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Alterations in *Nicotiana tabacum* L. cv Xanthi Cell Membrane Function following Treatment with an Ethylene Biosynthesis-Inducing Endoxylanase

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ABSTRACT

An ethylene biosynthesis-inducing xylanase (EIX) produced by the fungus *Trichoderma viride* elicited enhanced ethylene biosynthesis and leakage of potassium and other cellular components when applied to leaf disks of tobacco (*Nicotiana tabacum* L. cv Xanthi). Suspension-cultured cells of Xanthi tobacco responded to EIX by rapid efflux of potassium, uptake of calcium, alkalization of the medium, inhibition of ethylene biosynthesis, and increased leakage of cellular components. EIX-treated cell suspensions released 1-aminocyclopropane-1-carboxylate (ACC) into the surrounding medium, resulting in a reduction of cellular pools of ACC. The responses of both cell suspensions and leaf disks were inhibited (50–80%) by the preincubation of the tissues with the calcium channel blocker La^{3+}. High concentrations of EGTA inhibited the alkalization of the medium by cell suspensions responding to EIX, but EGTA alone caused extensive loss of K^+ and ACC and inhibited ethylene biosynthesis by tobacco cells. Alterations in membrane function appear to be important in the mode of action of EIX in Xanthi cells.

Most of the studies on the effects of EIX[2] on plant tissues have concentrated on the protein's ability to induce ethylene biosynthesis (11, 13–15, 18, 19). Recent observations that EIX, a β-1,4 endoxylanase, elicits electrolyte leakage and necrosis in whole tobacco tissues (6, 7) as well as biosynthesis of pathogenesis-related proteins (24) support the hypothesis that EIX functions as an elicitor of plant defense responses.

The list of biologically active molecules capable of eliciting plant cell defense responses is long and includes products of plant and pathogen cell wall breakdown (20) and proteins (1, 9) with and without carbohydrate side chains. EIX is a 22,000-D protein with a small carbohydrate component (13). Xylanases produce biologically active wall fragments in some plant systems (10). We have been unable to identify digestion products of EIX activity that contain biological activity. On the contrary, EIX may carry its biological activity as a separate component from the xylanase activity (J.F.D. Dean, personal communication).

Most of the plant defense responses in whole tissues attributed to EIX action require more than 1 h to quantify. These include electrolyte leakage (6), necrosis (6, 7), ethylene biosynthesis (6, 7, 18, 19), secondary product biosynthesis (17), and pathogenesis-related protein biosynthesis (24). The use of cell suspensions has allowed more rapid and sensitive detection of biological responses in many instances (3, 8, 12, 16, 21, 25, 29). We have used cell suspensions of tobacco (*Nicotiana tabacum* L.) as a tool for better understanding the immediate responses of tobacco to EIX.

MATERIALS AND METHODS

Chemicals and Enzymes

The chemicals used were of commercial origin. The EIX was purified as previously described (13) from xylan-induced *Trichoderma viride* cultures.

Plant Materials

Tobacco plants (*Nicotiana tabacum* L. cv Xanthi) were grown under greenhouse conditions. Detached tobacco leaves were incubated for 14 h in a humid chamber in the dark under an atmosphere of 120 μL/L of ethylene (6) prior to use in leaf disk assays. The leaf disc assays were carried out on six leaf disks (1 cm diameter, average total weight 85 mg) in 1 mL of assay buffer (250 mM sorbitol, 0.1 mM $CaCl_2$, 10 mM Mes at pH 6.0) in 25-mL Erlenmeyer flasks.

Xanthi cell-suspension cultures were originally produced by placing whole seedlings in Schenk and Hildebrandt medium (28) (pH 6.0) supplemented with 2.2 μM 2,4-D, 10.7 μM *p*-chlorophenoxyacetic acid, and 0.46 μM kinetin. The cell suspensions were maintained on a rotary shaker (200 rpm) and transferred to fresh medium on a 7-d schedule. Cell suspensions 5 or 6 d old were washed in assay buffer by filtration. The cell-suspension assays were carried out on 200 mg of cells in 1 mL of assay buffer as described for the leaf disk assays. All the data points presented for leaf disk and cell-suspension assays represent the mean of three replicates. Each experiment was repeated at least twice.

Tissue Treatments

Additions (0.1–10.0 mM) of $LaCl_3$ and EGTA were made to assays 30 min prior to treatment with EIX. During experi-

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[2] Abbreviations: EIX, ethylene biosynthesis-inducing xylanase; ACC, 1-aminocyclopropane-1-carboxylate.

ments with EGTA, the assay buffer was removed after the 30-min preincubation and replaced with similar assay medium plus or minus EIX to maximize removal of Ca^{2+} and other metals by chelation. The $CaCl_2$ was left out of the assay medium during the EGTA experiments. $LaCl_3$ was vacuum infiltrated into leaf disks immediately after addition to assays.

EIX was applied directly to the assay medium in concentrations of 0 to 5000 ng. Immediately after EIX application, the flasks were sealed with rubber septa and incubated at room temperature in the light for up to 20 h on a rotary shaker (100 rpm).

Ethylene Measurement

A 3-mL gas sample was taken from each flask prior to removing the rubber septum. Ethylene production was quantified by GC (22) and is expressed as nL of ethylene produced/g of tissue.

Medium Alkalization, Chloride Efflux, and Absorbance

The assay medium for each sample was removed from the flask, leaving behind as much tissue as possible, and centrifuged 5 min in a table-top centrifuge to remove remaining cell debris. Assay medium from each sample was split into two fractions. One fraction from each sample was used to determine medium pH and absorbance or, in some cases, Cl^- content, which was determined with an Orion[3] model 96–17B combination chloride electrode (3). The other fraction was used to determine K^+ concentration.

The pH of the medium was assayed directly, and the sample was centrifuged for 5 min. The supernatant was heated in a boiling water bath for 5 min and centrifuged again. The absorbance of the supernatant was determined at 200, 260, 280, 320, and 500 nm in a Hewlett-Packard 8452A Diode Array spectrophotometer. The absorbance values reported are recorded as absorbance at 280 nm minus absorbance at 500 nm.

K^+ Efflux

The fractions for K^+ concentration determination were prepared as described above for the A_{280} measurements. Suitable sample aliquots were diluted with demineralized water and spiked with the equivalent of 1 mg of $CsCl_2$/g of tissue prior to analysis of K^+. Potassium analysis was performed with an air-acetylene flame in an Instrumentation Laboratory model 257 atomic absorption spectrophotometer. The concentration of K^+ is expressed as μmol of K^+ released/g of tissue.

Measurement of ACC

The samples used to determine ACC content were collected by replacing the assay buffer with extraction buffer (100 mM Hepes, 4 mM DTT, 2.5 μM pyridoxal 5′-phosphate, pH 8.0) at various time points after EIX treatment, freezing the cells with liquid nitrogen, and grinding the cells on ice in a mortar and pestle. The cell debris was removed from samples by centrifugation, and ACC content was determined by conversion to ethylene by the methods of Lizada and Yang (23). The ACC content of the assay medium was determined similarly. The ACC content is expressed as nmol of ACC/g of tissue.

Ca^{2+} Uptake

Radioactive Ca^{2+} ($^{45}Ca^{2+}$) was used to quantify the uptake of calcium by cell suspensions. The isotope $^{45}Ca^{2+}$ (0.5 μCi/assay) was added to the assay medium 15 min prior to addition of EIX. The uptake of $^{45}Ca^{2+}$ was stopped by adding 5 mL of assay buffer containing 10 mM $CaCl_2$. The cells were filtered onto glass-fiber filters and washed four times with 5 mL of the stop buffer, allowing 2 min between filtering for Ca^{2+} exchange. The washed cells were placed in 20 mL of Beckman Ready Safe liquid scintillation cocktail and kept in the dark overnight before they were counted. Ca^{2+} uptake is expressed as nmol of Ca^{2+} taken up/g of tissue.

RESULTS

Cell-Suspension Responses to EIX Addition

The addition of EIX to cell suspensions resulted in a rapid increase in the uptake of Ca^{2+}, which was detectable 15 min after application of EIX (Fig. 1A). The rate of uptake exceeded 3.7 nmol g^{-1} of tissue min^{-7} over the first 30 min after EIX treatment. During this time, the efflux of K^+ increased from near zero to 530 nmol g^{-1} of tissue min^{-1} (Fig. 1B). An increase in K^+ efflux was detectable within 7.5 min of EIX application. The rates of both Ca^{2+} uptake and K^+ efflux declined by 60 min posttreatment.

Cell suspensions responded to EIX by rapid alkalization of the surrounding medium (Fig. 2A) in a concentration-dependent manner, but the response was saturated at 1 μg of EIX/200 mg of cells. Alkalization of the medium was detectable after application of EIX concentrations as low as 8 ng/200 mg of cells. The absorbance of the medium at 280 nm (Fig. 2B) increased in response to EIX in a manner similar to alkalization of the medium. Ethylene production (Fig. 2C) was rapidly inhibited in cell suspensions when EIX was applied. The inhibition of ethylene accumulation associated with EIX treatment was correlated with the reduction of cellular ACC pools and the accumulation of ACC in the medium (Fig. 3). Leakage of ACC along with changes in the medium absorbance and ethylene production were not affected by 8 ng of EIX/200 mg of cells, although alkalization of the medium was consistently observed at this level of EIX.

La^{3+}, a Ca^{2+} channel blocker, inhibited all of the measured EIX-induced responses of cell suspensions at concentrations as low as 0.1 mM (Fig. 4). Ca^{2+} uptake was strongly inhibited by 0.1 mM $LaCl_3$ (Fig. 4A). $LaCl_3$ alone had little effect on any of the cell responses in control tissues (non-EIX-treated). Although washing cells with the chelator EGTA inhibited alkalization of the medium in response to EIX (Fig. 5), the EGTA treatment alone increased K^+ efflux (Fig. 5B) and ACC

[3] Mention of a 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.

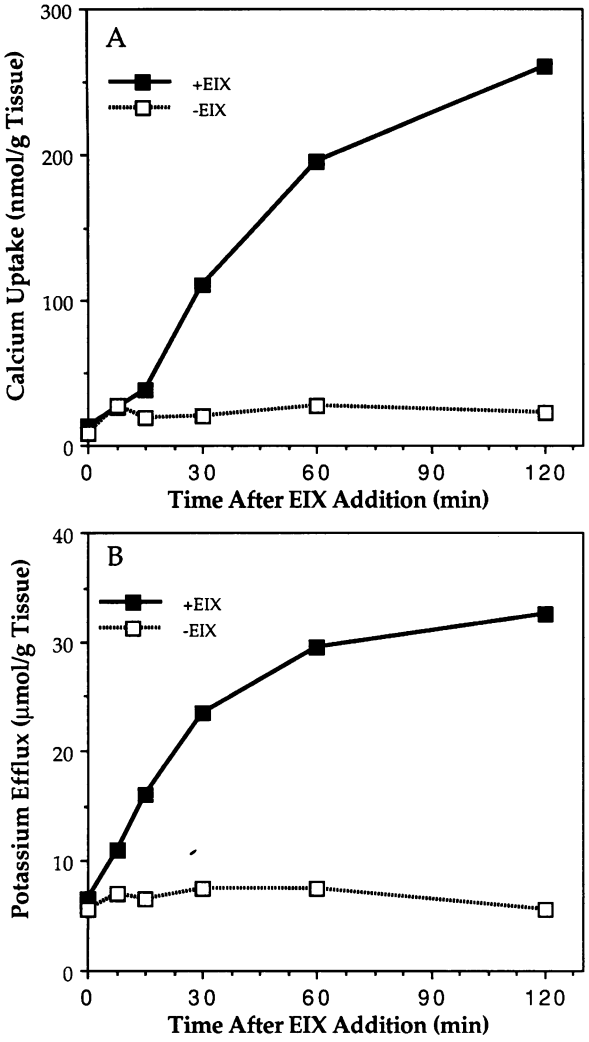

Figure 1. Effect of EIX on Ca^{2+} uptake (A) and K^+ efflux (B) by Xanthi cell suspensions. Xanthi cells (200 mg) were treated with 1 μg of EIX in 1 mL of assay medium. Ca^{2+} uptake and K^+ efflux were determined 0, 7.5, 15, 30, 60, and 120 min after application of EIX.

leakage (Fig. 5D) and decreased ethylene production (Fig. 5C) by cell suspensions.

Leaf Disc Responses

Leaf discs responded to EIX by increased K^+ efflux, ethylene biosynthesis, and release of A_{280}-absorbing compounds into the medium (Fig. 6). The responses developed more slowly than the responses of cell suspensions. We were unable to detect alkalization of the medium in the leaf disc assays despite considerable effort to do so (data not shown). Leakage of ACC was not observed in leaf disc assays within 8 h (data not shown).

The addition of 0.5 mM $LaCl_3$ to the leaf disc assays resulted in partial inhibition of all the measured responses to EIX over the first 4 h of the assays (Fig. 6). After 4 h, the leaf disc responses to EIX in the presence of $LaCl_3$ increased and, in the case of K^+ efflux, exceeded the response to EIX in the absence of La^{3+}.

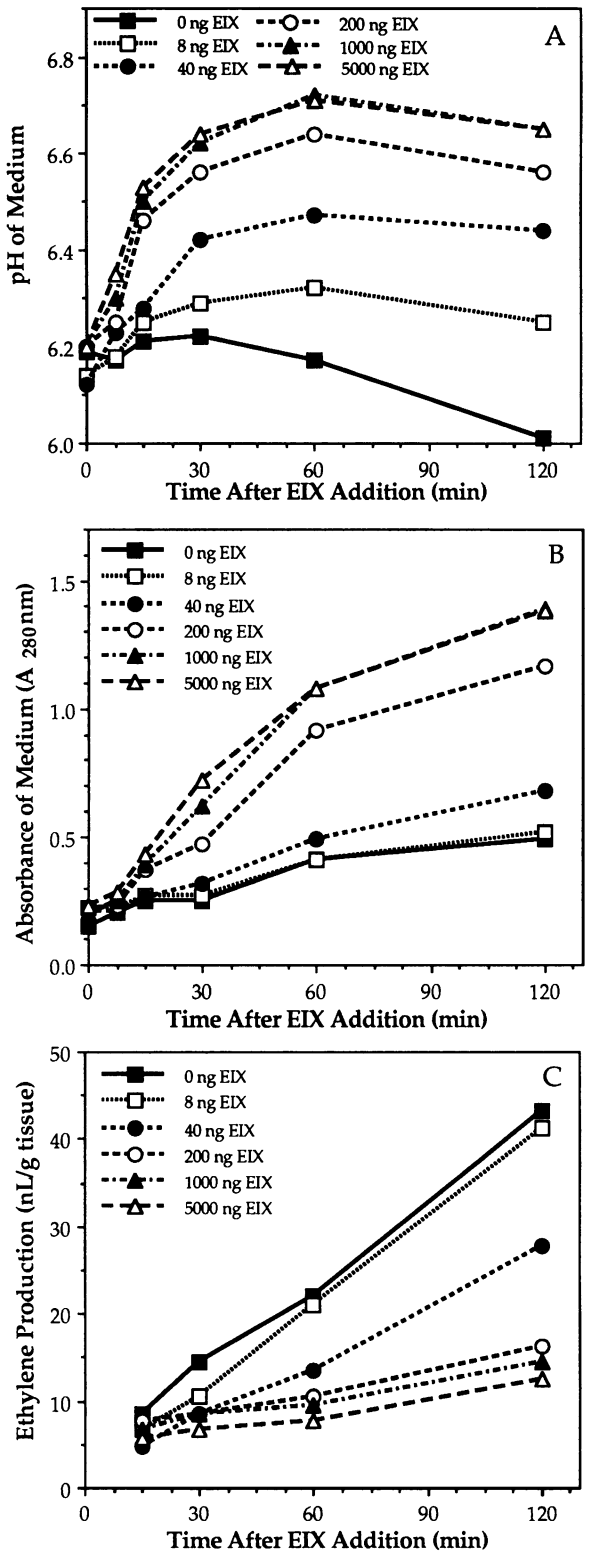

Figure 2. Effect of varying concentrations of EIX on alkalization of the medium (A), changes in medium absorbance (B), and ethylene production (C) by Xanthi cell suspensions. Cells (200 mg) were treated with 0, 8, 40, 200, 1000, and 5000 ng of EIX in 1 mL of assay medium. Measurements were made 0, 7.5, 15, 30, 60, and 120 min after application of EIX.

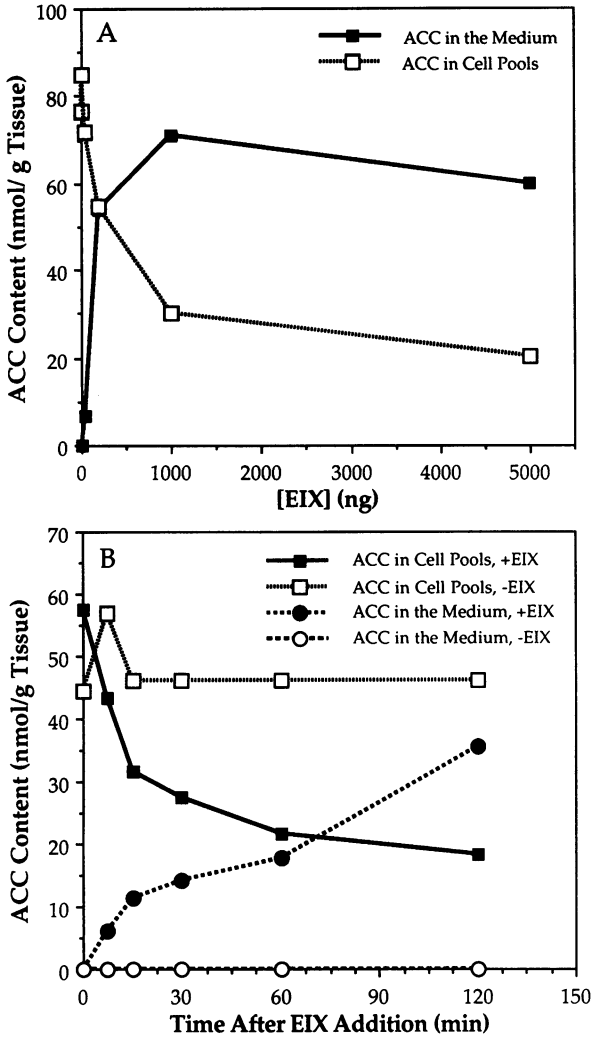

Figure 3. The leakage of ACC and changes in cellular pools of ACC in Xanthi cell suspensions treated with EIX. A, The effect of varying concentrations of EIX (0, 8, 40, 200, 1000, 5000 ng) were measured 2 h after EIX application to 200 mg of cells in 1 mL of assay medium. B, The time dependence of the response was determined at 0, 7.5, 15, 30, 60, and 120 min after EIX application (1 μg/mL of assay buffer).

Cl^- Loss

Leakage of Cl^- (3.39 μmol g^{-1} of tissue h^{-1}) from cell suspensions (Fig. 7A) treated with EIX occurred at approximately 1/10th the rate of K^+ leakage (34.5 μmol g^{-1} of tissue h^{-1}). Cl^- loss (0.231 μmol g^{-1} of tissue 8 h^{-1}) from leaf discs (Fig. 7B) treated with EIX was very near the limits of detection occurring at less than 1/15th the rate of K^+ loss (3.54 μmol g^{-1} of tissue 8 h^{-1}).

DISCUSSION

Although cell suspensions, leaf discs, and whole Xanthi plants (6, 7) are sensitive to EIX, they respond differently. Leaf discs showed enhanced ethylene production in response to EIX, and cell suspensions produced less ethylene in response to EIX. This may reflect EIX-induced leakage of ACC, the precursor of ethylene, and a resultant lowering of cellular pools of ACC. Little is known about transport of ACC out of cells, but transport of ACC across the plasmalemma and into the vacuole is energy dependent (27). Factors that interfere with cell metabolism, such as metabolic uncouplers or anaerobic conditions, block transport of ACC into the vacuole (27) and allow net loss of ACC out of the cell by diffusion (B. Saftner, personal communication). The importance of H^+ ATPase activity in plant defense responses has been demonstrated (2, 8). It has previously been shown that leaf discs accumulate ACC when treated with the cell wall-degrading enzyme mix Cellulysin, which was the original source of EIX (11).

A second inconsistency is the alkalization of the medium by cultured cells but not by leaf discs after treatment with EIX. Leaf discs cut from EIX-treated detached Xanthi leaves exhibited electrolyte leakage and extracellular acidification when placed in water (6). However, the acidification was also associated with widespread necrosis, which has not been observed in leaf discs placed in assay medium containing osmoticum. Atkinson et al. (5) observed alkalization of the medium by both cell suspensions and leaf discs of tobacco undergoing a hypersensitive response, although the rate was greatly reduced in leaf disc assays. The differences between responses of cultured cells and leaf discs may result from the differences in state of cell differentiation between the two tissues. The remaining responses, medum A_{280}, K^+ efflux, and the effect of La^{3+}, were observed in both cell suspensions and leaf discs.

All the measured responses of cell suspensions to EIX were detectable within 15 min of EIX treatment and can be attributed, at least in part, to alterations in membrane function. The alkalization of the medium by Xanthi cell culture is stimulated by 8 ng of EIX, but leakage of cell ACC, inhibition of ethylene production, and increased absorption of the medium are not stimulated to a measurable extent by this amount of EIX. We have not determined the identity or origin of the compounds responsible for the increase in absorbance of the medium. The compounds may be released from the cell wall in response to EIX-stimulated Ca^{2+} and K^+ fluxes, or they may be released through the plasmalemma in response to EIX action. EGTA had many of the same effects on cells as EIX, but it did not cause alkalization of the medium, suggesting that alkalization is independent of ACC leakage and reduced ethylene production. Some of the side effects of EGTA on tobacco cells have been previously noted (5).

Because La^{3+} inhibits Ca^{2+} uptake, as well as all the other responses of tobacco to EIX, Ca^{2+} channel-mediated influx may be required for EIX responses. Thus, early responses of Xanthi tobacco cells to EIX may include stimulation of Ca^{2+} and K^+ channels and alteration of net H^+ transport. These observations are similar to responses characterized as part of the hypersensitive response in tobacco. The hypersensitive response of tobacco to *Pseudomonas syringae* pathovars involves K^+ efflux and H^+ and Ca^{2+} influx to the exclusion of other ions (4, 5), leading to the hypothesis that specific ion channels are involved. The low level of Cl^- leakage observed is a measure of nonspecific electrolyte loss (3) and suggests that a limited amount of the observed electrolyte leakage in

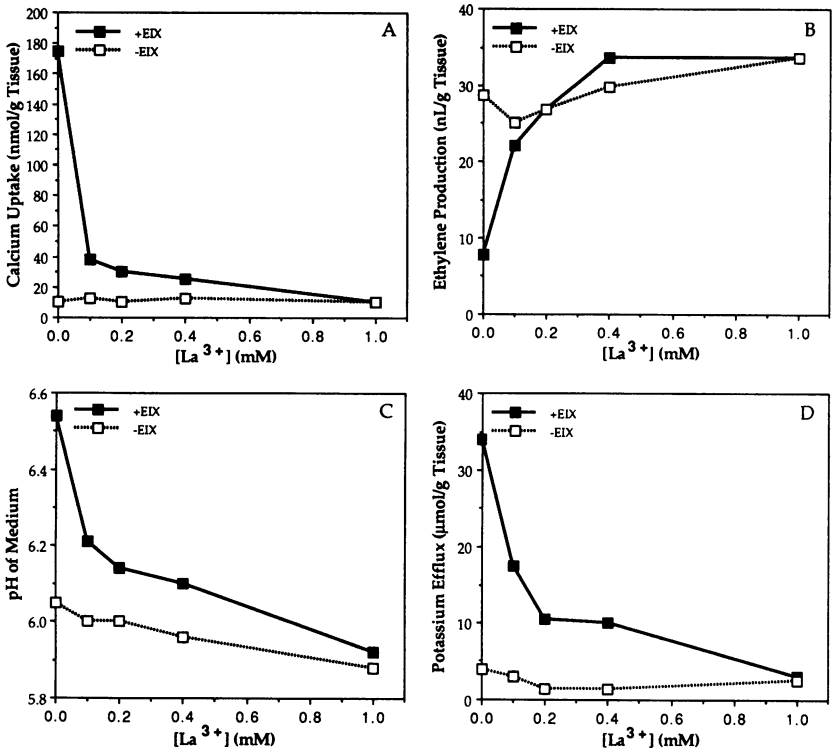

Figure 4. Effect of varying concentrations of $LaCl_3$ on cell suspensions responding to EIX application. The cells (200 mg) were placed in 1 mL of assay medium containing 0, 0.1, 0.2, 0.4, and 1.0 mM $LaCl_2$ 30 min prior to application of EIX (1 µg). Measurements were made 2 h after application of EIX for Ca^{2+} uptake (A), ethylene biosynthesis (B), alkalization of the medium (C), and K^+ efflux (D).

response to EIX may result from general membrane damage. Electrolyte leakage has been noted when tobacco cells were treated with other proteinaceous elicitors of fungal origin (8, 12, 26).

EIX is a well-characterized protein with β-1,4 endoxylanase activity. Some xylanases produce cell wall fragments with elicitor activity (10), but when EIX was tested under these same conditions it was ineffective at eliciting defense responses (S. Kauffmann, personal communication). Similarly, a xylanase isolated from *Magnaporthe grisea* cultures (a gift from S. Kauffmann), which is known to produce wall fragments with elicitor activity, did not elicit plant defense re-

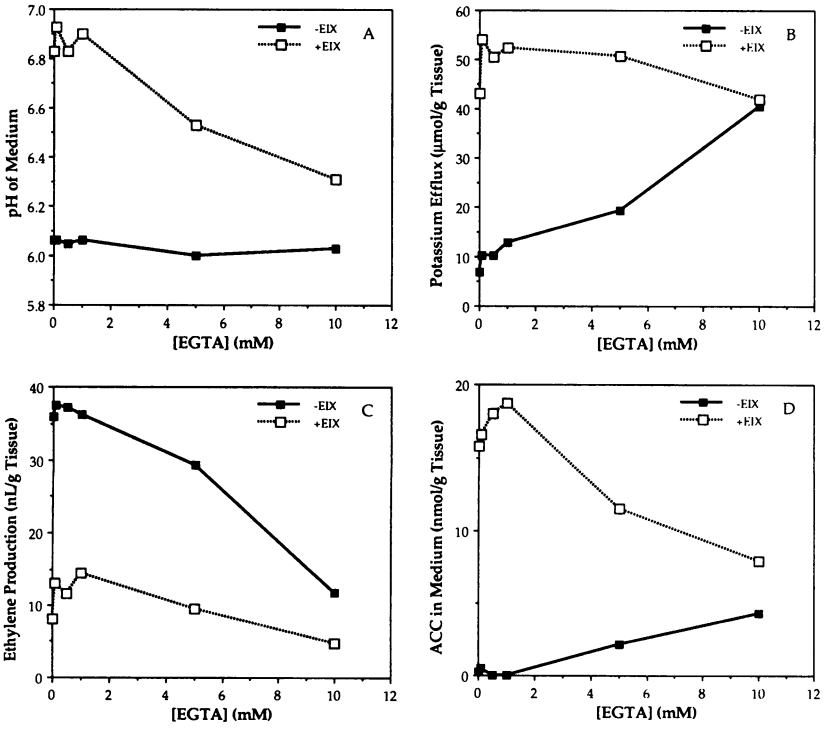

Figure 5. Effect of varying concentrations of EGTA on cell suspensions responding to EIX application. The cells (200 mg) were placed in 1 mL of assay medium minus $CaCl_2$ containing 0, 0.1, 0.5, 1.0, 5.0, and 10.0 mM EGTA 30 min prior to application of EIX (1 µg). Measurements were made 2 h after application of EIX for alkalization of the medium (A), K^+ efflux (B), ethylene production (C), and leakage of ACC into the medium (D).

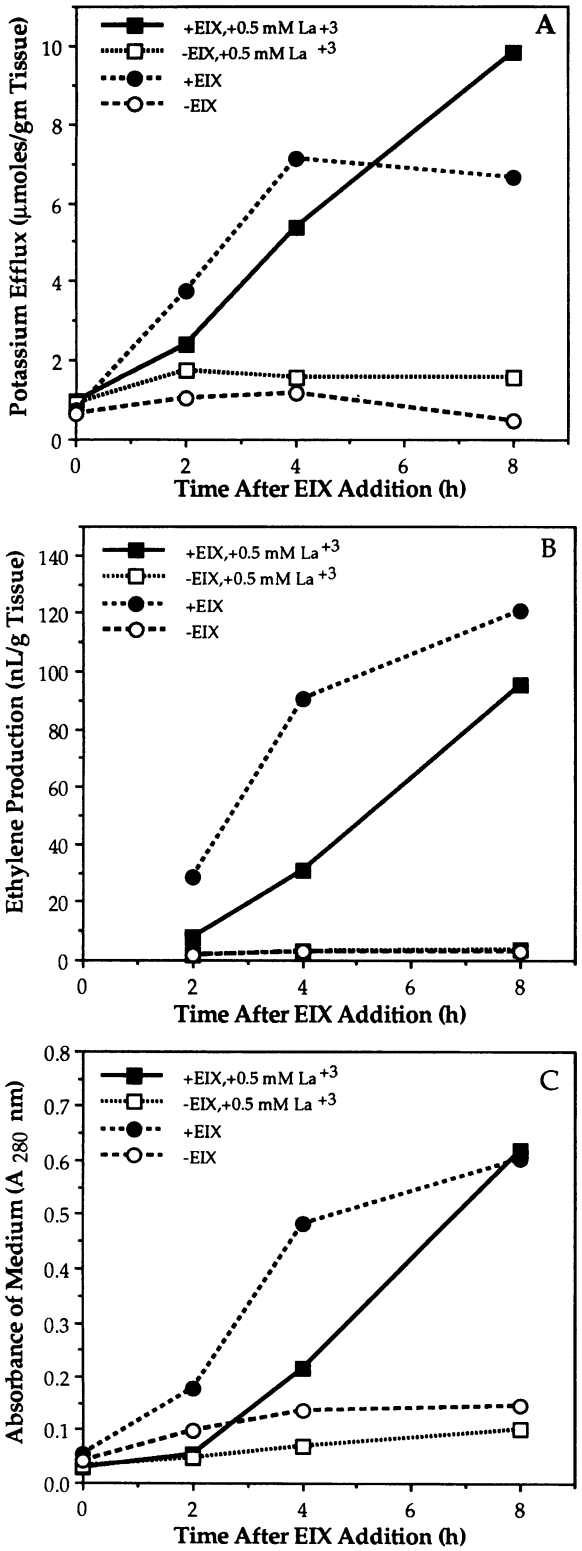

Figure 6. Effect $LaCl_3$ on Xanthi leaf discs responding to EIX application. The $LaCl_3$ (0 or 0.5 mM) in assay medium was vacuum infiltrated into leaf discs 30 min prior to application of EIX (1 μg). Measurements were made 0, 2, 4, and 8 h after application of EIX for K^+ efflux (A), ethylene production (B), and absorbance of the medium (C).

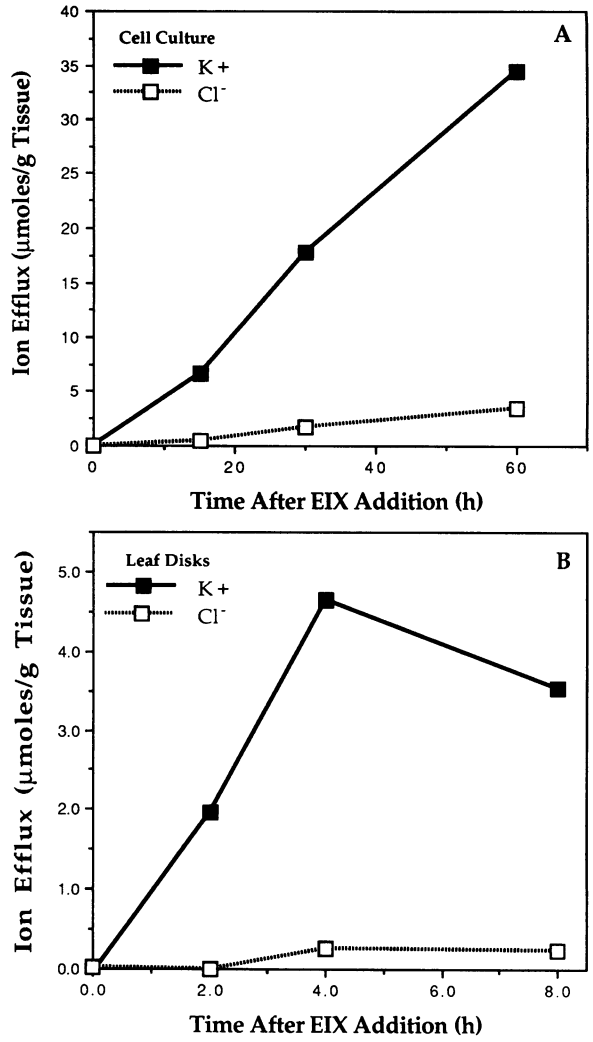

Figure 7. A comparison of the leakage of Cl^- and K^+ from cell suspensions (A) and leaf discs (B) in response to treatment with EIX (1 μg EIX/mL of assay medium). The reported values represent the means of differences between EIX-treated and untreated samples at each time point.

sponses in Xanthi tobacco (data not shown). Evidence suggests that EIX carries its elicitor activity as a separate function from the xylanase activity (J.F.D. Dean, personal communication). Evidence has been provided, in the case of cryptogein, that a proteinaceous elicitor may stimulate cell responses through receptor-mediated interactions (8). We remain intrigued by the possibility that a cell wall-degrading enzyme may function as an elicitor by directly interacting with cell membranes in a receptor-mediated response.

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