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Excess Ca^{2+} does not alleviate but increases the toxicity of Hg^{2+} to photosystem II in *Synechocystis* sp. (Cyanophyta)

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ABSTRACT

This study demonstrated that excess Ca^{2+} increased the toxicity of Hg^{2+} to PSII of cyanobacterium *Synechocystis* sp. using fast rise chlorophyll fluorescence test. Excess Ca^{2+} increased the inhibitory effect of Hg^{2+} on O_2 evolution. Exposure to Hg^{2+} caused increase in functional antenna size (ABS/RC), trapping rate of reaction center (TR_0/RC), dissipated energy flux per reaction center (DI_0/RC) and maximum quantum yield of non-photochemical deexcitation (φ_{D_o}), indicating that some reaction centers were transformed to dissipation sinks under Hg^{2+} stress. Hg^{2+} stress slowed down electron transport on both donor side and acceptor side and caused accumulation of P_{680}^{+}. Excess Ca^{2+} intensified all the Hg^{2+} toxic effects on PSII function and led to dysfunction of PSII. The number of reaction centers that were transformed into dissipation sinks increased with increasing Ca^{2+} concentration.

1. Introduction

Mercury (Hg) is highly toxic to organisms even at low concentration. Hg can inhibit growth, mineral nutrient uptake, photosynthesis and transpiration of plant (Patra and Sharma, 2000). Photosynthetic apparatus is susceptible to Hg toxicity, and photosystem II (PSII) is the most sensitive site (De Fillipis et al., 1981). Both the donor side (Bernier and Carpentier, 1995) and the acceptor side (Prokowski, 1993) of PSII can be adversely impacted by Hg.

Calcium (Ca) is an essential element for the photosynthesis in higher plants and algae (Bharti et al., 1996). Oxidation of H_2O by PSII requires Ca as obligatory activators/cofactors of the reaction (Chen et al., 1995). All S-state transitions require Ca^{2+}. A few studies reported that Ca^{2+} could protect against the toxicity of various heavy metals including Cu^{2+}, Ni^{2+}, Pb^{2+} and Cd^{2+} to photosynthesis (Skorzynska-Polit et al., 1998; Ouzounidou et al., 2006; Drążkiewicz and Baszyński, 2008; Andosch et al., 2012; Chen et al., 2012; Farzadfar et al., 2013). However, effects of Ca^{2+} on Hg^{2+}-induced toxicity to PSII and the underlying mechanisms are still unclear.

Various chlorophyll fluorescence tests have been proven to be a rapid, non-invasive and reliable method for evaluation of photosynthetic performance under environmental stresses (Brack and Frank, 1998; Kobbia et al., 2001; Baumann et al., 2009; Pokora and Tukaj, 2010; Wang et al., 2012; Wang and Pan, 2012; Wang et al., 2013). The OJIP fast fluorescence induction curve provides valuable information about the function of PSII (Strasser and Strasser, 1995; Lazár, 2006). Upon the triggering of strong actinic light, the rise of Chl fluorescence of dark-adapted photosynthetic materials will typically form a triphasic kinetic curve from initial level (F_0), to two intermediate steps (F_J and F_I) and its maximal level (F_M or F_P) (Strasser and Govindjee, 1992; Strasser and Strasser, 1995; Lazár, 2006). The JIP test analysis (Strasser and Strasser, 1995) based on the OJIP curve can be used to analyze changes in electron transfer reaction on both donor (Delsome and Joliot, 2002) and acceptor side of PSII (Strasser and Govindjee, 1992). JIP-test analysis has been extensively used to quantify responses of PSII activities to various environmental stresses (Joshi and Mohanty, 2004; Pan et al., 2008; Pan et al., 2009; Wang et al., 2012; Wang and Pan, 2012). In southwest China, there are large areas of karstic water bodies where the calcium ions are rich. Mercury contamination in karstic lakes due to mining activities has been frequently reported (Zhang et al., 2009). *Synechocystis*, which is ubiquitous in karstic waters, was used for toxicity tests in the present study because our previous studies showed that this species is very sensitive to various contaminants. We hope that we can use this common cyanobacterium species as model microorganism to examine the effects of excess Ca^{2+} on the toxicity of mercury to photosynthetic apparatus, i.e., whether

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excess Ca alleviates or aggravates the toxicity of mercury. The aim of the present study was to examine the effects of excess Ca^{2+} on toxicity of Hg^{2+} to PSII in cyanobacterium *Synechocystis* sp. using the OJIP chlorophyll *a* fluorescence test. Our study showed that excess Ca^{2+} increased the toxicity of Hg^{2+} to PSII.

2. Materials and methods

2.1. Culture of cyanobacterium

The cyanobacterium *Synechocystis* sp. (#FACH898) was purchased from the Institute of Hydrobiology, Chinese Academy of Sciences. The cyanobacterial cells were precultured photoautotrophically in BG-11 medium (Rippka et al., 1979) at 25 °C under 55 μmol photons m^{-2} s^{-1} fluorescent white light. BG-11 medium contains about 0.25 mM Ca^{2+}. The cyanobacterial cells at exponentially growing phase were diluted with fresh BG-11 medium to a chlorophyll *a* density of 5 μg ml^{-1} and cultured in 10 mm × 10 mm plastic cuvettes for chlorophyll fluorescence tests.

2.2. Preparation of Hg^{2+} and Ca^{2+} solutions

$HgCl_2$ stock solution and $CaCl_2$ stock solution were prepared by dissolving $HgCl_2 \cdot 2H_2O$ and $CaCl_2 \cdot 6H_2O$ of analytical grade in deionized water, respectively. The stock solutions were stored in the dark at 4 °C until use.

2.3. Hg^{2+} and Ca^{2+} treatments

$HgCl_2$ stock solution, $CaCl_2$ stock solution and deionized water were added into the cyanobacteria cultures to make a final Hg^{2+} concentration of 5.0 μM and final Ca^{2+} concentrations of 0.5, 1.0, 2.5 and 5.0 mM in the BG-11 medium. Our previous studies showed that Ca^{2+} concentrations up to 5.0 mM were not harmful to *Synechocystis* sp. (#FACH898) (Zhang et al., 2008). The medium without addition of $HgCl_2$ solution and $CaCl_2$ solution was used as the control. The volumes of all the samples were kept the same by addition of deionized water. All the samples were incubated at 25 °C under fluorescent white light with illumination of 55 μmol photons m^{-2} s^{-1}.

2.4. Measurement of O_2 evolution

After 6 h of treatment with Hg^{2+} or/and Ca^{2+}, the photosynthetic O_2 evolution rate of the *Synechocystis* sp. cells was measured using a Clark oxygen microelectrode (Unisense, Denmark) at 25 °C under illumination of 500 μmol m^{-2} s^{-1} white light.

2.4.1. OJIP Chl a fluorescence test

The polyphasic fluorescence transient was recorded by a double-modulation fluorometer FL3500 (PSI, Brno, Czech). All the samples were dark-adapted for 5 min before each measurement. The JIP test (Strasser and Strasser, 1995) was employed to analyze the OJIP chlorophyll *a* fluorescence transient. F_0, F_J and F_M from the original measurements were used. F_0 was the minimal fluorescence intensity when all the reaction centers are open or in oxidized state. F_0 was determined at 50 μs after the onset of the actinic light. F_J was the fluorescence intensity measured at 2 ms. F_M, the maximal fluorescence intensity, denotes the values when all the reaction centers are physiologically closed. $TF_{300\ \mu s}$ was required for the calculation of the initial slope (M_o) of the relative variable fluorescence kinetics. JIP-test parameters and their meaning were listed in Table 1 (Strasser et al., 2000).

2.5. Statistics

Measurement of O_2 evolution and fluorescence tests were conducted in triplicate. The data for O_2 evolutin was presented as mean value and standard errors. All data of JIP-test parameters for Hg or/and Ca treated samples were expressed as the percentage of the control. The mean values were used.

3. Results

3.1. Oxygen evolution

Compared to the control, the O_2 evolution rate of *Synechocystis* sp. was clearly reduced under stress of Hg^{2+} alone or Hg^{2+} plus various concentrations of Ca^{2+} (Fig. 1). The O_2 evolution of *Synechocystis* sp. was reduced by 52.8% after 6 h of exposure to 5.0 μM Hg^{2+}. The relative O_2 evolution rate drastically decreased with increasing Ca^{2+} concentration when excess Ca^{2+} was added together with Hg^{2+}. The relative O_2 evolution rate of *Synechocystis* sp. treated with 5.0 μM Hg^{2+} plus 5.0 mM Ca^{2+} was only 2.21% of the control.

3.2. Fluorescence rise OJIP kinetics and JIP test analysis

Fig. 2 showed the exemplified OJIP curves of the samples after 6 h of exposure to 5.0 μM Hg^{2+} plus various concentrations of Ca^{2+}. It was found that the chlorophyll fluorescence was quenched in various treatments. Moreover, fluorescence intensities at O, J, I and P steps decreased as Ca^{2+} concentration increased from 0.25 mM to 5.0 mM. F_V, F_V/F_M and PI_{ABS} generally decreased with increasing Ca^{2+} concentration (Fig. 3).

Fig. 4 showed the effects of Hg^{2+} plus various concentrations of Ca^{2+} on the specific energy fluxes through PSII at the RCs. At 5.0 μM Hg^{2+}, the functional antenna size (ABS/RC) and the trapping rate of the RC (TR_0/RC) increased as Ca^{2+} concentration increased. The drastic increase of ABS/RC and the small increase of TR_0/RC resulted in drastic increases of DI_0/RC and maximum quantum yield of non-photochemical deexcitation (φ_{D_o}) with increasing Ca^{2+} concentration.

Table 1
Formulae and terms used in the JIP-test (Strasser et al., 2000).

Formulae and terms	Illustrations
$F_V/F_M=(F_M-F_0)/F_M$	Maximal quantum yield of PSII photochemistry
$V_J=(F_{2\ ms}-F_0)/(F_M-F_0)$	Relative variable fluorescence intensity at the J-step
$M_o=4(F_{300\ \mu s}-F_0)/(F_M-F_0)$	Approximated initial slope of the fluorescence transient
Quantum efficiencies or flux ratios	
$\varphi_{P_o}=TR_0/ABS=[1-(F_0/F_M)]=F_V/F_M$	Maximum quantum yield for primary photochemistry (at $t=0$)
$\varphi_{E_o}=ET_0/ABS=[1-(F_0/F_M)]\bullet\Psi_o$	Quantum yield for electron transport (at $t=0$)
$\varphi_{D_o}=DI_0/ABS=1-\varphi_{P_o}=F_0/F_M$	Maximum quantum yield of non-photochemical deexcitation
$\Psi o=ET_0/TR_0=(1-V_J)$	Probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A (at $t=0$)
Specific fluxes or specific activities	
$ABS/RC=M_o\bullet(1/V_J)\bullet(1/\varphi_{P_o})$	Absorption flux per reaction center
$TR_o/RC=M_o\bullet(1/V_J)$	Trapped energy flux per reaction center (at $t=0$)
$ET_o/RC=M_o\bullet(1/V_J)\bullet\Psi o$	Electron transport flux per reaction center (at $t=0$)
$DI_o/RC=(ABS/RC)-(TR_o/RC)$	Dissipated energy flux per reaction center (at $t=0$)
Density of reaction centers	
$RC/ABS=\varphi_{P_o}\bullet(V_J/M_o)\bullet(ABS/RC)$	Active PSII reactive centers per absorption
Performance index	
$PI_{ABS}=(RC/ABS)\bullet[\varphi_{P_o}/(1-\varphi_{P_o})]\bullet[\Psi_o/(1-\Psi_o)]$	Performance index on absorption basis

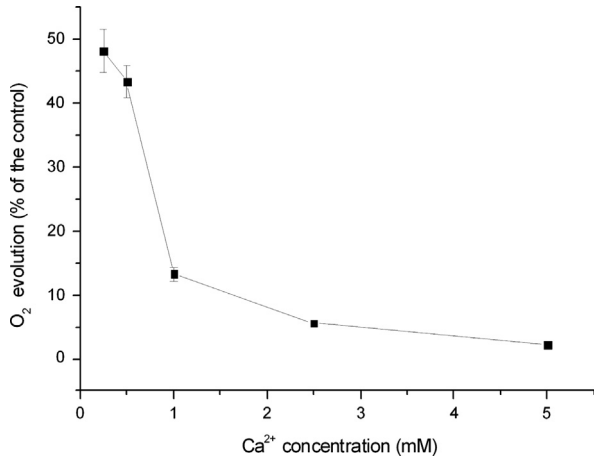

Fig. 1. The relative O_2 evolution activity for *Synechocystis* sp. under Hg stress for 6 h in the presence of various concentrations of Ca^{2+}. All the values were expressed as the percentage of the control.

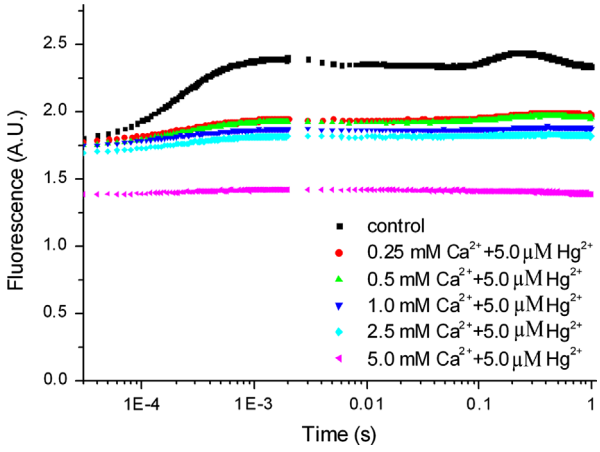

Fig. 2. Exemplified OJIP curves for the control and samples treated with Hg^{2+} in the presence of various concentrations of Ca^{2+}.

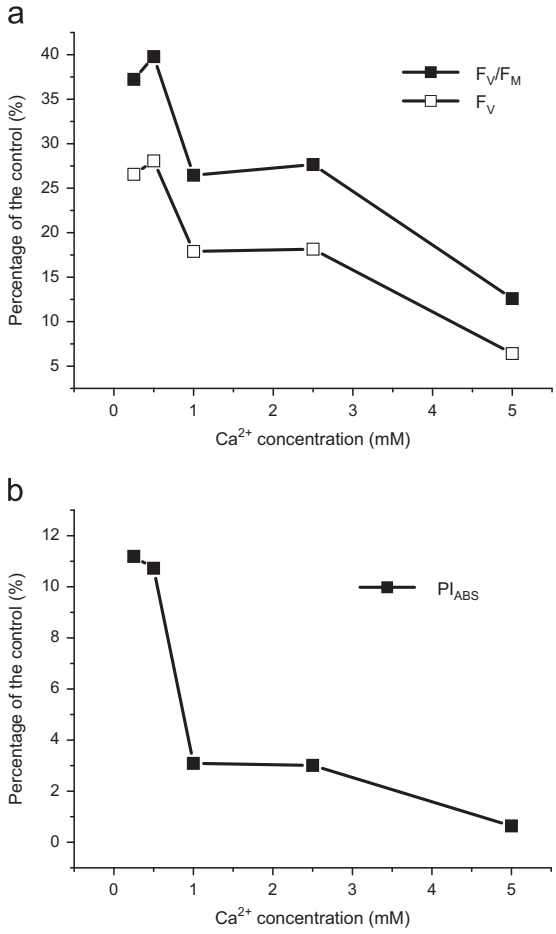

Fig. 3. (a) The maximum quantum yield for primary photochemistry ($\varphi_{P_o}=F_V/F_M$) and variable fluorescence (Fv), and (b) the photosynthetic performance index PI_{ABS} of the 6-h Hg^{2+}-treated samples in the presence of various concentrations of Ca^{2+}. All the values of the parameters were expressed as the percentage of the control.

The response of electron transport flux to 5.0 μM Hg^{2+} plus various concentrations of Ca^{2+} was shown in Fig. 5. Treatment with 5.0 μM Hg^{2+} for 6 h increased ET_0/RC by 13.9% compared to the control. However, ET_0/RC markedly decreased when Ca^{2+} concentration increased from 0.25 mM to 1.0 mM, and then changed slightly as Ca^{2+} concentration increased to 5.0 mM (Fig. 5a). However, treatment with 5.0 μM Hg^{2+} plus 1.0 mM Ca^{2+} caused a decrease in Ψ_0 by 28.2%. Ψ_0 changed slightly as Ca^{2+} concentration increased further. The yield for electron transport (φ_{E_o}) decreased under Hg^{2+} stress and excess Ca^{2+} worsened the toxic effect of Hg^{2+}. After treatment with 5.0 μM Hg^{2+} for 6 h, V_J decreased a little. Addition of excess Ca^{2+} along with Hg^{2+} caused rise of V_J (Fig. 5c).

4. Discussion

This study clearly demonstrated that Ca^{2+} aggravated the Hg^{2+}-induced toxicity to PSII of *Synechocystis* sp., which is contrary to the protective role of excess Ca^{2+} against the toxicity of other heavy metals. A few studies showed excess Ca^{2+} alleviated the toxicity of heavy metals such as Cu^{2+} to photosynthetic apparatus (Maksymiec and Baszyński, 1999), Pb^{2+} (Rashid and Popovic, 1990), Cd^{2+} (Skorzynska-Polit et al., 1998) and Ni^{2+} (Ouzounidou et al., 2006). Only one previous study reported similar results. Bernier et al. (1993) showed that calcium could not reverse the inhibitory effect of mercury on PSII prepared from *Hordeum vulgate*, whereas chloride significantly reversed the inhibitory effect of mercury.

In the present study, under the stress of Hg^{2+}, the relative O_2 evolution of *Synechocystis* sp. drastically decreased with increased Ca^{2+} concentration (Fig. 1). On the contrary, a few previous studies reported that toxic effects of other metals on oxygen evolution system were alleviated in the presence of excess Ca^{2+} (Rashid and Popovic, 1990). Rashid and Popovic (1990) reported that addition of 15 mM Ca^{2+} along with Pb^{2+} significantly weakened the inhibitory effect of Pb^{2+} on O_2 evolution. The mechanism involved in the protective role of Ca^{2+} against the toxicity of heavy metals were attributed to the competition of Ca^{2+} with heavy metal ions for binding to the active sites in the vicinity of water splitting complex (Rashid and Popovic, 1990; Wan et al., 2011; Andosch et al., 2012). The binding possibilities of heavy metals can be restricted when excess Ca^{2+} is present, and excess Ca^{2+} thus ameliorates the inhibition of heavy metal ions. Recently, it has been demonstrated that Ca^{2+} near the plasma membrane alleviates Cd toxicity by reducing the cell-surface negativity and competing for Cd^{2+} ion influx (Wan et al., 2011). Drążkiewicz and Baszyński (2008) confirmed a protective effect of 10 mM Ca on D1, D2 and 17 kDa proteins in PSII complex treated with 250 μM Cd, and on 43 kDa protein in the complex of *Phaseolus coccineus* exposed to 500 μM Cd. The toxicity of 500 μM Cd to the 43, 47 and 17 kDa proteins and the harmful effect of 1000 μM Cd on 47 and 17 kDa ones were counteracted by 20 mM Ca. Recently, it has been demonstrated that

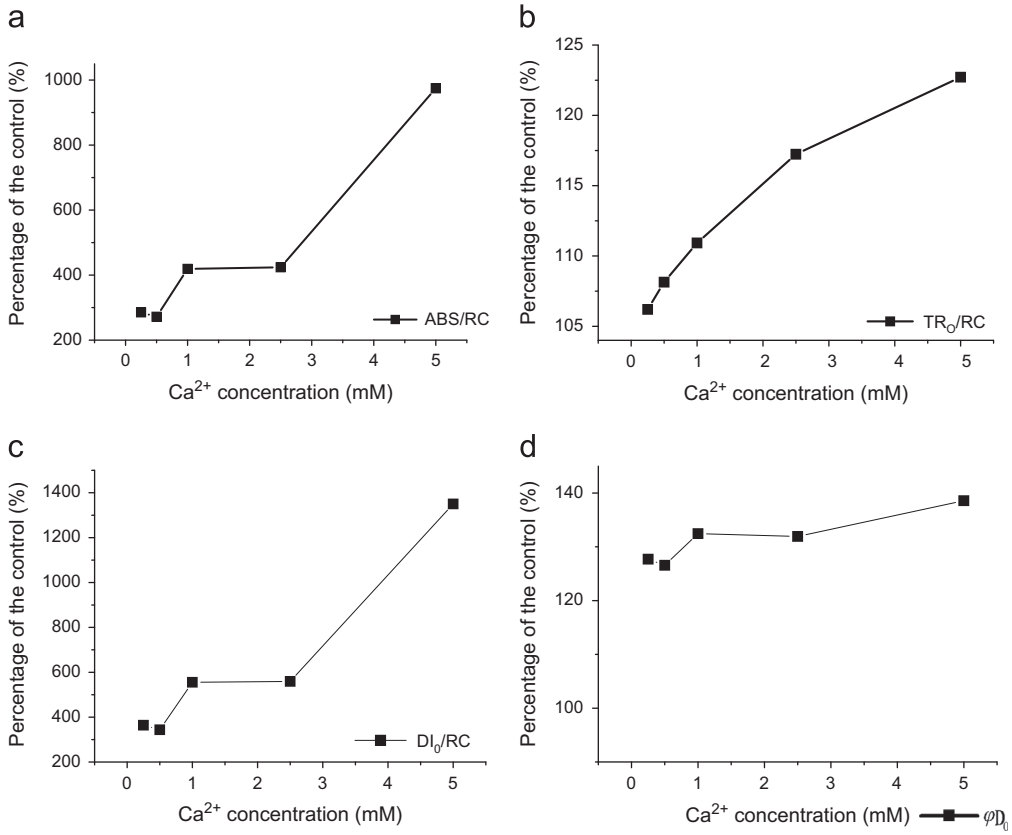

Fig. 4. (a) the functional antenna size (ABS/RC), (b) the trapping rate of the RC (TR_0/RC), (c) the dissipated energy flux per reaction center (DI_0/RC), and (d) the maximum quantum yield of non-photochemical deexcitation (φ_{D_0}) of the 6-h Hg^{2+}-treated samples in the presence of various concentrations of Ca^{2+}. All the values of the parameters were expressed as the percentage of the control.

Ca can prevent the damage of Cd to structure of chloroplast and physiological function of *Micrasterias* (Andosch et al., 2012). However, in the present study, excess Ca aggravate the toxicity of Hg to PSII function, implying that the role of Ca in alleviating or aggravating toxic effects of heavy metals on photosynthetic apparatus is dependent on the heavy metal species.

Fluorescence intensities at O, J, I and P steps were markedly reduced under Hg^{2+} stress, and the quenching effect increased with increasing Ca^{2+} concentration (Fig. 2). Similarly, Boucher and Carpentier (1999) reported that the values of F_0, F_M and F_V/F_M of thylakoid membranes isolated from *Spinacia oleracea* leaves significantly decreased due to toxicity of Hg^{2+}. The decrease of F_M might be ascribed to the inhibition of the donor side of PSII by Hg^{2+} and interpreted as an increase of closed PSII reaction centers (RCs) that do not participate in electron transport. The decrease of F_0 with increasing Ca^{2+} concentration indicates that more fluorescence-quenching centers in PSII were formed (Pfündel, 2003) and structural changes occurred in the antenna pigments (Murthy et al., 1990) in presence of excess Ca^{2+}.

F_V, F_V/F_M and PI_{ABS} also showed decreasing trends as Ca^{2+} concentration increased (Fig. 3), implying that Hg^{2+} plus excess Ca^{2+} synergistically inhibited photosynthetic performance. The decrease of F_V suggests that PSII capacity to reduce plastoquinone was inhibited because of the disturbance of the PSII donor side (Bukhov et al., 1987) or the damage to the oxygen evolving system.

Exposure to Hg^{2+} alone or Hg^{2+} plus excess Ca^{2+} caused a drastic increase in the functional antenna size (ABS/RC) and a small increase in the trapping rate of the RC (TR_0/RC), which caused drastic increases in DI_0/RC and maximum quantum yield of non-photochemical deexcitation (φ_{D_0}) (Fig. 4). This implies that the cells are unable to regulate the light-harvesting capacity to adapt to Hg^{2+} stress (Adams and Demmig-Adams, 2004), and PSII RCs are consequently transformed into dissipative sinks for excitation energy (Tevini et al., 1988). The inhibitory effect increased with increasing Ca^{2+} concentration. This was consistent with the decreasing F_0 with increasing Ca^{2+} concentration, which suggests that nonfunctional PSII centers act as dissipative sinks (Öquist et al., 1992). A few previous studies also reported the disturbed energy transfer due to Hg^{2+} (Boucher and Carpentier, 1999; Lu et al., 2000). Boucher and Carpentier (1999) demonstrated that photosynthetic energy storage of spinach thylakoids decreased by about 80% after exposure to Hg^{2+}. Lu et al. (2000) also reported that the efficiency of excitation energy captured by the open PSII reaction centers of maize (*Zea mays* L. cv. Yedan 13) and wheat (*Tritium aestivum* L. cv. Shannong 229) was reduced under Hg^{2+} stress. Murthy et al. (1989) showed that Hg^{2+} at low concentrations affect the transfer of energy within phycobilisomes. Our study shows that excess Ca^{2+} can increase the adverse effect of Hg^{2+} on energy transfer in PSII.

The change of ET_0/RC suggests that the reoxidation of reduced Q_A via electron transport in RC is promoted by Hg^{2+} but is significantly inhibited by Hg^{2+} plus excess Ca^{2+} (Fig. 5). The drastic decreases in the yield for electron transport (φ_{E_0}) and the probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A (Ψ_0) in the presence of excess Ca^{2+} (Fig. 5) indicates that electron transport is one of the primary targeted sites for Hg^{2+} and excess Ca^{2+} makes the electron transport chain more vulnerable to the toxicity of Hg^{2+}. Lu et al. (2000) reported that Hg^{2+} stress led to a decrease of quantum yield of PSII electron transport.

The heterogeneity of PSII was significantly altered under Hg^{2+} stress. The increase of V_J might indicate a rise of the proportion of closed PSII RCs and consequently an increase of the proportion of reduced Q_A at J step (Fig. 5). Similarly, Lu et al. (2000) showed that

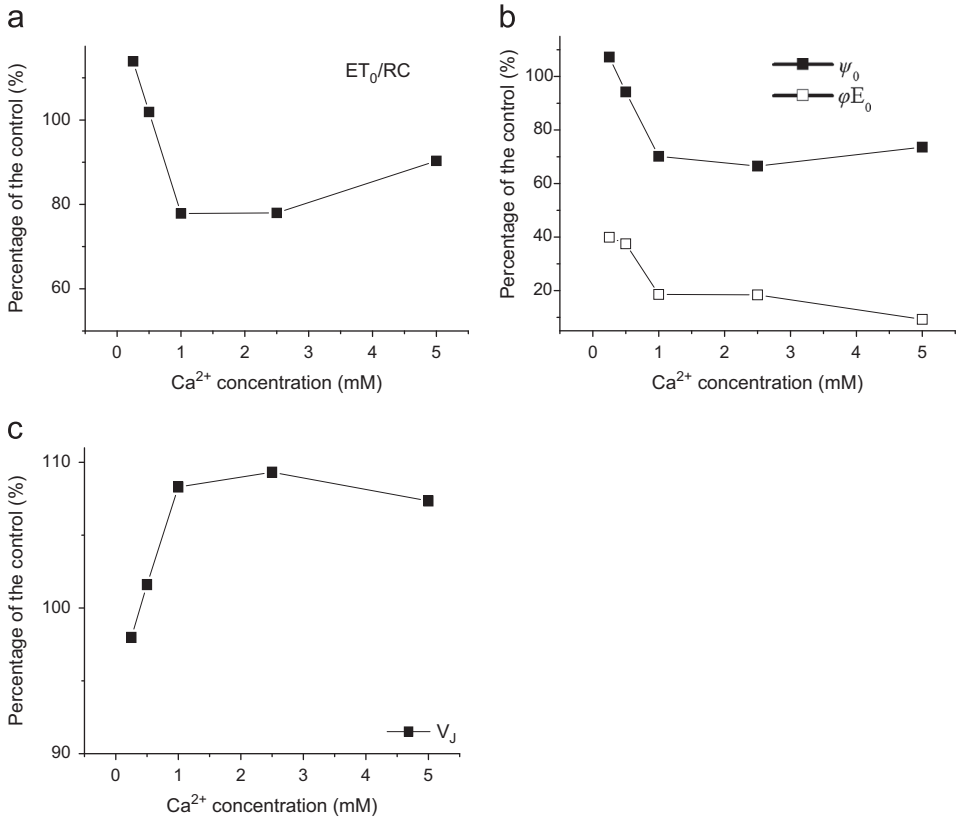

Fig. 5. (a) electron transport flux per reaction center (ET_o/RC), (b) probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A (Ψo) and quantum yield for electron transport (φ_{E_o}). All the values of the parameters were expressed as the percentage of the control, and (c) V_J. All the values of the parameters were expressed as the percentage of the control.

Hg^{2+} induced a significant increase in the proportion of the Q_B-non-reducing PSII reaction centers. High ABS/RC and high V_J at high levels of Ca^{2+} suggest that excess Ca^{2+} enhances the adverse effect of Hg^{2+} on PSII heterogeneity. In other words, when excess Ca^{2+} and Hg^{2+} are present simultaneously, the number of closed PSII RCs increased but efficiency of excitation energy capture decreased.

Since Ca^{2+} is one of the major cations in the water, its aggravating effect on Hg^{2+}-induced toxicity to PSII implies that the harmful effect of Hg^{2+} on the photosynthetic organisms in aquatic environments with high Ca^{2+} hardness, e.g., in the karstic areas where Ca^{2+} concentration can be up to several hundreds or thousands of mg L^{-1}, may be magnified. The mechanisms underlying need further study.

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