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The Elicitation of Ethylene Biosynthesis by a *Trichoderma* Xylanase Is Not Related to the Cell Wall Degradation Activity of the Enzyme[1]

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A β-1,4-endoxylanase (EIX) isolated from *Trichoderma viride* elicits plant defense responses in certain tobacco (*Nicotiana tabacum* L.) cultivars in addition to its xylan degradation activity. It was not clear whether elicitation occurs by cell wall fragments released by the enzymic activity or by the xylanase protein interacting directly with the plant cells. We used protoplasts isolated from tobacco leaves to test whether the cell wall is required for the stimulation of ethylene biosynthesis by EIX. Protoplasts of tobacco (cv Xanthi) responded to treatment with the EIX, as indicated by an increased production of ethylene and the loss of protoplast viability. Protoplasts prepared from ethylene-pretreated leaves produced more ethylene and had higher rates of cell death in response to EIX than protoplasts prepared from nonethylene-treated leaves. Protoplasts of an EIX-insensitive cultivar of tobacco (Hicks) were insensitive to high concentrations of EIX. The addition of a crude cell wall preparation to protoplasts during incubation with EIX did not enhance the induction of ethylene biosynthesis by nonsaturating as well as saturating concentrations of EIX. These data indicate that the xylanase activity of EIX is unrelated to the elicitation of ethylene biosynthesis through the production of some cell wall fragment, since the protein per se appears capable of eliciting ethylene biosynthesis in protoplasts.

EIX is a 22-kD protein isolated from the fungus *Trichoderma viride* (Fuchs et al., 1989; Dean and Anderson, 1991). When applied to tobacco (*Nicotiana tabacum* L. cv Xanthi) leaf tissue, EIX induces ethylene formation and hypersensitive-type necrosis as well as other plant defense responses (Anderson et al., 1990; Bailey et al., 1990, 1992). Previous work in this laboratory showed that EIX is translocated in the xylem tissues when applied through a cut petiole and induces symptoms in leaves both above and below the point of its application (Bailey et al., 1991; Sharon et al., 1992). When EIX is injected into the leaf mesophyll intercellular spaces, induction of necrosis and elicitation of PR protein and capsidiol accumulation are restricted to the injected area (Lotan and Fluhr, 1990; Fluhr et al., 1991; Sharon et al., 1992). Proteinaceous elicitors exhibiting hydrolytic enzymic activity have been isolated from different fungi, and the assumption in these cases was that specific elicitor compounds are released from the cell wall of the plant or the pathogen due to the enzymic activity of the protein (Walker-Simmons et al., 1984; Hahn et al., 1989; Bucheli et al., 1990). With EIX, however, we were unable to detect any heat-stable elicitor from the xylanase and tobacco leaf interaction (Fuchs et al., 1989) or xylan (Dean et al., 1989), suggesting that elicitation of plant responses by EIX may result from a direct interaction of the peptide with the cell and does not involve intermediate compounds.

Protoplasts have been used successfully in many systems to study plant responses to external stimuli, including studies of plant responses to elicitor treatments (Anderson et al., 1979; Fisher, 1979; Guy and Kende, 1984; Dangl et al., 1987; Bossen et al., 1988; Schnitzler and Seitz, 1989; Jouanneau et al., 1991). It also has been reported that proteinaceous fungal elicitors are capable of inducing ethylene synthesis and other defense responses in plants (Toppan and Esquerré-Tugayé, 1984; Horn et al., 1989; Bucheli et al., 1990; Blein et al., 1991; Felix et al., 1991). In the present study, we used protoplasts to test whether cell walls are required for the elicitation of ethylene biosynthesis by EIX.

MATERIALS AND METHODS

Plant Material

Two tobacco (*Nicotiana tabacum*) cultivars were used: Xanthi and Hicks. Plants were grown in a greenhouse until they were 25 to 35 cm tall. Young, fully expanded leaves were cut and incubated for 14 h in an atmosphere of 120 μL/L of ethylene or in an atmosphere purged of ethylene, as previously described (Chalutz et al., 1984).

Preparation of Protoplasts

The preparation of protoplasts from tobacco leaves was modified from Zelcer and Galun (1976) and was done asep-

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Abbreviation: EIX, ethylene biosynthesis-inducing xylanase.

tically in a laminar flow hood. Wash medium contained 0.55 M mannitol, 10 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KNO_3, 5 mM Mes (free acid), pH 5.8. The cell wall digestion mix included 0.04% Cellulase (Worthington)[4], 0.08% Driselase (Sigma), 0.03% Macerozyme R-10 (Yakult Honsha Co.). None of the enzymes used contained measurable amounts of EIX on western blots; however, they are known to induce ethylene biosynthesis. Enzymes were dissolved in the wash medium and filter sterilized and 15 mL were distributed in each 10-cm sterile plastic Petri dish. Tobacco leaves were surface sterilized with 30% Clorox followed by 70% ethanol and rinsed with sterile water and then cut into disks (90 mm in diameter), pricked (with a multiprickle device), and floated on the maceration fluid. The leaves were then cut into 1-mm-wide strips and incubated for 14 h at 25°C.

Up to this point, all operations were done under aseptic conditions. Protoplasts were then sieved (190-mesh metal sieve) and centrifuged in 30-mL Corex glass test tubes (5 min at 100*g*); the supernatant was removed and the pellet was resuspended in 25 mL of wash medium and centrifuged again as before. After removal of the supernatant, the protoplasts were suspended according to Kaiser et al. (1982) in 30% Percoll (Sigma) in wash medium, then 3 mL of 20% Percoll in wash medium were layered on top of the protoplast suspension, and finally, 1 mL of the wash solution was layered on top. This gradient was centrifuged at 100*g* for 5 min and the band of protoplasts at the 0 and 20% Percoll interface was collected with a Pasteur pipet. The protoplasts were washed three times by resuspending them in 25 mL of wash medium devoid of calcium (incubation medium) and centrifuged as above.

In preliminary experiments, we found that the presence of Ca^{2+} ions in the medium reduced the amount of ethylene that was produced by protoplasts in response to EIX. Washed protoplasts were diluted in incubation medium and the number of protoplasts was determined using a hemocytometer.

Treatment of Protoplasts with EIX

EIX was purified from xylan-induced cultures of *Trichoderma viride* as previously described (Dean at al., 1991) and desalted over an Excellulose GF-5 minicolumn (Pierce). Protoplasts were diluted in incubation medium to the desired concentration and 1 mL was placed in a 10-mL flask. EIX was added to the medium and the flasks were closed with rubber vaccine caps and placed on an orbital shaker operating at 70 rpm at room temperature. Preliminary experiments have shown that boiled EIX has no effect on the response of the protoplasts; thus, control treatments included equal volumes of incubation medium only. Head space was sampled after 4 h and ethylene levels were determined using a gas chromatograph as previously described (Lieberman et al., 1966). To measure ethylene production between 4 and 16 h, the experiment was carried out under aseptic conditions throughout. The flasks were opened after 4 h, then they were closed again and left for an additional 12 h, after which ethylene was measured.

Viability Staining

The viability of protoplasts was based on the ability of live protoplasts to exclude Evans blue. Dead or damaged protoplasts stained an intense blue. Protoplasts were incubated with a 0.01% solution of Evans blue in incubation medium for 20 min, and then 200 protoplasts were counted in each replication.

Addition of Cell Wall Material to Protoplasts

Leaf tissue of the Xanthi cultivar (1 g) was ground in 10 mL of cold wash medium. The extract was then centrifuged at 10,000*g* for 15 min and the supernatant was removed and discarded. The pellet containing cell walls and other cellular debris was resuspended in 10 mL of wash medium. Preliminary experiments showed that the pellet contained about 10 mg/mL of crude cell wall material. To confirm this, a sample was removed for dry weight determination (48 h at 80°C). One hundred microliters of the crude cell wall preparation were added to each 1 mL of protoplast suspensions in 10-mL flasks. Preincubation of the Xanthi cell wall-containing pellet, with or without EIX, was conducted for 3 h, in the dark, at 30°C prior to addition of the protoplast suspension.

Calcofluor-White Staining

Protoplasts were stained with calcofluor-white (Galbraith, 1981) to determine whether cell wall material was either left on protoplasts following protoplast isolation or formed during the incubation after digestion. Protoplasts were incubated for 1 min with a 0.01% solution of calcofluor-white in incubation medium and then washed five times, each with 1 mL of incubation medium. Protoplasts were viewed using a fluorescence microscope (Nikon inverted microscope, Diaphot-TMD with Epi-Fluorescence attachment TMD-EF) with 395 excitation and 420 barrier filters.

RESULTS AND DISCUSSION

EIX Induction of Ethylene Biosynthesis

The sensitivity of protoplasts to EIX was tested by measuring ethylene production in response to EIX treatment. We found that protoplasts of the Xanthi cultivar responded to EIX by enhanced production of ethylene in a concentration-dependent manner (Fig. 1). The amount of ethylene produced by protoplasts in response to EIX was affected by leaf conditions (e.g. age, nutrition level, etc.) and ranged in different experiments between 120 and 200% of that produced by control protoplasts.

Less than 50 ng/mL of protein were required for ethylene biosynthesis induction, and the response was saturated between 0.5 and 1.0 μg/mL of protein. The synthesis of ethylene induced by EIX could be completely blocked by 0.1 mM aminoethoxy vinyl glycine, whereas the addition of 0.1 mM ACC enhanced ethylene evolution (data not shown).

These results are similar to those obtained with leaf tissue

[4] Mention of trademark, proprietary product, or vendor does not constitute a guarantee of warranty by the U.S. Department of Agriculture and does not imply approval to the exclusion of other products or vendors that may also be suitable.

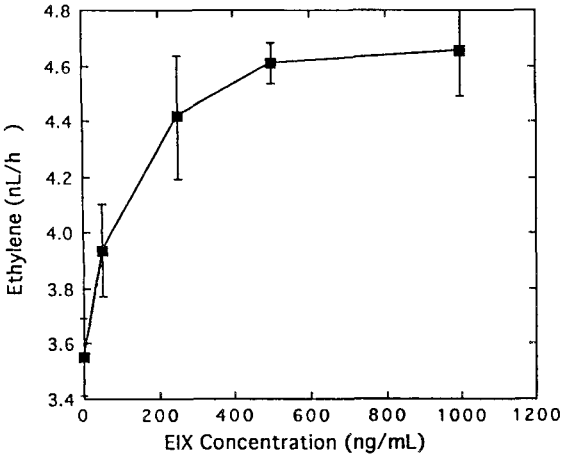

Figure 1. Effect of EIX concentration on ethylene production by tobacco protoplasts (3.5×10^5/mL). Protoplasts were prepared from ethylene-pretreated leaves and were incubated with EIX for 4 h at room temperature. Control protoplasts were treated with incubation medium only. Rates of ethylene production were calculated per 10^6 protoplasts.

(Bailey et al., 1990) and indicate that the biosynthetic pathway induced by EIX is identical in leaves and in protoplasts. In preliminary studies, we found that the amounts of ethylene induced by 1 μg/mL of EIX (4.5 nL h^{-1} 10^6 protoplasts^{-1}) were similar to those induced by 0.1 mM $CuSO_4$, which is known to elicit high rates of ethylene biosynthesis in tobacco (Mattoo et al., 1992).

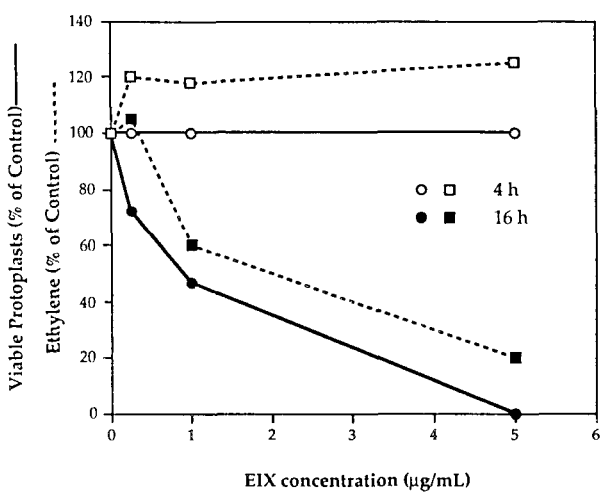

Figure 2. Effect of EIX concentration on protoplast viability and ethylene production. Protoplasts (3.5×10^5/mL) were prepared from ethylene-pretreated Xanthi leaves. Viability (based on Evans blue staining) and ethylene production were determined after 4 and 16 h. Rates of ethylene production were calculated per 10^6 protoplasts. Control protoplasts produced 2.3 nL/h ethylene during the 4-h incubation and 0.5 nL/h during the last 12 h of incubation, and 95% of the initial number of untreated (control) protoplasts were alive after 4 h and 92% of them were still alive after 16 h.

Effect of EIX on Protoplast Viability

In leaf tissue pretreated with ethylene, EIX induces measurable ethylene production within 1 h of treatment and necrosis is visible within 4 to 8 h (Bailey et al., 1990). Treatment of cell suspensions with EIX results in a decrease in ethylene biosynthesis within 15 min (Bailey et al., 1992) and cell death occurs within 4 h. Evans blue staining of protoplasts 4 h after application of EIX indicated no change in protoplast viability (Fig. 2, open symbols), yet measurable amounts of ethylene were produced. Death of the protoplasts could be detected between 4 and 16 h, and the number of dead protoplasts increased with EIX concentration. Ethylene production decreased as the EIX concentration was increased (Fig. 2, solid symbols). When treated with 5 μg/mL of EIX, all the protoplasts were dead after 16 h, and following treatment with 0.25 μg/mL of EIX, 25% of the protoplast population was dead. Untreated protoplasts lost about 5% of their viability during the same period of time. The loss of protoplast viability caused by EIX seems to be different than that caused by heat-stable phytotoxic factors released during protoplast production of cell cultures (Hahne and Lorz, 1988).

It was previously shown that leaves treated with ethylene became more sensitive to EIX, i.e. they produced more eth-

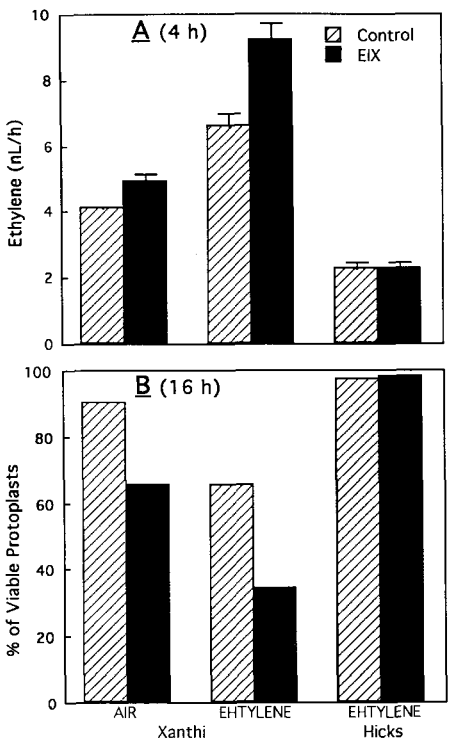

Figure 3. Effect of ethylene pretreatment and tobacco cultivar on the sensitivity of protoplasts to EIX. Protoplasts were prepared from air- and ethylene-pretreated leaves of Xanthi and from ethylene-pretreated leaves of Hicks. Xanthi and Hicks protoplasts were treated with 1 and 5 μg/mL of EIX, respectively. Control protoplasts were incubated in incubation medium only. Ethylene (A) was determined after 4 h and the percent of viable protoplasts (B) was determined after 16 h based on Evans blue staining. Rates of ethylene production were calculated per 10^6 protoplasts.

ylene and exhibited earlier necrosis (Bailey et al., 1990). This phenomenon was retained in the protoplast. Protoplasts prepared from ethylene-pretreated leaves were more sensitive to EIX than protoplasts prepared from fresh leaves, i.e. they produced more ethylene (Fig. 3A) and a higher percentage of the protoplasts were killed in response to EIX treatment (Fig. 3B). Recently, Bailey et al. (1993) reported that the tobacco cv Hicks is insensitive to EIX. We tested the sensitivity of protoplasts of cv Hicks and found that they too were insensitive to EIX. Even 100 times higher concentrations of EIX (5 μg/mL) did not induce ethylene production or cause death of the Hicks protoplasts (Fig. 3). This same phenomenon was found in intact leaf tissue.

The timing of ethylene biosynthesis induction and cell death in isolated protoplasts resembles that of leaf tissue much more than that of cell suspensions, although cell suspensions contain cell walls. In addition, protoplasts were shown to retain many other leaf characteristics (e.g. increased sensitivity by ethylene pretreatment and cultivar specificity). The similarity between protoplasts and leaf tissues was interpreted to mean that cell walls are not required for these EIX-induced responses and that the perception of EIX occurs elsewhere in the cell.

Addition of Cell Walls with EIX

The above data strongly suggest that the elicitation of ethylene biosynthesis by EIX occurs by direct interaction of EIX with the cells and is not the result of xylanase-released cell-wall components, as occurs in other systems (Hahn et al., 1989; Bucheli et al., 1990). Cell walls could not be detected on protoplasts that were stained with calcofluor-white 1 to 4 h after preparation (data not shown). However, remnants of cell walls may have remained in the protoplast's incubation medium, which could serve as substrate for the xylanase, even after our extensive washing. To test this possibility, a crude cell wall preparation was isolated and added to protoplasts along with EIX to test whether the cell walls, or other components present in this crude preparation, would enhance EIX-induced ethylene biosynthesis. The addition of this cell wall preparation did not enhance the response of protoplasts to a nonsaturating (50 ng/mL) or saturating (1 μg/mL) concentration of EIX (Table I). The addition of leaf cell wall preparation alone caused some inhibition of ethylene production but had no effect on the net increase of ethylene production elicited by EIX. Moreover, preincubating the crude cell wall preparation for 3 h with 50 ng/mL of EIX did not enhance the induction. Similarly, the addition of the crude suspension together with a saturating concentration of EIX (1 μg/mL) had no effect on the net increase of ethylene production elicited by EIX.

In conclusion, we have presented evidence that the elicitor activity associated with the β-1,4-endoxylanase from *T. viride* is not related to the production of cell wall-derived elicitor activity associated with ethylene biosynthesis. This conclusion is based on our finding that protoplasts respond to EIX, indicating that EIX interacts with other components of the cell (e.g. plasma membrane or other sites). Previously, we showed that the EIX is translocated in the xylem tissues and reaches the mesophyll cells of the leaf (Bailey et al., 1991; Sharon et al., 1992). To the best of our knowledge, this is the first report of a proteinaceous elicitor whose demonstrated enzymic activity is unrelated to its elicitor activity. The mode of action of this elicitor is not clear. It is possible that it interacts with a specific membrane receptor, but other modes of action, such as disruption of membrane integrity by integration into the membrane (Boman and Hultmark, 1987) or penetration and toxification of the cell (Takemoto, 1992), should also be considered.

Table I. *Effect of Xanthi tobacco leaf cell wall preparation on the response of Xanthi protoplasts (3.5 × 10^5/mL) to EIX treatment*

Rates of ethylene production were calculated per 10^6 protoplasts. Protoplasts were prepared from ethylene-pretreated Xanthi leaves.

A. Effect on ethylene production with a nonsaturating concentration of EIX

Treatment[a]	Ethylene (nL/h ± SD)
1. Control (assay medium only)	2.70 ± 0.16
2. EIX (50 ng/mL)	3.20 ± 0.19
3. Leaf cell wall only	1.96 ± 0.10
4. EIX (50 ng/mL) + leaf cell wall	2.30 ± 0.22
5. EIX (50 ng/mL) + leaf cell wall	2.50 ± 0.36

B. Effect on ethylene production with a saturating concentration of EIX

Treatment	Ethylene (nL/h ± SD)	
	No Cell Wall	Plus Cell Wall
Control	3.03 ± 0.07	2.80 ± 0.06
EIX (1 μg/mL)	4.20 ± 0.07	3.87 ± 0.10

[a] All treatments except for treatment no. 5 were preincubated in the dark at 30°C for 3 h before they were added to the protoplasts for 4 h of incubation. In treatment no. 5, EIX and the leaf cell wall preparation were added separately to the protoplasts just before the 4-h incubation.

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