

A new elicitor of the hypersensitive response in tobacco: a fungal glycoprotein elicits cell death, expression of defence genes, production of salicylic acid, and induction of systemic acquired resistance

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Summary

A 32 kDa glycoprotein whose effects in tobacco and other Nicotianae mimic a typical hypersensitive response, was isolated from *Phytophthora megasperma*. Infiltration of a few nanograms of the protein into leaves caused the formation of lesions that closely resemble hypersensitive response lesions. Transcripts of genes encoding enzymes of the phenylpropanoid and sesquiterpenoid pathways accumulated rapidly after elicitor application followed by salicylic acid production. Cellular damage, restricted to the infiltrated zone, occurred only several hours later, at a time when expression of PR protein genes was activated. After several days systemic acquired resistance was also induced. Thus, tobacco plant cells that perceived the glycoprotein generated a cascade of signals acting at local, short, and long distances, and causing the coordinate expression of specific defence responses in a way similar to hypersensitivity to tobacco mosaic virus. The glycoprotein represents a powerful tool to investigate further the signals and their transduction pathways involved in induced disease resistance. It may also be useful to engineer broad disease protection in a Nicotianae and possibly into crop plant species.

Introduction

One of the most efficient mechanisms of induced disease resistance in plants is the hypersensitive response (HR) whose induction results from a specific recognition event occurring between a component of the host and a component of the pathogen (Keen, 1990). HR is a multifaceted defence mechanism active against viruses, fungi and bacteria (Klement, 1982). Rapid and localized death of a few plant cells at the site of attempted penetration by the pathogen is the early macroscopic event of HR. This early

cell death has often been emphasized as a mechanism of inhibition of pathogen development.

Activation of defence genes and production of antimicrobial substances also occur. For instance, in tobacco plants reacting hypersensitively to TMV, synthesis of a large array of defence proteins including the PR proteins is induced (Bowles, 1990; Stintzi *et al.*, 1993). A strong stimulation of secondary metabolisms like the phenylpropanoid and sesquiterpenoid metabolisms also occurs (Nicholson and Hammerschmidt, 1992). They provide the plant cell with precursors of cell wall barriers, that is, lignin, and molecules with antimicrobial activity, like the phytoalexins. So far, it is not well understood which of these changes is responsible, *per se*, for the restriction of an invading pathogen. Rather it is their superimposition and their synergistic effects that contribute to the confinement of the pathogen.

During HR, endogenous signals are also produced. Each of them probably has the potential to regulate the expression of a given set of defence responses. Salicylic acid (SA) has been implicated as one of these signals (reviewed by Malamy and Klessig, 1992, and Raskin, 1992). There is compelling evidence that SA is a key signal molecule involved in the phenomenon of systemic acquired resistance, SAR (Gaffney *et al.*, 1993), which develops subsequent to local HR in uninfected parts of the plant and provides resistance to further infection by pathogens (Ross, 1961). Ross also indicated that SAR was progressively induced if necrotic lesions were formed by pathogen infection and not if necrotizing chemicals, such as salts, were applied to the plants. Recently, it was also postulated that SA plays a central role in the induction of general disease resistance (Delaney *et al.*, 1994).

The host signal molecules and transduction pathways involved in HR development are still not fully understood. The dissection of such a cascade is difficult in the biological system of an intricate plant-pathogen interaction. A simplified system in which plant cells would be synchronously challenged with a single molecular inducer of HR, should greatly facilitate the study of HR development. Elicitors, which can be microbe- or plant-derived molecules, have therefore been sought and used as tools to study these molecular events linking initial perception of a pathogen and expression of plant defence responses.

A wide variety of elicitors have been shown to trigger some of the defence responses. However, most of them

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do not cause plant cell death and thus, have been called non-HR elicitors (Atkinson, 1993). Some have been characterized as oligosaccharides (Darvill *et al.*, 1992; Ryan and Farmer, 1991). Other are proteins (Ricci *et al.*, 1993) and glycoproteins (Anderson, 1989). A glycoprotein of molecular mass 46 kDa and isolated from *Phytophthora nicotianae* was shown to elicit phytoalexin accumulation in tobacco callus (Farmer and Helgeson, 1987). A 42 kDa glycoprotein purified from *P. megasperma* f.sp. *glycinea* was shown to stimulate various defence responses in cultured parsley cells including ion fluxes, oxidative burst, expression of defence-related genes and phytoalexin accumulation (Nürberger *et al.*, 1994). However, parsley leaves did not respond to the pure glycoprotein (Parker *et al.*, 1991).

Although compounds of pathogen origin have been reported to cause plant tissue necrosis and also, in some instances, to induce various plant responses, these biological activities do not evoke HR necessarily. For instance, necrosis-inducing peptides secreted by the fungus *Rhynchosporium secalis* have been shown to display such activities which were not associated with HR (Hahn *et al.*, 1993; Wevelslep *et al.*, 1991). A protein of molecular mass 60 kDa isolated from the bacteria *Pseudomonas solanacearum* was described as an inducer of the hypersensitive response in potato based on its capacity to elicit callus browning when applied at very low concentrations (Huang *et al.*, 1989). Harpins and PopA1 are proteins isolated from *Pseudomonas* species which induce necrotic zones when infiltrated into tobacco leaves, similar to the hypersensitive necrosis induced by the incompatible bacteria from which they are secreted (Arlat *et al.*, 1994; He *et al.*, 1993). Race-specific peptides encoded by the avirulence genes *Avr9* and *Avr4* of *Cladosporium fulvum* elicit necrosis and PR protein accumulation in tomato plants containing the corresponding resistance genes *Cf9* and *Cf4* (Honée *et al.*, 1994). Elicitins are small extracellular holoproteins produced by many species of *Phytophthora*. Upon application to tobacco plants they were shown to induce tissue necrosis (Billard *et al.*, 1988; Ricci *et al.*, 1989), as well as production of ethylene and the phytoalexin capsidiol (Milat *et al.*, 1991). Protein phosphorylation (Viard *et al.*, 1994), and increase in extracellular pH and conductivity (Blein *et al.*, 1991) were measured when elicitors were applied to suspension-cultured cells.

In this report, we describe the isolation and characterization of a new proteinaceous elicitor from the culture filtrate of *P. megasperma* which is distinct from elicitors, and whose biological effects on tobacco induce the following responses of a typical HR: (i) induction of rapid plant cell death; (ii) induction of defence responses; (iii) production of endogenous signal(s) that are known to participate in the triggering of defence responses; and (iv) subsequent appearance of systemic acquired resistance. This elicitor may have a potential as a tool to engineer disease

resistance against a broad spectrum of pathogens by manipulating the HR in transgenic tobacco plants.

Results

Purification from P. megasperma of α and β elicitors and a 32 kDa protein with necrosis-inducing activity on tobacco leaves

The concentrated and desalting medium of a 4-week-old culture of *P. megasperma* H20 was fractionated by anion-exchange chromatography. Necrosis-inducing activity was found bound to the column and in the flow through as well, indicating the presence of distinct acidic and basic active molecules.

Active fractions eluted from the anion-exchange column after application of a salt gradient (data not shown), were subjected to hydrophobic interaction chromatography. The elution profile is shown in Figure 1(a). Necrosis-inducing activity was resolved as two major peaks of proteins (compare Figure 1a and c). SDS-PAGE analysis followed by silver nitrate staining (Figure 1b) was performed on the protein peaks as designated in Figure 1(a). Analysis of the second peak of activity (labelled 5 in Figure 1a) revealed a major band corresponding to an acidic elicitor, called α -megaspermin, together with a few contaminants present in minute amounts (Figure 1b, lane 5). The first peak of necrosis-inducing activity (protein peak labelled 3 in Figure 1a) contained two major proteins (Figure 1b, lane 3) clearly distinct from α -megaspermin. The major proteins of fractions 3 and 5 were further purified by molecular sieving chromatography, and homogeneous α -megaspermin and a protein of molecular mass of 32 kDa were obtained (Figure 2a, lanes 1 and 2). Both proteins had a high capacity to induce necrosis on tobacco leaves. The 32 kDa protein appeared as a slightly broader band after SDS-PAGE and silver nitrate staining suggesting the presence of an oligosaccharide moiety.

The unbound protein fraction with necrosis-inducing activity from the first chromatographic step on Q-Sepharose was subjected to cation-exchange chromatography. All necrosis-inducing activity bound to the column and was eluted as a single peak. Active fractions were pooled and further purified by hydrophobic interaction chromatography. At this step of the purification, necrosis-inducing activity was associated with a single silver-stained band detected after SDS-PAGE (Figure 2a, lane 3). This product is a basic elicitor, called β -megaspermin. The two elicitor isoforms have slightly different electrophoretic mobilities (compare lanes 2 and 3 of Figure 2a) indicating slightly different molecular masses in the range of 10 kDa.

Detection of oligosaccharide moieties

The three purified proteins were electrophoresed under denaturing conditions (Figure 2a) and were blotted on to

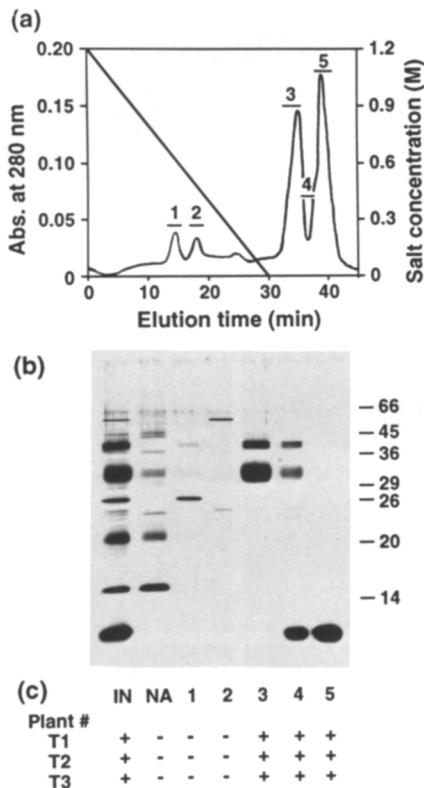


Figure 1. Separation of different *P. megasperma* acidic proteins with necrosis-inducing activity in tobacco.

(a) Elution profile upon hydrophobic interaction chromatography on a FPLC Phenyl-Superose column. Three milligrams of proteins present in the active fraction issuing from anion-exchange chromatography were loaded on to the column. Column fractions analyzed further are marked 1–5.
 (b) SDS-PAGE analysis. Lane IN, aliquot of initial fraction loaded on to the column; lane NA, unbound fraction; lanes 1–5, pooled fractions of protein peaks as designated in (a). Proteins were stained with silver nitrate. Molecular weight ($\times 10^{-3}$) of standard proteins is indicated.

(c) Necrosis-inducing activity of the same fractions as those analysed by SDS-PAGE in (b). A 5000-fold dilution in water of each fraction was infiltrated into leaves of tobacco plants. One leaf from each of three plants, labelled T1, T2, T3, was treated. All fractions, in addition to the control sample containing only water, were infiltrated into the same leaf of each plant. The induction of necrotic symptoms was scored as + or -, that is, occurrence or absence, after 24 h of incubation.

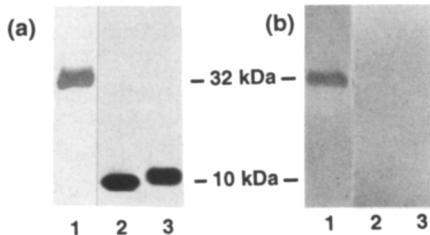


Figure 2. Analysis of the purified necrosis-inducing proteins.

(a) SDS-PAGE analysis. Lane 1, 32 kDa protein; lane 2, α -megaspermin; lane 3, β -megaspermin. Proteins were stained with silver nitrate. The apparent molecular mass of each protein is indicated.
 (b) Detection of oligosaccharide moieties. After SDS-PAGE, purified proteins were blotted onto nitrocellulose. Oligosaccharidic side-chains were detected with the method described in Experimental procedures. Lanes 1, 2, 3, as in (a).

a nitro-cellulose membrane. Detection of oligosaccharide moieties was carried out *in situ* by specific periodate oxidation of the sugar residues followed by biotin hydrazide labelling. Proteins carrying covalently linked biotin were revealed by adding extravidin conjugated to alkaline phosphatase. The α - and β -megaspermin, like previously characterized elicitors (Ricci *et al.*, 1989), were devoid of oligosaccharide side-chains. However, the results presented in Figure 2(b) clearly indicate that the 32 kDa protein contains a sugar moiety.

The molecular mass and glycosidic side-chains clearly distinguish the 32 kDa protein from elicitors. Similar proteins, to our knowledge, have not been reported in *Phytophthora* species. Therefore, we further investigated the biological activity of this new elicitor of necrosis, in particular whether the elicitor-induced tissue necrosis corresponded to the macroscopic symptoms of a typical HR which is accompanied by production of endogenous signals, like salicylic acid, induction of several defence genes, accumulation of defence proteins, like PR proteins, and by subsequent induction of systemic acquired resistance.

Characterization of the biological activity of the 32 kDa glycoprotein

Infiltration of the purified 32 kDa glycoprotein into the mesophyll of tobacco leaves resulted in rapid macroscopic changes. The first symptoms appeared 1–3 h after the treatment. The tissue became slightly bright, then became necrotic about 20 h post-infiltration. Browning and complete dryness were observed after about 24–30 h of incubation (Figure 3a). The induced necrosis was not found to expand the infiltrated zone, even when high concentrations of the glycoprotein were applied, suggesting that the elicitor remained at the site of infiltration. Further experiments with radiolabelled, active glycoprotein (data not shown) revealed only trace amounts of radioactivity 1–2 mm beyond the infiltrated area. The glycoprotein was highly active since nanomolar concentrations were sufficient to induce necrotic symptoms. For subsequent studies, a concentration of 50–60 nM was routinely used.

Reduction followed by alkylation of the glycoprotein resulted in a complete loss of the biological activity, suggesting that the necrosis-inducing activity was borne by the polypeptide chain and not by the oligosaccharide moiety or a polysaccharide contaminant. It also indicated that the native conformation was essential for the activity of the glycoprotein. A similar observation was reported for elicitors (Ricci *et al.*, 1989).

The glycoprotein also induced necrotic symptoms when infiltrated in leaves of other tobacco cultivars such as cv. Samsun, cv. Xanthi nc, cv. Petite Havana SR1, and other

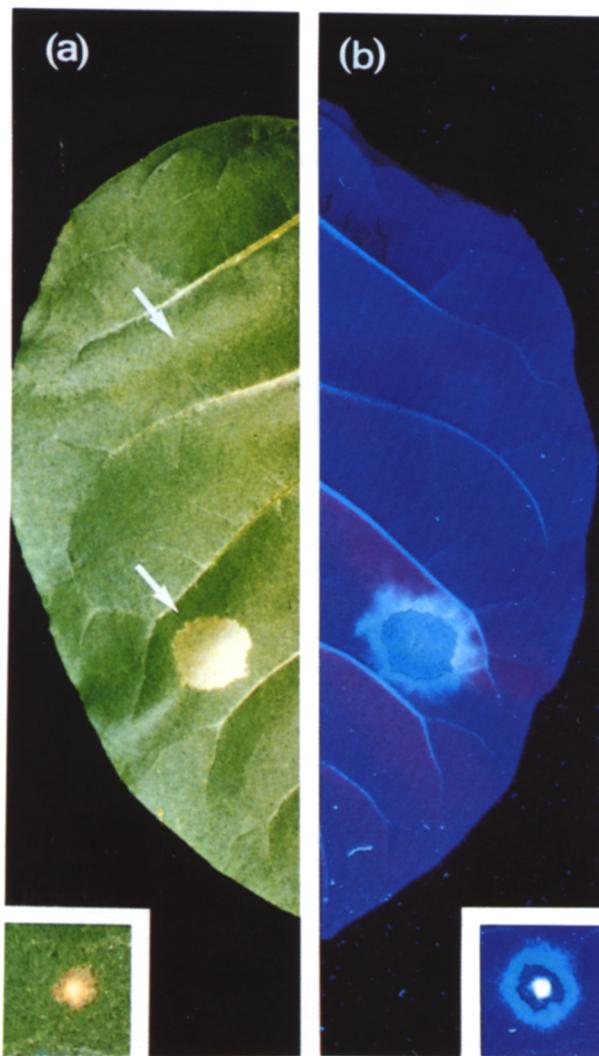


Figure 3. Symptoms induced on tobacco leaves by the 32 kDa glycoprotein. Tobacco leaves were infiltrated with the protein (bottom arrow) at a concentration of 60 nM, or with water (top arrow), or inoculated with tobacco mosaic virus. Symptoms were observed 3 days after elicitor and water treatment or 7 days after virus inoculation.
 (a) Leaf was photographed under white light. The inset shows a TMV lesion.
 (b) As (a) but viewed under UV light.

Nicotianae species such as *N. sylvestris* and *N. glutinosa* (data not shown).

Observation under UV light of tobacco leaves infiltrated with the 32 kDa glycoprotein revealed a strong epifluorescence located in cells surrounding the necrotic lesion (Figure 3b). Similar symptoms occur on tobacco leaves during the hypersensitive reaction to TMV (inset of Figure 3b). The epifluorescence is due to the accumulation of compounds derived from the phenylpropanoid pathway which is highly stimulated during HR (Fritig *et al.*, 1972). Moreover, cells surrounding viral necrotic lesions undergo considerable alteration in gene expression. Defence-related genes, such as PR proteins genes, are preferentially expressed (Fritig *et al.*, 1987; Heitz *et al.*, 1994a).

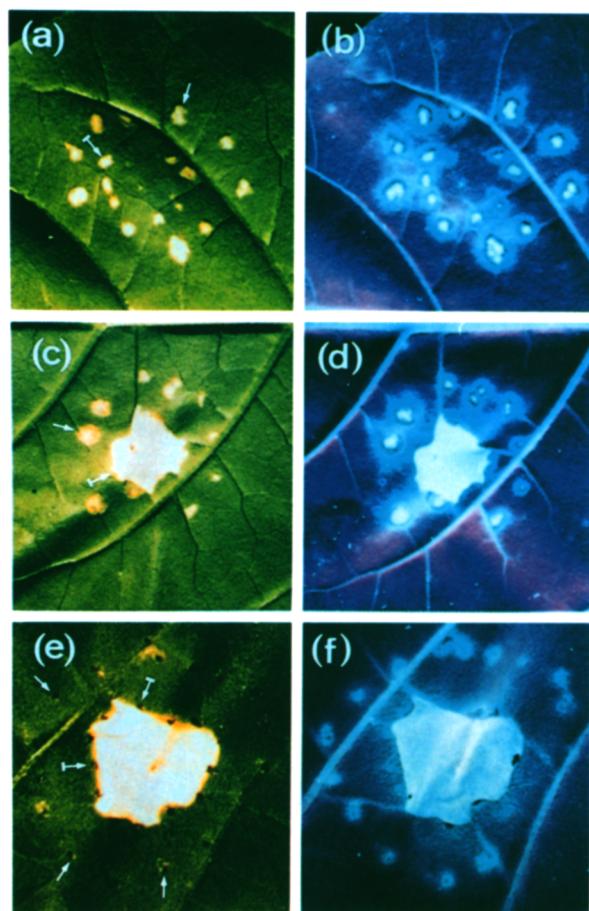


Figure 4. Antiviral resistance induced in tobacco leaves after infiltration with the fungal glycoprotein.

Tobacco leaves were infiltrated with the protein at a concentration of 50 nM, or with water. Subsequently, TMV was micro-inoculated (arrows). Virus lesions were observed under white light (a), (c), (e), or UV light (b), (d), (f), 7 days after the challenge inoculation with the virus. Virus inoculation was performed 14 days after water infiltration (a and b), 2 days (c and d) or 14 days (e and f) after infiltration with the glycoprotein.

Induction of systemic acquired resistance

We tested whether infiltration of the glycoprotein into tobacco leaves would result in the establishment of systemic acquired resistance (SAR) in tissue free of glycoprotein and at a distance from the infiltrated site. Samsun NN leaves were infiltrated with the glycoprotein and TMV was subsequently micro-inoculated. When inoculation was performed 2 days after elicitor treatment, viral lesions observed 7 days later (Figure 4c and d) were of the same size as those found on control leaves infiltrated with water (Figure 4a and b). Inoculation of virus 14 days after infiltration of the glycoprotein, resulted in the formation of almost invisible lesions (Figure 4e). The presence of TMV lesions was detected when the leaf was observed under UV light (Figure 4f), thus indicating a high level of acquired antiviral resistance. Smaller lesions were also observed when virus was inoculated in the upper non-elicitor-treated leaf (data not shown).

Ross (1961) reported that no gradient in the size of TMV lesions was evident when the challenge inoculation was performed at short or long distances from the first inoculation sites, and that in tobacco leaves expressing SAR, many local infections never progressed to enough cells to result in visible necrosis. These lesions were revealed only if the leaf was observed under UV light. The primary action of SAR, thus, appeared to be on lesion size and not on the establishment of infection. In conclusion, our results indicate that infiltration of tobacco plants with the glycoprotein resulted in the establishment of SAR.

The similarity of induced symptoms between HR to TMV and the response to the fungal glycoprotein (Figure 3), and induction by the glycoprotein of acquired resistance against a challenge infection with TMV (Figure 4), suggested that treatment with the glycoprotein somehow mimicked a typical HR.

Accumulation of pathogenesis-related proteins

Tobacco leaves were infiltrated with the 32 kDa glycoprotein at a concentration of 60 nM or with water in order to obtain several treated zones (1–2 cm²) per leaf. At various times after infiltration, leaves were divided into four equal parts which were used separately to extract PR protein, total RNA, salicylic acid, or to visualize necrosis and epifluorescence.

PR proteins were analysed by SDS-PAGE followed by immunoblotting. The different sera used have been shown previously to be specific to a given family of PR proteins. Anti-PR-1 recognizes the three acidic members of the PR-1 family (Heitz *et al.*, 1994a) shown recently, along with their basic counterpart, to have antifungal activity *in vitro* (Niderman *et al.*, 1995). Anti-PR-2 detects 1,3-β-glucanases of classes I and II (Kauffmann *et al.*, 1987), anti-PR-3 detects chitinase-lysozymes of classes I and II (Legrand *et al.*, 1987), anti-PR-5 detects the acidic and basic members of the thaumatin-like PR proteins (Kauffmann *et al.*, 1990; Stintzi *et al.*, 1991), and anti-PR-11 detects a new class of chitinase-lysozymes (Heitz *et al.*, 1994b). A separate electrophoretic analysis was performed (Figure 5b) in order to detect PR-6, an inhibitor of microbial proteases of much lower molecular weight (Geoffroy *et al.*, 1990).

Figure 5 shows that members of the different PR-families assayed, that is the PR-1, PR-2, PR-3, PR-5, PR-6, and PR-11 families, accumulated in high amounts in tobacco leaves upon treatment with the glycoprotein (Elicitor lanes). In control leaves, that is non-treated leaves and leaves infiltrated with water, PR proteins were hardly detectable, except some members of the PR-2 and PR-3 families, although in minute amounts (NT and Water lanes). These particular proteins, most probably, corresponded to the basic isoforms which have been shown to occur in small amounts in fully developed healthy leaves (Heitz *et al.*,

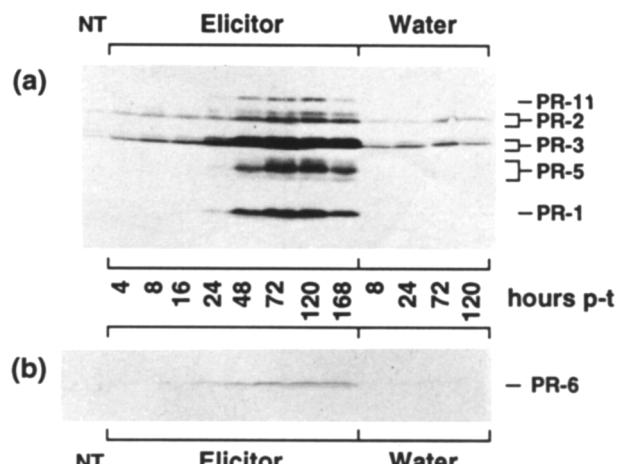


Figure 5. Kinetics of pathogenesis-related protein accumulation in tobacco leaves infiltrated with the fungal glycoprotein.

Leaves were infiltrated at several discrete spots with the protein at a 60 nM concentration or with water. Proteins of leaf tissue (1 g) from treated and non-treated plants (lane NT) were extracted at various indicated times after treatment, electrophoresed under denaturing conditions, blotted onto nitro-cellulose. Immunodetection was performed with a mixture of antibodies raised against PR-1, PR-2, PR-3, PR-5, and PR-11 (a) or with an antiserum specific for PR-6 (b).

1994a). Conditions used in Figure 5 do not distinguish members within a given family. Thus, we also performed electrophoresis of some of the samples under native conditions which resolved the different acidic members of families PR-1, -2, -3, and -5. We observed (data not shown) that the 10 acidic PR proteins typically induced by TMV infection were also induced in the glycoprotein-treated leaf tissue, namely, PR-1a, -1b, -1c, -2a, -2b, -2c, -3a, -3b, -5a, and -5b. The combined data obtained under denaturing and non-denaturing conditions clearly indicated a close parallelism in the qualitative pattern of induced PR proteins between the response to the 32 kDa glycoprotein and HR to TMV.

In leaves treated with the fungal glycoprotein, PR proteins were first detected at 24 h post-infiltration (Figure 5) which also corresponded to the time point when the induced necrosis was well established. PR protein accumulation was maximum at 3 days and remained high 7 days after the infiltration with the glycoprotein. These results indicated that PR proteins of the different families exhibited very similar kinetics of accumulation in tobacco leaves, thus suggesting a correlated expression of their corresponding genes in response to the treatment with the fungal glycoprotein.

Expression of defence-related genes

Accumulation of defence-related mRNAs was analysed by Northern hybridization. The cDNAs used as probes were both PR protein genes and other defence-related genes

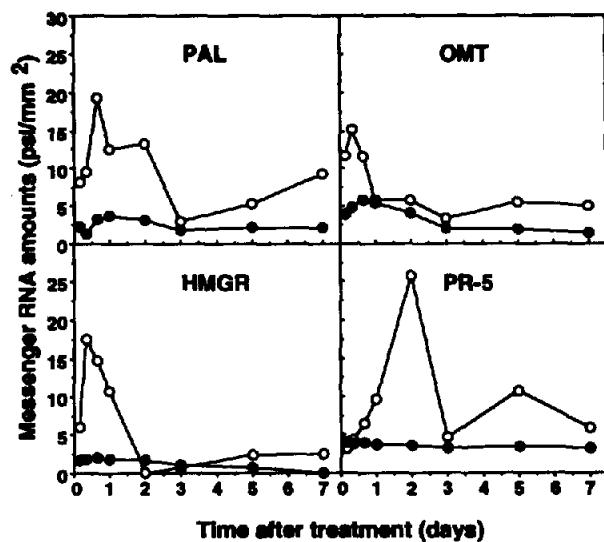


Figure 6. Kinetics of defence-related mRNA accumulation in tobacco leaves infiltrated with the fungal glycoprotein.

Tobacco leaves were treated as indicated in Figure 5 and total RNA was extracted. Twelve micrograms of RNA was electrophoresed and blotted on to a nylon membrane. The blot was hybridized with the different ^{32}P -labelled cDNA probes indicated in the figure, and radioactivity quantified as described in Experimental procedures. Closed symbols refer to RNA from water-infiltrated leaves, and open symbols to RNA from glycoprotein-infiltrated leaves.

known to be activated during HR to TMV infection. The selected probes are specific to:

- (i) two enzymes of the phenylpropanoid pathway, phenylalanine ammonia-lyase, PAL (Pellegrini et al., 1994) and O-methyltransferase, OMT (Pellegrini et al., 1993);
- (ii) hydroxymethylglutarylCoA reductase, HMGR (Genschick et al., 1992) a key enzyme of the sesquiterpenoid pathway leading to the synthesis of capsidiol known as the major phytoalexin produced by tobacco;
- (iii) PR-5a protein (Brederode et al., 1991) which was chosen as a typical member of the PR proteins of tobacco according to the results of the immunoblotting experiments.

The quantitated steady-state levels of mRNAs are presented in Figure 6. Plants infiltrated with water did not show significant differences in the steady-state levels of the different mRNAs. Messenger RNAs corresponding to PAL, OMT, and HMGR started to accumulate as early as 4 h after the treatment with the glycoprotein. Maximum accumulation was observed at 8–16 h post-infiltration for PAL, OMT and HMGR mRNAs. Afterwards, a decrease in the steady-state levels of these mRNA occurred. The RNA induction pattern for PR-5 gene was shown to be different in that increase in mRNA accumulation started only 16 h post-infiltration with the glycoprotein. The maximum of steady-state level was reached at 48 h and then a decline in mRNA amount was observed.

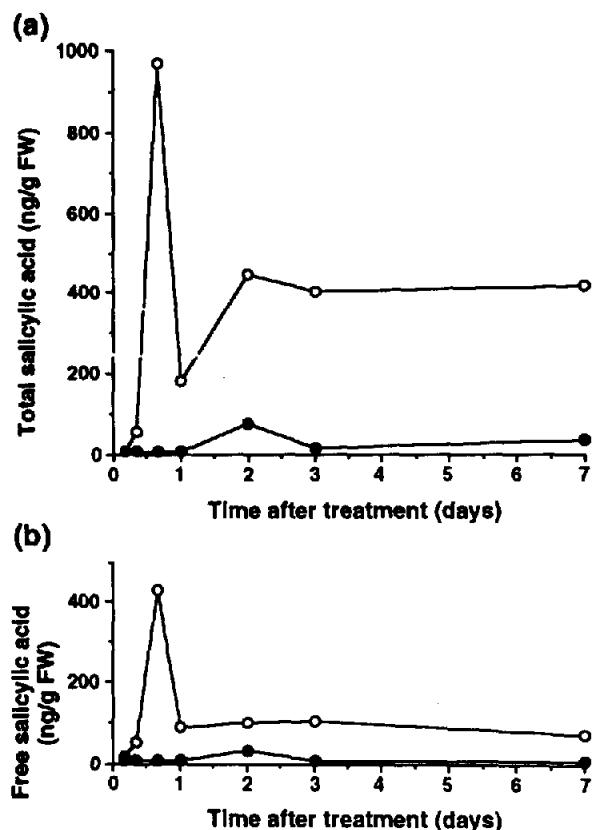


Figure 7. Kinetics of salicylic acid accumulation in tobacco leaves infiltrated with the fungal glycoprotein.

Tobacco leaves were treated as indicated in Figure 5, and SA was extracted and analyzed by HPLC. Since the detection limit of assay was 10 ng SA per gram of fresh weight, we assigned this value to extracts in which no SA was detected. Closed symbols refer to SA from water-infiltrated leaves, and open symbols to SA from glycoprotein-infiltrated leaves. Total (a) and free (b) SA were analysed.

Thus, two classes of genes could be clearly distinguished, depending on their kinetics of expression. Genes of the first class, which comprise PAL, OMT, and HMGR genes, were characterized by a rather rapid activation, preceding visible necrosis, of their expression in leaves upon treatment with the glycoprotein. The second class of genes comprising PR protein genes were characterized by a delayed stimulation of expression in leaves infiltrated with the glycoprotein, occurring at a time when the necrosis of the infiltrated zone was well established.

Production of salicylic acid

SA accumulation was measured in tobacco leaves treated with the 32 kDa glycoprotein (Figure 7, open symbols). Levels of total SA were low during the first 8 h post-infiltration, then increased sharply between 8 and 16 h after the treatment, and then decreased to a constant level. When free SA was analysed, a similar pattern of accumulation was observed, but with significantly lower

levels. In control leaves infiltrated with water (Figure 7, closed symbols) there was no significant accumulation of free or total SA, except for the 48 h time points, but it was much lower than in those corresponding to the glycoprotein-treated samples. Thus, production of SA, another typical feature of the HR, could also be identified as one of the responses of tobacco leaves to the glycoprotein treatment.

Discussion

An extensive analysis of the *P. megasperma* H20 culture filtrate for compounds inducing rapid tissue necrosis after infiltration of tobacco leaves led to the isolation of a 32 kDa glycoprotein and two isoforms of elicitors. The new protein was highly active; a few nanograms were sufficient to induce rapid leaf tissue necrosis. The new elicitor molecule had peculiar biochemical properties which distinguished it clearly from elicitors. It is a glycoprotein of apparent molecular mass of 32 kDa, while elicitors are known as 10 kDa holoproteins. However, as for elicitors (Ricci *et al.*, 1989), the native conformation of the glycoprotein, most probably maintained by disulphide bridge(s), and not the oligosaccharide moiety, is essential for the necrosis-inducing activity.

Rapid plant cell death is one aspect of the defence mechanisms that plants have against incompatible pathogens. They also include the induction of a large set of biochemical defence responses (Fritig *et al.*, 1987; Lamb *et al.*, 1989). The present study demonstrated that the 32 kDa glycoprotein stimulated the transcription of a large variety of defence-related genes including genes encoding enzymes of the phenylpropanoid (PAL and OMT) and sesquiterpenoid (HMGR) pathways and also PR protein genes of the different families. Accumulation of PR proteins was observed. Activity of defence-related enzymes, such as enzymes of the phenylpropanoid pathway was inferred from fluorescence surrounding the elicitor-induced lesions.

Expression of the various biochemical defence responses in plants is known to be coordinately regulated (Graham and Graham, 1991; Lamb *et al.*, 1989). For instance, in tobacco, PAL and OMT gene expression is induced about 24 h after TMV inoculation (Pellegrini *et al.*, 1993, 1994), while PR protein gene transcription starts only 2–3 days post-infection (Brederode *et al.*, 1991; Ward *et al.*, 1991). Similar differential kinetics of gene expression were observed in the present study after treatment with the 32 kDa glycoprotein. Expression of class I genes, including PAL, OMT, and HMGR genes was induced soon after infiltration with the elicitor. Class II genes, comprising PR protein genes, were induced only at a time when the amount of class I transcripts were decreasing, and when the induced necrosis was clearly established. This suggests that application of the purified glycoprotein elicitor led to

the production of endogenous signal molecules responsible for the activation of class II genes, since no glycoprotein was found beyond the infiltrated tissue which was undergoing necrosis.

A large body of evidence indicates that SA is a signal molecule triggering some of the plant defence responses, such as PR protein production (reviewed by Malamy and Klessig, 1992). Application of the 32 kDa glycoprotein resulted in the production of large quantities of SA. Free and conjugated SA were present in a ratio similar to that described in the HR to TMV (Malamy *et al.*, 1992). Interestingly, accumulation of SA occurred between activation of class I and class II genes. It is hypothesized that SA is a substituted benzoic acid resulting from the phenylpropanoid pathway, whose first committed step is catalysed by PAL (Raskin, 1992). Our results on the kinetics of PAL and SA induction agree with this hypothesis. The kinetics of SA and PR protein gene induction also agree with the data of Malamy *et al.* (1992) who have shown that tobacco plants reacting hypersensitively to TMV accumulate SA before expression of PR-1 genes. Whether SA produced upon elicitor treatment is the endogenous signal for PR protein gene induction remains, however, to be determined.

Application of the glycoprotein to tobacco plants also led to induction of SAR as described by Ross (1961) which results from the production and activity of a plant signal moving systemically. Although SA was shown to be a key signal molecule involved in the phenomenon of SAR (Gaffney *et al.*, 1993), it is not the translocated signal responsible for inducing SAR (Vernooy *et al.*, 1994). Therefore, another consequence of infiltration of the glycoprotein into tobacco leaves is the production of such an endogenous signal. The acquired resistance that developed after application of elicitors on the top of decapitated tobacco plants was, in that case, different from SAR in Ross (1961) since it was due to the systemic movement of the fungal elicitor producing therefore a systemic elicitor effect (Keller *et al.*, 1994).

Considered as a whole, the results of the present study strongly suggest that the effect of the 32 kDa glycoprotein on tobacco plants mimics a typical HR which is viewed as rapid plant cell death, coordinate expression of biochemical defence responses under the control of endogenous signal molecules such as SA, and induction of SAR. The glycoprotein may act as an avirulence factor in the *Phytophthora*-tobacco interaction contributing, with elicitors, to the incompatible interaction between these two species. Further studies have shown that similar glycoproteins are produced by various species of *Phytophthora* (manuscript, in preparation).

The glycoprotein represents a molecular tool to understand the role of cell death in limiting a challenging pathogen and in providing the neighbouring cells with

signals inducing defence responses. Since it can be applied in a synchronous manner to many cells at a predetermined zone of leaf tissue (in contrast to what happens with elicitors released by a whole infecting micro-organism), an accurate spatiotemporal analysis of signals and defence responses is possible in the elicitor-treated zone, its immediate vicinity, and in more distant zones undergoing systemic acquired resistance. Such a system also makes it possible to assay accurately the antimicrobial potential, *in vivo*, of these clearly delimited zones in challenge infections with various tobacco pathogens. Finally, cloning of the sequences coding for the fungal elicitor and their expression in transgenic plants under the control of promoters inducible by compatible pathogens could lead to manipulation of the HR and confer broad disease resistance in tobacco in a manner similar to the *avr 9/Cf 9* cassette strategy proposed to confer broad disease resistance in tomato (De Wit, 1992).

Experimental procedures

Biological assays

Necrosis-inducing activity was assayed on 3-month-old tobacco plants, *Nicotiana tabacum* cv. Samsun NN, grown in a glasshouse under controlled conditions. Solutions were infiltrated into mesophyll tissue of fully developed leaves with a syringe equipped with a fine needle. Routinely, 50–100 µl of solution were infiltrated in order to cover areas of 1–2 cm². Necrosis-inducing activity was also tested in similar conditions against *N. tabacum* cv. Samsun, *N. tabacum* cv. Xanthi nc, *N. tabacum* cv. Petite Havana SR1, *N. sylvestris* and *N. glutinosa*.

Induction of systemic acquired resistance was assayed on *N. tabacum* cv. Samsun NN plants infiltrated with elicitor or water solutions. After 2 and 14 days of incubation at 22°C in a growth chamber with a day/night period of 16 h/8 h, TMV was inoculated using a micro-inoculation procedure developed in the laboratory (Konate and Fritig, 1984); this method allows one to study virus lesion development at predetermined infection sites. The plants were then incubated for 7 more days before observation of virus lesions.

Purification of the necrosis-inducing proteins of *P. megasperma* H20

Proteins responsible for the necrosis-inducing activity were purified from the culture medium of the fungus *Phytophthora megasperma* H20 (PmgH20). This strain, a pathogen of Douglas fir but a non-pathogen of tobacco, is a kind gift of Professor M. G. Hahn (Complex Carbohydrate Research Center, University of Georgia, USA) and was maintained at room temperature in the dark on V8 juice agar plates.

PmgH20 grown for 4 weeks (Ham *et al.*, 1991) was filtrated through a sintered glass funnel. The culture filtrate, about 1 l, was poured into dialysis tubing (6000–8000 molecular weight cut-off) and polyethylene glycol (15 000–20 000 molecular weight) was poured over them and allowed to sit at 4°C until the volume had been reduced about 10-fold. The concentrate was then collected by rinsing with 20 mM Tris-HCl, pH 8.0, and applied to a Sephadex

G25 column equilibrated in the same Tris-HCl buffer. The fraction that voided the column was loaded on to a Q-Sepharose column (Pharmacia) equilibrated in the Tris-HCl buffer. Proteins were eluted with a 0–0.5 M linear gradient of NaCl. Eluted active fractions were adjusted to 1.2 M ammonium sulphate and applied to a Phenyl-Superose column (Pharmacia) equilibrated in the same salt. Proteins were eluted using a decreasing gradient (1.2–0 M) of the same salt. Gel permeation chromatography on a FPLC Superdex 75 (Pharmacia) column equilibrated in 3-(N-morpholino)propanesulphonic (Mops) buffer (50 mM, pH 6.8, NaCl 100 mM) was performed as the last purification step.

The protein fraction that remained unbound from the Q-Sepharose column was dialysed against 20 mM Na-acetate, pH 5.2, and then subjected to cation-exchange chromatography on an S-Sepharose column (Pharmacia) that had been equilibrated in the Na-acetate buffer. The proteins were eluted with a 0–0.5 M linear gradient of NaCl. Active fractions were purified further by chromatography on a FPLC Phenyl-Superose (Pharmacia) column equilibrated as above. Elution of the proteins was performed as previously.

Protein analysis

Quantitation of proteins was performed according to the method of Smith *et al.* (1985), using BSA (Sigma) as standard. Methods for SDS-PAGE, silver staining, and immunoblotting have been described elsewhere (Ham *et al.*, 1991). Protein alkylation was performed on the purified protein which was reduced with dithiothreitol and then S-carboxymethylated with iodoacetamide following the procedure described by Stone *et al.* (1989). Detection of oligosaccharide moieties was performed on purified proteins blotted onto nitrocellulose membrane after SDS-PAGE following the method described by Bayer *et al.* (1990). Biotinamidocaproylhydrazide (9.3 µg ml⁻¹, Sigma) was used as reporter molecule for labelling of oligosaccharide moieties. Extraction of defence proteins from tobacco leaves was performed as described previously (Kauffmann *et al.*, 1990). Antisera used to probe the presence of various PR proteins have been raised against PR-1b (Heitz *et al.*, 1994a), PR-2c (Kauffmann *et al.*, 1987), PR-3a (Legrand *et al.*, 1987), PR-5a (Kauffmann *et al.*, 1990), PR-6 (Geoffroy *et al.*, 1990), and PR-11 (Heitz *et al.*, 1994b).

RNA analysis

Leaf tissues were collected at various times after elicitor or water infiltration. Total RNA was extracted from 0.5–1 g of plant material. Procedures of extraction and Northern hybridization have been described by Heitz *et al.* (1994b). Quantitation of Northern blots was performed using a Fujix Bio-Imaging analyser, model BAS 1000. Quantified radioactivity was expressed as PSL mm⁻², and no background corrections were made. PSL (photostimulated luminescence) is proportional to the level of radioactivity. Five different probes were used. The tobacco cDNA clones encoding phenylalanine ammonia-lyase, PAL (Pellegrini *et al.*, 1994) and class II o-diphenol-O-methyltransferase, OMT (Pellegrini *et al.*, 1993) were kindly supplied by Dr L. Pellegrini. The tobacco cDNA clone encoding PR-5a (Brederode *et al.*, 1991) was obtained from Dr J. Bol (Leiden). Dr E. Jamet (IBMP, Strasbourg) kindly provided us with a cDNA clone isolated from *N. sylvestris* and encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase (Genschick *et al.*, 1992).

Salicylic acid analysis

Leaf tissues were collected at various times after elicitor or water infiltration. Tissue samples (0.5 g) were ground in 1 ml of precooled 90% methanol directly in microfuge tubes. [¹⁴C]Salicylic acid (1 nCi, 54 mCi mmol⁻¹, NEN, UK) was then added as tracer to each tube. After centrifugation, the residue was extracted again with 100% methanol (1 ml) and the combined extracts were evaporated to dryness under nitrogen. For each sample, the dried extract was resuspended in 2 ml of hot (80°C) water and split into two equal parts; one part was processed for free SA analysis and one part for total SA (free SA plus SA conjugate) according to Malamy *et al.* (1992). Final samples were resuspended in 150 µl of 90% acetonitrile in 20 mM sodium acetate, pH 5.0. Injections of 100 µl were made on a 4.6 mm i.d. × 250 mm column containing Merck Lichrospher 100 RP-18 5 µm reversed-phase packing (Interchim, Montluçon, France). Chromatography was achieved at room temperature using a three-step gradient of acetonitrile in the Na-acetate buffer at a flow rate of 0.8 ml min⁻¹. The different steps of the gradient consisted of 6% acetonitrile for 12 min, 6–10% for 10 min, 10–80% for 1 min and final re-equilibration in 6% acetonitrile. SA was detected by fluorescence ($\lambda_{\text{ex}} 313 \text{ nm}$, $\lambda_{\text{em}} 405 \text{ nm}$). Radioactivity was determined by liquid scintillation counting of an aliquot sample. Recoveries of the internal standard [¹⁴C]-labelled salicylic acid were 37±6% ($n=15$), and corrections for losses were made for the individual samples.

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