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Pea lectin receptor-like kinase promotes high salinity stress tolerance in bacteria and expresses in response to stress *in planta*

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Abstract The plant lectin receptor-like kinases (LecRLKs) are involved in various signaling pathways but their role in salinity stress tolerance has not heretofore been well described. Salinity stress negatively affects plant growth/productivity and threatens food security worldwide. Based on functional gene-mining assay, we have isolated 34 salinity tolerant genes out of one million *Escherichia coli* (SOLR) transformants containing pea cDNAs grown in 0.8 M NaCl. Sequence analysis of one of these revealed homology to LecRLK, which possesses N-myristilation and N-glycosylation sites thus corroborating the protein to be a glycoconjugate. The homology based computational modeling of the kinase domain suggested high degree of conservation with the protein already known to be stress responsive in plants. The NaCl tolerance provided by PsLecRLK to the above bacteria was further confirmed in *E. coli* (DH5α). *In planta* studies showed that the expression of *PsLecRLK* cDNA was significantly upregulated in response to NaCl as compared to K^+ and Li^+ ions, suggesting the Na^+ ion specific response. Transcript of the *PsLecRLK* gene accumulates mainly in roots and shoots. The purified 47 kDa recombinant PsLecRLK-KD (kinase domain) protein has been shown to phosphorylate general substrates like MBP and casein. This study not only suggests the conservation of the cellular response to high salinity stress across prokaryotes and plant kingdom but also provides impetus to develop novel concepts for better understanding of mechanism of stress tolerance in bacteria and plants. It also opens up new avenues for studying practical aspects of plant salinity tolerance for enhanced agricultural productivity.

Keywords *Escherichia coli* · Functional screening · Glycoconjugates · Lectin receptor-like kinase · *Pisum sativum* · Salinity stress tolerance

Abbreviations

CSR	cellular stress response
E. coli	*Escherichia coli*
IPTG	isopropyl thio-β-D-galactopyranoside
LecRLK	lectin receptor-like kinase
MBP	Mylein basic protein

Introduction

Many proteins of plasma membrane are known to play important role in perception and transduction of both developmental and environmental signals in plants [1, 2]. Some of these proteins are plant receptor-like protein kinases (RLKs), which play a fundamental role in sensing external signals to regulate gene expression. Structurally the plant RLKs are similar to animal receptor kinases, because both consist of an extracellular N-terminal ligand-binding domain, a hydrophobic transmembrane domain and an intracellular C-terminal kinase catalytic domain [3, 4]. RLKs constitute one of the largest gene families in plant genomes with at least 610 members found in Arabidopsis all showing Ser/Thr kinase specificity [5]. A new class of receptor kinases with a legume-like extracellular domain

Notes Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers: EU041719 (*PsLecRLK* cDNA)

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known as lectin receptor-like kinases (LecRLKs) has been originally described from Arabidopsis [6, 7], which have structure similar to other plant RLKs [5]. Forty five such LecRLKs related sequences have been identified in Arabidopsis genome and they are N-linked glycoconjugates. The members of this family are known to be involved in disease resistance, hormone signaling, legume-rhizobium symbiosis, pollen development, senescence and in wounding stress responses [4, 8–10]. In one study the induction of AtLecRK2 in response to salt was shown to be regulated by ethylene signaling pathway [11]. However, the role of higher plant LecRLKs in high salinity stress tolerance in bacteria has not been reported so far and its role in salt stress signaling in plants has not yet been elucidated.

Plant growth and productivity are adversely affected by various abiotic and biotic stress factors and thus prevents them from reaching their full genetic potential. Abiotic stress in fact, is the principal cause of crop failure world wide, decreasing average yields for most major crops by more than 50% [12]. To cope with these unpredictable external environmental conditions, plants have evolved a plethora of adaptive mechanisms that are more complex than those of other organisms [13]. Among abiotic stresses, the high salinity stress is the major cause for reducing the crop yield [14, 15]. In response to high salinity stress, plants undergo a stress adaptive response and regulate a variety of genes whose products are involved in signal transduction, transcriptional regulation and induction of stress tolerance. These adaptive changes are made to ensure a high photosynthetic response under non optimal conditions. In spite of the complexity of signal transduction responses present in plants, the cellular stress response (CSR) is most likely similar in prokaryotes, lower eukaryotes and plants [16]. At the cellular level this response may be defined as a defense reaction against the stress imposed by environmental factors and it is activated when an organism is exposed to sub-lethal conditions. In salt stress CSR is involved in protecting cellular components against transient toxic intracellular Na^+ level and it also helps in cross-protection, *i.e.* increased tolerance to a stress by previous exposure to another apparently unrelated stress [17, 18]. There are few reports of functional screening of the plant stress genes by their random overexpression in yeast [19–21] and *E. coli* [22–25]. It has also been demonstrated that the overexpression of some conserved stress-induced genes confers increased plant stress tolerance [26].

The isolation and identification of new salinity stress induced genes with agronomic importance has been one of the focal points in plant biotechnology. These genes will be very useful in developing salinity stress tolerant crop plants. Therefore, the aim of the present study is to isolate some unidentified genes that may have a role in salinity stress from plants like pea (*Pisum sativum*), whose genome has not yet been sequenced. We therefore constructed a pea cDNA library and undertook functional screening for gene mining, using *E. coli* as the host organism. One of the interesting genes identified as p*ea lectin receptor-like kinase* (*PsLecRLK*) was further analyzed for its role in stress tolerance in bacteria, which was not elucidated earlier. In planta, the expression profile of *PsLecRLK* showed up regulation in response to high salinity stress. This study brings new directions in studying the mode of salt tolerance both in plants and bacteria.

Materials and methods

Plant materials, growth conditions and stress treatments

Pea seeds were surface-sterilized in a solution of Clorox plus 0.05% Triton X-100 for 10 min, washed with sterilized water three times and imbibed in sterilized water for at least 4 h. These presoaked seeds were grown under controlled conditions (14/10 h light/dark) in white light at an intensity of 100 μmol m^{-2} s^{-1} PAR in vermiculite moistened with tap water or on wet germination paper (for treatment) under a 14 / 10-h light / dark cycle at 25°C for 7 days. Seven days old pea seedlings were provided with different stress conditions. The individual stress treatments given were as follows. For the cold treatment, the seedlings were grown under continuous white light and were transferred to the 4°C cold chamber. For drought treatment, the seedlings were carefully removed and dehydrated on the filter paper as described by Yamaguchi and Shinozaki [27]. For the salt treatment the roots of the seedlings were dipped at 200 mM NaCl concentration. The pea seedlings were given abiotic stresses for 6 h each. For time kinetics studies related to salt stress, the uniformly developed seedlings were transferred into media either containing indicated concentrations of 150 mM and 300 mM NaCl, 150 mM KCl and 150 mM LiCl. The stress treatment given was for zero, 6 h, 12 h or 24 h each. For organ specific expression roots, stems, leaves and tendril of pea plantlets were harvested directly into liquid nitrogen and stored at −80°C. The control plants were kept at 25°C in growth chamber under constant light conditions. Screening and functional analysis of plant stress-related cDNAs by their random expression in high salt concentrations has been described below.

Construction of the cDNA library and functional screening of salinity tolerant genes

A cDNA library was constructed from 5 μg of poly(A) + RNA (isolated from top four leaves of 7-day-grown pea seedlings) in Uni-Zap XR vector using Zap-cDNA synthe-

sis kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. The resulting phage library contained 1×10^9 plaque forming units/ml. The insert size of library ranged from 500 bps to 3,000 bps. Using an *in vivo* excision system the library was converted to phagemids and transferred in SOLR *E. coli* cells, according to the manufacture's protocol (Stratagene, La Jolla, CA, USA). Incubation times for mass excision were kept strictly as per the manufacturer's protocol so as not to alter clonal representation.

Plasmids, pBluescript SK- (pBSK) containing cDNA inserts were mass-excised from phage stock of the *P. sativum* cDNA library using ExAssist helper phage and propagated in SOLR *E. coli* cells (Stratagene, La Jolla, USA) according to the manual provided by the manufacturer. The cDNAs of *P. sativum* were cloned downstream of the *lac* promoter of pBSK plasmids thus allowing the expression of recombinant proteins upon isopropyl-β-D-thiogalactopyranoside (IPTG) induction. Over one million *E coli* recombinant cells from the same bacterial culture were plated on LB agar containing 50 μg/ml kanamycin, 50 μg/ml ampicillin, 1 mM IPTG and increasing concentrations of salt such as 0.4 M, 0.6 M and 0.8 M NaCl (concentration not permissible for the host bacterial growth). As a control the cells were also grown in the above medium with no extra salt included. The plates were incubated at 37°C for 12 h to 16 h.

Bacterial clones, which were able to grow on LB plates supplementing with 1 mM IPTG and 800 mM NaCl at 37°C [23], were isolated and streaked on the same medium to confirm their abilities to tolerate high concentration of salt. *E. coli* cells with pBSK vector used as negative controls. To further confirm the effective contribution of plant cDNAs to bacterial NaCl survival and to exclude any association of the observed phenotype with unpredictable chromosomal mutations, the plasmids were purified from these over-expressing colonies (SOLR) and reintroduced into a different *E. coli* strain (DH5α) and re-streaked in 0.8 M NaCl plates. The plasmids from positive colonies were sequenced and further characterized.

DNA sequencing and data analysis

cDNAs from plasmids were sequenced on both strands by the dideoxy chain termination method, using Sequenase Version 2.0 (US Biochemicals, Cleveland, OH, USA). The clones of the expression library were found to be in frame with the *LacZalfa* gene, which is driving expression in pBSK plasmid. Sequences were compared to GenBank database, using BLASTN or BLASTX software (BLAST 2.0). The deduced amino acid sequences of cDNAs and other plant homologues were aligned using the CLUSTALW algorithm. The sequences were exposed to protein motif analysis software (*InterProScan*) and the protein motifs were deduced. Since the functional analysis of the genes was done in the bacterial system.

Phylogenetic analysis

The amino acid sequences of *PsLecRLK* and its homolog in higher plants were aligned using the CLUSTALW algorithm [28] and phylogenetic analysis was conducted with the Fast minimum evolution method [29].

Survival of recombinant bacterial cells at high NaCl stress

To further substantiate role of *PsLecRLK* in providing salinity tolerance this clone was further grown in liquid LB media containing higher concentration of NaCl salt. Briefly, the recombinant *E. coli* DH5α cells, transformed with pBSK plasmid carrying the *PsLecRLK* cDNA (pBSK-*PsLecRLK*) or with the empty pBSK vector as control, were grown at 37°C up to 1.0 OD600 and then diluted to 0.1 OD600 with LB medium, supplemented with ampicillin 50 μg/ml, glucose 2% and 1 mM IPTG. Three hours after IPTG induction (0.4–0.6 OD600), salt (NaCl) was added to a final concentration of 0.8 M. Cell aliquots (1 ml) were taken after 0 min, 30 min, 60 min, 120 min and 180 min. The OD represents the mean of three replicates of at least two independent recombinant bacterial cultures and results were represented by plotting a graph. To confirm the salt-stress effect more clearly, the *E. coli* transformants, containing empty pBSK vector as control and pBSK-*PsLecRLK* plasmid cDNA were grown under NaCl stress using a different approach as described below. As the optimum response of *E coli* transformants carrying the pBSK-*PsLecRLK* plasmid DNA to salt stress (0.8 M NaCl) was seen at 60 min and 120 min as observed from the above experiment, these transformed cells were incubated with 0.8 M NaCl for 120 min. Serial dilutions, 1:10, were made from these *E coli* transformants, and were spotted equally on 2YT agar plates supplemented with 171 mM NaCl and 50 μg/ml ampicillin. Spotted agar plates were incubated at 37°C. Quantitative estimation of survivability of *E. coli* DH5α transformants was also done by counting the number of colony-forming units (CFU), after overnight incubation of the plates at 37°C. Cell survivability is expressed in terms of CFU/ml under induced conditions (IPTG), in presence of 0, and 0.8 M NaCl. Cell survival values represent the mean of three replicates of at least two independent recombinant bacterial cultures. In order to check whether the lectin domain of PsLecRLK protein is responsible for imparting the salinity stress tolerance to bacteria, we have also cloned the only kinase domain, pET28a-*PsLecRLK-KD*, and cheched for salinity tolerance in bacteria as described above.

Endogenous sodium ion content of transformed bacterial cells grown under normal and in presence 0.8 M NaCl

To validate the salt (Na^+ ions) uptake during the salinity stress, the bacterial cells (DH5α) transformed with empty pBSK vector (control) and pBSK-*PsLecRLK* plasmid were grown in normal LB medium and transformed with pBSK-*PsLecRLK* plasmid grown in LB containing 0.8 M NaCl. After 16 h of growth, the cells were pelleted by centrifugation and washed thrice with phosphate buffer saline. The cell pellet was dried at 60°C. Equal amount of dried bacterial cells pellet was treated with concentrated HNO_3 and 10% of H_2O_2 for 5 h at 65°C. Samples then were treated with 2 N HCl and analyzed for Na + content by using simultaneous inductively coupled argon-plasma emission spectrometry (ICP trace analyzer, Labtam, Braeside, Australia).

Reverse transcription-PCR (RT-PCR) analysis

Total RNA was extracted with TRIzol (Gibco-BRL, Gaithersburg, MD, USA) from the 7 days old pea seedlings, which were processed as per the details described in the earlier section of "Materials and methods" (Plant materials, growth conditions and stress treatments). For preparation of cDNA, approximately 5 μg of the total RNA from all the stressed and non-stressed samples were reverse transcribed by using oligo(dT) primer and the first strand cDNA Synthesis Kit (Invitrogen,CA,USA). The RT-PCRs reactions were performed with gene specific primers design from the cytoplasmic kinase domain (1. PsLecRLK-F: 5′-CAGGAATTCGGATCCCAGGAGAA GAGAAAGAGAG-3′ and 2. PsLecRLK-R: 5′-CAGCTCGAGCCTACCCTCAACTAAAGATCCAG-3). The recommended thermal profile followed was: 95°C for 5 min, 25 cycles of 95°C for 45 s, 60°C for 1 min and 72°C for 2 min The final extension was carried out at 72°C for 10 min. The expected size of the RT-PCR amplified product was 1.1 Kb. Similarly total RNAs from recombinant *E. coli* strain (DH5α) carrying pBSK vector only and pBSK-*PsLecRLK* was extracted with TRIzol after induction with 1 mM IPTG for 120 min. Two μg of total RNA from bacteria after DNAse I treatment, were used for semi-quantitative RT-PCR analysis with the Superscript II One Step RT-PCR System (Invitrogen) according to the manufacturer's protocol. The *P. sativum* Alaska α-tubulin (*TubA1*) gene (Accession number PSU12589)was taken as internal standard using tubulin gene specific primers (1. TubA1-F: 5′-ATGAGG GAGTGCATTTCG-3′ and 2. TubA1-R: 5′-CTAG TACTCTTCCTCACC-3′). The PCR products were separated on 1.2% agarose gel and quantified using ChemiDoc-XRS (Bio-Rad, USA). The transcript levels (DNA intensity ratio) were estimated by ChemiDoc-XRS (Bio-Rad, USA).

Construction of plasmids for expression of cytoplasmic kinase domain of PsLecRLK protein

The cytoplasmic region of the PsLecRLK protein containing putative kinase domain (KD) of the PsLecRLK was amplified from original plasmid *PsLecRLK* by PCR with primers harboring restriction sites (BamH1 and Xho1) and cloned in frame into the same sites of pET28a vector(+) (Novagen, Madison, WI). (Note: In this case the expressed PsLecRLK protein will contain a His-tag in the N-terminal region). This resulted in the construction of plasmid pET28a-*PsLecRLK-KD* whose sequence was verified before it was used for protein expression. The sequence of the gene specific primers for *PsLecRLK-KD* are: 1. forward primer: 5′ CAG GAA TTC GGA TCC CAG GAG AAG AGA AAG AGA G 3′ containing a BamH1 site (underlined) and 2. reverse primer: 5′CAG CTC GAG CCT ACC CTC AAC TAA AGA TCC AG 3′ containing a Xho1 site (underlined).

Expression and purification of recombinant PsLecRLK proteins

The plasmid pET28a-*PsLecRLK-KD* was transformed into *E. coli* Rosetta (DE3) cells (Novagen). Fresh culture of the *E. coli* transformant from the overnight culture of the same was grown in Luria–Bertani (LB) medium containing 50 μg/ml kanamycin (until the A600 reached 0.6), induced by IPTG (1 mM) and harvested by centrifugation. All of the purification steps were performed at 4°C. The resulting cell pellet was resuspended in ice-cold sonication buffer containing 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM PMSF, 0.5% Triton X100, 10 mg/ml lysozyme. The cell suspension was incubated at 37°C for 20 min followed by sonication for 8 min in 30 s pulse. The resulting lysed cells were centrifuged at 8,000 rpm for 30 min at 4°C to separate the pellet and clear supernatant fractions. The PsLecRLK-KD protein was found to be present in both the soluble as well as pellet fractions. The recombinant PsLecRLK protein from the supernatant fraction was further purified to apparent homogeneity using Ni^{2+}–NTA–agarose (Qiagen) column chromatography following the manufacturer's instructions. The purity of the recombinant protein (histidine-tagged) was checked by SDS-PAGE and Western blotting with anti-His antibodies using standard procedures and this pure protein was used for all assays.

Preparation of nuclear extract

Nuclear extract was prepared from 50 g pea leaves as described earlier [30]. Briefly, the pea seedlings 7 days to

8 days old were harvested in cold buffer containing 0.55 M sucrose, 50 mM Tris-HCl pH 8.0 and 10 mM MgCl2 (STM buffer). The tissue was washed once and ground in STM buffer. Thereafter the homogenate was passed through two layers of cheesecloth and two layers of Miracloth (Cal Biochem, San Diego, CA, USA). The filtrate was then centrifuged at 1,000 g for 10 min at 4°C in a Sorvall RC 5B centrifuge. The pellet was slowly resuspended in STM containing 2.5% Triton 3 100, and incubated at 4°C with slow shaking followed by centrifugation at 2,000 g for 30 min at 4°C. The resulting nuclear pellet was then resuspended in a buffer containing 600 mM KCl, 50 mM Tris-HCl pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 0.5 mM PMSF, 50 mM leupeptin and 1 mM pepstatin, and homogenized in a Potterelvehjham homogenizer. Then the homogenate was spun at 12,000 g for 30 min at 4°C, and the clear supernatant (nuclear extract) was dialyzed against buffer containing 50 mM KCl, 50 mM Tris-HCl pH 8, 20% glycerol and protease inhibitors, and stored at −80°C.

Protein kinase assay

Phosphorylation was measured as the incorporation of radioactivity from γ^{32}P-ATP into the PsLecRLK (auto-phosphorylation) or into the substrate proteins. The purified recombinant PsLecRLK protein alone or in the presence of the substrate (Mylein Basic Protein [MBP], Casein or nuclear extract) was incubated in the kinase buffer containing 20 mM HEPES pH 7.5, 5 mM $MgCl_2$, 2 mM $MnCl_2$ and ATP mix (4.8 µl 0.1 mM ATP, 3.3 µl of 10 µCi- γ^{32}P-ATP and 51.9 µl H_2O; used 1 µl per reaction), for 30 min at 30°C. The reaction was terminated by addition of EDTA to a final concentration of 10 mM. The phosphorylated protein was subjected to 12% sodium dodecyle sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by autoradiography.

Homology based protein modeling

To create homology based protein models automated homology modeling program ESyPred3D (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/) was used. The method uses neural networks for increased alignment performance. The program works by combining, weighting and screening the results of several multiple alignment programs. The final three dimensional structures were built using special software named Pymol, which has well developed functions for manipulating structures. PyMol is a molecular graphics program intended for the visualization of proteins, nucleic acids and small molecules. The program reads in molecular coordinate files and interactively displays the molecule on the screen in a variety of representations and color schemes. Further conserved domain search of the proteins, to validate the protein models, was done using NCBI's conserved domain search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Results

Functional screening of plant salinity stress-related cDNAs by random overexpression in *E. coli* cells (SOLR)

E. coli cells were transformed with phagemids derived from a cDNA library (made from RNA isolated from stressed seedlings of *P. sativum*) as described in "Materials and methods". A total of over one million recombinant bacterial cells (SOLR *E. coli* cells) were selected on a medium supplemented with NaCl concentrations not permissive for bacterial cell growth. For control, the same numbers of cells were grown on LB medium (which normally contains 171 mM NaCl) and the result showed a lawn of colonies on the plate (Fig. 1a). When same number of recombinant bacterial cells was plated using 0.4 M, 0.6 M and 0.8 M NaCl concentrations, the number of colonies reduced to ~1500, ~500 and about 34 healthy colonies, respectively (Fig. 1b–d). It was noted that the healthy colonies observed at 0.8 M NaCl concentration took 16 h to 20 h to grow and were smaller in size as compared to colonies grown on normal media. Plasmids were rescued from the 0.8 M NaCl grown colonies and the insert of these cDNAs were sequenced and the insert sizes ranged from 300 bps to ~3,000 bps. One of these healthy grown bacterial colonies was selected for further studies regarding its ability to impart salinity stress tolerance in bacterial system and to study its expression level in planta in response to abiotic stress including salinity stress. The sequence analysis of this clone showed that it was homologous to a lectin receptor-like kinase (*LecRLK*; accession no. EU 041719).

The identity of all the 34 cDNAs and their details are shown in Table 1. Based on sequence homology search with the known database all these 34 cDNAs are classified into five groups according to their putative function in the plant system (Fig. 1e). Figure 1e shows that 68% of these cDNAs matched with annotated proteins in EMBL/GenBank databases, whereas 32% of them were categorized into unknown proteins. Out of 68% known proteins the majority of them (38%) were stress responsive. Other groups included proteins involved in miscellaneous/signaling (15%), metabolism (12%) and gene expression (3%). Some sequences were present in multiple copies like chlorophyll a/b binding protein was present in four copies, while 60S ribosomal protein was present in two copies (Table 1). Most of them were unique including our gene of interest *i.e. LecRLK*.

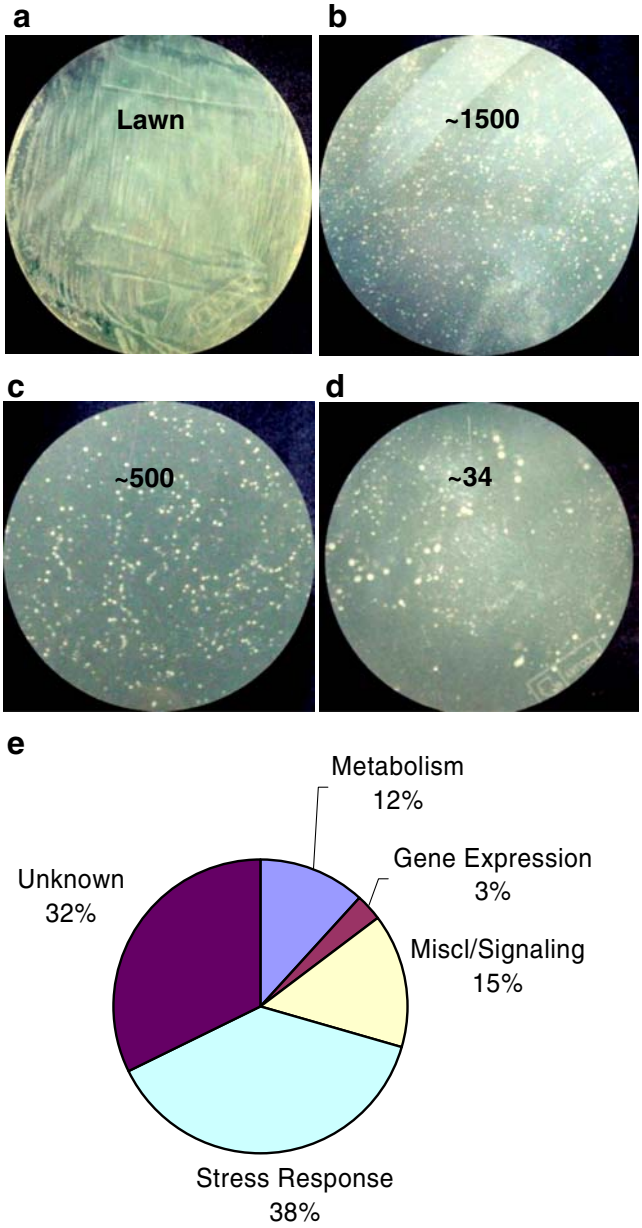

Fig. 1 Functional screening for salt tolerant genes from *Pisum sativum* using *E. coli* system as a host for overexpression. For screening a total of over one million recombinant *E. coli* cells were selected on medium supplemented with NaCl concentrations not permissive for bacterial cell growth. **a** In control conditions the recombinant *E. coli* cells were grown on normal LB medium (171 mM NaCl) and the result showed a lawn of colonies on the plate. **b** At 0.4 M NaCl concentrations, the lawn was much reduced and around 1,500 colonies were observed. **c** At 0.6 M NaCl concentrations, the colonies reduced to about 500 colonies. **d** Finally, at 0.8 M NaCl concentrations, about 34 healthy colonies were observed. **e** Classification of high salinity tolerant cDNA sequences from *Pisum sativum* according to their putative functions bases on sequence homology

Sequence analysis of salinity stress-induced *P. sativum* lectin receptor-like kinase (*PsLecRLK*) cDNA clone

This homologue of *LecRLK* from pea encodes a partial cDNA, which is 1992 bps in length. About 57 bps to 75 bps (~19–25 amino acids) are missing from the 5′ end. Similar to other receptor lectin kinases, the PsLecRLK contained three main domains such as extracellular (267 amino acids), transmembrane (24 amino acids) and cytoplasmic region containing putative kinase domain (372 amino acids). The legume type of lectin domain is present in the extracellular region, while ATP-binding domain and Ser/Thr protein kinase active-site domains are present in the cytoplasmic region of the protein (Fig. 2a). The amino acid alignment of PsLecRLK with its corresponding units in other plants is also shown in Fig. 2a. The PsLecRLK is 59% and 52% identical to lectin kinases from *Arabidopsis thaliana* (NP567277 and NP194564, respectively) and shares 61% identity to *Nicotiana tabacum* (BAF47279). Predicted domain structure of PsLecRLK representing its three domains is shown in Fig. 2b. A phylogenetic analysis of pea lectin kinase revealed that it is closest to lectin kinase from *Lotus japonicas*, *Vitis vinifera*, *Nicotiana tabacum* and *Arabidopsis thaliana* (Fig. 2c).

Salinity stress tolerance of *E. coli* (DH5α) overexpressing *PsLecRLK* cDNA

Since the role of the *PsLecRLK* gene has not been well studied in salinity stress, therefore we made an effort to prove the effective contribution of this plant cDNA to salt tolerance in bacterial system. For this purpose the plasmids purified from the *E coli* transformants (expressing in SOLR cells) and empty pBSK vector (as a control) transformed into DH5α cells were used. This was done to exclude the possibility of unpredictable chromosomal mutations in SOLR cells, which might have resulted in providing salt tolerance. These bacterial cells (DH5α) overexpressing the *PsLecRLK* cDNA were induced by IPTG and subjected to salt (NaCl) stress by growing them in LB agar plates containing 0.8 M NaCl. The *E. coli* DH5α bacterial cells transformed with *PsLecRLK* and *pBSK* empty vector were streaked in solid media with 0.8 M NaCl concentration. The results show that *pBSK* empty vector could not grow (Fig. 3a [1, left panel]), while the *pBSK-PsLecRLK* could sustain growth under such a high salt concentration (Fig. 3a [2, right panel]).

Further, *PsLecRLK* overexpressing bacterial cell lines as well as empty *pBSK* were induced with 1 mM IPTG and subjected to salt stress for 120 min in liquid medium containing 0.8 M NaCl. After salt stress, the whole cell RNA was extracted. RT-PCR was done by using gene specific primers as mentioned in "Materials and methods".

Table 1 The putative identity of high salinity stress induced cDNA clones from *Pisum sativum*

S. No.	Clone name	Partial/Full length	Homologous to	Accession no. of matching sequence
1	S-4R	Partial	Cryptochrome 1 (CRY1) [*Pisum sativum*]	AY161310
2	S-5	Unknown	Unknown	Unknown
3	S-5R	Full-length	Chlorophyll a/b binding protein [*Pisum sativum*]	ABN49454
4	S-6	Partial	Phosphatidylcholine transfer protein [*Ricinus communis*]	XP_002518934
5	S-8	Full-length	Chlorophyll a/b binding protein [*Pisum sativum*]	ABN49454
6	S-13B	Partial	Kinase-related protein [*Glycine max*]	ACM89527
7	S-15	Full-length	Chlorophyll a/b binding protein [*Pisum sativum*]	ABN49454
8	S-15B	Unknown	Unknown	Unknown
9	S-20	Partial	F-box protein PP2-A12 [*Populus trichocarpa*]	XP_002298062
10	S-25R	Unknown	Unknown	Unknown
11	S-26	Full-length	Aspartate aminotransferase [*Laccaria bicolor*]	XP_001874806
12	S-32	Partial	Dehydrin [*Pisum sativum*]	CAA44789
13	S-33B	Genomic sequence	Clone mth2-18h17 [*Medicago truncatula*]	AC140914
14	S-38B	Full-length	60S ribosome protein [*Vitis vinifera*]	XP_002281850
15	S-42	Full-length	Pathogenesis-related protein PR10 [*Pisum sativum*]	AAB07451
16	S-50B	Partial	Hypothetical protein [*Ricinus communis*]	XP_002530231
17	S-69	Unknown	Unknown	Unknown
18	S-75	Partial	Protein kinase[*Medicago truncatula*]	ABN08751
19	S-82	Unknown	Unknown	Unknown
20	S-83B	Partial	Unknown [*Populus trichocarpa*]	ABK94463
21	S-86	Unknown	Unknown	Unknown
22	S-88B	Genomic sequence	Chromosome 5 clone mth2-146m15 [*Medicago truncatula*]	CT571264
23	S-90B	Partial	Leucine-rich repeat protein, putative [*Ricinus communis*]	XP_002509547
24	S-113	Full-length	Small nuclear ribonucleoprotein[*Ricinus communis*]	XP_002520764
25	S-124B	Partial	60S ribosomal protein L37a, putative [*Ricinus communis*]	XP_002517507
26	S-126B	Unknown	Unknown	Unknown
27	S-135	Partial	Ultraviolet-B-inducible auxin-related protein *[Pisum sativum*]	AY065659
28	S-140B	Partial	Glycolytic glyceraldehyde 3-phosphate dehydrogenase [*Antirrhinum majus*]	CAA42103
29	S-158	Partial	Flavonoid glycosyltransferase [*Medicago truncatula*]	ABI94024
30	S-163	Partial	Sterol delta-7 reductase DWF5 [*Gossypium hirsutum*]	ABA01480
31	S-131	Full-length	Lectin receptor like kinase [*Arabidopsis thaliana*]	NP567277
32	S-130	Full-length	Fiddlehead like protein [*Gossypium hirsutum*]	AAL67993
33	S-25	Full-length	Ribosomal protein L30 [*Medicago truncatula*]	ABPO2866
34	S-22	Full-length	Chlorophyll a/b binding protein [*Pisum sativum*]	AAW31511

The upper panel (Fig. 3b; lane 2) shows accumulation of *PsLecRLK* transcript (1.1 Kb) in transformed bacterial cells, while there was no transcript observed in empty *pBSK* vector (Fig. 3b; lane 1). The lower panel in Fig. 3b shows the equal loading of RNAs. In a similar type of experiment, the *PsLecRLK* gene transformants (overexpressing clone in *E. coli* [DH5α]) was also grown in liquid medium containing 0.8 M NaCl at 0 min, 30 min, 60 min, 120 min and 180 min time interval. Upon salt stress, cell survival rate (expressed as bacterial O.D.) decreased in control *i.e.* cells transformed with the empty vector (Fig. 3c). However, the growth of the bacteria over expressing the *PsLecRLK* gene was partially recovered 30 min post stress. The O.D. of bacterial cells harboring the *PsLecRLK* cDNA increased with time period and even at 180 min it was quite high as compared to the control. These data suggest that the gene product of pea *PsLecRLK* cDNA is likely to be a part of the general CSR to NaCl stress, which is conserved in prokaryotes and plants.

In order to check whether lectin domain of PsLecRLK protein is responsible for imparting the salinity stress tolerance to bacteria, the only kinase domain (lacking lectin

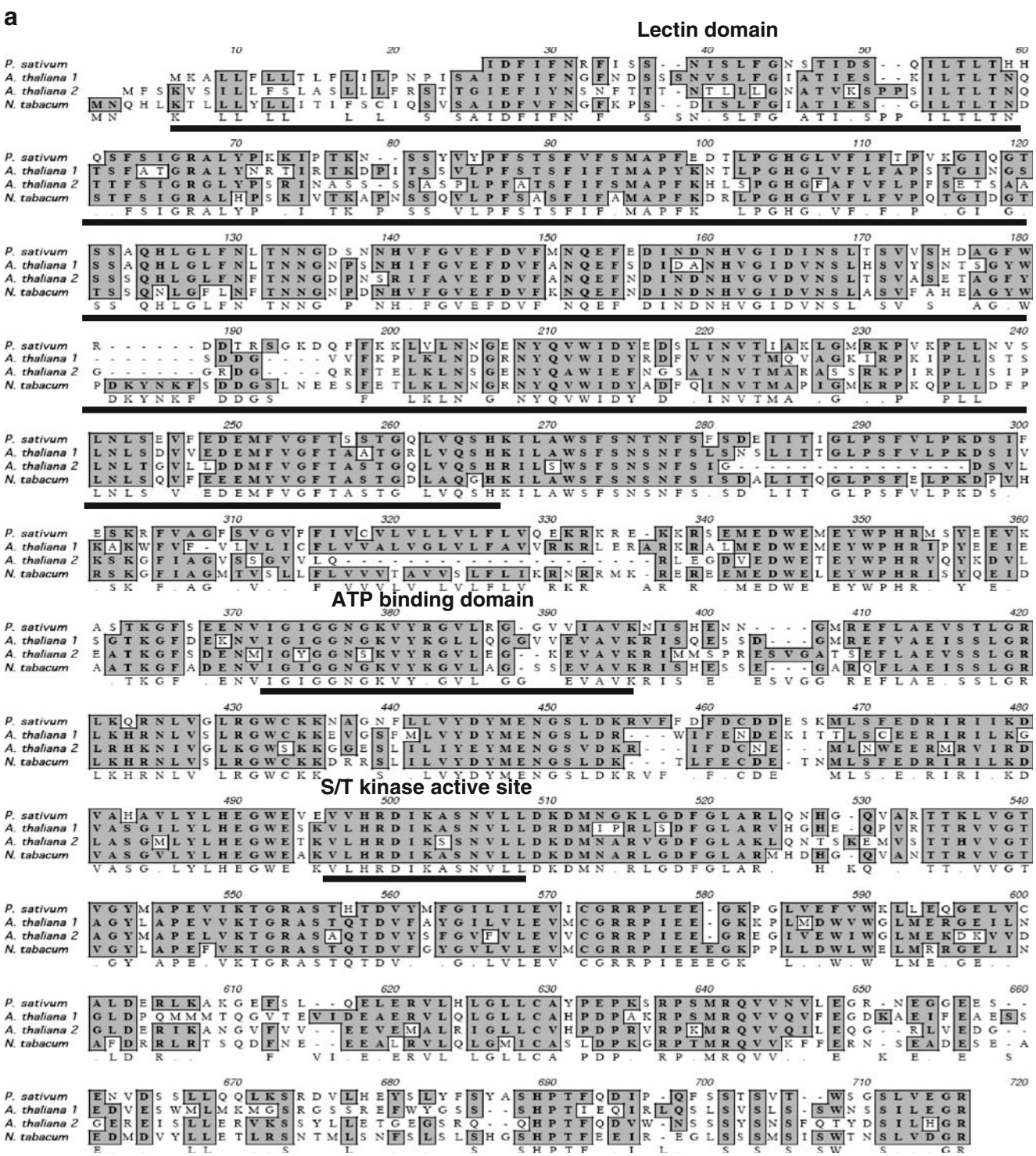

Fig. 2 The bioinformatical properties of *Pisum sativum* lectin receptor-like kinase (*PsLecRLK*). **a** Alignment of the deduced amino acid sequence of the *PsLecRLK* cDNA with other plant homologues. GenBank accession nos.: *Pisum sativum* (ABU75307), *Arabidopsis thaliana* 1 (NP_567277), *Arabidopsis thaliana* 2 (NP_194564), *Nicotiana tabacum* (BAF47279). The underlined portion in the sequence represents conserved domains like lectin domain, ATP-binding domain and Ser/Thr (S/T) protein kinases domain. **b** A linear schematic representation of PsLecRK denoting three domains comprising this polypeptide; TM denotes transmembrane region **c** Phylogenetic tree of *PsLecRLK*. Dendrogram showing the phylogenetic relationship between the nucleotide sequence of *PsLecRL* with other *LecRLK*. The names and accession numbers of the sources are mentioned in the figure

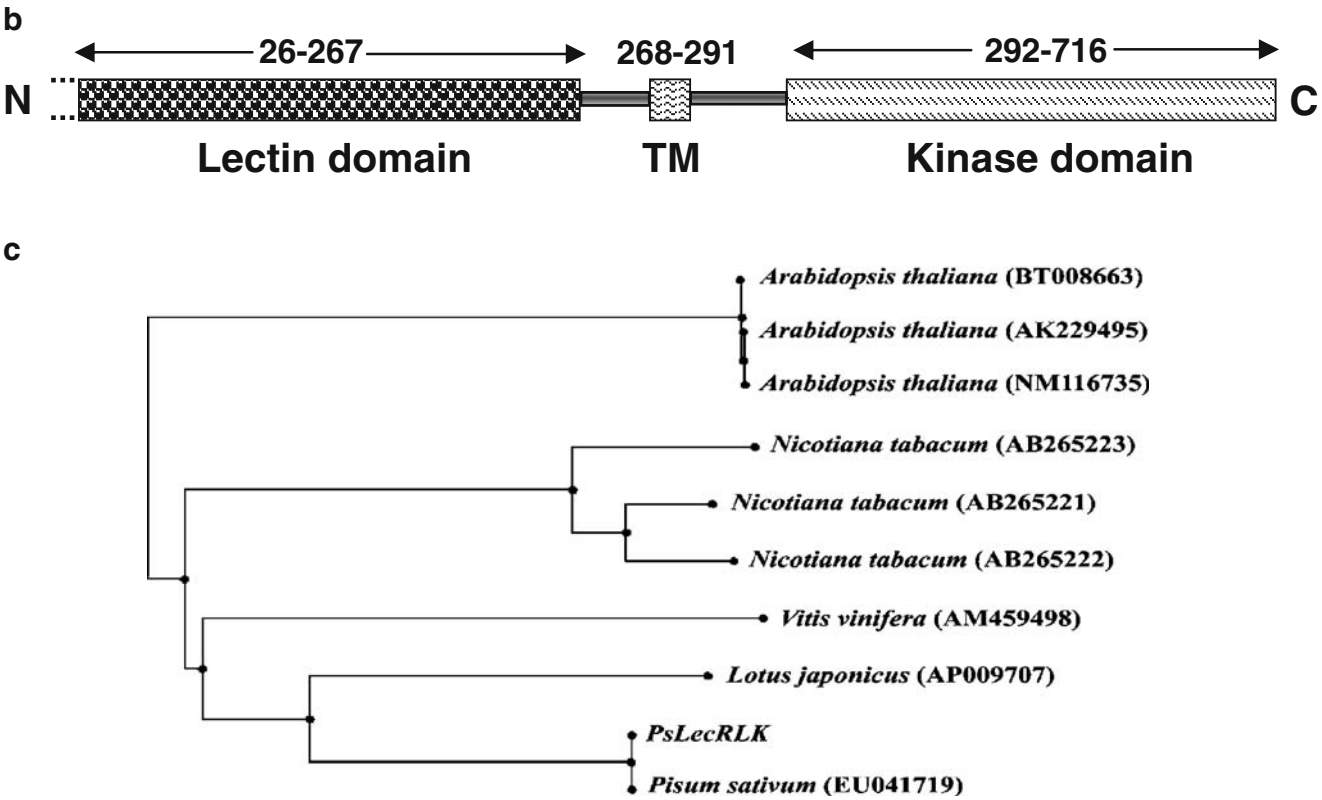

Fig. 2 (continued)

domain) was cloned in the protein expression vector pET28a and were transformed into Rosetta *E. coli* cells. The transformed cells were streaked on LB Agar plates with and without 0.8 M NaCl along with 50 mg/ml kanamycin. The results showed that the bacterial cells congaing vector having a kinase domain (pET28a-PsLecRLK-KD) as well as the empty vector (only pET28a as control) grew happily in LB agar plate containing no extra salt (Fig. 3d-1 and 2). However, both could not survive the 0.8 M stress (Fig. 3e-1 and 2). This was in direct contrast to the results that was obtained with the PsLecRLK containing both the lectin domain (carbohydrate binding moiety) and the kinase domain (see Fig. 3a), which grew at 0.8 M NaCl.

The salt tolerance and the cellular death kinetics were assessed in *E. coli* DH5α cells transformed with constructs containing empty vector *pBSK* and *PsLecRLK* by spot test (Fig. 4a) and by colonies counting (Fig. 4b). Growth of the transformants in LB media containing 0.8 M NaCl under IPTG induction was measured on solid LB media with increasing dilutions at 120 min time period. The transformed cells grew almost to the same extent at 171 mM NaCl at 120 min. With 0.8 M NaCl in the growth media and with the increasing dilution, steady inhibition of growth was observed in case of an empty vector, while the *E. coli* transformants carrying *PsLecRLK* cDNAs showed very little growth inhibition of transformed cells after 120 min of incubation time (Fig. 4a). Such growth pattern and survival of cells under conditions of increasing salinity were quantitated by tracking cell growth using plating and colony counts (Fig. 4b). As apparent, the *PsLecRLK* transformants showed better survival in comparison to the empty vector at 0.8 M NaCl, stress with 120 min of incubation time. Although extent of growth or survivability was reduced even in case of *PsLecRLK* transformed cells at 0.8 M NaCl, in case of empty vector transformants, virtually no growth was observed at this salt concentration (Fig. 4b).

Endogenous sodium ion content of bacterial transformants cells

To examine the basic mechanism resulting in salt-tolerant phenotypes of *PsLecRLK*-overexpressing bacterial transformants cells, we measured the endogenous (intracellular) Na^+ ion levels in pBSK empty vector (control) and pBSK-PsLecRLK grown 16 h at 37°C under normal LB medium and pBSK-PsLecRLK grown under 0.8 M NaCl in the LB medium. The results showed that intracellular Na^+ ion concentrations were almost same whether the *E. coli* DH5α transformants (pBSK-PsLecRLK) were grown in normal condition (no extra salt in LB) or in the presence of high

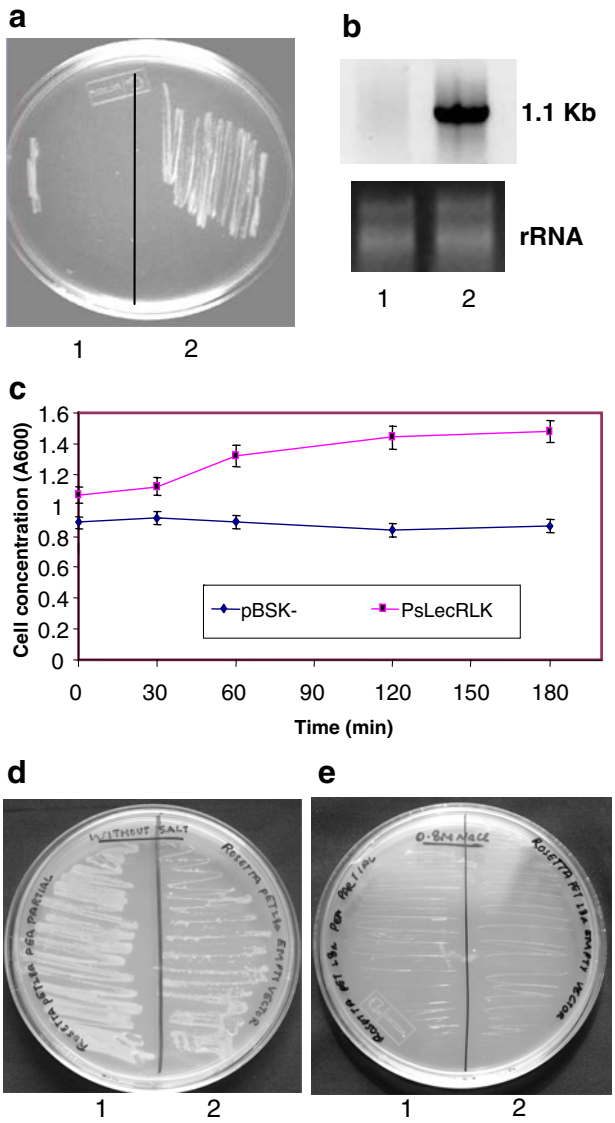

Fig. 3 **a** Growth of *E. coli* DH5 α transformed with pBluescript SK-▶ (pBSK) vector as control (1, *left side*) and pBSK-*PsLecRLK* plasmid DNA (2, *right side*) on solid LB agar, supplemented with 50 μg/ml ampicillin under IPTG induction and in presence of 0.8 M concentrations of NaCl at 37°C **b** RT-PCR using total bacterial RNA from *E. coli* cells transformed with pBSK vector alone (1) or pBSK-*PsLecRLK* cDNA (2). Gene specific primers were used for PCR amplification as described in "Materials and methods". The upper panel shows the gel picture PCR product and the bottom panel shows the rRNA as loading control. **c** Survival rate of *E. coli* DH5α transformants in liquid LB medium after NaCl treatment at various time intervals. pBSK-*PsLecRLK* cDNA and the empty pBSK vector as control, were grown at 37°C up to 1.0 OD600 and then diluted to 0.1 OD600 with LB medium. Cell aliquots (1 ml) were taken after 0 min, 30 min, 60 min, 120 min and 180 min. Cell survival was estimated by taking OD. The OD represents the mean of three replicates of at least two independent recombinant bacterial cultures. **d** Growth of *E. coli* Rosetta transformed with pET-28a vector as control (2, *right side*) and pET-28a-PsLecRLK-kinase domain DNA (1, *left side*) on solid LB agar, supplemented with 50 μg/ml kanamycin under IPTG induction grown at 37°C. (e) Growth of *E. coli* Rosetta transformed with pET-28a vector as control (2, *right side*) and pET-28a-PsLecRLK-kinase domain DNA (1, *left side*) on solid LB agar, supplemented with 50 μg/ml kanamycin under IPTG induction and in presence of 0.8 M NaCl at 37°C

salt (0.8 M NaCl) (Fig. 4c). As a control the intracellular Na^+ ion concentration of empty vector (pBSK) was similar to that of pBSK-PsLecRLK grown in normal LB medium (Fig. 4c). However, the empty vector pBSK transformants could not grow under the high salt conditions.

Abiotic stress induced and tissue specific expression level of *PsLecRLK* gene in plant

To analyze the expression of *PsLecRLK* gene in various plant organs and under abiotic conditions including drought, cold and various salt stress (NaCl, KCl and LiCl), 7-day-old pea seedlings were given various treatments as mentioned in "Materials and methods". Total RNAs were extracted from control and treated tissues and RT-PCR was done using gene specific primers. Tubulin was used as an internal control. Overall, it was found that *PsLecRLK* was induced in salt (200 mM NaCl) stress (Fig. 5a). In case of cold and dehydration stress the expression levels went up as compared to the control but maximum level of transcript increase was found in salt (NaCl) stress (Fig. 5a). The expression level of Tubulin did not change throughout the experiment (Fig. 5b). We also wanted to evaluate the expression profiles of *PsLecRLK* under different plant organ type. Therefore, we processed the various plant organs as discussed in the "Materials and methods". We found that the *PsLecRLK* showed high expression in root, shoot and a low expression in leaves and tendrils (Fig. 5c). rRNA was used as a loading control (Fig. 5d).

To observe the optimum effect of NaCl stress on the plants the NaCl stress was also given at 150 mM as well as 300 mM. The KCl and LiCl stress was given at 150 mM salt concentration only. For this the 7- to 8-day-old pea seedlings were treated with salt concentrations, as mentioned above, for variable time periods (6 h, 12 h and 24 h) and their transcript levels were analyzed. The maximum transcript level was observed in 12 h time periods in both the 150 mM and 300 mM NaCl concentrations (Fig. 6a and c). The overall pattern of expression of *PsLecRLK* was found to be similar in response to 150 mM as well as 300 mM NaCl treatments (Fig. 6a and c). The expression levels in *PsLecRLK* at 150 mM NaCl showed upregulation upto twofold at 6 h as compared to the control at 0 h, and a further three fold at 12 h. The expression level fell down drastically 24 h post salt stress. (Fig. 6a). However, at 300 mM NaCl tenfold increase was observed at 6 h and at 12 h a further about tenfold increases was observed. At 24 h time point the expression level of the gene came down

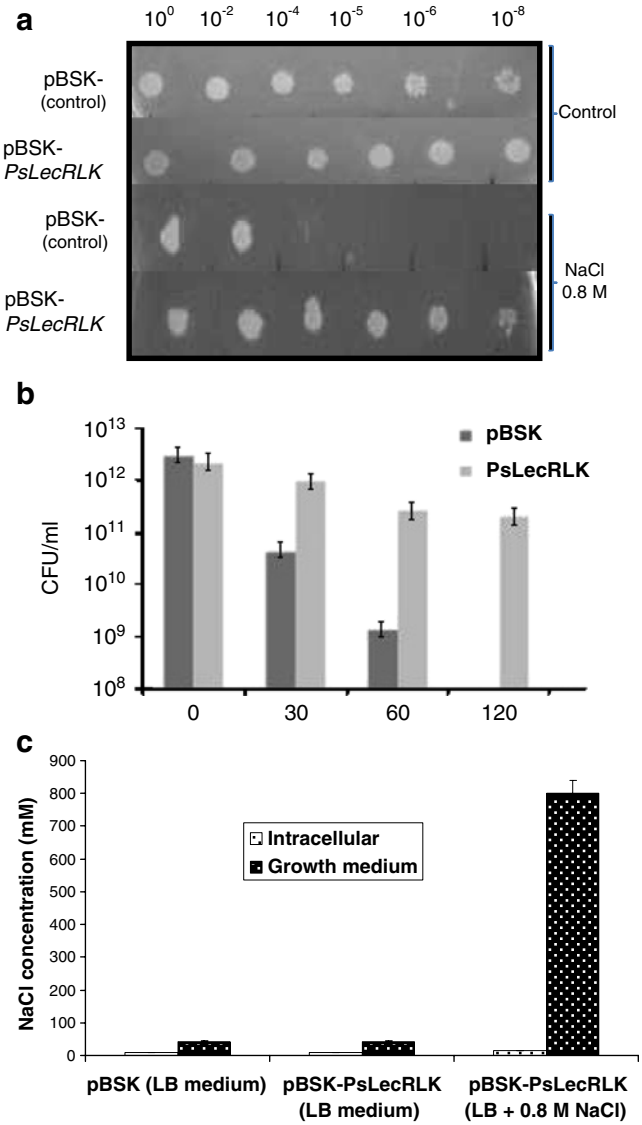

Fig. 4 **a** Protection of salt induced growth inhibition of *E. coli* by *PsLecRLK* expression. The *E. coli* DH5α transformants carrying the pBSK and pBSK-*PsLecRLK* constructs were induced with IPTG and grown in LB media containing on normal (171 mM NaCl) as control and salt stressed (0.8 M NaCl). The dilutions were spotted on solid LB agar, supplemented with 50 mg/ml ampicillin under IPTG induction and grown. Plates were photographed after incubation at 37°C for 16 h. **b** Quantitative estimation of survivability of *E. coli* DH5α transformed pBSK and pBSK-*PsLecRLK* constructs in presence of 0.8 M NaCl concentration and increasing dilutions at 120 min. Cell survivability is expressed in terms of colony forming unit/ml under induced conditions. *Error bars* indicate standard deviation from triplicate experiments. **c** Quantification of endogenous sodium ion content in *E.coli* DH5α transformants namely pBSK plasmid (control) and pBSK-PsLecRLK grown under normal LB medium and pBSK-PsLecRLK grown under 0.8 M NaCl in the medium. After 16 h of growth at 37°C, the Na + ion concentration was measured and plotted as histogram as described in "Materials and methods"

drastically (Fig. 6c). Tubulin was used as an internal control and its expression did not change throughout the experiment (Fig. 6b and d).

To check for the specific role of sodium ions in comparison to other ionic stressants (potassium and lithium ions) we analyzed the expression levels of *PsLecRLK* gene under 150 mM KCl and 150 mM LiCl treatments to the pea seedlings for 6 h, 12 h and 24 h. The expression levels of *PsLecRLK* did not show any specific pattern of expression in transcript, although little increase in the transcript level was observed with respect to control (Fig. 7a and c). However, this increase was many less fold as compared to the one observed in case of NaCl stress. Tubulin was used as an internal control and its expression did not change throughout the experiment (Fig. 7b and d).

Expression and purification of PsLecRLK-KD

Since the cytoplasmic region of the PsLecRLK protein possesses a putative kinase domain (PsLecRLK-KD), we expressed this domain as His-tagged protein in an bacterial system. The *PsLecRLK-KD* was cloned into the expression vector pET28a and the recombinant protein was expressed in *E. coli*. SDS/PAGE analysis showed a highly expressed 47 kDa additional polypeptide for PsLecRLK-KD in IPTG induced fraction as compared to uninduced (data not shown) fraction. The recombinant PsLecRLK-KD was purified from the soluble fraction through a single Ni^{2+}–NTA–agarose column chromatography step. The protein, purified to near homogeneity, showed a 47 kDa (Fig. 8a, lane 1). In western blotting the purified PsLecRLK-KD protein was also recognized by anti-His antibody (Fig. 8b).

In-vitro assay of recombinant PsLecRLK-KD protein for kinase activity

To determine whether PsLecRLK-KD is a functional protein kinase, the autophosphorylation and substrate phosphorylation activities of the enzyme were checked in an *in-vitro* kinase assay by incubating the enzyme with [γ-^{32}P]ATP in the absence or presence of the substrates. After incubation, the phosphorylation of the proteins was examined by SDS/PAGE (Fig. 4c) followed by autoradiography (Fig. 8d). The results showed that the PsLecRLK-KD was unable to autophosphorylate (Fig. 8d; lane 3). To check whether the PsLecRLK-KD can phosphorylate other protein substrates, we used Mylein Basic Protein (MBP) and casein as substrates. The results show that the PsLecRLK-KD protein phosphorylates these protein substrates (Fig. 8d lane 1 and 2). These protein substrates were not getting phosphorylated in absence of PsLecRLK-KD protein (Fig. 8d, lanes 6 and 7). Differential kinase activity of

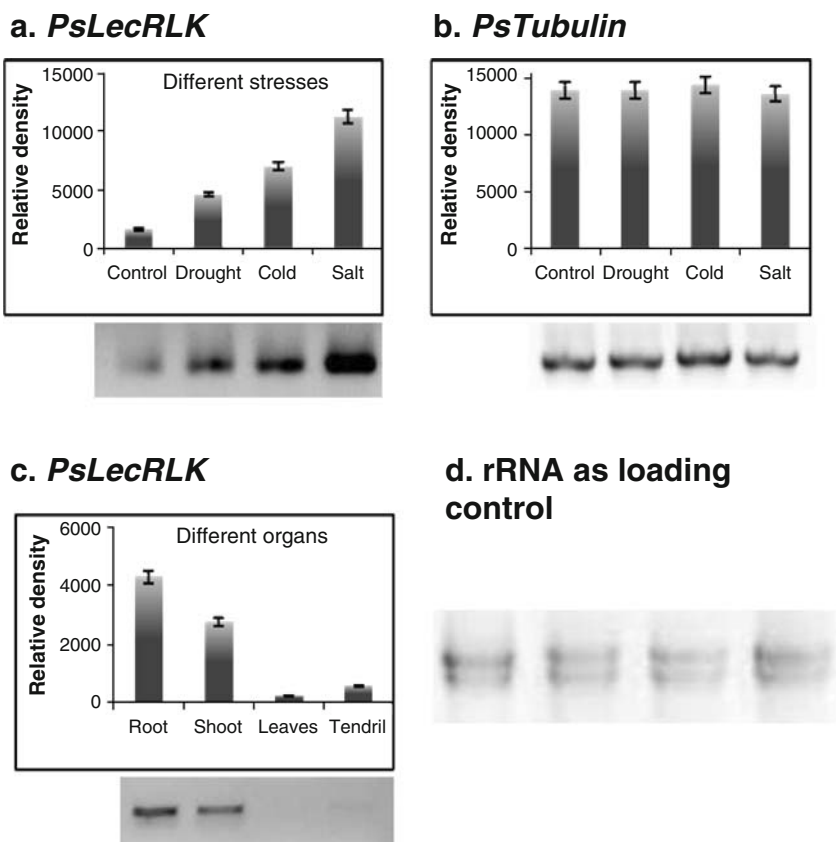

Fig. 5 Transcript levels of the *PsLecRLK* gene in response to different stresses including drought, cold and salt (**a**) and different organs including root, shoot, leaf and tendril (**c**) of *Pisum sativum*. Tubulin (**b**) gene and rRNA (**d**) were used as internal controls. The total RNAs were extracted from 7 day old pea seedlings after stress treatment. RT-PCR was done in triplicate to check the expression level of the transcripts. In panel (**a**) lane 1 is the control without any treatment while lanes 2, 3 and 4 are the transcript levels after giving drought, cold and salt (200 mM NaCl) stresses, respectively. In panel (**b**), lane 1 is root tissue, lane 2 is shoot tissue, lane 3 is leaf tissue and lane 4 is tendril tissue. In each of the above panels the upper part shows (**a**, **b** and **c**) the quantitative data, while the lower shows the RT gel picture

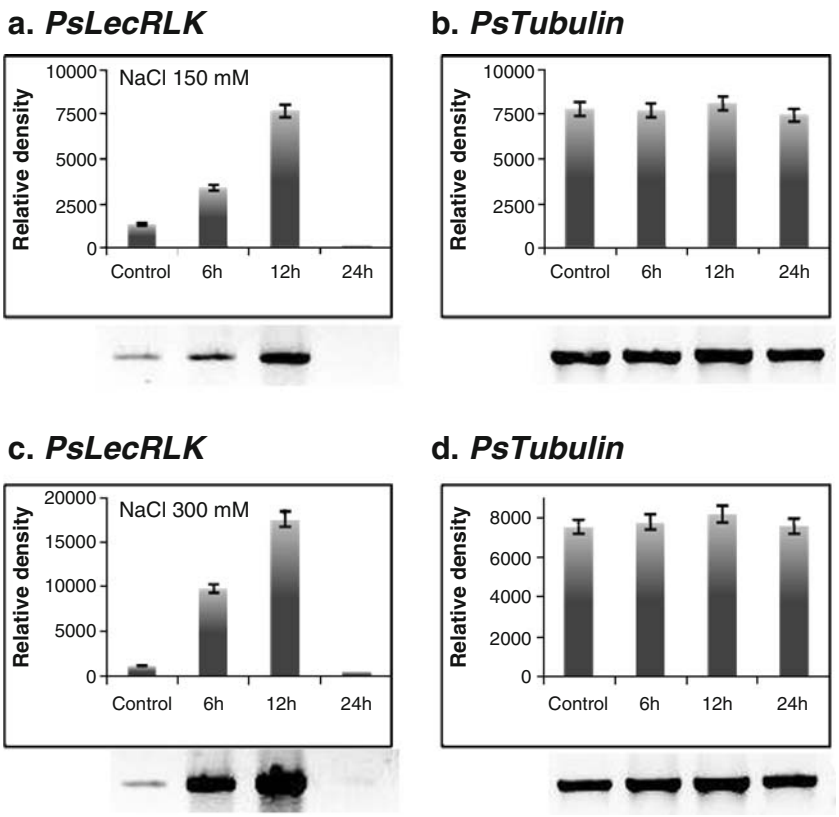

Fig. 6 Transcript levels of *PsLecRLK* in response to 150 mM NaCl (**a**) and 300 mM NaCl (**c**) salt stress at various time intervals. Tubulin (**b** and **d**) gene was used as an internal control. The total RNAs were extracted from 7 day old pea seedlings after stress treatment. RT-PCR was done in triplicate to check the expression level of the transcripts. In each panel lane 1 is the control without any treatment while lanes 2, 3 and 4 are the RNA samples collected after 6 h, 12 h and 24 h of stress treatments, respectively. In each of the above panels the upper part shows the quantitative data, while the lower shows the RT gel picture

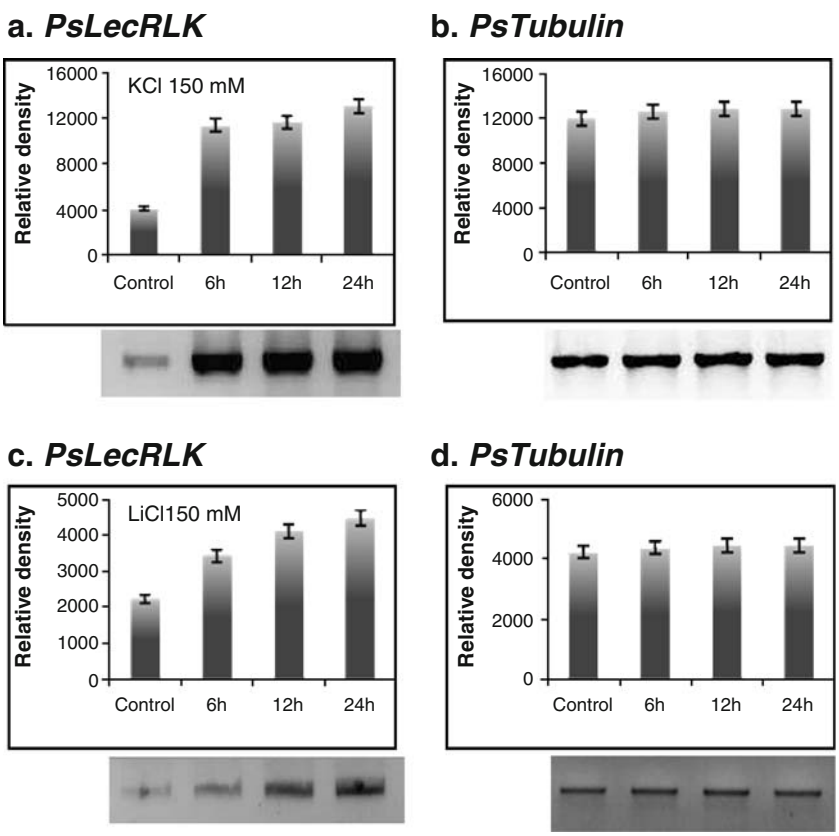

Fig. 7 Transcript levels of *PsLecRLK* in response to 150 mM KCl (**a**) and 150 mM LiCl (**c**) at various time intervals. Tubulin gene (**c** and **d**) was used as an internal control. The total RNAs were extracted from 7 day old pea seedlings after stress treatment. RT-PCR was done in triplicate to check the expression level of the transcripts. In each panel lane 1 is the control without any treatment while lanes 2, 3 and 4 are the RNA samples collected after 6 h, 12 h and 24 h of stress treatments, respectively. In each of the above panels the upper part shows the quantitative data, while the lower shows the RT gel picture

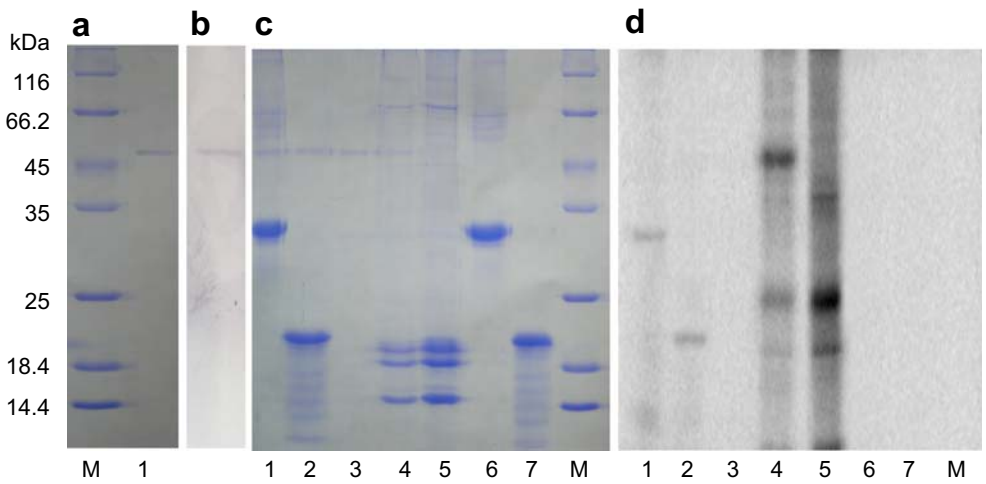

Fig. 8 Protein purification and phosphorylation property of kinase domain of recombinant PsLecRLK protein. **a** Purification of overexpressed PsLecRLK in *E. coli* is shown on SDS/PAGE. Lane M, molecular weight marker; lane 1, 47 kDa kinase domain of PsLecRLK purified (PsLecRLK-KD) from soluble fraction through Ni^{2+}–NTA–agarose column chromatography. The protein size markers are indicated at the left side of the gel. **b** Western blot analysis of the same 47 kDa purified protein fraction by using polyclonal anti-His antiserum. (**c** and **d**) Autophosphorylation of PsLecRLK and phosphorylation of MBP and casein by PsLecRLK-KD protein. After the kinase reaction the samples were electrophoresed on SDS / PAGE and were stained with Coomassie blue (**c**). The phosphorylated products were visualized by autoradiography (**d**). In both c and d, the lane 1: MBP with PsLecRK-KD enzyme, lane 2: casein with PsLecRK-KD enzyme, lane 3: PsLecRK-KD enzyme alone for autophosphorylation, lane 4: pea nuclear extract with PsLecRK-KD enzyme, lane 5: pea nuclear extract without PsLecRK-KD enzyme, lane 6 casein without PsLecRK-KD enzyme, lane 7: MBP without PsLecRK-KD enzyme, lane M, molecular weight marker

PsLecRLK-KD on pea nuclear extract was also carried out, which revealed presence of an extra phosphorylated band (Fig. 8d, lane 4), which was not seen when nuclear extract was incubated with label in the absence of PsLecRLK-KD (Fig. 8d, lane 5). These results suggest that PsLecRLK-KD may participate in signaling by phosphorylation of target proteins.

Homology based modeling of PsLecRK protein

The three dimensional modeling of protein was done by taking the atomic coordinates of the protein towards which the protein of interests exhibited maximum homology. Homology modeling of the kinase domain of PsLecRK using AvrPto protein (2QKW chain 'B') as template protein is shown as a ribbon diagram and a Connolly diagram in Fig. 9a and b, respectively. Both the proteins showed the presence of highly conserved ATP binding sequence typical of a protein kinase along with Ser/Thr protein kinase active site signature. Pointed arrows show proximity of the two sites to each other.

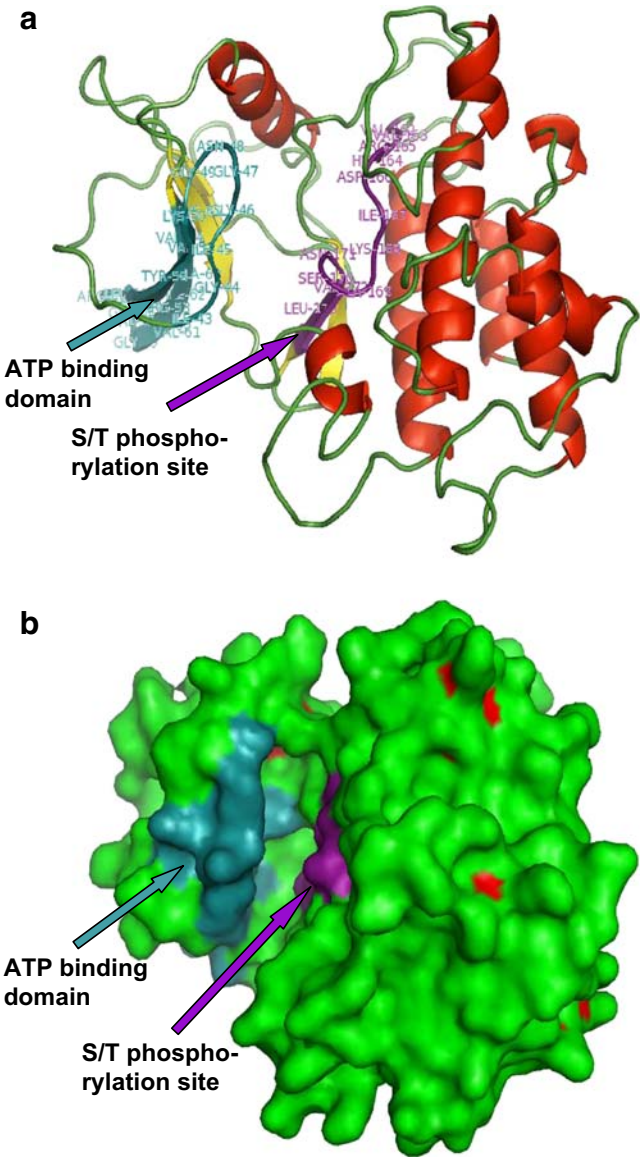

Fig. 9 Homology modeling of the kinase domain of PsLec RK. Comparative modeling was done using EsyPred and PyMol was used as the visualization software. The figure shows a kinase specific signature on the structures. ATP binding site is shown in blue color whereas the catalytic domain (Ser/Thr Phosphorylation site) is shown in purple color (**a**) Ribbon model for the kinase domain of PsLecRK (**b**) Connolly surface of kinase domain of PsLecRK showing accessible surface area. α-helices are denoted by red color, β- sheets by yellow color and loops are colored in green. The structure was constructed using AvrPto protein as a template

Discussion

Salinity stress decreases crop productivity worldwide and generates substantial economic losses each year. Yet innovative research on crop yield improvement under stress condition can reveal cost-effective ways in which farmers can increase both their productivity and their income. The development of high salinity tolerant useful, bacteria of agronomic importance would therefore have a great application in agricultural biotechnology. With this aim we have isolated new salinity stress tolerant genes which can be used to develop salinity stress tolerant crop plants and useful bacteria, which may ultimately play a role in imparting salinity tolerance to plants.

Plant growth and development are mediated by a complex array of signaling pathways that regulate all phases of growth. The ability to adapt to salinity stress exists in all living systems with minor modifications from bacteria to higher plants. Understanding the mechanism of stress tolerance along with a plethora of genes involved in stress signaling network is important to improve salinity stress tolerance in crops plants. Various stress-induced genes involved in plant stress response have been identified by differential screening, differential display or microarray analysis [31–33]. Current data have established that functionally analogous salinity stress tolerance genes exist in both unicellular organisms and plants, suggesting that common mechanisms for salinity tolerance are emerging across the phylogenetic spectrum [24, 25, 34–36]. Therefore, isolating novel salinity stress-induced genes from plant and checking their salinity tolerance in bacteria could be a faster approach for the functional validation of genes in salt tolerance. Previously, the *E. coli* has been used for functionally screening plant cDNAs involved in plant drought and salt tolerance [22, 23, 25] and to prove the key role of specific plant gene products in enhancing stress tolerance, as found for the HSP17.5 of *Castanea sativa* [37].

In the present study, a total of 34 cDNA sequences from *Pisum sativum* were isolated from E.coli cells that survived growth on high salt (0.8 M NaCl) containing medium and assigned to six groups. Among these are putative genes that are stress related to be stress responsive in literature. The cDNAs that fall into this category includes those encoded for proteins involved in photosynthesis like chlorophyll a/b binding and those involved in translational machinery like ribosomal proteins. There were also certain cDNAs encoding for DNA binding proteins such as F-box protein and small nuclear ribonucleo protein. Other like phosphatidylcholine transfer protein, aspartate aminotransferase, glycolytic glyceraldehyde 3-phosphate dehydrogenase, flavonoid glycosyltransferase and sterol delta-7 reductase DWF5 were known to be involved in metabolism. Some proteins like fiddlehead proteins, kinases, cryptochromes and leucine-rich repeat protein belong to miscellaneous/signaling categories. The presence of unknown proteins may attribute to possibilities that many unique salinity tolerant genes have not been annotated in the databases. These proteins may represent new salinity tolerant gene candidates from *Pisum sativum*. The elucidation of their functions may shed light on new mechanisms of salinity tolerance in this plant. The functions of photosynthesis related proteins in non-photosynthetic bacteria is unknown, however, this was not an isolated case as we have also isolated a cDNA clone for light harvesting protein (chlorophyll a/b binding protein) from Pisum sativum that could confer salinity tolerance to bacteria [25].

Here, we have described in detail the isolation of a *LecRLK* cDNA from *Pisum sativum* by functional screening of pea cDNA library under salt stress and shown its functional validation in high salinity stress tolerance in bacteria and its upregulation in planta in response to stress. *LecRLKs* are a large family of receptor-like transmembrane kinases with an extracellular legume lectin-like domain [10]. In Arabidopsis, it is reported to be a highly glycosylated integral plasma membrane protein [6]. We have also been able to isolate a gene, which shows a high homology to lectin receptor like kinases. It also has an extracellular legume lectin-like domain, and a cytoplasmic kinase moiety. The legume lectins have been reported in the seeds of leguminous plants and have been shown to bind complex sugars [38]. It is reported that legume lectin motifs in RLKs are similar to those of *Ulex* lectin II, a chitobiose-binding lectin [39]. This suggests that these RLKs may also bind to chitobiose, a cell wall component of fungi, insects, and nematodes. In plants lectin-like RLKs play important roles in signal transduction especially in perceiving extracellular signal through its lectin-like domain present at the N-terminal region and finally in transducing the signals to the downstream components [8, 11]. By using OGPET software (http://ogpet.utep.edu/OGPET/), which is an algorithm to predict potential O-glycosylation sites in proteins, a potential glycosylation site of PsLecRLK could be predicted at position 77 (K-n-S-S-Y-n-Y-P). Though not much of work has been done on carbohydrate binding ability of lectin domain of LecRLKs, it is postulated, owing to resemblance with the lectin proteins, that this protein has strong ability to bind to carbohydrates similar to earlier reported plant proteins [6, 7, 9]. Though the carbohydrate binding moiety is essential (Fig. 3) for the function of PsLecRLK in salinity stress tolerance in bacteria, but the mechanism behind this has still to be studied.

Though there are about 45 LecRLKs in *Arabidopsis thaliana*, but relatively little is known about the functions of members of the *LecRLK* family. A number of studies implicated members of this family in various functions, such as disease resistance, hormone signaling, and legume-rhizobium symbiosis [see 10]. Sasabe *et al*. [40] have reported that the tobacco elicitin-responsive lectin-like receptor kinases are membrane-located protein kinases that are induced during defense responses in BY-2 cells. Recently, Wan *et al*. [10] have reported the role of lectin receptor-like kinase in the pollen development in Arabidopsis. As per the findings of the present study *PsLecRLK* transcript shows maximum expression in response to NaCl stress. It also gives the recombinant bacteria, the maximum survival rate in high NaCl stress conditions. The gene is shown to be highly conserved all across the taxonomic lineage. Homology modeling of kinase domain of LecRK was done using AvrPto protein, which is also a kinase. AvrPto phosphorylates Pto kinase rendering it inactive. The homology model along with conserved domain search for kinase domain of lectin kinase revealed the presence of a cleft, on one side of which sits the ATP binding site and on the other side sits the active site (Ser/Thr Phosphorylation site). Though in the sequence the two sites are separated by hundreds of amino acids, in spatial arrangement these two domains face each other in such a way that after ATP binding at the ATP binding site, the phosphate group could be easily transferred by the catalytic domain to the substrate without facing any spatial constraints.

The present study predicts that the *PsLecRLK* may have a novel role in high salinity stress tolerance. A hypothetical model describing the role of lectin kinase in stress tolerance is shown in Fig. 10. The stress signal is perceived probably by the ligand binding region of the lectin kinase present in the extracellular domain, which in turn may activate its protein kinase domain. The activated domain then phosphorylates some intermediate substrates, which then directly or indirectly regulates the salinity stress tolerance.

Since *PsLecRLK* is present in the cell membrane it is an interesting speculation as to how it would be helping the bacteria to survive the very high salt stress conditions. As

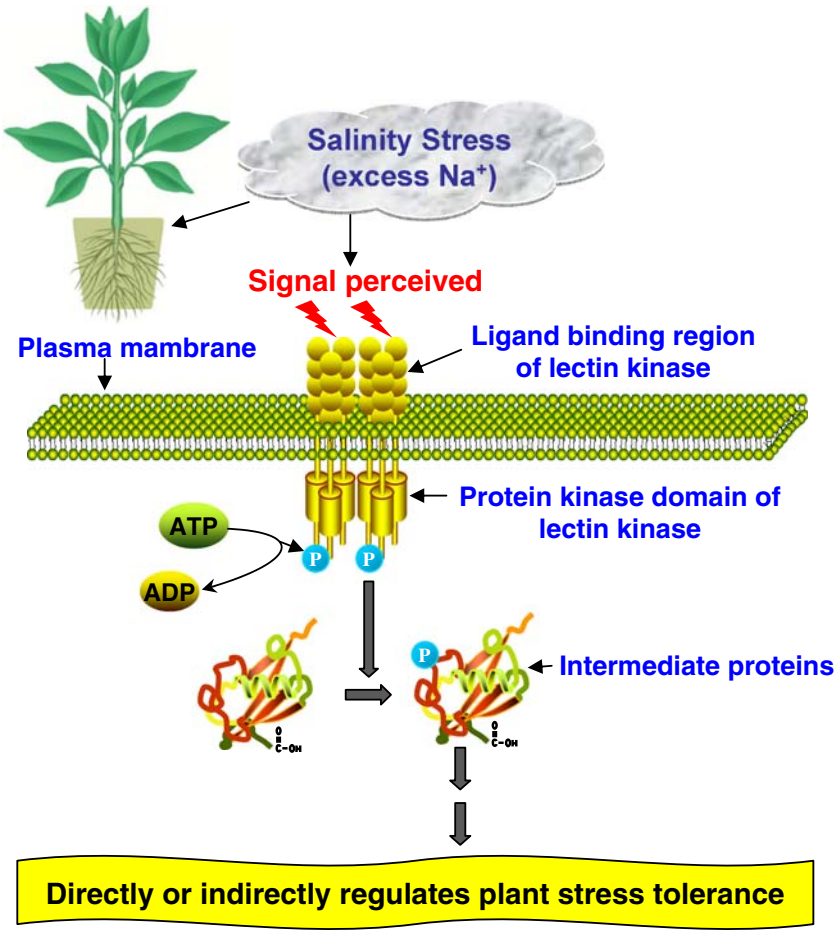

Fig. 10 A possible hypothetical model for role of plant lectin kinase in salinity stress tolerance. The lectine-like RLK has an extracellular domain sharing sequence similarity with legume lectin which can bind various hydrophobic molecules including oligosaccharides and plant hormones. This lectin-like domain can perceive extracellular stress signals and transduce them to the downstream components through similar biochemical pathways. After activation of lectin-like RLK via the stress signal(s) it may phosphorylate some intermediate proteins which can directly or indirectly regulate the stress tolerance. However a detailed model for mechanisms and role of lectin kinase mediated response has yet to emerge

the lectin kinase is conserved taxonomically, it probably plays a role in some stress signaling pathway present in bacteria, which still needs to be defined. Probably it phosporylates and thereby activates some important proteins which overall protects the integrity of the cell membrane in high salt stress conditions and allows the cell to survive. The kinase activity of the purified PsLecRLK-KD protein suggested that it may be participating in the signal transduction pathway by phosphorylation of the target proteins. The results of intracellular Na^+ ions measurement in bacterial transformants cells of pBSK-PsLecRLK grown under normal LB medium and 0.8 M NaCl in the LB medium showed that there was no significant difference in both the conditions. With these results we concluded that PsLecRLK could be membrane resident and somehow functions by regulating sodium ion uptake from medium. This protein probably interacts, directly or indirectly, with some antiporter proteins, which then regulate Na^+ ion concentration making conditions amenable for survival of the bacteria. WoLF PSORT software (http://wolfpsort.org/) was used to predict whether the PsLecRLK protein is localized in the membrane or not. This software has the ability to specifically predict transmembrane helices of gram negative bacteria. The result also predicted that the protein is a membrane protein with three transmembrane helices. The other speculation could be that the PsLecRLK may play a role in stress signaling in bacteria similar to the trehalose pathway [41]. The disaccharide trehalose is widely distributed in nature and can be found in many organisms, including bacteria, fungi, plants, invertebrates and mammals. Due to its particular physical features, trehalose is able to protect the integrity of the cell against a variety of environmental injuries and nutritional limitations. The *PsLecRLK* may be activating the trehalose pathway and thereby helping the bacteria to survive high salt concentration. However, the link between this kinase and trehalose pathway has still to be defined.

As there is always a cross-talk between the stresses, resistance to one abiotic stress may confer cross tolerance to several other stresses [13, 42–44]. The data presented here also show that the salinity-induced *LecRLK* gene may also be responsible for giving cold or drought stress tolerance, as its expression level induces in these stresses also. The *PsLecRLK* also showed organ specific expression as the transcript accumulated maximum in roots and shoots whereas only trace amounts were found in leaves and tendrils. To check for the specificity of Na^+ ion induced

expression of *PsLecRLK* as compared to other ionic stressants like KCl and LiCl causing salinity stress, the expression levels of the *PsLecRLK* gene were also studied for different time periods under various salt stressants as mentioned above. We observed that induction of the gene in response to Na^+ ion was much more than Li^+ and K^+ ions, suggesting the Na^+ ion-specific response.

Overall, here we present the first direct evidence for the role of pea LecRLK in salinity stress tolerance in bacteria and its upregulation *in planta* under abiotic stress including salinity. Also, our findings provide an excellent starting point to investigate the potential roles of other members of the LecRLK family in plant stress tolerance. This study shall provide a significant contribution for our better understanding of mechanism of stress tolerance in bacteria and plants. These observations also give us new insights into its application in the plant biotechnology field and provide a new tool for developing stress tolerant crop plants and bacteria. It remains to be tested whether the expression of these genes will confer durable resistance to high salinity tolerance in crops, but the successful identification of the salinity stress induced gene reveals a clear new pathway for the direction for further experimentation.

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