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Gene Note

Hormonal regulation of the expression of cysteine proteinase genes in germinated cotyledons of common bean seeds

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Abstract Storage proteins in cotyledons of legume plants are degraded by proteinases after germination. We examined whether abscisic acid (ABA), gibberellin (GA) and brassinosteroid (BR) are involved in the expression of cysteine proteinases in cotyledons of common bean seeds with RNA blotting using cDNAs for five papain-like proteinases, EP-C1, CP1, CP2, CP3, CP4 and two legumain-like proteinases, LLP1 and LLP2 as probes. Six genes, except for CP4, are activated after seed imbibition. These germination-induced cysteine proteinase genes (*GICPs*) are repressed by exogenously applied (+)-5′α, 8′-cyclo-ABA which is more resistant to hydroxylation than ABA. Either GA_3 or epi-BR activated *GICPs* in the presence of ABA, suggesting that both of GA and BR are involved in the expression of *GICPs*. It is possible that GA biosynthesis is required for the activation of *GICPs*, because either GA-biosynthesis inhibitors, chloroethyltrimethylammonium chloride or prohexadione calcium repressed *GICPs* and this repression was restored by exogenously applied GA_3.

Key words: Abscisic acid, brassinosteroid, gibberellin, *Phaseolus vulgaris*, proteinase.

Abscisic acid (ABA) and gibberellin (GA) play antagonistic roles in the regulation of seed dormancy and germination (Bewley and Black 1994). ABA represses seed germination and several ABA-insensitive mutants of *Arabidopsis* exhibited reduced seed dormancy (Koornneef et al. 1984). Analysis of GA- and ABA-insensitive mutants indicated that GA is needed to break ABA-induced dormancy (Steber et al. 1998). Brassinosteroids (BRs) are known as plant steroid hormones and partially rescue the germination of GA-biosynthetic and GA-insensitive mutants (Steber and McCourt 2001). Furthermore, either BR or GA broke the ABA-induced dormancy of tobacco seeds (Leubner-Metzger 2001). Thus, ABA, GA and BR are involved in seed germination.

The synthesis of several groups of hydrolytic enzymes including proteinase and amylase is induced in seeds after imbibition (Bewley and Black 1994). Sugars, amino acids and small peptides resulting from the hydrolysis of seed nutrients support the growth of seedlings. In cereal seeds, storage reserves are mainly present in the endosperm. Hydrolases involved in the degradation of the reserves are secreted from the aleurone layer, and their syntheses are induced by GAs. ABA antagonizes the GA-induced expression of genes for hydrolases. In leguminous seeds, nutrient reserves are stored in the cotyledons of embryos (Bewley and Black 1994). During germination, the reserves in the cotyledons are hydrolyzed, and mobilized to the embryonic axis to support the growth of the axis. Proteinases play a significant role in protein degradation. Two types of cysteine proteinases, papain- and legumain-like families, are involved in the degradation of seed storage protein in leguminous seeds (Fisher et al. 2000, Senyuk et al. 1998); however, it is unclear which hormone is involved in the regulation of the gene expression of proteinase for storage protein degradation. Ethylene was shown to activate the gene for a proteinase in germinated cotyledons of chick peas (Cervantes et al. 1994) and peas (Cercòs et al. 1999). Previously, we indicated that the synthesis of a papain-like proteinase, SH-EP in *Vigna mungo* seeds was regulated by ABA and GA (Taneyama et al. 1996, 2001); however, it is unclear whether transcriptional regulation is involved in the hormone response.

Tanaka et al. (1991) isolated a cDNA clone for a papain-like proteinase, designated EP-C1, from common bean pods. EP-C1 was found to be expressed in germinated cotyledons of seeds (Tanaka et al. 1993). Immunoblot and RNA blot analyses indicated that the

Abbreviations: ABA, abscisic acid; BR, brassinosteroid; CCC, chloroethyltrimethylammonium chloride, cyclo-ABA, (+)-5′α,8′-cyclo-ABA; GA, gibberellin; GICP, germination-induced cysteine proteinase; PHD, prohexadione calcium.
This article can be found at http://www.jspcmb.jp/

synthesis of EP-C1 in germinated cotyledons was regulated at its mRNA level (Terasaki et al. 1995). A genomic clone for EP-C1 was isolated and sequenced (Ogushi et al. 1992). We constructed a plasmid containing the 5′-upstream region of the EP-C1 gene fused to the β-glucuronidase coding sequence (Terasaki et al. 1995). A transient expression assay for the plasmid using particle bombardment showed that the region downstream of position −340 of the gene was sufficient for gene expression in the cotyledons of germinated seeds of common beans. Chimeric genes were also introduced into tobacco plants to examine the transcription regulation of the gene in tobacco seeds (Yamauchi et al. 1996). The region downstream of nucleotide position −340 was sufficient for gene expression in the endosperm of tobacco seeds but the region between −1170 and −340 was required for expression in the cotyledons of tobacco seeds (Yamauchi et al. 1996). The RY element, having a typical sequence of CATGCATG, is conserved in the promoter regions of seed storage protein genes (Dickinson et al. 1988). A transcription factor, PvALF, activated the promoters of seed storage protein genes, phaseolin and lectin, through the RY element in their promoters (Bobb et al. 1995). The RY element CATGCAAG is also located at −340 in the EP-C1 promoter (Ogushi et al. 1992). We indicated that PvALF suppressed EP-C1 gene expression through the RY element in maturing seeds (Yamauchi 2003).

Six cDNAs for cysteine proteinases, CP1, CP2, CP3, CP4, LLP1 and LLP2, as shown in Table 1, were cloned from germinated common bean seeds by Senyuk et al. (Accession nos. Z99952–Z99957). In this study we used these cDNAs in addition to the cDNA for EP-C1 (Tanaka et al. 1991) as probes for RNA blot analyses. Proteinases in *Vicia sativa* seeds were well characterized and were compared to proteinases in common bean seeds (Table 1). CPR1, CPR2, CPR4 and proteinase A belong to a papain family and both proteinase B and VsPB2 are included in a legumain family (Fisher et al. 2000). CPR2, CPR4 and VsPB2, homologs to CP2, CP4 and LLP2, respectively, are synthesized in maturing seeds and contained in protein bodies in dry seeds, initiating storage globulin mobilization after germination (Schlereth et al. 2001). CPR 1 and CPR2, homologs to CP1 and CP2, respectively, are thought to initiate the breakdown of a major seed protein, globulin, after germination (Fischer et al. 2000). Proteinase A, a homolog to EP-C1, is the most abundant proteinase in germinated cotyledons of *V. sativa* seeds and takes part in the hydrolysis of seed globulin during the latter stage of germination (Becker et al. 1997). Proteinase B and VsPB2, homologs to LLP1 and LLP2, belong to legumain-like cystein proteinase (Fisher et al. 2000). Both proteinase B and LLP1 have activity to degrade globulin *in vitro*, and are involved in storage protein mobilization (Fisher et al. 2000; Senyuk et al. 1998). As the gene for VsPB2 is expressed during maturation, and its amino acid sequence is similar to β-VPE involved in processing a precursor of storage globulin (Hara-Nishimura et al. 1993), VsPB2 is thought to process precursors of various vacuolar proteins. These suggest that two types of cysteine proteinase act on the degradation of storage protein in common bean seeds, synergistically.

Table 1. Probes for RNA blot analyses

Type	Name	Size (bp)	Homolog from *Vicia sativa*
papain	EP-C1	1,221	Proteinase A
	CP1	1,351	CPR1
	CP2	1,376	CPR2
	CP3	1,680	
	CP4	1,286	CPR4
legumain	LLP1	1,850	Proteinase B
	LLP2	1,652	VsPB2

In this study, we analyzed temporal changes in the levels of mRNAs for cysteine proteinases during the germination of common bean seeds by RNA blot with seven cDNA probes, as cited in Table 1. We also examined the effects of hormones and hormone biosynthesis inhibitors on gene expressions for cysteine proteinases in germinated cotyledons.

Common bean seeds (*Phaseolus vulgaris* L. cv Gold Star) were obtained from Sakata Seed, Yokohama. After the seeds were soaked for 6 h, they were germinated on 2 layers of wet filter paper in a Petri dish at 27°C in darkness. To examine the effects of chemicals, seeds were germinated with the solution described below instead of water.

A total RNA fraction was prepared by SDS-phenol method as described by Zhong et al. (1983). After denaturation with glyoxal and dimethyl sulfoxide, a 10 μg portion of the RNA fraction was separated on 1.4% agarose gel, blotted onto a nylon membrane Hybond-N (Amersham Biosciences, Buckinghamshire, UK), and hybridized with ^{32}P-labeled cDNA probes in a solution containing 50% formamide, as described by Sambrook et al. (1989). We used seven cDNA probes, as cited in Table 1. As the highest identity between the nucleotide sequences of each probe was approximate 51%, cross-hybridization did not occur.

Epibrassinolide (epi-BR; 22R,23R,24R-2α,3α,22,23-tetrahydroxy-B-homo-7-oxa-5α-ergostan-6-one) was purchased from Sigma (St. Louis, MI USA). (+)-5′α, 8′-Cyclo-ABA (cyclo-ABA) was kindly provided by Dr. Todoroki, Shizuoka University (Todoroki et al. 1996). Cyclo-ABA was resolved with a small amount of acetone and then added to water. ABA or GA_3 was resolved with ethanol and added to water. Chloroethyltrimethylammonium chloride (CCC) or

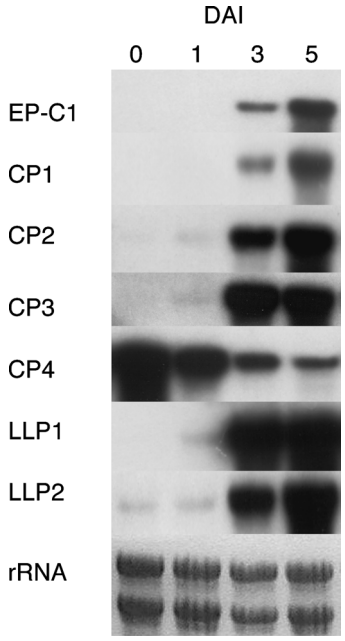

Figure 1. Temporal change of mRNA levels for cysteine proteinases in cotyledons of common beans. Total RNA fractions were extracted from cotyledons of common bean seeds. Numbers indicate days after the start of imbibition (DAI). Probes of cDNA for EP-C1 (EP-C1), CP1 (CP1), CP2 (CP2), CP3 (CP3), CP4 (CP4), LLP1 (LLP1) and LLP2 (LLP2) were used for hybridization. rRNA was stained with methylene blue (rRNA).

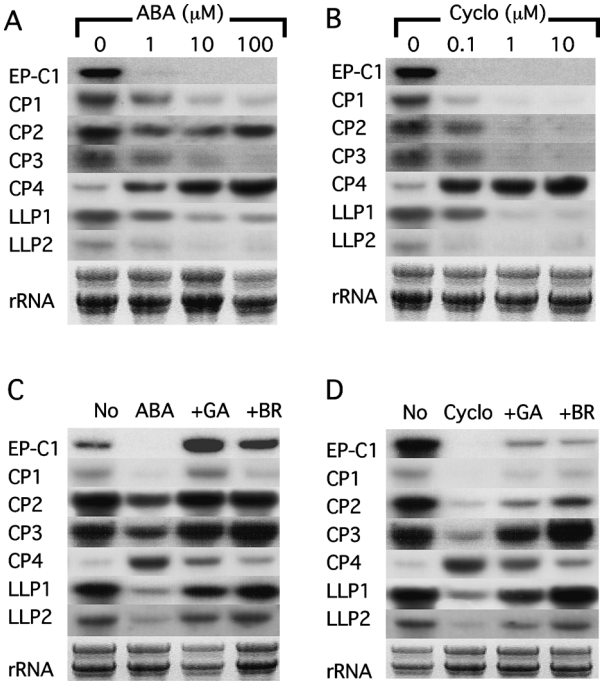

Figure 2. Effect of ABA and cyclo-ABA on expression of cysteine proteinase genes in germinated cotyledons. (A) Common bean seeds were allowed to imbibe 0 to 100 μM ABA and were incubated for 3 days. (B) Common bean seeds were allowed to imbibe 0 to 10 μM cyclo-ABA and were incubated for 3 days. (C) Common bean seeds were allowed to imbibe water (No), 10 μM ABA (ABA), 10 μM ABA and 10 μM GA_3 (+GA), or 10 μM ABA and 1 μM epi-BR (+BR). (D) Common bean seeds were allowed to imbibe water (No), 1 μM cyclo-ABA (Cyclo), 1 μM cyclo-ABA and 10 μM GA_3 (+GA), or 1 μM cyclo-ABA and 1 μM epi-BR (+BR). The cDNA probes and rRNA detection are described in the legend of Figure 1.

prohexadione calcium (PHD) was resolved in dimethyl sulfoxide and then added to water.

We examined RNA blot analysis with seven cDNA probes, as cited in Table 1. EP-C1, CP1, CP2, CP3 and CP4 belong to the papain family, and LLP1 and LLP2 are legumain-like cysteine proteinases. Temporal changes of mRNA levels during the germination of common bean seeds were analyzed as shown in Figure 1. Except for CP4, the levels of the six mRNAs in dry seeds were very low and increased during imbibition. After imbibition, EP-C1 mRNA appeared at 3 DAI. This pattern was similar to the other five mRNAs, except for CP4. CP4 mRNA was abundant in dry seeds and its mRNA level decreased after imbibition. Here, the six germination-induced cysteine proteinase genes were designated germination-induced cysteine proteinases (*GICPs*).

We examined whether ABA repressed the expression of *GICPs* in common bean seeds (Figure 2A). EP-C1 mRNA could not be detected in cotyledons treated with 1 μM ABA, and the others, except for CP2 and CP4, were repressed by exogenously applied ABA at a concentration of 10 μM; however, both CP2 and CP4 mRNAs were detected in 100 μM ABA-treated seeds (Figure 2A). As CP4 mRNA was abundant in dry seeds, we presumed that the gene expression was activated by ABA at late embryogenesis. As CP4 is a homolog to CPR4 in *Vicia sativa* involved in the initiation of storage globulin mobilization (Schlereth et al. 2001), the function of CP4 in common bean seeds may be similar to CPR4, and CP4 may be required for storage globulin degradation in early germination. In addition, it is known that ABA is related to drought stress which induces the expression of genes for cysteine proteinases (Yamaguchi-Shinozaki et al. 1995). It is possible that drought stress activates *CP4*, and that the proteinase acts to degrade polypeptides denatured by desiccation stress, although the function of desiccation-inducible proteinase has not been elucidated.

ABA is known to be inactivated by hydroxylation during germination of *Arabidopsis* seed (Kushiro et al. 2003; Saito et al. 2004). We supposed that exogenously applied ABA was inactivated in the cotyledons of common bean seeds and did not repress the expression of *CP2*. Todoroki et al. (1996) developed cyclo-ABA resistant to hydroxylation; therefore, we examined whether cyclo-ABA repressed germination-induced genes (Figure 2B). mRNAs for *GICPs* except for CP4 were not detected in cotyledons treated with 1 μM cyclo-ABA. The EP-C1 gene was most sensitive to cyclo-ABA, because EP-C1 mRNA could not be detected at 0.1 μM concentration. Taken together, these results suggest that *GICPs* including *CP2* were repressed by ABA.

GA and BR activated the germination of tobacco seeds treated with ABA in the dark (Leubner-Metzger 2001). We examined the effect of GA and BR on the expression of *GICPs* in common bean seeds treated with 10 μM ABA (Figure 2C). Levels of mRNAs for GICPs with treatment of 10 μM GA_3 or 1 μM epi-BR treatment were higher than treatment with 10 μM ABA treatment alone; thus, either GA or BR overcame repression by ABA. We also used cyclo-ABA to avoid the effect of ABA catabolism (Figure 2D). *GICPs* were classified into two types: one group, including the genes for CP3, LLP1 and LLP2, showed that repression by cyclo-ABA was partly restored by GA and completely by BR. The other group, containing the genes for EP-C1, CP1 and CP2 indicated that either GA or BR partially activated the expression of those genes in seeds treated with cyclo-ABA. Although there is difference between the two groups, these results suggest that activation of *GICPs* by GA is antagonized by ABA in common bean seeds as well as cereal seeds (Bewley and Black 1994).

We examined the effect of GA biosynthesis inhibitors on the expression of *GICPs* in common bean seeds. A GA biosynthesis inhibitor, CCC, blocks *ent*-kaurene synthesis (Rademacher 2000). Exogenously applied CCC repressed *GICPs* (Figure 3A). This inhibition was restored by exogenously applied GA_3 (Figure 3B). We also examined another GA biosynthesis inhibitor, PHD (Nakayama et al. 1990), which blocked the steps at the 3β- and 2β-hydroxylation of GAs, on the expression of *GICPs*. PHD also repressed *GICPs* (Figure 3C). Exogenously applied 10 μM GA_3 restored the expression of *GICPs*, but 1 μM epi-BR did not activate these genes (Figure 3D). This result suggests that GA is required for the expression of *GICPs* and BR did not activate *GICPs* without *de-novo* GA biosynthesis. A high concentration of the GA biosynthesis inhibitors showed a lesser effect on repression than a lower concentration (CCC, CP3 and LLP1, Figure 3A; PHD, CP2, CP3 and LLP2, Figure 3C). GA biosynthesis inhibitors are known to cause side effects (Rademacher 2000); for example, cytokinins were considerably elevated in wheat shoots by exogenously applied 100 μM PHD (Grossmann et al. 1994). Although it is unclear whether cytokinins activate *GICPs*, it is possible that side effects were occurred by the high concentration of the inhibitors in bean seeds and lowered the repressive effects.

Recently we sequenced the gene for *CP2* (Accession No. AB270920). To identify putative hormone response elements, both promoter regions of *EP-C1* and *CP2* were compared. Sequences corresponding to GARE TAACAG/A (Skriver et al. 1991) were found in both promoter regions of *EP-C1* and *CP2*. It is possible that this motif is involved in the GA response of the genes. Interestingly, DRE and G-box related to drought stress and ABA response (Yamaguchi-Shinozaki et al. 1995) were found in the promoter region of *CP2*. The amino acid sequence of CP2 is similar to drought-inducible cysteine proteinases, *Arabidopsis* RD19 (Koizumi et al. 1993) and pea 15a (Guerrero et al. 1990). These facts suggest that drought stress may activate *CP2*. The mRNA level of CP2 was increased slightly by 100 μM ABA (Figure 2A), but cyclo-ABA repressed *CP2* (Figure 2B), suggesting that ABA cannot activate *CP2*. A higher concentration of ABA might induce the expression of genes for DRE binding factors (Yamaguchi-Shinozaki et al. 1995), activating *CP2*. Examination of the stress response of genes for cysteine proteinases is required to clarify the roles of proteinases.

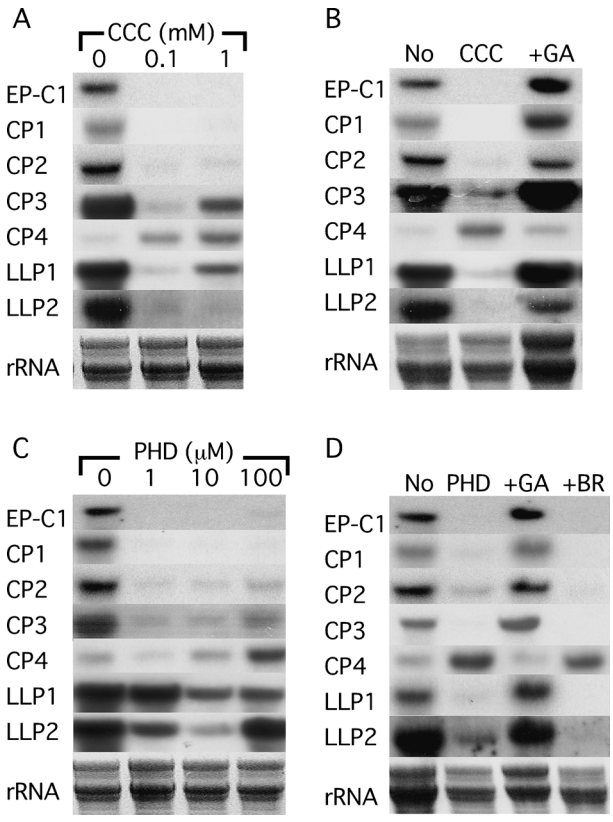

Figure 3. Effect of GA biosynthesis inhibitors on the expression of cysteine proteinase genes in germinated cotyledons. (A) Common bean seeds were allowed to imbibe 0 to 1 mM CCC and were incubated for 3 days. (B) Common bean seeds were allowed to imbibe water (No), 0.1 mM CCC (CCC) or 0.1 mM CCC and 10 μM GA_3 (+GA). (C) Common bean seeds were allowed to imbibe 0 to 100 μM PHD and were incubated for 3 days. (D) Common bean seeds were allowed to imbibe water (No), 10 μM PHD (PHD), 10 μM PHD and 10 μM GA_3 (+GA) or 10 μM PHD and 1 μM epi-BR (+BR). The cDNA probes and rRNA detection are described in the legend of Figure 1.

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