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Structural Characterization of *Hordeum vulgare* L. Chloroplast by Ozone

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The effects of ozone on chloroplast development in barley seedlings during greening was investigated based on ultrastructural changes in the chloroplasts and band pattern changes in the chloroplast thylakoid membrane proteins.

In the analysis of the chloroplast thylakoid membrane thylakoid protein band pattern by SDS-PAGE, none of the 24-hour greening bands included were clearer than the control. This means that the ozone treatment produced a delay in chloroplast development and decreased the amount of thylakoid membrane proteins. LHC II chloroplast band of developing barley seedlings treated with 0.5 and 1.0 ppm ozone during the last 4 hours of the 24-hour greening period was weaker than the other bands. This result indicates that ozone affects the LHC II protein complex of the chloroplast thylakoid membrane. When investigating the ultastructural changes in ozone-treated chloroplast, the main site affected by 0.5 ppm ozone was the chloroplast grana, thereby explaining the delayed chloroplast development during the early phase of greening. In addition, there was also a structural change in the stromal grana of the ozone treated chloroplast during the middle phase of greening. The effects of ozone on the chloroplast of barley seedlings during the last phase of 48-hour greening were more functionally inhibiting than structural changes.

Key words : barley, ozone, thylakoid membrane, SDS-PAGE, TEM

1. Introduction

Ozone, a major air pollutant in big cities, is increasing in density with the ever increasing number of cars[1]. Ozone enters cells by diffusion through the stomata in the leaves, resolves the cell walls and membranes[2], and then creates various virulent substances which are transferred into the cells[3]. A high density of ozone and light in the atmosphere also causes visual damage such as waterlogging, bronzing, and silvering[4~6]. Even though many studies have been performed on the effect of ozone on plants, there is still intense debate on its effect on chloroplast and how it operates on the photosynthetic plant. organism. It has been previously reported that light increases the photosynthetic activity in a plant body since chlorophyll is precipitously generated in the etioplast and a photosynthesis organism is developed. Furthermore, PS I activity has a relation with the formation of stroma lamella in chlorophyll and PS II activity is correlated with the formation of grana[7]. The effect of ozone on a green plant body, its target region, and method of operation, is believed to differ from its effect while the etiolated plant body is greening and the chloroplast is developing. Therefore, in order to establish which photosynthetic region in the plant body is primarily suppressed by ozone, the effect of high-concentrated ozone treatment was investigated during the young stage when the plant body is sensitive to ozone and the photosynthesis organism is rapidly developing.

To investigate the phenomena caused by ozone treatment based on the degree of chloroplast development in etiolated barley seedlings under weak light, this study compared and analyzed the band pattern of the thylakoid membrane proteins

using SDS-PAGE and the chloroplast ultrastructure using a TEM (transmission electron microscope).

2. Materials and Methods

2.1. Plant Materials

The barley(*Hordeum vulgare* L.), supplied by an agricultural agent in Seung-ju Kun, was sown after being washed in distilled water and then grown at 22±2 ℃, 70±5% in the dark.

2.2. Ozone Treatment

In order to investigate the effects of ozone based on the degree of the chloroplast development, two etiolated barley seedlings were exposed to 0.5 and 1.0 ppm ozone for 4 to 12 hours, irradiated for 60 μmol $m^{-2}s^{-1}$, and then greened for 24 and 48 hours. The ozone was created by an ozone generator through a high voltage discharge, and the ozone density was measured with a UV photometric O_3 analyser (Model: Dasibi 1108) and controlled at ±0.05 ppm. The ozone treatment and greening was conducted in a growth room of 49cm × 30cm × 41cm with a temperature of 22±2 ℃ and 70± 5%(relative humidity).

2.3. Extraction of Chlorophyll Thylakoid Membrane and SDS-PAGE

The extraction of the chlorophyll thylakoid membrane was performed using a modification of Mayfield and Huff's method[8]. Isolated chlorophyll was suspended in a 50 mM Hepes buffer(pH 7.8) and centrifuged at 1,000g for 10 min. The sediment was dissolved with a sample buffer [0.0625 M Tris-HCl buffer(pH 6.8, 10%(v/v)glycerol, 5%(v/v) β-mercaptoethanol, 2,3%(v/v) SDS included), which was added to make SDS : Chl = 10 : 1, dissolved for 10 min at room temperature, and then centrifuged at 4,000g for 10 min. The supernatant was used as the electrophoresis sample.

The gel was made based on a modification of Laemmli's method[9]. Where a 12% separating gel and 5% stacking gel were electrophoresized in the dark for 6 hours, and the resulting gel was dyed with 0.2% Coomassie Brilliant Blue R-250 for 12 hours at room temperature. A decolorizer(Red Rocker, Hoefer) was used to make the gel transparent with 7% acetic acid, then the gel was dried by a gel dryer(Slab gel dryer; Moder 1160, Hoefer) and preserved. Cytochrome C was used as the standard for the SDS-PAGE marker.

2.4. Observation of Microscopic Structure of Chloroplast

After the prefixation of a barley leaf in a 0.1 M phosphate buffer(pH 6.8, 2% glutaraldehyde, 0.33% paraformaldehyde), it was washed 3 times with the 0.1 M phosphate buffer, and then postfixed with a 0.1 M phosphaste buffer(pH 6.8, 2% osmium tetroxide) for 90 minutes. The material washed with the 0.1 M phosphaste buffer was processed with dehydratin including ethyl alcohol and embredded using an Epok 812 kit. Thereafter, the embredded material was made into a 60-100 nm-thick slice using an ultramicrotome(Reichert Supernova). The resulting silver slice was attached to a copper grid(200 mesh), double-dyed with uranyle acetate and lead citrate, and finally observed using a transmission electron microscope (Hitachi H-7100).

3. Results and Discussion

3.1. Band Pattern of Chloroplast Thylakoid Membrane Protein Using SDS-PAGE

Fig. 1 shows the SDS-PAGE result of the chlorophyll thylakoid membrane that was separated after ozone treatment during the first 4 hours of the greening period of the etiolated barley seedlings. The molecular weight of the protein standard cytochrome C from Sigrist and Staehelin's study[10] was used for LHC II(28kD) and CF(56 kD, 59 kD) where the band was clear. The CF band (56 kD, 59 kD) already appeared before greening(Fig. 1A). All the bands resulting from the 24-hour greening period with 0.5 ppm ozone were similar to the control, however, the LHC II, CF band and all the other bands were weaker than the control when treated with 1.0 ppm ozone. However, the 48-hour greening period showed an ozone treated band similar to that of the control. Therefore, this result could imply that the 0.5 ppm ozone treatment had only a minimal effect on the protein of the chloroplast thylakoid membrane. Whereas, weaker band resulting from the 1.0 ppm ozone treatment suggests that a delay in the chloroplast development

produced a quantitative decrease in the chloroplast membrane protein. Lee *et al.*[11] found that when rape cotyledons were greened for 48 hours, the rate of LHC/CP(chlorophyll-protein complex) was fixed and the formation of the chlorophyll-protein complex became stable. This result indicates that, when exposed to 48 hours of light, the ozone-treated band pattern is similar to that of the control. Therefore, although the chloroplast development was initially delayed because of the ozone treatment, when the greening period was prolonged, the chloroplast development was completed.

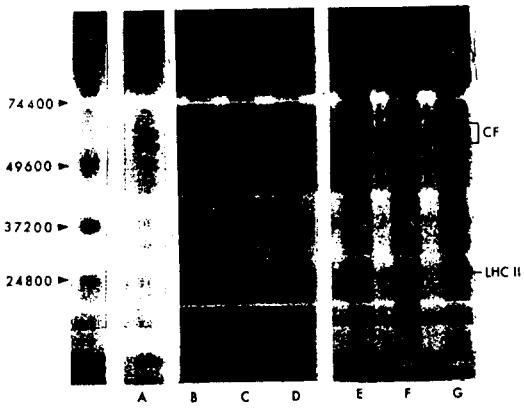


Fig. 1. Effects of the various concentration of ozone on the chloroplast protein band pattern. Ozone was treated to the barley seedling for the first 4 h during greening period. B-D, 24 h greening period; E-G, 48 h greening period; A, etiolated; B, E, control; C, F, 0.5 ppm ozone; D, G, 1.0 ppm ozone.

Fig. 2 shows the SDS-PAGE result of the chlorophyll thylakoid membrane that was separated after ozone treatment during the last 4 hours of the greening period. The LHC II band treated with 0.5 and 1.0 ppm ozone during a 24-hour greening period was particularly weak and the one treated with 1.0 ppm ozone was weaker than that of control. A 48-hour greening period also produced a weaker LHC II band than the control. However, the other bands including the CF band were all similar to the control. This result indicates that the ozone treatment had the greatest effect on the LHC II protein complex of the chlorophyll thylakoid membrane. It has been previously reported that the treatment and electrophoresis of RuBPCase with a concentration of 0.15 ppm ozone, that is lower than in this study, had no effect on the band pattern yet rather changed the subunit[3]. Accordingly, when considering Park's report[12], under the same experimental conditions of a 4-hour treatment period with 0.5 ppm ozone, a suppressed PS II activity by 20% and decreased electron transport activity preceded the change of band pattern in the thylakoid membrane protein.

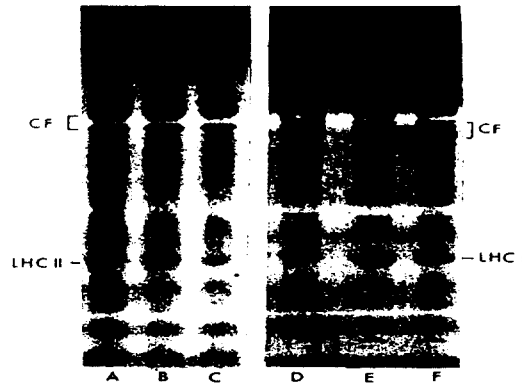
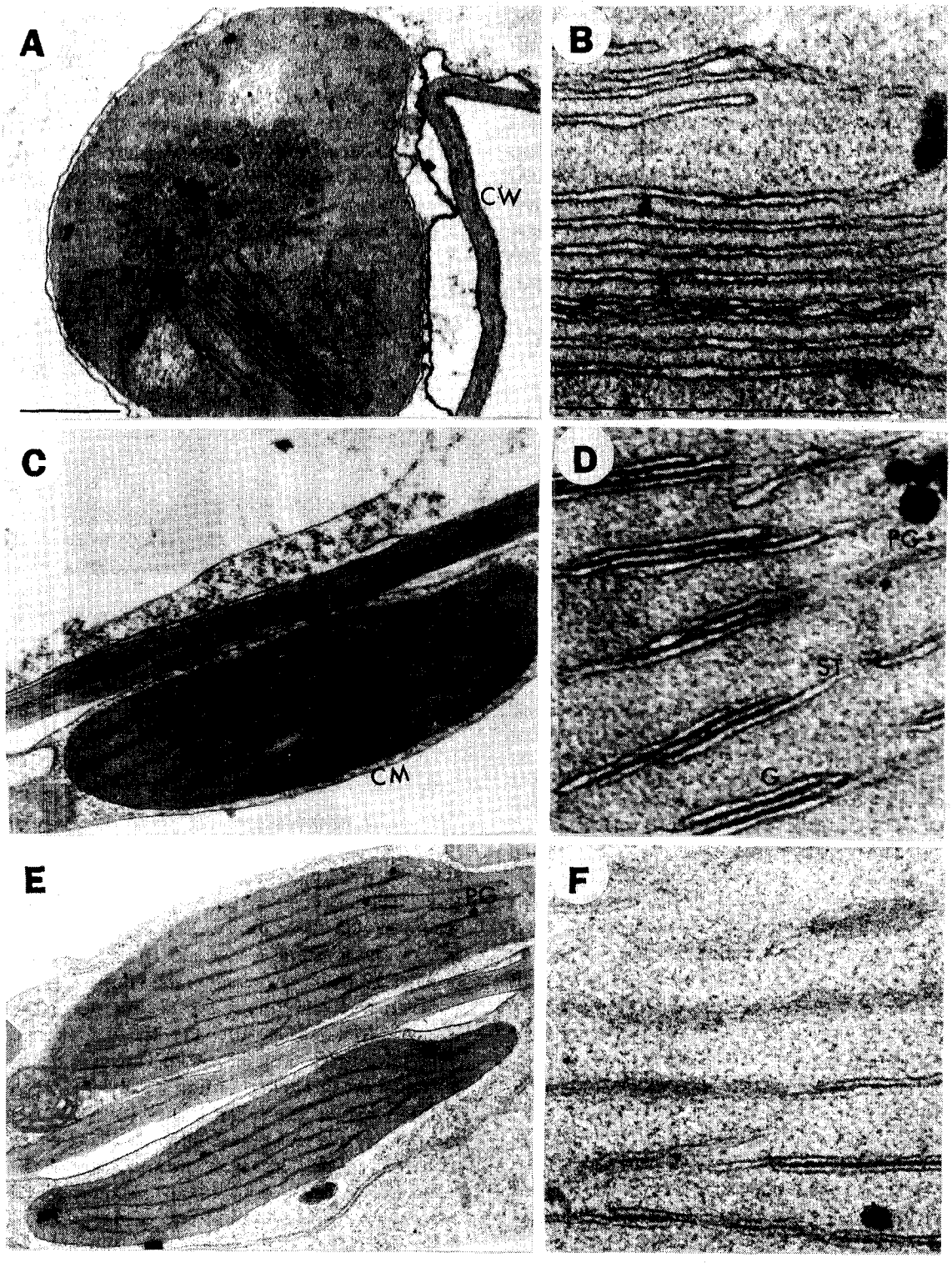


Fig. 2. Effects of the various concentration of ozone on the chloroplast protein band pattern. Ozone was treated to the barley seedling for the last 4 h during greening period. A-C, 24 h greening period; D-F, 48 h greening period; A, D, control; B, E, 0.5 ppm ozone; C, F, 1.0 ppm ozone.

3.2. Ultrastructure of Chloroplast

In order to investigate the effect of 0.5 ppm ozone on barley seedlings, the chloroplast ultrastructure was observed using a transmission electron microscope after treating etiolated barley seedlings with ozone for 4 to 12 hours(60 μ mol $\cdot$ $m^{-2}s^{-1}$), and greening for 24 and 48 hours(Fig. 3).

After 12 hours of greening, the etioplast in the etiolated barley seedlings had already developed stroma thylakoid(Fig. 3A and B) and two or three-fold of grana(Fig. 3C and D). However, ozone treatment for this period of time hardly developed or destroyed the grana(Fig. 3E and F). A 24-hour greening period developed three or four-fold of grana(Fig. 3C and D). Ozone treatment during the early 4-hour phase of a 24-hour greening period delayed the development of two or three-fold of grana and damaged the stroma grana(Fig. 3I and J), while treatment during the early 8-hour phase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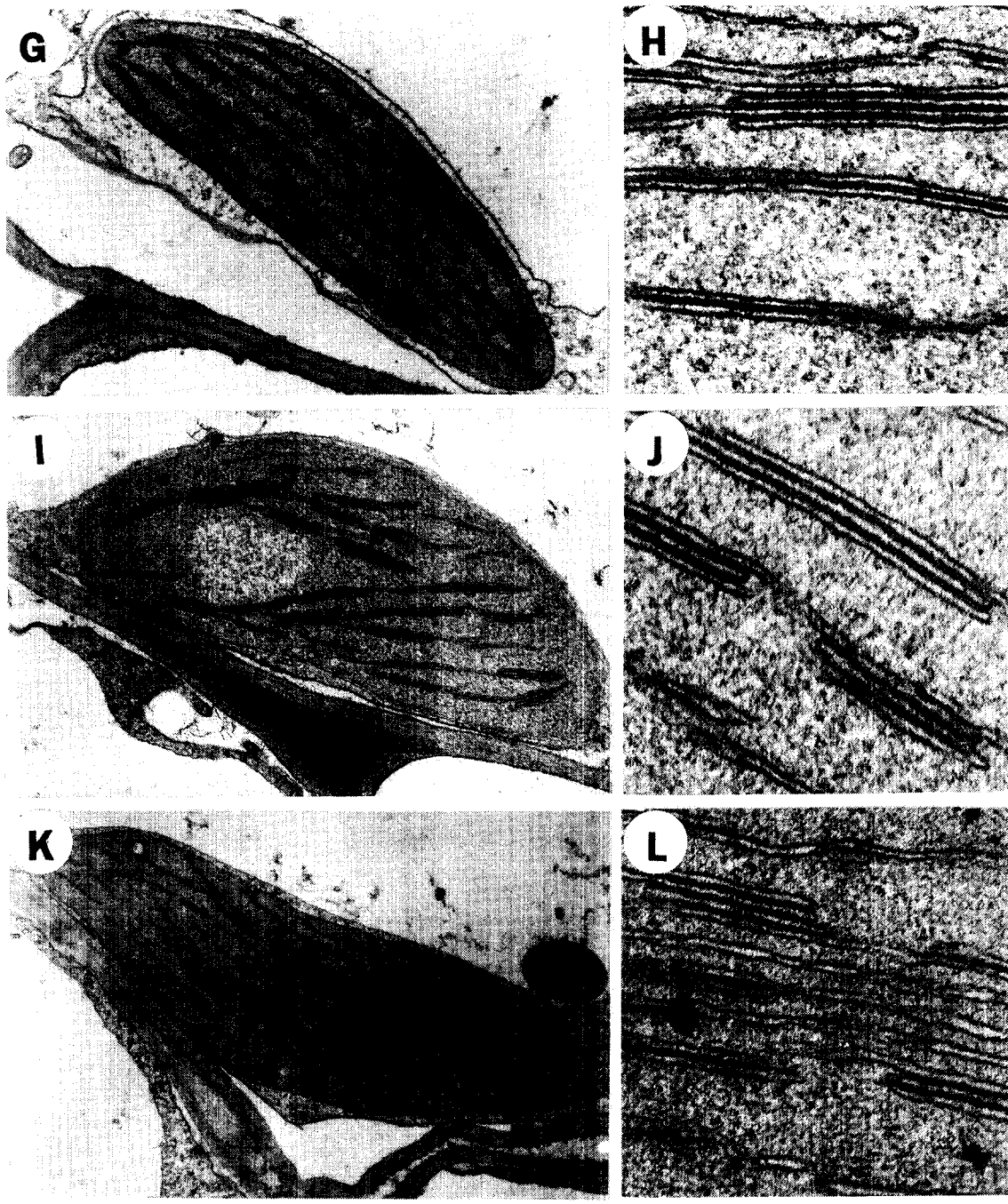
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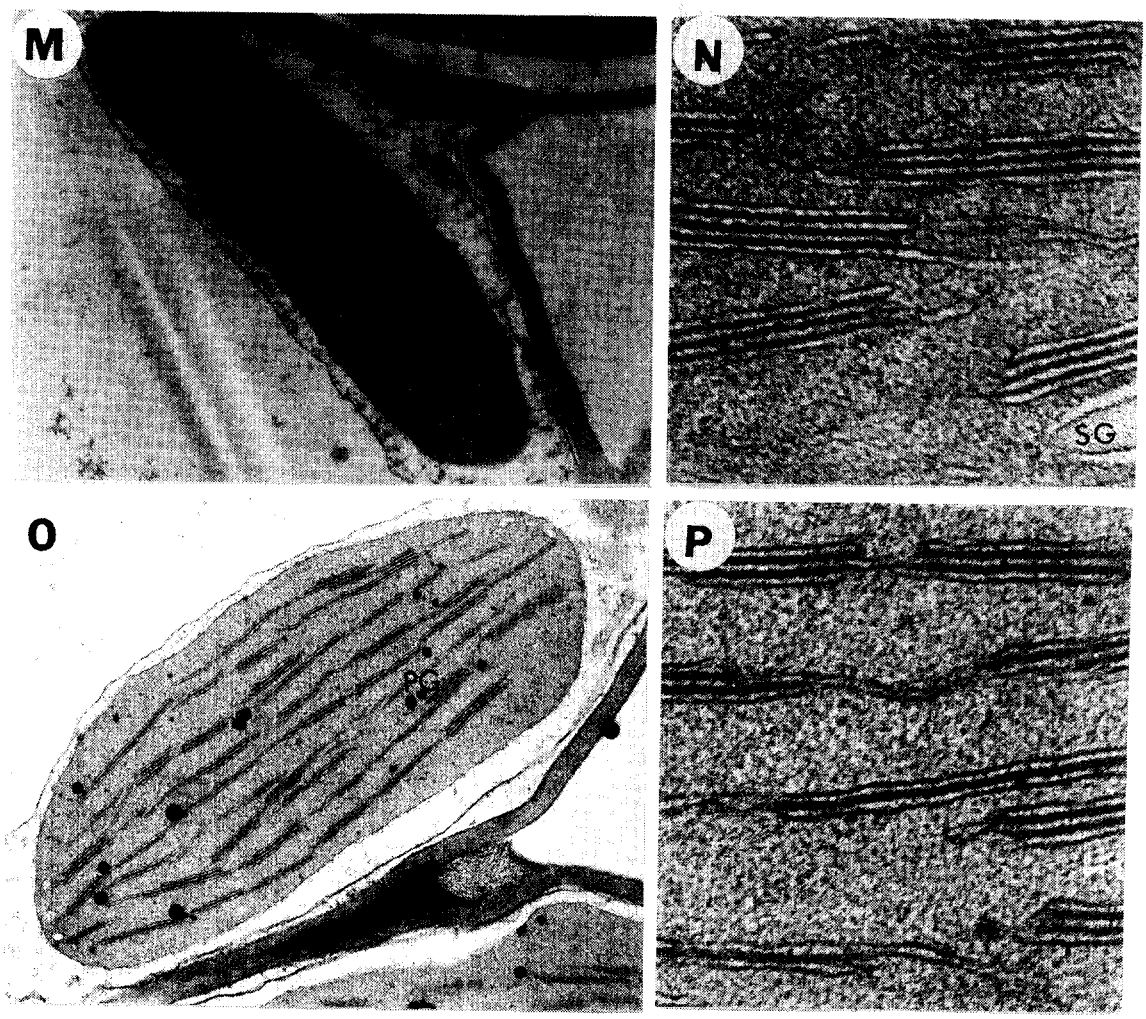
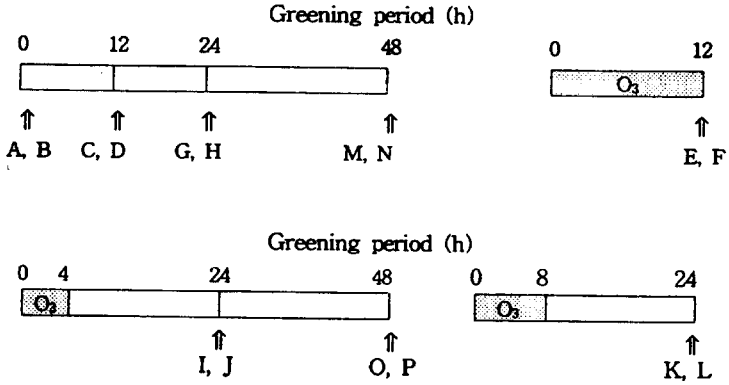
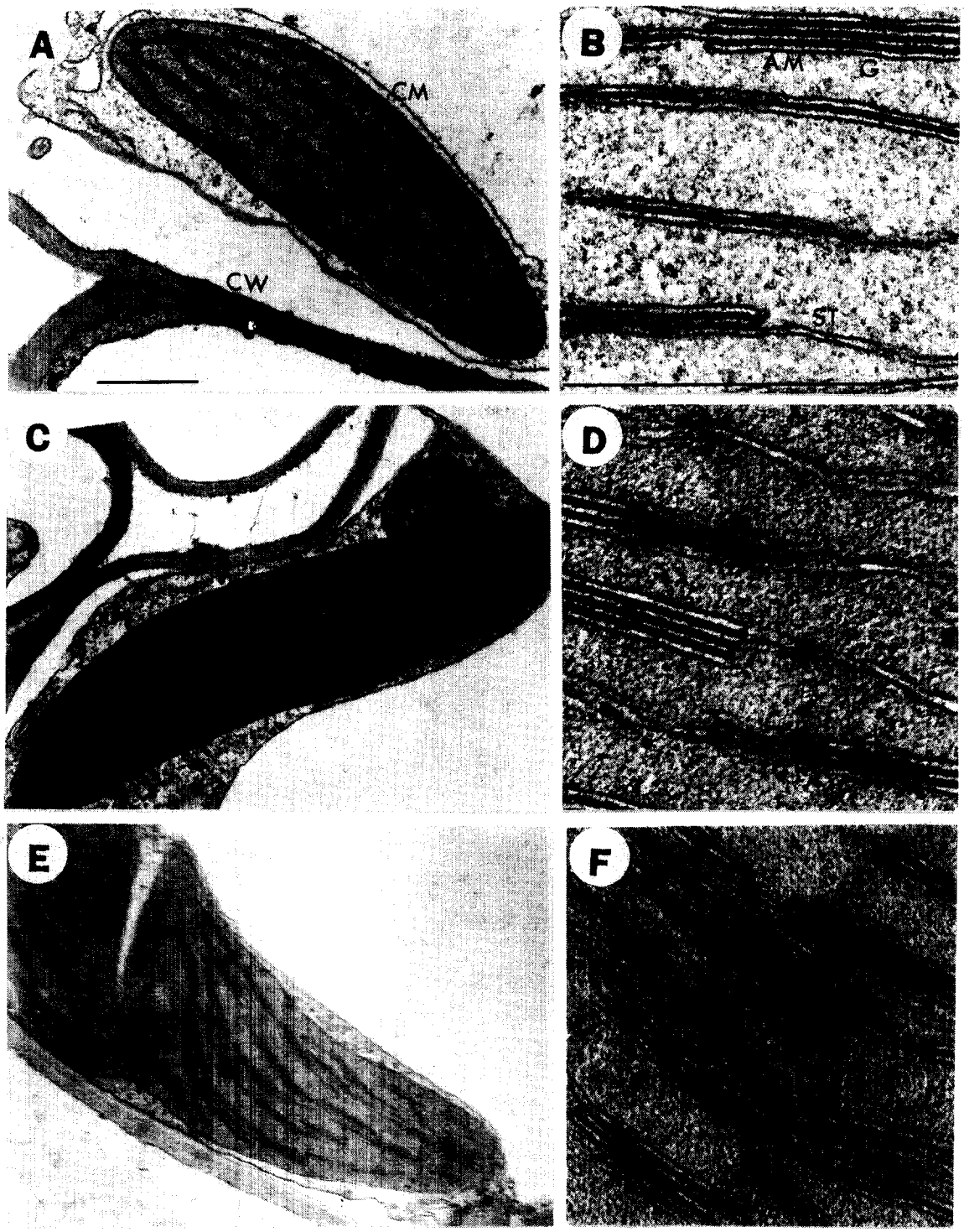


Fig. 3. Electron micrographs of chloroplast obtained from barley seedling treated 0.5 ppm ozone during greening period. A, etioplast; B, stroma thylakoid; C, D, 12 h of greening period; G, H 24 h of greening period; Q, R, 48 h of greening period. C, E, G, I, K, M, O, chloroplast; D, F, H, J, L, N, P thylakoid membrane. SG, starch grain; PG, plastoglobuli; G, grana; ST, stroma thylakoid; CW, cell wall; CM, chloroplast membrane. Bar = 1 μm.

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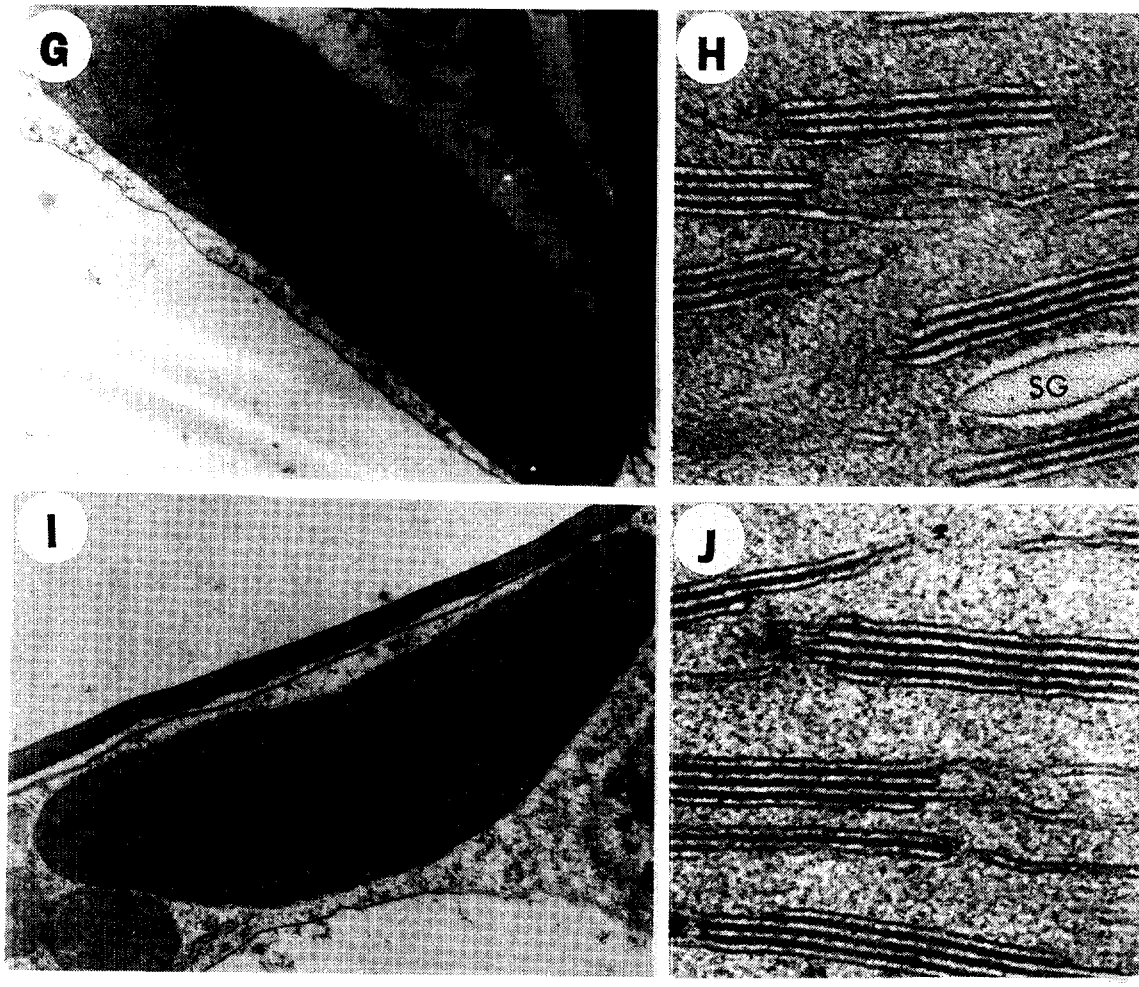
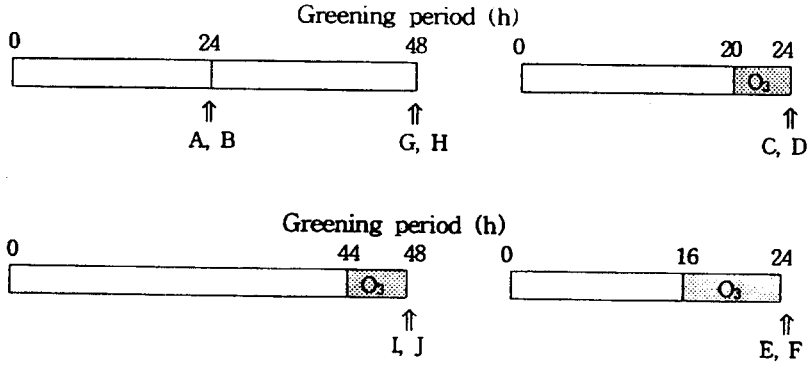


Fig. 4. Electron micrographs of chloroplast obtained from barley seedling treated 0.5 ppm ozone during greening period. A, B, h of greening period; G, H, 48 h of greening period. A, C, E, G,, I, chlorplast; B, D, F, H, J, thylakoid membrane. SG, starch grain; PG, plastoglobuli; ST, stroma thylakoid; CW, cell wall; CM, chloroplast membrane; AM, appressed mambrane; NAM, non-appressed membrane. Bar=1 μm.



of a 24-hour greening period resulted in no grana development and much of the stroma grana was destroyed(Fig. 3K and L). Ozone treatment during a 48-hour greening period developed four-fold of grana and starch grain(Fig. 3M and N), and treatment during the early 4-hour phase during this greening period delayed the development two or three-fold of grana and no stroma grana was formed (Fig. 3O and P). Chinn[13,14] reported that when a starch grain appears as a green ginkgo seedling, its photosynthesis device develops and the photosynthesis is performed smoothly. Therefore, this indicates that ozone treatment at an early phase of greening affects the grana of chloroplast by delaying its development and photosynthesis, and as a result, there is no formation of starch grains.

In order to observe the extent of the effect of ozone on barley seedlings during the period of chloroplast development, the ultrastructure of the chloroplast was observed using a transmission electronic microscope after treating etiolated barley seedlings with ozone for 4 to 8 hours(60 μ mol · $m^{-2}s^{-1}$), and greening for 16, 20, and 48 hours (Fig. 4).

A 24-hour greening period developed 3 to 4-fold of grana and normal stroma grana(Fig. 4A and B). However, ozone treatment during the last 4 hours developed a similar extent of grana as the control and destroyed a part of the stroma grana (Fig. 4C and D). Also, ozone treatment during the last 8 hours delayed the development of 2 to 3-fold of grana, and even exhibited non-oppressed parts[15] including those of stroma grana because of the destruction of the grana(Fig. 4E and F). A greening of 48 hours developed about 4-fold of grana and starch grain(Fig 4E and F). Ozone treatment during the last 4 hours, however, developed a similar degree of grana to the control and no starch grain. Therefore, this indicates that ozone has less effect after the chloroplast development is completed.

The above results show that, ozone treatment at an early phase delays the chloroplast development, whereas during the middle phase it destroys the chloroplast thylakoid membrane protein, and finally after greening, a functional restraint rather than structural destruction occurs.

Acknowledgements

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