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Sensitivity to an Ethylene Biosynthesis-Inducing Endoxylanase in *Nicotiana tabacum* L. cv Xanthi Is Controlled by a Single Dominant Gene

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The ethylene biosynthesis-inducing xylanase (EIX) is known to be a potent elicitor of ethylene biosynthesis and other responses when applied to leaf tissue of *Nicotiana tabacum* L. cv Xanthi. In contrast, leaf tissue of the tobacco cultivar Hicks was insensitive to EIX at concentrations 100-fold higher than was needed to elicit responses from Xanthi. Cell-suspension cultures of Xanthi and Hicks showed similar differences in sensitivity to EIX. Equivalent levels of ethylene production were elicited in leaf discs of both cultivars after treatment with $CuSO_4$. The F_1 and Xanthi backcross progeny of Hicks and Xanthi crosses were all sensitive to EIX, whereas the F_2 and Hicks backcross progeny segregated for sensitivity to EIX. Individual plants from the F_2 and Hicks backcross that were insensitive to EIX produced only insensitive progeny when they were self-pollinated. Progeny from sensitive plants either segregated for sensitivity to EIX or produced all sensitive progeny (an F_2 plant). Sensitivity to EIX is controlled by a single dominant gene, based on chi-square analysis of segregation ratios.

EIX is a 22-kD β-1,4-endoxylanase isolated from filtrates of xylan-induced *Trichoderma viride* liquid cultures (Dean and Anderson, 1991). Similar xylanases have been identified in xylan-induced culture filtrates of plant pathogenic fungi (Dean et al., 1989). EIX elicits enhanced ethylene biosynthesis, altered membrane permeability, and necrosis when applied to leaves of *Nicotiana tabacum* L. cv Xanthi (Bailey et al., 1990, 1991). Membrane permeability is also altered in suspension-cultured Xanthi cells treated with EIX, but ethylene production is inhibited because ACC leaks out of treated cells (Bailey et al., 1992b). Most of the responses to EIX identified in Xanthi tobacco are characteristic of plant responses to exogenously applied elicitors (Toppan and Esquerre-Tugaye, 1984; De Wit et al., 1985; Kurosaki et al., 1987; Daniel et al., 1990; Keen et al., 1990; Blein et al., 1991; Conrath et al., 1991), as well as of plant resistance to infection by pathogenic organisms (De Wit et al., 1985; Atkinson et al., 1990).

Xylanases have been reported to have elicitor activity on a number of plant species (Farmer and Helgeson, 1987; Ishi, 1988; Bucheli et al., 1991; Felix et al., 1991; Ronen et al., 1991). A xylanase isolated from *Magnaporthe grisea*, the causal agent of rice blast, has been shown to produce cell wall fragments that kill plant cells (Bucheli et al., 1991). We have been unable to identify active xylan fragments in the EIX interaction with Xanthi tobacco (Dean et al., 1990). Polypeptide elicitors devoid of any known enzyme activity (Toppan and Esquerre-Tugaye, 1984; De Wit et al., 1985; Blein et al., 1991; van den Ackerveken et al., 1991) can produce symptoms similar to those observed in EIX-treated plant tissues, presumably by direct interaction with cell membranes (Blein et al., 1991).

We report here that EIX does not elicit defense responses in all tobacco cultivars. This raises the possibility that the elicitor effect of EIX is dependent on the genetic makeup of the cultivar. van den Ackerveken et al. (1991) have isolated low mol wt proteins from the *Cladosporium fulvum* interaction with tomato that elicit defense responses in a cultivar-specific manner. We have characterized the differences in sensitivity between the insensitive tobacco cultivar Hicks and the sensitive Xanthi. To determine the genetic control of sensitivity to EIX, various crosses were made involving Xanthi and Hicks as well as their progeny. Progeny of these crosses were evaluated as to their sensitivity to EIX using ethylene biosynthesis and tissue necrosis as the measured responses (Bailey et al., 1990, 1991).

MATERIALS AND METHODS

Chemicals and Enzymes

The EIX was purified as previously described from xylan-induced cultures of *Trichoderma viride* (Dean and Anderson, 1991). All chemicals used were commercially available.

Growth and Handling of Plant Materials

The tobacco cultivars Hicks and Xanthi (*Nicotiana tobaccum* L.) were grown under greenhouse conditions until they were 20 to 30 cm tall. Leaf disks (1 cm in diameter, approximately 85 mg) cut from detached leaves of Hicks or Xanthi tobacco that had been pretreated with ethylene (120 μL/L, 14 h) were placed in 25-mL flasks, each containing 1 mL of assay medium (10 mM Mes, pH 6.0, 250 mM sorbitol). Cell cultures of Xanthi and Hicks tobacco maintained on SH medium (Schenk and Hildebrandt, 1972) were washed with assay medium and transferred to 25-mL flasks (200 mg of cells/flask), each containing 1 mL of assay medium.

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Abbreviation: EIX, ethylene biosynthesis-inducing xylanase.

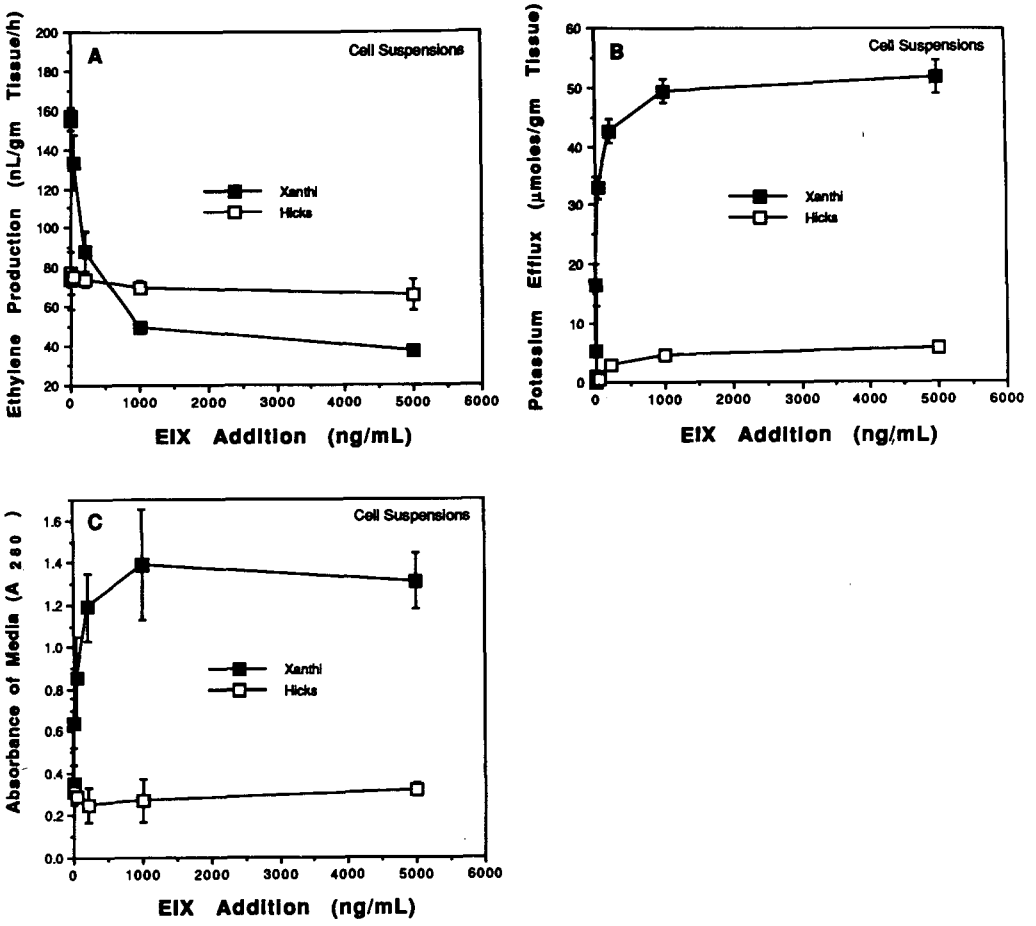

Figure 1. A comparison of the effects of EIX (0–5000 ng) on ethylene biosynthesis (A), potassium efflux (B), and changes in medium A_{280} (C) by Xanthi and Hicks tobacco cell cultures (200 mg). Samples (three replications/point) were taken 2 h after EIX application.

EIX was applied directly to the assay medium in concentrations that varied from 0 to 5000 ng/mL. In separate experiments, leaf discs were treated with $CuSO_4$ (10 mM) in assay medium (pH 5.0). Immediately after EIX or $CuSO_4$ addition, the flasks were sealed with rubber septa and incubated in the light for up to 20 h at room temperature on a rotary shaker (100 rpm).

Measurement of Tissue Responses

Gas samples (3 mL) were taken at varying times from the flask head space for determination of ethylene production by GC (Lieberman et al., 1966). The assay medium was removed from the flask and medium pH, absorbance, and K^+ concentration were determined (Bailey et al., 1992b). The pH of the medium was assayed directly. The absorbance of the medium was determined at 280 and 500 nm using a diode array spectrophotometer (Hewlett-Packard[1] model 8452A). The absorbance values reported are recorded as absorbance at 280 nm minus absorbance at 500 nm (A_{280}). The K^+ concentration for each sample was determined with an air-acetylene flame using an atomic absorption spectrophotometer (Instrumentation Laboratory model 257). The concentration of K^+ is expressed as μmol of K^+/g of tissue.

Scheme for Genetic Evaluation

The genetic control of sensitivity to EIX was studied using progeny of crosses between Hicks and Xanthi cultivars. Reciprocal crosses were made between Hicks and Xanthi to produce an F_1 population. Individuals from the F_1 generation were self-pollinated to produce the F_2 population, and others were backcrossed to parental Xanthi or Hicks. Individual plants from the F_2 generation, as well as Hicks backcross progeny, were self-pollinated after having been classified as either sensitive or insensitive to EIX. The sensitivity of F_1, F_2, and backcross progeny to EIX was determined by measuring ethylene production in 20-h leaf disc assays as described above. The total ethylene produced was rounded off to the nearest nL g^{-1} of tissue h^{-1}. Plants that produced less than 0.5 nL of ethylene g^{-1} of tissue h^{-1} were classified as insen-

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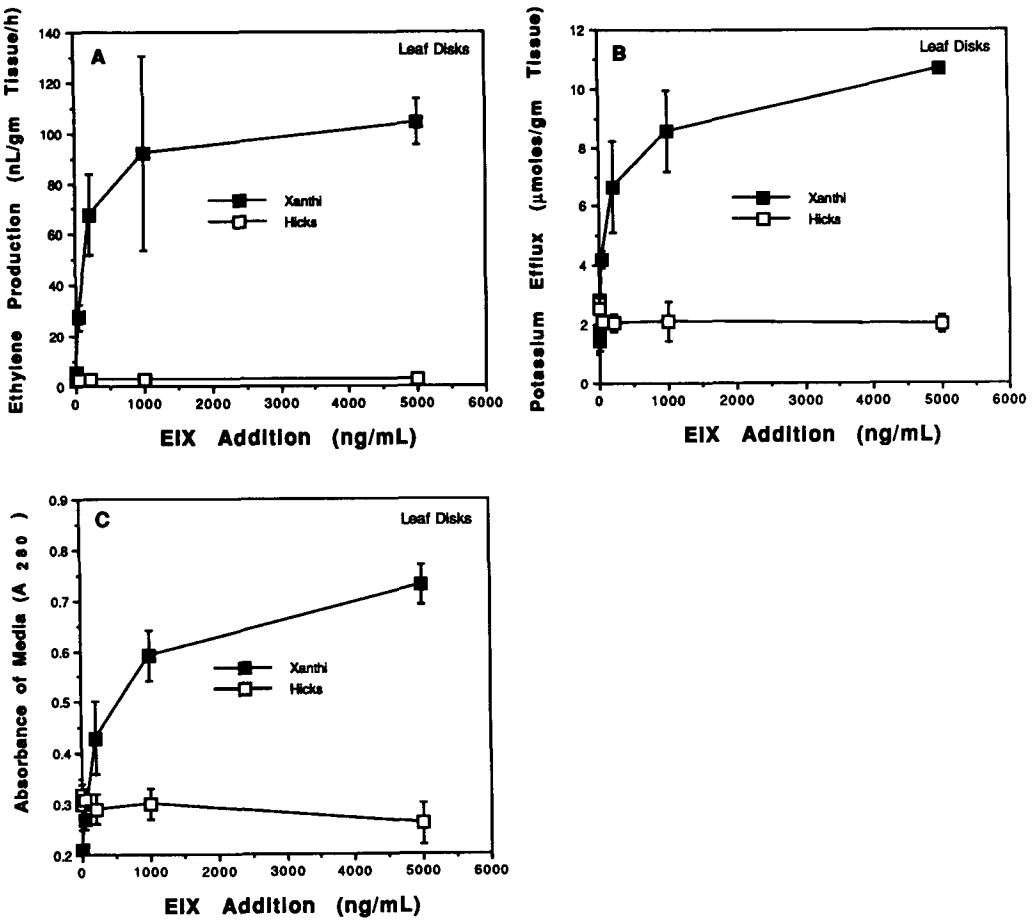

Figure 2. A comparison of the effects of EIX (0–5000 ng) on ethylene biosynthesis (A), potassium efflux (B), and changes in medium A_{280} (C) by Xanthi and Hicks tobacco leaf discs. Samples (three replications/point) were taken 4 h after EIX applications.

sitive to EIX for the purpose of further study. The sensitivity of F_3 and self-pollinated Hicks backcross progeny were determined by infiltrating a single leaf on intact plants with a solution of 1 μg of EIX/mL of water and observing necrotic/chlorotic symptom production 4 d after treatment. Water alone or EIX boiled in water for 10 min was used to infiltrate control plants.

RESULTS

EIX had very little effect on ethylene production, K^+ efflux, or A_{280} of the medium when applied to cell cultures of the Hicks cultivar (Fig. 1). Similar treatment of Xanthi cell cultures resulted in efflux of K^+, release of compounds absorbing at A_{280}, and inhibition of ethylene production (Bailey et al., 1992b). EIX failed to elicit any of these responses when applied to leaf discs of the Hicks cultivar (Fig. 2) at concentrations that saturated the responses in Xanthi. Both cultivars responded to $CuSO_4$ treatment with enhanced ethylene production (Fig. 3).

Infiltration with a solution of 1 μg of EIX/mL of water of leaves on tobacco plants in the greenhouse resulted in necrosis of Xanthi leaf tissue within 4 d of treatment (Fig. 4). Hicks leaf tissue was unaffected by infiltration with solutions containing EIX.

All Xanthi plants were sensitive to EIX as measured by increased ethylene production, whereas individual plants of the Hicks cultivar uniformly failed to produce any measurable ethylene in response to EIX (Fig. 5A). The F_1 population of the Hicks × Xanthi cross were all sensitive (produced ethylene) to EIX (Fig. 5B). The response of F_1 plants was not affected by which parent served as the female. The F_2 population segregated for sensitivity to EIX (Fig. 5C). All progeny from F_1 backcrosses onto Xanthi were sensitive to EIX (Fig. 5D), whereas approximately one-half of the progeny from each backcross onto Hicks were sensitive to EIX. The total variability in ethylene production was large in all populations other than Hicks itself.

The developmental stage of leaves and plants apparently has a large effect on the total ethylene produced by a particular leaf on a particular plant. This is borne out by the observation that F_2 plants that produced only 1 or 2 nL of ethylene g^{-1} of tissue h^{-1} in initial evaluations produced up to 5-fold higher levels of ethylene when evaluated 4 weeks later (Fig. 6). Plants that produced less than 0.5 nL of ethylene

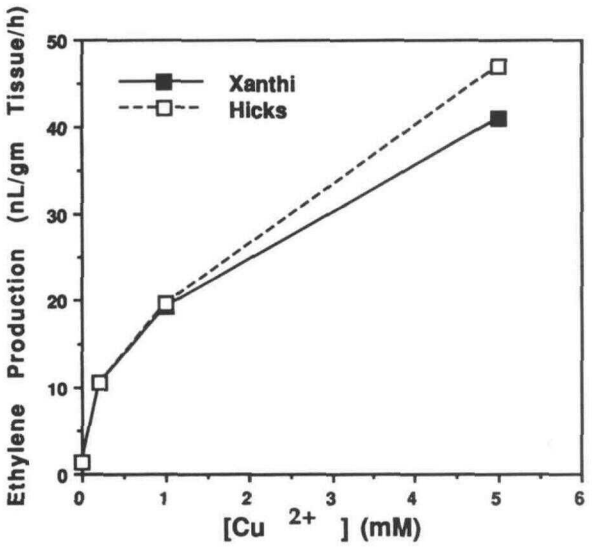

Figure 3. A comparison of the effects of $CuSO_4$ on ethylene biosynthesis by Xanthi and Hicks tobacco leaf discs. Leaf discs were treated with pH 5.0 assay buffer with or without $CuSO_4$. Samples (three replications/point) were taken 4 h after EIX applications.

g^{-1} of tissue h^{-1} in initial evaluations uniformly failed to respond to EIX when evaluated a second time 4 weeks later.

The F_2 and Hicks backcross progeny that were classified as insensitive to EIX based on lack of an ethylene response uniformly produced offspring that were insensitive (no necrotic/chlorotic symptoms) to EIX (Table I). Offspring from Hicks backcross progeny that produced ethylene in response to EIX segregated for sensitivity (necrotic/chlorotic) to EIX. Offspring (F_3) from sensitive F_2 progeny either segregated for sensitivity to EIX or, in one case, were all sensitive. Plants that necrosed in response to EIX also produced ethylene when treated with EIX as leaf discs (Fig. 7), whereas those plants that did not necrose in response to EIX infiltration also failed to produce ethylene. Chi-square analysis (Gardner, 1984) of the results from the various generations of Hicks and Xanthi crosses indicates that the segregation ratios are consistent with the hypothesis that a single dominant gene confers sensitivity to EIX in Xanthi tobacco (Table II).

DISCUSSION

Dramatic differences in sensitivity to EIX exist between the Hicks and Xanthi tobacco cultivars and are consistent between cell cultures and leaf discs. EIX moves easily through the vascular tissues of both Hicks (Sharon et al., 1992) and Xanthi (Bailey et al., 1990, 1991; Sharon et al., 1992). The apparent molecular mass of EIX (Dean and Anderson, 1991) under nondenaturing conditions (9.2 kD) is small enough that EIX should be capable of penetrating the cell wall (Carpita, 1982) and interacting directly with the plasmalemma. Leaf discs and cell cultures allow direct access of EIX to the intercellular spaces, so it is unlikely that the difference in sensitivity observed between the two cultivars is due to variable penetration of EIX. Both cultivars are capable of producing ethylene at high rates, at least in response to $CuSO_4$ treatment. It has been demonstrated that induction of ethylene in tobacco by Cu^{2+} (Mattoo et al., 1992), as well as by EIX (Bailey et al., 1990), is dependent on ACC synthase (Bailey et al., 1992a) activity. Xanthi is not the only tobacco cultivar that is sensitive to EIX (Lotan and Fluhr, 1990), although the comparative degree of sensitivity in other cultivars has not been determined. There is some evidence that other species of plants may be sensitive to EIX (Felix et al., 1991; Ronen et al., 1991), but the nature and specificity of observed responses are unknown.

The data indicate that the difference in sensitivity to EIX between Hicks and Xanthi is conferred by a single dominant gene for sensitivity carried by Xanthi. Plants carrying the gene respond to EIX by induction of ethylene biosynthesis and necrosis, suggesting that all responses to EIX are under the control of one gene. The ethylene response, as might be expected, was highly variable and strongly affected by factors other than EIX, which include plant age. Other factors are likely to include tissue age and nutritional level, as well as environmental conditions. Despite the variability of the ethylene response in sensitive plants, plants classified as insen-

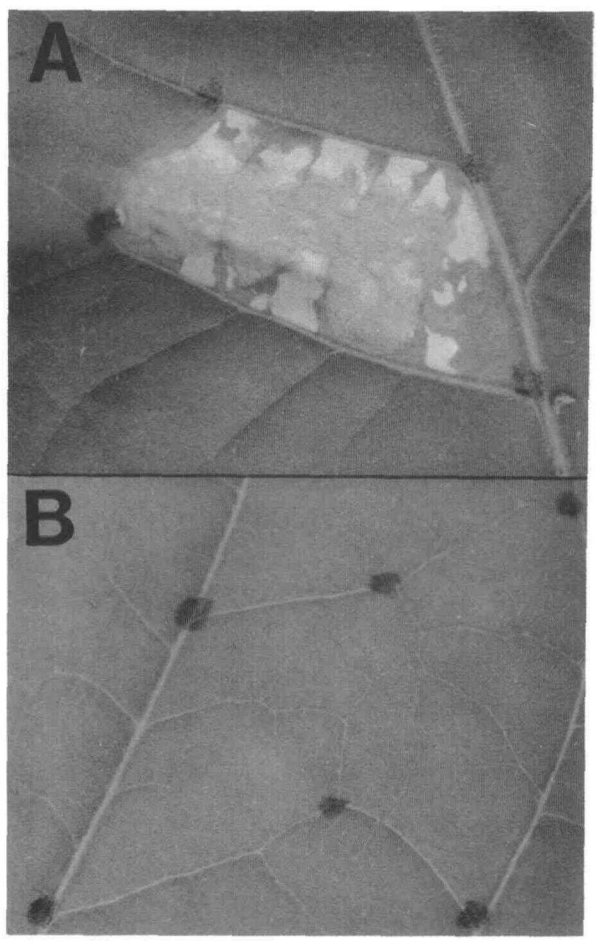

Figure 4. Elicitation of necrosis by infiltration of tobacco leaves with a solution of 1 μg of EIX/mL of water. A, Xanthi. B, Hicks. Photos were taken 4 d after treatment.

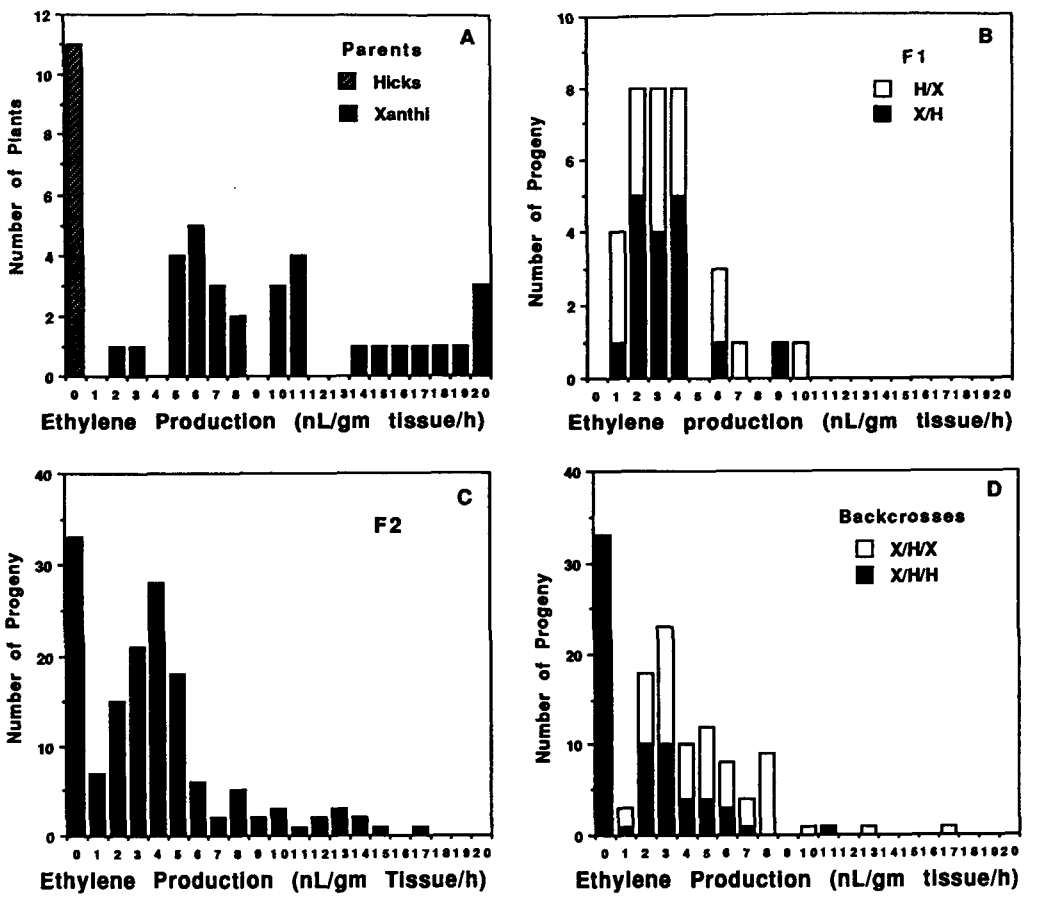

Figure 5. Ethylene production by leaf discs of Hicks and Xanthi (A), F_1 (B), F_2 (C), and backcross progeny (D) in response to EIX treatment. The F_1 generation was made up of progeny from Hicks by Xanthi crosses using Hicks (H/X) or Xanthi (X/H) as the pollen source. The F_2 generation consisted of self-pollinated F_1 plants. F_1 plants were backcrossed onto both Hicks (X/H/H) and Xanthi (X/H/X). Ethylene measurements were made 20 h after treatment with EIX (1 μg of EIX/mL of assay buffer). Values represent the difference between EIX-treated samples and controls treated with either boiled EIX or distilled water.

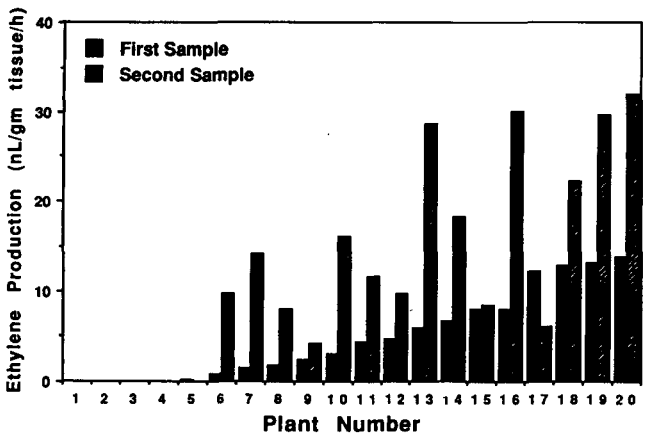

Figure 6. The effect of sampling time on response of F_2 plants to EIX treatment. Samples were taken 4 weeks apart on fully expanded leaves. Ethylene measurements were made 20 h after treatment with EIX (1 μg of EIX/mL of assay buffer). Values represent the difference between EIX-treated samples and controls treated with either boiled EIX or distilled water.

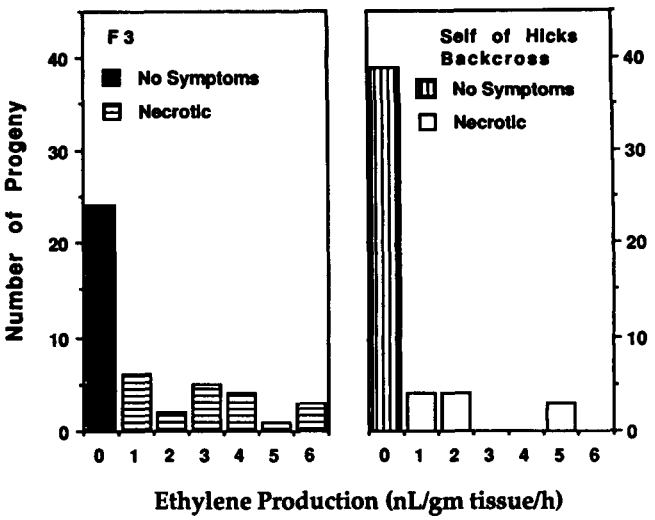

Figure 7. Elicitation of ethylene biosynthesis in leaf discs from F_3 and self of Hicks backcross progeny. The F_3 consists of progeny from self-pollinated F_2 plants that had previously been classified as either sensitive or insensitive to EIX. Progeny from the F_1 backcross onto Hicks were self-pollinated to give the self of Hicks backcross progeny. Plants were initially classified as sensitive (symptoms) or insensitive (no symptoms) to EIX based on the necrotic response to EIX infiltration. Leaf discs were then cut from ethylene-primed leaves from each plant and treated with EIX (1 μg of EIX/mL of assay buffer). Ethylene measurements were made 20 h after EIX treatment.

Table I. *Elicitation of necrosis/chlorosis (Nec/Chl) by EIX in progeny of F_2 and Hicks backcross parents*

Plants 20 to 30 cm tall were infiltrated with EIX (1 μg of EIX/mL of H_2O) and observed for symptom development after 4 d.

Parental Line Number	Response		
	Parent	Progeny[b]	
	(C_2H_4[a])	Nec/Chl	No symptoms
	nL g^{-1} tissue h^{-1}	*No. of progeny*	
F_2			
1	0	0	20
2	0	0	20
3	0	0	20
4	0	0	20
5	0	0	20
6	1	14	6
7	2	15	5
8	4	16	2
9	6	15	5
10	7	57	0
11	13	13	7
Hicks backcross			
1	0	0	20
2	0	0	20
3	0	0	20
4	0	0	20
5	3	15	4
6	4	13	5
7	5	17	3

[a] Leaf disks from F_2 and Hicks backcross were treated with EIX (1 μg/mL of assay buffer). Ethylene production was measured 20 h after treatment. [b] Individual plants from the F_2 and Hicks backcross were self-pollinated after being classified as either sensitive or insensitive to EIX based on the ethylene response. Progeny were then evaluated for sensitivity to EIX using tissue necrosis/chlorosis as the measured response.

sitive to EIX in initial studies remained insensitive to EIX and produced only insensitive progeny. The necrosis/chlorosis response was very distinct, with little variation with repeated sampling. The total ethylene production of sensitive F_2 and backcross parents had no detectable influence on the segregation ratios of progeny in the necrosis/chlorosis evaluation.

Sensitivity to EIX appears to be regulated in a manner similar to the hypersensitive response in plant-pathogen interactions, where single dominant genes predominate as the controlling factor (Gabriel, 1989). It is possible that EIX generates a cell wall fragment (Bucheli et al., 1991) that Xanthi recognizes and Hicks does not. However, we have been unable to demonstrate the existence of biologically active wall fragments (Dean et al., 1990). The response of Xanthi tobacco to EIX is very similar to the response of plant tissues to other proteinaceous elicitors where no known enzyme activity has been identified (Toppan and Esquerre-Tugaye, 1984; De Wit et al., 1985; Ricci et al., 1989; Blein et al., 1991). The similarities do not end there, since EIX has a relatively small native conformation (9.2 kD based on size exclusion column chromatography) and a high isoelectric point (9.1) (Dean and Anderson, 1991), characteristics that are very similar to those of another proteinaceous elicitor, cryptogein (Ricci et al., 1989). We are intrigued by the observation that sensitivity to a β-1,4-endoxylanase isolated from *T. viride* is controlled by a single dominant gene in Xanthi tobacco.

Table II. *Chi-square (χ^2) analysis of data from crosses involving Hicks and Xanthi*

The data are consistent with the hypothesis that a single dominant gene confers the difference in sensitivity observed between Hicks (H) and Xanthi (X). Sen, Sensitive; Insen, insensitive.

Cross	Ratio	*n*	Expected (Sen:Insen)	Observed (Sen:Insen)	χ^2	P
	Sensitivity based on ability of EIX to induce ethylene production					
F_1 (H/X)	1:0	33	33:0	33:0		
Xanthi backcross (H/X/X)	1:0	62	62:0	62:0		
Hicks backcross (H/X/H)	1:1	67	33.5:33.5	34:33	0.015	0.95–0.8
F_2 (H/X Self)	3:1	150	112.5:37.5	117:33	0.787	0.4–0.3
	Sensitivity based on ability of EIX to induce necrosis					
H/X/H Self						
Sensitive	3:1	59	44.3:14.7	47:12	0.684	0.45–0.35
Insensitive	0:1	80	0:80	0:80		
F_3 (F_2 Self)						
Sensitive (Ho[a])	1:0	57	57:0	57:0		
Sensitive (Het[a])	3:1	95	71.3:23.7	70:25	0.088	0.75–0.8
Insensitive	0:1	100	0:100	0:100		

[a] The sensitive F_2 is expected to be made up of two-thirds heterozygous (Het) and one-third homozygous (Ho) plants for sensitivity to EIX if a single gene confers sensitivity. Of six sensitive F_2 plants selfed and carried to the F_3 generation, only one appears homozygous, with 57 of 57 plants tested being sensitive to EIX.

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