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High-affinity binding site for ethylene-inducing xylanase elicitor on *Nicotiana tabacum* membranes

Uri Hanania and Adi Avni*
Botany Department, Tel-Aviv University, Tel-Aviv 69978, Israel

Summary

Challenge of *Nicotiana tabacum* cv Xanthi with the ethylene-inducing xylanase (EIX) from *Trichoderma viride* causes rapid induction of plant defense responses leading to hypersensitive necrosis. This phenomenon is cultivar-specific; no response is detected when *N. tabacum* cv Hicks is similarly treated. The responsiveness is determined in tobacco and tomato by a single dominant gene. EIX was labeled with fluorescein-isothiocyanate and incubated with cell suspension cultures, protoplasts or microsomal membranes. Binding of EIX to the microsomal membranes was found to be specific and saturable, with a dissociation constant of 6.2 nM. Using confocal laser microscopy, the EIX binding site was localized to the plasma membrane. Binding of EIX to its high-affinity site occurred in responsive species. These results demonstrate the existence of a high-affinity binding site for EIX on the plasma membrane of responsive cultivars. Chemical cross-linking of EIX to microsomal membranes from responding plants revealed a 66 kDa protein complex. This protein may function as the receptor that mediates the hypersensitive response induced by EIX binding.

Introduction

The interaction between plants and incompatible pathogens induces a multi-component defense response. This inducible reaction results either from transcriptional activation of defense-related genes (Avni *et al.*, 1994b; De Wit *et al.*, 1986; Dixon and Lamb, 1990; Keen, 1992; Lotan and Fluhr, 1990) or from specific enzymatic activation (Bradley *et al.*, 1992; Kauss *et al.*, 1989). These reactions are frequently associated with highly localized responses leading to hypersensitive cell death (Atkinson, 1993; Jakobek and Lindgren, 1993). Initiation of these events is triggered by a signal, usually generated by organic compounds termed elicitors, but similar responses can also be triggered by synthetic compounds.

Elicitors that trigger plant defense responses have been isolated from a variety of phytopathogenic and non-pathogenic micro-organisms (Ebel and Cosio, 1994; Fuchs *et al.*, 1989; Ricci *et al.*, 1993). The ability to induce plant defense responses by elicitors at nanomolar concentrations suggests the involvement of elicitor recognition proteins, i.e. receptors (Ebel and Cosio, 1994). In several cases, elicitor-binding proteins have been characterized (Basse *et al.*, 1993; Cote *et al.*, 1995; Hahn *et al.*, 1994; Kooman-Gersmann *et al.*, 1996; Nurnberger *et al.*, 1994).

A highly purified fungal 22 kDa protein (β-1,4-endoxylanase), referred to as ethylene-inducing xylanase (EIX), was isolated from *Trichoderma viride* cultures (Dean *et al.*, 1989; Fuchs *et al.*, 1989). EIX is very active in inducing ethylene biosynthesis in *Nicotiana tabacum* (Bailey *et al.*, 1990), tomato and pepper (Avni *et al.*, 1994a). EIX induces ethylene production, accumulation of pathogen related (PR) proteins and phytoalexins, electrolyte leakage and hypersensitive responses (HR) in *N. tabacum* cv Xanthi (Bailey *et al.*, 1990, 1992; Lotan and Fluhr, 1990) and tomato leaf tissue (Avni *et al.*, 1994a). These biochemical changes are all common responses of plants to exogenously applied elicitors (Blein *et al.*, 1991; Bottin *et al.*, 1994; Felix *et al.*, 1993; Keen, 1992; Keen *et al.*, 1990). The xylanase activity of EIX is probably not required for the elicitation process (Sharon *et al.*, 1993). If so, some other recognition mechanism involving EIX is responsible for the various defense responses.

Here we report the existence of a high-affinity binding site for the EIX elicitor in *Nicotiana tabacum* and *Lycopersicon esculentum* membranes. High-affinity binding occurs only in *N. tabacum* cv Xanthi or *Lycopersicon esculentum* cv M82 varieties which are genetically capable of responding to the elicitor.

Results

Binding of labeled elicitor to plant cells and protoplasts

EIX was labeled with fluorescein isothiocyanate (FITC) as a first step to characterize possible interactions with plant cells. Labeling EIX with FITC does not alter its elicitor activity in leaf disks (Table 1). The induction of ethylene biosynthesis and xylanase activity by FITC–EIX was indistinguishable from that of unlabeled EIX (Table 1). FITC–EIX was incubated with cells derived from EIX-responsive *N. tabacum* cv Xanthi or EIX-non-responsive *N. tabacum* cv Hicks. Cells derived from suspension cultures of each variety looked normal and proliferated at the same rate; however, FITC–EIX interacted only with the cv Xanthi cells (Figure 1).

Further characterization of FITC–EIX binding was per-

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*For correspondence (fax +972 3 640 9380; e-mail lpavni@ccsg.tau.ac.il).

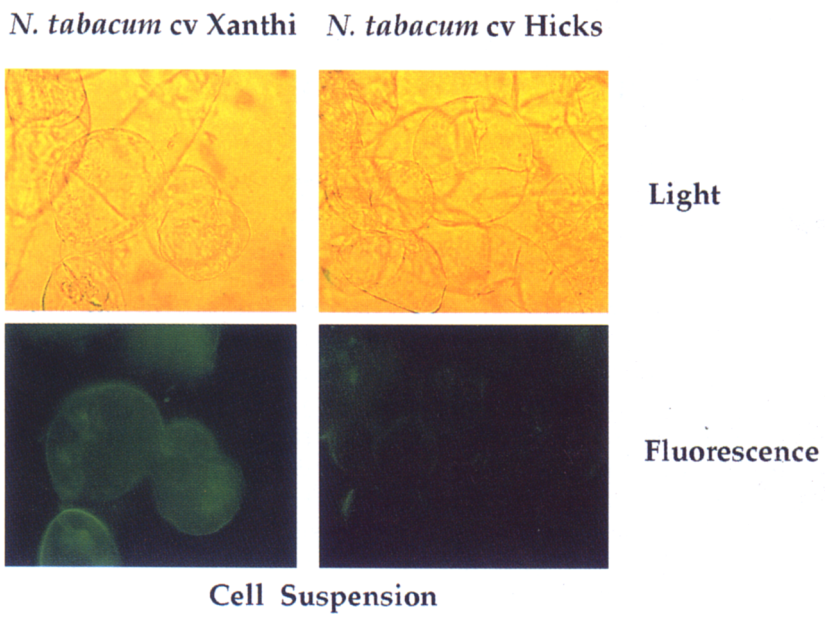

Figure 1. Binding of FITC–EIX to tobacco cells.
Cells were incubated with 20 nM FITC-labeled EIX for 30 min. Two type of cells were used: *N. tabacum* cv Xanthi (EIX-responsive) and *N. tabacum* cv Hicks (EIX-non-responsive). Cells were washed three times and examined under light and fluorescence microscopy.

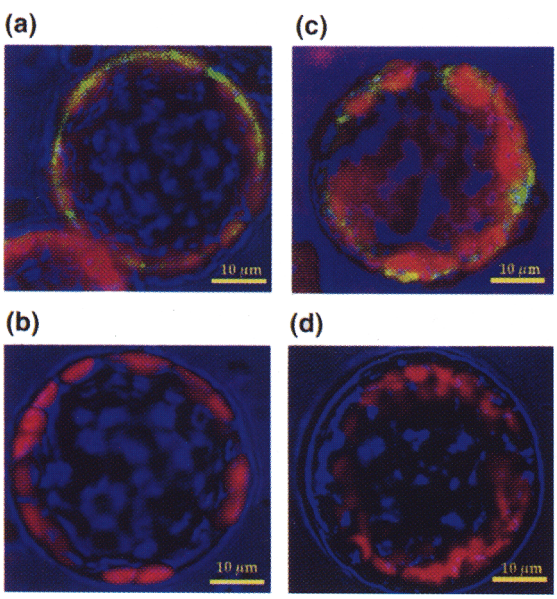

Figure 2. Binding of FITC–EIX to *N. tabacum* protoplasts.
Protoplasts were incubated with 20 nM FITC-labeled EIX. Folowing incubation for 30 min, protoplasts were washed twice and examined with confocal laser-scanning microscope. Chloroplasts are colored due to chlorophyll fluorescence (seen as red), FITC–EIX labeling appears green and Nomarski images are blue. A 10 μm bar is indicated. (a) *N. tabacum* cv Xanthi; (b) *N. tabacum* cv Hicks; (c) *L. esculentum* cv M82; (d) *L. esculentum* cv IL90.

formed with protoplasts, using confocal laser-scanning microscopy (CLSM). Protoplasts of *N. tabacum* cv Xanthi and *L. esculentum* cv M82 showed intense fluorescent staining of EIX on the plasma membrane (Figure 2a,c), while no EIX fluorescence was detected on protoplasts

Table 1. Endo-1,4-β-xylanase activity and induction of ethylene biosynthesis by FITC–EIX and EIX

Elicitor	Endo-1,4-β-xylanase activity[a]	Ethylene biosynthesis (nl h^{-1} g^{-1})[b]
EIX	0.121 ± 0.004	10.25 ± 2.47
FITC–EIX	0.127 ± 0.003	10.02 ± 1.20

[a]Endo-1,4-β-xylanase activity was measured at A_{595} as described in Experimental procedures using 1 μg EIX.
[b]The induction of ethylene biosynthesis was measured as described by Bailey *et al.* (1990) using leaf disks from *N. tabacum* cv Xanthi.

isolated from cv Hicks and cv IL90 (Figure 2b,d). Fluorescence-activated cell sorting (FACS) showed that FITC–EIX was bound to 98% and 83% of the protoplasts isolated from the Xanthi and M82 cultivars respectively (Figure 3a,c), but to less than 6% of those from the non-responding Hicks and IL90 cultivars (Figure 3b,d). The amount of fluorescence detected on protoplasts isolated from the FITC–EIX-treated Hicks and IL90 cultivars was not significantly higher than that of the non-treated controls. FACS analysis showed that treatment of protoplast isolated from Xanthi caltivar with 60 μg ml^{-1} proteinase K for 2 h abolished the binding of FITC–EIX (Figure 3e), suggesting that the EIX binding site is a protein.

Binding of FITC-labeled elicitor to tobacco membranes

The binding of EIX to the plasma membrane was characterized by measuring florescence of washed microsomal

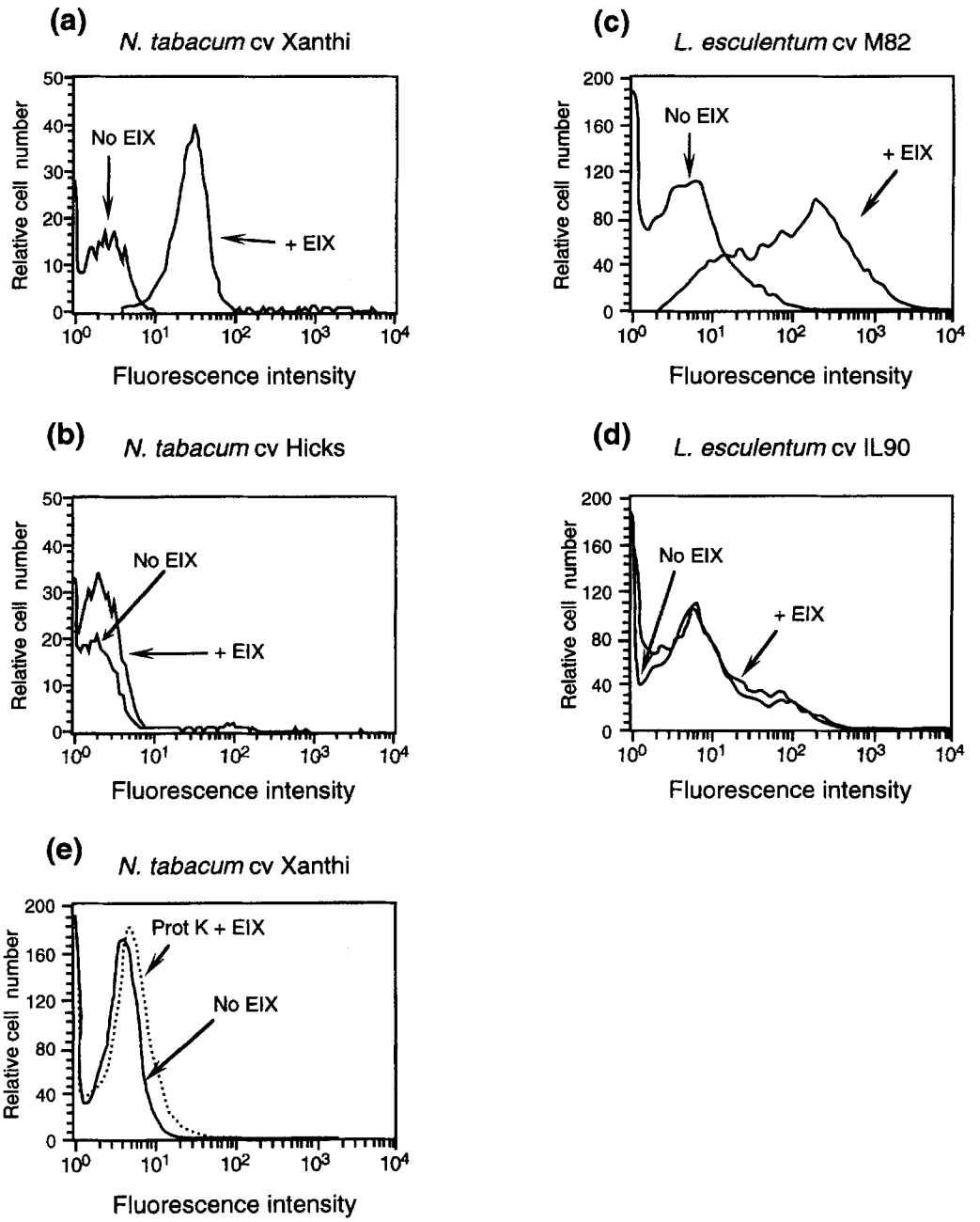

Figure 3. Sorting of tobacco and tomato protoplasts.
Protoplasts were incubated with 20 nM FITC-labeled EIX for 30 min. Protoplasts were from (a) *N. tabacum* cv Xanthi (EIX-responsive); (b) *N. tabacum* cv Hicks (EIX-non-responsive); (c) *L. esculentum* cv M82 (EIX-responsive); (d) *L. esculentum* cv IL90 (EIX-non-responsive). (e) Protoplasts of *N. tabacum* cv Xanthi were incubated with 60 μg ml^{-1} proteinase K for 2 h followed by incubation with 20 nM FITC-labeled EIX for 30 min as indicated. Protoplasts were washed twice and then analyzed by FACS as described in Experimental procedures based on their fluorescence signal.

membranes following incubation with FITC–EIX (Figure 4). The parameters of EIX binding were determined by several criteria as described by Hulme and Birdsall (1992). For saturation analysis, microsomal preparations were incubated with increasing concentrations (1–50 nM) of FITC-labeled EIX. Saturation of specific binding, calculated by deducting non-specific binding from the total binding, occurred at an EIX concentration of 20 nM. Total binding in the saturation range continued to increase due to an increase in non-specific binding (Figure 4a,c). Linearization of the data in a Scatchard plot (Figure 4e) and a Hill plot (Figure 4f) indicated the existence of a high-affinity, single-class binding site for EIX in microsomal membranes from *N. tabacum* cv Xanthi (Figure 4e; K_d 6.2 nM) and EIX-

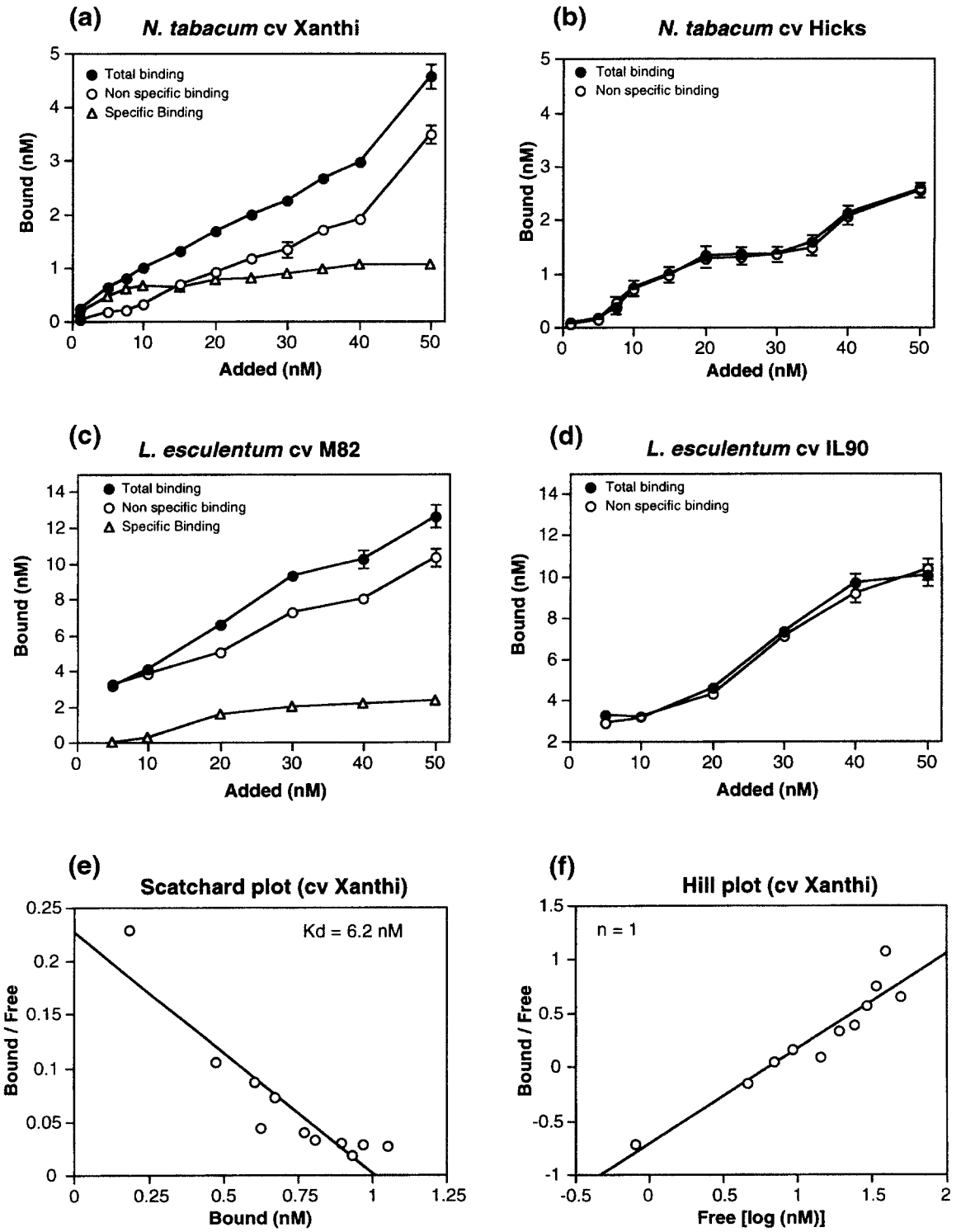

Figure 4. Binding of FITC–EIX to membranes.
Assays were initiated by incubating microsomes with different concentration of FITC-labeled EIX as indicated. Specific binding was obtained by subtracting non-specific binding (open circles) from total binding (closed circles). Non-specific binding was determined in the presence of 1 μM unlabeled EIX. Each data point represents the average of three repeats. Membranes isolated from (a) *N. tabacum* cv Xanthi; (b) *N. tabacum* cv Hicks; (c) *L. esculentum* cv M82 and (d) *L. esculentum* cv IL90 were used. The scatchard plot (e) and Hill plot (f) are of the binding data shown in (a).

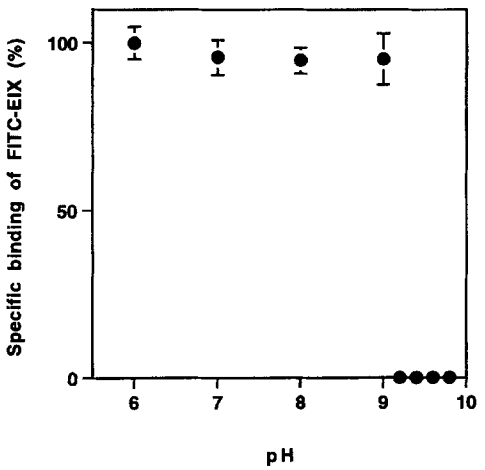

Figure 5. Specific binding at different pH values.
Microsomal membranes (300 μg) were incubated with 20 nM FITC-labeled EIX for 30 min. Specific binding at pH 7.0 was defined as 100% binding, obtained by subtracting non-specific binding from total binding. Each data point represents the average of three repeats. At pH values of 6.0 to 9.0, binding was performed in bis-tris propane buffer, and at pH values of 9.0 to 9.8 in CHES buffer. At pH 9.0, binding was performed in both buffers.

responsive *Lycopersicon esculentum* cv M82 (Figure 4c). No binding activity was detected in membranes isolated from *N. tabacum* cv Hicks (Figure 4b) or non-responding *L. esculentum* cv IL90 (Figure 4d). The specific binding activity reached saturation within 10 min. Figure 5 shows that binding is optimal between pH 6 and 9.0. Increasing the pH of the binding buffer above pH 9.0 abolished the binding activity. Varying the salt concentration (0.01–1.0 M NaCl) did not significantly affect specific binding (data not shown). Pre-treatment of tobacco membranes with proteinase K, or boiling the membranes for 10 min completely abolished the specific binding capability of the membranes, suggesting that the EIX-binding site is a protein (data not shown).

Cross-linking of EIX to a membrane protein from the responsive cultivar

Microsomal membranes isolated from *N. tabacum* cv Xanthi and cv Hicks, and *L. esculentum* cv M82 and cv IL90 were incubated with EIX. Unbound EIX was removed by centrifugation and the cross-linker reagent bis-(sulfosuccinimidyl)-suberate (BS^3) was added to the resuspended membranes. Cross-linked proteins were solubilized in SDS loading buffer, subjected to SDS–PAGE and subsequently analyzed by immunoblotting with antibodies against EIX. Figure 6 shows that BS^3 treatment resulted in the appearance of one major band corresponding to an EIX–protein complex with an apparent molecular mass of 66 kDa. This complex could be visualized only when EIX was pre-incubated with microsomal membranes from *N. tabacum* cv Xanthi and *L. esculentum* cv M82 . With membranes

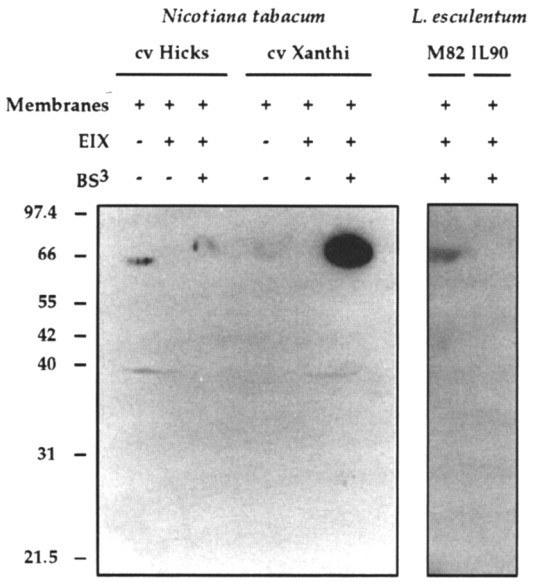

Figure 6. Cross-linking of EIX to a membrane protein.
Membranes (300 μg) isolated from *N. tabacum* cv Xanthi and cv Hicks, and *L. esculentum* cv M82 and cv IL90 were incubated with 20 nM EIX as indicated. The cross-linker BS^3 was added, as indicated, to a final concentration of 10 mM. Cross-linked material was analyzed by immunoblotting as described in Experimental procedures.

derived from *N. tabacum* cv Hicks or *L. esculentum* cv IL90, or when the cross-linker and/or EIX were not added, the band was not visualized (Figure 6).

Discussion

We describe here a high-affinity binding site for the EIX elicitor on the plasma membrane of tobacco cells. The EIX binding site fulfils several criteria expected from a receptor (Hulme and Birdsall, 1992), i.e. binding was saturable and highly specific with respect to the origin of the membranes (membranes isolated from EIX-responding or non-responding plants).

Ligand saturation analysis performed with tobacco membranes revealed the existence of a single class of binding sites for EIX. The K_d of 6.2 nM is similar to that for carbohydrate elicitors (Cheong and Hahn, 1991; Cosio *et al.*, 1990; Shibuya *et al.*, 1993) and for a protein elicitor isolated from *Phytophthora megasperma* (Nurnberger *et al.*, 1994). A similar level of affinity between ligand and receptors is found in mammalian systems.

Most of the elicitors for which high-affinity binding sites were demonstrated are carbohydrates (Cheong and Hahn, 1991; Cosio *et al.*, 1990; Shibuya *et al.*, 1993) or glycopeptides (Basse *et al.*, 1993). Our findings, however, are similar to those of Nurnberger *et al.* (1994) who identified a high-affinity binding site for a protein elicitor from *P. megasperma,* and those of Koomam-Gersmann *et al.* (1996) who identified a high-affinity binding site for the

elicitor AVR9. The binding in each case is established within minutes, in the same time frame as occurrence of the typical biological responses (Bailey *et al.*, 1992).

The effects of elicitors on ion fluxes across the plasma membrane are considered to be part of an elicitor-specific signal transduction leading to a plant defense response in several plant–pathogen interactions (Ebel and Cosio, 1994). EIX was previously shown to induce ion fluxes and other plant defense responses in a species-specific manner (Bailey *et al.*, 1992), similar to the interaction with its high-affinity binding site shown here. Moreover, two tomato isogenic lines that differ in their response to EIX (Avni *et al.*, 1994a; Eshed *et al.*, 1992) also differ in EIX binding, with the high-affinity binding site present only in the responding tomato variety (Figures 2–4). Indeed, the response to EIX is controlled by a single dominant gene in tobacco and tomato (Avni *et al.*, 1994a; Bailey *et al.*, 1993).

All plants that respond to EIX treatment by induction of ethylene biosynthesis and hypersensitive response bind EIX, while plants that do not respond to EIX treatment do not bind EIX. The binding of EIX to cells, protoplasts or membranes is correlated with the genetic ability of the plant to respond to EIX (i.e. induction of ethylene biosynthesis and hypersensitive response). This suggests that the gene controlling the response to EIX controls the ability of the elicitor to bind to its receptor. Thus, the gene controlling the response to EIX might encode the EIX receptor. However, it is also possible that separate genes exist for the EIX receptor and for the protein controlling the phenotype response.

Recently, Kooman-Gersmann *et al.* (1996) have shown that the binding of AVR9, a peptide elicitor which determines race specificity in the tomato pathogen *Cladosporium fulvum* (Van Den Ackerveken *et al.*, 1992), binds equally to microsomal membranes isolated from resistant (MM-Cf9) and susceptible (MM-Cf0) tomato genotypes. The correlation between the response to EIX and the binding activity may indicate that EIX operates in a different manner to the AVR9 elicitor. The induction of plant defense responses by EIX is correlated with binding of EIX to microsomal membranes, suggesting that in this case there is a direct interaction of the elicitor with a specific receptor. There is also the possibility that in EIX-non-responding plants, EIX binds with very low affinity that we cannot detect.

Chemical cross-linking is widely applicable for identifying receptors in different systems, including receptors for insulin, angiotensin, interferon γ and the phytopathogen *P. megasperma* elicitor (Nurnberger *et al.*, 1995). We have used the general cross-linker BS^3 to covalently bind unlabeled EIX to its binding site in tobacco and tomato membranes. As in the binding experiments, the cross-linked material could be visualized, using antibodies specific for EIX, only in membranes isolated from plants responding to EIX. Several non-related bands were visualized in the cross-linking experiment with the anti EIX antibodies (Figure 6), perhaps due to non-specific binding of the antibodies, since some of the bands appear in samples that do not contain EIX. The apparent size of the cross-linked material is 66 kDa. If we subtract EIX (22 kDa), the estimated size of the binding protein is about 44 kDa. Other receptors or resistant gene products seems to be of higher molecular weight (Cote *et al.*, 1995; Jones *et al.*, 1994; Nurnberger *et al.*, 1995; Staskawicz *et al.*, 1995; Whitham *et al.*, 1994). Thus, it might be that the receptor for EIX is a multi-subunit complex, with EIX binding to only one of these subunits.

The isolation of a receptor for EIX will provide important information on the molecular structure and mode of action of these proteins and will contribute to our understanding of signal transduction in the interaction between plant and micro-organism.

Experimental procedures

Preparation and purification of EIX

EIX was a kind gift from Dr J.D. Anderson (USDA, Beltsville, MD) and had been purified as previously described from xylan-induced cultures of *Trichoderma viride* (Dean and Anderson, 1991) .

Plant material and protoplast preparation

Tobacco plants (*Nicotiana tabacum* L cv Xanthi and cv Hicks, *L. esculentum* cv M82 and cv IL90) were grown under greenhouse conditions. Cell-suspension cultures were originally produced from calli derived from seedlings. The cell suspensions were maintained at 25°C in Schenk and Hildebrandt medium (1972) supplemented with 2.2 μM 2,4-D, 10.7 μM *p*-chlorophenoxyacetic acid and 0.46 μM kinetin. Cells were subcultured at intervals of 3 weeks. Protoplast were prepared as described by Sharon *et al.* (1993).

Endo-1,4-β-xylanase assay

Xylanase activity was determined as described by Biely *et al.* (1985). Enzyme activity was determined with 1 mg ml^{-1} Remazol Brilliant Blue Xylan (Sigma) in 0.05 M acetate buffer (pH 5.4) at 30°C for 60 min. The reaction was terminated by the addition of 2 vol of 96% ethanol. Insoluble material was removed by centrifugation at 2000 ***g*** for 5 min. The absorbance of the supernatant was measured at 595 nm.

Labeling of EIX with fluorescein isothiocyanate (FITC)

Protein was labeled with fluorescein isothiocyanate (FITC) as described by The and Feltkamp (1970). Briefly, EIX (1 mg ml^{-1}) was mixed with FITC, dissolved in 50 mM HEPES–KOH (pH 9.9) at a molar ratio of 1:400 (EIX/FITC) and incubated for 5 days at room temperature, followed by 80% ammonium sulfate precipitation. The labeled protein was dissolved in 25 mM HEPES–KOH (pH 7.5) and dialyzed against the same buffer.

Binding of FITC-labeled EIX to plant cells and protoplasts

Cell clumps were dispersed by passing them through a Teflon homogenizer. Cells were washed once with PBS. Binding was initiated by addition of FITC–EIX and incubated at 25°C for 30 min. The cells were then washed three times in PBS containing 0.1% BSA and examined by light and fluorescence microscopy. Binding of FITC–EIX to protoplasts was determined as described for cells using binding buffer (25 mM HEPES–KOH (pH 7.5), 12 mM MgCl2, 400 mM mannitol, 5 mM EGTA and 1 mM PMSF) containing 0.1% BSA. Protoplasts were washed twice in the binding buffer. Fluorochrome-labeled protoplasts were examined with a Zeiss 1000 CLSM 410 confocal laser-scanning microscope with the following configuration: 25 mW Ar and He–Ne lasers with 488, 514 and 543 maximum lines. Fluorescent and Nomarski images were generated. When comparing the fluorescence intensity, we used identical parameters for each image (scanning line, laser light, contrast and brightness).

Flow cytometry

All experiments were performed with a FACSort fluorescence-activated cell sorter (Beckton-Dickinson, Mountain View, CA). Binding of FITC–EIX to protoplasts was initiated by addition of FITC–EIX and incubation at 25°C for 30 min in binding buffer (25 mM HEPES–KOH (pH 7.5), 12 mM $MgCl_2$, 400 mM mannitol, 5 mM EGTA and 1 mM PMSF) containing 0.1% BSA. Protoplasts were washed twice in binding buffer. Protoplasts (2×10^6/ml) were separated two-dimensionally according to 'Forward Scatter' and 'Side Scatter' and dead cells were gated out: 10 000 intact protoplasts were analyzed (Parks *et al.*, 1986). The percentage of stained protoplasts and fluorescence label per protoplast were analyzed using a 535 nm FL1 filter. Data management was done using CellQuest software (Beckton-Dickinson, Mountain View, CA).

Microsomal membrane preparation

Microsomal membranes were obtained following the methods described by Wolfersberger *et al.* (1987) Thirty grams of leaf tissue were ground in 150 ml ice cold buffer (17 mM HEPES–KOH (pH 7.5), 300 mM mannitol, 5 mM EGTA and 1 mM PMSF), and an equal volume of 24 mM $MgCl_2$ solution was added to the membranes. Following 30 min incubation, the cell debris was sedimented at 2500 ***g*** for 15 min at 4°C. The resulting supernatants were centrifuged at 38 000 ***g*** for 60 min at 4°C. The pellet was resuspended in binding buffer (25 mM HEPES–KOH (pH 7.5), 12 mM $MgCl_2$, 150 mM mannitol, 5 mM EGTA and 1 mM PMSF). Protein content was determined according to Jahnen-Dechent and Richard (1990), using BSA as a standard.

Binding of FITC-labeled EIX to plant microsomal membranes

Microsomal membranes (300 μg) suspended in 50 μl binding buffer containing 150 mM mannitol, 0.1% BSA were used for the binding assay. Binding was initiated by addition of FITC–EIX to the suspension and incubation at 25°C for 30 min. Membranes were centrifuged at 20 000 ***g*** for 10 min and washed twice with PBS. Non-specific binding was determined in the presence of 1 μM unlabeled EIX. Fluorescence was measured in a Kontron spectrofluorometer, model SFM 23 (excitation 470 nm, emission 510 nm) in 3 ml cuvette. The absorption of the membranes was less than 0.1 OD at 470 and 510 nm.

Chemical cross-linking

Binding was performed as described for microsomal membranes, using unlabeled EIX. Following the binding, membranes were incubated for 30 min at 25°C in binding buffer containing 10 mM cross-linking reagent bis-(sulfosuccinimidyl)-suberate (BS^3; Pierce). Cross-linked proteins were analyzed by 12% polyacrylamide gel electrophoresis in the presence of SDS (Laemmli, 1970) followed by immunoblotting and probing with antibodies against EIX kindly supplied by J.D. Anderson, USDA, Beltsville, MD (Dean *et al.*, 1989).

Acknowledgments

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