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RESEARCH PAPER

Molecular analysis of *SCARECROW* genes expressed in white lupin cluster roots

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Abstract

The Scarecrow (*SCR*) transcription factor plays a crucial role in root cell radial patterning and is required for maintenance of the quiescent centre and differentiation of the endodermis. In response to phosphorus (P) deficiency, white lupin (*Lupinus albus* L.) root surface area increases some 50-fold to 70-fold due to the development of cluster (proteoid) roots. Previously it was reported that *SCR*-like expressed sequence tags (ESTs) were expressed during early cluster root development. Here the cloning of two white lupin SCR genes, *LaSCR1* and *LaSCR2*, is reported. The predicted amino acid sequences of both *LaSCR* gene products are highly similar to AtSCR and contain C-terminal conserved GRAS family domains. *LaSCR1* and *LaSCR2* transcript accumulation localized to the endodermis of both normal and cluster roots as shown by *in situ* hybridization and gene promoter::reporter staining. Transcript analysis as evaluated by quantitative real-time-PCR (qRT-PCR) and RNA gel hybridization indicated that the two *LaSCR* genes are expressed predominantly in roots. Expression of *LaSCR* genes was not directly responsive to the P status of the plant but was a function of cluster root development. Suppression of *LaSCR1* in transformed roots of lupin and *Medicago* via RNAi (RNA interference) delivered through *Agrobacterium rhizogenes* resulted in decreased root numbers, reflecting the potential role of *LaSCR1* in maintaining root growth in these species. The results suggest that the functional orthologues of *AtSCR* have been characterized.

Key words: Cluster roots, endodermis, interfering RNA, *Lupinus albus*, scarecrow, short roots.

Introduction

White lupin (*Lupinus albus* L.) is considered an ideal legume species in which to assess plant adaptation to phosphorus (P) deficiency (Skene, 2001; Neumann and Martinoia, 2002; Vance *et al.*, 2003; Lambers *et al.*, 2006). In response to P deficiency, white lupin develops specialized roots called cluster or proteoid roots characterized by dense clusters of rootlets with determinate growth (Dinkelaker *et al.*, 1995; Johnson *et al.*, 1996; Watt and Evans, 1999). Cluster roots display several adaptations for acquiring P. They exude copious amounts of citrate and malate into the rhizosphere which increase P availability to the plant by solubilizing P bound to aluminium, iron, and calcium (Dinkelaker *et al.*, 1995; Neumann *et al.*, 1999; Shane and Lambers, 2005). Cluster roots also exude acid phosphatases (Gilbert *et al.*, 2000; Miller *et al.*, 2001) and protons (Marschner *et al.*, 1986; Neumann *et al.*, 1999) which facilitate P availability. The expression of a number of genes related to P acquisition is enhanced in cluster roots, including Pi transporters (Liu *et al.*, 2001), acid phosphatase (Miller *et al.*, 2001), several ion channels and MATEs (multidrug and toxic compound extrusion) (Uhde-Stone *et al.*, 2003*b*), and genes involved in carbon metabolism and signalling (Uhde-Stone *et al.*, 2003*a*; Tesfaye *et al.*, 2007).

A genomics-based approach assessing expressed sequence tags (ESTs) in white lupin cluster roots identified several genes involved in root development (Uhde-Stone *et al.*, 2003*b*). Among the ESTs evaluated, some highly similar to an *Arabidopsis SCARECROW* (*AtSCR*) gene were detected. *SCARECROW* encodes a GRAS transcription factor (Pysh *et al.*, 1999) important in radial cell patterning and stem cell maintenance in *Arabidopsis*, maize (*Zea mays* L.), and rice (*Oryzae sativa* L.) (Scheres *et al.*, 1995; DiLaurenzio *et al.*, 1996; Lim *et al.*, 2000; Wysocka-Diller *et al.*, 2000; Kamiya *et al.*, 2003; Sabatini *et al.*, 2003; Heidstra *et al.*, 2004). In all species examined to date, SCR expression in roots is found in the endodermis and the quiescent centre (QC) (Pysh *et al.*, 1999; Sassa *et al.*, 2001; Kamiya *et al.*, 2003; Sabatini *et al.*, 2003; Laajanen *et al.*, 2007). *Arabidopsis SCR* mutants have reduced root growth and impaired maintenance of the QC stem cell niche (Scheres *et al.*, 1995; Sabatini *et al.*, 2003; Heidstra *et al.*, 2004). Cui *et al.* (2007) have recently reported that *SCR* also functions in the establishment of the root endodermis in *Arabidopsis* and rice. SCARECROW interacts with SHORTROOT (*SHR*), sequestering SHR to the nucleus of endodermal cells and thereby preventing further movement to other root cell layers. When *SCR* is silenced, SHR can move into multiple cell layers which express endodermal markers (Cui *et al.*, 2007). *SCR* expression is not only under transcriptional regulation but is also affected by microRNA (miRNA). Llave *et al.* (2002) demonstrated cleavage of *SCR*-like mRNAs by the miRNA 170 family.

In efforts to understand the molecular regulation of P stress-induced cluster root development in white lupin, it was hypothesized that transcription factors such as *SCR* may be important in cluster root morphogenesis. The overall objective was to characterize one or more root expressed *SCR* genes and evaluate expression of *SCR* genes during development of cluster roots. In work reported here: (i) two white lupin *SCR* genes (*LaSCR1* and *LaSCR2*) that appear to be orthologous to *AtSCR* are cloned; (ii) the expression of the two *LaSCR* genes in P-sufficient and P-deficient plants is analysed; (iii) root cellular expression patterns for *LaSCR* are identified through *in situ* hybridization and *LaSCR* promoter::reporter staining patterns in transgenic roots; and (iv) *LaSCR* expression is impaired via RNA interference (RNAi).

Materials and methods

Plant material and growth conditions

Lupinus albus L. (var. Ultra) plants were grown in the growth chamber in sand culture at 20/15 °C with 16/8 h light/dark cycles (Johnson *et al.*, 1996). The plants were watered every second day with 500 ml of the appropriate nutrient solution (Johnson *et al.*, 1996). The control plants were grown under P-sufficient conditions while the P-stressed plants received 0.5 mM $CaSO_4$ instead of 0.5 mM $Ca(HPO_4)_2$. Tissues were harvested 14 d after emergence (DAE). Normal roots and proteoid roots (divided into zones 1–5) were collected from P-deficient and P-sufficient plants, frozen in liquid nitrogen, and stored at –80 °C until used for RNA extraction.

RNA and DNA gel blot analysis

Lupin total RNA was isolated as previously described by Uhde-Stone *et al.* (2003*a*) for use in northern blot analysis. Total RNA was electrophoresed in an agarose gel system containing formaldehyde, then transferred to Zetaprobe membrane (Bio-Rad). RNA blots were probed using random primed cDNA inserts labelled with [^{32}P]dCTP and washed under high stringency conditions using the formamide protocol as per the manufacturer's instructions (Bio-Rad). Smaller amounts of RNA were isolated for quantitative real-time-PCR (qRT-PCR) using an RNeasy plant mini kit (Qiagen, Valencia, CA, USA). Lupin total genomic DNA (10 μg) was digested with either *Eco*RI or *Hin*dIII and electrophoresed on a 0.8% (w/v) agarose gel. The DNA blot was generated by transfer of the DNA to Immobilon NY+ membrane (Millipore, Bedford MA, USA) and then hybridized at 65 °C and washed under high stringency conditions (65 °C, 0.1× SSC, 0.1% SDS).

Screening of lupin cDNA and genomic libraries

Lupin cDNA libraries were constructed in the excision vector λZAPII as previously described from P-stressed pre-emergent, 7- to 10-day-old or 12- to 14-day-old proteoid root tissues (Miller *et al.*, 2001). BLAST analysis of an EST database generated from these libraries revealed a partial clone encoding an *LaSCR1*. This insert was used to rescreen the 7- to 10-day library to obtain a full-length cDNA clone. Use of the *LaSCR1* cDNA insert to screen an *Eco*RI lupin genomic library described by Liu *et al.* (2001) yielded genomic clones encoding two *SCR* genes, 1 and 2. The genomic *LaSCR2* clone DNA was used to probe the mixed cDNA libraries for the full-length *LaSCR2* cDNA. The genomic clones were subcloned into pBSKS+ (Stratagene, Cedar Creek, TX, USA) for restriction mapping and sequencing. Sequence data for the *LaSCR* sequences can be found in the GenBank data library under accession numbers FJ236985 (*LaSCR1* cDNA), FJ236986 (*LaSCR2* cDNA), FJ236987 (*LaSCR1* genomic), and FJ236988 (*LaSCR2* genomic).

qRT-PCR and reverse transcription PCR (RT-PCR) analysis

Total RNA from three biological replicates of lupin cluster root developmental zones and normal roots was treated with DNase I to remove any contaminating DNA, according to the manufacturers' instructions using the DNA-free kit from Ambion Inc. (Austin, TX, USA). First-strand cDNA was prepared from 2 μg of total RNA with the Superscript RT II enzyme (Invitrogen, Carlsbad, CA, USA) and oligo(dT_{17}) primer for 1 h at 42 °C.

Gene-specific primers for the qRT-PCR analysis were designed after comparison of the two sequences by hand, selecting areas with unique bases at the 3′ end of the primers since the *LaSCR1* and *LaSCR2* cDNA sequences are highly similar, and generating 183 bp and 163 bp products for SCR1 and SCR2, respectively. The SCR1 and SCR2 primer sequences are as follows: SCR1F, 5′-ACACTAGTGTCCCACAGTAG-3′; SCR1R, 5′-AACAGTCCTGGCCATTGAAG-3′; SCR2F, 5′-TTTCCTCGTCGAAGCAGCAG-3′; and SCR2R, 5′-GGGGTTTGGAACAACATGGG-3′. A PCR master mix (iTaq SYBR Green Supermix with ROX, Bio-Rad, Hercules, CA, USA) was mixed with 5 μl of diluted first-strand cDNA as template and 40 pmol of each primer for a final volume of 25 μl per reaction. The ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) was used for the PCR and the detection of the fluorescent signal. Cycle conditions were one cycle for 50 °C for 2 min and one cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The analysis was performed in triplicate. The specificity of the RT-PCR products was confirmed by running a heat dissociation curve at the end of the PCR and agarose gel electrophoresis. Primers specific for lupin tubulin were used as an endogenous control to account for variability in the initial concentration and quality of the total RNA. Gene expression was normalized to the tubulin expression levels for data analysis of each sample. The calibrator sample in real-time PCR was the cDNA from +P normal roots. The threshold cycle (ΔΔCt) method of comparing expression data was applied and the relative quantitative value were expressed as $2^{-\Delta\Delta Ct}$.

RT-PCR analysis was performed on cDNA samples synthesized as described for real-time PCR. The cDNA pools were normalized by PCR amplification of actin. For *LaSCR1*, a 500 bp fragment was amplified using the primers LaSCR1-F, 5′-CAACACTAGTGTCCCACAGTAG-3′ and LaSCR1-R, 5′-GGAATGTTGTAACCAACCAATGGAC-3′. For *LaSCR2*, a 650 bp fragment was amplified using the primers LaSCR2-F, 5′-CTCACTCACCTTGGCTTCTCT-3′ and LaSCR2-R, 5′-CTCAACAGTAGCACCGGTACTAG-3′. The SCR PCRs were run for 27 cycles using an annealing temperature of 55 °C.

Phylogenetic analysis of LaSCR *sequences*

The gene sequences for *LaSCR1* and *2* were blasted against the *Arabidopsis* genome (www.arabidopsis.org) and GenBank non-redundant databases to find 31 *Arabidopsis* gene members of the GRAS family and sequences of *SCR* from five additional plant species. The sequences were aligned using the ClustalW program (www.ebi.ac.uk/clustalw) with the default gap penalties option (Thompson *et al.*, 1997). The phylogenetic analysis was performed using the MAFFT program version 6.

In situ *hybridization*

A 1068 bp fragment of *LaSCR1* cDNA (bp 1224–2292) corresponding to the most conserved domains of the SCR sequence (leucine heptad I and II, VHIID, and an extended PFYRE motif) was PCR amplified, ligated into pGEMTeasy vector (Promega, Madison, WI, USA), then subcloned into pBSSK+ to use for digoxigenin (DIG) labelling. The PCR primer sequences were as follows: SCR-F, 5′-CAATGTGCTGAAGCAGTTTCAGC-3′ and SCR-R, 5′-GGGAGGTTCTCCAGCAGAAAG-3′.

Primary root tips (0.3–0.5 mm lengths) were excised from developing lupin seedlings. Tissue was fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.2. The tissue was dehydrated in a graded ethanol series followed by xylene replacement, and subsequently embedded in paraplast. Embedded tissue was sectioned to a thickness of 10 μM and adhered onto poly-L-lysine-coated slides. DIG-labelled RNA probes were generated in both sense and antisense orientation by *in vitro* transcription using the DIG-11-UTP-labelled nucleotide (Roche Applied Science, Mannheim, Germany). An overnight hybridization of the labelled probe on the tissue sections was incubated at 42 °C with a final wash of 2× SSC at 42 °C. Immunological detection using the DIG Nucleic Acid Detection Kit (Roche Applied Science) was performed according to the manufacturer's description. The anti-DIG antibody was used at a concentration of 1:100 for an overnight incubation. The signal was detected using the alkaline phosphatase substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

Construction of chimeric reporter genes and RNAi plasmid

A 1152 bp or a 1172 bp sequence upstream of the translational start codon corresponding to the promoters of *LaSCR1*or *LaSCR2*, respectively, was PCR amplified from the corresponding lupin genomic clones and inserted into pBI101.2 (Clontech, Palo Alto, CA, USA) in-frame with the ATG start codon of β-glucuronidase (GUS) for plant transformation. The recombinant plasmids were introduced separately into *Agrobacterium rhizogenes* strain A4TC24 (Quandt *et al.*, 1993) for lupin or strain ARqual for *Medicago truncatula* transformation by electroporation. The pBI101.2 promoterless GUS vector was used as a negative control.

A fragment of the 3′ end of the *LaSCR1* cDNA was amplified by PCR using the primers LaSCR1i-F, 5′-AAGCTGTTGCTGTCCATTGG-3′ and *LaSCR*1i-R, CAACCAAGTTTGAGGATGC-3′. The product was introduced into the RNAi-inducing pHellsgate 8 vector (Helliwell *et al.*, 2002) using the GATEWAY system (Invitrogen), creating the *LaSCRi* (*SCRi*) construct. The Hellsgate 8 vector with a fragment of human myosin (*MYOi*) was used as a control. Proper insertion of the PCR products into the vector was verified through sequencing. The *LaSCRi* and the *MYOi* construct were electroporated into *A. rhizogenes* strains A4TC24 and ARqual for transformation into lupin and *M. truncatula*, respectively.

Agrobacterium rhizogenes*-mediated plant transformation and analysis*

Lupin plants were transformed using two procedures, radicle dipping previously described (Uhde-Stone *et al.*, 2005) and hypocotyl injection. For injection, lupin seeds were surface sterilized with 90% ethanol for 10 min followed by several rinses with sterile water. The seeds were then placed in 10% bleach for 10 min followed by several rinses with sterile water then germination in the dark on 0.6% agarose medium. When the emerging radicles reached a length of ~10 mm, the seedlings were transferred to moist vermiculite and grown in trays in the dark at room temperature for 3 d followed by transfer into the light at 4 °C for an additional 2–3 d. The plants were then injected with a syringe (25 gauge needle) filled with a suspension of *A. rhizogenes* harbouring the construct of interest (grown on tryptone-yeast extract medium containing 100 mM acetosyringone and 1% glucose) diluted in 2 ml of phosphate-buffered saline solution. The plants were potted in vermiculite and grown under the same growth chamber conditions described previously. After 3 weeks, the untransformed normal roots (below the injection site) were trimmed off and the plants were planted to the injection site in fresh vermiculite (the transgenic roots were covered) and grown for an additional 2 weeks. The SCRi and MYOi plants were harvested at either 5 or 7 weeks for plants transformed by hypocotyl or radicle inoculation, respectively. Only the hairy roots emerging from the injection site in the case of hypocotyl injection were harvested and analysed. The number of hairy roots emerging from the RNAi transformant plants was counted.

Medicago truncatula A17 or L416 (A17 containing a PRO_{ENOD11}-GUS construct (Journet *et al.*, 2001) seeds were used for the transformation for GUS reporter constructs or RNAi constructs, respectively. The *LaSCRi* DNA sequence was used to search the *M. truncatula* Gene Index (compbio.dfci.harvard.edu/tgi/) for similar sequences, and TC127458 was identified as 80% identical. Specific primers matching a 405 bp fragment were designed from the *Medicago* sequence (MtSCR-F, 5′-CGTAACGTACTTGCAGTTGG-3′ and MtSCR-R, 5′-ACCGTCCGATCTTCATCAAC-3′) to verify silencing of *SCR* by RT-PCR in these transformants. The number of hairy roots emerging from the RNAi transformant plants was determined.

For histochemical GUS activity detection, fresh lupin and *Medicago* transgenic root samples were incubated for various times (see Results) at 37 °C in GUS assay buffer, using 5-bromo-4-chloro-3-indoyl glucuronide as a substrate (Jefferson, 1989).

Results

Cloning of LaSCR *cDNAs*

A white lupin EST clone (GenBank accession no. CA41147) highly similar to *Arabidopsis* SCR (AtSCR) was used to screen a lupin cDNA (Uhde-Stone *et al.*, 2003*a*) and a lupin genomic (Miller *et al.*, 2001) library. Two cDNA clones having high similarity to *AtSCR* were identified and fully sequenced. *LaSCR1* contains a 2581 bp open reading frame (ORF) encoding a deduced polypeptide of 776 amino acids. *LaSCR2* contains a 2579 bp ORF encoding a deduced polypeptide of 770 amino acids. Comparison of the deduced amino acid sequence of LaSCR1 and LaSCR2 with that of AtSCR shows extensive sequence identity over their C-terminal region (from Leu401 to Trp761 for LaSCR1 and from Leu399 to Trp757 for LaSCR2) (Fig. 1). Overall,

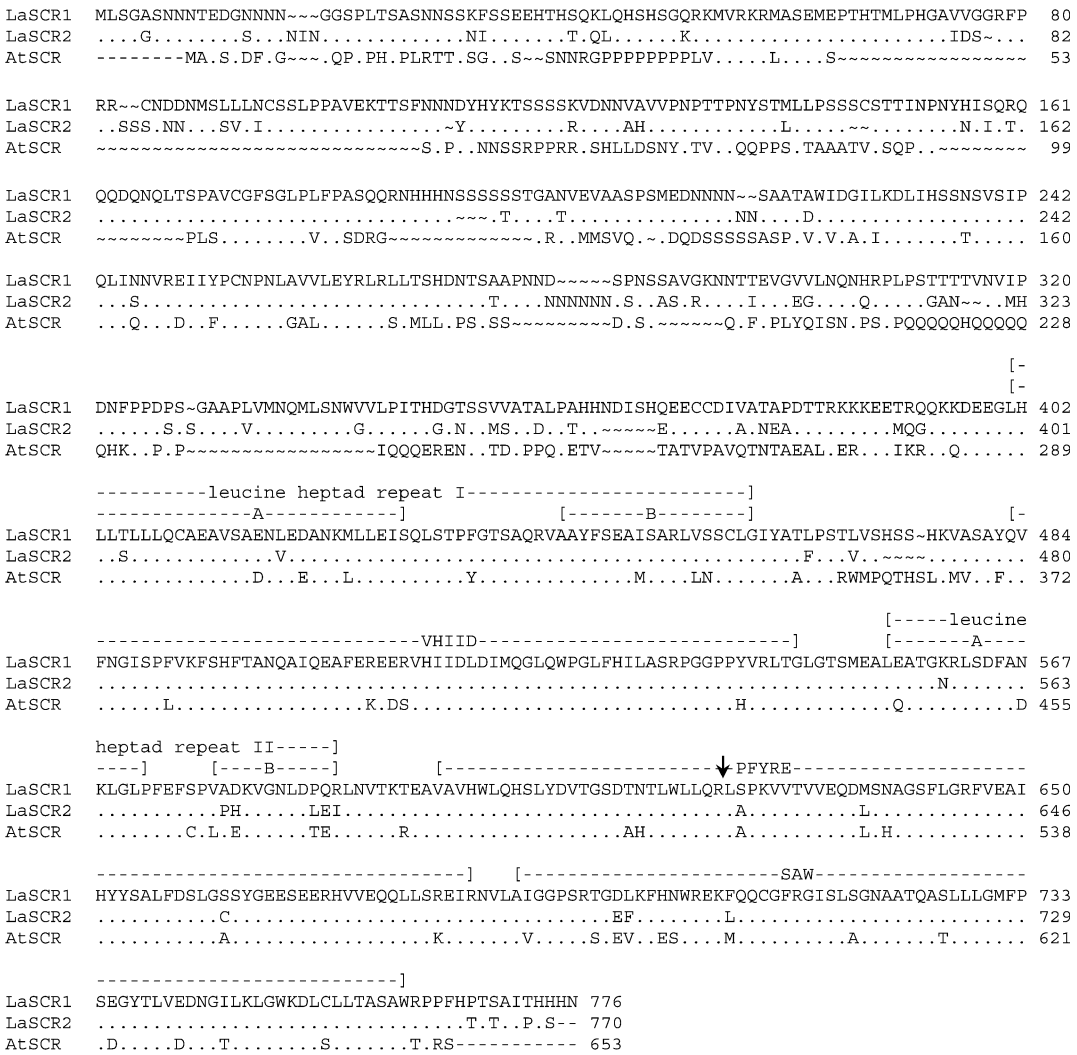

Fig. 1. Comparison of the deduced amino acid sequences of lupin LaSCR1 and LaSCR2 with that of *Arabidopsis* SCR. Alignment of the amino acid sequences of LaSCR1, LaSCR2, and AtSCR using the Clustal W program (http://bioportal.bic.nus.edu.sg/clustalw/clustalw.html). Identical residues are indicated by dots (.). Wavy lines (~) indicate gaps introduced in the sequences to maximize similarity. Amino acid position numbers are indicated to the right of the sequence. The arrowhead between the R-622 and L-623 amino acid residues shows the position of the single conserved intron in the three genes. Broken lines (- -) above amino acid residues show the position of the GRAS protein family elements (leucine heptad repeats I and II, VHIID, PFYRE, and SAW motifs). GenBank accession numbers are as follows: LaSCR1, FJ236985; LaSCR2, FJ236986; AtSCR, At3g54220. Comparative analysis of the motifs between the three amino acid sequences was performed with the PHYLIP 3.66 package using the PRODIST program (http://evolution.genetics.washington.edu/phylip.html).

LaSCR1 and *LaSCR2* share 85% identity throughout their deduced amino acid sequence. The N-terminal domain of *LaSCR2* shows, as compared with *LaSCR1*, 60 amino acid substitutions, 13 amino acid insertions, and 14 amino acid deletions. In the C-terminal domain, *LaSCR1* and *LaSCR2* are more similar; *LaSCR2* shows 21 amino acid substitutions, one amino acid insertion, and six amino acid deletions as compared with *LaSCR1*. The comparison of the two white lupin SCR sequences with the functional domains of AtSCR shows that they are divergent at the N-terminal domain and highly conserved at the C-terminal domain (Table 1). The high similarity of LaSCR1 and LaSCR2 coding sequences to AtSCR suggests that the two lupin SCR isoforms may be orthologues of *AtSCR*.

Analysis of the LaSCR1 and LaSCR2 proteins using protein structure prediction (http://www.predictprotein.org) revealed the presence of several important motifs predictive of post-translational modification including tyrosine kinase phosphorylation, *N*-myristoylation, leucine zipper, protein kinase C phosphorylation, *N*-glycosylation, and cAMP- and cGMP-dependent protein kinase phosphorylation.

Both LaSCR1 and LaSCR2 contain the five distinct motifs characteristic of the GRAS family proteins (DiLaurenzio *et al.*, 1996; Pysh *et al.*, 1999): two leucine heptad repeats LHRI and LHRII; the VHIID motif; the PFYRE motif; and the SAW motif. The LaSCR1 and LaSCR2 deduced amino acid sequences for these motifs range from 75% to 93% similar to comparable motifs in AtSCR (Table 1). The

Table 1. Amino acid similarity of conserved GRAS motifs between lupin *SCR1, SCR2*, and *Arabidopsis SCR*

Motif	% Similarity to AtSCR	
	LaSCR1	***LaSCR2***
Homopolymeric repeats	40.4	42.0
Leucine heptad I	88.6	86.5
VHIID	93.4	93.4
Leucine heptad II	81.8	75.7
PFYRE	93.4	93.5
SAW	86.0	87.7

homopolymeric repeat region of the two LaSCRs is more divergent, showing only 40–42% similarity to AtSCR. The divergence in sequence of LaSCR1 and LaSCR2 compared with that of AtSCR between residues 1 and 347 is due in part to the numerous asparagine residues in the LaSCRs as compared with the glutamine and serine residues in AtSCR. The presence of LHR motifs in GRAS family proteins suggests that these proteins may function as multimers (Hurst, 1994; Pysh *et al.*, 1999). In the AtSCR protein, the LHRI, LHRII, and VHIID motifs were reported to mediate protein–protein interactions between SCR and SHORTROOT (Cui *et al.*, 2007).

Phylogenetic analysis of LaSCR1, LaSCR2, and other GRAS family members

The relationships between the lupin LaSCR proteins and other GRAS family members were investigated using their full-length ORF sequences. A phylogenetic analysis of LaSCR1 and LaSCR2 compared with all the known SCR deduced amino acids from *Arabidopsis thaliana* (AtSCR), *Pisum sativum* (PsSCR), *Oryza sativa* (OsSCR), *Zea mays* (ZmSCR), *Cucumis sativa* (CsSCR), and *Pinus sylvestris* (PysSCR) was performed (Fig. 2). The comparison also included 31 *Arabidopsis* GRAS family members. An unrooted tree was generated displaying nine GRAS groups: SCR, DELLA, SHR, SCL3, HAM, SCL4/7, PAT1, LAS, and SCL9 (Fig. 2). These findings agree with previous reports (Bolle, 2004; Lim *et al.*, 2005; Lee *et al.*, 2008). LaSCR1 and LaSCR2 are positioned in the SCR clade with the SCR genes from the six species and also SCARECROW Like 23 (SCL23) which was described to be the GRAS gene most closely related to the SCR group.

Isolation and characterization of LaSCR *genes*

The full-length *LaSCR1* and *LaSCR2* cDNA fragments isolated were used to screen an *Eco*RI lupin genomic library (Miller *et al.*, 2001). The corresponding isolated genomic clones were cloned into pBSKS and fully sequenced. Both *LaSCR1* and *LaSCR2* contain a single intron at a conserved position compared with *AtSCR*; at position Arg623:Leu624 for LaSCR1 and at position Arg619:Leu620 for LaSCR2 (Fig. 1 and Supplementary Fig. S1 available at *JXB* online). The *LaSCR* introns are unusually large, with 1022 bp and 2404 bp for *LaSCR1* and *LaSCR2*, respectively. It is noteworthy that two overlapping regions in the introns share significant identity. One region of 142 bp is 82% identical, while another region of 1068 bp is 50% identical. Analysis of the intron sequences of *LaSCR1*/*LaSCR2* reveals repetition of 5–6 bp motifs. These motifs may be due to the polyploid nature of white lupin. The identity between LaSCR1 and LaSCR2 intronic and exonic sequences may reflect genome duplication some 56 million years ago followed by divergence (Phan *et al.*, 2007).

A DNA gel blot analysis was performed to verify the genomic organization and gene copy numbers of *LaSCR1* and *LaSCR2* (Supplementary Fig. S2 at *JXB* online). The hybridization was performed under highly stringent conditions, and three bands were detected when genomic DNA was digested with *Eco*RI and probed with LaSCR1 cDNA. *LaSCR1* has an internal *Eco*RI restriction site. When genomic DNA was digested with *Hin*dIII, two bands of equal intensity were detected with *LaSCR1*. Because *LaSCR1* has no internal *Hin*dIII site, this suggests that white lupin may have two *SCR1* genes. When genomic DNA digests were probed with *LaSCR2*, a single band was detected, indicating that lupin has a single *SCR2* gene.

Cis-*regulatory elements in* Lupinus albus LaSCR1 *and* LaSCR2 *promoters*

Several reports have documented that transcription factor (TF) genes respond to P stress, including members of the MYB, zinc finger, WRKY, β-HLH (helix–loop–helix), and Nit2 families (Hammond *et al.*, 2003; Mission *et al.*, 2005; Graham *et al.*, 2006; Muller *et al.*, 2007). Genetic studies with *Arabidopsis* have demonstrated that at least four TF genes, *AtPHR1* (MYB family), *AtWRKY75*, *AtZat6* (zinc finger), and *AtBHLH*, are involved in P signalling. Mission *et al.* (2005) identified the *AtPHR1*-binding element P1BS sequence over-represented in the promoter of numerous P stress-induced genes. Moreover, Muller *et al.* (2007) found that the AGTTTT motif was enriched in *Arabidopsis* P-repressed genes while the motif GAATAT was over-represented in 16 P-induced genes. As shown in Table 1 and Supplementary Fig. S1 at *JXB* online, scanning of the 5′-upstream putative promoter region of *LaSCR1* and *LaSCR2* revealed several *cis*-elements including HLH, Nit2, AGTTTT, and GAATAT. These elements are also found in the *AtSCR* genes. It is noteworthy that the *PHR1*-binding motif P1BS (Rubio *et al.*, 2001) element is absent from both of the *LaSCR* genes sequenced and also *AtSCR*.

Expression of LaSCR1 *and* LaSCR2 *in lupin roots and leaves*

The expression of *LaSCR1* and *LaSCR2* in cluster roots of both P-deficient and P-sufficient plants was evaluated by qRT-PCR (Fig. 3). In Fig. 3A the five developmental zones of cluster roots selected for gene expression studies are clearly shown. The zones selected are: Z1, the primary root tip; Z2, the area where cluster root primordia emerge from the pericycle; Z3, typified by elongating cluster root initials in the root cortex (Johnson *et al.*, 1996; Watt and Evans,

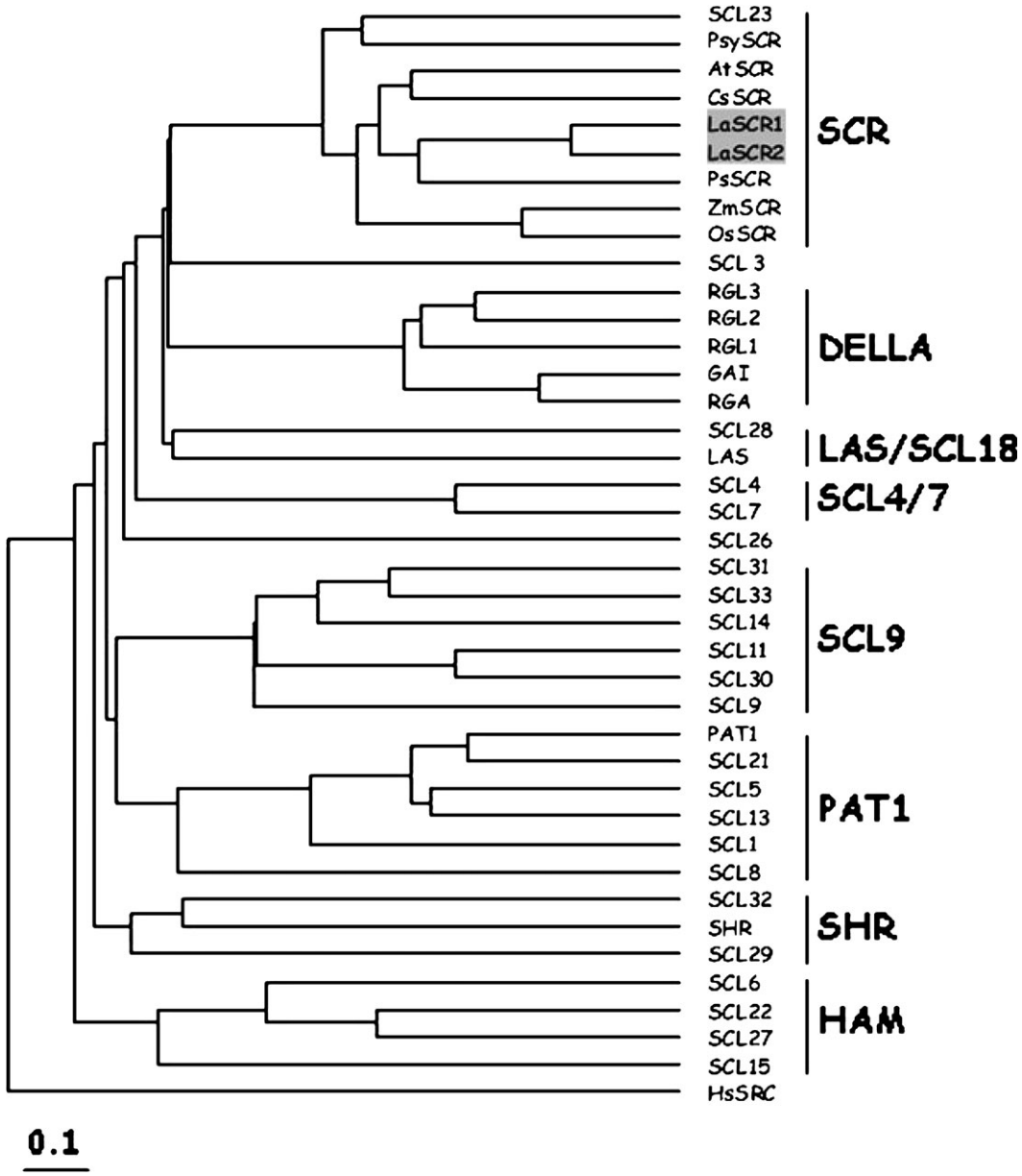

Fig. 2. Phylogenetic relationship of *LaSCR1*, *LaSCR2*, five *SCR* genes, and 31 *Arabidopsis thaliana* GRAS genes. The rooted tree was constructed using the Neighbor–Joining method using the program MAFFT version 6. The human STAT protein (HsSRC; NP004374) was used as an outgroup (Bolle, 2004; Lim *et al.*, 2005). The analysis was performed using amino acid sequences extracted from the public databases. NCBI (www.ncbi.nlm.nih.gov) for: Zea mays ZmSCR (AAG13663); *Oryza sativa* OsSCR (BAD22576); *Pisum sativum* PsSCR (BAB39155); *Pinus sylvestris* PsySCR (ABH85406); and *Cucumis sativa* CsSCR (CAI30893). The TAIR (www.arabidopsis.org) for the *Arabidopsis* GRAS genes: AtSCR (At3g54220); AtSCL23 (At5g41920); AtSCL3 (At1g50420); AtRGL1 (At1g66350); AtRGL2 (At3g03450); AtRGL3 (At5g17490); AtGAI (At1g14920); AtRGA (At2g01570); AtSCL28 (At1g63100); AtLAS/SCL18 (At1g55580); AtSCL4 (At5g66770); AtSCL7 (At3g50650); AtSCL26 (At4g08250); AtSCL31 (At1g07520); AtSCL33 (At2g29060); AtSCL14 (At1g07530); AtSCL11 (At5g59450); AtSCL30 (At3g46600); AtSCL9 (At2g37650); AtPAT1 (At5g48150); AtSCL21 (At2g04890); AtSCL5 (At1g50600); AtSCL13 (At4g17230); AtSCL1 (At1g21450); AtSCL8 (At5g52510); AtSCL32 (At3g49950); AtSHR (At4g37650); AtSCL29 (At3g13840); AtSCL6 (At4g00150); AtSCL22 (At3g60630); AtSCL27 (At2g45160); AtSCL15 (At4g36710). The different groups are defined according to the literature (Bolle, 2004; Lim *et al.*, 2005). The bar indicates the genetic distance corresponding to the score of pairwise global alignment.

1999); Z4, characterized by newly emerged and premature cluster roots still elongating; and Z5, with fully mature cluster roots that have ceased to elongate due to their determinancy (Neumann *et al.*, 1999).

Results for qRT-PCR expression analysis from three independent experiments as presented in Fig. 3B show that the *LaSCR1* and *LaSCR2* mRNA expression pattern is similar in both P-sufficient and P-deficient plants. However, expression patterns of both *LaSCR* genes during cluster root development were modulated according to the stage of development. *LaSCR1* and *LaSCR2* expression irrespective of P status appeared to increase in Z2 and Z3 areas where

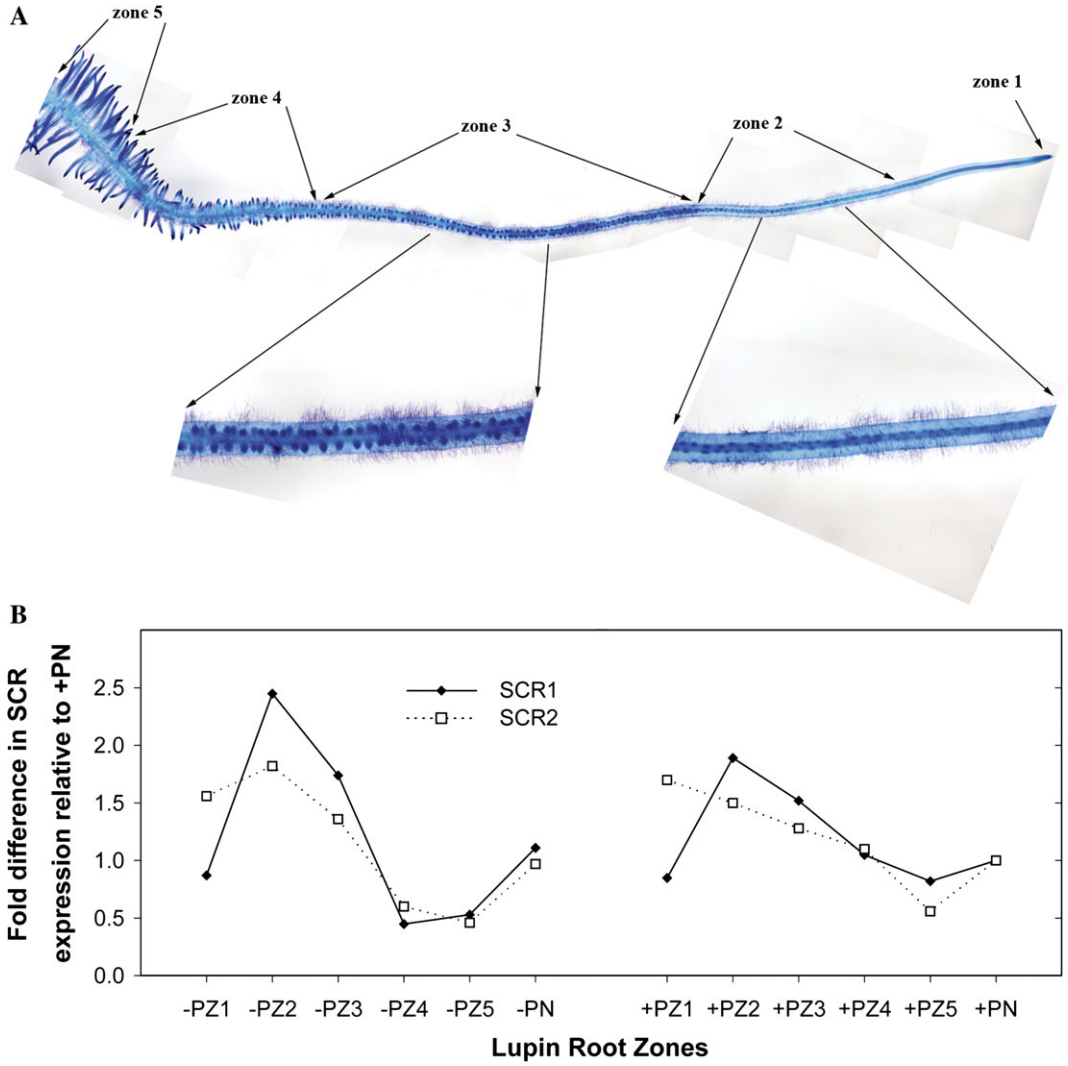

Fig. 3. Cluster root developmental zones and *LaSCR1* and *LaSCR2* transcript expression. (A) Phosphorus stress-induced lupin cluster root stained with methylene blue to show stages of rootlet development. Zone 1, primary root tip; zone 2, cluster root initials developing from the pericycle; zone 3, elongating cluster root primordia within the cortex; zone 4, newly emerged and premature cluster roots protruding through the epidermis; and zone 5, fully mature cluster roots. Inserts are magnified areas of zones 2 and 3. (B) Quantitative real-time PCR on first-strand cDNA generated from P-sufficient (+P) and P-deficient (–P) lupin root zones 1–5 (Z1–Z5) and normal roots using specific primer pairs for *LaSCR1* and *LaSCR2*. The calibrator sample was the cDNA from +P normal roots (+PN). The data points, expressed as the fold difference in *SCR* expression relative to the calibrator sample, represent the mean of three biological replicates. Three separate experiments gave similar patterns of expression. Total RNA was extracted from each of the zones noted in A.

cluster root meristems are forming and are growing through the cortex. In comparison, transcript expression of both *LaSCR* genes is lower in Z4 and Z5 areas where cluster roots are elongating and development becomes determinate. Leaves had very low to no detectable expression of *LaSCR1* and *LaSCR2* (data not shown).

Localization of LaSCR1 *transcripts in root endodermis*

To determine cellular localization of lupin SCR transcripts in normal root tips, *in situ* hybridization was performed using the DIG-labelled antisense and sense RNA of *LaSCR1* as a riboprobe (Fig. 4A, B). The 1 kb fragment of *LaSCR1* cDNA used was 93% identical to *LaSCR2* cDNA. In white lupin primary root tips, *LaSCR1* mRNA was consistently detected in a single layer of endodermis through the QC. The localization pattern for *LaSCR1* transcripts is similar to that reported for several other plant species (Malamy and Benfey, 1997; Lim *et al.*, 2000; Sassa *et al.*, 2001; Kamiya *et al.*, 2003; Laajanen *et al.*, 2007). Reduced expression of *LaSCR1* was also noted in the daughter cortex cells (LS, BB, and CV, unpublished) similar to that reported in rice (DiLaurenzio *et al.*, 1996; Kamiya *et al.*, 2003).

LaSCR2 *promoter::reporter gene expression*

To assess the timing and cellular localization pattern of *LaSCR* in white lupin and *M. truncatula*, an ~1200 bp fragment 5′ upstream of *LaSCR1* and *LaSCR2* corresponding to the putative promoter was ligated to the GUS reporter

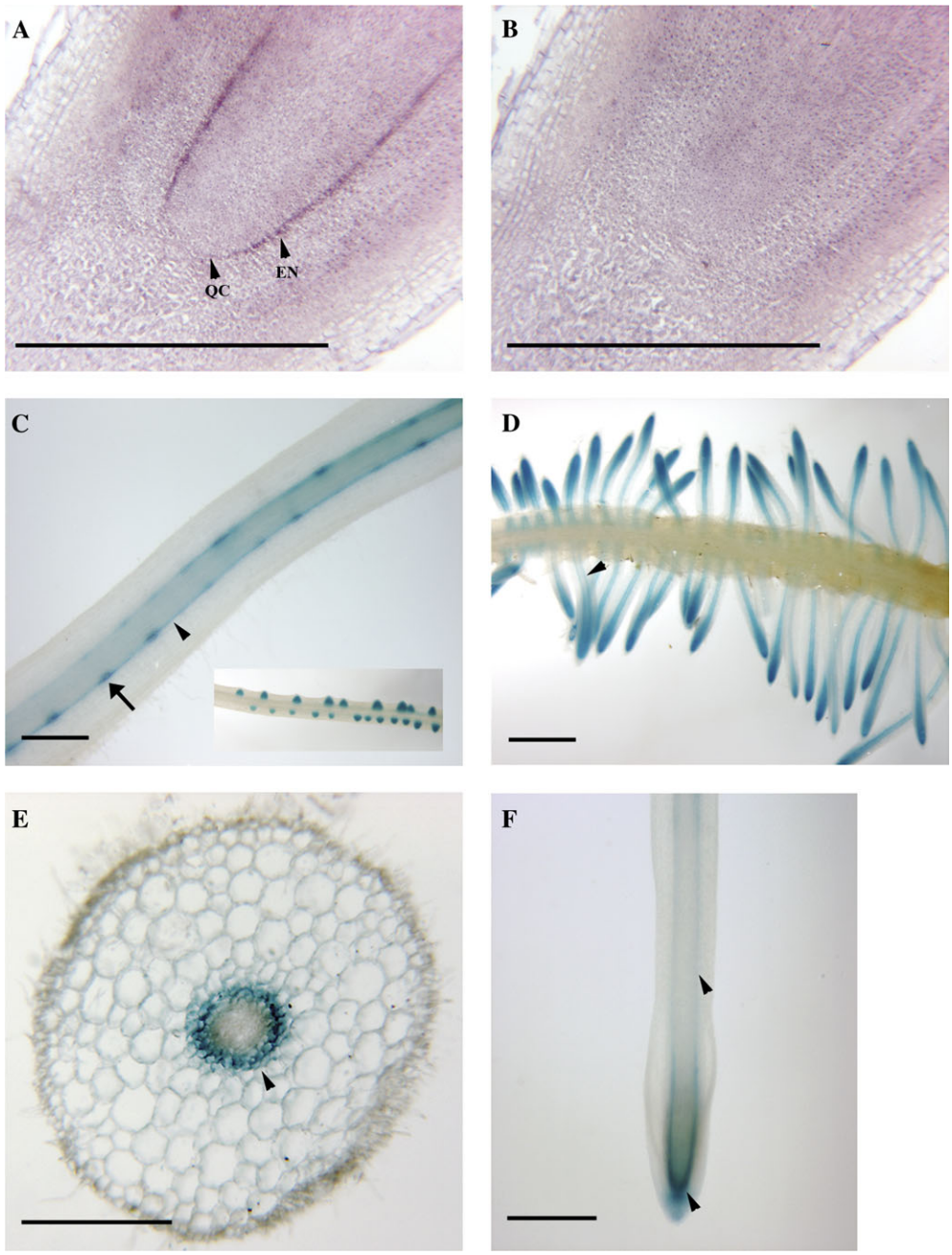

Fig. 4. Localization of *LaSCR* gene expression in white lupin. (A) *LaSCR1* transcripts are localized to the endodermis (EN, arrow head) and quiescent centre (QC, arrowhead) of white lupin roots. *LaSCR1* antisense probe is labelled with DIG-11-UTP. (B) *LaSCR1* sense labelled control shows no DIG staining. (C) Zone 2 cluster root initials, SCR2::GUS staining visible within root initials (arrow) and along the endodermis (arrowhead). Insert, zone 3 cluster root primordia within cortex shows GUS staining. (D) Zones 4 and 5 elongating and mature cluster roots. SCR2::GUS staining is visible in elongating and mature cluster roots and along the endodermis (arrowhead). (E) Transverse section of normal root showing GUS staining in endodermis (arrowhead). (F) Cluster root tip showing SCR1::GUS staining originating near the quiescent centre and extending along the endodermis (arrowheads). A and B magnified 100×; C and D magnified 10×; E and F magnified 30×. Scale bar for A and B=0.5 mm, for C, D, E, and F=1 mm.

gene. Both promoters were used in these experiments to confirm that both were expressed similarly. White lupin and *M. truncatula* were transformed with *A. rhizogenes* harbouring the *pLaSCR1::GUS* constructs (Uhde-Stone *et al.*, 2005; Chabaud *et al.*, 2006). Transformation with *A. rhizogenes* results in composite plants having transformed roots but not shoots. Each regenerate root is considered a single event. Plants transformed with a promoterless GUS construct were generated as negative controls to account for positional effects. In >10 independently transformed white lupin roots, GUS staining was visible within 30 min of incubation with the substrate X-Gluc. As evidenced in Fig. 4C–F, staining was visible in primary roots as well as cluster roots in the endodermis and the QC, but absent in the vascular tissue.

Reporter gene staining was evident at all stages of cluster root development including at the very early stage of meristem initiation. Similar to *LaSCR1* reporter gene staining in white lupin, *M. truncatula* roots containing the *pLaSCR1::GUS* reporter construct also showed rapid GUS staining in the endodermis of both primary and lateral roots (Supplementary Fig. S3 at *JXB* online).

RNAi base silencing of LaSCR1

To elucidate the function of the *SCR1* gene in white lupin roots RNAi methods were used to silence expression of the gene in transgenic roots. Lupin roots were transformed with *A. rhizogenes* harbouring the *LaSCR1::RNAi* (*SCRi*) construct. A 463 bp fragment spanning bp 1876–2338 of the cDNA was cloned into pENTR/D-TOPO and introduced in the sense and antisense orientation into pHELLSGATE8 by the GATEWAY LR Clonase reaction (Invitrogen) as described by Uhde-Stone *et al.* (2005). The 463 bp fragment of *LaSCR1* used for the RNAi construct was 92% identical to the corresponding fragment in *LaSCR2*. The human myosin (*Myo*) gene RNAi construct was used as the control (Cnt). Transformation of white lupin roots with *A. rhizogenes* containing *LaSCR1::RNAi* and *Myoi::RNAi* yields transgenic roots but not transgenic shoots. Six independent experiments were performed, with each yielding at least 10 plants with transgenic roots. Each transgenic root is an independent transgene event. The most noticeable phenotype observed with both lupin and *Medicago* as seen in Fig. 5A and B was a reduction in root numbers accompanied by a slight decrease in root length and weight. The phenotype of LaSCRi plants was variable, with <50% producing a reduced number of roots. This phenotype was more pronounced in *M. truncatula LaSCR1i* roots. The nucleotide sequence of the LaSCR gene RNAi construct is 85% identical to the comparable region in *Medicago* SCR sequences. To confirm that transformation of white lupin and *Medicago* roots with *LaSCR1i* was effective in reducing expression of *SCR1*, RT-PCR was performed on transgenic roots. As shown in Fig. 5C and D, both white lupin and *M. truncatula* roots transformed with the *LaSCR1::RNAi* construct had much reduced *SCR1* transcript. Transcript abundance of *LaSCR2* was also reduced in transgenic lupin roots (Supplementary Fig. S4 at *JXB* online). Quantitative analysis of LaSCRi transcripts via qRT-PCR of seven transgenic roots showed silencing ranging from 20% to 95% (data not shown). Although variable, the reduction of SCR transcripts and root numbers suggests a conserved role for *SCR* in root development among legume species. The visual phenotype observed in *Lupinus albus* and *M. truncatula* RNAi plants is similar to that previously reported for the *A. thaliana* T-DNA mutant *scr-1* and *scr-2* (DiLaurenzio *et al.*, 1996) and *Arabidopsis* RNAi transgenic lines *SCRi-1* and *SCRi-2* (Cui *et al.*, 2007).

Discussion

In this report the understanding of white lupin cluster root development has been extended by: (i) isolating and characterizing two lupin *SCR* genes and showing that the genes are expressed at the earliest stages of cluster root development; (ii) demonstrating through *in situ* hybridization and promoter::GUS reporter staining that the expression of the *SCR* genes is localized to the root endodermis and QC; (iii) showing that *SCR* expression appears to be related to cluster root development rather than plant P status; and (iv) revealing that silencing the expression of *LaSCR* in white lupin and *Medicago* roots can result in reduced root growth.

Cluster roots are characterized by synchronous development and emergence of tertiary lateral roots opposite every protoxylem point within a cluster root area (Skene, 2000). In comparison, typical lateral roots in dicot plants emerge at regular intervals opposite alternating protoxylem points. Under P stress conditions, cluster roots can comprise upwards of 75% of the total root mass of P-deficient white lupin (Johnson *et al.*, 1996; Neumann *et al.*, 1999). Cluster roots can also form under P-sufficient conditions but comprise <5% of the total root mass. Thus, under P deficiency, cluster root formation can increase root surface area by 50-fold to 70-fold providing for intense mining of nutrients in depleted soils. Another unique feature of cluster roots is that they are determinate in growth and elongation, attaining lengths of 0.4–1.0 cm (Skene, 2001). In comparison, typical lateral roots are indeterminate and attain lengths >10 cm.

Because we first detected *LaSCR* genes in ESTs derived from the early zone 2–3 (Fig. 4A) of P-stressed roots and *LaSCR* genes appeared to be over-represented in this library (Uhde-Stone *et al.*, 2005), it was proposed that *LaSCR*s may play a role in cluster root formation and adaptation to P stress. However, a comparison of *LaSCR* expression via qRT-PCR (Fig. 3B) showed that transcript expression was not affected by plant P status (Fig. 3B). However, *LaSCR* expression appeared to be more closely related to the stage of development of cluster roots than to P stress, showing higher expression in zones 2–3 as compared with zones 4–5. The reason for increased *LaSCR* expression in zones 2–3 is that these zones contain a multitude of newly initiated lateral root meristems undergoing cell division, elongation, and differentiation of the endodermis. In comparison, as cluster roots become determinate, cells cease elongation and root meristems cease division. This interpretation is supported by promoter::GUS reporter staining of cluster root meristems at the earliest stages of differentiation in zones 2–3 (Fig. 4C). Any difference in *LaSCR* transcript expression related to P status would be due to the strikingly greater number of cluster roots in P-deficient as compared with P-sufficient plants. As noted above, cluster roots comprise much more of the roots of P-stressed plants than P-sufficient plants. Low to no expression of *LaSCR1* and *2* was detected in both P-sufficient and P-deficient white lupin leaves.

Several lines of evidence suggest that *LaSCR* genes are orthologues of *AtSCR*. The present phylogenetic comparison of 39 GRAS proteins showed that LaSCR1 and 2 reside within the *AtSCR* clade. The *LaSCR* genes appear most closely related to a legume *SCR* from pea (*P. sativum*). Similar to pea SCR, the N-terminal half of the LaSCRs contains numerous asparagine-rich regions but lacks homopolymeric

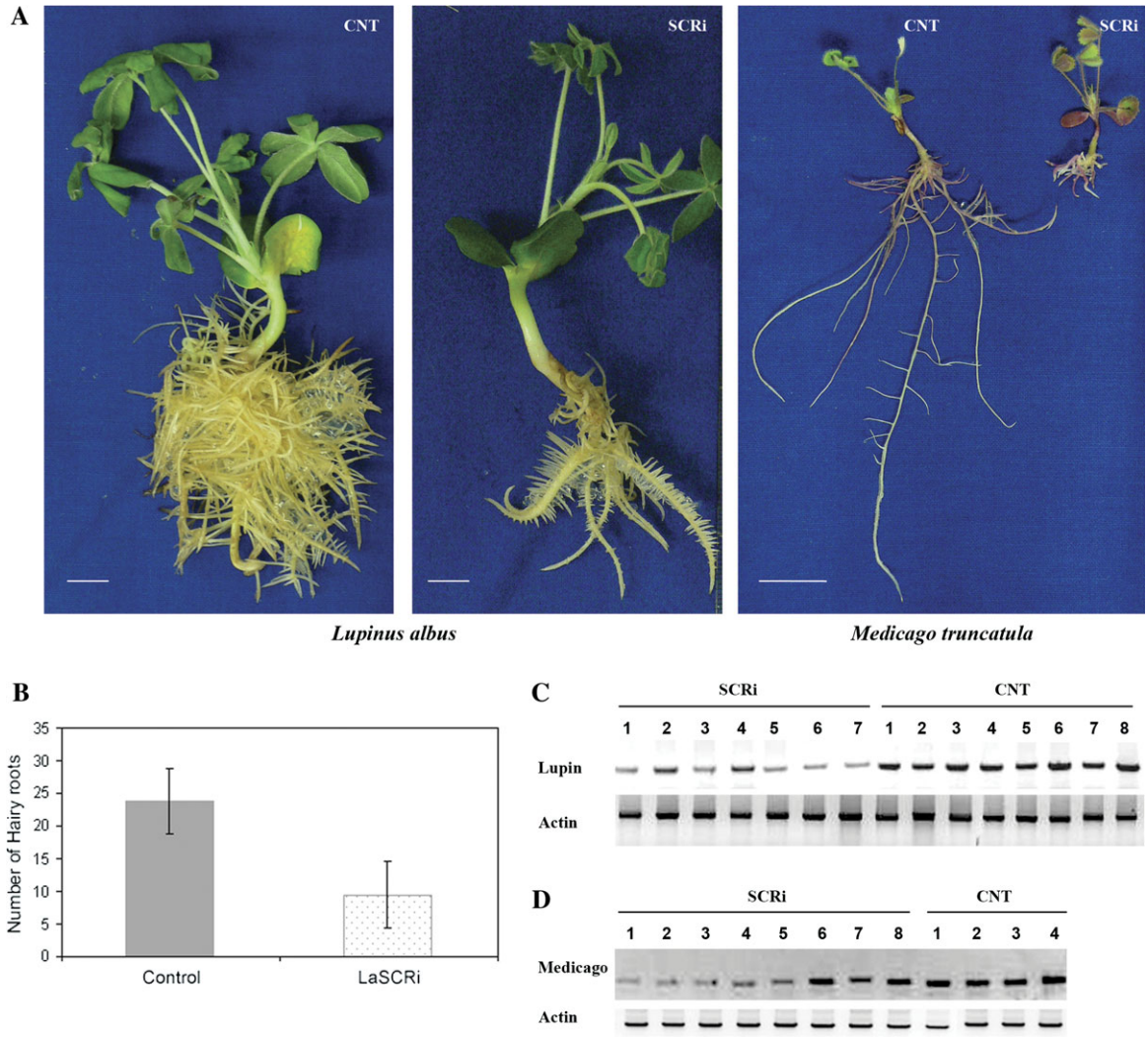

Fig. 5. Silencing of *LaSCR1* gene expression in *Lupinus albus* and *Medicago truncatula*. A white lupin *SCR1* RNAi construct was used to transform lupin and *Medicago* roots. The RNAi construct was introduced via *Agrobacterium rhizogenes*. Roots carrying the RNAi transgene were identified by PCR. Shoots are not transformed in this system. Each transgenic root represents an independent event. Human myosin RNAi (*Myoi*) was used to transform control plants (CNT). (A) Transgenic roots of *LaSCRi* RNAi-silenced lupin and *Medicago*. Note the reduction in root number of RNAi-silenced roots. Scale bar=1 cm. (B) Average number of roots developing on white lupin RNAi plants as compared with plants transformed with myosin control RNAi. Error bars indicate the standard deviation. (C) RT-PCR of RNA from *SCRi*-silenced lupin plants and controls. Actin was used in the RT-PCR as the internal control to ensure uniform PCR conditions. Note the reduction in *LaSCR1* gene expression in SCR-silenced (*SCRi*) lupin roots as compared with controls (CNT). (D) RT-PCR of RNA from *SCRi*-silenced *Medicago* plants and controls. Actin was used as the internal control. Note that the *LaSCRi* RNAi construct was effective in silencing *SCR1* expression (*SCRi*) in *Medicago* roots as compared with controls. Each well in C and D reflects an RT-PCR run on a single transgenic root from each transgenic root system.

stretches of glutamine and proline characteristic of *AtSCR* (Sassa *et al.*, 2001). The asparagine-rich regions were more abundant in LaSCR2 as compared with LaSCR1. Further support for *LaSCR* orthology is demonstrated by conservation of root tissue gene expression found in the endodermis of lupin and *M. truncatula* similar to that seen in *Arabidopsis* and pea. Moreover, the single intron found in *LaSCR1* and *2* is located in the same position as that in *AtSCR* and pea *SCR* (Supplementary Fig. S1 at *JXB* online). Lastly, although *LaSCR* promoter::GUS staining in *Arabidopsis* was not tested, the reporter construct drove expression in *M. truncatula* root endodermis as in lupin.

In *Arabidopsis*, rice, and maize, *SCR* genes play a key role in regulation of asymmetric cell division and radial root patterning (DiLaurenzio *et al.*, 1996; Wysocka-Diller *et al.*, 2000; Kamiya *et al.*, 2003; Sabatini *et al.*, 2003; Heidstra *et al.*, 2004; Lim *et al.*, 2005). In *Arabidopsis* and rice roots, *SCR* was recently shown to sequester the SHORTROOT (SHR) protein into a single cell layer comprising the endodermis, the cortex/endodermal initial cells, and the QC (Cui *et al.*, 2007). The sequestration of SHR by SCR to a single cell layer may explain why all plants have a single layer endodermis. Mutation in SCR results in reduced root growth (Scheres *et al.*, 1995; Sabatini *et al.*, 2003) because

Table 2. Potential phosphate cis-regulatory elements in *Lupinus albus LaSCR1* and *LaSCR2*

Element	Sequence	Position in LaSCR1	Position in LaSCR2
Helix–loop–helix	CA(T/G)(A/C)TG	–887, –1393	–611
WRKY box	(T)(T)TGAC(C/T)	None	None
Zing finger	(A/T)GATA(A/G)	–433, –737, –905, –921	–160, –468, –994, –1053
P1BS (PHR1)	GNATATNC	None	None

SCR is required for root stem cell maintenance and continued root growth. Although the present success was variable, complete silencing of SCR can result in reduced root numbers in both white lupin and *M. truncatula*. Reduced root number and growth is consistent with the phenotype described for *Arabidopsis SCR* mutants and provides additional support for a role for SCR in maintaining root growth. In *Arabidopsis*, SCR1 and SCR2 RNAi mutants showed supernumerary cell layers between the pericycle and epidermis (Cui *et al.*, 2007). Whether this has occurred in *LaSCR::RNAi* roots remains to be determined.

During P stress white lupin forms massive amounts of cluster roots which enhance P acquisition potential. Two *LaSCR* genes are expressed throughout cluster root development but their expression appears only indirectly related to P stress through increased cluster root formation. The lupin *SCR* GRAS transcription factors appear to be more related to cluster root development and growth similar to their role in more typical roots. Future studies directed at other white lupin transcription factors may define genes involved in both P stress and cluster root development.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Gene structure and promoter sequences of *LaSCR1* and *LaSCR2*. (A) Gene structure of lupin LaSCR1 and LaSCR2 compared with that of *Arabidopsis* AtSCR. Note the N-terminal extension of the LaSCRs as compared with AtSCR and the conserved large intron of LaSCR1 and LaSCR2 as compared with AtSCR. L (leucine) and R (arginine) designate the amino acids on either side of the single intron. (B) The 1427 bp promoter sequence upstream from the transcription start of *LaSCR1*. (C) The 1176 bp promoter sequence upstream from the transcription start of *LaSCR2*. The shaded boxes define potential *cis*-element motifs in both promoter sequences. The start ATG codon of each sequence is underlined.

Fig. S2. DNA blot analysis of lupin genomic DNA to evaluate SCR gene copy number. Total genomic DNA digested with *Eco*RI and *Hin*dIII was subjected to DNA blot analysis with *LaSCR1* and *LaSCR2* full-length cDNAs as probes.

Fig. S3. *Medicago truncatula* primary root tip showing SCR::GUS staining originating near the quiescent centre and extending along the endodermis (arrow heads). Scale bar=1 mm.

Fig. S4. Expression of lupin *LaSCR1* and *LaSCR2* in *LaSCR1::RNAi*-transformed lupin plants (*SCRi* 1–5) and in controls (CNT 1–4). Specific primer pairs were used to amplify a 500 bp *LaSCR1* fragment and a 650 bp *LaSCR2* fragment. Actin was used as an internal control to standardize PCR conditions. Note the silencing of both *LaSCR1* and *LaSCR2* by the *LaSCRi::RNAi* construct. The plants were transformed using the hypocotyl injection procedure.

Acknowledgements

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