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RESEARCH ARTICLE

Cloning and In silico analysis of Casparian strip membrane domain protein (CASP) from rice

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ABSTRACT

Casparian strip (CS) seals the adjacent endodermal cells to form a tight junction and thereby regulate the movement of water and salts across the endodermis. Casparian strip membrane domain proteins (CASPs) play an important role in the formation of the CS. Among the CASP proteins, the role of CASP1 was much emphasized in the making of a protein scaffold on the surface of endodermal cells that inturn leads to the formation of CS. In this study, full-length cDNA of CASP1 was cloned from the roots of salt-stressed rice cultivar ASD16 using specific primers derived from the corresponding reference gene sequence in GenBank. The cDNA fragment of 813 bp obtained through RT-PCR was cloned in an intermediary cloning vector pJET1.2. The recombinant clone carrying CASP1 was characterized by restriction analysis and sequencing. Further, in silico analysis of CASP1 revealed that it is a transmembrane protein consisting of five transverse helices as per secondary and tertiary structure analysis. 3D structure of CASP1 protein was obtained by I TASSER online server. The structure was validated using PROCHECK structural analysis tool.

Keywords:

INTRODUCTION

Plants have acquired several adaptive measures to cope up with stressed environments. One such adaptive measure is the development of endodermal barriers to keep out toxic elements. The solutes and water absorbed by the root hairs move radially through the roots *via* a combination of apoplastic, symplastic and transcellular pathways (Steudle 2000). Plasma membrane selectively uptake ions through specific transporters and hence act as the first line of a barrier for symplastic and transcellular movement of water and ions (Marschner 2011). The apoplastic pathway is the major pathway for the entry of solutes and water into the xylem vessels of roots (Enstone *et al.,* 2002). On the other hand, the movement of solutes through the apoplastic route is regulated by apoplastic barriers *viz*.Casparian strip (CS) which is present in the endodermis of all vascular plants for efficient and selective sorting of nutrients and ions.

CS is a belt-like radial thickening observed in the transversal - anticlinal cell wall of exodermis and endodermis of plant roots. CS was reported for the first time by Robert Caspary in 1865, who described them as "protective sheath" ("Schutzscheide") while (Kroemer 1903) called them as a Casparian strip. CS spans the cell wall of adjacent endodermal cells to form a tight junction that blocks extracellular diffusion across the endodermis (Alassimone *et al.,* 2010). This junction is composed of lignin along with Casparian strip domain proteins (CASPs), thus leading to the establishment of a diffusional barrier between the inner and outer sides of endodermis. CASPs form a protein scaffold in the plasma membrane at the site where the CS forms. CASPs, according to (Roppolo *et al.,* 2011) are transmembrane spanning proteins, with short cytoplasmic domains. They specifically earmark the prospective site of CS deposition in the root endodermis, aggregate in a central ring-like formation referred to as the Casparian strip domain (CSD). Under CASPs, a family of proteins were identified *viz*., CASP1 to CASP5, which establishes CSD and helps to localize CSD at the place of CS formation. It has been demonstrated that among the CASPs, CASP1 and CASP3 are required for correct positioning of CS, as the *casp1* and *casp3* mutants showed defects in CS formation (Roppolo *et al.,* 2011).

Salt stress majorly affects rice growth and productivity worldwide. Na^+ is the causal ion, which imposes ionic and osmotic toxicity under salt stress. The apoplast is a dominant pathway for entry of Na^+ ions in rice shoots. There is a correlation between the accumulation of Na^+ ions in shoots and reinforcement of apoplastic barriers in rice. Salt

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tolerant rice cultivar *Pokkali* developed an extensive apoplastic barrier as compared to sensitive genotypes upon salt treatment (Krishnamurthy *et al.,* 2009), suggesting the relevance of apoplastic barriers in salt tolerant genotype, *Pokkali*. Cai *et al.* (2011), showed that when two weeks old rice cultivars were subjected to 200 mM salt stress for 12 hrs, the rate of CS cells development in the radial walls of endodermis was significantly higher in salt tolerant cultivar Liaohan 109 in the regions up to 20 mm and 30 mm from the root tip as compared to salt susceptible cultivar Nipponbare.

In this direction, genetic manipulation of crops using genes involved in CS development has been thought of a prospective strategy towards the development of salt-tolerant plants (Foster and Miklavcic 2017). Therefore, cloning of the genes involved in CS strip development would lay the basis for engineering salt tolerance in rice. In this study, we chose to clone and characterize Casparian strip membrane domain protein 1 (CASP1) given its importance in CS development. Given gene was cloned from salt susceptible rice variety ASD16, cloning of a gene from salt-tolerant variety is underway. CASP1 gene sequence comparison between salt susceptible and tolerant varieties to be taken up.

MATERIAL AND METHODS

An elite rice cultivar ASD16 maintained in Paddy Breeding Station, TNAU was used in this experiment.

In vitro culture of rice seedlings

Hydroponics culture system based on Yoshida medium (Yoshida 1976) was used for growing rice *in vitro*. The hydroponics assembly included, perforated thermocol sheets with nylon mesh support fitted onto plastic trays filled with Yoshida medium. Seeds were directly germinated on the nylon mesh and the seedlings grown under glass house condition. The pH of Yoshida medium was maintained at 5.0 and medium was changed every six days. Twenty days old seedlings were stressed with 75 mM Sodium chloride for three days and the roots harvested.

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from roots of salt-stressed plants using TRI-reagent. RNA was converted into cDNA using Revert Aid First Strand cDNA synthesis kit (Thermo Fisher Scientific India) using OligodT primers. The cDNA was amplified by *CASP1* gene-specific primers (Table 1) with Q5 polymerase master mix. PCR program used was as follows, denaturation at 98 °C for 3 min, followed by 35 cycles of 98°C for 30 sec, annealing at 59 °C for 30 sec and an extension of 72 °C for 45 sec, with a final extension of 72 °C for 5 min. A 20 µL of PCR mixture contained; 1 µL of each primer (100 pmol), 10 µL of 2X Q5 polymerase (High Fidelity Polymerase) master mix (NEB, England), 1 µL of cDNA, and 7 µL of DNase free water. The RT-PCR product was separated on 1 % agarose gel and target band was eluted using gel elution kit (BioBasic Canada Inc.). The amplified product was ligated in pJET 1.2 cloning vector harboring ampicillin resistance selectable marker. The recombinant vector was transformed into to *E. coli* DH5α competent cells which were plated onto LB agar medium plate supplemented with ampicillin (100 mg/ml). Transformants were characterized by colony PCR with gene-specific primers and by restriction digestion of the recombinant plasmid with *Bgl*II enzyme for the release of the cloned insert. Subsequently, the putative recombinant clone(s) was sequenced.

Table 1. Primers used for amplifying the cDNA encoding CASP1 gene

Primer Name	Primer sequence (5'—3')
CASP1-F	CTATCTCGCAGCCAACCTGT
CASP1-R	CGCGCATGCAGATAGAAATCAA

Sequence and structure analysis

The amino acid sequence of CASP1 from *Oryza sativa, Zea mays, Panicum miliaceum, Panicum hallii, Sorghum bicolor, Aegilops tauschii* subsp. *Tauschii, Setaria italica*, were retrieved from the UniProt database. Multiple sequence alignment was performed using the Clustal W program and MEGA X software tool was used to construct the phylogenetic tree by the neighbor-joining method.

Hydropathicity plot of CASP1 amino acid sequence from *Oryza sativa* (Unit prot Id. Q7XPU9) was plotted with a window of 19 amino acids by Protscale program (http://ca.expasy.org/cgi-bin/protscale.pl). Transmembrane domain prediction was done by TMHMM (http://www.cbs.dtu.dk). Secondary structure was predicted using SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl).

Tertiary structure modeling of CASP1 sequence from *Oryza sativa* (Unit prot Id. Q7XPU9) was done using online threading server I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). Further structure validation was carried out using PROCHECK (https://servicesn.mbi.ucla.edu/PROCHECK/)

RESULTS AND DISCUSSION

Cloning of CASP1

The 813 bp cDNA fragment of *CASP1* was cloned from the roots of an elite rice cultivar ASD16 using gene-specific primers derived from the reference sequence (Gene ID: XM_015778983.1) of cv. Nipponbare. The expression of *CASP1* was earlier reported from young and differentiating roots of rice (Krishnamurthy *et al.,* 2009, Cai *et al.,* 2011). In rice, CS develops near to root tips during salt stress

Table 2. Ramachandran Plot statistics for CASP1 model

	No.of residues	percentage
Most favoured regions [A,B,L]	142	71.7 %
Additional allowed regions [a,b,l,p]	47	23.7 %
Generously allowed regions [~a,~b,~l,~p]	3	1.5 %
Disallowed regions [XX]	6	3.0 %
Non-glycine and non-proline residues	198	100 %

conditions and deposition was wider in the case of salt tolerant genotypes (Cai *et al.,* 2011). Taking cues from earlier reports, rice seedlings were grown for a three weeks period under *in vitro* condition in Yoshida nutrient solution and subjected to 75 mM NaCl salt stress for a three days period before the roots were harvested for total RNA extraction. The *CASP1* RT-PCR product of 813 bp from ASD16 as resolved on agarose gel electrophoresis is given in figure 1. Subsequent restriction digestion with *Bgl*II enzyme confirmed the presence of an insert DNA of the expected size (i.e) of about 813 bp (Fig. 2). The putative recombinant clone was further characterized by sequencing, and the *CASP1* from ASD16 was aligned with the *CASP1* from the Nipponbare reference genome and ensured the correctness of the cloned *CASP1* sequence.

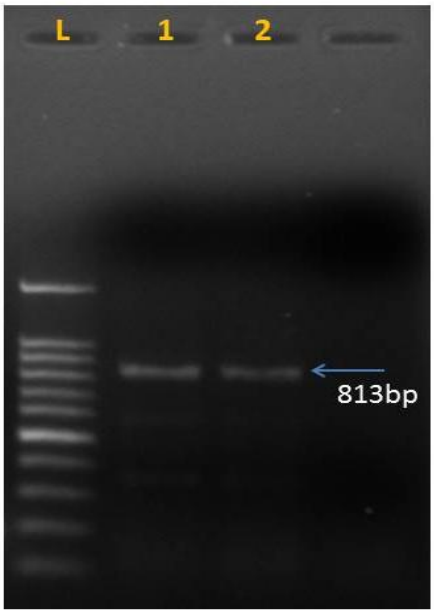

Figure 1 RT-PCR product of CASP1 gene
(L: 100bp ladder,1-2: RT-PCR product of CASP1 gene)

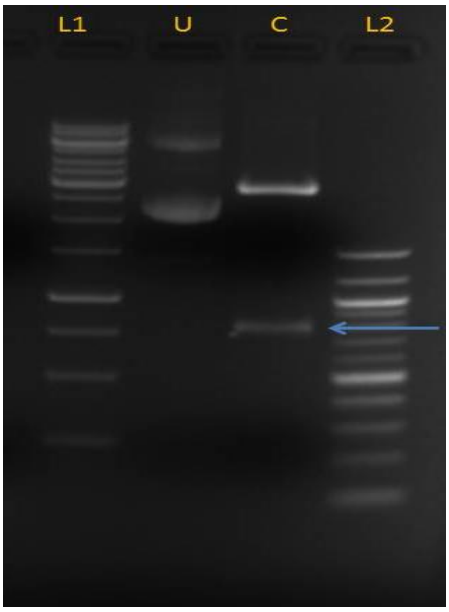

Figure 2 Restriction digestion of isolated plasmid by BglII restriction enzyme (L1:1kb ladder, U:Uncut plasmid, C:Cut plasmid, L2: 100 bp ladder)

***In silico* sequence analysis**

Multiple sequence alignment of CASP1 amino acid sequences of *Oryza sativa, Zea mays, Panicum miliaceum, Panicum hallii, Sorghum bicolor, Aegilops tauschii subsp. Tauschii, Setaria italica* (Fig. 3) showed highly conserved regions across the members of the cereal family. Further phylogenetic analysis using revealed that CASP1 amino acid sequences from *Zea mays* and *Panicum halli* are clustered together. And, *Oryza sativa* appears to group with the above (Fig. 4).

Kyte Dolittle plot analysis of rice CASP1 amino acid sequence showed a highly hydrophobic pattern (Fig. 5). Further, the transmembrane nature of CASP1 was predicted using the TMHMM program which revealed the possibility of at least five transmembrane domains spanning 106 amino acid residues (Fig. 6). The probability of N-terminal lying on the internal or cytoplasmic side was 24.59 %. This suggested that the N-terminal of CASP1 might lie on the exterior side of the membrane of the endodermal cells. Apart, the expected number of amino acids in transmembrane helices in the first 60 amino acids of the protein is 16.37, therefore it is predicted that CASP1 might have a signal peptide on its N- terminal.

The hydrophobic nature of the CS helps to protect plants from various environmental stress conditions (Karahara *et al.,* 2004). CS was earlier thought to be made up of lignin and suberin but (Naseer *et al.,* 2012) established that it is solely made up of lignin. This finding is functionally significant as CS do not completely seal the cell membrane like suberin lamellae and in turn might be regulating ion and water uptake.

Secondary and tertiary structure analysis

Secondary structure of CASP1 revealed that it is primarily made of helices to an extent of 51.9 %. Rest of the region is composed of beta turns (4.02 %), extended strands (14.73 %) and random coil (29.46 %). Beta turns and coils might facilitate the formation

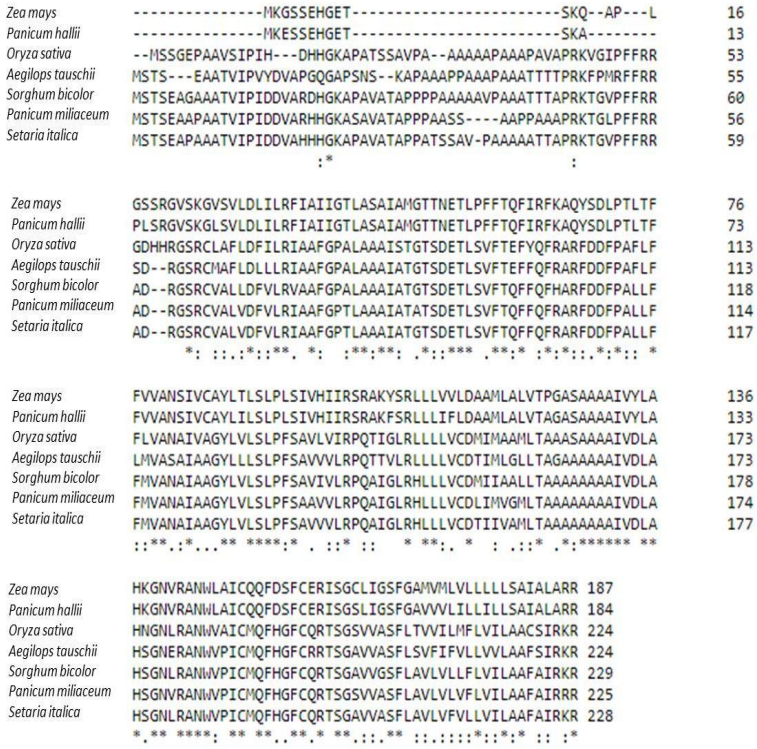

Figure 3. Amino acid sequence alignment of *CASP1* from members of the cereal family

of helix-loop-helix motifs and in turn the transmembrane domain of CASP1. Thethree-dimensional structure of CASP1 was performed by threading approach in an online server I-Tasser.

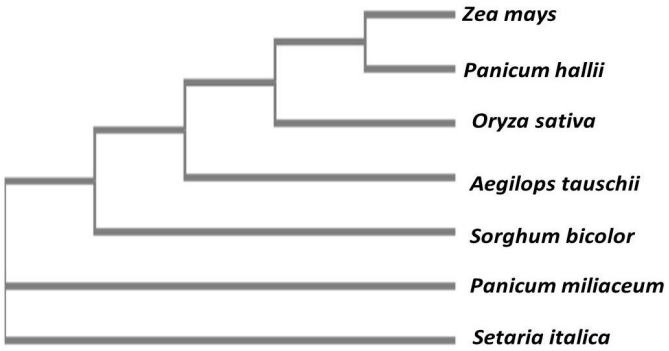

Figure 4 Phylogenetic relationship of CASP1 from members of the cereal family

Five 3D models were obtained from I TASSER, out of which best model was selected by structural validation using PROCHECK, a structure analysis

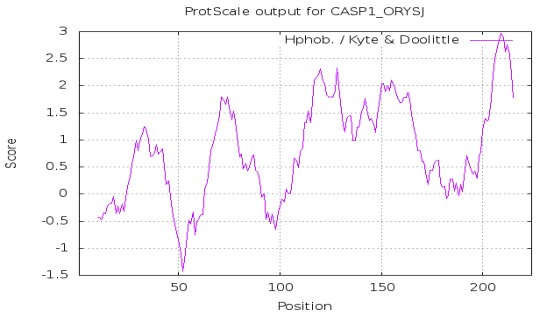

Figure 5. Kyte-Dolittle hydrophobicity plot for CASP1 amino acid sequence

tool which analyzes model on basis of parameters like Ramachandran plot, peptide bond polarity, bad

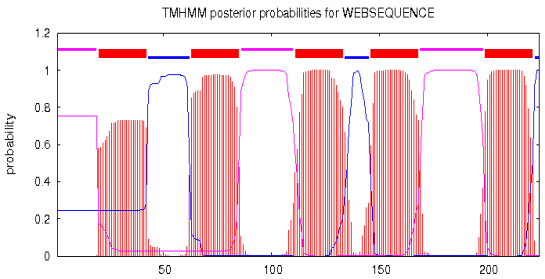

Figure 6 The CASP1 plot showing the posterior probabilities of inside/outside/TM helix.

non bonded interactions, main chain hydrogen bond energy, C-alpha chain clarity and overall G factor. Percentage of amino acid residues in the desired regions were around 71.7 % (Table 2). Five transmembrane helices were found in the predicted tertiary fold given that the best fitting model obtained in this study (Fig. 7). Further improvement in the

model is required to have a detailed functional insight.

Figure 7. Predicted 3D structure of Oryza sativa Casparian strip membrane domain protein 1

The CASP1 in *Arabidopsis* is known to have four transmembrane domains with N-terminal segment on the cytoplasmic side (Roppolo *et al.,* 2014).

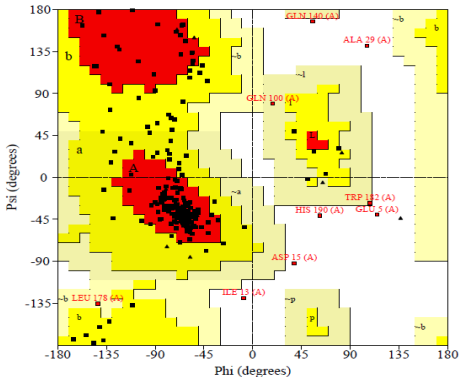

Figure 8. Ramachandran Plot of CASP1 model

Transmembrane domain analysis and structure prediction revealed five transmembrane domains in rice CASP1 unlike *Arabidopsis* and N-terminal end lies on the extracellular side. The extracellular loops are important characteristics of CASP's which have to be investigated further.

CONCLUSION

Increased salinity of the arable lands necessitates the development of salt tolerant crop varieties. In this direction, CS appears to be an adaptive mechanism of the salt-tolerant crop varieties wherein, reduced uptake of the Na^+ ions enhanced the developmental ability of the crops to withstand salt stress. Genes like *CASP1* are known to be important in CS development. Such genes can be exploited in the development of transgenic plants under the control of suitable promoter paving way for engineering plants showing earliness in the development of CS, in turn conferring an effective apoplastic barrier to Na^+ uptake. In the present study, *CASP1* gene was successfully cloned which can be used to genetically engineer rice varieties for better performance under salinity stress.

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