Journ. Fac. Sci., Hokkaido Univ. Sev. V (Botany), **14**(2) 159-167, 1988.

# In vitro translation of long-lived mRNA from dry wheat seeds

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The mRNAs from germinated and ungerminated wheat seeds were purified and translated in both wheat germ extract and rabbit reticulocyte lysate systems. The translation activity of poly(A)$^+$RNA from dry aleurone layers was less than those of seedlings and dry embryos in both systems.

The poly(A)$^+$RNAs from seedlings and dry embryos were translated into high molecular weight, whereas that from aleurone layers was translated into low molecular weight. The polypeptide synthesized *in vitro* was immunoprecipitated using rabbit anti-wheat $\alpha$-amylase antibody and goat anti-rabbit IgG antibody. Fluorography after SDS-polyacrylamide gel electrophoresis of the immunoprecipitated translation products of poly(A)$^+$RNAs from seedlings and dry embryos showed a band of molecular weight 43,000 (the same as that of the $\alpha$-amylase precursor polypeptide), but that from aleurone layers was not. Ratios of immunoprecipitate to the total translation products were 0.9% using goat anti-rabbit IgG antibody and 0.4% using Protein A-Sepharose.

Various dry seeds or embryos are shown to contain long-lived mRNA, but little is known about its functions. Although the synthesis of new mRNA occurs rapidly after the onset of imbibition, the level of early protein synthesis is largely sustained by stored mRNA (THOMPSON and LANE, 1980). ASPART *et al.* (1984), using radish embroyos, reported that the polypeptides synthesized during the first 15 min of germination are encoded by stored mRNA, and also that some stored mRNAs are not remnants of mRNA present during embryogenesis, but code for polypeptides playing a particular role in early germination.

Several groups have identified the translation products of mRNA which are conserved in dry embryos, and they include lectin (PEUMANS *et al.*, 1980), mung bean albumin (CARLIER *et al.*, 1980) and wheat ribosome proteins (CUMING and LANE, 1979). TANEJA and SACHAR (1976) suggested from experiments using inhibitors of RNA synthesis that three enzymes (*o*-diphenolase, peroxidase, and RNase) are translated from stored mRNA.

In this study, poly(A)$^+$RNA was isolated from 48-h germinated seedlings, dry embryos, and aleurone layers. Poly(A)$^+$RNAs were translated with *in*

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*vitro* translation systems, and products were analyzed by electrophoresis and immunoprecipitation. The results indicated the existence of $\alpha$-amylase mRNA in the seedlings and the dry embryos.

## Materials and Methods

*Plant materials*

Winter wheat seeds (*Triticum aestivum* L. cv. Mukakomugi) were sterilized in 0.05% $HgCl_2$ solution for 15 min, rinsed with running water, and germinated in the dark at 24°C. After 48 h germination, the seedlings containing scutella were separated manually from the endosperms. Dry embryos were separated according to the method of JOHNSTON and STERN (1957). Aleurone layers with seed coats were separated by grinding dry seeds in an Analytical Mill and then sieving. The separated seedlings, dry embryos, and aleurone layers were stored frozen at −70°C until use.

*Preparation of RNA*

Total RNA was extracted as described by HARRIS and DURE (1978) with some modifications. Frozen material was ground to a powder in a Universal Mill under liquid nitrogen, and homogenized with extraction buffer (0.1 M Tris-HCl, pH 9.0, 0.1 M NaCl, 1 mM EDTA, 0.5% (w/v) SDS, 10 mM ribonucleoside vanadyl complex). An equal volume of phenol : chloroform : isoamylalcohol (25 : 24 : 1) mixture was added to the homogenate and the mixture was shaken for 20 min at room temperature. After centrifugation at 10,000×$g$ for 10 min, the aqueous phase was removed. The phenol phase and interface layer were reextracted with 0.5 volume of extraction buffer. The aqueous phases were combined and again extracted with phenol-chloroform mixture. The aqueous solution was made up to 2 M LiCl, 1 mM Tris-HCl (pH 7.4), 10 mM NaCl and allowed to stand at 0°C for 4 h. The RNA precipitate was collected by centrifugation and washed twice with 2 M LiCl buffer. The RNA pellet was dissolved in sterile water, and precipitated with 0.3 M Na-acetate and 2.5 volumes ethanol overnight at −20°C. The precipitate was collected, rinsed twice with cold 70% ethanol, dried under vacuum, and redissolved in sterile water.

Poly(A)$^+$RNA was prepared from total RNA by oligo(dT)-cellulose chromatography (AVIV and and LEDER, 1972). Poly(A)$^+$RNA was precipitated with ethanol and dissolved in sterile water at 4 $\mu$g RNA/$\mu l$, and used for *in vitro* protein synthesis.

*In vitro protein synthesis*

Purified poly(A)$^+$RNA was translated in rabbit reticulocyte and wheat germ cell-free protein-synthesizing systems (Amersham). The rabbit reticu-

locyte lysate system contained 9 $\mu l$ lysate, 4 $\mu$g poly(A)$^+$RNA and 25 $\mu$Ci [$^{35}$S]methionine (1255 Ci/mmol) in a final volume of 10 $\mu l$ and was incubated at 30°C for 60 min. The wheat germ cell-free system (30 $\mu l$) consisted of 15 $\mu l$ wheat germ extract, 0.07 mM each of 19 amino acids except for methionine, 80 mM K-acetate, 20 $\mu$Ci [$^{35}$S]methionine (1225 Ci/mmol) and 6 $\mu$g poly(A)$^+$RNA. The incubation was carried out at 30°C for 90 min. Incorporation of methionine into protein was assessed by pipetting aliquots (3 to 10 $\mu l$) of the incubation mixture on to glass filter discs (Whatman GF/C). The filter discs were washed in a series of TCA solutions containing cold methionine as described by ROBERTS and PATERSON (1973). Radioactivities were counted in toluene-based scintillant.

The *in vitro* translation products were fractionated by electrophoresis on 12.5% (w/v) polyacrylamide slab gels containing SDS using the method of LAEMMLI (1970). The incubation mixture were added to an equal volume sample buffer at a two-fold concentration, and after heating in a boiling -water bath, the samples were applied to the gel slots. After electrophoresis, the gels were stained with 0.05% Coomassie brilliant blue, destained, dried and fluorographed using Fuji X-ray film.

*Immunoprecipitation*

Antibody against $\alpha$-amylase was obtained from rabbits immunized with wheat $\alpha$-amylase purified from 7-day germinated seedlings as described in a previous report (FUJIOKA *et al.*, 1984). Immunoprecipitation was carried out using the anti-wheat $\alpha$-amylase antibody and either secondary antibody (goat anti-rabbit IgG antibody, Miles Laboratories, Inc.) or Protein A-Sepharose CL 4B (Pharmacia Fine Chemicals). In the case of secondary antibody (HALL *et al.*, 1978; MOTOJIMA and SAKAGUCHI, 1982), 20 $\mu l$ of each *in vitro* translation reaction mixture was diluted with 1 m$l$ of immunoprecipitation buffer (10 mM Tris-HCl, pH 7.5, 30 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate) then centrifuged. The supernatant was mixed with 10 $\mu l$ (2 $\mu g/\mu l$) of anti-$\alpha$-amylase antiserum and the mixture was incubated for 60 min at 37°C. The mixture was incubated with 15 $\mu l$ (2 $\mu g/\mu l$) of goat anti -rabbit IgG antibody for 15 min at 37°C and placed at 4°C overnight. The immunoprecipitate was collected by centrifugation, and washed with immunoprecipitation buffer 3 times and with cold 70% ethanol twice. After the immunoprecipitate was dried and dissolved in sterile water, it was analyzed by SDS polyacrylamide gel electrophoresis, or counted for radioactivity as described above. Immunoprecipitation using Protein A-Sepharose CL 4B was carried out according to the procedure of MIYATA *et al.* (1981).

## Results

*Isolation of poly(A)$^+$RNA from dry embryos and aleurone layers*

As shown in Table 1, about 300 $\mu$g of poly(A)$^+$RNA was isolated from 4 g of dry embryos, whereas only about 70 $\mu$g was isolated from 130 g of dry

**Table 1.** Yield of poly(A)$^+$RNA from dry embryos, aleurone layers, and 48-h seedlings.

| | Fresh weight | Total RNA | Poly(A)$^+$ RNA | $\frac{\text{Poly(A)}^+\text{RNA}}{\text{Total RNA}} \times 100$ |
|---|---|---|---|---|
| | (g) | (mg) | ($\mu$g) | (%) |
| Seedlings | 75 | 17.9 | 202.3 | 1.13 |
| Embryos | 4 | 47.5 | 302.5 | 0.64 |
| Aleurone layers | 130 | 12.6 | 71.3 | 0.56 |

Extraction and purification of total and poly(A)$^+$RNA were carried out as described in **Materials and Methods**.

**Table 2.** Translation activities of poly(A)$^+$RNA from dry embryos, aleurone layers, and 48-h seedlings in rabbit reticulocyte lysate and wheat germ extract systems measured as incorporation of [$^{35}$S]methionine.

| Poly(A)$^+$RNA | Rabbit reticulocyte lysate | | | | | |
|---|---|---|---|---|---|---|
| | Exp. 1 | | Exp. 2 | | Exp. 3 | |
| | Activity | Percent | Activity | Percent | Activity | Percent |
| | (cpm) | (%) | (cpm) | (%) | (cpm) | (%) |
| Endogenous | 889 | | 492 | | 810 | |
| Seedlings | 15,753 | 100 | 13,254 | 100 | 6,712 | 100 |
| Embryos | 11,034 | 70 | 8,298 | 63 | — | — |
| Aleurone layers | 6,423 | 41 | — | — | 3,784 | 56 |

| Poly(A)$^+$RNA | Wheat germ extract | | | | | |
|---|---|---|---|---|---|---|
| | Exp. 1 | | Exp. 2 | | Exp. 3 | |
| | Activity | Percent | Activity | Percent | Activity | Percent |
| | (cpm) | (%) | (cpm) | (%) | (cpm) | (%) |
| Endogenous | 1,683 | | 230 | | 884 | |
| Seedlings | 17,705 | 100 | 3,731 | 100 | 25,590 | 100 |
| Embryos | 17,707 | 100 | — | — | 25,502 | 100 |
| Aleurone layers | 11,491 | 65 | 2,677 | 72 | — | — |

Samples were prepared as described in **Materials and Methods**.

aleurone layers with seed coats. The ratio of poly(A)$^+$RNA to the total RNA in dry embryos was 0.64%, which was signifhcantly higher than in aleurone layers (0.56%). The ratio in 48-h seedlings was more than 1%.

*Translation of poly(A)$^+$RNA*

The poly(A)$^+$RNA was translated in both wheat germ extract and rabbit reticulocyte lysate systems (Table 2). Incorporation of [$^{35}$S]methionine into TCA-insoluble material directed by poly(A)$^+$RNA isolated from dry embryos or 48-h seedlings was more than 10-fold above endogenous activity in both systems. The translation activity of poly(A)$^+$RNA from aleurone layers was less than that of dry embryos or 48-h seedlings. While the translation activity of poly(A)$^+$RNA from dry embryos was about 63 to 70% of that of 48-h seedlings in the rabbit reticulocyte lysate system, both activities were equal in the wheat germ extract system. However, the activity of poly(A)$^+$RNA from aleurone layers was only 41 to 56% of that of seedlings in the rabbit reticulocyte lysate system, and no more than 65 to 72% even in the wheat germ extract system.

The *in vitro* translation products of poly(A)$^+$RNAs from 48-h seedlings, dry embryos, and aleurone layers were separated on SDS-polyacrylamide slab gels and detected by fluorography (Fig. 1, A, C, and E). Poly(A)$^+$RNA from 48-h seedlings and dry embryos were translated mostly into high molecular weight proteins. However, relatively low molecular weight proteins were detected in the products of poly(A)$^+$RNA from aleurone layers. There were no differences in the translation products synthesized by the wheat germ extract and the rabbit reticulocyte lysate systems.

*Translation of α-amylase by long-lived mRNA*

*In vitro* translation products of poly(A)$^+$RNAs in wheat germ extract were precipitated by double immunoreaction using rabbit anti-wheat α-amylase IgG and goat anti-rabbit IgG antibody. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 1, B, D, and F). Significant amounts of radioactivity were immunoprecipitated from the products of poly(A)$^+$RNA from 48-h seedlings and from dry embryos. The main band of radioactivity appeared at molecular weight 43,000, identical to that of α-amylase precursor protein (BOSTON *et al.*, 1982). Consequently, α-amylase mRNA was conserved in the dry embryos, but not in the aleurone layers, since no radioactive immunoprecipitates were detected in cell-free translation products synthesized from aleurone mRNA.

*Contents of the long-lived mRNA for α-amylase*

As shown in Table 3, about 4.7% of the total *in vitro* translation products were immunoprecipitated with α-amylase antibody by double immunoreac-

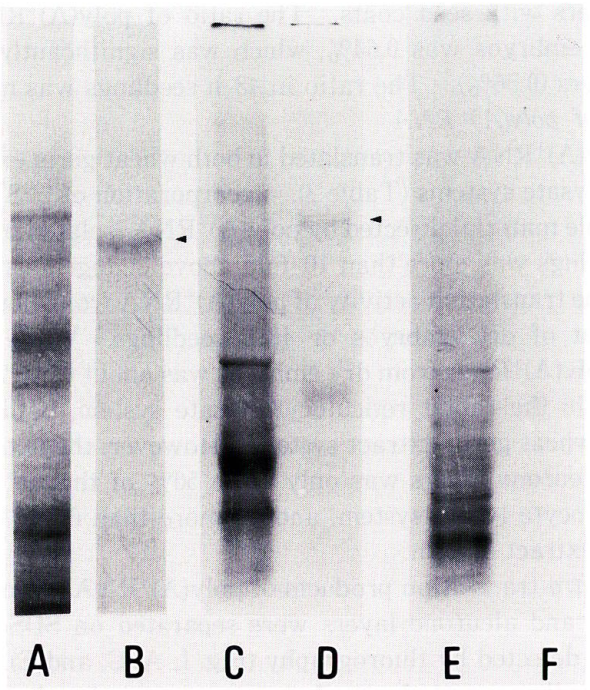


**Fig. 1.** Fluorograms of SDS-polyacrylamide gels of total translation products and immunoprecipitates. Poly(A)$^+$RNA were translated in wheat germ extract system and the products were immunoprecipitated by double immunoreactions. The translation products and the immunoprecipitates were fractionated by SDS-polyacrylamide gel electrophoresis. Translation products of poly(A)$^+$RNA from 48-h seedlings (A), dry embryos (C), and aleurone layers (E). Immunoprecipitates of translation products of poly(A)$^+$RNA from 48-h seedlings (B), dry embryos (D), and aleurone layers (F).

**Table 3.** Incorporation of [$^{35}$S]methionine into TCA-insoluble and immunoprecipitated materials.

| TCA-insoluble materials | Immunoprecipitation | | Ratios estimated from Fig. 3 |
|---|---|---|---|
| | Goat anti-rabbit IgG antibody | Protein A-Sepharose | |
| (cpm/$\mu l$) | (cpm/$\mu l$) | | (%) |
| 10,145.3 | 478.5(4.7%) | | 0.94 |
| 12,768.0 | | 950.1(7.4%) | 0.41 |

Poly(A)$^+$RNA from dry embryos was translated in wheat germ extract system. The products were immunoprecipitated with rabbit anti-wheat $\alpha$-amylase antibody and goat anti-rabbit IgG antibody or Protein A-Sepharose linked antibody as described in **Materials and Methods**.

tion, and about 7.4% of the products were immunoprecipitated by the antibody liked to Protein A-Sepharose. However, since the immunoprecipitates contained some radioactive proteins precipitated non-specifically which were visible in the fluorograph (Fig. 2), the ratios of a main band of radioactivity at molecular weight 43,000 to total immunoprecipitates were determined by densitometric scanning at 590 nm on fluorographs of SDS-polyacrylamide gels of immunoprecipitates (Fig. 3). The ratios of $\alpha$-amylase were estimated at about 0.94% of total proteins synthesized *in vitro* recovered by the double immunoreaction method, and about 0.41% of the total products recovered by antibody linked to Protein A -Sepharose.

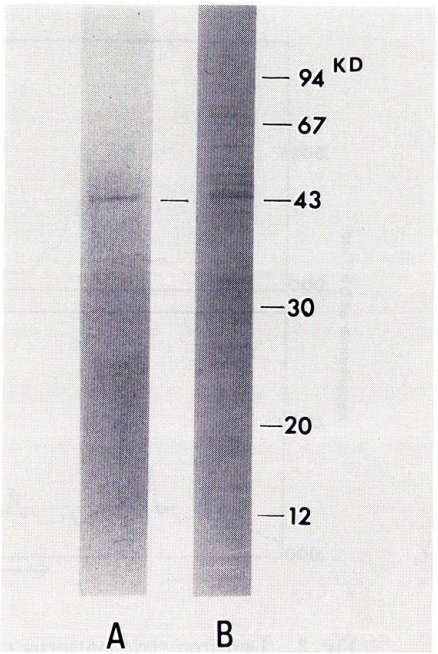


**Fig. 2.** Fluorograms of SDS-polyacrylamide gels of immunoprecipitated translation products. *In vitro* translation products of poly(A)$^+$ RNA from dry embryos were immunoprecipitated either by double immunoreaction (A) or Protein A -Sepharose linked antibody (B) and fractionated by SDS-polyacrylamide gel electrophoresis.

## Discussion

Dry wheat embryos contain long-lived mRNA ; however, neither species nor function of the proteins encoded by the mRNA have been definitively established. Are they only remnants of mRNA specific to embryogenesis or do they play a particular role during early germination ?

The poly(A)$^+$RNAs prepared from dry embryos and aleurone layers were shown to be mRNA by their ability serve as templates in cell-free translation systems. However, translation activities of the mRNA from different sources were quite different ; mRNA from embryos translated *in vitro* produced proteins having a wide range of molecular weight, whereas mRNA from the aleurone layers translated into only low molecular weight proteins. $\alpha$-Amylase mRNA is present in the long-lived mRNA from dry wheat embryos in this study, the long-lived mRNA coding for enzymes is unlikely

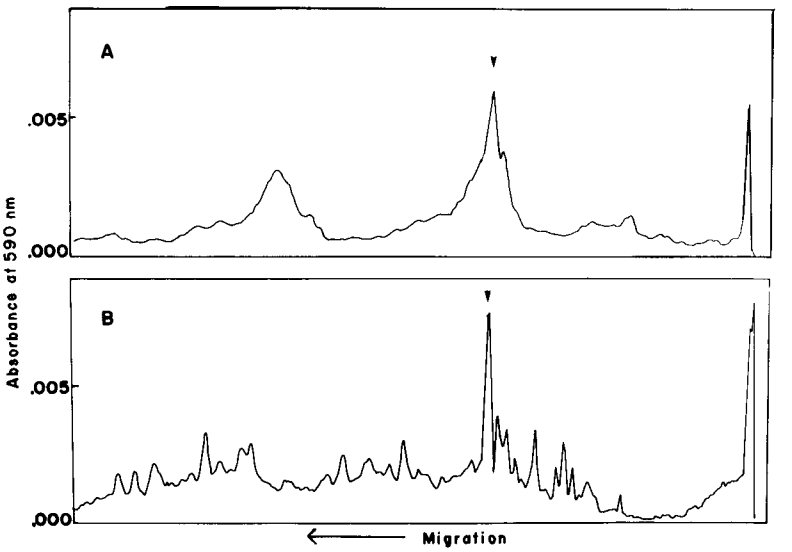


**Fig. 3.** Densitometric patterns of fluorograms shown in Fig. 2, A and B. Fluorograms shown in Fig. 2, A and B were scanned at 590 nm. The arrows indicated migration of $\alpha$-amylase precursor.

to be only remnants of mRNA translated during late embryogenesis. ASPART *et al.* (1984) have shown that stored mRNA in dry radish embryos synthesize polypeptides that may play a particular role during early germination.

PEUMANS *et al.* (1980) have reported that pea lectin is one of the products coded by stored mRNA from dry primary axes and lectin mRNA represents about 0.1% of the total population of the stored mRNA. In the present study we have shown that $\alpha$-amylase mRNA is present in the long-lived mRNA from dry wheat embryos and the ratio of $\alpha$-amylase mRNA to the total long -lived mRNA is no less than 0.41%

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