Water stress inducible proline transporter from Indica rice

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ABSTRACT

The accumulation of osmoprotectant such as proline is an important process for the adaptation to adverse environmental conditions. The increase of osmoprotectants is achieved either by altering metabolism or by its transport. Plant proline transporters belong to the amino acid/auxin permease (AAAP) sub family of plant amino acid transporter (AAT) super family. We identified four putative proline transporters in indica rice by sequence homology search. With an objective to clone proline transporter genes and cDNAs from rice, we used a drought tolerant indica cultivar N22 and carried out a transcript expression profiling after imposing water stress. A 1.2kb genomic fragment and a 900bp cDNA of two different indica rice proline transporters was PCR/RTPCR amplified, cloned in pGM-T-easy vector, sequenced and characterized.

Key words : Proline transporter, rice, water stress.

Understanding the molecular responses of plants exposed to different abiotic stresses is of utmost importance today. There are many cellular mechanisms by which organism ameliorate the effects of environmental stresses. In plants, under abiotic stress, low molecular weight osmoprotectants and osmolytes such as proline, glycine betaine, sugar alcohols are synthesized and accumulated and their synthesis and degradation have been studied well (Kiyosue et al., 1996 and Ishitani et al., 1993). Very high accumulation of cellular proline (upto 80% of the amino acid pool under stress and 5% under normal condition) has been documented in many plant species (Widodo et al., 2009 and Choudhary et al., 2005). The increase in osmoprotectants is achieved either by altering metabolism (increasing biosynthesis and/or decreasing degradation) or by transport (increase uptake and/or decrease export) which also depends on the species and the extent of stress. In plants proline is synthesized from glutamate as well from arginine/ornithine. Unlike other amino acids, proline has cyclized amino nitrogen that has significant influence on the conformation of polypeptides. Proline is also a major component of structural proteins in animals and plants besides being a known osmoprotectant capable of mitigating the impacts of drought, salt, and temperature stress in plants (Rodriguez and Redman, 2005).

Proline has diverse role under osmotic stress condition, such as stabilization of proteins, membranes and other sub cellular structure, and protecting cellular function by scavenging reactive oxygen species (Rodriguez and Redman, 2005; Bohnert, 1999 and Vanrensburg et al., 1993). Current studies revealed high concentrations of Pro in plant organs during endogenously controlled dehydration, e.g. in pollen (Lehmann et al., 2010) and a high leaf-to-root ratio for Pro in several plant species including Arabidopsis, lentil and common bean (Misra and Saxena, 2009; Sanchez et al., 2001 and Hua et al., 1997) has been reported. Proline is also found in foliar, extra floral and floral nectar (Nepi et al., 2009 and Carter et al., 2006). Schmidt et al. (2007) reported high proline level in Arabidopsis seeds.
However, the highest levels of Pro accumulation are typically seen in response to low water potential with lower levels accumulating in response to salt or cold (Sharma et al., 2011).

Chen and Dickmen (2005) showed the ability of Pro to function as a potent antioxidant scavenger and inhibitor of programmed cell death in the fungal pathogen Colletotrichum trifolii and budding yeast. Sharma et al., (2011) demonstrated that both Pro synthesis and catabolism were required for optimal growth at low water potential. During low water potential Pro synthesis in the photosynthetic tissue regenerates NADP while Pro catabolism in meristematic and expanding cells is needed to sustain growth.

Recently, the importance of proline transport and distribution was reported at the tissue level. Proline transport into cells is mediated by both high and low affinity transport systems coupled with H⁺ co-transport. The low affinity system consists of some amino acid permeases, which have broad substrate specificity for various amino acids. A proline specific transporter (ProT) functions as a high affinity uptake system, and it is important for rapid distribution of proline under water stress (Ueda et al., 2008).

Amino acid transporters (AATs) are the integral membrane proteins which mediate the transport of amino acids across cellular membranes in higher plants, and play an indispensable role in various processes of plant growth and development, including long distance amino acid transport, response to pathogen and abiotic stresses (Zhao et al., 2012). By heterologous expression systems and database screening with known transporters, more than 60 distinct AAT genes have been identified in Arabidopsis (Tegeder, 2012 and Rentsch et al., 2007). Zhao et al. (2012) carried out a genome wide survey and expression analysis of 85 AAT genes in rice and classified them into eleven distinct groups. In plant AATs family includes two main families that belong to the amino acid-polyamine-choline (APC) transporter superfamily: the amino acid/auxin permease (AAAP) family and the APC family (Saier et al., 2009 and Ortiz et al., 2000). There are at least six subfamilies in the AAAP family; proline transporters (ProTs), including amino acid permeases (AAPs), lysine and histidine transporters (LHTs), c-aminobutyric acid transporters (GATs), auxin transporters (AUXs), aromatic and neutral amino acid transporters (ANTS) (Zhao et al., 2012). The APC family in plants is grouped into three subfamilies: cationic amino acid transporters (CATs), amino acid/choline transporters (ACTs) and polyamine H⁺-symporters (PHSs) (Okumoto and Pilot, 2011, Hunt et al., 2010).

Within plant super families of amino acid transporters, the transporters of proline transport proline only and no other amino acids and they have been identified in different plants (Lehmann et al., 2010). Proline transporters (ProTs) were first isolated from Arabidopsis as highly selective transporters for proline (Rentsch et al., 1996). AtProTs were responsible for transporting proline, glycine betaine and GABA, and their expression patterns were complementary (Grallath et al., 2005). There are very few reports on transport of Pro in various tissues. In one of the research report by Ueda et al., (2008) balance of Pro translocation between source and sink tissue was analysed in transgenic Arabidopsis plants by altered expression of a barley proline transporter using a constitutive and root cap specific promoter in Arabidopsis. Their results showed the importance of proline distribution at the tissue level during vegetative development.

Towards investigating ProTs from indica rice we have carried out transcript expression analysis of rice ProT (OsProT) in tissues under water stress at seedling stage and also cloned and analysed the cDNA of (OsProT) and a genomic fragment of another putative amino acid permease/transporter.

**MATERIALS AND METHODS**

The present study was carried out in a laboratory experiment in the 2008-2010 periods at National Research Centre on Plant Biotechnology, New Delhi.

**Sequence identification, retrieval of ProTs**

Various indica rice genome sequence databases such as BGI, Gramene and NCBI,
RGAP-MSU nucleotide databases were searched with ProT as the keyword and sequences for four ProTs (OsPrT1-4) carrying locuses were downloaded and used for primer designing.

**Plant material and water stress treatments**

Seeds of *Oryza sativa* cv Nagina 22 were germinated in pots containing autoclaved soilrite under 25°C in a culture room for 7 days for genomic DNA isolation. For total RNA isolation 7 day old seedlings were exposed to water stress by withholding water for 7 days and samples were collected on 14th day and stored at -80°C freezer for further analysis.

**Chemicals**

Chemicals for molecular biology work were obtained from Sigma (USA) and BioBasic (Canada), and Hi media Laboratories (India).

**Kits and enzymes**

PCR -Gel extraction kit and TA cloning vectors were obtained from Promega, Madison (USA). Restriction Enzymes, Taq DNA Polymerases, DNA Ladders from New England Bio Lab (USA). Superscript III first strand cDNA kit, DNase I amp grade, Triazol were obtained from Invitrogen (USA). Primers were obtained from Sigma (USA) and Gene Script (China).

**Isolation of DNA and PCR**

DNA was isolated from fresh leaf sample using CTAB (cetyl trimethyl ammonium bromide) extraction method (Murray and Thompson, 1983). In brief, 100 mg of leaf tissue was ground thoroughly in liquid nitrogen. Freshly prepared extraction buffer containing β-mercaptoethanol was added. The suspension was incubated at 60°C for 30 min with intermittent mixing followed by addition of equal volume of chloroform:isoamylalcohol (24:1) and centrifuged at 12000 rpm for 5 min. DNA from aqueous layer was precipitated by adding 0.6 volume of isopropanol. The mixture was centrifuged at 13000 rpm for 10 min to collect the pellet. DNA was washed with 75% ethanol, centrifuged at 12000 rpm for 5 min and dried for 10 min and finally dissolved in RNAse free water and incubated at 55°C for 10 min and checked on a 1.2% gel with RNA loading dye. 1 ug RNA after nanodrop quantification was used to synthesize first-strand cDNA using superscript III reverse transcriptase using manufacturer's protocol. 2ul of cDNA mix was used as template in PCR reaction using the primers OsProT2 having forward sequence 5’-ATGGTCCCTTTAGGCTGGATTGGT-3’ and reverse sequence 5’-TCCACTGCCGAATCTTGTGTCCAA-3’ in final volume of 50ul reaction mixture. The following thermocycler program used was: initial denaturation at 95°C for 3 min followed by 35 cycles of 94°C for 45 sec, 65°C for 45 sec, 72°C for 1 min.
for 90 sec with a final extension of 8 min at 72°C. A 900 bp RT-PCR amplicon was observed by running a 1.2% agarose gel which was PCR-gel purified and sequenced.

**Cloning and sequencing of OsProTs**

The purified amplicons of 1.2 kb of genomic DNA fragment and 900 bp cDNA fragment obtained by PCR and RTPCR respectively were ligated into pGEM easy vector following the supplier’s instruction. The ligated vector was introduced into competent *Escherichia coli* strain XL1 blue following heat shock protocol and blue white screening. White colonies were picked up from the respective plates of LB agar-ampicillin-IPTG-X-Gal and clones were confirmed by colony PCR screening protocol using the specific primer for both the fragments and also by restriction digestion with EcoRI enzyme. Confirmed colonies were further sequenced at Ocimum Biosolution (Hyderabad).

**In silico analysis**

The resultant sequences were analyzed using BLAST (www.ncbi.nlm.nih.gov) for sequence homology and confirmed as respective proline transporters. The multiple sequence alignment of the deduced proteins was generated using the Clustal W (www.ebi.ac.uk). The features were added using the Adobe Illustrator software. Putative transmembrane regions were predicted by using the TMHMM server (www.cbs.dtu.dk).

**RESULTS AND DISCUSSION**

A continuing challenge in understanding plant responses to low water potential is to identify the adaptive metabolic changes and determine how they contribute to drought resistance. Besides acting as an excellent osmolyte, proline plays three major roles during stress, i.e., as a metal chelator, an antioxidative defense molecule and a signaling molecule (Hayat et al., 2012).

In this research we have identified four transporters of proline by sequence homology search and after transcript profiling by water withholding in pots cloned partial fragments of one proline transporter cDNA from indica rice viz. OsProT2 by RTPCR (Fig. 1). Genomic fragment of another transporter OsProT4 was also PCR amplified (Fig. 2). The fragments were gel purified, cloned in TA cloning vector and transformed to *E. coli*. After confirmation of cloning by colony PCR and restriction digestion the fragments were sequenced and confirmed as respective ProTs (Figs. 3, 4 and 5). Subsequently they were submitted to NCBI with the accession numbers GQ225740 and GQ246189 respectively.
Multiple sequence alignment of partial OsProT2 with other reported rice proline transporter 2 and one monocot barley proline transporter showed 99-100% and 84% homology respectively (Fig. 6). The deduced amino acid sequence of the rice ProT2 protein (OsProT2) also showed 68.8% homology to the ProT protein 1 from Arabidopsis thaliana and 59.6% homology to Arabidopsis thaliana 193
that from *Lycopersicon esculentum* and is located in chromosome three of the rice genome with nine predicted transmembrane domains (Fig. 7). The *OsProT4* partial genomic fragment was located to chromosome ten of rice genome and harboured amino acid, polyamine transporter motifs.

The proline biosynthetic pathway was outlined 60 years before in *Escherichia coli* (Vogel et al., 1952). Singh et al., (1972) was probably the first to assign a correlation between proline accumulation and drought resistance in barley cultivars. In the glutamate pathway proline is synthesized from glutamic acid via intermediate Δ'-pyrroline-5-carboxylate (P 5C). The reaction is catalyzed by Δ'-pyrroline-5-carboxylate synthetase (P 5CS) and Δ'-pyrroline-5-carboxylate reductase (P 5CR). Proline catabolism occurs in mitochondria by means of the chronological action of proline dehydrogenase or proline oxidase (PDH or POX) producing P 5C from proline and P5C dehydrogenase (P 5CDH) which converts P 5C to glutamate. Intercellular transport of proline occurs between cytosol, chloroplasts and mitochondria as implied by compartmentalization of proline metabolism. Uptake of proline in mitochondria is also an active process with existence of specific amino acid transporters (Hayat et al., 2012, Munns 2005).

Transgenic approaches using various proline biosynthetic and degradation genes have demonstrated the role of proline as a regulator of osmotic adjustment (Kishor et al., 1995 and Nanjo et al., 1999b).

Only a few papers have reported about the effects of genetically modifying amino acid transporters on plant growth. Effect of ectopic expression of *Vicia faba* amino acid permease was investigated in *Vicia narbonensis* and pea with seed specific expression using the LeB4 promoter (Rolletschek et al., 2005). In both transformed *Vicia narbonensis* and pea, individual seed size was increased due to improved nitrogen status. Taken together with the results of the root cap specific promoter derived expression of HvProT in transgenic Arabidopsis plants, (Ueda et al., 2008) it seems to be useful agronomic trait to design amino acid accumulation with tissue specific promoters.

Genetic engineering of a ProT combined with modulation of proline biosynthesis at tissue level will be helpful in developing targeted tolerant plants for a resilient agriculture.

![Fig. 7. Nine predicted transmembrane domains of rice proline transporter2 (OsProT2)](image-url)
REFERENCES


broad specificity amino acid permeases identified by suppression of a yeast amino acid permease-targeting mutant. Plant Cell 8: 1437-1446.


