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PHOTOSYNTHETIC ELECTRON TRANSPORT IN *HYDRILLA VERTICILLATA* (L.) IS INSENSITIVE TO METHYLVIOLOGEN (PARAQUAT) INHIBITION

S. R. Mishra and S. C. Sabat

Environmental Biology Unit, Institute of Life Sciences, 301, Sahid Nagar, Bhubaneswar - 751 007, ORISSA, INDIA

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Effect of paraquat (methylviologen) was investigated on photosynthetic electron transport activity of a submerged aquatic angiosperm *Hydrilla verticillata* following polarographic and fluorimetric techniques. The plant showed resistance for electron acceptance by paraquat both in isolated chloroplasts and in intact leaves. The *in vitro* (isolated chloroplasts) and *in vivo* (intact leaf) electron transport analysis indicated that the failure of paraquat to intercept electrons from photosynthetic electron transport chain is for the existence of some rate limiting steps at the acceptor side of photosystem I complex. *Hydrilla verticillata*, as reported here for the first time, is a true paraquat resistant bio-type where the resistance for the herbicide paraquat can be observed both in isolated chloroplasts and also in intact leaf.

Methylviologen (MeV) a bipyridylium herbicide (paraquat), acts as an inhibitor of photosynthetic electron transport by accepting electron from photosystem (PS) I. The photosynthetically reduced MeV (MeV radical) siphons electron to molecular O_2 forming superoxide radical [1, 2 3], which eventually metabolized to H_2O_2 ($H_2O + 1/2\ O_2$ (MeV, light, chloroplast, $2e^-$)----->H_2O_2). Many bio-types of plant species (species of *Conyza*, *Hordeum*, *Poa*, *Erigeron*, *Arctotheca* and *Lolium*) are resistant to MeV electron acceptance [4, 5, 6, 7, 8]. The underlying mechanism for the resistance to MeV toxicity has been suggested to be the increased levels of the constitutive enzymes that detoxify the active O_2 species [9, 10]. However, this claim has again been disputed [11]. In resistant bio-types although MeV action is transiently seen at intact leaf level, the herbicide manifests identical sensitivity in isolated chloroplasts as in chloroplasts of MeV sensitive bio-types [5]. We, however, encountered a complete different situation in the chloroplasts of a submerged aquatic angiosperm, *Hydrilla verticillata* with respect to MeV electron acceptance. The compound failed to intercept electron from PS I both in isolated chloroplasts and also in intact leaf.

MATERIALS AND METHODS

Healthy *Hydrilla* twigs were collected from the culture vat and thoroughly washed to remove surface deposited inorganic and organic materials. Chloroplasts were isolated from these washed twigs following [12]. Chlorophyll (chl) estimation was done as in [13]. Photosynthetic electron transport rates were measured in terms of O_2 evolution or consumption in a polarographic assembly. The light intensity was rate saturating (1500 $\mu E\ m^{-2}\ s^{-1}$). The reaction temperature was maintained at 25 ± 1^0 C. Electron transport measurements in intact leaf was measured directly taking the leaf samples in the polarographic cuvette. Chl *a* fluorescence intensity of isolated chloroplasts were measured at room temperature (25 ^{0}C). The excitation light (600 nm) was obtained through a monochromator with xenon lamp as exciting

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source. The emission was collected at 685 nm. The fluorescence data have been presented as the relative fluorescence intensity in arbitrary units. Other details of the polarographic and fluorimetric measurements are given in figure legends. To avoid the senescence effect, all measurements were completed within 2h of chloroplast isolation.

RESULTS AND DISCUSSION

Hydrilla chloroplasts, in presence of PS II electron acceptor like dichlorobenzoquinone (DCBQ) or phenyl-para-benzoquione (pPBQ) showed appreciable O_2 evolution activity (Figs. 1 a & b) The whole-chain (PS II + PS I) electron transport activity , when measured, using MeV as terminal electron acceptor no detectable change in light induced O_2 activity was noticed (Fig. 1 c). The small amount of O_2 consumption found in dark remained unaffected in light. Anthraquinone sulfonate (AQ), when used as PS I acceptor [3], a substantial light induced O_2 consumption was observed (Fig. 1 d, broken line). This O_2 consumption was not due to the chloroplastic electron transport reaction. Addition of AQ to the reaction mixture devoid of chloroplast membrane, also showed an equal extent of O_2 consumption in light (Fig. 1 e, solid line).The photosensitized AQ can reduce O_2 to superoxide radical thus resulting the decline in O_2 tension (data not shown). Furthermore, the AQ supported O_2 consumption was diuron (DCMU) insensitive (Fig. 1 f, dotted line). No such light dependent O_2 consumption was noticed with MeV alone in the reaction mixture (Fig.1 g). These results suggest that MeV or AQ do not trap the photosynthetically generated electrons from PS I of *Hydrilla* chloroplasts.

More than one reasons could be assigned for the failure of MeV siphoning electrons to O_2 in *Hydrilla* chloroplasts. One of the reasons may be the isolation dependent leaching of plastocyanin (PC) from the membranes (PS I donor side impairment). Plastocyanin a copper containing polypeptide links the electron flow between cytochrome b_6/f complex and P_{700} (PS I reaction centre). Alternatively, the failure may have been arisen due to the isolation dependent alterations of the electron transport component at the acceptor side of PS I; a site where MeV intercepts electron.

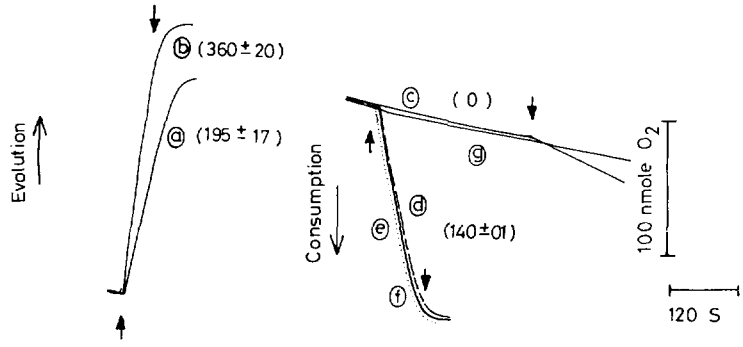

Figure 1. Polarographic tracings of light induced O_2 activities of isolated *Hydrilla* chloroplasts in presence of pPBQ (a), DCBQ (b), Mev (c) and AQ (d). O_2 consumption activity of photosensitized AQ has been shown in tracing (e., solid lines). Fig 1 (f) shows the effect of 10 μM DCMU. The tracings in (g) indicate the photo-reaction with MeV alone in the reaction mixture. The basal reaction ingradients in 1 ml contained 100 mM sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 20 mM $CaCl_2$, and 20 mM TRICINE - NaOH (PH 7.5). The pPBQ or DCBQ concentration was 400 μM. The whole chain electron flow when measured in the basal reaction mixture also included 100 μM MeV/100 mM AQ and 1 mM NaN_3. The chlorophyll concentration in all the measurements was 20 ug ml^{-1}. The reaction was monitored at 25^0 C under saturating light intensity (1500 μE m^{-2} S^{-1}). Figures in brackets show the rate of O_2 evolution or consumption $(mgchl)^{-1}$ h^{-1}. The values represent the SD of rate obtained from 3 or 4 independent batches of chloroplast isolation. The solid arrow marks given upward (↑) and downward (↓) here and in other figures represent the point of illumination and darkening of the sample.

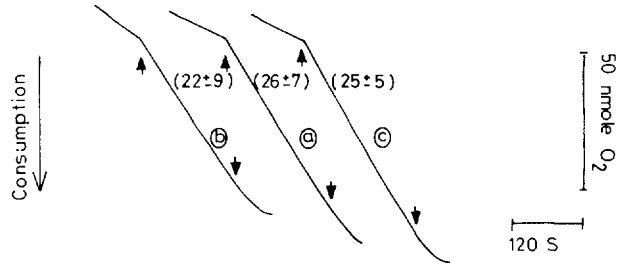

Figure 2. O_2 consumption tracings in *Hydrilla* chloroplasts supported by 1 mM reduced DCIP. Tracing (a) denotes the O_2 consumption activity in presence of 100 μM MeV. Tracing (b) shows the same but in absence of MeV .PS I catalyzed O_2 consumption activity with high concentration of MeV has been shown in tracing (c). The reaction was done in the same basal reaction medium (see Fig. 1) but additionally containing 10 μM DCMU, 1 mM DCIP, 5 mM sodium ascorbate, 1 mM NaN_3. The chl. concentration was 15 μg ml^{-1}.

To locate the possible existence of impairment at the acceptor side of PS I, the PS I catalyzed electron transport activity was investigated using ascorbate reduced dichlorophenolindophenol ($DCIPH_2$) as electron donor with MeV as electron acceptor. $DCIPH_2$ can donate electron to PS I either via PC (low Km) or directly to P_{700} (high Km) in a concentration dependent manner [14, 15] .At low concentration (100 μM) the major site of electron donation is cytochrome b_6/f complex (via PC). It can also donate electron to P_{700} (thus by-passing the electron flow via PC) at high concentration (1 mM). At 1 mM $DCIPH_2$, the *Hydrilla* chloroplasts showed a substantial low rate of PS I catalyzed O_2 consumption activity (Fig. 2 a). But without added MeV there also existed an inherent O_2 consumption activity of chloroplasts (Fig. 2 b), which was roughly equal to the rate obtained with MeV (Fig. 2 a). These results suggest that the terminal electron acceptor in *Hydrilla* chloroplast is the molecular O_2 and the chloroplasts do not entertain MeV to function as transient artificial Hill-acceptor. We, further verified the

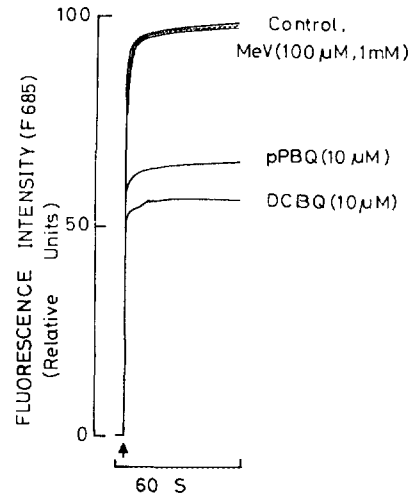

Figure 3. Room temperature chl a fluorescence yield in presence and absence (control) of electron acceptors like MeV (100 μM & 1 mM), pPBQ (10 μM),DCBQ (10 μM). The chloroplasts were dark adapted for 5 min before exposing them to light. The membranes were excited by 600 nm light and fluorescence emission was collected at 685 nm. The excitation and emission slit were maintained at 5 nm each. The chloroplasts were suspended in the basal reaction mixture containing 7 μg chl ml^{-1}.

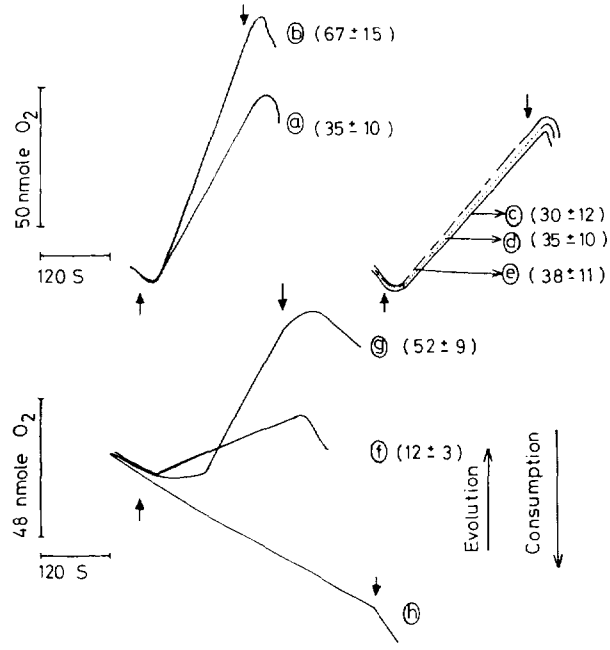

Figure 4. O_2 evolution tracings of *Hydrilla* leaf samples in absence (a, c) and in presence (b) of 10 mM HCO_3^-. The O_2 evolution activity in presence of MeV (1 mM) or 1 mM MeV incubated leaf samples is presented in tracings designated as (d) and (e), respectively. Tracings shown as f and g denote respectively the O_2 evolution activities of spinach leaf samples with and without infiltrated HCO_3^- (10 mM). Tracing (h) shows the effect of infiltration of 1 mM MeV (combination with 10 mM HCO_3^-) on O_2 evolution activity of spinach leaf.

acceptor limitation by assaying the reaction at 10 fold elevated concentrations (from 100 μM to 1 mM) of MeV. The light dependent O_2 consumption activity was identical both at 100 μM and 1 mM MeV (Figs. 2 a & c). Therefore, it is the intrinsic but not the barrier property of chloroplast membrane that is responsible for failure of MeV accepting electrons from PS I.

The ineffectiveness of MeV to accept electron can be further verified from acceptor dependent quenching of room temperature chl *a* fluorescence intensity. Under saturating intensity the fluorescence intensity of chl *a*, without any added electron acceptor represents the maximum reduction of Q_A [16]. The variable part of the fluorescence can be quenched by addition of electron acceptor due to the siphoning of electrons from Q_A thus keeping a significant proportion of Q_A oxidized . Fig. 3 shows the effect of different electron acceptors on the alterations of the steady state level of chl *a* fluorescence intensity measured at 685 nm (F_{685}). The fluorescence intensity of control chloroplasts (without acceptor) remained unaffected in presence of 100 μM and 1 mM MeV. Presence of 10 μM DCBQ or pPBQ brought about 35% (pPBQ) and 45% (DCBQ) lowering of control chl *a* fluorescence. Although the steady state fluoresence has been measured, it is believed that the DCBQ and pPBQ mediated quenching of chl *a* fluorescence was mostly due to the loss of variable fluorescence and was not due to the lowering of the constant background, the so called Fo level. Thus, these observations also suggest that MeV in *Hydrilla* chloroplasts does not support the whole-chain electron flow.

To substantiate our observation on the ineffectiveness of MeV to intercept electrons from PS I, the electron transport activities of *Hydrilla* chloroplasts were further studied using intact leaf samples.

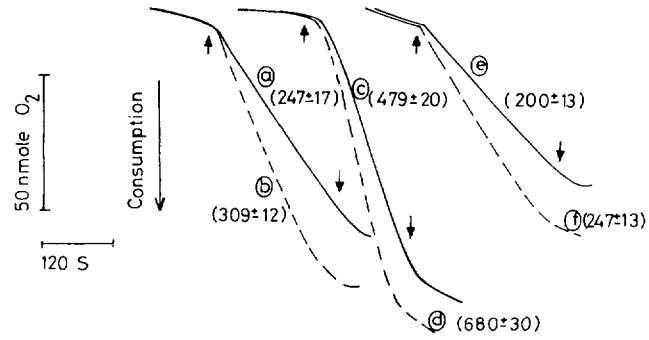

Figure 5. Effect of heat treatment on the MeV (solid tracings) and AQ (broken tracings) supported PS I electron transport activity of spinach chloroplast membranes incubated at 50 ° C (5 min dark, 500 μg chl ml^{-1}) in presence (e & f) or in absence (c & d) of 50 % ethylene glycol. The control PS I catalyzed electron transport activities supported by MeV and AQ are presented in tracings designated as (a) and (b). The basal reaction mixture (Fig.1) included DCIP 100 μM, DCMU 10 μM, sodium ascorbate 2 mM, NaN_3 2 mM and MeV/AQ 100 uM. The chl concentration was 10 μg ml^{-1}.

This step was taken basing on the reports that MeV can enter into the intact leaf [5, 6]. The entry of the compound in *Hydrilla* leaf is still easier because of the ecological features of the leaf. The leaf in *Hydrilla* plant, unlike the leaves of higher terrestrial plant, lack stomata and the whole plant surface is exposed for absorption of nutrients from water medium [17,18]. However,for comparison, we have also done the experiment in intact spinach (*Beta vulgaris*) leaves. For studying the electron transport in intact spinach leaf the test compound was infiltrated under vacuum.

The *Hydrilla* leaf showed light dependent O_2 evolution which was stimulated nearly twice in presence of 10 mM HCO_3^- (Figs. 4 a & b); suggesting that there exist a linear electron flow from H_2O to the physiological electron acceptor NADP. Surprisingly when MeV was added, the leaf showed O_2 evolution activity (Fig. 4 c ,solid line), identical to the rates obtained without MeV (Fig. 4 e, broken line). Similar observations were made in leaf samples incubated with 1 mM MeV for about 30 min (Fig. 4 d, dotted line). Similar to *Hydrilla* the O_2 evolution rate in spinach leaf showed about 4 fold stimulation in presence of 10 mM HCO_3^- (Figs. 4 g) over the rates obtained without HCO_3^- (Fig. 4 f). The O_2 evolution activity became suppressed when the leaf samples were infiltrated with 1 mM MeV along with 10 mM HCO_3^- (Fig. 4 h).Therefore, it could be suggested that the inability of MeV to oxidize the electron transport component in *Hydrilla* chloroplast as postulated earlier (Fig. 1 c), was not due to the leaching of PC but due to the presence of some rate limiting sites at the acceptor side of PS I complex; possibly the site where MeV intercepts electrons in isolated chloroplasts.

Methylviologen a low potential (E_0' = - 0.44 V) compound exclusively accepts electrons from PS I [3]. The major identified site of electron acceptance is Fe-S centre F_B, although the acceptor can also accept electron poorly from Fe-S centre F_A [19]. . The redox centre F_A and F_B are housed in a low molecular (8.9 kDa) PS I C polypeptide [20]. This polypeptide can be removed from the chloroplast membrane by various treatments like a) lithium dodecyl sulphate [21], b) chaotropic agents [22], c) $HgCl_2$ treatment [19] or d) even treatment with hot ethylene glycol [23]. Removal of PS I C polypeptide by hot ethylene glycol also leads to the decline of MeV electron acceptance capacity [23, also see Fig. 5].Although unknown is whether F_A or F_B serves as the physiological electron donor to NADP via ferredoxin under *in vivo* condition; in isolated spinach chloroplasts NADP has been shown to accept

electrons largely from F_B [19]. However, in *Hydrilla* chloroplasts the electron acceptance sites of NADP and MeV seems to be different; an aspect needs further probe. The primary structure of PS I C polypeptide is highly conserved during evolution [24]. The *Hydrilla* plant may be an exception in this respect. It could only be postulated for the present time that changes in the redox property of PS I C protein in *Hydrilla* chloroplasts may have caused the plant resistant to MeV.

Leaving aside the mechanism of resistance, the *Hydrilla* can be regarded as a true paraquat resistant plant. The resistance, unlike in the other reported paraquat bio-types [4], can be visualized in *Hydrilla* both at isolated chloroplast and intact leaf level.

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