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Research article

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

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ABSTRACT

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

1. Introduction

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

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

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detailed, especially in non-model species (Pacurar et al., 2014). Yucca (*YUC*) and Tryptophan Aminotransferase of *Arabidopsis* (*TAA*) genes integrate the main auxin biosynthesis pathway from tryptophan in *Arabidopsis* (Won et al., 2011). Auxins are mainly synthesized in the shoot apex and young leaves, being redistributed inside the plant through phloem parenchyma cells equipped with various transporters. Influx (Auxin Resistant 1/Like AUX - AUX/LAX) and efflux (PIN-Formed - PIN) carriers play roles in auxin basipetal transport through the stem, allowing local accumulation of the phytohormone at the stem base (Da Costa et al., 2013). Auxin Response Factors *ARF6* and *ARF8* were characterized as positive regulators of adventitious root formation in *Arabidopsis* (Gutierrez et al., 2009). In the proposed model, adventitious root initiation is controlled by a complex mechanism between the activators *ARF6* and *ARF8*, the repressor *ARF17*, and its posttranscriptional regulators miR160 (*ARF17*) and miR167 (*ARF6* and *ARF8*), acting with *AGO1* (*Argonaute1*) in silencing. *ARF6* also regulates *ARF8* transcript abundance and can be controlled by light (Gutierrez et al., 2009).

Carbohydrates not only provide energy and carbon, which are essential for adventitious rooting, but can also act as signaling molecules, interacting with phytohormones, and regulating many developmental processes in plants, including adventitious rooting in *Eucalyptus* (Corrêa et al., 2005). The control of adventitious rooting by carbohydrates might be direct, via their signaling pathway, or indirect, by activating the biosynthesis or transport of phytohormones (Takahashi et al., 2003). Efficient utilization and partitioning of carbohydrates may be modulated by light and can be key factors for adventitious rooting in cuttings (Koplotek et al., 2010).

Availability, intensity and quality of light may influence rooting ability and quality, although results vary according to species (Fett-Neto et al., 2001; Corrêa et al., 2005). *Eucalyptus globulus* is a commercially relevant species with rooting recalcitrance (Negishi et al., 2014), apparently related to higher cambium-specific expression of rooting inhibitory genes (*TPL*, *IAA12*, *ARR1*) and relatively lower indole-3-acetic acid content compared to easy-to-root *Eucalyptus grandis* (De Almeida et al., 2015). Far-red light exposure of donor-plants of *E. globulus* originates microcuttings with significantly improved rooting competence, when comparing with white light control. Percent rooting without exogenous auxin increased from 20% to 51%. A high root/shoot ratio of carbohydrates was observed in microcuttings derived from donor-plants exposed to far-red light. The light treatment had no effect when applied to microcuttings (Ruedell et al., 2013). Interactions between light quality, carbohydrate and auxin metabolism may be part of the mechanisms resulting in reduced rooting recalcitrance in *E. globulus*.

In this study, we investigated the expression of genes involved in auxin and carbohydrate metabolism underlying the higher adventitious rooting competence in *E. globulus* microcuttings derived from donor plants exposed to a far-red light enriched environment. To that end, we analyzed the relative expression profile of these genes in donor plants and throughout the rooting process.

2. Materials and methods

2.1. Plant material

Seeds of *E. globulus* (kindly provided by CMPC Celulose Riograndense S.A., Guaíba, RS, Brazil) were surface sterilized and cultivated as previously described (Fett-Neto et al., 2001). Essentially, seeds and donor plants derived from them were grown in half-strength MS salts, with 0.6% (w/v) agar and devoid of sucrose (germination medium). Rooting of tip microcuttings (approximately 3 cm long) was carried out in two steps, always without auxin: four-day root induction (0.3× MS salts, 100 mg l^{-1} myo-inositol, 0.4 mg l^{-1} thiamine HCl, 88 mM sucrose and 0.6% agar), followed by root formation medium (exact same composition but added of 1 g l^{-1} activated charcoal). Reagents used were of analytical grade, media were prepared with distilled water and sterilized by autoclaving (121 °C and 0.11 MPa) for 25 min.

Approximately fifteen seeds were sown in 300 ml glass jars (covered with a double layer of aluminum foil or transparent plastic film for irradiance experiments) with 60 ml of germination medium. Jars were incubated in a growth room at 25 ± 2 °C and 16 h of daily light (45 μmol m^{-2} s^{-1}). After 3.5 months, tip microcuttings were obtained from the donor plants and transferred to rooting medium as described above. Microcuttings were incubated in the same conditions of donor plants under white light only (see below).

2.2. Effect of light environment on donor-plants

Seedlings of *E. globulus* growing on germination medium for 2.5 months were exposed to white (control) or far-red light enrichment for 1 additional month. Spectral quality modification was provided by filtering the output of the white fluorescent tubes using double cellophane sheets. Green sheets were used for far-red light enrichment and transparent sheets for white light. The transmittance spectra of the filters were measured and recorded in a Cintra 5 spectrophotometer (GBC, Victoria, Australia) (Ruedell et al., 2013). Filters were replaced every five days to avoid minor changes in spectral quality of the transmitted light due to possible color fading. A set of 6 donor-plants per treatment was harvested upon completion of the one month period of light enrichment treatments. All samples were frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Microcuttings (about 3-cm-long tip cuttings) were excised from donor-plants and used for in vitro rooting experiments, being harvested for RNA extraction as described in the next section.

2.3. In vitro adventitious rooting

For qPCR analyses, microcuttings were harvested at days 2 and 4 of induction phase and 1, 5 and 10 days after transfer to formation phase. Microcuttings remained for 4 days in the induction medium before transfer to formation medium. All samples were frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Each harvest point included a pool of 6 whole microcuttings. All experiments were independently repeated three times (biological replicates) and each experiment included technical replicates. All of the independent experiments had similar results and data presented are the means of the three experiments.

2.4. Total RNA extraction and first strand cDNA synthesis

Total RNA was extracted and purified using NucleoSpin RNA Plant Kit (*Machery-Nagel*), following the manufacturer recommendations, with modifications as described by De Almeida et al. (2010). Quant-It™ RBA Assay Kit and Qubit fluorometer (*Invitrogen*) were used to determine total RNA concentration, whereas RNA quality was monitored by electrophoresis in 1% agarose gel (data not show). One independent cDNA synthesis was performed for each sample, starting from 100 ng of total RNA. First strand 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 Milli-Q water prior to use in qPCR.