

Phylogenetic Classification of Dof-type Transcription Factors in Pea (*Pisum sativum*)

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Received 2 October 2002; accepted 1 April 2003

Abstract

The Dof protein family is a group of plant transcription factors carrying highly conserved 52 residues referred to as the Dof domain, and belongs to the C4 zinc-finger transcription factors. We isolated various *PsDof* genes by PCR using the Dof-domain nucleotide sequence of the *PsDof1* gene from a cDNA library of elicitor-treated pea epicotyls, and these isolated *PsDof* genes were then classified phylogenetically. Since the obtained genes (*PsDof1* to *PsDof7*) were scattered over various positions of the phylogenetic tree, they were expected to perform various functions as Dof-type transcription factors. From their positions in the tree, it is expected that the *PsDof2* and *PsDof5* genes are defense related, as is the *PsDof1* gene.

Accession numbers: AB087831, AB087832, AB087833, AB087834, AB087835, AB087836, AB087837, AB087838, AB087839, AB087840, AB087841, AB087842, AB087843, AB087844, AB087845, AB087846, AB087847, AB087848, AB087849, AB087850, AB087851, AB087852.

Key words: elicitor, *Mycosphaerella pinodes*, phylogenetic tree, *Pisum sativum*, *PsDof* genes, transcription factor.

In plant-pathogen interactions, a plant recognizes elicitors produced by pathogens and immediately implements various defense responses (Dixon *et al.*, 1994; Knogge, 1996). Pathogen-produced elicitors induce various early defense responses: hypersensitive-cell death, the production of pathogenesis-related proteins and phytoalexins, and the occurrence of an oxidative burst and ion flux. Plant defense genes also produce glucanase, chitinase, proteinase inhibitors, peroxidase, chalcone synthase, and phenylalanine ammonia lyase in response to pathogens (Dixon *et al.*, 1994; Hammond-Kosack and Jones, 1996).

In our previous studies to understand the defense mechanisms via *de novo* gene activation, we used by differential screening to identify and isolate *hsr203J* (hypersensitivity-related) and *PsDof1* genes as candidates for elicitor-inducible genes (Table 1) (Ichinose *et al.*, 1999; Seki *et al.*, 2002). The elicitor was prepared from the pycnospore germination fluid of *Mycosphaerella pinodes* (Yamada *et al.*, 1989). It was certain that the *PsDof1* gene

carrying the conserved 156-bp Dof (DNA binding with one finger) domain was related to plant defense responses, as discussed in a previous report (Seki *et al.*, 2002).

Defense-gene regulation by defense-related transcription factors is important for understanding plant-pathogen interactions. Considerable information has been accumulated on general *trans*-acting factors regarding the particular WRKY and ERF families. These families also carry the factors working on defense-gene regulation (Rushton and Somssich, 1999; Ohme-Takagi *et al.*, 2000; Singh *et al.*, 2002).

The Dof family is a group of plant-specific transcription factors (like the WRKY and AP2/EREBP families), and they do not exist in *Drosophila*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae* genomes. In *Arabidopsis*, genomic sequence analyses indicated the presence of 37 members of the Dof gene family (Eulgem *et al.*, 2000; Riechmann *et al.*, 2000; Yanagisawa, 2002). Dof proteins contain a highly conserved CX₂CX₂

Table 1 cDNA clones isolated as elicitor-inducible genes in pea by differential screening.

Sequence significance ¹⁾	Accession No.
<i>ADH</i> (alcohol dehydrogenase)	AB087831 ²⁾
<i>chitinase</i> (class 1)	AB087832
<i>GRP</i> (glycine-rich protein)	AB087833 ²⁾
<i>HRGP-1</i> (hydroxyproline-rich glycoprotein 1)	AB087834
<i>HRGP-2</i> (hydroxyproline-rich glycoprotein 2)	AB087835
<i>HRGP-3</i> (hydroxyproline-rich glycoprotein 3)	AB087836
<i>hsr203J</i>	AB026296 ³⁾
<i>In2-1</i> (<i>GST</i> ; glutathione <i>S</i> -transferase)	AB087837
<i>PRX</i> (peroxidase)	AB087838
<i>PGIP</i> (polygalacturonase-inhibiting protein)	AB087839
<i>sadA</i> (short-chain alcohol dehydrogenase A)	AB087840
<i>SAMDC</i> (<i>S</i> -adenosylmethionine decarboxylase)	AB087841
<i>SAMS-1</i> (<i>S</i> -adenosylmethionine synthetase 1)	AB087842
<i>SAMS-2</i> (<i>S</i> -adenosylmethionine synthetase 2)	AB087843
<i>SAMS-3</i> (<i>S</i> -adenosylmethionine synthetase 3)	AB087844
<i>SOD</i> (superoxide dimutase)	AB087845
<i>SIR</i> (ferredoxin: sulfite reductase)	AB087846
zinc-finger protein (ring H2 type)	AB018422 ³⁾
zinc-finger protein (<i>PsDof1</i>)	AB026297 ³⁾

¹⁾ The sequence significance was analyzed using BLAST search of the National Center for Biotechnology Information (Altschul *et al.*, 1997).

²⁾ Incomplete cDNA sequences which were isolated by differential screening have been registered as expressed sequence tags in the DDBJ.

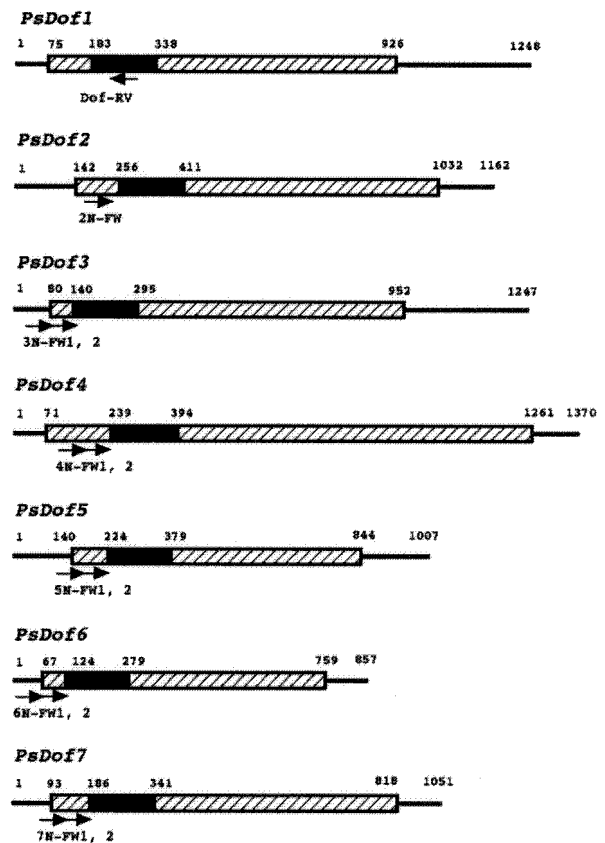
³⁾ The accession numbers AB026296, AB018422, and AB026297 were reported in Ichinose *et al.* (1999), Ichinose *et al.* (2001), and Seki *et al.* (2002), respectively.

CX₂C putative zinc-finger motif in respective N-terminal regions, and diverse amino acid sequences outside the Dof domain (Yanagisawa, 1996; Yanagisawa and Sheen, 1998). Although many Dof-type transcription factors have been identified and isolated already, their functions have not yet been fully clarified.

In the study reported here, we isolated various *PsDof* genes by PCR to examine the possible existence of defense-related Dof-type transcription factors in pea.

The PCRs were carried out using a T3 primer positioned on the 5'-upstream side of cDNAs and with the Dof-RV primer from the cDNA library elicitor treated as a template. We designed the Dof-RV primer in the *Dof* domain of the revealed *PsDof1* gene for cloning of the 5' part of other unknown *PsDof* genes (Fig. 1A and Table 2). The pea (*Pisum sativum* L. cv. Midoriusui) cDNA library was constructed from fungal elicitor-treated epicotyls for 5 h as described previously (Ichinose *et al.*, 2001). Six clones (*PsDof2* to *PsDof7*) with

A



B

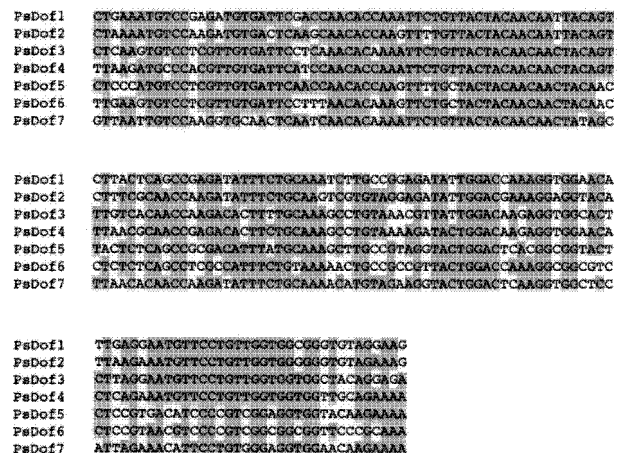


Fig. 1 (A) Schematic diagram of *PsDof* cDNAs. Nucleotide positions in each cDNA are numbered from the 5' end. Hatched and black boxes indicate coding regions and *Dof* domains, respectively. Horizontal bars indicate both untranslated regions in each transcript. Arrows indicate primer positions used for common PCR reactions. (B) Alignment of nucleotide sequences corresponding to *Dof* domains. Dark shading on the alignment indicates homologous nucleotides. The nucleotide sequences of the *PsDof* genes (*PsDof2* to *PsDof7*) have been registered in DDBJ, GenBank, and EMBL databases under accession numbers AB087847 to AB087852, respectively.

Table 2 Primer sequences used for PCR.

Primer name	Sequence
T3	5' - AATTAACCCTCACTAAAGGG - 3'
T7	5' - GTAATACGACTCACTATAGGGC - 3'
Dof-RV	5' - GTTGTAGTAACAGAATTTGGTG - 3'
2N-FW	5' - CCAGTCATTGGAGAGCATGT - 3'
3N-FW1	5' - TAGATGATGGTAAGATAATGG - 3'
3N-FW2	5' - CAAGGTCAAGAGCTTCTGC - 3'
4N-FW1	5' - GGACACAGAGTGAGATAGAT - 3'
4N-FW2	5' - TCAATGGCTTCTTCAACCGG - 3'
5N-FW1	5' - CAATGCCGTCATCGGACTCT - 3'
5N-FW2	5' - AATCAAACCACAGAACCGCC - 3'
6N-FW1	5' - CTCCATCGAAGGCGTTAATC - 3'
6N-FW2	5' - GGCGTTAATCATCATCAACA - 3'
7N-FW1	5' - CCATGGACACAACCTCAATGG - 3'
7N-FW2	5' - CAGGAGATTATGGTGAAGCC - 3'

an approximate length of 200 bp including the Dof domain were identified.

Based on the respective sequence information of the 5' parts, FW-series primers were designed for general and nested PCRs: 2N-FW, 3N-FW1, 3N-FW2, 4N-FW1, 4N-FW2, 5N-FW1, 5N-FW2, 6N-FW1, 6N-FW2, and 7N-FW1 and 7N-FW2 (**Fig. 1A** and **Table 1**). The general and nested PCRs were carried out by AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystems, Foster City, CA, USA) using the FW-series primers and with a T7 primer positioned on the 3'-downstream side of cDNAs from the cDNA library elicitor treated as a template (**Fig. 1A**). The nested PCRs were carried out for 30 cycles each. FW2-series primers and the T7 primer were used in the first PCR, then FW1-series primers and the T7 primer were used in the second PCR. The entire sequences of the corresponding *PsDof2* to *PsDof7* cDNA clones were determined with a DNA sequencer (ABI PRISM 310, PE Applied Biosystems, Chiba, Japan). Other nucleic-acid procedures were performed according to a molecular cloning protocol (Sambrook *et al.*, 1989).

As shown in **Fig. 1A**, the open reading frames of *PsDof* genes deduced from nucleotide sequences encode a 31.6-kDa polypeptide of 283 amino acids (*PsDof1*) (Seki *et al.*, 2002), a 33.2-kDa polypeptide of 298 amino acids (*PsDof2*), a 31.7-kDa polypeptide of 290 amino acids (*PsDof3*), a 43.1-kDa polypeptide of 396 amino acids (*PsDof4*), a 24.6-kDa polypeptide of 234 amino acids (*PsDof5*), a 25.5-kDa polypeptide of 230 amino acids (*PsDof6*) and a 26.7-kDa polypeptide of 241 amino acids (*PsDof7*). A serine/threonine-rich region is

well conserved in all seven deduced *PsDof* proteins, and especially the deduced *PsDof3* and *PsDof4* proteins near the C-terminus have a glycine-rich domain. Moreover, the deduced *PsDof4* protein also carries an asparagine-rich domain. The respective *PsDof* proteins carry a highly conserved Dof domain near the N-terminus. However, none of the *PsDof* proteins show any obvious homology with each other except for the Dof domain.

Dof proteins in three databases - DDBJ (DNA Data Bank of Japan), GenBank, and EMBL - were classified phylogenetically using the highly conserved 52 residues of Dof domains, and the presumed functions of *PsDof* proteins according to the phylogenetic tree (**Fig. 2**). The tree, which has 52 components, includes 26 nonredundant *Arabidopsis Dof* genes out of the 37 registered in the databases (Riechmann *et al.*, 2000).

OsDof3 of rice regulates the expression of the *CPD3* (type-III carboxypeptidase) gene under the control of gibberellin (Washio, 2001). *NtBBF1* of tobacco regulates *rolB* oncogene expression in T-DNA (De Paolis *et al.*, 1996; Baumann *et al.*, 1999). *Arabidopsis DAGs* regulate seed germination (Papi *et al.*, 2000), and *Arabidopsis OBP1* and *OBP2* genes are likely to have distinct functions in specific parts of the plant. Particular *Arabidopsis OBP1* genes are involved in defense responses via the *Arabidopsis GST6* gene. Generally, OBP proteins are related to the *ocs* (a stress-response) element, which is induced by auxin, salicylic acid, or cycloheximide. The *OBP3* gene is related to plant growth and development (Zhang *et al.*, 1995; Chen *et al.*, 1996; Kang and Singh, 2000). *ZmDof1* and *ZmDof2* genes regulate the transcription of genes responsible for light sensitivity, such as *CAPEPC* (C4 photosynthetic phosphoenolpyruvate carboxylase) of maize (Yanagisawa and Sheen, 1998). Pumpkin *AOBP* protein binds to the silencer region of the pumpkin ascorbate oxidase gene (Kisu *et al.*, 1998). The *StDof1* gene of potato interacts in a sequence-specific manner with a promoter of the *KST1* gene, which encodes a K⁺-influx channel of the guard cell (Plesch *et al.*, 2001). *PBFs* (prolamin-box binding factors) of maize, wheat, and barley activate gene expression for prolamin seed storage proteins during endosperm development (Vicente-Carbajosa *et al.*, 1997; Mena *et al.*, 1998).

It was certain that the *PsDof1* gene carrying the conserved Dof domain was related with plant defense responses, as discussed in a previous report (Seki *et al.*, 2002). The *PsDof2* gene is positioned near the *PsDof1* gene of the tree, and the *PsDof5* gene is positioned near the *Arabidopsis OBP1* gene of the tree, which is involved in plant defense

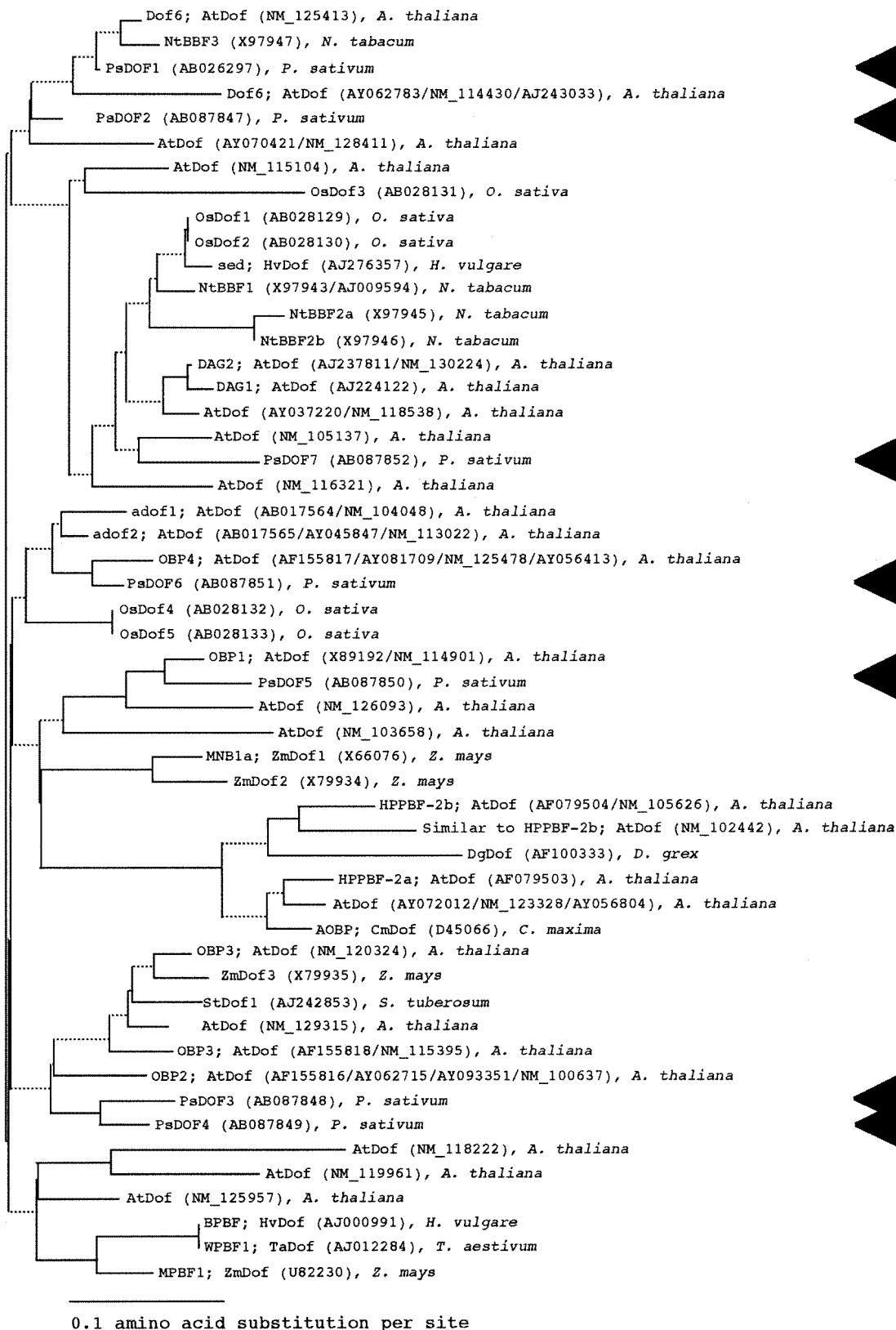


Fig. 2 Phylogenetic tree of the Dof domains.

The tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using GENETYX-MAC software (version 11.0.2, Software Development, Tokyo, Japan). The 45 Dof domains registered in the databases and the seven Dof domains of pea newly characterized were used to calculate the phylogenetic distances. Three pairs (OsDof1 and OsDof2, OsDof4 and OsDof5, and BPBF and WPBF) out of the 52 have the same amino acid sequences of the Dof domains, but they are different *Dof* genes. Node bootstrap values of less than 50% are indicated by broken lines. Software was not used for the alignment, because all Dof-domain sequences have been conserved highly, and gaps did not exist in all domains. Accession numbers of genes in the databases are shown in parentheses. Arrowheads indicate respective PsDof domains. The scale bar at the bottom indicates 0.1 amino acid substitution per site

responses via the *Arabidopsis GST6* gene (Fig. 2). Therefore, as for the *PsDof1* gene, it is expected that the *PsDof2* and *PsDof5* genes are defense related.

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (No. 12460023) from the Ministry of Education, Science, and Culture of Japan.

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