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Salt stress enhances proline utilization in the apical region of barley roots

Akihiro Ueda *, Yuko Yamamoto-Yamane, Tetsuko Takabe

Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan

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Abstract

Accumulation of an osmoprotectant, proline, is enhanced in response to salinity in plants. Here, by immunohistochemical analysis, we demonstrated that proline transporter (HvProT) was highly expressed in the apical region of barley roots under salt stress. Free proline was accumulated more in the basal region than in the apical region of barley roots under salt stress, although expression level of HvProT was higher in the apical region. On the other hand, salt stress increased proline and hydroxyproline contents in the cell wall fraction of the root apical region, suggesting increment of proline utilization. Expression of the genes encoding cell wall proteins (proline rich protein and extensin) and cellulose synthase was induced in barley roots by salt stress. These findings indicated that free proline transported by HvProT presumably behaved as a component of cell wall synthesis in the apical region of barley roots under salt stress.

Keywords: Barley; Cell wall; *Hordeum vulgare*; HvProT; Hydroxyproline; Proline; Proline transporter; Salt stress

Plants are often exposed to environmental stresses through a life cycle. Water stress caused by high salinity, drought, or both, is one of the serious factors to limit plant productivity. Furthermore, plants usually face the dilemma between CO_2 uptake for photosynthesis and water loss by transpiration from stomata. To overcome water deficit, plants have developed the mechanisms of physiological adaptation, such as improvement of water use efficiency by regulation of stomatal closure, development of root system to acquire water, accumulation of osmoprotectants and control of water permeability by aquaporins. Especially, genetic engineering was applied to enhanced accumulation of osmoprotectants [1]. The strategy to acquire much water is essential for plant growth under water deficit conditions.

Proline is one of the well-known osmoprotectants and its accumulation is widely observed in various organisms under salt stress. Proline accumulation is regulated by multiple factors, such as its synthesis, catabolism, utilization for protein synthesis and transport from other tissues. Expression of *Δ^1-pyrroline-5-carboxylate synthetase (P5CS)* and *proline dehydrogenase (PDH)*, the key genes in proline synthesis and catabolism, respectively, is regulated with a coordinate manner in response to environmental stress cues [2]. Improved accumulation of free proline conferred salt and drought tolerance in tobacco and this result supported that free proline functions to regulate cellular osmotic balance [3]. Additionally, proline is also utilized for protein synthesis, and large part of hydroxyproline, a derivative of proline through hydroxylation, is found in structural proteins, such as collagen in animals or hydroxyproline rich protein in plants [4,5]. Proline and hydroxyproline in structural proteins are clearly distinguished from free proline, which serves to regulate osmotic adjustment.

A proline specific transporter (ProT) contributes to tissue specific proline deposition under salt stress or during development program [6,7]. Increased accumulation of free proline was enhanced in the root apical region in response to water stress [8,9]. In the growing zone of maize root, net free proline deposition is considered to serve to decline cellular osmotic potential at a lower water potential condition. Notwithstanding the evidence of free proline

* Corresponding author. Present address: Department of Chemical Engineering, Texas A&M University, College Station, TX 77843-3122, USA. Fax: +1 979 845 6446.
E-mail address: aueda@neo.tamu.edu (A. Ueda).

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accumulation in the root apical region, it is still unknown the direct relationship between proline accumulation and ProT under water stress.

Previously, we identified a proline transporter (HvProT) gene in barley roots by screening of salt-inducible genes [7]. HvProT mediated proline transport from outside to inside cells and its activity was dependent on the pH gradient between outer and inner membranes [7]. Here, we demonstrated that HvProT protein highly expressed in the root cap cells under salt stress, but P5CS activity was not significantly increased in the root apical region. This indicated that HvProT is a substantial contributor to proline accumulation rather than P5CS under salt stress. Despite expression level of HvProT protein is much higher in the apical region than in the basal region, free proline was more accumulated in the basal region under salt stress. Indeed, proline content was increased in the insoluble fraction of the root apical region. This is the novel finding that proline serves as not only osmotic adjustment, but also a component of cell wall proteins in the root apical region under salt stress.

Materials and methods

Plant material and growth conditions. Barley (*Hordeum vulgare* L. cv Haruna-nijyo) was hydroponically grown with the modified Hoagland solution in a growth chamber under 13 h light (25 °C, 100 μmol m^{-2} s^{-1}, humidity 70%)/11 h dark (22 °C, humidity 75%) condition [10]. Seven-day-old plants were subjected to 100 mM NaCl stress for 2 days, and then 200 mM NaCl stress for 2 days. After total 4 days of salt stress, barley roots were dissected into 5-mm sections from the root apex (0–5 mm, 5–10 mm, and 10–15 mm), and used for further analyses.

Immunohistochemistry. For immunohistochemical analysis, rabbit polyclonal anti-HvProT antibody was raised against a KLH-conjugated HvProT synthetic peptide. HvProT specific antibody was purified using HiTrap NHS-activated HP Column coupled with a synthetic peptide of HvProT protein (Amersham Bioscience, Uppsala, Sweden). Barley root tips were fixed in 4% paraformaldehyde and 0.25% glutalaldehyde solution at 4 °C. After dehydration in ethanol/buthanol series and embedding into paraffin, the samples were sliced 10 μm in thickness. After incubation with the purified anti-HvProT antibody at 4 °C overnight, the root samples were treated with the secondary antibody at room temperature for 2 h (goat anti-rabbit IgG-AP; Zymed Laboratories Inc., South San Francisco, CA). Signal was detected with NBT/BCIP solution.

Determination of proline and hydroxyproline contents. To determine proline content, 100 tips of barley roots were pooled and homogenized in the extraction buffer (100 mM Tris–HCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5) at 4 °C. After centrifugation at 15,000*g* for 10 min at 4 °C, supernatant and precipitate were used for determination of free proline content and extraction of the insoluble protein, respectively. The insoluble fraction of each root section was washed five times with the extraction buffer, twice with 0.5% Triton X-100, three times with ddH_2O, twice with 1 M NaCl, three times ddH_2O, and twice with acetone as described previously [11] and then hydrolyzed with 6 N HCl at 110 °C for 24 h. DL-Norleucine was used as an internal standard. Proline and hydroxyproline contents were measured using an amino acid analyzer (JLC-500/V, JEOL Ltd., Tokyo, Japan).

Northern blot analysis. Total RNA was extracted using a TRIZOL reagent (Invitrogen, Carlsbad, CA). Ten microgram total RNA was separated on 1.2% agarose gel containing 0.66 M formaldehyde and blotted onto a nylon membrane (Hybond -N; Amersham Bioscience). Northern blot analysis was performed as described previously [12].

Measurement of P5CS activity. P5CS activity was determined as described previously [13]. Briefly, ATP- and NADPH-dependent P5CS activity was monitored as decrease of NADPH in absorption at 340 nm in a reaction mixture at 25 °C (100 mM Tris–HCl, 25 mM $MgCl_2$, 75 mM glutamate, 5 mM ATP, and 0.4 mM NADPH, pH 7.2). One unit of P5CS activity is defined as a 1 nmol NADPH reduction min^{-1}. Total protein amount was determined as described by Bradford (1976) with bovine serum albumin as a standard [14].

Results

HvProT protein is highly accumulated in the root cap cells

Plant ProTs mediate proline entry into cells with H^+ cotransport [6,7]. Therefore, the target for proline transport is expected to be the cells that highly expressed ProT protein in plants. In barley, the localization of HvProT protein was determined in non-stressed and salt-stressed roots by immunohistochemical analysis (Fig. 1). Even without a salt stress cue, signal of HvProT protein was weakly detectable in the root cap cells (Fig. 1B). After 4 days of salt stress, HvProT protein was highly expressed in the root cap cells (Fig. 1C and D). Accumulation of HvProT protein was also found in the root cortical and stelar cells of the 10–15 mm sections under salt stress (Fig. 1E). Immunohistochemical analysis displayed intense signals of HvProT in the root cap cells than in the cortical and stelar cells, suggesting that activity of proline transport mediated by HvProT might be higher in the apical region rather than in the basal region under salt stress (Fig. 1D and E). Any signal was not detectable in the root tips by incubation with the pre-immune serum (Fig. 1A). These results were corresponding with the observation of *HvProT* mRNA localization by *in situ* hybridization [7], indicating that HvProT functions *bona fide* in the root cap and cortical cells, and activity of proline transport to the root apical region is enhanced by salt stress.

Salt stress enhances free proline accumulation in the basal region of barley roots

In order to assess contribution of HvProT to polar transport of proline in barley roots, we investigated changes of free proline accumulation in the root tips under salt stress. Contents of free proline were significantly increased in the 5–10 mm and 10–15 mm sections by 2 days of 100 mM NaCl treatment (0.04 ± 0.02 nmol/mg FW, 0.20 ± 0.05 nmol/mg FW, and 0.33 ± 0.04 nmol/mg FW in the 0–5 mm, 5–10 mm, and 10–15 mm sections, respectively) (Fig. 2). Additional 2 days treatment with 200 mM NaCl greatly enhanced free proline accumulation (2.22 ± 0.32 nmol/mg FW, 2.08 ± 0.52 nmol/mg FW, and 2.95 ± 0.47 nmol/mg FW in the 0–5 mm, 5–10 mm, and 10–15 mm sections, respectively), whereas any increase was not seen in non-stressed roots. It is notable that, at both 2 and 4 days of salt stress treatment, increment of free proline accumulation was higher in the 10–15 mm section than in the 0–5 mm section. These observations implied

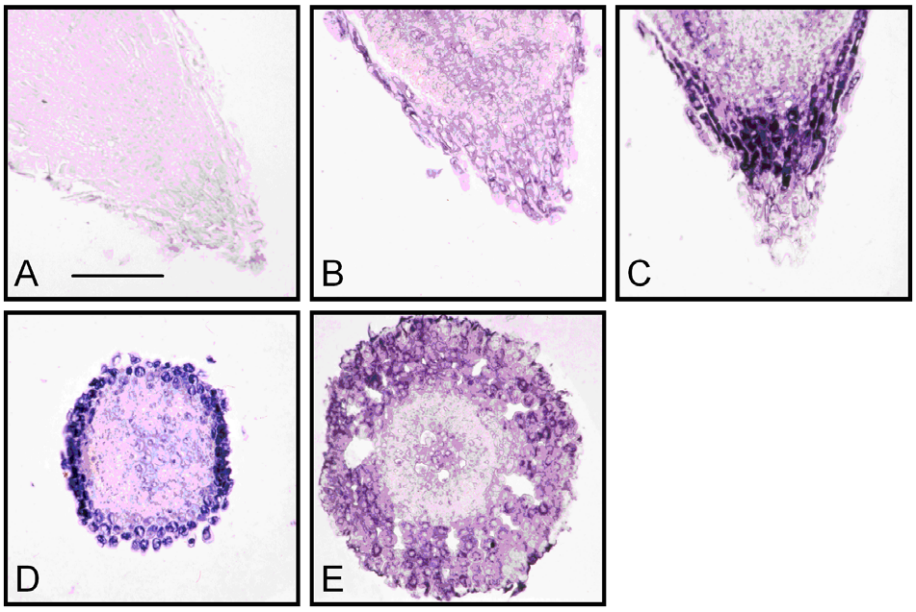

Fig. 1. Localization of HvProT protein in barley roots. HvProT protein was detected with the pre-immune serum (A) or anti-HvProT antibody (B–E). Seven-day-old plants were treated without (B) or with 100 mM NaCl for 2 days, and then 200 mM NaCl for 2 days (A,C–E). Immunohistochemical analysis was performed using the longitudinal sections of the 0–5 mm regions (A–C) and transverse sections of the 0–5 mm (D) and 10–15 mm (E) regions. Scale bar showed 100 μm.

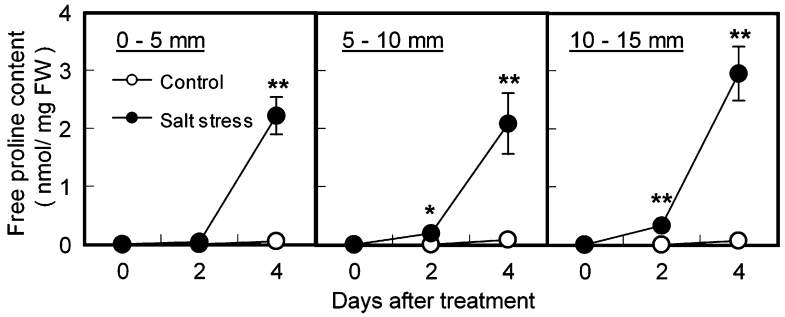

Fig. 2. Changes in free proline content of barley roots during 4 days of salt stress. Seven-day-old plants were treated without (open circle) or with 100 mM NaCl for 2 days, and then 200 mM NaCl for 2 days (closed circle). Barley roots were dissected into 5-mm sections from the root apex. Data showed the average ± SE of three-independent experiments. Asterisks indicated significant differences between control and salt stress ($^{*}P < 0.05$; $^{**}P < 0.01$).

that deposition of free proline was increased in the root basal region rather than in the apical region under salt stress.

Increased proline utilization in the root apical region under salt stress

We also studied changes in proline utilization under salt stress. In comparison to control condition, proline content in the insoluble fraction of the root extracts was increased to 1.6-fold in the 0–5 mm section at 4 days of salt stress, whereas no significant difference was observed in the 5–10 mm and the 10–15 mm sections (Fig. 3). Hydroxyproline content was also increased in the 0–5 mm section of salt-stressed roots than control roots (Fig. 4). Elevated hydroxyproline level also suggested enhanced utilization of proline as a component of cell wall because hydroxyl-

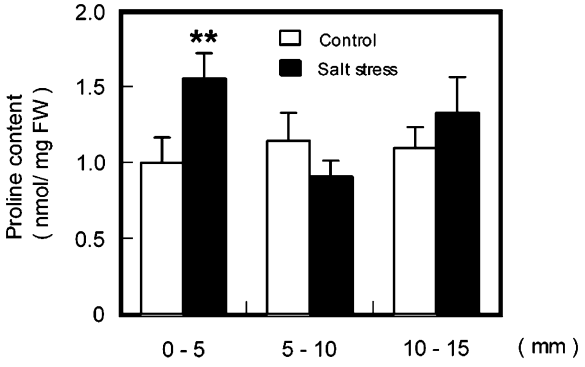

Fig. 3. Proline content in the insoluble fraction of barley root extracts (open bar, control; closed bar, salt stress). After 4 days treatment of salt stress, barley root tips were dissected into 5-mm sections. Data showed the average ± SE of five-independent experiments. Asterisks indicated significant differences between control and salt stress ($^{**}P < 0.01$).

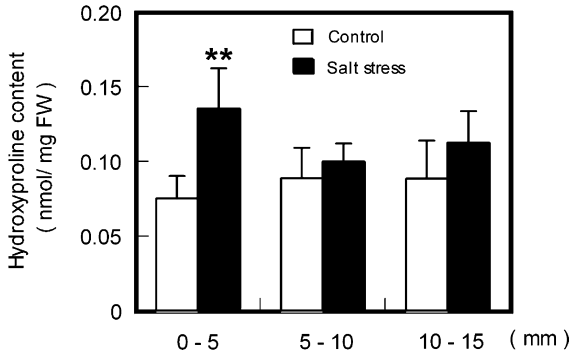

Fig. 4. Hydroxyproline content in the insoluble fraction of barley root extracts (open bar, control; closed bar, salt stress). After 4 days treatment of salt stress, barley root tips were dissected into 5-mm sections. Data showed the average ± SE of five-independent experiments. Asterisks indicated significant differences between control and salt stress ($^{**}P < 0.01$).

ation of proline follows proline incorporation into synthesis of cell wall proteins. These results indicated that utilization of free proline for synthesis of insoluble cell wall proteins was increased in the root apical region, but not in the basal region in response to salt stress.

Salt stress induced expression of cell wall-related genes

As seen in Figs. 3 and 4, salt stress enhanced proline incorporation in synthesis of cell wall proteins in the root apical region. Plant cell wall consists of cellulose, hemicellulose, and many kinds of matrix proteins, such as proline rich protein (PRP) and extensin (hydroxyproline rich protein). To examine expression of the genes encoding cell wall-related proteins in barley roots under salt stress, amount of *PRP*, *extensin*, *cellulose synthase*, and *P5CS* transcripts was determined by Northern blot analysis. Expression of *PRP* gene was highly increased by salt stress treatment (Fig. 5). Up-regulation of expression of *extensin*, *cellulose synthase*, and *P5CS* was also observed under salt

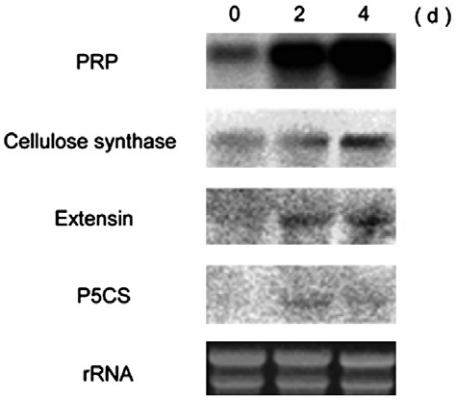

Fig. 5. Northern blot analysis of *PRP*, *cellulose synthase*, *extensin*, and *P5CS* genes in barley roots. Seven-day-old plants were treated with 100 mM NaCl for 2 days, and then 200 mM NaCl for 2 days. Ten micrograms of total RNA was used for Northern blot analysis.

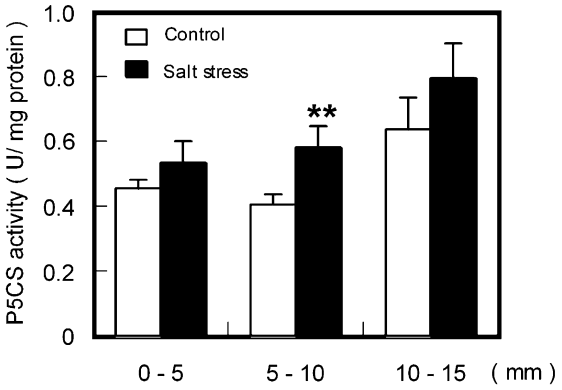

Fig. 6. P5CS activity of barley roots under salt stress. Seven-day-old plants were treated with 100 mM NaCl for 2 days, and then 200 mM NaCl for 2 days. After 4 days treatment of salt stress, barley root tips were dissected into 5-mm sections. Data showed the average ± SE of five-independent experiments. Asterisks indicated significant differences between control and salt stress ($^{**}P < 0.01$).

stress. These observations supported that salt stress increased utilization of free proline for synthesis of cell wall proteins.

P5CS activity

Expression of *P5CS*, a rate-limiting step in proline biosynthesis, was slightly induced in barley roots by salt stress (Fig. 5). In order to estimate contribution to proline accumulation in the root tip region, P5CS activity was investigated in each root segment after 4 days of salt stress. Despite weak induction of *P5CS* expression, ATP- and NADPH-dependent P5CS activity was not significantly increased in both the 0–5 mm and 10–15 mm sections of barley roots (Fig. 6). In the 5–10 mm section of the roots, 1.4-fold increase in P5CS activity was observed under salt stress.

Discussion

In this research, we demonstrated proline distribution in barley root tip regions under salt stress. As shown in Figs. 3 and 4, the results indicating increased proline and hydroxyproline contents in the insoluble fraction give the novel insight into the function of proline under salt stress. Since proline as a free form, but not as an insoluble form, can contribute to regulation of osmotic balance, *in vivo* function of proline is proposed to be the major osmoprotectant to adjust cellular osmotic potential in stressed plants [3,15]. Together with the results of Northern blot analysis (Fig. 5), evidence for increased proline utilization suggests increased incorporation of proline into synthesis of cell wall proteins. This might be involved in morphological changes, which is one of the well-known responses under salt stress. For instance, thickening roots and sloughing out of the root epidermis cells are enhanced by salt stress [16]. PRP and hydroxyproline rich glycoprotein

participates in the secondary cell wall formation and developmental program [4,17]. Although a function of these cell wall proteins is unknown under salt stress, increased synthesis or reconstitution of cell wall components may be the adaptive response to mechanical and injury stresses, secondarily caused by salt stress.

HvProT protein was highly expressed in barley root apical region, such as the root cap cells under salt stress (Fig. 1C). This is the direct evidence that plant ProT protein localizes in the root cap cells. In spite of abundance of HvProT protein in the root apical region (0–5 mm section), free proline tended to be more accumulated in the basal region (5–10 mm or 10–15 mm section) at 2 days and 4 days of salt stress (Fig. 2). As seen in Figs. 3 and 4, proline also behaves as a component of cell wall proteins as well as of osmotic adjustment in the root apical region. This may be one of the reasons why the root apical region accumulated less amount of free proline under salt stress, although transport of free proline is probably accelerated to the root tip region by HvProT under salt stress. By contrast to the root apical region, the root basal region is developmentally matured in thickening of cell wall and formation of large vacuole. Hence, altered pattern of free proline accumulation and utilization might be due to developmental difference in the root apical and basal regions.

Unlike the salt-inducible feature in roots, expression level of *HvProT* was down-regulated under osmotic or salt stress in barley leaves [12,18]. Based on the expression analyses of *HvProT*, activity of proline transport is enhanced in roots rather than in leaves under water stress. Loading of proline in the phloem was significantly increased by water deficiency [19]. Proline requirement in barley roots is possibly supported by phloem translocation from source leaves during water stress, and then HvProT facilitates proline uptake in the root cap or cortical cells of the apical region. Tissue specific proline accumulation is governed by coordinate regulation of *P5CS* and *PDH* at transcription level and induction of *P5CS* and suppression of *PDH* were often observed in response to salt stress [2]. However, this mechanism might be ruled out in proline accumulation of the root tip region. Some lines of evidence were reported on increased accumulation of free proline in the apical region of maize root at low water potential. Within 2 h after treatment of low water potential, proline synthesis from neither glutamine nor ornithine was a substantial contributor to increased proline accumulation in the root apical region [8,9]. In barley, induction of *HvProT* expression was observed earlier than that of *HvP5CS* expression in roots by salt stress treatment [7], and increment of P5CS activity was found in only the 5–10 mm section of salt-stressed roots. These findings suggested that *de novo* proline synthesis seemed not to serve as a source of net proline deposition in the 0–5 mm section of barley roots under water stress conditions.

Contribution of solute accumulation to maintenance of cellular osmotic balance is a consensus idea in plant stress physiology [20], because, in fact, enhanced proline accumulation increased salt tolerance [3]. On the other hand, the potential functions of osmoprotectants were shown by scavenging radicals or protection of mitochondria complex II under stress conditions [21,22]. Besides osmoregulation, these potential functions of proline probably serve to improve salt tolerance in plant cells. In this paper, we demonstrated HvProT might participate in proline transport to the root apical region, and transported proline serves for utilization of cell wall synthesis in the root apical region, but not basal region, in response to salt stress.

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