Differential Effects of Herbicidal Compounds on Cytoplasmic Leakages of Green– and White – Maize Leaf Segments

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Using maize green - and white - leaf tissues, we have examined the effect of various chemicals on cytoplasmic leakage with respect to the light requirement or chloroplast targeting for their activities. Oxyfluorfen, oxadiazon, diuron, and paraquat, which are known as representative herbicides acting on plant chloroplasts, caused the electrolyte leakage only in the green tissues, whereas 2, 4-dinitrophenol, rose bengal (singlet oxygen producing chemical) and methyl-jasmoante (senescence-stimulating chemical) play a role both in green- and white - tissues. Benzoyl(a) pyrene, generating superoxide radical upon light illumination, functions only in white tissues. Tralkoxydim, metsulfuron-methyl and norflurazon showed no effect in two tested plant samples. In terms of light requirement in electrolyte leakage activity, diuron, oxyfluorfen, oxadiazon, rose bengal, and benzoyl(a) pyrene absolutely require the light for their functions, but other chemicals did not. Based on these results, we could classify into four different response types according to whether chemicals require light or chloroplasts for their action. This classification is likely to be applied to simply and rapidly identify the requirement of light and chloroplasts for the actions of chemicals, thereby it makes easy to characterize many new herbicides that their action mechanisms are unclear, and to elucidate the mode of action of them.

Key words: Chloroplast - targeting, electrolyte leakage, herbicide screening, light requirement, white leaf

INTRODUCTION

Plant chloroplast have been a good target organelle for developing a herbicide because of its presence only in the plant system and many herbicides targeted to the organelle have been commercialized up to date [1]. To know whether certain chemicals are acting on the chloroplast, the metabolic status peculiar to the organelle, such as photosynthetic Hill reaction, contents of chlorophyll biosynthetic intermediates or the activities of chloroplast enzymes, has been investigated in general. However, these biochemical methods are relatively complicated and time-consuming. If there is any a simple bioassay that may be achieved by a single subjection of tissue segments, it may be useful in screening many new chloroplast-targeting compounds easily and elucidating their action modes. For this purpose, a simple investigation method should be set up primarily. Practically the change in chlorophyll content [2] and cytoplasm leakage [3] have been used as the simple index for the chloroplast damage, because chloroplast-targeting compounds, directly and indirectly, have an effect on the change of chlorophyll contents and herbicides such as oxyfluorfen, diuron and paraquat, producing active oxygen species (ROS) in chloroplast, cause chlorophyll breakdown as well as the cytoplasm leakage by the peroxidation of membrane [4-8].

However, since non-chloroplast targeting chemicals can also affect, secondary or tertiary, the chlorophyll content and cytoplasm leakage, both indices are not absolute values in a single and one step assay system in order to find out compounds acting directly to chloroplast. To overcome this problem, we tried to use the cytoplasmic leakage as the investigation index and two different tissues, green- and white leaf segments. It is expected that chloroplast-acting chemicals appear to be effective only on grana-containing green tissue, whereas chemicals that act on any membrane effective on both green- and white tissues. In addition, photosensitizer causing cellular leakage may be also classified by a series of experiment, an incubation of chemical-treated two different tissues in the dark followed by light condition.

The important factor in this system is how uniform white tissues are obtained easily. Instead of using white mutants which their production is limited, white tissues generated by inhibiting the carotenoid biosynthesis were used in this experiment. Carotenoids protect photosystems from ROSs produced normally during photosynthesis in the chloroplast [1,9]. Therefore, the inhibition of carotenoid biosynthesis during leaf development develops white tissues in which photosynthesis doesn't occur because of loss of chlorophyll, breakdown of thylakoid membrane and inactivation of ribulose carboxylase [10-12]. In the white tissue, the nitrate reductase is also inactivated, but there is no effect on photomorphogenesis and activities of cytoplasmic enzymes [15,16]. The white tissue that can not absorb light energy produces little ROSs, thereby

they can grow for certain period of time if adequate nutrients are supplied under the moderate light intensity.

In this study, the possibility and availability of this new strategy as a simple bioassay to identify the chloroplast targeting herbicides as well as to search photosensitizers that require light for their functions was investigated.

MATERIALS AND METHODS

Plant material   Green- and white - seedlings were obtained by dipping seeds in distilled water or 150 μM of fluridone for 1 day followed incubated in the growth chamber (14 h light / 10 h dark, 70 μmolm^{-2}s^{-1}) for 7 days, respectively. The tissues used in this experiment were sampled from the second leaf blade of maize (*Zea mays* cv. Suwon 19).

Chemicals   Three groups of chemicals were selected for this experiment: chloroplast-targeting chemicals, such as diuron, oxyfluorfen, oxadiazon and paraquat; nonchloroplast-targeting chemicals, such as 2, 4-dinitrophenol, methyl-jasmonate, rose bengal and benzo(*a*) pyrene; chemicals that do not cause cytoplasmic leakage, such as metsulfuron-methyl, tralkoxydim and norflurazon. All chemicals were dissolved in either distilled water or 1% acetone containing 0.01% Tween - 20.

Experimental procedures   Leaf tissues were cut into 5 mm in length after mid-rib was removed and weighed to 0.15 g for each treatment. The leaf segments were incubated on petri-dish containing 7 m*l* of 1 mM MES buffer, pH 6.5, which is supplemented with either 1% or 0.015% sucrose, for 12 h under the darkness. Then, the tissues were transferred to light condition (110 μmolm^{-2}s^{-1}) and the amount of leaked electrolytes was measured with conductance meter (AOC-10, DKK Co.) at the indicated time. The difference in the amount of leaked electrolytes between control and treatment has calculated and all experiments replicate three times under 25°C.

RESULTS AND DISCUSSION

Responses to chloroplast-targeting chemicals

Three types of chemicals targeting and functioning actively in chloroplasts were tested : chlorophyll biosynthesis inhibitors (oxyfluorfen and oxadiazon), photosystem (PS)I inhibitor (paraquat) and PSII inhibitor (diuron) (Fig. 1). In the case of treatment of either oxyfluorfen or oxadiazon, no electrolyte leakage was observed in both green- and white - leaf segments under the darkness (Fig. 1A and B). In contrary, large amounts of electrolyte leakage were detected in the green-leaf segments when the tissue segments were transferred to light, whereas not in the white tissues. These two chemicals are inhibitors of protoporphyrinogen oxidase (PROTOX), which is one of enzymes catalyzing chlorophyll biosynthesis [17]. Inhibition of PROTOX reduces chlorophyll biosynthesis, causing the accumulation of its substrate, protoporphyrinogen IX. The
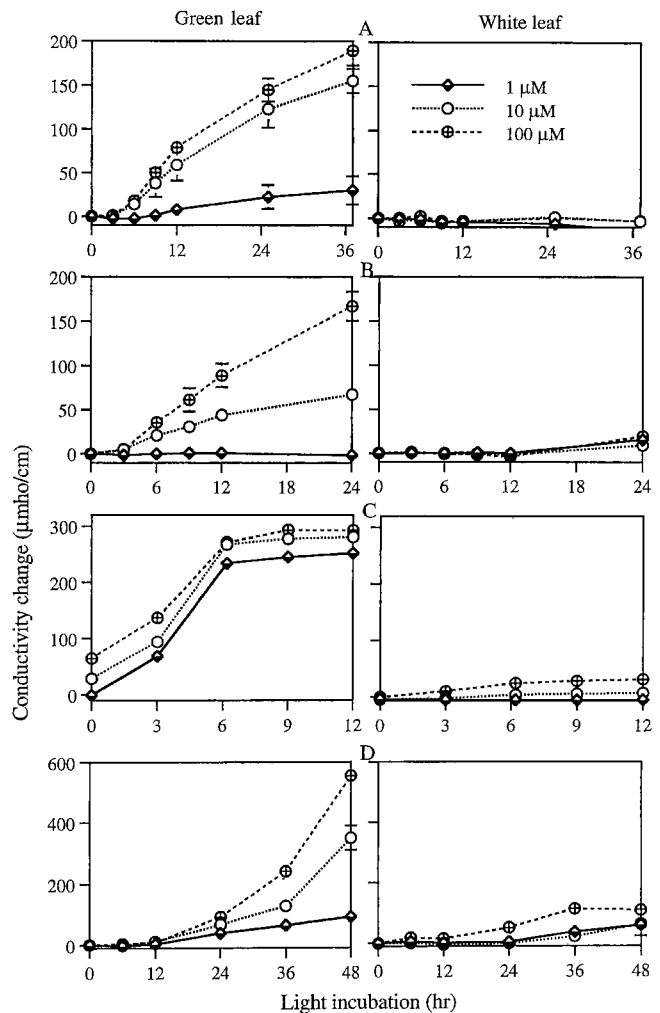

Figure 1. Effects of oxyfluorfen (A), oxadiazone (B), paraquat (C) and diuron (D) on electrolyte leakages of green- or white - maize leaf segments. Bars represent standard errors from the means of three replicates.

protoporphyrinogen IX accumulated is transferred out of chloroplasts [18] and then oxidized into protoporphyrin IX by the herbicide-resistant PROTOX homolog present on the plasma membrane [19,20]. Thus, abnormally accumulated protoporphyrin IX can not be involved in chlorophyll biosynthesis. Instead, they generate singlet oxygens upon light illumination, resulting in membrane lipid peroxidation [4,8] and necrosis. Therefore, it is not surprising that there is no response observed in the white tissues by those two chemical treatment, while distinct leakage detected in the green tissues. The no response in the white tissues may be due to extremely low capacity for chlorophyll biosynthesis by inhibition of glutamate supply, based on the reports that neither nitrate reductase activity nor nitrite reductase activity was observed in the white tissues [13,14] and protochlorophyllide synthesis was inhibited in carotenoid-deficient plants even under extremely low light intensity [11], whereas certain amounts of Mg-

protoporphyrin IX methyl ester were accumulated in the white tissues even under darkness when 5-aminolevulinic acid was added exogenously [unpublished data].

Paraquat treatment in green tissues caused a relatively low level of electrolyte leakage in the dark, but dramatic effects were observed with the concentration-dependent pattern after the incubation under light (Fig. 1C). In contrary, paraquat in white tissues had no response in the dark and only little effects were observed in the treatment of 100 µM paraquat under light illumination. These results imply that the paraquat activity requires green tissues, but absolutely not depends on light illumination. Paraquat became radical state by absorbing electrons from ferredoxine in photosystem I and then, disrupted cell membranes [6,7,21]. Therefore, the herbicidal effect of paraquat was pronounced in the green tissues under the light, but little in the white tissues which has no normal photosystems [37,38]. A little effect of paraquat in the white tissues under darkness seems to be the result of the radicalization of the paraquat by receiving electrons from cytochrome P450 enzyme system, which has relatively low affinity to the chemical [22].

Diuron treatment did not cause the electrolyte leakage both in green- and white - tissues during darkness, but increased the leakage with 12-h lag time upon light illumination especially in green tissue (Fig. 1D). Whereas, there was little effects observed in white tissues. Diuron blocks the electron transport by binding to the Qb site of D1 polypeptide in PSII [23,24]. The excited chlorophylls present in the diuron-treated tissues generate ROSs, which breakdown cell membranes [5]. Therefore, diuron may cause pronounced leakage of electrolyte from the green tissues, while relatively low effects in the white tissues in which photosynthetic electron transport does not work normally. Compared to others treatments, diuron showed higher electrolyte leakage (Fig. 1D). We speculate that the high leakage might be due to lowering the concentration of sucrose in the test solution from 1% to 0.015%. It has been known that the relative high sucrose concentration in the buffer solution will reduce the photosynthetic electron transport due to the phosphate deficiency [25,26] and decrease the electrolyte leakage itself by increasing the osmotic pressure. The latter case was proven by that the leakage in 1% mannitol containing solution was lower than in 0.01% solution (data not shown).

Responses to non-chloroplast-targeting chemicals

The effects of rose bengal, 2, 4-dinitrophenol, methyl-jasmonate, and benzo(a) pyrene on the leakage of electrolytes were investigated in maize green- and white - leaf segments. Rose bengal that generates singlet oxygen species itself by the absorption of light [27] rapidly induced the electrolyte leakage from both green and white tissues (Fig. 2A). The slightly increased leakage by 100 µM rose bengal treatment in the dark may be due to green-safe light exposed during chemical treatment. An uncoupler of the electron transport system, 2, 4-dinitrophenol [28], showed similar effects on both tissues (Fig. 2C). Methyl-jasmonate, a chemical causing senescence
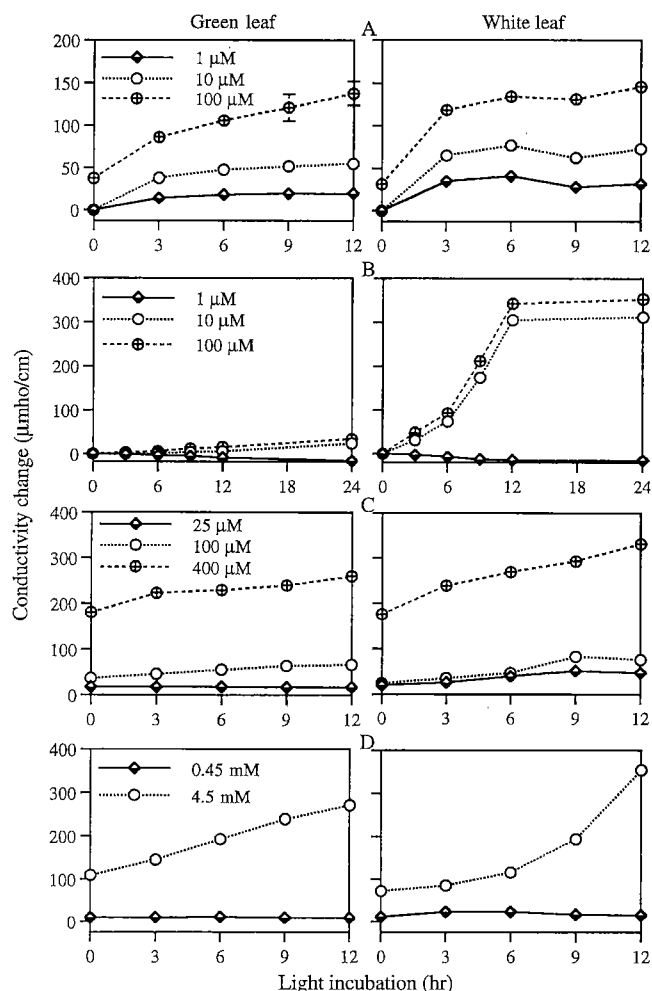

Figure 2. Effects of rose bengal (A), benzo(a) pyrene (B), 2,4-dinitrophenol (C), and methyl-jasmonate (D) on the electrolyte leakages of green- or white-maize leaf segments. Bars represent standard errors from the means of three replicates.

and membrane peroxidation by ethylene production [29,30], also induced a significant electrolyte leakage, even in the darkness if high concentration was applied (Fig. 2D).

Benzo(a) pyrene is a photosensitizer generating superoxide radicals by light illumination [31]. This chemical was very effective upon the light illumination, especially in the white tissues (Fig. 2B). This result might be due to that the chemical has been trapped and inactivated by chlorophyll molecules in the green tissue. In fact, there were reports that chlorophyllin and natural porphyrins showed anti-cancer activity by functioning as the interceptor molecules of mutagens in animal system [39]. However, the mechanism underlined its action has to be elucidated in plant system. Taken together, non-chloroplast targeting chemicals exerted their activities even in the white tissues.

Responses to other chemicals

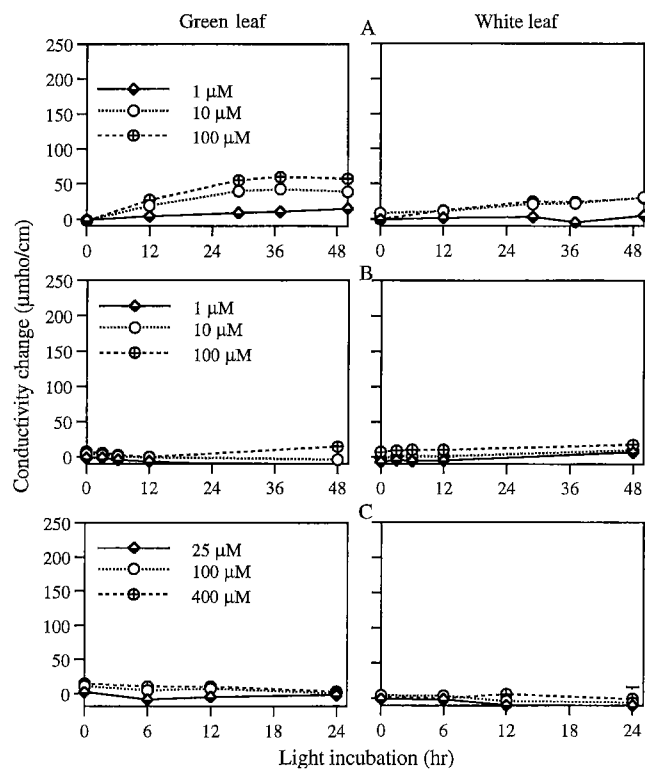Some chemicals having herbicidal effects, such as norflurazon,

Figure 3. Effects of metsulfuron-methyl (A), tralkoxydim (B) and norflurazon (C) on the electrolyte leakages of green- or white-maize leaf segments. Bars represent standard errors from the means of three replicates.
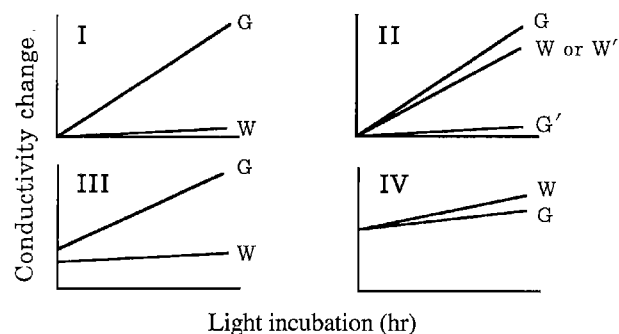

Figure 4. Summary for action types of chemicals in relation to electrolyte leakage. G and W represent green- and white-leaf segments.

Table 1. Classification of the tested chemicals based on different response patterns of electrolyte leakage between green- and white-maize leaf segments

Types	Chloroplast-targeting	Light-dependence	Chemicals
I	Yes	Yes	oxyfluorfen, diuron, oxadiazon
II	No	Yes	rose bengal, benzo(a) pyrene
III	Yes	Partial	paraquat
IV	No	No	2,4-dinitrophenol, methyl-jasmonate

tralkoxydim, and metsulfuron - methyl, showed no distinguishable result (Fig. 3). Metsulfuron - methyl, a sulfonylurea compound, inhibits acetolactate synthase, thereby causing to stop growth and inducing chlorosis and necrosis slowly [36]. This chemical plays a role especially in the actively differenciating tissue. As shown in Fig. 3A, the effect of this chemical on the membrane leakage was not so dramatic as in chloroplast-targeting chemicals. Only little effects were observed in the green tissues treated with over 10 μM. Tralkoxydim, an inhibitor of acetyl CoA carboxylase [33], caused the treated plants to stop growth and to start chlorosis and necrosis slowly [34,35]. It also functions higher in the actively differenciating tissue. This chemical did not show any leakage of electrolytes in tested conditions (Fig. 3B). Norflurazon, an inhibitor of phytoene desaturase, caused a bleaching (whitening) symptom by in-hibiting the carotenoid biosynthesis in young developing leaves [32]. Norflurazon showed no effect in both green- and chlorotic-tissue, because the chemical did not function in mature and differentiated tissues [10,12,16].

All results obtained in this experiment were exactly consistent with the known characterisitcs of tested chemicals in terms of action mechanisms. Although variuos responses were observed, we can classified them into four types depending on chloroplast-targeting and light requirements (Fig. 4, Table 1). Chemicals showing no effect have been omitted in the classification. The

first type includes chloroplast-targeting and light requirement, like diuron, oxyfluorfen and oxadiazon. The second type requires light for their action, but functions in both green- and white-tissues. Rose bengal and benzo(a) pyrene belong to this type. The third type includes paraquat that appears to be chloroplast-targeting, but not require light. Instead, under the light condition its activity was higher in the green tissue than in white one. The fourth type chemicals caused the leakage in all the tested conditions and tissues, impling that they need neither chloroplasts nor light. They include 2, 4-dinitrophenol and methyl-jasmonate. Accordingly, this classification is likely to be applied to simply and rapidly identify the requirement of light and chloroplasts for chemicals' action, thereby it makes easy to characterize many new herbicides that their action mechanisms are unclear, and elucidate the action mode of them.

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