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Differential fine-regulation of enzyme driven ROS detoxification network imparts salt tolerance in contrasting peanut genotypes

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ABSTRACT

The present study was aimed to identify the major ROS detoxification pathway in peanut under salinity stress. Pot experiment was conducted with six peanut genotypes (differing in salt-sensitivity) and four levels of salt stress. Higher level of salt stress led to severe plant mortality and reduction in membrane stability especially in sensitive genotypes. Higher ROS accumulation in sensitive genotypes (NRCG 357 and TMV 2) as compared to the tolerant ones (Somnath, TPG 41, CS 240) was confirmed by both spectrometry and *in situ* histo-chemical staining. Salinity stress changed the cellular antioxidant pool, where the levels of total ascorbate and proline increased in all the genotypes, but the total glutathione content showed significant reduction with more pronounced effect in sensitive genotypes. Major changes in POD and CAT activities was observed in response to salt stress, indicating POD as the major H_2O_2 detoxifying enzyme in tolerant genotypes. The POD activity was supplemented by CAT activity in sensitive genotypes, where there was relatively higher ROS load. The SOD showed minimal up-regulation under salt stress with undistinguishable difference between tolerant and sensitive genotypes, while APX and GR showed almost no induction, suggesting nominal association of these enzymes with overall salt tolerance in peanut.

1. Introduction

Salinity is one of the major abiotic stresses limiting plant growth and productivity as nearly 7% of the total territorial area and 20% of the irrigated arable land is affected by soil salinity globally (Parihar et al., 2015). Peanut (*Arachis hypogaea* L.), an important legume, preferred both as oilseed and confectionary purposes globally, has been reported to be moderately salt sensitive and shows restriction in growth and yield after crossing the threshold level of soil salinity (Singh et al., 2008). As peanut is cultivated mostly in marginal and resource poor soil, so developing salinity tolerant genotypes would definitely help to expand its area of cultivation in non-traditional saline soils. Although, considerable efforts were made in the past for developing salt-tolerant peanut cultivars, but very limited success was tasted mostly due to lack of understanding of key mechanisms of salt tolerance in this crop. As we know, salt tolerance is a complex trait consisting of various mechanisms; hence detailed understanding of each of the components is absolutely essential for developing salt tolerant genotypes (Flowers, 2004).

Even under normal environmental conditions plants tend to produce reactive oxygen species (ROS) essentially as byproducts of photosynthesis, respiration and photorespiration (Apel and Hirt, 2004; Mittler, 2002). Environmental stresses including salinity further aggravate the production of ROS, which causes severe oxidative damage to the plants growing in saline environment (Gupta and Huang, 2014). Salt stress can affect plant growth and metabolism due to both osmotic (dehydration) and ionic (Na^+ and Cl^- toxicity) effects (Flowers, 2004). Due to the lowering of water status, plants tend to close their stomata partially in order to prevent higher water loss through transpiration under salt stress, resulting in reduced CO_2 supply to leaves and unfavourable CO_2/O_2 ratio in chloroplasts (Remorini et al., 2009). Such deprivation of internal CO_2 concentration and increased rate of photorespiration induces the oxygenase activity of Rubisco resulting in toxic superoxide radical formation (Hsu and Kao, 2003) and higher H_2O_2 production in the leaf tissue (Hernandez et al., 2000). The most commonly occurring ROS in plants includes hydrogen peroxide

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; GR, glutathione reductase; GSSG, oxidized glutathione; GSH, reduced glutathione; GPX, glutathione peroxidase; MSI, membrane stability index; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; SOR, superoxide radical; TBARS, thiobarbituric acid reactive substances.

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(H_2O_2), superoxide anions (O^{-2}), hydroxyl radicals ($^{\bullet}OH$) and singlet oxygen ($^1O_2$), which interferes with normal cellular metabolism by oxidizing proteins, lipids and DNA and other cellular macromolecules under salt stress (Ahmad et al., 2016).

To counterbalance these, plants have well-defined network of ROS detoxification system having an array of enzymes and low molecular weight organic metabolites either working independently or in combination. Superoxide in chloroplast is dismutated by superoxide dismutase (SOD) into H_2O_2, which is decomposed by a variety of peroxidases such as ascorbate peroxidase (APX), glutathione peroxidase (GPX) and phenol peroxidase (Asada, 1999) into water by using various reducing agents. On the other hand, H_2O_2 produced in the peroxisome as a result of photorespiration is decomposed primarily by catalase (CAT) (Dat et al., 2000). Both peroxidases (APX and POD) and CAT decompose H_2O_2, but in general peroxidases have much higher affinity to H_2O_2 than CAT (Mittler, 2002; Abogadallah, 2010), indicating the fact that peroxidases could be associated with ROS scavenging at lower cellular H_2O_2 concentration, while CAT removes H_2O_2 at much higher concentration. Under salt stress, higher ROS accumulation could cause severe cell damages, however, lower levels of ROS particularly H_2O_2 is suggested to work as secondary signals that activate the stress tolerance mechanisms (Desikan et al., 2001).

Different plant species respond differentially by modulating their antioxidative machinery. For instance, salinity stress in cotton, increased the activities of SOD, peroxidase (POD), glutathione reductase (GR) but decreased the activities of CAT and APX (Gossett et al., 1994); where as in rice, strong up-regulation of SOD, APX and GPX activities were observed in response to salt stress with little change in GR and decline in CAT activities (Lee et al., 2001). Higher induction of SOD, APX and POD was observed in tomato with increasing salinity levels (Mittova et al., 2004). Abogadallah et al. (2010) reported crucial role of SOD, POD, APX and GR in salt tolerance mechanism of C_4 barnyard grass, while CAT activity although induced under salt stress, but had negligible association with salt tolerance. Under salt stress, the antioxidant system of the halophyte, *Limonium sinense*, is activated by up-regulation of CAT, SOD and POD and effectively scavenges reactive oxygen species in order to maintain growth under high external Na^+ concentration (Zhang et al., 2014).

From the existing evidences, it seems that plants do differ in executing the antioxidant defense strategy at the mechanistic level under different abiotic stresses. Not only that, even under salt stress every species does not necessarily require to up-regulate the full set of antioxidant enzymes for achieving salt tolerance. Rather one or few specific component of the whole defense system plays crucial role, which may well vary from one crop to another. Thus, it is very much pertinent to identify crop specific components of ROS detoxification pathway, if any, that are involved in imparting salt tolerance in peanut. To the best of our knowledge, specific information on salt tolerance mechanism of peanut in terms of fine regulation of ROS detoxification pathway has not been well worked out till date. Such intricate knowledge will definitely help in understanding salinity tolerance mechanism in peanut and intern would benefit in developing salt tolerant genotypes. Hence, the present study was aimed to know (i) how the specific component (s) of antioxidant defense system imparts salinity tolerance in peanut through ROS detoxification? and (ii) is the role of specific component(s) differing between sensitive and tolerant peanut genotypes?

2. Materials and methods

2.1. Experimental condition and plant material

For the present study, a pot experiment was conducted in summer (dry season) 2015 at ICAR-Directorate of Groundnut Research, Junagadh, India. Based on our initial field/lab screening (data not shown), six genotypes *viz.* 'NRCG 357', 'CS 240', 'TMV 2', 'Girnar 1', 'TPG 41' and 'Somnath' were selected in the present study, having enough diversity amongst themselves to accommodate the maximum representation of cultivated peanut genotypes (see Supplementary Table S1 for individual genotype character).

The plants were subjected to four different levels of salt treatment (0, 25, 50 & 100 mM NaCl) through irrigation water starting from 2 weeks after sowing (after early establishment of the seedlings). The plants were watered every alternate day to maintain soil moisture level at field capacity. The whole experiment was conducted in two-factor completely randomized design with 10 replicates (in the form of pots) per treatment combination, where 5 plants were kept per pot to maintain an uniform plant stand throughout the experimental period. Prolonged salt treatment for six weeks resulted in significant development of soil salinity leading to severe plant mortality in sensitive genotypes under 100 mM NaCl treatment. As we lost almost all the plants under 100 mM NaCl treatment, the final experiment was restricted to only three levels of salt stress (0, 25 & 50 mM).

All the studied parameters under present experiment were investigated at 60 days after sowing (about 6 weeks of salt treatment). For this, uniform samples were collected from third fully matured leaf on the main axis randomly for all the physiological and biochemical estimations and total RNA extraction for gene expression studies.

2.2. Estimation of soil parameters and membrane stability index

To study the level of stress developed during the experimental period, soil samples were collected at 60 days after sowing (DAS), oven dried and finely ground. For estimation of Na^+ content, the extraction was done in neutral 1 N ammonium acetate solution (Hanway and Heidel, 1952) and the pH and electrical conductivity of the soil samples were measured using portable Hanna-make pH-EC meter in saturation extract with distilled water at a ratio of 1:2.5.

Membrane stability index (MSI) was estimated by measuring the electrical conductivity of leaf samples (100 mg) in 10 mL double distilled water by heating at 40 °C for 30 min and 100 °C for 10 min as described by Chakraborty et al. (2012).

2.3. Determination of superoxide radical, H_2O_2 content and lipid peroxidation

To determine the level of oxidative stress, different key components like superoxide and hydrogen peroxide content and lipid peroxidation level was measured. Superoxide radical content was estimated by its capacity to reduce nitroblue tetrazolium chloride (NBT) and the absorption of end product was measured at 540 nm (Chaitanya and Naithani, 1994). Hydrogen peroxide was estimated by forming titanium-hydro peroxide complex (Rao et al., 1997). One gram leaf was ground with liquid nitrogen and the fine powder was mixed with 10 mL cooled acetone in a cold room. The filtered mixture was added with 4 mL titanium reagent and 5 mL ammonium solution to precipitate the titanium-hydro peroxide complex which was further dissolved 10 mL of 2 M H_2SO_4 and absorbance was recorded at 415 nm against blank.

The level of lipid peroxidation was measured in terms of thiobarbituric acid reactive substances (TBARS) content (Heath and Packer, 1968). Leaf sample (0.5 g) was homogenized in 10 mL 0.1% trichloro-acetic acid (TCA) and centrifuged at 15000*g* for 15 min. One mL of supernatant was mixed with 4 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA and heated at 95 °C for 30 min followed by cooling in ice bath. After centrifugation at 10,000*g* for