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Possible Involvement of AAAG Motif and PsDof1 in Elicitor-Induced Gene Expression in Pea

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Recently, we, isolated cDNA clone, *PsDof1,* from elicitor-treated pea cDNA library. The putative gene product, a PsDof1, encodes DNA binding protein that specifically binds the DNA fragment containing AAAG core sequence. In this paper we report that GST-PsDof1 fusion protein specifically binds to the promoter region containing AAAG core sequence(s) of *PsCHS1*, one of the elicitor-inducible genes encoding chalcone synthase (CHS). Furthermore the addition of DNA fragment containing AAAG motif to the 35S minimal promoter provided the elicitor-responsibility in transient transfection assay using pea protoplasts. These results suggest that PsDof1 might be involved in defense responses by activating the transcription by a binding to AAAG core sequence in the promoter of the defense-related genes in pea.

Key words : *cis*-element, DNA binding proteins, Dof protein, Elicitor

Introduction

Gene expression is mostly regulated at the stage of transcription. Transcriptional regulation of gene expression mediates the recognition of promoter elements by the transcription factors. In plant defense response, the study of transcriptional regulation is very important to elucidate the mechanism for defense gene expression and enhance disease resistance. Recently, we isolated a cDNA clone, *E84* that encodes a putative transcription factor[8]. Deduced amino acid sequence of *E84* revealed that *E84* is partly homologous to Dof (DNA binding with one finger) type transcription factors. Dof proteins belong to zinc finger transcription factor proteins. Although many zinc finger proteins possess multiple zinc finger motifs that are possible to bind specific DNA sequence, Dof proteins possess only one zinc finger motif near the N-terminus. Dof proteins conserve a Dof domain consisting of a 52-amino-acid stretch with $CX_2CX_{21}CX_2C$; in contrast, the region outside the Dof domain has little or no homology to any sequence found in protein databases. Because Dof proteins were known to be composed of a multigene family, *E84* was renamed as *PsDof1*[8]. Interestingly, Dof type transcription factors were found only in plant species[13], thus Dof proteins are thought to regulate transcription for plant-specific functions.

PsDof1-cDNA was isolated from the cDNA library that was constructed from fungal elicitor-treated pea epicotyls[8]. The mRNA for *PsDof1* accumulated slightly by the elicitor-treatment; therefore, *E84* would be a likely regulator for the elicitor-responsive gene expression as a DNA-binding protein. We produced recombinant fusion GST-PsDof1 protein, and then the DNA sequences for PsDof1 binding were identified by random binding site selection assay. The results showed that PsDof1 bound to specific DNA sequences with an AAAG core. For the last decade, we have isolated elicitor-responsive genes such as phenylalanine ammonia-lyase (PAL) and chalcoen synthase (CHS) from pea, and investigated their transcriptional regulation[9]. Thus, we identified five elicitor-responsive *PsCHS* genes and two *PsPAL* genes[4,12].

In this study, we have surveyed the existence of the possible PsDof1-binding sequences in the promoter region of the elicitor-responsive genes, and we investigated the binding activity for PsDof1 to the promoter of one elicitor-responsive gene, *PsCHS1*. Furthermore, the activity as an elicitor-responsive *cis*-regulatory element of the core sequence, AAAG

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Abbreviations
CaMV, cauliflower mosaic virus; CAT, chloramphenicol acetyl transferase; CHS, chalcone synthase; PAL, phenylalanine ammonia-lyase; PsDof1, *Pisum sativum* DNA binding with one finger protein 1.

was assessed by transient transfection assay with pea protoplasts.

Materials and Methods

Preparation of recombinant PsDof1 protein

The open reading frame for PsDof1 protein was inserted into pGEX-5X-1 plasmid (Amersham Pharmacia Biotech), and produced fusion protein of glutathione *S*-transferase (GST) and PsDof1 in *E. coli* as described[8].

Preparation and labeling of DNA probes and Gel mobility shift assay

For gel mobility shift assay, DNA fragments were prepared from *PsCHS1* promoter region after PCR amplification and extraction from polyacrylamide gels subjected to electrophoresis, then labeled with ^{32}P-ATP by T4 polynucleotide kinase. As competitive DNA fragments, 30-fold molar excess of unlabeled DNA fragments A or B and synthetic double-stranded oligonucleotides (BS4/37-wt: 5'-TCATTTAAAGTG TTTT-3'; BS4/37-m2: 5'-TCATTTAgAcTGTTTT-3') were used.

Plant Material and CAT transient transfection assay

Pea (*Pisum sativum* L. cv. Midoriusui) suspension culture was described previously[3]. For transient transfection assay, protoplasts were prepared from pea suspension cultured cells, then plasmid DNAs possessing chimeric gene were introduced into the protoplasts by electroporation[3]. The activity of chloramphenicol acetyl transferase (CAT) was measured as described[3,7]. After electroporation of the reporter gene into pea protoplasts, elicitor or water was added. Elicitor was prepared from the pycnospore germination fluid of *Mycosphaerella pinodes* as described[11], and used at 100 μg/ml as glucose equivalent as a final concentration.

Plasmid construction

Reporter plasmids containing a gene encoding a bacterial CAT and elicitor-responsive promoter of *PsCHS1* gene (−242 to +78) were previously described[6,7]. We also found that synthetic double-stranded 16 bp oligonucleotide, BS4/37 (5'-TCAT TT*AAAG*TGTTTT-3'), was one optimal sequence for PsDof1 binding[8]. Therefore to assess the function of BS4/37 sequence for elicitor responsibility, we tetramerized an AAAG-containing BS4/37, and fused it to minimal CaMV 35S promoter (−44 to +4).

Results

Existence of potential PsDof1 binding sites in PsCHS and PsPAL genes

The potential PsDof1 binding sequences, AAAG and CTTT, were surveyed in the proximal sequences in the promoter of elicitor-responsive *PsCHS1-5* and *PsPAL1, 2* genes (Fig. 1). For example, the promoter sequence of *PsCHS1* contains one AAAG and four CTTT sequences. Other promoters also contain plural potential sites, especially around TATA-box and transcription starting points or at the 5'-noncoding regions. The multiple existence of AAAG and CTTT sequences might indicate the potential involvement of these sequences in the elicitor-induced activation of elicitor-responsive genes.

E84 binds to the fragments of the PsCHS1 promoter region

We revealed that the promoter region from −242 to +78 is sufficient for maximal elicitor-mediated activation of *PsCHS1*, a member of elicitor-responsive chalcone synthase genes in pea, by transient transfection assay[6,7]. Thus, it was interesting to see if the recombinant PsDof1 showed any affinity for promoter fragments of *PsCHS1*. Three similar-size DNA fragments, fragment A (−242 to −137), B (−137 to −45) and C (−44 to +36) were PCR-amplified from *PsCHS1* promoter region, and then used as probes in

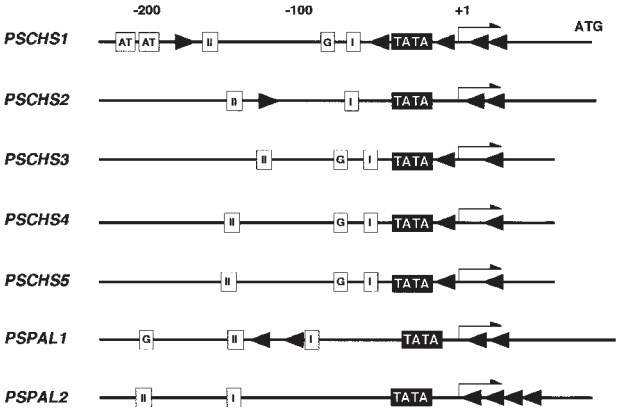

Fig. 1 The position and orientation of the potential PsDof1 binding sites on the elicitor-responsive pea *CHS* and *PAL* gene promoters. The horizontal lines indicate the nucleotide sequence from the 5'-upstream region to the putative translation start site, ATG. The small arrows indicate the position of the transcription starting points and direction of the transcription. TATA boxes are shown by black boxes. AAAG or its complementary sequence, CTTT are shown by arrowheads. The putative elicitor-mediated *cis*-acting elements such as AT (AT-rich sequence), II (Box II), G (G-Box) and I (Box I) are also indicated.

gel retardation assay. As shown in Fig. 2A, AAAG-containing sequence(s) exists in fragment A and C, but not in fragment B.

Upon incubation with the GST-PsDof1 and labeled fragment A, two retarded bands were observed (Fig. 2B, left). However, the higher migrating complex (indicated by open arrowheads) might not be due to the GST-PsDof1, since this complex formation was not affected by the addition of unlabeled BS4/37-wt in the next competition assay (Fig. 2C, lane 4 in left panel), and the same retarded band was observed also with fragment B which has no AAAG sequence (Fig. 2B, center). Specific binding of GST-PsDof1 to AAAG-containing sequence in fragment A was demonstrated by competition assay (Fig. 2C, left panel). Addition of 30-fold molar excess of unlabeled BS4/37-wt clearly inhibited the formation of lower migrating complex (lane 4); in contrast, the same molar excess of BS4/37-m2, possessing two base substitutions in AAAG core sequence, had no effect (lane 5). In fragment C, besides the major shifted complex (indicated by closed arrowheads), higher molecular weight shifted complex (indicated by closed circle) was also observed with increased amount of GST-PsDof1 (Fig. 2B, right). This might be due to the binding of more than one GST-PsDof1 molecule to the probe, because fragment C contains multiple AAAG sequences (see Fig. 2A). The right panel of Fig. 2C displays the results of competition experiment with fragment C as a probe. Again, addition of unlabeled BS4/37-wt clearly inhibited the formation of lower migrating complexes (lane 4); in contrast, BS4/37-m2 had no effect (lane 5). These results indicate that PsDof1 binds specifically to the AAAG-containing sequences in *PsCHS1* gene promoter.

Elicitor activates AAAG-repeated promoter in transient transfection assay

To evaluate the significance of AAAG sequence on the promotion of elicitor-mediated transcription, we fused tetramerized AAAG core sequence to CaMV 35S minimal promoter (−44), and carried out transient transfection assay using pea protoplasts. As shown in Fig. 3, *PsCHS1* promoter (−242 to +78) containing elicitor-responsive *cis*-acting elements, AT-rich sequences, Box-II, G-Box and Box-I, showed basal transcriptional activity. Furthermore, the promoter gave about two-fold CAT induction in response to elicitor-treatment. On the other hand, 35S minimal promoter resulted in little CAT activity regardless of elicitor-treatment. It is noteworthy that tetramerized units of AAAG motif conferred the same level of basal expression and elicitor-induced expression as we had

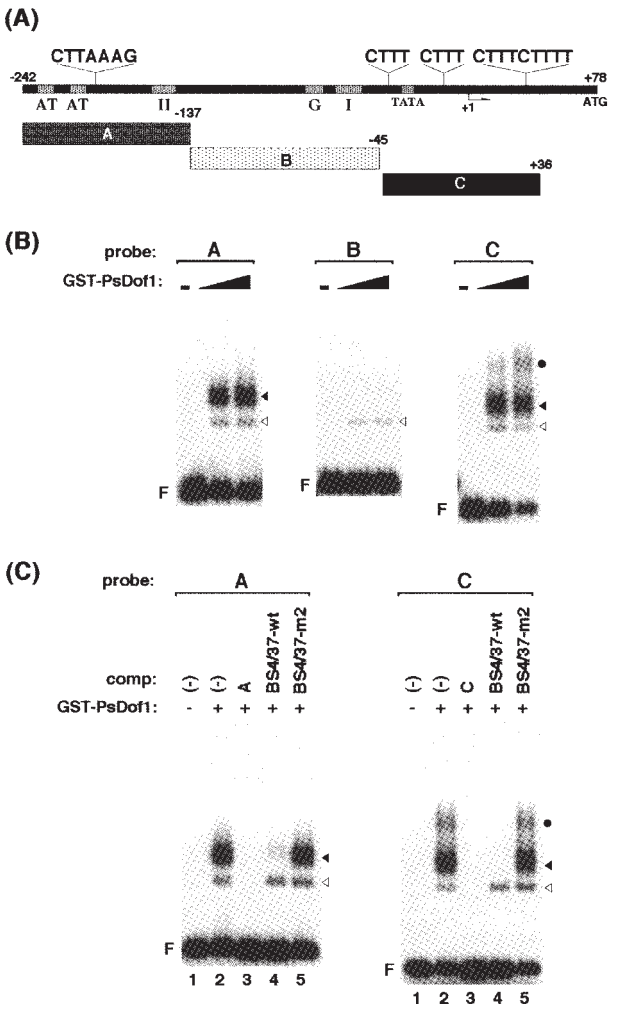

Fig. 2 Specific DNA-binding of GST-PsDof1 to *PsCHS1* promoter fragments. (A) Schematic representation of the *PsCHS1* promoter fragments (A, B and C) used in gel retardation assay. A map of the *PsCHS1* promoter from −242 to +78 is shown at the top, and potential PsDof1 binding sites (AAAG or its complementary sequence, CTTT) are indicated as inserts. The other marks are indicated in Fig. 1. (B) Gel retardation assay using labeled each fragment with absence (−) or increasing amount of purified GST-PsDof1 fusion protein. Specific retarded bands due to GST-PsDof1 fusion protein are indicated by closed circle and arrowheads. Non-specific complexes are indicated by open arrowheads. F denotes the free probes. (C) Binding competition assay using the radiolabeled fragments A (left panel) and C (right panel) as probes. Thirty-fold excess of each competitor DNA was added to probe DNA. Double stranded synthetic oligonucleotides, BS4/37-WT and BS4/37-m2 are putative optimal binding sequence and its modified sequence without binding ability for PsDof1, respectively. The marks are referred to the legend in Fig. 2B.

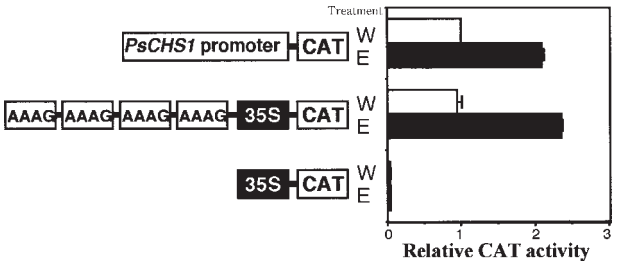

Fig. 3 Elicitor-responsive transcriptional activation of the repeat sequence of AAAG motif. Chimeric constructs were electroporated into pea protoplasts, and CAT activity was examined 12h after the treatment with water (W) or elicitor (E). *PsCHS* promoter was used as an elicitor-inducible promoter for positive control, whereas CaMV 35S minimal promoter (−44 to +4) was used for negative control. Tetramerized units of AAAG sequence motif (BS4/37-WT) were connected with CaMV 35S minimal promoter.

observed in wild type *PsCHS1* promoter.

Discussion

In the DNA binding site selection assay for PsDof1 protein, we isolated relatively many oligonucleotides that possess two sets of AAAG or CTTT motifs[8], suggesting that plural units of AAAG and CTTT sequences might enhance binding affinity to PsDof1 protein. However, in the ordinary DNA sequence, AAAG and CTTT motifs appeared relatively frequently. Thus, it remains unclear how the individual Dof proteins recognize their specific target genes *in vivo*. Different preferences of each Dof protein in the flanking sequence around the AAAG core may participate in determining the specificity. However, the effect of flanking sequences is limited. Yanagisawa and Schmidt reported that maize Dof1, 2 and PBF were able to recognize some identical sequences (e.g. GTTTAAAGGGG) as optimal[15]. Thus, the flanking sequences cannot be the only determinant of specificity for target genes, and hence it is likely that other factors also contribute to specific interaction of Dof proteins with their target genes *in vivo*. One possibility is that Dof proteins recognize their target genes through the interaction with other regulatory factors. In fact, it has already been reported that some Dof proteins interact with other regulatory factors, and these may modulate protein-DNA interactions. The *Arabidopsis* Dof protein OBP1 interacts with OBF proteins, bZIP factors that bind *ocs* element in the CaMV 35S promoter and related sequence in the *Arabidopsis* glutathione *S*-transferase-6 (GST6) gene promoter, and stimulates the binding of OBF proteins to *ocs* element[1,17]. *Ocs* elements are known to be activated by auxin as well as by the plant defense signals, salicylic acid and hydrogen peroxide[1,16]. In both the CaMV 35S and *GST6* promoters, the binding sites for OBP1 and its interacting OBF proteins are closely located. The maize PBF also interacts with a bZIP protein, Opaque2 (O2). Again, the binding sites of O2 and PBF are closely located in the target 22-kDa zein gene promoter[10]. The maize Dof1 interacts with HMG1 protein[14]. Thus the interaction with specific partners might produce diversity and specificity for the target promoters *in vivo*.

In this study, we demonstrated that PsDof1 specifically binds to AAAG containing sequences in the *PsCHS1* promoter (Fig. 2). We have previously revealed by transient transfection assays, that maximal elicitor-mediated activation of *PsCHS1* requires the combined effects of several distinct *cis*-acting elements, the unit of adjacent G-Box and Box I (homologous to H-Box in bean *chs15* gene promoter[2]), Box II (homologous to Box P in parsley *PAL* gene promoters[5]) and AT-rich repeated sequences[6,7]. We have also found that these *cis*-acting elements directed maximal elicitor-mediated activation only in combination with minimal *PsCHS1* promoter (−44 to +78), whereas their combination with the minimal CaMV 35S promoter (−44 to +4) resulted in greatly reduced elicitor-responsiveness[6]. This strongly suggests that the region from −44 to +78 (encompassing the fragment C in Fig. 2A; region from −44 to +36) also contains important regulatory sequence(s) involved in elicitor-mediated activation of *PsCHS1*. Interestingly, the results of gel retardation assays showed that multiple, (at least two), PsDof1 binding sites are located in this region (Fig. 2B). In addition, possible PsDof1-binding sequences, that is, AAAG-containing sequences are found at similar positions in the promoter of other members of elicitor-responsive pea *CHS* and *PAL* genes (Fig. 1). These findings imply that PsDof1 binding sequences might be regulatory elements involved in elicitor-mediated activation of *PsCHS1*; however, other sequences are also critical for the regulation of this complex promoter. Recently, a soybean cDNA encoding a novel bZIP protein, G/HBF-1, which binds to both the G-Box and the adjacent H-box in the bean *chs15* promoter has been cloned. During pathogen-induced activation of *chs15* gene, the transcript and protein levels of G/HBF-1 do not change; however it is rapidly phosphorylated and phosphorylation enhances binding to the *chs15* promoter[2]. Therefore, G/HBF-1 is thought to direct the elicitor-mediated transcriptional activation through the interaction with adjacent G-box and H-Box. A

similar structure, a unit of adjacent G-box and Box I (highly homologous to H-Box), in the *PsCHS1* promoter has also been demonstrated to be important for elicitor-mediated activation[6]. Interestingly, we can find the PsDof1 binding site about 20 bp downstream of the Box I motif (Figs. 1 and 2). These findings allow us to speculate that G/HBF-1 homolog and PsDof1 interact with each other on the *PsCHS1* promoter, although at this stage it is not known whether G/HBF-1 homolog exists in pea. Thus, analyses on protein-protein interactions should provide new insights for specific interaction between PsDof1 and target promoter(s) *in vivo*.

Interestingly, tetramerized units of AGGG motif responded to elicitor treatment, and conferred CAT activation (Fig. 3). The result indicated that positive regulatory factor binds to the sequence of tetramerized units of AGGG motif. Presently, it is not clear whether PsDof1 binds promoter sequence of *PsCHS1* and activate transcription *in vivo*, because Dof type transcription factors consist of multigene family, and almost all Dof proteins characterized so far have binding activity to AAAG motif. Recently, we also isolated six novel cDNAs encoding different Dof proteins from pea (data not shown). Further studies are needed to investigate the transcriptional regulation and possible involvement of Dof proteins in plant defense genes.

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エンドウのエリシター誘導性遺伝子発現における AAAG モチーフと PsDof1 タンパク質の関与

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エリシターを処理したエンドウ上胚軸由来の RNA から作成された cDNA ライブラリーからエリシター処理により発現が増高する遺伝子候補の cDNA として *PsDof1* が単離された．大腸菌で生産された GST-PsDof 1 融合タンパク質は AAAG 配列をコアとする DNA に結合することが明らかにされている．本論文では GST-PsDof 1 がエリシター応答性遺伝子の一つ，*PsCHS1* のプロモーター上の AAAG または CTTT 配列を有する断片に特異的に結合することを明らかにした．更に AAAG 配列のエリシター応答性シスエレメントとしての機能を解析するため，AAAG 配列を 4 回繰り返したユニットを CaMV 35 S の最小プロモーターと CAT レポーター遺伝子に連結したキメラ遺伝子を構築し，エンドウプロトプラストにエレクトロポレーション法により導入した．CAT 活性を指標にプロモーター活性を調べたところ，AAAG 配列を有するプロモーターは，エリシター処理により活性化されることが明らかとなった．これらの結果は PsDof 1 がエリシター応答性防御遺伝子のプロモーター上の AAAG 配列に結合し，転写を活性化させる可能性を示唆している．