Altered expression of barley proline transporter causes different growth responses in Arabidopsis

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ABSTRACT

A compatible solute, proline is accumulated in various kinds of plants and microorganisms under environmental stresses. The function of proline is thought to be an osmotic regulator under water stress, and its transport into cells is mediated by a proline transporter. Here, we report the effects of expressing the barley proline transporter (*HvProT*) under the control of either the CaMV35S promoter (35Sp) or a root cap promoter (RCp), on Arabidopsis growth. In Arabidopsis, transformed HvProT functions in the plasma membrane, like other amino acid transporters. Reduction in biomass production was observed in aerial parts of 35Sp-*HvProT* plants, and it was accompanied with decreased proline accumulation in leaves. Impaired growth of 35Sp-*HvProT* plants was restored by exogenously adding L-proline. These results suggested that growth reduction was caused by a deficiency of endogenous proline. In 35Sp-*HvProT* plants, the amount of proline dehydrogenase (PDH) transcript was increased compared to wild type plants, with a consequent enhancement of the activity of PDH. On the other hand, transgenic RCp-*HvProT* plants accumulated 2- to 3-fold more proline in the root tip region compared to WT, and root elongation was enhanced at the same time. Thus, different physiological responses were caused by the different location in accumulation of proline using two different promoters for heterologous expression of *HvProT*. These results indicate the importance of proline distribution at the tissue level during vegetative development.

Key Words: Arabidopsis, Barley, HvProT, Proline, Proline transporter, Root cap

INTRODUCTION

In response to water stress, plants accumulate low molecular weight compounds (compatible solutes), such as proline, betaine and sugar alcohols (Munns 2005). Accumulation of these compounds contributes to enhancing water stress tolerance by generating an osmotic driving force. Therefore, much recent research has been focused on improving the ability to produce proline (de Ronde et al., 2000; Sawahel and Hassan, 2002; Han and Hwang, 2003; Hmida-Sayari et al. 2005), trehalose (Holmström et al. 1996) and betaine (Hayashi et al. 1997). Among these osmoprotectants, proline has been extensively studied in recent years. Proline is synthesized via either the glutamate pathway or the ornithine pathway (Delauney and Verma 1993). The former has been well-studied for proline accumulation under stress conditions (Kishor et al. 1995). Proline accumulation is regulated by the balance of synthesis and degradation in response to environmental stresses or relief from stress conditions. P5CS, Δ^1-pyrroline-5-carboxylate synthetase, is the rate-limiting enzyme in proline synthesis from glutamate, and overexpression of *P5CS* gene confers drought tolerance in tobacco (Kishor et al. 1995). Antisense-suppression of proline dehydrogenase (PDH), the key enzyme in proline degradation, also leads to a weak improvement in proline accumulation in Arabidopsis (Nanjo et al. 1999a; Mani et al. 2002). Considering the results obtained through the studies on improving the pool of free proline, increased proline accumulation is one of the advantageous adaptations under water stress.

What is the benefit of proline accumulation for plants? Transgenic approaches revealed that proline plays a role as a regulator of osmotic adjustment (Kishor et al. 1995; Hmida-Sayari et al. 2005). Additionally, by *in vitro* analyses, some lines of evidence on the potential function of proline were reported as a radical scavenger (Smirnoff and Cumbes 1989), a destabilizer of DNA helices (Rajendrakumar et al. 1997), a suppressor of ribulose-1,5-bisphosphate carboxylase/oxygenase activity (Sivakumar et al. 1998) or a protector of complex II of the mitochondrial electron transport system (Hamilton and Heckathorn 2001). Certainly, proline accumulation could contribute to enhancing tolerance under stress conditions. However, consensus was not achieved on the effectiveness of proline overaccumulation by genetic modification. Whereas proline overaccumulation has no effects on root growth and seed yields in tobacco (Kishor et al. 1995), it induced growth reduction, and increases in ploidy

level and vacuolation in budding yeast under non-stress conditions (Maggio et al. 2002). Therefore, further analysis is required to give insight into effective utilization of proline accumulation for stress tolerance.

Recently, the importance of proline transport and distribution was reported at the tissue level. Since pollen often undergoes drought stress during the process of pollination, it is reasonable to find proline is accumulated at higher level. However, in tomato pollen, proline accumulation was not accompanied with the induction of *P5CS* expression (Fujita et al. 1998) and a proline specific transporter is thought to contribute to proline deposition in pollen (Schwacke et al. 1999). 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 (Frommer et al. 1993). A proline specific transporter (ProT) functions as a high affinity uptake system, and it is important for rapid distribution of proline under water stress. In barley, the proline transporter (*HvProT*) was identified as a salt inducible gene by differential display. It was highly expressed under salt stress in the root tips, especially the root cap and cortex cells (Ueda et al. 2001; Ueda et al. 2002). In maize roots, increased accumulation of proline was often observed in the growing zone of the root tip (Verslues and Sharp 1999; Raymond and Smirnoff 2002). Such accumulation of proline in the tip region rather than in the mature roots was shown to be achieved by proline transport, but not proline *de novo* synthesis. Because the root tip region is active in cell division and elongation, its protection may be urgent upon water deficit conditions. Thus, localization of proline through the function of ProT is regulated at tissue level, depending on a developmental program or surrounding conditions.

Limited information is available on genetic engineering of osmoprotectant transporters, although that of osmoprotectant accumulation has been established. Antisense suppression of H^+/amino acid permease with 35Sp caused 50% reduction of free amino acid content in potato tuber (Koch et al. 2003). This indicated the possibility of altered accumulation of amino acids by genetic modification of amino acid transporters. If genetic engineering of an amino acid transporter enables to design tissue specific accumulation of amino acids, it would be expected to combine with modulation of amino acid synthetic activity for further improvement of plant functions. In this research, to examine the importance of proline

distribution at the tissue level, we designed two kinds of transgenic Arabidopsis plants expressing *HvProT* gene with CaMV35S promoter (35Sp) or root cap promoter (RCp). Additionally, usage of HvProT, but not Arabidopsis ProTs, is expected to enhance the effect of ectopic ProT expression on Arabidopsis growth, because HvProT is a proline specific transporter and has a high affinity to L-proline than Arabidopsis ProTs (Ueda et al., 2001). On the other hand, Arabidopsis ProTs have broad substrate specificity to not only proline, but also betaine and γ-amino butylic acid (Grallath et al., 2005). Transgenic Arabidopsis plants expressing *HvProT* with 35Sp or RCp exhibited growth suppression by addition of excess exogenous proline. 35Sp-*HvProT* transgenic plants showed reduction in biomass production with decreased proline accumulation in leaves, and this was reversibly recovered by adding appropriate concentration of exogenous proline. Enhanced PDH activity was found in leaves by overexpressing the *HvProT* gene, suggesting that growth suppression in the transgenic plants may be due to activation of the proline degradation pathway mediated by unfavorable proline flow in leaves. Conversely, RCp-*HvProT* transgenic plants showed enhanced root growth in comparison to that of WT plants. Through the analyses of transgenic Arabidopsis enhancing ProT activity with two different promoters, we discuss the importance of proline distribution at the tissue level in plants.

MATERIALS AND METHODS

Plant material and growth condition

In this study, we have constructed *HvProT*-expressing cassettes with two types of promoter. For overexpression under the control of 35Sp, *HvProT* cDNA (Accession No. AB073084) was inserted into the *Hind*III/*Eco*RI site of pBI121 binary vector with replacement of the GUS fragment. To regulate *HvProT* expression specifically in the root cap cells, RCp was cloned using *Pfx* DNA Polymerase (Invitrogen, Carlsbad, CA) as described previously (Tsugeki and Fedoroff 1999). The *Sca*I digested RCp fragment (1.4 kb) was inserted into the upstream of *HvProT* cDNA in pBI101 binary vector. Arabidopsis plants (ecotype Columbia) were transformed with *Agrobacterium tumefaciens* (strain C58) carrying pBI121-*HvProT* or pBI101-RCp-*HvProT* by the floral dipping method (Clough and Bent 1998). Transformants were selected on MS medium (1x MS salts, 3% sucrose, 0.5 g l^{-1} MES, 0.8% agar, pH 5.7) with

50 μg ml^{-1} kanamycin and 100 μg ml^{-1} carbenicillin. Seeds of T_3 homozygous plants were used for further analysis. Seeds were surface-sterilized with 1% sodium hypochlorite solution for 5 min, washed with ddH_2O several times and then incubated at 4°C for 3 d. Arabidopsis plants were grown on MS medium with or without the indicated concentration of L- or D-proline under continuous light (50 μmol m^{-2} s^{-1}) at 23°C. For the measurement of rosette diameter and leaf number, plants were grown in a pot with vermiculite and watered with 1000 times diluted hyponex solution under continuous light (50 μmol m^{-2} s^{-1}) at 23°C. Rosette diameter and leaf number were determined at 30 days and at the time of inflorescence emergence.

Northern blot analysis

Total RNA was extracted by the ATA-method. Northern blot analysis was performed as described previously (Ueda et al. 2001). The probe cDNA was obtained by PCR with the following primers: *AtP5CS1* (Accession No. D32138) forward (5'-ttccgagtgtgtgtttgtgt-3') and reverse (5'-gatcagaaatgtgtaggtagc-3'), *AtPDH* (Accession No. NM_133981) forward (5'-tatgagaaccggggaatgat-3') and reverse (5'-gcatttttattgataaggtga-3').

Whole mount *in situ* hybridization

Antisense *HvProT* probe was transcribed by T7 RNA polymerase with incorporation of digoxygenin (DIG) -UTP. Root tip (0 - 5 mm) of 7-d-old plants were fixed as described previously (Ueda et al. 2001). Prior to hybridization, root tips were treated with 40 μg ml^{-1} proteinase K at 37°C for 15 min and 2 mg ml^{-1} glycine at room temperature for 5 min. These were then fixed in 5% (w/v) paraformaldehyde for 30 min. Prehybridization was carried out in a hybridization buffer [50% (w/v) deionized formamide, 5x saline sodium citrate] at 60°C for 1 h. Root tips were incubated with 2 μg DIG-labeled probe and 100 μg herring sperm DNA at 60°C overnight with gentle shaking. After washing with hybridization buffer at 55°C for 30 min then RNase A (40 μg ml^{-1}) treatment to digest excess RNAs, anti-DIG alkaline phosphatase (1:2000; Roche, Switzerland) was incubated at 4°C overnight. Signals were detected by incubation with NBT/BCIP solution.

Subcellular localization of HvProT protein

The *HvProT* cDNA was ligated in frame to N- or C-terminal of *sGFP*. The resulting constructs, *HvProT-GFP* and *GFP-HvProT* fusion cDNAs were inserted into the pBI121 binary vector. Transformation and screening of transformants were carried out as described above. Using 5-d-old transformants, GFP fluorescence was observed with a confocal laser scanning microscopy (LSM5 PASCAL; Carl Zeiss, Germany).

Western blot analysis

Plant materials were homogenized in the ice-cold extraction buffer [30 mM Tris-HCl, 400 mM mannitol, 10% (v/v) glycerol, 0.5% (w/v) BSA, 5 mM EDTA, 5 mM $MgSO_4$, 2 mM DTT, 1 mM PMSF, 5% (w/v) PVP-40, pH 8.0], and then centrifuged at 10,000 *g* for 20 min at 4°C. The precipitate was discarded and the crude membrane fraction was collected by centrifugation at 100,000 *g* for 3 h at 4°C. Thirty microgram of protein was separated by SDS-PAGE and then blotted onto a PVDF membrane. Rabbit polyclonal anti-HvProT antibody was raised against KLH-conjugated synthetic peptide derived from the N-terminal sequence of HvProT (MPPAEKVIVVDANPSKNGHG). Affinity purified anti-HvProT antibody was used for detection of HvProT protein. After incubation with the primary and secondary antibodies (horseradish peroxidase, donkey anti-rabbit IgG; Pierce, Rockford, IL), the signal was detected using ECL Western blotting system (Amersham Bioscience, Uppsala, Sweden).

Measurement of PDH activity and proline content

Leaves, petioles and roots were dissected and homogenized in the ice-cold extraction buffer (100 mM sodium phosphate, 1 mM cysteine, 0.1 mM EDTA, pH 8.0). After centrifugation at 15,000 *g* for 10 min at 4°C, the supernatant was used for enzyme assay. PDH activity was measured as described by Rena and Splittstoesser (1975). Briefly, crude extract was incubated in the reaction buffer (100 mM Na_2CO_3-$NaHCO_3$, 10 mM NAD, 20 mM L-proline, pH 10.3) at 32°C, and then PDH dependent NAD reduction was monitored at OD_{340}. One unit of PDH activity was defined as 1 nmol NAD reduction min^{-1}. Protein content was determined as described by Bradford (1976) with bovine serum albumin as a standard. Proline was extracted in 3% (w/v) sulfosalicylic acid, and the supernatant was used for determination of proline content according to Bates et al (1973). To examine proline content in the root tip region, the

root tips (0 - 2 mm and 2 - 4 mm regions) were dissected and 20 - 30 tips were pooled. Proline content in the root tip was indicated as nmol per one root tip.

RESULTS

Characterization of *HvProT* overexpressing plants

The amount of HvProT protein in transgenic Arabidopsis was examined by Western blot analysis. Signals were detected in the crude membrane fraction of the transgenic plants, but not in WT plants (Fig. 1a). HvProT protein was more abundant in S11 and S4 plants than in S8 plants. Anti-HvProT antibody was raised against the N-terminal hydrophilic region of HvProT protein, and no significant similarity was not found in the sequence of N-terminal regions between HvProT and Arabidopsis ProTs. Actually, signal was detected in the transgenic plants, but not WT by Western blot analysis, suggesting that observed signals were due to heterologous expression of HvProT, but not Arabidopsis ProTs. To examine subcellular localization of HvProT protein, we have introduced HvProT-GFP fusion construct in Arabidopsis mediated by *Agrobacterium tumefaciens*. A typical signal peptide for organelle targeting was not found in the amino acid sequence of HvProT. When GFP was expressed alone, a green fluorescent signal was widely detectable in the cytosol (Fig. 1d). On the other hand, HvProT-GFP fusion protein was exclusively localized in the plasma membrane (Fig. 1e). The same result was obtained using a GFP-HvProT construct in which GFP is fused to the N-terminal of HvProT protein (data not shown).

Overexpressing *HvProT* caused impaired growth and altered sensitivity to exogenous proline

By ectopic expression of *HvProT* in Arabidopsis, an obvious difference was observed in the size of aerial parts in soil-grown WT and S11 plants (Fig. 2a). Rosette diameter and number of leaves were investigated at the end of the vegetative growth stage. Both S4 and S11 plants exhibited a significant decrease in rosette diameter (Table 1). Furthermore, the leaf number of three transgenic plants was reduced during vegetative growth stage, suggesting that overexpression of *HvProT* led to delayed development in Arabidopsis (Table 1). On the other hand, there was no difference in elongation of the primary root (Fig. 2b). By yeast mutant analysis, it was shown that HvProT has a high affinity for not only L-proline, but also for its

analogs, such as D-proline, hydroxyproline, azetidine-2-carboxylate and dehydroproline (Ueda et al. 2001). To test the activity of proline transport, 1 mM D-proline, a toxic analog of L-proline, was added in MS medium and then growth was monitored. In the presence of D-proline, growth of S11 plants was severely inhibited by 72% compared with WT growth (Fig. 2c). Similarly, S4 and S8 plants also displayed a sensitive response to D-proline, suggesting that introduced HvProT was functional in all of S4, S8 and S11 plants. That its activity was higher in S11 plants is consistent with the result of Western blot analysis.

Then, we tested the effects of exogenously added L-proline on Arabidopsis growth. As seen in Fig. 2d, S11 and S4 plants showed 20% reduction of biomass production in the absence of exogenous proline. However, reduced growth was recovered to the same level of WT plants by adding optimal concentration of L-proline, 0.1 mM for S11 plants and 1 mM for S4 plants, suggesting that impaired growth in S11 and S4 plants was caused by deficiency of endogenous proline. At increased concentrations of exogenous L-proline, growth of all plants was suppressed. Especially, S11 and S4 plants were more sensitive, and they could not survive in presence of 25 mM and 50 mM L-proline, respectively. Thus, higher concentrations of L-proline also acted negatively for Arabidopsis growth, as reported previously (Hellmann et al. 2000). S11 and S4 plants accumulated much higher amounts of proline under 10 mM or 25 mM L-proline than wild type plants did (Fig. 2e); this fact explains the increased sensitivity of these plants to exogenous L-proline. On the other hand, proline content of the whole plant was lower than that of wild type in Arabidopsis expressing *HvProT* when it was grown on regular MS medium (Fig. 2e).

To investigate the distribution of endogenous proline, we have dissected the proline content at the tissue level in S11 plants which showed conspicuous phenotype in this research. In the leaves of S11 plants, proline accumulated much less than in leaves of WT, whereas no significant difference was found in petioles and roots (Fig. 3a). This implied that reduced growth of aerial parts is due to decreased proline accumulation in leaf blade, but not in roots, of S11 plants.

Enhanced PDH activity in HvProT expressing plants

The lower content of proline in leaves of S11 plants (Fig. 3a) could be due either to suppression

of proline synthesis or to enhancement of proline degradation. To test which occurs, we investigated the amount of *AtP5CS* and *AtPDH* transcripts that are the rate limiting step in each pathway. As a result of Northern blot analysis, expression of *AtPDH* was induced to a greater extent in S4, S8 and S11 plants than in WT (Fig. 3b). In leaves of S11 plants, the enzyme activity of PDH was also significantly increased to 1.4-fold that of WT (Fig. 3c). By contrast, no significant difference was found in the PDH activity of petiole and root between WT and the transgenic plants. On the other hand, *AtP5CS* expression was uniform in each plant. Treatment with 10 mM L-proline induced intense expression of PDH gene in all plants (Fig. 3d), suggesting that signaling pathway for proline degradation is activated by excess proline supplement in all transgenic plants as well as WT plants.

Enhanced root growth by accumulating proline in the root tip

RCp was isolated by screening the pool of enhancer trap lines (Tsugeki and Fedoroff 1999). To express *HvProT* gene only in the root cap cells, RCp was used to produce the transgenic plants. We have chosen two lines, R1 (strongly expressed) and R5 (weakly expressed) plants for phenotypic identification. In R1 plants, *HvProT* mRNA was exclusively localized in the root cap cells, whereas its expression was strongly observed in the upper region of S11 roots (Fig. 4a). In Arabidopsis, activity in production and release of the root cap is much less than in cereal crops such as maize or barley, and it is difficult to collect the root cap (border) cells. Therefore, we estimated the effect of *HvProT* expression on proline accumulation through the comparison of the 0 - 2 mm and 2 - 4 mm tip regions in WT and the transgenic plants. In R1 and R5 plants, 3- and 2-fold more proline was accumulated in the 0 - 2 mm tip region in comparison to that of WT and S11 plants, respectively (Fig. 4b). In spite of increasing proline accumulation in the root tip region, no difference was found in the 2 - 4 mm region of WT and the transgenic plants. These results indicated that RCp-driven HvProT works functionally to accumulate proline in the root cap cells. Interestingly, R1 and R5 plants showed 20% and 15% increased elongation of the primary roots (Fig. 4c). Treatment with 1 mM D-proline suppressed root growth severely in R1 and S11 and mildly in R5 plants (Fig. 4c). Similar results were seen when L-proline was exogenously added in the growth medium.

DISCUSSION

In this research, we have demonstrated that heterologous expression of HvProT with 35Sp or RCp had the different impacts on Arabidopsis growth. Similar to the localization of CAT5 and CAT8, a member of the cationic amino acid transporter family (Su et al. 2004), GFP-fused HvProT was exclusively found in the plasma membrane (Fig. 1b). This means that HvProT probably transports proline from outside into cells under the control of 35Sp or RCp. Root elongation of R1 and R5 plants was enhanced with increased proline accumulation in the root tip. On the other hand, overexpression of *HvProT* with 35Sp was the cause of impaired growth in Arabidopsis. This difference might be caused due to altered proline accumulation mediated by two different promoters.

Effects of lesser proline accumulation on Arabidopsis growth

Accumulation of proline is widely observed in many plants and the mechanism of proline accumulation is well-studied in response to environmental stresses or release from stress conditions (Delauney and Verma 1993; Yoshiba et al. 1997). Metabolic engineering to enhance the free proline pool has been achieved by overexpressing the *P5CS* gene (Kishor et al. 1995; Zhu et al. 1998). Removing feedback regulation of P5CS activity by proline also contributed to more effective accumulation of proline (Hong et al. 2000). However, limited information is available on the effect of lesser accumulation of proline in plants. In this research, we described that an impaired growth phenotype was induced by overexpression of *HvProT* gene in Arabidopsis. This must have been caused by deficiency of endogenous proline, because it was prevented by addition of exogenous L-proline (Fig. 2d). Antisense suppression of the *P5CS* gene induced severe proline deficiency in Arabidopsis and consequent morphological alternations (Nanjo et al. 1999b). Similarly to our results, morphological abnormalities in *P5CS*-antisense transgenic plants were suppressed by exogenously adding L-proline. Thus the results of both studies indicate that an appropriate concentration of proline is essential for normal development.

On the other hand, the extent of observed growth reduction caused by proline deficiency was different between *HvProT*-overexpressing and *P5CS*-antisense Arabidopsis plants. This could be explained by differences in the level of proline deficiency. The TF3

plants, one of the *P5CS*-antisense transgenic plants, accumulated less than 10% of free proline in comparison to that of WT plants and the amount of proline and hydroxyproline in cell wall components was also significantly affected (Nanjo et al. 1999b). In *HvProT*-overexpressing plants, the reduction in proline amount was only 30% (Fig. 2e). Therefore, the impact of proline deficiency on Arabidopsis growth must have been less significant than *P5CS*-antisense transgenic plants, although we could not exclude the possibility of significant modulation in metabolic pathways regulated by proline.

Decreased proline accumulation was possibly regulated by enhanced PDH activity

As shown in Fig. 3b and 3c, we have shown up-regulation of both PDH gene expression and enzyme activity in leaves of S11 plants. Generally, the genes involved in proline synthesis/degradation are coordinately regulated by environmental cues. For example, in response to osmotic stress, expression of *P5CS* gene is up-regulated and *PDH* gene is synchronously down-regulated, leading to proline accumulation. By rehydration, expression of *PDH* gene is activated and accumulated proline is catabolized, permitting recycling of nitrogen. Proline treatment could also trigger *PDH* expression (Kiyosue et al. 1996). In this research, Arabidopsis plants did not undergo stress treatment and relief. Therefore, it is possible that enhanced PDH activity is due to overaccumulation of proline in leaves of S11 plants. On the other hand, S11 plants accumulated much more amount of proline in presence of 10 mM L-proline (Fig. 2e). Even though 10 mM proline is enough to induce intense expression of PDH in S11 plants as well as WT plants (Fig. 3d), proline degradation activity may not be sufficient for maintenance of proline homeostasis. Consequently, growth of S11 plants was suppressed in presence of 10 mM L-proline.

The Arabidopsis genome encodes three proline transporters (*AtProT1*, *AtProT2* and *AtProT3*), and their localizations were investigated by GUS-promoter analysis (Grallath et al. 2005). In leaves, GUS activity of *AtProTs* was detectable strongly in veins, especially phloem and phloem parenchyma cells, and weakly in leaf mesophyll cells. By quantitative RT-PCR, the expression level of *AtProTs* was higher in stems and flowers than in source leaves, implying that intrinsic activity of proline transport may be low in leaves, especially in mesophyll cells, but high in leaf veins. Therefore, unfavorable proline flow into the leaves from other tissues was

possibly prompted by overexpressing *HvProT* under control of the 35Sp. Whereas a remarkable difference was seen in the growth of the aerial part, roots of *HvProT*-overexpressing plants grew normally, as in the WT (Fig. 2b). In roots, there was also no significant difference in proline accumulation and PDH activity between WT and the transgenic plants. One possibility why proline deficiency-induced phenotype was not seen in roots might be that *AtProTs* are highly expressed endogenously in roots. The relative expression level of *AtProT1* and *AtProT2* in root vascular and cortex cells, is much higher than in leaves (Grallath et al. 2005). Therefore, heterologously expressed *HvProT* does not strongly affect proline homeostasis in roots due to the higher basal expression level of *AtProT1* and *AtProT2*.

Mild increase of proline accumulation in the root tip accelerated root elongation

Enhanced root growth was achieved by increased proline accumulation (2- to 3-fold increase) in the root cap cells (Fig. 4c). This increment is mild in comparison with the case of Arabidopsis *esk1* mutant (30-fold accumulation) or mutated *P5CS*-expressing yeast (60-fold accumulation). However, in both cases, growth was suppressed because of proline overaccumulation (Xin and Browse 1998; Maggio et al. 2002). On the other hand, enhanced growth was also observed by mild increase of proline accumulation in tobacco BY-2 cells (Tateishi et al. 2005). By antisense suppression of *PDH* gene, 1.2- to 3.0-fold increase in proline accumulation was observed in BY-2 cells. Taken together, in the active cells such as BY-2 cells or the root cap cells, a mild increase of proline might be able to enhance plant growth without causing a stress adaptive status. Under salt stress, *HvProT* mRNA was strongly expressed in the root tip region (Ueda et al. 2001), therefore, we tested the effect of proline accumulation in the root cap cells on salt tolerance. However, improved salt tolerance was not observed in R1 and R5 plants under 50 - 150 mM NaCl conditions (data not shown). In a maize root tip, proline level was increased at a lower water potential due to proline transport (Verslues and Sharp 1999). It is considered that proline accumulation in the tip region is essential to maintain root growth under stress conditions. In RCp-transgenic plants, *HvProT* mRNA is expressing constitutively, but not in a stress-inducible manner. Therefore, stress-inducible transport in the root cap cells would be preferable to examine the contribution to improve stress tolerance. In RCp-transgenic plants, proline content was increased in the tip

region, indicating that proline degradation pathway may not be significantly activated. This may have caused the difference in growth response mediated by 35Sp and RCp. By GUS analysis of *PDH* promoter, basal expression level of *PDH* gene was found to be high in roots and very low in leaves (Nakashima et al. 1998). After L-proline treatment, intense *PDH* expression was induced in leaves (Fig. 3d). This means that the difference in basal and L-proline induced expressions is greater in leaves, and regulation of proline content is strictly controlled in response to increased amount of L-proline. In the root tip, GUS signal of *PDH* gene was found in the root cap and elongation zone. However, its expression was strongly induced in the root meristematic cells, but not in the root cap and elongation zone (Nakashima et al. 1998). Proline accumulation in the root cap cells of RCp-transgenic plants may be achieved by weak response of *PDH* expression.

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 RCp-transgenic plants, it seems to be useful to design amino acid accumulation with tissue specific promoters. And it is also important to understand the balance of amino acid translocation between sources and sink tissues.

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FIGURE LEGEND

Fig. 1 (a) Amount of HvProT protein in the transgenic Arabidopsis. Thirty microgram of crude membrane protein was used for Western blot analysis. (b–e) Subcellular localization of HvProT protein in Arabidopsis. Arabidopsis plants expressing GFP or HvProT-GFP were grown on MS medium for 7 d. Nomarski image of Arabidopsis root transformed with (b) GFP alone and (c) HvProT-GFP. GFP fluorescence of (d) mock and (e) HvProT-GFP plant was scanned by a confocal laser scanning microscopy.

Fig. 2 Phenotype of *HvProT*-overexpressing Arabidopsis. (a) Reduction in growth of S11 plant during vegetative stage. WT and S11 plants were grown in a soil for 35 d with 1000 times diluted hyponex solution. Scale bar showed 1 cm. (b) Root length of WT, S4, S8 and S11 plants. Three-d-old plants were transformed onto MS medium, and then length of the primary root was measured after a week. (c) Growth of WT, S4, S8 and S11 plants in the presence of 1 mM D-proline for 25 d. (d) Dose response of biomass of WT, S4, S8 and S11 plants to exogenous L-proline. Plants were grown on MS medium with various concentrations of L-proline for 25 d. (e) Proline content of WT, S4, S8 and S11 plants grown on MS medium with or without exogenous L-proline for 25 d. ***S11 plants could not survive on MS medium with 25 mM L-proline. All data showed the average ± S.E. of at least 4 independent experiments. Significant difference between WT and the transgenic plants was indicated with * ($P<0.05$) or ** ($P<0.01$).

Fig. 3 Relationship between proline content and PDH activity at each tissue. Plants were grown on MS medium for 25 d, and then dissected tissues from 5 plants were pooled. (a) Tissue specific accumulation of proline in WT and S11 plants. Data showed the average ± S.E. of 5 independent experiments. (b) Expression of *AtP5CS* and *AtPDH* genes. RNAs extracted from shoot were used for Northern blot analysis. Expression of α-tubulin was shown as a loading control. (c) PDH activity in leaf, petiole and root of WT and S11 plants. Enzyme activity was shown as a percentage of PDH activity in WT plants. Actual activity was as follows, 13.3 ± 1.5, 39.3 ± 7.3 and 36.5 ± 9.1 (unit/ mg protein) in leaf, petiole and root, respectively. Significant difference between WT and the transgenic plants was indicated with *

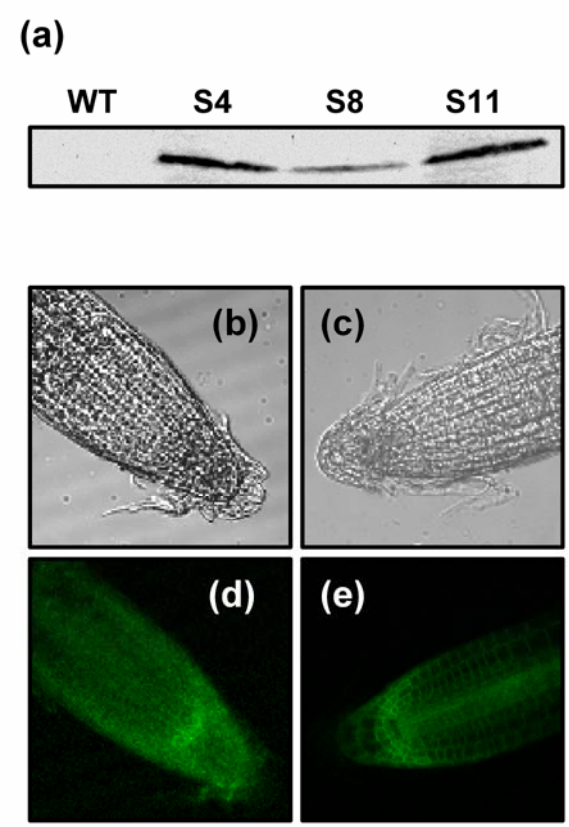
(P<0.05) or ** (P<0.01). (d) Expression of *AtPDH* gene in presence of 10 mM L-proline. RNAs extracted from shoot were used for Northern blot analysis. Expression of α-tubulin was shown as a loading control.

Fig. 4 Enhanced root elongation by expressing HvProT in the root cap cells. (a) Confirmation of *HvProT* mRNA expression by whole mount *in situ* hybridization. (b) Proline content of WT and the transgenic plants in the root tip region. Plants were grown on MS medium for 7 d. Two sections of the root tips were dissected from the root apex (0 - 2 mm and 2 - 4 mm), and then the pool of 20 - 30 tips was used for determination of proline content. (c) Root elongation of WT and the transgenic plants without (Con) or with 1 mM D-proline (1D). Three-d-old plants were transformed on MS medium with or without 1 mM D-proline, and then grown for 7 d. All data showed the average ± S.E. of 4 - 5 independent experiments. Significant difference between WT and the transgenic plants was indicated with * (P<0.05) or **(P<0.01).

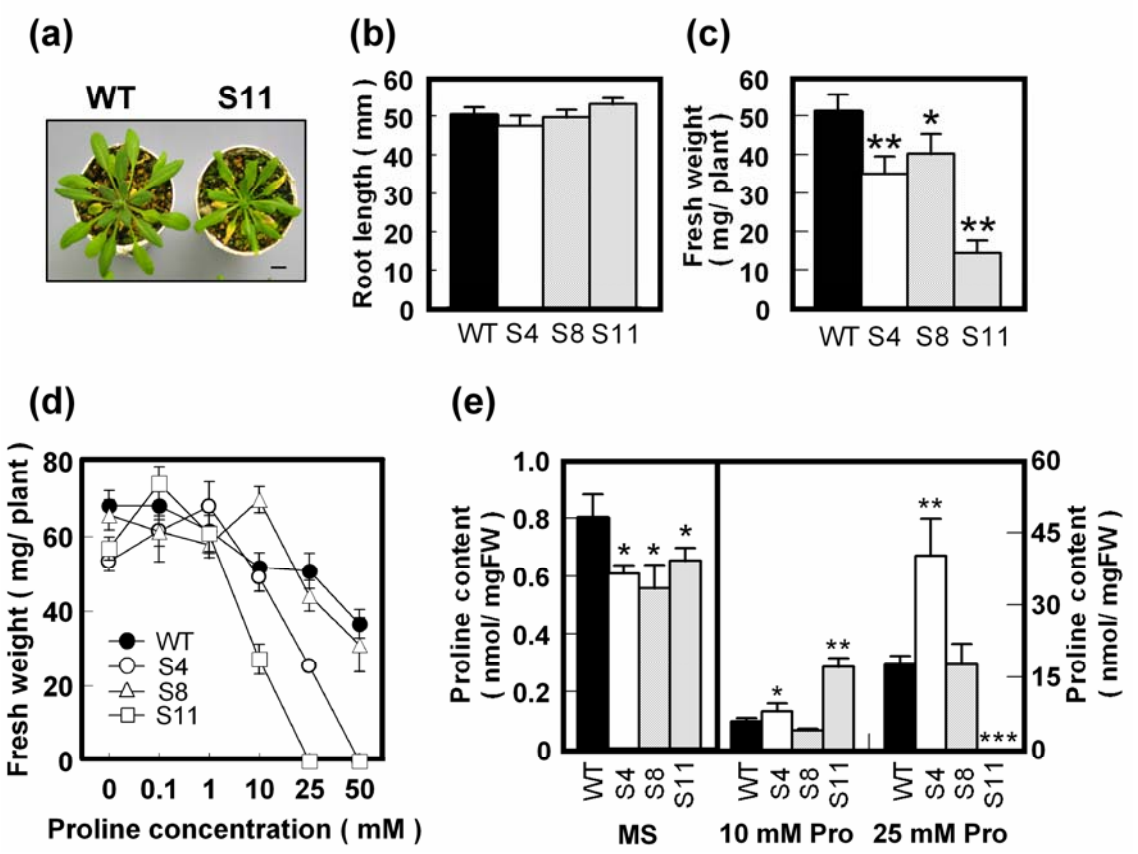
Table 1. Growth phenotype of WT, S4, S8 and S11 plants during vegetative stage. Rosette diameter was determined using soil-grown 30-d-old plants. Leaf number was counted at the time of inflorescence emergence. Data showed the average ± S.E. of three independent experiments.

	WT	S4	S8	S11
Rosette diameter (mm)	77.7 ± 5.8	67.1 ± 1.5*	69.4 ± 1.6	58.3 ± 2.7**
Leaf number	21.1 ± 0.6	19.0 ± 0.5**	19.6 ± 0.7*	18.9 ± 0.4**

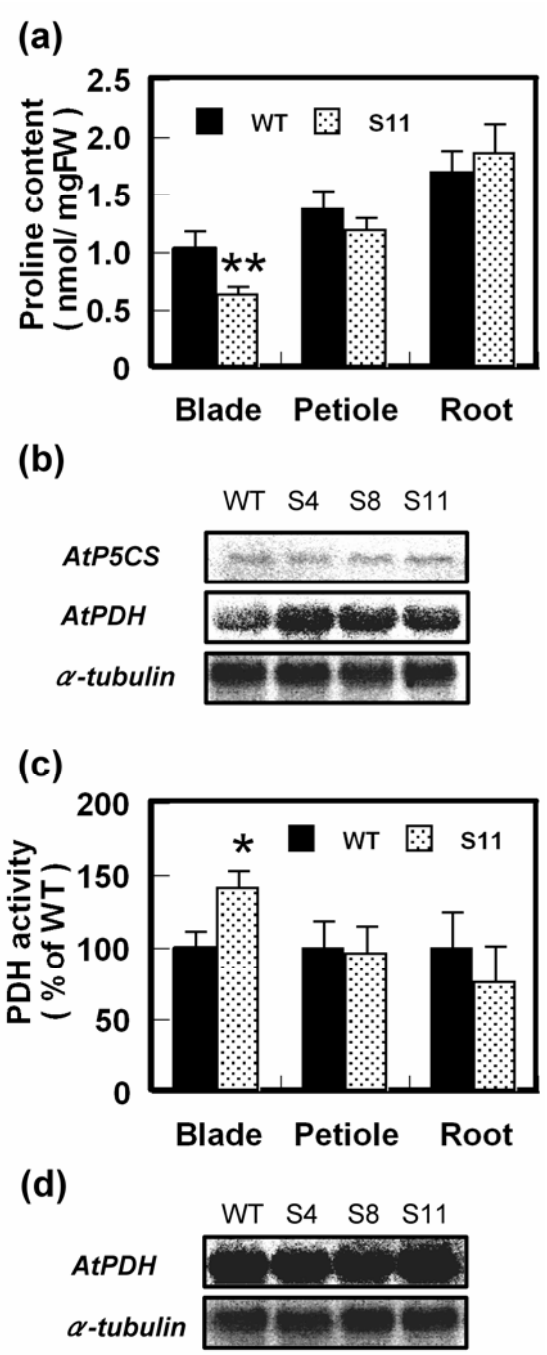
Asterisk showed significant differences between WT and the transgenic plants (*, $P<0.05$; **, $P<0.01$).



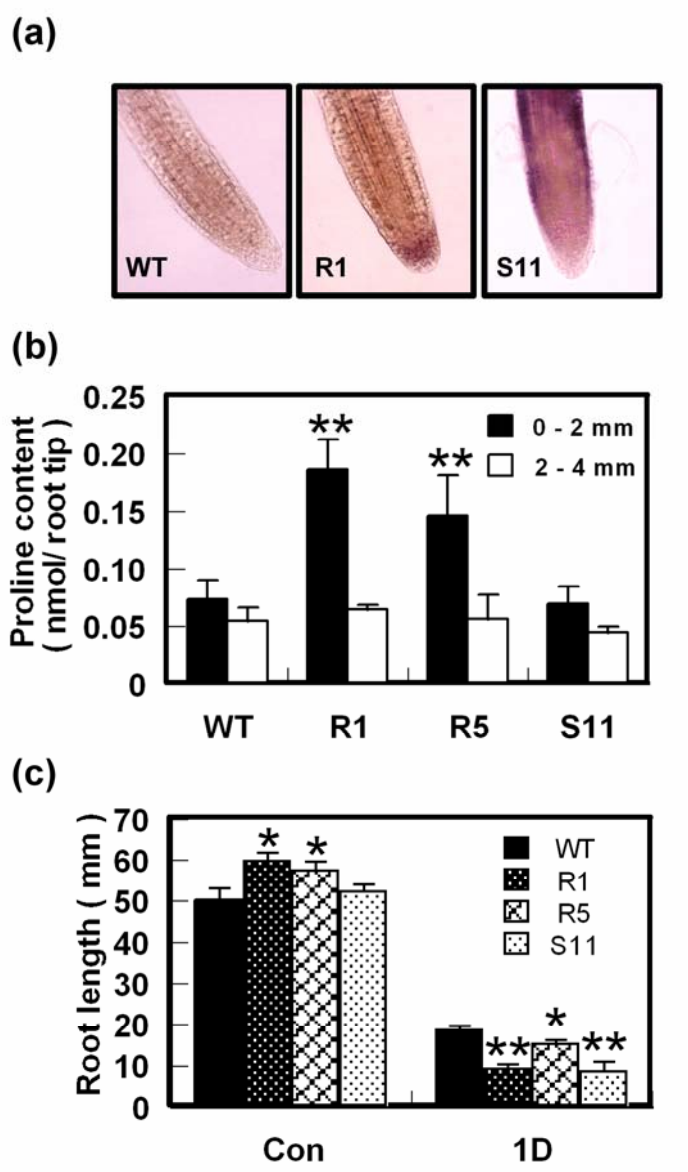
Ueda *et al.* Figure 1



Ueda *et al.* Figure 2



Ueda *et al.* Figure 3



Ueda *et al.* Figure 4