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PHOSPHATIDYLSERINE-SENSITIVE CALCIUM DEPENDENT PROTEIN KINASE IN RICE EMBRYO

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Key Word Index—*Oryza sativa*; Gramineae; rice embryos; protein phosphorylation; protein kinase activity.

Abstract—Protein kinase activity in the embryos of rice (*Oryza sativa* L. cv. Nipponbare) during seed soaking in water was assessed under various conditions. This activity was increased by the addition of Ca^{2+} and phosphatidylserine (PS), but not by EGTA or Ca^{2+} alone. The potent phorbol ester did not augment this increase. The M_r of the protein kinases were 55 000 and 56 000. The phosphorylated proteins were separated by two-dimensional gel electrophoresis. Two proteins with M_r 40 000 and isoelectric points of 8.9 and 7.6 were significantly phosphorylated with Ca^{2+} and PS *in vitro*. They were first observed after 2 days of soaking, accumulated thereafter and disappeared after 4 days. When the membrane fraction was incubated in Ca^{2+} and PS, proteins in the fraction were phosphorylated. Phosphorylation of the proteins with Ca^{2+} and PS was inhibited by staurosporine and H-7, but not by W-7 or okadaic acid. The proteins were also found in wheat embryos, but wheat proteins were phosphorylated by Ca^{2+} alone. The present data suggest that activation of PS-sensitve Ca^{2+}-dependent protein kinase associated with membrane and subsequent protein phosphorylation may be involved in regulating the early stages of rice development.

INTRODUCTION

In higher plants, as in mammalian cells, calcium (Ca^{2+}) is important as a second messenger and may be involved in cell elongation and division, protoplasmic streaming, and hormone action [1–3]. In mammalian cells, two classes of Ca^{2+}-dependent protein kinases have been observed: Ca^{2+}/calmodulin-dependent protein kinase and Ca^{2+}/phospholipid-dependent protein kinase (PKC) [4–6]. Members of these two classes of protein kinases are not active in the presence of Ca^{2+} alone, but require effector molecules, either calmodulin or phospholipid, in addition to Ca^{2+} for activation [4–6]. In plants, Schafer *et al.* [7] demonstrated that a PKC-like enzyme can be partially purified from courgette. Subsequently, Olah and Kiss [8] reported the partial purification and characterization of a PKC-like enzyme from *in vitro* cultured wheat tissue and showed that several endogenous proteins can serve as substrates for this enzyme. Elliott and Skinner [9] suggest that an enzyme resembling PKC may be present in plants. It follows then that changes in Ca^{2+}, whether early or late in transduction, probably have a profound effect on cellular functions. However, in plant cells, no molecular mechanism of PKC-catalysed protein phosphorylation has been reported.

Protein phosphorylation occurs in plant cells, and phosphorylated proteins and protein kinases have been shown to be present in all cellular compartments of plants [10]. To elucidate the signal transduction mechanism in plant cells, we examined protein kinase-catalysed protein phosphorylation in rice embryos in relation to Ca^{2+}- and phospholipid-dependent protein kinase activity. It was shown by the present data that the activation of phosphatidylserine (PS)-sensitive Ca^{2+}-dependent protein kinase associated with membrane and subsequent protein phosphorylation may be involved in regulating the early stages of rice development.

RESULTS AND DISCUSSION

In embryos of rice seeds soaked in water, the substrates of protein kinases were shown to be present by phosphorylating protein under various conditions and they were subsequently separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Proteins from embryos could be clearly fractionated

12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), two proteins with M_r 40 000 and pI, 8.9 and 7.6 (40-k proteins) were phosphorylated *in vitro*, compared with the incubated cytosol fraction (Fig. 1I).

Calcium and PS affected protein phosphorylation (Fig. 2F), in contrast to EGTA, PS or Ca^{2+} alone (Fig. 2A, C or E). PS fulfils the lipid requirement for Ca^{2+}- and phospholipid-dependent protein kinase in mammalian cells. TPA containing Ca^{2+} and PS during protein phosphorylation failed to augment stimulation (Fig. 2G). TPA as the potent phorbol ester is a stimulator of Ca^{2+}- and phospholipid-dependent protein kinase in mammalian cells.

Protein kinase activity in the embryos of rice seeds soaked in water was assessed under various conditions. Protein kinase activity was determined based on ^{32}P-incorporation into the substrate, histone III-S. It was increased by the addition of Ca^{2+} and PS, but not by EGTA or Ca^{2+} alone. TPA caused no further increase. Protein kinase activity was mainly present in the membrane fraction (Fig. 3). When histone III-S was present in the reaction buffer, phosphorylation of the 40-k proteins was not stimulated by Ca^{2+} and PS.

Phosphorylation of the proteins with Ca^{2+} and PS was inhibited by 5 nM staurosporine (Fig. 4B) and 5 μM 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H-7) (Fig. 4C), but not by 5 or 50 μM *N*-(6-aminohexyl)-chloro-1-naphthalenesulphonamide hydrochloride (W-7) (Fig. 4D or E) or 0.1 μM okadaic acid (Fig. 4F). Staurosporine binds the catalytic domain of Ca^{2+}- and phospholipid-dependent protein kinase; thus H-7 is the most potent of all derivatives for inhibiting Ca^{2+}- and phospholipid-dependent protein kinase in mammalian cells and W-7 is specific to

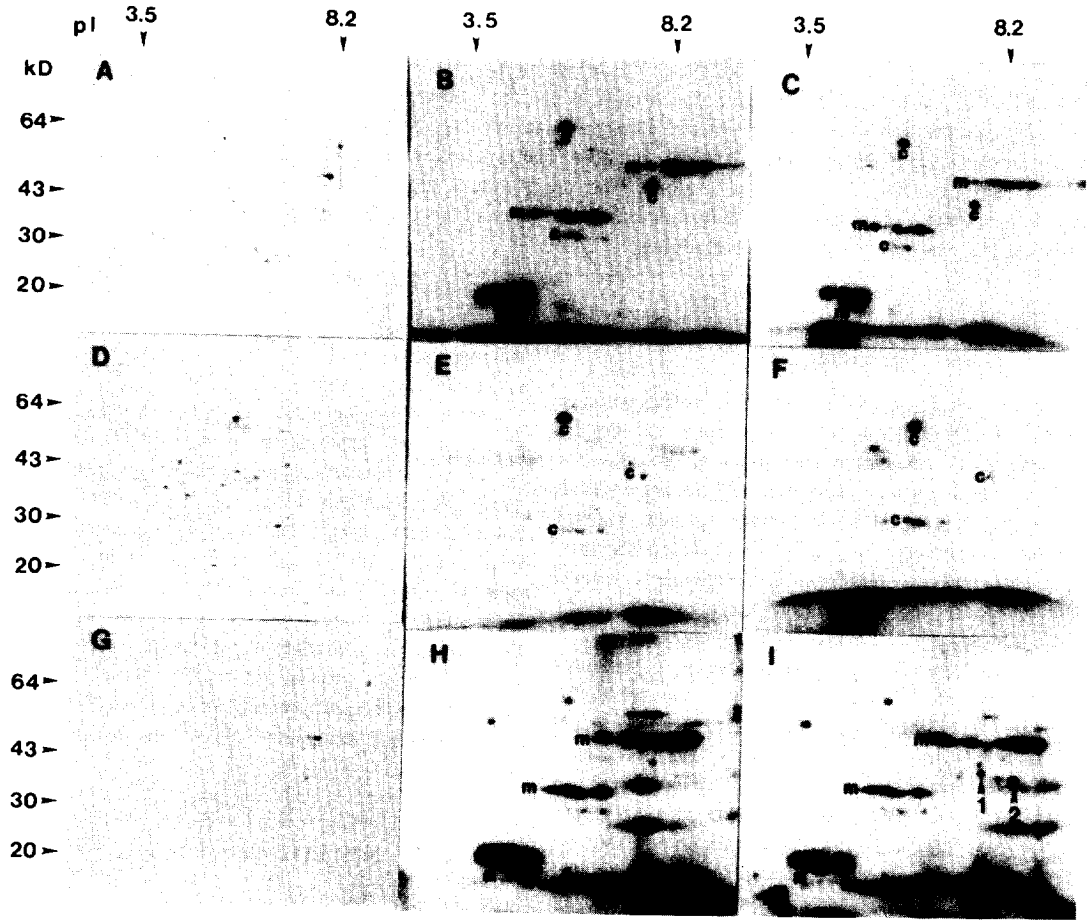


Fig. 1. Subcellular distribution of embryo protein phosphorylated *in vitro*. The samples were collected after soaking the seeds for 3 days in water. Rice embryos were homogenized and subcellular fractionation was performed as described by Komatsu and Hirano [13]. A, B and C: extract. D, E and F: cytosol fraction. G, H and I: membrane fraction. A, D and G: proteins detected with CBB staining. B, E and H: phosphoproteins reacted with EGTA. C, F and I: phosphoproteins reacted with Ca^{2+}/PS/TPA. Right to left: IEF for the first dimension. Top to bottom: SDS-PAGE for the second dimension.

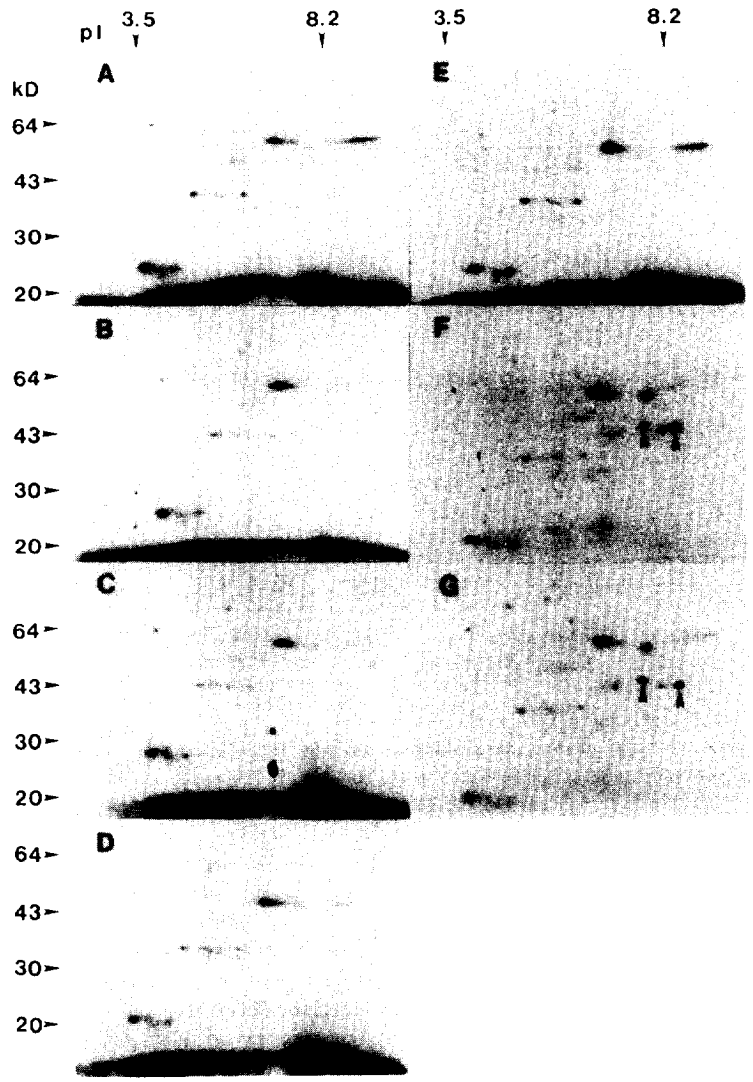


Fig. 2. *In vitro* phosphorylation under various conditions. Membrane fractions were collected after soaking the seeds for 3 days in water. *In vitro* phosphorylation was carried out in the presence of EGTA (A), water (B), PS (C), TPA (D), Ca^{2+} (E), Ca^{2+}/PS (F) and Ca^{2+}/PS/TPA (G).

calmodulin antagonists. Okadaic acid is a phosphatase inhibitor.

The presence of the substrates of protein kinases was confirmed by phosphorylating protein with Ca^{2+} and PS. Two proteins with *Mr* 40 000 and pI 8.9 and 7.6, were significantly phosphorylated *in vitro* (Fig. 2). The 40-k proteins in the membrane fraction were predominantly phosphorylated (Fig. 1). Protein kinase activity and protein phosphorylation in the membrane fraction were increased by Ca^{2+} and PS (Fig. 3). Their phosphorylation with Ca^{2+} and PS was inhibited by staurosporine and H-7, but not W-7, a specific calmodulin antagonist, or okadaic acid, a phosphatase inhibitor (Fig. 4).

peak at 0.1–0.3 M NaCl showed high protein kinase activity. The samples were separated by SDS–polyacrylamide gel containing histone III-S. The gel was incubated with [γ-^{32}P]ATP, Ca^{2+} and PS. In the absence of Ca^{2+}, these protein kinase fractions did not phosphorylate histone III-S. However, in the presence of Ca^{2+} and PS, two bands with M_r 55 000 and 56 000 (Fig. 6) were observed.

Ca^{2+} and PS were affected protein kinase activity, compared with EGTA or Ca^{2+} alone. PS provides the lipid requirement for Ca^{2+}- and phospholipid-dependent protein kinase in mammalian cells. TPA processing Ca^{2+} and PS in the protein kinase assay failed to enhance the stimulation of protein kinase with Ca^{2+}

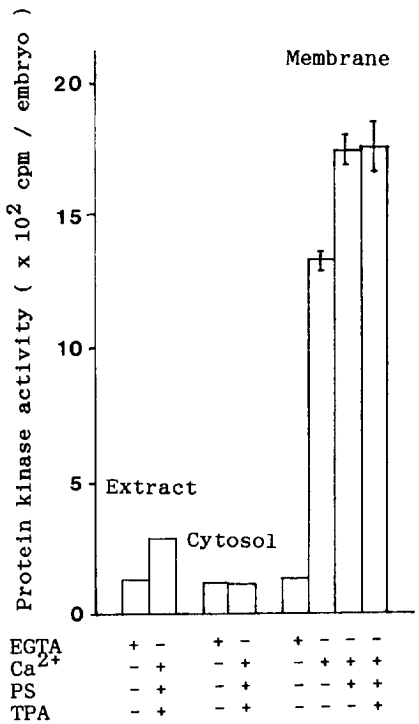


Fig. 3. Protein kinase activity of rice embryos after soaking the seeds for 3 days in water. Rice embryos were homogenized and subcellular fraction was performed as described by Komatsu and Hirano [13]. The results are the mean values of triplicate assays. The bars represent SE.

mammalian cells. Rice protein kinase activity was associated with the membrane fraction (Fig. 3). Ca^{2+}- and phospholipid-dependent protein kinase in rice has biochemical properties that differ from those of Ca^{2+}- and phospholipid-dependent protein kinase associated with the cytosol in mammalian cells [6]. Ca^{2+}-dependent protein kinase was stimulated at lower Ca^{2+} concentration (2 μM) but not by PS [11]. A protein kinase associated with the plasma membrane in oats has been shown to express maximum activity in the present of 100 μM Ca^{2+} and lipid [12]. The protein kinase from oat was associated with plasma membranes and stimulated by some specific lipids but not by PS [12]. The protein kinases from rice described in this paper (Fig. 5) were stimulated by PS, which implies that they differ from the oat protein kinase. Protein kinases with M_r 55 000 and 56 000 (Fig. 6) may be present in rice embryos.

Phosphorylation of the 40-k proteins, as in rice with M_r and pI, also occurred in the membrane fraction of wheat embryos. However, in wheat embryos, protein with M_r 40 000 and pI 8.9 was phosphorylated by Ca^{2+} alone (Fig. 7, B). No 40 k-proteins, as in the case of rice, were observed in soybean, barley or maize.

Phosphorylated proteins and protein kinases have phytohormones or other signals. We described here the characterization of protein kinase in the early stages of development of rice and the roles of several endogenous proteins as substrates for this enzyme. The 40-k proteins are present in wheat embryos (Fig. 7). However, PS does not stimulate the wheat kinase. The Ca^{2+}/PS stimulation may be an unusual feature of rice. The present data support that the activation of PS-sensitive Ca^{2+}-dependent protein kinase associated with the membrane and subsequent protein phosphorylation are involved in regulation of the early stages of development in rice and wheat. PS-sensitive Ca^{2+}-dependent protein kinase in rice may have biochemical properties unlike those of Ca^{2+}- and phospholipid-dependent protein kinases in mammalian cells.

EXPERIMENTAL

Plant material. Rice (*Oryza sativa* L.) cv. Nipponbare were soaked in water and embryos were isolated from the seeds.

Chemicals. [γ-^{32}P]ATP was purchased from Amersham. TPA, PS, EDTA and EGTA were from Sigma. H-7 and W-7 were from Seikagaku Kogyo (Tokyo, Japan).

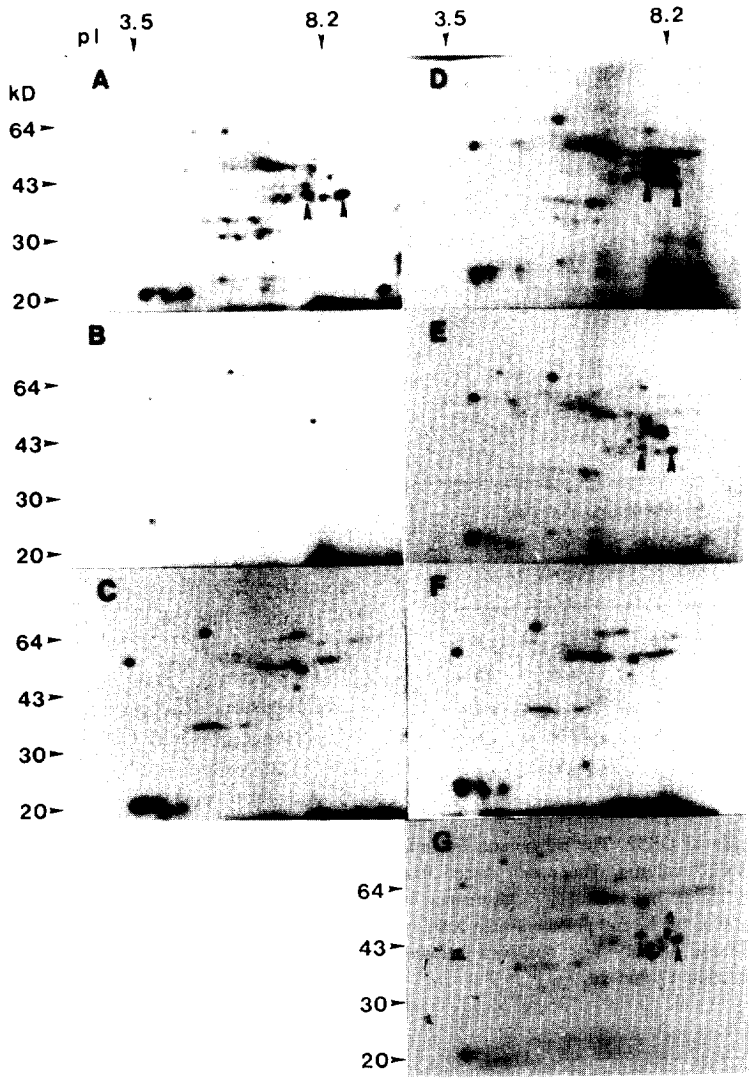
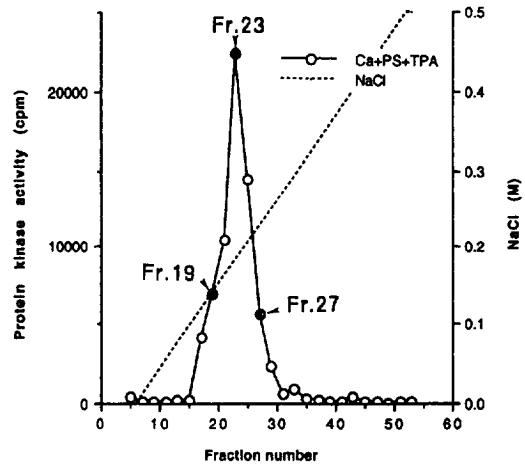


Fig. 4. Inhibition of PS-sensitive Ca^{2+}-dependent protein phosphorylation. Membrane fractions were collected after soaking the seeds for 3 days in water. *In vitro* phosphorylation was carried out in the presence of Ca^{2+}/PS (A), Ca^{2+}/PS/5 nM staurosporine (B), Ca^{2+}/PS/5 μM H-7 (C), Ca^{2+}/PS/5 μM W-7 (D), Ca^{2+}/PS/50 μM W-7 (E) and 0.1 μM okadaic acid (F).

of the embryos was removed and homogenized with 1 ml extraction buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Na deoxycholate, 1 mM EDTA, 5 μM Na vanadate and 1 mM PMSF. The homogenate was centrifuged at 15 000 *g* for 5 min and the supernatant served as the rice-embryo extract.

Preparation of the subcellular fraction. Rice embryos (250 mg) were suspended in 1 ml homogenization buffer containing 20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 10 mM EGTA, 1 mM PMSF and 1 mM DTT. The homogenates were centrifuged at 600 *g* for 5 min. The supernatants were centrifuged at 356 000 *g* for cytosol fraction was obtained by collecting the supernatant. The pellet was resuspended in 500 μl homogenization buffer and washed by centrifugation at 356 000 *g* for 15 min. After removing the supernatant, the pellet was resuspended in 50 μl membrane-solubilizing buffer containing 1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and 50 mM 2-mercaptoethanol, and solubilized for 30 min on ice. The membrane fr. was obtained from the supernatant after centrifugation at 356 000 *g* for 7 min [13].

Gel electrophoresis. The sample was added into a lysis buffer containing 8 M urea, 2% Triton X-100, 2% Ampholine and 10% PVP-40, and subjected to 2D-



Fraction number	Protein kinase activity (nmol PI/mg protein ± S.E.) Fr. 19	Fr. 23	Fr. 27
EGTA (4mM)	2.0±0.8	1.8±0.1	1.4±0.2
none	0.9±0.0	7.4±3.5	0.9±0.1
Ca^{2+} (0.2mM)	16.0±0.5	68.0±7.4	27.6±0.4
Ca^{2+}/PS (25 μg ml^{-1})	40.0±0.9	116.0±8.9	35.4±7.1
Ca^{2+}/PS/TPA (4 μg ml^{-1})	35.3±1.6	104.0±5.4	50.8±6.5

Fig. 5. Separation profile of DE52. The membrane fractions were applied onto a DE52 column. The rough dotted line, dotted line and open circle indicate A_{280} (absorbance at 280 nm), NaCl gradient 0 to 0.5 M and protein kinase activity, respectively. Protein kinase activity was observed between fraction 17 and 29.

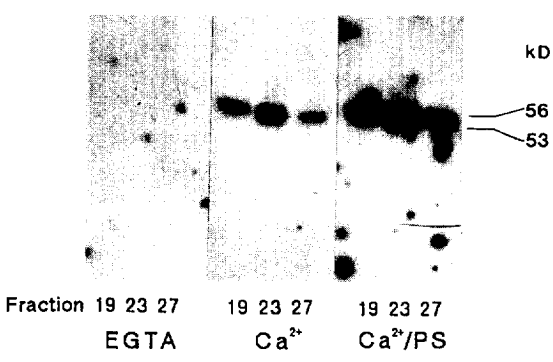


Fig. 6. Phosphorylation in SDS-polyacrylamide gel containing histone III-S. The membrane fractions were separated using a

250 (CBB) or silver staining. The pI and M_r of the proteins sepd were determined using pI marker proteins and M_r marker proteins (Pharmacia).

In vitro *phosphorylation of endogenous protein by protein kinase. In vitro* phosphorylation assay was carried out in a reaction mixt. (40 μl) containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 25 μg ml^{-1} PS, 4 μg ml^{-1} TPA, 39 μM [γ-^{32}P]ATP (0.44 TBq mmol^{-1}) and 5 μl of rice sample [14]. The basal level of phosphorylation was measured in the presence of 4 mM EGTA instead of $CaCl_2$, PS or TPA. The reaction mixt. was incubated for 10 min at 30° and terminated by the addition of lysis buffer. After *in vitro* phosphorylation, the proteins were sepd by 2D-PAGE and stained with CBB. The gel was destained, dried and exposed to autoradiography on X-ray film (Kodak) at −80° for 2 days.

Assay of protein kinase activity. Protein kinase activity was assayed in a reaction mixt. of 100 μl containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.4 μg ml^{-1} histone III-S, 0.2 mM $CaCl_2$, 25 μg ml^{-1} PS, 4 μg ml^{-1} TPA, 6 μM [γ-^{32}P]ATP (0.11 TBq mmol^{-1}) and 5 μl of sample [14]. The basal level of histone III-S phosphorylation was measured in the presence of 4 mM EGTA instead of $CaCl_2$, PS and TPA. The reaction mixt. was incubated for 10 min at 30° and terminated by cooling to 0°. A portion (75 μl) of reaction mixt. was applied onto a P-81 filter (Whatman). The filters were washed with H_2O, then EtOH and dried. Radioactivity was determined by Cherenkov counting.

DE-52 fraction. The membrane frs were applied onto a DE52 column (30 ml) equilibrated with elution buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 1 mM DTT and 10% glycerol. The enzyme was eluted from the column by a linear gradient of 0–0.5 M NaCl elution buffer. All frs were assayed for protein kinase activity and the active frs were pooled.

Detection of protein kinase in SDS-polyacrylamide gel containing histone III-S. The samples were sepd by 15% SDS-PAGE containing 2 mg ml^{-1} histone III-S, as substrate, in a separating gel. After electrophoresis, the gel was incubated with 20% 2-PrOH/50 mM Tris-HCl (pH 8) for 60 min and washed with buffer A containing 5 mM 2-mercaptoethanol/50 mM Tris-HCl (pH 8) for 60 min. The gel was incubated in buffer A containing 6 M guanidine hydrochloride for 60 min and washed with buffer A containing 0.05% Tween 40 for 12 hr. The gel was then incubated with buffer B containing 40 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 2 mM DTT and 4 mM EGTA or 0.2 mM $CaCl_2$ and PS for 30 min at 30°. Reaction was initiated by 50 μM [γ-^{32}P]ATP (3.7 MBq) and the gel was incubated for 60 min at 30°. The gel was washed with 5%(W/V) TCA/1%(W/V) NaPi soln until background radioactivity decreased

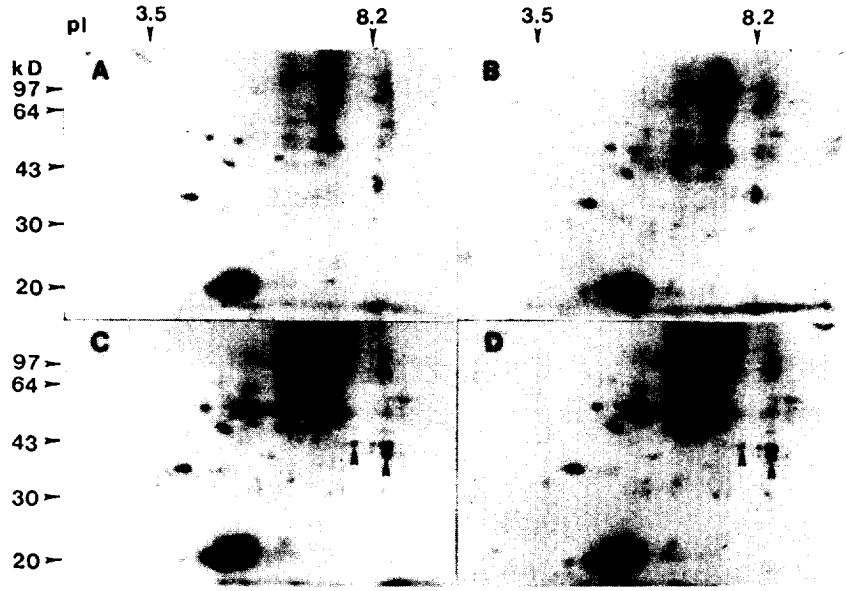


Fig. 7. *In vitro* phosphorylation in the membrane fraction of wheat embryo after soaking the seeds for 3 days in water. *In vitro* phosphorylation was carried out in the presence of EGTA (A), Ca^{2+} (B), Ca^{2+}/PS (C) and Ca^{2+}/PS/TPA (D).

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