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Isolation of the Gene for EILP, an Elicitor-Inducible LRR Receptor-Like Protein, from Tobacco by Differential Display

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We screened tobacco genes, which are differentially expressed in response to a fungal elicitor, and have isolated a gene which codes for a leucine-rich repeat (LRR) protein closely related to Cf genes in tomato. The *EILP* (elicitor inducible LRR protein) gene encodes 95 kDa protein, which consists of a putative membrane spanning region, 28 leucine-rich repeats and some N-linked glycosylation sites, and shows high homology to *Cf-2/Cf-5* family genes. Southern blot analysis revealed the presence of some genes homologous to *EILP* in tobacco, like *Cf* genes in tomato. The expression of *EILP* was low at the basal level and increased by treatment with elicitor, implying that *EILP* is involved in both preexisting and inducible surveillance systems. The expression of *EILP* was activated by a non-pathogen, *Pseudomonas syringae* pv. *glycinea*, and in a delayed fashion by the tobacco pathogen *P. syringae* pv. *tabaci*, suggesting that the product of *EILP* may be involved in non-host disease resistance in tobacco.

Key words: Differential display — Elicitor-inducible gene — LRR protein — *Pseudomonas syringae* — Tobacco.

Plants attacked by pathogenic microbes respond with a number of protective biochemical changes. Such responses include hypersensitive cell death, the production of reactive oxygen species, cell wall fortification, accumulation of pathogenesis-related (PR) proteins and other anti-microbial proteins, and biosynthesis of low molecular weight antimicrobial compounds called phytoalexins (Hammond-Kosack and Jones 1996). Genes activated in plant disease resistance can be divided into three classes. One class consists of genes encoding transcriptional regulators such as BPF-1 and WRKY1–3 (da Costa e Silva et al. 1993, Rushton et al. 1996). The expression of these regulatory genes is activated rapidly, and their products bind to and activate the cis-acting elements of other genes involved in halting the growth and spread of pathogens. Another class of genes encodes enzymes that are involved in secondary metabolic pathways. These enzymes catalyze the biosynthesis of wall appositions and phytoalexins, which are accumulated in cells that are in direct contact with the pathogen and in the surrounding cells. The third class of defense genes is induced at a late stage in the defense responses, not only in the invaded region, but also systemically. These genes include genes for PR-proteins. Some kinds of PR-proteins, such as chitinase and β-1,3-glucanase, have enzymatic activities, and have been shown to possess antifungal and antibacterial properties by their ability to degrade microbial cell wall structural polysaccharides. It was also suggested that the other PR-proteins have the anti-microbial activities (Niderman et al. 1995, Ponstein et al. 1994). The expression of genes encoding PR-proteins is generally used as an index of disease response in plants (Stintzi et al. 1993).

As described above, various physiological changes associated with defense responses are induced by the expression of various genes. As in the example of PR-proteins, the cloning and characterization of genes expressed in plant disease resistance could be an initial step toward understanding the molecular mechanisms. To achieve new insights into disease responses induced by transcriptional activation, we used the differential display method with random primers to compare fungal elicitor-treated plants with water-treated plants. Differential display is easier and more sensitive than subtractive or differential hybridization procedures, and allows the detection of relatively scarce mRNAs (Yoshida et al. 1994). Now, we describe a tobacco gene encoding an elicitor-inducible LRR receptor-like protein, the *EILP* gene, that is closely related to the *Cf* genes of tomato. These latter genes confer resistance to the leaf mold pathogen *Cladosporium fulvum* (Dixon et al. 1996, 1998). In this study, we showed that the transcription of the *EILP* was activated by treatment of salicylic acid or inoculation of *Pseudomonas syringae* pv. *glycinea*. This raises the possibility that the *EILP* product amplifies the sensitivity to disease stress and induces the subsequent disease responses.

Abbreviations: *EILP*, gene encoding an elicitor-inducible LRR protein; HWC, hyphal wall components; LRR, leucine-rich repeat; PR-protein, pathogenesis related protein.

The nucleotide sequence reported in this paper has been submitted to the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB029327.

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Materials and Methods

Plants and bacterial strains—Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were cultivated at 25°C for 6 or 8 weeks after sowing. *P. syringae* pv. *glycinea* 801 and *P. syringae* pv. *tabaci* 274 were provided by Dr. Yuichi Takigawa, Faculty of Agriculture at Shizuoka University, Japan. The bacteria were cultured at 25°C in King's B medium, harvested by centrifugation, resuspended in water, and used for inoculation.

Elicitor treatment and wounding of tobacco leaves—Hyphal wall components (HWC) elicitor was prepared from mycelia of *Phytophthora infestans* that had been grown in liquid medium for 2 weeks at 20°C in darkness as described previously (Doke and Tomiyama 1980). Detached leaves of 8-week-old tobacco plants or intact leaves of 6-week-old tobacco plants were injected with water, 1 mg ml^{-1} HWC, 0.5 mM salicylic acid or *P. syringae* pv. *glycinea* or pv. *tabaci* at 3×10^6 c.f.u. ml^{-1}. Solutions were injected into the intercellular space of tobacco leaf using a syringe without a needle. The injected areas were harvested at defined times for RNA extraction. For the wounding experiment, intact leaf of 6-week-old tobacco plants was abraded with carborundum (600 mesh). Plants were left in a room held at 25°C, and leaves were harvested at defined times. Detached leaves were incubated at 25°C in a translucent box lined with moist filter paper until harvesting at defined times.

Differential display and RT-PCR method—RT-PCR for differential display (Yoshida et al. 1994) was carried out using the RT-PCR high plus Kit (TOYOBO, Japan). Of the total RNA, 0.5 μg was added to 12.5 μl RT-PCR solution containing 1x reaction buffer, 300 μM dNTPs, 2.5 mM $Mn(OAc)_2$, 0.4 unit RNase inhibitor and 0.1 unit rTth DNA Polymerase. Each random primer (DNA Oligomer (12) Set, A01-12, A21-32, A41-52, A61-72, A81-92, B01-12, B21-32, B41-52, B61-72, D01-D12, D21-32, D41-52, Wako Pure Chemical Industries, Japan) at 0.8 μM was added to the RT-PCR mixture. The amplification was taken through one cycle of 30 min at 60°C and 2 min at 94°C followed by 40 cycles of 1 min at 94°C and 1.5 min at X°C ($X = 4 \times (C+G) + 2 \times (A+T) - 10$). After the last cycle, one final extension step was performed for 7 min at X°C. RT-PCR for amplification of the D44-12 fragment or the 3′-untranslated region of the EILP gene was performed under the same conditions with each set of specific primers, D44-F1 (5′-GAGAATACAATTATTCTG-3′) and D44-R1 (5′-CAGCTGAGAAGCAATTTG-3′) derived from the D44-12 fragment or EILP-UTR-F (5′-TATCAGAACTTCAGATGT-3′) and EILP-UTR-R (5′-AAGGATCAATCAATTCATC-3′) derived from the 3′-untranslated region of the EILP cDNA.

Northern hybridization—Twenty μg of total RNA was separated on a 1% formaldehyde agarose gel and transferred onto a Hybond N (+) membrane (Amersham). The cDNAs for EILP and basic PR-3 (Neale et al. 1990) were labeled with [α-^{32}P]dCTP using a random-primed DNA labeling kit (Megaprime; Amersham). Hybridizations were performed at 42°C for 20 h in 5× SSPE (20× SSPE; 3 M NaCl, 173 mM $NaH_2PO_4 \cdot 2H_2O$, 25 mM EDTA), 50% formamide, 5× Denhardt's solution, 1% SDS and 100 μg ml^{-1} denatured salmon sperm DNA. Membranes were washed with 1× SSPE and 0.2% SDS at 20°C for 30 min, and 0.1× SSPE and 0.1% SDS at 65°C for 5 min and then subjected to autoradiography.

Genomic Southern hybridization—Ten μg of tobacco DNA was digested with *Eco*RI, *Eco*RV or *Hin*dIII, and the digests were fractionated on a 0.8% agarose gel and transferred onto a Hybond N (+) membrane (Amersham). The full-length cDNA for *EILP* were labeled with [α-^{32}P]dCTP using a random-primed DNA labeling kit (Megaprime; Amersham). Hybridization was performed at 42°C (high-stringency) or 38°C (low-stringency) for 20 h in 5× SSPE (20× SSPE; 3 M NaCl, 173 mM $NaH_2PO_4 \cdot 2H_2O$, 25 mM EDTA), 50% formamide, 5× Denhardt's solution, 1% SDS and 100 μg ml^{-1} denatured salmon sperm DNA. Membranes were washed with 2× SSPE and 0.2% SDS at 60°C for 30 min, and 1× SSPE and 0.1% SDS at 60°C for 10 min and then subjected to autoradiography.

Determination of EILP gene sequence—Inverse polymerase chain reaction (IPCR) was carried out as follows (Ochman et al. 1988). After total digestion of tobacco DNA by *Apa*I, *Pvu*II, *Sal*I, *Sma*I or *Xba*I, 1 μg of digested DNA was ligated at 16°C for 16 h in a total volume of 1 ml, containing 66 mM Tris-HCl, pH7.6, 6.6 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 10 units of T4 DNA ligase. Of the ligated DNA, 100 ng was added to 20 μl of PCR solution containing 1× LA PCR buffer II (TaKaRa, Japan), 2.5 mM $MgCl_2$, 250 μM dNTPs and 1 unit LA Taq Polymerase (TaKaRa, Japan). Two gene-specific primers derived from the D44-12 fragment were added to the PCR mixture at 1 μM each. The amplification was taken through one cycle of 5 min at 95°C followed by 30 cycles of 1 min at 95°C, 2 min at 47°C and 3 min at 72°C. After the last cycle, one final extension step was performed for 7 min at 47°C. One μl of the resulting PCR products was subjected to nested PCR with two newly designed specific primers. The amplified fragment was cloned into the pGEM-T Easy plasmid vector (Promega, U.S.A.) using TA cloning. To determine the polyadenylation site of *EILP*, PCR was performed using a cDNA library prepared from a tobacco leaf 12 h after HWC treatment as a template with primers corresponding to the 3′ end of the deduced open reading frame and a vector sequence of the cDNA library. To confirm that the sequences gained by these PCR methods were derived from a single gene, the full length of the *EILP* gene was obtained by PCR using the cDNA library as a template with primers corresponding to the 5′ end of the deduced open reading frame and upstream region of the polyadenylation site.

Nucleotide sequencing and data analysis—DNA sequence analysis was performed with an automatic DNA sequencer (model 373A; Perkin Elmer/Applied Biosystems, U.S.A.) according to the manufacturer's instructions. The nucleotide and deduced amino acid sequences were analyzed with DNA analytical software (DNASYS; Hitachi Software, Japan). The amino acid sequences were aligned using the CLUSTAL W program (Thompson et al. 1994).

Results and Discussion

Previous studies have shown that treatment of potato (Takemoto et al. 1999a) and tobacco (Takemoto et al. 1999b) with HWC elicitor induced resistance responses, such as cell death and accumulation of PR-proteins. In order to obtain differentially expressed genes during the resistance responses, we compared mRNAs of tobacco leaves treated with HWC elicitor and water by the RAPD/RT-PCR differential display method (Yoshida et al. 1994). The total RNAs were amplified by RT-PCR with single random primers (12-mers) and the PCR products were separated by agarose gel electrophoresis. The RAPD/RT-PCR were performed with each of 132 primers individually, and the PCR products were separated by agarose gel electrophoresis. The number of amplified fragment varied

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from 6 to about 30, averaging 20 fragments per primer. Among the primers tested, 16 gave products specific to tobacco leaf treated with HWC. The 16 PCR fragments were subcloned and subjected to Northern hybridization for RNAs from tobacco leaf treated with HWC. As a result, the expression of only one gene, which encoded cytochrome P450, was detected by Northern hybridization as elicitor-inducible gene (Takemoto et al. 1999b). Furthermore, we performed a RT-PCR analysis with newly designed specific primers derived from the elicitor inducible fragments and confirmed the induction of the *D44-12* gene,which was amplified with the D44 primer (5′-GGCA-GATGTCAG-3′), by the HWC elicitor (Fig. 1). Expression of *D44-12* mRNA increased for 12 h after treatment with the HWC elicitor, and declined after 24 h (Fig. 1B, bottom), whereas a little increase of the transcript was detected in tobacco leaves in the control (Fig. 1B, top). A low level of expression of the *D44-12* gene was detected at all times, and the slight constitutive expression of *D44-12* was visualized clearly by RT-PCR using an additional 15 cycles of amplification (data not shown).

The full-length sequence of *D44-12* was obtained by the inverse PCR method. When compared with other available sequences, the D44-12 product showed homology to LRR receptor-like genes, especially to the tomato *Cf-2* and *Cf-5* genes (Dixon et al. 1996, 1998). We have designated this clone *EILP* for a gene encoding an elicitor-inducible LRR protein. The *Cf-2/Cf-5* family genes of tomato more resembled the *EILP* gene of tobacco than the *Cf-4/9* family genes of tomato. Presumably, each progenitor sequence for *Cf-4/Cf-9* and *Cf-2/Cf-5* had been present when the ancestor of solanaceous plants differentiated into tomato and tobacco. EILP was divided into seven domains (A–G) as is the case in Cf-2/Cf-5 family genes (Fig. 2A). Domain A is a putative signal peptide of 25 amino acids and domain B is presumed to be the NH_2-terminus of the mature EILP product. Domain C consists entirely of 28 LRRs. Compared with the highly reiterated structures of Cf-2 and Cf-5, the domain C of EILP contains various types of LRRs. There are structural similarities in LRRs of domain C between the Cf-2/Cf-5 and EILP genes. Domain F is a potential membrane-spanning region containing 24 uncharged amino acids. Domains E and G are rich in acidic and basic amino acids, respectively, which is consistent with a role in anchoring and orienting the protein within the cell membrane. There are 22 potential N-linked glycosylation sites within domains B to E. Comparison of each domain in EILP with those of Cf-2/Cf-5 family genes revealed that domains A to F were very similar to each other, while domain G of EILP was remarkably different from that of Cf2/Cf-5 family genes (Fig. 2B). The *EILP* gene shared a high degree of identity with *Cf-2/Cf-5* family genes within the protein coding regions, whereas there was no similarity between the sequences of the 3′ untranslated regions of *EILP* and *Cf-2/Cf-5* family genes. From DNA sequence analyses of inverse PCR and 3′ rapid amplification of cDNA end (RACE) products, an intron sequence was identified in the mRNA downstream of the translation termination signal (data not shown). Interestingly, the intron position within the *EILP* gene is approximately the same as that of the single intron in *Cf-2, Cf-4, Cf-5* and *Cf-9* genes (Dixon et al. 1996, 1998, Thomas et al. 1997, Jones et al. 1994) in spite of the absence of any similarity between the nucleic acid sequence of the 3′ untranslated regions of the *EILP* and *Cf* genes.

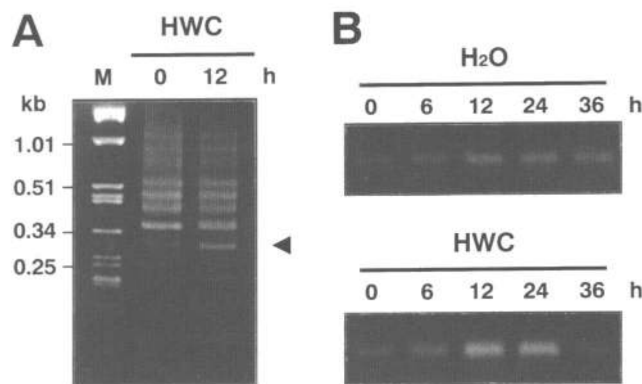

Fig. 1 Isolation of elicitor-inducible D44-12 fragment by differential display. (A) Agarose gel electrophoresis of RT-PCR products amplified with total RNAs from tobacco leaves and D44 primer. Tobacco leaves treated with 2 mg ml^{-1} HWC were harvested at 0 and 12 h after treatment and total RNAs were extracted. Arrow indicates the position of the D44-12 fragment. (B) RT-PCR analysis of D44-12 expression in response to HWC. Tobacco leaves treated with water (left) or 2 mg ml^{-1} HWC (right) were harvested at the indicated time after treatment and total RNAs were extracted. Amplifications were performed with specific primers for D44-12 fragment (D44-F1 and D44-R1).

Tobacco genomic DNAs separately digested with *Hin*dIII, *Eco*RV or *Eco*RI were subjected to Southern hybridization with the cDNA for EILP as a probe. Two or three strongly hybridizing bands were found in each lane under high-stringency condition (Fig. 3, high). A lot of additional bands were detected at low-stringency condition (Fig. 3, low), indicating that a large number of genes related to the *EILP* gene are present in tobacco, as occurs with the *Cf* genes in tomato. As expected from the restriction map of the cDNA for EILP, bands of 1.16 (lane H), 0.65 (lane EV) and 0.41 (lane EV) kb were detected (Fig. 3 arrowheads).

Accumulation of *EILP* transcript in tobacco leaves of intact plants subjected to different forms of stress was investigated by Northern hybridization. Upon inoculation with *P. syringae* pv. *glycinea* (which is non-pathogenic to tobacco), *EILP* transcripts were weakly detected within 6 h, and slightly increased to a peak at 24 h (Fig. 4). The estimated size of the hybridized band correlated well with the

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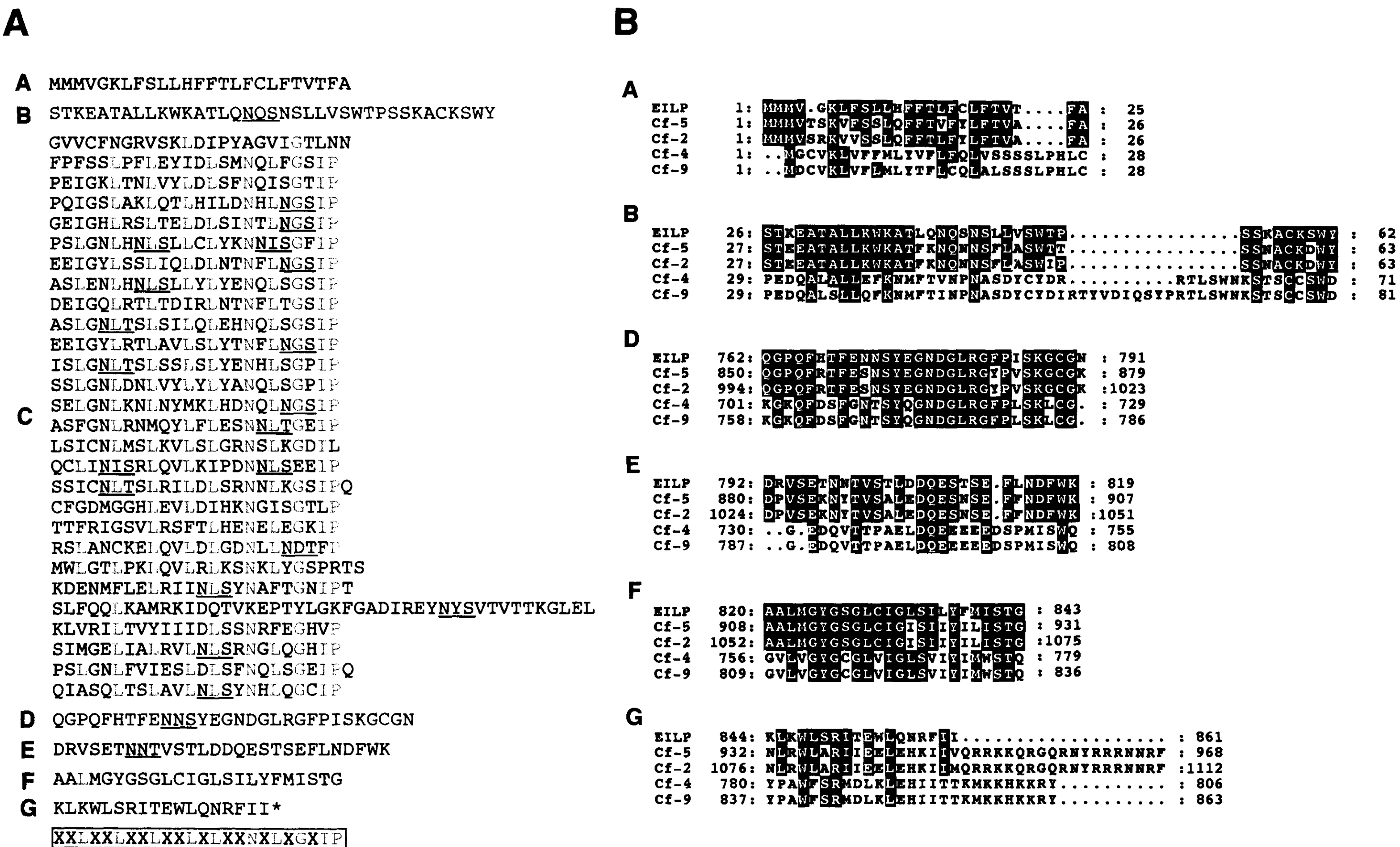

Fig. 2 EILP amino acid sequence analysis. (A) Deduced primary structure of EILP protein. The sequence is divided into seven domains (A–G) to emphasize the structural similarities with Cf-2 and Cf-5 (Dixon et al. 1996, Dixon et al. 1998), as described in the text. The consensus sequence for extra-cytoplasmic LRR proteins is shown boxed and aligned below the EILP sequence and the conserved amino acids are shown in gray. Potential NXS/T glycosylation sequences are underlined. (B) Comparison of domains A, B, D, E, F and G for EILP with those for Cf-2.2 (Dixon et al. 1996) Cf-4 (Thomas et al. 1997) Cf-5 (Dixon et al. 1998) and Cf-9 (Jones et al. 1994). Identical amino acids are shown in reverse. The sequences were aligned using the CLUSTAL W program (Thompson et al. 1994).

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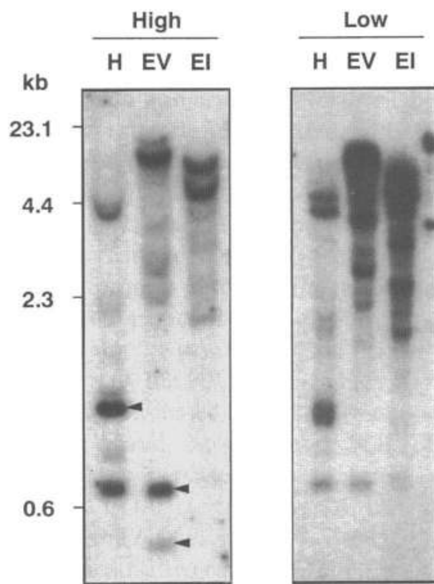

Fig. 3 Southern blot analysis of the EILP gene in tobacco. Ten μg of total DNAs prepared from a tobacco leaf were digested with *Hin*dIII (H), *Eco*RV (EV) or *Eco*RI (EI) fractionated on a 0.8% agarose gel and transferred to a nylon membrane. The membrane was allowed to hybridize with the cDNA for EILP under low- or high-stringency condition. Sizes of DNA markers are indicated on the left. Arrowheads indicated the bands expected from the restriction map of the cDNA for EILP.

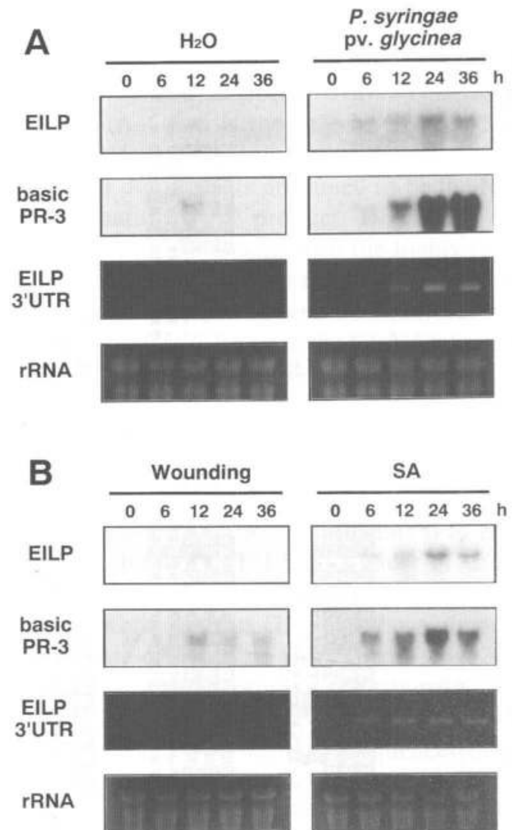

Fig. 4 Analysis of the expression of the EILP and PR-protein genes induced by different types of stress. (A) Tobacco leaves of intact plants were injected with water (H_2O), or *P. syringae* pv. *glycinea* (3×10^6 c.f.u. ml^{-1}). They were harvested at the indicated times and total RNAs were extracted. Twenty μg of total RNAs were fractionated on a 1% agarose gel containing formaldehyde and then transferred to a nylon membrane. The membrane was allowed to hybridize with the cDNAs for EILP or basic PR-3 (Neale et al. 1990). RT-PCR analysis of EILP expression (EILP-3′UTR) were performed with specific primers for the 3′-untranslated region of the EILP gene (EILP-UTR-F and EILP-UTR-R). (B) Tobacco leaves of intact plants were injected with 0.5 mM salicylic acid (SA) or wounded with carborundum (Wounding). Northern hybridization and RT-PCR analysis were performed as described in (A).

size of the cloned *EILP* cDNA. The time course of *EILP* mRNA abundance was similar to that in tobacco leaves treated with salicylic acid, whereas no signal was detected in the case of water-treated or wounded leaves (Fig. 4). Basic *PR-3* transcript was also accumulated by treatment with SA or inoculation with *P. syringae* pv. *glycinea*. The expression pattern of the *EILP* gene resembled that of basic *PR-3* gene except for the magnitude (Fig. 4). It is indicated that the *EILP* gene is a new member of a family of genes that are induced at a late stage in defense responses. It was reported that SA inhibits the induction of basic PR-proteins (Niki et al. 1998). Whereas, there were also reports that SA induces expression of basic PR-protein genes (Bol and Linthorst 1990, Ward et al. 1991). Thus, the relationship between SA and induction of basic PR-protein genes remains as matter for debate.

As shown in Fig. 3, a large number of *EILP*-like genes exist in tobacco. In order to check whether the results of Northern hybridization reflect the expression of the *EILP* gene, RT-PCR analysis was carried out using specific primers derived from the 3′-untranslated region of the *EILP* gene. The result of the RT-PCR experiment agreed with that of Northern hybridization (Fig. 4).

Additionally, induction of *EILP* by pathogenic and non-pathogenic bacteria was compared. Detached tobacco leaves were inoculated with *P. syringae* pv. *glycinea*, which is non-pathogenic to tobacco, or *P. syringae* pv. *tabaci*, which is the causative agent of tobacco wildfire disease. Expression of the *EILP* gene increased within 12 h and then decreased gradually in response to *P. syringae* pv. *glycinea*, whereas it occurred later when leaves were inoculated with the pathogen *P. syringae* pv. *tabaci* (Fig. 5). This indicates that the expression of *EILP* is regulated by a signal transduction pathway that defines the nature of the resistance of tobacco to avirulent bacteria.

A key event of plant responses to pathogen attack lies on the ability of the plant to recognize the pathogen and quickly mount the battery of inducible defensive measures. In the past several years, dominant or semidominant re-

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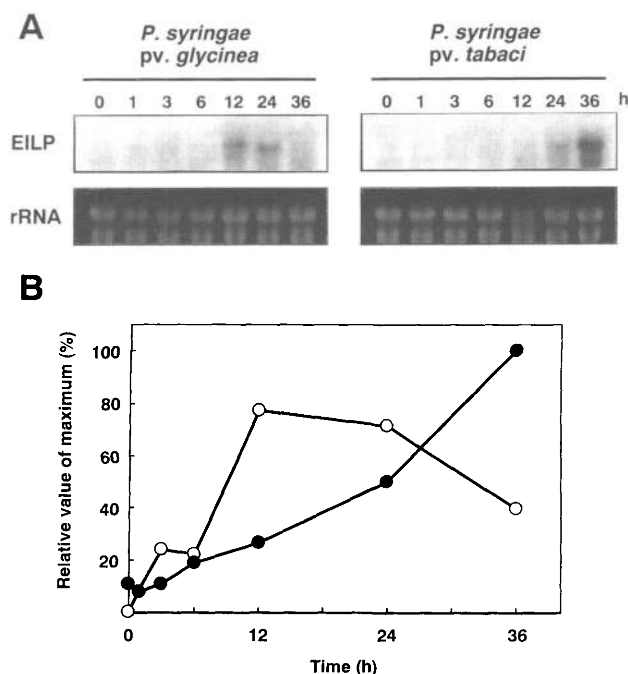

Fig. 5 Accumulation of *EILP* transcript in response to *P. syringae* pathovars. (A) Detached tobacco leaves infiltrated with 3×10^{6} c.f.u. ml^{-1} of *P. syringae* pv. *glycinea* (left) or pv. *tabaci* (right) were harvested at the indicated times. For control, rRNAs were stained with acridine orange. Other details are as described in the legend of Fig. 4. (B) Intensities of hybridized bands in (A) obtained by densitometric scanning. The amount of *EILP* transcripts was normalized by the densities of rRNA stained with acridine orange and plotted relative to the highest value. Symbols indicated the relative amount of *EILP* transcript in tobacco leaves inoculated with *P. syringae* pv. *glycinea* (○) or pv. *tabaci* (●).

sistance (R) genes conforming to Flor's gene for gene hypothesis (Flor 1971) were isolated from several plant species (Staskawicz et al. 1995, Bent 1996). Many of these R gene products share structural motifs such as leucine-rich repeat (LRR), nucleotide binding site and protein kinase domain, strongly indicating that the R gene products are involved in recognition of phytopathogen and initiation of following signal transduction. R genes frequently occur in tightly linked clusters and these loci contain arrays of related genes, including *Pto, Cf* and *Mi* from tomato (Jia et al. 1997, Parniske et al. 1997, Dixon et al. 1998, Milligan et al. 1998), *Dm* in lettuce (Meyers et al. 1998), *Xa 21* in rice (Song et al. 1997), *M* in flax (Anderson et al. 1997) and *RPP* in *Arabidopsis* (Botella et al. 1998). *EILP* gene was strongly homologous to genes for LRR receptor-like protein, especially to *Cf* genes of tomato (Fig. 2). Additionally, *EILP* have a number of related genes (Fig. 3). These results indicated that *EILP* is one of a large number of LRR receptor genes as *Cf* gene family. It was known that pathogen inoculation induced the expression of genes for receptor-like protein kinases of *Arabidopsis* (Wak-1 and At-RLK3) (He et al. 1999, Czernic et al. 1999) and *Brassica* (SFR2) (Pastuglia et al. 1997). It was also reported that altered environmental condition induced the expression of some disease resistance genes as *Xa-1* and *Pib* (Yoshimura et al. 1998, Wang et al. 1999). Wang et al. showed that the expression of *Pib* homologue was also induced upon altered conditions, indicating that transcriptional activation of receptor-like gene by stimulation is a general response of plants to resist various stresses. It may be likely that the transcription of a lot of receptor like genes, including *EILP*, is activated in tobacco plant under stress conditions. However, it should be noted that the expression of *EILP* was activated by a nonpathogen, in a delayed fashion by the tobacco pathogen, and was not activated by wounding (Fig. 5). This indicated that the expression of *EILP* would correlate with disease resistance and the product of *EILP* might be involved in disease resistance in tobacco. Actually, the expressions of genes involved in disease resistance are induced in this manner (Alfano and Collmer 1996). While induction of the *EILP* gene by disease stress is intrigues us, the function of *EILP* is not yet clear. Additional investigations, such as characterization of *EILP* gene family, are needed to elucidate the involvement of the EILP in disease resistance of tobacco plant.

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References

Alfano, J.R. and Collmer, A. (1996) Bacterial pathogen in plants: life up against the wall. *Plant Cell* 8: 1683–1698.

Anderson, P.A., Lawrence, G.J., Morrish, B.C., Ayliffe, M.A., Finnegan, E.J. and Ellis, J.G. (1997) Inactivation of the flax rust resistance gene M associated with loss of a repeated unit within the leucine-rich repeat coding region. *Plant Cell* 9: 641–651.

Bent, A.F. (1996) Plant disease resistance genes: function meets structure. *Plant Cell* 8: 1757–1771.

Bol, J.F. and Linthorst H., J., M. (1990) Plant pathogenesis-related proteins induced by virus infection. *Annu. Rev. Phytopathol.* 28: 113–138.

Botella, M.A., Parker, J.E., Frost, L.N., Bittner-Eddy, P.D., Beynon, J.L., Daniels, M.J., Holub, E.B. and Jones, J.D. (1998) Three genes of the *Arabidopsis* RPP1 complex resistance locus recognize distinct *Peronospora parasitica* avirulence determinants. *Plant Cell* 10: 1847–1860.

Czernic, P., Visser, B., Sun, W., Savoure, A., Deslandes, L., Marco, Y., Van Montagu, M. and Verbruggen, N. (1999) Characterization of an *Arabidopsis thaliana* receptor-like protein kinase gene activated by oxi-

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dative stress and pathogen attack. *Plant J.* 18: 321–327.
da Costa e Silva, O., Klein, L., Schmelzer, E., Trezzini, G.F. and Hahlbrock, K. (1993) BPF-1, a pathogen-induced DNA-binding protein involved in the plant defense response. *Plant J.* 4: 125–135.
Dixon, M.S., Hatzixanthis, K., Jones, D.A., Harrison, K. and Jones, J.D. (1998) The tomato Cf-5 disease resistance gene and six homologs show pronounced allelic variation in leucine-rich repeat copy number. *Plant Cell* 10: 1915–1926.
Dixon, M.S., Jones, D.A., Keddie, J.S., Thomas, C.M., Harrison, K. and Jones, J.D. (1996) The tomato Cf-2 disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. *Cell* 84: 451–459.
Doke, N. and Tomiyama, K. (1980) Effect of hyphal wall components from *Phytophthora infestans* on protoplasts of potato tuber tissues. *Physiol. Plant Pathol.* 16: 169–176.
Flor, H.H. (1971) Current status of the gene for gene concept. *Annu. Rev. Phytopathol.* 9: 275–296.
Hammond-Kosack, K.E. and Jones, J.D. (1996) Resistance gene-dependent plant defense responses. *Plant Cell* 8: 1773–1791.
He, Z.H., Cheeseman, I., He, D. and Kohorn, B.D. (1999) A cluster of five cell wall-associated receptor kinase genes, Wak1-5, are expressed in specific organs of *Arabidopsis*. *Plant Mol. Biol.* 39: 1189–1196.
Jia, Y., Loh, Y.T., Zhou, J. and Martin, G.B. (1997) Alleles of Pto and Fen occur in bacterial speck-susceptible and fenthion-insensitive tomato cultivars and encode active protein kinases. *Plant Cell* 9: 61–73.
Jones, D.A., Thomas, C.M., Hammond-Kosack, K.E., Balint-Kurti, P.J. and Jones, J.D. (1994) Isolation of the tomato Cf-9 gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science* 266: 789–793.
Meyers, B.C., Chin, D.B., Shen, K.A., Sivaramakrishnan, S., Lavelle, D.O., Zhang, Z. and Michelmore, R.W. (1998) The major resistance gene cluster in lettuce is highly duplicated and spans several megabases. *Plant Cell* 10: 1817–1832.
Milligan, S.B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P. and Williamson, V.M. (1998) The root knot nematode resistance gene Mi from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. *Plant Cell* 10: 1307–1319.
Neale, A.D., Wahleithner, J.A., Lund, M., Bonnett, H.T., Kelly, A., Meeks-Wagner, D.R., Peacock, W.J. and Dennis, E.S. (1990) Chitinase, beta-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. *Plant Cell* 2: 673–684.
Niderman, T., Genetet, I., Bruyere, T., Gees, R., Stintzi, A., Legrand, M., Fritig, B. and Mosinger, E. (1995) Pathogenesis-related PR-1 proteins are antifungal. Isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against *Phytophthora infestans*. *Plant Physiol.* 108: 17–27.
Niki, T., Mitsuhara, I., Seo, S., Ohtsubo, N. and Ohashi, Y. (1998) Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. *Plant Cell Physiol.* 39: 500–507.
Ochman, H., Gerber, A.S. and Hartl, D.L. (1988) Genetic application of an inverse polymerase chain reaction. *Genetics* 120: 621–625.
Parniske, M., Hammond-Kosack, K.E., Golstein, C., Thomas, C.M., Jones, D.A., Harrison, K., Wulff, B.B. and Jones, J.D. (1997) Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the Cf-4/9 locus of tomato. *Cell* 91: 821–832.
Pastuglia, M., Roby, D., Dumas, C. and Cock, J.M. (1997) Rapid induction by wounding and bacterial infection of an S gene family receptor-like kinase gene in *Brassica oleracea*. *Plant Cell* 9: 49–60.
Ponstein, A.S., Bres-Vloemans, S.A., Sela-Buurlage, M.B., van den Elzen, P.J., Melchers, L.S. and Cornelissen, B.J. (1994) A novel pathogen- and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. *Plant Physiol.* 104: 109–118.
Rushton, P.J., Torres, J.T., Parniske, M., Wernert, P., Hahlbrock, K. and Somssich, I.E. (1996) Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes. *EMBO J.* 15: 5690–5700.
Song, W.Y., Pi, L.Y., Wang, G.L., Gardner, J., Holsten, T. and Ronald, P.C. (1997) Evolution of the rice Xa21 disease resistance gene family. *Plant Cell* 9: 1279–1287.
Staskawicz, B.J., Ausubel, F.M., Baker, B.J., Ellis, J.G. and Jones, J.D. (1995) Molecular genetics of plant disease resistance. *Science* 268: 661–667.
Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdinoglu, S., Kauffmann, S., Geoffroy, P., Legrand, M. and Fritig, B. (1993) Plant 'pathogenesis-related' proteins and their role in defense against pathogens. *Biochimie* 75: 687–706.
Takemoto, D., Hayashi, M., Doke, N., Nishimura, M. and Kawakita, K. (1999b) Molecular cloning of a defense-response-related cytochrome P450 gene from tobacco. *Plant Cell Physiol.* 40: 1232–1242.
Takemoto, D., Maeda, H., Yoshioka, H., Doke, N. and Kawakita, K. (1999a) Effect of cytochalasin D on defense responses of potato tuber discs treated with hyphal wall components of *Phytophthora infestans*. *Plant Sci.* 141: 219–226.
Thomas, C.M., Jones, D.A., Parniske, M., Harrison, K., Balint-Kurti, P.J., Hatzixanthis, K. and Jones, J.D. (1997) Characterization of the tomato Cf-4 gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognitional specificity in Cf-4 and Cf-9. *Plant Cell* 9: 2209–2224.
Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* 22: 4673–4680.
Wang, Z.-X., Yano, M., Yamanouchi, U., Iwamoto, M., Manna, L., Hayasaka, H., Katayose, Y. and Sasaki, T. (1999) The Pib gene for rice blast resistance belongs to the nucleotide binding and leucine-rich repeat class of plant disease resistance genes. *Plant J.* 19: 55–64.
Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J.-P. and Ryals, J. (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3: 1085–1094.
Yoshida, K.T., Naito, S. and Takeda, G. (1994) cDNA cloning of regeneration-specific genes in rice by differential screening of randomly amplified cDNAs using RAPD primers. *Plant Cell Physiol.* 35: 1003–1009.
Yoshimura, S., Yamanouchi, U., Katayose, Y., Toki, S., Wang, Z.-X., Kono, I., Kurata, N., Yano, M., Iwata, N. and Sasaki, T. (1998) Expression of Xa1, a bacterial blight-resistance gene in rice, is induced by bacterial inoculation. *Proc. Natl. Acad. Sci. USA* 95: 1663–1668.

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