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Expression of 5-amino levulinic acid induced photodynamic damage to the thylakoid membranes in dark sensitized by brief pre-illumination

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Abstract. The 5-amino levulinic acid treated cucumber (*Cucumis sativus* L., CV. Pointsette) plants upon exposure to light (≃ 30,000 lux) wilted within 6 h and died after 36 h due to photodynamic reactions. Thylakoid membranes, the site of accumulation of porphyrins, were damaged due to photodynamic reactions leading to the inhibition of membrane linked functions of photosystem II, photosystem I and the whole chain electron transport. Photosystem II was more susceptible to photodynamic damage than photosystem I. The exogenous electron donors Mn^{2+}, diphenyl carbazide and NH_2OH failed to donate electrons to photosystem II suggesting that the damage has taken place close to P680. The 5-amino levulinic acid treated plants exposed to 30 min of light did not show any damage to the thylakoid membranes. However, when the above plants were transferred to dark for 12 h there was substantial damage to the thylakoid membrane system.

Keywords. 5-Amino levulinic acid; thylakoid membrane; photosystem.

Introduction

Biological tissues, which accumulate tetrapyrroles, are sensitive to photodynamic action of light (Rebeiz *et al*., 1984, 1987a, b; Witkowski and Hailing, 1988). 5-Amino levulinic acid (ALA) is a metabolite present in all living cells. The chlorophyll (Chl) molecules are synthesized from ALA *via* predominantly monovinyl and divinyl monocarboxylic acid routes (Tripathy and Rebeiz, 1986, 1988). The plants treated with ALA in dark accumulate an excess amount of tetrapyrroles of protoporphyrin IX, Mg protoporphyrin IX and protochlorophyllide (Rebeiz *et al*., 1984, 1987a, b). When the ALA treated plants are exposed to light these excess tetrapyrroles absorb light that is not utilized in the photochemical reactions; rather it may be utilized to photosensitize the production of singlet oxygen (Hopf and Whitten, 1978). It is proposed that the latter oxidizes the unsaturated membrane lipids and generates free radicals leading to membrane peroxidation and death of the plants (Haworth and Hess, 1988). Recently it has been reported that the treatment of plants with the herbicides, diphenyl ether and oxadiazone causes excess accumulation of protoporphyrin IX, the photodynamic pigment (Lydon and Duke, 1988; Becerril and Duke, 1989; Duke *et al*., 1989). This pigment may act as sensitizer for the production of singlet oxygen in the plants leading to their death.

The primary target of the photodynamic damage should be the thylakoid membranes where most of the tetrapyrroles are localized. In the present paper, we demonstrate that the ALA induced photodynamic reactions damage the thylakoid membranes and this damage can be accomplished by exposing the ALA treated plants to light for a brief period followed by darkness.

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Abbreviations used: ALA, 5-Amino levulinic acid; Chl, chlorophyll; PS, photosystem; DCIP, 2,6-dichlorophenol-indophenol; MV, methyl viologen; DPC, diphenyl carbazide.

Materials and methods

Cucumber (*Cucumis sativus* L. CV. Pointsette) seeds were grown in petri plates (14·5 cm diameters) on moist germination paper at 25°C as described before (Tripathy and Mohanty, 1980). Five ml of aqueous ALA solution (20 mM, pH 4·8) was spread on each petri plate having 6–8-day old cucumber plants. A glass sprayer (atomizer) was used for spraying ALA. After the ALA spray, the plants were kept in the dark for 15 h. The control plants were sprayed with distilled water (pH 4·8) and kept for the same period in dark. After 15 h of dark period, the ALA treated as well as the control plants were exposed to light (≃ 30,000 lux) at 25°C in a plant growth chamber (Heraeus Votsch VEPHO 5/2000). The cotyledons were harvested after the desired time period for chloroplast isolation as described before (Tripathy and Mohanty, 1980). Chlorophyll was determined according to Arnon (1949). Carotenoids were estimated according to Wellburn and Lichenthaler (1984). Protein content was estimated according to the method of Bradford (1976).

The electron transport activity through photosystem II (PSII) was monitored spectrophotometrically as well as polarographically. The spectrophotometric assay of dichlorophenol indophenol (DCIP) photoreduction was measured at 600 nm as described before (Tripathy and Mohanty, 1980). $K_3Fe(CN)_6$ and *p*-phenylene diamine supported oxygen evolution was measured polarographically by YS I model 53 clark type oxygen electrode connected to a recorder (Tripathy and Mohanty, 1980). Electron transport through the whole chain of photosynthesis ***i.e.***, from H_2O to methyl viologen (MV) (O_2 uptake) was measured polarographically (Tripathy and Mohanty, 1980). The partial electron transport chain through PSI (ascorbate/DCIP to MV) was also measured polarographically in terms of O_2 uptake (Tripathy and Mohanty, 1980). The Chl fluorescence induction kinetics of the dark adapted chloroplasts were measured after Renganathan and Bose (1989) in a laboratory built apparatus.

ALA, DCIP, 3-(3,4-dichlorophenyl)-1, 1-dimethyl urea (DCMU), 4-(2-hydroxymethyl)- l-piperazineethanesulfonic acid (Hepes) and NH_2OH were obtained from Sigma (USA). DPC was obtained from E. Merck (FRG).

Results

There was no damage to the ALA treated cucumber plants kept in dark for 36 h. Upon transfer to light (≃ 30,000 lux) the above plants wilted within 6 h and died after 36 h.

Effect of photodynamic damage on pigments and protein content

Upon exposure of ALA treated plants incubated in dark for 15 h, the leaves wilted followed by the appearance of prominent necrotic patches. Due to desiccation, the moisture content of the leaves reduced by 4–20% after 6–12 h of light exposure. The estimation of the pigment (per g fresh weight) in light exposed ALA treated plants was corrected for the decrease in fresh weight. The total Chl content reduced by 33–49% after 4–12 h of exposure (figure 1). However, the loss of Chl a was higher (56%) than that of Chl b (30%) after 12 h of light exposure. Hence, the Chl a/b ratio decreased from 2·8 at 0 h to 1·8 after 12 h of light treatment. The

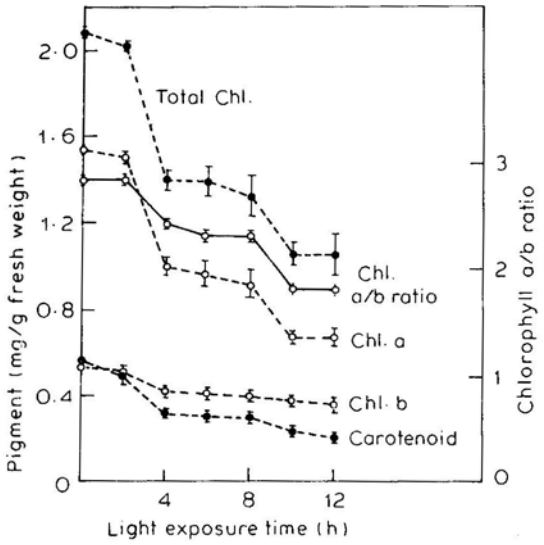

Figure 1. Effect of photodynamic damage on pigment content of the leaves as a function of time of exposure to light (≃ 30,000 lux) at 25°C. The error bars represent standard deviation.

carotenoid content declined by 59% after 12 h. Under identical conditions, there was no decline in Chl and carotenoid content of control plants (data not shown).

The protein content of the leaves in control plants was almost the same throughout the treatment. However, in ALA treated plants, the protein content of the leaves declined by 13–45% after 4–12 h of light exposure (figure 2). The protein content was also corrected for the decrease in fresh weight due to photodynamic damage.

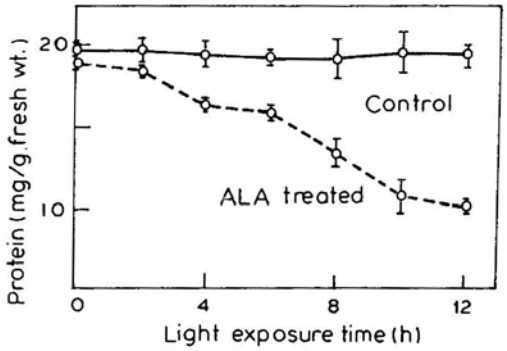

Figure 2. Effect of light exposure for various time period on protein content of control (———) and ALA treated (– – – –) cucumber plants. The error bars represent SD.

Photodynamic damage to thylakoid membrane linked functions

PSI and PSII are two major functional units of thylakoid membrane. Therefore, any damage to the thylakoid membrane is likely to affect the activities of the above

photosystems. Table 1 shows the electron transport rate in chloroplasts isolated from control and treated plants exposed to light for 6 and 12 h. The K_3 $Fe(CN)_6$-supported PSII dependent O_2 evolution inhibited by 35% within 6 h of light exposure. After 12 h of exposure the PSII activity reduced by 70%. The PSI was less susceptible to photodynamic damage. The PSI activity reduced by 18 and 30% after 6 and 12 h respectively. The whole-chain electron transport measured in terms of O_2 uptake reduced by 38 and 65% after 6 and 12 h of light exposure respectively.

Table 1. Effect of photodynamic damage on the photochemical reactions of the thylakoid membrane.

Photochemical reactions	Control	T6	T12
		μmol O_2/mg Chl/h	
PSII assay			
$H_2O \rightarrow K_3$ $Fe(CN)_6$	45 ± 2	29 ± 1(35)	14 ± 1(70)
PSI assay			
$DCIPH_2 \rightarrow MV$	327 ± 14	239 ± 19(27)	199 ± 17(39)
Whole chain assay			
$H_2O \rightarrow MV$	34 ± 5	21 ± 1(38)	11 ± 1(65)

The cucumber plants sprayed with ALA were incubated in dark for 15 h. They were exposed to light (≃ 30,000 lux) for 6 h (T6) and 12 h (T12) at 25°C. The cotyledons were harvested immediately after the light treatment and their chloroplasts were isolated. Polarographic measurement of electron transport through PSII (O_2 evolution), PSI (O_2 uptake)· and the whole chain (O_2 uptake) were measured as described before. The per cent inhibitions are given in parentheses. Each observations is the mean of 3 replicates.

The water splitting enzyme system of PSII is labile and most likely destroyed due to the photodynamic damage of the thylakoid membranes. To assess if the damage of PSII activity was only due to the impairment of the water splitting enzyme system or also due to the inactivation of PSII reaction centre, the effect of exogenous electron donors on PSII-supported DCIP reduction was measured, Mn^{2+}, DPC and NH_2OH donate electrons at the oxidizing side of PSII at different sites (Ghanotakis and Babcock, 1983; Tripathy and Mohanty, 1980). None of the above electron donors could restore the PSII mediated DCIP-photoreduction in photodynamically damaged chloroplasts (data not shown). DPC also failed to restore PSII-supported DCIP reduction in the above chloroplasts treated with heat as before (Tripathy and Mohanty, 1980). The variable Chl a fluorescence level of chloroplasts decreased due to the photodynamic damage to the thylakoid membranes. As expected from the electron transport data the exogenous electron donors, Mn^{2+}, DPC and NH_2OH could not restore the lost variable fluorescence (data not shown).

The ALA treated plants exposed to light (≃ 30,000 lux) for 10–30 min did not exhibit any visual symptom of injury or any damage to the thylakoid membrane functions. Upon return of 10 min light treated plants to darkness for 12 h, as

expected, there was no damage to the thylakoid membrane functions. However, upon dark incubation of 30 min light exposed plants, the plants wilted in 8–12 h. The assay of photochemical functions revealed no inhibition of photochemical reactions after 1 h of dark treatment. The PSII, PSI and whole-chain electron transport activities damaged by 55, 50 and 27% respectively after 8 h of dark incubation (table 2). After 12 h in the dark, PSII, PSI and whole-chain electron transport rates damaged by 65, 60 and 35% respectively. The extent of above damage to the thylakoid membrane was slightly less than in continuous light treatment for 12 h.

Table 2. Post dark treatment of 30 min light exposed ALA sprayed cucumber plants.

	Control	TO	DT1	DT8	DT12
Assay condition	μmol O_2/mg Chl/h				
PSII assay $H_2O \rightarrow K_3FE(CN)_6$	82 ± 6	82 ± 6	80 ± 5	37 ± 4(54)	29 ± 1(65)
PSI assay $DCIPH_2 \rightarrow MV$	191 ± 20	187 ± 10	190 ± 10	136 ± 12(27)	117 ± 8(37)
Whole chain assay $H_2O \rightarrow MV$	44 ± 2	47 ± 2	48 ± 1	23 ± 2(50)	19 ± 1(60)

The cucumber plants sprayed with ALA were incubated in dark for 15 h. They were then exposed to light (≃ 30,000 lux) for 30 min (TO) followed by dark treatment of 1 h (DT1), 8 h (DT8) and 12 h (DT12). The photochemical reactions of isolated chloroplasts were measured as described before. The per cent inhibitions were calculated from TO and are given in parentheses. Each observation is the mean of 3 replicates.

Discussion

There is significant loss of Chl due to photodynamic damage. The loss of Chl a is higher than that of Chl b. The rate of Chl b degradation is also slower, which results in the progressive decrease in Chl a/b ratio. This suggests preferential loss of Chl a enriched light harvesting Chl-protein (LHCP) complex I over Chl b enriched LHCP complex II (Staehelin, 1986). The loss of protein due to photodynamic damage suggests the destruction of primary structure of protein.

The inhibition of PSII and PSI electron transport suggests that the photodynamic action damages the thylakoid membranes. PSII is more susceptible than PSI. The latter is usually more resistant to stresses such as drought, heavy metal etc. (Hsiao, 1973; Tripathy *et al*., 1981, 1983). NH_2OH at low concentration inhibits not only water oxidizing enzyme system but also the site between Z and P680. Higher concentration of NH_2OH donates electron to PSII and its donating site is after the inhibition site between Z and P680 (Ghanotakis and Babcock, 1983). The failure of NH_2OH to donate electrons to PSII suggests that the damage occurs either to P680 or extremely close to P680.

The damage to the thylakoid membrane in the dark following sensitization by 30 min preillumination is of interest. The loss of photochemical reactions appears after 1 h of dark treatment. This suggests that membrane peroxidation reactions go

on in the dark following the production of singlet oxygen in the presence of light. The extent of damage to the thylakoid membrane in the dark is slightly lower than that in continuous light treatment. This suggests that production of a certain amount of active species (singlet oxygen and free radicals) is enough to damage the thylakoid membrane. The absence of damage to the thylakoid membrane in 10 min light exposed ALA treated plants suggests that a certain threshold level of active species should be produced for the onset of photodynamic membrane damage. The mechanism of cell and plant death is under investigation.

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