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Isolation of a novel SUMO protein from tomato that suppresses EIX-induced cell death

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Summary

Challenging tomato or tobacco varieties with ethylene-inducing xylanase (EIX) from the fungus *Trichoderma viride* causes rapid induction of plant defence responses leading to programmed cell death. Using the yeast two-hybrid system, we isolated a novel protein, tomato small ubiquitin-related modifier protein (T-SUMO), which specifically interacts with EIX. T-SUMO, a cytoplasmic protein, is a member of the ubiquitin-like protein family. It shows homology to human protein sentrin/SUMO1, which suppresses tumour necrosis factor-induced cell death. Transgenic plants that express T-SUMO in the sense orientation suppress EIX induction of ethylene biosynthesis and cell death, while in the antisense orientation they enhance EIX-induced ethylene biosynthesis. These results indicate that T-SUMO is involved in mediating the signal generated by EIX that leads to induction of plant defence responses.

Introduction

Elicitors that trigger plant defence responses have been isolated from a variety of phytopathogenic (Blein *et al.*, 1991; Bottin *et al.*, 1994; He *et al.*, 1993) and non-pathogenic micro-organisms (Ebel and Cosio, 1994; Fuchs *et al.*, 1989; Ricci *et al.*, 1993). The defence response includes cell-wall strengthening, phytoalexin production, ethylene biosynthesis, expression of pathogenesis-related (PR) proteins and localized cell death (Atkinson, 1993; Greenberg, 1997; Jakobek and Lindgren, 1993; Morel and Dangl, 1997).

Defence responses in plants have been compared to apoptosis phenomena in mammalian cells (Mittler and Lam, 1996; Mittler *et al.*, 1997; O'Brien *et al.*, 1998). The first event in triggering programmed cell death is probably recognition of the elicitor by the plant cells (Diekmann *et al.*, 1994; Hanania and Avni, 1997; Scofield *et al.*, 1997; Tang *et al.*, 1997). Molecular characterization of plant resistance genes is an important step in understanding plant defence mechanisms. Several disease resistance genes have been isolated (Staskawicz *et al.*, 1995) and similarities have been found between them and mammalian genes (Whitham *et al.*, 1994; Zhou *et al.*, 1995).

A highly purified 22 kDa protein (β-1–4-endoxylanase), referred to as ethylene-inducing xylanase (EIX), was isolated from the fungus *Trichoderma viride* (Dean *et al.*, 1989; Fuchs *et al.*, 1989). EIX is very active in inducing ethylene biosynthesis in tobacco (Bailey *et al.*, 1990), tomato and pepper (Ron *et al.*, 1999). In addition, EIX induces accumulation of PR proteins, phytoalexins, electrolyte leakage and programmed cell death in sensitive strains of tobacco (Bailey *et al.*, 1990; 1992; Lotan and Fluhr, 1990) and tomato (Ron *et al.*, 1999). These biochemical defence responses are common responses of plants challenged by exogenously applied elicitors (Blein *et al.*, 1991; Bottin *et al.*, 1994; Felix *et al.*, 1993; Keen *et al.*, 1990; Keen, 1992). Induction of defence responses by EIX is controlled by a single dominant gene in both tobacco and tomato (Bailey *et al.*, 1993; Ron *et al.*, 1999). A high-affinity binding site was found in tomato and tobacco for the EIX elicitor and binding occurs only in varieties genetically capable of responding to the elicitor (Hanania and Avni, 1997).

In mammalian cells, tumour necrosis factor (TNF) induces programmed cell death by binding to the Fas/Apo-1 receptor. The presence of a 'death domain' in the cytoplasmic region of the receptor is responsible for transducing the death signal (Itoh and Nagata, 1993; Nagata, 1997). Using the death domain as bait in the two-hybrid interaction assay, Okura *et al.* (1996) isolated a novel protein, sentrin. Sentrin shows amino acid similarity to ubiquitin, Nedd8 and a *Saccharomyces cerevisiae* protein, Smt3. When over-expressed, sentrin provides protection against both anti-Fas/APO-1 and TNF-induced cell death. The SUMO-1 protein, which appears to be involved in protein targeting, is identical to sentrin (Mahajan *et al.*, 1997; Okura *et al.*, 1996).

The signal transduction pathway by which the EIX elicitor induces cell death is not well defined. We hypothesized that proteins involved in this pathway will associate with each other in the cell. We therefore used the yeast two-hybrid system to clone genes encoding proteins that interact physically with EIX. Here we report the isolation of a cDNA encoding a tomato homologue of human protein sentrin/SUMO-1. The tomato small ubiquitin-related modifier protein (T-SUMO) is shown to suppress the induction of defence responses by the EIX elicitor in transgenic plants.

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Results

Two-hybrid screen for EIX-interacting proteins

The cDNA encoding the EIX protein was isolated from a library prepared from *Trichoderma viride* (Furman-Matarasso *et al.*, 1999). The cDNA encoding the mature protein was fused in-frame to the C-terminus of the DNA-binding domain of LexA in the 'bait' plasmid pEG202 and transformed into the yeast strain EGY48 (Gyuris *et al.*, 1993). We verified the expression of the full-length fusion protein encoded by the bait construct in yeast cells by immunoblot analysis using anti-EIX antibodies (Figure 1a). The EIX bait was transformed into the yeast strain EGY48 containing an arbitrary DNA fragment (insert in pJG4-5; Figure 1a) in order to ensure that it did not activate transcription of the reporter genes. Moreover, the EIX bait was introduced into EGY48 containing pJK101 to ensure that the LexA–EIX fusion protein translocated into the nucleus. For 'prey', we used a tomato-leaf cDNA library (Zhou *et al.*, 1995) and screened approximately 5×10^6 independent clones. The initial screen recovered approximately 5000 leucine-prototrophic colonies. Of these, four colonies appeared blue on galactose medium containing X-gal. One was homologous to ribosomal RNA while two had small insert fragments that did not show homology to the sequence databank. The fourth colony, designated tomato small ubiquitin-related modifier protein (T-SUMO), was fully characterized and is described below.

Yeast strains carrying the EIX bait and T-SUMO prey grew in the absence of leucine, indicating LEU2 reporter gene activation (Figure 1b). When grown on X-gal plates, these yeast cells were blue as a result of LacZ reporter gene activation. By contrast, a control yeast strain expressing the arbitrary bait, Bicoid (LexA fused to a transcriptionally inert fragment of the *Drosophila* Bicoid product) and the T-SUMO prey did not activate the LEU2 or LacZ reporter genes (Figure 1b). Expression was dependent upon growth on galactose medium, indicating that expression of T-SUMO was required for expression of the reporter genes. Thus, T-SUMO encodes a protein that physically interacts with EIX protein in the yeast two-hybrid system.

An *in vitro* interaction assay was conducted to assess whether the interaction between T-SUMO and EIX observed in the two-hybrid system is a direct one. A GST–T-SUMO fusion protein was engineered and expressed in *E. coli*. GST or GST–T-SUMO proteins were used to precipitate the EIX protein elicitor *in vitro*. EIX was incubated with resin-bound GST–T-SUMO or resin-bound GST. After incubation, the resins were washed extensively and the bound proteins separated by SDS–PAGE, transferred to a nitrocellulose membrane and probed with anti-EIX antiserum. The results (Figure 1c) showed that GST–T-SUMO, but not GST, specifically precipitates the EIX protein. Thus, the findings from the *in vitro* interaction are consistent with those observed in the yeast two-hybrid system.

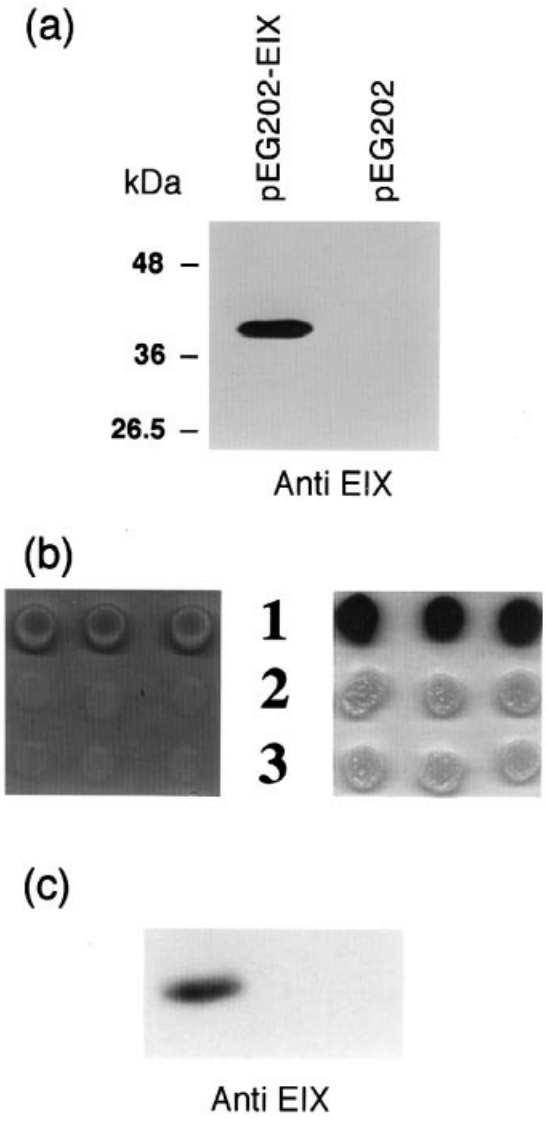

Figure 1. Interaction of EIX with T-SUMO.
(a) EGT48 yeast cells were transformed with pEG202 or pEG202–EIX. Total yeast proteins (10 μg lane^{-1}) were separated on 12% acrylamide SDS–PAGE. The expression of bait–EIX fusion protein was analysed by immunoblotting using anti-EIX antibodies (Dean and Anderson, 1991). (b) EGY48 yeast cells containing (1) EIX (in pEG202) and T-SUMO (in pJG4-5), (2) Bicoid (in pEG202) and T-SUMO (in pJG4-5), or (3) EIX (in pEG202) and arbitrary insert (in pJG4-5), were all grown on galactose medium lacking uracil and the amino acids Leu, His and Trp (left) or the same but supplied with Leu and X-gal (right). (c) EIX was incubated with resin-bound GST–T-SUMO and GST as described in Experimental procedures. Bound proteins were separated on a 15% acrylamide SDS–PAGE gel, transferred to nitrocellulose filter and probed with anti-EIX antibodies.

T-SUMO is similar to ubiquitin-like proteins

The entire nucleotide sequence of the T-SUMO clone (EMBL accession no. AJ012717) was determined. The

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T-SUMO      MS...ASGGT  GD.EDKKPND  QMVHINLKVK  26
Sentrin/SUMO1 MSDQEAKPST  EDLGDKK.EG  E..YIKLKVI  27

T-SUMO      GQDGNEVFFR  IKRSTQMRKL  MNAYCDRQSV  56
Sentrin/SUMO1 GQDSSEIHFK  VKMTTHLKKL  KESYCQRQGV  57

T-SUMO      DMNSIAFLFD  GRRLRAE.QT  PDELEMEEGD  85
Sentrin/SUMO1 PMNSLRFLFE  GQRI.ADNHT  PKELGMEEED  86

T-SUMO      EIDAMLH.QT  GG.SCCTCFS  NF  105
Sentrin/SUMO1 VIE.VYQEQT  GGHS..TV..  ..  101
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Figure 2. Similarity of T-SUMO to the human protein sentrin/SUMO-1.
The bestfit and pretty box programs (GCG sequence analysis software package, version 9.0; University of Wisconsin, Madison, Wisconsin, USA) were used to align the T-SUMO amino acid sequence (EMBL accession no. AJ012717) with that of sentrin/SUMO-1 (Okura *et al.*, 1996). Identical amino acids are shaded in black and conservative substitutions are shaded in grey.

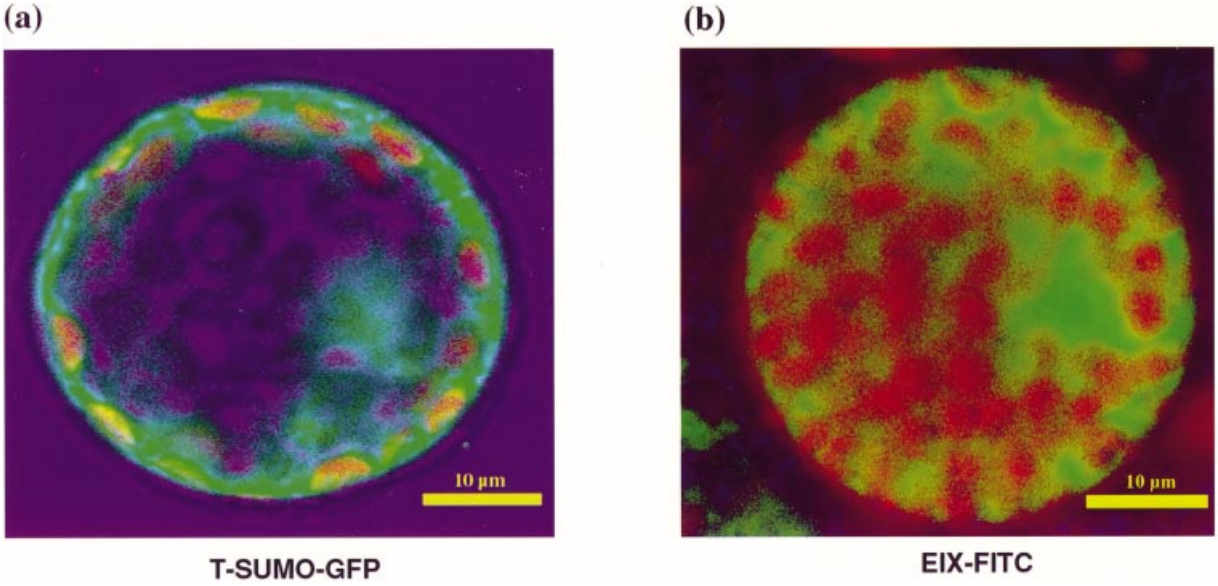

Figure 3. Localization of T-SUMO and EIX.
(a) Protoplasts derived form *N. tabacum* cv Samsun were transformed with T-SUMO–GFP genes under the control of the 35S-Ω promoter as described in Experimental procedures. Transient expression of T-SUMO–GFP was monitored by confocal laser-scanning microscopy 48 h after transformation. Subcellular components were identified by fluorescense and Nomarski microscopy. Chloroplasts are coloured red, over-expressed T-SUMO–GFP protein is green, and other components are blue.
(b) Protoplasts were incubated with 20 nM FITC-labelled EIX. Following incubation for 60 min, protoplasts were washed three times and examined by confocal laser-scanning microscopy. Chloroplasts are red, FITC–EIX protein is green, and other components are blue. A 10 μm bar is indicated.

0.53 kb cDNA clone contains an open reading frame of 105 amino acids (Figure 2). The first ATG of the open reading frame is preceded in-frame by two stop codons at –18 to –13 and a purine (A) at position –3. This is a conserved context for initiation of translation in eukaryotes (Kozak, 1989). A search of the database with the deduced amino acids of T-SUMO revealed significant identity to ESTs from *Arabidopsis thaliana* (90%; accession no. x99609) and *Oryza sativa* (88%; accession no. x99608). There was also 64% similarity and 47% identity with the human gene sentrin/SUMO-1 (Figure 2; Mahajan *et al.*, 1997; Okura *et al.*, 1996). This conservation extended to the two glycine residues (positions 96, 97) near the C-terminus. The similarity from humans to tomato suggests that that SUMO is an evolutionarily conserved protein that has a specialized function in cellular metabolism.

Cellular localization of T-SUMO and movement of EIX into the cytoplasm

We used transient expression of T-SUMO–GFP fusion protein in tobacco protoplasts to locate the T-SUMO

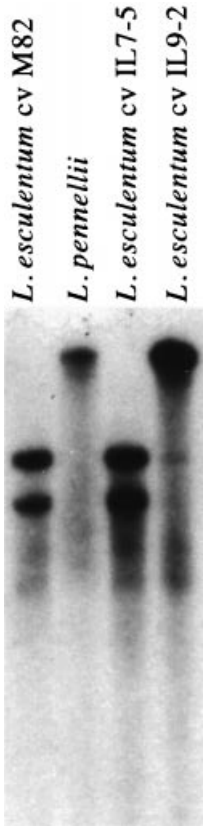

Figure 4. Mapping the T-SUMO gene.
Genomic DNA (10 μg lane^{-1}) from *Lycopersicon esculentum* cv M82, *L. esculentum* cv IL7-5, *L. esculentum* cv IL9-2 and *L. pennellii* was digested with *Eco*RI, separated on a 1% agarose gel, transferred to a nylon membrane and hybridized with a T-SUMO probe.

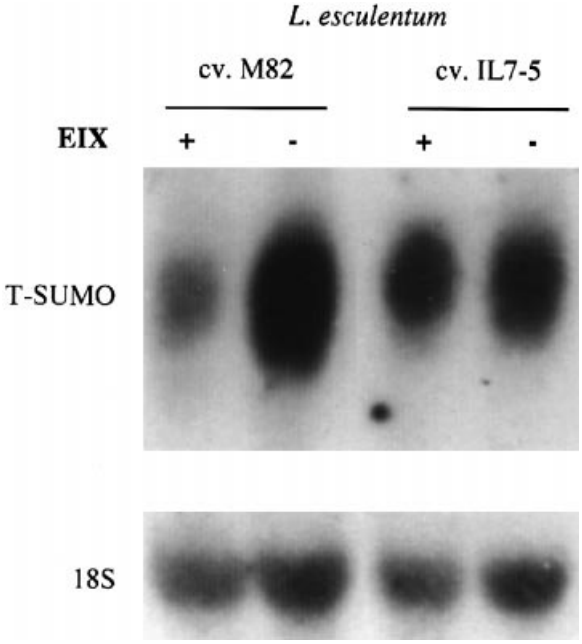

Figure 5. Expression the of T-SUMO gene.
Lycopersicon esculentum cv M82 and cv IL 7-5 were treated with EIX for 4 h and RNA was isolated as described in Experimental procedures. Total RNA (10 μg lane^{-1}) was separated on a 1% agarose gel, blotted to nylon membranes and hybridized with T-SUMO DNA and rDNA probes. A representative experiment of three repeats is shown.

protein in the cell. Confocal laser-scanning microscopy revealed that the expressed T-SUMO–GFP fusion protein is localized to the cytoplasm (Figure 3a). This raised an apparent paradox: T-SUMO is a cytoplasmic protein while EIX is known to interact with cell membranes (Hanania and Avni, 1997). To resolve this, we examined the possibility that EIX after binding to the plant membranes is transported into the cytoplasm, where it exerts its effects. EIX was labelled with FITC and incubated with protoplast of *N. tabacum* cv Samsun for 60 min. The protoplasts were then extensively washed and FITC–EIX localized by confocal laser microscopy. The labelled protoplasts clearly showed intense fluorescent staining of EIX in the cytoplasm (Figure 3b). Thus, after binding to the plant membranes, EIX is able to enter the cytoplasm.

Mapping T-SUMO on the tomato genome

The response of tomato or tobacco plants to EIX treatment is controlled by a single gene mapped in tomato to the short arm of chromosome 7 (Bailey *et al.*, 1993; Ron *et al.*, 1999). In order to determine whether the T-SUMO gene co-segregates with the gene controlling this trait, we mapped the T-SUMO gene on the tomato genomic map. Genomic DNAs were isolated from *L. esculentum* cv M82, *L. pennellii* and from 52 introgression lines (Eshed *et al.*, 1992). The DNAs were digested with *Eco*RI separated on 1% agarose gel, blotted on nylon filter and hybridized with the T-SUMO clone. All of the introgression lines (see example, *L. esculentum* cv IL7-5) showed polymorphic patterns similar to *L. esculentum* cv M82, except for *L. esculentum* cv IL9-2, which showed the polymorphic pattern of *L. pennellii* (Figure 4). Furthermore, we have shown that *L. esculentum* cv M82 and cv IL9-2 extensively respond to EIX treatment while *L. esculentum* cv IL7-5 and *L. pennellii* do not respond at all (Ron *et al.*, 1999). Thus, T-SUMO maps to the short arm of chromosome 9 and is distinct from the gene on chromosome 7 controlling the response to EIX treatment (Ron *et al.*, 1999). Finally, Southern blot analysis under stringent conditions using the entire cDNA as a probe detected only a few fragments in tomato genomic DNA (Figure 4) suggesting that T-SUMO is a low copy number gene.

T-SUMO and EIX: mutual effects

When *L. esculentum* cv M82 and *L. esculentum* cv IL7-5 were treated with EIX for 4 h and total RNA isolated, we found a reduction in the amount of RNA coding for T-SUMO

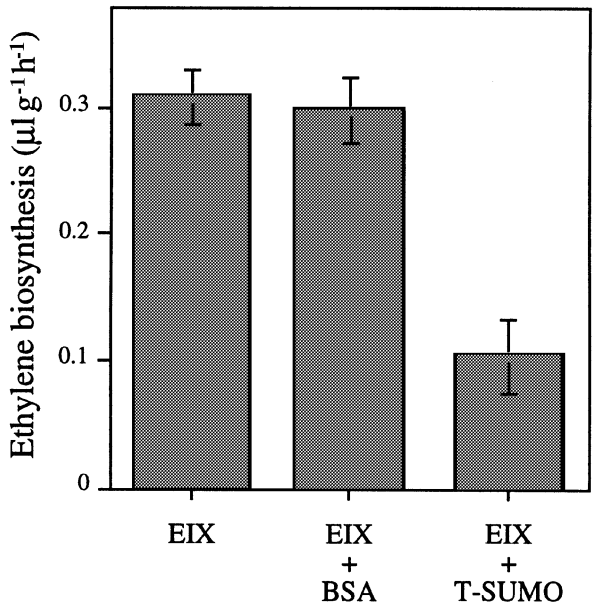

Figure 6. *In vitro* effect of T-SUMO protein on ethylene biosynthesis.
Six leaf disks of *N. tabacum* cv Samsun were treated with EIX (1 µg ml^{-1}), EIX + BSA (1 µg ml^{-1}) or EIX + T-SUMO (1 µg ml^{-1}) for 4 h. Ethylene production was measured by gas chromatography as described elsewhere (Avni *et al.*, 1994). The results shown are the average of five independent experiments. Standard errors are indicated.

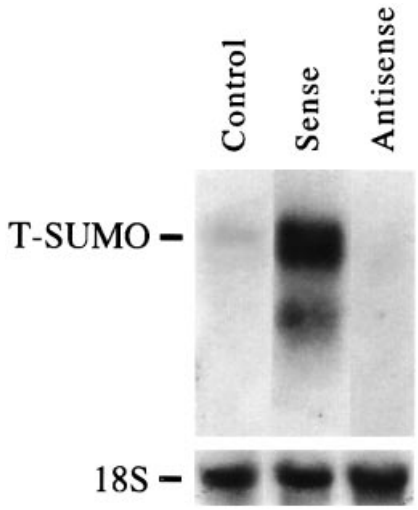

Figure 7. Expression of T-SUMO mRNA in transgenic plants.
Total RNA was isolated from the leaves of transgenic tobacco plants. RNA gel-blot analysis was performed on controls (containing only vector), T-SUMO in sense orientation or T-SUMO in antisense orientation. Total RNA (10 µg lane^{-1}) were separated on a 1% agarose gel, transferred to a nylon filter and hybridized with T-SUMO DNA and rDNA probes.

only in EIX-responding plants (Figure 5). This suggests that T-SUMO mediates the signal generated by EIX.

The effect of T-SUMO on the induction of ethylene biosynthesis by EIX was also determined. We found that T-SUMO, but not BSA, drastically reduced the amount of ethylene induced by EIX (Figure 6). This suggests that T-SUMO may function as a repressor of the defence response.

Expression of T-SUMO in transgenic tobacco plants suppresses the induction of the defence response by EIX

To test the biological relevance of T-SUMO gene expression, we introduced the T-SUMO gene into tobacco cultivar Samsun under the control of a strong constitutive promoter. The T-SUMO open reading frame was inserted into pBIN19$^+$ (van Engelen *et al.*, 1995) in the sense and antisense orientation under the control of the cauliflower mosaic virus 35S promoter and the Ω translation enhancer signal (Mitsuhara *et al.*, 1996). Transgenic *N. tabacum* cv Samsun plants were generated using *Agrobacterium*-mediated transformation. Eight independent transgenic plants were regenerated from each construct, and transformation confirmed by antibiotic resistance and DNA blot analysis. Southern blot analysis showed that there are several copies of the T-SUMO gene in the sense orientation and in the antisense orientation. Northern blot analysis indicated that in transgenic plants harboring the sense construct there is a high level of T-SUMO RNA compared to wild-type plants, while in transgenic plants harboring the antisense construct the level of T-SUMO RNA is reduced to below the level in wild-type plants (Figure 7).

In tobacco, EIX induces cell death within 6–48 h after treatment (Bailey *et al.*, 1995; Avni, unpublished data) and there is complete tissue desiccation within 96 h. We examined the induction of cell death in 12 independent transgenic plants (six in sense orientation and six in antisense orientation). In the transgenic sense plants, the induction of cell death by EIX was clearly suppressed compared to control plants transformed with pBIN19$^+$ alone (Figure 8a), while with the antisense transgenes, induction was as in controls (not shown). We further inoculated 12 independent transgenic plants (six sense and six antisense) with EIX and measured the induction of ethylene biosynthesis 4 h after treatment. Ethylene induction in the transgenic sense plants was consistently reduced, and in the antisense transgenes consistently enhanced, compared to control plants transformed with pBIN19$^+$ alone (Figure 8b). The antisense results indicate a direct involvement of T-SUMO protein in the EIX signal transduction pathway leading to induction of programmed cell death.

In order to analyse the specificity of the EIX–T-SUMO interaction, we assayed the induction of ethylene biosynthesis and cell death in six independent transgenic plants (three sense and three antisense) by a non-host 24 kDa elicitor isolated from *Fusarium oxysporum* sp. Erythroxylum coca (Bailey, 1995). We found no difference in the response (induction of ethylene biosynthesis and

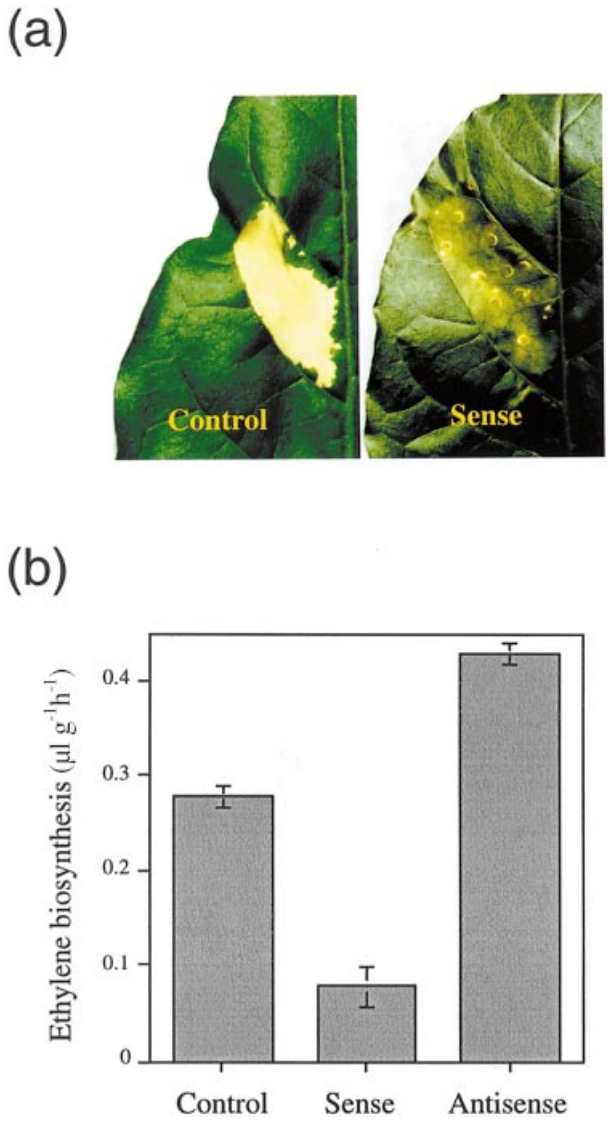

Figure 8. Induction of ethylene and cell death by EIX in transgenic tobacco plants.
(a) Young, fully expanded leaves from a transgenic plant harbouring T-SUMO in sense orientation, or control containing only vector, were injected with EIX (2.5 μg g^{-1} tissue) as described by Bailey *et al.* (1990). Development of cell death was monitored 96 h after treatment with EIX. (b) Leaf disks of from transgenic plants harbouring T-SUMO in sense or antisense orientations, or control (containing only vector), were treated with EIX (1 μg ml^{-1}) for 4 h. Ethylene production was measured by gas chromatography as described previously (Avni *et al.*, 1994). The results shown are the average of five independent experiments. Standard errors are indicated.

induction of cell death) of the sense, antisense and control plants to this elicitor.

Discussion

In order to understand the downstream signalling events involved in induction of the EIX defence response, we used the yeast two-hybrid system to isolate T-SUMO, a plant cDNA that encodes a protein physically interacting with the EIX fungal elicitor. EIX or T-SUMO alone, or either one in combination with a non-specific clone, could not activate the reporter genes. The interaction of the two, either in yeast cells or *in vitro*, proved necessary for a response. T-SUMO presumably acts downstream of the gene (Ron *et al.*, 1999) controlling the response of the plant to EIX treatment (Figure 4). In this regard, *N. tabacum* cv SR1, an EIX-insensitive cultivar, transformed to harbour T-SUMO in the sense or antisense orientation, does not respond to EIX treatment (Avni, unpublished data). This suggests that EIX, which binds to the cell membrane (Hanania and Avni, 1997), has to be first recognized by the plant cell and only then can T-SUMO function in the signal transduction pathway. Indeed, 60 min after EIX treatment, EIX can be localized to the cytoplasm of responding plants (Figure 3b).

We assessed the significance of our findings by testing the effects of EIX–T-SUMO interaction *in vivo* in transgenic plants. We showed that the level of T-SUMO mRNA drops after EIX treatment only in plants responding to EIX treatment (Figure 5). As EIX may influence the activity of a large set of genes, its effect on T-SUMO mRNA may be indirect. Alternatively, as a result of EIX application, gene(s) such as T-SUMO which act as suppressors for induction of the defence response maybe directly inhibited.

The mechanism of T-SUMO action is not clear. T-SUMO might inhibit the defence response in tobacco by blocking transmission of a death signal. Alternatively, as SUMO proteins are members of the ubiquitin-like protein family (Gong *et al.*, 1997; Kamitani *et al.*, 1998; Mahajan *et al.*, 1997), T-SUMO might exert its suppressive effect through modification of other proteins. Indeed, SUMO-1/sentrin can modify other proteins in a process similar to ubiquitination (Hershko and Ciechanover, 1998; Kamitani *et al.*, 1998). Interestingly, tobacco plants perturbed in their ubiquitin system have a tendency to form necrotic lesions resembling a typical defence response (Bachmair *et al.*, 1990). Moreover, expression of mutated ubiquitin influences the response of the plants to viral infection (Becker *et al.*, 1993).

Several fungal pathogens secrete endoxylanases during infection of plant tissue (Wu *et al.*, 1997). The impact of those enzymes on pathogenicity is unclear. *Magnaporthe grisea* appears capable of secreting additional xylanases when the major expressing xylanase has been eliminated (Wu *et al.*, 1997), suggesting that xylanases might have a role in pathogenicity. On the other hand, EIX may function as an avirulence gene product by binding to a specific plant protein and inducing the plant defence response.

Is the EIX-response system specific? For this we tested the response of transgenic plants to a non-host elicitor

isolated from *Fusarium oxysporum* sp. Erythroxylum coca (Bailey, 1995). Similar to EIX (Bailey *et al.*, 1990), this elicitor induces ethylene production and cell death in many plants including tobacco. However, there was no difference in the response of control or transgenic plants (sense and antisense) to this elicitor. Our working hypothesis is that the EIX system is a specific system following the gene-for-gene model. It is similar to the Pto system where fenthion does not recognize the Pto gene product and AvrPto does not recognize the Fen gene product (Frederick *et al.*, 1998).

Experimental procedures

Plant material and EIX purification

Tobacco plants were grown under greenhouse conditions. EIX, a kind gift of Dr J.D. Anderson (USDA, Beltsville, Maryland, USA), was purified as previously described from xylan-induced cultures of *Trichoderma viride* (Dean and Anderson, 1991).

Yeast two-hybrid interaction and cDNA library screening

The plasmids (pEG202, pJG4-5, pSH18-34, pRSHM1 and pJK101) and yeast strain EGY48 (ura3, his3, trp1, lexApo-leu2) were kindly provided by R. Brent (Massachusetts General Hospital, Boston, Massachusetts, USA). The basic procedure for the two-hybrid system was according to Gyuris *et al.* (1993). To create the in-frame LexA–EIX fusion construct (bait), we cloned the mature EIX protein from a cDNA clone encoding the EIX gene (Furman-Matarasso *et al.*, 1999) into the pEG202 vector. The bait vector construct was confirmed by DNA sequencing. A tomato cDNA prey library in pJG4-5 (Zhou *et al.*, 1995) was used for interaction screening. Screening was done as described previously (Zhou *et al.*, 1995).

Expression of GST fusion proteins and in vitro *interaction assay*

Expression and purification of GST fusion proteins were performed as described by Kaelin *et al.* (1991). The full-length open reading frame of T-SUMO was fused to GST in the expression vector pGEX-3X (Pharmecia) to generate the GST–T-SUMO clone. *E. coli* XL1-blue cells transformed with GST–T-SUMO construct were cultured in 500 ml LB supplied with 100 $\mu g\ ml^{-1}$ of ampicillin. At $OD_{600} = 0.3$, IPTG was added to a final concentration of 0.1 mM. After 2 h incubation, the culture was harvested, washed in cold PBS and suspended in 10 ml PBS, 1 mM DTT and 0.1 mM PMSF. After overnight storage at –20°C, the cells were disrupted in a French press followed by centrifugation for 20 min at 15 000 *g*. The supernatant was incubated with Glutathione–Sepharose beads at 4°C for 4 h, and the resin washed five times with 10 ml of PBS. Solution of EIX (1 $\mu g\ ml^{-1}$) and BSA (1 $\mu g\ ml^{-1}$) were incubated overnight at 4°C with the GST–T-SUMO–glutathion–Sepharose or GST–glutathion–Sepharose beads in PBS containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$, followed by five washes with 1 ml of 100 mM NaCl, 50 mM Tris–HCl (pH 8.0). Proteins were eluted with the same buffer with addition of 50 mM reduced glutathione. Bound proteins were analysed by 15% polyacrylamide gel electrophoresis in the presence of SDS (Laemmli, 1970), followed by immunoblotting and probing with antibodies against EIX (Dean and Anderson, 1991).

DNA and RNA analysis

DNA isolation, restriction digestion, electrophoresis on agarose gel, Southern blots, hybridization and autoradiography were performed as described by Bernatzky and Tanksley (1986) except that filters were probed with random hexamer-labelled plasmids (Feinberg and Vogelstein, 1983). Sequencing was performed by the dideoxy chain termination method (Sanger, 1981). Sequence analysis was performed using the GCG version 9.0 sequence analysis software package (University of Wisconsin, Madison, Wisconsin, USA). Fully expanded tobacco leaves were harvested and total RNA was extracted using the RNeasy plant kit (Qiagen) as recommended by the manufacturers. RNA hybridizations were performed as described by Avni *et al.* (1994).

Tissue treatment

EIX (1 $\mu g\ ml^{-1}$) was applied to attached leaves by infiltration into the intercellular space with the blunt end of a 1 ml syringe and observed for induction of cell death development for 96 h. To measure induction of ethylene biosynthesis, six leaf disks were floated on 1 ml assay medium (10 mM MES pH 6.0, 250 mM sorbitol) containing 1 $\mu g\ ml^{-1}$ EIX for 4 h. Ethylene biosynthesis was assayed as described in Avni *et al.* (1994).

Tobacco transformation and microscope analysis

The GFP gene fused in-frame behind T-SUMO, under the control of 35S-Ω promoter (Mitsuhara *et al.*, 1996), was cloned into pUC19. The resulting construct was introduced into protoplasts isolated from *N. tabacum* cv Samsun as described elsewhere (Aviv and Galun, 1985). Fluorochrome-labelled protoplasts were examined with a Zeiss 1000 CLSM 410 confocal laser-scanning microscope with the following configurations: 25 mW Ar and HeNe lasers with 488, 514 and 543 maximum lines. Fluorescence and Nomarski images were generated. When comparing the fluorescence intensity, we used identical parameters for each image (scanning line, laser light, contrast and brightness). EIX was labelled with fluorescein isothiocyanate (FITC) as described by Hanania and Avni (1997).

The T-SUMO gene was ligated in the sense and antisense orientation between the 35S-Ω promoter containing the translation enhancer signal and the Nos terminator (isolated from the plasmid pBI121; Clontech Laboratories, Palo Alto, California, USA). These fragments were introduced into pBINplus vector (van Engelen *et al.*, 1995). The resulting constructs were electroporated into *A. tumefaciens* LBA4404 and transformed into *N. tabacum* cv Samsun and cv SR1 as described by Thilmony *et al.* (1995).

Acknowledgements

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