UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

Programa de Pós-Graduação em Biologia Celular e Molecular

Dissertação de Mestrado

GENES-REFERÊNCIA E EXPRESSÃO DE TRANSPORTADORES DE AUXINA DURANTE A RIZOGÊNESE ADVENTÍCIA EM *Eucalyptus* sp.

Márcia Rodrigues de Almeida

Orientador: Arthur Germano Fett-Neto

Dissertação submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS como requisito parcial para a obtenção do grau de Mestre em Biologia Celular e Molecular

Porto Alegre

Outubro, 2011

AGRADECIMENTOS

À minha família querida, especialmente Mãe, Pai, Ana, Gabi e Tia Lana... pelo amor, compreensão e apoio em todas as escolhas, por entenderem minhas eventuais ausências, pelas palavras de carinho nos momentos difíceis e por toda a vibração diante de cada conquista... vocês são meu porto seguro, meu acalento e meu maior incentivo... Amo vocês...

Ao meu orientador Arthur, pela amizade, exemplo e confiança e principalmente por acreditar na minha capacidade quando às vezes eu mesma não acreditava... obrigada por permitir que eu continue mais um tempo aprendendo contigo...

À Carol, pelo aprendizado desde a época da iniciação científica, pela ética e dedicação que me serviram de exemplo desde o início, pelos conselhos (experimentais e, principalmente, de vida), pela parceria dentro e fora do laboratório e principalmente por me dar a chance de te ter como amiga... quisera eu ter um coração tão grande... Te adoro...

À Joséli, eterna "orientadora" e ponto de referência... *"quando eu crescer quero ser como tu"*... obrigada pela paciência no início e pela amizade que tomou força ao longo destes anos... Adoro muito...

Às demais meninas e amigas queridas do Laboratório: Anna, Cibele, Fer, Janieli, Kaka, Kelly, Lívia e Palomita... pela amizade, pela troca de experiências, por compartilharem tanto momentos de descontração como de tensão no laboratório, pelos almoços seguidos de chazinhos maravilhosos e conversas intermináveis ... é muito bom saber que posso contar com vocês...

Aos meninos do Lab que valem por mil: Helinho, rei herdeiro das gambiarras que salvam o dia e Felipe, gênio e fonte obrigatória de consulta ... figuras raras, sempre dispostos a ajudar, a dar um conselho e, principalmente, donos de palpites muito certeiros... o laboratório certamente não seria o mesmo sem vocês...

Ao Raul, pela ajuda nos primeiros passos do PCR em tempo Real, pela disponibilidade de sempre, pela humildade e pelas piadas, às vezes absurdas, mas que rendiam grandes gargalhadas...

Ao Álvaro, pelo amor, carinho e cumplicidade, por agüentar no osso meus momentos de estresse e mau-humor, pela torcida, pelos pensamentos positivos e principalmente por me fazer tão feliz... Te Amo...

Às amigas de sempre, Lu, Pati e Sô pelo carinho, confiança e amizade indiscutíveis... mesmo longe a gente sabe que a torcida é mútua, a saudade é gigante e que o tempo nunca vai diminuir isso... Adoro vocês demais...

Ao professor Giancarlo Pasquali e seus alunos do Laboratório de Genética Molecular de Plantas - UFRGS, pela ajuda e empréstimo de materiais e equipamentos em tantos momentos...

Ao Luciano e à Sílvia, pela simpatia e ajuda na secretaria...

Aos tantos professores pelos ensinamentos...

À empresa Celulose Riograndense pela doação de sementes...

E às agências de fomento CNPq, Capes e Fapergs por permitirem a condução desta pesquisa.

SUMÁRIO

RESUMO

ABSTRACT

1 INTRODUÇÃO **07**

1.1 O Eucalipto **07**

1.2 Enraizamento adventício e auxina **08**

1.3 *Eucalyptus globulus* e *Eucalyptus grandis* **12**

1.4 PCR Quantitativo em Tempo Real e genes-referência **13**

2 OBJETIVO GERAL **17**

2.1 Objetivos Específicos **17**

3 Reference gene selection for quantitative reverse transcription-polymerase chain reaction normalization during *in vitro* adventitious rooting in *Eucalyptus globulus* **18**

Abstract 19

Background 20

Results 20

Discusssion 23

Conclusions 27

Methods 27

4 Expression of auxin transporter genes during *in vitro* adventitious rooting in *Eucalyptus globulus* Labill and *Eucalyptus grandis* Hill ex Maiden **31**

Abstract 33

Introduction 34

Material and methods 36

Results 43

Discussion 51

Conclusion 54

5 CONCLUSÕES GERAIS E PERSPECTIVAS **59**

6 REFERÊNCIAS BIBLIOGRÁFICAS **61**

RESUMO

O uso industrial do eucalipto é uma atividade que está em franca expansão principalmente devido ao seu potencial como matéria-prima para a produção de papel e celulose. As florestas comerciais de eucalipto são geralmente constituídas por meio da propagação vegetativa de materiais elite, sendo necessário um enraizamento adventício eficaz para o estabelecimento das plantas. Dentre os vários fatores que podem afetar a competência ao enraizamento adventício, a auxina tem um papel central, principalmente associado ao conteúdo endógeno e taxa de transporte deste fitormônio. *Eucalyptus globulus* e seus híbridos interessam à indústria de papel e celulose do Sul do Brasil devido a características como o baixo teor de lignina, facilitando a obtenção de celulose, e a relativa tolerância à geada, comum na região. Entretanto, o enraizamento desta espécie é recalcitrante, sendo necessária a aplicação de auxina exógena para a obtenção de enraizamento satisfatório. *Eucalyptus grandis* é a espécie mais plantada no Brasil atualmente e possui entre suas características uma boa propensão ao enraizamento espontâneo sem suprimento de auxina exógena, sendo utilizada neste estudo com fins comparativos. Como parte inicial de uma investigação para analisar a base molecular da recalcitrância de *E. globulus* à propagação clonal, foi realizada uma análise para identificar genes adequados ao uso como controles endógenos em estudos de expressão gênica durante o enraizamento utilizando qPCR. Esta análise permitiu a realização de estudos subseqüentes acerca do padrão de expressão de genes sabidamente relacionados com ação de auxina e enraizamento adventício em outras espécies de plantas, notadamente *Arabidopsis thaliana*. Os resultados indicaram a utilização das combinações dos genes codificantes da *Histona H2B* e da *Alfa-Tubulina* e da *Histona H2B* e da *Actina 2/7* como os mais adequados para serem utilizados como referências em estudos com *E. globulus* e *E. grandis*, respectivamente. Estes resultados foram validados através da análise da expressão do gene codificador da proteína *Argonaute 1*, envolvida no metabolismo de auxinas endógenas e regulação de fatores de transcrição responsivos à auxina em *Arabidopsis*. O perfil de expressão do gene codificador do transportador de influxo de auxina *AUX1* em *E. globulus* indicou que provavelmente este gene não é crítico para o processo de enraizamento adventício. O fator limitante parece ser o transporte de efluxo da auxina da célula para redistribuição e focalização deste fitormônio na base das microestacas, como ficou evidenciado pelo padrão de expressão do gene codificador do transportador de efluxo de auxina *PIN1*. O aumento na expressão de *PIN1* no início do processo de enraizamento em microestacas de *E. globulus* submetidas a aporte de auxina exógena, sugere a ação deste transportador para concentrar a auxina e permitir o estabelecimento de novos meristemas de raiz. Em *E. grandis*, o aumento na expressão de *PIN1* em microestacas submetidas a 96h de exposição à auxina exógena provavelmente se deve à necessidade de remobilização e re-localização da auxina excedente. Análises comparativas do teor endógeno de auxina nestas espécies, bem como investigação da expressão de genes e proteínas envolvidos na sua biossíntese e sinalização, poderão contribuir para um melhor entendimento do papel deste fitormônio no fenótipo rizogênico de *Eucalyptus*.

ABSTRACT

The industrial use of *Eucalyptus* is an expanding activity, mainly due to its use for paper and cellulose production. Commercial eucalyptus forests are generally the product of vegetative propagation of elite materials, requiring effective adventitious rooting for plant establishment. Among the many factors that may affect adventitious rooting competence, auxin plays a key role, particularly related to its endogenous content and transport rate. *Eucalyptus globulus* and its hybrids are targets for forestry industries in Southern Brazil, mainly due to characteristics such as low lignin content and relative frost tolerance. However, this species is recalcitrant to rooting, requiring the application of exogenous auxin to obtain consistent adventitious root development. *Eucalyptus grandis* is currently the most planted species in Brazil, rooting promptly from microcuttings without exogenous auxin, and used in this research for comparative purposes. As an initial step of an investigation to analyze the molecular basis of *E. globulus* recalcitrance to clonal propagation, an analysis was carried out to identify adequate genes to use as endogenous controls in gene expression studies during adventitious rooting monitored by qPCR. This analysis allowed subsequent studies concerning the expression pattern of genes related to auxin action and adventitious rooting in other plant species, particularly *Arabidopsis thaliana*. The results indicated that combinations of genes encoding *Histone H2B* and *Alpha-Tubulin* and *Histone H2B* and *Actin 2/7*, could be used as reliable expression normalizers in rooting studies with *E. globulus* and *E. grandis*, respectively. These normalizers were validated by analyzing the expression of the gene encoding the *Argonaute 1* protein, related to the metabolism of endogenous auxins and regulation of auxin response transcriptions factors in *Arabidopsis*. Expression of the gene encoding the auxin influx carrier *AUX1* in *E. globulus* indicated that this gene is probably not critical to the adventitious rooting process. The limiting factor seems to be the expression of the auxin efflux transporter, necessary for auxin removal from the cell and presumably to allow redistribution and focalization of the phytohormone at the target tissues in the microccuting basis. This was supported by the expression profile of the gene encoding the auxin efflux carrier *PIN1*. The increase in *PIN1* expression at the beginning of the rooting process in *E. globulus* microcuttings under exogenous auxin supply suggested the requirement of this carrier to concentrate auxin and to allow the establishment of new root meristems. In *E. grandis*, however, the late increase of *PIN1* expression in microcuttings submitted to 96h of exogenous auxin exposure is probably due to the need for remobilization and re-location of auxin surplus. Comparative analysis of the auxin endogenous content in these species, as well concerted investigations of expression profiles of transcripts and proteins involved with auxin biosynthesis and signaling, may help achieve a better understanding of the role of this phytohormone in determining adventitious rooting phenotypes in *Eucalyptus*.

INTRODUÇÃO

1.1 O Eucalipto

O eucalipto é uma árvore originária da Austrália, pertencente à família Myrtaceae. Seu uso industrial está em expansão no setor florestal mundial, sendo encontradas grandes plantações em países como África do Sul, Angola, Austrália, Brasil, China, Espanha, Índia e Portugal, devido à ampla capacidade do eucalipto de se adaptar a ambientes entre as latitudes 45°C Sul e 40°C Norte (ELDRIDGE *et al,* 1997). Além do seu potencial como matéria-prima para a indústria de papel e celulose, o eucalipto também é muito utilizado na indústria madeireira, na produção de carvão para siderurgia, na produção de mel e extração de taninos e óleos essenciais utilizados em produtos de limpeza, aromatizantes e na indústria farmacêutica (TURNBULL, 1999; SOUZA & LORENZI, 2008). No Brasil, os produtos de base florestal representam aproximadamente 3,5% do Produto Interno Bruto (ABRAF, 2010), sendo que o país ocupa a quarta posição em produção mundial de polpa para celulose, tendo uma área plantada de florestas de eucalipto que somam aproximadamente 4,5 milhões de ha, o que corresponde a apenas 0,6% do território nacional (ABRAF, 2010).

Devido ao seu rápido crescimento (cerca de seis a oito anos), florestas de eucalipto são tidas como uma alternativa para suprir a demanda por biomassa lenhosa, evitando a devastação de florestas nativas (FOGAÇA, 2003). Além disso, as plantações florestais comerciais contribuem para a remoção do excesso de dióxido de carbono da atmosfera, já que são compostas apenas por árvores jovens, com alta taxa de fotossíntese, mitigando um dos principais gases causadores do aquecimento global (DEWAR, 1990).

A eucaliptocultura brasileira é baseada principalmente em florestas clonais formadas a partir de materiais elite (MORA & GARCIA, 2000). Características encontradas nas árvores de plantações comerciais, como troncos retos com baixa densidade de ramificações, além da excelente qualidade das fibras e adaptabilidade a diversos tipos de

solo, fazem do eucalipto uma arbórea ideal para cultivos comerciais (POKE *et al,* 2005). O desenvolvimento de florestas clonais permite a preservação de genótipos de interesse, resultando em clones com melhor qualidade e homogeneidade, fatores importantes para a manutenção da vantagem competitiva da indústria nacional de papel e celulose (FOGAÇA, 2003).

1.2 Enraizamento adventício e auxina

A propagação vegetativa é uma das técnicas mais amplamente utilizadas para multiplicar plantas-elite, tanto aquelas obtidas em programas de cruzamento como as selecionadas a partir de populações naturais (HARTMANN *et al,* 1990). Esta estratégia de propagação tem o enraizamento adventício como uma etapa indispensável (DE KLERK *et al,* 1999), sendo particularmente importante para espécies lenhosas de interesse econômico, como o eucalipto (FETT-NETO *et al,* 2001).

O enraizamento adventício é baseado nos princípios de regeneração, processo que, em plantas, corresponde à formação de uma nova parte aérea, raiz ou embrião a partir de tecidos sem um meristema pré-existente (De KLERK, 2002). Este processo pode ser dividido em duas fases principais, as quais possuem diferentes requerimentos hormonais: (1) indução, correspondendo a eventos moleculares e bioquímicos, sem mudança morfológica visível, e (2) formação, que compreende divisões celulares envolvidas na organização do meristema da raiz e estabelecimento do primórdio radical, seguido do alongamento e emergência da raiz (FETT-NETO *et al,* 2001).

A formação de raízes adventícias é comum a todas as plantas vasculares, podendo se desenvolver em diversos pontos ao longo do corpo da planta. Em caules jovens, geralmente se originam do parênquima vascular ou cortical e, antes de emergirem, desenvolvem promeristema, coifa, cilindro vascular e córtex. É o sistema radical característico de estacas para propagação vegetativa, ao contrário de plântulas, as quais

apresentam raízes pivotantes fortemente gravitrópicas, com raízes primárias, oriundas do meristema embrionário, a partir das quais surgem as raízes secundárias, oriundas do periciclo de raízes primárias (ESAU, 1974). Muitas vezes, plantas com raízes adventícias são mais sujeitas ao tombamento, já que este tipo de arquitetura radical proporciona menor capacidade de sustentação. Portanto, o desenvolvimento de raízes adventícias com arquiteturas mais adequadas também é tema de grande interesse.

Múltiplos fatores podem interferir no enraizamento adventício. Fitormônios, compostos fenólicos, condição nutricional, características genéticas (HAND, 1994), bem como fatores externos como luz e temperatura (TAKAHASHI *et al.,* 2003; CORRÊA & FETT-NETO, 2004) e respostas associadas ao estresse modulam este tipo de enraizamento, o que o torna bastante complexo. Um aspecto que trouxe inúmeros avanços ao estudo da rizogênese adventícia foi a utilização do cultivo *in vitro*. Além de possibilitar experimentos com explantes menores, como segmentos de caule, esta técnica viabiliza o fornecimento de fitormônios e vitaminas e a adição controlada de componentes inorgânicos e carboidratos, além de diminuir significativamente a chance de degradação dos mesmos por ação microbiana (VAN DER KRIEKEN *et al,* 1993).

Dentre os fitormônios, a classe das auxinas é essencial para o desenvolvimento vegetal, atuando na divisão, expansão, diferenciação e crescimento celular, desenvolvimento do embrião, formação de novos ramos laterais, indução de diferenciação vascular, alongamento do caule, dominância apical, desenvolvimento de flores e frutos e controle de tropismos (revisado em TEALE *et al,* (2006) e De RYBEL *et al,* (2009)). Além disso, auxinas desempenham um papel fundamental na determinação da capacidade de enraizamento; porém, concentrações que são favoráveis para a indução deste processo bloqueiam o alongamento das raízes, exigindo um eficaz controle dos níveis de auxina ao longo do processo rizogênico. 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. Em plântulas, porém, praticamente todos os órgãos são capazes de sintetizar IAA (Ácido indol-acético - *Indole-3-Acetic-Acid* – auxina natural mais abundante em plantas) (revisado em NORMANLY, 2010). Várias rotas de biossíntese de auxinas são possíveis e estudos fisiológicos, de genética molecular e utilizando marcação com isótopos estáveis permitiram o estabelecimento do triptofano como um precursor para a biossíntese *de novo* de IAA em plantas. Além disso, todas as rotas de biossíntese deste fitormônio definidas para microorganismos são triptofano-dependentes (revisado em BARTEL, 1997). Contudo, em 1992, Wright e colaboradores proporcionaram a melhor evidência experimental da existência de uma rota de síntese de auxina independente de triptofano em plantas. Ao estudarem um mutante de milho com a rota de biossíntese de triptofano bloqueada, os autores perceberam que, apesar deste bloqueio, o mutante apresentava concentrações de IAA 50 vezes maiores do que as plantas selvagens. Estudos posteriores mostraram que o ponto de ramificação para a biossíntese de IAA independente de triptofano é o indol-3-glicerol-fosfato, porém o precursor imediato de IAA nessa rota ainda é desconhecido, sendo o IAN (Indol-3-acetonitrila - *indole-3-acetonitrile*) um candidato para esta função (NORMANLY *et al*, 1993).

Embora nenhuma rota biossintética de auxina tenha sido completamente estabelecida até o momento em plantas, evidências apontam que as mais comuns sejam as rotas da triptamina (TAM) e do ácido-indol-3-pirúvico (IPA), sendo que ambas são dependentes de triptofano e parecem ser bem distribuídas no reino vegetal (revisado em ZHAO, 2010). A rota TAM inicia com a descarboxilação do triptofano para formar triptamina, sendo que estudos *in vitro* em *Arabidopsis* mostram que a triptamina é posteriormente convertida em N-hidroxil-triptamina através da ação de uma enzima codificada pelo gene *YUC1* ou outros membros da família, seguindo até IAA por intermediários ainda não elucidados (ZHAO *et al,* 2001; KIM *et al*, 2007). A importância desta rota se baseia no papel dos genes *YUC* na embriogênese, crescimento da plântula, iniciação de folhas e flores e formação vascular. Além disso, a superexpressão destes genes leva à superprodução de auxina e mutantes do tipo perda-de-função apresentam importantes defeitos no

desenvolvimento (revisado em ZHAO, 2010). Na rota IPA, o triptofano é desaminado para formar o ácido-indol-3-pirúvico, o qual é descarboxilado para formar indol-3-acetaldeído, sendo então convertido em IAA. Estudos com *Arabidopsis* demonstraram a presença de uma aminotransferase, codificada pelo gene *TAA1* (*Tryptophan Aminotransferase of Arabidopsis 1*), a qual é responsável pelo primeiro passo da rota (STEPANOVA *et al,* 2008; TAO *et al,* 2008). Várias evidências indicam o envolvimento de *TAA1* na biossíntese de auxina: mutantes *taa1* contem 60% menos IAA livre e apresentam baixo padrão de expressão de alguns genes sabidamente induzidos por auxina (TAO *et al,* 2008), além de serem resistentes ao inibidor de transporte de auxina NPA, o qual inibe o alongamento de raízes de *Arabidopsis* (YAMADA *et al,* 2009).

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). A atividade de IAA envolve a ligação à proteína do tipo F-box TIR1, desencadeando a degradação via proteasoma de proteínas 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 (WOODWARD & BARTEL, 2005; DHARMASIRI *et al,* 2005). A proteína AGO1 (*Argonaute 1*) está envolvida no complexo de regulação de miRNAs, e mutantes deficientes nessa proteína apresentam severas limitações na rizogênese adventícia devido ao acúmulo de uma classe de ARFs inibidores de transcrição - *ARF17*, o qual não interage adequadamente com proteínas Aux/IAA e pode bloquear promotores de genes responsivos à auxina - além de afetar o aumento de atividade de proteínas GH3, indutoras de conjugação de auxinas (SORIN *et al.,* 2005), levando-as a um estado inativo. Um mecanismo alternativo, o qual não envolve degradação de Aux/IAA e parece estar mais relacionado com resposta a IBA (Ácido indol-butírico - *Indole Butyric Acid*) se dá através da proteína tipo fosfatase de dupla especificidade IBR5

(receptor 5 de ácido indol butírico), a qual parece agir *downstream* ao reconhecimento da auxina pelo complexo $SCF^{TIR1/AFB}$-Aux/IAA (STRADER *et al,* 2008).

A exposição a auxinas exógenas acarreta em aumento do número de primórdios de raízes (LOIO *et al,* 2008), e a maioria dos programas comerciais de propagação utiliza IBA para o enraizamento adventício de estacas. IAA também é usado, porém em menor extensão, assim como a auxina sintética NAA (Ácido naftaleno-acético - *Naphtalene Acetic Acid*) (FOGAÇA e FETT-NETO, 2005). A eficácia de cada tipo de auxina depende da afinidade pelo receptor envolvido no enraizamento, da concentração de auxina livre disponível, da concentração de auxina endógena e da estabilidade metabólica, sendo esta baixa em IAA, intermediária em IBA e alta em NAA (DE KLERK *et al,* 1999).

1.3 *Eucalyptus globulus* e *Eucalyptus grandis*

O interesse da indústria de papel e celulose do sul do Brasil em *Eucalyptus globulus* se deve às características de baixo teor de lignina e relativa tolerância à geada, apresentadas tanto por essa espécie, como por vários de seus híbridos interespecíficos, quando comparada a espécies de eucaliptos tropicais. A geada é um fenômeno característico da região sul, que pode ter efeitos bastante severos sobre as plantas enquanto a lignina interfere negativamente na extração da celulose (CHIANG, 2002), aumentando significativamente os custos deste processo. Entretanto, *E. globulus* é considerado recalcitrante ao enraizamento (LE ROUX & VAN STADEN, 1991 e SERRANO *et al.,* 1996), com mudas difíceis de propagar. Neste caso, a aplicação de auxina exógena é necessária para um bom desenvolvimento radical (FOGAÇA e FETT-NETO, 2005), porém não se sabe se isto se deve a alguma alteração no mecanismo de transporte e/ou sinalização deste fitormônio, ou se o conteúdo de auxina endógena é insuficiente nesta espécie para desencadear todas as respostas necessárias ao enraizamento.

Eucalyptus grandis é a espécie mais comumente plantada no Brasil (CANETTIERI *et al.,* 2007), possuindo madeira relativamente macia e menos densa que outras espécies de eucalipto, ideal para diferentes utilidades, incluindo papel e celulose, carvão, postes de iluminação e madeira para construção (ELDRIDGE *et al.,* 1997). É muito utilizada para produção de híbridos na indústria de papel e celulose e tem como características interessantes adaptabilidade e boa qualidade de fibra (ALFENAS *et al.,* 2004). É considerada propensa ao enraizamento, sendo sua propagação clonal também dependente de enraizamento adventício.

Estudos comparativos de espécies com diferentes capacidades de enraizamento representam uma estratégia apropriada para estudos que visam analisar parâmetros fisiológicos e moleculares relevantes ao controle deste processo de desenvolvimento. A compreensão dos mecanismos do enraizamento adventício pode trazer importantes avanços no controle deste processo, tão importante para a viabilidade das florestas plantadas comerciais, podendo resultar em aumento na produtividade das mesmas.

1.4 PCR Quantitativo em Tempo Real e genes-referência

Em plantas lenhosas, a formação do meristema radical ainda é pouco compreendida no âmbito da expressão gênica (LI *et al.,* 2009), sendo poucos os trabalhos que se referem à formação de raízes adventícias. Consequentemente, pouco se sabe sobre os genes envolvidos neste processo em árvores. SANCHEZ e colaboradores (2007) verificaram o padrão de expressão de dois genes *Scarecrow-like* em duas espécies arbóreas não-relacionadas (*Pinus radiata* e *Castanea sativa*) e constataram que esses genes são altamente expressos em raízes e são induzidos por auxina exógena, ocorrendo um aumento nos níveis de mRNA nas primeiras 24h do processo de indução de raízes. Os autores concluem que esses genes podem desempenhar papéis importantes nos estágios inicias do enraizamento adventício. MACEDO e colaboradores (2009) analisaram o envolvimento de

genes *AOX* (*Alternative Oxidase*) na indução de raízes em plântulas de oliveira e verificaram um aumento na expressão de *AOX2* no momento da indução dos primórdios radicais. Estudos subseqüentes do grupo objetivam analisar *AOX* como um possível marcador para seleção de árvores com indução de raízes mais eficiente.

Diversos estudos visando melhorar o entendimento da fisiologia do processo de rizogênese adventícia em *Eucalyptus* sp. foram conduzidos (FETT-NETO *et al,* 2001; CORRÊA e FETT-NETO, 2004; FOGAÇA e FETT-NETO, 2005; SCHWAMBACH *et al,* 2005; SCHWAMBACH *et al,* 2008), porém ainda não estão disponíveis estudos acerca deste tema em nível molecular. Neste sentido, a técnica de PCR quantitativo em tempo real (qPCR) se mostra como uma boa estratégia, permitindo analisar o padrão de expressão de genes de interesse durante um determinado processo de desenvolvimento.

O qPCR é baseado no processo de transcrição reversa (RT) seguida da Reação em Cadeia da DNA-polimerase (PCR) com a incorporação de moléculas fluorescentes covalentemente ligadas ou não a nucleotídeos, as quais podem ser quantificadas durante a cinética da reação. Os produtos formados são monitorados a cada ciclo, o que permite uma detecção rápida e precisa dos produtos de amplificação. Atualmente é um dos métodos de maior sensibilidade e especificidade na análise de transcritos (GACHON *et al*, 2004, VAN GUILDER *et al,* 2008).

No entanto, esta técnica necessita de normalização para uma correta interpretação dos dados obtidos. O propósito desta etapa é “padronizar” eventuais variações associadas com os vários passos do procedimento experimental, como diferenças na quantidade inicial de amostra, integridade do RNA, eficiência na síntese do cDNA e diferenças na atividade transcricional nos tecidos e células analisados (EXPÓSITO-RODRÍGUEZ *et al*, 2008). A normalização é realizada com base em genes que possuam expressão uniforme na maioria das células do organismo de estudo, bem como durante as várias fases de desenvolvimento e sob diferentes condições ambientais. Estes são os chamados genes-referência, constitutivos, controles endógenos ou *housekeeping genes*. Considerando que tanto a sequência do gene-referência como a sequência do gene de interesse estarão presentes na

amostra testada, ambos apresentarão o mesmo padrão de variação dentro do experimento, exceto em relação ao nível de expressão (EXPÓSITO-RODRÍGUEZ *et al,* 2008). Em geral são escolhidos genes envolvidos em processos celulares básicos como manutenção da estrutura celular e metabolismo primário (CZECHOWSKI *et al,* 2005).

Embora vários genes tenham sido amplamente utilizados como constitutivos em estudos com plantas, como codificadores do rRNA 18S, gliceraldeído-3-fosfato desidrogenase, fator de alongamento 1α, actina, α- tubulina e β-tubulina, trabalhos desenvolvidos na última década demonstraram a necessidade de uma determinação específica para cada espécie e processo de estudo (BRUNNER *et al,* 2004; CZECHOWSKI *et al,* 2005; GUTIERREZ *et al.,* 2008), uma vez que um gene-referência adequado para uma espécie ou processo estudado pode não apresentar expressão estável em outra situação, bem como entre tecidos e genótipos, o que invalida sua utilização como referência universal para esta finalidade.

Tendo em vista a importância de determinar a estabilidade de expressão de genes candidatos a controles internos em experimentos de qPCR, vários trabalhos têm sido desenvolvidos nos últimos anos, com as mais variadas espécies. Tal avanço se deve, em parte, ao desenvolvimento de ferramentas que permitem a identificação estatística dos melhores controles internos de um grupo de candidatos para um dado experimento. Dentre as mais utilizadas e bem sucedidas ferramentas encontram-se o software *geNorm* (VANDESOMPELE *et al,* 2002), e o software *NormFinder* (ANDERSEN *et al,* 2004). *geNorm* é uma Aplicação Básica Visual (VBA) para Microsoft Excel que calcula automaticamente uma medida (M) de estabilidade de expressão para cada gene controle em um dado grupo de amostras. Além disso, o software indica o número mínimo de genes necessário para uma normalização acurada dos dados, uma vez que seus autores sugerem a utilização de dois ou mais genes para normalização ao invés de apenas um. *Normfinder* também é um aplicativo para Microsoft Excel e leva em consideração variações intra- e inter-grupo para o cálculo dos fatores de normalização, sendo que seus resultados não são afetados devido à ocasional utilização de genes co-regulados, ao contrário de *geNorm*. De

acordo com *NormFinder*, o gene mais estável será aquele que apresentar um valor de variação inter-grupo mais próximo de zero possível e que tenha, ao mesmo tempo, as menores barras de erros, sendo que o programa também indica a melhor combinação de dois genes a serem usados em conjunto para a normalização.

Dentre as plantas arbóreas, a mais representada em estudos com genes-referência e sobre a qual existem resultados mais consistentes é *Populus* sp (BRUNNER *et al,* 2004; GUTIERREZ *et al,* 2008). Um estudo recente analisou a estabilidade de genes tradicionais e oriundos de estudos de microarranjo em folhas, flores e xilema em seis espécies de *Eucalyptus* crescidas a campo. Os resultados indicaram três novos genes, derivados das análises de microarranjo, como os mais indicados para normalização em estudos de expressão gênica com espécies de eucalipto a campo (BRETON, 2011). Até a presente dissertação, no entanto, nenhum estudo de normalização havia sido realizado com plantas *in vitro* e/ou durante o processo de enraizamento adventício em eucalipto.

2 OBJETIVO GERAL

Melhorar o entendimento do processo de rizogênese adventícia através da análise cinética e quantitativa da expressão de transportadores de auxina ao longo do processo de enraizamento adventício *in vitro* de *E. globulus* e *E. grandis.*

Nossa hipótese de trabalho é que a recalcitrância de *E. globulus* ao enraizamento adventício está, ao menos em parte, relacionada a problemas no transporte e localização de auxina em tecidos-alvo envolvidos no processo.

2.1 Objetivos Específicos

- Determinar genes-referência adequados para estudos de expressão gênica durante o enraizamento adventício *in vitro* em *E. globulus* e *E. grandis*, utilizando ferramentas específicas para estudos subseqüentes com qPCR.

- Analisar a expressão de ortólogos de *AUX1* e *PIN1* – típicos transportadores de influxo e efluxo de auxina, respectivamente, conhecidos na planta modelo *Arabidopsis thaliana* – em duas espécies de *Eucalyptus* com diferentes capacidades de enraizamento, usando qPCR.

3. Reference gene selection for quantitative reverse transcription-polymerase chain reaction normalization during *in vitro* adventitious rooting in *Eucalyptus globulus*

Artigo publicado no periódico *BMC Molecular Biology*, no ano de 2010.

de Almeida *et al. BMC Molecular Biology* 2010, **11**:73
http://www.biomedcentral.com/1471-2199/11/73

BMC Molecular Biology

RESEARCH ARTICLE Open Access

Reference gene selection for quantitative reverse transcription-polymerase chain reaction normalization during *in vitro* adventitious rooting in *Eucalyptus globulus* Labill

Márcia R de Almeida[1], Carolina M Ruedell[2], Felipe K Ricachenevsky[1], Raul A Sperotto[1], Giancarlo Pasquali[1], Arthur G Fett-Neto[1,2*]

Abstract

Background: *Eucalyptus globulus* and its hybrids are very important for the cellulose and paper industry mainly due to their low lignin content and frost resistance. However, rooting of cuttings of this species is recalcitrant and exogenous auxin application is often necessary for good root development. To date one of the most accurate methods available for gene expression analysis is quantitative reverse transcription-polymerase chain reaction (qPCR); however, reliable use of this technique requires reference genes for normalization. There is no single reference gene that can be regarded as universal for all experiments and biological materials. Thus, the identification of reliable reference genes must be done for every species and experimental approach. The present study aimed at identifying suitable control genes for normalization of gene expression associated with adventitious rooting in *E. globulus* microcuttings.

Results: By the use of two distinct algorithms, *geNorm* and *NormFinder*, we have assessed gene expression stability of eleven candidate reference genes in *E. globulus*: *18S, ACT2, EF2, EUC12, H2B, IDH, SAND, TIP41, TUA, UBI* and *33380*. The candidate reference genes were evaluated in microccuttings rooted *in vitro*, in presence or absence of auxin, along six time-points spanning the process of adventitious rooting. Overall, the stability profiles of these genes determined with each one of the algorithms were very similar. Slight differences were observed in the most stable pair of genes indicated by each program: *IDH* and *SAND* for *geNorm*, and *H2B* and *TUA* for *NormFinder*. Both programs indentified *UBI* and *18S* as the most variable genes. To validate these results and select the most suitable reference genes, the expression profile of the *ARGONAUTE1* gene was evaluated in relation to the most stable candidate genes indicated by each algorithm.

Conclusion: Our study showed that expression stability varied between putative reference genes tested in *E. globulus*. Based on the *AGO1* relative expression profile obtained using the genes suggested by the algorithms, *H2B* and *TUA* were considered as the most suitable reference genes for expression studies in *E. globulus* adventitious rooting. *UBI* and *18S* were unsuitable for use as controls in qPCR related to this process. These findings will enable more accurate and reliable normalization of qPCR results for gene expression studies in this economically important woody plant, particularly related to rooting and clonal propagation.

* Correspondence: fettneto@cbiot.ufrgs.br
[1]Center for Biotechnology, Federal University of Rio Grande do Sul, P.O. Box 15005, 91501-970 Porto Alegre, RS, Brazil
Full list of author information is available at the end of the article

BioMed Central

© 2010 de Almeida et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Background

Eucalyptus sp. is one of the most planted hardwood genus in the world [1], mainly because of its applications for generating wood and paper products. Brazil is the largest world producer of eucalypts for short fiber pulp [2], thanks to clonal forests developed from elite material with high productivity [3]. In southern Brazil and temperate areas, especially Mediterranean Europe, Portugal and Chile, *Eucalyptus globulus* and its hybrids are of interest for the cellulose industry due to their relatively high frost resistance and low lignin content, which facilitates cellulose extraction [4]. On the other hand, *E. globulus* is generally considered recalcitrant to rooting [5,6]. The commercial eucalypt forests are generally formed through vegetative propagation which has adventitious rooting as a key step [7]. This developmental process can be divided in two main steps, each with its own requirements and characteristics: (1) induction step, which involves biochemical and molecular events, without visible morphological changes; and (2) formation step, which consists of cellular divisions involved in both root meristem organization and primordium establishment, followed by root elongation and emergence out of the cutting [8].

Multiple factors can interfere with adventitious rooting, such as phytohormones, phenolic compounds, nutritional conditions and genetic characteristics [9]. Among phytohormones, auxins play a central role in rooting capacity [10], particularly its endogenous content and transport rate [11]. However, concentrations that are beneficial to root induction can block its elongation [7]. The shoot apex is the main source of endogenous auxin. Stems have a specific basipetal active transport through vascular parenchyma carried out by both influx (AUX1) and efflux (PIN) carriers [12,13].

Eucalyptus globulus needs exogenous auxin application for an adequate root development *in vitro* [8], but it is unknown if this occurs because of defficiencies in auxin content, transport or perception mechanisms, or due to some other non-auxin related reason that can block spontaneous rooting responses. At gene expression level, little is known about the formation of root meristems in woody plants and even less concerning the effect of auxins on this process [14].

To evaluate changes in gene expression, reverse-transcription followed by quantitative, real-time polymerase chain reaction (qPCR) is one of the most widely used methods. Some of the advantages of qPCR are high sensitivity and specificity, speed, ease of use and capacity to carry out simultaneous measurements of gene expression in several different samples for a limited number of genes [15-17]. To accurately and reproducibly quantify gene expression, some factors should be taken into account, such as quality and amount of starting material, presence of inhibitors in different samples, primer design, RNA extraction and reverse-transcription efficiencies [16]. Therefore, selection of an appropriate normalization strategy is essential for obtaining an accurate and reliable quantification of gene expression levels [15,16,18]. The most commonly applied approach for normalization of qPCR is the use of one or more endogenous reference genes [18-20], which will undergo the same type of variation and preparation steps throughout the assay as the target genes.

An ideal reference gene, also known as housekeeping or internal control gene, is assumed to be constitutive, i. e., it should be expressed at a constant level in the majority of the study organism cells, through different developmental stages and in different organs. Besides, its expression is assumed to be unaffected by experimental parameters [21]. Genes involved in basic cellular processes, such as cell structure maintenance or primary metabolism, are often chosen as normalizers [22]. However, the transcript levels of these putative reference genes are not always stable and their systematic use without previous validation may lead to the misinterpretation of results. Indeed, the previous assurance of at least one stable gene expression in all variable situations tested is recommended to accurately validate the expression of genes under analysis [22-27]. In recent years, a large number of reference gene validation attempts have been reported for plants, but most of them have covered model and crop species: rice [28,29], sugarcane [30], *Arabidopsis thaliana* [22,31], potato [26], grape [32], barley [33], *Brachypodium* sp. [34], soybean [35,36], tomato [37], *Brachiaria* sp. [38], coffee [39], peach [40], wheat [41], chicory [42], cotton [43], cucumber [44], longan tree [45], *Petunia* sp. [46], ryegrass [47], rapeseed [48] and tobacco [49]. Just a few studies have focused on forest genera, such as poplar [25,27] and *Eucalyptus* sp. [50]; however, there is a lack of investigations covering *in vitro* growth or adventitious rooting.

The aim of this study was to evaluate the stability of 11 putative reference genes for the purpose of normalization in studying *E. globulus* gene expression during *in vitro* adventitious rooting (Figure 1). Statistical methods implemented in *geNorm* [24] and *NormFinder* [51] were used and compared in this evaluation. In addition, to further validate the reference genes, an expression analysis of a putative *E. globulus* orthologue of the *A. thaliana ARGONAUTE1 (AGO1)* gene during rooting of microcuttings was carried out.

Results

To identify the most stable genes in *E. globulus* microcuttings rooted *in vitro*, we selected 11 candidate reference

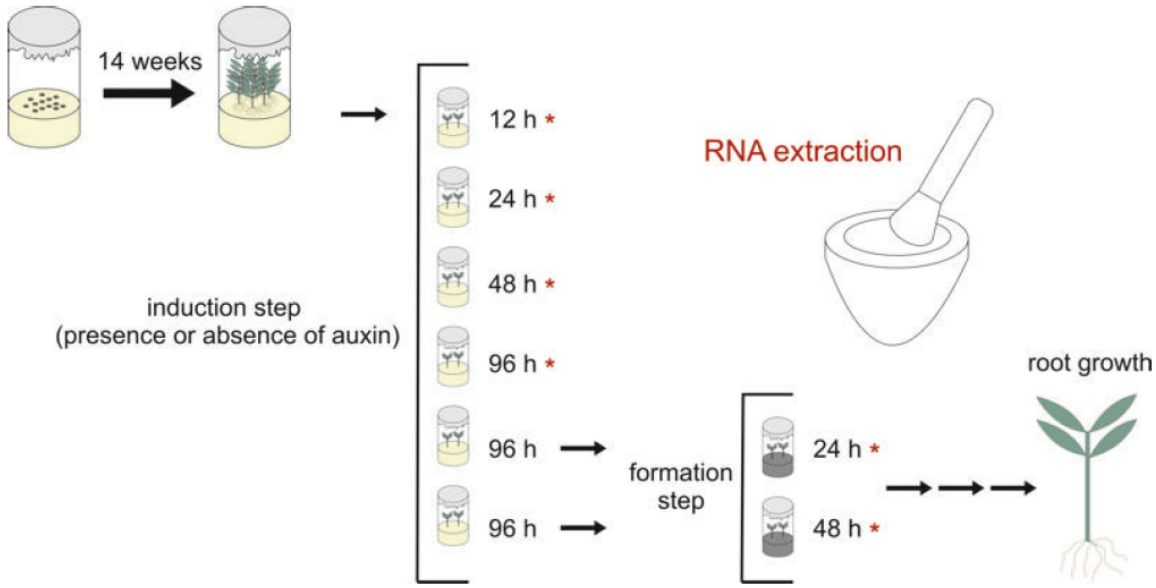


Figure 1 Stages of *in vitro* adventitious rooting in *Eucalyptus globulus*. After seeding, seedlings remained 14 weeks in germination medium. After this time, apical microcuttings were obtained and used in adventitious rooting experiments. The microcuttings were kept in induction medium (presence or absence of 10 mg l^{-1} IBA) for 12, 24, 48 or 96 h, depending on the harvest time. For the harvest points in formation medium, the microcuttings remained 96 h in induction medium and after that were transferred to formation medium (devoid of auxin and with activated charcoal). In this case, the samples were collected after 24 and 48 h. The samples harvested at 12, 24, 48 and 96 h of induction step and at 24 and 48 h of formation step were submitted to RNA extraction and used for the analyses.

genes to validate by qPCR. The gene expression stability of the candidates was evaluated in a set of 12 samples covering six different stages along adventitious rooting time in the presence or absence of the phytohormone auxin. The genes included: 18S ribosomal RNA (*18S*) [25-27,40], Actin 2/7 (*ACT2*) [24,40], Translation elongation factor 2 (*EF2*) [40], Histone H2B (*H2B*) [52], NADP Isocitrate Dehydrogenase (*IDH*) [53-55], Polyubiquitin (*UBI*) [25,27], SAND protein (*SAND*) [22,31], TIP41-like protein (*TIP41*) [22,27,31,37], Alpha-tubulin (*TUA*) [25], an *Eucalyptus* ortholog of *Arabidopsis thaliana* expressed protein without determined function (*33380*) [22,27,31], and a putative RNA binding protein (*EUC12*) previously shown to be constitutive in different *Eucalyptus* organs (unpublished results). The characteristics of each of the listed genes, such as gene name, *A. thaliana* ortholog locus, *A. thaliana* locus description, *GenBank* accession number, function and *E-value* are provided in Table 1. All *Arabidopsis* genes were used simply to identify the corresponding orthologs in *Eucalyptus* using the *Genolyptus project* data base. The specificity of the amplifications was confirmed by the presence of a single band of expected size for each primer pair in agarose gel electrophoresis after PCRs employing either cDNA or chromosomal DNA as templates, and by the observation of single-peak melting curves of the qPCR products (data not shown). No primer dimers or other products resulted from non-specific amplification. Amplification efficiencies of PCRs ranged from 1.790 for *EF2* to 1.972 for *H2B* (Table 2).

Expression levels of reference gene candidates

Not surprisingly, some variations on Cq values, i.e. number of cycles needed for the amplification-related fluorescence to reach a specific threshold level of detection, occurred among the reference genes tested. Transcripts of *18S* were the most abundant (mean Cq = 12.48 ± 1.87), followed by *H2B, 33380, ACT2, IDH, UBI, TUA, TIP41, EF2* and *SAND*, whereas *EUC12* was the least abundant (mean Cq = 25.30 ± 0.63) (Figure 2A). The expression profiles of all candidate reference genes along the adventitious rooting process are depicted in Figure 2B.

Expression stability of reference gene candidates

Two programs were used to evaluate the stability of the 11 candidate reference genes: *geNorm* [24] and *NormFinder* [52]. Cq data were collected for all samples and transformed to relative quantities using the delta-Cq method developed by Livak and Schmittgen [56] (see Material and Methods section for details).

Table 1 Description of reference genes used for qPCR in *Eucalyptus globulus*

Gene symbol	Gene name	*Arabidopsis thaliana*'s best hit locus	*Arabidopsis thaliana* locus description	Putative *E. globulus* orthologs GeneBank accession number	Function	E-value (tblastx)
18S	RNA ribosomal 18S	At3g41768	RNA ribosomal 18S	HO048251	Cytosolic small ribosomal subunit, translation	0,0
ACT2	Actin 2/7	At5g09810	Actin 2/7	HO048249	Structural constituent of cytoskeleton	0,0
EF2	Translation elongation factor 2	At1g56070	Translation elongation factor 2, putative	HO048253	Translation factor activity, nucleic acid binding	e-176
EUC12	Putative RNA bindig protein	*	*	HO048248	Unknown	e-78
H2B	Histone H2B	At5g59910	Histone H2B, putative	HO048243	Strutural constituent of the eukaryotic nucleosome core	e-66
IDH	NADP-Isocitrate dehydrogenase	**	**	HO048252	Carbohydrate metabolism	**
UBI	Polyubiquitin	At4g05050	Polyubiquitin gene, belongs to a subtype group with UBQ10 and UBQ14	HO048245	Signalling complexes for protein degradation, translation control, DNA repair, endocytosis regulation, protein traffic	e-144
SAND	SAND protein	At2g28390	SAND family protein	HO048246	Intracellular vesicular transport, biogenesis and vacuole signalling	e-68
TIP41	TIP41-like protein	At4g34270	TIP41-like family protein	HO048247	Unknown	e-78
TUA	Alpha-tubulin	At5g19780	Alpha tubulin-5	HO048250	Structural constituent of cytoskeleton, microtubule-based processes	e-134
33380	Expressed protein	At4g33380	Expressed sequence	HO048244	Unknown	e-74

* Gene assumed as constitutive in microarray studies employing leaf and vascular tissues of fully grown *E. globulus* and *E. grandis* trees (unpublished results).
** Gene obtained from Carvalho *et al.* (2008) [55].

geNorm analysis

The average expression stability (*M* value) of all genes was calculated by *geNorm* (version 3.5). The *M* value is defined as the average pairwise variation of a particular gene with all other potential reference genes. The average *M* values of the candidate reference genes tested are shown in Figure 3 and summarized in Table 3. The *geNorm* program recommends using an *M* value below the threshold of 1.5 to identify reference genes with stable expression, but some authors propose the maximum value of 0.5 to obtain more accurate results [57,27]. Our results indicate *IDH* and *SAND* as the most stably expressed genes (i. e., with the lowest *M* value = 0.320) and *18S* as the least (*M* value = 0.935). To evaluate the optimal number of reference genes for reliable normalization, *geNorm* calculates the pairwise variation V_n/V_{n+1} between the sequential ranked normalization factors NF_n and NF_{n+1} to determine the effect of adding the next reference gene in normalization. The normalization factor is calculated based on the geometric average among the three most stable genes relative quantities and stepwise inclusion of the other genes in the order of their expression stability. A large pairwise variation implies that the added reference gene has a significant effect on normalization and should be included for calculation of a reliable normalization factor [24]. Considering the *cut-off* value of 0.15 proposed by Vandesompele *et al.* [24], below which the inclusion of an additional reference gene is not necessary, the use of the two most stably expressed genes (*IDH* and *SAND*) is sufficient for accurate normalization ($V_{2/3}$ = 0.107) in *E. globulus in vitro* rooting (Figure 4). If needed, the *EUC12* gene can be added to the analysis, resulting in a pairwise variation value of 0.087 ($V_{3/4}$ = 0.087) (Figure 4).

NormFinder analysis

In addition to the analysis by the *geNorm* software, the expression stability of the candidate reference genes on the experimental samples was also evaluated by *NormFinder*. This program takes into account the intra- and inter-group variations for normalization factor calculation and the results are not affected by occasional co-regulated genes. The best candidate will be the one with the inter-group variation as close to zero as possible, and, at the same time, having the smallest errors bars possible [51]. A change in the indication of most stable genes was observed after *NormFinder* analysis when compared to *geNorm*, with *H2B* showing the most stable

Table 2 Primer sequences and amplicon characteristics for each of the 11 reference genes and for the *Argonaute 1* gene of interest

Gene symbol	Primer sequence (5' → 3') Forward/Reverse	Amplicon length (bp)	Annealing temperatures (°C)	Amplification efficiency
18S	TGACGGAGAATTAGGGTTCG/ CCGTGTCAGGATTGGGTAAT	100	60	1.860
ACT2	TCCACCATGTTCCCTGGTAT/ ACCTCCCAATCCAGACACTG	124	60	1.935
EF2	GCGTTCCCTCAGTGTGTCTT/ GGTCATCTGCTCCTTCAAGC	126	60	1.790
EUC12	GCGTGGTTCTTGGATCACTA/ TGGTGACAAAGTCAGGTGCT	114	59	1.807
H2B	GAAGAAGCGGGTGAAGAAGA/ GGCGAGTTTCTCGAAGATGT	145	60	1.972
IDH *	CTGTTGAGTCTGGAAGATGAC/ CATTTAATTCCTCCCCAACAAA	271	60	1.892
UBI	AGAAGGAATCGACCCTCCAC/ CCTTGACGTTGTCAATGGTG	126	60	1.888
SAND	CCATTCAACACTCTCCGACA/ TGTGTGACCCAGCAGAGTAAT	143	59	1.919
TIP41	GAACAAAAGCTCGGGACATC/ CAACCAGCAAGAGCATCAAA	122	60	1.938
TUA	ACCGGTTGATCTCTCAGGTG/ TAAGGGACCAGGTTGGTCTG	103	60	1.845
33380	TCCAGAGTGCAATGCTGAAC/ CCCCTCGTCTGGCATACTTA	134	60	1.935
AGO1	TCTTGGGCTCGTTTCTCAGT GGAATTCGCCTAGACAGTGC	144	60	1.887

* Primer obtained from Carvalho *et al.* (2008) [55].

expression (near zero variation value and small error bars), followed by *TUA*. These two genes were also among the most stably expressed genes based on *geNorm* analysis, displaying *M* values below 0.5 (Figure 3). *UBI* and *18S* were the least stable genes (both with variation values farther from zero and larger error bars) (Table 3). When defining the best combination of two genes using *NormFinder*, once again both *H2B* and *TUA* were the genes of choice, with a stability value of 0.045 when used together (data not show).

Relative expression profile of *AGO1*

To further validate the control genes and for determining the best choice of genes to use, the relative expression of a putative ortholog of *A. thaliana ARGONAUTE1* (*AGO1*) gene in *E. globulus* was investigated during rooting. *AGO1* encodes a micro RNA binding protein [58] involved in regulating the expression of *ARF17* (*Auxin Response Factor 17*), an auxin response transcription factor with inhibitory action, possibly controling genes related to auxin homeostasis and adventitious root development in *A. thalina* [10]. The two gene pairs indicated by *geNorm* and *Normfinder* were used as references to evaluate *AGO1* relative expression in eight samples, covering time points of adventitious root induction and formation, both under presence and absence of auxin during the induction step.

Using *IDH* and *SAND* as references (indicated by *geNorm*) *AGO1* relative expression profile was not significantly different among the adventitious rooting time points analysed both in presence or absence of auxin (Figure 5A). When *H2B* and *TUA* were used as references (indicated by *NormFinder*), *AGO1* relative expression at 48 h after transfer to formation step (48 h form) was significantly higher in absence of auxin (control), whereas no differences were detected for the auxin exposure treatment (Figure 5B). Moreover, when comparing presence and absence of auxin within each time point, *AGO1* relative expression after 48 h of culture on root induction medium (48 h ind) and at 24 h after transfer to root formation medium (24 h form) was higher in absence of auxin (control) than in presence of auxin (Figure 5B). Thus, the control gene combinations suggested by each program yielded different relative expression profiles of a gene of interest.

Discussion

In gene expression studies, qPCR is often the method of choice for a target gene expression profile investigation

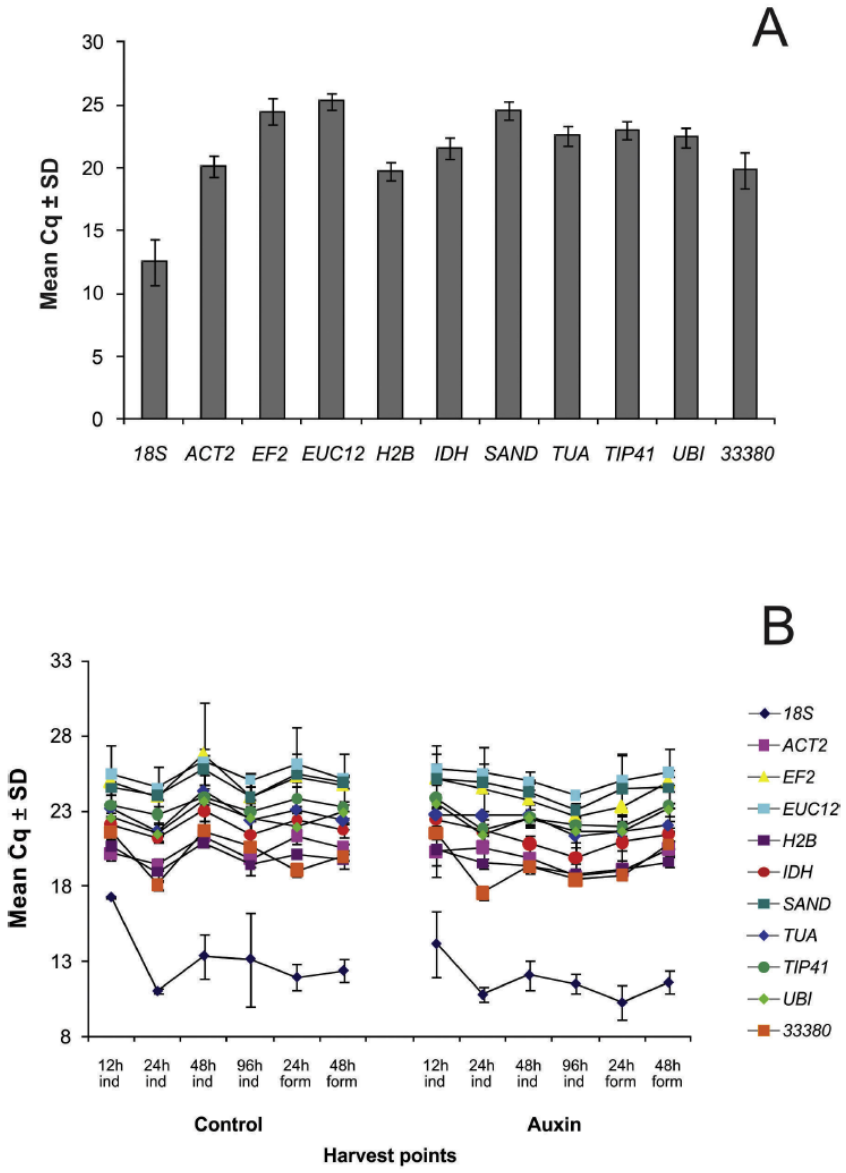


Figure 2 RNA transcription level of reference genes tested, presented as Cq mean value ± Standard Deviation (SD). (A) Mean Cq of each reference gene. (B) Transcription level profile of reference genes along the adventitious rooting process. ind: induction step; form: formation step. Auxin: indicates addition of 10 mg l^{-1} Indol Butyric Acid (IBA) in induction step culture medium; control: indicates absence of IBA in induction step culture medium.

because it is currently one of the most sensitive techniques available [15-17,20]. In this context, a correct normalization is a pre-requisite for the reliability of the final results [59,60], especially considering possible biological relevance of small differences in gene expression or in the study of different tissues and organ samples [18].

In the present work, candidate reference genes for expression studies on adventitious rooting in *E. globulus* microcuttings were selected based on previous reports of normalization in plants, mainly with *A. thaliana*, *Populus* sp. and *Eucalyptus* sp., taking into account the model plant status of the first species and the woody habit of the last two genera. Additional genes frequently

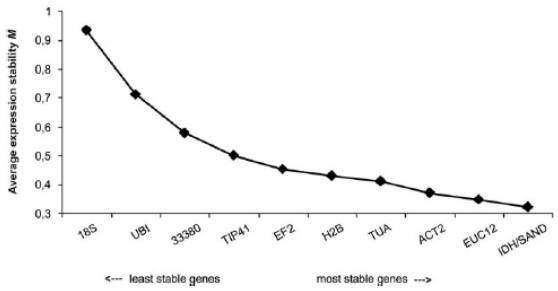


Figure 3 Gene expression stability (*M*) and ranking of the 11 reference genes as calculated by *geNorm* in *Eucalyptus globulus* microcuttings during *in vitro* adventitious rooting. The microcuttings were rooted in presence or absence of 10 mg l^{-1} Indol Butyric Acid (IBA) in the induction step. A lower average expression stability *M* value indicates more stable expression.

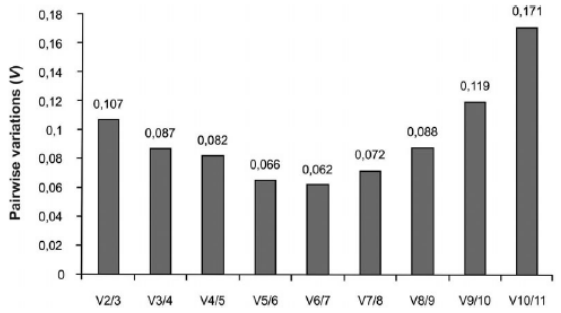


Figure 4 Determination of the optimal reference gene number as calculated by *geNorm* for accurate normalization during *Eucalyptus globulus in vitro* adventitious rooting. *geNorm* pairwise variation values (V values) are calculated by an algorithm which measures pairwise variation (Vn/n + 1) between two sequential normalization factors NFn and NFn + 1, where n is the number of genes involved in the normalization factor.

used for normalization in qPCR studies in other plant species were also evaluated.

The use of a single reference gene has been avoided to minimize the chances of erroneous expression estimates [24,57]. In fact, the use of multiple reference genes is becoming the golden standard in expression studies [19]. In order to select the most suitable reference genes for use in adventitious rooting, we investigated the relative expression profile of a gene of interest (*AGO1*) during this process, using the pairs of reference genes indicated as most stable by each one of the programs (*geNorm* and *NormFinder*).

An increase in *AGO1* expression would be expected during microcutting root development considering its function in regulating auxin metabolism-related genes during adventitious rooting in *Arabidopsis* [10]. The higher *AGO1* expression in the absence of auxin may be due to a lower requirement for the regulation of auxin-metabolism related genes in presence of exogenous auxin.

Table 3 Ranking of candidate reference genes in decreasing order of expression stability calculated by *geNorm* and *NormFinder*

Ranking order	*geNorm* (*M* value)	*NormFinder* (Stability value ± EB*)
1	*IDH/SAND (0.320)*	*H2B (0.031 ± 0.006)*
2		*TUA (0.084 ± 0.067)*
3	*EUC12 (0.346)*	*IDH (0.116 ± 0.082)*
4	*ACT2 (0.370)*	*EUC12 (0.121 ± 0.087)*
5	*TUA (0.410)*	*TIP41 (0.123 ± 0.091)*
6	*H2B (0.429)*	*SAND (0.143 ± 0.124)*
7	*EF2 (0.452)*	*33380 (0.161 ± 0.156)*
8	*TIP41 (0.500)*	*EF2 (0.174 ± 0.183)*
9	*33380 (0.579)*	*ACT2 (0.186 ± 0.215)*
10	*UBI (0.712)*	*UBI (0.306 ± 0.566)*
11	*18S (0.935)*	*18S (0.547 ± 1.833)*

* EB = Error Bars

Therefore, *NormFinder* seemed to be better suited for the selection of the most stable reference genes for monitoring gene expression during adventitious rooting, detecting subtle differences that can be regarded as important for the adventitious rooting process. Recent studies have reported some discrepancies between different approaches for selection of reference genes and have chosen *NormFinder* [43,45,61,62] as data analysis tool. This is mainly due to the wide applicability of this tool in any kind of experimental design, especially when involving different cell types and experimental stages associated with high variation of gene expression [45,51]. Hence, we suggest *H2B* and *TUA* as the most adequate reference genes for expression studies during the rooting of *E. globulus* microcuttings.

H2B (Histone H2B) is one of the four core histones that form the mononucleosome, the fundamental repeating unit of chromatin in eukaryotic cells [63], being a key component for genetic material structure [52]. Two works have motivated us to include *H2B* in our analysis. Hays et al. [64] employed it as reference in Northern blot analysis to assess differential gene expression between alfalfa (*Medicago sativa* L.) varieties exhibiting long- or short-stalked glandular hairs. Later, Sterky et al. [65], through a transcriptomics approach, showed that transcripts for histones (and ribosomal proteins) were among the most ubiquitous gene products present in different tissues and species of *Populus*. More recently, a histone H3 gene was included when defining reference genes for the analysis of gene expression in longan tree somatic embryogenesis by qPCR [45]. Nevertheless, histone genes have been employed as

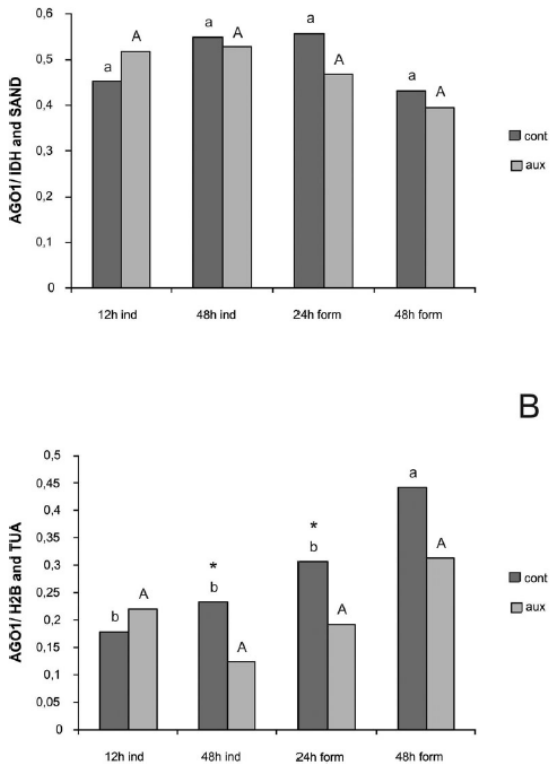


Figure 5 *AGO1* relative expression profile during *Eucalyptus globulus in vitro* adventitious rooting. The *AGO1* (*Argonaute 1*) expression profile was investigated relative to the best combination of reference genes indicated by both *geNorm* (A) and *NormFinder* (B) programs. Aux: indicates addition of 10 mg l^{-1} Indol Butyric Acid (IBA) in induction step culture medium; cont: indicates absence of IBA in induction step culture medium; Columns sharing the same letter are not different by ANOVA followed by Duncan test ($P \leq 0.05$); small letters correspond to analysis of variance (ANOVA) performed for control treatment samples; capital letters correspond to analysis of variance (ANOVA) performed for samples treated with auxin. *: indicates significant difference between treatments within time points by *t-test* ($P \leq 0.05$).

references mostly for expression studies in animal systems [66,67].

The high expression stability showed in our experiments for *TUA* corroborates data of Brunner *et al.* [24] in a study with poplar. On the other hand, Tong and collaborators found *TUA* as one of the most variable genes for studies with peach, except when comparing genotypes or different storage time of fruit [40].

Data to date point to *IDH* as the most used control gene for normalization in *Eucalyptus* sp. [53-55], but without indication of a previous reference-gene detailed analysis. Although elected as the most stably expressed gene in *Eucalyptus* clones under biotic and abiotic stress [50], results from the present study did not support ranking *IDH* as one of the best reference genes because, together with SAND, it appears less sensitive to detect slight differences in the expression profile of an adventitious rooting-related gene of interest. This fact reinforces the need of detailed reference gene analysis for specific experimental conditions and processes.

Adventitious rooting is a very complex process, regulated by both environmental and endogenous factors, but the molecular mechanisms by which adventitious root formation is regulated are still poorly understood. Important advances are upcoming with genetic and microarray studies in *Populus*, especially after the availability of the poplar genome [68]. Studying gene expression during rooting in *Pinus contorta*, Brinker *et al.* [69] reported an increased expression of genes involved in protein synthesis and a decreased expression of genes related to protein degradation for the first 3 days after auxin treatment, whereas an opposite trend was observed during root formation and elongation. Sorin *et al.* also identified proteins with altered expression during adventitious rooting in *Arabidopsis* mutants, and, among these, there were some related to protein degradation [70]. These observations may help explain the low expression stability of the gene for polyubiquitin (*UBI*) found in our experiments (Figure 3 and Table 3), since the product of this gene is known to function in protein degradation [71].

The least stably expressed gene was *18S*, both by *geNorm* and *NormFinder* analysis (Figure 3 and Table 3). The use of rRNA as reference gene was previously considered a good strategy in gene expression studies [21] but recent works suggest that this is often not the case [24,72,29,40,44,45]. This is possibly due to the high abundance of *18S rRNA* compared with target mRNA transcripts, making it difficult to accurately subtract the baseline value in qPCR data analysis [24]. Besides, *18S rRNA* can not be used for this purpose when reverse transcription is carried out using oligo-dT primers or only mRNA is used as template [29].

The most stably expressed genes identified in this study, *H2B and TUA*, were effective references in both phases of the rooting process, induction and formation, and also in presence or absence of auxin, i.e. with conditions leading to profuse and scarce root development, respectively (Figure 2B and Figure 5B). This is a rather important feature because it allows the use of these genes as internal controls in evaluating the expression of putative key genes in the rooting process that are likely to vary between phases and in conditions leading or not to root development [73]. These key genes may include those encoding auxin transporters, auxin metabolism enzymes, auxin receptors and selective proteolysis-related proteins [74].

Conclusions

This work constitutes the first in-depth study to validate optimal control genes for the quantification of transcript levels in *E. globulus* during *in vitro* adventitious rooting.

Considering that both programs used are based on distinct statistical algorithms, potential discrepancies could be expected. The comparative *AGO1* expression profile, using the combinations of reference genes indicated by each program, allowed us to suggest the most adequate combination of control genes, based on the functions described in the literature for this gene of interest. Hence, the *NormFinder* program was considered more appropriate for our experiments, indicating *H2B* and *TUA* as suitable reference genes for normalization in *E. globulus* microcuttings rooted *in vitro*. Besides, *UBI* and *18S* are not indicated for use as internal controls in this species under the conditions tested.

In summary, these findings represent useful tools for normalization of qPCR results and will enable more accurate and reliable gene expression studies related to the vital process of clonal propagation by rooting in this important woody species.

Methods

Plant material

Seeds of *Eucalyptus globulus* Labill (batches from Chile and kindly supplied by Celulose Riograndense S.A., Guaíba, RS, Brazil) were surface-sterilized in 70% (v/v) ethanol (1 min) and 1.5% (v/v) NaClO (20 min) followed by 4 washes in sterile distilled water. Fifteen seeds were planted in 300 ml glass flasks (capped with a double layer of aluminum foil) containing 60 ml of medium as follows: half-strength modified MS salts [75] 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 (extra pure microbiological grade, *Merck*) [8]. The medium was sterilized in autoclave for 20 min at 121°C. The growth room conditions were 16 h photoperiod, 30 μmol $m^{-2}s^{-1}$ of photosynthetically active radiation (provided by white light fluorescent tubes) and temperature of 23 ± 2°C. After 14 weeks, tip microcuttings (about 3 cm in length) were excised from the seedlings and used for *in vitro* rooting experiments.

In vitro adventitious rooting experiments

The culture system consisted of a two-step protocol: an initial step of induction, which lasted 96 h (induction medium composition: 0.3x MS salt concentration, 0.4 mg l^{-1} thiamine HCl, 100 mg l^{-1} inositol, zero (control) or 10 mg l^{-1} indolyl-butyric acid (leading to rooting), equivalent to 49.3 μM, 30 g l^{-1} sucrose and 6 g l^{-1} agar, adjusted to pH 5.8 ± 1 before autoclaving), followed by a formation step (same composition of induction medium except without added auxin and supplemented with 1 g l^{-1} activated charcoal) [8].

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 with the same conditions described above for seed germination.

The expression analysis of the selected genes was monitored along the rooting process and the harvest of microcuttings for RNA extraction was at 12, 24, 48 and 96 h of exposure to induction medium and 24 and 48 h after transfer to formation medium (formation step), for both treatments (with and without auxin in the first step). For the formation step harvest, the microcuttings remained for 96 h in the induction medium before transfer to formation medium (see Figure 1 for details). The microcuttings were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Each point of harvest combined six microcuttings (approximately 200 mg of homogenized tissue fresh weight). The experimental design was completely randomized and the experiments were independently repeated three times with similar results.

Total RNA extraction and first strand cDNA synthesis

Total RNA was isolated using NucleoSpin RNA Plant Kit (*Macherey-Nagel*) including DNAse I treatment, following the manufacturer recommendations, with modifications as follows: 200 mg of homogenized tissue fresh weight from each sample was divided in two fractions of 100 mg. Each fraction of 100 mg had its RNA extracted separately. After the extraction procedure, the corresponding fractions were joined again for RNA quantification. Total RNA concentration was determined using Quant-iT™ RNA Assay Kit and the Qubit fluorometer (*Invitrogen*) and quality was monitored by electrophoresis in 1% agarose gel (data not show). One independent cDNA synthesis was performed for all of the samples starting from 100 ng total RNA. First strand cDNA synthesis was performed using oligo-dT primers and reverse transcriptase M-MLV (*Invitrogen*) in a final volume of 20 μl. The final cDNA products were diluted 10-fold in RNAse-free distilled water prior to use in qPCR.

PCR primer design

The sequences used to design the primer pairs, except *IDH*, whose primers were obtained from Carvalho *et al.* [55], represent putative orthologs in eucalypt of genes chosen for this study and are described in Table 1. For that, sequences placed in the *Arabidopsis thaliana*

Database (http://www.arabidopsis.org) were subjected to a tBlastx against the *Genolyptus Project* Database (http://www.lge.ibi.unicamp.br/eucalyptus/) and the sequence with highest homology was selected. For all genes, primer pairs were designed using Oligo Perfect™ Designer software (*Invitrogen*) and are described in Table 2. The specifications were: melting temperatures (Tm) of 58 - 62°C, primer lengths of 20 nucleotides, guanine-cytosine contents of 45-55% and PCR amplicon lengths of 100-150 base pairs.

qPCR conditions

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. All of the reactions were carried out in quadruplicates for each cDNA sample and contained 10 μl 10-fold diluted cDNA template, 4.25 μl sterile water, 2 μl 10× PCR Buffer (*Invitrogen*), 1.2 μl of 25 mM $MgCl_2$ (*Invitrogen*), 0.1 μl of 10 mM dNTP (*Invitrogen*), 2 μl SYBR Green (1:10,000, Molecular Probes, *Applied Biosystems*), 0.2 μl of each of the 10 μM forward and reverse gene-specific primers and 0.05 μl Platinum® Taq DNA polymerase (5 U/μl, *Invitrogen*) in a final volume of 20 μl. This experiment was repeated three times in independent runs for all selected genes per time sample.

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 sec, 60°C for 10 sec, and 72°C for 15 sec. 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. This procedure allowed us to verify that the resulting fluorescence originated from a single PCR product and did not represent primer dimerization formed during PCR or a non-specific product.

Determination of reference gene expression stability

Two publicly available software tools, *geNorm* v. 3.5 [24] and *NormFinder* [51] were used to evaluate gene expression stability. Both tools require the transformation of Cq (quantification cycle) values to linear scale expression quantities. The Cq values were converted into relative quantities by the delta-Cq method [56] using the sample with the lowest Cq as calibrator and taking into account the amplification efficiencies for each primer pair obtained with *LinReg PCR software* [76]. The measures were then exported to Microsoft Excel.

geNorm approach

The *geNorm* software is a Visual Basic Application (VBA) tool for Microsoft Excel and relies on the principle that the expression ratio of two ideal reference genes should be constant throughout the different experimental conditions or cell types [24]. This program estimates an expression stability value (M) for each gene, defined as the average pairwise variation of a certain gene with all other control genes in a given panel of cDNA samples. Genes with the lowest M values have the most stable expression and an ideal M-value must be preferably <0.5 [57,27]. Reference genes are ranked by *geNorm* through the elimination of the worst-scoring candidate control gene (the one with the highest M value) and recalculating of new M values for the remaining genes. At the end of this procedure, two candidate genes are always top-ranked because expression ratios are required for gene-stability measurements [24]. The *geNorm* program also allows the establishment of minimal number of control genes required for calculating an accurate normalization factor, as the geometric mean of their relative quantities. A pairwise variation of 0.15 is accepted as *cut-off* [24] below which the inclusion of an additional control gene is not required for reliable normalization.

NormFinder approach

The *NormFinder* software [51], another VBA applet, uses a model-based approach for identifying the optimal normalization gene(s) among a set of candidates. This strategy is based on a mathematical model of gene expression that enables estimation of the intra- and inter-group variations, which are combined into a stability value. Candidate control genes with the minimal intra-group variation will have the lowest stability value and will be top ranked [51]. For adequate application of the *NormFinder* program, the sample sets were subdivided in two groups (presence and absence of auxin in the induction step of adventitious rooting) with each of them containing 6 samples.

Determination of *AGO1* expression profile

The putative *A. thaliana AGO1* ortholog in *E. globulus* had its expression profile investigated in eight samples among the twelve analyzed in this study (12 and 24 h after inoculation in induction medium, and 24 and 48 h after transfer to formation medium, both in presence and absence of auxin in induction medium). The experiment was carried out with three biological replicates and the qPCR method and primer design strategy followed the same parameters used for the analysis of reference genes. The relative expression profile analysis was obtained through the delta-Cq method [56]. Analysis of Variance (ANOVA), followed by Duncan's test when appropriate ($P \leq 0.05$), were applied for samples of the same treatment (presence or absence of auxin). To compare the same time point of different treatments, a *t-test* was used ($P \leq 0.05$).

List of abbreviations

AGO1: Argonaute 1; AUX1: Auxin Resistant 1; PIN: *Arabidopsis thaliana* PIN-Formed proteins; qPCR: reverse-transcription followed by quantitative real-time Polymerase Chain Reaction; RNA: Ribonucleic Acid; PCR: Polymerase Chain Reaction; Cq: Quantification cycle; Tm: melting temperature; S.D: Standard Deviation; VBA: Visual Basic Application; NF: Normalization Factor; NaClO: Sodium Hypochloride; cDNA: complementary Deoxyribonucleic Acid.

Authors' contributions

MRA performed all the experimental procedures, data analysis and drafted the manuscript. CMR assisted on the execution of experimental procedures and data analysis. FKR and RAS helped designing primers and in data analysis. GP assisted in gene selection and sequence identification at the Genolyptus database. AGFN conceived and supervised the study and finalized the paper. All authors read and approved the final manuscript.

Acknowledgements

This research was funded by the Brazilian agencies National Council for Scientific and Technological Development (CNPq) and National Commission for Graduate Program Funding and Evaluation (CAPES). Seeds of *E. globulus* were a gift from Celulose Riograndense (Guaíba, RS, Brazil).

Author details

[1]Center for Biotechnology, Federal University of Rio Grande do Sul, P.O. Box 15005, 91501-970 Porto Alegre, RS, Brazil. [2]Department of Botany, Federal University of Rio Grande do Sul, P.O. Box 15005, 91501-970 Porto Alegre, RS, Brazil.

Received: 5 April 2010 Accepted: 20 September 2010
Published: 20 September 2010

References

1. Del Lungo A, Ball J, Carle J: **Global planted forests thematic study: results and analysis.** *Planted Forests and Trees Working Paper 38* FAO (Food and Agriculture Organization of the United Nations - Forestry Department) 2006.
2. **Statistical yearbook from ABRAF.** Brazilian Association of Foresters (Associação Brasileira de Produtores de Florestas Plantadas) 2009.
3. Mora AL, Garcia CH: **The *Eucalyptus* Culture in Brazil (A Cultura do Eucalipto no Brasil).** SBS, São Paulo 2000, 112.
4. Chiang V: **From rags to riches.** *Nat Biotechnol* 2002, **20**:557-558.
5. Le Roux JJ, Van Staden J: **Micropropagation and tissue culture of *Eucalyptus* - a review.** *Tree Physiol* 1991, **9**:435-477.
6. Serrano L, Rochange F, Semblant JP, Marque C, Teulières C, Boudet AM: **Genetic transformation of *Eucalyptus globulus* through biolistics: complementary development of procedures for organogenesis from zygotic embryos and stable transformation of corresponding proliferating tissue.** *J Exp Bot* 1996, **45**:285-290.
7. De Klerk GJ, Van Der Krieken W, De Jong JC: **The formation of adventitious roots: New concepts, new possibilities.** *In Vitro Cell Dev-Pl* 1999, **35(3)**:189-199.
8. Fett-Neto AG, Fett JP, Goulart LWV, Pasquali G, Termignoni RR, Ferreira AG: **Distinct effects of auxin and light on adventitious root development in *Eucalyptus saligna* and *Eucalyptus globulus*.** *Tree Physiol* 2001, **21**:457-464.
9. Ruedell CM, Schwambach J, Corrêa LR, Fett-Neto AG: **Strategies for adventitious rooting in clonal propagation of *Eucalyptus*.** In *Adventitious root formation of forest trees and horticultural plants - From genes to applications*. Edited by: Niemi K, Scagel C. Kerala: Research Signpost; 2009:337-358.
10. Sorin C, Bussell JD, Camus I, Ljung K, Kowalczyk M, Geiss G, Mckhann H, Garcion C, Vaucheret H, Sandberg G, Bellini C: **Auxin and light control of adventitious rooting in *Arabidopsis* require ARGONAUTE 1.** *Plant Cell* 2005, **17**:1343-1359.
11. Fogaça CM, Fett-Neto AG: **Role of auxin and its modulators in the adventitious rooting of *Eucalyptus* species differing in recalcitrance.** *Plant Growth Regul* 2005, **45**:1-10.
12. Muday GK, Delong A: **Polar auxin transport: controlling where and how much.** *Trends Plant Sci* 2001, **6**:535-542.
13. Benjamins R, Scheres B: **Auxin: the looping star in plant development.** *Annu Rev Plant Biol* 2008, **59**:443-465.
14. Li S-W, Xue L, Xu S, Feng H, An L: **Mediators, genes and signaling in adventitious rooting.** *Bot Rev* 2009, **75**:230-247.
15. Bustin SA: **Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems.** *J Mol Endocrinol* 2002, **29**:23-39.
16. Ginzinger DG: **Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream.** *Exp Hematol* 2002, **30(6)**:503-512.
17. Gachon C, Mingam A, Charrier B: **Real time PCR: what relevance to plant studies?** *J Exp Bot* 2004, **55(402)**:1445-1454.
18. Huggett J, Dheda K, Bustin S, Zumla A: **Real-time RT-PCR normalisation; strategies and considerations.** *Genes Immun* 2005, 1-6.
19. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT: **The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments.** *Clin Chem* 2009, **55(4)**:611-622.
20. Van Guilder HD, Vrana KE, Freeman WM: **Twenty-five years of quantitative PCR for gene expression analysis.** *BioTechniques* 2008, **44**:619-626.
21. Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E: **Housekeeping genes as internal standards: use and limits.** *J Biotechnol* 1999, **75**:291-295.
22. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W: **Genome-wide identification and testing of superior reference-genes for transcript normalization in *Arabidopsis*.** *Plant Physiol* 2005, **139**:5-17.
23. Schmittgen TD, Zakrajsek BA: **Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR.** *J Biochem Bioph Meth* 2000, **46**:69-81.
24. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F: **Accurate normalisation of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.** *Genome Biol* 2002, **3**:1-11.
25. Brunner AM, Yakovlev IA, Strauss SH: **Validating internal controls for quantitative plant gene expression studies.** *BMC Plant Biol* 2004, **4**:14.
26. Nicot N, Hausman J, Hoffman L, Evers D: **Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress.** *J Exp Bot* 2005, **56(421)**:2907-2914.
27. Gutierrez L, Mauriat M, Guénin S, Pelloux J, Lefebvre J, Louvet R, Rusterucci C, Moritz T, Guerineau F, Bellini C, Van Wuytswinkel O: **The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants.** *Plant Biotechnol J* 2008, **6**:609-618.
28. Kim BR, Nam HY, Kim SU, Kim SI, Chang YJ: **Normalization of reverse transcription quantitative-PCR with housekeeping genes in rice.** *Biotechnol Lett* 2003, **25(21)**:1869-72.
29. Jain M, Nijhawan A, Tyagi AK, Khurana JP: **Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR.** *Biochem Bioph Res Co* 2006, **345**:646-651.
30. Iskandar HM, Simpson RS, Casu RE, Bonnett GD, Maclean DJ, Manner JM: **Comparison of reference genes for quantitative real-time polymerase chain reaction analysis of gene expression in sugarcane.** *Plant Mol Biol Rep* 2004, **22**:325-337.
31. Remans T, Smeets K, Opdenakker K, Mathijsen D, Vangronsveld J, Cuypers A: **Normalization of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations.** *Planta* 2008, **227**:1343-1349.
32. Reid KE, Olsson N, Schlosser J, Peng F, Lund ST: **An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development.** *BMC Plant Biol* 2006, **6**:27.
33. Faccioli P, Ciceri GP, Provero P, Stanca AM, Morcia C, Terzi V: **A combined strategy of "in silico" transcriptome analysis and web search engine optimization allows an agile identification of reference genes suitable for normalization in gene expression studies.** *Plant Mol Biol* 2007, **63**:679-688.
34. Hong S, Seo PJ, Yang MS, Xiang F, Park CM: **Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR.** *BMC Plant Biol* 2008, **8**:112.
35. Jian B, Liu B, Bi Y, Hou W, Wu C, Han T: **Validation of internal control for gene expression study in soybean by quantitative real-time PCR.** *BMC Mol Biol* 2008, **9**:59.
36. Libault M, Thibivilliers S, Bilgin DD, Radwan O, Benitez M, Clough SJ, Stacey G: **Identification of four soybean reference genes for gene expression normalization.** *Plant Genome* 2008, **1**:44-54.
37. Expósito-Rodríguez M, Borges AA, Borges-Pérez A, Pérez JA: **Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process.** *BMC Plant Biol* 2008, **8**:131.

38. Silveira ED, Alves-Ferreira M, Guimarães LA, Silva FR, Carneiro VTC: **Selection of reference genes for quantitative real-time PCR expression studies in the apomictic and sexual grass *Brachiaria brizantha*.** *BMC Plant Biol* 2009, **9**:84.
39. Barsalobres-Cavallari CF, Severino FE, Maluf MP, Maia IG: **Identification of suitable internal control genes for expression studies in *Coffea arabica* under different experimental conditions.** *BMC Molecular Biol* 2009, **10**:1.
40. Tong Z, Gao Z, Wang F, Zhou J, Zhang Z: **Selection of reliable reference genes for gene expression studies in peach using real-time PCR.** *BMC Mol Biol* 2009, **10**:71.
41. Paolacci AR, Tanzarella OA, Porceddu E, Ciaffi M: **Identification and validation of reference genes for quantitative RT-PCR normalization in wheat.** *BMC Mol Biol* 2009, **10**:11.
42. Maroufi A, Van Bockstaele E, De Loose M: **Validation of reference genes for gene expression analysis in chicory (*Cichorium intybus*) using quantitative real-time PCR.** *BMC Mol Biol* 2010, **11**:15.
43. Artico S, Nardeli SM, Brilhante O, Maria Grossi-de-Sá F, Alves-Ferreira M: **Identification and evaluation of new reference genes in *Gossypium hirsutum* for accurate normalization of real-time quantitative RT-PCR data.** *BMC Plant Biol* 2010, **10**:49.
44. Wan H, Zhao Z, Qian C, Sui Y, Malik AA, Chen J: **Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber.** *Anal Biochem* 2010, **399**:257-261.
45. Lin YL, Lai ZX: **Reference gene selection for qPCR analysis during somatic embryogenesis in longan tree.** *Plant Science* 2010, **178**:359-365.
46. Mallona I, Lischewski S, Weiss J, Hause B, Egea-Cortines M: **Validation of reference genes for quantitative real-time PCR during leaf and flower development in *Petunia hybrida*.** *BMC Plant Biol* 2010, **10**:4.
47. Lee JM, Roche JR, Donaghy DJ, Thrush A, Sathish P: **Validation of reference genes for quantitative RT-PCR studies on gene expression in perennial ryegrass (*Lolium perenne* L.).** *BMC Mol Biol* 2010, **11**:8.
48. Wu G, Zhang L, Wu Y, Cao Y, Lu C: **Comparison of five endogenous reference genes for specific PCR detection and quantification of *Brassica napus*.** *J Agric Food Chem* 2010, **58**:2812-2817.
49. Schmidt GW, Delaney SK: **Stable internal reference genes for normalization of real-time RT-PCR in tobacco (*Nicotiana tabacum*) during development and abiotic stress.** *Mol Genet Genomics* 2010, **283**:233-241.
50. Boava LP, Laia ML, Jacob TR, Dabbas KM, Gonçalves JF, Ferro JA, Ferro MIT, Furtado EL: **Selection of endogenous genes for gene expression studies in *Eucalyptus* under biotic (*Puccinia psidii*) and abiotic (acibenzolar-S-methyl) stresses using RTqPCR.** *BMC Res Notes* 2010, **3**:43.
51. Andersen CL, Jensen JL, Orntoft TF: **Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets.** *Cancer Res* 2004, **64**:5245-5250.
52. Campos EI, Reinberg D: **Histones: annotating chromatin.** *Annu Rev Genet* 2009, **43**:559-99.
53. Goicoechea M, Lacombe E, Legay S, Mihaljevic S, Rech P, Jauneau A, Lapierre C, Pollet B, Verhaegen D, Chaubet-Gigot N, Grima-Pettenati J: **EgMYB2, a new transcriptional activator from *Eucalyptus* xylem, regulates secondary cell wall formation and lignin biosynthesis.** *Plant Journal* 2005, **43**:553-567.
54. Legay S, Lacombe E, Goicoechea M, Brièrea C, Séguind A, Mackayc J, Grima-Pettenati J: **Molecular characterization of EgMYB1, a putative transcriptional repressor of the lignin biosynthetic pathway.** *Plant Sci* 2007, **173(5)**:542-549.
55. Carvalho MCCG, Caldas DGG, Carneiro RT, Moon DH, Salvatierra GR, Franceschini LM, Andrade A, Celedon PAF, Oda S, Labate CA: **SAGE transcript profiling of the juvenile cambial region of *Eucalyptus grandis*.** *Tree Physiol* 2008, **28**:905-919.
56. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}$CT method.** *Methods* 2001, **25**:402-408.
57. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J: **qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data.** *Genome Biol* 2007, **8**:19.
58. Chen X: **Small RNAs and their roles in plant development.** *Annu Rev Cell Dev Biol* 2009, **25**:21-44.
59. Dheda K, Huggett JF, Chang JS, Kima LU, Bustin SA, Johnson MA, Rook GAW, Zumla A: **The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization.** *Anal Biochem* 2005, **344**:141-143.
60. Guénin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C, Gutierrez L: **Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references.** *J Exp Bot* 2009, **60(2)**:487-493.
61. Pernot F, Dorandeu F, Beaup C, Peinnequin A: **Selection of reference genes for real-time quantitative reverse transcription-polymerase chain reaction in hippocampal structure in a murine model of temporal lobe epilepsy with focal seizures.** *Journal of Neuroscience Research* 2010, **88**:1000-1008.
62. Steiger MG, Mach RL, Mach-Aigner AS: **An accurate normalization strategy for RT-qPCR in *Hipocrea jecorina* (*Trichoderma reesei*).** *Journal of Biotechnology* 2010, **145**:30-37.
63. Chatterjee C, Muir TW: **Chemical approaches for studying histone modifications.** *J Biol Chem* 2010, **285**:11045-11050.
64. Hays DB, Skinner DZ: **Development of an expressed sequence tag (EST) library for *Medicago sativa*.** *Plant Sci* 2001, **161**:517-526.
65. Sterky F, Bhalerao RR, Unneberg P, Segerman B, Nilsson P, Brunner AM, Charbonnel-Campaa L, Lindvall JJ, Tandre K, Strauss SH, Sundberg B, Gustafsson P, Uhlén M, Bhalerao RP, Nilsson O, Sandberg G, Karlsson J, Lundeberg J, Jansson S: **A *Populus* EST resource for plant functional genomics.** *P Natl Acad Sci USA* 2004, **101**:13951-13956.
66. Goossens K, Van Poucke M, Van Soom A, Vandesompele J, Van Zeveren A, Peelman LJ: **Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos.** *BMC Dev Biol* 2005, **5**:27.
67. Sindelka R, Ferjentsik Z, Jonák J: **Developmental expression profiles of *Xenopus laevis* reference genes.** *Dev Dynam* 2006, **235**:754-758.
68. Busov V, Yordanov YS, Meilan R: **Discovery of genes involved in adventitious root formation using *Populus* as a model.** In *Adventitious root formation of forest trees and horticultural plants - From genes to applications*. Edited by: Niemi K, Scagel C. Kerala: Research Signpost; 2009:85-103.
69. Brinker M, Van Zyl L, Liu W, Craig D, Sederoff RR, Clapham DH, Von Arnold S: **Microarray analyses of gene expression during adventitious root development in *Pinus contorta*.** *Plant Physiol* 2004, **135**:1526-1539.
70. Sorin C, Negroni L, Balliau T, Corti H, Jacquemot M, Davanture M, Sandberg G, Zivy M, Bellini C: **Proteomic analysis of different mutant genotypes of *Arabidopsis* led to the identification of 11 proteins correlating with adventitious root development.** *Plant Physiol* 2006, **140**:349-364.
71. Schrader EK, Harstad KG, Matouschek A: **Targeting proteins for degradation.** *Nat Chem Biol* 2009, **5**:815-822.
72. Gonçalves S, Cairney J, Maroco J, Oliveira MM, Miguel C: **Evaluation of control transcripts in real-time RT-PCR expression analysis during maritime pine embryogenesis.** *Planta* 2005, **222**:556-563.
73. Ludwig-Müller J: **Molecular basis for the role of auxins in adventitious rooting.** In *Adventitious root formation of forest trees and horticultural plants - From genes to applications*. Edited by: Niemi K, Scagel C. Kerala: Research Signpost; 2009:1-29.
74. Woodward AW, Bartel B: **A receptor for auxin.** *Plant Cell* 2005, **17**:2425-2429.
75. Murashigue T, Skoog F: **A revised medium for rapid growth and bioassay with tobacco tissue cultures.** *Physiol Plant* 1962, **15**:473-498.
76. Ramakers C, Ruijtera JM, Deprez RHL, Moorman AFM: **Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data.** *Neurosci Lett* 2003, **339**:62-66.

doi:10.1186/1471-2199-11-73
Cite this article as: de Almeida *et al.*: Reference gene selection for quantitative reverse transcription-polymerase chain reaction normalization during *in vitro* adventitious rooting in *Eucalyptus globulus* Labill. *BMC Molecular Biology* 2010 **11**:73.

4. Expression of auxin transporter genes during *in vitro* adventitious rooting in *Eucalyptus globulus* Labill and *Eucalyptus grandis* Hill ex Maiden

Artigo a ser submetido ao periódico *Tree Physiology*

Expression of auxin transporter genes during *in vitro* adventitious rooting in *Eucalyptus globulus* Labill and *Eucalyptus grandis* Hill ex Maiden

Márcia R. de Almeida [1], Carolina M. Ruedell [1], and Arthur G. Fett-Neto [1*]

[1] Plant Physiology Laboratory, Center for Biotechnology and Department of Botany, Federal University of Rio Grande do Sul, P.O. Box 15005, 91501-970 Porto Alegre, RS, Brazil.

* Corresponding author: fettneto@cbiot.ufrgs.br

Abstract

Eucalyptus sp. is currently one of the most planted tree genera used for wood industry as well for paper and cellulose production. *Eucalyptus* forests are produced through vegetative propagation of elite materials, being adventitious rooting a key step in this process. *Eucalyptus globulus* and its hybrids are of interest for Southern Brazilian paper and cellulose industries due to characteristics such as low lignin content and relative frost tolerance. However, this species is recalcitrant to rooting and often requires exogenous auxin application for adequate rooting. The formation of new root meristems in woody plants is currently not well understood in the context of gene expression. In this work we analyzed the expression of auxin influx (*AUX1*) and efflux (*PIN1*) transporters during *in vitro E. globulus* adventitious rooting. *AUX1* seems to be not so critical for this process, unlike *PIN1*, which appears to be required to redistribute and perhaps concentrate auxin in specific areas of the base of microcuttings early in the process upon exposure to exogenous auxin, the only condition that leads to significant root development in this difficult-to-root species. In order to better interpret these findings, we also evaluated an easy-to-root species (*E. grandis*). Initially, the expression stability of eight candidates to reference genes for expression studies in *E. grandis* was determined. The analysis indicated Histone H2B and Actin 2/7 as the best combination of genes to be used as reference, which was further validated by examining the *Argonaute 1* gene expression pattern. The *PIN1* expression pattern during adventitious rooting of *E. grandis* was then analyzed, revealing a late increase in expression under conditions of auxin exogenous supply, indicating a possible requirement of *PIN1* to enable supplied auxin relocation, promoting the development of additional roots. Although more studies are needed for understanding the roles of auxin in determining the rooting competence in *Eucalyptus*, it seems that differences in *PIN1* gene expression may at least partly explain rooting recalcitrance phenotypes, presumably being required for early auxin focusing in target tissues of recalcitrant species to allow rooting to take place.

Keywords: gene expression; auxin carriers; reference genes; *Eucalyptus*

Introduction

Adventitious rooting is a process based on the principles of regeneration where a new shoot, root or embryo can be formed from tissues without a pre-existing meristem (De Klerk, 2002). This developmental event is a key step to the success of vegetative propagation of woody plants like eucalypts (De Klerk et. al. 1999). Adventitious rooting can be divided in two main sequential steps, each with its own requirements and characteristics: (1) induction step, which involves biochemical and molecular events, without visible morphological changes; and (2) formation step, which consists of cellular divisions involved in both root meristem organization and primordia establishment, followed by root elongation and emergence out of the cutting (Fett-Neto et. al. 2001).

Several factors can influence adventitious root development, such as phytohormones, phenolic compounds, nutritional conditions and genetic characteristics (Ruedell et. al. 2009). Among phytohormones, auxin has a very important role in determining rooting capacity (Sorin et. al. 2005), mainly related to its endogenous content and transport rate (Fogaça and Fett-Neto, 2005). Meanwhile, concentrations that benefit root induction can block its elongation (De Klerk et. al 1999). The shoot apex is the main source of endogenous auxin. Stems have a specific basipetal active transport through vascular parenchyma carried out by both influx (AUX1) and efflux (PIN) carriers (Muday and Delong, 2001; Benjamins and Scheres, 2008).

Eucalyptus sp. is one of the most planted genus currently (Del Lungo, 2006), mainly because of its use as wood as well as raw material for the paper and cellulose industries. Brazil developed highly productive eucalypt clonal forests by clonal propagation of elite genotypes, allowing the country to hold the first position on short fiber cellulose pulp production (ABRAF, 2010). *Eucalyptus globulus* is a very attractive species to industries in the south of Brazil due to its relative tolerance to frost and low lignin content, which facilitates cellulose extraction (Chiang, 2002). However, this species and its hybrids show rooting recalcitrance (Le Roux and Van Staden, 1991; Serrano et. al. 1996) and often need application of exogenous auxin to develop adventitious roots *in vitro* (Fett-Neto et. al. 2001;

Fogaça and Fett-Neto, 2005). It is possible that some alteration in auxin transport or signaling may occur in *E. globulus*, or that auxin endogenous content might be insufficient, yielding the rooting recalcitrance phenotype.

Considering that little is known about the formation of root meristems in woody plants and even less concerning the effect of auxins on this process at gene expression level (Li et. al. 2009), reverse-transcription followed by quantitative, real-time polymerase chain reaction (qPCR) could be used to verify the expression pattern of auxin-related genes during rooting, shedding light on the molecular basis of this process. Because of its high sensitivity and specificity, speed, ease of use and capacity of simultaneous measurements of gene expression in several different samples (Bustin et. al. 2002; Ginzinger, 2002, Gachon et. al. 2004), qPCR still is one of the most widely used methods for monitoring gene expression. However, several factors influence the accuracy of the experiments, such as quality and amount of starting material, presence of inhibitors in different samples, primer design, RNA extraction, reverse-transcription efficiencies, quantification methods (Ginzinger, 2002; Demeke and Jenkins, 2010) and most importantly, selection of adequate reference-genes (Huggett et. al. 2005) that will undergo the same type of variation and preparation steps throughout the assay as the target genes.

An ideal reference gene, also known as housekeeping gene, internal control gene or constitutive, should be expressed at a constant level in the majority of the study organism cells, throughout different developmental stages and in different organs. Genes involved in basic cellular processes, such as cell structure maintenance or primary metabolism, are often chosen as references (Czechowski et. al. 2005). On the other hand, Gutierrez et. al. (2008) an Guénin et. al. (2009) reinforced the necessity of previous validation of reference genes to every species and experimental condition to avoid misinterpretation of the results.

As part of a study to elucidate the causes of recalcitrance to rooting in *E. globulus*, the aim of this work was analyse the expression pattern of auxin transporters during *in vitro* adventitious rooting. To better understand the results obtained with *E. globulus*, we included *Eucalyptus grandis* in this investigation since this species is highly competent to rooting,

even in the absence of exogenous auxin. To that end, we evaluated the stability of eight candidate reference genes in *E. grandis* under same experimental conditions to which *E. globulus* was submitted. The statistical methods implemented in *geNorm* (Vandesompele et. al. 2002) and *NormFinder* (Andersen et. al. 2004) were used and compared. The expression pattern of *Argonaute 1* gene was analysed to validate of the results, as previously described (De Almeida et. al. 2010).

Material and methods

Plant Material

Seeds of *Eucalyptus globulus* and *Eucalyptus grandis* (kindly supplied by Celulose Riograndense S.A., Guaíba, RS, Brazil) were surface-sterilized in 70% (v/v) ethanol (1 min) and 1.5% (v/v) NaClO (20 min) followed by 4 washes in sterile distilled water. Fifteen seeds were planted in 300 ml glass flasks (capped with a double layer of aluminum foil) containing 60 ml of medium as follows: half-strength modified MS salts (Murashigue and Skoog, 1962) 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 (extra pure microbiological grade, Merck) (Fett-Neto et.al. 2001). The medium was sterilized in autoclave for 20 min at 121°C. The growth room conditions were 16 h photoperiod, 30 µmol $m^{-2}s^{-1}$ of photosynthetically active radiation (provided by white light fluorescent tubes) and temperature of 23 ± 2°C. After 14 (*E. globulus*) and 16 (*E. grandis*) weeks, tip microcuttings (about three cm in length) were excised from the seedlings and used for *in vitro* adventitious rooting experiments.

In vitro adventitious rooting conditions

The culture system consisted of a two-step protocol: an initial step of induction, which lasted 96 h (induction medium composition: 0.3x MS salt concentration, 0.4 mg l^{-1} thiamine HCl, 100 mg l^{-1} inositol, zero (control) or 10 mg l^{-1} indolyl-butyric acid (leading to rooting), equivalent to 49.3 µM, 30 g l^{-1} sucrose and 6 g l^{-1} agar, adjusted to pH 5.8 ± 1 before autoclaving, followed by a formation step (same composition of induction medium except

without added auxin and supplemented with 1 g l^{-1} activated charcoal) (Fett-Neto et. al. 2001).

Experiments were carried out in 20 ml glass vials containing 6 ml of medium, 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 with the same conditions described above for seed germination.

The expression analysis of the selected genes was monitored along the rooting process and the harvest of microcuttings for RNA extraction was after 12, 24, 48 and 96 h of exposure to induction medium (induction step) and 24 and 48 h after transfer to formation medium (formation step), for both treatments (with and without auxin in the induction step). For the formation step harvest, the microcuttings remained for 96 h in the induction medium before transfer to formation medium. For monitoring the expression of auxin transporter genes, the microcuttings were harvested also after 6h of exposure to induction medium (Figure 1). The microcuttings were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Each point of harvest combined six microcuttings (approximately 200 mg of homogenized tissue fresh weight). The experimental design was completely randomized and the experiments were independently repeated three times.

Total RNA extraction and first strand cDNA synthesis

Total RNA was isolated using NucleoSpin RNA Plant Kit (*Macherey-Nagel*) including DNAse I treatment (following manufacturer instructions) and first strand cDNA synthesis was performed using oligo-dT primers and reverse transcriptase M-MLV (Invitrogen) as described in De Almeida et. al. (2010).

qPCR primer design

The sequences used to design the primer pairs, except *IDH* and *Eucons08*, which were respectively obtained from Carvalho et. al. (2008) and kindly provided by Dr. Giancarlo Pasquali (Plant Molecular Biology Laboratory – Center for Biotechnology, Federal University

of Rio Grande do Sul, Brazil), represent putative orthologs in eucalypt of genes chosen for this study and are described in Table 1. Sequences of interest from the *Arabidopsis thaliana* Database (http://www.arabidopsis.org) were subjected to a tBlastx against the Genolyptus Project Database (http://www.lge.ibi.unicamp.br/eucalyptus/) and the sequence with highest identity was selected. For all genes, primer pairs were designed using Oligo Perfect™ Designer software (*Invitrogen*) and are described in Table 2. The specifications were: melting temperatures (Tm) of 58 - 62°C, primer lengths of 20 nucleotides, guanine-cytosine contents of 45-55% and PCR amplicon lengths of 100-150 base pairs.

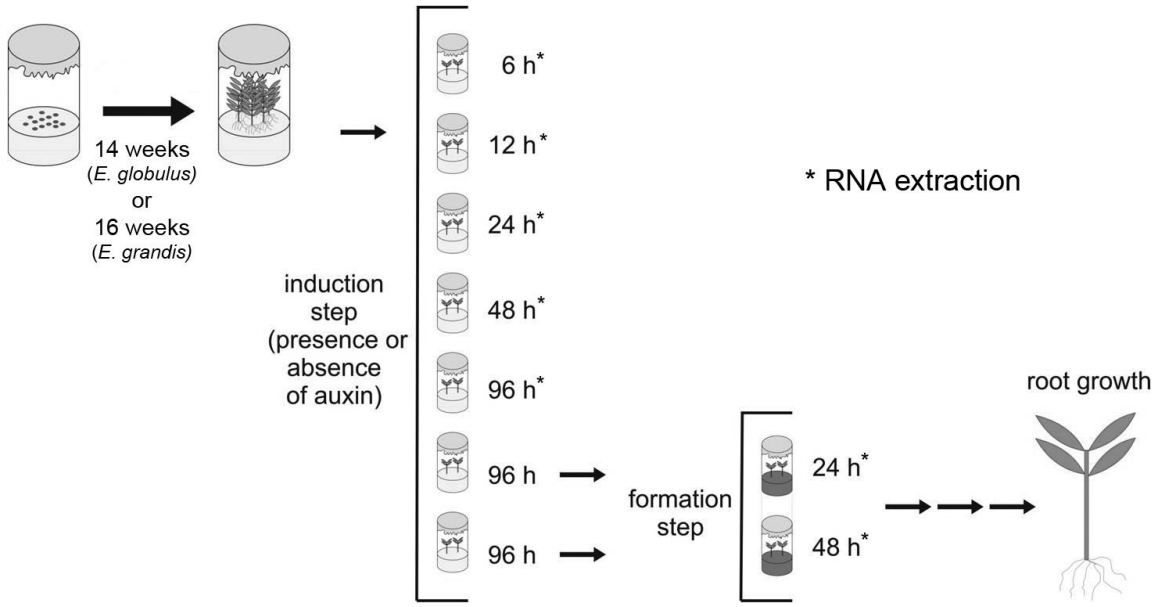


Figure 1. Adventitious rooting stages in *Eucalyptus globulus* and *Eucalyptus grandis* and experiment details. After seeding, seedlings remained 14 or 16 weeks (depending on species) in germination medium. After this time, apical microcuttings were obtained and used in adventitious rooting experiments. The microcuttings were kept in induction medium (presence or absence of 10 mg l^{-1} IBA) for 12, 24, 48 or 96 h, depending on the harvest time. For the harvest points in formation medium, the microcuttings remained 96 h in induction medium and after that were transferred to formation medium (devoid of auxin and with activated charcoal). In this case, the samples were collected after 24 and 48 h. The samples harvested at 12, 24, 48 and 96 h of induction step and at 24 and 48 h of formation step were submitted to RNA extraction and used for the analyses of reference-genes. For the analysis of auxin-related genes, samples were also harvested at 6 h of induction step. Modified from De Almeida et.al. (2010).

Table 1. Description of auxin-related and reference genes used for qPCR in *Eucalyptus globulus* and *Eucalyptus grandis*.

Gene symbol	Gene name	*Arabidopsis thaliana*´s best hit locus	*Arabidopsis thaliana* locus description	Function	E-value (tblastx)
AUX1	Auxin Resistant 1	At2g38120	Auxin influx transporter	Auxin influx transmembrane transporter activity; auxin polar transport; influences lateral root initiation and positioning	e-75
PIN1	PIN Formed 1	At1g73590	Auxin efflux transporter	Auxin efflux transmembrane transporter activity; auxin polar transport; involved in shoot and root development	e-72
AGO1	Argonaute 1	At1g48410	Encodes an RNA Slicer that selectively recruits microRNAs and siRNAs	Regulates genes involved at the cross talk between auxin and light signaling during adventitious root development	e-88
At4g33380	Expressed protein	At4g33380	Expressed sequence	Unknown	e-74
ACT2	Actin 2/7	At5g09810	Actin 2/7	Structural constituent of cytoskeleton	0,0
Eucons08	Putative Transcription Elongation Factor	*	*	Unknown	*
H2B	Histone H2B	At5g59910	Histone H2B, putative	Strutural constituent of the eukaryotic nucleosome core	e-66
IDH	NADP-Isocitrate dehydroge-nase	**	**	Carbohydrate metabolism	**
SAND	SAND protein	At2g28390	SAND family protein	Intracellular vesicular transport, biogenesis and vacuole signalling	e-68
TIP41	TIP41-like protein	At4g34270	TIP41-like family protein	Unknown	e-78
TUA	Alpha-tubulin	At5g19780	Alpha tubulin-5	Structural constituent of cytoskeleton , microtubule-based process	e-134

* Gene assumed as constitutive in microarray studies employing leaf and vascular tissues of fully grown *E. globulus* and *E. grandis* trees (unpublished results).

** Gene obtained from Carvalho et. al. (2008).

Table 2. Primer sequences and amplicon characteristics for each of the three auxin-related genes and eight reference genes.

Gene symbol	Primer sequence (5' → 3') Forward/Reverse	Amplicon length (bp)	Annealing temperatures (°C)	Amplification efficiency
AUX1	ATATCTGATCGCCACGCTCT/ GAAGGCGTTGGAGTGGTAGA	103	60	1.97
PIN1	GACATGGCAGGGTCGATAGT/ GCATTGGACCTCCTCACTGT	134	60	1.95
AGO1	TCTTGGGCTCGTTTCTCAGT/ GGAATTCGCCTAGACAGTGC	144	60	1.92
At4g33380	TCCAGAGTGCAATGCTGAAC/ CCCCTCGTCTGGCATACTTA	134	60	1.99
ACT2	TCCACCATGTTCCCTGGTAT/ ACCTCCCAATCCAGACACTG	124	60	1.91
*Eucons08**	TCCAATCCGAGTCGCTGTCATTGT/ TGATGAGCCTCTCTGGTTTGACCT	152	60	1.92
H2B	GAAGAAGCGGGTGAAGAAGA/ GGCGAGTTTCTCGAAGATGT	145	60	1.91
*IDH***	CTGTTGAGTCTGGAAGATGAC/ CATTTAATTCCTCCCCAACAAA	271	60	1.86
SAND	CCATTCAACACTCTCCGACA/ TGTGTGACCCAGCAGAGTAAT	143	59	1.80
TIP41	GAACAAAAGCTCGGGACATC/ CAACCAGCAAGAGCATCAAA	122	60	1.77
TUA	ACCGGTTGATCTCTCAGGTG/ TAAGGGACCAGGTTGGTCTG	103	60	1.77

* Gene assumed as constitutive in microarray studies employing leaf and vascular tissues of fully grown *E. globulus* and *E. grandis* trees (unpublished results).

** Gene obtained from Carvalho et. al. (2008).

qPCR conditions

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. All of the reactions were carried out in quadruplicates for each cDNA sample and were conducted as in De Almeida et. al. (2010).

Determination of reference gene expression stability in Eucalyptus grandis

Two publicly available software tools, *geNorm* v. 3.5 (Vandesompele et. al., 2002) and *NormFinder* (Andersen et. al., 2004) were used to evaluate gene expression stability. For both tools were necessary the transformation of Cq (quantification cycle) values to

relative quantities, what was done by the delta-Cq method (Livak and Scmittgen, 2001) using the sample with the lowest Cq as calibrator. The transformation also took into account the amplification efficiencies for each primer pair obtained with LinReg PCR software (Ramakers et. al., 2003). The measures were then exported to Microsoft Excel.

geNorm

The *geNorm* software is a Visual Basic Application (VBA) tool for Microsoft Excel and relies on the principle that the expression ratio of two ideal reference genes should be constant throughout the different experimental conditions or cell types (Vandesompele et. al., 2002). The program estimates an expression stability value (M) for each gene, defined as the average pairwise variation of a certain gene with all other control genes in a given panel of cDNA samples. Genes with the lowest M values have the most stable expression and an ideal M-value must be preferably <0.5 (Hellemans et. al., 2007; Gutierrez et. al., 2008). Reference genes are ranked by *geNorm* through the elimination of the worst-scoring candidate control gene (the one with the highest M value) and recalculating of new M values for the remaining genes. At the end, two candidate genes are top-ranked because expression ratios are required for gene-stability measurements (Vandesompele et. al., 2002). The *geNorm* program also establishes the minimal number of control genes required for calculating an accurate normalization factor, as the geometric mean of their relative quantities. A pairwise variation of 0.15 is accepted as cut-off (Vandesompele et. al., 2002) below which the inclusion of an additional control gene is not required for reliable normalization.

NormFinder

The *NormFinder* software (Andersen et. al., 2004), another VBA applet, uses a strategy based on a mathematical model of gene expression that enables estimation of the intra- and inter-group variations, which are combined into a stability value for identifying the optimal normalization gene(s) among a set of candidates. Candidate control genes with the

minimal intra-group variation will have the lowest stability value and will be top ranked (Andersen et. al., 2004). For adequate application of the *NormFinder* program, the sample sets were subdivided in two groups (presence and absence of auxin in the induction step of adventitious rooting) with each of them containing six samples. This allowed the program to indicate the best combination of genes to be used as references.

Determination of AGO1 expression profile in Eucalyptus grandis

The putative *A. thaliana AGO1* ortholog in *E. grandis* had its expression profile investigated in eight samples among the twelve analyzed for determination of best reference genes (12 and 48 h after inoculation in induction medium, and 24 and 48 h after transfer to formation medium, both in presence and absence of auxin in induction medium). The experiment was carried out with three biological replicates and the qPCR method and primer design strategy followed the same parameters used for the analysis of reference genes. The relative expression profile analysis was obtained through the Comparative Cq method (Pfaffl, 2001), using as references the set of genes indicated by *geNorm* and by *NormFinder*.

Determination of auxin carrier expression profile in E. globulus and E. grandis

The expression profiles of putative *A. thaliana PIN1* and *AUX1* orthologues in *E. globulus* and of a *PIN1* ortholog in *E. grandis* were monitored along the adventitious rooting process, in presence or absence of auxin in the induction step. The experiments were carried out with three biological replicates and the qPCR method and primer design strategy followed the same parameters used for the analysis of reference genes. The relative expression profile analysis was obtained through the Comparative Cq method (Pfaffl, 2001), using both *Histone H2B* (*H2B*) and *Alpha-Tubulin* (*TUA*) as reference genes for *E. globulus* (De Almeida et. al., 2010) and both *Histone H2B* (*H2B*) and *Actin 2/7* (*ACT2*) for *E. grandis*, as indicated by *NormFinder* approach on the present work.

Statistical analysis

For analysis of *AGO1, PIN1* and *AUX1* expression profile, Levene's Test of Homogenity of Variance was applied and when not significant (P≥ 0.05), a One-Way ANOVA, followed by Duncan test (P≤0.05) was carried out. When Levene's Test was significant (P≤0.05), Welch's ANOVA followed by Dunnet's C test (P≤ 0.05) were the tests of choice. These tests were conducted for samples of the same treatment (presence or absence of auxin). To compare the same time point of different treatments, a Student's t-test was used (P ≤ 0.05).

Results

Expression profile of auxin transporter genes in E. globulus

As part of a larger investigation to identify the causes of low rooting rate in *E. globulus* without exogenous auxin supply, we investigated the expression profiles of *AUX1* and *PIN1* auxin transporters along the process of *in vitro* adventitious rooting using qPCR. The characteristics of each gene, such as gene name, *A. thaliana* ortholog locus, *A. thaliana* locus description, function and E-value are provided in Table 1. We evaluated a set of 14 samples, covering seven different stages along adventitious rooting time in the presence or absence of the phytohormone auxin during induction step. The gene encoding the auxin influx transporter (*AUX1*) did not show differences in expression profile between treatments (with and without exogenous auxin) (Figure 2A). The auxin efflux transporter gene encoding *PIN1* showed increased expression during the first 24 hours of the induction step in microcuttings of *E. globulus* exposed to exogenous auxin when compared with the control treatment (without exogenous auxin supply) (Figure 2B).

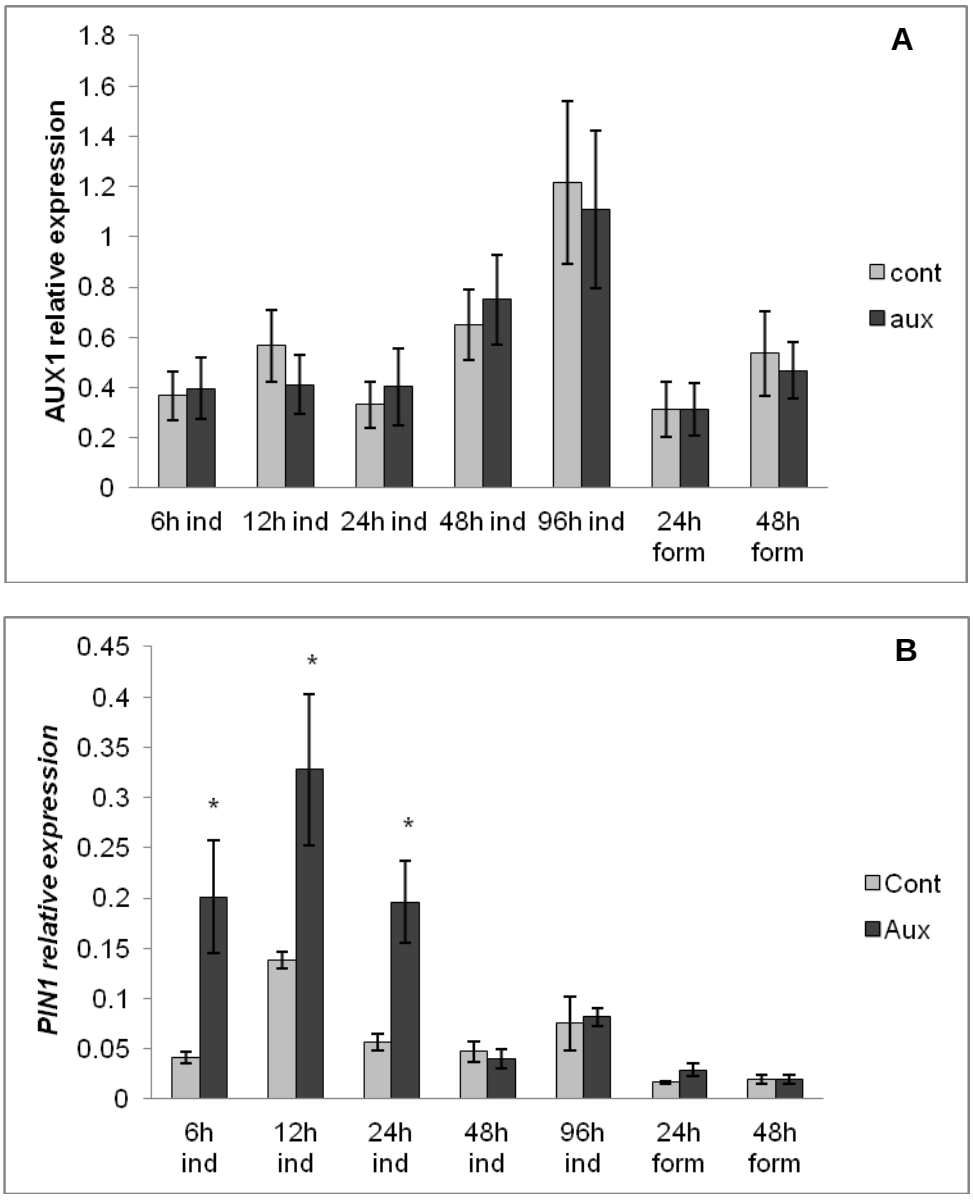


Figure 2. Relative expression levels of *AUX1* (A) and *PIN1* (B) during *in vitro* adventitious rooting of *Eucalyptus globulus*. "Ind" indicates induction step and "form" indicates formation step. Cont: without auxin in induction step. Aux: presence of 10 mg l^{-1} IBA in induction step. "*" indicates significant difference between different conditions within a time-point (Student's t-test with P≤ 0.05).

Determination of reference genes in E. grandis

To better understand the results obtained for *PIN1* gene expression in *E. globulus*, we decided to analyse the expression profile of this auxin efflux transporter in *E grandis*, a relatively easy to root species, for comparative purposes. However, before conducting a qPCR analysis it is necessary to define appropriate reference genes to allow relative

expression determination. Considering the absence of available studies focusing control genes for *E. grandis*, we evaluated the expression stability of eight candidates with both *geNorm* and *NormFinder* approaches.

The analysis included 12 samples, covering six different stages along the process of adventitious rooting in *E. grandis*, in the presence or absence of auxin, similarly to what was done by De Almeida et. al. (2010). The selected genes included: Actin 2/7 (*ACT2*) (Vandesompele et. al. 2002; Tong et. al. 2009), Histone H2B (*H2B*) (Campos and Reinberg, 2009), NADP Isocitrate Dehydrogenase (*IDH*) (Goicoechea et. al. 2005; Legay et. al. 2007; Carvalho et. al. 2008), SAND protein (*SAND*) (Czechovski et. al. 2005; Remans et. al. 2008), TIP41-like protein (*TIP41*) (Czechovski et. al. 2005; Gutierrez et. al. 2008; Remans et. al. 2008; Expósito-Rodriguez et. al. 2008), Alpha-tubulin (*TUA*) (Brunner et. al. 2004), an *Eucalyptus* ortholog of *Arabidopsis thaliana* expressed protein without determined function (*At4g33380*) (Czechovski et. al. 2005; Gutierrez et. al. 2008; Remans et. al. 2008) and *Eucons08*, a putative transcription elongation factor found in *Eucalyptus* microarray analysis (G. Pasquali, UFRGS, personal communication). The characteristics of each of the listed genes are described in Table 1. No primer dimers or other products resulted from non-specific amplification. Amplification efficiencies of PCRs ranged from 1.77 for *TIP41* to 1.99 for *At4g33380* (Table 2).

For both *geNorm* and *NormFinder* tools, Cq data were collected for all samples and transformed to relative quantities using the delta-Cq method (Livak and Schmittgen, 2001). Using *geNorm* analysis, results indicated *At4g33380* and *TIP41* as the most stable genes (M value = 0.44) and the SAND gene as the least (M value = 0.70) (Table 3). To evaluate the optimal number of reference genes for reliable normalization, *geNorm* calculates the pairwise variation V_n/V_{n+1} between the sequential ranked normalization factors NF_n and NF_{n+1} to determine the effect of adding the next reference gene in normalization. Considering the cut-off value of 0.15 proposed by Vandesompele et. al. (2002), below which the inclusion of an additional reference gene is not necessary, the use of the two most stably expressed genes (*At4g33380* and *TIP41*) is sufficient for accurate normalization (V2/3 = 0.146) in *E. grandis in*

vitro rooting (Figure 3). If needed, the *ACT2* gene can be added to the analysis, resulting in a pairwise variation value of 0.130 (V3/4 = 0.130) (Figure 3).

A change in the indication of most stable genes was observed after *NormFinder* analysis when compared to *geNorm*, with *H2B* showing the most stable expression (near zero variation value and small error bars), followed by *ACT2*. *IDH* and *SAND* were the least stable genes (both with variation values farther from zero and larger error bars) (Table 3). When defining the best combination of two genes using *NormFinder*, once again both *H2B* and *ACT2* were the genes of choice, with a stability value of 0.058 when used together (data not show).

Table 3. Ranking of candidate reference genes in decreasing order of expression stability calculated by *geNorm* and *NormFinder.*

Ranking order	***geNorm*** **(M value)**	***NormFinder*** **(Stability values ± EB*)**
1	*At4g33380/TIP41* (0.44)	*H2B* (0.059 ± 0.0315)
2		*ACT2* (0.094 ± 0.0541)
3	*ACT2* (0.47)	*TIP41* (0.120 ± 0.129)
4	*Eucons08* (0.52)	*At4g33380* (0.136 ± 0.118)
5	*H2B* (0.57)	*Eucons08* (0.138 ± 0.114)
6	*TUA* (0.60)	*TUA* (0.153 ± 0.141)
7	*IDH* (0.65)	*IDH* (0.166 ± 0.165)
8	*SAND* (0.70)	*SAND* (0.208 ± 0.259)

* Error Bars

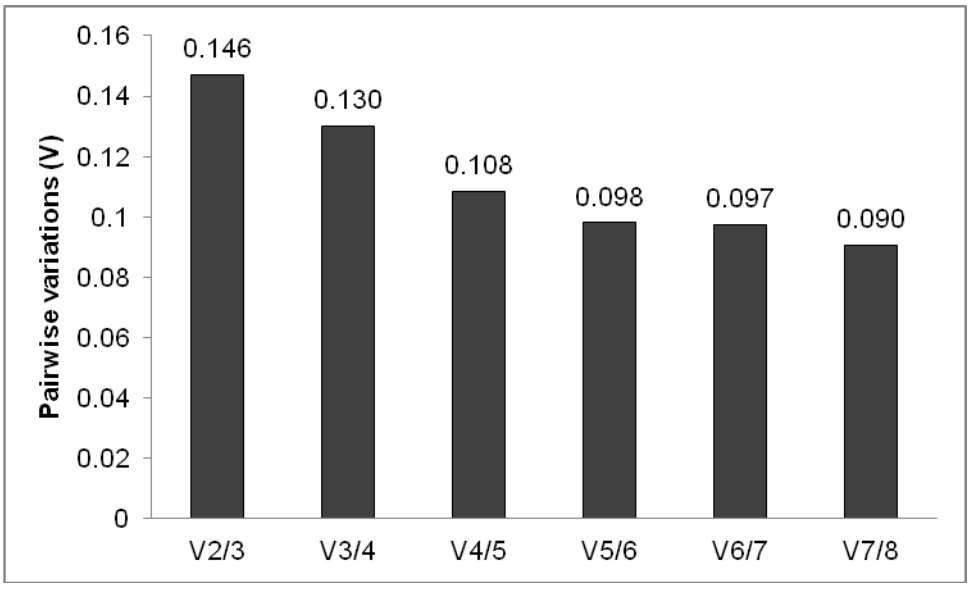


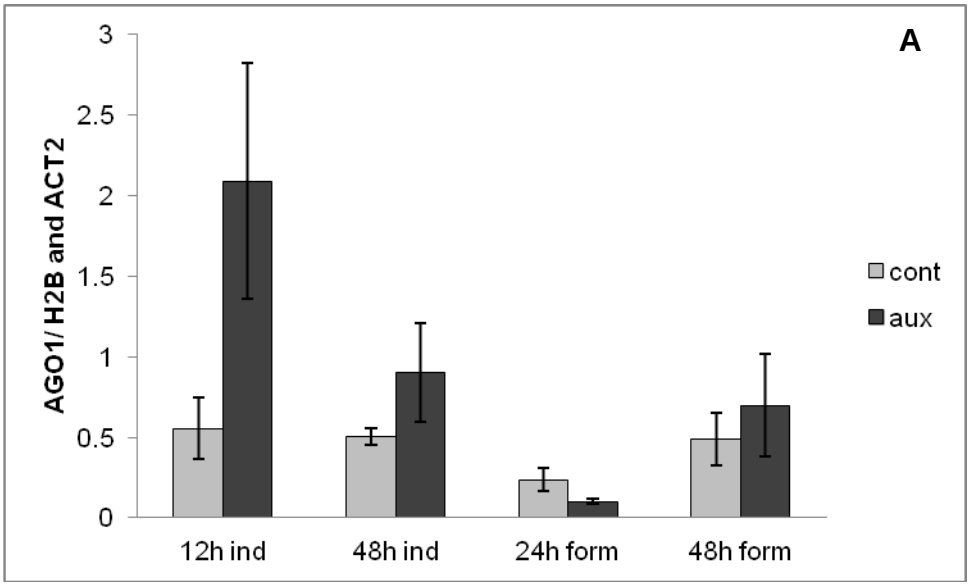
Figure 3. Determination of the optimal reference gene number calculated by *geNorm* for accurate normalization during *in vitro* adventitious rooting in *Eucalyptus grandis*. *geNorm* pairwise variation values (V values) between two sequential normalization factors NFn and NFn + 1, where n is the number of genes involved in the normalization factor. Cut-off ≤ 0.15.

Relative expression profile of AGO1 in E. grandis

To allow validation of the reference-genes found in this study and determining the best choice of genes to use as reference, we examined the relative expression of a putative ortholog of *A. thaliana ARGONAUTE1* (*AGO1*) gene in *E. grandis* during adventitious rooting. *AGO1* encodes a micro RNA binding protein (Chen, 2009) that regulates the expression of *ARF17* (Auxin Response Factor 17), an auxin response transcription factor with inhibitory action on transcription of auxin-related genes, presumably controlling genes related to auxin homeostasis and adventitious root development in *A. thalina* (Sorin et. al. 2005). The two gene pairs indicated by *geNorm* and *NormFinder* were used as references to evaluate *AGO1* relative expression in eight samples, covering four time points of adventitious root induction and formation, both under presence and absence of auxin during the induction step.

Using *At4g33380* and *TIP41* as references (as indicated by *geNorm*) or *H2B* and *ACT2* (as indicated by *NormFinder*), no significant difference was detected on *AGO1*

expression profile during adventitious rooting time points analysed in *E. grandis* (Figure 4). However, a trend for increase on *AGO1* expression at 12h of induction step under exogenous auxin supply, followed by a decreasing on subsequent steps was detected when *H2B* and *ACT2* were used as references (Figure 4A). This result is the opposite of what we found for *E. globulus* (De Almeida et. al. 2010), a species recalcitrant to rooting, and seems to fit the relatively easy-to-root phenotype of *E. grandis* (Table 4). Since the control gene combinations suggested by each program yielded different relative expression profiles of our gene of interest, the genes indicated by *NormFinder* (*H2B* and *ACT2*) were selected as references for further analysis with *E. grandis* due to fact that the resulting *AGO1* expression profile relative to these genes was more consistent with the characteristics of this species and *AGO1* function, than that found when using *At4g33380* and *TIP41* as control genes (Figure 4B).



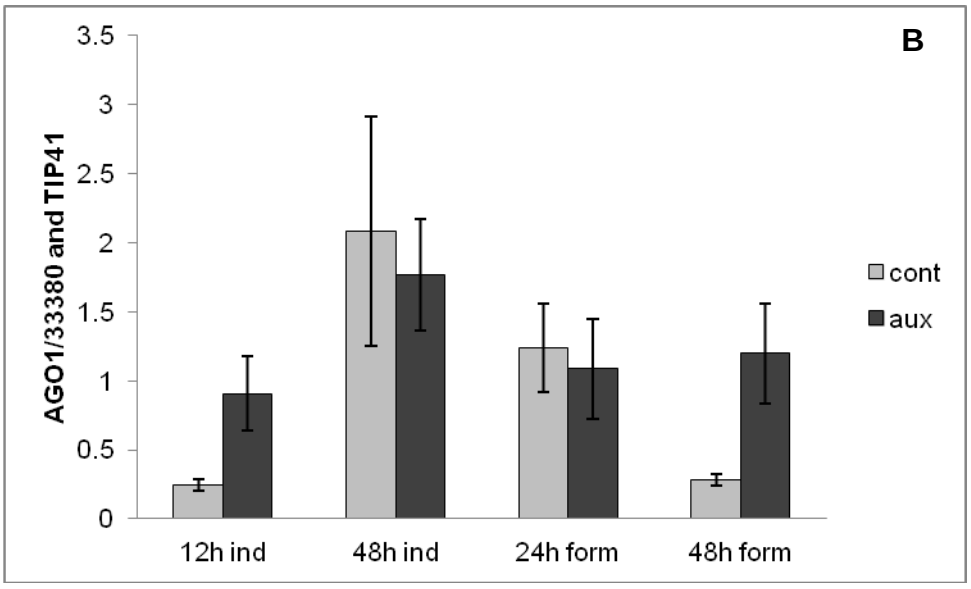


Figure 4. *Argonaute 1* (*AGO1*) relative expression profile during in vitro adventitious rooting in *Eucalyptus grandis*. *AGO1* expression profile relative to the best combination of genes indicated by *Normfinder* (A) and *geNorm* (B). "Ind" indicates induction step and "form" indicates formation step. Aux: indicates addition of 10 mg l^{-1} Indol Butyric Acid (IBA) in induction step culture medium; cont: indicates absence of IBA in induction step culture medium.

Relative expression of PIN1 in E. grandis

Considering the expression profile obtained for *PIN1* in *E. globulus* (Figure 2B), we decided to analyse the expression of this gene in *E. grandis*, aiming at a better understanding of the function of *PIN1* in adventitious rooting process. The results indicated a basal expression along all control samples (without exogenous auxin supply in induction step). In the case of samples treated with exogenous auxin (aux samples), the expression was similar, except by a pronounced increase at 96h of induction step (Figure 5). Moreover, when compared to *E. globulus*, the *PIN1* expression level was higher in both conditions (Figures 2B and 5; Table 4).

Table 4. Mean expression level (fold compared to respective normalizer genes) of auxin-related genes (Argonaute 1 and Pinformed 1) during induction (Ind) and formation (Form) phases of *in vitro* adventitious rooting in difficult-to-root *Eucalyptus globulus* and easy-to-root *E. grandis*, both under control (Cont) and exogenous auxin (Aux) exposure conditions.

	AGO1				***PIN1***			
	Cont		**Aux**		**Cont**		**Aux**	
	Ind	**Form**	**Ind**	**Form**	**Ind**	**Form**	**Ind**	**Form**
E. globulus	0.21*	0.37*	0.17*	0.25*	0.07	0.02	0.17	0.03
E. grandis	0.53	0.36	1.49	0.40	0.34	0.36	1.13	0.55

* Data from De Almeida et al. (2010).

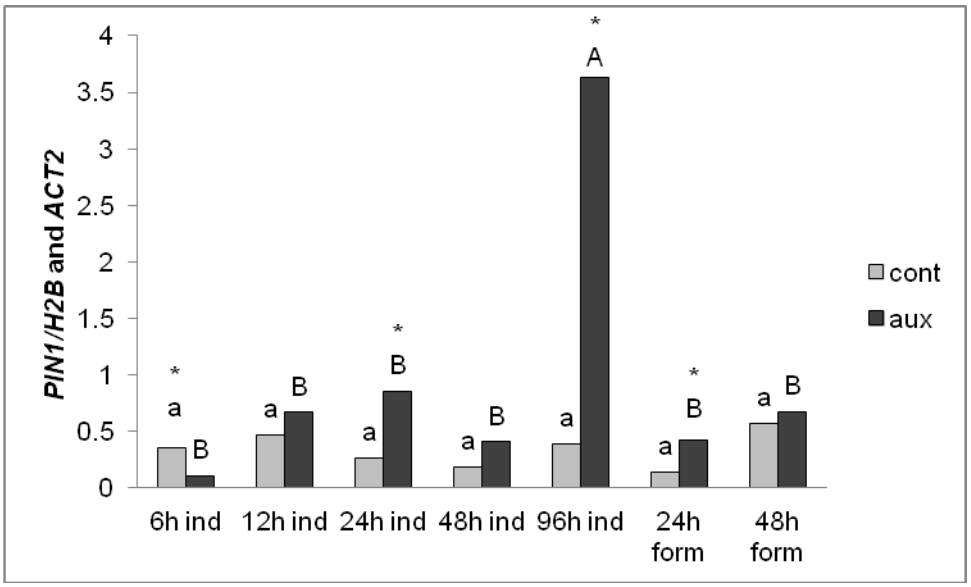


Figure 5. *PIN1* relative expression during *in vitro* adventitious rooting *Eucalyptus grandis.* "Ind" indicates induction step and "form" indicates formation step. Cont: without auxin in induction step. Aux: presence of 10 mg l^{-1} IBA in induction step. Columns sharing the same letter are not different by ANOVA followed by Duncan test ($P \leq 0.05$); small letters correspond to analysis of variance (ANOVA) performed for control treatment samples; upper case letters correspond to analysis of variance (ANOVA) performed for samples treated with auxin. "*" indicates significant difference between different conditions within a time-point (Student's t-test with $P \leq 0.05$).

Discussion

Although it is well established that auxin is essential for adventitious root development, its specific mechanisms of action in this context remain unclear, particularly in woody species. In order to help clarifying this problem, we decided to analyze auxin-related gene expression as an initial approach. Considering the importance of auxin transport in rooting capacity (Fogaça and Fett-Neto, 2005; Overvoorde et. al. 2010), we initially analyzed the expression patterns of the main auxin influx and efflux transporters along the process of adventitious rooting in *Eucalyptus globulus*, a recalcitrant to root species of significant economic importance. The gene encoding the auxin influx transporter *AUX1* (*Auxin Resistant 1*) did not show differences in expression profile between treatments (Figure 2A), suggesting that *AUX1* does not play an essencial role in the process of adventitious rooting promoted by exogenous auxins in microcuttings. Auxin molecules can enter cells passively due to pH gradients existing between extra- and intra-cellular environments. Besides the anionic form in neutral or basic pH, auxin molecules can occur in a proton-associated form under acidic environments, such as the apoplast, being able to enter the cell by lipophilic diffusion (passive movement) across the plasma membrane (Zazimalová et. al. 2010). The need for active auxin-uptake (through AUX1) arises mostly in cells where high and rapid auxin influx is needed, such as in the lateral root cap where AUX1 plays a major role in redirecting polar auxin streams (Kramer and Bennett 2006). Thus, in the case of *E. globulus* microcuttings, exogenous auxin may be entering cells passively, without major participation of AUX1. Besides, the rate of endogenous auxin transport is probably less limiting under exogenous auxin supply.

The rate of auxin export from cells seems to be more relevant for adventitious rooting in *E. globulus*. The expression of the gene encoding the auxin efflux transporter *PIN1* (*PIN Formed 1*) increased in the first 24 h of the induction step in microcuttings under exogenous auxin supply (Figure 2B). This seems to indicate a requirement of *PIN1* to redistribute and perhaps concentrate auxin in specific areas of the base of microcuttings (target tissues), in order to allow root development. Previous studies of PIN expression and auxin distribution in

pin mutants showed that PIN proteins are the major responsible for directional distribution networks that mediate auxin maxima and gradients during different developmental processes (review – Vieten et. al. 2007). Moreover, Dubrovsky et. al. (2008), in a study about lateral root organogenesis, revealed an absolute spatial and temporal correlation of auxin maxima with developmental reprogramming, resulting in organ initiation. These and other studies support a view for dynamic roles and re-locations for PIN proteins in cell membranes, both along developmental programmes and upon specific environmental stimuli, such as gravity and light, to allow auxin re-distribution and focusing. Taken together, these observations lead us to hypothesize that *E. globulus* has lower concentrations of endogenous auxin in its microcuttings bases or that this auxin is not well distributed to allow adventitious root formation in the absence of exogenous auxin supply. On the other hand, although IBA is a natural auxin recognized as an IAA (indolyl-acetic acid) precursor, its use as exogenous auxin instead of IAA, a common practice in clonal propagation by cuttings, may have also involved the expression of other sets of recently described IBA-specific transporters (Strader and Bartel, 2011).

To facilitate understanding the *PIN1* expression pattern in *E. globulus*, we decided to compare the expression profile of this gene in a relatively easy-to-root species, *E. grandis*, the most planted species from this genus in Brazil (Canettieri et. al. 2007). Since no previous study was available indicating reference genes appropriate for gene expression analysis during rooting of *E. grandis*, we investigated the expression stability of eight putative constitutive genes during *in vitro* adventitious rooting. Two distinct statistical approaches were used for this: *geNorm* and *NormFinder*, which indicated different combination of genes as better choices. As the two programs are based in distinct mathematical calculations, this was not unexpected.

To validate the most suitable reference genes for use in our experiments, the relative expression profile of *AGO1* (*Argonaute 1*) was investigated, using the pairs of reference genes indicated as most stable by each one of the programs. An increase in *AGO1* expression would be expected during microcutting root development considering its function

in regulating auxin metabolism-related genes during adventitious rooting in *Arabidopsis* (Sorin et. al. 2005). In fact, an overall trend for increase in *AGO1* expression with time was observed in microcuttings of *E. globulus* both with and without exogenous auxin. The relatively lower *AGO1* expression in presence of auxin may be due to a reduced requirement for the regulation of auxin metabolism related genes under external auxin supply (De Almeida et. al. 2010), which, in contrast, is probably at a less than ideal concentration in control microcuttings. The overall increasing *AGO1* expression trend with time may also reflect the need for tighter auxin homeostasis control along the rooting process. However, In *E. grandis*, an increase in *AGO1* expression in an early stage of adventitious rooting occurred under exogenous auxin supply, showing an overall trend for decrease with time (Figure 4A); this profile seems to match with the easy-to-root characteristic of this species. Since *E. grandis* does not require exogenous auxin to develop adventitious roots, this early increment in *AGO1* under exposure to this phytohormone is probably occurring to allow homeostasis of a likely excessive auxin concentration at the beginning of the process. In addition, mean levels of expression of *AGO1* in both treatments were higher in *E. grandis* compared to *E. globulus*, in agreement with a more efficient auxin homeostasis control in the former species (Table 4).

Taking the reference gene results together, *NormFinder* was selected as better suited for analysis of gene expression stability during adventitious rooting, in agreement with other studies by Artico et. al. (2010), De Almeida et. al (2010), Lin et. al. (2010), Pernot et. al. (2010) and Steiger et. al.(2010). This tool is considered more widely applicable, mainly when involving different cell types and experimental stages associated with high variation of gene expression (Andersen et. al. 2004; Lin et. al. 2010). Therefore, we chose *H2B* and *ACT2* as the most adequate reference genes for expression studies during the rooting of *E. grandis* microcuttings. Although both *IDH* and *SAND* genes were indicated as good reference genes by previous works (Goicoechea et. al. 2005; Legay et. al. 2007; Carvalho et. al. 2008; Boava et. al. 2010 and Czechowsky et. al. 2005; Remans et. al. 2008, respectively) they were found as the least stable genes indicated by both programs in the present work. This reinforces the

need for selecting suitable reference genes for every species and experimental condition (Gutierrez et. al. 2008; Guénin et. al. 2009).

The selection of the better reference genes allowed us to investigate the expression profile of *PIN1* in *E. grandis*. The results showed a transiently expression of *PIN1* along adventitious rooting in this species, except by a pronounced increase at 96 h in microcuttings exposed to exogenous auxin. As in *E. globulus*, this may indicate that *PIN1* is necessary to remobilize and relocate auxin to induce formation of root meristems. On the other hand, in *E. grandis* this increase in *PIN1* expression took place at a later time. This probably occurs to allow the establishment of several additional root primordial. Although *E.grandis* roots at much higher percentages than *E. globulus* without exogenous auxin and develops considerably more adventitious roots both in presence or absence of auxin supply than *E. globulus* (data not show), the added exogenous auxin also has a positive effect on root number in *E. grandis* (samples treated with auxin – 13.2 roots per sample; control samples – 2.3 roots per sample – unpublished data). This hypothesis finds further support on the higher *PIN1* expression levels in *E. grandis* compared to *E. globulus* under both conditions of the experiment (Table 4). Considering the rooting phenotype of untreated microcuttings of *E. grandis*, this increase in *PIN1* expression does not appear to be as essential as in *E. globulus*. Short auxin treatments activate transcription of different *PIN* genes in various tissues and long-term treatments with high concentrations of auxin can lead to an increased turnover of some *PIN* proteins (Vieten et. al. 2005). Thus, the same treatment of exogenous auxin may result in different molecular and biochemical balances in distinct species.

Conclusion

Adventitious rooting is a very complex process, regulated by both environmental and endogenous factors, but the molecular mechanisms regulating adventitious root formation are still poorly understood. This work constitutes a first step to elucidate the causes of recalcitrance to rooting in *E. globulus*. The process of auxin exit from the cells, probably related to auxin focusing in target tissues, seems to be important since *PIN1* expression is

activated in the early stages of the process in presence of exogenous auxin, which correlates to the adventitious root development in this difficult-to-root species. The same early rise in *PIN1* is not observed in easy-to-root *E. grandis*, which displays mean levels of auxin efflux transporter expression that are higher than those observed in *E. globulus*. Obviously more studies are necessary to better understand the role of auxin in this process and how appropriate treatmens and genetic modifications can make *E. globulus* more competent to rooting and, consequently, generate more productive clonal forests.

Acknowledgments

The authors thank Celulose Riograndense (Guaíba, RS, Brazil) for donation of *E. globulus* and *E. grandis* seeds, and Dr. Giancarlo Pasquali (Plant Molecular Biology Lab – Center for Biotechnology, Federal University of Rio Grande do Sul – Brazil) for the kind gift of primer pairs for *Eucons08* and assistance in acessing the *Genolyptus* database.

Funding

This research was funded by the Brazilian agencies National Council for Scientific and Technological Development (CNPq) and National Commission for Graduate Program Funding and Evaluation (CAPES).

References

ABRAF - Statistical yearbook from ABRAF. 2010. Brazilian Association of Foresters (Associação Brasileira de Produtores de Florestas Plantadas).

Andersen, C.L., Jensen, J.L., Orntoft, T.F. 2004. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64:5245-5250.

Artico, S., Nardeli, SM., Brilhante, O., Grossi-de-Sá, F. M., Alves-Ferreira, M. 2010. Identification and evaluation of new reference genes in Gossypium hirsutum for accurate normalization of real-time quantitative RT-PCR data. BMC Plant Biol. 10:49.

Benjamins, R., Scheres, B. 2008. Auxin: the looping star in plant development. Annu. Rev. Plant Biol. 59:443-465.

Boava, L.P., Laia, M.L., Jacob, T.R., Dabbas, K.M., Gonçalves, J.F., Ferro, J.A., Ferro, M.I.T., Furtado, E.L. 2010. Selection of endogenous genes for gene expression studies in *Eucalyptus* under biotic (*Puccinia psidii*) and abiotic (acibenzolar-Smethyl) stresses using RTqPCR. BMC Res. Notes 3:43.

Brunner, A.M., Yakovlev, I.A., Strauss, S.H. 2004. Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol. 4:14.

Bustin, S.A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol. Endocrinol. 29:23-39.

Campos, E.I., Reinberg, D. 2009. Histones: annotating chromatin. Annu Rev Genet. 43:559-99.

Canettieri, E.V., Rocha, G.J.M., Carvalho Jr., J.A., Silva, J.B.A. 2007. Optimization of acid hydrolysis from the hemicellulosic fraction of *Eucalyptus grandis* residue using response surface methodology. Bioresource Technology 98: 422-428.

Carvalho, M.C.C.G., Caldas, D.G.G., Carneiro, R.T., Moon, D.H., Salvatierra, G.R., Franceschini, L.M., Andrade, A., Celedon, P.A.F., Oda, S., Labate, C.A. 2008. SAGE transcript profiling of the juvenile cambial region of *Eucalyptus grandis*. Tree Physiol. 28:905-919.

Chen, X. 2009. Small RNAs and their roles in plant development. Annu. Rev. Cell. Dev. Biol. 25:21-44.

Chiang, V. 2002. From rags to riches. Nat. Biotechnol. 20:557-558.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., Scheible, W. 2005. Genome-wide identification and testing of superior reference-genes for transcript normalization in Arabidopsis. Plant Physiol. 139:5-17.

De Almeida, M.R., Ruedell, C.M., Ricachenevsky, F.K., Sperotto, R.A., Pasquali, G. and Fett-Neto, A.G. 2010. Reference gene selection for quantitative reverse transcription-polymerase chain reaction normalization during *in vitro* adventitious rooting in *Eucalyptus globulus* Labill. BMC Molecular Biology 11:73.

De Klerk, G.J., Van Der Krieken, W., De Jong, J.C. 1999. The formation of adventitious roots: New concepts, new possibilities. In Vitro Cell Dev. Biol. Plant 35(3):189-199.

De Klerk, G.J. 2002. Rooting of microcuttins: theory and practice. In Vitro Cellular & Developmental Biology-Plant, 38: 415-422, Del Lungo

Demeke, T.; Jenkins, G. R. Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. 2010. Anal Bioanal. Chem. 396(6):1977-90.

Dubrovsky, J.G., Sauer, M., Napsucialy-Mendivil, S., Ivanchenko, M., Friml, J., Shishkova, S., Celenza, J., and Benková, E. 2008. Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. Proc. Natl. Acad. Sci. USA 105, 8790–8794.

Expósito-Rodríguez, M., Borges, A.A., Borges-Pérez, A., Pérez, J.A. 2008. Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol. 8:131.

Fett-Neto, A.G., Fett, J.P., Goulart, L.W.V., Pasquali, G., Termignoni, R.R., Ferreira, A.G. 2001. Distinct effects of auxin and light on adventitious root development in *Eucalyptus saligna* and *Eucalyptus globulus*. Tree Physiol. 21:457-464.

Fogaça, C.M., Fett-Neto, A.G. 2005. Role of auxin and its modulators in the adventitious rooting of Eucalyptus species differing in recalcitrance. Plant Growth Regul. 45:1-10.

Gachon, C., Mingam, A., Charrier, B. 2004. Real time PCR: what relevance to plant studies? J. Exp. Bot. 55(402):1445-1454.

Ginzinger, D.G. 2002. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. Exp. Hematol. 30(6):503-512.

Goicoechea, M., Lacombe, E., Legay, S., Mihaljevic, S., Rech, P., Jauneau, A., Lapierre, C., Pollet, B., Verhaegen, D., Chaubet-Gigot, N., Grima-Pettenati, J. 2005. EgMYB2, a new transcriptional activator from *Eucalyptus* xylem, regulates secondary cell wall formation and lignin biosynthesis. Plant Journal 43:553-567.

Guénin, S., Mauriat, M., Pelloux, J., Van Wuytswinkel, O., Bellini, C., Gutierrez, L. 2009. Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. J. Exp. Bot. 60(2):487-493.

Gutierrez, L., Mauriat, M., Guénin, S., Pelloux, J., Lefebvre, J., Louvet, R., Rusterucci, C., Moritz, T., Guerineau, F., Bellini, C., Van Wuytswinkel, O. 2008. The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant Biotechnol. J. 6:609-618.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8:19.

Huggett, J., Dheda, K., Bustin, S., Zumla, A. 2005. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun. 1-6.

Kramer, E.M., Bennett, M.J. 2006. Auxin transport: A field in flux. Trends Plant Sci. 11: 382–386.

Legay, S., Lacombe, E., Goicoechea, M., Brièrea, C., Séguind, A., Mackayc, J., Grima-Pettenati, J. 2007. Molecular characterization of EgMYB1, a putative transcriptional repressor of the lignin biosynthetic pathway. Plant Sci. 173(5):542-549.

Le Roux, J.J., Van Staden, J. 1991. Micropropagation and tissue culture of *Eucalyptus* - a review. Tree Physiol. 9:435-477.

Li, S-W., Xue, L., Xu, S, Feng, H., An, L. 2009. Mediators, genes and signaling in adventitious rooting. Bot. Rev. 75:230-247.

Lin, Y.L., Lai, Z.X. 2010. Reference gene selection for qPCR analysis during somatic embryogenesis in longan tree. Plant Science 178:359-365.

Livak, K.J., Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}$CT method. Methods 25:402-408.

Muday, G.K., Delong, A. 2001. Polar auxin transport: controlling where and how much. Trends Plant Sci. 6:535-542.

Murashigue, T., Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15:473-498.

Overvoorde P., Fukaki, H., Beeckman, T. 2010. Auxin control of root development. Cold Spring Harb. Perspect. Biol. 2:a001537

Pernot, .F, Dorandeu, F., Beaup, C., Peinnequin, A. 2010. Selection of reference genes for real-time quantitative reverse transcription-polymerase chain reaction in hippocampal structure in a murine model of temporal lobe epilepsy with focal seizures. Journal of Neuroscience Research, 88:1000-1008.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29 (9):2002-2007.

Ramakers, C., Ruijtera, J.M., Deprez, R..HL., Moorman, A.F.M. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339:62-66.

Remans, T., Smeets, K., Opdenakker, K., Mathijsen, D., Vangronsveld, J., Cuypers, A. 2008. Normalization of real-time RT-PCR gene expression measurements in Arabidopsis thaliana exposed to increased metal concentrations. Planta 227:1343-1349.

Ruedell, C.M., Schwambach, J., Corrêa, L.R., Fett-Neto, A.G. 2009. Strategies for adventitious rooting in clonal propagation of *Eucalyptus*. In Adventitious root formation of forest trees and horticultural plants - From genes to applications. Edited by: Niemi K, Scagel C. Kerala: Research Signpost: 337-358.

Serrano, L., Rochange, F., Semblant, J.P., Marque, C., Teulières, C., Boudet, A.M. 1996. Genetic transformation of Eucalyptus globulus through biolistics: complementary development of procedures for organogenesis from zygotic embryos and stable transformation of corresponding proliferating tissue. J. Exp. Bot. 45:285-290.

Sorin, C., Bussell, J.D., Camus, I., Ljung, K., Kowalczyk, M., Geiss, G., Mckhann, H., Garcion, C., Vaucheret, H., Sandberg, G., Bellini, C. 2005. Auxin and light control of adventitious rooting in *Arabidopsis* require *ARGONAUTE 1*. Plant Cell, 17:1343-1359.

Steiger, M.G., Mach, R.L., Mach-Aigner, A.S. 2010. An accurate normalization strategy for RT-qPCR in Hipocrea jecorina (Trichoderma reesei). Journal of Biotechnology 145:30-37.

Strader, L.C., Bartel, B. 2011. Transport and metabolism of the endogenous auxin precursor Indole-3-Butyric Acid. Molecular Plant 1-10.

Tong, Z., Gao, Z., Wang, F., Zhou, J., Zhang, Z. 2009. Selection of reliable reference genes for gene expression studies in peach using real-time PCR. BMC Mol Biol. 10:71.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F. 2002. Accurate normalisation of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3:1-11.

Vieten, A., Vanneste, S., Wiśniewska, J., Benková, E., Benjamins, R., Beeckman, T., Luschnig, C., and Friml, J. 2005. Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. Development 132, 4521–4531.

Vieten, A., Sauer, M., Brewer, P.B., and Friml, J. 2007. Molecular and cellular aspects auxin-transport-mediated development. Trends Plant Sci. 12 160–168.

Zazimalová, E., Murphy, A.S., Yang, H. et. al. 2010. Auxin Transporters—Why So Many? Cold Spring Harb. Perspect. Biol. 2: 1-14.

5 CONCLUSÕES GERAIS E PERSPECTIVAS

Considerando o processo de enraizamento adventício *in vitro* de espécies lenhosas, este trabalho se caracteriza como o sendo o primeiro estudo com enfoque molecular para o entendimento deste fenômeno em uma espécie de grande interesse econômico como o eucalipto. A utilização da técnica de qPCR se mostrou adequada para análise do padrão de expressão de genes possivelmente relacionados com a rizogênese adventícia, porém a seleção de genes referência adequados foi de grande importância para a interpretação dos dados obtidos. As análises indicaram *H2B* (Histona H2B) e *TUA* (Alfa-tubulina) e *H2B* e *ACT2* (Actina 2/7) como genes constitutivos adequados para estudos subseqüentes de expressão gênica *in vitro* em *Eucalyptus globulus* e *Eucalyptus grandis*, respectivamente. Estes resultados corroboram estudos recentes que afirmam a necessidade de validação de genes referência para cada espécie e condição experimental. Em relação às diferentes ferramentas utilizadas para seleção dos genes constitutivos, o software *NormFinder* se mostrou mais adequado, indicando genes controle em relação aos quais a expressão de genes de interesse se mostrou mais condizente com a sua função já descrita em outras espécies.

Dois dos três genes relacionados a transporte e ação de auxina que foram analisados parecem ter papel relevante na modulação do processo de enraizamento adventício em *Eucalyptus*, apresentando distintos padrões de expressão nas diferentes condições experimentais e espécies estudadas. Este resultado era esperado, uma vez que *E. globulus* e *E. grandis* possuem competências ao enraizamento distintas, portanto apresentando necessidades e regulação metabólica de auxinas diferentes. *AGO1* (*Argonaute 1*), utilizado na validação dos genes-referência, parece aumentar sua expressão ao longo do enraizamento em *E. globulus*, apresentando maior expressão em plantas não sujeitas ao efeito de auxina exógena, as quais apresentam enraizamento ineficiente nesta espécie. Esta observação corrobora o papel deste gene na homeostase de auxinas, atuando como um estimulador de enraizamento, já descrito para *Arabidopsis.* No caso de exposição

à auxina exógena, a necessidade de expressão de *AGO1* seria reduzida na espécie recalcitrante. *AUX1* (*Auxin Resistant 1*), codificador do transportador de influxo de auxina, parece não ter papel crítico no enraizamento em eucalipto. Entretanto, o processo de saída de auxina da célula e redistribuição deste fitormônio se mostrou mais limitante, como verificado pelo aumento na expressão do codificador do transportador de efluxo de auxina *PIN1* (*PIN Formed 1*), em plantas sob efeito de auxina exógena. Além disso, o efeito de *PIN1* provavelmente é mais determinante na espécie recalcitrante *E. globulus* do que na espécie propensa ao enraizamento *E. grandis*, pois seu nível de expressão é, em média, maior na última. Em concordância com a mesma linha de raciocínio, o nível de expressão médio de *AGO1* também foi maior na espécie de fácil enraizamento.

Estes resultados servirão de ponto de partida para estudos mais aprofundados acerca das causas da recalcitrância ao enraizamento encontrada em *E. globulus*. Genes envolvidos na biossíntese e sinalização de auxina deverão ser analisados, bem como aspectos relacionados à concentração e distribuição de auxinas endógenas na planta. Quando estes desafios forem transpostos, abordagens de engenharia genética, melhoramento e/ou manejo fisiológico poderão ser utilizadas na tentativa de tornar essa espécie mais competente ao enraizamento e consequentemente, conferir aumento na produtividade das florestas clonais de *E. globulus*.

6 REFERÊNCIAS BIBLIOGRÁFICAS

ABRAF - **Anuário Estatístico da ABRAF** - Associação Brasileira de Produtores de Florestas Plantadas, 2010.

ALFENAS, A.C.; ZAUZA, E.A.V.; MÁFIA, R.G.; ASSIS, T.F. **Clonagem e Doenças do Eucalipto.** UFV Editora, Universidade Federal de Viçosa, Brasil. p. 154, 2004.

ANDERSEN CL, JENSEN JL, ORNTOFT TF. **Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets.** Cancer Res 64:5245-5250, 2004.

BARTEL, B. **Auxin biosynthesis.** Ann. Rev. Plant Physiol. Plant Mol. Biol. 48: 51-66, 1997.

BRETON, M. C. **Estudo do transcriptoma em flores de *Eucalyptus grandis*.** Tese de Doutorado. Programa de Pós Graduação em Biologia Celular e Molecular. Universidade Federal do Rio Grande do Sul. Brasil. 2011.

BRUNNER, A., M., YAKOVLEV, I., A. & STRAUSS, S., H. **Validating internal controls for quantitative plant gene expression studies.** BMC Plant Biology 4:14, 2004

CANETTIERI, E.V., ROCHA, G.J.M., CARVALHO Jr., J.A., SILVA, J.B.A. **Optimization of acid hydrolysis from the hemicellulosic fraction of Eucalyptus grandis residue using response surface methodology.** Bioresource Technology 98: 422-428, 2007

CHIANG, V. **From rags to riches.** Nature Biotechnology 20: 557-558, 2002.

CORREA, L. DA R.; FETT-NETO AG. **Effects of temperature on adventitous root development in microcuttings of *Eucalyptus saligna* and *E. globulus*.** Journal of Thermal Biology 29: 315-324, 2004.

CZECHOWSKI, T., STIIT, M, ALTMANN, T., UDVARDI, M. K. & SCHEIBLE, W. **Genome-wild identification and testing of superior reference-genes for transcript normalization in *Arabidopsis*.** Plant Physiology 139: 5-17, 2005.

DE KLERK, G.J., VAN DER KRIEKEN W. & DE JONG J.C. **The formation of adventitious roots: New concepts, new possibilities.** In Vitro Cellular & Developmental Biology-Plant. 35(3): 189-199 MAY-JUN, 1999.

DE KLERK, G.J. **Rooting of microcuttins: theory and practice.** In Vitro Cellular & Developmental Biology-Plant, 38: 415-422, 2002.

DEWAR, C. R. **A model of carbon storage in forests and forest products.** Tree Physiology 6: 417-428, 1990.

De RYBEL, B., AUDENAERT, D., BEECKMAN, T., KEPINSKI, S. **The past, present and future of chemical biology in auxin research.** ACS Chemical Biology 4: 987-998, 2009.

DHARMASIRI, N.; DHARMASIRI, S.; ESTELLE, M. **The F-box protein TIR1 is an auxin receptor.** Nature 435: 441-445, 2005.

ELDRIGE, K., DAVIDSON, J., HARDWOOD, C. & VAN WYK, G. **Eucalypt domestication and breeding.** Oxford Science Publications. 1997.

ESAU, K. **Anatomia das plantas com sementes.** São Paulo, Edgard Blücher, Ed. da Universidade de São Paulo, 1974.

EXPÓSITO-RODRÍGUEZ, M., BORGES, A. A., BORGES-PÉREZ, A. & PÉREZ, J. A. **Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process.** BMC Plant Biology 8:131, 2008.

FETT-NETO AG ; FETT, J. P. ; GOULART, L. W. ; PASQUALI, G ; TERMIGNONI, R. R. ; FERREIRA, A. G. **Distinct effects of auxin and light on adventitious root development in Eucalyptus saligna Smith and Eucalyptus globulus Labill**. Tree Physiology 21: 457-464, 2001.

FOGAÇA, C.M. **Papel de Auxinas e seus Moduladores na Rizogênese de *Eucalyptus globulus* Labill e *Eucalyptus saligna* Smith.** Dissertação de Mestrado. Programa de Pós Graduação em Botânica. Universidade Federal do Rio Grande do Sul. Brasil. 2003.

FOGAÇA, C. M.; FETT-NETO A.G . **Role of auxin and its modulators in the adventitious rooting of *Eucalyptus* species differing in recalcitrance.** Plant Growth Regulation 45: 1-10, 2005.

GACHON, C., MINGAM, A., CHARRIER, B. **Real time PCR: what relevance to plant studies?** Journal of Experimental Botany, 55 (402): 1445-1454, 2004.

GUTIERREZ, L., MAURIAT, M., GUÉNIN, S., PELLOUX, J., LEFEBVRE, J., LOUVET, R., RUSTERUCCI, C., MORITZ, T., GUERINEAU, F., BELLINI, C. & VAN WUYTSWINKEL, O. **The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants.** Plant Biotechnology Journal 6: 609-618, 2008.

HAND, P. **Biochemical and molecular markers of cellular competence for adventitious rooting.** In Biology of adventitious root formation. Eds. T.D. Davis and Haissig, B.E. Basic Life Sciences 62. Plenum Press. New York. pp 111- 121, 1994.

HARTMANN, H.T., KESTER D.E. & DAVIES, F.T. **Plant propagation: principles and practices.** Englewood Cliffs, NJ: Prentice Hall. 1990.

KIM, J.I.; SHARKHUNN, A.; JIN, J.B.; LI, P.; JEONG, J.C.; BAECK, D.; LEE, S.Y.; BLAKESLEE, J.J.; MURPHY, A.S.; BOHNERT, H.J. et al. ***yucca6*, a dominant mutation in *Arabidopsis* affects auxin accumulation and auxin-related phenotypes.** Plant Physiol. 145:722-735, 2007.

LI, S-W., XUE, L., XU, S., FENG, H., AN, L. **Mediators, genes and signaling in adventitious rooting.** Botanical Reviews 75: 230-247, 2009.

LE ROUX, J.J. & VAN STADEN, J. **Micropropagation and tissue culture of *Eucalyptus* – a review.** Tree Physiol. 9: 435-477, 1991.

LOIO, R.D., NAKAMURA, K., MOUBAYIDIN, L., PERILLI, S., TANIGUCHI, M., MORITA, M.T., AOYAMA, T., COSTANTINO, P., SABATINI, S. **A genetic framework for the control of cell division and differentiation in the root meristem.** Science 322: 1381–1384, 2008.

MACEDO, E.S., CARDOSO, H.G., HERNÁNDEZ, A., PEIXE, A.A., POLIDOROS, A., FERREIRA, A., CORDEIRO, A., ARNHOLDT-SCHMITT, B. **Physiologic responses and gene diversity indicate olive alternative oxidase as a potential source for markers**

involved in efficient adventitious root induction. Physiologia Plantarum 137: 532–552, 2009

MORA A.L. & GARCIA C.H. **A Cultura do Eucalipto no Brasil.** 112 p. SBS, São Paulo, 2000.

MUDAY, G.K.; DELONG, A. **Polar auxin transport: controlling where and how much.** Trends in Plant Science 6: 535-542, 2001.

NORMANLY, J.; COHEN, J.D.; FINK, G.R. ***Arabidopsis thaliana*** **auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid.** Proc. Natl. Acad. Sci. 90: 10355-10359, 1993.

NORMANLY, J. **Approaching cellular and molecular resolution of auxin biosynthesis and metabolism.** Cold Spring Harb. Perspect. Biol. 1-17, 2010.

POKE, F. S., VAILLANCOURT, R. E., POTTS, B. M., REID, J. B. **Genomic research in *Eucalyptus*.** Genetica 125: 79-101, 2005.

SANCHEZ, C., VIELBA, J.M., FERRO, E., COVELO, G., SOLÉ, A., ABARCA, D., DE MIER, B.S., DÍAZ-SALA, C. **Two SCARECROW-LIKE genes are induced in response to exogenous auxin in rooting-competent cuttings of distantly related forest species.** Tree Physiology 27: 1459–1470, 2007.

SCHWAMBACH, J.; FADANELLI, C.; FETT-NETO A.G. **Mineral nutrition and adventitious rooting in microcuttings of *Eucalyptus globulus*.** Tree Physiology 25: 487- 494, 2005

SCHWAMBACH, J.; RUEDELL, Carolina Michels ; ALMEIDA, Márcia Rodrigues de ; PENCHEL FILHO, R. M. ; ARAUJO, E. F. ; FETT-NETO AG . **Adventitious rooting of *Eucalyptus globulus x maidennii* mini-cuttings derived from mini-stumps grown in sand bed and intermittent flooding trays: a comparative study.** New Forests 36: 261-271, 2008.

SERRANO, L., ROCHANGE, F., SEMBLANT, J.P., MARQUE, C., TEULIÈRES, C. & BOUDET A.M. **Genetic transformation of *Eucalyptus globulus* trought biolistics: complementary development of procedures for organogenesis from zygotic embryos and stable transformation of corresponding proliferating tissue.** J. Exp. Bot. 45: 285-290, 1996.

SORIN, C., BUSSELL, J. D., CAMUS, I., LJUNG, K., KOWALCZYK, M., GEISS, G., McKHANN, H., GARCION, C., VAUCHERET, H., SANDBERG, G., BELLINI, C. **Auxin and light control of adventitious rooting in *Arabidopsis* require ARGONAUTE1.** The Plant Cell, 17: 1343-1359, 2005.

SOUZA, V. C. & LORENZI, H. **Botânica Sistemática: guia ilustrado para identificação das famílias de Fanerógamas nativas e exóticas no Brasil, baseado em APG II.** 2ª ed. Nova Odessa, SP: Instituto Plantarum, 2008.

STEPANOVA AN, ROBERTSON-HOYT J, YUN J, BENAVENTE LM, XIE DY. **TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development.** Cell 133:177–91, 2008.

STRADER, L. C., MONROE-AUGUSTUS, M., BARTEL, B. **The IBR5 phosphatase promotes *Arabidopsis* auxin responses through a novel mechanism distinct from TIR1-mediated repressor degradation.** BMC Plant Biology 8:41, 2008.

TAKAHASHI, F. SATO-NARA, K. KOBAYASHI, K. SUZUKI, M. SUZUKI, H. **Sugar induced adventitious root in Arabidopsis seedlings**. Journal of Plant Research 116: 83-91, 2003.

TAO Y, FERRER JL, LJUNG K, POJER F, HONG F. **Rapid synthesis of auxin via a new tryptophandependent pathway is required for shade avoidance in plants.** Cell 133:164–76, 2008.

TEALE W.D., PAPONOV, I. A., PALME, K. **Auxin in action: signalling, transport and the control of plant growth and development** - Nature Reviews 7: 847-859, 2006.

TURNBULL, J.W. **Eucalypt plantations.** New Forests. 17:37-52, 1999.

VAN DER KRIEKEN, W.M., BRETELER, H., VISSER, M.H.M. & MAVRIDOU, D. **The role of the conversation of IBA into IAA on root regeneration in apple: introduction of a test system.** Plant Cell Rep. 12: 203-206, 1993.

VANDESOMPELE, J., De PRETER, K., PATTYN, F., POPPE, B., VAN ROY, N., De PAEPE, A. & SPELEMAN, F. **Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.** Genome Biology 3:7, 2002.

VAN GUILDER, H.D., VRANA, K.E., FREEMAN WM: **Twenty-five years of quantitative PCR for gene expression analysis.** BioTechniques 44:619-626, 2008.

WOODWARD, A.W. & BARTEL B. **A receptor for auxin.** Plant Cell, 17: 2425-2429, 2005.

WRIGHT, A., MOEHLENKAMP, C.A., PERROT, G.H., NEUFFER, M.G., CONE, K.C. **The maize auxotrophic mutant orange *pericarp* is defective in duplicate genes for tryptophan synthase beta**. Plant Cell 4: 711-719, 1992.

YAMADA M, GREENHAM K, PRIGGE MJ, JENSEN PJ, ESTELLE M. **The TRANSPORT INHIBITOR RESPONSE2 (TIR2) gene is required for auxin synthesis and diverse aspects of plant development.** Plant Physiol. 151:168–79, 2009.

ZHAO, Y.; CHRISTENSEN, S. K.; FANKHAUSER, C.; CASHMAN, J.R.; COHEN, J.D.; WEIGEL, D.; CHORY, J. **A role for flavin monooxygenase-like enzymes in auxin biosynthesis.** Science 291: 306-309, 2001.

ZHAO, Y. **Auxin biosynthesis and its role in plant development**. Ann. Rev. Plant Biol. 61: 49-64, 2010.