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LIGHT-DEPENDENT CELLULAR LEAKAGE FROM CUCUMBER COTYLEDON DISCS TREATED WITH δ-AMINOLEVULINIC ACID, OXYFLUORFEN, AND ROSE BENGAL

HEE JAE LEE* and KWANG YUN CHO
Division of Screening Research, Korea Research Institute of Chemical Technology,
Taejon, 305-606, Korea

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Abstract — When cucumber (*Cucumis sativus* L.) cotyledon discs were floated on δ-aminolevulinic acid, oxyfluorfen, or rose bengal solution under light condition following 20 h dark incubation, rapid electrolyte leakage from the tissues occurred. The electrolyte leakage from the tissues was dependent on the compounds treated, their concentrations, and the duration of light exposure to the tissues. Dark incubation before exposure to continuous white light enhanced electrolyte leakage from the tissues treated with the compounds and reduced lag period for the activity of the compounds. Electrolyte leakage from the treated tissues was greatly influenced by the light intensity to which they were exposed. Higher light intensities stimulated electrolyte leakage and reduced lag period. Porphyrin biosynthesis inhibitors, gabaculine and 4,6-dioxoheptanoic acid, completely inhibited electrolyte leakage from the oxyfluorfen-treated tissues. Protection against the activity of δ-aminolevulinic acid from electrolyte leakage was complete with 4,6-dioxoheptanoic acid, but not with gabaculine. However, gabaculine and 4,6-dioxoheptanoic acid gave no such protection against rose bengal activity. In summary, our results indicate that δ-aminolevulinic acid, oxyfluorfen, and rose bengal exert their effects by causing electrolyte leakage from the treated tissues in a similar manner, except that oxyfluorfen has an apparent lag period for its action on electrolyte leakage increase. All above compounds require preincubation of treated tissues in darkness and subsequent light exposure with a high intensity for their maximal activities. Our results also support that in the presence of light, δ-aminolevulinic acid and oxyfluorfen cause cellular damage through the indirect generation of singlet oxygen from accumulated tetrapyrroles of porphyrin pathway, whereas rose bengal causes cellular damage through the direct generation of singlet oxygen.

INTRODUCTION

Deregulation of porphyrin pathway would result in the accumulation of the pathway intermediates. For example, δ-aminolevulinic acid (ALA)† treatment to green plants in darkness causes massive accumulation of tetrapyrroles of porphyrin pathway, protoporphyrin IX (Proto), Mg-Proto, and protochlorophyllide.[1-3] Certain diphenyl ethers, such as oxyfluorfen, and structurally related herbicides also cause accumulation of abnormally high levels of Proto.[4-8] The accumulation is the result of inhibition of protoporphyrinogen oxidase (Protox), the last common enzyme in the biosynthesis of both heme and chlorophylls.[9-16] The accumulated tetrapyrroles act as photodynamic pigments, generating singlet oxygen in the presence of light and molecular oxygen.[1,2,6,17] In addition, xanthene dyes such as rose bengal and eosin, are known to be photodynamic sensitizers acting through the generation of singlet oxygen.[18-20] Singlet oxygen triggers a photodynamic membrane lipid peroxidation, resulting in cellular leakage and ultimate cellular death.[13,14,21-24] However, little work is available for the direct comparison of the effects of ALA, diphenyl ether herbicides, and xanthene dyes on plant tissues.

Cellular leakage is one of the first symptoms of peroxidative membrane damage due to environmental stresses and several photodynamic herbicides. Cellular leakage can be determined by measuring leakage of specific endogenous cellular contents (e.g.,

* To whom correspondence should be addressed.
†*Abbreviations* : ALA, δ-aminolevulinic acid; DHA, 4,6-dioxoheptanoic acid; GAB, gabaculine; Proto, protoporphyrin IX; Protogen, protoporphyrinogen IX; Protox, protoporphyriongen oxidase.

amino acids or phenolic compounds),[25] fluorescent compounds, radiolabeled compounds that they have been taken up by the cells,[26] or endogenous electrolytes.[27,28] Among these, electrolyte leakage can be determined continuously with a conductivity probe and without destructive sampling.[27-29] The measurement is relatively simple and quick. Furthermore, electrolyte leakage has successfully been employed to evaluate cellular damage, since it correlates well with other parameters of cellular damage.[5,14,30,31]

Objective of this study is to compare cellular damage, as measured by electrolyte leakage, in cucumber cotyledon discs treated with ALA, oxyfluorfen, rose bengal, or Proto, which are all known to generate singlet oxygen either indirectly (via accumulated tetrapyrroles) or directly. Effects of the compounds on cellular leakage are evaluated under different light conditions. Furthermore, the effects of porphyrin biosynthesis inhibitors, gabaculine (GAB) and 4,6-dioxoheptanoic acid (DHA), with the combination of the compounds are also determined.

MATERIALS AND METHODS

Plant material. Seeds of cucumber (*Cucumis sativus* L. cv. Baekmibaektataki) were germinated in flats in a commercial greenhouse substrate (Boo-Nong Soil; Seoul, Korea) and watered with tap water. Plants were grown in a greenhouse at 30 ± 3/20 ± 3°C, day/night temperature with a 14-h photoperiod for 7 days.

Chemicals. Rose bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Technical grade herbicide oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene] was provided by Dr. J. J. Lee. All other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Organic solvents were obtained from Oriental Chemical Industry (Seoul, Korea).

Chemical treatment. Tissues were treated with compounds depicted in Figure 1 as before[27] by cutting fifty 6-mm diameter cotyledon discs (approximately 0.36 g fresh weight) with a cork borer, and then placing them in a 6-cm diameter polystyrene Petri dish containing 7 ml of 1% sucrose, 1 m*M* 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5) with or without the test compound dissolved in an appropriate solvent. Oxyfluorfen and Proto were dissolved in acetone and 20% ethanol containing 10 m*M* KOH, respectively. When oxyfluorfen or Proto was used, control contained the same amount of the solvent without the compound and the final concentration of the solvent in the dishes was 1% (v/v). No detectable effects of the either solvent alone on tissues were observed during the experiments (data not shown). In some experiments, GAB or DHA was added to the dishes. The tissues were incubated in a growth chamber at 25°C in darkness for various time periods and then exposed to continuous white light at various light intensities for up to 10 h. The light intensity to which the tissues were exposed was set at 30, 60, or 120 μmol/m²/s photosynthetically active radiation by adjusting the height of shelf from the light source. Light intensity was measured by using Leaf Chamber Analysis System (type LCA-3) incorporated with Parkinson Leaf Chamber (type PLC-3) (Analytical Development Co., Ltd., Hoddesdon, England).

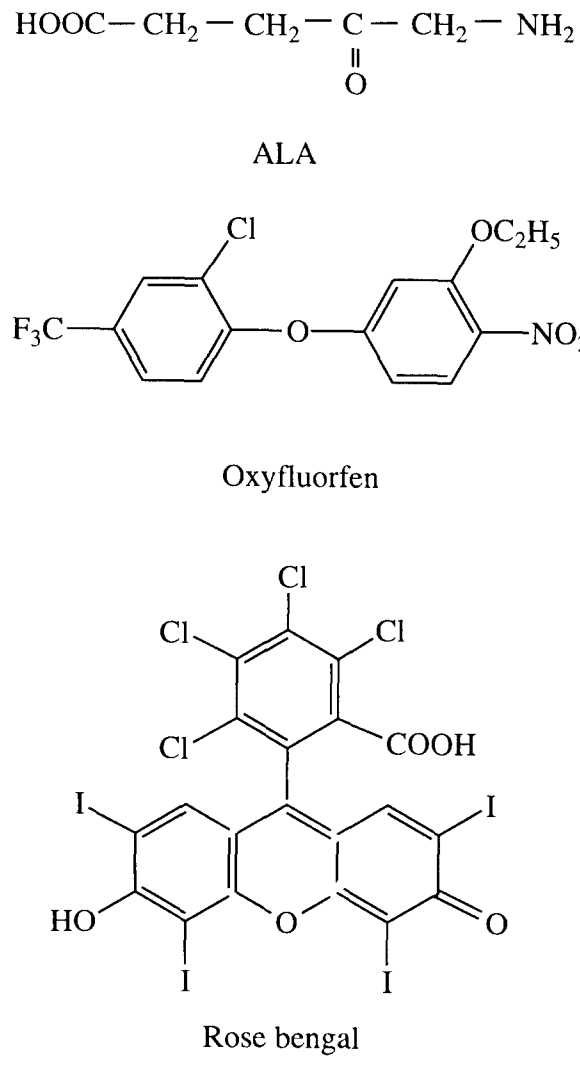

Figure 1. Structures of chemical compounds used in studies.

Cellular damage. Cellular damage was determined periodically by detection of electrolyte leakage into the bathing medium with a conductivity meter (Denki Kagaku Keiki Co., Ltd., Musashino, Japan) as described previously.[27] Because of differences in background conductivity of different treatment solutions, results were expressed as changes in conductivity from initial measurement. The experiments were repeated and results shown are means of triplicate treatments from representative experiments.

RESULTS

Dose response effects of ALA, oxyfluorfen, and rose bengal. When cucumber cotyledon discs were incubated with ALA, oxyfluorfen, or rose bengal under light condition following 20 h dark incubation, significant electrolyte leakage from the tissues into the bathing medium occurred (Fig. 2). The treatment compounds did not cause electrolyte leakage during the 20 h dark incubation (data not shown). However, the electrolyte leakage began to increase upon the exposure to continuous light, indicating that light is an absolute requirement for inducing cellular damage of plant tissues treated with any of those test compounds. The change of electrolyte leakage was proportional to the concentration of the above compounds and the duration of light exposure to the tissues. In general, rose bengal had about one order of magnitude more effect on causing electrolyte leakage than oxyfluorfen. Conversely, ALA had about one order of magnitude less effect than oxyfluorfen. At concentrations of 100 μM and higher, however, ALA was more stimulatory for causing electrolyte leakage than oxyfluorfen, suggesting that ALA has a threshold concentration causing cellular damage. Proto was also treated to the tissues to examine whether Proto has any direct effect on causing electrolyte leakage. However, Proto did not cause any significant electrolyte leakage from the treated tissues at a concentration range from 0.1 to 100 μM (data not shown).

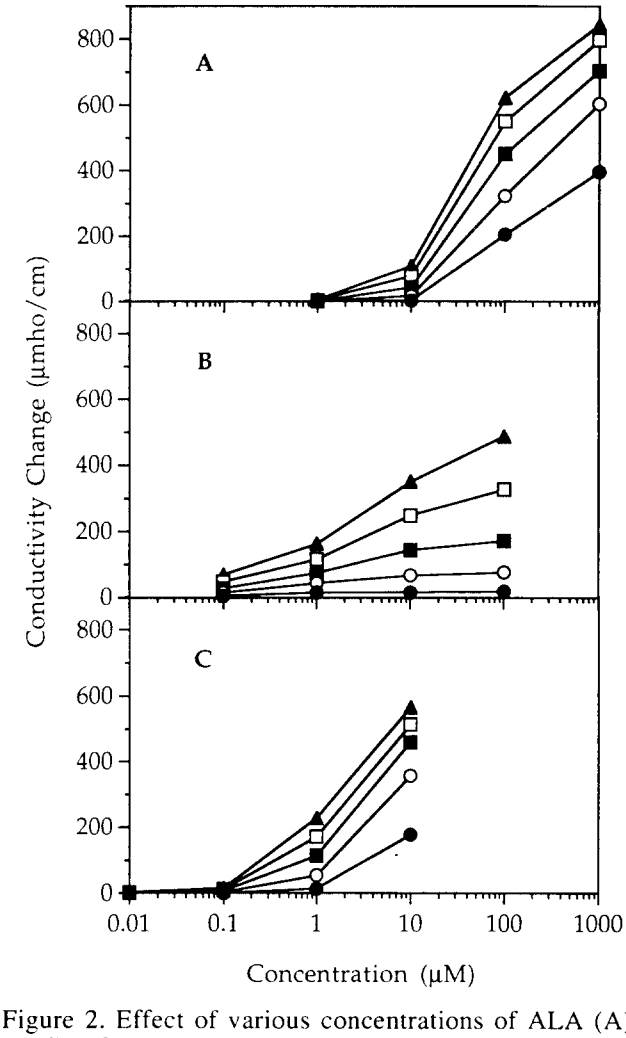

Figure 2. Effect of various concentrations of ALA (A), oxyfluorfen (B), or rose bengal (C) on cellular leakage from cucumber cotyledon discs upon exposure to continuous light at 60 $\mu mol/m^2/s$ at 25°C following 20 h dark incubation. Values are differences between treated and control tissues. Symbols are as follows: ●, 2 h; ○, 4 h; ■, 6 h; □, 8 h; ▲, 10 h after the light exposure.

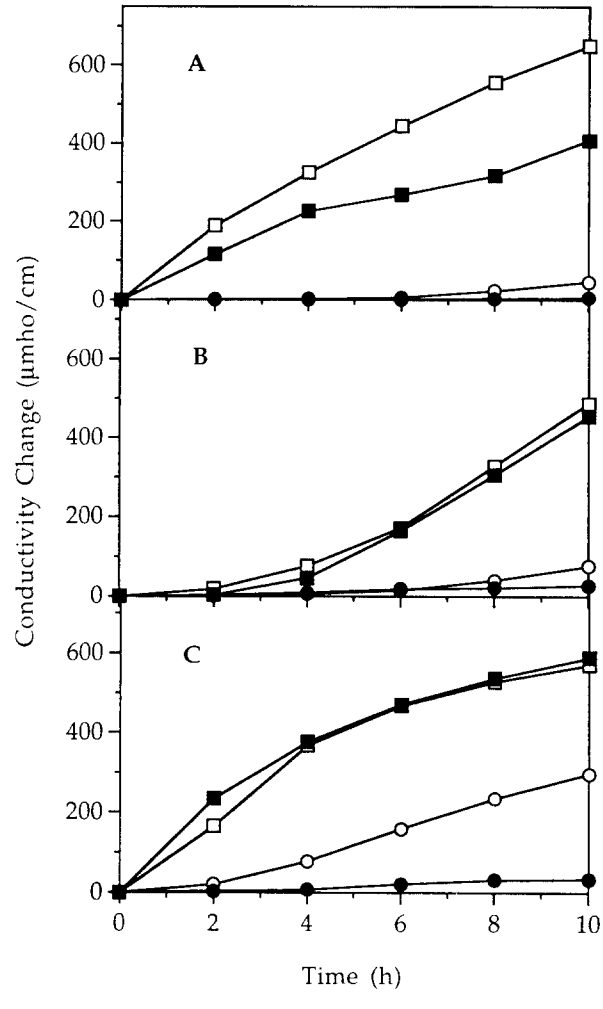

Figure 3. Effect of dark incubation period on cellular leakage from cucumber cotyledon discs treated with 100 μM ALA (A) 100 μM oxyfluorfen (B), or 10 μM rose bengal (C) before exposure to continuous light at 60 $\mu mol/m^2/s$ at 25°C. Values are differences between treated and control tissues. Symbols are as follows: ●, total darkness; ○, 0 h; ■, 10 h; □, 20 h dark incubation.

Dark incubation effects. Electrolyte leakage from the tissues treated with the test compounds was greatly influenced by dark incubation period before exposure to light (Fig. 3). No electrolyte leakage occurred when the treated tissues were incubated in total darkness. Without the dark incubation, electrolyte leakage in the light was not considerable except from the tissues treated with 10 μM rose bengal. However, electrolyte leakage rapidly increased upon the exposure to continuous light after 10 or 20 h dark incubation. No significant differences in electrolyte leakage from the tissues with 100 μM oxyfluorfen or 10 μM rose bengal were found between the 10 and 20 h dark incubation treatments. When the tissues were treated with 100 μM ALA, however, electrolyte leakage from the tissues was much higher with the 20 h dark incubation before exposure to light than with the 10 h dark incubation. Lag period in electrolyte leakage increase was obvious from the tissues treated with 100 μM oxyfluorfen, whereas sharp increase in electrolyte leakage without lag period was detected from the tissues treated with 100 μM ALA or 10 μM rose bengal. Changes in electrolyte leakage from 10 μM rose bengal-treated tissues were almost saturated after 10 h exposure to continuous light following 10 or 20 h dark incubation.

Light intensity effects. Electrolyte leakage from the tissues treated with the test compounds was also greatly affected by the light intensity to which they were exposed. With increasing the light intensities, electrolyte leakage from the tissues treated with any of the test compounds increased more rapidly and the magnitudes of the electrolyte leakage were also increased (Fig. 4). Lag periods which were usually observed under light condition at 30 μmol/m^2/s were greatly reduced or eliminated with the higher light intensities. With a light intensity of 120 μmol/m^2/s, the sharpest increases in electrolyte leakage were found at initial 2 h exposure to the light in 100 μM ALA- or 10 μM rose bengal-treated tissues. When 100 μM oxyfluorfen was treated to the tissues, however, the lag period was not completely eliminated even with a light intensity of 120 μmol/m^2/s. The increases of electrolyte leakage from the tissues caused by the compounds after light exposure were positively related to the light intensity to which they were exposed. The representative relationships resulted

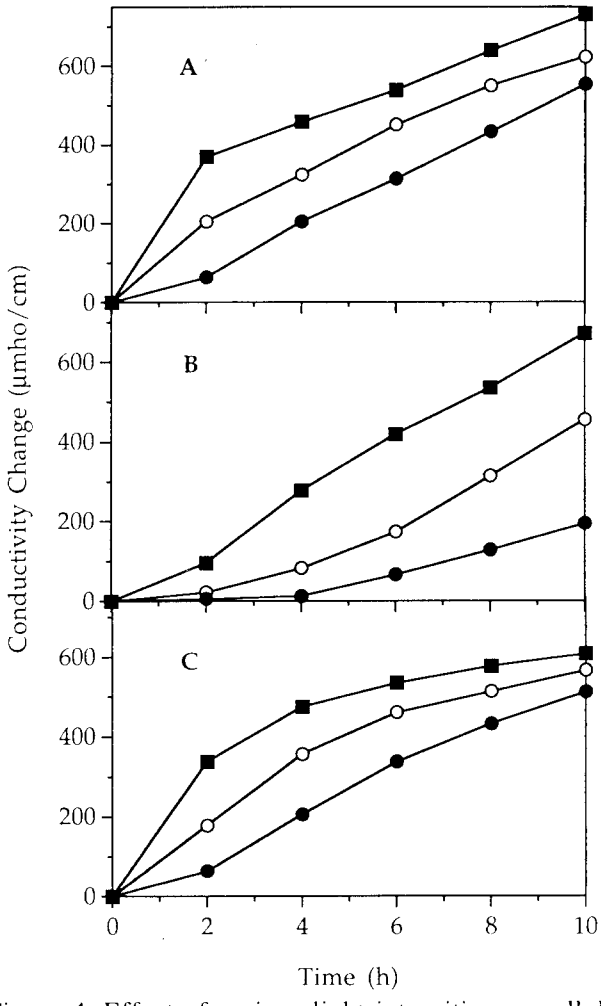

Figure 4. Effect of various light intensities on cellular leakage from cucumber cotyledon discs treated with 100 μM ALA (A), 100 μM oxyfluorfen (B), or 10 μM rose bengal (C) upon exposure to continuous light at 25°C following 20 h dark incubaton. Values are differences between treated and control tissues. Symbols are as follows: ●, 30 μmol/m^2/s; ○, 60 μmol/m^2/s; ■, 120 μmol/m^2/s of light intensity.

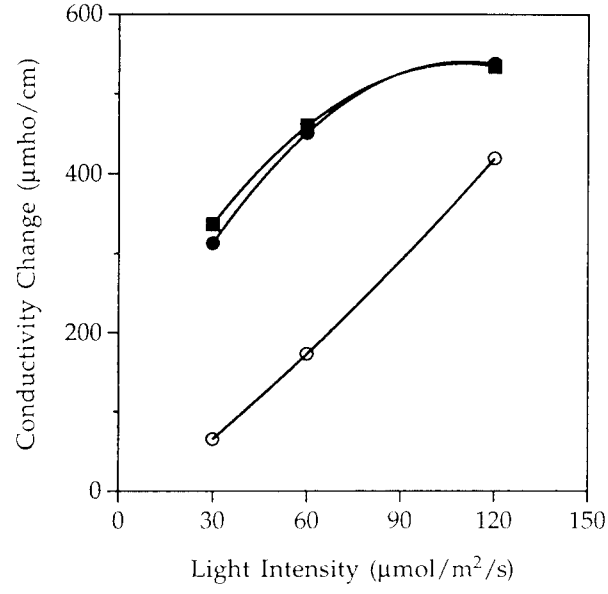

Figure 5. Effects of ALA, oxyfluorfen, or rose bengal on cellular leakage after dark incubation for 20 h and then exposed to continuous light at 25°C for 6 h under different light intensities. Values are from Figure 4. Symbols are as follows: ●, 100 μM ALA; ○, 100 μM oxyfluorfen; ■,10 μM rose bengal.

from each compound treatment were shown in Figure 5.

Porphyrin biosynthesis inhibitor effects. GAB and DHA inhibit porphyrin biosynthesis by inhibiting the biosynthesis of ALA[32,33] and the conversion of ALA to porphobilinogen catalyzed by ALA dehydratase,[34] respectively. GAB or DHA at a concentration of 1 m*M* completely protected the 100 *μM* oxyfluorfen-treated tissues from electrolyte leakage (Fig. 6), confirming the previous findings that the herbicidal activity of oxyfluorfen is dependent on porphyrin biosynthesis.[6,13,14] However, GAB or DHA gave no such protection against rose bengal activity. A complete protection against ALA activity was achieved with the exogenous DHA. It is unexpected that some protection against ALA activity was observed with GAB, since ALA dehydratase would not be inhibited by GAB.[33] GAB or DHA when applied alone had no or little effect on electrolyte leakage from the tissues under dark or light condition (data not shown).

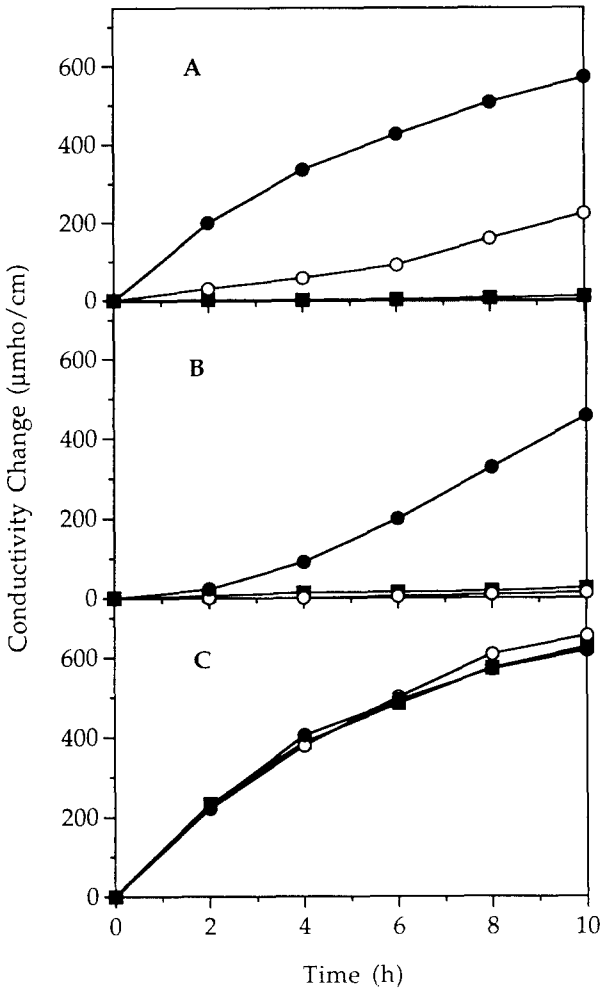

Figure 6. Effect of GAB and DHA in the presence of 100 *μM* ALA (A), 100 *μM* oxyfluorfen (B), or 10 *μM* rose bengal (C) on cellular leakage from cucumber cotyledon discs upon exposure to continuous light at 60 μmol/m^2/s at 25°C following 20 h dark incubation. Values are differences between treated and control tissues. Symbols are as follows: ●, no inhibitor; ○, 1 m*M* GAB; ■, 1 m*M* DHA.

DISCUSSION

Our results indicate that ALA, oxyfluorfen, or rose bengal causes significant cellular damage in the light, but not in the dark. The light-dependent activity of the compounds is due to the generation of singlet oxygen. Although rose bengal is known to generate singlet oxygen directly in the presence of light,[19] ALA or oxyfluorfen could produce the singlet oxygen only through the accumulation of tetrapyrroles of porphyrin pathway.[1,2,6,17,23] It is well known that plant tissues have a capability to accumulate tetrapyrroles in response to exogenous ALA or oxyfluorfen in darkness. Proto, Mg-Proto, and protochlorophyllide are known to be accumulated in ALA-treated tissues,[1-3] whereas Proto is the main tetrapyrrole accumulated in the herbicide-treated tissues.[4-8] Massive accumulation of Proto in the herbicide-treated tissues is due to inhibition of Protox, plastid enzyme, which catalyzes the conversion of protoporphyrinogen IX (Protogen) to Proto in porphyrin pathway.[9-16] When plastid Protox is inhibited by the herbicide, Protogen, the substrate of Protox, is exported from the plastid[35] and rapidly oxidized to Proto by plasma membrane-bound herbicide-resistant Protox-like activity.[15,16] Proto, which is relatively lipophilic, accumulates in plasma membrane and/or other extraplastidic membranes.[13,15]

Upon the exposure of light, the abnormally high levels of tetrapyrroles accumulated act as singlet oxygen-generating photodynamic pigments. Singlet oxygen initiates a peroxidation process of unsaturated membrane lipid by hydrogen abstraction, resulting in the formation of lipid hydroperoxides.[22,36] The consequence of the process is membrane destruction, which could easily be determined by the detection of electrolyte leakage. Membrane destruction may be one of the primary effect to occur in response to exogenous ALA, oxyfluorfen, or rose bengal, since electrolyte leakage increased rapidly following light exposure. However, there was a typical lag period before any significant increase in electrolyte leakage could be detected (Figs. 2, 3. and 4). The lag period was more apparent in oxyfluorfen-treated tissues than in ALA- or rose bengal-treated tissues. This lag period may be due to the fact that the natural protective mechanisms, such as detoxifying enzymes and endogenous antioxidants, are capable of scavenging singlet oxygen until the mechanisms are

overloaded.

Exogenously supplied Proto was not found to be effective to cause cellular leakage from the treated tissues (data not shown) as in the previous report.[25] This could be explained by poor cellular uptake of this relatively complex molecule into the tissues, since it is known to be lipophilic.[15,37]

Effect of ALA, oxyfluorfen, or rose bengal on causing cellular damage in the light was remarkably increased by dark incubation of the treated tissues before exposure to light (Fig. 3). Dark incubation increased the magnitude of electrolyte leakage from the treated tissues in the light and reduced lag period for the activities of the compounds. This is consistent with the previous reports that long dark incubation was necessary for maximal activities of ALA[5,38,39] and diphenyl ether herbicides[7,26,28,40] in the light. In addition, rose bengal is known to be subject to photodegradation which reduces its capacity to generate singlet oxygen.[19,41] The requirement of dark incubation may be related to uptake of the compounds, increasing effective concentration in the tissues. Furthermore, dark incubation seems to be necessary to accumulate enough tetrapyrroles for photodynamic cellular damage in ALA- or oxyfluorfen-treated tissues.

Electrolyte leakage from the tissues treated with the compounds was highly dependent on the light intensity to which they were exposed (Fig. 4). With higher light intensities, more electrolyte leakage occurred and lag period was greatly reduced or eliminated. Almost linear relationships were found between electrolyte leakage increases from the compound-treated tissues and the light intensity exposed (Fig. 5).

Blockage of porphyrin pathway at ALA dehydratase by 1 m*M* DHA completely reduced the effect of ALA or oxyfluorfen on causing cellular leakage from the treated tissues (Fig. 6). Similarly, GAB at a concentration of 1 m*M* completely protected the oxyfluorfen-treated tissues from cellular leakage. These findings confirm that the activities of ALA and oxyfluorfen are absolutely dependent on tetrapyrrole accumulation of porphyrin pathway.[14,34,42] However, it is surprising that some protection against ALA activity was observed with GAB (Fig. 6). Possible explanations for this observation are that reduced endogenous ALA production caused by GAB may contribute to the decrease of cellular leakage and/or 1 m*M* GAB concentration used in this study may be detrimental to ALA dehydratase activity.

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