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Biosynthesis of α-Amylase in *Vigna mungo* Cotyledon[1]

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ABSTRACT

***In vitro* translation of RNA extracted from *Vigna mungo* cotyledons showed that α-amylase is synthesized as a polypeptide with a molecular mass of 45,000, while cotyledons contain a form of α-amylase with a molecular mass of 43,000. To find out whether the 45,000 molecular mass polypeptide is a precursor to the 43,000 found *in vivo*, the cell free translation systems were supplemented with canine microsomal membrane; when mRNA was translated in the wheat germ system supplemented with canine microsomes, the 45,000 molecular mass form was not processed to a smaller form but the precursor form was partly processed in the membrane-supplemented reticulocyte lysate system. When *V. mungo* RNA was translated in *Xenopus* oocyte system, only the smaller form (molecular mass 43,000) was detected. Involvement of cotranslational glycosylation in the maturating process of the α-amylase was ruled out because there was no effect of tunicamycin, and the polypeptide was resistant to endo-β-H or endo-β-D digestion. We interpret these results to mean that the 45,000 molecular mass form is a precursor with a signal peptide or transit sequence, and that the 43,000 molecular mass is the mature form of the protein.**

Numerous investigations have been carried out on the mechanism of α-amylase synthesis and transport in both microorganisms and eukaryotes. In all cases, the enzyme appears to be a secretory protein. Investigations with plants (wheat, barley, and rice) showed that α-amylase is synthesized as a precursor polypeptide. In wheat, the larger precursor was processed to the mature form in *Xenopus* oocytes and by canine microsomes (2), but not by the RER obtained from aleurone layers (15). In barley, newly synthesized α-amylase is associated with the ER (6, 9). In rice, extensive glycosylation is preceded by proteolytic processing during the synthesis of α-amylase (12). Recently, cDNA complementary to mRNA sequence of wheat and barley α-amylase was obtained, and nucleotide sequencing showed that the enzymes have typical signal sequences at their N-terminal ends (16, 17). When cloned cDNA against the wheat amylase mRNA was inserted into a yeast expression vector, the transformed yeast cells synthesized α-amylase of mature size and secreted it out of the yeast cells in an active form (17). In contrast to these findings with cereals, nothing is known about the mechanism of the α-amylase synthesis in legumes seeds. In a previous paper (18), we demonstrated by histochemistry that α-amylase appears in *Vigna mungo* cotyledons in all the cells, and that there is no specific tissue from which α-amylase is secreted. To understand the processing of newly synthesized α-amylase in *V. mungo* cotyledons, we employed both a *Xenopus* oocyte system and cell-free translation systems coupled with canine microsome membrane. We found that in the cotyledons, α-amylase is synthesized as a large precursor by membrane-bound polysomes, cleaved to mature form in cell-free translation systems supplemented with canine pancreas microsome, and in *Xenopus* oocyte system. In addition, mature α-amylase is not glycosylated. Possible mechanisms of intracellular transport of α-amylase in *V. mungo* cotyledons are discussed.

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MATERIALS AND METHODS

Plant Material for RNA Preparation. We used only detached cotyledons because of their high ability of α-amylase synthesis as described previously (7). *Vigna mungo* dry cotyledons detached from their embryonic axis were immersed in water for 6 h and then surface sterilized in 1% NaOCl solution for 5 min, rinsed with sterile water, and incubated under aseptic conditions for 4 or 5 d as described previously (7). The cotyledons were harvested, frozen with liquid N_2, and stored at −20°C until used.

Preparation of Poly A⁺ RNA. All procedures were carried out in aseptic conditions according to the method of Watanabe et al. (19) with some modifications. Cotyledons were homogenized in liquid N_2 to obtain a frozen powder. Usually, about 2.5 g portion of frozen powder was homogenized in 20 ml of RNA extraction buffer consisting of 50 mM Tris-HCl buffer, pH 9.0, containing 1% SDS (w/v) and an equal volume of 85% phenol. The aqueous phase was separated and reextracted by adding an equal volume of 85% phenol in the same way several times until the interphase emulsion had almost disappeared. The resultant phenolic and middle phase were also reextracted by adding RNA extraction buffer. Finally, the aqueous phases were combined and 10 M LiCl solution added to give 0.1 M final concentration. Total RNA was precipitated with two volumes of cold ethanol and left for at least 4 h at −20°C. The precipitate obtained by centrifugation was dissolved in sterile water. This solution was mixed with one-fourth volume of 10 M LiCl and left overnight at 4°C. High mol wt RNA recovered as a precipitate was dissolved in sterile water. The RNA was precipitated by adding 2 volumes of cold ethanol. After centrifugation, the pellet was washed with 80% ethanol and then with 100% ethanol. After drying, the RNA was dissolved in a small amount of sterile water to yield the total RNA fraction.

Poly A⁺ RNA was fractionated from total RNA by affinity chromatography on oligo(dT)-cellulose (P-L Biochemicals, type 7A) essentially as described by Aviv and Leder (1). The total RNA fraction, dissolved in 50 mM Tris-HCl buffer, pH 7.4, containing 0.5 M LiCl and 0.5% SDS, was passed through the column and poly A⁺ RNA was eluted with sterile water. The poly A⁺ RNA was precipitated by adding 2 volumes of ethanol. After washing with ethanol and drying, the precipitate was dissolved in sterile water to yield the poly A⁺ RNA fraction. The concentration of total and poly A⁺ RNA was estimated by A_{260} measurement (1 mg/ml = 22 A_{260} units).

Extraction of RNA from 4,000*g* Pellet, Membrane Bound and Free Polysomes. Preparation of membrane-bound and free polysomes basically followed the methods of Mösinger and Schopfer

(14), and Jackson and Larkins (5). About 6 g of frozen powder of the detached 5-d-old cotyledons were ground in 20 ml of cold extraction buffer (0.2 M Tris-HCl buffer, pH 8.5, 0.4 M KCl, 50 mM Na-acetate, 25 mM EGTA, 0.25 M sucrose, 5 mM DTT). The homogenate was centrifuged at 4,000*g* for 30 min (4,000*g* pellet). The supernatant was centrifuged at 37,000*g* for 10 min to separate membrane-bound polysomes from free polysomes. The supernatant (free polysomes) was mixed with Triton X-100 to a 1% (v/v) final concentration and the pellet (membrane-bound polysomes) was resuspended in 4 ml of extraction buffer, containing 1% (v/v) Triton X-100. The resuspended polysomes were centrifuged at 37,000*g* for 10 min, the supernatants layered on a cushion of extraction buffer containing 1.5 M sucrose, and centrifuged at 160,000*g* for 1.5 h. From the 4,000*g* pellet and polysome pellets, RNA was extracted by the phenol method as described above.

Translation in Wheat Germ and Reticulocyte Lysate Cell-Free System. Wheat germ extract was prepared by the procedure of Marcu and Dudock (10), except that preincubation of the extract was omitted, because of the low translational activity of the endogenous mRNA. The reaction mixture contained in a final volume of 50 μl: 20 mM Hepes-KOH (pH 8.0), 50 mM KCl, 0.5 mM spermidine, 1 mM ATP, 8 mM creatine phosphate, 0.2 mM GTP, 50 μg/ml creatine kinase, 0.1 mM DTT, 50 μM each of 19 amino acids excluding leucine or methionine, 5 μCi [^{3}H]leucine (52 Ci/mmol) or 5 μCi [^{35}S]methionine (1098 Ci/mmol), 15 μl wheat germ extract and an appropriate amount of total (0.5 mg/ml), poly A$^+$ (60 μg/ml) or polysomal RNA (0.4 mg/ml). The mixture was incubated at 22°C and a 2.5 μl sample was withdrawn onto a filter paper disc after 30 min and 60 min of incubation. The paper was immersed in 5% TCA and boiled for 10 min. After washing with 5% TCA 3 times and once with ethanol-ether (1:1), the paper was dried and the radioactivity was determined with a Beckman liquid scintillation spectrometer CPM-200. Reticulocyte lysate cell-free translation system from New England Nuclear was used with [^{35}S]methionine.

Protein Synthesis in Cell-Free Translation Systems and in *Xenopus* Oocytes. Cell-free translation products were processed by supplement with canine pancreatic microsome purchased from New England Nuclear (NEK-019) in the reaction mixture of both the wheat germ system and the reticulocyte lysate system. Canine pancreatic microsomes were added to the translation reaction mixture to a final concentration of 4 A_{260} units, and then the reaction of the translation was started.

Synthesis of α-amylase by *Xenopus* oocytes was studied using the methods of Izumi *et al.* (4). Each *Xenopus laevis* oocyte was injected with 50 nl of a solution containing 7.3 mg/ml RNA obtained from the 4,000*g* pellet. The injection procedures and the culture of the oocytes was as described by Gurdon (3). Groups of 10 oocytes were incubated with 50 μl of sterile Barth's medium containing 25 μCi of [^{35}S]methionine for 48 h at 18°C. At the end of the incubation, the oocytes were homogenized with 0.5 ml of 0.02 M Tris-HCl containing 0.15 M NaCl, 0.5% Triton X-100, and 0.5% sodium deoxycholate, pH 7.5, and centrifuged at 100,000*g* for 30 min. The supernatant was used as a source of protein for the immunodetection of α-amylase as described below.

Immunological Isolation of α-Amylase Synthesized *in Vitro*. Quantitative isolations of α-amylase synthesized in the wheat germ and reticulocyte lysate cell-free systems were performed using protein A Sepharose gel according to a slightly modified method of Miyata *et al.* (11). The reaction mixture (50 μl) at the end of the *in vitro* synthesis was made 2% with respect to SDS, heated to 80°C for 3 min and then mixed with Triton X-100 to 4%. One ml of TTBS (20 mM Tris-HCl, pH 7.5, containing 0.1% Triton X-100 and 0.15 M NaCl) was added. In the case of reticulocyte lysate, this mixture was centrifuged at 100,000*g* for 30 min and the supernatant was used for the immunoreactive procedure. After addition of 50 μg of anti-α-amylase antiserum (7), the mixture was incubated for 1 h at 30°C. The mixture was applied to a small column of protein A Sepharose (10 μl bed volume) repeatedly for 30 min at 30°C. After successive washes (3 times with 1 ml of TTBS, 1 ml of 1 M NaCl, and 0.5 ml of water), immunocomplexes with protein A were eluted with 30 μl SDS sample buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 0.002% bromophenol blue, and 20% glycerol).

***In Vivo* Synthesis of α-Amylase and Effect of Tunicamycin.** Ten pairs of the detached cotyledons incubated for 3 d were sectioned transversely into four parts. The sections were preincubated in water (control) or 30 μg/ml tunicamycin solution at 27°C for 30 min. After wiping off the water or tunicamycin solution, each section was incubated with 3 μl [^{3}H]leucine (3 μCi, 52 Ci/mmol), containing 30 μg/ml tunicamycin or not, and incubated for 4 to 5 h at 27°C. The entire procedure was done in a Petri dish. The sections were washed with water, then homogenized with 3 ml of TTBS, and the homogenate was centrifuged at 100,000*g* for 40 min. From the supernatant, α-amylase was collected by immunological method using protein A Sepharose, as described above.

***In Vitro* Digestion of Purified α-Amylase with Endo-β-H and Endo-β-D.** Endo-β-H and endo-β-D were purchased from Seikagakukogyo Co., Ltd. Each glycosidase (0.4 m units) was added to 1 μg purified α-amylase solution in citrate phosphate buffer, pH 5.0 (endo-β-H) or 6.5 (endo-β-D). The mixture was incubated for 30 min at 37°C, the samples were analyzed with SDS-PAGE and gel was stained with Coomassie brilliant blue.

SDS-PAGE and Fluorography. SDS polyacrylamide slab gel electrophoresis was carried out according to the method of Laemmli (8) using 12.5 or 10% polyacrylamide gel with 1 mm thickness. Protein samples were treated with the SDS sample buffer for 2 min in boiling water. Electrophoresis was performed at 25 amp constant current. After staining for protein, the gel was prepared for fluorography using Amplify (Amersham) or Enhance (New England Nuclear) and exposed to an x-ray film as described (7).

RESULTS AND DISCUSSION

Comparison *in Vitro* Synthesized α-Amylase with *in Vivo* Synthesized α-Amylase. Figure 1 shows that poly A$^+$ RNA directed the synthesis of α-amylase in a wheat germ cell-free system with a larger mol wt (M_r 45,000) than α-amylase synthesized *in vivo* (M_r 43,000). This result indicates that there may be signal sequence in the α-amylase precursor, so we investigated the intracellular site of the α-amylase synthesis in *V. mungo* cotyledons. From the 4,000*g* pellet (starch grains, cell debris, nuclei, etc.), membrane bound polysomes and free polysomes, RNA were prepared and used to program the synthesis of α-amylase in the wheat germ cell-free translation system. The same aliquots (20 μl) of translation mixture was loaded on each sample and SDS-PAGE was performed. The α-amylase was synthesized on membrane bound polysomes and a little on free polysomes, and, unexpectedly, a lot of the α-amylase mRNA was present in the 4,000*g* fraction (Fig. 2b). Comparing the total synthesis products, α-amylase appeared to be enriched in the products from the RNA of the 4,000*g* pellet with respect to the other bands (Fig. 2a). This result suggested not only that α-amylase mRNA of *V. mungo* cotyledon was bound to RER, but also that the mRNA might be associated with some larger structure which sedimented at 4,000*g*. This possibility will be discussed later.

Processing of α-Amylase Synthesized in Cell-Free Translation System by Microsomal Membranes and in Oocytes. The α-amylase detected by the immunological method and synthesized in the wheat germ system in the presence (lane 2) and the absence

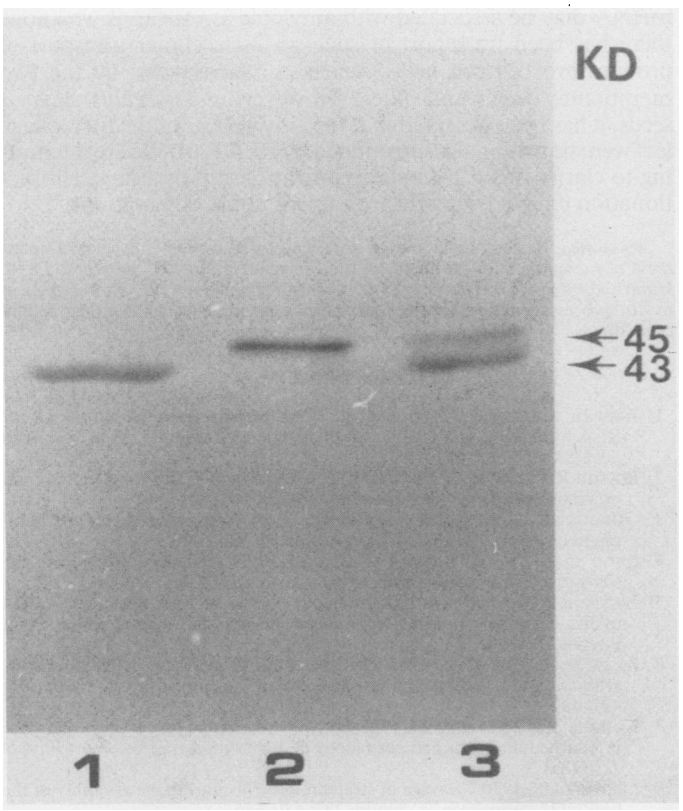

FIG. 1. Fluorogram of SDS-PAGE of immunoprecipitates of *in vivo* and *in vitro* synthesized α-amylase of *V. mungo* cotyledons. (1), *In vivo* synthesized α-amylase labeled with [^{3}H]leucine in 3-d detached cotyledons; (2), *in vitro* labeled α-amylase directed by poly A$^+$ RNA from 3-d cotyledons in wheat germ translation system with [^{3}H]leucine; (3), mixture of (1) and (2). Top arrow indicates the mol wt of 45,000 D, and bottom arrow indicates 43,000 D.

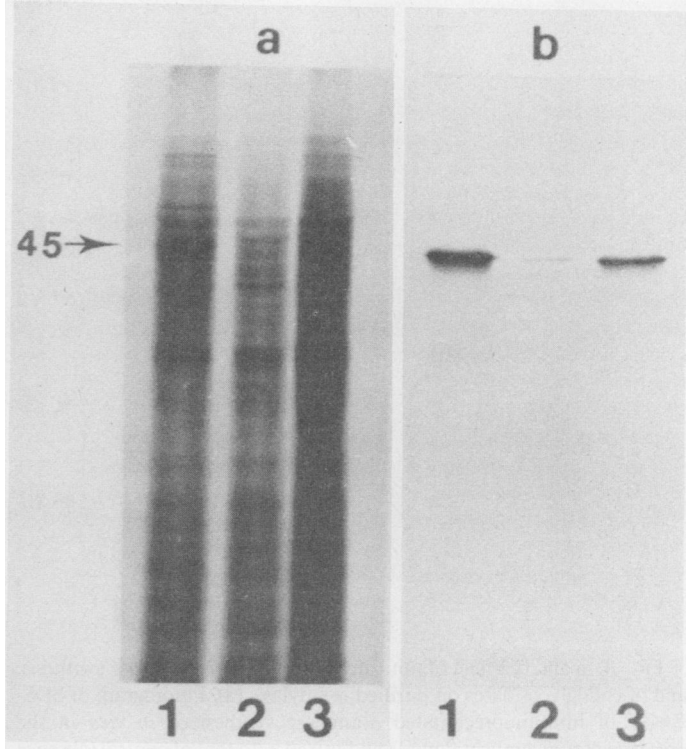

FIG. 2. a and b, Fluorogram of SDS-PAGE of total and immunoreactive product directed by mRNA of subcellular fractions. a, Total products synthesized by an in *in vitro* wheat germ translation system and directed by RNA extracted from: (lane 1), 4,000*g* pellet; (lane 2), free polysomal RNA; and (lane 3), membrane bound polysomal RNA. b, α-Amylase synthesized by an *in vitro* wheat germ translation system and directed by RNA extracted from: (lane 1), 4,000*g* pellets; (lane 2), free polysomes; and (lane 3), membrane bound polysomes. Arrow indicates the position of α-amylase synthesized *in vitro* (M_r 45,000).

(lane 1) of canine pancreas microsomes is shown in Figure 3a. Whether supplemented by microsomal membranes or not, α-amylase ran as a polypeptide of 45,000 D. This result was not affected by increasing the dose of microsomes (lane 3). However, in the case of the reticulocyte lysate system supplemented with microsomes, two bands were detected (Fig. 3b, lane 2); one migrating as a 45,000 D polypeptide and the other as a 43,000 D polypeptide. This indicated that the precursor was partly processed to mature form in this system. There is no clear answer yet to the question why the precursor could not cleave in the wheat germ system, because wheat α-amylase was cleaved to mature form in wheat germ cell-free translation system by canine pancreas microsome (2).

When the RNA was directly injected into *Xenopus* oocytes, only the M_r 43,000 band was detected which corresponded to that of purified α-amylase (Fig. 3c, lane 2). In the control (no cotyledon RNA injected), no band was detected (lane 1). These results strongly suggest that the α-amylase had a signal peptide and was processed to mature form in RER when the protein was translocated in the lumen of ER.

Absence of Glycosylation in the Course of α-Amylase Synthesis. In general, cotranslational glycosylation occurs in many secretory proteins, although the biological meaning of this has not yet been clarified. With respect to α-amylase of plant seeds, there are wide variations in the degree of glycosylation. In α-amylase secreted by rice scutellum, the glycosylation was found to be very large (13), but in wheat, this modification does not occur (2). In the present result, when tunicamycin (30 μg/ml), which inhibits asparagine-dependent cotranslational glycosylation, was fed to the detached cotyledon *in vivo*, the α-amylase synthesized had the same mol wt as authentic α-amylase in Figure 4a. This result was consistent with the observation that the endoglycosidases endo-β-H or endo-β-D, which are known to cleave at the base of high mannose and hybrid sugar chains

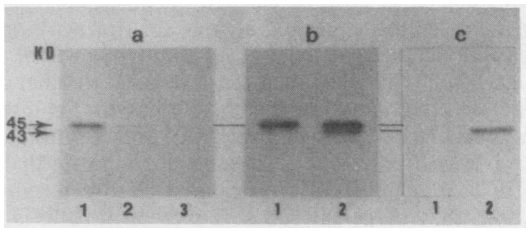

FIG. 3. a to c, Processing of a larger α-amylase protein molecule in *in vitro* translation systems and in a *Xenopus* oocytes. (a), Wheat germ cell-free translation system contained no membranes (lane 1) or contained 4 (lane 2) and 8 (lane 3) A_{260} units of canine pancreas microsomes. (b), Reticulocyte lysate cell-free translation contained no membranes (lane 1) or contained 4 A_{260} units of canine pancreas microsomes (lane 2). RNA was obtained from 4,000*g* pellet of 5-d detached cotyledons. (c), Translation product was detected in *Xenopus* oocyte system. After RNA obtained from 4,000*g* pellet of 5-d detached cotyledons were injected, the oocytes were incubated in a medium containing [^{35}S] methionine and produced α-amylase which was immunologically detected. Oocytes were stimulated to synthesize protein, but RNA was not injected (lane 1). Oocytes were stimulated and injected with RNA (350 ng/egg) (lane 2). Arrows indicate the positions of the unprocessed (M_r 45,000) and the processed (M_r 43,000) forms of α-amylase.

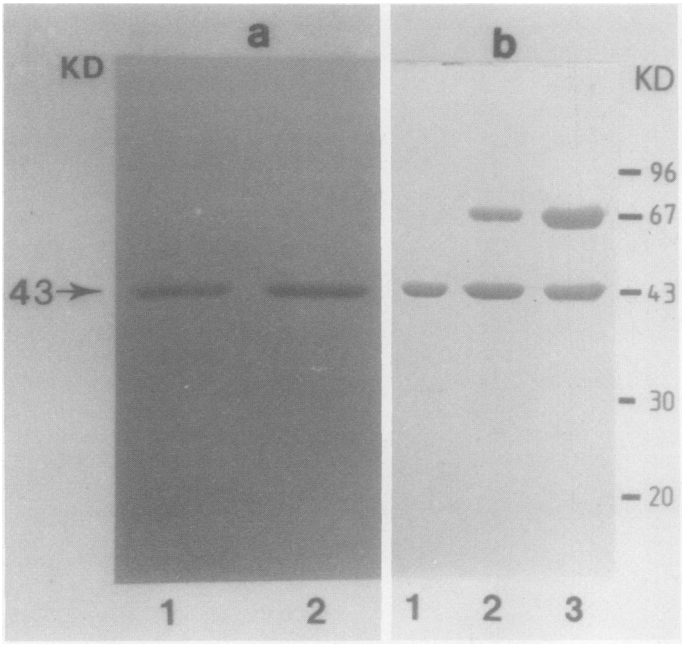

FIG. 4. a and b, Effect of tunicamycin on *in vivo* α-amylase synthesis, and of endoglycosidases on purified α-amylase. (a), Fluorogram of SDS-PAGE of immunoprecipitated α-amylase synthesized *in vivo* in the presence of tunicamycin (lane 1) and mixed with that of control (absence of tunicamycin) (lane 2). (b), SDS-PAGE of purified α-amylase treated *in vitro* with endo-β-H (lane 2) and endo-β-D (lane 3), and purified α-amylase (M_r 43,000) (lane 1). The band of M_r 67,000 is BSA included in the endoglycosidase samples.

of glycoproteins, respectively, did not alter the mol wt of α-amylase protein (Fig. 4b). From these results, we suggest that *V. mungo* α-amylase has no N-glycosylation.

The Possibility of Intracellular Transport of the α-Amylase. In the previous paper (18), we showed the possibility that in the *V. mungo* cotyledon no specific area existed for synthesis of α-amylase, so it can be expected that the α-amylase will be synthesized in each cell of cotyledon, and not secreted to other cells. In this study, we presented evidence consistent with the interpretation that α-amylase has a signal sequence and is synthesized on membrane bound polysomes. These results indicate that the α-amylase in *V. mungo* cotyledon is characterized as a transported protein like that of cereal seeds. To reconcile this apparent inconsistency, we propose a testable hypothesis: α-amylase in *V. mungo* cotyledon, synthesized on membrane bound polysomes as a larger precursor with a signal sequence, is cotranslationally processed by proteolytic cleavage of signal peptide, and is then transported to the amyloplasts through the lumen of ER. Our finding that α-amylase mRNA is present abundantly in the 4,000*g* pellet fraction, indicates that ER bearing α-amylase mRNA may be associated with amyloplasts. As far as we know, there has been no report of such an intracellular transport of protein into cell organelles which are surrounded by the two membranes (inner and outer). However, in *Phaseolus vulgaris* seeds, it has been observed that the amyloplasts of the first foliage leaf were transitionally surrounded by RER (20). We are attempting to clarify this ER-amyloplast relationship by using cell fractionation coupled with electron microscopic observations.

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