

# Spermidine-mediated in vitro phosphorylation of transcriptional regulator OSBZ8 by SNF1-type serine/threonine protein kinase SAPK4 homolog in indica rice

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**Abstract** Plants respond to abiotic stresses such as salinity, extreme temperature and drought by the activation of complex intracellular signaling cascades that regulate acclimatory biochemical and physiological changes. Protein kinases are major signal transduction factors that play a central role in mediating acclimation to environmental changes in eukaryotic organisms. It is well known that changes in abiotic conditions such as the concentration of ions, temperature and humidity lead to modulation of polyamine contents in plants. However, little is known about the relevant part these polyamines play in abiotic stress responses. Here, we address a specific role of spermidine during high salt stress by studying its interaction with OSPDK, a sucrose nonfermenting 1-related protein

kinase2 (SnRK2)-type serine/threonine protein kinase SAPK4 homolog in indica rice. In this report, we demonstrate that spermidine mediates in vitro phosphorylation of OSBZ8, a bZIP class of ABRE-binding transcription factor, by OSPDK. Our results give a first-hand indication of the pivotal role played by polyamines in abiotic stress cell signaling in plants.

**Keywords** Abiotic stress · ABRE · EMSA · Polyamine · Serine/threonine kinase · Spermidine · OSBZ8

## Abbreviations

ABA	Abscisic acid
ABRE	Abscisic acid responsive element
bZIP	Basic leucine zipper
EMSA	Electrophoretic mobility shift assay
GST	Glutathione S-transferase
MBP	Myelin basic protein
PA	Polyamine
Put	Putrescine
SNF-1	Sucrose non-fermenting protein 1
SnRK2	SNF1-related protein kinase 2
Spd	Spermidine
Spm	Spermine

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## Introduction

Environmental stresses like drought, heat, cold and salinity severely affect plant growth and productivity worldwide. It has been estimated that two-thirds of the yield potential of major crops are routinely lost due to the unfavorable environmental factors (Alcázar et al. 2011; Gill and Tuteja 2010; Quinet et al. 2010). On the other hand, the world

population is estimated to reach about 10 billion by 2050, which will witness serious food shortages. Therefore, staple crops like rice with enhanced vigor and high tolerance to various environmental factors should be developed to feed the increasing world population (Vinocur and Altman 2005). Maintaining crop yields under adverse environmental stresses is probably the major challenge facing modern agriculture where polyamines (PAs) can play important role. PAs, including the diamine putrescine ( $\text{Put}^{2+}$ ), triamine spermidine ( $\text{Spd}^{3+}$ ) and tetramine spermine ( $\text{Spm}^{4+}$ ), are ubiquitous, low molecular weight, straight chain aliphatic amines, involved in various biochemical and physiological processes related to the regulation of plant growth and development. Because of their polycationic nature at physiological pH, PAs are able to interact with nucleic acids, proteins, membrane phospholipids and cell wall constituents, thereby stabilizing these molecules (Gill and Tuteja 2010; Liu et al. 2007; Alcazar et al. 2006; Roy et al. 2005). During the last few years, genetic, transcriptomic and metabolomic approaches have unraveled key functions of different polyamines in the regulation of abiotic stress tolerance. In recent years, much attention has also been devoted to the involvement of PAs as second messengers in response to different environmental stresses such as osmotic stress, drought, heat, chilling, high light intensity, mineral nutrient deficiency, heavy metals, pH variation, UV irradiation and exposure to pollutants (Hussain et al. 2011; Roychoudhury et al. 2011; Jiménez-Bremont et al. 2007; Liu et al. 2007). Nevertheless, the precise molecular mechanism(s) by which PAs control plant responses to stress stimuli are largely unknown. Recent studies indicate that PAs may act as cellular signals in intricate cross talk with hormonal pathways, including abscisic acid (ABA) regulation of abiotic stress responses (Gupta et al. 2012; Alcázar et al. 2010).

Despite the rapidly growing body of evidence that the concentration of PAs change during the course of such diverse phenomena as development, cell division, stress response and senescence, there are few, if any, studies showing whether these fluctuations are responses or determinants of the associated physiological events. There is some information about the covalent interactions between PAs and proteins (Serafini-Fracassini et al. 1995); however, little is known on the noncovalent binding of PAs to membrane proteins, although this may represent one of the first steps of their action at the cellular level. The interaction between PAs and membranes is suggested to be an intermediate in important cellular events such as membrane fusion (Janicka-Russak et al. 2010; Roy et al. 2005; Schuber 1989) and transmission of receptor-mediated signals (Koenig et al. 1983). The distance between the cellular outer membrane and the nucleus seems to be tiny, only 20  $\mu\text{m}$ . Yet that minute distance encompasses a major

mystery: how do the cells of higher organisms respond to the diverse array of signals that they receive from the environment? The answer is crucial to understanding such long-standing questions in cell biology. It has been well established that growth factors (mitogens) bind to specific receptors located on the cellular membrane. The mitogen-receptor complexes then trigger a cascade of molecular events. The activation of protein kinases is considered to be the next obvious step in cellular signal transduction phenomenon. PAs appear to be involved in signaling pathways that regulate synthesis of transcription factors or modulate their binding activity via phosphorylation (Igarashi and Kashiwagi 2000). That the PAs can differentially modulate the transcription of growth-associated genes has been demonstrated by Celano et al. (1989). It is well known that many transcription factors are modulated by their phosphorylation status. One way that changes in PA levels might alter gene expression is by stimulating phosphorylation of regulatory proteins. We have recently reported the phosphorylation of NaCl induced 42-kDa protein in rice by Spd and ABA (Gupta et al. 2012). Two types of PA-sensitive protein kinases have been characterized in the last few years. The best known in molecular terms is the widespread casein kinase G (also termed casein kinase II), which represents a multifunctional protein kinase, at present classified as a messenger-independent activity. The other is a PA-dependent nuclear ornithine decarboxylase kinase characterized in *Physarum polycephalum* and several mammalian tissues (Kuehn et al. 1979). Both these protein kinases are activated by PAs in vitro at concentrations compatible with a physiological role, by a mechanism, which most likely also involves an effect through the protein substrate conformation modulation. A novel class of protein kinase activity may thus be defined as PA-mediated phosphorylation system, for which PAs may function as intracellular messengers (Cochet and Chambaz 1983; Datta et al. 1987).

Since there is very little available information on the actual intracellular target(s) that is recognized by PA or their biological functions, the main objective of our study was to explore the possibility of interaction of Spd with a salt and ABA-inducible SNF-1 group of kinase in rice. Kobayashi et al. (2004) have identified and analyzed ten SnRK2 protein kinases from rice, which were activated by hyperosmotic stress and three of them also by ABA. The activation was found to be regulated via phosphorylation. Their results clearly suggest that this family of protein kinases has acquired distinct regulatory properties, including ABA responsiveness by modifying the C-terminal as well as the kinase domain. Our earlier report has shown a 42-kDa  $\text{Ca}^{2+}$ -independent SnRK2 from roots of 3- and 12-day-old salt-sensitive (M-1-48) and salt-tolerant (Nonabokra) indica rice cultivars to be activated by NaCl/

ABA/spermidine (Gupta et al. 2012). To our knowledge, there has been no previous report on the differential activation of an SnRK2 by a naturally occurring polyamine specifically active in the rice roots under hyperosmotic stress.

In this report we have described the cloning of full-length cDNA of a sucrose nonfermenting 1-related protein kinase2 (SnRK2)-type serine/threonine protein kinase SAPK4 homolog from indica rice (OSPDK, *Oryza sativa* Spd-mediated protein kinase) and characterized its function by overexpressing it in *E. coli*. Interestingly, we found that in addition to salt stress, OSPDK was autophosphorylated by ABA and Spd treatments. The novel finding in this study was that Spd was found to play an important role in the implementation of phosphorylation and activation of OSPDK. Vlad et al. (2008) had confirmed that SnRK2-10 in *Arabidopsis thaliana* phosphorylated in vitro and also predicted the phosphorylation site as LXRXXS, which is conserved in dehydrins. Earlier reports from our laboratory had hinted on elevated binding of OSBZ8, a bZIP class of transcription factor, to ABRE (*cis* elements present in many ABA-dependent salt signaling pathway genes) by phosphorylation (Mukherjee et al. 2006; Roychoudhury et al. 2008). Here, we show that an ABA-activated member of the rice SnRK2 family is involved in the gene-regulation pathway of ABA signaling, and provide evidence that this kinase phosphorylates OSBZ8 directly to activate transcription in response to ABA. This study is the first report of specific activation of OSBZ8 by a kinase in rice and hence is important in elucidating the signaling cascade mediated by Spd as well as ABA in salinity stress.

## Materials and methods

### Plant materials, growth conditions and stress treatments

Seeds of *Oryza sativa* L. cv. M-I-48 were obtained from IRRI (Manila, Philippines); Nonabokra seeds were procured from Central Soil Salinity Research Institute (Canning, West Bengal, India), and both multiplied in the Madhyamgram Experimental Farm of Bose Institute. Seeds were surface sterilized with 0.1% (w/v) HgCl<sub>2</sub> for 20 min, washed extensively, imbibed in deionized water for 6–8 h and allowed to germinate over water-soaked sterile gauge in Petri dishes at 37°C in dark for 3 days. The germinated seedlings were grown in the presence of 0.25× MS medium (Murashige and Skoog complete media, Sigma, St Louis, USA) at 32°C under 16-h light and 8-h dark photoperiodic cycle with 50% relative humidity in a plant growth chamber (NIPPON, LHP-100-RDS, Tokyo, Japan) for three more days. Plants were then treated with fresh 0.25× MS medium alone, with 150 mM NaCl/100 μM

ABA or 1 mM Spd in fresh 0.25× MS medium for 16 h. Plants (roots and lamina) were harvested and washed thoroughly with deionized water; samples of equal fresh weight were frozen in liquid nitrogen and immediately homogenized for the preparation of total RNA or protein.

### Preparation of nuclei and nuclear extract fraction from rice

Nuclei were prepared from equal amounts of tissue (100 g of root) of 3-day-old germinated Nonabokra rice seedlings after stress treatment (150 mM NaCl, 16 h) according to Gupta et al. (1998), except that the crude nuclear pellet was washed with washing buffer (25 mM Tris-Cl, pH 7.5, 0.44 M sucrose, 10 mM MgCl<sub>2</sub>, 10 mM βME). Each pellet was resuspended in high salt buffer (2 ml per 100 g freshly harvested root), containing 100 mM KCl, 15 mM HEPES-KOH of pH 7.6, 5 mM MgCl<sub>2</sub>, 1 mM EDTA of pH 8.0, 1 mM DTT and 5 μg ml<sup>-1</sup> leupeptin and lysed by incubating on ice for 45 min with vigorous shaking; 3 M KCl was added dropwise to a final concentration of 485 mM with occasional stirring of the tubes kept on ice. Then it was centrifuged for 20 min at 15,000×g at 4°C. The final supernatant was dialyzed against 1,000 volumes of dialysis buffer (Gupta et al. 1998) and centrifuged at 15,000×g at 4°C. The supernatant was lyophilized at -50°C, aliquoted and kept at -70°C. The protein content was estimated following the method of Bradford (1976).

### Search for ASK1 homolog in rice and subsequent sequence analysis

The amino acid sequence of Arabidopsis SK1 (Swiss Prot accession no. P43291.1) was used as the query probe to search the Rice Genome Database of China through the tBLASTn algorithm program. The rice genomic contigs having higher homology with the probe were obtained for prediction, and assembling of putative ORFs was done by WISE-2 tool (version 2.1.20 stable) available online in <http://www.ebi.ac.uk/Tools/Wise2>. The sequence of the ORF was further confirmed by the ESTs existing in the rice EST division of Genbank. Based on the assembled results, oligonucleotide primers (OSPDK5 and OSPDK3; Table 1) were designed to clone the cDNA of OSPDK from rice.

The functional domain of the putative protein was analyzed using online server program SMART 4.0 (<http://smart.embl-heidelberg.de/>). The molecular weight of the deduced protein of OSPDK was calculated using the online server program ProtScale (<http://au.expasy.org/cgi-bin/protscale.pl>). The motif analysis was performed using the online server program MotifScan ([http://myhits.isb-sib.ch/cgi-bin/motif\\_scan](http://myhits.isb-sib.ch/cgi-bin/motif_scan)) and NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>). The secondary structure of the

**Table 1** Oligonucleotides used for our work

DNA/cDNA	Complementary oligos (nucleotide sequences)
OSBZ8 cDNA	OSBZ8 5 (43 mer) 5'-TG TAGATCTCAACAATGGGAAATGACGAAGCTGTAGTTACTCA-3'
	OSBZ8 3 (36 mer) 5'-GAGAGATCTTTACTTGAGGCTACAGCATCAGTCGC-3'
OSPDK cDNA	OSPDK 5 (31 mer) 5'-CGCGGATCCACATGGAGAAGTACGAGGCGGT-3'
	OSPDK 3 (32 mer) 5'-GGCGAATTCGCTCATATGCGCAGTGAGTCAT-3'
1XABRE	ABRE 5 (26 mer) 5'-AGCTTGCCGGACACGTGGCGCTCTAG-3'
	(Annealed with ABRE 3 to form 1X ABRE used in EMSA as probe or competitor)
	ABRE 3 (26 mer) 5'-CTAGAGCGCCACGTGTCCGCAAGCT-3'
Actin cDNA	RAC 5 (26 mer) 5'-CTTCATAGGAATGGAAGCTGCGGGTA-3'
	RAC 3 (26 mer) 5'-CGACCACCTTGATCTTCATGCTGCTA-3'

putative protein was predicted using Garnier protein secondary structure version 2.0u66 September 1998 ([http://fasta.bioch.virginia.edu/o\\_fasta](http://fasta.bioch.virginia.edu/o_fasta)).

#### Isolation of total RNA, RT-PCR and cloning of OSPDK full-length cDNA

All standard cloning procedures used in this study were carried out as described by Sambrook and Russell (2001). Total RNA was isolated from about 10 g of roots salt-treated (150 mM NaCl, 16 h) 3-day-old Nonabokra rice seedlings by the GITC method (Chomczynski and Sacchi 1987). RT was done using Superscript RT (Life technologies, USA) to produce first-strand cDNA at 42°C for 50 min from PolyA<sup>+</sup> RNA obtained from Nonabokra roots using oligo dT cellulose (Amersham Pharmacia Biotech, NJ, USA). PCR was done with OSPDK5 and OSPDK3 primers (Table 1) designed from the two extreme ends of the open reading frame (ORF) of previously ascertained cDNA sequence of a rice serine/threonine-specific protein kinase, which was activated in osmotic stress as well as by ABA. The PCR reaction mix was incubated at 94°C for 3 min and then 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min in a thermocycler (Perkin Elmer, 2400, USA). After agarose gel electrophoresis analysis of the amplified *OSPDK* PCR product, the 1.083 kb band was cut carefully from the gel and purified through cartridge (Qiagen, Valencia, CA, USA), cloned into BamHI-EcoRI sites of pGEX-2T (Amersham Pharmacia Biotech) and the recombinant clone was selected by transforming into *E. coli* strain BL21. The newly cloned cDNA sequence was submitted to GenBank (Accession number DQ408431).

A ~66-kDa polypeptide was produced after 1 mM IPTG (isopropyl thiogalactoside)-mediated induction (recombinant *OSPDK* fusion protein, now designated as *rOSPDK*). The GST fusion protein was purified using Glutathione-Sepharose affinity resin (Amersham Pharmacia Biotech) following manufacturer's protocol.

#### Cloning of OSBZ8 full-length cDNA

The full-length OSBZ8 cDNA from indica rice was cloned (Table 1), sequenced (GenBank Accession number AY606941) and expressed in *E. coli* (recombinant OSBZ8 fusion protein, now designated as *rOSBZ8*), and the recombinant purified protein was obtained according to Mukherjee et al. (2006) and RoyChoudhury et al. (2008).

#### In vitro phosphorylation assay

Purified *rOSPDK* and *rOSBZ8* proteins were used for phosphorylation studies. Protein kinase assays were performed according to Dasgupta (1994) with some modifications. Column purified *rOSPDK* and *rOSBZ8* proteins (200 ng each) were incubated in phosphorylation buffer (25 mM Tris–MES [pH 7.8], 5 mM MgCl<sub>2</sub>, 50 μM DTT, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>) in the presence or absence of 150 mM NaCl/100 μM ABA/1 mM Spd as and when required. The phosphorylation reaction was initiated with the addition of 1 μCi of [<sup>32</sup>P]ATP (Sp. Act. 6,000 Ci mmol<sup>-1</sup>) at 30°C. After incubation for 1 h at 30°C, the reaction was stopped by adding SDS-sample buffer (125 mM Tris, 4% SDS, 20% glycerol, 10 mM β-mercaptoethanol, 2 mM EDTA, 0.04% bromophenol blue, pH 6.8). Samples were boiled for

5 min prior to loading onto polyacrylamide gels and separation by 10% SDS-PAGE. Gels were finally autoradiographed by exposure to Kodak X-AR films.

#### In-gel kinase assay

In-gel kinase activity assay was performed according to Ohmura et al. (1987) with some modifications. Column-purified rOSPDK protein (200 ng), pre-incubated with either 150 mM NaCl/100  $\mu$ M ABA or 1 mM Spd, along with pre-stained protein molecular weight marker was electrophoresed in 10% SDS PAGE that contained one of the following co-polymerized protein substrates: 0.25 mg ml<sup>-1</sup> myelin basic protein (MBP), 0.5 mg ml<sup>-1</sup> histone, 0.5 mg ml<sup>-1</sup> casein, 0.5 mg ml<sup>-1</sup> casein + 100  $\mu$ g ml<sup>-1</sup> or 0.5 mg ml<sup>-1</sup> rOSBZ8. After electrophoresis, SDS was removed and the protein denatured by washing the gels for three times in buffer (25 mM Tris-Cl [pH 7.5], 6 M guanidium-HCl, 0.5 mM DTT, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5 mg ml<sup>-1</sup> BSA, 0.1% Triton X-100 and 5 mM NaF) with gentle rocking at room temperature followed by renaturation in buffer (25 mM Tris-Cl [pH 7.5], 0.5 mM DTT, 0.1 mM Na<sub>3</sub>VO<sub>4</sub> and 5 mM NaF) at 4°C overnight with three changes. Subsequently, the gels were incubated for 60 min at 30°C in reaction buffer (25 mM Tris-Cl [pH 7.5] containing 12 mM MgCl<sub>2</sub>, 2 mM EGTA, 1 mM DTT, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 200 nM ATP) supplemented with 50  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP (3,000 Ci mmol<sup>-1</sup>). The reaction was terminated by transferring the gels into a bath of 5% trichloroacetic acid and 1% sodium phosphate. The unincorporated [ $\gamma$ -<sup>32</sup>P]ATP was removed by washing the gels in the bath solution until the washing solution was determined to contain no more radioactive signal. The gel was dried and exposed for autoradiography.

#### Autophosphorylation assay

For autophosphorylation study, 1  $\mu$ g of purified rOSPDK or GST protein was pre-incubated with either 150 mM NaCl/100  $\mu$ M ABA or 1 mM Spd and was then separated by 10% SDS-PAGE. Subsequently, it was transferred to PVDF membrane at 4°C for 90 min at 5 V cm<sup>-1</sup>. The membrane, after treatment with methanol, was equilibrated in cold Tris-glycine transfer buffer for 10 min followed by denaturation in buffer (7 M guanidium-HCl in 50 mM Tris-Cl [pH 7.4] with 50 mM DTT, 2 mM EDTA [pH 8.3]) for 1 h at 30°C. After washing with TBS [pH 7.4] several times, renaturation buffer (140 mM NaCl, 10 mM Tris-HCl [pH 7.4] containing 2 mM DTT, 2 mM EDTA, 1% BSA and 0.1% NP 40) was added and the blot incubated overnight at 4°C with gentle rocking. The blot was then treated with blocking buffer (30 mM Tris-HCl [pH 7.4], 5% BSA) followed by in situ phosphorylation with

50  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (Sp. Act. 3,000 Ci mmol<sup>-1</sup>) according to Ohmura et al. (1987). The blot was further processed as described by Dasgupta (1994).

#### In vitro phosphorylation assay using different kinase inhibitors

In vitro phosphorylation assay of rOSPDK, pre-incubated or activated with either 150 mM NaCl/100  $\mu$ M ABA or 1 mM Spd, was carried out as described earlier in the presence of the following kinase inhibitors: 4.5  $\mu$ l of 1  $\mu$ M DHBAS (X)—PKC inhibitor, or 6  $\mu$ l of 1  $\mu$ M TFP (Y)—calmodulin chelator, or 3  $\mu$ l of 1  $\mu$ M compound R24571 (Z)—CDPK inhibitor. rOSPDK protein along with these kinase inhibitors were incubated in ice for 2 h prior to phosphorylation with 1  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (Sp. Act. 6,000 Ci mmol<sup>-1</sup>) at 30°C. The reaction was stopped by adding SDS sample buffer followed by 10% SDS-PAGE analysis and autoradiography.

#### Preparation of [<sup>32</sup>P]-DNA probe for EMSA experiments

Twenty pmoles each of ABRE5 and ABRE3 oligo, containing EmIa sequence (CACGTG) from *Em* gene of wheat (Marcotte et al. 1989), were separately labeled in microcentrifuge tubes at the 5' termini by T4 Polynucleotide Kinase (New England Biolabs, MA, USA) using [ $\gamma$ -<sup>32</sup>P]ATP (100  $\mu$ Ci for each oligo of specific activity 6,000 Ci mmol<sup>-1</sup> from JONAKI, BRIT, INDIA), by incubating at 37°C for 45 min. They were then heated to 68°C to inactivate the PNK. The contents of the two tubes were then mixed together and again heated to 75–80°C for 10 min. Finally, it was allowed to cool slowly to room temperature so that the two ABRE oligos hybridize to make double stranded 1XABRE (Absciscic acid responsive element) DNA probe (Table 1) (Sambrook and Russell 2001). The probes were purified using Sephadex G-50 spin column (Roche) and incorporation was measured by Scintillation Counter (Beckman, CA, USA). Cold 1XABRE as competitor was similarly prepared without radiolabeling.

#### EMSA reactions using 1XABRE [<sup>32</sup>P]-DNA probe

EMSA reactions were carried out as standardized earlier in our laboratory (RoyChoudhury et al. 2008; Mukherjee et al. 2006; Gupta et al. 1998) with root nuclear protein (20  $\mu$ g per lane) [from the salt-treated (150 mM NaCl, 16 h) seedlings of salt-tolerant Nonabokra rice cultivar], rOSBZ8 (200 ng per lane), rOSPDK (200 ng per lane, pre-incubated with 1 mM Spd), BSA (200 ng per lane) or 1 mM Spd as and when required. Substrate molecules

mentioned above were mixed with 5  $\mu\text{g}$  poly (dI:dC), 80 mM  $\text{MgCl}_2$  and 10 mM NaPi/1 mM EDTA (pH 8.0). The reaction mixtures were incubated on ice for 15 min; radioactive probe (1XABRE) was added (80,000 CPM per reaction) and incubated at 25°C for 30 min. Further DNA–protein interaction study was done by incubating increasing concentration of 1 mM Spd treated rOSPDK (50 ng, 100 ng, 200 ng) with 1XABRE (80,000 CPM per reaction) and rOSBZ8 (200 ng). For kinase inhibitor study, 1  $\mu\text{g ml}^{-1}$  heparin (casein kinase II inhibitor) was incubated with rOSBZ8 and rOSPDK before the addition of the probe. The DNA:protein complex was separated from free probe by carrying out electrophoresis in a native, 6% gel at a constant 100 V for 4 h at 25°C in 0.5 $\times$  TBE running buffer. The gel was dried and X-Omat film (Kodak) was exposed for autoradiography for 7–8 h. The autoradiograms were scanned by Gel-Doc 1000 (BioRad).

#### Semi-quantitative RT-PCR

Total RNA was isolated from the roots of control, NaCl, ABA or Spd-treated rice plants as described earlier in “Materials and methods”. RNA integrity was checked on a 1.5% formaldehyde agarose gel. RNA was treated with DNase (10 U/2.5  $\mu\text{g}$  of RNA) to remove genomic DNA contamination before use in reverse transcriptase reaction. Five micrograms of total RNA was used to synthesize first strand cDNA with anchored oligo dT primer using Superscript II reverse transcriptase (Invitrogen, 200 U/2.5  $\mu\text{g}$  RNA) as per manufacturer’s protocol. The cDNAs were subsequently treated with *E. coli* RNase H (2 U) and used for PCR amplification. For semi-quantitative RT-PCR (SQ RT-PCR) analysis of *OSPDK* gene expression with *OSPDK5* and *OSPDK3* primers (Table 1), 500 ng of total RNA from the roots of M-1-48 and Nonabokra rice plants grown for 3 days under control (C), 150 mM NaCl/100  $\mu\text{M}$  ABA or 1 mM Spd-treated conditions was used. *Actin* cDNA was amplified as a control. For *Actin* amplification, the primers RAC5 and RAC3 were used (GenBank Accession number X16280) (Table 1). All SQ RT-PCR reactions were performed for 30 cycles by DreamTaq™ DNA polymerase (Fermentas Inc., USA) and the amplified fragments were electrophoresed on 1.2% (w/v) agarose gels and then visualized by ethidium bromide staining.

#### Statistical analysis of *OSPDK* gene expression

At least five repetitions with individual biological sample sets were used for the statistical treatment of the data. The data are expressed as mean values; error bars indicate the standard error. To evaluate the significance of differences

of data, one-way ANOVA was performed with “Dunnett’s multiple comparison test”.

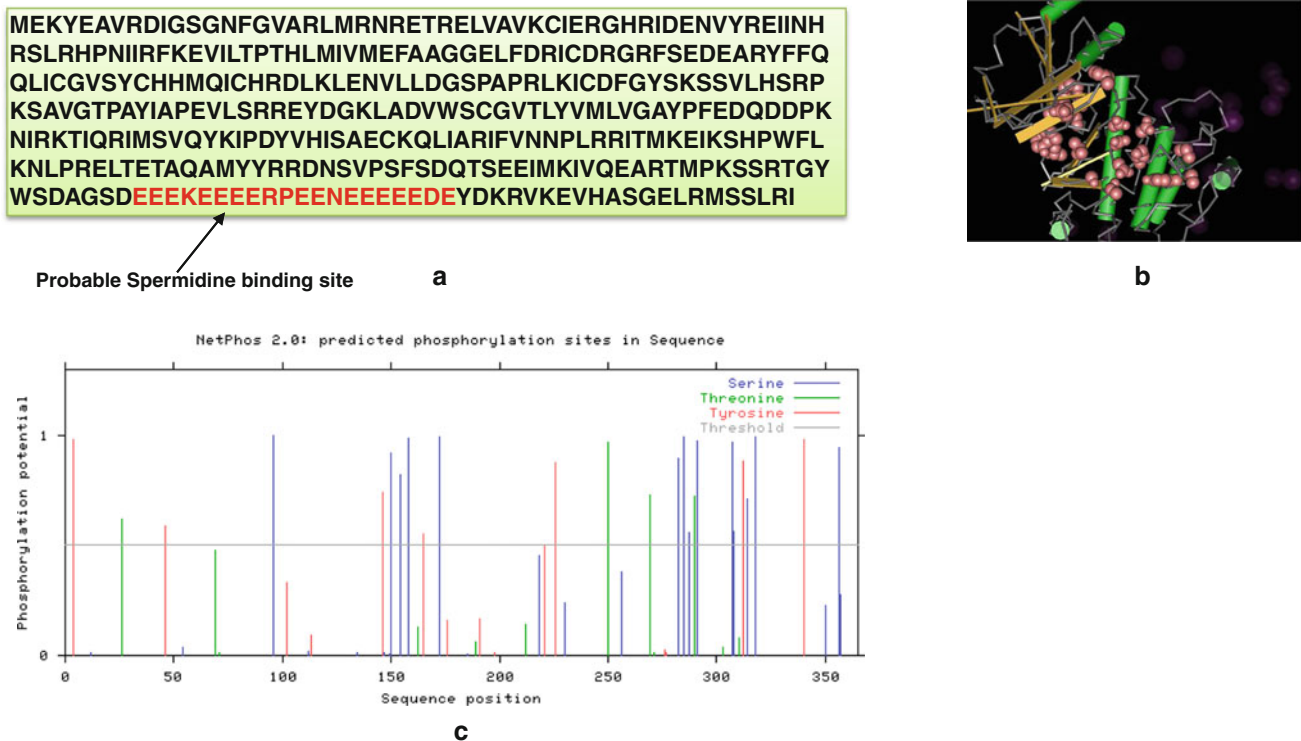
## Results

In silico approach to find out the sequence information of ASK1 homolog in rice

The aim of this bioinformatic study was to find out the kinase sequence in rice, which would give us the starting material having similar properties like *Nicotiana tabacum* osmotic stress-activated protein kinase (NtOSAK) or *Arabidopsis* serine/threonine kinase 1 (ASK1). Sequence information from *Arabidopsis* SK1 (Mikoajczyk et al. 2000) was utilized to fish out the rice homolog from ‘Rice Genome Database of China’ through tBLASTn algorithm program. The rice genomic contigs having higher homology with the probe were obtained for prediction. Assembling of putative ORFs was done by feeding the obtained putative sequence and ASK1 amino acid sequence in the WISE-2 tool (version 2.1.20 stable) available online in <http://www.ebi.ac.uk/Tools/Wise2>, which compares the gene and amino acid sequence to find out the exons and introns. The sequence of the ORF was further confirmed by the ESTs existing in the rice EST division of Genbank. The amino acid sequence deduced from the gene sequence was further analyzed by ‘GFSelector’ to predict its putative function. The study revealed that the amino acid sequence was that of a serine/threonine-specific protein kinase, which was activated in osmotic stress as well as by ABA. The obtained in silico polypeptide sequence, which is a homolog of ASK1, is referred to as OSPDK.

#### Deduced amino acid sequence characterization of OSPDK

The analysis of the amino acid sequence indicated that the predicted protein had a molecular weight of 41.9 kDa with  $\text{pI} = 6.06$ . Further analysis of the predicted amino acid sequence of OSPDK using GFselector revealed GLU\_R-ICH (Glutamic acid-rich) 320–339 amino acid region. Using SMART online software, the amino acid sequence including a serine/threonine protein kinase catalytic domain and protein kinase domain was analyzed. There was a protein kinase ATP-binding signature region between position 10 and 33 with a serine/threonine protein kinase active-site signature within position 119–131. Predicted by the method of Garnier et al. (1978), the secondary structure indicated it was a protein kinase with N-terminal catalytic domain and C-terminal regulatory domain (Fig. 1). It also showed that the protein (OSPDK) may



**Fig. 1** In silico analysis of putative OSPDK protein. **a** The amino acid sequence of OSPDK (GenBank Accession number DQ408431) depicting C-terminal GLU\_RICH region (red) showing putative Spd binding site. **b** Schematic representation of the predicted secondary structure of OSPDK; the structure was predicted by Garnier protein

secondary structure version 2.0u66 ([http://fasta.bioch.virginia.edu/o\\_fasta](http://fasta.bioch.virginia.edu/o_fasta)). **c** Predicted phosphorylation sites in the putative OSPDK polypeptide using NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>) (colour figure online)

contain helix conformation 44.5%, sheet 21.5%, turn 20.1% and coil 18.6%.

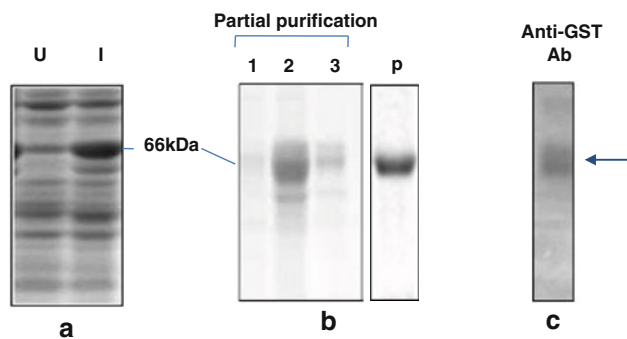
#### Bacterial expression and purification of OSPDK and OSBZ8

The gene-specific primers were designed from in silico GenBank sequence obtained earlier for PCR amplification and cloning the rice homolog *OSPDK* from 3 days old Nonabokra root (Table 1). RNA isolated from the salt-treated 3 days old Nonabokra roots were reverse transcribed to produce the cDNA and finally PCR amplified to yield the *OSPDK* full-length cDNA (1,083 bp). No band was detected in the RT<sup>-</sup> control indicating that the PCR product was the result of amplification of cDNA and not due to the contaminating genomic DNA. To further investigate its functions and study the possible inter and intramolecular interactions, both *OSPDK* and *OSBZ8* (Mukherjee et al. 2006; RoyChoudhury et al. 2008) cDNAs were cloned into pGEX 2T and pRSET expression vectors, respectively. The cloned *OSPDK* cDNA was sequenced and analyzed further to confirm its homology with the silicon ASK1 sequence. The recombinant plasmids were subsequently transformed in *E. coli* BL21 cells and subjected to induction by 1 mM

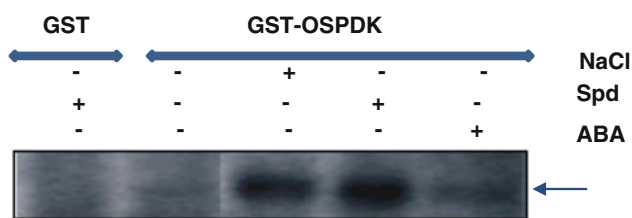
IPTG. Both these overexpressed fusion proteins (GST-OSPDK and 6XHis-OSBZ8, respectively) were found predominantly in the soluble fractions after sonication and lysozyme treatments of *E. coli* cells and purified to 90% homogeneity (Fig. 2a; RoyChoudhury et al. 2008). We were able to purify between 800 µg and 1 mg of recombinant protein per liter of culture using standard expression and purification procedures. Figure 2b shows the proteins from different steps of purification: lane 1 shows the flow through after the overexpressed recombinant protein was bound to the GST column, lane 2 contains the semipurified elute, and lane 3 contains the protein in the wash buffer after elution. The preparations were analyzed by 10% SDS-PAGE. In lane 2, bulk amount of the expressed protein with minor contamination of some other proteins was observed. The purified kinase (rOSPDK) was subjected to Western blot analysis against anti-GST antibody. Results clearly show the expression of the GST fusion protein (Fig. 2c).

#### In vitro phosphorylation of the expressed GST-OSPDK fusion protein

To ascertain and characterize the kinase activity of rOSPDK, equal amounts of recombinant protein (200 ng in



**Fig. 2** Production of purified recombinant OSPDK protein. **a** The GST-OSPDK protein expressed in *E. coli* after IPTG-mediated induction (I) and run in a 10% SDS–polyacrylamide gel (lane 2). No expression of protein was detected in lysates of uninduced (U) bacterial cells loaded in lane 1. **b** The partially purified recombinant OSPDK (rOSPDK) protein of 66 kDa is shown in lane 1, 2, and 3 containing flow through, elute, and wash, respectively, and purified recombinant OSPDK (rOSPDK) protein of 66 kDa (P) were analyzed through 10% SDS–polyacrylamide gel. **c** Purified GST-OSPDK protein was analyzed by immunoblotting with anti-GST antibody



**Fig. 3** Autoradiogram showing the activation (in vitro phosphorylation) of the 66-kDa GST-OSPDK protein in the presence of 150 mM NaCl (lane 3), 1 mM Spd (lane 4), and 100  $\mu$ M ABA (lane 5). GST alone (lane 1) or GST-OSPDK in the absence of any exogenous elicitors (lane 2) did not show any kinase activity as is evident from the absence of any phosphorylating band; 200 ng of purified protein was incubated with 1  $\mu$ Ci [ $\gamma$ <sup>32</sup>P]ATP (Sp. Act. 6,000 Ci mmol<sup>-1</sup>), and separated by 10% SDS-PAGE. Phosphorylated proteins were then visualized by autoradiography. Three replicate experiments were done with similar results. The photograph was made from the autoradiogram developed after 3 days of exposure

each lane) were subjected to in vitro phosphorylation assay using 1  $\mu$ Ci [ $\gamma$ <sup>32</sup>P]ATP (Sp. Act. 6,000 Ci mmol<sup>-1</sup>). Results show phosphorylation of a 66-kDa protein [GST-OSPDK = 24 + 42 kDa], in presence of 150 mM NaCl (lane 3), 1 mM Spd (lane 4) and 100  $\mu$ M ABA (lane 5) (Fig. 3). The phosphorylation signal was totally absent in the protein expressed from induced non-recombinant (lane 1 of Fig. 3) pGEX-2T, thereby showing that the protein phosphorylated was that of GST fusion rOSPDK protein. Absence of any phosphorylation signal in untreated rOSPDK (not pre-incubated with 150 mM NaCl/1 mM Spd/100  $\mu$ M ABA), loaded in lane 2 of the gel, clearly suggests that the kinase is activated in vitro in the presence of exogenous NaCl, Spd and ABA. Similar in vitro

phosphorylation studies on the purified rOSPDK protein after incubation with variable concentrations of other monovalent or divalent salts such as lithium chloride, potassium chloride, calcium chloride, manganese chloride and magnesium chloride did not invoke any positive signal corroborating this protein's specificity for sodium chloride (data not shown).

#### OSPDK substrate specificity and analysis of different kinase inhibitors

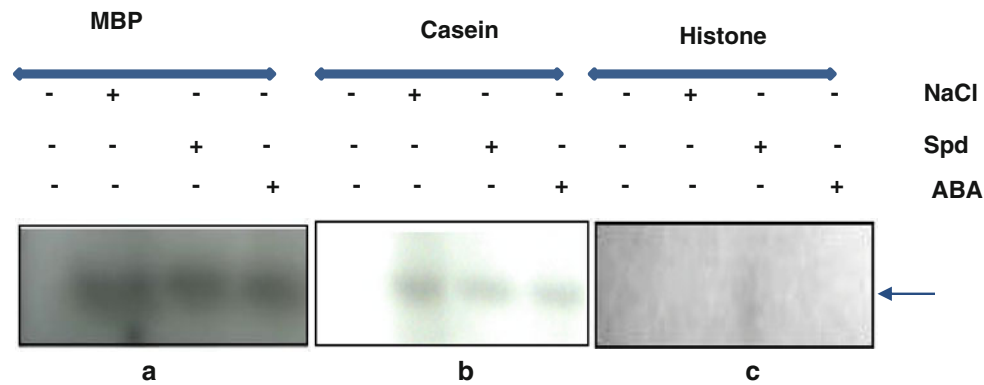
The activity of the 66-kDa GST fusion protein kinase was analyzed with different substrates to determine its specificity. The result, with equal amount of rOSPDK (200 ng each), of in-gel kinase activity study with MBP (Fig. 4a), casein (Fig. 4b) and histone (Fig. 4c) revealed that while the kinase actively phosphorylated MBP and casein, heparin, a potent casein kinase inhibitor, completely abolished its activity (Fig. not shown) and histone was not its preferred substrate.

Specific protein kinase inhibitors prove to be useful in monitoring the activity and functional analysis of different kinases. Therefore, several compounds known to be potent inhibitors of different protein kinases were analyzed for their potential effects on the rOSPDK's protein kinase activity. These inhibitors include DHBAS (X)—PKC inhibitor, TFP (Y)—calmodulin chelator and compound R24571 (Z)—CDPK inhibitor. None of the above-mentioned inhibitors, when applied in optimum quantity, could abolish the protein kinase activity (Fig. 5), thereby suggesting that the expressed OSPDK protein belonged to the SNF group of kinase family of protein kinase as reported earlier from *Arabidopsis* exhibiting similar kind of unusual kinase properties (Mikoajczyk et al. 2000).

#### OSPDK is an autophosphorylating kinase

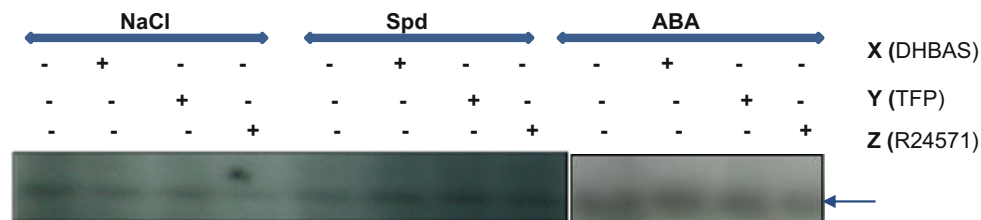
To determine autophosphorylating activity of rOSPDK protein, equal amount (1  $\mu$ g each) of control (i.e., purified GST) and rOSPDK were analyzed for autophosphorylation assay. The proteins were subjected to 10% SDS-PAGE and were subsequently transferred to PVDF membrane. The transferred proteins in the membrane were then subjected to denaturation followed by renaturation and then phosphorylated as described in "Materials and methods". The presence of 66-kDa phosphorylated bands (Fig. 6) in the lanes where rOSPDK was pre-incubated with 150 mM NaCl (lane 2), 1 mM Spd (lane 3) and 100  $\mu$ M ABA (lane 4) but absent in lane 1 with GST protein, proves it to be an autoregulatory kinase similar to *Nicotiana tabacum* osmotic stress-activated protein kinase (NtOSAK) (Kelner et al. 2004; Mikoajczyk et al. 2000).





**Fig. 4** **a** Substrate phosphorylation activity of the NaCl, Spd or ABA-induced 66-kDa purified GST-OSPDK protein; 200 ng of the purified protein was separated by 10% SDS-PAGE in the presence of  $0.25 \text{ mg ml}^{-1}$  MBP. After electrophoresis, SDS was removed and the protein denatured by washing the gels in a buffer containing 6 M guanidium-HCl followed by renaturation. Subsequently, the gels were incubated in phosphorylation buffer supplemented with  $50 \mu\text{Ci } [\gamma^{32}\text{P}]\text{ATP}$  ( $3,000 \text{ Ci mmol}^{-1}$ ). The reaction was terminated by 5% trichloroacetic acid and 1% sodium phosphate. The gel was then dried and exposed for autoradiography. The 66-kDa protein was found to phosphorylate MBP in the gel. Three replicate experiments were done

with similar results. The photograph was made from the autoradiogram developed after 7 days of exposure. **b** Substrate phosphorylation activity or activity gel assay of purified GST-OSPDK protein (200 ng in each lane), using  $0.5 \text{ mg ml}^{-1}$  casein as substrate, was performed to determine the nature of the 66-kDa protein. The experiment was done in a way similar to that with MBP as substrate and was repeated three times. The 66-kDa protein was found to phosphorylate casein in the gel. The photograph was made from the autoradiogram developed after 7 days of exposure. **c** A similar experiment was repeated with the same amount of purified GST-OSPDK protein using  $0.5 \text{ mg ml}^{-1}$  histone as substrate. No phosphorylating band was observed



**Fig. 5** Autoradiogram showing the effect of different inhibitors on the activity of 66-kDa protein; 200 ng each of purified GST-OSPDK protein were incubated in ice for 2 h with or without  $4.5 \mu\text{l}$  of  $1 \mu\text{M}$  DHBAS (X) (PKC inhibitor) or  $6 \mu\text{l}$  of  $1 \mu\text{M}$  TFP (Y) (calmodulin chelator), or  $3 \mu\text{l}$  of  $1 \mu\text{M}$  compound R24571 (Z) (CDPK inhibitor) followed by phosphorylation assay in the presence of  $150 \text{ mM}$  NaCl (lane 1–4),  $1 \text{ mM}$  Spd (lane 5–8), and  $100 \mu\text{M}$  ABA (lane 9–12)

using  $1 \mu\text{Ci } [\gamma^{32}\text{P}]\text{ATP}$  (Sp. Act.  $6,000 \text{ Ci mmol}^{-1}$ ). Inhibitors were absent in lanes 1, 5, and 9. The protein was then separated by 10% SDS-PAGE. Phosphorylated proteins were visualized by autoradiography. Three replicate experiments were done with similar results. The photograph was made from the autoradiogram developed after 5 days of exposure

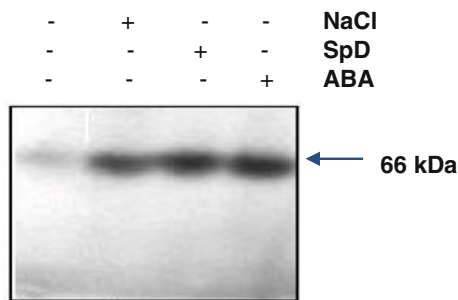
#### Expression patterns of the *OSPDK* gene in roots of salt-sensitive and salt-tolerant rice cultivars

To investigate the expression patterns of the *OSPDK* gene under stress conditions, semi quantitative RT-PCR was carried out. Figure 7 shows *OSPDK* transcript accumulation in salt-sensitive (M-1-48) and salt-tolerant Nonabokra exposed to in vivo NaCl, ABA and Spd-treated (16 h) rice roots. The mRNA levels were upregulated in the presence of NaCl, ABA and Spd. As expected, transcription of *OSPDK* was high in salt-tolerant Nonabokra rice cultivar as compared to salt-sensitive M-1-48. The *OSPDK* transcript in control roots was present in substantially less amount in M-1-48 than in Nonabokra. The NaCl, ABA and Spd-induced accumulation of the *OSPDK* transcript was more pronounced in M-1-48 than in Nonabokra, indicating

their possible involvement in *OSPDK* gene expression during salinity stress. *Actin* gene expression was used as a loading control. Representative results for the biological replicates are given in Supplementary Fig. S1.

#### *OSPDK* phosphorylates OSBZ8 in vitro

Nakagawa et al. (1996) and Mukherjee et al. (2006) have already shown that *OSBZ8* expression is upregulated by both NaCl and ABA. Phosphorylation is a frequently used mechanism to regulate the activity of transcription factors throughout the biological system. The ability of *OSPDK* to phosphorylate a transcriptional regulator OSBZ8 was then tested in vitro using recombinant fusion protein prepared from *E. coli* BL21 cells containing ‘pRSETA:OSBZ8 full-length cDNA’ recombinant plasmid (RoyChoudhury et al.



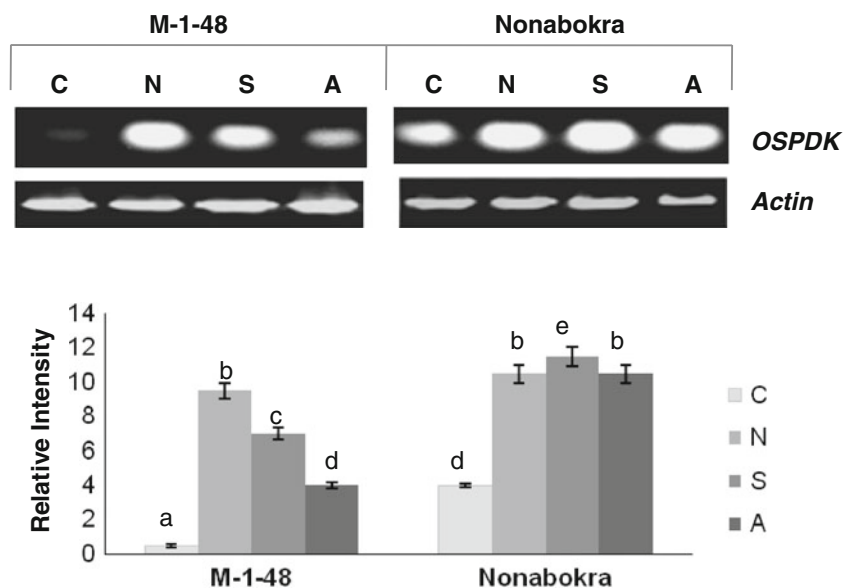
**Fig. 6** Autoradiogram showing the autophosphorylation of the purified 66-kDa protein; 800 ng each of the purified protein (as indicated) was separated in 10% SDS-PAGE and subsequently transferred to the PVDF membrane. The membrane was treated with buffers as described in “Materials and methods” followed by in situ phosphorylation with 50  $\mu\text{Ci}$  [ $\gamma^{32}\text{P}$ ]ATP (Sp. Act. 6,000 Ci  $\text{mmol}^{-1}$ ). Phosphorylated proteins were visualized by autoradiography. The phosphorylating bands at 66-kDa position denote that the protein is an autophosphorylating protein. Three replicate experiments were done with similar results. The photograph was obtained from the autoradiogram developed after 4 days of exposure

2008) and purified GST (lane 1)/GST-OSPDK (lane 2), in the presence of 1 mM Spd, using [ $\gamma^{32}\text{P}$ ]ATP as phosphate donor (Fig. 8a). Result shows phosphorylation bands of the 66-kDa protein (GST-OSPDK = 24 + 42 kDa) as well as of 42-kDa protein (6XHis-OSBZ8 = 3 + 39 kDa) in (Fig. 8a; lane 2). Absence of any band in lane 1 thus confirms that the phosphorylation is not due to GST.

As shown in Fig. 8b, a 66-kDa phosphorylated band appeared in activity gel assay using OSBZ8 as substrate ( $0.5 \text{ mg ml}^{-1}$ ), in the presence of purified rOSPDK protein pre-incubated with 150 mM NaCl, 1 mM Spd or 100  $\mu\text{M}$  ABA using 50  $\mu\text{Ci}$  [ $\gamma^{32}\text{P}$ ]ATP as phosphate donor. The band intensity of rOSBZ8 phosphorylation increased considerably in the presence of NaCl, Spd and ABA in comparison to control (without pre-incubation with these substrates). The above experimental data, taken together, indicate that OSBZ8 is, in fact, phosphorylated in an Spd, ABA and NaCl-dependent manner.

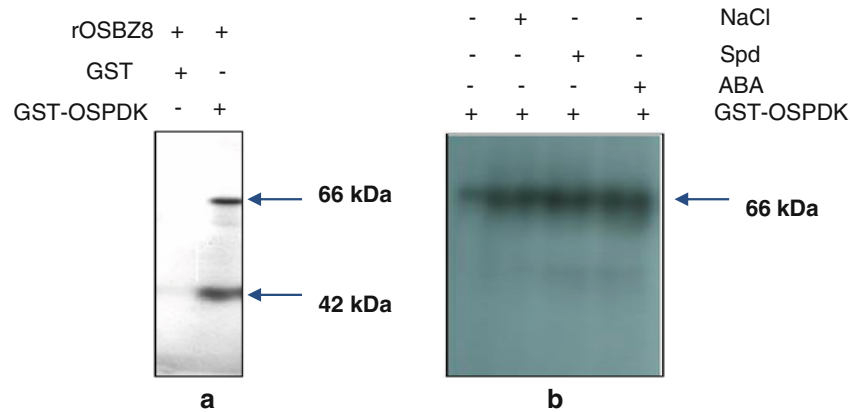
Comparative phosphorylation efficiency of MBP, casein and OSBZ8 by OSPDK

The results described so far have pointed toward the unusual feature of OSPDK protein. This SNF group of kinase shows the characters of both MAPK (by phosphorylating MBP) and casein kinase (by phosphorylating casein and being inhibited by heparin). Figure 8a, b also show OSBZ8 to be the substrate of OSPDK. Figure 9 analyzes the comparative phosphorylation efficiency of OSBZ8 with that of MBP, casein and histone. The band intensity or phosphorylation efficiency of OSBZ8 is much higher (1.5 times) than that of MBP or casein, showing clearly that the transacting factor OSBZ8 is one of the OSPDK's preferred substrates.



**Fig. 7** *OSPDK* SQ RT-PCR analysis. Changes in the abundance of *OSPDK* mRNA level in roots of 3 days old salt-sensitive (M-1-48) and salt-tolerant (Nonabokra) rice cultivars in control and following 150 mM NaCl (N), 1 mM Spd (S), and 100  $\mu\text{M}$  ABA (A) treatments for 16 h (lanes 1–4; upper panel). mRNA levels of rice *Actin* under various conditions used as internal control (lower panel) (GenBank

Accession number X16280). Each bar represents the mean of values obtained by the densitometric scanning of each lane from five separate experiments ( $n = 5$ )  $\pm 1\%$  standard error. *OSPDK* gene expression from untreated roots served as a control. Means followed by different small letters are significantly different ( $P < 0.05$ )



**Fig. 8** Autoradiogram showing the interaction between rOSPDK and rOSBZ8. **a** In vitro phosphorylation analysis using purified GST-OSPDK, 6XHis-OSBZ8, and GST proteins (200 ng each) as depicted in the autoradiogram. All the proteins were pre-incubated with 1 mM Spd and phosphorylation assay done with 1  $\mu$ Ci [ $\gamma$  $^{32}$ P]ATP (Sp. Act. 6,000 Ci  $\text{mmol}^{-1}$ ) and separated by 10% SDS-PAGE. **b** Substrate phosphorylation activity or activity gel assay of purified GST-OSPDK

protein (200 ng in each lane), using 0.5  $\text{mg ml}^{-1}$  6XHis-OSBZ8 as substrate (in presence or absence of 150 mM NaCl, 1 mM Spd and 100  $\mu$ M ABA), was performed to determine their interaction. The experiment was done in a way similar to that with MBP as substrate. The 66-kDa protein was found to phosphorylate rOSBZ8 in the gel. The photograph was made from the autoradiogram developed after 7 days of exposure

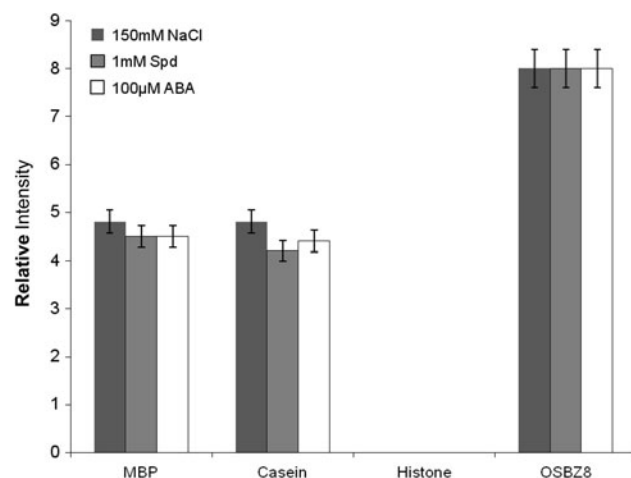
#### Bacterial overexpressed rice SnRK2 enhances the DNA-binding ability of recombinant full-length OSBZ8 to 1XABRE

To confirm our previous observation, EMSA using rOSBZ8 and rOSPDK was performed. Equal amount of full-length protein (200 ng/lane) was incubated with equal counts (80,000 CPM) of 1XABRE probe and run in native 6% polyacrylamide gel. Oligo duplex of 26 bp 1XABRE was designed according to the *Emla* sequence by annealing two oligonucleotides [5'end  $^{32}$ P-labeled], complementary to each other and containing *Emla* sequence, which was used as probe. Binding was observed and the mobility

of the complexes coincided with that from Nonabokra root nuclear extract (Fig. 10a; lanes 2 and 3).

The binding intensity of rOSBZ8 protein with 1XABRE (Fig. 10b; lane 2) was enhanced twofold when 200 ng of purified activated rOSPDK in the presence of Spd (Fig. 10b; lane 5) was pre-incubated with the rOSBZ8 (200 ng). However, when only purified rOSPDK (200 ng) without pre-activation with Spd (lane 3) was used, no significant change in binding intensity could be noticed. Spd alone with the free probe (lane 4) did not show any binding, signifying that its presence alone was not responsible for the increase in the binding capacity.

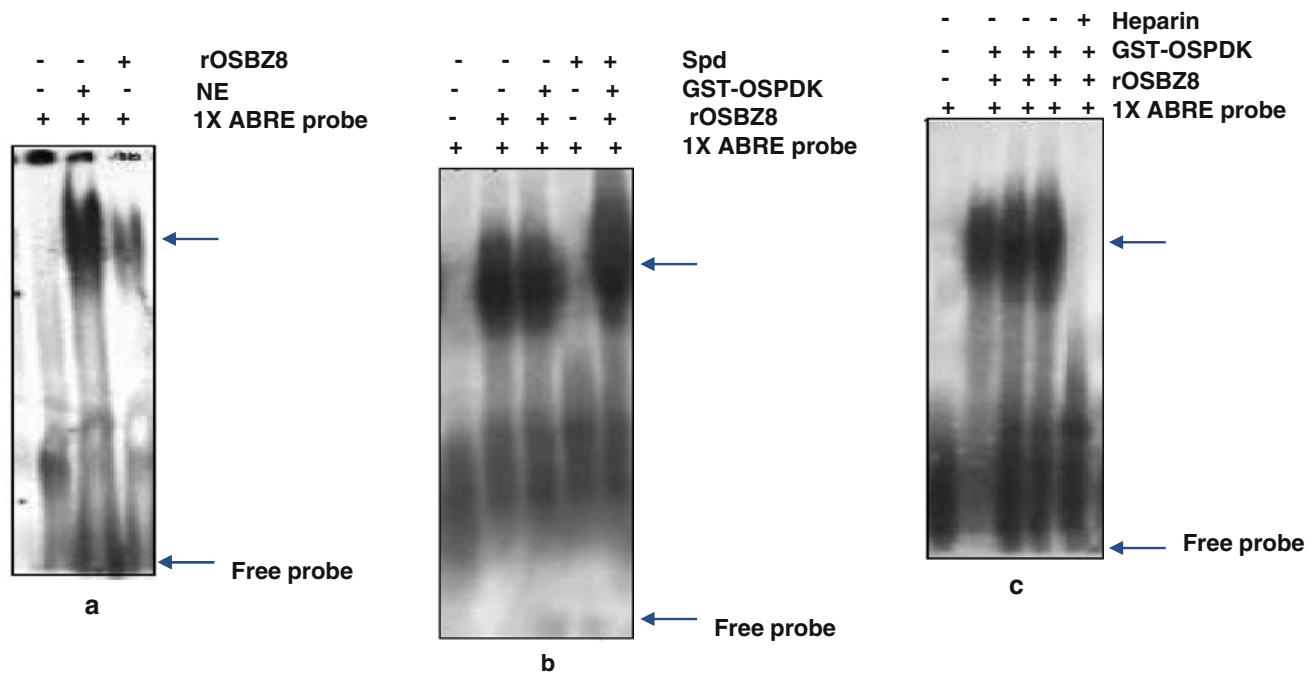
To further fortify our finding regarding a specific activation of rOSBZ8 by rOSPDK, a concentration kinetics (Fig. 10c) was done with increasing amount [50 ng (lane 2), 100 ng (lane 3) and 200 ng (lane 4)] of rOSPDK. A distinct increase in complex formation was observed with increasing concentration of rOSPDK. Heparin again abolished the complex formation (lane 5). Lane 1 in all the figures is for free probe. All these results reveal that OSPDK, rice SnRK2 protein, is involved in activating the transcription factor OSBZ8 in the presence of NaCl, Spd and ABA. Hence, this kinase is an important candidate for further analysis to establish ABA-dependent signaling pathway during hyperosmotic stress.



**Fig. 9** Bar diagram depicting the comparative phosphorylation efficiency of MBP, casein, histone, and OSBZ8 by OSPDK. OSBZ8, MBP, and casein are shown to be the preferred substrates of OSPDK

#### Discussion

Understanding the mechanisms by which living organisms perceive environmental signals and transmit the signals to cellular machinery to activate adaptive responses is of fundamental importance to biology. Knowledge about



**Fig. 10** EMSA of 26-mer  $[^{32}\text{P}]$ -ABRE with purified, recombinant full-length 6X His: OSBZ8 (rOSBZ8) protein, showing varying binding intensities. **a** Equal amount of rOSBZ8 protein (200 ng) was incubated with equal amount and equal count of  $[^{32}\text{P}]$ -ABRE (80,000 CPM) in lane 3. As positive control, nuclear extract (NE) from Nonabokra (Stress) instead of rOSBZ8, was included in lane 2. **b** EMSA showing the enhancement of binding intensity of rOSBZ8 with 26 bp  $[^{32}\text{P}]$ -ABRE after addition of rOSPDK. rOSBZ8 was treated with  $[^{32}\text{P}]$ -ABRE (80,000 CPM) alone in lane 2. rOSBZ8 was pre-incubated, with purified rOSPDK in lane 3 and rOSPDK (activated in presence of Spd) in lane 4, respectively. Absence of

any DNA-binding property in Spd alone (i.e., without rOSBZ8) is shown in lane 4. **c** EMSA showing the increase in the binding intensity of rOSBZ8 with 26 bp  $[^{32}\text{P}]$ -ABRE with the addition of increasing amount of rOSPDK 50 ng (lane 2), 100 ng (lane 3), and 200 ng (lane 4). Complete absence of DNA–protein complex in lane 5 is observed, which contains rOSBZ8 + rOSPDK (activated in presence of Spd) + heparin. Free ABRE probe is shown in lane 1 in all the four autorads. The photographs **a**, **b** and **c** were obtained from the autoradiogram developed after 7 days and 24 h of exposure, respectively

stress signal transduction is vital for continued development of rational breeding and transgenic strategies to improve stress tolerance in crops. Plants combat abiotic stress by the accumulation of osmolytes, cellular elevation of natural polyamines, producing specific hormones like ABA, expressing different enzymes and transcription factors, regulating the function of different protein kinases, etc. All these molecules collaborate together to formulate an intricate network of cell signaling pathways (Zhu 2002). Among these signaling molecules, both PAs and protein kinases are known to be involved in the plant responses to external environmental stimuli including light, temperature, pathogen infection, growth regulation factor and nutrition deficiency. Generally, spermidine and spermine are present in millimolar concentrations, whereas putrescine levels are slightly lower (Krishnamurthy and Bhagwat 1989; Basu and Ghosh 1991; Igarashi and Kashiwagi 2000). Most polyamines within cells are bound to nucleic acids and other negatively charged structures; hence, their free and potentially “reactive” concentration is much lower than total concentration. Their titer varies from

approximately micromolar to more than millimolar, and depends greatly on environmental conditions, especially stress. Protein kinases play a key role in many stress-induced signaling cascades. They differ in their phosphoryl amino acid substrate specificity: some act at Ser/Thr residues, and some at Tyr residues. Over the past few years, many serine/threonine protein kinase genes involved in plant responses to abiotic stresses have been cloned from the higher plants (Batistic and Kudla 2004; Zhang and Lu 2003).

In our recent report (Gupta et al. 2012), we have shown the phosphorylation of a polyamine, NaCl and ABA induced 42-kDa protein in rice. As a follow-up work, we tried to identify the signaling pathway(s) involved in salinity stress, triggered by Spd and/or ABA. The present report concerns a protein kinase OSPDK, cloned from rice, which shares a high degree of homology with the reported plant protein kinase ASK1 (*Arabidopsis*), SAPK4 (*Oryza sativa* var. japonica) and NtOSAK (tobacco) (Mikoajczyk et al. 2000). Sequence analysis using GF Selector software revealed the presence GLU\_RICH (320–339 amino acid,

glutamic acid-rich) region with a serine/threonine protein kinase signature active site within the 119–131 AA position. This acidic GLU\_RICH region is assumed to be the probable polycationic spermidine-binding site. X-ray crystallographic and other biophysical studies will perhaps demonstrate the exact mode of interaction(s) between OSPDK and these exogenously applied chemicals (NaCl, ABA, and Spd). The results of SQ RT-PCR consistently showed that Nonabokra plants have four to five times higher level of Spd-induced OSPDK transcript in comparison to M-1-48. The distinct difference in expression pattern of *OSPDK* gene in the presence of NaCl, Spd and ABA in roots of salt-sensitive and salt-tolerant rice cultivars throws light on its positive regulatory role in ABA-induced salinity stress signaling pathway as well. The NaCl, Spd and ABA-induced accumulation of the transcript was more pronounced in M-1-48 than in Nonabokra, suggesting a regulation of expression of *OSPDK* gene at the transcription level. Roychoudhury et al. (2011) have demonstrated that the salt injuries, encountered in M-1-48 and Gobindohog, both of which showed greater susceptibility to salinity stress, were more pronouncedly alleviated and counteracted by the PAs (such as Spd) than the salt-tolerant Nonabokra. This might also explain the pronounced accumulation of *OSPDK* transcript in salt-sensitive M-1-48 than the salt-tolerant Nonabokra. In vitro phosphorylation studies (using [ $\gamma$ <sup>32</sup>P]ATP) of the expressed rOSPDK protein showed that this kinase was indeed autophosphorylated in the presence of NaCl/Spd/ABA. Inhibitor study, with different potent kinase inhibitors, and activity gel assay with MBP, histone and casein of the expressed kinase (rOSPDK) confirmed that the kinase belongs to SNF family of protein kinase involved in NaCl/ABA/Spd-mediated signaling pathway. Thus, *OSPDK* gene expression is regulated both at the transcriptional and translational level and Spd seems to play a vital role in both the central dogma processes. To our knowledge, there is no previous report of polyamine-mediated regulation of gene expression (other than genes involved in PA biosynthetic pathway in rice or other plants). Further experiments will reveal the exact mechanism by which Spd triggers the upregulation of *OSPDK* gene expression.

There are reports of protein kinases and phosphatases that participate in ABA signaling and regulate constitutively bound transcription factors on ABA-inducible genes by phosphorylation or dephosphorylation (Fujii et al. 2007). Lee et al. (1997) showed that an increase of ABA content was responsible for the cold-induced increase of Put content in rice plants. Moreover, the inhibition of ABA synthesis also reduced arginine decarboxylase (ADC) activity and Put content in these plants. The effect of ABA on PA levels was also demonstrated in ABA-treated sugarcane (Nieves et al. 2001), chickpea (Bueno and Matilla

1992) and wheat (Kovács et al. 2010). In the same way, a reduction in endogenous ABA levels, either as a result of mutation or due to the use of a chemical inhibitor, led to a decrease in PA levels in maize (Liu et al. 2005). Modulation of PA metabolism at transcriptional and metabolite level by ABA in response to water stress has been reported (Alcazar et al. 2006). All these data indicate that ABA controls PA levels, and this regulatory effect may be involved in the protective effect of ABA against cold and other abiotic stresses. Molecular dissection, including sequence comparison, and functional analyses like deletion and linker-scanning studies of most promoters of ABA-responsive genes, such as *Em*, *Osem* and *Rab16A*, have narrowed these elements down to ABA-responsive elements (ABREs), sequences of 8–10 bp with a core ACGT sequence (homologous to the G-box) located within 300 bp upstream of the transcription start sites and capable of mediating ABA-inducible transcription (Marcotte et al. 1989; Mundy et al. 1990). ABRE elements present in the sequence of *Em1a* (from *Em* gene of wheat) are considered as the strongest G-box element. In plants, a variety of basic leucine zipper (bZIP) class of trans-acting factors, comprising the largest known family of DNA-binding regulatory proteins, specifically bind to the cis-acting elements in vitro as dimers along with partner proteins and up-regulate a whole array of genes under its control (Jakoby et al. 2002). Such factors may be either present in pre-synthesized inactive conditions or they are synthesized de novo and then regulate transcription of their target genes (Shinozaki and Yamaguchi-Shinozaki 1997). Several such factors that have been identified and cloned include: wheat *EmBP-1*; rice *RITA-1* (in developing endosperm, especially in aleurone layer cells), *OSBZ8*, *osZIP-1a*, and *TRAB-1*; tobacco *TAF-1*; Arabidopsis *TGA1*, *ABFs* and so on. ABA-dependent post-transcriptional activation of such factors probably occurs through multisite phosphorylation by ABA-activated kinases at Ser/Thr residues in the conserved regions (Furihata et al. 2006).

We earlier reported post-translational activation of ABRE (from *Em* gene)-binding factor from the enhancement of complex formation in EMSA after the addition of spermidine, proline or ATP/GTP to the control nuclear extract from Pokkali (Gupta et al. 1998), from which we concluded that phosphorylation/dephosphorylation mechanism might be involved. In our previous communication (Mukherjee et al. 2006), we suggested that the ABRE-binding factor *OSBZ8* is highly expressed in salt-tolerant indica rice cultivars than in salt-sensitive varieties, and a positive correlation between the expression pattern of *OSBZ8* and salt tolerance was proposed. So, it was hypothesized that *OSBZ8* can be considered as a key factor interacting with ABRE-based promoter and thereby regulating the ABA or abiotic stress-inducible genes in

vegetative tissues. We also considered OSBZ8 (38.5 kDa as reported by Nakagawa et al. 1996) as the prime factor that targets Rab16A promoter (RoyChoudhury et al. 2008). With these information and our already established and published work on an ABRE-binding factor OSBZ8 (Mukherjee et al. 2006; RoyChoudhury et al. 2008), we proceeded with the project of exploring the possibility of phosphorylation of OSBZ8, an ABA- and NaCl-activated ABRE-binding bZIP class of transcription factor in rice, with our cloned SAPK4 homolog OSPDK. An interesting factor which motivated our study is that there are hardly reports, if any, of a common Spd and ABA-mediated signaling pathway in response to salinity stress in rice. Since rOSPDK, a rice SnRK2, was shown to be activated with Spd, NaCl and ABA, we wanted to explore the possibility of its interaction with OSBZ8. This kinase was indeed able to phosphorylate rOSBZ8 as evident from in-gel kinase and in vitro phosphorylation studies, which showed distinct phosphorylating bands. EMSA studies with 1XABRE probe showed that the DNA-binding activity of rOSBZ8 was also enhanced when pre-incubated with Spd-treated rOSPDK. Similar EMSA experiment with 4XDRE (dehydration responsive element) showed no complex formation (data not shown), thereby revealing its specificity for the ABRE *cis*-element. Heparin, a potent casein kinase II inhibitor, inhibited the 1XABRE–rOSBZ8 complex formation indicating that phosphorylation was indeed involved in this increase of binding capacity. In the control reaction, Spd alone did not show any complex formation. These experiments were repeated with purified GST protein (data not shown) to exclude the possibility of artifacts and all the results were negative. In vitro phosphorylation studies on rOSPDK after incubation with different concentrations of other monovalent and divalent cations did not give any positive signal pointing toward this protein's specificity for sodium chloride (data not shown). The activation of rOSPDK with Spd, ABA and NaCl under in vitro conditions leads us to assume that these compounds' interaction modulates the tertiary structure of rOSPDK kinase to induce phosphorylation. Since rOSPDK is activated in vitro by Spd, ABA and NaCl in the absence of other proteins, we presume that this rOSPDK protein kinase activity is directly regulated by the presence or absence of these three precursors. It is very surprising to note that three completely different compounds like Spd, ABA and NaCl, with variable ionic and structural properties, could give similar phosphorylation signal. Further biophysical experiments will perhaps throw light on this unique behavior of OSPDK. To the best of our knowledge, there is no previous report of such activation. Interestingly, we got exactly similar result with a 42-kDa protein kinase as reported earlier (Gupta et al. 2012). In our report, we had

presented the partial characterization of an SAPK from indica rice, which was activated both in vivo and in vitro by Spd, ABA and NaCl, but we had predicted it to be one among SAPK8-10. Surprisingly, OSPDK with similar molecular weight (42 kDa) to our earlier described protein shares its cDNA sequence homology with japonica rice SAPK4. This finding has led us to speculate whether Spd plays a similar regulatory role for other members of SAPK family and maybe other proteins. From the present study, it can be hypothesized that salinity stress, which causes an increase in the level of Spd and ABA in vivo, differentially activates members of SnRK2 group of protein kinases, which in turn upregulates the expression of many stress-responsive genes through stimulation of a wide array of transcription factors. One such mechanism is by phosphorylation of different trans-acting factors, as evident in this report by the activation of OSBZ8 by OSPDK. Similar activation of TRAB1 by SAPK10 was reported by Kobayashi et al. (2004), but unfortunately PAs' role which would have given a better picture was not studied. Very few researchers have studied the molecular role of higher polyamines in combatting abiotic stress. Most of the findings deal with the physiological or biochemical analysis (Roychoudhury et al. 2011). Although research findings on abiotic stress physiology have come up with the easy availability of genomics, proteomics, transcriptomics and metabolomics data, it seems to be a tip of an enormous iceberg. Co-relating all these data into a signal network model will be an uphill task and solving this will give a clearer picture of the intricate abiotic stress signaling network in the plant kingdom (Vinocur and Altman 2005). Critical to these findings will be to have a global analysis of gene expression of in vivo polyamine-treated plants.

All the results in the present study, although in vitro, clearly indicate that higher polyamines do play an important part in regulating the abiotic stress signaling of plants. This is the first report of polyamine-mediated phosphorylation of transcriptional regulator OSBZ8 by SNF1-type serine/threonine protein kinase SAPK4 homolog in indica rice. Overexpression of OSBZ8 and/or OSPDK in salt-sensitive rice cultivar or their downregulation in salt-tolerant rice cultivars will perhaps give more answers than raising questions on their regulatory role in rendering abiotic stress tolerance in rice.

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**Authors' contribution** BG performed the cloning, bacterial over-expression and purification of full length OSBZ8 and OSPDK cDNAs, SQ RT-PCR of OSPDK, all gel shift experiments, and also participated in activity and in vitro phosphorylation assays. KG designed and performed all the phosphorylation assays, performed necessary bioinformatics and statistical analysis and assisted BG in cloning and expression analysis. DNSG assisted in design of experiment, coordinated the study and procured research fund. BG and KG, both contributed equally in drafting the manuscript in its final format. All authors read and approved the manuscript.

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