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BRIEF COMMUNICATION

Photosystem 2-activity and thylakoid membrane polypeptides of *in vitro* cultured chrysanthemum as affected by NaCl

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Abstract

Long-term (30 d) effects of 100, 200, 300, and 400 mM NaCl on photosystem 2 (PS 2)-mediated electron transport activity and content of D1 protein in the thylakoid membranes of chrysanthemum (*Dendranthema grandiflorum*) cultured *in vitro* at low irradiance 20 μmol(photon) m^{-2} s^{-1} were investigated. 100 mM NaCl increased contents of chlorophylls (Chl) *a* and *b*, carotenoids (Car; xanthophylls + carotenes), and the ratio of Chl *a*/*b*, and Car/Chl *a*+*b*. However, further increase in NaCl concentration led to the significant reduction in the contents of Chl *a*, and Chl *b*, and increase in the ratio of Chl *a*/*b* and Car/Chl *a*+*b*. NaCl treatment decreased the PS 2-mediated electron transport activity and contents of various thylakoid membrane polypeptides including D1 protein.

Additional key words: *Dendranthema grandiflorum*, D1 protein, electron transport rate, low irradiance, salt stress.

During salt stress photosynthetic capacity of many plant species is decreased, due to changes in the structural organization (Flowers *et al*. 1985). Photosystem 2 (PS 2) is multi-subunit complex consisting of more than 30 polypeptides in the thylakoid membranes of chloroplasts, and is responsible for water oxidation reaction of photosynthesis (Henmi *et al*. 2003). It was described that the maximal photochemical activity of PS 2 was not affected by salt-stress (Brugnoli and Björkman 1992, Allakhverdiev *et al*. 1999, Lu *et al*. 2003). On the other hand it was also mentioned that salt stress inhibited the PS 2 activity (Belkhodja *et al*. 1994, Everard *et al*. 1994) that resulted in inactivation of both PS 2 and PS 1 mediated electron transport and damage of the oxygen-evolving machinery of PS 2 (Allakhverdiev *et al*. 2000a,b). Decrease in the maximum quantum efficiency of PS 2, a variable to maximum fluorescence ratio, and the photochemical quantum yield of PS 2 was reported in *Cucumis sativus* leaves under salt stress (Stępień and Kłobus 2006). It was discussed that salt stress alone had little effect on PS 2 photochemistry at low irradiance, but induce photodamage to PS 2 when salt-stressed plants were exposed to high irradiance (Neale and Melis, 1989, Mishra *et al*. 1991, Masojidek *et al*. 1992, Belkhodja *et al*. 1994, Allakhverdiev *et al*. 2002). Molecular mechanisms about the salt-induced inactivation of the PS 2 under dark and light stress, *in vitro* and *in vivo* have been described by Allakhverdiev *et al*. (2005). Effects of salt stress on PS 2 under high irradiance have already been carried out (*e.g*. Sharma and Hall 1991), but long-term salt stress effect on the thylakoid membranes in higher plants under low irradiance is still a matter of uncertainty. Chrysanthemum is an important ornamental plant (a winter floral crop) that is grown under low irradiance and salt stress very often appeared under the natural conditions. The effects of 34.2 mM NaCl for 7 and 14 d on the

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Abbreviations: Car - carotenoids (xanthophylls + carotenes); Chl - chlorophyll; DCPIP - 2,6-dichlorophenol indophenol; DTT - dithiothreitol; LDS - lithium dodecylsulphate; OEC - oxygen evolving complexes; PS 1 - photosystem 1; PS 2 - photosystem 2; RC - reaction center; ROS - reactive oxygen species; SDS/urea-PAGE - sodium dodecylsulphate-urea polyacrylamide gel electrophoresis; TBS - tris-buffered saline.
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growth and development of callus of *Chrysanthemum morifolium* have been reported (Lombardi and Angelini 2005). Therefore, in the present study we have measured the effect of salt stress on the photosynthetic pigments, PS 2-mediated electron transport activity and the accumulation of D1 protein in the thylakoid membrane of chrysanthemum cultured *in vitro* at low irradiance.

Chrysanthemum [*Dendranthema grandiflorum* (Ramat.) Kitam.] shoots were cultured *in vitro* on full-strength solid (1.1 % agar) Murashige and Skoog (1962; MS) medium supplemented with different NaCl concentrations (0 - control, 100, 200, 300, and 400 mM) for 30 d under the low irradiance 20 μmol(photon) m^{-2} s^{-1} with 16-h photoperiod at temperature of 24 ± 1 °C. The contents of chlorophylls (Chl) *a* and *b*, carotenoids (Car; xanthophylls + carotenes) were determined by the method of Wellburn (1994). Isolation of thylakoid membrane was done according to the method of Suzuki *et al*. (2004), with some modifications. Leaves (0.8 g) were macerated with a 1 cm^3 grinding medium containing 50 mM MES-NaOH buffer (pH 6.0), 25 % glycerol and 5 mM $CaCl_2$. The homogenate was centrifuged (*Hanil Science Industrial Co.*, Incheon, South Korea) at 1 000 *g* and 4 °C for 3 min. The resultant supernatant was centrifuged at 14 000 *g* for 40 min. The pellet (thylakoid membranes) was resuspended in 0.050 cm^3 of medium containing 50 mM MES-NaOH buffer (pH 6.5), and 25 % glycerol and kept at -80 °C. The PS 2-mediated electron transport activity in terms of DCPIP photoreduction was measured in thylakoid membranes following the procedures of Prasad *et al*. (1991) and Parida *et al*. (2003). Sample preparation for the SDS-urea-PAGE was done, according to the method of Kashino *et al*. (2001). Briefly, thylakoid membrane suspension [0.25 mg(Chl *a*) cm^{-3}] was dissolved in a denaturing buffer [5.2 % lithium dodecyl sulfate (LDS), 172 mM Tris-HCl buffer, pH 8.0; 40 mM DTT, 0.5 M sucrose, 0.1 % bromophenol blue] by a stirrer, heated at 98 °C for 1 min and centrifuged at 12 000 *g* and 4 °C for 15 min and super-natant was used for the SDS-urea-PAGE. Electrophoresis conditions were similar as in case of 20 % acrylamide gel but containing 6 M urea (Kashino *et al*. 2001). The gel was stained with 0.15 % Coomassie Brilliant Blue R-250 in a solution of 50 % methanol and 10 % acetic acid, and destained by the solution of 25 % methanol and 7.5 % acetic acid. It was scanned with a *Gel Documentation System* (*Core Bio System, Digital UV Transilluminator*). Proteins were transferred to *Hybond-P PVDF* membrane (*Amersham Biosciences*, Little Chalfont, UK) overnight by passive transfer using blotting buffer [100 mM Tris, 192 mM glycine, 0.02 % (m/v) SDS and 5 % (v/v) methanol] as described by Kashino *et al*. (2001). Membrane was pre-wetted in 100 % (v/v) methanol, washed with distilled water for 5 min, and equilibrated in transfer buffer for at least 10 min before blotting. For immunoblotting, the membrane was incubated for 1 h at 25 °C with primary anti-*psbA* global polyclonal antibody raised against a peptide target conserved in PsbA/D1 protein from hen (*AgriSera*, Vännäs, Sweden). Anti-chicken IgG peroxidase conjugate antibody developed in rabbit (*Sigma*, St. Louis, USA) was used as a secondary antibody. Band corresponding to D1 protein was detected with ECL advance Western blotting detection kit (*Amersham Biosciences*, Little Chalfont, UK) and exposed to autoradiography *ECL Hyperfilm* (*Amersham Biosciences*) in a cassette for 15 s and developed, while another autoradiography film was exposed for 30 min. Phosphate buffered saline containing 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5, and 0.1 % (v/v) *Tween 20* was used as a base solution. Developed film was washed, dried and scanned with a *Gel Documentation System* and quantified densitometrically. Each parameter was repeated at least three times independently. Data was analysed using LSD test.

NaCl at low concentration (100 mM) increased contents of Chl *a* and Chl *b* compared with control, but further increase in NaCl concentration led to the reduction in Chl contents (Table 1). Similar results have been reported by Chen *et al*. (2003), where treatment of 50 mM NaCl increased the Chl content, while decreased it dramatically when concentration was above 50 mM. Also, decreases in the Chl content in the leaves of apple (*Malus domestica* Borkh) under 200 mM NaCl stress (Sotiropoulos 2007) and in the shoots of sweet cherry

Table 1. Long-term (30 d) effects of 0, 100, 200, 300, and 400 mM NaCl on the photosynthetic pigments in the leaves and the PS 2 actvity in terms of DCPIP photoreduction in isolated thylakoid membranes of chrysanthemum cultured *in vitro* at irradiance of 20 μmol m^{-2} s^{-1}. Each value represents the mean of three independent measurements ± SE. *, ** - differences significant at $P \leq 0.05$ and 0.01, respectively.

Parameter	Control	100 mM	200 mM	300 mM	400 mM
Chl *a* [g kg^{-1} (d.m.)]	2.85 ± 0.00	4.07 ± 0.01*	3.25 ± 0.07	0.84 ± 0.01**	0.56 ± 0.01**
Chl *b* [g kg^{-1} (d.m.)]	1.12 ± 0.00	1.40 ± 0.01	1.05 ± 0.01	0.21 ± 0.00**	0.12 ± 0.00**
Cars [g kg^{-1} (d.m.)]	0.51 ± 0.01	0.73 ± 0.00*	0.70 ± 0.02*	0.19 ± 0.00**	0.15 ± 0.00**
Chl *a/b*	2.53	2.90	3.09	3.90	4.55
Cars/Chl (*a+b*)	0.13	0.13	0.16	0.18**	0.21**
PS 2 activity [mM(DCPIP) $kg^{-1}(Chl^{-1})$ s^{-1}]	19.0 ± 0.41	15.51 ± 0.32	11.71 ± 0.29*	9.25 ± 0.45**	6.14 ± 0.34**

rootstock Gisela 5 (*Prunus cerasus* × *Prunus canescens*) under 150 mM NaCl stress (Erturk *et al*. 2007) were reported. Under salt stress, a decrease in Mg^{2+} absorption could be responsible for decreased Chl content (Leidi *et al*. 1991). In the present study, with the increase of NaCl concentration the ratio of Chl *a*/*b* increased significantly, indicating a decrease of light-harvesting complex of PS 2 (LHC 2) even at the low irradiance 20 μmol m^{-2} s^{-1}. Our result indicated that inter-conversion of Chl *a* and *b* plays a significant role in the establishment of required Chl *a*/*b* ratio during the adaptation of leaves to environmental stresses (Ito *et al*. 1993). Also in *Artemisia*, high salinity led to an increase in the Chl *a*/*b* ratio, but no change in the ratio of Car/(Chl *a*+*b*) were found (Lu *et al*. 2003). Car have several important functions in photosynthetic energy transduction pathways. For example lutein stabilizes the 3D structure in LHC 2 and xanthophyll cycle pigments allow the dissipation of excess radiation. In our study, Car content increased with increasing NaCl concentration, indicating that this energy dissipation mechanism helped to decrease the energetic overcharges in PS 2 and PS 1 (Ramalho *et al*. 2000, Lu *et al*. 2003, Pandey *et al*. 2005).

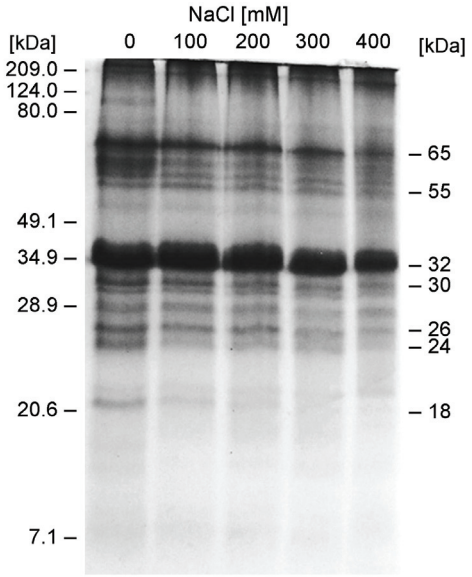

Fig. 1. Long-term (30 d) effect of 0, 100, 200, 300, and 400 mM NaCl on the thylakoid membrane polypeptides *Dendranthema grandiflorum* cultured *in vitro* at irradiance of 20 μmol m^{-2} s^{-1}. The SDS-urea/PAGE was done with 3 μg Chl *a* per lane.

Proper alignment of thylakoid membranes is essential for the functional integrity of photosystems and optimal light energy harvesting. Salt stress caused structural changes in photosynthetic membranes, hampering their normal function in the harvesting of light energy and utilization (Parida *et al*. 2003). Therefore, to study the extent of damage by imposed stress, we have measured the PS 2-mediated electron transport activity in terms of DCPIP photoreduction. Significant decrease in the rate of DCPIP photoreduction after NaCl treatments even under low irradiance might be due changes in the proper alignment of thylakoid membranes and dissociation of some extrinsic proteins (Table 1). NaCl induced loss in PS 2 mediated electron transport activity has already been reported (Tiwari *et al*. 1997, Allakhverdiev *et al*. 2000a,b, Parida *et al*. 2003).

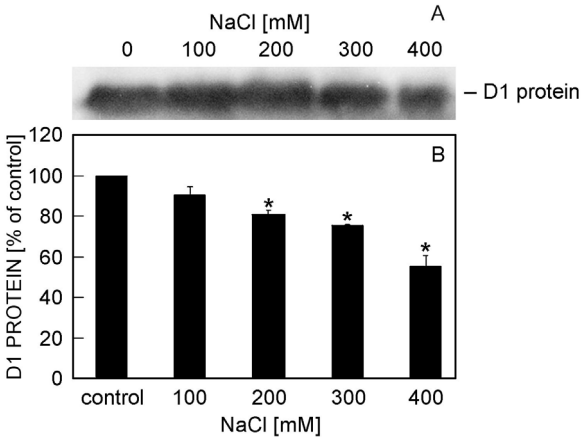

Fig. 2. Long-term (30 d) effects of 0, 100, 200, 300, and 400 mM NaCl on D1 protein accumulation in thylakoids isolated from *Dendranthema grandiflorum* cultured *in vitro* at low irradiance. *A* - Western blot of anti-PsbA global polyclonal antibody; *B* - changes in the content (mean ± SE) of 32-kDa D1 protein quantified densitometrically from three independent Western blots (* - differences significant at $P \leq 0.05$).

SDS-urea-PAGE of thylakoid membranes revealed that the contents of various polypeptides decreased during the long-term NaCl treatment as compared to control. Although this decrease was observed at 100 mM NaCl, maximum reduction in the contents of 65, 55, 32, 30, 28, 26, 24 and 18 kDa polypeptides was observed at 400 mM NaCl (Fig. 1). Similarly, immunoblotting analysis of D1 protein indicated a significant degradation of D1 protein under NaCl stress, and maximum degradation was observed at 400 mM NaCl (Fig. 2*A*,*B*). This result suggests that target site of NaCl stress might be the dissociation of certain thylakoid polypeptide. Similar studies indicated that NaCl treatment caused not only the degradation of D1 protein but also the degradation of other polypeptides (Enami *et al*. 1989, Suzuki *et al*. 2003, 2004). NaCl-induced inactivation of PS 2 and PS 1 corresponded to the time course of osmotic stress-induced inactivation (Allakhverdiev *et al*. 2000b), suggesting that the rapid decline in the activities of PS 2 and PS 1 might have been caused by osmotic stress. Allakhverdiev and Murata (2004) reported that light-induced damage to PS 2 was not affected by salt stress but rather the rate of replacement of damaged D1 protein by newly synthesized D1. In the present study under low irradiance, it is possible that D1 protein might have better repair capacity, compared to previous studies. Similarly, Allakhverdiev *et al*. (2005) reported that the rate of repair was depended

on irradiance and reached a maximum at 300 μmol m^{-2} s^{-1}. Reactive oxygen species (ROS) that are generated in PS 2 might be responsible for the photo-oxidative damage to the D1 protein (Nishiyama *et al*. 2001, Henmi *et al*. 2004). Therefore, in present study it is possible that *in vivo* ROS generated inside PS 2 membranes under salt stress may cause the photodamage to the photosynthetic machinery even at low irradiance.

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