European Journal of Plant Pathology **107:** 249–261, 2001.

Molecular characterization of the incompatible interaction of *Vitis vinifera* leaves with *Pseudomonas syringae* pv. *pisi*: Expression of genes coding for stilbene synthase and class 10 PR protein

Nadia Robert[1], Jean Ferran[1], Colette Breda[1], Pierre Coutos-Thévenot[2,*], Michel Boulay[2,**], Dominique Buffard[1,3] and Robert Esnault[1,3,4,†]
[1]*Institut des Sciences Végétales, C.N.R.S., 91198 Gif sur Yvette cedex, France (Fax: 33 1 69823768);* [2]*L.V.M.H. Recherche, France;* [*]*Present address: Physiologie et Biochimie Végétale, Université de Poitiers, Poitiers, France;* [**]*Present address: Moët & Chandon Recherche, 51 333 Epernay, France;* [3]*Université Paris 7;* [4]*Emeritus Professor, Université Paris 7;* [†]*Author for correspondence (E-mail: esnault@isv.cnrs-gif.fr)*

Accepted 2 January 2001

Key words: grapevine leaf, incompatible interaction, PR10, *Pseudomonas syringae* pv. *pisi*, resveratrol, stilbene synthase

Abstract

The interaction between *Vitis vinifera* and *Pseudomonas syringae* pv. *pisi* was examined at the pathological and molecular levels. Leaves infiltrated with the bacterial suspension developed necrotic regions which remained restricted to the infiltrated areas. In the infiltrated zone the number of bacteria decreased around 24 h after inoculation whilst no bacteria could be isolated from the non-infiltrated zone. At the molecular level, two genes, stilbene synthase (SS) and a PR10 gene, encoding putative defense proteins, were analyzed. Expression of the SS gene, measured by the analysis of transcript accumulation, was shown to be highly induced and was followed by the accumulation of resveratrol (peaking at approximately 48 h post-inoculation), considered as one of the major phytolaexins in the *Vitis* species. We report for the first time the isolation of a genomic clone (*VvPR10-1*) coding for a PR10 protein from this plant. The accumulation of the corresponding mRNA (0.8 kb) was observed from 3 to 96 h post-inoculation, peaking at 24–48 h, and was followed by the accumulation (between 24 and, at least, 96 h after inoculation) of the encoded polypeptide as detected by immuno-blotting. These results indicate that our experimental system based on an interaction of the non-host plant *V. vinifera* leaves with *P.s.* pv. *pisi*, has to be considered as an HR-like response and is well suited for the analysis of the defense reaction of this economically important species.

Abbreviations: HR – hypersensitive response; nt – nucleotide; PR – pathogenesis-related; *P.s.* – *Pseudomonas syringae*; SS – stilbene synthase

Introduction

The cultivated *Vitis vinifera* is a plant of great economical importance and large quantities of phytochemicals are used to prevent or reduce the impact of pathogen infection in the vineyard. Grapevine is vulnerable to a large number of diseases caused by viruses, bacteria or fungi. While no efficient long lasting treatment has been found for several important diseases (eutypa dieback (*Eutypa lata*), grey mould (*Botrytis cinerea*), fanleaf virus (grapevine fanleaf virus), berry rot (*Rhizopus stolonifer*), etc.), treatments of other diseases like downy (*Plasmopora viticola*) or powdery mildew (*Uncinula necator*) require the application of large quantities of chemicals resulting in negative effects on the environment and potential problems for wine quality. In spite of the important economical aspects of the pathogen attack, the defense mechanisms

in grapevine are not well characterized. Consequently, diversifying the means of controlling pathogen attacks may be considered as an important objective which requires a better knowledge of the defense reactions of this plant.

Analysis of the defense mechanisms in *V. vinifera* is complicated by the absence of gene to gene functional relationships corresponding to well defined incompatible (resistant plant/avirulent pathogen couples) or compatible (susceptible plant/virulent pathogen) interactions, preventing the characterization of avirulence and resistance genes. Moreover, defense reactions induced by pathogenic fungi appear at different times after pathogen recognition and at various distances from the infected area (Mansfield, 1990). To take into account these difficulties three strategies have been developed: (i) use of a simplified system consisting of the treatment of cell suspension cultures with elicitors (see, for e.g., Calderon et al., 1993), (ii) infection with fungal spore suspensions of leaves/ berries from cultivars considered as 'susceptible' (e.g. *Vitis vinifera*) or 'resistant' (e.g. *V. labrusca* or *V. riparia*) (Bavaresco et al., 1997; Jeandet et al., 1991; Langcake, 1981; Sarig et al., 1997) or (iii) fungal isolates differing in their virulence towards a given cultivar (Derkel et al., 1999).

Production of phytoalexins such as stilbene, is the best characterized defense reaction in grapevine and is controlled by a key enzyme, stilbene synthase (SS) (Schröder and Schröder, 1990). Condensation of 3 Malonyl CoA and 1 Coumaryl CoA molecules catalyzed by SS gives rise to resveratrol, a diphenol molecule, as well as to various metabolites such as ε-viniferin (dimerization product), pterostilbene (methylated resveratrol) and piceid (a glucosidic conjugated form), which represent the major forms of phytoalexins in grapevine (Bavaresco et al., 1999; Langcake, 1981). In response to pathogen ingress, elicitors, UV treatment or wounding, resveratrol and its metabolites are accumulated (Bavaresco et al., 1997; Calderon et al., 1993; Jeandet et al., 1991; Langcake and Pryce, 1976; 1977; Sarig et al., 1997). From veraison to the last stages of maturation of berries a negative correlation has been reported between the capability of the fruit to synthesize resveratrol and the marked changes in the susceptibility of berries to pathogenic fungi, such as *B. cinerea* (Jeandet et al., 1991). SS is encoded by a small multigene family, several members of which have been cloned (Goodwin et al., 2000; Melchior and Kindl, 1990; 1991; Schröder et al., 1988; Wiese et al., 1994). Their expression analysis in grapevine was limited to cell suspension cultures elicited with fungal cell preparations where a marked induction of some of these genes has been observed (Goodwin et al., 2000; Melchior and Kindl, 1991). Curiously, *in planta* analyses dealt only with heterologous species transformed with constructs based on SS promoter regions or full SS genes. Expression of SS has been reported in transgenic barley, oilseed rape, potato, rice, tobacco, tomato and wheat, resulting in a significant increase of resistance to a number of phytopathogenic fungi (Hain et al., 1993; Leckband and Lorz, 1998; Schubert et al., 1997; Stark-Lorenzen et al., 1997; Thomzik et al., 1997).

Recently, two classes of PR proteins from leaves or berries have been detected, analyzed and/or cloned (cDNA) namely chitinases, by far the most studied proteins of this class (Busam et al., 1997; Derkel et al., 1996; 1998; 1999; Robinson et al., 1997), β-1,3-glucanases (Derkel et al., 1998; 1999; Kraeva et al., 1998; Renault et al., 1996; Robinson et al., 1997; Salzman et al., 1998) and thaumatin-like proteins (Salzman et al., 1998; Tattersall et al., 1997). Two fundamental conclusions can be drawn from these studies: many of these proteins exist as isoforms (up to 13 were detected for chitinases (Derkel et al., 1996)) and they exhibit a differential expression (measured at the protein or transcript levels). Moreover, the fact that certain of these proteins showed antifungal properties, raises the question whether their accumulation might be sufficient to bring full protection against pathogens. It should be noted, however, that field observations have suggested that grape berries are increasingly more susceptible to *B. cinerea* attacks as they mature.

The development of efficient tools for the regeneration of *Vitis* plants via somatic embryogenesis and genetic transformation of embryogenic cells (Coutos-Thévenot et al., 1992; Mauro et al., 1995) allowed us to embark on a programme aimed at increasing the resistance of grapevine to pathogens through genetic transformation. The first step of our strategy relies on the availability of a controlled system to induce defense reactions, leading to a hypersensitive response (HR) and which does not depend on environmental factors, such as humidity which is important for fungal spore germination. For *V. vinifera*, we have chosen an approach analogous to that used successfully for analysing the defense reactions of *Cucumis sativus* (Rasmussen et al., 1991) and *Medicago sativa* (Esnault et al., 1993). This is based on a non-host plant/bacterium interaction following infiltration of

leaves with a suspension of *Pseudomonas syringae* pv. *syringae* and *pisi*, respectively.

In this paper, characterization of the *Vitis*/*P.s.* pv. *pisi* interaction at the pathological and molecular levels is presented. We show that a characteristic hypersensitivity response as well as expression of marker genes involved in defense reactions are induced. We have analyzed the expression of the SS gene as a reference marker as well as a gene encoding a class 10 PR protein, isolated for the first time from grapevine. Although the accumulation of transcripts from defense-related genes has been well characterized, it has also been shown that the increase of transcription does not always correlate with a significant increase in extractable enzymatic activities involved in the synthesis of phytolaxins, such as isoflavonoids (Sallaud et al., 1997), or lignin and wall-esterified phenolic compounds (Ni et al., 1996). Consequently, we analyzed the expression of the *SS* and *PR10* genes both at transcript as well as 'product' levels, i.e. resveratrol accumulation in the case of SS and immunodetection of the polypeptide for the PR10. In our experimental conditions we demonstrated a strong accumulation *in planta* of the corresponding transcripts, followed by the production of resveratrol and the PR10 protein. Taken together, our data allowed us to conclude that this experimental system is well suited for the analysis of the *V. vinifera* defense reactions.

Materials and methods

Plant materials

In vitro plantlets of *Vitis vinifera* L. cv. Ugni Blanc (gift from 'Moët et Chandon' Research Center) were obtained by multiplication through *in vitro* microcuttings on modified Murashige and Skoog medium pH 5.8 (half concentration of macroelements and glycine at 2 ng μl^{-1} as a nitrogen source), supplemented with 20 g l^{-1} sucrose, 7 g l^{-1} agar and active 0.5 g l^{-1} charcoal. Cultures were maintained at 27 °C, 80% relative humidity and 16/8 h day (at 230 $\mu E\ m^{-2}\ s^{-1}$) and night cycles. Three weeks old plantlets were acclimatized in a growth chamber (22 °C, relative humidity of 70% , photoperiod 16 h day at 90 $\mu E\ m^{-2}\ s^{-1}$), grown on a soil-vermiculite mixture (2 : 1, v/v) to a height of 25cm prior to use for experiments on leaves.

Bacterial infection

Pseudomonas syringae pv. *pisi* race 2 (strain 11 from the collection of the Phytpathology Department, INRA, Versailles) was grown and prepared as previously described (Esnault et al., 1993). The bacterial suspension (10^9 cells per milliliter in 10 mM $MgCl_2$) was infiltrated (3–6 infiltration spots per leaf depending on leaf size) into the three upper leaves of grapevine plants using a syringe. Control leaves were infiltrated with 10 mM $MgCl_2$. Leaves were collected 1.5–96 h after infiltration and were immediately frozen in liquid nitrogen.

Monitoring bacterial growth

The number of viable bacteria per square centimeter of leaf was determined by punching out disks of leaf tissue with a cork borer (0.5 cm in diameter) from within the inoculated area (outlined with a non-toxic marker immediately after infiltration) or from a zone adjacent to the inoculated area. The disks were ground with a mortar and pestle in 10 mM $MgCl_2$, and the number of viable bacteria was determined by plating on YDA solid medium.

RNA isolation

RNA extraction was performed using the extraction buffer as described by Chang et al. (1995) and was followed by a chloroform purification. Each aqueous phase was adjusted to 8 ml with extraction buffer containing also 0.2 g ml^{-1} of $CsCl_2$, laid on 3 ml of $CsCl_2$ cushion (5.7 M $CsCl_2$, 10 mM Na_2EDTA and 10 mM Tris–HCl pH 7.5) and spun off at 35,000 rpm, in a SW41Ti rotor (Beckman), at 22 °C for 18 h. The RNA pellets were dissolved in DEPC-treated water and further purified through a phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v) extraction followed by chloroform extraction prior to ethanol precipitation.

Northern analysis

Total RNA (10 μg/track, as estimated by A_{260}) was denatured and fractionated on agarose gel as described by Sallaud et al. (1995) and vacuum-blotted on nylon membrane (Nx-Hybond, Amersham). Fixation and hybridization of the membranes were performed as advised by the manufacturer. The quality and the

amount of RNAs were verified by staining the membrane with methylene blue. Probes were prepared by random-priming or PCR labeling with α ^{32}P dCTP (3000 Ci/mmol). Final washes were in SSPE 0.5×, 0.1% SDS at 50 °C or 65 °C for SS or PR10 hybridization, respectively. Blots were exposed on Kodak X-OMAT AR films with an intensifying screen at −80 °C.

PR10 cDNA cloning through RT-PCR amplification

Degenerate oligonucleotides (Esnault et al., 1993) were used in a RT-PCR amplification (Access RT-PCR System, Promega), containing 1 µg of total RNA extracted from leaves harvested 24 h after infection with *P.s.* pv. *pisi*. Cycles were as recommended by the manufacturer with an annealing temperature of 45 °C. After electrophoresis on an agarose gel, the band of the expected size was excised and the amplification products extracted and purified by using a QIAquick Gel extraction Kit (Qiagen). The products were blunt-ended with the T4 DNA polymerase, phosphorylated and ligated into Bluescript SK+, previously digested by *Sm*aI. *E. coli* DH5α cells were used for transformation.

PR10 gene cloning

We used the Universal Genome Walker Kit (Clontech) implying two successive PCR amplifications from a pool of adaptor-ligated genomic DNA fragments obtained by various restriction enzymes. The first amplification step needs an outer adaptor primer (AP1) and an outer gene-specific primer (GSP1) whereas the second, made from a dilution of the first, involved 'nested' primers, AP2 and GSP2, respectively. Primers AP1 and AP2 were provided by the manufacturer. This procedure was applied to extend the known cDNA sequence (see Figure 4) towards the 3′ end using the oligonucleotides O1 (GSP1: 5′-{807}-GAACTTCACATTCAGCTACACTGTGG-{831}-3′) and O2 (GSP2: 5′-{829}-GTGGTTGACGGAGATGTTTTGACGGG-{854}-3′). For the sake of clarity, the oligonucleotide positions, given in brackets, correspond to the numbering of the genomic sequence. Towards the 5′ end, the oligonucleotides O3 (GSP1: 3′-{832}-CAACTGCCTCTACAAAATGCCCGCC-{857} - 5′) and O4 (GSP2: 3′-{810}-GAAGTGTAAGTCGATGTGACACCAAC-{835}-5′) were used. The two fragments generated were isolated and sequenced, and from these sequences a new set of primers O5 (5′-{1}-TTCCTGTTGTGCTCTACATATCGTT-{25}-3′) and O6 (3′-{1211}-TCATAAACACTACAAGATAACGGTA-{1235}-5′) were designed in order to ascertain colinearity of the 5′ and 3′ fragments.

DNA sequencing

Sequencing was performed by using an 'Applied Biosystems 373A' apparatus (Perkin-Elmer) and the Big Dye Terminator detection system.

Protein analysis and immunological procedures

The method developed for grapevine tissues (Tattersall et al., 1997) was used, except that protein dissolution (final step), was made with 100 mM KOH. Protein concentration was determined by using the Bradford protocol. SDS PAGE was performed by using Tris–Tricine 16.5% gels (Mini Protean II Ready Gels, Biorad). After blotting, the proteins were visualized by dipping the membrane into 15 ml of 50% ethanol/10% acetic acid supplemented with 100 µl of Amido black solution (1% in methanol). Immunodetection used primary antibodies (at 1/1000) directed against a recombinant PR10 from *Medicago sativa*, obtained as described by Sikorski (1997), followed by biotinylated anti-rabbit Ig (RPN1004, Amersham Life Science) and a streptavidine alkaline phosphatase conjugate (RPN1234, Amersham Life Science) with a BCIP/NBT substrate solution (NEL937, from NEN).

Isolation of phenylpropanoid derivatives

Leaves (200 mg) were frozen and ground in liquid nitrogen. The resulting powder was mixed with 10 ml of pre-cooled medium consisting of 8 volume of ethanol and 2 volume of extracting buffer (0.1 M Na acetate + 20% ammonium sulfate, pH 3.4 (Fleuriet and Macheix, 1972)). After 30 min at 4 °C with gentle mixing and subsequent centrifugation at 3000 × g, the supernatant was dried with a N_2 gas stream (50 °C). The dried compounds were dissolved in 1 ml of 80% ethanol and passed through C_{18} Maxi-Clean cartridges. The phenylpropanoid derivatives were recovered in the flow through (1.5 ml of 80% ethanol), dried with N_2 (50 °C) and dissolved in 2 ml of extracting buffer. This solution was then extracted three times with equal volumes of diethyl ether followed by ethyl acetate; the organic

phases were evaporated under a gas stream and the pellets dissolved in 100 µl of solvent A (see below). The remaining aqueous phase (containing the hydrophilic compounds, i.e., the aglycone conjugated forms) was also evaporated to dryness and the compounds dissolved in 500 µl of solvent A.

Analytical HPLC and resveratrol quantification

Aliquots (20 µl) were loaded onto a Shimadzu HPLC system fitted with a 7.5 × 250 mm Hypersil C18-5 µm column, preceded by a guard column and kept at constant temperature through a column cooler (Model 7955 from Jones Chromatography). Elution was at 1 ml min^{-1} from 0 to 60 min with a linear gradient from 20 : 80 : 0.1 (solvent A) to 80 : 20 : 0.1 (solvent B) methanol : water : trifluoroacetic acid. Eluted compounds were monitored with a diode array detector (SPD-M10A, from Shimadzu).

For resveratrol quantification, resveratrol, supplied by the Extrasynthèse SA (BP62, 69726 Genay, France), was used as an external standard and analysed in the same conditions.

Results

Phytopathological analysis

Infiltration of *V.vinifera* leaves with a suspension of *P.s.* pv. *pisi* at a concentration of 10^9 cells ml^{-1} gave rise to symptoms expected for an incompatible interaction. Infiltrated leaf tissue began to collapse around 9 h after inoculation and necrosis of the inoculated zone was clearly evident within 24 h (Figure 1). The necrosis remained restricted to the infiltrated area up to at least 7 days after infiltration (data not shown). The control plant did not show any symptoms, except for a slight wounding response.

The growth of the bacterial population was examined for up to 9 days in both infiltrated and noninfiltrated zones, as described in the Materials and Methods. As shown in Figure 2, the number of bacteria did not vary significantly during the first day. Thereafter the number of viable *P.s.* pv. *pisi* cells decreased until two days after infiltration when no bacteria could be reisolated from the infiltrated area. Moreover, it appeared that the bacterial population was strictly limited to the infiltrated area, as expected for incompatible pathogenic bacteria.

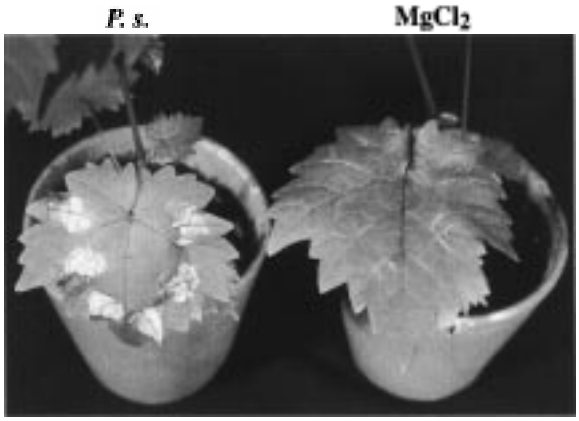

Figure 1. Reactions of *Vitis vinifera* leaf to injection with *P.s.* pv. *pisi*. Leaf blades were infiltrated with either bacterial suspensions (10^9 cfu ml^{-1} in 10 mM $MgCl_2$) or with 10 mM $MgCl_2$ as control. Photographs were taken 24 h after injection.

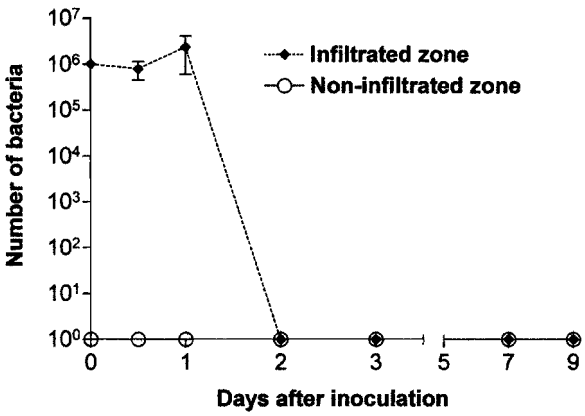

Figure 2. Growth of *P.s.* pv. *pisi* populations in *V. vinifera* leaves. Leaves were injected with this bacterium at 10^9 cfu ml^{-1} in 10 mM $MgCl_2$; leaf disks were punched from the infiltrated and the non-infiltrated zone and ground in 10 mM $MgCl_2$. The number of colony-forming units per disk was determined by serial dilution and plating and expressed per square centimeter. Due to the logarithmic scale the error bars appear asymmetric.

Molecular analysis

Accumulation of stilbene synthase transcripts and resveratrol during V. vinifera P.s. *pv.* pisi *interaction*

In order to gain a first insight into the possible involvement of the grapevine phytoalexin production pathway in the incompatible interaction, we monitored this process using a SS gene (*Vst1*) as a probe (kindly supplied by Dr H. Kindl). A time-course experiment was carried

out with leaves infiltrated with either $MgCl_2$ or *P.s.* pv. *pisi*, and the pattern of the SS transcript accumulation was determined by northern analysis. As shown in Figure 3A, the accumulation of the SS transcripts (1.5 kb) were clearly detectable 6 h after bacterial injection and remained at a high level through the assay period. In addition, a lower hybridizing band was also observable and may correspond to the expression of related genes, such as chalcone synthase which shares nearly 80% similarity with the SS.

To determine whether this transcript accumulation was followed by the production of resveratrol, we conducted an HPLC analysis by using extracts from leaves harvested 6–96 h after infiltration with $MgCl_2$ or the *Pseudomonas* suspension. Our extraction protocol, based on sequential extractions (see Material and methods), allowed us to separate the aglycones, split into apolar compounds soluble in diethyl ether (such as free resveratrol) and compounds of intermediate polarity (soluble in ethyl acetate) from polar molecules remaining in the water phase and represented by aglycone conjugated forms. As shown in Figure 4A, analysis of leaves extracted 48 h after infiltration with $MgCl_2$ (Ctrl) or with *P.s.* pv. *pisi* (+ P.s.), revealed the accumulation of high levels of a compound identified as resveratrol by virtue of its UV spectrum and co-chromatography with resveratrol standard (having a retention time (Rt), in our experimental conditions of 32.3 min) in the bacterium-infiltrated leaves only. No marked differences were detected in the ethyl acetate or water phases. The time course of resveratrol accumulation is illustrated in Figure 4B. Resveratrol began to accumulate at around 9 h post-inoculation and reached a maximum at around 48 h; thereafter the resveratrol amount remained at a high level until at least 96 h post-inoculation.

PR10 cloning and expression

Assuming that genes encoding pathogenesis-related proteins exist in *V. vinifera*, we developed a PCR approach using a cDNA population synthesized from RNA of leaves infiltrated with the suspension of *P.s.* pv. *pisi*. Given that no grapevine sequence encoding PR10 protein was available, we used degenerate oligonucleotides which had been successfully used for the isolation of the alfalfa PR10 clones (Breda et al., 1996; Esnault et al., 1993).

RNA extracted from leaves harvested 24 h post-inoculation was used for RT-PCR analysis. The PCR products from 5 out of the 14 detected positive clones were double strand sequenced. These clones were found to correspond to fragments of 298 nucleotides, ranging from 99% to 100% identity and encoding aminoacid sequences which, from a Fasta search and

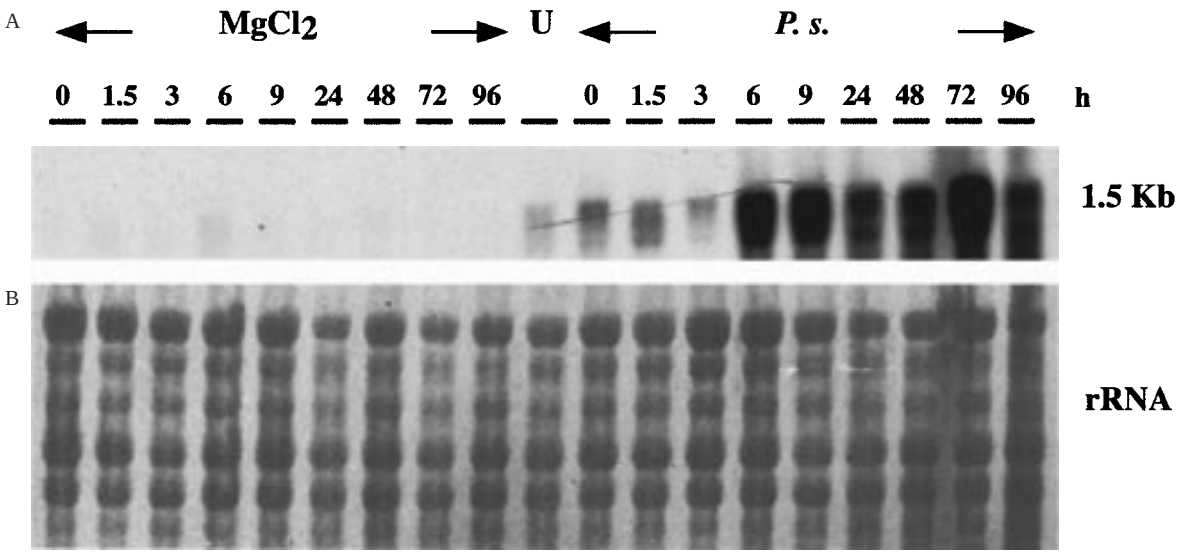

Figure 3. Time course of the accumulation of the SS transcripts upon infection with *P.s.* pv. *pisi* determined by a northern hybridization. Total RNA extracted from leaves 1.5 to 96 h after infection with the bacterial suspension (*P.s.*), infiltration with 10 mM $MgCl_2$ or untreated leaves (U) was used for northern hybridization (10 μg of RNA per well). (A) Hybridization was carried out by means of the *Vst1* gene. (B) For loading comparison, the amounts of rRNA loaded onto the lanes are shown as methylene blue staining.

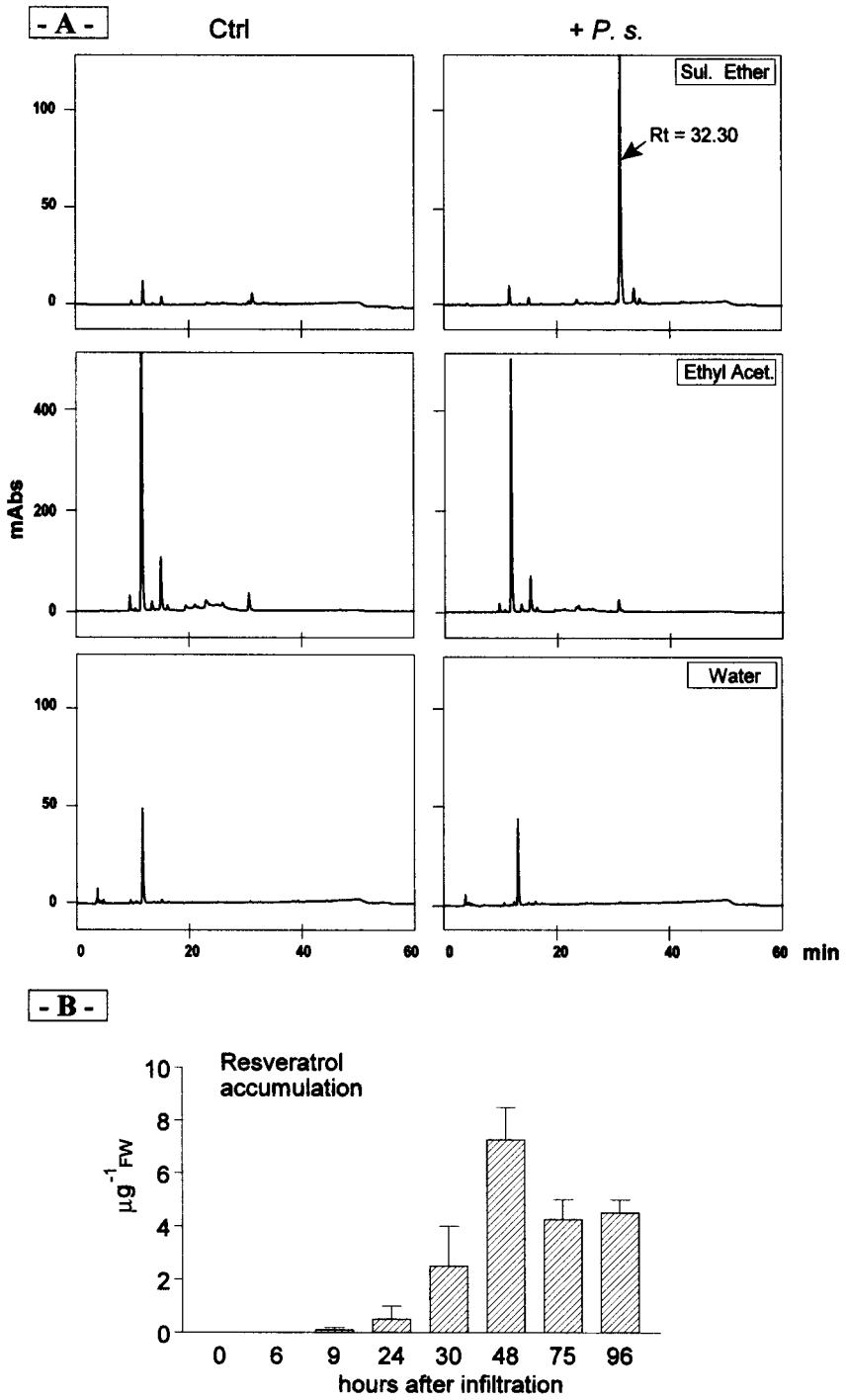

Figure 4. HPLC analysis of extracts from leaves infiltrated with $MgCl_2$ or with *P.s.* pv. *pisi* (-A-) and time course accumulation of resveratrol (-B-). (-A-), Chromatograms of extracts from leaves harvested 48 h after infiltration with $MgCl_2$ (Ctrl) or with a suspension of *P.s.* pv. *pisi* (+*P.s.*) and split into compounds soluble in diethyl ether, ethyl acetate or water. Slope of the gradient used for chromatographic profiles is always 20 : 80 : 0.1 to 80 : 20.1 (v/v/v) methanol : water : trifluoroacetic acid in 0–60 min. The retention time (Rt) is indicated in minutes (-B-), Kinetics of the relative accumulation of resveratrol in bacterium-infiltrated leaves. The amount of resveratrol is calculated from the peak size area from the HPLC chromatograms, using an external standard (authentic *trans*-resveratrol) and expressed in µg of resveratrol per g of fresh weight. Error bars indicate standard deviations, from 3 independent experiments.

a ClustalW alignment (data not shown), were homologous to *PR10* gene products. Four of these amino acid sequences were identical and the fifth differed by one amino acid due to a variation of the second nucleotide (in position 40 of the cDNA, see Figure 5) of the corresponding codon, i.e., AGT/AAT (serine/asparagine). The nucleotide and deduced amino amino acid sequences of one of these clones is presented in Figure 5.

For isolating a genomic sequence which might correspond to the cDNA depicted in Figure 5, we used the Universal GenomeWalker™ Kit (Promega) with oligonucleotides which were designed (see Table 1, Material and Methods) from the known sequences and allowing to extend it in both directions (towards the 5′ and the 3′ regions) leading to slightly overlapping sequences. To ensure colinearity of the two independent PCR products, another PCR was performed using oligonucleotides corresponding to the known extremities. Sequencing of this product confirmed the colinearity as well as showing nearly full identity with that of the cDNA. The genomic sequence and the cDNA differed by 2 out of 298 nucleotides; a conservating change of T to C was found at positions 539 and 759 (numbering of the genomic sequence, Figure 5). This genomic sequence, thereafter designated as *VvPR10-1*, is 1235 nt in length and includes a promoter region of around 380 nt with a TATA box as well as 3 putative CAP sites, an intron of 121 nt and a coding region of 477 nt (encoding a polypeptide of 159 aa). An alignment of the deduced full amino acid sequence with around 50 other PR10 proteins from various species confirmed that the deduced sequence belonged to this class. The *Vitis* PR10 protein is related to proteins expressed in some deciduous trees, belonging to *Betaluceae, Fagaceae* or *Rosaceae* families. However, it is more closely related to the PR10 expressed in *Fagus silvatica* as the two proteins exhibit 67.5% identity and 80.6% similarity, taking into account the equivalence of amino acids {F/Y, K/Q/R, S/T, V/I/L/(M)} (Risler et al., 1988).

Expression analysis of the PR10 gene(s) was conducted by using the cDNA clone (the sequence shown in Figure 5) as a probe for northern hybridization. The time course experiment was between 0 and 96 h after infiltration with either $MgCl_2$ or with the *P.s.* pv. *pisi* suspension. The Northern blot (Figure 6) shows that PR mRNA (around 0.8 kb) was expressed in leaves infiltrated with the bacterial suspension; in this experiment no attempt was made to distinguish between infiltrated and non-infiltrated zones. Its level was already high 6 h post-inoculation, and its accumulation further increased up to 48 h post-inoculation. In contrast, this transcript was practically undetectable in untreated leaves but accumulated slightly and transiently (peaking at around 6 h) in $MgCl_2$-infiltrated leaves.

Analysis at the transcriptional level was complemented by Western immunoblotting. Protein extracts were prepared from leaves harvested from 24 to 96 h post-inoculation and analyzed by immunoblotting using polyclonal antibodies directed against the *M. sativa* PR10 protein. Two controls were included: the recombinant protein used to raise the antibodies and a protein extract from *M. sativa* leaves harvested 96 h after infection with *P.s.* pv. *pisi*. As shown in Figure 7, no polypeptide reacting with these antibodies was detectable in the $MgCl_2$-infiltrated leaves whereas the leaves infiltrated with the bacterium suspension produced a single band corresponding to a polypeptide of approximately 17 kDa, the accumulation of which remained high up to, at least, 96 h post-inoculation. The immunodetected PR10 protein from *V. vinifera* was clearly above the PR10 protein detected from *M. sativa*, corroborated the theoretical values calculated from the amino acid sequences; the M_r values were 17,233 and 16,625 Da (Breda et al., 1996), respectively. One may conclude that the accumulation of transcripts coding for the PR10 protein was followed by the production of the encoded polypeptide.

Discussion

Pathological studies in *Vitis vinifera* have previously been focused on interactions with fungi or on cell suspension cultures treated with elicitors and as such a characteristic incompatible interaction was lacking. In this study, we have developed a system based on a non-host interaction, i.e., infiltration of the leaf blade with a suspension of *Pseudomonas syringae* pv. *pisi*. To date, there is no report on the analysis of the interaction of *V. vinifera* leaves with any bacterial suspension. To the best of our knowledge, the only report where bacterial effects were studied concerns the analysis of a chitinase mRNA accumulation following the addition of a suspension of *P.s.* pv. *syringae* to cultured cells of *V. vinifera* (Busam et al., 1997). In our experimental conditions, the *P.s.* pv. *pisi* isolate, infiltrated at a

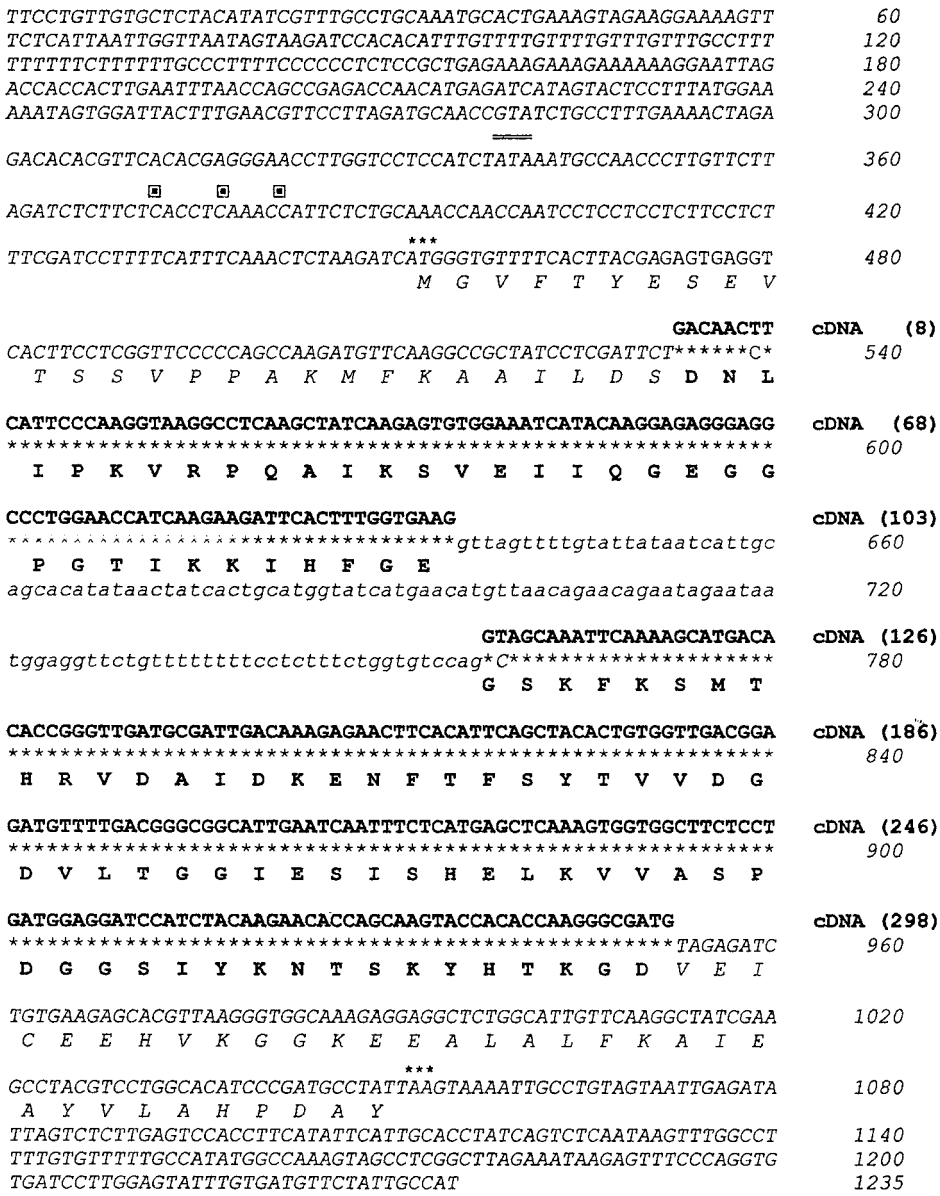

Figure 5. Nucleotide sequences of cDNA and genomic clones from *V. vinifera* encoding a PR10 protein. The cDNA (bold capitals) was isolated by RT-PCR and the genomic clone (italic capitals, except for the intron in small letters) was obtained by using the GenomeWalker Kit (Promega). Identical nucleotides, from the genomic clone, are indicated by *. The deduced amino acid sequences are in Roman (cDNA) or italic (gene). For the genomic sequence, a TATA box is superlined whereas the putative start transcription sites (determined with the PC-Gene software) are indicated by [•]. The start and stop codons are indicated by ***.

concentration of 10^9 cfu ml^{-1}, rapidly elicited (within 24 h) localized necrotic lesions typical of a HR (Figure 1) whilst the bacteria were unable to spread into the leaf blade (Figure 2). These effects are characteristic of an incompatible interaction, as observed after leaf infiltration with the same bacterium into another non-host plant, *M. sativa* (Esnault et al., 1993). Our system is to our knowledge the first example of a bacterial infection leading to an incompatible interaction with the cultivated *Vitis.* Based on an *in planta* interaction

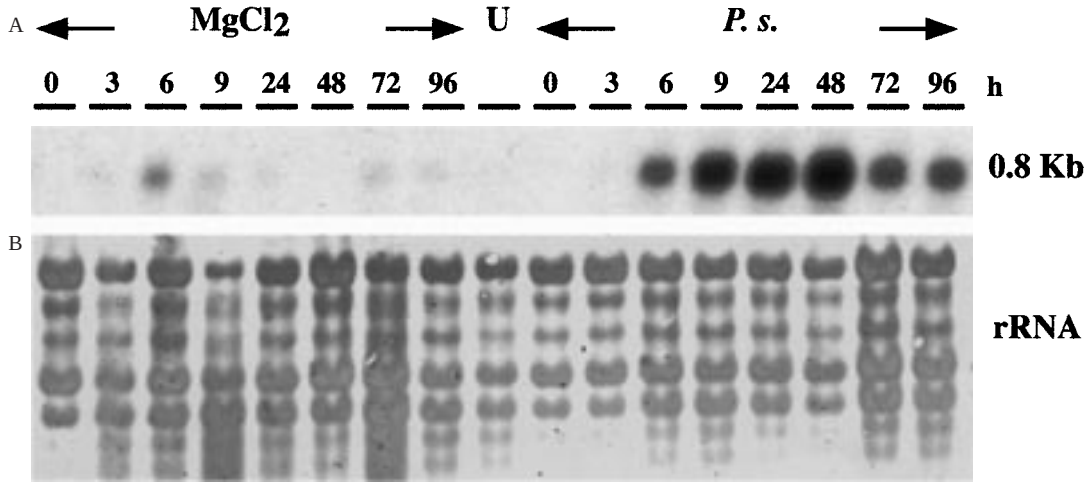

Figure 6. PR10 transcript accumulation in grapevine leaves infected with *P.s.* pv. *pisi*. Total RNA (10 μg per lane) isolated from leaves harvested 3–96 h after infiltration with *P.s.* pv. *pisi* or $MgCl_2$ or from untreated leaves (U) was used for northern hybridization were loaded. (A) Hybridization g with the cDNA clone illustrated in Figure 5. (B) For loading comparison, the amounts of rRNA loaded onto the lanes are shown as methylene blue staining.

this system does not suffer from the drawbacks of the cell culture suspension. Moreover, it is independent of variations in environmental factors which often make it difficult to control fungal diseases evoked by, for example, *Plasmopora viticola* and *Uncinula necator*, the causal agents of downy and powdery mildew diseases to which grapevine is particularly sensitive in Europe.

To further characterize this interaction, we have analyzed the expression of two defense-related genes, one encoding SS involved in the *Vitis* phytoalexin synthesis and the second coding for a newly identified PR protein of class 10 which might present a ribonucleolytic activity (Moiseyev et al., 1997).

Using the *Vst1* gene as a probe we have shown a marked SS transcript accumulation upon *P.s.*pv. *pisi* infiltration. The transcript level was already high 6 h post-inoculation and remained at a high level up to at least 96 h. Comparable data have been obtained with transgenic tomato leaves harboring two *V. vinifera* SS genes and infected with *Phytophthora infestans* (Thomzik et al., 1997). More rapid kinetics has been detected in cell suspensions from *Vitis* sp. (Melchior and Kindl, 1991; Wiese et al., 1994) and from transgenic tobacco transformed with the construct used also for tomato (Hain et al., 1993), and treated with elicitors. In these cases, accumulations were observed at 6–8 h. These kinetic differences may be due to the model systems analyzed: cell-suspension cultures cannot reflect all aspects of the complexity of plant-microbe interactions and in particular differences in the signal transduction pathways following elicitor or whole pathogen recognition by the plant cells might be involved. Furthermore, SS is encoded by a small multigene family (Goodwin et al., 2000; Melchior and Kindl, 1991; Wiese et al., 1994) and it seems that all members of this family are elicitor responsive, albeit with marked differences in the kinetics and intensity of their expression (Wiese et al., 1994). For the analysis of the SS transcript accumulation during the *V. vinifera*/*P. syringae* pv. *pisi* interaction, probes were generated with the *Vst1* gene and consequently we cannot ascertain whether related genes were expressed. Moreover, an induction of SS late responsive gene(s) might also account for differences in the kinetics of expression.

SS is the key enzyme for the production of stilbenes which are natural compounds occurring in a number of plant families, including the *Vitaceae*. Grapevine stilbenes behave like phytoalexins when the plant is attacked by fungi, such as grey mould (Bavaresco et al., 1997; Jeandet et al., 1991; 1995; Langcake, 1981), downy mildew (Dercks and Creasy, 1989; Langcake et al., 1979) or berry rot (Sarig et al., 1997). In most cases, the detectable stilbenes are *trans*-resveratrol, ε-viniferin, α-viniferin and pterostilbene produced in leaves or berries, the viniferins sometimes accumulating to higher level than resveratrol (Jeandet et al., 1991; Langcake, 1981). Moreover, disease-resistant

grapevine (interspecies hybrids or American species) show a rapid and high level of accumulation of phytoalexins in leaves (and berries) after elicitation, while in susceptible plants (*V. vinifera*) phytoalexin synthesis is rather slow and limited (Bavaresco et al., 1999). Consequently, it was important to determine whether in our experimental system the SS transcript accumulation was followed by an enhanced resveratrol production. Our HPLC analyses indicate (Figure 4) that the recognition of *P.s.* pv. *pisi* by the leaf blade cells led to resveratrol production, which was detectable 9 h post-inoculation and reached its maximum at around 48 h, in agreement with data obtained from transgenic tomato harboring stilbene synthase genes from *Vitis* (Thomzik et al., 1997). The highest value detected under our experimental conditions was around 8 μg per g of fresh weight which is comparable with resveratrol accumulation in *V. vinifera* or *V. riparia* leaves in response to infection with *B. cinerea* or *P. viticola*, respectively: 7–12 $\mu g\ g^{-1}$ (fresh weight) (Langcake, 1981). However, these values are far lower than those determined after UV irrradiation of leaves: values ranging from 75 (Langcake, 1981) to around 300 $\mu g\ g^{-1}$ (fresh weight) (Jeandet et al., 1991) were obtained. Nevertheless, in our experimental conditions, the major accumulated compound was by far the resveratrol. Viniferins, having higher retention times than resveratrol, were barely detectable (see Figure 4). Our experimental procedure was focused on resveratrol analysis and we cannot exlude that extraction and/or analysis methods adapted to the study of the viniferins would lead to the same conclusions. Moreover, our results might also reflect the physiological and molecular particularities of a plant/bacterium interaction *versus* interactions implying attacks by a given fungus.

As a second marker we have chosen a putative defense gene encoding a class 10 PR proteins (Van Loon et al., 1994). This class includes genes which have been identified from a wide range of flowering plants including both dicots and monocots and are homologous to the *Betv1* gene, encoding the 17 kDa major allergenic protein of birch pollen (Gajhede et al., 1997). In all the analyzed species they exist as a multigene family (Wen et al., 1997). We succeeded in isolating a PCR amplification product, allowing us to clone the corresponding gene, named *VvPR10-1*. The amino acid sequence deduced from this genomic sequence consists of 159 aa, with a M_r of 17 233 kDa, and presents the 'Pathogenesis-BETV1' signature (Prosite). Its homology with the PR10 proteins found in some deciduous trees is significantly higher (67.3% identity and 80.5% similarity with the PR10 from *Fagus sylvatica*) than, for example, with PR10 proteins from herbaceous species (such as *M. sativa):* 53.4% identity and 63.5% similarity with the *Ms*PR10-1. This genomic sequence (*VvPR10-1*) contains only a relatively short promoter region (around 380 bp) and an intron of 121 bp. The promoter region of *VvPR10-1* is too short to reveal putative *cis* elements but a comparison of the organization of the *VvPR10-1* and the *Betv-1* homologues indicated that the *VvPR10-1* intron is located in a position conserved in all the PR10 genes (Wen et al., 1997).

Using the cloned RT-PCR-amplified product as a hybridization probe (Figure 6), RNA blot analyses demonstrated that the level of a 0.8 kb mRNA increased during the incompatible interaction of leaves with *P.s.* pv. *pisi*. The kinetics of its accumulation are in accordance with the results obtained during the interaction of pea pods (Daniels et al., 1987) or alfalfa leaves (Esnault et al., 1993) infected with *P.s.* pv. *pisi*. The expression of the alfalfa PR gene exhibited its maximum level at around 48 h and decreased thereafter, albeit remaining at a fairly high level. Given that we have not used a specific probe for the Northern analyses, we cannot exlude the possibility that several genes were expressed.

Despite the relatively low homology and the difference in the Mr between the VvPR10-1 and MsPR10-1 polypeptides, the antibodies directed against the *Ms*PR10-1 cross reacted with the *Vitis* protein (Figure 7). This recognition strongly suggest that the *VvPR10-1* gene expressed efficiently not only at transcriptional but also translational level, as indicated by the accumulation of the encoded protein in the leaf blade cells.

All the presented data indicate that our experimental system, based on the non-host interaction of grapevine with the bacterium *P.s.* pv. *pisi* is perfectly suitable for the analysis of the defense reactions of this economically important plant. This is particularly important for studies aimed at strenthening the endogenous defense capabilities of the plant through genetic transformation. In our opinion, this system is well suited for identifying endogenous gene(s) expressed at the vicinity of the pathogen ingress and the encoded proteins which may have antipathogenic properties. The information which may be obtained from these studies will provide the means to analyse the plant reaction in vineyard conditions, when either leaves or berries are attacked by pathogens. Comparison of the expression of defense genes in our experimental conditions and in

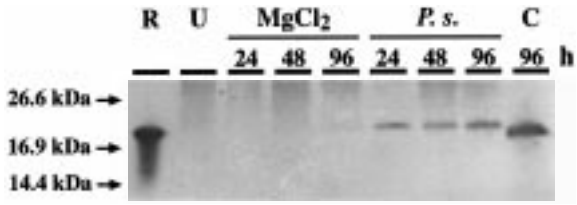

Figure 7. Immunodetection of PR10 proteins in leaf extracts after infiltration with $MgCl_2$ or *P.s.* pv. *pisi* (*P.s.*). Approximately 45 μg of protein extracted from untreated leaves (U) or leaves harvested 24, 48 or 96 h after infiltration with the $MgCl_2$ solution or the bacterial suspension (*P.s.*) were deposited in each well. Lane R contains 1.5 ng of *Ms*PR10-1 recombinant protein whereas lane C contains 10 μg of a protein extract from *M. sativa* leaves harvested 96 h after infiltration with the same bacterial suspension. The antibodies used for this immunostaining were directed against the recombinant *M. sativa* protein. Size of molecular mass markers are indicated in kilodaltons.

interactions occurring in the vineyard may be useful for searching genes which can reinforce the defense capabilities of cultivated *Vitis.*

Acknowledgements

The authors wish to express their gratitude to Dr M.M. Sikorski (Institute of Biological Chemistry, Poznan, Poland) for preparing the recombinant PR10 protein from *M. sativa,* to Dr H. Kindl (University of Marburg, Germany) for providing the DNA probe for stilbene synthase, to Dr James M. Bauly for a careful checking of the final English version, to the members of the "Centre de Recherche Moët et Chandon", Epernay, for their help and useful discussions. We are indebted to the "Ministère de l'Education Nationale, de la Recherche et de la Technologie" for research support (Biotechnologies, N°97C.0138 and 97C.0139) and to Dr A. Kondorosi, Director of the I.S.V., for providing the facilities to carry out this research.

References

Bavaresco L, Petegolli D, Cantu E, Fregoni M, Chiusa G and Trevisan M (1997) Elicitation and accumulation of stilbene phytoalexin in grapevine berries infected by *Botrytis cinerea*. Vitis 36: 77–83

Bavaresco L, Fregoni C, Cantu E and Trevisan M (1999) Stilbene compounds: from the grapevine to wine. Drugs Exptl Clin Res XXV: 57–63

Breda C, Sallaud C, El-Turk J, Buffard D, de Kozak I, Esnault R and Kondorosi A (1996) Defense reaction in *Medicago sativa*: a gene encoding a class 10 PR protein is expressed in vascular bundles. Molecular Plant–Microbe Interactions 9: 713–719

Busam G, Kassemyer H-H and Matern U (1997) Differential expression of chitinases in *Vitis vinifera* L. responding to systemic acquired resistance activators or fungal challenge. Plant Physiology 115: 1029–1038

Calderon AA, Zapata JM, Munoz R, Pedreno MA and Barcelo AR (1993) Resveratrol production as a part of the hypersensitive-like response of grapevine cells to an elicitor from *Trichoderma viride*. New Phytology 124: 455–463

Chang M-M, Horovitz D, Culley D and Hadwiger LA (1995) Molecular cloning and characterization of pea chitinase gene expressed in response to wounding, fungal infection and the elicitor chitosan. Plant Mol Biol 28: 105–111

Coutos-Thévenot P, Goebel-Tourand I, Mauro MC, Jouanneau JP, Boulay M, Deloire A and Guern J (1992) Somatic embryogenesis from grapevine cells. I – Improvement of embryo developments by changes in culture conditions. Plant Cell Tissue Organ Cult 29: 125–133

Daniels CH, Fristensky B, Wagoner W and Hadwiger KA (1987) Pea genes associated with non-host disease resistance to *Fusarium* are also active in race-specific disease resistance to *Pseudomonas*. Plant Mol Biol 8: 309–316

Dercks W and Creasy LL (1989) The significance of stilbene phytoalexins in the *Plasmopora*-grapevine interaction. Physiol Mol Plant Pathol 34: 189–202

Derkel JP, Legendre L, Audran JC, Haye B and Lambert B (1996) Chitinases of the grapevine (*Vitis vinifera* L.): five isoforms induced in leaves by salicylic acid are constitutively expressed in other tissues. Plant Sci 119: 31–37

Derkel JP, Audran JC, Haye B, Lambert B and Legendre L (1998) Characterization, induction by wounding and salicylic acid, and activity against *Botrytis cinerea* of chitinases and β-1,3-glucanases of ripening grape berries. Physiol Plant 104: 56–64

Derkel JP, Baillieul F, Manteau S, Audran JC, Haye B, Lambert B and Legendre L (1999) Differential induction of grapevine defenses by two strains of *Botrytis cinerea*. Phytopathology 89: 197–203

Esnault R, Buffard D, Breda C, Sallaud C, El Turk J and Kondorosi A (1993) Pathological and molecular characterizations of alfalfa interactions with compatible and incompatible bacteria, *Xanthomonas campestris* pv. *alfalfae* and *Pseudomonas syringae* pv. *pisi*. Mol Plant-Microbe Interact 6: 655–664

Fleuriet A and Macheix JJ (1972) Séparation et dosage par chromatographie en phase gazeuse de l'acide chlorogénique et des catechines des fruits. J Chromatogr 74: 339–345

Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, van Neerven RJJ, Schou C, Lowenstein H and Spangfort MD (1997) X-ray and NMR structure of Betv1, the origin of birch pollen allergy. Nature Struct Biol 3: 1040–1045

Goodwin PH, Hsiang T and Erickson L (2000) A comparison of stilbene and chalcone synthases including a new stilbene synthase gene from *Vitis riparia* cv. Gloire de Montpellier. Plant Sciences 151: 1–8

Hain R, Reif HJ, Krause E, Langebartels R, Kindl H, Vorman B, Wiese W, Schmelzer E, Schreier PH, Stocker RH and Stenzel K (1993) Disease resistance results from foreign phytoalexin expression in a novel plant. Nature 361: 153–156

Jeandet P, Bessis R and Gautheron B (1991) The production of resveratrol (3,5,4′-trihydroxystilbene) by grape berries in different development stages. Am J Enol Vitic 42: 41–46

Jeandet P, Bessis R, Sbagghi M and Meunier P (1995) Production of the phytoalexin resveratrol by grapes as a response to *Botrytis* attack under natural conditions. J Phytopathol 143: 135–139

Kraeva E, Tesnière C, Terrier N, Romieu C, Sauvage FX, Bierne J and Deloire A (1998) Transcription of a β-1,3-glucanase gene in grape berries in a late developmental period, or earlier after wounding treatments. Vitis 37: 107–111

Langcake P and Pryce RJ (1976) The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury. Physiol Plant Pathol 9: 77–86

Langcake P and Pryce RJ (1977) The production of resveratrol and the viniferins by grapevines in response to ultraviolet irradiation. Phytochem 16: 1193–1196

Langcake P, Cornford CA and Pryce RJ P (1979) Identification of pterostilbene as a phytoalexin from *Vitis vinifera* leaves. Phytochem 18: 1025–1027

Langcake P (1981) Disease resistance of *Vitis spp* and the production of resveratrol, ε-viniferin, α-viviferin and pterostilbene. Physiol Mol Plant Pathol 18: 213–226

Leckband G and Lorz H (1998) Transformation and expression of a stilbene synthase gene of *Vitis vinifera* L. in barley and wheat for increased fungal resistance. Theor Appl Genet 96: 1004–1013

Mansfield J (1990) Recognition and response in plante/fungus interactions. Recognition and Response in Plant-Virus Interactions. Fraser RSS. Berlin. Springer-Verlag. H41. 31–52.

Mauro MC, Toutain S, Walter B, Pinck L, Otten L, Coutos-Thévenot P, Deloire A and Barbier P (1995) High efficiency regeneration of grapevine plants transformed with the GFLV coat protein gene. Plant Sci 112: 97–106

Melchior F and Kindl H (1990) Grapevine stilbene synthase cDNA only slightly differing from chalcone synthase cDNA is expressed in *Escherichia coli* into a catalytically active enzyme. FEBS Lett 268: 17–20

Melchior F and Kindl H (1991) Coordinate- and elicitor-dependent expression of stilbene synthase and phenylalanine ammonia-lyase genes in *Vitis* cv. Optima. Arch Biochem Biophys 288: 552–557

Moiseyev GP, Fedoreyeva LI, Zhuravlev YN, Yasnetskaya E, Jekel PA and Beintema JJ (1997) Primary structures of two ribonucleases from ginseng calluses: New members of the PR-10 family of intracellular pathogenesis-related plant proteins. FEBS Lett 407: 207–210

Ni W, Sewalt VJH, Korth KL, Blount JW, Ballance GM and Dixon RA (1996) Stress response in alfalfa. XXI. Activation of caffeic acid 3-*O*-methyltransferase and caffeoyl coenzyme A 3-*O*-methyltransferase genes does not contribute to changes in metabolite accumulation in elicitor-treated cell-suspension cultures. Plant Physiol 112: 717–726

Rasmussen JB, Hammerschmidt R and Zook MN (1991) Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. *syringae*. Plant Physiol 97: 1342–1347

Renault A-S, Deloire A and Bierne J (1996) Pathogenesis-related proteins in grapevine induced by salicylic acid and *Botrytis cinerea*. Vitis 35: 49–52

Risler JL, Delorme MO, Delavroix H and Hénaut A (1988) Amino acid substitutions in structurally related proteins as a pattern recognition approach. J Mol Biol 11: 1019–1029

Robinson SP, Jacobs AK and Dry IB (1997) A class IV chitinase is highly expressed in grape berries during ripening. Plant Physiol 114: 771–778

Sallaud C, El Turk J, Breda C, Buffard D, de Kozak I, Esnault R and Kondorosi A (1995) Differential expression of cDNA coding for chalcone reductase, a key enzyme of the 5-deoxyflavonoid pathway, under various stress conditions in *Medicago sativa*. Plant Sciences 109: 179–190

Sallaud C, Zuanazzi J, El-Turk J, Leymarie J, Breda C, Buffard D, de Kozak I, Ratet P, Husson P, Kondorosi A and Esnbault R (1997) Gene expression is not systematically linked to phytoalexin production during alfalfa leaf interaction with pathogenic bacteria. Mol Plant-Microbe Interact 10: 257–267

Salzman RA, Tikhonova I, Bordelon BP, Hasegawa PM and Bressan RA (1998) Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defense response during fruit ripening in grape. Plant Physiol 117: 465–472

Sarig P, Zutkhi Y, Monjauze A, Lisker N and Ben-Arie R (1997) Phytoalexin elicitation in grape berries and their susceptibility to *Rhizopus stolonifer*. Physiol Mol Plant Pathol 50: 337–347

Schröder G, Brown JWS and Schröder J (1988) Molecular analysis of resveratrol synthase: cDNA, genomic clones and relationship with chalcone synthase. Eur J Biochem 172: 161–169

Schröder J and Schröder G (1990) Stilbene and chalcone synthase: related enzymes with key functions in plants-specific pathways. Z Naturforsch 45c: 1–8

Schubert R, Fischer R, Hain R, Schreier PH, Bahnweg G, Ernst D and Sandermann H (1997) An ozone-responsive region of the grapevine resveratrol synthase promoter differs from the basal pathogen-responsive sequence. Plant Mol Biol 34: 417–426

Sikorski MM (1997) Expression of *Lupinus luteus* cDNA coding for PR10 protein in *Escherichia coli*: purification of the recombinant protein for structural and functional studies. Acta Biochim Pol 44: 565–578

Stark-Lorenzen P, Nelke B, Hänssler G, Mühlbach HP and Thomzik JE (1997) Transfer of grapevine stilbene synthase gene to rice (*Oryza sativa* L.). Plant Cell Reports 16: 668–673

Tattersall DB, van Heeswijck R and Hoj PB (1997) Identification and characterization of a fruit-specific thaumatin-like protein that accumulates at very high level in conjunction with the onset of sugar accumulation and berry softening in grapes. Plant Physiol 114: 759–769

Thomzik JE, Stenzel K, Schreier PH, Hain R and Stahl DJ (1997) Synthesis of grapevine phytoalexin in transgenic tomatoes (*Lycopersicum esculentum* Mill.) conditions resistance against *Phytophthora infestans*. Physiol Mol Plant Pathol 51: 265–278

Van Loon LC, Pierpoint WS, Boller T and Conejero V (1994) Recommendations for naming plant pathogenenesis-related proteins. Plant Mol Biol Reporter 12: 245–264

Wen J, Vanek-Krebitz M, Hoffmann-Sommerbruger K, Scheiner O and Breiteneder H (1997) The potential of *Betv1* homologues, a nuclear multigene family, as phylogenetic markers in flowering plants. Mol Phylogen Evol 8: 317–333

Wiese W, Vornam B, Krause E and Kindl H (1994) Structural organization and differential expression of three stilbene synthase genes located on a 13 kb grapevine DNA fragment. Plant Mol Biol 26: 667–677