Theor Appl Genet (1999) 98: 1304–1310 

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# Phosphorylation upon cold stress in rice (*Oryza sativa* L.) seedlings

Received: 7 July 1998 / Accepted: 19 December 1998

**Abstract** The response of plants to cold stress is not well understood at the biochemical level, although it has been studied extensively at the ecological level. To investigate whether protein phosphorylation may play an important role in cold stress, we exposed rice seedlings to low temperatures, prepared protein extracts from the leaves and incubated these in the presence of [$\gamma$-$^{32}$P]ATP. The proteins were then separated by two-dimensional polyacrylamide gel electrophoresis. While several proteins were found to be phosphorylated upon cold stress one protein, pp35, which has an isoelectric point of 8.0, was more phosphorylated than the others. The pp35 protein was found to be phosphorylated when rice seedlings were incubated for 6 h at 5°C before the leaf protein extract was prepared and radioactive labeling was performed. The pp35 was, however, significantly more phosphorylated in cold-tolerant rice varieties. Antibodies were raised against purified pp35 in adult rabbits. Using this pp35 antibody, which can recognize the RuBisCO large-chain subunit (LSU), and from amino acid sequencing of pp35, we were able to identify and confirm the pp35 protein as the fragment of RuBisCO LSU (EC 4.1.1.39). Phosphorylation of the RuBisCO LSU may be important in cold tolerance.

**Key words** Cold stress · Leaf proteins · Protein phosphorylation · Rice · 2D-PAGE

Communicated by K. Oono

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## Introduction

Crop plants in tropical and sub-tropical regions are seriously injured by temperatures below 12°C but above the freezing point (Lyons 1973). A primary, if not exclusive, effect of chilling is considered to be the phase transition of membrane lipids at the critical temperatures (Lyons 1973; Raison et al. 1971). It has also been reported that, in rice plants, growth rate and metabolism are already markedly inhibited at temperatures above the chilling temperature, in the range of 15°–20°C (Kabaki et al. 1982; Takanashi et al. 1987), but the mechanism for the effects of low temperature stress on growth and the accompanying metabolic changes remains unclear. It is generally recognized that the protein synthesis patterns and mRNA levels change when plants are exposed to cold temperatures (Thomashow 1990; Koga-ban et al. 1991).

At the initial stage of cold acclimatization, phosphorylation of cellular proteins or activation of protein kinases has been detected (Garbarino et al. 1991; Monroy et al. 1993), while the genes of low temperature-inducible putative kinase have been reported for *Arabidopsis* (Holappa and Walker-Simmons 1995). We previously reported that cold-sensitive rice varieties showed similarity in their leaf protein phosphorylation patterns in contrast to the cold-tolerant varieties (Komatsu and Kato 1997). In the study reported here we investigated 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 the 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.

## Materials and methods

### Plant materials

Three kinds of rice (*Oryza sativa* L.) were used for the experiments: a Japonica-type ('Kitaibuki', 'Nipponbare'), Javanica-type ('Ketan Nangka', 'Silewah') and Indica-type ('IR36', 'Er Jiu-Qing', 'Jamuna' and 'Kele'). Seedlings were grown for 2 weeks under fluorescent light (about 600 $\mu mol\, m^{-2}\, s^{-1}$, 16-h light period/day) at 24°–26°C and 60–90% relative humidity. For cold treatments, plants were transferred to a growth chamber set at a low temperature and incubated for varying times. Leaf samples were collected 10 days after germination.

### Preparation of protein extracts

A portion of the leaves (250 mg) was removed and homogenized with 1 ml extraction buffer containing 50 m*M* TRIS-HCl (pH 7.4), 150 m*M* NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 m*M* EDTA, 5 $\mu M$ sodium vanadate and 1 m*M* phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 15 000 *g* for 5 min in a TMA-4 rotor (Tomy, Tokyo), and the supernatant was used directly as the rice protein extract.

### In vitro protein phosphorylation

The in vitro protein phosphorylation assay was carried out as described by Komatsu and Hirano (1993). Leaf extracts were incubated in a reaction mixture (40 µl) containing 20 m*M* TRIS-HCl (pH 7.5), 10 m*M* $MgCl_2$ and 39 $\mu M$ [$\gamma$-$^{32}$P] ATP (11.1 CTBg $mol^{-1}$, 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 polyacrylamide gel electrophoresis (2D-PAGE) (O'Farrell 1975). The sample was separated in the first dimension by iso-electric focusing (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, N.Y.) at − 80°C for 2 days.

### In vivo protein phosphorylation

Leaf segments (250 mg) from the leaves of rice seedlings grown as above were incubated in the presence of 37 MBg/ml [$^{32}$P]orthophosphate (Amersham) for 6 h at 5°C. The radiolabeled leaf segments were washed three times with phosphate buffered saline and extracted on ice in 1 ml of lysis buffer. After centrifugation at 15 000 *g* for 10 min, 20 µl of the supernatant was analyzed by 2D-PAGE as described above. The gel was stained with CBB, destained, dried and exposed to autoradiography on X-ray film at − 80°C for 6 hr.

### Purification of protein

Rice protein extracts were separated by 2D-PAGE and stained with CBB. Gel pieces containing the protein were removed, the protein was electroeluted from the gel pieces using an electrophoretic concentrator (M 1759, ISCO, Lincoln) at 2 W of constant power for 2 h and dried.

### N-terminal and internal amino acid sequence analysis

Protein was dissolved in 20 µl of SDS sample buffer (pH 6.8) and overlaid with 20 µl of a solution containing 10 µl of *Staphylococcus aureus* V8 protease (0.1 µg/µl, Pierce, Rockford) in deionized $H_2O$ and 10 µl of SDS sample buffer (pH 6.8) containing 0.001% Bromophenol Blue. Electrophoresis was initially performed until the sample was stacked in the upper gel and then interrupted for 30 min for digestion of the protein (Cleveland et al. 1977). Electrophoresis was initially continued and the separated 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.

### Preparation of antibody and immunoblotting

Protein (pp35) was separated by 2D-PAGE and electroeluted. The protein solution was dialyzed against deionized $H_2O$ for 2 days and dried. Using this purified protein, we raised an antibody against protein in adult rabbits applying Bailey's method (1985). Briefly, the protein solution containing TRIS-HCl buffer as described above, and an equal volume of a complete adjuvant was injected into the rabbits three times at 1-month intervals. The antiserum obtained was used directly in the protein-blotting experiment. For immunoblotting experiments, the extracted proteins were separated by 2D-PAGE and electroblotted onto a PVDF membrane. The blotted spot, which cross-reacted with the antibody against the protein, was detected by the peroxidase enzyme immunoassay (Komatsu and Hirano 1992).

## Results and discussion

### Protein phosphorylation in rice upon cold stress

Subjecting rice seedlings to low temperatures results in a variety of biochemical changes at the cellular level. In the present study an effort was made to determine the changes in phosphorylation patterns in rice seedlings in a range of rice varieties growing in temperate to tropical regions. All three types of rice were used in this study, Japonica, Javanica and Indica (data not shown for the Javanica-type rice varieties in Fig. 1 as the phosphorylation pattern was the same as that of the Indica-type rice varieties). From Fig. 1, it is evident that the major phosphorylated protein, pp35, indicated by an arrow, is prominent in the low temperature-tolerant Japonica variety 'Kitaibuki' and also in the moderately low temperature-tolerant Japonica variety 'Nipponbare' after exposure to a cold stress of 5°C for 6 h. This protein was highly phosphorylated to almost an equal degree in both the moderately low temperature-tolerant variety 'Nipponbare' and the extremely low temperature-tolerant variety 'Kitaibuki' after a 5°C stress. However, as can be seen from Fig. 1A and C, this protein was already phosphorylated in the Nipponbare seedlings kept at control temperatures as compared to no or low phosphorylation levels in the 'Kitaibuki' seedlings. On the other hand, this protein showed no

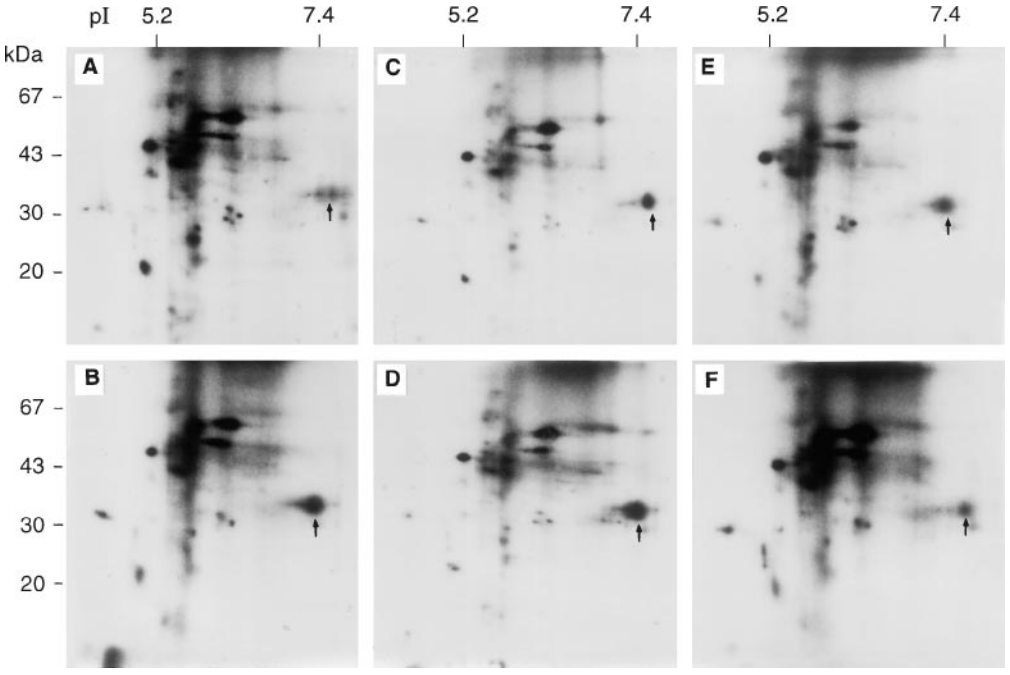


**Fig. 1A–F** In vitro phosphorylation patterns of rice seedling leaf proteins after cold stress (5°C for 6 h). The samples were collected at 10 days after germination. 2D-PAGE was performed after in vitro phosphorylation. **A, B** 'Kitaibuki', **C, D** 'Nipponbare', **E, F** 'IR36'. **A, C, E** 25°C for 6 h; **B, D, F** 5°C for 6 h

significant changes in its phosphorylation pattern in the low temperature-sensitive Indica variety 'IR36'. Thus, from this preliminary result, it can be said that among the various proteins being phosphorylated in the leaves of rice seedlings, a major change in phosphorylation of pp35 was observed after a 5°C stress.

An in vivo phosphorylation assay was performed to determine whether the same proteins were being phosphorylated in vitro and in vivo. In vivo phosphorylation was performed using leaf segments of the extremely low temperature-tolerant variety 'Kitaibuki' as this variety showed the major change in phosphorylation after the 5°C stress. Results presented in Fig. 2 show that in vitro and in vivo phosphorylation were identical and that the same proteins were being phosphorylated after 5°C stress in var 'Kitaibuki'. An arrow marks the position of the pp35 protein.

## Autoradiography and internal amino acid sequence of pp35

We next proceeded to analyze the pp35 protein and to determine its character. Rice leaf protein extracts were prepared as described in the Materials and methods, in vitro phosphorylation was performed and the gels were subjected to 2D-PAGE. After the gels were stained with CBB, they were dried and subjected to autoradiography. It can be seen from Fig. 3 that the major phosphorylated protein is a basic protein with a molecular mass of approximately 35 kDa (namely pp35). This pp35 protein has an isoelectric point of 8.0. The next step in the experiments was to determine the amino acid sequence of pp35. Internal sequence analysis revealed a high homology to the N-terminal region of RuBisCO large-chain subunit (LSU) (Fig. 4). This was an interesting result. The first question that came to mind was why is the RuBisCO LSU fragment being phosphorylated after an exposure to the 5°C cold stress and, in particular, why in the extremely low temperature- tolerant Japonica rice variety 'Kitaibuki'. One explanation is that RuBisCO synthesis appears to be very sensitive to a variety of environmental factors, low temperature being one of these factors. This was also found in the 1989 study of Hahn and Walbot who showed that both of the RuBisCO subunits were strongly affected by extended cold treatments in rice. Furthermore, among the three rice types analyzed in our study, only the Japonica varieties showed increased phosphorylation upon a 5°C cold stress, and of these the extremely low temperature-tolerant variety 'Kitaibuki' showed the highest phosphorylation (Fig. 1).

## Immunoblotting with anti-pp35 antibody and amino acid sequence

In our next experiment we examined the presence of pp35 using antibodies raised against pp35 (see Materials and methods). The pp35 antibody (anti-pp35 antibody) recognizes the RuBisCO LSU. Green and etiolated leaves of the extremely low temperature-tolerant Japonica rice variety 'Kitaibuki', were removed after a 6-h-long 5°C cold stress, proteins were separated by 2D-PAGE and immunoblotting was performed. Green leaf proteins with a molecular mass

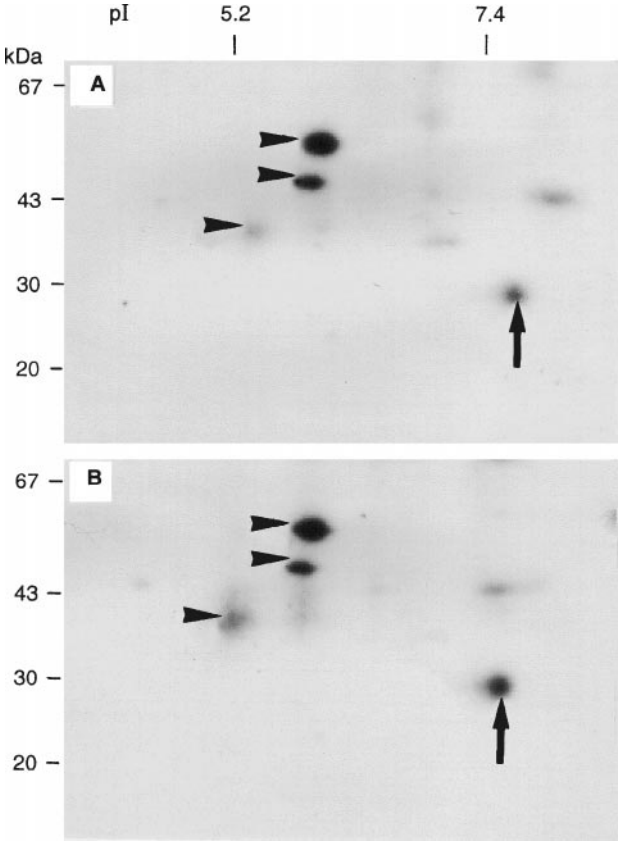


**Fig. 2A, B** In vivo phosphorylation patterns of the rice seedling leaf proteins in the low-temperature-tolerant rice variety 'Kitaibuki'. 2D-PAGE was performed as described in the Materials and methods and subjected to autoradiography. **A** 25°C, **B** 5°C. The *arrow* shows the position of pp35

of approximately 35 kDa and six other high-to-low-molecular-weight spots on 2D-PAGE cross-reacted with the pp35 antibody, indicating their immunological similarity (Fig. 5). On the other hand, etiolated leaf

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RuBisCO large chain (P28396 : EC4.1.1.39)
                    31                                  49
VGFKAGVKDY KLTYYTPEYE TKHTDILAAF---------PPHGIQVE GDKLNKYGRP---
                     ::::::::::                         ::::::::::
pp35                 (E)TKHTDILAAF                      (E)GDKLNKYGRP
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**Fig. 4** The amino acid sequence of the pp35 protein and its homology with the RuBisCO large chain (P28396: EC 4.1.1.39)

proteins did not show any cross-reactivity with the pp35 antibody. Further, upon sequencing each of these seven proteins, we found the amino acid sequences to be identical to the N-terminal region and internal regions of RuBisCO LSU (data not shown). These results indicate that when rice seedlings are subjected to a 5°C stress, with the exception of the initially observed phosphorylation of pp35 in the rice variety 'Kitaibuki', there is additionally a breakdown or degradation of RuBisCO LSU, this was confirmed by immunoblot experiments. We suggest that upon cold stress, there is either a very early activation of proteases, which results in degradation of the RuBisCO LSU, or there is an increase in the production of free radicals, which are known to fragment RuBisCO LSU (Ishida et al. 1997). By means of immunostainning, these researchers also observed the degradation of RuBisCO LSU into a major 37-kDa fragment in isolated wheat chloroplasts upon light stress. We have shown here the presence of a major degraded RuBisCO LSU fragment, approximately 35 kDa in size, following the exposure of rice seedlings to a 5°C stress, which is in good correlation to the expected size of the major breakdown product of RuBisCO LSU. In addition, our results also indicate that under a variety of stress conditions, the RuBisCO LSU is targeted for degradation. On the other hand, Hahn and Walbot (1989) argue that cold treatment simply puts some aspects of cell differentiation on hold without accelerating protein degradation. This observation held up well for their experimental setup in

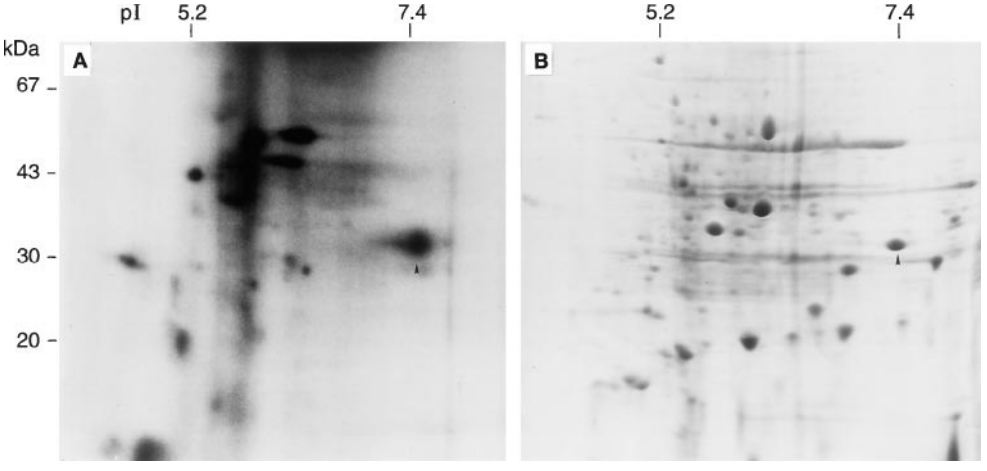


**Fig. 3A, B** The phosphorylated leaf protein pattern in rice variety 'Kitaibuki' after 2D-PAGE and CBB staining. The *arrowhead* shows the position of the pp35 protein. **A** Phosphorylation pattern, **B** CBB staining. Molecular weight markers are indicated on the *left-hand side*, and the isoelectric point markers are given at the *top* of the gel

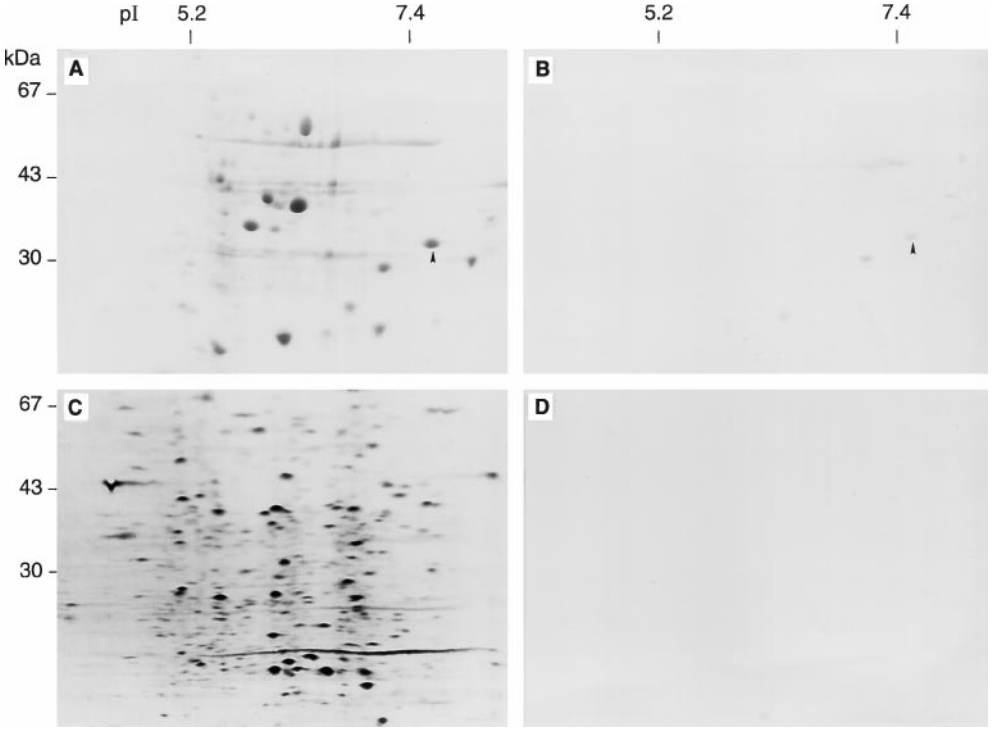


**Fig. 5A–D** Immunoblotting results of pp35 with anti-RuBisCO large-chain antibody. **A, B** Green leaf, **C, D** etiolated leaf. **A, C** CBB staining, **B, D** immunoblot. The *arrowhead* shows the position of pp35

which they used extended periods (7 days) of cold on rice seedlings at temperatures of 15°C or 11°C. In our experimental design, however, a 5°C cold stress to rice seedlings for 6 h resulted in the clear degradation of RuBisCO LSU and increased phosphorylation of the major degraded protein, namely pp35. The questions still remain as to exactly how and why.

## Amount of RuBisCO LSU and pp35 in the three rice types

We conducted experiments on a variety of rice genotypes differing in their cold sensitivity or tolerance. As the cold-tolerant rice variety, we selected the well-known cold-tolerant variety 'Kitaibuki', and the results obtained here on this variety clearly indicate a difference in the level of phosphorylation (Fig. 1). Our data, however, need to be checked in other cold-tolerant Japonica rice varieties, and these experiments are planned for the future. The presence of pp35 protein as well as amounts of the RuBisCO LSU in varieties of all three rice types were examined (Fig. 6). In the extremely low temperature-tolerant Japonica-type rice variety 'Kitaibuki', RuBisCO LSU was not present following a 5°C cold stress, instead a prominent pp35 protein spot and other protein spots similar to the ones shown in Fig. 5 were found. However, in the moderately low temperature-tolerant 'Nipponbare', both the RuBisCO LSU as well as the pp35 protein spot were present. On the contrary, in the Javanica- and Indica-type rice varieties, RuBisCO LSU was present in normal amounts, with only a small spot corresponding to the pp35 protein (Fig. 6). Using an image analyzer (Toyobo, Osaka), we determined the relative amounts of protein (pp35) in the different rice varieties, the extremely low temperature-tolerant rice variety 'Kitaibuki' was found to have the highest amount of pp35 protein (Fig. 7).

What is clear from these results is that cold stress to the rice variety 'Kitaibuki' results in fragmentation of the RuBisCO LSU in the leaf tissues and that a major fragment, pp35, is phosphorylated just after 6 h at 5°C. However, in the moderately low temperature-tolerant rice variety 'Nipponbare', the RuBisCO LSU is present in leaf tissues together with lower levels of the pp35 fragment. We therefore suggest that the low temperature-tolerant rice varieties (in this study) are characterized by early fragmentation of the RuBisCO LSU and phosphorylation of the pp35 protein. Moreover, this effect on RuBisCO fragmentation and subsequent pp35 phosphorylation decreases with the decreasing degree of cold tolerance of the rice varieties, the effect being maximum in cold area (temperate) varieties and minimum or not observed at all in varieties from areas of high temperatures (tropical). (Note that the Japonica-type rice varieties are grown more in temperate regions and that Javanica- and Indica-type rice varieties are grown in tropical regions).

On the other hand, Hahn and Walbot (1989) demonstrated that the synthesis of RuBisCO was drastically reduced after 7 days of cold stress and that cold-sensitive rice cultivars responded with a drastic reduction in LSU and SSU (small subunit) synthesis after 7 days at

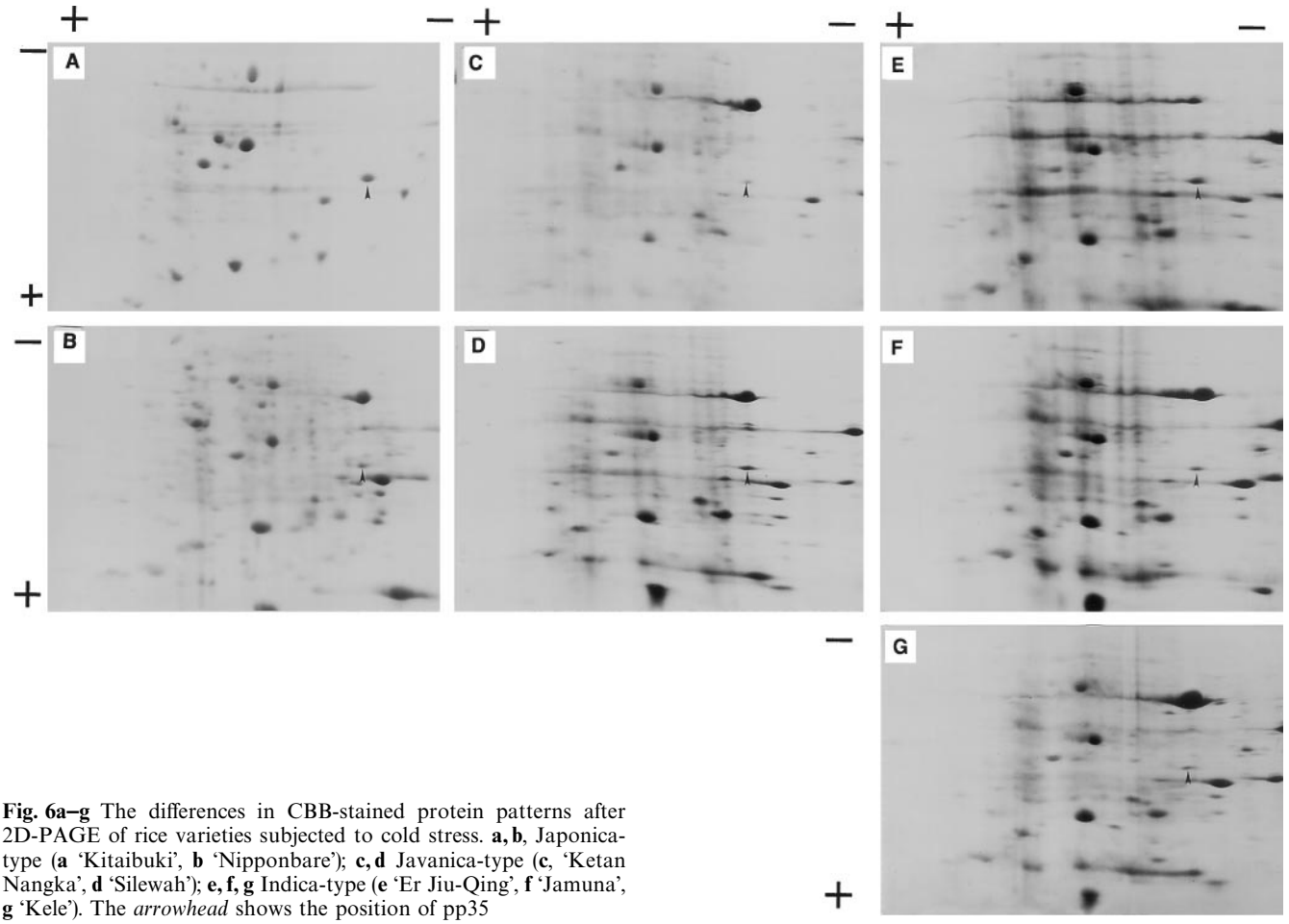


**Fig. 6a–g** The differences in CBB-stained protein patterns after 2D-PAGE of rice varieties subjected to cold stress. **a, b**, Japonica-type (**a** 'Kitaibuki', **b** 'Nipponbare'); **c, d** Javanica-type (**c**, 'Ketan Nangka', **d** 'Silewah'); **e, f, g** Indica-type (**e** 'Er Jiu-Qing', **f** 'Jamuna', **g** 'Kele'). The *arrowhead* shows the position of pp35

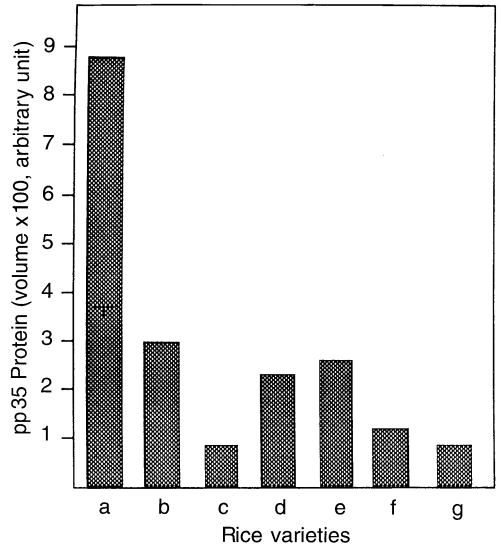


**Fig. 7** The differences in CBB-stained protein patterns after 2D-PAGE of rice varieties subjected to cold stress: the relative amount of pp35 protein in the rice leaf as determined using an Image analyzer (Toyobo, Japan). *a,b* Japonica-type (*a* 'Kitaibuki, *b* 'Nipponbare'); *c,d* Javanica-type (*c*, 'Ketan Nangka', *d*, 'Silewah'); *e, f, g* Indica-type (*e* 'Er Jiu-Qing', *f* 'Jamuna', *g* 'Kele')

15°C or 11°C while cold-tolerant cultivars maintained higher levels of RuBisCO synthesis. These researchers suggest that the alterations in total protein and RuBisCO synthesis seem to be correlated with the degree of cold stress experienced by the plant. The data from our present experiment argues for a similar correlation between RuBisCO and cold sensitivity/tolerance in rice. Our results do not fundamentally disagree with those of Hahn and Walbot's (1989). Firstly, the difference lies in the focus of our experiment, which detailed clear and significant differences in the phosphorylation patterns among the rice cultivars used, at an early time period of 6 h and an extreme cold stress condition of 5°C. Secondly, the possibility of varietal/cultivar differences cannot be overlooked, especially with respect to the highly sensitive phosphorylation assay used in our experiments. Further experiments will be needed to show the intriguing relationship between cold stress and RuBisCO synthesis and/or phosphorylation using a large number of cold-tolerant rice varieties from the Japonica-type rice, after both a short and extended cold stress. It would be particularly interesting to study the cold-tolerant Indica-type rice varieties, given the local

cultivation of Indica-type rice in the cold Himalayan belt of the Indian subcontinent.

Thus, it can be concluded on the basis of these experiments, that rice types growing in temperate or cold climates are more sensitive to cold stress and characterized by an early fragmentation of the RuBisCO LSU and pp35 phosphorylation than rice types growing in tropical or hot regions. This early effect on the RuBisCO LSU and pp35 phosphorylation in the leaves of rice variety 'Kitaibuki' might be related in a way yet unexplained to the cold-tolerating ability of this cultivar.

**Acknowledgments** We wish to thank Dr. A. Kato for encouraging the initiation of this study.

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