Proteomic Analysis of Embryonic Proteins Synthesized from Long-Lived mRNAs During Germination of Rice Seeds

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Dry seeds contain translatable, long-lived mRNAs that are stored during seed maturation. Early studies using transcriptional inhibitors supported the view that protein synthesis during the initial phase of germination occurs on long-lived mRNA templates. Rice seeds were treated with the transcriptional inhibitor actinomycin D (Act D), and the embryonic proteins translated from long-lived mRNAs during germination were identified using a proteomic analysis. De novo transcription was not required for germination of rice seeds, since >80% of seeds germinated when transcription was prevented by treatment with Act D. In contrast, germination was completely inhibited in the presence of cycloheximide, an inhibitor of translation. Thus, de novo protein synthesis is necessary for germination of rice seeds. The proteomic analysis revealed that 20 proteins are up-regulated during germination, even after Act D treatment. Many of the up-regulated proteins are involved in carbohydrate metabolism and cytoskeleton formation. These results indicate that some of the germination-specific proteins involved in energy production and maintenance of cell structure in rice seeds are synthesized from long-lived mRNAs. The timing of translation of eight up-regulated proteins was clearly later than that of the other up-regulated proteins under conditions in which translation was inhibited by Act D, suggesting that translation of long-lived mRNAs in rice seeds is regulated according to the germination phase.

Keywords: Actinomycin D • cycloheximide • Long-lived mRNAs • Oryza sativa • Proteome • Seed germination.

Abbreviations: ACN, acetonitrile; Act D, actinomycin D; CHX, cycloheximide; DAI, days after imbibition; 2-DE, two-dimensional gel electrophoresis; DTT, dithiothreitol; IEF, isoelectric focusing; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RBP, RNA-binding protein.
The use of transcriptional inhibitors in conjunction with proteomic techniques is an effective strategy for the global characterization of proteins synthesized from long-lived mRNAs in seeds. Sánchez de Jiménez and Aguilar (1984) used two-dimensional gel electrophoresis (2-DE) to analyze proteins synthesized in the presence and absence of α-amanitin in germinating maize seeds. They found that the 2-DE pattern of proteins synthesized during the early stage of germination in seeds treated with α-amanitin is very similar to that in untreated seeds, indicating that most proteins synthesized during this period are translated without de novo transcription from stored mRNAs. However, they did not identify any of the proteins synthesized from stored mRNAs. In addition, they reported that some of the proteins synthesized from stored mRNAs are not translated until late in germination, suggesting that a selective mechanism exists that modulates the translation of stored mRNAs in seed cells.

Rajou et al. (2004) identified the translation products of long-lived mRNAs in Arabidopsis using a proteomic approach in conjunction with α-amanitin treatment. They reported that the majority of proteins translated from stored mRNAs in seeds treated with α-amanitin correspond to proteins normally synthesized during seed development, such as 12S globulin subunits and members of the dehydrin family. However, they did not attempt to elucidate the selective translation mechanism for long-lived mRNAs during germination.

Rice is both an important staple food crop in Asia (Fresco et al. 2005) and a model monocot plant for use in molecular studies. However, little is known about the synthesis of proteins from long-lived mRNAs in rice seeds. The rice seed is composed of two distinct but interconnected parts, the embryo and endosperm. The embryo contains many important proteins involved in the induction of germination, whereas the endosperm contains storage proteins that serve as energy reserves (Dure and Waters 1965, Bewley 1997, Kim et al. 2009). The storage proteins are found in high abundance in rice seeds and are important for seedling development. Ju et al. (2001) reported that >99% of the proteins contained in defatted rice flour are storage proteins. It is well known that these high-abundance proteins are the primary limiting factor in detection of low-abundance proteins using 2-DE proteomic analyses (Masaki et al. 2008). Using embryos dissected from germinating seeds for their proteomic analysis, Kim et al. (2009) identified many key proteins involved in germination, such as glyceraldehyde 3-phosphate dehydrogenase, caffeoyl-CoA 3-O-methyltransferase and ascorbate peroxidase b. Therefore, embryos removed from endosperm containing high-abundance seed storage proteins are an ideal material for 2-DE proteomic analyses aimed at identifying key proteins synthesized from long-lived mRNAs during germination of rice seeds. In addition, it has been shown that rice seeds can germinate after being soaked in water containing α-amanitin (He et al. 2011a), further indicating that they are an ideal material for analysis of protein expression during germination.

In this study, embryos dissected from germinating rice seeds treated with a transcriptional inhibitor were examined using a proteomic approach in order to identify long-lived mRNA translational products. We also discuss the selective machinery employed by seeds to modulate the translation of long-lived mRNAs.

Results

Effect of actinomycin D on the germination of rice seeds and de novo transcription in rice seed embryos

To confirm that de novo transcription is not required for the germination of rice seeds, germination tests were carried out in the presence and absence of transcriptional and translational inhibitors. More than 90% of seeds soaked in water without inhibitors germinated 6 days after imbibition (DAI) (Fig. 1A). However, germination of seeds imbied in the presence 100 μM CHX was completely inhibited for 6 d (Fig. 1A), indicating that de novo protein synthesis is necessary for the germination of rice seeds. In contrast, >80% of seeds germinated when they imbibed water containing 2–200 μM actinomycin D (Act D), which incorporates into the DNA template and inhibits transcription (Goldberg and Friedman 1971). At 6 DAI, the germination rate of seeds soaked in water containing 200 μM Act D was 89% (Fig. 1A), although the process of germination was slightly slower than that of seeds soaked in water without inhibitors. Statistical analysis using the Tukey–Kramer test indicated that at 6 DAI there was no significant difference in the germination rate of seeds treated with Act D and that of untreated seeds.

To examine the inhibitory effect of Act D on the synthesis of RNA during seed germination, the total RNA content of embryos dissected from seeds soaked in water with and without Act D was determined (Fig. 1B). The total RNA content of embryos incubated in water only was slightly elevated at 2 DAI, and then increased dramatically at 4 DAI. At 6 DAI, the total RNA content in embryos from water-soaked seeds was approximately 2.2-fold greater than that of embryos dissected from dry seeds. In contrast, Act D treatment significantly inhibited the increase in total RNA content (Fig. 1B). In seeds treated with 2 μM Act D, the total RNA content was slightly elevated at 4 DAI; however, the total RNA content of embryos treated with 20 μM Act D was only about 0.7-fold of that of embryos dissected from dry seed at 4 DAI. Similar results were obtained for embryos treated with 100 or 200 μM Act D. These results indicated that Act D at concentrations ranging from 20 to 200 μM significantly inhibits de novo transcription during germination in rice embryos. Taken together, these data suggest that de novo transcription is not necessary for germination of rice seeds, since seeds can germinate even when de novo RNA synthesis is almost completely suppressed by an excess of Act D.
Influence of Act D on rice seedling growth

Although we found that Act D had no significant effect on the rate of seed germination at 6 DAI, it has been reported that transcriptional inhibitors suppress seedling growth after germination (Rajjou et al. 2004, He et al. 2011a). Therefore, we also examined the effect of Act D on rice seedling establishment. In water alone, the seedling coleoptile had grown slightly by 2 DAI, and had developed dramatically by 4 DAI (Fig. 2A). By 6 DAI, the coleoptile reached a length of 2.7 cm (Fig. 2B). In addition, the radicle had clearly elongated by 4 DAI (Fig. 2A). However, Act D had a significant dose-dependent inhibitory effect on the growth of rice seedlings. In seedlings treated with 200 μM Act D, the coleoptile reached only about 2 mm by 6 DAI (Fig. 2B). Moreover, emergence of the radicle could not be detected at 6 DAI (Fig. 2A). These results suggest that rice seedlings cannot be established through long-lived mRNAs alone, and that de novo transcription is necessary for growth after germination.

Proteomic analysis at 2 DAI of embryos from seeds treated with Act D

A proteomic analysis of embryos dissected from germinating rice seeds treated with 200 μM Act D was carried out in order to identify proteins up-regulated under conditions in which de novo transcription is inhibited. Total protein was extracted from embryos dissected at 2 DAI from seedlings treated with H2O alone or with Act D, and then analyzed using 2-DE (Fig. 3). A comparison of 2-DE protein profiles indicated that global

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**Fig. 1** Effect of Act D on germination and total RNA content in germinating embryos. (A) Effect of Act D and CHX on the germination of rice seeds. A total of 50 dehulled seeds were incubated at 28°C in water with or without inhibitors. The germination rate values are means of three replicates ± SE determined at 6 DAI. Identical letters represent no significant difference at P < 0.01 (Tukey-Kramer test). ND, not detected. (B) Change in total RNA content in germinating embryos after imbibition in water or Act D, assessed using a Nano Drop 1000 spectrophotometer. Values are means of three replicates ± SE.

**Fig. 2** Influence of Act D and CHX on rice seedling growth. (A) Photographs show dry seeds and growth of seedlings at 2, 4 and 6 DAI with water in the presence or absence of inhibitors. Bar = 2 mm. (B) Effect of Act D on coleoptile length. Values are means of 10 replicates ± SE determined at 2, 4 and 6 DAI.
protein synthesis during seed germination is inhibited by Act D. Several of the down-regulated proteins that were identified in Act D-treated embryos, such as putative globulin (spot 19) and cupin (spot 22), are seed storage proteins (Supplementary Table S1, Supplementary Fig. S1). The constitutively expressed proteins included those involved in metabolism (spot 26: glyoxalase I) and stress responses (spot 25: 16.9 kDa heat shock protein) (Supplementary Table S2, Supplementary Fig. S2).

A number of proteins up-regulated in expression were also detected on 2-DE gels of embryos from seeds treated with Act D. When seeds were soaked in 200 μM Act D for 2 d, the expression of 12 proteins apparently increased in the germinating embryos (Table 1). Representative expression profiles of several of the proteins that were up-regulated at 2 DAI are shown in Fig. 4. The levels of spots 4, 5 and 14 were >2-fold greater in embryos of seeds treated with Act D for 2 d than they were in dry embryos, despite the fact that de novo transcription was inhibited. Moreover, up-regulation of these 12 proteins was also detected in embryos from seeds that were imbibed in water without inhibitor. These results indicate that translation of these 12 proteins is induced in embryos during the early phase of normal germination.

Seven of the 12 up-regulated proteins are involved in carbohydrate metabolism (Table 1). Enolase (spot 4), putative phosphoglycerate mutase (spot 5), triosephosphate isomerase (spot 6) and fructokinase-1 (spot 12) play important roles in glycolysis (Alberts et al. 2010). Enolase (spot 4) is an essential enzyme, catalyzing the interconversion of 2-phosphoglycerate and phosphoenolpyruvate. Putative phosphoglycerate mutase (spot 5) mediates interconversion of the phosphate group between the C-3 carbon of 3-phosphoglycerate and the C-2 carbon of 2-phosphoglycerate. Triosephosphate isomerase (spot 6) catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, while fructokinase-1 (spot 12) catalyzes the conversion of fructose to fructose-6-phosphate, which is an entry point into glycolysis via conversion into glucose-6-phosphate. Spots 3 and 15 were identified as a putative α-amylase precursor and vacuolar ATPase B subunit, respectively, which are involved in energy production and carbohydrate metabolism (Guglielminetti et al. 1995, Kim et al. 2009). Actin-7 (spot 13) and tubulin α-1 chain (spot 14) are associated with cytoskeleton formation (Alberts et al. 2010). These results reveal that some germination-specific proteins involved in glycolysis, energy production and cell structure are translated under conditions in which de novo transcription is strongly inhibited by Act D, suggesting that these proteins are synthesized from long-lived mRNAs during the initial phase of rice seed germination.

**Proteomic analysis at 4 DAI of embryos from seeds treated with Act D**

At 4 DAI, slight growth of the seedlings was detected even under conditions in which RNA synthesis was strongly inhibited by 200 μM Act D. It has been reported that in
Arabidopsis, mRNAs encoding different types of proteins are translated in different developmental phases of seed germination (Gallardo et al. 2001). Therefore, it is possible that de novo translation of long-lived mRNAs distinct from those encoding the 12 proteins synthesized at 2 DAI may occur at 4 DAI. In order to examine this possibility, a proteomic analysis of embryos from seeds imbibed in the presence of Act D was carried out.

The expression of eight proteins was significantly up-regulated in the germinating embryos at 4 DAI in the presence of Act D (Table 2), suggesting that these proteins are also translated from long-lived mRNAs. Representative expression profiles for several of these up-regulated proteins are shown in Fig. 5. The expression of several proteins (spots 2, 9 and 10) in embryos from Act D-treated seeds did not increase until 2 DAI, but the expression of these proteins was >2-fold greater in dry embryos. The expression pattern in gels of embryos examined at 4 DAI was clearly different from that of up-regulated proteins identified in embryos examined at 2 DAI (Fig. 4). Up-regulation of these eight proteins was also observed when the seeds were soaked in water without inhibitor, indicating that they are synthesized in embryos during the normal germination process.

Most of the eight proteins identified as being up-regulated at 4 DAI are involved in metabolism (Table 2). Putative pyrophosphate-dependent phosphofructokinase (spots 8 and 10) and enolase (spot 16) play indispensable roles in glycolysis. Putative pyrophosphate-dependent phosphofructokinase catalyzes the transfer of a phosphate group from diphosphate to fructose 6-phosphate, yielding fructose 1,6-bisphosphate (Alberts et al. 2010). The proteins associated with spots 9, 11 and 27 were identified as putative phosphoglycerate dehydrogenase, mitochondrial aldehyde dehydrogenase ALDH2a and cytoplasmic malate dehydrogenase, respectively. Putative phosphoglycerate dehydrogenase plays a key role in serine biosynthesis (Ho et al. 1999), while mitochondrial aldehyde dehydrogenase ALDH2a is involved in alcoholic fermentation (Nakazono et al. 2000), and cytoplasmic malate dehydrogenase is part of the gluconeogenesis pathway (Hung et al. 2004). The protein associated with spot 2 was identified as tubulin β-1 chain, which is a constituent of the cytoskeleton (Alberts et al. 2010). These metabolism- and cell structure-related proteins are notably up-regulated in embryos at 4 DAI but not at 2 DAI, even under conditions in which de novo transcription is strongly inhibited, suggesting that some long-lived mRNAs are selectively translated depending upon the stage of germination.

### Table 1 Identification of proteins up-regulated at 2 DAI in germinating embryos imbibed with Act D

<table>
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<tr>
<th>Spot No.</th>
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<th>Exp./Theo.</th>
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<th>Cov. (%)</th>
<th>Relative ratio</th>
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<th>Function</th>
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<td></td>
<td></td>
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*Consistent with the spot numbers in Fig. 3.

The relative ratio of the protein amount determined using Polyans 2D during germination in each treatment compared with that of dry seeds.

Accession number of the matched protein in the NCBI database (http://www.ncbi.nlm.nih.gov/).

**Table 2**

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*Accession number of the matched protein in the NCBI database (http://www.ncbi.nlm.nih.gov/).*
**Fig. 4** Representative expression profiles of proteins up-regulated at 2 DAI in germinating embryos imbibed with Act D. (A) Quantitative changes in up-regulated proteins on images of the 2-DE gels shown in **Fig. 3**. (B) Relative protein levels were calculated with Polyans 2D software using the ovalbumin band in the marker proteins on the 2-DE gel as an internal control. Values are means of three replicates ± SE.

**Table 2** Identification of proteins up-regulated at 4 DAI in germinating embryos imbibed with Act D

<table>
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<th>Spot No.</th>
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</table>

aConsistent with the spot numbers in **Fig. 3**.
bExp./Theo. pI, experimental isolectric point/theoretical isolectric point.
cExp./Theo. Mr (kDa), experimental molecular size/theoretical molecular size.
dThe protein score is $-10 \times \log(P)$, where $P$ is the probability that the observed match is a random event. Protein scores >64 are significant ($P < 0.05$).
eSequence coverage (%).
fThe relative ratio of the protein amount determined using Polyans 2D during germination in each treatment compared with that of dry seeds.
gAccession number of the matched protein in the NCBI database (http://www.ncbi.nlm.nih.gov/).
Discussion

De novo translation of proteins from long-lived mRNAs in germinating rice embryos

The present study clearly demonstrates that the expression of 20 proteins is up-regulated in germinating embryos detached from seeds imbibed in water containing 200 μM Act D (Fig. 3), and that most of these proteins are involved in carbohydrate metabolism and formation of the cytoskeleton (Tables 1, 2). Treating seeds with Act D at concentrations >20 μM significantly reduced the accumulation of total RNA in germinating embryos (Fig. 1B), suggesting that the 20 up-regulated proteins were synthesized from previously existing mRNA templates. In addition, synthesis of these proteins might be critical during the early phase of normal germination since our data indicate that they are also up-regulated in untreated control embryos (Fig. 3). Taken together, these results strongly suggest that the 20 up-regulated proteins are translated from long-lived mRNAs during the initial phase of rice seed germination.

Proteins involved in carbohydrate metabolism and formation of the cytoskeleton during seed germination

It has been reported that most of the proteins synthesized in the early stage of maize seed germination might be formed from long-lived mRNAs because the 2-DE patterns of proteins expressed during germination with and without α-amanitin are very similar (Sánchez de Jiménez and Aguilar 1984). However, the authors of that study did not identify the proteins involved. Recent proteomic studies of Arabidopsis seeds have identified a number of proteins synthesized during the early phase of normal germination, many of which are key enzymes involved in carbohydrate metabolism (Gallardo et al. 2001). Because the quiescent embryonic cells in dry seeds shift into a metabolically active state and rapidly reinitiate tissue formation upon seed germination, it is likely that the proteins synthesized during this period play important roles in the process. Previous reports have also indicated that proteins involved in carbohydrate metabolism are up-regulated during normal germination of rice seeds (Yang et al. 2007, Kim et al. 2009). In addition, treating rice seeds with NaF, an inhibitor of glycolysis, prevents their germination (Siegel et al. 1967). These reports support the hypothesis that metabolic processes such as glycolysis play a central role in energy production during the early stage of rice seed germination. We also detected an increase in expression of α-amylase precursor (spot 3) in the embryo. The Rice Expression Profile Database (RiceXPro) (http://ricexpro.dna.affrc.go.jp/) indicates that the transcript of this α-amylase precursor (accession No. 46805736) is specifically stored in mature embryos. Moreover, it is known that α-amylase activity is

Fig. 5 Representative expression profiles of the proteins up-regulated at 4 DAI in germinating embryos imbibed with Act D. (A) Quantitative changes in up-regulated proteins on images of the 2-DE gels shown in Fig. 3. (B) Relative protein levels were calculated with Polyans 2D software using the ovalbumin band in the marker proteins on the 2-DE gel as an internal control. Values are means of three replicates ± SE.
detected in the rice embryo at the early germinating stage (Okamoto and Akazawa 1979). Thus, this enzyme may be involved in energy production in the embryo during the early stage of rice seed germination.

Members of the tubulin family of proteins are reportedly involved in cell division and cell enlargement processes during seed germination. For example, β-tubulin has been shown to accumulate during early germination of tomato seeds (De Castro et al. 2000). It has also been reported that proteins involved in formation of the cytoskeleton structure may play important roles in cell structure biosynthesis during the germination of rice seeds (Kim et al. 2009). The findings presented in this report indicate that some of the proteins that play key roles in carbohydrate metabolism and formation of the cytoskeleton during the initial phase of seed germination are synthesized from long-lived mRNA templates.

Long-lived mRNAs stored in dry seeds

Assays of the germination of rice seeds treated with Act D demonstrated that de novo transcription is not required for completion of germination (Figs. 1A, 2A). However, our results also indicate that de novo protein synthesis is necessary for germination of rice seeds since germination was completely inhibited in the presence of CHX (Figs. 1A, 2A). These results are consistent with previous studies of germination in cotton, Arabidopsis and rice (Dure and Waters 1965, Rajjou et al. 2004, He et al. 2011a), and suggest that proteins required for germination are synthesized from long-lived mRNAs previously produced and stored in dry seeds.

Reports have demonstrated that dry rice seeds contain >17,000 stored mRNAs (Howell et al. 2009) and that dry Arabidopsis seeds contain >12,000 stored mRNAs (Nakabayashi et al. 2005). These mRNAs are thought to be stored during seed maturation (Dure and Waters 1965, Hammett and Katterman 1975). Nakashima et al. (2009) reported that numerous genes in dry seeds of the severely ABA-insensitive snrk2d/e/i triple mutant Arabidopsis are transcribed to different degrees compared with their transcription in wild-type seeds, and that the mutant could not germinate after dry storage. In addition, it was reported that both the total RNA content and germination capacity decrease in Pisum sativum seeds as the seeds age (Kranner et al. 2011). These reports support the hypothesis that proper accumulation and translation of long-lived mRNAs in dry seeds is necessary for the completion of normal germination. However, little is known about when the mRNAs needed for germination accumulate during seed development or how their structural integrity is maintained during the long periods of severe desiccation that mature dry seeds may be subjected to prior to germination. Further research should be planned to clarify these points.

The effect of Act D on rice seed germination and seedling development

Dure and Waters (1965) examined the effect of Act D on de novo RNA synthesis in germinated cotton embryos by analyzing the incorporation of 32P into nuclear RNA. They assumed that 20 μg ml−1 (16 μM) Act D effectively inhibits de novo mRNA synthesis. Measurements of [3H]uridine incorporation into newly synthesized rRNA indicated that treatment with 30 μg ml−1 (24 μM) Act D almost completely inhibits RNA synthesis during the early stage of germination in pea cotyledons (Gumilevskaya et al. 1982). In our experiment, treatment with Act D at concentrations >20 μM significantly reduced the accumulation of total RNA in germinating embryos (Fig. 1B). These data suggest that de novo transcription during germination in rice embryos is almost completely suppressed by Act D in excess of 200 μM.

Although treatment with Act D had not prevented germination of rice seeds by 6 DAI, the rate of the germination process was slightly reduced. The germination rate of seeds that were not treated with inhibitors reached 90% at 2 DAI, but that of seeds soaked in water containing 200 μM Act D was only 70% (Supplementary Fig. S3). This result is consistent with those of previous studies in Arabidopsis and rice (Rajjou et al. 2004, He et al. 2011a). In addition, Act D markedly inhibited the growth of seedlings after germination. Elongation of the coleoptiles but no protrusion of the radicles was observed in seedlings germinated from seeds treated with 200 μM Act D (Fig. 2A). This finding agrees well with those of Rajjou et al. (2004) and He et al. (2011a). Observation of abnormal morphosis during germination of artificially aged P. sativum seeds was also reported (Kraner et al. 2011). These aged seeds could protrude the radicles but not the shoots after imbibition, which might have been due to abnormal transcription during germination since the DNA in the artificially aged seeds had begun to degrade. From these findings, it has been suggested that de novo transcription is necessary for normal morphogenesis of seedlings after germination.

Rajjou et al. (2004) reported that during the germination of Arabidopsis seeds de novo synthesis of several enzymes involved in reserve mobilization and resumption of metabolic activity is strongly repressed by α-amanitin after imbibition of seeds, and that the expression levels of these proteins might be transcriptionally regulated during germination. In our experiments, the level of de novo synthesis of proteins in germinating embryos from seeds treated with Act D was about half of that in embryos from seeds soaked in water alone (Tables 1, 2). In addition, some up-regulated proteins detected in 2-DE gels of embryos from seeds imbibed in water alone were not detected in germinating embryos treated with the inhibitor (Fig. 3). The up-regulation of these proteins appears to depend on newly synthesized mRNAs.

Can long-lived mRNAs be selectively translated?

In our 2-DE analysis, the expression of 20 proteins was up-regulated following treatment with Act D, and these proteins are thought to be synthesized from long-lived mRNA templates. Significant up-regulation in the expression of 12 of these proteins was observed at 2 DAI (Table 1). The expression
of the remaining eight proteins was either slightly up-regulated or unchanged compared with controls at 2 DAI, but dramatically increased at 4 DAI (Table 2). Thus, the timing of the translation of these eight proteins was clearly different from that of the 12 proteins up-regulated at 2 DAI. Since transcription was strongly inhibited by Act D, the mRNAs for all 20 proteins must be present in the embryo before imbibition. Therefore, the stored mRNAs encoding the eight proteins significantly up-regulated at 4 DAI were probably maintained in an inactive state until needed at 4 DAI. Our data indicate that the long-lived mRNAs present in rice seed are selectively translated depending upon the germination phase. Similar results demonstrating that mRNAs stored in maize seeds might be selectively translated during germination were reported by Sánchez de Jiménez and Aguilar (1984), who used 2-DE to examine differences in the patterns of proteins synthesized during the early and late germination periods in seeds treated with a-amanitin. Their results imply that the machinery that selects stored mRNAs for translation during germination is present in maize seeds.

Rajjou et al. (2004) reported that treatment of Arabidopsis seeds with a-amanitin induces the synthesis of proteins that are nearly undetectable in seeds germinated normally without inhibitor treatment. Several of the a-amanitin-induced proteins they identified are involved in seed maturation, such as the 12S globulin subunit and members of the dehydrin family, which are normally synthesized during seed development and then degraded during germination. These findings suggest that the translatable stored mRNAs can be selected by treatment with a-amanitin and that the synthesis of seed maturation proteins is recapitulated in Arabidopsis seeds. Re-induction of translation of seed development-related proteins following Act D treatment could not be detected under our experimental conditions. In any case, Arabidopsis seeds also contain the necessary machinery for the selection of long-lived mRNAs for translation.

Regulation of the selective translation of long-lived mRNAs

Our experimental results and previous reports indicate that selective translation of long-lived mRNAs occurs during seed germination. Moreover, it is known that large amounts of seed storage proteins accumulate in mature dry seeds, and that both transcription and translation of these proteins decrease during the germination process (Li et al. 2007, He et al. 2011b). The mRNAs for these storage proteins are residual mRNAs produced during seed development; they are not essential for germination and may alternatively be degraded during germination. The findings in our experiments and previous reports (Bewley and Black 1994) regarding the alternative degradation of stored mRNAs highlight a long unanswered question, namely how do the cells of imbibed seeds discriminate between mRNAs to be utilized in germination and those that are to be destroyed?

It is known that RNA-binding proteins (RBPs) govern many aspects of RNA metabolism, including pre-mRNA processing, transport, stability/decay and translation (Lorkovic 2009, Ambroson et al. 2011). In Arabidopsis, the pentatricopeptide repeat (PPR) protein (CRR4), which contains an RNA recognition motif (RRM), regulates the function of plastid ndh1 mRNA by C-to-U RNA editing to create the translational initiation codon (Okuda et al. 2006), whereas the Arabidopsis KH domain-containing proteins FLK and FPA are required for processing the mRNA for the flowering repressor, FLC (Quesada et al. 2005). RBPs involved in mRNA stability have been described in Arabidopsis (Lambermon et al. 2002). The UBP1-associated (UBA) proteins UBA1a and UBA2a are nuclear RBPs that may act as components of a complex that recognizes U-rich sequences in plant 3′ untranslated regions, contributing to the stabilization of mRNAs in the nucleus. In addition, induction of the expression of three UBA2 genes, UBA2a, UBA2b and UBA2c, stimulates leaf yellowing and cell death phenotypes through senescence and defense response pathways. These RBPs probably stabilize transcripts specifically involved in the progression of stress-induced senescence and cell death (Kim et al. 2008). Recently, it was shown that these RBPs are present in seeds. In the developing seeds of rice, 257 RBPs have been experimentally identified to date (Morris et al. 2011). Masaki et al. (2008) reported that the putative RBP and glycine-rich RBP appear to be associated with seed germination in rice. In maize embryonic axes, expression of two mRNA CAP-binding protein isoforms, eukaryotic initiation factors (eIF) 4E and elfiso4E, appears to count for part of the selective translation of stored mRNAs (Dinkova et al. 1999, Dinkova et al. 2000, Dinkova et al. 2003). These proteins are probably involved in recognition of transcript-specific CAP structures or the absence of CAP in the mRNAs in germinating maize embryonic axes, and produce distinctive translation patterns through mRNA CAP dependency. Decapping of mRNAs is one way in which mRNAs are marked for degradation. The AtDCP2 protein is an active subunit of the decapping RBP complex in Arabidopsis. While capped mRNAs accumulate in AtDCP2 mutants, some mRNAs are specifically depleted. Furthermore, post-embryonic development is blocked in Arabidopsis AtDCP2 mutants, indicating that decapping is important for embryo to seedling developmental transition (Goeres et al. 2007). The machinery involved may also be associated with discriminating mRNAs that are to be destroyed. The evidence strongly suggests that RBPs are involved in the selective translation of long-lived mRNAs. Further studies focusing on RBPs should be undertaken in order to obtain a better understanding of the mechanism that regulates the selective translation of long-lived mRNAs during seed germination.

Materials and Methods

Germination tests

A total of 50 dehulled rice (Oryza sativa cv. Nipponbare) seeds were pre-incubated in distilled water alone, various
concentrations of aqueous Act D (Wako, Japan) or 100 μM CHX (Wako) in water at 4°C for 2 d under dark conditions. The pre-incubated seeds were then transferred to a dark chamber maintained at 28°C and incubated for 6 d. The imbibition solutions were replaced with fresh solution every other day. Germination assays were carried out on three replicates of 50 seeds each at 6 DAI at 28°C. The significance of statistical differences in the germination rate for each treatment was examined using the Tukey–Kramer test. The average coleoptile length of 10 germinated seedlings was measured at 2, 4 and 6 DAI in germinating seeds imbibed in distilled water or various concentrations of Act D.

**Extraction and quantification of total embryo RNA**

Dry embryos were separated from dehulled seeds using a surgical blade. Embryos germinating in seeds imbibed in distilled water or solutions containing various concentrations of Act D were separated from dehulled seeds at 2, 4 and 6 DAI. Total RNA was extracted from 20 embryos using Fruit-mate™ for RNA Purification (TAKARA BIO INC.) and RNAiso Plus (TAKARA BIO INC.). All reagent kits and instruments were used according to the manufacturer’s instructions. The quantity and quality of extracted RNA was assessed at wavelengths of 230, 260 and 280 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

**Extraction of protein from embryos**

Total protein was extracted from 100 germinating embryos using Mg/NP-40 buffer [0.5 M Tris–HCl (pH 8.3), 2% (v/v) NP-40, 20 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 2% (v/v) 2-mercaptoethanol and 1% (w/v) polyvinylpyrrolidone K-90], and the extracted proteins were centrifuged at 20,000×g for 30 min. A 200 μl volume of the supernatant was added to 800 μl of ice-cold 10% trichloroacetic acid in acetone with 20 mM dithiothreitol (DTT) and kept overnight at -20°C. Precipitated proteins were recovered by centrifugation at 12,000×g for 20 min, after which the pellet was washed twice with ice-cold acetone containing 20 mM DTT, centrifuged, and then vacuum-dried. The dried protein powder was dissolved in 200 μl lysis buffer [7 M urea, 2 M thiourea, 3% (w/v) CHAPS, 1% (v/v) Trition X-100, 0.5% (v/v) IPG buffer (GE Healthcare) and 1% (w/v) DTT].

**Two-dimensional electrophoresis (2-DE) analysis**

First dimension isoelectric focusing (IEF) separation was performed using gel strips forming an immobilized linear pH gradient from 4 to 7 (Immobiline DryStrip, 13 cm, GE Healthcare). The strips were loaded with protein samples and rehydrated for 12 h at 20°C on an IPGphor system (GE Healthcare). IEF was performed as follows: 1 h at 500 V, 1 h at 1,000 V and 13 h at 8,000 V. The strips were equilibrated for 20 min in equilibration buffer [50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS and 0.01% (w/v) bromophenol blue] containing 10 mg ml⁻¹ DTT. Second dimension SDS–PAGE separation was performed using a 15% polyacrylamide gel. Polypeptide spots were visualized using Coomassie Brilliant Blue staining. The 2-DE analyses were repeated three times to ensure the reliability of the results. Quantitative changes detected in images of the spot pattern of 2-DE gels were analyzed using Polymaps 2D (2-Dimensional Polyacrylamide Gel Electrophoresis Analysis System), a software developed by us and provided as freeware at https://www.kazusa.or.jp/polymaps2d. Relative protein levels were determined by comparing protein spot intensities with the intensity of a marker protein (approximately 1.5 μg of ovalbumin; SDS–PAGE Molecular Weight Standards, Low Range) (Bio-Rad).

**In-gel digestion and MALDI-TOF-MS analysis**

Proteins were identified by peptide mass fingerprinting (PMF) using matrix-associated laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Protein spots were excised from Coomassie-stained gels and destained for 20 min at 25°C in 100 mM (NH₄)₂CO₃ in 50% (v/v) acetonitrile (ACN), then dried by vacuum centrifugation. Dried gel pieces were treated with reduction solution [25 mM (NH₄)₂CO₃ in 10 mM DTT] for 60 min at 56°C followed by alkylation solution [25 mM (NH₄)₂CO₃ in 1% (w/v) iodoacetamide]. The gel pieces were washed with 100% ACN and subjected to overnight in-gel digestion at 37°C with sequence grade modified trypsin (Promega KK). The resulting peptides were purified with a ZipTip C18 pipet tip (Millipore) and mixed with 1 μl of α-cyano-4-hydroxycinnamic acid. Peptide masses were determined using a Voyager-DE STR workstation (Applied Biosystems). The resulting monoisotopic peptide masses were matched to the NCBI nr database using the Mascot sequence database search program, available at http://www.matrixscience.com. The search parameters allowed for one missed cleavage and variable modifications of methionine oxidation and cysteine carboxamidomethylation, with a peptide mass tolerance of 100 p.p.m. The following criteria were set for positive match selection: the protein score must be greater than 64 and the protein sequence coverage as determined by Mascot should not be less than 19%.

**Supplementary data**

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