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Differential enzymatic defense mechanisms in leaves and roots of two true mangrove species under long- term salt stress

Md. Daud Hossain[a,b], Masashi Inafuku[a], Hironori Iwasaki[a], Naoyuki Taira[a], Mohammad Golam Mostofa[c], Hirosuke Oku[a,*]

[a] Tropical Biosphere Research Center, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan
[b] Farm Division, Bangladesh Agricultural Research Institute, Joydebpur, Gazipur, 1701, Bangladesh
[c] Laboratory of Plant Stress Responses, Faculty of Agriculture, Kagawa University, Kagawa 761-0795, Japan

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ABSTRACT

To elucidate the role of antioxidative defense system against salt stresses, the levels of antioxidative enzymes and lipid peroxidation were quantified in leaves and roots of 2-year old *Kandelia candel* and *Rhizophora stylosa* seedlings subjected to 0, 5, 15 and 30 parts per thousand (ppt) of salt concentrations during one and two months treatments (1MT and 2MT). The data revealed differential responses between the species to different salt concentrations, while no significant differences observed between the treatment periods.

In leaves, higher salinity decreased superoxide dismutase (SOD) and monodehydroascorbate reductase (MDHAR) activities in both species and catalase (CAT) activity in *K. candel*, while ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) activities in both species and CAT in *R. stylosa* were increased. In roots, salt stresses increased the activities of most of the tested enzymes. Except for the roots *K. candel*, the malondialdehyde (MDA) contents were decreased significantly at higher salt concentrations.

In *K. candel* leaves, moderate to strong negative correlations existed between APX, GPX and DHAR activities with MDA content, while SOD, CAT and MDHAR showed positive correlations. Contrarily, in *R. stylosa* leaves, CAT, APX and sometimes GPX and DHAR showed inverse correlations with respective MDA, while other enzymes showed reverse correlations. With few exceptions, all tested enzymes in roots showed negative correlations with MDA. These results suggested that most of the antioxidant enzymes in roots acted coordinately for effective stress mitigation, while leaf tissues might adopt many other mechanisms for salt tolerance of mangrove plants where antioxidant enzymes played minor role.

1. Introduction

Soil salinity constitutes a major factor limiting crop production because it affects plant growth and survival (Munns and Tester, 2008). Various environmental stresses including salt induce oxidative stress in plants through an enhanced generation of superoxide radical ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$) and singlet oxygen ($^1O_2$), collectively termed as reactive oxygen species (ROS) (Kathiresan and Bingham, 2001; Jithesh et al., 2006; Ardestani and Yazdanparast, 2007). These cytotoxic ROS can seriously disrupt normal metabolism of plant cell through oxidative damage to lipids, protein and nucleic acids (De Vos and Schat, 1991; Mehta et al., 1992; Parida et al., 2004; Parida and Jha, 2010) when ROS are not kept under control (Mittler, 2002). Therefore, excessive ROS are considered as indicators of stresses, and it is crucial for plants to balance the generation and elimination of ROS during exposure to salty environments, especially for long-term periods (Light et al., 2005).

Plants possess specific mechanisms to detoxify the ROS which include activation of antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione/guaiacol peroxidase (GPX, EC 1.11.1.7) and those of the ascorbate-glutathione cycle (Noctor and Foyer, 1998; Smirnoff, 2005) as well as non-enzymatic antioxidants. In ascorbate-glutathione cycle, four enzymes are known which take part in a series of coupled redox reactions, i.e., ascorbate peroxidase (APX, EC 1.1.1.11), glutathione reductase (GR, EC 1.6.4.2), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; FW, fresh weight; GPX, glutathione/guaiacol peroxidase; GR, glutathione reductase; GSH, glutathione; GSSH, glutathione disulfide; KPB, K-phosphate buffer; MDA, malondialdehyde; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; PVPP, polyvinylpolypyrrolidone; ROS, reactive oxygen species; SOD, superoxide dismutase

* Corresponding author.
E-mail address: okuhiros@comb.u-ryukyu.ac.jp (H. Oku).

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dehydroascorbate reductase (DHAR, EC 1.8.5.1) (Smirnoff, 2005).

'Resistance' and 'tolerance' are the terms used to denote the ability of the plant to manage a particular stress, be it biotic or abiotic. Resistance is an absolute term where the plant completely immunizes itself to a particular stress while tolerance is rather a relative term and it is also man-made to some extent. In agriculture, salt resistance usually refers to a genetic mechanism in the plant to counteract the adverse effect of salt. One the other hand, salt tolerance is the ability of plants to withstand the effects of high salt concentrations in the root zone or in the leaves without a significant adverse effect. Mangroves are a special kind of forest occurring in the intertidal zones of tropical and subtropical coastlines. They are mainly facultative halophytes, tolerant to both high and fluctuating salinity and are regarded to be more salt tolerant than any other species (Zhu et al., 2011). Their cells are well protected against the detrimental effects of ROS by a complex antioxidant system comprising non-enzymatic and enzymatic antioxidants (Tam and Yao, 2002; Zhang et al., 2007a,b; Agoramoorthy et al., 2008; Rahim et al., 2008). Kandelia candel (L.) Druce *and Rhizophora stylosa* Griff. are two true, non-secretor mangroves. *K. candel* found around the coasts of south and southeast Asia, from western India to Borneo whereas *R. stylosa* grows naturally in Japan, China, Taiwan, Cambodia, Vietnam, Malaysia and Queensland (Duke et al., 2002; Sheue et al., 2003). They also dominant in the southernmost Iriomote Island, Okinawa, Japan (24°20′N, 123°49′E), and are considered to be representative of all mangrove species (Kathirem and Baba, 1999; Takeuchi et al., 2001). Therefore, these two species are good materials to clarify the molecular mechanism of salt tolerance in mangrove plants. However, to our knowledge some information are available about the changes of antioxidative enzymes in *K. candel* and *R. stylosa* under salt stress but most of the works included only a few enzymes at a time, and they rarely analyzed and systematically discussed the contribution of individual enzymes specially in relation to long- term duration of salt stress. Furthermore, the correlation between activities of antioxidant enzymes with content of lipid peroxidation has not been systematically addressed to date. Therefore, to understand the salt-tolerance mechanism of mangrove plants, we did a comparative analysis of changes of the activities of six antioxidant enzymes and their relationship to malondialdehyde (MDA), as a marker of lipid peroxidation in the leaves and roots of two-year-old *K. candel and R. stylosa* seedlings treated with 0, 5, 15 and 30 parts per thousand (ppt) salts for one and two months periods. This work suggests that, adaptation of mangrove plants to salty environment associated with higher enzymatic activities along with lower content of lipid peroxidation, and for effective detoxification of ROS, antioxidant enzymes in root tissues played more crucial role than those in leaf tissues.

2. Materials and methods

2.1. Salt stress treatments

Seedlings used in this experiment had been grown in Wagner pots (two seedlings per pot) with salt free sand for two years under freshwater condition. The pots containing uniform seedlings (about 50 cm height) were selected and thereafter transferred in a glass house with natural temperature and sunlight. After one week of acclimatization, the seedlings were allowed to expose to different salinity treatments by replacing the fresh water of the pots with 0, 5, 15 and 30 ppt salt solutions directly. Each treatment had three replicated pots. Seawater for each treatment was prepared by dissolving commercial salt powder (Tetra marine salt Pro, Rakuten Inc., Tokyo, Japan) in tap water. In each treatment, the salt concentration was checked once weekly by an S/Mill-E salinity refractometer (Atago Co., Ltd., Tokyo, Japan), and adjusted by adding tap water to compensate for the water loss through evapotranspiration. The seedlings were allowed to grow normally (without any nutrients). After one month of treatment (1MT), the leaves and roots of one seedling of each pot (without disturbing the rest ones) were harvested and washed separately, subsequently ground to fine powder in liquid nitrogen and stored at −80 °C until use. After two months of treatment (2MT), same work was performed using the remaining seedling of each pot.

2.2. Enzymes extraction

For the measurement of SOD and GR activities, one gram of frozen leaf or root tissue materials were homogenized in 10 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.8), 0.1 mM EDTA, 0.2% (v/v) TritonX-100, 1 mM PMSF, 2 mM DTT, and 1% polyvinylpolypyrrolidone (PVPP). On the other hand, for GPX measurement, 50 mM ice-cold K-phosphate buffer (KPB) (pH 5.5) containing 1% PVPP, and for APX, CAT, MDHAR and DHAR measurements, 50 mM ice-cold KPB (pH 7) containing 1 mM ascorbate (AsA), 1 mM EDTA, 0.1% (v/v) Triton X-100 and 1% PVPP were used as extraction buffers. The whole homogenization process was carried out in an ice bath. Homogenates were centrifuged at 13.000 x g for 15 min at 4 °C. Supernatants were stored at 4 °C until assay of enzyme activity and protein content. Total soluble protein content was determined by a DC Protein Assay kit (Bio-Rad).

2.3. Activity assays

Unless otherwise noted, all activities were measured spectrophotometrically using one milliliter of reaction mixture run at 25 °C. Total SOD activity was estimated based on a xanthine–xanthine oxidase system (El-Shabrawi et al., 2010). One unit of activity was defined as the amount of enzyme required to inhibit NBT reduction by 50%. CAT activity was measured according to the method of Hossain et al. (2010). GPX activity was measured by following the method of Tatiana et al. (1999) using guaiacol as the hydrogen donor. Total APX and DHAR activities were assayed following the methods of Nakano and Asada (1981). MDHAR activity was measured according to Hossain et al. (1984).

2.4. Lipid peroxidation (MDA) measurement

The level of lipid peroxidation was measured by estimating MDA, a decomposition product of the peroxidized polyunsaturated fatty acid component of the membrane lipid, using thiobarbituric acid as the reactive material following the method of Heath and Packer (1968) with slight modifications (Hossain et al., 2010). The concentration of MDA was calculated by using $\Sigma = 155\ mM^{-1}\ cm^{-1}$ and expressed as nmol of MDA g^{-1} FW.

2.5. Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) followed by Dunnett's test for comparisons of means using XLSTAT 2015 statistical software. Relationships between the activities of SOD, CAT, APX, GPX, MDHAR and DHAR with the subsequent MDA contents were ascertained by means of Pearson's correlation coefficient (n = 12) using XLSTAT 2015.5.01.23164 software. Fig. 4 was prepared using Cytoscape 3.2.1 software.

3. Results

3.1. Alterations in antioxidant enzymes activities under long- term salinity

3.1.1. Alterations in antioxidant enzymes activities in leaves

To gain insight into the response of *K. candel* and *R. stylosa* seedlings to salt stress, the activities of SOD, CAT, GPX, APX, MDHAR and DHAR were assayed under diferent salinity levels after 1MT and 2MT. The activities of antioxidant enzymes in leaves of *K. candel* and *R. stylosa* showed somewhat differential response patterns to salinity in terms of concentration as well as duration (Fig. 1). The overall plant growth was

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