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QUARTERLY

High performance liquid chromatography and photodiode array detection of ferulic acid in *Rubus* protoplasts elicited by O-glycans from *Fusarium* sp. M7-1✪

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So far only little data have been available concerning the eliciting capacity of well defined glycan molecules isolated from plant pathogens. This study brings new information about changes in plant cells caused by fungal pathogens. Sugar fractions derived from glycoproteins isolated from the fungus *Fusarium* sp. M7-1 have been tested here as signaling molecules. The ability of three O-glycan fractions (named in this work inducer I, II, III) to trigger responses in *Rubus* protoplasts has been examined. It was found that inducer III was the most efficient as it elicited changes in the levels of phenylpropanoid pathway intermediates in relation to phenylalanine-ammonia lyase (PAL) activation.

Plants have developed efficient defense mechanisms to cope with pathogens. Plant cells have the ability to rapidly perceive the pathogenic organisms, and to trigger metabolic changes resulting in the activation of transcription of specific genes. Many of the genes encoding proteins or enzymes involved in plant defense reactions are those encoding peroxidases (Chittoor *et al.,* 1997), pathogenesis-related proteins, e.g. glucanases, chitinases, proteinase inhibitors (Chang *et al.,* 1995; Pinto & Ricardo, 1995) or those encoding phenolic biosynthetic enzymes. The enzymes of phenolic pathways generate prod-

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Abbreviations: PAD, photodiode array detector; PAL, phenylalanine-ammonia lyase.

ucts with numerous functions in plant defense, such as lignins which reinforce cell-walls, phytoalexins with antimicrobial properties (Bell *et al.,* 1986), or salicylic acid with a signaling function (Ryals *et al.,* 1996).

The fungus *Fusarium* causes many physiological or genetic disorders, nutrient deficiencies, and environmental stress both in plants and in animals, including humans. It can produce lytic enzymes, e.g. pectinases, glucanases, xylanases (Benhamou *et al.,* 1990; Alconada & Martinez, 1995; Christakopoulos *et al.,* 1996), and large amounts of biologically active metabolites such as carotenoids, bikaverins, mycotoxins, phytotropins, gibberellins and oestrogens (Bruckner *et al.,* 1989). Plants react to penetration by *Fusarium* by accumulation of callose or plant cell-wall components (Rodriguez-Galvez & Mendgen, 1995), by an increase of the steady-state mRNA level of phenylpropanoid pathway enzymes (Ni *et al.,* 1996) or pathogenesis-related like proteins (Casacuberta *et al.,* 1992). Plants infested by the fungus also elicit chitinases (Koga *et al.,* 1992) and accumulate phytoalexins (Stevenson *et al.,* 1997). Little is known, though, about the *Fusarium* component(s) which act(s) as the natural elicitors of the above responses.

MATERIALS AND METHODS

Materials. Suspensions cultures of *Rubus fruticosus* L. (raspberry) originally derived from cambial explants from twigs, were grown as described by Hustache *et al.* (1975).

The inducers I, II and III were prepared and identified according to Jikibara *et al.* (1992a; 1992b), Iwahara *et al.* (1992; 1995). Briefly, glycoproteins were isolated from the mycelium of *Fusarium* sp. M7-1, and the O-glycosidic fractions released by alkaline borohydride treatment were purified by gel filtration chromatography and high performance liquid chromatography (HPLC). The primary structure of the oligosaccharides was resolved by nuclear magnetic resonance spectrometry (^{1}H) in combination with methylation mass spectrometry and fast-atom bombardment mass spectrometry. Inducer I is an acidic polysaccharide with a M_r of about 50000. The chain backbone is a galactan linked through $\beta(1\rightarrow6)$ galactofuranoside linkages, with Man $\beta(1\rightarrow4)$ GlcA, Glc, and Man$\beta(1\rightarrow2)$ Man$\beta(1\rightarrow4)$ GlcAα1 as main side chains. Inducer II (with an average M_r of 1500) consists of neutral oligosaccharides with Man-ol, Man $\beta(1\rightarrow2)$ Man-ol as the main sugars. Inducer III, a Dowex 1-X2-bound fraction, is a mixture of acidic oligosaccharides, having the average M_r of 1000, with the following structures: (a) Man $\beta(1\rightarrow4)$ GlcA $\alpha(1\rightarrow2)$ Gal*f* $\beta(1\rightarrow6)$ Man $\alpha(1\rightarrow2)$ Man-ol and Man $\beta(1\rightarrow4)$ GlcA $\alpha(1\rightarrow2)$ Gal*f* $\beta(1\rightarrow6)$ Rha $\alpha(1\rightarrow2)$ Man $\alpha(1\rightarrow2)$ Man-ol; (b) GlcNAc $\alpha(1\rightarrow4)$ GlcA $\alpha(1\rightarrow2)$ GlcNac $\alpha(1\rightarrow4)$ GlcA $\alpha(1\rightarrow2)$ Gal*f* $\beta(1\rightarrow6)$ Rha α $(1\rightarrow2)$ Man $\alpha(1\rightarrow2)$ Man-ol; (c) GlcNAc $\alpha(1\rightarrow4)$ GlcA $\alpha(1\rightarrow2)$ GlcNac $\alpha(1\rightarrow4)$ GlcA $\alpha(1\rightarrow2)$ Gal*f* $\beta(1\rightarrow6)$ Rha $\alpha(1\rightarrow2)$ Man $\alpha(1\rightarrow2)$ Man-ol; (d) Rha $\alpha(1\rightarrow2)$ Man $\alpha(1\rightarrow2)$ Man-ol-P, Man α $(1\rightarrow2)$ Man $\alpha(1\rightarrow2)$ Man-ol-P, Man $\alpha(1\rightarrow2)$ Man-ol-P. In each experimental set, the concentration of inducers II and III is expressed as molarity, and the molar concentration of inducer I is reported by reference to its monomer unit.

Preparation of protoplasts. Cells of *Rubus fruticosus* L. (40 g fresh mass) were collected in the exponential growth phase (15 to 18 days after subculturing). They were incubated overnight at room temperature in 300 ml of Heller's medium (pH 5.9) supplemented with 0.56 M mannitol, 0.25% (w/v) caylase 345 and 0.01% (w/v) caylase M3. The released protoplasts were filtered through a 100 μm nylon mesh, washed twice with the incubation medium with no cell-wall degrading enzymes, recovered by centrifugation (500 × ***g***, 5 min) and then suspended in 25 mM Tris/HCl buffer, pH 4.8, containing 0.56 M mannitol, 0.06 M sucrose, 1 mM KCl, 1 mM $CaCl_2$, and 6% (w/v) Ficoll 400. The purified protoplasts were isolated by centrifugation at 100 × ***g*** for

8 min at 4°C. Finally, before use, they were suspended in the above buffer without Ficoll. Protoplast yields ranged from 70 to 85% of the initial number of treated cells. The viability of protoplasts was controlled by using Evan's blue indicator.

Elicitation experiments for PAL assays. *Rubus* protoplasts (2×10^6) or cells (4×10^6) were suspended for 0 to 30 h in 25 ml buffer, pH 4.8 (25 mM Bis-Tris/HCl containing 1 mM KCl, 1 mM $CaCl_2$, 0.06 M sucrose, 0.56 M mannitol supplemented or not with cycloheximide (1 μM)) on a roller mixer in the presence or not (in controls) of 100 nM fungal inducer I, II or III. When appropriate, the elicitation medium was removed by centrifugation (500 × ***g***, 5 min, 4°C), and the protoplasts were subjected to PAL analysis. At least, four replicates were monitored from three independent elicitation sets.

PAL assays. The protoplasts (cells) were subjected to PAL assays carried out according to the modified procedure of Hagendoorn *et al.* (1991). Briefly, the protoplasts (cells) were sonicated on ice (70 W, 20 s) with a Vibra cell (Bioblock) in 0.1 M sodium borate buffer (pH 8.8) containing 2 mM dithiothreitol. The homogenate was centrifuged (5000 × ***g***, 15 min), and the supernatant and then mixed with 100 mg of Dowex-8 (200–400 mesh) for 10 min at 4°C. The mixture was centrifuged for 5 min at 5000 × ***g***, and the supernatant was added to an equal volume of 0.05% polyvinylpyrrolidone (w/v). The mixture was kept for 10 min at 4°C and then centrifuged for 5 min at 5000 × ***g***, and the supernatant was dialyzed and concentrated with Ultrafree units equipped with a 10 kDa molecular-mass cut-off membrane (Ultrafree Millipore, Bedford, MA, U.S.A.). The crude enzymes were subjected to PAL assays at 40°C as in Zucker (1965). Kinetics were developed at 290 nm using a Beckman DU 640 spectrophotometer: changes in A_{290} of the reaction mixture containing enzyme extract (6 μg protein/ml according to the method of Bradford (1976)), L-phenylalanine substrate (up to 3 mM) in 0.1 M sodium borate, pH 8.8, supplemented with 2 mM dithiothreitol were followed up to 30 min. At least four kinetic curves per experimental condition were developed, and experiments were monitored from three independent protoplast preparations. The data between 2 and 10 min were fitted with second-order polynomial regressions giving an equation, and a coefficient r^2 value of 0.82–0.88. The slope (ΔA_{290} /min) gave the PAL activity expressed as μkat/kg protein, 1 μkat being 1 μmol product formed per sec. The enzyme activation is expressed by R value (the slopes of fitted curves obtained from induced protoplasts *versus* slopes of fitted curves obtained from controls).

Extraction of PAL reaction products. PAL was assayed using 2.25 μM L-phenylalanine substrate, at 37°C for up to 4 h as detailed above. The enzyme reaction was stopped by adding 30 μl of 2 M HCl, and the PAL products extracted with 500 μl ethyl acetate according to Khan and Vaidyanathan (1986) were dried by evaporation. The residue was then dissolved in a known volume of the starting solvent used for HPLC, and an aliquot of 100 μl was injected into the chromatograph.

HPLC-PAD analysis. HPLC analyses were carried out as described in Chevolot *et al.* (1997) but using a Kontron Liquid Chromatograph (Kontron Instruments, Milano, Italy) instead of a Waters one. The system encompassed a manual Rheodyne model 9125 injector, an HPLC 325 pump system, and a 440 photodiode array detector (PAD). All these modules were controlled by a microcomputer equipped with KromaSystem 2000 software (Kontron Instruments, Milano, Italy) which also stored and processed the data. Guard column (LichroCART 4-4, RP-18) and analytical column (Lichrospher 100 RP-18; 125 × 4 mm; 5 μm particle size) were from Merck (Darmstadt, Germany). The conditions of elution are given in the legend to Fig. 3. The detection wavelength range was 230–350 nm and wavelength resolution was 4 nm.

The phenolics were identified by comparison of their retention time (R_t values) and their UV spectra with those of authentic compounds using the comparison software available in KromaSystem 2000. Chromatograms were recorded at selected wavelengths (see Figs. legends). Quantification was performed at the most favorable wavelength (310 nm) by external calibration, and the data were expressed in nmole. The R_t values were the mean values of at least three runs from two biological samples prepared from two independent elicitation sets.

Controls. Kinetic curves were developed from elicited samples and from non-induced ones assayed in parallel. The controls consisted of protoplasts (cells) incubated in buffer without inducer, in the presence or absence of an effector, e.g. cycloheximide. The viability of protoplasts (cells) was controlled during each experimental set. Blanks for enzyme assays were performed with boiled enzyme or without L-phenylalanine substrate.

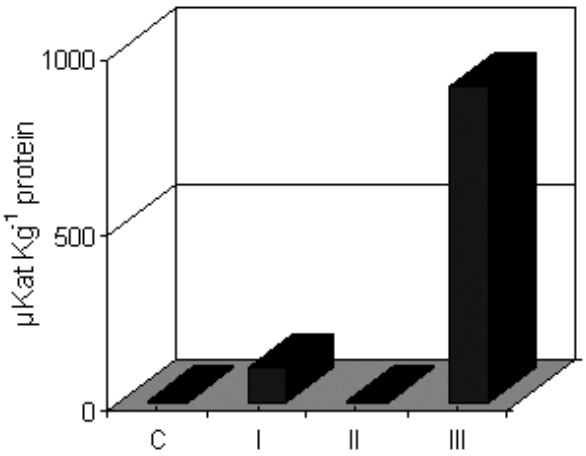

Figure 1. PAL response in *Rubus* protoplasts.

PAL activity of 2×10^6 protoplasts challenged for 30 min with 100 nM inducer I, II or III or in non-induced protoplasts (C) was determined at 290 nm. The values are expressed as relative rates of reaction products (μkat/kg protein). All experiments were carried out in triplicate.

RESULTS

Activation of PAL by inducers I, II and III

Rubus protoplasts treated or not with 100 nM inducer I, II or III for 30 min or up to 30 h were subjected to PAL extraction. It was verified that the viability of protoplasts (cells) was not affected by the inducer since it remained as high as in controls (85 or 95%). Briefly, the response to the inducer was then followed as changes in absorbance at 290 nm of reaction mixtures containing the substrate L-phenylalanine used at the optimal concentration of 2.25 μM, and the enzyme extract prepared from treated or untreated protoplasts. Data in Fig. 1 clearly show that inducer III is the most efficient with an activity of 900 μkat/kg protein while inducer I exhibits an activity of 101 μkat/kg protein only, and inducer II is not active. During 30 h of treatment, the response to inducer III was biphasic with respect to the kinetics (Fig. 2, curve b), and PAL activity in controls did not change over 20 h (Fig. 2, curve a). Initially, the treatment with inducer III resulted in a rapid and transient increase of PAL activity which peaked at 30 min approximately with the activity of 900 μkat/kg protein, giving the R value of 108. Incubation for a longer time resulted in a second initiation of PAL activity with the level of 775 μkat/kg protein, equivalent to the R value of 90 at 30 h. In the presence of 1 μM cycloheximide, the early response was maintained at R value of approximately 88 whereas the long-term induction was markedly attenuated since it decreased to the value of 20 at 30 h (Fig. 2, curve c). After 30 h the slope was increasing slowly (only for elicited protoplasts) and after 35 h the presence of contaminants was observed. The data revealed that the treatment for up to 5 h affects the specific enzyme activity since it did not significantly change the total amount of extractable proteins. In a marked contrast, the long term-treatment promoted a large increase of protein content, thus suggesting involvement of *de novo* synthesized proteins in

the delayed response (not shown). It was observed that when suspended cells were monitored instead of protoplasts, the detected responses from inducer III were largely attenuated, by about 35–40%.

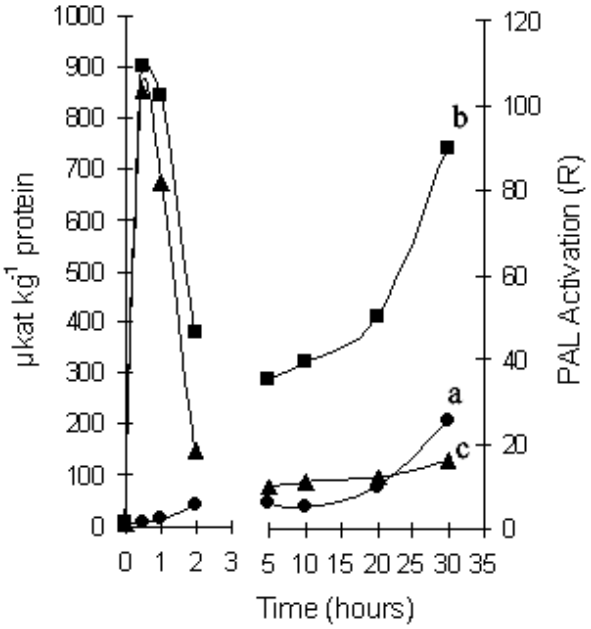

Figure 2. Time-course for PAL activity (PAL activation) in protoplasts challenged with 100 nM inducer III only (curve b) or 100 nM inducer III plus 1 μM cycloheximide (curve c); curve a shows the response in non-induced protoplasts.

2×10^6 protoplasts were elicited for up to 30 h, and PAL extracts were then assayed at 290 nm. The slope of kinetic curves obtained by linear-regression fits of the data from four assays performed in three independent sets gave the relative rate of reaction products expressed in μkat/ kg protein or by the R value (the activity in treated protoplasts *vs* controls).

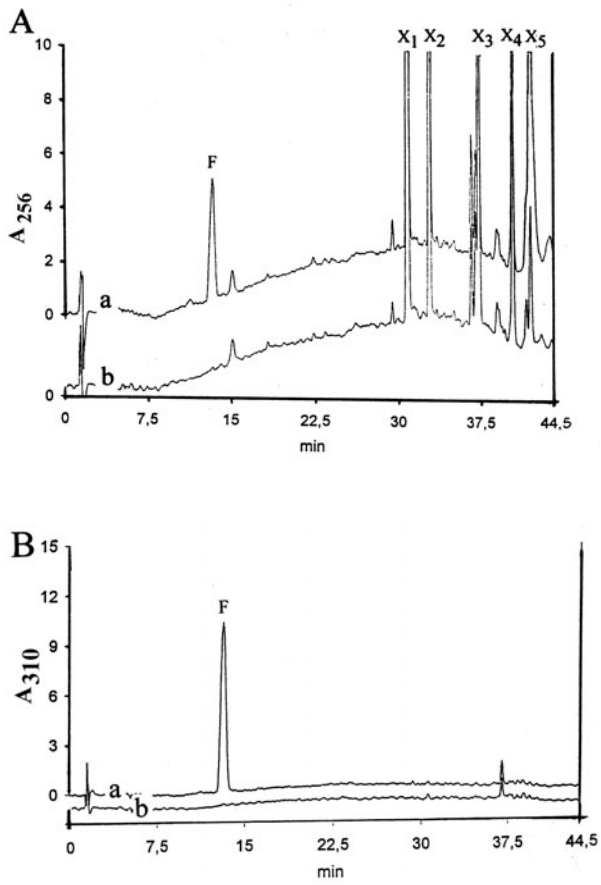

Figure 3. HPLC chromatograms of phenolics in PAL reaction mixtures derived from *Rubus* protoplasts elicited for 30 min with 100 nM inducer III (curve b) or from controls (curve a).

The phenolics in the reaction mixtures were eluted on RP-18 column, and the mobile phase was solvent A (5% (v/v) acetic acid in Milli-Q water) and solvent B (MeOH). Elution programme was: 0–5 min, isocratic 15% B in A; 5–35 min, linear gradient 15% B in A to 80% B in A; 35–45 min, isocratic 80% B in A. The flow rate was 1 ml/min. The detection and quantification were carried out with PAD at 256 nm in (A) or at 310 nm in (B) from 100 μl of injected sample. Peak F was identified as ferulic acid.

Changes in the phenylpropanoid pathway elicited by inducer III

HPLC-PAD investigations of PAL extracts prepared from elicited protoplasts or from controls were run. Typical HPLC profiles of standards with detection at 256 and 310 nm were firstly achieved as in Chevolot *et al.* (1997).

The chromatograms at 256 nm for the samples obtained from inducer treatment (Fig. 3A, curve a) showed the presence of major peaks F, X_1, X_2, X_3, X_4, X_5 with the R_t values of 13.45, 31.07, 33.1, 37.60, 40.74 and 42.37 min, respectively. The profiles from control protoplasts (Fig. 3 A, curve b) did not exhibit the same pattern since the peak F (R_t value of 13.45) is absent and lesser amounts of X_5 and higher amounts of X_1, X_2, X_3 and X_4 were detected.

The phenolic compound F was identified as ferulic acid by comparison of its UV spectrum (λ_{max} at 234 nm, shoulder at 325 nm) and especially by the identity of its R_t value with the authentic compound. The chromatograms at 310 nm indicated also that the peak F can be identified as ferulic acid (Fig. 3B, curve a), and that it is not detected in controls (Fig. 3B, curve b). Quantification of the phenolics at 310 nm revealed that the treatment of 2×10^6 protoplasts for 30 min with 100 nM inducer III promoted the accumulation of 1.3 nmols of ferulic acid.

Up to now, we failed to establish the identity of the other eluted products. For unknown compounds, comparison of peak areas at 256 nm indicated that X_5 increased by a factor of l.7 but X_1, X_2, X_3 and X_4 decreased by a factor of 0.4, 0.45, 0.42 and 0.37, respectively.

DISCUSSION

O-Glycan fractions obtained from *Fusarium* glycoproteins were used here as potential signaling molecules in raspberry protoplast suspensions. The biochemical study focused on the characterization of PAL activation generated by inducer III. This inducer, an acidic oligosaccharide fraction derived from uronic acid-containing glycoproteins from *Fusarium* sp. M7-1, has a chemical structure similar to that of the glycan part of cell-wall glycoproteins from *Gibberella* (Takegawa *et al.*, 1991; 1997). Our data indicate that inducer III elicits early events such as the induction of a PAL complex. Following the inducer treatment, the PAL response exhibited a maximal intensity after 30 min. Detection of oligosaccharides by protoplasts has also been previously reported in a number of plant systems (Dangl *et al.*, 1987; Kauss *et al.*, 1989; Patier *et al.*, 1995), and the fact that the protoplasts responded here better than intact cells indicated that the signal of *Fusarium* origin is attenuated by plant cell walls.

Following the application of inducer III, very high levels of PAL activity were rapidly detected in protoplasts. The response is transient and it is maintained in the presence of cycloheximide. This suggested involvement of a signal cascade associated with plasma-membranes as in Nurnberger *et al.* (1997), and brought evidence for a post-translational regulation of the enzyme. A delayed PAL response was detected: it decreased in the presence of cycloheximide, and therefore involved late changes in PAL mRNA translation levels. An elevation of PAL response, favored as an indicator of plant resistance, has often been reported to occur at the transcriptional level. But it is worth noting that the levels of PAL mRNA did not always increase proportionally to the enzyme activity measured (Lee *et al.*, 1992), thus suggesting that PAL can also be affected by post-transcriptional modifications (Shaw *et al.*, 1990). Extraction of phenolics from PAL reaction mixtures by suitable organic phase followed by HPLC-PAD revealed the presence of ferulic acid, thus suggesting the activation of a PAL complex including PAL, cinnamate 4-hydroxylase, 4-coumarate CoA ligase and caffeoyl 3-*O*-methyltransferase, directly channeling *t*-cinnamic acid to ferulic acid. The presence of a PAL complex directing the flow of secondary compounds after hormone or elicitor treatment has been reported previously. The enzymes of branch pathways for phenylpropanoids, benzoates, or for flavonoids are known to be differentially expressed in response to environmental factors (Habereder *et al.*, 1989; Funk & Brodelius, 1992; Chevolot *et al.*, 1997). Likewise, specific enzymes, such as 3-*O*-methyltransferases acting at the terminal step in the biosynthesis of phenylpropanoids or of isoflavonoids, increased in response to elicitor or to pathogen (Busam *et al.*, 1997; Grimmig & Matern, 1997; Preisig *et al.*, 1991). *O*-Methyltransferases are thought to be a target of modulation in the systemic acquired resistance predisposition of tissues for en-

hanced lignification (Busam *et al.*, 1997). Caffeic acid, ferulic acid or its derivative curcumin exhibited inhibitory effect on ornithine decarboxylase (Huang *et al.*, 1988).

In conclusion, the results revealed that a carbohydrate fraction of *Fusarium* origin can be a signaling molecule that causes, at a post-translational level, changes in phenolic metabolism. Based on the elicitation of ferulic acid, it can be proposed that the pathogen *Fusarium* affected the oxidant system of the host. Up to now, enzymes of the phenolic pathway had been often described as playing a central role in the orchestration of hypersensitive responses in plants, but here they are shown for the first time to be dependent on the application of a well defined elicitor from *Fusarium*.

REFERENCES

Alconada TM, Martinez MJ. (1995) Isolation of protoplasts from vegetable tissus using extracellular lytic enzymes from *Fusarium oxysporum* f. sp. *Rev Argent Microbiol.*; **27**: 191–7.

Bell JN, Ryder TB, Wingate VP, Bailey JA, Lamb CJ. (1986) Differential accumulation of plant defense gene transcripts in a compatible and an incompatible plant-pathogen interaction. *Mol Cell Biol.*; **6**: 1615–23.

Benhamou N, Chamberland H, Pauze FJ. (1990) Implication of pectic components in cells surface interactions between tomato root cells and *Fusarium oxysporum* f. sp. *radicis-lycopersici. Plant Physiol.*; **92**: 995–1003.

Bradford MM. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.;* **72**: 248–54.

Bruckner B, Blechschmidt D, Schubert B. (1989) *Fusarium moniliforme* Sheld, a fungus producing a broad spectrum of bioactive metabolites. *Zentralbl Mikrobiol.*; **144**: 3–12.

Busam G, Junghanns KJ, Kneusel RE, Kassemeyer HH, Matern U. (1997) Characterization and expression of caffeoyl-coenzyme A 3-*O*-methyltransferase proposed for the induced resistance response of *Vitis vinifera* L. *Plant Physiol.*; **115**: 1039–48.

Casacuberta JM, Raventos D, Puigdomenech P, San Segundo B. (1992) Expression of the gene encoding the PR-like protein PRms in germinating maize embryos. *Mol Gen Genet.*; **234**: 97–104.

Chang MM, Horovitz D, Culley D, Hadwiger LA. (1995) Molecular cloning and characterization of a pea chitinase gene expressed in response to wounding, fungal infection and the elicitor chitosan. *Plant Mol Biol.*; **28**: 105–11.

Chevolot L, Dubois-Dauphin R, Yvin JC, Liénart Y. (1997) High performance liquid chromatography and photodiode array detection of phenylpropanoids and benzoates in *Rubus* protoplasts elicited by kinetin. *Phytochem Anal.*; **8**: 22–6.

Chittoor JM, Leach JE, White FF. (1997) Differential induction of a peroxidase gene family during infection of rice by *Xanthomonas oryzae* pv. *oryza. Mol Plant Microbe Interact.*; **10**: 861–71.

Christakopoulos P, Nerinck W, Krkos D, Macris B, Claeyssens M. (1996) Purification and characterization of two low molecular mass alkaline xylanases from *Fusarium oxysporum* F3. *J Biotechnol.*; **51**: 181–9.

Dangl JL, Hauff S, Lipphardt S, Hahlbrock H, Sheel D. (1987) Parsley protoplasts retain differential responsiveness to u.v. light and fungal elicitor. *EMBO J.*; **6**: 2551–6.

Funk C, Brodelius PE. (1992) Phenylpropanoid metabolism in suspension cultures of *Vanilla planifolia*. IV. Induction of vanillic acid formation. *Plant Physiol.*; **99**: 256–62.

Grimmig B, Matern U. (1997) Structure of the parsley caffeoyl-CoA *O*-methyltransferase gene, harbouring a novel elicitor responsive cis-acting element. *Plant Mol Biol.*; **33**: 323–41.

Habereder H, Schröder G, Ebel J. (1989) Rapid induction of ammonia-lyase and chalcone synthase mRNA during fungus infection of soybean (*Glycine max* L.) roots of elicitor

treatment of soybean cell cultures at the onset of phytoalexin synthesis. *Planta.*; **177**: 58–65.

Hagendoorn MJM, Poortinga AM, Sang HW, van der Plas LH, van Walraven HS. (1991) Effect of elicitor on the plasma membrane of *Petunia hybrida* cell suspensions. *Plant Physiol.*; **96**: 1261–7.

Huang MT, Smart RC, Wong CQ, Conney AH. (1988) Inhibitory effect of curcumin, chlorogenic acid, caffeic acid, and ferulic acid on tumor promotion in mouse skin by 12-*O*-tetradecanoylphorbol-13-acetate. *Cancer Res.*; **21**: 5941–6.

Hustache G, Mollard A, Barnoud F. (1975) Culture illimitée d'une souche anergiée de *Rosa glauca* par la technique des suspensions cellulaires. *C R Acad Sci Paris D.*; **281**: 1381–4.

Iwahara S, Maeyama T, Mishima T, Jikibara T, Takegawa K, Iwamoto H. (1992) Studies on the uronic acid-containing glycoproteins of *Fusarium* sp. M7-1. IV. Isolation and identification of four novel oligosaccharide units derived from the acidic polysaccharide chain. *J Biochem (Tokyo).*; **3**: 355–9.

Iwahara S, Suemori N, Ramli N, Takegawa K. (1995) Isolation and identification of novel acidic oligosaccharides derived from glycoproteins of *Fusarium* sp. M7-1. *Biosci Biotech Biochem.*; **59**: 1082–5.

Jikibara T, Takegawa K, Iwahara S. (1992a) Studies on the uronic acid-containing glycoproteins of *Fusarium* sp. M7-1. III. The primary structures of the acidic polysaccharides of the glycoproteins. *J Biochem (Tokyo).*; **112**: 236–43.

Jikibara T, Takegawa K, Iwahara S. (1992b) Studies on the uronic acid-containing glycoproteins of *Fusarium* sp. M7-1. I. Isolation and some properties of the glycoproteins. *J Biochem (Tokyo).*; **111**: 225–9.

Kauss H, Jeblick W, Domard A. (1989) The degrees of polymerisation and *N*-acetylation of chitosan determine its ability to elicit callose formation in suspension cells and protoplasts of *Catharanthus roseus. Planta.*; **178**: 385–92.

Khan NU, Vaidyanathan CS. (1986) A new simple spectrophotometric assay of phenylalanine ammonia-lyase. *Curr Sci.*; **55**: 391–3.

Koga D, Hirata T, Sueshige N, Tanaka S, Ide A. (1992) Induction patterns of chitinases in yam callus by inoculation with autoclaved *Fusarium oxysporum*, ethylene and chitine and chitosan oligosaccharides. *Biosci Biotech Biochem.*; **56**: 280–5.

Lee SW, Nazar RN, Powell DA, Robb J. (1992) Reduced PAL gene suppression in *Verticillium*-infected resistant tomatoes. *Plant Mol Biol.*; **2**: 345–52.

Ni W, Fahrendorf T, Ballance GM, Lamb CJ, Dixon RA. (1996) Stress responses in alfalfa (*Medicago sativa* L.). Transcriptional activation of phenylpropanoid pathway genes in elicitor-induced cell suspension cultures. *Plant Mol Biol.*; **30**: 427–38.

Nurnberger T, Wirtz W, Nennstiel D, Hahlbrock K, Jabs T, Zimmermann S, Scheel D. (1997) Signal perception and intracellular signal transduction in plant pathogen defense. *J Recept Signal Transduct Res.*; **17**: 127–36.

Patier P, Potin P, Rochas C, Kloareg B, Yvin JC, Liénart Y. (1995) Free or silica bound oligokappa carrageenans elicit laminarinase activity in *Rubus* cells and protoplasts. *Plant Sci.*; **110**: 27–35.

Pinto MP, Ricardo CP. (1995) *Lupinus albus* L. pathogenesis-related proteins that show similarity to PR-10 proteins. *Plant Physiol.*; **109**: 1345–51.

Preisig CL, van Etten HD, Moreau RA. (1991) Induction of hydroxymackiain 3-*O*-methyltransferase and phenylalanine ammonia-lyase mRNA translation activities during the biosynthesis of pisatin. *Arch Biochem Biophys.*; **290**: 468–73.

Rodriguez-Galvez E, Mendgen K. (1995) Cell-wall synthesis in cotton roots after infection with *Fusarium oxysporum.* The deposition of callose, arabinogalactans, xyloglucans, and pectic components into walls, wall appositions, cell plates and plasmodesmata. *Planta.*; **197**: 535–45.

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD. (1996) Systemic acquired resistance. *Plant Cell.*; **8**: 1809–19.

Shaw NM, Bolwell GP, Smith C. (1990) Wound-induced phenylalanine ammonia-lyase in potato (*Solanum tuberosum*) tuber discs. Significance of glycosylation and immunolocalization of enzyme subunits. *Biochem J.*; **267**: 163–70.

Stevenson PC, Turner HC, Haware MP. (1997) Phytoalexin accumulation in the roots of chickpea (*Cicer arietinum* L.) seedlings associated with resistance to *Fusarium* wilt. *Physiol Mol Plant Pathol.*; **50**: 167–78.

Takegawa K, Yamaguchi S, Miki S, Jikibara T, Iwahara S. (1991) Purification and characterization of a novel lyase from *Cellulomonas* sp. that degrades *Fusarium* and *Gibberella* acidic polysaccharides. *Agric Biol Chem.*; **55**: 1969–75.

Takegawa K, Satoh K, Ramli N, Jikibara T, Iwahara S. (1997) Production and characterization of extracellular uronic acid-containing glycoproteins from *Fusarium oxysporum* L. *J Ferment Bioeng.*; **83**: 197–200.

Zucker M. (1965) Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. *Plant Physiol.*; **40**: 779–84.