RESEARCH PAPER

Involvement of EIN3 homologues in basic PR gene expression and flower development in tobacco plants

Tadaharu Hibi1,2,3,*, Shunichi Kosugi1,2, Takayoshi Iwai1,2, Motoshige Kawata3, Shigemi Seo1,2, Ichiro Mitsuhara1,2 and Yuko Ohashi1,2

1 National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan
2 Program for Promotion of Basic Research Activities of Innovative Bioscience, Toranomon Minato-ku, Tokyo, Japan
3 Hokuriku Research Centre, National Agricultural Research Centre. 1-2-1 Inada Joetsu Niigata 943-0193, Japan

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Abstract

The TEIL (Tobacco EIN3-Like) gene is a tobacco homologue of arabidopsis Ethylene Insensitive 3 (EIN3), and the gene product binds an 8 bp sequence in the tobacco PR1a promoter in a sequence specific manner. It was found here that accumulation of TEIL transcript was induced by wounding and preceded basic PR gene expression. To study the downstream signalling pathway of TEIL, TEIL was overexpressed under the control of the constitutive 35S promoter in tobacco plants. In 35S::TEIL lines, basic PR genes, which are wound-, jasmonate- and ethylene-inducible, were expressed constitutively. Next, the conserved 781 bp sequence among tobacco EIN3-like (EIL) protein genes was introduced as an inverted-repeat (IR) into tobacco to suppress expression of these genes. In two independent IRTEIL lines, the TEIL transcript was not found and transcripts of other tobacco EILs, NtEIL3, and NtEIL5, were reduced. In IRTEIL plants, wound-, jasmonate- and ACC-induced accumulation of basic PR gene transcripts was significantly inhibited. These results indicate that TEIL functions upstream of tobacco basic PR genes in wound signalling via not only ethylene but also jasmonate. In 35S::TEIL plants, the pistil length of the flower was longer with a slight protrusion of the stigma compared with the control. In IRTEIL plants, the length of the stamens was shorter than the control with significant protrusion of the stigma in the flower. These observations indicate the involvement of tobacco EILs in flower development.

Key words: basic PR, EIN3, ethylene, flower development, gene expression, jasmonic acid, pistil, stamen, tobacco, wound.

Introduction

EIN3 and EIN3-like (EIL) protein genes encode key transcriptional factors of ethylene signalling, and homologues have been identified in many plant species. They function downstream of ethylene receptors (Guo and Ecker, 2004), CTR1, a negative regulator downstream of the receptors encoding a protein with homology to the Raf family of Ser/Thr protein kinases (Kieber et al., 1993; Huang et al., 2003), and EIN2, a member of the Nramp metal ion transporter family (Alonso et al., 1999) in Arabidopsis. EIN3/EILs reportedly regulate expression of the GCC-box-binding transcription factors (Solano et al., 1998), whose products act as the activators or repressors of downstream ethylene-responsive genes such as basic pathogenesis-related (PR) genes, which contain GCC-boxes in their tobacco promoters (Ohme-Takagi et al., 2000). In the Arabidopsis ein3 mutant, expression of the ERF1 and a basic chitinase gene was reduced (Solano et al., 1998), and ethylene response phenotypes were lost. However, the overexpression of Arabidopsis EIL1 or 2 cancelled this effect, indicating the functional redundancy of EILs (Chao et al., 1997; Solano et al., 1998). The TEIL (Tobacco EIN3-Like) gene has been isolated from tobacco (Nicotiana tabacum cv. Samsun NN), whose gene product binds the promoter of the tobacco PR1a gene as a putative negative transacting factor (Kosugi and Ohashi, 2000). TEIL shares 60% identity in its amino acid sequence with * To whom correspondence should be addressed. E-mail: hibiharu@affrc.go.jp

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**Materials and methods**

**Plant materials and wound- and chemical treatments**

Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were grown in a growth chamber at 25 °C with a 16 h light cycle, and fully developed upper leaves from 2-month-old plants were used unless stated otherwise. For wound-treatment, leaves were cut into approximately 1 cm square sections, floated on 10 mM phosphate buffer (pH 7.0) or phosphate buffer containing methyl jasmonic acid (MeJA) or 1-amino cyclopropane-1-carboxylate (ACC) at 50 μM, respectively, and incubated at 25 °C under approximately 1000 lx. Samples for RNA preparations were quick-frozen in liquid nitrogen and stored at –80 °C until use.

**RNA gel blot analysis**

Total RNA was isolated by an acid guanidium-phenol-chloroform (AGPC) method (Chomczynski and Sacchi, 1987), and 20 μg RNA per lane was separated on agarose gels containing formaldehyde, transferred to nylon membranes, and hybridized with a 32P-labelled specific probe containing the 3’ untranslated region (UTR) of each gene. Equal loading of RNA was confirmed by monitoring the levels of ribosomal RNA (rRNA) stained by ethidium bromide (EtBr).

**Generation of 35S::TEIL plants and TEIL-suppressed plants**

The TEIL cDNA was inserted downstream of the Cauliflower mosaic virus (CaMV) 35S promoter, and introduced into binary vector pCEP5 (Kosugi and Ohashi, 2000). For the inverted repeat (IR) constructs of TEIL, the binary vector E12-omega-MCS was used (Mitsuhashi et al., 1996) that confers 10-fold higher expression compared with the CaMV 35S promoter. The 781 bp fragment from 363–1143 bp, which corresponds to the conserved N-terminal region of tobacco EIL family proteins, was amplified by PCR, using primers 5’-GATTATGGCATTGGAGGAGAT-3’ (forward), 5’-GAAAACCTTCTCTTCTGATT-GA-3’ (reverse), and inserted in sense and antisense orientations using a 120 bp fragment originating from the first intron of the rice ppxA gene as the spacer. The construct was introduced into *Agrobacterium tumefaciens* LBA4404, and transferred to tobacco cv. Samsun NN according to the procedure of Ohshima et al. (1990).

**Analysis of gene expression in transgenic tobacco plants by RT-PCR**

Total RNA extracted from tobacco samples was used for first-strand cDNA synthesis using Ready-to-Go you-prime first-strand beads kit (Amersham Biosciences, UK) and oligo (dT) primer. Specific primers were designed based on the sequences of each gene (Kosugi and Ohashi, 2000; Rieu et al., 2003). Actin mix primers were derived from consensus sequences of tobacco actin genes Tob25, Tob53, and Tob66 (Moniz and Drouin, 1996; Dhondt et al. 2000). Using sense primers (actin mix: 5’-GATATGGAGGAATATTG-CTCAYAC-3’, TEIL: 5’-GGGATTACAGAGGATAACATGCG-GAA-3’, NiEIL3: 5’-GCCACTACATGGGAATTTACCTT-3’, NiEIL5: 5’-GATCTTAGCAAATCAAACATCGGTC-3’) and antisense primers (actin mix: 5’-GTTCCTGCAATTTGCAATCGCA-3’) and anti-sense primers (actin mix: 5’-GATTATGGCATTGGAGGAGAT-3’, TEIL: 5’-GAACTACATGGGAATTTACTT-3’, NiEIL3: 5’-ACTGATAAAACTGCGAGATGGAAC3’-3’, NiEIL5: 5’-AGGTTAAGAGCATATTTCTCAATACG-3’), PCR was performed at 94 °C for 2 min followed by 25–30 cycles of 94 °C for 1 min, 55–60 °C for 1 min, 72 °C for 1 min, and then one cycle of 72 °C for 5 min using Takara PCR Thermal Cycler Personal (Takara, Japan). Amplified products were subjected to electrophoresis in 2% agarose gels and stained with ethidium bromide.

**Results**

**Wound-induced expression of a tobacco EIN3 homologue**

Ethylene is known as a wound signal, and the wound response of the *Tobacco EIN3*-like (TEIL) gene was studied using fully expanded upper tobacco leaves of 6-week-old plants. Leaves were cut into pieces, floated on phosphate buffer under light, and then subjected for RNA gel blot analysis. The transcript was detected at a basal level in healthy leaves, decreased at 0.5–1 h slightly and increased at 3–6 h after wounding, and then maintained the same level up to 24 h (Fig. 1). Wound-induced expression of tobacco basic pathogenesis related (PR)1 and 5 genes, which are wound- and jasmonic acid/ethylene-inducible (Niki et al., 1998), was emphasized, and related proteins at the protein level.

By contrast, the role of the transcriptional regulation of EIN3 on induction of downstream genes is not fully understood. Solano et al. (1998) indicated that overexpression of EIN3 resulted in constitutive expression of the basic chitinase and PDF1.2 genes. Developmentally regulated or ethylene-induced expression of carnation EILs (Waki et al., 2001; Iordachescu and Verlinden, 2005) was reported, while the effect of EIL expression on downstream genes was not clearly shown.

Using tobacco, it was found here that the expression of TEIL is regulated by wounding, indicating that the level of TEIL transcript is also important for the expression of the downstream genes. It is shown that the expression of basic PR genes, which are wound-, ethylene-, and jasmonate-inducible, was enhanced in TEIL-overexpressing plants, and repressed in TEIL/EILs-suppressed tobacco plants, indicating that basic PR genes are downstream genes of TEIL, and TEIL/EILs regulate basic PR gene expression not only via ethylene signalling but also via jasmonate signalling. Furthermore, it was found that TEIL/EILs control flower development. This is the first report of a role of TEIL/EILs using a loss of function strategy in tobacco.

**EIN3 from Arabidopsis.** TEIL cDNA overexpression resulted in constitutive triple response phenotypes in *Arabidopsis* seedlings, and TEIL has a DNA binding activity to TEIL binding sites (tbs) A(A/C)G(A/T)A(A/C)CT, which are found in the promoters of many defence-related genes (Kosugi and Ohashi, 2000). Five tobacco EIL family members (NtEILs) were isolated from an ovary cDNA library of *Nicotiana tabacum* L. cv. Petit Havana SR1 and were reported to be expressed at different levels in ovary tissues (Rieu et al., 2003).

Recent studies showed that the stability of the EIN3 protein was enhanced by ethylene treatment and inhibited in the presence of glucose (Yanagisawa et al., 2003), emphasizing the regulation of EIN3 and related proteins at the protein level.

Furthermore, it was found that expression not only via ethylene signalling but also via jasmonate signalling. This is the first report of the regulation of EIN3 by wounding, indicating that the level of downstream genes was not clearly shown. Using sense primers (actin mix: 5’-GATATGGAGGAATATTG-CTCAYAC-3’, TEIL: 5’-GGGATTACAGAGGATAACATGCG-GAA-3’, NiEIL3: 5’-GCCACTACATGGGAATTTACCTT-3’, NiEIL5: 5’-GATCTTAGCAAATCAAACATCGGTC-3’) and anti-sense primers (actin mix: 5’-GTTCCTGCAATTTGCAATCGCA-3’) and anti-sense primers (actin mix: 5’-GATTATGGCATTGGAGGAGAT-3’, TEIL: 5’-GAACTACATGGGAATTTACTT-3’, NiEIL3: 5’-ACTGATAAAACTGCGAGATGGAAC3’-3’, NiEIL5: 5’-AGGTTAAGAGCATATTTCTCAATACG-3’), PCR was performed at 94 °C for 2 min followed by 25–30 cycles of 94 °C for 1 min, 55–60 °C for 1 min, 72 °C for 1 min, and then one cycle of 72 °C for 5 min using Takara PCR Thermal Cycler Personal (Takara, Japan). Amplified products were subjected to electrophoresis in 2% agarose gels and stained with ethidium bromide.

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detected at 3 h and slightly increased at 6 h and thereafter (Fig. 1). Similar results were obtained in replicate experiments. These results indicate accumulation of both TEIL and basic PR transcripts was induced by wounding and that of TEIL was followed by that of basic PR genes.

Expression of TEIL gene in various organs
The characteristic TEIL expression in various organs was studied. In healthy tobacco plants, the TEIL transcript was found in all organs examined, but the level varied; it was lower in flowers and higher in stems and roots compared with the mature leaves (Fig. 2). A low transcript level in fruit compared with leaves was reported in tomato by Tieman et al. (2001).

Analysis of PR gene expression in 35S::TEIL transgenic tobacco plants
To study the function of TEIL, TEIL cDNA was overexpressed under the control of the CaMV 35S promoter by introduction of a 35S::TEIL gene into tobacco. In three independent 2nd generation lines, high levels of TEIL transcript was detected. Two 35S::TEIL lines were used (two plants each per line), and one order of magnitude higher levels of TEIL and basic PR5 transcripts were found in mature leaves compared with control plants (Fig. 3). By contrast, the transcript of acidic PR2, which is salicylic acid-inducible, was not detected in any 35S::TEIL transgenic tobacco plants examined. These results indicate that TEIL regulates basic PR gene expression positively in tobacco leaves.

Analysis of PR gene expression in TEIL-suppressed tobacco lines
To study the role of TEIL by a loss of function strategy, an inverted repeat (IR) sequence, corresponding to the conserved DNA binding domain among tobacco EILs, was introduced into tobacco. The DNA binding domains among TEIL and NtEIL family proteins are located in the N-terminus, and are highly conserved (Fig. 4A, framed in red). The conserved region (781 bp, which encodes amino acid residues 41–300, shown in red) was used to prepare the inverted repeat (IR) sequence. An intron sequence (120 bp) was inserted in the centre between the sense and antisense sequences, and the entire sequence was expressed under the control of a strong constitutive promoter, El2Ω (Mitsuhashi et al., 1996) (Fig. 4B). Nine
kanamycin-resistant transgenic plants were selected, and the insertion of the transgene was confirmed by PCR in six independent lines. Using the 2nd generation, reduced TEIL expression could be detected in four of the six lines, and selected lines 01242 and 0216 as two representatives IRTEIL lines. In mature leaves of 2-month-old two transgenic 2nd generation lines, no TEIL transcript signal was detected in healthy or wounded leaves by RT-PCR using specific primers for each gene (Fig. 4C). Transcript levels of NtEIL3 and NtEIL5, which are homologues of TEIL (Fig. 4A), were considerably reduced in both lines, 01242 and 0216 (Fig. 4C), while the reduction was less compared to TEIL.

Using IRTEIL lines, in which TEIL family genes were suppressed, the response of PR genes to some treatments was studied (Fig. 4D). In control plants, treatments with wound signal molecules methyl jasmonate (MeJA) and 1-aminoacyclopropane-1-carboxylic acid (ACC), which is the precursor of ethylene, induced little accumulation of TEIL transcript compared with phosphate buffer-treated controls at 48 h. Instead, expression of both basic PR5 and 1 genes was induced by wounding, and enhanced in the presence of MeJA and ACC. Acidic PR1α gene expression was not induced in IRTEIL plants, indicating not only TEIL but also other unknown molecules must be necessary to repress PR1α promoter activity (Hagiwara et al., 1993). In IRTEIL line 01242, expression of the TEIL gene was markedly reduced in both healthy and wounded leaves, and MeJA- and ACC-treated leaves. Similar results were obtained in the other IRTEIL line, 0216. Interestingly, both basic PR1 and 5 transcripts were not found in IRTEIL lines at all. Because basic PR genes are candidate genes downstream of TEIL in the ethylene signalling pathway, these findings are consistent with the proposal that TEIL regulates basic PR gene expression induced by ethylene and jasmonate. Because the transcripts of not only TEIL but also NtEIL3 and 5 were reduced in IRTEIL lines, the phenomena found in the lines would come from the sum result of individual suppression of multiple EILs. However, in addition to the findings from 35S::TEIL plants (Fig. 3), the results in IRTEIL lines clearly show that TEIL at least regulates the expression of basic PR genes, and the level of the TEIL transcript is important for downstream gene expression in tobacco plants.

Altered flower morphology in tobacco plants in which TEIL expression was up-regulated or suppressed
While a high level of TEIL transcript accumulated, 35S::TEIL lines grew with almost normal phenotypes in the vegetative stage and were fertile. However, the flower phenotypes just before flowering (developmental stage 11) (Koltunow et al., 1990) was slightly different from those of controls (Fig. 5A). The relative position of the top of the stigma (green arrows in Fig. 5A, illustrated as S in Fig. 5C right) to the positions of the anthers (yellow arrows, illustrated as H and L) was statistically higher than in control plants.

IRTEIL plants were successively generated, but were not fully fertile producing only small amounts of seeds by open-pollination. The flowers of IRTEIL plants from the second generation looked slim with clearly protruding pistils compared with control and 35S::TEIL plants (Fig. 5B). The distance between the top of the pistil and the top of the peduncle (A–S) was longest in IRTEIL plants, while the length of both H and L stamens was shorter than 35S::TEIL and control plants, highlighting the protrusion of the pistils in IRTEIL plants (Fig. 5B, C). This heterostylos phenomenon was likely to be caused by the relative length of the pistil to other flower constituents. These results indicate not only TEIL but also other NtEILs participate in the regulation of flower shape development.

Discussion
Expression of EIN3/EIL transcription factor genes was reportedly not affected by exogenous ethylene in Arabidopsis, tomato, tobacco, and mung bean (Chao et al., 1997; Tiemann et al., 2001; Lee and Kim, 2003; Rieu et al., 2003), indicating their regulation is mainly controlled at the post-transcriptional level. In Arabidopsis, the stability of EIN3 was reported to be regulated positively by ethylene and negatively by glucose (Yanagisawa et al., 2003), and the involvement of the ubiquitin/proteasome pathway in the regulation of EIN3 protein degradation was suggested (Guo and Ecker, 2003; Potuschak et al., 2003). These data presented here additively proposed the importance of transcriptional regulation of TEIL after wounding for induced expression of the predicted target

Fig. 4. Generation of TEIL-suppressed tobacco plants and the analysis of the characteristics. (A) Alignment of the derived peptide sequences of TEIL (Kosugi and Ohashi, 2000) and NtEIL1, 2, 3, 4, and 5 (Rieu et al., 2003). The conserved amino acids residues are framed in black, and conserved regions among the six genes are boxed in red. (B) Schematic representation of the introduced gene for generation of IRTEIL plants. For the inverted repeat (IR) construct, the conserved region (781 bp, framed in red) among TEIL, NtEIL1, 2, 3, 4, and 5 genes was used. An intron (120 bp) from the rice pcox gene was used in the centre of the sense and antisense sequences, and the IR construct was driven by a high expression promoter, El2Ω (Mituhara et al., 1996). (C) Expression of TEIL family genes in IRTEIL tobacco plants. Total RNA isolated from healthy (0 h) and wounded (48 h) leaves was subjected to RT-PCR analysis. For wound treatment, leaf discs were floated on 10 mM phosphate buffer (pH 7.0) at 25 °C under light for 48 h. RT-PCR was performed with specific primers for each gene and mix primers for tobacco actin genes. The gels were stained with ethidium bromide. (D) Expression pattern of PR genes in IRTEIL transgenic plants. RNA samples were isolated from healthy leaves (0 h), and leaf discs that were floated on 10 mM phosphate buffer (pH 7.0) for 48 h (Cont), or on a solution of MeJA (50 uM) or ACC (50 uM) at 25 °C under light. RNA gel blot analysis was done as described in the legend of Fig. 3. Similar results were obtained in replicate experiments.
35S::TEIL plants, TEIL overexpression resulted in constitutive expression of basic PR genes (Fig. 3A). In IRTEIL plants, the suppression of TEIL and related genes induced suppression of basic PR gene expression (Fig. 4D). These results show that at least TEIL positively controls the accumulation of basic PR transcripts in tobacco, although the individual role of other EILs should be studied in the future. It is shown here that TEIL also regulates jasmonate-induced expression of basic PR genes. The evidence was interesting in relation to the following reports; ERF1 for ethylene responsive factor is regulated via the jasmonate signalling pathway and the GCC-box in PDF1.2 has been identified as a JA-responsive element, indicating ERF1 might be an essential factor for both ethylene and jasmonate signals (Lorenzo et al., 2003; Guo and Ecker, 2004). JA- and ACC-induced expression of basic PRI and 5 genes was decreased in IRTEIL plants (Fig. 4D), indicating TEIL and its closely related genes might be essential factors in both ethylene and JA signalling as well as ERF1.

TEIL binds a sequence containing the 8 bp TEIL-binding site (tebs) as a putative negative transcriptional factor of the acidic PRIa promoter, and this binding
activity was enhanced by ethylene treatment (Hagiwara et al., 1993; Kosugi and Ohashi, 2000). In IRTEIL lines, no constitutive PR1a expression was found, indicating TEIL requires co-operation with other factors to repress the PR1a promoter. The tebs sequence was also found in the promoter regions of a number of basic PR genes, indicating the possible direct activation of basic PR promoters without the mediation of ERF1-related factor, which was reported to regulate GCC-box containing promoter sequences in Arabidopsis (Solano et al., 1998). Recently, it was reported that EIL3 bind tebs via a DNA-binding domain in Arabidopsis (Yamasaki et al., 2005), and that EILs in mung bean can also bind tebs and overexpression in tobacco resulted in constitutive expression of basic PRs (Lee and Kim, 2003). These reports support our results, suggesting conserved functions of EILs among various plants species.

For effective inhibition of ethylene responses, introduction of the antisense sequence of a single LeEIL (tomato EIN3 homologue) to tomato was not sufficient, and inhibition of the expression of multiple LeEIL genes was required (Tieman et al., 2001). In this study, IRTEIL plants were generated using a construct containing the conserved region among TEIL and NtEILs (Fig. 4A, B), and succeeded in suppressing TEIL gene expression. The homologuey in this region is high; TEIL has 93.9% and 87.1% identity to NtEIL3, and 85.8% and 77.7% to NtEIL5 in its amino acids sequence and nucleotide sequence, respectively. Thus, a significant decrease in TEIL expression and a reduced expression of NtEIL3 and NtEIL5 found in IRTEIL lines would be reasonable from the homologuey of the introduced sequence (Fig. 4C).

It was found that TEIL also functions in the regulation of flower development. In 35S::TEIL flowers, the top of the pistil protruded (Fig. 5A), which was consistent with the previous report that EIN3 over-expressing Arabidopsis exhibited a similar projecting pistil phenotype (Guo and Ecker, 2003). Flowers of IRTEIL plants exhibited pistil protrusion with elongated pistils and shortened stamens (Fig. 5B, C). These results were consistent with previous reports; overexpression of a melon ethylene receptor gene, Cm-ERS1/H69A, under the control of the 35S promoter resulted in flowers with projecting pistils, which was also observed in wild-type tobacco treated with silver thiosulphate (STS), an inhibitor of ethylene action, suggesting that the phenotype was due to their reduced ethylene sensitivity (Takada et al., 2005, 2006). The flower phenotype with projecting pistil was observed in both 35S::TEIL and IRTEIL plants, in which TEIL expression was enhanced and suppressed, respectively. These phenomena were apparently contradictory considering the expression of TEIL. In this study, the length of the whole flower, pistil, and the highest and lowest stamens in 35S::TEIL and IRTEIL lines were determined, and statistically compared with those from control lines. These results indicated the role of TEIL in flower development was not only concerned with the projected pistil phenotype, but also with the length of each organ. Expression not only of TEIL but also NtEIL3 and 5 was considerably suppressed in IRTEIL plants indicating that other NtEILs in addition to TEIL may also function in a complex manner in flower development in tobacco plants. While the mechanism of the protrusion of the pistil may not be simple, the involvement of TEIL was clearly shown in this study. The functions of other NtEILs in flower shape development should be evaluated in future studies. IRTEIL plants produced few seeds and were dependent on the low efficiency of pollination of the flower between elongated pistils and shortened anthers.

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