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QUARTERLY

Novel oligosaccharides isolated from *Fusarium oxysporum* L. rapidly induce PAL activity in *Rubus* cells✪

Mihai Nita-Lazar✉, Alain Heyraud, Claude Gey, Isabelle Braccini and Yvette Lienart

Centre de Recherches sur les Macromolécules Végétales (CERMAV)-CNRS, Grenoble, France

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Activation of the phenolic pathway is known to be part of a defense response against cell wall-derived elicitors from pathogens. Many examples of a defense response by increasing the synthesis of phenolic compound against the elicitor were demonstrated in the past, but the elicitor structure has so far been poorly characterized.

Our results indicate that a disaccharide fraction containing the following structure: **α-D-mannopyranosyl (1→2)α/β-D-glucopyranosyl and α-D-mannopyranosyl (1→x) inositol, isolated from *Fusarium oxysporum* L., promotes rapid and transient phenylalanine ammonia lyase activity in *Rubus fructicosus* cells at nanomolar concentration. The disaccharides were isolated by size-exclusion chromatography directly from extracts obtained by alkaline treatment of *F. oxysporum* mycelium. Their structure was determined by 500-MHz-^{1}H-NMR spectroscopy combined with methylation analysis and fast atom bombardment mass spectrometry.**

The *Fusarium* fungus causes physiological or genetic disorders, nutrient deficiencies, and environmental stress both in plants and in animals, including humans. It induces the

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✉Corresponding author and present address: Department of Biochemistry and Cell Biology, 450 Life Sciences Bldg., SUNY at Stony Brook, NY 11794, U.S.A.; tel.: (1 631) 632 8567; fax: (1 631) 632 8575; e-mail: mnitalazar@notes.cc.sunysb.edu

Abbreviations: COSY, correlation spectroscopy; FAB-MS, fast atom bombardment mass spectrometry; HMBC, heteronuclear multi bound correlation; HMQC, heteronuclear multi quantum coherence; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; PAL, phenylalanine ammonia lyase; TOCSY, total correlation spectroscopy.

production of lytic enzymes like pectinases, glucanases, xylanases (Benhamou *et al.*, 1990; Alconada *et al.*, 1995; Christakopoulos *et al.*, 1996), and large amounts of biologically active metabolites such as carotenoids, bikaverins, mycotoxins, phytotropins, gibberellins and estrogens (Bruckner *et al.*, 1989). Plants react against *Fusarium* penetration by accumulating callose or plant cell-wall components (Rodriguez-Galvez & Mendgen, 1995; El-Gendy *et al.*, 2001), by increasing the steady-state mRNA level of phenylpropanoid pathway enzymes (Ni *et al.*, 1996) or pathogenesis-related proteins (Casacuberta *et al.*, 1992). *Fusarium* also produces acidic polysaccharides as components of the cell wall, or as exopolymers in culture medium. Among the carbohydrate components of fungal cell walls, β (1→3)[1→6] D-glucans have been described as inhibitors of virus infection of *Nicotiana tabacum* (Rouhier *et al.*, 1995). The uronic acid-containing glycoprotein glycans of *Fusarium* sp. M7-1 are conjugated to serine and/or threonine residues (Iwahara *et al.*, 1992; 1995; Jikibara *et al.*, 1992a; 1992b), and they are involved as *Fusarium* allergens in the development of respiratory diseases (Backman *et al.*, 1995). In addition, the treatment of suspended plant cells or protoplasts by the carbohydrate enriched fractions isolated from uronic acid-containing glycoproteins from *Fusarium* sp. M7-1 indicated an ability of fungal oligosaccharides to elicit early plant defense reactions (Nita-Lazar *et al.*, 2000). To get insight into the mechanism of action of *Fusarium* carbohydrates which may be signaling molecules in plants, an attempt has been made to identify the structure of the minimum molecular size of O-glycans required for the expression of a biological activity. In this study, we report the isolation and characterization of disaccharides which promote elicitor activity. The carbohydrates were examined here in terms of their ability to trigger early reactions in *Rubus fructicosus* suspended cells.

MATERIALS AND METHODS

Materials. Suspension cultures of *Rubus fructicosus*, originally derived from cambial explant from twigs, were grown as described by Hustache *et al.* (1975).

Size-exclusion chromatography fractionation. Dried mycelium of *Fusarium oxysporum* L. (100 g) was extracted overnight with acetone (250 ml at 20°C) and the residual powder was subjected to alkaline treatment in 100 ml 0.1 M NaOH at 60°C for 2 h. The supernatant collected by centrifugation (15 000 × ***g***, 15 min) and neutralized to pH 7 with 50% acetic acid was stored overnight at 4°C. The resulting sample was centrifuged (15 000 × ***g***, 20 min at 20°C), and an aliquot (1 ml) of supernatant was applied to an HW 40F/50F column (50 cm × 2.5 cm; Interchim) and eluted with 0.1 M $NaNO_3$ at a flow rate of 2 ml/min. Detection was run both at 206 nm and 280 nm using a Beckman DU 640 spectrophotometer, and the refractive index was measured with a Waters 410 differential refractometer. The presence of carbohydrates or proteins in the isolated fractions was determined (independently of the HPLC detection system) according to Bradford (1976) and Dubois (1956).

Carbohydrate analysis. All ^1H and ^{13}C NMR spectra, with the exception mentioned below, were recorded on a Varian Unity Plus 500 spectrometer equipped with a 5-mm pulse field gradient (X) probe in D_2O solution (10 mg/ml (^1H) and 20–50 mg/ml (^{13}C)). Chemical shifts were expressed in p.p.m. relative to the methyl signal of acetone used as internal standard, and were taken to be 2.04 (^1H) and 31.45 (^{13}C) with respect to the signals for Me_4Si at 323 K (^1H) or 303 K (^{13}C). ^1H-NMR (1D) spectra were recorded using 45° pulses (8 μs) at 2,400 Hz spectral width, 16 K data points, 2.7 s acquisition time and 32 scans. ^1H-NMR (2D) COSY experiments were carried out using standard Varian Unity Plus 500 sequence, a 256 × 1024 points time do-

main matrix over 1000 Hz along the F1 and F2 directions, with F1 zero filled to 1024 points. Sine-bell window functions ($\pi/10$ phase-shifted) were used in both directions prior to Fourier transformation. ^{1}H-NMR(1D) TOCSY experiments were performed using the standard pulse sequence with a selective shaped pulse, 16 to 128 scans were performed for each experiment with the mixing time adjusted from 5 to 110 ms. ^{13}C-NMR (1D) spectra (75 MHz) were recorded on a Bruker AC 300 spectrometer using 30° pulse length (2 μs), 10000 Hz spectral width, 16 K data points and 3000 scans. ^{13}C-^{1}H shift-correlation 2D experiments were performed on a Varian Unity Plus 500 spectrometer by conventional HMQC and HMBC sequences. The sequences used a 128 × 512 points time-domain matrix, with spectral width of 1000 Hz (^{1}H) and 10000 Hz (^{13}C), which gave digital resolutions of 7.8 Hz/point (^{1}H) or 3.9 Hz/point (^{13}C) after zero-filling in F1. Two-hundred and fifty-six acquisition scans in inverse mode detection were obtained using a mixing delay *taumb* of 1.8 m at an interscan delay of 1.2 s. FAB-MS spectra were obtained on both an MS 50 KRATOS-AEI instrument (Manchester, U.K.) and a Nermag R 1010C mass spectrometer (Model 2000, Nermag, Rueil- Malmaison, France) equipped with an M Scan Wallis-type gun (8 kV, 20 mA). The samples were first dissolved in a glycerol matrix and submitted to Xe (9 kV) bombardment.

Elicitation experiments. *Rubus* cells (4×10^{6}) were suspended for 0 to 30 h in 25 ml of a pH 4.8 buffer (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 absence (controls) of fungal elicitor. In each experimental set, the fungal elicitor isolated from *Fusarium* was added up to 0.2 μg/ml to the medium. The elicitation medium was removed by centrifugation (500 × ***g***, 5 min, 4°C), and the cells were submitted to PAL activity analysis. At least four replicates were monitored from three independent elicitation sets.

PAL assay. The cells were subjected to PAL assays carried out according to the modified procedure of Hagendoorn *et al.* (1991). Briefly, the 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 was 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 0.05% (w/v) polyvinylpyrrolidone was added to the supernatant. The mixture was incubated for 10 min at 4°C and then centrifuged for 5 min at 5000 × ***g***. The supernatant was dialyzed and concentrated with an Ultrafree unit equipped with a 10 kDa molecular mass cut-off membrane (Ultrafree ™Millipore, Bedford, MA, U.S.A.). The crude enzyme extracts were then subjected to PAL assays according to Zucker *et al.* (1965). The reaction mixtures containing the L-phenylalanine substrate (2.25 μM) and enzyme (final concentration 6 μg protein/ml) in 0.1 M sodium borate, pH 8.8, supplemented with 2 mM dithiothreitol were incubated at 40°C. Kinetics were developed at 290 nm for 30 min using a Beckman DU 640 spectrophotometer and kinetic curves were drawn and fitted with second-order polynomial regressions; the statistical significance was set at 5%. The initial velocity of PAL reaction (ΔA_{290} min^{-1}) was calculated from the regression equation using Excel software and PAL activity was expressed as μkat/kg protein (1 μkat representing the formation of 1 μmol of product per s). Enzyme activation was expressed by the R value, i.e. the ratio of fitted-curve slope obtained with elicited cells *versus* controls.

Controls. Kinetic curves were developed in parallel from elicited samples and from non-induced ones. Controls consisted of cells incubated in a buffer without the elicitor in the presence or absence of an effector, like cycloheximide. The viability of cells was con-

trolled during each experimental set using Evan's Blue indicator. Blanks for enzyme assays were performed with boiled enzyme or in the absence of L-phenylalanine.

RESULTS

Elicitor activity

Alkaline extract (about 1 g) from the acetone powder was fractionated by size-exclusion chromatography into three fractions with different retention times (Fig. 1). About 400 mg of lyophilized powder of fraction I, 80 mg of fraction II and 100 mg of fraction III were obtained. Independently of the HPLC elution profiles protein and carbohydrate analysis

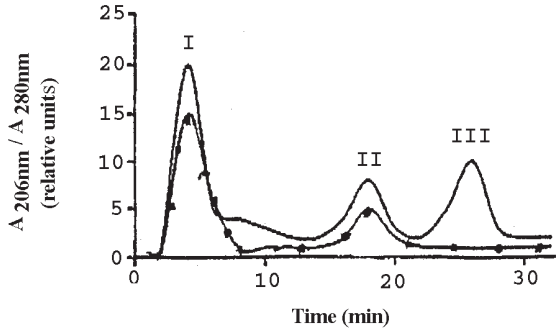

Figure 1. Size-exclusion chromatography elution profile of final extract of *Fusarium oxysporum* L.

I, II and III: fractions I, II, III, respectively. HW 40F/50F column (2.5 × 50 cm); elution with 0.1 M $NaNO_3$ (flow rate: 2 ml/min); detection at 206 nm (¦) and 280 nm (—).

showed that fractions I and II contained both carbohydrates and proteins, while fraction III contained carbohydrates only. The three fractions were used as elicitor to trigger mainly the PAL activity using *Rubus* cells in suspension for up to 30 min in at least 4 independent experiments.

A study of the ability of the three fractions to elicit PAL activity in 30 min showed a significant activity of 150 μkat/kg protein for fraction III, a lower activity of 95 μkat/kg protein for fraction I while fraction II had no activity (Fig. 2). On the basis of these results, we focused our interest on fraction III to characterize the PAL response and also to identify the signaling structure.

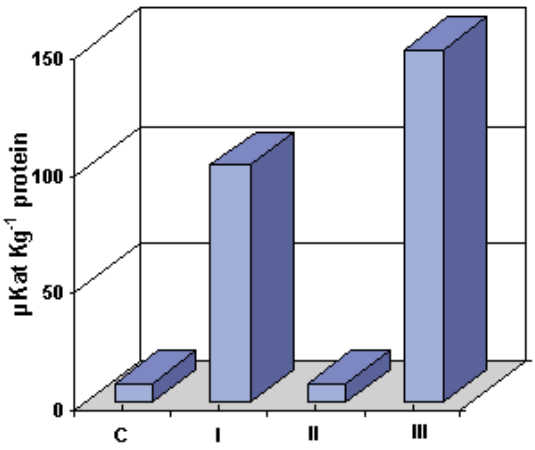

Figure 2. PAL responses in *Rubus* cells.

The cells (4×10^6) were challenged for 30 min with 3–6 μg elicitor I, II, III. Plot C represents non-induced cells (control). In ordinate: relative rates of reaction products expressed in μkat/kg protein. I, II and III: fractions I, II, III, respectively. Experiments were carried out in triplicate and the standard deviation was between ±1.5 to 2.7% of each plot.

Three to six micrograms of fraction III was applied to *Rubus* suspended cells for up to 30 h in at least 4 independent experiments. The PAL response was followed as changes in absorbance at 290 nm of reaction mixtures containing 2.25 mM L-Phe and the enzyme extract prepared from treated cells or from controls. We verified that the viability of cells was not affected by the elicitor application since it remained as high as in controls (85–95%). The induced response was biphasic with respect to the kinetics shown in Fig. 3 (curve a). After 30 h the slope increased only slowly and after 35 h the presence of contaminations was observed. The first response peaked at 30 min with the activity of 150 μkat/kg protein, giving an R value of 18, and the second one reaching at 30 h the level of 191.6 μkat/kg protein, equivalent to the R value of 23. In contrast, PAL activity in ab-

sence of elicitor remained unchanged over 15 h (Fig. 3, curve b). In the presence of 1 μM cycloheximide, the early response maintained the R value of 17 while the long-term response was strongly attenuated since it decreased to the R value of 5 at 30 h (Fig. 3, curve c). These data reveal that the treatment for up to 5 h affects the specific enzyme activity since it did not change significantly the total amount of extractable proteins. In a marked contrast, the long term-treatment promoted a large increase of the total extractable protein content, suggesting on involvement of a *de novo* synthesis of proteins in the delayed response (not shown).

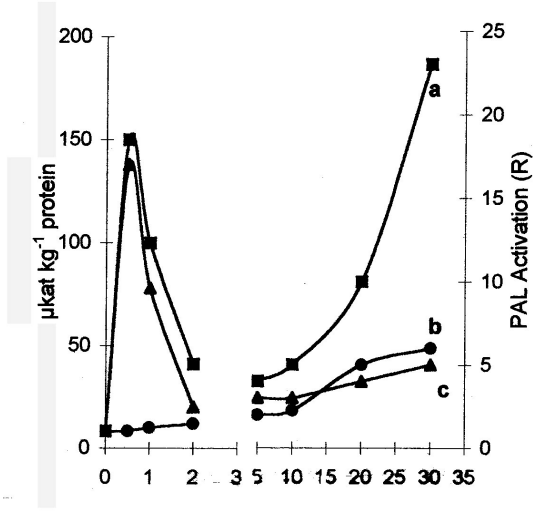

Figure 3. Time-course for PAL response in *Rubus* cells.

The cells (4×10^6) were challenged with elicitor only (curve a) or with elicitor plus 1 μM cycloheximide (curve c). Curve b is the control response. The cells were challenged by 3–6 μg elicitor up to 30 h, and PAL extracts were then assayed at 290 nm. In ordinate: relative rate of reaction products expressed in μkat/kg protein or by the R value (activity in treated cells *vs.* controls). Experiments were carried out in triplicate.

Carbohydrate analysis

The ^{1}H spectrum of the oligosaccharide fraction III displayed 5 signals in the anomeric region at 5.37, 5.18, 5.11, 4.95 and 4.65 p.p.m. (Fig. 4) and other very weak signals (not assigned) which represented no more than 1% of the total integrated resonance.

The doublets at 5.37 and 4.65 p.p.m., based on chemical shifts and coupling constants, were attributed to the α- and β-anomeric protons of a reducing end glucose unit. The integration of these resonances yielded an anomeric equilibrium (%) α/β: 52/48. The $^3J_{1,2}$ coupling constants displayed (about 2 Hz) by the two remaining signals, at 5.18 and 4.95 p.p.m., revealed the α-configuration of mannose. The α-configuration is supported by the internal H-1/H-2 NOE effect (not shown).

This result was confirmed by TOCSY, ^{1}H-^{1}H COSY and two-steps relayed COSY, spectra (Fig. 5) for the configurational assignments of the sugar units, by consideration of the vicinal (^{3}J) coupling constant value displayed in its J connectivities (Table 1).

The ^{1}H-^{13}C heteronuclear spectrum (HMQC) of the compound allowed the assignment of the ^{13}C chemical shift (Table 2).

The relatively low-field resonance of the proton of C-2 from α-glucopyranosyl and β-glucopyranosyl was caused by glycosylation and revealed the O-2 substitution. Moreover, the HMBC spectrum (Fig. 6) clearly showed a long-range correlation between the H-1 protons at 5.18 and 4.95 p.p.m. and the C-2 at 76.02 p.p.m. (α-glucopyranosyl) and 80.08 p.p.m. (β-glucopyranosyl), indicating that the distinct ^{1}H resonances displayed by the two α-mannopyranosyl units are a consequence of the proximity between the anomeric center of the reducing end residue and the glycosidic linkage.

The presence of a minor compound may be correlated with the weak signal observed at 5.11 p.p.m. in the anomeric region of the ^{1}H spectrum (9.2% of the total anomeric resonance). The FAB-MS spectrum of methylated fraction III indicated the existence of a minor mannopyranosyl-cyclichexitol, demonstrated by the presence of fragments at *m/z* 491 $[M+Na]^+$ and *m/z* 469 $[M+H]^+$ (not shown).

The presence of α-mannopyranosyl-(1→x)-inositol can be also correlated with the occurrence of the anomeric proton of D-mannopyranosyl observed at 5.11 p.p.m.

These data unambiguously characterize the disaccharide: α-D-mannopyranosyl-(1→2)-α/β-D-glucopyranosyl as the major compound (90.8% of fraction III) and suggest the presence of α-mannopyranosyl-(1→x)-inositol as a minor compound (9.2% of the fraction). The peaks at *m/z* 477 $[M+Na]^+$ and *m/z* 455 $[M+H]^+$ obtained by FAB-MS (not shown) additionally confirm the presence of these major compounds.

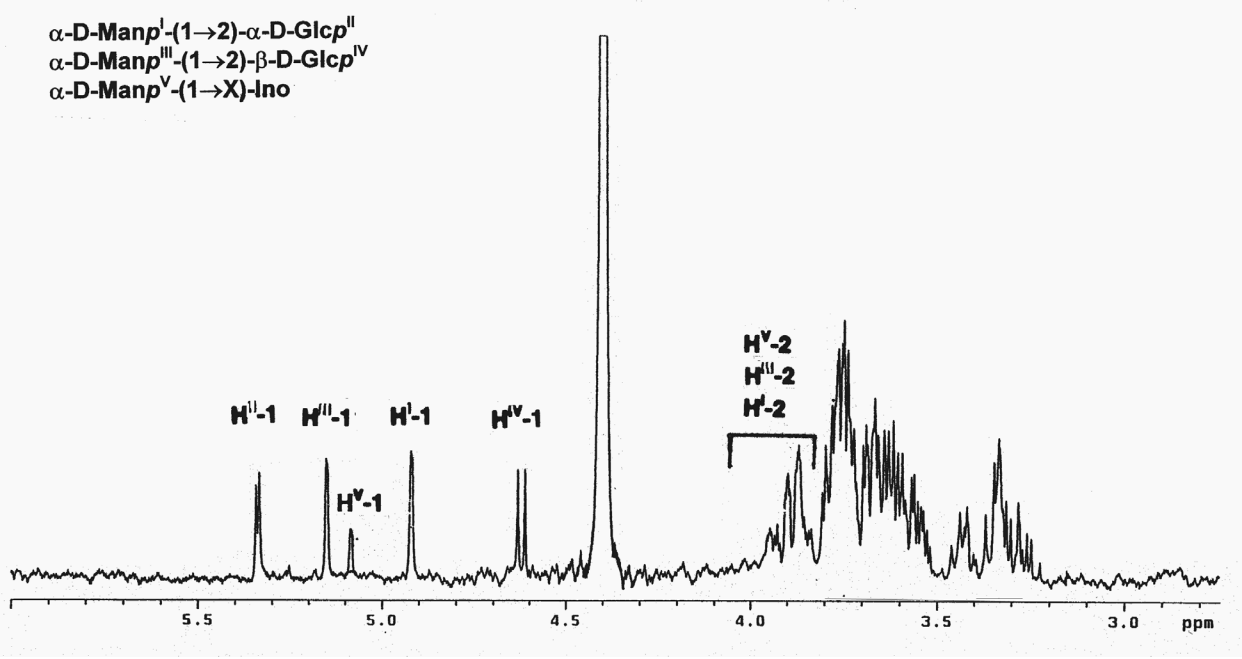

Figure 4. ^{1}H-NMR spectrum of oligosaccharide fraction III.

DISCUSSION

A carbohydrate signaling molecule

Fusarium oxysporum like many fungi contain in its cell wall various compounds such as proteins, glycoproteins (Jikibara *et al.*, 1992a), lipids (Iwahara *et al.*, 1996) and free oligosaccharides (Bruneteau, 1992). Previous extensive structural studies carried out on the carbohydrate *Fusarium* cell wall components indicated the presence of β-D-(1→3) [1→6] linked glucose- and uronic acid-containing glycoproteins (Iwahara *et al.*, 1995; Jikibara *et al.*, 1992a; 1992c). Some sequences like β-D-mannopyranosyl-(1→2)-β-D-mannopyranosyl, α-D-glucuronic acid-(1→2)-β-D-galactofuranosyl, β-D-galactofuranosyl-(1→6)-β-D-mannopyranosyl and β-D-galactofuranosyl-(1→6)-β-D-galactofuranosyl are common to different *Fusarium* species (Jikibara *et al.*, 1992b; Iwahara *et al.*, 1995) including *F. oxysporum.* The oligosaccharides from *Fusarium* were mainly composed of mannose residues with an α(1→2) linkage, but neither linkages with glucose nor inositol have been detected up to now. To our knowledge, the structure α-D-mannopyranosyl-(1→2)-α/β-D-glucopyranosyl derived from our biological source is reported here for the first time. This structure is identical to that of a disaccharide methylglycoside previously described by Shashkov *et al.* (1993) and synthesized with the aim of developing a computerized approach for the structural elucidation of regular branched polysaccharides.

Oligosaccharides have been often described as active molecules in plant defense reactions. It is known that some oligosaccharides originating from fungal cell wall components

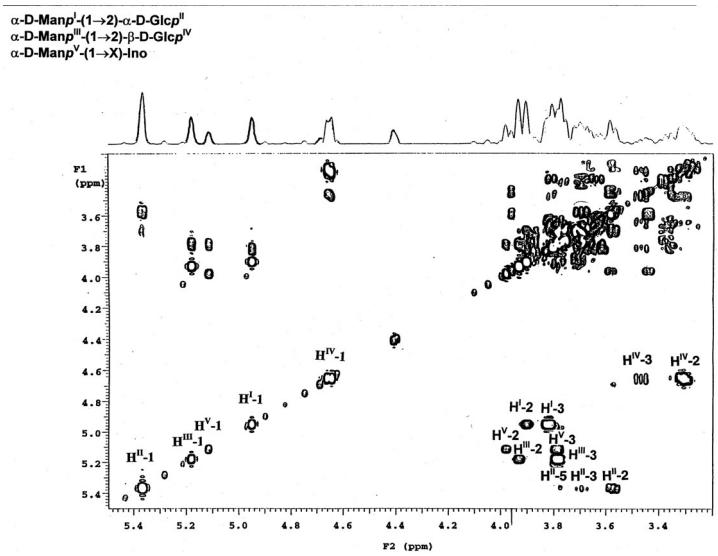

Figure 5. Two-step relayed COSY spectrum of oligosaccharide fraction III.

elicit phytoalexin accumulation and lignin or callose formation in plants (Kauss *et al.*, 1989; Lesney, 1989). Furthermore, the system recognition of an oligosaccharide signal is highly sensitive and selective (Prome, 1996). The present study reveals the induction of PAL in *Rubus* cells within a few minutes after the application of the disaccharide by Nurnberger *et al.* (1997), and provides evidence for post-translational regulation of the enzyme. In addition, changes in PAL activity in the presence of different elicitors were compared. The oligosaccharide fraction originating from *Fusarium* sp. M7-1 glycoproteins exhibits an R value of 108 (Nita-Lazar *et al.*, 2002). According to our observations when

Table 1. 500 MHz ^{1}H chemical shift data[a] (p.p.m.) of carbohydrate fraction III isolated from *Fusarium oxysporum* L. ($D_2$0, 50°C).

[a] δ in p.p.m. (J_{HH} in Hz) n.a indicates a resonance not assigned.

	H1	H2	H3	H4	H5	H6, H6'
α-D-Man*p*^{I}-(1→2)-α-D-Glc*p*^{II}						
α-D-Glc*p*^{II}	5.37 (3.9)	3.58	3.69	3.37	3.76	3.73, 3.69
α-D-Man*p*^{I}	4.95 (1.9)	3.90	3.82	3.62	n.a	n.a, n.a
α-D-Man*p*^{III}-(1→2)-β-D-Glc*p*^{IV}						
β-D-Glc*p*^{IV}	4.65 (8.0)	3.31	3.47	3.36	3.38	3.81, 3.64
α-D-Man*p*^{III}	5.18 (3.9)	3.93	3.78	3.66	n.a	n.a, n.a

α-D-Man*p*, α-D-mannopyranosyl; α/β-D-Glc*p*, α/β-D-glucopyranosyl.

elicitor. These very rapid responses are transient and they are maintained in the presence of cycloheximide. This suggests an early signal transduction cascade, which may be associated with plasma-membranes as suggested cells instead of protoplasts were monitored, the detected response was attenuated by 35–40% but this can not be the only explanation of the difference in the PAL response between the data presented here and those from

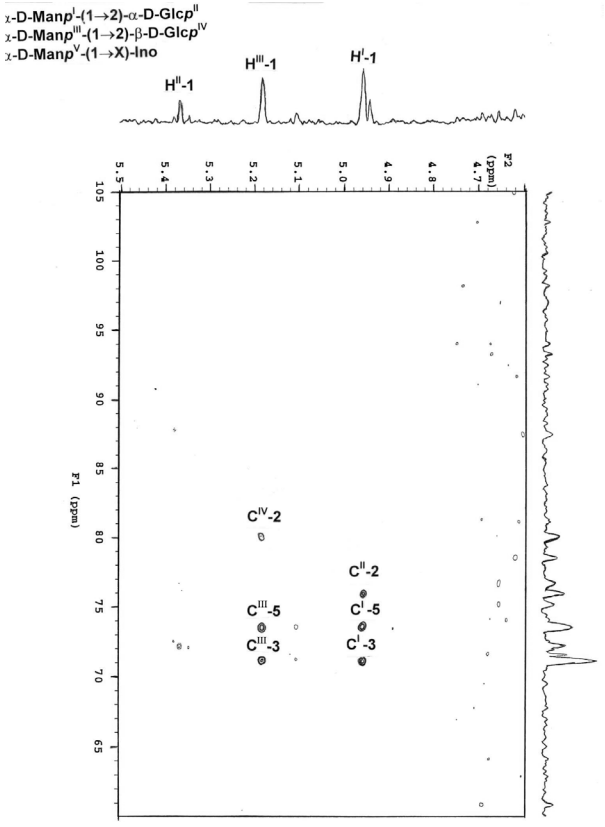

Figure 6. HMBC spectrum of oligosaccharide fraction III.

Nita-Lazar *et al.* (2002). A different *Fusarium* species was previously used, but the main difference came from the fact that the previous elicitor fraction was a complex mixture of glycans of different length and carbohydrate units in which it was very difficult to identify the active compound and the specific elicitor response. In the present work we made important steps forward in addressing this question.

A delayed PAL response which decreased in the presence of cycloheximide, and therefore involved late changes in PAL mRNA translation levels, has also been detected. PAL elevation, as an indicator of plant resistance, has often been reported to occur at the transcriptional level. It is worth noting that the level of PAL mRNA does not always increase proportionally to the measured enzyme activity (Lee *et al.*, 1992), thus suggesting that PAL can also be affected at post-transcriptional stages (Shaw *et al.*, 1990).

Up to now, enzymes of the phenol pathway have often been described as playing a central role in the orchestration of hypersensitive responses in plants, but they are shown here for the first time to be dependent on the application of a well-identified elicitor from *Fusarium*.

We thank Prof. Jean Montreuil and Dr. Gerard Strecker for fruitful discussions.

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Table 2. ^{13}C data for oligosaccharide fraction III isolated from *Fusarium oxysporum* L.

	C1	C2	C3	C4	C5	C6
α-D-Man*p*^{I}-(1→2)-α-D-Glc*p*^{II}						
α-D-Glc*p*^{II}	90.2	76.0	72.3	70.5	72.2	61.6
α-D-Man*p*^{I}	98.3	71.2	71.2	67.6	73.8	61.6
α-D-Man*p*^{III}-(1→2)-β-D-Glc*p*^{IV}						
β-D-Glc*p*^{IV}	97.2	80.8	75.4	70.7	76.8	61.6
α-D-Man*p*^{III}	100.8	71.2	71.2	67.6	73.8	61.6

α-D-Man*p*, α-D-mannopyranosyl; α/β-D-Glc*p*, α/β-D-glucopyranosyl.

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