J. Gen. Plant Pathol. 67 : 89-96 (2001)

Characterization of Elicitor-inducible Tobacco Genes Isolated by Differential Hybridization

Daigo TAKEMOTO[1,2]*, Noriyuki DOKE[1] and Kazuhito KAWAKITA[1]

ABSTRACT

Inducible responses in plants against pathogen attack play a major role in resistance to disease. The defense responses are mostly associated with the expression of various kinds of inducible genes. We employed differential hybridization to isolate elicitor-inducible genes (EIGs) of tobacco (*Nicotiana tabacum* cv. Samsun NN) using the tobacco-fungal elicitor system. A cDNA library was constructed from tobacco leaves treated for 12 hr with hyphal wall components (HWC) prepared from *Phytophthora infestans*, and six EIGs were identified. Expression of all EIGs was induced after inoculation with the soybean pathogen *Pseudomonas syringae* pv. *glycinea* (nonpathogenic on tobacco) or treatment with salicylic acid, and a variety of expression patterns of EIG mRNAs was observed. Sequence analysis of EIG cDNAs revealed similarities to genes for SAR8.2 (*EIG-B39* and *EIG-D14*), glycine-rich protein (*EIG-G7*), extensin (*EIG-I30*), acyltransferase (*EIG-I24*) and unknown protein (*EIG-J7*). Possible roles of EIG products in disease resistance are discussed.

(Received August 30, 2000 ; Accepted November 30, 2000)

Key words : differential hybridization, elicitor-inducible genes, hyphal wall components, *Pseudomonas syringae* pv. *glycinea*, salicylic acid, tobacco.

INTRODUCTION

Plants have evolved an array of defense strategies to combat attack from microbes. They have developed physical barriers and antimicrobial compounds that are preformed in advance of pathogen attack[21]. After plants are attacked by microbes, these constitutive defenses are accompanied by a variety of induced resistance mechanisms. Typically, this response includes superoxide anion generation ; hypersensitive cell death, which is triggered to isolate the pathogens from the healthy part of the plant ; cell wall fortification ; accumulation of pathogenesis-related (PR) proteins and other antimicrobial proteins ; and biosynthesis of phytoalexins, low molecular-weight antimicrobial compounds. The defense responses cooperatively act to limit invasion of pathogens[11].

Various physiological changes associated with defense responses are induced at the transcriptional level. It is well known that the transcription of various genes encoding enzymes involved in secondary metabolism, including phenylalanine ammonia-lyase, cinnamate 4-hydroxylase and 5-epi-aristolochene synthase, are activated during plant defense responses[6,9,14,16]. These enzymes in secondary metabolism catalyze biosynthesis of wall appositions and phytoalexins that accumulate in cells that are in direct contact with the pathogen and in the surrounding cells.

Genes for PR-proteins are well characterized as disease resistance-inducible genes. Some kinds of PR-proteins, such as chitinase (PR-3) and β-1, 3-glucanase (PR-2), have enzymatic activities and have been shown to possess antifungal and antibacterial properties from the degradation of cell wall structural polysaccharides[15]. Other PR-proteins may also have antimicrobial activities[18,20]. The expression of genes encoding PR-proteins is generally used as an index of disease responses in plants[26].

The characterization of genes expressed in a plant that is activating disease resistance responses is an initial step towards understanding the protective changes induced in plants. For example, some elicitor-inducible cytochrome P450 genes, cloned by PCR-based differential screening, were identified as enzymes involved in phytoalexin biosynthesis[1,23].

Tobacco plants injected with hyphal wall components

[1] Plant Pathology Laboratory, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan

[2] Plant Cell Biology, Research School of Biological Sciences, Australian National University, Canberra ACT 2601, Australia

* Corresponding author (E-mail : takemoto@rsbs.anu.edu.au)

(HWC) prepared from *Phytophthora infestans*, the potato late blight fungus, have typical defense responses[27]. After this elicitor treatment, differential hybridization was used to isolate six elicitor-inducible genes (EIGs). The expression of these genes was also induced by inoculation of the soybean pathogen *Pseudomonas syringae* pv. *glycinea*, which is avirulent on tobacco. In this paper, we report the use of differential hybridization to isolate elicitor-inducible genes of tobacco. We compare the expression profiles of these genes in several stress responses in tobacco and discuss the possible roles of the cloned EIGs during the activation of plant disease resistance.

MATERIALS AND METHODS

Plants and bacterial strains Tobacco plants carrying the *N* gene (*Nicotiana tabacum* cv. Samsun NN) were cultivated at 25°C for 6 weeks after sowing in continuous light. *P. syringae* pv. *glycinea* 801 was provided by Dr. Yuichi Takigawa, Faculty of Agriculture at Shizuoka University, Japan. The bacteria were cultured at 25°C in King's B medium[5], harvested by centrifugation, resuspended in water and used for inoculation.

Elicitor treatment and wounding of tobacco leaves HWC were prepared from mycelia of *P. infestans* that had been grown in liquid medium for 2 weeks at 20°C in darkness as described previously[7]. Leaves of 6-week-old tobacco plants were injected with water, 1 mg/ml HWC, 0.5 mM salicylic acid (SA) or *P. syringae* pv. *glycinea* at 3×10^6 cfu/ml. Solutions were injected into the intercellular space of tobacco leaves using a syringe without a needle. Leaves were superficially wounded with a needle to improve injection. The injected areas were harvested at defined times for RNA extraction. For the wounding experiment, intact leaves of 6-week-old tobacco plant were wounded by rubbing the surface of the leaves with carborundum (600 mesh). Tobacco plants were incubated at 25°C until harvesting over a time course.

Northern hybridization Twenty μg of total RNA was separated on a 1% formaldehyde agarose gel and transferred onto a Hybond N (+) membrane (Amersham Pharmacia, Sweden). The isolated EIG cDNAs, cDNA for the 3′-untranslated region of *PR-2*[13] and *PR-P*[19] were labeled with [α-^{32}P] dCTP using a random-primed DNA labeling kit (Megaprime ; Amersham Pharmacia). Hybridizations were performed at 42°C for 20 hr in 5× SSPE (20× SSPE ; 3 M NaCl, 173 mM $NaH_2PO_4\cdot2H_2O$, 25 mM EDTA), 50% formamide, 5× Denhardt's solution, 1% SDS and 100 μg/ml denatured salmon sperm DNA. Membranes were washed with 1× SSPE and 0.2% SDS at 60°C for 30 min, and 0.1× SSPE and 0.1% SDS at 60°C for 5 min and then subjected to autoradiography.

Construction of a cDNA library and differential hybridization Poly(A)$^+$ RNA was prepared from tobacco leaf tissue by chromatography on oligo(dT)-cellulose 12 hr after HWC treatment as described by Aviv and Leder[3]. A cDNA library in pBluescript II SK (Stratagene, USA) was constructed with poly (A)$^+$ RNA and the vector-primer as described previously[17]. *Escherichia coli* cells (DH5α) were transformed with the library. Plasmid DNA prepared from each colony containing cDNA was isolated, and 200 μg of the plasmid DNA was transferred onto a Hybond N (+) membrane (Amersham Pharmacia). Probe cDNA was synthesized in a 20-μl reaction containing 1× RNA buffer (Takara, Japan), 1 mM dNTP, 5 mM $MgCl_2$, 1 unit/μl RNase inhibitor, 1 μg of total RNA and 0.125 μM oligo dT-adapterprimer (Takara) and 0.25 unit/μl AMV reverse transcriptase XL (Takara) at 45°C for 30 min. Unincorporated dNTP and primers were removed using Suprec-02 (Takara). Labeling of probes, hybridization and autoradiography were performed under the same conditions as described for northern hybridization.

Nucleotide sequencing and data analysis DNA sequence analysis was performed with an automatic DNA sequencer (model 373A ; Perkin Elmer/Applied Biosystems, USA) according to the manufacturer's instructions. The nucleotide and the deduced amino acid sequences were analyzed with DNA analytical software (DNASYS ; Hitachi Software, Japan). The alignments of amino acid sequences were made using the CLUSTAL W program[28].

RESULTS AND DISCUSSION

Differential screening for tobacco EIGs

A bacterial cDNA library made from tobacco leaves treated with HWC for 12 hr was used for differential screening. Plasmid DNAs prepared from each colony containing a cDNA were isolated, and 200 μg of plasmid DNAs were transferred onto nylon membranes. Duplicate membranes were hybridized with either the control probe (made from a mixture of RNA prepared from tobacco leaves treated with water for 6 and 12 hr) or the post-induction probe (made from a mixture of RNA prepared from tobacco leaves treated with HWC for 6 and 12 hr). Eight out of 200 cDNA clones hybridized stronger to the cDNA probe from HWC-treated leaves than to the probe from water-treated leaves. A typical example is shown in Fig. 1. To confirm that the expression of eight cDNA clones was actually induced by HWC treatment, accumulation of these transcripts in tobacco leaves treated with HWC was investigated by northern hybridization. The expression of six clones was activated by HWC, while two clones were identified as false positives (data not shown). These differentially hybridizing cDNAs appeared to be

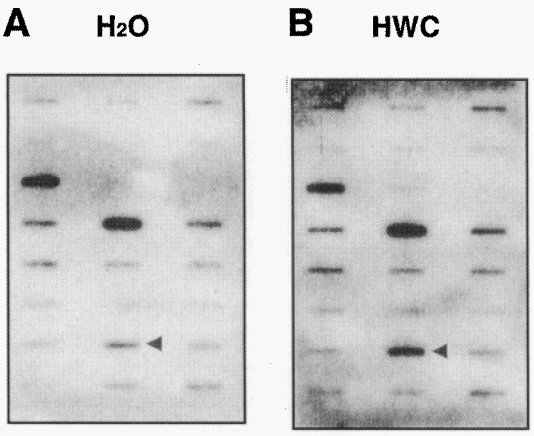

Fig. 1. Representative result of differential hybridization. Randomly chosen cDNAs from a cDNA library from tobacco leaves treated with HWC were blotted onto a couple of nylon membranes. The membranes were hybridized with labeled cDNAs from tobacco leaves treated with water (H_2O) (A) or HWC (B) for 6 and 12 hr. The position of the cloned elicitor-inducible gene (*EIG-I30*) is indicated by the arrowhead.

specifically expressed in tobacco leaves treated with HWC elicitor, designated EIGs (Elicitor-Inducible Genes). The estimated size of hybridized bands for EIGs correlated well with the size of the cloned EIGs cDNAs.

The expression and sequence analysis of EIGs

The expression of cloned EIGs in tobacco leaves inoculated with nonpathogen, *P. syringae* pv. *glycinea*, was examined by northern hybridization. For comparison, the accumulation of *PR-2* (β-1,3-glucanase) and *PR-3* (chitinase) transcripts was also demonstrated. We confirmed that the expression of six EIGs was actually induced during disease resistance responses activated in the tobacco plant (Fig. 2).

The nucleic acid sequences of six EIG clones were determined and subjected to a database homology search for similarities to registered plant genes. General features of the EIG clones, including their nearest match with other sequences in the databases, are given in Table 1.

EIG-B39* and *-D14

The expression pattern of *EIG-B39* and *-D14* genes resembled each other. Expression of *EIG-B39* and *-D14* increased, almost reach a maximum within 6 hr, and remained at the same level through 36 hr in response to *P. syringae* pv. *glycinea* (Fig. 2). The *EIG-B39* and *-D14* cDNAs encodes putative small, highly basic proteins containing N-terminal hydrophobic signal peptides and a cysteine-rich C-terminal domain and shows significant sequence homology with *SAR8.2* genes isolated from tobacco (Table 1).

SAR8.2 genes were isolated as genes induced systemically in tobacco plants inoculated with tobacco mosaic virus (TMV)[2,30]. The *SAR8.2*-gene family is also known to consist of 10–12 members[2]. Interestingly, although there are a number of *SAR8.2*-family in tobacco, there is no report on any other homologous sequences to *SAR8.2* genes in other plants. The nucleic acid sequence of *EIG-B39* is 100% identical with *SAR8.2m* except for an additional 19-base extension at the 5′ end of cDNA and five bases at the 3′ end (data not shown). This result indicated that *EIG-B39* cDNA is a longer version of *SAR8.2m* cDNA. The *EIG-D14* sequence has a deletion of 59 bases relative to *SAR8.2c* in the 3′ UTR, and the overlapping sequences have 98% identity, indicating that *EIG-D14* is a new member of the SAR8.2 gene family (Fig. 3A). It is interesting that the N-terminal of the amino acid sequences of *EIG-B39* and *-D14* has similarity with those of cell wall proteins that will be discussed next (Fig. 3B). The function of the products of *SAR8.2* genes, however, has not been identified.

EIG-G7

Within 24 hr of inoculation with *P. syringae* pv. *glycinea*, *EIG-G7* transcripts were detected and had slightly increased by 36 hr (Fig. 2). The *EIG-G7* cDNA encodes a putative protein of 109 amino acids with an N-terminal signal peptide and high proportion of glycine (21%). For *EIG-G7* cDNA, significant identity was found

Table 1. Summary of elicitor-inducible genes isolated by differential hybridization

Clone	Length	M.W.[a]	Matching sequence from database (Accession nos.)	Origin of matching sequence	DNA % match	Protein % match	Accession nos.
EIG-B39	487 bp	7184 Da	SAR8.2m (U89604)	*N. tabacum*	94.9	100	AB040408
EIG-D14	482 bp	10137 Da	SAR8.2c (M97360)	*N. tabacum*	85.2	98.9	AB040145
EIG-G7	572 bp	11586 Da	glycine-rich protein (M37152)	*N. tabacum*	94.8	99.1	AB041513
EIG-I24	1410 bp	50009 Da	putative protein (AAF24555)	*A. thaliana*	61.8	55.5	AB041514
EIG-I30	714 bp	15589 Da	extensin-like protein (D83226)	*P. nigra*	53.4	64.9	AB041516
EIG-J7	743 bp	19578 Da	TMV-induced protein I (AF242731)	*C. annuum*	60.3	58.2	AB040407

a) Molecular weight calculated from protein sequence deduced from the cDNA sequence.

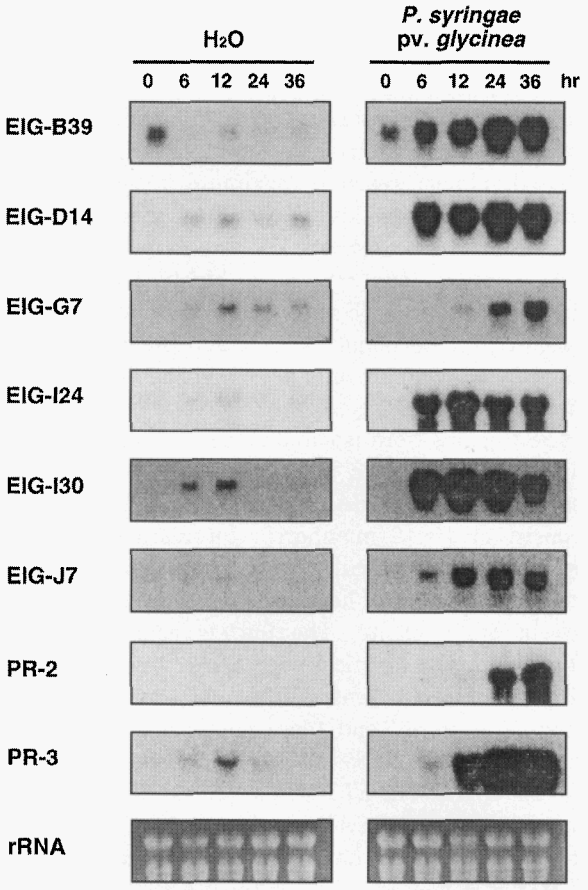

Fig. 2. The expression of EIGs in nonpathogen inoculated leaves. Whole areas of tobacco leaves, treated with water (H_2O) or inoculated with *P. syringae* pv. *glycinea* (3×10^6 cfu/ ml), were harvested at indicated times and total RNAs were extracted. Twenty μg of the total RNAs was fractionated on a 1% agarose gel containing formaldehyde and then transferred to a nylon membrane. The membrane was allowed to hybridize with individual cDNA probes indicated on the left of panels. The rRNA band stained with acridine orange is shown to verify that similar amounts of RNA were loaded per lane.

with the glycine-rich protein (GRP) of tobacco (Table 1). Van Kan *et al.*[29] reported that the tobacco genome contains approximately eight GRP genes, indicating *EIG-G7* is a new member of the GRP gene family in tobacco. Amino acid sequence similarity for *EIG-G7* was also found with GRP of petunia and tyrosine- and lysine-rich protein (TLRP) of tomato, with 65% and 53% identity respectively[8,12]. It was also shown that TLRP is localized in the cell wall[8]. Because of the high similarity of *EIG-G7* with TLRP at the N-terminal of the amino acid sequences, *EIG-G7* may be a cell wall component (Fig. 3B). The GRPs are most likely structural proteins that may have an important function with respect to the plant's vascular system and wound healing[24]. GRPs were also reported to be expressed in response to a variety of stress conditions, including wounding, drought stress, viral infection and SA treatment. The *EIG-G7* protein that accumulates in the cell wall is presumably involved in repair of the injured wall or reinforcement of the wall structure for disease resistance.

EIG-I24

Expression of *EIG-I24* increased within 6 hr, rose to a peak at 12 hr and slightly decreased after nonpathogen inoculation (Fig. 2). The deduced amino acid sequence of *EIG-I24* displays the highest degree of homology with *Arabidopsis thaliana* sequences for which no function have yet been determined (Table 1). *EIG-I24* also has low degrees of amino acid identity with tobacco hsr201 (induced after inoculation with phytopathogenic bacteria) (26% identity)[4], carnation hydroxycinnamolyl/benzoyltransferase (the enzyme for phytoalexin biosynthesis) (25% identity)[31], taxadienol acetyl transferase of yew tree (38%, accession no. AF190130) and many other hypothetical proteins of *A. thaliana*. The amino acid sequence of *EIG-I24* contains a conserved sequence among plant acyltransferases, suggesting that *EIG-I24* encodes a novel acyltransferase of tobacco (Fig. 3D). However, we were unable to propose an enzymatic activity for *EIG-I24* based on the gene structure.

EIG-I30

The *EIG-I30* transcripts increased rapidly, reached a maximum within 6 hr and decreased at 36 hr in response to nonpathogenic bacteria (Fig. 2). The *EIG-I30* cDNA encodes a deduced protein of 148 amino acids with a high proportion of proline (13%) and an N-portion motif containing proline, serine, tyrosine, histidine lysine and threonine. The highest identity was found with extensin-like protein of *Populus nigra* (Poplar) (Sakuma *et al.* 1996, unpublished data) (Fig. 3C). The *EIG-I30* has no significant amino acid similarity with tobacco extensin, suggesting *EIG-I30* is a gene for a novel extensin (or proline-rich protein) of tobacco. Extensins, hydroxyproline-rich glycoproteins in the cell walls of higher plants, have been proposed to be structural proteins that may function in development, wound healing and plant defense[24]. After elicitor treatment of cultured bean cells, extensin in the cell walls was rapidly cross linked, suggesting that extensin is involved in impeding pathogen infection by contributing to a more impenetrable cell wall barrier[24]. The expression of extensin genes is also known to be induced by various kinds of stresses, such as wounding, fungal infection, viral infection, endogenous elicitors, ethylene and heat shock[24].

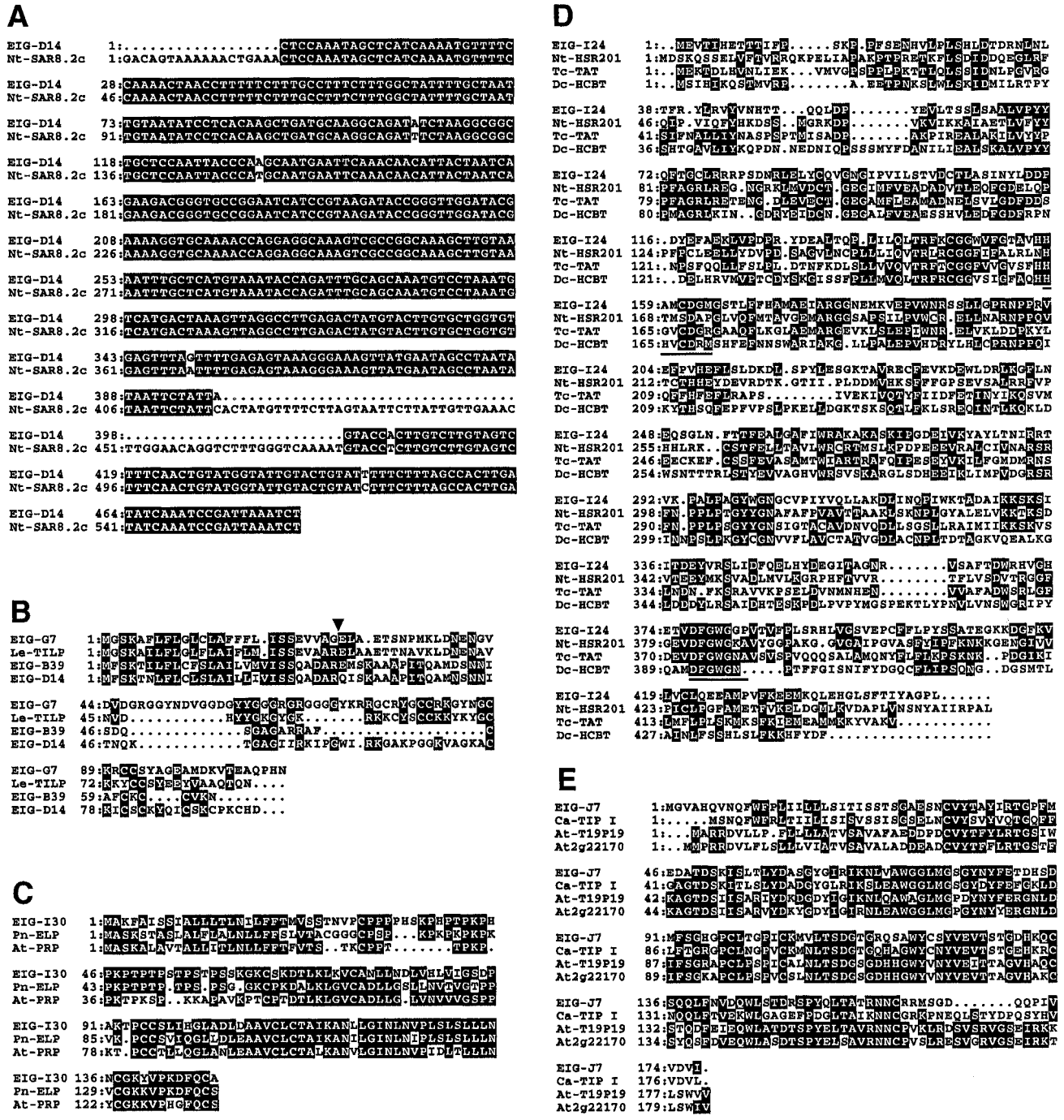

Fig. 3. A) Alignment of nucleotide sequences of *EIG-D14* and *SAR8.2c*[2]. B) Comparison of putative amino acid sequences of *EIG-G7*, *EIG-B39*, *EIG-D14* and tomato tyrosine- and lysine-rich protein (Le-TLRP)[8]. C) Comparison of putative amino acid sequences of *EIG-I30*, poplar extensin-like protein (Pn-ELP) and Arabidopsis putative proline-rich protein (At-PRP). D) Comparison of putative amino acid sequences of *EIG-I24*, tobacco hsr201[4], yew tree taxadienol acetyl transferase (Tc-TAT) and carnation hydroxycinnamoyl/benzoyltransferase (Dc-HCBT)[31]. E) Comparison of putative amino acid sequences of *EIG-J7*, hot pepper TMV-induced protein I (Ca-TIP I) and Arabidopsis T19P19 and At2g22170. Identical nucleotides or amino acids are shown in reverse. The sequences were aligned using the CLUSTAL W program[28].

EIG-J7

The *EIG-J7* transcripts, weakly detected within 6 hr, had slightly increased to a peak by 24 hr in tobacco leaf inoculated with a nonpathogen (Fig. 2). The putative amino acid sequence of *EIG-J7* displays 58% identity with TMV-induced protein I isolated from the hot pepper *Capsicum annuum* (Shin *et al.* 2000, unpublished data) (Table 1, Fig. 3E). In addition, 43 and 41% amino acid identities were found with two hypothetical proteins of *A. thaliana* (Fig. 3E). However, the amino acid sequence of *EIG-J7* has no similarity with any functional motifs in the database.

Expression patterns of EIGs in response to wounding or SA

The expression patterns of EIGs in tobacco leaves either wounded or treated with SA were examined (Fig. 4). Upon wounding, the transcripts of five EIGs (*EIG-B39, -D14, -G7, -I24* and *-J7*) and *PR* genes increased slightly. This increase was transient, as in the control, whereas the expression of *EIG-I30* increased considerably with a maximum at 12–24 hr after wounding (Fig. 4). The *EIG-I30* cDNA encodes a putative cell wall protein, implying the product of *EIG-I30* is involved in healing of injured cell wall. SA is well known as an important component of the signal transduction pathway leading to disease resistance and can lead to the elaboration of systemic signals that immunize distal parts of the plant to pathogen infection[10,22]. The SA treatment increased the expression of all cloned EIGs, supporting the central role of SA in regulating disease stress-inducible genes (Fig. 4). The induction of *EIG-B39* and *-D14* by SA had a pattern quite similar to that after inoculation with the nonpathogen, *P. syringae* pv. *glycinea*. This result indicated the expression of *EIG-B39* and *-D14* gene is SA-dependent like acidic *PR* genes. The expression pattern of *EIG-G7* after treatment with SA also resembled that after a nonpathogen (Fig. 4). In contrast, the induction pattern of *EIG-I24, -I30* and *-J7* by SA was unlike that after a nonpathogen. The expression of *EIG-J7* was induced strongly within 6 hr after SA treatment, while the expression of this gene at the same time was slight in the case of nonpathogen, implying that there is positive and negative regulation of *EIG-J7* expression. The individual expression pattern of EIGs varies during disease responses, indicating that transcriptional activation of EIGs is regulated by a complex of various signals and the products of EIGs are involved in different processes at different stages in the plant defense responses. It is also possible that the results of the northern hybridizations for EIGs reflect the expression of more than one homologous gene. Additional investigation, such as characterization of each EIG family, is needed to elucidate the regulation of EIG expression in disease resistance of tobacco plant.

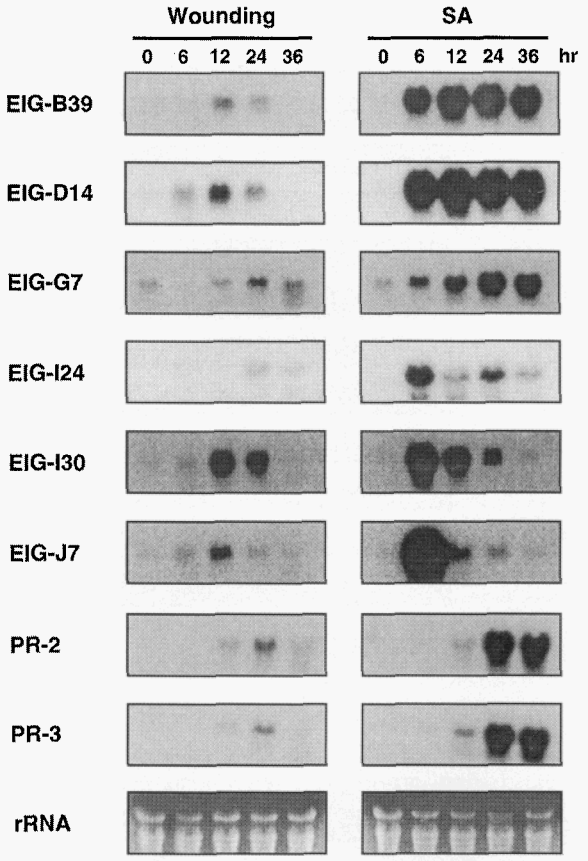

Fig. 4. The expression of EIGs in wounded or salicylic acid treated leaves. Whole areas of tobacco leaves were wounded with carborundum (Wounding) or treated with 0.5 mM salicylic acid (SA). They were harvested at indicated times and total RNAs were extracted. Twenty μg of total RNAs was fractionated on a 1% agarose gel containing formaldehyde and then transferred to a nylon membrane. The membrane was allowed to hybridize with individual cDNA probes indicated on the left. The rRNA band stained with acridine orange is shown to verify that similar amounts of RNA were loaded per lane.

Activation of plant defense genes is an important element in the outcome of a plant-pathogen interaction. Isolation of elicitor-inducible genes, characterization of activation profiles and identification of their putative functions make these genes good candidates for understanding early and late events in plant disease resistance responses. However, further investigation is necessary to reveal the function of the EIGs products.

ACKNOWLEDGMENTS

The authors thank Dr. Yuko Ohashi, Department of Molecular Biology at National Institute of Agrobiological Resources for providing the cDNA probes for tobacco PR-2 and Dr. Yuichi Takigawa, Faculty of Agriculture at Shizuoka University, for the gift of *P. syringae* pv. *glycinea* 801. The authors thank Dr. Hitoshi Mori, Laboratory of Developmental and Genetic Regulation at Nagoya University, for technical advice, Drs. Hirofumi Yoshioka and Takashi Tsuge in our laboratory for valuable suggestions, and the members of the Radioisotope Research Center, Nagoya University, for technical assistance. The authors are grateful to Ryan Wilson, Research School of Biological Sciences at Australian National University for reading the manuscript. This work was supported in part by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists and also by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

LITERATURE CITED

1. Akashi, T., Aoki, T. and Ayabe, S. (1998). CYP81E1, a cytochrome P450 cDNA of licorice (*Glycyrrhiza echinata* L.), encodes isoflavone 2′-hydroxylase. Biochem. Biophys. Res. Commun. 251 : 67-70.
2. Alexander, D., Stinson, J., Pear, J., Glascock, C., Ward, E., Goodman, R.M. and Ryals, J. (1992). A new multigene family inducible by tobacco mosaic virus or salicylic acid in tobacco. Mol. Plant-Microbe Interact 5 : 513-515.
3. Aviv, H. and Leder, P. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA 69 : 1408-1412.
4. Czernic, P., Huang, H.C. and Marco, Y. (1996). Characterization of *hsr201* and *hsr515*, two tobacco genes preferentially expressed during the hypersensitive reaction provoked by phytopathogenic bacteria. Plant Mol. Biol. 31 : 255-265.
5. Dhingra, O.D. and Sinclair, J.B. (1995). Basic Plant Pathology Methods, 2nd ed., pp. 354, CRC Press, Boca Raton, FL.
6. Dixon, R.A. and Paiva, N.L. (1995). Stress-induced phenylpropanoid metabolism. Plant Cell 7 : 1085-1097.
7. 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.
8. Domingo, C., Gomez, M.D., Canas, L., Hernandez-Yago, J., Conejero, V. and Vera, P. (1994). A novel extracellular matrix protein from tomato associated with lignified secondary cell walls. Plant Cell 6 : 1035-1047.
9. Facchini, P.J. and Chappell, J. (1992). Gene family for an elicitor-induced sesquiterpene cyclase in tobacco. Proc. Natl. Acad. Sci. USA 89 : 11088-11092.
10. Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261 : 754-756.
11. Hammond-Kosack, K.E. and Jones, J.D. (1996). Resistance gene-dependent plant defense responses. Plant Cell 8 : 1773-1791.
12. Linthorst, H.J., van Loon, L.C., Memelink, J. and Bol, J.F. (1990). Characterization of cDNA clones for a virus-inducible, glycine-rich protein from petunia. Plant Mol. Biol. 15 : 521-523.
13. Linthorst, H.J., Melchers, L.S., Mayer, A., van Roekel, J.S., Cornelissen, B.J. and Bol, J.F. (1990). Analysis of gene families encoding acidic and basic beta-1,3-glucanases of tobacco. Proc. Natl. Acad. Sci. USA 87 : 8756-8760.
14. Lois, R., Dietrich, A., Hahlbrock, K. and Schulz, W. (1989). A phenylalanine ammonia-lyase gene from parsley : structure, regulation and identification of elicitor and light responsive cis-acting elements. EMBO J. 8 : 1641-1648.
15. Mauch, F., Mauch-Mani, B. and Boller, T. (1988). Antifungal hydrolases in pea tissue. Plant Physiol. 88 : 936-942.
16. Mizutani, M., Ward, E., Dimaio, J., Ohta, D., Ryals, J. and Sato, R. (1993). Molecular cloning and sequencing of a cDNA encoding mung bean cytochrome P450 (P450C4H) possessing cinnamate 4-hydroxylase activity. Biochem. Biophys. Res. Commun. 190 : 875-880.
17. Mori, H., Takeda-Yoshikawa, Y., Hara-Nishimura, I. and Nishimura, M. (1991). Pumpkin malate synthase. Cloning and sequencing of the cDNA and northern blot analysis. Eur. J. Biochem. 197 : 331-336.
18. 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.
19. Payne, G., Ahl, P., Moyer, M., Harper, A., Beck, J., Meins, F. Jr. and Ryals, J. (1990). Isolation of complementary DNA clones encoding pathogenesis-related proteins P and Q, two acidic chitinases from tobacco. Proc. Natl. Acad. Sci. USA 87 : 98-102.
20. 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.
21. Ride, J.P. (1985). Non-host resistance to fungi. *In* Mechanisms of resistance to plant disease (Junk, W., ed.). pp. 29-61, R.S.S. Fraser, Dordrecht.
22. Ryals, J.A., Neuenschwander, U.H., Willits, M.G.,

Molina, A., Steiner, H.-Y. and Michelle, D.H. (1996). Systemic acquired resistance. Plant Cell 8 : 1809-1819.
23. Schopfer, C.R., Kochs, G., Lottspeich, F. and Ebel, J. (1998). Molecular characterization and functional expression of dihydroxypterocarpan 6a-hydroxylase, an enzyme specific for pterocarpanoid phytoalexin biosynthesis in soybean (*Glycine max* L.). FEBS Lett. 432 : 182-186.
24. Showalter, A.M. (1993). Structure and function of plant cell wall proteins. Plant Cell 5 : 9-23.
25. Singh, N.K., Nelson, D.E., Kuhn, D., Hasegawa, P.M. and Bressan, R.A. (1989). Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. Plant Physiol. 90 : 1096-1101.
26. 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.
27. Takemoto, D., Hayashi, M., Doke, N., Nishimura, M. and Kawakita, K. (1999). Molecular cloning of a defense-response-related cytochrome P450 gene from tobacco. Plant Cell Physiol. 40 : 1232-1242.
28. 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. Nucleic Acids Res. 22 : 4673-4680.
29. Van Kan, J.A.L., Cornelissen, B.J.C. and Bol, J.F. (1988). A virus-inducible tobacco gene encoding a glycine-rich protein shares putative regulatory elements with the ribulose bisphosphate carboxylase small subunit gene. Mol. Plant-Microbe Interact. 1 : 107-112.
30. 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.
31. Yang, Q., Reinhard, K., Schiltz, E. and Matern, U. (1997). Characterization and heterologous expression of hydroxycinnamoyl/benzoyl-CoA : anthranilate N-hydroxycinnamoyl/benzoyltransferase from elicited cell cultures of carnation, *Dianthus caryophyllus* L. Plant Mol. Biol. 35 : 777-789.