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# The Receptor for the Fungal Elicitor Ethylene-Inducing Xylanase Is a Member of a Resistance-Like Gene Family in Tomato

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**An ethylene-inducing xylanase (EIX) is a potent elicitor of plant defense responses in specific cultivars of tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum*). The *LeEix* locus in tomatoes was characterized by map-based cloning, which led to the identification of a novel gene cluster from which two members (*LeEix1* and *LeEix2*) were isolated. Similar to the tomato *Ve* resistance genes in tomato plants, the deduced amino acid sequences encoded by *LeEix1* and *LeEix2* contain a Leu zipper, an extracellular Leu-rich repeat domain with glycosylation signals, a transmembrane domain, and a C-terminal domain with a mammalian endocytosis signal. Silencing expression of the *LeEix* genes prevented the binding of EIX to cells of an EIX-responsive plant and thus inhibited the hypersensitive response. Overexpression of either *LeEix1* or *LeEix2* genes in EIX-nonresponsive tobacco plants enabled the binding of EIX, although only *LeEix2* could transmit the signal that induced the hypersensitive response. Overexpressing *LeEix2* in mammalian COS-7 cells enables binding of EIX, indicating physical interaction between the EIX elicitor and *LeEix2* gene product. Structural analysis of the LeEix proteins suggests that they belong to a class of cell-surface glycoproteins with a signal for receptor-mediated endocytosis. Mutating the endocytosis signal in *LeEix2* (Tyr 993 to Ala) abolished its ability to induce the hypersensitive response, suggesting that endocytosis plays a key role in the signal transduction pathway.**

## INTRODUCTION

Plants are constantly under attack by such pathogens as bacteria, fungi, viruses, and nematodes, which can potentially cause significant crop losses. Plants have evolved numerous defense mechanisms to protect themselves from pathogens (Yang et al., 1997). These include the strengthening of mechanical barriers, oxidative burst, de novo production of antimicrobial compounds such as pathogenesis-related proteins and phytoalexins, and the induction of the hypersensitive response (HR) mechanism, where the tissue surrounding the infection site dies and confines pathogen growth (Dangl and Holub, 1997; Greenberg, 1997; Morel and Dangl, 1997; Somssich and Hahlbrock, 1998).

These defense mechanisms are triggered when a pathogen-derived signal (an *Avr* gene product) or other organic component (termed elicitor) is recognized by a plant disease resistance protein (R-protein). The elicitors belong to a diverse range of molecular types (Boller, 1995). Many do not appear to be determinants of race-specific cultivars and are considered non-race-specific elicitors (Ebel and Cosio, 1994; Boller, 1995). The ligand–receptor interaction has been used to explain the gene for gene specificity model. In this model, the R-protein acts as a receptor for the elicitor (Gabriel and Rolfe, 1990).

More than 40 *R*-genes have been isolated from different plant species, including both monocots and dicots, out of which the corresponding *Avr* factors have been isolated only for some. These *R*-genes are effective against bacterial, viral, nematode, and fungal pathogens (Jones et al., 1994; Bent, 1996; Baker et al., 1997; Martin et al., 2003). *R*-genes encode proteins with certain common motifs and have been divided into five classes (Dangl and Jones, 2001; Martin et al., 2003): (1) intracellular protein kinases (e.g., Pto); (2) receptor-like protein kinases with an extracellular Leu-rich repeat (LRR) domain (e.g., Xa21 and FLS2); (3) intracellular proteins with a region of LRRs, a nucleotide binding site, and a Leu zipper motif (e.g., Pi-ta, RPS2, and RPM1); (4) intracellular nucleotide binding site-LRR proteins with a region similar to the Toll and interleukin-1 receptor proteins (e.g., N, L6, and RPP5); and (5) LRR proteins that encode membrane-bound extracellular proteins (e.g., Cf-2 and Cf-9). Proteins with these motifs are known to play important roles in signal recognition and transduction in mammals. Despite these important insights into R-protein structures, much remains to be elucidated regarding the molecular mechanisms by which proteins encoded by *R*-genes recognize pathogen elicitors and transduce this information in the plant cell to initiate a cascade of defense response events.

The physical interaction between *Avr* gene products and their corresponding *R*-gene products has been demonstrated in a few cases (Scofield et al., 1996; Tang et al., 1996; Jia et al., 2000; Leister and Katagiri, 2000). Pto kinase interacts physically with

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AvrPto, and mutations in the AvrPto or Pto that disrupt the interaction were also found to abolish the induction of disease resistance (Scofield et al., 1996; Tang et al., 1996). Similarly, interaction between the cytoplasmic receptor Pi-ta and the corresponding AVR-Pita is required to induce resistance response (Jia et al., 2000). While these interactions are crucial for plant defense induction, the Avr9 elicitor exhibits high affinity binding to plasma membranes isolated from both resistant and susceptible tomato (*Lycopersicon esculentum*) cultivars (Kooman-Gersmann et al., 1996; Luderer et al., 2001).

The 22-kD fungal protein ethylene-inducing xylanase (EIX; Fuchs et al., 1989) induces ethylene biosynthesis, electrolyte leakage, pathogensis-related protein expression, and HR in specific plant species and/or varieties (Bailey et al., 1990, 1993; Ron et al., 2000; Elbaz et al., 2002). Analysis of EIX mutants lacking enzymatic activity (β-1-4-endoxylanase) but retaining elicitation activity showed that the xylanase activity is not required for the HR elicitation process because the protein per se functions as the elicitor (Enkerli et al., 1999; Furman-Matarasso et al., 1999; Rotblat et al., 2002). In this system, the EIX was shown to specifically bind to the plasma membrane of both the tomato and tobacco (*Nicotiana tabacum*) responding cultivars (Hanania and Avni, 1997).

The response to EIX in tobacco and tomato cultivars is controlled by a single dominant locus (Bailey et al., 1993; Ron et al., 2000). The EIX-responding locus (*LeEix*) was mapped to the short arm of chromosome 7 of the tomato cultivar, and a yeast artificial chromosome (YAC) clone carrying a 300-kb DNA segment, derived from the *LeEix* region (Ron et al., 2000), was isolated. Mapping the ends of this YAC clone showed that it spans the *LeEix* locus (Ron et al., 2000).

This article describes the identification of a novel gene family corresponding to the *LeEix* locus of tomato. Two members of this family (*LeEix1* and *LeEix2*) were isolated and show homology to the *Ve* and *Cf* resistance genes in tomato. Using silencing and complementation experiments, we show that both these genes

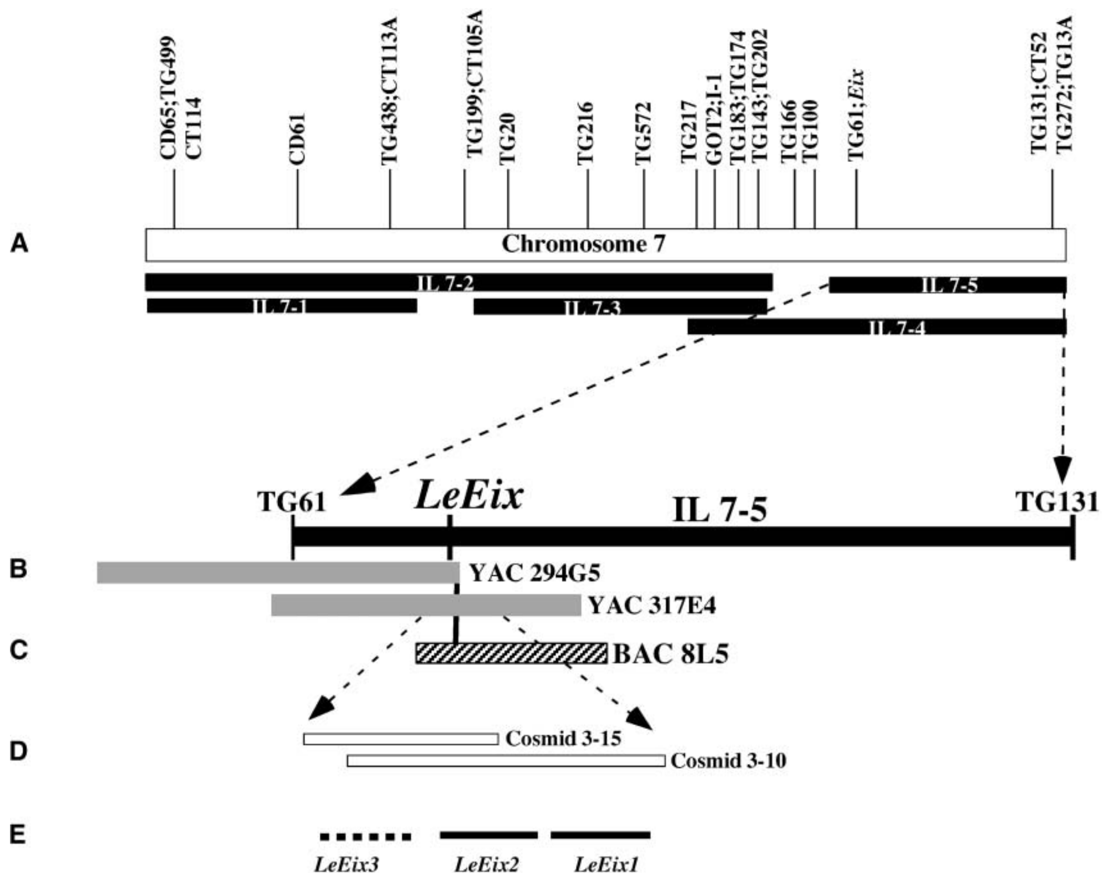


**Figure 1.** Schematic, Genetic, and Physical Representation of the *LeEix* Region.

**(A)** Genetic linkage map of chromosome 7, adapted from Eshed and Zamir (1995). The introgression regions of the introgression lines (ILs) are shown in black. Chromosome walking was initiated with the single-copy RFLP marker TG-61.

**(B)** YAC contig spanning the *LeEix* region (Ron et al., 2000); YACs are shown in gray.

**(C)** BiBAC clone isolated by hybridization with the right end clone of YAC 294G5 (294R). The BiBAC left end clone (L5L) cosegregates with the *LeEix* locus.

**(D)** Cosmid clones derived from YAC 317E4. Only cosmids hybridized to both 294R and L5L are shown.

**(E)** Position of *LeEix1*, *LeEix2*, and *LeEix3* genes.

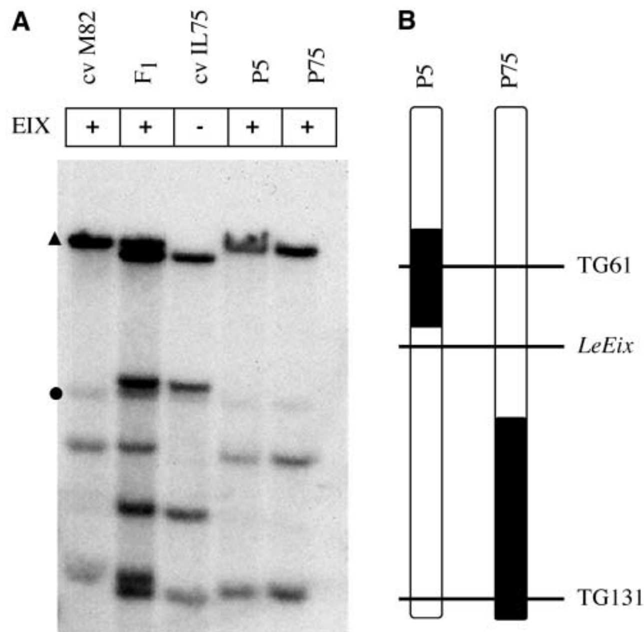


**Figure 2.** Genetic Map of the *LeEix* Locus.

**(A)** The full-length cDNA of *LeEix1* was hybridized to DNA from the near-isogenic lines *L. esculentum* cv M82, cv IL 7-5, F1 hybrid, and the two recombinant plants P5 and P75, which had been digested with *Hind*III. The responsiveness of the above lines to EIX is indicated above each lane. The triangle represents the band hybridizing with the 3′ UTR of *LeEix1*; the circle represents the band hybridizing with the 3′ UTR of *LeEix2*.

**(B)** A schematic representation of the *LeEix* gene family on the genetic map of chromosome 7. Black represents *L. pennellii* DNA and white *L. esculentum* DNA.

are capable of binding the EIX elicitor independently. However, only *LeEix2* can transmit the HR induction signal. Furthermore, induction of HR is dependent on the endocytosis signal.

## RESULTS

### Identification of a Cluster of *Cf* Homologs at the *LeEix* Locus

The *LeEix* tomato locus was previously mapped to the short arm of chromosome 7, between restriction fragment length polymorphism (RFLP) markers TG61 and TG131 (Ron et al., 2000). Two YACs, YAC 317E4 and YAC 294G5, containing tomato genomic DNA were isolated from this region. We showed that YAC 317E4 encompasses the *LeEix* locus, whereas the end of YAC 294G5 cosegregates with the *LeEix* locus (Ron et al., 2000). Positional cloning of the *LeEix* locus was performed as schematically shown in Figure 1. Using the end of YAC 294G5 (294R), we screened a tomato binary BAC2 (BiBAC2) library (Hamilton, 1997) and identified a single BAC (8L5) containing the 294R marker (Ron et al., 2000). Mapping the BAC end clones on the recombinant population showed the left end of BAC 8L5 (L5L) cosegregated with the *LeEix*, whereas the right end of the clone cosegregated with the RFLP marker TG131 (Figure 1). We searched the DNA databank using the sequence of the two clones (L5L and 294R). The two clones showed homology to many plant resistance genes and particularly to a family of extracellular transmembrane LRRs (e.g., *Cf-9*, *Cf-2*, *Cf-5*, *Ve*, and *HcrVf*). The L5L clone showed homology to the 3′ end of these genes, whereas 294R showed homology to their 5′ end (data not shown). To obtain a full-length cDNA clone of the candidate *LeEix* gene(s), we screened two different cDNA

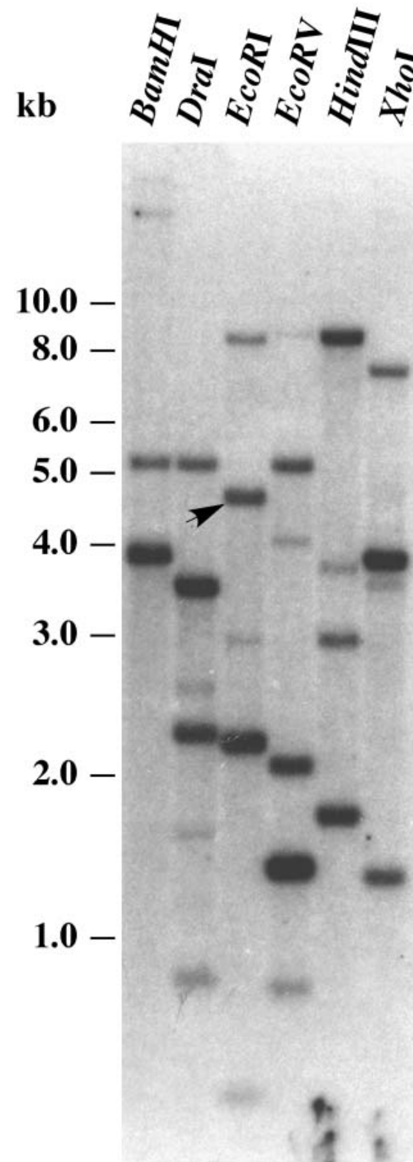


**Figure 3.** Physical Map of the *LeEix* Region.

*L. esculentum* cv M82 genomic DNA (5 μg/lane) was digested with several restriction enzymes as indicated. Fragments were separated on a 1.0% agarose gel, transferred to a nylon membrane, and hybridized with a mixture of *LeEix1*, *LeEix2*, and the partial *LeEix3* clones. The arrow represents the band hybridizing with the 3′ UTR of *LeEix1* and *LeEix2*.

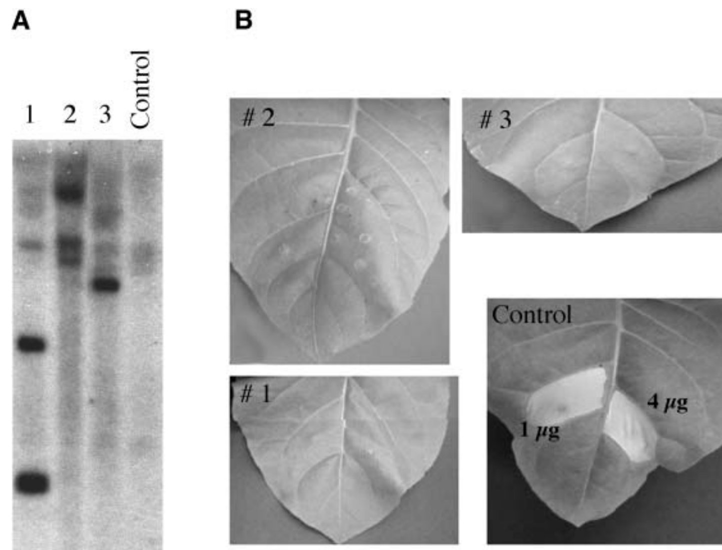


**Figure 4.** *LeEix* Gene Silencing Suppresses EIX-Induced Cell Death.

**(A)** Genomic DNA (10 μg/lane) from three different transgenic tobacco plants (lanes 1 to 3) and a control (*N. tabacum* cv Samsun) was digested using *Xho*I, separated on a 1% agarose gel, transferred to a nylon membrane, and hybridized with the 684-bp fragment of *LeEix1* used to create the silencing construct.

**(B)** Young, fully expanded leaves from three independent transgenic plants harboring the pKANNIBAL-*LeEix1* or from control plants (*N. tabacum* cv Samsun) were injected with EIX (1 or 4 μg/mL) as described in Methods. Development of cell death was monitored 96 h after EIX treatment.

libraries generated from leaves of *L. esculentum* ($10^6$ clones from each library) using both probes. Seven full-length cDNA clones, hybridized to L5L and 294R, were identified. Partial sequencing of these clones and restriction analysis with several enzymes showed them to be identical (designated *LeEix1*).

*LeEix1* comprises 3247 nucleotides, has an open reading frame (ORF) of 1031 amino acids, and a 151-bp-long 3′ untranslated region (UTR). The *LeEix1* cDNA deduced amino acid sequence shares 48% similarity and 31% identity with the Cf-2 resistance protein (Dixon et al., 1996) and 45% similarity and

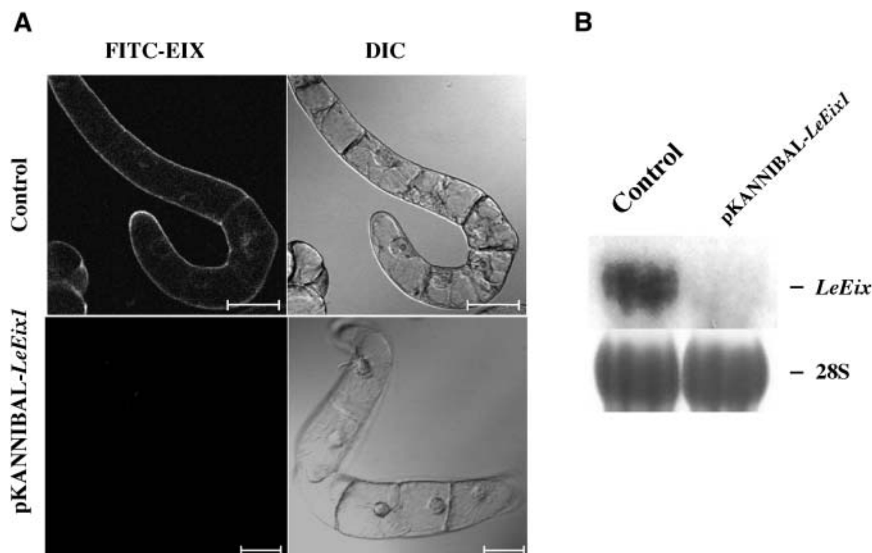


**Figure 5.** *LeEix* Gene Silencing Abolishes FITC-EIX Binding to Tobacco Cells.

**(A)** Cell suspensions derived from the control (*N. tabacum* cv Samsun) or silenced plant (pKANNIBAL-*LeEix1*) were incubated with FITC-labeled EIX (0.5 μg/mL) for 30 min. Cells were washed three times and examined with a confocal laser-scanning microscope. DIC, Nomarsky differential interference contrast image; FITC-EIX labeling appears in white. Bars = 50 μm.

**(B)** Total RNA (15 μg/lane) was isolated from control (*N. tabacum* cv Samsun) and silenced cell suspensions, separated on a 1% agarose gel, transferred to a nylon filter, and hybridized to the full-length *LeEix1* clone. Methylene blue staining of the 28S is shown.

30% identity with the tomato Ve1 resistance protein (Kawchuk et al., 2001).

The isolated cDNA clone shares 87% identity with L5L and 294R clones, suggesting that they are different genes. When the *LeEix1* gene was mapped, it cosegregated with the *LeEix* locus (Figure 2). Several polymorphic fragments were detected when hybridizing the full-length *LeEix1* cDNA with genomic DNA from both EIX-responding and EIX-nonresponding plants (Figure 2). This suggests that the *LeEix1* clone may contain exons spanning a large region or a family of related genes. We tested whether the genomic and *LeEix1* cDNA share a similar structure. Amplification of genomic DNA with *LeEix1*-specific primers amplified a DNA fragment with the same size as the cDNA, suggesting that the *LeEix1* gene does not have introns (data not shown). The detection of several polymorphic bands in the DNA gel blot analysis therefore suggests that it represents a family of related genes, which cosegregate with the *LeEix* locus (Figures 2 and 3). In our attempts to identify additional members of the *LeEix* cluster, a cosmid library generated from YAC 317E4 DNA (Ron et al., 2000) was screened using the 294R and L5L markers as probes. Two of the hybridizing cosmids (Figure 1) were partially sequenced, and a new ORF was identified. Using specific primers, the new gene (*LeEix2*) was amplified from the cosmid and sequenced.

*LeEix2* comprises 3213 nucleotides, has an ORF of 1021 amino acids, and a 144-bp-long 3′ UTR. The amino acid sequences of LeEix1 and LeEix2 are 81.4% identical and 85.1% similar. DNA gel blot analysis with the 3′ UTR of the two genes mapped them to different regions of the *LeEix* locus (Figure 2). Sequence analysis revealed that marker 294R is identical to *LeEix2*; however, the L5L marker showed 87% similarity to *LeEix1* and 84% similarity to *LeEix2*, suggesting that it belongs to a third gene of the *LeEix* family (*LeEix3*).

To estimate the number of genes in the *LeEix* locus, total genomic DNA from *L. esculentum* was digested with *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xho*I, separated on an agarose gel, blotted onto a nylon filter, and probed with a mixture of *LeEix1*, *LeEix2*, and the partial clone *LeEix3* (Figure 3). The combined size of all of the hybridizing bands ranged between 15 and 18 kb. The 3′ UTR of *LeEix1* and *LeEix2* hybridized to the same 4.8-kb *Eco*RI DNA fragment (Figure 3). Restriction map analysis of *LeEix1* and *LeEix2* combined with the hybridization data suggest that the distance between *LeEix1* and *LeEix2* is 4 kb. The combined size of *LeEix1* and *LeEix2* is 6 kb, and they are separated by a 4-kb region. We estimate that the size of *LeEix3* is 3 kb. Therefore, we suggest that there are three genes in the *LeEix* locus.

### Silencing the *LeEix* Gene Family Abolishes the Response to EIX Treatment

Our mapping experiments suggest that one or more members of the *LeEix1* gene family control the plant's response to EIX treatment. To verify this hypothesis, a gene suppression approach using RNA interference (RNAi) was chosen. A segment of the LRR region of *LeEix1* (684 bp from residue 1871 to residue

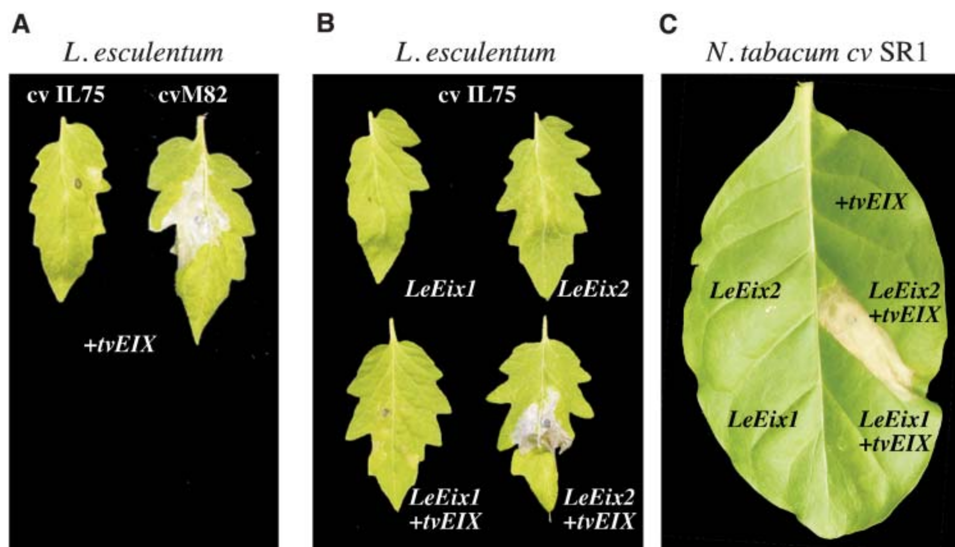


**Figure 6.** In Vivo Functional Analysis of *LeEix* Genes in Tomato and Tobacco.

**(A)** *L. esculentum* cv M82 and cv IL7-5 were injected with a suspension of Agrobacterium strain GV3101 ($OD_{600} = 0.1$) carrying a binary vector with *tvEix* driven by the 35S promoter of *Cauliflower mosaic virus*.

**(B)** *L. esculentum* cv IL7-5 was infiltrated with Agrobacterium GV3101 ($OD_{600} = 0.1$) strains containing either *Pro35S:LeEix1* construct, *Pro35S:LeEix2* construct, or a mixture of either *Pro35S:LeEix1* construct or *Pro35S:LeEix2* stains with Agrobacterium GV3101 strain containing *Pro35S:tvEIX*.

**(C)** *N. tabacum* cv SR1 were injected with a suspension of Agrobacterium GV3101 ($OD_{600} = 0.1$) strains containing either *Pro35S:tvEIX* construct, *Pro35S:LeEix1* construct, or *Pro35S:LeEix2* construct or a mixture of either *Pro35S:LeEix1* construct or *Pro35S:LeEix2* stains with Agrobacterium GV3101 strain containing *Pro35S:tvEIX*.

Development of HR was monitored 72 h after injection.

2554) was cloned in the pKANNIBAL vector in both sense and antisense orientation, flanking the Pdk intron (Wesley et al., 2001). This construct was subcloned into the binary vector pART27 (Gleave, 1992) and used for transforming *N. tabacum* cv Samsun (EIX-responding plants). Several independent transgenic plants harboring the RNAi construct were generated, and antibiotic resistance and DNA gel blot analysis confirmed transformation (Figure 4A). In responsive tobacco cultivars, EIX induces cell death within 48 h of treatment (Bailey et al., 1990) and complete tissue desiccation within 96 h. We examined the induction of cell death in 11 independent transgenic plants. Cell death by EIX was clearly suppressed compared with control plants (Figure 4B). Furthermore, fluorescin isothiocyanate labeled EIX (FITC-EIX) interacts only with the wild-type cells but not with the cells derived from the silenced transgenic plant (Figure 5A). RNA gel blot analysis indicated that the level of *LeEix* RNA in the transgenic cell suspension harboring the RNAi construct was reduced to levels below that found in wild-type plants (Figure 5B).

These two experiments thus indicate that at least one of the *LeEix* genes controls the response to the elicitor.

### Genetic Complementation

Transient expression assays (Bendahmane et al., 2000; Van der Hoorn et al., 2000) were conducted to identify which member(s) of the *LeEix* gene family control(s) the plant's response to the EIX elicitor. *LeEix1* and *LeLeEix2* cDNAs were cloned independently in the sense orientation into the binary vector pBINPLUS between the 35S-Ω promoter containing the translation enhancer signal and the Nos terminator. The cDNA encoding the EIX elicitor (*tvEIX*; Furman-Matarasso et al., 1999) was cloned in a similar vector. The constructs were electroporated into *Agrobacterium tumefaciens* GV3101 and the bacteria used for transient expression assays.

To test the specificity of the transient expression system, Agrobacterium GV3101 harboring $Pro_{35S}$:*tvEIX* was injected into

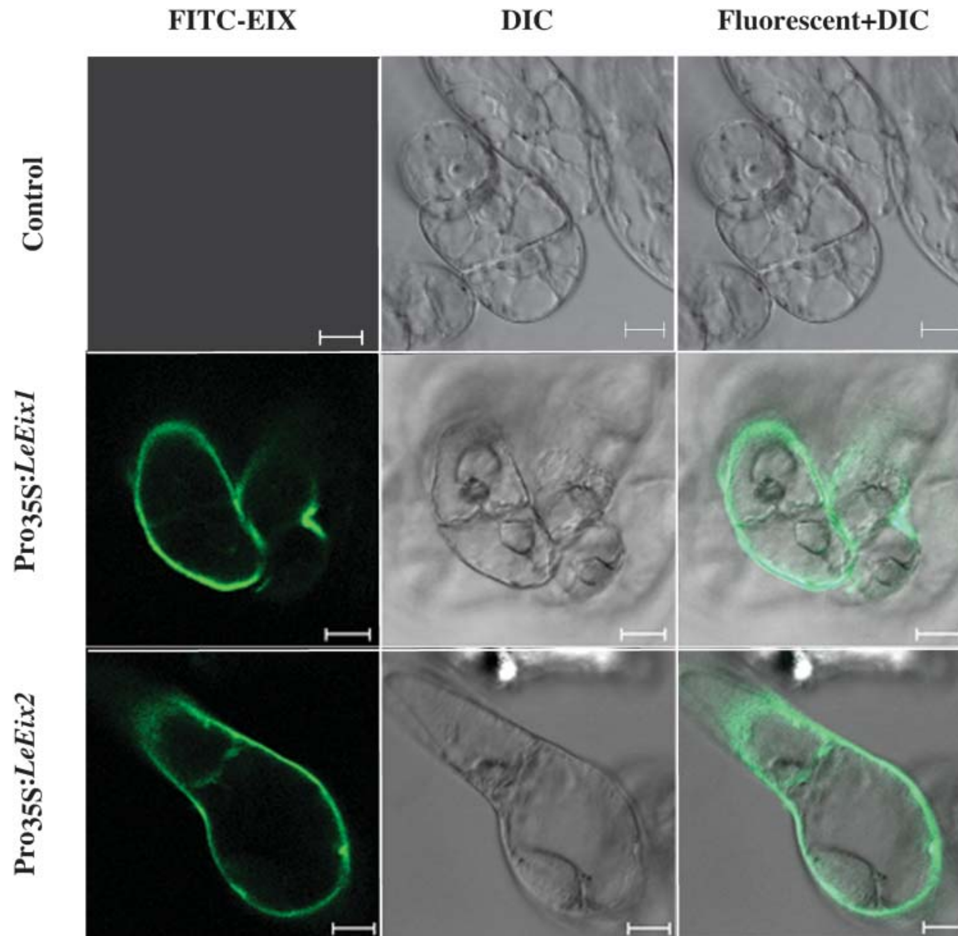


**Figure 7.** *LeEix* Gene Expression Restores FITC-EIX Binding to Transgenic Cells.

Cell suspensions of control (*N. tabacum* cv BY2) or BY2 transgenic cells overexpressing either $Pro_{35S}$:*LeEix1* or $Pro_{35S}$:*LeEix2* were incubated with FITC-labeled EIX (0.5 μg/mL) for 30 min. Cells were washed three times and examined using a confocal laser-scanning microscope. FITC-EIX labeling appears in green; DIC, Nomarsky differential interference contrast image; Fluorescent+DIC, overlay of FITC-EIX and differential interference contrast images. Bars = 20 μm.

*L. esculentum* cv M82 (EIX-responding plants) and cv IL7-5 (EIX-nonresponding plants) (Figure 6A) or *N. tabacum* cv SR1 (EIX-nonresponding plants; Figure 6C). Leaves of the M82 tomato cultivar exhibited HR within 48 h of infiltration, whereas those of the EIX-nonresponding cultivar (IL7-5 and SR1) did not (Figures 6A and 6C). Two Agrobacterium GV3101 strains containing either a $Pro_{35S}$:*LeEix1* construct or a $Pro_{35S}$:*LeEix2* construct were each mixed separately with an Agrobacterium GV3101 strain containing $Pro_{35S}$:*tvEIX* and then infiltrated into leaves of *L. esculentum* cv IL7-5. Tomato leaves infiltrated with a mixture of $Pro_{35S}$:*LeEix1* and $Pro_{35S}$:*tvEIX* exhibited no response, whereas leaves infiltrated with a mixture of $Pro_{35S}$:*LeEix2* and $Pro_{35S}$:*tvEIX* developed HR within 48 h (Figure 6B). Similarly, we examined the genetic complementation in *N. tabacum* cv SR1 (EIX-nonresponding cultivar). *N. tabacum* cv SR1 leaves infiltrated with a mixture of $Pro_{35S}$:*LeEix1* and $Pro_{35S}$:*tvEIX* exhibited no response, whereas leaves infiltrated with a mixture of $Pro_{35S}$:*LeEix2* and $Pro_{35S}$:*tvEIX* developed HR within 48 h (Figure 6C).

To further define the interaction among *LeEix1*, *LeEix2*, and the EIX elicitor, we used the above Agrobacterium strains carrying $Pro_{35S}$:*LeEix1* and $Pro_{35S}$:*LeEix2* to transform *N. tabacum* cv BY2 cell lines (an EIX-nonresponding cultivar). The resulting transgenic cells were tested for their ability to bind the EIX elicitor. Transgenic cells harboring $Pro_{35S}$:*LeEix1* and those harboring $Pro_{35S}$:*LeEix2* exhibited binding to the EIX elicitor, whereas the control BY2 cell suspension did not (Figure 7). These experiments indicate that both *LeEix1* and *LeEix2* can restore the binding of the EIX elicitor, although only *LeEix2* can transmit the signal necessary to induce the HR response.

### Binding between EIX and Mammalian COS-7 Cells Overexpressing *LeEix2*

Our data suggests physical interaction between the EIX elicitor and the LeEix proteins. *LeEix2* was overexpressed in COS-7 cells to allow binding studies between the EIX elicitor and LeEix2 protein in the absence of other plant proteins. COS-7 cells were transfected with *LeEix2* fused to a mammalian Igκ signal peptide. The resulting transgenic COS-7 cells were tested for their ability to bind the FITC-EIX elicitor. Transgenic cells transfected with *LeEix2* exhibited binding to the EIX elicitor, whereas no such binding was detected when the cells were transfected with empty vector (Figure 8). These experiments indicate that binding between EIX elicitor and LeEix2 protein is not dependent on other plant proteins, suggesting direct interaction between the two proteins.

### Deduced Primary Structure of *LeEix1* and *LeEix2*

On average, the protein sequences of the *LeEix1* and *LeEix2* genes revealed a 31% identity with the tomato Cf family disease resistance proteins and shared the same domains as those predicted for the *Cf9*, *Cf4*, *Cf2*, and *Cf5* resistance genes (Jones et al., 1994; Dixon et al., 1996, 1998) and the tomato *Ve* genes (Kawchuk et al., 2001). The predicted domains for *LeEix1* and *LeEix2* are shown in Figure 9. Domain A consists of the signal peptide and its cleavage site at the N terminus of the protein, whereas domain B consists of the predicted $NH_2$ terminus of the mature protein and a Leu zipper motif. The LRR domain C contains 31 imperfect repeats of the consensus sequence LxxLxxLxxLxLSxNxLGxIP (Jones et al., 1994), often associated with protein–protein interaction and ligand binding. The presence of the amino acid Gly within the consensus sequence is consistent with the LRR region being extracytoplasmic, a location that facilitates the recognition of an extracellular ligand (Jones et al., 1994; Song et al., 1995). Within the predicted LRR region, 18 sequences, matching the *N*-glycosylation consensus sequence NX(S/T) were observed in *LeEix1* and 17 in *LeEix2*. As often observed with membrane-spanning proteins, a hydrophobic sequence with a predicted α-helix secondary structure, predicted to be the transmembrane domain of the protein (domain E), was flanked by a negatively charged extracytoplasmic domain D and a positively charged cytoplasmic domain F. These domains are predicted to play a role in the orientation and anchoring of the protein to the cell membrane. The cytoplasmic

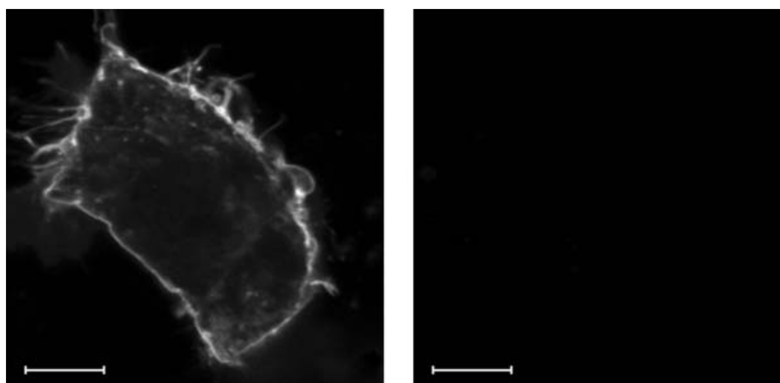

**Figure 8.** Binding of FITC-EIX to COS-7 Cells Overexpressing *LeEix2*.

COS-7 cells were either transfected with *pSeqTaq-LeEix2* (left panel) or mock transfected (right panel). Two days after transfection, FITC-labeled EIX (250 ng/mL) was added to COS-7 cells for 4 h. Cells were washed three times with PBS, fixed with 4% paraformaldehyde, and visualized by confocal laser microscopy. FITC-EIX labeling appears in white. Bars = 10 μm.

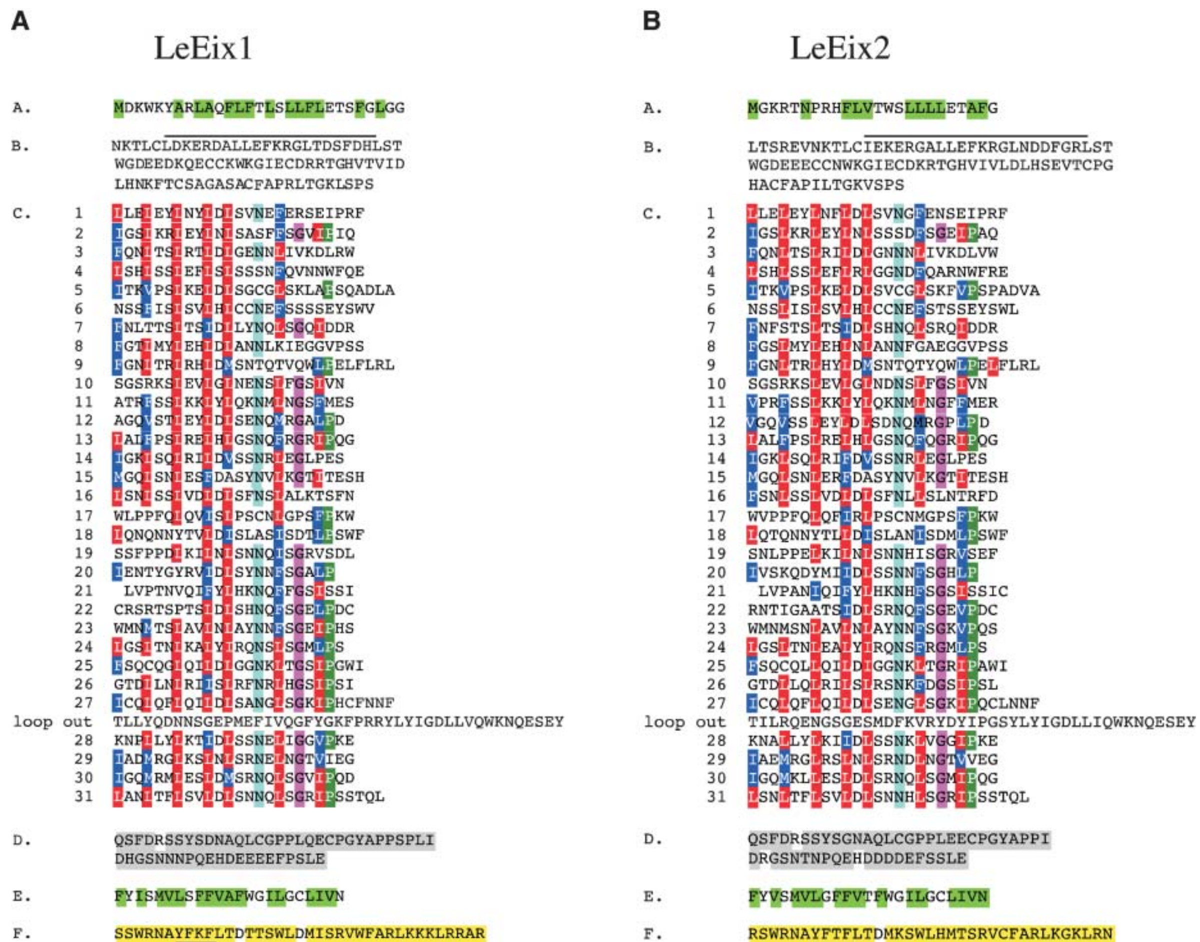


**Figure 9.** Primary Structures of LeEix Proteins.

LeEix1 **(A)** and LeEix2 **(B)** proteins deduced from the cDNA sequence. The polypeptides were divided into domains A to F as described in the text. A line is shown above the putative N-terminal Leu zipper in domain B and below the endocytosis signal in domain F. Hydrophobic amino acids (light green) of the putative signal peptide domain A and membrane-associated domain E are highlighted. In domain C, the conserved L of the LRRs (red) is often replaced by I, F, or V and occasionally by M (blue), whereas the conserved I (red) is replaced by L, F, or V (blue). Conserved N are highlighted in light blue, conserved G in purple, and conserved P in green. Neutral and acidic amino acids of domain D are highlighted in gray and neutral and basic amino acids of domain F in yellow.

domains possess a Tyr YXXø signal sequence, where ø is an amino acid with a hydrophobic side chain, which stimulates receptor-mediated endocytosis and degradation of mammalian cell-surface receptors (Letourneur and Klausner, 1992; Bonifacino and Traub, 2003). Thus, as with the *Cf* and *Ve* resistance genes, the *LeEix1* and *LeEix2* genes are likely to encode the extracytoplasmic glycoproteins anchored to the cell membrane, with the majority of the extracytoplasmic domain consisting of LRR motifs.

## Mutating the Endocytosis Signal Abolishes HR

In mammalian, receptor-mediated endocytosis acts as the mechanism for transducing the signal of an external stimuli (Goldstein et al., 1985; Ceresa and Schmid, 2000; Bonifacino and Traub, 2003). In soybean (*Glycine max*) cell culture, the *Verticillium* elicitor was shown to enter the cell by an endocytosis process (Horn et al., 1989). Interestingly, the tomato Ve2 resistance protein contains the conserved endocytosis signal (YXXø) within the short cytoplasmic domain (Kawchuk et al., 2001).

Hanania et al. (1999) showed that after binding the plant membrane, EIX is transported into the cytoplasm. The LeEix proteins contain the YXXø motif (Tyr at position 993 in *LeEix2*) within the short cytoplasmic domain similar to the Ve2 protein. The endocytosis motif was mutagenized to test the hypothesis that endocytosis plays a role in HR induction. Site-directed mutagenesis was used to modify YFTF, the epitope present in the LeEix2 protein, to AFTF. The mutated *LeEix2-Y993A* ORF was cloned into the binary vector pBINPLUS between the 35S-Ω promoter containing the translation enhancer signal and the Nos terminator. The construct was electroporated into Agrobacterium GV3101 and the bacteria used for transient expression assays. Two Agrobacterium GV3101 strains containing either a $Pro_{35S}$:*LeEix2* construct or a $Pro_{35S}$:*LeEix2-Y993A* construct

were mixed separately with an Agrobacterium GV3101 strain containing *Pro$_{35S}$:tvEIX* and then infiltrated into leaves of *N. benthamiana* (EIX-nonresponding plant). Leaves infiltrated with a mixture of *Pro$_{35S}$:LeEix2-Y993A* and *Pro$_{35S}$:tvEIX* exhibited no response, whereas leaves infiltrated with a mixture of *Pro$_{35S}$:LeEix2* and *Pro$_{35S}$:tvEIX* developed HR within 48 h (Figure 10A). RT-PCR analysis indicated that the two genes are expressed in leaf sections infiltrated with *Pro$_{35S}$:LeEix2-Y993A* and *Pro$_{35S}$:LeEix2* compared with control leaf section infiltrated with medium (Figure 10B). These results indicate that endocytosis plays a key role in mediating the signal generated by EIX that leads to HR induction.

## DISCUSSION

Two *LeEix* genes from tomato, whose products act as receptors for the fungal elicitor EIX were positionally cloned, and the following was observed: *LeEix* gene family members cosegregate with the plant's response to the EIX elicitor; suppression of the *LeEix* genes expression abrogated the response to EIX; the *LeEix2* complemented the EIX elicitor response in vivo; and both *LeEix1* and *LeEix2* proteins restored the binding of EIX. Partial sequencing of the *LeEix* locus and DNA gel blot analysis suggests the presence of three different genes in this locus.

*LeEix* genes show homology to genes encoding plant proteins with LRRs, including disease resistance genes that encode cytoplasmic proteins and membrane bound proteins (Dangl and Jones, 2001; Martin et al., 2003). Like the *Ve* resistance genes (Kawchuk et al., 2001), *LeEix* genes contain an endocytosis signal and a Leu zipper. Leu zippers are present in the cytoplasmic class of *Arabidopsis thaliana* resistance genes *RPS2* and *RPM1* for *Pseudomonas syringae* and can facilitate the dimerization of proteins through the formation of coiled-coil structures (Lupas et al., 1991). However, unlike the *Ve* resistance genes, the *LeEix* genes do not contain the PEST sequence that characterizes some rapidly degraded proteins (Rechsteiner and Rogers, 1996).

Physical interaction between the R-protein and its corresponding elicitor has been demonstrated only for a few cases (Scofield et al., 1996; Tang et al., 1996; Jia et al., 2000; Leister and Katagiri, 2000; Martin et al., 2003). The EIX elicitor was found to specifically bind to the membranes of EIX-responsive plants (Hanania and Avni, 1997).

The COS-7 cell expression system has previously been shown to be suitable for expression of various functional receptor proteins (Mathews and Vale, 1991; Kieffer et al., 1992). Furthermore, Luderer et al. (2001) demonstrated that COS-7 cells, transfected with the *Cf-9* gene, presented the protein on the plasma membrane. We showed that expression of *LeEix2* in the COS-7 cells enables the binding of EIX in the absence of other plant proteins and hence indicates direct binding between the EIX and the LeEix2 protein. Thus, *LeEix2* acts as a functional receptor for the EIX elicitor.

Recent studies suggest that R-proteins are part of multiprotein complexes (Bogdanove, 2002; Ellis et al., 2002). The inability of the LeEix1 protein to induce HR may be because of poor interaction with other membrane-associated proteins, resulting in a malfunctioning complex. Alternatively, differences in the cytoplasmic domain between *LeEix1* and *LeEix2* may account for the inability of *LeEix1* to transduce the signal, which triggers HR induction.

All eukaryotic cells exhibit receptor-mediated endocytosis as a mechanism of communication for external stimuli and as regulation of signal transduction (Goldstein et al., 1985; Bonifacino and Traub, 2003). In plant cells, indirect evidence of receptor-mediated endocytosis and the presence of clathrin-coated

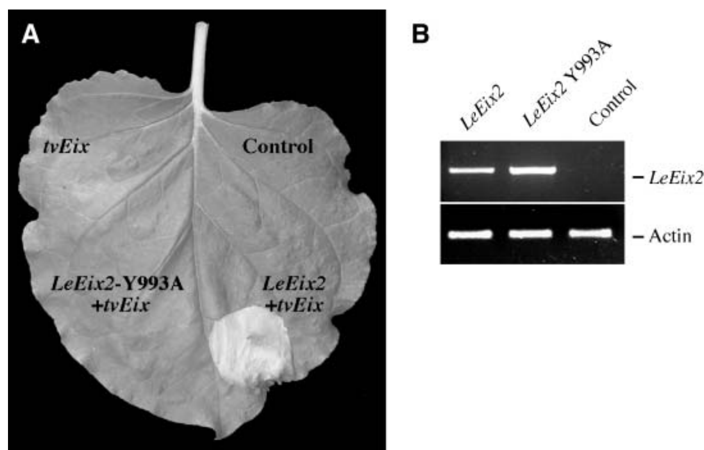


**Figure 10.** Mutation in the Endocytosis Signal Abolish HR Induction.

**(A)** *N. benthamiana* (EIX-nonresponding plant) was injected with a suspension of Agrobacterium GV3101 ($OD_{600} = 0.1$) strains containing either *Pro$_{35S}$:tvEIX* construct or induction medium (control) or a mixture of either *Pro$_{35S}$:LeEix2* construct or *Pro$_{35S}$:LeEix2Y993A* stains with Agrobacterium GV3101 strain containing *Pro$_{35S}$:tvEIX*. Development of HR was monitored 72 h after injection.

**(B)** Total RNA was isolated from leaves infiltrated with *Pro$_{35S}$:LeEix2-Y993A*, *Pro$_{35S}$:LeEix2*, or medium and was used to generate first-strand cDNA. The cDNA was used in RT-PCR reactions using specific primers to *LeEix2* or *N. tabacum* actin genes. RT-PCR products were separated on an agarose gel and stained with ethidium bromide.

pits has been obtained (Battey et al., 1999). The YXXø motif has been found in mammalian endocytic receptors (Bonifacino and Traub, 2003). The presence of YFKF and YFTF sequences in the short cytoplasmic domains of *LeEix1* and *LeEix2* suggests that plant and mammalian cell-surface receptors share similar endocytosis signals (Mellman, 1996; Bonifacino and Traub, 2003).

Compartmentalization may play a crucial role in the initiation of *R*-gene dependent signaling. Following the demonstration that mutating the endocytosis signal inhibits HR induction and that the EIX elicitor interacts with the cytoplasmic protein T-SUMO (Hanania et al., 1999), we propose that the binding of the EIX elicitor to the LeEix2 protein causes ligand-induced conformational change spreading from the extracytoplasmic to the cytoplasmic domain. These conformational changes may induce signals similar to those suggested for the Cf-9 resistance protein (Romeis et al., 1999) and the Clavata receptor (Trotochaud et al., 1999). Alternatively, the binding of EIX to LeEix2 protein may induce receptor-mediated endocytosis, thus allowing the receptor and/or EIX to interact with the cytoplasmic proteins and hence generate a signal to induce the defense response.

The identification of the EIX receptor provides the biochemical foundation for future experiments aimed at understanding the regulatory pathway leading to induction of plant defense responses.

## METHODS

### Plant and Cell Culture Material and Growth Conditions

*Nicotiana tabacum* cv Samsun and cv SR1 and *N. benthamiana* and *Lycopersicon esculentum* cv M82 and cv IL7-5 plants (Eshed and Zamir, 1995) were grown from seeds under greenhouse conditions. *N. tabacum* cv Samsun and cv Bright Yellow 2 (BY2) cells were maintained by weekly dilution in fresh MS medium (Sigma, St. Louis, MO) supplemented with 100 μg/L of 2,4-D and 30 g/L of sucrose for *N. tabacum* or 200 mg/L of $KH_2PO_4$, 1 mg/L of thiamine HCl, 100 mg/L of myoinositol, 0.2 mg/L of 2,4 D, and 30 g/L of sucrose for BY2. The medium was adjusted to pH 5.7. The cells were maintained with shaking at 110 rpm at 25°C.

### DNA and RNA Analysis

Standard methods were used for DNA and RNA manipulation (Bernatzky and Tanksley, 1986; Ausubel et al., 1987; Sambrook et al., 1989). The cosmid library was generated from the YAC 317E4 (Ron et al., 2000). YAC DNA was isolated from agarose gel, partially digested with *Sau*3AI, and inserted into the binary cosmid vector pCLD04541 (Jones et al., 1992). DNA packaging was performed using commercial extracts (Gigapack; Strategene, La Jolla, CA) as per the manufacturer's instructions. The *L. esculentum* mixed elicitors (T1297) and *Pseudomonas syringae* resistance (T1080) cDNA libraries screening was performed as described previously (Sambrook et al., 1989).

For RT-PCR analysis, total RNA was extracted from leaves 28 h after *Agrobacterium tumefaciens* infiltration. Two micrograms of RNA were converted to cDNA using M-MLV reverse transcriptase (Promega, Madison, WI). One microliter of each RT reaction was used as template in a 50-μL PCR reaction containing *LeEix2*-specific primers or Actin-specific primers as control.

### Sequence Analysis

DNA sequencing was performed using the dideoxy chain termination method (Sanger, 1981). Sequence analysis was performed using the GCG sequence analysis software package (version 10.0; Accelrys, San Diego, CA). The BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) was used to search the DNA and protein databases for similarity. Motifs were identified using the SMART program (http://smart.embl-heidelberg.de/) and the PROSITE program (http://www.expasy.ch/prosite).

### Construction of Silencing and Complementation Plasmids

The pKANNIBAL vector (Wesley et al., 2001) was used for silencing. A fragment of 684 bp from the LRR region of the *LeEix1* gene was amplified by PCR using the following primers: 5′-CTCGAGGATCCATCTCTTC-AATTTGTCG-3′ and 5′-GGTACCATCGATTCTCATATCAGCTATTTCTT-TAG-3′.

The sense and antisense orientations were cloned into a pKANNIBAL vector (Wesley et al., 2001). The pKANNIBAL containing the sense and antisense fragments was digested with *Not*I, and the fragment was subcloned into the binary vector pART27 (Gleave, 1992). The resulting construct was introduced by electroporation into Agrobacterium GV3101.

For the complementation assays, the cDNAs of *LeEix1* and *LeEix2* and the cDNA encoding the EIX elicitor (Furman-Matarasso et al., 1999) were cloned separately into pBINPLUS (van Engelen et al., 1995) between the 35S-Ω promoter containing the translation enhancer signal and the Nos terminator (Hanania et al., 1999). The resulting constructs were electroporated into Agrobacterium GV3101.

For mutagenesis, we applied the mega-primer method (Sarkar and Sommer, 1990). Site-directed mutagenesis was generated by PCR, using *LeEix2* as template and the primer 5′-GCCGCTTTCACATTCTTAAC-AG-3′ to change Tyr 993 into Ala. The resulting clone was sequenced to verify the mutation. *LeEix2*-Y993A was then cloned into pBINPLUS as described above. The resulting construct was electroporated into Agrobacterium GV3101.

### Plant and Cell Suspension Transformation

*N. tabacum* cv Samsun was transformed as previously described (Horsch et al., 1985). *N. tabacum* cv BY2 cell suspension were transformed as described by Savaldi-Goldstein et al. (2003).

For transient expression assays, the *LeEix1* and *LeEix2* constructs that were cloned in pBINPLUS (van Engelen et al., 1995) were introduced by electroporation into Agrobacterium strain GV3101. Agrobacterium was grown in LB medium overnight, diluted into an induction medium (50 mM Mes, pH 5.6, 0.5% (w/v) glucose, 1.7 mM $NaH_2PO_4$, 20 mM $NH_4Cl$, 1.2 mM $MgSO_4$, 2 mM KCl, 17 μM $FeSO_4$, 70 μM $CaCl_2$, and 200 μM acetosyringone) and grown for an additional 6 h until $OD_{600}$ reached 0.4 to 0.5. The Agrobacterium culture was diluted to $OD_{600} = 0.1$, and the suspensions expressing $Pro_{35S}$:*tvEIX*, $Pro_{35S}$:*LeEix1*, $Pro_{35S}$:*LeEix2*, or a mixture thereof were injected with a needleless syringe into the leaves of 7- to 8-week-old *L. esculentum* plants. Leaves were observed for HR induction 48 to 96 h after injection.

### Generation of Cell Cultures from Transgenic Plants

The leaves or stems of the transgenic plants were washed in 70% ethanol for 30 s, followed by surface sterilization using 3% bleach and 0.1% Tween-20 for 20 min, and rinsed three times with sterile water. Leaf disks or stem disks were spread on MS plates supplemented with 2 mg/L of 2,4-D and 0.2 mg/L of 6-benzylaminopurine riboside or 3 mg/L of a-naphtalene acetic acid and 1 mg/L 6-benzylaminopurine riboside. The plates were incubated at 25°C until the formation of calli. The calli were transferred to a fresh liquid MS medium supplemented with 1:5 of the original hormones and maintained with shaking at 110 rpm at 25°C. Cell cultures were further maintained as described for the *N. tabacum* cv Samsun.

**EIX Treatments**

Xylanase (Fluka, Milwaukee, WI) was purified as previously described (Dean and Anderson, 1991). In the plants, EIX (1 to 4 μg/mL) was injected into leaves using needleless syringes as previously described (Hanania et al., 1999). The development of cell death was monitored 24 to 96 h after treatment.

**Binding of FITC-Labeled EIX to Plant Cells**

EIX was labeled with FITC as previously described (Hanania and Avni, 1997). Cells were incubated for 30 min with 0.1% BSA followed by the addition of EIX-FITC (500 ng/mL) for 30 min. The cells were subsequently washed three times in washing buffer (10 mM phosphate buffer, pH 7, and 0.1% BSA) and visualized by confocal laser microscopy. Confocal imaging was performed using a Zeiss LSM510 confocal laser-scanning microscope (Jena, Germany). Excitation was performed using an argon laser set to 488 nm, and emission was detected with a 525 nm ± 15 nm bandpath filter. Image analysis was performed using Zeiss CLSM-5 and Adobe Photoshop 7.0 (Mountain View, CA).

**COS-7 Cell Culture Transfection and EIX Binding**

COS-7 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Paz et al., 2001). For transfections, cells were plated on 20 × 20-mm cover slides in a six-well plate. Cells were transfected with 0.5 μg of DNA (pSeqTag vector containing *LeEix2*; Invitrogen, Carlsbad, CA) by dextran (Amersham Biosciences, Uppsala, Sweden) as previously described (Seed and Aruffo, 1987). Two days after transfection, 0.1% BSA was added to the medium 30 min before adding FITC-labeled EIX (250 ng/mL). Cells were further incubated for 4 h at 37°C in the presence of the FITC-EIX followed by 3 washes with PBS. Cells were fixed with 4% paraformaldehyde and visualized by confocal laser microscopy.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY359965 (*LeEix1*) and AY359966 (*LeEix2*).

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