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Immunodetection of PR-1-like proteins in grapevine leaves infected with *Oidium tuckeri* and in elicited suspension cell cultures

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Summary

Three pathogenesis-related (PR-1-like) proteins extractable at pH 8.0 were found to accumulate in grapevine leaves after fungal pathogen (*Oidium tuckeri*) infection. These proteins were called gPR-1 (grapevine pathogenesis-related) proteins. Estimated molecular masses in SDS-containing gels were: gPR-1a 15.5 kDa; gPR-1b 16.8 kDa; gPR-1c 17.7 kDa. Antiserum raised against tobacco PR-1a reacted specifically with grapevine counterparts. Likewise, stimulation of gPR-1 protein accumulation was observed when a set of prototype elicitors was added to grapevine cell suspension cultures. Results with inducing elicitors also showed that the extracellular PR-1-like proteins represent the only isoforms of this prominent group of pathogenesis-related proteins found in grapevine.

Key words: *Vitis vinifera* L., Limberger, protein, elicitor, defense, Western blotting.

Introduction

Pathogenesis-related (PR) proteins accumulate as part of a multicomponent defense response in plants exposed to pathogens and various external stimuli (review: Rigden and Coutts 1988). The best characterized PR proteins are the tobacco PR-1 proteins, a group of three closely related members, PR-1a, PR-1b and PR-1c.

PR-1 proteins represent a dominant group of PRs and their expression is commonly used as a marker of systemic acquired resistance (SAR, Sticher *et al.* 1997). Since their discovery in tobacco cultivars exhibiting the hypersensitive response (HR) after TMV infection (Gianinazzi *et al.* 1970; Van Loon and Van Kammen 1970), numerous researchers have attempted to assess the function of PR-1 proteins in plants, but without much success (Buchel and Linthorst 1999). The PR-1 isoforms have been well characterized in terms of sequence (Matsuoka *et al.* 1987), gene regulation (Beilmann *et al.* 1991), synthesis (Ohashi and Matsuoka 1985), and cellular localization (Carr *et al.* 1987). Moreover, PR-1 proteins are induced under non-pathogenic, developmentally regulated events *e.g.* flowering (Lotan *et al.* 1989) and cytokinin fluctuation (Memelink *et al.* 1987). The structure of tomato PR-1b (P14a) was solved recently by nuclear magnetic resonance and found to represent a unique molecular architecture (Fernandez *et al.* 1997). Likewise, homology searches using the protein database network service revealed no significant homology to any plant gene. A limited homology exists to a subset of very specific proteins from yeast, insects and vertebrates (Van Loon and Van Strien 1999) indicating that the PR-1 family makes a distinct and highly conserved group of proteins.

Although the PR-1 proteins of plants were the subject of much of the earlier work on PRPs and continue to be intensively studied, their apparent biological function(s) remain unknown. Nevertheless, the distribution and location of the PR-1 proteins suggest at least 4 potential functions. First, these proteins may have an interferon-like and/or antifungal activity which induces changes in surrounding healthy plant tissues (Gianinazzi and Kassanis 1974). The study of transgenic tobaccos constitutively expressing the PR-1b gene did not support the latter statement. Cutt *et al.* (1989) and Carr *et al.* (1989) independently demonstrated that the PR-1b protein of tobacco was not sufficient for TMV resistance and thus, the PR-1 proteins may not function as unique antiviral factors. Similarly, Alexander *et al.* (1993) demonstrated that constitutive high-level expression of PR-1a in transgenic tobacco results in tolerance to infection by two oomycete pathogens, *Peronospora tabacina* and *Phytophtora parasitica* var. *nicotianae*. Second, the PR-1 protein may alter the extracellular environment in such a way as to inhibit pathogen infection with the cells and/or tissues. Since the PR-1 proteins have a very limited antifungal activity (Van Loon and Van Strien 1999), it must be concluded that this is not their primary biological function. Third, they may have no direct effect on pathogen replication itself but may be induced as part of a generalized response to plant disease status. Perhaps the PR-1 genes encode proteins involved in protecting plants against many, although not all, kinds of environmental stresses since the expression of the PR-1b gene can be dissociated from TMV resistance (Coutts and Waghi 1983, Asselin *et al.* 1985, Ohashi and Matsuoka 1985). Fourth, they may not play a direct role in the resistance response but may rather function as stress proteins which help to limit the damage caused by pathogens or the host's own response to the pathogens or both. In this context, two wheat cDNAs that encode proteins PR-1.1 and PR-1.2 have recently been cloned by Molina *et al.* (1999)

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who demonstrated that although expression of both of these genes was induced upon infection by *Erysiphe graminis*, the genes did not respond to activators of systemic acquired resistance (SAR), such as SA, benzothiadiazole (BTH), or isonicotinic acid (INA). Alternatively, the novel dark regulation of the PR-1 transcript (EYAL *et al.* 1992) may point to additional nonpathogenesis-related roles for these genes in plant-environment interaction. Taken together, the high extent of sequence conservation of the plant PR-1 proteins from different families is remarkable, but to define their actual function(s) in plants, new strategies need to be devised.

In spite of the considerable amount of data available on PR-proteins in herbaceous plants, to our knowledge there are only a few reports giving some informations on PR-proteins in woody plants. In grapevine, RENAULT *et al.* (1996) and DELOIRE *et al.* (1997) have identified grapevine PR-2 (β-1,3-glucanase) and PR-3 (chitinase) type proteins by immunoblotting tests with tobacco anti-PR-2 and anti-PR-3 antibodies, respectively. The gene encoding the PR-2 protein has been recently cloned and its expression was studied in grape berries at different developmental stages and in wounded berries with or without salicylic acid elicitation (KRAEVA *et al.* 1998).

Even though to date, PR-1 proteins have been identified in approximately 25 plant species, virtually nothing is known about this group of PRs in grapevine. Since the identification and characterization of grapevine PRs with respect to their putative biological function(s) in resistance are our long-term goal, experiments concerning PR-1-like proteins have been initiated. In this paper we present the first information, to our knowledge, about the presence of PR-1-like proteins in pathogen and/or elicitor-treated grapevine leaves and cultured suspension cells, respectively.

Material and Methods

Plant material and cell cultures: Grapevine (*Vitis vinifera* L. cv. Limberger, covar. *orientalis*) plants were grown in a growth chamber at 28 ± 1 °C (RH 60 %) with a 14 h light period (130 W m^{-2}). Alternatively, the rapidly growing cell suspension line D1 (photosynthetically active), derived from a stably propagated Limberger callus culture, was grown in a modified Murashige-Skoog (MS/D, pH 5.8) medium containing 3 % sucrose (w/v), 1 mg l^{-1} NAA and 0.2 mg l^{-1} BAP. Multiplication subcultures were carried out in 250 ml flasks agitated at 110 rpm in an orbitary shaker. Cells were used for experiments 4 d after subculture.

Preparation of elicitors: The fungal cell wall elicitor was prepared from grey mold (*Botrytis cinerea* Pers. *et* Fries, a grapevine isolate) according to the published protocol of MALOLEPSZA and URBANEK (1994). The elicitor was used at a final concentration of 2.4 μg of glucose equivalents (2.2 μg of protein) per ml cell suspension culture.

Crab-shell chitosan (Sigma, Deisenhofen, Germany) was ground to a fine powder and purified following the procedure of EL GHAOUTH *et al.* (1992). Salicylic acid (300 μM, Duchefa, Haarlem, The Netherlands) was prepared in DMSO (0.5 % final concentration) and titrated to pH 5.7. Methyl jasmonate (50 μM MeJA, Duchefa, Haarlem, The Netherlands) was prepared from a stock solution in ethanol (0.1 % final concentration). Both 0.1 % ethanol (EtOH) and 0.5 % DMSO alone were used as control.

Pathogen inoculation and elicitor treatments: Powdery mildew, *Uncinula necator* (Schein.) Burr, a natural isolate, was propagated on grapevine plants in a cabinet with computer-controlled environment. The conidia (*Oidium tuckeri* Berk) were sprayed on grapevine leaves using the KenAir air duster (Kenro, Swindon, UK) and treated plants were transferred to the controlled environment (28 ± 1 °C, RH 60 %, 16 h photoperiod at 130 W m^{-2}). A control experiment was carried out by spraying sterile distilled water instead of the conidia inoculum on the leaves.

Four days after subculture log phase cells were used and treatment with the elicitors was performed in the original flasks in the light to avoid any stress associated with the transfer. Six days after the treatments, the cells and the spent medium were harvested separately for protein extraction.

Preparation of protein extracts: To prepare extracts from control and treated plant material, leaves or cells were mixed with 5 volumes (w/v) of ice-cold TRISEPAC extraction buffer (50 mM Tris-HCl, pH 8.0, 500 mM sucrose, 1 mM EDTA, 0.2 % insoluble PVP, 6 mM ascorbic acid and 0.1 % cysteine) and homogenized using a prechilled mortar and pestle. Homogenates were centrifuged at 20,000 *g* for 10 min at 4 °C, the supernatant was concentrated using solid PEG 8000 and stored at -20 °C until further use.

Extracellular proteins were isolated from the spent medium immediately after the elicitation protocol was completed. Cells were separated from the medium to which solid ammonium sulphate was added to reach 90 % saturation. The precipitated proteins were collected by centrifugation (20 min, 20,000 *g*), dissolved in buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA) and the suspension dialysed against several changes of the same buffer. The protein solution was concentrated and stored as above.

Powdery mildew mycelia (2.0 g) were suspended in ice-cold TRISEPAC buffer (10 ml) and homogenized using a motor-driven Diax 900 homogenizer (Heidolph, Keilheim, Germany) for 5 min at maximum speed. The extract was further processed and stored as described above.

The protein concentration in the extracts was determined colorimetrically by the method of BRADFORD (1976), using BSA as standard.

Analytical SDS-PAGE: SDS-PAGE was performed by the method of LAEMMLI (1970) with a 12.5 % resolving gel and a 5 % stacking gel in a SE 600 apparatus (Hoefer, San Francisco, USA). Each lane of the gel was loaded with equal amounts of protein and the gel was sil-

ver-stained by the procedure of BLUM *et al.* (1987). Prestained size markers (Bio-Rad, Richmond, USA) were used to calculate the size of the PR-1 proteins.

Immunoblotting and SIBA-ECL test: For immunodetection, proteins were transferred from other gels onto nitrocellulose membrane (PROTRAN BA-85, Schleicher und Schüll, Dassel, Germany) in Bio-Rad blotting apparatus. The transfer was carried out at 10 V for 24 h in 40 mM Na-phosphate (pH 6.5). After electrophoretic transfer of the proteins, the blots were immunoprocessed basically according to REPKA (1999). The immunospecific signal was developed with the chemiluminescent substrate from the SuperSignal West Dura kit (Pierce, Rockford, USA) and visualized using the ECL-Hyperfilm (Amersham, Buckinghamshire, UK).

The elicitor-stimulated expression of the PR-1-like gene products was estimated following the SIBA-ECL protocol (REPKA *et al.* 1996). Individual samples equivalent to 5 µg of total proteins were slotted onto PROTRAN BA-85 nitrocellulose membrane using the Slot Blot PR648 apparatus (Hoefer, San Francisco, USA). After loading the samples, the membrane was immunodecorated either with anti-tobacco PR-1a serum or with anti-petunia chalcone isomerase (CHI) serum both diluted 1:1,000. Serological reactions were detected by chemiluminescence as described for immunoblots.

Results

Changes in protein composition following powdery mildew (*Oidium tuckeri*) infection of grapevine leaves were analyzed by SDS-PAGE (Fig. 1, A). From the protein patterns obtained after extraction with a pH 8.0 buffer, at least 6 new, low-molecular mass proteins were identified. The protein bands induced by fungal infection are numbered according to their apparent molecular masses on the SDS-PAGE gel (Fig. 1, A, lane I, numbers 1-6). Since none of these proteins were detectable either in healthy grapevine or in fungal mycelium extract, we consider them as true PR-proteins.

In the immunological blotting test, three of the 6 proteins induced by fungal infection reacted specifically with the antiserum raised against PR-1a purified from TMV-inoculated *Nicotiana tabacum* leaves (Fig. 1, B). The immunoreactive proteins were designated gPR-1a (15.5 kDa), gPR-1b (16.8 kDa) and gPR-1c (17.7 kDa) in order of decreasing mobility, using the nomenclature recently proposed by VAN LOON and VAN STRIEN (1999).

The accumulation pattern of gPR-1 in response to several potent elicitors was analyzed by SIBA - ECL blot analysis, using the tobacco anti-PR-1a serum. Accumulation of gPR-1 protein in the cultivation medium was substantially induced by fungal elicitor, salicylic acid, chitosan and methyl jasmonate, albeit to a various extent (Fig. 2). Surprisingly, a low-level accumulation of gPR-1 was detected also in EtOH serving as the MeJA control, while neither DMSO control nor unelicited controls (0 and 6 d after the onset of elicitor treatment) accumulated respective protein.

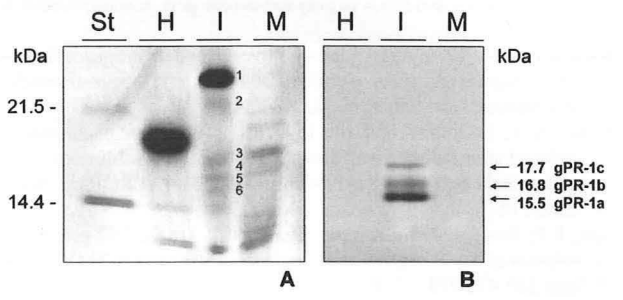

Fig. 1: Electrophoretic patterns on polyacrylamide gel and corresponding immunoblot of crude extracts from untreated grapevine leaves (H), leaves treated with *Oidium tuckeri* (I), *Oidium tuckeri* mycelium (M). **A**: silver stained 12.5 % SDS-PAGE of soluble proteins extracted at pH 8.0. Protein bands induced by fungal infection are numbered according to their apparent molecular mass. The molecular mass standards (St) were soybean trypsin inhibitor (21,500) and hen egg white lysozyme (14,400). **B**: the same extracts were subjected to 12.5 % SDS-PAGE and then transferred to a nitrocellulose membrane and immunodetected with the rabbit anti-tobacco-PR-1a serum. Each lane of the gel was loaded either with 5 or 25 µg and stained with silver or immunodecorated, respectively.

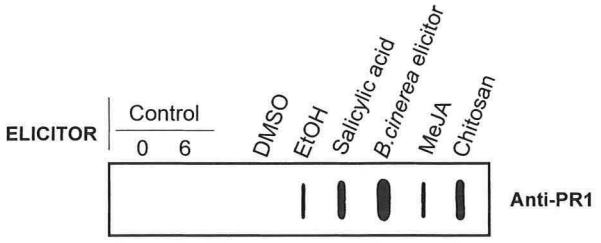

Fig. 2: Slot-immunoblot analysis of gPR-1 protein accumulation upon pretreatment of cultured grapevine cells with various elicitors. Cells were preincubated for 6 d with the indicated compounds. After the treatment was completed, equal amounts of the spent medium corresponding to 5 µg of total proteins were blotted and immunoprocessed using antiserum specific to tobacco PR-1a protein. The immunopositive signal was detected using the chemiluminescence substrate.

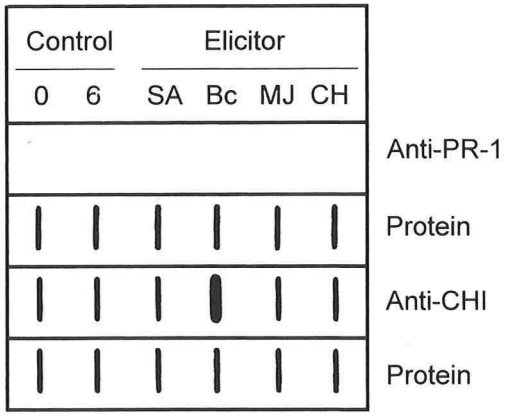

Fig. 3: Slot-immunoblot analysis of gPR-1 and CHI protein accumulation in grapevine cells pretreated with various elicitors. Cells pretreated as indicated in Fig. 2 were assayed either for accumulation of gPR-1 protein or for CHI protein serving as an intracellular marker. Equal amounts corresponding to 5 µg of total proteins were blotted and immunoprocessed either with anti-tobacco PR-1a serum or with anti-petunia CHI serum. The immunopositive signal was detected using the chemiluminescence substrate. SA - salicylic acid, Bc - *Botrytis cinerea* elicitor, MJ - methyl jasmonate, CH - chitosan.

In order to confirm an extracellular compartmentalization of gPR-1 products, protein extracts from control and elicited cell suspension cultures were analysed for the presence of immunoreactive signal using the SIBA-ECL blotting test. Simultaneously, equal amounts of the respective protein extracts were immunodecorated either with tobacco anti-PR-1a serum or with petunia anti-chalcone isomerase (CHI) serum serving as an intracellular protein marker. Fig. 3 shows that none of these elicitors had a significant effect on gPR-1 accumulation when compared with that typical for CHI accumulation. These results suggest that only extracellularly compartmentalized isoforms of PR-1-like proteins exist in pathogen and/or elicitor-treated grapevines.

Discussion

The present report describes the identification and accumulation of three grapevine PR-1-like proteins, namely gPR-1a, gPR-1b and gPR-1c. As far as we know this is the first report on expression of grapevine and even of woody plant PR-1-like proteins. Firstly, we have shown that gPR-1 proteins are induced in grapevine leaves by fungal infection. The three gPR-1 proteins separated by one-dimensional SDS-PAGE have similar molecular masses (15,500-17,700), which perfectly fits the size of the PR-1 family proteins that had been shown previously to be present in the intercellular fluid of tobacco leaves infected with a tobacco mosaic virus (Antoniw and Pierpoint 1978; Matsuoka and Ohashi 1984). Secondly, grapevine gPR-1 proteins belong to the group of tobacco PR-1 proteins as shown by their serological relationships with these proteins.

Tobacco leaf PR-1 proteins have been considered to be induced not only by pathogen infection, but also by chemical treatments (Van Loon 1983). To address this question in grapevine, various potent biotic and abiotic elicitors were tested by immunoblotting in suspension cell cultures. Treatment of grapevine cells with the *Botrytis cinerea* elicitor resulted in a massive accumulation of gPR-1 protein (Fig. 2). Moreover, two other popular elicitors of plant defense genes expression, salicylic acid (SA) and chitosan (CH), have shown to be almost equally effective in induction of gPR-1 protein accumulation. One of the most surprising and puzzling aspect of our study was the accumulation of the gPR-1 protein we observed after methyl jasmonate (MeJA) application. However, based on the fact that there is no report on MeJA-induced PR-1 expression in plants and on the coincidence of the relative signal intensities for both MeJA and EtOH (the MeJA solubilizer), we suggest that the accumulation of gPR-1 protein is rather due to EtOH than to MeJA itself. Thus, the grapevine PR-1 are stress-related proteins because the present work showed that these proteins can in fact be induced by stress other than pathogen infection. In contrast, the findings that heat-shock did not induce the expression of tobacco PR-1 protein (Ohashi and Matsuoka 1985) and on the other hand, the expression of this protein *via* stress-independent factor(s) (Eyal *et al.* 1992), provide further support for the existence of a second pathway for PR-1 gene induction.

We have demonstrated that all three grapevine PR-1 proteins are predominantly localized in the extracellular space when compared with the accumulation of an intracellular marker protein (Fig. 3). This finding correlates very well with the localization experiments performed by Carr *et al.* (1987) who also immunolocalized these proteins to the extracellular spaces predominantly in regions adjacent to viral lesions.

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