Identification of Novel Putative Regulatory Genes Induced During Alfalfa Nodule Development with a Cold-Plaque Screening Procedure

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Until now very few plant genes with possible regulatory functions during nodule development have been isolated. We have used a modified cold-plaque screening method to identify new transcripts expressed at low levels that are induced during nodulation. Several clones were isolated and characterized by their mRNA expression patterns during nodule development and in spontaneous nodules. Sequence homology with known genes of other organisms indicated that transcripts corresponded to (i) “basic” genes probably required during the growth of the nodule organ (e.g., structural proteins), (ii) genes related to the metabolic adaptations taking place during nodule morphogenesis and function (e.g., carbonic anhydrase), and (iii) genes containing regulatory motifs and/or homologies (three clones out of the 20 identified). The latter genes encode a zinc-finger-containing protein, a putative protein kinase, and a Wilm’s tumor (WT) suppressor homologue, respectively. Expression of the kinase and WT suppressor homologues was induced early in nodulation, although the latter was activated transiently. Accumulation of the Zn-finger gene transcripts was detected at a later stage of development and seems to be regulated in a complex manner. Hence, using a cold-plaque screening procedure, we could identify genes that may play regulatory roles in the signal transduction pathways activated during nodule development.

Additional keyword: nodulins.

Bacteria belonging to the genus *Rhizobium* are able to induce nodules on legume roots. The formation of these new organs relies on the exchange of highly specific molecular signals between plant and bacteria for a spatio-temporal coor-

dination of gene expression of both partners (see Mylona et al. 1995 and references therein). In response to flavonoids secreted by the plant host, the nodulation (*nod*) genes of *Rhizobium* are induced, leading to Nod factor synthesis (Schultze et al. 1994). These lipochitooligosaccharide signals alone are able to trigger nodule development. Nevertheless, this developmental program might preexist in legumes since certain *Medicago sativa* (alfalfa) cultivars have the capacity to form related organs, the spontaneous nodules, even in the absence of *Rhizobium* (NAR⁺ phenotype; Truchet et al. 1989). Genes whose expression is induced during nodule development and in nitrogen-fixing nodules (respectively, early and late nodulin genes) are suspected to play roles in this process. Nodulin genes were initially isolated by differential screening approaches with cDNA probes derived from root mRNAs versus young or mature nodule mRNAs to screen stage-specific nodule cDNA libraries (Mylona et al. 1995 and references therein). We have previously used this screening procedure to identify new nodulin genes by comparing spontaneous nodule versus root cDNAs (Crespi et al. 1994; Coba de la Pena et al. 1997), since certain genes involved in symbiotic nodule formation ought to be common with those acting during spontaneous nodule organogenesis.

To identify new, differentially expressed transcripts, several recently developed techniques have been applied to nodule tissues, such as subtractive library screening procedures (Cook et al. 1995; Gamas et al. 1996) and differential display reverse transcription–polymerase chain reaction (DDRT-PCR) approaches (Goormachtig et al. 1995; Heidstra et al. 1997). Despite the clearly improved sensitivity of these two techniques, problems have been reported (Vögeli-Lange et al. 1996; Wan et al. 1996), in particular when using closely related tissues. Moreover, only a few regulatory genes have been identified in these cases. An alternative method is cold-plaque screening, which has been successfully used in plants for the isolation of low- or medium-abundant, anther-specific transcripts (Hodge et al. 1992).

In this paper, we report the identification of new genes expressed differentially during nodule development, through the use of a modified cold-plaque screening procedure. The expression of these genes was analyzed during nodule development and in spontaneous nodules. Sequence analyses of 20 cDNA clones indicate that three of them code for alfalfa

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Nucleotide and/or amino acid sequence data are to be found at the Genbank and EMBL data bases as accession numbers X93312 (*Mscp4*), AA735021 (*Mscp5*), AA735022 (*Mscp6*), AA735023 (*Mscp7*), AA735024 (*Mscp8*), AA735025 (*Mscp9*), AA735026 (*Mscp10*), AA735027 (*Mscp11*), AA735028 (*Mscp12*), AA735029 (*Mscp13*), AA735030 (*Mscp14*), AA735031 (*Mscp15*), Y16130 (*Mscp16*), and Y16131 (*Mscp17*).

homologues of a zinc-finger-containing putative transcription factor, a human tumor suppressor gene, and a novel type of plant protein kinase, respectively. Thus, we presumably have access to an mRNA population in which good candidates for putative regulatory factors involved in nodule development were found.

RESULTS

Identification of an mRNA population containing low-abundance transcripts differentially expressed in nodule tissues.

Spontaneous nodule versus root cDNAs were used in a conventional differential screening (Crespi et al. 1994). Under our conditions, a strong hybridization signal with either of the two cDNA probes was seen for around half of the clones ("hot" plaques), while the rest appeared as "cold" plaques (*Mscp* clones, for *Medicago sativa* cold-plaque), i.e., phage lysis plaques showing no or faint hybridization signals. In the original method, the cold-plaque clones were directly tested by Northern (RNA) dot blots (Hodge et al. 1992). In our case, we performed an additional, secondary screening step with the same probes on a group of 250 clones (*Mscp* population), based on PCR amplification (Fig. 1A and B). To assess the tissue-specificity of the expression of these clones, we also hybridized the PCR blot to cDNA probes derived from other tissues, such as symbiotic nodules, stems, flowers, and leaves. Less than 5% of the whole *Mscp* population appeared to be constitutive genes (data not shown). Thus, two subpopulations were identified: true *Mscp* clones showing no hybridization signal in any blot (subpopulation A), and *Mscp* clones showing a differential expression with PCR blotting (subpopulation B; Fig. 1A).

Expression analysis of *Mscp* transcripts in different nodule types.

The expression of 21 selected *Mscp* clones (subpopulation B) as well as three *Mscp* clones selected from subpopulation A was studied during nodule development with Northern blots. Inserts were purified from all the clones and individually labeled. Total RNAs extracted from roots inoculated with a Nod⁻ strain of *R. meliloti* (ZB138), from immature and young nitrogen-fixing nodules induced by the wild-type *R. meliloti*, and from spontaneous nodules were assayed. A summary of the Northern blot experiments for different *Mscp* clones is presented in Table 1. As expected, the three clones of *Mscp* subpopulation A, *Mscp1*, 2, and 3, gave no signal in any of the organs tested, even after long over-exposure of the Northern blots. On the other hand, several *Mscp* clones of subpopulation B (*Mscp4*, 5, 7, 10, 11, 12, 13, 14, 15, and 17) showed differential expression between root and either spontaneous or symbiotic nodule samples (selected examples are shown in Figure 2A and B). Nevertheless, in several cases (*Mscp6*, 8, 9, and 16), no signal was detected with Northern analysis, not allowing any further conclusion about their differential expression observed before the use of PCR-blotting (as illustrated in Figure 2C). Most of the clones exhibited a weak signal on Northern blots for the spontaneous nodule sample, indicating that the identified genes correspond to middle- or low-abundant transcripts in this organ. Finally, the remaining seven clones turned out to be expressed at variable

levels in all tissues tested (data not shown), and were therefore not analyzed further.

To study the expression of these genes during the initial stages of nodule development, we used *R. meliloti* spot-inoculated on alfalfa roots. In this way, only RNA isolated from the root region containing emerging nodules was analyzed. Since the recovered amount of RNA is too low to perform several Northern blots, the *Mscp* clones were tested by PCR dot blotting. PolyA⁺ mRNAs isolated from *R. meliloti* spot-inoculated roots at different times (0.25, 1, 2, and 4 days post infection [d.p.i.]) were used for the generation of cDNA probes and hybridized to dot blots of the different clones. Quantification of the intensities of the hybridization signals was done for each transcript in independent experiments. The results are reported in relation to the constitutive gene *Msc27* (Pay et al. 1992), which was included as an internal standard to monitor the efficiency of cDNA probe synthesis. Early induction during nodule formation was detected for certain clones, such as *Mscp7*, *Mscp12*, and *Mscp15*, in the 1 d.p.i. sample and *Mscp4* at 2 d.p.i. (Fig. 3). The latter gene was the only one that did not show a detectable level of expression in root tissues. Despite a weak hybridization signal, the *Mscp15* transcript was transiently activated during nodule initiation. For the other clones, no expression was detected at these early time points.

These results indicate that we have isolated low-expressing early and late nodulin genes.

Sequence analysis of differentially expressed *Mscp* transcripts revealed novel genes containing regulatory motifs.

The inserts in the isolated phage clones were amplified by PCR and their sequences were determined. Similarities with known sequences in data banks were searched for with Blast and Fasta algorithms. Sequence analysis of six randomly chosen clones in the true *Mscp* subpopulation A did not reveal any significant homologies in data bases (*Mscp1* to 3, Table 1). This subpopulation probably represents genes expressed at very low levels, and therefore homologous genes are difficult to find. On the other hand, sequence analysis of the clones from subpopulation B revealed homologies with previously identified genes in most of the cases (Table 1, *Mscp4* to 17). The presence of the first methionine was detected in six out of 14 *Mscp* clones exhibiting similarities to known genes in the data banks. Only one of the *Mscp* clones (*Mscp4*) was previously identified as an alfalfa early nodulin gene, the carbonic anhydrase *MscA1* (Gamas et al. 1996; Coba de la Pena et al. 1997), and another clone, *Mscp5*, showed similarity to the early nodulin GRP5 from another legume species, *Vicia faba* (Schröder et al. 1997). In addition, *Mscp12* is homologous to glutamine synthetase, a gene already identified as a late nodulin in alfalfa (Stanford et al. 1993). The small number of already known nodulins identified further confirmed that a different mRNA population was selected in the library with the cold-plaque screening procedure.

Based on the homologies determined with the Blast and Fasta algorithms, we defined three groups of transcripts (Table 1):

Mscp5 to 8. Genes that may be required during the growth of the nodule organ: ribosomal protein L3 homologue (*Mscp7*); a homologue of the elongation factor Tu (*Mscp8*); and two glycine-rich proteins (GRPs), *Mscp5* and *Mscp6*, that may be associated with cell wall structures.

Fig. 1. A, Cold-plaque screening strategy. Distribution of clones during initial screening, and after polymerase chain reaction (PCR) and Northern (RNA) blotting experiments, is always depicted as percent in relation to the initial cDNA population. A group of 250 clones was selected as our original *Mscp* population (see text for definition). PCR amplification products were detected for more than 95% of the clones ranging in size from 0.5 to 2.5 kb. Blots of amplified phage inserts were hybridized to spontaneous nodules versus root cDNA probes to increase the probability of identifying differentially expressed clones ("PCR blotting" illustrated in **B**). A hybridization signal detectable with at least one of the cDNA probes was observed for the majority of the clones, whereas the rest still gave no signal (true *Mscp*, subpopulation A). Comparison of PCR blots showed that 21 of the *Mscp* clones (subpopulation B) exhibited a differential hybridization signal, e.g., induction or repression in the spontaneous nodule and root samples, in relation to constitutive controls. Northern analysis (Table 1) confirmed the differential expression of half of the selected clones (5% of the initial *Mscp* clones). **B,** PCR blotting experiment showing hybridization of selected *Mscp* clones. Blots were hybridized with cDNA probes derived from polyA⁺ RNAs of either roots or spontaneous nodules. Inserts were amplified directly from the phage by PCR. Similar amounts of each insert were deposited on the gel. D indicates differentially expressed *Mscp* clones; CP indicates true cold-plaque clones (no hybridization signal detected with either probe). Arrowheads indicate localization of PCR products from D clones. Stars show location of PCR products from CP clones. As a control, PCR products amplified from "hot" plaques were also hybridized with the same probes and showed at least a 10-fold higher signal than those of the *Mscp* clones (data not shown).

Mscp9 to *12*. Genes that may have metabolic roles during nodule morphogenesis and function (e.g., glutamine synthetase, *Mscp12*). Interestingly, several genes seem to be related to the carbon metabolism, an important physiological feature of spontaneous nodules (Truchet et al. 1989; Coda de la Pena et al. 1997): carbonic anhydrase (*Mscp4*), UTP-glucose glucosyl transferase (*Mscp9*), pyruvate decarboxylase (*Mscp10*), and tagatose/fructose biphosphate aldolase (*Mscp11*). In addition, novel metabolic functions that may play roles in nodulation were identified: methionine sulfoxide reductase (*Mscp13*), an enzyme that functions as an antioxidant in yeast (Moskovitz et al. 1997), and 3-oxoacyl-(acyl carrier protein) reductase (*Mscp14*), which catalyzes the first reduction step in the fatty acid biosynthetic pathway (Slabas et al. 1992).

Mscp15 to *17*. Genes that might code for potential regulatory proteins of developmental processes: a Wilm's tumor suppressor homologue (*Mscp15*, Fig. 4A), a putative protein kinase (*Mscp16*, Fig. 4B), and a Zn-finger-containing protein (*Mscp17*, Fig. 4C). The protein kinase domain of *Mscp16* shows higher level of similarity to those of *pk6* (Feng et al. 1993) from soybean, *ctrl* (Kieber et al. 1993) and *tmk1* (Chang et al. 1992) from *Arabidopsis thaliana*. The *Mscp16* kinase domain is fused to a 5' region divergent from any known sequence in several independent clones, suggesting that *Mscp16* encodes a novel plant protein kinase. The putative product of *Mscp17* contains Zn-finger motifs of the Krüppel type (Rosenberg et al. 1986) and the plant homologues are the *epf2* family expressed in *Petunia* flowers (Takatsuji et al. 1994), *stz* (Lippuner et al. 1996) and *zat10* (Meissner and Michael, 1997) from *A. thaliana* (these two sequences are almost identical, except for one amino acid residue), and *wzfl* from wheat (Sakamoto et al. 1993). The same spacing between the two Zn fingers, which might be important in the recognition

of their target DNA (Takatsuji et al. 1994), was found in these genes, except for the wheat homologue (Fig. 4C).

The three putative regulatory genes are differentially regulated during nodulation.

Due to their homologies to regulatory genes, the differential expression of genes belonging to group 3 was further analyzed. Transient expression of the Wilm's tumor suppressor homologue was found in independent experiments with either Northern blots (Fig. 2A, *Mscp15*) or dot blots (Fig. 3). This activation, occurring early during nodulation, seems to be associated with the growing phase of the organ.

The protein kinase gene showed a very low level of expression that could not be detected on Northern blots. Thus, we used RT-PCR and specific oligonucleotides to study its expression. This gene was induced as early as 3 days after initiation of symbiotic nodule development and in the mature nitrogen-fixing nodule (Fig. 2C). In this case, spot-inoculation of *R. meliloti* was used to precisely excise the responding region of the root since we could detect a basal level of the transcript in root tissues by RT-PCR. In addition, transcripts corresponding to *Mscp16* were detected at higher levels in the spontaneous nodules, compared with roots (Fig. 2C), as previously observed in PCR blots (Table 1).

The Zn-finger protein gene was strongly expressed in the spontaneous nodule sample and was induced late in symbiotic nodule development (Table 1), although a basal level was detected in root tissues. To better determine the conditions of its induction, we compared roots grown for 7 days in the presence of nitrate and roots grown in nitrogen-limiting conditions for 7 days after inoculation with either the wild-type or Nod⁻ *R. meliloti* strains. In the former case, nodules were visible in these plants and root parts were separated from nodules. Northern analysis of these samples showed that the Zn-finger

Table 1. Sequence homologies and summary of expression analysis of selected *Mscp* (*Medicago sativa* cold-plaque) clones

Clone name	Best homology	Statistical significance (5' end / 3' end) ^a	Initial selection ^b	Root ^c	Nodule ^c		
					3 days	6 days	Spontaneous
<i>Mscp1</i>	—	—	CP	—	—	—	—
<i>Mscp2</i>	—	—	CP	—	—	—	—
<i>Mscp3</i>	—	—	CP	—	—	—	—
<i>Mscp4</i> (= <i>Mscp1</i>)	Carbonic anhydrase	1,8.10 ⁻⁵¹ / 1,2.10 ⁻³⁰	D	—	+	+	++
<i>Mscp5</i>	Early nodulin GRP5	1,4.10 ⁻³² / 1,8.10 ⁻⁷	D	—	—	+++	++
<i>Mscp6</i>	Glycine-rich protein (GRP) ?	2,4.10 ⁻² / —	D	—	—	—	—
<i>Mscp7</i>	Ribosomal protein L3	8.10 ⁻¹²⁴ / 1,1.10 ⁻⁴¹	D	+/-	+	—	++
<i>Mscp8</i>	Elongation factor Tu	6,2.10 ⁻⁸³ / 3,6.10 ⁻²¹	D	—	—	—	—
<i>Mscp9</i>	UTP glucose glucosyl transferase	2,7.10 ⁻⁴² / 8,8.10 ⁻⁸	D	—	—	—	—
<i>Mscp10</i>	Pyruvate decarboxylase	1.10 ⁻⁸⁷ / 9,8.10 ⁻³³	D	—	—	—	++
<i>Mscp11</i>	Tagatose / fructose biphosphate aldolase	1,3.10 ⁻³² / 1.10 ⁻¹¹	D	—	—	—	+
<i>Mscp12</i>	Glutamine synthetase	5,8.10 ⁻⁷⁰ / 5,5.10 ⁻⁴⁷	D	—	+	+	—
<i>Mscp13</i>	Methionine sulfoxide reductase	6,8.10 ⁻⁶⁰ / 5,5.10 ⁻¹⁹	D	—	—	+	—
<i>Mscp14</i>	3-oxoacyl-(acyl carrier protein) reductase	1,2.10 ⁻⁵⁴ / —	D	—	—	+	—
<i>Mscp15</i>	Wilm's putative tumor suppressor QM homologue	2,5.10 ⁻¹¹⁹ / 5,1.10 ⁻²⁴	D	+/-	+	+/-	+/-
<i>Mscp16</i>	Protein kinase ^d	2,2.10 ⁻¹¹ / 2,5.10 ⁻²⁶	D	—	—	—	—
<i>Mscp17</i>	Zinc-finger protein	1,7.10 ⁻¹⁶ / 2,5.10 ⁻⁸	D	+/-	+/-	++	++

^a Statistical significance, indicating results from a search in the nonredundant protein data base with the Blastx algorithm (Gish and States 1993). Sequence analysis was initially done on the 5' end of the clones (about 500 to 700 nucleotides) and homologies were confirmed by also sequencing the 3' end of the cDNA clones (as indicated: 5' end / 3' end).

^b Initial selection was carried out on polymerase chain reaction (PCR) blots as shown in Figure 1B. *Mscp1* to *3*: true *Mscp* clones (CP); *Mscp4* to *17*: differentially expressed *Mscp* clones (D).

^c Northern (RNA) blots were performed for all selected clones and examples are shown in Figure 2A, B, and C. RNAs were prepared from aerobically grown plants. Quantification of band intensities in different samples (roots, nodules 3 and 6 days post inoculation, spontaneous nodules) in relation to the *Msc27* value, is indicated by the following: —, +/-, +, ++, +++, meaning undetectable to strong mRNA expression level, respectively.

^d Differential expression of this clone was tested with reverse transcription PCR (Fig. 2C).

protein gene was strongly induced in the *Rm41*-treated roots 7 d.p.i. whereas the corresponding nodules showed a lower level of expression (Fig. 2B). Nevertheless, a clear induction was observed when comparing nodule and non-nodulating roots (Root Nod⁻) of the same age with *Msc27* as a constitutive control (Fig. 2B). This indicates that this regulatory gene is induced during nodulation although its expression might be regulated in a complex manner during development.

DISCUSSION

Using a modified cold-plaque screening method, we have identified new genes induced during nodule development. Rare mRNAs represent approximately 99% of the different transcripts, but only 50% of the total mRNA mass; the other half is constituted by a relatively small number of abundant mRNAs (Wan et al. 1996). The population of cold-plaque

clones may represent a sample of rare nodule mRNAs in which potential regulatory developmental elements and factors related to signal transduction (e.g., transcription factors or protein kinases) might be present. Previously, this method has been used to identify anther-specific transcripts by comparing anthers versus seedlings (Hodge et al. 1992). The use of a second screening step based on insert PCR amplification allowed further selection of differentially expressed clones, with a relatively low number of false positives (less than 50%). Alternative procedures based on subtractive hybridization and DDRT-PCR (Cook et al. 1995; Goormachtig et al. 1995; Gamas et al. 1996; Heidstra et al. 1997) have previously been used to identify genes induced during nodule development. In the differential display method, the proportion of false positive clones can be high (Vögeli-Lange et al. 1996), at least in *M. sativa* when roots and nodule primordia are used (E. Kondorosi and A. Cebolla, *personal communication*). In addition,

Fig. 2. Expression analysis of *Msc* clones. Northern (RNA) blots containing total RNAs (10 µg) from the following: **A**, Roots (inoculated with a Nod⁻ *R. meliloti* strain), and *Rhizobium*-induced nodules 3 days post inoculation (d.p.i.) and 6 d.p.i. (nitrogen-fixing), respectively, as well as spontaneous nodules. Probes used are indicated. **B**, Roots grown on nitrate 7 days, Nod⁻ *Rhizobium*-infected roots 7 d.p.i., wild-type *Rhizobium*-induced roots and their corresponding excised nodules 7 d.p.i., as well as spontaneous nodules. Probes used are indicated. **C**, Roots, *Rhizobium*-induced nodules 7 and 20 d.p.i., as well as spontaneous nodules. Probes used are indicated. Reverse transcription–polymerase chain reaction (RT-PCR) assays were done with total RNAs from roots, *Rhizobium*-induced nodules 3, 6, and 20 d.p.i., spontaneous nodule, and water control with oligonucleotides specific for the *Msc16* and *Msc27* sequences. Probes used for detection of PCR products are indicated.

Fig. 3. Expression of selected *Mscp* genes (see text for definition) during early nodule development. RNAs from plants grown on agar and spot-inoculated with *Rhizobium* were prepared at 0, 0.25 (6 h), 1, 2, and 4 days post inoculation (d.p.i.). Polymerase chain reaction (PCR) amplification products of *Mscp* clones (1 µg) were hybridized to cDNA probes derived from polyA⁺ RNAs from spot-inoculated roots. Intensity of hybridization signals was normalized to the *Msc27* value in each case and is expressed in arbitrary units (a.u.). At least two independent experiments were done for each time point.

cloning of the differentially expressed transcripts needs careful verification to show that the cloned gene is the appropriate mRNA. On the other hand, subtractive hybridization has been successfully used to identify several new genes induced during nodule development in *M. truncatula* (Gamas et al. 1996). This latter procedure may nevertheless have disadvantages since only induction or repression of particular transcripts can be detected in the same experiment and primarily abundant differentially expressed mRNAs are identified (Wan et al. 1996). Cold-plaque screening might therefore be a simpler alternative procedure for the identification of new genes differentially expressed in plant-microbe systems.

Several new genes induced in spontaneous nodules have been cloned and their expression studied during nodule development. In the first group of genes, *Mscp7*, a homologue of a component of the large subunit of cytoplasmic ribosomes (the L3 ribosomal protein) was shown to be induced in the developing nodule (1 to 4 d.p.i.), and in spontaneous nodules. This activation may correspond to specific requirements of the translation machinery of the developing organ under growth conditions in which nitrogen (and hence the amino acid pool) is limiting. Growth-related gene expression has been found for an L2 ribosomal protein gene in tobacco protoplasts (Marty et al. 1993). Another gene of this group, *Mscp5*, is an alfalfa homologue of the *Vicia faba* early nodulin GRP5 gene (Schröder et al. 1997) and showed an analogous expression pattern in *Medicago sativa*. These GRPs are good candidates to be associated with cell walls in specific regions of the nodule (Schröder et al. 1997).

In the second group, several genes related to carbon metabolism were identified to be differentially expressed in spontaneous nodules (*Mscp4*, 9, 10, and 11). However, their expression could not be detected during symbiotic nodulation except for carbonic anhydrase (Gamas et al. 1996; Coba de la Pena et al. 1997). This gene changes its cell-specific expression during nodule development and showed different transcript levels in symbiotic and spontaneous nodules (Coba de la Pena et al. 1997). In addition, glutamine synthetase (*Mscp12*), a well-known late nodulin, was shown to be expressed very early during nodule formation. Such early induction of another late nodulin gene, leghemoglobin, has also recently been found in *Vicia sativa* (Heidstra et al. 1997) and *Sesbania rostrata* (Goormachtig et al. 1995). This indicates that genes required in the functional nodule might be induced at the beginning of organ formation.

Finally, three genes containing regulatory motifs were identified. A clone showing homology to the human Wilm's putative tumor suppressor *QM* gene, *Mscp15*, was found to be early and transiently induced during nodule development. This temporal control suggests that it may be associated with the initial events of organogenesis. A plant homologue identified in *Nicotiana tabacum* is highly expressed during the growth phase of suspension cell cultures and in young leaves (Marty et al. 1993). Other homologues among different eukaryotes are highly conserved, and transfection of the human *QM* gene into a Wilm's tumor cell line suppressed the tumorigenic phenotype (Dowdy et al. 1991; Koller et al. 1996), indicating a role in the control of mammalian cell differentiation. Recently, a yeast homologue was also found based on its involvement in growth control (Koller et al. 1996). A second gene of interest, *Mscp16*, codes for a novel protein kinase not yet identified in

Fig. 4. Sequence homologies of *Mscp* clones (see text for definition) from group 3. Alignment of predicted amino acid sequences of (A) *Mscp15* with other members of the Wilm's tumor suppressor/QM family; (B) protein kinase domain of *Mscp16* with closest homologous kinase genes; and (C) Zn-finger region of *Mscp17* with other plant members of this Krüppel type family. Gaps (points) were inserted to optimize alignment (PileUP, GCG, University of Wisconsin, Madison, 1994). Black boxes represent identical amino acids. Sequences were obtained from GenBank: A- *mus* (P45634, *Mus musculus*), *homo* (P27635, *Homo sapiens*), *solanum* (AB001891, *Solanum tuberosum*), *ara* (Q08770, *Arabidopsis thaliana*), *zea* (P45633, *Zea mays*), *oryza* (U55048, *Oryza sativa*). The first 120 amino acids of the *Mscp15* (partial sequence) were used for the comparison. B- *ctrl* (Q05609, *Arabidopsis thaliana*), *dpyk1* (A35670, *Dictyostelium discoideum*), *pk6* (S27760, *Glycine max*), *tmk1* (P43298, *Arabidopsis thaliana*). Deduced amino acid sequence starting from amino acid 199 to 458 of *Mscp16* was compared with catalytic domains of homologous kinases showing the highest degree of conservation. I to XI indicate conserved kinase subdomains (Hanks et al. 1998). "Consensus" depicts conserved amino acids in at least half of the sequences. C- *stz* (X98671, *Arabidopsis thaliana*), *zat10* (X95573, *Arabidopsis thaliana*), *epf2-7* (D26086, *Petunia hybrida*). Deduced amino acid sequence of *Mscp17* starting from amino acid 78 to 165 carrying the two zinc fingers was compared with equivalent domain of other Krüppel-type genes.

plants showing a very low level of expression. According to the homology searches, the gene contains the 11 kinase subdomains and motifs fitting with the consensus of a serine/threonine kinase (Hanks et al. 1988) were found in subdomains VIb and VIII. Transcripts of this gene are induced in the developing nodule despite a basal level of expression in root tissues. It will be of interest to test whether the kinase enzymatic activity is also controlled in the early steps of the interaction. A third putative regulatory gene, *Mscp17*, is homologous to a Zn-finger transcription factor of the Krüppel type (Rosenberg et al. 1986). Complete sequencing of this clone confirmed that it is the first Krüppel-type, Zn-finger regulatory gene expressed in nodules. The *epf2* petunia homologues have been shown to be organ-specific transcription factors in flowers, recognizing the spacing of specific sequences in target DNA (Takatsuji et al. 1994). This gene exhibited a relatively late induction in nodule development, starting at 7 d.p.i., and expression was even stronger in spontaneous nodules and in roots infected with bacteria producing active Nod factors, further suggesting a role in nodule differentiation processes.

In summary, with the use of a modified cold-plaque screening procedure, different new genes induced during nodule development have been found. Three of them contained regulatory motifs and showed specific expression patterns, suggesting that they may play roles in nodule growth and differentiation. Further molecular characterization of the identified genes together with transgenic approaches will serve to explore their function in nodule development.

MATERIALS AND METHODS

Bacterial strains and plant material.

Rhizobium meliloti wild-type strain *Rm41* was used to nodulate plants and strain *ZB138*, a Nod⁻ strain derived from *Rm41* (Kondorosi et al. 1984), was used as inoculum in control experiments. Both strains were grown in GTS medium supplemented with biotin as described in Kondorosi et al. (1984).

Alfalfa (*Medicago sativa* subsp. *sativa* cv. Sitel) seeds were sterilized in 20% Bayrochlor for 1 h at 28°C under agitation, rinsed 3 times with water, incubated overnight at 28°C in 200 mg of augmentin per l, rinsed 3 times, and germinated for 2 days on 1% water agar plates. Plantlets were grown under aeroponic conditions (Crespi et al. 1994) to isolate nodules 3, 6, and 20 days after *Rhizobium* inoculation (d.p.i.). Control roots were obtained under the same conditions after inoculation with the *ZB138* strain. Spontaneous nodules were obtained from *M. sativa* subsp. *varia* genotype A2 NAR⁺ plants (Crespi et al. 1994).

Early nodule development was studied with 3-day-old seedlings spot inoculated on nitrogen-free Gibson medium (Coba de la Pena et al. 1997) with *Rhizobium* strains (*Rm41* and *ZB138*, respectively). Spot-inoculated root zones were collected from 30 plants per time point, at 6 h post infection (0.25 d.p.i.) and 1, 2, and 4 d.p.i. PolyA⁺ RNAs extracted from the spot-inoculated region (about 1 cm in length) were used to prepare cDNA probes for dot blot hybridization. Under these conditions, nodule development was retarded in comparison with aeroponic growth conditions (nodules appeared at 4 to 5 d.p.i. instead of 3 d.p.i., respectively).

In the experiment depicted in Figure 2B, plants were grown in parallel in three aeroponic tanks for 3 days in the presence of nitrate. Then, in one tank the nitrate medium was kept whereas in the other two tanks it was replaced with nitrogen-free medium and the plants were inoculated with wild-type and the Nod⁻ *R. meliloti* strains, respectively. After 7 days, the plant roots from the three tanks were collected and used for RNA preparation. In the case of *R. meliloti* *Rm41*-infected plants, excised developing nodules and the remaining roots were processed separately.

Cold-plaque screening and "PCR blotting".

Differential screening was performed as in Crespi et al. (1994) with spontaneous nodule versus root cDNA probes on a cDNA library of young *M. sativa* A2 nodules. Isolated non-hybridizing clones (250 *Mscp* clones) were selected. Amplification of the inserts was done directly on λ ZAP phages (Stratagene, La Jolla, CA), with T3/T7 primers and standard PCR conditions (5 min, 95°C, and then 1 min, 95°C; 1 min, 55°C; 1 min, 72°C; 30 cycles). PCR products were run on 1% agarose gels, blotted onto nylon membranes (Hybond N, Amersham, Little Chalfont, England) and hybridized overnight at 65°C with the cDNA probes (Sambrook et al. 1989). cDNA probes were prepared with Superscript reverse transcriptase (Stratagene, La Jolla, CA). The membranes were subsequently washed for 30 min each with 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) / 0.1% sodium dodecyl sulfate (SDS) and 1 $\times$ SSC / 0.1% SDS and finally for 15 min with 0.1 $\times$ SSC / 0.1% SDS. Membranes were then also hybridized to cDNA probes derived from symbiotic nodules, stems, flowers, and leaves to eliminate constitutive clones.

Expression analysis.

Northern blots were done with 10 μ g of total RNAs from each tissue (isolated from plants grown under aeroponic conditions), extracted as described in Sambrook et al. (1989) from 0.2 to 1 g of frozen plant material, and hybridized to the different cDNA probes (derived from roots and nodules at different stages after infection) under high stringency conditions (similar to the PCR blots, see above). *Msc27* was used as a control for RNA loading (Pay et al. 1992).

RT-PCR experiments were done with total RNAs from root and nodules at different stages after infection for cDNA preparation. Amplification was done on 6 ng of cDNAs for 20 PCR cycles in parallel with oligonucleotides specific of the *Msc27* clone as described (Crespi et al. 1994).

Dot blots were done with a dot blot apparatus (Bio-Rad, Hercules, CA) and filters were hybridized with cDNA probes derived from polyA⁺ RNAs (mRNA capture kit, Boehringer Mannheim, Mannheim, Germany). Blotting and hybridization procedures were similar to PCR blots (see above). Quantification of the intensities of the hybridization signals was done by densitometric scanning of the blots with the image analysis software "NIH Image" (W. Rasband; NIH Image is available on-line from the National Institutes of Health). Mean and standard deviations of the hybridization signal intensities were calculated from independent experiments.

Sequence analysis.

Sequencing was performed with an automatic sequencer 373A (Applied Biosystems, Foster City, CA) with the dideoxy

chain termination method (Pharmacia kit, Uppsala, Sweden). Primers T3 and T7 of pBluescript SK were used. Homology searches in data bases was done with the GCG software package (Program Manual for the Wisconsin Package, Version 8, University of Wisconsin, Madison, WI), with Blast (Gish and States 1993) and Fasta (Pearson and Lipman 1989) algorithms, on both 5' and 3' ends.

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LITERATURE CITED

- Chang, C., Schaller, G. E., Patterson, S. E., Kwok, S. F., Meyerowitz, E. M., and Bleecker, A. B. 1992. The TMK1 gene from *Arabidopsis* codes for a protein with structural and biochemical characteristics of a receptor protein kinase. *Plant Cell* 4:1263-1271.
- Coba de la Pena, T., Frugier, F., McKhann, H. I., Bauer, P., Brown, S., Kondorosi, A., and Crespi, M. 1997. A carbonic anhydrase gene is induced in the nodule primordium and its cell-specific expression is controlled by the presence of *Rhizobium* during development. *Plant J.* 11:407-420.
- Cook, D., Dreyer, D., Bonnet, D., Howell, M., Nony, E., and Vanden-Bosch, K. 1995. Transient induction of a peroxidase gene in *Medicago truncatula* precedes infection by *Rhizobium meliloti*. *Plant Cell* 7:43-55.
- Crespi, M. D., Jurkevitch, E., Poirier, M., D'Aubenton-Carafa, Y., Petrovics, G., Kondorosi, E., and Kondorosi, A. 1994. *enod40*, a gene expressed during nodule organogenesis, codes for a non-translatable RNA involved in plant growth. *EMBO J.* 13:5099-5112.
- Dowdy, S. F., Lai, K. M., Weissman, B. E., Matsui, Y., Hogan, B. L. M., and Stanbridge, E. J. 1991. The isolation and characterization of a novel cDNA demonstrating an altered mRNA level in nontumorigenic Wilm's microcell hybrid cells. *Nucleic Acids Res.* 19:5763-5769.
- Feng, X. H., Zhao, Y., Bottino, P. J., and Kung, S. D. 1993. Cloning and characterization of a novel member of protein kinase family from soybean. *Biochem. Biophys. Acta* 1172:200-204.
- Gamas, P., de Carvalho Niebel, F., Lescure, N., and Cullimore, J. V. 1996. Use of a subtractive hybridization approach to identify new *Medicago truncatula* genes induced during root nodule development. *Mol. Plant-Microbe Interact.* 9: 233-242.
- Gish, W., and States, D. J. 1993 Identification of protein coding regions by database similarity search. *Nature Genet.* 3:266-272.
- Goormachtig, S., Valerio-Lepiniec, M., Szczyglowski, K., Van Montagu, M., Holsters, M., and de Bruijn, F. J. 1995. Use of differential display to identify novel *Sesbania rostrata* genes enhanced by *Azorhizobium caulinodans* infection. *Mol. Plant-Microbe Interact.* 8:816-824.
- Hanks, S. K., Quinn, A. M., and Hunter, T. 1988. The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science* 241:42-52.
- Heidstra, R., Nilsen, G., Martinez-Abarca, F., van Kammen, A., and Bisseling, T. 1997. Nod factor-induced expression of leghemoglobin to study the mechanism of NH_4NO_3 inhibition on root hair deformation. *Mol. Plant-Microbe Interact.* 10:215-220.
- Hodge, R., Paul, W., Draper, J., and Scott, R. 1992. Cold-plaque screening: A simple technique for the isolation of low abundance, differentially expressed transcripts from conventional cDNA libraries. *Plant J.* 2:257-260.
- Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A., and Ecker, J. R. 1993. *CTR1*, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the raf family of protein kinases. *Cell* 72:427-441.
- Koller, H. T., Klade, T., Ellinger, A., and Breitenbach, M. 1996. The yeast growth control gene *GRC5* is highly homologous to the mammalian putative tumor suppressor gene *QM*. *Yeast* 12:53-65.
- Kondorosi, E., Banfalvi, Z., and Kondorosi, A. 1984. Physical and genetic analysis of a symbiotic region of *Rhizobium meliloti*: Identification of nodulation genes. *Mol. Gen. Genet.* 193:445-452.
- Lippuner, V., Cyert, M. S., and Gasser, C. S. 1996. Two classes of plant cDNA clones differentially complement yeast calcineurin mutants and increase salt tolerance of wild-type yeast. *J. Biol. Chem.* 271:12859-12866.
- Marty, I., Brugidou, C., Chartier, Y., and Meyer, Y. 1993. Growth-related gene expression in *Nicotiana tabacum* mesophyll protoplasts. *Plant J.* 4:265-278.
- Meissner, R. C., and Michael, A. J. 1997. Isolation and characterisation of a diverse family of *Arabidopsis* two and three-fingered C_2H_2 zinc finger protein genes and cDNAs. *Plant Mol. Biol.* 33:615-624.
- Moskovitz, J., Berlett, B. S., Poston, J. M., and Stadtman, E. R. 1997. The yeast peptide-methionine sulfoxide reductase functions as an antioxidant *in vivo*. *Proc. Natl. Acad. Sci. USA* 94:9585-9589.
- Mylona, P., Pawlowski, K., and Bisseling, T. 1995. Symbiotic nitrogen fixation. *Plant Cell* 7:869-885.
- Pay, A., Heberle-Boers, E., and Hirt, H. 1992. An alfalfa cDNA encodes a protein with homology to translationally controlled human tumor protein. *Plant Mol. Biol.* 19, 501-503.
- Pearson, W. R., and Lipman, D. J. 1989. Improved tools for biological sequences comparison. *Proc. Natl. Acad. Sci. USA* 85:2444-2448.
- Rosenberg, U. B., Schröder, C., Preiss, A., Kienlin, A., Coté, S., Riede, I., and Jäcke, H. 1986. Structural homology of the product of the *Drosophila Krüppel* gene with *Xenopus* transcription factor IIIA. *Nature* 319:336-339.
- Sakamoto, A., Minami, M., Huh, G. H., and Iwabuchi, M. 1993. The putative zinc-finger WZF1 interacts with a *cis*-acting element of wheat histone genes. *Eur. J. Biochem.* 217:1049-1056.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Schröder, G., Frühling, M., Pühler, A., and Perlick, A. M. 1997. The temporal and spatial transcription pattern in root nodules of *Vicia faba* nodulin genes encoding glycine-rich proteins. *Plant Mol. Biol.* 33: 113-123.
- Schultze, M., Kondorosi, E., Ratet, P., Buiré, M., and Kondorosi, A. 1994. Cell and molecular biology of *Rhizobium*-plant interactions. *Int. Rev. Cytol.* 156:1-75.
- Slabas, A. R., Chase, D., Nishida, I., Murata, N., Sidebottom, C., Saford, R., Sheldon, P. S., Kekwick, R. G. O., Hardie, D. G., and Mackintosh, R. W. 1992. Molecular cloning of higher-plant 3-oxoacyl-(acyl carrier protein) reductase. *Biochem. J.* 283:321-326.
- Stanford, A. C., Larsen, K., Barker, D. G., and Cullimore, J. V. 1993. Differential expression within the glutamine synthetase gene family of the model legume *Medicago truncatula*. *Plant Physiol.* 103:73-81.
- Takatsuji, H., Nakamura, N., and Katsumoto, Y. 1994. A new family of zinc finger proteins in *Petunia*: Structure, DNA sequence recognition, and floral organ-specific expression. *Plant Cell* 6:947-958.
- Truchet, G., Barker, D. G., Camut, S., Billy, F. D., Vasse, J., and Huguet, T. 1989. Alfalfa nodulation in the absence of *Rhizobium*. *Mol. Gen. Genet.* 219:65-68.
- Vögeli-Lange, R., Bürckert, N., Boller, T., and Wiemken, A. 1996. Rapid selection and classification of positive clones generated by mRNA differential display. *Nucleic Acids Res.* 24:1385-1386.
- Wan, J. S., Sharp, S. J., Poirier, G. M. C., Wagaman, P. C., Chambers, J., Pyati, J., Hom, Y. L., Galindo, J. E., Huvar, A., Peterson, P. A., Jackson, M. R., and Erlander, M. G. 1996. Cloning differentially expressed mRNAs. *Nature Biotech.* 14:1685-1691.