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Biochemical And Molecular Defence Mechanism In Pigeon Pea Genotypes Against Salinity Stress

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Abstract

Salinity being a critical factor to sustainable pigeon pea production addresses limitations to meet the demands of resource-poor people where it is grown. Biochemical studies-antioxidant potential (AO), lipid peroxidation (LP) and total phenolic contents (TPC) were carried out in root and shoot tissues of screened 6 genotypes. Based on morphological and biochemical expressions, ICP7 and ICP1071 were found to be most salt-tolerant and salt-susceptible pigeon pea genotypes, respectively. Partial CDS for pigeon pea stress-responsive genes viz., hybrid proline-rich protein (*CcHyPRP*,218 bp), cold, drought salt regulatory protein (*CcCDR*,207 bp) and cyclophilin protein (*CcCYP*,323 bp) conferring abiotic tolerance were cloned in to *E.* coli DH5α from ICP7 genotype. The semi-quantitative (RT-PCR) expression analysis of these isolated genes were conducted in root and shoot tissues of contrasting pigeon pea genotype and found to be expressed in both root and shoot tissues. These findings shall go a long way not only in understanding the important molecular and biochemical mechanisms underlying the salinity tolerance of the pigeon but also can be utilized in providing important leads to the breeders to evolve improved varieties of pigeon pea with higher salinity tolerance levels.

Key words : Salinity,lipid peroxidation (LP) and total phenolic contents (TPC), Hybrid proline-rich protein,RT-PCR, cloning, BLAST.

Introduction

Food and nutritional insecurities are perhaps the most pressing and intractable social issues now being faced by Indian agriculture and abiotic stresses are one of the major contributing factors for this embarrassing situation. Pigeon pea is well known for food and nutritional security of the poor people, as it is a rich source of proteins (28%), minerals and vitamins. More than 85% of the world's pigeon pea is produced and consumed in India where it is a key crop for food and nutritional security of the people. Pigeon pea being suitable crop as compare to other pulses for sustainable agriculture production (Saxena, 2008) shares about 15% of the total legume production in India. Pigeon pea remains one of the most drought-tolerant legumes (Valenzuela and Smith, 2002). Pigeon pea is highly sensitive to salt stress and salinity poses a major constraint to its production

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(Chauhan, 1987). In India, an area of nearly 9.38 million ha is occupied by salt-affected soils.Salt stress is posing a serious threat to pigeon peacrop producing areas and isencouragingresearch community to work on it. Our studies included morphological variations, estimation of total phenolic content (TPC), lipid peroxidation (LP), antioxidant activity (AO), cloning and characterization of abiotic stress responsive-CcHyPRP, CcCYP and CcCDR genes after 7 days of 250 Mm salt stress imposition under controlled conditions.

Material and methods

I.Screening of pigeon pea genotypes

Seeds were surface sterilized with 0.1 % mercuric chloride ($HgCl_2$) solution. Salt stress of 250mM NaCl for 7 days of stress was found to be optimum concentration and duration for further studies. Salt stress (250 mMNaCl) in half strength Hoagland solution was imposed on two weeks old plants. Three biological samples of shoot and root in triplicate were harvested on 7^{th} day after salt treatment (DAT) for biochemical and molecular observations.

II.Biochemical studies

A.Determination of total phenolic content (TPC)

Total Phenolic Content (TPC) was determined by adding 0.5 ml aqueous extract to 2.5 ml of 10% Folin-Ciocalteu reagent (v/v) and 2 ml of 7.5% sodium carbonate. The reaction mixture was incubated at 45°C for 40 min and the absorbance was measured at 765 nm in the spectrophotometer. Gallic acid was used as a standard phenol (Singleton *et al.,* 1999). The mean of three readings was used and the TPC was expressed as milligrams of gallic acid equivalents/g extract.

B. Determination of lipid peroxidation (LP)

Lipid peroxidation (LP) was measured as MDA in the shoot and root, according to Cakmak and Horst (1991) method. Tissues (1.0g) were ground properly in 20 ml of 0.1% TCA solution and centrifuged for 10 min at 12000 g. One ml of the supernatant was reacted with 4 ml of 20% TCA solution comprising 0.6% thiobarbituric acid and then it was incubated for 30 min at 95°C in a water- bath and then immediately cooled on ice. The absorbance of the supernatant was read at 532 and 600 nm respectively on a UV-VIS Spectrophotometer.

C.Determination of total antioxidant activity (AO)

The antioxidant activities (AO) of the tissue extracts were measured using the stable DPPH radical according to the method of Hatano*et al*., (1988). The alcoholic solution of DPPH radical (0.5 ml, 0.2mM) was added to the 100 µl of sample solution and the mixture was shaken vigorously to left stand for 30 min in the dark. The absorbance was measured at 517 nm.

III.Molecular studies

A.RNA isolation and cDNA Preparation

Total RNA was isolated from control and treated root and shoot samples of these two contrasting pigeon pea genotypes according to protocol given in a manual of Plant RNA isolation kit (Nucleospin). Complimentary DNA (cDNA) was synthesized by a standard protocol of Revert aid kit (Fermentas).

B. Semi-quantitative amplification (RT-PCR)

The cDNA synthesized were used for the semi-quantitative RT-PCR analysis using gene

specific primers and pigeon pea tubulin were used as a reference gene.A 25µl reaction volume was prepared for cyclic amplification against *CcHyPRP, CcCDR*and *CcCYP*gene specific primers. Amplified product were run on 1% agarose gel and then desiredamplicons (DNA band) were excised from the agarose gel and eluted as per manufacturer's protocol of Qiagen gel extraction kit (QIAX II).

C. Molecular cloning of stress responsive genes

Gel eluted PCR amplified product (*CcHyPRP*, *CcCDR, CcCYP*) were ligated with pGEMT easy cloning vector (Promega) using DNA ligase enzyme (Fermentas). Then vector containing target gene introduce in to competent bacterial cell using heat shock method.Plasmid DNA was isolated from white transformed colonies by using QIAprep spin miniprep kit (Qiagen) as per manufacturer's protocol. Digested products by FastDigest*Eco*RI enzyme showed release of DNA insert of our targeted genes (*CcHyPRP*, *CcCDR* and *CcCYP*) and sequenced them commercially using universal primers (M13) (Fig.3). Sequences were characterized by BLAST search homology tool.

Result and Discussion

Plant adaptation to salinity is a complex mechanism, involving far more changes than just an attenuated growth. Adverse effects of salinity on plant growth may be due to ion cytotoxicity and osmotic stress (Chinnusamy*et al*., 2005). It implies, at the cellular level, regulation of gene expression, such as of those encoding transporter proteins, accumulation of compatible solutes and increased levels of antioxidant pathways (Bartels and Sunkar, 2005, Chaves *et al*., 2009). Besides imposing toxic effects on the integrity of cellular membranes (Wolf *et al*., 2012), salt stress also generates reactive oxygen species-ROS (Skopelitis*et al*., 2006). As a response plants trigger the production of several stress related proteins and compatible osmolytes (Zhu *et al*., 1997). The increase in lipid peroxidation (LP) level in salt-stressed tissues, may be due to oxidative damage of membrane.The most salt-susceptible genotype (ICP1071) showed ~20% increase in their LP levels in the treated tissues of both the root and shoot regions, while the most salt-tolerant genotype (ICP7) showed lowest LP levels in both control and treated samples. LP levels were observed ~2 folds more in the shoot regions than the roots in all the contrasting genotypes (Fig 1). The lower LP level in salt-stressed tolerant genotype than control is important in terms of providing salt tolerance to the plant and confirming their tolerance to salinity stress (Ruiz *et al*., 2005; Koca*et al*., 2007; Jaleel*et al*., 2007). The possibility of LP/MDA the involvement in combating salt tolerance (Khan and Panda, 2008) has been reported and our findings are in accordance to these reports. The reduction in growth might be due to toxicity of the ions or low osmotic potential as well as a decrease in wall extensibility (Grieve *et al*., 2001; Haplerin and Lynch, 2003). Tolerance to salt stress in higher plants showed a direct linkage between the level of antioxidant system and phenolic substrates (Jahnke and white, 2003; Athar*et al*., 2008). Most salt-tolerant genotype- ICP7 showed ~ 15% higher TPC due to salt-stress imposition in

both root and shoot regions. In the case of most salt susceptible genotypes (ICP1071) root regions showed ~30% reduction in TPC content (Fig. 2). To overcome the effects of salinity induced oxidative stress, plants make use of a complex antioxidant (AO) system in strong correlation with accumulation of total phenolic content (TPC) as osmolyte and LP as well. A positive correlation between TPCs and antioxidant (AO) activity (r = 0.98) indicated (Rao*et al*., 2013) that AO activity is highly contributed by phenols (Trust *et al*., 2005. Our results also agree that salt-tolerant (ICP6815, ICP7, ICP8860) pigeon pea genotype contain low level of MDA accumulation as compared to salt-susceptible (ICP1071, ICP14155, ICP15185) genotypes (Fig. 1). Isolation and characterization of pigeon pea CcHyPRP, CcCYP and CcCDR genes, and their over-expression in Arabidopsis had been reported to impart multiple abiotic stress tolerance in the plants (Sekhar*et al*., 2010; Priyanka*et al*., 2010; Tamirisa*et al*., 2014). CYP genes from maize, bean, Solanumcommersonii and S. tuberosum were also found to confer multiple stress tolerance during drought, salinity and extreme temperatures (Godoy *et al*., 2000).

These results found to be in accordance to above research information and indicated that, they are helping roots by reducing the uptake of salt from the soil, while in the shoots they will be assisting in the efflux/removal of salt ions through vacuoles. However, there is a need to further analyze plant salt-stress responses at molecular level due to complexity of events associated with the sensing of salt stress and the activation of specific pathways. Thus, identified and validated candidate genes will enable scientific community to use them as molecular markers in the production of improved crops through allele mining of superior alleles present in germplasm collection.

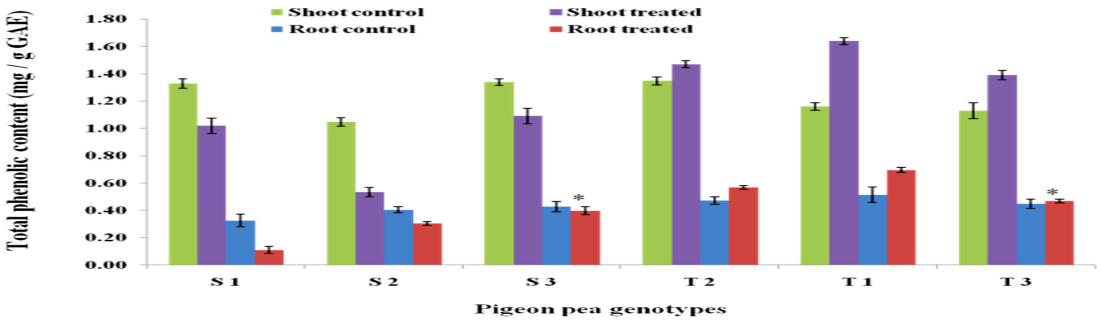

Figure 1- Comparative analysis of differential variation in total phenolic content (TPC) in shoot and root region of contrasting pigeon pea genotypes- Salt susceptible (S_1, S_2, S_3) and Salt tolerant (T_1, T_2, T_3) after 7 days of 250 mM salt stress. * indicates non-significant differences ($P > 0.05$) and rest are showing significant differences in comparison with control at $P < 0.05$.

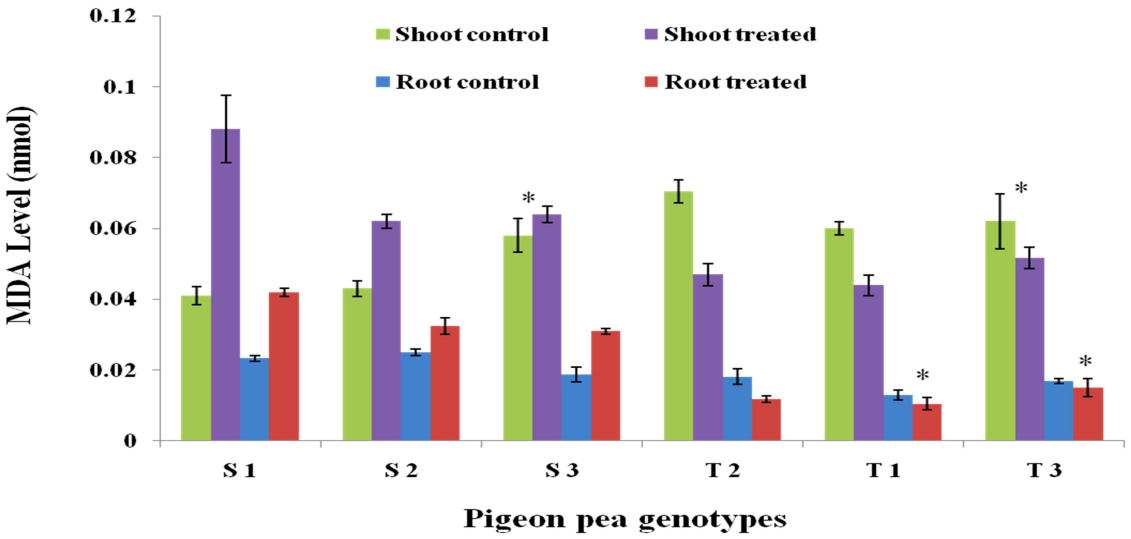

Figure 2- Comparative analysis of differential variation in Lipid Peroxidation (LP) in shoot and root region of contrasting pigeon pea genotypes- Salt susceptible (S_1, S_2, S_3) and Salt tolerant (T_1, T_2, T_3) after 7 days of 250 mM salt stress. * indicates non-significant differences ($P > 0.05$) and rest are showing significant differences in comparison with control at $P < 0.05$.

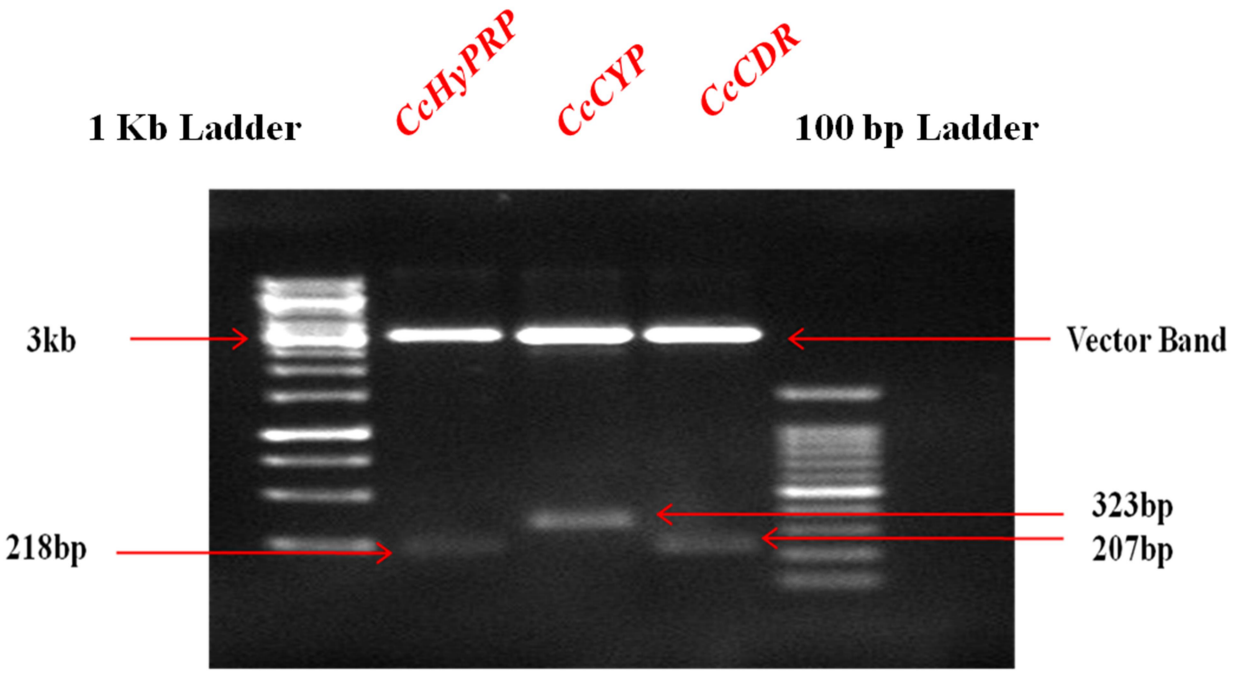

Figure3- Restriction digestion of isolated plasmid DNA with *Eco*RI.

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