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Effect of seed priming with spermine/spermidine on transcriptional regulation of stress-responsive genes in salt-stressed seedlings of an aromatic rice cultivar

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ABSTRACT

Gobindobhog, the indigenous aromatic rice cultivar from West Bengal, India, is well-known for its magnificent flavor and intense pleasant aroma. Earlier observation established the highly salt-susceptible nature of this cultivar and the potential role of exogenous spermidine (Spd) and spermine (Spm) in the amelioration of salt stress. In the present study, the effect of seed priming with Spd and Spm on salt-stressed Gobindobhog seedlings has been studied with respect to the regulation of expression of genes involved in multiple metabolic pathways controlling salt tolerance. The expression profiling of key genes encoding non-enzymatic and enzymatic antioxidants (*ANS*, *CAT*, *SOD*, *APX*, *GR*), osmolyte (*P5CS*, *PDH*, *BADH1*), ABA biosynthetic enzyme (*NCED3*), transcription factors (*TRAB-1*, *WRKY-71*), *LEA* (*Osem*), ion transporter (*NHX1*), PA metabolic enzymes (*SAMDC*, *SPDS*, *SPMS*, *DAO*, *PAO*), enzyme for RuBisCo (*RbcS*), and content of endogenous PAs, responsible for stress tolerance were studied both in the shoots and roots of Gobindobhog seedlings. Both Spm and Spd priming enhanced the expression of antioxidant genes in shoots and roots with respect to stressed seedlings. However, the enhanced expression was more pronounced with Spm priming. The genes for osmolyte and ABA biosynthesis were significantly enhanced by both the PAs, together with increased expression of ABA-inducible transcription factors and *LEA* gene in shoots and roots of stressed seedlings. However, better expression was noted with Spm. Priming also altered the expression of ion transporter gene *NHX1* during stress. Salt stress did not increase the (Spm + Spd)/Put ratio; however, Spm pre-treatment slightly altered this ratio and also led to increased expression of PA biosynthetic genes and decreased expression of PA catabolic genes like *DAO* and *PAO* in shoots. Seed pre-treatment with Spd however decreased the (Spm + Spd)/Put ratio, as well as leading to higher expression of *PAO* in shoots, thereby indicating that the overall levels of Spm and Spd did not increase and that the biosynthesis and degradation processes were mutually regulated. However, the expression of *DAO* and *PAO* was decreased in roots. The metabolic readjustment in the seedlings brought about by the coordinated expression of a diverse array of genes during pre-treatment could significantly restore the down regulated *RbcS* expression noted under stress. Our results highlight the potentiality of priming technique in improving the overall performance of the aromatic cultivar during salt stress by controlling stress-tolerant determinants of multiple metabolic pathways.

1. Introduction

Fragrance and aroma adds value to the quality of rice, making it more demanding worldwide. However, one of the premier causes of reduced yield and poor agronomic trait of aromatic rice is their extreme susceptibility to environmental stress. Gobindobhog is a popular, indigenous aromatic rice variety, cultivated in several districts of West Bengal in India, and is highly prized for its magnificent aroma and soft white texture. Previous reports from our group have shown this variety to be highly affected by salt stress at the seedling stage (Roychoudhury et al., 2008a). The salt-sensitive nature of Gobindobhog was reflected by its high chlorophyll damage, increased concentration of leaf Na^+ ion, elevation of lipoxygenase activity and increased accumulation of malondialdehyde and H_2O_2 content during salt stress (Roychoudhury et al., 2008a).

Salinity causes hyperosmotic stress by reducing soil water potential and leads to the generation of Reactive Oxygen Species (ROS), such as O_2^-, H_2O_2 and hydroxyl radical. Additionally, it causes hyperionic stress by accumulating excess amount of intracellular Na^+ ion, resulting in inhibition of overall growth and metabolism. The ROS can cause

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lipid peroxidation and consequently membrane injury, protein degradation, enzyme inactivation and DNA damage (You and Chan, 2015; AbdElgawad et al., 2016; Hossain and Dietz, 2016). However, various defense mechanisms are adopted by the plants to ameliorate salt damages, including triggering of the expression of genes encoding antioxidative enzymes, viz., catalase (*CAT*), superoxide dismutase (*SOD*), ascorbate peroxidase (*APX*), glutathione reductase (*GR*) or non-enzymatic antioxidants like ascorbate, glutathione, anthocyanin (*anthocyanin synthase*, *ANS*) etc. (Gill and Tuteja, 2010; Das and Roychoudhury, 2014), genes responsible for biosynthesis of osmolytes like glycine betaine (*betaine aldehyde dehydrogenase 1* or *BADH1*) and proline (Δ^1-*pyrroline-5-carboxylate synthetase* or *P5CS*), ion transporters like Na^+/H^+ antiporter (*NHX1*) (Fukuda et al., 2004), abscisic acid (ABA) biosynthesis (*NCED3*) and various groups of ABA-inducible transcription factors such as basic leucine zipper (bZIP, such as *TRAB-1*, *OSBZ8*, *RITA-1*); WRKY group (*OsWRKY-71*, *OsWRKY-54*, *AtWRKY-8*), along with *late embryogenesis abundant* (*LEA*) genes like *Osem* and *Rab16*. Salinity stress also induces the level of polyamines (PAs) by up regulating the genes encoding PA-biosynthetic enzymes (Liu et al., 2015).

PAs like spermidine (Spd), spermine (Spm) and their diamine precursor, putrescine (Put) are polycationic molecules that can regulate *in vivo* cellular processes including plant protection against abiotic stress (Shi and Chan, 2014). Transgenic plants overexpressing PA biosynthetic genes, viz., *arginine decarboxylase* (*ADC*), *ornithine decarboxylase* (*ODC*), *S-adenosylmethionine decarboxylase* (*SAMDC*), *spermidine synthase* (*SPDS*) and *spermine synthase* (*SPMS*) from different plant sources showed increased accumulation of endogenous PA levels which conferred tolerance to various abiotic stresses including salinity (Moschou et al., 2012; Roychoudhury and Das, 2014). Our previous study has shown that exogenous application of Spm and Spd modulated the endogenous PA content during salt stress and eventually enhanced tolerance of rice to salt stress at the seedling stage (Roychoudhury et al., 2011). Abscisic acid (ABA), the universal stress hormone is also connected with PA regulation, since the exogenous application of ABA up regulates the expression of *ADC2*, *SPDS1* and *SPMS*, indicating the involvement of ABA in PA metabolism at transcriptional level (Alcázar et al., 2010). The effect of PAs on expression of several groups of genes could be explained due to their ability to bind to nucleic acids and proteins so that these molecules can stabilize and remodel the chromatin structure (Basu et al., 1992; Childs et al., 2003). PAs have also shown to modulate the rate of transcription by mediating changes in DNA structure (Kumar et al., 2009; Miller-Fleming et al., 2015). Moreover, studies have shown that PAs promote the binding between proteins and DNA, thus modulating gene expression (Panagiotidis et al., 1995).

Although the effects of exogenous application of Spm or Spd in the amelioration of salt stress- induced damages in Gobindobhog has been shown earlier (Roychoudhury et al., 2011), the effect of seed priming with Spm/Spd on the transcript level of various groups of genes encoding enzymatic and non-enzymatic antioxidants, osmolytes, ion transporter, ABA biosynthetic gene, transcription factors, LEA, and endogenous PA level in any aromatic rice cultivar has not been shown earlier. Hence, the objective of this study was to understand the effect of seed priming with Spm or Spd on PA metabolism and the molecular responses involved in salt tolerance in the aromatic cultivar Gobindobhog. Transcriptome analysis of genes for antioxidants (*CAT*, *SOD*, *APX*, *GR*, *ANS*), PA biosynthesis (*SAMDC*, *SPDS*, *SPMS*) and catabolism (*DAO* and *PAO*), osmolytes (*P5CS*, *BADH1* and *PDH*), ion transporter (*NHX1*), ABA synthesis (*NCED3*) and ABA-inducible transcription factors and LEA (*TRAB-1*, *WRKY-71*, *Osem*) and endogenous contents of PAs (Put, Spd and Spm) were studied. Our study highlights the effect of seed priming with Spm/Spd upon the regulation of expression of genes responsible for stress tolerance in Gobindobhog, thereby showing the possible cross-talk between higher PAs and the genes involved in different metabolic pathways.

2. Materials and methods

2.1. Plant material and growth conditions

The seeds of Gobindobhog (GB) were obtained from Chinsurah Rice Research Station, Hooghly, West Bengal, India. The seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ solution for 10 min and then washed extensively with sterilized distilled water to remove the traces of the disinfectant. The sterilized seeds were primed with 2.5 mM of spermine (Spm) or 5 mM of spermidine (Spd) separately at 15 °C in darkness for 8 h. The concentration of the priming agents was selected based on earlier standardization by our group (Paul and Roychoudhury, 2016; Paul et al., 2017) as well as by Iqbal et al. (2006). The seeds were dried back to their original moisture content at room temperature (25 °C). The unprimed (water imbibed) dried seeds were used as control. After priming, fifty seeds of each treatment were placed on two layers of filter paper in Petri dish and supplemented with 75 mM NaCl for stress treatment, while distilled water was used as control (untreated). Solutions were renewed every two days. Preliminary experiments were conducted to standardize the concentration of NaCl to be employed for stress imposition to rice seedlings. NaCl concentration beyond 75 mM was critical for seedling growth and yielded only insufficient plant biomass required for analyses. The seeds were incubated in plant growth chamber (NIPPON, LHP-100-RDS, Tokyo, Japan) at 25 °C under alternating cycles of 16 h illumination with a light intensity of 700 μmol photons $m^{-2} s^{-1}$ and 8 h darkness with 50% relative humidity for 10 days. The treatment sets were (i) control seeds without salt stress (− Spm/− Spd − NaCl), (ii) control seeds with salt stress (− Spm/− Spd + NaCl), (iii) spermine (2.5 mM)/spermidine (5 mM)-primed seeds without salt stress (Spm/Spd – NaCl), (iv) spermine (2.5 mM)/spermidine (5 mM)-primed seeds with salt stress (Spm/Spd + NaCl). Each treatment set was maintained in three replicates. The shoots and roots were harvested after 10 days of stress, immediately frozen in liquid N_2 and stored at − 80 °C until the initiation of experiments. The seedlings from all the experimental sets were at the same developmental stages, so that the monitored gene expression changes were due to salt stress and not due to differences in plant development stages.

2.2. RNA extraction and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

The shoot and root tissues (100 mg) from each of the experimental sets were used for RNA isolation. Total RNA was isolated using RNAiso plus (Takara, Japan) following the manufacturer's instructions and the RNA concentration and purity was checked spectrophotometrically. The primers for *CAT*, *GR*, *SOD*, *APX*, *ANS*, *BADH1*, *P5CS*, *PDH*, *SAMDC*, *SPDS*, *SPMS*, *DAO*, *PAO*, *NHX1*, *NCED3*, *TRAB-1*, *WRKY-71*, *Osem*, *Rbcs* and *actin* genes were designed using online NCBI Primer-blast (http://www.ncbi.nlm.gov/tools/primer-blast/index) software. The accession number and the sequence of primer set of each of the genes (including internal control) have been shown in Table 1. Total RNA was treated with DNase I (Thermo Scientific) to remove DNA contamination. About 5 μg of total RNA was reverse-transcribed using Maxima First Strand cDNA synthesis kit (Thermo Scientific). About 100 ng of cDNA was used as a template for semi-quantitative RT-PCR using gene-specific primers, with *actin* as internal standard. Following densitometric scanning of band intensity of each transcript, the derived value for gene expression level at respective treatment was normalized by dividing the value with that of the internal standard. Three biological replicates were used for expression study. The average data from semi-quantitative RT-PCR analysis was imported into TM4 microarray software suite for heat map analysis (Saeed et al., 2003).