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Reactive oxygen species dynamics in roots of salt sensitive and salt tolerant cultivars of rice

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ABSTRACT

Salinity stress is one of the major constraints for growth and survival of plants that affects rice productivity worldwide. Hence, in the present study, roots of two contrasting salinity sensitive cultivars, IR64 (IR64, salt sensitive) and Luna Suvarna (LS, salt tolerant) were compared with regard to the levels of reactive oxygen species (ROS) to derive clues for their differential salt stress adaptation mechanisms. In our investigation, the tolerant cultivar exhibited longer primary roots, more lateral roots, higher root number leading to increased root biomass, with respect to IR64. It was observed that LS roots maintained higher level of H_2O_2 in comparison to IR64. The activities of various enzymes involved in enzymatic antioxidant defense mechanism (SOD, CAT, GPX, DHAR and MDHAR) were found to be greater in LS roots. Further, the higher transcript level accumulation of genes encoding ROS generating (*RbohA, RbohD* and *RbohE)* and scavenging enzymes (*Fe-SOD*, *Chloroplastic Cu/Zn-SOD, CAT* and *DHAR)* were noticed in the roots of tolerant cultivar, LS. Moreover, the content of other stress markers such as total protein and proline were also elevated in LS roots. While, the expression of proline biosynthesis gene (*P5CS)* and proline catabolism gene (*PDH)* was observed to be lower in LS.

Introduction

Rice is an important food crop that immensely contributes toward global food security [1,2]. Due to increase in world population there has been a growing demand to further enhance its productivity [1,3]. However, the shrinking cultivable land and persistence of unfavorable environmental conditions are the major challenges towards achieving this target. Salinity which covers approximately 30% area of world's agricultural land and accounts for the loss of US $ 12 billion of global agriculture is undoubtedly one of the major threats to rice production [4,5].

Salt stress affects the plant at phenotypic, biochemical, physiological, cellular and molecular level [6]. Further, different parts of the plant such as shoot and root might respond differentially to salt stress due to the difference in the basic nature of their intrinsic factors [7]. Roots are the primary target site for perception of salt stress signal and could act as an early warning system for the plant [8]. Upon exposure to stress, roots initiate a triggering effect which could lead to the activation of various stress adaptive mechanisms in plants [9].

Oxidative stress in plant is a resultant of various extrinsic factors including environmental stresses [10]. It regulates various biological functions and hence its modulation has been an area of research interest in many laboratories across the world [10]. Among various mechanisms involved in stress tolerance, the role of reactive oxygen species (ROS) is considered as critical [11–13]. ROS such as superoxide radical (O^{2-}), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) are known to be involved in regulating various signaling cascades associated with several biological functions of the plants [14,15]. They are normally produced at a low concentration in plants as a result of various metabolic activities such as photorespiration, photosynthesis and mitochondrial respiration [16]. NADPH oxidases in plants which are known as respiratory burst oxidase homologs (Rbohs) are one of the key enzymes that generate extracellular free radical O^{2-}, through catalyzing the transfer of electrons from NADPH to molecular oxygen (O_2), thus producing ROS [17,18]. They occupy a unique position in plants due to their involvement in different signaling pathways regulating plant growth, development and stress related responses [18]. Similarly, ROS scavenging enzymes readily convert the ROS produced by NADPH oxidases into H_2O_2, H_2O and O_2 [19]. At threshold level, H_2O_2 acts as a signaling molecule playing versatile roles in normal plant physiological processes and offers tolerance against various stresses [12,20,21].

For developing a suitable strategy for abiotic stress management in

Abbreviations: DAB, 3,3-Diaminobenzidine; NBT, Nitro blue tetrazolium; H_2DCFDA, 2′ 7′-Dichlorodihydrofluorescein diacetate; DHAR, Dehydroascorbate reductase; MDHAR, Monodehydroascorbate reductase; Rboh, Respiratory burst oxidase homologs; P5CS, Delta pyrroline-5-carboxylate synthase; PDH, Proline dehydrogenase

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plants, it is not only important to monitor the changes by subjecting plants to stress, but also critical to analyze the comparative responses in stress sensitive and tolerant cultivars [22]. Therefore, in the present study, ROS dynamics which is central to many abiotic stress related responses is evaluated in the salt sensitive (IR64) and salt tolerant, Luna Suvarna (LS) rice cultivar with the aim to obtain necessary inputs in understanding the acquisition of salt tolerance mechanisms in an important crop like rice. To the best of our knowledge, this is the first report where a comparative study on the role of ROS dynamics has been dissected in roots of two contrasting salt sensitive cultivars of rice.

Materials and Methods

Plant material

For the present investigation healthy seeds of salt sensitive and salt tolerant cultivars of rice were collected. The certified seeds of *Oryza sativa* (var. IR64) were procured from Punjab Agriculture University (PAU), Ludhiana, Punjab, India and LS (CR Dhan 403) were taken from the Central Rice Research Institute (CRRI), Cuttack, India.

Growth conditions

Rice seeds of cultivar IR64 and LS were washed with distilled water and tween-20 for 15 min. Surface sterilization of the seeds was performed using 70% ethanol for 1 min and were dipped in 0.4% sodium hypochlorite solution with a drop of Tween-20 for 20 min. The seeds were then washed thrice with autoclaved distilled water. Seeds were grown in sterile sand saturated with autoclaved double distilled water at 70–80% relative humidity (day/night), 25 °C (day/night) and 14 h photoperiod for 14 days in the greenhouse and were then harvested.

Morphological parameters

For phenotypic analysis, length of the roots was examined using a meter scale and observation for fresh weight of the roots was also made. Root number for each of the seedlings was noted. Roots were then placed in an oven at 70 °C till a constant weight was attained and then the dry weight (mg/seedling) was measured. The experiment was conducted thrice with three biological replicates using a representative lot of 10 samples (n = 10) each.

Histochemical determination of ROS

Roots of IR64 and LS were cut with a razor blade and dipped into 10 mM sodium citrate buffer (pH 6.0) containing 6 mM nitroblue tetrazolium (NBT) for 8 h under light at 25 °C for the detection of superoxide radicals (O^{2-}) [23]. As the solution entered through the excised roots, NBT reacted with O^{2-} radicals forming a dark blue insoluble compound, formazan. The roots were observed under stereomicroscope and were also photographed.

The production of hydrogen peroxide (H_2O_2) in IR64 and LS roots was visualized histochemically using 3, 3-diaminobenzidine (DAB) as a substrate [23]. Roots were excised using a razor blade from plants and were incubated in 1 mg ml^{-1} solution of DAB (pH 3.8) under dark at 25 °C for 6 h. After the reaction of DAB with H_2O_2 the deep brown polymerization product was clearly visible in roots under a stereomicroscope and photographed. For 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) assay, the excised roots of IR64 and LS were vacuum infiltrated for 5 min with its 10 μM concentration. The roots were cleaned with double distilled water and laser beam of excitation 488 nm was used to observe the roots under confocal microscope [24,25]. For histochemical detection of ROS 3 samples (n = 3) were taken and the experiment was conducted thrice with three biological replicates.

Estimation of aqueous H_2O_2 content

Ferrous oxidation-xylenol orange (FOX) assay was conducted to determine the aqueous H_2O_2 content in IR64 and LS roots of 14 days old plants [26]. Activated charcoal (0.1 g) prepared in 5 ml of 5% trichloroacetic acid was used to prepare the extract from 1 g of fresh roots. Whatmann filter No.1 was used to filter the homogenate and centrifugation was performed at 8000 rpm for 10 min. The supernatant was collected and used to estimate the aqueous H_2O_2 content. The composition of FOX reagent included 1 ml of reagent 'a' containing 25 mM ammonium ferrous sulfate prepared in 2.5 M sulfuric acid, 50 μl of reagent 'b' (0.25 M xylenol orange synthesized in HPLC-grade methanol), while reagent 'c' comprised 9.69 mg of butylated hydroxytoluene (BHT) dissolved in 90 ml of HPLC grade methanol. The volume of the mixture was raised to 100 ml by addition of distilled water. To measure H_2O_2 content, 0.2 ml of root extract was taken in a test tube and 1 ml of FOX reagent was added into it. The reaction was mixed thoroughly and was kept for 15 min at room temperature. The absorbance at 560 nm was recorded to estimate the concentration of H_2O_2. The H_2O_2 concentration was measured by plotting a standard curve taking different known concentrations of H_2O_2. The experiment was conducted thrice with three biological replicates using a representative lot of 3 samples (n = 3).

NADPH oxidase activity

Isolation of plasma membrane proteins

The proteins from the plasma membrane were extracted using 3 g of fresh root tissue [26]. The roots were thoroughly mixed with 10 ml of protein extraction buffer which contained 50 mM HEPES (pH 7.2), 3.6 mM L-cysteine, 0.25 M sucrose, 3 mM ethylene diamine tetraacetic acid (EDTA), 0.1 mM $MgCl_2$, 0.6% PVP, 1 mM dithiothreitol (DTT) and PIC (Protease Inhibitory Cocktail). Two layers of muslin cloth were used to filter the homogenate so obtained. The filtered solution was centrifuged at 10,000 g for 45 min at 4 °C. The supernatant was collected and ultracentrifuged at 1,80,000 rpm for 1 h at 4 °C. The pellet so obtained was resuspended in a chilled 10 mM Tris-HCl, while the supernatant was discarded. The concentration of the protein was measured using Bradford method taking bovine serum albumin (BSA) as a standard [27].

Spectrophotometric assay of NADPH oxidase activity

The NADPH oxidase activity was measured using Kaundal's method [26]. The mixture comprised of 50 mM Tris-HCl buffer (pH 7.5), 1 mM XTT (sodium, 3-[1-[phenylamino-carbonyl]-3, 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate), 1 mM NADPH and 20 μg of membrane protein. An extinction coefficient of 2.16×10^4 M^{-1} cm^{-1} was used to evaluate the rate of reduction of XTT by O^{2-} at 492 nm spectrophotometrically. NADPH oxidase activity was measured thrice with three biological replicates using a representative lot of 3 samples (n = 3).

Antioxidant enzyme assays

Isolation of protein

The 14-days old seedlings were harvested, their roots were separated and crushed in liquid nitrogen. The roots were homogenized in ice chilled 50 mM phosphate buffer (pH 7.8) containing 2 mM EDTA, 0.5% (v/v) triton X-100, 10% (w/v) PVP-40, 1 mM dithiothreitol (DTT) and 1 mM phenylmethane sulfonylfluoride (PMSF). The mixture was centrifuged at 12,000 rpm for 20 min at 4 °C, the supernatant was collected and used for determining protein concentration. Protein concentrations of the samples were determined using Bradford assay [27] taking bovine serum albumin (BSA) as a standard. The supernatant was also used for the antioxidant enzyme assays. For antioxidant enzyme assays and