# Rice gibberellin-binding phosphoprotein structurally related to ribulose-1,5-bisphosphate carboxylase/oxygenase activase

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**Abstract A gibberellin A (GA)-binding protein was identified from rice (*Oryza sativa* L.) leaves by a ligand-binding assay. The dissociation constant of GA-binding protein and GA complex was about 100 nM. This protein has a relative molecular mass of 47 000 and an isoelectric point of 5.1. The partial amino acid sequence of the protein was determined for 54 residues from both the N-terminal and internal regions. A sequence homology search indicated that the amino acid sequence of GA-binding protein was homologous to that of the ribulose-1,5-bisphosphate carboxylase/oxygenase activase from barley, *Arabidopsis*, spinach and *Chlamydomonas*. The GA-binding protein was immunologically detected in two polypeptides in the protein extract from leaves. The GA-binding protein identified was phosphorylated with $Ca^{2+}$, $Mg^{2+}$ and ATP in the leaf protein extracts of rice grown in the presence of exogenous GA.**

*Key words:* Gibberellin-binding protein; Protein phosphorylation; Rice leaf; Ribulose-1,5-bisphosphate carboxylase/oxygenase activase

## 1. Introduction

Gibberellin (GA) is a class of plant hormones which regulates growth and development, including stem elongation, flowering and seed germination. The cellular responses to GAs are thought to be mediated by GA receptors. A few GA-binding proteins have been identified as candidates for GA receptors by using a variety of techniques. For instance, Hooley et al. [1] found a soluble 50 kDa GA-binding protein in *Avena fatua* aleurone by $GA_4$ photoaffinity labeling. Keith and Rappart [2] reported that at least two high molecular weight proteins in the cytosol of the leaf sheath of maize bind to $GA_1$. Nakajima et al. [3] partially purified a soluble binding protein specific for $GA_4$, $GA_7$ and $GA_9$ from mung bean hypocotyls. However, the function of these GA-binding proteins remains to be resolved.

Murthy and Render [4] showed that phosphoinositides were involved in $GA_3$-induced synthesis and secretion of α-amylase in the aleurone cells of barley seeds. Ladedgenskaya et al. [5] showed that $GA_3$ increased activity of $Ca^{2+}$- and phospholipid-dependent protein kinase-like enzyme in potato tubers. These reports suggest that the GA signal transduction system may involve protein phosphorylation through protein kinases.

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*Abbreviations:* GA, gibberellin; PVDF, polyvinylidene difluoride; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis.

In this study, we tried to detect plant GA-binding proteins by a ligand-binding assay, and identified a protein in rice leaves which was capable of binding to GA. The amino acid sequence was partially determined and a sequence homology search indicated that the amino acid sequence of the GA-binding protein was homologous to that of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activase from barley [6], *Arabidopsis* [7], spinach [8] and *Chlamydomonas* [9]. We found that the GA-binding protein identified was phosphorylated with $Ca^{2+}$, $Mg^{2+}$ and ATP.

## 2. Materials and methods

### 2.1. Plant materials

Rice (*Oryza sativa* L.) cv. Nipponbare was used in this study. Rice plants were grown in vessels containing commercial soils blended with chemical fertilizer and cultivated in an air-controlled greenhouse under natural light. The developing leaves from field-grown plants were collected.

### 2.2. Chemicals

[1,2(*n*)-$^3$H]$GA_1$ was purchased from Amersham, Buckinghamshire. $GA_1$ was a gift of Dr. M. Koshioka, and $GA_3$, $GA_4$, 3-indoleacetic acid, abscisic acid and digoxigenin were purchased from Wako, Osaka. Other reagents used were analytical grade.

### 2.3. Preparation of protein extract

A portion (250 mg) of the 3rd leaves was removed and homogenized with 1 ml of extraction buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, 5 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 15 000 rpm for 5 min in a TMA-4 rotor (Tomy, Tokyo). The supernatant was used as rice leaf extracts.

### 2.4. Detection of hormone-binding proteins and competition binding assay

Proteins capable of binding to [$^3$H]$GA_1$ were detected by a ligand-binding assay as described in [10]. GA-binding protein was separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [11] and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. After transfer of proteins to the PVDF membrane, the membrane was dried at 37°C for 5 min. It was then soaked at 4°C, first, for 30 min in saline (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.5 mg/ml sodium azide) supplemented with 3% Nonidet P-40; second, for 2 h in saline containing 1% bovine serum albumin (BSA); and finally, for 10 min in saline containing 0.1% Tween 20. The PVDF membrane was then sealed in a plastic bag with 400 000 cpm [$^3$H]$GA_1$ with 3 ml saline containing 1% BSA and 0.1% Tween 20. The PVDF membrane was incubated overnight at 4°C. The PVDF membrane was washed twice for 15 min at 4°C in saline containing 0.1% Tween 20, and then 3 times for 15 min in saline. The blot was dried and exposed for 2 weeks at −70°C to X-ray film.

GA-binding protein (200 pmol) electroblotted onto the PVDF membrane was incubated for 30 min at 4°C with increasing concentrations of unlabeled $GA_1$, $GA_3$, $GA_4$, 3-indoleacetic acid, abscisic acid or digoxigenin. Then the amount of [$^3$H]$GA_1$ bound to protein was measured and the dissociation constant was calculated as described in [12].


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*2.5. Isolation of a protein capable of binding to gibberellin*

Rice GA-binding protein was separated by 2D-PAGE and electro-eluted. After electroelution, the protein solution was dialyzed against deionized water for 2 days and dried. The collected protein was applied to a reverse-phase column (Wakosil-II 5C18 AR, 4.6×250 mm) for reverse-phase HPLC. The separation was made using a linear acetonitrile gradient (10–60%, 40 min) at 4.0 ml/min. The gradient was created by mixing 1.0% aqueous trifluoroacetic acid and acetonitrile containing 0.1% trifluoroacetic acid. The protein of interest was eluted at an acetonitrile concentration of about 50%.

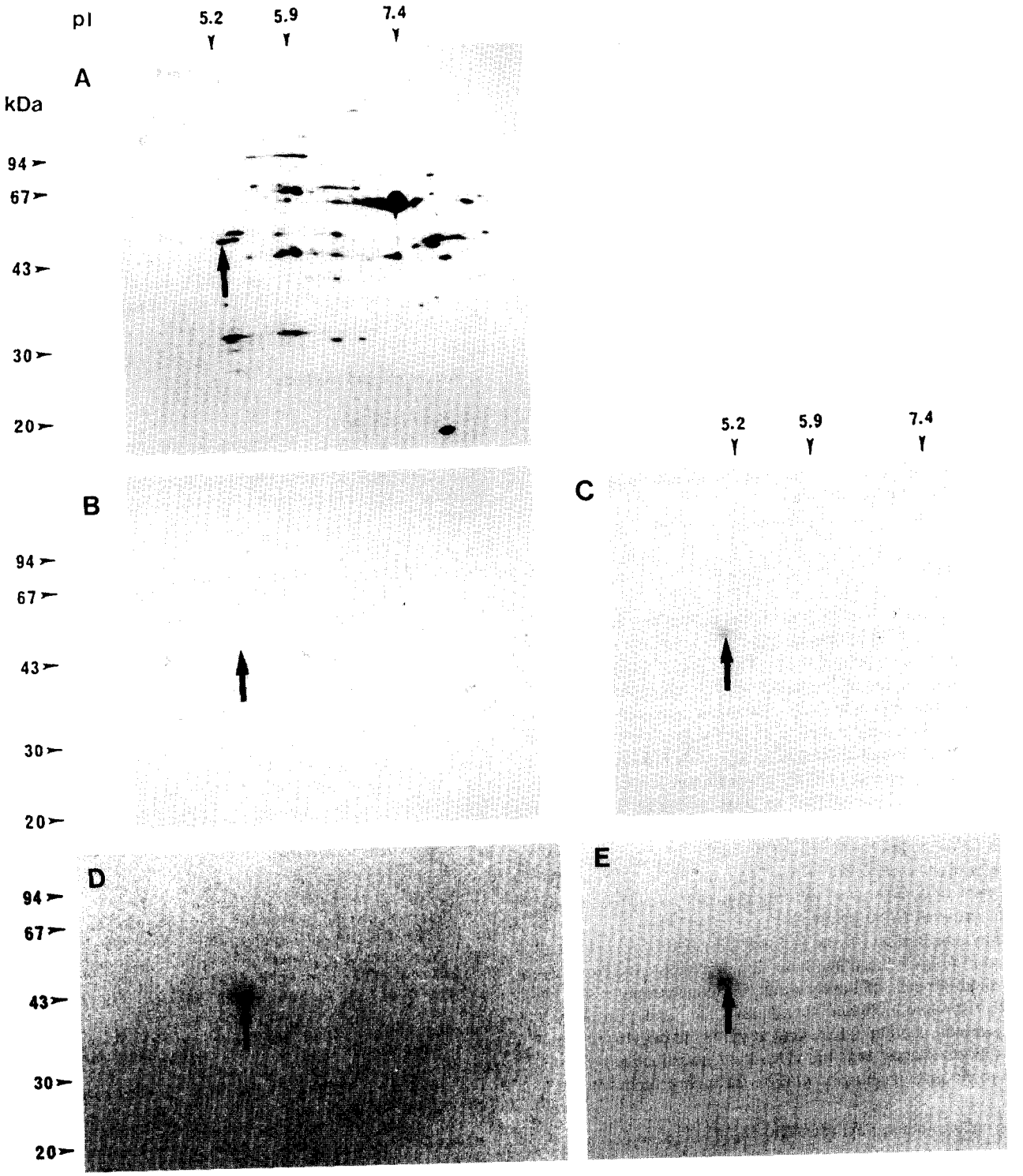


Fig. 1. GA-binding protein shown in the 2D-PAGE pattern of the rice leaf extract. Western ligand blotting was performed as described in section 2. Right to left: isoelectric focusing for first dimension, top to bottom: SDS-PAGE for second dimension. (A) Proteins detected with silver staining; (B) protein detected with [$^3$H]$GA_1$ containing unlabeled $GA_1$; (C) protein detected with [$^3$H]$GA_1$; (D) protein detected with [$^3$H]$GA_1$ containing unlabeled 3-indoleacetic acid; (E) protein detected with [$^3$H]$GA_1$ containing unlabeled digoxigenin. Arrow indicates the position of the GA-binding protein which has a relative molecular mass of 47000 and an isoelectric point of 5.1.

*2.6. N-terminal and internal amino acid sequence analysis*

The collected protein was electroblotted onto the PVDF membrane and subjected to amino acid sequence analysis [13]. The internal amino acid sequences of the protein were determined as described in [14]. The amino acid sequence analysis of proteins on the PVDF membrane was performed by a gas-phase protein sequencer (477A, Applied Biosystems, CA) [15].

*2.7. Preparation of polyclonal antibody and immunoblot*

Using collected protein, an antibody against the GA-binding protein was raised in adult rabbit [16]. Proteins extracted were separated by 2D-PAGE and electroblotted onto a PVDF membrane. The blotted spots which cross-reacted with antibodies raised against GA-binding protein, were detected by peroxidase enzyme immunoassay [17].

*2.8. In vitro phosphorylation assay*

In vitro phosphorylation assay was carried out on the reaction mixture of 40 μl containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 39 μM [$\gamma$-$^{32}$P]ATP (0.44 TBq/nmol) and 5 μl of rice sample [18]. The basal level of phosphorylation was measured in the presence of 4 mM EGTA instead of $CaCl_2$. The reaction mixture was incubated for 10 min at 30°C and terminated by the addition of lysis buffer. After in vitro phosphorylation, the proteins were separated by 2D-PAGE and stained with CBB. The gel was destained, dried and exposed to autoradiography on X-ray film at −80°C for 3 days.

## 3. Results and discussion

Proteins extracted from endosperm, embryo, callus, root or leaf of rice were separated by 2D-PAGE and electroblotted from the gels onto a PVDF membrane, and the blotted proteins were exposed to [$^3$H]$GA_1$. Only one protein with a relative molecular mass of 47 000 and an isoelectric point of 5.1 (Fig. 1A) from the rice leaves was found to bind with [$^3$H]$GA_1$ (Fig. 1C).

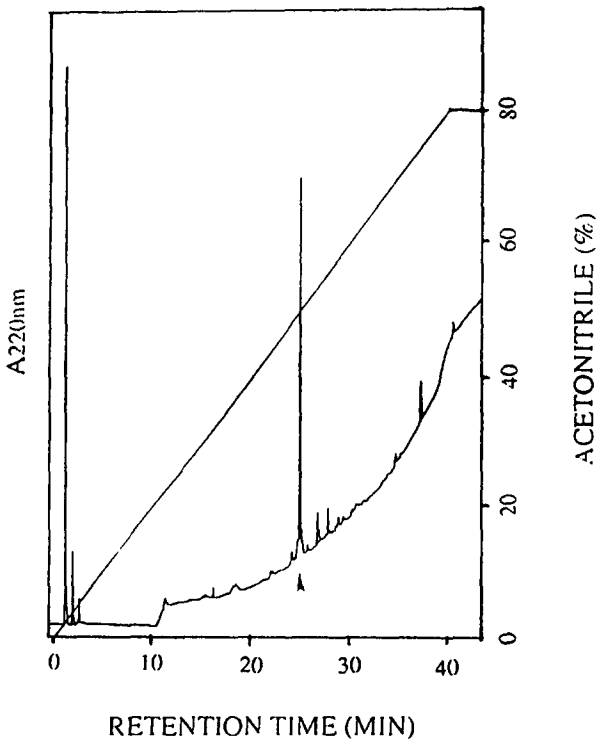


Fig. 2. HPLC profile of GA-binding protein. Arrowhead indicates a peak corresponding to the GA-binding protein. The protein was chromatographed on a reverse-phase column in a solvent system of 0.1% trifluoroacetic acid in acetonitrile. The flow rate was 1.0 ml/min.

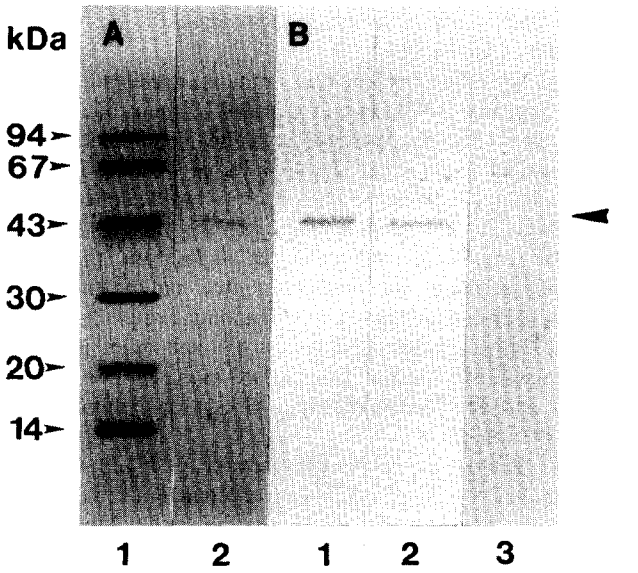


Fig. 3. (A) SDS-PAGE analysis of the purified GA-binding protein and (B) binding of [$^3$H]$GA_1$ to the GA-binding protein in the presence or absence of $GA_1$ probed by ligand blotting. (A) Lane 1, molecular marker proteins; lane 2, purified GA-binding protein. (B) Lane 1, no $GA_1$ added to the incubation buffer; lane 2, $GA_1$ is present at a concentration of 0.1 μM; lane 3, $GA_1$ is present at a concentration of 10 μM. Arrowhead indicates the position of the blotted GA-binding protein.

To examine the specificity of the binding of this protein to GA, the contribution of nonspecific binding with [$^3$H]$GA_1$ was measured in the presence of excess unlabeled $GA_1$, $GA_3$, $GA_4$, 3-indoleacetic acid or abscisic acid to saturate specific binding sites. Nonspecific binding was not detected on the PVDF membrane (Fig. 1B) in the presence of excess unlabeled $GA_1$, $GA_3$ or $GA_4$. This GA-binding was not inhibited by other plant hormones, which are 3-indoleacetic acid or abscisic acid (Fig. 1D), and polycyclic hydrophobic compound, which is digoxigenin (Fig. 1E). The collected protein was separated by reverse-phase HPLC. The main peak corresponded to GA-binding protein (Fig. 2). The preliminary competitive binding assay showed that the dissociation constant of GA and GA-binding protein complex was about 100 nM (Fig. 3).

Rice leaf proteins were separated by 2D-PAGE, and the GA-binding protein was electroeluted from the gel. The GA-binding protein was collected by HPLC and re-electrophoresed on a SDS-gel, electroblotted onto the PVDF membrane and directly sequenced by Edman degradation in a gas-phase sequencer. The sequence of 22 residues from the N-terminus of GA-binding protein was determined (Fig. 4). After electroelution from the 2D-PAGE gels, the GA-binding protein was digested with *S. aureus* V8 protease on the SDS-PAGE gel and electroblotted from the gel onto the PVDF membrane to determine the internal amino acid sequences. Seven major fragments of the GA-binding protein were obtained: two from the N-terminal region and the others from the internal regions of the GA-binding protein. The amino acid sequence for 54 residues was determined in this experiment (Fig. 4). A sequence homology search indicated that the amino acid sequence of the GA-binding protein was homologous to that of the RuBisCO activase from barley [6], *Arabidopsis* [7], spinach [8] or *Chlamydomonas* [9]. RuBisCO acti-

vase is one of the enzymes that catalyzes the activation of RuBisCO in vivo [19].

The GA-binding protein was immunologically detected in two polypeptides (47 kDa and 43 kDa) in the protein extract from leaves (Fig. 5). Werneke et al. [6] reported that antibodies eluted from either the 45 kDa or 41 kDa RuBisCO activase in spinach reacted with both polypeptides on an immunoblot. The two polypeptides may be derived from the same, or very similar molecules. In *Arabidopsis* and barley, RuBisCO activase also comprises two polypeptides [7,8]. This result indicates that the GA-binding protein is structurally similar to RuBisCO activase. On the other hand, the physiological significance of the two polypeptides was not clear. It was observed that only a 47 kDa polypeptide bound to GA in rice leaves.

When rice was grown in the presence of 5 μM $GA_1$, the addition of $Ca^{2+}$, $Mg^{2+}$, ATP into the reaction mixture stimulated phosphorylation of a 47 kDa protein showing the same electrophoretic mobility as the GA-binding protein in 2D-PAGE (Fig. 6C), while the stimulative effect was not observed with the addition of EGTA (Fig. 6D). Simultaneously, the PVDF membrane was immunodecorated with the anti-GA-binding protein antibody. On the PVDF membrane, the phosphorylated 47 kDa protein cross-reacted with the antiserum raised against the GA-binding protein (Fig. 6E). These experiments confirmed that the 47 kDa protein is identical to the GA-binding protein.

Activation involves binding of an activator $CO_2$, followed by $Mg^{2+}$, on the ε-amino group of a Lys residue on the RuBisCO large subunit near the active site [19]. Although this process can occur spontaneously in vitro, RuBisCO activation in vivo is catalyzed by RuBisCO activase [20] in an ATP-dependent reaction [21]. Activation of the GA-binding

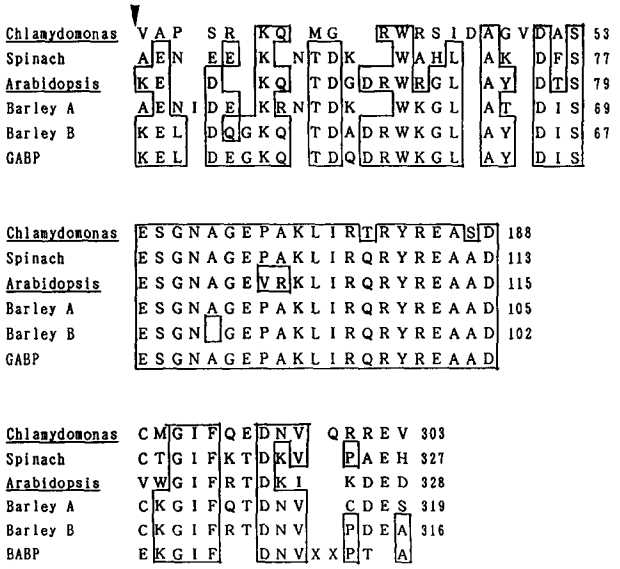


Fig. 4. Amino acid sequence comparisons of RuBisCO activase polypeptides from *Chlamydomonas*, spinach, *Arabidopsis*, barley (A: RcaA and B: RcaB) and GABP (GA-binding protein). Boxes indicate amino acids identical to those of GA-binding protein. Spaces between parentheses represent arbitrary deletions, which were added for optimal alignment. Arrowhead indicates the putative site of transit peptide cleavage. Numbers on the right indicate the amino acid residue number of each polypeptide. *Chlamydomonas*: Roesler and Ogren [9]. Spinach: Werneke et al. [8]. *Arabidopsis*: Werneke and Ogren [7]. Barley: Rundle and Xielinski [6].

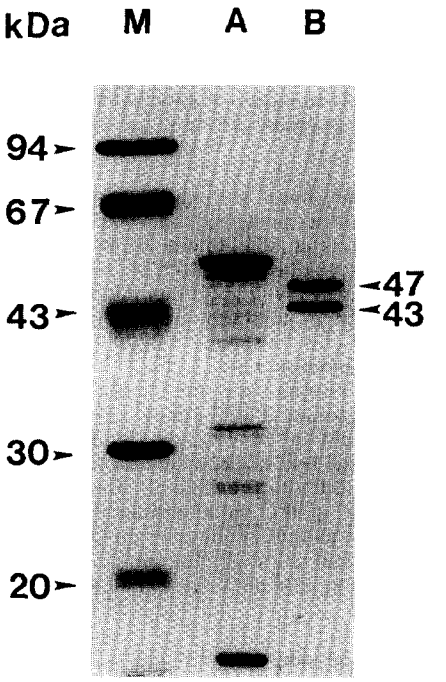


Fig. 5. Western immunoblot detection of GA-binding protein in rice protein extracts. Proteins isolated from 10-day-old rice seedlings were subjected to SDS-PAGE. Gels were either stained with Coomassie blue (A) or blotted onto PVDF membrane and probed with anti-GA-binding protein antibodies (B). Polypeptide sizes are indicated in kDa.

protein, which is RuBisCO activase, may be obtained in the presence of $Ca^{2+}$, $Mg^{2+}$ and ATP.

Protein phosphorylation is a widespread phenomenon and phosphorylated proteins and protein kinases have been detected in all compartments of plant cells [22]. The data obtained in this experiment suggest that regulation of the GA-binding protein in rice leaf, which is RuBisCO activase, may be caused by activation of protein kinase and subsequent phosphorylation of the GA-binding protein.

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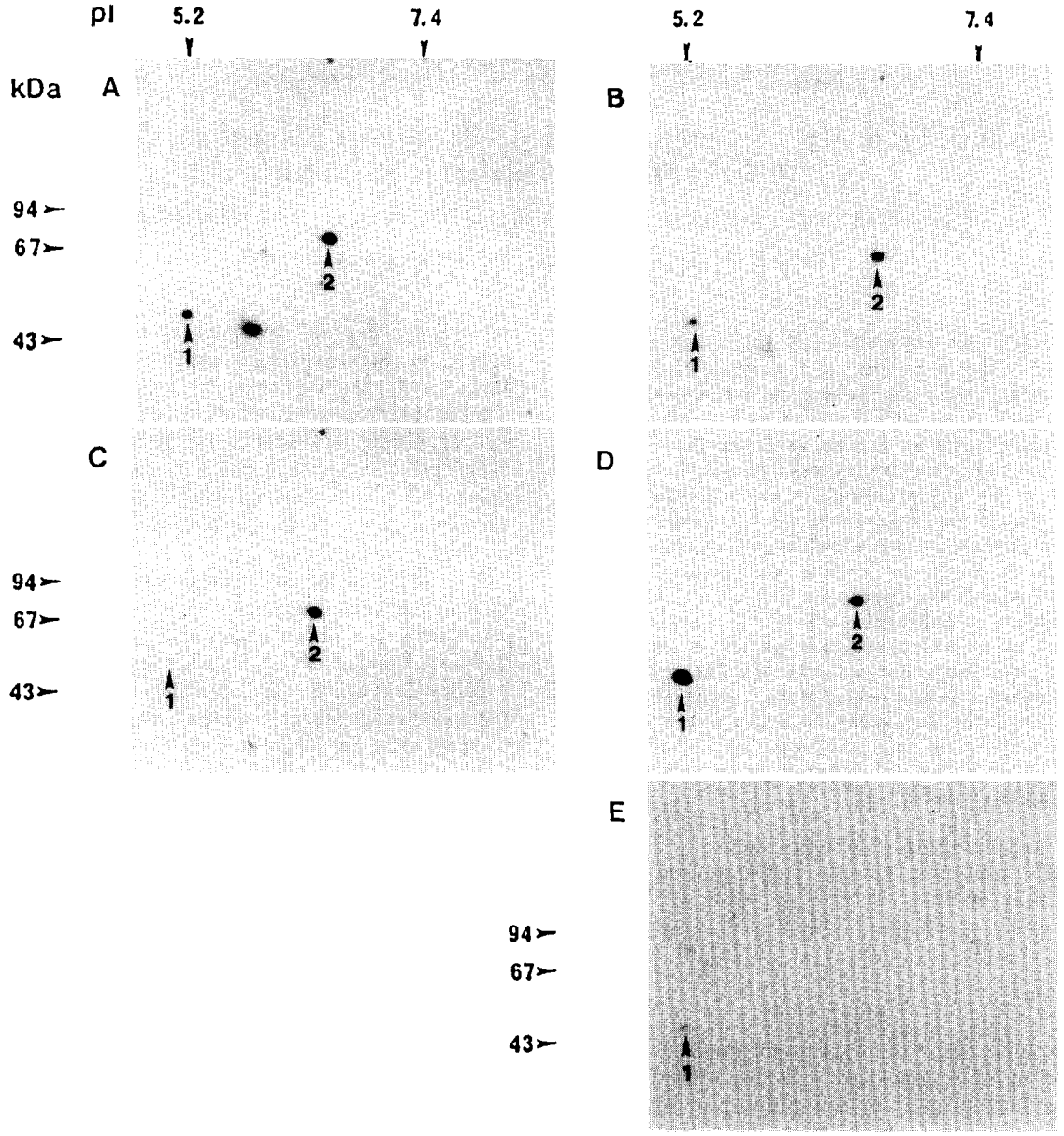


Fig. 6. 2D-PAGE pattern of in vitro labeled phosphoproteins in the rice leaf extract. Rice plants were grown in the presence of 5 μM $GA_1$ (C and D) or without 5 μM $GA_1$ (A and B). Rice leaf extracts were treated with EGTA (A and C), and $Ca^{2+}/Mg^{2+}$ (B and D). E shows the immunoreactive protein with anti-GA-binding protein antibody. Right to left: isoelectric focusing for first dimension, top to bottom: SDS-PAGE for second dimension. Numbers in the figure show positions of proteins having a relative molecular mass of 47000 and an isoelectric point of 5.1 (1) and 73000 and 6.0 (2).

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