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Gene Note

Jasmonate- and wounding-induced expression of a tobacco gene encoding a BTB domain protein

Hisabumi Takase[a,b], Koji Inai[b], Takashi Hashimoto*

Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara 630-0192, Japan
* E-mail: hasimoto@bs.naist.jp Tel: +81-743-72-5520 Fax: +81-743-72-5529

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Abstract Nicotine is synthesized in the root of tobacco (*Nicotiana tabacum*) and transported to aerial parts where this alkaloid functions as an insect repellant. Nicotine biosynthesis is regulated by jasmonate and specific regulatory *Nic* loci, but molecular components in the jasmonate- or *Nic*-dependent signaling pathways have not been well characterized. We here identified a tobacco gene encoding a novel protein *JEI1* (*Jasmonate Early Inducible 1*) containing a BTB domain and a WD40-like repeat. The *JEI1* expression was regulated by the *NIC* loci. In the leaf, application of jasmonate or wounding rapidly and transiently induced the *JEI1* expression, with an induction pattern distinct from a wound-inducible protein kinase *WIPK*. This tobacco gene might be involved in wound-elicited signaling pathways.

Key words: BTB domain, jasmonate, nicotine, tobacco, wounding.

Nicotine is a major alkaloid accumulating in most commercial varieties of tobacco (*Nicotiana tabacum*). In this species and its ancestor *N. sylvestris*, nicotine is synthesized exclusively in the root, and distributed to whole plant parts by transport through xylem, resulting in enriched accumulation in young tissues of aerial parts where this alkaloid acts as an insect repellent (Katoh et al. 2005). Leaf damage caused by insect herbivory or wounding rapidly increases synthesis of jasmonic acid, and after a 90-min delay, jasmonate levels in the root also increase (Zhang and Baldwin 1997). In the tobacco root, elevated levels of jasmonates enhance nicotine formation, apparently by transcriptional up-regulation of genes involved in nicotine biosynthesis (Shoji et al. 2000; Shoji et al. 2002). Genes encoding enzymes for nicotine formation, such as putrescine *N*-methyltransfease (PMT) and an oxidoreductase A622, are specifically expressed in the root and up-regulated after wounding or treatment with exogenous methyl jasmonate (MeJA).

Previous studies have shown that these and other genes involved in nicotine biosynthesis are positively regulated by two genetic loci (*NIC1* and *NIC2*) that appear to specifically control nicotine levels in tobacco (Legg 1984; Hibi et al. 1994; Reed and Jelesko 2004; Cane et al. 2005). Indeed, *PMT* and *A622* genes have been initially isolated based on their differential expression patterns in the roots of wild-type tobacco and the *nic1nic2* double mutant (Hibi et al. 1994). In this study, we extended our expression analysis to the MeJA-treated tobacco leaves to see whether any tobacco genes are under the control of the *NIC* regulatory loci in the leaf.

We compared gene expression profiles of MeJA-treated wild-type tobacco leaves and *nic1nic2* mutant leaves by using a fluorescent differential display technique. Eight-week-old tobacco plantlets were grown on an agar culture medium in sealed containers in which small cotton balls soaked with 0.5 ml of a 100 μM MeJA solution were placed beside the plantlets, as described (Shoji et al. 2000). Leaf samples were harvested after 0 h and 1 h, and total RNAs were prepared. Details of the display procedures will be reported elsewhere. By using 48 PCR primer combinations, we obtained a cDNA clone named *JEI1* (*Jasmonate Early Inducible 1*) whose transcript was down-regulated in the *nic* mutant leaf treated with MeJA for 1 h. A full length cDNA clone of *JEI1* was obtained by 5′RACE using a SMART RACE cDNA Amplification kit (Clontech, Mountain View, USA), and its nucleotide sequence (accession number AB300776) has been deposited in GenBank/DDBJ. *JEI1* is a 51 kDa-protein with 463 amino acid residues, and contains a BTB-POZ (Bric a brac, Tramtrack and Broad complex/Pox virus and Zinc finger) domain at the N

Abbreviations: BTB, Bric a brac, Tramtrack and Broad complex; JEI1, jasmonate early inducible 1; MeJA, methyljasmonate; PMT, putrescine *N*-methyltransferase; WIPK, wound-inducible protein kinase.

[a] Present address: Faculty of Bioenvironmental Science, Kyoto Gakuen University, Kyoto 621-8555

[b] These authors contributed equally.

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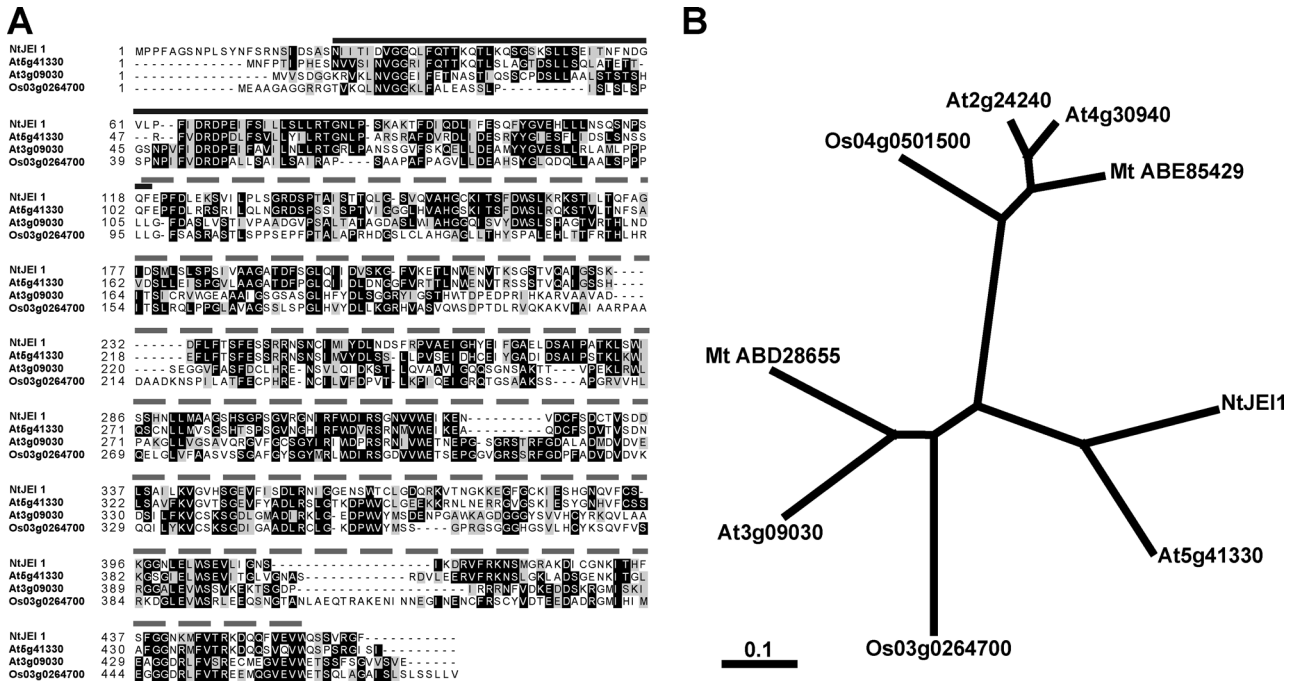

Figure 1. Comparison of the *JEI1* amino acid sequence with Class XIa BTB-domain proteins. (A) Identical residues are boxed in black, and conserved residues are shaded. The BTB domain and the WD40-like domain are shown above the sequences with a solid line and a dotted line, respectively. (B) Phylogenetic relationship of *JEI1* with Class XIa BTB-domain proteins. At; *Arabidopsis thaliana*, Os; *Oryza sativa*, and Mt; *Medicago truncatula*.

terminus and a WD40-like domain at the C-terminus (Figure 1A). The best characterized function of the BTB domain proteins is that some BTB domain proteins interact with Cullin3 to form ubiquitin protein E3 ligases (Petroski and Deshaies 2005). Arabidopsis contains at least 76 BTB domain proteins belonging to 11 major families. *JEI1* belongs to the Class XIa, which possesses a WD40-like domain downstream of the BTB domain (Dieterle et al. 2005). The BTB domain in this subfamily is also related to the potassium channel tetramerization domain, and functions of the Class XIa BTB proteins have not been characterized. Homology search showed that *JEI1* is most closely related to At5g41330, among the four Arabidopsis XIa-class BTB domain proteins (Figure 1B).

Genomic DNA blot analysis was done as described (Hashimoto et al. 1998), and showed that a *JEI1* cDNA probe hybridized one or two genomic DNA fragments that had been digested with either *Bam*HI, *Eco*RI, or *Xba*I (Figure 2). Two *Eco*RI fragments of the allotetraploid *N. tabacum* were detected in either of the two presumed progenitor species, *N. sylvestris* and *N. tomentosiformis*. These results indicated that *JEI1* is a single gene in the diploid tobacco species, and that the two *JEI* genes originating from the progenitors are still retained in the current tobacco genome.

Induction of *JEI1* by MeJA was analyzed in wild type and *nic1nic2*, by RNA gel blot (Figure 3A, D) and by RT-PCR (Figure 3B, C) as described (Shoji et al. 2000; Shoji et al. 2002), which gave similar results. For the RT-PCR experiments, we used the following primers; *JEI1* (5′-GTGGGAGATAAAGGAAAATG-3′ and 5′-GAACGAAATGAAGTGAAAGG-3′), *S-Adenosylmethinine Synthase* (*SAMS*; 5′-

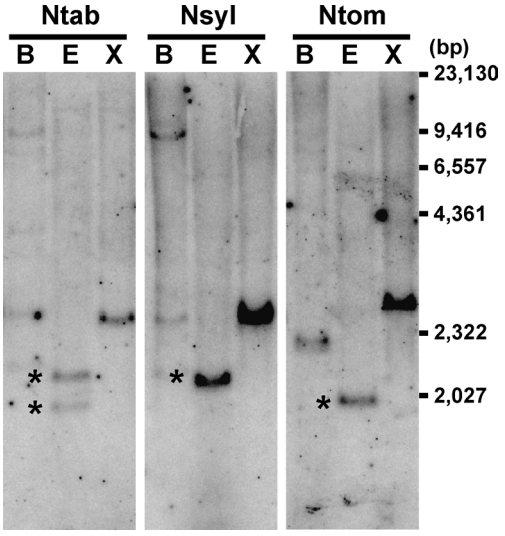

Figure 2. Genomic DNA blot analysis. Genomic DNA samples from *N. tabacum* (Ntab), *N. sylvestris* (Nsyl), and *N. tomentosiformis* (Ntom) were digested with *Bam*HI (B), *Eco*RI (E), or *Xba*I (X), subjected to hybridization using a radio-labeled *JEI1* cDNA probe, and washed under high stringency conditions. The positions of molecular weight standards are shown on the right. Asterisks show informative genomic fragments (see the text).

CCAGATTGCCCAGGACTTGA-3′ and 5′-GAGAAGTCGGGGTCATCACG-3′), and *18S rRNA* (5′-CCAGGTCCAGACATAGTAAGG-3′ and 5′-GATGACTCGCGCTTACTAGG-3′). Two *nic1nic2* double mutant lines with different genetic backgrounds were used to confirm the *Nic* dependency. In healthy wild-type leaves of Burley 21 (BL21) and NC95 backgrounds, *JEI1* transcripts were very low but rapidly accumulated within 1 h after MeJA treatment. In *nic1nic2* mutant leaves of the two genetic backgrounds, *JEI1* transcripts were hardly detectable at 1 h after the treatment (Figure 3A–C). In the roots of both wild type and the mutant, *JEI1* transcripts were detectable before the MeJA treatment and increased within 1 h after the treatment (Figure 3D). The time course of the induction appeared to be slightly slower in *nic1nic2* roots than in wild-type roots. Expression of *PMT* was clearly dependent of the functional *NIC* genes in the root, as reported previously (Hibi et al. 1994). These results suggest that *NIC* genes are required for rapid induction of *JEI1* transcript accumulation by MeJA in the leaf, and that this *NIC* requirement is largely compensated for by other factors in the root.

We next examined the time courses of *JEI1* induction by RT-PCR in the wild-type tobacco leaf of the Burley 21 background in response to MeJA or mechanical damages, and compared the induction patterns with those of other MeJA- and wound-inducible genes (Figure 4). Mechanical damages to the tobacco leaf were done as described (Seo et al. 2003), and the PCR primers for *Wound-Inducible Protein Kinase WIPK* (5′-ATCCTCGCCAGCAGTTAGCA-3′ and 5′-GGTCCGAGCAAGAAAATCA-3′), *Proteinase-*

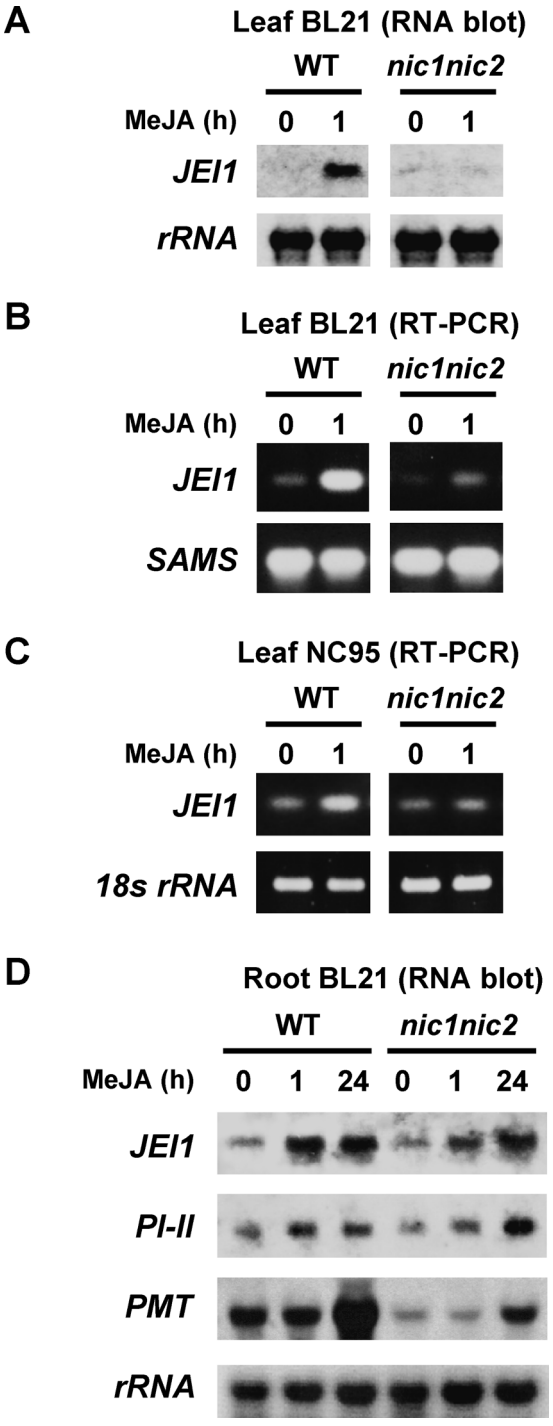

Figure 3. Induction of *JEI1* by MeJA in wild-type tobacco and *nic1nic2* mutants. After two-month-old tobacco plants were treated with MeJA vapor for the indicated periods, *JEI1* expression was analyzed in the leaf (A–C) and in the root (D) by RNA gel blot analysis (A, D) or by RT-PCR analysis (B, C). Tobacco plants of the Burley 21 variety (BL21) were used in A, B, and D, whereas tobacco plants of the NC95 variety were used in C.

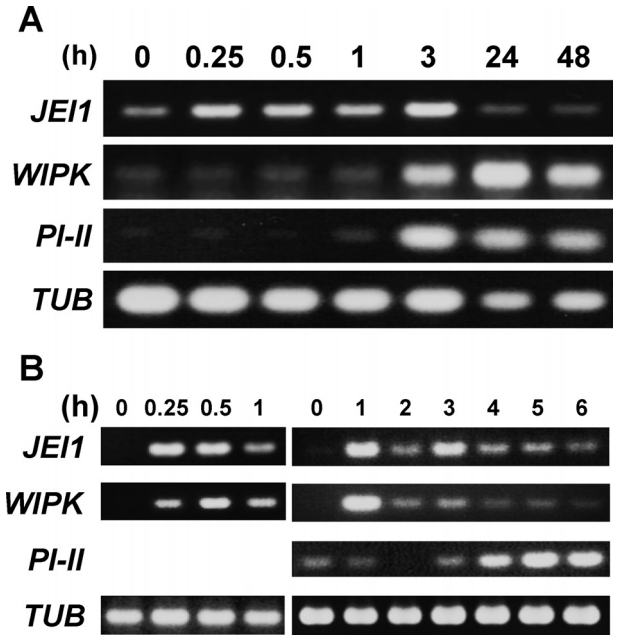

Figure 4. Time-courses of *JEI1* induction after MeJA treatment and wounding. One-month-old wild-type tobacco plantlets were treated with MeJA vapor (A) or mechanically wounded (B) for the indicated periods, and abundance of *JEI1*, *WIPK*, *PI-II* and tubulin (TUB) transcripts was analyzed by RT-PCR. In (B), target cDNAs were amplified by PCR for 23 cycles on the left column (0–1 h) and for 25 cycles on the right column (0–6 h).

Inhibitor-II (*PI-II*; 5′-AGTTAGTTTCGTCGCTCATC-3′ and 5′-AAACGGGCAACTTATGGTAG-3′) and *α-Tubulin* (*TUB*; 5′-AGTTGGAGGAGGTGATGATG-3′ and 5′-TATGTGGGTCGCTCAATGTC-3′) were used. In the tobacco leaf treated with MeJA vapor, the transcript level of *JEI1* showed a bi-phase pattern; it clearly increased within 15 min, thereafter gradually decreased, peaked again at around 3 h, and returned to the basal level before 24 h (Figure 4A). *WIPK* and *PI-II* transcripts were low immediately after the MeJA application, and were later induced at the period between 1 h and 3 h after the treatment. When the tobacco leaf was wounded by mechanical damages, the *JEI1* trascripts changed in a pattern similar to the MeJA treatment; the transcripts rapidly accumulated within 15 min, then gradually decreased, and peaked again at 3 h (Figure 4B). In the wounded leaf, the *WIPK* transcript level also rapidly increased within 15 min, peaked at 30 min, and decreased thereafter, whereas the *PI-II* transcripts started to increase after 3 h. These results demonstrate that *JEI1* is an early inducible gene that responds to MeJA and wounding, and that the transcriptional induction patterns of *JEI1* are distinct from those of *WIPK*, a well-characterized wound-inducible mitogen-activated protein kinase (Seo et al. 1999; Kumar and Klessig 2000). Wounding rapidly and transiently activates WIPK, which subsequently caused an increase in jasmonate synthesis. Future studies should address whether the transcriptional induction of *JEI1* requires the activity of WIPK, and whether *JEI1* is required for the wound- and jasmonate-induced expression of genes involved in nicotine biosynthesis or general defense responses.

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