Original Paper Plant Biotechnology, **20** (3), 187–194 (2003)

Phosphate Modulates Ca^{2+} Uptake and α-Amylase Secretion in Suspension-Cultured Cells of Rice

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Received 21 October 2002; accepted 10 February 2003

Abstract

Effects of phosphate on the Ca^{2+} uptake and the sucrose-controlled secretion of α-amylase molecules in cultured rice cells were investigated. Phosphate markedly stimulated Ca^{2+} uptake into rice cells, particularly at the outer cell layer of the cell cluster. Phosphate also increased the synthesis and extracellular liberation of α-amylase II-4 in sucrose-supplemented cells. The distribution pattern of enzyme in rice cell clusters induced by phosphate was similar to that of Ca^{2+} uptake. Phosphate did not increase the level of mRNA of α-amylase II-4, indicating that phosphate stimulates the translation and posttranslational secretory processes of α-amylase II-4 in the presence of sucrose. Furthermore, phosphate enhanced both the Ca^{2+} uptake and α-amylase II-4 synthesis in the microsomes. These results strongly suggested that the ratio of phosphate to sugar is important for regulating the Ca^{2+} uptake, and that phosphate and sugar precisely coordinate the Ca^{2+}-mediated synthesis and extracellular liberation of α-amylase II-4.

Key words: α-amylase, Ca^{2+}, cell culture, *Oryza sativa* L, phosphates, sucrose.

Introduction

Phosphorus is one of the most important elements that significantly affect plant growth (Marschner, 1995). Phosphate plays an important role in root architecture, photosynthesis, respiration, energy metabolism and biosynthesis of nucleic acids and membranes, and regulation of a number of enzymes (Duff *et al.*, 1991; Loffler *et al.*, 1992; Plaxton, 1996; Raghothama, 1999; Paul and Foyer, 2001; Williamson *et al.*, 2001). In addition, it has been reported that phosphate modulates the transcription of several sugar-induced genes from soybean and potato (Sadka *et al.*, 1994; Sheen, 2001).

Phosphorus is stored in plant seeds as phytin (insoluble mixture of potassium, magnesium and calcium salt of phytic acid) during seed development. Phytin is associated with the aleurone grains in the aleurone layer and is more or less absent from the protein bodies of the starchy endosperm in rice seed, although in soybeans it is deposited in protein bodies. In germinating rice seeds, phosphate is an important metabolic intermediate as well as sugars. Upon seed germination, hydrolysis of phytin reserves provides nutrients for the rapidly growing seedlings (Marschner, 1995).

α-Amylase (EC 3.2.1.1.), a key enzyme for germination and subsequent seedling growth of cereal seeds, is a multigene-encoded enzyme (Mitsui and Itoh, 1997). Recently, rice α-amylase isoforms have been classified into classes I and II. α-Amylase I-1 is encoded by *RAmy1A* (*ORYsa*; *Amy1* ; *1*, CPGN nomenclature, http://mbclserver.rutgers.edu/CPGN), α-amylase II-3 by *RAmy3E* (*ORYsa*; *Amy1*; *4*), and α-amylase II-4 by *RAmy3D* (*ORYsa*; *Amy1*; *3*). The expression of α-amylase isoforms in class-I and class-II are differentially regulated by phytohormones and metabolic sugars (Kashem *et al.*, 1998, 2000).

The scutellar tissue is important for rice seed germination. Actually, the scutellar epithelial cells synthesize and secrete several hydrolytic enzymes, particularly α-amylase, into the storage organ

endosperm. The hydrolysates of storage materials, glucose from the starchy endosperm and Pi from the aleurone grains in the aleurone layer, are absorbed by the scutellar tissue. Furthermore, glucose is converted into sucrose in these tissues, and then sucrose is transported to the growing root and shoot tissues. The suspension-cultured cells derived from the rice seed embryos retain some properties of the scutellar tissue, such as the abilities to secrete α-amylase and convert glucose to sucrose under adequate conditions (Kashem *et al.*, 1998), and thus can be used as a model system of the scutellar tissues.

By controlling the Ca^{2+} uptake, metabolic sugars such as glucose and sucrose prevent the *de novo* synthesis and intracellular transport of α-amylase molecules and stimulate its protein turnover in suspension-cultured rice cells (Mitsui *et al.*, 1999a, b). In the present communication, we report that phosphate stimulates the Ca^{2+} uptake and the sucrose-controlled secretion of α-amylase II-4 in suspension-cultured rice cells, and discuss a possible role of phosphate in germinating rice seeds.

Materials and methods

Plant materials

Cells derived from the embryo of rice seed (*Oryza sativa* L. cv. Nipponkai) were suspension-cultured in Murashige-Skoog (MS) medium as described previously (Mitsui *et al.*, 1999b). Rice cells cultured for 7 days at 28 ℃ were used in this study. The standard MS medium contained 3 mM Ca^{2+}, 1.5 mM Mg^{2+}, 1.25 mM phosphate (Pi) and 88 mM (3%, w/v) sucrose.

Preparation of microsomes

Microsomes were prepared by the procedure described previously (Mikami *et al.*, 2001). Rice cells were gently homogenized with 50 mM glycylglycine-NaOH (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.5 M mannitol in a mortar with a pestle. The homogenate was passed through two layers of gauze and centrifuged at 1,000*g* for 20 min and at 10,000*g* for 30 min, sequentially. The supernatant was layered on a 15% (w/w) sucrose layer and 50% sucrose cushion containing 50 mM glycylglycine-NaOH (pH 7.0) and further centrifuged at 100,000*g* using a Beckman Type 55.2 Ti rotor for 3 h. The membrane fraction trapped on the 50% sucrose cushion was used as the microsome preparation.

Measurements of Ca^{2+}, Mg^{2+} and phosphate contents

The contents of two divalent cations in rice cells and microsomes were analyzed by atomic absorption analysis. Rice cell samples were frozen in liquid N_2 and lyophilized. Fifty mg of the powdered sample was then suspended in a solution containing 0.5 ml of HNO_3 and 0.4 ml of $HClO_4$ (60%) solution and incubated for 5 h. The mixture was heated at 100 ℃ for 1 h, 0.2 ml of H_2O_2 (31%) was added, and the whole mixture heated again at 100 ℃ for 2 h. After being dried at 210 ℃, the sample was dissolved in 5 ml of HCl (9%) and heated at 100 ℃ for 5 min. The sample was cooled and filled up to 10 ml with distilled water. The microsomes prepared from 10 g rice cells were lyophilized and treated with HNO_3 and $HClO_4$, H_2O_2, and HCl sequentially, as described the above. Atomic absorption spectrophotometry (Hitachi Z-8200, Tokyo, Japan) was performed on appropriately diluted samples containing 0.1 mM lanthanum oxide. An aliquot of the above sample was neutralized with NaOH, diluted to 10 ml, and mixed with one-tenth volume of 2.5% (w/v) ammonium molybdate-28% (v/v) H_2SO_4 to determine the phosphate content by the molybdenum blue method. The sample was incubated with 100 μl of 1.25% (w/v) $SnCl_2$-9% HCl and filled up to 25 ml. Absorbance at 660 nm was measured after incubation for 10 min. KH_2PO_4 was used as the standard phosphate.

Electron probe microanalyzer (EPMA) study

The distribution and accumulation of Ca^{2+} in rice cells were analyzed using an Electron probe microanalyzer (EPMA) (Shimadzu EPMA-8705, Kyoto, Japan). Two different sample preparations were used: (1) Rice cell samples were immediately frozen and embedded in optimal cutting temperature (O.C.T.) compound. The section specimens (5 μm) were prepared using Coldtome (Sakura, Tokyo, Japan) and placed on an EPMA sample carbon block (20x25x20 mm) directly. (2) Rice cell samples were frozen, cracked with a razor in liquid N_2, and finally lyophilized. The cracked surface was coated with carbon by using a carbon vaporizer (Shimadzu CC50). Mapping conditions were as follows: acceleration voltage, 15 kV; sample current intensity, 0.05 mA; step width, 0.001-0.005 mm per (x, y); data points, 256x256; measuring time, 0.08 s per step.

In vivo and in vitro labeling

In the standard *in vivo* pulse labeling system, 20 mg of rice cells were incubated in 1 ml of MS media containing [^{35}S] Met (400 kBq, 37 TBq mmol^{-1}) for 18 h at 30 ℃ on a reciprocal shaker operated at 130 rpm with amplitude of 30 mm. After pulse incubation, cells were removed and homogenized in a

small mortar using 1 ml of MS medium containing 0.1% (v/v) Triton X-100, and centrifuged at 15,000*g* for 20 min to collect the cell extracts. On the other hand, microsomes were prepared from the cells as described above. Further details and alterations of the incubation conditions are given in each figure legend.

Total RNA was isolated from rice as described previously (Mitsui *et al.*, 1999b). The amount of translatable mRNA in the cells was estimated by translating the isolated RNA (4 μg) using the reticulocyte lysate translation system (Amersham Pharmacia Biotech, Tokyo, Japan) with [^{35}S] Met (400 kBq, 29.6 TBq mmol^{-1}) according to the manufacturer's protocol. When RNA isolated from rice cells incubated without sucrose for 18 h at 30 ℃ was used, radioactivity incorporated into translation products was approximately 50 kBq.

[^{35}S]-labeled α-amylase II-4 molecules synthesized *in vivo* and *in vitro* were subjected to the quantitative immunoprecipitation. Aliquots (100 to 500 μl) of the above [^{35}S]-labeled proteins were made to 1% (w/v) with 10% SDS, boiled for 1 min, and then made to 1.5% (w/v) with 20% Triton X-100. After adding 5 volumes of buffer A (20 mM Tris-HCl (pH 7.5), 0.14 M NaCl, 1 mM EDTA) containing 0.1% Triton X-100, the mixture was incubated with 25 μg each of polyclonal anti-α-amylase II-4 IgG (Mitsui *et al.*, 1996) for 1 h at 30 ℃. Ten μl of Protein A-Sepharose swollen gel was added, and the whole mixture incubated for 1 h with vigorous shaking. The suspension was then applied to a small column to retain the Protein A-Sepharose-immunoprecipitate complex, and the column was washed sequentially with buffer A with 0.1% Triton X-100, buffer A with 1 M NaCl, buffer A, and distilled water. Finally the immunoprecipitate was eluted from the column with 70 μl of SDS sample buffer consisting of 10 mM Tris-HCl (pH 6.8), 2% SDS, 5% (v/v) mercaptoethanol, 10% (w/v) glycerol, and 0.005% (w/v) bromophenol blue. Eluates were boiled for 1 min to dissociate α-amylase molecules from IgG, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE), followed by autoradiography using a radioisotope imaging analyzer (BAS-5000, Fuji Film, Tokyo, Japan) (Mitsui *et al.*, 1999a).

Immunohistochemical study

Rice cell samples were immediately frozen and embedded in O.C.T. compound. The sections (5 μm) prepared as described above were placed on a slide glass. The sections were fixed with cold acetone for 10 min at 4 ℃, stained with anti-α-amylase II-4 IgG (primary antibody) and [^{35}S]-labeled anti-rabbit IgG IgG (secondary antibody, 18.5 kBq ml^{-1}) in a solution consisting of 1% (w/v) skimmed milk, 0.05% (w/v) Tween 20 and phosphate buffered saline (PBS), and finally analyzed with BAS-5000.

Northern blot analysis

Northern blot analysis was carried out as follows: The isolated RNA (20 μg) was loaded onto 1.4% (w/v) agarose-formaldehyde gels and blotted onto Hybond N+ membrane according to the manufacturer's protocol (Amersham). The nylon membrane blotted with RNA was incubated with prehybridization solution consisting of 5x SSPE, 5x Denhardt's solution, 0.5% SDS and 0.02% (w/v) denatured salmon sperm DNA for 1 h at 65 ℃, and then hybridized with [^{33}P]-labeled DNA probes in the prehybridization solution for 16 h at 65 ℃. The α-amylase II-4 (*RAmy3D*) gene-specific DNA was amplified by PCR with 5'-TCCGCGTCCCGGCGGGGCGGCACCT-3' and 5'-CTGCATCCTGAACCTGAC-3' primers (Sheu *et al.*, 1996) and the rice actin 1 DNA by 5'-CATGCTATCCCTCGTCTCGACCT-3' and 5'-CGCACTTCATGATGGAGTTGTATG-3' primers (Kashem *et al.*, 2000). The DNA probes were labeled with DNA-labeling beads with [α-^{33}P] dCTP (1.85 MBq, 110 TBq mmol^{-1}) according to the manufacturer's protocol (Amersham). After hybridization, the membrane was washed twice with 2x SSPE and 0.1% SDS at room temperature and finally washed with 1x SSPE and 0.1% SDS for 15 min at 65 ℃. The air dried membrane was subjected to autoradiography.

Results

Our previous study demonstrated that the Ca^{2+} uptake into rice cells is controlled by sugars, such as glucose and sucrose, and that sugar supplementation reduces the Ca^{2+} uptake (Mitsui *et al.*, 1999a). **Table 1** shows the effects of phosphates on uptake of both Ca^{2+} and Mg^{2+} into suspension-cultured rice cells. Supplementation of 10 mM phosphate (Pi) stimulated the Ca^{2+} uptake into rice cells in the presence of 44 mM sucrose, while Mg^{2+} uptake was not significantly affected. Higher concentrations of Pi up to 50 mM further increased the uptake of Ca^{2+}. The external concentration of Pi (1.25-50 mM) had no effect on the uptake of Pi. Addition of 10 mM pyrophosphate (PPi) had the same effect as that of Pi on the uptake of both divalent cation and phosphate. Mg^{2+} uptake decreased with the increase in the concentration of PPi from 10 to 50 mM. The stimulating effect of tripolyphosphate (PPPi, 10

Table 1 Effects of phosphates on uptake of divalent cation into rice cultured cells

Incubation condition	Ca (mg per g dry cells)	Mg (mg per g dry cells)	Pi (mg per g dry cells)
control 1 (MS with 0 mM sucrose)	2.73 ± 0.35	6.44 ± 0.49	13.4 ± 1.0
control 2 (MS with 44 mM sucrose)	1.00 ± 0.14 (100%)	4.55 ± 0.64 (100%)	13.1 ± 2.1 (100%)
control 3 (MS with 88 mM sucrose)	0.83 ± 0.04	4.85 ± 0.51	13.3 ± 2.5
control 2 + 10 mM Pi	2.13 ± 0.24 (213%)	5.11 ± 0.34 (112%)	12.1 ± 1.7 (92%)
control 2 + 20 mM Pi	2.84 ± 0.24 (284%)	7.01 ± 0.89 (154%)	14.4 ± 2.7 (110%)
control 2 + 50 mM Pi	5.22 ± 0.72 (522%)	6.41 ± 0.59 (141%)	14.6 ± 1.8 (111%)
control 2 + 10 mM PPi	2.56 ± 0.24 (256%)	4.61 ± 0.66 (101%)	14.7 ± 1.1 (112%)
control 2 + 20 mM PPi	2.10 ± 0.16 (210%)	1.95 ± 0.16 (43%)	10.4 ± 1.9 (79%)
control 2 + 50 mM PPi	2.33 ± 0.25 (233%)	1.31 ± 0.14 (29%)	14.7 ± 2.8 (112%)
control 2 + 10 mM PPPi	6.04 ± 0.88 (604%)	4.79 ± 0.96 (105%)	17.0 ± 2.1 (130%)
control 2 + 20 mM PPPi	4.10 ± 0.36 (410%)	2.75 ± 0.57 (60%)	17.5 ± 2.0 (134%)
control 2 + 50 mM PPPi	2.06 ± 0.15 (206%)	1.05 ± 0.14 (23%)	22.5 ± 4.4 (172%)

Rice cells were incubated in MS culture media supplemented with sucrose (0-88 mM) and different phosphates (0-50 mM) for 18 h at 30 ℃. The basal MS medium contained 3 mM Ca^{2+}, 1.5 mM Mg^{2+}, and 1.25 mM phosphate (Pi). The data represent the average of duplicate experiments.

mM) on Ca^{2+} uptake was the greatest, although a higher concentration of PPPi (>20 mM) was toxic and reduced the uptake of both Ca^{2+} and Mg^{2+}. These results indicate that phosphate specifically stimulated the uptake of Ca^{2+} into rice cells.

The distribution of Ca^{2+} accumulated in Pi-supplemented cells was examined using EPMA. As shown in **Fig. 1**, the increase of Pi concentration stimulated the accumulation of Ca^{2+}, and Ca^{2+} accumulated in the outer cell layer of the rice cell cluster. **Fig. 2** shows that Ca^{2+} was accumulated and localized inside the cell not being restricted to the cell walls, although some morphological change occurred by Pi treatment. Ca^{2+} was accumulated and distributed in the Pi- and sucrose-supplemented cells in a pattern similar to that seen in sugar-starved cells (Mitsui *et al.* 1999b). We further examined the reversibility of Pi-induced Ca^{2+} accumulation. By removal of the excessive external Pi from the cell culture, the Pi-induced Ca^{2+} accumulation completely disappeared (**Fig. 3**), and cells resumed normal growth (data not shown).

The suspension-cultured rice cells actively synthesize and secrete α-amylase II-4 encoded by *RAmy3D*, which is a dominant isoform in the cells (Mitsui *et al.*, 1996). The expression of α-amylase II-4 is regulated by sugar at both transcriptional and posttranscriptional steps (Mitsui *et al.*, 1999b). Sucrose supplementation strongly prevented the synthesis and extracellular liberation of α-amylase II-4 in rice cell culture (Mitsui *et al.*, 1999b; **Fig. 4**). The effect of phosphate on the synthesis and extracellular liberation of α-amylase II-4 was examined by *in vivo* labeling of α-amylase II-4 molecules in rice cells with [^{35}S] Met in the presence or absence of phosphate. Supplementation of Pi, PPi, or PPPi at 10 mM markedly stimulated the incorporation of [^{35}S] Met into α-amylase II-4 molecules and its extracellular liberation in the presence of sucrose, although these phosphates scarcely affected the [^{35}S] Met incorporation under the sugar-depleted condition (**Fig. 4**). Immunohistochemical studies using anti-α-amylase II-4 antisera confirmed that Pi, PPi and PPPi induce α-amylase II-4 expression in the sucrose-supplemented cells (**Fig. 5**). The quantitative analysis showed that the phosphate-induced expression of α-amylase II-4 molecules exclusively occurs at the outer cell layer of the cell cluster (**Fig. 5**). The distribution of phosphate-induced α-amylase II-4 expression resembled that of Ca^{2+} accumulation (**Fig. 1** and **5**).

Fig. 6 shows that Pi increased the accumulation of Ca^{2+} into micorosomes in the sucrose-supplemented cells. The stimulating effect was gradually and weakly enhanced with the increase in Pi concentration from 10 to 50 mM, although the accumulation of Ca^{2+} into the cells caused by 50 mM Pi was 2.5-fold higher than that caused by 10 mM Pi (**Table 1**). Pi also induced the synthesis of [^{35}S]-labeled α-amylase II-4 in microsomes in the sucrose-supplemented cells. EGTA treatment completely inhibited the Pi-induced α-amylase II-4 synthesis (**Fig. 6**). The level of α-amylase II-4

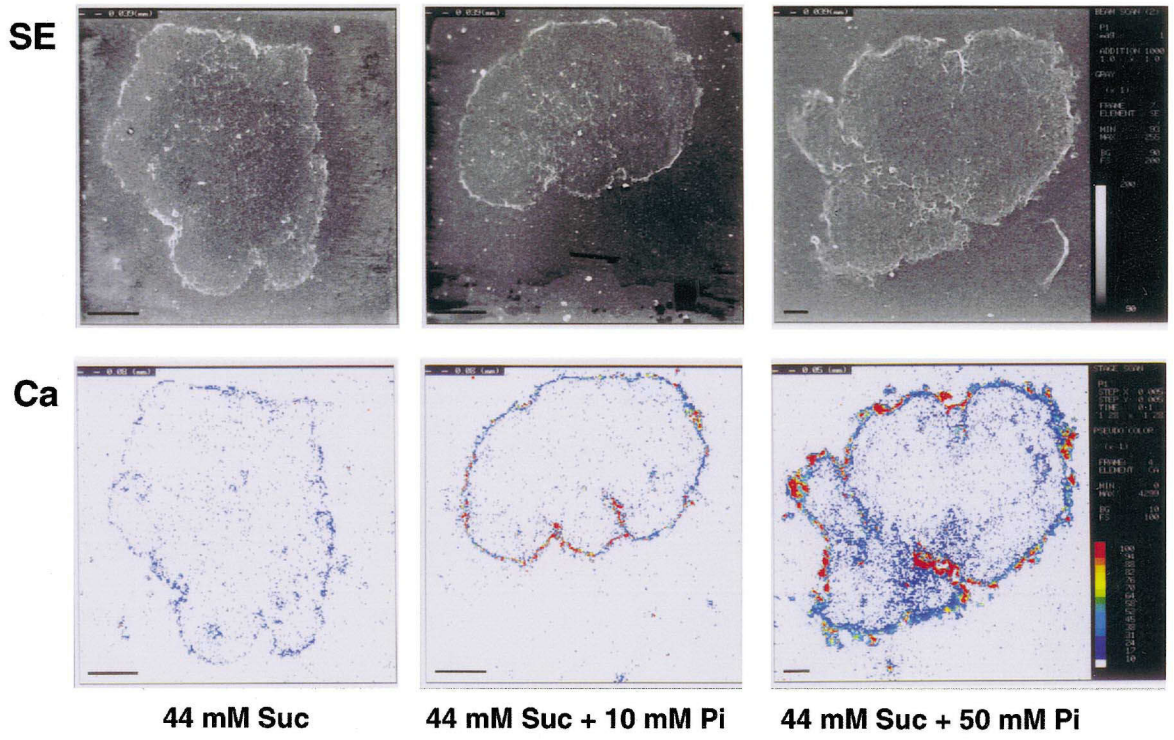

Fig. 1 Accumulation and distribution of Ca^{2+} in rice cell cluster under Pi-supplemented conditions. Rice cells incubated for 18 h at 30°C in the MS culture media supplemented with 44 mM sucrose under different Pi-supplemented conditions. (0, 10 and 50 mM) were immediately frozen and embedded in O.C.T. compound, and the sections (5 μm) were scanned with EPMA. Relative amount of Ca is presented by pseudocolor coded in all the images according to the color bar on the right. SE ; secondary electron image, Ca ; mapping pattern of calcium. Bars ; 200 μm.

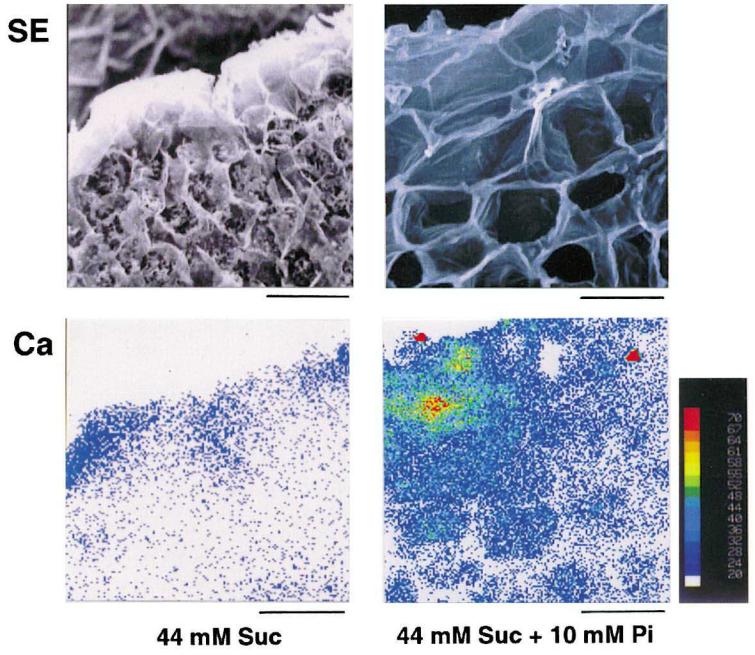

Fig. 2 Localization of Ca^{2+} accumulated in rice cells in the presence of 10 mM Pi. Rice cells incubated for 18 h at 30°C in the 44 mM sucrose-supplemented MS media with or without 10 mM Pi were immediately frozen and cracked with a razor in liquid N_2. The cracked surfaces were scanned with EPMA. Relative amount of Ca is presented by pseudocolor according to the color bar on the right. SE ; secondary electron image, Ca ; mapping pattern of calcium. Bars ; 50 μm.

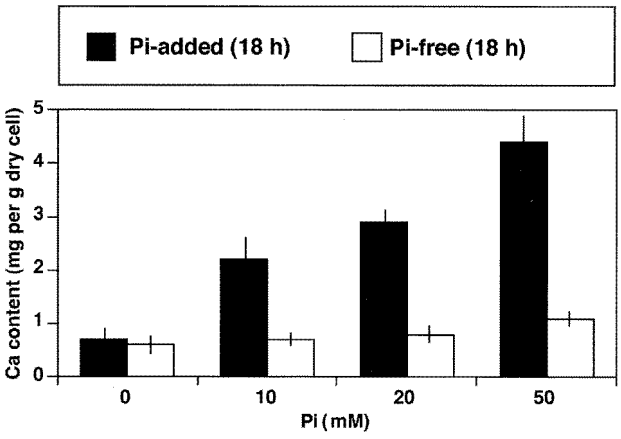

Fig. 3 Reversibility of Pi-induced accumulation of Ca^{2+} in rice cells. Rice cells were incubated in MS media with 44 mM sucrose under different Pi-supplemented conditions (0, 10, 20 and 50 mM) for 18 h at 30 ℃ (Pi-added), and further incubated in MS media with 40 mM sucrose for 18 h at 30 ℃ (Pi-free). These treated cells were subjected to atomic absorption analysis to determine the Ca^{2+} content.

synthesis in microsomes induced by 10 mM Pi was approximately half of that induced by sugar starvation. This is coincident with the fact that the intracellular amount of α-amylase II-4 synthesized in the sucrose- and Pi-supplemented cells (**Fig. 4**, lane 5) was approximately 2-fold lower than that in the sugar-depleted cells (**Fig. 4**, lane 1).

To determine the effect of Pi on the level of mRNA of α-amylase II-4, we obtained total RNA from rice cells incubated with or without 10 mM Pi and subjected it to both Northern blot and *in vitro* translation analyses. As can be seen in **Fig. 7**, Pi did not increase the accumulation of α-amylase II-4 mRNA in rice cells. In addition, PPi also did not increase the mRNA level (data not shown).

Discussion

The present study showed that phosphate markedly stimulated the Ca^{2+} uptake into rice cells (**Table 1**, **Fig. 1** and **2**), and that phosphate induced the synthesis and extracellular liberation of α-amylase II-4 molecules in the sucrose-fed cells (**Fig. 4**). The distribution of the enzyme in the rice cell cluster was similar to that of the Ca^{2+} uptake induced by phosphate (**Fig. 1** and **5**). Surprisingly, phosphate did not increase the mRNA level of α-amylase II-4 (**Fig. 7**). Judging from all these experimental results, we concluded that phosphate stimulates the synthesis and extracellular liberation of α-amylase II-4 without enhancing the mRNA level, mediated by Ca^{2+}. We also observed that phosphate stimulates the secretion of α-amylase activity from

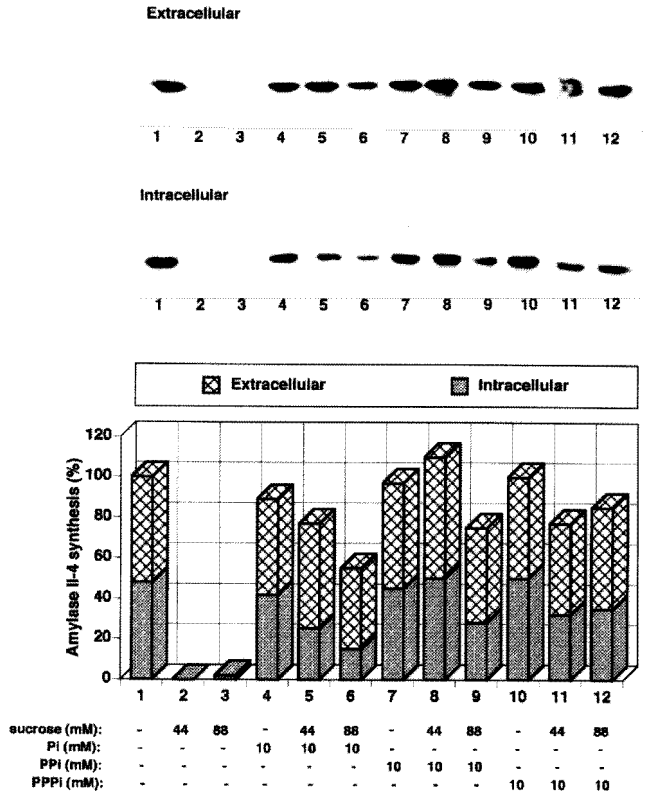

Fig. 4 Effects of Pi, PPi, and PPPi on the synthesis and extracellular liberation of α-amylase II-4 molecules in the sucrose-supplemented rice cells. Rice cells were incubated with [^{35}S] Met in the MS media supplemented with 0 mM phosphate (lane 1-3), 10 mM Pi (lane 4-6), PPi (lane 7-9) or PPPi (lane 10-12) in the presence of different concentrations of sucrose (0, 44 and 88 mM) for 18 h at 30 ℃, followed by the quantitative immunoprecipitation of extracellular and intracellular [^{35}S] α-amylase II-4 molecules using anti-α-amylase II-4 antibodies. Upper panel: The autoradiograms, Lower panel: The quantitative results. Total radioactivity of α-amylase II-4 synthesis in control 1 (9940 PSL) was regarded as 100%. Photo-stimulated luminescence (PSL) value shows the unit of radiation obtained by autoradiography. The data represent the average of triplicate experiments and the SD did not exceed 15%.

the rice scutellar tissues of germinating rice seeds (data not shown). Drozdowicz and Jones (1995) reported that phytohormones regulate the release of acid from barley aleurone layers. Gibberellin stimulated the release of phosphoric acid from the aleurone, but abscisic acid reduced it. In addition, it has been revealed that the level of inorganic phosphate in rice seed increases at the early stage of germination (Marschner, 1995). It is likely that the secretion of α-amylase in the rice scutellar tissues is precisely controlled by several factors including phytohormones, metabolic sugars and phosphate.

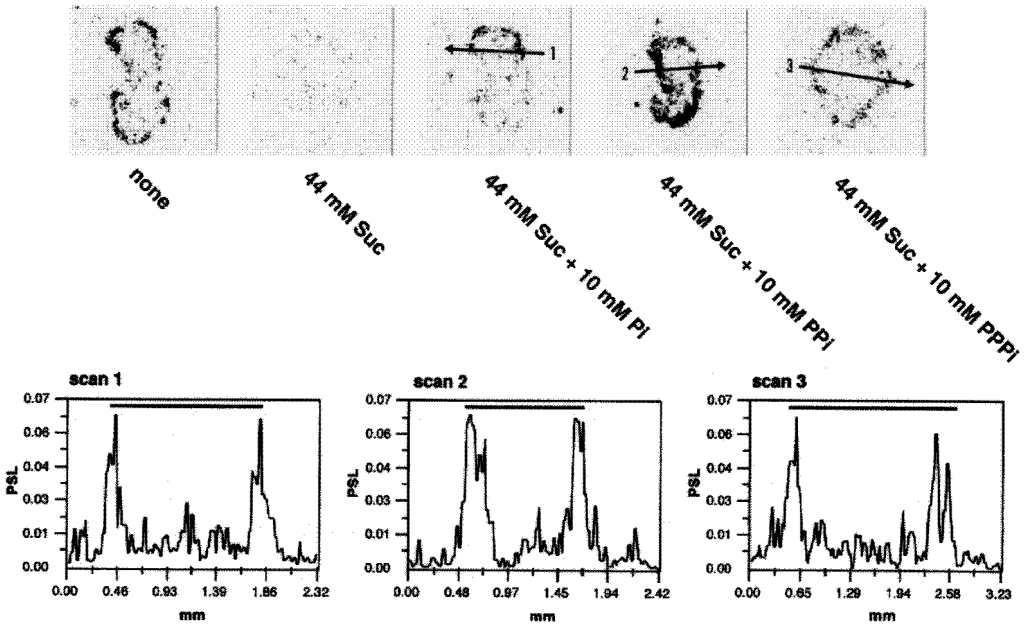

Fig. 5 Distribution and accumulation of α-amylase II-4 molecules in rice cell cluster under Pi-supplemented conditions. Rice cells incubated for 18 h at 30 ℃ in the MS culture media with or without 44 mM sucrose under different phosphates-supplemented conditions (10 mM Pi, PPi or PPPi) were immediately frozen and embedded in O.C.T. compound. Radio-immunodetection of α-amylase II-4 molecules in the sections (5 μm) was carried out as described in the text. Autoradiograms were analyzed with BAS 5000. (Upper panel) The autoradiografic images: arrows represent scanning directions for quantitative analyses. (Lower panel) The quantitative results: the bars shown in scanning results represent the sizes of the cell clusters.

There is a possibility that phosphate forms an insoluble $CaPO_4$ complex with Ca^{2+} and that the stable compound is deposited at the apoplast. However, the reversibility of the phosphate-induced accumulation of Ca^{2+} with or without the external phosphate (**Fig. 3**) and the fact that neither formation nor increase of insoluble and/or crystallitic materials was observed in the cell culture with 10-50 mM Pi (data not shown) clearly ruled out this possibility.

The mRNA of α-amylase II-4 was hardly detectable in the rice cells cultured with 44 mM sucrose and 10 mM Pi for 18 h (**Fig. 7**). The strength of protein expression usually depends on its mRNA level, so that the phosphate-stimulated expression of α-amylase II-4 molecules is a strange phenomenon. Therefore, we further examined the α-amylase II-4 expression in the sucrose- and Pi-supplemented cells during the next 18 h incubation. In this stage, the phosphate-stimulated expression of [^{35}S]-labeled α-amylase II-4 was also detected, but total expression was lower compared with that during the first 18 h (data not shown). These results indicate that indeed the significant depletion of mRNA caused by a long incubation affects the expression of the enzyme molecules. However there is no doubt that the phosphate-stimulated expression of α-amylase II-4 molecules occurred independent of its mRNA decrease. We infer that the mRNA remaining in the sucrose- and Pi-supplemented cells during the first 18 h of incubation is enough for the usual protein synthesis.

In soybean cell culture, the transcription of sucrose-inducible genes, such as vacuolar acid phosphatase (*VspB*), lipoxygenase A, and chalcone synthase was inhibited by phosphate (Sadka *et al.*, 1994). On the other hand, phosphate did not block the repression of *cabZm5-cat* caused by glucose in maize protoplasts (Jang and Sheen, 1994). Sucrose strongly inhibited the expression of α-amylase II-4 (*RAmy3D*) in cultured rice cells at both the transcription and posttranscriptional steps (Hwang *et al.*, 1998; Lu *et al.*, 1998; Toyofuku *et al.*, 1998; Mitsui *et al.*, 1999b). Interestingly, phosphate induced the protein expression of α-amylase II-4 in the sucrose-supplemented cells, although it did not overcome the repression of α-amylase II-4 gene transcription caused by sucrose. This indicates that phosphate is involved not only in the transcriptional regulation but also in the posttranscriptional regulation of enzyme expression in plant cells.

Phosphate enhanced both the Ca^{2+} accumulation and α-amylase II-4 synthesis in the microsomes (**Fig. 6**), indicating that the Ca^{2+} inpoured into the

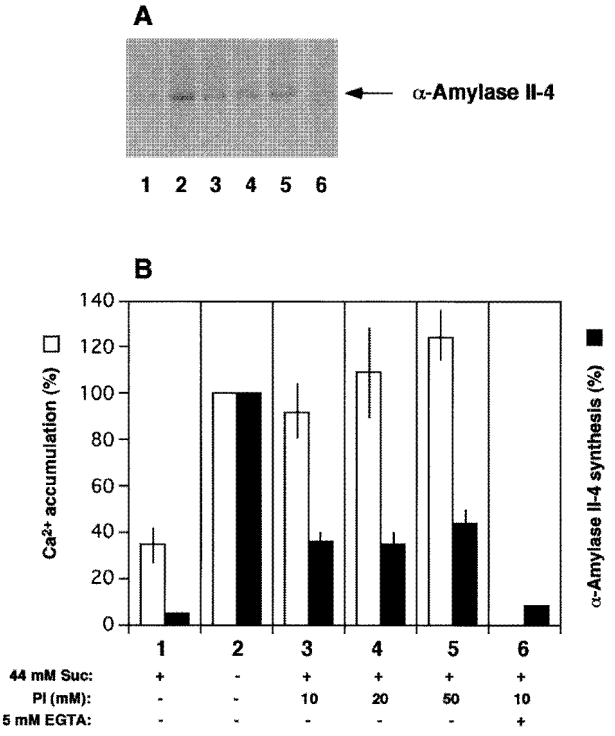

Fig. 6 Effects of Pi on Ca^{2+} accumulation and α-amylase II-4 synthesis in microsomes. Rice cells were incubated with [^{35}S] Met in the MS media supplemented with sucrose (0 or 44 mM), Pi (0-50 mM) and ethylene glycol-bis(α-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA, 0 or 5 mM) as indicated in the figure for 18 h at 30 ℃, followed by the quantitative immunoprecipitation of [^{35}S] α-amylase II-4 molecules in microsomes using anti-α-amylase II-4 antibodies. The Ca^{2+} accumulation was determined in microsomes, by incubating rice cells in the same condition without [^{35}S] Met. (A) The autoradiogram of [^{35}S] α-amylase II-4 molecules synthesized. (B) The quantitative results: the radioactivity of α-amylase II-4 synthesis (416 PSL) and the Ca^{2+} content (92 ng (mg protein)$^{-1}$) in lane 2 were regarded as 100%. The Ca^{2+} content with EGTA was not determined (lane 6).

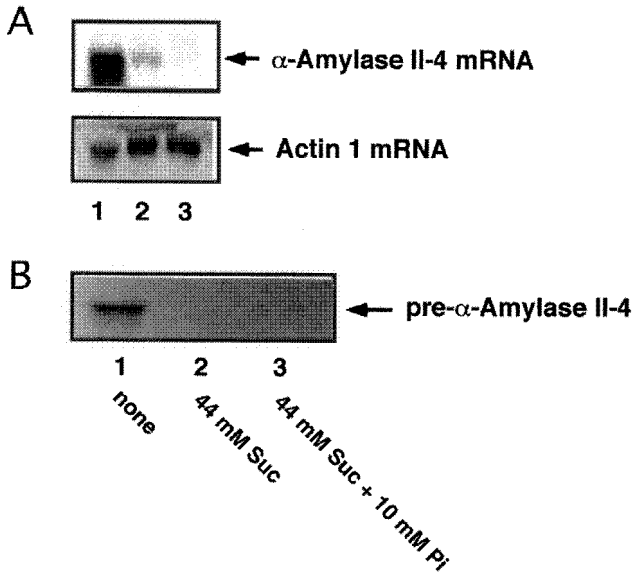

Fig. 7 Effects of Pi on the level of mRNA for α-amylase II-4. (A) Total RNA prepared from rice cells incubated in the MS media containing no sucrose and Pi (lane 1), 44 mM sucrose (lane 2) or 44 mM sucrose and 10 mM Pi (lane 3) for 18 h at 30 ℃ was subjected to the northern blot analysis with [^{33}P]-labeled α-amylase II-4 and actin 1 DNA probes. (B) The above RNA preparation was translated *in vitro* by reticulocyte lysates with [^{35}S] Met, followed by the quantitative immunoprecipitation of [^{35}S] α-amylase II-4 precursor using polyclonal anti-α-amylase II-4 antibodies.

cells further reached the microsomes. The protein synthesis induced by phosphate was also disappeared completely under the Ca^{2+}-free condition (**Fig. 6**). Cereal α-amylase is a Ca^{2+}-containing metalloenzyme (Kadziola *et al.*, 1998), and the exocytotic secretion of α-amylase requires Ca^{2+} (Zorec and Tester, 1992; Mitsui and Itoh, 1997). Indeed, Ca^{2+} depletion completely prevented the synthesis and extracellular liberation of α-amylase molecules in typical rice secretory cells (Mitsui *et al.*, 1999a; Kashem *et al.*, 2000). The present experimental results clearly suggest that phosphate causes Ca^{2+} to permeate across the plasma membranes into the cytoplasm, and that the intracellularly accumulated Ca^{2+} stimulates the protein expression and the post-translational secretory processes of α-amylase II-4 molecules in rice cells. Treatments with PPi and PPPi at adequate concentrations stimulated the uptake of Ca^{2+} and expression of α-amylase similar to or more than that with Pi (**Table 1**, **Fig. 4**). Calcium polyphosphate is reportedly a component of a non-proteinaceous Ca^{2+} channel existing in the plasma membrane of *E. coli*, which can permeate Ca^{2+} but not Mg^{2+} (Reush *et al.*, 1995). The patterns of Ca^{2+} and Mg^{2+} uptake observed in the phosphates-supplemented rice cells (**Table 1**) were similar to the phenomenon observed in *E. coli*, suggesting that a channel-like complex is formed on the plasma membranes in phosphate and Ca^{2+}-supplemented rice cells.

Furuichi *et al.* (2001) who employed Ca^{2+}-dependent photoprotein aequorin-transformed *Arabidopsis thaliana* reported that sugars increase cytosolic Ca^{2+} in the root and leaves, although the reverse phenomenon is observed in rice cells (Mitsui *et al.*, 1999a). They suggested that a voltage-gated Ca^{2+} channel on the plasma membrane is involved in the increase of $[Ca^{2+}]_{cyt}$. In mammalian skeletal muscle, the regulation of the Ca^{2+} release

channel by inorganic phosphate has been discussed (Fruen *et al.*, 1994; Balog *et al.*, 2000). To answer the interesting question whether inorganic phosphate stimulates a Ca^{2+} channel at the plasma membranes of rice cells or not, further experiments are underway.

Acknowledgments

This research was supported by Grant-in-Aid no. 10640628 (to T.M) from the Japan Society for the Promotion of Science, and a grant of Rice Genome Project PR-1102 (to T.M), MAFF, Japan. We wish to thank A. Ode and A. Itoh for their excellent technical assistance.

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