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RESEARCH PAPER

Local and systemic mycorrhiza-induced protection against the ectoparasitic nematode *Xiphinema index* involves priming of defence gene responses in grapevine

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Abstract

The ectoparasitic dagger nematode (*Xiphinema index*), vector of *Grapevine fanleaf virus* (GFLV), provokes gall formation and can cause severe damage to the root system of grapevines. Mycorrhiza formation by *Glomus* (syn. *Rhizophagus*) *intraradices* BEG141 reduced both gall formation on roots of the grapevine rootstock SO4 (*Vitis berlandieri*×*V. riparia*) and nematode number in the surrounding soil. Suppressive effects increased with time and were greater when the nematode was post-inoculated rather than co-inoculated with the arbuscular mycorrhizal (AM) fungus. Using a split-root system, decreased *X. index* development was shown in mycorrhizal and non-mycorrhizal parts of mycorrhizal root systems, indicating that both local and systemic induced bioprotection mechanisms were active against the ectoparasitic nematode. Expression analyses of ESTs (expressed sequence tags) generated in an SSH (subtractive suppressive hybridization) library, representing plant genes up-regulated during mycorrhiza-induced control of *X. index*, and of described grapevine defence genes showed activation of chitinase 1b, pathogenesis-related 10, glutathione *S*-transferase, stilbene synthase 1, 5-enolpyruvyl shikimate-3-phosphate synthase, and a heat shock proein 70-interacting protein in association with the observed local and/or systemic induced bioprotection against the nematode. Overall, the data suggest priming of grapevine defence responses by the AM fungus and transmission of a plant-mediated signal to non-mycorrhizal tissues. Grapevine gene responses during AM-induced local and systemic bioprotection against *X. index* point to biological processes that are related either to direct effects on the nematode or to protection against nematode-imposed stress to maintain root tissue integrity.

Key words: Arbuscular mycorrhiza, bioprotection, defence gene expression, grapevine, split-root system, *Xiphinema index*.

Introduction

Grapes (*Vitis.* spp) represent an important fruit production of high economic value across the world. Amongst pathogens that can affect grapevines, the widely occurring soil-borne dagger nematode *Xiphinema index* can cause severe damage to root systems (Brown and Trudgill, 1989; Jawhar *et al.*, 2006; Tzortzakakis *et al.*, 2006; Leopold *et al.*, 2007) and it is recognized as the primary vector for transmission of the *Grapevine fanleaf virus* (GFLV) (Hewitt *et al.*, 1958), which is considered to be a major threat to the grapevine industry (Andret-Link *et al.*, 2004). *Xiphinema index* is a migratory ectoparasitic nematode which establishes feeding sites preferentially in young zones of roots, inducing cell hypertrophy and necrosis, which result in the formation of galls at the feeding sites (Weischer and Wyss, 1976; Rumpenhorst and Weischer, 1978). The dagger nematode can survive in vineyard soils for many years with or without

host plants (Demangeat *et al.*, 2005) and the use of nematicides and fumigants to control the nematode has not been highly effective (Raski and Goheen, 1988). In addition, the acute toxicity of these agrochemicals has rendered their use unacceptable in several countries because of potential adverse environmental effects (Abawi and Widmer, 2000).

Beneficial soil microorganisms such as arbuscular mycorrhizal (AM) fungi have been proposed as a potential alternative to chemical control (Pozo and Azcón-Aguilar, 2007; Shoresh *et al.*, 2010). AM fungi are widespread root symbionts in plants, colonizing species belonging to >80% of all plant families (Wang and Qiu, 2006). They have been reported to enhance plant uptake of phosphate (P) and other mineral nutrients by grapevine under certain conditions (Mortimer *et al.*, 2005; Karagiannidis *et al.*, 2007). Root colonization by AM fungi is also known to increase tolerance or induce resistance to fungal pathogens and reduce nematode development in different plants, including grapevine (Pinochet *et al.*, 1996; Li *et al.*, 2006; Camprubi *et al.*, 2008; Nogales *et al.*, 2009). Evidence exists that AM-induced protection against root pathogens involves not only local but also systemic induced resistance, with reduction in root infection in mycorrhizal and non-mycorrhizal parts of mycorrhizal root systems, suggesting the existence at a distance of signal-mediated phenomena (Rosendahl 1985; Cordier *et al.*, 1998; Slezack *et al.*, 1999; Zhu and Yao, 2004; Khaosaad *et al.*, 2007; Elsen *et al.*, 2008).

Research on AM fungi–nematode interactions has mainly focused on specific groups of endoparasitic nematodes, such as root-knot (*Meloidogyne*) and root-lesion (*Pratylenchus*, *Radopholus*) nematodes (reviewed by Hol and Cook, 2005). Effects vary with the environment, plant genotype, nematode species, and fungal isolates (Pinochet *et al.*, 1996; Hol and Cook, 2005; de la Pena *et al.*, 2006; Camprubi *et al.*, 2008). Interactions between AM fungi and ectoparasitic nematodes have received very little attention. Three studies of the ectoparasite *Tylenchorhynchus* spp. have shown that AM fungi can compensate for negative effects of root damage although the nematode population may remain unaffected or increase (Kassab and Taha, 1991; Jain *et al.*, 1998*a*, *b*). However, interactions between AM fungi and the ectoparasitic nematode *X. index* have not been reported. Likewise, the cellular and molecular mechanisms involved in nematode control in mycorrhizal root systems are unknown.

It has been suggested that mechanisms underlying mycorrhiza-induced resistance or tolerance to plant pathogens are probably multiple and synergistic, involving enhanced or altered plant growth and changes in root system morphology, nutrition status, and rhizosphere microbe populations (Azcón-Aguilar and Barea, 1996). While some studies on fungal root pathogens have reported a reduction in damage after co-inoculation with an AM fungus (e.g. Caron *et al.*, 1986), others have clearly shown that intracellular arbuscule formation in a well-established AM symbiosis is necessary for bioprotection (Cordier *et al.*, 1998; Slezack *et al.*, 2000; Pozo *et al.*, 2002). Few investigations have focused on the cellular or molecular bases of bioprotection in mycorrhizal root systems, although it has been suggested that this may also be exerted through the activation of plant defence systems (Gianinazzi-Pearson *et al.*, 1996; Pozo and Azcón-Aguilar, 2007). Formation of the mycorrhizal symbiosis is associated with a weak activation of host plant defence which has been suggested to predispose mycorrhizal roots to respond rapidly to plant pathogens through a mechanism analogous to sensitization or priming (Gianinazzi, 1991; Dumas-Gaudot *et al.*, 2000; Garcia-Garrido and Ocampo, 2002; Conrath *et al.*, 2006; Pozo *et al.*, 2009). Decreased bacterial or fungal development in mycorrhizal plants is associated with local and systemic root defence responses involving cell wall depositions, accumulation of phenolics and callose, lytic enzyme activities, and pathogenesis-related (PR) protein gene activation (Cordier *et al.*, 1998; Dumas-Gaudot *et al.*, 2000; Pozo *et al.*, 2002; Zhu and Yao, 2004; Li *et al.*, 2006).

In the present study, the first evidence for bioprotection against *X. index* in mycorrhizal grapevines is provided and initial steps towards the molecular characterization of local and systemic nematode control in mycorrhizal root systems are described. Whole or split-root systems of grapevine rootstock SO4 (*Vitis berlandieri*×*V. riparia*) were inoculated with the AM fungus *Glomus intraradices* and co-inoculated, or post-inoculated after mycorrhizal development, with the nematode *X. index*. Plant gene activation associated with bioprotection was investigated using the non-targeted technique of subtractive suppressive hybridization (SSH; Diatchenko *et al.*, 1996), and a targeted approach based on expression profiling of key plant defence genes encoding phenylalanine ammonia lyase (*PAL*), stilbene synthase 1 (*STS*), lipoxygenase (*LOX*), glutathione *S*-transferase (*GST*), chitinase 1b (*CHI*), protease inhibitor PR6 (*PIN*), and two nematode-specific resistance genes (*HS* and *HERO*).

Materials and methods

Biological material

Herbaceous two node cuttings (~10 cm long) were used to propagate the grapevine rootstock SO4 (*V. berlandieri*×*V. riparia*), which is extensively cultivated in the Burgundy region of France. The cuttings were inserted into small cellulose sponges with peat, and placed on an intermittent mist propagation bed. After 4–5 weeks, uniform rooted cuttings were gently washed from the peat and transferred to a 1:1 (v/v) mixture of terragreen® (OilDri-US special, Mettman, Germany) (180 °C, 6 h) and clay–loam soil (γ-irradiated) with the following properties: pH_{H2O} 7.96; 14.4 g kg^{-1} organic matter; 29 mg kg^{-1} Olsen-P; 1.46 mg kg^{-1} NaOH-extractable N; 0.241 g kg^{-1} NH_4Ac-exchangeable K.

The AM fungus *G. intraradices* (Schenck & Smith) (isolate BEG141, syn. *Rhizophagus intraradices*) was propagated in pot culture on onion (*Allium cepa* L.) plants in the clay–loam soil for 10 weeks. Inoculum from pot cultures (spores, mycelium, soil, and root fragments) was used at a rate of 1:7 (v:v) in the growth medium for mycorrhizal treatments. In non-mycorrhizal treatments, inoculum was replaced by sterilized inoculum, plus a filtered water suspension of the inoculum in order to provide a similar microflora in the absence of the mycorrhizal fungus.

Xiphinema index Thorne & Allen (1950) was collected from vineyards in the Burgundy region and reared under greenhouse conditions on *Ficus carica* L. to provide a permanent source of virus-free nematodes (Coiro and Brown, 1984). Nematodes were

extracted from 250 ml of soil using an Oostenbrink elutriator and collected using 50 mm sieves. The sievings containing nematodes were rinsed with water and placed on moist cellulose paper in a Petri dish containing water. Active nematodes were recovered in the bottom of the Petri dish after 48 h; adults and juveniles were counted in the final suspension with an etched grid. Nematode inoculation consisted of dispensing a water suspension of 100 nematodes (10 nematodes ml^{-1}) into evenly spaced 6–8 cm deep holes around plants; non-inoculated plants received an equivalent volume of water.

Experimental design

To determine the dynamics of bioprotection against *X. index* by *G. intraradices*, rooted grapevine cuttings were transferred to 800 ml of growth substrate in 1.0 l pots and subjected to four treatments: control (no *G. intraradices*, no *X. index*); inoculation with *G. intraradices* only, at transplanting; inoculation with *X. index* only, 21 d after transplanting plants; amd inoculation with *G. intraradices* at transplanting then with *X. index* 21 d later. Four plants from each treatment were harvested and the corresponding soil collected at 0, 7, 14, 21, and 35 d after inoculation with *X. index*. Subsamples of root systems were stored in liquid nitrogen for gene expression analyses.

The effect of co-inoculation of *X. index* with *G. intraradices* or post-inoculation of AM plants on nematode development was investigated. Rooted grapevine cuttings were transferred to 800 ml of growth substrate in 1.0 l pots and subjected to six treatments: control (no *G. intraradices*, no *X. index*); inoculation with *G. intraradices* only at transplanting; inoculation with *X. index* only 21 d after transplanting; inoculation with *G. intraradices* at transplanting then with *X. index* 21 d later; inoculation with *X. index* at transplanting; and co-inoculation with *G. intraradices* and *X. index* at transplanting. Four plants from each treatment were harvested and the corresponding soil collected at 0 d and 35 d after inoculation with *X. index*.

Systemic and local bioprotection against *X. index* in mycorrhizal grapevine roots was analysed by planting root system halves into adjacent pot compartments containing 400 ml of substrate. This split-root experiment consisted of four treatments (Fig. 1): one root system half inoculated with *X. index* (C/N); one root system half inoculated with *G. intraradices* then the other with *X. index* 21 d later (M/N) (systemic effect); one root system half inoculated with *G. intraradices* and with *X. index* 21 d later, the other non-–inoculated (C/M+N) (local effect); and one root system half inoculated with *G. intraradices*, the other with *G. intraradices* then with *X. index* 21 d later (M/M+N) (combined effect). In a first experiment, shoots and root system halves of four plants per C/N and M/N treatments were harvested and time course induction of systemic bioprotection was monitored at 0, 7, 14, 21, and 35 d after inoculation with *X. index*. In a second experiment, root system halves of three plants were harvested for each of the four treatments at 0 d and 35 d after inoculation with *X. index*, systemic and local effects of mycorrhiza on nematode development were estimated, and roots were stored in liquid nitrogen for gene expression analyses.

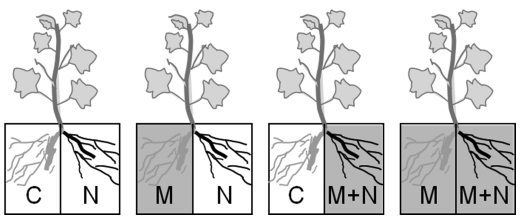

Fig. 1. Experimental design of the split-root system used to investigate local and systemic bioprotection against *X. index* induced by *G. intraradices* in grapevine plants. C, no *G. intraradices* or *X. index*; N, *X. index*; M, *G. intraradices*; M+N, *G. intraradices* and *X. index*.

The role of mycorrhiza-mediated phosphate effects in bioprotection against *X. index* was investigated by growing grapevine plants in soil with higher available P (60 mg kg^{-1} Olsen-P) and amended with phosphate fertilizer. The experiment consisted of four treatments: control (no AM fungus, no P, no *X. index*), supplied with 200 mM P in Long Ashton nutrient solution each week; inoculation after 21 d with *X. index*; and supplied with 200 mM P and inoculated after 21 d with *X. index*. Nematode development was estimated 35 d after inoculation.

For each experiment, pots were arranged in a completely randomized design under glasshouse conditions (17–23 °C, 16 h photoperiod with supplementary lighting). Plants were watered daily (deionized water) to field capacity and received 10 ml per week of a modified (P/10) Long Ashton nutrient solution (Hewitt, 1966).

Mycorrhiza and nematode development

Root systems were carefully washed with pre-cooled (4 °C) deionized water, and fresh shoot and root mass recorded. Nematode development was analysed by counting gall numbers on roots and by estimating nematode numbers in soil using an Oostenbrink elutriator and sieving as described above. Subsamples of fresh roots were stained by the ink–vinegar method (Vierheilig *et al.*, 1998) and mycorrhizal root colonization was quantified microscopically according to Trouvelot *et al.* (1986; www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

RNA extraction and cDNA synthesis

Root samples from each of three plants per treatment were ground in liquid nitrogen and added to pre-warmed (65 °C) extraction buffer [2% cetyltrimethylammonium bromide (CTAB), 2% polyvinylpolypyrrolidone (PVPP), 100 mM TRIS-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% β-mercaptoethanol] at 20 ml g^{-1} tissue. After incubation for 10 min at 65°C, the mixture was extracted twice in equal volumes of chloroform:isoamyl alcohol (24:1), centrifuged (6000 *g*, 15 min, 20 °C), and the aqueous layer transferred to 1 vol. of 5 M LiCl. Nucleic acids were precipitated overnight at 4 °C, centrifuged down (10 000 *g*, 30 min, 4 °C) and the pellet dissolved in 600 µl of 1 M NaCl, 0.5% SDS, 10 mM TRIS-HCl (pH 8.0), and 1 mM EDTA, then incubated for 3 min at 65 °C. The solution was extracted twice with equal volumes of chloroform:isoamyl alcohol (24:1), centrifuged for 15 min at 14 000 *g* at 20°C, the aqueous layer transferred to 3 vols of 0.5% ethanol was added, and nucleic acids were precipitated overnight at –20 °C. After centrifugation (13 000 *g*, 30 min, 4 °C), pellets were washed twice with ice-cold 70% ethanol, air dried, and dissolved in 50 µl of water. A 25 mg aliquot of total RNA samples was treated in a 60 ml DNase solution for 30 min at 37 °C [40 U of RNase inhibitor, 25 U of RNase-free DNase (Promega), 6 ml of 10× buffer provided with the enzyme, and diethylpyrocabonate (DEPC)-treated water]. RNA quantity and quality were estimated by 260/280 nm absorbance and 1% denaturating gel electrophoresis. Replicate extractions of RNA were performed from three independent experiments. cDNA synthesis from DNase I-treated RNAs [1 mg of RNA, 2 mM dNTP, 0.25 mg l^{-1} oligo$(dT)_{15}$ (Promega)] was performed in 5 ml of M-MLV RT buffer (50 mM TRIS-HCl of pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 10 mM dithiothreitol), 1 ml of RNasin, and 1.5 ml of reverse transcriptase (M-MLV), in a PCR thermocycler (Biometra 2000) (15 min at 25 °C, 1 h at 42 °C, 2 min at 96 °C).

Subtractive suppressive hybridization (SSH) and library construction

Grapevine roots from four treatments: control (C), inoculation with *G. intraradices* at transplanting (M), inoculation with *X. index* after 21 d (N), or inoculation with *G. intraradices* then *X. index* (M+N), were harvested at 35 d, pooled, and used to construct an SSH library using the PCR Select system according to the protocol

provided by the supplier (Clontech Laboratories, Palo Alto, CA, USA). Concentration and 260/280 nm ratios of mRNA purified from total RNA using the Dynabeads mRNA Direct kit (Dynal) were determined before and after DNase I digestion with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the integrity of the RNA was checked on 1% denatured agarose gels.

For SSH library construction, 2 mg of mRNA from the mycorrhizal- and nematode-inoculated treatment (M+N) was subtracted with a mixture of 2 mg of mRNA from the non-inoculated treatment (C), 2 mg from the mycorrhizal treatment (M), and 2 mg from the nematode-inoculated treatment (N). The resulting cDNA fragments, corresponding to differentially expressed transcripts from the M+N treatment, were amplified and cloned into the pGEM-T vector (Promega France, Charbonnières-les-Bains, France). Subtraction efficiency was checked using expression of the constitutively expressed grapevine *GAPDH* gene (encoding a glyceraldehyde phosphate dehydrogenase) according to the PCR select system protocol using a 1:10 dilution of the subtracted and non-subtracted cDNA samples as template and *GAPDH* for (TATGAGCAAGTTAAAGCTGC) and *GAPDH* rev (AAGAACTTCTCGTTGAGGGC) primers (Reid *et al.*, 2006).

Expressed sequence tag (EST) clones from the SSH library were screened by northern blot hybridization. Each clone was amplified in a 50 ml reaction volume containing 1 ml of 1:100 cDNA, 0.5 U of *Taq* polymerase (Qbiogene), 125 mM dNTP, and 0.5 mM of each primer (18.1for GTCACGACGTTGTAAAACG and 18.2rev AGCTATGACCA TGATTACG), with the program 1 min at 95 °C; 30 cycles of 1 min at 56 °C and 1 min 30 s at 72 °C; 5 min at 72 °C. PCR products were separated on 1.2% agarose gels, transferred to Hybond-XL (Amersham Bioscience, Orsay, France) by capillary blotting, and fixed under UV light (70 000 J cm^{-2}). cDNA from 2.5 mg of total RNA of the four treatments was ^{32}P labeled by RT-PCR and purified on ProbeQuant G-50 Micro Columns (Amersham Bioscience) before denaturing for 5 min at 95 °C. Membranes were pre-hybridized for 1 h at 60 °C and hybridized with probes overnight at 60 °C in Church buffer (Church and Gilbert, 1984), then washed twice for 5 min in 2× SSC (0.30 M NaCl, 0.03 M sodium citrate)/0.1% SDS at room temperature, twice for 20 min in 0.5× SSC/0.1% SDS at 60 °C, and twice for 20 min in 0.5× SSC/0.1% SDS at 65 °C. Hybridization signals were quantified in a Storm 860 phosphorimager with ImageQuant software (Molecular Dynamics, Amersham Bioscience) and normalized using the *GAPDH* gene. Hybridizations were repeated twice.

ESTs corresponding to genes showing >2-fold induction in M+N roots as compared with M, N, or control roots were cloned into the pCR®4TOPO® vector for sequencing (Invitrogen) according to the manufacturer's instruction. Plasmids were extracted from transformed clones with the NucleoSpin® Plasmid extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction, sequenced (MWG Biotech AG, Ebersberg, Germany), and gene similarities identified by blastn or blast-x sequence comparisons (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were defined for transcript quantification of selected genes (Table 1).

Monitoring of defence-related gene transcripts

Previously published primers (Trouvelot *et al.*, 2008), given in Table 2, were used to monitor expression of the following defence-related vine genes: *CHI* (Busam *et al.*, 1997), *PAL*, *STS* (Sparvoli *et al.*, 1994), *GST*, *LOX*, *PIN* (Aziz *et al.*, 2003), and the constitutively expressed reference gene coding the *GAPDH* gene (Reid *et al.*, 2006). Primers were also designed for the heterologous sequences of the tomato nematode defence-related genes $Hs1^{pro-1}$ and *Hero* (XM_002268530.1 and XM_002265532.1) (Table 2). Primer specificity was confirmed by direct PCR (1 ml of pure cDNA, 14.9 ml of DEPC water, 2 ml of *Taq* DNA polymerase 10× buffer, 1 ml of each primer pair, and 0.5 U of *Taq* DNA polymerase) using the PCR program: 2 min at 95 °C, 30 cycles of 30 s at 94 °C, 30 s at 72 °C, 5 min at 72 °C. PCR products were cloned and sequenced as described above and sequence identity confirmed by blastn or blast-x comparisons.

Quantitative absolute real-time PCR

Transcripts were quantified using the ABsolute™ QPCR SYBR® green ROX mix (ABgene, Epsom, UK) and an ABI PRISM 7900 apparatus (Applied BioSystems, Foster City, CA, USA). Three biological repetitions were used for each treatment, and PCRs were carried out in triplicate per sample, using 1 ml of 100× diluted cDNA as template in a final volume of 20 ml containing 1× SYBR green mix, and 20 nM of each gene-specific primer, as recommended by the manufacturer. To calculate the number of transcripts present in original samples, TOPO plasmids containing each amplicon were quantified by UV absorbance spectroscopy (eppendorf BioPhotometer) and linearized by *Not*I (Promega) digestion overnight at 37 °C in 50 ml final volume (300 ng of plasmid, 5 ml of 10× buffer, 1 U of *Not*I, qsp ultrapure water). 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. To verify amplification of each target cDNA, a melting curve analysis was included at the end of each PCR run. The generated data were analysed by SDS 2.2 software (Applied Biosystems). Target gene expression data from real-time RT-PCR were plotted as $2^{(40-CT)}/10$, as described in Czechowski *et al.* (2004), and normalized against the reference *GADPH* gene.

Table 1. Characteristics of SSH-generated ESTs from mycorrhizal, *X. index*-challenged grapevine roots

EST	Predicted function (NCBI accession no.)	e-value (blastn/ NCBI)	Forward primer	Reverse primer	Annealing temperature (°C)	Amplicon size (bp)
48	*Vitis vinifera* misc_RNA (XR_077542)	e^{-133}	GCTCTTCCAATCATTGTGAAGC	TTGCGACACTGTCATCAGTAGG	53	128
82	Calcium-binding protein CML27 (XM_002282039)	e^{-19}	AGGCGCATGGATTCTTGG	TTTCCAACGCACACGAGC	53	194
91	*Vitis* hybrid cultivar pathogenesis-related protein 10 (FQ_388330)	$5e^{-75}$	CGCTGAGGTCTGTGAAGAGC	GGTCCACACTTTGACTGATGC	59	184
104	*Vitis vinifera* 5-enolpyruvyl shikimate-3-phosphate synthase (XM_002280886)	$4e^{-88}$	AAGACTCGCCACTGTGACAACC	AATGAGCCCTGGCCAGCA	58	106
120	*Vitis vinifera* unknown protein (XM_002271856)	$3e^{-80}$	CCGTGCAGCTATTCAAACG	CAATGTTTAATCACGACTGG	56	143
129	*Vitis labrusca* Hsp70-interacting protein 1 (EU404167)	0.0	CTTTGACGCCAAGTGGAGATCC	AAGCCCAATGCCGCTATCC	58	171

Table 2. Defence-related marker gene characteristics for targeted expression analysis.

Gene	Accession no.	Forward primer	Reverse primer	Annealing temperature (°C)	Amplicon size (bp)
CHI	Z54234	CCCAAGCCTTCCTGCCATA	TGTGATAACACCAAAACCGGG	60	96
GST	AY156048	TGCATGGAGGAGGAGTTCGT	CAAGGCTATATCCCCATTTTCTTC	60	98
LOX	AY159556	CCCTTCTTGGCATCTCCCTTA	TGTTGTGTCCAGGGTCCATTC	56	101
PAL	X75967	TCCTCCCGGAAAACAGCTG	TCCTCCAAATGCCTCAAATCA	56	101
PIN	AY156047	AGTTCAGGGAGAGGTTGCTG	CACCAACCCAATGAGTCTATCC	59	185
STS	X76892	AGGAAGCAGCATTGAAGGCTC	TGCACCAGGCATTTCTACACC	55	101
HERO	XM_002265532.1	CGGAAGAAAATGGGATGGAAGAG	ATGAGCAATAAGTCGGCGAGGG	59	188
HS	XM_002268530.1	GCTGTACCGCGAAAAGGTAG	AGATTTGAGACAACGAGTCC	57	188

CHI, chitinase 1b; *GST*, glutathione-S-transferase; *LOX*, lipoxygenase; *PAL*, phenylalanine ammonia lyase; *PIN*, pathogenesis-related proteinase inhihibitor PR6; *STS*, stilbene synthase; *HERO* and *HS*, tomato nematode defence-related genes.

Table 3. Effect of mycorrhiza on grapevine growth and induced systemic or localized root protection against X. index 35 d after inoculation in a split-root experiment based on four treatments (*Fig. 1):* C/N, root system halves non-inoculated (C) or *X. index* challenged (N); M/N, root system halves mycorrhizal (M) or *X. index* challenged (N); C/M+N, root system halves non-inoculated (C) or mycorrhizal, *X. index* challenged (M+N); M/M+N, root system halves mycorrhizal (M) or mycorrhizal, *X. index* challenged (M+N).

Plant treatment	Shoot fresh weight (g)	Root compartment treatment	Mycorrhizal colonization (%)	Root system half fresh weight (g)	Gall no./ plant	Nematode no./pot
C/N	3.008 b	C	0 b	1.755 a	0 d	0 c
		N	0 b	1.580 a	18.6 a	93.3 a
M/N	5.164 a	M	62.9 a	2.124 a	0 d	0 c
		N	0 b	1.884 a	13.3 b	52.5 b
C/M+N	4.868 a	C	0 b	1.539 a	0 d	0 c
		M+N	65.2 a	1.603 a	9.0 b,c	49.5 b
M/M+N	5.729 a	M	72.5 a	1.845 a	0 d	0 c
		M+N	63.0 a	1.854 a	6.7 c	46.6 b

Different letters indicate significant differences (P <0.05, n=4) between treatments after one-way ANOVA and LSD test.

Statistical analyses

All data were analysed by analysis of variance (ANOVA) using the SPSS 11.0 package. Significant differences between means were established by calculation of the least significant difference (LSD) at the 5% level. Prior to analyses, mycorrhizal colonization data were arcsin transformed.

Results

Root colonization by G. intraradices *and plant growth*

No AM fungal colonization was observed in non-inoculated roots of grapevine plants. In all the experiments, mycorrhizal colonization was ~50–60% of the root systems 21 d after inoculation with *G. intraradices*, before inoculation with *X. index*. Mycorrhiza levels remained high, characterized by abundant arbuscules and vesicles, up to 35 d later, and the presence of *X. index* did not significantly affect *G. intraradices* development within plant roots in any treatment (see Table 3). No growth differences in fresh mass were observed between mycorrhizal and non-mycorrhizal grapevine plants up to 21 d after planting, before nematode inoculation, but AM fungal colonization significantly increased shoot mass thereafter, and this mycorrhizal growth effect was unaffected by inoculation with *X. index* (Fig. 2A, Table 3). Root fresh mass showed a similar trend (Fig. 2B).

Xiphinema index *development in roots and soil*

Xiphinema index was not found in soil or roots of treatments not inoculated with the nematode. An initial slight decrease in nematode numbers in soil was observed in all treatments at 7 d after inoculation. This was followed by an increase with time, especially in the non-mycorrhizal treatments, due to the formation of a new generation of larvae from eggs laid by the inoculated adults (Fig. 2C). Nematode proliferation was attenuated around roots of mycorrhizal plants so that at 35 d after *X. index* inoculation, nematode populations were at a significantly lower level ($P \leqslant 0.05$) than in soil from around non-mycorrhizal grapevine roots (Fig. 2C). Gall induction on roots by *X. index* followed a similar trend, with a pronounced bioprotective effect of mycorrhization. During early stages, up to 14 d after nematode inoculation, no galls were found on roots of either non-mycorrhizal or mycorrhizal grapevine plants (Fig. 2D). At 21 d and 35 d, the presence of *G. intraradices* in root systems significantly reduced nematode gall formation ($P \leqslant 0.05$) (Fig. 2D). The supply of P to non-mycorrhizal plants inoculated with *X. index* had no effect on gall formation or nematode development (data not shown).

Co-inoculation of *G. intraradices* and *X. index* at transplanting of the rooted grapevines had no effect on nematode propagation in soil up to 21 d later as compared with plants

inoculated with the nematode alone (results not shown), but nematode numbers in soil and gall induction were reduced after 35 d in the presence of *G. intraradices* (Fig. 3A, B), although this bioprotective effect was less than when the nematode was post-inoculated onto roots of mycorrhizal plants.

Systemic and local induced bioprotection against X. index *in mycorrhizal root systems*

Time course monitoring of plant growth in the split root experimental systems confirmed the growth promotion by *G. intraradices* in *X. index*-challenged plants (Fig. 4A, B). Mycorrhizal development in one half of the root system significantly decreased *X. index* proliferation associated with the non-mycorrhizal half. This systemic effect of mycorrhization impacted both on nematode numbers in the soil and on gall formation on roots, which decreased between 21 d and 35 d after nematode inoculation (M+N versus C+N, Fig. 4C, D), as in entire root systems (Fig. 2). When systemic and local effects of mycorrhiza on nematode development were compared, an induced bioprotection against *X. index* was also observed locally in mycorrhizal root parts where gall and nematode numbers were significantly lower than in non-mycorrhizal halves of root systems (C/N versus C/M+N, Table 3). Maximum reduction in nematode development occurred with combined systemic and local mycorrhizal effects when the whole root system was colonized by *G. intraradices* (M/M+N, Table 3).

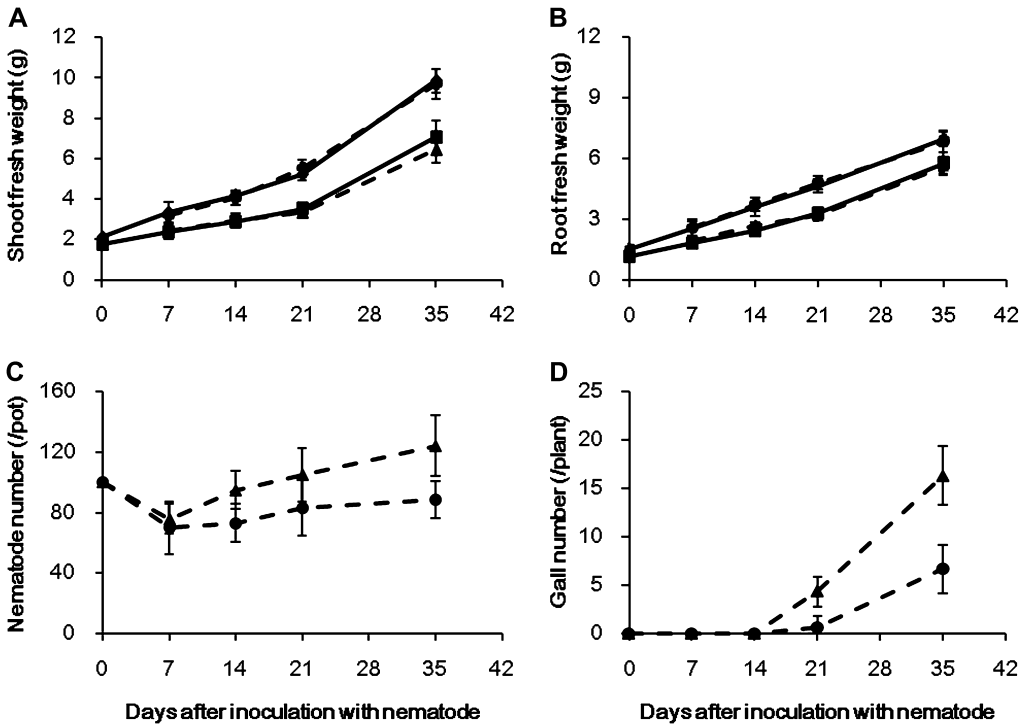

Fig. 2. Time course monitoring of shoot (A) and root (B) fresh weight, soil nematode number per pot (C), and gall number per plant (D) of control (filled squares), mycorrhizal (filled diamonds), nematode-challenged non-mycorrhizal (filled triangles), and mycorrhizal (filled circles) grapevine plants. Bars indicate the standard errors (n=4).

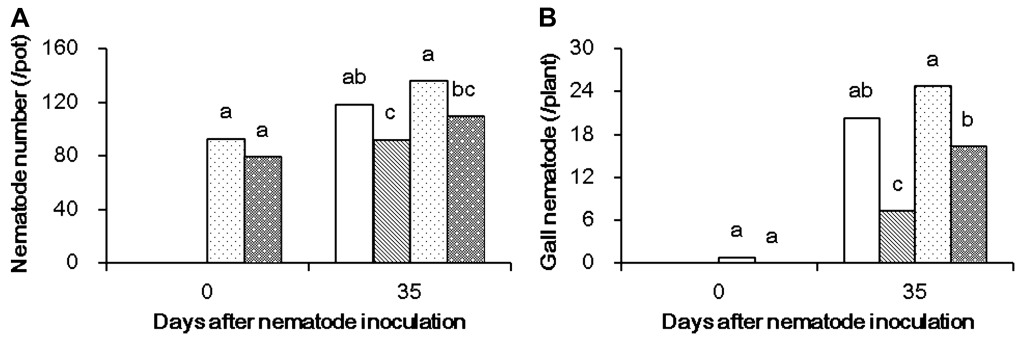

Fig. 3. Soil nematode number per pot (A) and gall number per plant (B) of grapevine plants at *X. index* inoculation (0) and 35 d later. Plants were either inoculated with *X. index* immediately (white dotted bars) or co-inoculated with *G. intraradices* and *X. index* (grey dotted bars) at transplanting (time 0), or post-inoculated with the nematode after 21 d growth (open bars, non-mycorrhizal; diagonally striped bars, mycorrhizal). Different letters indicate significant differences ($P < 0.05$, n=4) between treatments after one-way ANOVA and LSD test.

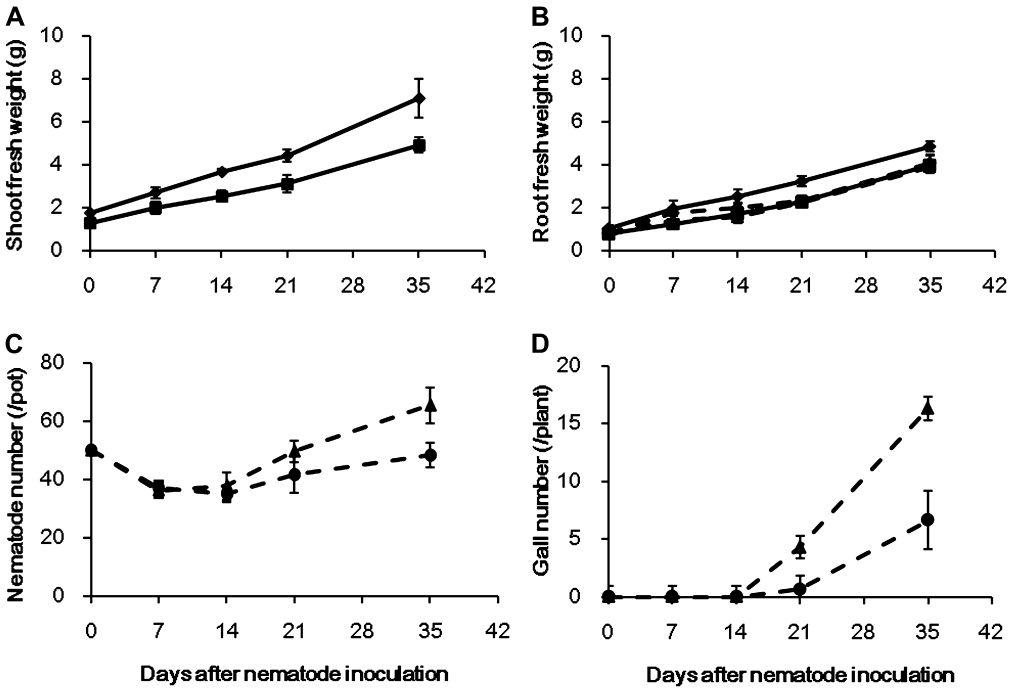

Fig. 4. Time course monitoring of shoot (A) and root (B) fresh weight, soil nematode number per pot (C), and gall number per plant (D) in a split-root experiment. The experimental design is given in Fig. 1. Shoot data correspond to *X. index*-challenged mycorrhizal (filled diamonds, M+N) and non mycorrhizal (filled squares, C+N) plants. Root and nematode data are given for root system halves: filled squares, non-inoculated control (C); filled triangles, *X. index*-challenged half of C+N plants; filled diamonds, mycorrhizal treatment (M); filled circles, *X. index*-challenged half of M+N plants. Bars indicate the standard errors (n=4).

Time course monitoring of plant gene responses associated with mycorrhiza-induced bioprotection against X. index *in whole root systems*

Screening of the SSH library for plant genes differentially expressed during bioprotection in nematode-challenged mycorrhizal grapevine roots gave 132/182 clones (72.2%) which clearly showed stronger signals with M+N extracts compared with other treatments (results not shown). Fourteen clones with 2-fold higher signals in the M+N treatment were selected and sequenced. Clustering resulted in six singletons representing *Vitis* genes (Table 1), of which three had significant sequence similarity to stress response genes: pathogenesis-related 10 (*PR10*, EST91), 5-enolpyruvyl shikimate-3-phosphate synthase (*ESPS*, EST104), and a heat shock protein 70 (Hsp70)-interacting protein (*HIP*, EST129). Sequences have been registered in the NCBI database (GenBank accession nos JK694186– JK694191). For all six genes, the level of expression was similar in roots from non-mycorrhizal, mycorrhizal, or nematode-infested non-mycorrhizal grapevine plants across the different time points (Fig. 5). However, except for one (calcium-binding protein CML27, EST82), consistently higher expression levels were confirmed for all the genes in nematode-challenged mycorrhizal roots which showed decreased *X. index* development and root attack. Expression of the genes encoding PR10 (EST91), ESPS (EST104), and HIP (EST129) sharply increased in mycorrhizal roots from 7 d to 14 d and 21 d in the presence of the nematode, and before bioprotection became evident (Fig. 2), then decreased after 21 d for ESPS and HIP but remained high for PR10 (Fig. 5). The unknown protein EST gene (EST120) showed increased expression at 21 d and 35 d in nematode-challenged mycorrhizal grapevine roots as compared with the other treatments. The expression of AM-induced bioprotection was accompanied by activation of the miscellaneous RNA-encoding gene (EST48) up to 35 d.

The defence-related marker genes showed a different trend in expression compared with the SSH-generated plant genes (Fig. 6). The *LOX*, *PAL*, *PIN*, *HERO*, and *HS* genes were clearly activated in response to the nematode itself from 7 d to 35 d after inoculation. This expression profile was unaffected by the presence of *G. intraradices* in mycorrhizal roots for *PAL*, *HERO*, and *HS*. In contrast, *PIN* was down-regulated in all mycorrhizal roots whether these were challenged or not by *X. index*, whereas LOX expression increased later in nematode-challenged mycorrhizal roots above that of control and mycorrhizal roots at 35 d. The *GST* gene also showed decreased expression in mycorrhizal root systems at 7 d and 14 d, then increased at 35 d, as compared with control or nematode-infested roots. The expression level of *CHI* remained lower in control roots as compared with other treatments from 7 d to 21 d, but showed an early peak of induction at 14 d after nematode inoculation in mycorrhizal roots (Fig. 6). The *STS* gene was the only defence-related marker gene to show consistently higher expression levels at 21 d and 35 d, as compared with other treatments, in nematode-challenged mycorrhizal roots which showed decreased *X. index* development and root

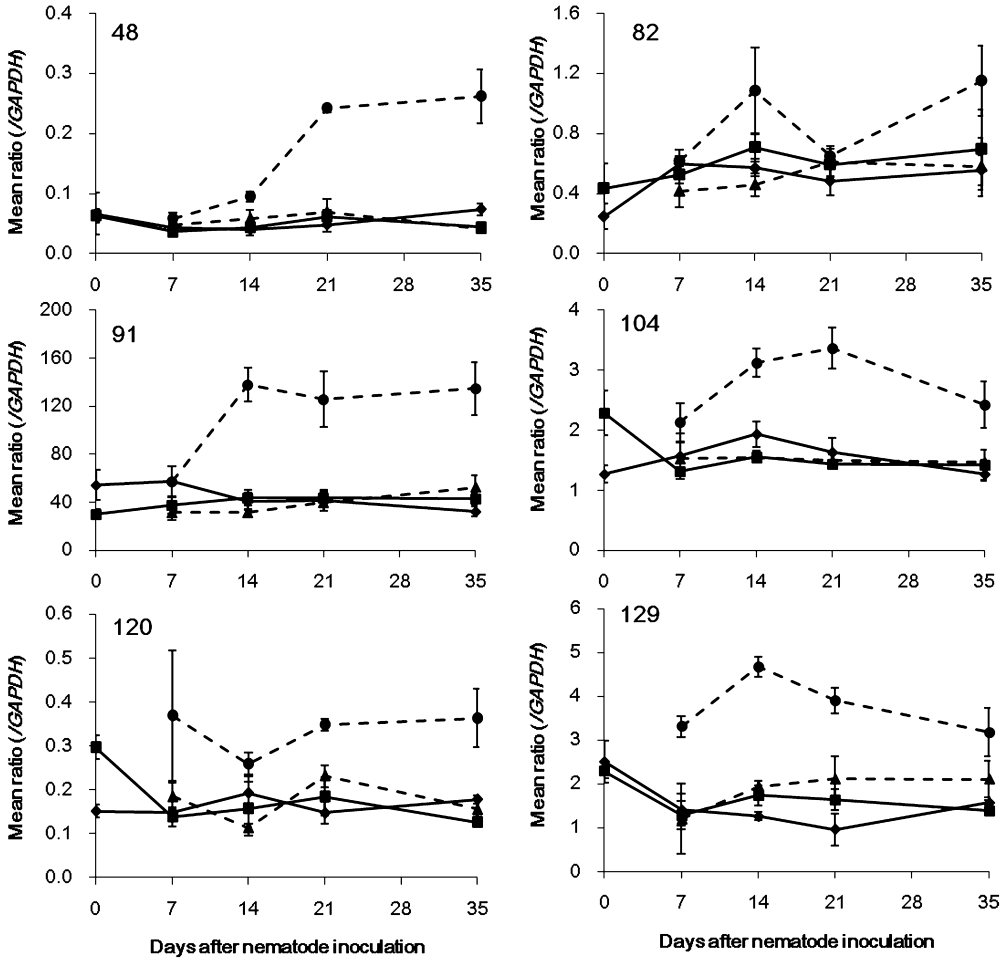

Fig. 5. Time course monitoring of gene expression corresponding to selected SSH-generated ESTs in control (filled squares), mycorrhizal (filled diamonds), nematode-infested (filled triangles), and nematode-challenged mycorrhizal (filled circles) grapevine roots. 48, miscellaneous RNA; 82, calcium-binding protein; 91, pathogenesis-related protein PR10; 104, 5-enolpyruvyl shikimate-3-phosphate synthase; 120, unknown protein; 129, Hsp70-interacting protein. Mean values are presented as the ratio to GAPDH gene expression used as a reference. Bars indicate the standard errors (n=3).

attack. This gene was activated by the presence of the nematode alone but at much lower levels.

Plant gene responses during systemic or local mycorrhiza-induced bioprotection against X. index

In the split-root experiment, inoculation with *X. index* alone did not induce expression of any of the SSH-selected genes in non-mycorrhizal halves of grapevine root systems (Fig. 7). Again, enhanced expression of the miscellaneous RNA-encoding gene (EST48) was consistently associated with mycorrhiza-induced bioprotection against the nematode whether this was systemic (M/N), local (C/M+N), or the two combined (M/M+N). A similar expression profile was observed for the genes encoding ESPS (EST104) and HIP (EST129), indicating that these two genes are also related to both systemic and local mycorrhiza-induced bioprotection against *X. index*. In contrast, increased expression of the calcium-binding protein gene (EST82) only occurred in the combined bioprotection treatment where both halves of the root system were colonized by *G. intraradices*; a relatively higher mycorrhizal colonization may be needed to achieve induction of this gene. The gene encoding PR10 (EST91) appeared to be uniquely linked to systemic mycorrhiza-induced bioprotection against *X. index*, as it was induced only in the nematode-inoculated half of the M/N treatment. No significant difference was detected between treatments in expression of the unknown protein gene (EST120) in this analysis.

Variations in defence-related marker gene expression monitored at 35 d after inoculation of *X. index* in the split root system experiment are presented in Fig. 8. The *HS* gene showed no clear profile in relation to nematode–mycorrhiza interactions at 35 d, and expression of the *LOX* and *PAL* genes did not differ between treatments, except for being higher in the root system half of the C/N treatment inoculated with *X. index* alone as compared with the mycorrhizal half of M/N plants. As observed in the time course experiment, the *PIN* gene was down-regulated in all mycorrhizal roots whether these were challenged or not by *X. index*, whilst the *HERO* gene was significantly activated by nematode attack alone (C/N) and to a similar extent as in the nematode-inoculated half of mycorrhizal root systems

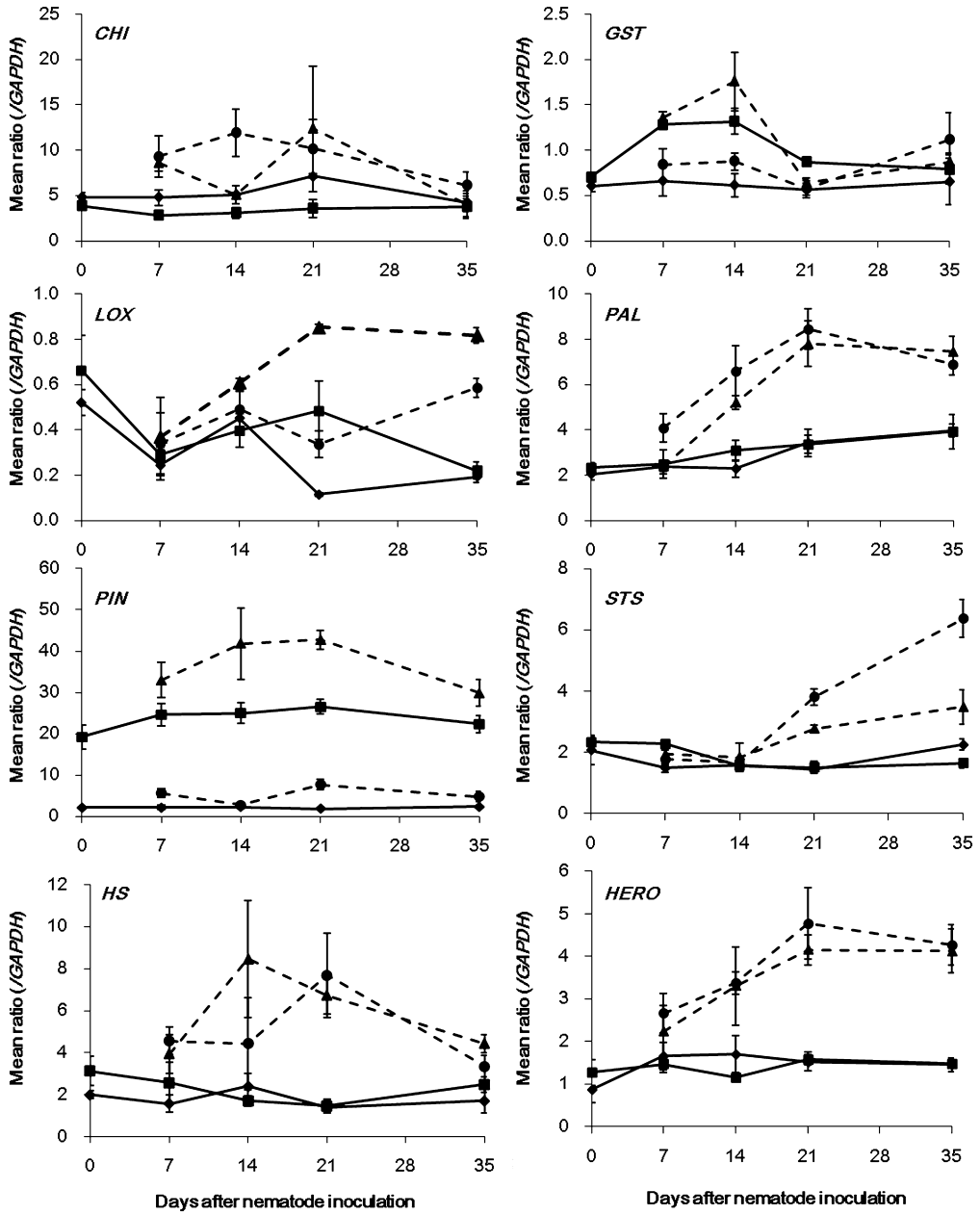

Fig. 6. Time course monitoring of defence-related marker gene expression in control (filled squares), mycorrhizal (filled diamonds), nematode-infested (filled triangles), and nematode-challenged mycorrhizal (filled circles) grapevine roots. *CHI*, chitinase 1b; *GST*, glutathione *S*-transferase; *LOX*, lipoxygenase; *PAL*, phenylalanine ammonia lyase; *PIN*, pathogenesis-related protein PR6; *STS*, stilbene synthase; and *HS* and *HERO*, tomato nematode defence-related genes. Mean values are presented as the ratio to GAPDH gene expression used as a reference. Bars indicate the standard errors (n=3).

in the M/N, C/M+N, or M/M+N treatments. Enhanced expression of the *CHI* gene was associated with systemic (M/N) and local (C/M+N) mycorrhiza-induced bioprotection against *X. index* development, whilst *GST* and *STS* were clearly more active in treatments corresponding to a local effect of *G. intraradices* in controlling *X. index* development (N/M+N and M/M+N).

Discussion

Data from the present experiments show that root colonization by the AM fungus *G. intraradices* BEG141 can reduce development of the ectoparasitic nematode *X. index* associated with grapevine rootstock SO4 (*V. berlandieri*× *V. riparia*), and that this bioprotective effect is not linked to an improved P status of the plants. The fact that a negative effect of the nematode on plant growth was not observe may be linked to the short duration of the experiments. The grapevine rootstock SO4 is susceptible to *X. index* infestation (McKenry and Anwar, 2006) and reduced plant growth due to root attack by the nematode has been observed after longer periods (Xu *et al.*, 2008). The split-root experimental system provides a demonstration that mycorrhiza-induced bioprotection against *X. index* acts

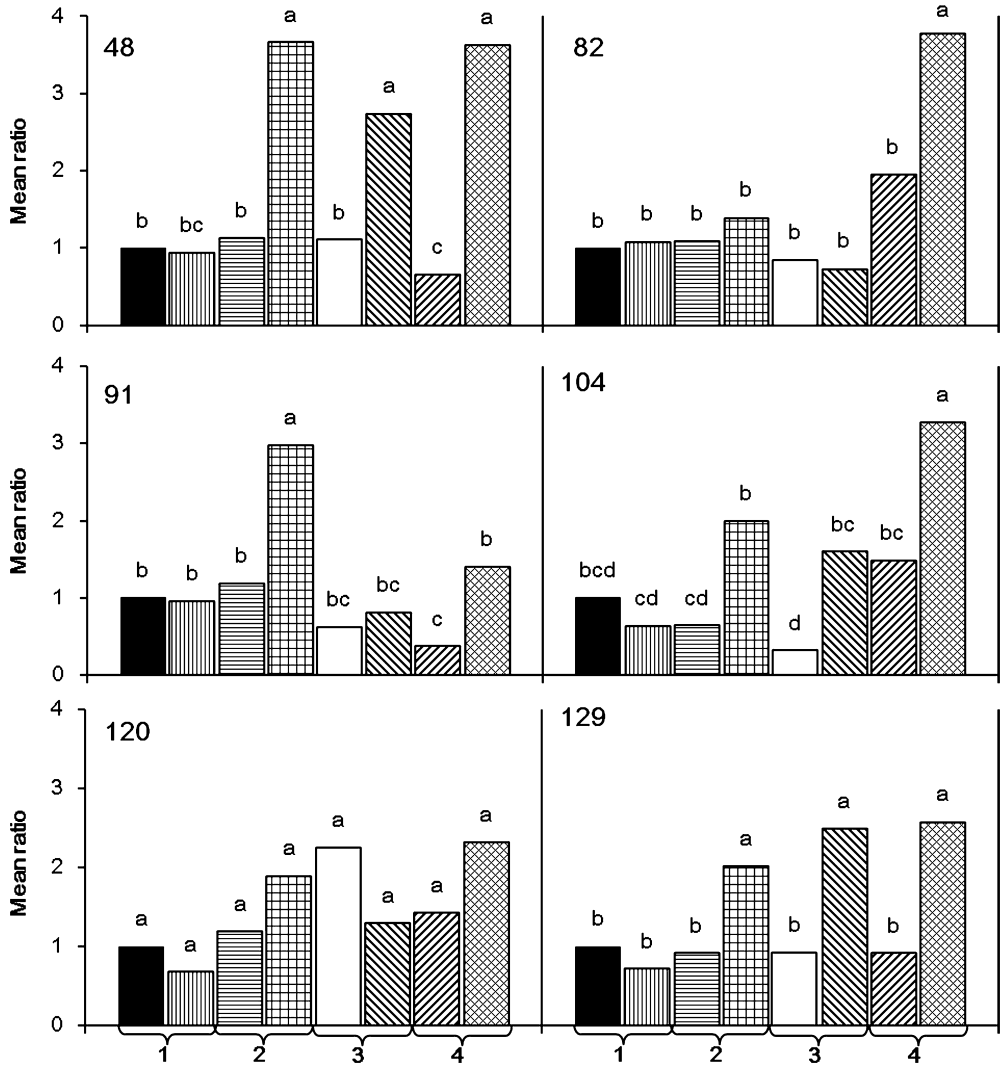

Fig. 7. Monitoring of gene expression corresponding to selected SSH-generated ESTs in grapevine roots showing systemic or local bioprotection against *X. index* induced by *G. intraradices* 35 d after inoculation of the nematode. The experimental design is given in Fig. 1. Data, expressed as a ratio of control treatments (C), are given for root system halves: (1) non-inoculated (filled bars) and *X. index*-challenged (vertically striped bars) in C/N plants; (2) mycorrhizal (horizontally striped bars) and *X. index*-challenged (cross-hatched bars) in M/N plants; (3) non-inoculated (open bars) and mycorrhizal, *X. index*-challenged (rightward sloping diagonal striped bars) in C/M+N plants; (4) mycorrhizal (leftward sloping diagonal striped bars) and mycorrhizal, *X. index*-challenged (diamond-filled bars) in M/M+N plants. 48, miscellaneous RNA; 82, calcium-binding protein; 91, pathogenesis-related protein PR10; 104, 5-enolpyruvyl shikimate-3-phosphate synthase; 120, unknown protein; 129, Hsp70-interacting protein. Different letters indicate significant differences ($P \leqslant 0.05$, $n=3$) between treatments after one-way ANOVA and LSD test.

through both local and systemic mechanisms. Systemic bioprotection by mycorrhiza against an ectoparasitic nematode complements similar observations on pathogenic bacteria (Zhu and Yao, 2004), fungal pathogens (Rosendahl, 1985; Cordier *et al.*, 1998; Pozo *et al.*, 2002; Khaosaad *et al.*, 2007), and an endoparasitic nematode (Elsen *et al.*, 2008). The decrease in pathogen development in non-mycorrhizal parts of mycorrhizal root systems points to a plant-mediated mechanism of bioprotection which must involve an induced systemic factor.

It has been clearly shown that root systems have to be well colonized by a mycorrhizal fungus before pathogen attack for AM-induced bioprotection to be effective (cf. Cordier *et al.*, 1998; Dumas-Gaudot *et al.*, 2000; Slezack *et al.*, 2000; Elsen *et al.*, 2008). The present observations suggest not only that bioprotection is conferred against *X. index* if roots are colonized by *G. intraradices* prior to contact with the nematode but also that co-inoculation of the AM fungus at the same time as the nematode can also reduce proliferation of the pathogen. Due to the complexity of the AM fungi–grapevine–nematode interactions, more fungal and rootstock species should be tested and the mechanisms need to be further elucidated. Furthermore, because nurse planting before transplanting can be a common practice in grapevine production, inoculation of appropriate AM fungi prior to transplanting could have practical implications for the control of root infestation by *X. index* in the field.

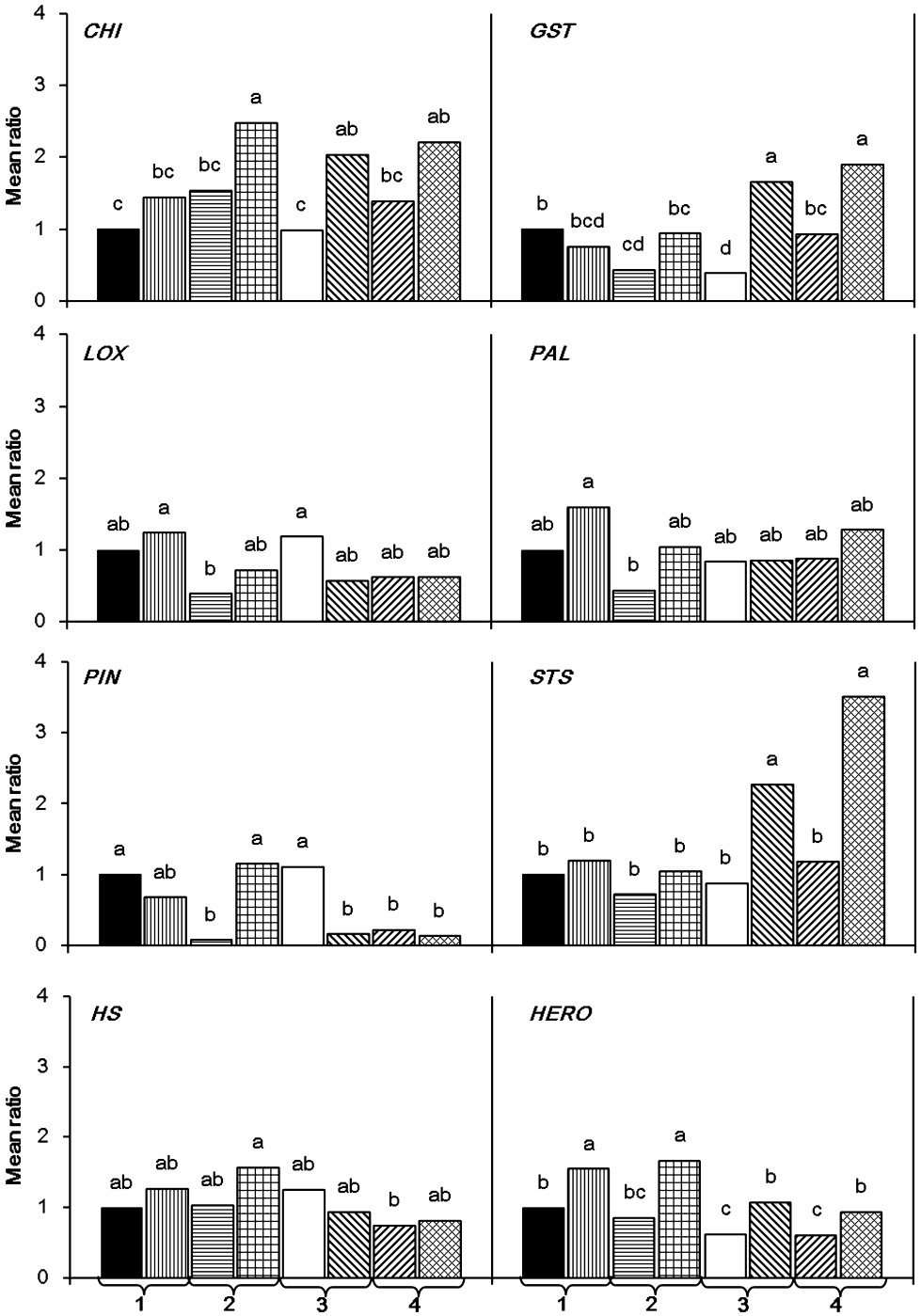

Fig. 8. Monitoring of defence-related marker gene expression in grapevine roots showing systemic or local bioprotection against *X. index* induced by *G. intraradices* 35 d after inoculation of the nematode. The experimental design is given in Fig. 1. Data, expressed as a ratio of control treatments (C), are given for root system halves: (1) non-inoculated (filled squares) or *X. index*-challenged (Mean ratio striped bars) in C/N plants; (2) mycorrhizal (horizontally striped bars) or *X. index*-challenged (cross-hatched bars) in M/N plants; (3) non-inoculated (open bars) or mycorrhizal, *X. index*-challenged (rightward sloping diagonal striped bars) in C/M+N plants; (4) mycorrhizal (leftward sloping diagonal striped bars) or mycorrhizal, *X. index*-challenged (diamond-filled bars) in M/M+N plants. *CHI*, chitinase 1b; *GST*, glutathione *S*-transferase; *LOX*, lipoxygenase; *PAL*, phenylalanine ammonia lyase; *PIN*, pathogenesis-related protein PR6; *STS*, stilbene synthase; and *HS* and *HERO*, tomato nematode defence-related genes. Different letters indicate significant differences ($P \leqslant 0.05$, $n=3$) between treatments after one-way ANOVA and LSD test.

Knowledge about the mechanisms of mycorrhiza-induced bioprotection against biotic stress and the processes involved still remains fragmentary, especially as far as mycorrhiza–nematode interactions are concerned. Basal plant defence processes that are weakly triggered by AM fungi are thought to predispose root tissues to an efficient activation of defence

mechanisms when mycorrhizal roots are challenged by a pathogen, in a way similar to priming (Gianinazzi, 1991; Dumas-Gaudot *et al.*, 2000; Pozo *et al.*, 2009). Localized and systemic mycorrhiza-induced bioprotection against fungal pathogens, demonstrated using split-root systems like that described here, is associated with callose synthesis and a higher accumulation of PR-1a, basic β-1, 3-glucanases, phenolic compounds, and derivatives than in non-mycorrhizal root systems (Cordier *et al.*, 1998; Pozo *et al.*, 2002; Yao *et al.*, 2003). Molecular studies of root tissue responses related to AM bioprotective effects against nematode attack are, in contrast, scarce.

Of the 14 *Vitis* genes studied here to identify transcriptional modifications in *X. index*–mycorrhiza interactions, seven (*CHI*, *GST*, *STS*, *PR10*, *ESPS*, *HIP*, and miscellaneous RNA) were consistently up-regulated during AM-induced bioprotection against *X. index*. Expression of the *CHI* gene encoding chitinase 1b clearly increased in root halves showing either systemic (M/N) or local (C/M+N) bioprotection by *G. intraradices* against *X. index*, but no significant response was observed to the mycorrhizal fungus alone. These observations suggest that the AM fungus may prime activation of the plant chitinase gene which then responds during bioprotection of the root tissues against *X. index*. In addition, coincidental enhanced *CHI* expression and systemically induced bioprotection indicate that the chitinase 1b gene responds to the nematode at a distance through a signal transmitted from the mycorrhizal tissues. Chitinases are amongst the most widely studied defence-related plant proteins, and there are many reports of their differential expression in mycorrhizal roots (Dumas-Gaudot *et al.*, 2000). Li *et al.* (2006) have described transcriptional activation of a class III chitinase gene in mycorrhizal grapevine roots which is further enhanced during a defence response against the root-knot nematode *Meloidogyne incognita*. Chitin is a component of nematode eggshells and it has been located in the nematode feeding apparatus (Veronico *et al.*, 2001). The local and systemic activation of chitinase 1b in *G. intraradices*-colonized grapevine root systems could decrease *X. index* vitality by interfering with both feeding and reproduction, and so contribute to the observed decreases in gall formation and nematode numbers associated with the mycorrhizal plants.

Enhanced transcriptional activity of the two defence-related genes *GST* and *STS* was detected only in mycorrhizal grapevine root system halves showing local (C/M+N) bioprotection by *G. intraradices* against *X. index*. There are several reports of AM fungi inducing *GST* transcription in roots of other plants, and more precisely in arbuscule-containing cells (cf. Strittmatter *et al.*, 1996; Wulf *et al.*, 2003; Brechenmacher *et al.*, 2004; Kutnetsova *et al.*, 2010). GST isoforms are involved in the detoxification of reactive oxygen species that can cause damage to living cells, and the presence of a higher GST expression in *X. index*-challenged mycorrhizal roots may reflect such a protective effect versus the stress imposed by cell hypertrophy and necrosis induced by the nematode. In this context, the gene has been associated with the protective effect conferred by AM symbiosis to *Medicago truncatula* grown in the presence of a cadmium stress (Aloui *et al.*, 2009). STS1 is a key enzyme in the phenyl propanoid pathway where it condenses malonyl-CoA molecules to produce the phytoalexin resveratrol, the accumulation of which is a typical defence response by grapevine to biotic or abiotic stresses (Langcake and Pryce, 1977; Adrian *et al.*, 1997; Coutos-Thévenot *et al.*, 2001). The lack of or very low induction of the *STS* gene by *G. intraradices* alone and its high local response in mycorrhizal roots to *X. index* is again suggestive of a priming phenomenon in grapevine tissues by the mycorrhizal fungus. Although resveratrol does not affect life fitness of the free-living nematode *Caenorhabditis elegans* (Gruber *et al.*, 2007), it inhibits growth of grapevine fungal pathogens (Coutos-Thévenot *et al.*, 2001) and its effect on dagger nematode vitality and root feeding needs to be investigated.

PR10 proteins belong to a large family which is widely distributed in higher plants (van Loon and van Strein, 1999; Liu and Ekramoddoullah, 2006), and several isoforms are active in vine roots (Lebel *et al.*, 2010). Genes encoding members of this group are expressed in early and late stages of root interactions with AM fungi (Ruiz-Lozano *et al.*, 1999; Brechenmacher *et al.*, 2004; Siciliano *et al.*, 2007). Activation is also associated with plant response to plant pathogens (Liu and Ekramoddoullah, 2006), but accumulation of PR10 protein did not accompany control of *Aphanomyces euteiches* infection in mycorrhizal roots (Colditz *et al.*, 2005). In contrast, a *PR10* isoform (EST91) from grapevine was induced early and prior to the observed bioprotective effects in mycorrhizal–*X. index* interactions, but not in roots colonized only by *G. intraradices* or the nematode. The split-root experiment clearly showed activation of this gene only during systemically induced bioprotection (C/N), indicating again, as for chitinase 1b, transmission of a plant-mediated signal from *G. intraradices*-colonized tissues to prime responses to the nematode at a distance. PR10 proteins have roles in biotic or abiotic stress responses through functions including antimicrobial activity and RNase activity (van Loon and van Strein, 1999; Liu and Ekramoddoullah, 2006). The action of the *Vitis PR10* gene in mycorrhiza-induced protection against a nematode is not known but, in this context, a PR10 protein has recently been purified from *Crotalaria pallida* which has nematostatic and nematicide effects against the root-knot nematode *M. incognita* through an action against the parasite's digestive proteinase (Andrade *et al.*, 2010).

The *ESPS* (EST104) and *HIP* (EST129) genes showed similar expression profiles in that up-regulation was clearly associated with both systemically and locally AM-induced bioprotection against *X. index* in grapevine roots. No gene response was observed in roots colonized only by *G. intraradices*, or the nematode, again pointing to the existence of a priming phenomenon in mycorrhizal tissues with transmission of a signal to non-mycorrhizal roots. The enzyme ESPS is involved in the shikimate pathway which produces the majority of plant aromatic compounds including the amino acids tyrosine, phenylalanine, and tryptophan, precursors for aromatic secondary metabolites

and some plant hormones (Tzin and Galili, 2010). Although ESPS is better known as the target of the herbicide glyphosate (Steinrücken and Amrhein, 1980), enhanced expression of the gene has been reported in *Arabidopsis thaliana* during defence responses elicited by oligosaccharide treatment to prime resistance against the fungal pathogen *Botrytis* cinerea (Ferrari *et al.*, 2007).

The grapevine *HIP* up-regulated during AM-induced bioprotection against *X. index* shows some similarity to the gene encoding a Hip-like protein, consisting of a Hip–thioredoxin chimera, reported from A. thaliana and *Vitis labrusca* (Webb *et al.*, 2001). Hip is one of several co-chaperones that regulate activities of the Hsp70 chaperone family in animals (Irmer and Höhfeld, 1997; Smith, 2000), but very little is known about their function in plants. Members of the plant Hsp70 family are implicated in protein folding (Marshall and Keegstra, 1992), bind to denatured proteins, and help re-establish their native configuration and reintegration into the membrane complex (Neumann *et al.*, 1994). Under stress, Hsp70 can prevent protein degradation (Hottiger *et al.*, 1992), and it has been suggested that activation of a *Hsp70* gene in mycorrhizal roots subjected to heavy metal stress (Cd) may be implicated in maintaining protein membrane integrity in arbuscule-containing cells and so contributing to symbiotic functioning and greater tolerance of AM plants in polluted soils (Rivera-Becerril *et al.*, 2005). Whether Hip regulation in mycorrhizal grapevine roots of Hsp70 during the biotic stress imposed by *X. index* is related to conservation of protein integrity in the presence of the pathogen or eventually in a signalling process in the mycorrhizal tissues needs to be investigated.

The expression profile of the miscellaneous RNA-encoding gene (EST48), which could reflect a general enhanced activity of root tissues, coincided with development of mycorrhiza-induced bioprotection against *X. index* in whole root systems and in tissues showing a systemic or local effect, whilst that of the unknown protein (EST120) showed enhancement at later stages which could not be clearly related to systemic or local bioprotection. Information is currently not available concerning the function of these genes. The gene encoding a calcium-binding protein (EST82) also did not present a clear profile, although expression appeared to be significantly enhanced at 35 d in whole mycorrhizal root systems challenged with the nematode. Calcium-binding protein can be a receptor in Ca^{2+} signalling within cells, which by conformational change and activity identifies and transfers specific Ca^{2+} signals downstream, causing a series of changes in cell morphology, gene expression, and regulation (Knight *et al.*, 1997).

In conclusion, local and systemic processes are active in the mycorrhiza-induced bioprotection of grapevine roots against the ectoparasitic nematode *X. index.* Decreased gall formation on mycorrhizal roots and reduced nematode reproduction in the surrounding soil suggest that the bioprotective effects target *X. index* feeding sites and/or nematode vitality. Causal mechanisms are not due to improved plant phosphate nutrition; plant gene expression analyses indicate that they are related rather to direct effects on the nematode or through protection against nematode-imposed stress to maintain root tissue integrity. The up-regulation of defence-related *Vitis* genes uniquely in *X. index*-challenged mycorrhizal roots expressing bioprotection, and not in roots inoculated with the mycorrhizal fungus alone, points to induction of a primed state by the AM fungus *G. intraradices*. Furthermore, stronger activation of some genes only during systemically induced bioprotection suggests the implication of a plant-mediated signal from *G. intraradices*-colonized tissues to prime responses to nematode attack at a distance.

Acknowledgements

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