ELSEVIER

Biochimica et Biophysica Acta 1520 (2001) 54–62

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Identification of Dof proteins with implication in the gibberellin-regulated expression of a peptidase gene following the germination of rice grains

Kenji Washio *

Laboratory of Environmental Molecular Biology, Division of Bioscience, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan

Received 14 February 2000; received in revised form 11 May 2001; accepted 16 May 2001

Abstract

Type III carboxypeptidase (CPD3) is one of the hydrolytic enzymes whose expression is up-regulated by gibberellins (GA) in the aleurones of germinated cereal grains. A number of pyrimidine boxes and a sequence resembling the gibberellic acid response element (GARE) are observed in the region upstream of the transcription initiation site of the CPD3 gene, showing a characteristic of cereal GA-responsive genes. Transient gene expression assays in germinated rice aleurone demonstrated that the CPD3 promoter was able to confer hormonally responses on the expression of the reporter gene. By southwestern screening, several cDNAs encoding the Dof class proteins were isolated from a rice aleurone library. Each mRNA accumulation for five novel members of Dof proteins (OsDof1–5) occurs with a different time course and in a tissue-specific manner following the germination of grains. Of these, the expression of the OsDof3 gene is abundant in aleurones where it precedes that of the CPD3 gene, implying that this is an early response gene of GA. The OsDof3 protein, expressed in *Escherichia coli*, selectively bound AAAG motifs of the pyrimidine boxes through the DNA-binding activity of its Dof domain. Co-expression experiments in aleurones suggested that the OsDof3 protein should play a regulatory role in the expression of the CPD3 gene under the control of GA.

Keywords: Carboxypeptidase; Dof protein; Germination; Gibberellin; Glycine-rich protein; Rice

1. Introduction

Gibberellins (GA) are potent phytohormones that play crucial roles in the development and growth of plants [1]. During the germination of cereal grains, aleurone cells respond to GA by synthesizing and secreting several kinds of hydrolytic enzymes that participate in the mobilization of storage molecules. The synthesis of these hydrolytic enzymes, such as α-amylase, is accompanied by activation of individual gene expression [2]. Attempts have been made to clarify the mechanism of such activation, with particular emphasis on the promoters of the cereal α-amylase genes. The GA-induced activities of the α-amylase promoters were proved to be achieved by the concerted actions of several promoter motifs composed of pyrimidine, TAACAA/GA, TATCCAC/T boxes and so on [3]. The TAACAA/GA box could alone confer GA responsiveness on the expression of the reporter gene in aleurone protoplasts [4] and has been recognized to be a potent gibberellic acid response element (GARE).

Gubler et al. identified a Myb-related protein (GAMYB) from barley [5], by referencing the fact that the core sequence of GARE contained the nucleotide sequence of the Myb-binding site. GAMYB could transactivate the promoter of a high-p*I* α-amylase gene by binding to GARE, and its own expression seemed to be up-regulated by GA at the early stage of germination. GA-responsive promoters of another class of hydrolytic enzymes, β-glucanase and cathepsin B-like protein, were similarly transactivated by GAMYB [6]. These evidences suggest that GAMYB plays a central role in the transcription activation of genes that mediates the GA actions governing the germination process. In addition to GAMYB, three putative transcription factors with a zinc finger motif, HRT [7], ABF-1, and ABF-2 [8], were identified in southwestern screening using the promoter fragments of α-amylase genes. HRT showed a repressor function on the expression of an α-amylase gene; however, the functions of ABF

* Fax: +81-11-706-2253.
E-mail address: washi@ees.hokudai.ac.jp (Kenji Washio).

0167-4781/01/$ – see front matter
PII: S0167-4781(01)00251-2

proteins remained unknown. Complex transcriptional machinery coordinated with these factors seems to be required for full activation of the GA-responsive promoter.

We previously determined the primary structure of a rice gene for a type III carboxypeptidase (CPD3) and studied its expression following the germination of grains [9]. The corresponding mRNA was abundant in germinated aleurones and the mode of expression of the gene seemed to be similar to that of a high-p*I* α-amylase. In this study, I evaluated in detail the activity of the CPD3 promoter influenced by the treatment of GA in germinated rice aleurones. The nature of the promoter region necessary for the GA response of the CPD3 gene was substantially similar to but characteristically dissimilar to those of previously reported GA-responsive promoters. An aberrant adenine-rich region, including the promoter motifs conserved among the GA-responsive genes, conferred a hormone responsiveness on the CPD3 promoter. By southwestern screening, I identified a Dof class protein present in germinated aleurones and confirmed relevant interaction with the CPD3 promoter region. The data allowed me to propose an additional model for the transcription response of GA, with the recruitment of the function of a Dof class transcription factor.

2. Materials and methods

2.1. Plant material

Rice grains (*Oryza sativa* L. cv. Yukihikari; kindly supplied from the Hokkaido Central Agricultural Experiment Station, Iwamizawa, Japan) and deembryonated half-grains were surface sterilized in 2.5% sodium hypochlorite for 20 min, rinsed throughly, immersed in water containing 20 mM $CaCl_2$, 10 μg/ml chloramphenicol, 10 μg/ml ampicillin, and 25 units/ml nystatin, and grown in the dark at 30°C. Germinated grains were dissected into leaves, roots, scutella, and endosperms containing the aleurone layer at appropriate times. Samples were immediately frozen in liquid nitrogen.

2.2. Southwestern screening

The poly(A)$^+$ RNA was prepared from deembryonated half-grains that had been grown with 10^{-6} M GA_3 for 2.5 days, by the previously described method [9]. An expression cDNA library was constructed in λgt11, as described previously [9].

The probe DNA was generated by ligation of the double-stranded oligonucleotide that corresponded to the CPD3 promoter sequence (nucleotide positions −190–−133). The concatenated oligonucleotide DNA was labelled with a nick translation kit (Takara Shuzo, Kyoto, Japan). Screening was performed by the method of Vinson et al. [10] with slight modifications. Nitrocellulose filters of plates were incubated with radiolabelled probe in a buffer (20 mM HEPES–KOH (pH 7.5), 50 mM KCl, 0.25 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 50 μg/ml sheared calf thymus DNA) at 4°C for 2 h. Each cDNA excised from positive clones was subcloned into a plasmid vector (pBluescript_II SK(+), Stratagene, CA, USA). Nucleotide sequences were determined using the Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech, UK) and the LI-COR 4000L DNA autosequencer (LI-COR, USA).

2.3. Northern analysis

Total RNA was extracted from frozen samples by the sodium dodecyl sulfate (SDS)–phenol method, as described previously [9]. Samples of RNA were fractionated on a 1.2% agarose gel that contained 0.66 M formaldehyde. After electrophoresis, RNAs were blotted onto nylon membranes (Hybond-N$^+$, Amersham Pharmacia) and hybridized with a radiolabelled probe prepared from each cDNA clone as follows. The *Pst*I–*Eco*RI (nucleotide positions 946–2316), the *Sph*I (1020–1425), the *Sac*I–*Eco*RI (608–1572), the *Sac*I (955–1331), the *Pst*I–*Nco*I (884–1174), and the *Xho*I–*Eco*RI (1237–1728) fragments were prepared from cDNA clones that encoded OsDof1, -2, -3, -4, -5, and CPD3, respectively, and each was labelled by the random priming procedure.

2.4. Expression of recombinant protein in Escherichia coli

A 1164 bp cDNA fragment, spanning from nucleotide positions 120 to 1283 in the OsDof3 cDNA clone, was amplified by PCR using oligonucleotide primers as follows: the forward primer was 5′-TGAGATATCGCGAGCGGCGGCGCATTATCC-3′ that incorporated an *Eco*RV site (underlined) on a first initiation codon by which the first ATG residue changed to Ile; the reverse primer was 5′-TCTGGATCCAACCTTGGCATGGTTGATTTG-3′ that added a new *Bam*HI site at the 3′ end of the PCR product. This PCR fragment was cloned into an *Eco*RV–*Bam*HI-digested pET32a vector (Novagen, USA) to generate the His-tagged thioredoxin (Trx) fusion protein.

E. coli strain BL21(DE3) was transformed with the expression plasmid. An overnight culture was grown for the middle log phase at 37°C. Expression of recombinant proteins was induced by the addition of 0.5 mM IPTG for 3 h at 37°C, and purification was achieved by Ni–agarose affinity chromatography following the manufacturer's instructions (Novagen). To remove the Trx domain, the fusion proteins were cleaved with enterokinase (Novagen).

2.5. Electrophoretic mobility shift assay

Nucleotide substitutions on the CPD3 promoter shown in Fig. 5A were generated by a PCR-based in vitro muta-

genesis according to the manufacturer's instructions (Takara Shuzo). The *Xho*I/*Dra*I fragment (−274–−94) of the CPD3 promoter and its derivatives were end-labelled by a fill-in reaction with a [α-^{32}P]dNTP mixture (3000 Ci/mM, ICN, USA) and Klenow enzyme, and gel purified. Reactions were performed in 20 μl of a mixture containing 20 mM HEPES–KOH (pH 7.9), 100 mM KCl, 1 mM DTT, 0.02% Nonidet P-40, 4% (w/v) Ficoll 400, 0.5 μg poly(dI-dC), 0.5 μg poly(dA-dT), 0.2 ng of radiolabelled probe (approximately 20 000 cpm) and 5.0 ng of the OsDof3 protein. When required, 1.0 ng of competitor DNA was added to the reaction as described in Fig. 5B. After incubation for 30 min at room temperature, the DNA–protein complexes were resolved on a 4% polyacrylamide gel with 0.5×TBE (1×TBE: 89 mM Tris–HCl, 2 mM EDTA) at 120 V for 3.5 h at 4°C. The gel was dried and subjected to autoradiography.

2.6. Plasmid constructions for the biolystic method

In a preliminary analysis, a reporter gene fused to the CPD3 promoter in a translational fusion failed to detect the reporter activity in aleurones; therefore, I investigated synergistic effects of the CPD3 promoter on the activity of a minimal CaMV35S promoter [11]. The *Xho*I–*Dra*I fragment (−274–−94) excised from the CPD3 gene [9] and its mutated derivatives were ligated with a gene cassette that carried a −90 region of the CaMV35S promoter, a firefly luciferase gene (F-luc, Promega, USA), and the 3′ terminator of a gene for nopaline synthetase (Nos-ter). The ligated DNA fragment was cloned between restriction sites for *Hin*dIII and *Eco*RI in the plasmid, pUC18. An effector plasmid was created by the insertion of the *Bgl*II fragment (100–1282) of the OsDof3 cDNA between the CaMV35S promoter and the Nos terminator of an expression vector pBI221 (Clontech, USA) in a correct orientation. Similarly a reference plasmid was generated by the insertion of a gene for a *Renilla* luciferase (Promega) between the −90CaMV35S promoter and the Nos terminator in pUC18. The −90CaMV35S promoter is lacking potential sites for binding of Dof proteins which are observed in the original CaMV35S promoter [12], and whose activity seems not to be influenced by any hormone treatment [4].

2.7. Transient gene expression of germinated rice aleurone

Particle bombardment was carried out with a biolystic helium gun device (IDERA GIE-III, Tanaka Co., Ltd., Sapporo, Japan). DNA-coated gold particles were simply prepared by mixing 20 μl of gold suspension (50 mg/ml; 1.5–3.0 μm in size) and 30 μl of plasmid solution including 1.0 μg of the reporter plasmid, 2.0 μg of the reference plasmid, and if needed, either 0.5 μg of the effector plasmid or an equal molar amount of pUC18. Coated gold particles were recovered by ethanol precipitations, and finally resuspended in 320 μl of ethanol. For the bombardment, a capture disk of Teflon mesh was used. 4 μl of particles, dispersed by brief sonication, were spotted onto the center of the mesh, and bombarded into tissues by mediating the flow of helium (vacuum, 600 mm Hg; pressure, 4.5 kgf/cm^2).

Embryo-less half-grains of rice were grown in the dark for 16 h at 30°C as described above. The pericarp of each grain was removed with forceps. Groups of 12 grains were twice bombarded with plasmid DNAs, and separated into subgroups of grains. Bombarded grains were incubated in the absence (no hormone), or in the presence of 10^{-6} M GA_3, or in the presence of 10^{-6} M GA_3 plus 10^{-5} M ABA for 3.5 days, at which time they were ground into powder in liquid nitrogen and then suspended in 100 μl of a lysis buffer (dual-luciferase reporter assay system, Promega). The cell homogenate was placed in an Eppendorf tube and centrifuged at 12 500 rpm for 5 min at 4°C. A 20 μl aliquot of the supernatant was subjected to a dual-luciferase assay of firefly and *Renilla* luciferase activities, following the manufacturer's instructions. Total light units emitted from each reporter reaction were counted by a Lumat LB9501 luminometer (Berthold, Germany). The relative firefly luciferase activities from each reporter plasmid, normalized with reference to *Renilla* luciferase activity due to the internal control, are shown. Each reporter plasmid was assayed at least three times and averaged.

3. Results and discussion

Several key motifs similar to the promoter sequences conserved among the GA-responsive genes, that is the pyrimidine and GARE-like sequences found in the nucleotide sequence near the transcription initiation site of the CPD3 gene, constitute a characteristic of the GA-responsive promoter (Fig. 1). To explore the promoter activities that were influenced by GA, helium gas-mediated transfections of a luciferase reporter gene into rice aleurones were carried out (Fig. 2). The promoter fragment of the CPD3 gene had a stimulatory effect on the expression of a reporter gene driven by a minimal region (−90CaMV35S) of the cauliflower mosaic virus 35S promoter (CaMV35S) [4]. The plasmid with the CPD3 promoter generated a 6.7-fold higher level of reporter activity in grains that were treated with exogenous GA_3 than that in the non-treated control. The GA induction was abolished by the addition of abscisic acids (ABA), antagonistic hormones against GA. The upstream portion of the CPD3 promoter (−274–−94) conferring hormonally responses contains the pyrimidine and GARE-like sequences, suggesting that the transcriptional machinery involved in the GA-dependent expression of the CPD3 gene is substantially identical to those proposed in many α-amylase genes.

In an attempt to isolate cDNA clones encoding proteins that bind the promoter region of the CPD3 gene, I carried out southwestern screening with a bait of the synthetic

```
ctcgagcagagaaatagaaccgtaagtagtagtacaatacgaatttaagcggaatcagctcagattccac   -206
 XhoI
acgaggacaaattcgtattcgagctcgaaaaggcaaaaaagaaaccaaaaaacagaaaaaaaaaaagaaaagg   -136
                      SacI        pyrimidine  GARE-like  pyrimidine
aatccggctgtgcttggattgcattggaagcaaagttcgtttaaaaaaaaaaactaaaactaataaacca   -66
                                      DraI
gtccgtttcaaacaccaaaaccgcgaacgcTATAAATTcggagggaaaacctctccgaatctccaAactc    +5

ctccaATGGCGACCGCGCGCGTCTCCCTCATCCTCCTCGTCGTCGTCCTCGCCGCCTCCGCCTGCGCGGA   +75
    M  A  T  A  R  V  S  L  I  L  L  V  V  V  L  A  A  S  A  C  A  E
```

Fig. 1. Nucleotide sequences near the initiation site for the transcription of the rice CPD3 gene. The sites of restriction enzymes used to release DNA fragments are underlined. Conserved sequences found in the GA-inducible genes are indicated in bold letters. A TATA box, the site of initiation of the transcription, and the translated regions are shown by capital letters. The databank accession number of the rice CPD3 gene is D10985.

oligonucleotide derived from the CPD3 promoter (−190–−133), closely associated with a high sensitivity to GA. Several clones were purified and confirmed to bind the probe with similar intensities. Sequence analysis of cDNA inserts indicated that two clones had identical cDNA fragment encoding a protein similar to the Dof proteins.

The Dof protein is a novel class of DNA-binding proteins possessing a plant-specific variant of the zinc finger motifs (Cys2–X21–Cys2), designating the Dof domain [13,14]. It has been recently shown that Dof proteins are real transcription factors regulating the expression of plant genes in response to a variety of factors including light [15], developmental stage [16], and hormone treatment [17]. Binding of the Dof proteins to promoter elements of target genes occurs with related DNA sequences including a CTTT (or an AAAG) core [18], that is capable of matching the conserved nucleotide sequence of the pyrimidine box (YCTTTTY) [19]. These observations raise the possibility that the Dof-related proteins are implicated in the GA-dependent expression of genes by binding to the pyrimidine box.

Based on this assumption, classification and expression pattern of the Dof proteins present in the germinated grains were investigated. 16 clones were isolated in the course of rescreening the library. Sequencing inserted cDNAs revealed that all of them encoded Dof proteins by which the predicted amino acid sequences were arranged into the five novel members of the Dof proteins, named OsDof1 (*O. sativa* Dof protein) to OsDof5. The extent of homologies through the Dof domains is high among the five proteins, sharing around 70% sequence identities. When considering the whole sequences, similarities are scarcely detectable besides the Dof domains, although there is a slight similarity (62% sequence identities) between OsDof1 and OsDof2. The diversities of amino acid residues outside the Dof domain suggest distinct roles for each member of OsDof proteins.

The mRNA accumulations of OsDofs were examined by Northern analysis of RNA samples isolated either at varying times or from different tissues of germinated grains. Each transcript of OsDof genes appeared at a different time course and in a tissue-specific manner (Fig. 3). The most notable change in mRNA levels is for OsDof3. An immediate increase in the mRNA level is observed at the early stage of germination (2 h after inhibition) with this

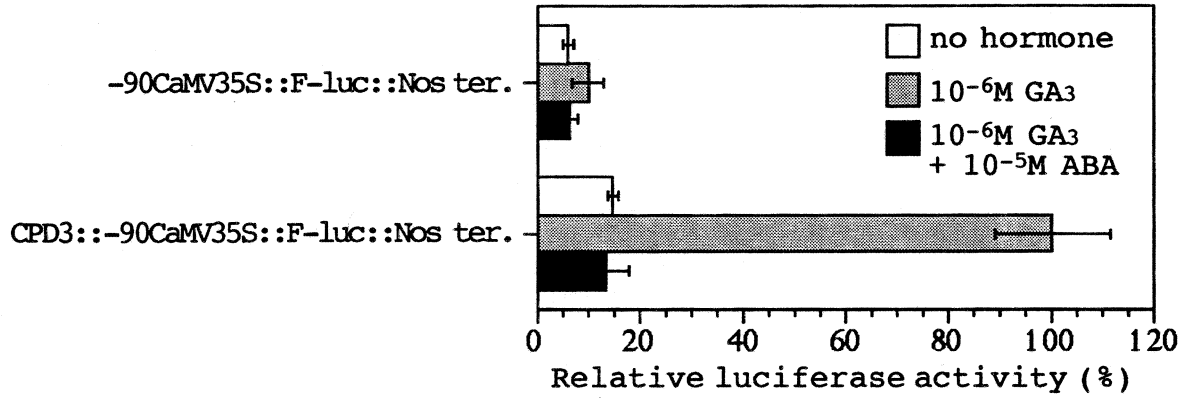

Fig. 2. Transient expression of the firefly luciferase reporter in rice aleurones. The reporter construct used in each transfection is schematically presented at the left of the panel. Relative firefly luciferase activities, estimated from cell lysate of deembryonated half-grains incubated in the absence and in the presence of 10^{-6} M GA_3, or in the presence of 10^{-6} M GA_3 plus 10^{-5} M ABA, are shown by open, shaded, and closed bars, respectively. Bars indicate standard errors. The luciferase activity from GA-treated half-grains transfected with the CPD3::−90CaMV35S::R-luc::Nos-ter was set to 100% in each case.

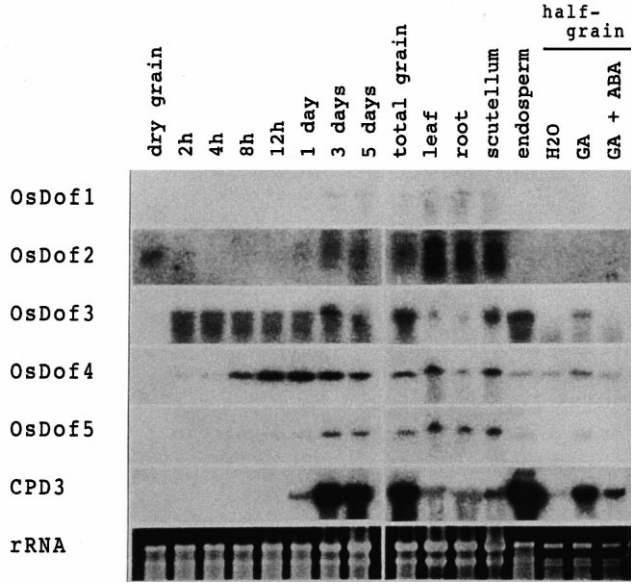

Fig. 3. Accumulation of the mRNAs for rice grain Dof proteins. Rice grains were incubated for different times (0 h, 2 h, 4 h, 8 h, 12 h, 1 day, 3 days, 5 days). Leaves, roots, scutellum, and endosperms were excised from 2.5-day-germinated grains. Deembryonated half-grains were incubated in the absence (H_2O), or in the presence of 10^{-6} M GA_3 (GA), or 10^{-6} M GA_3 plus 10^{-5} M ABA (GA+ABA). Total RNA (20 μg/lane for OsDofs, or 5 μg/lane for the CPD3 and rRNA) from each type of tissues was subjected to electrophoresis, blotted, and subjected to hybridizations with the probe indicated to the left of each panel. Ethidium bromide-stained RNAs are indicated to ensure the equal loading on each lane. The OsDof1–5 accession numbers are AB028129, AB028130, AB028131, AB028132, AB028133.

level remaining at the later stage. In 2.5-day-germinated grains, the mRNA accumulation of OsDof3 is conspicuous in the scutellum and endosperm containing aleurones. The spatial distribution of the OsDof3 gene expression parallels that of the CPD3 gene, and the temporal accumulation of the OsDof3 mRNA occurs before the expression of the CPD3 gene. Taken together, fluctuating amounts of the OsDof3 mRNA in deembryonated half-grain upon treatment of phytohormones and that OsDof3 seems to be an immediate early gene suggest an involvement of transcription responses of GA in germinated grains.

Fig. 4 shows the nucleotide and deduced amino acid sequences of the OsDof3 cDNA. The OsDof3 protein (OSDOF3) contains 371 amino acids with a predicted molecular mass of 37 030 Da. Near its N-terminal end, a 52 amino acid stretch typical of Dof proteins, spanning the Cys2–X21–Cys2 zinc finger, is observed. OSDOF3 has a number of interesting features in its C-terminal portion after the Dof domain. A high content of alanine and glycine residues (15.3 and 19.3%, respectively) exhibits a moderate hydrophobicity to this protein. A proline-rich region is also located. I did not found any significant homology between any of the other Dof proteins outside the Dof domain, except between cereal PBF proteins [16], that are Dof proteins specifically expressed in developing endosperms, mostly due to the clustering of glycine residues. OsDof3 seems to be a member of grain-specific Dof proteins in small cereal plants.

OSDOF3 was overexpressed as a His-tagged Trx fusion protein in *E. coli* from the pET32a vector and purified through Ni affinity chromatography. After removal of the Trx peptide, its ability to bind the promoter fragment of the CPD3 gene was tested in electrophoretic mobility shift assay (EMSA, Fig. 5). Incubation with a 68-mer oligonucleotide used in southwestern screening resulted in formation of several retarded bands (Fig. 5B), due in part to secondary digestions of the fusion proteins with enterokinase, or because of the presence of multiple Dof-binding sites in the probe. This binding was effectively competed by excess molar amounts of unlabelled CPD3 promoter fragment (*Xho*I–*Dra*I, −274–−94). The *Xho*I–*Sac*I fragment, namely the first half (−274– −180), did not affect the interaction, whereas the second half (*Sac*I–*Dra*I, −185–−94) was a good competitor. Moreover, the binding ability of OSDOF3 was prevented in the presence of the metal chelator 1,10-phenanthroline. This compound effectively inhibits the DNA binding of the zinc finger of Dof protein [14]. Interaction should occur in the second half of the CPD3 promoter through the Dof domain of OSDOF3.

To further evaluate the specificities of binding of OSDOF3, I sequentially introduced nucleotide substitutions in the CPD3 promoter (Fig. 5A). The CPD3 promoter has two sets of tandem repeated binding sites for Dof proteins within an adenine-rich region. Each set of AAAG motifs, namely the first pyrimidine and the second pyrimidine boxes, had been mutated to ACAT. This version of a mutation proved to prevent the binding of several Dof proteins [20]. The binding of OSDOF3 was significantly reduced by the mutation on the first pyrimidine box (CM-1), while considerable amounts of binding were observed in the incubation with the CM-3 probe, in which the second pyrimidine box had been lost (Fig. 5C). There was no binding observed when a 2 bp mutation had occurred in all the AAAG motifs, demonstrating a way of binding to multiple AAAG motifs favoring the first pyrimidine box.

Transient expressions from reporter plasmids differ from the parent construct (CPD3) only in the presence of mutations on the promoter were assayed in order to address whether the promoter motifs observed in the CPD3 promoter were vital (Fig. 6). Distinct mutation on two pyrimidine boxes (CM-1, CM-3) resulted in similar reductions (40% reductions) in reporter activities in aleurones upon treatment of GA. Combinative mutation on two pyrimidine boxes (CM-1,3) significantly decreased both the absolute level of expression and the effect of GA. The most profound effects were observed with the mutation on a GARE-like sequence regardless of whether it occurred alone (CM-2) or present with the other mutations (CM-1,2,3). These results indicate vital activities of all the identified promoter motifs, and support an idea

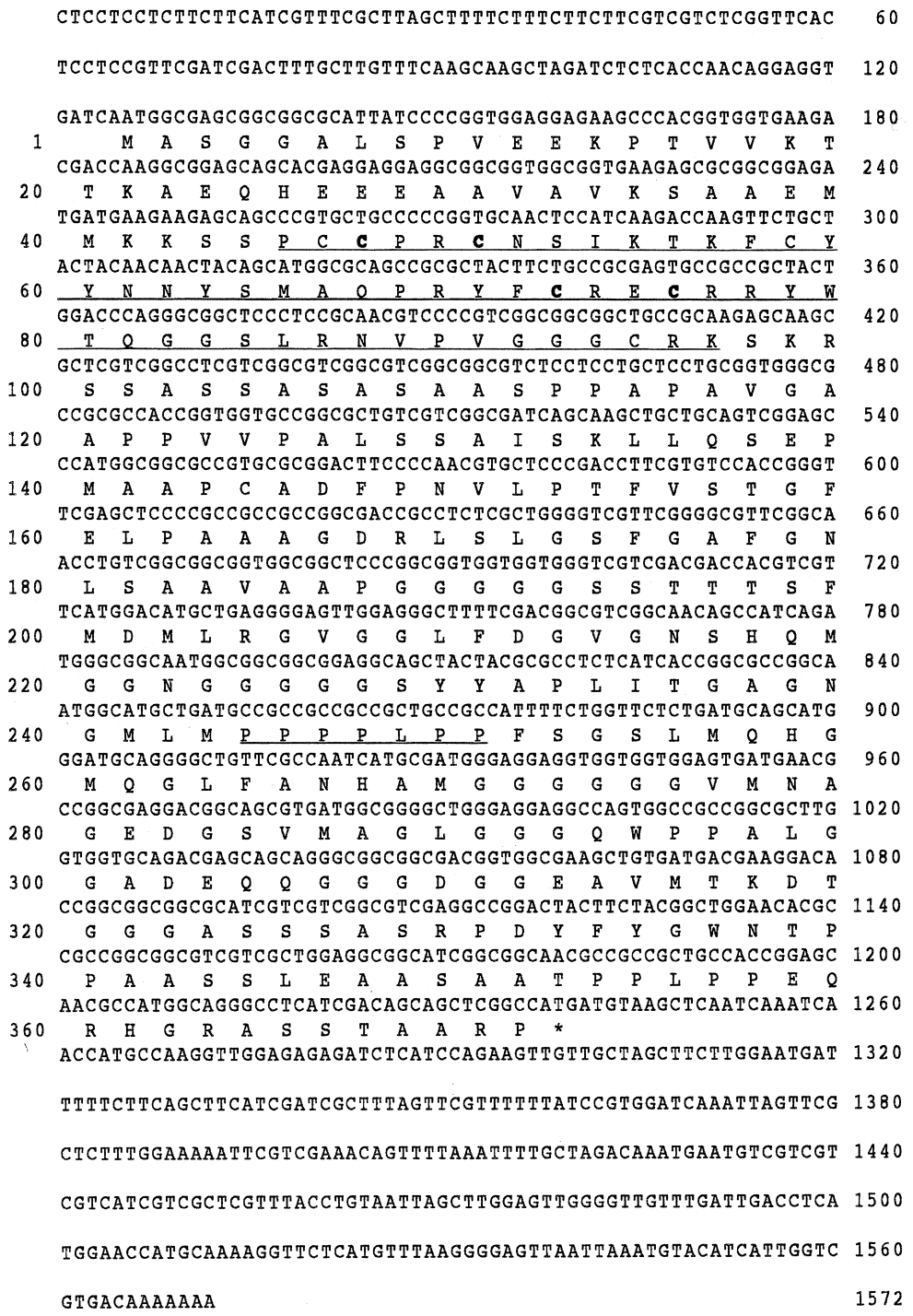

Fig. 4. Nucleotide sequence and the predicted amino acid sequence of the OsDof3 cDNA. The Dof domain in the N-terminus and a proline-rich region are underlined. The four cysteine residues putatively involved in the formation of a zinc finger are bolded. The OsDof3 accession number is AB028131.

that the GA-dependent activations of the CPD3 promoter are coordinated with the functions of multiple *cis* elements composed of a GARE-like sequence and perhaps the pyrimidine boxes.

The functional relevance of the interaction observed in EMSA between OSDOF3 and the AAAG motifs was investigated by assessing the effects of OSDOF3 on the activity of the CPD3 promoter in co-bombarded rice aleurones. An effector plasmid was designated to express OSDOF3 under the control of the CaMV35 promoter. The effects of OSDOF3 on the transcription of the CPD3 promoter were complex, since co-expression of OSDOF3 repressed the absolute levels of the reporter activities in the GA-treated aleurones. There are a number of possible explanations for these results, including unequal coating of gold particles in comparison with those in the

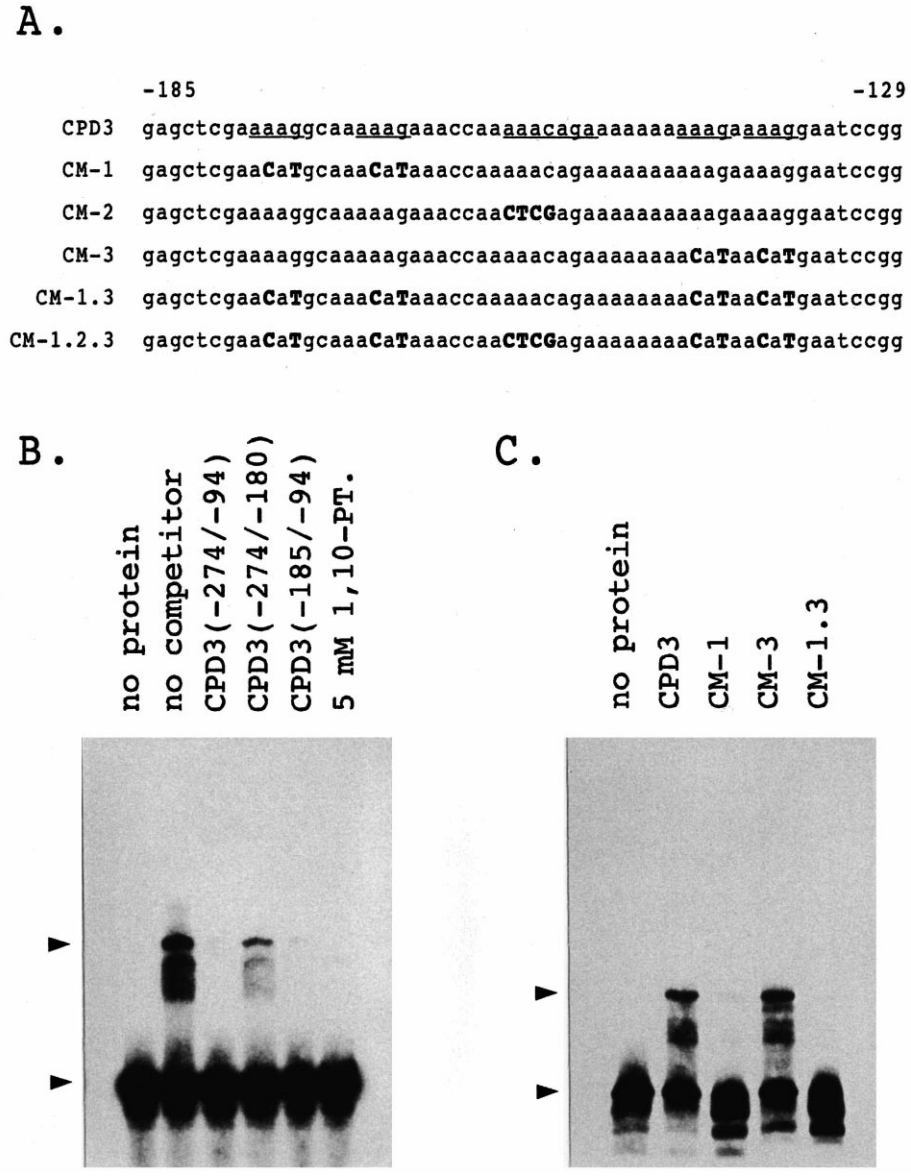

Fig. 5. Electrophoretic mobility shift assay (EMSA) with the recombinant OsDof3 protein. A: Nucleotide sequences of the wild-type promoter (CPD3) and mutated (CM) regions of the CPD3 gene (−185–−129) are shown. Four potential sites for the Dof recognition (AAAG) and GARE-like (AAACA-GA) sequences are underlined. Nucleotide changes introduced in the mutant promoters are indicated by bolded capital letters. B: DNA-binding activity of recombinant OsDof3 protein. Binding reactions were performed without competitor DNA (no competitor), or with excess amounts of non-labelled promoter fragments identified above each lane, or in the presence of 5 mM 1,10-phenanthroline (5 mM 1,10-PT). Free and bound probes are indicated by arrows. C: Binding specificities of the OsDof3 protein. Binding reactions were performed with 5.0 ng of OSDOF3 and 0.2 ng of radiolabelled promoter fragments identified above each of the lanes.

non-effector experiments, overall reduction of GA responses by proliferating aberrant amounts of OSDOF3 in the cells, and/or this protein is not a transcription activator. The exogenous OSDOF3 reduced the GA-induced level of expression from a mutated form of the CPD3 promoter, even though it had no recognition sites for Dof proteins (CM-1,3 pro+OsDof3), by 35.9% compared with the non-effector control (CM-1,3 pro). This result suggests that overexpression of OSDOF3 causes transcriptional repression through non-specific toxicity to the cells, indeed overexpression of an *Arabidopsis* Dof protein (OBP3) resulted in severe morphological defects in the transgenic plants [20]. Precise analysis is necessary to fully evaluate the role of OSDOF3 in the GA-dependent expression of the CPD3 gene in other experimental procedures.

The promoter region conferring the GA responses on the CPD3 promoter is characteristically different from other GA-responsive promoters; that is, it is very rich in adenine residues. My analysis of the CPD3 promoter indicates that multiple promoter elements are necessary for high-level hormonal regulation of expression in rice aleurones. The result shows the existence of at least three regions of the promoter that potentially contain active *cis* elements. These are two pyrimidine boxes and a GARE-

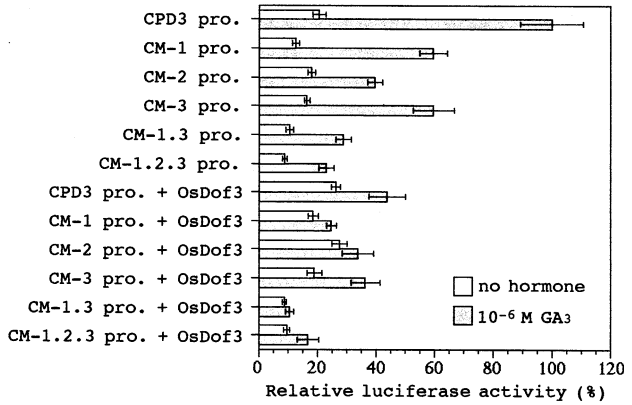

Fig. 6. Transient expression affected by mutations on the CPD3 promoter and by co-expression of the OsDof3 protein in rice aleurones. The reporter and effector constructs used in each transfection are schematically indicated at the left of the panel. Relative luciferase activities from the samples incubated in the absence (no hormone), or in the presence of 10^{-6} M GA_3 are shown by open or shaded bars, respectively. The luciferase activity from the GA-treated sample transfected with only the wild-type reporter construct was set to 100% in each case.

like sequence. The data of transient gene expressions suggest that these elements act in concert, in that mutations of any one region cause severe reduction of the reporter expression, and this is consistent with the general concept that different GA response complexes may exist in a diverse group of GA-responsive promoters sharing common promoter motifs such as GARE [3]. There is no evidence as to whether GAMYB virtually binds to the CPD3 promoter; however, the most effective decline in the reporter activity achieved by mutation on a GARE-like sequence indicates the possibility that GAMYB could transactivate the CPD3 promoter by binding a variant sequence of GARE.

Although the pyrimidine box is generally found in the promoter region of cereal GA-responsive genes [19], it has been obscure what the real function of this promoter motif is in the GA-dependent expression of genes. Mutation on the pyrimidine box of some GA-responsive promoters resulted in slight reductions in the reporter activities in transient expressions, with a lesser magnitude than that observed by mutation on GARE [21,22]. Oligonucleotides containing the pyrimidine box alone failed to confer a GA responsiveness onto the minimal CaMV35S promoter [4]. These observations suggest an accessory role of the pyrimidine box in the expression of the GA-responsive genes, coupled with GARE-associating factor(s), GAMYB.

In this study I have shown that several Dof proteins are present in germinated rice aleurones and encode proteins capable of binding AAAG motifs. These experimental results prolong assigning conclusively the roles of the pyrimidine box. The fact that OSDOF3, expressed in *E. coli*, binds to the pyrimidine boxes of the CPD3 promoter in a sequence-specific manner, together with a correct matching between the Dof recognition and the pyrimidine box sequences, strongly supports an idea that Dof class protein is a pyrimidine box-binding protein. Despite the preferential binding of OSDOF3 to the first pyrimidine box of the CPD3 promoter, the presence of the second pyrimidine box further sustains additional promoter activities without a native first pyrimidine box. This suggests that multiple interactions between distinct class of Dof proteins and separate Dof-binding sites including pyrimidine boxes might lead to increased levels of the CPD3 gene expression; in fact, Northern analysis indicates appearance of other Dof proteins such as OSDOF4 and OSDOF5 in germinated aleurones.

The expression profile of the OsDof3 mRNA, showing features of an early response gene of GA, associates a positive role in the CPD3 gene expression; contrarily, co-expression experiments in GA-treated aleurones presented no vital roles on the induction of the CPD3 promoter. It seems difficult to speculate native functions of a protein generated by artificial expressions such as the biolystic method, but similar procedures showed reliable functions of Dof proteins that participated in the expression of corresponding target genes. Two maize Dof proteins, Dof1 and Dof2, were reported to have distinct transcriptional activities in transfected leaf protoplasts [15]. Dof1 activated the light response of the C4PEPC gene promoter, while the effect of Dof1 was eliminated by Dof2. Previously described barley PBF that transfected in developing endosperms using particle bombardment could increase B-hordein expression [16]. These data are consistent with a model in which each Dof protein acts itself as an autonomous transcriptional regulator, but the possibility that its effect might be due to recruitment of another factor(s) having pivotal function(s) on transcription can not be ruled out. Relevant interaction between the maize PBF and the b-ZIP protein Opaque-2 was experimentally confirmed [23].

The C-terminal part of OSDOF3 besides the Dof domain contains glycine-rich regions, accounting for an overall content of 16.2%. The glycine content of OSDOF3 is lower than those of the other typical glycine-rich proteins (GRPs, 20–70%) [24]. In plants, at least three types of glycine-rich repeats, namely GGGX, GGXXXGG, and GXGX (X is any amino acid), can be observed in a set of GRPs such as cell wall-located, pathogen-related, stress-induced, and RNA-binding proteins. A lower content and incomplete clustering of glycine residues in OSDOF3 make it difficult to categorize this protein into a specific group of GRPs. The biological function of GRPs is still obscure but the high flexibility of glycine-rich peptides is thought to make GRP a good candidate for interaction with other protein(s). In this context, OSDOF3, binding to the pyrimidine box, may potentiate transcriptional activation of another transcription factor(s) such as GAMYB, through protein/protein interaction. This accessory role is in agreement with the presumed function of the pyrimidine box described by researchers [21]. Further

investigations are needed to clarify the correct functions of this Dof protein on the transcription response of GA.

References

[1] R. Hooley, Gibberellin: perception, transduction and responses, Plant Mol. Biol. 26 (1994) 1529–1555.

[2] J.V. Jacobsen, L.R. Beach, Control of transcription of α-amylase and rRNA genes in barley aleurone protoplasts by gibberellic acid and abscisic acid, Nature 316 (1985) 275–277.

[3] J.V. Jacobsen, F. Gubler, P.M. Chandler, Gibberellin action in germinated cereal grains, in: P.J. Davies (Ed.), Plant Hormones, Kluwer Academic Publishers, Dordrecht, 1995, pp. 246–271.

[4] K. Skriver, F.L. Olsen, J.C. Rogers, J. Mundy, *cis*-Acting DNA elements responsive to gibberellin and its antagonist abscisic acid, Proc. Natl. Acad. Sci. USA 88 (1991) 7266–7270.

[5] F. Gubler, R. Kalla, J.K. Roberts, J.K. Jacobsen, Gibberellin-regulated expression of a *myb* gene in barley aleurone cells: Evidence for Myb transactivation of a high-pI α-amylase gene promoter, Plant Cell 7 (1995) 1879–1891.

[6] F. Gubler, D. Raventos, M. Keys, R. Watts, J. Mundy, J.V. Jacobsen, Target genes and regulatory domains of the GAMYB transcriptional activator in cereal aleurone, Plant J. 17 (1999) 1–9.

[7] D. Raventós, K. Skriver, M. Schlein, K. Karnahl, S.W. Rogers, J.C. Rogers, J. Mundy, HRT, a novel zinc finger, transcriptional repressor from barley, J. Biol. Chem. 273 (1998) 23313–23320.

[8] P.J. Rushton, H. Macdonald, A.K. Huttly, C.M. Lazarus, R. Hooley, Members of a new family of DNA-binding proteins bind to a conserved *cis*-element in the promoter of α-*Amy2* genes, Plant Mol. Biol. 29 (1995) 691–702.

[9] K. Washio, K. Ishikawa, Structure and expression during the germination of rice seeds of the gene for a carboxypeptidase, Plant Mol. Biol. 19 (1992) 631–640.

[10] C.R. Vinson, K.L. LaMarco, P.F. Johnson, W.H. Landschulz, S.L. McKnight, In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage, Genes Dev. 2 (1988) 801–806.

[11] P.N. Benfey, L. Ren, N.H. Chua, The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns, EMBO J. 8 (1989) 2195–2202.

[12] S. Yanagisawa, K. Izui, Molecular cloning of two DNA-binding proteins of maize that are structurally different but interact with the same sequence motif, J. Biol. Chem. 268 (1993) 16028–16036.

[13] S. Yanagisawa, Dof DNA-binding proteins contain a novel zinc finger motif, Trends Plant Sci. 1 (1996) 213–214.

[14] S. Yanagisawa, A novel DNA-binding domain that may form a single zinc finger motif, Nucleic Acids Res. 23 (1995) 3403–3410.

[15] S. Yanagisawa, J. Sheen, Involvement of maize Dof zinc finger proteins in tissue-specific and light-regulated gene expression, Plant Cell 10 (1998) 75–89.

[16] M. Mena, J. Vicente-Carbajosa, R.J. Schmidt, P. Carbonero, An endosperm-specific DOF protein from barley, highly conserved in wheat, binds to and activates transcription from the prolamin-box of a native B-hordein promoter in barley endosperm, Plant J. 16 (1998) 53–62.

[17] K. Baumann, A. De Paolis, P. Costantino, G. Gualberti, The DNA binding site of the Dof protein NtBBF1 is essential for tissue-specific and auxin-regulated expression of the *rolB* oncogene in plants, Plant Cell 11 (1999) 323–333.

[18] S. Yanagisawa, R.J. Schmidt, Diversity and similarity among recognition sequences of Dof transcription factors, Plant J. 17 (1999) 209–214.

[19] N. Huang, T.D. Sutliff, J.C. Litts, R.L. Rodriguez, Classification and characterization of the rice α-amylase multigene family, Plant Mol. Biol. 14 (1990) 655–668.

[20] H.G. Kang, K.B. Singh, Characterization of salicylic acid-responsive, Arabidopsis Dof domain proteins: overexpression of OBP3 leads to growth defects, Plant J. 21 (2000) 329–339.

[21] M.B. Lanahan, T.H.D. Ho, S.W. Rogers, J.C. Rogers, A gibberellin response complex in cereal α-amylase gene promoters, Plant Cell 4 (1992) 203–211.

[22] M. Cercós, A. Gómez-Cadenas, T.H.D. Ho, Hormonal regulation of a cysteine proteinase gene, *EPB-1*, in barley aleurone layers: cis- and trans-acting elements involved in the co-ordinated gene expression regulated by gibberellins and abscisic acid, Plant J. 19 (1999) 107–118.

[23] J. Vicente-Carbajosa, S.P. Moose, R.L. Parsons, R.J. Schmidt, A maize zinc-finger protein binds the prolamin box in zein gene promoters and interacts with the basic leucine zipper transcriptional activator Opaque2, Proc. Natl. Acad. Sci. USA 94 (1997) 7685–7690.

[24] G. Sachetto, L.O. Franco, D.E. de Oliveira, Plant glycine-rich proteins: a family or just proteins with a common motif?, Biochim. Biophys. Acta 1492 (2000) 1–14.