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Structural and Expression Analysis of Salinity Stress Responsive Phosphoserine Phosphatase from *Brassica juncea* (L.)

Ram Singh Purty*, Madhav Sachar and Sayan Chatterjee

University School of Biotechnology, Guru Gobind Singh Indraprastha University, New Delhi, India

Abstract

Plants exposed to environment generally encounter various abiotic stresses which include drought, salinity, mineral deficiency, high and low temperature leading to reduction in growth and productivity. Phosphoserine phosphatase (EC 3.1.3.3) belongs to Haloacid Dehalogenase (HAD) superfamily and it catalyzes the last step in the phosphorylated pathway for the biosynthesis of L-Ser. In the present investigation, the full-length cDNA coding for phosphoserine phosphatase was cloned and purified from *Brassica juncea* L. Sequence analysis indicates that it encodes a protein of 296 amino acids and revealed high homology to PSP of *Arabidopsis thaliana*. Multiple sequence alignment analysis showed the presences of the three short conserved sequence motifs. Molecular modelling of BjPSP showed core α/β domain of Rossmann fold which is the characteristic of members of HAD superfamily. Western blot analysis showed that the level of BjPSP protein increased under different concentration of salinity stresses indicating its possible role during salinity stress in *B. juncea* L.

Keywords: Abiotic stress; Brassica; Amphidiploid; L-phosphoserine; Serine biosynthesis; Phosphorylated pathway

Introduction

Amino acid L-Ser plays an important role as building blocks of protein synthesis as well as acts as a precursor of a number of compounds required for cell proliferation, including amino acids, nitrogenous bases, phospholipids and sphingolipids [1]. L-Ser also participates as single-carbon (C1) donor in Tetrahydrofolate (THF) metabolism in plants [2]. Besides its involvement in metabolism, additional non-metabolic functions in plants and mammals have been documented. In mammals, L-Ser is the precursor of D-Ser, a neuromodulator [3]. In plants, D-Ser plays crucial role in signaling between the male gametophyte and pistil communication [4]. Recent findings have shown that deficiencies in molecules derived from L-Ser have drastic consequences. In plants, deficiencies in phosphatidylserine resulted in alteration in microspore development and high embryo abortion rate in *Arabidopsis thaliana* [5]. Therefore, for each of these metabolic pathways appropriate levels of L-Ser in all tissues is required to ensure complete plant development.

Biosynthesis of L-Ser in plants is carried out by three different pathways, the glycolate pathway, phosphorylated pathway and glycerate pathway. The glycolate pathway occurs in the mitochondria and is associated with photorespiration whereas the other two are nonphotorespiratory pathways [6]. In the glycolate pathway, glycine formed in peroxisomes enters mitochondrion where 2 molecules of glycine react to form one molecule of L-Ser. The Glycine Decarboxylase Multienzyme Complex (GDC), along with the enzyme Serine Hydroxymethyltransferase (SHMT), is responsible for the respiratory conversion of glycine to yield L-Ser, CO_2, NH_3 and NADH [1,7,8].

In nonphotorespiratory glycerate pathway, synthesis of L-Ser takes place by dephosphorylation of 3-phosphoglycerate (3-PGA) and includes the reverse sequence of reaction from part of the photorespiratory cycle, from 3-PGA to L-Ser (3-PGA-glycerate-hydroxypyruvate-Ser). These reactions are catalyzed by enzyme 3-PGA phosphatase, glycerate dehydrogenase, Ala-hydroxypyruvate aminotransferase, Gly hydroxypyruvate aminotransferase [9]. Some of the genes encoding these enzymes have been cloned as well as their activity has been reported [9,10]. However, the extent to which this pathway is functionally important in plants remains unknown.

In second nonphotorespiratory phosphorylated pathway, synthesis of L-Ser from 3-PGA occurs in the plastids. It involves three enzymes that catalyze three sequential reactions, e.g., 3-phosphoglycerate dehydrogenase (PGDH), 3-Phosphoserine Aminotransferase (PSAT) and 3-Phosphoserine Phosphatase (PSP). In non-photosynthetic tissues (e.g. roots, developing and germinating seeds, etc.), the glycolytic intermediate, 3PGA is oxidised by PGDH to form 3-phosphohydroxypyruvate (3PHP), which in turn is converted to 3- phosphoserine by PSAT. In the final step, dephosphorylation of 3-phosphoserine is catalyzed by 3-phosphoserine phosphatase [11]. The phosphorylated pathway is conserved in mammals, bacteria and plants where it defines a branching point for 3-PGA from glycolysis.

Out of the three pathways for L-Ser, glycolate pathway has been considered to be of major importance, whereas nonphotorespiratory phosphorylated pathway has been completely neglected. Genetic and physiological evidences were lacking until recently, when the functional characterization of the first and last enzyme of phosphorylated pathway was achieved. Expression of these genes in both photosynthetic and non-photosynthetic organs like root-apical meristem, vasculature, embryo, anthers, stigma and pollen grains, indicates that phosphorylated pathway is the only source of L-Ser for these cells [12-14]. These findings on the biological function of the phosphorylated pathway indicate that it has an important function in plant metabolism and development. However, the significance of the phosphorylated pathway under different environmental conditions like salinity, drought, high and low temperature is still unknown.

Therefore, in order to study the response of gene involved in

***Corresponding author:** Ram Singh Purty, Ph.D., University School of Biotechnology, Guru Gobind Singh Indraprastha University, New Delhi, India, Tel: +91-9818584097; Fax: +91-11-25302304; E-mail: rspurty@ipu.ac.in

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the phosphorylated pathway under salinity stress, we targeted Phosphoserine Phosphatase (PSP) (EC 3.1.3.3), a metalloprotein, that catalyzes the last step in the biosynthesis of L-Ser. It belongs to the Haloacid Dehalogenase (HAD) superfamily. All the members of the HAD superfamily are characterized by three short conserved sequence motifs. The residues of these motifs cluster together to form the active site. It utilizes Mg^{2+} ion to catalyze the hydrolysis of L-phosphoserine [6,15]. In the present investigation, full length cDNA encoding phosphoserine phosphatase has been cloned and purified from *Brassica juncea* L. *In silico* characterization of phosphoserine phosphatase based on structural homology with known crystal structure as well as its expression under different concentration salinity stress was also studied.

Materials and Methods

Plant material and stress treatment

Seeds of *Brassica juncea* L. were pretreated with antifungal agent and surface sterilized with 0.1% $HgCl_2$ prior to germination in a hydroponic system for 48 h in darkness. For further growth, seedlings were germinated under control condition, i.e., (25 ± 2°C, 12 h light and dark cycle). For salinity stress treatment, 7 days old seedlings were treated with 200 mM NaCl for 24 h using a hydroponic system. Simultaneously, seedlings maintained in de-ionized water were taken as control. After treatment, seedlings were harvested for extraction of total RNA.

PCR cloning of full length cDNA of PSP from *Brassica juncea*

Total RNA was isolated from 7 d old salinity stressed seedlings using the TRIzol method [16]. For cDNA synthesis, the total RNA was used for first-strand cDNA synthesis following the manufacturer's protocol (Fermentas, EU). Sequence-specific primers were designed and used for PCR cloning of full-length PSP cDNA from *B. juncea*. The primer sequences used for PCR cloning were 5'-ATGGAAGCGTTACTTACTTC-3' as forward and 5'-TTAGTCCAACGAGTTTAGGA -3'as the reverse primer. PCR amplification was carried out in a 0.5 ml PCR tube (Axygen Inc., USA) containing 100 ng template cDNA, 50 ng each of forward and reverse primers, 200 µM dNTPs (Fermentas, EU), 1.5 mM $MgCl_2$ and 1U Taq DNA polymerase (Fermentas, EU) in 25 µl of the reaction volume in a thermal cycler (iCycler®, BioRad Laboratories Inc., USA). PCR amplification was carried out using the following conditions: Initial denaturation for 5 min at 94°C, followed by 35 cycles each having denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. Final extension at 72°C for 7 min at the end. The PCR amplification product obtained was separated in 1% agarose gel containing ethidium bromide (10 µg/ml). PCR amplified fragment from agarose gel was eluted and cloned in TA cloning vector (Fermentas, EU). Positive clones were confirmed using colony PCR as well as by restriction digestion. Plasmid DNA was isolated from the positive clone and sequenced using universal M13 forward primer.

Sequence and phylogenetic tree analysis

Sequence analysis, translation and molecular mass calculation, amino acid composition, pI value prediction were carried using ProtParam (http://us.expasy.org/tools/protparam.html). NCBI server was used for BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). To study the sequence homology and its relationship with other PSP including plants and *Homo sapiens*, multiple sequence alignment was carried out in MEGA6 software using MUSCLE algorithm [17]. The final figure of the alignment was prepared using a multiple sequence alignment editor, Jalview. Phosphoserine phosphatase sequences from plants included *Brassica juncea* (ADM15724.2), *Arabidopsis thaliana* (BAA33806), *Oryza sativa indica* (EEC69328), *Populus trichocarpa* (XP002318590), *Zea mays* (ACG28371), *Sorghum bicolor* (AAD27569) and *Homo sapiens* (CAA71318). In the present study, the aligned sequences were used to construct phylogenetic tree by applying the neighbour-joining method in MEGA6 software [17].

Prediction of secondary structure and subcellular localization

Secondary structure prediction of a *BjPSP* protein was carried out using SOPMA [18]. Prediction of protein subcellular localization was carried out with WoLF PSORT (http://wolfpsort.org/), Cell eFP Browser (http://142.150.214.117/cell_efp/cgi-bin/cell_efp.cgi), TargetP 1.1 prediction (http://www.cbs.dtu.dk/services/TargetP/) and ChloroP1.1 analysis (http://www.cbs.dtu.dk/services/ChloroP/).

Homology modelling of *BjPSP* protein

The peptide sequences of PSP from *Brassica juncea*, (NCBI GenBank accession no. ADM15724.2; UniProt accession No. E1ACV8) and other sequences examined in this study were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) and UniProt (http://www.uniprot.org/). Structurally homologous subsets of the experimentally determined 3D structures of the PSP proteins were retrieved from the PDB database [19] via SWISSMODEL [20-22] server at Swiss Institute of Bioinformatics, Switzerland. Template search with BLAST [23] and HHBlits [24] was performed against the SWISSMODEL Template Library (SMTL). For each identified template, the template's quality was predicted from features of the target template alignment. The templates with the highest quality were then selected for model building. Models were built based on the target template alignment using PromodII. Coordinates conserved between the target and the templates were copied from the template to the model. Insertions and deletions were remodelled using a fragment library. Side chains were then rebuilt. Finally, the geometry of the resulting model was regularized by using a force field. In case loop modelling with ProModII [25] does not give satisfactory results, an alternative model is built with MODELLER [26]. Multiple tools were used in validation of protein structure and models by verifying the parameters like Ramachandran plot quality [27] at RAMPAGE [28] and Verify 3D [29]. The binding sites on the *BjPSP* protein were also studied using 3D LigandSite [30].

Purification and confirmation of recombinant *BjPSP* protein by Western blot analysis

For directional cloning of *BjPSP* cDNA into pET 28b expression vector (Novagen, Madison, WI, USA), sequence specific primers carrying the restriction sites were designed. The primer sequences used for PCR cloning were 5'-GCTAGCATGGAAGCGTTACTTACTTC-3' as forward having Nhe I site and 5'-GCGGCCGCTTAGTCCAACGAGTTTAGGA -3' as the reverse primer having Not I. All the conditions for PCR cloning and the protocol for gel elution were same as describe above. The eluted product was digested stepwise with specific restriction enzyme and cloned into pET 28b expression vector. The construct *BjPSP* cDNA: pET 28b was stabilized first in *E. coli* strain XL-1 blue and then cloned into *E. coli* strain BL21 (DE3) competent cells. For expression of the recombinant protein in BL21, the condition used was 0.5 mM IPTG, at 20°C, overnight. Cells were harvested by centrifugation at 8000 rpm and lysed by resuspending in lysis buffer containing 50 mM Tris. HCl pH 7.5, 200 mM NaCl. Lysate obtained was passed through a Ni-NTA column. The pET 28b expression vector used in the present study carries an N-terminal 6xHis-Tag which was used for purification by Ni-NTA

affinity column. The bound proteins were eluted with elution buffer containing 50 mM Tris. HCl pH 8.0, 200 mM NaCl, 250 mM Imidazole. The different fractions obtained were dialysed three times with dialysis buffer (50 mM Tris. HCl pH 8.0, 200 mM NaCl) and separated in 12% SDS-PAGE. The gel was stained with 0.125% w/v coomassie brilliant blue R-250 (Sigma-Aldrich, USA) in 50% v/v methanol and 10% v/v acetic acid, and destained in 50% methanol containing 10% acetic acid. The positive fractions were concentrated using Amicon ultra 15 centrifugal filter units (EMD Millipore, USA) and further confirmed using Western blot analysis.

For Western blot analysis, recombinant protein fractions obtained were estimated following the Quick Start™ Bradford protein assay instruction manual (Bio-Rad Laboratories, CA, USA) and separated in 12% SDS-PAGE. Each lane was loaded with 80 µg total proteins and run at 100 V for 2 h. Proteins separated were electro-transferred to Hybond LFP (PVDF) membrane and reacted with PSPH (3G12) monoclonal antibody (SantaCruz Biotech. Inc) which was raised against recombinant phosphoserine phosphatase of human then visualized by means of goat anti-mouse IgG-HRP (Santa Cruz Biotech. Inc., USA). Prestained molecular weight markers were used (Fermentas, EU).

Immunodetection of *BjPSP* protein under salinity stress

Western blot analysis was performed as above using PSPH (3G12) monoclonal antibody for immunodetection of *BjPSP* protein under salinity stress in the 7 days old seedlings subjected to 100, 200, 300 and 400 mM NaCl for 24 h and compared with the unstressed seedlings. Prestained molecular weight markers were used (Fermentas, EU). Beta-actin was used as the loading control. The blots were imaged with AlphaImager HP system (Alpha Innotech, CA, USA) and the densitometric analysis of protein bands was carried out using Alpha View® imaging and analysis software. The data shown are representative of three independent experiments. Data are presented as the mean ± SD of three independent experiments.

Results

PCR cloning of *BjPSP* cDNA and sequence analysis

Brassica juncea seedlings treated with 200 mM NaCl for 24 h wilted whereas unstressed seedlings remained greener and healthier. Total RNA isolated from salinity stress treated seedlings was used for cDNA synthesis. Using sequence specific primers, PCR amplification was carried out and an amplification product of molecular weight ~891bp was obtained when separated in 1% agarose gel. Amplification product was cloned and the presence of the insert in the positive clone was confirmed though restriction digestion and sequencing. The sequence obtained was submitted to GenBank with the accession number HM627514.

Sequence analysis showed the full-length cDNA sequence to be 891 bp long which encodes a deduced polypeptide of 296 amino acids with a predicted molecular mass of 32.25 kD and a pI value of 7.49. The most frequent amino acid in deduced *BjPSP* protein was Leu (11.5% by frequency), followed by Ala (9.1%), Val (8.8%), and Gly (8.4%) and Ser (8.1%). Negatively charged residues (Asp+Glu) constituted 13.01% of the polypeptide, while positively charged residues (Arg+Lys) constituted 12.16% of the polypeptide. The protein was computed to have an instability index of 39.09, which classified the protein as stable.

Homology search and phylogeny analysis

Proteins homologous to *BjPSP* protein using the BLASTP algorithm against a non-redundant protein database, showed several sequence homology with the *BjPSP* protein sequence, out of which maximum of 17 hits are of *Homo sapiens* and in plants, 9 hits were of *Arabidopsis thaliana*, 10 hits of *Oryza sativa* cv. japonica, 2 hits of *Oryza sativa* cv. indica, 3 hits each of *Populus trichocarpa*, *Sorghum bicolor* and *Zea mays*. Multiple sequence alignment exhibited high levels of similarity and the presence of conserved Haloacid Dehalogenase (HAD) like domain (Figure 1a). *BjPSP* protein showed the presence of three short

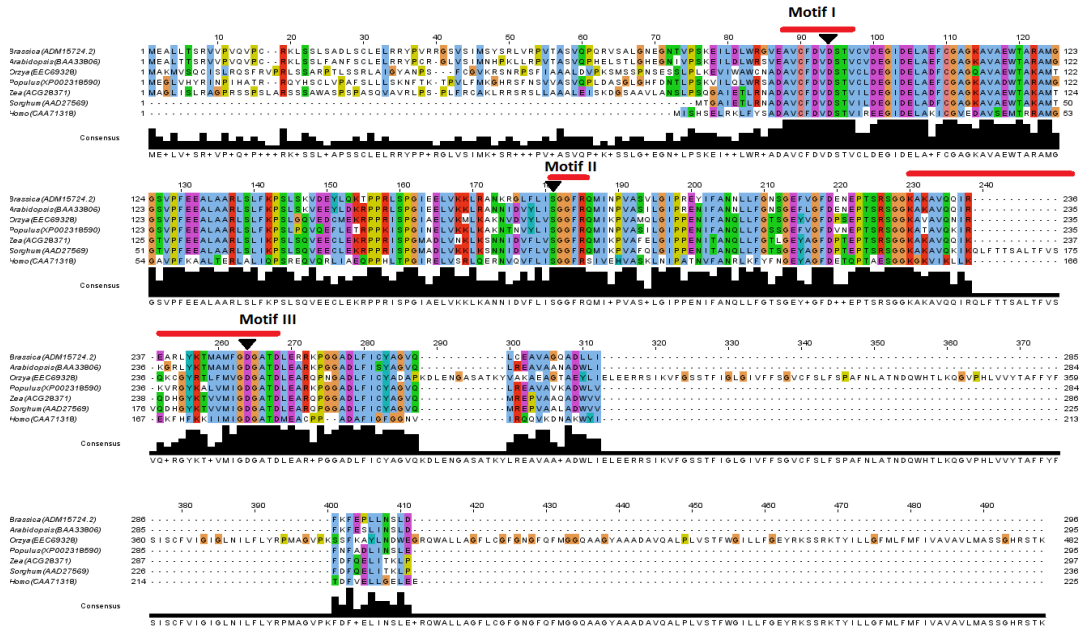

Figure 1a: Multiple alignment of the deduced amino acid sequences of *BjPSP* protein (ADM15724.2) and other phosphoserine phosphatase (PSPs). Amino acid sequences were aligned as follows: *Arabidopsis thaliana* (BAA33806), *Oryza sativa indica* (EEC69328), *Populus trichocarpa* (XP002318590), *Zea mays* (ACG28371), *Sorghum bicolor* (AAD27569) and *Homo sapiens* (CAA71318). The completely identical amino acids were indicated with identical color. Conserved motifs I, II and III are indicated by single red line on the top of the alignment. Active site contains conserved residue is indicated by black triangles.

conserved sequence motifs, in which aspartate residue is present in the conserved D*X*DST motif I, Ser179 in motif II and the third GDG*XX*D motif showed the presence of an aspartate residue at 249 positions. BLASTP analysis showed that *BjPSP* protein had 47% homology to PSP of *Homo sapiens* (CAA71318) whereas with other plants PSPs which includes *Arabidopsis thaliana* (BAA33806), *Oryza sativa* cv. *indica* (EEC69328), *Populus trichocarpa* (XP002318590), *Zea mays* (ACG28371) and *Sorghum bicolor* (AAD27569) showed 86, 53, 66, 57, 64% similarity, respectively.

The phylogenetic tree was generated using the deduced amino acid sequence of *BjPSP* gene. Neighbor-joining evolutionary tree showed the relationship of PSP from *Brassica juncea* (HM627514) with *Arabidopsis thaliana* (BAA33806), *Oryza sativa* cv. *indica* (EEC69328), *Populus trichocarpa* (XP002318590), *Zea mays* (ACG28371), *Sorghum bicolor* (AAD27569) and *Homo sapiens* (CAA71318). The tree indicated that the PSP of *Brassica juncea* is closely related to *Arabidopsis thaliana* and *Populus trichocarpa* (Figure 1b).

Secondary structure and subcellular localization prediction of *BjPSP* protein

The secondary structure of *BjPSP* protein was analyzed by SOPMA and the result showed that the putative *BjPSP* peptide consisted of 49.66% alpha helix, 12.84% extended strand, 7.43% beta turn, and 30.07% random coil. The random coil constituted the major part of the secondary structure, while alpha helix was the basic element of both N and C terminal parts. WoLF PSORT and Cell eFP Browser prediction showed the subcellular localization of *BjPSP* protein in chloroplast region of the cell (Supplementary Figure S1). This was further confirmed by TargetP 1.1 prediction (http://www.cbs.dtu.dk/services/TargetP/) and ChloroP1.1 analysis (http://www.cbs.dtu.dk/services/ChloroP/) which showed that *BjPSP* protein contained an N-terminal chloroplast transit peptide of about 54 residues in length with a CS score of 2.576, suggesting that this protein may be located in the chloroplast.

Comparative modelling of *BjPSP* protein

The structure of phosphoserine phosphatase in *Brassica juncea* is not available in PDB. Therefore, we predicted the structure based on template based homology modelling with the peptide sequence of *BjPSP* protein (GenBank Acc. No. ADM15724.2). Molecular modelling was done using SWISS-MODEL and the obtained structure was validated using Ramachandran plot and Verify 3D. A total of 696 templates were found to match the peptide sequence of *BjPSP* protein. This list was filtered by a heuristic down to 65 and the top twelve templates were ranked by their sequence identity. The prospective templates thus were 1nnl (Human Phosphoserine Phosphatase; Resolution: 1.53 Å; R-plot outliers: 0), 1l8l (conformational rearrangement of Human Phosphoserine Phosphatase; Resolution: 2.51 Å; R-plot outliers: 6.1%) and 1l8o (conformational rearrangement of Human Phosphoserine Phosphatase; Resolution: 2.80 Å; R-plot outliers: 5.9%). Among this 1nnl showed the highest sequence similarity by BLAST, the best resolution and no outliers in the R-plot. The template 1nnl showed the maximum sequence identity of 47.53% and coverage of 0.75 ranging from 72-296 with the target *BjPSP* peptide sequence. Thus 1 nnl was chosen further as a template for the molecular modelling. The structure modelling was done using SWISS MODEL server and resulted into a high resolution structure of *BjPSP* protein. Validation of obtained model was carried out using Ramachandran plot and Verify 3D. The Phi/Psi angles of the amino acids that determine the secondary structural property of the hypothetical proteins were computed and represented as Ramachandran plot. The residues were classified according to its regions in the quadrangle. The Ramachandran map of *BjPSP* protein and the plot statistics showed zero outliers in R-plot. The Ramachandran plot showed that 95.4% of the residues were located in the most favoured regions and 4.6% in the additional allowed regions (Figure 2). No residues were found in the generously allowed regions or in the disallowed regions. The modelled structure for *BjPSP* protein showed 100% of the residues scored ≥ 0.26 in the 3D-1D profile and thus qualifying as a very good structure w.r.t. passing these structure quality checks (Supplementary Figure S2).

Model of *BjPSP* protein was built based on the alignment of peptide sequence of *BjPSP* protein and 1nnl template (Figure 3). One monomer of *BjPSP* protein contains 296 residues and has a relative molecular mass of 32.25 kDa. The ribbon diagram shows that a monomer of

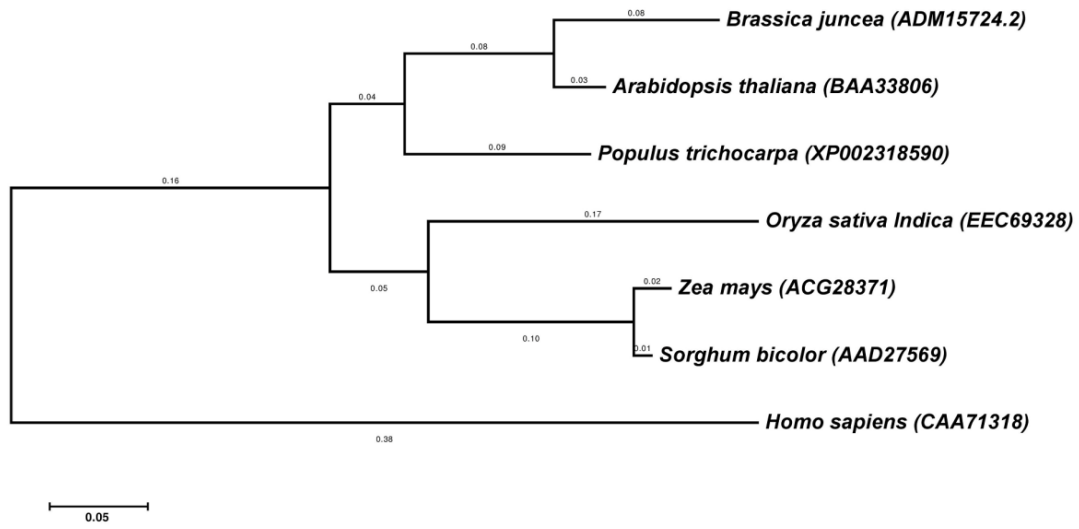

Figure 1b: Neighbor-joining evolutionary tree showing relationship of phosphoserine phosphatase from *Brassica juncea* (ADM15724.2) with *Arabidopsis thaliana* (BAA33806), *Oryza sativa Indica* (EEC69328), *Populus trichocarpa* (XP002318590), *Zea mays* (ACG28371), *Sorghum bicolor* (AAD27569) and *Homo sapiens* (CAA71318). It showed PSP of *Brassica juncea* is closely related to *Arabidopsis thaliana* and *Populus trichocarpa*.

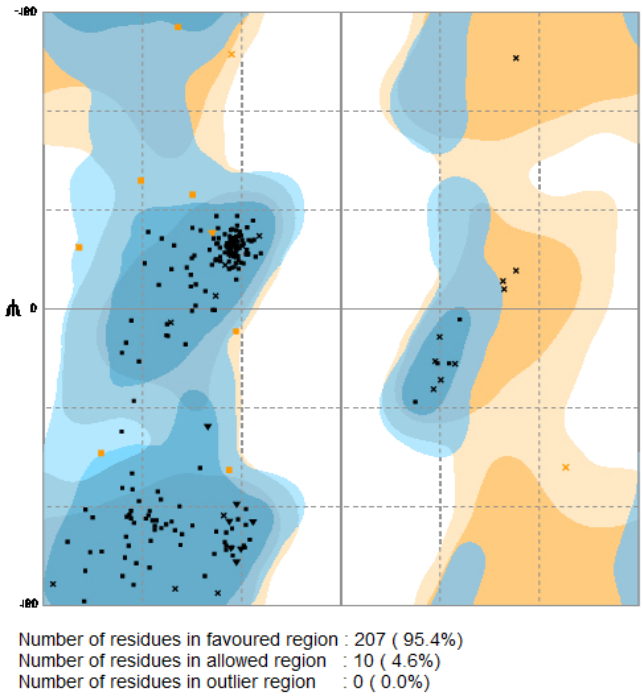

Figure 2: Ramchandran plot of *BjPSP* protein (ADM15724.2) showing zero outlier. No residues are found in generously allowed or in additional allowed region.

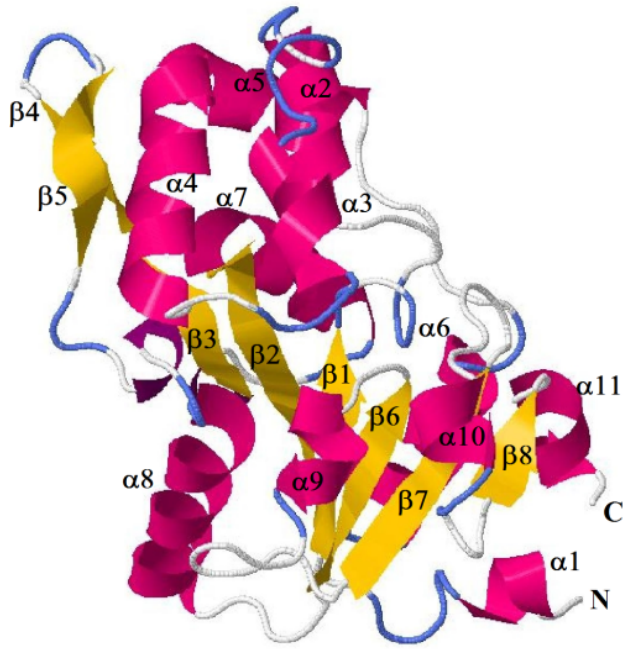

Figure 3: Ribbon diagram of the overall three-dimensional structure of *BjPSP* protein. The β-strands are numbered *β1-β8* and the α-helices *α1-α11*. The helices on top *α2, α4* and *α5* form the helix bundle and the dmer interface *β4-β5*. The lower part contains the core domain which resembles a Rossmann fold.

BjPSP protein consisting of two major domains. One domain resembles a Rossmann fold [31] and the second domain consists of the dimer-interface part and a helix bundle. The core α/β domain, which resembles a Rossmann fold, displays a six-stranded parallel β-sheet (β1-β3 and β6-β8) surrounded by three α-helices on one side (α8-10) and four α-helices on the other side (α1, α6, α7, α11). The dimer-interface part is located in strands β4 and β5 and the helix bundle of *BjPSP* protein consist of α2, α4 and α5. The binding sites on the *BjPSP* protein was also studied using 3DLigandSite (Supplementary Figure S3a-S3g). *BjPSP* protein showed binding sites for Mg^{2+}, Ca^{2+} and phosphate along with its interacting residues (Table 1).

Purification of recombinant *BjPSP* protein

Expression of the recombinant protein was carried out in *E. coli* strain BL21 (DE3). For induction of the recombinant protein, 0.5 mM of IPTG concentration at 20°C was found to be optimum (Supplementary Figure S4). For large scale purification, the same condition was followed and the eluted fractions upon separation in 12% SDS-PAGE revealed an induced recombinant protein band with a molecular weight of around 35 kDa (Figure 4a). Further, using monoclonal antibody raised against phosphoserine phosphatase of human (PSPH) the induced recombinant protein was confirmed to be *BjPSP* protein through western blot analysis. The antibodies cross reacted with the recombinant *BjPSP* protein of around 35 kDa indicated the possible structural homology between PSPH and *BjPSP* protein (Figure 4b).

Accumulation of *BjPSP* protein under salinity stress

Western blot analysis using PSPH monoclonal antibody revealed the presence of PSP in the total protein fractions of the salinity stresses seedlings. Accumulation of PSP in unstressed seedlings was found to less as when compared to salinity stresses seedlings. Signals indicated expression of PSP in 100, 200 and 300 mM was higher than when compared to seedlings treated with 400 mM NaCl (Figure 5).

Discussion

Cloning and purification of phosposerine phophatase of *Brassica juncea*

In the plant kingdom molecular cloning of PSP from *Arabidopsis* was successfully achieved by functional complementation of *E. coli ser B* mutant SK472 [32]. The open reading frame of *AtPSP* was found to be 885 nucleotides, encoding 295 amino acids with a putative organelle targeting pre-sequence in its N-terminal region [32]. In the present investigation, the full-length cDNA sequence of *BjPSP* was found to be 891 bp long which encodes for a 296 amino acid protein. Subcellular localization prediction of *BjPSP* protein showed the presence of N-terminal chloroplast transit peptide of about 54 residues in length indicating its presence in the chloroplast region of the cell. BLASTP analysis showed high sequence homology to PSP of *Homo sapiens*, *Arabidopsis thaliana, Oryza sativa*, *Populus trichocarpa*, *Sorghum bicolor* and *Zea mays* indicating that all are structurally similar and display a conserved fold [31]. The neighbour-joining evolutionary tree analysis showed PSP of *Brassica juncea* to be closely related to PSP of *Arabidopsis thaliana.*

All members of Haloacid Dehalogenase (HAD) superfamily are characterized by the presence of three conserved sequence motifs i.e., motif I, DX(D/T/Y)X(T/V)(L/V); motif II, (S/T); and motif III, K(G/S)(D/S)XXX(D/N) and the residues of these motifs cluster together to form the active site [15,33]. Sequence analysis of *BjPSP* protein showed the presence of the three short conserved sequence motifs and aspartate residue in the conserved D*X*DST motif. A nucleophilic aspartate residue present in the first conserved D*X*DST motif is used in

Site	Residues	Avg. MAMMOTH scores (7)	Ligand	Reference structure (S)	Reference figure
1	92ASP,179SER, 180GLY,181GLY	23.2	ADP	1t5t_A,2zbd_A,2oa0_A,3fps_A	Figure S3a
			MG	1t5t_A,1t5s_A,2zbd_A,3b8c_A,2c8k_A	
			CA	3ba6_A	
2	92ASP, 249ASP	23.9	MG	2zbg_A,3fgo_B,3fpb_A,1wpg_D,2o9j_A, 3b9r_B	Figure S3b
3	116GLU,123GLY,182PHE	26.3	ADP	1wpg_D	Figure S3c
			ATP	3fpb_A	
4	249ASP	24.3	MG	2zxe_A,3a3y_A	Figure S3d
5	250GLY,252THR	24.1	MG	2dqs_A	Figure S3e
6	252THR	23.5	MG	2by4_A	Figure S3f
7	249ASP	22.6	MG	1xp5_A	Figure S3g

Table 1: Ligand binding and interacting residues of *BjPSP* protein.

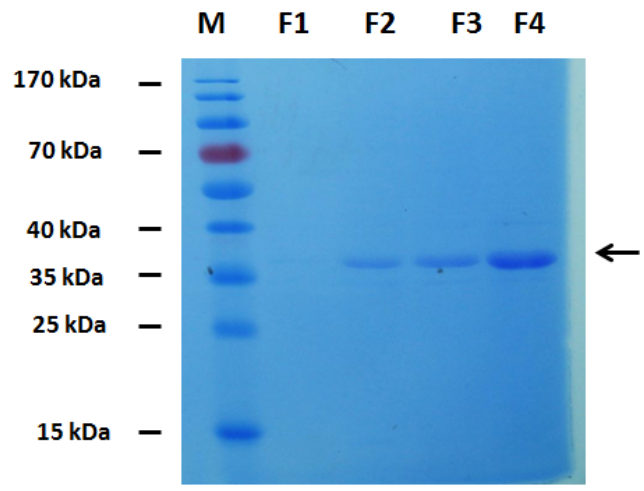

Figure 4a: SDS PAGE gel showing the separation of purified *BjPSP* protein of around 35 kDa indicated by arrow. Proteins were separated in 12% gel and stained with Coomassie Brilliant Blue. Lane 1: Prestained protein ladder (M), Lane 2-5: Purified protein fraction (F1-F4).

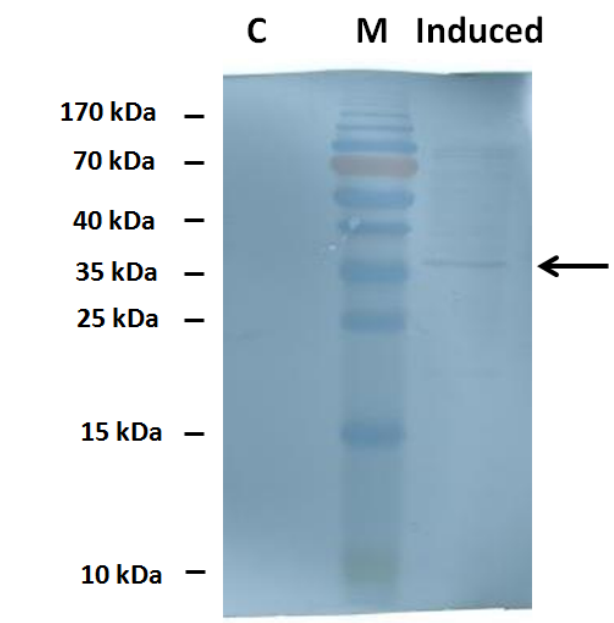

Figure 4b: Western blot analysis of recombinant *BjPSP* protein using PSPH monoclonal antibody. Colorimetric detection showing a good signal around 35 kDa (indicated in arrow) confirms the presence PSP in *Brassica juncea*. Prestained protein ladder (M) and un-induced control (C) are indicated.

the phosphoryl transfer reaction [15]. Replacement of the first aspartate of the DXDST motif by asparagine or glutamate resulted in complete inactivation of the enzyme, suggesting that the first aspartate of this motif is the phosphorylated residue in human PSP [15]. The second motif of *BjPSP* protein contains a conserved Ser179 residue which is important for catalytic functions. Mg^{2+} is required for catalytic activity. The third GDGXXD motif contains a strictly conserved aspartate and lysine residue. In the case of *BjPSP* protein the third GDGXXD motif showed the presence of an aspartate residue at 249 positions. Mutagenesis studies on these conserved residues show that all three motifs play an important role in the catalytic process [34,35]. Members of the HAD superfamily are sometimes referred to as belonging to the DDDD superfamily of phosphohydrolases. The conserved regions of *BjPSP* protein (DXDST and GDGXXD), showed high similarity to amino acid sequences of PSP from other organisms.

Upon cloning and expression of phosphoserine phosphatase from human in *E. coli*, it was found that the recombinant PSPH is of 25 kDa [36]. In the present study, a heterologous expression system was used for expression of *BjPSP* cDNA in *E. coli* strain BL21 and further facilitated to obtain highly purified recombinant *BjPSP* protein by Ni-NTA purification system. To confirm the expressed recombinant protein, Western blot analysis was carried out with monoclonal antibody raised against phosphoserine phosphatase of human (PSPH). The developed blot showed the presence of a single band of around 35 kDa indicating the possible structural homology between PSPH and *BjPSP* protein. Sequence and BLASTP analysis already showed that *BjPSP* protein had 47% homology to PSP of *Homo sapiens* (CAA71318). Sequence analysis showed the full-length *BjPSP* cDNA sequence to be 891 bp long which encodes a deduced polypeptide of 296 amino acids with a predicted molecular mass of 32.25 kD. This increase in molecular mass of recombinant *BjPSP* protein could be due to the presence of hexa histidine tag in an N-terminal in pET 28b expression vector used in the present study.

Structural analysis of phosposerine phophatase of *Brassica juncea*

The crystal structure of plant PSP is not available till date. It was first reported from *Methanococcus jannaschii* [37,38] and later in *Homo sapiens* [39,40]. Therefore, in the present investigation, we predicted the structure of PSP of *Brassica juncea* for the first time based on template based homology modelling using the peptide sequence

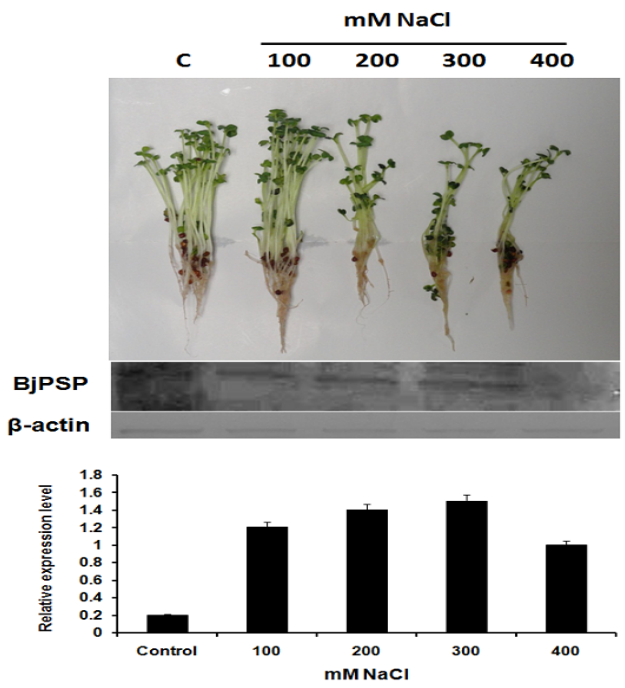

Figure 5: Western blot analysis of total proteins from seedlings subjected to various concentration of NaCl, using PSPH monoclonal antibody. Lane 1: Unstressed Control (C), Lane 2: 100, Lane 3: 200, Lane 4: 300 and Lane 5: 400 NaCl (mM). Colorimetric detection showing a signal (indicated by arrow) revealed the accumulation of *BjPSP* protein in response to all concentration of NaCl treatment for 24h. Beta-actin was used as the loading control. The data shown are representative of three independent experiments. Data are presented as the mean ± SD of three independent experiments.

of *BjPSP* (GenBank Acc. No. ADM15724.2). Multiple sequence alignment analysis of PSP sequence from *Brassica juncea* with other plant species showed high sequence homology whereas it showed only 47% similarity with HPSP. All known structures of enzymes belong to HAD superfamily display a conserved Rossmann fold [31]. Indeed, 2-haloacid dehalogenase from *Pseudomonas* sp. YL and *Xanthobacter autotrophicus* [41,42], phosphonoacetaldehyde hydrolase from *Bacillus cereus* [43], soluble epoxide hydrolase [44] the Ca^{2+}-P-type ATPase [45], β-phosphoglucomutase from *Lactococcus lactis* [46], phosphoserine phosphatase (PSP) from *Methanococcus jannaschii* [37,38] and HPSP [39,40], all have a core α/β domain resembling the NAD(P)-binding Rossmann fold. In the present studies, predicted structure of *BjPSP* protein also showed the core α/β domain, which resembles a Rossmann fold, displays a six-stranded parallel β-sheet (β1- β3 and β6- β8) surrounded by three α-helices on one side (α8-10) and four α-helices on the other side (α1, α6, α7, α11). The HAD superfamily members, except for 2-haloacid dehalogenases [41,42], utilize Mg^{2+} as a cofactor during catalysis. In the present study, *BjPSP* protein showed the binding sites for Mg^{2+}, Ca^{2+}, phosphate using 3DLigandSite indicating that despite the low overall sequence homology with HPSP all the members of the HAD superfamily are structurally similar and display a conserved fold.

Effect of salinity stress on the expression of PSP of *Brassica juncea*

Plants growing in the field are constantly exposed to various environmental stresses which include drought, salinity, high and low temperature. Of the various abiotic stresses plant perceive both osmotic and as well ionic stress when subjected to salinity stress [47,48]. The response to these stresses can be seen to occur at cellular, biochemical or molecular level. Cellular responses include modifications of the cell wall structure and undergo changes in cell division. Biochemical response includes production of compatible solutes like proline and glycine betaine that are able to stabilize proteins and cellular structures and/or to maintain cell turgor by osmotic adjustment [49], At the molecular level the expression of genes either gets up-regulated or down regulated resulting in the tolerance or susceptibility of plant [50,51]. Under extreme environmental condition plants accumulate higher amounts L-Ser since it is a precursor of many compounds as well as a building block for protein synthesis [6]. In the present investigation, the expression of *BjPSP* protein was altered when the seedlings were subjected to different concentration of salinity. In comparison to unstressed treated seedlings, the level of expression drastically increased upon 24 h of salinity stress treatment indicating that *BjPSP* protein to be stress inducible. The role of phosposerine phosphatase in embryo, pollen, and root development has been well documented and lack of its activity has affected the pollen and tapetum development in *Arabidopsis thaliana* [52,53]. In the present investigation, the level of *BjPSP* protein accumulation varied from 5-3 fold increase under the different salinity stress treatment. Based on their relatively high abundance and their rapid inducibility and accumulation of protein in the seedling during salinity stress treatment indicate that phosphoserine phosphatase, which catalyses the last and irreversible step in the phosphorylated pathway for the biosynthesis of L-Ser, may play major role during salinity stress tolerance.

Conclusion

Plant abiotic stress tolerance is due to cumulative responses at physiological, molecular and biochemical levels, which are often considered to be a complex process. The molecular responses are mainly based on the modulation of transcriptional activity of stress-related genes which are induced under stress conditions and protect the plants during extreme environmental condition. In the present investigation, we have cloned and purified phosphoserine phosphatase from *Brassica juncea* from salinity stressed 7 days old seedlings. Immunodetection analysis showed that it is inducible under different concentration of salinity, indicating its role during salinity stress. Further, we have predicted the tertiary structure of *BjPSP* protein and showed its interacting partners. Phosphoserine phosphatase of *Brassica juncea* showed sequence and structural similarities with other members of the HAD superfamily. Structural studies of stress inducible protein are important because it will help us in understanding the molecular mechanism of abiotic stress tolerance in plants.

Author Contributions

MS and RSP did the cloning experiment. SC and RSP did the *in silico* analysis. RSP designed the research project, performed the majority of the experiments and wrote the manuscript.

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