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., 2003b), and genes involved in carbon metabolism and signalling (Uhde-Stone et al., 2003a; 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., 2003a). 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 2015 °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 CaSO4 instead of 0.5 mM Ca(HPO4)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. (2003a) 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 [32P]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 RNAeasy plant mini kit (Qiagen, Valencia, CA, USA). Lupin total genomic DNA (10 μg) was digested with either EcoRI or HindIII 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 LaScri. This insert was used to rescreen the 7- to 10-day-old library to obtain a full-length cDNA clone. Use of the LaSCR1 cDNA insert to screen an EcoRI 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+ (Strategene, 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’-ACACTAGTGTCCCAACAGTAG-3’; SCR1R, 5’-AACAGTCCCTGGCCACAT-TGAAG-3’; SCR2F, 5’-TTTCTCTGTCGAAGCAGCAG-3’; and SCR2R, 5’-GGGTTGGGAAACATGGG-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-ΔΔCt.
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′-CAACATAGT-GTCCACAGTAC-3′ and LaSCR1-R, 5′-GGAATTCTGTTA-CCAACTATGGAC-3′. For LaSCR2, a 650 bp fragment was amplified using the primers LaSCR2-F, 5′-CTCACCTACCTTG-GCTTCTC-3′ and LaSCR2-R, 5′-CTCAACAGTACGACC-GTACTAG-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 pGEMEasy 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′-CAATGTGCTGAAGCTTCCG-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 paraffin. 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 A4T24 (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 LaSCR1-F, 5′-AAGCTTGGTGCTGCT ATTGG-3′ and LaSCR1-R, CAACCAAGTTGGAGATGC-3′. The product was introduced into the RNAi-inducing pHellsgate 8 vector (Hellwell et al., 2002) using the GATEWAY system (Invitrogen), creating the LaScrI (SCRi) construct. The Hellsgate 8 vector with a fragment of human myosin (MYOH) 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 A4T2C4 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 acetylsyringone 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 SSCRi 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 infection site in the case of hypocotyl infection 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 PROENOD11-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/tg1) for similar sequences, and TC127458 was identified as 80% identical. Specific primers matching a 405 bp fragment were designed from the Medicago sequence (MiSCRF, 5′-CTGAACCTCTTGACGTTGCACG-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-indolyl 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., 2003a) 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 776 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,
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 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 protein family elements: 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).
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 LHR1, LHRII, and VHIID motifs were reported to mediate protein–protein interactions between SCR and SHORTROOT (Cui et al., 2007).

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

<table>
<thead>
<tr>
<th>Motif</th>
<th>% Similarity to AtSCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>LaSCR1</td>
<td>LaSCR2</td>
</tr>
<tr>
<td>Homopolymeric repeats</td>
<td>40.4</td>
</tr>
<tr>
<td>Leucine heptad I</td>
<td>88.6</td>
</tr>
<tr>
<td>VHIID</td>
<td>93.4</td>
</tr>
<tr>
<td>Leucine heptad II</td>
<td>81.8</td>
</tr>
<tr>
<td>PFYRE</td>
<td>93.4</td>
</tr>
<tr>
<td>SAW</td>
<td>86.0</td>
</tr>
</tbody>
</table>

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 according to previous studies. Both LaSCR1 and LaSCR2 are highly conserved and have 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 EcoRI and probed with LaSCR1 cDNA. LaSCR1 has an internal EcoRI restriction site. When genomic DNA was digested with HindIII, two bands of equal intensity were detected with LaSCR1. Because LaSCR1 has no internal HindIII 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 Nrt2 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 overrepresented 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, Nrt2, AGTFTT, 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,
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 determinacy (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
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
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.

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.
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 *SCRI* 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 *LaSCR* 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 Myo::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 mass accompanying 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 *SCRI*, 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 *SCRI* 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 (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 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* genes 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 *SCRF* from pea (*P. sativum*). Similar to pea SCR, the N-terminal half of the LaSCRs contains numerous asparagine-rich regions but lacks homopolymeric

**Discussion**

In this report the understanding of white lupin cluster root development has been extended by: (i) isolating and charac-
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
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-terminus extension of the *LaSCR* genes 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 EcoRI and HindIII 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 (*SCR*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 *LaSCR::RNAi* construct. The plants were transformed using the hypocotyl injection procedure.

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