TWO *SCARECROW-LIKE* GENES FROM DISTANTLY-RELATED FOREST SPECIES ARE INDUCED IN RESPONSE TO EXOGENOUS AUXIN IN ROOTING-COMPETENT CUTTINGS

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Running Head: Scarecrow-like gene expression during adventitious rooting

Abbreviations: Ct, Threshold cycle; IBA, indol-3-butyric acid; QRT-PCR, quantitative reverse transcription-PCR; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR; SCL, Scarecrow-like; SCR, Scarecrow; SHR, Short-Root

ABSTRACT

This paper reports for the first time the identification of *SCARECROW-LIKE* genes from two distantly-related forest species, *Pinus radiata* and *Castanea sativa*, which are auxin-induced in rooting-competent cuttings before the activation of cell division that gives rise to adventitious root formation. The predicted protein sequences contain domains characteristic of the GRAS protein family, and show a strong similarity to the SCARECROW-LIKE proteins, which indicates conserved functions of these proteins. Quantitative RT-PCR analysis shows that these genes are expressed at relatively high levels in roots. In addition, an induction of the mRNA levels in rooting-competent cuttings from *P. radiata* and *C. sativa* in response to exogenous auxin is observed within the first 24 h of the root induction process, a time when cell reorganization takes place, prior to the resumption of cell division and to the appearance of adventitious root primordia. These results suggest that *SCARECROW-LIKE* genes may play a role at very early stages of adventitious root formation.

Key words: vegetative propagation, pine, chestnut, root meristem

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INTRODUCTION

Adventitious rooting is a postembryonic organogenic process in which root meristems are organized, in general from differentiated cells, at positions where roots are not originated in normal development. Adventitious root formation can be induced in intact plants in some species, or on stem cuttings by a regulated process of cell proliferation and redifferentiation of new cells into root initials. The ability to form adventitious roots from stem cuttings is a key step for propagation of selected adult trees. However, in many forest species, the regeneration capacity of adult cells is limited, especially at maturation, an age-related developmental process that also affects morphology and growth rate (Díaz-Sala et al., 1990; Sánchez and Vieitez, 1991; Greenwood and Hutchison, 1993; Day et al., 2002). The rate and extent of rooting capacity is species-dependent. In *Larix laricinia*, for example, the frequency of cuttings that root declines by 50% during the course of 20 years (Greenwood et al., 1989); in contrast, loss of rooting ability occurs abruptly and early in *Pinus taeda* (1- to 2- year-old plants) (Greenwood and Weir, 1995). The rapid decline in rooting ability in *P. taeda* stem cuttings provided the opportunity to develop experimental systems for the study of adventitious rooting in pine species. Díaz-Sala et al. (1996) showed that whereas hypocotyl cuttings from 20- or 50- day-old seedlings rapidly form roots in the presence of exogenous auxin, epicotyl cuttings from 50-day-old seedlings root poorly. The difference in the rooting capacity of hypocotyl and epicotyl cuttings was also observed in *Pinus radiata* (Díaz-Sala, unpublished). In *Castanea sativa*, the rooting capacity of cuttings and *in vitro* shoot cultures is also closely related to juvenility. Chestnut cuttings from juvenile plants (3 to 6.5 month-old plants) and shoot cultures derived from juvenile-like material form roots easily (rooting rate over 90%), whereas cuttings from mature chestnut trees, as well as mature shoot derived

cultures, are very difficult to root (rooting rate under 12%) (Vieitez, 1974; Vieitez et al., 1987; Sánchez and Vieitez, 1991). The similar rooting response of the *Pinus* and *Castanea* systems makes them suitable for the study of the mechanisms involved in the organization of adventitious root meristems in stem cuttings from distantly-related forest species.

The mechanisms underlying rooting ability in forest species are unknown. Auxins, such as indol-3-butyric acid (IBA), or 1-naphtalenacetic acid (NAA), have been shown to be effective inducers of adventitious root formation in many forest tree species (Grönroos and Von Arnold, 1985; Selby et al., 1992; Díaz- Sala et al., 1996; Goldfarb et al., 1998; Ballester et al., 1999). While auxins are required to induce roots, they do not seem to be the limiting factor for the ability to form adventitious roots. Changes in auxin uptake, metabolism, transport and distribution do not correlate with differences in the capacity of cells to form adventitious roots in *Pinus sylvestris, P. taeda, C. sativa* or *Quercus robur* (Grönroos and Von Arnold, 1988; Díaz- Sala et al., 1996; Ballester et al., 1999; Vidal et al., 2003). In *P. taeda*, cells competent to form adventitious roots are confined to the vascular parenchyma region of the hypocotyl, centrifugal to the resin canal (Díaz-Sala et al., 1996). In *C. sativa*, root primordia are initiated directly from cambial derivates in shoot cultures derived from both juvenile and basal sprouts (Ballester et al., 1999). In both *P. taeda* and *C. sativa*, histological analysis in rooting-competent and –incompetent cuttings during auxin treatment have shown that cells form roots in competent cuttings, and are also sensitive to auxin in rooting-incompetent cuttings - they show cell division but are unable to undergo the organized cell divisions required for adventitious root initiation (Díaz-Sala et al., 1996; Ballester et al., 1999). Goldfarb et al. (1998) have concluded that the regulation of auxin-induced cell division may be a separate process from that of root meristem organization. These observations suggest that the loss of rooting ability is a result of a loss of cellular capacity to induce adventitious roots by auxin. It

is not known whether this is due to the loss of a specific cell type, the inability of individual cells to perceive auxin signals that are specific for root meristem organization, or the suppression of gene expression needed for cells to enter the root-formation pathway (Hutchison et al., 1999). Although genes related to the induction of adventitious roots in forest species have been described (Hutchison et al., 1999; Lindroth et al. 2001 a, b; Goldfarb et al. 2003; Gil et al., 2003; Busov et al., 2004), there is little understanding about how root meristems are organized in stem cuttings during adventitious root formation.

In an attempt to obtain information from genes expressed during adventitious root formation, we have identified expressed sequence tags (ESTs) associated to adventitious rooting by sequencing cDNA subtractive libraries from rooting-competent cuttings under induction conditions of *P. radiata* and *C. sativa*. One clone from *C. sativa* and another from *P. radiata* show homology with the *SCARECROW* gene, which is involved in the establishment of root meristem cell identity in *Arabidospis.* Scarecrow (SCR) is a putative transcription factor that belongs to the group of GRAS proteins that are expressed in the cortex/endodermis initials, and it is required for the asymmetric cell division that gives rise to the cortex and endodermis, and to other tissues in aerial organs of *Arabidopsis thaliana* (Di Laurenzio et al., 1996; Wysocka-Diller et al., 2000; Heidstra et al., 2004). *Arabidospis SCR* (*AtSCR*) is also involved in the establishment of quiescent center identity, and in the maintenance of the stem cell status of the surrounding initial cells during the embryonic pattern formation and postembryonary development (Sabatini et al., 2003), and its expression is associated to the polar auxin transport and the auxin distribution in the root apical meristem (Di Laurenzio et al., 1996; Sabatini et al., 1999). On the other hand, *AtSCR* plays a pivotal role in the regeneration of root tip after laser ablation (Xu et al., 2006). Orthologous genes to *AtSCR* have been described in maize (*ZmSCR*), rice *(OsSCR*) and pea (*PsSCR*) (Lim et al., 2000, 2005; Kamiya et al., 2003;

Sassa et al., 2001). Expression of *ZmSCR* has also been associated with the regeneration of the root tip after whole or partial excision (Lim et al., 2000). In addition to its role in the establishment of root apical meristem identity, in *Arabidopsis*, Malamy and Benfey (1997) described that the *SCR* reporter line END 199 expresses GUS activity in the central cells of the outer layer after the first round of cell division of the pericycle cells during lateral root formation, which suggests that *SCR* may play an important role in the organization of lateral root primordia at very early stages. Recently, Konishi and Sugiyama (2006) have described that the END 199 line also expresses GUS in association with the auxin-induced initiation of adventitious root primordia from pericycle cells in excised hypocotyls from young seedlings at a very early stage of the root primordia organization, when the initiation of cell division is observed. However, the role of *SCR* genes in root meristem organization and root regeneration from cells at positions other than the root tip, such as stem cuttings, is unkown in *Arabidopsis* and other plant species.

In this paper we show for the first time the characterization of two *SCARECROW-LIKE* genes from *P. radiata* (pine) and *C. sativa* (chestnut), which are induced in rooting-competent cuttings in response to exogenous auxin during the early stages of adventitious root formation.

MATERIAL AND METHODS

Plant material

Pine (*Pinus radiata* L.) seeds were provided by Oihanberri (Vitoria, Spain). Seed were germinated and seedlings were grown as previously described (Díaz-Sala et al., 1996), except for the light/dark temperatures that were 25 ºC and 20 ºC, respectively.

Stock shoot cultures of chestnut (*Castanea sativa* Mill) were initiated from basal sprouts of an 80-year-old tree (clone P2) and maintained *in vitro* for more than 15 years as described by

Sánchez and Vieitez (1991). Proliferating shoot cultures were maintained in Gresshoff and Doy (1972) (GD) medium supplemented with 0.44 μM 6-benzyladenine (BA), 30 $g.l^{-1}$ sucrose and 0.7% Bacto Difco Agar. At the end of the 4-week multiplication cycle, newly developed 2.5 to 3-cm-long shoots were harvested for subculture, rooting or RNA isolation.

Adventitious root induction

In pine, root induction was performed as described by Díaz-Sala (1996). Briefly, hypocotyls cuttings from 21-day-old pine seedlings including the intact epicotyl were used for rooting assays. Cuttings 3.5 cm long were prepared by severing the hypocotyl at its base and trimming it to a lenght of 2.5 cm from the cotyledons. Root induction was conducted by exposing the cuttings to 10 μM indole-3-butyric-acid (IBA) continuosly. Cuttings without IBA treatment were used as controls. IBA was obtained from Sigma (St. Louis, MO, USA) as IBA-K and dissolved in distilled water. Conditions for root induction were the same described for seedling growth.

In chestnut, root induction was performed as described by Ballester et al. (1999). Briefly, the basal end of the shoots was dipped in a 4.9 mM solution of IBA for 1 min, and subsequently the shoots were transferred to 300 ml jars, each containing 60 ml of auxin-free medium (GD with 1/3 strength macronutrients). Shoots without IBA treatment were used as controls.

RNA extraction, quantification and cDNA synthesis

Pine total RNA was extracted from the bottom 1 cm segment of the hypocotyl cuttings. Segments from 30 cuttings were pooled for each treatment and time point as it is specified in each experiment, immediately frozen in liquid nitrogen and stored at -70º C until used for RNA isolation. Total RNA was extracted using the Plant Concert RNA Reagent (Invitrogen

Corporation, Carlsbad, CA, USA) following the manufacturer´s instructions. RNA was also extracted from different organs of plant seedlings as it is specified in each experiment.

Basal segments (1 cm) were collected from 20 chestnut microshoots for each treatment and time point as it is specified in each experiment, immediately frozen in liquid nitrogen and stored at -70º C until used for RNA isolation. Chestnut total RNA was isolated as described by Chang et al. (1993). RNA was also extracted from different organs of *in vitro* microshoots as it is specified in each experiment. RNA (4 μg) was digested with RQ1 RNase-free DNaseI (Promega, Madison, WI, USA) according to the manufacturer´s instructions. Digestion was performed for 1.5 h at 37º C, and then purified using the Microcon YM-100 (Millipore Corporation, Billerica, MA, USA). RNA concentration was measured in spectrophotometer, and RNA integrity was checked in a 1% (w/v) agarose gel prior to and after DNaseI digestion. RNA from pine and chestnut was prepared from at least three biological repetitions. cDNA synthesis was performed using 1 μg of total RNA. For quantitative RT-PCR, RT reaction was performed using 200 ng random primers with SuperScript™II reverse transcriptase (Invitrogene Corporation, Carlsbad, CA, USA) according to the manufacturer´s instructions. For 5´ and 3´-RACE, a modified oligodT and PowerScript Reverse Transcriptase (Clontech, BD Bioscience Clontech, Palo Alto, CA, USA) was used according to the instructions of the SMART-RACE cDNA amplification kit (Clontech).

5´- and 3´-Rapid amplification of cDNA ends (RACE)

Near full-length cDNA clones were constructed in a two-step procedure. We carried out 3´- and 5´- cDNA amplification using *PrSCL1* (*Pinus radiata SCARECROW-LIKE* gene1) and *CsSCL1* (*Castanea sativa SCARECROW-LIKE* gene1) specific primers designed based on the sequence of pine and chestnut ESTs obtained in our lab (unpublished). 3´- and 5´-RACE were

performed with the SMART-RACE cDNA amplification kit (Clontech, BD Bioscience Clontech, Palo Alto, CA, USA) following the manufacturer´s instructions. Two independent RNA extractions were used as templates for reverse transcription. For 3´- RACE, reverse transcription was carried out in the presence of a modified oligo(dT) primer and, for the 5´-RACE the reaction was performed with the modified oligo(dT) primer and the SMART II primer (see list of primers below). Double stranded cDNA was synthesized by PCR amplification using the Advantage Taq Polymerase (Clontech) in the presence of UPM and forward and reverse *PrSCL1* or *CsSCL1* specific primers (see list of primers below). Cycling conditions were: initial denaturation at 94º C for 5 min, then 94º C for 30 s; 66º C for 30 s; and 72º C for 2 min for 40 cycles. After a final extension at 72º C for 10 min, the reactions were cooled to 4º C. The reaction products were purified from agarose gels using the Quiaquick Gel Extraction Kit (Quiagen Inc., Germany), and cloned using the TA Cloning kit from Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA). DNA was sequenced using the ABI-PRISM® 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Clones from each experiment and species were sequenced at least twice. Nucleotide sequences were loaded into the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) for BLAST-X search. Each sequence was also compared (BLAST-N) to loblolly pine or poplar ESTs available at the TIGR (www.tigr.org) database.

Primers:

SMART II 5´-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3´

UPM 5´-CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT-3´

Pine

PrSCL1For 5´- TCTGGCAAATCGTCCTGGTGGT-3´

PrSCL1Rev 5′- CGAGGATTCATGCTCCGAATCTT-3′

Chestnut

CsSCL1For 5′- GTGCATTCCGGATGAAGAACCTAC-3′

CsSCL1Rev 5′- GTGCTTCTCGGAACCTCGTAAGAA-3′.

Phylogenetic analysis

The conserved C-terminal region of PrSCL1 (*Pinus radiata* SCARECROW-LIKE protein1), CsSCL1 (*Castanea sativa* SCARECROW-LIKE protein1) and other GRAS proteins was used for phylogenetic analysis as previously described (Bolle, 2004; Lim et al., 2005). The polypeptides were aligned with ClustalW, and subsequent analyses were done using programs from the PHYLIP package (Phylogeny Interference Package, version 3.6a3, Department of Genetics, University of Washington, Seattle). Bootstrap analysis was performed with SEQBOOT and generated 1000 replicates that were used to obtain a set of distance matrices with PROTDIST using the Dayhoff PAMmatrix algorithm. A set of unrooted trees was generated by the neighbor-joining method using NEIGHBOR, and a consensus tree was obtained with CONSENSE. A putative SCL encoded by a *Physcomitrella patens* EST (Nishiyama et al., 2003) was used as an outgroup. The tree was drawn using Phylodendron (D.G. Gilbert, University of Indiana, version 0.8d).

Quantitative RT-PCR (qRT-PCR)

RNA extraction, quantification and cDNA synthesis were as previously described. Primers for qRT-PCR were designed using Primer Express version 2.0.0 (Applied Biosystems) following the standard parameters for primer design. A 18S rRNA gene (*Ri18S*) was used as control. Primers were designed based on the sequence of *Pinus wallichiana* 18S rRNA (gi: 403026),

and confirmed in both pine and chestnut. *PrSCL1* and *CsSCL1* specific primers were designed based on the pine or chestnut RACE sequences (see list of primers below).

PCR efficiency was estimated using template dilutions and the equation $E=10^{(-1/slope)}-1$, as described by Pfaffl (2001). Polymerase chain reactions were performed in an optical 96-well plate with an ABI-PRISM® 7000 Sequence Detection System (Applied Biosystems), using SYBR® Green to monitor dsDNA synthesis. Reactions containing 2xSYBR® Green Master Mix reagent (Applied Biosystems), 12.5 ng cDNA and 200 nM *(Ri18S)* or 400 nM (*PrSCL1* and *CsSCL1*) of primers in a final volume of 25 µl were subjected to the standard thermal profile 50 ºC for 2 min, 95 ºC for 10 min, 40 cycles of 95 ºC for 15 sec and 60 ºC for 1 min. Three technical repetitions from each treatment and time point were subjected to each PCR run. In order to compare data from different PCR runs or cDNA samples, the average of C_T values of the three technical repetitions were normalized to the average of C_T value of *Ri18S*. *SCR* gene expression was normalized to that of *Ri18S* by subtracting the C_T value of *Ri18S* from the C_T value of the *SCR* gene. Expression ratios were then obtained from the equation $2^{\wedge-\Delta\Delta CT}$, where $\Delta\Delta C_T$ represents ΔC_T of *SCR* for each treatment and time point minus ΔC_T of *SCR* at time 0 for rooting time course or specific organs for spatial expression (Applied Biosystems, Technical Bulletin #2). Results are expressed as mean values $\pm$ standard error from at least three biological repetitions.

Primers:

Ribosomal gene

Ri18StrFor 5´-GCGAAAGCATTTGCCAAGG-3´

Ri18StrRev 5´-ATTCCTGGTCGGCATCGT TTA-3´

Pine

PrSCL1trFor 5´-TCAATGTCTGGCAAATCGTCC-3´

PrSCL1trRev 5´-GCGCCCAGTCTCTTCAATTCT-3´

Chestnut

CsSCL1trFor 5´-AGCAGCTCCCTTTGAACCAAG-3´

Cs*SCL1trRev* 5´-GCATAGACAATCCGGCCTTTC-3´

Statistical analysis

Comparison between two groups were done by Student´s *t* test, and comparison among multiple groups were done by ANOVA. Results were considered to be statistically significant at $p \leq 0.05$.

The nucleotide sequence of *PrSCL1* and *CsSCL1* have been deposited in GenBank database under accession numbers DQ683567 and DQ683579, respectively.

RESULTS

***PrSCL1* and *CsSCL1* are members of the GRAS gene family**

To isolate genes with homology to the *Arabidopsis SCR* gene in forest species, we identified several ESTs from pine and chestnut roots as well as from auxin-induced rooting-competent cuttings for which the predicted protein sequence showed a high degree of sequence similarity to SCR. By using 5´- and 3´ rapid amplification of cDNA ends (RACE), two near full-length cDNAs of 3.1 Kb and 2.5 Kb from pine and chestnut, respectively, were identified. The cDNAs contained a predicted protein sequence of 810 amino acids (Fig.1) in pine, and 767 amino acids in chestnut (Fig. 2), designated PrSCL1 (*Pinus radiata* SCARECROW-LIKE protein1) and CsSCL1 (*Castanea sativa* SCARECROW-LIKE protein1). The comparison of the amino acid sequence of the conserved region showed a 60% of identity and 84% of similarity between the two predicted proteins, and the similarity of both predicted protein

sequences with other GRAS proteins extends throughout their entire length (data not shown). Comparison of the amino acid sequence with previously described proteins showed that both contain domains characteristic of the SCARECROW-LIKE proteins, which belong to the GRAS protein family. The analysis of the predicted sequence revealed the presence of the highly conserved VHIID, PFYRE and SAW motifs within the carboxyl-terminal region of both proteins (Fig. 3). Two leucine repeats (LHRI and LHRII) were also identified in the carboxy terminus. In addition, the LXXLL motif and several additional amino acid residues conserved in other known GRAS members of the protein family, such as the RVER or the LRITG motifs, were also identified. The SAW motif contains the pairs of conserved residues RX_4E, WX_7G, $WX_{10}W$. Although typical nuclear localization signal (NLS) or tyrosine phosphorylation motifs were not identified within PrSCL1 or CsSCL1, the BRI and BRII highly basic regions found in LlSCL (*Lilium longifolium* SCL), predicted to serve as the nuclear localizing signal (Moroshasi et al., 2003), were also found in both PrSCL1 and CsSCL1 (Fig. 1, Fig. 2). In the amino-terminal domain, acidic residue-rich regions, similar to those found in LlSCL, were also found in both PrSCL1 and CsSCL1. Stretches of serine were found in PrSCL1 (Fig. 1) and CsSCL1 (Fig. 2).

A phylogenetic analysis was performed in order to analyze evolutionary relationships of PrSCL1 and CsSCL1 with other members of the GRAS family. For comparisons, representatives of different GRAS protein subfamilies (Bolle, 2004), *Arabidopsis thaliana* DELLA proteins (AtGAI and AtRGA), *A. thaliana* AtSCR, *Licopersicon esculentum* LATERAL SUPRESSOR (LsLS), *Petunia hybrida* HAIRY MERISTEM (PhHAM), *A. thaliana* SHORT-ROOT (AtSHR) and LlSCL branches, were selected. The predicted sequence of a putative GRAS protein encoded by a *Physcomitrella patens* EST (Nishiyama et al., 2003) was used as an outgroup. Results indicate that PrSCL1 and CsSCL1 do not

originate a separate branch in the phylogenetic tree of known GRAS proteins. They formed a single clade with the previously described GRAS proteins included in the LlSCL branch, to which they showed the highest similarity (Fig. 4).

PrSCL1 and *CsSCL1* are expressed in roots and aerial organs

To determine the spatial expression of *PrSCL1* and *CsSCL1*, the expression pattern of both genes was analyzed in different organs of pine and chestnut. QRT-PCR was conducted using RNA from roots, shoot apex (including apical meristem and young needles), hypocotyls, and cotyledons from pine 35-day-old seedlings, and RNA from roots, shoot apex (including primary leaves), stems containing 4-5 axillary buds, and shoot internodes from chestnut 30-day-old *in vitro* microshoots. Results are expressed as relative values to the expression in cotyledons (pine) or shoot internodes (chestnut). Both transcripts accumulated to relatively high levels in roots (significant at $p< 0.01$) (Fig. 5A, B). *CsSCL1* was also highly expressed, at equal levels to those detected in roots, in the shoot apex, which was mostly apical meristem and leaf primordia (Fig. 5B). Considerable expression was also detected in pine shoot apex, which was mostly apical meristem and young needles, chestnut stem, which includes axillary buds, and pine hypocotyl (Fig 5A, B). Expression in pine cotyledons and chestnut shoot internodes (without axillary buds) was comparatively lower (Fig. 5A, B).

PrSCL1 and *CsSCL1* are induced in the presence of exogenous auxin in rooting-competent cuttings

Adventitious root formation in pine and chestnut is dependent on the application of exogenous auxin (Sánchez and Vieitez, 1991; Díaz-Sala et al., 1996). In order to approach the functional role of *PrSCL1* and *CsSCL1* in adventitious root formation, the expression profile

of both genes in rooting-competent cuttings of pine and chestnut during the early stages of adventitious root formation and their response to the application of exogenous auxin were examined. Quantitative RT-PCR was performed using RNA isolated from hypocotyl cuttings from pine and from basal-sprout microshoots from chestnut treated with IBA. The analysis was performed during the first 48 h of the root induction process, time necessary for root determination (Díaz-Sala et al., 1996, Ballester et al., 1999). Non-treated cuttings were used as controls. RNA was extracted at different times from IBA-treated and –untreated cuttings, and results are expressed as relative values to time 0. *PrSCL1* and *CsSCL1* mRNA levels increased significantly ($p<0.01$) within the first 24 h in response to the application of exogenous auxin (Fig. 6 A, B). A slight increase was also measured in the pine segments in the absence of auxin. The expression was induced as early as 6 h in chestnut and 8 h in pine, peaked at 12 h in chestnut, and extended during 24 h in pine, after which there was a slow decline in *PrSCL1* and *CsSCL1* mRNA levels, indicating a transient response to auxin. Slight differences in the expression levels of *SCL1* genes were detected in chestnut and pine. A higher induction of gene expression in response to auxin, with regard to the controls, was generally detected in chestnut than in pine, with a clear peak at 12 h in chestnut, whereas in pine the mRNA levels were similar at 8, 16 and 24 h. These results indicate that *PrSCL1* and *CsSCL1* are clearly induced in response to exogenous auxin in rooting-competent cuttings during the early stages of adventitious root formation.

DISCUSSION

GRAS proteins are conserved in forest species

In this study we have isolated and characterized for the first time genes encoding members of the GRAS protein family from two distantly-related forest species, that are auxin-induced in

rooting-competent cuttings during the early stages of adventitious root formation. GRAS proteins are a family of putative transcription factors involved in several aspects of plant growth and development ranging from meristem maintenance to hormone and light signalling (Silverstone et al., 1998; Schumacher et al., 1999; Bolle et al., 2000; Stuurman et al., 2002). The deduced amino acid sequence of PrSCL1 and CsSCL1 (Fig. 3) showed a complete conservation at the carboxyl-terminal region with the characteristic features of SCR and other members of the GRAS protein family that point to a potential role as transcriptional activators (Pysh et al., 1999; Bolle, 2004). These features include the basic region, which has been involved in nuclear localization (Morohashi et al., 2003), the leucine-rich repeats and VHIID motifs, involved in protein-protein and DNA-protein interacions (Ellenberger et al., 1992), and the PFYRE and SAW domains. The LXXLL motif, which has been demonstrated to mediate the binding of steroid receptor co-activator complexes to the respective steroid receptor in animal systems (Heery et al., 1997), and other motifs are also conserved. Based on the evolutionary position of the forest species, these results indicate that GRAS proteins are an ancient family of proteins as already pointed out by Nishiyama et al. (2003), who described the detection of ESTs with sequence similarity to GRAS proteins in bryophytes. The first basic region of PrSCL1 and CsSCL1 shares similarity with the BRI basic region of LlSCL (Murohashi et al., 2003), and with the basic region of AtSCR (Pysh et al., 1999); however the similarity of the second basic region of PrSCL1 and CsSCL1 is lower when compared to the LlSCL and is not found in AtSCR. These regions could be involved in the intracellular localization of the proteins. Similarly to AtSCR, AtPAT1 (Phytocrome A signal Transduction 1-1) (Bolle et al., 2000), LlSCL or *Brassica napus* SCL (BnSCL) (Gao et al., 2004), the NLS of the RGA protein (Silverstone et al., 1998) was not found in PrSCL1 or CsSCL1. Although the GRAS proteins share highly conserved domains in the carboxy

terminus, the amino-terminal region of members of this family are of varying length and are not similar in sequence. The divergent amino terminus may determine the specificity of function of these regulatory proteins. PrSCL1 and CsSCL1 (Fig. 1, Fig. 2) do not share the DELLA domain of the RGA proteins, which is required for their specific function in the gibberellin response (Itoh et al., 2002). The presence of stretches of serine in both proteins is similar to that found in LlSCL, AtSCR, ZmSCR and PsSCR (Di Laurenzio et al., 1996; Lim et al., 2000, Sassa et al., 2001, Morohashi et al., 2003). However, the characteristic stretches of glutamine, alanine or asparagine found in AtSCR, ZmSCR or PsSCR were not found in PrSCL1 or CsSCL1. The sequenced amino-terminal region of these proteins is rich in acidic residues, and shares homology with the amino terminus of LlSCL (Morohashi et al., 2003). Except for the DELLA domain, the biological function of the amino terminus of the GRAS proteins has not been described (Bolle, 2004). Specific acidic and neutral regions of the amino terminus of LlSCL protein have shown strong activity as transcriptional regulators (Morohashi et al., 2003), as was also observed in yeast cells (Triezenberg, 1995). The similarity of the amino terminus of PrSCL1, CsSCL1 and LlSCL could indicate a similar mechanism of transcriptional activation by the amino terminus that is evolutionally conserved.

It is noteworthy that PrSCL1 and CsSCL1 share a highly similar sequence despite the fact that pine and chestnut are distantly-related species. This result could indicate that both proteins have equivalent functions. *PrSCL1* and *CsSCL1* have been isolated from roots and auxin-induced rooting-competent cuttings, and could be functionally related to root initiation or development as described for SCR proteins (Bolle, 2004). However, an analysis of their evolutionary relationship (Fig.4) with GRAS proteins belonging to different subfamilies (Bolle, 2004; Tian et al., 2004), revealed that PrSCL1 and CsSCL1 are closely related to

LlSCL, a protein expressed in the early stages of microsporogenesis in lily (Morohashi et al., 2003), and several *Arabidopsis* Scarecrow-like proteins (SCLs) of unknown function, such as AtSCL9, AtSCL11, AtSCL14, AtSCL30, AtSCL31, AtSCL33a, or AtSCL33b (Bolle, 2004). Although AtSCL9, AtSCL11 and AtSCL14 are expressed in roots (Psych et al., 1999), indicating a role in some root-related process, the grouping of both PrSCL1 and CsSCL1 is distinct from those proteins previously characterized as being involved in root pattern formation, such as SCR or SHR (Di Laurenzio et al., 1996; Helariutta et al., 2000; Lim et al., 2000; Sassa et al., 2001; Sabatini et al., 2003, Levesque et al., 2006). However, they do not represent a separated branch in the phylogenetic tree of GRAS proteins. Gene duplication has greatly expanded the GRAS gene family. Nearly 30 species have ESTs matching the conserved domains of GRAS proteins. In addition, more than 30 GRAS genes from *Arabidopsis* and more than 50 from rice have been identified (Tian et al., 2004). According to Tian et al. (2004), the lineage-specific groups might represent genes with major diversifications towards species-specific functions, genes that may become highly specialized in a specific function common to different species, or genes lost from other species. For example, *PAT1* functions in the phytocrome signalling in *Arabidopsis* (Bolle et al., 2000), and *CIGR1* and *CIGR2*, which group with *PAT1*, are proposed to act as transcriptional regulators in elicitor-induced defense response in rice (Day et al., 2003). In addition, *BnSCL1*, which groups with *AtSCL15* and *PhHAM*, is regulated by auxin and is functionally related to root development similarly to *SCR* (Gao et al., 2004). Similar results have been described in the close relationship within the DELLA subfamily, which forms a different clade over plant species (Tian et al., 2004).

These results indicate that PrSCL1 and CsSCL1 encode for proteins that belong to the GRAS family. The similarity between PrSCL1 and CsSCL1 in two distantly-related forest species,

their high expression in roots and auxin-induced rooting competent cuttings, and the evolutionary relationships to the GRAS proteins, some of which have been involved in division, auxin signaling and root meristem specification, suggest a role of the identified genes in auxin signaling and regulation of root induction and development.

PrSCL1 and *CsSCL1* are predominantly expressed in roots

Gene expression analysis is an instrument of studying gene function. *PrSCL1* and *CsSCL1* are expressed in both roots and aerial organs, which indicates a potential function of these genes in both types of organs. It is remarkable that the expresion is higher in roots and aerial organs with proliferative meristems or active dividing cells (Fig. 5 A,B), such as the pine hypocotyl which is a root-related organ that had entered secondary growth at the age it was taken for analysis (Díaz-Sala et al., 1996). These results are consistent with the data obtained for *SCL* genes in other plant species. In addition to the expression and the pivotal role of *SCR* genes in root cell identity and patterning (Di Laurenzio et al., 1996), most *SCR*, as well as *SCL*, genes are also expressed in aerial organs (Wysocka-Diller et al., 2000, Sassa et al., 2001, Kamiya et al. 2003, Gao et al., 2004, Lim et al., 2005). Wysocka-Diller et al., (2000) have described expression of *AtSCR* in hypocotyls, shoot apical meristem, leaf primordium, bundle sheath cells of leaves, and inflorescence stems supporting the hypothesis of the role of this gene in asymetric cell divisions. A similar expression profile were found in maize (Lim et al., 2005), *Brassica napus* (Gao et al., 2004), pea (Sassa et al., 2001), or rice (Kamiya et al. 2003). It is remarkable that *PsSCR* (Sassa et al., 2001), similarly to *CsSCL1,* is expressed in upper shoot and root tip at equal levels. However, the gene encoding LlSCL, which shows the highest similarity to PrSCL1 and CsSCL, is barely expressed in vegetative tissues (Moroshashi et al., 2003). The similarities in the gene expression patterns of *PrSCL1* and *CsSCL1* with those

described for *SCR* genes in other plant species suggest that *PrSCL1* and *CsSCL1* might have similar roles to those reported for SCR in other plant species. It is possible that the divergent sequence (Fig. 4), especially in the N-terminal region, could be related to different modes of transcriptional activation that might be specific for the characteristic features of tissues of forest species.

PrSCL1 **and** ***CsSCL1*** **genes are regulated by auxin during the early stages of adventitious root formation in cuttings**

Adventitious root formation in pine and chestnut is dependent on the application of exogenous auxin and, in the case of pine, is also sensitive to NPA inhibition, indicating a role of polar auxin transport in adventitious rooting (Sánchez and Vieitez, 1991; Díaz-Sala et al., 1996; Ballester et al., 1999). *PrSCL1* and *CsSCL1* mRNA levels increase in response to exogenous auxin within the first 24 h of the root induction process (Fig. 6A, B). The differences detected in the expression levels of *PrSCL1* and *CsSCL1* may be a consequence of the different root induction treatment applied to pine and chestnut. The level of expression in the pine segments in the absence of auxin may represent a basal expression level or induction by the endogenous pool of auxin, which accumulates at the base of the cutting. The increase of *PrSCL1* mRNA levels in response to exogenous auxin is not a result of a generalized increase in transcription. For example, expression analysis of a *SHORT-ROOT* gene from pine showed an overall increase in mRNA levels during the course of the experiments, whereas the expression of a gene encoding glyceraldehyde-3-phosphate dehydrogenase C subunit (GapC) from pine (Accesion number BE761936) was not induced by auxin (data not shown). *PrSCL1* and *CsSCL1* expression increases during the first 2 days and is maximal within the first 24 h of the root induction process in pine and chestnut. Cell reorganization was observed in both

species during this time, a time when the rooting process is also sensitive to NPA inhibition in pine and prior to the resumption of cell division (Díaz-Sala et al., 1996; Goldfarb et al., 1998; Ballester et al., 1999; Greenwood et al., 2001). Genes encoding expansin from pine were also associated with adventitious root formation during the first 24 h of the root induction process (Hutchison et al., 1999). The auxin-regulated expresion, and the timing of the *PrSCL1* and *CsSCL1* induction, together with the sequence similarity of both proteins and their expression in roots, suggest a conserved function of the proteins in the auxin signaling associated to adventitious root formation in rooting-competent cuttings between the two distantly-related species. The role of GRAS proteins in auxin signaling and root development has already been described (Bolle, 2004). It is noteworthy that AtSCR, the first GRAS family member identified, is essential to maintain the stem cell status of the root apical meristem and for the asymmetric division associated with the establishment of the radial pattern of the root and other tissues (Di Laurenzio et al., 1996; Wysocka-Diller et al., 2000). *AtSCR* expression pattern is associated with auxin distribution and polar auxin transport, which affect cell division and specification of cell fate, in the root apical meristem (Di Laurenzio et al., 1996; Sabatini et al., 1999, 2003). Studies of homologous genes in maize, rice and pea showed that *ZmSCR*, *OsSCR* and *PsSCR*, as well as many AtSCLs, have expression patterns similar to *AtSCR* in roots (Pysch et al., 1999, Lim et al., 2000; Sassa et al., 2001; Kamiya et al., 2003), and another GRAS gene isolated, *BnSCL1,* is induced by auxin and is predominantly expressed in roots (Gao et al., 2004). On the other hand, *ZmSCR* and *AtSCR* expression has also been involved in root tip regeneration after wounding or laser ablation (Lim et al., 2000; Xu et al., 2006), and *AtSCR* is expressed during the auxin-induced adventitious root formation in excised hypocotyls from young *Arabidopsis* plants (Konishi y Sugiyama, 2006), and during lateral root formation in intact plants (Malamy and Benfey, 1997). These data, in conjunction

with the structural features of the proteins and their high expression in roots, suggest a similar regulation and a putative role for *PrSCL1* and *CsSCL1* in adventitious root formation from stem cuttings. It is remarkable that both genes are expressed before the activation of cell division, which is observed at 48-72 h in pine (Díaz-Sala et al., 1996; Greenwood et al., 2001) and at 24 h in chestnut (Ballester et al., 1999). Assuming that *PrSCL1* and *CsSCL1* have a role in root meristem formation similar to that described for *SCR* genes, these data suggest that early stages of root initials are determined during the first 24 h before the organization of root primordia. A similar assumption was pointed out by Brinker et al (2004) who found a 2.6-fold increase of the *PINHEAD/ZWILLE* expression during the early stages of adventitious root formation in *Pinus contorta.* Rooting competence is ultimately a function of differential expression of genes affecting all phases of root meristem development (Greenwood et al., 2001), and depends specifically on the respecification of adult cells into initials and the establishment of root pattern and polarity. Many genes have been associated to cell reorganization and the onset of cell division during adventitious root formation, such as primary cell wall, cytoskeleton or cell cycle genes (Sánchez et al., 1995, Díaz-Sala et al., 1997, Hutchison et al., 1999, Brinker et al., 2004). These genes could be potential targets of SCR.

The results of this study demonstrate that SCARECROW-LIKE proteins are found in forest species, are highly conserved, and are induced in response to exogenous auxin in rooting-competent cuttings before the activation of cell division that gives rise to adventitious root meristems, suggesting that they may play a role at very early stages of adventitious root formation.

Further studies will be needed to elucidate the biological function of these proteins in adventitious root formation. Because of limitations to conduct reverse genetics analysis for

studies of gene function using the pine or chestnut systems, functional analyses using *PrSCL1* and *CsSCL1* homologs in Arabidopsis could help to define the biological function of these genes in adventitious root formation. The function of most genes encoding Scarecrow-like proteins from *Arabidopsis* is unknown (Pysch et al., 1999). However, the biological role of *SCR* in root meristem identity and root patterning is well studied in the root apical meristem (Di Laurenzio et al., 1996; Wysocka-Diller et al., 2000; Heidstra et al., 2004), and expression data suggest a putative role in adventitious root formation as well (Konishi and Sugiyama, 2006). Studies of the function of *SCR* in adventitious root formation in Arabidospis, and complementation analysis of the *SCR* mutation with *PrSCL1* and *CsSCL1* to confirm if pine and chestnut genes are true functional orthologs of the *SCR* gene, as well as their expression in non-rooting competent cuttings in response to auxin are underway.

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FIGURE LEGENDS

Figure 1.- The nucleotide and deduced amino acid sequence of *PrSCL1* cDNA from pine. Basic amino acid regions are included in open boxes. Acidic amino acids are underlined and stretches of serine are double underlined. The termination codon is marked with an asterisk.

Figure 2.- The nucleotide and deduced amino acid sequence of *CsSCL1* cDNA from chestnut. Basic amino acid regions are included in open boxes. Acidic amino acids are underlined and stretches of serine are double underlined. The termination codon is marked with an asterisk.

Figure 3.- Alignment of *PrSCL1* (DQ683567) and *CsSCL1* (DQ683579) carboxyl-terminal region with *Lilium longiflorum* LlSCl (AB106274), *Arabidopsis thaliana* AtSCR (U62798), *Zea mays* ZmSCR (AF263457), and *Pisum sativum* PsSCR (AB048713). Conserved amino acids are displayed in black and grey boxes. Amino acids displayed in black boxes represent positions where 100% of residues are similar, amino acids displayed in grey boxes represent positions where at least 50% of residues are similar. Characteristic domains of the GRAS proteins are indicated. Conserved domains are double underlined. Characteristic residues of the specific domains are indicated with an asterisk.

Figure 4.- Phylogenetic tree of GRAS proteins from different plant species (accession no. in parentheses): *Oryza sativa* OsSCR (AB180961), *Zea mays* ZmSCR (AF263457), *Cucumis sativus* CusSCR (AJ870306), *Pisum sativum* PsSCR (AB048713), *Arabidopsis thaliana* AtSCR (U62798), *Castanea sativa* CsSCL (DQ683579), *A. thaliana* AtSCL9 (NM_129321),

Pinus radiata PrSCL (DQ683567), *Lilium longiflorum* LlSCL (AB106274), *A. thaliana* AtSCL7 (NM_114925), *A. thaliana* AtGAI (Y11337), *A. thaliana* AtRGL3 (NM_121755), *Lycopersicon esculentum* LsLS (AF098674), *Brassica napus* BnSCL1 (AY664405), *Petunia hybrida* PhHAM (AF481952), *A. thaliana* AtSHR (NM_119928), *Physcomitrella patens* PpSCL (BJ976460). Bootstrap values of 1000 replicates are indicated.

Figure 5.- Expression of *PrSCL1* and *CsSCL1* in different organs. a) QRT-PCR of root, hypocotyl, shoot apex or cotyledon RNAs from pine. One μg RNA was reverse transcribed, and 12.5 ng of cDNA was amplified with 400 nM of *PrSCL1* specific primers. Pine *Ri18S* was used as control. Results are expressed as mean values of relative expression to cotyledons ± SE from at least three biological repetitions. b) QRT-PCR of root, stem, shoot internode or shoot apex RNAs from chestnut. One μg RNA was reverse transcribed, and 12.5 ng of cDNA was amplified with 400 nM of *CsSCL1* specific primers. Chestnut *Ri18S* was used as control. Results are expressed as mean values of relative expression to shoot internodes ± SE from at least three biological repetitions. The increase of gene expression was significant at $p< 0.01$ for both species.

Figure 6.- Expression of *PrSCL1* and *CsSCL1* during adventitious root formation. a) QRT-PCR of hypocotyl RNA from pine. RNA was extracted from the base of hypocotyl cuttings treated with or without 10 μM IBA at the indicated times. One μg RNA was reverse transcribed, and 12.5 ng of cDNA was amplified with 400 nM of *PrSCL1* specific primers. Pine *Ri18S* was used as control. Results are expressed as mean values of relative expression to time 0± SE from at least three biological repetitions. b) QRT-PCR of basal microshoot RNA

from chestnut. RNA was extracted from the base of microshoots treated with or without 4.9 mM IBA for one minute and placed in auxin-free medium for the indicated times. One µg RNA was reverse transcribed, and 12.5 ng of cDNA was amplified with 400 nM of *CsSCL1* specific primers. Chestnut *Ri18S* was used as control. Results are expressed as mean values of relative expression to time 0± SE from at least three biological repetitions. The increase of gene expression within the first 24 h in the presence of IBA at each time was significant at $p < 0.01$ for both species. The increase of gene expression over the 24 h period was significant at $p < 0.01$ for both species. The interaction time x treatment was significant at $p < 0.01$ for both species.

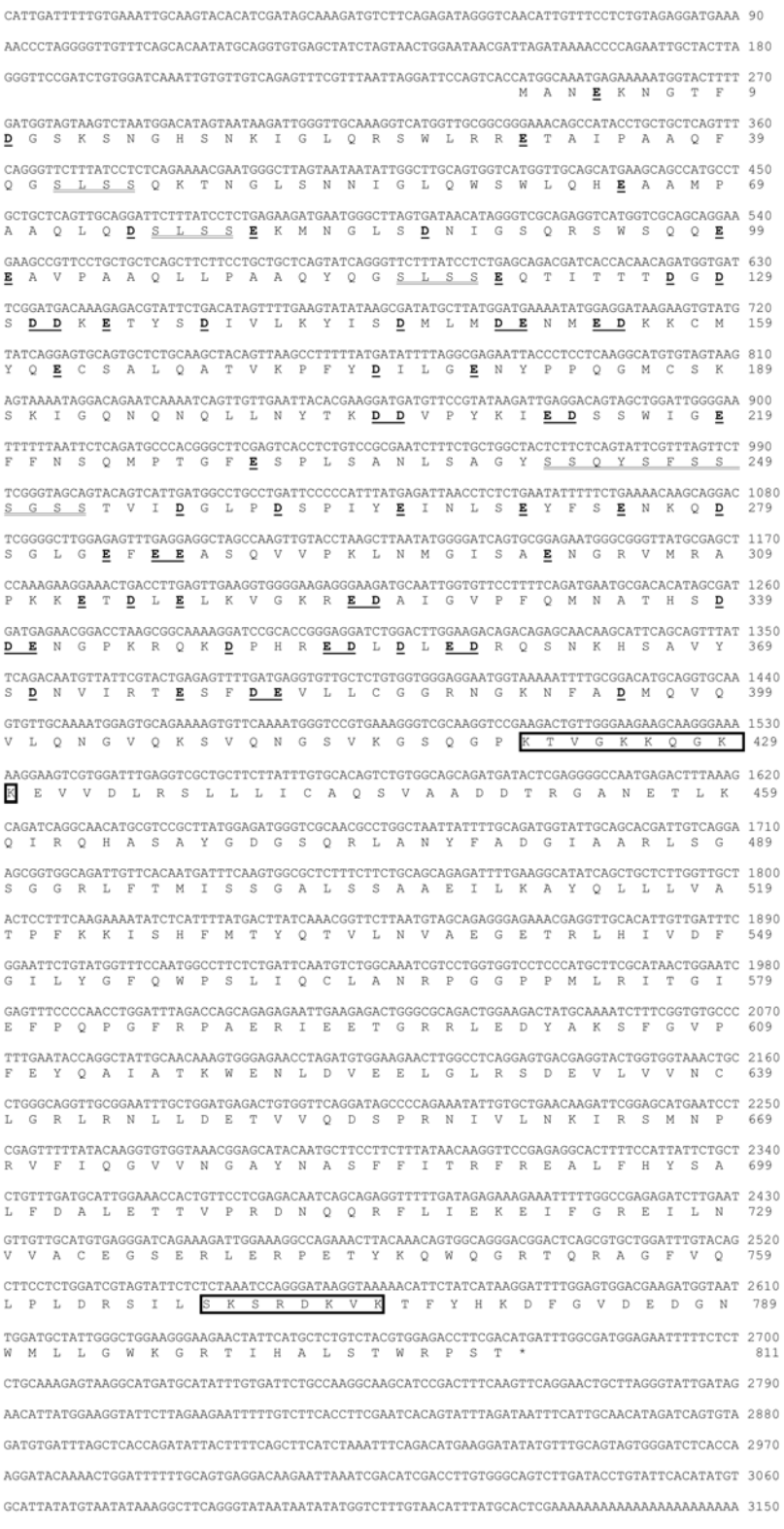


Figure 1 Sánchez et al.

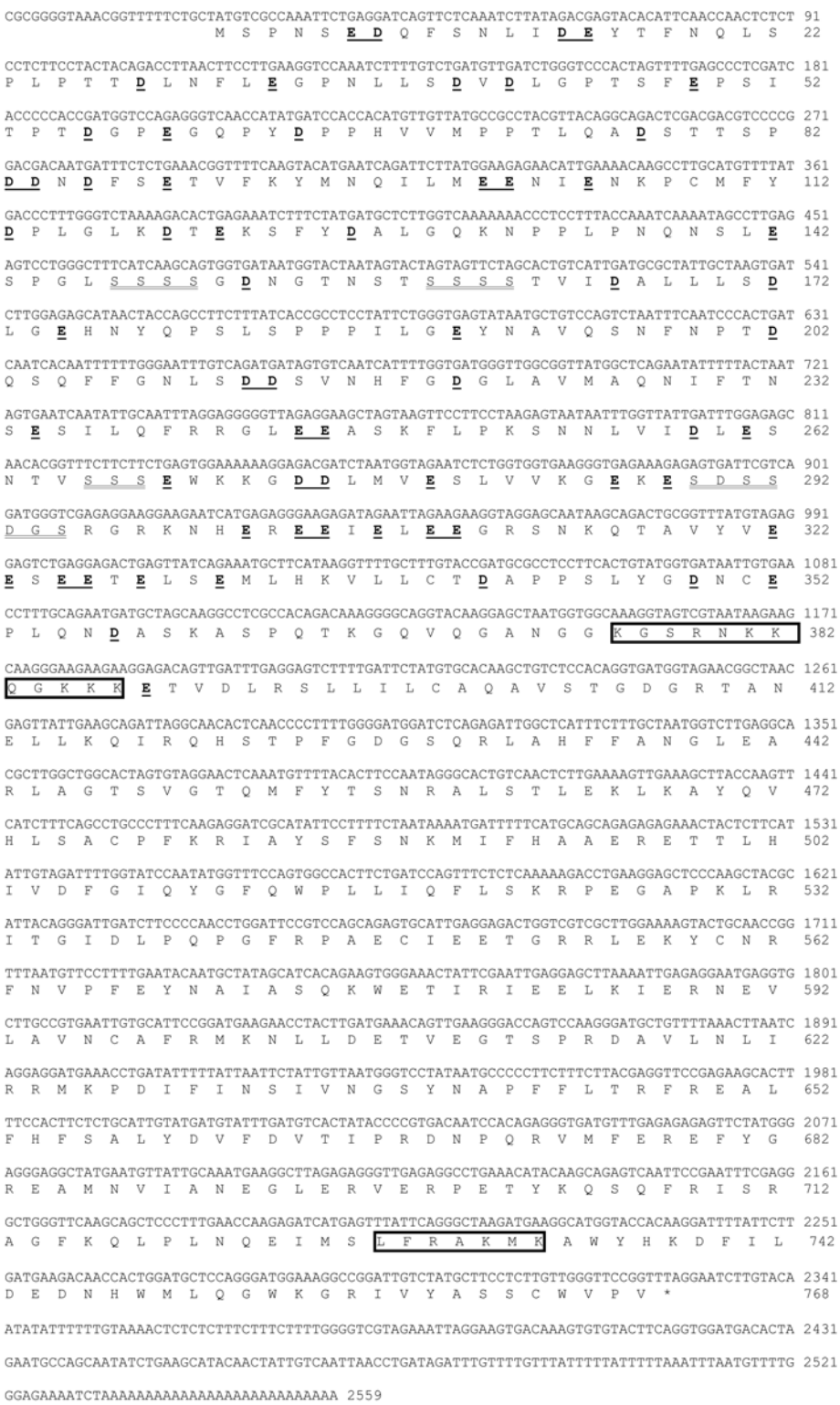


Figure 2 Sánchez et al.

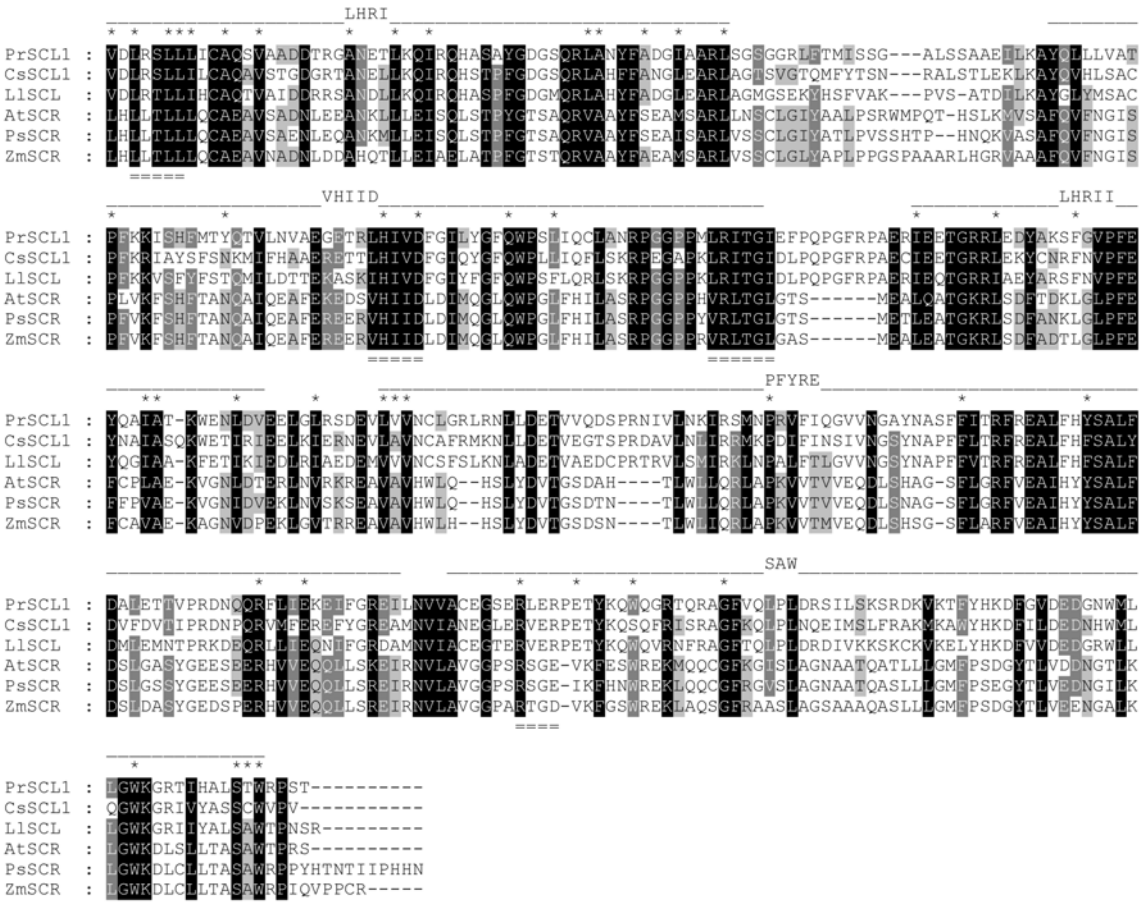


Figure 3 Sánchez et al.

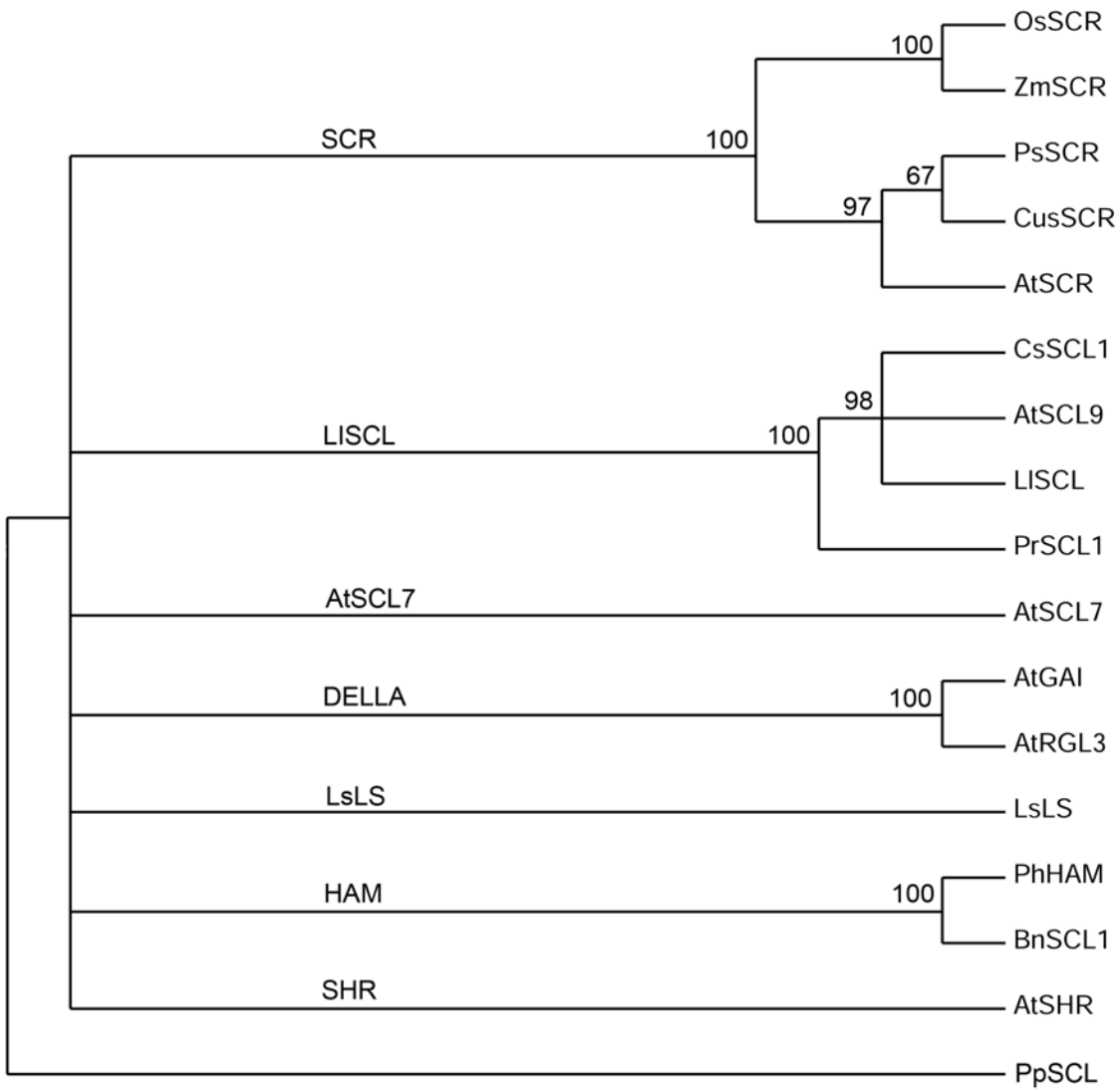
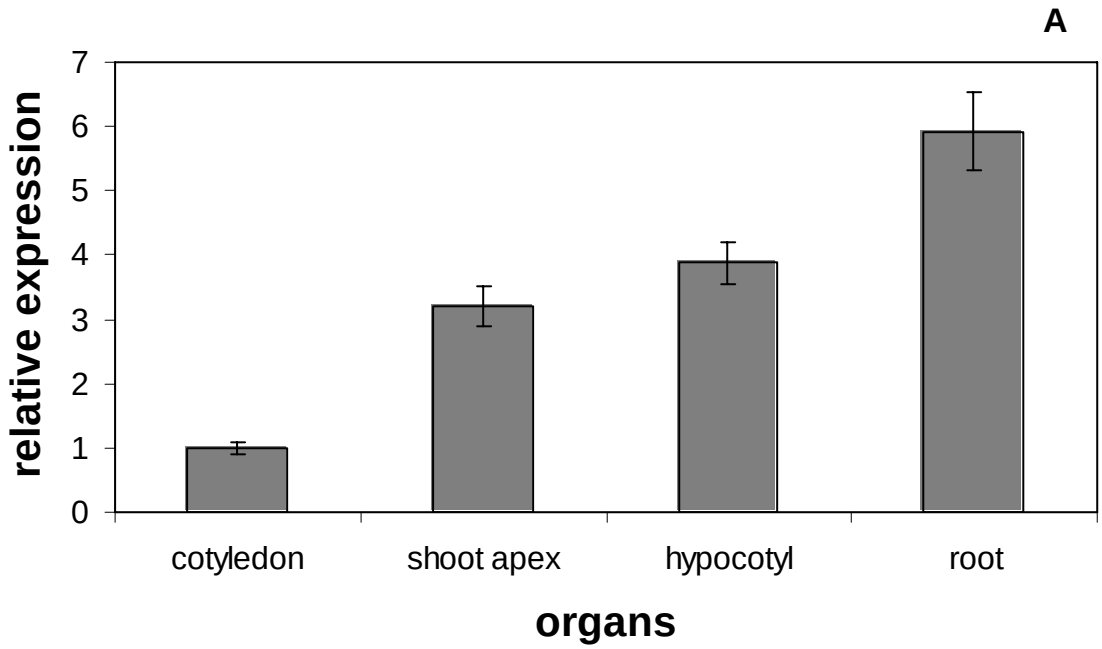


Figure 4 Sánchez et al.



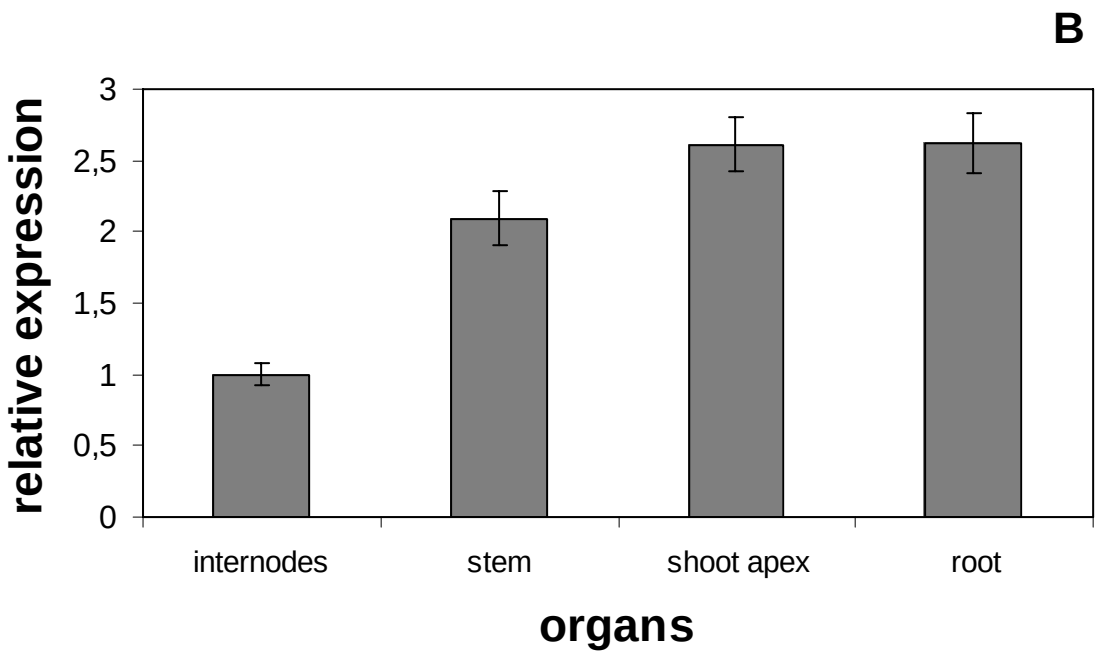


Figure 5 Sánchez et al.

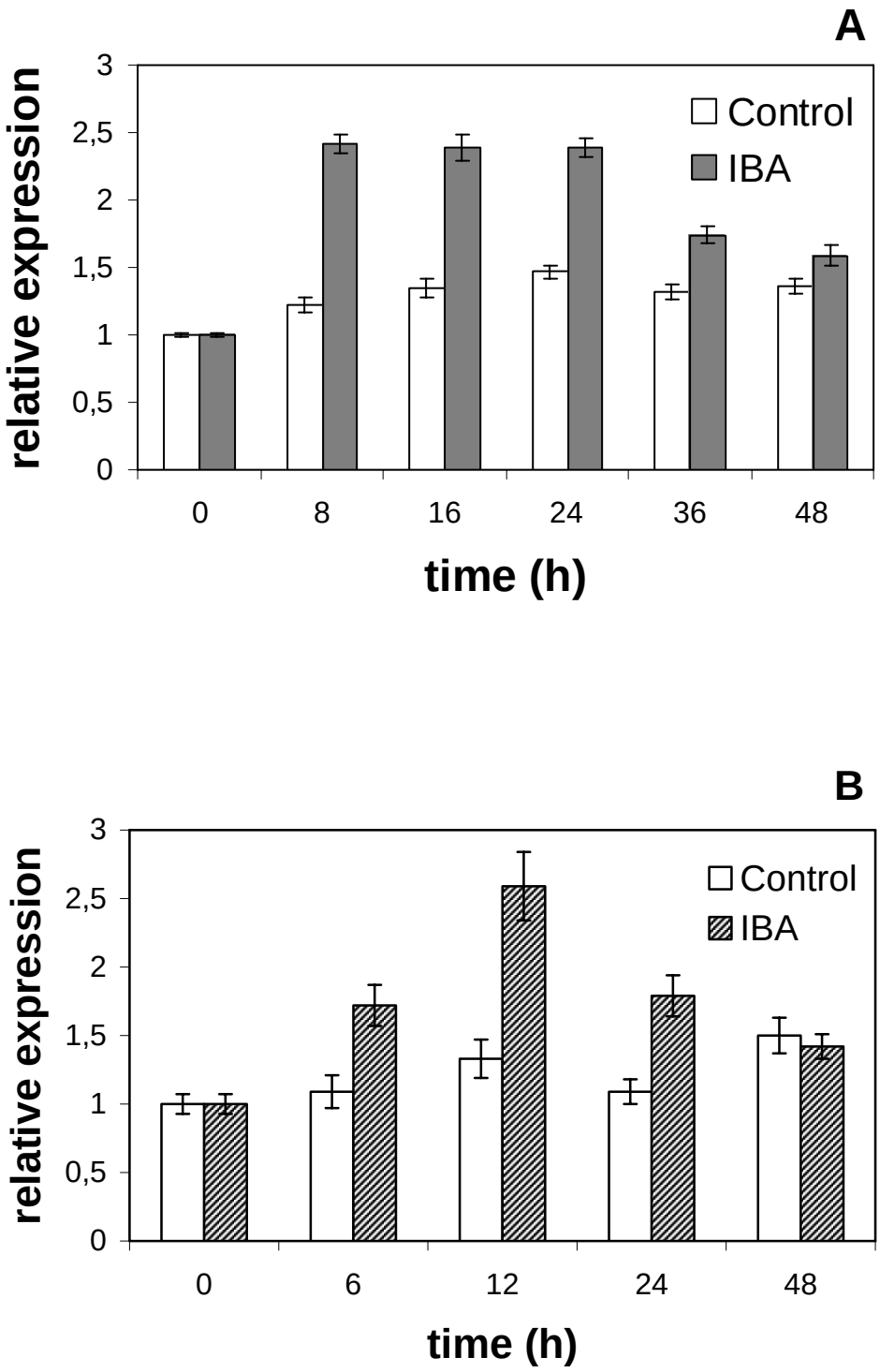


Figure 6 Sánchez et al.