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Short Communication

Regulation of Gene Expression of a Cysteine Proteinase, EP-C1, by a VIVIPAROUS1-like Factor from Common Bean

Daisuke Yamauchi [1]

Department of Life Science, Himeji Institute of Technology, 2167 Shosha, Himeji-shi, Hyogo, 671-2201 Japan

A VIVIPAROUS1-like transcription factor, PvALF, is known to activate storage protein genes through the RY element in their promoters in maturing common bean seeds. This element also exists in the promoter of the gene for a cysteine proteinase, EP-C1, which is expressed in a germination-specific manner. We show that the EP-C1 gene expression is suppressed through interaction of PvALF with the RY element in the EP-C1 promoter.

Keywords: Germination — *Phaseolus vulgaris* — Proteinase — PvALF — VIVIPAROUS1.

Abbreviations: CaMV, cauliflower mosaic virus; DAI, days after imbibition started; GUS, glucuronidase.

Seed reserve accumulation is followed by desiccation and dormancy. Mutation of the gene *Viviparous-1* (*VP1*) in maize disrupts these processes (Robertson 1955). The seeds of the mutant reduce their storage reserves and lose tolerance to desiccation and dormancy. The seeds also show precocious germination in planta. The *VP1* gene encodes a transcription factor (McCarty et al. 1991). Overexpression of VP1 has been shown to activate the promoter of an ABA-induced gene, *Em*, and reduce the gibberellin-induced expression of a gene for α-amylase (Hoecker et al. 1995). Genes for VP1-like factors have been cloned from rice, *Arabidopsis thaliana*, and common bean (Bobb et al. 1995, Giraudat et al. 1992, Hattori et al. 1994). The comparison of these amino acid sequences indicated four conserved domains: an acidic activation domain named A1 and three basic amino-acid-rich domains designated as B1, B2, and B3. Domain A1 is sufficient for the activation of transcription, because the GAL4 DNA-binding domain fused to domain A1 activated transcription through the GAL4 binding sequence (McCarty et al. 1991). Both B1 and B2 domains are required for the interaction of bZIP transcription factor TRAB1, which binds to ABRE and is involved in ABA- or VP1-activated gene expression (Hobo et al. 1999). The B3 domain has been shown to be required for interaction with the RY element conserved in the promoter region of seed-specific genes (Suzuki et al. 1997).

In leguminous seeds, nutrient reserves are stored in the cotyledons of the embryos (Bewley and Black 1994). During germination, these reserves are hydrolyzed and mobilized to the embryonic axis to support its growth. Proteinases play a significant role in the protein degradation in the cotyledons. A major endopeptidase designated EP-C1 is expressed in the germinated cotyledons of common bean seeds (Tanaka et al. 1993). The deduced amino acid sequence from cDNA of EP-C1 was shown to be a papain-like cysteine proteinase (Tanaka et al. 1991). Immunoblot and Northern blot analyses indicated that the synthesis of EP-C1 in germinated cotyledons was regulated at the level of its mRNA (Terasaki et al. 1995). The introduction of a plasmid containing the 5′-upstream region of the EP-C1 gene fused to the β-glucuronidase (GUS) coding sequence into the germinated cotyledons of common bean showed that the region downstream of position –340 of the gene was sufficient for gene expression in the germinated cotyledons (Terasaki et al. 1995).

The RY element, having a typical sequence CATGCATG, is conserved in the promoter regions of the genes of legume seed storage proteins (Dickinson et al. 1988). A VP1-like factor in common bean, PvALF, activated the promoters of the genes of the seed storage proteins phaseolin and lectin through the RY element in their promoters (Bobb et al. 1997). Previously, we found the RY element in the promoters of the genes for protease and α-amylase, which were expressed in germinated seeds of *Vigna mungo* (Yamauchi et al. 1994). The RY element CATGCAAG is also located at position –340 in the EP-C1 promoter (Ogushi et al. 1992). In the study reported here, we employed a transient assay using particle bombardment to examine whether PvALF regulated EP-C1 gene expression through the RY element.

RNA blot analysis was carried out to estimate the PvALF mRNA level during seed germination (Fig. 1A). This mRNA was detected in the cotyledons of maturing seeds, and it remained in dry seeds. PvALF mRNA was also detected 1 d after imbibition started (DAI), and the additional lower band appeared. The upper band disappeared and the intensity of the lower band decreased at 3 DAI. At 5 DAI the PvALF mRNA disappeared. It is possible that the lower band is a degraded product from PvALF mRNA or a product derived by alternative RNA splicing. In contrast, mRNA for a cysteine proteinase, EP-C1, appeared at 3 DAI during germination and its level increased at 5 DAI.

[1] Corresponding author: E-mail, dyamauch@sci.himeji-tech.ac.jp; Fax, +81-792-67-4920.

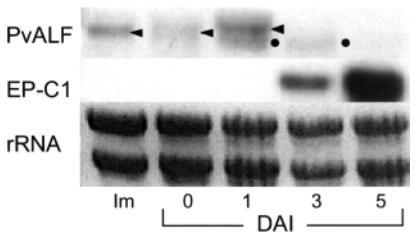

Fig. 1 Northern blot analysis of mRNAs for PvALF and EP-C1 in common bean seeds. RNAs (20 μg) were separated on a 1.4% agarose gel and transferred onto a nylon membrane. Hybridization was carried out with ^{32}P-labeled cDNAs for PvALF and EP-C1. The membrane was exposed to X-ray film for either 1 week (PvALF) or 2 d (EP-C1). Arrowheads and dots correspond to upper and lower bands, respectively, of RNA detected by PvALF cDNA as a probe. After electrophoresis, RNA was stained with methylene blue (rRNA). Numbers indicate days after imbibition started (DAI). Im, immature seeds.

We constructed the effector plasmid designated p35S-PvALF for overexpression of PvALF with cauliflower mosaic virus (CaMV) 35S RNA promoter to examine whether the transient expression of PvALF repressed the promoter activity of the EP-C1 gene (Fig. 2A). First we carried out transfection with p35S-PvALF and the GUS reporter gene fused to the phaseolin promoter, because its promoter is known to be activated by PvALF (Bobb et al. 1997). This effector increased by about 7-fold the GUS activity derived from the phaseolin promoter in germinated cotyledons of common bean seeds (data not shown). In contrast, the GUS activity derived from the EP-C1 promoter of pEP221 was reduced by 77% by cotransfection of p35S-PvALF (Fig. 2B). This result suggests that the transcription factor PvALF enhanced the expression of seed storage protein genes and suppresses protease gene expression in maturing seeds.

Deletion analysis of the RY element of the EP-C1 promoter was performed to examine whether this motif was involved in the repression by PvALF. We deleted the nucleotide sequence CATG from the RY element CATGCAAG and constructed a reporter plasmid pEPdRY1 from pEP221 (Fig. 2A). Transient expression of this reporter with p35S-PvALF produced no reduction in the GUS activity in the germinated seeds (Fig. 2C), which indicates that the RY element in the EP-C1 promoter participates in the repression by PvALF. Furthermore, the GUS activity of pEPdRY1 was about 6.7-fold higher than that of pEP221 in the germinated cotyledons. It remains a possibility that other negative factors repress the EP-C1 gene through the RY element.

Hoecker et al. (1995) showed that the region between the B1 and B2 domains of VP1 was required for the repression of gibberellin-inducible α-amylase expression. Furthermore, the A1 domain and the B3 DNA-binding domain were unnecessary for this repression. Fig. 3 shows three constructs that we made for the overexpression of mutated PvALF. Deletion of the A1 domain (p35S-PvALF28/146) reduced the suppression of the EP-C1 promoter activity. Internal deletion between the 339th and 476th residues of Pro and Gly, respectively (p35S-PvALF338/477), resulted in the total loss of repression of the EP-C1 promoter activity. The deletion of the C-terminal region (p35S-PvALF590) caused less repression of the EP-C1 pro-

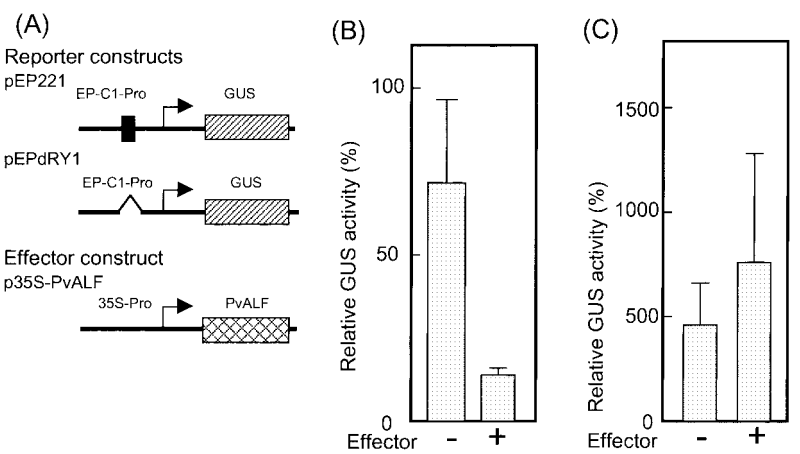

Fig. 2 Effects of overexpression of PvALF on regulation of the promoter of the EP-C1 gene. (A) Constructs of the reporter plasmids and the effector plasmid. The reporter pEP221 contains the GUS coding region (shaded box) fused to the EP-C1 promoter from –1,173 to +15 (EP-C1-Pro) including the RY element (solid box). For construction of another reporter plasmid, designated pEPdRY1, the RY element was deleted from the promoter region from –1,173 to +15 of the EP-C1 gene (EP-C1-Pro). The effector p35S-PvALF includes PvALF cDNA (hatched box) driven by CaMV 35S RNA promoter (35S-Pro). (B) Repression of the EP-C1 promoter activity by PvALF. We delivered the chimeric GUS gene, pEP221, in the presence (+) or absence (–) of the effector, p35S-PvALF, into the cotyledons of common bean seeds 4 DAI using particle bombardment. Relative GUS activity was calculated by dividing the GUS to luciferase ratio from each construct by the GUS to luciferase ratio obtained from pBI221. The results shown are means and standard errors from four independent bombardments. (C) Effect of deletion of the RY element in the EP-C1 promoter on gene expression. The transfection of plasmid pEPdRY1 was carried out in the presence (+) or absence (–) of the effector, p35S-PvALF, as described above. The presentation of the relative GUS activity is also as described above.

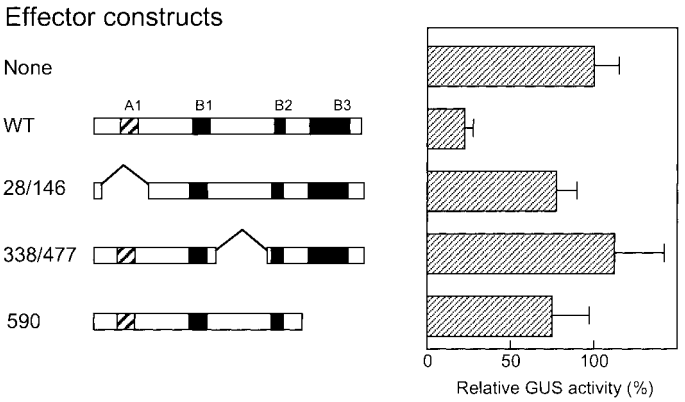

Fig. 3 Effects of deletion of the domain of PvALF on the expression of the EP-C1 gene. The effector plasmids are listed on the left, and the boxes correspond to the polypeptide of PvALF. Shaded boxes and solid boxes show the acidic domain A1 and the basic domains B1, B2, and B3, respectively. The effectors were as follows: none, no effector; WT (wild type), p35S-PvALF; 28/146, p35S-PvALF28/146; 338/477, p35S-PvALF338/477; and 590, p35S-PvALF590. The reporter, pEP221, was introduced into cotyledons of common bean on 4 DAI. Data represent activities relative to that with no effector. The results shown are means and standard errors from four independent bombardments.

moter than wild-type PvALF. In addition to the region between the B1 and B2 domains, the A1 and B3 domains were required for the repression of the EP-C1 promoter.

In this report, we have shown that EP-C1 gene expression is repressed by PvALF, a homolog of maize VP1 and *Arabidopsis* ABI3. Furthermore, this repression was lost by the deletion of the RY element in the EP-C1 promoter (Fig. 2C). The C-terminal DNA binding domain was required for the repression of the EP-C1 gene (Fig. 3). Taken together, these results suggest that PvALF binds to the RY element in the EP-C1 promoter through the DNA binding domain and represses the EP-C1 gene expression. Many reports have indicated that the RY element is involved in seed maturation-specific gene expression (Bobb et al. 1997, Chamberland et al. 1992, Ezcurra et al. 1999, Lelievre et al. 1992). However, this is the first report showing that the RY element acts as a negative regulatory element in a germination-specific gene. RY elements are found in the promoters of the genes for a cysteine proteinase, SH-EP, and α-amylase in *V. mungo* (Yamauchi et al. 1994). It is probable that PvALF-related factors are involved in the repression of the genes.

The region between B1 and B2 is known to be required for the repression of α-amylase gene expression by VP1 (Hoecker et al. 1995). This region of PvALF was also necessary for the repression of the EP-C1 gene (Fig. 3). However, there is low homology in the regions (data not shown). The prediction of the secondary structures indicates that the regions have a β-turn (Chou and Fasman 1978) – this structure may be involved in the repression. Furthermore, the repression was required for the N-terminal acidic domain A1. This domain was able to activate transcription in yeast cells and common bean cotyledons (Bobb et al. 1995). It is known that steroid hormone receptors regulate the activation and repression of transcription (Weatherman et al. 1999). A coactivator and a corepressor are involved in activation and repression of the transcription, respectively, and these two factors compete with each other over a common or overlapping binding site of a steroid receptor (Nagy et al. 1999). Therefore, it is possible that a coactivator and a corepressor interacting with domain A1 of PvALF regulate activation and repression, respectively.

Seeds of common bean (*Phaseolus vulgaris* L. cv. Gold Star) were obtained from Sakata Seed Company (Yokohama, Japan). The seeds were germinated in wet vermiculite at 27°C in darkness. Plants were grown at the experimental farm of Tokyo Metropolitan University. Cotyledons were collected from maturing fruits and stored at –20°C.

Preparations of total RNA and RNA blot analysis were carried out as described by Terasaki et al. (1995). Polyadenylated RNA was prepared from total RNA with Oligotex-dT (Roche Applied Science, Mannheim, Germany). The genomic DNA was prepared from leaves of common bean as described by Yamauchi et al. (1989).

PvALF cDNA was amplified from polyadenylated RNA of immature seeds of common bean by the RT-PCR method, and cloned into pCR (Invitrogen Corp., Carlsbad, CA, U.S.A.). The sequence was reported by Kobayashi et al. (2002; accession no. AB085836). The cDNA insert was subcloned into pBC (Stratagene, La Jolla, CA, U.S.A.), and the resultant plasmid was designated pBCPvALF1. The cDNA insert cut by *Eco*RV and *Sac*I was inserted to sites between *Sma*I and *Sac*I of pBI221 to replace the GUS coding region (Jefferson et al. 1987). The resultant plasmid was named p35S-PvALF. To delete the domain between the 29th residue of Glu and the 145th residue of Met, the pBCPvALF1 was digested with *Nco*I and self-ligated. The plasmid for the overexpression of this mutated PvALF was constructed as described above and the resultant plasmid was designated p35S-PvALF28/146. To remove the region between the 339th residue of Pro and the 476th residue of Gly from PvALF, the 475th residue of His and the 476th residue of Gly were changed to Gln and Asp by creating a *Bam*HI site as described below. A fragment of approximately 1 kb was amplified from pBCPvALF1 by PCR with a reverse sequencing primer and a IntBamH1 primer (5′-TTTCAGGATCCTGGTGATAGGTTGATGAGG-3′). This fragment was digested with *Bam*HI and *Sac*I, and ligated to a large fragment of pBCPvALF1 generated by this digestion. The mutated cDNA obtained was inserted into pBI221 and the resultant plasmid was termed p35S-PvALF338/477. To delete the C-terminal region of PvALF, mutation of the 591st residue of Arg to the stop codon and creation of its downstream *Sac*I site were

achieved by PCR of p35-PvALF with 35S sequencing primer (5′-GATTGATGTGATATCTCCAC-3′) and StopSac primer (5′-GATGGAGCTCAGTTCTGATGAGAATTC-3′). The amplified fragment cut by *Spe*I and *Sac*I was inserted into the *Spe*I and *Sac*I sites of p35S-PvALF. The resultant plasmid was designated p35S-PvALF590.

The reporter plasmid pEP221 was prepared as described by Terasaki et al. (1995). For deletion of the RY element in the EP-C1 promoter, pEP221 was cut by *Bam*HI and *Eco*RI and the obtained fragment containing the GUS fusion gene was inserted into pBC. The resultant plasmid was cut with *Sph*I and both ends were blunted with T4 DNA polymerase, then self-ligated. The resultant plasmid was named pEPdRY1. The promoter region from –314 to +30 of the phaseolin gene (Doyle et al. 1986) was generated from the genomic DNA of common bean by PCR, and *Hin*dIII and *Bam*HI sites were created at both ends. The amplified fragment was fused to the GUS coding region of pBI221 and the resultant plasmid was designated pPH221.

Nucleotide sequences of the deleted constructs described above were confirmed with an automatic DNA sequencer (Prism model 310, Applied Biosystems, Foster City, CA, U.S.A.).

To normalize the GUS activity, pDO432 (Ow et al. 1986) — in which the CaMV 35S promoter is fused to the luciferase gene — was used as an internal control. All plasmids were mixed at a molar ratio of 6 : 3 : 2 (reporter : effector : internal control). Reporters and effectors were introduced into germinated cotyledons of common bean seeds at 4 DAI by particle bombardment as described previously (Yamauchi 1997). After bombardment, the cotyledons were incubated on wet filter paper at 27°C in darkness for 24 h. The enzyme activity was measured as described by Yamamoto et al. (1995).

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