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Induction by α-L-Arabinose and α-L-Rhamnose of Endopolygalacturonase Gene Expression in *Colletotrichum lindemuthianum*

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The production of endopolygalacturonase (endoPG) by *Colletotrichum lindemuthianum*, a fungal pathogen causing anthracnose on bean seedlings, was enhanced when the fungus was grown in liquid medium with L-arabinose or L-rhamnose as the sole carbon source. These two neutral sugars are present in plant cell wall pectic polysaccharides. The endolytic nature of the enzyme was demonstrated by its specific interaction with the polygalacturonase-inhibiting protein of the host plant as well as by sugar analysis of the products released from its action on oligogalacturonides. Additional characterization of the protein was achieved with an antiserum raised against the pure endoPG of the fungus. Induction by arabinose and rhamnose was more prolonged and led to a level of enzyme activity at least five times higher than that on pectin. Northern blot experiments showed that this effect was correlated to the induction of a 1.6-kb transcript. A dose-response study indicated that the endoPG transcript level was already increased at a concentration of each sugar as low as 2.75 mM in the medium and was maximum at 55 mM arabinose and 28 mM rhamnose. Glucose, the main plant cell wall sugar residue which is also present in the apoplast, prevented endoPG gene expression, partially when added to pectin at concentrations ranging from 5 to 110 mM and totally when added at 55 mM to arabinose. Inhibition by glucose of the rhamnose-induced endoPG was correlated to nonuptake of rhamnose. This is the first report that arabinose and rhamnose stimulate endoPG gene expression in a fungus. The possible involvement of these various sugars on endoPG gene expression during pathogenesis is discussed.

Pectin, a complex galacturonic acid-containing polysaccharide, is one of the main polymers of the primary plant cell wall and middle lamella (10). As such, it represents a mechanical barrier as well as a carbon source for many microbial plant pathogens. In order to degrade pectin, these microorganisms secrete a wide array of pectinases (15). Since these enzymes are able to cause tissue maceration, they have long been regarded as a major factor of pathogenicity of bacteria and fungi (15, 30). In addition, research on pectinases has been widened by the finding that the pectic fragments that they release from their substrate induce numerous physiological effects in plants, notably defense responses (18, 41).

In bacteria, several pectinase clones have been isolated, and regulatory loci have been characterized (16). The molecular regulation of pectinases is less documented in fungi than in bacteria. However, the strong applied interest in these enzymes has led to the isolation of a battery of genes from *Aspergillus* species encoding polygalacturonases (PGs) (8, 11, 29, 42) as well as pectin lyase (24) and pectate lyase (19). By comparison, only a small number of genes have been isolated from filamentous phytopathogenic fungi; they include the genes encoding endopolygalacturonases (endoPGs) of *Cochliobolus carbonum* (43), *Fusarium moniliforme* (9), and *Sclerotinia sclerotiorum* (39) and pectate lyases from *Fusarium solani* f. sp. *pisi* (23). Most of them appear to be substrate inducible and catabolite repressed by glucose and other sugar monomers, thus confirming the long-reported effects of these carbon sources on pectinases activity in *Verticillium albo-atrum*, *Fusarium oxysporum* f. sp. *lycopersici* (17), and *Botrytis cinerea* (35).

This work deals with the endoPG (EC 3.2.1.15) of *Colletotrichum lindemuthianum*, the causal agent of bean anthracnose. The enzyme which is produced in vitro has been isolated and partly characterized at the biochemical and molecular levels (5, 21, 27, 28). It randomly cleaves the α-1,4-D-galacturonosyl bonds of homogalacturonan and pectic polymers, producing mono-, di-, and trigalacturonic acid residues as the final hydrolysis products; these two oligomers cannot be further degraded by the enzyme. EndoPG activity is inhibited by the bean cell wall PG-inhibiting protein (PGIP) (2, 13, 33), a molecule whose leucine-rich repeats resemble those of protein receptors (20). We recently reported that the pure endoPG of *C. lindemuthianum* race β elicits the biosynthesis of PR proteins, notably β-1,3-glucanases, in a cultivar-specific manner in bean cuttings (34) and that its action on host cell walls releases pectic fragments which can modulate hydroxyproline-rich glycoprotein gene expression (6). Thus, the enzyme seems to play a key role in the interaction, and the aim of this research was to look for plant components which might regulate its expression.

Gene expression studies have recently shown that pectin and glucose regulate endoPG production at the transcript level when the fungus is grown in vitro (27). In this work, we compare the effects of different sugar monomers present in the apoplastic compartment of plant cells on endoPG production during in vitro culture of the fungus. We describe the hitherto unreported stimulatory effects of arabinose and rhamnose on endoPG gene expression.

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MATERIALS AND METHODS

Fungal material. *C. lindemuthianum* (Sacc. et Mgn) Briosi and Cav., race β, was maintained on synthetic agar (Difco) medium (3). For endoPG production, liquid cultures were prepared in 250-ml Erlenmeyer flasks containing 50 ml of synthetic medium, as described by Barthe et al. (5), supplemented with different carbon sources. D-(+)-glucose, D-(+)-galactose, D-(+)-xylose, D-(+)-fructose, L-(+)-arabinose, L-(−)-rhamnose, L-(−)-fucose, D-(+)-mannose, myoinositol, α-D-galacturonic acid, polygalacturonic acid, and apple pectin were purchased from Sigma; sucrose was purchased from Merck. After inoculation of the medium with a suspension of conidia in distilled water to a final concentration of 2×10^3 conidia per ml, the cultures were incubated in the dark at 23°C. In some experiments, the fungus was first grown on glucose for 4 days and then washed with sterile water before being transferred to the growth media. After culturing, the mycelium was filtered through a previously dried filter paper, washed with distilled water, and dried at 100°C overnight to constant weight.

Purification of endoPG. *C. lindemuthianum* endoPG was purified as previously described (5) and was used as a control in Western blot analysis.

Assay for PG activity. Aliquots of the liquid medium were withdrawn from the culture flask and were immediately dialyzed against 50 mM sodium acetate buffer (pH 5.2) for 5 h at 4°C; three flasks were sampled for each assay. PG activity was determined by monitoring the increase in reducing groups released from polygalacturonic acid for 30 min at 30°C as described by Somogyi (44) and was expressed in nanokatals, 1 nkat corresponding to the release of 1 nmol of reducing group equivalent per second. α-D-galacturonic acid (Sigma) was used as a standard. The data were expressed either in nanokatals per milligram (dry weight) or in nanokatals per flask (i.e., 50 ml of culture filtrate) in time course experiments because the amount of mycelium was too low for accurate measurement at the beginning of the culture.

The PG assay allowed us to measure endoPG as well as exopolygalacturonase (exoPG) activity. To discriminate between endoPG and exoPG activities, each sample corresponding to 0.138 nkat of PG activity was preincubated with or without 2.6 μg of PGIP, a specific inhibitor of the endoPG enzyme purified as described previously (33). This amount of PGIP is sufficient to inhibit 0.138 nkat of the pure endoPG of *C. lindemuthianum*, and has no effect on exoPG, as previously shown by Albersheim and Anderson (2) and also confirmed by us.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. Culture filtrates were dialyzed against cold distilled water and subjected to gel electrophoresis under denaturing conditions in a 12.5% polyacrylamide gel as described by Laemmli (32). Depending on the carbon source, the samples were analyzed either on a protein basis or on a mycelium dry weight basis when the presence of pectin in the culture filtrate precluded protein measurement. After migration, the proteins were transferred to nitrocellulose by using an LKB 2005 transphor apparatus at a constant current (1.8 mA cm^{-2} of membrane). The membrane was then incubated for 10 min in a Tris-buffered saline solution (50 mM Tris-HCl [pH 5.5] buffer, 150 mM NaCl). After saturation with blocking reagent 11 (Bio-Rad) for 45 min at room temperature, the membrane was incubated with the endoPG antiserum, which was used at a dilution of 10,000 in Tris-buffered saline. The antigen-antibody complex was visualized by colorimetric detection using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G as instructed by the supplier (Bio-Rad).

Sugar analysis. The sugars present in the culture filtrate of the fungus were analyzed by anion-exchange chromatography using a Dionex (Sunnyvale, Calif.) high-pressure liquid chromatography (HPLC) system equipped with a CarboPac PA1 column (4 by 250 mm) as described by Hotchkiss and Hicks (26). This method was also used for measuring the products released from 1 mg of the oligogalacturonide $(GalA)_3$ (Sigma) per ml by culture filtrate samples, corresponding to 0.5 nkat of PG activity.

Protein determination. Protein concentration in the culture filtrates was estimated by the method of Bradford (7).

RNA isolation and gel blot analysis. Total RNA was isolated from the mycelium by the method of Haffner et al. (25). Aliquots corresponding to 10 μg of total RNA were denatured with formamide-formaldehyde and subjected to electrophoresis in a 1.2% (wt/vol) agarose gel containing formaldehyde. Equal loading was checked by ethidium bromide staining of the gel. The gels were transferred to nitrocellulose membranes and fixed by backing for 2 h at 80°C under vacuum. The membranes were then prehybridized for 2 h at 42°C in 50% formamide–0.1% SDS–1× Denhardt's solution–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50 ng of denaturated calf thymus DNA per ml. After addition of the *Eco*RI insert from pCC63, corresponding to the 1.3-kb endoPG cDNA of *Cochliobolus carbonum*, kindly provided by Scott-Craig et al. (43), hybridization was carried out overnight under the same conditions. The insert was radiolabeled with [α-^{32}P]dCTP by random priming as described previously (22). The membranes were washed twice with 2× SSC–0.1% SDS at 53°C before autoradiography.

RESULTS

Effect of carbon source on PG production and mycelium growth of *C. lindemuthianum* race β. In this study, the main sugar monomers of plant cell walls and apoplastic fluids were used as carbon sources in liquid cultures of *C. lindemuthianum*. To determine their effects on PG production, the fungus was grown for 13 days on liquid media containing each carbon source at 10 g per liter. As shown in Table 1, the mycelium developed on all media. Measurement of the fungal biomass indicated that pectin was the most efficient, and myoinositol the poorest, carbon source. The fungus grew equally well on sucrose, fructose, glucose, or mannose and, to a somewhat lesser extent, on galactose, xylose, arabinose, rhamnose, or galacturonic acid. PG activity was detected in most culture filtrates but was very low on glucose, galacturonic acid, and polygalacturonic acid and was not detectable on fucose. The effect of arabinose and rhamnose was particularly remarkable, since on a mycelium dry weight basis, the enzyme activity was five to seven times higher than on pectin. It was previously shown that induction on pectin was maximum at earlier stages of growth (6 days) and declined with the release of galacturonic acid in the medium (27). At maximum induction on pectin, it appeared that the addition of galacturonic acid at 11 g per liter (55 mM) to the pectin medium lowered by about one-third the extent of PG induction.

TABLE 1. Effects of various carbon sources on *C. lindemuthianum* mycelium growth and endoPG production after 13 days of culture on liquid media containing each carbon source at 10 g/liter[a]

Carbon source	Mycelium dry wt (mg/flask)	PG activity (nkat/mg [dry wt])	PG inhibition (%)	$(GalA)_3$ hydrolysis (%)
Glucose	35 ± 6	0.6 ± 0.5	97.8 ± 2.5	<20
Fructose	35.2 ± 3.4	1.1 ± 0.7	80 ± 6.7	<20
Galactose	16.4 ± 0.3	3.4 ± 0.3	97 ± 1	<20
Xylose	21 ± 4	2.9 ± 1.1	70 ± 8	<20
Arabinose	22.5 ± 3.3	17 ± 1.9	97 ± 1	<20
Rhamnose	17.2 ± 4.8	12.8 ± 4	95.5 ± 3.5	<20
Myoinositol	7.7 ± 2	2.9 ± 0.5	83	<20
Mannose	35.5 ± 5	2 ± 0.5	95	<20
Sucrose	35.5 ± 5.6	2 ± 1.07	100	<20
Galacturonic acid	16.5 ± 3.8	1.7 ± 0.3	0	ND[c]
PGA[b]	33 ± 5	0.4 ± 0.2	0	ND
Pectin	52 ± 2	1.8 ± 0.2	47.5 ± 7.5	>90

[a] The proportion of total PG activity (endoPG plus exoPG) which was inhibited by PGIP corresponds to endoPG. The proportion of hydrolyzed $(GalA)_3$ reflects the presence of exoPG. The data are means of three independent experiments ± standard deviations of the means.
[b] PGA, polygalacturonic acid.
[c] ND, not determined.

The enzyme activity which was induced on arabinose and rhamnose was further characterized. To discriminate between endoPG and exoPG, the PGIP isolated from bean cell walls, which inhibits the fungal endoPG but not the exoPG (33), was used. It appeared that both arabinose and rhamnose stimulated mainly endoPG production, as the use of PGIP inhibited about 95% of the total PG activity (Table 1). In the other neutral sugar-containing media, endoPG was also responsible for most of the PG activity. In contrast, inhibition of PG activity was not observed when *Colletotrichum* was grown on galacturonic acid or polygalacturonic acid at 10 g/liter and reached only 50% when the fungus was grown on pectin, thereby suggesting that exoPG was produced on these media at that stage of growth (Table 1). At maximum induction on pectin, i.e., 6 days, it was previously showed that endoPG accounted for the major activity (27). The prevalence of endoPG activity in most media and the presence of exoPG activity when the fungus was grown for 13 days on pectin was checked by Dionex HPLC analysis of galacturonic acid re-

leased from the oligogalacturonide $(GalA)_3$. Only the exoPG can degrade it, whereas the endoPG of *C. lindemuthianum* cannot release galacturonic acid from this small oligomer, as indicated by standard experiments performed with the two pure enzymes (data not shown). As indicated in Table 1, only a small proportion of $(GalA)_3$ was degraded by the culture filtrate of the fungus grown on neutral sugar monomers, thereby indicating that the exoPG activity was low in these media. In contrast, $(GalA)_3$ was almost completely degraded by the filtrate of the fungus grown on pectin, which confirmed the presence of exoPG in this medium.

Induction of PG by growth on pectin is a well-known effect which has been reported for several saprophytic and parasitic fungi, including *C. lindemuthianum* (27). In contrast, this is the first report that arabinose and rhamnose, two sugar monomers present in pectin and in hemicellulose, have a high stimulatory effect on endoPG. A study was then undertaken to better characterize and compare the effects of arabinose, rhamnose, and pectin.

Comparative study of the effects of arabinose, rhamnose, and pectin on PG production. A time course measurement of PG production in the culture filtrate of the fungus grown on arabinose, rhamnose, or pectin was undertaken. Every 2 or 3 days, PG activity in a sample of the culture filtrate was monitored, and the mycelium was dried and weighed. The amount of carbon source remaining in the culture filtrate was simultaneously determined by Dionex HPLC sugar analysis. As previously shown (27), induction on pectin was early and transient; the enzyme reached maximum activity of around 200 nkat per flask, e.g., 12.3 nkat per mg (dry weight), on day 6 of culture (Fig. 1A) and was at a very low level after 14 days (data not shown). For this reason, the sampling of pectin broth was not extended further. By comparison, the production of PG on arabinose- and rhamnose-containing media was somewhat delayed but much more prolonged and increased than on pectin, reaching maximum activity at around 800 nkat per flask, e.g., 21.7 nkat per mg (dry weight), at 20 to 25 days of culture. The prevalence of endoPG activity in both media at 24 days of culture was observed (data not shown). The mycelium growth was slower on rhamnose and arabinose than on pectin at the beginning of the culture, which correlated well with the delayed induction of endoPG activity (Fig. 1B). Induction on rhamnose and arabinose occurred while the amounts of the two sugars were still high in the media (Fig. 1C), thereby indicating that it did not result from carbon source limitation. Since endoPG activity was maximum at 6 days on pectin and already significant at 6 to 13 days on arabinose and rhamnose, most experiments were then performed on cultures ranging from 6 to 13 days.

In planta, one can expect different carbon sources to be available at the same time and in different concentrations. To determine the minimum amount of arabinose and rhamnose required for endoPG induction, each sugar was added to the medium at concentrations ranging from 2.75 to 100 mM. In this case, the fungus was grown for 8 days, in order to avoid carbon source limitation when the sugars were present at low concentrations. Control experiments were simultaneously performed on glucose- and on pectin-containing media. It appeared that even a low concentration (2.75 mM) of arabinose and rhamnose was sufficient to stimulate PG production, i.e., endoPG, compared to glucose (Fig. 2A). The enzyme activity was maximum at concentrations of either sugar ranging from 27.5 to 55 mM; higher concentrations were less stimulatory. Hence the concentration of 55 mM was used for further experiments. When arabinose at 55 mM and rhamnose at 55 mM were added simultaneously, we did not notice a cumulative

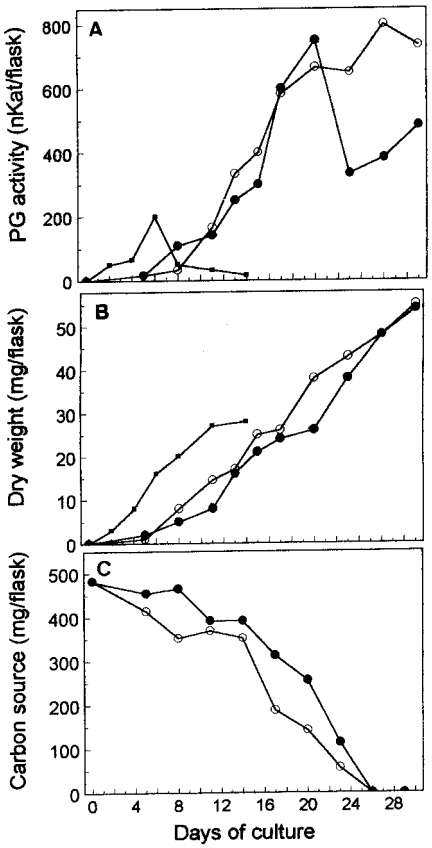

FIG. 1. Time course measurements of PG activity (A), mycelium growth (B), and carbon source consumption (C) of *C. lindemuthianum* grown on pectin (squares), arabinose (closed circles), or rhamnose (open circles) at 10 g/liter as the sole carbon source. Data represent the means of triplicates within a typical experiment.

effect (Fig. 2B). Similarly, addition of pectin at 10 g/liter to arabinose at 55 mM did not lead to a synergistic effect of the two carbon sources (data not shown). In the presence of glucose at 55 mM, i.e., 10 g/liter, in the arabinose- or rhamnose-containing medium, *C. lindemuthianum* failed to develop a significant level of endoPG activity (Fig. 2B). Induction by pectin of the enzyme activity was also lowered by about 50% when glucose was added to the medium at a concentration as low as 5 mM (Fig. 2C). From these data, it follows that arabinose and rhamnose act by enhancing the amount of active endoPG and that glucose acts by lowering it. The effects of arabinose, rhamnose, pectin, and glucose were then analyzed at the molecular level.

Molecular study of endoPG gene expression. On the basis of previous work (27), an antiserum raised against the pure endoPG (42 kDa) of *C. lindemuthianum* and a cDNA of *Cochliobolus carbonum* encoding an endoPG which cross-reacts with the antiserum against the protein of *C. lindemuthianum* were used for this study.

In a first step, the culture filtrates of the fungus grown on the different carbon sources were analyzed by SDS-PAGE. Silver staining of the gel (data not shown) indicated that several proteins are secreted by the fungus, particularly when grown on arabinose alone or on arabinose plus glucose or rhamnose, and that a number of proteins are clearly diminished in the presence of glucose in the media. Western blot analysis of the gel with the antiserum against *C. lindemuthianum* endoPG

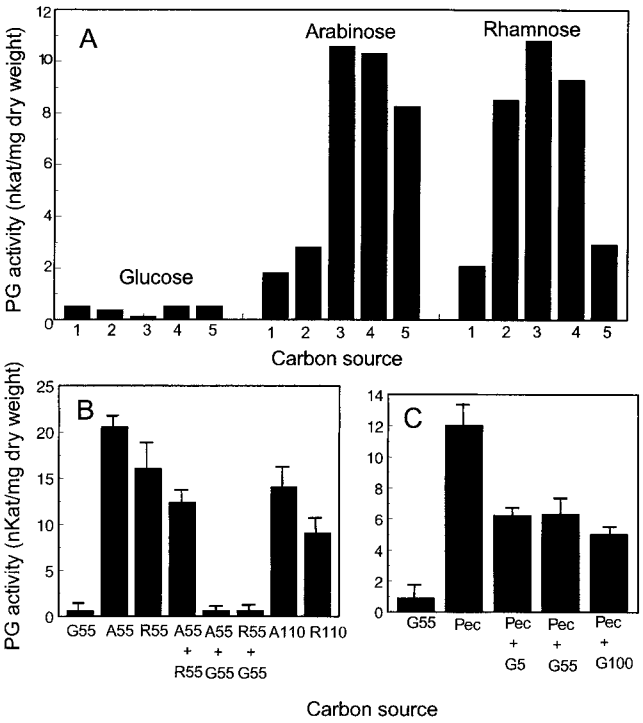

FIG. 2. Effects of different concentrations of arabinose, rhamnose, and glucose, either alone (A) or in mixtures with each other (B) or with pectin (C). (A) PG activity recovered from the culture filtrate of the fungus grown for 8 days on 2.75 (column 1), 5.5 (column 2), 27.5 (column 3), 55 (column 4), and 110 (column 5) mM glucose, arabinose, or rhamnose. (B) PG activity of the culture filtrate of the fungus grown for 13 days on arabinose or rhamnose at 55 or 110 mM alone (A55, R55, A110, or R110) or on mixtures with each other (A55 + R55) or with glucose at 55 mM (A55 + G55 and R55 + G55). (C) PG activity of the culture filtrate of the fungus grown for 6 days on pectin alone (Pec) or on pectin plus glucose at 5 mM (G5), 55 mM (G55), or 100 mM (G100). The data represent the means of triplicates within a typical experiment (A) or the means of three independent experiments with the standard deviations indicated by error bars (B and C).

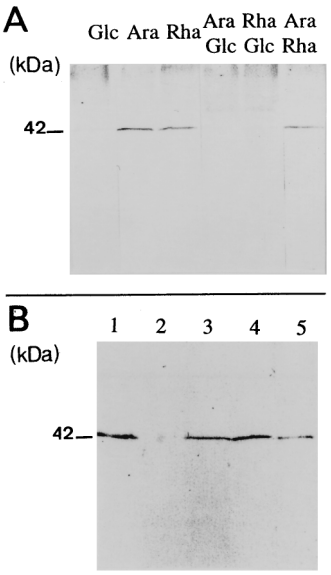

FIG. 3. SDS-PAGE and Western blot analysis of the culture filtrate of *C. lindemuthianum*. (A) The fungus was grown for 13 days on arabinose (Ara), rhamnose (Rha), or glucose (Glc) as the sole carbon source (55 mM) or on mixtures of these sugars; aliquots of the culture filtrates corresponding to 1 μg of protein were electrophoresed and revealed by Western blotting with the antiserum raised against the endoPG of the fungus. From left to right, the samples corresponded to 350, 80, 70, 300, 400, and 58 μg of mycelium dry weight. (B) Western blot analysis of the culture filtrates of the fungus grown for 6 days on glucose (55 mM) (lanes 2 and 5) or on pectin (10 g/liter) with (lane 3) or without (lane 4) glucose (55 mM). Aliquots corresponding to equal amounts of mycelium dry weight (62.5 μg; lanes 2 to 4) or to 300 μg (lane 5) were loaded on the gel, electrophoresed, and revealed with the anti-endoPG antiserum. EndoPG staining was enhanced by lengthening the duration of immunodetection with peroxidase. The pure endoPG of the fungus was loaded in lane 1. Sizes are indicated in kilodaltons.

(Fig. 3A) revealed one polypeptide band of 42 kDa which was present only in the culture filtrate of the fungus grown on arabinose, rhamnose, and arabinose plus rhamnose and was not detected when glucose was added to the culture. Thus, the increased endoPG activity of the fungus grown on arabinose or rhamnose resulted from an increase in the amount of the corresponding protein. Essentially the same data were obtained when the fungus was grown on pectin (Fig. 3B). The 42-kDa protein which was revealed in the culture filtrate by the anti-endoPG antiserum (lane 4) comigrated with the pure endoPG used as a standard (lane 1). On a constant mycelium dry weight basis (62.5 μg), this endoPG was decreased (lane 3) or not detected (lane 2) in glucose-containing media. However, loading the gel with a more concentrated sample (lane 5) allowed the detection of a constitutive low level of endoPG protein, even when the fungus was grown on glucose alone.

In a second step, Northern blot experiments were performed on total RNA isolated from *Colletotrichum*. It was previously found that pectin induces a 1.6-kb endoPG transcript (27). Figure 4A shows that a transcript of the same size was induced when the fungus was grown on arabinose or rhamnose and was almost undetectable when glucose was the carbon source. Maximum induction was observed at concentrations of 27.5 mM rhamnose and 55 mM arabinose, which was in good agreement with the dose-response effect found for endoPG activity. The amount of transcript was not enhanced by growing the fungus on arabinose plus rhamnose, thereby indicating the absence of a cumulative effect (Fig. 4B). In contrast, addition of glucose to arabinose or rhamnose up to the final total sugar concentration of 28 mM led to a strong decrease in the level of the endoPG transcript (Fig. 4B). A similar effect was observed when the mycelium was first grown on glucose, to maximize biomass, before being transferred to pectin. The endoPG gene expression which was recorded in this case was also partly repressed by glucose (Fig. 4C). Taken together, these results show that arabinose and rhamnose lead to an increase of endoPG protein and enzyme activity via an increase in transcript level.

From the observation that in mixture with arabinose and rhamnose, glucose led to a strong decrease of endoPG induction and that there was no cumulative effect between the two sugars, we were interested in knowing which sugars were consumed during the experiment. The remaining amount of each monomer used as a carbon source was measured in the culture filtrate after 13 days of culture with a Dionex HPLC system. We observed that when glucose was in mixture with arabinose, the proportions of the sugars which were taken up were similar, e.g., 44% for arabinose and 38% for glucose. In contrast, when glucose was in mixture with rhamnose, glucose was preferentially taken up (39%) compared to rhamnose (7%). When arabinose and rhamnose were in mixture, the two sugars were consumed (30 and 18%, respectively) but with a greater disappearance of arabinose. These results suggest that glucose completely prevents the effect of arabinose and rhamnose on endoPG gene expression by different mechanisms.

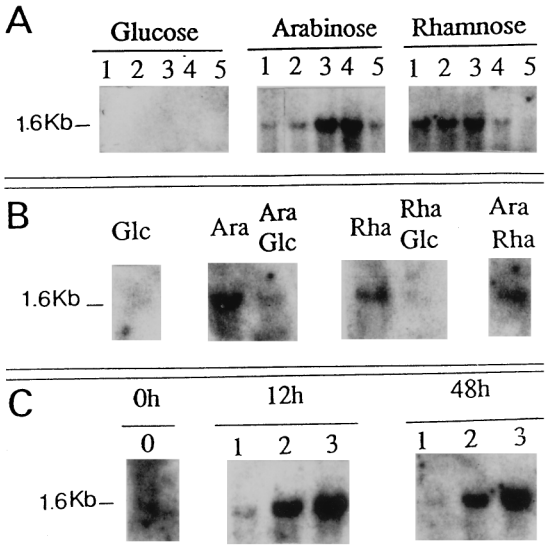

FIG. 4. Northern blot analysis of total RNA (10 μg) isolated from *C. lindemuthianum* and probed with the endoPG cDNA insert from pCC63. (A) The fungus was grown for 7 days on glucose, arabinose, or rhamnose at increasing concentrations: 2.75 mM (lane 1), 5.5 mM (lane 2), 27.5 mM (lane 3), 55 mM (lane 4), and 110 mM (lane 5). (B) The fungus was grown for 7 days on glucose, arabinose, or rhamnose as the sole carbon source, or on mixtures of these sugars, at 14 mM each. (C) The fungus was first grown on glucose for 4 days and then transferred for the indicated times to media containing either glucose (55 mM) alone (lanes 0 and 1), pectin (10 g/liter) alone (lane 3), or pectin plus glucose (lane 2).

DISCUSSION

The aim of this work was to determine the effects of various carbon sources on endoPG gene expression in *C. lindemuthianum* grown in vitro. The results allowed us to show for the first time the stimulatory effect of arabinose and rhamnose on endoPG. This effect was demonstrated at the levels of endoPG transcript, endoPG protein, and enzyme activity.

Rhamnose and arabinose are present in the hairy, rhamnogalacturonan I and II domains of pectins (10). Only L-(+)-arabinofuranose, the natural form of arabinose in plant cell walls, was found to induce the production of endoPG, while the D-(+) form of the sugar had no effect. Galacturonic acid and galactose, two other main sugar monomers of pectin, were not stimulatory. Galacturonic acid was reported to induce pectinase production in fungal pathogens such as *V. albo-atrum* and *B. cinerea* (17, 35) when used at concentrations lower than 20 mM and to repress it at higher concentrations, notably in *B. cinerea* and *Aspergillus niger* (1, 35).

Compared to induction of endoPG on pectin, the effect of arabinose and rhamnose was slower at the beginning of the culture but then started to increase, reaching total enzyme activity values five to seven times higher than those on pectin alone. In addition, this increase lasted for a longer time, i.e., 21 days, compared with 6 to 7 days on pectin. It would be interesting to know whether other pectinases in *Colletotrichum*, and in other fungi as well, are also increased in response to the two sugar monomers.

An essential question is the availability of arabinose and rhamnose in planta and the potential significance of their effect on the outcome of the interaction between endoPG and its substrate during pathogenesis. The two sugars are found in cell wall pectic polysaccharides and hemicelluloses. The arabinofuranosidase activity which has been measured in the growth media of several fungi, notably *Colletotrichum*, and in diseased tissues (reference 38 and data not shown) might result in a substantial release of arabinose. On the other hand, it has been shown that *Aspergillus aculeatus*, a fungal saprotroph, releases rhamnose from pectin through the sequential action of rhamnogalacturonase and rhamnanase (37); the presence of rhamnogalacturonase in the fungal pathogen *B. cinerea* was recently reported (14). Thus, it is likely that arabinose and rhamnose are released during in vitro culture of the fungus and account, at least in part, for the induction of endoPG when the fungus is given bean cell walls as the sole nutrient source (unpublished results). Their availability to *Colletotrichum* during its biotrophic phase of pathogenesis might significantly contribute to the outcome of the disease. On the one hand, they could be sufficient to induce the production of a certain amount of endoPG, which would then allow the fungus to degrade pectin in the cell walls and synthesize more enzyme. On the other hand, arabinose and rhamnose would sustain endoPG gene expression in conditions where inhibition of the enzyme by PGIP and down regulation by galacturonic acid, the final hydrolysis product released from pectin by PGs, would otherwise lead to extinction of endoPG action. One may assume that endoPG is one of the components allowing the fungus to enter the necrotrophic phase of pathogenesis. Besides these predictable roles, it would be of interest to know whether these two sugars induce other pathogenic traits in *C. lindemuthianum.*

The effect of rhamnose and arabinose provides an additional example of the activity displayed by small molecules at the host-pathogen interface. Recently, a pectic fragment isolated from carrot extracts was shown to induce the expression of a putative PG gene in *Agrobacterium tumefaciens*; the presence of arabinosyl residues was required for this induction (40).

The mechanism(s) by which arabinose and rhamnose regulate endoPG gene expression remains to be elucidated. Several promoter elements are involved in the regulation of gene expression in filamentous fungi (36). One of them mediates the repressor effect of glucose. In various fungi, it has been shown that this readily utilizable sugar represses transcription of the genes encoding the permeases and enzymes required to use other carbon sources (31). The protein CreA mediates the negative regulation exerted by glucose. When added to the growth medium of *Colletotrichum*, this sugar diminishes endoPG gene expression to a very low, almost undetectable level. Our data suggest that glucose prevents the entrance of rhamnose and abolishes the effect of arabinose on endoPG gene expression in *C. lindemuthianum*. If these effects involve CreA, one may hypothesize that arabinose and rhamnose, or their catabolic products, derepress endoPG gene expression at this level. However, other upstream regulatory elements have been found in pectinase genes (8, 23), but the proteins interacting with these regions have not yet been identified. Thus, an alternative interpretation of our data might be that arabinose and rhamnose or their degradation products induce endoPG gene expression via an effect targeted to such elements. Finally, one cannot exclude that increased enzyme activity might also reflect increased stability of the active protein. Future work will be aimed at understanding the regulatory effects of arabinose and rhamnose on *clpg1* and *clpg2*, the two endoPG genes which were recently isolated from *C. lindemuthianum* in a parallel work (reference 12 and unpublished research). Use of *clpg1* as a probe fully confirmed the data obtained with the *Cochliobolus* endoPG cDNA reported in this study. Considering the low endoPG gene expression in the presence of glucose under certain growth conditions, it will be of interest to know whether the two genes are differently regulated by glucose and the other carbon sources.

In conclusion, this work opens new avenues of research. It

extends the list of carbon sources involved in the regulation of pectinase gene expression and widens the model based on substrate induction and carbon catabolite repression. Use of arabinose and rhamnose allows the production of one category of PG only, namely, endoPG, not exoPG, and facilitates enzyme recovery and purification. Provided that the effect of arabinose and rhamnose holds true for other fungi, these findings may have wide implications in plant pathology and pectinase biotechnology.

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REFERENCES

1. **Aguilar, G., and C. Huitron.** 1993. Conidial and mycelial bound exo pectinase of *Aspergillus* sp. FEMS Microbiol. Lett. **108:**127–132.
2. **Albersheim, P., and A. J. Anderson.** 1971. Proteins from plant cell walls inhibit polygalacturonases secreted by plant-pathogens. Proc. Natl. Acad. Sci. USA **68:**1815–1819.
3. **Bannerot, H.** 1965. Résultats de l'infection d'une collection de haricots par six races physiologiques d'anthracnose. Ann. Amelior. Plant. **15:**201–222.
4. **Barras, F., F. van Gijsegem, and A. K. Chatterjee.** 1994. Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. Annu. Rev. Phytopathol. **32:** 201–234.
5. **Barthe, J. P., D. Cantenys, and A. Touzé.** 1981. Purification and characterization of two polygalacturonases secreted by *Colletotrichum lindemuthianum*. Phytopathol. Z. **100:**161–171.
6. **Boudart, G., G. Dechamp-Guillaume, C. Lafitte, G. Ricart, J. P. Barthe, D. Mazau, and M. T. Esquerré-Tugayé.** 1995. Elicitors and suppressors of hydroxyproline-rich glycoprotein accumulation are solubilized from plant cell walls by endopolygalacturonase. Eur. J. Biochem. **232:**449–457.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. **72:**24–254.
8. **Bussink, J. D., F. P. Buxton, B. A. Fraaye, L. de Graaf, and J. Visser.** 1992. The polygalacturonases of *Aspergillus niger* are encoded by a family of diverged genes. Eur. J. Biochem. **208:**83–90.
9. **Caprari, C., R. A. Bergmann, S. Lo Cicero, G. Salvi, F. Cervone, and G. De Lorenzo.** 1993. Cloning and characterization of the gene encoding the endopolygalacturonase of *Fusarium moniliforme*. Mycol. Res. **97:**497–505.
10. **Carpita, N. C., and D. M. Gibeaut.** 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J. **3:**1–30.
11. **Cary, J. W., R. Brown, T. E. Cleveland, M. Whitehead, and R. A. Dean.** 1995. Cloning and characterization of a novel polygalacturonase-encoding gene from *Aspergillus parasiticus*. Gene **153:**129–133.
12. **Centis, S., B. Dumas, J. Fournier, M. Marolda, and M. T. Esquerré-Tugayé.** 1996. Isolation and sequence analysis of *Cplg1*, a gene coding for an endopolygalacturonase of the phytopathogenic fungus *Colletotrichum lindemuthianum*. Gene **170:**125–129.
13. **Cervone, F., G. De Lorenzo, L. Degra, G. Salvi, and M. Bergami.** 1987. Purification and characterization of a polygalacturonase-inhibiting protein from *Phaseolus vulgaris* L. Plant Physiol. **85:**631–637.
14. **Chen, H. J., D. A. Starren, D. Zhou, M. L. Tucker, and K. C. Gross.** 1996. Cloning and characterization of rhamnogalacturonase A from *Botrytis cinerea*, poster J-6. Presented at the 8th International Congress "Molecular Plant Microbe Interactions,", Knoxville, Tenn.
15. **Collmer, A., and N. T. Keen.** 1986. The role of pectic enzymes in plant pathogenesis. Annu. Rev. Phytopathol. **24:**1389–1409.
16. **Collmer, A., D. W. Bauer, S. Y. He, M. Lindeberg, S. Kelemu, P. Rodriguez-Palenzuela, T. J. Burr, and A. K. Chatterjee.** 1991. Pectic enzyme production and bacterial plant pathogenicity. Adv. Mol. Genet. Plant-Microbe Interact. **1:**65–72.
17. **Cooper, R. M., and R. K. S. Wood.** 1975. Regulation of synthesis of cell wall degrading enzymes by *Verticillium albo-atrum* and *Fusarium oxysporum* f.sp. *lycopersici*. Physiol. Plant Pathol. **5:**135–156.
18. **Darvill, A., C. Augur, R. W. Carlson, J. J. Cheong, S. Eberhard, M. G. Hahn, V. M. Lo, V. Marfa, B. Meyer, D. Mohnen, M. A. O'Neil, M. D. Spiro, H. van Halbeek, W. S. York, and P. Albersheim.** 1992. Oligosaccharins: oligosaccharides that regulate growth, development and defence responses in plants. Glycobiology **2:**181–198.
19. **Dean, R. A., and W. E. Timberlake.** 1989. Regulation of the *Aspergillus nidulans* pectate lyase gene (*pelA*). Plant Cell **1:**275–284.
20. **De Lorenzo, G., F. Cervone, D. Bellincampi, C. Caprari, A. J. Clark, A. Desiderio, A. Devoto, R. Forrest, F. Leckie, and L. Nuss.** 1994. Polygalacturonase, PGIP and oligogalacturonides in cell-cell communication. Biochem. Soc. Trans. **22:**394–397.
21. **English, P. D., A. Maglotin, K. Keegstra, and P. Albersheim.** 1972. A cell-wall degrading endopolygalacturonase secreted by *Colletotrichum lindemuthianum*. Plant Physiol. **49:**293–297.
22. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for labelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
23. **Gonzalez-Candelas, L., and P. R. Kolattukudy.** 1992. Isolation and analysis of a novel inducible pectate lyase gene from the phytopathogenic fungus *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*, mating population VI). J. Bacteriol. **174:**6343–6349.
24. **Gysler, C., J. A. M. Harmsen, H. C. M. Kester, J. Visser, and J. Heim.** 1990. Isolation and structure of the pectin lyase-D encoding gene from *Aspergillus niger*. Gene **89:**101–108.
25. **Haffner, M. H., M. B. Chin, and B. G. Lane.** 1978. Wheat embryo ribonucleases. XII. Formal characterization of terminal and penultimate nucleoside residues at 5′ end of "capped" RNA from imbibing wheat embryos. Can. J. Biochem. **56:**729–733.
26. **Hotchkiss, A. T., and B. Hicks.** 1990. Analysis of oligogalacturonic acids with 50 or fewer residues by high performance anion-exchange chromatography and pulsed amperometric detection. Anal. Biochem. **184:**200–206.
27. **Hugouvieux, V., S. Centis, C. Lafitte, and M. T. Esquerré-Tugayé.** 1995. Characterization of *Colletotrichum lindemuthianum* endopolygalacturonase with molecular probes. C. R. Acad. Sci. (Paris) **318:**113–120.
28. **Keon, J. P. R., G. Waksman, and J. A. Bailey.** 1990. A comparison of the biochemical and physiological properties of polygalacturonase from two races of *Colletotrichum lindemuthianum*. Physiol. Mol. Plant Pathol. **37:**193–206.
29. **Kitamoto, N., T. Kimura, Y. Kito, K. Ohmiya, and N. Tsukagoshi.** 1993. Structural features of a polygalacturonase gene cloned from *Aspergillus oryzae* KBN616. FEMS Microbiol. Lett. **111:**37–42.
30. **Kotoujansky, A.** 1987. Molecular genetics of pathogenesis by soft-rot *Erwinias*. Annu. Rev. Phytopathol. **25:**405–430.
31. **Kulmburg, P., M. Mathieu, C. Dowzer, J. Kelly, and B. Felenbok.** 1993. Specific binding sites in the *alcR* and *alcA* promoters of the ethanol regulon for the CREA repressor mediating carbon catabolite repression in *Aspergillus nidulans*. Mol. Microbiol. **7:**847–857.
32. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
33. **Lafitte, C., J. P. Barthe, J. L. Montillet, and A. Touzé.** 1984. Glycoprotein inhibitors of *Colletotrichum lindemuthianum* endopolygalacturonase in near isogenic lines of *Phaseolus vulgaris* resistant and susceptible to anthracnose. Physiol. Plant Pathol. **25:**39–53.
34. **Lafitte, C., J. P. Barthe, X. Gansel, G. Dechamp-Guillaume, C. Faucher, D. Mazau, and M. T. Esquerré-Tugayé.** 1993. Differential induction by endopolygalacturonase of β-1,3-glucanase in *Phaseolus vulgaris* isolines susceptible and resistant to *Colletotrichum lindemuthianum* race β. Mol. Plant-Microbe Interact. **6:**628–634.
35. **Leone, G., and J. van den Heuvel.** 1987. Regulation by carbohydrates of the sequential *in vitro* production of pectic enzymes by *Botrytis cinerea*. Can. J. Bot. **65:**2133–2141.
36. **MacKenzie, D. A., D. J. Jeenes, N. J. Belshaw, and D. B. Archer.** 1993. Regulation of secreted protein production by filamentous fungi: recent developments and perspectives. J. Gen. Microbiol. **139:**2295–2307.
37. **Mutter, M., I. J. Colquhoun, H. A. Schols, G. Beldman, and G. J. Voragen.** 1996. Rhamnogalacturonase B from *Aspergillus aculeatus* is a rhamnogalacturonan α-L-rhamnopyranosyl-(1-4)-α-D-galactopyranosyluronide lyase. Plant Physiol. **110:**73–77.
38. **Rehnstrom, A. L., S. J. Free, and R. G. Pratt.** 1994. Isolation, characterization and pathogenicity of *Sclerotinia trifoliorum* arabinofuranosidase-deficient mutants. Physiol. Mol. Plant Pathol. **44:**199–206.
39. **Reymond, P., G. Deleage, C. Rascle, and M. Fevre.** 1994. Cloning and sequence analysis of a polygalacturonase-encoding gene from the phytopathogenic fungus *Sclerotinia sclerotiorum*. Gene **146:**233–237.
40. **Rong, L., N. C. Carpita, A. Mort, and S. B. Gelvin.** 1994. Soluble cell wall compounds from carrot roots induce the *picA* and *pgl* loci of *Agrobacterium tumefaciens*. Mol. Plant-Microbe Interact. **7:**6–14.
41. **Ryan, C. A., and E. Farmer.** 1991. Oligosaccharide signals in plants: a current assessment. Annu. Rev. Plant Physiol. Mol. Biol. **42:**651–674.
42. **Ruttkowski, E., R. Labitzke, N. Q. Khanh, F. Löffler, M. Gottshalk, and K. D. Jany.** 1990. Cloning and DNA sequence analysis of a polygalacturonase cDNA from *Aspergillus niger* RH5344. Biochim. Biophys. Acta **1087:** 104–106.
43. **Scott-Graig, J. S., D. G. Panacione, F. Cervone, and J. D. Walton.** 1990. Endopolygalacturonase is not required for pathogenicity of *Cochliobolus carbonum* on maize. Plant Cell **2:**1191–1200.
44. **Somogyi, M.** 1952. Notes on sugar determination. J. Biol. Chem. **195:**19–23.