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Phosphorylation of Myo-inositol by Isolated Aleurone Particles of Rice

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When aleurone particles isolated from rice grains were incubated with ^{32}P-orthophosphate or ^{3}H-myo-inositol, both radioactivities were incorporated into an acid-stable phosphate ester. As the reaction product, myo-inositol monophosphate was recognized by ion exchange column chromatography. The phosphorylation activity was highest at the bran which corresponded to the aleurone layer. These observations suggest that the phosphorylation site of myo-inositol in the rice grain is the aleurone particles.

The phosphorylation of myo-inositol was enhanced by the existence of ATP. The optimum pH and temperature for the phosphorylation were 7.9 and 30°C, respectively.

It is well established that during the ripening period of plant seed, phosphate binds to myo-inositol to form phytic acid, which comprises over 80% of the total phosphorus existing in the grains. Previous work in our laboratory demonstrated the formation of phytic acid in ripening rice and wheat grains from ^{32}P-orthophosphate and ^{3}H-myo-inositol administered to grains through stalks.[1] Using microautoradiography and electron microprobe X-ray analysis, we demonstrated that the site of accumulation of phytic acid is the particles in the aleurone layer.[2,3] This conclusion was further supported by the chemical analysis of particles isolated from the aleurone layer.[4]

This work is intended to clarify, whether aleurone particles are the site of phosphorylation of myo-inositol or not. The results indicate that aleurone particles are not only the site of accumulation of phytic acid but also that phosphorylation of myo-inositol.

MATERIALS AND METHODS

Preparation of aleurone particles. Rice aleurone particles were isolated in accordance with the method described in the previous paper.[5] Isolated particles were free from other particles such as protein bodies and starch granules and were kept in a desiccator over silica gel at room temperature without loss of phosphorylation activity for at least two months. The contents of orthophosphate and free myo-inositol were 60 μmoles and 1.4 μmoles per gram of the isolated particles, respectively.

Standard reaction conditions for esterification of ^{32}P-orthophosphate and ^{3}H-myo-inositol. The reaction mixture consisted of 10 mg of aleurone particles, 50-μmoles of tricine-KOH (pH 8.0 at 27°C), 10 μmoles of myo-inositol, 10 μmoles of $MgCl_2$, 10 μmoles of KF, and 10.6 μmoles of ^{32}P-orthophosphate (0.56 μCi) in a total volume of 1 ml. After the reaction at 27°C, the reaction mixture was heated for 20 min with 10 ml of 10% perchloric acid in boiling water. Under these conditions, acid-labile phosphorus compounds, such as ATP, were hydrolysed. Subsequently, ^{32}P-orthophosphate was removed with benzene: *n*-butanol mixture in accordance with the procedure of Asada *et al.*[6] Acid-stable ^{32}P was estimated by using the Čerenkov radiation in a liquid scintillation counter.

Basal reaction mixture for phosphorylation of ^{3}H-myo-inositol contained 5 mg of aleurone particles, 5.0 μmoles tricine-KOH (pH 8.0 at 27°C), 1.0 μmole $MgCl_2$, 10 μmoles KF, 1.0 μmole KH_2PO_4, 0.1 μmole ATP, and 2.23 nmoles of 0.14 μCi ^{3}H-myo-inositol in a total volume of 0.2 ml. After the reaction was carried out at 27°C, a strip of ion exchange paper (Amberlite SB-2, Cl^-) was immersed in the reaction mixture. The paper strip was washed thoroughly with distilled water to remove unreacted ^{3}H-myo-inositol. Then, the paper was dried and offered for estimation of ^{3}H with a liquid scintillation counter by dipping into the scintillator.

Ion-exchange column chromatography. Myo-inositol phosphate was separated by ion exchange column chro-

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matography. Ba-precipitate of 10% perchloric acid extracts was dissolved by Amberlite IR-120 (H^+). After the resin was removed by filtration, the filtrate was neutralized by 0.1 N NaOH and charged onto a Dowex-1 (Cl^-) or Bio-Rad AG 1×8 (Cl^-) column (1.1×28 cm). The columns were washed successively with 600 ml of distilled water. Phosphorus compounds from Dowex-column were eluted by a linear gradient elution of HCl (1.5 liters, 0 to 1 M). For elution from a Bio-Rad AG 1×8 (Cl^-) column, a mixing chamber containing 115 ml of 0.1 N NH_4OH and a reservoir chamber containing 115 ml of 0.25 N NaCl in 0.1 N NH_4OH were used, substantially in accordance with the method of Cosgrove.[6] Radioactivity was determined with a liquid scintillation counter. For ^{32}P estimation, Čerenkov radiation was used. Estimation of myo-inositol in the eluate from Bio-Rad AG 1×8 column (Fig. 4) was carried out with *Kloekera apiculata* after myo-inositol phosphate was hydrolyzed as described previously.[7]

Radioactive compounds, myo-inositol monophosphate and acid phosphatase. ^{32}P-Orthophosphate was purchased from the Japan Isotope Association, Tokyo, and purified with Dowex-1 (Cl^-) column by the method of Suelter *et al.*[5] Myo-inositol-2-^3H was purchased from The Radiochemical Center, Amersham, England.

Myo-inositol monophosphate was prepared from the hydrolyzate of Na-phytate by rice bran phytase as described in a previous paper.[1]

To extract acid phosphatase, isolated aleurone particles were suspended in 0.1 M acetate buffer, pH 4.8. From the extract, a precipitated protein fraction was obtained, saturated 30 to 60% with ammonium sulfate. The precipitated protein was dialysed against 0.1 M acetate buffer and used as acid phosphatase preparation.

RESULTS

Esterification of ^{32}P-orthophosphate and ^3H-myo-inositol in aleurone particles

When ^{32}P-phosphate was incubated with aleurone particles of rice grains, incorporation of ^{32}P into acid-stable fraction was observed (Fig. 1). This reaction progressed linearly at least for 1 hr and reached a maximum after about 10 hr. The incorporation of ^{32}P increased with the amount of aleurone particles added. However, the incorporation did not occur with 10 min-boiled aleurone particles. When ^3H-myo-inositol was incubated with aleurone particles and ATP, ^3H-myo-inositol was converted into an anionic form. Figure 2 illustrates the progress of the conversion.

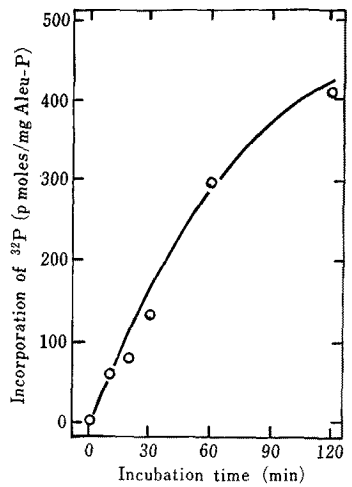

FIG. 1. Incorporation of ^{32}P-Phosphate into Acid-stable Phosphate.

Aleu-P stands for aleurone particles. This abbreviation is used in the following Figs.

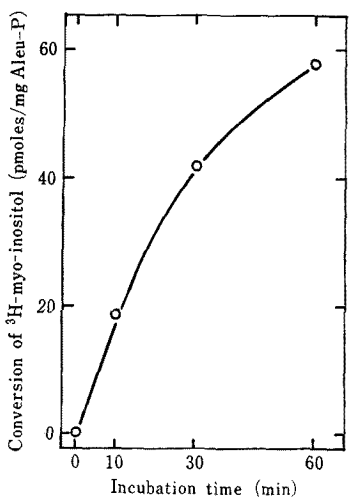

FIG. 2. Conversion of ^3H-Myo-inositol into Anionic Form by Isolated Aleurone Particles.

Fractionation of esterified products

Fractionation of esterified products of ^{32}P-phosphate reveals that about 74% existed in an acid-stable fraction (Table I). Most of the ^{32}P incorporated into the acid-soluble fraction was acid-stable, indicating that a greater part of the acid-soluble phosphorylated product was myo-inositol phosphate. ^{32}P in the acid-stable fraction was completely released as orthophosphate when it was treated with acid phosphatase. A fairly high level of ^{32}P activity was seen in the nucleotide fraction, but little

TABLE I. INCORPORATION OF ^{32}P-ORTHOPHOSPHATE AND ^{3}H-MYO-INOSITOL INTO EACH FRACTION

Fractions	^{32}P Incorporated[a] (per 200 mg Aleurone particles)	^{3}H-Myo-inositol[b] incorporated
	($\times 10^{-4}$ cpm)	($\times 10^{-3}$ cpm)
Acid-soluble		
Acid-stable	15.6	
Acid-labile	2.1	
Ba-precipitatable		87.4
Charcoal-adsorbed	2.99	10.9
Acid-insoluble	0.39	0.17

a) The reaction mixture consisted of 200 mg of aleurone particles, 250 μmoles of tricine-KOH (pH 8.0 at 27°C), 50 μmoles of $MgCl_2$, 50 μmoles of KF, 50 μmoles of myo-inositol, and 53.0 nmoles of 5.6 μCi ^{32}P-orthophosphate in a total volume of 5 ml. The reaction was carried out at 27°C for 60 min. After the addition of 10 ml of 10% perchloric acid, the reaction mixture was centrifuged at 10,000$\times g$ for 20 min. The sediment was further washed 4 times with 10 ml of 10% perchloric acid. The sediment was finally suspended in 15 ml of distilled water and offered for ^{32}P estimation as acid-insoluble fraction. The supernatant and washings were combined and 4 g of charcoal was added to remove nucleotides. The charcoal was centrifuged at 10,000$\times g$ for 20 min. The supernatant fluid was divided into two portions and one portion was heated by boiling water for 20 min to hydrolyze acid-labile phosphate linkages. From aliquots of both portions ^{32}P-orthophosphate was extracted with organic solvent in accordance with the procedure of Asada *et al.*[9] The two portions of water layers were offered for ^{32}P estimation. One was ^{32}P in total acid-soluble phosphate and the other, ^{32}P in acid-stable phosphate. ^{32}P adsorbed on charcoal was extracted three times with 15 ml of 60% ethyl alcohol containing 1% NH_4OH.

b) The reaction mixture consisted of 200 mg of isolated aleurone particles, 250 μmoles tricine-KOH (pH 8.0 at 27°C), 50 μmoles of $MgCl_2$, 50 μmoles of KF, 50 μmoles of orthophosphate, 5 μmoles of ATP, and 6.69 nmoles of 0.42 μCi ^{3}H-myo-inositol, in a total volume of 5.0 ml. The reaction was carried out at 27°C for 60 min and stopped by adding 10 ml of 10% perchloric acid. The reaction mixture was centrifuged at 10,000$\times g$ for 20 min. The supernatant fluid was taken by decantation, and the residue was further washed four times by centrifugation with 10 ml of 5% perchloric acid. The supernatant and washings of perchloric acid were assembled, and 4 g of charcoal was added to remove nucleotides. The charcoal was centrifuged off at 10,000$\times g$, for 20 min. To the supernatant was added 3 ml of 1 M barium acetate, and pH was brought to 10 with 1 M NaOH. The white precipitate formed was collected by centrifugation and to it was added Amberlite IR-120 (H^+)* ^{32}P was observed in the acid-insoluble fraction.

Fractionation of products of ^{3}H-myo-inositol is also shown in the Table. By incubation for 60 min, about 10% of myo-inositol was changed into Ba-precipitatable compound, indicating the formation of myo-inositol phosphate. ^{3}H in this fraction was also completely recovered as ^{3}H-myo-inositol when this fraction was treated with acid phosphatase. ^{3}H in the supernatant of the Ba-precipitate formed existed only as ^{3}H-myo-inositol, and no other form of ^{3}H compound was detectable by

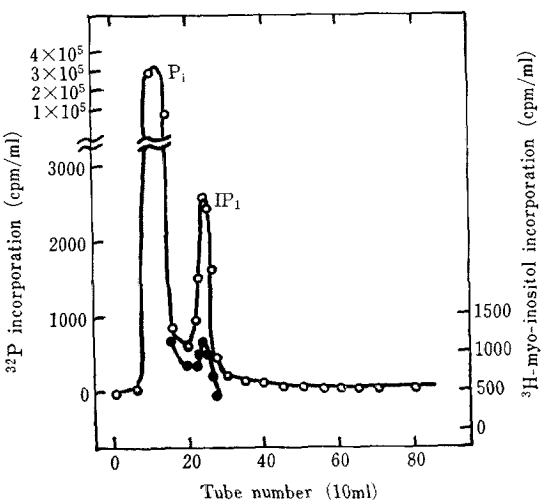

FIG. 3. Dowex-1 (Cl^-) Column Chromatogram of Phosphorylated Product by Isolated Aleurone Particles of Rice.

The reaction mixture and the procedure were the same as those for the fractionation of myo-inositol phosphate as described in the legend of Table I, b), except for 50 μmoles orthophosphate which was replaced by 53.0 nmoles of 5.6 μCi ^{32}P-orthophosphate, and 6.69 nmoles of 0.42 μCi ^{3}H-myo-inositol which was replaced by 2.23 nmoles of 0.14 μCi ^{3}H-myo-inositol. The phosphorylated product was separated by a linear gradient elution as described in the text. ○, ^{32}P; ●, ^{3}H; P_i; orthophosphate, IP_1; inositol monophosphate.

*resin to remove Ba^{2+}. After the resin was removed by filtration, and aliquot of the filtrate was offered for ^{3}H estimation using liquid scintillation counter. ^{3}H in charcoal-adsorbed fraction was obtained by extracting the charcoal five times with 15 ml of 60% ethyl alcohol containing 1% NH_4OH. ^{3}H in acid-insoluble fraction was obtained by adding 50 ml of chloroform: methanol (1: 1) to the residue, which had been obtained after the perchloric acid extraction of the reaction mixture.

paperchromatography.

Identification of esterified product

^{32}P-Phosphate and ^{3}H-myo-inositol were incubated together with aleurone particles and the phosphorylated product was separated by Dowex-1 (Cl^-) column chromatography, as shown in Fig. 3. ^{32}P and ^{3}H were eluted from the column corresponding to inositol monophosphate, but, not found in myo-inositol di-, tri-, tetra-, penta- or hexaphosphate. This pattern of incorporation of ^{32}P into myo-inositol phosphate was similar to that observed when ^{32}P-phosphate was administered to ripening grains of rice,[1] except that ^{32}P was incorporated into both myo-inositol mono- and hexaphosphates in that case. Formation of myo-inositol monophosphate by aleurone particles was further confirmed by Bio-Rad AG 1×8 column chromatography using authentic myo-inositol monophosphate. As shown in Fig. 4, ^{32}P was found in myo-inositol monophosphate.

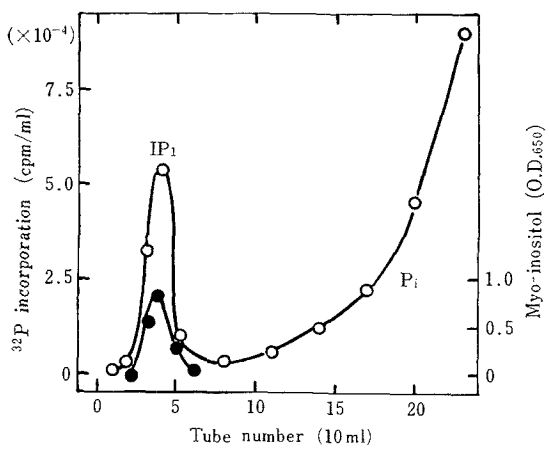

FIG. 4. Bio-Rad AG 1×8 (Cl^-) Column Chromatogram of Phosphorylated Product by Isolated Aleurone Particles of Rice.

The reaction mixture and the procedure for fractionation of myo-inositol phosphate were the same as those described in the legend of Table I, b), except for 50 μmoles orthophosphate which was replaced by 530 nmoles of 56μCi ^{32}P-orthophosphate, and 6.69 nmoles of 0.42 μCi ^{3}H-myo-inositol which was replaced by 50 μmoles of myo-inositol. The Ba-precipitatable fraction and 10 mg of myo-inositol monophosphate were charged onto a column of Bio-Rad AG 1×8 (Cl^-), and eluted by a linear gradient of NaCl as described in the text. ○, ^{32}P; ●, myo-inositol; P_i, orthophosphate; IP_1, myo-inositol monophosphate.

Distribution of phosphorylation activity

Figure 5 shows that phosphorylation activity was highest in the bran obtained at about 2% bran fraction, which roughly corresponded to the aleurone layer. A low phosphorylation activity of the bran at the beginning of the polish means that phosphorylation activity was not dependent on the germ adhering on the surface of the rice grains. This observation suggests that phosphorylation of myo-inositol proceeded in the aleurone layer.

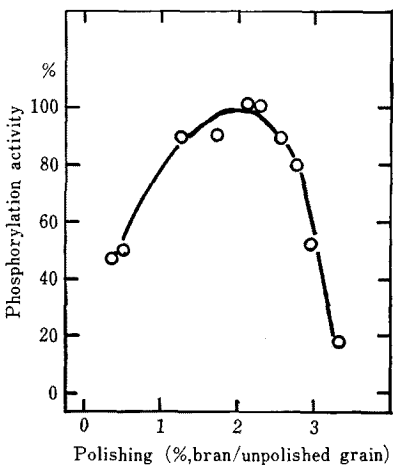

FIG. 5. Distribution of Phosphorylation Activity.

The polishing process was as shown in the previous paper.[5] The reaction was carried out for 30 min in accordance with the procedure described in the standard incubation, except that aleurone particles were replaced by rice bran. ^{32}P-ester formation was measured.

Requirement and properties of phosphorylation by aleurone particles

Phosphorylation of myo-inositol in aleurone particles required divalent cations. Addition of Mg^{2+}, Ca^{2+} or Mn^{2+} at 10 mM accelerated the phosphorylation about 3.5 times. ATP at 1 mM reinforced the phosphorylation of ^{3}H-myo-inositol twice (Fig. 6). Although requirements on metals and nucleotides for this phosphorylation system must be examined more accurately and in detail, as far as Fig. 6 is concerned, the addition of 1 mM ATP was nearly satisfactory for the phosphorylation of myo-inositol.

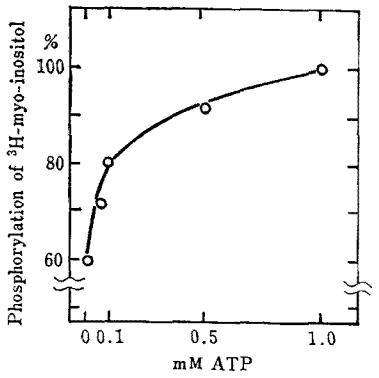

FIG. 6. Effect of ATP on the Phosphorylation of ^{3}H-Myo-inositol by Isolated Aleurone Particles of Rice.

The reaction was carried out for 30 min in accordance with the procedure described in the standard incubation using ^{3}H-myo-inositol, except for the addition of ATP as indicated.

The optimum pH and temperature for phosphorylation in aleurone particles were about 7.9 and 30°C, respectively (Figs. 7, 8). The phosphorylation activity was strongly depressed when the reaction was carried out at about 40°C. The association of phytase activity with this system had also been demonstrated.[10] However, the optimum pH and temperature for the hydrolytic reaction were 4.5 and 45°C, respectively. The optimum conditions for hydrolysis and phosphorylation were thus widely different. The maximum phosphorylation activity was obtained at

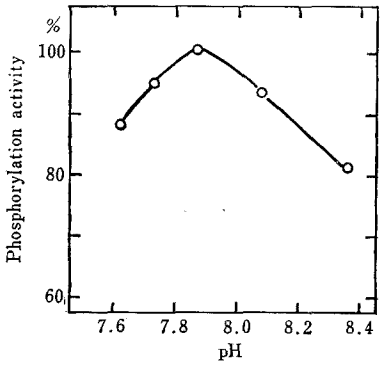

FIG. 7. Effect of pH on ^{32}P-Phosphate Ester Formation by Isolated Aleurone Particles.

The reaction was carried out for 30 min in accordance with the procedure described in the standard incubation, except that the pH of Tricine was changed as indicated.

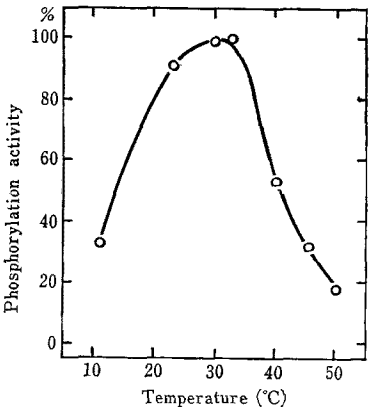

FIG. 8. Effect of Temperature on Phosphorylation Activity of Aleurone Particles.

The reaction was carried out for 30 min in accordance with the procedure described in the standard incubation, except for the temperature which was changed as indicated.

10 mM PO_4^-.

DISCUSSION

The mechanisms of phosphorylation of myo-inositol had been studied by many workers. Hoffman-Ostenhof first demonstrated the myo-inositol kinase in *E. coli*,[11] and Albersheim also isolated myo-inositol kinase from mung bean to form myo-inositol monophosphate.[12,13] Following them, Biswas *et al.* demonstrated a kinase of myo-inositol in mung bean seed to form highly phosphorylated myo-inositol phosphates, which finally led to phytic acid.[14]

In our previous study, we showed that when ^{32}P-phosphate was administered to ripening rice grains, ^{32}P appeared only in inositol monophosphate and phytic acid but not in any other inositol phosphate, such as inositol di-, tri-, tetra or penta-phosphate.[1] This observation induced the following conclusions; (i) Inositol di-, tri-, tetra-, and pentaphosphates are not the intermediates for the formation of phytic acid in the ripening rice grains or (ii) The lower phosphorylated inositols could not be detected because of their low contents and rapid turnover rate.

In spite of these works, which were concerned with the phosphorylation mechanism

of myo-inositol, no experiments intended to demonstrate the phosphorylation site of myo-inositol have been done. The highest phosphorylation activity observed in the 2% bran fraction in Fig. 5 suggested that the phosphorylation of myo-inositol might proceed in the aleurone layer but not in any inner part of the endosperm, including the subaleurone layer. Consequently, protein bodies are hardly considered the phosphorylation site of myoinositol. Actually, incorporation of ^{32}P-phosphate into myo-inositol phosphate and phosphorylation of ^{3}H-myo-inositol by isolated aleurone particles were demonstrated (Figs. 3, 4).

The nucleotide requirement for phosphorylation of myo-inositol by aleurone particle was not clear. As shown in Fig. 1, incorporation of ^{32}P-phosphate into myo-inositol phosphate fraction was observed without addition of nucleotides. However, for formation of ^{3}H-myo-inositol phosphate from ^{3}H-myo-inositol, addition of ATP proved effective (Fig. 7). To clarify the detailed mechanism of myo-inositol phosphate formation, purification of the enzyme, which catalyzes the phosphorylation of myo-inositol, is required.

The structure of myo-inositol monophosphate formed by isolated aleurone particle was not identified. However, in the previous works of our laboratory, only myo-inositol-2-monophosphate was detected, but no other myo-inositol monophosphate in the ripening rice grains.[7] A suggestion that myo-inositol-2-monophosphate could be the precursor of the formation of phytic acid was endorsed by administering ^{14}C-myo-inositol-2-monophosphate to ripening rice grain. On the other hand, Asada *et al.* showed that myo-inositol was formed from glucose,[15] and Kurasawa *et al.* demonstrated the occurrence of glucose-6-phosphate cyclase in ripening rice grains.[16] Both results imply the existence of myo-inositol-1-monophosphate, with the mechanism of myo-inositol formation from glucose taken into account.[17,18] At present, although myo-inositol-2-monophosphate and myo-inositol-1-monophosphate are highly probable candidates of the phosphorylated product, precise structure of myo-inositol monophosphate formed by isolated aleurone particles is not yet clear.

Formation of myo-inositol hexa-phosphate was not demonstrated by using either ^{32}P-orthophosphate or ^{3}H-myo-inositol as the precursors. The failure in the demonstration of myo-inositol hexa-phosphate formation might be explained by the following reasons. 1) Isolated aleurone particles were isolated from matured rice grains. Matured rice grains may have no ability to form phytic acid. If aleurone particles, isolated from ripening rice grains, had been used, incorporation of ^{32}P and ^{3}H-myo-inositol into phytic acid might have been observed. 2) Isolated aleurone particles were not intact as the phosphorylation system of myo-inositol. A phosphorylation activity, only for formation of myo-inositol monophosphate, was retained in the aleurone particles. In this case, the phosphorylation must be carried out with such aleurone particles isolated by another method as in aqueous media. 3) Phosphorylation of myo-inositol required an other system, such as ATP generation system. When phosphorylation by isolated aleurone particles is carried out, coupled with appropriate ATP generation system, formation of phytic acid may be observed. As discussed above, phosphorylation system of myo-inositol is incomplete with respect to the formation of phytic acid. How to complete the system is an important aspect of the future work.

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