Mol Gen Genet (1997) 256: 37–44


ORIGINAL PAPER

F. Martin-Laurent · D. van Tuinen · E. Dumas-Gaudot
V. Gianinazzi-Pearson · S. Gianinazzi · P. Franken

# Differential display analysis of RNA accumulation in arbuscular mycorrhiza of pea and isolation of a novel symbiosis-regulated plant gene

Received: 3 March 1997 / Accepted: 12 May 1997

**Abstract** Differential RNA display was used to analyze gene expression during the early steps of mycorrhiza development on *Pisum sativum* following inoculation with *Glomus mosseae*. Seven out of 118 differentially displayed cDNA fragments were subcloned and sequenced. One fragment corresponded to part of the fungal 25S ribosomal RNA gene and a second one showed similarity to a human Alu element. The others were derived from plant genes of unknown function. One of the fragments was used for the isolation of a full-length cDNA clone. It corresponded to a single-copy gene (*psam1*) which is induced during early symbiotic interactions, and codes for a putative transmembrane protein. Northern and RNA dot blot analyses revealed enhanced accumulation of *psam1* RNA after inoculation with *G. mosseae* of wild-type pea and an isogenic mutant deficient for nodule development ($Nod^-$, $Myc^+$).

**Key words** *Glomus mosseae* · *Pisum sativum* L. · Transmembrane protein

## Introduction

Fungi that develop arbuscular mycorrhiza (AM) belong to the order Glomales (Zygomycetes) and are obligate symbionts. They are able to colonize roots of the large majority of higher plant species (Newman and Reddel 1987), forming, in general, mutually beneficial associations. Four distinct stages can be recognized in AM development: (i) induction of spore germination and hyphal growth by plant signals in root exudates; (ii) fungal contact with the root surface, recognition, and differentiation of appressoria; (iii) hyphal penetration of root tissues and formation of specialized intracellular structures, the arbuscules; and (iv) establishment of a functional symbiosis (for review see Gianinazzi-Pearson 1996). The morphological and physiological changes induced in the plant and in the fungus during development of the AM symbiosis are considered to result from the production in, and perception by, both partners of signals which spatially and temporally regulate the expression of symbiosis-related (SR) genes (Franken et al. 1996).

Molecular investigations have shown that colonization of root tissues by AM fungi is accompanied by a weak and transient induction of plant defense-related genes (for review see Gianinazzi-Pearson et al. 1996). Novel genes, however, have not yet been identified, although the isolation of isogenic pea mutants altered in their ability to form AM has confirmed the hypothesis that specific plant genes are involved in establishing this symbiosis (Duc et al. 1989; Gianinazzi-Pearson et al. 1996). Moreover, two-dimensional gel electrophoresis has revealed the appearance of new gene products during development of mycorrhiza (Garcia-Garrido et al. 1993; Dumas-Gaudot et al. 1994; Samra et al. 1997).

Differential RNA display (Differential Display Reverse Transcription, DDRT; Liang and Pardee 1992) was used to identify new genes that are differentially expressed during early stages of mycorrhizal development between pea plants and *Glomus mosseae* (Martin-Laurent et al. 1995). Seven out of 113 differentially displayed cDNA fragments were confirmed as being differentially expressed, cloned and sequenced. Their origin was determined by PCR analysis, and one plant-encoded fragment was chosen for further analysis of the corresponding gene. A full-length cDNA was isolated and sequenced. For expression analysis, RNA accumulation was quantified at different time points after mycorrhiza inoculation.

Communicated by J. Schell

F. Martin-Laurent · D. van Tuinen · E. Dumas-Gaudot
V. Gianinazzi-Pearson · S. Gianinazzi · P. Franken
Laboratoire de Phytoparasitologie,
INRA/CNRS, CMSE-INRA,
BV 1540, F-21034 Dijon Cedex, France

P. Franken (✉)
Max-Planck-Institut für terrestrische Mikrobiologie,
Abteilung Biochemie, Karl-von-Frisch-Straße,
D-35043 Marburg, Germany
Fax: +49-6421-178309; e-mail: frankenp@mailer.uni-marburg.de

## Materials and methods

### Plant growth and inoculation

Wild-type pea (*Pisum sativum* L. cv Frisson, $Myc^+$ $Nod^+$) and an isogenic nodulation-resistant ($Myc^+$ $Nod^-$) mutant $P_{56}$ (Duc et al. 1989) were grown in the presence or absence of the fungal isolate *G. mosseae* (Nicol and Gerd) Gerd. and Trappe (BEG 12) as described previously (Dumas-Gaudot et al. 1994). Roots were harvested for RNA analysis at 3, 6, 9, 12 or 20 days after inoculation (dai) and parameters of mycorrhizal colonization, including the number of appressoria, were determined on root samples according to Trouvelot et al. (1986). Briefly, roots stained with trypan blue were cut into 1-cm pieces and mounted on slides. The colonization intensity (M%) was calculated as the proportion of colonized to total root length and relative arbuscule development (A%) as abundance of arbuscules per colonized root system.

### DNA and RNA isolation

Genomic DNA was extracted from uninoculated and *G. mosseae*-inoculated (12 dai) pea roots following the method of Sambrook et al. (1989). Spores of *G. mosseae* were isolated from the soil by wet sieving and collected on a Percoll gradient (Hosny et al. 1996). Genomic DNA was extracted from the spores as described by Zézé et al. (1994). Total RNA was isolated from pea roots according to the method of Franken and Gnädinger (1994) and subjected to DNase treatment (Bauer et al. 1993). Poly(A)$^+$ RNA was prepared using Dynabeads (Dynal) oligo(dT)$_{25}$ according to the manufacturer's recommendations. Concentrations of genomic DNA, of total RNA and of poly(A)$^+$RNA were determined from absorption values at 260 and 280 nm.

### Differential RNA display

DDRT-PCR was carried out on total RNA extracted from roots of uninoculated and *G. mosseae*-inoculated (6 dai) pea roots using four different oligo(dT)$_{11}$MN [MN = AA; CA; GA; GG] and 20 random 10mer oligonucleotides (RAPD primer set from Bioprobe), according to the method of Liang and Pardee (1992). Total RNA (2 µg) served as the substrate for reverse transcription by the MMLV enzyme (Promega) in the presence of the four oligo(dT)MN primers in a volume of 25 µl of the buffer recommended by the enzyme supplier. A 2.5-µl aliquot of the first-strand reaction was used as the template for amplification by PCR, in a total volume of 20 µl containing 2.5 µM oligo(dT)$_{11}$ MN, 1 µM random primers, 1 µM dNTPs, 2 µCi [$\alpha$-$^{32}$P]dCTP (Amersham), 70 mM TRIS-HCl, pH 8.8, 15 mM $(NH_4)_2SO_4$, 7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1.8 µg bovine serum albumin (buffer according to Gorman and Steinberg 1989) and 0.2 U Taq polymerase (Appligene). After a denaturation step for 4 min at 94° C, amplification was carried out for 30 cycles of 30 s at 94° C, 2 min at 40° C and 30 s at 72° C, and an additional extension period of 5 min at 72° C. PCR products were separated by electrophoresis on a 6% denaturing polyacrylamide gel and gels transferred onto Whatman paper, dried and exposed overnight to Kodak X-RAY-OMAT. The analysis was repeated twice in two separate experiments. Fragments of interest were cut out of the gel and differential expression of cDNA fragments was verified as previously described (Martin-Laurent et al. 1995).

### Cloning and sequencing of cDNA fragments

cDNA fragments were cloned into the pGEM plasmid (Promega) and recombinant plasmids used to transform competent *E. coli* XL1-Blue cells. Positive clones were grown and DNA was isolated according to Sambrook et al. (1989). Sequencing was carried out using the dideoxy sequencing method of Sanger et al. (1977), with fluorescent dye-linked universal primers (T7 and T3) and an Applied Biosystems model 370A DNA sequencer, by ESGS (EuroSéquences Gènes Services, Paris, France).

### Accession numbers

The sequences reported here will appear in the EMBL/Genbank under the accession numbers U90031 (AA02), U90032 (CA14), U90033 (CA18), U43402 (GA12a), U90034 (GG01), U43403 (GG02) and U43401 (*psam1*).

### PCR reactions

Genomic DNA from pea roots and *G. mosseae* spores (100 ng) served as templates for PCR (20 µl total volume) with 1 µM concentrations of each primer and 200 pM of dNTPs, using 0.5 U of Taq polymerase and the corresponding buffer supplied with the enzyme (Gibco/BRL). After denaturation for 5 min at 94° C, amplification was performed for 30 cycles of 30 s at 94° C, 1 min at 50° C and 30 s at 72° C, and an additional extension period for 5 min at 72° C. PCR products were separated on 2.5% agarose gels and photographed after ethidium bromide staining.

### Construction and screening of a cDNA library

A cDNA library was constructed in λZAPII (Stratagene) using 0.75 µg of poly(A)$^+$ RNA isolated from mycorrhizal roots. After reverse transcription, double-stranded cDNAs were ligated to *Eco*RI and *Xho*I adapters and inserted into the vector following Stratagene's recommendations. After packaging and plating, a total of $10^7$ recombinants was obtained. The library was screened with a probe labeled as described for Southern blot analysis. Positive recombinant phagemids were excised in vivo and used to transform competent XL1-Blue cells. Sequencing was carried out as described for the cloning of the DDRT fragments.

### Genomic southern blots

Genomic DNA from pea roots and *G. mosseae* spores (10 µg) was digested with *Eco*RI, separated by electrophoresis on a 1% agarose gel and capillary-transferred onto a nitrocellulose membrane. cDNA fragments were labeled by random priming with [$\alpha$-$^{32}$P]dATP (Random Primers DNA Labeling System, Gibco-BRL) and hybridized to the genomic DNA at 65° C (Sambrook et al. 1989).

### Northern and dot blot analyses

Total RNA (5 µg) was blotted directly with the Gibco BRL dot blot apparatus, or after electrophoresis on 1.4% agarose containing 2.2 M formaldehyde by capillary transfer (Sambrook et al. 1989), onto Nytran membranes (N13, Schleicher and Schuell). Blots were hybridized and washed at 65° C under standard conditions (Sambrook et al. 1989) with the cDNA clone of the *psam1* gene and subsequently with an rRNA probe (Franken and Gianinazzi-Pearson 1996). Probes were labeled as indicated for the genomic blots. Signals on the autoradiograms from the dot blots were quantified by image analysis (Alcatel-TITN image analysis system, Grenoble, France).

### Computer analysis

Homology searches were carried out in the EMBL databank using the FASTA program package (Pearson et al. 1993). The amino acid sequence was analyzed for structural features by the two MacIntosh programs Protean (prediction algorithms of Garnier et al. 1978, and Kyte and Doolittle 1982) and TopBred (method of Sipos and von Heijne 1993).

## Results

### Mycorrhiza development and total RNA levels

Table 1 shows the intensity of colonization, the frequency of appressoria and the abundance of arbuscules, in roots of pea plants inoculated with *G. mosseae*, at different time points. The $Myc^+$ $Nod^+$ wild type and the $Myc^-$ $Nod^+$ $P_{56}$ mutant showed typical and similar patterns of mycorrhiza development. Appressoria were the more frequent fungal structure at 3–6 dai, and arbuscules predominated when the symbiosis was fully established (12–20 dai). The nodulation-deficient mutant $P_{56}$ was colonized more rapidly – by 9 dai. Total RNA extracted at each time point gave in all cases a yield of approximately 300 μg per g fresh weight of roots.

### Differential RNA display

Total RNA from wild-type pea isolated at 6 dai was treated with DNase, checked for integrity by native gel electrophoresis (data not shown) and used for DDRT-PCR experiments. Comparison of the DDRT-PCR banding patterns obtained using 80 combinations of oligo(dT)MN and random primers revealed four types of differences. Some cDNA bands were detected only in mycorrhizal RNA samples, others were specifically visualized in uninoculated root samples and, finally, some bands were detected in both samples but their intensity was enhanced or reduced (Fig. 1). We focused our attention on additional bands revealed in root samples. Some 5600 bands were obtained with the 80 primer combinations, and differences were observed in 113 of these (2%). In all, 57 cDNA fragments were amplified only from mycorrhizal and 61 cDNA fragments from non-mycorrhizal samples.

The 113 differentially represented bands were cut out from the denaturing polyacrylamide gels, eluted and reamplified with the same pairs of primers as used for the original DDRT-PCR. All resulting fragments showed the size expected from the polyacrylamide gel analysis, and were tested by Southern blotting using as probes $^{32}$P-labeled cDNAs synthesized from RNA of non-mycorrhizal (Fig. 2A) and mycorrhizal root samples at 6 dai (Fig. 2B). Differential screening confirmed seven out of the 113 cDNA fragments as corresponding to differentially expressed genes. One was unique (lanes 7), four were enhanced (lanes 5, 8, 10 and 11) and two were repressed (lanes 2 and 4) in mycorrhizal samples. These represent 6% of the cDNA variations detected during the early stages (6 dai) of the AM symbiosis and 0.1% of all cDNA fragments obtained.

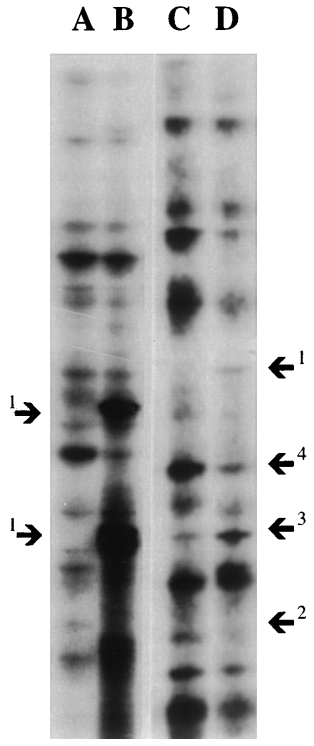


**Fig. 1** DDRT gel electrophoresis. RNA from control (lanes A and C) and mycorrhizal samples (lanes B and D) were analyzed by DDRT using oligo(dT)$_{11}$GA and random primer 12 (lanes A and B) or oligo(dT)$_{11}$CA and random primer 14 (lanes C and D). Products were separated on 6% polyacrylamide gels and analyzed by autoradiography. Bands which occur differentially are indicated by *arrows*. Some bands are present in mycorrhizal (1) or nonmycorrhizal samples (2) only, others show enhanced (3) or reduced (4) intensity after inoculation

**Table 1** Development of *G. mosseae* in roots of wild type and an isogenic mutant of *P. sativum* at different time points after inoculation

| Plant line | Colonization | Days after inoculation | | | | |
|---|---|---|---|---|---|---|
| | | 3 | 6 | 9 | 12 | 20 |
| Wild type | App/cm[a] | 13.3 | 20.7 | 24.7 | 30.6 | 36.3 |
| | M%[b] | 4.6 | 16.2 | 19.0 | 71.7 | 84.2 |
| | A%[c] | 0.0 | 15.0 | 15.8 | 46.4 | 57.8 |
| $P_{56}$ | App/cm | 15.4 | 21.4 | 27.9 | 34.7 | 45.4 |
| | M% | 5.5 | 13.9 | 46.3 | 67.8 | 78.1 |
| | A% | 1.0 | 11.7 | 31.3 | 48.5 | 50.1 |

[a] Number of appressoria per cm root length
[b] Colonization intensity
[c] Arbuscule abundance

### Sequencing and genomic PCR analyses

The seven verified cDNA fragments were cloned and sequenced. (Fig. 3 shows the sequences of those fragments for which no full-length clones were isolated.) Different clones of one fragment were identical, indicating that at least these PCR bands represented single products. The random primer was localized at both ends in six cases and only one fragment, GA12b, carried the anchored oligo(dT) (see Fig. 6). In comparisons with

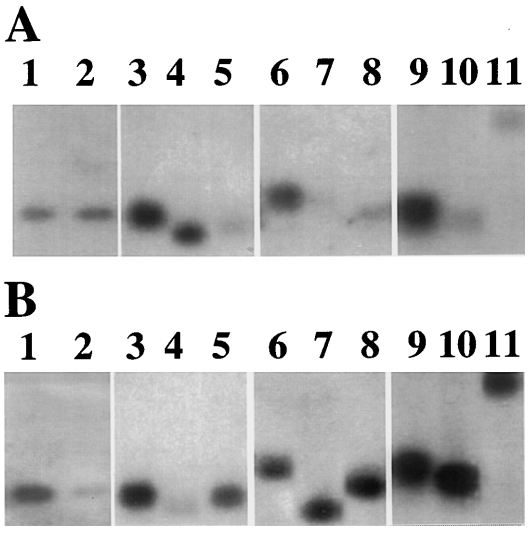


**Fig. 2A, B** Screening of DDRT fragments. Differentially occurring fragments were cut out of the polyacrylamide gels, eluted and reamplified. For verification, the resulting PCR fragments were run on two agarose gels (**A** and **B**), transferred to nitrocellulose filters and hybridized to labeled cDNA synthesized from RNA of control (**A**) and of mycorrhizal roots (**B**). Fragments which gave positive results are AA02 (lane 2), CA14 (lane 4), CA18 (lane 5), Ga12a (lane 7), GA12b (lane 8), GG01 (lane 10) and GG02 (lane 11). In the other lanes (1, 3, 6 and 9) examples are given of fragments which did not hybridize differentially

sequences in the EMBL databank, one fragment (GA12a) showed similarity (93%) to the 25S rRNA gene of *Phytophthora megasperma* (Van der Auwera 1994) and one-half of a second one (GG02) was homologous (83%) to a human Alu element (Hillier et al., Genbank accession number R99034). No significant matches were obtained for the other five sequences.

Genomic DNA from *G. mosseae* and from pea served as templates for PCR using the DDRT fragment-specific primers (Figs. 4 and 6). As expected from the hybridization to cDNA of RNA from nonmycorrhizal pea roots, the primer pairs for the fragments AA02, CA14, CA18, GA12b, GG01 and GG02 only gave PCR products with the plant DNA (Fig. 4A), while those specific for GA12a only amplified the fungal DNA (Fig. 4B).

## cDNA cloning and analysis

One of the fragments putatively derived from a differentially expressed gene was chosen for further analysis. A cDNA library was constructed from RNA of mycorrhizal root samples and $5 \times 10^5$ clones were screened using the GA12b fragment as probe. Three positive clones were isolated. The insert of the largest clone, *psam1* (*P. sativum* arbuscular mycorrhiza-induced), corresponded to the 1440-nucleotide length of the respective transcript as observed in Northern blots. Southern blot analysis using this insert as probe detected one *Eco*RI fragment of 4 kb (Fig. 5), indicating the presence of a single-copy gene in the pea genome. The

**AA02**

```
  1 TGCCGAGCTGCAGTGACAGATACAACAT TCCATGTAAGG
 41 CACAACACTAACCCTAACAACGAAAAAAATGCAACATACT
 81 GCAATGTTCGAAGGTGAATTGGAATTTCATTTGTGATATC
121 ATGATGAATAATTGGGAAAAATGGTGGCCAGTTCTTCTCC
161 TCAATAACAATTCCAGCTCGGCA
```

**CA14**

```
  1 TCTGTGCTGGCTTCAGTGTATTCAAACAATTATTCAACCT
 41 AACAAGTAATTCACCCAGTGCTTTCGATGAATCAGTTGCA
 81 CCACTCTCTCCTCATTTCTGAATTTATTAGGAATTCCCAT
121 GTATCTATTTTTTTAAAGTCCAGTATTAGAATCAAACATT
161 CCTTATATACTTTATGCTACTTGAGGTAATGCTCTTACCC
201 CAGCACAGA
```

**CA18**

```
  1 TTCCTAATGGATTGATTAAGTATTGTTTATGATTGTAGCT
 41 AACACAATGACAAGATAACATATAGTTACAGTATTTTCTT
 81 ATGAATAGTCCTAGCATGAAAAACAGAAAAATAAAAACAA
121 AAGGCTATGAAAATAATATAATTTAGGTAACACTTCCCAT
161 AATTGGCCAAGTGTTTGAAAATGCAGACTATTAACAGTCA
201 CATCTTTTAGTAGTCTAGAGATCCATTAGGAA
```

**GA12a**

```
          ... .. ............ .............
  1 TCGGCGATAGCAGTTATGAGTACGAACGAGGGTGCGAATA
    ....... ..................... .. .......
 41 AATCTCTAACCAGGATTTTCAAGGGCTGTCATGTGCGCGC
    ............... ................... ....
 81 AGGACACTTCAAAAACTAAAGTGCTTTGCCAAGGCTTCCT
    .. ........
121 CCCTATCGCCGA
```

**GG01**

```
  1 CAGGCCCTTCTCCAATGAGTCCTTCTTGTTCATGTTGTTG
 41 TCTGTTCACTGTATTCAGACAGATGATTTTCATATTTTGT
 81 TCAATTATTTTATTGCTATAGATAAGGATACTGGTTTAAT
121 ATAAGTTACATTTTTCCATTCACAAGTAGAAAGCTTTTCT
161 TCTTATATAATTGAGTTTTAAAATCTATTTTCTTATACTT
201 TATGGAAGTTTTCCTATATGTGGCTATTTCCTTGAAGGGC
241 CTG
```

**GG02**

```
  1 TGCCGAGCTGTAACCCATAAAACTATTTCTGTATAACACT
 41 CCCATTTTCAGTCTATAAATACCATTCACGTTGTGAAATG
 81 AAGCATGAAGCTCTCTGAACCACTTCCGGTTCTGGTTCTG
121 AGTACTGCCTGATTCATAAACAGTTCTTTGCCCAAAGCGA
161 CTCTGTTAATTTTTATGTTATTTTATTTACTTATTTATTT
     ...... ..... ..      ............... ...
201 ATTCTTTGAGACGAAGTCTCGCTCTGTCGCCCAGGCTGGA
    ......... ....... ..... ................
241 GTGCAGTGGTGTGATCTTAGCTCGCTGCAACCTCCACCTC
    .. . ... .  ....... ......... ....... ..
281 CCAGATTCCAGAGATTCTCCTGCCTCAGCATCCTGAGGAG
    .....  .......... .......   . ..... ....
321 CTGGGATTACAGGCACGCACCACCACATCTGGCTAATTTT
    . . ......... ....  ............... ......
401 TGTATTTTTAGTGGAGACAGGGTTTCACCATGTTGGCCAG
    ......... ..... ................ ...   ..
441 GCTGGTCTCGAACTTCTGACCTCAAGTGATCCGCCCAGCT
    .....
481 CGGCA
```

**Fig. 3** Sequences of DDRT fragments. Sequences of the DDRT fragments (except GA12b) are shown. Fragment GA12a is compared with the 25S rRNA of *Phytophthora megasperma* (EMBL accession number X75631), and fragment GG02 with a human Alu element (EMBL accession number R99034). Identical nucleotides are indicated as *dots* above the sequences. Sequences which served as fragment-specific primers for PCR with genomic DNA are *underlined*

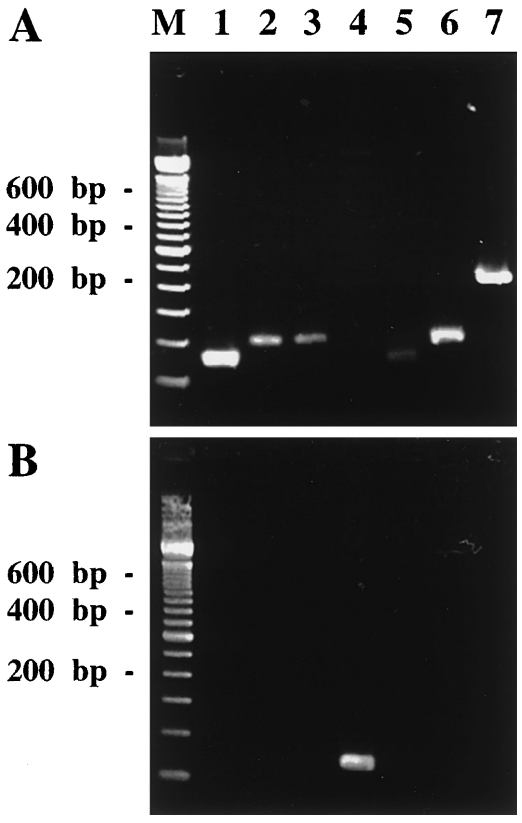


**Fig. 4A, B** PCR with genomic DNA. Genomic DNA from pea (**A**) or from *G. mosseae* (**B**) served as template for PCR with primers specific for the fragments AA02 (lane 1), CA14 (lane 2), CA18 (lane 3), Ga12a (lane 4), GA12b (lane 5), GG01 (lane 6) and GG02 (lane 7)

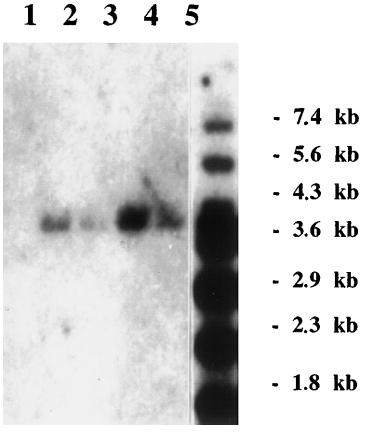


**Fig. 5** Southern blot analysis. The $^{32}$P-labeled insert of the clone *psam1* was hybridized to *Eco*RI-digested genomic DNA from *G. mosseae* spores (lane 1), uninoculated (lane 2) and *G. mosseae*-inoculated (lane 3) wild-type pea and uninoculated (lane 4) and inoculated mutant P56 (lane 5) roots. The sizes of the marker DNA fragments are given on the right

sequence of the *psam1* cDNA includes an ORF coding for a putative peptide (PSAM1) of 108 amino acids (Fig. 6). It showed no homology to any sequence in the database, but deduction of the amino acid sequence

```
1    AATTCGGCACGAGTTGAAAAACCTATACTGGCCTCCCTCC
41   CTGCTACTCCTCTTCGCTCTACACCTTCACGAATCAAAAC
81   CCCCGGCGCCGTCAACCAAAACGAAACGCCACGAACGAAA
121  ATCAACCACCAAGGACGCGATCTCCTCTTCCTTTAAGCTC
161  CAACCTAATGCTCACCGGACGCTTTCCAATTTGAACGAGG
201  GAGGTTACGACGTAACAAACTCAACGCGTTTCCAAGACCA
241  AACAAAATGCCGAAATTGATTCTGATTCGAAGCCCACACT
1          M  P  K  L  I  L  I  R  S  P  H  S
281  CACTTCCTCGCGCGAGTAATCTCGAATATGAACAATTCAC
13     L  P  R  A  S  N  L  E  Y  E  Q  F  T
321  GACCTCAACCTCAACCTCTCATGAAGAATCCAGTATGACA
26    T  S  T  S  T  S  H  E  E  S  S  M  T
361  AACGAACCCGCGCCCAAACACAACACCAGATTGAAATCCT
39   N  E  P  A  P  K  H  N  T*  R  L  K  S*  S*
401  CACCGAAAGTGTGCGCGAATGCAAGAAAATCAGGGGAAAA
53     P  K  V  C  A  N  A  R  K  S*  G  E  K
441  GAAACTCACCGAATTTGACGTTCTGATGTGGTTGTTCAAT
66    K  L  T*  E  F  D  V  L  M  W  L  F  N
481  TGGTGCATTTGTCGTTCTTCGATCGCTACGCCCTGTTGTA
79   W  C  I  C  R  S  S  I  A  T  P  C  C  M
521  TGATTCGTATGCGTTTCGCTGCATCGTCGAATGGAGGTGA
93     I  R  M  R  F  A  A  S  S  N  G  G  E
561  AGGTTTGTGTTAGACTTTGTGGCGTTGCAGGACGAGGAAT
106   G  L  C
601  GGAAATGGTAGTTCGTGCGCTCACTACTGATCCGAGATGA
641  AGAGTGAATTGGTTGATTCGTCTTGAGGTGAAGAATGTGG
681  GATATTGCAGAGTGAATCCTGGAAACATTGTTGTACTCAT
721  GAGCCGATGAAGGACCGATGAAGGAACAGAGACGTGAATG
761  AGCTCGTGACGAAGACGATTTTGCCGTTGAGGAAGAAGAA
801  TGAAGCTTGTGTTCGTTACTGATTAAAACGAGGACAAATC
841  CCTCGTTTCTGTTGTTCCATTTTCTGCACATTGTTGATTA
881  TTCCCCGCGTTTCTTTTCCCCCCTTCTCTTTCTGTTTCCG
921  TTTTTGTTGGTGGAGTTTGTTGAACTGGTTTTCTGCAGGA
961  TTATCGTGAACTGTTGGAAGAAGTTGGAAGTTGGAGGTGT
1001 TGGCAGAAGAAGATGGGGTTATGGAGTTTGGGTTGGCTCA
1041 TCATTTGTTATAGGTTCTGTTATGGTGATAATGAAGATGA
1081 AGTGTGGAGTATGAAGATGGAGGAAGGTGAAAAACGATTA
1121 TGCAGTGAAAAGCTTTTTCTGTTTGTGTGAAAATGATGAA
1161 TATCTTTTCCCTCAATCACAGACATGATCAATAGCAGGTA
1201 TTGAATTCCCATGCTAAAAATGGGATAGCGGCTGTGAAAC
1241 TCAAATGGGTGGACATGTAATGTGAAAATGGTGGTGGTTG
1281 TCAAGATAAACAAATGGGCAAAAGCTTCTCAATTCTCAAA
1321 GTCATCCCATGTATAGTTAATTAATGATTCAATGTAAAGT
1361 TAATTAATTAAAAAATTTAGGGATGCAATGTAAAGTGAAC
1401 CACTATTTGTGCTAAAACCAAATTCCTCTTGTTTTCCTCA
1441 AAAAAAAAAA
```

**Fig. 6** Sequence of the cDNA clone *psam1*. The complete nucleotide sequence of the isolated clone *psam1* is shown. Start and stop codons, putative polyadenylation signals, as well as the binding sites of the random primer and the anchored oligo(dT) for the DDRT reaction are *underlined*. Sequences which served as specific primers for PCR with genomic DNA are *underlined with dots*. The amino acid sequence deduced from the ORF are shown *below* the nucleotide sequence. The putative phosphorylation sites are marked with *asterisks* and the part of the peptide which might be located in a membrane is *underlined*

predicted several phosphorylation sites and a hydrophobic helix between residues 70 and 92, which is sufficiently long to span a membrane.

## Northern and dot blot analyses

RNA extracted at five time points from mycorrhiza of the two genotypes and from control wild-type roots was hybridized in Northern blot experiments with the DDRT fragment GA12b and, subsequently, to an rRNA probe (Fig. 7). In order to quantify the data for

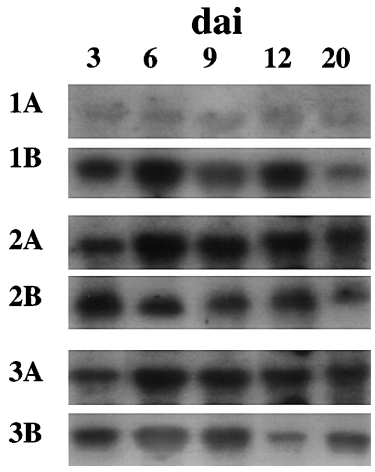


**Fig. 7** Northern blot analysis of the gene *psam1*. Total RNA extracted on various days after inoculation (dai) from wild-type control roots (**1**) and *G. mosseae*-inoculated roots (**2**), as well as from $P_{56}$ mutant *G. mosseae*-inoculated roots (**3**) was separated by gel electrophoresis, blotted and hybridized to the $^{32}$P-labeled cDNA insert (**A**). After autoradiography, membranes were stripped and rehybridized with an rRNA probe (**B**)

transcript levels, dot blot experiments were carried out on three RNA preparations and analyzed by image analysis. The results were similar to those of the Northern blot experiments. The values derived from the hybridizations with the mycorrhiza samples were normalized with reference to the data for the rRNA probe and expressed as percentages of the highest value obtained (Table 2).

The results shown in Fig. 7 and Table 2 revealed that a modulation of RNA accumulation occurs over time. While in the uninoculated controls, the amount of RNA remained at a very low level over the whole period (Fig. 7), during mycorrhiza formation *psam1* transcript levels increased and reached a maximum at 6 dai (Table 2).

## Discussion

Differential RNA display was chosen as an untargeted approach to analyze the AM symbiosis at the molecular level because it can give a broad overview of changes in the expression of different genes, as has already been shown in studies of other developmental processes in plants and in fungi (Appleyard et al. 1995; Goormachtig et al. 1995; Tseng et al. 1995; Wilkinson et al. 1995). At 6 days after inoculation of pea roots with *G. mosseae*, when infection was at an early stage (appressorium formation predominating), differences in 2% of the cDNA fragments revealed by DDRT already indicated qualitative modifications in gene expression.

Differential screening of these displayed cDNAs led to the isolation of seven cDNA fragments which represent up- or down-regulated genes in the *G. mosseae*-inoculated pea roots. These fragments represent a proportion of only 1 in 800 observed on the polyacrylamide gels. It is, however, probable that this does not reflect the relative number of genes affected during early steps of mycorrhizal development, since there may be other transcripts which are not detected by this method. In addition, differentially occurring bands could be obscured by constitutively expressed bands, as was observed by Vögeli-Lange et al. (1996), or one transcript might be represented by several DDRT fragments. A higher frequency of polypeptide modifications has been detected by two-dimensional gel electrophoresis in mycorrhizal pea roots (Samra et al. 1997).

PCR analysis with genomic DNA showed only one fragment to be of fungal origin, and its sequence was homologous to the highly expressed large ribosomal RNA gene. Such a low yield of fungus-derived cDNA results probably from the relatively low fungal biomass associated with the pea roots during the early stages of interactions (6 dai), so that for the isolation of fungal protein-encoding genes, it will be necessary to analyze later stages of the symbiosis. Among the plant-derived cDNA fragments obtained, only the fragment GG02 showed similarities to known sequences – human Alu sequences. In humans, Alu repeats can be detected in 5% of fully spliced cDNAs, predominantly in untranslated regions (Yulug et al. 1995), and accumulation of corresponding mRNAs seems to be a general stress response (Liu et al. 1995). In pea, enhanced expression of the corresponding gene is detected in the early stages of mycorrhizal interactions, which are dominated by appressorium formation and may initially be perceived as a stress by plant cells.

Sequencing of the DDRT fragments revealed that a large proportion (6 out of 7) did not contain the anchored oligo(dT), which contrasts with other published results (Tseng et al. 1995) and could result from the use of slightly different conditions for the PCR reactions. For this reason, it is not certain that these fragments are derived from the 3′ regions of the respective transcripts. Therefore, the absence of similarity to known genes cannot necessarily be explained merely by assuming that the fragments represent the untranslated parts of the mRNAs.

A complete cDNA clone, *psam1*, was obtained for the DDRT fragment GA12b. It possesses an ORF for 108 amino acids and presents no homology to known genes. Transcripts of the *psam1* gene accumulated during the

**Table 2** Relative changes in levels of *psam1* mRNA during mycorrhiza development

| Plant line | Days after inoculation[a] | | | | |
|---|---|---|---|---|---|
| | 3 | 6 | 9 | 12 | 20 |
| Wild type | 43 (±6) | 92 (±3) | 73 (±5) | 48 (±8) | 38 (±8) |
| $P_{56}$ | 50 (±7) | 100 (±3) | 78 (±3) | 67 (±5) | 48 (±7) |

[a] The values are expressed as percentages of the highest value ($P_{56}$ 6 dai) in %

symbiotic interactions in the $Myc^+$ wild-type genotype and in the non-nodulating mutant $P_{56}$. The similarity in the patterns in both genotypes indicates that induction of the gene is specifically linked to mycorrhiza formation, and is not due to the presence of rhizobia. In order to relate the pattern of RNA accumulation to specific developmental events, it will be necessary to localize by in situ hybridization those tissue or cell domains in which induction or repression of the gene is occurring, as has been done for defense genes (Harrison and Dixon 1994). However, using prediction methods for protein secondary structure (Garnier et al. 1978; Kyte and Doolittle 1982; Sipos and von Heijne 1993), it is possible to propose a model which would group the putative polypeptide PSAM1 into a class of transmembrane proteins with a regulatory function. One other member of this class is phospholamban in vertebrates. Like PSAM1, it is a small protein encoded by a relatively long mRNA, it possesses a transmembrane alpha-helix, has several phosphorylation sites in the cytoplasmatic N-terminal region and is transcriptionally regulated (Fujii et al. 1991; Arkin et al. 1994; Hu et al. 1995). It has been shown that when phospholamban in the dephosphorylated state becomes associated with an $Ca^{2+}$-ATPase, the activity of this enzyme is repressed by 50%. After phosphorylation, phospholamban is detached from the ATPase, which thereupon becomes fully active, thus allowing maximum transport of $Ca^{2+}$ ions into the endoplasmic reticulum (Reddy et al. 1995). It may be postulated that, in a similar way, production of PSAM1 following induced RNA synthesis may regulate the activity of another transmembrane protein. $Ca^{2+}$-ATPase would be an interesting candidate, since it is known that $Ca^{2+}$ plays an important role in intra- and intercellular signaling (Bush 1995), including interactions between plant cells and pathogens (Scheel et al. 1991). Future investigation of PSAM1 with respect to its localization and its phosphorylation states will help to elucidate the role of the corresponding gene *psam1*, which is the first plant gene of unknown function to be characterized in arbuscular mycorrhiza and not known to function in any other developmental or interactive process in plants.

**Acknowledgments** We thank J. F. Bonavent, G. Aubert, C. Schneider and F. Jacquin for their technical assistance. P. Franken was supported by the Deutsche Forschungsgemeinschaft, and F. Martin-Laurent by a Conseil Regional de Bourgogne/INRA grant (Contract 5315B) and PROCOPE (Contract 93134).

## References

Appleyard VCL, Unkles SE, Legg M, Kinghorn JR (1995) Secondary metabolite production in filamentous fungi displayed. Mol Gen Genet 247:338–342

Arkin IT, Adams PD, Mackenzie KR, Lemmon MA, Brunger AT, Engelman DM (1994) Structural organization of the pentameric transmembrane alpha-helices of phospholamban. EMBO J 13:4757–4764

Bauer D, Müller H, Reich J, Riedel H, Ahrenkiel V, Warthoe P, Strauss M (1993) Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). Nucleic Acids Res 21:4272–4280

Bush DS (1995) Calcium regulation in plant cells and its role in signaling. Annu Rev Plant Physiol Plant Mol Biol 46:95–122

Duc G, Trouvelot A, Gianinazzi-Pearson V, Gianinazzi S (1989) First report of non-mycorrhizal mutants (myc-) obtained in pea (*Pisum sativum* L.) and fababean (*Vicia faba* L.). Plant Sci 60:215–222

Dumas-Gaudot E, Guillaume P, Tahiri-Alaoui A, Gianinazzi-Pearson V, Gianinazzi S (1994) Changes in polypeptide patterns in tobacco roots colonized by two *Glomus* species. Mycorrhiza 4:215–221

Franken P, Gianinazzi-Pearson V (1996) Phage cloning of ribosomal RNA genes from the arbuscular mycorrhizal fungi *Glomus mosseae* and *Scutellospora castanea*. Mycorrhiza 6:167–173

Franken P, Gnädinger F (1994) Analysis of parsley arbuscular endomycorrhiza: infection development and mRNA levels of defense-related genes. Mol Plant Microbe Interact 7:612–620

Franken P, van Tuinen D, Martin-Laurent F, Gianinazzi-Pearson V (1996) Molecular characterization and gene analysis of arbuscular mycorrhizal fungi. In: Azcon-Aguilar C, Barea JM (eds) Mycorrhizas in integrated systems. From genes to plant development. European Commission, EUR 16 728, Luxembourg, pp 226–230

Fujii J, Zrain-Herzberg J, Willard H, Tada M, MacLennan DH (1991) Structure of the rabbit phospholamban gene, cloning of the human cDNA, and assignment of the gene to human chromosome 6. J Biol Chem 266:11669–11675

Garcia-Garrido JM, Toro N, Ocampo JA (1993) Presence of specific polypeptides in onion roots colonized by *Glomus mosseae*. Mycorrhiza 2:175–177

Garnier J, Osguthorpe D, Robson B (1978) Analysis of the accuracy and implication of simple methods for predicting the secondary structure of globular proteins. J Mol Biol 120:97–120

Gianinazzi-Pearson V (1996) Plant cell response to arbuscular mycorrhizal fungi: getting to the roots of the symbiosis. Plant Cell 8:1871–1883

Gianinazzi-Pearson V, Dumas-Gaudot E, Gollotte A, Tahhiri-Alaoui A, Gianinazzi S (1996) Cellular and molecular defence-related root response to invasion by arbuscular mycorrhizal fungi. New Phytol 133:45–57

Goormachtig S, Valerio-Lepiniec M.K.S, Van Montagu M, Holsters M, De Bruijn FJ (1995) Use of differential display to identify novel *Sesbania rostrata* genes enhanced by *Azorhizobium caulinodans* infection. Mol Plant Microbe Interact 8:816–824

Gorman KB, Steinberg RA (1989) Simplified method of selective amplification and direct sequencing of cDNAs. Bio Techniques 7:326–331

Harrison MJ, Dixon RA (1994) Spatial patterns of expression of flavonoid/isoflavonoid pathway genes during interactions between roots of *Medicago truncatula* and the mycorrhizal fungus *Glomus versiforme*. Plant J 6:9–20

Hosny M, Dulieu H, Gianinazzi-Pearson V (1996) A simple and rapid method for collecting Glomales spores from soil. In: Azcon-Aguilar C, Barea JM (eds) Mycorrhizas in integrated systems. From genes to plant development. European Commission, EUR 16 728, Luxembourg, pp 541–542

Hu P, Zhang KM, Wright LD, Nixon TE, Wechsler AS, Spratt JA, Briggs FN (1995) Transcriptional regulation of phospholamban gene and translational regulation of SERCA2 gene produces coordinate expression of these two sarcoplasmic reticulum proteins during skeletal muscle phenotype switching. J Biol Chem 270:11619–11622

Kyte J, Doolittle RF (1982) A simple method for display the hydrophobic character of a protein. J Mol Biol 157:105–132

Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257:967–971

Liu WM, Chu WM, Choudary PV, Schmid CW (1995) Cell stress and translational inhibitors transiently increase the abundance of mammalian Sine transcripts. Nucleic Acids Res 23:1758–1765

Martin-Laurent FA, Franken P, Gianinazzi S (1995) Screening of cDNA fragments generated by differential RNA display. Anal Biochem 228:182–184

Newman EI, Reddel P (1987) The distribution of mycorrhizas among families of vascular plants. New Phytol 106:745–751

Pearson RP, Mitchelhill KI, Kemp BE (1993) Studies of protein kinase/phosphatase specificity using synthetic peptides. In: Hardie DG (ed) Protein phosphorylation. Oxford University Press, New York, pp 269–291

Reddy LG, Jones LR, Cala SE, Obrian JJ, Tatulian SA, Stokes DL (1995) Functional reconstitution of recombinant phospholamban with rabbit skeletal $Ca^{2+}$-ATPase. J Biol Chem 270:9390–9397

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual ($2^{nd}$ edn). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Samra A, Dumas-Gaudot E, Gianinazzi S (1997) Detection of new polypeptides during the early recognition stages and the establishment of arbuscular mycorrhiza between *Glomus mosseae* and *Pisum sativum* L. roots. New Phytol 135:711–722

Sanger F, Nicklen S, Coulsen AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Scheel D, Colling C, Hedrich R, Kawalleck P, Parker JE, Sachs WR, Somssich IE, Hahlbrock K (1991) Signals in plant defense gene activation. In: Hennecke H, Verma DPS (eds) Advances in molecular genetics of plant-microbe-interactions, vol 1. Kluwer Academic Publishers, Dordrecht, pp 373–380

Sipos L, von Heijne G (1993) Topology of eukaryotic proteins. Eur J Biochem 213:1333–1340

Trouvelot A, Kough JL, Gianinazzi-Pearson V (1986) Mesure du taux de mycorhization VA d'un système radiculaire. Recherche des méthodes d'estimation ayant une signification fonctionnelle. In: Gianinazzi-Pearson V, Gianinazzi S (eds) Les mycorhizes: Physiologie et génétique. INRA Presse, Paris, pp 217–221

Tseng T-C, Tsai T-H, Lue M-Y, Lee H-T (1995) Identification of sucrose-regulated genes in cultured rice cells using mRNA differential display. Gene 161:179–182

Van der Auwera G, Chapelle S, De Wachter R (1994) Structure of the large ribosomal subunit RNA of *Phytophthora megasperma*, and phylogeny of the oomycetes. FEBS letters 338:133–136

Vögeli-Lange R, Bürckert N, Boller T, Wiemken A (1996) Rapid selection and classification of positive clones generated by mRNA differential display. Nucleic Acids Res 24:1385–1386

Wilkinson JQ, Lanahan MB, Conner TW, Klee HJ (1995) Identification of mRNAs with enhanced expression in ripening strawberry fruit using polymerase chain reaction differential display. Plant Mol Biol 27:1097–1108

Yulug IG, Yulug A, Fisher EMC (1995) The frequency and position of Alu repeats in cDNAs, as determined by database searching. Genomics 27:544–548

Zézé A, Dulieu H, Gianinazzi-Pearson V (1994) DNA cloning and screening of a partial genomic library from an arbuscular mycorrhizal fungus, *Scutellospora castanea*. Mycorrhiza 4:251–254