Accumulation of Protoporphyrinogen IX Induced by Acifluorfen Methyl

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Confocal fluorescence microscopic images were used to investigate the accumulation site of protoporphyrin IX (PPIX) within liverwort cells *(Marchantia polymorpha L.)* treated with peroxidizing herbicides as acifluorfen methyl (AFM). A high level of PPIX accumulation was observed in the cells during 12 h to 24 h after the addition of AFM. The results obtained from confocal fluorescence microscopic images gave clear evidence that the accumulation of PPIX occurred only in the chloroplasts, but was not observed in the cytosol or at the plasma membrane. The presence of PPIX in the chloroplasts strongly suggests that protoporphyrinogen (Protogen) accumulates by inhibition of protoporphyrinogen oxidase (Protox) which is the target enzyme for peroxidizing herbicides. The plastidic occurrence of PPIX provides evidence of either the presence of an additional herbicide-resistant Protox or of a non-enzymatic Protogen-oxidation system in the *Marchantia* chloroplast.

Introduction

A number of laboratories have reported that peroxidizing herbicides such as acifluorfen, chlorophthalim and 7-[2chloro-6-fluoro-4-(trifluoromethyl)phenoxy]-2,4-dimethyl-2H,4H-1,4-benzoxazine-3-one and its derivatives cause accumulation of PPIX in the plant cells, rapid photobleaching, and the accumulation of PPIX which correlates to their herbicidal activity (Sandmann and Böger, 1988; Witkowski and Halling, 1989; Matringe *et al.,* 1989; Matsumoto and Duke, 1990; Watanabe *et al.,* 1992; Sumida *et al.,* 1995). PPIX is also recognized as a potent photo-sensitizer (Carlson *et al.,* 1984; Rebeiz *et al.,* 1987). These observations have led to the hypothesis that PPIX produces activated oxygen in the light that results in peroxidation of membrane lipids and the following membrane disruptions are responsible for the major herbicidal effects. We have also reported that PPIX may be transformed to a pigment fluorescing at 590 nm (590FP) under the oxidative condition induced by these herbicides and that 590FP causes phytotoxicity in the light in liverwort cells (Iwata *et al.,*1992; Iwata *et al.,* 1994). On the other hand, it has been demonstrated that the primary effect of these peroxidizing herbicides to algae and plants is inhibition of protoporphyrinogen oxidase in chloroplasts and mitochondria, and peroxidizing herbicides lead to an accumulation of PPIX *in vivo,* the product of the enzyme reaction (Matringe and Scalla 1988a; Matringe and Scalla 1988b). The mechanism of PPIX accumulation is not yet fully elucidated, but it is generally assumed that unprocessed protoporphyrinogen molecules diffuse out from their site of synthesis and then oxidize to PPIX in the stroma and cytosol. Recently, Jacobs *et al.,* (1991) have found Protox forms much less sensitive to inhibition by peroxidizing herbicides at a nonplastidic site, such as the plasma membrane or the cytoplasm. Due to herbicide-insensitive Protox enzymes within plant cells, one possible mechanism of PPIX accumulation is explained that protoporphyrinogen, which is synthesized by the chloroplast, may diffuse out of the organelle and is

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oxidized to PPIX at the plasma membrane or in the cytoplasm. This explanation is not in accordance that accumulated protoporphyrinogen IX was not observed to diffuse substantially away from the chloroplast into the cytoplasm or to the plasma membrane.

The objective of the present study was to determine experimentally the site of tetrapyrrole biosynthesis affected by AFM in liverwort cells using a confocal laser scanning fluorescence microscope. Compared to conventional light microscopy, confocal scanning microscopy has the superior capability to directly visualizing the three-dimensional structure inside the cell. The PPIX-specific fluorescence images obtained demonstrated the location site of PPIX inside the cellular organelle induced by AFM. It also showed clearly that PPIX increased transiently in certain regions of treated liverwort cells suggesting that excess protoporphyrinogen IX does not diffuse from the chloroplast into the cytoplasm, but may be converted to PPIX in the chloroplast.

Materials and Methods

Chemicals

Acifluorfen methyl (AFM, methyl 5-[2-chloro-4-(trifluoromethyl)-phenoxy]-2-nitrobenzoate) was synthesized according to the method of Johnson (1977). PPIX was purchased from Sigma Chemical Co. (St. Louis, USA). Other chemicals were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Cell culture and herbicide treatment

A cell suspension culture of *Marchantia polymorpha L.*, has been routinely subcultured every two weeks using a modified medium of Murashige and Skoog (1962). The suspension cultures were grown in 100-ml flasks containing 40 ml of medium on a rotary shaker (Model LR-3, Iwashiya K. Sawada Co., Tokyo) at 110 rpm at 25°C. The cultures were continuously illuminated from the bottom by fluorescent lamps with an average light intensity of about 9 W/m^2 at the bottom of the flasks. Dark suspension cultures were grown by placing the flasks in black bags.

For experimental purposes, 6-day old cells of the exponential growth phase, called "immature cells", or 20-day old cells of the stationary growth phase, called "mature cells", were adjusted to a cell density of 1 mg dry weight/ml culture medium. Aliquots were treated with herbicides at the concentrations indicated and incubated either in dark or light at 25°C.

Extractions and determination of chlorophyll and PPIX

Cells of 1-ml culture aliquots were collected by centrifugation (5 min at 3000 x *g*) and the pellet was extracted 3 times with boiling 90% methanol. Absorbances at 653 nm and 666 nm were measured and the total chlorophyll concentration was calculated using the method of Iwamura *et al.*, (1970). Extraction and estimation of the fluorescent PPIX was carried out according to the method of Lee *et al.*, (1991). Cells of 1-ml culture aliquots collected by centrifugation were suspended in 1 ml of water and extracted with 5 ml of a mixture of cold acetone: 0.1 N NH_4OH (9:1, v/v) overnight. After centrifugation at 1500 x *g* for 5 min at 5°C, the clear supernatant was collected and washed twice with the same volume of hexane. The acetone fraction contained the fluorescent pigments and protochlorophyllide; their fluorescence was measured with a Hitachi F3000 fluorometer, using an excitation wavelength of 410 and emission wavelength of 633 nm.

Cell preparation and confocal fluorescence microscopy

Six-days old cells were treated with AFM at the concentrations indicated and incubated either in the dark or the light at 25°C. Microscopic images of cultured liverwort cells and AFM-treated liverwort cells were taken by a confocal scanning fluorescence microscope system (MRC-600; Bio-Rad, Cambridge, MA) with an inverted epifluorescence microscope (Nikon TMD-EFQ; Tokyo). The temperature of the observation chamber was controlled at 37°C. To observe the accumulation of PPIX in liverwort cells, two filter systems ranging from 560 to 630 nm (PPIX fluorescence) and above 665 nm (chlorophyll fluorescence) were combined on the confocal fluorescence microscopic system. The preparations of liverwort cells

were excited at 488 nm using an argon-ion laser and the fluorescence emission of PPIX and chlorophyll were observed at the range of 560–630 nm and above 665 nm, respectively.

Results and Discussion

Accumulation of protoporphyrin IX (PPIX) in AFM-treated cells

PPIX can be visualized by fluorescence microscopy with the typical fluorescence at 630 nm in liverwort cells after cultivation with AFM in the light or darkness. In the experiment of Fig. 1, growth of liverwort cells of the exponential phase under the light was completely inhibited by 10 μM of AFM within 4 days and chlorophyll decreased to zero after 6 or 7 days. The cellular disruption was complete with 10 μM AFM treatment for 4 days, and plasmalemma and chloroplast membrane disruption had been occurred in most cells as visualized by light microscopy. Fig. 1 also shows that AFM induced a high level of PPIX accumulation in the cultured cells in the light, reaching a peak after 15 h and decreasing rapidly the following 24 h. When liverwort cells were incubated with AFM in the dark, PPIX also accumulated rapidly and attained a high level in the cells after 15 h.

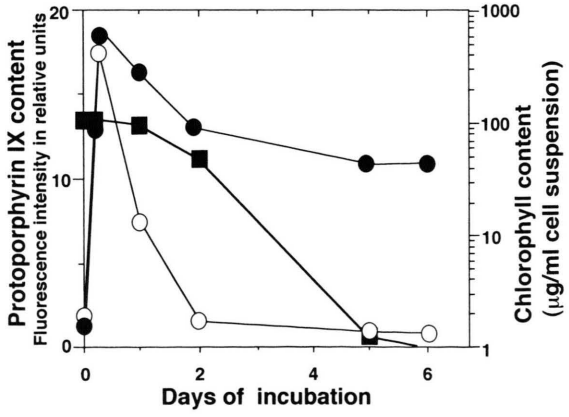

Fig. 1. Effect of a peroxidizing herbicide on chlorophyll and PPIX of cultured cells. Liverwort cells were treated with 10 μM AFM in the light or dark. Chlorophyll content (■) of the cells treated in the light. PPIX contents of the cells after treatment in the light (○) or the dark (●).

Location of PPIX in liverwort cells

A concentration of 10 μM was enough to induce the maximum accumulation level of PPIX in the cells during 24 h under the light or dark. We then measured the fluorescence image in liverwort cells before and after 15 h and 48 h treated with AFM in the dark using the confocal fluorescence microscope. To catch the specific fluorescence of PPIX we used the filters with a transmission emission range of 575–630 nm. Fig. 2 shows a typical example before and after incubation with AFM. By AFM treatment for 15 h and 48 h, fluorescence intensity of PPIX increased gradually as demonstrated by Fig. 2 B, C and confocal fluorescence microscopic image of PPIX in the cells was homogeneous. The regions of bright fluorescence in the cells after a 15 h treatment with AFM were present in the chloroplasts and the accumulated bright fluorescence diffused homogeneously within the chloroplast. This fact indicates that build-up of protoporphyrinogen IX by inhibition of protoporphyrinogen oxidase and its conversion to PPIX occurred in chloroplasts exclusively, but not in the cytoplasm and at the cytoplasm membrane. Although cultivation for 48 h in the dark with AFM present led to no loss of chlorophyll in the cells and confocal fluorescence microscopic image of these cells exhibited a bright fluorescence due to the PPIX accumulation, PPIX seemingly leaks out from chloroplast and is dispersed to the cytoplasm. When the cells treated for 48 h with AFM in the dark were exposed to the light, immediately the chlorophyll content decreased and membrane disruption of chloroplasts occurred. Recently, Jacobs *et al.* (1991) also observed the appearance of the typical porphyrin fluorescence in the intact cells of plant tissue with etioplasts by treating it with a peroxidizing herbicide.

For understanding the site of action of peroxidizing herbicide, more detailed experimental proof is required whether excess protoporphyrinogen is easily exported and enzymatically oxidized by Protox located outside the chloroplast or is not exported to other organelles in plant cell. The liverwort cell culture system can contribute to understand the site of action of peroxidizing herbicides, because these cells are very sensitive and show a quick response to herbicides. Especially, accumulation of PPIX and other tetrapyrroles af-

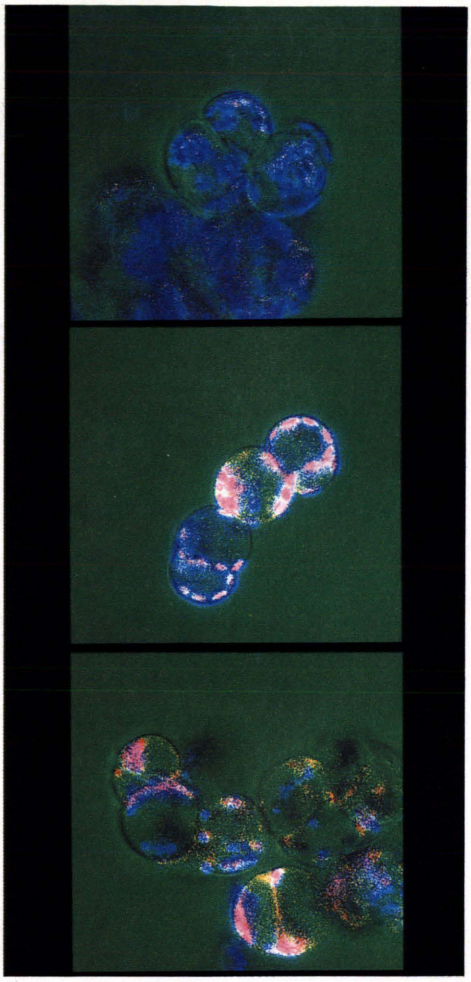

Fig. 2. Confocal fluorescence microscopic images of liverwort cells treated with AFM for 15 and 48 hours in the dark.
Liverwort cells were cultured for 6 days as mentioned in MATERIALS AND METHODS and transferred to new media with 10 μM AFM and incubated for 0, 15 and 48 hours in the dark. The accumulation of PPIX in the cells was observed by a confocal fluorescence microscopic system using two filter systems ranged 560 to 630 nm (PPIX fluorescence) and above 665 nm (chlorophyll fluorescence). In these photographs, the blue color indicates the fluorescence of chlorophyll and pink that of PPIX. The intact liverwort cells before treatment contain several chloroplasts without PPIX (A). In cells treated with AFM and cultivated for 15 h in the dark a bright fluorescence (pink) due to PPIX develops in the chloroplast only, but not in cytoplasm or plasma membrane (B). After a 48-h cultivation with AFM in the dark, PPIX is seen in the cytoplasm (C).

ter treatment of peroxidizing herbicides can be followed easily and quantitatively.

These results suggests the possibility that at least in liverwort excess protoporphyrinogen formed as a result of Protox inhibition does not diffuse away from the chloroplast, but oxidizes and converts to PPIX within the chloroplast by a nonenzymatic or unknown oxidative process.

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