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High-resolution linkage analysis and physical characterization of the EIX-responding locus in tomato

Abstract An ethylene-inducing xylanase (EIX) from *Tricohoderma viride* is a potent elicitor of ethylene biosynthesis, localized cell death and other defense responses in specific cultivars of tobacco (*Nicotiana tabacum)* and tomato (*Lycopersicon esculentum*). Wild species of tomato, such as *Lycopersicon cheesmanii* and *Lycopersicon pennellii*, do not respond to EIX treatment. The F_1 progeny of a *L. esculentum×L. cheesmanii* and a *L. esculentum×L. pennellii* cross responded to EIX treatment with an increase in ethylene biosynthesis and the induction of localized cell death. The F_2 progeny of the above mentioned crosses segregated 3:1 (responding:non-responding). We mapped the EIX-responding locus (*Eix*) to the short arm of chromosome 7 using a population of introgression lines (ILs), containing small RFLP-defined chromosome segments of *L. pennellii* introgressed into *L. esculentum*. RFLP analysis of 990 F_2 plants that segregated for the introgressed segment mapped the *Eix* locus 0.1 cM and 0.9 cM from the flanking markers TG61 and TG131, respectively. Using the marker TG61 we isolated a yeast artificial chromosome (YAC) clone that carries 300-kb DNA segments derived from the *Eix* region. By mapping the ends of this YAC clone we show that it spans the *Eix* locus. Thus, positional cloning of the *Eix* locus appears feasible.

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Introduction

Plants are equipped with multi-component defense responses to protect themselves from pathogens (Keen et al. 1990; Blein et al. 1991; Felix et al 1993). The defense reaction includes cell-wall strengthening, phytoalexin production, ethylene biosynthesis, expression of pathogenesis-related (PR) proteins, and rapid localized cell death termed the hypersensitive response (HR) (Atkinson 1993; Jakobek and Lindgren 1993; Greenberg 1997, Morel and Dangl 1997). Initiation of these events is sometimes triggered by a pathogen-derived signal generated by organic compounds termed elicitors. Elicitors that trigger plant defense responses have been isolated from a variety of phytopathogenic and nonpathogenic types (Fuchs et al. 1989; Ricci et al. 1993; Ebel and Cosio 1994).

A 22-kDa fungal protein (β-1-4-endoxylanase), referred to as the ethylene-inducing xylanase (EIX), was isolated from xylan-induced *Trichoderma viride* cultures (Dean et al. 1989; Fuchs et al. 1989). Similar xylanases have been identified in xylan-induced filtrates of phytopathogenic fungi (Dean et al. 1989; Wu et al. 1997). When applied to cut roots or petioles EIX was translocated in the xylem tissues and induced symptoms in leaves both above and below the point of application (Bailey et al. 1991; Sharon et al. 1992).. Injection of EIX into the leaf-mesophyll intercellular spaces induced ethylene production, localized cell death as well as other plant defense responses in *Nicotiana tabacum* cv Xanthi (Bailey et al. 1990; Lotan and Fluhr 1990; Avni et al. 1994) and cell suspensions (Bailey et al. 1992; Felix et al. 1993; Yano et al. 1998). These are characteristic responses of plants to exogenously applied elicitors (Keen et al. 1990; Blein et al. 1991; Felix et al. 1993).

Gene-for-gene interactions, in which plant disease resistance involves a single resistance (R) gene in the plant

that responds specifically to an avirulence gene in the pathogen, have been described for numerous plant-pathogen pairs (Staskawicz et al. 1995). EIX induces ethylene biosynthesis, electrolyte linkage, PR protein expression and limited necrosis only in a specific plant species and/or variety (Bailey et al. 1993). On the other hand, a general stress inducer, like $CuSO_4$, induces ethylene biosynthesis in both EIX-responsive and EIX non-responsive plants (Bailey et al. 1993; Avni et al. 1994). Furthermore, evidence has been reported indicating that a single dominant locus controls the plant response to EIX in *N. tabacum* (Bailey et al. 1993).

The present study describes the genetic and physical characterization of a region on tomato chromosome 7 that contains the xylanase-responding gene (*Eix*). The data presented indicate that positional cloning of this *Eix* locus appears feasible.

Materials and methods

Preparation and purification of EIX

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

Plant material and tissue treatment

L. esculentum plants were grown from seeds under greenhouse conditions. EIX was applied as a hanging drop to freshly cut petioles of detached leaves (2.5 µg /g tissue) as previously described (Bailey et al. 1990). After the solution was absorbed, the leaf petioles were placed in an assay medium containing 10 mM Mes (pH 6.0) and 250 mM sorbitol and placed in jars (100 ml). Ethylene biosynthesis was assayed as described in Avni et al. (1994). Alternatively, EIX (2 µg/ml) was injected in to leaf tissue and the development of cell death was analyzed after 96 h. The response to EIX treatment was determined in F_2 and F_3 progenies.

DNA-extraction RFLP analysis

DNA isolation, restriction digests, electrophoresis on agarose gel, Southern blots, hybridization and autoradiography were performed as described by Bernatzky and Tanksley (1986) with the exception that filters were probed with random hexamer-labeled plasmids (Feinberg and Vogelstein 1983).

Linkage maps were constructed using the MapMaker program (Lander et al. 1987), employing the Kosambi mapping function. Linkage was considered significant if the LOD score was equal to, or greater than, 3.0. Additional statistical analysis was performed using DataDesk™ software for the Macintosh™ (Velleman and Pratt 1989).

Yeast DNA isolation CHEF fractionation, transfer and hybridization

Total yeast DNA was isolated according to Hoffman and Winston (1987) for the isolation of YAC end clones. Megabase-size DNA was prepared according to Gerring et al. (1991). Contour-clamped homogeneous electric field (CHEF; Chu et al. 1986) electrophoresis was performed in a CHEF-DR III apparatus (Bio-Rad). Gels were prepared in 0.5× TBE buffer (0.5×=0.045 M Tris-borate, 0.001 M EDTA) at an agarose concentration of 1%, electophoresed in 0.5×TBE buffer for 24 h at 120° and 14°C, at 6 V/cm, with an initial pulse time of 50 s and a final pulse time of 90 s. Yeast chromosomal DNA (strain AB1380) was used as a size marker (Burke et al. 1987). Gels were stained with 0.5 µg/ml of ethidium bromide in running buffer for 30 min and photographed. The DNA was nicked by UV-irradiation (254 nm) for 3 min prior to capillary transfer onto nylon membrane. Transfer and hybridization were performed as for RFLP analysis.

YAC end isolation

The YAC end-clones were isolated by the vectorette method (Riley et al. 1990) or by PCR walking (Devic et al. 1997). For isolation of the left-end clones of a YAC, total yeast DNA was digested with *Dra*I. Anchor bubble adaptor was ligated to the digested DNA followed by amplification with specific primers for the bubble and the pYAC4 vector (Riley et al. 1990). For isolation of the right-end clones of a YAC, total yeast DNA was digested with *Eco*RV or *Sca*I, followed by ligation with the GenomeWalker adaptor (Devic et al. 1997). Amplification of the right-end YAC was done by using specific primers to the adaptor and the pYAC4 vector (Devic et al. 1997). Each YAC end-clone was sequenced.

Results and discussion

The responsiveness to EIX treatment is controlled by a single dominant locus

To understand the genetic basis of the plant response to EIX, we studied the responsiveness of several different plant species in which EIX induces ethylene biosynthesis and localized cell death (Table 1). We first examined the responsiveness of different species of *Lycopersicon* to EIX treatment. *L. esculentum* cv. Floradade, cv M82, cv Moneymaker, as well as many other commercial tomato varieties, responded to EIX treatment (Table 1). In contrast, wild tomato species such as *L. cheesmanii* or

Table 1. Responsiveness of plant species to EIX treatment

Plant species	Responsiveness to EIX treatment[a]
Capsicum annuum. L Bell Pepper	+
Fragaria chiloensis Duchesne	−
Lycopersicon chmielewskii	−
Lycopersicon esculentum cv Elsa Creg	+
Lycopersicon esculentum cv Money Maker	+
Lycopersicon esculentum cv Pik-Red	+
Lycopersicon esculentum cv Rio Grande	+
Lycopersicon esculentum cv Rutgers	+
Solanum tuberosum L. cv Desireé	−
Vitis vinifera	+
Lycopersicon esculentum cv M82	+
Lycopersicon pennellii	−
F_1 (*L. esculentum* cv M82×*L. pennellii*)	+
Lycopersicon esculentum cv Floradade	+
Lycopersicon cheesmanii	−
F_1 (*L. esculentum* cv Floradade×*L. cheesmanii*)	+

[a] Responsiveness was measured by the ability to induce ethylene biosynthesis and cell death by EIX. Ethylene biosynthesis was measured 4 h after applying EIX (2.5 µg/g tissue) to the petiole (Bailey et al. 1990). Cell death was measured 3 days after injecting a solution of 2 µg/ml of EIX in H_2O into the leaf

Fig. 1 Response of F_2 progeny to EIX treatment. Leaves from F_2 plants (selfing of the cross between *L. esculentum* and *L. cheesmanii*) were injected with a solution of 2 μg/ml of EIX in H_2O. On the *left*, a leaf from an EIX non-responding F_2 plant; on the *right*, a leaf from an EIX-responding plant. Marked leaves were monitored on the plants 2 days after injection

L. pennellii did not respond to EIX treatment (Table 1). All the F_1 plants generated by crossing EIX-responsive *L. esculentum* cultivars with *L. cheesmanii* and *L. pennellii* responded to EIX treatment (i.e. resulting in ethylene production and the induction of cell death.

In addition, F_2 plants generated by selfing of the above F_1 plants derived from the cross of *L. esculentum* cv Floradade and *L. cheesmanii* were also examined for their response to EIX (Fig. 1). From a total of 138 F_2 plants, 106 plants were EIX-responsive whereas 32 plants were EIX non-responsive. Thus the F_2 progeny segregates 3:1 responding:non-responding ($\chi^2=0.242$; 70%>P>50%) which indicates the response to EIX in tomato is controlled by a single dominant locus *Eix*, similar to that of *N. tabacum* (Bailey et al. 1993).

The *Eix*-locus maps to chromosome 7 of the tomato genome

High-resolution mapping is necessary in order to isolate a gene based on its map position. To expedite such mapping a population was developed of introgression lines that contain small RFLP-defined chromosome segments of *L. pennellii* introgressed into *L. esculentum* cv M82 (Eshed et al. 1992; Eshed and Zamir 1995). This mapping resource provides a high level of DNA polymorphism in the targeted region coupled with an overall *L. esculentum* background for the rest of the genome. This latter aspect is important since some of the morphological markers are difficult to score in segregating generations of interspecific crosses.

Fifty two different ILs of the above population, which covers the entire tomato genome, were screened for their responsiveness to EIX. Fifty lines showed a response to EIX while two lines, IL 7-4 and IL 7-5, did not respond

to EIX treatment (Fig. 2). These non-responsive lines both carry an introgression segment from *L. pennellii* derived from chromosome 7. In IL 7-4 the introgression of the *L. pennellii* chromosome is from the proximal end of the chromosome up to RFLP marker TG217, whilst in IL 7-5 the introgression is from the proximal end of the chromosome up to RFLP marker TG61 (Eshed and Zamir 1995). These data also demonstrate that the inheritance of the response to EIX in tomato is determined by a single locus located on the short arm of tomato chromosome 7.

To increase the mapping resolution, a large F_2 mapping population of 990 individuals was developed from a cross between M82×IL7-5. The mapping population was used to more-precisely place the *Eix* locus with respect to the tomato RFLP markers, TG61 and TG131, and the potato markers, CP43 and CP52 (Gebhardt et al. 1994). The mapping results showed that the *Eix* locus is located 0.1 cM from TG61, away from the centromere, and flanked by TG131 on the other side at 0.9 cM (Fig. 2). The potato RFLP markers co-segregated with the TG131 RFLP marker (data not shown).

Since there were no other RFLP markers closer to the *Eix* locus, we used a targeted marker-identification strategy employing RAPD technology (Williams et al. 1990) to identify more markers. We screened 280 RAPD oligos (Operon) using DNA from *L. esculentum* cv M82 (EIX responsive) and IL 7-5 (EIX non-responsive), which differ only for the region surrounding the *Eix* locus. Only a single oligo, OPH2, showed a polymorphic phenotype. However, OPH2 co-segregated with the RFLP marker TG131. The difficulty in identifying additional molecular markers in the vicinity of the *Eix* locus might indicate that the physical distance between TG131 and TG61 is not very large.

Screening of a tomato YAC library

We screened a tomato YAC library (Martin et al. 1992) with the RFLP marker TG61. A total of 315 YAC DNA pools, each containing 96 individual YAC clones, were screened by PCR with specific primers isolated from the TG61 marker (Green and Olson 1990). Thus 30,240 YACs were screened which, based on an average YAC size of 150 kb, corresponds to 4.2 tomato genome equivalents (Martin et al. 1992). Three YAC pools were identified (RG294, RG317 and RG348) from which a PCR product amplified corresponding to TG61. Each one of these YAC pools was hybridized with TG61 in order to identify the individual YAC clones. By using this approach three YACs were identified (294G5, 317E4, 348E2) as having a DNA segment hybridizing with TG61.

The YACs were separated on CHEF gels, photographed, blotted and hybridized to the TG61 DNA marker (Fig. 3). The tomato genomic DNA in the YACs ranges from 295 to 600 kilobase pairs (Fig. 3), based on a comparison with yeast chromosome sizes (Burke et al. 1987).

End-specific probes corresponding to the right and left arms of the different YACs were isolated by the vecto-

Fig. 2A, B Linkage map of tomato chromosome 7 showing location of the *Eix* locus. **A** Genetic linkage map of chromosome 7, adapted from Eshed and Zamir (1995). The introgression regions of the ILs are shown in *black*. **B** Linkage map developed from an F_2 population derived from cross between *L. esculentum* cv M82 and *L. esculentum* cv IL75 (Eshed and Zamir 1995), in which 990 segregating F_2 populations were analyzed by RFLP mapping. Linkage maps were constructed using the MapMaker program (Lander et al. 1987), employing the Kosambi mapping function
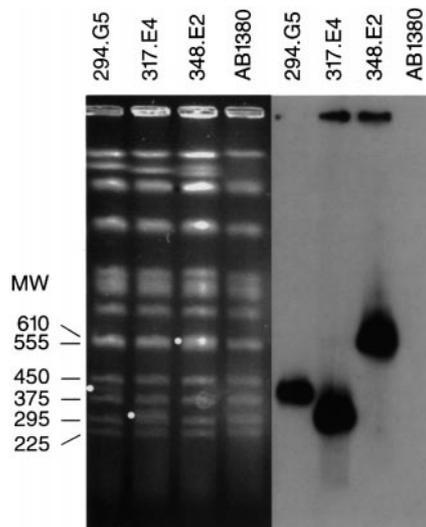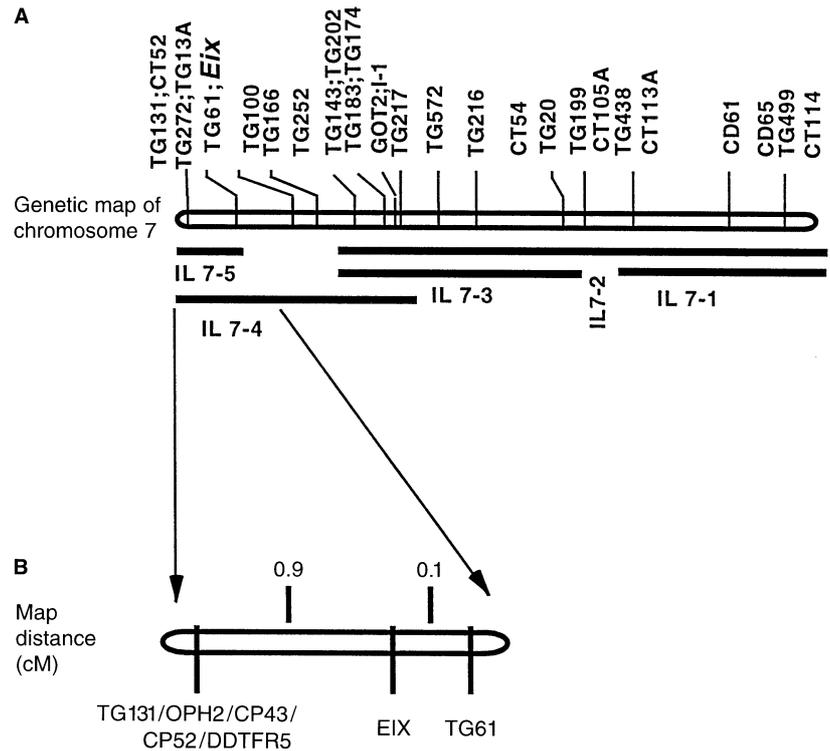

Fig. 3 Pulse-field-gel electrophoresis analysis of tomato yeast artificial chromosomes (YACs). Total megabase yeast DNA of yeast containing YACs was separated by the CHEF method on a 1% agarose gel (see Materials and methods). Yeast chromosomes were stained with ethidium-bromide. The different YACs are marked with a *white dot* (*left*). Yeast chromosomes were transferred to a nylon filter and hybridized with the RFLP marker TG61 (*right*)
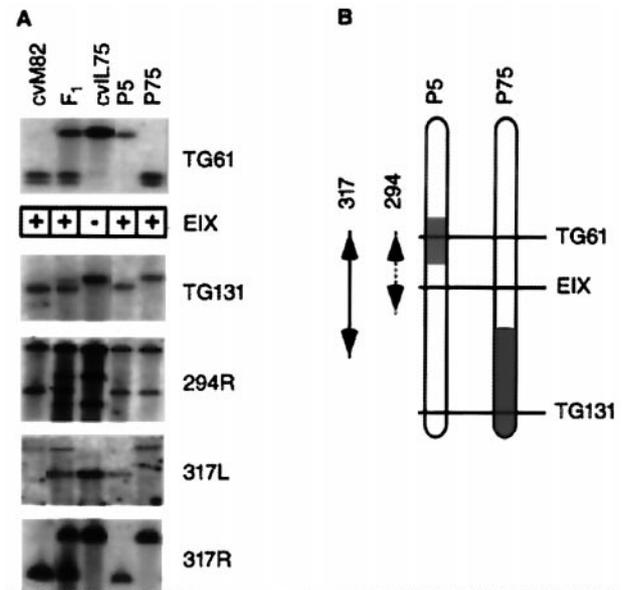
Fig. 4A, B The genetic map of the *Eix* region. A. The RFLP markers TG61, TG131 and the YAC end clones were hybridized to DNA from the near-isogenic lines *L. esculentum* cv M82, cv IL 7–5, the F_1 hybrid and two recombinant plants cv P5 and cv P75 digested with polymorphic restriction enzymes. The responsiveness of the above line to EIX treatment was determined. **B** Schematic representation of the YAC's position on chromosome 7. *Gray* represents *L. pennellii* DNA and white *L. esculentum* DNA. The approximate position of the YACs on the genetic map were estimated by mapping the YAC ends

rette or PCR-walking methods (Riley et al. 1990; Devic et al. 1997). The sizes of the end-specific clones range from 250 to 900 bp. All of the specific probes detected a low-copy sequence in the tomato genome (Fig. 4A).

In order to confirm that the isolated YAC encompasses the *Eix* locus, it was necessary to identify a plant with a recombination event between the end of the YAC clone and the *Eix* locus. Therefore, we placed the end-specific probes on the high-resolution linkage map of the region. Probe 348R was mapped on the introgression IL7–5 while probe 348L did not map on lines IL7-5 or IL7-4. Therefore we considered the YAC 348E2 to be a chimeric clone. Both probes 294L and 317L co-segregate with the marker TG61 (Fig. 4). Probe 294R co-segregates with the *Eix* locus, while probe 317R co-segregates with the TG131 marker on one of the recombinant plants (Fig. 4; a recombinant between *Eix* and TG131). Our results indicate that the YAC 317 clone, having an insert of 300 kb, encompasses *Eix* locus. Since 294R co-segregated with the *Eix*, it is the closest marker to the *Eix* locus. In conclusion, our genetic and physical analysis suggests that the *Eix* locus is located within a region of the tomato genome amenable to successful chromosome landing. The isolation of the gene controlling the plant responses to EIX will contribute to our understanding of signal transduction in the interaction between plant and microorganism.

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