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

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A B S T R A C T

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

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1. Introduction

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

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

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

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

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

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

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

2. Material and methods

2.1. Plant material

Seeds of *E. globulus* Labill and *E. grandis* Hill ex Maiden were surface sterilized with 70% ethanol and 1.5% sodium hypochlorite, followed by four washings with sterilized distilled water. Fifteen seeds were sown in 300 ml glass jars containing 60 ml of solid culture medium, consisting of 0.5X MS salts [29], 2% sucrose, 0.6% agar and pH adjusted to 5.8 [3]. Medium was autoclaved at 121 °C for 20 min. The plants were kept under controlled conditions of light and temperature, with photoperiod of 16 h, 50 μmol $m^{-2}s^{-1}$ light intensity (provided by white fluorescent lamps) and temperature of 23 ± 2 °C. After 14 (*E. globulus*) or 16 weeks (*E. grandis*), apical microcuttings (~3 cm-long) were obtained, which were used in the *in vitro* adventitious rooting experiments.

2.2. In vitro adventitious rooting

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

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

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

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

2.3. Whole plant gene expression

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

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

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