Grapevine Downy Mildew *Plasmopara viticola* Infection Elicits the Expression of Allergenic Pathogenesis-Related Proteins

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Key Words

Grapevine · *Vitis vinifera* · Downy mildew · *Plasmopara viticola* · Pathogenesis-related proteins · Proteomics · Mass spectrometry · Harpin-binding protein · β -1,3-glucanase

Abstract

Background: Downy mildews are a group of microorganisms belonging to the Chromista kingdom that can infect specific plants. When growing on plant tissues these microbes can elicit the expression of pathogenesis-related proteins (PRs), a group of stress-induced proteins frequently described as allergens in many plant species. Our aim was to verify by a proteomic approach whether the allergic reactions experienced by a farmer working in a vineyard infected by *Plasmopara viticola* (Pv), the etiological agent of downy mildew, are elicited by PRs expressed by the grapevine upon infection or by allergens present in Pv. **Methods:** A skin prick test and prick-to-prick test with infected field grapevine leaves and control leaves were carried out. Field leaves and ad hoc Pv-inoculated leaves were compared by SDS-PAGE and IgE-immunoblotting with extracts from control leaves

and Pv sporangia. IgE-binding proteins were further separated by two-dimensional electrophoresis and the positive spots analyzed by nanoHPLC-Chip and tandem mass spectrometry (MS/MS) for identification. **Results:** Only infected leaves showed IgE-binding protein bands at 42 and 36 kDa. This agreed with the positive skin prick test experienced by the patient only with the infected leaves extract. Two-dimensional electrophoresis followed by MS/MS analysis led to the identification of PR-2 (β -1,3-glucanase) and harpin-binding protein 1 as putative allergens, the latter having never been reported before. **Conclusion:** The results indicate that Pv infection might represent a new source of plant allergens.

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Introduction

Cultivated grapevine is nowadays represented by the species *Vitis vinifera*, a plant native to Caucasus which has evolved over more than 100 million years. However, the history of viticulture is much more recent and began only

10,000 years ago. In terms of fruit production, grapevine represents one of the most cultivated fruit crops in the world.

Grapevine can mediate type-1 allergic diseases, albeit less frequently than other plant species. Vine-associated respiratory symptoms have been described as being induced by pollens spread by vines during the pollination period in spring [1–3]. In a case report of a patient simultaneously allergic to vine pollen and berry, the IgE-binding proteins extracted from pollen were found to cross-react with grape proteins. However, vine allergens, inducing respiratory reactions, have not been identified so far. Besides pollen, aeroallergens associated with vine can be derived from different sources. A case report has been published of a patient experiencing respiratory symptoms induced by the vine-infecting mildew *Plasmopara viticola* (Pv), the agent of downy mildew [4]. The patient was probably sensitized through working with the mildew in a greenhouse.

Although the authors demonstrated that the allergenic proteins were derived from Pv, we cannot exclude that molds might induce the expression of allergenic proteins in vine. Indeed, it has recently been demonstrated that Pv induces the expression of pathogenesis-related proteins (PRs) in vine-infected leaves [5]. The objective of the present work was to verify by a proteomic approach whether the allergenic reactions experienced by a farmer working in a vineyard infected by Pv were elicited by PRs expressed upon infection or by allergens present in Pv.

Materials and Methods

Patient

A 54-year-old man who worked in a vineyard infected with *P. viticola* and reported asthma episodes in August was recruited by the allergy division of the hospital of Pordenone. The patient tolerated the ingestion of grape berries as well as all varieties of wine. The patient underwent a clinical investigation by skin prick test (SPT) with a large range of commercial food (milk, egg, soy, wheat, chestnut, hazelnut, fish, shrimp, peach) and inhalant allergen extracts (grass, mugwort, ragweed, pellitory, plantain, birch, olive, cypress, mites, *Alternaria alternata*, cat and dog; Lofarma, Milan, Italy), by specific IgE assays for the most common pollen (ragweed, mugwort, grass, pellitory, plantain, birch, olive, cypress, saltwort, London plane) and mold (*Aspergillus fumigatus*, *A. alternata*, *Cladosporium herbarum*, *Botrytis cinerea*) allergens, grape berries, peach nsLTP (Pru p 3) and PR-10 (Bet v 1; Thermofisher Scientific, Uppsala, Sweden), and by prick-to-prick test using infected and uninfected vine leaves previously ground and resuspended in a physiological solution.

Plant Material, Inoculum and Pathogen Infections

Infected and uninfected field vine leaves were collected in the vineyards close to the University building. Grapevine plants (cv pinot noir) were grown in vitro (27°C, 16-hour photoperiod, 50 µE/m²/s) as previously described [5]. The Pv inoculum was collected from infected field leaves and used for several artificial infection cycles on surface-sterilized leaves before the final inoculum was stored as sporangia at –20°C.

Leaves from in vitro plants were detached and placed in Petri dishes, abaxial side upwards, on a Whatman 3MM paper disk soaked in sterile distilled water. Each leaf was inoculated with a suspension of 30,000 sporangia per milliliter of sterile distilled water. Samples were incubated in a growth chamber (24°C, 16-hour photoperiod, 50 µE/m²/s) and two leaves from three different plants were collected at 96 h following infection. Control leaves were treated with water and maintained under the same conditions as inoculated leaves, and collected at the same time points. Samples were stored at –80°C before processing.

Protein Extraction

The extraction of plant proteins was performed as previously described [5], with minor modifications, starting with both field- and in vitro-cultivated leaves. Briefly, infected and uninfected leaves were ground under liquid nitrogen in the presence of a protease inhibitor cocktail (1× Complete Tablet; Roche Molecular Biochemicals, Mannheim, Germany) and 5% PVPP (polyvinylpyrrolidone) to remove phenolic compounds. Proteins were extracted in five volumes of 7 M urea, 2 M thiourea, 80 mM citric acid, 3% CHAPS, and incubated on ice for 2 h with regular vortexing and then centrifuged at 15,000 g for 30 min at 4°C. The supernatant was then collected. Extracted proteins were precipitated with 4 volumes of cold acetone (–20°C) overnight and pelleted by centrifugation (15,000 g for 15 min at 4°C). Pellets were washed in acetone and then dissolved in 7 M urea, 2 M thiourea, 3% CHAPS, 40 mM Tris, 1% DTT (dithiothreitol) and 1% pH 3–10 ampholytes. The solutions were incubated on ice for 1 h and centrifuged (15,000 g for 15 min at 4°C) to remove debris and nucleic acids. Sporangia were extracted with 1% SDS–5% β-mercaptoethanol. Protein concentrations were estimated using Bradford assay (Sigma) by a microplate reader (Bio-Tek).

SDS-PAGE

The protein extracts were mixed with 2× loading buffer [62.5 mM Tris-HCl, pH 6.8, 1.3% (w/v) SDS, and 10% (w/v) glycerol, 4% β-mercaptoethanol] and heated at 100°C for 5 min. Proteins (50 µg/cm of lane width) were separated in 16% SDS-PAGE with a 20-mA current by means of a Mini Protean III apparatus (Bio-Rad, Hercules, Calif., USA) in 25 mM Tris, 192 mM glycine and 0.1% SDS (pH 8.4). The gels were stained with Coomassie R250 or alternatively blotted as described below.

Two-Dimensional Electrophoresis (IPG X SDS-PAGE)

Two-dimensional electrophoresis (2DE) separation was performed using 7-cm-long dry IPG strips (pH 3–10; Bio-Rad). The strips were actively rehydrated at 20°C by a Protean IEF Cell (Bio-Rad) for 12 h with a buffer containing 100 µg of protein from the field-infected leaves. The focusing was then carried out until 25 kVh was reached. After focusing, the IPG gel strips were placed in a screw-cap tube containing 10 ml of equilibration buffer (0.375 M Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 20 mM DTT)

and incubated for 15 min. The solution was replaced by 10 ml of 50 mM iodoacetamide-containing equilibration buffer. The strips were equilibrated for a further 15 min. The second dimension SDS-PAGE was performed using a 12% polyacrylamide gel as previously described. Gels were alternatively stained with Coomassie G (EZBlue; Sigma Aldrich, St. Louis, Mo., USA) or blotted as described below.

Immunoblotting

Proteins separated by SDS-PAGE or 2DE were electroblotted onto polyvinylidene fluoride membranes (Millipore, Billerica, Mass., USA) at a constant voltage (50 V for 150 min) and immunodetected with the patient's serum diluted 1:10 in blocking solution (3% w/v of skimmed dry milk and 0.05% Tween-20 in PBS) overnight. As secondary antibody, a horseradish peroxidase-conjugated anti-human IgE (Southern Biotech, Birmingham, Ala., USA) diluted 1:1500 was used. Bound IgE was detected by chemiluminescence (Millipore) using a Chemidoc XRS (Bio-Rad) image-acquiring device.

Mass Spectrometry

In-Gel Digestion

Spots giving a positive signal by IgE-immunoblotting were carefully cut out from 2D Coomassie-stained gels and subjected to in-gel trypsin digestion according to Shevchenko et al. [6] with minor modifications. The gel pieces were swollen in a digestion buffer containing 50 mM NH_4HCO_3 and 12.5 ng/ μl of porcine trypsin (Promega, Madison, Wisc., USA) in an ice bath. After 30 min the supernatant was removed and discarded, 20 μl of 50 mM NH_4HCO_3 were added to the gel pieces and digestion was allowed to proceed at 37°C overnight. The supernatant-containing tryptic peptides were dried by vacuum centrifugation. Prior to mass spectrometric analysis, the peptide mixtures were redissolved in 5 μl of 2% acetonitrile and 0.1% formic acid.

Peptide Sequencing by nanoHPLC-Chip Tandem Mass Spectrometry

Peptides from 5 μl of each sample were separated by reverse-phase nanoHPLC-Chip technology (Agilent Technologies, Palo Alto, Calif., USA) online coupled with a 3D ion trap mass spectrometer (Esquire 6000; Bruker Daltonics, Bremen, Germany). The chip was composed of a Zorbax 300SB-C18 (150 mm $\times$ 75 μm , with a 5- μm particle size) analytical column and a Zorbax 300SB-C18 (40 nl, 5 μm) enrichment column. The complete system was fully controlled by ChemStation (Agilent Technologies) and EsquireControl (Bruker Daltonics) software. The scan range used was from 300 to 1,800 m/z. For tandem mass spectrometry (MS/MS) experiments, the system was operated with automatic switching between MS and MS/MS modes. The three most abundant peptides of each m/z were selected to be further isolated and fragmented. The MS/MS scanning was performed in the normal resolution mode at a scan rate of 13,000 m/z per second. A total of five scans were averaged to obtain an MS/MS spectrum. Database searches were conducted using the MS/MS ion search of Mascot against all entries of the nonredundant NCBI database with the following parameters: specific trypsin digestion, up to one missed cleavage; fixed and variable modifications, carbamidomethyl-Cys and oxidated-Met, respectively; peptide and fragment tolerances, ± 0.9 and ± 0.9 Da, respectively, and peptide charges, +1, +2 and +3. The protein spots were identified as being a 'significant hit'

Fig. 1. SDS-PAGE and IgE immunoblotting of the extracts. 1 = Pv; 2 and 3 = uninfected and infected leaves (from field plants); 4 and 5 = uninfected and infected leaves (from in vitro plants).

($p < 0.05$) based on the individual peptide ion score. These peptide ion scores were automatically calculated by the Mascot program as $-10 \times \log(P)$, where P is the probability that the observed match is a random event.

Results

Clinical Data

SPT and specific IgE assays tested positive only for birch pollen and for its major allergen (PR-10; Bet v 1). The prick-to-prick test carried out with the infected leaves was positive while the uninfected leaves failed to induce skin reactions.

The Infection of P. viticola Induces the Expression of Different IgE-Binding Proteins

The extracts obtained from the infected and uninfected vine leaves (both cultivated in the field and in in vitro) and from the mildew sporangia were compared by SDS-PAGE and IgE immunoblotting with the patient serum (fig. 1). The results indicate that the allergens are intensely expressed by the plant upon infection (lanes 3 and 5) since no signals were detected in the Pv extract (lane 1) and only traces were found in uninfected leaves (lanes 2 and 4). Two major bands were detected by IgE at 42 and 35 kDa, showing the same detection pattern for the in-

Fig. 2. 2DE of the infected leaves from field plants: Coomassie staining (**a**) and IgE immunoblotting with the patient's serum (**b**). pI = Isoelectric point.

Table 1. MS/MS results

Spot	Protein name	Mr (theor.), Da	Mascot score	NCBI acc. No.
A	β -1,3-glucanase (<i>V. riparia</i>)	36,718	312	gi:37992763
B	β -1,3-glucanase (<i>V. riparia</i>)	36,718	235	gi:37992763
C	HBP1 (<i>Vitis</i> sp. NL-2003)	31,360	193	gi:38679335
D	HBP1 (<i>Vitis</i> sp. NL-2003)	31,360	176	gi:38679335
E	β -1,3-glucanase (<i>V. riparia</i>)	36,718	165	gi:37992763
F	HBP1 (<i>Vitis</i> sp. NL-2003)	31,360	147	gi:38679335
	β -1,3-glucanase (<i>V. riparia</i>)	36,718	128	gi:37992763
	HBP1 (<i>Vitis</i> sp. NL-2003)	31,360	159	gi:38679335

infected leaves collected from the field and after in vitro culture. A faint 24-kDa band was detected in all vine samples, indicating that the related protein is not involved in the allergy manifestations. Interestingly, no bands at 20 kDa, typical of Bet v 1 homologues, have been detected despite birch SPT having tested positive.

The IgE-Binding Proteins Are Defense Proteins

The analysis of the 2D map of the extract of the infected leaves showed IgE-binding spots at different molecular masses. Due to the better resolving capacity of this ap-

proach it was possible to distinguish two spot trains at 35.6 and 34 kDa (fig. 2), whereas in the previous SDS-PAGE these spots were comigrating in one band.

The results of the MS/MS analysis of the spots showing a positive signal in the immunoblotting are summarized in table 1. The data indicate that the spot train at 35.6 kDa (spots A and B) contains essentially β -1,3-glucanase (PR-2), while the 34-kDa train (spots C–F) is mainly composed of harpin-binding protein 1 (HBP1), even if some of the spots (spots D and E) contain β -1,3-glucanase, probably due to an imperfect separation of the two pro-

teins (spot D and E). The MS analysis of the 42-kDa spot did not lead to any identification, probably as a consequence of the scarce protein concentration.

Discussion

PRs are part of an inducible plant defense system against pathogen attacks (e.g. bacteria, fungi, etc.) [7, 8] or abiotic stresses [9]. Different PR families have been described so far and, in parallel, it has been demonstrated that several of these families include proteins capable of eliciting allergic reactions. This is probably due to their higher stability and resistance to proteases in comparison to ordinary proteins [10]. In the present work we demonstrated that upon infection with *P. viticola*, specific IgE-binding proteins belonging to the PR-2 family (β -1,3-glucanases) and – to the best of our knowledge for the first time – to HBP1, are expressed. While β -1,3-glucanases have been extensively described as allergens, no record describing the potential allergenicity of HBPs has been found. The targets of HBP1 are harpins [11], protein elicitors produced by a number of phytopathogenic bacteria [11, 12], that are considered virulence factors and can induce a strong hypersensitive reaction in plant tissues. HBPs are expressed upon bacterial infection, but, in general, their upregulation can be also be a consequence of different stress conditions or treatments independent of the presence of harpin [13, 14]. This correlates with our findings since Pv infection carried out under controlled conditions, and thus in the absence of evident bacterial growth, produced the same results observed in the field-cultivated leaves. It is interesting that the action of dufulin, an inducer of systemic acquired resistance in plants, is based on the activation of HBP1 [15]. The same ap-

proach has been described to enhance the expression of PRs like PR-1, PR-2 and PR-5 in the very commonly used model plant *Arabidopsis thaliana* [16]. For this reason, we cannot exclude that the use of such resistance inducers can increase the allergenic potency of some crops.

Despite the great number of works describing the induction of PRs due to biotic and abiotic stresses, only a few of them analyzed the IgE binding pattern and the allergenic potency of these proteins, and the results are in some cases contrasting. Sanchez-Monge et al. [17] described the induction of a class 1 chitinase after ethylene treatment of green bean. Similarly, ethylene associated to tissue wounding provoked an increase of the allergenic potency of tur-nip by inducing the expression of a prohevein [18]. Another example is given by the work of Mayer et al. [19], who demonstrated the increase of Mal d 1 gene transcription and protein accumulation after infection by *Erwinia amylovora*. In contrast, a recent publication [20] showed that the infection of tomato by Pepino mosaic virus, despite inducing the overexpression of specific allergenic proteins, did not lead to a parallel increase of the allergenic potency of the fruit, analyzed by basophil activation test.

In our case, an SPT performed with the extracts of the infected leaves clearly induced a skin reaction in comparison to the uninfected leaves, suggesting that the IgE-binding proteins contained are also characterized by allergenic potency. The sensitization route remains elusive. We can suggest that this might be mediated by skin contact, as frequently described for β -1,3-glucanase in latex-fruit syndrome [21]. We also cannot exclude that some leaf particles, perhaps from the necrotic tissues very prone to fragment and characterized by the highest allergen concentration, might have been inhaled by the subject during operations such as harvesting, thus representing the most likely means of exposure.

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