Pergamon

PII: S0031-9422(97)00163-5

Phytochemistry, Vol. 45, No. 7, pp. 1329–1335, 1997

0031-9422/97 $17.00+0.00

VARIETAL DIFFERENCES IN PROTEIN PHOSPHORYLATION DURING COLD TREATMENT OF RICE LEAVES

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(*Received in revised form* 2 *January* 1997)

Key Word Index—Low temperature; rice (*Oryza sativa* L.); protein phosphorylation.

Abstract—The effects of cold stress on protein phosphorylation in leaves of rice (*Oryza sativa*) seedlings were investigated. Two-week-old rice seedlings were exposed to low temperature (5°) for 6 hr at a relative humidity of 100%. The leaf protein extracts were phosphorylated *in vitro*. Cold stress stimulated the phosphorylation of a 60 kDa protein in the cold-sensitive rice variety, IR36. In the cold-tolerant rice variety, Kitaibuki, this protein had already been phosphorylated. For greater clarificating of these changes, the effects of cold on rice varieties differing in cold susceptibility were examined. Cold-sensitive rice varieties showed similar the protein phosphorylation in contrast to the cold-tolerant rice varieties.

INTRODUCTION

Crop plants in tropical and sub-tropical regions are seriously injured by temperatures below 12° but above the freezing point [1, 2]. A primary, if not exclusive, effect of chilling is considered to be the phase transition of membrane lipids at critical temperatures [1, 3].

Sterols, which inhibit the phase transition of lipids [4], reduce chilling injury to rice seedlings [5]. Therefore, the above hypothesis could be applicable to chilling injury also in rice plants. However, it has been reported that, in rice plants, growth rate and metabolism are markedly inhibited already at temperatures above the chilling temperature in the range of 15–20° [6, 7], but the mechanism for the effects of low-temperature stress on growth and the accompanying metabolic changes is unclear. It is generally recognized that patterns of protein synthesis and mRNA level change when plants are exposed to cold [8. 9].

At the initial stages of cold acclimation, phosphorylation of cellular proteins or activation of protein kinases has been detected [10, 11], and the genes of low-temperature-inducible putative kinase regulator have been reported for *Arabidopsis* [12]. Thus, in this study, examination was made of the effects of low temperature on protein phosphorylation in developing green leaves of two rice varieties differing in sensitivity to low temperature, namely, the low-temperature sensitive variety, Indica type IR36, and low-temperature tolerant variety, Japonica type Kitaibuki. For greater clarification of these changes, the effects of cold on rice varieties differing in cold susceptibility were examined.

RESULTS AND DISCUSSION

Leaves of rice seedlings were found to respond to cold stress, accompanied by definite changes in protein phosphorylation. Within cells, protein kinases and protein phosphatases phosphorylate and dephosphorylate proteins [13]. Protein kinase in rice leaves may possibly be involved in cell regulation through phosphorylation of protein kinase catalysed substrates [14]. In the early stages after germination, changes in phosphorylation and dephosphorylation could be detected in the leaf protein extract. Changes in phosphorylation could not be found in the leaf protein extract at 10 days after germination (Fig. 1). Cold treatment was thus carried out on leaves 10 days after germination.

Changes in proteins were made more apparent by 2D-PAGE analysis of leaf protein extracts after cold stress (Fig. 2). More than 300 polypeptide spots could be identified. The basic protein of 30 kDa was markedly induced by cold treatment in the leaves, in the rice variety, Kitaibuki. However, in the rice variety, IR36, this protein was always present.

Rice protein extracts were separated by 2D-PAGE and a 30 kDa protein was removed by electroelution.

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‡ Abbreviations: 2D-PAGE, two dimensional-PAGE; CBB, Coomassie Brilliant Blue.

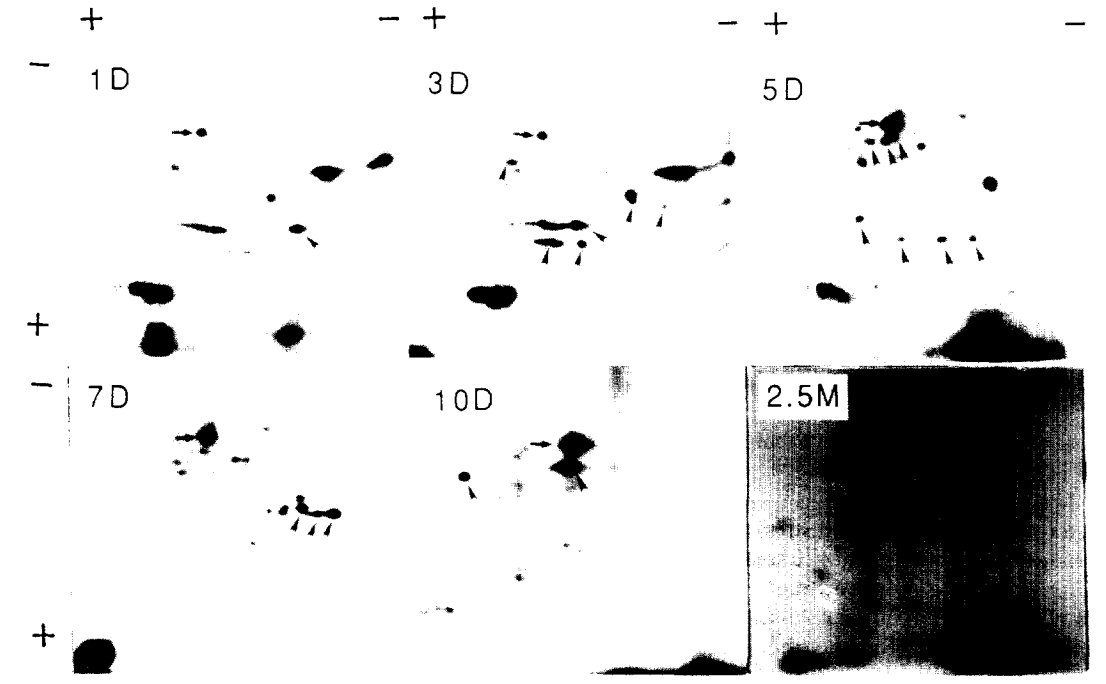

Fig. 1. *In vitro* phosphorylation using embryos and leaves in the early stages after germination of rice (cultivar Nipponbare). The samples were collected 1 day (embryo), 3 day (embryo), 5 day (embryo), 7 day (leaf), 10 day (leaf) and 2.5 months (leaf) after germination. Protein extracts were incubated in a reaction mixture containing [γ-^{32}P]ATP. After *in vitro* phosphorylation, phosphorylated proteins were subjected to 2D-PAGE. Right to left, IEF for first dimension; top to bottom, SDS-PAGE for second dimension. The arrow shows the position of the 60 kDa protein. Arrowheads show changed phosphoprotein during the germination.

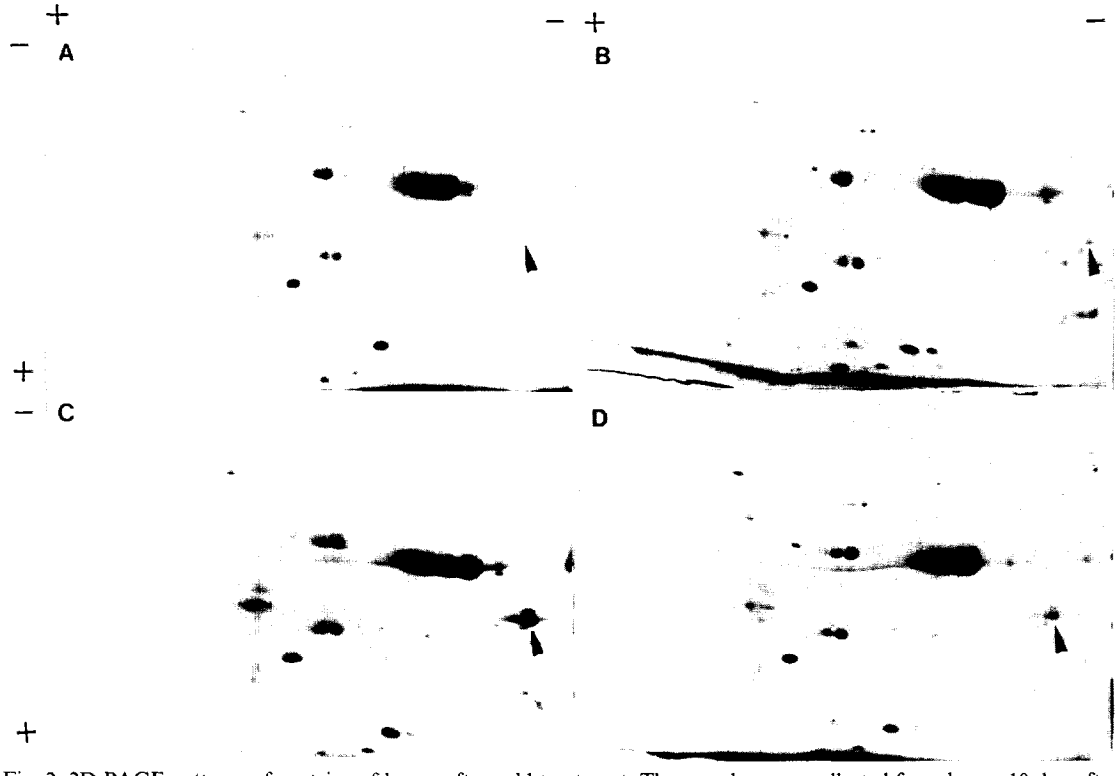

Fig. 2. 2D-PAGE patterns of proteins of leaves after cold treatment. The samples were collected from leaves 10 day after germination. After 2D-PAGE, the gels were stained with CBB. A and B, Japonica cultivar (Kitaibuki); C and D, Indica cultivar (IR36). A and C, 25° for 6 hr; B and D, 5° for 6 hr. The arrowhead shows the position of the 30 kDa protein.

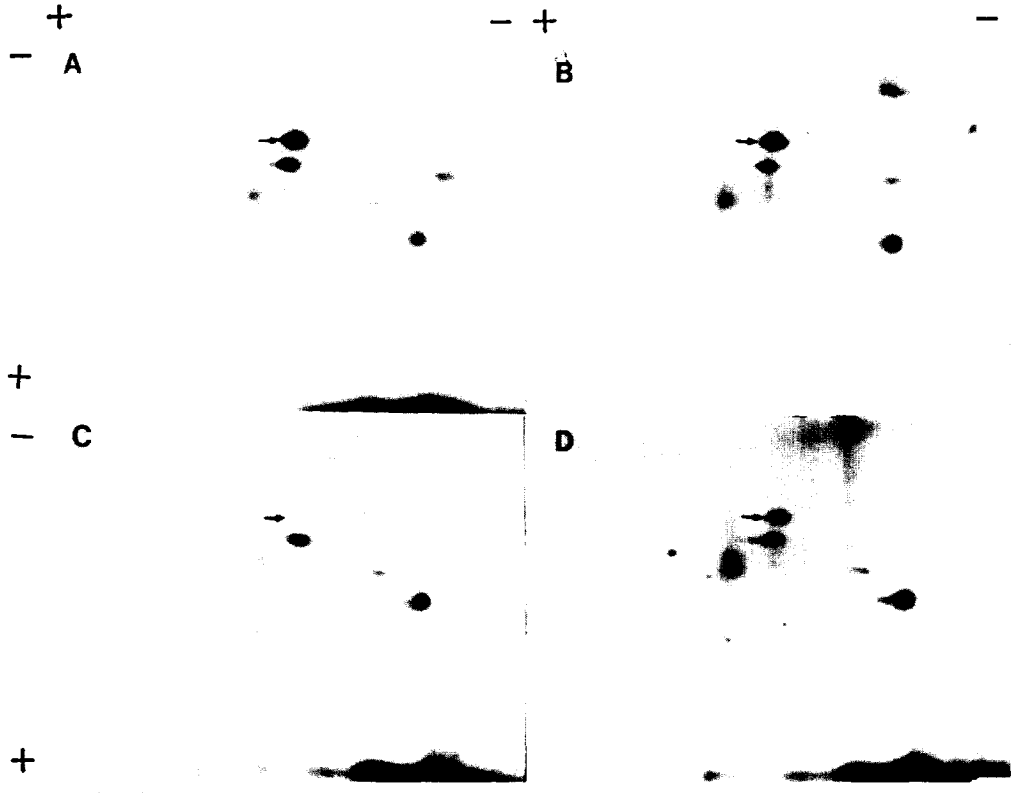

Fig. 3. *In vitro* phosphorylation patters of proteins of leaves after cold treatment. The samples were collected from leaves at 10 days after germination. After *in vitro* phosphorylation, phosphorylated proteins were subjected to 2D-PAGE. A and B, Japonica cultivar (Kitaibuki); C and D, Indica cultivar (IR36). A and C, 25° for 6 hr; B and D, 5° for 6 hr. The arrow shows the position of the 60 kDa protein.

The Cleveland mapping technique [15] was carried out to determine the amino acid sequences of the internal regions of proteins. The amino acid sequence of this protein was partially determined. The partial amino acid sequences of this protein were TKDTDILAAF and RDKLNKYGRP. The amino acid sequence was compared with that of the PIR data-base. The result was found homologous to that of ribulose bisphosphate carboxylase large chain (Accession no. EC 4.1.1.39). However, ribulose bisphosphate carboxylase large chain should be approx. 50 kDa. Some protease may be increased in response to cold stress and thus explain the 'induction' of the 30 kDa protein, which is a fragment of riburose bisphosphate carboxylase large chain.

Cold treatment stimulated the phosphorylation of a 60 kDa protein in the rice variety, IR36 (Fig. 3). The 60 kDa protein was most extensively phosphorylated at 5°, compared with the phosphorylation at 25° or 15° (Fig. 4). This protein was rapidly phosphorylated within 6 hr (Fig. 5).

To determine whether the observed protein phosphorylation is specific for cold seedlings, it was compared with that of labeled protein from seedlings subjected to light change or $CaCl_2$-treatment. The phosphorylation of this protein did not depend on Ca^{2+} (Fig. 6), nor on light (Fig. 7). Neither of these treatments resulted in responses similar to those obtained after cold treatment. At this point, the biochemical mechanisms of phosphorylation of the protein having the M_r of 60000 differ from those for the 30 kDa protein.

In order to understand better the nature of these changes, we compared the effects of cold on rice varieties differing in cold-susceptibility. There were definite differences in electrophoresis patterns of the 60 kDa protein, depending on variety. The 60 protein in 10 varieties or species of rice (Kitaibuki, Nipponbare, Bamba 1, Ketan Nangka, IR36, Er Jiu-Qing, Jamuna, Dular, Kele and Silewah) was separated by 2D-PAGE (Fig. 8). For this analysis, 10 varieties including Japonica, Javanica and Indica-type were used. Japonica and Javanica-types were generally (but not always) cold-tolerant as compared to Indica-type. However, the extent of chilling injury may depend on the test conditions. The phosphorylation pattern of all three Japonica varieties were similar to each other (group I) and three Indica varieties also showed another type of pattern (group II), but the two Javanica varieties showed a third type of pattern (group III) in Silewah or group I-type of pattern in Ketan Nangka.

In this study, we identified a specific 60 kDa protein which is phosphorylated in the leaf protein when the plants are exposed to cold. The response should not be related to desiccation stress caused by cold treatment since the plants were treated at saturating level of

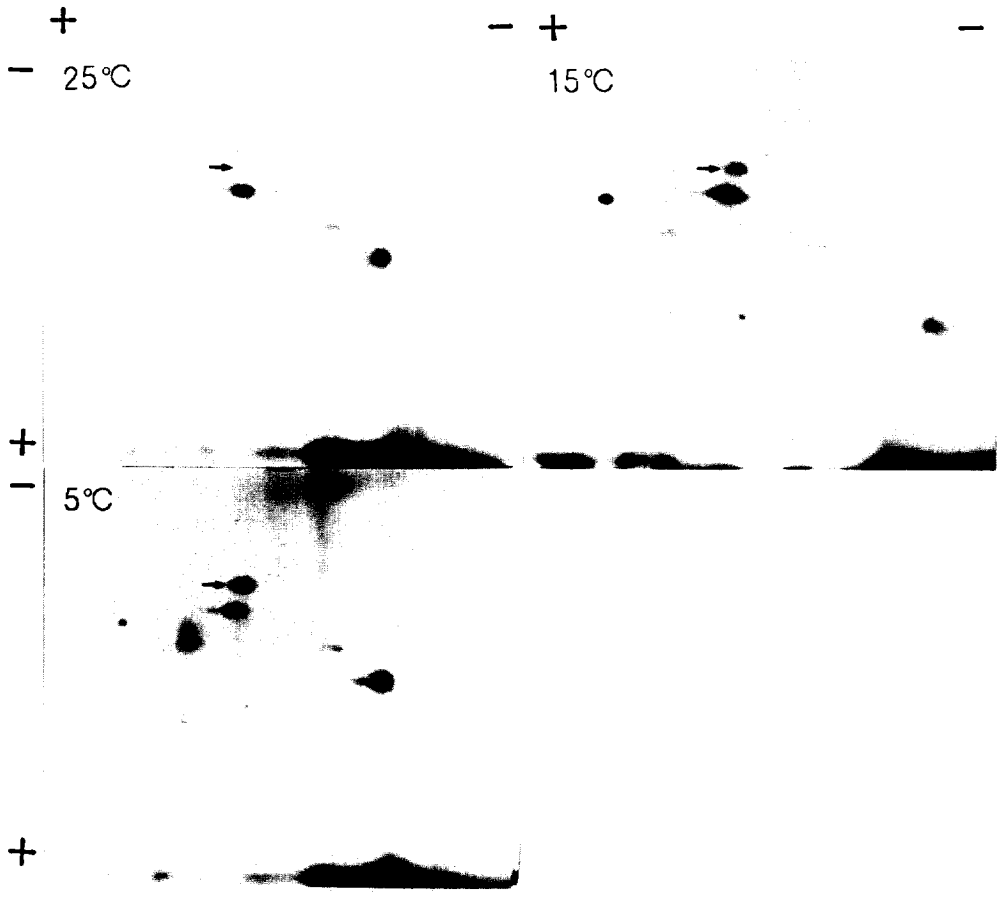

Fig. 4. Effect of low temperature on protein phosphorylation. The rice (indica cultivar IR36) was treated at 5°, 15° and 25° for 6 hr. The arrow shows the position of the 60 kDa protein.

relative humidity. The protein is phosphorylated in cold tolerant Japonica varieties irrespective cold treatment but the phosphorylation is induced in cold sensitive Indica varieties. Javanica varieties are a subgroup of Japonica-types and relatively cold tolerant, and in agreement with this, the phosphorylation pattern in Javanica was similar to Japonica in Ketan Nangka, though Silewah showed a special pattern. The relationship between the increase of phosphorylating activity in leaf protein extracts in cold-sensitive Indica varieties and chilling injuries needs to be analysed genetically, and F2 plants of Japonica and Indica varieties may be useful for the study.

EXPERIMENTAL

Plant materials. Seedlings were grown for 2 weeks under fluorescent light (about 600 μmol m^{-2} S^{-1}, 16 hr light period/day) at 24–26° and 60–90% relative humidity. For cold treatment, plants were transferred to a growth chamber set at low temp. and incubated for different times.

Samples were collected at 1 day (embryo), 3 day (embryo), 5 day (embryo), 7 day (leaf), 10 day (leaf) and 2.5 months (leaf) after germination.

Preparation of protein extract. A portion (250 mg) of the leaves were removed and homogenized with 1 ml 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 vanadate and 1 mM phenylmethylsulphonyl fluoride. The homogenate was centrifuged at 15000 *g* for 5 min in a TMA-4 rotor(Tomy, Tokyo). The supernatant was used as the rice protein extract.

In vitro *protein phosphorylation. In vitro* phosphorylation assay was carried out in the reaction mixt. described by Komatsu and Hirano [14]. Leaf extracts were incubated in a reaction mixt. (40 μl) containing 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ and 39 μM [γ-^{32}P]ATP (300 mCi mol^{-1}, Amersham, Buckinghamshire). After *in vitro* phosphorylation, the sample was added to a lysis buffer containing 8 M urea, 2% Triton X-100, 2% ampholine, 10% PVP-40, and subjected to two dimensional-PAGE (2D-PAGE) according to O'Farrell [16] with some modifications. The sample was sepd in the first dimension by IEF, and in the second dimension by SDS-PAGE using 15% polyacrylamide gels. The gel was stained with Coomassie Brilliant Blue (CBB), destained, dried and exposed to autoradiography on X-ray film (Kodak, NY) at −80° for 2 day.

Isolation of protein. Rice protein extracts were sepd by 2D-PAGE and stained with CBB. Gel pieces containing the protein were removed and the protein elec-

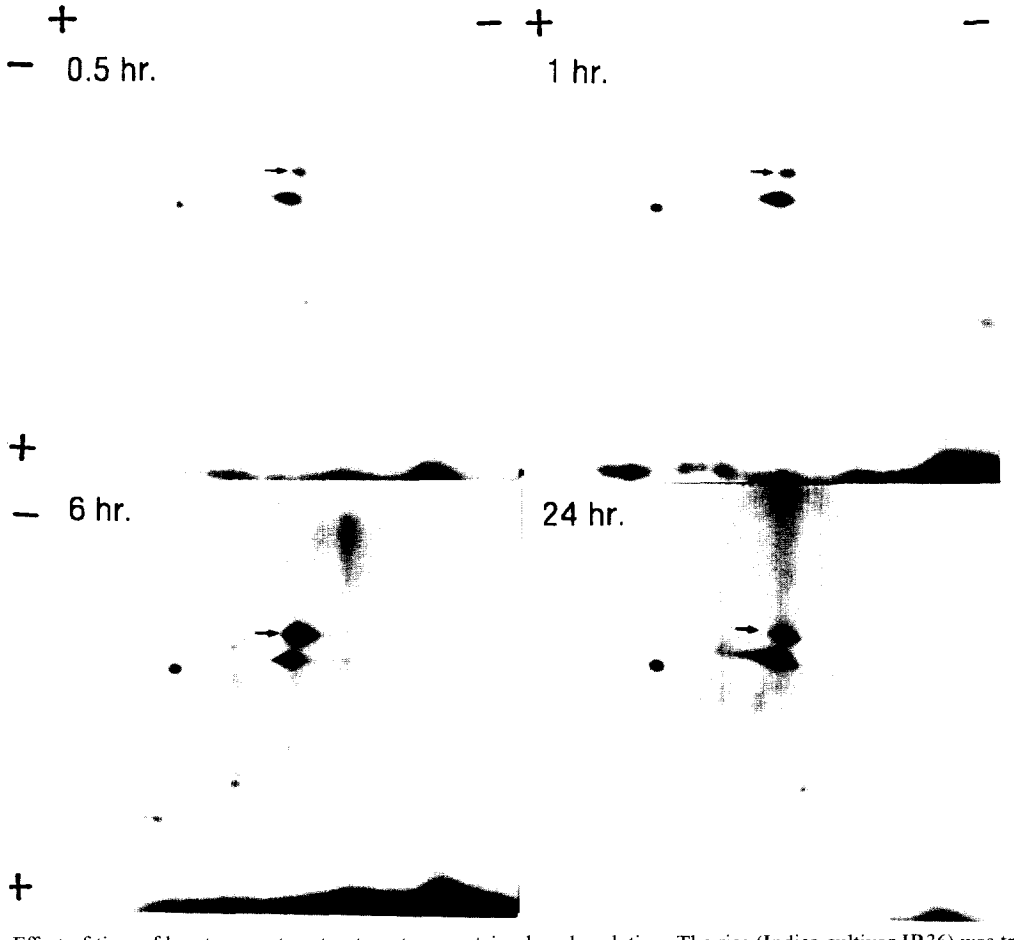

Fig. 5. Effect of time of low temperature treatment on protein phosphorylation. The rice (Indica cultivar IR36) was treated at 5° for 30 min, 1 hr, 6 hr and 1 day. The arrow shows the position of the 60 kDa protein.

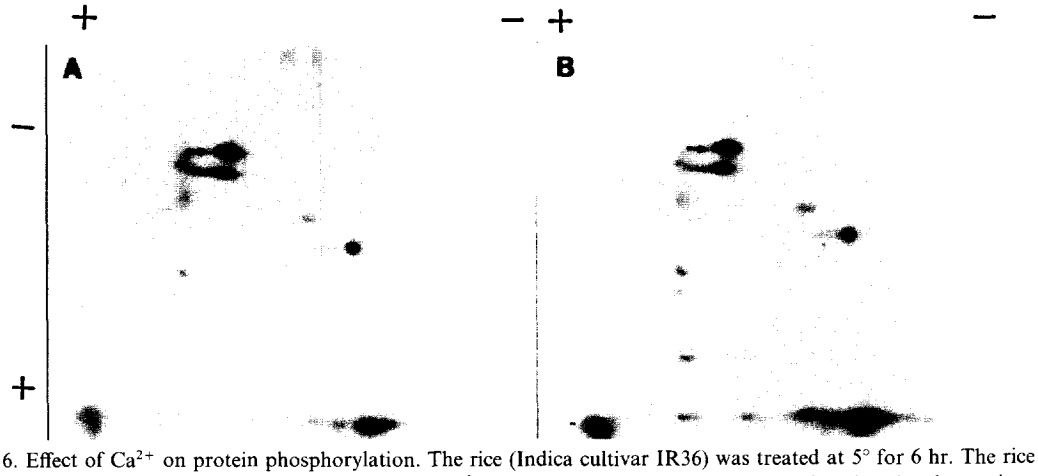

Fig. 6. Effect of Ca^{2+} on protein phosphorylation. The rice (Indica cultivar IR36) was treated at 5° for 6 hr. The rice leaf protein extracts were incubated in the presence of Ca^{2+}. After *in vitro* phosphorylation, phosphorylated proteins were subjected to 2D-PAGE. A, protein phosphorylated in the presence of EGTA; B, protein phosphorylated in the presence of Ca^{2+}. The arrow shows the position of the 60 kDa protein.

troeluted from the gel pieces using an Electrophoretic Concentrator (M1759, ISCO, Lincoln) at 2 W constant power for 2 hr. After electroelution, the protein soln was dialysed against deionized H_2O for 2 day and dried.

N-terminal and internal amino acid sequence analysis. Proteins were dissolved in 20 μl of SDS sample buffer (pH 6.8) and overlaid with 20 μl of a soln containing 10 μl of *Staphylococcus aureus* V8 protease (Pierce, Rockford) (0.1 $\mu g\ \mu l^{-1}$) in deionized H_2O and 10 μl of SDS sample buffer (pH 6.8) containing 0.001% Bromophenol Blue. Electrophoresis was per-

Fig. 7. Effect of light on the protein phosphorylation. The rice (Indica cultivar IR36) was treated at 5° for 6 hr in the dark (D) or in the light (B). The arrow shows the position of the 60 kDa protein.

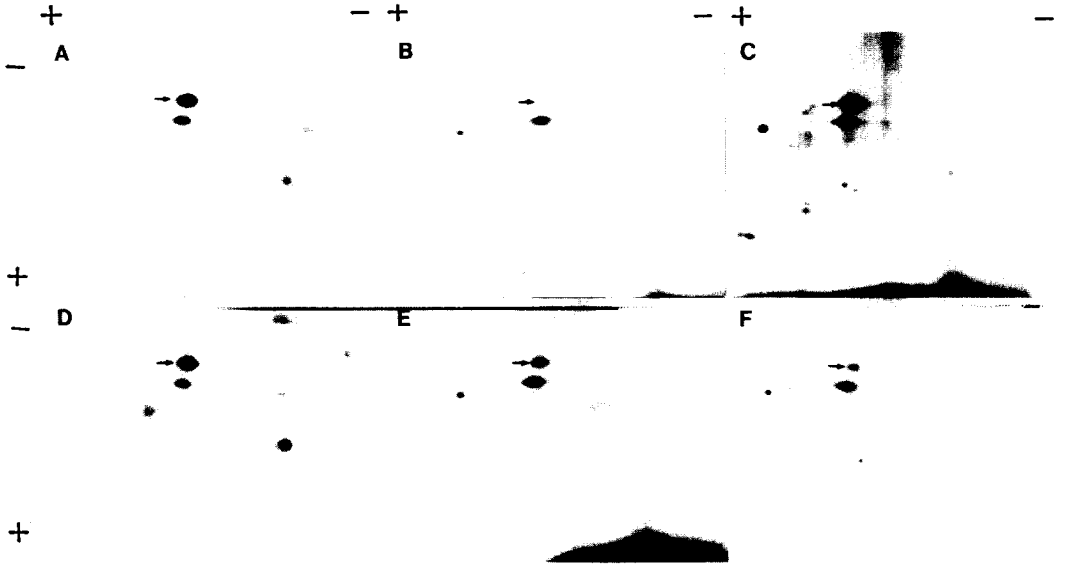

Fig. 8. *In vitro* phosphorylation patterns. The rice was grown at 10 day after germination and treated at 5°, 15° and 25° for 30 min, 1 hr, 6 hr and 1 day. After *in vitro* phosphorylation of leaf protein extract, phosphorylated proteins were subjected to 2D-PAGE. A, B and C, 25° treatment; D, E and F, 5° treatment. A and D, group I; B and E, group II; C and F, group III. The arrow shows the position of the 60 kDa protein.

formed until the sample was stacked in the upper gel and then interrupted for 30 min for digestion of the protein [15]. Electrophoresis was then continued and the sepd digest was electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Applied Biosystems, Foster City), dried, and subjected to gas-phase sequencing. The PVDF membrane was applied to the upper glass block of the reaction chamber in a gas-phase protein sequencer (477A and 473A, Applied Biosystems). Edman degradation was performed according to the standard program supplied by Applied Biosystems.

REFERENCES

1. Lyons, J. M., *Annual Review of Plant Physiology*, 1973, **24**, 445.
2. Raison, J. K. and Lyons, J. M., *Plant Cell Environment*, 1986, **9**, 685.
3. Raison, J. K., Lyons, J. M. and Keith, A. D., *Journal of Biological Chemistry*, 1971, **246**, 4036.
4. Lippert, J. L. and Peticolas, W. L., *Proceedings of the National Academy of Science of the USA*, 1971, **68**, 1572.
5. Tajima, K., Amemiya, A. and Kabaki, N., *Bulletin of the National Institute of Agricultural Science*, 1983, **D32**, 69.
6. Kabaki, N., Yoneyama, T. and Tajima, K., *Journal of Crop Science*, 1982, **51**, 82.
7. Takanashi, J., Maruyama, S., Kabaki, N. and Tajima, K., *Japanese Journal of Crop Science*, 1987, **56**, 44.
8. Thomashow, M. F., *Advances in Genetics*, 1990, **28**, 99.
9. Koba-Ban, Y., Abe, M. and Kitagawa, Y., *Plant Cell Physiology*, 1991, **32**, 901.
10. Garbarino, J. E., Hurkman, W. J., Janaka, C. K. and DuPont, E. M., *Plant Physiology*, 1991, **95**, 1219.
11. Monroy, A. F., Sarhan, F. and Dhindsa, R. S., *Plant Physiology*, 1993, **102**, 1227.
12. Holappa, L. D. and Walker-Simmons, M. K., *Plant Physiology*, 1995, **108**, 1203.
13. Ranjeva, R. and Boudet, A. M., *Annual Review of Plant Physiology*, 1987, **38**, 73.
14. Komatsu, S. and Hirano, H., *Plant Science*, 1993, **94**, 127.
15. Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K., *Journal of Biological Chemistry*, 1977, **252**, 1102.
16. O'Farrell, P. F., *Journal of Biological Chemistry*, 1975, **250**, 4007.