Plant Cell Physiol. 38(11): 1242-1248 (1997)

Hormonal Regulation of Expression of Two Cysteine Endopeptidase Genes in Rice Seedlings

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Two cDNA probes (pRP60 and pRP80) were used to examine the hormonal regulation of expression of two cysteine endopeptidase genes in germinated whole seeds and de-embryonated seeds of rice. The pRP60 protein is a major rice cysteine endopeptidase named REP-1 that digests rice glutelin, the main seed storage protein. The pRP80 protein has features of a cysteine endopeptidase but has not yet been confirmed to be one. Neither mRNA was detectable in dry seeds, and the levels per seed increased sharply upon imbibition, reached peaks at d 6 to d 9, and then decreased. Both mRNAs were expressed in a seed-specific manner, but the accumulation of pRP60 mRNA was about ten times greater than the other. When seeds were de-embryonated and incubated, the amounts of both mRNAs were reduced to very low levels, but in the presence of 0.01 to 1 μM gibberellic acid (GA_3) both again reached high levels. Addition of abscisic acid (ABA) or uniconazole, an inhibitor of gibberellin biosynthesis, partly eliminated the effect of GA_3. Protein immunoblot analysis showed that the accumulation of the REP-1 protein in seeds was regulated by GA_3 and ABA similarly to that of pRP60 mRNA, but the change in pRP60 mRNA with time after the onset of imbibition preceded that of the REP-1 protein.

Key words: Abscisic acid — Cysteine endopeptidase — Germination (seed) — Gibberellic acid — Rice (*Oryza sativa*) — Seed storage protein.

Cereal grains such as rice, barley and wheat store reserve protein in the aleurone layer and the starchy endosperm. During seed germination and early seedling growth, the reserve protein is digested by proteases and mobilized to supply amino acids that support the growth of the embryo. A number of reports have shown that endopeptidases play a major role in the seed protein mobilization process (Koehler and Ho 1988, 1990a, de Barros and Larkins 1990, Wrobel and Jones 1992). Endopeptidases in cereal grains are synthesized primarily in the aleurone layer after the onset of imbibition. EP-A and EP-B, two major cysteine proteases from barley seeds, have been well studied for hormonal regulation of their expression, processing and secretion events (Koehler and Ho 1988, 1990a, b). Another cysteine protease, aleurain, was isolated from barley plants as a non-secreted aminopeptidase (Holwerda and Rogers 1992). Using a cDNA for aleurain as the probe (Rogers et al. 1985), Watanabe et al. (1991) selected three cDNA clones, oryzain α, β and γ, from a cDNA library of rice seeds. Although the accumulation of oryzain mRNAs in rice seeds was reported to be GA_3-inducible, little information on their functions has been available. In contrast to extensive studies of proteases from barley seeds, possible hormonal regulation of the development of protease activities in germinated rice grains has not been well studied.

In previous studies (Kato and Minamikawa 1996), we separated two major cysteine endopeptidases, termed REP-1 and REP-2, from extracts of rice seeds by hydrophobic chromatography. REP-1 was first characterized as a major cysteine endopeptidase that digests glutelin, the main rice storage protein which accounts for about 80% of the total seed protein. We then selected a cDNA clone (pRP60) for REP-1 from a cDNA expression library of rice seeds and sequenced the cDNA insert. The deduced amino acid sequence of REP-1 showed strong similarity (68 and 72%) to the mature proteins of two other plant cysteine proteases, SH-EP from *Vigna mungo* (Akasofu et al. 1989) and EP-B from barley (Koehler and Ho 1990b). REP-1, but not REP-2, was immunoreactive to an antiserum raised against SH-EP. A long prosequence of REP-1 suggests that the large precursor of this enzyme is posttranslationally processed through multiple steps to form the mature protein in a fashion analogous to those for SH-EP and EP-B. We also earlier isolated a full-length cDNA clone, called pRP80, for a putative cysteine endopeptidase from rice seeds and determined its nucleotide sequence (Shintani et al. 1995). The putative mature protein of pRP80 showed 64 to 67% similarity to those of SH-EP and EP-B, having 63 and 52% similarity in terms of the amino acid sequence of mature form and preproform, respectively, to the REP-1 protein. We have not, however, examined whether or not this is actually expressed as a protease in rice seeds.

In this study, using the RNA gel blot technique, we examined the hormonal regulation of expression of pRP60 and pRP80 mRNAs in rice seeds during germination and early seedling growth. Results showed that both mRNAs were detectable only in seeds and similarly accumulated in

Abbreviations: BSA, bovine serum albumin; CBB, Coomassic brilliant blue; EP-B, cysteine protease from barley; REP, rice cysteine endopeptidase; SH-EP, cysteine protease from *Vigna mungo*.

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de-embryonated seeds in response to exogenously supplied GA_3. Differences were seen in the time-course and quantity of accumulation between the two mRNAs in response to GA_3 and ABA. We also examined the hormonal response of the REP-1 protein using protein immunoblot analysis.

Materials and Methods

Plant materials—Rice seeds (*Oryza sativa* L. cv. Koshihikari) were purchased from the Agricultural Cooperative Association of Shibata-shi, Japan. Dehulled seeds were surface-sterilized in 1% NaClO for 30 min, rinsed thoroughly with sterile water, and allowed to germinate and grow at 27°C in darkness. Hulled whole seeds and de-embryonated seeds were surface-sterilized in 1% NaClO for 15 min, rinsed thoroughly with sterile water, and incubated as above. When necessary, seedlings were dissected into shoots, roots and seeds (endosperms plus aleurone layers) at indicated times. Samples were immediately frozen in liquid nitrogen and stored at −80°C.

Isolation of RNA—Total RNA was prepared by the SDS-phenol method of Karrer et al. (1991). Plant samples (2.5 g) were ground to powder in liquid nitrogen and homogenized with 15 ml of TLE buffer (0.2 M Tris-HCl, pH 8.2, 0.1 M LiCl, 5 mM EDTA and 1% SDS) containing 2 mM aurintricarboxylic acid, 50 mM 2-mercaptoethanol and 10 ml of 85% phenol in an Ultra-disperser (model LK-21; Yamato Scientific, Japan) for 3 min. The homogenate was centrifuged at 3,000 × *g* for 10 min at 4°C. The aqueous phase was removed and stirred with an equal volume of phenol/chloroform (1 : 1) for 10 min. This procedure was repeated two or three times. The subsequent treatment was followed as described previously (Suzuki and Minamikawa 1985).

RNA blot hybridization—RNA blot analysis was performed as described by Thomas (1983) using pRP6 cDNA (a 0.8 kbp cDNA corresponding to full-length pRP60 cDNA) or pRP80 cDNA as a probe. The glyoxalated RNA was separated by electrophoresis on a 1.4% agarose gel and blotted onto a Hybond-N nylon membrane (Amersham Life Science, Buckinghamshire, U.K.). Hybridization with the ^{32}P-labeled cDNA insert was performed at 42°C in 50% formamide, 5 × SSPE (1 × SSPE: 0.15 M NaCl, 10 mM NaH_2PO_4, pH 7.4, 1 mM EDTA), 5 × Denhardt's (100 × Denhardt's: 2% BSA, 2% Ficoll, 2% polyvinylpyrrolidone), 0.5% SDS and 0.1 mg ml^{-1} denatured salmon sperm DNA. The membrane was washed with 2 × SSC (1 × SSC: 0.15 M NaCl and 15 mM sodium citrate, pH 7.0) containing 0.1% SDS at 65°C for 30 min and 0.2 × SSC containing 0.1% SDS at 65°C for 30 min before autoradiography. With the exception for comparison of relative levels of mRNAs for pRP60 and pRP80 (Fig. 2), the same blot was stripped in 0.1% SDS at 100°C before reprobing with the other probe. For comparison of pRP60 and pRP80 mRNAs, the two blots were subjected to hybridization with probes of identical specific activity and to autoradiography under identical conditions.

Synthesis of REP-1 in Escherichia coli cells and preparation of antiserum against REP-1—The full-length cDNA (pRP60) for REP-1 was prepared as described elsewhere (Kato and Minamikawa 1996). For the large-scale production of REP-1 in *E. coli* cells, the nucleotide sequence corresponding to the mature form of REP-1 was amplified by PCR technique using two mutation primers and pRP60 as a template. A 5′ primer (5′-GAGGGCGCCATGGACCTCCCCCGCGCC-3′) and a 3′ primer (5′-ATGGCGCCGGATCCATCACTGGGTTTC-3′) were designed to make restriction enzyme sites for *Nco*I and *Bam*HI, respectively (the sites underlined). The amplified product was inserted into the *Nco*I and *Bam*HI sites of the pET16b expression vector (Novagen, Madison, WI, U.S.A.), and its expression was induced in *E. coli* BL21 (DE3) cells by the addition of 1 mM isopropyl β-D-(−)-thiogalactopyranoside (IPTG; Sambrook et al. 1989). Inclusion bodies were purified from *E. coli* lysate by the method of Kuhelj et al. (1995) with minor modifications. The inclusion body fraction was further purified on a column of DEAE-cellulose (DE-52, Whatman). The eluate that contained the recombinant protein was separated by SDS-PAGE, and the band of the target protein was cut from the gel. Determination of the amino-terminal amino acid sequence indicated that the recombinant protein had nine amino-terminal amino acids identical with those of REP-1 except for the addition of the initial Met at the amino terminus of the former. REP-1 (0.2 mg per injection) synthesized in *E. coli* cells was emulsified with Freund's complete adjuvant and injected subcutaneously into a rabbit three times at intervals of two weeks.

Protein immunoblotting—To study the change over time in the level of REP-1 protein in germinated seeds, rice seeds were allow to imbibe for 3 to 21 d. One hundred whole seedlings at each indicated growth stage were homogenized with 20 ml of 50 mM sodium acetate (pH 6.0) containing 0.1 M 2-mercaptoethanol. The homogenate was centrifuged at 20,000 × *g*, and solid ammonium sulfate was added to the supernatant to 80% saturation. After centrifugation, the precipitate was dissolved in 0.5 ml of 50 mM sodium acetate (pH 6.0) containing 10 mM 2-mercaptoethanol. To examine the hormonal effects on the level of the REP-1 protein, d-5 whole or de-embryonated rice seeds (200 pieces, ca. 3.8 g) were incubated for 0 to 9 d in 20 ml sterile water in darkness in the presence or absence of plant hormones. After incubation, 1 ml of 1 M sodium acetate (pH 6.0) containing 0.1 M 2-mercaptoethanol was added to the incubation medium, and the seeds were homogenized. Following centrifugation, solid ammonium sulfate was added to the supernatant to 80% saturation, and the precipitate was dissolved in a small volume of the 50 mM sodium acetate buffer. The gel was transferred onto a nitrocellulose membrane after SDS-PAGE (Laemmli 1970), and protein immunoblot analysis was performed using the antiserum raised against REP-1. Protein bands were visualized by enhanced chemiluminescence (ECL) reagents (Amersham).

As comparisons, d-3 dark-grown seeds (endosperms plus aleurone layers) of barley (cv. Kashima) and wheat (cv. Norin 61) were also subjected to immunoblot analysis. These seeds (20 pieces, 0.94 to 1.14 g) were homogenized with 5 ml of 50 mM sodium citrate buffer (pH 5.0) containing 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 13,000 × *g* for 20 min and the supernatant was used as an extract. Protein content was determined by the method of Bradford (1976) using BSA as the standard.

Results

Changes in levels of mRNAs for pRP80 and pRP60—Total RNA was prepared from dry seeds and 3 to 21-d germinated whole seeds and analyzed by RNA gel blotting with pRP80 and pRP6 cDNAs as probes. The autoradiogram was scanned with a densitometer, and relative amounts of mRNAs were quantified (Fig. 1). Amount of total RNA per seed increased slightly during 3 d of imbibition and then steadily decreased. The amount was half of the maximum value on d 12. pRP80 mRNA was not detecta-

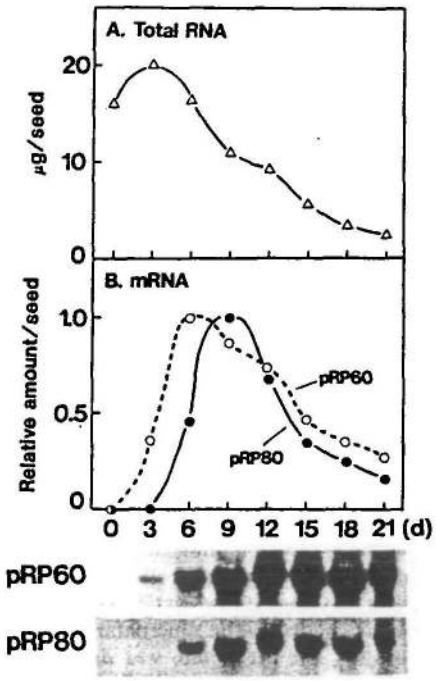

Fig. 1 Changes over time in total RNA content (A) and relative levels of pRP60 and pRP80 mRNAs (B) during germination and early seedling growth. Total RNA (15 μg per lane) prepared from d-0 to d-21 germinated whole seedlings was analyzed by RNA gel blotting with the pRP6 or pRP80 cDNA probe. Autoradiographs (*lower panels*) were scanned with a densitometer (Model CS-930; Shimadzu, Kyoto, Japan), and relative amounts of mRNA per seed were calculated. The highest mRNA levels are set at 1.0.

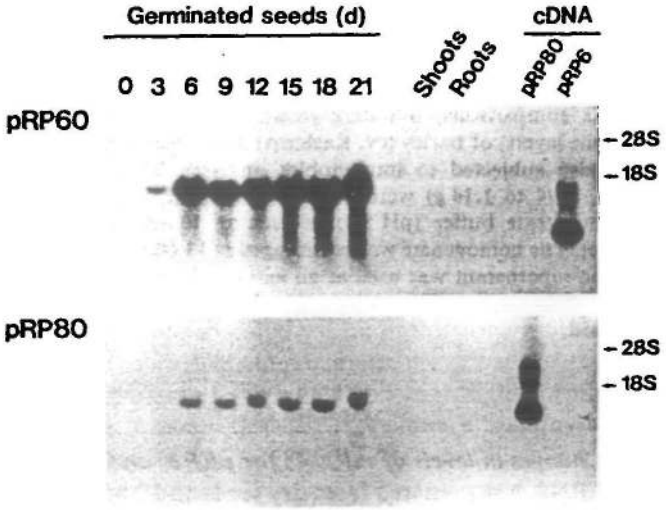

Fig. 2 Comparison of relative levels of mRNAs for pRP60 and pRP80 during germination and early seedling growth. Total RNA was prepared from d-0 to d-21 germinated whole seedlings, shoots and roots of d-9 seedilngs, and 10 μg of RNA was subjected to RNA gel blotting with the pRP6 or pRP80 cDNA probe. The pRP6 and pRP80 cDNA inserts (0.5 ng each) were electrophoresed as hybridization controls.

ble in d-0 or d-3 seeds, but the level expressed per seed increased after d 6, reached a peak on d 9, and fell thereafter. pRP60 mRNA was also not detected in dry seeds either, but became detectable and reached a peak level about 3 d earlier than pRP80 mRNA during the course of germination and early seedling growth. To compare relative levels of pRP60 and pRP80 mRNAs, total RNAs from d-0 to d-21 whole seeds were subjected to RNA gel blot analysis using pRP80 and pRP6 as probes with the same specific radioactivities. Hybridization, washes of membrane and autoradiography for both probes were carried out concurrently under the same conditions. The analytical results indicated that about ten times as much pRP60 mRNA accumulated as pRP80 mRNA throughout the d 6 to 21 period (Fig. 2). Neither mRNA was detected in the shoot or root of d-9 seedlings (Fig. 2).

Response of mRNA levels to gibbrellic acid—When whole seeds were allowed to imbibe water and germinate for 3 d, a slight amount of pRP80 mRNA and a much higher amount of pRP60 mRNA were detected on RNA blots (Fig. 3A). When de-embryonated seeds were incubat-

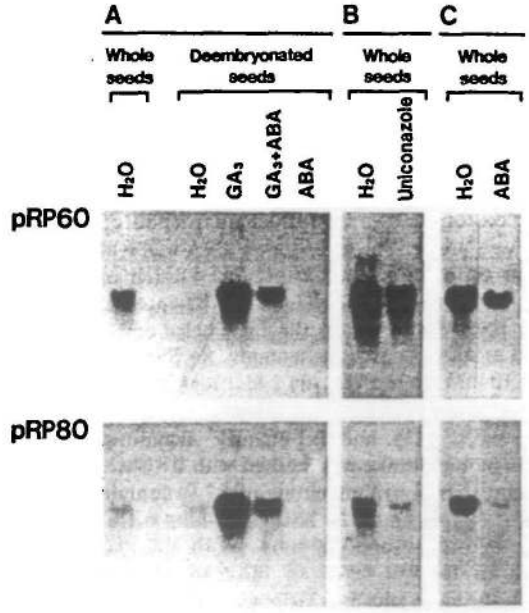

Fig. 3 Effects of GA_3, ABA and uniconazole on levels of mRNAs for pRP60 and pRP80 in germinated and de-embryonated seeds. (A) Whole seeds were allowed to germinate for 3 d in the absence (H_2O) of GA_3. De-embryonated seeds were incubated for 3 d in the absence (H_2O) and presence of 0.05 μM GA_3 (GA_3), 0.05 μM GA_3 plus 50 μM ABA (GA_3+ABA) or 50 μM ABA (ABA). (B) Whole seeds were allowed to germinate for 5 d in the absence (H_2O) and presence of 50 μM uniconazole (Uniconazole). (C) Whole seeds were allowed to germinate for 5 d in the absence (H_2O) and presence of 50 μM ABA (ABA). Total RNA (10 μg per lane) prepared from the seeds was analyzed by RNA gel blotting with the pRP6 or pRP80 cDNA probe. Experimental details are described in 'Materials and Methods'.

ed for 3 d in this manner, the amounts of pRP80 and pRP60 mRNAs were very low (about 10% and 15% of whole seeds, respectively; Fig. 3A). However, when de-embryonated seeds were incubated for 3 d in the presence of 0.05 μM GA_3, there were high levels of both pRP60 and pRP80 mRNAs. This effect of GA_3 in increasing mRNA levels was partly eliminated by the addition of 50 μM ABA to the GA_3 medium. Incubation of embryo-less seeds for 3 d in the presence of 50 μM ABA resulted in only a slight amount of pRP60 mRNA and an undetectable amount of pRP80 mRNA (Fig. 3A). When whole seeds were germinated for 3 d in the presence of 50 μM uniconazole, a triazole-type growth retardant which inhibits the biosynthesis of gibberellins at the conversion of *ent*-kaurene to *ent*-kaurenoic acid (Izumi et al. 1985), the level of pRP80 mRNA was clearly lower than the control; the level of pRP60 mRNA fell less effectively (Fig. 3B). The estimation of the relative mRNA levels by scanning radioautograph with a densitometer indicated that, in the presence of uniconazole, the amounts of pRP60 and pRP80 mRNAs were reduced to 60 and 20% of the control values, respectively. In the presence of 50 μM ABA, the levels of both mRNAs decreased, and it was noted that ABA affected the level of pRP80 mRNA more strongly than that of pRP60 (Fig. 3C).

De-embryonated seeds were incubated for 5 d in the presence of GA_3 at various concentrations. RNA gel blots showed there was a marked effect of GA_3 at 0.01 to 1 μM, increasing the amount of both mRNAs. GA_3 at 1 nM was only slightly effective (Fig. 4). Both pRP60 and pRP80 mRNAs seemed to be similarly sensitive to GA_3 at the concentrations used.

To examine the possible difference in response of pRP60 and pRP80 mRNAs to GA_3, de-embryonated seeds were incubated for 0, 3 and 5 d in the presence of GA_3, and the time-course of mRNA accumulation was analyzed (Fig. 5). Slight amounts of both mRNAs were present in de-embryonated seeds incubated for 3 to 5 d in the absence of GA_3. However, exogenously supplied GA_3 at 1 μM was effective in restoring the levels to those of whole seeds. In the presence of GA_3, the amounts of both pRP60 and pRP80 mRNAs increased to high levels on d 3, although, on d 1, the levels were at the limit of detection. The amounts of both mRNAs seemed to increase further on d 5. Densitometric measurement indicated that levels of pRP60 and pRP80 mRNAs on d 3 increased 3.5 to 4.0 fold, respectively, with the addition of 1 μM GA_3 to the incubation medium. Essentially no conspicuous difference was observed in the time-course response of these two mRNAs to GA_3.

Effects of GA_3 and ABA on REP-1 expression—Using protein immunoblot analysis, we examined the hormonal response of REP-1 protein, the translation product of pRP60 mRNA. To obtain an antiserum against REP-1, we synthesized the REP-1 protein in *E. coli* cells. The recombinant REP-1 protein had the same mobility as REP-1 from rice seedlings on an SDS-polyacrylamide gel and had the same nine amino-terminal amino acids as REP-1 except for the addition of Met^1 (Fig. 6). Antiserum raised against the recombinant REP-1 was similarly immunoreactive to REP-1 from rice, but not to REP-2, the other major cysteine

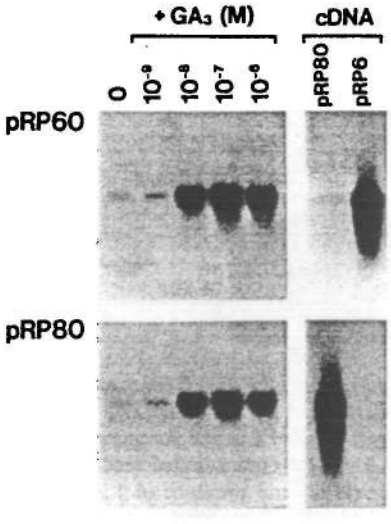

Fig. 4 Effects of GA_3 concentrations on levels of mRNAs for pRP6 and pRP80 in de-embryonated seeds. De-embryonated seeds were incubated for 5 d in the presence of GA_3 at various concentrations. Total RNA (10 μg per lane) prepared from the seeds was analyzed by RNA gel blotting with the pRP6 or pRP80 cDNA probe. The pRP80 and pRP6 cDNA inserts (0.5 ng each) were electrophoresed as positive hybridization controls.

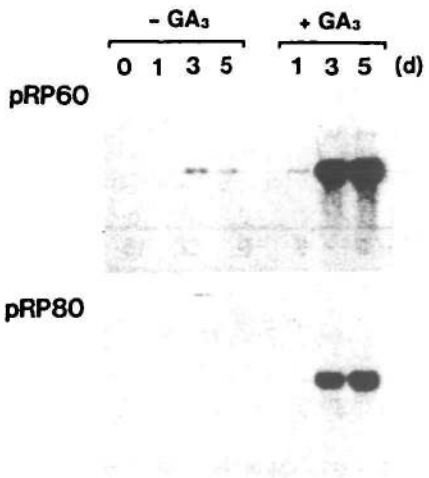

Fig. 5 Change over time in levels of mRNAs for pRP60 and pRP80 in de-embryonated seeds incubated in the presence of GA_3. De-embryonated seeds were incubated for 0, 3 and 5 d in the presence (+) and absence (−) of 1 μM GA_3. Total RNA (10 μg per lane) prepared from the seeds was analyzed by RNA blotting with the pRP6 or pRP80 cDNA probe.

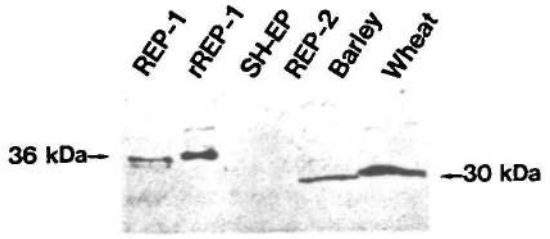

Fig. 6 Immunoreactivity of REP-1 and related proteins. Immunoreactivity of REP-1 and related proteins was analyzed by SDS-PAGE/immunoblotting using the antiserum against REP-1 that was synthesized in *E. coli*. *REP-1*, REP-1 isolated from germinated rice seeds (0.5 μg of protein); *rREP-1*, REP-1 synthesized in *E. coli* cells (0.5 μg); *SH-EP*, a major cysteine endopeptidase from *V. mungo* seedlings (1 μg); *REP-2*, the other major cysteine endopeptidase partially purified from germinated rice seeds (3 μg; Kato and Minamikawa 1996); *Barley*, extracts from d-3 germinated barley seeds (6 μg); and *Wheat*, extracts from d-3 germinated wheat seeds (8 μg). Experimental details are described in 'Materials and Methods'.

endopeptidase from rice seedlings (Kato and Minamikawa 1996). The antiserum was immunoreactive to a 30-kDa polypeptide corresponding to barley EP-B (Koehler and Ho 1990b) but not to *V.mungo* SH-EP (Mitsuhashi and Minamikawa 1989). The deduced nucleotide sequence of the REP-1 mature enzyme has 72% identity with that of EP-B. The presence of a 30-kDa polypeptide on the immunoblot of extracts from wheat seeds suggests the occurrence of a wheat cysteine protease in the same category as EP-B.

The change over time in relative levels of the REP-1 protein was measured by scanning immunoreactive bands on an immunoblot with a densitometer (Fig. 7). No detectable amounts of the REP-1 protein were found in extracts from dry (quiescent) or d-3 seeds. The level, expressed per seed, increased notably after d 6, reached a maximum on d 9 and decreased thereafter. This pattern of change in REP-1 level seemed similar to that of pRP60 mRNA (Fig. 1) but occurred about 3 d later. When de-embryonated seeds were incubated in the absence of GA_3, no immunoreactive polypeptide corresponding to 36-kDa REP-1 was detected on the gel 3 d after the onset of imbibition, but weak and then stronger bands of 36 kDa were observed on d 5 and 7, respectively (Fig. 8A). In contrast, in the presence of 1 μM GA_3, the polypeptide was detected even on d 1, and stronger bands were detected on d 3 to 7 with the strongest band on d 5. The effect of GA_3 in increasing the level of the 36-kDa polypeptide was clearly diminished when 10 μM ABA was added to the GA_3 medium (Fig. 8B). Supplying 10 μM ABA to whole seeds had little effect on the level of

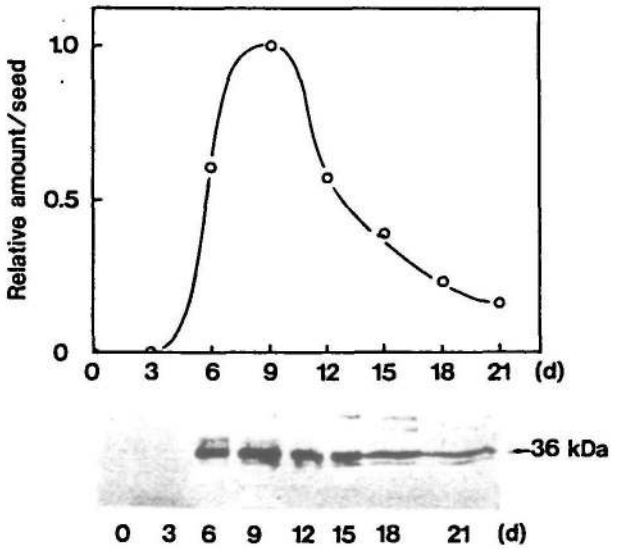

Fig. 7 Change over time in the relative level of the REP-1 protein during germination and early seedling growth. Extracts (10 μl per lane) prepared from d-3 to d-21 seedlings (100 pieces each) were analyzed by SDS-PAGE/immunoblotting using the antiserum against REP-1. Relative amounts of the REP-1 protein per seed were semi-quantified by scanning the immunoreactive bands (*lower panel*) with a densitometer, and the highest level was set at 1.0.

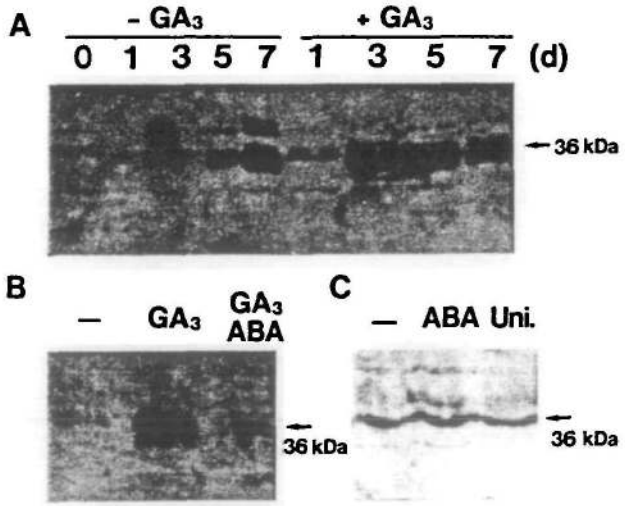

Fig. 8 Effects of GA_3, ABA and uniconazole on levels of REP-1 in de-embryonated or whole seeds. (A) De-embryonated seeds were incubated for 1 to 7 d in the absence (H_2O) and presence of 1 μM GA_3 (GA_3). (B) De-embryonated seeds were incubated for 5 d in the absence (H_2O) and presence of 1 μM GA_3 (GA_3) or 1 μM GA_3 plus 10 μM ABA (GA_3-ABA). (C) Whole seeds were incubated for 5 d in the absence (H_2O) and presence of 10 μM ABA (ABA) or 10 μM uniconazole (Uni.). After the incubation, the protein was extracted from the seeds and analyzed by SDS-PAGE/immunoblotting using the antiserum against REP-1. The extract corresponding to 20 μg (A, B) or 100 μg (C) of protein was loaded on a lane. Experimental details are described in 'Materials and Methods'.

the 36-kDa polypeptide, and the addition of 10 μM uniconazole partly reduced this level on d 5 (Fig. 8C). These incomplete effects of ABA and uniconazole on the amount of the 36-kDa polypeptide coincided with those on pRP60 mRNA (Fig. 3C). When de-embryonated seeds were incubated for 5 to 7 d in the absence of GA_3, an immunoreactive band with a larger molecular mass was observed on the immunoblot (Fig. 8A). This polypeptide was also observed when whole seeds were incubated in the presence of ABA (Fig. 8C). The possibility that the polypeptide is an intermediate of mature REP-1 remains to be examined.

Discussion

pRP60 and pRP80 mRNAs were detected only in germinated rice seeds but not in dry seeds (Fig. 1), indicating that these are newly transcribed after the onset of imbibition. Total endopeptidase activity in seed extracts was barely detectable until d 6 of post-imbibition, after which it increased and reached a maximum at d 15 to 18 (Kato and Minamikawa 1996). pRP60 mRNA increased on d 3 of post-imbibition, reached a peak on d 6, and then decreased. pRP60 mRNA showed a time-course profile similar to pRP80 mRNA but was 3 d later (Fig. 2). These results imply that the accumulation of pRP60 mRNA, a mRNA for REP-1, as well as pRP80 mRNA, largely preceded those of endopeptidase activity during germination and early seedling growth. Comparison of amounts of the two mRNAs showed that pRP60 mRNA accumulated at about ten-fold higher levels than did pRP80 mRNA (Fig. 2). This coincides with our previous observation that RER-1 is a major endopeptidase which acts to degrade seed storage glutelin during germination and early seedling growth (Kato and Minamikawa 1996). A preliminary genomic DNA analysis suggested that each of the pRP60 and pRP80 mRNAs is derived from a single-copy gene (Shintani et al. unpublished data).

It has been well decumented that, during early stages of germination of cereal grains, gibberellins are synthesized in the embryo, diffuse to the aleurone layers through the scutellar epithelium septum and induce the synthesis and secretion of hydrolytic enzymes such as α-amylases and proteases (Akazawa and Hara-Nishimura 1985, Koehler and Ho 1990a, b, Bewley and Black 1994) which degrade endosperm nutrients and thus support the seedling growth. Consistent with those results, the present study shows the dependence of pRP60 and pRP80 mRNA expression on GA_3. High levels of expression of both pRP60 and pRP80 mRNAs observed in germinated rice seeds were strongly reduced when seeds were de-embryonated and incubated. Exogenously supplied 0.01 to 1 μM GA_3 compensated for the removal of the embryo with respect to the mRNA levels (Fig. 4). Inhibition of the accumulation of pRP60 and pRP80 mRNAs by ABA or uniconazole in intact seeds also supported the involvement of endogenous gibberellin in the high level expression of the mRNAs. Accumulation of both mRNAs in de-embryonated seeds that had been exogenously supplied with GA_3 was induced more rapidly than that in germinated seeds (Fig. 3).

Two major barley cysteine proteases, EP-A and EP-B, are differentially regulated with respect to their temporal and hormonally induced expression (Koehler and Ho 1988, 1990b). In the present study, pRP60 and pRP80 mRNAs appeared to respond similarly to exogenously supplied GA_3 at various concentrations (Fig. 4, 5). The GA_3 concentrations most effectively inducing these mRNAs were in the range of those for barley EP-B mRNAs (Koehler and Ho 1990b). Both pRP60 and pRP80 mRNAs were expressed specifically in seeds but not in shoots or roots (Fig. 2). Protein immunoblot analysis showed that the responses of REP-1 expression to exogenously supplied GA_3, ABA and uniconazole were analogous to those of pRP60 mRNA in de-embryonated seeds (Fig. 3, 8). However, the time-course of change in the REP-1 protein level per seed after the onset of imbibition lagged behind that of the pRP60 mRNA level by about 3 d (Fig. 7). This could indicate that the expression of the REP-1 gene is controlled not only at the transcriptional level but also by posttranscriptional events such posttranslational processing and secretion of REP-1, which might also be affected by plant hormones.

Analysis using d-5 whole seeds indicated that the expression of pRP80 mRNA was more sensitive to both antagonistic action of ABA and to inhibition by uniconazole (Fig. 3). This might be partly reflected by the temporal difference in expression between the two mRNAs during germination and early seedling growth (Fig. 1). The expression of pRP80 mRNA appeared to differ from that of pRP60 mRNA in terms of sensitivity to ABA and uniconazole and also of the time-course profile after the onset of imbibition. Although the function of the protein corresponding to pRP80 mRNA has not yet been demonstrated, the deduced amino acid sequence suggests that, like REP-1, the putative pRP80 protein has features of a cysteine protease and has a long prosequence following the signal sequence (Shintani et al. 1995). The relevance of the putative pRP80 protein to REP-2, the other major cysteine endopeptidase separated from rice seedlings, remains to be elucidated.

This work was supported in part by Grants-in-Aid for Scientific Research (nos. 08044217 and 09640776) from the Ministry of Education, Science and Culture of Japan.

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(Received May 28, 1997; Accepted September 8, 1997)