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Characterization of a type-A response regulator differentially expressed during adventitious caulogenesis in *Pinus pinaster*

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ABSTRACT

The molecular cloning and characterization of PipsRR1, a type-A response regulator in *Pinus pinaster*, is reported here. Type-A response regulators mediate downstream responses to cytokinin and act as negative feedback regulators of the signal transduction pathway. Some type-A response regulators in *Arabidopsis* have been related to de novo meristem formation. However, little information exists in *Pinus* spp. The *PipsRR1* gene contains 5 exons, as do all type-A response regulators in *Arabidopsis*, and the deduced protein contains a receiver domain with the conserved DDK residues and a short C terminal extension. Expression analysis showed that the *PipsRR1* gene is differentially expressed during the first phases of adventitious caulogenesis induced by benzyladenine in *P. pinaster* cotyledons, suggesting that PipsRR1 plays a role in caulogenesis in conifers. Additionally, a binary vector carrying the *PipsRR1* promoter driving *GFP:GUS* expression was constructed to analyze the promoter activity in *P. pinaster* somatic embryos. The results of genetic transformation showed GUS activity during somatic embryo mass proliferation and embryo maturation.

Introduction

Adventitious caulogenesis in conifers can be induced by culturing cotyledons in the presence of cytokinins (Thorpe, 1993), and it is particularly useful to study the mode of action of cytokinins (Alonso et al., 2007; Cortizo et al., 2009; Moncaleán et al., 2005). The system in maritime pine (*Pinus pinaster*) is based on the induction of direct adventitious shoot formation in cotyledons excised from mature embryos and cultured in the presence of benzyladenine (BA) (Álvarez et al., 2009a; Calixto and Pais, 1997; David et al., 1982; Tereso et al., 2006). These explants are fully competent and do not require de-differentiation (callus phase) as do other models, such as *Arabidopsis thaliana* (Flinn et al., 1988). In recent years, a model integrating the mechanism of cytokinin signal transduction and meristem formation in *Arabidopsis* has been proposed (Gordon et al., 2009; Sablowski, 2009). The cytokinin signal is transduced via a two-component phosphorelay system to response regulators (RRs), nuclear proteins responsible for cytokinin effects on gene and protein function (Appleby et al., 1996; Goulian, 2010; Wuichet et al., 2010). Response regulators are divided into three classes based on phylogenetic and functional analysis: type-A, type-B and type-C. All of them carry a cytokinin receiver domain and a variable length C-terminal domain. Type-B response regulators have a large C-terminal extension with DNA-binding domains and act as transcriptional regulators. Type-A response regulators are small and contain short N- and C-terminal extensions. Type-C response regulators also contain a short C-terminal but are not grouped in the same clade as type-A RRs (Schaller et al., 2008). While type-B RRs are transcription factors expressed constitutively, type-A RRs, which do not have a DNA-interacting domain, are transcribed rapidly after cytokinin treatment without the requirement for previous protein synthesis (D'Agostino et al., 2000; To and Kieber, 2008). The type-A response regulators mediate downstream responses to cytokinin and act as negative regulators of the initial signal transduction pathway (Cheng et al., 2010; Hirose et al., 2007; To et al., 2007). With respect to de novo meristem formation, some type-A RRs have been shown to negatively regulate adventitious shoot induction in *Arabidopsis*, acting via feedback inhibition on meristem-promoting genes (Che et al., 2007). The stem cell population of shoot meristems in *Arabidopsis* is maintained by a regulatory loop between the *CLAVATA (CLV)* and *WUSCHEL (WUS)* genes. This *WUS/CLV* regulatory module is linked to the organism-wide cytokinin signaling system by direct transcriptional control of A-type *ARR* genes by *WUS* (Leibfried et al., 2005).

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However, the differences in the adventitious caulogenesis models found between two evolutionary distant genera, i.e. *Arabidopsis* (angiosperms) and *Pinus* (gymnosperms), call for a specific analysis of the latter. In a previous study, we identified a type-A RR in *Pinus pinea* (*PipiRR1*; GenBank: FJ717710; Cortizo et al., 2010). *PipiRR1* was upregulated in *P. pinea* cotyledons after exposure to BA, suggesting that this gene could play a crucial role in adventitious meristem formation in pines. The predicted protein showed that the structural features observed in other plant type-A RRs are at least partially conserved in conifers.

Given the key role played by cytokinins in caulogenesis, in this study we aimed to identify and characterize a type-A response regulator gene in *P. pinaster* during caulogenic induction in cotyledons after exposure to BA. Using *PipiRR1* as a reference, we obtained the full coding sequence, the genomic sequence and a promoter fragment of a type-A RR in *P. pinaster*. We studied its expression during the first stages of adventitious bud formation and its promoter activity in *P. pinaster* using *Agrobaterium*-mediated genetic transformation.

Materials and methods

Cloning and characterization of a type-A RR in Pinus pinaster

Plant material and culture conditions

Mature seeds from open-pollinated *P. pinaster* Ait. trees (Atlantic provenance) were treated according to Álvarez et al. (2009a).

RNA extraction and cDNA synthesis

Tissue was ground in liquid nitrogen and RNA was extracted using the Nucleospin RNA plant kit (Macherey-Nagel, Germany) following the manufacturer's instructions. The RNA concentration was quantified using a Picodrop microliter spectrophotometer (Picodrop Limited, United Kingdom) and the RNA integrity was tested in a 1% agarose gel. One microgram of total RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems Inc., Foster City, CA, USA) following the manufacturer's protocol.

Molecular cloning and sequence analysis

The *PipiRR1* CDS from *Pinus pinea* L. (GenBank FJ717710; Cortizo et al., 2010) was used to design primers for Rapid Amplification of cDNA Ends (RACE). RACE was performed using the FirstChoice® RLM-RACE kit (Ambion, Applied Biosystems Inc.) following the manufacturer's instructions, using SuperTaq Plus® Taq polymerase for PCR amplification (Ambion, Applied Biosystems Inc.). Resulting bands were gel-extracted with the NucleoSpin EXTRACT II kit (Macherey-Nagel), cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced (at least three clones per band) at the Oviedo University DNA Analysis Facility (Spain). After obtaining the full coding sequence and UTRs, primers were designed to amplify the genomic sequence. DNA from *P. pinaster* embryos was extracted using the NucleoSpin® Plant II kit (Macherey-Nagel). The sequence was called *PipsRR1*. The putative protein sequence encoded was analyzed using the InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and Prosite (http://prosite.expasy.org/) tools. Similarity searches in the National Center for Biotechnology Information (NCBI) public database were performed with the BLASTX program (Altschul et al., 1997). Multiple sequence alignment was performed with the CLUSTALW software (Thompson et al., 1994). Sequence annotations were performed with the Geneious software (Biomatters Ltd., New Zealand). An unrooted amino acid sequence similarity tree with the receiver domain of PipsRR1, PipiRR1, all *Arabidopsis* type-A RRs and all rice (*Oryza sativa*) type-A RRs was generated by the UPGMA method and the Jukes-Cantor genetic distance model using the Geneious software. The tree was re-sampled by the bootstrap method.

Quantitative RT-PCR

To examine *PipsRR1* expression, excised cotyledons were harvested at 0 h and after 12 h, 1, 2, 4 and 6 days of culture on TE medium (Tang et al., 1998) or bud induction medium (TEB), consisting of TE plus 10 μM BA. Batches of 100 mg of random cotyledons from each treatment were frozen in liquid nitrogen and stored at −80 °C until use. RNA extraction and cDNA synthesis were performed as indicated above.

The gene expression analysis was performed by real-time PCR with an ABI PRISM 7900HT instrument (Applied Biosystems Inc.) and the Fast SYBR® Green Master Mix (Applied Biosystems Inc.). Approximately 10 ng cDNA were used per well. Reactions (10 μL) were performed using the following standard protocol: 95 °C 20 s; 45 cycles of 95 °C 1 s and 60 °C 20 s. Real-time PCR specificity was assessed using negative controls (no template), RT-control (non-retrotranscribed RNA), a melting curve analysis and by gel electrophoresis of a group of selected reactions. Three biological and 2 technical replicates were used for analysis. Primers for *PipsRR1* were designed with Primer3 software (Rozen and Skaletsky, 2000) to amplify a 101 bp fragment of the target cDNA. The relative expression ratio of the cotyledons treated with BA compared to the controls for each time tested was computed by the comparative Ct method (Livak and Schmittgen, 2001), incorporating the mean PCR efficiencies and fluorescence group threshold values as described in Ruijter et al. (2009). The expression of *PipsRR1* was normalized to the mean value of expression of two reference genes in each sample. Primers for reference genes were designed from GenBank sequences AF461687 and AY172979 (Supplementary data 1) (Alonso et al., 2007). Results are expressed as mean expression ratios ± standard error of three biological and two technical replicates.

Binary vector construction and genetic transformation mediated by Agrobacterium tumefaciens

P. pinaster RR1 promoter cloning and vector construction

The upstream PipsRR1 sequence was obtained using the GenomeWalker kit (Clontech Laboratories Inc., CA, USA), following the manufacturer's instructions. The PCR products obtained were sequenced and aligned with the *PipsRR1* sequence. The upstream sequence was verified by performing a PCR with upstream sequence- and gene-specific primers, and a band of the expected length was obtained. A fragment of 1568 bp upstream of the start codon including 97 bp of the 5′UTR was cloned into a Gateway® pENTR/D TOPO vector (Gateway® Recombination Cloning, Invitrogen, Life technologies Corporation, CA, USA) and introduced by *att* site LR recombination into the pKGWFS7.0 destination vector (Karimi et al., 2002). The resulting vector was called pPipsRR1-GFP:GUS and carried a T-DNA 6491 bp in length, containing a *PipsRR1* promoter fragment driving the expression of a *GFP:GUS* fusion and the *nptII* kanamicyn resistance gene under the regulation of the *nos* promoter (Supplementary data 2).

Plant material

Embryogenic mass was induced according to Lelu-Walter et al. (2006), but using WV5 salts as induction medium (Coke, 1996). The embryogenic line P5LV41 was selected for transformation due to its high growth rate and good maturation capacity.

Transformation procedure

The disarmed *A. tumefaciens* strain AGL1 (Lazo et al., 1991) was selected based on previous experiments (data not shown) for genetic transformation. The binary vector pPipsRR1-GFP:GUS was