

## Molecular Cloning of cDNA for a Novel Pea Dof Protein, PsDof1, and Its DNA-Binding Activity to the Promoter of *PsDof1* Gene

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### Abstract

A cDNA clone, designated *PsDof1*, encoding a novel member of the Dof DNA-binding protein family was isolated from pea. Random-binding-site selection and gel retardation experiments showed that the GST-PsDof1 recombinant protein bound to specific DNA sequences with an AAAG core. A metal-chelating agent, 1,10-phenanthroline, abolished binding of GST-PsDof1 to DNA, but the addition of Zn<sup>2+</sup> restored it, indicating that zinc ions are required for its DNA-binding activity. Interestingly, there are possible sites for binding PsDof1 in its promoter sequence. Indeed, GST-PsDof1 binds to the AAAG-containing promoter sequence of the *PsDof1* gene with higher affinity. These results suggest that PsDof1 is a novel transcription factor that is involved in its own transcriptional regulation.

**Key words:** Autoregulation, DNA-binding protein, Dof protein, *Pisum sativum*, Promoter, Transcription factor, Zinc-finger protein

### Abbreviations

Dof, DNA binding with one finger; GST, glutathione S-transferase; PCR, polymerase chain reaction

### Introduction

Dof (DNA binding with one finger) proteins belong to the family of zinc-finger transcription factors in plants. All Dof proteins that have been analyzed conserve a 52-amino-acid stretch called the Dof domain that contains a CX<sub>2</sub>CX<sub>21</sub>CX<sub>2</sub>C putative zinc-finger motif near the N-terminus; in contrast, the regions outside the Dof domain have little or no homology to each other and show no significant homology to any sequence found in protein databases (Kisu *et al.*, 1998; Takatsuji, 1998). Interestingly, genes encoding Dof proteins (*Dof* genes) are found only in higher plants – they are not found in either yeast or *Caenorhabditis elegans* genomes (Riechmann *et al.*, 2000). These findings suggest that Dof proteins regulate the genes required for a wide variety of plant-specific functions, including metabolism and differentiation.

Therefore, characterization of the Dof protein attracted us to the study of the transcriptional regulation of plant-specific functions.

The first member of this protein family, Dof1, was identified from maize as the protein that specifically bound to an AAGG motif in the cauliflower mosaic virus 35S promoter (Yanagisawa and Izui, 1993). Since this finding, the role of Dof proteins has been studied in only a few members of that family. Several functions of Dof proteins have been reported: involvement in light-regulated gene expression in maize Dof1 and Dof2 (Yanagisawa and Sheen, 1998), gene activation of storage proteins in maize and barley PBF (Vicente-Carbajosa *et al.*, 1997; Mena *et al.*, 1998), regulation of *rolB* oncogene expression in tobacco BBF (De Paolis *et al.*, 1996; Baumann *et al.*, 1999), regulation of stress-related genes in *Arabidopsis* OBP1 (Kang and Singh, 2000), and regulation of seed germination in *Arabidopsis* DAG1 (Papi *et al.*, 2000). Genomic-sequence analysis in *Arabidopsis* revealed the presence of 37 members of the *Dof* gene family (Riechmann *et al.*, 2000). However, the function of gene products has been investigated in only several *Dof* genes, such as OBP1 and DAG1 (Kang and

Singh, 2000; Papi *et al.*, 2000); thus most *Dof* genes remain to be analyzed. Furthermore, no promoter analysis of *Dof* genes has yet been performed. Because *Dof* proteins are transcription factors, analysis of the transcriptional regulation of *Dof* genes should provide novel information for elucidation of the transcriptional hierarchy.

In this study, we identified the pea *Dof* gene *PsDof1* from an elicitor-treated cDNA library. We further characterized the DNA-binding activity of PsDof1 protein. We found that PsDof1 protein binds to both sequences with an AAAG core and to the AAAG-containing promoter sequence of the *PsDof1* gene itself, which suggests transcriptional autoregulation. Because the corresponding cDNA clone was isolated from elicitor-treated pea epicotyls, we also discuss the possible function of PsDof1 in defense responses.

## Materials and Methods

### Isolation of cDNA and genomic clones for *PsDof1* gene

A pea (*Pisum sativum* L. cv. Midoriusui) cDNA library was constructed from fungal elicitor-treated epicotyls for 5 h as previously described (Ichinose *et al.*, 2001). The *E84* cDNA clone was isolated as a candidate for elicitor-inducible cDNA clone by differential screening (Ichinose *et al.*, 2001), and renamed *PsDof1* according to its sequencing information.

The *PsDof1* gene was initially isolated from a commercially available pea genomic DNA library (Stratagene, La Jolla, CA, USA). To further isolate the *PsDof1* gene from the pea cultivar Midoriusui, polymerase chain reaction (PCR) was carried out using genomic DNA as the template. PCR primers (P1: 5'-GCCCAAAGCAAATAATATCAG-3'; P2: 5'-CTTCCTACACCCGCCACCAA-3') were designed by the sequence in the *PsDof1* gene from the genomic DNA library.

The nucleotide sequences were determined with a DNA sequencer (ABI PRISM™ 310, PE Applied Biosystems, Chiba, Japan) using the BIG Dye™ Terminator Cycle Sequencing kit. The homology search was performed with the BLAST search protocol.

### Expression of recombinant *PsDof1* in *Escherichia coli*

The recombinant plasmid for expression of a fusion protein of glutathione S-transferase (GST) and PsDof1 was constructed using the pGEX-5X-1 vector (Amersham Pharmacia Biotech, Tokyo, Japan). The entire coding region of *PsDof1* was

amplified by PCR with the following primers: 5'-*ccgaattc**ATGGATCCTTCTTCTCAGCA*-3' and 5'-*cccctcgag**CATTAGAGGACTATGAAGAA*-3'; lower-case nucleotides were added to restrict digestion after PCR amplification, underlined sequences are the recognition sites for *EcoRI* and *XhoI* (the ATG codon for the first methionine in *PsDof1* is in italics). The resulting DNA fragment was inserted into a pGEX-5X-1 vector, then transformed into the *E. coli* strain BL21.

*E. coli* cells containing the chimeric construct were grown in 300 ml of 2 x YT medium at 37 °C to the mid-log phase. The expression of fusion protein was induced with 1 mM isopropyl  $\beta$ -D-thiogalactopyranoside at 30 °C for 2 h. Cells were harvested by centrifugation, resuspended in 10 ml of PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>; pH 7.3). Phenylmethylsulfonyl fluoride and Triton X-100 were added to final concentrations of 1 mM and 1%, respectively. The cells were sonicated five times each for 15 s on ice, and centrifuged at 12,000 g for 15 min. The supernatant was mixed with 300  $\mu$ l of 50% glutathione-Sepharose 4B (Amersham Pharmacia Biotech) slurry, and incubated at 4 °C for 30 min. The Sepharose beads were recovered by low centrifugation (<1,000 g, 3 min) and washed three times with PBS buffer. Then the GST-PsDof1 fusion protein bound to glutathione-Sepharose beads was resuspended in 1 x binding buffer (25 mM HEPES-KOH, 100 mM KCl, 10  $\mu$ M ZnSO<sub>4</sub>, 1 mM DTT, 0.1% NP40, 5% glycerol; pH 7.5) and used directly for random binding-site selection assay. For the electrophoretic mobility-shift assay, GST-PsDof1 fusion protein was eluted from the beads by incubation for 20 min with 50 mM Tris-HCl (pH 8.0) containing 10 mM reduced glutathione.

### Binding-site selection assay

A 56-bp DNA mixture was made with 10-ng of 56-mer synthetic DNA (5'-AAGCTTCTAGAGATCCGTCA-N<sub>16</sub>-CACAAATCCCCTATCCTTCG-3') as an initial template, two 0.2  $\mu$ M primers (F: 5'-AAGCTTCTAGAGATCCGTCA-3'; R: 5'-CGAAGGATAGTGGGATTGTG-3'), and 47  $\mu$ l of Super Taq premix kit (SAWADY, Tokyo, Japan). The amplified DNA sequences contained 16 random nucleotides flanked by the defined sequence, and they were used as the source of the initial PsDof1 for the binding-site selection assay. Amplification was carried out for 25 cycles in a volume of 50  $\mu$ l, and then the first round of capture was initiated using the amplified dsDNA without further purification. For the first round of capture, 2  $\mu$ l of amplified PCR mixture was mixed with 10  $\mu$ g of

GST-PsDof1 bound to glutathione-Sepharose beads in 100  $\mu$ l of 1 x binding buffer (25 mM HEPES-KOH, 100 mM KCl, 10  $\mu$ M ZnSO<sub>4</sub>, 1 mM DTT, 0.1% NP40, 5% glycerol; pH 7.5) containing 5  $\mu$ g of poly (dI-dC:dI-dC), which was included in all of the rounds of capture as a nonspecific competitor. This mixture was incubated at 4 °C for 30 min whilst rotating continuously. The GST-PsDof1-DNA complexes on beads were collected by centrifugation at 1,000 g for 30 s; the pellet was then resuspended and washed seven times with 1 ml of 1 x binding buffer. The bound DNA was extracted from the GST-PsDof1 on beads as described by Hyde-DeRuyscher *et al.* (1995) with slight modifications. The DNA was resuspended in 20  $\mu$ l of H<sub>2</sub>O, and 1  $\mu$ l of this was used for amplification. After six rounds of selection, the amplified DNA was cloned into a pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA), and 16 independent clones were sequenced. Strikingly, all selected DNAs contained an AAAG sequence (data not shown), suggesting that this sequence is the recognition core of PsDof1 protein. Thus, to investigate whether the PsDof1 exhibited binding preferences for the sequences flanking the AAAG core, we carried out a second binding-site selection experiment with DNAs carrying a prefixed AAAG core flanked by six random nucleotides. A 57-mer synthetic DNA (5'-AAGCTTCTAGAGATCCGTCAN<sub>6</sub>-AAAG-N<sub>6</sub>-GTCGCACGACCCTGGTCGTAA-3') containing an AAAG core flanked by six random nucleotides on each side and two primers (F and R': 5'-TTACGACCAGGGTCGTGCGAC-3') was used to generate a 57-bp DNA mixture by PCR amplification. Fifty-one individual clones were obtained after six rounds of selection, and the inserted sequences were determined.

#### Gel retardation assay

A gel retardation assay was carried out as previously described (Seki *et al.*, 1996). Probe DNAs were labeled with [ $\gamma$ -<sup>32</sup>P]-ATP and T4 polynucleotide kinase, then incubated with 500 or 1,000 ng of purified GST-PsDof1 fusion protein in a 1 x binding buffer (25 mM HEPES-KOH, 100 mM KCl, 1 mM DTT, 0.1% NP40, 5% glycerol; pH 7.5) containing 2  $\mu$ g of poly (dI-dC:dI-dC) in a total volume of 10  $\mu$ l. Mixtures were fractionated on a 4% polyacrylamide gel (acrylamide:bisacrylamide, 19:1) with 0.5 x TBE buffer at 100 V at 4 °C. Gels were dried under a vacuum and exposed to X-ray film at room temperature for 17-24 h. For competition assays, a 30- or 90-fold excess of unlabeled synthetic DNAs was added to the binding mixture. For inhibition assay by the metal chelator, 2, 5, or

10 mM 1,10-phenanthroline was added to the binding mixture. To prepare DNA fragments corresponding to the promoter region of *PsDof1* gene, we amplified DNA fragments with two sets of DNA primers (P1 and P3: 5'-TGAAATGAGGTTGGTAGTGGAGAG-3'; P2 and P4: 5'-CATTTCCTCACTCTCTCCATTCCTC-3'), then digested them with *Eco*RI, *Alu*I, *Afa*I, and *Bam*HI. After separation of each DNA fragment with polyacrylamide gel electrophoresis, DNA fragments F1-F4 were extracted and labeled prior to the binding assay.

## Results

### Structural analysis of a cDNA clone for *PsDof1* and its gene expression

To analyze the molecular mechanism of elicitor-induced transcription of defense-related genes, we constructed a cDNA library from pea epicotyls treated with an elicitor from *Mycosphaerella pinodes* for 5 h and found a unique cDNA clone, *E84*. As shown in **Fig. 1A**, DNA sequence analysis of *E84* revealed that the cDNA insert is 1,269 bp long, and includes a 21-nucleotide poly-A tail. The *E84* cDNA clone seems to possess the entire coding region (or almost all of it), because the size of the cDNA is consistent with the estimated size of the mRNA detected in a Northern-blot hybridization experiment (described below). The largest open reading frame spans 283 amino acids, from which a protein of 31.6 kDa is predicted. Database searches revealed that the deduced amino acid sequence of *E84* exhibits high homology to a recently discovered Dof class of DNA-binding proteins (Yanagisawa, 1996); thus, *E84* was renamed *PsDof1*. The Dof domain in *PsDof1* is located near the N-terminus of a 52-amino-acid stretch (37-88 in **Fig. 1**), like other Dof proteins. The amino acid sequence of the Dof domain was highly conserved with other Dof domains (**Fig. 1B**), whereas the sequence outside the Dof domain did not share high homology with any other proteins in the database. The most homologous protein is a putative Dof protein encoded by At2g28510 of *A. thaliana*, which was registered by the genomic sequencing project. *PsDof1* and At2g28510 showed 35.9% identity and 71.4% similarity in amino acid sequences, including the sequence of the conserved Dof domain. The function and expression of At2g28510 is not yet characterized.

The organ-specific expression of *PsDof1* was examined by Northern-blot hybridization. *PsDof1* mRNA (ca. 1.3 kb) was most abundant in stems, then in roots and leaves (data not shown). Although the cDNA clone for *PsDof1* was initially isolated as

**A**

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CATTTTCACTCTCTCCATCTCTCTCTTTGTCTCTTTAATATCCCACTCCAACGACGAAATTTGTTGTATGAAATGGATCCTTCTCTC 90
                                     M D P S S Q [6]
AGCAAATGTCARAGCAATCATCAATGAAACATGTGGTTTGTTCAAAAGAACAGCAAGAAAGCAAACCCAGCCTCAGCCGGAACAG 180
Q H S R Q S S H E N M L V C S K E Q Q E S K F K F Q P E Q A [36]
CTCTGAAATGTCGGAGATGTGATTCGACCAACACCAAAATTCGTACTACAACAATTACAGTCTTACTCAGCCGAGATATTTCTGCAAA 270
L K (C) P R (C) D S T N T K F C Y Y N N Y S L T Q P R Y Y F (C) K S [66]
CTTGCCGGAGATATTTGGACCAAGGTGGAACATTTGAGGAATGTTCTCTGTGGTGGCGGTGTAGGAAGAACAARAGATCATCGTCTTCGA 360
(C) R R Y W T K G G T L R N V P V G G G C R K N K R S S S S K [96]
AGAGGATTCAGATCAAGCATTTCGGCCGAATCATAATCCGTTTCGAACCTACCTCATTGATCAATCTAATGATTTGCGTTACGTT 450
R I Q D Q A F A P N H N P F S N L P H F D Q S N D F A L R L [126]
TGGCTAGGCTTCAGAAACAAATCATGTGGTGGACAAATGGGTTATGATGAAACGAGCTTTCATGTTGGGAAATAGTGGATCAAGCATGA 540
A R L Q K Q S C G G Q M G Y D E N E L S M L G N S G S S M N [156]
ACCACTCGATGAGCATAAATATGGGTTTATGGATTCGATGAGAAGTGGTTTATCTCTGGAAATGGAATGCATATAATAATAGTGTTC 630
Q S M S I N H G F M D A A I R S G L F L G N G M H Y N N S V Q [186]
AAAATATGATGTGGTGGCTATGGAAATGGTGACAATGGAGAAGTGAATAGTGGAAACAACAATTTGGGGTTAGTGAAGAGATGATGT 720
N M Y G G G Y G N G D N G E V N S G N N N C G V S E E M M F [216]
TTAATATGATCAAGAAATGACCAATGCATGCTGATTAACATGAAGCAAGAGCAAAAGTGAAGTAGGGTTTTGTTGGTGGTTTCCAT 810
N Y D Q E M S H C N A A I R S M K Q E S E S R V F G G F P W [246]
GGCAGATAAATGGAGAAATGGAAACATGGGTGAAGTTGAACAAGCAAGCAAGTTGGAATAATGTTTCACTACACCTTCTTGGCAG 900
Q I N G G I G N M G E V E Q A R A S W N N G F T T P S W Q G [276]
GGCTTCTCATAGTCCCTCAATGATAGAAATCAAAGATAAATGATTTTAAATTCGGGTCTCGACTGAAGTTGAGAAATGCAGCTACTAC 990
L L H S P L M * [283]
AGATGAAGTTGTCATTGCAGATGCAATTTAGAAATTTAGAAGTGGTGGTATAAATTTGCCATAATGTTGACAAAATATATATGGTTA 1080
GGGTTAATTAATTTTCAGTCTCTTGGATGAAATGGGTTGATAGAGATTTGATGCATTTAGAAATGGATCAAATTCATTCTCTACCT 1170
TGTTTTGTTCTTTTAAATTTCTTCTTTTATTAGGTTACCAAATGTTACATTTTTTTTGTGTGAAGAAAGTTTATGTAATAAAAAA 1260
AAAAA 1269
    
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**B**

		Identity	Reference
PsDof1	LKPCRCDSNTNKFCYNNYSLTQPRYFKSCRRYWTKGGTLRNVVGGGCRK		
NtBBF1	IN***N*****S*****T*****D**S*****S**	(84.6%)	[4]
AtOBP1	*P*****S*****NFS***H***A*****H*****D*****T**	(80.8%)	[24]
AtDAG1	*N*****A*****G*****E**S*****S**	(86.5%)	[11]
ZmDof1	DP****A*RD*****NTS***H***G*****T**	(78.8%)	[20]
ZmDof2	DP****G*RD*****NTS***H*****S*****T**	(76.9%)	[18]
ZmDof3	*****S*****H**RA*****R**A*****YR*	(86.5%)	[18]
ZmPBF	*****N*****MS*****A*****H*****I*****	(88.5%)	[16]
BPBF	VE****K*G*****MS*****A*****H**S*****I*****	(80.8%)	[10]
AOBP	*P*****ME*****NVN***H***A*Q****E***I*****A*R**	(73.1%)	[9]

**Fig. 1** Structural analysis of *PsDof1* cDNA. (A) The nucleotide sequence of *PsDof1* cDNA numbered from the 5' end. The deduced amino acid sequence of PsDof1 protein is shown below the nucleotide sequence, and is numbered in parentheses. The Dof domain is underlined, and the four cysteine residues putatively involved in Zn<sup>2+</sup> coordination are circled. The asterisk indicates the stop codon. (B) Sequence conservation through the Dof domain. The amino acid sequence of the Dof domain of PsDof1 is shown aligned with other Dof proteins. Asterisks are the amino acids that are identical to the corresponding amino acid of PsDof1. Numbers in parentheses on the right indicate the percentage of sequence identity with PsDof1 within the Dof domain. The four cysteine residues that putatively coordinate to Zn<sup>2+</sup> are shown by arrowheads. The nucleotide sequence of *PsDof1* cDNA has been submitted to DDBJ, GenBank, and EMBL databases under accession number AB026297.

a candidate for an elicitor-inducible gene, *PsDof1* was expressed slightly after 6 h treatment with elicitor (data not shown).

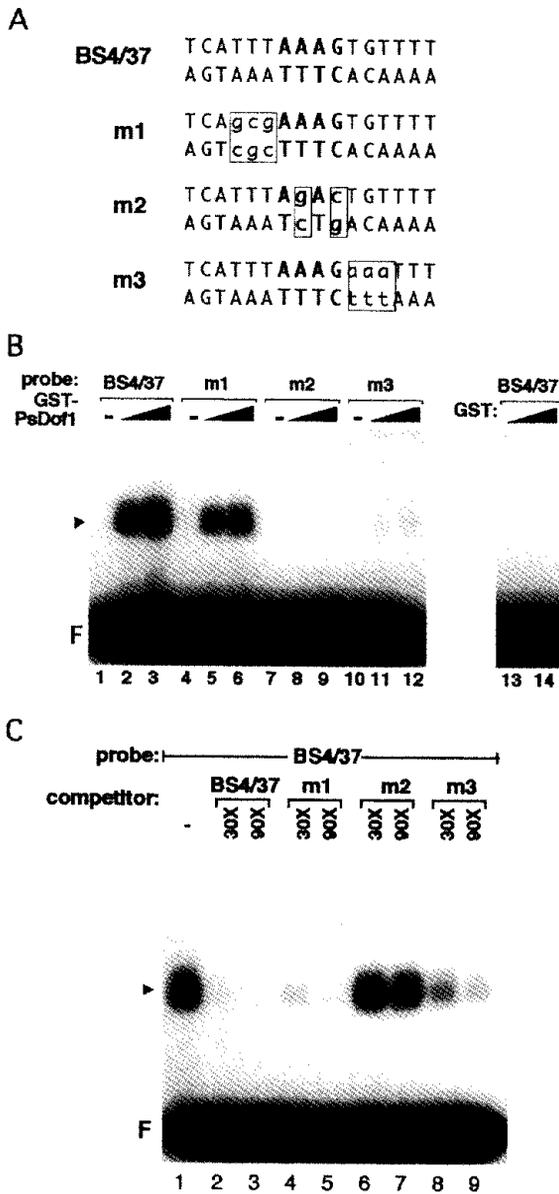
*DNA-binding sequence of PsDof1 protein*

To examine the DNA-binding ability of PsDof1 protein and to identify its binding motif, GST-PsDof1 fusion protein was prepared using an *E. coli* expression system as described in the Materials and Methods. Two rounds of a random binding-site selection assay revealed that GST-PsDof1 protein binds 51 individual sequences. The sequences of selected DNAs are listed in Fig. 2A, where they are aligned to match the sequence with an AAAG core.

The putative optimal PsDof1 binding sequence deduced from the 51 independent sequences is TTT/cT/cT/gT/aAAAGTGT/cTTT (Fig. 2B). There are T/C-rich sequences around the AAAG-core motif, and AAAG or its complementary sequence CTTT were found frequently beside the central AAAG-core sequence (in 25 out of 51 clones, Fig. 2A).

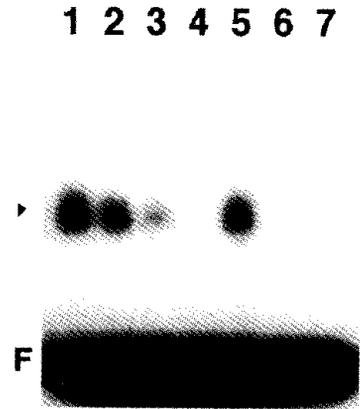
A gel retardation assay was performed to determine if PsDof1 binds specifically with the AAAG-containing sequences. The binding-site selection experiment (Fig. 2A) showed that individually isolated clones (BS4 and BS37) have an identical sequence, which shows high homology to the puta-





**Fig. 3** DNA-binding specificity of GST-PsDof1. (A) Synthetic DNA sequence corresponding to the clones BS4 and BS37 and three different derivatives are shown as BS4/37, m1, m2, and m3, respectively. The substituted nucleotides were boxed. (B) Gel retardation assay using each synthetic DNA shown above as a probe. Labeled synthetic DNAs were incubated in the absence (-) or with increasing (500 and 1,000 ng, indicated by triangles) amounts of purified GST-PsDof1 or GST control protein (lanes 13 and 14). (C) Binding competition assay using BS4/37 as a probe. A 30- or 90-fold excess of each synthetic competitor was added to the probe DNA. Arrowhead and F denote the positions of the retarded band and free probe, respectively.

involved in the DNA-binding activity of this protein. We tested the inhibition of complex formation



**Fig. 4** Inhibition of DNA-binding of PsDof1 by 1,10-phenanthroline. The labeled DNA fragment, BS4/37, and GST-PsDof1 (500 ng) were incubated without (lane 1) or with 1,10-phenanthroline (lane 2: 2 mM; lane 3: 5 mM; lane 4: 10 mM). The loss of DNA-binding activity caused by 1,10-phenanthroline (lane 4) was restored by the addition of 10 mM ZnCl<sub>2</sub> (lane 5) but not by 10 mM MgCl<sub>2</sub> (lane 6) or 10 mM CaCl<sub>2</sub> (lane 7). Closed arrowhead and F denote the positions of the retarded band and free probe, respectively.

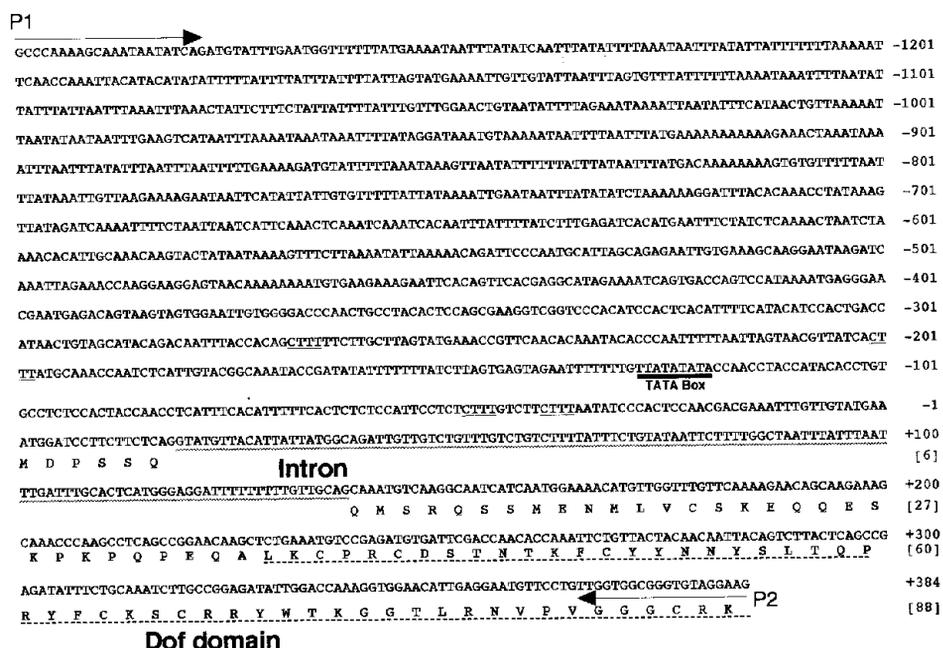
by a metal chelator, 1,10-phenanthroline that prefers Zn<sup>2+</sup> in the gel retardation assay. As shown in **Fig. 4**, the formation of the complex of GST-PsDof1 and BS4/37 was effectively inhibited by the addition of 1,10-phenanthroline. We confirmed that the chelator did not inhibit the binding activity via a nonspecific mechanism: the DNA-binding activity could be restored by the addition of Zn<sup>2+</sup> after inhibition by the chelator (**Fig. 4**, lane 5), but not by the addition of Mg<sup>2+</sup> or Ca<sup>2+</sup> (**Fig. 4**, lanes 6 and 7). Thus, zinc coordination is required for DNA-binding activity of PsDof1.

*Promoter region of PsDof1 gene*

To characterize the structure of the *PsDof1* gene, we first isolated the corresponding genomic phage clone from the commercially available pea genomic DNA library using the *PsDof1* cDNA sequence as a probe. Then, using the DNA sequence information obtained, we further isolated the corresponding region from the pea cultivar Midoriusui by PCR. As shown in **Fig. 5**, the 1,684-bp sequence of a PCR-amplified fragment containing the approximately 1.4-kb promoter region was determined. There is a 120-bp intron between the sixth and seventh amino acids.

*Specific binding of PsDof1 to PsDof1 promoter*

To investigate the possible interaction between PsDof1 and the promoter region of the *PsDof1* gene,



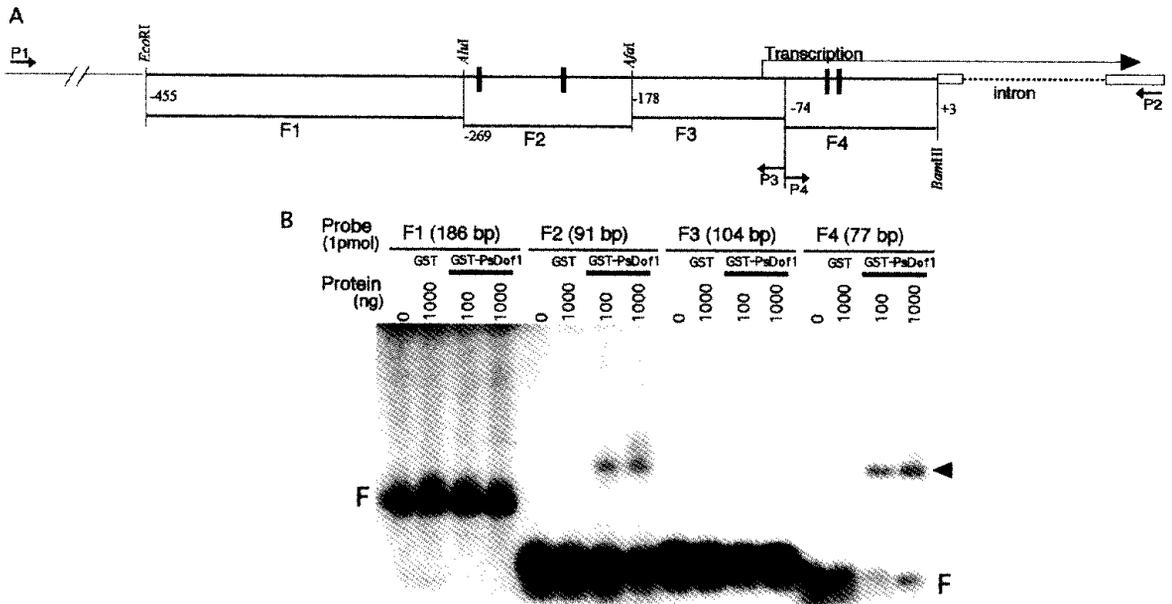
**Fig. 5** Structural analysis of *PsDof1* gene. The nucleotide sequence of the *PsDof1* gene containing the promoter region is shown. The nucleotides are numbered from the putative translation start site, A, as +1. The 5' end of *PsDof1* cDNA (E84) is indicated by the dot at position -74. The amino acid sequence of *PsDof1* is also shown below the nucleotide sequence, and is numbered in parentheses. The nucleotide sequences for the TATA box and intron and amino acid sequence for the Dof domain are indicated by a thick line, a wavy line, and a dotted line, respectively. The thin underlines indicate the CTTT sequences indicated in Fig. 6A as bold vertical lines. The two arrows represent the primer sequences used for PCR-mediated amplification of this region as described in the Materials and Methods. The nucleotide sequence of the *PsDof1* gene has been submitted to DDBJ, GenBank, and EMBL databases under accession number AB074443.

we carried out the DNA-protein binding assay with gel retardation experiments. Four labeled DNA fragments containing either promoter or 5'-untranslated regions of the *PsDof1* gene were prepared (Fig. 6A). The results clearly demonstrate that the fragments F2 and F4 formed complexes with GST-PsDof1 fusion proteins, but not with GST proteins (Fig. 6B). As indicated in Fig. 6A, both F2 and F4 contained putative PsDof1-binding sequences (AAAG or CTTT), but neither F1 nor F3 contained such sequences. Thus, these sequences were thought to be involved in the formation of the complex.

## Discussion

In this study, we isolated a cDNA clone designated *PsDof1*. This gene encodes a novel type of transcription factor that has a Dof DNA-binding domain with DNA-binding ability. Random binding-site selection and gel retardation assays demonstrated that PsDof1 binds specifically to DNA sequences with an AAAG-core motif, and sequences flanking the AAAG core can also affect the binding affinity (Fig. 2 and 3). Because the Dof

domain was identified as the DNA-binding domain in maize Dof1 (Yanagisawa, 1995), and the Dof domain is highly conserved among the Dof proteins (Fig. 1B), all members of the Dof protein family may recognize similar DNA sequences. Indeed, maize Dof proteins Dof1, Dof2, and Dof3 recognize the (A/T)AAAG sequence as the core motif (Yanagisawa, 1996). The target sequences of *Arabidopsis* Dof protein OBP1 also contain the AAAG motif (Zhang *et al.*, 1995; Chen *et al.*, 1996). Another maize Dof protein, PBF, and its barley homolog, BPBF, bind a P-Box motif: TGTAAAG (Vicente-Carbajosa *et al.*, 1997; Mena *et al.*, 1998). The tobacco Dof protein BFB1 binds a TAAAGT sequence (De Paolis *et al.*, 1996). Our gel shift-retardation assay revealed that not only the AAAG-core motif but also a TGT sequence located just downstream of the core motif are essential for DNA binding of PsDof1 (Fig. 3). Furthermore, in random binding-site selection assays, we frequently found a CTTT sequence motif beside the AAAG-core sequence (Fig. 2). This result indicates that the presence of two potential binding sites could increase the binding efficiency to PsDof1 proteins.



**Fig. 6** DNA binding of PsDof1 to its promoter sequence. (A) The promoter and 5'-untranslated region of *PsDof1* was divided into four fragments, designated F1-F4. Fragments F2 and F4 contain two putative PsDof1 binding sequences, CTTT, and are indicated as bold vertical lines. The position of the translation start site, A, is numbered as +1. The regions for open reading frames in two exons are indicated as boxes, and the intron is shown as a dotted line. (B) The DNA-binding activity of each DNA fragment was examined by gel retardation assay. Each 1 pmol of labeled DNA was incubated in the absence or presence (100 or 1,000 ng) of purified GST-PsDof1 or GST control protein. Arrowhead and F denote DNA-protein complexes and free DNA probes, respectively. The preparation of each DNA fragment is described in the Materials and Methods.

Although all Dof proteins recognize highly similar sequences, as described above, Dof proteins have been shown to be involved in a variety of signal-responsive and/or tissue-specific gene expressions in plants. For example, the maize Dof1 and Dof2 proteins are implicated in the light-regulated activation of the gene for the C4-type phosphoenolpyruvate carboxylase of maize (Yanagisawa and Sheen, 1998). Another maize Dof protein, PBF, and its barley homolog, BPBF, are involved in the endosperm-specific expression of seed-storage protein genes (Vicente-Carbajosa *et al.*, 1997; Mena *et al.*, 1998). The tobacco Dof protein BBF1 is involved in the tissue-specific and auxin-responsive expression of the *rolB* gene of *Agrobacterium rhizogenes* (De Paolis *et al.*, 1996; Baumann *et al.*, 1999), and the *Arabidopsis* DAG1 gene controls seed germination (Papi *et al.*, 2000). Recently it was reported that the expression of *Arabidopsis* Dof proteins, called OBP1, -2, and -3, were induced by the plant-defense signaling compound salicylic acid (Kang and Singh, 2000). Furthermore, Chen *et al.* (1996) also found that OBPs could interact with OBF4 protein, a bZIP factor that binds *ocs* elements, and stimulate the binding of OBF4 to the

*ocs* element in the *Arabidopsis* glutathione S-transferase-6 (*GST6*) gene promoter. *Ocs* elements are activated by salicylic acid (Zhang and Singh, 1994; Yang *et al.*, 1997), and an *ocs* element in the *Arabidopsis* *GST6* gene promoter has also been shown to be involved in the responsiveness of this gene to salicylic acid (Chen and Singh, 1999). These observations suggest that certain members of the Dof-type DNA-binding proteins are an emerging group of transcription factors that play a role in the regulation of defense genes in a wide range of plant species. Although *PsDof1* cDNA was isolated from the library of elicitor-treated pea epicotyls, the induction of *PsDof1* by the elicitor was not clearly observed. Thus, it is still unclear whether PsDof1 contributes to the transcriptional activation of some elicitor-inducible genes.

Interestingly, we found that PsDof1 protein strongly binds *in vitro* to AAAG-containing sequences present in the promoter region of *PsDof1* itself. The evidence suggests that the *PsDof1* gene is regulated by PsDof1 protein itself. Such autoregulation has been reported in some plant genes. For example, in response to a fungal elicitor, one of the plant-specific zinc-finger-type transcription fac-

tors, parsley WRKY1 protein, binds W-box promoter elements in not only *PR-10* but also *WRKY1* genes, and activates their transcriptions (Eulgem *et al.*, 1999). On the other hand, a TFIIIA-type transcription factor, wheat WZF-1, was reported as a transcriptional repressor for the *WZF-1* gene (Sakamoto *et al.*, 1996). A transient transfection assay revealed that WZF-1 activates the histone H3 promoter via WZF-1 binding elements in wheat, whereas the results of the cotransfection experiments showed that the expression of WZF-1 by an effector plasmid significantly repressed *WZF-1* promoter-mediated reporter gene expression in tobacco (Sakamoto *et al.*, 1996). Thus, WZF-1 might possess bifunctional activities by regulating plant genes both positively and negatively.

Further studies are needed on the role of the *PsDof1* gene in plant gene regulation, especially in defense responses. The identification of the target genes of PsDof1 protein and the transcription factors that regulate *PsDof1* gene expression will reveal the downstream and upstream signal transduction pathways, respectively.

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