Physiological and Structural Changes in Tobacco Leaves Treated with Cryptogein, a Proteinaceous Elicitor from *Phytophthora cryptogea*

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ABSTRACT

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Cryptogein was applied on the petiole section of excised tobacco leaves. It elicited necroses that can be correlated with histological alterations, such as rapid chloroplast breakdown and a collapse of cells leading to disorganization of the parenchyma tissue. In addition, it induced ethylene production and accumulation of capsidiol. In order to detect an early response, we analyzed the kinetics of chlorophyll fluorescence induction. When tobacco leaves were treated with cryptogein (more than 1 μg per leaf), the level P of fluorescence (F_P) lowered progressively, indicating a decrease in the variable fluorescence. In order to rule out variations due to optical heterogeneity between leaves, the value of F_P was related to that of F_O (constant fluorescence, level O) and F_I (intermediary fluorescence, level I), which remained constant throughout the experiment. These ratios could thus be used as a nondestructive test to detect the induced stress resulting from elicitation before necrotic areas were visible. On the basis of these results, cryptogein can be considered an elicitor of defense mechanisms.

Additional keywords: hypersensitive response, *Nicotiana tabacum*, phytoalexins.

The hypersensitive reaction (HR) is a common response of a plant to an incompatible pathogen. It is characterized by rapid localized death of cells at the site of attempted invasion and often leads to some protection against otherwise compatible pathogens (23). Among plant cell responses associated with the HR, the accumulation of phytoalexins (12) and the induction of an enhanced ethylene synthesis (9,22) have been reported. However, the biochemical processes involved in the HR are not understood and the initiation mechanism of the hypersensitive cell death remains to be determined.

The initial event involves an elicitor from the incompatible pathogen, but evidence of defined microbial elicitors, inducing a typical HR, is limited. A major limitation in studying plant responses to microorganisms is the few fully characterized elicitors available. Recently, it has been reported that the phytopathogenic fungus *Phytophthora cryptogea* Pethybr. & Lafferty, when inoculated to the stem of a decapitated tobacco (nonhost) plant, causes a restricted fungal colonization associated with the induction of necroses at the inoculation court and also at a distance on the leaves (4). We believe that both local and distant necroses are of the HR type (hypersensitivelike), despite the unusual location of the latter, because 1) they occur soon after inoculation (24–36 h) and do not evolve afterwards; 2) they affect limited areas and do not lead to plant death; and 3) no similar necroses occur in the interaction between tobacco and the compatible pathogen *P. parasitica* Dastur var. *nicotianae* (Breda de Haan) Tucker.

From the culture filtrate of *P. cryptogea*, an extracellular protein (MW 10,323 D) named cryptogein has been isolated. It elicits similar stem and leaf necroses and pathogenesis-related (PR) protein accumulation, and it protects tobacco plants against invasion by *P. p.* var. *nicotianae* (4,6). This protein was purified and its amino acid sequence determined (21). It does not show any protease or β-glucanase activity. We studied the response of cultured tobacco cells to cryptogein in order to provide a certain physiological basis for the biochemical analysis (3). Cryptogein induces an increase in pH and conductivity of the extracellular medium and also triggers ethylene and capsidiol production. Binding experiments suggest the presence of elicitor-binding sites with high affinity for cryptogein (3).

In this paper we examine the physiological and structural changes induced by cryptogein in excised tobacco leaves in order to compare these responses with those already reported for HR (ethylene and phytoalexin production), and to determine if the first levels of fluorescence induction could be used to detect the induced-stress resulting from elicitation (kinetics of chlorophyll fluorescence). In addition, we describe the histological alterations observed in light and electron microscopy. The results allow that cryptogein can be considered an elicitor of HR.

MATERIALS AND METHODS

Materials. Tobacco plants (*Nicotiana tabacum* L. 'Xanthi') were planted in the greenhouse. Five weeks later, they were placed in a controlled environment chamber (20 C, 70–80% relative humidity, 16,000 lx, 16 h of light). Pots were watered daily with a nutrient solution (24). The upper leaves were used for the bioassays 50–60 days after sowing. They weighed 3–5 g (fresh weight determined before treatment).

P. cryptogea was grown on a synthetic medium as described by Bonnet et al (7), and cryptogein was purified according to the method published by Ricci et al (21). Cryptogein was applied to the petiole of excised tobacco leaves, using a pulse of an aqueous solution (5 μl) with a water chase (3 × 5 μl). Petioles were placed in water and leaves were kept at room temperature in the dark. Using ^{125}I-radiolabeled cryptogein (3), we verified that the protein was not released in water and spread to the whole leaf in a few minutes. Western blot analysis confirmed that cryptogein was not metabolized.

Ethylene determination. Excised leaves were placed in 125-ml Erlenmeyer flasks sealed with rubber caps. Samples of internal gas (1 ml) were withdrawn and analyzed by gas chromatography (GC) using Delsi chromatograph model DI 700 (Delsi Instruments, Suresnes, France) equipped with a flame ionization detec-

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tor (FID) and a packed column (Porapak Q 80-100, 2 m × 3 mm, Delsi; N_2 carrier gas, 30 ml/min; injector and detector, 120 C; column, 35 C). A calibration curve was established with ethylene standards (Aldrich-Chimie, Strasbourg, France).

Capsidiol extraction and analysis. Tobacco leaves were frozen in liquid nitrogen, ground in a mortar, and then extracted with 2 × 30 ml of a 50% H_2O/MeOH mixture. The solid material was removed by vacuum filtration on Whatman No. 3 filter paper, the extracts were reduced to half volume by evaporation, and the phytoalexins were extracted with 3 × 20 ml of dichloromethane. The organic extracts were pooled and dried before being redissolved in 200 μl of dichloromethane. These fractions were purified by thin-layer chromatography (TLC) on silica gel (Kieselgel 60, Merck-Clévenot SA, Nogent-sur-Marne, France) developed with ethyl acetate/hexane (2:1, v/v). Capsidiol was visualized by spraying vanillin reagent (1% in concentrated H_2SO_4) on a separated lane. Capsidiol was eluted from the silica gel with ethyl acetate. The sample was dried, then dissolved in 500 μl ethanol, and finally analyzed by GC (Delsi DI 700 equipped with an FID and a packed column [3% OV-225 on Chromosorb W 100-120, 1.5 m × 3 mm; N_2, 30 ml/min; injector, 235 C; detector, 255 C; column, 180 C]). Quantitative analysis of capsidiol was obtained by measuring peak areas relative to those of methyl erucate (Merck-Clévenot SA), which was used as internal standard. The response factor was determined by using a mixture of capsidiol and methyl erucate.

Chlorophyll fluorescence measurement. Fluorescence measurements were performed as previously reported (13). The beam from a slide projector, with a DC power supply, was focused through a broad band blue and anticaloric filter (400–600 nm) on a vertical slit (0.5 × 2 cm) punched into a black screen. The light intensity was approximately 100 $\mu E/m^2 \cdot s$. Fluorescence emission from the upper side of the leaves was conveyed by an optical fiber to a photomultiplier through complementary red filters (> 680 nm). Leaves, maintained in the dark for at least 2 h before measurements, were placed behind the screen and were held by a magnet against the slit. The fluorescence signal was digitized and stored as 5,000 × 1 ms channels, using an Analog to Digital conversion card plugged into an Apple II microcomputer. Channel 1 was defined as the first detection of fluorescence signal. Shutter on the excitation beam was fully opened within 3 ms, so that level O (constant fluorescence, F_O) was estimated at half-opening time (channel 2) from signal values at channels 3 and 4. The level I (F_I), corresponding to the photochemical rise from O to I, was obtained by averaging signal values between 100 and 120 ms. The level P (F_P), detected as the maximum of signal reached, was averaged over 100 ms.

Preparation and fixation of tissues for microscopy. Twenty hours after treatment with cryptogein, a drop of fixative was layered on top of the necrotic spots before the leaf tissue was segmented into strips approximately 0.5 mm wide to allow better penetration of the fixative. For a single spot, central necrotic regions and healthy peripheral areas were present in the same strip of tissue for direct comparison. Healthy tissues from untreated plants also were fixed for controls. The fixative solution consisted of 3% glutaraldehyde and 1.5% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.3. Fixation was done at room temperature for 2 h. Then leaf segments were rinsed three times (20 min) with 0.2 M sucrose in 0.1 M cacodylate buffer.

Tissues were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.3 for 1 h. They then were dehydrated in an ethanol series followed by propylene oxide and embedded in Araldite-Epon (Fluka, Mulhouse, France). Sections were cut on a Sorvall MT-2B ultramicrotome (Jose Delville, St-Germain en Laye, France) and 2-μm thin sections were dry mounted on glass slides, stained with a 1% toluidine blue/1% methylene blue borax mixture, and viewed in a Zeiss Axiophot light microscope (Carl Zeiss SARL, Le Pecq, France). Silver-gray ultrathin sections were mounted on copper grids and stained in 2.5% uranyl acetate for 15 min followed by lead citrate for 5 min. Sections were viewed in a Hitachi H 600 electron microscope (Elexience, Verriéres-le-Buisson, France) at an accelerating voltage of 75 kV.

RESULTS

Structural modifications of tobacco leaves treated with cryptogein. Twenty hours after treatment, the leaf sections showed healthy and altered areas, depending on their location from the veins. Signs of cellular alterations were seen in close vicinity of minor veins in the mesophyll of treated leaves. A few cells of the spongy parenchyma had lost their turgidity and had collapsed, giving rise to a small translucent depression in the mesophyll. In other areas, cells from the upper epidermis, palisade parenchyma, and spongy parenchyma were disorganized. In these cells, the vacuolar volume had drastically decreased and the original cell shape was no longer maintained. The cell walls were crumpled. There was some evidence of cytoplasm and vacuolar sap mixing. The cell content bound basic stains, as observed in the light microscope (data not shown).

Detailed observations, using the electron microscope, confirmed the general features of the collapsed cells. The protoplasm was pulled away from the corrugated cell walls, and an electron-clear space was left between the wall and the still-recognizable plasma membrane (Fig. 1B and C). Although the vacuole seemed flaccid, the tonoplast was still present even in very late stages of alterations of the protoplasm (Fig. 1B and C). The cytoplasm was unevenly disturbed, but the most evident alterations were seen in the chloroplasts. The intrathylakoid space was swollen and the grana were less regularly stacked (Fig. 1B, arrows). In many damaged cells, the envelope membranes of the chloroplasts were ruptured and the stroma had merged in the cytosol (Fig. 1C, arrows). The other organelles—e.g., mitochondria, Golgi apparatus, endoplasmic reticulum—were severely altered, although remnants of membranes could be seen even in totally disorganized cells.

Ethylene production by tobacco leaves treated with cryptogein. When assayed on tobacco leaves, cryptogein induced ethylene production. The dose-dependency of ethylene production is shown in Figure 2. Maximum ethylene production occurred at about 250 ng of cryptogein per gram FW and reached a plateau in this range. The time course of ethylene production for 260 ng of cryptogein per gram FW (1 μg per leaf) is shown in Figure 3: production started after a lag period (2–3 h), then increased linearly.

Capsidiol accumulation in tobacco leaves treated with cryptogein. Tobacco leaves accumulated capsidiol as a function of cryptogein concentration (Fig. 4). Usually tobacco leaves were treated for 18 h, with variable cryptogein amounts, then sesquiterpenoid compounds were extracted and capsidiol production was analyzed. Contrary to ethylene production, which leveled off for less than 1 μg of cryptogein per gram FW, capsidiol accumulation continuously increased over the range of concentrations assayed that extended up to 2.8 μg of cryptogein per gram FW. The production of capsidiol increased as a function of time (Fig. 5). We did not detect any capsidiol production in control leaves nor at 3 h in treated leaves.

Changes in chlorophyll fluorescence parameters of tobacco leaves treated with cryptogein. Upon illumination of a dark-adapted tobacco leaf, fluorescence increases biphasically from level O to level I, then increases more slowly to level P, which is close to the maximum level (F_M) when the excitation light is strong enough (for a review, see 18). In order to detect an early response in tobacco leaves treated with cryptogein, we studied the kinetics of chlorophyll fluorescence induced by light. Experiments were carried out during 20–25 h, using tobacco leaves of about 3–5 g FW.

In control leaves, the different fluorescence characteristics (F_O, F_I, and F_P) did not change significantly during 20 h in the dark (Table 1). In leaves treated with cryptogein, F_O and F_I also remained unchanged, but F_P decreased. Great variations in absolute values of F_O, F_I, and F_P were observed due to optical heterogeneity between leaf samples. In order to correct this variation, F_P was related to F_O, as classically done (Table 1), or to F_I (Fig. 6). A more accurate discrimination between treated and control leaves was obtained using the F_P/F_I rather than the F_P/F_O ratio, due to the averaging of F_I over 20 signal sampling values, whereas

F_O was estimated from the values of the first two signals. Work in progress (using an IBM-compatible computer) shows that a better accuracy for F_O measurement can be obtained by extrapolating the O to I rise of kinetics to the half-opening time of the optical shutter. Therefore, F_P/F_I can be taken as a working index to detect the action of cryptogein after a 10-h treatment, whereas macroscopic necrosis appeared more than 20 h after treatment (Fig. 6).

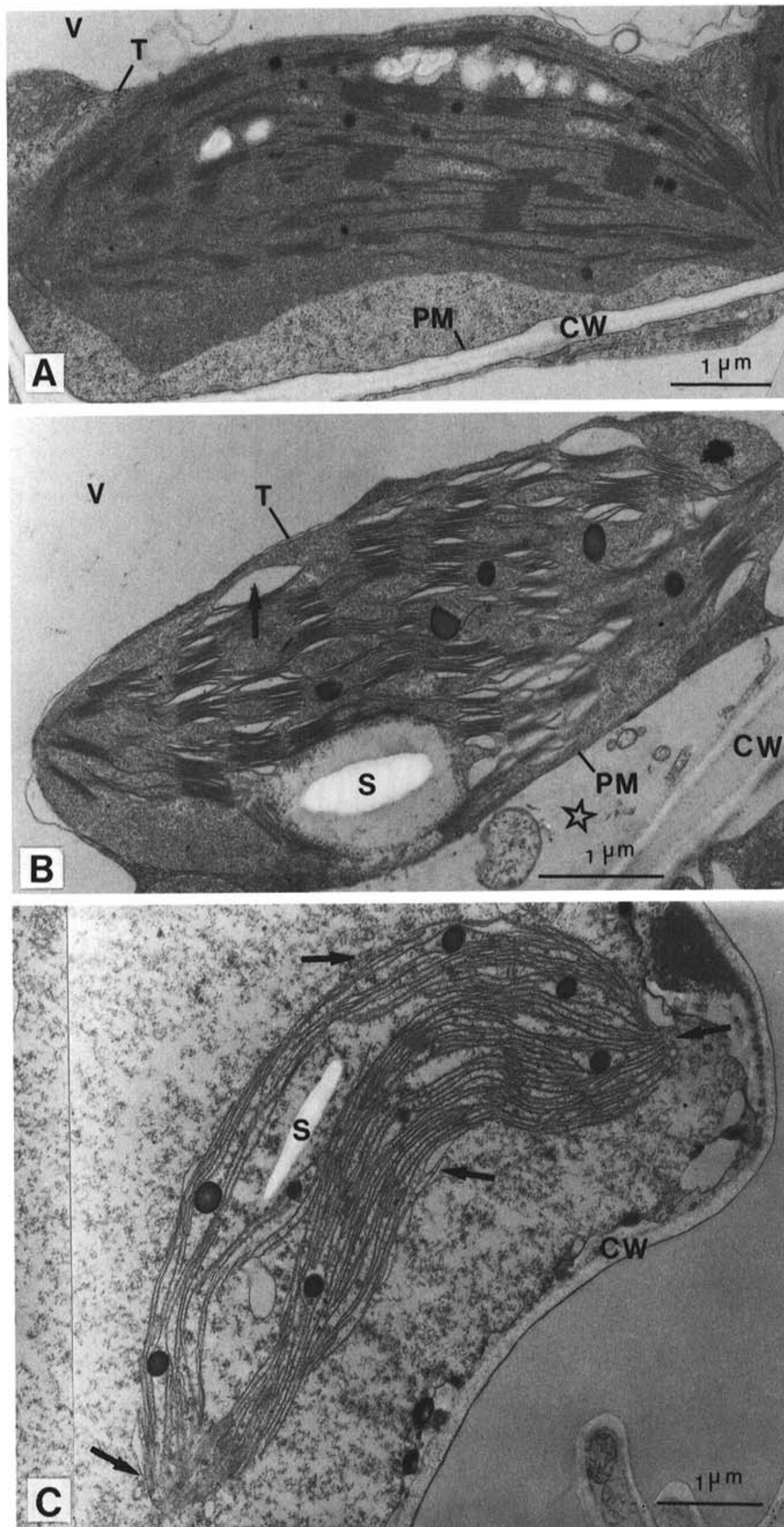

Fig. 1. Electron micrographs of **A,** healthy tissue, and **B** and **C,** cryptogein-treated leaves, of *Nicotiana tabacum* 'Xanthi'. cw, Cell wall; pm, plasmalemma; S, starch; T, tonoplast; V, vacuole; ★, electron-clear space between the wall and the plasmalemma.

The decrease in F_P value corresponds to a decrease in the variable fluorescence (F_V) and of the F_V/F_O ratio, which reflects a lower efficiency of excitation capture by open Photosystem II centers (15). The decrease in the variable fluorescence with almost no change in the constant fluorescence is similar to what is observed when thylakoids are placed in a Mg^{2+}-depleted medium (1,8). Hence, the decrease in F_P can be ascribed to an unstacking of grana induced quite rapidly by cryptogein, which is in agreement with observations by microscope (Fig. 1B).

DISCUSSION

We report here the responses of tobacco leaves to a well-characterized proteinaceous elicitor (3,21). This elicitor induces accumulation of a phytoalexin (capsidiol) and production of ethylene together with cell disorganization. On whole tobacco plants, it has been shown to elicit necrosis, the accumulation of the defense protein PR1a, and protection against the pathogen *P. p.* var. *nicotianae* (5,21).

We previously reported the effect of cryptogein on a cellular suspension of tobacco (3,19): cryptogein induced ethylene and capsidiol production over time similarly as in tobacco leaves, i.e., these responses appeared several hours after treatment. These results are comparable to those obtained using other elicitor preparations, such as crude extracts or glycoproteins from mycelial walls of several *Phytophthora* species (10,11,14).

Besides these late responses, cell suspensions that undergo HR show initial phenomena, such as an increase in pH and conductivity of the extracellular medium (2,16). Cryptogein also induced a very rapid increase in pH and conductivity, which appeared within the first few minutes after treatment, without any obvious alteration of the plasma membrane integrity (3). Such responses cannot be measured on leaves. We examined the changes in chlorophyll fluorescence induction as an early marker of the

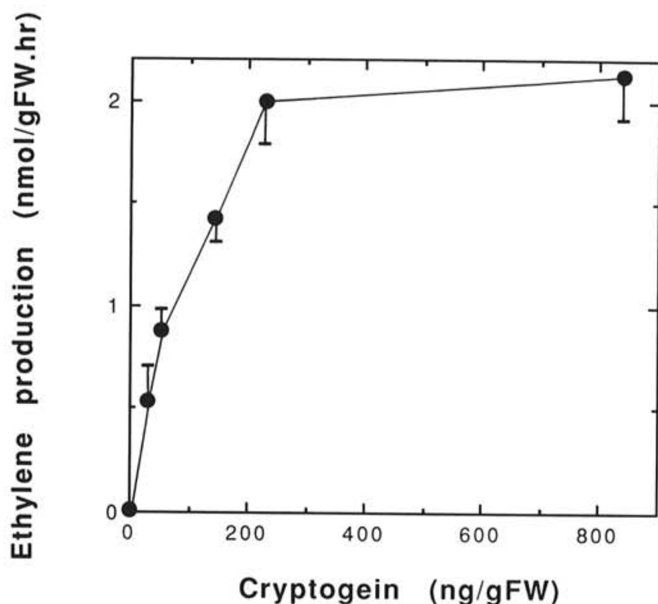

Fig. 2. Rates of ethylene production by tobacco leaves elicited with cryptogein, calculated from linear parts of time course experiments. The average of the initial fresh weight of leaves was 3.8 ± 0.4 g. Vertical bars represent standard deviation ($N = 3$).

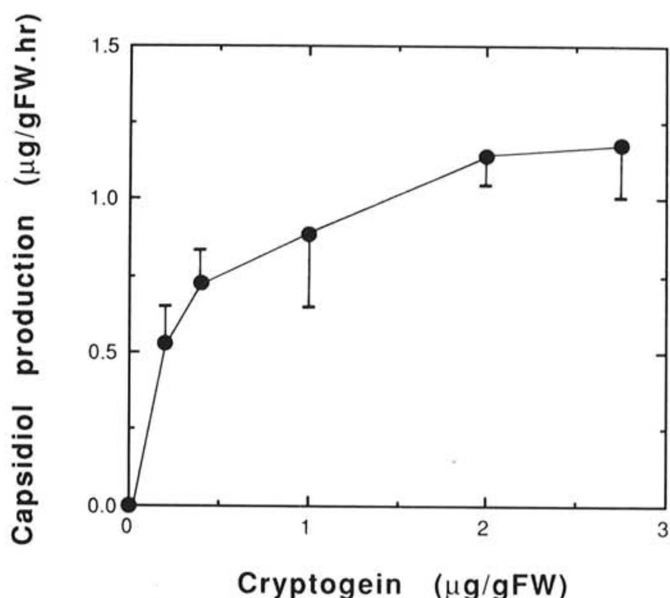

Fig. 4. Rate of capsidiol accumulation in tobacco leaves elicited with cryptogein, determined after 18-h incubation with variable cryptogein concentrations. The average of the initial fresh weight of leaves was 3.3 ± 0.3 g. Vertical bars represent standard deviation ($N = 4$).

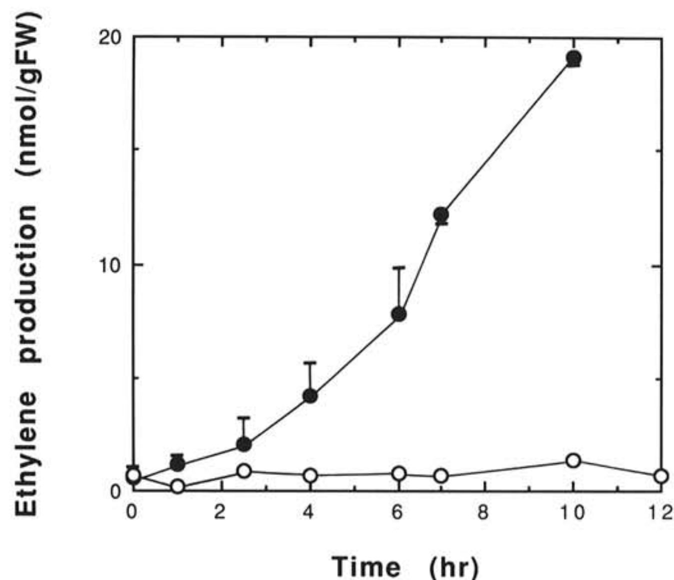

Fig. 3. Time course of ethylene production by tobacco leaves elicited with cryptogein. ○, Control; ●, 260 ng of cryptogein per gram FW (1 µg per leaf). The average of the initial fresh weight of leaves was 3.8 ± 0.4 g. Vertical bars represent standard deviation ($N = 3$).

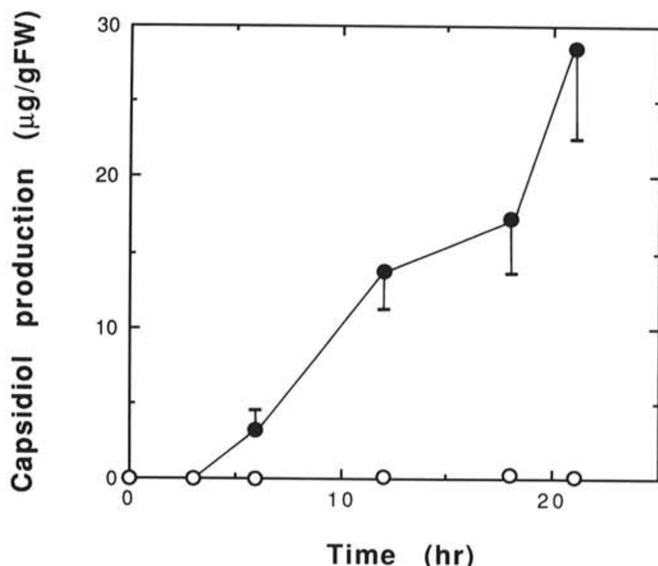

Fig. 5. Time course of capsidiol production by tobacco leaves elicited with cryptogein. ○, Control; ●, 2.8 µg of cryptogein per gram FW (10 µg per leaf). The average of the initial fresh weight of leaves was 3.6 ± 0.2 g. Vertical bars represent standard deviation ($N = 4$).

TABLE 1. Changes in fluorescence parameters induced by cryptogein from kinetics experiments during a 20-h period

Cryptogein (μg/leaf)	F_O[a]	F_I	F_P	A	R
0	40 ± 7 (17)[b]	102 ± 17 (17)	255 ± 47 (17)	6.0 10^{-6} (17)	NS[c]
0.01	41 ± 3 (17)	104 ± 12 (17)	276 ± 27 (17)	2.0 10^{-4} (17)	NS
0.1	43 ± 3 (17)	107 ± 4 (17)	284 ± 27 (17)	−1.5 10^{-4} (17)	NS
1	49 ± 2 (11)	124 ± 10 (11)	decreasing	−2.4 10^{-3} (11)	0.94
10	46 ± 6 (10)	115 ± 5 (10)	decreasing	−4.1 10^{-3} (10)	0.96

[a] F_O, constant fluorescence (level O); F_I, intermediary fluorescence (level I); F_P, maximal fluorescence reached (level P). A represents the variation by time unit (h) of F_V/F_P, which is the ratio between the variable fluorescence ($F_V = F_P - F_O$) and the maximal fluorescence reached.
[b] Figures in brackets represent the number of points analyzed. The average of the initial fresh weight of leaves was 3.6 ± 0.4 g.
[c] No significant correlation between F_V/F_P to time.

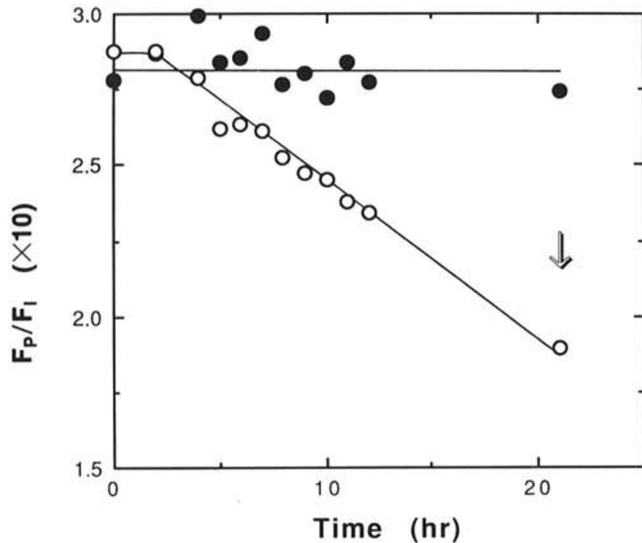

Fig. 6. Time course of the F_P/F_I ratio evolution after application of cryptogein. ●, Control; ○, leaves treated with 10 μg of cryptogein; ↓, appearance of necroses. Typical experiment from a series of three repetitions.

effect of cryptogein on organized tissues. This method has been described previously in the detection of other induced-stress responses (17,20). Results shown in Figure 6 demonstrate that a decrease of the ratio F_P/F_I provides an early indication that an elicitation process has been triggered. Although it is necessary to wait several hours to discriminate between control and cryptogein-treated leaves, this method allows different parts of an elicited plant to be easily monitored without deterioration of the plant material. It is possible to compare HR induction over time in different leaves of a single treated plant.

On the basis of the results reported here, cryptogein is an elicitor of defense mechanisms. The use of this purified protein should facilitate investigations on the physiological modifications occurring when the hypersensitive reaction begins. In addition, the analysis of chlorophyll fluorescence induction appears to be a convenient and nondestructive method to detect the induced stress resulting from elicitation.

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