Identification of an essential component of the elicitation active site of the EIX protein elicitor

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Summary

Defense mechanisms of plants against pathogens often entail cell wall strengthening, ethylene biosynthesis, expression of pathogen-related proteins and hypersensitive responses (HR). Pathogen-derived elicitors trigger these defense responses. The Elicitor Ethylene-inducing Xylanase (EIX) elicits HR and other plant defense responses in some tobacco and tomato cultivars independently of its xylan degradation activity. The elicitation epitope on the EIX protein responsible for inducing the HR response has been elucidated. Through the generation of EIX-specific polyclonal antibodies and screening of combinatorial phage display peptide libraries an essential sequence of the EIX elicitation activity has been identified. This sequence consists of the pentapeptide TKLGE mapped to an exposed β-strand of the EIX protein. Substitution of the pentapeptide TKLGE to VKGT inhibited the elicitation activity but not the β-1-4-endoxylanase activity of the EIX protein further demonstrating that elicitation and enzyme activity are independent properties. Elucidation of a peptide sequence that is essential for elicitation of HR creates the opportunity to understand the control and signaling of plant defense.

Keywords: elicitor, hypersensitive response, phage display peptide, xyalanase.

Introduction

When plants encounter pathogens (e.g. bacteria and fungi) they respond by a variety of localized reactions including cell wall strengthening, phytoalexins production, ethylene biosynthesis, expression of pathogenesis-related proteins and cell death (Atkinson, 1993; Greenberg, 1997; Jakobek and Lindgren, 1993; Morel and Dangl, 1997). These defense mechanisms are triggered by pathogen-derived molecules that have been defined *elicitor*s. Some of these elicitors are microorganism-derived proteins.

How the plant perceives the presence of elicitors is still not understood. Nurnberger *et al.* (1994) showed that an oligopeptide segment of the 42 kDa glycoprotein elicitor from the fungus *P. megasperma* can stimulate a complex defense response in parsley cells and bind to plant cell membranes. Another example is the AVR9 elicitor for which a specific oligopeptide derived from this elicitor was shown to induce the defense response in tomatoes (Kooman-Gersmann *et al.*, 1997). Moreover, two distinct regions on the AvrPto elicitor determine the recognition specificity of this elicitor on tomato and tobacco (Shan *et al.*,

2000). All of these examples illustrated that segments of elicitors proteins can function as 'elicitor active sites' and bind to corresponding receptors.

Our research has focused on the inter-relationship between the ethylene-inducing xyalanase (EIX) *elicitor* and tobacco plants. EIX, a 22-kDa protein from the fungus *Trichoderma vride*, elicits HR and other defense responses in such plants as tomato and tobacco (Bailey *et al.*, 1992; Felix *et al.*, 1993; Ron *et al.*, 2000; Yano *et al.*, 1998). Data presented by us and others (Enkerli *et al.*, 1999; Furman-Matarasso *et al.*, 1999; Sharon *et al.*, 1993) indicate that the enzymatic xylanase activity of EIX is unrelated to the elicitation process. The elicitation process is, however, dependent on the binding of EIX to a host protein (Hanania and Avni, 1997).

Combinatorial phage display peptide libraries provide an effective means to study protein–protein interactions and particularly in the task of epitope discovery and analysis. They are used extensively to discover bioactive peptides, epitopes for selected antibodies and ligands for receptors

(Cortese *et al.*, 1994, 1995, 1996; Lane and Stephen, 1993; Scott and Smith, 1990). These libraries display random peptides on the filamentous bacteriophage pIII or pVIII surface proteins (Enshell-Seijffers *et al.*, 2001a; Sibille and Strosberg, 1997).

In the present study, we have discovered an essential component of the elicitation active site of EIX. This has been accomplished by first producing antibodies that specifically inhibit the elicitation activity of EIX. We then mapped the epitope of the inhibitory antibody by combinatorial phage display peptide analysis. The contribution of the identified EIX peptide to the elicitation activity was then confirmed by site directed mutagenesis.

Results

Inhibition of EIX elicitor activity by anti-EIX antibodies

Two rabbits were injected with EIX preparations to generate polyclonal antibodies against the antigen. These antibodies were shown to bind the original antigen EIX and another xylanase, XynII that also induces the HR (Furman-Matarasso *et al.*, 1999). The two sera did not recognize XynI, a non-inductive xylanse (Figure 1a). We tested the ability of the two sera (designated anti-EIX-1 and -EIX-2, respectively) to inhibit the EIX activity. The EIX (0.1 μg) protein was incubated with the anti-EIX-1 and -EIX-2 polyclonal sera for 2 h followed by tobacco leaf injections. HR induction was monitored 4 days after injection. As seen in Figure 1(b) development of HR was completely inhibited by the anti-EIX-1 serum, while the anti-EIX-2 serum did not inhibit HR development (incubating the EIX with the control pre-immune serum did not show inhibition as well, Figure 1b).

FITC-EIX was previously shown to bind specifically to tobacco cells in suspension (Hanania and Avni, 1997). FITC-labeled EIX (0.2 μg) was incubated with the anti-EIX-1, anti-EIX-2 antibodies or pre-immune serum for 1 h, followed by incubation with a *Nicotiana tabacum* cv Xanthi cell suspension for 2 h. Cells were examined by fluorescence microscopy (Figure 1c). The anti-EIX-1 serum completely inhibited the binding of the FITC-labeled EIX to the cell, while anti-EIX-2 serum did not inhibit the binding. These two experiments thus indicate the specificity of the anti-EIX activity of the EIX-1 polyclonal serum, suggesting that in the anti-EIX-1 polyclonal serum, antibodies exist that bind to the EIX recognition site.

Isolating epitopes based on combinatorial phage display peptide library screening

In order to find peptides mimicking the epitopes situated on the EIX protein responsible for HR induction, we screened a
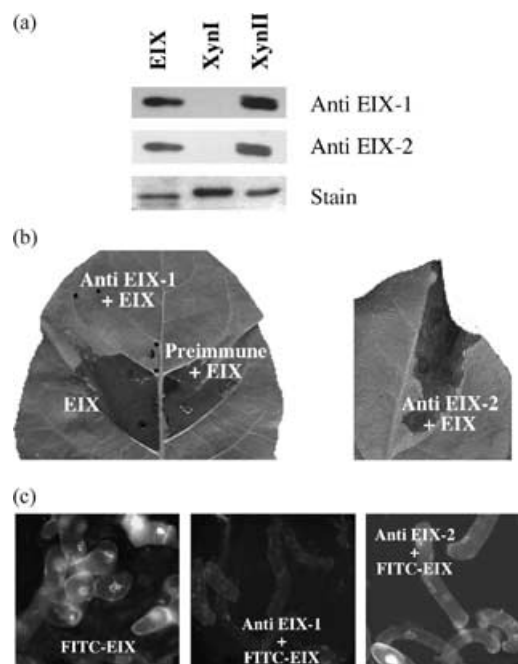

Figure 1. Inhibition of elicitor activity by anti-EIX serum.
(a) EIX, XynI and XynII (100 μg each) were separated on 12% (w/v) SDS-polyacrilamide gel, stained with co-omassie blue or transferred to a nitro-cellulose filter and probed with anti-EIX-1 or anti-EIX-2 sera, as indicated.
(b) *N. tabacum* cv Xanthi leaf was injected with EIX, EIX pre-incubated with pre-immune serum or EIX pre-incubated with anti-EIX-1 or anti-EIX-2 sera as described in experimental procedures. Development of HR was monitored 96 h after injection. Black dots label the injection area.
(c) *N. tabacum* cv Xanthi cell suspension was incubated with FITC-EIX (200 ng) or FITC-EIX (200 ng) pre-incubated with anti-EIX-1 or anti-EIX-2 sera as described in experimental procedures. Cells were washed three times and examined under fluorescence microscopy.

combinatorial phage display peptide library. The phage display library expressed 10^9 random 12mer peptides flanked by constant cyceine residues expressed at the N-terminal of the PVIII major coat phage-protein (Enshell-Seijffers *et al.*, 2001a). A single round of biopanning was performed and a total of 192 phages were picked and screened as dot blots against the original serum. Thirty-four positive phages were identified. The corresponding inserts of the 14 phages that gave the highest signal in a dot-blot binding assay (Enshell-Seijffers *et al.*, 2001a,b) were sequenced. Ten clones presented peptides with sequence homologies among themselves (Figure 2a the remaining four sequences could not be ascribed to any defined sequence related to EIX). The sequence analysis of the 10 displayed peptides generated a common linear motif of a pentameric peptide TKLGE (Figure 2a). This motif can be mapped to an exposed β-strand of the EIX protein (Figure 2b). This motif, identified by the anti-EIX serum, is conserved between the two xylanases EIX and XYNII that induce the HR and it is different in the XYNI, the non-HR-inducing xylanase (Furman-Matarasso *et al.*, 1999).
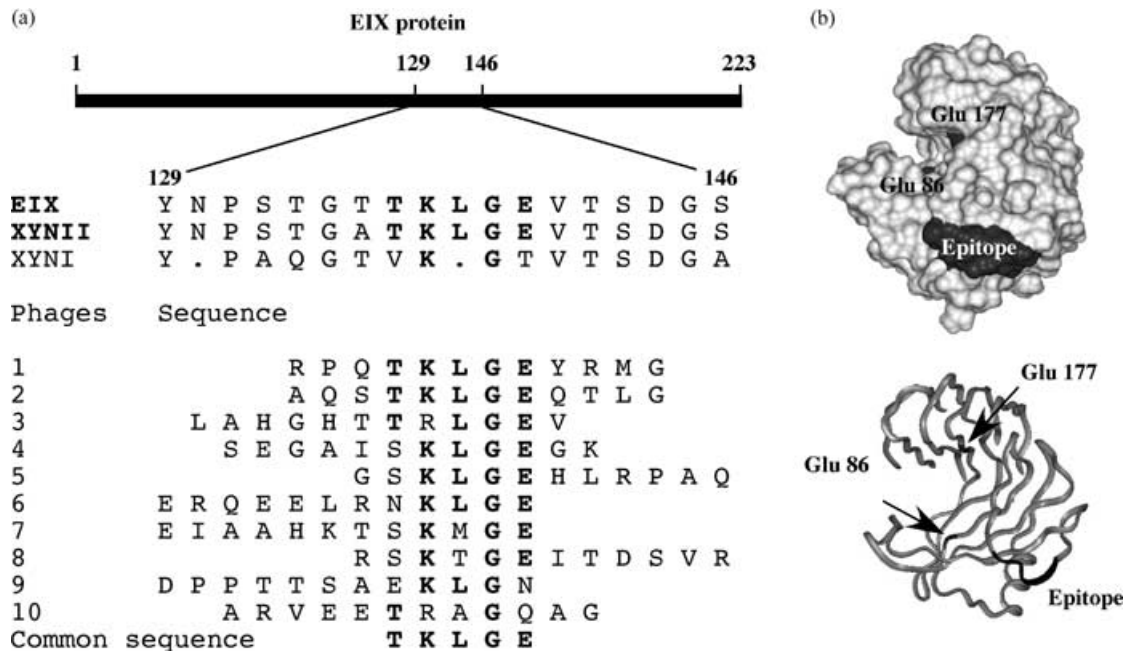
Figure 2. Mimotope sequence analysis.
(a) Alignment and sequence of phage display peptide selected by the anti-EIX serum. The phage peptides isolated are aligned with EIX, XynII and XynI (Furman-Matarasso *et al.*, 1999; Torronen and Rouvinen, 1995). The peptide sequences were aligned according to the PileUp sequence analysis program (Genetics Computer Group, version 10, Madison, WI). Single letter codons are used for amino acid residues, bold letters indicate identical residue to the EIX protein sequence, and dot indicate gaps introduced to allow optimal alignment of the sequences.
(b) Schematic representation of the XynII three-dimensional structure according to Torronen and Rouvinen (1995). Residues E-86, E-177 of the enzyme active site and the identified epitope are marked.

Competition for anti-EIX antibodies binding between the mimotopes and EIX

Pre-incubation of EIX with the anti-EIX serum followed by its injection into responsive plants show inhibition of the activity of the EIX elicitor (Figure 1b). Our assumption was that the polyclonal serum binds the inductive epitope and inhibits the binding of EIX to the plant cells. Positive phages recognized by the antibodies specific to the inductive epitope, should inhibit the activity of the anti-EIX serum. Therefore, we tested whether or not positive phages could compete with EIX for the binding of the HR inhibiting antibodies. Three different positive phages (2.5 × 10¹¹ pfu) were pre-incubated individually with the anti-EIX serum. EIX (100 ng) was then added, to the mixture. The solution-containing EIX, serum and phages was then injected to tobacco leaves. Development of HR was monitored 4 days after injection (Figure 3a). Four positive phages recognized by the polyclonal serum were tested as inhibitors of the anti-EIX activity. Phages 1, 2 and 5 have sequences containing the TKLGE motif whereas phage #11 served as a negative control as its insert (TRRTIEMEKSNC) shows no homology with EIX. Phages 1, 2 and 5 (Figure 2a) counteracted the inhibition effect of the anti-EIX serum, while a phage 11 did not (Figure 3a).

In order to confirm that the antibodies that react with the TKLGE epitope are those that inhibit the elicitation activity, we affinity purified phage-specific antibodies from the polyclonal serum. Specific anti-TKLGE antibodies were purified using phage number-1 (Figure 2a). The affinity-purified antibodies reacted with EIX, but did not recognize (Figure 4a) a mutated EIX protein (see below). EIX protein (100 ng) was incubated with phage-1 affinity purified antibodies for 2 h followed by injection into tobacco leaves. Induction of HR was monitored 4 days after injection. Development of HR was completely inhibited by the affinity-purified antibodies (Figure 3b), suggesting that the TKLGE epitope on the EIX protein contributes and is essential to the induction of HR in tobacco leaves.

Modifying the EIX protein based on the mimotope sequence

Comparing the three xylanases XynI, XynII and EIX revealed that the identified epitope is identical in the HR-inducing xylanases: EIX and XynII while it is different in the XynI (Figure 2a). Therefore, site directed mutagenesis was used to modify the epitope TKLGE presented in the EIX protein to the VKGT sequence present on XynI (Figure 2a). The mutated EIX gene open-reading frame was cloned
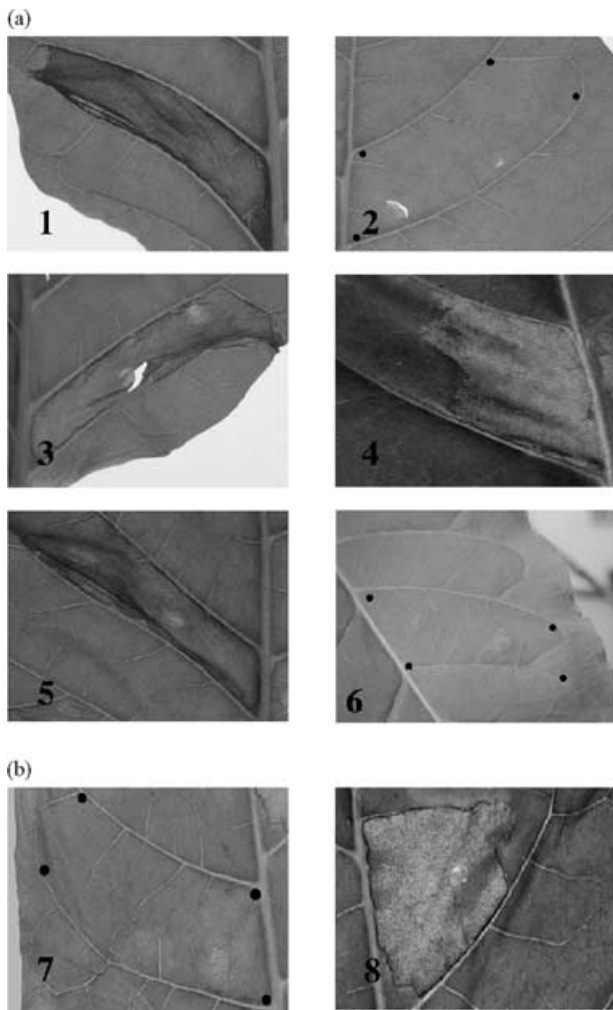
Figure 3. Neutralization of antibody by specific phage-display peptides.
(a) Six tobacco leaves were injected with EIX, either alone (1) or with anti-EIX serum (2–6) in the presence of positive reacting phages 1, 2, 5 (3–5) or the non-related phage-11 displaying the peptide TRRTIEMEKSNC (6). Development of HR was monitored 96 h after injection.
(b) Two tobacco leaves were injected with EIX with (7) or without (8) the presence of affinity purified anti-phage-1 antibodies as described in experimental procedures. Development of HR was monitored 96 h after injection. Black dots label the injection area.

over-expressed protein (Figure 4b). The mutated protein was injected to *N. tabacum* leaves and the induction of HR was monitored 4 days after injection (Figure 5a). We injected 11 different leaves on five different plants and in all cases the mutant protein was unable to induce HR. The result of a representative single leaf is shown in Figure 5(a). Furthermore, the induction of ethylene biosynthesis, another measure of defense response, was used to test the induction activity of the mutant EIX as compared to the wild types. Less than 1% of the wild-type activity could be measured for the mutated EIX (Figure 5b).

Our data clearly illustrated the necessity for the intact pentamer TKLGE for inducing HR activity by the EIX elicitor. In order to evaluate whether or not this sequence is sufficient to elicit the HR effect on its own a corresponding synthetic pentameric peptide was prepared. Application of this synthetic peptide has no effect on tobacco leaves. Whereas these results might indicate that the TKLGE sequence is not sufficient for HR induction this point is further being investigated.

Discussion

The aim of this study was to identify elements of the EIX protein that are responsible for its elicitation activity. The binding of EIX to the responsive plant cell membrane plays a key roll in the activation of the plant defense response (Hanania and Avni, 1997; Ron *et al.*, 2000). We generated two different polyclonal sera against the native EIX protein (anti-EIX-1 and EIX-2). These antibodies recognized only the HR-inducing xylanases (EIX and XYNII; Furman-Matarasso *et al.* 1999) and did not recognize the non-inducing xylanase (XYNI; Figure 1). We tested the ability of the two sera to inhibit the EIX activity. Only the anti-EIX-1 serum inhibited the HR-inducing activity, while the anti-EIX-2 serum did not (Figure 1b). Furthermore, the anti-EIX-1 serum inhibited the binding of FITC-labeled EIX to cultured tobacco cells, while anti-EIX-2 serum did not inhibit the binding (Figure 1c). In view of our observation that only one of the polyclonal sera showed the surprising and clear inhibition of EIX activity we pursued this discovery. Moreover, the existence of the second polyclonal serum emphasized that mere binding to EIX is not in itself inhibitory. We speculated that the HR inhibition was due to the binding of antibodies to the elicitation active site (or part of it). Therefore, in an effort to identify this specific active surface of the EIX responsible for HR induction, phage display technology was employed.

Polyclonal serum presents somewhat of a challenge since there are numerous potential epitopes that could be recognized by various antibody species. Nonetheless, by comparing the serum-specific phages amongst themselves one can identify common motifs of discrete antigens. Fortunately, in this study screening such a phage display

down stream to the strong polyhedrin promoter of the baculovirus, and the protein was expressed in sf9 insect cells. Immunoblots were used to identify the over-expressed EIX protein in the sf9 cells (Figure 4a). The modified protein reacted with the whole serum but did not react with the phage-1 purified antibodies (Figure 4a). Similar amounts of over-expressed mutated proteins as determined by immunoblots were used to test the xylanase and elicitor activities. The xylanase enzymatic activity (β-1-4-endoxylanase) of the mutated protein showed similar activity to the wild type

Figure 4. Expression of a mutated EIX protein.
(a) Mutated EIX (1) and wild-type EIX (2) proteins, expressed in sf9 cells (5 μg each) were separated on 12% (w/v) SDS-polyacrylamide gel, transferred to a nitrocellulose filter and probed with anti-EIX serum or phage 1-specific antibodies.
(b) β-1-4-Endoxylanase activity of different amounts of EIX (□) and the mutated EIX protein (■) was performed as described in experimental procedures.
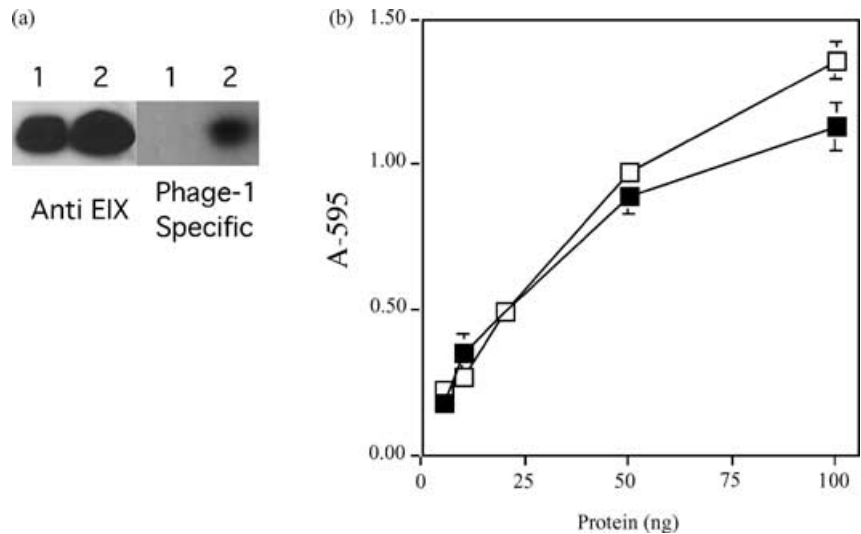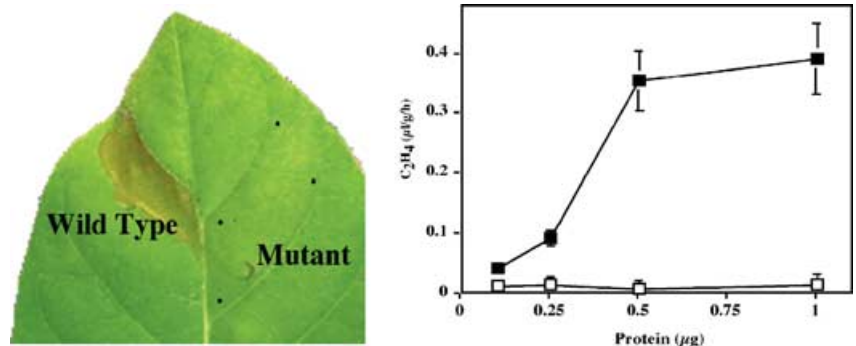

Figure 5. Induction of HR and ethylene biosynthesis of the sf9 expressed EIX proteins.
(a) Tobacco leaves were injected with EIX (wild type) or with mutated protein (1 μg ml^{-1}). Development of HR was monitored 96 h after injection.
(b) Six tobacco leaf discs were treated with different amounts of EIX (■) or with the mutated EIX protein (□) for 4 h. Ethylene production was measured by gas chromatograph as described in experimental procedures. Black dots label the injection area.

library allowed us to characterize a dominant protein structure. The motif, TKLGE, was discovered and found to correspond to a linear epitope of EIX. Comparing the motif with the sequences of the three xylanases (EIX, XYNII and XYNI) revealed that the motif is homologous to EIX and XYNII and differs at three positions from XYNI. The epitope is mapped to a surface exposed β-strand on EIX, a possible candidate for participation in a receptor–ligand interaction. In some cases, mutations made in the catalytic cavity interfered with the inductive activity of EIX (Furman-Matarasso *et al.*, 1999), and we speculated that the inductive epitope is close to the catalytic cavity. Indeed, the position of the proposed epitope supports this assumption.

To verify that the identified epitope has a role in the induction of HR by the EIX elicitor we performed competition assays. Three phages displaying epitopes recognized by this serum successfully competed with EIX on binding the HR inhibiting antibodies *in vivo*. Our conclusion could be further supported using affinity chromatography, to purify specific antibodies against the TKLGE epitope. The affinity-purified antibodies were able to inhibit the elicitation activity of the EIX protein (Figure 3b), suggesting that

the active epitope on the EIX protein contains the TKLGE motif. Verification of the critical role of TKLGE was achieved by mutating the EIX protein. Note wording is the fact that the mutated version did not loose its enzymatic activity (Figure 4). However, this protein did not react with phage-1 affinity purified antibodies (specific for TKLGE) suggesting that the mutated protein is similar to the XynI protein. Moreover, the mutated protein lost the ability to induce HR and ethylene biosynthesis. The changes in the protein were only in the TKLGE epitope suggesting that this is an essential component of the elicitation active site of the EIX protein. Application of the TKLGE peptide onto tobacco leaves did not induce HR, suggesting that the epitope might not be sufficient for inducing HR. Nonetheless, this is still open question. The conformation of the synthetic peptide may differ from the native conformation of this sequence in the context of the intact EIX protein. Therefore further experiments in this direction are currently being pursued.

We used combinatorial phage display screening in combination with site directed muatgenesis to identify the epitope on the EIX elicitor protein that is required for the elicitation activity of the protein. We identified a surface

exposed β-strand harboring the motif TKLGE which is responsible for the elicitation activity of the EIX elicitor. This epitope is conserved on the HR-inducing xylanase and is not present on the non-inducing xylanase.

Experimental procedures

Polyclonal antibodies

EIX antigen was purified from crude xylanase (Worthington) as previously described by Dean and Anderson (1991). A 6-month-old rabbit was immunized with EIX according to the following protocol. The rabbit was first prebled and then received an initial immunization of 1 mg of EIX purified protein suspended in water and mixed 1 : 1.5 with complete Feund's adjuvant (Sigma Inc.) and administered subcutaneously along the animal back. Two months later, the rabbit was boosted with 500 μg of EIX suspended in water and mixed with incomplete Freund's adjuvant (1 : 1). Two weeks later, it was bled and polyclonal serum was prepared and used in the experiments described in the Results section.

Plant material and tissue treatment

Tobacco cell suspension cultures were grown as described by Hanania and Avni (1997). *N. tabacum* L cv Xanthi were grown under greenhouse conditions until they were 25–35 cm tall. For elicitation activity assays EIX was injected into leaf tissue and necrosis development was analyzed after 96 h (Bailey *et al.*, 1990).

Alternatively, EIX (1 μg ml^{-1}) was applied to 1 cm leaf disks floated on assay medium (10 mM MES (pH 6.0) and 250 mM sorbitol) as previously described (Avni *et al.*, 1994). Ethylene production (measured by gas chromatography) was quantified 4 h after induction (Avni *et al.*, 1994).

Neutralization of EIX activity was tested by incubating 10 μl of anti-EIX polyclonal serum for 2 h with EIX (50 ng ml^{-1}) at room temperature followed by injection into leaf tissue.

Binding of FITC-labeled EIX to plant cells

EIX was labeled with fluorescein-isothiocyanate (FITC) and its binding to plant cells were performed as described by Hanania and Avni (1997). Briefly, FITC-labeled EIX (EIX-FITC; 200 ng) was pre-incubated at room temperature with or without anti-EIX serum (diluted 1 : 50 v/v) for 2 h followed by incubation for 1 h with tobacco cells. The cells were washed three times in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and visualized by fluorescence microscopy (Zeiss, Germany).

Affinity selection of phages

Selection of phages was done according to the methods described by Enshell-Seijffers *et al.* (2001a, 2001b). Anti-EIX polyclonal sera were diluted 1 : 100 in Tris-buffered saline (TBS) containing 0.25% (w/v) gelatin (TBSG). Protein G (Sigma) was used to coat the bottom of a 35-mm Petri dish overnight (70 μg ml^{-1} in 0.7 ml TBS, 4°C). After discarding the excess solution, the dish was blocked with TBSG for 2 h at room temperature, washed rapidly five times with TBS, incubated with the diluted serum (total volume 0.7 ml), and rocked gently at room temperature for 4 h. After washing with TBS, biopanning was accomplished by adding

10^{11} transducing units of phages from the library to the dish in 0.7 ml TBSG and incubated at 4°C overnight. Unbound phages were removed and the dish was washed extensively five times with TBS. Bound phages were eluted with 400 μl of elution buffer (0.1 M HCl adjusted to pH 2.2 with glycine, 1 mg ml^{-1} BSA) for 10 min at room temperature with gentle agitation. The eluate was transferred into a 1.5-ml Microfuge tube and neutralized with 75 μl of neutralizing buffer (1 M Tris–HCl pH 9.1).

Immunoscreening

DH5αF′ bacteria were infected with the affinity-selected phages (see above), plated on Luria–Broth (LB) plates containing 10 μg ml^{-1} tetracycline, and grown at 37°C overnight. Single colonies were used to inoculate 200 μl LB in U-bottom 96-well plates. After overnight culture, the plates were centrifuged at 3000 **g** for 30 min at room temperature; 125 μl of the supernatant from each well was transferred to a flat-bottom, 96-well plate already containing 50 μl per well of polyethylene glycol (PEG)/NaCl solution (33% PEG, 3.3 M NaCl). The flat-bottom plates were incubated at 4°C for 2 h and centrifuged. The precipitated phages were re-suspended in total of 100 μl TBS and applied via a vacuum manifold to nitrocellulose filters. Immuno-screening of isolated phages were performed as described by Enshell-Seijffers *et al.* (2001a,b).

Affinity purification of anti-EIX antibodies

Five hundred microliters of phages were incubated with 1.0 g CNBr-activated Sepharose 4B (Pharmacia) in coupling buffer (0.2 M NaH$_2$CO$_3$; 0.5 M NaCl pH 8.8) overnight at 4°C. To block any remaining active groups, the gel was incubated with 1 M ethanolamine (pH 9.0) and then washed. The EIX-specific polyclonal serum (5 ml) was incubated with the matrix overnight at 4°C. After extensive washing with 0.9% (w/v) NaCl, bound antibodies were eluted with 1 ml elution buffer (100 mM glycine pH 2.5). Eluted fractions were immediately neutralized with 1 ml buffer (400 mM glycine; 10 mM Tris pH 7.0). Quantity and quality of eluants were checked with dot-blots.

Inhibition of antibody activity by phages

The serum from the immunized rabbits was used for neutralization assays using tobacco plants as described above. Anti-EIX serum was pre-incubated for 2 h with EIX (50 ng ml^{-1}) and 100 μl of various phages, at room temperature followed by injection into leaf tissue. Development of hypersensitive response (HR) was assessed after 96 h.

Modifying the EIX gene

The open-reading frame of the EIX gene was cloned into the *Bam*HI–*Sac*I sites of the baculovirus transfer vector pFastBac1 (GibcoBRL). Recombinant baculoviruses and protein expression were performed as described by Furman-Matarasso *et al.* (1999).

Site directed mutagenesis was performed according to the QuickChange™ Site-Directed mutagenesis Kit (Stratagene). Site directed mutagenesis was performed on the pFastBac1 harboring the EIX gene with the following primers: 5′-GGCACCGTGAAGGG-TACCGTCACCTCTGACGG-3′ and 5′-CCGTCAGAGGTCACGGTA-CCCTTCACGGTGCC-3′. We sequenced the entire EIX open-reading frame and confirmed that the only change in the mutated gene was in the TKLGE epitope.

Endo-1,4-β-xylanase assay

Xylanase activity was determined as described by Biely *et al.* (1985). Enzyme activity was determined with $1\,\text{mg}\,\text{ml}^{-1}$ 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 two volumes of 96% ethanol. Insoluble material was removed by centrifugation at 2000 *g* for 5 min The absorbency of the supernatant was measured at 595 nm.

Acknowledgments

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