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Differential Expression of Eight Chitinase Genes in *Medicago truncatula* Roots During Mycorrhiza Formation, Nodulation, and Pathogen Infection

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Expression of eight different chitinase genes, representing members of five chitinase classes, was studied in *Medicago truncatula* roots during formation of arbuscular mycorrhiza with *Glomus intraradices*, nodulation with *Rhizobium meliloti*, and pathogen attack by *Phytophthora megasperma* f. sp. *medicaginis*, *Fusarium solani* f. sp. *phaseoli* (compatible interactions with root rot symptoms), *Aschochyta pisi* (compatible, symptomless), and *F. solani* f. sp. *pisi* (incompatible, nonhost interaction). In the compatible plant-pathogen interactions, expression of class I, II, and IV chitinase genes was enhanced. The same genes were induced during nodulation. Transcripts of class I and II chitinase genes accumulated transiently during early stages of the interaction, and transcripts of the class IV chitinase gene accumulated in mature nodules. The pattern of chitinase gene expression in mycorrhizal roots was markedly different: Expression of class I, II, and IV chitinase genes was not enhanced, whereas expression of three class III chitinase genes, with almost no basal expression, was strongly induced. Two of these three (*Mtchitinase* III-2 and *Mtchitinase* III-3) were not induced at all in interactions with pathogens and rhizobia. Thus, the expression of two mycorrhiza-specific class III chitinase genes can be considered a hallmark for the establishment of arbuscular mycorrhiza in *Medicago truncatula*.

Additional keywords: chalcone synthase, trehalase.

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Nucleotide and/or amino acid sequence data have been submitted to the National Center for Biotechnology Information (NCBI) Genome Survey Sequence data base (GSSdb) as the following accession numbers: *Mtchitinase* I, AF167322; *Mtchitinase* II, AF167323; *Mtchitinase* III-1, AF167324; *Mtchitinase* III-2, AF167325; *Mtchitinase* III-3, AF167326; *Mtchitinase* III-4, AF167327; *Mtchitinase* IV, AF167328; *Mtchitinase* V, AF167329. Sequence data for trehalase and ubiquitin were deposited at the EMBL gene bank as accession number AJ238651 for the trehalase gene, AJ245511 for the ubiquitin gene fragment of *Medicago truncatula*, and AJ245512 for the ubiquitin gene fragment of *Glomus mosseae*.

Medicago truncatula is a diploid autogamous legume with a short generation time that can be readily transformed with *Agrobacterium tumefaciens* (Cook et al. 1997). While the well-known model plant *Arabidopsis thaliana* only interacts antagonistically with microorganisms and has no known mutualistic symbionts, *M. truncatula*, like most legumes, has the potential to entertain two different mutualistic symbioses in its roots, namely, with fungi involved in arbuscular mycorrhiza (AM) (Harrison and Dixon 1994; Salzer et al. 1999) and with rhizobia involved in nodule formation and nitrogen fixation (Cook et al. 1995, 1997). This allows comparisons between different types of plant-symbiont and plant-pathogen interactions, and *M. truncatula* has therefore been proposed as a model plant to study interactions with microorganisms (Cook 1999).

Chitinases have received particular attention in plant-microbe interactions and have been the subject of many reviews (Boller 1987; Collinge et al. 1993; Meins et al. 1992, 1994). They are grouped into six classes (I–VI): each class is characterized by a common primary structure of the protein (Collinge et al. 1993; Meins et al. 1992, 1994; Melchers et al. 1994) and by consensus sequences (Levorson and Chlan 1997). Chitinases are hydrolytic enzymes (E.C. 3.2.1.14) that are able to cleave β-1,4-glycosidic bonds between *N*-acetyl glucosamine residues of chitin. This polysaccharide is a primary structural component of the wall of all true fungi, including fungi involved in AM and ectomycorrhiza (EM), as well as many pathogens, indicating that chitinases are important in plant-fungal interactions (see Boller 1987).

The role of chitinases as part of the inducible plant defense response is well documented (Boller 1987; Collinge et al. 1993). For example, class I and V chitinases were shown to have anti-fungal activity in vitro (Arlorio et al. 1992; Mauch et al. 1988; Melchers et al. 1994; Schlumbaum et al. 1986), and tobacco plants constitutively expressing class I chitinases showed increased resistance to the pathogenic fungus *Rhizoctonia solani* (Broglie et al. 1991; Vierheilig et al. 1993).

The role of chitinases in mutualistic symbioses is less clear. With regard to the nodule symbiosis, chitinases have been shown to differentially cleave Nod factors, and they have therefore been proposed to modulate the activity of these key morphogenetic signals during symbiotic development

(Schultze et al. 1998; Staehelin et al. 1994a, 1994b). In EM, plant chitinases may function to degrade chitin fragments, released from walls of the symbiotic fungus, that would otherwise elicit host defense responses (Salzer et al. 1997a, 1997b). Interestingly, growth of fungi involved in EM and AM formation were not inhibited by class I chitinases that were antifungal against pathogens (Arlorio et al. 1991; Salzer et al. 1997b; Vierheilig et al. 1993). Taken together, these results suggest differential effects of chitinases in symbiotic versus pathogenic interactions.

Generally, chitinase activity is at a low level in plants but is induced in response to various stimuli, e.g., the phytohormone ethylene (Chen and Bleeker 1995; Shinshi et al. 1995; Siefert et al. 1994; Xie et al. 1996), salicylic acid (Jung et al. 1993; Margis-Pinheiro et al. 1994), virus infections (Lawton et al. 1992; Ohme-Takagi et al. 1998; Payne et al. 1990), and various pathogenic microorganisms (Kästner et al. 1998; Mohr et al. 1998; Münch-Garthoff et al. 1997; Vad et al. 1993). With respect to the mutualistic AM symbiosis, studied in leek infected with *G. mosseae*, chitinase activity increased during early stages of the interaction and was suppressed in later stages (Spanu et al. 1989). Similar results have been obtained in the alfalfa-*G. intraradices* interaction (Volpin et al. 1994).

There are indications that the induction of various isoforms of chitinases is differentially regulated at the level of gene expression (Collinge et al. 1993; Meins et al. 1992). The same stimulus may trigger up-regulation of certain chitinase isoforms and down-regulation of others. In tobacco, for example, two acidic isoforms of chitinase were induced and expression of a basic chitinase gene suppressed by the AM fungus *Glomus intraradices* (David et al. 1998; Dumas-Gaudot et al. 1992).

Here, we establish *M. truncatula* as a model for the functional analysis of chitinases in roots interacting with pathogenic fungi, rhizobia, and mycorrhizal fungi. We provide a comprehensive picture of the expression of individual chitinase genes in these different plant-microbe interactions, and we describe the induction of two class III chitinase genes in *M. truncatula* as a hallmark for the establishment of the AM symbiosis.

RESULTS

Infection of *M. truncatula* roots with pathogenic fungi.

As a basis for a comparison of chitinase gene expression between *M. truncatula* roots colonized by mutualistic symbionts and pathogenic fungi, we first established pathosystems for *M. truncatula* roots consisting of compatible interactions with strong root rot symptoms, compatible, symptomless biotrophic interactions, and incompatible nonhost interactions. Inoculation of *M. truncatula* roots with *Fusarium solani* f. sp. *phaseoli*, the causal agent of dry root rot of bean (Vögeli-Lange et al. 1995), produced necrotic lesions on roots of *M. truncatula*, accompanied by the collapse of cortical cells. Within 5 days, the infection spread along the primary and secondary roots (Fig. 1A) and the fungus formed typical, sickle–shaped macro-conidia within the root tissue (Fig. 2A). Infection with *Phytophthora megasperma* f. sp. *medicaginis*, the causal agent of alfalfa root rot, also caused browning of the lateral roots (Fig. 1B). In the infected areas of the root, clusters of young oogonia with paragyn antheridia (Fig. 2B) as well as sporangia (Fig. 2C) were formed, indicating that this fungus completed its life cycle within *M. truncatula* roots. *Ascochyta pisi* caused no obvious disease symptoms 5 days after inoculation (Fig. 1D). However, microscopic examination of the roots revealed that this fungus colonized the rhizodermis and root hairs and formed chains of chlamydospores within the plant tissue (Fig. 2D, E). In contrast to the compatible interactions described above, *Fusarium solani* f. sp. *pisi* caused no disease symptoms (Fig. 1C) and failed to penetrate the roots. Although inoculated chlamydospores attached to the root surface, fungal structures could not be detected within the root tissue 5 days after inoculation (not shown).

Cloning of chitinase genes.

To obtain chitinase genes from *M. truncatula*, we compared DNA sequences for over 80 plant, fungal, animal, and bacterial genes (data not shown). Conserved regions for each of the subclasses that contained plant chitinase genes were identified and used to design degenerate oligonucleotide primers for polymerase chain reaction (PCR; Table 1). Following amplification of genomic DNA from *M. truncatula*, DNA sequencing of the cloned amplification products confirmed that we had cloned PCR fragments for eight distinct chitinase genes, representing five chitinase subclasses. One gene each was identified for subclasses I, II, IV, and V, while four distinct genes were identified for subclass III (Fig. 3). By means of Southern blot analysis, we determined that each of these eight genes did not cross-hybridize with any of the other chitinase genes (data not shown). We recovered the corresponding full-length genomic clones from an *M. truncatula* BAC (bacterial artificial chromosome) genomic library (Nam et al. 1999), as indicated in Table 1.

Use of ubiquitin genes and a trehalase gene as controls in RT-PCR.

To compare gene expression by reverse transcription (RT)-PCR between noninfected roots and roots infected by fungi, it is necessary to use an internal standard to monitor the amount of cDNA derived from the plant. For this purpose we selected the ubiquitin gene, which had been demonstrated to be constitutively expressed in pea, and therefore seemed to be useful as an internal control (Albrecht et al. 1998). To design primers specific for *M. truncatula* ubiquitin, *M. truncatula* and *Glomus mosseae* genomic DNA was amplified by PCR with primers corresponding to conserved regions of ubiquitin (Heidstra et al. 1997). Sequencing of the cloned fragments revealed 77% homology at the nucleotide level (Fig. 4A) and 96% homology at the protein level (data not shown) between the *M. truncatula* and *G. mosseae* genes. Using sequence alignment, we designed oligonucleotide primers against regions of the *M. truncatula* gene that diverged considerably from the *G. mosseae* gene (Fig. 4A). These primers readily amplified the ubiquitin template from total *M. truncatula* genomic DNA, but not from genomic DNA of the fungal symbiont *G. intraradices*, or from the pathogenic fungi *Phytophthora megasperma* f. sp. *medicaginis*, *Fusarium solani* f. sp. *phaseoli*, or *Fusarium solani* f. sp. *pisi* (Fig. 4B). Although an amplification product was obtained from *A. pisi* DNA, the PCR product had a larger size and thus was easily distinguished from *M. truncatula* upon agarose gel electrophoresis (Fig. 4B). Similar results were obtained by RT-PCR with

RNA from *M. truncatula* and the different fungi (data not shown).

Furthermore, there is a potential risk of genomic DNA contaminations in RT-PCR studies, although the RNA preparations are routinely treated with DNase I before RT. To visualize potential contaminations by genomic DNA, we used a trehalase gene from *M. truncatula* that contained an intron, and we designed primers spanning the intron.

Induction of chitinase gene expression by pathogenic fungi.

In the compatible interactions with *F. solani* f. sp. *phaseoli* and *P. megasperma* f. sp. *medicaginis*, which led to strong disease symptoms, expression of *Mtchitinase* I, II, III-1, and IV genes was enhanced, with the strongest increase occurring in expression of *Mtchitinase* IV (Fig. 5). In the compatible biotrophic interaction with *A. pisi*, expression of *Mtchitinase* I and III-1 was enhanced. In addition, *Mtchitinase* III-4 was expressed at a low level (Fig. 5). On the other hand, in the incompatible interaction with *F. solani* f. sp. *pisi*, only expression of *Mtchitinase* IV was stimulated (Fig. 5).

Induction of different chitinase genes during early and late stages of nodule formation.

In contrast to infection by pathogenic fungi, formation of mutualistic symbioses in *M. truncatula* is a process that demands several weeks. Three days after inoculation with *Rhizobium meliloti*, nodule meristems were apparent; after 10 days, white nodules have developed; and after 20 days, the entire root system harbored red, functional nodules. Chitinase gene expression differed at various stages of the nodulation process. At early stages, about 3 days after inoculation, expression of *Mtchitinase* I and II was transiently increased (Fig. 6). At later stages, expression of these genes was hardly detectable but transcripts of *Mtchitinase* IV chitinase strongly accumulated in nodules, particularly in the most mature ones. In addition, expression of *Mtchitinase* III-4 was slightly induced (Fig. 6).

Expression of class III chitinase genes in AM formed by *G. intraradices* on roots of *M. truncatula*.

To perform studies on gene expression in early and later stages of the mycorrhiza formation, we employed two different inoculation procedures. For early interactions, the inoculum was directly dropped on the roots to provide contact with the fungus from the beginning of the experiment. To obtain even colonization of the whole root system in later stages, the inoculum was mixed with the substratum before the seedlings were planted.

In early stages, before arbuscules were formed, the *M. truncatula* roots showed only very little response to *G. intraradices*. Expression of *Mtchitinase* II, III-1, and IV, as well as expression of a chalcone synthase gene, was only slightly enhanced, and expression of *Mtchitinase* III-2, III-3, and III-4 could not be detected (Fig. 7). In fully mycorrhizal roots at a later stage, however, where about 60% of the root length was colonized (in 1 cm of colonized roots up to 100 arbuscules and 10 vesicles were found), expression of these three class III chitinase genes, namely, *Mtchitinase* III-2, III-3, and III-4, was strongly induced (Fig. 8). In contrast, expression levels of *Mtchitinase* I, II, III-1, and IV, which were increased in response to pathogenic fungi and rhizobia, were similar or somewhat lower than in nonmycorrhizal controls (Fig. 8).

DISCUSSION

By establishing four different pathosystems for *M. truncatula* roots, in conjunction with the already well-described mutualistic symbioses with rhizobia and AM fungi, we are able to provide a comprehensive picture of chitinase gene expression in one plant in response to various kinds of interactions with microorganisms. Our first surprising result is that chitinase gene expression during nodulation with rhizobia is more related to interactions with pathogenic fungi than to formation of AM. Even more unexpected is our finding that chitinase gene expression in mycorrhizal roots is different from that in all the other types of interactions and involves a mycorrhiza-specific expression of two class III chitinase genes.

Chitinase gene expression in response to pathogenic fungi.

Induction of chitinase activity and of genes encoding various isoforms of chitinase has been extensively studied in plant-pathogen interactions, and the expression patterns we observed with our newly established pathosystems for *M. truncatula* roots corresponded to what was known from other plant pathogen interactions (Table 2). For example, the induction of *Mtchitinase* I, II, III-1, and IV in response to the different *Fusarium solani* strains closely resembled their induction in broad bean (Mohr et al. 1998), and induced expression of a class I chitinase gene in response to *A. pisi* was also found in pea (Vad et al. 1993). On the other hand, *Mtchitinase* III-4 was differentially expressed in response to virulent and avirulent fungi. This gene was only expressed in interactions with those pathogenic fungi that caused no disease symptoms in *M. truncatula* roots.

Differential induction of chitinases in *M. truncatula* during early and late stages of nodulation.

Expression of different chitinase genes occurred in *M. truncatula* during early and later stages of the nodule formation with *R. meliloti*. During early stages of the symbiosis, when first nodule primordia became obvious, the expression of *Mtchitinase* I and II transiently increased. Because these same chitinase genes were induced in *M. truncatula* during infection by pathogenic fungi, and class I and II chitinase genes are commonly induced by pathogenic fungi (Table 2), it seems plausible that induction of these genes during nodulation may represent a transient defense response to compatible *R. meliloti*. In fact, Vasse et al. (1993) documented the accumulation of the pathogenesis-related (PR) proteins P and Q (corresponding to class II chitinases) during the alfalfa-*R. meliloti* symbiosis, and determined that these epitopes localized to necrotic cells that also contained arrested rhizobial infections, reminiscent of a defense response. The transient character of the chitinase expression in *M. truncatula* roots probably reflected the transient action of Nod factors, which can also act as elicitors. In roots of soybean, for example, Nod factors from *Bradyrhizobium japonicum* were sufficient to induce enhanced chitinase activity (Xie et al. 1999).

In contrast to the transient expression of class I and II chitinases, class IV chitinase transcripts were detected as early

as 1 day post inoculation, and continued to increase throughout the 20-day time course of nodulation. Moreover, transcript levels were elevated both in nodules and in areas of the roots that lacked nodules. Staehelin et al. (1992) determined that, within soybean nodules, chitinase was localized in the nodule endodermis. Thus, in soybean nodules the chitinase activity is unlikely to interact directly with the bacterial symbiont, but may instead provide a preformed barrier to fungal infection.

Interestingly, expression of class III chitinase genes in *M. truncatula* during nodulation differed from that of other legumes. While in *Sesbania rostrata* a class III chitinase is supposed to be a early nodulin (Goormachtig et al. 1998) and in *Vicia faba* two proteins (Nvf32-A1, Nvf32-A2) with some sequence homology to class III chitinases accumulated during nodule formation (Perlick et al. 1996), in no stage of the interaction with *R. meliloti* did strong induction of a class III chitinase gene occur in *M. truncatula* roots.

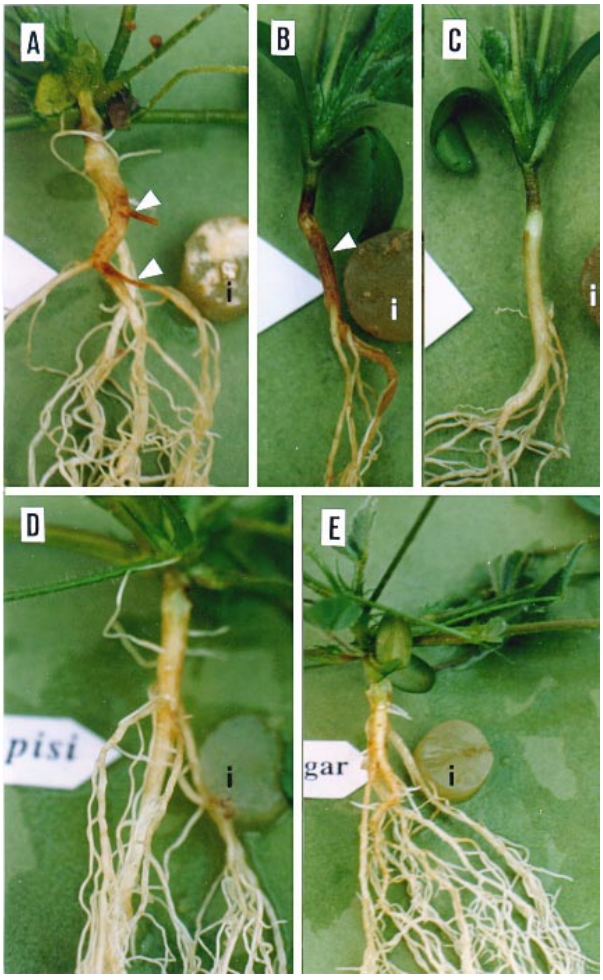

Fig. 1. Macroscopic disease symptoms of *Medicago truncatula* roots infected with pathogenic fungi. V8-agar disks with fungal inoculum were brought into contact with the root surface of 3- to 5-week-old *M. truncatula* plants. Five days after inoculation with **(A)** *Fusarium solani* f. sp. *phaseoli* and **(B)** *Phytophthora megasperma* f. sp. *medicaginis*, browning of roots became obvious (indicated by arrowheads). No browning was observed after inoculation with **(D)** *Ascochyta pisi*, **(C)** *F. solani* f. sp. *pisi*, and **(E)** V8 agar disks without fungus. Similar observations were made in five independent plants in two independent experimental series for each of the pathogenic fungi.

Expression of mycorrhiza-specific class III chitinase genes in AM of *M. truncatula*.

Chitinase expression in mycorrhizal *M. truncatula* roots was completely different from that in roots infected with rhizobia or pathogenic fungi (Fig. 9). In roots colonized by *G. intraradices*, expression of *Mtchitinase* III-2, III-3, and III-4 was strongly induced in mature mycorrhizae. The expression of *Mtchitinase* III-2 and III-3 was mycorrhiza specific, because they were not expressed in any other type of interaction. On the other hand, expression of those chitinase genes that are induced during pathogen attack or nodulation, namely, *Mtchitinase* I, II, and IV, was not increased in functioning mycorrhizae. These mycorrhiza-specific changes in chitinase gene expression are unexpected, because numerous previous studies had indicated that similar genes, proteins, and signal transduction pathways were activated during AM formation and nodulation (Albrecht et al. 1998; Frühling et al. 1997; Wyss et al. 1990; Xie et al. 1999). Apparently, to maintain a functional AM association with *G. intraradices*, the expression of a unique set of chitinase genes is required: In AM of *M. truncatula* with *G. intraradices* all class III chitinase genes are expressed, and the expression of the defense-related *Mtchitinase* I, II, and IV is kept at a low, basal level or even suppressed (Fig. 9).

The function of the class III-2 and III-3 chitinases, which are specifically induced in AM of *M. truncatula*, is unclear. However, it is conceivable that expression of these class III chitinases is involved in suppression of plant defense reactions in the later stages of the AM as observed in bean, leek, and alfalfa (Lambais and Mehdy 1993; Spanu et al. 1989; Volpin et al. 1994). The chitinases, which are induced by the AM fungus, could cleave the fungal elicitors and, in turn, attenuate defense responses. The following arguments support this hypothesis: (i) in various cell culture systems, e.g., tomato (Felix et al. 1993), rice (Inui et al. 1996, 1997), alfalfa (Savouré et al. 1997), and spruce (Hebe et al. 1999), *N*-acetyl glucosamine oligomers were demonstrated to induce defense responses; (ii) chitin-related elicitors were also released from walls of the AM fungus *G. intraradices* (Salzer and Boller 2000); (iii) chitinases from spruce cells were able to cleave elicitors from various EM fungi in the same way as chitin elicitors (Salzer et al. 1997a, 1997b); and (iv) treatment of EM elicitors with chitinases reduced their ability to induce defense responses in plant cells (Salzer et al. 1997a, 1997b).

MATERIALS AND METHODS

Plant culture, synthesis of AM, and infection with rhizobia and pathogenic fungi.

The *Medicago truncatula* cv. Jemalong strain A 17 was germinated on agar and grown in Terra Green as previously described (Salzer et al. 1999), with the exception that the plants were continuously fertilized with B&D medium (Broughton and John 1979) containing 0.5 mM inorganic potassium phosphates and 2 mM KNO_3. These culture conditions were continued after inoculation of the plants with all the different microorganisms.

Culture of the AM-forming fungus *Glomus intraradices* Schenk & Smith, inoculation, and synthesis of AM were performed as previously described (Salzer et al. 1999).

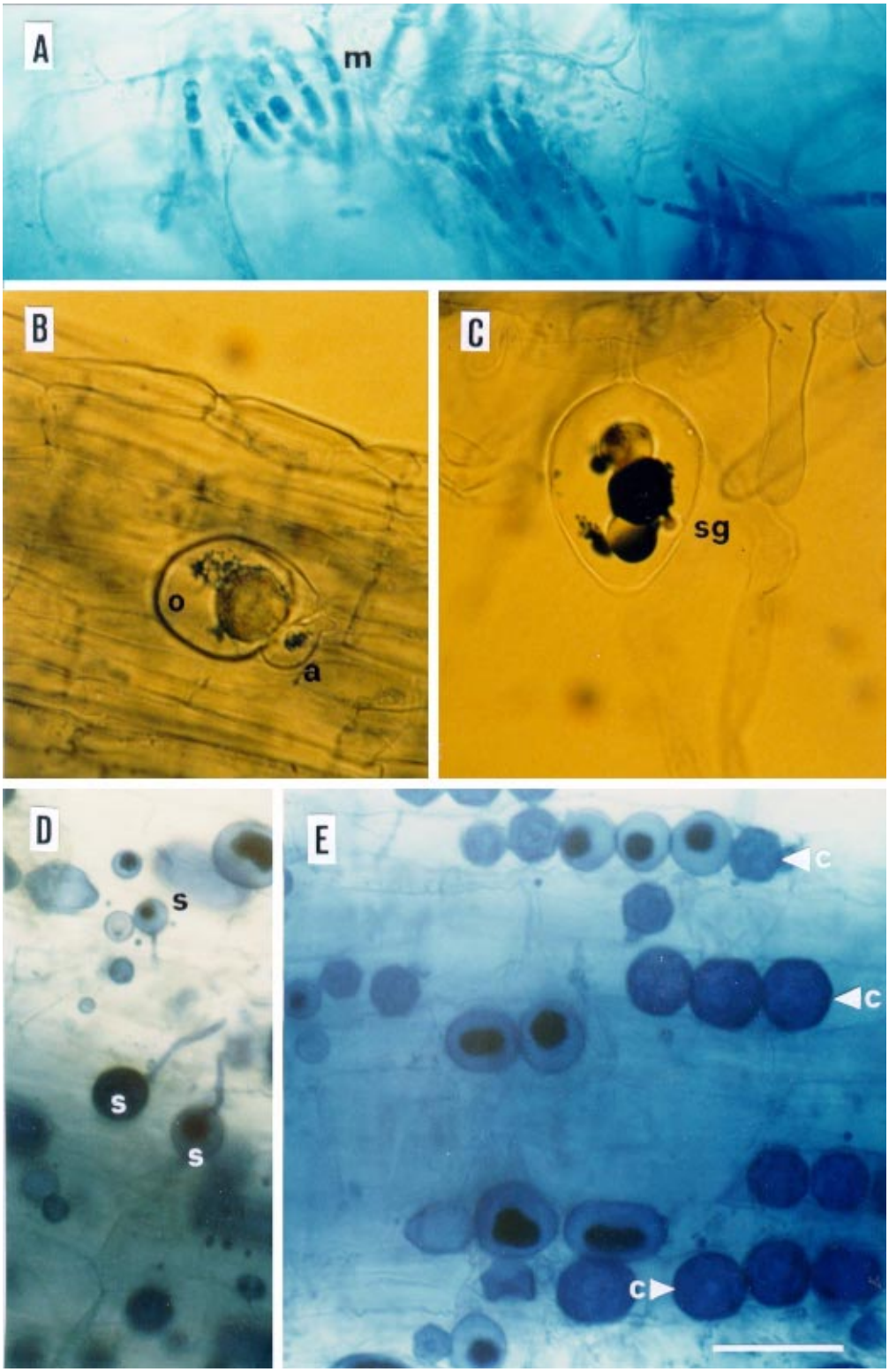

Fig. 2. Reproductive structures of pathogenic fungi in *Medicago truncatula* roots. **A,** Sickle-shaped macroconidia (m) of *Fusarium solani* f. sp. *phaseoli* within *M. truncatula* root tissue. **B,** Oogonium (o) with a paragyn antheridium (a), and **(C)** an ovoid sporangium (sg) of *Phytophthora megasperma* f. sp. *medicaginis* protruding from root surface of *M. truncatula*. **D,** Germinating spores (s) of *Ascochyta pisi* on surface and chains of chlamydospores (c) within rhizodermal cells of *M. truncatula*. Structures shown were observed 5 days after inoculation. Bar = 20 µm. With each fungus, similar observations were made on five separately cultured plants in two independent experiments.

For nodulation, 3 ml of a *Rhizobium meliloti* (strain 1021, kindly provided by P. Curioni, ETH Zürich, Switzerland) suspension was added to 6- to 10-week-old plants. Before inoculation, the rhizobia had been cultured at 27°C for 3 days in succinate minimal medium (Schmidt et al. 1992). Control plants were mock inoculated with 3 ml of the bacterial culture medium.

The following pathogenic fungi were used to inoculate 3- to 6-week-old *M. truncatula* plants: *Fusarium solani* f. sp. *phaseoli* (strain W-8), *F. solani* f. sp. *pisi* (teleomorph *Nectria haematococca* mating population VI strain 77-104), *Phytophthora megasperma* f. sp. *medicaginis* (kindly provided by the Novartis fungal pathogen collection, Basel, Switzerland) and *Ascochyta pisi* Lib. (from the house collection of the Botanical Institute, Basel). Before inoculation, the fungi were grown on V8 agar at room temperature for 12 days. Then, agar disks (0.5 cm in diameter) were cut from the margins of the fungal mycelium and placed close to the root. In control experiments, plants were mock inoculated with V8 agar disks without fungi.

All procedures described above were performed under sterile conditions.

Infection analyses.

For microscopic studies, roots were cleared with 10% KOH and stained with trypan blue in lactophenol (Phillips and

Table 1. Degenerate primer sequences used for polymerase chain reaction cloning of *Medicago truncatula* genes[a]

Conserved amino acid motifs	Oligonucleotide sequence	Product size	BAC clone[b]
Chitinase classes			
I			
LCCS(QKEN)(FY)G(WF)CG	TTATGCTGYWSIVAITWYGGITKBTGYGG	330 bp	08P19
QTSHET(TM)G	GACTTACCIRTNGTYTCRTGISWIGTYT		
II			
VNNPDLVATD	GTCAAYAAYCCIGAYYTNGTIGCNACIGA	310 bp	52E20
NLDC(YN)NQ(RK)NF	GTTRTTIYKYTGRTTGTWTCARTCIARRT		
III			
WVW(VI)QFYNNP	TGGGTCTGGGTICARTTYTAYAAYAAYCC	220 bp	05N18, 51J12
YGGYMLW(SDN)RF	GAACGCTHCCAIARCATIACNCCNCCRTA		72H13, 80G10
IV			
LCCS(QNRK)(FY)G(FY)CG	CTITGYTGYWSIMRITWYGGITWYTGYGG	310 bp	46B22
FAH(VAFI)(TS)HETGH	TGTCCIGTATCRTGISWIRHRTGIGCRAA		
V			
KLVLG(IM)P(FMI)YG	AAATTGGTIYTIGGIATNCCIQTNTAYGG	250 bp	53L15
WISYDDTQSV	ACAGTTTGIGTRTCRTCRTANSWIATCCA		
Trehalase			
GQQWDFPNG	CARCARTGGGAYTTYCCNAAAAYG	245 bp	
PQTGFGWSNG	CCNCARACNGGNTTYGGNTGGTCNAAYGGN		

[a] Degenerate nucleotides: R = A or G; Y = C or T; B = not A; H = not G; N = A, C, G or T; W = A or T; S = G or C; K = G or T; M = A or C. Amino acids in parentheses represent variation at a single position.
[b] Bacterial artificial chromosome.

Fig. 3. Nucleotide sequence of chitinase gene fragments amplified from a *Medicago truncatula* genomic bacterial artificial chromosome (BAC) library (Nam et al. 1999) by polymerase chain reaction with degenerate oligonucleotide primers, then cloned and sequenced. Arrows indicate primer sequences used for semiquantitative reverse transcription-PCR and their orientation.

Hayman 1970). The degree of colonization with *G. intraradices* was determined according to Giovanetti and Mosse (1980). Infection with pathogenic fungi was examined on longitudinal sections of the infected root area with a Zeiss Axioplan microscope under bright-field conditions. Pictures were taken with a Zeiss M 35 F camera coupled to a Zeiss MC 63 light detection system with Kodak EPJ 320T films.

mRNA expression studies.

Plant material (100 mg fresh weight) was homogenized in liquid nitrogen and RNA extracted with a Plant RNeasy Extraction Kit (Qiagen, Basel, Switzerland) with RLT-lysis buffer, and a 3-min incubation at 56°C. Then, the RNA preparation was treated with DNase I (Message Clean Kit; GeneHunter, Nashville, TN), following the manufacturer's instructions. Reverse transcription of 1 µg of RNA was performed with the Reverse Transcription System (Promega, Madison, WI) in a total volume of 20 µl with oligo $(dT)_{15}$ primers at 42°C for 15 min. Semiquantitative PCR was carried out with a dilution series (1:1, 1:4, 1:16, 1:64, 1:256) of the template cDNA. One microliter of cDNA was used per 20 µl of PCR mix, containing 2 µl of 10× PCR buffer (Amersham Pharmacia Biotech Europe, Dübendorf, Switzerland, 1.6 µl of dNTPs (2.5 mM concentrations of each; Amersham Pharmacia), 0.1

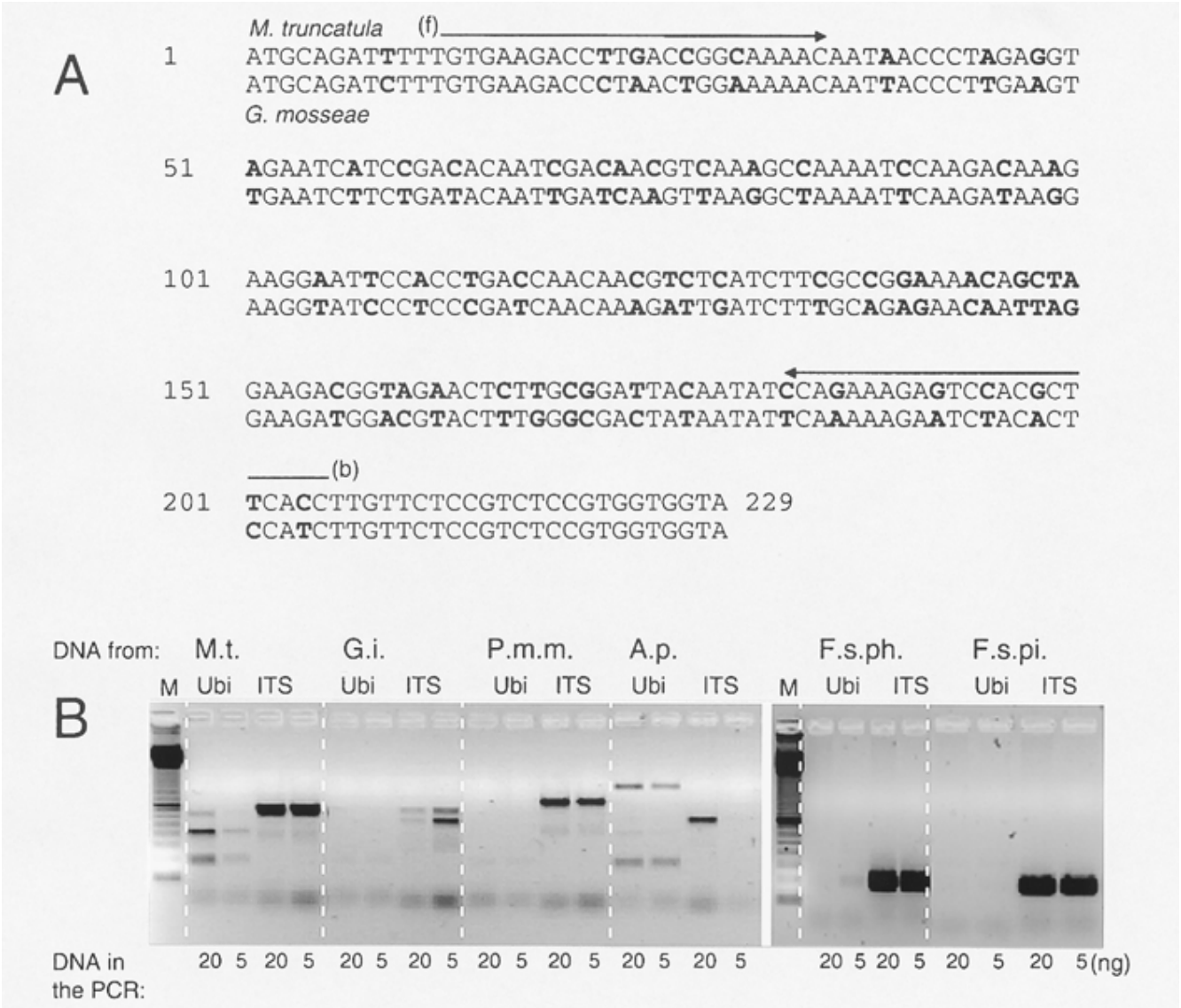

Fig. 4. Specific detection of ubiquitin from *Medicago truncatula.* **A,** Alignment of nucleotide sequences from ubiquitin from *M. truncatula* (upper line) and *Glomus mosseae* genomic DNA (lower line). Different nucleotides at corresponding positions are typed in bold. Arrows indicate position and orientation of *M. truncatula*-specific primers designed for reverse transcription-polymerase chain reaction (RT-PCR); (f) indicates their forward, (b) their backward orientation. **B,** PCR on genomic DNA with *M. truncatula*-specific primers. PCR only amplified ubiquitin fragments from *M. truncatula* (M.t.), not from *G. intraradices* (G.i.), *Phytophthora megasperma* f. sp. *medicaginis* (P.m.m.), *Fusarium solani* f. sp. *phaseoli* (F.s.ph.), or *F. solani* f. sp. *pisi* (F.s.pi.). Fragments amplified from *Ascochyta pisi* differed in size from those of *M. truncatula,* allowing recognition of *M. truncatula* ubiquitin by different size. Size differences of 100 bp are indicated by bands of the 100-bp ladder, which served as marker (M). PCR products obtained with ITS1/ITS4 primer combinations served as positive control for presence of fungal or plant DNA. With the exception of the *Fusarium* spp., where ITS1/ITS4 primer combinations specific for *Fusarium* spp. were used, primers described by Heidstra et al. (1997) were employed. The same results were obtained in two independent experiments.

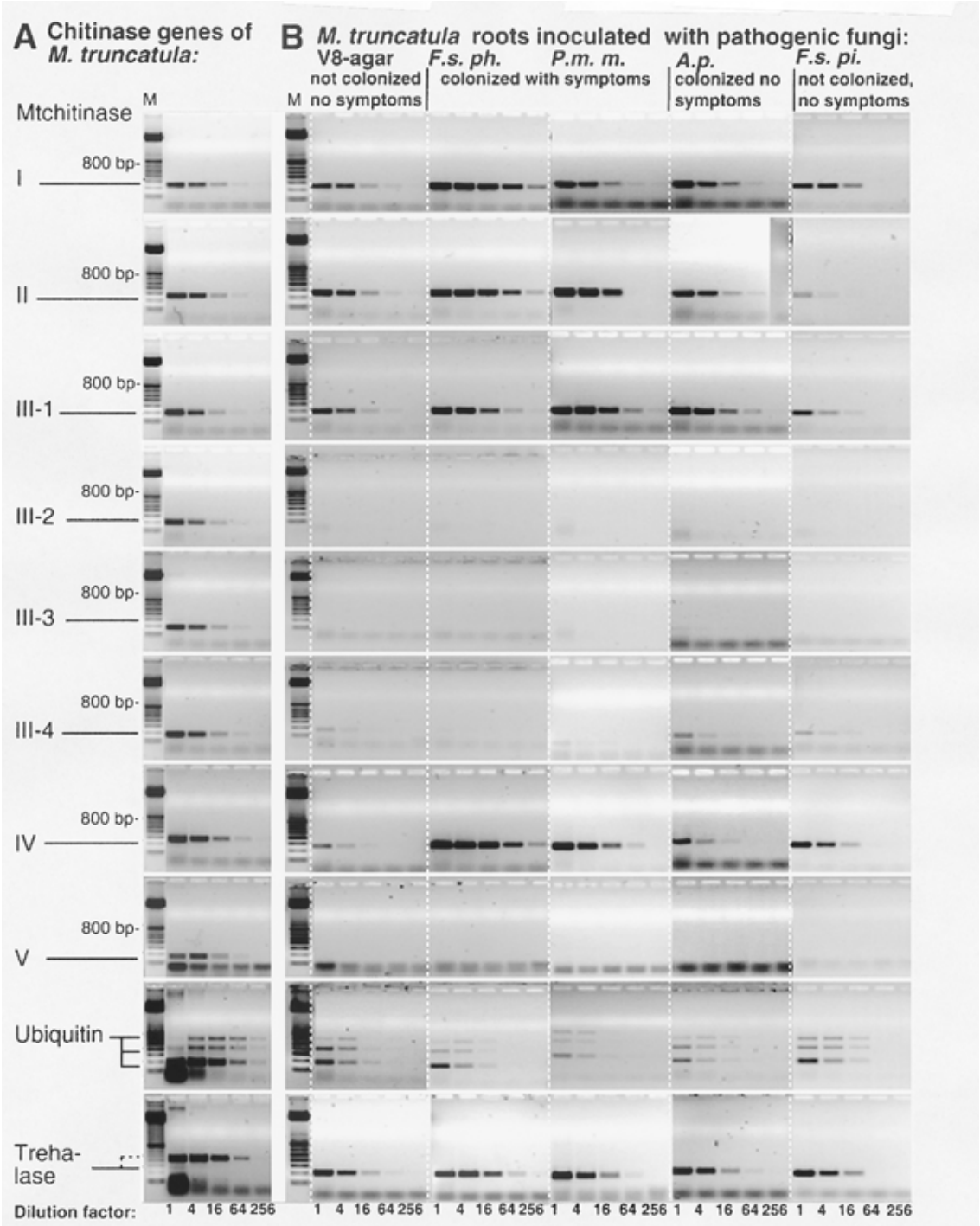

Fig. 5. Chitinase gene expression in plant-pathogen interactions. **A,** Semiquantitative polymerase chain reaction (PCR) performed with a dilution series of genomic DNA from *Medicago truncatula* with *Mtchitinase* primers, *M. truncatula*-specific ubiquitin primers, and intron-spanning trehalase primers. Dilution factors 1, 4, 16, 64, and 256 correspond to 5, 1.25, 0.31, 0.08, and 0.02 ng of DNA subjected to PCR. As marker (M) a 100-bp ladder was used with its 800-bp fragment more intensively stained. Position of labeling indicates size of expected PCR products. For trehalase, PCR product obtained with intron-containing genomic DNA is labeled by a dashed line; corresponding RT-PCR product obtained with cDNA by a plain line. Signals on the gel bottoms are due to primer dimer formation. **B,** Semiquantitative RT-PCR was performed with a dilution series of cDNA obtained from RNA extracted from *M. truncatula* roots 5 days after inoculation with *Fusarium solani* f. sp. *phaseoli* (F.s.ph.), *Phytophthora megasperma* f. sp. *medicaginis* (P.m.m.), *Ascochyta pisi* (A.p.), or *F. solani* f. sp. *pisi* (F.s.pi.), or mock inoculation with V8 agar. *Mtchitinase* I and III-1 are induced in all the compatible interactions, whereas strong induction of *Mtchitinase* II occurred only in response to *F. solani* f. sp. *phaseoli*. Slight expression of *Mtchitinase* III-4 was only apparent in roots inoculated with V8 agar or challenged with the avirulent fungi. Similar intensity of *M. truncatula*-specific ubiquitin signal indicates equal introduction of cDNA into the PCR. Exclusive appearance of a 245-bp trehalase PCR product confirms that no genomic DNA was amplified in the PCR. Identical results were obtained in two independent experimental series.

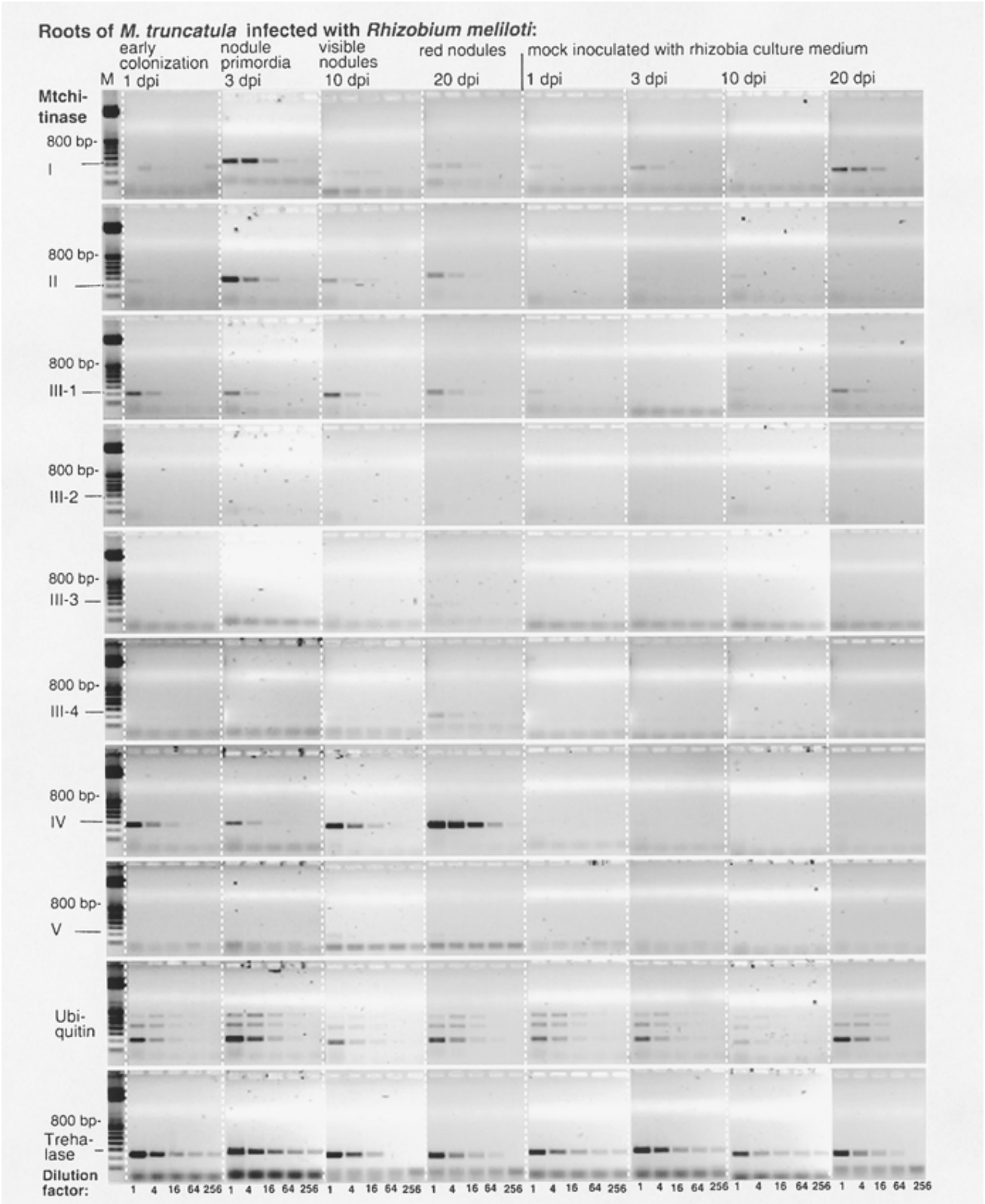

Fig. 6. Biphasic induction of chitinase genes during nodulation with *Rhizobium meliloti*. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) performed with a dilution series of cDNA obtained from roots of *Medicago truncatula* inoculated with *R. meliloti* and from control roots mock inoculated with bacterial culture medium. For the time points 10 and 20 days, root sectors have been selected that harbored 15 to 30 nodules per cm of root length. *Mtchitinase* I and II were transiently increased during early stages, expression of *Mtchitinase* IV was most induced in mature nodules. Similar results were obtained in two independent experimental series. A 100-bp ladder was used as marker (M).

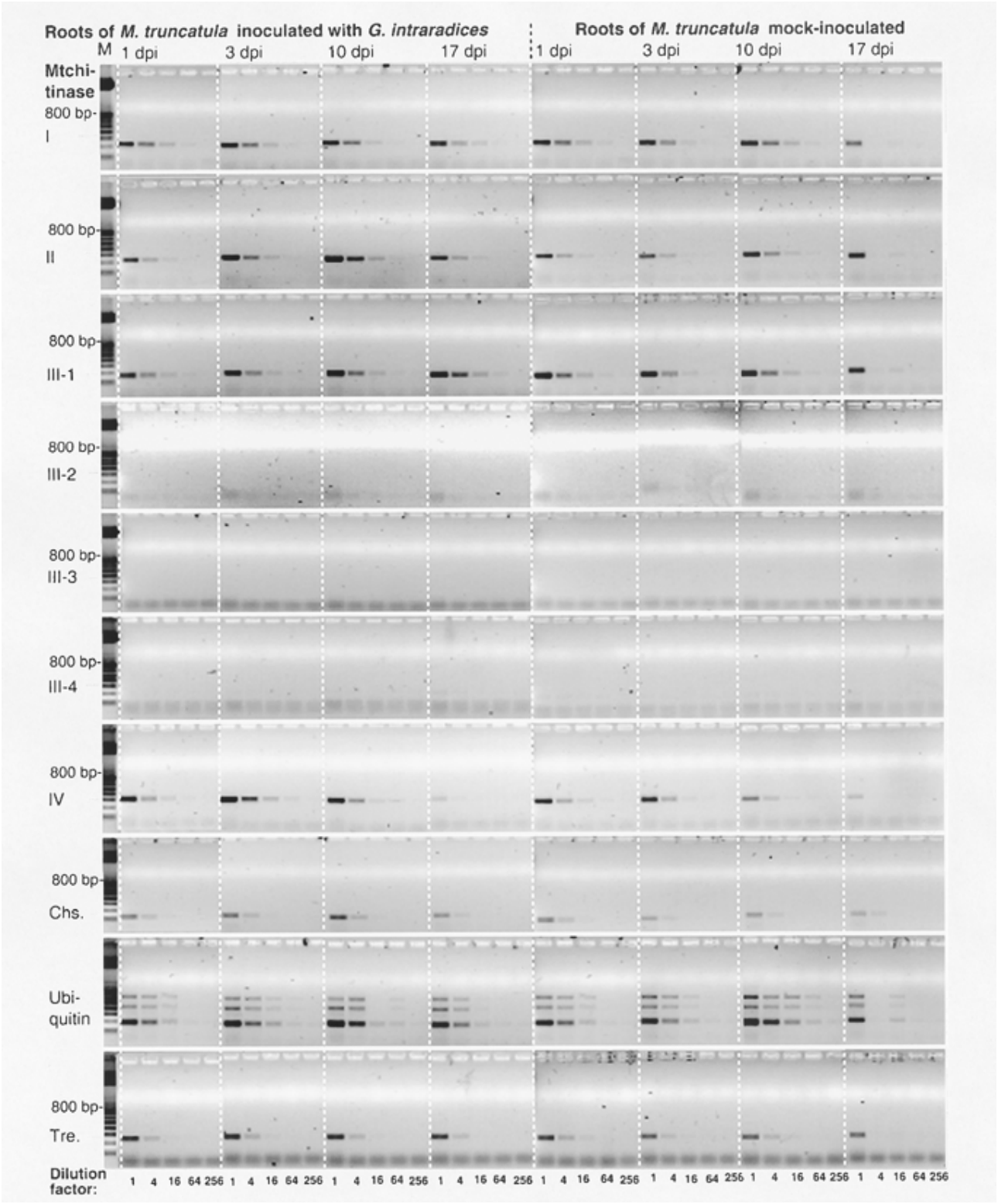

Fig. 7. Chitinase gene expression during early stages of mycorrhiza formation. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) performed with a dilution series of cDNA obtained from RNA extracted from seedlings of *Medicago truncatula* that have been inoculated with spores (five per root) and hyphae from *G. intraradices*. Three days after inoculation there was no physical contact between germinated spores and roots established. First internal hyphae, but no arbuscules and vesicles, were observed 10 days after inoculation. Internal hyphae and a few arbuscules but no vesicles were found at day 17 after inoculation. Expression of *Mtchitinase* II, III-1, and IV as well as of the chalcone synthase (Chs.) gene is already slightly induced before arbuscules were formed. In contrast, expression of *Mtchitinase* III-2, III-3, and III-4 could not be detected during the early stages of colonization. Similar results were obtained in two independent experimental series. A 100-bp ladder was used as marker (M).

µl of *Taq* DNA polymerase (0.5 U; Amersham Pharmacia), 11.3 µl of water, 2 µl of forward primers (2.5 µM) and 2 µl of backward primers (2.5 µM). The primer sequences and annealing temperatures are summarized in Table 3. Before cycling, samples were incubated at 95°C for 2 min, followed by 28 cycles (55°C, 30 s; 72°C, 40 s; 95°C, 15 s; and a final extension at 72°C for 7 min). Finally, the reaction was cooled to 4°C and stopped by addition of 5 µl of DNA-loading buffer consisting of 50% vol/vol glycerol, 100 mM EDTA, pH 8.0, and 0.05% (wt/vol) xylene cyanol.

PCR products were separated by electrophoresis on 1.8% agarose gels in Tris-acetic acid-EDTA (TAE) buffer containing 0.24 µg · ml^{-1} ethidium bromide. Documentation of the gels occurred under UV-light with a Bioprint image documentation system (Vilber Lourmat, Marne La Vallée, France). Final assembly of the pictures was done with Adobe Photoshop 3.0 (Mountain View, CA) and Claris Draw (Apple Computer Corp.) on a Macintosh G3.

DNA was isolated from axenically grown *M. truncatula* roots and fungi after homogenization in liquid nitrogen with a Nucleon Phytopure Plant DNA Extraction Kit (Amersham Pharmacia).

Cloning chitinase and ubiquitin fragments from *M. truncatula* by PCR.

Chitinase. Degenerate oligonucleotides were designed for five plant chitinase subclasses based on conserved amino acid motifs, as shown in Table 1. To avoid size variation that would be introduced by intron sequences, conserved amino acid motifs were selected to reside within a single exon. The degenerate primer sets yielded amplified products of the expected sizes, as indicated in Table 1. Typical PCR conditions were 35 cycles, with a 45 s, 94°C denaturation step; a 30 s, 55°C annealing step; and a 45 s, 72°C extension step. PCR products were cloned into pPCR-Script-Amp vector (Stratagene, La Jolla, CA) and sequenced by means of a 377 DNA sequencer (Perkin Elmer Applied Biosystems, Foster City, CA).

Probes for Southern hybridization were prepared by digesting the PCR clones with the following enzyme combinations: *Mtchitinase* I, II, IV, and V, *Not*I and *Bam*HI; *Mtchitinase* III-1, *Bsg*I and *Dde*I; *Mtchitinase* III-2, *Bsg*I and *Drd*I; *Mtchitinase* III-3, *Not*I and *Drd*I, *Mtchitinase* III-4, *Bsg*I and *Drd*I.

Ubiquitin. From genomic DNA of *M. truncatula* and *G. mosseae* ubiquitin fragments were amplified by PCR with the primers described for *Vicia faba* (Heidstra et al. 1997). The PCR fragments were cloned into the pCRTM Vector (Invitrogen, Leek, The Netherlands) and sequenced. Sequence analysis was performed with the GCG software package (Genetics Computer Group, Madison, WI).

Primers for ITS1 and ITS4 specific for *Fusarium solani* were used according to J. Lange (*unpublished*). The primers

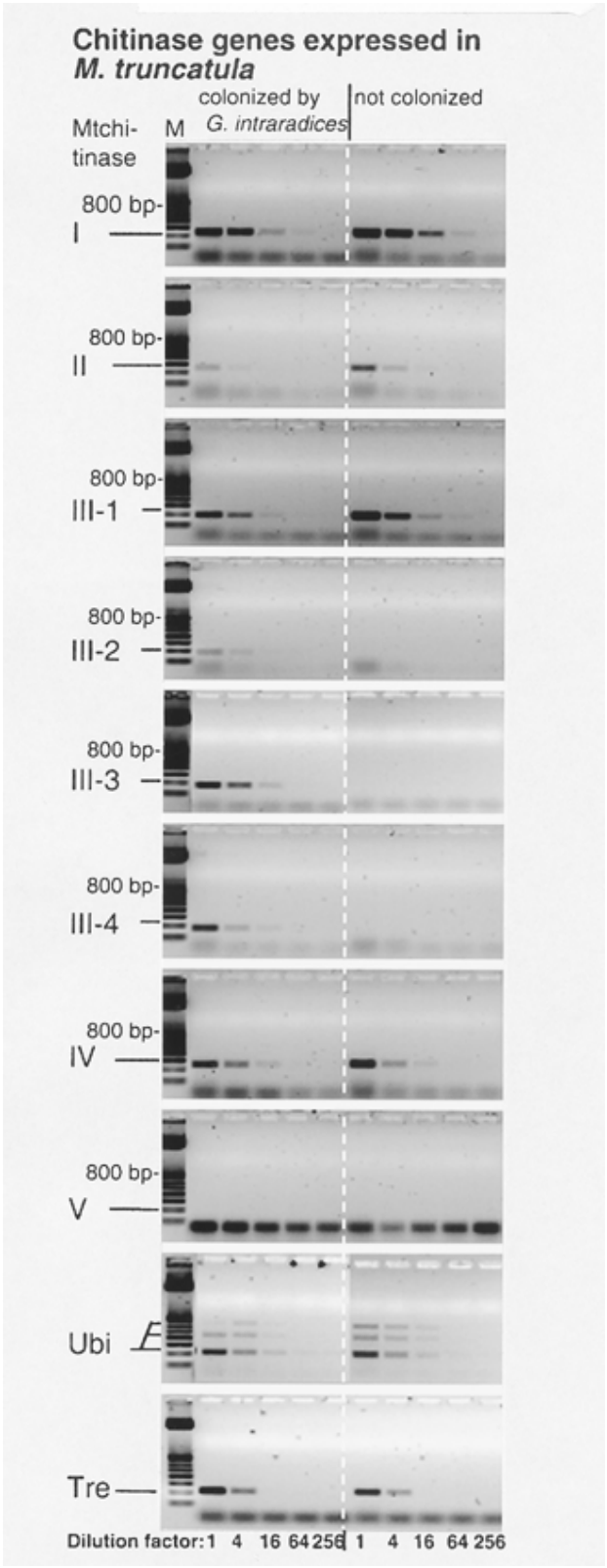

Fig. 8. Induction of chitinase class III-2, III-3, and III-4 gene expression in arbuscular mycorrhiza (AM) formed by *Glomus intraradices* on *Medicago truncatula* roots. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) performed with a dilution series of cDNA obtained from *M. truncatula* roots colonized (about 60% of the root length with arbuscules, vesicles, or intraradical hyphae 5 weeks after inoculation) and noncolonized with *G. intraradices*. Only in mycorrhizal roots is expression of *Mtchitinase* III-2, III-3, and III-4 induced. Induction of *Mtchitinase* III-2 and III-3 is specific for a functioning mycorrhiza. The same results were obtained in at least three independent experimental series. A 100-bp ladder was used as marker (M).

Table 2. Chitinase activity and gene expression induced in plants by microorganisms and their elicitors

Plant species		Microorganism[a]	Chitinases induced	References
Allium porrum	AM	*Glomus mosseae*	Activity, transiently increased, roots	Spanu et al. 1989
Brassica campestris	P	*Plasmodiophora brassicae*	Activity, in roots	Ludwig-Müller et al. 1994
Cucumis melo	P	*Colletotrichum lagenarium*	Immuno detection, seedlings	Roby and Esquerre-Tugaye 1987
Cucurbita pepo	P	*C. lagenarium*	Class III mRNA, etiolated hypocotyls	Kästner et al. 1998
Eucalyptus globulus	P	*Phytophthora cinnamomi*	Activity in gels increased, roots	Albrecht et al. 1994
	EM	*Pisolithus tinctorius*	New band in activity gels, roots	Albrecht et al. 1994
Glycine max	R	*Bradyrhizobium japonicum*	Activity, roots	Staehelin et al. 1992
	R	*B. japonicum* exo B mutant	Activity, nodules	Parniske et al. 1994
	AM	*Glomus mosseae*	Activity gels, roots	Xie et al. 1999
	R	*B. japonicum*	Activity gels, nodules	Xie et al. 1999
	R	*Rhizobium* sp. strain NGR234	Activity gels, nodules	Xie et al. 1999
Hordeum vulgare	P	*Erysiphe graminis* f. sp. *hordei*	Immuno detection, class II protein, leaves	Ignatius et al. 1994; Kragh et al. 1997
Lycopersicon esculentum	P	*Cladosporium fulvum*	Activity, leaves	Joosten and De Wit 1989
Medicago sativa	AM	*Glomus intraradices*	Activity, roots	Volpin et al. 1994
	R	*Rhizobium meliloti*	PR-P, PR-Q, class II, cortical root cells	Vasse et al. 1993
Medicago truncatula	AM	*G. intraradices*	Class III-2, III-3, III-4, mRNA, roots	This study
	P	*Ascochyta pisi*	Class I, II, III-1, III-4, mRNA, roots	This study
	P	*Fusarium solani* f. sp. *phaseoli*	Class I, II, III-1, IV, mRNA, roots	This study
	P	*F. solani* f. sp. *pisi*	Class I, III-4, IV, mRNA, roots	This study
	P	*Phytophthora megasperma* f. sp. *medicaginis*	Class I, II, III-1, IV, mRNA, roots	This study
	R	*R. meliloti*	Class I, II, mRNA, transient, roots	This study
	R	*R. meliloti*	Class IV, III-4, mRNA, nodules	This study
Nicotiana tabacum	AM	*Glomus versiforme*	Acidic chitinase, activity gels, roots	Dumas-Gaudot et al. 1992
	AM	*G. intraradices*	Acidic chitinase, activity gels, roots	Dumas-Gaudot et al. 1992
	AM	*Glomus fasciculatum*	Acidic chitinase, activity gels, roots	Dumas-Gaudot et al. 1992
	P	*Chalara elegans*	Acidic chitinase, activity gels, roots	Dumas-Gaudot et al. 1992
	P	*Tobacco mosaic virus*	PR-P, PR-Q equivalent to class II, leaves	Payne et al. 1990
	P	*Tobacco mosaic virus*	Acidic and basic class III, leaves	Lawton et al. 1992
	P	*Phytophthora infestans* (elicitor)	Class I basic, class II acidic, mRNA, cells	Suzuki et al. 1995
	P	*Phytophthora parasitica*	Class I basic, mRNA, leaves	Meins and Ahl 1989
	P	*Pseudomonas tabaci*	Class I basic, mRNA, leaves	Meins and Ahl 1989
Oryza sativa	P	*Rhizoctonia solani*	35 kDa, 28 kDa protein, sheath	Anuratha et al. 1996
Phaseolus vulgaris	AM	*G. intraradices*	Activity, class I, and II, mRNA, roots	Lambais and Mehdy 1993
	AM	*G. intraradices*	mRNA (in situ) in cells with arbuscules	Blee and Anderson 1996
	P	*F. solani* f. sp. *phaseoli*	Class I, II, IV mRNA, roots	Mohr et al. 1998; Lange et al. 1996
	P	*Colletotrichum lindemuthianum*, fungus and elicitors	mRNA, hypocotyl and cells	Hedrick et al. 1988
Picea abies	EM	*Amanita muscaria*, elicitors	Activity, cells	Sauter and Hager 1989
	EM	*Hebeloma crustuliniforme*	Activity gels, roots	Salzer et al. 1997b
Pisum sativum	AM	*G. mosseae*	Acidic chitinase, activity gel, roots	Dumas-Gaudot et al. 1994
	P	*A. pisi*	Class I mRNA, leaves	Vad et al. 1993
Sesbania rostrata	R	*Azorhizobium caulinodans*	Srchi13, homolog to class III, stem	Goormachtig et al. 1998
Solanum tuberosum	P	*Phytophthora infestans* fungus and elicitors	Class I, II mRNA, leaves	Büchter et al. 1997
Triticum aestivum	P	*Puccinia graminis* f. sp. *tritici*	Class I, mRNA, leaves	Liao et al. 1994; Münch-Garthoff et al. 1997
Vicia faba	R	*Rhizobium leguminosarum* bv. *viciae*	Nvf32-A1, Nvf32-A2, homology to class III chitinase proteins, roots	Perlick et al. 1996

[a] P = pathogens, AM = arbuscular mycorrhizae, EM = ectomycorrhizae, R = rhizobia.

used to amplify ITS1 and ITS4 in *M. truncatula*, *G. intraradices*, *Ascochyta pisi*, and *Phytophthora megasperma* f. sp. *medicaginis* were described by Heidstra et al. (1997).

Chalcone synthase. Primers for chalcone synthase were designed after cloning and sequencing a cDNA band that was detected by differential display to be induced in mycorrhizal roots of *M. truncatula* (Vögeli-Lange et al. 1996).

Trehalase. From genomic DNA of *M. truncatula*, a PCR fragment was amplified with degenerate primers mapping to conserved regions of a trehalase gene in *Glycine max* (Aeschbacher et al. 1999). Cloning and sequencing of this fragment revealed that it contained an intron. For RT-PCR, the forward primer was positioned upstream and the reversed primer downstream of the intron.

The sequences of the chitinase gene fragments were deposited in the National Center for Biotechnology Information (NCBI) Genome Survey Sequence data base (GSSdb) with the following accession numbers: *Mtchitinase* I, AF167322; *Mtchitinase* II, AF167323; *Mtchitinase* III-1, AF167324; *Mtchitinase* III-2, AF167325; *Mtchitinase* III-3, AF167326; *Mtchitinase* III-4, AF167327; *Mtchitinase* IV, AF167328, and *Mtchitinase* V, AF167329. Sequence data for trehalase and

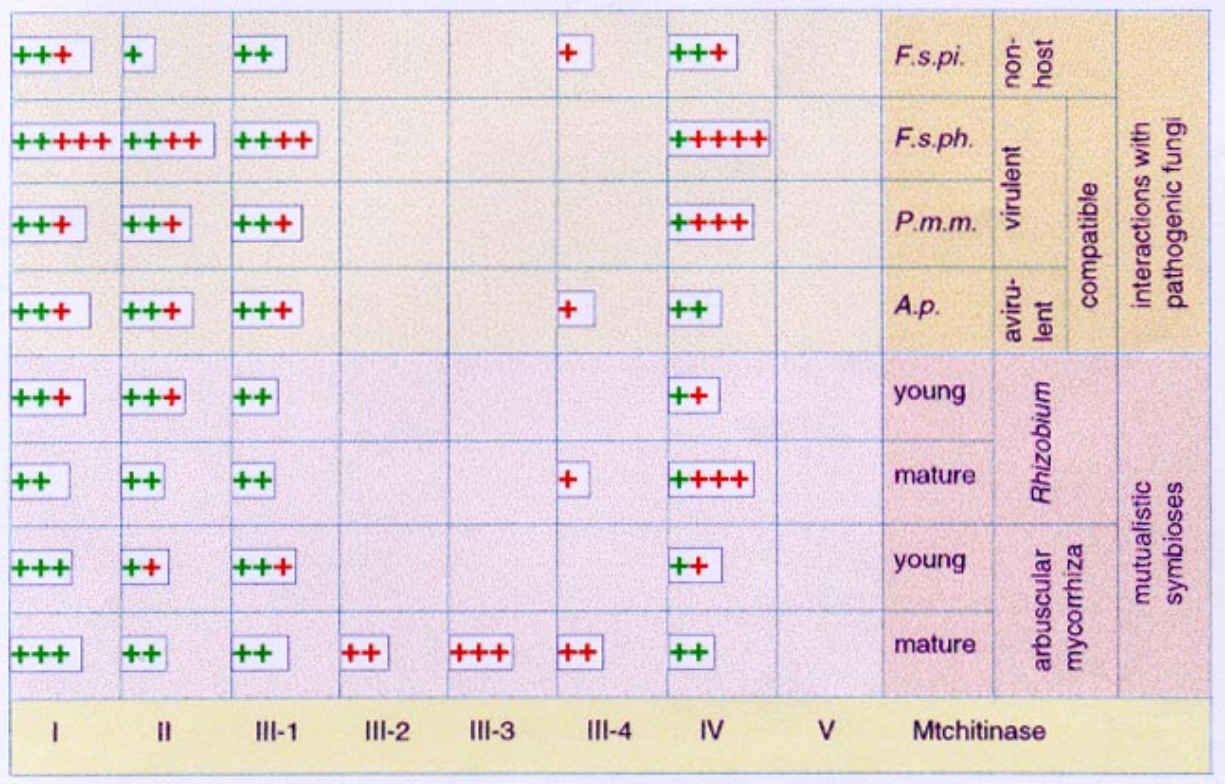

Fig. 9. Comprehensive survey of *Mtchitinase* expression in roots of *Medicago truncatula* infected with rhizobia and mycorrhizal and pathogenic fungi. Total number of crosses (green plus red) resembles level of gene expression of a certain *Mtchitinase*. Red symbols highlight level of expression, which is due to induction by the microorganism. Green symbols give expression level in corresponding axenic control roots. For mutualistic symbioses, the strongest expression level at an early (young) or later stage (mature) of the interaction is shown. Number of crosses corresponds to average number of bands detected in dilution series of two independent experimental series. Half numbers are rounded off and shown as a cross.

Table 3. Primer sequences used for reverse transcription-polymerase chain reaction[a]

Mtchitinase	Primer sequence	Tm
I	(f) 5′-TCAACCGGCGACTACTGTGGTGATG-3′	67.4°C
	(b) 5′-CCCAAGAAAGCAGCAATCTCTC-3′	64.8°C
II	(f) 5′-CCCAACTGTATCATTCAAGACAGCC-3′	65.5°C
	(b) 5′-CACCAGGACTCACTCCCAATATTTGAC-3′	66.3°C
III-1	(f) 5′-CCCTGGTGCTTGTAACTTTGTTTC-3′	64.5°C
	(b) 5′-GGTGAAGGCTTAACAATAGGCAGC-3′	65.6°C
III-2	(f) 5′-CCTGAATGTGACTATAGTGAAAGCGAG-3′	64.6°C
	(b) 5′-CGCGATATCCTTAAAACAGG-3′	62.6°C
III-3	(f) 5′-CCTTGTCAATACAATCCTGGTG-3′	63.4°C
	(b) 5′-GCAGAACCTTTAATAGCTGG-3′	62.6°C
III-4	(f) 5′-CCCTGATGCATTTATGAAC-3′	61.1°C
	(b) 5′-CCGTCTTTGGAAGAACCTTTAATAACTGG-3′	64.8°C
IV	(f) 5′-GGTGATGCATATTGTGGCACAGGG-3′	66.6°C
	(b) 5′-GCAGCAGCAACCTCACGTTTGGAG-3′	67.4°C
V	(f) 5′-AAGAGTTGGAAGCTTCAGGATCCG-3′	65.6°C
	(b) 5′-CCCACTATATGAATAAACCGATCCGG-3′	65.3°C
MtUbiquitin	(f) 5′-GTGAAGACCTTGACCGGCAAAAC-3′	61.9°C
	(b) 5′-GGTGAAGCGTGGACTCTTTCTGG-3′	63.5°C
MtTrehalase	(f) 5′-CGGTTGGGCTCCACTTCAACAC-3′	62.1°C
	(b) 5′-GGACCATCCAAAWCCSGTCTG-3′	59.0°C
Chalcone synthase	(f) 5′-CGAAAAGATGAATGCAAC-3′	47.5°C
	(b) 5′-CCAAAACCAAATAACACAC-3′	48.4°C

[a] Tm gives annealing temperature, (f) designates forward and (b) backward orientation of primers. In the degenerate primers W stands for A and T, S for G and C.

ubiquitin were deposited in the European Molecular Biology Laboratories (EMBL) gene bank and are available by accession number AJ238651 for the trehalase gene, AJ245511 for the ubiquitin gene fragment of *Medicago truncatula*, and AJ245512 for the ubiquitin gene fragment of *Glomus mosseae*.

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