MPMI Vol. 9, No. 3, 1996, pp. 214–216. Publication no. M-1996-0126-03N.

Research Note

Occurrence among *Phytophthora* Species of a Glycoprotein Eliciting a Hypersensitive Response in Tobacco and Its Relationships with Elicitins

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Received 3 October 1995. Accepted 24 November 1995.

Elicitins and a glycoprotein produced by the H20 strain of *Phytophthora megasperma* are elicitors of the hypersensitive response in tobacco. Structural relationships between elicitins and the glycoprotein were detected by immunoblotting. Like elicitins, the glycoprotein was found to be produced by several *Phytophthora* species, and might contribute to a reduction in their pathogenicity.

Additional keywords: Nicotiana tabacum L. cv. Samsun NN, serology

The oomycete *Phytophthora megasperma* Drechs. H20 (PmH20), a pathogen of Douglas fir and a nonhost of tobacco, secretes α- and β-megaspermins that are α-type and β-type elicitins, respectively (Baillieul et al. 1995). We have shown (Kauffmann et al. 1993; Baillieul et al. 1995) that this strain also secretes a 32-kDa glycoprotein whose infiltration into tobacco leaves at ng levels causes the coordinate expression of defense responses including induction of cell death, differential expression of various defense genes, accumulation of salicylic acid, and induction of systemic acquired resistance as defined by Ross (1961). These responses evoke a hypersensitive response (HR) similar to the typical HR developed by N-gene–containing tobacco plants infected with tobacco mosaic virus (TMV). Elicitins are a family of 10-kDa holoproteins produced by many species of *Phytophthora*. They were shown to induce general defense-related responses when applied to tobacco plants and tobacco cell cultures (reviewed by Yu 1995). Two structural classes of elicitins can be distinguished by their isoelectric point and their biological effects after application to decapitated stems or petioles: α-type elicitins are acidic proteins whereas β-type are basic, and α-elicitins have to be applied at a 50- to 100-fold higher concentration than β-elicitins to induce comparable necrotic symptoms (Nespoulos et al. 1992). Recently, elicitins secreted by the fungus *Pythium vexans* were shown to carry an oligosaccharide moiety (Huet et al. 1995). They exhibit a molecular mass of 15 kDa when determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and ranging from 12,693 to 13,260 Da when determined by mass spectrometry.

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We show here that the PmH20 glycoprotein and the α- and β-megaspermins display similar biological activity if they are infiltrated into tobacco (*Nicotiana tabacum* L. cv. Samsun NN) leaves. Treatment with a 60 nM solution of each purified protein caused tissue necrosis after 24 h (Fig. 1A). Under UV light the cells surrounding the three necrotic lesions exhibited comparable epifluorescence (Fig. 1B), due to the accumulation of compounds derived from the phenylpropanoid pathway. Similar symptoms occur on tobacco leaves during the HR to TMV (inset of Figure 1). Thus, although the apparent molecular mass and the presence of an oligosaccharide moiety clearly distinguish the glycoprotein from the megaspermins, they exhibit identical biological activities in the infiltration assay, suggesting possible structural relationships. This was investigated with an immunochemical approach that was extended to search for the occurrence in the culture filtrate of various *Phytophthora* species of homologs of the PmH20 glycoprotein.

The two isoforms of megaspermin and the glycoprotein were purified according to Baillieul et al. (1995), and used to raise specific rabbit antibodies following the procedure described elsewhere (Geoffroy et al. 1990). The three purified elicitor proteins were submitted to SDS-PAGE and revealed by silver staining (Fig. 2A) or immunodetection according to the method described by Geoffroy et al. (1990). The glycoprotein antiserum failed to cross-react with both α- and β-megaspermins (Fig. 2B), indicating a strict specificity to the homolog antigen. Similarly, the β-megaspermin antiserum only recognized the β-megaspermin antigen (Fig. 2D). Ricci and co-workers (1992) reported that an antiserum raised against the β-type elicitin cryptogein cross-reacted in immunoblotting with the α-type elicitin parasiticein. Whereas our antibodies directed against the β-type elicitin β-megaspermin were able to cross-react with cryptogein (Fig. 3B, lane cryptogea), they failed to recognize parasiticein (Fig. 3B, lane parasitica) as well as other α-type elicitins (Fig. 3B, lanes capsici and nicotianae 3034). The α-megaspermin antiserum immunodetected the three antigens (Fig. 2C), indicating that common epitopes have been revealed with these antibodies. The glycoprotein elicitor may, therefore, share with

elicitins common structural features that were recognized only by the α-megaspermin antiserum. Further evidence should be obtained through the determination of the primary structure of the glycoprotein. The antibodies produced against α-megaspermin also cross-reacted with α and β elicitins produced by other species of *Phytophthora* (Fig. 3B).

We tested whether a protein homolog to the PmH20 glycoprotein would be also produced by other *Phytophthora* species. Protein extracts from the culture filtrates of *P. cryptogea* Pethybr. & Lafferty, *P. cinnamomi* Rands, *P. sojae, P. capsici* Leonian, *P. parasitica*, and *P. parasitica* var. *nicotianae* isolate 3034 (Ppn3034) and isolate A1 (PpnA1) were analyzed by SDS-PAGE followed by silver staining (Fig. 3A) or immunoblotting (Fig. 3B). Elicitins were present in all culture filtrates except in the PpnA1 filtrate (Fig. 3). Under these conditions, the β-megaspermin antiserum cross-reacted only with the β-megaspermin antigen and slightly with another β-elicitin, cryptogein (Fig. 3B). In the crude extracts, the α-megaspermin serum immunodetected α-type elicitins and β-type elicitins (Fig. 3B), as well as the glycoprotein homologs together with very few faint bands. The PmH20 glycoprotein displayed a typical yellow-brown color after silver staining, which clearly distinguished it from other proteins on the gel. A protein of similar color and electrophoretic mobility was observed in the protein extracts obtained from all *Phytophthora* species tested except *P. sojae* (Fig. 3A). Antibodies directed against the PmH20 glycoprotein immunoreacted only with this latter protein and with a protein of slightly lower electrophoretic mobility (Fig. 3B). Homologs of the 32-kDa glycoprotein are, thus, produced by several *Phytophthora* species except *P. sojae*. The procedure of glycoprotein purification applied to the culture filtrate of *P. sojae* confirmed that this fungus does not produce any glycoprotein homolog (data not shown). PpnA1, which is pathogenic on tobacco, produced a homolog of the PmH20 glycoprotein, although in much lower amount than that present in the culture filtrate of PmH20. Infiltration into tobacco leaves of the protein extract issuing from the culture filtrate of this isolate, which does not synthesize elicitins, led to HR-like symptoms similar to those produced by infiltration of the purified PmH20 glycoprotein (data not shown), suggesting that the PpnA1 glycoprotein homolog is biologically active. Some *P. parasitica* var. *nicotianae* isolates, the causal agents of tobacco black shank, were found to produce elicitins (Bonnet et al. 1994; Kamoun et al. 1994), for instance the isolate Ppn3034. These isolates, however, exhibited lower virulence on tobacco than the isolates that did not produce elicitins. Furthermore, elicitins purified from these tobacco isolates

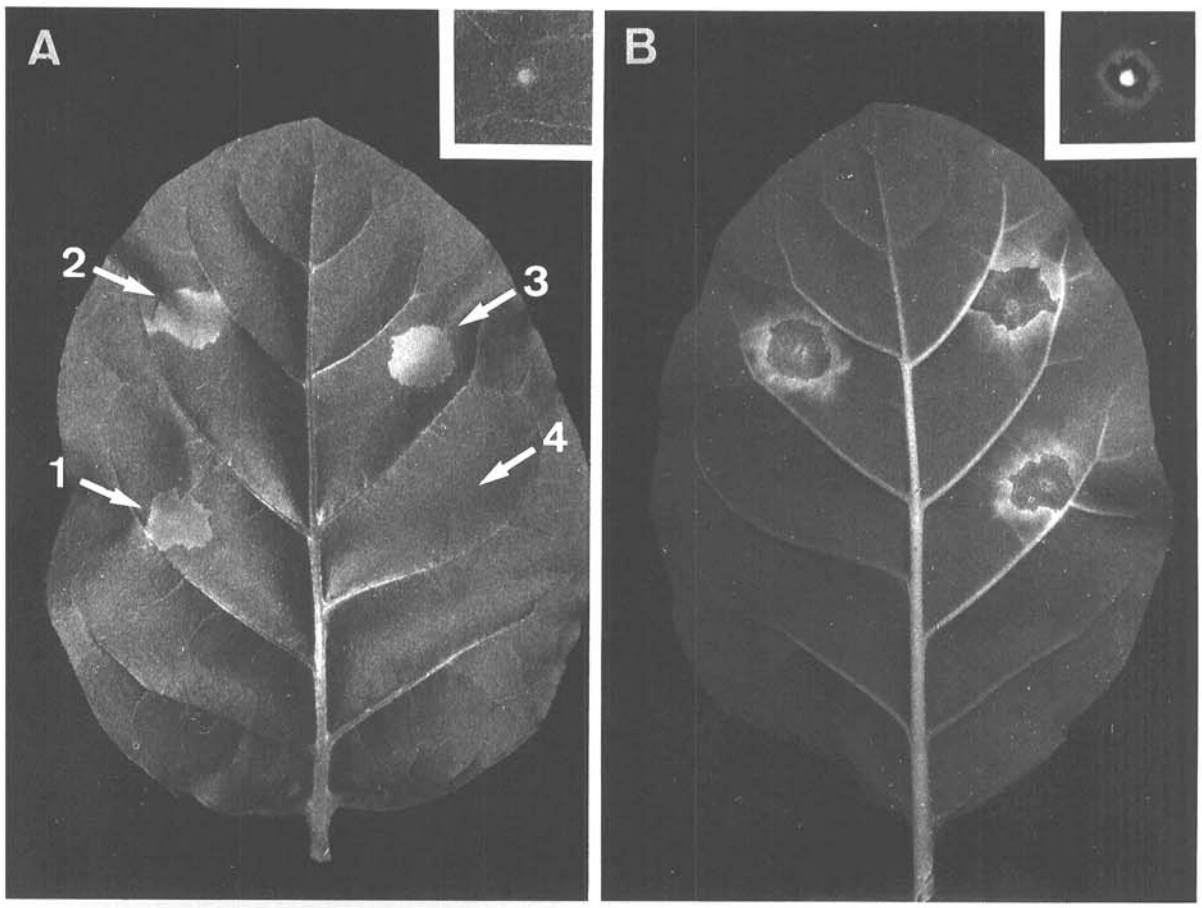

Fig. 1. Symptoms induced on a tobacco leaf by the glycoprotein and α and β isoforms of megaspermin isolated from *Phytophthora megasperma* H20. The tobacco leaf was infiltrated with 60 nM of the purified proteins (1: β-megaspermin; 2: α–megaspermin; 3: glycoprotein) or with water (4), or inoculated with tobacco mosaic virus (insets). Symptoms were observed 3 days after elicitor and water treatment or 7 days after virus inoculation. **A,** leaf photographed under white light. **B,** as **A** but viewed under UV light.

displayed the same elicitor activity as elicitins purified from non-tobacco isolates (Mouton-Perronnet et al. 1995). Elicitins were, thus, associated with a reduction of virulence of *Phytophthora* interacting with tobacco. Since an HR-inducing glycoprotein homolog was found in the culture filtrate of PpnA1, which also shows reduced virulence on tobacco, this elicitor could, thus, function similarly to elicitins.

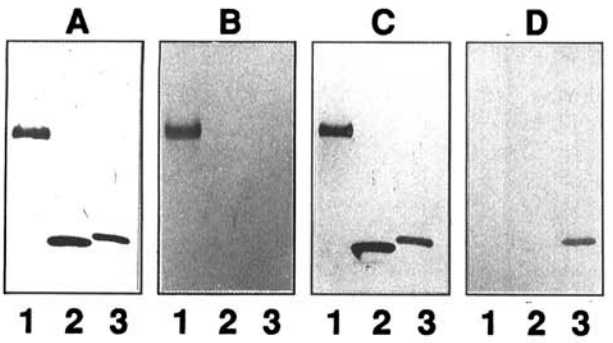

Fig. 2. Serological relationships between the *Phytophthora megasperma* H20 glycoprotein and α and β isoforms of megaspermin. 300 ng of the glycoprotein (lanes 1), of α-megaspermin (lanes 2), and of β-megaspermin (lanes 3) were submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with silver nitrate (**A**), or transferred onto nitrocellulose sheets and assayed for immunodetection with antisera raised against the glycoprotein (**B**), α-megaspermin (**C**), and β-megaspermin (**D**).

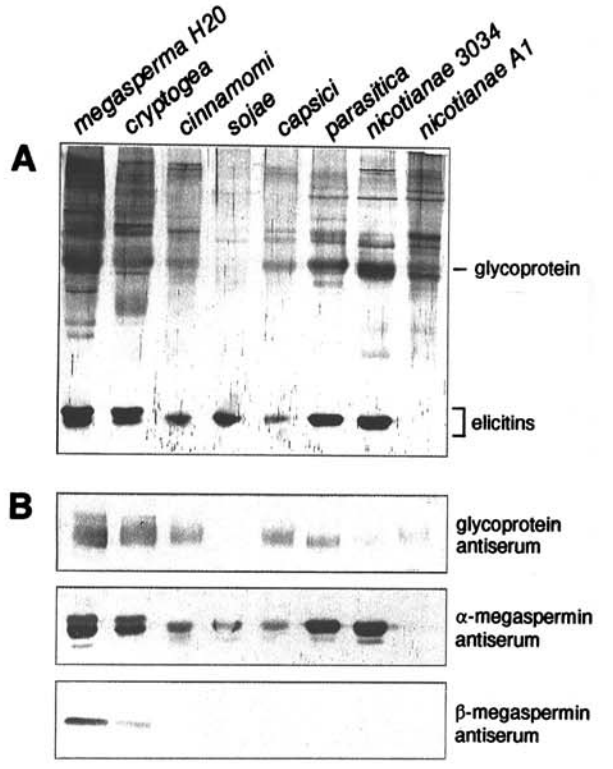

Fig. 3. Occurrence among different species of *Phytophthora* of homologs to the glycoprotein. 8 μg of protein issuing from culture filtrates of different species (indicated on top of the panel) were submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by staining with silver nitrate (**A**), or by transfer onto nitrocellulose sheets and incubation with antisera raised against the glycoprotein, α-megaspermin, and β-megaspermin, as indicated in **B**.

Results of the present study and of Baillieul et al. (1995) indicate that *Phytophthora* species secrete two classes of proteins inducing HR on tobacco plants. One class is composed of elicitins that are highly conserved 10-kDa holoproteins, and the second class contains glycoproteins of apparent molecular mass of 32 kDa. While some *Phytophthora* species produce both classes of elicitor, other species secrete either elicitins or the glycoprotein. The two classes of elicitors are related by some structural features revealed only by antibodies directed against an α-type elicitin. Determination of the amino acid sequence of the PmH20 glycoprotein is in progress and should provide further insight into the structural relationships between these two classes of elicitors.

ACKNOWLEDGMENTS

The authors are particularly thankful to Pierrette Geoffroy for skillful technical assistance. *Phytophthora megasperma* H20 was obtained from M. G. Hahn. *Phytophthora cryptogea*, *P. cinnamomi*, *P. capsici*, *P. parasitica*, and *P. nicotianae* 3034 were obtained from G. Bompeix and P. Saindrenan. *Phytophthora sojae* was obtained from J Ebel. *Phytophthora nicotianae* A1 was obtained from J.-C. Pernollet. The authors are thankful for all these kind gifts. This research was supported in part by grant no. 92-T-0200 from the French Ministry of Research.

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