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BRIEF COMMUNICATION

Response of antioxidant enzymes to high NaCl concentration in different salt-tolerant plants

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Abstract

The effects of NaCl on the H_2O_2 content and the activities of catalase (CAT) and superoxide dismutase (SOD) were studied in diverse group of plants, such as a unicellular alga, *Chlorella* sp., an aquatic macrophyte, *Najas graminea*, and a mangrove plant, *Suaeda maritima*, all showing high tolerance to NaCl. Significant accumulation of H_2O_2 was observed in all the tested plants upon their exposure to 255 mM NaCl. The activity of both CAT and SOD increased significantly in response to the NaCl treatment. Growing the plants in presence of 255 mM NaCl also resulted in the synthesis of new isoforms of both CAT and SOD.

Additional key words: catalase, *Chlorella* sp., NaCl stress, *Najas graminea, Suaeda maritima*, superoxide dismutase.

The antioxidative enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) play the key role in removal of reactive oxygen species (ROS) produced in plant cells as byproducts of normal cell metabolism or as a result of disturbance in the cell metabolic processes under abiotic stresses (Shaw *et al*. 2004). Their activity, particularly of CAT and SOD, gets accelerated in plants facing abiotic stresses (Rout and Shaw 2001a, Mutlu *et al*. 2009, Sahu and Shaw 2009a). Studies on the effect of abiotic stresses on the enzyme isoform composition are, however, limited (Jebara *et al*. 2005, Fedina *et al*. 2009). Salinity is widely studied abiotic stress. The immediate toxic effect may be reflected as ionic imbalance or cell/tissue dehydration (Rout and Shaw 2001b, Ermawati *et al*. 2009, Sahu and Shaw 2009b). Salinity also induces ROS production and oxidative damage (Rout and Shaw 2001a, He and Zhu 2008, Aghaleh *et al*. 2009) and several attempts have been made to improve salt tolerance of plants by changes in antioxidant enzymes in transgenic plants (Prashanth *et al*. 2007, Tseng *et al*. 2007). However, the basis of the selection of such transgenes has always been hypothetical, rather than empirical, as the transgenes selected were never tested beforehand for their salt responsiveness. The present study, hence, was designed to look for salt responsiveness of CAT and SOD at the isoform level taking morphologically and taxonomically diverse salt-tolerant plant species. APX was not considered as in an earlier experiment it was seen that the enzyme was not salt responsive in aquatic plants (Rout and Shaw 2001a).

The test species selected for the experiment were *Chlorella* sp. (a unicellular alga isolated from rice field), *Najas graminea* Delile (a submerged brackish water macrophyte) and *Suaeda maritima* L. (a mangrove plant) all showing tolerance to high concentration of NaCl. These were exposed to Na^+ for short (12 h) or long (10 d) duration. For short-duration exposure of the alga, the NaCl concentration of the individual flasks containing the alga grown for 15 d in 1 dm^3 mineral standard medium (MSM; Ogawa and Terui 1970) was initially raised to 50 mM by adding NaCl, except of the flask meant for control, which contained 4 mM NaCl. After 1 h of incubation in dark, NaCl concentration was raised to 85 mM, 255 mM or 425 mM and the flasks were exposed to irradiance of 250 μmol m^{-2} s^{-1} in a culture room at

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; H_2O_2 - hydrogen peroxide; KCN - potassium ferricyanide; NBT - nitroblue tetrazolium; $O_2^{\bullet-}$ - superoxide; $^{\bullet}OH$ - hydroxyl radical; PAGE - polyacrylamide gel electrophoresis; ROS - reactive oxygen species; SOD - superoxide dismutase.
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temperature of 22 ± 2 °C. In the case of *S. maritima*, the seedlings (about 6 cm with lateral branches) grown in sterilized garden soil in pots under natural irradiance in a greenhouse (temperature 24 ± 3 °C, relative humidity 70 - 75 %) were initially treated with 150 cm^3 of 0.5 % NaCl prepared in 1/10 strength Hoagland's solution. After incubation for 1 h in dark, NaCl concentration was increased to 85, 255 or 425 mM, respectively (Sahu and Shaw 2009a,b). Na^+ treatment of *N. graminea*, grown under shade for 20 - 25 d in winter in 100 dm^3 concrete tanks (maximum tank temperature recorded was 24 °C), was done by applying concentrated solution of NaCl in the individual tanks, except in that meant for control, to raise the NaCl concentration to 50 mM. After 1 h, more NaCl solution was added to raise the Na^+ concentration to 85, 255 or 425 mM. The algal mass, the leaves of *S. maritima* and the branch tips (about 6 cm) of *N. graminea* were collected after 12 h of initial Na^+ exposure for estimation of H_2O_2 and assay of CAT and SOD activities. Only 255 mM NaCl concentration was used for the long duration exposure. The treatment was done as described above, but by applying NaCl for consecutive 5 d in the evening raising its concentration by 51 mM every day. The samples for analyses were collected as described above after 10 d of the initial application of NaCl. In all the cases, the samples were preserved in liquid N_2 until analysis.

H_2O_2 content of the plant samples was determined using 4-(-pyridylazo)resorcinol and potassium titanium oxalate as the colorimetric reagent (Patterson *et al*. 1984). The enzyme extract was prepared as described in Rout and Shaw (2001a) and the activity of CAT (EC 1.11.1.6) and SOD (EC 1.15.1.1) in the supernatant was measured following methods of Chance and Maehly (1955) and Beyer and Fridovich (1987), respectively, with some modification (Rout and Shaw 2001a). Protein in the supernatants was quantified following Bradford (1976). Statistical analysis of the data was done by Duncan's multiple range test for unequal sample size (Blis 1967). The homogenizing buffer (Rout and Shaw 2001a) for the isoenzyme analysis contained 10 % glycerol (Mittler and Zilinskas 1993). Native polyacrylamide gel was casted and run at 4 °C. The isoforms of CAT and SOD were visualized following Woodbury *et al*. (1971) and Beauchamp and Fridovich (1971), respectively.

In all the test species, the cellular H_2O_2 content and CAT activity were significantly higher at 255 mM Na^+ treatment compared to the control and *S. maritima* showed the maximum increase (Table 1). In *N. graminea* and *Chlorella* sp, the CAT activity decreased significantly at 425 mM treatment. Unlike CAT, SOD activity exhibited maximum response in *Chlorella* sp. showing significant increase at 85 and 255 mM NaCl (Table 1). In *N. graminea* and *S. maritima*, significant increase in the SOD activity was observed at 255 mM and 425 mM NaCl, respectively.

Activity staining of the native gel did not show any change in number of CAT isoforms in *S. maritima* and *N. graminea* in response to NaCl, although some increase in intensity of bands was observed (Fig. 1). *Chlorella* sp., on the other hand, showed appearance of at least three new CAT isoforms when grown in the NaCl-supplemented medium, besides increase in the intensity of the band 2. Both *N. graminea* and *S. maritima* grown at 255 mM NaCl showed the appearance of a new isoform of SOD (Fig. 2, band 2 in *S. maritima* and band

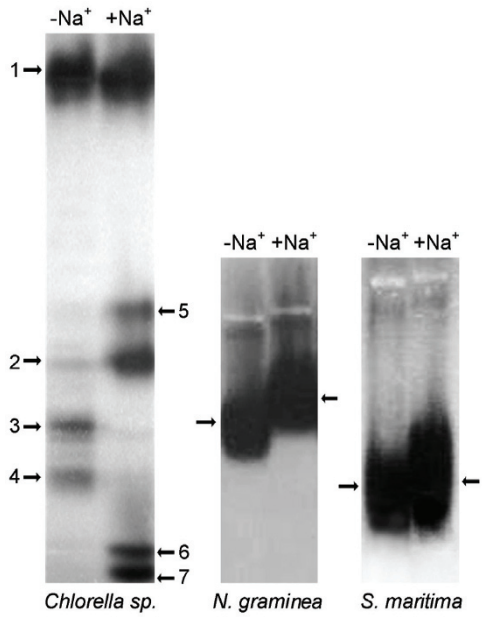

Fig. 1. Native PAGE showing CAT isoforms in the three test species grown for 10 d in the medium containing negligible Na^+ (control, $-Na^+$) and that containing 255 mM Na^+ (treated, $+Na^+$).

4 in *N. graminea*). KCN inhibition study revealed these to be CuZn-SOD isoform. Under the control condition, *S. maritima* had only one isoform, a CuZn-SOD, and *N. graminea* had two isoforms, both Mn-SOD (Fig. 2), as revealed by KCN and H_2O_2 inhibition studies. In contrast, *Chlorella* sp. grown in NaCl-supplemented medium showed appearance of 10 new SOD isoforms. The alga under control condition had 4 SOD isoforms. KCN inhibition study showed the presence of no CuZn-SOD isoform. H_2O_2 inhibition study revealed the bands 1, 2 and 3 (present originally), 5 and 6 (NaCl-induced) to be Fe-SOD. The remaining isoforms were Mn-SOD, most of which were NaCl-induced, except the band 4.

NaCl-induced increase in the cellular H_2O_2 content in the tested species (Table 1) is a circumstantial evidence of generation of $O_2^{\bullet-}$ in them under NaCl stress, as H_2O_2 is generated mainly by dismutation of $O_2^{\bullet-}$ catalyzed by SOD. H_2O_2 accumulation has been considered as a sign of oxidative stress, as its reaction with $O_2^{\bullet-}$ leads to the formation of highly reactive hydroxyl radical ($^{\bullet}OH$) causing peroxidative damage of biomolecules (Shaw *et al*. 2004, Kim *et al*. 2005). Hence, it is a requirement for plants to keep content of both H_2O_2 and $O_2^{\bullet-}$ to minimum in order to prevent the Fenton reaction to proceed (Shaw *et al*. 2004). Significant increase in the activity of

Table 1. Changes in cellular H_2O_2 content [nmol g^{-1}(f.m.)] and the specific activities of CAT and SOD [U mg^{-1} (protein)] in *N. graminea*, *Chlorella* sp. and *S. maritima* treated with various concentrations of NaCl for 12 h. Means ± standard deviation of at least four independent estimations. The means for a species with same letter are not significantly different at $P \leq 0.05$, as determined by Duncan's multiple range test for unequal sample size. A unit enzyme activity is the amount of protein in the enzyme extract required to decompose 1 μmol of H_2O_2 in 1 min (for CAT) or to inhibit 50 % colour development in the reaction (for SOD).

NaCl	H_2O_2 content			CAT activity			SOD activity		
[mM]	*N. graminea*	*Chlorella*	*S. maritima*	*N. graminea*	*Chlorella*	*S. maritima*	*N. graminea*	*Chlorella*	*S. maritima*
Control	9.94±1.43^{b}	15.22±2.31^{b}	10.13±1.96^{b}	310.6±19.8^{b}	240.5±40.0^{b}	291.3±21.5^{b}	10.19±1.65^{c}	12.24±1.48^{c}	6.80±1.26^{c}
85	13.14±3.98^{ab}	15.28±1.96^{b}	12.69±3.26^{b}	372.2±25.1^{a}	225.5±16.9^{bc}	382.4±51.3^{a}	11.63±1.27^{ab}	15.12±1.87^{a}	5.93±1.18^{c}
255	16.25±1.69^{a}	19.21±2.39^{a}	23.31±6.54^{b}	360.4±15.6^{a}	315.4±37.3^{a}	414.6±23.6^{a}	13.23±1.55^{a}	17.50±1.99^{a}	8.90±0.90^{ab}
425	6.01±1.68^{c}	16.81±2.31^{ab}	20.40±3.65^{b}	260.7±37.5^{c}	190.7±18.8^{c}	392.3±28.0^{a}	9.43±0.67^{c}	14.40±1.51^{ab}	9.53±0.85^{a}

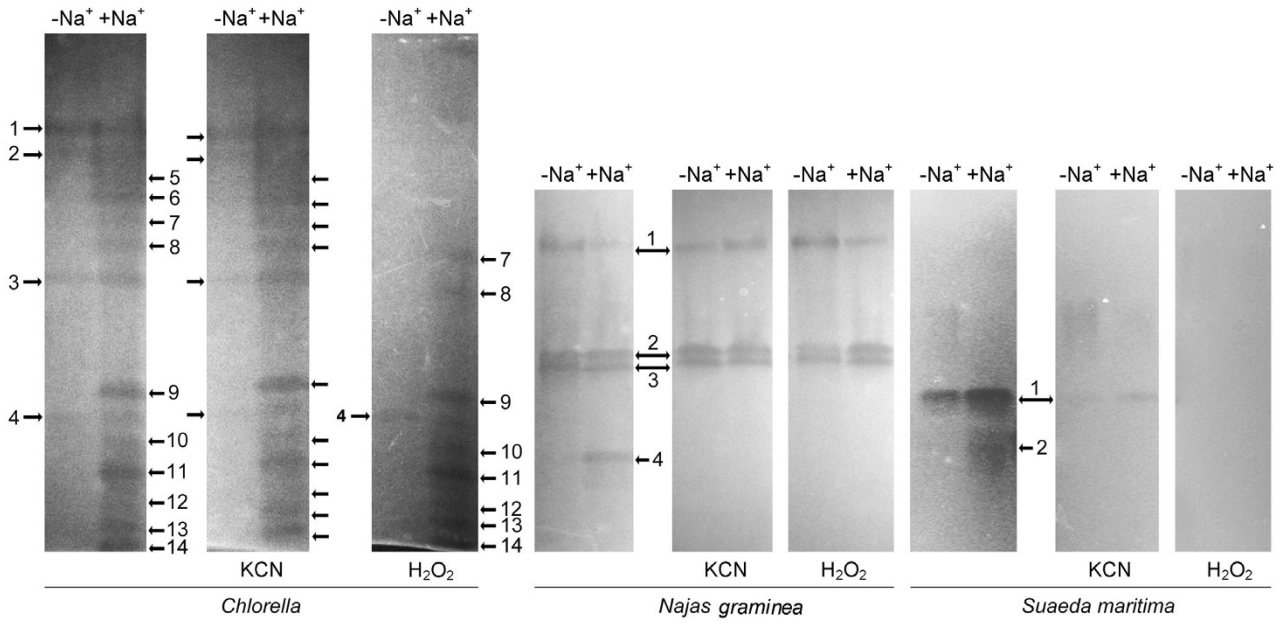

Fig. 2. Native PAGE showing SOD isoforms in the test species grown for 10 d in the medium containing negligible Na^+ (control, -Na^+) and that containing 255 mM Na^+ (treated, +Na^+).

both SOD and CAT in the test species in response to NaCl treatment (Table 1) protected the plants from NaCl-induced oxidative stress. Stimulating effect of NaCl on SOD and CAT activity has been reported for many other plant species, particularly the salt-tolerant ones (Dionisio-Sese and Tobita 1998, Rout and Shaw 2001a, Jebara *et al*. 2005, Kim *et al*. 2005, Sekmen *et al*. 2007, Fedina *et al*. 2009). Furthermore, Dionisio-Sese and Tobita (1998) and Sekmen *et al*. (2007) suggested greater increase in SOD activity in the salt tolerant plants than in the non-tolerant ones to be the reason of less lipid peroxidation in the former than in the latter upon their exposure to NaCl. It has also been reported that in glycophytes, like rice and cotton, the activity of CAT generally decreases in response to NaCl treatment (Gossett *et al*. 1994, Lee *et al*. 2001). Such studies in aquatic plants are, however, limited (Rout and Shaw 2001a). This study thus suggests increase in the activity of CAT and SOD in plants in response to NaCl to be a salt adaptive response. Somewhat direct evidence of involvement of both CAT and SOD in salt tolerance, nevertheless, comes from greater salt-tolerance of transgenic plants overexpressing CAT and SOD genes (Tseng *et al*. 2007).

NaCl-induced increase in the activity of CAT in the tested species is also reflected in the increase in intensity of its bands in *N. graminea* and *S. maritima*, and in addition from appearance of its new isoforms in *Chlorella* sp. (Fig. 1) upon their long-duration exposure to Na^+. Report on salt-induced changes in CAT isoform is scant (Kholova et al. 2009), particularly with regard to the distinct changes observed in the enzyme isoform in the present study. Similar to that of CAT, study on the isoform-specific response of SOD to Na^+ exposure is also scant, and to the best of our knowledge only Kim *et al*. (2005) and Kholova *et al*. (2009) observed salt-induced appearance of the enzyme isoforms in barley and maize, respectively, but these were not characterized for their metal co-factor. Thus, the present study for the first time

reports Na+-induced appearance as many as ten isoforms of SOD comprising Fe-SOD and Mn-SOD, in *Chlorella* sp. (Fig. 2). The Na^+-induced appearance of new Fe-SOD isoforms in *Chlorella* sp. is of much significance from the point of view of providing the alga protection against Na^+-induced oxidative stress as the transgenic plant overproducing Fe-SOD has been reported to be more tolerant to oxidative stress than that overproducing Mn-SOD (Van Camp *et al*. 1996). The absence of CuZn-SOD in *Chlorella* sp. is in agreement with the report that most eukaryotic algae lack CuZn-SOD (Asada *et al*. 1977). However, the absence of Mn-SOD, as found in *S. maritima*, has so far not been reported in any plant species.

The study thus indicated that the presence of excess NaCl in the environment leads to oxidative stress build-up in all kinds of photosynthetic plants, including the salt tolerant ones. Besides, the antioxidative enzymes like CAT and SOD could be important in providing the plant tolerance to salinity by mitigating the oxidative stress build-up. In this regard, the appearance of multiple isoforms of both CAT and SOD in *Chlorella* sp., a freshwater inhabitant, could be of much significance. A clear understanding on the regulatory aspects of the salt/Na^+-inducible isoforms of the enzymes may allow scientists to adapt appropriate biotechnological approach for improvement of salt tolerance in the species of interest.

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