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Studies on Ultrastructure and Function of Photosynthetic Apparatus in Rice Cells

IV. Effect of low dose and intermittent fumigation of ozone on the ultrastructure of chloroplasts in rice leaf cells

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Abstract : Young rice plants (*Oryza sativa* L. cv. Koshihikari), which are 4 week old plants grown in a potting soil under natural light at 28/27°C in day/night, were intermittently fumigated with 0.1 ppm ozone for 4 h (AM 10-14)/day for 4 days, and the ultrastructural changes of chloroplasts in leaf cells were examined by electron microscopy. A visual symptom of small reddish brown flecks appeared on an abaxial leaf surface fumigated for 2 days. After fumigation for 3 days or more, the flecks increased in both size and number and also brightness of green leaves slightly diminished. On observation by electron microscopy, the first indication of ozone injury was swelling of the thylakoid membranes in chloroplasts of abaxial leaf cells fumigated for 2 days. The swelling began first at the outermost thylakoid in grana stacks and was followed by a distortion in the arrangement of internal thylakoids. After fumigation for 3 days or more, the changes of thylakoid membranes became serious and then vacuolization occurred in chloroplast stroma. In leaves which were fumigated for 4 days and afterward kept in a fumigation chamber for a day, destruction of chloroplast envelope was observed. The ultrastructural changes occurred first on the leaf tip area and then extended to the middle region. Ozone injury was more serious in abaxial leaf cells than in adaxial ones. A characteristic was no increase of osmiophilic globules in chloroplasts throughout this ozone fumigation.
Key words : Chloroplasts, *Oryza sativa* L., Ozone fumigation, Swelling, Thylakoid membranes, Ultrastructure.

イネの光合成器官の微細構造と機能に関する研究　第4報　イネの葉細胞における葉緑体の微細構造に及ぼす低濃度オゾン暴露の影響：遠山　益*・吉田みどり*・仁木輝緒**・大橋　毅***・小山　功***（*お茶の水女子大学理学部・**拓殖大学工学部・***東京都環境科学研究所大気部）

要　旨：イネの種子を鉢中の土壌にまき，27—28°C で自然光の下で育てた．4週間後の若いイネを0.1 ppmのオゾンに1日4時間（AM 10—14）暴露し，これを4日間繰り返した．このような処置をしたイネの葉緑体の微細構造の経日的変化を電子顕微鏡で試験した．1日間暴露では，イネの葉に可視症状は現われなかったが，2日間暴露すると，葉の背面に赤紫色の斑点が現われた．3日間またはそれ以上暴露すると，斑点の大きさも数も増加した．しかし，葉の緑色は僅かの減少にとどまった．オゾン暴露を終了したイネを温室内に移した後でも，その成長はやや抑えられた．電子顕微鏡による観察では，オゾンによる被害の最初の徴候は，2日間暴露した背面葉細胞における葉緑体のチラコイド膜系の膨潤であった．膨潤はまずグラナスタックの最外部のチラコイドで始まり，次第に内部のチラコイドへ波及した．3日間またはそれ以上の暴露後には，チラコイド膜系の膨潤と配列の乱れはさらに激しくなり，ストロマ部には空胞化が生じた。4日間暴露し，翌日1日間暴露チャンバー内に置いたイネでは，葉緑体包膜の破壊が観察された．オゾン被害は細胞のエイジングと密接に関係し，4日間暴露した葉の先端部では葉緑体の構造破壊が顕著であったが，基部ではそれはほとんど観察されなかった．また，葉の背面における細胞では被害は大きかったが，腹面では軽微であった．この暴露実験を通して，葉緑体内の好オスミウム果粒の大きさも数も増加しないことが特徴的であった．
キーワード：イネ，オゾン暴露，チラコイド膜，微細構造，膨潤，葉緑体．

Ozone is a major component of photochemical air pollutants and generated when NO_x and hydrocarbons released into the atomosphere react with sunlight[16]. Ozone exposure reduces productivity in many plant species including agronomically important crops by the following ways: induction of stomatal closure, reduction of photosynthetic activity, and induction of necrosis and chlorosis[6,7]. Consequently, previous studies have strongly

focused on the injury of structure and function of chloroplasts in higher plants. Electron microscopic observations of cell membranes and organelles in ozone-fumigated plants have shown ultrastructural alterations before appearance of visible symptoms, especially deformation and disintegration of the thylakoid membranes in chloroplasts[1,15,22,23,25]. On the other hand, many biochemical and physiological studies have shown that molecular organization of photosynthesizing cells is altered by ozone[2,13,19,20], including oxidative degradation of lipids and proteins in membranes and breakdown of photosynthetic pigments. Although the mechanism by which ozone causes injury to plant cells remains unclear, it has been reported that an alteration of cell membrane permeability is most likely the initial step leading to injury[4,21].

The present study was performed to obtain further information of the rice chloroplast injury by ozone fumigation. Fumigation experiments were carried out under strictly regulated conditions, because ozone injury of plants are closely connected not only with the surface structure and aging of plant leaves, but also with ozone concentration, temperature and light intensity during fumigation.

Materials and Methods

Seeds of rice (*Oryza sativa* L. cv. Koshihikari) were sowed in a potting soil under natural light at 28/27°C in day/night. After 4 weeks, fully expanded 3rd leaves were used for experiments. Before ozone treatment, the potted plants were placed for a day in a naturally-lit environmentally controlled glass chamber [2 m (D) ×2 m (W) ×2 m (H)] equipped with activated chacoal filters. The conditions in the chamber were maintained at 30°C and 80/90% in day/night for relative humidity. On the next day, the plants were fumigated with ozone in the chamber.

Ozone was generated by passing air through a high voltage discharging tube and introduced into the naturally-lit environmentally controlled glass chamber in which the plants were placed. The plants were fumigated with ozone for 4 h (AM 10—14)/day for 4 days. The environmental conditions during ozone fumigation are shown in Table 1. The concentration of ozone in the chamber was maintained at about 100 ppb and monitored with a UV absorption ozone analyzer, although it took about 1 h to obtain the desired ozone concentration. On the last day, the plants were placed in the chamber for a day without ozone fumigation.

The materials for electron microscopy were collected from three sites, tip, middle and base, of a 3rd leaf (18—23 cm tall) ; 1.5—2.5, 4—5 and 15—20 cm respectively from the tip. Samples were collected everyday at the end of fumigation and immediately fixed in 4% glutaraldehyde for 16 h at 4°C. After washing in cacodylate buffer (pH 7.2), samples were postfixed in 2% OsO_4 in 0.05 M sodium cacodylate buffer (pH 7.2) for 16 h at 4°C. Samples were continuously dehydrated with ethanol series and embedded in Spurr's resin. Ultrathin sections were stained with a saturated solution of uranyl acetate and Reynolds's lead citrate solution, and observed by a Hitachi H-600 electron microscope.

Results

In rice leaves fumigated with about 100 ppb ozone for 2 days, the first visible symptom was small reddish brown flecks on the tip and middle region in the abaxial surface of the expanded 3rd leaves grown in a potting soil under natural light for 4 weeks (Fig. 1). On fumigation for 3 days or more, these flecks, which were irregular in shape and about 1—2 mm in diameter, increased in both size and number. Brightness in green leaves was slightly diminished. Subsequent growth of plants which were fumigated for 4 days and then transferred into a green house, was slow compared with that of untreated plants. The plants, however, still appeared healthy.

Figs. 2 and 3 show normal chloroplasts in untreated leaves, which show typical arrangements of grana- and stroma-thylakoid membranes running parallel to a chloroplast long axis. The grana stacks are composed of 5—10 thylakoids or more. The thylakoid membranes did not swell at all and intact membranes of chloroplast envelope were clearly observed (Fig. 3, arrow). The first indication of ultrastructural injury in chloroplasts caused by ozone fumigation was swelling of grana thylakoids, which was first detected in chloroplasts fumigated for 2 days (Fig. 4). These changes became more conspicuous after fumigation for 3 days (Figs. 5 and 6). The swelling appeared

Table 1. Environmental conditions of ozone fumigation.

	DATE 87/08/17 S−1					DATE 87/08/18 S−1					DATE 87/08/19 S−1					DATE 87/08/20 S−1				
	°C	%	MJ/m²	O_3 ppb	NO_2 ppb	°C	%	MJ/m²	O_3 ppb	NO_2 ppb	°C	%	MJ/m²	O_3 ppb	NO_2 ppb	°C	%	MJ/m²	O_3 ppb	NO_2 ppb
[1]	25.2	92	0.00	1	0	[1]	25.3	94	0.00	1	0	[1]	25.4	95	0.00	1	0	[1]	25.3	94	0.00	1	0
[2]	25.2	93	0.00	1	0	[2]	25.3	94	0.00	1	0	[2]	25.4	95	0.00	1	0	[2]	25.3	94	0.00	1	0
[3]	25.3	94	0.00	1	0	[3]	25.3	94	0.00	1	0	[3]	25.4	95	0.00	1	0	[3]	25.3	94	0.00	1	0
[4]	25.3	94	0.00	1	0	[4]	25.3	94	0.00	1	0	[4]	25.4	95	0.00	1	0	[4]	25.3	94	0.00	1	0
[5]	25.3	94	0.00	1	0	[5]	25.3	93	0.00	1	0	[5]	25.4	94	0.00	1	0	[5]	25.3	94	0.00	1	0
[6]	25.3	93	0.05	1	0	[6]	25.3	93	0.03	1	0	[6]	25.4	93	0.08	1	0	[6]	25.3	94	0.08	1	0
[7]	27.8	95	0.39	1	0	[7]	28.0	93	0.14	1	0	[7]	26.0	93	0.39	1	0	[7]	27.9	94	0.41	1	0
[8]	30.1	89	0.69	1	0	[8]	30.4	90	0.22	1	0	[8]	30.2	89	0.74	1	0	[8]	30.1	86	0.98	1	0
[9]	29.9	84	0.92	1	0	[9]	30.3	85	0.98	1	0	[9]	29.9	85	1.13	1	0	[9]	29.9	83	1.48	2	0
[10]	29.8	82	1.70	15	0	[10]	29.8	83	1.34	15	0	[10]	30.0	84	0.93	15	0	[10]	29.8	83	1.78	15	0
[11]	29.6	83	2.04	93	0	[11]	29.5	84	2.18	93	0	[11]	29.7	84	1.43	92	0	[11]	29.6	84	2.48	92	0
[12]	29.5	84	2.39	102	0	[12]	29.5	83	2.49	104	0	[12]	29.9	84	1.17	103	0	[12]	29.6	84	2.61	103	0
[13]	29.7	84	2.33	102	0	[13]	29.6	83	1.81	101	0	[13]	29.9	83	1.22	103	0	[13]	29.7	84	2.71	99	0
[14]	29.6	84	1.74	101	0	[14]	29.9	85	0.70	102	0	[14]	29.6	83	1.76	100	0	[14]	29.5	84	2.19	103	0
[15]	29.7	83	1.44	77	0	[15]	30.2	86	0.22	76	0	[15]	29.6	84	1.05	77	0	[15]	29.4	84	1.90	77	0
[16]	29.8	82	0.80	9	0	[16]	30.2	84	0.26	8	0	[16]	30.3	87	0.33	9	0	[16]	29.7	84	1.17	5	0
[17]	30.0	85	0.36	2	0	[17]	30.4	83	0.05	1	0	[17]	30.4	86	0.10	1	0	[17]	30.0	85	0.57	2	0
[18]	27.4	87	0.11	1	0	[18]	27.7	87	0.00	1	0	[18]	27.7	88	0.06	1	0	[18]	27.5	88	0.08	1	0
[19]	25.0	93	0.01	1	0	[19]	25.3	96	0.00	1	0	[19]	25.2	95	0.00	1	0	[19]	25.1	94	0.00	1	0
[20]	25.1	93	0.00	1	0	[20]	25.3	97	0.00	1	0	[20]	25.2	95	0.00	1	0	[20]	25.2	95	0.00	1	0
[21]	25.2	93	0.00	1	0	[21]	25.4	96	0.00	1	0	[21]	25.3	95	0.00	1	0	[21]	25.2	94	0.00	1	0
[22]	25.2	92	0.00	1	0	[22]	25.5	96	0.00	1	0	[22]	25.3	95	0.00	1	0	[22]	25.3	94	0.00	1	0
[23]	25.3	93	0.00	1	0	[23]	25.5	94	0.00	1	0	[23]	25.3	94	0.00	1	0	[23]	25.3	94	0.00	1	0
[24]	25.3	94	0.00	1	0	[24]	25.5	94	0.00	1	0	[24]	25.4	94	0.00	1	0	[24]	25.3	94	0.00	1	0

°C : temperature, % : percent of relative humidity, MJ/m² : microjoule per m² of light intensity, ppb : parts per billion of O_3 and NO_2 concentration.

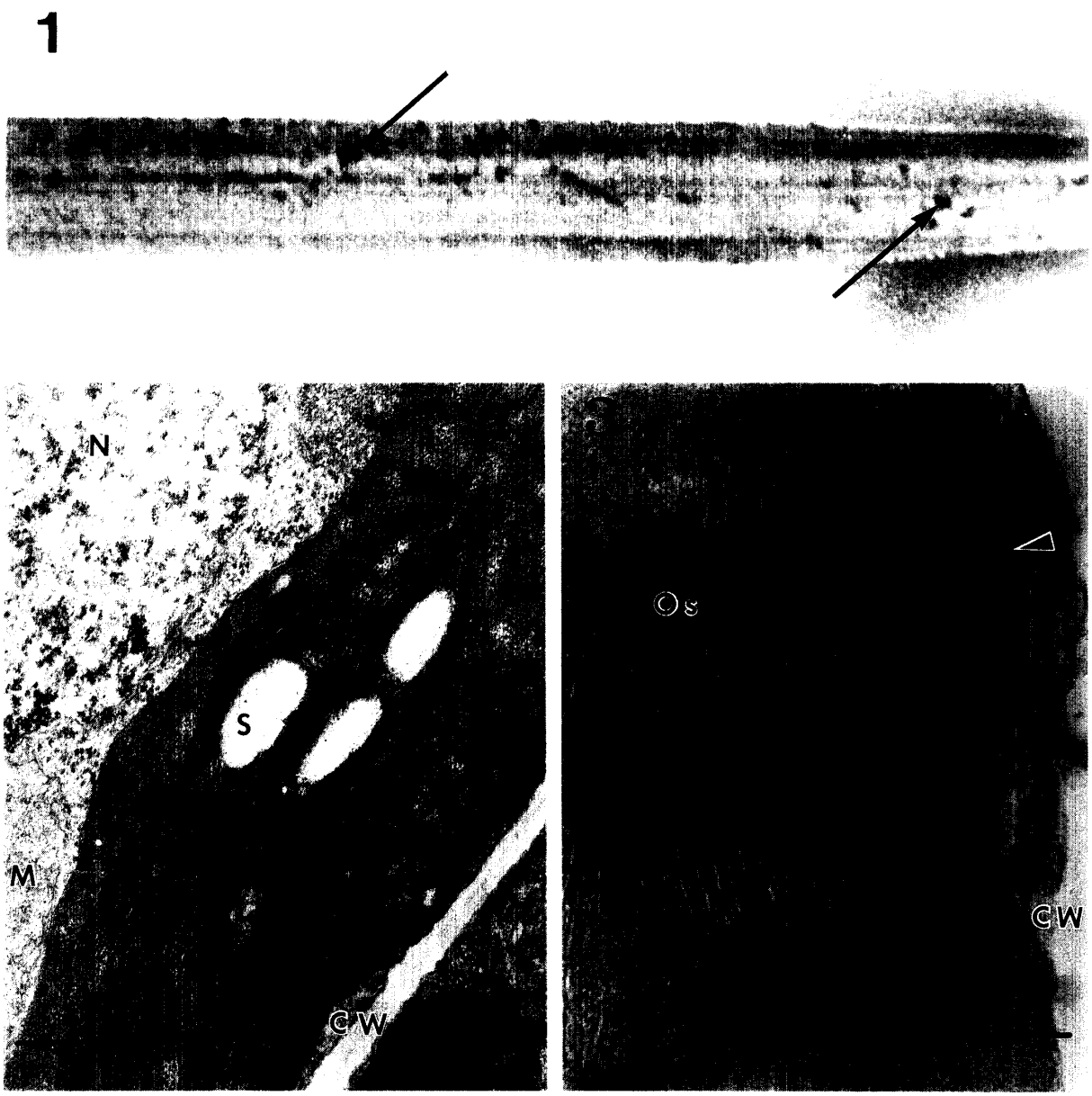

Fig. 1. Abaxial leaf surface of a 4-week old rice plant which was fumigated with 0.1 ppm ozone for 4 h/day for 4 days. Dark spots (arrows) on a leaf surface show reddish brown flecks caused with ozone fumigation. This picture was solarized from a color slide. Figs. 2 and 3. Normal chloroplasts in a 3rd leaf of 4-week old rice before fumigation with ozone. An arrow in Fig. 3 shows membranes of chloroplast envelope. CW : cell wall, M : mitochondria, N : nucleus, Os : osmiophilic globule, S : starch. A bar length in Fig. 2 and Fig. 3 is 1 μm and 0.5 μm respectively.

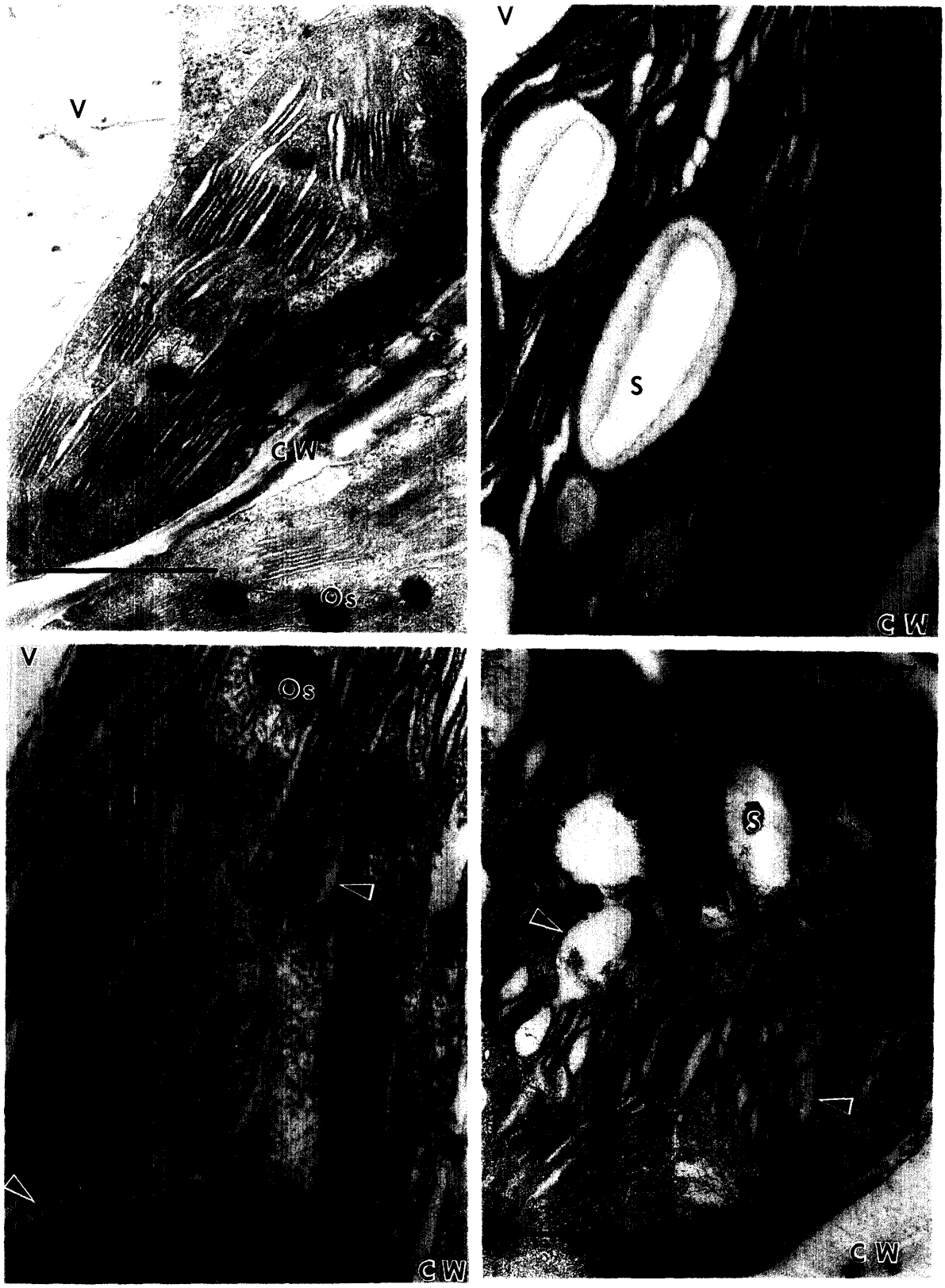

Fig. 4. A chloroplast in a leaf fumigated for 2 days in which thylakoid membranes began to swell. V : vacuole. Figs. 5 and 6. A part of chloroplast in a leaf fumigated for 3 days respectively. Swelling of thylakoid membranes occurred seriously, especially the outermost thylakoids in grana stacks (arrows in Fig. 6). Fig. 7. A chloroplast in a leaf fumigated for 4 days. Note the serious swelling of thylakoid membranes and their distortion in arrangement. Arrows show microvesicles in stroma resulted from ozone fumigation. A bar leagth in Figs. 4, 5, 7 and Fig. 6 is 1 μm and 0.5μm respectively.

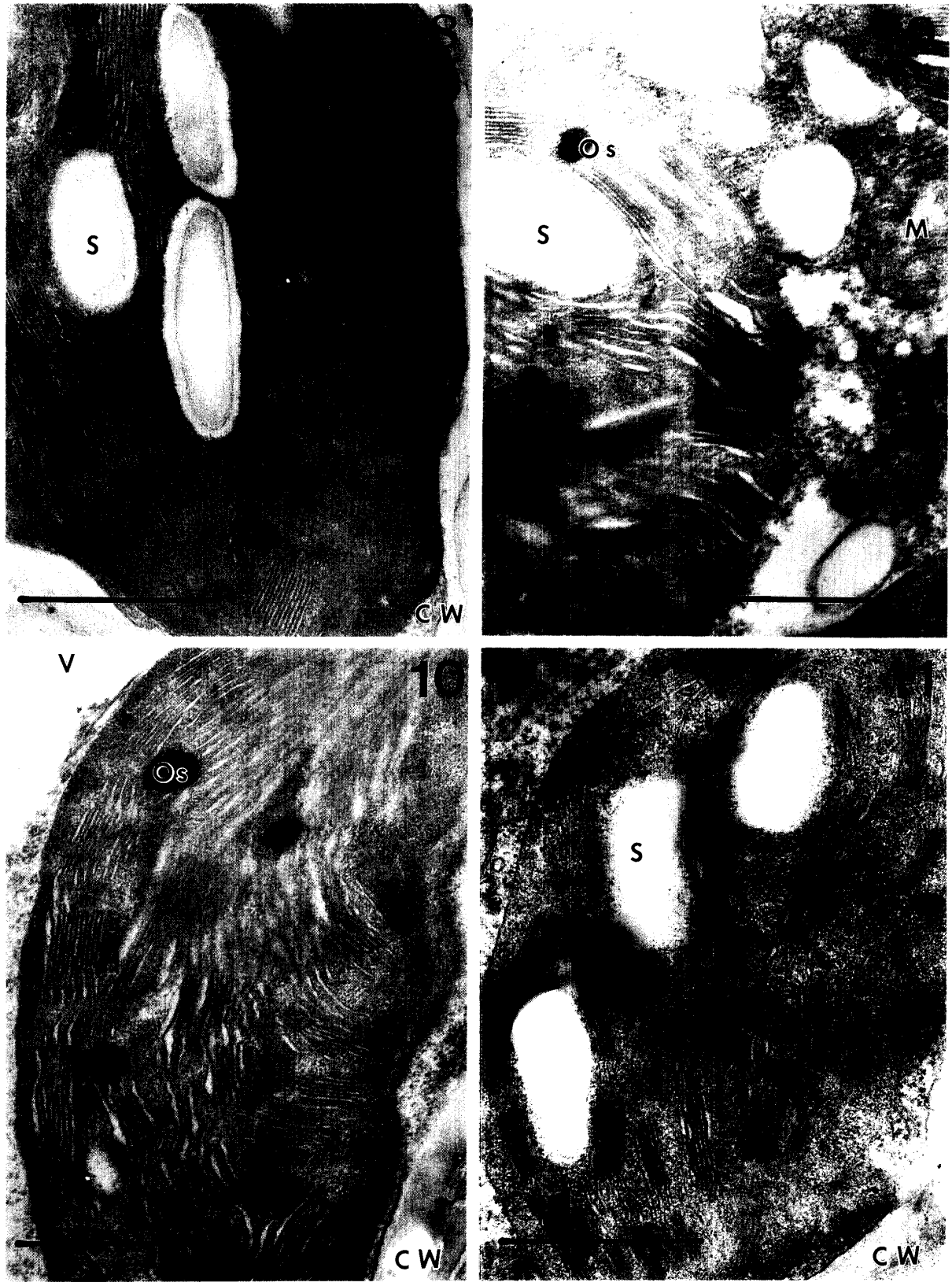

Fig. 8. A part of chloroplast in a adaxial leaf surface fumigated for 4 days. Note the normal arrangement and compact structure of thylakoid membrane system. Fig. 9. A part of chloroplast in a leaf which was fumigated for 4 days and afterward kept in a fumigation chamber for a day. The serious disintegration of thylakoid systems and chloroplast envelope is observed. Fig. 10. A chloroplast in a leaf tip fumigated for a day in which slight swelling of thylakoid membranes is observed. Fig. 11. A chloroplast in a base region of leaf which was fumigated for 4 days and afterward kept in a fumigation chamber for a day. The serious changes of chloroplast ultrastructure are not observed. A bar length in Figs. 8, 9, 10 and 11 is 1 μm.

at the outermost thylakoid in grana stacks (Fig. 6, arrow) and then spread to internal thylakoids in grana stacks after fumigation for 4 days or more (Figs. 7 and 9). The orientation of the thylakoid membrane system was seriously disturbed with increasing fumigation. The swelling of the outermost thylakoid occurred prior to the microvesicle formation in stroma (Fig. 7, arrow). On the next day after 4-day fumigation, envelope membranes of some chloroplasts were disintegrated (Fig. 9).

Although these changes began in the chloroplasts of abaxial leaf cells after fumigation for 2 days, but not in the chloroplasts of adaxial leaf cells even after fumigation for 4 days (Fig. 8), and the orientation of membrane arrangement was not disturbed (Fig. 8). In old cells of leaf tip region, swelling of thylakoid membranes was observed even when fumigated for only a day (Fig. 10). On the other hand, in young cells of leaf base region, these ultrastructural changes were not so marked even after fumigation for 4 days (Fig. 11). Thus, the sensitivity of rice cells to ozone fumigation was high with increasing maturity of leaf cells. Osmiophilic globules were commonly observed in chloroplasts of both unfumigated (Fig. 3) and fumigated leaves (Fig. 6) with ozone, but not increased in number and size with continuing fumigation.

Discussion

Ozone reacts preferentially with lipids and proteins of cell membrane which is first exposed to ozone. It has been suggested that oxidation of fatty acids in cell membranes led to an alteration of the membrane permeability[5]. Rich and Tomlinson[18] reported that oxidation of protein sulf-hydryl groups was a primary reaction leading to cell membrane injury and that lipid oxidation was a consequence rather than a cause of injury. This proposal is supported by the recent studies with bean leaves[3] and *Euglena gracilis*[2] exposed to ozone. However, it is not simple to predict "*in vivo*" ozone injury from "*in vitro*" studies, because the action of ozone on plant cells is affected by the interior and exterior conditions of cellular environment[7,21]. It has been known that antioxidants such as vitamins C and E or glutathione limit the extent of oxidative degradation of cell components[11,14]. Mehlhorn et al.[14] reported that fir needles had a 1.9 fold and 3 fold higher content of vitamin E and glutathione respectively, after exposure to a mixture of SO_2 and O_3 than needles exposed to filtrated air.

Appearance of visible foliar injury is closely related with aging and surface structure of fumigated leaves : many reddish brown flecks appeared only on the abaxial surface and tip region of rice leaves. Visible ozone injury is reported to involve in the accumulation of flavonoid pigment[9,10]. On the other hand, Sakaki *et al*[19]. proposed a sequence of loss of O_2^- scavenger activity, O_2^- accumulation and pigment degradation (visible injury) using spinach leaves fumigated with ozone. Therefore, small reddish brown flecks observed in this study are most likely a result of the accumulation of flavonoid pigments and degradation of photosynthetic pigments.

The aim of this study was to make clear the ultrastructural changes of chloroplasts in rice mesophyll cells fumigated with ozone. In this study, swelling of thylakoids, reorientation of thylakoid system, loss of stroma and rupture of chloroplast envelope were found by electron microscopy. Two cases are known on the swelling of thylakoid membranes. One is a reversible change of thylakoid constriction and swelling induced by coupling with energy exchange processes in photosynthesis[17]. Another is a equivocal alteration as a response of plant cells to senescing and/or uncomfortable conditions[24,26]. The sequence of ultrastructural changes in rice chloroplasts fumigated with ozone was similar to that in chloroplasts of naturally senescing or detached aging leaves. It is well known that the ultrastructural changes during plant senescence are usually expressed first in chloroplasts[8,24]. And the conspicuous indicators of chloroplast senescence are appearance of osmiophilic globules, swelling of thylakoids, loss of starch grains, disappearance of plastid ribosomes and loss of chloroplast envelope[24,26].

The formation of osmiophilic globules is the first and the most remarkable modification of these indicators. During chloroplast degradation, osmiophilic globules increase in number and size as thylakoid membranes break down[8,12]. These globules, however, did not increase either in number and size in rice chloroplasts during ozone fumigation. This was a striking difference between ozone

fumigated chloroplasts and naturally senescing ones. This difference depends most likely on a speed and violence of alteration accelerated by ozone fumigation. Cellular degradation is more rapid in detached aging leaves than in naturally senescing leaves[8]. This is also quite true with the degradation of cell organelles. More osmiophilic globules are formed in chloroplasts of naturally senescing leaves than in those of detached leaves where thylakoid degradation was not marked[8]. In chloroplasts of detached aging leaves, the chloroplast envelope is lost before thylakoid break down, but in chloroplasts of naturally senescing leaves, the envelope remains intact until terminal stages of senescence[8]. On the other hand, a main component of osmiophilic globules is composed of lipids released from thylakoids[12,26]. Therefore, no growth in number and size of osmiophilic globules in ozone fumigated rice chloroplasts may be attributed to a rapid decomposition of lipids prior the formation of osmiophilic globules with ozone fumigation.

Plants have usually some repair mechanisms for ozone fumigated membrane injury[14,21]. Sutton and Ting[21] reported that this repair was retarded by cold or continuous dark treatment, enhanced by continuous light, and greatly increased by glucose applied to the leaves. These results suggest that ozone injury at the cellular level can be repaired by energy-dependent processes so that necrosis of the leaf tissue does not occur, and also that conditions and treatments after ozone fumigation can alter the degree of ozone injury. There are only a few reports on the plant resistance and counteraction mechanisms to ozone injury[14,19]. It is important to make clear a remedy mechanism of plant cells for ozone injury with endogenous antioxidants ; ascorbic acid, reduced glutathion and tocopherol etc.

In summary, ultrastructural alterations of chloroplasts in ozone fumigated rice leaves may be regarded as a rapid and violent senescence caused by ozone exposure.

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