Mycorrhiza (2010) 20:427–443
DOI 10.1007/s00572-009-0292-8

ORIGINAL PAPER

Symbiosis-related pea genes modulate fungal and plant gene expression during the arbuscule stage of mycorrhiza with *Glomus intraradices*

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Received: 12 October 2009 / Accepted: 11 December 2009 / Published online: 22 January 2010

Abstract The arbuscular mycorrhiza association results from a successful interaction between genomes of the plant and fungal symbiotic partners. In this study, we analyzed the effect of inactivation of late-stage symbiosis-related pea genes on symbiosis-associated fungal and plant molecular responses in order to gain insight into their role in the functional mycorrhizal association. The expression of a subset of ten fungal and eight plant genes, previously reported to be activated during mycorrhiza development, was compared in *Glomus intraradices*-inoculated wild-type and isogenic genotypes of pea mutated for the *PsSym36*, *PsSym33*, and *PsSym40* genes where arbuscule formation is inhibited or fungal turnover modulated, respectively. Microdissection was used to corroborate arbuscule-related fungal gene expression. Molecular responses varied between pea genotypes and with fungal development. Most of the fungal genes were downregulated when arbuscule formation was defective, and several were upregulated with more rapid fungal development. Some of the plant genes were also affected by inactivation of the *PsSym36*, *PsSym33*, and *PsSym40* loci, but in a more time-dependent way during root colonization by *G. intraradices*. Results indicate a role of the late-stage symbiosis-related pea genes not only in mycorrhiza development but also in the symbiotic functioning of arbuscule-containing cells.

Keywords *Glomus intraradices* · *Pisum sativum* · Symbiosis-related plant mutants · Gene expression · Laser microdissection

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Introduction

Development of the arbuscular mycorrhiza (AM) symbiosis requires significant morphological and physiological changes in host and fungal cells. Being obligate biotrophs, AM fungi depend on a symbiotic relationship with host plants to complete their life cycle, their asymbiotic development being restricted to spore germination, and production of a limited amount of mycelium in the absence of a host root system (Balestrini and Lanfranco 2006). In the *Arum* type of root colonization (Dickson 2004), typical for pea, AM fungi form appressoria (hyphopodia) at the root surface, then penetrate the root via intercellular hyphae, and in the parenchymal cortex give rise to the highly branched intracellular arbuscules which play a key role in reciprocal nutrient and signal exchange between the partners. AM fungal morphogenesis within host tissues is accompanied by modifications in their wall structure and composition and by extensive cellular rearrangements in the protoplast of the colonized root cells (Bonfante-Fasolo and Scannerini 1992; Lemoine et al. 1995; Gianinazzi-Pearson 1996; Balestrini and Lanfranco 2006).

Colonization of root cortical cells is preceded by the differentiation of a prepenetration apparatus (Genre et al.

2008), and then a periarbuscular membrane is formed de novo to completely surround the developing arbuscule, resulting in the creation of a symbiotic interface (Gianinazzi-Pearson 1996). The presence of the fungus in the host cell induces rearrangements of cytoskeleton elements, decrease in size and fragmentation of the vacuole, disappearance of amyloplasts, proliferation of organelles (plastids, mitochondria, dictyosomes), and movement of the plant nucleus which lodges between arbuscule branches (Gianinazzi-Pearson 1996; Genre and Bonfante 2002). The nucleus of arbuscule-containing cells undergoes hypertrophy and is characterized by an increase in chromatin decondensation reflecting greater transcriptional activity of the plant genome (Balestrini et al. 1992; Gianinazzi-Pearson 1996). Specific activation of H^+-ATPase and phosphate transporter genes in arbuscule-containing cells and the location of corresponding protein in the periarbuscular membrane suggest that specific transmembrane transport activities exist at the symbiotic interface (Gianinazzi-Pearson et al. 2000; Harrison et al. 2002; Bucher 2007).

Investigations of plant and fungal genes regulated during the establishment and functioning of the AM symbiosis have provided information about molecular modifications underlying interactions between the partners. Exploitation of molecular techniques such as suppressive subtractive hybridization, macro-/microarrays, and, more recently, microdissection has resulted in a list of plant and fungal genes predicted to be induced in the AM symbiosis and which are promising candidates for monitoring symbiosis-related events during plant–fungal interactions (Liu et al. 2003; Wulf et al. 2003; Brechenmacher et al. 2004; Breuninger and Requena 2004; Küster et al. 2004; Manthey et al. 2004; Weidmann et al. 2004; Frenzel et al. 2005; Hohnjec et al. 2005; Sanchez et al. 2005; Balestrini et al. 2007; Gomez et al. 2009). In legumes, transcriptome profiling studies have mainly focused on the model species *Medicago truncatula* and *Lotus japonicus* for which a large majority of genome sequences are available (http://www.medicago.org/genome/; http://www.kazusa.or.jp/lotus/). Moreover, genetical analyses of mutants of *M. truncatula*, *L. japonicus*, or *Pisum sativum* that either prevent or enhance AM formation has led to the functional identification of eight plant genes which regulate symbiosis development (Schnabel et al. 2005; Parniske 2008).

The exploitation of mycorrhiza-deficient mutants through comparisons of symbiosis-related gene expression between wild-type and mutant genotypes, mainly in *M. truncatula* and *L. japonicus*, has started to pinpoint cellular events that are regulated by plant *SYM* genes, and plant genes have been identified which are modulated during the early stages of plant–fungal compatibility (Gianinazzi-Pearson et al. 2007). By focusing on mutants blocked at the appressorium stage of fungal development, designated Myc^{-1} by Duc et al. (1989), studies have identified plant responses indicative of signaling pathways and essential to early plant–fungal compatibility (Kosuta et al. 2003; Weidmann et al. 2004; Sanchez et al. 2005). Very recently, Seddas et al. (2009) provided first evidence that plant gene inactivation in such mutants also negatively impacts on AM fungal gene expression prior to and after contact with the root surface.

Although most transcriptional analyses have targeted plant or fungal genes modulated during the fully developed and functional symbiosis (Balestrini and Lanfranco 2006), few plant mutants have been reported that are affected for the later steps of AM interactions. In this context, the morphological phenotype of three pea mutants *Pssym33*, *Pssym36*, and *Pssym40* has been characterized in detail at the cellular level. The mutant *Pssym36* is defective for arbuscule formation (Myc^{-2} phenotype; Duc et al. 1989), and plasma membrane ATPase, typical of a functional symbiosis, is not active at the intracellular plant–fungal interface (Gianinazzi-Pearson et al. 1991, 1995); a number of pea genes have been reported to show differential expression between wild-type and *Pssym36* genotypes (Lapopin et al. 1999; Grunwald et al. 2004). The *Pssym33* and *Pssym40* mutants show decreased (Rmd^- phenotype) and increased (Rmd^{++} phenotype) rates of AM development and arbuscule turnover, respectively (Borisov et al. 2004). Although nothing is known about the effect of the mutations on symbiont gene expression, they do not appear to block the symbiotic process (Jacobi et al. 2003a, b). The corresponding genes *PsSym36*, *PsSym33*, and *PsSym40* in wild-type pea have not yet been cloned so that their precise functions remain unknown. In order to gain better insight into the role of these pea genes in the mycorrhizal association, we have analyzed the effect of their inactivation in mutant plants on symbiosis-associated fungal and plant molecular responses by monitoring the expression of a subset of fungal and plant genes considered to be markers of a functional AM symbiosis.

Materials and methods

Plant and fungal material

Two wild-type pea (*P. sativum* L.) genotypes (*P. sativum* cv. Finale and SGE) and their symbiotic mutants *Pssym36* (previously denoted RisNod24; Engvild 1987), *Pssym33* and *Pssym40* (previously denoted $SGEFix^-$-2 and $SGE-Fix^-$-1, respectively; Jacobi et al. 2003a, b), were used throughout this study. Seeds of *P. sativum* cv. Finale and *Pssym36* were provided by G. Duc (INRA Dijon, France) and of SGE, *Pssym40* and *Pssym33* by ARRIAM (St.

Petersburg, Russia). All pea mutants and their parental lines were inoculated with the fungal isolate *Glomus intraradices* Schenck & Smith BEG 141 provided by the International Bank for the Glomeromycota (Dijon, France).

Growth and inoculation of plants

Seeds were surface-disinfected 10 min in 3% Na hypochlorite for *P. sativum* cv. Finale and *Pssym36* and 25 min in 98% sulfuric acid for SGE, *Pssym33*, and *Pssym40* and then germinated on humid filter paper in Petri dishes at 24°C in the dark. Five-day-old seedlings were individually transplanted into 200 g of a 1:1 (*v*/*v*) sand/clay loam soil (pH 7.8, 28 ppm Olsen P) mix as growth substrate. Soil was gamma-irradiated (10 kGy) and sand (Special Aquarium, Quartz, Nr. 3, Zolux, France) was washed before heat sterilizing both 4 h at 180°C. Mycorrhizal plants were produced by replacing soil with a soil-based inoculum of *G. intraradices* from 10-week-old leek pot cultures. Noninoculated controls received a filtered (Whatman no. 2) water suspension of inoculum to introduce the associated microflora. Plants were grown in a constant environment chamber (24/19°C, 16 h light, 420 µmol m^{-2} s^{-1}, 70% relative humidity) and fertilized with 10 ml Long Ashton solution (Hewitt 1966) without phosphorus three times a week.

Plants were harvested at 21 and 28 days after inoculation (dai). Root systems were washed in cold water, root and shoot fresh weights were recorded, and roots were stored in liquid nitrogen for RNA extraction. A sample from each root system was directly stained either with ink (Vieheilig et al. 1998) or for fungal alkaline phosphatase activity (Tisserant et al. 1993) and mycorrhiza parameters estimated using the MYCOCALC program (www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html; Trouvelot et al. 1986).

Gene selection

Ten protein-encoding genes from the Glomeromycota were selected for monitoring the effect of pea SYM gene inactivation on fungal gene expression. The *G. intraradices* primers published by Seddas et al. (2009) were used for eight of these: putative Rho/GDP dissociation inhibitor (*RHO*), peptidylprolyl isomerase (*PEPISOM*), 26S proteasome subunit 2 (*26S*), 26S proteasome regulatory subunit (*26SREG*), stearoyl-CoA desaturase (*DESAT*), thioredoxin peroxidase (*THIO*), superoxide dismutase (*SOD*), and hypothetical vacuolar import protein (*VACU*). The genes have predicted functions in transcription, protein turnover, primary or secondary metabolism, and are active in *G. intraradices* at the arbuscule stage of root colonization (Seddas et al. 2008, 2009). Primers for two other *G. intraradices* genes active in intraradical mycelium, alkaline phosphatase (*ALP*) and H^+*-ATPase*, were taken from Aono et al. (2004) or deduced from the *GiHA5* sequence (accession number AF420481, deposited by Nielsen et al.), respectively (Table 1). Eight protein-encoding plant

Table 1 Newly designed primers for transcript analyses of *G. intraradices* and *P. sativum* genes: predicted gene function, ID accession number of target sequences for primer design, oligonucleotide sequences, annealing temperatures (T_m), and amplicon size

Protein-encoding gene	Primer sequences	T_m (°C)	Amplicon size (bp)
G. intraradices			
H^+*-ATPase* (*H^+ATP*; AF420481)	TTGAGGATTGGCAAATGAGTGC GTCCAAGACGCCATTTATCAGG	55	126
Alkaline phosphatase (*ALP*; AB114298.1)	GCTCGTCAAGTTTCCGATCTAC CTCCGATTCCTAATCCGCTAC	55	163
P. sativum			
Plasma membrane ATPase (*ATP*; AJ132892/JO13158009)	TACAGGTGAGTCCCTGCCG CCCAATTGCAGTCAAGACCT	60	252
Inorganic phosphate transporter (*PT*; AY116210/AF305623)	CTTCACGTGCCATGTTCATC GCGTCGGAAACAGCTCC	58	273
Blue copper binding protein (*BCOP*; Z25471)	GTTGGGTGATTGGTGGTGA AAGAGGAATGGTTGTCGCAC	55	187
Glutathione-*S*-transferase (*GST*; AY134608/AY062941)	ACCAAAAGGTGCCTGTGTTC CTAGCTTGTGCGCGATCATA	58	133
MAP kinase (*MAPK*; AJ308148)	GCACTGGCACACCCTTACTTG GGGGTTAAATGCTAGAGCTTCTCTG	60	154
Serine protease (*SERPROT*; MtC91337)	AGAGTAGCCAAAGGTCAAGCAGT GACCTCTTGATGAAAATGAAGCA	58	253
Glyceraldehyde phosphate dehydrogenase (*GAPDH*; AY308154)	AAGAACGACGAACTCACCG TTGGCACCACCCTTCAAATG	55 to 60	187

genes were selected among those reported in the literature to be upregulated during AM interactions or in arbuscule-containing cells of *P. sativum* or *M. truncatula* (Krajinski et al. 2002; Wulf et al. 2003; Liu et al. 2003; Brechenmacher et al. 2004; Grunwald et al. 2004; Massoumou et al. 2007). They are putatively involved in membrane transport, defense/cell rescue, signaling, or protein synthesis. Published primers (Grunwald et al. 2004) were used for pea genes encoding the pathogenesis-related protein pI230 (*DRP*) and trypsin inhibitor (*TI*). Blue copper protein (*BCOP*), mitogen-activated protein kinase (*MAPK*), and glyceraldehyde phosphate dehydrogenase (*GAPDH*) primers were deduced from database pea sequences. Since nucleotide sequences were not available for pea genes encoding plasma membrane H^+-ATPase (*H^+ATPase*), inorganic phosphate transporter (*PT4*), glutathione-S-transferase (*GST*), or serine protease (*SERPROT*), primers were designed from alignments with *M. truncatula* and other plant nucleotide sequences available in public databases (Table 1).

RNA extraction

Total RNA was extracted from *G. intraradices*-inoculated or noninoculated roots with the LiCl method (Franken and Gnädinger 1994). Samples were treated with DNase I using SV Total RNA Isolation System (Promega, Madison, WI, USA) and used for fungal and plant transcript analyses. cDNA was synthesized from total DNaseI-treated RNA (1 μg) using 1 μg oligo$(dT)_{15}$ primer (Promega), 300 U M-MLV Reverse transcriptase RNase H Minus (Promega), and 40 U RNasine RNA inhibitor (Promega) under conditions recommended by Weidmann et al. (2004; 25°C for 15 min, 42°C for 1 h, and 96°C for 2 min). Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) was carried out in 25 μl PCR mix (Invitrogen) supplemented with 1.5 mM $MgCl_2$, containing 0.5 U of *Taq* polymerase (Invitrogen), 125 μM dNTP, and 0.5 μM of each gene-specific primer using 1 μl of diluted 1:5 cDNA for both fungal and plant genes. Absolute real-time RT-PCR was performed in 25 μl PCR mix (QPCR SYBR Green kit; ABgene, UK), the same sets of primers as for RT-PCR, and 1 μl of diluted 1:5 cDNA for both fungal and plant genes.

Transcript analyses

Primer specificity was checked by semiquantitative RT-PCR, cloning, and sequencing. Transcripts were amplified in a Biometra thermocycler (Göttingen, Germany) at 94°C for 1 min, followed by 26 cycles at 94°C for 45 s, the appropriate primer annealing temperature for 45 s, 72°C for 45 s, and a final extension at 72°C for 1 min. Amplification products were analyzed by 1.4% (wt/vol) agarose gel electrophoresis in 0.1 Tris–acetate–EDTA (4 mM Tris, 0.1 mM EDTA, 2 mM acetic acid; Fluka, St. Gallen, Switzerland), stained with ethidium bromide for 10 min, and observed under UV light using GelDoc EQ apparatus (Weidmann et al. 2004). Amplified cDNA fragments were cloned into the TOPO vector (Promega), sequenced (MWG, Ebersberg, Germany) and identity verified by TBLASTX analyses in public databases.

Transcripts were quantified by real-time RT-PCR using the Absolute QPCR SYBR Green kit (ABgene, UK) and an ABI PRISM 7900 real-time cycler (Applied Biosystems, Foster City, CA, USA). Briefly, after 10 min denaturation at 95°C, 40 amplification cycles (95°C for 15 s, primer annealing temperature (see Tables 1 and 2) for 30 s and 72°C for 30 s) were followed by a melting curve program included at the end of each PCR run according to the thermal profile suggested by the manufacturer (95°C for 15 s, primer annealing temperature for 15 s, 95°C for 15 s) to verify specific amplification of each target cDNA. The expression of each gene was assayed in three technical replicates and analyzed by the SDS 2.2 program (Applied Biosystems, Foster City, USA), as described by Seddas et al. (2009). To calculate the amount of transcripts present in original samples, plasmid DNA containing each amplicon was prepared using the Macherey-Nagel kit (Macherey-Nagel, Düren, Germany), quantified by UV absorbance spectroscopy, and linearized by restriction enzyme digestion with *Eco*RI for MtBC plasmids for eight of the fungal genes (Journet et al. 2002) and *Not*1 for the Topo plasmids (Promega Corp) for two of the fungal genes and for all the plant genes. Standard amplification curves were determined from duplicate samples of plasmid DNA at 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 copies for each assay.

Relative amounts of transcripts for each gene were calculated as a ratio to the amount of translation elongation factor 1 α-subunit (*TEF1*) transcripts as the AM fungal reference gene (Seddas et al. 2009) or of glyceraldehyde phosphate dehydrogenase (*GAPDH*) transcripts as the plant reference gene (Weidmann et al. 2004; Sanchez et al. 2005). Real-time RT-PCR analyses of each gene were repeated in three biologically independent RNA batches from *G. intraradices*-inoculated or noninoculated roots of each wild-type pea genotype (*P. sativum* cv. Finale, SGE) and the corresponding symbiosis-defective mutants (*Pssym36*, *Pssym33*, *Pssym40*).

Laser capture microdissection

Mycorrhizal roots from *Pssym40* were cut into 1-cm pieces, fixed in freshly prepared Farmer's fixative solution (acetic acid/absolute ethanol, 1:3 (*v*/*v*)) overnight at 4°C, and transferred to 96% ethanol. They were then incubated 15 min at room temperature in ethanol/eosin (861006

Table 2 Mycorrhization parameters of symbiosis-defective pea mutants *Pssym36*, *Pssym33*, and *Pssym40* in comparison to parental wild-type (wt) lines at 21 and 28 days after inoculation with *G. intraradices* (BEG 141)

Plant line	Days after inoculation	Ink staining		Alkaline phosphatase active fungal structures	
		M%	A%	M%	A%
Finale (wt)	21	19.7±3.6	3.9±0.9	1.2±0.4	0.1±0.1
	28	47.6±7.0	9.8±2.1	9.1±2.7	2.0±1.0
Pssym36	21	5.6±1.8[a]	0.2±0.10[a]	0.4±0.1	0
	28	11.6±3.0[a]	0.6±0.2[a]	6.0±0.9	0.8±0.1
SGE (wt)	21	34.5±4.8	10.8±2.9	16.8±2.1	5.3±1.3
	28	36.3±5.6	9.1±3.9	15.0±3.2	3.8±1.3
Pssym33	21	24.1±1.1	5.1±0.5	10.9±1.6[a]	3.1±0.7
	28	27.7±3.0	7.0±1.1	11.6±1.6	2.7±0.8
Pssym40	21	44.2±9.9	16.2±6.5	24.5±5.7	8.0±2.9
	28	29.3±1.9	7.5±1.4	29.5±4.3[a]	7.3±1.6[a]

M% colonization intensity of root systems, *A%* arbuscule abundance in root systems (stunted arbuscules in *Pssym36*)

[a] Indicates significantly different values between mutant and wild-type genotypes

solution, SIGMA ALDRICH, in 0.1% diethyl pyrocarbonate water), rinsed in 100% ethanol, and incubated 1 h in 100% ethanol. After the last ethanol step, root tissues were treated at room temperature with a series of ethanol 100%/histoclear II solutions (3:1, 1:1, and 1:3 (*v*/*v*)) for 30 min each and then embedded in paraffin (paraplast plus SIGMA ref P 3683) through a series of histoclear II (VWR ref: 370720601)/paraffin solutions in which the histoclear II was gradually replaced by paraffin. Roots were incubated in each paraffin-containing solution at 55°C for one to several hours and finally overnight in pure paraffin. Embedded samples were stored in pure paraffin at 4°C in small Petri dishes until use.

Sectioning was performed at room temperature using a Jung RM 2065 microtome (Leica) and RNAse-free tools. Sections of 10 μm thickness were placed on slides, stretched in a few drops of distilled RNase–DNase-free water and kept in a slide warmer at 42°C overnight. The sections were deparaffinized just before use by gradually replacing histoclear II with 100% ethanol. Deparaffinized slides were placed in a desiccator for 1 h before microdissection.

Dissection of arbuscule-containing cells

Dissection was performed using an Arcturus™ Microdissection instrument (ARCTURUS NIKON inverse XT100). Slides with sections were visualized on a computer monitor through a video camera; selected cells were marked using graphical software, isolated using an IR laser (810 nm, laser spot size of 10 μm, laser power of 69 mw, laser pulse duration of 25 ms) and captured automatically into CapSure HS laser capture microdissection caps (Arcturus). Harvested cells were transferred into RNA extraction buffer within a maximum of 30 min after dissection had started (see II.6.4). RNA was extracted using the Picopure Kit (Arcturus). Briefly, cell extracts were loaded from the CapSure caps onto a preconditioned purification column and washed with washing buffer. After a DNase treatment (RNase-Free DNase Set, Qiagen), the membrane was washed twice with washing buffer, and the RNA was eluted into a fresh centrifuge tube using a low ionic strength buffer. The quality of the RNA was estimated using a Bio-Rad Experion HighSens Analysis kit following the manufacturer's protocol on a Bio-Rad Experion System and quantified using a Nanodrop Spectrophotometer ND-100 (Nanodrop Technologies, Willington, DE, USA). RNA was stored at −80°C until use.

aRNA amplification

RNA extracted from laser microdissected arbuscule-containing cells was amplified using TargetAmp™2-Round antisense RNA (aRNA) amplification kit (Epicentre Biotechnologies, Madison, WI, USA) and 40–200 pg of total RNA in the following protocol. The first reverse transcription reaction was primed with a synthetic T7-oligo(dT) containing a phage T7 RNA Polymerase promoter sequence at its 5′-end. The primer was annealed to the RNA template at 65°C for 5 min then chilled on ice for 1 min. First-strand cDNA synthesis was performed at 50°C for 30 min using a premix containing primer and RNA, 50 U of SuperScript III reverse transcriptase (Invitrogen), reverse transcription premix (supplied with the amplification kit), and dithiothreitol (DTT). The RNA component of the resulting cDNA/RNA hybrid was digested by RNase H into small

RNA fragments that were primed for second-strand cDNA synthesis. For this purpose, DNA polymerase and DNA polymerase mix from the amplification kit was added to the reaction mix and incubated for 10 min at 65°C and for 3 min at 80°C. The reaction was stopped by adding finishing solution from the amplification kit. In vitro production of aRNA was obtained by adding T7 RNA polymerase, T7 transcriptional buffer, in vitro transcriptional premix, and DTT. The reaction was performed at 42°C for 4 h followed by a DNase I treatment at 37°C for 15 min. Qiagen RNeasy MinElute Cleanup kit was used to purify aRNA according to the manufacturer's protocol. A second reverse transcription reaction was catalyzed by SuperScript II reverse transcriptase (Invitrogen). After adding random sequence hexamer primers, the volume of mix was adjusted to 3 μl by speed vacuum centrifugation without heating, and the reaction was incubated at 65°C for 5 min. The first-strand cDNA synthesis mix was combined with the RNA-primer mix, 50 U of SuperScript II reverse transcriptase (Invitrogen), reverse transcription premix, and DTT. Reaction was incubated for 10 min at room temperature and for 1 h at 37°C. The RNA component of the cDNA/RNA hybrid was digested with RNase H, as in the first round of amplification. Double-stranded cDNA was produced using a T7-Oligo(dT) primer, DNA polymerase, and DNA polymerase premix supplied by the amplification kit. The reaction was incubated for 10 min at 37°C, for 3 min at 80°C, chilled on ice, and stored at −20°C. The cDNA obtained was used for semiquantitative and real-time PCR.

Statistical analyses

Real-time RT-PCR data were statistically compared between treatments at each time point using one-way ANOVA for each fungal gene and two-way ANOVA for mycorrhiza × mutant interactions for each plant gene (SPSS 16.0 package, SPSS Inc., Chicago, IL, USA); significant differences between means were established by Tukey at $P \leq 0.05$. Where ANOVA gave marginally significant ($P \leq 0.1$) differences across treatments, data between *G. intraradices*-inoculated and noninoculated roots were compared separately for each genotype using the Student's *t* test at $P \leq 0.05$.

Results

Effect of mutation of *PsSym36*, *PsSym33*, and *PsSym40* genes on AM development

The parameter F%, indicating inoculum infectivity, was high in all treatments (94–100%), but fungal development within the root systems differed between parent and mutant pea genotypes. Mycorrhization parameters, estimated by ink staining and alkaline phosphatase-active fungal structures, are indicated in Table 2 for the pea genotypes Finale, SGE, *Pssym36*, *Pssym33*, and *Pssym40* at 21 and 28 dai with *G. intraradices*. The AM fungus developed rapidly to 21 dai in the root systems of the SGE line and corresponding mutants *Pssym33* and *Pssym40*, which all showed consistently higher values for overall root colonization (M%) and arbuscule abundance (A%) at 21 dai than in either *P. sativum* cv. Finale or *Pssym36*. From 21 to 28 dai, mycorrhiza development more than doubled in *P. sativum* cv. Finale to reach values close to those in the 28-day-old SGE line. Fungal spread within roots was persistently lowest in the *Pssym36* mutant, only aborted arbuscules were observed within cortical cells, and no vesicles developed. From 21 to 28 dai, root colonization levels remained constant for SGE and *Pssym33* and slowed down in *Pssym40* root systems to levels comparable to those in *Pssym33*. The proportion of overall alkaline phosphatase active as compared to total (ink-stained) mycelium in roots at 21 dai was considerably higher in parent and mutant SGE lines (45–55% of ink-stained structures) than in either *P. sativum* cv. Finale (6%) or *Pssym36* (7%) roots. This remained fairly constant up to 28 dai for SGE and *Pssym33* but increased to 100% in *Pssym40*, 51% in *Pssym36*, and 19% in *P. sativum* cv. Finale. Relatively few active well-developed arbuscules could be detected in roots of *P. sativum* cv. Finale while all arbuscules appeared to be active at 28 dai in *Pssym40* mycorrhiza and 40% in SGE and *Pssym33* mycorrhizal roots. No mycorrhiza effect on growth was observed between inoculated and noninoculated plants for any of the pea genotypes at 21 and 28 dai (data not shown).

Fungal gene expression in *G. intraradices*-colonized roots of parent and mutant pea genotypes

Nucleotide sequences of amplification products for the *G. intraradices* genes *RHO*, *PEPISOM*, *26S*, *26SREG*, *DESAT*, *THIO*, *SOD*, and *VACU* were identical to those used in the MENS database to design primers (Seddas et al. 2009). The sequences for fungal *ALP* and H^+*ATPase* showed high similarity to the *GiALP* (AB114298.1, 6e-70) and *GiHA5* (AF420481, 1e-48) genes of *G. intraradices*, respectively.

P. sativum cv. Finale vs. *Pssym36*

Real-time quantification of transcript abundance clearly indicated that mutation of the *PsSym36* pea gene affected expression of the *G. intraradices* genes in colonized roots (Fig. 1). Eight of the ten selected AM fungal genes were

downregulated in roots of the *Pssym36* mutant as compared to wild-type *P. sativum* cv. Finale. At 21 dai, decreases in transcript abundance were significant for six genes (*RHO*, *PEPISOM*, *DESAT*, *THIO*, *SOD*, *VACU*). Differences in *G. intraradices* gene expression between *P. sativum* cv. Finale and *Pssym36* roots were the greatest for the *PEPISOM* (4.7-fold), *DESAT* (39-fold), *THIO* (5.6-fold), and *SOD* (8.3-fold) genes. The fungal genes generally had lower expression levels in *P. sativum* cv. Finale roots at 28 dai than at 21 dai, except for *DESAT*, and *26S* transcript levels were extremely low in roots of both pea genotypes. Significant decreases (100-fold) persisted for *DESAT* in *Pssym36*-colonized roots at 28 dai, and the expression of fungal H^+*-ATPase* and *26SREG* was also downregulated, respectively, 3.2- and 10-fold at this time point. *ALP* was the only gene of *G. intraradices* to show significantly enhanced transcript accumulation (4.5-fold) in mutant as compared to *P. sativum* cv. Finale-colonized roots.

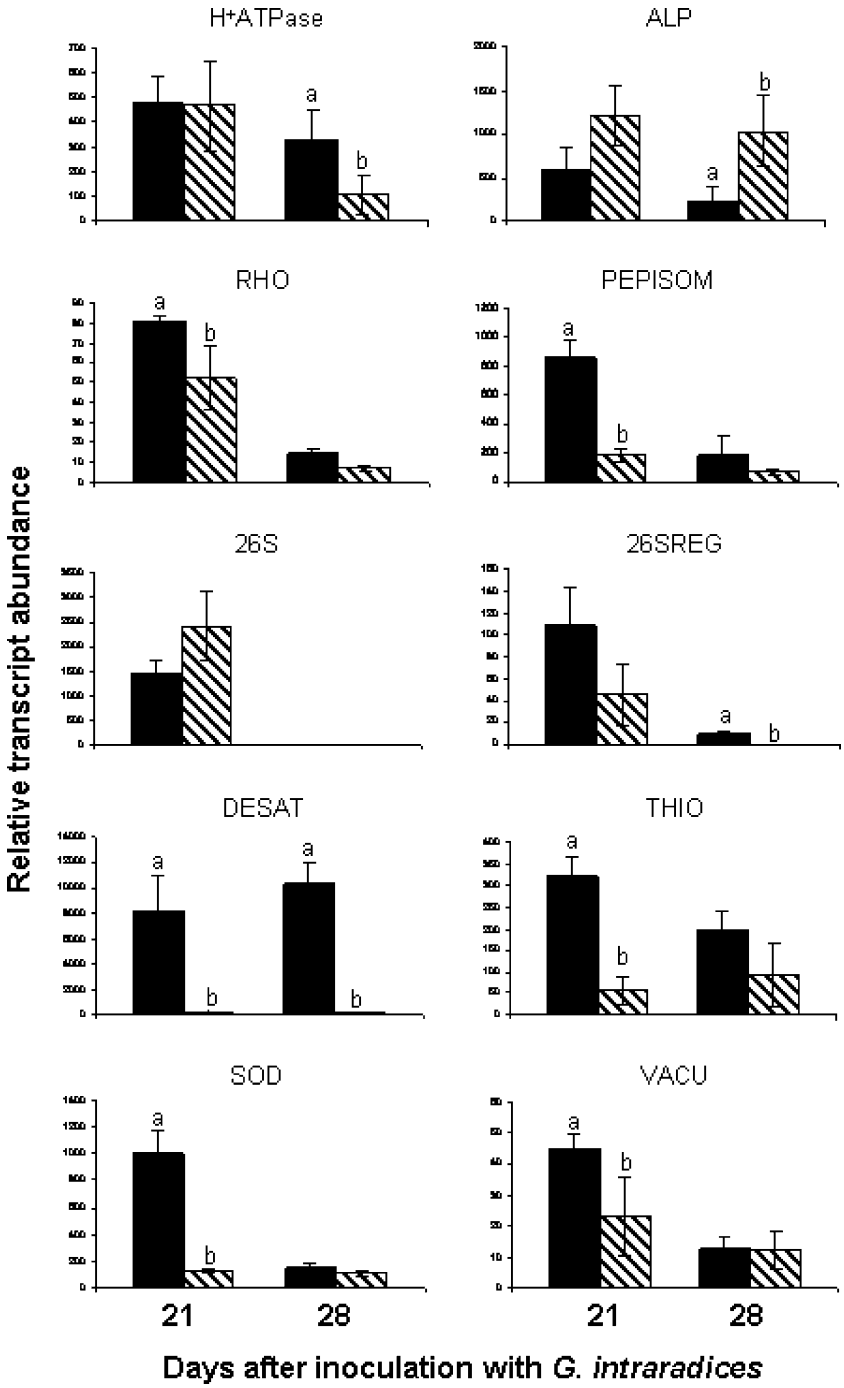

Fig. 1 Expression of fungal genes in *G. intraradices*-colonized roots of wild-type *P. sativum* cv. Finale (*black bar*) and mutant *Pssym36* (*striped bar*) genotypes, 21 and 28 days after inoculation. Transcript abundance of each gene, measured by real-time RT-PCR, is expressed relative to that of the house-keeping fungal *TEF* gene. *Letters* indicate significant differences between genotypes at one time point ($P \leq 0.05$, $n=3$). *Abbreviations* for genes are defined in "Materials and methods" section

SGE line vs. *Pssym33* and *Pssym40*

Inactivation of the *PsSym33* or *PsSym40* pea genes had less pronounced effects on *G. intraradices* gene expression than inactivation of *PsSym36* (Fig. 2). Only expression of the *ALP* and *26S* genes was significantly higher in mycorrhizal roots of *Pssym33* as compared to the *SGE* line at 21 dai, while at 28 dai, no differences in transcript levels were observed between any of the fungal genes in roots of the two pea genotypes. At 21 dai, fungal transcript levels did not differ significantly between colonized roots of the wild-type and *Pssym40* mutant genotypes for H^+-*ATPase*, *ALP*, *PEPISOM*, *THIO*, *SOD*, or *VACU*, while expression of the *RHO*, *26S*, *26SREG*, and *DESAT* genes increased in

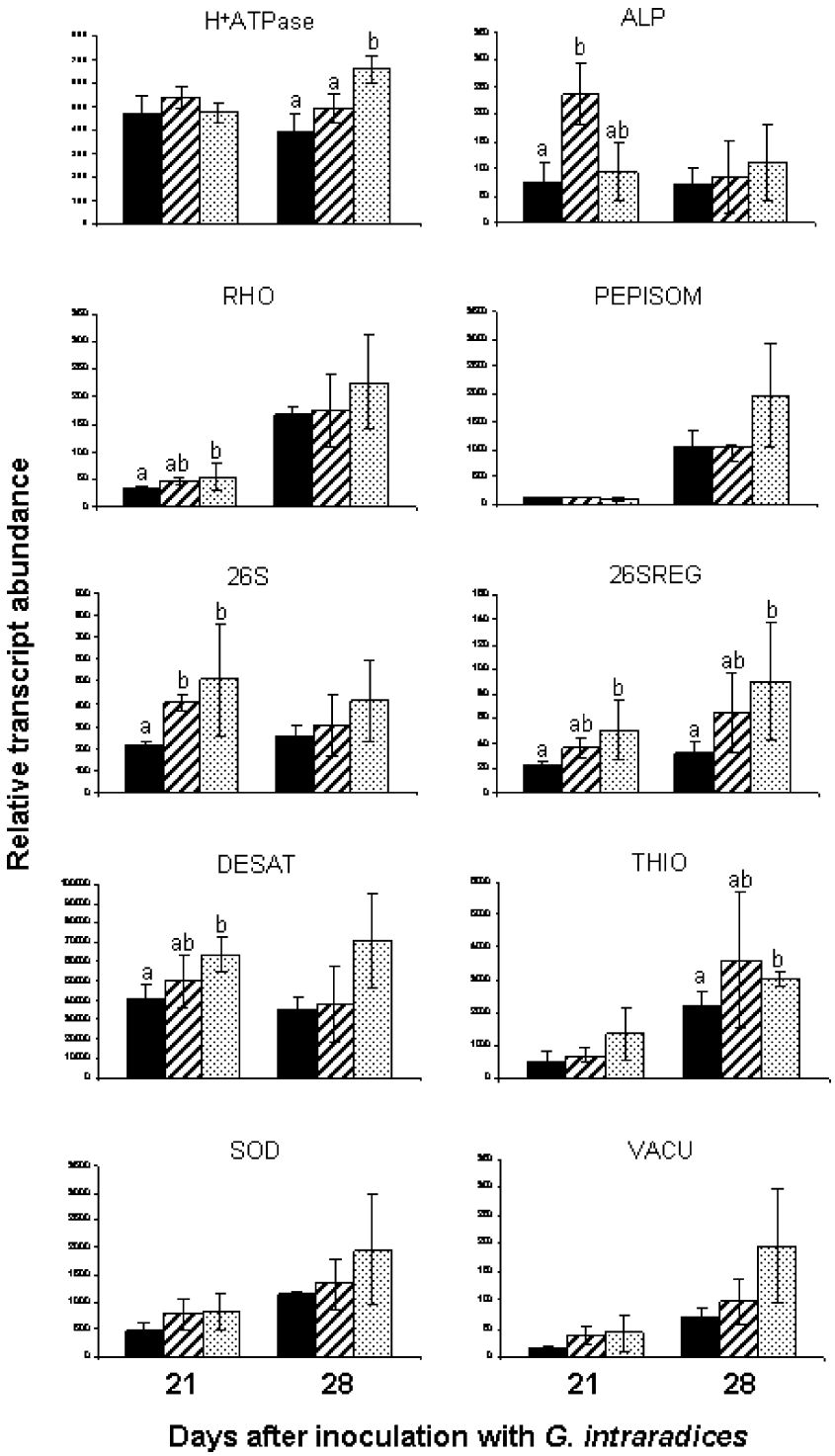

Fig. 2 Expression of fungal genes in *G. intraradices*-colonized roots of the wild-type SGE genotype (*black bar*) and of the mutants *Pssym33* (*striped bar*) and *Pssym40* (*dotted bar*) of *P. sativum*, 21 and 28 days after inoculation. Transcript abundance of each gene, measured by real-time RT-PCR, is expressed relative to that of the house-keeping fungal *TEF* gene. *Letters* indicate significant differences between genotypes at one time point ($P \leq 0.05$, $n=3$). *Abbreviations* for genes are defined in "Materials and methods" section

colonized mutant roots. In general, *RHO*, *PEPISOM*, *26SREG*, *THIO*, *SOD*, and expression increased from 21 to 28 dai in colonized roots of all three pea genotypes and especially in *Pssym40*. However, only *THIO*, *26SREG*, and *H^+-ATPase* transcript abundance levels gave significantly higher values in *Pssym40* at 28 dai as compared to SGE mycorrhizal root systems.

Fungal gene expression in arbuscule-containing cells

The strong downregulation of eight *G. intraradices* genes in colonized roots of *Pssym36* as compared to wild-type, *Pssym33*, or *Pssym40* mycorrhiza suggests that these genes may be preferentially expressed when complete arbuscules develop in pea cells. To verify this, transcript accumulation of three genes showing greatest differences (*DESAT*, *PEPISOM*, *SOD*) was analyzed in 40 whole sections (~240 arbuscule-containing cells) of *G. intraradices*-colonized roots of *Pssym40* and compared to contents of microdissected arbuscule-containing cells (637±58). Results confirmed an enrichment of transcripts for all three genes in the isolated fungal arbuscules (Table 3).

Influence of *G. intraradices* colonization on plant gene expression in roots of parent and mutant pea genotypes

The nucleotide sequences obtained by RT-PCR for *TI* and *DRP* showed high similarity to pea genes encoding a kunitz-like trypsin inhibitor (AJ308163, 1e-70) and the cysteine-rich disease resistance response protein pI230 (L01578, 2e-203). Sequences obtained using primers from other plant species gave closest homologies to *Vicia faba* H^+ATPase *vha4* (AJ310523.1, 1e-79), *M. truncatula* phosphate transporter *MtPt4* (AY116211, 9e-35), *P. sativum* blue copper protein (Z25471, 1e-37), *M. truncatula* glutathione-*S*-transferase (AY134608, 2e-15), *P. sativum* MAP kinase homolog (X70703, 8e-28), and a putative *M. truncatula* serine protease (AL382601, 7e-40). The new pea sequences have been deposited in the EMBL database under the accession numbers FN554871-76.

Expression of seven of the eight selected plant genes was affected by mycorrhiza formation in *P. sativum* cv. Finale roots, compared to noninoculated roots (Fig. 3; Table 4). At 21 dai with *G. intraradices*, *TI* and *GST* genes were upregulated, while the *DRP*, *MAPK*, and *SERPROT* genes were all downregulated. Similar trends persisted for the *TI*, *GST*, and *MAPK* genes at 28 dai, while *DRP* became upregulated in mycorrhizal roots. At this time point, development of *G. intraradices* within *P. sativum* cv. Finale roots significantly repressed expression of the *BCOP* gene and activated the *PT4* gene. In roots of the mutant *Pssym36*, only *GST* expression was significantly modified (upregulated) by *G. intraradices* colonization at 21 dai; no significant responses were detected in this mutant genotype for any of the other seven plant genes at either time point. The effect of mutation of the *PsSym36* gene on plant responses was confirmed by significant ($P<0.05$) or marginally significant ($P\leq0.1$) mycorrhiza × mutant interactions for expression of most of the plant genes (Table 4).

When plant gene responses to *G. intraradices* in the SGE line and the *Pssym33* or *Pssym40* mutant genotypes were compared (Figs. 4 and 5), significant modifications in gene expression were of the same tendency for the wild-type genotype in the two analyses: *PT4*, *TI*, *GST*, and *SERPROT* were upregulated and *MAPK* was downregulated in mycorrhizal roots. The overall responses of *TI*, *GST*, and *MAPK* to *G. intraradices* colonization were similar to those in mycorrhizal *P. sativum* cv. Finale. Neither *H^+ATPase* nor *BCOP* expression was affected by *G. intraradices* in the SGE line. Mutation in the *PsSym33* or *PsSym40* gene did not alter the pattern of transcript accumulation in the *PT4*, *TI*, *GST*, or *SERPROT* genes at 21 dai as compared to the SGE line, although responses to the mycorrhizal fungus tended to be lower in *Pssym33* roots. Expression of the *MAPK* gene was not affected by fungal colonization in *Pssym33* or *Pssym40* roots, while transcripts of the *DRP* gene were not detected in roots of any genotype at 21 dai. Increases in *GST* and *PT4* expression persisted at 28 dai in mycorrhizal SGE and *Pssym40* but were attenuated in *G. intraradices*-colonized *Pssym33* roots. No clear expression patterns could be observed for the *DRP*, *TI*, or *MAPK* genes at this time point. As for *P. sativum* cv. Finale and *Pssym36*, *H^+ATPase* or *BCOP* transcript accumulation did not differ significantly between nonmycorrhizal and *G. intraradices*-colonized roots of SGE, *Pssym33*, or *Pssym40* roots either at 21 or 28 dai. Significant or marginally significant

Table 3 Relative transcript abundance of three fungal genes in whole sections of *G. intraradices*-colonized roots or microdissected arbuscule-containing cells of the *sym40* pea mutant

Material analyzed	Stearoyl-CoA desaturase	Peptidylprolyl isomerase	Superoxidase dismutase
Whole root sections	77.0a	0.7a	17.8a
Dissected arbuscule-containing cells	466.6b	999.0b	1,083.7b

Letters indicate significant differences between values in columns ($P\leq0.001$, $n=3$)

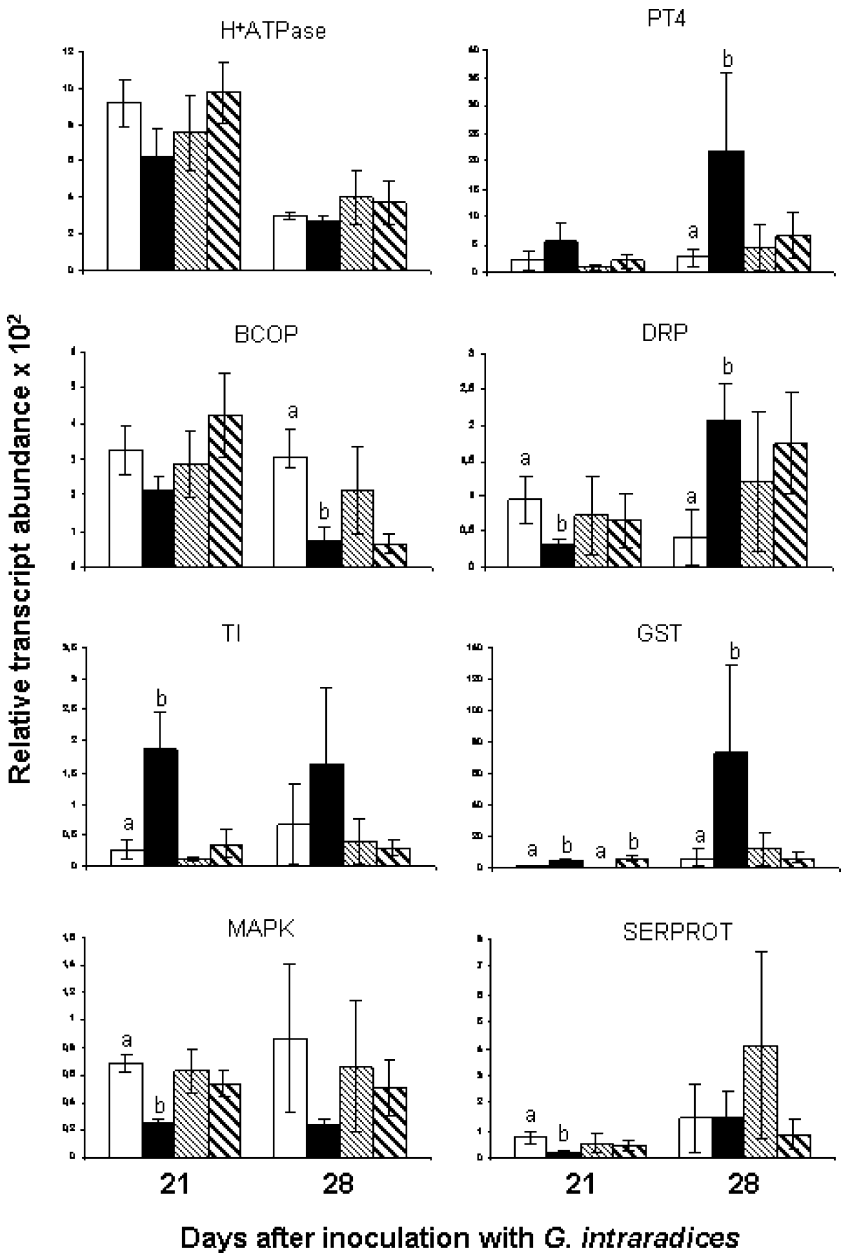

Fig. 3 Expression of pea genes in nonmycorrhizal (*white bar*) and mycorrhizal (*black bar*) roots of wild-type *P. sativum* cv. Finale and in noncolonized (*thin striped bar*) and colonized (*thick striped bar*) roots of the mutant *Pssym36*, 21 and 28 days after inoculation with *G. intraradices*. Transcript abundance of each gene, measured by real-time RT-PCR, is expressed relative to that of the house-keeping pea *GAPDH* gene. *Letters* indicate significant differences between treatments at one time point ($P \leq 0.05$, $n=3$). *Abbreviations* for genes are defined in "Materials and methods" section

mycorrhiza × mutant interactions were only observed for *GST* and *TI* expression in *Pssym33* roots and *MAPK* in *Pssym40* roots (Table 4).

Discussion

Plant mutants are key tools for defining *SYM* genes involved in distinct morphological stages of AM symbiosis development and for the identification of plant or fungal gene responses that are part of the molecular network underlying the symbiotic interactions. Comparisons of symbiosis-related gene expression between wild-type and mutant genotypes has previously focused on plant mutants, mainly in *M. truncatula* and *L. japonicus*, that are affected in the earliest stages of symbiosis development, after appressorium formation and preventing penetration of fungi into the root (Myc^{-1} phenotype; Duc et al. 1989). Plant mutants which do not fully block the symbiotic process but are altered for arbuscule formation (Myc^{-2} phenotype; Gianinazzi-Pearson et al. 1991) or in the rate of AM symbiosis development (Rmd^{-} and Rmd^{++} phenotypes; Jacobi et al. 2003a, b) are less frequent, and contrary to Myc^{-1} mutants, the function of the corresponding *SYM* genes has not yet been identified. In the present study, we investigated how inactivation of these *SYM* genes in pea affects expression of symbiosis-related fungal and plant genes in order to gain some insight into their implication in the cellular events of

Table 4 Two-way ANOVA for influence of the factors mycorrhiza (Myc), pea genotype (mutant), and an interaction between both factors (Myc × mutant) on plant gene expression 21 and 28 dai by *G. intraradices* in roots of *P. sativum* cv. Finale vs. *Pssym36*, line SGE vs. *Pssym33*, and line SGE vs. *Pssym40*

Plant gene	H+ATPase	PT4	BCOP	Drp	TI	GST	MAPK	SERPROT
Finale/*sym36*								
21 dai								
Myc	0.678	*0.098*	0.815	*0.092*	**0.001**	**<0.001**	**0.001**	**0.049**
Mutant	0.348	*0.077*	0.114	0.770	**0.002**	0.502	*0.063*	0.849
Myc × mutant	**0.027**	0.317	**0.036**	0.206	**0.006**	0.175	**0.014**	0.120
28 dai								
Myc	0.594	**0.050**	**0.003**	**0.027**	0.348	*0.100*	0.110	0.302
Mutant	0.115	0.184	0.266	0.577	*0.086*	*0.096*	0.903	0.497
Myc × mutant	0.988	*0.098*	0.373	0.205	0.240	*0.063*	0.311	0.299
SGE/*sym33*								
21 dai								
Myc	0.402	**0.003**	0.270	–	**0.010**	**<0.001**	**0.025**	**0.031**
Mutant	0.260	0.467	**0.010**	–	**0.219**	*0.059*	0.542	0.339
Myc × mutant	0.667	0.435	0.987	–	0.208	**0.046**	0.254	0.399
28 dai								
Myc	0.081	*0.060*	0.139	–	*0.065*	**0.021**	0.182	0.251
Mutant	0.491	0.606	0.128	–	0.377	0.140	0.215	0.495
Myc × mutant	0.660	0.231	0.751	–	*0.096*	**0.026**	0.220	0.221
SGE/*sym40*								
21 dai								
Myc	0.114	**0.007**	0.873	–	**0.002**	**0.031**	**0.049**	**0.033**
Mutant	0.593	0.411	**0.001**	–	0.994	0.308	0.158	0.562
Myc × mutant	0.787	0.387	0.431	–	0.954	0.821	**0.034**	0.824
28 dai								
Myc	0.326	**0.028**	0.422	**0.027**	0.165	**0.012**	0.238	0.384
Mutant	0.669	0.410	**0.048**	0.509	0.597	*0.074*	0.324	0.309
Myc × mutant	0.708	0.496	0.660	0.663	0.233	0.361	0.317	0.125

Significant ($P<0.05$) or marginally significant ($P\leq0.1$) P values are indicated in bold or italics, respectively

arbuscule formation and function. Mutation of *PsSym36* had a greater impact on *G. intraradices*–pea root interactions than inactivation of *PsSym33* or *PsSym40* not only at the level of fungal development within roots but also fungal and plant gene responses.

Root colonization by *G. intraradices* was significantly less in the *Pssym36* mutant than in the wild-type parent *P. sativum* cv. Finale, arbuscules were truncated, and no vesicles were observed (Myc^{-2} phenotype). These data are in agreement with those published earlier for *Glomus mosseae* (Lapopin et al. 1999; Grunwald et al. 2004) and suggest that the *Pssym36* mutant provides suboptimal conditions for fungal development inside the root. This was reflected in the considerably lower expression of most of the *G. intraradices* genes monitored in roots of the arbuscule-defective Myc^{-2} mutant. In contrast and as previously reported by Jacobi et al. (2003a, b), mutation of the *PsSym40* gene led to more rapid root colonization and arbuscule formation than in the other pea genotypes (Rmd^{++} phenotype). None of the targeted fungal genes were repressed in this interaction, and the Rmd^{++} phenotype was associated with a higher expression of several *G. intraradices* genes during root colonization. The Rmd^{-} pea mutant *Pssym33* was characterized by a phenotype with slower root colonization (cf. Jacobi et al. 2003a, b) than the SGE wild-type or *Pssym40* mutant and a level of fungal gene expression which tended to be situated between the two. No mycorrhizal effect on plant biomass was observed in any of the pea genotypes up to 28 dai. Rivera-Becceril et al. (2002) made similar observations for *P. sativum* cv. Finale up to 42 dai, while Jacobi et al. (2003a) reported accelerated plant development in mycorrhizal SGE, *Psym33*, and *Pssym40* but over a longer growth period up to pod ripening.

All the studied fungal genes were expressed in mycorrhizal roots of the wild-type pea genotypes (*P. sativum* cv. Finale, SGE). Since eight of the *G. intraradices* genes are active both in appressoria and established mycorrhiza in wild-type *M. truncatula* (Seddas et al. 2009), they may be related to more general processes of symbiotic structure differentiation in the mycorrhizal fungus. For the SGE background genotypes, the relative expression of several fungal genes increased from 21 to 28 dai, but there was little relationship with the mycorrhization parameters;

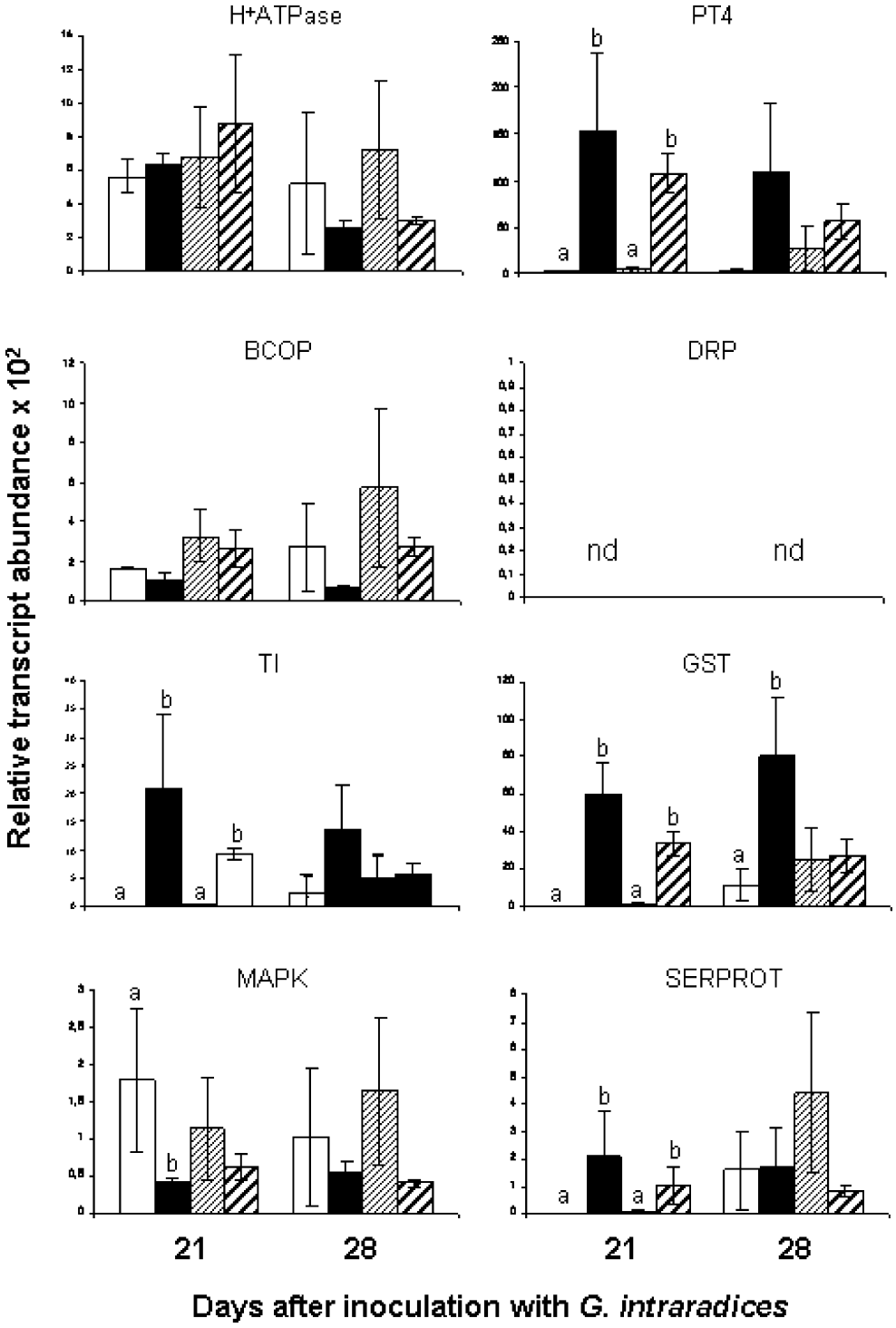

Fig. 4 Expression of pea genes in nonmycorrhizal and mycorrhizal roots of the wild-type SGE genotype (*white and black bars*) and of the *Pssym33* mutant (*thin and thick striped bars*), 21 and 28 days after inoculation with *G. intraradices*. Transcript abundance of each gene, measured by real-time RT-PCR, is expressed relative to that of the house-keeping pea *GAPDH* gene. *Letters* indicate significant differences between treatments at one time point ($P \leq 0.05$, $n=3$). *Abbreviations* for genes are defined in "Material and methods"

fungal transcript levels were generally the highest in mycorrhizal roots of *Pssym40*, the lowest in SGE, and intermediate in *Pssym33*, although colonization was the slowest in the latter. Fungal gene expression did not follow the same pattern in the *P. sativum* cv. Finale background. Root colonization by *G. intraradices* more than doubled between 21 and 28 dai in wild-type roots but, except for *DESAT*, relative transcript abundance of the fungal genes tended to decrease. In contrast to Janoušková et al. (2009), no correlation was found between *ALP* gene expression and the amount of enzyme-active mycelium detected in mycorrhizal roots of the different pea genotypes. However, *ALP* expression and the proportion of ALP-active mycelium appeared to increase with intraradical development of *G.*

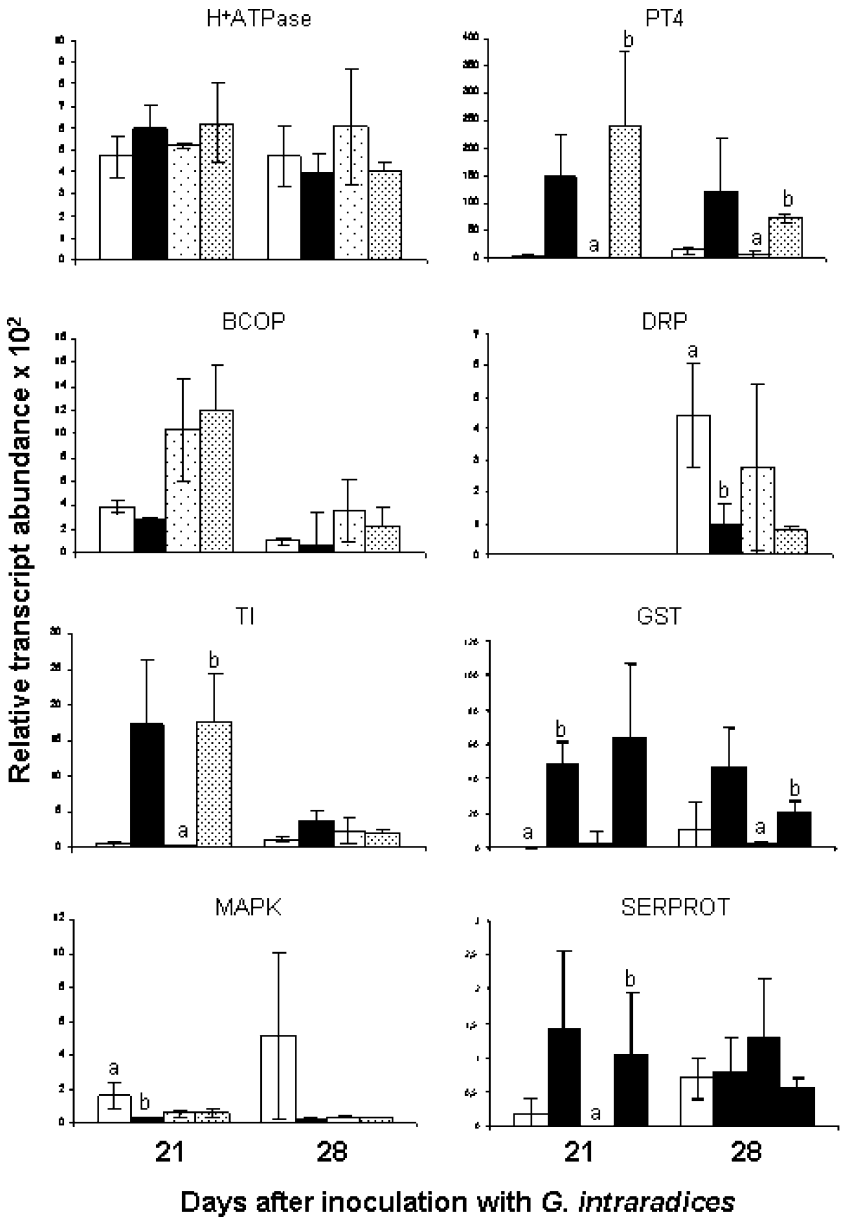

Fig. 5 Expression of pea genes in nonmycorrhizal and mycorrhizal roots of the wild-type SGE genotype (*white and black bars*) and of the *Pssym40* mutant (*large and small dotted bars*), 21 and 28 days after inoculation with *G. intraradices*. Transcript abundance of each gene, measured by real-time RT-PCR, is expressed relative to that of the house-keeping pea *GAPDH* gene. *Letters* indicate significant differences between treatments at one time point ($P \leq 0.05$, $n=3$). *Abbreviations* for genes are defined in "Materials and methods" section

intraradices in roots of *Pssym36* and *Pssym33*, although mycorrhiza levels were lower, as compared to wild-type mycorrhiza. Because purification of the *ALP* enzyme from AM fungi has so far been unsuccessful, little is known about its enzymatic characteristics or its function in the AM symbiosis (Kojima et al. 2001). Microorganisms produce alkaline phosphatases in response not only to phosphate but also to various environmental stresses (Lim et al. 1996), and the upregulation of *G. intraradices ALP* expression in *Pssym36* and *Pssym33* roots may be indicative of a stress to the fungus in these genotypes.

The lower transcript levels of most of the studied *G. intraradices* genes in colonized *Pssym36* roots indicates regulation by *PsSym36* of fungal processes associated with arbuscule development and function. Such a control of plant genes over fungal gene activity was recently hypothesized for *M. truncatula SYM* genes during the early stages of symbiotic interactions leading to the initiation of AM establishment (Seddas et al. 2009). The strong down-regulation of the *DESAT* gene of *G. intraradices* in *Pssym36* roots suggests modifications in fungal lipid metabolism associated with reduced biosynthesis of fatty acids which would result from a limited active uptake of photosynthetic carbon in the absence of a functional arbuscule interface. This is reflected in a decreased expression in fungal membrane H^+*ATPase* when arbuscule formation is incomplete in *Pssym36* roots and which correlates with the lack of enzyme activity on the fungal plasma membrane bordering aborted arbuscules branches in the same pea genotype (Gianinazzi-Pearson et al. 1995).

Reduced carbon acquisition would explain the lower root colonization and lack of vesicle development by *G. intraradices* in the *Pssym36* mutant roots. Carbon and phosphate exchange at the symbiotic interface have for a long while been considered interlinked processes in AM (Woolhouse 1975). Furthermore, there is recent evidence that PT4 transporter activity is essential for the maintenance of arbuscules (Javot et al. 2007) and lysophosphatidylcholine has been proposed as a possible signal molecule at the symbiotic interface to maintain this activity (Drissner et al. 2007). In this context, the pea phosphate transporter homolog was not activated in *G. intraradices*-colonized roots of the *Pssym36* mutant, in contrast to its upregulation in mycorrhizal roots of the wild-type, *Pssym33*, and *Pssym40* pea genotypes where expression of fungal *DESAT* and *H^+ATPase* was also maintained, or increased. Arbuscule-related accumulation of fungal *DESAT* transcripts in fully developed mycorrhizal tissues has previously been monitored by in situ RT-PCR (Seddas et al. 2008), and this was confirmed in the present transcript analysis of microdissected arbuscule-containing cells from *Pssym40* pea roots.

Microdissection also revealed the preferential accumulation of fungal *PEPISOM* and *SOD* transcripts in arbuscule-containing cells of pea roots. Using an in situ RT-PCR technique, Seddas et al. (2008) previously reported presence of *PEPISOM* but absence of *SOD* gene expression in arbuscules formed in *M. truncatula* roots. This discrepancy could be due to a greater detection sensitivity of *SOD* transcripts resulting from RNA amplification after microdissection, contrary to direct in situ RT-PCR, but it may also suggest an influence of the host plant over AM fungal gene expression. Apart from the present study and that by Seddas et al. (2009), there are no data about how AM fungal gene expression can vary in interactions with different host genotypes. However, the complexity of fungal–plant interactions is illustrated by the variations reported between AM fungi in *SOD* transcript accumulation within symbiotic tissues (Lanfranco et al. 2005; Liu et al. 2003) and the differential effects that AM fungal species can have on plant gene activation in a same host (Massoumou et al. 2007).

The weaker expression of the fungal *RHO* gene, encoding a putative Rho/GDP dissociation inhibitor, in *G. intraradices*-colonized *Pssym36* roots could indicate effects on cell signaling events at the fungal–plant interface, as was suggested for mutations in two *M. truncatula* SYM genes which similarly repressed *RHO* expression in appressoria (Seddas et al. 2009). Such inhibitors regulate GTP–GDP conversion on Rho/GTPase proteins which are essential to basic cell functions including metabolism, proliferation, and differentiation (Groysman et al. 2002). Likewise, lower transcript levels in colonized *Pssym36* roots of the *PEPISOM* and *26SREG* genes which are implicated in cellular recognition (Miele et al. 2003) and metabolic regulation/cell cycle progression (Murray 1995), respectively, may contribute to or result from defective arbuscule development in the pea mutant. In this context, inactivation of *PsSym33* or *PsSym40* was not detrimental to the expression of these three pea genes or to arbuscule development.

The fungal genes *THIO* and *SOD* encoding functions in antioxidative stress metabolism were active in mycorrhizal roots of the wild-type, *Pssym33*, and *sym40* pea genotypes. Activation of these two genes has been reported for mycorrhiza of other plant/fungal combinations where arbuscules are fully developed (Brechenmacher et al. 2004; Seddas et al. 2009). Such gene activation may enable the AM fungus to contend with plant defense/stress responses which are typically activated within arbuscule-containing cells (Gianinazzi-Pearson et al. 1996) and which are evidenced in the mycorrhizal roots of the wild-type, *Pssym33*, and *Pssym40* pea genotypes by the upregulation of the plant genes encoding the pI230 disease resistance response protein, a trypsin inhibitor, and glutathione-*S*-transferase. Expression of the latter gene has previously been localized in arbuscule-containing cells of potato and *M. truncatula* roots (Strittmatter et al. 1996; Wulf et al. 2003). This hypothesis is further strengthened by the fact that neither the fungal *THIO* and *SOD* genes nor the plant *DRP* or *TI* genes are activated by *G. intraradices* colonization of the arbuscule-defective *sym36* pea mutant, which suggests that arbuscule formation induces a stress situation within the colonized plant cells.

The MAP kinase-encoding pea gene was consistently downregulated in mycorrhizal roots of the wild-type pea genotypes, but expression was not significantly affected in the three mutants. Liu et al. (2003) also reported down-regulation of two *M. truncatula* MAP kinase genes as *Glomus versiforme* developed within roots, although the genes were transiently upregulated during the initial stages of symbiosis establishment, while Grunwald et al. (2004) found that a MAP kinase gene was upregulated in mycorrhizal *P. sativum* cv. Finale roots and unaffected in *Pssym36*. MAP kinases belong to a gene family which interacts with a large number of receptors making it difficult to hypothesize as to a specific function in mycorrhizal interactions since distinct MAP kinase pathways are involved in a wide variety of biological processes including cell growth and death, differentiation, the cell cycle, and stress responses (Jonak et al. 1999). Neither the pea *H^+ATPase* gene nor the *BCOP* gene was upregulated in mycorrhizal interactions of any of the pea genotypes which is in contrast to that reported for genes in *M. truncatula*, even though the *PT4* gene is activated in the two plants. Both genes again belong to large gene families (the pea

H^+ATPase gene showed highest homology to *vha4*), and isoforms may behave differently in response to signals or external stimuli in different plant species.

In conclusion, the pea genes *PsSym36*, *PsSym33*, and *PsSym40* not only control root colonization and morphological differentiation of an AM fungus but they also influence gene responses linked to symbiotic interactions between the partners. Results indicate that fungal and plant factors essential to arbuscule morphophysiology are affected by inactivation of these *SYM* genes, but to different extents. Gene expression patterns are coherent with a role of the *PsSym36* gene in the coordinated cellular development in plant and fungus to elaborate a functional interface in arbuscule-containing cells. The influence of *PsSym33* and *PsSym40* appears more subtle since fungal gene expression tended to increase when they were inactivated and plant gene responses were little affected by their mutation. The use of symbiotic plant mutants in genome-wide studies of fungal and plant gene responses will give more insight into the metabolic circuits controlled by *SYM* genes and driving symbiotic interactions. An initial approach which has been undertaken for steps in early interactions using *M. truncatula* chips (Seddas et al. 2009) could be extended to fully established mycorrhiza in this species. DNA sequencing of *G. intraradices* is underway in order to prepare for large-scale investigations (Martin et al. 2008), but studies of pea transcriptional programs are hampered by the lack of genome-wide information in this species. The extensive conserved synteny between pea and *M. truncatula* genomes (Zhu et al. 2005) could be exploited to circumvent this obstacle by probing *M. truncatula* microarrays to profile, at least partially, the mycorrhiza-related transcriptome in pea.

Acknowledgments This work was supported by the French Foreign Affairs Ministry (doctoral grant to E. Kuznetsova), the Regional Council of Burgundy (Project no. 07 9201 AAO40 S3623), RFBR (10-04-01026, 10-04-01146), and the Russian Ministry of Science and Education (02.512.11.2254). Authors thank G. Duc for seeds of *P. sativum* cv. Finale and *Pssym36* pea genotypes, C. Desmetz and Z. Hao for assistance respectively in RNA control and statistical analyses, and A. Colombet and V. Monfort for technical assistance.

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