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A GLYCOPROTEIN FROM *CERATOCYSTIS FIMBRIATA* F. SP. *PLATANI* TRIGGERS PHYTOALEXIN SYNTHESIS IN *PLATANUS* × *ACERIFOLIA* CELL-SUSPENSION CULTURES

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Key Word Index—*Platanus acerifolia*; Platanaceae; plane tree; *Ceratocystis fimbriata* f.sp. *platani*; phytoalexines; elicitor; glycoprotein.

Abstract—Treatment of cell-suspension cultures of *Platanus* × *acerifolia* with a crude elicitor preparation from *Ceratocystis fimbriata* f. sp. *platani* germlings induced the synthesis of the hydroxycoumarin phytoalexins, scopoletin and umbelliferone, and their accumulation in the growth medium. Only the protein-containing fraction of the culture filtrate was involved in cell response. By ultrafiltration of this last fraction, a major eliciting glycoprotein able to induce 80% coumarin synthesis was isolated. The glycoprotein was substituted by N-glycan(s) containing terminally linked mannose as revealed by lectin immunoblotting. The molecular mass of the eliciting compound is 66 kD (SDS-PAGE) and the native conformation was necessary for elicitor recognition by plane tree cells and thereby phytoalexin synthesis. We discuss the possible involvement of the GP 66 elicitor from the canker stain agent of the plane tree in the activation of the phenolic metabolism of the plant.

INTRODUCTION

Plants defend themselves from infection by invasive pathogenic microorganisms by a combination of constitutive as well as induced defense responses. The activation of plant defense mechanisms is considered to be consequent upon an initial recognition event in which the plant detects molecular components, referred to as elicitors, released from fungal and plant cell walls [1, 2]. The perception of pathogen signals by the host is followed by signal transduction inducing specific gene activation which leads to a wide spectrum of defense responses [3, 4] aimed at stopping the infection process. The interaction of the London plane tree (*Platanus* × *acerifolia* (Ait) Willd) with the canker stain agent, the fungus *Ceratocystis fimbriata* f. sp. *platani* (Ell and Halst) Walter (*Cfp*), has recently received considerable attention because this disease is potentially the most dangerous for the future of the plane tree. This pathogenic fungus enters through wounds in the roots, trunk and branches and causes foliar withering accompanied by trunk canker. A large vigourous tree may be destroyed within 4–7 years [5]. Although the mechanisms of plant infection are relatively well known [5], the plants reactions are poorly understood. The exposure of young plants of *P. acerifolia* to *Cfp* conidia leads to rapid localized necrosis and to the accumulation of preexisting phenolic compounds [6] and two hydroxycoumarin phytoalexins, scopoletin and umbelliferone, at the infection site [7, 8]. It is clear that triggering the synthesis of phytoalexins is one of the first defense responses taking place in the pathosystem and is related to the resistance of *P. occidentalis* and the susceptibility of *P. acerifolia* [8]. In numerous pathosystems, the synthesis of phytoalexins in plant tissues challenged by pathogenic microorganisms has been demonstrated and is related to elicitor-induced activation of phenolic metabolism [9]. To date, in the plane tree -*Cfp* interaction, no compounds of fungal origin able to induce phytoalexin synthesis in the host have been identified.

In the present paper, we describe the isolation and the partial characterization of a glycoprotein elicitor from *C. fimbriata* f. sp. *platani* and we show that using a model of reduced complexity, cell-suspension cultures of *P. acerifolia*, the fungal component triggers phytoalexin synthesis in cultured cells

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RESULTS

Response of cell cultures to treatment with eliciting preparations

Treatment of cultured cells with the various fractions from the *Cfp* filtrate culture induced the synthesis of the two hydroxycoumarin phytoalexins, scopoletin and umbelliferone, and their accumulation in the growth medium (Fig. 1). Only the supernatant collected after protein precipitation did not induce phytoalexin synthesis (Fig. 1). The time course of hydroxycoumarin accumulation was similar with the different eliciting fractions, a rapid increase in phenolic accumulation was observed up to 48 h after elicitation followed by a slow decrease (Fig. 1). However, the amounts of umbelliferone accumulated were slightly higher than those of scopoletin (Fig. 1). With the crude elicitor preparation (CEP), the level of phytoalexin accumulation was similar to that of cells elicited with the total protein fraction. By contrast, when cells were treated with the GP 66 glycoprotein obtained on ultrafiltration of the protein fraction, the amount of coumarins was 80% of that of cells elicited either with CEP or with the total protein fraction. However, for an equivalent amount of protein (0.5 μg per ml culture medium), the filtrate collected after GP 66 isolation induced only a very slight response of plane tree cells (Fig. 1).

Treatment of GP 66 with periodate or trypsin in order to inactivate, respectively, the carbohydrate or the protein moiety of this glycoprotein, suppressed the eliciting activity by about 85% (Fig. 2). Heat treatment of GP 66 at 100° for 10 min slightly reduced the eliciting activity, while a 90% decrease in phytoalexin accumulation was obtained after a 30 min treatment (Fig. 2).

In all cases, cell viability was about 85%, as found with unelicited cells. In unelicited cell cultures, phytoalexins were only detected as trace amount.

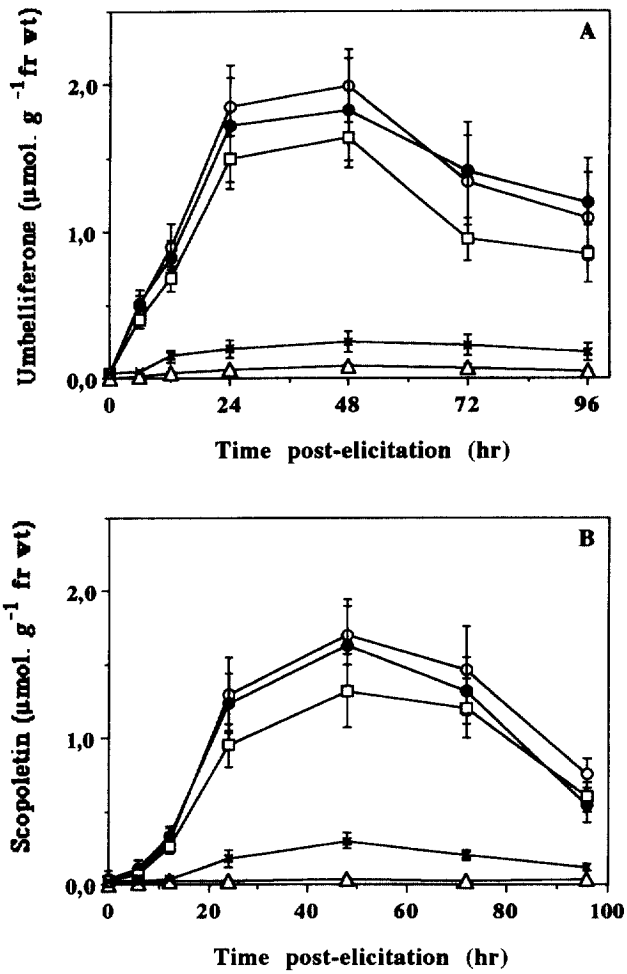

Fig. 1. Time course of umbelliferone (A) and scopoletin (B) accumulation in growth medium of *P. acerifolia* suspension cultures elicited (0.5 μg protein equivalents ml^{-1} culture medium) on day 12 after subculture. -○-, Crude elicitor preparation (CEP); -●-, protein fraction from CEP; -△-, supernatant from CEP after protein precipitation; -□-, GP 66 and -×-, Filtrate from protein fraction subjected to ultrafiltration.

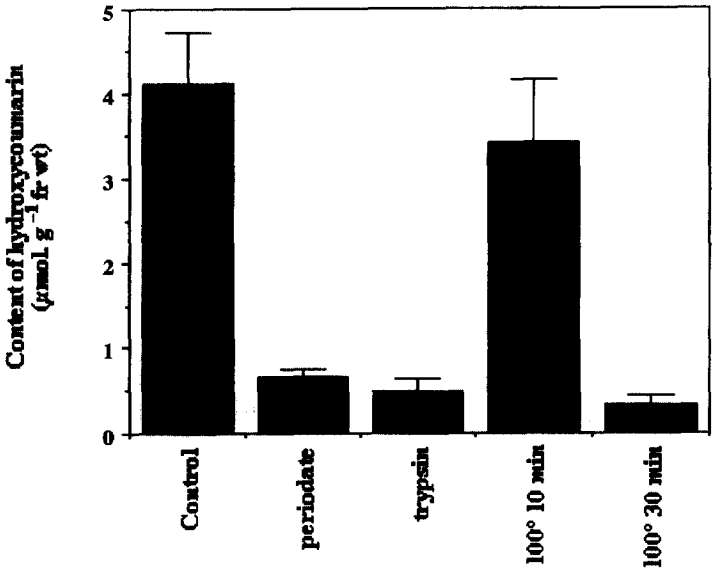

Fig. 2. Effect of different treatments on the eliciting activity of GP 66: periodate, trypsin and heat (100° for 10 and 30 min). Accumulation of hydroxycoumarins is given for the growth medium of elicited *P. acerifolia* suspension cultures.

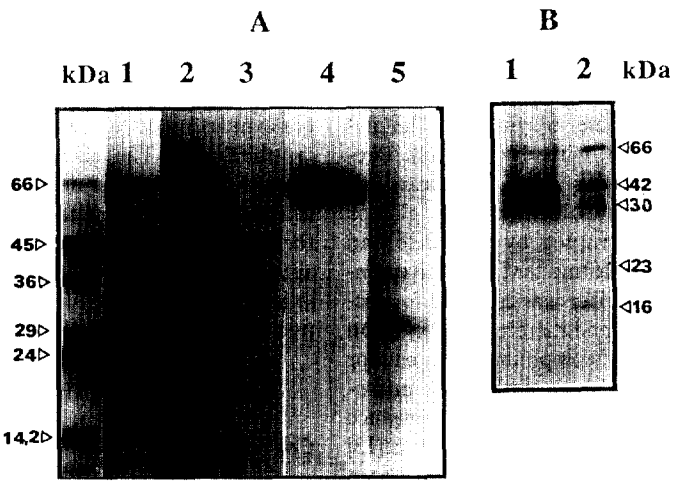

Fig. 3. (A). SDS-PAGE of eliciting preparations stained with silver nitrate. Lane 1: crude elicitor preparation (CEP); Lane 2: protein fraction from CEP; Lane 3: supernatant from CEP after protein precipitation; Lane 4: upper fraction from protein fraction subjected to ultrafiltration (GP 66); Lane 5: filtrate after ultrafiltration to recover (GP 66). (B). Western blot and N-glycan detection with lectin GNA digoxigenin labelled, and anti DIG Fab fragments alkaline phosphatase conjugated (recognizing mannose terminally linked to mannose). Lane 1: crude elicitor preparation (CEP); Lane 2: protein fraction of CEP.

Electrophoresis analysis of the eliciting preparations

SDS-PAGE analysis (denaturing conditions) of the crude elicitor preparation (CEP) and the protein fractions (described below) followed by silver nitrate staining, revealed five bands with electrophoretic mobilities of 66 kD, 42 kD, 30 kD, 23 kD and 16 kD (Fig. 3(A), lane 1 and 2). Only traces of protein (42 kD and 30 kD) were detected in the supernatant collected after protein precipitation (Fig. 3 (A), lane 3). SDS-PAGE analysis of the upper fraction collected from the protein fraction subjected to ultrafiltration revealed only one band with a 66 kD molecular mass similar to that previously detected in CEP (Fig. 3(A), lane 4). Glycosylation of this protein, so called GP 66, was suggested by periodic acid–silver nitrate staining which is specific for glycoproteins [10] (data not shown).

Glycan detection with Galanthus nivalis *agglutinin (GNA)*

Positive staining of the 66 kD, 42 kD, 30 kD, 23 kD and 16 kD proteins clearly showed that they were substituted by N-glycan(s) and contained mannose at the non-reducing end of carbohydrate chain (Fig. 3(B)). Indeed, GNA recognizes terminal non-reducing mannose α(1–3), α(1–6) or α(1–2) linked to mannose [11] and would essentially reveal only oligomannosides.

DISCUSSION AND CONCLUSION

In this study, we showed that the protein extract from a crude elicitor preparation from germlings of *Ceratocystis fimbriata* f.sp. *platani*, canker stain agent of *P. acerifolia* induced a strong stimulation of secondary metabolism of cultured cells of plane tree. The synthesis of scopoletin and umbelliferone was found in treated cells and a large amount of these hydroxycoumarin phytoalexins accumulated in the growth medium. The non-protein fraction was clearly inactive. Among the five glycoproteins detected in the crude elicitor preparation from *Cfp* culture, the glycoprotein with a molecular mass of 66 kD, so called GP 66, can be considered as the major eliciting compound since it induces 80% phytoalexin synthesis. The glycoprotein was substituted with N-glycan(s), presumably an oligomannosidic type N-glycan, as revealed by lectin immunoblotting. The elicitor activity of GP 66 was disrupted by periodate and trypsin treatments demonstrating that the native conformation of the GP 66 elicitor was essential for recognition by plane tree cells and thereby the elicitation process. Numerous cell-free compounds with elicitor activity have been isolated from pathogenic microorganisms [12]. Many fungi are known to produce extracellular glycoproteins in liquid culture media [13] and a wide variety of glycoprotein elicitors have been characterized and shown to activate plant defense genes [14] leading, in numerous herbaceous plants, to phenolic compound biosynthesis *de novo* [2, 15, 16]. In contrast, elicitors from pathogens of trees are less well documented excepted for the elicitor-active glycoprotein of the Dutch Elm Disease agent, *Ophiostoma ulmi* [17].

In the *P. acerifolia–C. fimbriata* f. sp. *platani* interaction, previous experiments have shown that the deposition of *Cfp* conidia in sterile distilled water droplets on leaves of *P. acerifolia* induced the synthesis of the same phytoalexins, scopoletin and umbelliferone. These coumarins were accumulated intra- and extra-cellularly to a significant extent in tissues situated under inoculum droplets [8]. Moreover, a preliminary bioassay using the direct application of the GP 66 elicitor on plane tree leaves showed a similar response characterized by coumarin accumulation. Therefore, elicitors are probably released from the conidia of *Cfp* during the earliest stages of germ-tube formation and later recognized by host cells.

The results of the present study strongly suggest that the treatment of plane tree cells with the GP 66 elicitor somehow mimics the modifications of phenolic metabolism displayed in plants during the establishment of host–pathogen interaction. Thus, it can be supposed that GP 66 is the major eliciting compound able to induce phytoalexin synthesis in cultured cells or in leaves of the plane tree. It is likely that under natural conditions, the release of elicitors starts at the formation of germ tubes, an event marking the beginning of the infectious phase in the life cycle of the pathogen. The presence of elicitors during these very early stages of the infectious process may be decisive in the perception of the pathogen by the host and the subsequent induction of an effective defense response such as phenolic metabolism activation leading to phytoalexin synthesis and accumulation.

Future works will focus on the immunolocalization of the GP 66 elicitor *in planta* after experimental infection with *Cfp* in order to confirm the release of GP 66 into the intercellular compartments of plane tree tissues during pathogenesis and to establish a correlation with phytoalexin synthesis in the susceptible plane tree (*P. acerifolia*) and the resistant one (*P. accidentalis*). Indeed, previous results have shown that in infected plane trees, a later and lower level of phytoalexin accumulation was correlated with plant susceptibility [8]. The elicitation of *P. acerifolia* cell suspension culture on day 12 with the elicitor-active glycoprotein GP 66 offers a good experimental system for the elucidation of the process of molecular interaction including the elicitor recognition and the regulation of *de novo* expression of genes involved in plane tree defense responses.

EXPERIMENTAL

Cell cultures

Calli of *Platanus* × *acerifolia* were generated from surface sterilized hypocotyl segments of 1-month-old plants on woody plant medium (WPM) [18] supplemented with 2.4 μM pyridoxine-HCl, 3 μM thiamine-HCl, 550 μM myoinositol, 4 μM nicotinic acid, 0.25 μM 2,4-D and kinetin, 20 g l^{-1} sucrose. 21-day-old calli were transferred into WPM liquid medium supplemented with 0.15 μM 2,4-D and kinetin. Culture flasks (250 ml) containing 100 ml of sterilized medium were inoculated with 10 g cells (fr. wt) and grown at 25° in continuous light (40 μE m^{-2} s^{-1}) under horizontal rotary shaking (95 rpm). Cultures were transferred to fresh medium every 12 days. Cell viability was monitored using the fluorescein diacetate method [19].

Elicitor preparation

A culture filtrate of *cfp* was prepared from conidial suspension (4×10^4 spores ml^{-1}) grown for 48 h at 25°

in sterile distilled water. This crude elicitor preparation (CEP) was filter sterilized then treated with 30% $(NH_4)_2SO_4$ (w/v), 30 min at 4° for protein precipitation. After centrifugation (12,000 g, 15 min), the protein pellet was dissolved in 10 mM K-Pi buffer (pH 7) then dialysed against the same buffer overnight at 4°. The supernatant was dialysed under similar conditions. The protein fraction was fractionated by ultrafiltration (Microsep 50 kDa, Filtron), then the filtrate and the upper fraction were separately collected. Protein content of the different fractions were estimated by the method of [20] using BSA as standard.

Electrophoresis

SDS-PAGE was carried out according to the method of Ref. [21] with 12% acrylamide for separating gel and 4% for stacking gel. The sample run under denaturing conditions were diluted 1 : 4 with a reducing sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) bromophenol blue, 0.7 M β-mercaptoethanol and boiled for 3 min. SDS-7 (Sigma) was used as a source of M_r markers. Proteins were stained either with silver nitrate reagent [22] or periodic acid–silver nitrate reagent for glycoproteins [10].

Protein electroblotting and glycan detection

After separation by SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Millipore) using the Milliblot-SDS Transfer System (Millipore) run (2.5 mA cm^{-2} for 35 min) at room temp. with the low ionic discontinuous buffer system given in the instructions: anode buffer 1 : 0.3 M Tris, 10% MeOH (pH 10.4), anode buffer 2 : 25 mM Tris, 10% MeOH (pH 10.4), cathode buffer : 25 mM Tris, 40 mM 6-aminohexanoïc acid, 20% MeOH (pH 9.4).

Galanthus nivalis agglutinin (GNA, diluted 1 : 1000) was used to identify "high mannose" or "complex-type" glycans (DIG glycan differentiation kit, Boehringer Mannheim). The interaction was revealed with the alkaline phosphatase conjugate of the anti-dioxigenin Fab fragments (Boehringer Mannheim). The standard buffer for the lectins was Tris-buffered saline (TBS) (50 mM Tris-HCl, 0.15 M NaCl, pH 7.5). The membrane was incubated overnight at 4° in blocking solution (TBS containing 0.1% Tween 20, 2% BSA and 0.05% NaN_3). The membrane was washed and incubated with the lectin for 1 h at 25°. The plant lectin was used as described previously [23] except that the TBS buffer contained 0.1% BSA and 0.1% Tween 20 [24]. Carboxypeptidase Y was used as positive control for the lectin blotting.

Elicitor treatments

The protein collected in the upper fraction on ultrafiltration of the protein fraction was submitted to various treatments. For carbohydrate moiety inactivation, 0.5 μg protein ml^{-1} was treated with 10 mM sodium periodate for 1 h at room temp. in the dark [25]. The reaction was stopped by ethylene glycol (20%, v/v) and the mixture was purified by ultrafiltration (Microsep 10 kDa, Filtron), washed × 3 with distilled water and at resuspended in distilled water to the original concentration. In the control sample distilled water was used instead of sodium periodate.

For trypsin digestion, 0.5 μg protein ml^{-1} was incubated in 40 mM Tris-HCl (pH 8.1), 10 mM $CaCl_2$ and trypsin (10%, w/v) for 24 h at 37°. In the control, the proteins were omitted.

Heat treatment of 0.5 μg protein ml^{-1} of distilled water was carried out by heating in a 100°-water bath for 10 and 30 min.

Elicitation of cell cultures

Eliciting activity of the following fractions were tested : (i) crude elicitor preparation (CEP), (ii) protein fraction from CEP, (iii) supernatant from CEP after protein precipitation, (iiii) upper fraction and filtrate collected on ultrafiltration of the protein fraction. Every fraction was added separately to cell cultures in early stationary phase (day 12) to give a final concentration in the medium of 0.50 μg ml^{-1} protein equivalents according to Ref. [26]. Unelicitated (control) cell cultures received only sterile distilled water or phosphate buffer or various control solutions used to study the effects of protein treatments on elicitation (see above). Afterwards, the cell cultures were incubated under standardized conditions.

Phytoalexin extraction and analysis

Phytoalexin extraction was only performed on the growth medium of cultured cells because more than 90% of these compounds were excreted into and accumulated in the cell medium [26]. At different points in time (6, 12, 24, 48, 72 and 96 h) following elicitation, 15 ml of cell suspension culture were harvested. The growth medium was collected by vacuum filtration and extracted twice with EtOAc containing 20% $(NH_4)_2SO_4$ (w/v) and 2% metaphosphoric acid (w/v). The organic phase was reduced to dryness *in vacuo* and the residue was dissolved in 1.5 ml MeOH. Afterwards, the methanolic extracts were analysed by HPLC for phytoalexin quantification. HPLC analysis (Waters 990 with a photodiode bar detector coupled with a Waters 420 fluorescence detector) was performed on a spherisorb C18 column (particle size 5 μm, 250 × 5 mm) and samples were eluted in a solvent consisting of MeCN and H_2O (pH 2.6) using a gradient of 5–40% MeCN for 45 min with 1 ml min^{-1} flow rate. Scopoletin and umbelliferone were identified by comparing their chromatographic and spectral characteristics to standards (Sigma) and the results were expressed in nmol of scopoletin and umbel-

liferone g^{-1} fr. wt (FW). The data correspond to 3 replicates with 3 flask samples.

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