Genes Encoding Nitrilase-like Proteins from Tobacco

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Abstract

Nitrilase (nitrile aminohydrolase, EC 3.5.5.1) catalyzes the hydrolysis of indole-3-acetonitrile (IAN) to indole-3-acetic acid (IAA). Arabidopsis thaliana genome has four nitrilase genes (NIT1, NIT2, NIT3 and NIT4). Three (NIT1, NIT2 and NIT3) of the four genes have high similarity. We have cloned two NIT4 homologs (TNIT4A and TNIT4B) from tobacco (Nicotiana tabacum). Genomic Southern hybridization, among other experiments, strongly suggests that tobacco has NIT4 homologs but not NIT1 to NIT3 homologs. Introduction of Arabidopsis NIT2 into tobacco conferred IAN-mediated growth inhibition, probably due to hydrolysis of IAN to IAA, while ectopic expression of TNIT4A had little effect on the sensitivity of transgenic plants to IAN. Nitrilase activity of TNIT4 proteins is discussed.

Key words: Nicotiana tabacum; nitrilase; indole-3-acetic acid; indole-3-acetonitrile; transgenic plant

1. Introduction

Several pathways for biosynthesis of indole-3-acetic acid (IAA), one of the major phytohormones, have been discussed, but they have not been definitively characterized in any plant species. One of the key intermediates is presumed to be indole-3-acetoaldehyde and another intermediate is indole-3-acetonitrile (IAN), both of which are derived from tryptophan. Recent work, however, has shown that, in tryptophan biosynthetic mutants of Arabidopsis thaliana and maize, the bulk of IAA was not derived from tryptophan, but rather from anthranilate and IAN was expected to be the immediate precursor of IAA. Thus, these different pathways might function in the same plant in a stage- or organ-specific manner.

Nitrilase (nitrile aminohydrolase, EC 3.5.5.1) catalyzes the hydrolysis of IAN to IAA. Four nitrilase genes (NIT1, NIT2, NIT3 and NIT4) were cloned from A. thaliana. Three (NIT1, NIT2 and NIT3: named the NIT1 group) of them have high similarity and are less similar to NIT4. All of the NIT proteins expressed in bacterial cells have been shown to convert IAN to IAA in vitro.

While nitrilase activities have been detected in extracts from some plant families, including Brassicaceae, Poaceae and Musaceae, no nitrilase activity was detected in 20 species from 17 families including tomato in Solanaceae. Growth of A. thaliana (Brassicaceae) seedlings is inhibited by IAN as well as IAA probably due to conversion of IAN to IAA. On the other hand, tobacco (Nicotiana tabacum) in Solanaceae is resistant to IAN, while introduction of NIT2 or any one of the NIT1 group conferred growth inhibition by IAN suggesting that tobacco has no ability to convert IAN to IAA. However, we recently found two NIT4 homologs from tobacco, determined their DNA sequences and named TNIT4A and TNIT4B (Accession no. D63331 and D83078, respectively). TNIT4 genes are expressed at very low level in all tissues tested (cotyledon, mature leaf, stem, root and flower). In this study, we describe in detail cloning of TNIT4 cDNAs and clarify that tobacco has only Arabidopsis NIT4 homologs and not the NIT1 group. Ectopic expression of TNIT4A had no effect on the sensitivity of transgenic plants to IAN.

2. Materials and Methods

2.1. Plant materials and growth conditions

N. tabacum SR-1 and its transgenic plants, and A. thaliana Columbia ecotype were grown as described previously.

2.2. Isolation of genomic DNA and total RNA, and gel blot analyses

DNA was isolated from leaf-powder according to the benzil chloride method and treated with RNase A. To-
2.3. Polymerase chain reaction (PCR) amplification of NIT homologs

To amplify internal regions of NIT homologs of tobacco, we designed a primer set of 5’AAAGTACCAGC-TTCTGC(T/C)ATT3’ (nucleotide positions 280-300; nucleotide position 1 was designated as A of the first codon of NITIP) and 5’(T/G)GTGCAATA(G/T)AG-TGTATA(C/G)CC3’ (408–388). Reverse Transcription (RT)-PCR was carried out using GeneAmp Thermostable rTth Reverse Transcriptase RNA PCR Kit (Perkin Elmer/Cetus) and total RNA (250 ng) from tobacco as a template. The PCR conditions were 35 cycles of 95°C for 1 min and 50°C for 1 min. Based on the sequence of an amplified 130-bp cDNA fragment, both ends of the cDNA were amplified using the Marathon cDNA Amplification Kit (Clontech). Gene-specific primers of the first PCR and the nested PCR for the 3’ end were 5’CCTGAGGTGATCCCTTGGCAGCAAT3’ (313–338) and 5’GTGTACTTAGTGATGGGTGTTATTGAC3’. The full length cDNA was cloned into pUC19.13 Sequencing revealed the presence of two very similar but clearly different clones. To confirm the presence of two very similar but clearly different clones.

2.4. Production of transgenic tobacco

The full length TNIT4A cDNA were cloned into pBI2113 to replace the β-glucuronidase gene. As a result, TNIT4A is under the control of cauliflower mosaic virus 35S promoter (CaMV-P35S). The resultant plasmid was designated pBI-TNIT4A. Transformation of N. tabacum SR-1 by Agrobacterium tumefaciens LBA4404 carrying pBI-TNIT4A and selection of transgenic tobacco were performed as described.10 Transgenic tobacco plants of NIT2 (pBI-NIT2) and NIT4 (pBI-NIT4) were previously described.10 In this study, we used transgenic tobacco plants, in which the transgenes are homozygous.

2.5. Immunodetection of nitrilases

NIT2, NIT4 and TNIT4 were recloned into the Nde I/BamHI site of pET16b (Novagen) and introduced to Escherichia coli BL21(DE3). NIT proteins with His-tag were overproduced in the presence of 1 mM IPTG at 37°C for 3 hr, and purified under denaturing conditions through the His-Bind metal chelation resin (Novagen) as recommended by the supplier. Rabbit anti-NIT2 antibody was generated by 4 times-inoculation every 2 weeks.

Frozen powder (100 μg) of leaves was solubilized in 1 ml lysis buffer and SDS-PAGE was carried out as described.15 Judging from protein bands (for example, the large subunit of ribulose-1,5-bisphosphate carboxylase) stained with Coomassie Brilliant Blue R-250, almost same amounts of total protein (10–30 μl) were loaded on the 12.5% polyacrylamide gel. After electrohoresis, NIT proteins were electro-blotted to an Immobilon PVDF membrane (Millipore) in 25 mM Tris-base, 192 mM glycine and 15% methanol. The immunodetection of NIT proteins were performed with anti-NIT2 antibody and ECF Western Blotting Regent Pack (Amersham Pharmacia Biotech). Reactions were carried out as recommended by the supplier. The intensity of fluorescence was determined with a FluorImager SI (Molecular Dynamics).

3. Results and Discussion

3.1. Cloning and sequencing of NIT homologs from tobacco

We obtained two different but similar nucleotide sequences from the cloned 5’ end. To confirm the presence of different kinds of cDNA for the nitrilase-homolog, two RT-PCR primers containing the putative initiation codon and the 3’ end were designed (see Materials and Methods). Thus, about a 1.3-kb cDNA fragment was produced by RT-PCR. Sequence analysis of these full-length cDNA revealed again two different but similar sequences. Since these most closely resemble NIT4 among A. thaliana nitrilase genes as mentioned above, we designated these cDNA TNIT4A (349 amino acid residues) and TNIT4B (348 amino acid residues). These cDNA sequences have 94.9% homology in their nucleotide sequences and 98.3% identity in their amino acid sequences. Since N. tabacum that we used is regarded to be amphidiploid of N. sylvestris × N. otophora or N. tomentosiformis,16 these sequences were derived from different ancestors. Figure 1 shows the alignment of putative amino acid sequences of nitrilases and nitrilase-like proteins from A. thaliana and tobacco, respectively. Although both N-terminal and C-terminal regions are variable, 188 residues (55% of NIT2) are identical among these six proteins. In particular, TNIT4A and TNIT4B resemble NIT4 (78% amino acid identity). A cysteine residue corresponding to Cys-186 of NIT1 is essential for enzymatic activity of Alcaligenes faecalis nitrilase.17 Gly-228 and Gly-277 are also required for NIT1 function.18 These amino acid residues are con-
Figure 1. Putative amino acid sequence alignment of nitrilases and nitrilase-like proteins. Identical amino acid residues to those of TNIT proteins are shown as white letters on a black background. Asterisks denote residues essential for enzymatic activity of nitrilases. 17

Nitrilases from *A. thaliana* are shown in NIT1 to NIT4.

Phylogenetic analyses were carried out using 11 amino acid sequences including bacterial nitrilases, a yeast nitrilase-like protein and the cyanide hydratase (Fig. 2). According to this tree, the *Arabidopsis* NIT1 group and *Arabidopsis* NIT4 are divided into different subgroups and tobacco nitrilase-like proteins belong to the same group as NIT4, although all NIT proteins from higher plants are included in the same group. Among prokaryotic nitrilases, *A. faecalis* nitrilase is highly specific for arylacetonitriles, such as IAN and benzyl cyanide, and cannot hydrolyze compounds with a cyano group directly bound to an aromatic or heterocyclic ring such as benzonitrile, which are substrates of other bacterial nitrilases. 17 These NIT4-like proteins might be widely distributed in higher plants, since a nitrilase-like protein found in the rice genome (Accession no. D15299) is a member of the NIT4 subfamily.

3.2. Genomic southern analysis of TNIT genes

*A. thaliana* has four NIT genes. To search for other genes of the NIT family in tobacco, we performed Southern blotting analysis of genomic DNAs from *N. tabacum*, *A. thaliana* and *Brassica campestris*, the latter belonging...
to the same family, Brassicaceae, as *A. thaliana*. These genomic DNAs were digested with *EcoRI* or *HindIII*, hybridized to *TNIT4A*, *NIT1* or *NIT4* cDNAs under the low stringent condition as shown in Figs. 4A, B or C, respectively. When *TNIT4A* was used as a probe (Fig. 4A), a major single *EcoRI* band (about 11-kb) and three *HindIII* bands (2.9-kb, 1.9-kb and 1.2-kb) were observed in the *N. tabacum* DNA digest. Also in *A. thaliana* DNA, weak but major bands (about 12 kb and 3.8 kb in the *EcoRI* digestion and the *HindIII* digestion, respectively) with faint bands (15 kb and 4.5 kb in the *EcoRI* digestion and 12 kb in the *HindIII* digestion) were detected.

After removal of hybridized *TNIT4A* DNA, the same membrane was rehybridized to labeled *NIT1* cDNA (Fig. 4B). In *A. thaliana*, DNA bands (15 kb and 4.5 kb in the *EcoRI* digestion and 12 kb in the *HindIII* digestion) hybridized faintly to the *TNIT4A* probe, hybridized strongly to *NIT1*, while previously weak but major bands (12 kb in the *EcoRI* digestion and 3.8 kb in the *HindIII* digestion) became weak. According to the restriction enzyme map of *NIT* genes, the DNA bands that hybridized strongly to *NIT1* contain *NIT1*, *NIT2* and *NIT3* (Fig. 3B), and the 12-kb *EcoRI* fragment and the 3.8-kb *HindIII* fragment contain *NIT4*. On the other hand, no notable bands were detected in tobacco DNA. Only a very faint 11-kb *EcoRI* band, which is the same one that hybridized to *TNIT4A*, was detected. Finally, the same membrane was rehybridized to *NIT4* cDNA. The same band patterns as seen when *TNIT4* was used as the probe were observed although the signals were strengthened in both *A. thaliana* and *B. campestris*. These results suggest that no gene similar to *NIT1* exists in the tobacco genome. Regardless of the kind of probes, the band patterns in *B. campestris* were very similar to those in *A. thaliana* although their molecular sizes differed. The composition of the *NIT* genes family and their arrangement on the chromosome might be similar to each other.

No DNA fragments resembling *NIT1* to *NIT3* could be cloned in PCR using mixed primers. Only *TNIT4* genes were isolated from about 400,000 clones of genomic DNA library (unpublished observation). Taken together with DNA blot analyses, we concluded that the tobacco genome has no homolog of the *NIT1* group.

### 3.3. Effect of overexpression of *TNIT4* on *IAN* sensitivity of tobacco

The amino acid sequences of *TNIT4* have more than 65% identity to those of four *A. thaliana* nitrilases, all of which can hydrolyze IAN to *IAA* in vitro when expressed in *E. coli*. In fact, growth of *A. thaliana* seedlings is inhibited by IAN. Tobacco is, however, resistant to IAN. Therefore, we introduced CaMV P35S-directed *TNIT4* into tobacco as well as *NIT2*. Figure 4A shows Southern blotting analysis of *HindIII*-digested genomic DNA to determine the copy numbers of the *TNIT4A* transgene. One clone (pBI-TNIT4A-3) has one extra DNA band in addition to three bands common to the parental strain. This suggests that the clone acquired one copy of the transgene, since *HindIII* cleaves a unique site between the *ntpl* gene and CaMV-P35S in the transgene. Similarly, it was confirmed that an *NIT2* transgenic tobacco (pBI-NIT2-22) acquired one copy of the transgene (data not shown). The mRNA levels of the transgenes were determined by Northern blotting. *TNIT4A* transgenic tobacco had a higher transcript level of the transgene than that of endogenous *TNIT4* in the wild-type tobacco (Fig. 4B). To obtain evidence for overproduction of *NIT* gene products, we used the antibody raised against *TNIT4* on IAN sensitivity of tobacco.

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Figure 3. Southern hybridization of genomic DNAs from A. thaliana, B. campestris and N. tabacum. Total DNAs from A. thaliana, B. campestris and N. tabacum (5 μg, 7 μg and 15 μg, respectively) were digested with EcoRI (lane E) and HindIII (lane H), and were separated by agarose gel electrophoresis. Plasmid DNAs containing NIT1 cDNA (lane A) and TNIT4A cDNA (lane T) were used as positive controls. DNA probes used were TNIT4A (A), NIT1 (B) and NIT4 (C) cDNAs. The size markers are given at the left.

Figure 4. Copy numbers and transcript levels of transgenes in transgenic tobacco. (A) Southern blot analysis of the TNIT4 gene. Twenty micrograms of genomic DNA from wild-type and TNIT4A transgenic tobacco was digested with HindIII and hybridized with 32P-labeled 1.3-kb TNIT4A cDNA (full-length). Closed arrowheads and an open arrowhead indicate endogenous TNIT4A and TNIT4B genes, and exogenous TNIT4A gene, respectively. (B) Northern blot analysis of TNIT4A mRNA and actin mRNA level. One hundred nanograms of poly(A)+ RNA from wild-type and TNIT4A transgenic tobacco leaves were hybridized with 32P-labeled 1.3-kb TNIT4A antisense RNA (full-length) or 0.5-kb actin antisense RNA. (C) Western blot analysis of purified NIT proteins and total proteins from the wild-type and transgenic tobacco. Total proteins from leaves (approximately 50 μg) and 20 ng each of purified NIT proteins were separated by SDS-PAGE and detected with anti-NIT2 antibody.

of the NIT2 transgenic tobacco was not feasible.

We tested the IAN sensitivity of seedling from these transgenic tobacco plants (Fig. 5). Elongation of both roots and hypocotyls was clearly inhibited by 20 μM IAN in NIT2 transgenic tobacco (pBI-NIT2-22) as shown in previous experiments.9,10 Similar findings were observed in three other NIT2 transgenic tobacco lines containing one copy of the transgene. On the other hand, TNIT4A transgenic tobacco (pBI-TNIT4A-3) was not inhibited even by 200 μM IAN, nor was the wild-type tobacco.
Another TNIT4A transgenic tobacco line containing two copies of the transgene was also not sensitive to IAN (data not shown). One possible explanation is that the proteins encoded by TNIT4A, and probably TNIT4B, have little or no ability to convert IAN to IAA. Arabidopsis NIT4 transgenic tobacco lines were also resistant to IAN, as was TNIT4A transgenic tobacco.\(^7\) However, it has been reported that the NT4 protein has an activity converting IAN to IAA.\(^8\) An alternative explanation for IAN-resistance of NIT4 and TNIT4A transgenic tobacco is that the enzymatic activities of these proteins are somehow masked. It was recently reported that TNIT4 protein interacts with ethylene-responsive element-binding proteins (EREBPs), and a model that the TNIT4 protein functions as a signal amplifier of defense gene expression via IAA and/or ethylene was presented.\(^9\) TNIT4 proteins might be inactivated by binding of EREBPs. If the TNIT4 protein has other substrate specificity than IAA, TNIT4 might play other roles in defense gene expression; producing a signal molecule other than IAA, participating more directly in regulation of defense gene expression through complex formation with EREBPs and so on. Purification of the TNIT4 protein should be performed to further clarify its function and its precise protein level in plant tissues.

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