Overexpression of Proline Transporter Gene Isolated from Halophyte
Confers Salt Tolerance in Arabidopsis

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Abstract: Proline is one of the most important and widespread osmolyte which functions in adaptation to adverse environmental stresses in many organisms. Also it is an important carbon and nitrogen resource in higher plants. Metabolism of proline has been elucidated in many plant species. However, transport of proline was poorly characterized although transport system plays an important role in proline distribution in different tissues. We isolated one full-length cDNA encoding proline transporter from the typical halophyte: *Atriplex hortensis* L. through cDNA library screening and 5'-RACE. The deduced amino acid sequence had eleven transmembrane domains, showed 60% - 69% similarities to other ProTs and the gene was designated *AhProT1*. In the phylogenetic tree higher plants ProTs e.g., *AhProT1* showed more similar to ProP from microorganisms than ProT from mammals. *AhProT1* gene was transformed into *Arabidopsis thaliana* under 3SS promoter. In MS medium containing 14C proline, *AhProT1* plants were able to accumulate much more radiolabeled proline in the roots than control plants. In MS medium containing different concentrations of NaCl, *AhProT1* plants could endure 200 mmol/L NaCl and keep development and biomass increase with proline supply whereas control plants died back at 150 mmol/L NaCl.

Key words: *Atriplex hortensis*: proline transporter; deposition: salt stress

Crops suffered from adverse environmental stresses such as salinity, drought and freezing. Salinity causes ion homeostasis and osmotic stress in plant cells, drought decreases water content and osmotic pressure in plant cells and freezing injury is thought to result primarily from membrane lesions caused by cellular dehydration. As adaptation to these stress conditions many plants can accumulate some highly soluble compounds to raise osmotic pressure in the cytoplasm and stabilize proteins and membranes. These compounds are called osmoprotectants or osmolytes including glycine betaine, proline (Pro), mannitol, etc., and proline is the most widespread osmolyte existed in all biological kingdoms. In plant tissues such as meristems and seeds, proline can also serve as important carbon and nitrogen resource. After decades of researches metabolism of Pro in different organisms now has been well characterized. In higher plants Pro is synthesized through two independent pathways from different precursors, mainly from Glu and partially from Orn. Two enzymes, P5CS and P5CR, catalyze the pathway from Glu. Stress conditions such as salinity can induce the expression of P5CS, P5CR and δ-OAT ( Orn pathway), reduce ProDH ( Pro decomposition pathway) transcript, and thus triggers the Pro accumulation in cytoplasm. However, not all the tissues accumulating Pro depend on Pro biosynthesis. In some tissues without chloroplast such as roots the restricted precursor resources of Glu and NADPH result in limitation of Pro biosynthesis, and in some tissues such as pollen and seeds that undergo dehydration during their maturation, neither the synthesis pathways nor decomposition pathway contributed to the higher concentrations of Pro (> 70% of free amino acids). Using labeled chemicals physiological researches pointed that metabolism of Pro in these tissues has only minor effect, whereas transport of Pro plays a major role in Pro accumulation. It is then necessary to make clear how the Pro transport system contributes to osmotic pressure adjustment in some tissues under salt stress.

For many years it has been a longstanding target of agricultural biotechnology to improve salt and drought resistance in crops. Plant genetic engineering of osmolytes had been viewed as the most effective pathway to achieve stress tolerance in crops, e.g., rice which was an osmolyte non-accumulating plants. In several reports introduction of foreign genes encoding biosynthesis enzymes of osmolytes into transgenic rice had led to modest accumulation of some osmolytes e.g., GlyBet and Pro apparently in consequence limited increase in stress tolerance. In previous reports we isolated several genes involved in osmolyte biosynthesis and performed further genetic engineering in tobacco rice and wheat. We also introduced *AhCMO* and *AhBADH* into tobacco and gained salt-tolerant transgenic plants. In *Atriplex hortensis*, a Pro and GlyBet accumulating halophyte of Chenopodiaceae.
biosynthesis of Pro and GlyBet was recently clarified\textsuperscript{[10-12, 13]}. However, mechanisms regarding the transport of Pro and GlyBet inside plant cells remained unknown. As to the osmolyte transporter functional reseachers were few. The first Pro transporter (ProT) was cloned from Arabidopsis thaliana\textsuperscript{[14-15]} and recently two other ProTs were characterized in tomato and rice\textsuperscript{[6, 18]}, respectively. All of these ProTs are categorized into the AAAP (amino acid/auxin permease) family, which includes over four dozen proteins from euakaryotes\textsuperscript{[17]}. However, only few of them were functionally characterized and till now there is no report characterizing possible function of Pro transporter in plant osmolyte engineering. To obtain the knowledge about Pro transport in a GlyBet natural accumulator under salt stress, we isolated a new ProT gene, AhProTI, from Atriplex hortensis, predicted the arrangement of secondary structures and investigated its phylogenetic tree. Particularly, overexpression of AhProTI could obviously promote salt tolerance in transgenic Arabidopsis and our result indicated new pathway of plant genetic engineering to improve crop resistance to osmotic stresses.

1 Materials and Methods

1.1 Plant materials and growth condition

Seeds of Atriplex hortensis L. were germinated hydroponically at 25 °C for 2 d and then grown in greenhouse at 22 °C in natural daylight during October and November. Mature plants were irrigated with solutions containing 400 mmol/L NaCl for 4 d as salt treatment before mRNA isolation and cDNA library construction. Arabidopsis plants (Arabidopsis thaliana ecotype Columbia) were grown in soil or MS plates at 21 °C with constant light (100 μmol photons·m\(^{-2}\)·s\(^{-1}\)) and 60% humidity.

1.2 Construction and screening of a cDNA library

Total RNA was isolated from leaves of A. hortensis after stressed with 400 mmol/L NaCl for 4 d and 2 μg of poly (A\(^+\)) RNA were used for cDNA library construction. mRNA purification and cDNA synthesis (Promega) followed manufacturer’s protocol, and pExCell vector and Ready-To-Go Kit (Amersham) were used for ligation and packaging. Approximately 250,000 plaques were screened with AtProTI cDNA as a probe. Positive plaques were obtained from the third round screening and then excised in vivo into pExCell plasmids and the plasmid with the longest insert was subjected to sequencing analysis.

1.3 Rapid amplification of cDNA end (5\textsuperscript{\prime}-RACE)

Three gene-specific primers were designed according to the partial sequences of the positive clone obtained by screening cDNA library. The SP1 (5\textsuperscript{\prime}-CTGATAAGTACTGGGGATCC-3\textsuperscript{\prime}) was used to reverse-transcribe the mRNA into first-strand cDNA, and the second nested primer SP2 (5\textsuperscript{\prime}-GCTAAGGTGATCAAGGGCC-3\textsuperscript{\prime}) located upstream of SP1 was used in combination with the adaptor primer for the PCR amplification of the 5\textsuperscript{\prime} terminus of this gene following the manufacturer’s instruction (Roche). The PCR product was ligated into pGEM-T easy vector (Promega) and subjected to transformation. Positive clones were identified by PCR with gene-specific primers SP2 and SP3 (5\textsuperscript{\prime}-AAACCTGATGATGATGTTG-3\textsuperscript{\prime}) and subjected to sequencing. Then a pair of primers (5\textsuperscript{\prime}-TGAACCCCTCTCTCTGCTGGG-3\textsuperscript{\prime}, 5\textsuperscript{\prime}-GGGAAAAATTTATGTTCCG-3\textsuperscript{\prime}) were designed based on the 5\textsuperscript{\prime} and 3\textsuperscript{\prime}-termini respectively, and used to amplify the full-length cDNA.

1.4 Data analysis

The nucleotide and amino acid sequences were compared with those released in GenBank databases by using the Gapped BLAST analysis program. The full-length sequence of AhProTI has been deposited in GenBank databases under the accession number AF274032. The alignment and phylogenetic tree reports were produced by software DNASTAR.

1.5 Arabidopsis transformation and cultivation

A Sma I / Sac I fragment encoding the full-length AtProTI was inserted downstream of the 35S promoter in the plant expression vector pBI121 (Clontech) and introduced into Arabidopsis plants by the vacuum infiltration technique. Nine independent homozygous transgenic lines were obtained after selection of T3 progeny on MS media containing 50 mg/L kanamycin and expression of transgene was confirmed by Northern blotting. Ten-day-old seedlings of nine transgenic progenies and wild type plants are cultured on MS medium and used for further analysis.

1.6 L-[\textsuperscript{14}C]-proline uptake and salt stress test with exogenous osmylates

Seedlings of wild type and transgenic lines were moved into MS bottles containing 0.01 μCi·mL\(^{-1}\) L-[\textsuperscript{14}C]-proline and cultured for 3 d. Radiolabeled plants were then washed carefully and dried on filter paper for fifteen minutes at room temperature, and analyzed by STORM 840 phosphor imager (Molecular Dynamics, USA) after 24 h exposure to a \textsuperscript{14}C-sensitive screen.

To test salt tolerance 0 mmol/L, 150 mmol/L, 175 mmol/L and 200 mmol/L NaCl was used, and seedlings of wild type and transgenic lines were moved into these MS medium containing 5 mmol/L proline. Photos were taken after cultured for four weeks.

2 Results

2.1 Isolation of AhProTI gene from Atriplex hortensis

A cDNA library was constructed using mRNA from salt-treated A. hortensis plants and screened with AtProTI cDNA. Four 5\textsuperscript{\prime}-truncated cDNA clones with different lengths were isolated. A 3\textsuperscript{\prime}-truncated fragment of 583 bp was successfully obtained by 5\textsuperscript{\prime}-RACE-PCR, which contains a 63 bp 5\textsuperscript{\prime}-untranslated region and 240 bp coding region overlapped with the previous clone obtained from cDNA library. Then the full-length cDNA of 1684 bp was generated by Reverse Transcription-PCR and verified by sequencing. Analysis of this cDNA sequence revealed an ORF of 1362 bp in length, which comprises 453 amino acid residues with a calculated molecular.
Fig. 1. Nucleotide and deduced amino acid sequence of AhProT1. Dashed line marks the segment of low compositional complexity. Solid lines indicate transmembrane segments. Secondary structure of AhProT1 was predicted by SMART program on EMBL website (http://smart.embl-heidelberg.de/), and the full-length sequence of AhProT1 has been deposited in GenBank databases under the accession number AF274032.

weight of 49.7 kD (Fig. 1). The deduced amino acid sequence was 69% identical to the Arabidopsis ProT1 protein, and it also exhibits significant sequence similarities to other characterized ProTs. Thus the gene corresponding to this cDNA was designated AhProT1. Secondary structure analysis of AhProT1 predicted totally eleven possible transmembrane domains and categorized AhProT1 into the AAAP (amino acid/auxin permease) family, which
Fig. 2. Phylogenetic analysis of AhProT1 with other Pro transporters and GlyBet transmitters from microorganisms, higher plants and mammals.

Genes and corresponding accession numbers are: EcProP (U75904), CgProP (Y12537), RsProT1 (P22587), HaProT1 (Q99884), RnBGTI (P48056), GgBGT1 (P27789), HaBGT1 (S68256), AtProT1 (X95737), AtProT2 (X95738), AtProT3 (A006919), LeProT1 (AF014808), LeProT2 (AF014809), LeProT3 (AF014810) and OsProT1 (AB022783).

Fig. 3. Uptake of [U-14C] proline in AhProT1+ and control plants. Numbers of 1, 2 and 3 indicate three independent transgenic T3 lines. CK means wild type plant. Seedlings were cultured for 3 days, then washed carefully and dried on filter paper 15 min at room temperature and analysis by STORM 840 phosphor imager after 24 h exposure to a 14C-sensitive screen. A. Four dried seedlings. B. The exposure image.

consists of many eukaryote proteins with similar transmembrane domains.

2.2 Phylogenetic analysis of ProTs

We performed phylogenetic analysis based on amino acid sequence alignment. Totally eight deduced ProTs from plants, two ProTs from microorganisms, two ProTs and two BGTs (Betaine/GABA Transporters) from mammals were analyzed. Figure 2 shows that ProTs in plants and ProTs in microorganisms were more similar to each other than ProTs in mammals in which proline and Betaine were transported by two independent protein systems. And Fig. 2 also shows that AhProT1, LeProT and AtProT in dicots were divergent from OsProT1 in monocots.

2.3 L-[U-14C] proline uptake

To characterize the proline uptake of AhProT1 in plants, AhProT1 was inserted into binary vector under 35S promoter and transformed into Arabidopsis thaliana. After homozygous T3 progenies were identified, nine independent T3 lines were obtained for the following study. Expression of AhProT1 in the nine seedlings was detected by Northern blotting (Data not shown). After the seedlings were cultured on MS medium containing 0.01 \( \mu \text{g} \cdot \text{ml}^{-1} \) L-[U-14C] proline for 3 days, the labeled exogenous proline was transported into the whole plants from roots to apices (Fig. 3). Deposition of proline in AhProT1+ plants and in control plants is different in roots.
but similar in stems or leaves. It has not yet been identified whether AbProT1 can increase similar deposition in flower organs as that in roots due to the limitation of our methods.

2.4 Salt stress on AbProT1+ plants

In order to figure out the effect of active foreign ProT in crops genetic engineering, we use Arabidopsis as a simple model. Wild type Arabidopsis plants respond to 50 mmol/L NaCl on molecular level, grow slowly in 100 mmol/L NaCl, wilt and die away in 150 mmol/L and higher NaCl. We thus set up salt gradient of 0, 150, 175 and 200 mmol/L NaCl, each with additional 5 mmol/L Pro supply. After transfer to salt medium for 4 weeks, AbProT1+ seedlings showed an obvious tolerance to salt stress compared with wild type seedlings not only in survival but also in biomass increase. Figure 4 shows the phenotype of AbProT1+ and wild type seedlings on different salt medium, and Fig. 5 listed the biomass of AbProT1+ and wild type seedlings after four-week-stress on MS medium containing salt. These data indicate that, with exogenous Pro, AbProT1+ seedlings kept growth at 175 mmol/L NaCl, even endured 200 mmol/L NaCl, whereas exogenous Pro had no obvious effect on wild type seedlings.

![Fig. 4. Salt tolerance of seedlings of AbProT1 transgenic plants and control plants grown on MS medium containing different concentrations of salt and additional osmolites. Photos were taken after culture for 4 weeks. A. 5 mmol/L proline and 0 mmol/L NaCl. B. 5 mmol/L proline and 150 mmol/L NaCl. C. 5 mmol/L proline and 175 mmol/L NaCl. D. 5 mmol/L proline and 200 mmol/L NaCl.](image)

3 Discussion

As one important amino acid, Pro exists in all biological kingdoms from microorganisms to higher plants and mammals. Metabolism and transport of Pro were well characterized in microorganisms and mammals. In microorganisms, Pro is transported by two systems of ProP and ProU, which also transport another important osmolyte GlyBet at high affinity. In higher plants, Pro and GlyBet transporter kept unknown till recently genes encoding ProTs were isolated from Arabidopsis and tomato. LeProT1 can transport both Pro and GlyBet[6], and AtProT2 can also transport Pro and GABA which was an important neurotransmitter in mammalian[15]. However, in mammalian cells, Pro transporters is quite different from GlyBet/GABA transporters, indicating an evolution divergence of Pro transporter[19,20]. In present study, we isolated the AbProT1 from a halophyte which accumulated both Pro and GlyBet as osmolites, and concluded the phylogenetic tree of Pro transporters as well as GlyBet transporters. Figure 2 shows that ProTs in plants are more similar to ProP/ProU in microorganism not only in precursor affinity but also in evolution relationship. In fact, some genes e.g. P5CRI of tomato were found to be a proplast homolog. Among the ProTs in plants, AbProT1 and the three ProTs in dicots were obvious divergent from OsProT1 although the sequence divergence was little.

![Fig. 5. The biomass (including roots) of seedlings of AbProT1+ and control plants. After culture for four weeks on salt medium as described in Materials and Methods, seedlings were weighed. Biomass of AbProT1+ seedlings (black) is obviously higher than control seedlings (white) on 150, 175 and 200 mmol/L NaCl medium.](image)

Plants accumulate Pro as carbon and nitrogen resource during development and as critical response to osmotic stress. In higher plants, two systems of Pro metabolism and transport contribute to the Pro deposition in different tissues, especially in tissues without redundant supply of Glu and NADPH. For example, in pollen cells that undergo dehydration process during maturation[6], or in root tip cells under low ψw[7], osmotic pressure was adjusted by increasing high concentration Pro in cytoplasm which was achieved through active transport of exogenous Pro instead of native Pro biosynthesis. We thus performed a radiolabeled experiment to investigate the exogenous Pro deposit when foreign Pro transporter


(AhProTII) was constitutively active not only in root but also in other tissues. 

Figure 3 shows that when cultured under non-stressed conditions, root has no advantage in exogenous Pro deposit in wild type Arabidopsis but in transgenic Arabidopsis overexpressed AhProTII, root obviously obtained more exogenous Pro than other tissues although AhProTII was constitutively expressed in most tissues under 35S promoter. Our result strongly suggested that Pro transporter itself could not afford the specific Pro accumulation in root tips, and Pro transport system consisting of ProT and other unknown proteins might be the reason for the Pro deposit in root although it is complex to verify this system in such experiment. In addition, current study of ProTs had made it clear that ProTs had no tissue specificity in root as well as in leaves or stems.

Chenopodiaceae plants such as spinach A. hortensis can endure adverse salt stress by accumulating high concentration of osmolytes like proline and glycine betaine in cytoplasm. In plant metabolic engineering, transgenic plants overexpressing P5CS has a phenotype of salt tolerance which indicates that strengthened Pro biosynthesis has obvious effect on salt tolerance in plants: on the other hand, suppressed P5CS with antisense P5CS under ubiquitin promoter caused death in barley seedlings (Preben B. Holm, personal communication), which suggests the necessity of Pro accumulation in reproductive tissues of plants. It is then of great interest to investigate the effect of active foreign Pro transporter on plant salt tolerance. In present report, Arabidopsis overexpressing AhProTII could endure as high as 200 mmol/L NaCl and kept growth and increased biomass in a salt gradient consisted of 150, 175 and 200 mmol/L NaCl. In contrast, wild type Arabidopsis grew slowly in MS containing 100 mmol/L NaCl, died away in 150 mmol/L NaCl, and perished in 175 or 200 mmol/L NaCl. Our data demonstrated that active AhProTII could increase Pro content in root tips and consequently promote salt-tolerance in transgenic plants obviously. Because Pro is widespread in nature, crops could obtain Pro of different forms from humus, fertilizer or soil. Our result firstly threw light on such new method to achieve salt-tolerance in crops instead of constructing a foreign biosynthesis pathway in plants.

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榆钱菠菜脯氨酸转运蛋白基因的克隆
及转基因拟南芥的耐盐性

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摘要：脯氨酸是自然界中分布最广泛，作用最重要的渗透保护剂之一，同时又是高等植物中一类重要的碳源和氮源物质。为了解环境胁迫下脯氨酸的转运机制，从一个典型的盐生植物榆钱菠菜（Atriplex hortensis L.）中通过 cDNA文库筛选和 5’- RACE 的方法获得了一个全长的 cDNA（AbProT1），其编码蛋白与脯氨酸转运蛋白有 60% - 69% 的同源性，含有 11 个跨膜结构域。聚类分析表明，微生物和高等植物的脯氨酸转运蛋白同源程度相对高于哺乳动物。为进一步分析脯氨酸转运蛋白在植物中的功能，将 AbProT1 置于 35S 启动子下转入拟南芥（Arabidopsis thaliana）, 通过同位素示踪法发现，与对照植物相比，转基因植物在根中积累更多的脯氨酸，在一系列不同浓度的盐胁迫试验中，转基因植株最高可耐受 200 mmol/L NaCl，并可持续生长，而对照植株在 150 mmol/L NaCl 下即已死亡。

关键词：榆钱菠菜；脯氨酸转运蛋白；沉积作用；盐胁迫

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