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Accumulation of pathogenesis-related proteins in the apoplast of a susceptible cultivar of apple (*Malus domestica* cv. Elstar) after infection by *Venturia inaequalis* and constitutive expression of PR genes in the resistant cultivar Remo

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Abstract

Leaves of apple (*Malus domestica* cv. Elstar) were infected with a cloned isolate of the apple scab *Venturia inaequalis.* The intercellular washing fluid (IWF) of these plants was collected and the variation in the composition of proteins in the IWF was analysed by SDS-PAGE and two-dimensional gel electrophoresis during and after the infection with *V. inaequalis*, the causal agent of apple scab. The subsequent analysis of induced proteins by electron spray ionization quadrupole time of flight mass spectroscopy revealed the presence of β-1,3-glucanase, chitinase, thaumatin-like protein and a cysteine-like protease in *M. domestica* leaves infected by *V. inaequalis*. These results were confirmed by immunoblotting with antibodies against some of these proteins. Moreover, a non-specific lipid transfer protein was identified in uninfected leaves: the amount declined to a non-detectable level within the first week after infection by *V. inaequalis.* The analysis of the IWF of *M. domestica* cv. Remo, bearing resistances to apple scab, powdery mildew and fire blight, showed a protein pattern comparable to that of the IWF from *V. inaequalis* infected leaves from cultivar Elstar indicating the constitutive production at least of some of the pathogenesis-related proteins in the resistant cultivar.

Abbreviations: ESI Q-TOF – electron spray ionization quadrupole time of flight mass spectroscopy; HSC – heat shock cognate protein; IWF – intercellular washing fluid; PDA – potato dextrose agar; PR – pathogenesis-related; TMV – tobacco mosaic virus.

Introduction

The apoplast forms a continuous, yet structured space between plant cells and constitutes a considerable part of the plant's body, namely between 5% and 10% of the plant's mass (Winter et al., 1993). The apoplast fluid is made up mainly by the transpiration stream and its chemical composition is most likely achieved via water, ion and protein transporters (Hoson, 1998; Sakurai, 1998; Sattelmacher, 2001) that may have a tissue-specific distribution. More recently, evidence has been obtained that the apoplast has an important function during plant defence and contains a number of proteins. More than 200 proteins (Robertson et al., 1997) are exported from the interior of the cells to their outside mostly via the endoplasmic reticulum and the plasma membrane, but also via different, and not completely unravelled routes via the plasma membrane as suggested for yeast (Gozalbo et al., 1992). In the other direction, bacteria and fungi try to invade and to explore the cell's interior via the apoplast either through the stomata or the cuticle. If this view is correct, the question arises as to how plants manage to protect themselves against microorganisms that try to invade them. By analogy to the well-studied system of vertebrates this might be attempted via the excretion of

proteins that exert a protective function. It is known that vertebrates defend their body openings against potential invaders by antibodies of the IgA- and IgE-type. Antibodies are lacking in plants, however, it is known that plants export and secrete proteins to the exterior including the important groups of pathogenesis-related proteins (PR proteins). Originally discovered by van Loon and van Kammen (1970), the PR proteins have been subdivided into up to 14 classes (Joosten and De Wit, 1989; Dudler, 1997; Somssich and Hahlbrock, 1998; van Loon and van Strien, 1999; Datta and Muthukrishnan, 1999; Odjakova and Hadjiivanova, 2001; Muthukrishnan et al., 2001; Blein et al., 2002). Thus, the question arose as to whether plants might use this compartment and its proteins as a front line in defence against microorganisms that try to invade the plant from the phyllosphere. Several research groups tried to find an approach to this problem by analysis of the apoplast proteins (Boller, 1995; Oka et al., 1997; Bolwell et al., 2001). The advantage of this attempt clearly is that the components of the apoplast can easily be collected by infiltration and centrifugation (Hogue and Asselin, 1987; Ruan et al., 1995; Lohaus et al., 2001) and separated from the rest of the plant's proteins (Lohaus et al., 2001).

In order to gain information for the plant pathogen system *Malus domestica* and the biotrophic fungus *Venturia inaequalis*, we decided to analyse the protein composition of the apoplast fluid of sterile grown apple plants prior to or after infection by the fungus *V. inaequalis*, the causal agent of apple scab. Our rationale was to understand infection of apple plants by apple scab and, more importantly, to unravel new ways to prevent this infection. We thought that this might be possible using induction and production of plant protective proteins of the apoplast by elicitors or by natural antagonists instead of chemicals. The analysis of the proteins in the apoplast fluid prior to and after infection with *V. inaequalis* in these plants should thus allow facilitate the study of the natural defence potential of a plant.

Materials and methods

Plant propagation

Malus domestica (cv. Elstar) were cultivated as described by Gau et al. (2002). These sterile and genetically identical plants were transferred to rooting media containing 1× Murashige and Skoog medium including vitamins, 3% sucrose, 1.5 μM indolebutyric acid and 0.7% plant agar. After rooting, the plants were planted into soil and adapted to normal environmental conditions. The plants were grown in a greenhouse at 22 °C under additional illumination with ∼250 μmoles m^{-2} s^{-1} with a light/dark rhythm of 12 h (Philips, SON-T-AGRO Na-high pressure lamps 400 W).

Cultivation of V. inaequalis *and inoculation of* M. domestica *(cv. Elstar)*

An isolate of *V. inaequalis*, isolated from a leaf of *M. domestica* (cv. Elstar) in Biologische Bundesanstalt (Dossenheim, Germany) and designated as strain no. 15, was grown as previously reported by Parker et al. (1995) on potato dextrose agar (PDA) Petri dishes covered by a cellophane membrane. For the infection of apple trees with *V. inaequalis*, conidia were harvested 7 days after propagation on PDA Petri dishes and the trees inoculated by spraying 10 ml conidia suspension (containing 1×10^6 conidia in water) per plant. On average the trees had approximately 30 leaves. Subsequently, the inoculated plants were stored for 3 days at 100% relative humidity under transparent plastic bags at 19 °C. During this period the plants were illuminated with fluorescent tubes (radium white) at ∼80 μmoles m^{-2} s^{-1} under a light/dark regime of 12 h. After 3 days the plastic bags were removed. Control plants were sprayed with 10 ml water.

Isolation of intercellular washing fluid (IWF)

The 20 youngest leaves of plants infected with *V. inaequalis* were selected for the isolation of apoplastic fluid. This was performed according to Hogue and Asselin (1987) with slight modifications. The leaves were cut off from the petiole and infiltrated for 10 min with PBS buffer under vacuum and were inserted into homemade holders between two brackets containing a deepening in the shape of the leaves. The leaves were orientated with the cutting area to the bottom of the centrifuge tube and centrifuged for 5 min at 50 × *g* in an HS-4 rotor (Sorvall) to remove the excess of liquid. The apoplastic fluid was collected by an additional centrifugation step for 10 min at 700 × *g* and finally stored at −20 °C.

Gel electrophoresis, silver staining and immunoblotting

SDS-PAGE and sample denaturation were carried out as described in Schägger and von Jagow (1987).

Silver staining was done according to Blum et al. (1987). Immunoblotting was performed following the method of Towbin et al. (1979) using 0.45 μm pore size nitrocellulose filters. Blots were blocked with 5% skim milk and incubated with selected antibodies against PR proteins (rabbit, 1 : 1000 dilution in milk), kindly provided by Dr. M. Legrand, IBMP, Strasbourg, France. The antibodies were detected by anti-rabbit-IgG from goat labelled with alkaline phosphatase (dilution 1 : 20 000, Sigma, Munich, Germany).

Isoelectric focusing

The apoplastic fluid was concentrated using a Vivaspin concentrator (cut off 5000 Da, Vivascience, Hannover, Germany). IEF was carried out with the PROTEAN IEF system (Bio-Rad, Munich, Germany) using IPG strips (11 cm) with pH gradients of 3–10 or 4–7, respectively. Apoplastic fluid sample containing 100 μg protein in 20 μl was mixed with 10 μl solubilization buffer containing 8 M urea, 4% CHAPS, 40 mM Tris, 100 mM DTT, incubated at room temperature for 1 h and subsequently centrifuged for 10 min at 10 000 × g. An aliquot of the supernatant (10 μl) was added to 340 μl rehydration buffer containing 8 M urea, 2% CHAPS, 0.28% DTT and applied to the strip. All further steps were performed according to the manufacturer's instructions.

Protein digestion and sequencing

Proteins were excised after Coomassie brilliant blue staining from SDS-PAGE gels and digested according to Jensen et al. (1998). Peptide fragments were extracted from the gels by washing several times with the same volume of extraction solution (50 μl of a 1 : 1 mixture of 5% formic acid and acetonitrile). The extraction solutions were collected and concentrated with ZipTips C18 (Millipore). *De novo* sequencing was done on a quadrupole/time-of-flight hybrid mass spectrometer (Q-TOF2 Micromass, Waters, Manchester, UK) in positive-ion mode.

Results

Isolation of IWF from M. domestica *and separation on SDS-PAGE*

The infiltration–centrifugation technique is a method suitable for the isolation of apoplastic fluid from apple leaves. This fluid was isolated from healthy and infected apple leaves using different gravity forces to verify whether the apoplastic fluid became contaminated by constituents of the plant cell during the isolation procedure. A careful consideration of the influence of the gravity force on cell wall breakage during the isolation was done according to Lohaus et al. (2001) with the result that no contaminations by cell breakage of *M. domestica* cv. Elstar were obtained when the gravity force was lower than 1500 × g. The purity of the apoplastic fluid from cytosolic contamination was confirmed by immunoblotting with an anti-serum against the constitutively expressed cytosolic heat shock cognate (HSC70) protein (Anderson et al., 1994, data not shown). The analysis of the apoplastic fluid from healthy leaves of *M. domestica* cv. Elstar by SDS-PAGE revealed the presence of proteins with an apparent molecular mass between 10 and 90 kD. The apoplastic fluid from infected leaves comprised several prominent proteins with apparent molecular masses between 22 and 32 kD that were translocated to the apoplast during the process of infection with *V. inaequalis* (Figure 1) in addition to those observed in the controls. Moreover, the decline of a low molecular weight polypeptide could be observed after the first week of infection of healthy leaves with *V. inaequalis*.

The apoplastic fluid from healthy leaves of the apple cultivar Remo (*M. domestica* cv. Remo) that bears resistances (V_f, V_r, V_m and VA) against different plant

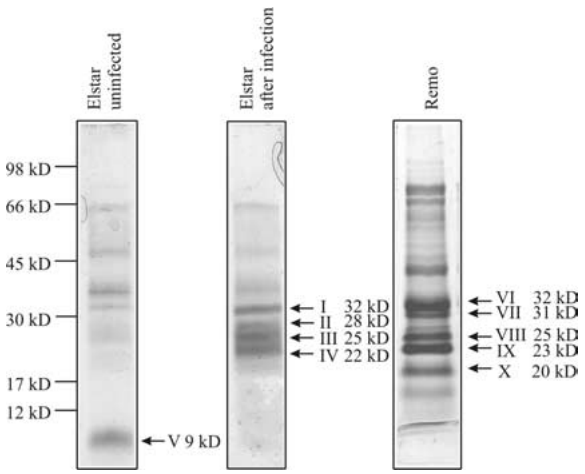

Figure 1. Preparative SDS-PAGE separation of apoplastic fluid from *M. domestica* cv. Elstar leaves, *V. inaequalis* infected Elstar leaves (21 dpi) and apoplastic fluid from the resistant *M. domestica* cv. Remo. I–X indicate bands that were excised for further analyses. Each lane was loaded with 50 μg protein.

Table 1. ESI Q-TOF mass spectroscopy of tryptic peptide fragments and identification of the derived peptide sequences from cultivar Elstar after apple scab infection by homology search

Band	Rel. mass (D)	Charge	Mass (D)	Derived amino acid sequence	Sequence similarity	Organism	Accession number
I	543.26	2+	1084.51	HWG(I/L)FSPNK	Acidic-β-1,3-glucanase	*Cucumis sativus*	Q9S9G2[#]
I	658.85	2+	1315.68	TYNSN(I/L)(I/L)QHVK	β-1,3-glucanase	*Vitis vinifera*	Q9SBY5[#]
I	759.39	2+	1516.76	(I/L)YEPNEAT(I/L)QA(I/L)R	β-1,3-glucanase	*Vitis vinifera*	CAB91554[§]
I	895.47	2+	1788.93	VSTA(I/L)DTGV(I/L)GNSFP(SP)K	β-1,3-glucanase	*Prunus persica*	AAL30426[§]
I	1014.51	2+	2027.00	YPS(I/L)FDA(I/L)(I/L)DAQYAA(I/L)(EK)	β-1,3-glucanase	*Vitis vinifera*	CAB91554[§]
II	504.28	2+	1006.55	AADGSV(I/L)SC*K	Thaumatin-like protein	*Malus sylvestris*	Q9FSG7[#]
II	506.32	2+	1010.61	VC*PA(P(I/L))(QV)K	Thaumatin-like protein	*Malus sylvestris*	Q9FSG7[#]
II	528.28	2+	1054.55	SAC*(I/L)AFGDSK	Thaumatin-like protein	*Malus sylvestris*	Q9FSG7[#]
III	443.31	2+	884.61	S(K/Q)V(I/L)PT(I/L)K	Acidic endochitinase	*Cucumis sativus*	P17541[#]
III	556.32	2+	1110.62	YGGVM*(I/L)WNR	Acidic chitinase	*Glycine max*	Q9SXM5[#]
III	1012.62	2+	2023.23	V(I/L)(I/L)S(I/L)GGAAGSYS(I/L)TSADDAR	Acidic endochitinase	*Malus domestica*	AAG25709[§]
IV	644.33	2+	1286.65	((TG)/(SA))VAAVEG(I/L)TE(I/L)K	Putative cysteine protease	*Arabidopsis thaliana*	AAF88120[§]
IV	692.82	2+	1383.63	EQGAVTPV(K/Q)D(K/Q)GR	Cysteine protease	*Arabidopsis thaliana*	NP_567489[§]
IV	1113.04	2+	2224.06	S(F/M*)(Q/K)P(I/L)SVA(I/L)VASQAFQAYK	Cysteine protease	*Arabidopsis thaliana*	NP_566634[§]
V	571.73	2+	1141.44	N(I/L)AGS(I/L)SGVNPN	Non-specific lipid transfer protein	*Malus domestica*	Q9M5X7[#]
V	591.23	2+	1180.44	(I/L)STSTNCATVK	Non-specific lipid transfer protein	*Malus domestica*	Q9M5X7[#]

*Oxidized amino acid residue.
[§]NCBI accession number.
[#]Swiss Prot accession number.

pathogens (apple scab, powdery mildew, fire blight) was also analysed (Figure 1). These leaves were collected from an orchard and had non-visible symptoms of an infection with plant pathogens. The polypeptide pattern of the apoplastic fluid of this resistant cultivar is comparable to that of the apoplastic fluid obtained from *V. inaequalis* infected leaves of the non-resistant cultivar (*M. domestica* cv. Elstar). Prominent protein bands with an apparent molecular mass between 20 and 32 kD were recognized.

Identification of polypeptide fragments by Q-TOF mass spectroscopy and comparison to known proteins

The bands with the additionally expressed proteins (presented in Figure 1 and numbered I–X) were excised from the gel, digested with trypsin and subsequently analysed by Q-TOF mass spectroscopy. The obtained molecular masses and the derived amino acid sequences were identified with the program PeptideSearch (EMBL, Heidelberg, Germany). A summary of the derived amino acid sequences and the identification of these polypeptides are given in Tables 1 and 2.

All 10 protein bands were identified by different derived amino acid sequences that were obtained by collision fragmentation of peptide bonds of small tryptic polypeptide fragments in the mass spectrometer. The PeptideSearch (EMBL, Heidelberg, Germany) program revealed sequence similarities of the contiguous 10–15 amino acid fragments. The homology search in databases of the peptide sequences for the protein bands I, III and IV shows high sequence similarity to β-1,3-glucanase, chitinase and cysteine protease of several known proteins from different other species (e.g. *Prunus persica*, *Cucumis sativa*, *Vitis vinifera*, *Glycine max*, *Arabidopsis thaliana*). The protein bands II and V were identified by sequence comparison homology as a thaumatin-like protein and

Table 2. ESI Q-TOF mass spectroscopy of tryptic peptide fragments of the apoplastic fluid from cultivar Remo and identification of the derived peptide sequences by homology search

Band	Rel. mass (D)	Charge	Mass (D)	Derived amino acid sequence	Sequence similarity	Organism	Accession number
VI	692.80	2+	1383.58	(I/L)YDPNAA(I/L) AA(I/L)R	β-1,3-glucanase♦	*Prunus persica*	AAL30425§
VI	859.37	2+	1788.72	VSTA(I/L)DTGV(I/L) GNSFPPSK	β-1,3-glucanase♦	*Prunus persica*	AAL30426§
VI	1014.51	2+	2027.00	YPS(I/L)FDA(I/L)(I/L) DAQYAA(I/L)(EK)	β-1,3-glucanase♦	*Prunus persica*	P52408#
VII	504.12	2+	1006.22	AADGSV(I/L)SC*K	Thaumatin-like protein♦	*Malus sylvestris*	JC7201§
VII	506.17	2+	1010.32	VC*PAP(I/L))QVK	Thaumatin-like protein♦	*Malus sylvestris*	Q9FSG7#
VII	528.13	2+	1054.24	SAC*(I/L)AFGDSK	Thaumatin-like protein♦	*Malus sylvestris*	Q9FSG7#
VII	579.65	2+	1157.28	VSDAPSPWSGR	Thaumatin-like protein♦	*Malus sylvestris*	Q9FSG7#
VIII	809.09	2+	1616.17	NSWGTSWGEDGYMK	Putative cysteine protease♦	*Lycopersicon esculentum*	AAM19209§
VIII	943.13	2+	1884.24	ASEANYPYQSSDGR	Putative cysteine protease♦	*Arabidopsis thaliana*	AAO42167§
IX	660.17	2+	1318.32	GQSWA(I/L)DVSAGTK	Osmotin-like protein	*Glycine max*	1906370A§
IX	711.14	2+	1420.26	APGGC*NNPC*TVFK	Osmotin-like protein	*Linum usitatissimum*	AAO13658§
X	644.68	2+	1287.34	QDY(I/L)NSHNAAR	PR1 protein	*Nicotiana tabacum*	P11670§
X	702.70	2+	1403.39	VC*GHYTQVVWR	PR1 protein	*Nicotiana tabacum*	P11670§
X	820.77	2+	1639.51	(I/L)GVGP(I/L)TWD DNVAR	PR1 protein	*Nicotiana tabacum*	P11670§

*Oxidized amino acid residue.
§NCBI accession number.
#Swiss Prot accession number.
♦Similar protein found in *M. domestica* cv. Elstar.

a non-specific lipid transfer protein from *M. sylvestris*. All identified polypeptides of infected leaves from the cultivar Elstar belong to the class of PR proteins (Figure 1 and Table 2). The protein bands VI and VIII from the apoplastic fluid of the resistant cultivar Remo have the same high sequence similarities to known β-1,3-glucanase and cysteine protease proteins from *P. persica* and *A. thaliana* as also found in the apple scab susceptible cultivar Elstar after infection. Also the thaumatin-like protein (protein band VII) from *M. sylvestris* is located in the apoplast fluid in the cultivar Remo. The protein bands IX and X show high sequence similarities to osmotin-like protein from *G. max* and to PR1 protein from *Nicotiana tabacum*, respectively. The latter two proteins were only found in apoplastic fluid of the resistant cultivar Remo.

Time-dependent accumulation of PR proteins in the apoplast and two-dimensional (2D) separation of IWF

The conspicuous variation of the apoplastic fluid composition during the infection of healthy leaves with *V. inaequalis* was thoroughly analysed in a time course experiment (Figure 2). One week after infection the *V. inaequalis* treated leaves show no detectable symptoms, but prominent protein bands in the apoplastic fluid with apparent molecular masses of 32, 28, 25 and 22 kD were found on SDS-PAGE. The amount of these protein bands in the apoplast remained constant during the experiment with the exception of a slight increase of a polypeptide with an apparent molecular mass of 18 kD that accumulated during the third week. Lesions caused by *V. inaequalis* treatment were observed 2 weeks after the infection with the plant pathogen. On the other hand, the low molecular mass non-specific lipid transfer protein with an apparent molecular mass of 9 kD declined during the first week after the infection to a non-detectable level on SDS-PAGE.

The 2D separation of apoplastic fluid revealed that majority of the 20 polypeptides in the apoplastic fluid of uninfected leaves had low isoelectric points in the range of pI 4–6. In infected leaves, the number of detectable proteins increased to 47. The additionally expressed proteins were predominantly found between pI 4 and 5. The comparison of the 2D separation pattern of uninfected and with *V. inaequalis* infected leaves revealed that the majority of detected

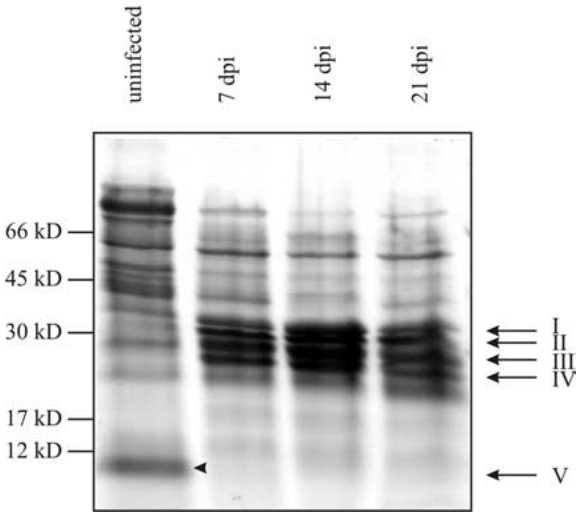

Figure 2. SDS-PAGE of apoplastic fluid from *M. domestica* cv. Elstar leaves and *V. inaequalis* infected leaves at three different times. Each lane contained 50 µg protein.

polypeptides were either newly synthesized or dramatically increased in their stationary concentration. Only four of the proteins that were expressed in infected leaves were identified by isoelectric focusing in the uninfected plant material (data not shown).

The verification of the differences between infected and uninfected leaves as a result of the artificial infection of *M. domestica* with *V. inaequalis* is presented in Figure 3. This figure compares the protein pattern of the artificially infected leaves with that of the natural infection of leaves by *V. inaequalis* observed in an orchard and with that by apple powdery mildew (*Podosphaera leucotricha*). The polypeptide composition of the apoplastic fluid from *V. inaequalis* as well as that of powdery mildew (*P. leucotricha*) infected leaves showed a comparable protein pattern. Moreover, the results revealed that the expression level of the 28 kD protein, that has been identified by artificial infection as thaumatin-like protein, is increased in the apoplastic fluid of leaves, obtained from an orchard, that were found to be infected with *V. inaequalis* by natural infection pressure.

Identification of constitutively expressed polypeptides in the IWF of the apple scab resistant cultivars M. domestica *cv. Remo and* M. domestica *cv. Pilot*

The identification of PR proteins in the apoplastic fluid of different apple cultivars by electron

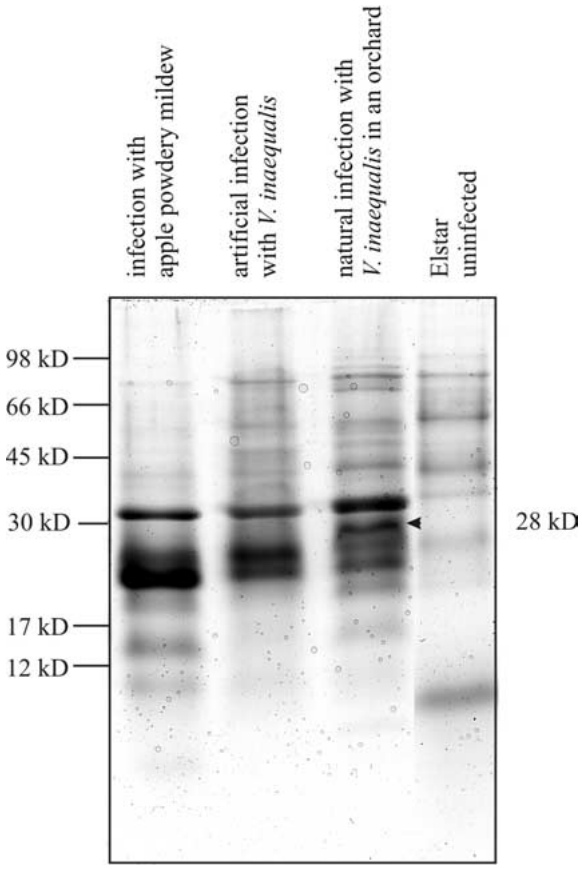

Figure 3. SDS-PAGE of apoplastic fluid from *M. domestica* cv. Elstar. Each lane contained 50 µg protein. Lane 4 has been merged by a graphic processing program to lanes 1–3.

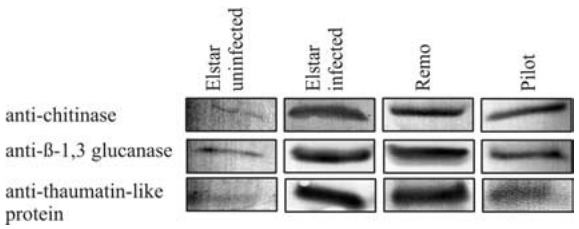

Figure 4. Immunoblots of apoplastic fluid from different *M. domestica* cultivars. Identification of PR proteins by immunoblotting. Each lane contained 50 µg protein. The anti-sera were diluted 1 : 500.

spray ionization quadrupole time of flight (ESI Q-TOF) mass spectroscopy and sequence homology search were confirmed by immunoblotting (Figure 4). The immunoblots with anti-sera against PR proteins (anti-β-1,3-glucanase, anti-chitinase

and anti-thaumatin-like protein) pointed out that β-1,3-glucanase, chitinase and thaumatin-like protein were highly expressed in infected leaves of the susceptible cultivar Elstar and in healthy leaves of the resistant cultivar Remo in comparison to the low amount of these proteins in uninfected Elstar leaves. Moreover, these proteins could also be detected in the cultivar Pilot which had an eliminated susceptibility against apple scab and mildew. The detectable levels of PR proteins in the cultivar Pilot were lower than the recognized PR protein levels of the resistant cultivar Remo or in the infected leaves of the susceptible cultivar Elstar. These data indicate the induced expression of PR genes in the infected cultivar Elstar and the constitutive expression of these genes in the resistant cultivars.

Discussion

The prominent finding of this study consists of the observation that after infection of apple leaves with *V. inaequalis* some proteins became predominant in the apoplastic fluid. The analyses by proteolysis and mass spectroscopy resulted in the identification of a number of these proteins indicating that they come from the apple tissue and not from *V. inaequalis*. The proteins that were identified are all induced by *V. inaequalis* and belong to the group of PR proteins. The expression of β-1,3-glucanase, chitinase, thaumatin-like protein and cysteine protease after the infection with *V. inaequalis* corresponds well to results of Sindelarova and Sindelar (2001) who showed the presence of PR1 protein, β-1,3-glucanase and chitinase in the apoplastic fluid of *N. tabacum* L. cv. Xanthi-nc after the infection with tobacco mosaic virus. Moreover, Rep et al. (2002) have shown the presence of PR proteins in the xylem sap of fungus-infected tomato leaves by mass spectroscopy.

The decline of the non-specific lipid transfer protein during the first week after the infection with *V. inaequalis* points to a functional role of this protein in the recognition of pathogens and in plant defence. A very similar result was recently described in the xylem sap during the infection of *Lycopersicon esculentum* by *Fusarium oxysporum* (Rep et al., 2003). To explain this relationship, Blein et al. (2002) have suggested that non-specific lipid transfer proteins bind to the elicitin receptor and consequently induce the hypersensitive cell death and non-specific systemic resistance. This cellular response, in turn, could be inhibited by a non-specific lipid transfer protein as an antagonist against to elicitin that is produced by most of the phytopathogenic fungi of the genus *Phytophthora*, and provokes in tobacco both, remote leaf necrosis and induction of a resistance against a subsequent attack by various microorganisms. It was proposed that the non-specific lipid transfer protein interacts with cutin monomers that were released by fungal cutinase activity, as a non-specific lipid transfer–cutin monomer complex triggering the plant defence responses (Blein et al., 2002). Maldonado et al. (2002) reported that an apoplastic lipid transfer protein interacts with lipid-derived molecules and operates as a mobile signal for a long distance signalling in the apoplast.

The protein pattern in the apoplast fluid that is observed after artificial infection by *V. inaequalis* is comparable to the protein pattern that were obtained after a natural *V. inaequalis* infection in an orchard as well as by powdery mildew infection indicating that both pathogens were recognized by *M. domestica* and released very similar plant defence mechanisms. This finding appears important as it indicates that the plants can prepare themselves to various degrees against an attack by a pathogen and might be explained by the assumption that an equilibrium exists between the cost of synthesis of these proteins and the chance to get into contact with the fungus. Such assumption might explain why the resistant cultivar Remo presents the same or very similar PR proteins constitutively in the apoplast as those that are induced in the cultivar Elstar by *V. inaequalis*. In addition to the β-1,3-glucanase, chitinase, thaumatin-like protein and cysteine protease the resistant cultivar Remo has located two additional constitutively expressed proteins, an osmotin-like protein an the PR1 protein, in the apoplast. Whether the anti-fungal activity of the PR1 protein as shown by Rauscher et al. (1999) for the inhibition of rust infection of broad bean or the presence of resistance genes like the V_f genes (Vinatzer et al., 2001) are responsible for the resistance of this cultivar against plant pathogens could not be answered yet.

The amount of the constitutively expressed PR proteins in the apoplast of *M. domestica* cv. Remo could be sufficient for a plant defence against pathogens during the early stage of infection with *V. inaequalis*, because only few hyphae penetrate the cuticle and were found located in the apoplast which could then rapidly be degraded by the PR enzymes. This could be a part of the plant's defence mechanisms of this cultivar that had been found resistant against *V. inaequalis* in earlier studies. The molecular mechanism of the constitutive expression of PR genes in the cultivar Remo is unclear and requires further investigation.

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