Plant cell growth and differentiation may involve GAP regulation of Rac activity

Søren Borg[1], Lone Pødenphant, Trine Juul Jensen[2], Carsten Poulsen*

Laboratory of Gene Expression, Department of Structural and Molecular Biology, University of Aarhus, Gustav Wieds Vej 10, DK-8000 Aarhus C, Denmark

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Abstract Two Rac GTPase cDNAs, *LjRac1* and *LjRac2*, were identified in the legume *Lotus japonicus*. Two-hybrid screening with dominant-constitutive mutations in the two Rac GTPases target three plant cDNAs, *LjRacGAP1*, *LjRacGAP2* and *LjRacGAP3*, that encode putative GTPase activating proteins of Rho-GTPase subfamily members. Employing Rac antiserum, purified recombinant LjRac GTPases and recombinant LjRacGAP1, for ligand overlay assays, in vitro GAP affinity assays and GTPase activation, we confirmed that eukaryote Rac/RacGAP interplay is conserved in plants. In this investigation we have developed some tools that can be used to characterize the role of enhanced *LjRac2* expression in developing root nodules.

Key words: Two-hybrid screening; Fusion protein expression; GTPase activating protein; Cdc42/Rac interactive binding element; Root nodule development; *Lotus japonicus*

1. Introduction

Biogenesis of legume root nodules containing dinitrogen-fixing *Rhizobium* symbiosomes require expression of a number of specific genes, coordinated with multiplication of bacterially colonized cells in root nodule primordia. A variety of signalling processes temporally control a specific developmental program [1–3]. Small GTP binding (SMG) proteins participate in the process [4].

In mammals and fungi, SMG proteins control processes such as growth, cell division, vesicular transport, nuclear import, cytoskeletal organization and apoptosis. Activation of important events through cognate effectors depend on the GTP bound form. Intrinsic GTPase activity turns off these activation events. Conversely, replacement of GDP by GTP in SMG protein reactivation is stimulated by guanine nucleotide exchange factors, induced by appropriate factors in the environment. The GTPase activity can be modulated by GTPase activating proteins (GAPs). This complex of reactions constitutes the GTPase switch, which relies also on association with guanine nucleotide dissociation inhibitors [5,6].

*Corresponding author. Fax: (45) 86201222.
E-mail: chp@mbio.aau.dk

[1] Present address: Research Center Flakkebjerg, Flakkebjerg, DK-4200 Slagelse, Denmark.
[2] Present address: John Innes Institute, Colney Lane, Norwich NR4 7UH, UK.

Abbreviations: SMG, small GTP binding; GAP, GTPase activating protein; CRIB, cdc42/Rac interactive binding; MBP, maltose binding protein; GST, glutathione *S*-transferase; GppNHp, guanylyl 5′-imidodiphosphate; PBS, phosphate buffered saline

In recent years, animal and fungal members of the Rho subfamily SMG proteins have received a lot of attention. The involvement of Rho GTPases in the control of cellular mobility and morphogenesis, signal transduction and pathogen defense activities has been extensively studied [7–10]. In plant cells very little is known about the function and regulation of Rho GTPases. In *Pisum sativum*, a Rho GTPase was implicated in cytoskeletal activities during growth of pollen tubes [11,12]. Several *Rho*, *Rac* and *Rop* genes have been described for *Arabidopsis thaliana* [13,14]. Expression patterns for two *Lotus japonicus Rac* genes [15] revealed low constitutive levels for the *LjRac1* mRNA, and increasing levels for the *LjRac2* mRNA during root nodule development.

To study targets for Rac function in this system, we employed a *L. japonicus* cDNA expression library in yeast two-hybrid screening. We report here our analysis of the *LjRacGAP1* cDNA and the ability of the corresponding protein to stimulate Rac GTPase activity To our knowledge, this is the first identification and demonstration of a Rho subfamily GTPase activating protein from a plant source.

2. Materials and methods

2.1. Techniques

The presented work was performed using molecular and protein techniques [16,17]. Some procedures were obtained from biotechnology companies providing essential materials. Other procedures were described previously [15,18] or below. All nucleotide sequences were confirmed on both strands.

2.2. In vitro mutagenesis

LjRac1 and *LjRac2* mRNA reading frames were inserted as *Eco*RI-*Sal*I trimmed PCR fragments into pBluescript SK$^+$. Site directed mutagenesis employed the USB mutagenesis kit (Pharmacia). We replaced the C-terminus proximal cysteine residue with a serine (S194 in LjRac1, S193 in LjRac2), to prevent isoprenyl modification and membrane anchorage. These mutations were paired with the dominant-constitutive V15 (valine for glycine) or L64 (leucine for glutamine) replacements, or the N20 (asparagine for threonine) dominant-negative mutation. Nucleotide sequencing confirmed the sequences.

2.3. Libraries and plasmids

For hybridization based screening, we used a random primed cDNA library in λZAPII [19]. An oligodT primed cDNA library in λHybriZap was used for two-hybrid screening. Libraries were made at Stratagene, from mRNA of *Mezorhizobium loti* infected *L. japonicus* roots/nodule primordia. Mutant bait pBDGAL4 constructions were used for the two-hybrid analyses in *Saccharomyces cerevisiae* YRG-2 (*Matα ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-538 LYS2::UAS_{GAL1}-$TATA_{GAL1}$-HIS3 URA3::$UAS_{GAL417mer(x3)}$-$TATA_{CYC1}$-lacZ*), as recommended by Stratagene.

Bait and target cDNA sequences were transferred to pGEX-5X-1 (Pharmacia) or pMAL-c2 (New England Biolabs) for fusion protein expression in *Escherichia coli*.

2.4. Hybridizations

We used poly(A)$^+$ RNA filters for transcript sizing, and gene copy

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numbers in *L. japonicus* were analyzed on Southern filters. Hybridizations were performed as before [15].

2.5. DNA primers

Oligonucleotide primers for sequencing, PCR, and mutagenesis were made by DNA Technology (Aarhus, Denmark). Information on the structure of the primers used with recombinant pBluescript, pGAL4BD, pGAL4AD, pMAL-c2 and pGEX-5X-1 gene fusions can be obtained upon request.

2.6. Expression and purification of fusion proteins

Glutathione *S*-transferase-Rac fusion proteins were expressed in *E. coli* BL-21 and isolated as described for the GST gene fusion system (Pharmacia). To improve protein folding cells were grown at 28°C, in the presence of 1 M sorbitol and 2.5 mM betaine [20].

pGAL4AD cDNA inserts were transferred as *Eco*RI-*Xho*I fragments into the *Eco*RI-*Sal*I sites of pMAL-c2. Rac interacting protein was expressed in *E. coli* TB-1 and isolated as an MBP fusion protein (Protein Fusion and Purification System, New England Biolabs).

Protein purity was evaluated by amido black staining of SDS-PAGE separated proteins blotted onto PVDF membrane. The Bradford reagent procedure, employing bovine serum albumin as a standard, was used for protein quantitation. Fusion proteins were cleaved with factor Xa (New England Biolabs).

2.7. GTP binding assay

GTP binding by recombinant Rac protein on PVDF membrane was performed essentially as previously described [18]. Each Western filter carried samples of 1 μg of GST-Rac and 1 μg of factor Xa cleaved fusion protein.

2.8. In vitro protein-protein interaction experiments

In vitro interactions between GST-Rac or Rac proteins with LjRacGAP1 were performed by overlay assays [21] and affinity based immune detection assays [22].

The former used 10 μg aliquots of MBP, GST, MBP-LjRacGAP1 and MBP-LjRacGAP1/factor Xa slot-blotted onto PVDF membrane. Unspecific protein binding was blocked in phosphate buffered saline (PBS), pH 7.3, 3% (w/v) skimmed milk powder. Air-dried membranes were incubated for 10 min at 20°C, in the presence of 0.5 μg of [α-^{32}P]GTP loaded (10 μCi, 3000 Ci/mmol) GST-LjRacs. Filters were further processed as described by others [21]. Autoradiography revealed protein-protein interaction.

Affinity purification of GST-LjRac2 employed 5 μl aliquots of MBP-GAP loaded amylose beads (2 μg of MBP-GAP). First, we used GST-LjRac2 protein directly from the glutathione column. Secondly, we used dialyzed GST-LjRac2 protein loaded with GTP or GDP. Loading reactions were performed as described [21]. The amylose-resin aliquots were subjected to 50 μl loading reactions containing 5 μg of GST-LjRac2 protein, incubated and processed as described by others [22]. Bound protein was boiled in 20 μl of SDS-PAGE sample buffer, separated by 12% SDS-PAGE, and the GST-LjRac2 was detected on blots using our anti-Rac serum.

In an experiment with LjRac1 we used 20 μg of factor Xa cleaved GST-LjRac1 in each 50 μl loading reaction. Four 5 μl aliquots of MBP-GAP amylose-resin, and one resin control containing 10 μg of MBP were used. Two GTPase samples were loaded with 2 mM GppNHp, one sample with 2 mM GDP and one sample had no nucleotide added.

The 50 μl loading reactions and amylose-resin aliquots were combined appropriately, and processed as before. Bound proteins were separated by 15% SDS-PAGE, and LjRac1 was detected on Western blots.

2.9. GTPase and GTPase activating assays

GTPase activity of LjRac1 was performed by [LjRac1-[γ-^{32}P]GTP] filter binding assays. LjRac1 (∼7 μg) samples were preloaded with [γ-^{32}P]GTP (10 μCi, 6000 Ci/mmol, Amersham) in 110 μl. $MgCl_2$ was added to 5 mM. The sample was split into two equal fractions and GTPase reactions were initiated, by addition of either buffer or LjRacGAP1 (∼0.3 μg) contained in buffer, as described elsewhere [23]. Reaction volumes were 200 μl. Aliquots of 20 μl were removed and diluted at appropriate timepoints, filtered onto nitrocellulose, and washed with 10 ml of ice-cold wash buffer. Radioactivity retained on filters was estimated by digital analyses of autoradiograms.

GTPase and GAP activity of the respective fusion proteins was precluded by these procedures.

2.10. Antibodies

Rabbit GST-Rac polyclonal antisera were raised by DAKO, Glostrup, Denmark, using affinity purified GST-LjRac1. AP conjugated secondary antibodies (DAKO) were used for detection.

3. Results

3.1. Two-hybrid screening

The two-hybrid screening of the *L. japonicus* pADGAL4 cDNA target library, with the pBDGAL4-encoded recombinant Rac2-L64 and -V15 activating mutant proteins, resulted in 71 His$^+$, LacZ$^+$ yeast colonies. A number of pADGAL4 recombinant cDNAs were analyzed by nucleotide sequencing. Database searches (www.ncbi.nlm.nih.gov) divided the cDNAs into five protein encoding groups: (1) three putative RacGAPs, (2) two putative Raf-homologous protein kinases, (3) a single protein related to the myosin superfamily, (4) eight known sequences without relation to Rac functions, (5) 12 unknown sequences. Some of the protein species encoded in groups 1, 2, and 3 were represented by multiple cDNA copies.

3.2. Analyses of RacGAP cDNAs

Two-hybrid interactions were confirmed when the three RacGAPs, LjRacGAP1, -GAP2 and -GAP3, were retested against mutant GAL4BD-LjRac1 and -LjRac2 fusion proteins after retransformation into yeast. All three LjRacGAPs interact only with the putative dominant-constitutive forms of both Racs, that mimic the GTP bound conformation, and not with the dominant-negative (GDP bound) forms (data not shown).

We screened 500 000 cDNAs of the random primed *L. japonicus* cDNA library with 5′ probes from the three pADGAL4 cDNAs. This resulted in a 2102 bp cDNA contig for the *LjRacGAP1* mRNA, whereas no extra sequence information was obtained for the other two GAP cDNAs. The 493 residue LjRacGAP1 is encoded by nucleotides 229–1707 and the *GAL4AD-LjRacGAP1* fusion contains nucleotides 225–2102.

Southern hybridizations of *L. japonicus* genomic DNA fragments, with a full-length *LjRacGAP1* cDNA probe under modest stringency conditions, indicated a single gene copy (data not shown).

Northern filters with root nodule poly(A)$^+$ RNA (data not shown) revealed an *LjRacGAP1*-mRNA of ∼2.1 kb, equalling the cDNA contig.

3.3. Structure of the deduced LjRacGAP1 protein

Fig. 1 depicts the deduced LjRacGAP1 primary structure, including comparison of the putative GAP domain with similar domains from known regulatory proteins. The GAP domains occupy 150–200 residues, and can be divided into three subdomains, centered around the motifs EGIF/YR, RELP, and FNKMN. The compared proteins are not homologous beyond the GAP domain. A short region of 16 residues (boxed) aligns with the CRIB element (IS.P.dF.H..HVgfd), a motif often found in other Cdc42/Rac interactive binding proteins [24]. No other important protein sequence motifs can be seen.

Thus, the *LjRacGAP1* mRNA encodes a plant protein, which is related to Rac-regulating proteins in other eukaryotes.

3.4. In vitro studies

Protein-protein interaction and Rac GTPase activation by LjRacGAP1 was pursued with biochemical experiments. LjRac proteins were expressed in the GST fusion proteins. Production of LjRac1 recombinant protein was unproblematic, whereas only a few μg of GST-LjRac2 protein could be isolated, sufficient for only a few experiments.

GTP binding by LjRac1 was demonstrated with assays, where [α-^{32}P]GTP was bound by GST-LjRac1 as well as factor Xa cleaved fusion protein on Western filters (data not shown).

For the overlay assay, LjRacGAP1 protein was expressed as MBP-LjRacGAP1. This protein was bound on filters, with or without factor Xa cleavage. The GST-LjRac proteins were loaded with [α-^{32}P]GTP and incubated with the filter. Fig. 2a shows that both [GST-LjRac1-GTP] and [GST-LjRac2-GTP] bound to the LjRacGAP1 filter spots, and not to the MBP or GST controls.

Affinity chromatography on the MBP-LjRacGAP1 amylose-resin and immunodetection demonstrates a trace of binding of GST-LjRac2, when no nucleotide is added (Fig. 2b, lane 1). Dialyzed and GTP loaded GST-Rac2 affinity for MBP-LjRacGAP1 increases about 20-fold (lane 2). GDP loaded GST-Rac2 protein did not bind (lane 3).

Dialyzed and factor Xa cleaved GST-Rac1 was used for affinity assays. To prevent GTP hydrolysis and retain Rac1 in the activated conformation, we introduced the GTP analog guanylyl 5′-imidodiphosphate (GppNHp). Only in the presence of the analog (GTP) does the antiserum detect the GTPase (Fig. 2c, lane 5). The resin control suggests that an unspecific 75 kDa protein detected on this filter (Fig. 2c, lane 6, is derived from the amylose-MBP-LjRacGAP1.

These data support the two-hybrid LjRac/LjRacGAP1 interaction.

3.5. GTPase and GTPase activating assays.

The graphs in Fig. 3a show that LjRacGAP1 is a GTPase activating protein. We measured time course dependent [γ-^{32}P]GTP binding under GTPase activity favoring conditions, employing two different batches of the LjRac1 and LjRacGAP1 proteins (circles and squares). LjRac1 has intrinsic GTP hydrolyzing activity (open symbols), which is linear for the first 25–30 min. Hereafter inhibition or loss of catalytic activity appears to stall hydrolysis. Addition of LjRacGAP1 to the other half-samples (closed symbols) leads to immediate increases in GTPase activity. Activity curves level off after 20–30 min, suggesting that the proteins lose activity, or that the GDP-Rac complex inhibits the GAP from associating

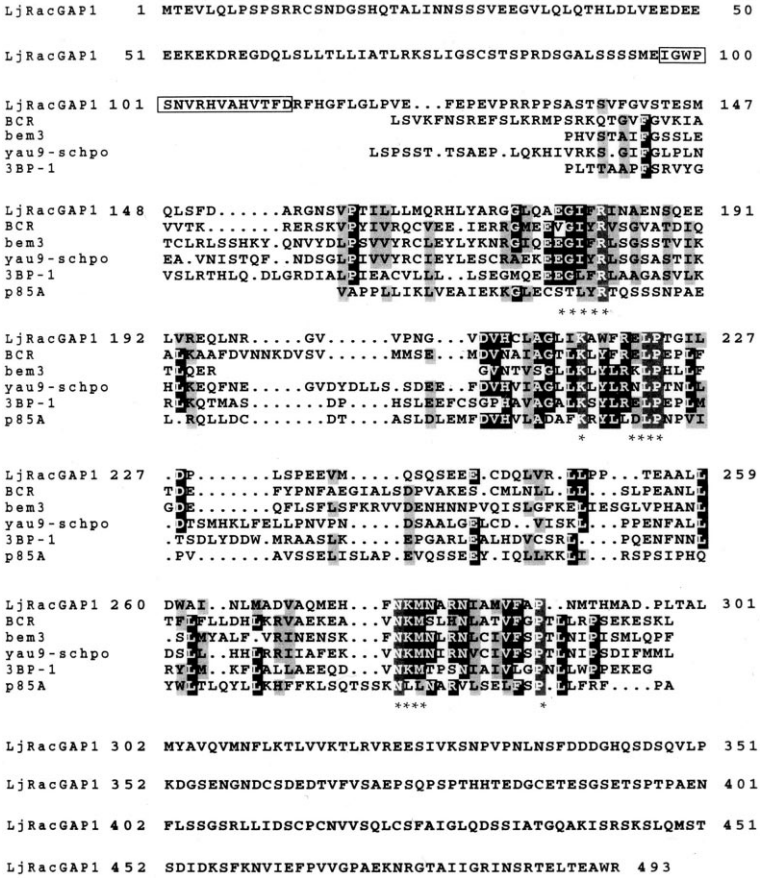

Fig. 1. The LjRacGAP1 amino acid sequence. The putative GAP domain is compared to five GAP domain sequences known from mammalian and fungal cells. Human BCR, AC: P11274, residues 1029–1221; yeast bem3, AC: P32873, residues 898—964 and 994–1099, *Schizosaccharomyces pombe* yau9, AC: Q10164, residues 1041–1238; mouse 3BP-1, AC: P55194, residues 183–362, and bovine PI3'K p85α, AC: P23727, residues 127–276. Points correspond to gaps that are introduced to maximize homology. Asterisks under the sequences designate core motifs of three GAP subdomains. The three *LjRacGAPs* sequences have been deposited at GenBank under accession numbers AF064787, AF064788 and AF064789.

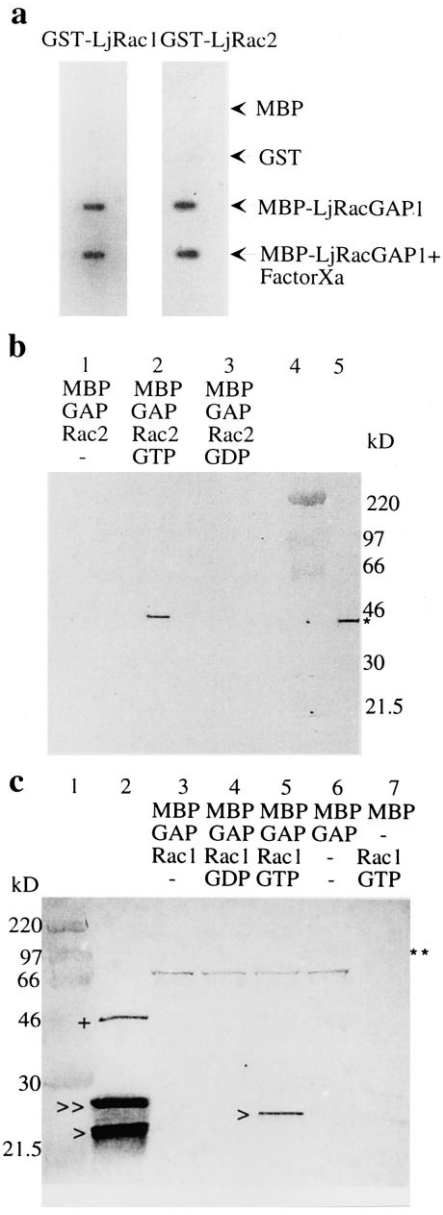

Fig. 2. LjRacGAP1 interacts with LjRac GTPases. a: Ligand overlay assays with [α-^{32}P]GTP loaded GST-LjRac1 and GST-LjRac2. Two PVDF filters, with recombinant protein bound as indicated, were subjected to the radioactively loaded GTP binding proteins. b: In vitro GAP1 interaction with GST-LjRac2. Lane 4, rainbow protein HMW marker (Amersham). Lane 5, 0.2 μg of purified GST-LjRac2 (*). c: The GTP dependent interaction between LjRac1 and GAP1. Lane 1, rainbow protein HMW-marker. Lane 2 contains 5 μg of the factor Xa cleaved GST-LjRac1. Fusion protein (+), LjRac1 (>), GST (>>) and MBP-LjRacGAP1 (**) gel positions are indicated. GTP = GppNHp.

with remaining GTP-Rac molecules. At the end of the activations, less than 5% of the initially bound GTP remains unhydrolyzed.

4. Discussion

We expect that a plant battery of Rho subfamily GTP binding proteins will activate cellular pathways of importance for developmental processes. Unfortunately, Rho gene multiplication and functional redundancy may prevent defects in cell differentiation and organ development, as caused by induced Rho GTPase malfunction from conventional mutagenesis programs.

Thus, to study how the two differently expressed LjRac proteins [15] might be involved in plant development, we isolated LjRac targets by yeast two-hybrid screening. Among the isolated targets, we identified three distinct mRNAs, encoding proteins containing CRIB elements and putative Rho subfamily GAP domains. To our knowledge, the CRIB/GAP constellation of elements has not been observed in other Rho subfamily GAPs. The yeast two-hybrid interaction depend on the GTP bound conformations, maintained through specific Rac amino acid replacements.

The GAP1 protein was expressed and purified from *E. coli*, and interaction with both LjRac GTPases was demonstrated in vitro. Purified LjRacGAP1 selects the GTP bound forms of the Rac GTPases and enhances the GTPase activity of LjRac1, establishing that the two LjRac proteins interact with LjRacGAP1 in a GTP dependent manner, and that eukaryotic Rac/RacGAP interplay is conserved in plant cells.

Lack of LjRac2 in enzymatically active form disabled biochemical experiments that address the question of GAP1 preference for either Rac GTPase. In the two-hybrid system, GAP1 had a slight preference for Rac2, and GAP1 interactions with both Racs were stronger than for GAP2 and

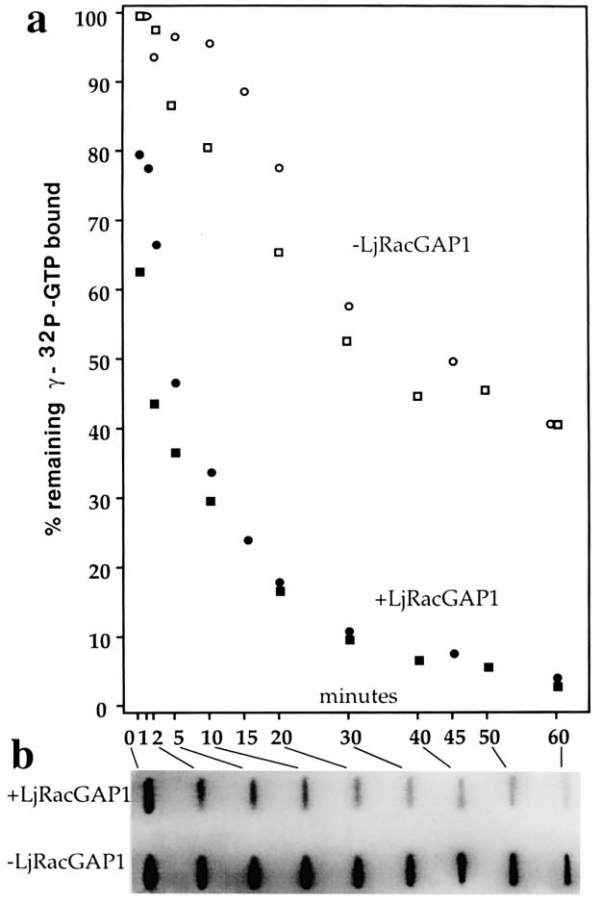

Fig. 3. GTPase and GTPase activation assays using recombinant LjRac1 and LjRacGAP1. a: Time course of GTPase activity, depicted as % remaining radioactivity bound. b: Nitrocellulose slot blot autoradiogram of the circle symbol experiment.

GAP3. This might be due to a lack of important sequence elements in these GAL4AD fusion proteins. Thus, we need to isolate these GAPs in full length, and we need to express active LjRac2 protein in a different system, so that these questions can be addressed in more detail.

Rac/RacGAP interplay preferences may have implications for downstream events. With this study, we have obtained some tools that allow us to analyze this further. First, we need to study expression patterns for the Racs and RacGAPs at the cellular level. Secondly, we aim to influence in vivo the GAP and Rac biological activities. Expression pattern changes may be accomplished by transgenic antisense approaches. Also, general or cell specific overexpression, and expression of the mutant Rac proteins reported here, might influence differentiation patterns, and thus could increase our understanding of the function of these proteins. With the *Lotus japonicus* experimental system [25], and the root nodule specific *LjRac2* expression [15], we have a unique opportunity to study this interplay.

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