Use of Differential Display to Identify Novel *Sesbania rostrata* Genes Enhanced by *Azorhizobium caulinodans* Infection

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Upon infection of the tropical legume *Sesbania rostrata* with *Azorhizobium caulinodans* ORS571, nodules are formed on the roots as well as on the stems. Stem nodules appear at multiple predetermined sites consisting of dormant root primordia, which are positioned in vertical rows along the stem of the plant. We used the differential display method to isolate and characterize three cDNA clones (*di*fferential *di*splay; didi-2, didi-13, and didi-20), corresponding to genes whose expression is enhanced in the dormant root primordia after inoculation. Database searches revealed that the deduced (partial) didi-2 gene product shares significant similarity with hydroxyproline-rich cell wall proteins. The (partial) didi-13 and didi-20 products are similar to chitinases and chalcone reductases, respectively. Transcripts corresponding to the cDNA clones didi-2 and didi-13 were first detectable 1 day after inoculation. In contrast, didi-20 transcripts were found at low levels in uninfected root primordia and were enhanced significantly around 3 days after inoculation. In addition, a cDNA was isolated (didi-42) that corresponds to the previously identified leghemoglobin gene *Srlb6*. These studies show that differential display is a useful method for the isolation of infection-related genes.

Bacteria belonging to the genera *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium* are able to induce nodules on legume plants. The formation of these new plant organs, where the symbiotic nitrogen fixation process takes place, involves a highly specific signal exchange between the two symbionts. First, flavonoid compounds, secreted by the plant host, induce the rhizobial nodulation (*nod*) genes. The products of the *nod* genes are responsible for the production of the Nod factor(s), which initiate nodule formation in the host plant (for review, see Fisher and Long 1992; Dénarié and Cullimore 1993).

Significant progress has been made in the isolation and characterization of plant genes and proteins that play a role in the nodulation and symbiotic nitrogen fixation processes. Proteins that are induced during rhizobial infection or specifically found in nodules and are not, or barely, detectable in other tissues of the plant, especially roots, are called nodulins (reviewed in Franssen et al. 1992). The nodulins and the corresponding nodulin genes are generally divided in two groups, according to the time course of their appearance.

The early nodulins are reported to be expressed during the infection process and in nodule ontogeny. Examples of the corresponding genes are *Enod40*, *Enod12*, *Enod5*, *Enod2*, *Enod8*, and *MtPRP4* (Kouchi and Hata 1993; Franssen et al. 1987; Scheres et al. 1990a, 1990b; Dickstein et al. 1993; Wilson et al. 1994). Although the expression pattern and transcript localization of these early nodulin genes has been well documented, their function is not known.

The late nodulins are involved in nodule functioning and maintenance, and the corresponding genes are generally induced around the time the nitrogen fixation process commences. The most abundant proteins belonging to this class are the leghemoglobins (see de Bruijn and Schell 1992).

Sesbania rostrata is a tropical legume that grows in the Sahel region of West Africa. A typical feature of this plant is that, after infection with the bacterium *Azorhizobium caulinodans*, nodules are formed not only on the roots but also on the stem of the plant (Dreyfus and Dommergues 1981; Duhoux 1984; de Bruijn 1989). The stem nodules are formed at predetermined sites, corresponding to dormant root primordia, which are organized in vertical rows along the stem. At the distal part of these primordia a dormant root meristem is located that becomes activated to form adventitious roots by submersion in water. In contrast, the nodule primordium originates from cortical cells at the base of the root primordia. During both stem and root nodule development, the bacteria enter the plant by intercellular invasion and establish intercellular infection pockets (see de Bruijn 1989). By subsequently formed infection threads, the bacteria are delivered to the central nodule meristem (Ndoye et al. 1994).

Since stem nodulation occurs at predetermined sites and, in general, all the dormant root primordia that have been infected with bacteria develop into nodules, significant quantities of infected plant material can be harvested very precisely and in a synchronized fashion. This constitutes a distinct ad-

vantage for studying the nature and expression of plant genes involved in the early stages of the nodulation process.

By comparing two-dimensional polyacrylamide gel electrophoresis patterns of in vitro translation products of poly(A)⁺ RNA from uninfected *S. rostrata* roots and stems with poly(A)⁺ RNA from infected roots or stems, at least 16 nodule-specific proteins have been detected and about 10 proteins appear to be nodule-enhanced (de Lajudie and Huguet 1988). On the gene/cDNA level, only the early nodulin gene *Enod2* and four leghemoglobin gene homologs have been isolated and characterized (Metz et al. 1988; Welters et al. 1989; Strittmatter et al. 1989; Dehio and de Bruijn 1992).

Until recently, the most common methods for identifying differentially accumulated mRNAs in particular plant tissues have been differential screening and subtractive hybridization (see for instance Scheres et al. 1990b; Kouchi and Hata 1993; Cook et al. 1995). Although the subtractive procedures are more sensitive than the differential hybridization protocols, several problems still exist. These methods often require a lot of biological material, and are generally labor intensive.

The recently developed differential display technique circumvents these problems (Liang and Pardee 1992). The mRNA of a certain cell type is visualized by first performing a reverse transcription using a modified oligo(dT) primer followed by a polymerase chain reaction (PCR) amplification with the same oligo(dT) primer and a random decamer primer in the presence of ³⁵S-labeled nucleotides (nt). The labeled cDNA fragments obtained are separated on a denaturing polyacrylamide gel. Thus, the patterns of the amplified cDNA products of different mRNA samples can be compared side by side and differentially expressed cDNAs can be identified. For this method little biological material is needed and it is, therefore, particularly suitable for studying gene expression during early stages of nodule formation.

We used this method to compare noninfected root primordia on *S. rostrata* stems with primordia infected with *A. caulinodans* ORS571 at different time points following inoculation. Here, we report the isolation and characterization of three new cDNAs, and one previously identified cDNA, the expression of which is strongly enhanced during early stages of stem nodule formation.

RESULTS

Differential display: Comparison of mRNAs from infected versus noninfected *S. rostrata* root primordia.

Stems of 10-week-old *S. rostrata* plants were inoculated with *A. caulinodans* ORS571 and infected root primordia were excised at 2, 3, and 4 days after inoculation. Total RNA was extracted from infected and uninfected control tissues and used for differential display experiments (see Materials and Methods). A range of input RNA concentrations was examined. The use of 0.5 μg total RNA per cDNA synthesis and 1/10 of the resulting samples for subsequent PCR reactions was found to yield optimal results. Reducing input RNA to 0.2 μg yielded similar results, but after longer gel exposures, while increasing the input RNA to 1 μg resulted in higher gel backgrounds (data not shown).

Normally, 24- to 48-h exposures of the gels to X-ray films resulted in clearly visible amplification products (bands), up to about 650 nt in length. An example of these experiments using four different primer combinations is shown in Figure 1. In each case, RNA from infected tissues harvested 2 (+2), 3 (+3), and 4 (+4) days after inoculation was compared with RNA from two independent uninfected samples (–). In the example shown in Figure 1, one differential display (didi) product could be identified that was clearly enhanced after infection (arrow) and was used for further studies (didi-2; see below). A control reaction, omitting the reverse transcriptase (C), was run alongside the other samples. Although PCR products could be observed at times in the control reactions, these products generally did not comigrate with the bands observed in the differential display lanes (see Figure 1; as previously observed by Liang et al. 1993).

An average of 80 bands could be detected per lane and bands smaller than 300 bp were found to migrate as double
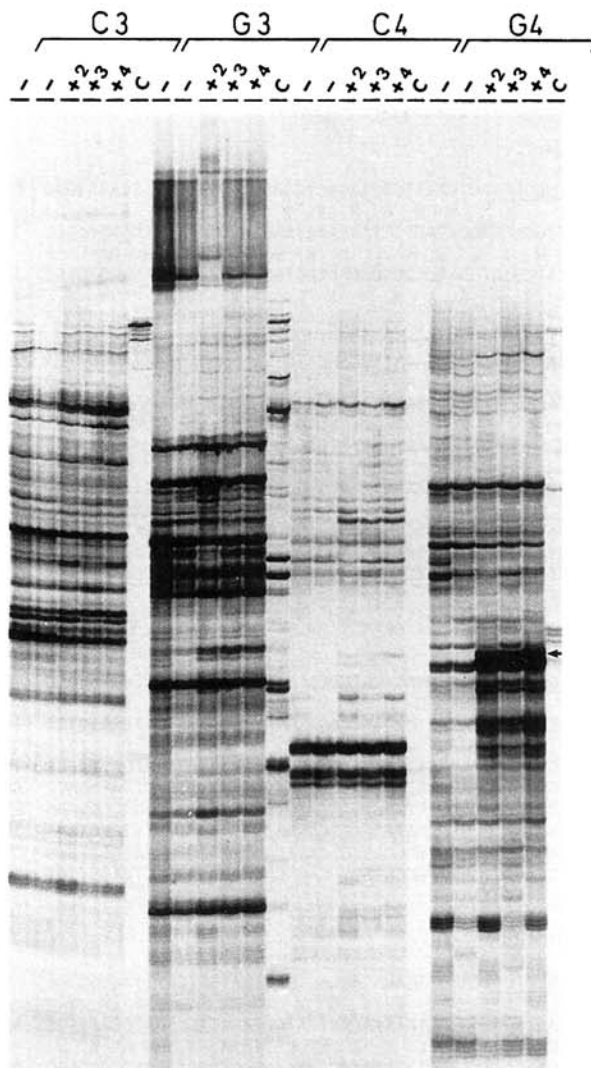

Fig. 1. Differential display of the results of 4 primer combinations. Two independent RNA amplification products of noninfected primordia (–) were run next to amplification products of infected root primordia harvested at 2 (+2), 3 (+3), and 4 (+4) days post inoculation. Lane C contains a control experiment, consisting of the same reaction as (+4) except that reverse transcriptase was omitted. C3, G3, C4, and G4 are designations of the primer combinations used. C = N and G = N of $T_{12}MN$ (see Materials and Methods); 3 and 4 denote different decamers (Ap-3 and Ap-4). The arrow indicates the differentially displayed product designated as didi-2.

bands or triplets, as described previously (Bauer et al. 1993; Liang et al. 1993).

In total, 35 different primer combinations were used and 45 bands that showed a clear enhancement or induction in the infected samples were identified. Seventeen of these bands were further processed. Fifteen of the samples yielded detectable DNA products after one round of reamplification. Subsequently, purified DNA from the reamplified bands was used as probe in Northern analyses to examine the expression pattern of the corresponding genes.

didi-2

```
TACTCCACCACACTACTATTATAAATCACCTCCTCCACCACCTTACCATC    50
        Y  Y  Y  K  S  P  P  P  P  P  Y  H
ATTAGATAGCGGACCTAAACAGAAAGTGTGAAAACAAAAGAAAGCCATGG   100
H  *
ACGAGAAGGTTATAAGATCGGGGATTCTTCATATATATTTCGTGATCAGT   150
ATTTGATTGTTGTTGAGAGGACTGTGTTTATTTACTTATTTTAATTGTGT   200
TGATGTTGTATTAGACAGTTGTAAGGCACTGCATGCCCCCTCATGTCTTG   250
ATCTGCAGTTGTCTATATGCATTGTAATTTCAAAGTTTGGTTTATCAAGC   300
TGACCAAGCCAAATAAATAAATATGTTATATTATTCCCTCCCGAAAAAAA   350
AAAAA                                               355
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didi-13

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CGTGGCAATACTCAAATGGCAACACTGGACCTCTTTTGGGGTCATGGGAC    50
          S  N  G  N  T  G  P  L  L  G  S  W  D
GCTTGGACATCCTTAGTTCTACCAAATAACACCGTATTCATGGGACTCCC   100
A  W  T  S  L  V  L  P  N  N  T  V  F  M  G  L  P
AGCATCGCGTGAAGCTGCTCCCAGTGGTGGTTATATACCACCAAATGTGC   150
A  S  R  E  A  A  P  S  G  G  Y  I  P  P  N  V
TGATTTCTGAGGTACTTCCTTACATAAAACAAGCTTCCAACTACGGAGGA   200
L  I  S  E  V  L  P  Y  I  K  Q  A  S  N  Y  G  G
ATTATGTTATGGAGCAGATTCCAAGATGTTACAAATCACTATAGCGATCA   250
I  M  L  W  S  R  F  Q  D  V  T  N  H  Y  S  D  Q
GATAAAGTATTATGTTACAAAATATATGTTGCGGTTTGTGAAGGCAGTTT   300
I  K  Y  Y  V  T  K  Y  M  L  R  F  V  K  A  V
CCAACGCAATATCTGACTGTGTGTCAGCAGCTTTGCACCGCTTCTTACCC   350
S  N  A  I  S  D  C  V  S  A  A  L  H  R  F  L  P
AAACCATATTGAAATAAAATATATATGCTGCTATATATCTATGAATGTAC   400
K  P  Y  *
GGAATAAGCAGCTTATTGTTGCCTTTGACTTCCTATGTTATATAGTATGT   450
CTGTGTAACCAGCGCGAATCCAAGCATGTTTGATCTTTTGTAATGGATTT   500
TGCTTTGAATAATAATCACTAGCTATTGACTATTGTGGTCCGAAAAAAAA   550
AAAA                                                554
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didi-20

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CGTGGCAATATACAGGAGACATGTGGCTATCTGAAAATGGGTCTTGCAAG    50
         T  G  D  M  W  L  S  E  N  G  S  C  K
ACCTTAGAAGAACTTTGGGATGGAGATGTGTGAAAATTTCTTCAAAGTCT   100
T  L  E  E  L  W  D  *
AAACTATATTTGTAGAATATATATTAGGAAAATAAACTTCAAGATTTGCT   150
CATGTATTAGCATGAGCAATCATGGTTGGCTCTATATTCATTTATAGACC   200
TAGCTAACCTCAGTATTTACTTCAGATGTGTCTTTCAATAAGACCTTGAG   250
TTGTGACTTGAGTTCAATAAGAGTGAGAATTTGACATTTGTGTGAGAGGA   300
GTGAGGATGTGGTGTTGCCACTGTCATTCAAATGTAGCCTCAGATATGTG   350
GTTCCATGGTCATGTGCCATGCTTCAAACATGTGAAAGCTCTATGGTTCT   400
CCAAAAAAAAAAAAA                                     414
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didi-42

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AGGGCCTGTTCATGTTCAGAAAGGAGTCACTGATGCTCATTTTGTGGTGG    50
         V  Q  K  G  V  T  D  A  H  F  V  V
TTAAAGAGGCACTGCTGAAAACAGTGAAGGAAGCCACAGGAGCCAAATGG   100
V  K  E  A  L  L  K  T  V  K  E  A  T  G  A  K  W
AGCGATGAGTTGAGCAATGCTTGGGAAGTAGCCTATGATGAATTGGCAGC   150
S  D  E  L  S  N  A  W  E  V  A  Y  D  E  L  A  A
TGCTATTAAGAAAGCGATGGGTTGAACTTGAAATCTATATTTTTTTGGTG   200
A  I  K  K  A  M  G  *
TAAAACTTCAAATCTATATCATATTGCCATAAAGGCATAAATAATTATAA   250
TAAATAATTTTTATTTCATTGAGTTGCCCCGTATAAATGTTCTTTAAGAT   300
GAGTATATTATCGTTTGGATAAACAATATAAAGTTATTTACGTTTGGCAA   350
AAAAAAAAAA                                          360
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Fig. 2. DNA sequence of the 4 differential display cDNAs (did-2, -13, -20, and -42) analyzed and their deduced amino acid sequence. The primers used for the differential display reaction are underlined.

Four reamplified didi products corresponded to nodule-enhanced mRNAs, five corresponded to constitutively expressed mRNAs, three yielded complex hybridization patterns, and three did not reveal any detectable hybridization signals. The latter may correspond to very low abundant mRNAs. The four nodule-enhanced didi products were cloned in a pBluescript KS (–) vector (Stratagene) and used for further analysis.

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1  SNGNTGPLLG  SWDAWTSLVL  PNNTVFMGLP  ASREAAPSGG
2  SSGNTQNLFD  SWNKWTT.SI  AAQKFFLGLP  AAPEAADS.G
3  SSGNINNIIN  SWNRWTT.SI  NAGKIFLGLP  AAPEAAGS.G
4  SNGNINNLVN  AWNQW.T.SS  QAKQVFLGVP  ASDAAAPSGG
5  SSGNTNDLIS  SWNQW.T.SS  QAKQLFLGVP  ASTAAAGS.G
6  A.DNADNLLS  SWNQW.T.AF  PTSKLYMGLP  AAREAAPSGG
```

```
1  YIPPNVLISE  VLPYIKQASN  YGGIMLWSRF  QDVTNHYSDQ
2  YIPPDVLTSQ  ILPTLKKSRK  YGGVMLWSKF  WDDKNGYSSS
3  YVPPDVLISR  ILPEIKKSPK  YGGVMLWSKF  YDDKNGYSSS
4  LIPADVLTSQ  VLPAIKTSPK  YGGVMIWDRF  NDAQSGYSNA
5  FIPADVLTSQ  VLPTIKGSSK  YGGVMLWDRF  NDGQSGYSGA
6  FIPADVLISQ  VLPTIKASSN  YGGVMLWSKA  FD..NGYSDS
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1  IKYYVTKYML  RFVKAVSNAI  SDCVSAALHR  FLPKPY*
2  ILASV.....  ..........  ..........  .......
3  ILDSV.....  ..........  ..........  .......
4  IKGSV.....  ..........  ..........  .......
5  IIGSV.....  ..........  ..........  .......
6  IKGSIG....  ..........  ..........  .......
```

Fig. 3. Amino acid comparison of the deduced differential display (didi)–13 protein product with different class III chitinases. 1 = didi-13; 2 = *Arabidopsis* chitinase (Samac et al. 1990); 3 = Para rubber tree chitinase (SP accession number P23472); 4 = chickpea chitinase (SP accession number P36908); 5 = adzuki bean chitinase (PIR accession number S36932); and 6 = cucumber chitinase (Metraux et al. 1989). The identical residues are highlighted.

```
TGGAAATAGCCACCATTCCTCCTGCAGTCAATCAGGTGGAAATGAACCCA   50
   E  I  A  T  I  P  P  A  V  N  Q  V  E  M  N  P
TCCTGGCAGCAAGGGAATCTCAGGGAGTTCTGCAAGCAGAAAGGAATTCA  100
S  W  Q  Q  R  N  L  R  E  F  C  K  Q  K  G  I  H
TGTTAGTGCTTGGTCACCTCTTGGAGCCTATAAGATATTTTGGGGTTCAG  150
   V  S  A  W  S  P  L  G  A  Y  K  I  F  W  G  S
GAGCTGTCATGGAGAATCAAATCCTCCAAGACATAGCAACAGCAAAAGGG  200
G  A  V  M  E  N  Q  I  L  Q  D  I  A  T  A  K  G
AAGACCATAGCTCAGGTTGCACTTAGATGGGTGTACCAACAGGGTCAGG   250
K  T  I  A  Q  V  A  L  R  W  V  Y  Q  Q  G  S  S
TGCAATGGCTAAAAGCTTCAACAAGGAGAGGATGAAACAAAACCTTGAAA  300
   A  M  A  K  S  F  N  K  E  R  M  K  Q  N  L  E
TATTTGACTTCGAGTTGAGTGAAGAAGAATTAGAGAAGATTAAGCAGATT  350
I  F  D  F  E  L  S  E  E  E  L  E  K  I  K  Q  I
CCACAGCGCCAGGCAATATACAGGAGACATGTGGCTATCTGAAAATGGGTC  400
   P  Q  R  R  Q  Y  T  G  D  M  W  L  S  E  N  G  S
TTGCAAGACCTTAGAAGAACTTTGGGATGGAGATGTGTGAAAATTTCTTC  450
   C  K  T  L  E  E  L  W  D  *
AAAGTCTAAAACTATATTTGTAGAATATATATTAGGAAAATAAACTTCAAG  500
ATTTGCTCATGTATTAGCATGAGCAATCATGGTTGGCTCTATATTCATTT  550
ATAGACCTAGCTAACCTCAGTATTTACTTCAGATGTGTCTTTCAATAAGA  600
CCTTGAGTTGTGACTTGAGTTCAATAAGAGTGAGAATTTGACATTTGTGT  650
GAGAGGAGTGGAGGATGTGGTGTTGCCACTGTCATTCAAATGTAGCCTCA  700
GATATGTGGTTCCATGGTCATGTGCCATGCTTCAAACATGTGAAAGCTCT  750
ATGGTTCTCCAAAAAAAAAAAAAA                            772
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Fig. 4. Extended DNA sequence of differential display (didi)–20 and its deduced amino acid sequence. The antisense primers used for the 5' rapid amplification of cDNA ends are highlighted.

DNA sequence analysis.

Since the reamplified didi products could be composed of more than one cDNA fragment, six individual transformants derived from the same excised band were used for DNA sequence analysis. In fact, in three out of the four cases examined, multiple DNA sequences were observed.

New Northern analyses were performed with the cloned inserts as probe to identify the cloned cDNAs corresponding to the induced mRNAs (see below). All these clones contained the decamer and the oligo(dT) primer used for the display and part of an open reading frame (Fig. 2).

Database searches (EMBL, Genbank, and Swissprot databanks) revealed the following similarities. At the nucleotide level, the 355-bp didi-2 fragment showed 76% identity with the gene corresponding to the hydroxyproline-rich glycoprotein (sbHRGP3) from soybean (Hong et al. 1994). The short carboxy-terminal part of the open reading frame (ORF) present in didi-2 contained a hydroxyproline-rich motif similar to the hydroxyproline-rich repeat from sbHRGP3 and hydroxyproline-rich proteins from other plant species (reviewed in Showalter 1993; data not shown).

At the nucleotide level, the 554-bp didi-13 fragment showed 71% identity with the chitinase CHI2 gene from cucumber (Metraux et al. 1989; Lawton et al. 1994). At the amino acid level 66% similarity was also found with the acidic class III chitinase from Arabidopsis (Samac et al. 1990). The amino acid comparison of the deduced didi-13 product with several class III chitinases is shown in Figure 3.

The didi-20 DNA sequence revealed no identity to known sequences in the database. To obtain further DNA sequence information about the ORF from the didi-20 clone, a 5' rapid amplification of cDNA ends (RACE) experiment was performed (see Materials and Methods). The DNA sequences corresponding to the antisense primers used for the RACE reaction are highlighted in Figure 4. RNA from 4-day-infected root primordia was used for reverse transcription. Figure 4 shows the extended partial sequence of the didi-20 cDNA. Similarity searches revealed that the deduced didi-20 product was 70% similar to chalcone reductases from soybean and alfalfa (Fig. 5A). Figure 5B shows the comparison between the sequence of the decamer used for didi-20 amplification and the sequence of the RACE fragment. The decamer hybridized to the sequence with two mismatches and with 1 nucleotide missing. This is in agreement with the results of Liang and Pardee (1992) who found, after consider-

A

```
1  ........EI ATIPPAVNQV EMNPSWQQGN LREFCKQKGI
2  VKKLENLLSV ATVLPAVNQV EMNLAWQQKK LREFCNANGI
3  VKKLENLLSV ATVLPAVNQV EMNLAWQQKK LREFCNAHGI
4  VKKLQNLLSV ATIRPVVDQV EMNLAWQQKK LREFCKENGI


1  HVSAWSPL.. GAYKIFWGSG AVMENQILQD IATAKGKTIA
2  VLTAFSPLRK GA...SRGPN EVMENDMLKE IADAHGKSVA
3  VLTAFSPVRK GA...SRGPN EVMENDMLKE IADAHGKSVA
4  IVTAFSPLRK GA...SRGPN EVMENDVLKE IAEAHGKSIA


1  QVALRWVYQQ GSSAMAKSFN KERMKQNLEI FDFELSEEEL
2  QISLRWLYEQ GVTFVPKSYD KERMNQNLCI FDWSLTKEDH
3  QISLRWLYEQ GVTFVPKSYD KERMNQNLRI FDWSLTKEDH
4  QVSLRWLYEQ GVTFVPKSYD KERMNQNLHI FDWALTEQDH


1  EKIKQIPQRR QYTGDMWLSE NGSCKTLEEL WDG*
2  EKIDQIKQNR LIPGPTK... ....PGLNDL YDD*
3  EKIAQIKQNR LIPGPTK... ....PGLNDL YDD*
4  HKISQISQSR LISGPTK... ....PQLADL WDDQI*
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B

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357         367
CGCAGGCAATA
CGTGG CAATA
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Fig. 5. Amino acid comparison of the deduced differential display (didi) –20 protein product with chalcone reductases from soybean and alfalfa. **A,** 1 = didi-20; 2 = alfalfa chalcone reductase (accession number U13925); 3 = alfalfa chalcone reductase (accession number U13924); and 4 = soybean chalcone reductase (Welle et al. 1991). Identical residues are highlighted. **B,** Comparison between the actual DNA sequence and the DNA sequence of the decamer used for didi-20 amplification.
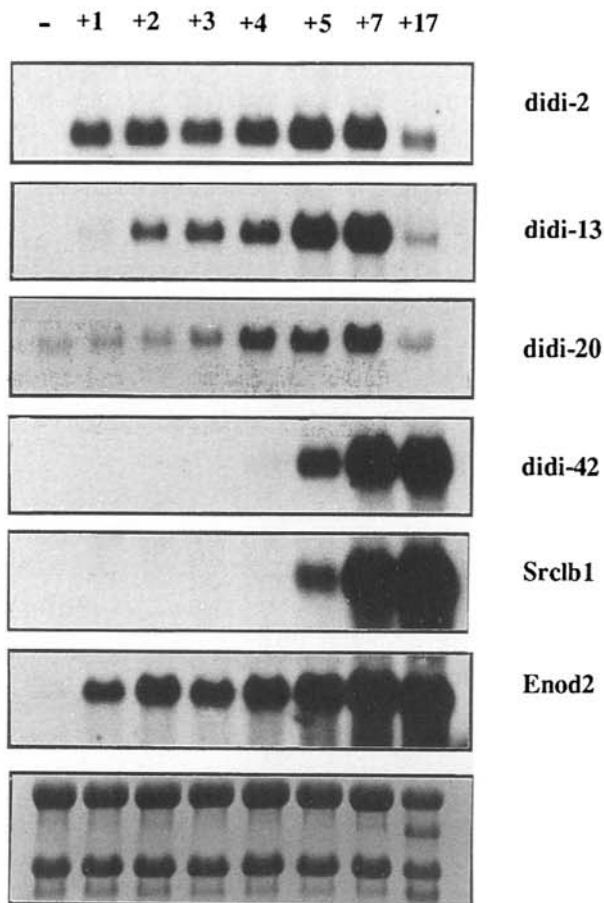

Fig. 6. Northern blot analysis of transcripts hybridizing to differential display (didi) and other nodulin gene probes. RNA from noninfected root primordia (–) was run next to RNA of primordia harvested at several days after inoculation (+1, +2, +3, +4, +5, +7, +17) and hybridized with the probes indicated on the right of the panels. Blots were stained with methylene blue, to show equal loading (Sambrook et al. 1989; bottom panel). *Srclb*1 corresponds to the *S. rostrata* genomic clone *Srglb*2.

ing theoretical calculations and experimental data, that during the PCR conditions the decamers hybridized in a degenerate fashion.

The didi-42 DNA sequence was found to be identical to leghemoglobin *Srlb*6 cDNA of *S. rostrata* (Strittmatter et al. 1989). Southern blot analysis revealed that the four didi products corresponded to *S. rostrata* genes (data not shown).

Temporal expression patterns of the genes corresponding to the didi cDNAs.

The time course of appearance of the mRNAs corresponding to the different didi clones during stem nodule development was determined by RNA Northern blot analysis. Identical blots containing total RNA from noninfected root primordia and infected primordia harvested 1, 2, 3, 4, 5, 7, and 17 days after inoculation were hybridized with the four didi cDNAs (didi-2, didi-13, didi-20, and didi-42). The *S. rostrata Srclb*1 cDNA, which corresponds to the leghemoglobin *Srglb*2 gene (Metz et al. 1988) and the *S. rostrata Enod*2 gene (Dehio and de Bruijn 1992) were included in the time course experiment as reference probes. The results of these experiments are shown in Figure 6.

*Enod*2 mRNA was found to be already enhanced 1 day after inoculation. Its level increased further up to 7 days, after which it remained constant. Overexposure of the Northern blot revealed that *Enod*2 mRNA is also present at a low level in noninfected root primordia. *Srclb*1 mRNA could be observed 5 days after inoculation and the hybridization signal increased during the entire time course experiment. No hybridization signal could be detected at earlier time points, not even after an extended exposure time.

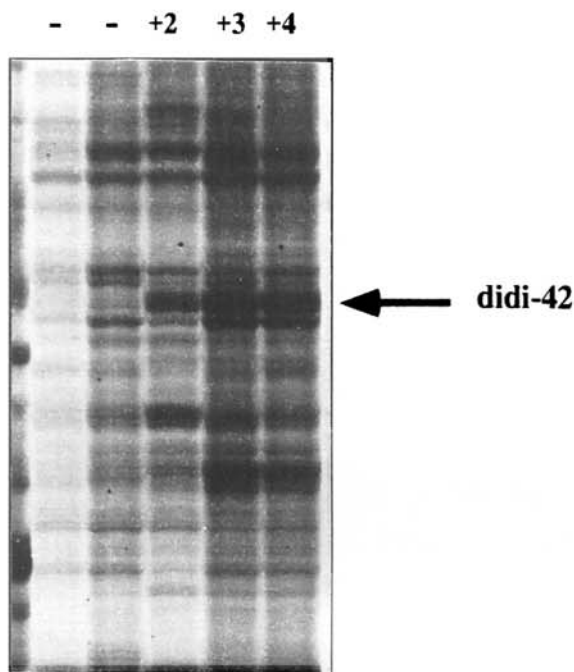The mRNA hybridizing with the didi-2 probe was found to be strongly enhanced 1 day after inoculation. The hybridiza-

tion signal remained constant up to 7 days after inoculation and then diminished. Overexposure of the Northern blot revealed that the mRNA hybridizing with didi-2 was also present at a low level in the noninfected root primordia. The didi-2 probe recognized a transcript of about 900 nt, indicating that about 550 nt were still missing from the DNA sequence shown in Figure 2.

The mRNA hybridizing to didi-13 was first observed 1 day after inoculation and increased up to day 7. The hybridization signal was strongly reduced in the 17-day-old nodule sample, as observed with didi-2. The size of the mRNA detected by didi-13 was found to be approximately 1,100 nt in length.

The mRNA hybridizing with didi-20 could be observed at a basal level in the noninfected root primordia. The hybridization signal increased slowly until day 7 and, as observed with didi-2 and didi-13, the didi-20 signal was significantly reduced in 17-day-old nodules. The size of the mRNA detected by didi-20 was found to be approximately 1,200 nt.

Finally, the mRNA hybridizing to didi-42, corresponding to the *Srlb*6 cDNA, could be observed 4 days after inoculation. However, on the didi gel, the didi-42 band was already clearly visible after 2 days (Figure 7).
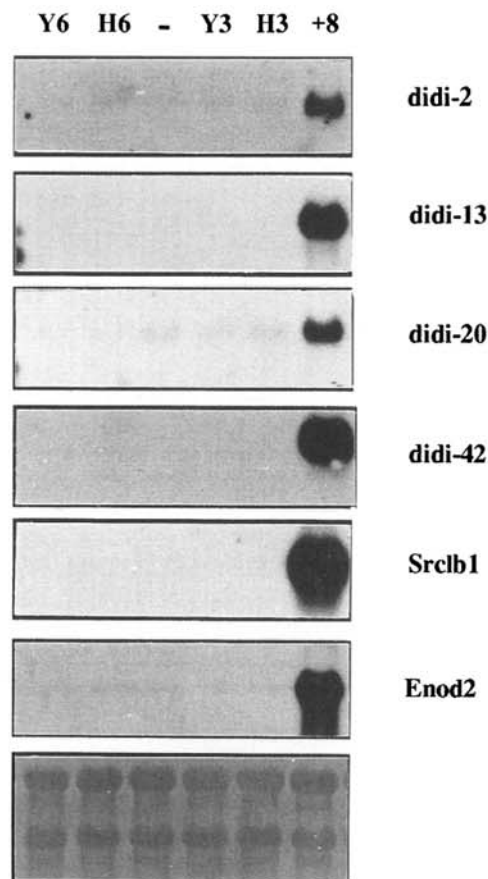
Fig. 8. Control Northern blot experiments. RNA from primordia inoculated with water alone (H; 3 and 6 days after treatment) and with water containing a residual amount of YEB medium (0.5% beef extract; 0.5% peptone; 0.1% yeast extract; 0.5% sucrose; 0.002 M MgSO₄), (Y; 3 and 6 days after treatment) was compared with RNA from nontreated root primordia (–) and 8-day-old nodules (+8). For other details see Figure 6 caption.

Fig. 7. Differential display of didi-42. Amplification products of RNA from noninfected primordia (–) were run next to samples isolated from infected primordia 2 (+2), 3 (+3), and 4 (+4) days after inoculation. The arrow indicates the didi-42 product.

To determine whether the observed enhancements of mRNA levels corresponding to the didi cDNAs could be correlated to the presence of the infecting bacteria, the following control experiments were carried out. Since dormant root primordia can develop adventitious roots upon submersion in water or exposure to humid conditions and since the root primordia were moistened during inoculation (see Materials and Methods), we examined whether the observed mRNA enhancements could be due to the effect of water (moisture) alone. Moreover, since it has been shown that commercially available yeast extract contains elicitors that can induce stress-related responses in tomato culture cells (Basse and Boller 1992) and three of the four isolated cDNAs could be correlated with stress response genes (for review, see Dixon and Lamb 1990), we examined whether the residual amount of YEB medium in the bacterial inoculum (see Materials and Methods) could be the activator of gene expression.

The results of these control experiments are shown in Figure 8. RNA isolated from untreated root primordia was compared with RNA from primordia treated with water or with water containing a residual amount of YEB medium. A lane with RNA from root primordia 8 days after A. caulinodans infection was included for comparison. Neither water alone nor water plus YEB medium was found to enhance the level of any of the four mRNAs hybridizing with the didi cDNAs, strongly suggesting that the expression patterns described in Figure 6 were caused by the presence of the bacteria.

DISCUSSION

Here, we report the isolation, using the differential display method (Liang and Pardee 1992), of four cDNAs corresponding to mRNAs that are enhanced at early stages of S. rostrata stem nodulation. This method has already been used successfully to isolate differentially expressed cDNAs from mammalian tissues, such as pre-implantation mouse embryos (Zimmermann and Schultz 1994), mammary tumor cells (Liang et al. 1992), and glucose-induced bovine aortic smooth muscle cells (Nishio et al. 1994). However, this is one of the first reports describing the use of this technique to isolate differentially expressed cDNAs from specific plant tissues.

The fact that S. rostrata stem nodulation occurs synchronously at predetermined sites (Dreyfus and Dommergues 1981; Duhoux 1984; de Bruijn 1989) provided us with a sensitive system to examine plant gene expression during early stages of nodule development. RNA of noninoculated root primordia could be directly compared with RNA from root primordia infected with A. caulinodans ORS571, isolated 2, 3, and 4 days after inoculation.

In total, 35 primer combinations were used and 45 bands were identified that were enhanced in the infected tissues. Fifteen of these didi products were further analyzed. Initially, the collection of didi products was screened for those corresponding to infection-enhanced genes by using their reamplified DNA as a probe in Northern analysis. Only those didi products hybridizing to a clearly induced or enhanced mRNA were selected for further analysis and cloned. The resulting individually cloned cDNAs (six per didi band) were then used again as probes for Northern blots in a second screening to identify those giving a similar expression pattern, as observed with the total didi band probe. Since the reamplified didi bands frequently contain a mixture of DNA fragments, the pre-screen was necessary to avoid missing interesting cDNAs.

Our subsequent DNA sequence similarity searches at the nucleotide and deduced amino acid levels generated several interesting findings. The didi-2 cDNA was found to share 76% identity at the nucleotide level with the hydroxyproline-rich glycoprotein sbHRGP3 of soybean (Hong et al. 1994). The short segment of the ORF present in the didi-2 cDNA contained a SerPro$_4$ sequence that has been observed in several extensin proteins in other plants (for review see Showalter 1993). The YYYK motif observed resembles the YXYK motif found in several other HRGPs. The Y residues at the first and third position are thought to be involved in the formation of intramolecular isodityrosine cross-links (Epstein and Lamport 1984).

The didi-13 cDNA was found to share 71% identity with the CHI2 gene of cucumber (Metraux et al. 1989; Lawton et al. 1994). In addition, at the amino acid level, a 66% similarity was observed with the acidic class III chitinase from Arabidopsis (Samac et al. 1990). As shown in Figure 3, the deduced didi-13 partial protein product has a carboxy-terminal extension of 31 amino acids, when compared with the other chitinase sequences. This is not completely unique, since one of the three genes that encode the isoforms of the class III chitinase in cucumber (CHI1) also contains a carboxy-terminal extension of 30 amino acids. It has been suggested that this sequence may target the protein to the vacuole (Lawton et al. 1994).

The deduced didi-20 product was found to share 70% similarity with chalcone reductases from soybean and alfalfa (see Fig. 5A). Significant similarities were also found with other reductases, mostly those active in carbohydrate metabolism (Welle et al. 1991).

The didi-42 cDNA was found to be identical to Srlb6 (Strittmatter et al. 1989). Surprisingly, the mRNA corresponding to didi-42 was found to be expressed quite early during stem nodule development, since the didi-42 band could already be clearly observed in the 2-day sample (Fig. 7). This finding contrasts with the general view that leghemoglobin genes are late nodulin genes. It has been shown previously that the soybean lb genes are induced biphasically, with a low level of induction at 7 to 8 days after inoculation and a major enhancement at around day 12 (Marcker et al. 1984). In our Northern blot experiments, mRNA hybridizing with didi-42 (Srlb6) could be clearly detected at day 5 after inoculation (Fig. 6). The detection of lb mRNA at day 2 using didi may simply reflect the higher sensitivity of this method to detect minor mRNA species. RT-PCR–based methods should therefore be used to further re-examine lb gene expression patterns. It is likely that the lb gene(s) may be induced at early stages of rhizobial infection.

The expression of the four induced genes corresponding to the four didi clones was investigated in noninfected root primordia and in primordia at several stages after infection, in comparison with the expression of the previously characterized S. rostrata Enod2 and Srclb1 clones (Metz et al. 1988; Dehio and de Bruijn 1992). Like Enod2 mRNA, mRNA corresponding to didi-2 was already significantly enhanced 1 day after bacterial inoculation, whereas mRNA corresponding to didi-13 only appeared slightly enhanced at this time. Didi-20 mRNA has a basal level in noninfected primordia and only

from day 3 on the expression level increased significantly. The level of mRNAs hybridizing to didi-2, didi-13, and didi-20 was observed to decrease in 17-day-old nodules. Although equal amounts of RNA were loaded, the sample from this time point contains considerably more bacterial RNA than samples isolated at earlier time points, as evidenced by the appearance of clear 16S and 23S mRNA bands (see Fig. 6). The relative reduction of plant mRNA in these samples could result in decreased hybridization levels relative to samples containing little or no bacterial RNA.

With regard to the potential role of the novel infection-enhanced *S. rostrata* genes, a number of speculations can be made. Chitinases, enzymes from the phenylpropanoid pathway and hydroxyproline-rich cell wall proteins, have been implicated in plant responses to pathogen attack (Dixon and Lamb 1990), but also in plant development. For example, chitinases are able to rescue a temperature-sensitive embryogenic carrot mutant line (De Jong et al. 1992) and flavonoid compounds can act as auxin transport inhibitors (Jacobs and Rubery 1988).

Three possible functions for nodule-enhanced chitinases have been proposed. Chitinase activity, detected in the nodule cortex of mature nodules (Staehelin et al. 1992), could play a role in protection against external pathogens. Secondly, chitinases could be involved in regulation of the nodulation, as part of a hypersensitive response that is induced when infection threads are arrested (Vasse et al. 1993). Finally, chitinases could control the quantity and biological activity of Nod factors by degrading them (Staehelin et al. 1994). Evidence has been presented by Schultze et al. (1994) showing that *Medicago* roots respond specifically to Nod factor by the induction of a Nod-factor-degrading activity.

Several proline-rich proteins have been shown to be involved in the symbiosis. The early nodulins ENOD2 and ENOD13 have been correlated with nodule morphogenesis (Franssen et al. 1987; Franssen et al. 1988; van de Wiel et al. 1990), while ENOD12 and MsENOD10 have been correlated with the nodule invasion process (Scheres et al. 1990a; Löbler and Hirsch 1993). Moreover a new proline-rich cDNA (MtPRP4) has been shown to be expressed in the persistent meristem of alfalfa nodules (Wilson et al. 1994). All these proteins contain the typical Pro_2 sequence, included in a penta-repeat motif. In contrast, the carboxy-terminal part of the didi-2 ORF was found to contain a $SerPro_4$ sequence, which is typical for glycosylated extensins, another group of hydroxyproline-rich proteins (for review, see Showalter 1993; Kieliszewski and Lamport 1994).

Proteins from the phenylpropanoid pathway could be involved in the enhanced synthesis of *nod*-gene-inducing flavonoids, as accentuated by the Ini effect (van Brussel et al. 1990; Recourt et al. 1991). Alternatively, the enzymes could produce defense-related phytoalexins to control nodulation (Vasse et al. 1993) or be responsible for the production of flavonoid compounds to regulate hormone balances (Yang et al. 1992).

In the case of *A. caulinodans* ORS571, the most effective *nod* gene inducer is 7,4′-dihydroxyflavanone (liquiritigenin; Messens et al. 1991). Chalcone reductase, in concert with chalcone synthase, opens the branched pathway toward the direction of the synthesis of this molecule (Welle et al. 1991) so it is tempting to speculate that the enhancement of the didi-20 mRNA reflects the previously described Ini effect.

Clearly, further investigations need to be carried out to elucidate the role of the novel genes and their expression patterns. Full-length cDNA clones will be isolated, biochemical studies will be carried out to verify the proposed functions of the didi-13 and didi-20 gene products, and in situ hybridizations will be performed to determine spatial expression patterns. It remains to be determined whether the induction or enhancement pattern of the genes described here is *A. caulinodans* specific. However, in preliminary experiments with the didi-13 clone, no enhancement of expression could be observed upon inoculation of *S. rostrata* with a NodA⁻ *A. caulinodans* strain (V44; Van den Eede et al. 1987).

Our experiments allow us to conclude that the differential display technology is likely to be extremely useful for the identification of novel infection/nodulation-enhanced genes. This observation has recently been extended using the model legume system *Lotus japonicus* (K. Szczyglowski, P. Kapranov, D. Hamburger, and F. J. de Bruijn, unpublished).

MATERIALS AND METHODS

Biological material.

Sesbania rostrata seeds were sterilized as described by Goethals et al. (1989), and were germinated on petri dishes in the dark at 28°C for 2 days. Next, the seedlings were transferred to pots containing 50% potting soil and 50% sand. The plants were grown at 28°C with a light period of 16 h for 2 to 3 months. The root primordia on the stems were infected with *A. caulinodans* ORS571 by painting the stem with the bacterial inoculum. The inoculum was prepared by growing a culture of ORS571 overnight in YEB medium (0.5% beef extract; 0.5% peptone; 0.1% yeast extract; 0.5% sucrose; 0.002 M $MgSO_4$), centrifuging the cells, and resuspending them in half of the starting volume in water. The plant material was harvested by peeling the root primordia off the stem and freezing the tissue directly in liquid nitrogen. For control experiments, the stems were painted with water alone or with a solution containing 1 ml of YEB medium per 50 ml of water.

RNA isolation.

RNA was extracted using a method based on LiCl precipitation. Plant material (300 mg) was ground in liquid nitrogen and transferred to a 1.5-ml microfuge tube. Extraction buffer (500 µl) (0.2 M Tris-HCl, pH 7.5; 0.1 M LiCl; 5 mM EDTA; 1% sodium dodecyl sulfate [SDS]) and 500 µl phenol:chloroform (1:1) was added, the tubes were vortexed for 30 s and centrifuged for 5 min. The extraction was repeated once and the RNA was precipitated by adding 1 volume of 6 M LiCl to the aqueous phase and incubating on ice for 4 h. Then the samples were centrifuged for 10 min and the pellets were rinsed with 3 M LiCl. After centrifugation the pellets were redissolved in diethylpyrocarbonate (DEPC)–treated water. The RNA was precipitated again by adding 0.1 vol. of 3 M NaOAc and 2 vol. of EtOH. The precipitation was allowed to proceed overnight at –20°C and subsequently the RNA was pelleted, washed with 70% ethanol, and redissolved in 50 µl of water. Subsequently, the RNA samples were DNaseI treated, as described by Sambrook et al. (1989), using RQ1 DNase (Promega, Madison, WI).

Differential display.

Total RNA (0.5 µg) was used for cDNA synthesis. All solutions and enzymes used, except the *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT), were included in the RNAmap system (Genhunter Corporation, Brookline, MA). The reverse transcription (RT) primer consists of $dT_{12}MN$ where M denotes a mixture of dA, dC, and dG, and N denotes one of the four nucleotides. The primer is degenerate with regard to the second nucleotide from the end since it was found that the final display patterns are independent of this nucleotide (Liang et al. 1993). The reactions were performed according to the instructions provided in the manual of the kit, except that after each PCR cycle an extension of 2 s was added to the elongation step. All the 20 decamers tested (Ap-1 to Ap-20) were used in combination with the $T_{12}MC$ and $T_{12}MG$ primers. Bands were purified from the polyacrylamide gels according to the instructions provided in the manual of the RNAmap kit. After reamplification of the bands of interest, the fragments were rendered blunt ended using the Klenow fragment of DNA polymerase (Boehringer, Mannheim, Germany) and phosphorylated with T_4 DNA kinase (Boehringer Mannheim), according to the protocols described by Sambrook et al. (1989). Subsequently they were cloned in a pBluescript KS (−) vector (Stratagene, La Jolla, CA).

Northern blot hybridizations.

Total RNA (7 µg in Figure 6 and 5 µg in Figure 8) was separated on a 1% agarose gel containing 2% formaldehyde and was transferred to Hybond-N filters (Amersham, Aylesbury, U.K.), as described by Sambrook et al. (1989). Prehybridization and hybridization experiments were performed according to Church and Gilbert (1984) with the following modifications: the (pre)hybridization solutions contained 7% SDS, 1% bovine serum albumin, and 0.5 M sodium phosphate buffer (pH 7.4) and both the prehybridization and hybridization were performed at 65°C. ^{32}P probes were generated using the "quickprime" kit (Pharmacia, Uppsala, Sweden). Washing steps were performed at 65°C; 2 × 15 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS; 2 × 15 min with 1× SSC, 0.1% SDS, and 1 × 15 min with 0.1× SSC, 0.1% SDS. Blots were exposed to Fuji film overnight to 3 days. To verify equal loading and transfer of RNA, filters were stained with methylene blue, according to Sambrook et al. (1989).

DNA sequence analysis.

DNA sequencing procedures were carried out essentially as described by Sanger et al. (1977). DNA sequence data were assembled and analyzed using the GCG package (version 7; Genetics Computer Group, Madison, WI). The percentage of similarity between sequences was determined using the GAP program of GCG.

5′ RACE reaction.

5′ rapid amplification of cDNA ends (RACE) reactions were performed as described in the manual from the corresponding kit (Gibco BRL, Gaithersburg, MD). Antisense primers were synthesized on a model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA), according to the manufacturer's recommendations. The RACE products were cloned into the pGEMT vector (Promega).

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