Early legume responses to inoculation with Rhizobium sp. NGR234

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ATP-ases; Plasma-membranes; Receptor kinases; Symbiosis; Vigna unguiculata

Summary

Interactions between legumes and rhizobia are controlled by the sequential exchange of symbiotic signals. Two different techniques, 2D-PAGE electrophoresis and differential display were used to study the effects of rhizobial signals on legume development. Application of variously substituted lipo-oligo-saccharidic Nod-factors to roots of Vigna unguiculata resulted in changes in the phosphorylation patterns of microsomal proteins. Reliable amino-acid sequences were obtained for one Nod-factor enhanced protein which was highly homologous to the 57-kDa subunit from Arabidopsis thaliana vacuolar membrane H+-ATPase. Immuno-blotting techniques demonstrated that Nod-factors cause rapid and massive increases of this enzyme in treated roots, suggesting that H+-ATPases play symbiotic roles. Concomitantly, we used differential display (DD) techniques on mRNA isolated from root-hairs to analyse early root responses to NGR234. Significant matches of several DD clones to known sequences were found. Clone D2.62 was homologous to a multitude of receptor kinases including S receptor-like kinases of A. thaliana and clone D4.1 showed similarities to Lotus japonicus phosphatidylinositol transfer-like protein III.
and late nodulin 16. Independent confirmatory analyses of these differentially expressed clones indicated expression at very low levels.

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Introduction

Symbiotic bacteria (rhizobia) interact with the roots of legumes, where they elicit the formation of nodules in which atmospheric nitrogen is reduced to ammonia. Rhizobia bind to receptive root-hairs of host plants, and provoke deformation (Hd) followed by curling (Hac) of the root-hairs, as well as formation of infection threads (for reviews see Broughton et al., 2000; Irving et al., 2000; Perret et al., 2000; Stougaard, 2001). Symbiotic interactions begin in response to flavonoids that are released from legume roots and recognised by rhizobial NodD-proteins. Flavonoid–NodD complexes form one level of specificity, as rhizobial NodD-proteins vary in their ability to interact with flavonoids of different plants (Kobayashi et al., 2004). Binding of flavonoids to the NodD-proteins results in expression of nodD-dependent nod-genes, some of which encode enzymes involved in the synthesis and excretion of Nod-factors. Nod-factors are N-acylated oligomers of β-1,4-linked N-acetyl-D-glucosamine (three to six sub-units) containing various substitutions. Purified Nod-factors provoke Hd and occasionally Hac of root-hairs in homologous legumes, and allow rhizobia to enter infection threads (Relić et al., 1993, 1994; D’Haese et al., 1998). Nod-factors also activate expression of specific plant genes (early nodulins), as well as cell-division in the root cortex. Ultra-structural studies suggest that plasma-membrane and cell-wall synthesis occurs during the early steps of nodule morphogenesis (Horvath et al., 1993). After perception of Nod-factors, numerous rapid plant responses in root-hairs including changes in cytosolic Ca2+ concentrations and calcium spiking have been detected (reviewed in Irving et al., 2000). Furthermore, Nod-factors modulate the activity and expression of G proteins, which are important in Hd of Vigna unguiculata (Kelly and Irving, 2003). In other words, a major re-direction of signal transduction pathways occurs in legumes following inoculation, a suggestion that has been supported by biochemical analyses of mutants (see Udvardi et al., 2005). Yet rather than relying on the randomness of mutants, we devised methods to analyse global changes in gene- and protein-expression in root-hairs following inoculation (or treatment with Nod-factors). Two methods were used—proteomic analyses of plasma-membranes and differential display (DD) which has been successfully applied in plants to isolate nodulation genes (e.g. Goormachtig et al., 1995; Wilkinson et al., 1995).

Materials and methods

Bacterial strains, plasmids and Nod-factors

A RifR derivative of Rhizobium sp. NGR234 (Stanley et al., 1988; Pueppke and Broughton, 1999) was raised under standard conditions at 26 ºC (Broughton et al., 1986). To inoculate seedlings, NGR234 was grown to an absorbance at 1cm A600 nm of about 1.7. After centrifugation, the bacterial pellet was suspended in sterile water to a concentration of 108 cfu mL-1, and sprayed onto the seedlings (Krause and Broughton, 1992, 1994). Nod-factors were isolated from cultures of NGR234 carrying extra copies of nodD1, the transcriptional activator of nodulation genes [NGR234(pA28)] or from a strain in which nodD2, a repressor of nod-genes, was mutated [NGR0nodD2—Fellay et al., 1998] using published procedures (Price et al., 1992). Only non-sulphated Nod-factors [NodNGR(Ac,OH)] were used, and these were prepared as stock solutions (10−4 M) in a 1:1:1 (vol/vol) mixture of DMSO/ethanol/H2O.

Plant material

Sterile seedlings of V. unguiculata (L) Walp var. Red Caloona (purchased from Wrightson Seeds, P.O. Box 357, Seven Hills, NSW 2147, Australia) raised on B & D agar (Broughton and Dilworth, 1971) were treated with Rhizobium sp. NGR234 or non-sulphated Nod-factors {NodNGR[Ac,OH]}(Price et al., 1992) as described previously (Krause and Broughton, 1992, 1994). Nodulation tests were performed in Magenta TM jars (Lewin et al., 1990).

Isolation and purification of the plasma-membrane fraction

Eighty grams of still frozen roots were added to 200 mL of chilled (Yoshida et al., 1983) extraction buffer and homogenised in a “Waring” blender (Dynamics Corporation of America, New Hartford,
CT 06057, USA). Then, the homogenate was filtered through cheese-cloth (mesh-size 100 µm), and the microsomal fraction recovered after successive centrifugations of the filtrate; first at 15,000g for 10 min, then 100,000g for 50 min (both at 4 °C). The microsomal pellet was re-suspended in 10 mM potassium phosphate buffer (pH 7.5), containing 0.5 M sorbitol. Plasma-membranes were purified from the microsomal fraction following a dextran–polyethylene glycol two-phase partition protocol (Yoshida et al., 1983) in which the concentration of both polymers was fixed at 5.9% (Fig. 1). After mixing, separation of the phases was achieved by centrifugation in a swinging-bucket rotor at 2500g for 30 min (4 °C). The upper phase was then diluted with an equal volume of the phosphate–sorbitol buffer, and the plasma-membranes recovered from it by ultra-centrifugation (100,000g for 1 h). After re-suspension in phosphate–sorbitol buffer, the plasma-membrane fraction was finally diluted to 2 µg protein µL⁻¹ and stored at −70 °C until use. Similarly, the lower phase of the two-phase systems (which is rich in tonoplasts) was transferred to at least two times its volume of 0.25 M sucrose, 4 mM KNO₃, 5 mM K₂HPO₄, 1 mM EDTA (pH 7.5) and spun at 100,000g for 1 h (4 °C). After re-suspension in the phosphate–sorbitol buffer, the microsomal fraction was also stored at −70 °C for further use.

Assay of the marker enzyme (K⁺-ATPase)

The activity of membrane-associated K⁺-ATPase that is predominantly found in plasma-membranes was used to assess the purity of the various subcellular fractions. Assays were performed in the absence or presence of Na₃VO₄ as described by Briskin et al. (1987), and the results presented as Na₃VO₄-sensitive ATPase activity. To determine the effectiveness of the different percentages of the Dextran/PEG phases used, the data were calculated as nmol of phosphate liberated per microgram of protein in the preparation per minute and expressed relative to both the microsomal and tonoplasl fractions.

In vitro phosphorylation and SDS-polyacrylamide gel-electrophoresis

Thio-phosphorylation of the plasma-membrane fraction was performed in vitro micro-centrifuge tubes as described by Farmer et al. (1991). To do so, 2 µg of plasma-membrane protein was added to 8.7 µL of phosphorylation buffer [20 mM MES pH 7.6 containing 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, 5 mM MgCl₂, 1 mM MnSO₄, 0.1 mM CaCl₂ and 0.4% (w/v) octaethylene glycol-dodecylether] containing 12.5 µCi adenosine 35S-(thio)-triphosphate. The reaction was initiated by adding [γ-35S]ATP (1000 Ci mmol⁻¹) to a final concentration of 55 nM, followed by vortex mixing for 5 s. After incubation at 30 °C for 10 min, the proteins were separated on 10% or 12% polyacrylamide-SDS-PAGE gels (Laemmli, 1970), then dried and exposed to X-ray film.

Two-dimensional gel-electrophoresis, N-terminal sequencing of spots

The proteins in plasma-membrane fractions were solubilised in 7 M urea, 2 M thiourea, 70 mM of the non-denaturing detergent 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS), 13 mM 1,4-dithiothreitol (DTT), and 1% (v/v) Ampholytes [a 2:3 (v/v) mixture of BDH (BDH Laboratory Supplies, Pool, Dorset, UK) Resolytes # 44333 3P (pH range 3.5–10) and BDH Resolyte # 44340 3C (pH range 4–8)]. Two-dimensional polyacrylamide gel-electrophoresis (2D-PAGE) was performed as described by O’Farrell (1975). Iso-electric focusing was carried out for 4 h 50 min at 300V, 5 h at 2000V and 6 h 30 min at 3000V. Following iso-electric focusing, gel-strips were equilibrated in 2% (w/v) SDS, 5 mM DTT, 62 mM Tris–HCl (pH 6.8) and 10% (v/v) glycerol. SDS-PAGE separation of the proteins in the strips was performed in 10% polyacrylamide gels, which were stained with either Coomassie Brilliant Blue G 250 or silver. After de-staining, the gels were dried and exposed to X-ray film. Phosphorylated spots were
excised from preparative 2D gels, and digested with trypsin. Phenylthiodantoin amino-acids were identified by on-line HPLC using standard sequencing procedures. Amino-acid sequence homology searches against GeneBank and Swiss-Prot databases were made with the FASTA programme (GCG package). Multiple alignments of amino-acid sequences were performed using the CLUSTAL W programme (version 1.5; Thompson et al., 1994).

**Immuno-(protein)-blotting**

PAGE gels were blotted on to Immobilon-P™ membranes (Millipore, Bedford, MA, USA) using the Trans-Blot™ Cell (Bio-Rad Laboratories, Hercules, CA, USA) (30 V and 110 mA overnight) and a transfer buffer comprising 25 mM Tris and 192 mM glycine (pH 8.3) as well as 15% (v/v) methanol. Non-specific binding sites on the membrane were blocked in 5% (w/v) non-fat milk suspended in PBS (5 mM NaH2PO4, 150 mM NaCl, pH 7.4), washed three times in PBS-T [0.05% (v/v) Tween-20 in PBS] for 5 min, and incubated with purified oat vacuolar H+-ATPase [Ward et al., 1992 (1:2000 dilution)] for 1 h. Afterwards, the membrane was washed as described above, and incubated (4°C for 1 h) with horseradish peroxidase-conjugated secondary antibody (A6154, Sigma-Aldrich, St. Louis, MO, USA) diluted 1:25,000 in PBS-T. After washing three times (each 15 min), in the same buffer, chemiluminescent methods were used to detect the bands (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) following the manufacturer’s instructions.

**Differential display**

mRNA DD was performed as described by Liang and Pardee (1992) using 13 combinations of 5′-arbitrary primers” 3′-oligo(dT) anchored primers 32P-dATP as radioactive nucleotide listed in Table 1. The cycling parameters were: 94°C for 40 s, 40°C for 2 min, 72°C for 30 s for 45 cycles followed by 72°C for 5 min. Amplified cDNAs were then separated on 6% acrylamide gels (Sambrook et al., 1989). Each gel was dried directly onto one of the running glass-plates and exposed to X-ray films. Selected sections of gels were re-hydrated with 5–7 µL of H2O. Fragments of acrylamide containing-cDNAs were transferred into 50 µL TE8 buffer, and incubated at 60°C for 1 h. Each cDNA was re-amplified and the PCR samples were run on a 1.2% agarose gel and stained with ethidium bromide. The remaining samples were stored at −20°C for cloning and sequencing.

**Cloning and sequencing of fragments retrieved from the DD gels**

The PCR fragments were cloned into the pCRT-TRAP® vector or pBluescript II KS+(+) Fragments that could not be cloned directly were re-amplified with Vent® polymerase (exo)® (New England Biolabs, Ipswich, MA, USA) generating blunt-ended fragments that facilitated cloning into either vector. The accession numbers of the 21 cDNA fragments that were sequenced are listed in Table 2.

**RNA gel blot analysis**

Poly(A)+ messenger RNA from root-hairs, hypocotyls, roots or nodules was purified using ca. 1 g of frozen plant tissue, oligo dT-latex beads and the Message Maker Kit (R&D Systems, Abingdon, Oxon, UK), Agarose/formaldehyde gel electrophoresis of V. unguiculata poly(A)+ RNA was performed essentially as described in Ausubel et al. (1990). The separated RNA was then transferred to Nylon membranes (Scheicher and Schuell, Dassel,
Germany) by capillarity. Radio-labelled probes were generated by PCR using [32P]-dCTP, and unincorporated nucleotides were removed by passage through Bio-Gel P-30 spin columns (BioRad, Hercules, CA, USA). Hybridisation was performed at 42°C in the presence of 50% formamide as denaturing agent (Sambrook et al., 1989). Filters were washed twice in 2×2 SSC/0.1% SDS for 10 min, and once more in 1×2 SSC/0.1% for 10 min at 30°C before autoradiography.

Quantitative reverse transcriptase PCR

mRNA samples [0.5μg of poly(A)+] were reverse transcribed using Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT) and 30 pmoL anti-sense gene specific primers (LTP2, specific to LTP and VRK9, specific to D2.62) under conditions prescribed by the vendor (described in Promega Technical Bulletin no. 545). One-fifth of the reaction mixture was used in the PCR reaction in combination with the corresponding sense and anti-sense gene specific primers. A 10μL aliquot of the PCR reaction was sampled at every 10 cycles up to 40 cycles. The PCR aliquots were separated on 1.4% agarose gels.

Proteins-concentrations and staining

Quantification of proteins present in the various fractions was performed using the Bradford assay (Bradford, 1976), using bovine serum albumin (BSA) as the standard. Proteins present in poly-acrylamide gels were visualised using either a modified Morrissey silver stain procedure (Merril et al., 1981; Morrissey, 1981) or by staining with Coomassie Brilliant Blue R-250 (CBB R-250 Staining Kit, BioRad Laboratories, Hercules, CA, USA).

Results

Optimisation of the plasma-membrane isolation procedure

Since a number of components of signal-transduction pathways reside in membranes, we optimised procedures for extracting them from V. unguiculata roots. Four different concentrations (5.7%, 5.8%, 5.9%, and 6.0%) of Dextran/PEG were tested in an aqueous two-polymer phase system for isolating sub-cellular components (see Yoshida et al., 1983). In this system, the lower (denser) fraction contains mainly tonoplasts that can be used as controls, as well as indicators of the degree of purification. K+-ATPase activity was used as a marker for the plasma-membrane fraction, and the enzyme activity was calculated as the amount of phosphorus liberated per unit protein (nmole Ph⁻/μg), providing a measure of the degree of purity. A Dextran/PEG concentration of 5.9% resulted in the highest yield of plasma-membranes and gave a seven- to nine-fold enrichment in the specific activity of the plasma-membrane marker (vanadate-inhibited K⁺-ATPase activity) when compared to microsomes (Table 3).

To check the integrity of proteins in each fraction, the various extracts were subjected to SDS-PAGE. Proteins in each of the extracts appeared intact (Fig. 1). Furthermore, the protein composition of the different fractions varied, with the largest spectrum of proteins being found in the microsomal fraction, followed by the tonoplast and plasma-membrane fractions, respectively. It thus seems that the aqueous two-polymer phase plasma-membrane purification system used here, not only yields sufficient proteins enriched in K⁺-ATPase activity, but that the distribution and quality of proteins in the other fractions is adequate for

Table 2. Designation, primer combination, length, and accession number for each of the cDNA clones identified through differential display analyses of Rhizobium sp. NGR234 inoculated and water sprayed Vigna unguiculata root-hairs

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proteomic-scale analyses. Interestingly, prior treatment of the roots with Nod-factors leads to the appearance of additional bands, particularly in the microsomal fractions (cf. lanes 2 and 3 in Fig. 1).

2D-SDS-PAGE profiles of plasma-membrane proteins

2D-SDS-PAGE resolved many plasma-membrane proteins (Fig. 2A). The iso-electric points (pIs) of these polypeptides ranged from 4 to 9 and their molecular masses from 27 to 150 kDa. To see if changes in the phosphorylation patterns occur, seedlings were sprayed with \(10^{-7}\) M NodNGR[Ac,OH] factors (or with H\(_2\)O containing 0.1% (v/v) ethanol as a control), and harvested 2 h later (see Materials and Methods). Plasma-membrane fractions were isolated, and phosphorylated in vitro. Following SDS-PAGE separation and exposure to X-ray film or a phospho-imager, a number of phosphorylated spots could be seen (Fig. 2B). Although the number of phosphorylated spots was far lower than the total, silver-stained spots, some of them appeared to be differentially phosphorylated (cf. Fig. 3A and B). One phosphorylated polypeptide was clearly visible in extracts from Nod-factor treated roots, but not from control roots. Two hours after treatment with Nod-factors, an acidic ca. 57 kDa protein was visible (Fig. 3B). This clear dependence on prior treatment with Nod-factors and separation from other radiolabelled spots made it an ideal candidate for N-terminal sequencing. Accordingly, the phosphorylated spot was excised, digested by trypsin and the N-terminal amino-acid sequence determined using standard procedures.

N-terminal sequencing and amino-acid analysis

Sequence alignment searches indicated that three peptides (QYPPINVLPSLR, TPVSDLMLGR and QYPPINVLPSLSR) from the Nod-factor enhanced phosphorylated spot were almost identical to parts of the 57 kDa \(\beta\)-subunit of the Arabidopsis thaliana vacuolar membrane H\(^+\)-ATPase (Table 4). This extra-ordinary sequence identity between proteins of two quite different plants—A. thaliana (Cruciferae) and V. unguiculata (Papilioioidae, a sub-family of the Leguminosae), prompted us to examine the relationships amongst the sequences of other vacuolar ATP synthases. Many are known, and vary in size from 483 aa (Hordeum vulgare L.) to the largest, 492 aa (A. thaliana). Apart from differences at the N-terminal end of the proteins, variation occurs in just over 30 of the amino-acids, and often these represent conservative- or semi-conservative substitutions. As more than 90% of the amino-acids are completely conserved along the sequence, we reasoned that it was unnecessary to isolate the corresponding gene from V. unguiculata. Rather,
since polyclonal antibodies to an oat (*H. vulgare*) vacuolar ATP synthase were available (Ward et al., 1992), we decided to test whether Nod-factors enhanced the expression of this enzyme using immuno-blotting procedures.

**Immuno-blot analyses**

Plasma-membrane proteins were isolated from control or Nod-factor treated roots and separated by SDS-PAGE (10% or 12%) as shown in Fig. 1. Then, the proteins were electro-blotted on to PVDF membranes and exposed to a polyclonal antibodies raised against the oat vacuolar ATPase. A 57 kDa band was visible in plasma-membranes extracted from Nod-factor treated roots, but not from control root preparations (Fig. 4A). These obviously higher amounts of vacuolar ATPase found in Nod-factor treated preparations cannot be attributed to unequal amounts of protein on the PVDF membrane since staining with Ponceau S showed that the amounts loaded were approximately equal (Fig. 4B). Furthermore, the band that is visible in Fig. 4 is not likely to be due to non-specific binding of the antibody to other proteins, since the mono-specific anti-oat antibody was used at a dilution of 1:2000. We thus conclude that Nod-factors rapidly induce (≤2 h) the accumulation of a H⁺-ATPase in the roots of *V. unguiculata*.

**Differentially expressed genes**

Thirteen different primer combinations were used in the DD experiment to compare gene-expression in root-hairs sprayed with NGR234 or water. Twenty-four cDNA bands were excised from the DD gels and successfully re-amplified. Twelve cDNA bands were obtained from root-hairs pre-treated with NGR234 (three unique to treatment with NGR234 and nine others whose expression was enhanced in the presence of NGR234). Seven cDNA bands found in non-inoculated root-hair samples were clearly repressed after treatment with NGR234, while two other bands were unaffected by treatment (selected to serve as controls). Eight of the 24 bands were easily cloned into the PCR-TRAP™ vector, while 12 other fragments had to be re-amplified using Vent® polymerase to generate blunt ended fragments and then cloned into either pCR-TRAP™ or pBluescript II KS(+) vectors. Four bands could not be cloned or re-amplified. Six individual colonies from each transformation were picked to confirm that the insert-size corresponded to that estimated from the DD gels, and three of these were sequenced. Only in one case (D2.6) were two clones of approximately the same length but with distinct sequences found (these were renamed D2.62 and D2.64). DNA sequences of the cloned DD fragments were deposited in the EST database at the National Centre for Biotechnology Information, NCBI (Table 3).

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**Table 4.** Placement of the amino-acid sequence generated from the Nod-factor enhanced phosphorylated spot seen on SDS-PAGE gels of plasma-membrane preparations of *Vigna unguiculata* root-hairs on the sequence of an *Arabidopsis thaliana* vacuolar ATP synthase subunit B

<table>
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Those amino-acids that were generated by N-terminal amino acid sequencing of the 57 kDa protein of *V. unguiculata* are shown in bold in the *A. thaliana* sequence.

**Figure 4.** Plasma-membrane preparations from roots of *Vigna unguiculata* immuno-blotted with polyclonal antibodies raised against the oat vacuolar ATPase: (A) proteins of plasma-membrane extracted from roots treated with water (PM-H₂O); or after 2 h of treatment with 10⁻⁷ M NodNGR[Ac,OH] factors (PM-NF). The ca. 57 kDa protein that cross-hybridises with the antibody raised against the oat vacuolar ATPase is marked with an arrow. (B) Similar membrane stained with Ponceau S to verify the amount of protein (ca. 10µg) loaded onto the gel.
All cDNA sequences contained the corresponding arbitrary primer and anchored oligo(dT) primer sequences at their 5’ and 3’ ends, respectively. Each clone was compared against sequences in the non-redundant and EST databases at the NCBI (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm (Altschul et al., 1997). A summary of the BLAST search analyses is given in Table 5.

Expression analyses

To independently validate the DD results, RNA blot analyses were performed on all cloned DD fragments using 10–20 μg total root-hair RNA extracted 24 h after inoculation with NGR234 (or after spraying with water). In all cases, no reliable hybridisation signal could be observed even after prolonged exposure (14 days). This finding indicates that all the cloned cDNAs represent transcripts that are only expressed at very low levels. As total RNA did not provide the sensitivity required for detection of any of these transcripts, subsequent RNA blots were performed using poly(A)+-enriched RNA and analyses were limited to clones with convincing matches to database entries.

A cDNA clone encoding a lipid transfer protein (LTP) was isolated from root-hairs of V. unguiculata by Krause et al. (1994). This clone, whose expression was enhanced in root-hairs challenged with

Table 5. Basic local alignment search tool (BLAST) matches of the cDNA clones identified by differential display analyses.

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<thead>
<tr>
<th>Clone</th>
<th>Root-hair treated witha</th>
<th>Best homology</th>
<th>Score</th>
<th>Expect valueb</th>
</tr>
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<tr>
<td>D1.1</td>
<td>NGR234</td>
<td><em>Arabidopsis thaliana</em> putative β-N-acetylhexosaminidase (accession no. AAN41320)</td>
<td>82</td>
<td>4e−15</td>
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<tr>
<td>D1.2</td>
<td>H₂O</td>
<td>nsf</td>
<td></td>
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<td>D1.3</td>
<td>NGR234</td>
<td><em>Vigna unguiculata</em> root-hair clone D3.2 (accession no. AI755295) Glycine max cDNA (accession no. CA935202)</td>
<td>176</td>
<td>5e−42</td>
</tr>
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<td>D1.C</td>
<td>NGR234</td>
<td>nsf</td>
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<td>D2.1</td>
<td>NGR234</td>
<td><em>A. thaliana</em> UDP-glucuronic acid epimerase 1 (accession no. AA06796)</td>
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<td>D2.2</td>
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<td><em>Oncidium cv. ‘Gover Ramsey’</em> carotenoid isomerase (accession no. AAX84688)</td>
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<td>D2.3</td>
<td>NGR234</td>
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<td><em>A. thaliana</em> putative receptor kinase (accession no. CAB77808)</td>
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<td>D2.64</td>
<td>H₂O</td>
<td><em>V. unguiculata</em> root-hair clone D5.3 (accession no. AI755300) Phaseolus vulgaris P- root EST (accession no. CV543754)</td>
<td>515</td>
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<td>H₂O</td>
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<td>H₂O</td>
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<td>515</td>
<td>1e−143</td>
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</table>

aRoot-hair treatment: inoculated with NGR234 or sprayed with water.
bProbability that such a match would occur merely by chance as given by BLAST.
cnsf, no significant match.
NGR234, served as a positive control to validate the hybridisation results (Fig. 5a). Signals corresponding to the size of the LTP transcript (~700 nt) were detected in inoculated, as well as in non-inoculated root-hairs. When the same filter was probed with the D1.1 clone (a homologue of β-N-acetylhexosaminidase of A. thaliana AAN41320), only weak signals were detected at ca. 1400 nt after 7 days exposure (Fig. 5b). This cDNA, which seemed unique to inoculated root-hairs in the DD experiment, hybridised about equally to poly(A)+ RNA derived from inoculated or control root-hairs.

D4.1 that was isolated from root-hairs treated with water, has similarity to a phosphatidylinositol transfer-like protein III (accession no. AAK63247) and late nodulin 16 (LjNOD16) of Lotus japonicus. Unlike the highly expressed nodule specific LjNOD16 (Kapranov et al., 1997), the transcript corresponding to D4.1 was only weakly expressed in all tissues tested, except in hypocotyls treated with water, where it increased (Fig. 6). It is clear from this experiment that D4.1 corresponds to a messenger of approximately 2500 nt in size that is similar in size to the phosphatidylinositol transfer-like gene family of L. japonicus homologues of LjNOD16 (Kapranov et al., 2001).

As mRNA blot analyses of the D2.62 clone did not yield detectable signals even after prolonged exposure (data not shown), the more sensitive quantitative RT-PCR technique was used to monitor the patterns of D2.62 expression in root-hairs (Fig. 7). Primers based on the conserved motifs of serine-threonine protein kinases (Walker, 1994) were designed to extend the sequence of D2.62, then used to amplify, clone and sequence the kinase domain of D2.62. In turn, this sequence facilitated the design of primer VRK9 (sense strand D2.62 specific primer) that was used in the RT-PCR reaction. Verification of the amplification patterns by DNA blot analysis (data not shown), confirmed that the LTP-transcript is present in both root-hairs challenged with NGR234 and those treated with...
water (Fig. 7), which is consistent with data of Fig. 5A. Although the expression levels of the D2.62 transcript were significantly lower than those of the LTP gene, they remain unchanged after inoculation with NGR234. Lange et al. (1999) reported a similar expression pattern of a receptor-like kinase of Phaseolus vulgaris in response to mutualistic microsymbionts including Rhizobium tropici and Nod-factors of NGR234. Interestingly, expression of this receptor-like kinase transiently and rapidly increases in response to pathogens, wounding and Nod-factors of R. tropici.

Discussion

We used two different methods to isolate symbiotic signals from legume root-hairs. By targeting the plasma-membranes which are a probable site of symbiotic signal-transduction and applying proteomic methods to them, we were able to identify a protein that almost certainly plays a role in symbiotic development. Methods based on varying RNA expression levels in differently treated tissues identified a number of putative symbiotic genes, some of which are involved in signal transduction.

The uptake and release of solutes across the vacuolar membrane is fundamental to many cellular processes, such as adaptation to osmotic stress, signal transduction, and metabolic regulation. Vacuolar H+-translocating ATPases (v-ATPases) are a family of ATP-dependent ion-pumps that play central roles in the growth and development of plant cells. Their function is to generate a proton electro-chemical gradient across membranes, without which cells cannot function. In mature cells, the vacuole is the largest intra-cellular compartment, occupying about 90% of the cell volume. The proton electrochemical gradient (acid inside) formed by the v-ATPase provides the primary driving force for the transport of numerous ions and metabolites against their electrochemical gradients (Michelet and Boutry, 1995). In plants, v-ATPases have been localised to vacuoles and other membranes of the secretory system, including the endoplasmic reticulum, Golgi, small vesicles and the plasma-membrane (Lüttge and Ratajczak, 1997; Sze et al., 2002). It is thus not surprising that a v-ATPase was found in the plasma-membranes of V. uniguiculata roots.

v-ATPases are composed of two distinct domains: a catalytic V-1 sector, in which ATP hydrolysis takes place, and the membrane-embedded sector, V-0, which functions in ion-conduction (Gruber, 2003). H+-ATPases are thus like master enzymes for energising cell membranes (Serrano, 1989), a fact that has often been exploited, for it is possible to follow both the enzyme activity and the generation of a proton gradient in a single preparation. Increasingly, H+-ATPases are implicated in plant-microbe interactions. Not only are they present in the fungal parasite Uromyces fabae (Struck et al., 1998), as well as the mycorrhizal fungus Glomus mosseae (Requena et al., 2003), but they are also found in both symbiotic and pathogenic bacteria (Freiberg et al., 1997; Viprey et al., 2000; Pozidis et al., 2003). Since the exchange of metabolites at the symbiotic interface between bacteria and plants permits control of nutrient exchange, it is not surprising that they have also been found in the peri-bacteroid membranes of L. japonicus nodules (Wienkoop and Saalbach, 2003).

Our findings that Nod-NGR-factors rapidly and dramatically increase the amounts of a v-ATPase in V. uniguiculata roots suggest that plant ATPases may also play symbiotic roles. As the increases seen here occur after the dramatic changes in the cytoskeleton structure that prepare root-hairs for renewed growth, we have initiated (Irving et al., 2000), this ATPase is more likely to be associated with the swelling, elongation and division of cells rather than nodule development per se. Obviously, elongation and swelling are driven by an increased osmotic pressure of the vacuole, and it is possible that the greater amounts of this particular v-ATPase are responsible for the solute influx. Activity of this enzyme seems to be exerted at two levels—synthesis and phosphorylation. Both seem to be under Nod-factor control, since the spots that lead to the identification of this v-ATPase were phosphorylated in vitro. Modulation of ATPase activity (de)phosphorylation is an attractive possibility, since yeast and plant H+-ATPases are naturally phosphorylated by a membrane-associated protein kinase (Michelet and Boutry, 1995; Venema and Palmgren, 1995). This is particularly interesting since we have found that NopL and NopP, which are thought to be injected into legumes, serve as substrates for plant protein-kinases (Bartsev et al., 2003, 2004; Skorpi et al., 2005). NopL, NopP and perhaps other rhizobial proteins could thus help modulate MAP kinase pathways in the plant including those involved in controlling v-ATPase activity.

DD identified candidate genes that are predominantly expressed at very low levels. Although the use of DD in the isolation of symbiotic genes has met with variable success (cf. Heidstra et al., 1997; de Carvalho Niebel et al., 1998), it is often a more sensitive technique than RNA blotting (e.g. Goormachtig et al., 1995; Wan et al., 1996; Kapranov
et al., 1997). And even though poor reproducibility is often encountered in DD (Liang and Pardee, 1995) resulting in putative transcripts that turn out to be false positives (Debouck, 1995; Wan et al., 1996), we were able to identify homologues of S receptor-like kinases, phosphatidylinositol transfer-like protein III, or late nodulin 16 in *V. unguiculata* root hairs. Interestingly, a soybean homologue of the phosphatidylinositol transfer protein was phosphorylated in response to various environmental stress conditions (including hyper-osmotic stress), suggesting a physiological role in plant osmoprotection (Kearns et al., 1998). Nodule-specific regulation of phosphatidylinositol transfer proteins has been described in *L. japonicus* (Kapranov et al., 2001). Thus, as the genes isolated via use of DD technique are also parts of signal transduction pathways, it seems that both methods used here are useful in identifying components of the symbiotic signal transduction pathway.

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