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Comparative studies of chilling stress on alterations of chloroplast ultrastructure and protein synthesis in the leaves of chilling-sensitive (mungbean) and -insensitive (pea) seedlings

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Abstract. The effects of chilling temperature at 4°C on 7 day-old light-grown seedlings of mungbean (*Vigna radiata* L.) and pea (*Pisum sativum* L. cv. Taichung No.9) were different. Exposure of mungbean seedlings to 4°C under light resulted wilting in leaves within 3 hours, and dried after 2 days treatment. However, under the same condition, pea seedlings showed no significant changes in leaves. In addition to these morphological changes, the chloroplast ultrastructure and patterns of protein synthesis were altered by chilling. Ultrastructural changes of chloroplast occurred in chilling-sensitive (mungbean) plants were of obvious by a short period low temperature treatment with accumulation of starch granules. This symptom did not occur at 4 days after chilling-insensitive pea plants. Prolonged treatment of pea with the low-temperature, the distoration and swelling of thylakoids, the formation of small vesicles and the rupture of chloroplasts started to occur. Electrophoretic patterns of soluble proteins form pea showed that a 19 KD protein associated with thylakoid membrane was synthesized under 4°C. However, this protein was not synthesized in mungbean unless the mungbean seedlings were cold acclimated at 8°C previously.

Key words: Chilling stress; Chloroplasts; Cold shock proteins; *Pisum sativum* seedlings; Ultrastructure; *Vigna radiata.*

Introduction

Chilling injury has been defined as injury at temperature low enough to cause damage but not cause freezing of water (Levett, 1980). Consideration of the time frame during which symptoms of chilling injury develop is important in determining the metabolic cause of the injury. These symptoms can either occur rapidly (within several hours) or be delayed (several hours to several days) depending on the severity and the duration of exposure to the chilling temperature and on the species of plants. Analysis of changes occurring in plants during chilling stress is expected to be usefull for understanding the mechanisms underlying freezing resistance and for devising means to improve freezing resistance of plants. Although it has been proposed that metabolic changes associated with chilling stress or cold acclimation of plants originate from the altered gene expression which relate to the acquisition of cold resistance (Weiser, 1970), there is still a general paucity of information on this subject.

There is a general agreement now that cell membranes are a principal site of susceptibility to environmental stress (Steponkus, 1984). The effects of light on

Abbreviations: BSA, Bovine Serum Albumin; EDTA, Ethylenediaminetetraacetic acid, EGTA, Ethyleneglycol-bis (β-aminoethylether) N, N, N′, N′-tetraacetic acid; PAGE, Polyacrylamide gel electrophoresis; PMSF, Phenylmethylsulfonyl fluoride; SDS, Sodium dodecyl sulfate; Tris, Tris (hydromethyl) amino methane.

chloroplast ultrastructure and development have been investigated in various pigment-deficient mutants (Homann and Schmid, 1967). Rapid changes occurring in ultrastructure or size of chloroplasts by light have also been described. However, the relationship between ultrastructural changes and cold resistance is still unknown.

In order to have a better comprehension of response to chilling stress in chilling-sensitive (mungbean) and -insensitive (pea) seedlings, we attempted to comparative studies of chilling effects of these two different plants on ultrastructural alterations in chloroplasts and changes in protein synthesis in leaf discs when they were exposed to chilling temperature (4°C) in light or darkness.

Materials and Methods

Plant Materials

Seeds of mungbean (*Vigna radiata* L.) and pea (*Pisum sativum* L. cv. Taichung No. 9) were immersed in running water overnight and then grown in vermiculite at 28°C for 7 days under light (12,000 lux). Seven-day old light grown seedlings were transferred to the low temperature growth chamber for various periods of time in light or darkness for chilling treatment. The seedlings grew continuously at 28°C in light or darkness as control.

Microscopy

Small leaf discs (1 mm^2) were fixed in 4% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h at 4°C, washed in two changes of cacodylate buffer (15 min, each), and postfixed for 2 h in 2% (w/v) OsO_4 in cacodylate buffer. The specimens were dehydrated through acetone for Spurr's resin. Thin sections (pale gold-silver reflectance) of the specimens were cut with glass knife with a MT-1 ultramicrotome and were mounted on copper grids for Hitachi-600 transmission electron microscopy.

Protein Labelling with ^{35}S-methionine

Leaf discs (1 cm in diameter) were harvested by cork border from leaves of control (grown at 28°C) or pre-chilled at 4°C for 24 h seedlings. Proteins were labeled by incubation of leaf discs in a buffer (5 mM potassium phosphate buffer, pH 6.0, 0.01% (v/v) Tween -20 and 1% (v/v) sucrose) containing ^{35}S-methionine (98.4 μCi/ml). After 3 h of incubation in shaking water bath at 28°C (control) or at 4°C (chilling treatment) under constant illumination (200 $J \cdot m^{-2} \cdot S^{-1}$), leaf discs were rinsed with 1 mM nonradioactive methionine. The ^{35}S-methionine-labeled leaf discs were used for isolation of chloroplasts or extraction of total proteins.

Isolation of Chloroplasts and Thylakoid Membranes

Leaf discs were homogenized by mortar and pestle with cold grinding medium containing 50 mM Hepes -KOH buffer (pH 8.3), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5% (w/v) BSA, 2 mM EGTA, 350 mM sorbitol and 4 mM ascorbic acid. The resulting homogenate was filtered through Miracloth and the filtrate was centrifuged at 1,500 xg for 3 minutes. The pellets were resuspended with the grinding medium, overlayed onto a discontinuous Percoll density gradient (3 ml of 20%, 40%, 60%, and 80% of each in a 15 ml of glass centrifugal tube) and centrifuged at 13,200 xg for 7 min. The procedures for isolation of intact chloroplasts were followed as described by Fish and Jagendorf (1982). The chloroplasts-enriched pellets were then swollen by suspension on five volume of distilled water. The thylakoid membrane was pelleted by centrifuged at 10,000 xg for 10 min.

Extraction of ^{35}S-proteins from Leaf Discs, Chloroplasts, and Thylakoid Membrane

For extraction of total proteins from leaf discs, 12 leaf discs were frozen with liquid nitrogen and were grinded into fine powder by mortar and pestle. The fine powder was mixed with 2 ml of protein extraction buffer containing 50 mM Tris-HCl buffer (pH 8.5), 2% SDS, 2% 2-mercaptoethanol and 1 mM PMSF and then incubated at 37°C for 1 h. The protein extract was collected by the centrifugation at 13,000 xg for 30 min and the supernatant was filtered through one layer of Miracloth.

The proteins in chloroplasts and thylakoid membranes were solubilized in a SDS-sample buffer containing 12% (w/v) sucrose, 50 mM Tris-HCl (pH 8.5), 2% (w/v) SDS and 50 mM dithioreitol. For SDS -gel electrophoresis, the protein extracts were precipitated with 5 volumes of acetone and stored at -20°C overnight. The precipitates were pelleted, dried, and dissolved in SDS sample buffer for gel electrophoresis.

Electrophoresis and Fluorography

An equal amount of protein sample (in radioactivity, cpm) was loaded on 12.5% SDS-slab gels (1.2 × 120 × 140 mm) with a 3.5% stacking gel and were subjected to electrophoresis under constant current of 25 mA per gel according to the method of Laemmli described (1970). At end of electrophoresis, the gels were removed and then fixed with 30% (v/v) methanol, 10% (v/v) TCA and 10% (v/v) acetic acid. The gels were immersed in EN^{3}HANCE (New England Nuclear) for 30 min, washed with water, and then were dried on Whatman filter paper by gel drier (BioRad Model 224). The protein patterns in gels were visualized by fluorography at -70°C on X-ray film (Dupont Cronex film).

Results

External Changes of Leaves in the Seedlings after Chilling Treatment

The effects of chilling temperature (at 4°C) on the morphology of leaves in the mungbean and pea seedlings were different. The most common visible symptom of chilling injury in mungbean seedlings was loss water, resulted in severe wilting during the chilling exposure. The chilling injury was more severe under light than in the dark during chilling treatment. If the chilling temperature has been sustained, death of the whole plant occurred. However, under the same condition, pea showed no significant effects (data not shown).

Ultrastructural Alterations of Chloroplasts in Leaf Cells of Mungbean and Pea Seedlings by Chilling Treatment

The ultrastructural changes of chloroplasts between the chilling-sensitive and -insensitive plants under 4°C chilling treatment were also different. The developmental changes of chloroplast ultrastructure in mungbean under low temperature treatment up to 24 hours are shown in Figs. 1 to 3. Ultrastructural changes occurred in chilling-sensitive seedlings (mungbean) firstly with accumulation of starch grains in chloroplasts; secondly the distoration of chloroplasts progressed in these plants when the chilling treatment was being continued. Prolonged low-temperature treatment of mungbean resulted in hydrolysis of the accumulated starch grains after a lag of 24 hours. When the starch grains were completely disappeared, the thylakoid membranes packed more close each other to reduce the interspace between them and the chloroplasts, as a whole, began to swell. While the swelling of the chloroplasts progressed, the thylakoid interspaces dilated markedly.

The effects of low temperature on the chloroplast ultrastructure in pea are shown in Figs. 4 to 6. Firstly, we observed starch grains accumulation at first 24 hours; secondly, the arrangement of thylakoid membranes in the chloroplasts remained normal. But under the long duration (more than 4 days) of chilling treatment, the chloroplasts became swelling and thylakoid membranes were dilated (data not shown). The differ-

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Fig. 1a. TEM of leaf cells from the 7-day old light grown mungbean seedlings at 28°C without chilling treatment (as control). The thylakoid membrane in chloroplasts were in normal regular arrangement. bar = 1 μm.

Fig. 2a. TEM of leaf cells from the 7-day old light grown mungbean seedlings at 28°C and then were chilled at 4°C for 3 h in light. bar = 1 μm.

Fig. 3a. TEM of leaf cells from the 7-day old light grown mungbean seedlings at 28°C and then were chilled at 4°C for 24 h in light. The starch granules accumulated in the chloroplasts were reduced. bar = 1 μm.

Figs. 1b, 2b, and 3b are a portion of grana magnified from a chloroplast of Figs. 1a, 2a and 3a, respectively. bar = 0.1 μm.

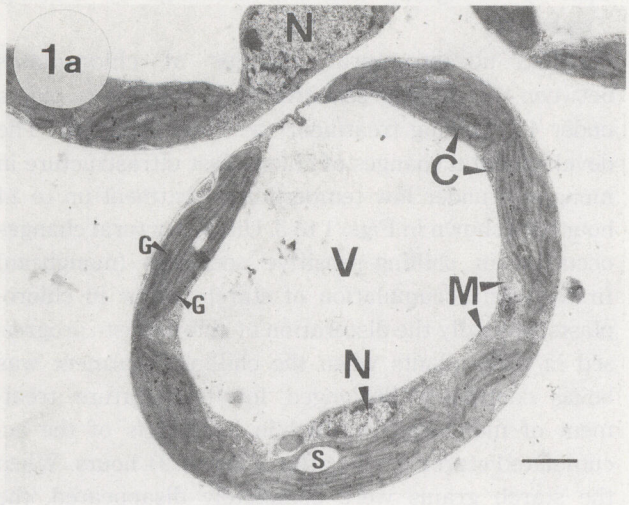
Fig. 4a. TEM of leaf cells from the light-grown 7-day old pea seedlings (as control). The starch granules were accumulated in the chloroplasts. bar = 1 μm.

Fig. 5a. TEM of leaf cells from the light-grown 7-day old pea seedlings at 28°C and then were chilled at 4°C for 3 h in light. bar = 1 μm.

Fig. 6a. TEM of leaf cells from the light-grown 7-day old pea seedling at 28°C and then were chilled at 4°C for 24 h in light. bar = 1 μm.

Figs. 4b, 5b, and 6b are a portion of grana magnified from a chloroplasts of Figs. 4a, 5a, and 6a, respectively. bar = 0.1 μm.

(Abbreviations in the figures: C: Chloroplast; CW: Cell wall; G: Granum; M: Mitochondrion; N: Nucleus; S: Starch granule; V: Vacuole.)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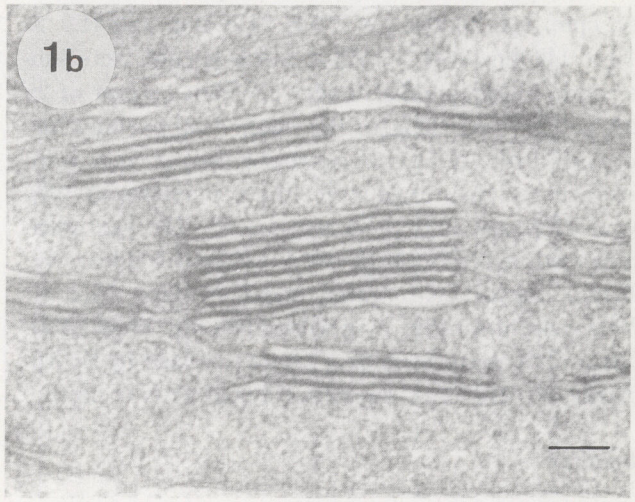
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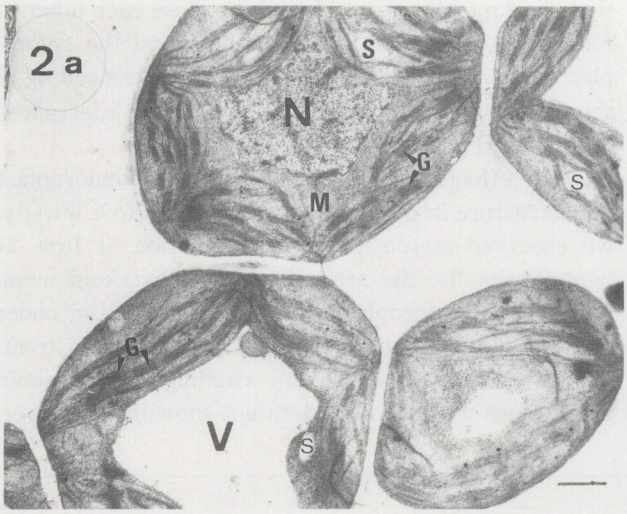
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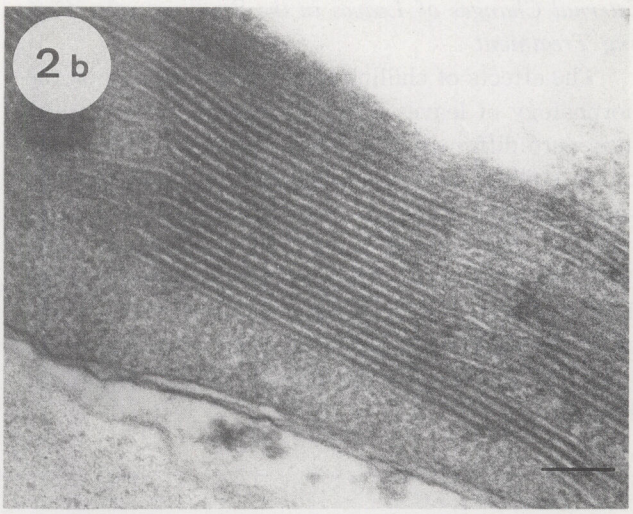
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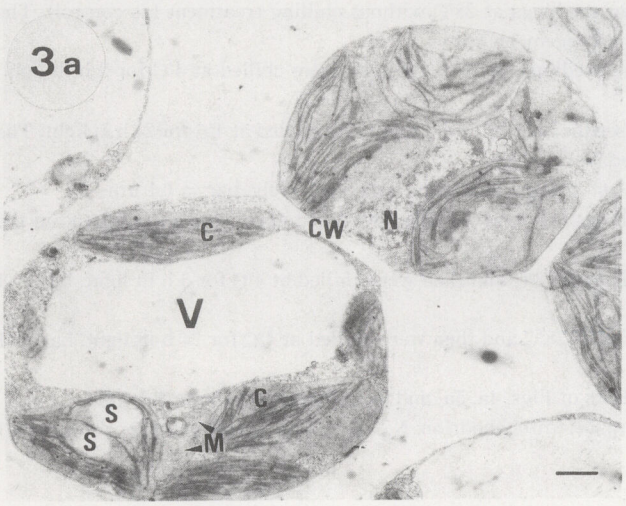
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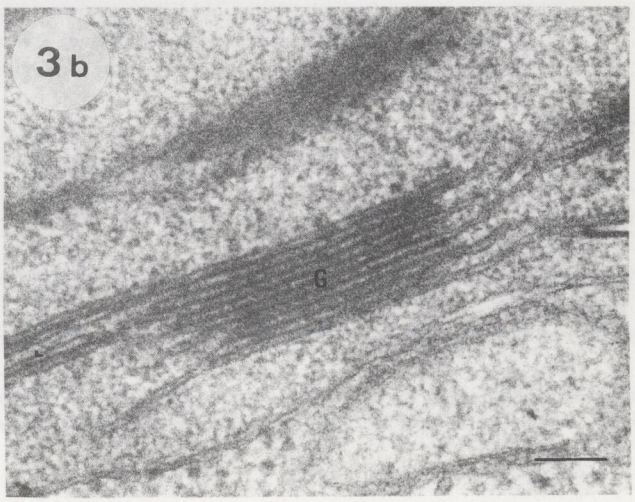
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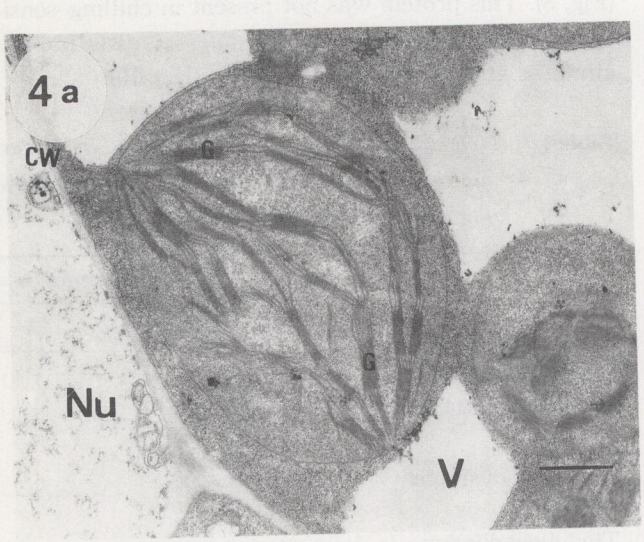
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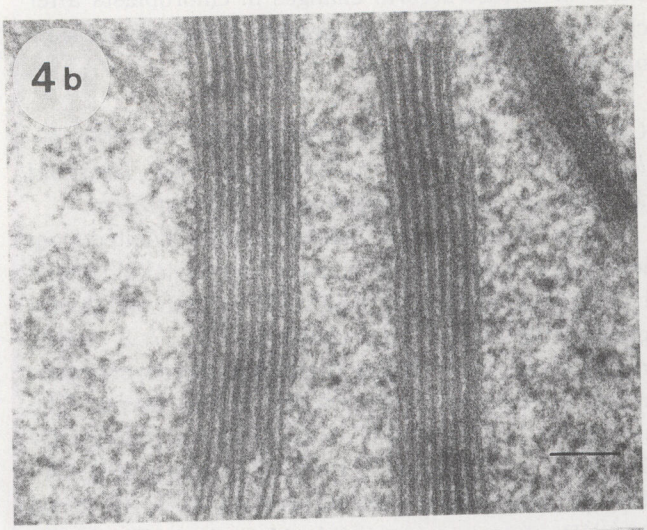
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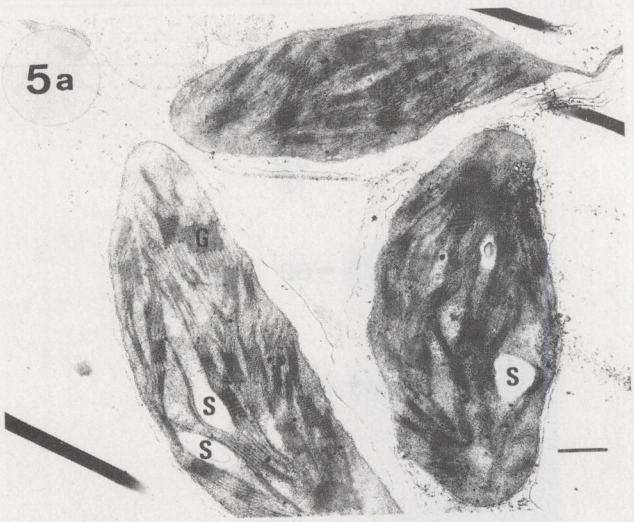
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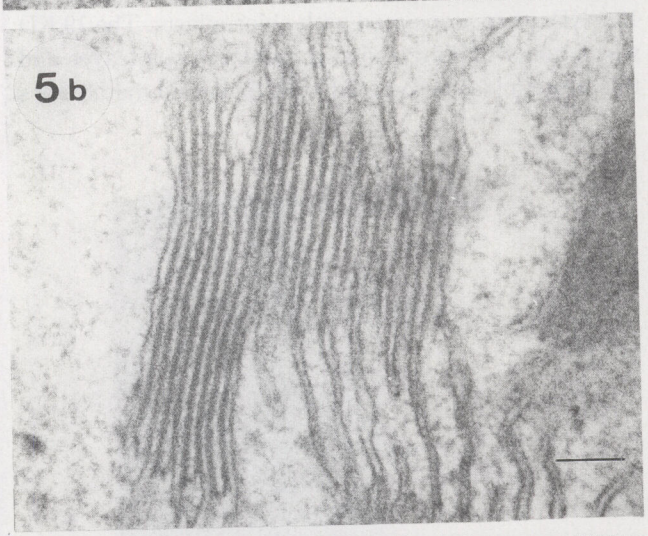
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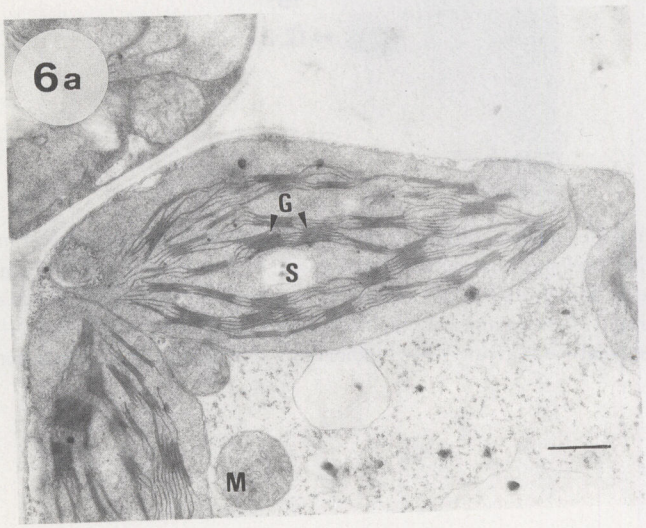
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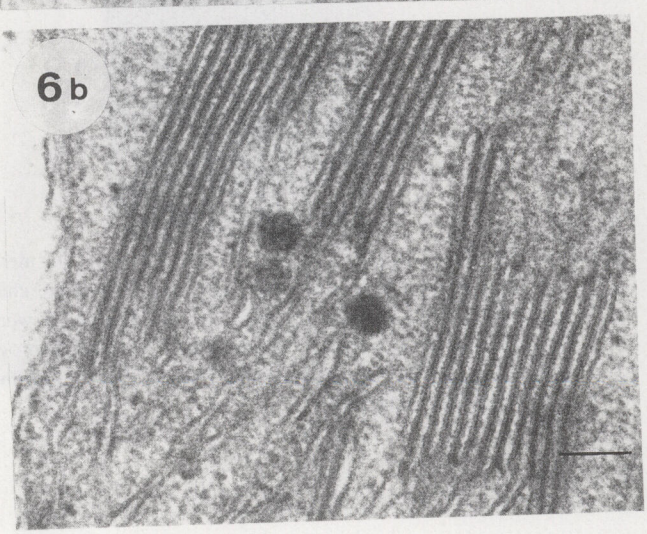
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ences of ultrastructural changes in chloroplasts after chilling treatment between pea and mungbean are summerized in Table 1.

Comparison of ^{35}S-methionine Labeled Proteins Isolated from Leaf Discs, Chloroplasts, and Thylakoid Membranes between Mungbean and Pea Seedlings

The ^{35}S-methionine-labeled proteins isolated from leaf discs, isolated chloroplasts, and thylakoid membranes were analyzed by 12.5% SDS-PAGE and fluorography, the results were shown in Figs. 7, 8, and 9, respectively. After chilling treatment, there were seven newly synthesized protein bands in pea leaves. The molecular weight of these chilling-induced proteins are 78, 58, 48, 38, 27, 19 and 12.3 KD, respectively. (Fig. 7A). Five of these chilling-induced proteins, 78, 58, 48, 19 and 12.3 KD, were associated with chloroplasts. The 19 KD was a major chilling-induced protein in pea leaves and this protein was associated with thylakoid membranes (Fig. 8). This protein was not present in chilling-sensitive mungbean leaves (Fig. 9), unless it was pre-acclimated at 8°C for at least 24 hours (Fig. 10).

Table 1. *Time course study of chilling injury in 7-day -old light grown mungbean and pea seedlings after chilling treatment at 4°C in light*

	Time of 4°C-chilling exposure (h)				
	3	6	9	12	24
Leaf wilting				----------	
Appearance of starch grains			———		
Disappear of starch grain					----------
Thylakoid dilation	———	———	———	———	———
Swollen of plastids		----------	----------	----------	----------

---------- Mungbean; ——— pea.

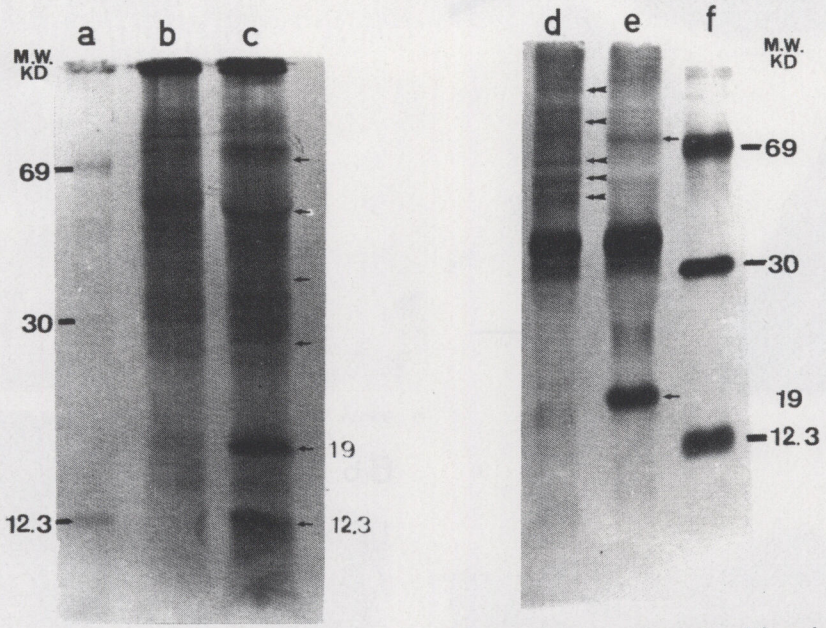

Fig. 7. 12.5% SDS-PAGE and fluorography analysis of ^{35}S-methionine-labeled proteins isolated from leaf and thylakoid membrane in pea seedlings. Leaf discs from 28°C control and 4°C-chilled seedlings for 24 h were labeled with ^{35}S-methionine at 28°C (b and d) or at 4°C (c and e) in light for 3 h. The ^{35}S-proteins were isolated from leaf discs (b and c) and thylakoid membrane (d and e) and then were analyzed by 12.5% SDS-PAGE and fluorography. Lanes a and f are the molecular weight standards. Arrow "←" indicated an increase of protein amounts after 4°C-chilled treatment; arrow " ⟵ " indicated a decrease of protein amounts after chilled treatment.

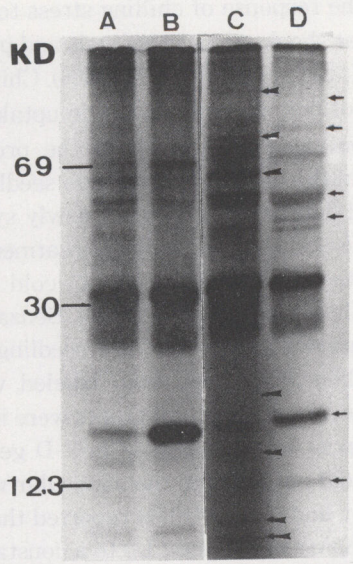

Fig. 8. Analysis of ^{35}S-proteins isolated from pea chloroplasts and chloroplasts and thylakoid membrane by 12.5% SDS-PAGE and fluorography. A, B: from thylakoid membrane; C, D: from chloroplasts; A and C: from 28°C control; B and D: from 4°C-chilled chloroplasts. The symbols indicated in the figure are same as in Fig. 7.

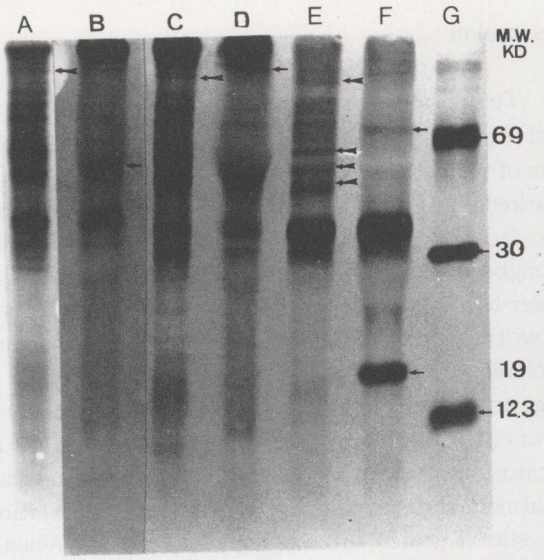

Fig. 9. Comparison of ^{35}S-proteins isolated from chloroplasts and thylakoid membranes of mungbean and pea analyzed by 12.5% SDS-PAGE and fluorography. A, B: Total chloroplast proteins from mungbean; C, D: Thylakoid membrane proteins from mungbean; E, F: Thylakoid membrane proteins from pea. A, C, and E were the 28°C control, B, D, and F were from 4°C-chilled leaves. The symbols indicated in the figure are same as in Fig. 7.

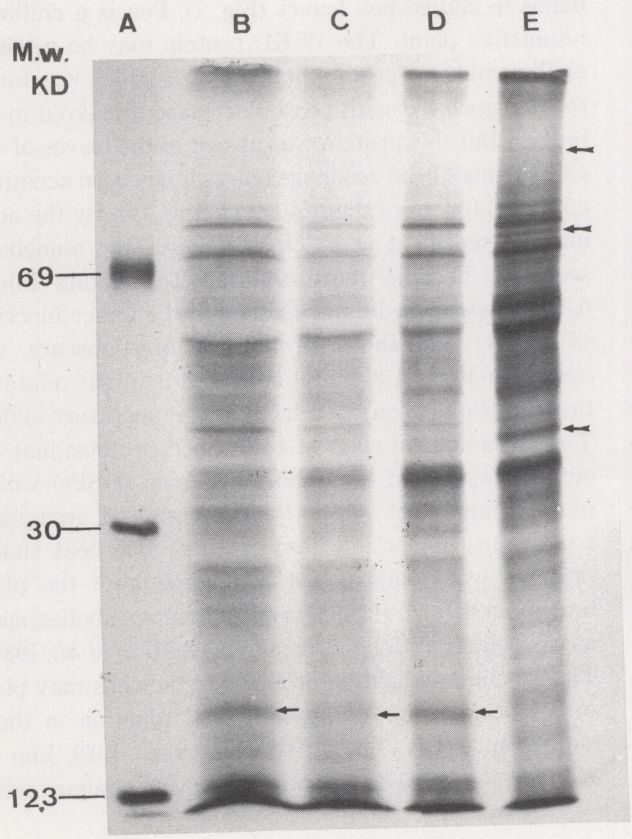

Fig. 10. Effects of 8°C-acclimation on the protein synthetic patterns in leaf discs of mungbean seedlings. After each of the following treatment, leaf discs were labeled with ^{35}S-methionine at 4°C for 3 h except in E at 28°C. ^{35}S-proteins were extracted from leaf discs and then were analyzed by 12.5% SDS-PAGE and fluorography. A: molecular weight standards; B: 8°C, 2 days→4°C, 1 day; C: 8°C, 2 days→28°C, 1 day→4°C, 1 day; D: 8°C, 2 days and were labeled with ^{35}S-methionine at 8°C for 3 h; E: 8°C, 1 day→28°C, 1 day. The symbols indicated in the figure are same as in Fig. 7.

Discussion

Temperature is one of the major environmental factor governing the growth, development and distribution of plants. Tropical and subtropical plants exhibit a marked physiological dysfunction when exposed to low non-freezing temperature below about 10°C to 12°C. Mungbean is a chilling-sensitive plant. When it grew under light and 4°C conditions for 12 h, the leaf appeared wilting (Chen, unplublished data). The ultrastructural changes in chloroplasts of mungbean leaf under chilling treatment indicate that plants obviously accumulated starch granules in a short period of low temperature treatment, but the prolonged low temperature treatment resulted in hydrolysis of the accumulation of the starch grains after a lag of 24 hours. When the starch grains were completely disappeared, the thylakoid membranes moved close each other to reduce the interspace between them and the chloroplasts began to swell (Figs. 1 to 3). The initial contraction of thylakoid interspaces was tentatively ascribed to an increase in the transmembrane hydrogen ion gradient which caused the movement of cations and undissociated organic acids from the thylakoid interspace in the stroma (Taylor and Craig, 1971). The swelling of the chloroplasts induced by the longer chilling treatment may be due to the enzyme responsible for the degradation of starch in the stroma of chloroplasts remaining active. This would lower the water potential of the stroma and caused and influx of water from the cytoplasm (Heldt and Rapley, 1970). The chilling treatment also caused the injury of cell membrane systems and increased the leakage of solutes and water from tissues. This phenomenon is similar to other environmental stress, such as heat shock (Wise *et al.*, 1983; Lin *et al.*, 1985). The envelopes of chloroplasts were disrupted in many places and thylakoid membranes were dilated, stack of grana were titled in relation to each other (Table 1). Dilation is a common feature of chilling injury in chilling-sensitive plants (Forde *et al.*, 1975). Under chilling stress, the accumulation of lipid have also been reported in the cytoplasm (Ilker *et al.*, 1976; Platt-Aloia and Thomson, 1976), in the plastids (Slack *et al.*, 1974) and in the cell wall and the plasmalemma (Platt-Aloia and Thomson, 1976).

Alteration of gene expression during environmental stress in plants has been reviewed by Sachs and Ho (1986). The response of chilling stress to plants is similar to heat shock stress in animals and plants (Schlesinger *et al.*, 1982; Sachs and Ho, 1986). Chilling treatment caused not only a decrease in the uptake of ^{35}S-methionine, but also a decrease in the protein synthetic activity in the etiolated mungbean seedlings (Chen, unpublished data). There were 15 newly synthesized protein bands induced by chilling treatment which coresponded to protein induced at 0°C, cold shock proteins (CSPs, Chen, unpublished data). Benza-Basso *et al.* (1986) reported that when the seedlings of rapeseed were chilled at 0°C and then labeled with ^{35}S-methionine, they found 14 protein spots were increased and 5 protein spots were decreased in 2-D gel fluorograms. But they did not indicate the molecular weight of these CSPs. Guy and Carter (1984) reported that exposure of spinash (*Spinacia oleraceia* L.) to a constant 5°C for several days induced a greater tolerance to a lower freezing temperature at -12°C. This treatment induced 5 newly synthesized protein bands in the leaves. The molecular weight of these proteins were 110, 82, 66, 55, and 13 KD, respectively. In the present study, we found three protein bands are similar to those of Guy and Carter (1984) among seven newly synthesized protein bands in chilled pea leaves (Fig. 7). Pea is a chilling-insensitive plant. The 19 KD protein may be a major chilling-induced protein in pea leaves which was found to be associated with pea chloroplast thylakoid membranes. But this protein was absent in the leaves of 4°C chilled-mungbean seedlings unless it has been acclimated at 8°C for more than 24 hours (Fig. 10). By the acclmation treatment at 8°C for 24 hours, the mungbean seedlings became more tolerant to chilling injury (Chen, unpublished data). Although the exact function of 19 KD cold shock protein remains obscure, the results of Figs. 7, 8, and 9 indicated it might relate to provide plants protection from low temperature injury. The physiological roles of cold shock proteins may be similar to that of heat shock proteins (HSPs) which play an important role in the protection of organisms from injury under low temperature. The heat shock proteins was found to be associated with the plasmalemma (Lin *et al.*, 1985) and cellular organelles, such as ribosomes, mitichondria, and nuclei (Lin *et al.*, 1984). The special localization of HSPs in the cells may play some special roles of physiological function in thermotolerance (Lin *et al.*, 1984; Chou *et al.*, 1989; Jinn *et al.*, 1989). Cold harden plants may have a similar mech-

anism which enables them to synthesize cold-tolerance proteins for specific localization. The presence of 19 KD protein in the thylakoid fraction suggests possible regulatory role associated with the metabolic changes known to occur during the onset of cold hardening (Perras and Sarhan, 1984). It is also possible that this protein may be involved in the processes leading to the plasma membrane alternations observed during cold hardening (Lynch and Steponkus, 1987).

Investigations are under way at the level of gene expression to understand the molecular and genetic basis of cold tolerance and the possible roles of the induced proteins during cold hardening.

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低溫逆境對耐寒和不耐寒植物之葉部葉綠體細微構造與蛋白質合成的比較研究

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在光照下七天的綠豆和豌豆幼，若同時放在 4°C 低溫下，其影響是不同的。綠豆苗在 4°C 光照下經三小時後就開始枯萎，兩天後整棵植株乾枯而死；但在相同條件下，豌豆則無顯著之變化，僅植株之生長較矮小。在低溫逆境下，植物幼苗除外部形態改變外，葉綠體的細微構造及蛋白質合成的類型亦會發生改變。以不耐寒的綠豆苗爲例，在低溫 (4°C) 下經 9 小時，葉綠體會有明顯的澱粉粒累積，但耐寒性的豌豆幼苗在同溫度下經過四天並不會發生此現象。但豌豆幼苗若長時間處於低溫 (4°C) 環境下，葉綠體內的類囊體 (thylakoids) 亦會變形及膨大，並形成小胞囊及破裂。從 4°C 低溫處理過的豌豆葉片所分離的可溶性蛋白質，在聚丙烯醯胺膠體電泳圖上有一條含量甚多的 19 KD 蛋白質帶，它會與類囊體結合，但綠豆幼苗在 4°C 溫度下並不會產生此蛋白質帶；除非綠豆幼苗先在 8°C 下做先前冷馴化處理。