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PR-1 protein inhibits the differentiation of rust infection hyphae in leaves of acquired resistant broad bean

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Summary

Treatment of broad bean leaves with salicylic acid (SA) or 2,6-dichloro-isonicotinic acid (DCINA) induces resistance against the rust fungus *Uromyces fabae* resulting in reduced rust pustule density. Light-microscopy studies showed that in induced resistant plants the rust fungus is inhibited immediately after penetration through the stomatal pore. The differentiation of infection structures growing within the intercellular space of the leaf, i.e. infection hyphae and haustorial mother cells, is inhibited. Furthermore, low-temperature scanning electron microscopy studies of freeze fractures revealed protrusions at the tips of infection hyphae growing in induced resistant broad bean leaves. Treatment of *in vitro*-differentiating rust infection structures with intercellular fluids (IFs) from induced resistant plants confirmed that the fungus is sensitive towards an apoplastic anti-fungal activity only after having formed appressoria. Other legume rusts such as *U. vignae* and *U. appendiculatus* were likewise inhibited in the presence of IF from SA-treated broad bean leaves. Heterologous antibodies were used to study changes in the extracellular pathogenesis-related (PR) protein pattern after resistance induction. Western blots indicated that chitinases and β-1,3-glucanases were present in both induced and control plants. In contrast, PR-1 proteins were newly synthesized in response to SA or DCINA application. The major induced PR-1 protein was purified and exhibited strong differentiation-inhibiting activity towards *U. fabae* infection structures. We conclude that the inhibition of rust infection hyphae in acquired resistant broad bean plants is mainly due to the anti-fungal activity of this induced basic PR-1 protein.

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Introduction

Plants have evolved diverse mechanisms to defend themselves against pathogen attack (Bowles, 1990; Brisson *et al.*, 1994; Vance *et al.*, 1980). Acquired resistance, one of the most elaborate responses to pathogens, consists of a whole array of defence reactions (Dean and Kuć, 1987; Hammerschmidt and Kuć, 1982; Sticher *et al.*, 1997; Stumm and Gessler, 1986) and is effective against various pathogens including viruses, bacteria and fungi (Schneider *et al.*, 1996). This type of resistance can be induced by pre-inoculation with virulent or avirulent pathogens (Kuć, 1982; Mauch-Mani and Slusarenko, 1994; Uknes *et al.*, 1993) or by application of resistance-inducing chemicals such as salicylic acid (SA), 2,6-dichloro-isonicotinic acid (DCINA) or benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (Görlach *et al.*, 1996; Kessmann *et al.*, 1994). Endogenous SA has been shown to play a key role in acquired resistance (Delaney *et al.*, 1994; Gaffney *et al.*, 1993).

In most plants investigated, acquired resistance is associated with the induction of pathogenesis-related (PR) proteins (Bol *et al.*, 1990; Ryals *et al.*, 1996; van Loon *et al.*, 1987; Ward *et al.*, 1991). Many of these proteins exhibit anti-fungal activity *in vitro* (Ji and Kuć, 1996; Niderman *et al.*, 1995; Ponstein *et al.*, 1994; Woloshuk *et al.*, 1991). Proteins belonging to the PR-2 family are known to be β-1,3-glucanases, and those classified as PR-3 proteins exhibit chitinolytic activity. Both enzymes act together in the lysis of fungal cell walls (Mauch *et al.*, 1988). The osmotin-like PR-5 proteins have been shown to permeabilize fungal plasma membranes (Abad *et al.*, 1996). In contrast, nothing is known about the mode of action of PR-1-type proteins.

Our work addressed the mechanisms underlying acquired resistance of broad bean to the rust fungus *Uromyces fabae* by investigating both time course and spatial distribution of factors acting against the fungus in chemically induced leaves. This obligate biotrophic rust fungus penetrates the host plant through stomatal openings, differentiates a series of infection structures in the intercellular space, and exhibits highly localized penetration of the host cell wall to establish a haustorium (Deising *et al.*, 1996; Mendgen *et al.*, 1996). All infection structures

up to the haustorial mother cell are also differentiated *in vitro* on thigmo-inductive polyethylene membranes (Deising *et al.*, 1991). This system can be used for bioassays and to test anti-fungal effects possibly involved in acquired resistance.

Our results show that one of the major mechanisms underlying acquired resistance of broad bean is the specific inhibition of rust infection structures invading the leaf. This effect is mainly due to the anti-fungal activity of a basic PR-1 protein present in the intercellular space of acquired resistant broad bean leaves. Low-temperature scanning electron microscopy (LTSEM) studies revealed that the occurrence of this PR-1 homologue is correlated with severe morphological anomalies of fungal structures growing within the leaf. For the first time we present evidence that rust infection hyphae are the target of an anti-fungal PR-1 protein.

Results

SA and DCINA are known to induce resistance against a broad spectrum of pathogenic micro-organisms in higher plants. Accordingly, infiltration of broad bean leaves with these chemicals resulted in resistance to the broad bean rust fungus *U. fabae* (Figure 1). On control leaves treated with distilled H_2O, rust pustules developed densely, indicating high susceptibility of the plant. On leaves infiltrated with SA, the pustule density was significantly reduced, but the upper untreated leaves of the plant were still susceptible. In contrast, after DCINA treatment, all leaves became resistant to *U. fabae*.

SA induced resistance in a concentration-dependent manner. Infiltration of leaves with 0.5 mM SA 1 day before inoculation with *U. fabae* urediniospores (Figure 2) resulted in a 69.1% reduction of pustule density. When plants were treated 4 days before inoculation, the level of resistance was lower, leading to pustule reduction by 7.1% only. In leaves treated with 50 µM DCINA 1 day before inoculation, a reduction of pustule density by 35.2% was found, in contrast to 59.9% reduction when DCINA was applied 4 days prior to inoculation. Concentrations exceeding 0.5 mM SA or 50 µM DCINA led to phytotoxic effects and were not used in our experiments.

To find out at which stage the rust fungus is affected in induced resistant broad bean plants, leaves were treated with SA, inoculated 1 day later and after another 24 h infection structures were counted (Figure 3). In the mesophyll of control plants, predominantly infection hyphae and haustorial mother cells were present. About 5% of the structures had formed haustoria in mesophyll cells at that time, and only about 8% of the fungal structures did not develop beyond the appressorial stage. By contrast, in SA-

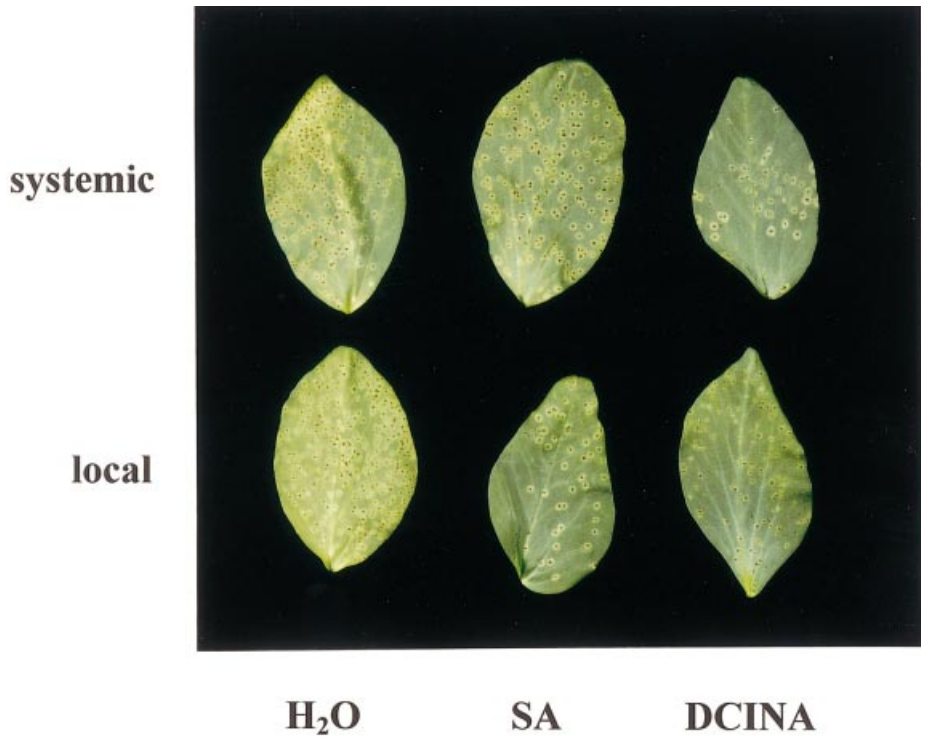

Figure 1. Rust pustule density on treated and upper untreated broad bean leaves.
Leaves were infiltrated with H_2O (control), 0.5 mM SA or 50 µM DCINA to induce resistance and plants were inoculated 1 day (SA) or 4 days (DCINA) after induction. Treated (local) and upper (systemic) leaves were excised and photographed 10 days after inoculation.

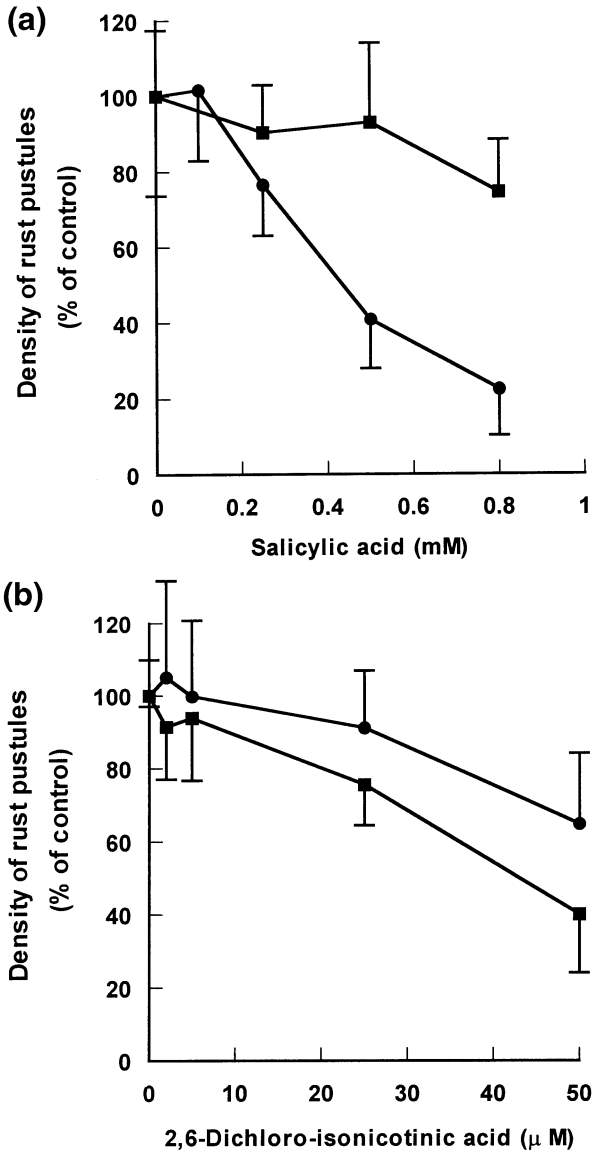

Figure 2. Rust pustule density after infiltration of broad bean leaves with different concentrations of SA and DCINA.
Inoculation was performed 1 day (●) or 4 days (■) after treatment. Pustules were counted 8 days after inoculation.

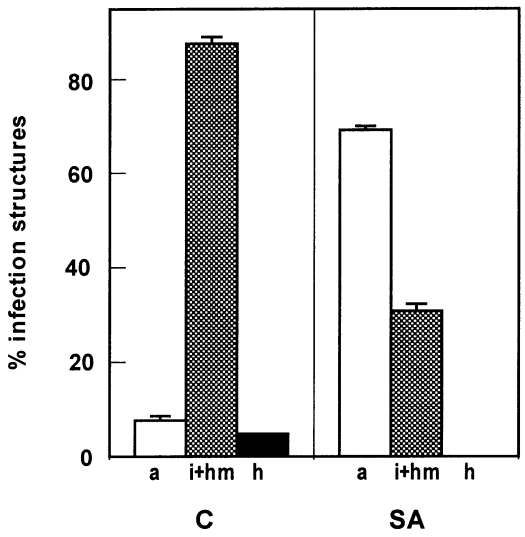

Figure 3. Comparison of infection structure differentiation in broad bean leaves treated with H_2O (C) or 0.5 mM SA (SA).
Fungal infection structures were counted 24 h after inoculation. a, appressoria; i + hm, infection hyphae (including sub-stomatal vesicles) and haustorial mother cells; h, haustoria.

treated plants, most fungal sporelings (69%) stopped development after formation of appressoria and no haustoria were found 24 h after inoculation.

LTSEM studies of freeze-fractured broad bean leaves confirmed the results of our light microscopy investigations (Figure 4). As expected, rust infection structures were found abundantly in the intercellular space of inoculated control leaves 24 h after inoculation. Differentiation of haustorial mother cells occurred in intimate contact with host mesophyll cells (Figure 4a). The cell walls of infection hyphae appeared smooth, with no signs of disturbance. By contrast, in freeze fractions of acquired resistant leaves, intercellular hyphae were scarce. The majority of these exhibited striking protrusions at their tips (Figure 4b,c). The cell-wall texture of hyphal apices was rough and these appeared to consist of coarse cell-wall material. Taken together, these microscopical studies suggested the presence of an inhibitory factor which affects fungal structures growing in the intercellular space of acquired resistant broad bean leaves.

To test whether this inhibitor can be extracted with intercellular fluids (IFs) from acquired resistant plants, we used a bioassay in which rust urediniospores were inoculated onto thigmo-inductive polyethylene membranes and sprayed with IFs isolated from SA-treated leaves or from control leaves. Microscopic analysis of trypan blue-stained infection structures revealed drastic differences in fungal development (Figure 5). In the presence of IFs from control plants, normal differentiation of *U. fabae* took place, with approximately 80% of the structures differentiating infection hyphae and haustorial mother cells. When IFs from induced resistant plants were applied, more than 65% of the structures arrested growth after developing appressoria. LTSEM studies revealed that after formation of appressoria in the presence of the inhibitor, hyphae exhibited deformed and collapsed tips (Figure 6). The growth of all subsequent infection structures was significantly inhibited by the anti-fungal activity present in IFs from SA-treated plants.

The inhibitory effect on *in vitro*-differentiating infection structures was not restricted to the broad bean rust fungus *U. fabae*. Two other legume rusts, the cowpea rust *U. vignae* and the bean rust *U. appendiculatus*, were likewise inhibited by IFs extracted from acquired resistant broad bean leaves. While differentiation proceeded up to the

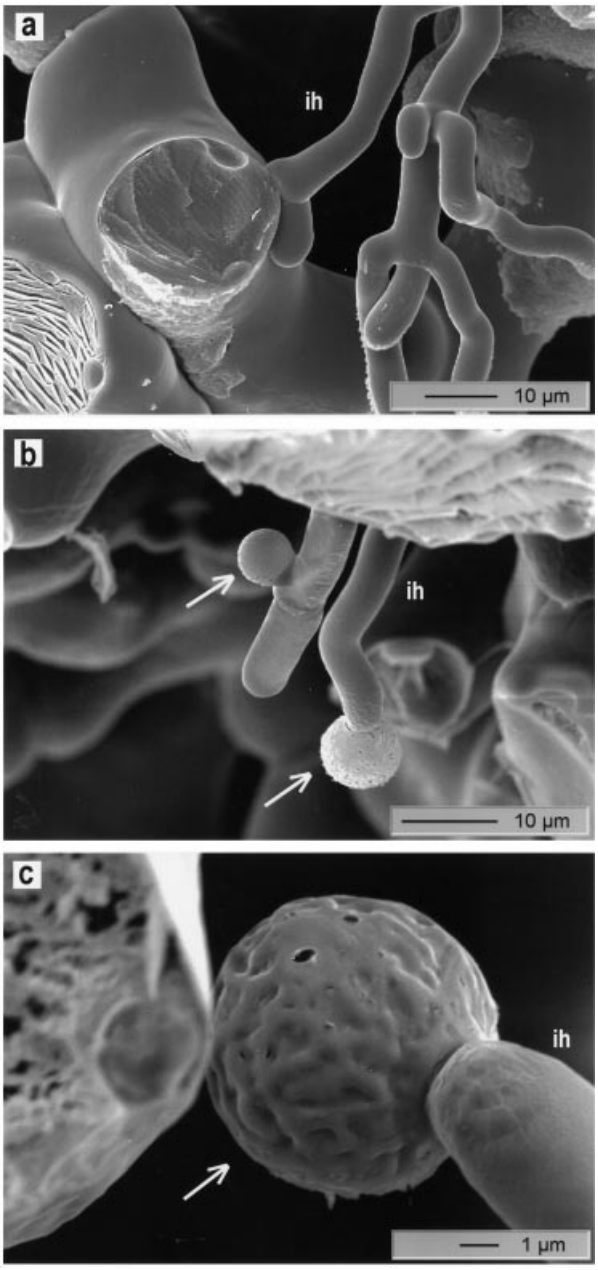

Figure 4. Freeze fractures of broad bean leaves treated with water (a) or 0.5 mM SA (b, c) and inoculated with *U. fabae* urediniospores 24 h later. Leaves were excised 1 day after inoculation. ih, infection hypha; protrusions are marked with arrows.

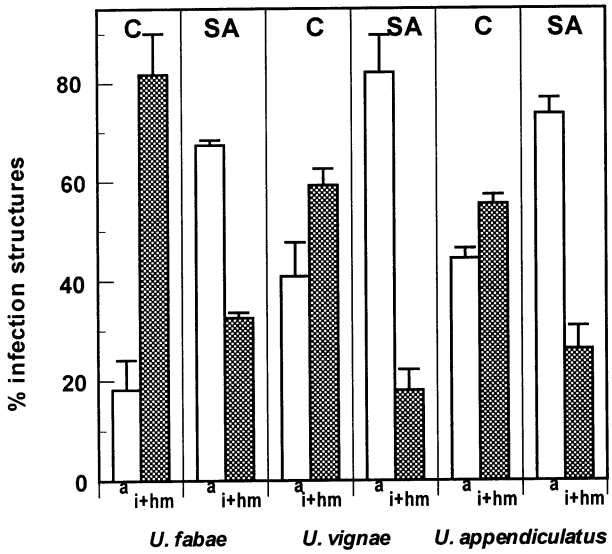

Figure 5. Infection structures formed by different legume rusts on thigmo-inductive membranes after treatment with IF isolated 1 day after treatment of leaves with distilled H_2O or SA.
a, appressoria; i + hm, infection hyphae (including sub-stomatal vesicles) and haustorial mother cells.

formation of infection hyphae and haustorial mother cells in the presence of IFs from control plants, fungal development stopped after formation of appressoria when IFs from SA-treated plants had been applied (Figure 5).

Dialysis and proteinase digestion experiments with IFs from SA-treated plants suggested that the inhibitor might be a small protein (data not shown). Therefore, it was of interest whether resistance-related proteins were present in IFs from acquired resistant broad bean leaves in comparison with water-treated or necrotized leaves. After electrophoretic separation and electrotransfer of apoplastic proteins, blots were probed with heterologous antibodies raised against known PR proteins (Figure 7). Interestingly, in IFs from both control and acquired resistant plants, antibodies to class I β-1,3-glucanase and chitinase from potato cross-reacted with protein bands of 38.7 and 38 kDa, respectively. In addition, an antibody specific to tobacco class III chitinase detected a 30.4 kDa band in IFs from induced as well as from non-induced plants. This was consistent with photometrically determined chitinase and β-1,3-glucanase activities, which were similar in SA-treated and control plants (data not shown). In contrast, antibodies raised against tobacco PR-1a and P14, a PR-1 like protein from tomato, cross-reacted with small proteins present in IFs from induced plants, but not for control plants – or in the case of the P14-antibody at much lower intensity. In IFs from SA- and DCINA-treated plants, two bands of 15 and 15.9 kDa cross-reacting with the PR-1a antibody were found and an additional band of 17 kDa appeared in IFs from plants necrotized by $AgNO_3$ treatment. An antibody raised to PR-1c from tobacco detected an 18 kDa protein in IFs from induced as well as from non-induced plants.

The presence of the broad bean PR-1a homologue coincided with the resistant state of the leaves (Figure 8, compare with Figure 1). The protein occurred in treated leaves 1 day after application of 0.5 mM SA but could not be detected 3 days later. When 50 µM DCINA was used, PR-1 was detectable 1 and 4 days after application, both in the treated leaves and in the upper untreated leaves. SA did

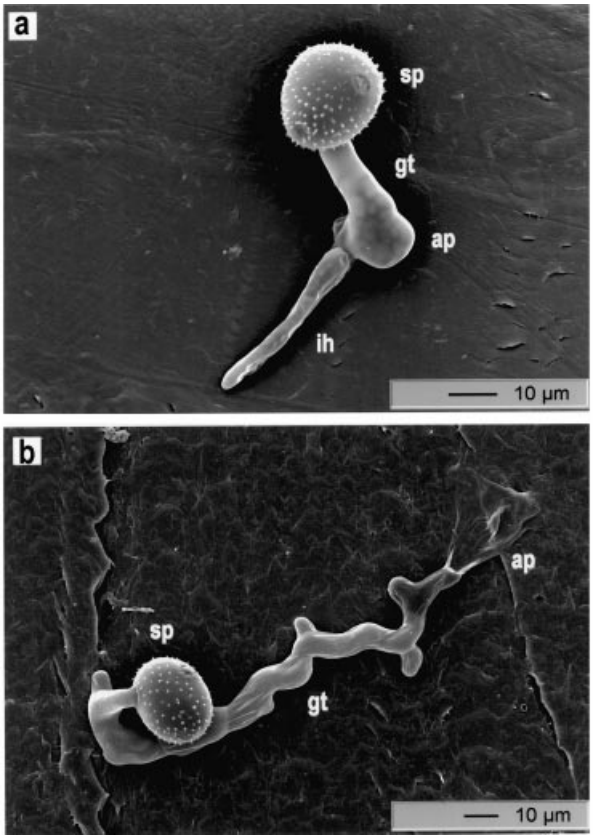

Figure 6. LTSEM of *in vitro*-differentiating infection structures. *U. fabae* urediniospores were inoculated onto thigmo-inductive membranes and sprayed with IF from H_2O-treated (a) or SA-treated (b) broad beans. Infection structures were visualised by LTSEM after 24 h. sp, spore; gt, germ tube; ap, appressorium; ih, infection hypha.

not induce systemic accumulation of PR-1. This corresponds to the inability of SA to reduce rust pustule densities in upper untreated leaves.

These results strongly suggested the involvement of PR-1 in acquired resistance of broad bean to *U. fabae*. We therefore purified the major induced PR-1a homologue by chromato-focusing and subsequent hydrophobic interaction chromatography. The purified protein (15.9 kDa, pI = 9.8) was applied to *in vitro*-differentiating *U. fabae* infection structures and exhibited strong anti-fungal and differentiation-inhibiting effects comparable to those of the IFs from induced resistant leaves (Figure 9). The formation of infection hyphae and haustorial mother cells was reduced by up to 59%. Appressoria frequently appeared collapsed. Fractions containing the highest amounts of PR-1 in some cases caused total lysis of infection structures.

Discussion

In various plant species, resistance can be induced with chemicals such as SA, DCINA and BTH (Kessmann *et al.*, 1994; Ryals *et al.*, 1996; Schneider *et al.*, 1996; Sticher *et al.*, 1997). In broad bean plants, treatment with these inducers efficiently reduces susceptibility to rust disease caused by *U. fabae*. While resistance induction by SA was restricted to the treated leaf, DCINA treatment resulted in systemic resistance. This difference is probably due to the rapid conversion of SA into its glucoside (SAG) (Hennig *et al.*, 1993; Lee and Raskin, 1998). Glucosylation could also account for the rather transient induction of resistance by SA, as compared to the longer persisting effect of DCINA. Yet, we used SA in most of our experiments as it has a key position in the natural signal transduction pathway of acquired resistance (Delaney *et al.*, 1994; Gaffney *et al.*, 1993; Métraux *et al.*, 1990). Whether or not SA can be considered a long distance signal is still controversial (Yang *et al.*, 1997). While grafting experiments indicated that this was not the case (Vernooij *et al.*, 1994), refined methods to follow SA transport have shown that up to 70% of the increase in SA concentration in upper non-treated leaves originated from the lower inoculated leaf (Mölders *et al.*, 1996; Shulaev *et al.*, 1995).

Acquired resistance is often characterized by the induction of marker genes or proteins mainly belonging to the group of pathogenesis-related (PR) proteins (Ward *et al.*, 1991). However, the pattern of markers differs significantly between plant species (Ryals *et al.*, 1996). Many PR proteins are known to act anti-microbially *in vitro* (Ji and Kuć, 1996; Mauch *et al.*, 1988; Niderman *et al.*, 1995; Ponstein *et al.*, 1994; Woloshuk *et al.*, 1991) and in transgenic plants. The over-expression of tobacco osmotin reduced growth of *Phytophthora infestans* in potato but not in tobacco (Liu *et al.*, 1994). Furthermore, synergistic effects of chitinases and β-1,3-glucanases have been shown in transgenic plants (Jach *et al.*, 1995). By contrast, over-expression of PR-1a (Linthorst *et al.*, 1989) or PR-1b (Cutt *et al.*, 1989) in tobacco did not influence the susceptibility to TMV, but significantly enhanced resistance against two oomycete pathogens was achieved by constitutive high-level expression of PR-1a in this plant (Alexander *et al.*, 1993). These results indicate that several PR proteins might be involved in induced defence responses.

To elucidate defence mechanisms acting against the biotrophic broad bean rust fungus in acquired resistant plants, we focused on the early stages of the interaction. Light microscopy indicated that fungal differentiation is blocked in the intercellular space of SA-treated leaves, in most cases immediately after formation of appressoria, i.e. when the fungus initiates invasion of the sub-stomatal cavity. LTSEM studies confirmed these results. In freeze fractions of SA-treated leaves, the morphology of rust infection structures was severely affected. Protrusions were found at the hyphal apices, where the cell wall structure was of altered appearance. This might be due to

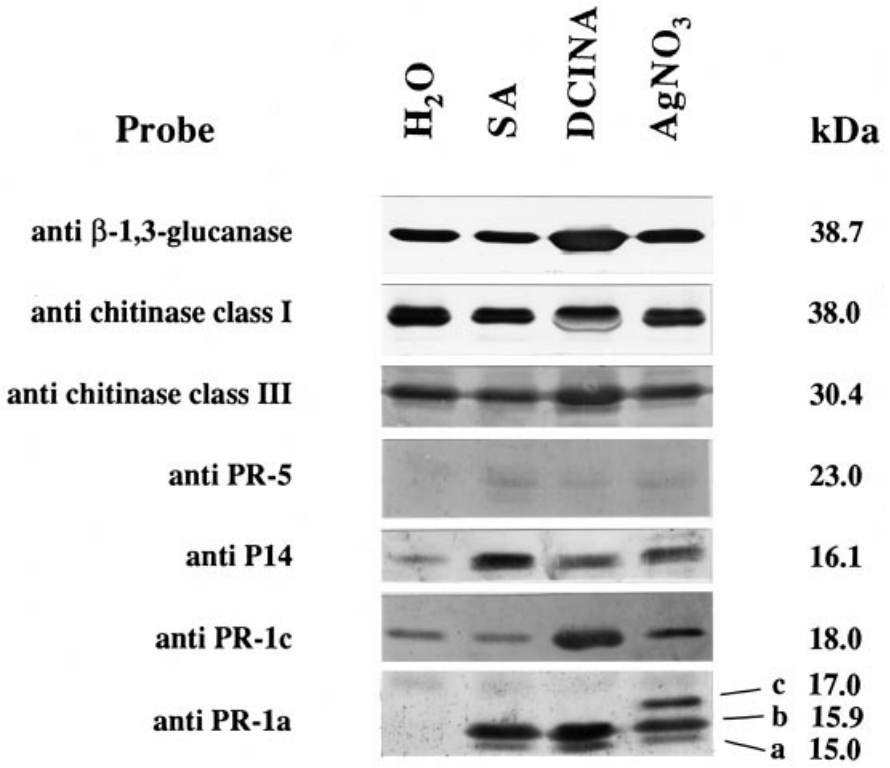

Figure 7. Western blots of apoplastic proteins from broad bean leaves treated with H_2O, SA, DCINA or the necrotizing agent $AgNO_3$.
IFs were isolated 1 day (SA, H_2O) or 4 days (DCINA; $AgNO_3$) after treatment. Heterologous antisera as indicated were used at 750-fold dilution to detect broad bean antigens.

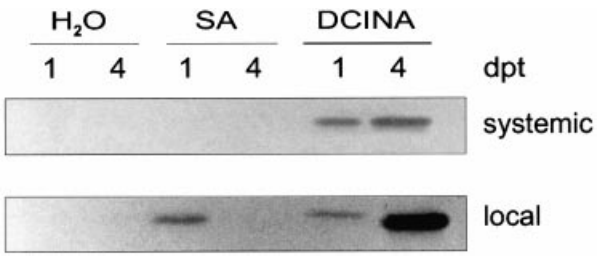

Figure 8. Western blots of apoplastic proteins from treated and upper untreated broad bean leaves.
IFs were isolated from leaves excised 1 or 4 days after treatment (dpt) with H_2O, SA or DCINA. Blots were probed with antibodies raised against tobacco PR-1a.

an impaired synthesis of structural polymers, which could result in reduced cell wall stability.

Since we assumed the presence of an inhibitory activity in the apoplast of chemically induced resistant broad bean leaves, we extracted IFs and analysed them for occurrence of PR proteins. Western blots probed with heterologous antibodies revealed PR-1 isoforms as marker proteins for acquired resistance in broad bean. Interestingly, chitinases and β-1,3-glucanases were present in large amounts in the intercellular space of control leaves and did not disturb fungal development. Previous studies have shown that *U. fabae* changes its surface properties when invading the sub-stomatal cavity, so that chitin is no longer exposed on the hyphal surface (Deising *et al.*, 1996; Kapooria and Mendgen, 1985). Work by Deising and Siegrist (1995) suggested that this was due to the action of chitin deacetylases, the synthesis of which is morphogenetically controlled in *U. fabae*. After conversion of chitin to chitosan, the polymer appears to be inaccessible to chitinase digestion (Ride and Barber, 1990). These findings explain unaffected fungal growth in host leaves containing large amounts of chitinases and β-1,3-glucanases.

The presence of the broad bean PR-1a homologue coincided with the resistant state of SA- or DCINA-induced plants. Furthermore, experiments in which *in vitro*-differentiating *U. fabae* infection structures were treated with the purified PR-1a homologue showed unambiguously that this basic protein exhibits strong anti-fungal activity resulting in differentiation inhibition at the appressorial stage. Anti-fungal effects of PR-1 proteins have already been described, e.g. inhibition of zoospore germination and mycelial growth of *Phytophthora infestans* (Niderman *et al.*, 1995). Yet the mode of action as well as molecular and cellular targets of PR-1 proteins are unknown. In induced resistant mycorrhizal tomato plants infected with

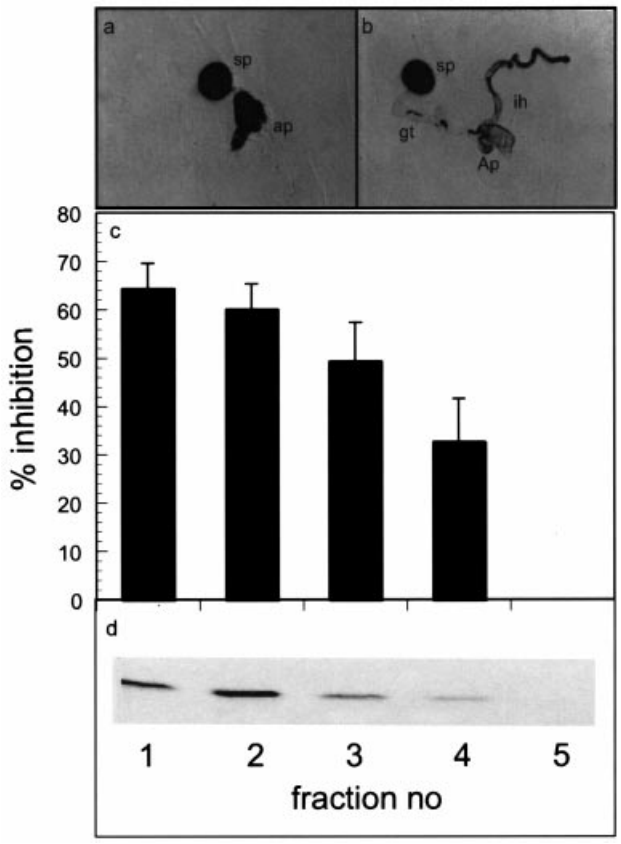

Figure 9. Inhibition of infection hypha differentiation by the purified broad bean PR-1a homologue.
In vitro-differentiating rust infection structures were treated with fractions of purified PR-1a homologue (a) or H_2O (b). The inhibition of infection hypha differentiation (% of control) was analyzed 24 h post-treatment (c) and correlated with the amount of PR-1 present in the column fractions as shown in Western blots (d).

Phytophthora parasitica, Cordier *et al.* (1998) immunolocalized PR-1 to the cell walls of the fungus. This corresponds with our observation that the fungal cell wall is altered in the presence of PR-1.

In conclusion, we describe for the first time the inhibition of fungal differentiation as a possible effect of PR-1 proteins, and present evidence that apices of infection structures might represent the target of these anti-fungal proteins.

Experimental procedures

Plant and fungal material, and induction of resistance

Broad bean (*Vicia faba* cv. *con amore*, Nickerson Zwaan, Edemissen, Germany) plants were grown in growth chambers under a 16 h:8 h light:dark regime at 22°C. For resistance induction, the second leaves of 16-day-old plants were infiltrated with 0.5 mM Na-salicylate (SA) or 50 μM 2,6-dichloro-isonicotinic acid (DCINA) if not indicated otherwise. Necrosis was induced by infiltration with 10 μM $AgNO_3$. To test for acquisition of resistance, plants were inoculated with a suspension of *U. fabae* urediniospores (0.1 mg urediniospores and 1 mg BSA per ml distilled H_2O).

Urediniospores of *U. fabae* [formerly referred to as *U. vicae-fabae* (Boerema and Verhoeven, 1979)] originated from a single spore line isolated from naturally infected plants and were propagated on *V. faba* cv. *con amore* as described previously (Deising *et al.*, 1991). Urediniospores were used immediately after harvesting or stored at –70°C. Urediniospores of other legume rusts were obtained from the stock collection of K. Mendgen.

Growth of *in vitro*-differentiating infection structures from urediniospores of *U. fabae* was performed on scratched polyethylene sheets as described earlier (Deising *et al.*, 1991). Structures were analysed 24 h after inoculation.

Staining and light microscopy

Microscopical techniques essentially followed the description of Deising *et al.* (1991). Infection structures growing in broad bean leaves were analysed as described by Rohringer *et al.* (1976).

Low-temperature scanning electron microscopy

LTSEM was essentially performed as described in Müller *et al.* (1991). Thigmo-inductive membranes with fungal infection structures or infected leaves were cut into pieces and mounted onto a Balzers specimen table using a low-temperature mounting medium and rapidly frozen by plunging into liquid nitrogen. After cryofixation, samples were transferred to a Balzers cryopreparation unit SCU 020 attached to a JEOL JSM 6300 scanning electron microscope. Infected leaves were freeze-fractured. Sputter coating with 20 nm gold was carried out in argon gas atmosphere. The samples were examined and photographed at a stage temperature of –165°C using an accelerating voltage between 10 and 25 kV.

Isolation of intercellular plant proteins

Intercellular fluids containing apoplastic proteins of broad bean leaves were isolated as follows. Leaves were infiltrated with 20 mM Na-phosphate buffer, pH 7.5, at 0–2°C and –800 mbar for 3 min, before the vacuum was slowly released over 3 min. The plant material was then blotted dry between paper towels and centrifuged at low speed (36 *g*, 20 min, 4°C).

The activity of the intracellular marker enzyme malate dehydrogenase (Deising *et al.*, 1995) in the IF was used to judge cell integrity. Only samples with less than 0.1% MDH activity as compared to that of leaf homogenates were used in our experiments.

SDS–PAGE and Western blots

SDS–PAGE was performed with 4% acrylamide/0.2% bis-acrylamide stacking and 12.5% acrylamide/0.33% bisacrylamide running gels in the buffer system described by Laemmli (1970). Gels were run at a constant current of 15 mA (stacking gel) and 30 mA (running gel).

After SDS–PAGE, the separated proteins were electroblotted onto PVDF membranes for 2 h at 1.5 mA/cm^2, using the semi-dry procedure and the buffer system described by Kyhse-Anderson (1984). Lanes containing marker proteins were cut off and stained in a solution of 0.1% (w/v) Coomassie brilliant blue R-250 in 50% (v/v) methanol, 10% (v/v) acetic acid (LeGendre and Matsudaira,

1989). The PVDF membranes were incubated in blocking buffer (2% (w/v) BSA and 3% (w/v) skimmed milk powder, 0.02% (w/v) NaN_3 in TBS (150 mM NaCl, 50 mM Tris/HCl, pH 7.2)) for at least 3 h. Then a 3 h incubation with rabbit antibodies to tobacco PR-1a (White *et al.*, 1987) or PR-1c (van Loon *et al.*, 1987), tomato P14, potato class I chitinase or β-1,3-glucanase (generous gift of E. Kombrinck, MPI Köln, Germany), tobacco class III chitinase (Legrand *et al.*, 1987), tomato P-2 (Joosten *et al.*, 1990) and tobacco PR-5 (Kauffmann *et al.*, 1990), each 750-fold diluted with blocking buffer, was performed. After three washes with TBS, the membranes were incubated with alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit, AP-conjugate; Sigma, Deisenhofen, Germany), diluted 5000-fold in blocking buffer for 45 min, followed by five washes in TBS. Finally the membranes were incubated in a staining solution containing 0.4 mM BCIP, 0.4 mM NBT in 100 mM Tris buffer, pH 9.5, 100 mM NaCl, until signals of desired intensity were obtained. The developed membranes were washed with TBS and dried.

Purification of the PR-1 protein

IF from SA-treated leaves was loaded onto a column containing PBE 118 resin equilibrated with 0.025 M triethylamine, pH 11. Pharmalyte buffer (diluted 1:45, pH 8; Pharmacia Biotech, Freiburg, Germany) was used to generate a pH gradient, and fractions of 2 ml volume were collected. Fractions containing PR-1 were adjusted to 1.8 M ammonium acetate and loaded onto a column containing the hydrophobic interaction resin Fractogel TSK Butyl 650 (M) (Merck, Darmstadt, Germany) equilibrated with 1.8 M ammonium acetate. After excessive washing with 1.8 M ammonium acetate, the PR-1 protein was eluted with 1.6 M ammonium acetate. This fraction contained a single protein band of 15.9 kDa as shown by SDS–PAGE and subsequent silver staining (Jungblut and Seifert, 1990).

Acknowledgements

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