5). Em contraponto a uma série histórica de dados de diferentes laboratórios ao redor do mundo, mutantes nulos de Arabidopsis gerados por edição genômica (CRISPR) não mostraram nenhum defeito óbvio de desenvolvimento sob condições normais de crescimento (Gao et al, 2015). Portanto, pode-se afirmar que o papel de ABP1 como receptor de auxina ainda é tema de debate.
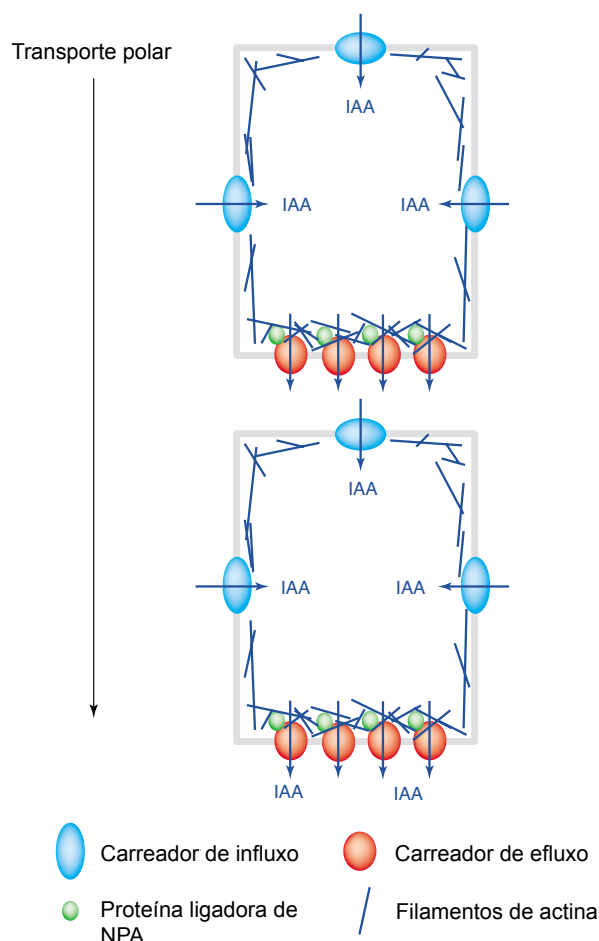
Figura 3. Transporte polar basípeto de auxina. IAA, ácido indol-3-acético; NPA, ácido naftil-p-talámico. A proteína ligadora de NPA é um componente catalítico integral de membrana que faz parte do complexo carreador de efluxo de auxina. Modificado de Muday & DeLong (2001).
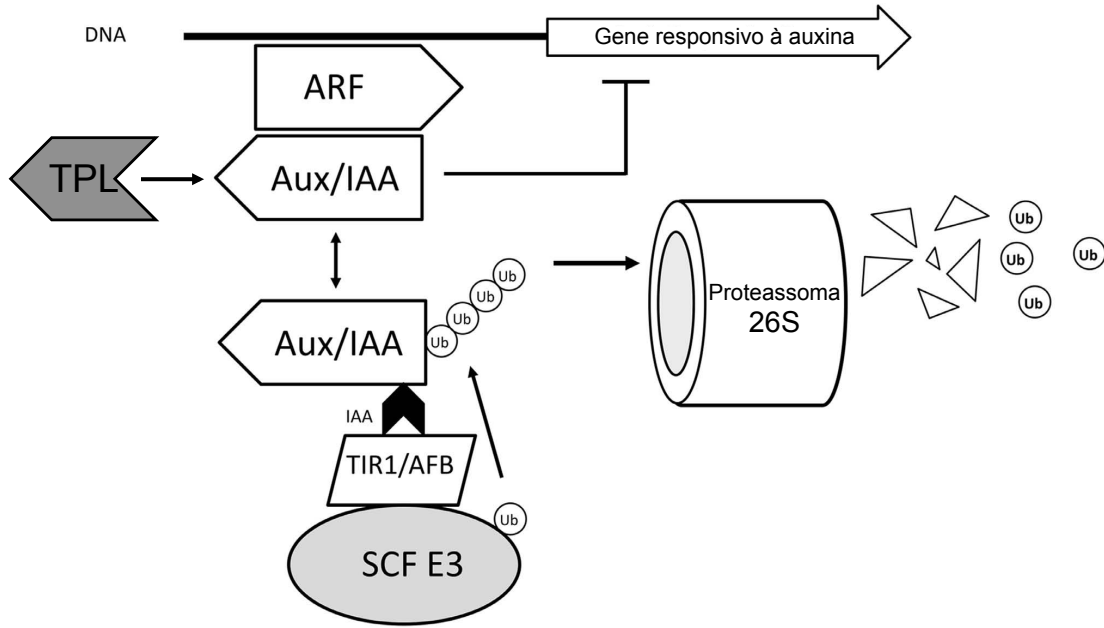
Figura 4. Percepção de auxina através da proteína F-box TIR1. Respostas lentas ao fitormônio. O co-repressor TPL interage fisicamente com a proteína Aux/IAA IAA12/BODENLOS, auxiliando na repressão da transcrição de genes responsivos à auxina. Em casos de alta concentração de auxina na célula, a ligação de auxina ao receptor TIR1 induz o recrutamento das proteínas Aux/IAA para degradação pelo proteassoma. ARF, fator de resposta à auxina; TPL, Ub, ubiquitina, IAA, ácido indol-3-acético; SCF E3, complexo de ubiquitinação SCF. Modificado de Da Costa et al (2013).
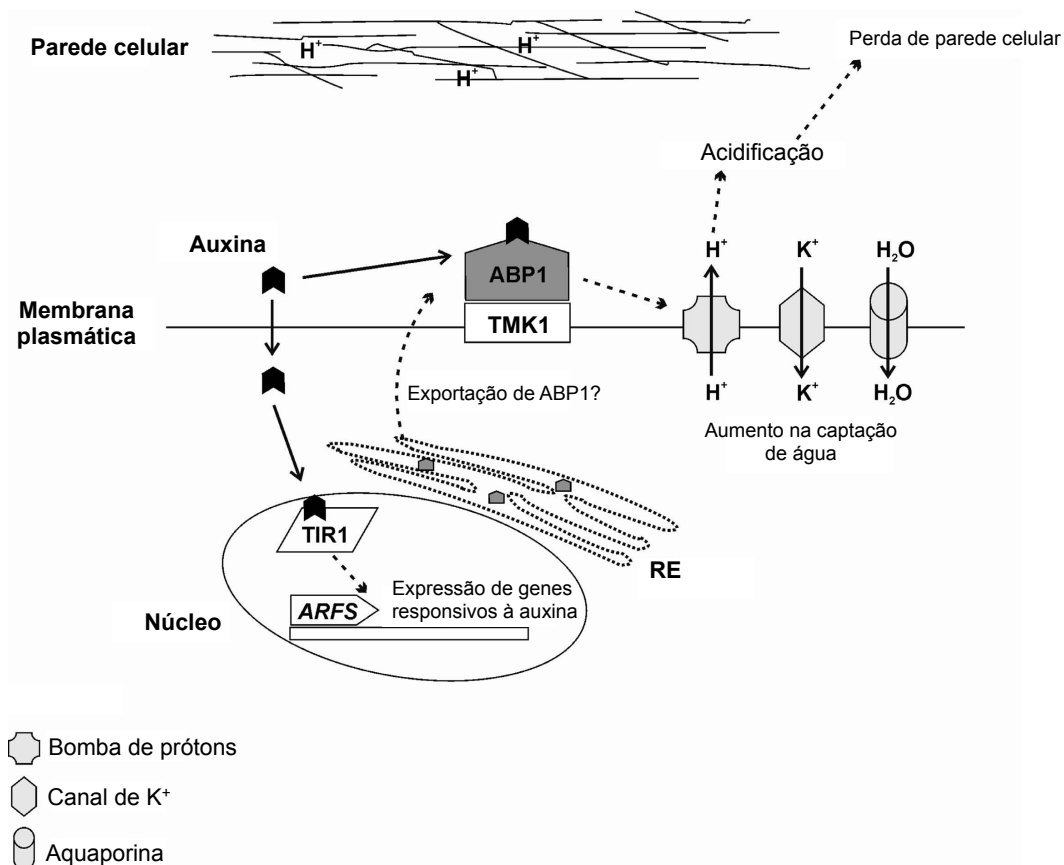
Figura 5. Percepção de auxina através da proteína ABP1. Respostas rápidas ao fitormônio. ABP1 está localizada no retículo endoplasmático e é exportada para a membrana plasmática (MP) através de um mecanismo ainda desconhecido. A proteína transmembrana TMK1 ancora ABP1 à MP. A ligação de auxina a ABP1 induz vários eventos, incluindo ativação de bombas de prótons, o que leva à acidificação e perda de parede celular. Há ainda a ativação de canais de potássio, aumentando a concentração intracelular de K^+ e induzindo a captação de água, levando à expansão celular. H^+, prótons; RE, retículo endoplasmático. Modificado de Da Costa et al (2013).

1.4. Expressão gênica e proteômica durante a rizogênese adventícia

Embora as pesquisas acerca do enraizamento adventício tenham avançado bastante nas últimas décadas, pouco ainda se sabe sobre este processo em nível molecular, principalmente no que diz respeito a plantas lenhosas. A maioria dos genes indicados como reguladores do processo de enraizamento estão relacionados com auxina, uma vez que esse é o fitormônio mais diretamente envolvido com o processo e também o mais estudado atualmente (Pacurar et al, 2014).

Considerando os primeiros eventos relacionados com o enraizamento adventício, três genes parecem ser bastante importantes: *SHORT-ROOT (SHR), SCARECROW (SCR)* e *AINTEGUMENTA LIKE1 (AIL1). SHR e SCR* atuam de forma coordenada para manter as células em estado meristemático ou competentes à divisão e sua ação é fortemente influenciada por auxina. Tanto genes *SHR* como *SCR-like* foram induzidos após tratamento com auxina em plantas competentes ao enraizamento de *Pinus radiata e Castanea sativa*. A indução ocorreu principalmente na região cambial e em células adjacentes, nas primeiras 24 horas do processo, antes da formação do primórdio radicular (Sanchez et al, 2007; Vielba et al, 2011). O gene *AIL1* foi isolado em *Populus* e está diretamente relacionado ao controle da formação do primórdio de raiz adventícia. Linhagens superexpressando este gene desenvolveram um maior número de raízes adventícias, enquanto que linhagens deficientes nesse gene geradas por meio de RNA de interferência (RNAi) apresentaram menos raízes (Rigal et al, 2012).

Alguns ARFs foram diretamente relacionados ao enraizamento adventício em Arabidopsis. *ARF17* foi indicado como um regulador negativo do processo, já que linhagens superexpressando este gene desenvolveram menos raízes do que o tipo selvagem (Sorin et al, 2005). *ARF6* e *ARF8*, por outro lado, foram considerados reguladores positivos do enraizamento adventício, uma vez que os respectivos mutantes nocaute desenvolveram menos raízes do que o tipo selvagem e mutantes superexpressando esses mesmos genes superaram o

tipo selvagem quanto ao número de raízes (Gutierrez et al, 2009). A expressão desses três ARFs é regulada por miRNAs e foi verificado que mutantes deficientes na proteína AGO1 (*Argonaute 1*), envolvida no complexo de regulação de miRNAs, acumulam altos níveis de ARF17 e, consequentemente, apresentam severas limitações na produção de raízes adventícias (Sorin et al, 2005).

O transportador de efluxo de auxina *ABCB19* parece ser importante no enraizamento adventício em hipocótilos de Arabidopsis, onde a formação de raízes foi significantemente afetada na linhagem mutante deficiente na expressão deste gene, enquanto que a linhagem superexpressando *ABCB19* produziu mais raízes que o tipo selvagem (Sukumar et a, 2013). Também em Arabidopsis, Della-Rovere et al (2013) verificaram o papel da auxina na manutenção do centro quiescente, necessário para garantir o crescimento indeterminado após a formação do primórdio da raiz adventícia. Os autores concluíram que as atividades dos transportadores de auxina *PIN1 e LAX3* (AUX-like) determinam o acúmulo de auxina no ápice do primórdio radicular, mesmo local de expressão de *WOX5*, responsável pela manutenção do centro quiescente. Além disso, a síntese local de auxina também é mantida através de *YUC6*.

Em *E. grandis*, análises do transcriptoma de estacas de plantas jovens e adultas mostraram que o processo de remodelamento de microtúbulos pode estar associado com a perda na capacidade rizogênica conforme as plantas envelhecem. Plantas adultas tratadas com um composto capaz de perturbar levemente a organização dos microtúbulos durante a fase de indução com auxinas tiveram a taxa de enraizamento aumentada de 10 para mais de 40% (Abu-Abied et al, 2014).

Em relação ao padrão de proteínas relacionadas ao enraizamento adventício, a literatura é ainda mais escassa. Em Arabidopsis, análises do perfil protéico de mutantes deficientes na produção de raízes adventícias identificou 11 proteínas envolvidas em processos como homeostase de auxina e rotas metabólicas associadas à fotossíntese. Dentre as proteínas identificadas estavam três GH3-like, correspondentes a genes induzidos por auxina, as quais foram correlacionadas com diferentes estágios do desenvolvimento das raízes adventícias (Sorin et al, 2006). Mais recentemente, em estudo com crisântemo, Liu et al (2013) analisaram proteínas diferencialmente expressas em bases de estacas durante o enraizamento adventício na tentativa de identificar proteínas relacionadas com a habilidade de formar raízes. Dentre várias proteínas relacionadas a diferentes processos, os autores identificaram a proteína induzida por auxina PCNT115 e a proteína ACC oxidase, relacionada

ao metabolismo de etileno, como positiva e negativamente correlacionadas ao enraizamento, respectivamente.

Considerando as últimas descobertas no campo do enraizamento adventício, ainda há muito a se avançar na pesquisa com plantas lenhosas. Nesse sentido, a comparação de plantas com diferentes capacidades de enraizamento é uma importante ferramenta para auxiliar na descoberta de novos componentes reguladores deste importante processo de desenvolvimento (Legué et al, 2014). Além disso, a expansão de florestas plantadas pode se tornar limitada devido a necessidade de maiores áreas para cultivo de plantas comestíveis e para produção de biocombustíveis ou, ainda, devido à pressão da população (Grattapaglia and Kirst, 2008). Sendo assim, a busca por novos conhecimentos que permitam o desenvolvimento de plantas e florestas mais robustas e produtivas, sem necessariamente aumentar de modo expressivo a área de plantio, é de extrema importância para a indústria florestal, tanto no ponto de vista econômico como ecológico.

2. Objetivo Geral

Caracterizar o processo de rizogênese adventícia em nível molecular, por meio da análise do papel do fitormônio auxina na determinação da capacidade de enraizamento na espécie recalcitrante *Eucalyptus globulus* Labill, visando contribuir para desvendar as causas da recalcitrância ao enraizamento e, consequentemente, tentar melhorar a propagação da espécie.

2.1 Objetivos específicos

- Analisar o padrão de expressão de genes envolvidos com biossíntese, transporte, sinalização e regulação de auxina ao longo do tempo de enraizamento;

- Analisar as alterações cito-histológicas e os tecidos envolvidos durante a formação de raízes adventícias;

- Realizar a imunolocalização da auxina em bases de micro-estacas em diferentes estágios de crescimento;

- Determinar genes expressos de forma tecido-específica na base de micro-estacas através de microdissecção a laser;

- Analisar o padrão de expressão de proteínas durante o enraizamento adventício.

Com exceção da análise proteômica, todos os experimentos foram realizados utilizando a espécie de interesse *E. globulus*, recalcitrante ao enraizamento, e a espécie propensa ao enraizamento *E. grandis,* de forma a permitir uma análise comparativa dos resultados.

3. Resultados

O presente trabalho foi dividido em três capítulos. O capítulo 1, intitulado "**Comparative transcriptional analysis provides new insights into the molecular basis of adventitious rooting recalcitrance in *Eucalyptus***", engloba análises morfológicas e de expressão gênica em estacas inteiras, doseamento de AIA endógeno, imunolocalização de auxina, anatomia durante o enraizamento e expressão gênica tecido-específica, indicando genes possivelmente importantes em cada fase do enraizamento adventício de *E. globulus* e *E. grandis*. O capítulo 2 , intitulado "**Proteomic profiles during adventitious rooting in *Eucalyptus globulus***" trata do perfil proteico encontrado nas fases de indução e formação do enraizamento adventício em *E. globulus*. Já o capítulo 3, intitulado "**Laser Capture Microdissection: Avoiding bias in analysis by selecting just what matters**" trata-se de um manuscrito para livro descrevendo a técnica de Microdissecção a laser em plantas, com protocolo detalhado.

3.1 Capítulo 1

Comparative transcriptional analysis provides new insights into the molecular basis of adventitious rooting recalcitrance in *Eucalyptus*

Márcia Rodrigues de Almeida, Daniela de Bastiani, Marcos Letaif Gaeta, Jorge Ernesto de Araújo Mariath, Fernanda de Costa, Jeffrey Retallick, Lana Nolan, Helen H Tai, Martina V Strömvik and Arthur Germano Fett-Neto.

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Comparative transcriptional analysis provides new insights into the molecular basis of adventitious rooting recalcitrance in *Eucalyptus*

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ABSTRACT

Adventitious rooting (AR) is essential in clonal propagation. *Eucalyptus globulus* is relevant for the cellulose industry due to its low lignin content. However, several useful clones are recalcitrant to AR, often requiring exogenous auxin, adding cost to clonal garden operations. In contrast, *E. grandis* is an easy-to-root species widely used in clonal forestry. Aiming at contributing to the elucidation of recalcitrance causes in *E. globulus*, we conducted a comparative analysis with these two species differing in rooting competence, combining gene expression and anatomical techniques. Recalcitrance in *E. globulus* is reversed by exposure to exogenous indole-3-acetic acid (IAA), which promotes important gene expression modifications in both species. The endogenous content of IAA was significantly higher in *E. grandis* than in *E. globulus*. The cambium zone was identified as an active area during AR, concentrating the first cell divisions. Immunolocalization assay showed auxin accumulation in cambium cells, further indicating the importance of this region for rooting. We then performed a cambium zone-specific gene expression analysis during AR using laser microdissection. The results indicated that the auxin-related genes *TOPLESS* and *IAA12/BODENLOS* and the cytokinin-related gene *ARR1* may act as negative regulators of AR, possibly contributing to the hard-to-root phenotype of *E. globulus*.

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1. Introduction

Adventitious rooting (AR) is a key process for vegetative propagation of economically important species. *Eucalyptus* sp. is one of the most planted genera worldwide [1], mainly because of its interesting features for the paper and cellulose industry and multipurpose wood properties. *Eucalyptus globulus* and its hybrids are of interest due to their low lignin content, which facilitates cellulose extraction [2]. However, this species is considered recalcitrant to rooting, making propagation often difficult [3]. Therefore, the characterization of rooting in *E. globulus* and the identification of mechanisms that cause rooting recalcitrance can help developing new strategies to overcome this obstacle and improve tree propagation.

Adventitious roots originate from organs other than roots, like leaves or stems [4]. AR can be divided in two main phases, each with its own specific requirements and characteristics: (1) induction, involving early biochemical and molecular events; and (2) formation, consisting in the first cellular divisions involved in root meristem organization and primordium establishment, followed by root elongation [3]. AR is complex and can be affected by multiple factors, such as phytohormones, light, temperature and mineral nutrition [4–6]. Auxins are the main phytohormones related to AR and high concentrations are beneficial to root induction but can block elongation [7]. Auxins are synthesized mainly in the shoot apex and are basipetally transported through Polar Auxin Transport (PAT). Indole-3-acetic-acid (IAA) is the most abundant naturally occurring auxin [8].

Along with local production, auxin transport is part of a redundant mechanism to allow the formation of effective auxin maxima [9]. The link between PAT, auxin peak and induction of AR was

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recently shown in *Petunia* cuttings [10]. The auxin influx to the cell is controlled by the AUX1/LAX family of amino acid permease-like proteins [11] and the efflux transport is carried out by members of the PIN Formed (PIN) family [12]. Once in the cell, IAA perception involves binding to the F-box protein TIR1, which recruits the Aux/IAA transcriptional repressor proteins for degradation via proteasome, thereby releasing Auxin Response Factors (ARFs) to modulate auxin-related gene expression [13]. The Auxin Binding Protein ABP1 is also thought to be an auxin receptor involved with early responses of cell growth [reviewed in 14], acting with transmembrane kinases (TMK) on the cell surface in an auxin-dependent manner [15].

The transcriptional co-repressor TOPLESS (TPL) is involved in auxin signaling through its physical interaction with IAA12/BODENLOS (IAA12/BDL), an Aux/IAA protein. This complex acts to repress auxin response genes and *TPL* is required for *IAA12/BDL* repressive activity [16]. Together with the TOPLESS-RELATED PROTEINS (TPRs), TPL participates in repression of auxin-related genes [17], likely affecting AR. Some ARFs are also involved in rooting. *ARF17* was postulated as a negative regulator of AR, integrating auxin and light signaling pathways [18]. On the other hand, *ARF6* and *ARF8* were considered positive regulators of AR. These three ARFs could modulate the balance between positive and negative regulators of AR through feedback loops driving the abundance of their respective regulatory miRNAs [19].

Although auxin is the most critical phytohormone for AR, a crosstalk involving several other phytohormones contributes to the success of the process [6,20]. Ethylene may act both as positive and negative regulator of AR, depending on the rooting phase and species [6]. The upregulation of *PtERF003*, a gene encoding a transcription factor of the AP2/ERF family of unknown function in poplar, had a positive effect on both adventitious and lateral root proliferation [21], probably as a result of the auxin-ethylene crosstalk. *AINTEGUMENTA LIKE1* (*PtAIL1*), another AP2 member, controls development of adventitious root primordia [22]. In poplar stem cuttings, the type-B cytokinin response regulator *PtRR13* was found to be a repressor of AR formation, in agreement with the postulated inhibitory effect of cytokinin on AR development [23]. Strigolactones have also been identified as negative regulators of AR, affecting auxin accumulation in the rooting zone, reducing auxin levels in the pericycle and leading to a decrease in root initiation [24].

Despite all the advances in AR research, still little is known about this process, particularly in woody plants. This is partly due to the slow life cycle and complications in transformation techniques of trees [25]. The use of *in vitro* cultured forest species can help obtaining results in shorter time. Another limitation is the use of whole plants or organs for the study of AR. It is well known that the majority of biological processes take place in subsets of specialized cells in a determined location within the plant body [26,27]. Tissue and cell type-specific transcriptomic studies have helped the elucidation of complex gene regulatory networks, and numerous techniques are available that allow higher resolution in biological sampling [28], yielding robust and accurate findings. Here we combined gene expression, anatomy, endogenous IAA measurements, and laser capture microdissection (LCM) to examine the role of auxin in AR and identify possible causes of rooting recalcitrance in *E. globulus* by comparing it to the easy-to-root species *Eucalyptus grandis*.

2. Material and methods

2.1. Plant material

Seeds of *E. globulus* Labill and *E. grandis* Hill ex Maiden were surface sterilized with 70% ethanol and 1.5% sodium hypochlorite,

followed by four washings with sterilized distilled water. Fifteen seeds were sown in 300 ml glass jars containing 60 ml of solid culture medium, consisting of 0.5X MS salts [29], 2% sucrose, 0.6% agar and pH adjusted to 5.8 [3]. Medium was autoclaved at 121 °C for 20 min. The plants were kept under controlled conditions of light and temperature, with photoperiod of 16 h, 50 μmol m^{-2}s^{-1} light intensity (provided by white fluorescent lamps) and temperature of 23 $\pm$ 2 °C. After 14 (*E. globulus*) or 16 weeks (*E. grandis*), apical microcuttings ($\sim$3 cm-long) were obtained, which were used in the *in vitro* adventitious rooting experiments.

2.2. In vitro adventitious rooting

The culture system consisted of two sequential steps: (1) induction, lasting 96 h in culture medium containing MS salts 0.3X, 0.4 mg l^{-1} thiamine, 100 mg l^{-1} inositol, 3% sucrose, presence or absence of 10 mg l^{-1} (57 μM) of IAA, 0.6% agar and pH 5.8; (2) formation, consisting in the same medium, but free of auxin and added of 0.1% of activated charcoal [3]. The media were sterilized by autoclaving at 121 °C for 20 min.

Experiments were carried out in 20 ml glass vials containing 6 ml of medium, which were capped with a double layer of aluminum foil, at a density of two explants per vial. Both treatments (presence and absence of auxin during the induction step) were carried out in a growth room under the same conditions described in Section 2.1.

The expression analysis of the selected genes was monitored along the rooting process and the harvests of microcuttings for RNA extraction were at 6, 24, 48 and 96 h of exposure to induction medium and 24, 48 h and 96 h after transfer to formation medium (formation step), for both treatments (*i.e.*, with or without exogenous auxin). The microcuttings were immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction. Each point of harvest combined eight microcuttings (approximately 200 mg of homogenized tissue fresh weight).

For the morphological analysis, the plants remained in the formation medium for 20 days. After this, the following parameters were analyzed: root number per rooted cutting, length of the longest root per rooted cutting and rooting percentage. For obtaining the mean rooting time, cuttings with visible roots were counted every other day, from day 2 to day 20 of the formation step, and these numbers were analyzed in relation to the final number of rooted cuttings. Mean rooting time was calculated as previously described [3]. A total of 20 microcuttings was used for each treatment (control and presence of exogenous auxin in induction medium). The experimental design was completely randomized and the experiments were independently repeated three times with similar results.

2.3. Whole plant gene expression

Total RNA was isolated from microcuttings using NucleoSpin RNA Plant Kit (Macherey-Nagel) including DNAse I treatment, following the manufacturer recommendations. Total RNA concentration was determined using a Nanodrop™ Spectrophotometer (Thermo Scientific). First strand cDNA synthesis was performed using 1 μg of total RNA, oligo-dT primers and reverse transcriptase M-MLV (Invitrogen) in a final volume of 20 μl. The final cDNA products were diluted 100 fold in RNAse-free distilled water prior to use in qPCR.

The qPCR analyses were performed in fast optical 48 well reaction Plates 0.1 ml (MicroAmp™—Applied Biosystems) using a StepOne™ Real-Time PCR System (Applied Biosystems), according to the manufacturer instructions. Reactions were incubated at 95 °C for 5 min to activate the Platinum® Taq DNA polymerase (Invitrogen), followed by 40 cycles of 95 °C for 15 s, 60 °C for 10 s, and

72 °C for 15 s, using SYBR® Green dye. The specificity of the PCR was confirmed with a heat dissociation curve (or melting curve) from 60 °C to 90 °C, following the final PCR cycle. The primers for the genes analyzed were designed with Primer 3 software [30,31] and are described in the Supplementary Table S1.The reference genes used were Histone H2B and Alpha Tubulin for *E. globulus* [32] and Histone H2B and Actin for *E. grandis* (unpublished data). The data were analyzed by the Comparative Ct method [33] and the relative expression was calculated using each reference gene separately, followed by the determination of the respective mean relative expression.

2.4. Histological analysis and immunolocalization of auxin

Microcutting stem bases were harvested after 24 and 48 h of incubation in adventitious root formation medium, for both control and auxin treated plants. The samples were fixed in 4% formalde-hyde solution in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h, followed by 3 washes of 30 min in sodium phosphate buffer and 2 washes of 30 min in distilled water. The samples were then submit-ted to dehydration in an ethanolic series, from 20% to 100% ethanol, remaining 30 min in each solution and under constant agitation. The pre-infiltration was made in solution containing LR White Resin (Sigma–Aldrich) and ethanol 1:1 (v/v) for 8 h under agitation, fol-lowed by infiltration in 100% LR White Resin under agitation (2 shifts of 8 h each). After this, the samples were embedded in LR White resin and submitted to polymerization in gelatin capsules for 24 h at 50 °C [34]. Sections of 3 μm were prepared in microtome RM2265 (Leica).

The sections were incubated as described in Sakata et al. [35] for IAA detection, using an anti-IAA monoclonal antibody (No A0855, Sigma–Aldrich, 1:1,000 dilution) and Alexa 488-conjugated goat anti-mouse IgG antibody (Invitrogen; 1:300 dilution). Negative controls were performed without adding anti-IAA antibody, and no fluorescence signal was detected (data not shown). The samples were observed on a Leica DMR fluorescence microscope (excitation filter 450–490 nm), coupled with digital capture system DFC500 (Leica). Sections were obtained and observed for at least five dif-ferent individuals in each treatment. The IAA fluorescence signal was quantified using Image J, according to the method described in Gavet and Pines [36].

2.5. Endogenous auxin quantification by HPLC

The quantification of endogenous auxin content was performed according to Kim et al. [37], with the following modifications: approximately 400 mg of fresh tissue (pool of whole cuttings har-vested at 24, 48 and 96 h of control induction phase without exogenous auxin and 48 and 96 h of formation phase of AR) were powdered with liquid N_2 and extracted with 100% methanol HPLC grade. After centrifugation, the supernatant was completely evap-orated in a Speed Vac (SC 110, Savant). The resulting pellet was resuspended in 200 μl of water and 200 μl of 100% *n*-butanol. After partitioning, the resulting lower aqueous phase was transferred to a new tube. The pH of the solution was adjusted to between 11 and 12 with 1 M KOH and then partitioned against 100% ethyl acetate. The lower aqueous phase was transferred to a new tube and the pH of the solution was lowered to between 1 and 2 with concentrated acetic acid to keep IAA in protonated form. 100% ethyl acetate was added to the acidic sample (1:1 v/v) and the resulting homogeneous solution was completely dried in Speed Vac (SC 110, Savant). The pellet was dissolved in 500 μl 100% HPLC grade methanol.

Samples were analysed in a Shimadzu SPD-20A HPLC sys-tem equipped with a Shimadzu C_{18} HPLC reverse-phase column (250 × 4.6 mm) with corresponding guard column, using a gradient system of three different mobile phases: Solution A: 10% methanol, 0.3% acetic acid; Solution B: 90% methanol, 0.3% acetic acid; Solu-tion C: 100% acetonitrile, as proposed by Kim et al. [37]. All solutions were previously filtered through 0.45 μm Millipore membranes and degassed. Flow rate of the mobile phase was 1.0 ml min^{-1} and detection was done with a Shimadzu RF-10A XL fluorescence detec-tor (Emission at 360 nm, Excitation at 282 nm). Solvents acetonitrile (Merck) and methanol (Merck) were HPLC grade, whereas all other chemicals and solvents were of analytical grade. To quantify IAA, 20 μl of each sample were injected and an external standard curve was generated using IAA (Acros Organics). The identification of IAA content from samples of *E. grandis* and *E. globulus* was based on retention time and co-chromatography with authentic IAA stan-dard. The contents of IAA in samples were expressed as nmol of IAA per gram of extracted fresh weight. Analyses herein described were performed in totally randomized layout and using biological quadruplicates.

2.6. Laser capture microdissection (LCM) and tissue specific gene expression

For LCM experiments, the plants were grown in a growth cham-ber under the same light and temperature conditions and in the same media described for the other experiments. Rooting tests were performed in 250 ml glass jars containing 25 ml of medium and at a density of four plants per flask. The harvest of the micro-cuttings stem bases (about 5 mm) was made in triplicate (each one with *n* = 8) for each treatment (control and IAA application) at dif-ferent times of the adventitious rooting process: 24, 48 and 96 h after transfer to formation medium.

The stem bases of microcuttings were submitted to a fixation process by infiltration in an Ethanol: Acetic Acid (EAA) solu-tion (75:25 v/v), according to Matas et al. [38]. The infiltration was performed on ice and under vacuum for 15 min, followed by a replacement with fresh solution and gentle agitation on ice overnight. Next, the samples were transferred to a 10% sucrose solution in PBS buffer and infiltrated in the same conditions above. After smooth agitation on ice for 30 min, the infiltration process was repeated with a 20% sucrose solution in PBS buffer, followed by another round of agitation on ice until the samples were fully submerged [38]. Once the fixation and infiltration processes were completed, the samples were embedded in Shandon cryochrome media (Fischer Scientific), frozen with liquid nitrogen and stored at −80 °C until sectioning.

For sectioning, 25 μm thick cross sections were prepared at −21 °C using a Thermo Electron Corporation Cryotome. The sec-tions were melted on polyethylene naphthalate (PEN) Membrane Frame Slides (Applied Biosystems), submitted to dehydration pro-cess in an ethanolic series and directly used for laser capture microdissection with an ArcturusXT™ Microdissection Instrument (Applied Biosystems). The microdissected tissues were captured on CapSure® Macro LCM caps (Molecular Devices, Mt. View, CA) with the settings at a spot size of 40 μm, UV cut length of 500 and UV cut speed of 500 [39]. The region selected consisted of cells from cambium zone, encompassing fascicular and interfascicular cambium.

The RNA from the microdissected tissues was extracted from the LCM caps using the Arcturus™ PicoPure® RNA isolation kit (Applied Biosystems), followed by a DNAse I treatment and ampli-fied to aRNA in 2 rounds using Arcturus™ RiboAmp® kit (Applied Biosystems), following manufacturer recommendations. The aRNA concentration and quality were assessed using a Nanodrop Spec-trophotometer (Thermo Scientific) and a Bioanalyzer 2100 (Agilent Technologies), respectively.

Gene expression analysis was performed using the nCounter® system (Nanostring Technologies) [40]. The analysis was carried

out using 100 ng of amplified RNA as template. The CodeSet contained specific probes for a multiplex assay of the eleven genes of interest and five reference genes. The reference genes were *Histone H2B, Alpha tubulin, Actin, IDH* and *TIP41* [32]. The geometric mean of the five genes was used for normalization. The sequences used for the CodeSet probes for the genes of interest were obtained from the *E. grandis* genome database available at the *Phytozome* website (http://phytozome.jgi.doe.gov/pz/portal.html). The sequences from the auxin-related genes in *Arabidopsis* were used as query for searching the respective orthologs in *Eucalyptus*. For the cytokinin-related gene *ARR1*, the search was performed using the *PtRR13* sequence from *Populus* as query, an orthologue from the *Arabidopsis ARR1*. The probes are described in Supplementary Table S2. The data were analyzed with the nSolver® Analysis Software version 2.0 (Nanostring Technologies).

3. Results

3.1. Exposure to exogenous auxin overcomes recalcitrance to rooting in E. globulus

The exogenous application of auxin (IAA) in the induction medium restored the rooting capacity in *E. globulus*, for which rooting percentage increased 4.7 fold when compared to control plants (absence of auxin) (Fig. 1A). Auxin treatment was also effective in decreasing the mean rooting time in both species (Fig. 1D). The easy-to-root feature of *E. grandis* was confirmed, since no difference was detected in rooting percentage with or without auxin supply (Fig. 1A). On the other hand, exogenous auxin improved root number and length in *E. grandis*, with no significant effect on these parameters being observed in *E. globulus* (Fig. 1B and C).

3.2. Gene expression of auxin related genes in microcuttings reveals differences between E. globulus and E. grandis

Considering auxin as the main phytohormone involved in adventitious rooting, as well as its positive effect on *Eucalyptus* rooting, we decided to analyze the expression pattern of genes associated with different processes involving auxin action in microcuttings. To that end we selected genes involved in auxin biosynthesis (*TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 – TAA1* and *YUCCA – YUC3*), perception (*ENDOPLASMIC RETICULUM AUXIN BINDING PROTEIN 1 – ABP1* and *TRANSPORT INHIBITOR RESPONSE 1 – TIR1*), signaling (*AUXIN RESPONSE FACTORS – ARF6* and *ARF17*) and transport (*AUXIN RESISTANT 1 – AUX1* and *PIN-FORMED 1 – PIN1*). Gene expression was analyzed during the process of AR in control and auxin-treated plants of both *E. globulus* and *E. grandis*.

The auxin treatment was effective for inducing the expression of some genes in both species analyzed, albeit in different ways. In *E. globulus*, the expression of auxin biosynthesis genes *TAA1* and *YUC3* was increased during formation phase in auxin-treated microcuttings, with no difference in control samples (Fig. 2A, C). In *E. grandis*, *TAA1* had higher expression in control than in auxin-treated plants during the induction phase; in addition, although in lower quantity compared to control, *TAA1* was induced over time along the induction phase in auxin-treated plants (Fig. 2B). At the end of *E. grandis* induction phase, *YUC3* was expressed at higher levels in IAA-treated when compared to control plants (Fig. 2D). The auxin receptor *TIR1* and the auxin response factor *ARF6* had both a peak of expression after 6 h of exposure to exogenous auxin in *E. globulus* (Fig. 2G and I). On the other hand, in *E. grandis*, *TIR1* was significantly reduced by auxin at 48 h in induction phase with no difference between treatments in the following time points (Fig. 2H). *ARF6* was also reduced by auxin treatment at 6 h of induction phase in *E. grandis* (Fig. 2J). *ABP1* was induced at mid to end of induction phase in
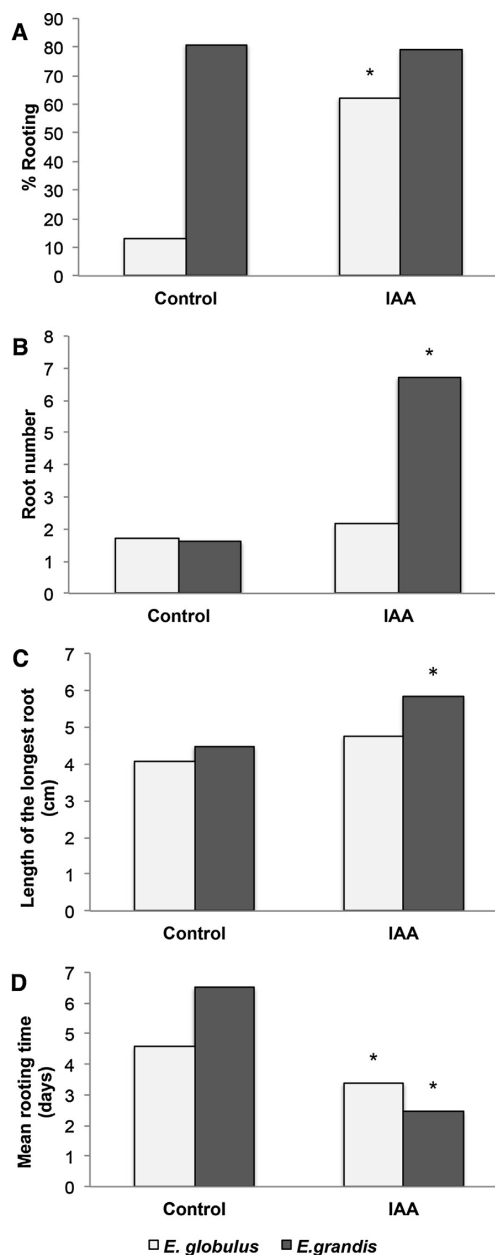
Fig. 1. Rooting performance in *E. globulus* and *E. grandis*. Asterisks indicate significant difference between treatments in the same species, according to Student's *t* test, with $P \leq 0.05$. The label "IAA" indicates presence of exogenous auxin in rooting induction medium. Values represent the means of three independent experiments. (C—evaluation done per rooted cutting).

both control and auxin-treated plants in *E. grandis*, but no significant difference was observed between treatments and time points in *E. globulus* (Fig. 2E and F). *ARF17* did not change significantly during the evaluated stages of AR in *E. globulus*, but a significant decrease in expression was observed at the end of induction and during the formation phase in *E. grandis* auxin-treated plants when comparing to the same time points in control plants (Fig. 2K and L). Also in *E. grandis*, the auxin carriers *AUX1* and *PIN1* were induced

Fig. 2. Gene expression in whole cuttings. (A, C, E, G, I, K, M, O—Graphics in left). Detail of gene expression in *E. globulus*. (B, D, F, H, J, L, N, P—Graphics in right) Detail of gene expression in *E. grandis*. Bars sharing the same letters are not different according to Tukey test with $P \le 0.05$. Small let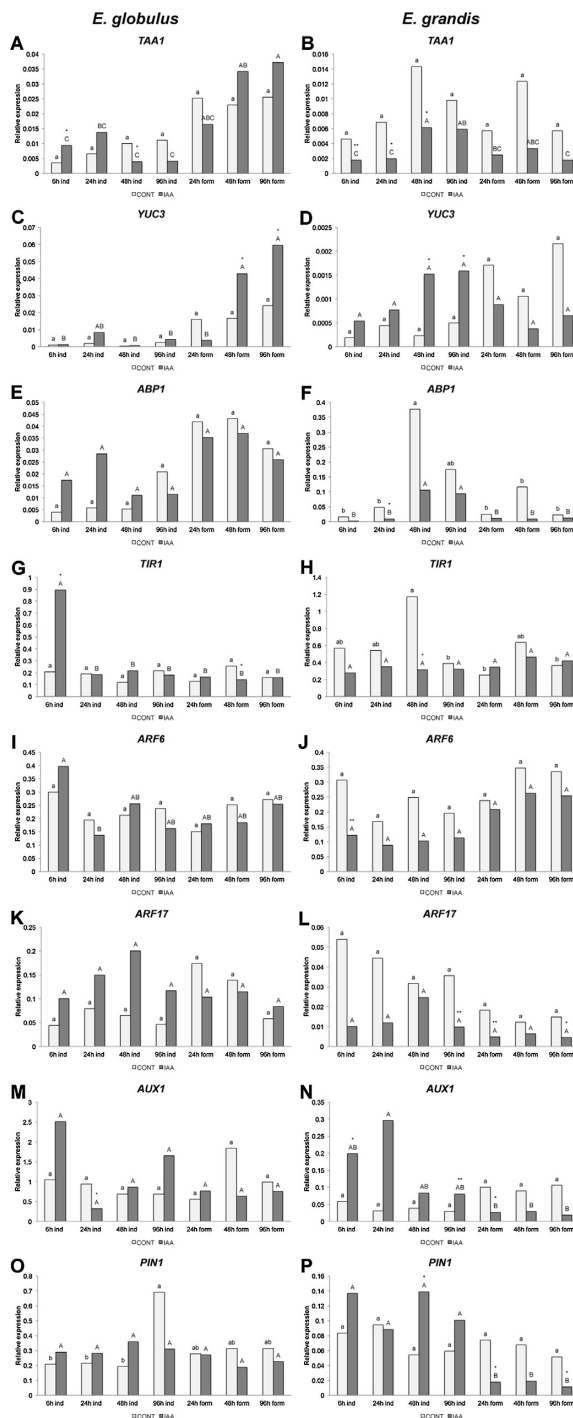ters correspond to ANOVA in control (CONT) samples. Capital letters correspond to ANOVA in auxin treated (IAA) samples. Asterisks indicate significant difference between treatments in the same time-point, according to Student's *t*-test, with $P \le 0.05$ (*) or $P \le 0.01$ (**). Values correspond to means of three independent experiments.

by auxin during the induction phase and repressed by this treatment in formation phase (Fig. 2N and P). In *E. globulus*, a decrease in *AUX1* expression was detected at 24 h of induction phase in auxin-treated plants (Fig. 2M). Furthermore, a peak of *PIN1* expression was observed at the end of induction phase in control plants of the same species (Fig. 2O). Despite the differences in gene expression patterns, these findings suggest that exogenous auxin application can induce modifications in gene expression to guarantee an appropriate homeostasis of auxin, leading to development of new roots in both species analyzed.

3.3. The cambium is an important region for adventitious root induction in Eucalyptus *and IAA endogenous content differs between* E. globulus *and* E. grandis

Adventitious roots can be formed from different tissues and cell types, depending on the species and the organ they develop from [41]. To better understand the factors involved in adventitious rooting in *Eucalyptus*, we also investigated the process from an anatomical point of view. The analysis was performed in control and auxin-treated plants. Through light microscopy of anatomical sections, the cambium zone was identified as an active area during adventitious root formation, concentrating the first cell divisions. Immunolocalization fluorescence microscopy showed that concentration of auxin in these cells was increased in *E. globulus* plants submitted to auxin treatment when compared to control plants (Fig. 3E, F, I). In *E. grandis*, auxin concentration in cambium cells was similar in both control and IAA treated plants and the later were comparable to IAA treated *E. globulus* plants (Fig. 3G–I). Quantification of immunofluorescence in images of the cambium zone indicated approximately five times higher content of IAA in *E. grandis* compared to *E. globulus* (Fig. 3I). These findings indicate the importance of auxin accumulation at the sites of root primordia formation for successful AR development. Indeed, the endogenous auxin content also seems to be very important for the competence to rooting. Considering the whole plant, the easy-to-root species (*E. grandis*) had twice the auxin concentration found in the hard-to-root one (*E. globulus*) (Fig. 3J).

3.4. Tissue-specific gene expression suggests inhibitory genes as major players in AR recalcitrance

In order to obtain more accurate results about the AR process, LCM was used for specifically selecting cambium cells for RNA extraction (Supplementary Fig. S1) followed by gene expression analysis with the nCounter technique (Fig. 4). The approach was applied to both *E. globulus* and *E. grandis* treated or not with exogenous auxin (IAA) during the induction phase of AR. In order to analyze gene expression patterns related to the formation and emergence of roots, we examined samples harvested at 24, 48 and 96 h after transfer to formation medium.

In this experiment, after removing genes with RNA counts lower than those of negative controls, a total of eleven genes were analyzed in more detail. In general, genes seem to be highly expressed in *E. globulus* when compared to *E. grandis* (Fig. 4A). However, in *E. grandis*, several genes related to auxin had a peak of expression at 96 h in formation medium in control samples (Fig. 4A).

In the recalcitrant species *E. globulus*, two genes known by their repressive functions in auxin signaling have shown interesting expression profiles. *TPL* was highly expressed at 24 and 48 h of formation phase in control samples, followed by decrease in expression at 96 h. In IAA treated samples, *TPL* was less expressed, as well as in *E. grandis* under both treatments (Fig. 4B). *IAA12* had a peak of expression at 24 h in control plants of *E. globulus*, with a decrease at 96 h in the same condition (Fig. 4C). In *E. grandis*, *IAA12* was significantly less expressed than in *E. globulus* at 24 h in
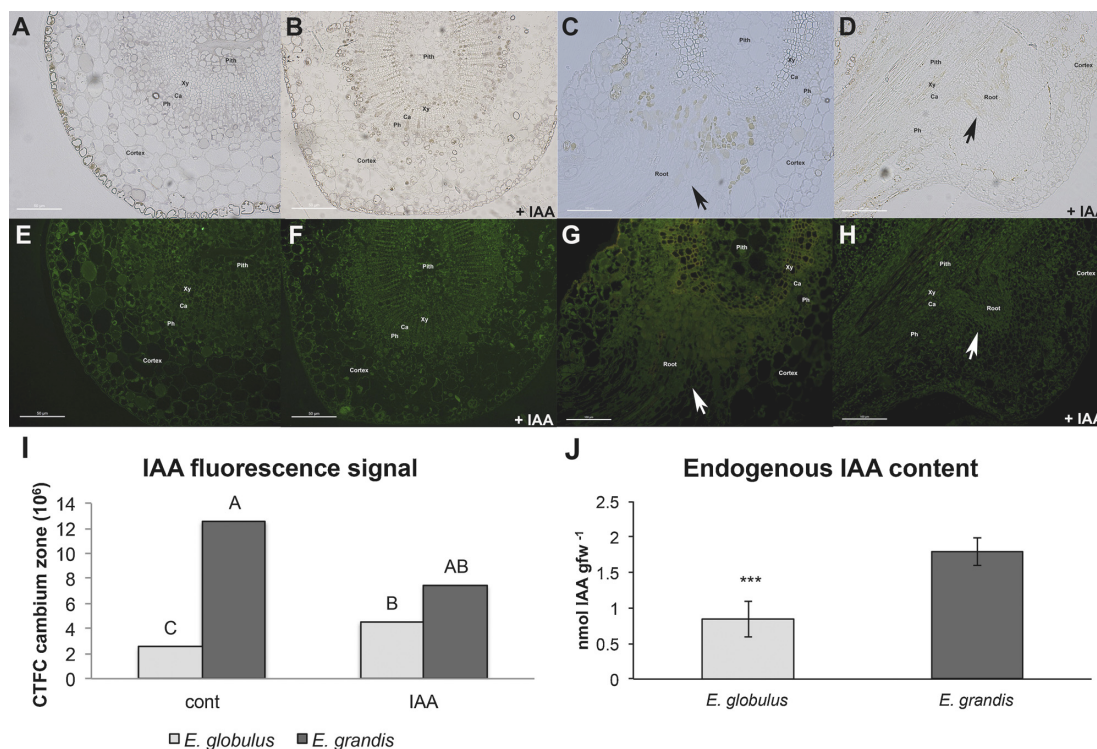
Fig. 3. Anatomy and auxin immunolocalization in stem bases of *Eucalyptus* after 48 h in formation medium. (A, B, E, F) Representative pictures from *E. globulus*—scale bars: 50 μm. (C, D, G, H) Representative pictures from *E. grandis*—scale bars: 100 μm. "+IAA" indicates treatment with Indol-3-Acetic Acid for 96 h before transfer to formation medium. Upper panels—bright field pictures. Bottom panels—correspondent anti-IAA immunofluorescence signal. A–C and E–G—cross sections, 20× magnification. D and H—longitudinal sections, 10× magnification. White and black arrows indicate adventitious root primordium. Xy—Xylem; Ca—Cambium; Ph—Phloem. (I) Quantification of the IAA fluorescence signal in cambium zone. Bars sharing the same letter are not different according to Dunnet-C test, with $P \leq 0.05$. (J) Endogenous auxin quantification in control plants of *E. globulus* and *E. grandis*. Asterisks indicate significant difference according to Student's *t*-test with $P \leq 0.001$. IAA levels in nmol correspond to 148.03 ng and 314.34 ng in *E. globulus* and *E. grandis*, respectively.

control plants, with similar expression in the other time points and auxin- treated plants.

The expression of the cytokinin type-B response regulator *ARR1* was higher in *E. globulus* control samples when compared to IAA-treated samples of the same species, except at 96 h, but the decreasing expression profile along time was similar in both conditions (Fig. 4A and D). In *E. grandis*, *TPL, IAA12* and *ARR1* were similarly expressed between control and treated samples (Fig. 4A–D), with low levels of expression of *TPL* and *ARR1*, in good agreement with the easy-to-root phenotype of this species.

4. Discussion

The improvement of rooting capacity can increase productivity of species in the planted areas by enabling clonal propagation of elite clones, which is essential for a competitive forest sector [42]. In *Eucalyptus* species, little is known about the molecular mechanisms involved in AR and, even less, about the basis of recalcitrance to rooting. Considering the relevance of this intriguing developmental process, more studies are necessary to provide new insights about AR in woody species. Based on the gene panel evaluated in these two *Eucalyptus* species, besides exogenous auxin-induced modifications in gene expression, the steady state transcriptome of untreated plants seems to be associated with the rooting phenotype.

E. grandis is considered easy-to-root and is the most planted eucalypt in Brazil due to its high adaptability and fiber quality [43],

whereas *E. globulus* is more recalcitrant to rooting [44]. The use of exogenous auxin is a common practice to induce rooting in cuttings of hard-to-root species [6]. Here we used indole-3-acetic-acid (IAA), a widespread natural auxin, rather than indole-3-butyric acid (IBA), more commonly employed in rooting of cuttings. Although IBA is also found naturally and may serve as a source of IAA, molecular aspects of its mechanisms of transport, sensing, signaling, and metabolism are less well defined [6]. The rooting recalcitrance of *E. globulus* compared to *E. grandis* was clear in the rooting tests, as well as the capacity of exogenous IAA to improve rooting performance of both species, particularly the former.

4.1. Exogenous auxin induces gene expression modifications in whole cuttings of both easy- and difficult-to-root Eucalyptus species

Both species were affected by exogenous auxin, but in different fashion. Whereas in the recalcitrant *E. globulus* supplied auxin essentially resulted in rooting of more cuttings, in *E. grandis*, the availability of IAA improved the rooting system *per se*, by increasing root number and length. As expected, these changes were accompanied by modifications in gene expression and different patterns were revealed in whole cuttings. *TAA1* and *YUCCA* seem to act in the same auxin biosynthetic pathway [8] and it is known that a decrease in IAA biosynthesis can lead to a reduced number of adventitious roots [41]. Both *TAA1* and *YUC3* genes were induced by auxin treatment in *E. globulus*, being more expressed during the
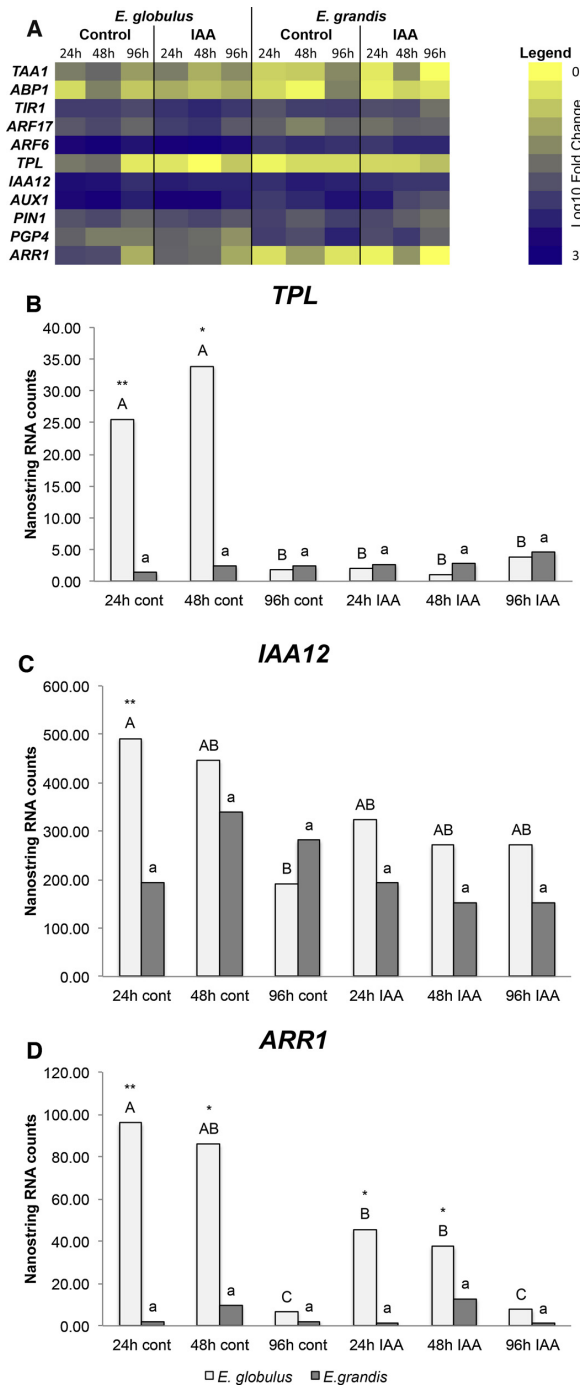
Fig. 4. Tissue-specific gene expression. (A) Heatmap comparing cambium-specific gene expression in *E. globulus* and *E. grandis* in control and IAA- treated plants. 24, 48 and 96 h correspond to time points in formation phase of AR. Yellow to Blue blocks correspond to the lowest and highest expression values, respectively. Expression values are represented as Log10. (B–D) Detail of gene expression pattern of root- inhibiting genes in control and IAA- treated conditions of *E. globulus* and *E. grandis*. Bars sharing the same letters are not different according to Dunnet-*C* test, with $P \leq 0.05$. Capital letters correspond to ANOVA in *E. globulus* and small letters correspond to ANOVA in *E. grandis*. Asterisks indicate significant differences between species within time points, according to Student's *t*-test, with $P \leq 0.05$ (*) or $P \leq 0.01$ (**). Values correspond to the means of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

formation phase. In *E. grandis*, on the other hand, the increase in *YUC3* expression by auxin treatment occurred at the end of induction phase (Figure 2). This difference in expression pattern of auxin biosynthetic genes upon exogenous auxin exposure between the two species may reflect the distinct contents of endogenous IAA, which are lower in the recalcitrant *E. globulus* (Fig. 3J).

In the present study, a pronounced increase in *TIR1* expression was evident after 6 h of exposure to exogenous auxin in *E. globulus* (Fig. 2), perhaps due to the increased levels of auxin in cells. *ARF6* is known as a positive regulator of AR in *Arabidopsis* [18] and its decreased expression at the beginning of induction phase in *E. grandis* auxin- treated plants was unexpected. Furthermore, the negative effect of *ARF17* on AR [18] was not clearly observed since this gene was expressed at similar levels in both control and auxin treated plants of *E. globulus* (Fig. 2K). On the other hand, in *E. grandis*, a consistent decrease in *ARF17* levels at late induction and during formation phase was observed in plants treated with auxin, what agrees with the higher amounts of adventitious roots in *arf17* loss of function mutants when comparing with the wild type [18]. However, as these genes are regulated by miRNAs [19], the analysis of expression of their respective regulators could lead to more conclusive information on the action of these transcription factors during AR in *Eucalyptus*.

In *E. grandis*, the increase in expression of *TIR1* and *ABP1* during induction phase in control plants can indicate an active perception of endogenous auxin levels to promote transcription of auxin-related genes and cell wall modifications necessary for development of roots. The higher number of roots after auxin exposure in this species (Fig. 1) can also reflect a more effective water uptake induced by increased levels of *ABP1* (Fig. 2), which can help cellular expansion [14] and, consequently, facilitate rooting.

The transport of auxin from the shoot apex is very important for auxin accumulation at the sites of primordia induction, which often requires a local auxin maximum [10]. Several AR studies with different species have shown the importance of Polar Auxin Transport (PAT) [10]. We have found very distinct expression patterns of auxin carriers for *E. globulus* and *E. grandis*. In the easy-to-root species, both influx and efflux carriers were induced by auxin application during induction phase (Fig. 2). In this case, increased levels of auxin at the stem bases due to exogenous application may require a more effective transport to allow the relocation of excess auxin to other parts of the plant. In turn, *AUX1* and *PIN1* overall expression essentially did not differ between control and treated plants of *E. globulus* (Fig. 2). In mango cotyledon segments, the expression of four members of the AUX family was increased in IBA-treated samples [45] but in *Medicago truncatula*, the expression of auxin carriers varied after treatment with IAA, and some members were up- while others were downregulated [46]. On the other hand, the exogenous auxin treatment led to an apparent decrease in *AUX1* levels at early induction phase in *E. globulus* and a significant decrease of both auxin transporters during formation phase in *E. grandis* (Fig. 2). IBA treatment in mango cotyledons also led to downregulation of *PIN1* at some points during adventitious rooting [45]. Similarly, in *Sorghum bicolor*, *PIN1* and *PIN5* were downregulated by IAA treatment [47]. In contrast, several reports have shown an increase in the expression of *PIN* genes after treatment with exogenous auxins [45,48,49], indicating that different PIN proteins can respond differently to auxin treatment in various species.

4.2. Auxin accumulation in cambium zone signals to adventitious root formation

One of the major differences between the formation of lateral and adventitious roots is that the former originate from pericycle cells and the latter generally derive from cambial or other meristematic cells [50]. AR studies in poplar and juvenile cuttings

of *E. grandis* have shown that most of the cell divisions leading to adventitious roots primordia occur in cambium cells [22, 51, respectively]. In agreement with these findings, the first cell divisions were concentrated in the cambium zone in both *E. globulus* and *E. grandis* microcuttings (Fig. 3). These cells were also the main auxin accumulation site, another evidence of the importance of the cambium zone in AR (Fig. 3I). Although some differences in gene expression were detected between the species analyzed (Fig. 2), the specific expression profile revealed by each one seemed to contribute to a final common scenario, *i.e.*, auxin focusing to generate the necessary peak for triggering the rooting process [52]. The lower concentration of both endogenous and immunodetected auxin in *E. globulus* when compared to *E. grandis* (Fig. 3) could contribute to the difficult-to-root phenotype of the former species. Negishi et al. [53] showed that the initial IAA content in an *E. globulus* easy-to-root line was twice that of a difficult-to-root one. The ability to root can also depend on the capacity to conjugate auxin to inactive forms. In the same study by Negishi et al. [53], conjugation of IAA to indole-3-acetyl aspartic acid at cutting base was higher in the easy-to-root genotype during rooting. Exogenous auxin application can facilitate the formation of auxin gradients and the results have shown that auxin concentration in cambium cells of *E. globulus* auxin-treated plants was higher than in respective control plants (Fig. 3I). Indeed, the cell to cell transport of auxin is determinant for rooting, since the use of auxin transport inhibitors, such as NPA, can abolish adventitious root formation in *E. globulus* independently of the plant competence to rooting [53]. In our study, the increased auxin levels driven by the treatment with exogenous auxin in *E. globulus*, coupled with changes in gene expression, could trigger the necessary modifications that result in new roots in this species.

4.3. The recalcitrance to rooting can be related to the higher expression of rooting inhibitory genes, as revealed by tissue-specific transcript profiles

Molecular analysis can be refined and strengthened when samples are of pure cell lines or specific tissue types. Laser capture microdissection (LCM) is a powerful tool for such precise tissue and cell-specific studies [26,27].

The RNA extracted from the cambium cells obtained from each harvest point was amplified to aRNA and directly used for gene expression analysis using nCounter® technique (Nanostring Technologies) [40]. This effective technology is very suitable for this kind of study because it is possible to obtain an absolute quantification of the expression of a determined gene using minimal amounts of sample (100 ng RNA) and without further amplification [40]. Although mostly used in studies with animal or human cells, particularly in oncology studies, recent papers have demonstrated its suitability for plant research as well [51,54,55].

The identification of genes that distinguish genotypes differing in their competence to AR has valuable practical applications to engineer plants and to be used as markers. Besides, this information can also provide insights about the variation of this process through evolution [56]. In the case of trees, the use of comparative studies is very important to help understanding molecular mechanisms involved in adventitious root development [50]. The use of cell and tissue specific approaches can be highly valuable to precisely determine the factors involved in a given developmental process. This is the first report about tissue-specific gene expression during AR in *Eucalyptus*. Here we have shown that LCM is a powerful technique for isolating specific cells or tissues without the need for specific markers or transformation protocols, being suitable for specific gene expression studies in woody plants. Using this approach, we were able to investigate in more detail the gene expression patterns directly related to the formation of adventitious roots. The differences in expression of some auxin-related genes in whole cuttings
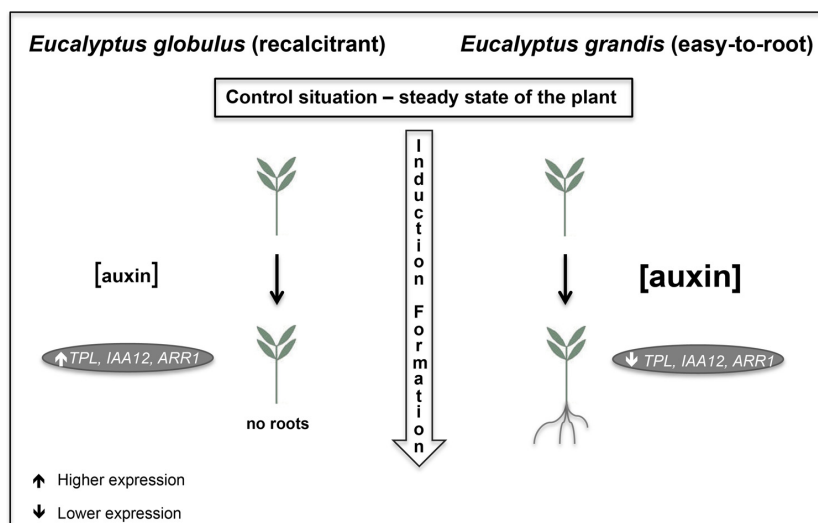
Fig. 5. Working model on the molecular basis of AR phenotypes in *E. globulus* and *E. grandis*.In the recalcitrant species, under control conditions, the lower endogenous IAA concentration and the higher levels of expression of auxin signaling- repressive genes and cytokinin- related gene *ARR1*during formation phase contribute to maintain the recalcitrant phenotype, yielding no roots. On the other hand, in the easy-to-root species, the higher endogenous content of IAA and the lower levels of expression of auxin signaling- repressive genes and cytokinin- related gene *ARR1*during formation phase result in rooted plants. The application of exogenous auxin induces modifications in the levels of expression of auxin-related genes in both species, including repression of *TPL, IAA2,* and *ARR1*in *E. globulus*. The gene expression profiles during the rooting phases under treatment with exogenous auxin result in increased number of rooted cuttings in *E. globulus* and improved adventitious root systems in *E. grandis* (higher number of roots and longer roots).

(Fig. 2) and in cambium cells (Fig. 4) were probably due to the influence of specific characteristics of the various tissues sampled in the cuttings on the final expression pattern.

The apparent higher gene expression in *E. globulus* when compared to *E. grandis*, as well as the earlier increase in expression in the former species, may reflect differences in the establishment and maintenance of auxin homeostasis. It is possible that the overall balance between repressors and promoters of AR development in *E. globulus* may result in slower responses and recalcitrance to rooting. The fact that the expression of several auxin-related genes peaked at later stages of the root formation phase in cambium cells of *E. grandis* control samples can be due to the fact that, in this species, several adventitious roots are already emerging at this point (data not shown), so there is a need for maintaining auxin homeostasis to allow the growth and development of roots and their ramifications. On the other hand, at about the same time in auxin treated samples, it is likely that exogenous auxin is being relocated to other parts of the plant body.

The lower expression of auxin signaling repressive genes in *E. globulus* plants treated with exogenous auxin (Fig. 4) was a striking finding and can help us better understand the causes of recalcitrance to rooting in this species. In good agreement, the rooting competent *E. grandis* had considerably low levels of expression of the same genes (Fig. 4). The results confirmed that the auxin signaling pathway plays an important role in AR, but also highlighted the importance of increasing investigation of crosstalk with other phytohormones, such as cytokinins, for a better knowledge of the process.

Transcriptional co-repressors play an important role in several plant developmental processes. TOPLESS (TPL) is a member of the Groucho (Gro)/Tup1 family of co-repressors and has been shown to be involved in a broad range of signaling pathways in plants [17]. These proteins interact with transcription factors to repress gene expression in different processes by the recruitment of histone deacetylases and modification of chromatin state to inactive [57]. The first well established function of *TPL* was reported in *Ara-*

bidopsis, where *TPL* interacts with Aux/IAA partners to suppress the expression of auxin-related genes, mediating the inhibitory action of these proteins on auxin signaling [16]. An Aux/IAA protein that interacts with *TPL* is *IAA12/BODENLOS*, which binds to *ARF5/MONOPTEROS (MP)* and represses its activity [58]. Considering that *TPL* is important for the repressive action of *IAA12* [16], the simultaneous higher levels of both *TPL* and *IAAI2* in control samples of *E. globulus* could lead to a more repressive action on auxin signaling pathway, impairing root formation and development.

In the present work, the pronounced decrease of *TPL* in cambium cells from *E. globulus* auxin-treated plants, coupled with the low level of expression detected in *E. grandis* in both experimental conditions, further suggests a role for this co-repressor in the regulation of AR in *Eucalyptus* (Fig. 4). Although no effect on AR has yet been determined for *IAA12*, this protein is known to participate in lateral root initiation, acting along with *ARF5/MP*. In this case, the auxin response controlled by the *IAA12/BDL-ARF5/MP* pair acts downstream of the response controlled by the complex formed by *SOLITARY ROOT (SLR)/IAA14-ARF7-ARF19* [59]. Druege et al. [60] found indication from transcriptome data of *Petunia* that expression of genes encoding Aux/IAA proteins may govern the phases of AR formation in cuttings. The different levels of expression of *IAA12* in *E. globulus* and *E. grandis* are in agreement with the profile of *TPL* in the two species. As *IAA12* needs *TPL* to exert its repressive action [16], the low level of *TPL* in the rooting competent *E. grandis* contributes to suppressing *IAA12* inhibitory action.

Besides auxin, genes related to other phytohormone signaling pathways are also important for AR. Cytokinins often inhibit adventitious rooting [41], and this has been previously shown for both *E. globulus* and *E. saligna* [44]. In poplar, a cytokinin type-B response regulator *PtRR13*was found to be a negative regulator of AR, inhibiting the transcription of two *APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF)* genes [23]. Recently, a member of AP2/ERF family *PtERF003*was determined as a positive regulator of AR in poplar, being upregulated by auxin [21]. These previous studies support our findings about the expression of the cytokinin

type-B response regulator *ARR1*, which was reduced by auxin treatment in the difficult-to-root species and remained basally expressed in *E. grandis*. However, since *ARR1*is part of a large gene family, investigation of other members should be done in order to confirm its negative role in AR. Taken together, our findings suggest that *TPL, IAA12* and *ARR1* may contribute for the difficult-to-root characteristic of *E. globulus*, reaffirming the importance of auxin homeostasis and its crosstalk to other phytohormones in AR.

Considering the overall results, a working model is proposed, where different auxin-related genes can act as AR positive regulators in each rooting phase of *E. globulus* and *E. grandis*. Furthermore, the recalcitrance to rooting in *E. globulus* seems to be related to the high expression of auxin signaling-repressive genes and the cytokinin- related gene *ARR1*, affecting the metabolism and action of both phytohormones (Figure 5). *E. grandis*, in turn, shows lower expression levels of these negative regulators. This feature, coupled with its higher level of endogenous auxin, can contribute to its increased competence to rooting. Future studies should address the viability of genetic transformation or genome editing strategies focusing on these recalcitrance-related genes in hard-to-root *E. globulus* and other relevant eucalypts, as tools to help overcoming AR barriers for improved clonal propagation.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.07. 022

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Supplementary information:
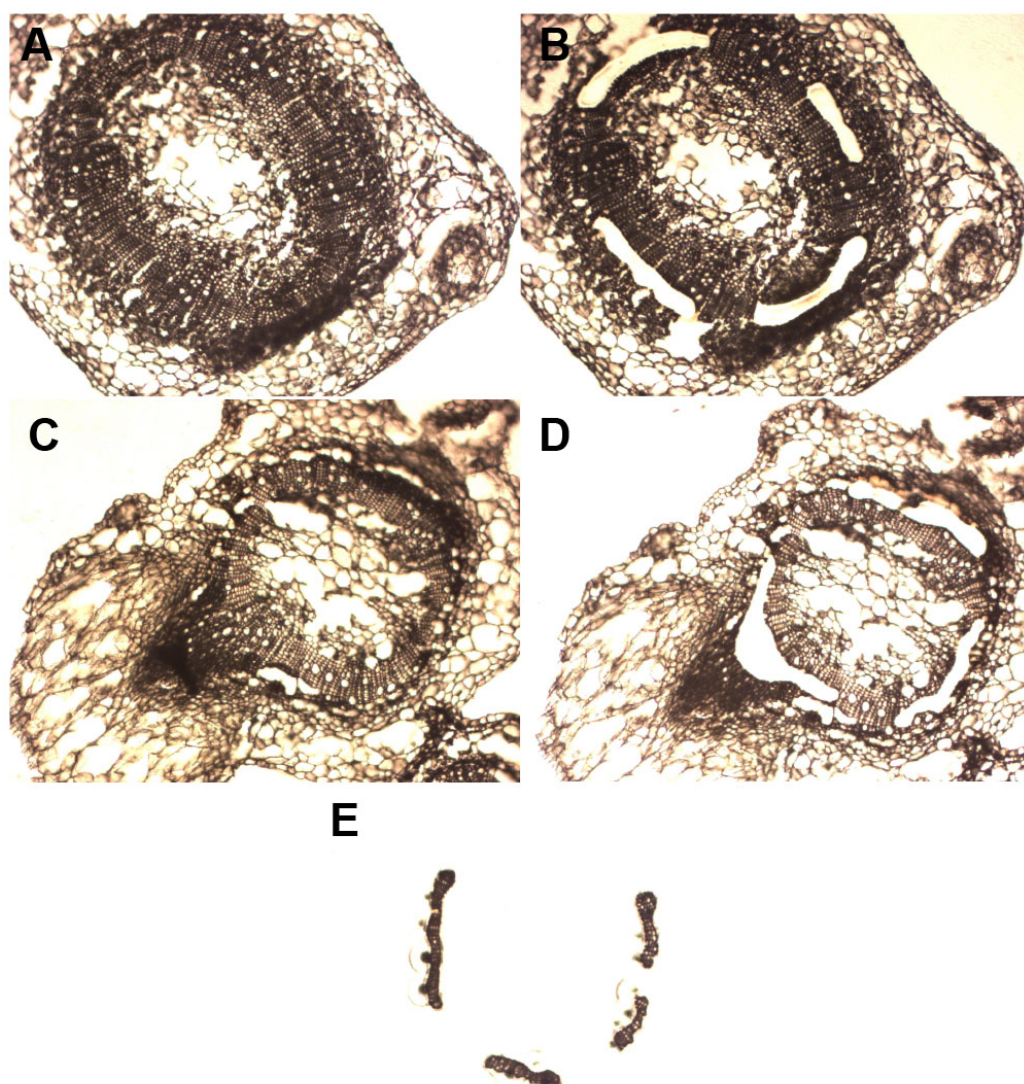
Supplementary Figure S1. Representative pictures from cambium cells selection by LCM. (A, B) *E. globulus* and (C, D) *E. grandis* stem transversal cryosections before – left panels - and after – right panels - LCM procedure. (E) Example of cambium cells collected on LCM caps. 10X magnification.

Supplementary Table S1. Sequences of genes and primer pairs used for qPCR analysis.

Gene symbol	Gene name	Function	Putative *Eucalyptus* ortholog (Phytozome)	Primer sequence (5' → 3') Forward/Reverse
TAA1	Tryptophan Aminotransferase of Arabidopsis 1	Involved in the shade-induced production of indole-3-pyruvate (IPA), a precursor to IAA.	Eucgr.H02519.1	GGGAACAGGTGACAAACTCAA/ TGAAGGCGGATTACATGGAT
YUC3	YUCCA 3	Oxidoreductase and flavin-containing monooxygenase activity; involved in auxin biosynthetic process.	Eucgr.H02874.1	AGCTTCACCTTCCAAAGCAA/ TGTCAAAGTGCCTGGCATAG
ABP1	Endoplasmic Reticulum Auxin Binding Protein 1	Auxin binding protein involved in cell elongation and cell division.	Eucgr.H01544.1	TTGCAGCCACTTTCTGACTG/ GATCAAACATCGGGGTATGC
TIR1	Transport Inhibitor Response 1	Auxin receptor that mediates auxin-regulated transcription.	Eucgr.K03439.1	GTGGCACTATGGTGTGGTGA/ AGCTCAGCCAAGTGCAAAAT
ARF6	Auxin Response Factor 6	Transcription factor; mediates auxin response via expression of auxin-regulated genes.	Eucgr.D00264.1	AGCTTGCTCGCATGTTTAGC/ GCCATCACCAAGGAGAAGACT
ARF17	Auxin Response Factor 17	Transcription factor; regulates early auxin responsive genes.	Eucgr.F04380.1	AAGAACTCGAAGGGCGACAT/ CCACTCTCCTCCTCTACCTTCA
AUX1	Auxin Resistant 1	Auxin influx transporter.	Eucgr.A00514.1	CAGCTTCAAGAACCACGTCA/ CGAAGAGGAGGAAAGTGCAG
PIN1	PIN-Formed 1	Auxin efflux transporter.	Eucgr.K02271.1	ACCTCATGGTCCAGATCGTC/ ACCTCGGATGGAGATGATGG
H2B	Histone H2B	Structural constituent of the eukaryotic nucleosome core.	HO048243 *	GAAGAAGCGGGTGAAGAAGA/ GGCGAGTTTCTCGAAGATGT
TUA	Alpha-tubulin 5	Structural constituent of cytoskeleton, microtubule-based processes.	HO048250 *	ACCGGTTGATCTCTCAGGTG/ TAAGGGACCAGGTTGGTCTG
ACT2	Actin 2/7	Structural constituent of cytoskeleton.	HO048249 *	TCCACCATGTTCCCTGGTAT/ ACCTCCCAATCCAGACACTG

* Gene Bank accession numbers. Primers from De Almeida et al, 2010 [32].

Supplementary Table S2. Genes and primer pairs used for tissue-specific gene expression analysis by nCounter (*Nanostring Technologies*).

Gene	Accession (*Phytozome*)	Description	Target Region	Target Sequence
ABP1	Eucgr_H01544.1	Endoplasmic Reticulum Auxin Binding Protein 1	657-756	TGACTGATAATGTCCCGTGGGGTGTTT AGGGGACATAAATGAGGGATGGAGGG AACTTGGAAATGCATTGTGTCCTTGTC CGATAACAGAACTTAGCCAG
ACT2	HO048249.1*	Actin 2/7	596-695	CTGGTATTGCAGACAGGATGAGCAAG GAGATTACTGCTCTTGCTCCAAGCAGC ATGAAGATTAAGGTGGTAGCACCGCCA GAGAGGAAATACAGTGTCTG
ARF17	Eucgr_F04380.1	Auxin Response Factor 17	1474-1573	TCTTCCATCCAGCTATTTGGTAAGACTA TTCATGCGATGAAGCCTGCTGAAAGCA ATTTAGATGGTGTTGTTAGCCCATCAG ATGATGGCTCCAAAAGGC
ARF6	Eucgr_D00264.1	Auxin Response Factor 6	2063-2162	ATTTAGAACCCTCTTCTCTTCTGATGCA AAATGGGATCTCGAGCCTAAGGGCCG TTGGTAGCGAAAGTGACTCAACATCCA TGCCCTTCCCTTCTAACTA
ARR1	Eucgr_A00189.1	Cytokinin type-B Response Regulator 1	1959-2058	GGGTATGCCGAGCTACGATATACTCCA TGACTTGCAGCAAATTAGATCCAATGA TTGGGAGTTTCAGAATGTGGGTGTGAC GTTTGATACATCGCAACCA
AUX1	Eucgr_A00514.1	Auxin Resistant 1	2175-2274	TCCTATTTTGTCCAAGTAGATCATGTAA CATATACATGGGGATAGTGGGCACTGG GCAATGATTCCCCTTCCCCTCCATGTG ATGTGTGCTGGAATTTCC
H2B	HO048243.1*	Histone H2B	175-274	AAGGCCATGGGCATCATGAACTCCTTC ATCAACGACATCTTCGAGAAACTCGCC CAGGAGGCCTCGAGGCTAGCCAGGTA CAACAAGAAGCCCACCATCA
IAA12	Eucgr_H02914.1	Indole-3-Acetic Acid Inducible 12/ Bodenlos	960-1059	TGTTCCTTGGGGGATGTTCCTCACTGC CGTCAAGAGACTAAGAATCATGAGGAC TTCTGAAGTGAATGGAATAGCTCCAAG ATTCCAACAAAAGAGCGAG
IDH	HO048252.1*	NADP- Isocitrate dehydrogenase	221-320	TTAGGAACATCTTGAATGGCACTGTCT TCAGAGAGCCAATAATGTGCAAAAATA TTCCCCGGCTTGTCCCAGGGTGGTCC AAGCCAATATGCATTGGAAG
PGP4	Eucgr_K00570.1	Multidrug Resistance 4, P-glycoprotein 4	3930-4029	AGCAGGTGGCCATCCCGGTTTTTCTTG CGGGTTTCATCATGAAAATTACCTGCG AGGTGATGAGAAAGAAAGCCAGAAGT GCATCTAAAACATGTGGCGG
PIN1	Eucgr_K02271.1	PIN Formed 1	1988-2087	GGAGGGCAGAAGGGGGAAGATAGAAT GCTTAATGTTTAAATGCGTTAAAAAGG GGAAGTTGTATAATCATGATCTTGGTA GTCAAAGGTAGGTTGAAAGA
TAA1	Eucgr_H02519.1	Tryptophan Aminotransferase of Arabidopsis 1	1153-1252	GGGAACAGGTGACAAACTCAAGTTGAT ATAGACAGAAAATTTTGTTGCAGCCTCT AGTTTCTAATCATGTAACCCCGAGGTG ATAGTTTGAATGGCCTAC
TIP41	HO048247.1*	TIP41-like family protein	82-181	TGAATAGTGGGACGTCAATCCATTACA ATGCTTTTGATGCTCTTGCTGGTTGGA AGCAGGAGGGTTTGCCACCCGTTGAA GTTCCTGCTGCGGCAAAATG

TIR1	Eucgr_K03439.1	Transport Inhibitor Response 1	2003-2102	AGCAGGTACATGCTCTTCACCGCCCTC TTTCATGCAGGTCTAATTTCTTTTTAGC TTAGTATTAGCAGCTGTTATTGGTGGT GACATTAGCTGTGGGAAG
TPL	Eucgr_C01368.1	Topless	2246-2345	GGGCAAGCCTGGTTGATAGAGGTGCT CCAGTGGCAGCAATGGTTAGTATGAAC AGTGAGAGCATGGCTGATGTGAAGCC CAAATTTGCTGATGAGTCAGG
TUA	HO048250.1*	Tubulin alpha	293-392	ATCTATGACATCTGCCGAAGATCCCTC GATATTGAGCGTCCCACATACACTAAT CTGAACCGGTTGATCTCTCAGGTGATC TCATCCTTGACTGCCTCTC

* Gene Bank accession numbers. Probes are amplifying the same region as in De Almeida et al, 2010 [32].

3.2 Capítulo 2

Proteomic profiles during adventitious rooting in *Eucalyptus globulus*

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Proteomic profiles during adventitious rooting in *Eucalyptus globulus*

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Abstract

Adventitious rooting (AR) is essential for vegetative propagation of forest species. The cellulose and paper industries are interested in *Eucalyptus globulus* and its hybrids due to their low lignin content, which facilitates cellulose extraction. However, this species, and often its hybrids, is recalcitrant to rooting, requiring auxin application. Here, we investigate the proteome changes during AR of *E. globulus* and the effects of exogenous auxin in different phases of the process, using 2-D electrophoresis. We found 108 spots in induction (8 specific of control plants and 29 of auxin-treated plants) and 165 spots in formation phase, being 50 specific of control and 17 of auxin-treated plants. By MS/MS analysis we identified 44 and 18 proteins during induction and formation, respectively. From those, 13 proteins were specifically present during induction phase and 3 during formation phase. The proteins are predicted to be involved in different biological pathways, mainly energy metabolism, oxidative stress, photosynthesis and plant defense, with some pathways possibly regulated by auxin. Interesting differences between induction and formation phases also include proteins involved in energy metabolism, besides active transport and cell cycle. These proteins can be used in future studies as potential markers of rooting phases and to evaluate rooting competence in different genotypes.

Keywords: 2-D electrophoresis, protein pattern, adventitious roots, auxin, *Eucalyptus*

1. Introduction

Eucalyptus is one of the most planted trees worldwide due to its fast growth, high quality of fibers and high adaptability to different kinds of soils. These features are very interesting for both wood and cellulose industries, increasing the economic importance of Eucalyptus species. *Eucalyptus globulus* is of high interest due to its low lignin content, which makes cellulose extraction easier, decreasing costs of this industrial process. However, this species and its hybrids are recalcitrant to rooting, making vegetative propagation difficult and causing loss of productivity.

Propagation of the majority of economically relevant forest, horticultural and ornamental plants is performed rooting leafy cuttings and establishing clonal gardens. The stem cuttings develop adventitious roots, which can be formed from organs such as leaves and stems and share important differences and similarities with lateral roots [1]. Adventitious rooting (AR) is a complex process known to be affected by several factors such as phytohormone concentration, phenolic compounds, light, wounding, genetic traits and nutritional conditions [2]. This process can be divided in two main phases: (1) induction, comprising the first molecular and biochemical events; and (2) formation, where the first cellular divisions occur, originating the root primordium, followed by root elongation [3].

Auxins are effective inducers of adventitious roots in different woody species [4]. The different phases of AR have different hormonal requirements. High concentrations of auxin are needed at rooting zone during induction, but a decrease in this concentration is required during formation phase, as auxins can impair root elongation [4]. Also, the accumulation and local concentration of auxin in the base of cuttings seems to be important for starting the rooting process [2]. In some recalcitrant to rooting *E. globulus* clones, the auxin concentration was found to be lower than in clones with higher rooting capabilities [5]. When comparing endogenous auxin content in stem cuttings of *E. globulus* and the easy-to-root species *E. grandis*, the concentration of the former is about half of the concentration of the latter. Exogenous auxin treatment can restore the capability of *E. globulus* to produce roots [6].

Although relatively well studied regarding physiological aspects, the genetic and molecular mechanisms involved in AR are still poorly known, especially concerning woody plants. Recent findings have indicated different genes as positive or negative regulators of AR [7-9], but little is known about the proteins involved in this process [10,11].

Proteomics is a relatively recent area of research in trees, as they are in general considered hard-to-work species for molecular studies. This is due to large life cycles,

difficulty in plant transformation and less availability of molecular tools. The proteome coverage in plant biology is still considered very low if compared to animal science. Challenges such as difficult protein extraction decrease the speed of data acquisition, and thus a low number of protein entries in databases is deposited [12]. Considering woody plants, the most studied species belong to the genus *Populus, Pinus, Picea, Fagus, Eucalyptus* and *Quercus.* Several studies have been developed in the past three decades, covering different biological aspects [12]. However, with a few exceptions, most protein-related research in trees is focused in descriptive and differential expression proteomics [12].

In *Eucalyptus,* the last decade was responsible for important advances in the proteomics field. Interesting findings include the identification of proteins related to lignification process [13], osmotic stress [14], water stress [15,16], heavy metals contamination in the soil [17], reproductive diversification in the genus [18], and pathogen infection [19]. On the other hand, the knowledge about protein pattern related to AR process is still scarce. Until now, only a few studies have focused in proteome investigation during AR. Sorin et al [20], studying the process in Arabidopsis, were able to identify 11 proteins involved in different processes, including auxin homeostasis and light-associated pathways. In chrysanthemum cuttings, Liu et al [10] have found a positive correlation of auxin-induced proteins with AR and a negative correlation of the ethylene-related protein CmACO (ACC oxidase) with the process. Besides those, other proteins related with several biological processes were identified. More recently, Han et al [11] have performed a joint analysis of transcriptome and proteome during AR in hybrid larch and have found that proteins related to polyamine synthesis and stress response can play important roles in adventitious roots development. And finally, investigating the proteins that influence the rooting process in etiolated juvenile branch cuttings of *Robinia pseudocacia*, Lu et al [21] have found proteins related to different biological processes, such as signaling, lignin synthesis, nucleoglucoproteins and phyllochlorin.

In an attempt to better understand the protein pattern related to the AR in *Eucalyptus globulus*, we performed a Mass Spectroscopy- based identification of the proteins that were present during induction and formation phases of the process in plants treated or not with the phytohormone auxin. We found several AR control and auxin-treatment-specific proteins, besides relevant differences among induction and formation phases. Our results shed further light on the mechanisms of AR and may provide a basis for identifying genotypes that are more prone to AR in *Eucalyptus globulus* by evaluating protein patterns.

2. Results

2.1. 2-DE maps of present proteins during AR

Proteins that were present during AR in control and auxin-treated plants of *E. globulus* were initially evaluated by two- dimension electrophoresis (2-DE) using a pH gradient from 3 to 10. As the resolution was very low (data not shown), we decided to perform the 2D gels using a narrower pH gradient, from 4 to 7. Using this approach, we were able to detect a total of 108 spots during induction phase and 165 spots during formation phase, considering both conditions analyzed (Figure 1). The spots were evaluated considering common and differential expression when comparing control and auxin-treatment conditions. During induction phase, 63 spots (58%) were common for both control and auxin treatment conditions, without difference in expression. From the spots differentially expressed, only one (1%) was highly expressed in auxin-treated plants and seven spots (7%) showed higher expression in control plants. Eight spots (7%) were specific from control plants and 29 spots (27%) were specific of auxin-treated plants. (Figure 2A). On the other hand, during formation, there were 83 (50%) common spots. Considering the differentially expressed spots, 15 (9%) were highly expressed in auxin-treated plants, 50 (31%) were specific of control and 17 (10%) were specific of auxin-treated plants (Figure 2B). We were able to select 104 spots from induction and 63 spots from formation phase gels. However, several spots from formation phase had poor quality spectra and were removed from analysis (data not shown).

2.2. Functional classification of identified proteins

We were able to identify 44 and 18 proteins during induction and formation, respectively. In total, the identified proteins belong to different biological pathways, with 4% corresponding to unknown proteins. However, 44% of the proteins did not meet any result from the databases and were grouped as "no hit". When different results were retrieved for the same spot, a correct identification was not possible. The protein mixture is a common event in proteomic analysis, as proteins with similar pI and mass can migrate together. Another possible explanation is cross contamination from neighbor spots or protein degradation [14, 22]. To avoid misinterpretation of results, the corresponding spots were grouped as "no confident identification/ hit" and correspond to 4% of total of analyzed proteins (Figure 3).

During induction phase of AR, most part of successfully identified common proteins was involved in oxidative stress and energy metabolism, followed by photosynthesis and plant defense (Table 1). From the differentially expressed proteins during induction, three proteins highly expressed in control plants were identified as involved in energy metabolism and oxidative stress biological pathways such as Fructose-bisphosphate aldolase (spot 45), Superoxide dismutase (spot 16) and Plastid-lipid-associated protein (spot 68) (Table 2). Considering the condition-specific proteins, five from the eight proteins that were identified as specific of control plants cover chaperones and proteins related to mRNA processing and photosynthesis. They include grpE protein (spot 73), 28 kDa ribonucleoprotein (spot 74), Oxygen-evolving enhancer protein 1 (spots 72 and 77) and Chloroplast photosystem II light harvesting complex (spot 79) (Table 2). In the case of proteins specifically expressed in auxin-treated plants, only six match consistent hits in the databases used, being one of them unknown. The hits correspond to proteins involved in energy metabolism, photosynthesis and protein destination, including Malate dehydrogenase (spot 83), Plastid-lipid-associated protein 6 (spot 87), Ribulose 1,5-bisphosphate carboxylase (spot 82), Chloroplast photosystem II light harvesting complex (spot 86) and Proteasome subunit beta type-6 (spot 84) (Table 2).

During formation phase of AR, most proteins identified were involved in energy metabolism and oxidative stress (Table 3), similarly to what was verified during induction phase (Table 1). When comparing the proteins with common expression during induction and formation, 13 proteins were found just during induction and 3 were specifically expressed during formation phase (see Tables 1 and 3). However, considering that the analysis of several spots from formation phase was not possible due to bad quality, it is possible that several proteins found in induction samples are also present in formation samples. So we can only confirm the specificity of the proteins found exclusively in formation phase. Among those are the energy metabolism- related protein enolase (Spot 2), the nucleosome core protein Histone H3 (Spot 19), involved in DNA packaging, and the ATP-binding cassete protein ABC transporter A (ABCA - Spot 3), responsible for translocation of substrates across cell membranes (Table 3).

3. Discussion

Most identified proteins in this work are involved in energy metabolism, oxidative stress, photosynthesis and plant defense biological pathways. Considering the AR process *per se*, the finding of proteins involved in stress and defense is expected, mainly due to the wound

caused by the separation of the cutting from the donor-plant [23]. The expression of the enzyme Superoxide dismutase (Table 1) indicates the production of hydrogen peroxide (H_2O_2) [24]. H_2O_2 might function as a signaling molecule involved in the formation and development of adventitious roots, as observed in mung bean [25] and olive cuttings [26]. 2-Cys peroxiredoxin BAS1 and thioredoxin (Table 1) act to avoid the toxic effect of H_2O_2, as these proteins are involved in H_2O_2 reduction and protection of photosynthetic apparatus against oxidative damage [27, 28]. Phenylcoumaran benzylic ether reductase (PCBER) (Table 1) and isoflavone reductase (Tables 1 and 3) are involved in the biosynthesis of several phenylpropanoid-derived plant defense compounds, including lignans. A function has been suggested for PCBER in reducing phenylpropanoid dimers to form antioxidants that protect the plant against oxidative damage [29]. Isoflavone reductase is also involved in the biosynthesis of isoflavonoids. The accumulation of phenolic acids and flavonoids was correlated with *in vitro* rooting, as these compounds can modulate peroxidase activity and prevent auxin degradation at cutting bases [4]. Isoflavone reductase was found in the cambium region of *E. grandis* [13], an important region for adventitious roots development [6]. These proteins are probably acting together in helping the plant control oxidative damage during AR, particularly during the acute phase of induction right after cutting severance.

The wound can also act as an enter point for pathogens or a signal for attack by herbivores, so the expression of different defense proteins is useful to restore plant integrity. The allergen Pru ar 1-like (Tables 1 and 3) is an enzyme involved in plant defense, causing oral allergy syndrome [30]. This allergen was also found in roots of *E. globulus* plants grown hydroponically, being triggered by environmental factors [14]. Cell wall pectinesterases are involved in cell wall extension, to facilitate water absorption and increase turgor pressure for cell growth [31]. This phenomenon is very important during development of root primordium and root elongation [2], in good agreement with its finding during formation phase of AR (Table 3). On the other hand, pectinesterases can also play a role in plant defense, modifying cell wall stability [32]. Considering the kinetics of AR process, this could be an explanation for its expression during induction phase (Table 1).

The energy metabolism pathway was also highly represented during AR in *Arabidopsis* and *Chrysanthemum* plants and may play important roles in carbon chain storage and use during this process [10, 20]. The enzyme Fructose-bisphosphate aldolase was negatively correlated with adventitious root number and free IAA content in both species [10, 20]. These results corroborate our findings, as this protein was highly expressed in control plants during induction phase, when comparing to auxin-treated plants (Table 2). Previous

results from our group confirmed that *E. globulus* control plants produce less roots and have less IAA content than auxin-treated plants [6].

Proteins specifically found in control plants include chaperones and a ribonucleoprotein (Table 2). Chaperones are responsible for folding, assembling, translocation and degradation of proteins, maintaining their functional conformations and preventing the aggregation of misfolded proteins [33]. This class of proteins was also found in the cambium region of 6 months- old *E. grandis* [13]. Together with proteins related with mRNA processing, chaperones can act as post-transcriptional regulators of adventitious root production [34]. Although not the same protein as the one identified here, a potential role for a ribonucleoprotein was reported during AR in *Chrysanthemum* cutting bases [10].

The auxin-treatment specific protein Proteasome subunit beta type-6, also known as 20S proteasome beta subunit A-1, is involved in protein fate and can indicate increase in protein degradation in auxin-treated plants. The 20S proteasome subunits were also found in *Arabidopsis*, although not correlated to AR [20]. On the other hand, the respective transcripts were described to be potentially associated with adventitious roots development in *Pinus contorta* [35].

Among proteins differentially expressed between control and auxin-treated plants, most chloroplast proteins were highly expressed or specific of control plants, which are recalcitrant to rooting (Table 2). Considering that the treatment with auxin can restore rooting capacity of *E. globulus* plants, this trend matches results found in *Arabidopsis*, where several plastid- encoded proteins were accumulated in the genotypes that produced less adventitious roots [20]. Brinker et al [35], in a microarray analysis during AR in *P. contorta*, proposed that the down-regulation of plastid proteins can be due to the loss of photosynthetic capacity of hypocotyl cells during adventitious roots formation.

In contrast to what we found during AR induction, during formation a higher number of spots were specific of control condition when compared with auxin-treated plants (Figure 2). These findings may indicate that important protein-mediated mechanisms are taking place during this process in control plants, which could be associated with rooting recalcitrance in *E. globulus*.

Besides the differentially expressed proteins between control and auxin-treated plants during induction, an interesting finding was related to proteins specifically expressed in formation when compared to induction phase (Tables 1 and 3). Enolase is an enzyme of the glycolytic pathway responsible for the conversion of 2-phosphoglycerate to phosphoenolpyruvate. The presence of enolase was also found in etiolated branches of

Robinia pseudocacia, exclusively at the ninth day of AR process [21]. In investigation of proteome of the cambium region in trees of *E. grandis*, Celedon et al [13] observed the expression of both ATP synthase and enolase. Considering the importance of cambium region in the formation of adventitious roots [6], these proteins may have important functions in providing energy compounds to maintain the activity of cambium cells during AR formation.

Other formation-specific proteins include the ATP-binding cassete protein ABC transporter A and the nucleosome core protein Histone H3 (Table 3). Members of ABC transporters are divided into subfamilies ABCA to ABCI, being the ABCH subfamily absent in plants. These ATP transporters have been implicated in several processes such as polar auxin transport, lipid catabolism, xenobiotic detoxification, disease resistance, and stomatal function. However, even if each subfamily has a predicted function for its members, many of them can deviate from those predictions [36]. The ABCA transporters are still poorly known in plants and are predicted to act in cellular lipid transport [36]. Lipid metabolism is not well studied during rooting, but has been pointed out as relevant in cuttings of different varieties of *Perle Noir* vines [37]. In *Chrysanthemum* base cuttings, the lipid metabolism-related protein Stearoyl-ACP desaturase was negatively correlated with AR [10], opening questions on the roles of lipids during rooting in different species. Considering its role in nucleosome structure and DNA packaging, Histone H3 can also be necessary for cell cycle progression. The expression of histone H3 transcripts was increased during development of adventitious root primordia in *P. contorta,* deepwater rice, and olive cuttings [35, 38, 39]. The expression of this gene indicates initiation of DNA synthesis in the cells right before division. Considering that during formation phase the root primordia starts to develop to emerge out of the cutting [3], these findings are in agreement with our results, as the expression of Histone H3 was only detected in formation phase harvested plants, probably acting during the first cellular divisions to form adventitious root primordia in *E. globulus*. The fact that this protein was expressed in both control and auxin-treated plants suggests plants in both conditions have the machinery to produce roots but, for some reason, these roots are not developed in control conditions. A recent study comparing *E. globulus* and the easy-to-root species *E. grandis* suggests that recalcitrance can be related to the expression of inhibitory genes involved in auxin and cytokinin pathways [6].

The proteomic analysis during AR in *E. globulus* plants treated or not with auxin allowed the identification of several proteins whose expression was related to the different phases of the process. Some proteins were correlated with the rooting phenotypes of auxin-treated and untreated plants (control). Interesting differences between induction and formation

phases include proteins involved in energy metabolism, active transport and nucleosome structure/ cell cycle, and can help unraveling the puzzle of post-transcriptional mechanisms controlling AR in woody plants. This is the first proteomic study in *E. globulus* during AR and the results herein described represent a key step in the knowledge about this developmental process at the protein level.

4. Experimental Section

4.1. Cutting source

Seeds from *E. globulus* were submitted to surface sterilization with ethanol 70% (1 min) and NaClO 1.5% (20 min), followed by four washes with autoclaved distilled water. Fifteen seeds were sown per glass flasks containing 60 mL of germination medium (MS salts 0.5X [40], with the concentration of calcium chloride reduced to 1/6, 2% (w/v) sucrose, pH adjusted to 5.8 ± 1 and 0.6% (w/v) agar). After 3.5 months, tip microcuttings were obtained and used for AR experiments.

4.2. Adventitious rooting conditions

Tip microcuttings were placed in rooting induction medium (MS salts 0.3X, 0.4 mg l^{-1} thyamin, 100 mg l^{-1} inositol and 30 g l^{-1} sucrose, presence (auxin treatment) or absence (control) of 10 mg l^{-1} indolyl-3-butyric acid (IBA), pH adjusted to 5.8 ± 1 and 0.6% (w/v) agar) [3, 41]. The plants were harvested after 2 and 4 days in induction medium and mixed for protein extraction. For analysis during the formation phase of AR, the plants were kept four days in induction medium and then transferred to formation medium (same medium as induction, but without auxin and containing 1 g l^{-1} activated charcoal). The plants were harvested after 2 and 4 days in formation medium and mixed for protein extraction. The experiments were repeated three times with similar results. Six plants were used for each time-point (days of harvesting), totalizing 12 plants in induction and 12 plants in formation phase, for both control and auxin-treatment conditions. Three independent experiments (replicates) were performed, totalizing 36 plants for each phase, for both conditions.

4.3. Protein extraction and 2D gel electrophoresis

The plants were pulverized with liquid N_2 and total protein was extracted with freshly made buffer (0.5M Tris HCl, 2M NaCl, 50mM $MgCl_2$, 4% CHAPS, 20mM DTT) added of Protease Inhibitor Cocktail (*Sigma*) (3.5 μl per 100 mg of pulverized plant material). Glass beads were used in the same quantity of tissue to facilitate the extraction. After 8 cycles of 1 minute of vortexing and 1 min in ice, the extracts were centrifuged at 4°C at 13,000 g for 10 min. The supernatant was submitted to dialysis for 96 hours, at 4°C and constant agitation. After dialysis the supernatant from each replicate (3) was joined, producing one extract for each sample: induction control, induction +AUX, formation control, formation +AUX. Next, the extracts were lyophilized at -60°C and stored at -80°C. The protein quantification was performed using the Bradford method [42], after a TCA: acetone (1:9 v/v) precipitation to eliminate the chlorophyll. About 200 μg of protein was used for two- dimension electrophoresis. The samples were submitted to the 2-D Clean-Up kit (*GE Healthcare*) following manufacturer recommendations, centrifuged and the supernatant was mixed to solubilization buffer (7M Urea, 2M Thiourea, 4% CHAPS, 1% DTT, 0.2% ampholyte, 0.001% bromophenol blue). After 40 min of incubation at 4°C, the samples were centrifuged at room temperature, at 8,000 g for 40 min. The isoelectric focusing was performed during 9 hours in 17 cm immobilized 4-7 pH strips, which were previously hydrated for 16 hours with the sample proteins. The 2-D electrophoresis was performed in 10% polyacrylamide gels. Three gels were performed for each sample. The protein spots were stained with Comassie Blue G-250 and analyzed with Image Master 2D Platinum 7.0 (*GE Healthcare*).

4.4. Spot selection and Trypsin digestion

Both common and condition-specific spots were selected for protein identification by MS/MS analysis. The gel pieces containing spots were treated with 50% (v/v) acetonitrile in 25 mM of NH_4HCO_3, pH 8.0, for 15 min, followed by 2 washes with the same solution. The pieces were then incubated in 100% acetonitrile for 5 min, dried in a vacuum centrifuge and the proteins were then digested in Trypsin solution (10 mg l^{-1} in 25mM NH_4HCO_3, pH 8.0) during 24 h at 37°C. Finally the resulting peptides were extracted twice by washing the gel pieces in solution containing 50% acetonitrile and 5% Trifluoroacetic Acid (TFA) during 30 min. Before analysis by MS/MS the samples were completely dried in a vacuum centrifuge.

4.5. Analysis and Protein identification

The samples were analyzed in a nano ACQUITY UPLC System (*Waters*) coupled with a Q-Tof micro^{TM} Mass Spectrometer (*Waters*), with the following parameters: acquisition time of 30 min, acquisition range from 200 to 2,000 Da, MS/MS range from 50 to 2,000 Da, ionic detection charge +2+3 and collision energy of 35 volts. The flow rate was 600 nl min^{-1}, in a non-linear gradient from 99% to 90% acetonitrile. The protein identification was performed using MASCOT search engine v3.5 - MASCOT MS/MS Ion Search software (www.matrixscience.com) (*Matrix Science Ltd*., London), with the following parameters: taxonomy restriction to Viridiplantae, one missed cleavage, 0.2 Da mass tolerance and 0.2 Da for MS/MS data, cysteine carbamidomethylation as a fixed modification and methionine oxidation as a variable modification. The search for the peptide sequences obtained from MS/MS spectra was performed in the NCBI nr databank. For spectra without a match in NCBI, we also searched Swiss Prot database.

Acknowledgments

Author contributions

Conceived and designed the experiments: JS, AGFN. Performed the experiments: MRA, JS, CF. Analyzed the data: MRA, JS, MRE. Contributed reagents/materials/analysis tools: AGFN, JS. Wrote the paper: MRA, JS, AGFN.

Conflicts of Interest

The authors declare no conflict of interest.

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Figure Captions:

Figure 1. Representative 2D electrophoresis from induction control (A), induction auxin-treated (B), formation control (C) and formation auxin-treated (D) *in vitro* microcuttings of *E. globulus*. The red circles indicate spots that were selected for protein identification through MS/MS analysis.

Figure 2. Spots detected during AR in *E. globulus*. A) Spots detected during induction phase. B) Spots detected during formation phase. The symbol ">" indicates spots that were highly expressed in each condition when compared to the other.

Figure 3. Classification of identified proteins expressed during induction and formation phases of AR in *E. globulus*.

Figure 1.

Figure 2.

Induction

- Common - same expression
- > Auxin-treated
- > Control
- Control- specific
- Auxin-treated- specific

27%

58%

7%

7%

1%

Formation

10%

50%

31%

9%

Figure 3.

1%

3%

4%

4%

12%

1%

17%

5%

4%

1%

2%

1%

1%

44%

- Aminoacid biosynthesis
- Cell wall stability
- Energy metabolism
- mRNA processing
- Oxidative stress
- Photosynthesis
- Plant defense
- Protein destination
- Protein folding
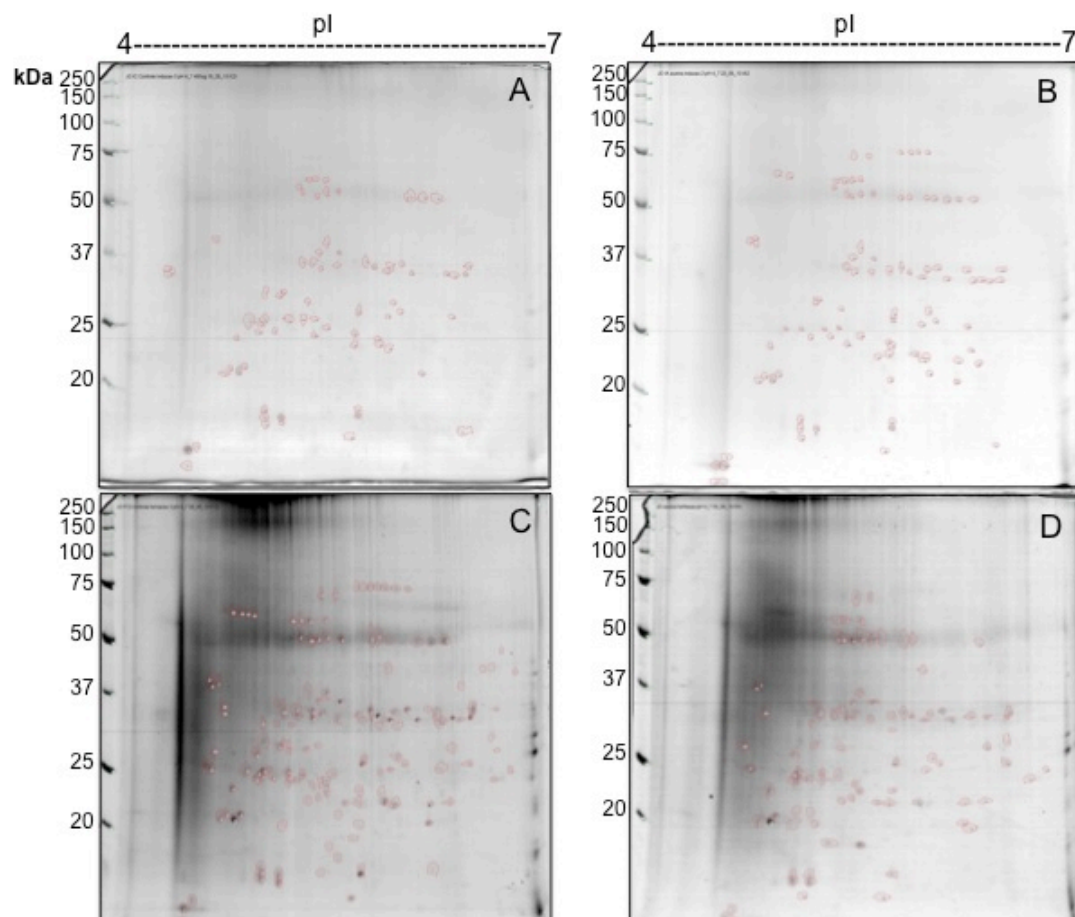- Nucleosome structure
- Transport
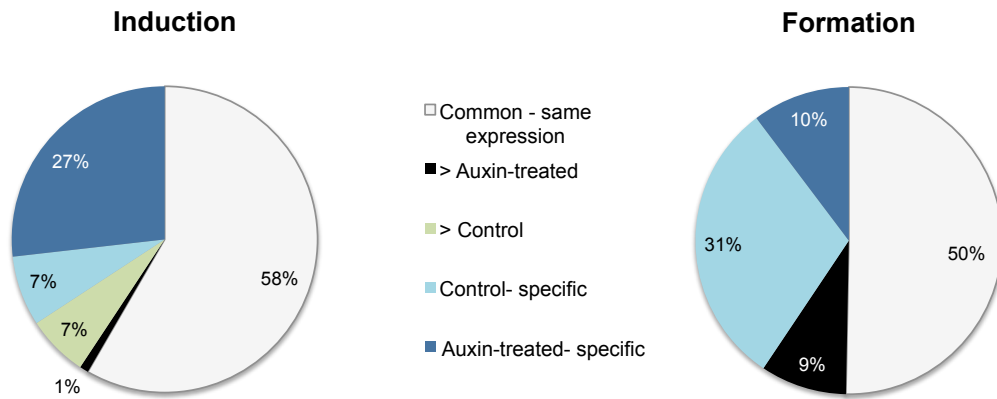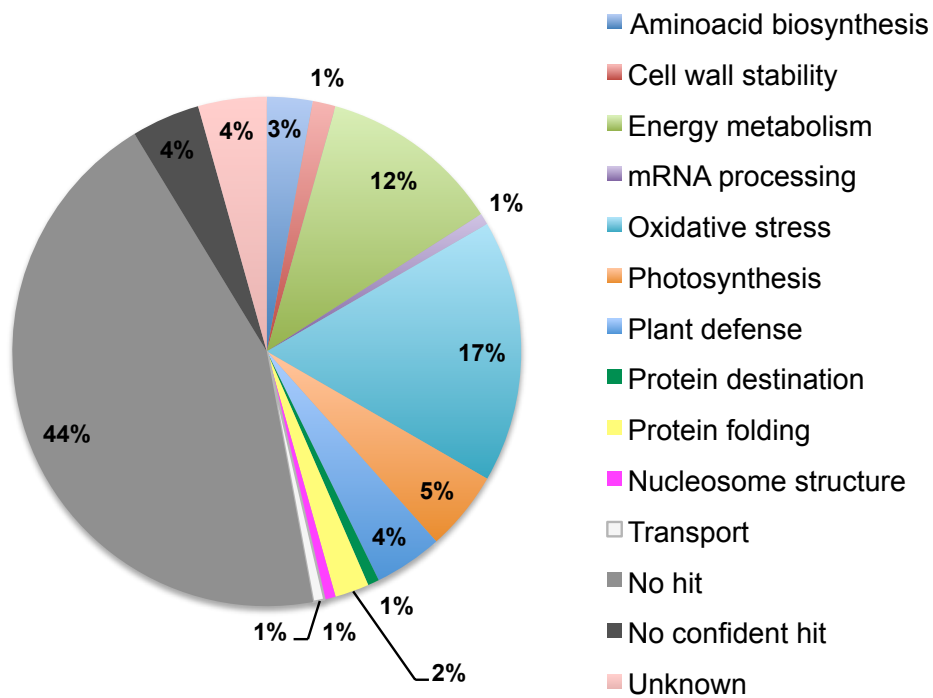- No hit
- No confident hit
- Unknown

Table 1. Common proteins expressed during induction phase of AR in *E. globulus*.

Category	Spot ID	Condition	Assignment Hit	Accession Code	Species	Mascot Score
Amino acid biosynthesis						
	6	auxin-treated	Aspartate aminotransferase	PAT_PINPS*	*Pinus pinaster*	37
	16	control	Aspartate aminotransferase	PAT_PINPS*	*Pinus pinaster*	31
		auxin-treated	Aspartate aminotransferase	PAT_PINPS*	*Pinus pinaster*	31
	47	auxin-treated	Cysteine synthase	gi|702444417	*Eucalyptus grandis*	259
	71	auxin-treated	Aspartate aminotransferase	PAT_PINPS*	*Pinus pinaster*	36
Cell wall stability						
	8	control	Pectinesterase-like	gi|702292007	*Eucalyptus grandis*	84
		auxin-treated	Pectinesterase-like	gi|702292007	*Eucalyptus grandis*	227
Energy metabolism						
	23	control	Fructose-bisphosphate aldolase 1, chloroplastic	gi|702404543	*Eucalyptus grandis*	167
	39	control	ATP synthase CF1 alpha subunit (chloroplast)	gi|108802628	*Eucalyptus globulus*	169
		auxin-treated	ATP synthase CF1 alpha subunit (chloroplast)	gi|108802628	*Eucalyptus globulus*	155
	43	auxin-treated	ATP synthase subunit alpha, chloroplastic	ATPA_AETCO*	*Aethionema cordifolium*	43
	44	control	Malate dehydrogenase, mitochondrial	gi|702377371	*Eucalyptus grandis*	315
		auxin-treated	Malate dehydrogenase family protein	gi|224114557	*Populus trcihocarpa*	84
	46	control	Malate dehydrogenase, mitochondrial	gi|702377371	*Eucalyptus grandis*	139
		auxin-treated	Malate dehydrogenase, mitochondrial	gi|1170898	*Eucalyptus gunnii*	84
	50	control	ATP synthase CF1 beta subunit (chloroplast)	gi|913021841	*Cinnamomum micranthum*	483
		auxin-treated	ATP synthase CF1 beta subunit (chloroplast)	gi|108802650	*Eucalyptus globulus*	738
	51	auxin-treated	ATP synthase CF1 beta subunit (chloroplast)	gi|108802650	*Eucalyptus globulus*	238
	52	control	ATP synthase subunit beta, mitochondrial	ATPBM_HEVBR*	*Hevea brasiliensis*	147
		auxin-treated	ATP synthase subunit beta, mitochondrial	gi|702274704	*Eucalyptus grandis*	337
	55	control	ATP synthase subunit beta, mitochondrial	ATPBM_HEVBR*	*Hevea brasiliensis*	35
		auxin-treated	ATP synthase CF1 alpha subunit (chloroplast)	gi|108802628	*Eucalyptus globulus*	357
Oxidative stress						
	1	control	Superoxide dismutase [Cu-Zn], chloroplastic	gi|702280541	*Eucalyptus grandis*	309
		auxin-treated	Superoxide dismutase [Cu-Zn], chloroplastic	gi|702280541	*Eucalyptus grandis*	236
	2	control	Superoxide dismutase [Cu-Zn], chloroplastic	gi|702280541	*Eucalyptus grandis*	68
	7	control	2-methylene-furan-3-one reductase-like	gi|702465432	*Eucalyptus grandis*	212
		auxin-treated	2-methylene-furan-3-one reductase-	gi|702465432	*Eucalyptus*	247

		like		*grandis*	
9	control	Putative Lactoylglutathione lyase	gi|702321779	*Eucalyptus grandis*	232
	auxin-treated	Putative Lactoylglutathione lyase	gi|702321779	*Eucalyptus grandis*	156
20	control	Phenylcoumaran benzylic ether reductase	gi|383081915	*Eucalyptus globulus*	233
	auxin-treated	Phenylcoumaran benzylic ether reductase	gi|383081915	*Eucalyptus globulus*	161
21	control	Isoflavone reductase-like protein	gi|702335628	*Eucalyptus grandis*	200
	auxin-treated	Isoflavone reductase-like protein	gi|702335653	*Eucalyptus grandis*	64
22	control	Isoflavone reductase-like protein	gi|702335628	*Eucalyptus grandis*	85
	auxin-treated	Isoflavone reductase-like protein	gi|702335628	*Eucalyptus grandis*	109
25	auxin-treated	Isoflavone reductase-like protein	gi|702335638	*Eucalyptus grandis*	111
26	control	Isoflavone reductase-like protein	gi|702335638	*Eucalyptus grandis*	127
	auxin-treated	Isoflavone reductase-like protein	gi|702335638	*Eucalyptus grandis*	97
29	control	Superoxide dismutase [Fe], chloroplastic	SODF_NICPL*	*Nicotiana plumbaginifolia*	41
30	control	2-Cys peroxiredoxin BAS1, chloroplastic	gi|702496718	*Eucalyptus grandis*	264
	auxin-treated	2-Cys peroxiredoxin BAS1, chloroplastic	gi|702496718	*Eucalyptus grandis*	251
31	control	2-Cys peroxiredoxin BAS1, chloroplastic	gi|702496718	*Eucalyptus grandis*	217
	auxin-treated	2-Cys peroxiredoxin BAS1, chloroplastic	gi|702496718	*Eucalyptus grandis*	187
32	control	2-Cys peroxiredoxin BAS1, chloroplastic	gi|702496718	*Eucalyptus grandis*	183
	auxin-treated	2-Cys peroxiredoxin BAS1, chloroplastic	gi|702496718	*Eucalyptus grandis*	137
33	control	2-Cys peroxiredoxin BAS1, chloroplastic	gi|702496718	*Eucalyptus grandis*	134
	auxin-treated	2-Cys peroxiredoxin BAS1, chloroplastic	gi|702496718	*Eucalyptus grandis*	183
38	control	Thioredoxin	gi|702377739	*Eucalyptus grandis*	105
	auxin-treated	Thioredoxin	gi|702377739	*Eucalyptus grandis*	86
70	auxin-treated	Probable plastid-lipid-associated protein 6, chloroplastic	gi|702432052	*Eucalyptus grandis*	137

Photosynthesis

3	auxin-treated	Ribulose-1,5-bisphosphate-carboxylase	gi|11478	*Euonymus bungeanus*	60
34	control	Chlorophyll a-b binding protein of LHCII type III, chloroplastic	CB23_HORVU*	*Hordeum vulgare*	40
	auxin-treated	Chlorophyll a-b binding protein 13, chloroplastic	CB23_SOLLC*	*Solanum lycopersicum*	31
54	control	RuBisCO large subunit-binding protein subunit beta, chloroplastic	RUBB_PEA*	*Pisum sativum*	118
	auxin-treated	RuBisCO large subunit-binding	gi|2506277	*Pisum sativum*	139

56	control	protein subunit beta, chloroplastic ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	gi|1518177	*Amaryllis belladonna*	80
	auxin-treated	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast)	gi|108802651	*Eucalyptus globulus*	87
57	auxin-treated	Ribulose bisphosphate carboxylase large chain	RBL_PACAQ*	*Pachira aquatica*	40
63	control	Oxygen-evolving enhancer protein 1, chloroplastic	gi|702462073	*Eucalyptus grandis*	136
	auxin-treated	Oxygen-evolving enhancer protein 1, chloroplastic	gi|702462073	*Eucalyptus grandis*	213
64	control	Oxygen-evolving enhancer protein 1, chloroplastic	gi|702462073	*Eucalyptus grandis*	217
Plant defense					
4	control	Major allergen Pru ar 1-like	gi|702446752	*Eucalyptus grandis*	360
	auxin-treated	Major allergen Pru ar 1-like	gi|702446752	*Eucalyptus grandis*	278
5	control	Major allergen Pru ar 1-like	gi|702446752	*Eucalyptus grandis*	210
	auxin-treated	Major allergen Pru ar 1-like	gi|702446752	*Eucalyptus grandis*	145
35	control	Major allergen Pru ar 1-like	gi|702446752	*Eucalyptus grandis*	251
	auxin-treated	Major allergen Pru ar 1-like	gi|702446752	*Eucalyptus grandis*	76
36	control	Major allergen Pru ar 1-like	gi|702446752	*Eucalyptus grandis*	239
	auxin-treated	Major allergen Pru ar 1-like	gi|702446752	*Eucalyptus grandis*	160
37	control	Major allergen Pru ar 1-like	gi|702446752	*Eucalyptus grandis*	181
Protein folding					
28	control	20 kDa chaperonin, chloroplastic	gi|702303436	*Eucalyptus grandis*	234
	auxin-treated	20 kDa chaperonin, chloroplastic	gi|702303436	*Eucalyptus grandis*	256
53	auxin-treated	Chaperonin CPN60-2, mitochondrial	gi|702441615	*Eucalyptus grandis*	68
No hit					
0	control	-	-	-	-
	auxin-treated	-	-	-	-
2	auxin-treated	-	-	-	-
6	control	-	-	-	-
10	control	-	-	-	-
	auxin-treated	-	-	-	-
11	control	-	-	-	-
	auxin-treated	-	-	-	-
14	control	-	-	-	-
	auxin-treated	-	-	-	-
15	auxin-treated	-	-	-	-
17	control	-	-	-	-
	auxin-treated	-	-	-	-
18	control	-	-	-	-
	auxin-treated	-	-	-	-
23	auxin-treated	-	-	-	-
25	control	-	-	-	-

29	auxin-treated	-	-	-	-
37	auxin-treated	-	-	-	-
40	control	-	-	-	-
	auxin-treated	-	-	-	-
41	auxin-treated	-	-	-	-
42	control	-	-	-	-
	auxin-treated	-	-	-	-
47	control	-	-	-	-
48	control	-	-	-	-
	auxin-treated	-	-	-	-
49	control	-	-	-	-
	auxin-treated	-	-	-	-
51	control	-	-	-	-
53	control	-	-	-	-
57	control	-	-	-	-
58	control	-	-	-	-
	auxin-treated	-	-	-	-
59	control	-	-	-	-
61	auxin-treated	-	-	-	-
62	control	-	-	-	-
	auxin-treated	-	-	-	-
64	auxin-treated	-	-	-	-
66	control	-	-	-	-
67	control	-	-	-	-
69	auxin-treated	-	-	-	-
71	control	-	-	-	-

* Accession code obtained from SwissProt database.

Table 2. Proteins differentially expressed during induction phase of AR in *E. globulus*.

	Category	Spot ID	Condition	Assignment Hit	Accession Code	Species	Mascot Score
> control (P <0.05)							
	Energy metabolism						
		45	control	Fructose-bisphosphate aldolase, chloroplastic	ALFC_SPIOL*	*Spinacia oleracea*	41
	Oxidative stress						
		16	control	Superoxide dismutase [Fe], chloroplastic (Fragment)	SODF_NICPL*	*Nicotiana plumbaginifolia*	31
			auxin-treated	Superoxide dismutase [Fe], chloroplastic (Fragment)	SODF_NICPL*	*Nicotiana plumbaginifolia*	31
		68	control	Probable Plastid-lipid-associated protein 6, chloroplastic	gi|702432052	*Eucalyptus grandis*	232
	No hit						
		13	control	-	-	-	-
			auxin-treated	-	-	-	-
		19	control	-	-	-	-
			auxin-treated	-	-	-	-
			control	-	-	-	-
		24	auxin-treated	-	-	-	-
		45	auxin-treated	-	-	-	-
		69	control	-	-	-	-
			auxin-treated	-	-	-	-
> auxin-treated (P <0.05)							
	No hit						
		12	control	-	-	-	-
			auxin-treated	-	-	-	-
Condition specific proteins							
	Energy metabolism						
		83	auxin-treated	Malate dehydrogenase, mitochondrial	gi|702365492	*Eucalyptus grandis*	254
	mRNA processing						
		74	control	28 kDa ribonucleoprotein, chloroplastic	gi|702256315	*Eucalyptus grandis*	87
	Oxidative stress						
		87	auxin-treated	Probable Plastid-lipid-associated protein 6, chloroplastic	gi|702432052	*Eucalyptus grandis*	128
	Photosynthesis						

72	control	Oxygen-evolving enhancer protein 1, chloroplastic	gi|470129332	*Fragaria vesca subsp. vesca*	126
77	control	Oxygen-evolving enhancer protein 1, chloroplastic	gi|470129332	*Fragaria vesca subsp. vesca*	87
79	control	Chloroplast photosystem II light harvesting complex protein type III, partial	gi|359754919	*Oxytropis maydelliana*	140
82	auxin-treated	Ribulose 1,5-bisphosphate carboxylase	gi|9909621	*Cannabis sativa*	74
86	auxin-treated	Chloroplast photosystem II light harvesting complex protein type III, partial	gi|359754919	*Oxytropis maydelliana*	131
Protein destination					
84	auxin-treated	Proteasome subunit beta type-6	gi|702298440	*Eucalyptus grandis*	201
Protein folding					
73	control	grpE protein homolog, mitochondrial isoform X1	gi|702492613	*Eucalyptus grandis*	160
Unknown					
95	auxin-treated	Uncharacterized protein LOC103705614	gi|672128283	*Phoenix dactylifera*	53
No hit					
75	control	-	-	-	-
76	control	-	-	-	-
78	control	-	-	-	-
80	auxin-treated	-	-	-	-
81	auxin-treated	-	-	-	-
88	auxin-treated	-	-	-	-
91	auxin-treated	-	-	-	-
92	auxin-treated	-	-	-	-
93	auxin-treated	-	-	-	-
96	auxin-treated	-	-	-	-
97	auxin-treated	-	-	-	-
98	auxin-treated	-	-	-	-
102	auxin-treated	-	-	-	-
103	auxin-treated	-	-	-	-

	104	auxin-treated	-	-	-	-
	106	auxin-treated	-	-	-	-
	107	auxin-treated	-	-	-	-
No confident identification						
	89	auxin-treated	-	-	-	-
	90	auxin-treated	-	-	-	-
	94	auxin-treated	-	-	-	-
	99	auxin-treated	-	-	-	-
	105	auxin-treated	-	-	-	-
	108	auxin-treated	-	-	-	-

* Accession code obtained from SwissProt database.

Table 3. Common proteins expressed during formation phase of AR in *E. globulus.*

Category	Spot ID	Condition	Assignment Hit	Accession Code	Species	Mascot Score
Cell wall stability						
	12	control	Pectinesterase-like	gi|702292007	*Eucalyptus grandis*	102
		auxin-treated	Pectinesterase-like	gi|702292007	*Eucalyptus grandis*	144
Energy metabolism						
	1	control	ATP synthase subunit beta, mitochondrial	gi|702274704	*Eucalyptus grandis*	124
		auxin-treated	ATP synthase subunit beta, mitochondrial	gi|702274704	*Eucalyptus grandis*	206
	2	control	Enolase-like	gi|502111700	*Cicer arietinum*	70
		auxin-treated	Enolase-like	gi|502111700	*Cicer arietinum*	75
	15	control	Malate dehydrogenase, mitochondrial (Fragments)	MDHM_CAPAA*	*Capsicum annuum var. annuum*	75
		auxin-treated	Malate dehydrogenase, mitochondrial (Fragments)	MDHM_CAPAA*	*Capsicum annuum var. annuum*	70
	18	control	Malate dehydrogenase, mitochondrial (Fragments)	MDHM_CAPAA*	*Capsicum annuum var. annuum*	49
		auxin-treated	Malate dehydrogenase, mitochondrial (Fragments)	MDHM_CAPAA*	*Capsicum annuum var. annuum*	52
	26	control	Fructose-bisphosphate aldolase 1, chloroplastic	gi|702404543	*Eucalyptus grandis*	78
		auxin-treated	Fructose-bisphosphate aldolase 1, chloroplastic	gi|702404543	*Eucalyptus grandis*	121
Nucleosome structure and Cell Cycle						
	19	control	Histone H3	gi|19611	*Medicago sativa*	80
		auxin-treated	Histone H3	gi|19611	*Medicago sativa*	87
Oxidative stress						
	11	control	Putative Lactoylglutathione lyase	gi|702321779	*Eucalyptus grandis*	152
		auxin-treated	Putative Lactoylglutathione lyase	gi|702321779	*Eucalyptus grandis*	73
	16	control	Isoflavone reductase homolog	IFRH_SOLTU*	*Solanum tuberosum*	39
		auxin-treated	Isoflavone reductase homolog	IFRH_SOLTU*	*Solanum tuberosum*	34
	17	control	Isoflavone reductase-like protein	gi|702335628	*Eucalyptus grandis*	70
		auxin-treated	Isoflavone reductase-like protein	gi|702335628	*Eucalyptus grandis*	64
	25	control	Isoflavone reductase-like protein	gi|702335628	*Eucalyptus*	120

		auxin-treated	Isoflavone reductase-like protein	gi|702335628	*grandis* *Eucalyptus grandis*	106
Plant defense						
	29	control	Major allergen Pru ar 1-like	gi|702446752	*Eucalyptus grandis*	205
		auxin-treated	Major allergen Pru ar 1-like	gi|702446752	*Eucalyptus grandis*	214
Transport						
	3	control	ABC transporter A family member 7-like	gi|573921721	*Oryza brachyantha*	49
		auxin-treated	ABC transporter A family member 7-like	gi|573921721	*Oryza brachyantha*	49
Unknown						
	5	auxin-treated	Uncharacterized protein LOC103705614	gi|672128283	*Phoenix dactylifera*	51
	7	auxin-treated	Uncharacterized protein LOC103705614	gi|672128283	*Phoenix dactylifera*	54
	8	auxin-treated	Uncharacterized protein LOC103705614	gi|672128283	*Phoenix dactylifera*	49
	14	control	Uncharacterized protein LOC103705614	gi|672128283	*Phoenix dactylifera*	54
	23	auxin-treated	Uncharacterized protein LOC103705614	gi|672128283	*Phoenix dactylifera*	52
No hit						
	5	control	-	-	-	-
	7	control	-	-	-	-
	8	control	-	-	-	-
	14	auxin-treated	-	-	-	-
	23	control	-	-	-	-
	27	control	-	-	-	-
		auxin-treated	-	-	-	-
	28	control	-	-	-	-
		auxin-treated	-	-	-	-

* Accession code obtained from SwissProt database.

3.3 Capítulo 3

Laser Capture Microdissection: Avoiding bias in analysis by selecting just what matters

Márcia R. de Almeida and Martina V. Strömvik

Capítulo publicado no livro *Biotechnology of Plant Secondary Metabolism: Methods and Protocols - Methods in Molecular Biology Series - Springer*.

Laser Capture Microdissection: Avoiding Bias in Analysis by Selecting Just What Matters

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Running head: "Laser Capture Microdissection"

Laser Capture Microdissection: Avoiding Bias in Analysis by Selecting Just What Matters

Márcia R. de Almeida and Martina V. Strömvik

Abstract

Laser capture microdissection (LCM) is a powerful technique for harvesting specific cells from a heterogeneous population. As each cell and tissue has its unique genetic, proteomic and metabolic profile, the use of homogeneous samples is important for a better understanding of complex processes in both animal and plant systems. In case of plants, LCM is very suitable as the highly regular tissue organization and stable cell walls from these organisms enable visual identification of various cell types without staining of tissue sections, which can prevent some downstream analysis. Considering the applicability of LCM to any plant species, here we provide a step-by-step protocol for selecting specific cells or tissues through this technology.

Key words: cell-specific technique, cryosections, plant cells, microgenomics, microscopy

1. Introduction

The majority of biological processes is asymmetrically distributed in higher plants and is dependent of specific trends, activities and interactions of subsets of specialized cells in determined locations (1, 2). Because of this, the genetic, proteomic and metabolic profiles of each cell are unique (3). In the actual large-scale data era, the advances in data generation and analysis have helped the understanding of correlations between transcriptome, proteome and metabolome profiles. However, at the same time, the complexity of several developmental processes, which are affected by both biotic and abiotic factors, highlights the necessity of more detailed analysis, to overcome the possible bias when analyzing samples composed by heterogeneous groups of cells (4). Considering this, the use of techniques for isolation of specific tissue or cell types is a good strategy. Several techniques have been developed in the last decades and, although primarily used in medical research with animal cells, they have been successfully used in plant studies as well (5-10).

The fluorescent activated cell sorting (FACS) allows the collection of specific cell types by tagging these cells with marker reporters, such as green fluorescent protein (GFP) or yellow fluorescent protein (YFP) (11). Similarly, the isolation of nuclei targets in specific cell types (INTACT) allows the purification of the nucleus from the cells of interest through the expression of a nuclear targeting fusion protein (NTF) containing GFP and a biotin acceptor peptide (12). These two techniques are very interesting but both have the limitation of needing transgenic plants, a difficult procedure still unsuccessful for many plant species and that requires a good knowledge of tissue specific promoters. INTACT has the additional limitation of being useful only for transcriptomic analysis.

Attempting to overcome the above-cited practical limitations, the laser microdissection (LM) has evolved as a powerful technique, which can be used for any species and for different purposes (1, 3). In this technology, a particular microscope is used to select specific cells from fixed samples, based on morphology or histology (5). LM can be divided in two major different techniques: Laser cutting and Laser capture microdissection (LCM). Several instruments are available on the market and all of them use one or both of these two strategies (1). In laser cutting, the target cell is cut free from tissue section (non contact) by UV laser and collected by different methods such as laser pressure catapulting, ejection downward a collection tube or blotting onto an adhesive cap (1). In LCM, the target cells are isolated from the tissue section by bonding them to a plastic film at sites activated by a near-IR laser, using a LCM cap as the collector device. The advantage of LCM when compared to other LM methods is that cells can be harvested onto the cap while their spatial relationships in the original section are preserved. With this, the inspection of LCM-isolated cells on the harvest cap is facilitated, allowing a detailed analysis of the harvested section after each event of collection. Furthermore, adjacent cells to the cells of interest are not damaged as in laser cutting, preserving them for future analysis (1). The contact of the sample with the plastic film can be avoided by the use of membrane frame slides, when it is possible to mount tissue samples onto a membrane that separates the tissue from the film.

The low concentrations of RNA, proteins and metabolites obtained from LCM captured cells and tissues are still a challenge. However, the development of highly sensitive detection methods and effective protocols for amplification of the extracted products are collaborating for increasing the application of this technology in plants, helping the elucidation of complex regulatory networks occurring at the cellular level (2, 13). Here we describe a general protocol for obtaining laser captured microdissected cells or tissues from

plant samples. The present protocol was successfully applied in soybean (9) and *Eucalyptus* sp. (**Fig. 1**) and can be used for transcriptomic, proteomic or metabolomic downstream studies. Cell and tissue specific biochemical and molecular microanalyses are particularly useful in addressing aspects of plant secondary metabolism for often the expression of different metabolic steps of its biochemical pathways take place in distinct cell types and tissues.

2. Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Ensure all utensils and glassware are sterile or have been previously cleaned and autoclaved (minimum of 20 min at 121°C). Prepare and store all reagents at room temperature (unless indicated otherwise). Ensure all waste disposal regulations will be followed when disposing waste materials. Ensure you are wearing powder free gloves during all the procedures.

2.1. Sample fixation and infiltration

1. Vacuum pump or any other equipment to produce vacuum in the samples.
2. Glass Buchner flasks with proper cap.
3. Tweezers and blades.
4. Fixation solution: 75% ethanol: 25% acetic acid solution v/v (*see* **Note 1**).
5. Shaker and Ice bath or any ice appropriate container
6. PBS Buffer: 137 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.3. Mix all the reagents in a glass beaker filled with about 1/3 of the final volume of ultrapure water. Set the final volume and adjust the pH. Ensure you are performing enough quantity of PBS Buffer for all of the following solutions. Store at 4°C.
7. 10% sucrose solution: Add 10 g of sucrose in 100 mL of PBS Buffer. Mix to dissolve.

8. 20% sucrose solution: Add 20 g of sucrose in 100 mL of PBS Buffer. Mix to dissolve.

9. Sterile plastic centrifuge tubes (15 mL or 50 mL), depending on the size of your sample.

2.2. Sample embedding in cryomedium

1. Petri dishes.

2. Clean wipes.

3. Cryocassettes (*see* **Note 2**).

4. Cryomedium.

5. Liquid nitrogen.

2.3. Cryosectioning

1. Cryotome

2. Tweezer and brushes.

3. Cryocassette.

4. UV treated PEN membrane frame slides (*see* **Note 3**).

5. Slides container or dry ice (*see* **Note 4**).

2.4. Dehydration

1. Ethanol solutions in ultrapure water: 70%, 75%, 95% and 100% (*see* **Note 5**).

2. Xylene.

3. 200 μL and 1000 μL pipettes.

4. RNAse free pipette tips.

2.5. Laser Capture Microdissection (LCM) procedure

1. LCM instrument

2. LCM caps

3. RNAse free glass support slides (*see* **Note 6**).

3. Methods

3.1. Sample fixation and infiltration

Proceed with the harvesting of the sample of interest. Use fresh samples. Any part of the plant can be used for the procedure. However, if you have a large sample, such as stems or leaves, you will probably want to cut it into small pieces to facilitate the fixation and infiltration processes. If necessary, gently wash the sample with autoclaved distilled water.

1. Fill one Buchner flask with enough Fixation solution to fully cover your sample. Put the sample in the flask and make sure all the sample is in contact with the solution. Close the flask and put it inside an ice bath or a container filled with ice. Connect the flask to the vacuum pump and leave the sample under vacuum for 15 min (*see* **Note 7**).

2. Put the sample in a sterile centrifuge tube and fill out the tube with fresh Fixation solution. Place the tube (horizontally oriented) in a container filled with ice. Completely cover the tube with another layer of ice and close the container. Place the container in the shaker and set the speed to allow a gentle agitation (not too slow and not too fast) (*see* **Note 8**). Keep shaking overnight (**Fig. 2**).

3. Transfer the sample to a Buchner flask filled with 10% sucrose solution. Place the flask inside an ice bath or a container filled with ice. Connect the flask to the vacuum pump and leave the sample under vacuum for 15 min.

4. Transfer the sample to a centrifuge tube filled with fresh 10% sucrose solution and place the tube in the same ice container used for step 2. Let agitate for 30 min or until the samples are fully submerged.

5. Repeat step 3 using the 20% sucrose solution for 15 min.

6. Repeat step 4 using the 20% sucrose solution for 30 min or until the samples are fully submerged.

3.2. Sample embedding in cryomedium

1. Gently dry the samples with a clean paper towel.

2. Cool the cryocassette pouring a small amount of liquid nitrogen on it. Use a cold block as a basis for the cryocassette or perform the procedure in a large styrofoam container, to avoid accidental contact with the liquid nitrogen.

3. Considering the size of the sample, pour an amount of cryomedium on the cold cryocassette to make a thick basis for your sample. Place the sample in the right orientation and immediately cover it with another thick layer of cryomedium (*see* **Note 9**).

4. Freeze immediately with liquid nitrogen. (*see* **note 10**). Detach from the cryocassette, wrap in aluminum foil and store at -80°C until ready for sectioning (*see* **Note 11**).

3.3. Cryosectioning

1. Ensure the cryotome is clean and in adequate temperature, which is -21°C (a range between -20 and -24 is ok). Keep blades, brushes, tweezers and any other material you think necessary inside the cryotome while making sections.

2. Adjust the cryotome settings according to your sample and purpose (*see* **Note 12**).

3. Allow the "cryocassette" to cool by keeping it on the cryobar for ~1 min.

4. Put a drop of cryomedium on the cryocassette and put the sample (which is already embedded) in the desired orientation (*see* **Note 13** and **Note 14**).

5. Place the cryocassette back on the cryobar and wait until the whole assembly is frozen.

6. Fit the cryocassette containing the sample in the "sample head" of the cryotome and adjust the angle of the blade to start sectioning.

7. When the sample is in line with the blade, start slicing off sections of the medium until you reach the sample.

8. When the desired region of the tissue is reached either pick up the section with a frozen, fine paintbrush and place it on the slide, which is at room temperature, or place the flat side of the slide directly on the section and allow it to melt on top of the slide. As soon as you get the first section, keep the slide inside the cryotome until finished (*see* **Note 15**). When using PEN membrane frame slides, you should place the samples in the flat side of the slide (**Fig. 3**). Try to put the sections as much in the center of the slide as possible (*see* **Note 16**).

9. When you finish taking samples to the slides, you are ready to proceed to the dehydration step. If you cannot proceed immediately to the next step, keep the slides with the cryosections in a closed container at -80°C until use (*see* **Note 17**).

3.4. Dehydration

Proceed with the dehydration directly on the slides. You can use immersion jars, Pasteur pipettes or electronic pipettes for each solution (*see* **Note 18**).

1. Ethanol 70% (at -20°C) for fixation – 30 s to 1 min (*see* **Note 19**).

2. RNAse free water to dissolve the cryomedium - 30 s to 1 min

3. Ethanol 75% - 30 s to 1 min

4. Ethanol 95% - 30 s to 1 min

5. Ethanol 100% - 30 s to 1 min

6. Xylene – 2 times of 2 min each

7. Air dry and use immediately for LCM (*see* **Note 20**).

3.5. Laser Capture Microdissection (LCM) procedure

Depending on the LCM instrument, the procedures regarding the orientation of slides and mode of cell capture can change. In this section we describe general procedures for instruments using Infrared (IR) and Ultra-Violet (UV) lasers for capture and cut of cells or tissue of interest, respectively. Here we also focus on the cell collection methodologies using specific caps.

1. For correct orientation of the slide in the LCM instrument, you should use RNAse free support glass slides (*see* **Note 6**) below the metal membrane frame slides (**Fig. 4**). Be sure your sections are completely dry before doing that.

2. Load slides and caps in their specific places in the instrument.

3. Adjust brightness and focus and search for your cells of interest. Once you find them, draw around the area of cells to be microdissected.

4. Place the cap so your cells of interest stay inside the coverage region of the cap.

5. Move to a clean part under the cap where there are no cells so you can manipulate the IR capture laser and UV cutting laser.

6. Once both lasers are settled you are ready to move to the drawn area to capture and cut the cells of interest. (*see* **Note 21**).

7. When finished sectioning, place the cap containing your samples in a 0.5 mL microcentrifuge tube already filled with the appropriate buffer for further downstream processes.

8. Invert the cap-microcentrifuge tube assembly. Tap the bottom of the tube to ensure all buffer is in contact with the captured cells.

9. Incubate following specific recommendations from your reagent's supplier considering the purpose of your downstream process.

4. Notes

1. Wear gloves and a mask when preparing this solution. Perform the procedure preferably in a fume hood.

2. Cryocassettes are specific sample holders for using in cryotomes. If not available for using during sample embedding you can alternatively use petri dishes. Just be sure the petri dishes are not going to crack when in contact with liquid nitrogen.

3. Perform an UV treatment (15 – 30 min) in the PEN membrane frame slides to aid in the adhesion of the sections on the polymer membrane. This treatment should be done on the slides preferably just a few days before the procedure. The treatment can be performed in air flow cabinets with UV light or using UV lamps. Be careful to avoid any direct contact of your eyes or skin with UV light.

4. While taking sections, keep the slides inside the cryotome or next to it on dry ice.

5. Stock in 70% Ethanol solution at -20°C until use.

6. In an air flow cabinet, treat the glass support slides with a specific solution for elimination of RNAses and rinse with ultrapure water. Air dry before use.

7. Test the pressure before start. You know the vacuum is working when you see little bubbles coming out the sample.

8. Ensure the samples are moving inside the tube and are in contact with the solution. If a cold chamber is available, the ice is not necessary and you can replace the shaker with a rotator.

9. It is very important that the cryocassette is cold before starting the embedding procedure. Alternatively you can use small petri dishes as a basis for the embedding. In this case, cool the petri dish with liquid nitrogen and fill it with cryomedium. Place your sample and cover with more cryomedium. The only issue is that it is harder to take the samples out the petri dishes than from the cryocassettes. You can also use specific cryomolds for this step.

10. You know the cryomedium is frozen when the color turns from clear to opaque.

11. Attempting to facilitate the sectioning, cut out the excess of cryomedium so you have flat sides in your sample. This is especially helpful for a good orientation of the sample to make cross sections.

12. The section thickness can vary depending on the purpose. Thin sections ($1 - 10$ μm) are better for visualization of structures and taking pictures. Thick sections ($20 - 30$ μm) are better for LCM, as you can take more cell layers at each time, but some structures can be difficult to detect in thick sections. We usually use 20 - 25 μm for LCM, but a previous test to define the better thickness for each case is advised.

13. If you prefer you can embed the sample just before sectioning, directly in the cryocassette that you are going to use in the cryotome. However, when you are working with more than one sample, it is better to proceed with the embedding right

after the sample fixation and infiltration to avoid degradation of the samples and save time.

14. Ensure you are manipulating your embedded sample inside the cryotome and that your tweezers and blades are also cooled, to avoid melting the cryomedium.

15. Remember to keep all the slides containing sections inside the cryotome or next to it, in a container filled with dry ice, so the sections do not melt.

16. Depending on your sample, you will probably need more than one slide with cryosections per sample. This is very important to ensure you will have enough samples to work with in your downstream processes. It is a good idea to make a prior test to know how much cells or tissue section you need per sample.

17. Take care during the transport of the slides to the -80°C to avoid melting the sections. This is very important to preserve RNA. We generally use a container filled with a small amount of liquid nitrogen. Then we take the slide box containing the slides off the cryotome and place it in contact with the liquid nitrogen for the transport. Avoid direct contact of the liquid nitrogen with the slides.

18. We prefer to use electronic pipettes during the dehydration steps since these are practical and reduce the chance of contamination if using sterilized tips. Be careful to avoid loosing sections during the procedure by gently applying each solution to the slide. If you prefer to use immersion jars, ensure they are clean and autoclaved and that you have enough jars for each solution. However, the chances of loosing sections using immersion jars do increase comparing to the use of pipettes.

19. To avoid melting of the cryosections apply the 70% ethanol (which was at -20°C) inside the cryotome or just after taking out the sections from -80°C. After this, proceed at room temperature.

20. If you see some white stains on the slide it means that the cryomedium was not completely dissolved, so you should start the whole procedure again.

21. If you use more than one cap to capture all the cells of interest, make sure to gather all the extracts to increase the concentration of your RNA, protein or metabolite of interest. If using column based downstream procedures, pass the extracts from each cap from a same sample through the same column.

Acknowledgments

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Figure captions

Figure 1. Laser capture microdissection (LCM) procedure in Soybean (a – c) and *Eucalyptus globulus* (d – e). (a) Soybean shoot tip before LCM; (b) Soybean shoot tip after capture of shoot tip meristem tissue; (c) Soybean shoot tip after capture of shoot tip meristem and epidermis; (d) *E. globulus* stem cross section before LCM; (e) *E. globulus* stem cross section after capture of cambium cells.

Figure 2. Sample agitation during fixation and infiltration process. Procedure scheme for sample agitation on ice. Samples were incubated with fixation and infiltration solutions in 15 mL centrifuge tubes and kept immersed in ice as described in step 2 from section 3.1.

Figure 3. Detail of a PEN membrane framed slide. Scheme showing the exact place where to put the cryosections.

Figure 4. Scheme showing the right orientation of a PEN membrane frame slide containing section on glass slide support. Oriented this way, the IR laser will allow the capture of the selected area by melting the thermoplastic transfer film in a region surrounding the selected

cells or tissue of interest, so the cells are attached to the cap. Then the UV laser is used to cut the drawn area, completing the dissecting of the cells of interest. The glass slide support is necessary so the weight of the cap does not change the curvature of the film in the PEN membrane frame slide. The change of curvature can modify the angle with which the lasers hit the sample, potentially damaging the capture and cutting procedure.
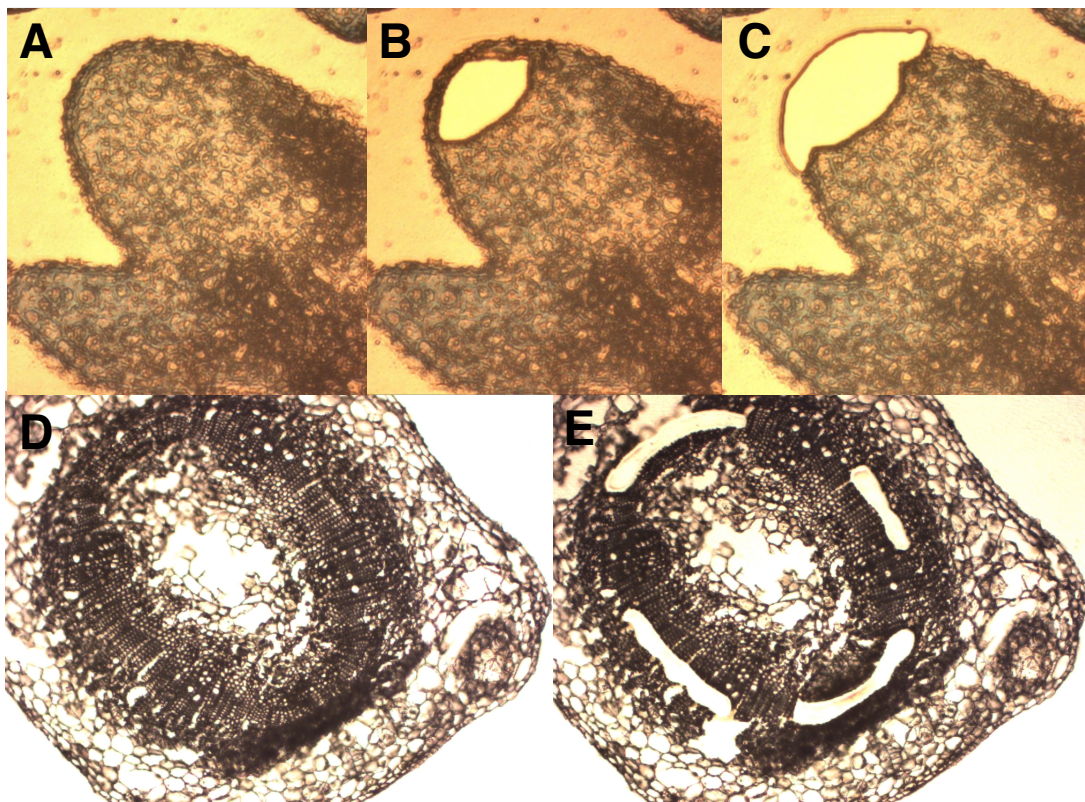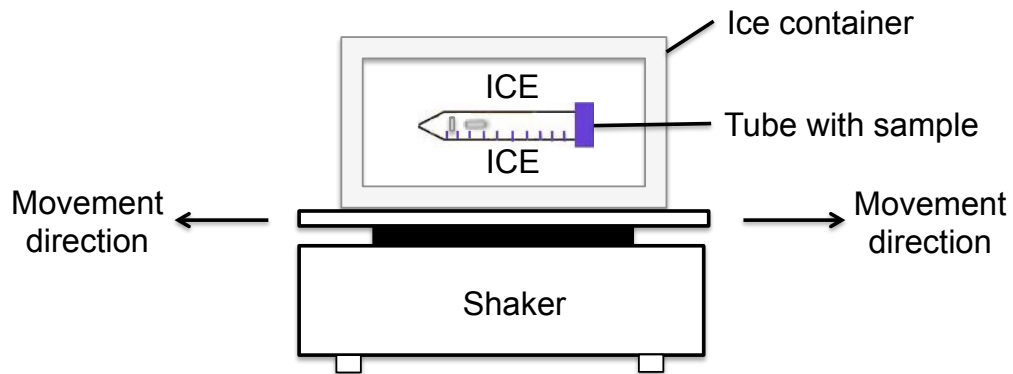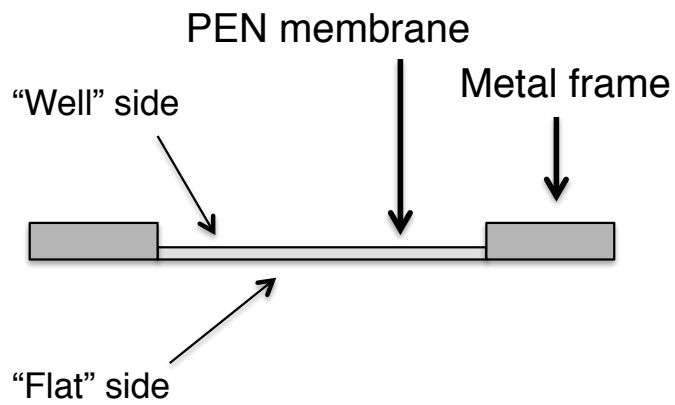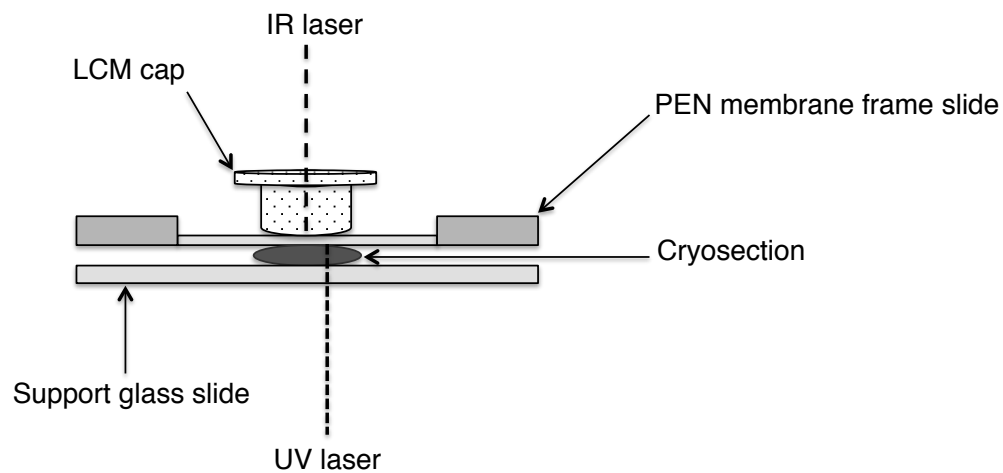
Figure 1.

Figure 2.

Figure 3.

Figure 4.

4. Conclusão e Considerações finais

O enraizamento adventício é um processo bastante complexo e muito importante para a propagação de espécies lenhosas e ornamentais. Embora bastante estudado, muitos aspectos ainda permanecem obscuros, principalmente no caso de lenhosas. O presente trabalho se caracteriza como pioneiro na utilização de microdissecção a laser no estudo da rizogênese adventícia e é também o primeiro trabalho a investigar o perfil proteico relacionado a este processo em eucalipto. A técnica de microdissecção a laser se mostrou uma ferramenta poderosa, permitindo análises com maior grau de detalhamento, o qual tem grande influência na investigação de mecanismos associados a processos de desenvolvimento. Através da utilização desta técnica, foi possível selecionar células do câmbio, região de onde as raízes adventícias se originam em eucalipto. Associada a ferramentas de análise de expressão gênica de alta sensibilidade como nCounter®, foi verificado que a alta expressão de genes com efeito inibitório nas vias de sinalização dos fitormônios auxina e citocinina em células do câmbio de *E. globulus,* quando comparado com *E. grandis*, parece contribuir para a recalcitrância ao enraizamento na primeira espécie. Os respectivos genes *TOPLESS, IAA12 e ARR1* foram também inibidos por auxina exógena aplicada em *E. globulus*, mais uma vez reforçando a ideia de que estes genes possam atuar como reguladores negativos do processo.

Outro aspecto bastante interessante foi verificado ao se comparar plantas tratadas ou não com auxina exógena. Embora a exposição à auxina tenha induzido modificações tanto na expressão de genes como de proteínas, o que já era esperado, a característica molecular natural das plantas não tratadas parece ser ainda mais determinante para o entendimento da recalcitrância ao enraizamento. A menor concentração de auxina endógena na planta e o consequente menor acúmulo de auxina na região do câmbio em *E. globulus* em relação à *E. grandis*, provavelmente contribui para a dificuldade na formação de raízes. Este resultado corrobora relatos de outras espécies, onde os níveis endógenos de auxina tem efeito importante na determinação da capacidade de enraizamento (Bellini et al, 2014).

Embora necessário para o sucesso da propagação de estacas, é sabido que o enraizamento adventício gera vários estresses na planta. Em relação às proteínas identificadas durante este processo em *E. globulus*, a maior parte se refere a proteínas relacionadas a estresse oxidativo, também encontradas em outras espécies durante o enraizamento. Além disso, o metabolismo energético também foi responsável por grande parte das proteínas identificadas. No entanto, considerando a essencialidade de processos como respiração e

geração de energia, este resultado já era esperado. Interessantemente, a cinética do enraizamento também parece contribuir para a competência ou não à produção de raízes. Diferenças importantes foram encontradas entre as duas fases do enraizamento, principalmente em análises do proteoma. Proteínas que foram expressas especificamente durante a fase de formação em *E. globulus* provavelmente indicam a ativação de vias de sinalização necessárias à formação dos primórdios radiculares e posterior desenvolvimento em raízes propriamente ditas. Estes resultados sugerem que tanto plantas não tratadas como plantas expostas à auxina exógena desenvolvem a maquinaria para a produção de raízes, porém estas raízes geralmente não se desenvolvem em plantas sem tratamento ou são abortadas antes de emergir.

Este trabalho representa importantes avanços no conhecimento sobre os mecanismos moleculares envolvidos no enraizamento adventício em eucalipto. Obviamente a extrapolação de resultados obtidos *in vitro* deve ser feita com cautela. Porém, os resultados obtidos nesta pesquisa abrem possibilidades para novos questionamentos acerca dos mecanismos de regulação deste importante processo de desenvolvimento. Muitos estudos ainda precisam ser realizados a fim de se desvendar as causas da recalcitrância ao enraizamento. O aprimoramento das análises do perfil proteico e a comparação com proteínas expressas na espécie de fácil enraizamento *E. grandis*, podem auxiliar na identificação de outros processos regulatórios importantes, em nível pós-transcricional. Além disso, através de técnicas de transformação genética e edição genômica, estudos focados na supressão dos possíveis reguladores negativos aqui identificados, poderão auxiliar na superação dos obstáculos ao enraizamento na tentativa de melhorar a propagação clonal em genótipos recalcitrantes, aumentando a sua produtividade.

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ANEXOS

Esta seção apresenta os trabalhos oriundos de colaborações e que foram publicados ao longo do período de doutoramento. As publicações estão listadas em ordem cronológica, iniciando pela mais recente.

ANEXO A - Artigo em co-autoria; fora do escopo da tese:
Ruedell, Carolina M.; **De Almeida, Márcia R.**; Fett-Neto, Arthur G. Concerted transcription of auxin and carbohydrate homeostasis-related genes underlies improved adventitious rooting of microcuttings derived from far-red treated *Eucalyptus globulus* Labill mother plants. Plant Physiology and Biochemistry, 97: 11-19, **2015**.

ANEXO B - Artigo em co-autoria; fora do escopo da tese:
Schwambach, Joséli; Ruedell, Carolina Michels; **De Almeida, Márcia Rodrigues**; Fett-Neto, Arthur G. Nitrogen Sources and Adventitious Root Development in *Eucalyptus globulus* Microcuttings. Journal of Plant Nutrition, 38(10): 1628-1638, **2015.**

ANEXO C - Artigo de revisão relacionado ao tema da tese:
Da Costa*, Cibele T.; **De Almeida*, Márcia R.**; Ruedell, Carolina M.; Schwambach, Joseli; Maraschin, Felipe S.; Fett-Neto, Arthur G. When stress and development go hand in hand: main hormonal controls of adventitious rooting in cuttings. Frontiers in Plant Science 4: 133, **2013**. * Contribuíram igualmente para este trabalho.

ANEXO D – Artigo em co-autoria; fora do escopo da tese:
Ruedell, Carolina Michels; **Almeida, Márcia Rodrigues**; Schwambach, Joséli; Posenato, Carina Fronza; Fett-Neto, Arthur Germano. Pre and post-severance effects of light quality on carbohydrate dynamics and microcutting adventitious rooting of two *Eucalyptus* species of contrasting recalcitrance. Plant Growth Regulation 69: 235-245, **2013.**

ANEXO E - Artigo em co-autoria; fora do escopo da tese:
Nascimento, Naíla Cannes; Menguer, Paloma Koprovski; Sperotto, Raul Antonio; **Almeida, Márcia Rodrigues**; Fett-Neto, Arthur Germano. Early Changes in Gene Expression Induced by Acute UV Exposure in Leaves of *Psychotria brachyceras*, a Bioactive Alkaloid Accumulating Plant. Molecular Biotechnology, 54(1): 79-91, **2013.**

ANEXO A

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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Research article

Concerted transcription of auxin and carbohydrate homeostasis-related genes underlies improved adventitious rooting of microcuttings derived from far-red treated *Eucalyptus globulus* Labill mother plants

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A R T I C L E   I N F O

A B S T R A C T

Economically important plant species, such as *Eucalyptus globulus*, are often rooting recalcitrant. We have previously shown that far-red light enrichment applied to *E. globulus* donor-plants improved micro-cutting rooting competence and increased rooting zone/shoot carbohydrate ratio. To better understand this developmental response, the relative expression profiles of genes involved in auxin signaling (*ARF6, ARF8, AGO1*), biosynthesis (*YUC3*) and transport (*AUX1, PIN1, PIN2*); sucrose cleavage (*SUS1, CWINV1*), transport (*SUC5*), hexose phosphorylation (*HXK1, FLN1*) and starch biosynthesis (*SS3*) were quantified during adventitious rooting of *E. globulus* microcuttings derived from donor plants exposed to far-red or white light. Expression of auxin transport-related genes increased in the first days of root induction. Far-red enrichment of donor plants induced *ARF6, ARF8* and *AGO1* in microcuttings. The first two gene products could activate *GH3* and other rooting related genes, whereas *AGO1* deregulation of the repressor *ARF17* may relief rooting inhibition. Increased sink strength at the basal stem with sucrose unloading in root tissue mediated by *SUC* and subsequent hydrolysis by *SUS1* were also supported by gene expression profile. Fructose phosphorylation and starch biosynthesis could also contribute to proper carbon allo-cation at the site of rooting, as evidenced by increased expression of related genes. These data are in good agreement with increased contents of hexoses and starch at the cutting base severed from far-red exposed donor plants. To sum up, pathways integrating auxin and carbohydrate metabolism were activated in microcuttings derived from donor plants exposed to far red light enrichment, thereby improving rooting response in *E. globulus*.

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1. Introduction

Successful clonal propagation depends on the formation of adventitious roots in a stem cutting, which involves recruitment and reprograming of cells to form a new radical meristem. This developmental process consists in a series of successive and interdependent phases (induction, formation, elongation), each with its own requirements and characteristics (Bellini et al., 2014). Adventitious rooting is influenced by endogenous factors, such as phytohormones and carbohydrate status, and environmental factors, including light, nutrition and temperature. In addition, the physiological status of the mother plant is of paramount impor-tance for cutting rooting (Da Costa et al., 2013). However, at present, the detailed molecular events involving this process are at best partially understood.

Although the importance of auxins in adventitious rooting control has already been described by many authors, the molecular signaling network involved in this process needs to be further

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ANEXO B

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NITROGEN SOURCES AND ADVENTITIOUS ROOT DEVELOPMENT IN *EUCALYPTUS GLOBULUS* MICROCUTTINGS

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☐ *In previous studies, it has been shown that nitrate supply may favor adventitious rooting in the rooting recalcitrant* Eucalyptus globulus. *Herein, the impact of various N sources on adventitious rooting and root branching in microcuttings of* E. globulus *was investigated. The positive effect of nitrate on adventitious root development was confirmed and extended to root branching. Urea yielded a rooting response comparable to that observed in presence of nitrate. Urease activity was observed, displaying two peaks: one at the root induction and another at the root formation step. The use of glutamic acid, glutamine or asparagine promoted higher root number, but yielded shorter roots. Rooted microcuttings derived from all nitrogen (N) sources were successfully acclimated to* ex vitro *conditions. The manipulation of N sources in adventitious rooting media can be a tool for improving new root density, length and branching in this species.*

Keywords: nitrate, urea, glutamate, urease activity, soil acclimatization

INTRODUCTION

In forestry, vegetative propagation is widely used to multiply elite individuals obtained in breeding programs or selected from natural populations (Hartmann et al., 1990). Adventitious root formation is a key step in vegetative propagation and is a developmental process consisting of a series of successive and interdependent phases (induction, initiation, formation), each with its own requirements and characteristics (De Klerk et al., 1999; Da Costa et al., 2013).

ANEXO C

When stress and development go hand in hand: main hormonal controls of adventitious rooting in cuttings

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Adventitious rooting (AR) is a multifactorial response leading to new roots at the base of stem cuttings, and the establishment of a complete and autonomous plant. AR has two main phases: (a) induction, with a requirement for higher auxin concentration; (b) formation, inhibited by high auxin and in which anatomical changes take place. The first stages of this process in severed organs necessarily include wounding and water stress responses which may trigger hormonal changes that contribute to reprogram target cells that are competent to respond to rooting stimuli. At severance, the roles of jasmonate and abscisic acid are critical for wound response and perhaps sink strength establishment, although their negative roles on the cell cycle may inhibit root induction. Strigolactones may also inhibit AR. A reduced concentration of cytokinins in cuttings results from the separation of the root system, whose tips are a relevant source of these root induction inhibitors. The combined increased accumulation of basipetally transported auxins from the shoot apex at the cutting base is often sufficient for AR in easy-to-root species. The role of peroxidases and phenolic compounds in auxin catabolism may be critical at these early stages right after wounding. The events leading to AR strongly depend on mother plant nutritional status, both in terms of minerals and carbohydrates, as well as on sink establishment at cutting bases. Auxins play a central role in AR. Auxin transporters control auxin canalization to target cells. There, auxins act primarily through selective proteolysis and cell wall loosening, via their receptor proteins TIR1 (transport inhibitor response 1) and ABP1 (Auxin-Binding Protein 1). A complex microRNA circuitry is involved in the control of auxin response factors essential for gene expression in AR. After root establishment, new hormonal controls take place, with auxins being required at lower concentrations for root meristem maintenance and cytokinins needed for root tissue differentiation.

Keywords: adventitious rooting, auxin, receptors, jasmonic acid, cytokinin, nutrition, microRNAs, hormonal crosstalk

INTRODUCTION

If flowering is a key developmental process for sexual reproduction in plants, adventitious rooting (AR) occupies a central role in asexual propagation. Forestry, horticulture, and fruit crops depend to a large extent on the successful establishment of roots in cuttings and other propagules. Clonal propagation is of particular relevance to forestry, since genetic improvement in long lived species with large generation cycles is often limiting. Genetic gains from interspecific hybridization, mutations, and transgenic events can be captured and multiplied faster and more efficiently based on clonal propagation through AR of cuttings. Overall, the main application of AR is propagation by cuttings and its derived techniques adapted to clonal garden greenhouses and *in vitro* cultures, minicuttings and microcuttings, respectively (Assis et al., 2004). Therefore, rather than looking into the examples of developmentally programmed AR in intact plants, the focus of the present review is on AR of severed organs or in response to stressful conditions, such as flooding.

Most research on AR has been centered on the role of phytohormones, mainly auxins, and cutting physiological conditions. The role of stress responses associated with cutting severance and the relevance of mother plant status has often received less attention, although a shift in focus has been clearly taking place in the last two decades or so. Wound responses associated with cutting severance are integrated, and often necessary, in the steps leading to AR, and mother plant status is a key determinant of rooting propensity of cuttings derived from it. Therefore, the control of environmental variables of stock plants is rather relevant for the clonal propagation process. Clearly, a fundamental aspect governing AR responses to external and internal stimuli is cellular competence to respond. This developmental capacity to respond is responsible for many of the failures to obtain AR in mature cuttings, even upon careful manipulation of environmental variables and phytohormones that can modulate rooting.

The concept of adventitious root is based essentially on anatomical origin. Adventitious roots are formed in stems, leaves and

ANEXO D

ORIGINAL PAPER

Pre and post-severance effects of light quality on carbohydrate dynamics and microcutting adventitious rooting of two *Eucalyptus* species of contrasting recalcitrance

Carolina Michels Ruedell · Márcia Rodrigues de Almeida ·
Joséli Schwambach · Carina Fronza Posenato ·
Arthur Germano Fett-Neto

Abstract Adventitious rooting is a complex developmental response affected by genetic and environmental factors. Radiation quality effects on adventitious rooting depend on characteristics such as species, growth stage, irradiance, spectral quality, and time of exposure. *Eucalyptus* is an essential genus for the paper industry, and high yield plantations depend on adventitious rooting of selected genotypes. This work addressed two hypotheses: (1) radiation quality equally affects adventitious rooting in *Eucalyptus* species of different recalcitrance; (2) adventitious rooting outcome depends on both donor plant and cutting radiation quality treatments. To that end, the easy-to-root *Eucalyptus grandis* and the recalcitrant *Eucalyptus globulus* were evaluated. The effect of white, blue, red and far-red radiation enrichment on microcuttings and donor plants of both species was evaluated in relation to rooting. There was no effect of radiation quality on adventitious rooting of *E. grandis* or when radiation treatments were applied to *E. globulus* microcuttings. In contrast, donor plants of *E. globulus*, grown in medium devoid of sucrose and exposed to far-red radiation, yielded microcuttings showing higher rooting percentage, even in the absence of exogenous auxin in the rooting medium. Sucrose in donor plant medium abolished the positive effect of far-red radiation. An increase in endogenous soluble sugars and starch contents in basal microcuttings was associated with far-red radiation treatment of donor plants. These results underline the importance of appropriate carbohydrate partitioning in donor plants for adventitious rooting of cuttings and provide a basis for understanding and overcoming rooting recalcitrance in *E. globulus* clones.

Keywords Adventitious rooting · Carbohydrates · Donor plant · *Eucalyptus* · Radiation quality

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Introduction

Eucalyptus is one of the most widely cultivated tree genera in the world due to its high adaptation ability to different environments (Eldridge et al. 1993). Most plantations are established to provide pulp for paper (Turnbull 1999). Brazil is one of the largest producers of eucalypt pulp and its plantations are based on the vegetative propagation of selected elite genotypes with high productivity (Mora and Garcia 2000; Schwambach et al. 2008).

Adventitious rooting is an essential step in the vegetative propagation of trees and may be divided in two main phases: (1) induction, corresponding to the molecular and biochemical events prior to any visible morphological changes, and (2) formation, comprising cell divisions involved in root meristem organization and radical primordia establishment, followed by root elongation and emergence (De Klerk et al. 1999; Fett-Neto et al. 2001; De Klerk 2002). This complex developmental process can be affected by internal and

ANEXO E

RESEARCH

Early Changes in Gene Expression Induced by Acute UV Exposure in Leaves of *Psychotria brachyceras*, a Bioactive Alkaloid Accumulating Plant

Naíla Cannes do Nascimento · Paloma Koprovski Menguer ·
Raul Antonio Sperotto · Márcia Rodrigues de Almeida ·
Arthur Germano Fett-Neto

Abstract   UV-B radiation can damage biomolecules, such as DNA, RNA, and proteins, halting essential cellular processes; this damage is partly due to ROS generation. Plant secondary metabolites may protect against UV-B. *Psychotria brachyceras* Müll. Arg. (Rubiaceae), a subtropical shrub, produces brachycerine, a monoterpene indole alkaloid mainly accumulated in leaf tissues, which displays antioxidant and antimutagenic activities. Exposure of *P. brachyceras* cuttings to UV-B radiation significantly increases leaf brachycerine concentration. It has been suggested that this alkaloid might contribute to protection against UV-B damage both through its quenching activity on ROS and as UV shield. To identify differentially expressed genes of *P. brachyceras* in response to UV-B and investigate a possible influence of this stimulus on putative brachycerine-related genes, suppressive subtractive hybridization was applied. Complementary DNA from UV-B-treated leaves for 24 h was used as tester, and cDNA from untreated leaves, as driver. After BLASTX alignments, 134 sequences matched plant genes. Using quantitative RT-PCR, selected genes potentially related to brachycerine showed significant increases in transcription after UV-B exposure: tryptophan decarboxylase, ACC oxidase, UDP-glucose glucosyltransferase, lipase, and serine/threonine kinase. Results suggest a possible involvement of brachycerine in acute UV-B responses and show that alkaloid accumulation seems at least partly regulated at transcriptional level.

Keywords   Ultraviolet light · *Psychotria* · Differential gene expression · Brachycerine · Alkaloid

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Introduction

Solar ultraviolet-B radiation (UV-B; 290–315 nm) is a small fraction of the solar spectrum that reaches ground level. In spite of its modest contribution to the total quantum flux, UV-B can be an important modulator of biological processes in terrestrial ecosystems [1–3]. UV-B has the potential of damaging biomolecules, such as DNA, RNA, proteins, and lipids, generating reactive oxygen species (ROS), and impairing cellular processes [3–6]. The effects of UV-B are multiple, affecting both morphological and physiological aspects of plants. The sessile life of plants requires effective adaptation to environmental changes, such as increased levels of UV radiation [4]. In the course of evolution, plants developed several adaptations to tolerate UV-B imposed stress [5, 7], including structural, enzymatic, and chemical defenses. The accumulation of UV-absorbing compounds is often observed as