***Armillaria mellea* Induces a Set of Defense Genes in Grapevine Roots and One of Them Codifies a Protein with Antifungal Activity**

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Grapevine root rot, caused by *Armillaria mellea*, is a serious disease in some grape-growing regions. Young grapevines start to show symptoms of *Armillaria* root rot from the second year after inoculation, suggesting a certain degree of resistance in young roots. We used a suppression subtractive hybridization approach to study grapevine's reactions to the first stages of *A. mellea* infection. We identified 24 genes that were upregulated in the roots of the rootstock Kober 5BB 24 h after *A. mellea* challenge. Real-time reverse-transcriptase polymerase chain reaction analysis confirmed the induction of genes encoding protease inhibitors, thaumatins, glutathione S-transferase, and aminocyclopropane carboxylate oxidase, as well as phase-change related, tumor-related, and proline-rich proteins, and gene markers of the ethylene and jasmonate signaling pathway. Gene modulation was generally stronger in Kober 5BB than in Pinot Noir plants, and in vitro inoculation induced higher modulation than in greenhouse *Armillaria* spp. treatments. The full-length coding sequences of seven of these genes were obtained and expressed as recombinant proteins. The grapevine homologue of the *Quercus* spp. phase-change-related protein inhibited the growth of *A. mellea* mycelia in vitro, suggesting that this protein may play an important role in the defense response against *A. mellea*.

Armillaria mellea (Vahl) P. Kumm. is the causal agent of root rot in several perennial woody forest plants (Fox 2000) and crops (Cox and Scherm 2006), including grapevine (Baumgartner 2004). Grapevine root rot is an emerging disease in some important grape-growing regions, such as California (Baumgartner and Rizzo 2002), Spain (Aguín-Casal et al. 2004), and Italy (Pertot et al. 2008). *A. mellea* is a saproparasitic basidiomycete that can survive for a long time on dead wood and root debris in the soil in the absence of any living host (Baumgartner and Rizzo 2006). For this reason, *Armillaria*

root disease is often observed in vineyards planted on land previously occupied by forest trees (Baumgartner and Rizzo 2002) or infected vineyards (Pertot et al. 2008).

A. mellea rhizomorphs (organized mycelial structures) penetrate healthy grapevine roots by mechanical and enzymatic means (Robinson et al. 2000). Once it has penetrated a root, *A. mellea* forms a mycelial fan underneath the bark and decomposes the underlying cambium, causing the decay of the secondary xylem (Baumgartner 2004). Infected grapevines show a decline in vigor, stunted shoots, dwarfed leaves, premature change of leaf color in autumn (Pertot et al. 2008), defoliation, and reduced quantity and quality of fruits (Baumgartner and Warnock 2006). Infected plants generally die some years after infection (Baumgartner and Warnock 2006; Pertot et al. 2008).

The commonly used *Vitis* rootstocks are susceptible to *A. mellea* (Aguín-Casal et al. 2004; Baumgartner and Rizzo 2006; Prodorutti et al. 2009). A lower frequency of *A. mellea* infection has been demonstrated only for the Teleki 5C rootstock (Prodorutti et al. 2009) and the *Vitis* hybrid Freedom (Baumgartner and Rizzo 2006). To date, post-infection control agents have not been available and agronomic practices, such as the careful removal of inoculum (elimination of infected plants and root debris), have been used to reduce the spread of *Armillaria* spp. in vineyards (Fox 2003). Soil fumigants cannot completely eradicate *Armillaria* spp. from soil (Aguín-Casal et al. 2006). Promising results have been reported for applications of antagonistic fungi (Otieno et al. 2003; Cox and Scherm 2006), bacteria (Baumgartner and Warnock 2006), and arbuscular mycorrhizae (Camprubí et al. 2008; Nogales et al. 2008). However, to date, none of these treatments have been found to be completely effective and none of these treatments have been commercialized. Therefore, the need for the development of efficient control methods is crucial.

Although the genetic diversity of *Armillaria* spp. and its distribution in forests (Coetzee et al. 2005; Bendel et al. 2006; Mwenje et al. 2006; Prospero et al. 2008) and vineyards (Aguín-Casal et al. 2004; Pertot et al. 2008) have been extensively studied, the plant mechanisms involved in the interaction between the pathogen and its host plants have not received sufficient attention. In pot trials, young grapevines (2 years old) start to show symptoms of *A. mellea* infection and hyphal root penetration only from the second year after pathogen inoculation (Prodorutti et al. 2009). These observations suggest the existence in young roots of a tolerance mechanism which delays root rot progress. This temporary resistance process could be an interesting starting point for the development of new disease-control approaches. Several studies have reported

This article is dedicated to the memory of Federica Bampi, who unexpectedly passed away. This work was part of her graduate thesis.

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*The e-Xtra logo stands for “electronic extra” and indicates that a supplementary table is published online.

plant defense reactions against *Armillaria* spp. infection, including the accumulation of reactive oxygen species (ROS) and pH changes (Víteček et al. 2005), the modulation of defense genes (Robinson et al. 2000, Wang et al. 2007), and the production of secondary metabolites (Isidorov et al. 2008). Also, in grapevine, the accumulation of free polyamines was observed nine months after *A. mellea* root inoculation, suggesting their possible role in signaling processes associated with the plant defense reaction (Nogales et al. 2008).

We provide evidence that a plant reaction is rapidly (24 h) activated in grapevine roots following *A. mellea* challenge. In particular, genes related to defense response, regulation of gene expression, and signal transduction are induced in the first stages of *A. mellea* attack. Moreover, the induction of genes involved in ethylene (ET) and jasmonate (JA) biosynthesis and response suggests the activation of the ET/JA signaling pathways in response to inoculation with this saproparasitic basidiomycete. We also demonstrate that the recombinant grapevine protein homologue of the *Quercus robur* phase-change-related protein can inhibit *A. mellea* growth in vitro.

RESULTS

Isolation of *A. mellea*-induced grapevine cDNA clones.

To study the early reaction of young grapevine roots to inoculation with *A. mellea* (24 h after inoculation), a subtracted cDNA library was constructed using a suppression subtractive hybridization (SSH) approach. The subtraction was done in forward, in order to enrich the library for cDNA sequences present in the *A. mellea*-inoculated Kober 5BB roots (tester) and absent from or less abundant in the untreated control roots

(driver). In total, 2,226 clones were collected and subjected to further analysis. To search for true positives, all clones were polymerase chain reaction (PCR) amplified, spotted on nylon membranes, and screened by hybridization with four different cDNA probes: tester, driver, forward, and reverse subtracted. This analysis detected 56 clones that were upregulated in the *A. mellea*-treated roots.

Sequence analysis and annotation.

The sequencing of the 56 selected clones yielded 47 good-quality sequences, which were cleaned of primer and vector contaminations, and nine low-quality sequences, which were discarded. Insert lengths varied from 59 to 363 bp, with an average length of 185 bp. The 47 sequences were clustered into contigs in order to group the sequences with significant overlapping regions together, indicating that these sequences probably originated from the same cDNA. This procedure defined six clusters, derived from 25 SSH sequences, plus 22 singletons. The clusters comprised two to seven SSH sequences and ranged from 154 to 335 bp in length, with an average length of 228 bp. The size of the singletons ranged from 59 to 447 bp; the average size was 194 bp.

When aligned with the TIGR Grape Gene Index database, the 28 unique sequences were found to correspond with 24 tentative consensus (TC) sequences (Table 1). For the 47 sequenced cDNAs, the overall library redundancy was 1.9. Functional annotation of the TC sequences according to the Gene Ontology (GO) categories revealed that they were involved in different biological processes, such as defense response (TC = 4), response to stress (TC = 1), hormone metabolism (TC = 1), signal transduction (TC = 3), regulation of gene expression

Table 1. Summary of the 28 suppression subtractive hybridization (SSH)-identified sequences isolated following *Armillaria mellea* treatment of Kober 5BB roots

SSH code ^a	Accession no. ^b	TC ^c	Pinot Noir gene ^d	Definition ^e	Homologous protein	E value
A08_2	GR911643	TC98523	fgenesh.VV78X248522.8_1	Protease inhibitor 1 (PIN1)	AAN85825	3.0E-34
A03_7	GR911640	TC97254	glimmer.VV78X012064.13_5	Protease inhibitor 2 (PIN2)	AAN85825	2.0E-19
A04_3	GR911641	TC91773	glimmer.VV78X176033.9_1	Thaumatococcus-like (TL)	AAD55090.1	1.0E-148
A10_3	GR911644	TC97635	No hit	Calmodulin (CaM)	AAQ63461.1	9.E-79
A12_6	GR911645	TC93391	glimmer.VV78X154025.18_1	Phase-change related protein (PCr)	CAB72442.1	5.0E-18
E08	GR911665	TC93391	No hit	PCr	CAB72442.1	5.0E-18
B03_4	GR911647	TC82945	glimmer.VV78X069566.6_1	Tumor-related protein (Tr)	AAC49969.1	9.0E-80
B08	GR911650	TC72587	glimmer.VV78X271907.6_1	Globulin (GLB)	AAM65577.1	5.0E-58
B12	GR911653	TC72587	glimmer.VV78X271907.6_1	GLB	AAM65577.1	5.0E-58
D05	GR911658	TC72587	glimmer.VV78X271907.6_1	GLB	AAM65577.1	5.0E-58
E03	GR911662	TC72587	No hit	GLB	AAM65577.1	5.0E-58
C05	GR911654	TC94117	glimmer.VV78X238526.9_6	Proline-rich protein (Pro-R)	CAB85624.1	6.0E-22
C11	GR911657	TC89821	glimmer.VV78X179831.13_1	Glutathione S-transferase (GST)	ABW34390.1	1.0E-96
D08	GR911659	TC75061	twinscan.VV78X191270.11_5	Aminocyclopropane carboxylate oxidase (ACO)	Q08507.1	3.0E-160
E04	GR911663	TC92611	fgenesh.VV78X217483.10_1	Transcription factor (TF)	CAA66481.1	3.0E-133
A01	GR911638	TC100086	fgenesh.VV78X086274.10_4	Receptor-like protein kinase-related	NP_566697	3.0E-64
A02	GR911639	TC84493	fgenesh.VV78X114032.9_1	Laccase	ACC78283.1	4.0E-125
A05	GR911642	TC86622	No hit	Unnamed protein product	CAO43937.1	7.0E-18
B02	GR911646	TC80320	No hit	RNA polymerase	ACF06506.1	1.0E-57
B04	GR911648	TC100606	No hit	Extensin (class I)	AAA34163	2.0E-28
B05	GR911649	TC87751	fgenesh.VV78X012423.11_2	Ca-dependent protein kinase	ABY28389	2.0E-75
B09	GR911651	TC87587	sim4.VV78X262027.9_2	Ubiquitin-protein ligase	NP_565147.1	4.0E-86
B10	GR911652	TC71862	fgenesh.VV78X135834.21_3	Pyroline-5-carboxylate synthetase	CAB40834	0.0
C09	GR911655	TC88813	glimmer.VV78X278114.3_1	Translation initiation factor	ACF06598.1	5.0E-78
C10	GR911656	TC77750	No hit	α -Expansin	AAR09170.1	1.0E-125
D04	GR911661	TC97529	No hit	Translation initiation factor eIF1	ACF06581.1	9.0E-55
D09	GR911660	TC82133	glimmer.VV78X236086.8_2	Short-chain dehydrogenase/reductase	NP_192713.1	5.0E-142
E05	GR911664	TC72567	sim4.VV78X096618.37_9	Aspartate aminotransferase	NP_196713.1	0.0

^a Codes of singletons and clusters of the SSH-identified sequences. The SSH code of each cluster is defined by the longest sequence, and the numbers following the underscore correspond to the number of sequences in the cluster.

^b National Center for Biotechnology Information (NCBI) Gene Bank accession numbers of the SSH sequences.

^c The corresponding grapevine tentative consensus (TC) sequences were identified by a nucleotide BLAST search against the TIGR database (version 6.0).

^d The corresponding Pinot Noir genes were identified by a nucleotide BLAST search against the IASMA Pinot Noir predicted genes (release 3) (Velasco et al. 2007).

^e Definitions of grapevine genes are based on the homologous proteins identified via BLAST-X of TC sequences against the NCBI nonredundant database. The abbreviations of genes further characterized in this work are shown in parentheses.

(TC = 4), protein modification (TC = 1), and cell wall organization (TC = 3). Specifically, two different protease inhibitors (PIN1 and PIN2), which are similar to the grapevine PIN (Aziz et al. 2007; Belhadj et al. 2008); a thaumatin (TL); and a tumor-related (Tr) protein that is homologous to a pathogen-induced trypsin inhibitor were identified. Moreover, the upregulation observed in the treated sample for genes that encode for enzymes involved in ethylene biosynthesis (aminocyclopropane-1-carboxylate oxidase) (ACO) and ROS detoxification (glutathione S-transferase) (GST) suggests that the defense reaction could be mediated by these signaling molecules. We also found genes coding for proteins involved in signal transduction pathways (one calmodulin and two protein kinases), in the regulation of gene expression (one transcription factor and one RNA polymerase), and in translation and protein modification (two translation initiation factors and one ubiquitin ligase). Genes coding for proteins associated with cell wall organization (one extensin, one expansin, and one laccase) also appeared to be induced.

Other induced genes whose functions were not directly linked to pathogen defense responses included a proline-rich (Pro-R)

protein, a phase change-related (PCr) protein, a globulin-like (GLB) protein, pyrroline-5-carboxylate synthetase, the short-chain dehydrogenase reductase, and an aspartate aminotransferase. We could not associate TC86622 with any GO biological process.

Expression analysis of *A. mellea*-induced genes.

In order to validate the SSH results, we measured the relative expression of 11 genes (Table 2) by quantitative real-time reverse-transcriptase PCR (RT-qPCR) in the RNA samples used to construct the SSH library and in roots treated with *A. mellea* under greenhouse (Kober 5BB and Pinot Noir) or in vitro conditions (Kober 5BB). These genes were selected because they were putatively related to plant defense response (*PIN1*, *PIN2*, *Tr*, *TL*, *GST*, *ACO*, *TF*, and *CaM*) or because they had never before been associated with this process (*GLB*, *Pro-R*, and *PCr*). The correct design of the primers was demonstrated by the expected size of the amplicon in RT-PCR experiments with grapevine cDNA. In addition, primer specificity was confirmed by the absence of amplification when using *A. mellea*-derived cDNA as template (data not shown).

Table 2. Primer sequences of the selected suppression subtractive hybridization (SSH)-identified genes and of some known pathogenesis-related (PR) markers and relative gene expression (fold change [FC]) of the SSH transcripts

SSH code	Definition	Abbreviation	RT-qPCR primer	FC ^a
A08_2	Protease inhibitor 1	<i>PIN1</i>	A08-2_for A08-2_rev ACCAACCCAATGAGCCTATCC	4.8 ± 0.3
A03_7	Protease inhibitor 2	<i>PIN2</i>	A03-7_for A03-7_rev CGCAACTCCTGATAGATTGGTG	4.1 ± 0.2
A04_3	Thaumatin-like	<i>TL</i>	A04-3_for A04-3_rev CTGGAGATGTATGGAAGCTGATAGTG	2.7 ± 0.1
A10_3	Calmodulin	<i>CaM</i>	A10-3_for A10-3_rev TATTCAGTAGTTGGGTGTAGTG	2.2 ± 0.2
A12_6	Phase change-related protein	<i>PCr</i>	A12-6_for A12-6_rev CTTCTCTTGCTGTTGTCTCATCC	5.9 ± 0.3
B03_4	Tumor-related protein	<i>Tr</i>	B03-4_for B03-4_rev ACTTCGCATCTCCACGCT	3.8 ± 0.2
C05	Proline-rich protein	<i>Pro-R</i>	C05_For C05_Rev CTCAGGACACAAGCCACCA	2.5 ± 0.1
C11	Glutathione S-transferase	<i>GST</i>	C11_For C11_Rev GCTTTATCTTTGGAGGTATCTT	2.2 ± 0.1
D05	Globulin	<i>GLB</i>	D05_for D05_rev CACAAAACCTACCAACCAAA	6.3 ± 0.3
D08	Aminocyclopropane carboxylate oxidase	<i>ACO</i>	D08_For D08_Rev GCCTCTCTCAATGTGGCTCC	2.3 ± 0.1
E04	Transcription factor	<i>TF</i>	E04_for E04_rev TGCTCTTTTGATCTTTGCC	2.3 ± 0.1
–	Thaumatin 1 ^b	<i>TL1</i>	TL1_For TL1_Rev TGGCGTGGCAAATGCGAAA	
–	Pathogenesis-related protein 1 ^c	<i>PR-1</i>	PR-1_For PR-1_Rev CGAGGTTATTGGGCTGGTTTA	
–	Pathogenesis-related protein 4 ^c	<i>PR-4</i>	PR-4_For PR-4_Rev ACTTGTGGGTGGGGAGAA	
–	Lipoxygenase 9 ^c	<i>LOX-9</i>	LOX9_For LOX9_Rev TGTTGTGTCAGAGTGGTTGGC	
–	Vegetative storage protein ^d	<i>VSP</i>	VSP_For VSP_Rev AACATGGCAAACACGTAAGTCT	
–	Pathogenesis-related protein 2 ^c	<i>PR-2</i>	PR-2_For PR-2_Rev TCCTTGCCTTGCCTCATCTAT	
–	Actin ^f	<i>Act</i>	ACT_For ACT_Rev CACCAATCACTCTCTGCTACAA	

^a Relative expression level (FC) of Kober 5BB genes in roots 24 h after inoculation with *Armillaria mellea* (tester) compared with the untreated root (driver) samples used for SSH library construction. Values are means and standard errors of three technical replicates.

^b Sequence of the antifungal protein thaumatin 1 (AF003007) as reported by Jayasankar and associates (2003).

^c *PR-1* (AJ536326, TC56938) was used as a marker gene for the salicylic acid (SA) pathway. *PR-4* (CF074510, TC58333) and *LOX-9* (AY159556, TC63584) served as marker genes for jasmonate (JA) signaling. Sequences were taken from Hamiduzzaman and associates (2005).

^d Grapevine *VSP* gene (TC64921), which is homologous to the JA-inducible *Arabidopsis thaliana* *VSP* (AT5G24780, Z18377.1) (van Wees et al. 1999), was identified through a BLAST-X search of the TIGR database (version 6.0).

^e Grapevine *PR-2* gene (TC64809), which is homologous to the SA-inducible *A. thaliana* *PR-2* (AT3G57260, M90509.1) (van Wees et al. 1999), was identified through a BLAST-X search of the TIGR database version 6.0.

^f Grapevine *Act* gene (TC100707) was used as a constitutive gene for normalization of the quantitative real-time reverse-transcriptase polymerase chain reaction (RT-qPCR) data (Gatto et al. 2008).

As expected, significant induction (more than twofold) of all of the selected grapevine genes was observed comparing the tester with the driver RNA samples used to construct the SSH library (Table 2).

Gene expression analyses performed in greenhouse-grown plants detected the significant upregulation of the *PIN1*, *PIN2*, *Tr*, *TL*, *GLB*, *PCr*, *Pro-R*, *GST*, and *ACO* genes and the control

gene, thaumatin 1 (*TL1*), in young roots of Kober 5BB and Pinot Noir 24 h after inoculation with *A. mellea* (Fig. 1). However, in Pinot Noir, the levels of induction of *PIN1*, *PIN2*, *Tr*, *GLB*, and *GST* and *ACO* expression were approximately half of those observed in Kober 5BB. The expression of the SSH-identified genes was not modulated in untreated grapevine roots (incubated in 10 mM MgCl₂ solution for 24 h), confirm-

Fig. 1. Quantitative real-time reverse-transcriptase polymerase chain reaction analysis of the expression of suppression subtractive hybridization-identified grapevine genes in roots of **A**, Kober 5BB rootstock and **B**, ungrafted Pinot Noir plants grown under controlled greenhouse conditions. Relative expression levels (fold change) of the specified genes 24 h after *Armillaria mellea* treatment (black) and 24 h after incubation in the MgCl₂ solution (gray), compared with untreated roots (white). Mean levels of relative expression and standard errors from three biological replicates of each treatment of one representative experiment are presented.

ing the absence of nonspecific gene modulation during the incubation of roots without the pathogen. No *A. mellea*-dependent induction of the *CaM* and *TF* genes was observed in the Kober 5BB or Pinot Noir roots.

To prolong the infection time and to confirm the results at 24 h under sterile conditions, in vitro-grown plants were inoculated with *A. mellea*. These same plants were also examined under a microscope, revealing that *A. mellea* mycelia had penetrated the root cortex 10 days after pathogen inoculation (Fig. 2).

Fig. 2. *Armillaria mellea* mycelium (arrows) in root sections of in vitro-grown Kober 5BB plants after FITC-WGA staining (Meyberg 1988), as viewed under a fluorescent microscope (10 days after inoculation with *A. mellea*). No green fluorescence was observed in root sections from untreated control plants that had been incubated in a 10 mM $MgCl_2$ solution for 10 days. The scale bar represents 100 μm .

The RT-qPCR analysis revealed that, at 24 h after inoculation, genes were more strongly induced in the in vitro experiments than in the greenhouse treatments (Fig. 3). In particular, *PIN2* and *Tr* were upregulated a hundred times more in the inoculated roots than in untreated control roots, and *PIN1*, *TL1*, *TL*, *GLB*, *GST*, and *ACO* genes were strongly induced (from 2- to 20-fold) as well. These genes were also highly expressed 96 h after *A. mellea* treatment; whereas the expression of *Pro-R* was induced only at 96 h, not at 24 h. The absence of gene modulation in the control sample after 24 and 96 h of incubation in 10 mM $MgCl_2$ solution indicates that the induction of these genes is a specific *A. mellea*-dependent reaction. The in vitro experiments confirmed the absence of modulation of *CaM* and *TF*, as had been indicated by the results of the greenhouse experiments.

In addition, the expression of four genes known as salicylic acid (SA)- or ET/JA-signaling markers was analyzed (Fig. 4). RT-qPCR data showed no modulation of the pathogenesis-related (PR) protein 1 gene (*PR-1*) or *PR-2*, which are both markers of the SA pathway. However, *A. mellea*-dependent induction of typical ET/JA signaling genes such as a lipoxigenase 9 (*LOX-9*) and a vegetative storage protein (*VSP*) was detected under all tested conditions, whereas no expression of *PR-4* was detected in the root samples.

Isolation of full-length cDNAs and their expression as recombinant proteins.

PIN 1, *PIN2*, *TL*, *Tr*, *PCr*, *GLB*, and *Pro-R* were further characterized to investigate their roles in the root response to *A. mellea*. We first obtained the full-length coding sequences (CDS) and subsequently cloned them into an expression vector to produce the corresponding recombinant proteins (Table 3).

The analysis of the short SSH sequences corresponding to the genes listed above revealed that the sequences encompassed the stop codon of the gene but were truncated at the 5'

Fig. 3. Quantitative real-time reverse-transcriptase polymerase chain reaction analysis of the expression of suppression subtractive hybridization-identified grapevine genes in roots of Kober 5BB plants grown in vitro. Relative expression levels (fold change) of the specified genes at 24 h (black) and 96 h (diagonal lines) after *Armillaria mellea* inoculation, and after 24 h (gray) and 96 h (horizontal lines) of incubation in the $MgCl_2$ solution, compared with untreated roots (white). Mean levels of relative expression and standard errors from three biological replicates of each treatment of one representative experiment are presented.

end and, therefore, were missing the ATG start codon. To clone the full-length CDS, we used a 5' rapid amplification of cDNA ends (RACE) RT-PCR protocol involving gene-specific primers (Supplementary Table 1). In parallel, the CDS of the Kober 5BB cystatin-1 gene (*CYS1*), which is homologous to the antifungal strawberry phytocystatin *Cyf1* (Martinez et al. 2005), was also obtained. The comparison of the cloned sequences with the SSH sequences and the homologous sequences from other species confirmed the successful isolation of the corresponding full-length gene. The presence of a putative signal peptide was predicted in the protein sequences of Tr, TL, PCr, GLB, Pro-R, and CYS1 (Table 3).

The sequences coding for the mature proteins were cloned for heterologous expression in *Escherichia coli* as fusion constructs with histidine tags. PIN1, PIN2, GLB, PCr, Pro-R, and CYS1 proteins were expressed at high levels in the soluble fraction and were purified to homogeneity by affinity chromatography (Table 3). Though different expression conditions were tested, we were not able to express the recombinant Tr protein and we could produce TL only as insoluble inclusion bodies.

Antimicrobial activity test of the recombinant proteins.

Tests of the antifungal activity of the six purified recombinant grapevine proteins demonstrated that PCr can significantly inhibit *A. mellea* growth in vitro ($30 \pm 4\%$ growth inhibition) when present at concentration of $60 \mu\text{mol}$. The dose-response study showed that PCr activity was concentration dependent (Fig. 5A) from $2.5 \mu\text{M}$ ($17 \mu\text{g/ml}$) through $400 \mu\text{M}$ ($46 \pm 4\%$ inhibition), with a subsequent saturation effect at higher dose. In contrast, no significant effects on *A. mellea* growth were observed for PIN1 ($4 \pm 3\%$ of inhibition), PIN2 ($4 \pm 3\%$), GLB ($4 \pm 2\%$), Pro-R ($3 \pm 3\%$), or CYS1 ($3 \pm 3\%$) at $120 \mu\text{M}$ (Fig. 5B) or lower concentrations (2.5 , 12.5 , and $60 \mu\text{M}$). The absence of any effects against *A. mellea* was confirmed for PIN1 ($5 \pm 3\%$ of inhibition), GLB ($8 \pm 5\%$), and CYS1 ($3 \pm 3\%$), also at $400 \mu\text{M}$. The absence of any synergistic effects between the purified proteins was demonstrated by applications of mixtures of the recombinant proteins (data not shown).

The in vitro antimicrobial activity of the proteins against another necrotrophic plant-pathogenic fungus and two gram-negative bacteria was evaluated. In particular, we examined the effects of these proteins against *Botrytis cinerea*, *E. coli*, and

Pseudomonas fluorescens. PCr, but not PINs, GLB, or Pro-R, was able to inhibit *B. cinerea* growth when applied at a high dosage ($200 \mu\text{g}$) (data not shown). A small zone of inhibition was also observed when the same amount of CYS1 was applied, suggesting that this grapevine protein might be slightly active

Fig. 4. Quantitative real-time reverse-transcriptase polymerase chain reaction analysis of the expression of gene markers of the salicylate (SA) and ethylene and jasmonate (ET/JA) signaling pathways in the roots of Kober 5BB plants grown under greenhouse (black) or in vitro (gray) conditions, and ungrafted Pinot Noir plants grown under greenhouse conditions (white). Relative expression levels (fold change [FC]) 24 h after *Armilaria mellea* inoculation were calculated with respect to control roots that had been incubated in 10 mM MgCl_2 solution for 24 h. Mean levels of relative expression and standard errors from three biological replicates of each treatment of one representative experiment are presented.

Table 3. Properties of the recombinant Kober 5BB mature proteins purified for functional characterization

Abbreviation	Accession no. ^b	Coding sequence (bp)	Signal peptide ^c	MW (kDa) ^d	SDS-PAGE ^a		Protein	
					Pellet	Soluble	Soluble (mg) ^e	Purified (mg) ^f
PIN1	GQ427141	216	—	7.8	+	+	24.0	1.2
PIN2	GQ427142	222	—	8.0	+	+	12.2	0.6
Tr	GQ427143	612	+	20.0	—	—	6.2	0
TL	GQ427144	720	+	23.9	+	—	6.4	0
PCr	GQ427145	279	+	6.8	+	+	15.0	0.9
GLB	GQ427146	1,076	—	38.5	+	+	22.0	1.8
Pro-R	GQ427147	402	+	12.0	+	+	19.7	0.3
CYS1 ^g	GQ427148	705	+	24.3	+	+	23.0	1.8
Empty vector ^h	—	—	—	—	—	—	27.9	0

^a Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) results for the presence (+) of the recombinant proteins in the soluble or insoluble fractions.

^b National Center for Biotechnology Information Gene Bank accession number of the sequences obtained by 5' rapid amplification of cDNA ends reverse-transcriptase polymerase chain reaction and containing the full-length coding sequences.

^c Presence of a putative N-terminal signal peptide is based on SignalP software and the number of amino acid residues is indicated in parentheses.

^d Translations to protein sequences and estimations of the molecular masses of the mature proteins were done using the ExPASy Proteomics Server, and these estimates include the first Met and exclude the fused His-tag.

^e Milligrams of total soluble protein obtained after centrifugation of lysate from 100 ml of bacterial culture (2 g of cells).

^f Milligrams of purified protein from 100 ml of bacterial culture (2 g of cells).

^g Sequence of Kober 5BB cystatin 1 (*CYS1*) was obtained by amplification with primers designed based on TC89650 (TIGR Grape database v. 6.0), the homologue of the strawberry *Cyf1* gene, which codes for a phytocystatin with antifungal properties (Martinez et al. 2005).

^h *Escherichia coli* cells transformed with the empty pET22b were used as a control to test the unspecific binding to the affinity chromatography gel.

against *B. cinerea* but is not active against *A. mellea*. PCr displayed antibacterial activity against *E. coli* TOP10 and *P. fluorescens* WCS417r (Fig. 6), with estimated half maximal inhibitory concentration (IC₅₀) values of 3 µg/ml (0.44 µM) and less

than 3 µg/ml, respectively. In contrast, PIN1, PIN2, GLB, Pro-R, and CYS1 did not display any antibacterial activity in this experiment.

DISCUSSION

The aim of this work was to characterize the response of young roots to *A. mellea* and to identify grapevine genes that are modulated during the first stages of this interaction. We employed a cDNA SSH approach (Diatchenko et al. 1996) to specifically isolate gene sequences whose expression is induced during the grapevine reaction to *A. mellea*. Kober 5BB plants were chosen in this study because this hybrid is a commonly used rootstock. Based on a report that cultured tobacco cells respond quickly to *A. mellea* elicitors (Víteček et al. 2005), root samples were collected 24 h after inoculation. As it was, the experimental set-up did not allow us to distinguish between the plant defense reaction to *A. mellea* elicitors and the more general stress response due to wounding or mechanical penetration. Our analysis of the SSH clones indicates that grapevine roots respond to *A. mellea* challenge with rapid upregulation of the expression of some genes. The annotation of this gene set suggests the activation of a whole defense process, because the set includes genes involved in signal transduction (calmodulin and protein kinases), hormone metabolism (*ACO*), transcription and translation (transcription factor, RNA polymerase, and translation initiation factors), protein modification (ubiquitin ligase), cell wall organization (extensin, expansin, and laccase), defense response (*PIN1*, *PIN2*, *TL*, and *Tr*), and response to stress (*GST*). Interestingly, among the induced transcripts, we also found sequences whose functions have not been previously linked to plant defense re-

Fig. 5. Test of antifungal activity against *Armillaria mellea* of **A**, different concentrations of the purified phase change-related (PCr) protein and **B**, a 120 µM solution of the purified grapevine recombinant proteins: PIN1, PIN2, PCr, GLB, Pro-R, and CYS. Mean percentage and standard error (six replicates) of live *A. mellea* fragments, calculated with respect to the total number of *A. mellea* fragments, are presented for each treatment. Results of one representative experiment are shown. Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

Fig. 6. Test of activity against *Escherichia coli* TOP10 (white) and *Pseudomonas fluorescens* WCS417r (black) incubated in the presence of different concentrations of the purified phase change-related (PCr) protein. For each treatment, the mean number of CFU and standard error from six replicates are presented. Results of one representative experiment are shown. Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

actions, such as PCr, *GLB*, and *Pro-R*; genes associated with cellular metabolism (coding for pyrroline-5-carboxylate synthetase and aspartate aminotransferase); as well as genes of unknown functions.

ET is known to contribute to plants' resistance to necrotrophic pathogens, whereas SA primarily mediates plant resistance to biotrophs (van Loon et al. 2006). ET usually exerts its resistance-stimulating activity in concert with JA, and ET/JA signals negatively affect the SA signaling pathway to finely tune plants' defense responses (Pieterse et al. 2009). During the grapevine reaction to *A. mellea*, we observed the modulation of transcripts that are involved in ET or JA biosynthesis as well as the modulation of genes known to be under ET/JA control in other systems. This group includes the gene encoding for the last enzyme of the ET biosynthetic pathway (ACO); two class V PR genes, *TL* and *TL1*, shown to be induced by ET (Jacobs et al. 1999); as well as the protease inhibitor genes *PIN* and *Tr*, which appeared to be modulated by JA (Tsukuda et al. 2006; Belhadj et al. 2008). The significant involvement of the ET/JA signals was also corroborated by the higher levels of *LOX-9* and *VSP* expression observed in the treated roots. These genes are markers of the JA signaling pathway in grapevine (Hamiduzzaman et al. 2005) and *Arabidopsis thaliana* (van Wees et al. 1999), respectively. Also, no induction of *PR-1* or *PR-2*, markers of the SA pathway (van Wees et al. 1999; Hamiduzzaman et al. 2005), was observed. Therefore, our results clearly indicate the activation of the ET/JA-mediated defense signaling pathways in grapevine roots following *A. mellea* challenge.

The accumulation of ROS is commonly observed during plant defense reactions, where they play multiple roles as toxic or signaling molecules (Apel and Hirt 2004) against necrotrophic pathogens, too (Shetty et al. 2008). The twofold upregulation of *GST* expression in *A. mellea*-treated roots, in both the greenhouse and in vitro experiments, suggests the possible accumulation of ROS during the grapevine reaction. Indeed, *GST* encode for ROS-detoxifying enzymes and their expression is induced by ROS (Lamb and Dixon 1997), and accumulation of ROS has also been observed in cultured tobacco cells 15 to 20 min after treatment with *A. mellea* extracts and lipids (Vítecek et al. 2005).

The downstream effect of the defense-related signals seems to be the upregulation of a set of defense genes belonging to well-known classes of PR genes, as mentioned above, as well as genes that have not yet been directly associated with any biological process. In all of the experiments, the highest level of *A. mellea*-dependent induction was observed for genes encoding protease inhibitors, such as *PIN1*, *PIN2*, and *Tr*. The serine-protease inhibitors encoded by *PIN* genes are small proteins belonging to the PR-6 class that display activity against fungal pathogens (Sels et al. 2008). Previous reports have shown that the grapevine *PIN* is activated by methyl jasmonate (Belhadj et al. 2008) and some elicitors of plant defense responses (Aziz et al. 2007), suggesting that it is the final effector of a network of signals. The grapevine *Tr* protein contains the typical Kunitz-type trypsin inhibitor motif and is very similar to a tumor-related protein found in tobacco and to a miraculin-like protein (LeMir) found in tomato. The tobacco *Tr* gene is able to elicit a hypersensitive response (HR) (Karrer et al. 1998) and LeMir was shown to be rapidly induced upon nematode infection and secreted into the surrounding root environment (Brenner et al. 1998). The high sequence homology and the root induction following exposure to soilborne microorganisms suggest that the grapevine *Tr* and LeMir genes may be functional homologues. Among the *A. mellea*-induced genes that belong to the PR-5 class, we found a *TL* gene that differs from the characterized *TL1* and *TL2* (Jacobs et al. 1999;

Jayasankar et al. 2003). Interestingly, the results of expressed sequence tag analysis indicate that the SSH-identified *TL* is expressed mainly in roots and in berries exposed to abiotic stress. In grapevine, the expression of *PR-5* genes is upregulated in leaves infected with powdery mildew (Jacobs et al. 1999), and *PR-5* proteins accumulate in berries and leaves that have been inoculated with *B. cinerea* or *Phomopsis viticola* (Monteiro et al. 2003). Activity tests have shown that these proteins display antifungal activity against biotrophic and necrotrophic grapevine pathogens (Jayasankar et al. 2003; Monteiro et al. 2003).

Although we observed *A. mellea*-dependent gene induction in both greenhouse- and in vitro-grown plants, the extent of modulation was generally much greater in the latter growth system. This could be due to a stronger reaction of the young root tissues or to more extensive contact with fungal inoculum in the in vitro experiments. The rapid penetration of the cortical plant tissues by the pathogen's hyphae, observed 10 days after in vitro *A. mellea* inoculation, supports both of these proposed hypotheses. However, the involvement of the same defense mechanisms in the in vitro- and greenhouse-grown Kober 5BB plants, as well as the Pinot Noir plants, seems very likely because, generally, the same genes were induced in all the experimental set-ups. Exceptions were the *Pro-R* and *PCr* genes, the first one being induced only in the greenhouse experiments and the second one not showing the increase in modulation usually observed when comparing in vitro and greenhouse gene expression results. Higher levels of expression of the defense genes in the non-*Vitis vinifera* species have been reported in previous studies and linked to the resistant phenotype (Kortekamp 2006; Figueiredo et al. 2008). A stronger gene induction was found in the hybrid Kober 5BB compared with *V. vinifera* Pinot Noir roots and this result is in line with recent findings showing that Kober 5BB displays a moderate resistance to *A. mellea* artificial inoculations (Prodorutti et al. 2009). Furthermore, the strong induction of the PR genes within the first 24 h following inoculation supports the hypothesized regulation of the encoded proteins at the transcriptional level in the early phases of the plants' response. Although a final conclusion will come only after protein quantification and characterization, several reports have suggested the possibility of large-scale transcriptional reprogramming of defense genes following pathogen recognition (Eulgem 2005).

An essential step in the investigation of the role of the SSH-identified genes in the grapevine response against *A. mellea* was the assay of their inhibitory effect on the growth of this fungus. PCr protein inhibited *A. mellea* growth already at 2.5 μ M (17 μ g/ml) and a significant effect (25% of inhibition) was measured at 60 μ M, displaying a dose dependence. Though apparently high, a similar range of active concentrations was reported for other antifungal proteins (Lee et al. 2008). The PCr amino acid sequence looks similar (>60%), among the others, to that of an antimicrobial peptide isolated from *Capsella bursa-pastoris* roots that has been named shepherdin. Shepherdins are cationic peptides rich in Gly and His residues, which probably exert their antimicrobial activity by interacting with the negative surfaces of cell membranes (Park et al. 2000). The *Capsella* shepherdin gene codes for two peptides, shepherdin I and II, whose maturation involves proteolytic cleavage (Park et al. 2000). The grapevine *PCr* codifies for a protein that is homologous to shepherdin II and includes a 26-amino acid putative signal peptide for secretion at its amino terminus. As previously demonstrated for the *Capsella* shepherdins, the purified grapevine PCr protein displayed strong antibacterial activity against gram-negative bacteria ($IC_{50} \leq 3$ μ g/ml) but it was also able to inhibit the growth of *B. cinerea*. Thus, PCr is not specifically active against *A. mellea* but it has a broad-

spectrum antimicrobial activity and it is likely part of a more general defense response mounted by the root system following pathogen attack.

The other recombinant proteins did not show any inhibitory activity against *A. mellea*. In the absence of further analyses, we can hypothesize that these other SSH-identified proteins might be involved in more general plant reaction processes or they might be active against other pathogens. This could be the case for CYS1-inhibited *B. cinerea* growth, and the PIN1 and PIN2 proteins, which are commonly activated by necrotrophs (Aziz et al. 2007). It is also possible that these proteins are involved in broad-spectrum defense mechanisms, as observed for Pro-R, which is highly similar to the Pro-R protein VvPRP2 that is expressed in grapevine roots and probably acts at the cell wall (Thomas et al. 2003). Pro-R proteins are often involved in reinforcing or restructuring the cell wall. They do not have any direct inhibitory effect on the invading pathogen but they do help to limit its penetration (Josè and Puigdomènech 1993). A role for GLB in the defense process is less clear, because its sequence resembles those of legumin-like proteins that accumulate in maize endosperm (Hunter et al. 2002). Whereas our results indicate that grapevine PCR could play an important role in the roots' reaction against *A. mellea*, the general root response observed in our experiments seems to be nonspecific. These findings, together with the lack of reports describing *Armillaria* spp.-resistant *Vitis* genotypes, support the hypothesis that grapevine (as well as many other woody plants) has not acquired any specific resistance traits against this pathogen during the course of its evolution.

MATERIALS AND METHODS

Plant and fungal materials.

Rooted cuttings of the grapevine rootstock Kober 5BB (*V. berlandieri* × *V. riparia*) and *V. vinifera* cv. Pinot Noir were grown for 2 months on 4-cm stone wool cubes (Grodan, Roermond, The Netherlands) under controlled greenhouse conditions at 20 ± 3°C and 70 ± 10% relative humidity. In vitro Kober 5BB plants were grown for 2 months in Magenta vessels (Sigma-Aldrich, St. Louis) on sterilized perlite with 20 ml of 50% Murashige-Skoog (MS) salt liquid medium (Duchefa, Haarlem, The Netherlands) in a growth chamber kept at 23 and 18 ± 1°C with a cycle of 16 and 8 h, day and night, respectively. *A. mellea* 7A (SafeCrop collection), which was originally isolated from an infected grapevine in an Italian vineyard (Pertot et al. 2008), was grown for 4 weeks at 20°C in darkness on 3% malt extract agar (MEA) (Oxoid, Basingstoke, U.K.). To obtain a mycelial suspension (Weitz et al. 2002), small pieces (3 mm in diameter) of *A. mellea* mycelia were grown for 4 weeks at 20°C in darkness on malt extract broth (MEB) (Oxoid) at 120 rpm.

Treatments of grapevine roots with *A. mellea*.

For library construction, the root systems of two Kober 5BB plants were each sandwiched between two petri dishes containing confluent mycelia of an *A. mellea* colony (tester). As a control treatment, the root systems of two other plants were each sandwiched between two dishes containing uninoculated MEA (driver). The root systems were kept this way for 24 h at 20 ± 2°C.

For gene expression analyses, the roots of each Kober 5BB or Pinot Noir plant (grown either in the greenhouse or in vitro) were incubated for 24 h at 20°C with 20 g of *A. mellea* mycelia suspension that had been previously washed four times with sterilized 10 mM MgCl₂. As control samples, the root systems of other plants were kept untreated or incubated in sterilized 10 mM MgCl₂ solution for 24 h at 20°C. In the greenhouse treatments, only the young roots that grew outside

the stone wool cubes were treated, in order to avoid abiotic stress to the plants. For the in vitro experiments, Kober 5BB plants were transferred into new, sterilized vessels containing 20 ml of fresh 50% MS. After 3 days, 10 ml of the nutrient medium was removed and the roots were treated with 20 g of washed *A. mellea* mycelia or 20 ml of 10 mM MgCl₂ solution. One experiment consisted of three biological replicates (plants) for each treatment (untreated roots, MgCl₂-treated, and *A. mellea*-treated at 24 or 96 h). Each experiment performed in the different inoculation conditions (Kober 5BB greenhouse, Pinot noir greenhouse, and Kober 5BB in vitro) was carried out in three independent repetitions.

Library construction by SSH.

Root samples of the two replica plants were pooled together and total RNA was extracted from 4 g of *A. mellea*-treated (tester) and control (driver) roots using the hot borate method (Moser et al. 2004). Poly (A)⁺ RNA was isolated using biotinylated oligo-dT (Promega Corp., Madison, WI, U.S.A.) and streptavidin-coupled magnetic M-280 dynabeads (Invitrogen, Carlsbad, CA, U.S.A.). Double-stranded cDNA was synthesized from 1 µg of Poly (A)⁺ RNA using the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen). The forward subtracted library (enriched with *A. mellea*-induced cDNAs) was obtained by hybridizing the tester cDNA with an excess of driver cDNA using the PCR-Select cDNA Subtraction kit (Clontech, Mountain View, CA, U.S.A.) for SSH (Diatchenko et al. 1996). The resulting subtracted cDNA was cloned into the pCR2.1 TOPO vector (Invitrogen) and mobilized into *E. coli* TOP10 (Invitrogen). Positive transformants, based on blue/white color selection, were picked and grown in Luria-Bertani (LB) broth (Sigma-Aldrich) supplemented with ampicillin at 100 mg/liter and 10% glycerol.

SSH library colony screening.

All positive clones of the SSH library were PCR amplified in a 384-well Geneamp 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, U.S.A.) using nested primers (Clontech). After electrophoresis on agarose and ethidium bromide gels, the PCR products were spotted in duplicate on Amersham Hybond-N⁺ membranes (GE Healthcare, Piscataway, NJ, U.S.A.) with a 96-pin manual replicator system (V&P Scientific, San Diego, CA, U.S.A.). Two different concentrations of insect nebulin cDNA were spotted as a control. Membranes were denatured (0.6 M NaOH and 2 M NaCl) and neutralized (0.6 M NaOH, pH 8.0, and 2 M NaCl) for 15 min, and DNA was then cross-linked for 2 h at 80°C. Digoxigenin (DIG)-labeled probes of tester, driver, forward (tester-driver), and reverse (driver-tester) cDNAs were generated using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Membranes were hybridized with 20 µl of each of the DIG-labeled probes and denatured at 70°C for 10 min, with 2 µl of labeled nebulin in 20 ml of hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.02% sodium dodecyl sulfate [SDS], 0.1% *N*-lauroylsarcosine, and 1% blocking reagent) at 65°C. After being washed twice with washing buffer 1 (2× SSC and 0.5% SDS) and twice with washing buffer 2 (0.2× SSC and 0.5% SDS) at 65°C, the membranes were incubated in washing buffer 3 (malic acid buffer and 5% Tween-20), and then with the blocking solution (10% blocking reagent in malic acid buffer). For detection, membranes were incubated in the antibody solution (1:20000 of anti-DIG-AP [Roche] in blocking solution) for 30 min and then washed twice with 100 ml of washing buffer 3. After equilibration in the detection buffer (1 M Tris-HCl, pH 9.5, and 1 M NaCl), each membrane was covered with 1 ml of CDP-STAR (Roche), a chemiluminescent substrate, and the signal was collected on

Kodak film (Sigma-Aldrich). Clones showing the most marked differences in their expression as revealed by the hybridization results were selected for sequencing.

Sequencing and sequence analysis.

The selected clones were single-pass sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems), starting from 100 ng of purified plasmid and 0.32 μ M of nested primer 1 (Clontech). The cloned gene CDS were sequenced in both directions using the M13 forward and reverse primers (Invitrogen). Sequencing reactions were performed using a 2-min initial denaturation step, followed by 40 cycles of: 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. After ethanol precipitation, samples were resuspended and HI-DI formamide (Applied Biosystems) was added before sequencing via capillary electrophoresis in an ABI PRISM 3730xl DNA analyzer (Applied Biosystems). Following base-calling performed using Chromas software 2.33 (Technelysium Pty Ltd., Tewantin, Australia), low-quality sequences were discarded and vector and adaptor sequences were removed. The sequences were clustered into contigs using the CAP3 assembly program (Huang and Madan 1999) with a minimum overlap length of 40 nucleotides and 95% identity for overlaps.

For annotation, the sequences of contigs and singletons were aligned using blastn (Altschul et al. 1997) against the TIGR grapevine gene index database (version 6.0) and the predicted genes of the Pinot Noir genome sequence (release 3) (Velasco et al. 2007). Best hits with *E* value < 10⁻⁵ were selected and annotated using blastx (Altschul et al. 1997) against the National Center for Biotechnology Information (NCBI) nonredundant protein database (July 2009). *E* value scores below 10⁻¹⁰ were considered significant and were used to find the homologous sequences and to classify them into GO functional categories.

Real-time RT-qPCR.

Total RNA from the treated and control grapevine roots, as well as from *A. mellea* mycelia, was extracted using the Spectrum Plant total RNA kit (Sigma-Aldrich). RNA was quantified and treated with DNase I (Invitrogen), and the first-strand cDNA was synthesized from 1.0 μ g of total RNA using Superscript III (Invitrogen) and oligo-dT primer. The qPCR reactions were carried out with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), and specific primers (Table 2, each primer sequence finds only one significant match on the Pinot Noir genome sequence) using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The qPCR conditions were 50°C for 2 min, 95°C for 2 min as initial steps, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Dissociation curves were analyzed to verify the specificity of each amplification reaction. ABI PRISM 7000 SDS software (Applied Biosystems) was used to extract cycle threshold values and LinReg software was used to calculate reaction efficiency (Ramakers et al. 2003). The relative expression of each gene was then calculated according to the Pfaffl (2001) equation, using untreated control roots as the calibrator and *actin* (TC100707) as the constitutive gene for normalization (Vandeleur et al. 2009), because its expression was not significantly affected by the treatments. For each treatment (untreated roots, MgCl₂-treated, and *A. mellea*-treated at 24 or 96 h), mean expression and standard error values were calculated on three biological replicates. The gene expression pattern was conserved in the three independent repetitions of the experiment.

Fluorescent microscopy.

Longitudinal sections (15 μ m thick) of grapevine roots were obtained using cryostat CM1510 (Leica, Wetzlar, Germany) and fungal hyphae were selectively stained using the FITC-

WGA method (Meyberg 1988). Root sections were immersed in 10% H₂O₂ for 10 min and then incubated for 2 h in staining solution: 10 mM phosphate buffer, pH 7.0, and FITC-WGA (Sigma-Aldrich) at 50 μ g/liter. The root sections were then washed in distilled water and images were taken using an Eclipse80i fluorescence microscope (Nikon Instruments EU, Amstelveen, The Netherlands) equipped with an FITC filter (EX 450-490, DM 505, BA 520) and a DS-Fi1 digital camera system (Nikon Instruments EU).

5'-RACE RT-PCR.

The full-length CDS of seven SSH-identified genes were obtained using the 5' RACE RT-PCR System (Invitrogen). First-strand cDNA was synthesized from the total RNA of *A. mellea*-treated Kober 5BB roots using the SuperScript II (Invitrogen) and specific reverse primers. After RNase Mix (Invitrogen) treatment, the cDNAs were purified using S.N.A.P. columns (Invitrogen) and a homopolymeric tail was added to their 3' ends by using the terminal deoxynucleotidyl transferase (Invitrogen) with dCTP. CDS were then amplified using the specific reverse primers and the oligo-dG anchor primer (Invitrogen). The PCR fragments were purified using NucleoSpin Extract II (Macherey-Nagel, Düren, Germany), cloned into pCR2.1-TOPO (Invitrogen), and sequenced. We used ClustalW (Larkin et al. 2007) and Blast (Altschul et al. 1997) software to compare the recovered sequences with the original SSH sequences and to confirm the presence of the full length of each sequence. The sequence of *CYS1* was amplified using specific primers designed based on TC89650 (TIGR Grape database v. 6.0), which is homologous to the strawberry gene *Cyfl* (Martinez et al. 2005).

Expression and purification of the recombinant proteins.

The presence of a putative signal peptide was detected using the SignalP v.3.0 program (Bendtsen et al. 2004). The sequences encoding for mature proteins were amplified using specific primers for subsequent cloning into the pET22b vector (Novagen, Madison, WI, U.S.A.) to achieve in-frame cytoplasmic expression of the peptides with the His tag. The amplified fragments were cloned into pCR2.1-TOPO (Invitrogen), sequenced, and introduced into pET22b (Novagen). The constructs were then mobilized into *E. coli* BL21 (Novagen).

Overexpressing bacterial cells were harvested by centrifugation, 20 h (5 h for PCR) following incubation at 28°C in LB broth (Sigma-Aldrich) supplemented with 1 mM isopropyl- β -D-thiogalactoside. Cells were resuspended in four volumes of extraction buffer: 50 mM phosphate buffer (pH 8.0), DNase (Sigma-Aldrich) at 50 μ g/ml, 10 mM MgCl₂, lysozyme (Sigma-Aldrich) at 500 μ g/ml, and 1 $\times$ protease inhibitor cocktail (Sigma-Aldrich). Protein extracts were obtained following six cycles of sonication by Sonoplus HD 2070 (Bandelin, Berlin) and centrifugation at 20,000 $\times$ g for 20 min at 4°C. Imidazole and NaCl were then added to final concentrations of 10 and 300 mM, respectively, and recombinant proteins were purified by affinity chromatography in batch using the HIS-select nickel affinity gel (Sigma-Aldrich) with an elution buffer (50 mM phosphate buffer, pH 8.0, 300 mM imidazole, and 300 mM NaCl). Proteins were dialyzed in 10 mM phosphate buffer, pH 6.5, concentrated using the Amicon Ultra 5K (Millipore, Billerica, MA, U.S.A.), sterilized by filtration (Millipore), quantified using Bradford reagent (Sigma-Aldrich), and analyzed by SDS polyacrylamide gel electrophoresis.

Antimicrobial activity assays.

Small fragments (1 mm in diameter) of fresh *A. mellea* mycelia were incubated for 72 h at 20°C under sterile conditions, in the presence of different concentrations (0 to 500 μ M) of

purified proteins. Six replicates of approximately 150 *A. mellea* fragments each were exposed to each protein concentration in a final volume of 200 µl in 10 mM phosphate buffer (pH 6.5). The *A. mellea* fragments were plated onto potato dextrose agar (PDA) (Oxoid) and the percentage of live *A. mellea* fragments (which produced white mycelia) on each plate was evaluated after 3 days of incubation at 20°C. The inhibition activity was calculated as a percentage of the growth of *A. mellea* in the presence of the control treatment, which was 10 mM phosphate buffer (pH 6.5).

Activity against *B. cinerea* was analyzed following the mycelial growth inhibition assay described by Goñi and associates (2009). Briefly, fresh *B. cinerea* mycelia were plated onto PDA and, following 24 h of incubation at 25°C, sterile filter paper discs (5 mm in diameter), with 30 µl of 200 µg of purified proteins in 10 mM phosphate buffer (pH 6.5) placed in front of the advancing fungal mycelium. As negative control, the same amount of bovine serum albumin (Sigma-Aldrich) was spotted. The assays were conducted in triplicate and inhibition zones were evaluated following 24 h of incubation at 25°C.

Activity against the bacteria *E. coli* TOP10 (Invitrogen) and *Pseudomonas fluorescens* WCS417r was determined as described by Park and associates (2000). Bacterial cells of the mid-exponential phase culture were centrifuged at 5,000 × *g* for 10 min at 4°C, then washed three times with cold 10 mM phosphate buffer (pH 6.5). The concentration of the bacterial suspension was measured using a Burkler chamber under a light microscope, and 200-µl aliquots of 10³ CFU/ml were incubated for 2 h at room temperature in the presence of different concentrations (0 to 6.3 µM) of the recombinant proteins, in six replicates. Suspensions were plated onto LB agar (Sigma-Aldrich) and the numbers of colonies were determined following 24 h of incubation at 37°C for *E. coli* or 28°C for *P. fluorescens*. The protein concentration required for a 50% reduction in the number of CFU relative to that of the control corresponds to the IC₅₀ value.

All experiments were repeated at least three times with similar results. Analysis of variance of the data was conducted using the Statistica 7.1 software (StatSoft, Tulsa, OK, U.S.A.) following validation of normal distribution of the data, to demonstrate insignificant treatment–experiment interactions (*P* > 0.05), and to compare the means of antimicrobial activity (Tukey's test).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

BLAST search page:
genomics.research.iasma.it/iasma/faces/BlastPage.jsp
 ExPASy Proteomics server: www.expasy.ch
 The Gene Index project grape gene index website:
compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=grape
 Gene Ontology website: www.geneontology.org
 NCBI home page: www.ncbi.nlm.nih.gov
 NCBI nonredundant BLAST database: blast.ncbi.nlm.nih.gov/Blast.cgi
 SignalP v.3.0 server: www.cbs.dtu.dk/services/SignalP