Vitis 35 (1), 49–52 (1996)

Pathogenesis-related proteins in grapevines induced by salicylic acid and *Botrytis cinerea*

by

ANNE-SOPHIE RENAULT, A. DELOIRE*) and J. BIERNE

Laboratoire de Biologie Cellulaire, Equipe d'Ampélologie, UR4R, UFR Sciences, Université de Reims Champagne-Ardenne, Reims, France

S u m m a r y : The grapevine pathogenesis-related proteins (PRs) were studied in order to determine the spatial localization and the temporal production of these inducible proteins. We used leaves of plants obtained from woody cuttings grown in greenhouse. Elicitations were done either with salicylic acid or with strains of *Botrytis cinerea*. Several PRs extractable at pH 2.8 were found to accumulate in grapevine leaves after salicylic acid treatment or *Botrytis* infection (SDS-PAGE, coomassie blue). Elicitation with salicylic acid has induced one new protein at about 32 kDa. *Botrytis* infection has resulted in the accumulation of four major acid-soluble proteins with apparent molecular weights of 27, 32, 34 and 38 kDa. Immunodetections using antisera raised against the tobacco PR-2 family have shown several bands, particularly two bands at 34 and 36 kDa revealed by the anti-2a and present both with salicylic acid and *Botrytis*.

K e y w o r d s : pathogenesis-related proteins, grapevine, Botrytis cinerea, salicylic acid, glucanases.

Introduction

Plants react to pathogens' attacks in a number of different ways. In particular, they may respond by synthesizing *de novo* specific products such as phytoalexins or characteristic proteins called pathogenesis-related proteins (PR proteins, PRs; VAN LOON *et al.* 1994). These PR proteins are induced by a wide range of pathogens including viruses, bacteria and fungi. They were discovered for the first time in tobacco by WHITE (1979) and were found lateron in several herbaceous species: tomato, potato, bean, maize, *Arabidopsis*, cucumber and celery, (ANTONIW and WHITE 1983; REDOLFI 1983; GRANELL *et al.* 1987; PARENT and ASSELIN, 1987; WALTER, *et al.* 1990. These proteins are also produced after treatments with chemicals such as salicylic acid (WHITE 1979). PR proteins have characteristic biochemical properties (VAN LOON *et al.* 1987). Extractable at low pH, they are predominantly localized in the intercellular spaces and appear to be resistant to proteolytic enzymes. It has been noticed that some PR proteins are also involved in developmental events such as flowering, leaf senescence and somatic embryogenesis (in grapevines, DELOIRE *et al.* unpubl.). Although PR proteins are now well-known in herbaceous species, they have not been really studied in woody plants. To our knowledge, no study has been published on grapevine PR proteins.

In fact, only a few mechanisms of resistance of grapevines to fungi are well-understood. These consist of preformed barriers and/or induced resistances.

Cuticular waxes, the thickness and chemical components of the skin constitute the preformed barriers and induce a mechanical resistance. Some grapevine varieties which are tolerant to *Botrytis* have these preformed barriers (DUBOS *et al.* 1993).

After infection grapevines synthesize several compounds such as phytoalexins (resveratrol and its derivatives, the viniferins), polyphenols and glycolic acid (JEANDET and BESSIS 1989; PEZET 1993). The preformed barriers and other compounds such as glycolic acid or polygalacturonase inhibitors in the ripening berries are more important in tolerant than in sensitive varieties. In mature berries these natural defense mechanisms decrease (DUBOS *et al.* 1993; PEZET 1993).

Resveratrol is not detectable in healthy leaves of grapevines but is induced after *Botrytis* infection (LISWIDOWATI *et al.* 1991). This induction can also be brought about by an artificial elicitor like UV light (SCHOEPPNER and KINDL 1979; FRITZEMEIER and KINDL 1981). The phytoalexin production has been correlated with resistance to *Botrytis cinerea* (resveratrol might inhibit the growth of fungi). Nevertheless, the observations are sometimes contradictory and the ability of grapevines to resist pathogens results probably from various defense mechanisms.

In the present study we have shown that in Chardonnay PR proteins can be induced by infection with *Botrytis* or elicitation with salicylic acid. Using Chardonnay we have developed an effective biological model and have started to identify β-1,3-glucanases amongst the inducible proteins.

Materials and methods

P l a n t m a t e r i a l : The *Vitis vinifera* variety Chardonnay was grown in a greenhouse at 25 ± 4 °C using a photoperiod of 16 h. Plants obtained from woody cuttings were used when 4-8 months old.

F u n g u s m a t e r i a l : The strains of *Botrytis cinerea* used were isolated from grapes of a Champagne

Correspondence to: Dr. ANNE-SOPHIE RENAULT, Université de Reims Champagne-Ardenne, Faculté des Sciences, Unité de Recherche "Reproduction, Régéneration, Reconstruction, Rajeunissement" (UR4R), Equipe d'Ampélologie Cellulaire et Moléculaire, B.P. 1039, F-51687 Reims Cedex 2, France. Fax: 26 05 32 79.

*) Present address: Dr. A. DELOIRE, ENSA-INRA, U.F.R. de Viticulture, 2 place Viala, F-34060 Montpellier Cedex 1, France.

vineyard and cultivated on a selective medium. The fungus was then propagated on solid potato dextrose medium at 25 °C in the dark with fresh subcultures every 15 d (BOUCHET unpubl.).

Induction of PR proteins: Elicitations with *Botrytis cinerea* were performed on leaves inoculated with droplets (20 µl) of a spore suspension ($5x10^4$ sp/ml) deposited on the upper surface. Plants were then incubated in a separated miniphytotron.

Elicitations were also performed by adding a 7.2 mM solution of salicylic acid (two droplets of 20 µl per leaf) after abrasion with a small disc of sandpaper. Salicylic acid was dissolved in a potassium-phosphate buffer (adjusted to pH 6 with KOH) and sterilized by filtration (0.22 µm; Sartorius). Two controls were used: healthy leaves, same size as those from elicited plants, control T_0; leaves abraded then treated with buffer, control T_1.

Extraction of proteins: PR proteins were extracted from plants 7 d after inoculation or elicitation. Extractions were carried out at 4 °C. 4 g of fresh material (half leaves without necrosis) were frozen in liquid nitrogen and ground with pestle and mortar. Citrate buffer (pH 2.8) was added on the powder in the proportion of 1.5 (v/w). The homogenate was centrifuged at 12000 *g* for 10 min. The supernatant was precipitated with 5 volumes of acetone (v/v) and dried.

Electrophoresis and blotting experiments: Protein concentration in extracts was quantified by direct assay at 230 nm using Bovine Serum Albumine (BSA) as standard. Proteins were separated on 13 % or 15 % SDS-PAGE according to LAEMMLI (1970) and then stained with coomassie blue.

For immuno-revelations, proteins were blotted onto nitrocellulose according to the procedure of THIRIET and ALBERT (1995). Membranes were blocked in PBS-T containing 1 % ovalbumine. They were then incubated for 1 h in PBS-T with antisera diluted 1:3000 (v/v). The antisera used were obtained from rabbits immunized against the tobacco PR-2 family (β-1,3-glucanases; KAUFFMANN *et al.* 1987; VAN LOON *et al.* 1994). The reactions were detected by using a peroxydase conjugated anti-rabbit IgG revealed with diaminobenzidine (DAB).

Results and discussion

Morphological consequences of elicitations: Inoculations with *Botrytis cinerea* have induced necrotic lesions. The lesion size evolved with the fungus development.

The injury by abrasion rapidly caused a necrosis (after 2 to 3 h). The addition of salicylic acid (SA) to the lesions induced necrotic reactions the sizes of which were directly linked to the quantity of SA deposited (results not shown). So elicitation results from both, SA and a necrotic reaction induced by the injury.

SA treatment, *Botrytis cinerea* infection and PR proteins expression: Changes in the protein pattern of grapevine leaves after fungal infection or SA treatment were analysed by electrophoresis on SDS-PAGE and coomassie staining.

A comparison between electrophoretic patterns of protein extracts from *Botrytis* inoculated and healthy leaves shows clear differences (Fig. 1). Proteins with apparent

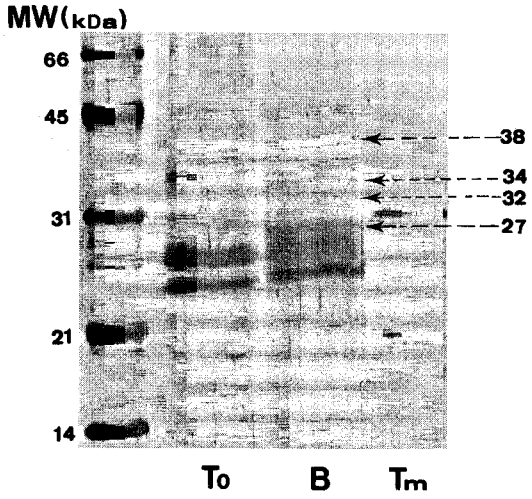

Fig. 1: Electrophoresis of acidosoluble proteins of healthy (T_0), *Botrytis* inoculated leaves (B) and *in vitro-Botrytis* mycelium (Tm). Arrows indicate the main alterations in the pattern of proteins as a result of infection.

molecular weights of 27, 32, 34 and 38 kDa accumulate in infected leaves (lane B). These proteins, extractable at pH 2.8, are induced by the interaction grapevine-*Botrytis*. In fact, they are revealed neither in healthy plants (lane T_0) nor in the mycelium of fungus growth *in vitro* (lane T_m). They are clearly produced after a pathogen infection and can therefore be called PR proteins (VAN LOON *et al.* 1994).

Elicitation with SA does not show the same electrophoretic pattern as with *Botrytis*. After coomassie blue staining only one new band of weak intensity is revealed at the apparent molecular weight of 32 kDa (Fig. 2).

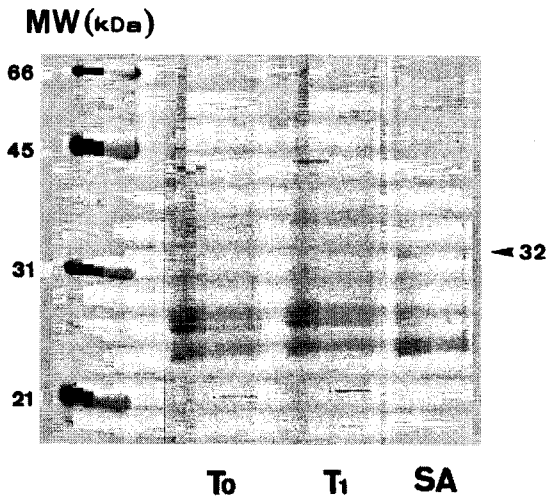

Fig. 2: Electrophoresis of acidosoluble proteins of healthy leaves (T_0), abraded leaves with buffer (T_1) and abraded leaves with salicylic acid (SA). Arrow indicates the main alteration in the pattern of proteins as the result of elicitation.

Immunodetections performed with tobacco PRs antisera have shown positive cross-reactions with PRs of many species (FRITIG, pers. comm). That is why the preliminary identifications of the grapevine PRs were performed by immuno-detections with these antisera.

Immunodetection of PR-2 proteins: We used antisera against two tabacco PR-2 proteins: PR-2a which is an acidic protein (pI=4.1) and PR-2e which is a basic protein (pI>7) as we can see in the table (VAN LOON *et al.* 1994).

Table

Tobacco PR-2 family (ß-1,3-glucanase)

Member	Molecular Weight (kDa)	Isoelectric point (pI)
a (II)	31	4.1
b (II)	33	4.6
c (II)	35	4.7
d (III)	35	5.3
e (I)	33	> 7

The use of the anti-2a serum (acidic group) reveals several bands for *B. cinerea* and SA elicitations (Fig. 3 a). In both cases a double band at 34 and 36 kDa is present however with a stronger intensity for *B. cinerea* elicitation. The confrontation with the pathogen induces three supplementary bands of weaker intensity at 28, 32 and 33 kDa.

The utilization of the anti-2e antiserum shows new bands only in the case of elicitation by *B. cinerea* at an apparent molecular weight of 27 and 32 kDa (Fig. 3 b). Furthermore, one basic protein PR-2e is present at significant background level on blots of controls (T_0 and T_1) and elicited leaves (SA and B) at the apparent molecular weight of 34 kDa: it is a constitutive type.

Thus, some of the PR proteins induced by *Botrytis* infection and SA treatment cross-react with antisera against tobacco PR-2 family (β-1,3-glucanases).

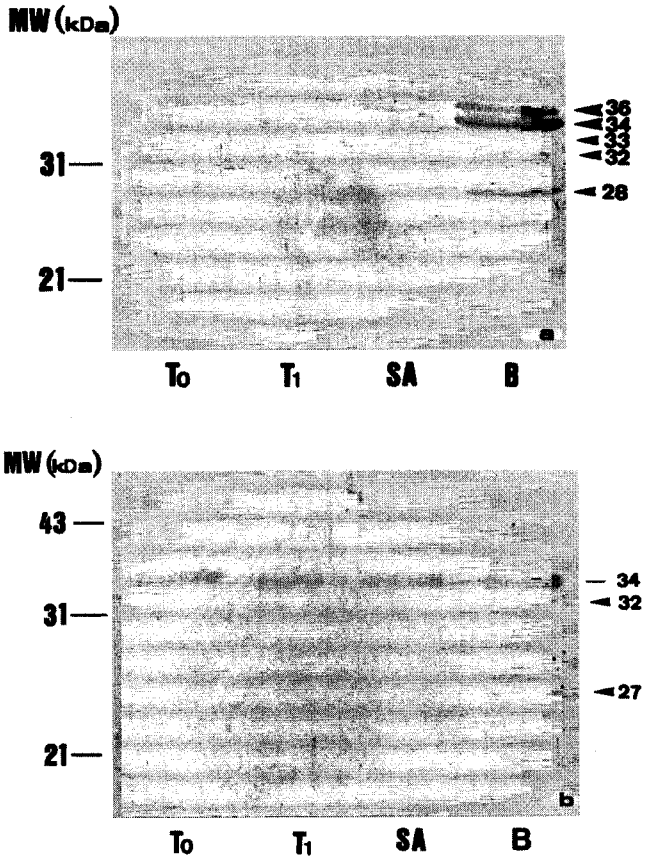

Fig. 3: Western blot analysis of acidosoluble leaf proteins. T_0, healthy leaves ; T_1, buffer added on abraded leaves; SA, salicylic acid solution added on abraded leaves; B, leaves inoculated with *Botrytis* spore suspension. **a** - antiserum anti-2a; **b** - antiserum anti-2e.

Conclusion

We have demonstrated that in Chardonnay an elicitation by salicylic acid or *B. cinerea* resulted in the synthesis of several PR proteins. Elicitation by SA, that induces only some of these PRs, copies partially *Botrytis* infection. Some of them are immunodetected by the anti-PR-2 antisera and are likely β-1-3-glucanases. Identification tests of the other PRs are to be done. Recently we have shown that an antichitinase antiserum (anti-PR-3b) revealed the band at 27 kDa induced after *Botrytis* infection (unpubl.). It is supposed that in grapevines, like in herbaceous species, glucanases and chitinases are involved in defense mechanisms.

We now need to answer several questions. First, we are going to study the ability of these PRs to inhibit the *Botrytis* development by *in vitro* and *in vivo* tests. Investigations on Systemic Acquired Resistance (SAR) are also in progress. Furthermore, homologous and heterologous probes will be used to compare the PR gene transcription in mRNA with the PR mRNA traduction in proteins.

Acknowledgements

We thank Prof. B. FRITIG (CNRS, Strasbourg, France) for the antisera; Prof. P. BOUCHET (UFR Pharmacie, URCA, Reims, France) for his help in *Botrytis* culture; E. BONNET (Lab. Biol. Cell., Reims, France) for technical assistance; MOËT et CHANDON (Epernay, France) for the grapevine material; the Agence Régionale pour la Recherche et le Développement (ARRD) and Europôl'Agro in Champagne-Ardenne for financial support.

Literature cited

ANTONIW, J. F.; WHITE, R. F.; 1983: Biochemical properties of the PR proteins from tobacco. Neth. J. Plant Pathol. **89**, 255-264.

DELOIRE, A.; KRAEVA, E.; COUTOS-THEVENOT, P.; MAURO, M. C.; BONNET, E.; BIERNE, J.: Immunodetection of chitinases-like and ß-1,3-glucanases-like amongst grapevine molecules secreted *in vitro* by respectively embryogenic and non embryogenic cells. Submitted to publication.

DUBOS, B.; PRUDET, S.; CHARRIER, L.; LE MENN, R.; 1993: Lutte contre le *Botrytis*. Difficultés, méthodes actuelles et perspectives. Vigneron Champenois (5), 84-93.

FRITZEMEIER, K. H.; KINDL, H.; 1981: Coordinate induction by UV light of stilbene synthase, phenylalanine ammonia-lyase and cinnamate 4-hydroxylase in leaves of Vitaceae. Planta. **151**, 48-52.

GRANELL, A.; BELLES, J. M.; CONEJERO, V.; 1987: Induction of PR proteins in tomato by citrus exocortis viroid, silver ion and ethephon. Physiol. Mol. Pl. Path. **31**, 83-90.

JEANDET, P.; BESSIS, R.; 1989: Une réflexion sur les mécanismes morphologiques et biochimiques de l'interaction vigne - *Botrytis*. Bull. O.I.V. **62**, 637-657.

KAUFFMANN, S.; LEGRAND, M.; GEOFFROY, P.; FRITIG, B.; 1987: Biological function of «pathogenesis-related» proteins: four PR proteins of tobacco have 1,3-glucanase activity. Embo J. **6**, 3209-3212.

LAEMMLI, U. K.; 1970: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685.

LISWIDOWATI; MELCHIOR, F.; HOHMANN, F.; SCHWER, B.; KINDL, H.; 1991: Induction of stilbene synthase by *Botrytis cinerea* in cultured grapevine cells. Planta **183**, 307-314.

PARENT, J. G.; ASSELIN, A.; 1987: Acidic and basic extracellular PR leaf proteins from fifteen potato cultivars. Phytopathology **77**, 1122-1125.

PEZET, R.; 1993: La pourriture grise des raisins. Le complexe plante-parasite. Vigneron Champenois (5), 65-83.

REDOLFI, P.; 1983: Occurrence of PR (b) and similar proteins in different plant species. Neth. J. Plant Pathol. **89**, 245-254.

SCHOEPPNER, A.; KINDL, H.; 1979: Stilbene synthase and its induction by UV light. FEBS LETTERS. **108**, 349-352.

THIRIET, C.; ALBERT, P.; 1995: Rapid and effective western blotting of histones from AUT and SDS polyacrylamide gels: two different approaches depending on the subsequent qualitative or quantitative analysis. Electrophoresis. **16**, 357-361.

VAN LOON, L. C. ; GERRITSEN, Y. A. M.; RITTER, C. E.; 1987: Identification, purification and characterization of pathogenesis-related proteins from virus infected Samsun NN tobacco leaves. Plant Mol. Biol. **9**, 593-609.

- -; PIERPOINT, W. S.; BOLLER, T.; CONEJERO, V.; 1994: Recommendations for naming plant PR proteins. Plant Mol. Biol. Reptr. **12** (3), 245-264.

WALTER, M.; LIU, J. W.; GRAND, C.; LAMB, C.; HESS, D.; 1990: Bean PR proteins deduced from elicitor induced transcripts are members of a ubiquitous new class of conserved PR proteins including pollen allergens. Mol. Gen. Genet. **222**, 353-360.

WHITE, R. F.; 1979: Acetylsalicylic acid (Aspirin) induces resistance to MV in tobacco. Virology **99**, 410-412.

Received October 10, 1995