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BRIEF COMMUNICATION

Fluoride affects distribution of absorbed excitation energy more in favour of photosystem 1

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Abstract

The effects of fluoride on the photosynthetic electron transport chain have been studied in spinach thylakoid membranes. Inhibition in photosystem (PS) 2 electron transport rates and a subsequent increase in PS 1 electron transport rate indicated a possibility of state transitions being a mechanism of fluoride action. This hypothesis was further confirmed by the increase in fluorescence emission $F_{735/685}$ at 77 K, a decrease in variable to maximum fluorescence ratio (F_v/F_m) at room temperature and increase in the absorption cross section of PS 1 suggesting that fluoride affects distribution of the excitation energy in favour of PS 1 at the expense of PS 2.

Additional key words: chlorophyll fluorescence, *Spinacea oleracea*, state transitions, thylakoid membranes.

Amongst all physiological processes in plants, photosynthesis is most susceptible to many types of abiotic stresses including water stress, high temperature, salinity, *etc*. (Cai *et al.* 2010). State transitions in photosynthesis are a short-term balancing mechanism of energy distribution between photosystem (PS) 1 and PS 2. PS 1 and PS 2 function in series. Each electron carrier is characterized by its redox potential which drives downhill flow of electrons through the Z-scheme structures. When PS 2 is preferentially excited (state 2) a pool of mobile light harvesting complex 2 (LHC 2) antenna proteins is thought to migrate from PS 2 to PS 1. Photosynthesis also depends on a set of proteins, enzymes and ions (both cations and anions) located in and around a highly organized thylakoid membranes. Amongst anions, chloride is a well known indispensible co-factor in oxygen evolution (Critchley 1985, Yocum 1992). Chloride can be replaced by other anions, which substitute Cl^- due to chemical properties such as ionic radius, hardness, pK_a, redox activity. Anions have also been reported to replace chloride due to its charge density (Critchley *et al.* 1982) and the order of anions replacing chloride being $Br^- > I^- > NO_2^-$, *etc*. Iodide supports a relatively high level of oxygen evolution in the chloride-depleted samples (Hasegawa *et al.* 2002). Chloride and iodide have also been reported to increase membrane fluidity by redistributing the surface charges (Jajoo and Kawamori 2006). In contrast, fluoride substituted samples have been reported to inhibit oxygen evolution rates by 10 - 20 % of the control (Baumgarten *et al.* 1990). Fluoride has been reported to completely inhibit oxygen evolution by preventing S_2 to S_3 transition which leads to considerable perturbation of the Mn cluster (Olesen and Andreasson 2003). Many Cl^- depletion treatments carried out in presence of F^- are thought to enhance Cl^- depletion in PS 2 (Homann 1987) indicating that fluoride may not be competing with Cl^-. NaF has been reported to presumably block the dephosphorylation of membrane proteins (particularly the chlorophyll *a*/*b* protein complex) phosphorylated by a light-activated kinase. Thus, fluoride facilitates the attainment of state 2 by interfering in state 1 to state 2 transitions (Canaani *et al.* 1984). The aim of the present study is to investigate the effect of fluoride on the distribution of absorbed excitation energy between the two photosystems.

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Abbreviations: Chl - chlorophyll; CP43, CP47 - core antenna subunits of PS 2; DCMU - 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; F_v - variable fluorescence; F_m - maximum fluorescence; F_j - fluorescence at the J step of OJIP curve; T_{fm} - time at which maximum fluorescence (F_m) is reached; PS - photosystem; P_{680} - primary electron donor; Q_A, Q_B - plastoquinones.

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Deveined market spinach (*Spinacea oleracea* L.) was used for the isolation of thylakoid membranes using the method of Kuwabara and Murata (1982). The grinding medium contained 50 mM phosphate buffer (pH 7.4), 0.35 mM NaCl, 2mM EDTA while the constituents of the suspending medium were 0.2 M saccharose, 20 mM N-morpholino propane sulphonic acid (MOPS; pH 6.8), 20 mM NaCl. Isolated thylakoid membranes were stored in 30 % glycerol in liquid nitrogen until further use. The chlorophyll concentration was estimated by the method of Vernon (1960).

The stored thylakoid membranes were thawed on ice and were washed with shock medium (50 mM HEPES-NaOH; pH 7.5) in order to give osmotic shock. The thylakoids were incubated for 3 min in shock medium in dark and centrifuged at 8 200 *g* for 15 min. They were then resuspended in isolation medium (IM; 0.33 M saccharose, 50 mM HEPES-NaOH, pH 7.5). Thylakoid membranes were further suspended in 20 mM fluoride and incubated in dark on ice for 10 min.

The PS 2 electron transfer rate was determines as photo-reduction of dichlorophenol-indophenol ($H_2O \rightarrow$ DCPIP) measured spectrophotometrically (*Perkin Elmer Bio 10*, USA) at 605 nm. The reaction mixture contained 35 μM DCPIP, 0.33 M saccharose, 50 mM HEPES-NaOH (pH 7.5), and thylakoid suspension equivalent to 10 μg(Chl) cm^{-3}. The irradiance used was 150 W m^{-2}.

The PS I activity was measured in terms of μmol(O_2 consumed) mg^{-1}(Chl) min^{-1} with $DCPIPH_2$ as the electron donor and methyl viologen as the terminal electron acceptor. These measurements were carried out with a Clark-type oxygen electrode (Yellow Springs, USA) at a constant temperature 25 ± 1 °C. The reaction mixture contained 100 mM saccharose, 20 mM HEPES-NaOH, 3 mM ascorbate, 0.1 mM $DCPIPH_2$, 5 μM DCMU, 5 mM sodium azide, 0.1 mM methyl viologen and thylakoid suspension equivalent to 20 μg(Chl) cm^{-3}. The saturating irradiance used was 150 W m^{-2}.

The Chl *a* fluorescence induction kinetics was measured using a *Plant Efficiency Analyzer* (*PEA, Hansatech*, King's Lynn, Norfolk, UK). The reaction mixture included 0.33 M saccharose, 50 mM HEPES-NaOH and thylakoid suspension equivalent to 10 μg(Chl) cm^{-3}.

The fluorescence emission spectra at room temperature as well as at low temperature (77 K) were measured with a spectrofluorometer (*Jasco FP-6300*, Tokyo, Japan). The reaction mixture contained 0.33 M saccharose, 50 mM HEPES-NaOH (pH 7.5) and thylakoid suspension of 10 μg(Chl) cm^{-3} while for low temperature fluorescence the same reaction mixture with an addition of 30 % glycerol was used. The excitation wavelength was 435 nm with 2.5 nm slit width of the excitation and emission monochromator. Wavelength calibration of the equipment was done using FITC (fluorescein isothiocyanate isomer I, *Sigma*, St. Louis, USA) and the wavelengths of the spectra have been corrected. The fluorescence excitation spectra (77 K) were measured at 735 nm. The reaction mixture and thylakoid suspension were the same as for the emission spectra. All experiments were performed thrice in triplicate.

To characterize the effects of fluoride on the photosynthetic electron transport chain, concentration response of fluoride on PS 2 and PS 1 mediated electron transfer reactions was studied using sodium fluoride as the fluoride salt. A decrease in the rates of PS 2 mediated electron transfer and a concomitant increase in PS 1 mediated electron transfer rates were observed in a concentration dependent manner (data not shown) and concentration of 20 mM NaF was chosen for further experiments. In the presence of 20 mM fluoride, rates of PS 2 electron transfer decreased by 41 %. These results are in agreement with earlier reports (Baumgarten *et al.* 1990, DeRose *et al.* 1994). This result was supported by the decrease in the fluorescence emission peak intensity at room temperature in the fluoride-treated samples (Fig. 1). There was an increase in PS 1 mediated electron transfer by about 38 % in the fluoride-treated samples in saturating irradiance (Table 1). A decrease in the PS 2 activity and increase in PS 1 activity could be because of the distribution of more energy towards PS 1 and thus state transitions. However, this increase could also depend on the number of PS 1 active centers or the electron transfer around PS 1 and not due to an increased antenna size as in case of state transitions. To investigate the role of state transitions in fluoride-treated samples, PS 1 mediated electron transfer rates were recorded in low irradiance (70 W m^{-2}). Adjustment of energy distribution is particularly important in low, limiting irradiance (Krause and Jahns 2004). There was an increase in the rates of electron transfer through PS 1 by 33 % even at limiting irradiance showing a possibility of state transitions (Table 1).

To test this possibility, Chl *a* fluorescence induction kinetics at room temperature was studied both in the absence and presence of DCMU. In the absence of DCMU, there was an increase in basic fluorescence (F_0) and a decrease in maximum fluorescence (F_m) observed

Table 1. Changes in electron transfer rates of PS 2 [μmol(DCPIP reduced) mg^{-1}(Chl) min^{-1}] and PS 1 [μmol(O_2 consumed) mg^{-1}(Chl) min^{-1}] in control and fluoride-treated thylakoid membranes. Means ± SE, n = 9. In all samples, means differ significantly from control at $P < 0.01$.

Parameter	Control	F-treated
Hill activity	1.46 ± 0.06	0.85 ± 0.00
Oxygen consumption at 150 W m^{-2}	1.98 ± 0.03	2.75 ± 0.07
Oxygen consumption at 70 W m^{-2}	1.26 ± 0.02	1.68 ± 0.03

Table 2. Parameters of chlorophyll *a* fluorescence (F_0 is the basic fluorescence level, F_m is the maximum fluorescence level, F_v/F_m is a ratio derived from the values of F_0 and F_m where $F_v = F_m - F_0$, F_j is the fluorescence at the J step of OJIP curve, T_{fm} is the time taken to reach F_m level and Area is the area above the fluorescence curve between F_0 and F_m which is proportional to the pool size of the electron acceptor Q_A on the reducing side of PS 2) in control and fluoride treated plants with and without addition of DCMU. Means ± SE, $n = 9$ (SE has been given only for the original experimental data). In all parameters means differ significantly from control at $P < 0.01$. Since we have done these experiments in ion deficient medium the values are rather low.

	F_0	F_m	F_v/F_m	F_j	T_{fm}	Area
Control	21.0 ± 0.54	39.0 ± 0.83	0.46 ± 0.01	35	1000	950
F-treated	25.0 ± 0.54	34.0 ± 0.70	0.26 ± 0.02	33	600	336
Control + DCMU	24.0 ± 0.89	42.0 ± 0.87	0.42 ± 0.02	41	67	21
F-treated + DCMU	26.0 ± 0.44	37.0 ± 1.30	0.30 ± 0.02	36	6	8

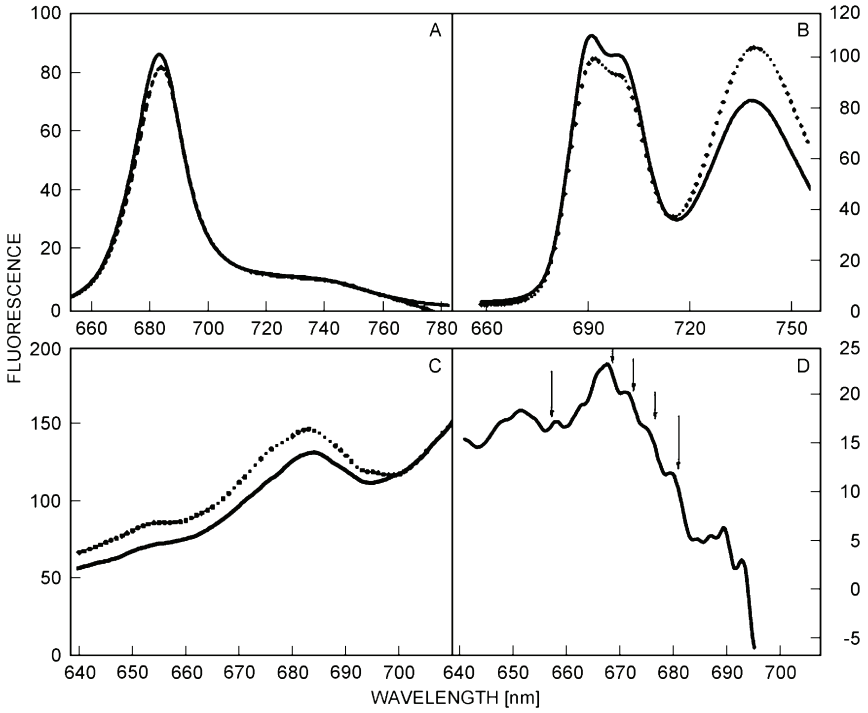

Fig. 1. *A* - room temperature Chl *a* fluorescence emission spectra of control (*full line*) and fluoride-treated (*hatched line*) thylakoid membranes; *B* - Chl *a* fluorescence emission spectra of control (*full line*) and fluoride-treated (*dotted line*) thylakoid membranes measured at 77 K (emission spectra were recorded at excitation wavelength of 435 nm and were normalized at 708 nm); *C* - fluorescence excitation (F_{735}) spectra of control (*full line*) and fluoride-treated (*dotted line*) thylakoid membranes (spectra have been normalized at 708 nm); *D* - difference excitation spectra for F735 of thylakoid membranes (the difference spectra were calculated for by subtracting the excitation spectra of control samples from excitation spectra of fluoride-treated samples).

in the fluoride-treated samples (Table 2). The increase in F_0 may reflect initially blocked (damaged) reaction centrum of PS 2 (Lazar 1999). The increase in F_0 and the decrease in PS 2 electron flow rate have been related to the perturbation of thylakoid membranes leading to the separation of LHC 2 from PS 2 core complex. F_m primarily depends on the amount of plastoquinone A (Q_A^-). It is maximal in the presence of DCMU where all Q_A exist as Q_A^- state. However a decrease in F_m even in the presence of DCMU indicates an inhibition at PS 2 donor side itself (Havaux 1993). The area above the fluorescence curve between F_0 and F_m is proportional to the pool size of the electron acceptor Q_A on the reducing side of PS 2 (Strasser 2004). A decrease of 64 % in the area above the curve suggested a significant decline in the pool size of Q_A. Another important parameter is F_j which is the fluorescence intensity at the J step of the OJIP fluorescence induction curve. The value of F_j in the control samples was higher than in the fluoride-treated samples. The increase in F_j indicates either the higher size of the effective antenna in control samples or the less electron transport rate. Since PS 2 electron transport rate decreased in fluoride-treated samples (Table 1), the latter does not seem to hold true. The decrease in the variable

to maximum fluorescence ratio (F_v/F_m) ratio in fluoride-treated thylakoid membranes suggested lowered quantum efficiency of the reaction centre. Also, the decrease in F_v/F_0 indicated structural alterations in PS 2 (Skorzynska and Baszynski 2000) in fluoride-treated thylakoid membranes. The parameter T_{fm} stands for the time at which F_m is reached. T_{fm} values are used to indicate stress which causes the F_m to be reached much earlier than expected. It is fastest in DCMU treated thylakoid membranes. In fluoride-treated samples, we observed a decline in the T_{fm}.

To ascertain the site of action of fluoride, Chl *a* fluorescence induction kinetics was measured in the presence of DCMU (Table 2). In the presence of DCMU, Q_A is reduced to Q_A^- resulting in maximum fluorescence. Hence, the PS 2 reaction center exists as $Z^+P_{680}PheoQ_A^-$ (Jajoo *et al*. 1998) In the presence of DCMU there was a decrease in F_m in fluoride-treated samples suggesting that fluoride inhibits electron donation from oxygen evolution complex (OEC) to P_{680}^+. These results suggested that the effect of fluoride is not on the acceptor side and could be due to state changes. Furthermore, the increase in PS 1 mediated electron transfer rate in low irradiance suggested an increase in PS 1 antenna size. This was confirmed by measuring Chl *a* fluorescence emission and excitation spectra at 77 K. A distinct decrease in the peak intensities at 685 nm [attributed to Chl *a* in core antenna subunit (CP) 43] and 695 nm (attributed to Chl *a* in CP 47) with a concomitant increase in peak intensity at 735 nm (attributed to PS I) (Govindjee 1995), was observed (Fig 2). The fluorescence emission ratio $F_{735/685}$ (PS 1/PS 2), which reflects the state of excitation energy distribution, was found to be 0.764 for the control samples while it was 1.042 for the fluoride-treated samples. Changes in the PS 1/PS 2 ratio have been mostly associated with the rearrangement of chlorophyll protein complexes inside thylakoid membranes (Garstka *et al.* 2005). Hence, decrease in the PS 2 functionality as judged from Chl *a* fluorescence parameters F_0 and F_m and a decline in peak intensity at 685 nm at 77 K (Allen 1992, Samson and Bruce 1995, Cardol *et al.* 2003) and a subsequent increase in PS 1 mediated electron transfer rates in both, saturating and limiting, irradiances supported a clear possibility of absorbed excitation energy being redistributed to PS 1 at the expense of PS 2.

To confirm this and to understand more about the components playing a role towards this energy distribution, the low temperature (77 K) excitation spectra were measured for emission at 735 nm. The fluoride-treated samples showed an increase in peak at 655 nm and 680 - 682 nm attributed to Chl *b* and Chl *a*, respectively of the LHC 2 (Fig. 1). These peaks also indicated that fluoride caused transfer of only the distal antenna of PS 2 or LHC 2 towards stroma or PS 1 (Telfer 1984). A new peak around 674 - 678 nm also supported these observations (Fig. 1). Peak at 687 nm which is the absorption maximum of the internal PS 2 antenna, peak around 668 - 670 nm which is the absorption maxima of CP 43 and peak around 695 nm (which belong to CP 47) also indicated the role of internal antenna of PS 2 reaction centre and also suggested that probably not only the state transitions but also spillover mechanism may be involved. This supports the contention, that fluoride redistributes the excitation energy towards PS 1 at the expense of PS 2 thereby increasing the cross section of PS 1. Presence of several other smaller peaks suggested probable participation of other forms of LHC 2 towards this increase in PS 1 absorption cross section (Ruban 1991).

Thus we suggest that the mechanism of action of fluoride in inhibiting PS 2 and increasing the rates of electron flow through PS 1 is by diverting the absorbed excitation energy more in favour of PS 1 at the expense of PS 2.

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