Genomic structure of the *NtPDR1* gene, harboring the two miniature inverted-repeat transposable elements, *NtToya1* and *NtStowaway101*

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(Received 1 July 2003, accepted 17 November 2003)

Here we report the genomic structure including the promoter sequence and coding region of *NtPDR1* (*Nicotiana tabacum* Pleiotropic Drug Resistance 1), which is an elicitor-responsive gene encoding an ATP binding cassette (ABC) transporter that might be involved in the defense response in tobacco, as we reported recently. The *NtPDR1* gene consists of 20 exons and 19 introns. Among the introns, the first and fifth are much larger than the others and harbor typical miniature inverted-repeat transposable elements (MITEs). One of the MITE elements in the first intron, termed *NtToya1*, belongs to the *Toya* family that was recently described in rice, while the other element in the fifth intron, termed *NtStowaway101*, shows high homology with the *Stowaway* elements of the *IS630-Tc1-mariner* family. Many of the genes we found to harbor *Toya* and *Stowaway* elements in *Nicotiana* species by BLAST search are also involved in stress responses or plant–pathogen interactions. The existence of putative *cis*-elements (a GCC box, three W boxes, and several JA-responsive elements) in the promoter region supports our previous finding that this gene is strongly inducible by elicitation and methyljasmonate, and that this ABC transporter might be essential for plant defense responses. Furthermore, Southern blot analysis and PCR amplification of the introns harboring the MITE-like elements from genomic DNA of three *Nicotiana* species suggests that *NtPDR1* originated from *N. sylvestris*.

Key words: ABC transporter, genomic structure, MITE, *Nicotiana tabacum*, *NtPDR1*

INTRODUCTION

ATP binding cassette (ABC) transporters form an ubiquitous superfamily and are well characterized in human and bacteria because of their potential to confer drug resistance and hence their importance in the medical treatment of cancer cells and infections. ABC transporters consist usually of four domains: two transmembrane domains (TMDs) and two nucleotide binding folds (NBFs) containing the ABC with the Walker A and B motifs as well as the ABC signature motif C (Higgins, 1992). These transporters are grouped into subfamilies based on the row of their domains. The multidrug-resistance proteins and multidrug-resistance-related protein subfam-

ilies show a [TMD-NBF]₂ structure, while the [NBF-TMD]₂ structure can be found in the pleiotropic drug resistance (PDR) subfamily (Theodoulou, 2000; Martinoia et al., 2002). Recently we described the cDNA of an ABC transporter from *Nicotiana tabacum*, *NtPDR1*, which is inducible by microbial elicitors and methyljasmonate (MeJA) (Sasabe et al., 2002). *NtPDR1* belongs to the PDR subfamily, and is most probably located in the plasma membrane (Sasabe et al., 2002). Three members of the PDR subfamily, *NpABC1* from *Nicotiana plumbaginifolia*, *SptUR2* from *Spirodela polyrrhiza*, and *AtPDR12* from *Arabidopsis thaliana* (accession nos. AJ404328, Z70524, and BK001011, respectively), are reported to be inducible by the antifungal diterpene sclareol, and have high homologies with the *NtPDR1* gene (Jasinski et al., 2001; van den Brûle and Smart, 2002; van den Brûle et al., 2002). *NpABC1* is also capable of transporting an analog of sclareol (Jasinski et al., 2001), while *OsPDR3* from *Oryza sativa* L. (accession no. BK001017)

Edited by Eiichi Ohtsubo

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is inducible by pathogen inoculation and elicitation (van den Brûle and Smart, 2002), analogous to several other ABC transporters being expressed during plant-pathogen interactions (Xiong et al., 2001; Sasabe et al., 2002). This suggests that plant PDR-type ABC transporters play an important role in the transportation of antibiotic secondary metabolites involved in plant defenses. On the other hand, some ABC transporters from plant pathogenic fungi have been reported to confer resistance to plant phytoalexins (Fleissner et al., 2002). Thus, ABC transporters appear to play an important role in the struggle for existence of both plants and pathogens.

We found miniature inverted-repeat transposable elements (MITEs) in the introns of the *NtPDR1* gene. Transposable elements could be classified into two groups, each having a unique mechanism for transposition: class I elements include retro-type elements that replicatively transpose via an RNA intermediate, while class II elements conservatively transpose through a cut-and-paste mechanism by recognition of their terminal-inverted repeats by the transposase. MITEs are small (60–700 bp) and have usually no open reading frame (Bureau and Wessler, 1992, 1994a, 1994b; Mao et al., 2000; Zhang et al., 2000). They are nonautonomous class II elements that depend on transacting transposases for recognition of their terminal-inverted repeats (Zhang et al., 2001; Turcotte and Bureau, 2002; Jiang et al., 2003; Kikuchi et al., 2003; Nakazaki et al., 2003).

Here we report the genomic structure of *NtPDR1* and show that this ABC transporter originated from *N. sylvestris*, which is one of the progenitors of *N. tabacum*. We also describe the identification of two MITEs (*NtToya1* and *NtStowaway101*) in the introns, and discuss the promoter sequence in terms of the observed induction of *NtPDR1* by elicitor and MeJA.

MATERIALS AND METHODS

Plant materials and DNA isolation. *N. tabacum* cv. Bright Yellow-2 (BY-2), *N. sylvestris*, and *N. tomentosiformis* were grown in a greenhouse, and tobacco suspension-cultured BY-2 cells were maintained as described previously (Sasabe et al., 2002). DNA was extracted from their foliage and from cultured cells by the hexadecyltrimethylammonium bromide method described by Murray and Thompson (1980).

Construction of genomic DNA library and isolation of *NtPDR1* gene. Genomic DNA from *N. tabacum* BY-2 cells was partially digested with *Sau3AI* and ligated into the Lambda Fix II vector, and a genomic DNA library was constructed with a Gigapack III Gold cloning kit (Stratagene, La Jolla, USA) following the manufacturer's instructions. The library was plated on *E. coli* XL1-Blue MRA(P2) and screened with probe I, which binds to the

5'-untranslated region (UTR) and the first exon of the *NtPDR1* gene (Fig. 1A). Hybridization was carried out as described below.

Polymerase chain reaction amplifications. All PCRs were carried out with the *PyroBest* DNA polymerase (TaKaRa, Kyoto, Japan). To obtain the complete sequence of the *NtPDR1* gene of *N. tabacum*, the 3'-terminal of *NtPDR1* was amplified using three primer pairs designed from the sequence of *NtPDR1* cDNA using a genomic DNA as the template. The following primer pairs were used to amplify the three regions of genomic DNA of *N. tabacum*: G1, 5'-TAGGAGCAAGAGCCAA-GATC-3' and 5'-GATGATACCATAAAACACAG-3'; G2, 5'-CTGTGTTTTATGGTATCATC-3' and 5'-GTTCCACAGTT.TCATTATCA-3'; and G3, 5'-TGATAATGAAACTGTG-GAAC-3' and 5'-TAACCAATGCTTCCTCAAAG-3'. Using genomic DNAs from *N. tabacum*, *N. sylvestris*, and *N. tomentosiformis* as templates, PCRs were carried out with the primer pair 5'-CTGATGAAGATAATGAGAAG-3' and 5'-CCCACATATGCATCTGCATC-3' for amplification of the first intron. To specifically amplify the PCR product of the fifth intron, we performed a nested PCR experiment with the outer primer pair 5'-GTCAAGAA-GAGAAAAAGC-3' and 5'-AAGAACATAATCTGTAAC-3' and the inner primer pair 5'-CCAGATGCTGATATTGAC-3' and 5'-AACATTGGCTTCTTGTC-3'.

Southern hybridization. Approximately 10 µg of genomic DNA was digested with appropriate restriction enzymes and separated on a 0.7% agarose gel in 1× TAE buffer. DNA was transferred to a Hybond N+ membrane in 10× SSC buffer, and hybridization was carried out at 42°C overnight in 5× SSC, 50% formamide, 0.02% SDS, 0.1% lauroylsarcosine, and 2% blocking reagent (Roche Diagnostics, Mannheim, Germany). The membranes were then washed at 68°C in 0.1% SDS and 0.1× SSC for 15 min twice. For the detection of *NtToya1* and *NtStowaway101* elements, the hybridization and washing were carried out at 37°C and 50°C, respectively. All probes used in the library screening (probe I) and Southern blot hybridization (probes II to IV) were labelled using PCR with the DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Probe I was generated by PCR with *NtPDR1* cDNA as the template and primers 5'-CAGAGAGAGAACTACATCTCTC-3' and 5'-CATCTCTAGATGAACGTGAG-3' (Sasabe et al., 2002). Probes II to IV were generated by PCR from a genomic subclone of *NtPDR1*. Primer pairs 5'-CTAAAGGGGAG-CACCAGACAC-3' and 5'-AAGGAAATTGCTCCCCAGGG-3' (including terminal inverted repeat) are for probe II, 5'-CTCCCCTCCGTTCCA-3' (binding in both terminal-inverted repeats) is for probe III, and 5'-CCTGGGGTAG-CAAAAATC-3' and 5'-GTACCAAGATCCCAGAAC-3' are for probe IV.

Fig. 1. Gene structure of *NtPDR1*. (A) Genomic DNA, cDNA, and protein structures. The exons in genomic DNA are indicated by hatched boxes, while the MITE-like elements (#1 and #3) and the repetitive sequence (#2) are shown by black boxes within the introns. The probes (I to IV) used in this study, and the PCR-amplified regions (G1, G2, and G3) are indicated by horizontal bars above the genomic structure. The mRNA refers to the *NtPDR1* cDNA (Sasabe et al., 2002). The deduced amino acid sequence is indicated by an open box. Nucleotide binding folds (NBFs) are indicated by black vertical bars, and transmembrane domains (TMDs) are indicated by gray vertical bars. (B) Schematic comparison of the coding regions of *NtPDR1*, *AtPDR12*, and *OsPDR3*. The introns are represented by vertical bars and numbered based on the *NtPDR1* gene. (C) A promoter sequence with putative *cis*-elements. All nucleotide positions refer to the putative transcription starting point, TSP (+1), which is determined by a conserved distance from a TATA-box-like sequence and the conserved adenosine in the TSP (+1) (Grec et al., 2003). The GCC box at position -74 to -84 is underlined and in boldface. The W boxes at positions -96 to -101, -1141 to -1146, and -1252 to -1257 are double underlined and in boldface. The hatched sequence from -341 to -355 resembles a sclareol box. The hatched and boldface sequence contains the putative five *cis*-elements closely related to various signal transduction pathways (see Fig. 6). The cytosine at position +31 is underlined as the 5'-terminal of the longest cDNA segment isolated. The putative TATA (at position -48 to -53) and putative CCAAT (at position -212 to -216) motifs, as well as the translation start codon ATG are indicated in boldface. The N-terminal part of the deduced amino acid sequence is shown below the nucleotide sequence.

DNA sequence and computer analysis. DNA sequencing was performed with a DNA sequencer (ABI PRISM TM310, PE Applied Biosystems, Foster City, CA). The phylogenetic trees of the MITEs were constructed by the neighbor-joining method (Saitou and Nei, 1987) and alignments were performed with the ClustalW program from the DDBJ. The BLAST protocol (Altschul et al., 1997) was used for homology searches of the DNA sequence.

RESULTS AND DISCUSSION

Gene structure of *NtPDR1*. A genomic clone obtained from the genomic library together with the PCR-amplified 3'-end of the *NtPDR1* provided information on the gene structure. The cloned fragment including the *NtPDR1* gene, which consists of 3.9-kb promoter and 8.0-kb transcribed regions, is 11835 bp long (accession no. AB075550). Based on the cDNA sequence and the GT/AG rule, we found that the *NtPDR1* gene consists of 20 exons and 19 introns, as indicated in Fig. 1A (Brown et al., 1996; Sasabe et al., 2002). All but the first and fifth introns show a relatively small conserved size of about 93 bp and an average A/T content of about 70%; the first and fifth are much larger than the others and harbor typical MITEs. The MITE-type element in the first intron, termed *NtToya1*, belongs to the *Toya* family that was recently described in rice, while the other element in the fifth intron, termed *NtStowaway101*, shows high homology with the *Stowaway* elements of the *IS630-Tc1-mariner* family. We compared the coding region structure of *NtPDR1* to two other closely related PDR-type ABC transporters, *AtPDR12* and *OsPDR3* (Fig. 1B), respectively which revealed that the intron positions among plant PDR-type ABC transporters are indeed highly conserved (van den Brle and Smart, 2002). However, at some positions we observed the absence of introns resulting in a different number of introns. The two corresponding third and eighteenth introns of *AtPDR12* were missing from *NtPDR1*. The two corresponding second and twelfth introns of *AtPDR12* were also missing from *OsPDR3*, but *OsPDR3* has obtained a novel one between seventh and eighth introns of *AtPDR12* (Fig. 1B). *AtPDR12* has 21 introns and *OsPDR3* has 20 introns. The existence of putative *cis*-elements (a GCC box, three W boxes, and a 30-bp segment concentrated *cis*-elements of several hormonal responses) in the promoter region supports our previous finding that this gene is strongly inducible by elicitation and MeJA. (Fig. 1C).

Two miniature inverted-repeat transposable elements exist in the first and fifth introns of *NtPDR1*. BLAST searches of the *NtPDR1* revealed the presence of MITE-like elements in the first and fifth introns. The *NtToya1* element in the first intron belongs

to the *Toya* family that was recently described in rice (Fig. 1A, #1). *NtToya1* is 174 bp long, harbors 10 bp of imperfect terminal-inverted repeats (5'-CAGAGGCGGA-3'), and 8 bp of imperfect target-site duplication (5'-TTGCTCCC-3') (Fig. 2A). The A/T content is high (71.3%), but the element is unlikely to form a hairpin-like structure. These characteristics are all similar to the *Toya* family of MITEs described in rice (Nagano et al., 2002). The *NtStowaway101* element in the fifth intron shows high homology with the *Stowaway* elements of the *IS630-Tc1-mariner* family (Fig. 1A, #3) (Bureau and Wessler, 1994b; Turcotte and Bureau, 2002). *NtStowaway101* is 248 bp long, harbors 11 bp of perfect terminal-inverted repeats (5'-CTCCCTCCGTT-3'), 2 bp of imperfect target-site duplication (5'-TA-3') (Fig. 2B). The *Stowaway* elements are thought to be associated with genes from monocotyledonous as well as dicotyledonous plants, have a short target-site duplication, high A/T content, high copy numbers in their genomes, and are able to form a stable secondary structure (Bureau and Wessler, 1994b). Therefore, we detected copy numbers of *Toya* elements and *Stowaway* elements in *Nicotiana* plants (Fig. 3A and B). *N. tabacum* and *N. sylvestris* genomes have not so high copy numbers of *Toya* elements, and the *N. tomentosiformis* genome does not seem to have the elements at all (Fig. 3A). On the other hand, the *N. tabacum* genome has high copy numbers of *Stowaway* elements, as well as the genomes from *N. sylvestris* and *N. tomentosiformis* (Fig. 3B). *NtStowaway101* was also inserted into another repetitive sequence (Fig. 1A, #2). The repetitive sequence divided by *NtStowaway101* element has been identified by BLAST search. Although it is reported that MITEs show also an insertion prefer-

- (A) cctagcgaaaaggaaa**TTGCTCCC****CAGGGGTGGAGCTAGCACAT**
ACATAGGGGAATGATTGAATCCAGTAATTTTAGTTCAAACCTTGT
ATTTGTCTTAAAAATTTATTAATATATACAAATTATTAATTTA
GAACCCAATAACTTATAACAAATTAATCTCGAACCTATAAGTT
TTAAATCCTAATCCGTTGCTG**GTGCTCCC**tttagaggattttt
- (B) tgctgtttttcttagtgtatattagtATAAAATTATGGGGCTT
 TTTTGTGTTTAGCCCGTGCTAGAACTATTTACATTCGGTAGC
 CGAGAAAGCGTATAAAATTT**CTCCCTCCGTTCCAATTTATGT**
GAATCTGTTTGA**CTGGGCACGGAGTTTAAGAAAAAATGAAGAC**
TTGGAATTTGTGGTCTTAACAAGTCCAAATGGGGCCAGAGTA
TTTGTGTGGTTATAAACCTTCTCATTAAATGGTAGAGTTGTAAC
TTTAAAGCTAAATTTGTTACCAATTTAGAAAGGGTTCAATTTT
TGGAACGGATCAAAAAGGAATAGGTTTCACATAAACTGGAACAG
AGGGAG**TA**TAATTTTGTATATAACATACAGAATGTATAAATAT
 ACAAAAAAGATATATATATTTTTCGGCTATTATTTTGAGAGC
 AGCTATACACGTCATTTTCTTAAattatttccctttt

Fig. 2. Sequence of the two MITEs. (A) *NtToya1* element in the first intron. (B) *NtStowaway101* element, and the splitted repetitive sequence in the fifth intron. The MITEs are indicated in boldface, and their terminal inverted repeats, TIR, are hatched. The repetitive sequence which is 190 bp long and has been splitted by the *NtStowaway101* element, is underlined. The imperfect target-site duplications, TSDs, are shown by open boxes.

Fig. 3. Southern blot analysis of MITEs. Genomic DNAs digested by *Bgl*II were hybridized with (A) probe II (the entire *NtToya1* element), and (B) probe III (the entire *NtStowaway101* element). Abbreviations are: tab, *N. tabacum*; syl, *N. sylvestris*; tom, *N. tomentosiformis*.

ence into other MITEs (Jiang and Wessler, 2001), the lack of confirmation of the target-site duplications or terminal-inverted repeats with the repetitive sequence makes it difficult to identify these nucleotide sequences as novel MITEs, (Fig. 1A, #2).

The origin of *NtPDR* genes. In addition to the previously reported *NtPDR1* cDNA (Sasabe et al., 2002), we obtained a different but highly similar ABC transporter cDNA, termed *NtPDR2*, from an elicitor-treated cDNA library. The partial *NtPDR2* cDNA (accession no. AB109389) is 3411 bp long and has 97.5% and 97.2%

homology with *NtPDR1* at the nucleotide and amino acid sequence level, respectively. *N. tabacum* is an amphidiploid plant, and *N. sylvestris* and *N. tomentosiformis* are its progenitors (Smith, 1974; Kenton et al., 1993). In order to assign the origin and copy number of *NtPDR1*, we performed Southern blot analysis with *Nde*I-digested genomic DNAs of three *Nicotiana* species, *N. tabacum*, *N. sylvestris*, and *N. tomentosiformis*, using exon 17 (Probe IV) of *NtPDR1* as a probe (Fig. 4A). Since the observed lower signal (3.3 kb) coincides with the restriction map of the *NtPDR1* gene, it is assumed that *NtPDR1* originated from *N. sylvestris*. Therefore, the upper signal (4.0 kb)

Fig. 4. Genetic derivation of *NtPDRs* among *Nicotiana* species. (A) Southern blot analysis to determine the genetic origin and copy number of *NtPDRs*. Genomic DNAs digested by *Nde*I were hybridized with probe IV (exon 17). (B) PCR analysis to determine the genetic derivation of *NtPDRs*. PCR amplification of the first intron and nested PCR amplification for the fifth intron were carried out with genomic DNAs from three *Nicotiana* species as templates: tab, *N. tabacum*; syl, *N. sylvestris*; tom, *N. tomentosiformis*.

is attributed to *NtPDR2*, assumed to originate from *N. tomentosiformis* (Fig. 4A) indicating that this gene is a single copy in *N. sylvestris* and *N. tomentosiformis*. Southern blots conducted with other restriction enzymes gave similar results (data not shown). We further confirmed the origin of *NtPDR1* by PCR amplification with the first and fifth introns of the three *Nicotiana* species. From the genomic nucleotide sequence of *NtPDR1*, we identified the 555-bp band for the first intron and 1321-bp band for the fifth intron corresponding to *NtPDR1* in the upper signals of *N. tabacum* and *N. sylvestris*. (The entire intronic sizes are 431 bp for the first intron and 1258 bp for fifth intron of *NtPDR1*.) From agarose gel

electrophoresis data, it is hypothesized that the approximate 200-bp band for the first intron and the approximate 900-bp band for the fifth intron correspond to *NtPDR2* in the lower signals of *N. tabacum* and *N. tomentosiformis*. The actual size of the lower bands for the fifth intron of *N. tabacum*, which was confirmed by cloning and sequencing of the PCR fragments, is 903 bp (sequence data not shown, Fig. 4B). (The entire intronic size is 840 bp for the fifth intron of *NtPDR2*.) It is assumed that the *NtPDR2* genes of *N. tabacum* and *N. tomentosiformis* do not have the MITE elements in the first and fifth introns. The sequences of the fifth introns of *NtPDR2* from *N. tabacum* and from *N. tomentosiformis*

Fig. 5. Phylogenetic trees of the MITEs. (A) *Toya*-like elements. (B) *Stowaway*-like elements. Respective numbers in (A) mean a series of *OsToya* elements. Family and species abbreviations: *Le*, *Lycopersicon esculentum*; *Ng*, *N. glutinosa*; *Np*, *N. plumbaginifolia*; *Ns*, *N. sylvestris*; *Nt*, *N. tabacum*; *Os*, *O. sativa*; *St*, *S. tuberosum*. The scale bars indicate 0.1 nucleotide substitutions per site.

were almost identical (99% homology). In *N. tabacum*, the sequence of the fifth intron of *NtPDR2* also shows high (80%) homology with the whole fifth intron of *NtPDR1*. The sizes of the fifth intron in *Nicotiana* species are larger than that in other ABC transporters, *AtPDR12*, of *Arabidopsis* *OsPDR3* and than the ABC transporter of *Lycopersicon esculentum* (accession no. BH016243).

Phylogenetic trees of miniature inverted-repeat transposable elements. We performed BLAST searches with *NtToya1* and *OsToya1* elements as template sequences to align *NtToya1* together with five other elements found in *Nicotiana* species, one element from potato, one from tomato, and twenty from rice. Eight out of twenty-eight elements belong to the *Solanaceae* family,

and a phylogenetic tree of these elements clearly shows that the elements from rice form a specific subgroup (Fig. 5A). *NtToya1* and the other elements have been grouped into the *Solanaceae* subgroup of *Toya* MITEs. This is the first description of *Toya* MITEs in dicotyledonous species. However, it is most intriguing that we were unable to detect *Toya* MITEs in *A. thaliana* on the databases. Moreover, *Toya* MITEs were scarcely detected in *N. tomentosiformis* genome by Southern blot (Fig. 3A). Although it may have been owing to their low homology with the *NtToya1* element that we do not have detected the *Toya* MITEs in *A. thaliana* in the databases, it seems that diversification of *Toya*-type MITEs in dicotyledonous species is now in progress. Although some of the *Stowaway* elements we found in *Nicotiana* species have not been described previously as MITEs, their char-

Table 1. Alignment of the identified *Toya* elements from the database.

element	TS and TIR**	associated gene	accession #	size
<i>NtToya1</i>	ttgctcccCAGGGGTGGA---TCCGTGTCTGgtgctccc	PDR1 ABC transporter (<i>NtPDR1</i>)	AB109388	174
<i>NtToya2</i>	aggggaatCAGGGGCGAA---TCCGCCTCTAagtggaaat	PSII oxygen evolving complex	X58910	159
<i>NtToya3</i>	atcattttcCAGGGGCGGA---TCCGCCTCCGatcatttt	thaumatin-like protein	AB029918	176
<i>NtToya4</i>	cttaaatCAAGGGGCGGA---TCCGC--TCTGcctatatc	peroxidase	D11396	175
<i>NsToya1</i>	atcagtgtCAGGGGCGGA---TCCGCCTCTGatcctgtgc	LHC a/b binding protein	AB012638	176
<i>NpToya1</i>	ttgttatcAGGGGCTAGA---TCCACATTTGgttgctgt	plasma membrane H ⁺ -ATPase	AF156684	175
<i>StToya1</i>	ttgaccagCAGGGTGGAA---TCTGCCTTTGttgaccaa	expressed sequence tag (EST)	BQ511215	177
<i>LeToya1</i>	cataatatTACGGGTAAA---TTCGCCTCTGctaaacat	polyphenol oxidase	Z12836	165
<i>OsToya1*</i>	catgtcacCAGTGGCGGA---TCCGCCACTGcatgtcac	PSII oxygen evolving protein	AP003203	176
<i>OsToya2*</i>	gatacttaCAGTGGTGGGA---TCCGCCATTGgatactta	unknown	AC084320	176
<i>OsToya3*</i>	gctcaagtAAGTGGCATA---TCCGCCACTGgcttaagt	DNA-directed RNA polymerase	AC084218	173
<i>OsToya4*</i>	gttaaaagCAGTGGCGGA---TCCGCCACTGgttaaaag	unknown	AC091734	175
<i>OsToya5</i>	attgttatTAGTGGCGGA---TCCGCCACTAattgttaa	unknown	AP003948	174
<i>Ostoya6</i>	cgtgtataAAGTGGCGGG---TCCGTAACCTgatgcatt	unknown	AL606651	166
<i>OsToya7</i>	catgtgtgCAGTGGCGGA---TCCGCCACTGcatgtgtg	unknown	AC093490	175
<i>OsToya8</i>	tgtatgtaCAGTGGTGGGA---TCCGCCACTTatgtatgtc	unknown	AL607000	177
<i>OsToya9</i>	gttctctcCAGTGGCGGA---TCCGCCACTGgttctctc	unknown	AL662997	175
<i>OsToya10</i>	ctgacctgCAGTGGCGGA---TCCGCCACTGctgacctt	unknown	AL606729	176
<i>OsToya11</i>	gtcaaatgCAGTGGCGGA---TCCGCCACTGataaaaaa	unknown	AC134241	183
<i>OsToya12</i>	gtgcacatCAGTGGTGGGA---TCCGCCACTGatgcacat	unknown	AP003315	175
<i>OsToya13</i>	attgcaagCAGTGGCAGA---TCCGCCACTAattggaaa	putative OsNAC protein	AC096687	173
<i>OsToya14</i>	attattgcCAGAGGCGGA---TCCACCACTTattaataa	unknown	AP006548	176
<i>OsToya15</i>	ctggggtgCA--TGCGCGGA---TCCGCCACCCgtgcatgt	unknown	AK108044	176
<i>OsToya16</i>	tcaccgcCAGTGGCGGA---TCCGCCACTCtcacctgc	unknown	AP003303	176
<i>OsToya17</i>	acctggggCAGTGGTGGGA---TCCGCTACTGcctaggg	unknown	AC118672	174
<i>OsToya18</i>	ggtgggCAGTGGCGGA---TCCGCCACTAggtgggct	unknown	AL935154	176
<i>OsToya19</i>	gtctccacCAGTGGCGGG---TTCGCCACTGgtctccac	unknown	AE017095	176
<i>OsToya20</i>	cctaccatCTGTGGCGAA---TCCGCCACTAcctacat	Cyt P450 Phenylurea dealkylase	AE017061	174

*These elements were previously reported (Nagano et. al., 2002).

**The target-site duplications, TSDs are indicated by small letters, while the terminal inverted repeats, TIRs are indicated by capitals.

Table 2. Alignment of the identified *Stowaway* elements from the database.

element	TSD and TIR**	associated gene	accession #	size
<i>NtStowaway1*</i>	t gCTCCCTCAGTT ----- AACGGAGGGAA t a	auxin-binding protein (<i>T85</i>)	X70902	225
<i>NtStowaway2*</i>	t aCTCCCTCCGTT ----- AACTGAGGGAA t a	class I chitinase B (<i>chn50</i>)	X64519	245
<i>NtStowaway3*</i>	t aCTCCATTTGTT ----- AACGGAGAGAG t a	nitrate reductase (<i>nia-2</i>)	X14059	249
<i>NtStowaway101</i>	t -CTCCCTCCGTT ----- AACAGAGGGAG t a	PDR ABC transporter (<i>NtPDR1</i>)	AB109388	248
<i>NtStowaway102</i>	t aCTCCCTTTATT ----- AACATAGGGAG t a	ACC syntase (<i>acs4</i>)	AJ131837	238
<i>NtStowaway103</i>	t aCTCCCTCCGTT ----- AATCGAGAGAG t a	lipid transferase (<i>ltp1</i>)	X62395	183
<i>NtStowaway104</i>	----- AACGATTAGTG t a	pathogen sensitivity marker gene	E16010	(217)
<i>NsStowaway101</i>	t aCTCCCTCTGTT ----- AACGGAGGGAG t a	RNA polymerase (<i>NsRpoT-B</i>)	AB058958	250
<i>NpStowaway1*</i>	t aCTCCCTC - CTT ----- GACAGCGAGAG t a	H ⁺ -ATPase (<i>pma1</i>)	M80489	247
<i>NgStowaway101</i>	t aCTCCATCCGTT ----- AACGGAGGGAA t a	virus resistance (<i>N-gene</i>)	U15605	248

*These elements were previously reported (Bureau and Wessler, 1994b).

**The target-site duplications, TSDs are indicated by small letters, while the terminal inverted repeats, TIRs are indicated by capitals.

acter and phylogenetic tree confirmed them as *Stowaway* elements (Fig. 5B). Many of the genes we found to harbor *Toya* and *Stowaway* elements in *Nicotiana* species by BLAST search are also involved in stress responses or plant-pathogen interactions (Table 1 and 2). These genes are *NtPDR1*, *thaumatin*-like protein and *peroxidase* in Table 1, and *chn50*, *NtPDR1*, pathogen sensitivity marker gene and *N-gene* in Table 2.

Putative *cis*-acting elements in the *NtPDR1* promoter. As reported previously, *NtPDR1* is inducible by various elicitors and MeJA, while SA and abscisic acid had no effect on gene expression (Sasabe et al., 2002). This observation could be explained by some of the putative *cis*-acting elements we found in the promoter region of *NtPDR1*. As indicated in Fig. 1C, we found a GCC box (5'-TAAGAGCCGCC-3') at position -74 to -84. The GCC box found in promoters of many basic *PR* genes from tobacco is an ethylene-responsive and also an elicitor-responsive element (Ohme-Takagi and Shinshi, 1995; Yamamoto et al., 1999). Both expression of *NtPDR1* and basic *PR* genes in tobacco are induced by MeJA but not by SA (Niki et al., 1998; Sasabe et al., 2002). Transcription factors binding to this *cis*-element are ethylene-responsive transcription factors belonging to the plant EREBP/AP2 family (Riechmann and Meyerowitz, 1998). Five tobacco EREBPs described previously

(Ohme-Takagi and Shinshi, 1995; Park et al., 2001) are putative candidates. We also found three W boxes (5'-TTGACY-3') at positions -96 to -101, -1141 to -1146, and -1252 to -1257. W boxes are targets for WRKY transcription factors, which are also described in *Nicotiana* (Eulgem et al., 2000). WRKY transcription factors belong to the immediate-early activated transcription factors that respond to wounding, elicitation, TMV infection, and SA treatment. The WRKY target sequences are also found in the promoter regions of some *PR* genes and in the WRKY promoter regions themselves (Eulgem et al., 2000). We found the sclareol box (5'-TTAAATACAG-TATTT-3') at position -341 to -355 (Grec et al., 2003), an element responsible for the induction of *NpABC1* by the diterpenes sclareol and sclareolide as well as MeJA.

One of the most interesting findings in the promoter sequence of *NtPDR1* is a 30-bp-long sequence at position -687 to -716 (Fig. 1C). This region contains five putative *cis*-elements closely related to various signal transduction pathways (Fig. 6). A sequence at position -687 to -706 is similar to *as-1*-like elements (Krawczyk et al., 2002). The *as-1* element is recognized by the plant bZIP (basic-region leucine zipper) TGA transcription factors, which are likely to be *ASF-1* (*as-1*-binding factor) and/or *SARP* (salicylic acid response protein) (Strompen et al., 1998; Niggeweg et al., 2000). The report of a synergistic effect of the GCC box and an *as-1* element in an artificial

-716	AGACCGTCAATGAGGTCATCAGTGACCCCA	-687	sequence from <i>NtPDR1</i>
-706	TGACGTCA nnnnn TGACGTCA	-687	<i>as-1</i> like-element
-702	GTC nnnn GTC A	-692	E17 / EIRE
-711	GTC nnnnnn GTC A	-699	E17 / EIRE
-712	CGTCAATGAA	-703	JASE1
-716	AGACCGCC	-709	JERE

Fig. 6. Alignment of a 30-bp-long sequence indicated in Fig. 1C, with interesting *cis*-element motifs. This sequence contains the putative five *cis*-elements closely related to various signal transduction pathways. Consensus nucleotide are indicated in boldface. Numbers are as in Fig. 1C.

promoter suggests that the expression of *NtPDR1* is regulated by interactions of a GCC-box-binding protein, an *as-1* binding protein, or other factors (Büttner et al., 1997). Sequences at positions -692 to -702 and -699 to -711 are similar to E17-like elements (Figs. 1C and 5) (Kirsch et al., 2001). This element is closely related to the W box, and consists of two inverted consensus sequences of the W box. Thus, the E17 sequence is thought to be very important for strong and rapid responsiveness to the elicitor (Kirsch et al., 2001). This element is very similar to the EIRE, described for the tobacco class I chitinase (Fukuda, 1997). A sequence at position -703 to -712 is the JASE1 (JA/senescence-responsive element 1), which is reported to be required for the upregulation of *OPR1* in *Arabidopsis* (He and Gan, 2001). A sequence at position -709 to -716 is the JERE (JA and elicitor-responsive element), which might contribute to the inducibility of the *NtPDR1* by elicitor or MeJA (Rushton et al., 2002; Sasabe et al., 2002). The JERE element shows striking similarities to the GCC box and is recognized by the ORCA (Octadecanoid-responsive *Catharanthus* AP) transcription factors described in *Catharanthus roseus*, which belong like the ERF transcription factors to the plant specific group of EREBP/AP2 transcription factors (van der Fits and Memelink, 2001), therefore being a possible target of both the ethylene and JA dependent signal transduction pathways. The *cis*-acting effects of the *NtPDR1* promoter will be investigated in the future.

The reported nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under accession numbers AB109388 for the *NtPDR1* gene and AB109389 for the *NtPDR2* cDNA.

This work was supported in part by Grants-in-Aid for Scientific Research on Priority Area (nos. 12052215 and 12052219) and for Scientific Research (no. 15108001) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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