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Changes in Protein Synthesis Induced by Chilling in Tomato Chloroplasts

Won-Il Kim*, Goo-Bok Jung, Min-Kyeong Kim, Kwang-Lai Park, Sun-Gang Yun
National Institute of Agricultural Science and Technology, RDA, Suwon 441-707, Korea
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Abstract : To find out the effect of low temperature on the regulation of tomato chloroplast genes, the optimization of the system in chloroplast protein synthesis and the identification of the changes in chloroplast protein synthesis induced by chilling were studied. Incorporation reaction occurred rapidly at the first 30 minutes and was constantly maintained after 60 minutes. A broad optimal temperature on protein synthesis was found around 20 to 30°C. No difference was shown in the chloroplast protein synthesis under high light intensity (1600 $\mu E/m^2/s$) as well as under low light intensity (400 $\mu E/m^2/s$) even darkness. K^+, Mg^{++} and ATP at an optimal concentration act as an activator, while DTT, chloramphenicol, cycloheximide, Ca^{++} and inorganic phosphate act as an inhibitor in the chloroplast protein synthesis. Synthesis of 15, 55 and 60 kd chloroplast encoded stromal proteins and 18, 24, 33 and 55 kd chloroplast encoded thylakoid membrane proteins were reduced by chilling, while 17 kd chloroplast encoded stromal protein and 16 kd chloroplast encoded thylakoid membrane protein was induced by chilling. It was expected that the 55 kd stromal protein would be the large subunit of rubisco and the 33 kd thylakoid membrane protein would be the D1 protein which was drastically reduced by chilling.

Key words : chilling, chloroplast, protein synthesis, tomato

INTRODUCTION

In chill sensitive plants, the exposure to low nonfreezing temperature (0<T<12°C) has adverse effects on growth[1,2]. Photosynthesis is one of the major metabolism inhibited. From the previous studies, Martin and Ort[2,3] concluded that the capacity of net photosynthesis in tomato seedling was inhibited about 60% by 16 hours of chilling in the dark and was more rapidly inhibited by chilling under the high light intensity (1000 $\mu E/m^2/s$). The majority of the inhibition on photosynthesis caused by chilling was the result of impaired chloroplast function.

Ort and his colleagues[4,5] also previously studied the effects of dark chilling on protein synthesis in chill sensitive tomato leaflets toward identifying potential sites of reduced net photosynthesis by chilling. They concluded that this reduction might be related with decreases in photosystem I and photostem II photochemical activities and changes of specific protein synthesis, such as a chlorophyll a/b binding protein of photosystem II, a novel 35 kd protein and other 3 proteins, and enzyme activity of Ribulose bisphosphate carboxylase/oxygenase (Rubisco), Fructose 1,6-bisphosphatase (FBPase), and photosynthetic stromal proteins.

However, the effect of low temperature on chloroplast gene expression has not been analyzed in detail. The impaired chloroplast function might be shown to involve the changes in gene expression of chloroplast genome. Thus, we are interested in assessing the changes specifically in only chloroplast DNA encoded proteins following chilling. As a first step in understanding the changes of chloroplast gene regulation using intact chloroplast by chilling, our works involve that the optimization of the *'in organello'* protein synthesis with intact chloroplasts, and the identification of the changes in chloroplast encoded chloroplast protein synthesis induced by chilling.

Corresponding author :
TeL : +82-31-290-0206 Fax : +82-31-290-0277
E-mail : wikim@rda.go.kr

MATERIALS AND METHODS

Plant materials

Tomatoes (*Lycopersicon esculentum* mill. cv Floramerica) were

grown under 14 hours, 30°C light period (400 $\mu E/m^2/s$) and 10 hours, 20°C dark period in growth chamber. When plants were 3.5 weeks old without stress, they were treated for 6 hours at 4~6°C and 1000 $\mu E/m^2/s$ as a light chilled leaflets and for 6 hours at 30°C and 1000 $\mu E/m^2/s$ as a light control leaflets, respectively[4].

Intact chloroplast isolation

Approximately 5 g of tomato leaflets were diced and briefly polytroned (1~3 sec bursts) in 100 mLs of ice-cold extraction buffer composed of 330 mM Sorbitol, 50 mM Tricine/KOH (pH 7.6), 10 mM NaCl, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM EDTA, 1% PVP-40, 0.5% BSA, 0.2% Na-ascorbate and 0.1% DTT. The homogenate was rapidly filtered through 4 layers of miracloth into 50 mL centrifuge tubes and centrifuged in a swinging bucket rotor at 1200 g for 2 minutes. The pellets were gently resuspended using a camel hair brush in a small volume of resuspension medium composed of 330 mM sorbitol, 50 mM Hepes-KOH (pH 7.6), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.5% BSA. The resuspended chloroplasts were then carefully layered over a discontinuous 40%/80% percoll gradient in resuspension buffer. The tubes were spun for 2 minutes at 1200 g, intact chloroplasts carefully removed from the 40%/80% interface, and washed and centrifuged once again in resuspension buffer. The pellet of intact chloroplasts was gently resuspended in a small volume of ice-cold resuspension medium[6]. Chlorophyll concentration was determined using the specific absorption coefficient for chlorophyll a and b determined by Graan and Ort[7].

Protein synthesis in intact chloroplasts

The basic protein synthesis mixture contained 330 mM sorbitol, 50 mM Hepes/KOH (pH 8.0), 10 mM $MgCl_2$, 60 mM KCl, 5 mM DTT, 10 mM ATP, 40 μM amino acid (-methionine), 70 μCi [^{35}S]-methionine and intact chloroplasts at 50 nmol chlorophyll. The mixture was incubated at room temperature for 30 minutes followed by centrifugation at 1200 g, 4°C for 1 minute to pellet chloroplasts. The supernatant was discarded, the pellet dissolved in 50 mL of ice-cold 0.02 M Na_2CO_3 solution containing 0.02 M DTT, the chloroplasts lysed by vigorous vortexing, and the suspension microfuged for 2 minutes. The supernatant was saved as the stromal fraction and the pellet was saved as the thylakoid fraction after 1~2 additional washings with 0.02 M Na_2CO_3 solution containing 0.02 M DTT[6].

Electrophoresis and autoradiography

The newly synthesized stromal and thylakoid protein samples were denatured in 2X laemmli's buffer with heating at 70°C for 2 minutes. Any insoluble material was then removed by microfuging. The amount of radioactivity incorporated into protein was determined by TCA precipitation technique. Between 20~80,000 cpms were loaded per lane on a 12~17.5% linear gradient sodium dodecyl sulfate polyacrylamide gel and the gel run at 17 mA for 16~17 hours. The gels were stained in 0.2% Coomassie blue in methanol, d-H_2O, glacial acetic acid (5:5:2, V/V/V) for 2~3 hours and destained in the same solution minus Coomassie blue until background is minimal. After the gel-drying, radioactively labeled proteins on the gel were visualized by autoradiography[4].

RESULTS & DISCUSSION

Optimization of the *'in organello'* protein synthesis with intact tomato chloroplasts

Effect of light intensity and temperature on chloroplast protein synthesis

Plant cells contain an unique organelle called the chloroplasts. Chloroplasts have a complete genome capable of expression of the genetic information in their DNA, like nuclei and mitochondria. Because the existence of such genetic systems in chloroplasts raises some questions as to their functions in photosynthesis, *'in organello'* protein synthesis using intact chloroplasts isolated from several plant leaves has been studied extensively and reviewed recently[8,9].

There are many general factors affecting chloroplast protein synthesis. For example, the effects of light, temperature, pH, monovalent and divalent cation, ATP and amino acid concentration, inorganic phosphate and polyamines on the chloroplast protein synthesis were investigated from several plants[6,8,10]. Using intact tomato chloroplasts isolated from 40~80% percoll gradient centrifugation, the optimal condition of the chloroplast protein synthesis in reaction mixture was examined.

In order to investigate the effect of light intensity and reaction temperature in chloroplast protein synthesis, reaction mixtures under the different light intensity (0, 400, 800, 1200 and 1600 $\mu E/m^2/s$) and different reaction temperature (5, 15, 25, 35 and 45°C) were treated. Fig. 1 shows that the TCA incorporation of [^{35}S]-methionine into protein at the different reaction time (A), reaction temperature (B) and light intensities

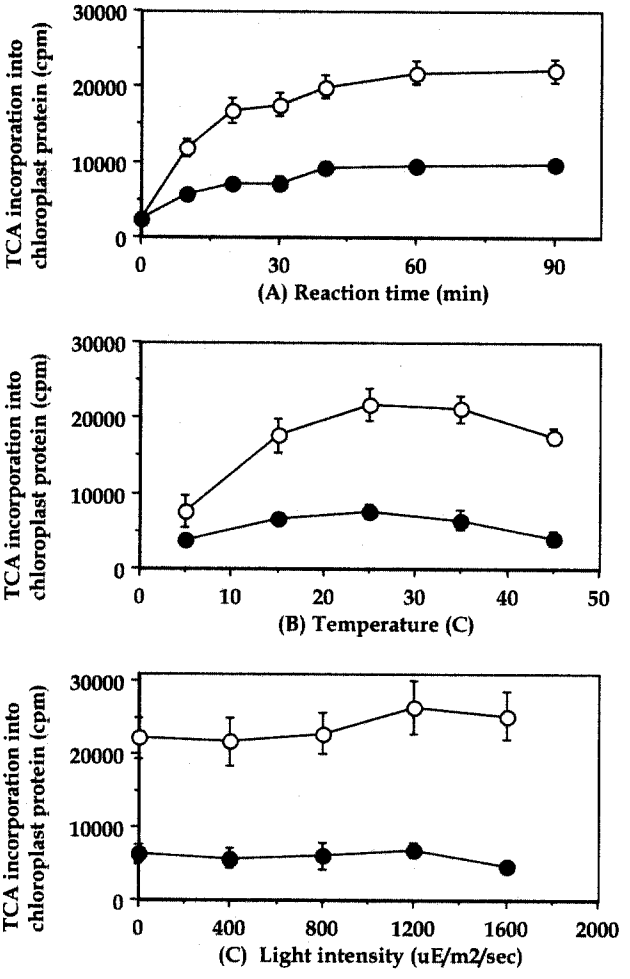

Fig. 1. Changes of *'in organello'* protein synthesis in intact chloroplasts isolated from tomato leaves at the different reaction time(A), reaction temperature(B) and light intensity(C). TCA incorporation cpm into [^{35}S]-methionine labeled chloroplast thylakoid membrane (*open*) and stromal(*closed*) protein were determined. Basic reaction condition was 30 minutes for reaction time, 25°C for reaction temperature, and 1000 μE/m^2/s for light intensity. The data means the average of three measurements + standard error.

(C). Incorporation reaction occurred rapidly at the first 30 minutes and was constantly maintained after 60 minutes. A broad optimal temperature on protein synthesis was found around 20 to 30°C. No difference was shown in the chloroplast protein synthesis high light intensity (1600 μE/m^2/s) as well as low light intensity (400 μE/m^2/s) even darkness. No difference were shown in the chloroplast stromal protein as well as thylakoid membrane protein synthesis between high light intensity and low light intensity even darkness. Chloroplast protein synthesis in reaction mixture at 25°C was relatively higher than any other reaction temperature. A broad optimum temperature was found between 20 and 30°C. The effect of temperature in chloroplast protein synthesis was greater than that of light intensity.

Effect of components of reaction mixture on chloroplast protein synthesis

In order to investigate the effect of components in chloroplast protein synthesis and improve the rates of TCA incorporation into chloroplast proteins, the relative incorporation of [^{35}S]-methionine into chloroplast stromal and thylakoid membrane protein with and without components in reaction mixture was determined. The control represents the TCA incorporation cpm of 100% and the other lanes represent the relative TCA incorporation cpm into chloroplast protein compared with the control (Table 1).

The rate of chloroplast protein synthesis was increased by K^+ and Mg^{++} of optimal concentration. K^+ at a 60 mM concentration activated 19% of stromal protein and 23% of thylakoid membrane protein. Mg^{++} at a 10 mM concentration activated 59% of stromal protein and 103% of thylakoid membrane protein. However, Ca^{++}, inorganic phosphate and dithiothreitol show as an inhibitor in chloroplast protein synthesis. Ca^{++} at a 20 mM concentration, inorganic phosphate at a 10 mM concentration and dithiothreitol at a 10 mM concentration inhibited TCA incorporation into 55%, 13%, and 15% of stromal protein and 55%, 11% and 27% of thylakoid membrane protein, respectively.

Effect of mono- and di-valent cations on chloroplast protein synthesis

Monovalent and divalent cations are important for protein synthesis by intact chloroplasts. Fig. 2 also shows the relative incorporation of [^{35}S]-methionine into chloroplast proteins with intact tomato chloroplasts at the different concentration of Mg^{++}, K^+ and ATP. Cation concentrations were mentioned to be critical for protein synthesis by chloroplasts. The incorporation into protein does not effect strongly by K^+. However, the effect of Mg^{++} on protein synthesis in intact chloroplast is larger than that of K^+. The protein synthesis was stimulated approximately 50% by additional 10 mM Mg^{++} concentration, but was strongly inhibited by excessive concentration. Stromal

Table 1. Relative TCA incorporation cpm(%) into [^{35}S]-methionine labeled chloroplast thylakoid membrane and stromal proteins in intact chloroplasts isolated from tomato leaves with components in chloroplast protein synthesis reaction mixture.

Proteins	Relative TCA incorporation cpm(%)							
	Control	K 60 mM	Mg 10 mM	Dithiothreitol 10 mM	Chloramphenicol 0.6 mM	Cycloheximide 0.7 mM	Ca 20 mM	PPi 10 mM
Thylakoid protein	100	122±15	203±11	73±3	86±6	33±2	45±4	89±0.3
Stromal protein	100	119±11	159±13	85±2	88±3	53±5	45±2	87±6

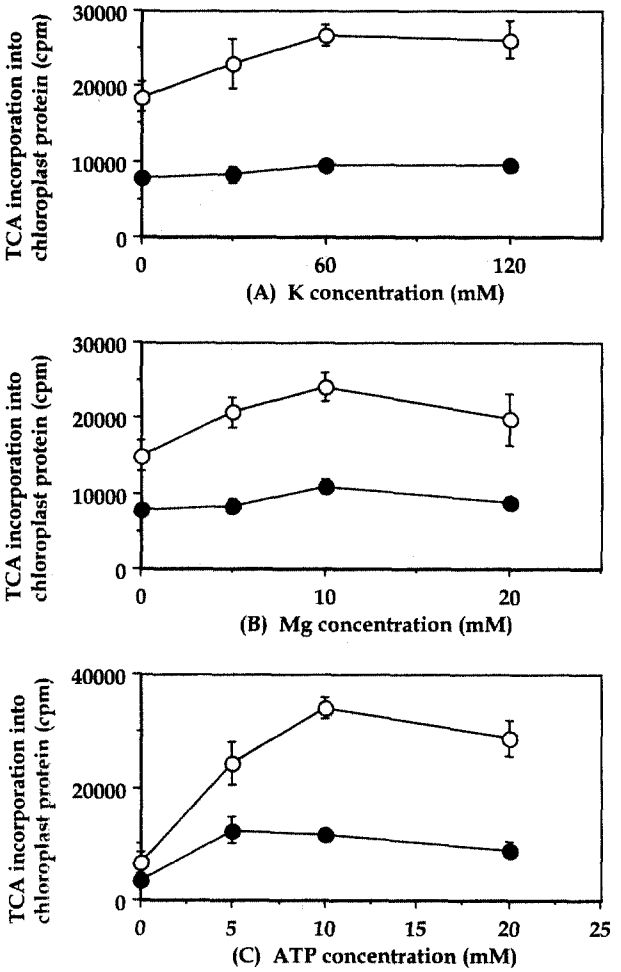

Fig. 2. Changes of protein synthesis in intact tomato chloroplasts isolated from tomato leaves at the different concentration of K^+(A), Mg^{++}(B) and ATP(C). TCA incorporation cpm into [^{35}S]-methionine labeled chloroplast thylakoid membrane protein(*open*) and stromal protein(*closed*) was determined.

protein as well as thylakoid membrane protein shows the same pattern of inhibition. The effect of ATP concentration on protein synthesis is very similar to that of Mg^{++}. An optimum reaction shows at the 10 mM of ATP concentration and about 67% lower at the no ATP.

Bhaya and Jagendorf[11] mentioned that a sharp optimum at 100 mM was found for K^+ with the rate 33% lower at 42 mM and progressive inhibition above 100 mM. Ellis[8] also reported K^+ was strongly required in the reaction mixture at 200 mM concentration. Bottomley[12] also reported the effect of other monovalent cations, such as Na^+ and NH_4^+. NH_4Cl at 80 mM gave the highest rates and NaCl was less effective in stimulating incorporation into chloroplast proteins. Nivison and Jagendorf[10] reported that light-driven protein synthesis in intact chloroplasts was stimulated 25% at 50~300 mM Mg concentration. Fish and Jagendorf[13] worked out the details and established the need for Mg^{++} addition for rapid ATP-driven protein synthesis in the dark. By supplying equimolar amount of Mg^{++} and ATP obtained greater rates of leucine incorporation[9].

Relationship between light and the product of light reaction

Possible relationship between light and the product of light reaction on photosynthesis, such as ATP and NADPH, was investigated (Fig. 3). The result showed that there was no clear difference in chloroplast protein synthesis between with ATP and without ATP in reaction mixture in light condition. However, the rate of chloroplast protein synthesis was rapidly increased in darkness if ATP was added in reaction mixture. ATP was found to be absolutely necessary for chloroplast protein synthesis in darkness. However, NADPH, which is another product of light reaction, did not have any effect in chloroplast protein synthesis. It concluded that light is not neces-

sary in chloroplast protein synthesis if ATP exists in reaction mixture.

High light intensity was used in chloroplast protein synthesis earlier. Bottomley[12] reported that the rates of protein synthesis in darkness in the presence of ATP were only 50% or less than those of the light driven synthesis. However, Nivison and Jagendorf[10] concluded that the total leucine incorporation into chloroplast protein was greater at lower light intensity (20~180 $\mu E/m^2/s$) than at high light intensity, even though the initial rate were shown to be lower at the lower light intensity. They thought the inhibitory effect of high light intensity might be due to damage to electron transport and ATP synthesis.

Function of chloroplast ribosome on chloroplast protein synthesis

In order to determine the function of chloroplast ribosome, two ribosomal inhibitors were added to reaction mixture. Cycloheximide, which inhibits 80S cytoplasmic ribosomes, has little effect on the rate of chloroplast protein synthesis. Cycloheximide at a 0.7 mM concentration inhibited the TCA incorporation of 12% of stromal protein and 14% of thylakoid membrane protein. However, chloramphenicol, which inhibits 70S chloroplast ribosomes, rapidly decreased the rate of chloroplast protein synthesis. Chloramphenicol at a 0.6 mM inhibited the TCA incorporation of 47% of stromal protein and 67% of thylakoid membrane protein (Table 1).

Fig. 4 also show the autoradiographic profile of newly synthesized chloroplast proteins in reaction mixture with cycloheximide and chloramphenicol. Cycloheximide has a little effect on the newly synthesized both chloroplast stromal proteins and thylakoid membrane proteins, while chloramphenicol rapidly inhibited in the chloroplast protein synthesis.

Changes in chloroplast protein synthesis induced by chilling

The exposure to chilling temperature is one of the major adverse effect on growth for several of the economically most significant plants. It has been recently reviewed that the exposure to chilling temperature has a deleterious effect on the several biochemical functions, such as changes in protein content, enzyme activity, metabolic modification and membrane structure[1]. Photosynthesis, which is a central process to plant growth, is one of the major metabolism inhibited. It was also

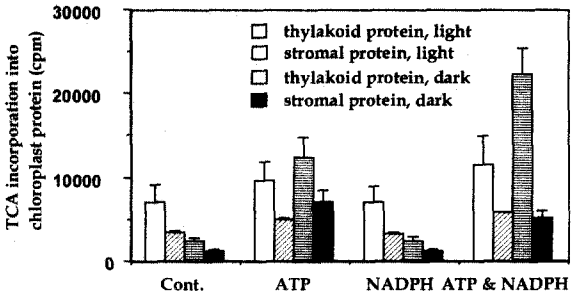

Fig. 3. TCA incorporation cpm into [^{35}S]-methionine labeled chloroplast thylakoid membrane and stromal proteins in intact chloroplasts isolated from tomato leaves with ATP and NADPH in chloroplast protein synthesis reaction mixture. Light in legend means that reaction occurred under 1000 $\mu E/m^2/s$ of light intensity whereas dark means that reaction occurred without light.

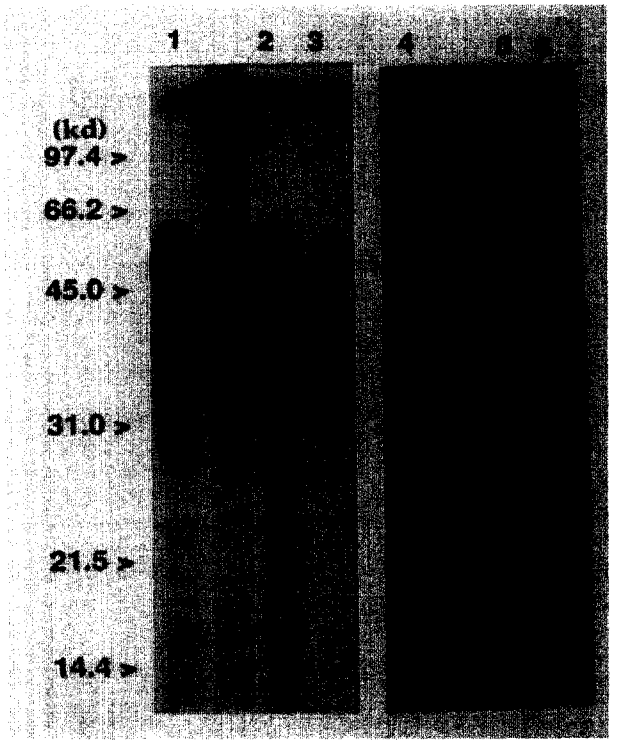

Fig. 4. Autoradiographic profiles of newly synthesized chloroplast stromal proteins and thylakoid membrane proteins treated with chloramphenicol and cycloheximide in intact chloroplasts isolated from tomato leaves. Same amounts of protein incorporation cpm were loaded per lane. Lanes; 1 to 3; stromal proteins, 4 to 6; thylakoid membrane proteins, 1,4; control, 2,5; chloramphenicol (0.6 mM), 3,6; cycloheximide (0.7 mM).

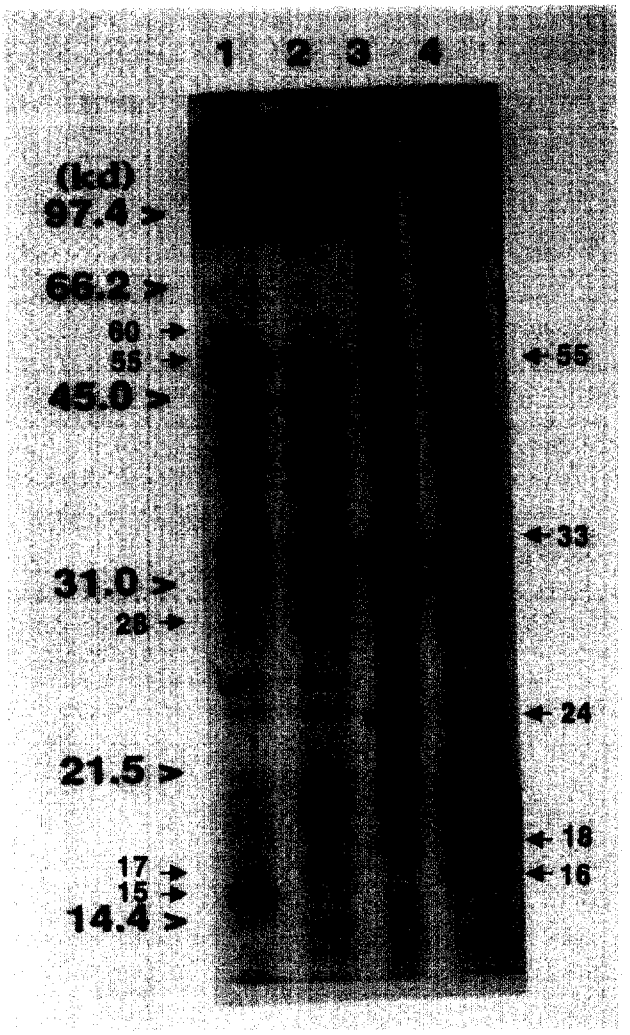

Fig. 5. Autoradiographic profiles of newly synthesized chloroplast thylakoid membrane and stromal proteins in isolated, intact tomato chloroplasts after light control for 6 hours at 30°C and light chilling for 6 hours at 4-6°C. control, stromal proteins (lane 1) : chilling, stromal proteins (lane 2) : control, thylakoid membrane proteins (lane 3) : chilling, thylakoid membrane proteins (lane 4).

reported that the alteration in characteristic spectrum of the photosynthetic stromal proteins, such as NADP malate dehydrogenase and pyruvate Pi dikinase, and the changes of enzymatic kinetics as a genetic adaption may result from effects of chilling and light intensity.

Alteration in protein synthesis induced by chilling has been studied in several plants, such as tomato[4,14,15], rice[16], wheat[17] and cucumber and spinach[18]. These changes are proposed to play a role of specific proteins in acclimation to non-freezing low temperature and useful for understanding the biochemical mechanism affecting chilling stress.

With optimal reaction mixture in chloroplast protein synthesis for efficient translation, Fig. 5 shows the effect of low temperature on *'in organello'* protein synthesis using intact chloroplasts isolated from tomato leaves. The synthesis of 17 kd stromal proteins was induced and the synthesis of 15, 55 and 60 kd proteins was reduced by chilling stress. In thylakoid proteins, the synthesis of 16kd protein was increased and the synthesis of 18, 24, 33 and 55 kd proteins was decreased by chilling stress. We expected that the 55 kd stromal protein would be the large subunit of rubisco and the 32 kd thylakoid membrane protein would be the D1 protein[6]. Both are the major chloroplast-encoded chloroplast proteins and their synthesis were drastically inhibited by chilling. Hahn and Walbot[16] investigated the effect of cold on protein and RNA metabolism in leaves of rice seedling. They reported that the synthesis of rubisco large and small subunit was drastically reduced over 80% after 7 days of cold. However, other proteins were not identified clearly and more detailed studies are needed.

Ort et al.[18] reported the changes in protein synthesis by chilling using *'in vivo'* labeling in several plants. The synthesis of 35 kd protein was induced by chilling in tomato and cucumber. While the synthesis of 27 kd, major chlorophyll a/b binding protein of the LHCP-II were substantially reduced after chilling in tomato plant, not cucumber. A chilling-insensitive spinach plant, however, showed no change of 27 and 35 kd protein to chilling stress. Giroux and Filion[15] also reported the appearance of a 35 kd protein in two different chillresponse tomato cultivars during the general temperature decrease and the cold shock and the appearance of a 27.5 kd LHCP protein during the gradual temperature decrease.

CONCLUSION

This study was conducted to optimize the reaction system in chloroplast protein synthesis, to identify the changes of protein profiles synthesized in tomato chloroplast, and to investigate the effect of low temperature on the regulation of chloroplast genes. The results can be summarized as below.

1. Incorporation reaction occurred rapidly at the first 30 minutes and was constantly maintained after 60 minutes. An optimal temperature on protein synthesis was found around

25°C. No difference was shown in the chloroplast protein synthesis under high light intensity (1600 $\mu E/m^2/s$) as well as darkness.

2. K^+, Mg^{++} and ATP at an optimal concentration act as an activator, while DTT, chloramphenicol, cycloheximide, Ca^{++} and inorganic phosphate act as an inhibitor in the chloroplast protein synthesis. Optimal concentrations of K^+, Mg^{++} and ATP in the reaction mixture were 60 mM, 10 mM, and 10 mM, respectively.

3. Cycloheximide, which inhibits 80S cytoplasmic ribosomes, has little effect on the rate of chloroplast protein synthesis, while chloramphenicol, which inhibits 70S chloroplast ribosomes, rapidly decreased the rate of chloroplast protein synthesis.

4. Synthesis of 15, 55 and 60 kd chloroplast encoded stromal proteins and 18, 24, 33 and 55 kd chloroplast encoded thylakoid membrane proteins were reduced by chilling, while 17 kd chloroplast encoded stromal protein and 16 kd chloroplast encoded thylakoid membrane protein was induced by chilling.

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