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Analysis of defense-related proteins in stem tissue of carnation inoculated with a virulent and avirulent race of *Fusarium oxysporum* f.sp. *dianthi*

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Abstract

The aim of this study was to learn more about the accumulation of defense-related proteins in stem tissue from carnation cultivar Pallas inoculated with 2 near-isogenic races, the avirulent race 1 and the virulent race 8 of *Fusarium oxysporum* f.sp. *dianthi*. Stem tissue was used, from which the epidermis, cortex and medulla were peeled off from the vascular cylinder. It appeared that chitinase activity was constitutively expressed in the intercellular fluids (IFs) of untreated leaves, stems and roots of carnation. The total chitinase activity in the IFs of stem tissue increased with time after inoculation. This increase was similar after inoculation with the virulent, the avirulent race and water. At least four chitinase isoenzymes, three acidic and one basic isoform, were detected in the IFs of inoculated plants. In contrast, total 1,3-β-glucanase activity was not detected in the IFs of untreated leaves, stems and roots. Furthermore, the increases in 1,3-β-glucanase activity in IFs of stem tissue were markedly higher in the compatible and incompatible interactions than in the water control, indicating that this activity is specially induced by elicitors common to both races 1 and 8 of *Fusarium oxysporum* f.sp. *dianthi*. Using an antiserum against 1,3-β-glucanase P3 of tomato, 2 bands were detected on immunoblots in the IFs of stem tissue inoculated with races 1 and 8. No bands were visible after inoculation with water. Total peroxidase activity increased with time in all combinations. One basic and one acidic peroxidase isoform were present in these IFs. Peroxidase activity in a cell wall fraction prepared from stem tissue was clearly higher, and its increase faster, than the activity in the soluble stem fraction. These increases were similar in the virulent, the avirulent race and the water control. The growth of the fungus *Trichoderma viride* was inhibited by the IFs obtained from stem tissue inoculated with the virulent and the avirulent race of *Fusarium oxysporum* f.sp. *dianthi*. However, the growth of *Fusarium oxysporum* f.sp. *dianthi* itself was not affected by these IFs.

Introduction

The fungus *Fusarium oxysporum* f.sp. *dianthi* (Fod) colonizes the vascular tissue of carnation and causes vascular wilt disease. The carnation cultivar Pallas shows a differential response to the races 1 and 8 of this fungus, developing an incompatible reaction with race 1 and a compatible reaction with race 8. Races 1 and 8 are near-isogenic and belong to the same vegetative compatibility group (VCG 0022) (Manicom and Baayen, 1993; Baayen et al., 1996). The mechanisms determining this differential response are yet unknown, but it has been suggested that plants utilize a large number of constitutive and inducible defense mechanisms to prevent colonization by pathogens. Accumulation of phenolics in cell lumina and lignification and suberization of walls of parenchyma cells have been described as defense responses in xylem vessels of roots and stems of carnation to restrict the growth of Fod (Baayen et al., 1996). Peroxidases are involved in the cross-linking of phenolics and in lignin and suberin biosynthesis in resistant cultivars (Ride, 1983; Mohan

and Kolattukudy, 1990; Bradley et al., 1992). In several crops, resistant cultivars produce higher quantities of specific peroxides isoenzymes upon infection than susceptible cultivars (Mohan and Kolattukudy, 1990; Reimers et al., 1992; Scott-Graig et al., 1995). In rice for instance, the accumulation of a cationic peroxidase is correlated with resistance. This peroxidase accumulates in the apoplastic fluid and cell walls of xylem vessels only during incompatible interactions with *Xanthomonas oryzae* p.v. *oryzae* (Young et al., 1995).

Besides peroxidases, the differential accumulation of PR proteins as a response to microbial attack has been studied in great detail in several plant species (Van Loon, 1985; Dixon and Lamb, 1990). Two groups of PR proteins, e.g. chitinases and 1,3-β-glucanases have attracted considerable interest because of their inhibitory activity *in vitro* and *in vivo* against fungi (Van den Elzen et al., 1993). In tomato leaves infected with *Cladosporium fulvum* the activity of these hydrolytic enzymes increases more rapidly in the apoplastic fluids of incompatible interactions as compared to compatible interactions (Joosten and De Wit, 1989). In susceptible cultivars of tomato infected by *Fusarium oxysporum* f.sp. *radicis-lycopersici*, 1,3-β-glucanases accumulate in areas close to fungal cells. In resistant cultivars 1,3-β-glucanases also accumulate in the uninvaded tissue, suggesting that differences in the spatial accumulation of these enzymes may play a role in resistance (Benhamou et al., 1989).

The objective of this study was to learn more about the accumulation of defense-related proteins in intercellular fluids obtained from stem tissue of carnation cultivar Pallas after stem inoculation with races 1 and 8. The advantage of inoculation directly into the stem lies in the consistency of timing and extent of infection. This inoculation method provides a way to compare the accumulation of defense-related proteins between different interactions. In the compatible interaction the fungus colonizes the vascular tissue and in the incompatible interaction the growth of the fungus is restricted by a localization response. This defense response has been localized by electron microcopy studies in the xylem tissue (Baayen et al., 1996). To study the accumulation of defense-related proteins in xylem tissue, stem tissue was used, from which the epidermis, cortex and medulla were peeled off from the vascular cylinder. Here, we describe the analysis of total protein, chitinase, 1,3-β-glucanase and peroxidase activities and their isozymes in stem tissue obtained from compatible and incompatible interactions of Fod with carnation cultivar Pallas.

Materials and methods

Inoculation of plants

Rooted cuttings of carnation (*Dianthus caryophyllus* L.) cultivar Pallas were obtained from Van Staaveren B.V., Aalsmeer, The Netherlands and grown in steamed soil in the greenhouse for 5 weeks at 18 °C. Monospore isolates of *Fusarium oxysporum* Schlecht. : Fr. f.sp. *dianthi* (Prill. & Delacr.) Snyder & Hansen race 1 (Garibaldi F101) and race 8 (Garibaldi F639) (Aloi and Baayen, 1993) were grown in potato dextrose broth (PDB) in shake cultures at 25 °C at 150 rpm. Four-day-old cultures were filtrated through a 48 μm filter (Nybolt 48/31) to remove the mycelium. Conidia were pelleted for 10 min at 1500 rpm (Hermle, BHG) and adjusted to a concentration of 10^7 conidia ml^{-1} with sterile tap water. Plants were stem inoculated with race 1, race 8 or sterile tapwater according to method III described by Baayen and Schrama (1990).

Preparation of the intercellular fluid

Intercellular fluids (IFs) were obtained from the stems at various times after inoculation. Two-cm-long stem segments directly above the inoculation site were removed and cut longitudinally. The epidermis, cortex and medulla were peeled off from the vascular cylinder. The remaining tissue, designated stem tissue, was weighed, placed on ice and washed with ice cold water to remove material leached from damaged surfaces. Subsequently, the sections were centrifuged immediately at 1000*g* for 20 min, per gram stem tissue 30–50 μl of IF was obtained. The IFs were precipitated with 4 volumes of acetone at −20 °C. The pellet obtained after centrifugation (5 min, 1500*g*) was washed twice with 80% (w/w) acetone and dried. The pellet was suspended in water (1/3 of the original volume) and stored at −20 °C. IFs from leaves and roots were obtained as described by De Wit and Spikman (1982). The presence of cytoplasmic contaminants from surrounding tissue was determined using the glucose-6-P-dehydrogenase assay described by Bergmeyer and Grass (1983).

Preparation of a cell wall fraction from stem tissue

Cell wall containing fractions were prepared as described by McDougall (1992). Stem tissue was weighed and frozen in liquid nitrogen and ground in 1 ml 1 M NaCl, 1% (v/v) Triton-X-100 and 1% (w/v) bovine serum albumin (BSA) per gram tissue. The extract was centrifuged for 5 min at 14 000*g* at 4 °C. The supernatant was dialyzed overnight (Spectra/Por NWCO 3500) against 50 mM phosphate buffer (pH 6.0) at 4 °C. The pellet was washed twice with ice cold 50 mM phosphate buffer (pH 6.0) and suspended in the same buffer. The pellet and supernatant were stored at −80 °C.

Protein determination

Protein determination was assayed according to the method described by Bradford (1970) using BSA as calibration standard.

PAGE and Western blot analysis

Polyacrylamide gelelectrophoresis (PAGE) of proteins with or without SDS were performed according to Laemmli (1970) on a Biorad Miniprotean II system. Eight µl aliquots of concentrated IF were loaded onto the gels. Proteins were visualized by silver staining. Isoelectric focusing was carried out on PhastSystem (Pharmacia/LKB) according to the manufacturers protocol. The pH gradient ranged from pH 3 to 9. An antiserum raised against 1,3-β-glucanase P3 of tomato (Joosten and De Wit, 1989) was used for immunodetection of proteins on nitrocellulose membranes. Antigens were visualized after incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase and with 5-bromo-4-chloro-3-indol phosphate/nitroblue tetrazolium as substrate.

1,3-β-glucanase assay

1,3-β-glucanase activity was assayed by measuring the rate of reducing sugar production with laminarin as substrate according to Mauch et al. (1984). The reaction mixture consisted of 0.2 ml of citric acid phosphate buffer (pH 5.6) containing 1 mg ml^{-1} laminarin and 0.05 ml of IF. After 30 min incubation at 37 °C, 0.25 ml of alkaline copper reagent was added and the mixture was heated at 100 °C for 10 min. After cooling on ice, 0.25 ml of arsenomolybdate reagent was added, followed by 1.5 ml H_2O after development of the blue color (Nelson, 1944). Glucose was used as a standard and enzyme and laminarin alone were included as controls. One Katal (Kat) was defined as the enzyme activity catalyzing the formation of 1 mol glucose equivalents per second.

Chitinase assay

The chitinase activity was determined as described by Boller et al. (1983) using crustacean chitin (Sigma) as substrate. The reaction mixture consisted of 0.3 ml IF,

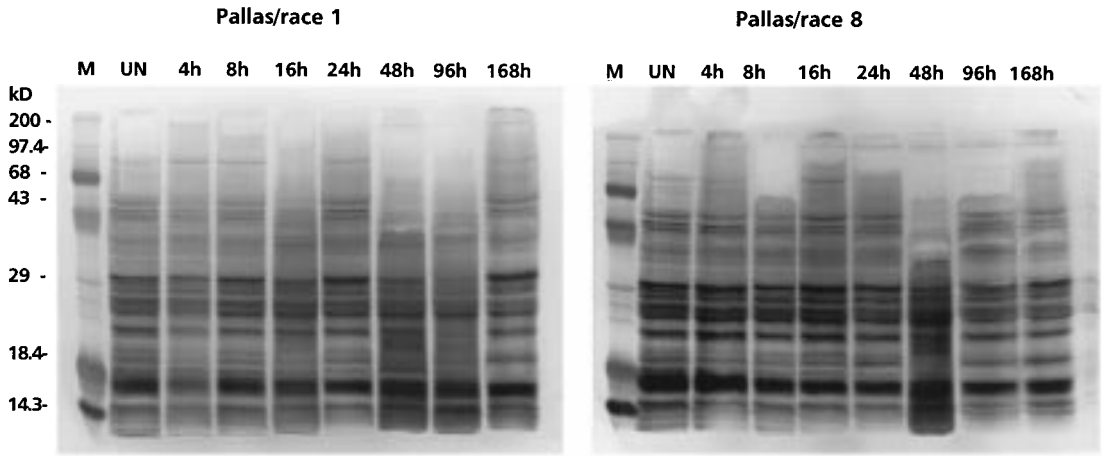

Figure 1. SDS-PAGE of proteins in IFs of stem tissue obtained from carnation cultivar Pallas inoculated with the avirulent race 1 and the virulent race 8 of Fod. Samples were collected after 4, 8, 16, 24, 48, 96 and 168 h. Lane marked M contained M_r markers. Lane UN contained IF from stem tissue of untreated cultivar Pallas.

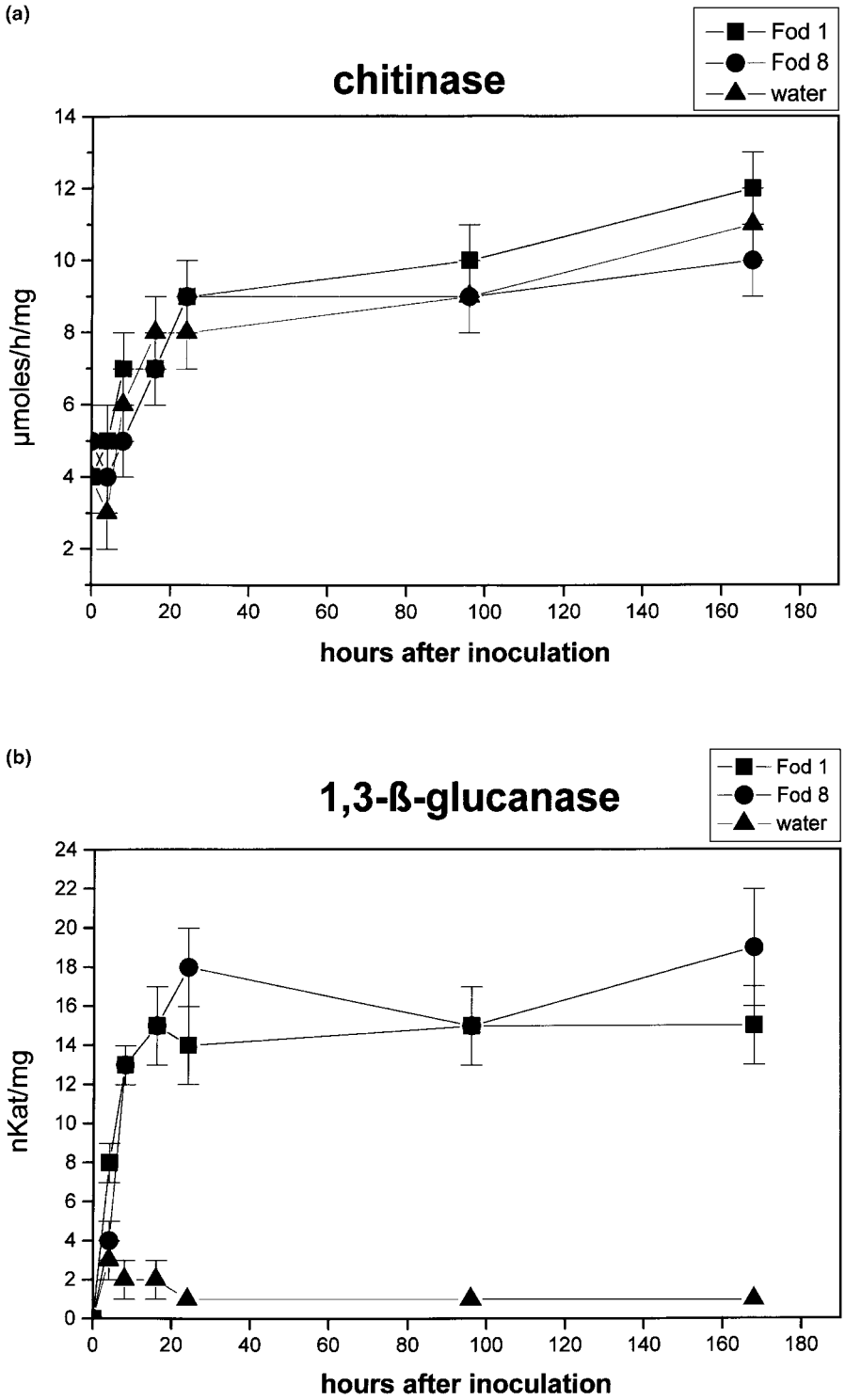

Figure 2. Time courses of chitinase (A), 1,3-β-glucanase (B) and peroxidase (C) activities per mg protein in the IFs of stem tissue obtained from cultivar Pallas inoculated with race 1, race 8, and water. The IFs were obtained 4, 8, 16, 24, 48, 96 and 168 h after inoculation. The data represent the mean value of 2 independent experiments done in duplicate (±SD).

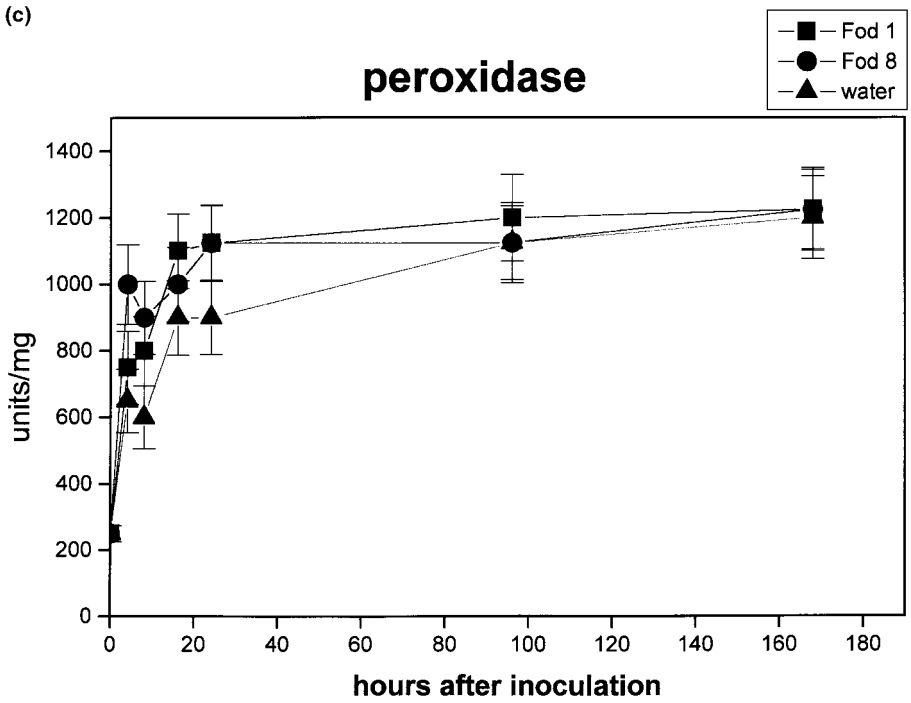

Figure 2. Continued.

0.3 ml 0.2 M Na-acetate buffer and 1 mg chitin ml^{-1}. After incubation for 3 h at 37 °C the reaction mixture was centrifuged for 20 min at 14 000*g*. The supernatant was incubated with 0.1% (w/v) of cytohelicase in 1 M phosphate buffer (pH 7.1) to hydrolyze solubilized chitin oligomers. The resulting N-acetylglucosamine was determined according to Reissig et al. (1955). Detection of chitinase activity after native PAGE at pH 8.8 on overlay gels with glycol chitin as substrate was performed as described by Pan et al. (1991).

Peroxidase assay

Peroxidase activity was measured by a continuous spectrophotometric assay using *ortho*-dianisidine as a hydrogen donor (Fry, 1990). Peroxidase activity was expressed as an increase in absorbance at 470 nm min^{-1} ml IF using a final concentration of 0.1 mg ml^{-1} *ortho*-dianisidine and 1 mM H_2O_2 or alternatively as an increase in absorbance per gram stem tissue. One unit is defined as the amount of enzyme that causes an increase in absorbance of 0.1 absorbance units min^{-1}. Isoelectric focusing gels were stained for peroxidase activity using 4-chloro-1-naphtol (Ye et al., 1990).

Mycelial growth inhibition

Assays on mycelial growth inhibition were performed as described by Ludwig and Boiler (1990). Conidia from *Trichoderma viride* and races 1 and 8 of Fod were obtained from PDB shaking cultures. PDA was added to 6 well microplates (Greiner), 100 µl aliquots of a conidial suspension (10^5 conidia ml^{-1}) were added to the wells containing potato dextrose agar and conidia were allowed to germinate overnight at 25 °C. After 16 h 100 µl aliquots of IF prepared from plants inoculated with races 1 and 8 sampled 7 days post inoculation were added to the wells. Growth inhibition was monitored by eye the following 2 days, after which the plates were photographed. As a control boiled IFs were used.

Results

Analysis of proteins in the IFs obtained from stem tissue

IFs were recovered from the compatible interaction Pallas/race 8, the incompatible interaction Pallas/race 1

and Pallas/water as a control. The glucose-6-P-dehydrogenase activity in the IFs remained low (data not shown), indicating that contamination with cytoplasmic contents had not occurred. The protein contents in the IFs remained fairly constant in both the compatible and the incompatible interaction as well as the water controls, and did not differ significantly from healthy plants (ca. 2 mg ml^{-1}). The total protein profiles on SDS-PAGE of the incompatible and compatible interactions are presented in Figure 1. Differences can be seen between lanes from one interaction in the high molecular weight regions. These differences were not reproducible and seemed to be due to protein handling. In the compatible and incompatible interactions and water controls (not shown) the same variation was observed. Infiltration of the stem tissue with 0.5 M NaCl during the IF isolation procedure did not result in qualitative changes in protein profiles (data not shown).

Chitinase, 1,3-β-glucanase and peroxidase activities in the IFs of stem tissue

Chitinase activity

The total chitinase activity in the IFs of stem tissue increased with time similar after inoculation with the virulent, the avirulent race and water (Figure 2A). At

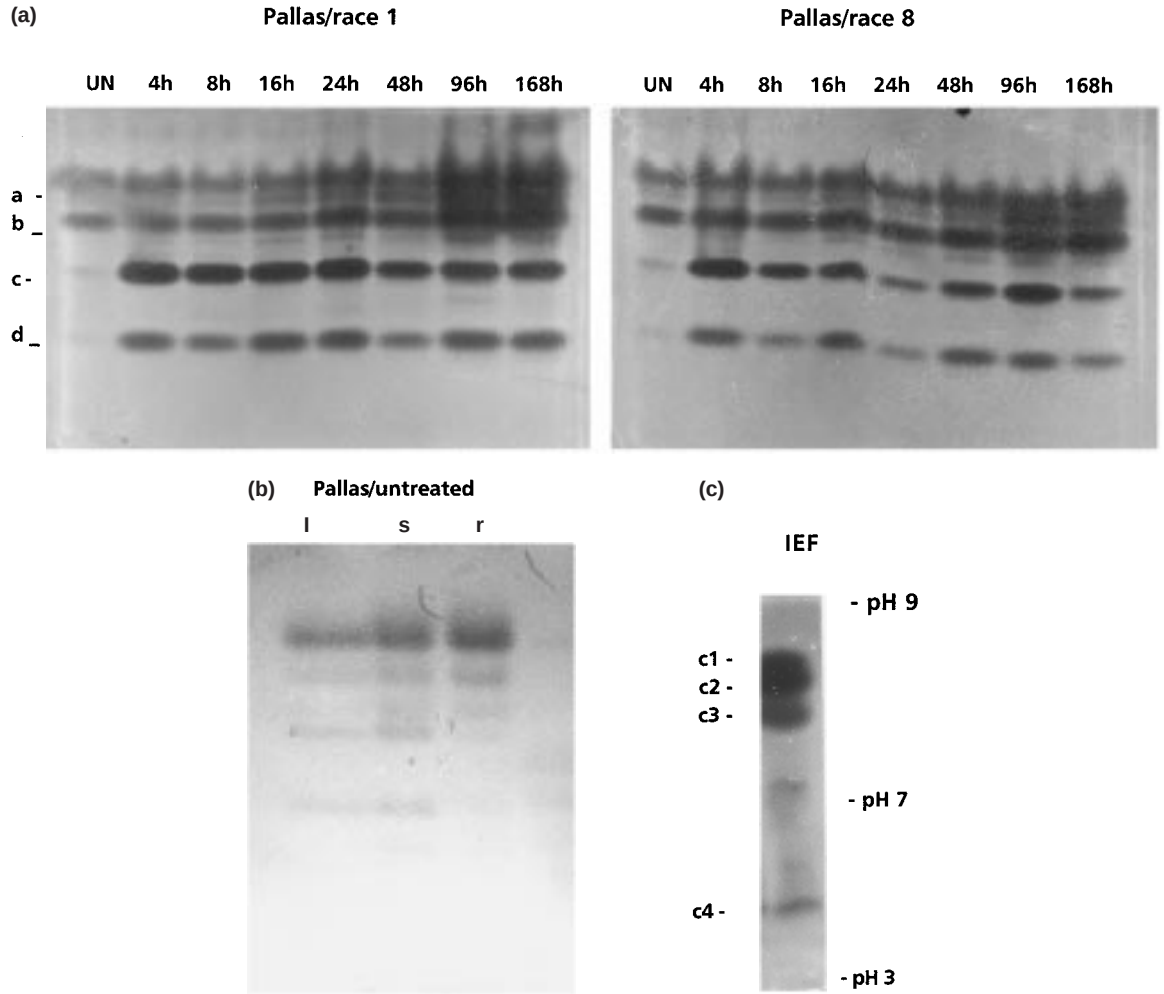

Figure 3. A. Chitinase isozyme patterns after native PAGE of proteins of IFs of stem tissue obtained from cultivar Pallas inoculated the avirulent race 1 and the virulent race 8 of Fod. Samples were collected after 4, 8, 16, 24, 48, 96 and 168 h. B. Chitinase isozyme banding patterns after native PAGE of proteins in IFs obtained from untreated leaves (l), stems (s) and roots (r). C. Chitinase isozyme banding pattern after isoelectric focusing of proteins of IFs of stem tissue from cultivar Pallas inoculated with the avirulent race 1 of Fod.

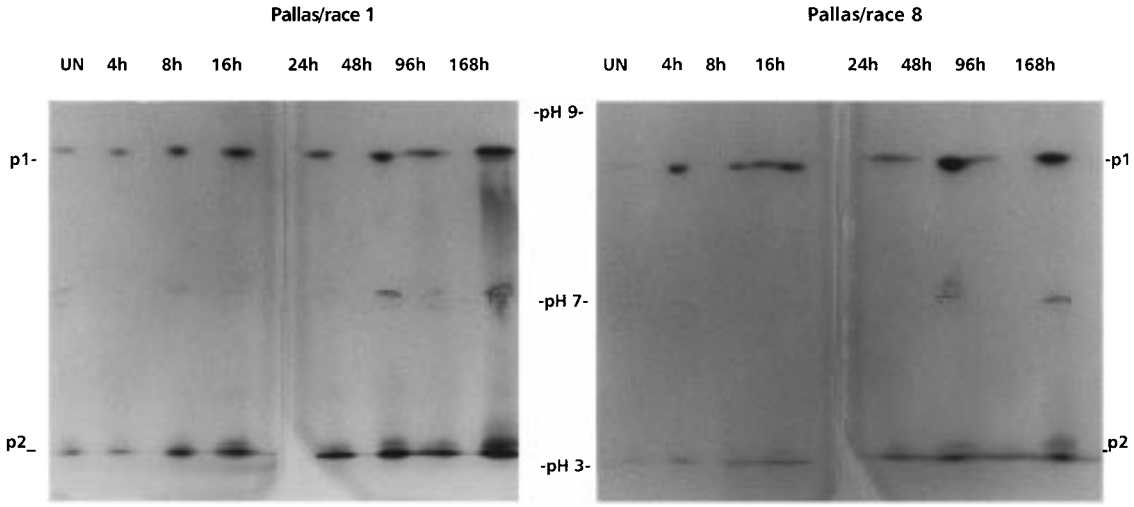

Figure 4. Peroxidase isozyme patterns after isoelectric focusing of proteins of IFs of stem tissue of stems obtained from cultivar Pallas inoculated with the avirulent race 1 and the virulent race 8 of Fod. Samples were collected after 4, 8, 16, 24, 48, 96 and 168 h.

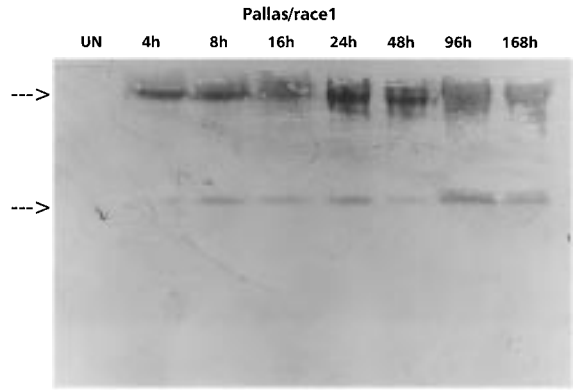

Figure 5. Western blots analysis of proteins in the IFs of stem tissue of stems obtained from cultivar Pallas inoculated with race 1. Samples were collected after 4, 8, 16, 24, 48, 96 and 168 h. The blot was probed with anti-1,3-β-glucanase P3 of tomato.

least 4 bands (designated a, b, c, d) with chitinase activity were detected on overlay gels after native PAGE in the IFs of all combinations (Figure 3A). Several bands with chitinase activity were also detected in IFs of untreated leaves, stems and roots of carnation (Figure 3B). After IEF of proteins from the IFs of inoculated plants, at least 4 bands were visible with chitinase activity, comprising 3 acidic isoforms (designated c1, c2 and c3) and one basic isoform (c4) (Figure 3C).

1,3-β-glucanase activity

The increases in total 1,3-β-glucanase activity in the IFs were markedly higher in the compatible and incompatible interactions than in the water control, but differences between the virulent and the avirulent race were not observed (Figure 2B). Total 1,3-β-glucanase activity could not be detected in the IFs of untreated leaves, stems and roots of carnation. Using the method described by Pan et al. (1991), 1,3-β-glucanase isoenzymes were not or at best very faintly detected in the IFs of carnations (data not shown).

Peroxidase activity

Total peroxidase activity increased with time in all combinations (Figure 2C). Two bands with peroxidase activity, an acidic isoform p1 and a basic isoform p2, were detected after isoelectric focusing of IF proteins. The intensity of both bands increased similarly in time in compatible, incompatible interactions (Figure 4) and water controls (data not shown).

Cross reaction of proteins present in the IFs of carnation with antiserum against a 1,3-β-glucanase from tomato

Since 1,3-β-glucanase isoenzymes could not be detected in IFs of carnation using the method described by Pan et al. (1991), an antiserum against 1,3-β-glucanase of tomato was used to analyze the presence of glucanases in the IFs. Western blots of nondenaturating gels loaded with IF proteins were probed with anti-1,3-β-glucanase P3 from tomato. With anti-1,3-β-glucanase two bands were detected both in the

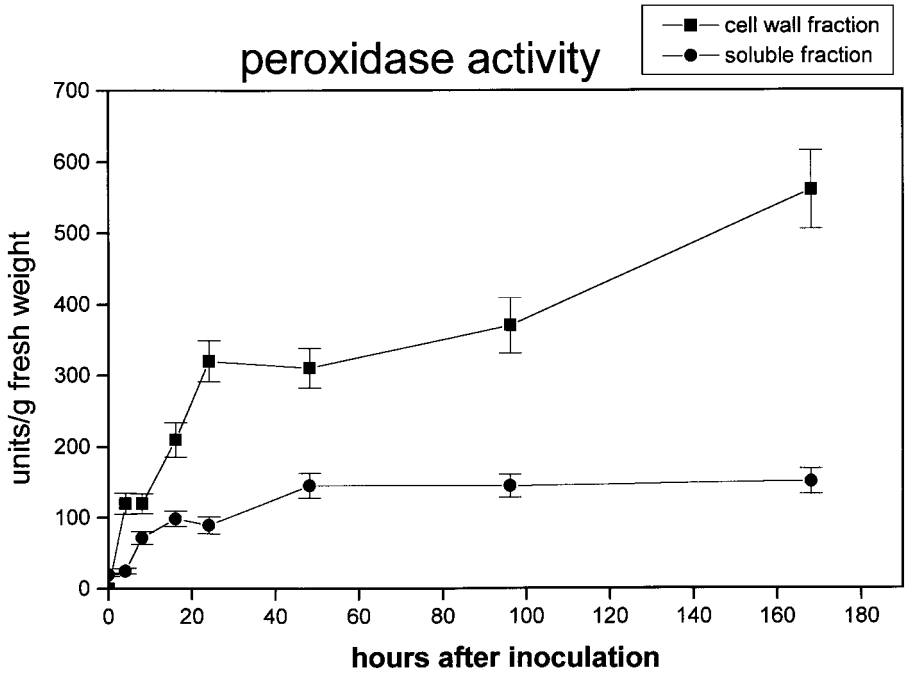

Figure 6. Peroxidase activity in a cell wall and soluble fraction of stem tissue obtained from cultivar Pallas prepared after 4, 8, 16, 24, 48, 96 and 168 h after inoculation of cultivar Pallas with race 1. The data represent the mean value of 2 independent experiments done in duplicate (±SD).

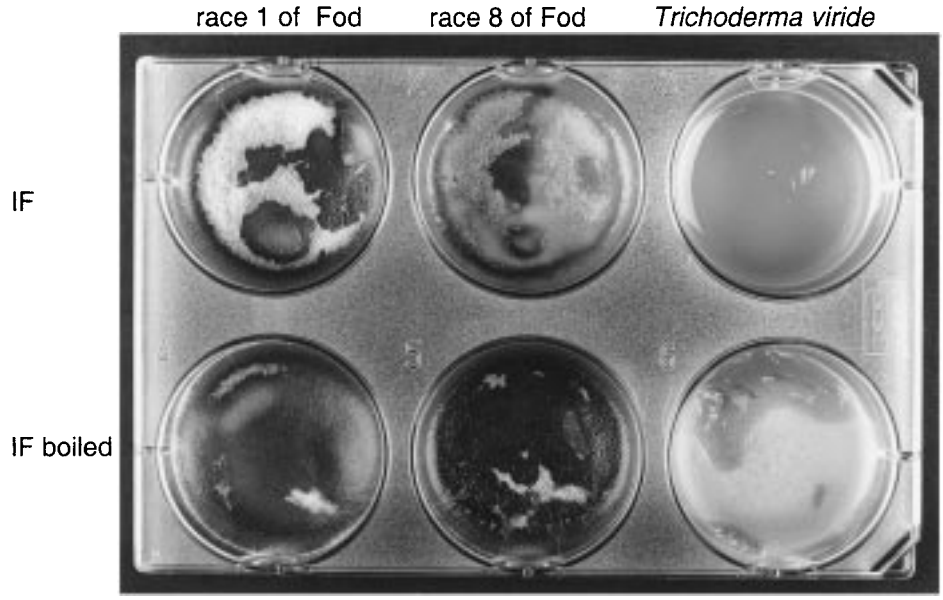

Figure 7. Mycelial growth inhibition of race 1 or 8 of Fod or *Trichoderma viride* by the IFs of Fod inoculated stem tissue obtained from cultivar Pallas.

incompatible (Figure 5) and compatible interaction, but not in the water controls (not shown).

Accumulation of cell wall bound peroxidase activity in compatible and incompatible interactions

Since it has been described that peroxidases, correlated with lignification are wall bound, the accumulation of peroxidase activity in a cell wall bound and a soluble fraction was investigated. The wall bound peroxidase activity was clearly higher, and its increase faster, than the activity in the soluble fraction (Figure 6). However no differences were observed after inoculation with the virulent, the avirulent race and water (not shown).

Mycelial growth inhibition

Growth of *Trichoderma viride*, which is very sensitive to hydrolases, was inhibited by the IFs obtained from the compatible and incompatible interaction. The growth of Fod was not affected by these IFs, even when they were concentrated 5 times. Boiled IF fractions did not inhibit the growth of *Trichoderma viride* and Fod (Figure 7).

Discussion

The aim of this study was to learn more about the induction of defense-related proteins in stem tissue of cultivar Pallas with races 1 and 8 of Fod and to investigate if there are defense responses that are related to the resistance phenotype. Differential accumulation of proteins in IFs from leaves of compatible and incompatible interactions of plants with pathogens has been described in several studies (Benhamou et al., 1989; Mohan and Kolattukudy, 1990; Scott-Graig et al., 1995; Joosten and De Wit, 1996). Results showed differences in protein patterns in the high molecular weight ranges in incompatible, compatible interactions and the water control. However, these differences were not reproducible and seemed to be due to protein handling. A fairly constant protein content was measured in the IFs during the time course experiments. This constant protein content may be due to damage during the preparation of the xylem tissue or the result of damage of cells due to infection. However, the glucose-6-P-dehydrogenase activity measured in the IFs remained low, indicating that the protein content in the IFs was not the result of contamination with intracellular proteins due to mechanical damage. Furthermore, histological observations showed that vascular tissue of carnations, colonized by races 1 and 8, members of VCG 0022 are virtually not degraded, while this is certainly the case with colonization by members of other VCGs and races (Baayen et al., 1988).

No differences were observed in the accumulation of chitinase and 1,3-β-glucanase activities or the chitinase isoenzyme banding pattern from compatible and incompatible interactions, indicating that these defense responses are not causally related to the resistance phenotype as has been described for the race/cultivar specific interaction of tomato with *Cladosporium fulvum* (Joosten and De Wit, 1989). The accumulation of chitinase activity in the compatible and incompatible interaction was similar to that in water controls, whereas the accumulation of 1,3-β-glucanase activity was much higher in the compatible and incompatible interaction as compared to the water control. These results indicate that 1,3-β-glucanase activity is specifically induced by elicitors common to races 1 and 8 of Fod, while chitinase activity increases should be categorized as a response to wounding itself. Interestingly, we observed that chitinase activity is constitutively expressed in untreated leaves, stems and roots of carnation, whereas no 1,3-β-glucanase activity is detected. For potato it has been suggested that constitutively expressed chitinases, which are localized in the outermost cell layer of the plant, may have a general protective function in defense against pathogens (Witte, 1991).

It has been demonstrated that basic chitinases and glucanases that accumulate in the vacuoles of tobacco leaves have *in vitro* antifungal activity towards Fod and *Trichoderma viride*, whereas the extracellular acidic chitinases and glucanases exhibited no growth inhibition (Melchers et al., 1994). Our results show that at least one basic chitinase is present in the IFs of stem tissue of carnation. The growth of *Trichoderma viride* could be inhibited by the IFs of stems of inoculated carnations, which may be due to the presence of this basic chitinase. The growth of Fod itself was not affected by the IFs from carnation.

In the case of peroxidase activities and isoenzyme banding patterns, time courses of compatible and incompatible interactions and water controls also indicate that peroxidase activities in the IFs were induced by wounding rather that elicitation. Furthermore, the increase of cell wall bound peroxidase activity, which

is suggested to be associated with lignification of walls (McDougall, 1993), a defense response of carnation against Fod, is also similar in the virulent, the avirulent race and the water control.

Further research is necessary to correlate differences observed in histological studies and bioassays between the compatible and incompatible interactions with differences encounted in properties as presently reported.

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