

Salt Stress-Induced Proline Transporters and Salt Stress-Repressed Broad Specificity Amino Acid Permeases Identified by Suppression of a Yeast Amino Acid Permease-Targeting Mutant

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A yeast mutant lacking *SHR3*, a protein specifically required for correct targeting of plasma membrane amino acid permeases, was used to study the targeting of plant transporters and as a tool to isolate new *SHR3*-independent amino acid transporters. For this purpose, an *shr3* mutant was transformed with an *Arabidopsis* cDNA library. Thirty-four clones were capable of growth under selective conditions, but none showed homology with *SHR3*. However, genes encoding eight different amino acid transporters belonging to three different transporter families were isolated. Five of these are members of the general amino acid permease (*AAP*) gene family, one is a member of the *NTR* family, encoding an oligopeptide transporter, and two belong to a new class of transporter genes. A functional analysis of the latter two genes revealed that they encode specific proline transporters (*ProT*) that are distantly related to the *AAP* gene family. *ProT1* was found to be expressed in all organs, but highest levels were found in roots, stems, and flowers. Expression in flowers was highest in the floral stalk phloem that enters the carpels and was downregulated after fertilization, indicating a specific role in supplying the ovules with proline. *ProT2* transcripts were found ubiquitously throughout the plant, but expression was strongly induced under water or salt stress, implying that *ProT2* plays an important role in nitrogen distribution during water stress, unlike members of the *AAP* gene family whose expression was repressed under the same conditions. These results corroborate the general finding that under water stress, amino acid export is impaired whereas proline export is increased.

INTRODUCTION

Transport processes play an important role in nitrogen allocation in higher plants. Under stress conditions, massive changes in partitioning of carbon and nitrogen take place. For example, under water stress, delivery of nitrate to the shoot is depressed (Shaner and Boyer, 1976). Carbon and nitrogen reduction are also impaired, and a reduction in phloem transport has been observed (Wardlaw, 1969; Tully et al., 1979). Concomitantly, the stressed plants accumulate osmotically active compounds, such as sugars, proline, and betaine, in the cytosol as a protective mechanism (Bohnert et al., 1995). In the case of proline, this is achieved by upregulating genes encoding enzymes involved in its biosynthesis (Verbruggen et al., 1993; Yoshida et al., 1995). However, the survival value of proline accumulation under water stress has been a matter of debate. Recent findings with transgenic tobacco overexpressing a key enzyme of proline biosynthesis (Δ^1 -pyrroline-5-carboxylate synthetase) have demonstrated that overproduc-

tion of proline results in increased tolerance to osmotic stress in plants (Kavi Kishor et al., 1995; Verma and Hong, 1996).

In contrast to metabolic events involved in proline accumulation, the stimulation of transport processes under these conditions has not been analyzed further (e.g., Delauney and Verma, 1993). To study these processes in detail, it seems appropriate to isolate the respective transporters and to study their regulation. Yeast mutants represent an excellent system for isolating amino acid transporters, but the approach is limited by the large number of endogenous transporters. Therefore, for the isolation of each specific amino acid transporter, specific mutants are required. To circumvent this problem, a mutant defective in multiple amino acid transport systems would be advantageous.

In yeast, mutations have been identified that pleiotropically affect amino acid uptake (*aap* [for amino acid permease], Surdin et al., 1965; *apf*, Grenson and Hennaut, 1971; *raa4*, McCusker and Haber, 1990; *shr3*, Ljungdahl et al., 1992). The *aap*, *apf*, and *shr3* mutations were shown to be allelic (Grenson and Hennaut, 1971; Ljungdahl et al., 1992). The *SHR3* gene was isolated by complementation of the respective mutant and

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Table 1. Transporters Isolated by Complementation of Yeast Mutants^a

Transporters	<i>shr3</i>	<i>hip1</i>	<i>put4/gap1</i>
ProT1	5	- ^b	-
ProT2	6	-	-
AAP1	6	5	7
AAP2	9	9	-
AAP3	1	1	-
AAP4	3	4	-
AAP5	-	1	-
AAP6	3	-	-
NTR1	1	1	-

^a Shown is the number of clones isolated by complementation of yeast strains carrying an *shr3* mutation and selection on proline and histidine (PLAS23-4B, Ljungdahl et al., 1992; this work), a mutation in the histidine permease gene (*hip1*) and selection on histidine (JT16, Tanaka and Fink, 1985; Kwart et al., 1993; Fischer et al., 1995), or mutations in the proline transporter and the general amino acid permease gene (*put4/gap1*) and selection on proline (22574d, Jauniaux et al., 1987; Frommer et al., 1993).

^b (-), no clone was identified.

encodes an endoplasmic reticulum membrane protein that seems to serve as a cargo specifier for plasma membrane amino acid permeases in endoplasmic reticulum vesicles (Ljungdahl et al., 1992).

Functional complementation of yeast amino acid transport mutants with several families of plant amino acid transporters indicates that the yeast targeting system recognizes the plant permeases and correctly targets them to the plasma membrane (Frommer et al., 1993, 1995; Fischer et al., 1995). To study the effect of a deficiency in SHR3 on the targeting of plant amino acid transporters and to identify plant homologs of *SHR3*, an *shr3* mutant yeast strain was transformed with a plant cDNA library. The isolated clones demonstrate that the mutant phenotype can be suppressed by expressing plant genes encoding amino acid permeases. Due to the reduced uptake capacity for amino acids in the mutant, *shr3* strains provide excellent systems to identify new transporters not affected by SHR3. Selection of transformants on medium containing proline and histidine led to the identification of both broad-specificity amino acid permeases and specific proline transporters. Expression of these transporters was studied to elucidate their function during water stress conditions.

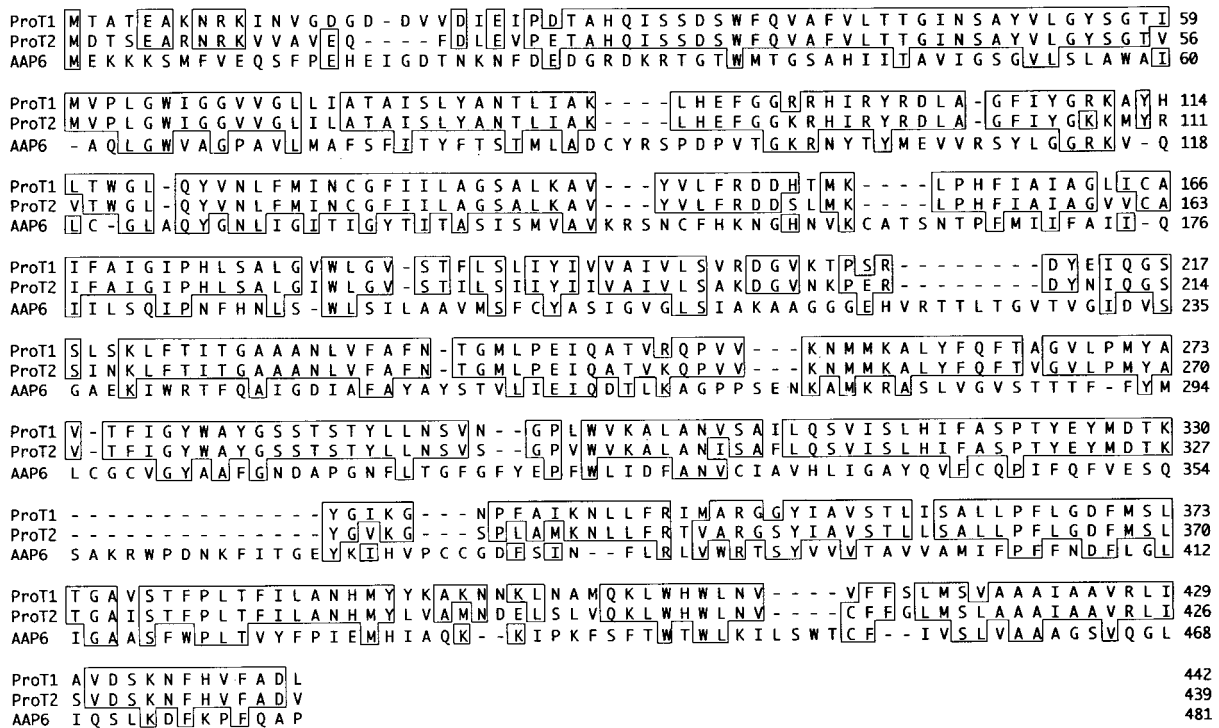


Figure 1. Comparison of the Deduced Amino Acid Sequences of ProT1, ProT2, and AAP6.

The first amino acid residue of the translation start site is designated as position 1. Analysis was performed using LASERGENE software (DNASTAR, London, UK). Identical amino acids are boxed. Dashes indicate gaps introduced for optimal alignment.

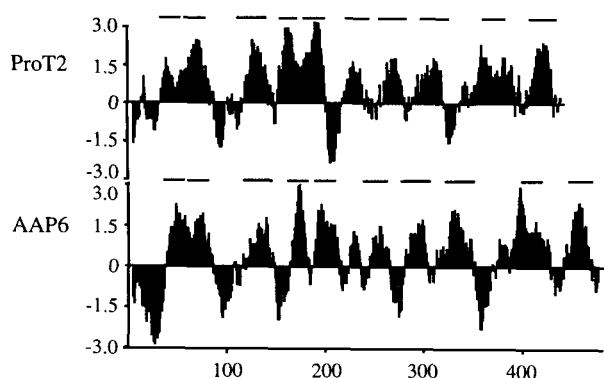


Figure 2. Prediction of Putative Membrane-Spanning Regions in the Amino Acid Sequence of ProT2 and AAP6.

Hydrophathy plots were performed according to Kyte and Doolittle (1982) with a window of 11 amino acids. Predictions of membrane-spanning regions were performed using the TMAP program (Milpetz and Argos, 1995) with the aligned sequences of AAP1-6 and ProT1-2. Hydrophobic amino acids are given a positive value.

RESULTS

Complementation of an *shr3* Mutant

The *Saccharomyces cerevisiae* strain PLAS23-4B carries a mutation in *SHR3*. The *SHR3* gene product is required for correct targeting of endogenous amino acid transporters to the plasma membrane. The *shr3* strain is resistant to high concentrations of histidine but shows only poor growth on minimal medium supplemented with 8.7 mM proline and 0.6 mM histidine (Ljungdahl et al., 1992). To identify plant *SHR3* homologs or amino acid transporters that suppress the mutant phenotype, PLAS23-4B was transformed with a cDNA library derived from *Arabidopsis* seedlings (Minet et al., 1992). After transformation, the cells were plated directly under selective conditions, and 34 independent clones mediating growth were identified. In all cases, we confirmed that no reversion or second-site mutations had occurred by reintroducing the recombinant plasmids into the original mutant.

Plasmid DNA was isolated and characterized by partial DNA sequence analysis. Several classes of clones were identified. In addition to already known members of the AAP family of amino acid transporters (Frommer et al., 1993; Kwart et al., 1993; Fischer et al., 1995) and the histidine-transporting oligopeptide transporter NTR1 (Frommer et al., 1994a; Rentsch et al., 1995), several clones different from the previously isolated transporter genes were identified and analyzed further (Table 1). Among the candidates that were able to grow, three different clones of ~1.7 kb enabled efficient growth of the mutant. To test whether these clones represented functional amino

acid transporters, yeast strain 22574d, deficient in three amino acid transport systems, was transformed (Jauniaux et al., 1987). All three clones (ProT1 [for proline transporter], ProT2, and AAP6) were able to complement proline uptake deficiency, indicating that transport proteins but not *SHR3* homologs were isolated.

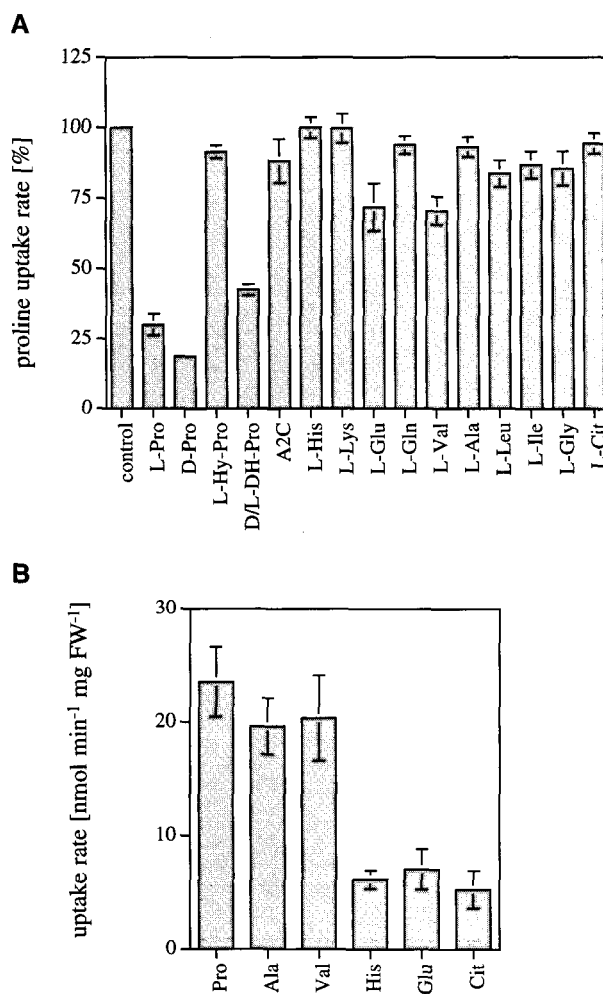


Figure 3. Biochemical Properties of ProTs in Comparison with AAP6.

(A) Competition of L - ^{14}C -proline uptake into yeast cells (PLY204) expressing *ProT2* in the presence of a 10-fold excess of the respective amino acids. The uncompetited uptake rate was taken as 100% correspondence with 60 pmol of L -proline $\text{min}^{-1} \text{mg}^{-1}$ of cells. The means of three independent competition experiments are shown. L -Hy-Pro, 4-hydroxy- L -proline; D/L -DH-Pro, 3,4-dehydro- D/L -proline; A2C, L -acetidine-2-carboxylic acid.

(B) Direct uptake of 0.1 mM radiolabeled L -amino acids (proline, alanine, valine, histidine, glutamic acid, and citrulline) into yeast cells (PLY204) expressing *AAP6*. Uptake by remaining endogenous activities obtained in the mutant transformed with the empty vector was subtracted as background. FW, fresh weight. Bars indicate SE ($n = 3$).

Table 2. pH Dependence and Inhibitor Sensitivity of L-¹⁴C-Proline Transport into PLY204 Expressing *ProT2*

Treatment	Uptake Rate (%) ^a
pH 4.5	100
pH 4.5 + 1 mM 2,4-DNP	4 ± 1
pH 5.5	25 ± 1
pH 6.5	4 ± 1

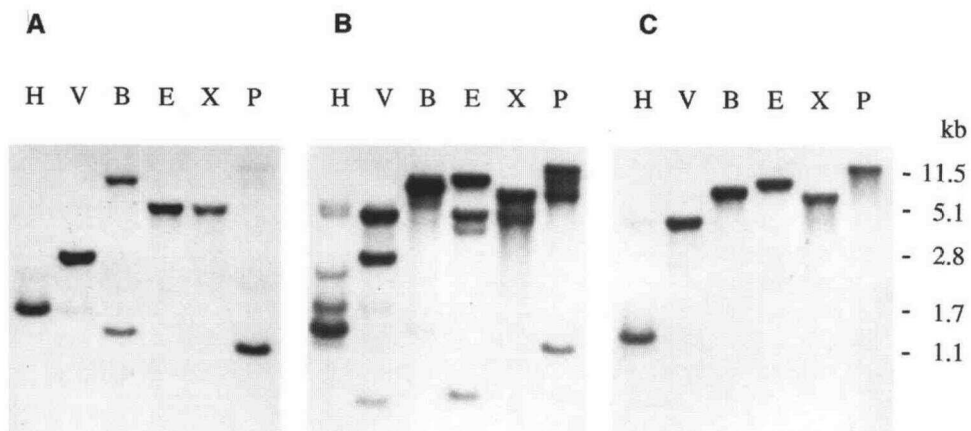
^a Uptake rates at pH 4.5 were taken as 100% and correspond to 48 pmol of L-proline min⁻¹ mg⁻¹ of cells. Data represent means ± SD (*n* = 3).

Sequence Analysis of *shr3* Suppressor Clones

Sequence comparisons showed that the two *ProT* clones are similar to each other and are distantly related to the *AAP* family (23 to 28% amino acid identity; Figure 1). The cDNAs encode polypeptides with a length of 442 and 439 amino acids and molecular masses of ~48 kD. The third new amino acid permease identified (*AAP6*; Figure 1) is closely related to previously identified members of the *AAP* gene family (Frommer et al., 1993; Kwart et al., 1993; Fischer et al., 1995). The *AAP6* protein is 73.3% identical to *AAP1*, the most closely related member of the *AAP* gene family. Among 34 clones characterized, none shared similarities with *SHR3* (Table 1). Analysis of the hydrophilicity indicated that the predicted *ProT* and *AAP* proteins are highly hydrophobic and contain 10 putative membrane-spanning regions (Figure 2; Milpetz and Argos, 1995).

Biochemical Characterization of *ProTs* and *AAP6*

Due to the reduced amount of amino acid permeases at the plasma membrane of *shr3* mutants, direct uptake experiments with ¹⁴C-labeled amino acids were performed to determine the biochemical properties of the plant permeases. However, neither L-¹⁴C-histidine nor glutamic acid was significantly taken up in *shr3* strains expressing *ProT1* or *ProT2*. In contrast, L-¹⁴C-proline was transported efficiently by both *ProT1* and *ProT2*. Competition experiments for L-proline uptake, using a 10-fold excess of different amino acids as competitors, showed that only D- and L-proline, and to a lesser extent 3,4-DL-dehydroproline, were able to reduce significantly L-proline uptake mediated by *ProT2* (Figure 3A). In contrast to the *AAPs*, proline transport mediated by *ProT2* was not stereospecific and thus was similar to transport activities described in the scutellum of barley grains (Väisänen and Sopanen, 1986). However, the *ProTs* seem to be different from proline transport systems described in *Arabidopsis* because the toxic proline analog azetidine-2-carboxylic acid did not compete for proline uptake mediated by the *ProTs* (Verbruggen et al., 1994). L-Proline transport was only slightly reduced by L-valine and L-glutamate, whereas none of the other amino acids tested significantly affected uptake rates. Unlike the broad specific *AAP* amino acid permeases, *ProT1* and *ProT2* represent highly specific proline transporters. The transport activity showed a Michaelis-Menten constant of 360 ± 15 μM. Transport rates increased with decreasing pH of the medium and could be inhibited by 2,4-dinitrophenol (2,4-DNP) (Table 2). The affinity, substrate specificity, and other parameters of proline uptake mediated by *ProT1* were basically identical (data not shown).

**Figure 4.** DNA Gel Blot Analysis of the Amino Acid Permease Genes *ProT1* and *ProT2*.

Genomic DNA (10 μg) from *Arabidopsis* was digested with different restriction enzymes and analyzed by DNA gel blot hybridization under stringent [(A) and (C)] and nonstringent [(B)] conditions, using ³²P-labeled full-length cDNAs as probes. Letters at top indicate restriction enzymes (B, BamHI; E, EcoRI; H, HindIII; P, PstI; V, EcoRV; X, XbaI). Numbers at right indicate length in kilobases.

(A) *ProT1*, stringent conditions.

(B) *ProT2*, nonstringent conditions.

(C) *ProT2*, stringent conditions.

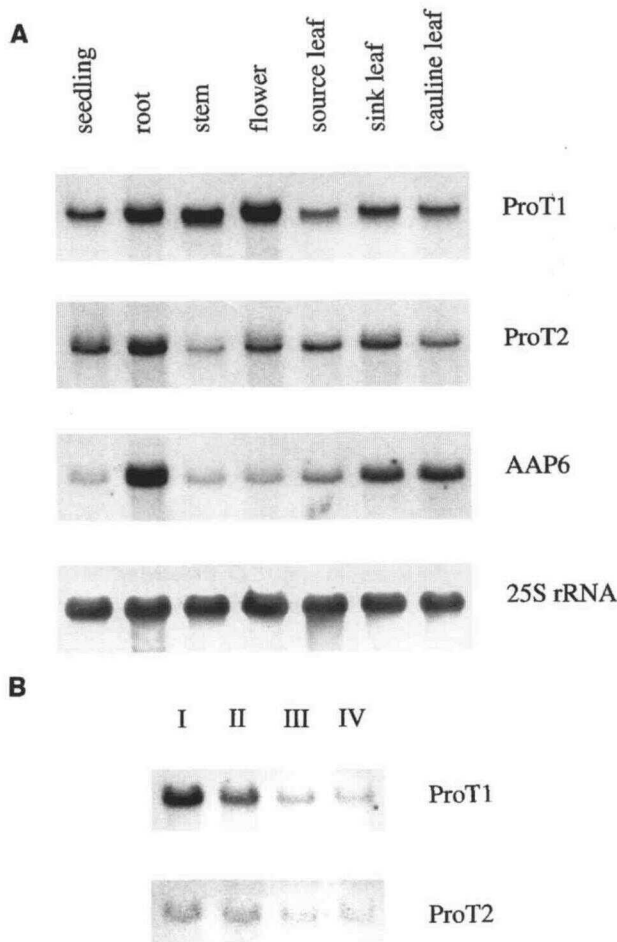


Figure 5. Expression of the Amino Acid Transporter Genes *ProT1*, *ProT2*, and *AAP6* in Arabidopsis.

(A) Determination of organ-specific expression. Total RNA from seedlings, developing leaves, mature leaves, cauline leaves, stems, flowers, and roots (20 μ g) was analyzed by RNA gel blot hybridization, using 32 P-labeled full-length cDNAs from *ProT1*, *ProT2*, and *AAP6* as probes. Controls were performed using cDNA for 25S rRNA from tomato.

(B) Developmental control of *ProT* expression in floral organs. Total RNA from flowers and siliques from different stages of development was analyzed by RNA gel blot hybridization as described in **(A)**. Stages of silique development were defined according to the stage of embryogenesis: I, open flowers; II, early embryos; III, heart-to-torpedo stage embryos; and IV, upturned U, walking stick-stage embryos.

Transport studies showed that *AAP6*, unlike *ProT1* and *ProT2*, transported proline in addition to a number of other amino acids. Neutral and acidic amino acids were efficiently transported by *AAP6*, whereas basic amino acids were marginally recognized (Figure 3B; data not shown). Thus, regarding substrate specificity, *AAP6* resembles *AAP1*, *AAP2*, and *AAP4* (Fischer et al., 1995). Because *AAP1*, *AAP2*, and *AAP4* were identified at least three times (Table 1), it is reasonable to as-

sume that from this cDNA library, these four clones are the only members of the *AAP* gene family that are capable of mediating growth under the selection conditions tested.

The *ProT* Gene Family from Arabidopsis

To obtain an estimate of the size of the *ProT* gene family, gel blot analyses (Figure 4) of Arabidopsis genomic DNA were performed under both stringent and nonstringent conditions, using *ProT1* and *ProT2* as probes. At high stringency, both genes showed a limited number of hybridizing fragments, indicating that no closely related genes are present. However, at reduced stringency, both genes showed a considerable degree of cross-hybridization. The additional hybridizing fragments either were derived from only slightly overlapping flanking regions that hybridized better at reduced stringency or perhaps indicated a third related gene.

Organ-Specific Expression of the Amino Acid Transporters

To analyze the expression profile of *ProT1*, *ProT2*, and *AAP6* in plants, RNA was isolated from different organs, and RNA gel blot analyses were performed at high stringency (Figure 5A). The results showed that *ProT1* and *ProT2* are expressed at low levels in all organs analyzed. Higher levels of *ProT1* mRNA were detected in roots, stems, and flowers. The expression of *ProT1* in flowers is downregulated during development (Figure 5B), indicating a specific role in flowers.

To determine where in the flower *ProT1* is expressed, RNA in situ hybridizations were performed. In general, overall higher expression was found in all floral tissues as compared with control hybridizations with the sense probe (Figures 6A and 6B). Highest levels of expression were found in the medial vascular strands of the carpels (Figure 6A) but also in the bundles of the pedicel and inflorescence stem (data not shown). Microscopic analysis of longitudinal sections showed that the expression is limited to the phloem.

AAP6 transcripts could be detected mainly in sink tissues such as roots and sink and cauline leaves (Figure 5A). This expression pattern is clearly different from that of other known members of the *AAP* gene family (Fischer et al., 1995). Together with *AAP6*, expression of the six members of the *AAP* gene family covers all organs analyzed, including sink leaves.

Regulation of Gene Expression by Osmotic Stress

In many plants, proline has been shown to accumulate under abiotic stresses (Delauney and Verma, 1993), and genes encoding proteins involved in proline biosynthesis are upregulated under these conditions (Verbruggen et al., 1993; Yoshida et al., 1995). To examine whether expression of proline transporter genes is also increased under water stress, Arabidopsis plants

were dehydrated for up to 26 hr or exposed to high-salt conditions. In Figure 7A, expression in response to drought is shown for *ProT2*, which encodes a specific proline transporter, and for *AAP4* and *AAP6*, encoding broad specificity amino acid permeases. With progressing desiccation, levels of *ProT2* transcripts increased slightly. In contrast, mRNA levels of *AAP6* and *AAP4* were drastically reduced under these conditions.

Similar results were obtained when plants were transferred to a solution containing 200 mM NaCl (Figure 7B). Accumulation of *ProT2* mRNA in response to salt stress was observed 4 hr after initiation of the treatment and increased with time. Expression of *P5CS* encoding Δ^1 -pyrroline-5-carboxylate synthetase (Hu et al., 1992), an enzyme catalyzing the first two steps of proline biosynthesis in plants, peaks 12 hr after the beginning of stress treatment, indicating that proline transport follows synthesis. Similar to the effects obtained under drought stress, mRNA levels of *AAP6* were reduced under salt stress.

DISCUSSION

Identification of Plant Amino Acid Transporters Independent of *SHR3* Control

The use of yeast mutants deficient in transport functions has allowed the isolation of genes for a number of plant transport

proteins, including permeases for ammonium, potassium, sucrose, and amino acids (reviewed in Frommer and Ninnemann, 1995). Three gene families encoding plant amino acid transporters were identified by complementation of yeast mutants defective in the uptake of proline, citrulline, or histidine (Frommer et al., 1994b). The increasing number of genes encoding plant amino acid permeases indicates that amino acid transport in plants might be as complex as transport in yeast (Grenson, 1992; Nelissen et al., 1995). Unlike yeast, however, little information is available on protein targeting to the membrane and regulation of amino acid transporters in plants.

Functional complementation of yeast amino acid transport mutants with plant amino acid transporters indicates that yeast secretory systems recognize the plant permeases and target them correctly to the plasma membrane. There is strong evidence that the targeting machinery for plasma membrane is conserved among different species and even kingdoms (Frommer and Ninnemann, 1995). Plant genes encoding proteins involved in intracellular targeting have been isolated by complementation of yeast mutants (*aPEP12*, Bassham et al., 1995; *STL2*, d'Enfert et al., 1992) or have been shown to complement the mutant phenotypes functionally in yeast (*aERD2*, Lee et al., 1993; *AtRAB6*, Bednarek et al., 1994). Other attempts to isolate plant homologs of yeast secretory proteins by this means were, however, unsuccessful (Welters et al., 1994). A yeast mutant deficient in a component specifically required for targeting of amino acid permeases to the plasma mem-

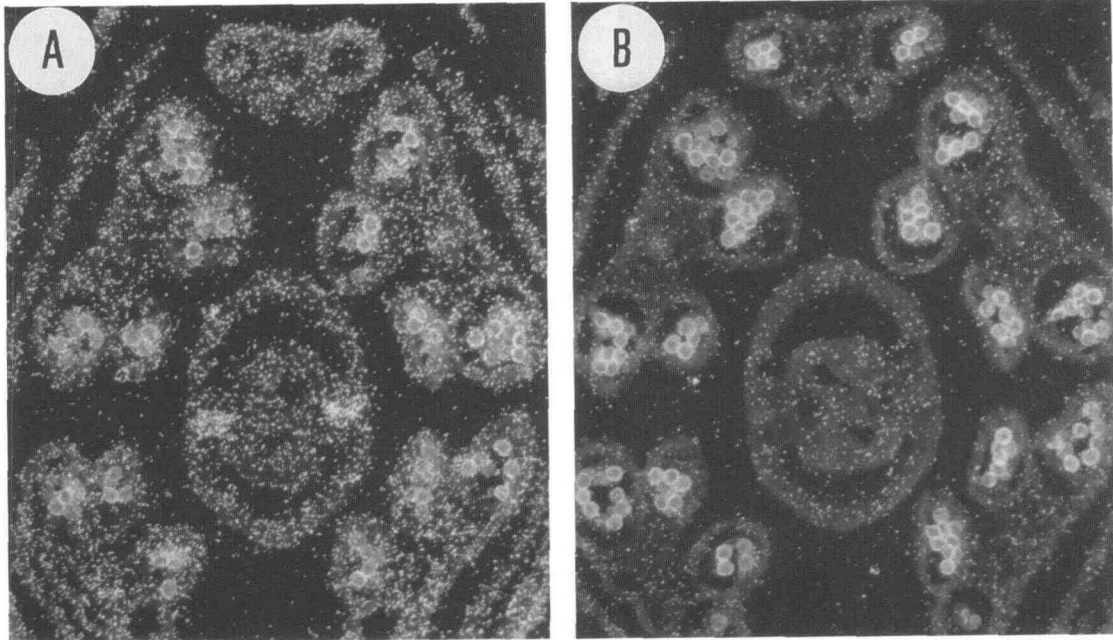


Figure 6. In Situ Localization of *ProT1* Expression in Flowers.

In situ hybridization of the proline transporter mRNA (*ProT1*) in flowers is shown.

(A) Cross-sections hybridized with antisense transcripts.

(B) Cross-sections hybridized with sense transcripts.

Carpel diameter is 90 μ m.

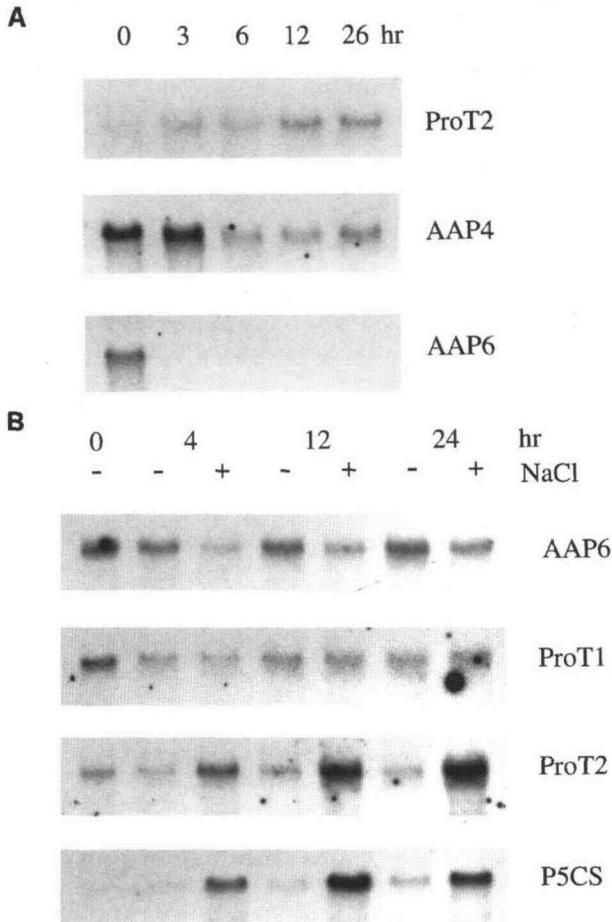


Figure 7. Regulation of *ProT* Gene Expression under Stress.

(A) Desiccation. Total RNA (20 μ g) from unbolted Arabidopsis plants subjected to various times of drought stress was analyzed by RNA gel blot hybridization, using 32 P-labeled full-length cDNAs from *ProT2*, *AAP4*, and *AAP6* as probes.

(B) High-salt conditions. Each lane was loaded with 20 μ g of total RNA prepared from Arabidopsis plants that had been transferred for the times indicated from agar plates to hydroponic growth medium in the presence (+) or absence (-) of 200 mM NaCl. Hybridization was performed using 32 P-labeled full-length cDNAs from *AAP6*, *ProT1*, *ProT2*, and *P5CS* as probes.

brane and thus lacking functional amino acid transport was used to analyze the targeting of plant amino acid permeases in yeast and potentially identify plant *SHR3* homologs. Although no functional plant homolog of *SHR3* encoding a higher order regulator of amino acid transport could be identified by heterologous complementation of an *shr3* strain, several plant amino acid transporter genes were able to suppress the mutant phenotype. Presumably, the plant transporters, although unrelated at the level of primary sequence to the yeast amino

acid permeases, can be targeted correctly to the plasma membrane even in the absence of *SHR3* function.

The transport proteins identified in this screen could be grouped into three families: members of the previously identified AAP family of broad specificity amino acid transporters, the histidine-transporting oligopeptide transporter NTR1, and a new class of proteins specifically transporting proline (Fischer et al., 1995; Rentsch et al., 1995). A novel member of the AAP family (*AAP6*) mediating efficient transport of proline, alanine, and valine was identified. The expression pattern of *AAP6* differs from that of the previously isolated *AAP* genes (Fischer et al., 1995). Similar to the tobacco hexose transporter MST1, *AAP6* seems to represent a sink-specific transport protein (Sauer and Stadler, 1993). Taken together with the newly identified *AAP6*, the six members of the AAP family isolated thus far would be sufficient to cover transport activities for all amino acids in all of the different organs analyzed.

Potential Functions of Proline Transporters during Flowering

A detailed characterization indicates that proline represents the actual substrate for *ProT1* and *ProT2*, whereas other amino acids do not seem to represent physiological substrates. Normal cytosolic and apoplastic proline concentrations in potato leaves range between 2 and 3 mM (Büssis, 1995). The affinity for proline of both proline transporters with several hundred micromolar is thus suitable for efficient import of proline into cells, that is, uptake into transport vessels either for long-distance transport or for loading into sink cells.

In reproductive tissues of Arabidopsis, proline represents 17 to 26% of total free amino acids, whereas in vegetative tissues, proline contributes only 1 to 3% (Chiang and Dandekar, 1995). Moreover, in kiwifruit, the onset of flowering seems to be associated with an accumulation of proline (Walton et al., 1991). Proline is supposed to derive from translocation from leaves to flowers (Savouré et al., 1995). Studies with bean imply that the accumulation of free proline in leaves plays a role in stimulating the production of generative organs due to transfer of proline from leaves to flowers (Venekamp and Koot, 1984). Direct evidence for the role of proline in the floral induction process stems from transgenic plants that overproduce proline. Increased levels of free proline have been correlated directly with flower formation (Kavi Kishor et al., 1995).

Under nonstressed conditions, both proline transporters were expressed at low levels in all tissues analyzed. *ProT1* expression, which is unaffected by various stress treatments, seems to serve as a general property of many cell types. Highest levels of transcript were found in roots, stems, and flowers, where expression was found mainly in the floral phloem supplying the ovules. The elevated expression of *ProT1* in flowers and stem agrees with an increased accumulation of proline in these organs. Therefore, *ProT1* might play a role in floral proline supply and in flower induction and development.

Potential Functions of Proline Transporters under Osmotic Stress Conditions

It has been well established that under various stress conditions, transport of nitrogenous compounds is altered and specific amino acids, such as proline and GABA (γ -aminobutyric acid), accumulate (Heineke et al., 1992; Breitreuz and Shelp, 1995). Under conditions of drought or salt stress, plants synthesize and accumulate osmotically active compounds, such as sugar alcohols, proline, and glycinebetaine (Tarczynski et al., 1993). Proline appears to be the most widely distributed osmoprotectant accumulating under stress conditions (Delauney and Verma, 1993). Direct evidence for a function of proline under osmotic stress has been provided by increasing the proline content due to overexpression of Δ^1 -pyrroline-5-carboxylate synthetase. Transgenic tobacco contained elevated levels of proline and exhibited increased tolerance of osmotic stress (Kavi Kishor et al., 1995; Verma and Hong, 1996). Under the assumption that proline accumulation is a cell-autonomous effect and a capacity of all cell types, researchers so far have concentrated on biosynthesis rather than translocation.

Our analysis of the responsiveness of *ProT* genes to desiccation and salt stress showed that although *ProT1* expression remains stable, *ProT2* is strongly induced in leaves under both water and salt stress. Because flower formation and proline accumulation in leaves are correlated, *ProT2* might be responsible for increased export of proline through the vascular system, whereas *ProT1* is responsible for import into the flower (Venekamp and Koot, 1984). In addition to increased *ProT2* expression, downregulation of the broad specificity AAPs might further enhance the relative transport of proline under water stress. This agrees with the finding that under water stress conditions, proline content and transport increase, whereas the export of other amino acids decreases (Tully et al., 1979). Additional experiments using transgenic plants with altered proline transport properties are required to confirm the significance of proline transport under normal and stress conditions.

METHODS

Plant Growth and Stress Treatments

Plants (*Arabidopsis thaliana* ecotype C24) subjected to drought stress were grown in the greenhouse for 4 to 5 weeks and harvested before bolting. Dehydration was performed as described by Yamaguchi-Shinozaki et al. (1992). Plants exposed to high-salt conditions were grown for 3 weeks on 0.5 \times MS (Murashige and Skoog, 1962) agar plates supplemented with 1% sucrose and subsequently grown hydroponically in the same medium containing 200 mM NaCl. Plants were frozen in liquid nitrogen after various times of the stress treatments.

Yeast Growth, Transformation, and Selection

Saccharomyces cerevisiae strains used in this study include 22574d (*MAT α* , *ura 3-1*, *gap 1-1*, *put 4-1*, *uga 4-1*; Jauniaux et al., 1987), JT16

(*MAT α* , *hip1-614*, *his4401*, *can1*, *ino1*, *ura3-52*; Tanaka and Fink, 1985), PLAS23-4B (*MAT α* , *shr3-23*, *ura3-52*, *his4 Δ 29*; Ljungdahl et al., 1992), and PLY204 (*MAT α* , *lys2 Δ 201* *ura3-52*, *ade2 Δ 1*, *hip1 Δ 2::ADE2*, *shr3 Δ 6*; P.O. Ljungdahl, unpublished results).

The yeast strain PLAS23-4B was transformed with an expression library derived from *Arabidopsis* seedlings (Dohmen et al., 1991; Ljungdahl et al., 1992; Minet et al., 1992). Transformants were selected on SPD medium supplemented with 0.6 mM histidine (Ljungdahl et al., 1992). Colonies that were able to grow were selected in liquid medium, and plasmid DNA was isolated and reintroduced into PLAS23-4B and PLY204. Candidates able to restore growth of the mutants under selective conditions were analyzed further. For nonselective conditions, the cells were grown in SUD medium (Ljungdahl et al., 1992).

Yeast strain 22574d was transformed with the cDNA clones *ProT1*, *ProT2*, and *AAP6* (Jauniaux et al., 1987). Transformants were selected on nitrogen-free medium supplemented with 10 mg of glucose mL⁻¹ and 0.5 mg of proline mL⁻¹.

DNA and RNA Manipulations and Sequence Analysis

The cDNAs were sequenced with T7 polymerase (Pharmacia, Freiburg, Germany) by using synthetic oligonucleotides. The nucleotide sequences have been submitted to the EMBL/GenBank/DBJ data base with accession numbers X95736 (*AAP6*), X95737 (*ProT1*), and X95738 (*ProT2*).

Genomic DNA from *Arabidopsis* was isolated according to Murray and Thomson (1980). RNA was extracted according to a method based on phenol extraction (Sambrook et al., 1989). DNA and RNA gel blot analyses were performed as described by Ninnemann et al. (1994) and Frommer et al. (1994a). DNA gel blot analyses under low-stringency conditions were performed with hybridization conditions of 16 hr at 50°C in a buffer containing 250 mM sodium phosphate, pH 7.2, 1% BSA, 7% SDS, and 1 mM EDTA; subsequently, the filters were washed twice for 20 min in 2 \times SSC (1 \times SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS at 68°C. High-stringency washes were subsequently performed twice for 20 min in 0.3 \times SSC, 0.5% SDS at 68°C.

Transport Assays

For standard uptake studies, yeast cells were grown to logarithmic phase. Cells were harvested at an OD₆₀₀ of 0.5, washed twice in water, and resuspended in buffer A (0.6 M sorbitol, 50 mM potassium phosphate, pH 4.5) to a final OD₆₀₀ of 6. Before the uptake measurements were made, the cells were supplemented with 100 mM glucose and incubated for 5 min at 30°C. To start the reaction, 100 μ L of this cell suspension was added to 100 μ L of the same buffer containing 18.5 kBq of ¹⁴C-labeled L-proline, L-valine, L-alanine, L-glutamic acid, L-citrulline, L-histidine (Amersham, Braunschweig, Germany), and 0.2 mM of the respective unlabeled amino acids. Because yeast strain PLY204 contains endogenous uptake activities for some of the amino acids tested, the transport activity of the yeast mutant PLY204 transformed with the empty vector pFL61 was subtracted as background from the observed rates. Samples were removed after 15, 60, 120, and 240 sec, transferred to 4 mL of ice-cold buffer A, filtered on glass fiber filters, and washed with 8 mL of buffer A. Competition for proline uptake was performed by adding a 10-fold molar excess of the respective competitors to the standard assay. The uptake of carbon-14 was determined by liquid scintillation spectrometry.

RNA in Situ Hybridization

Arabidopsis siliques were fixed in 3.7% formaldehyde, 5% acetic acid, and 50% ethanol (Cox and Goldberg, 1988), dehydrated in ethanol,

and embedded in Fibrowax (Plano, Marburg, Germany). Sections (10 μm) were mounted on precoated slides (Star Frost, Wiesbaden, Germany). Before hybridization, sections were soaked in xylene to remove paraffin, rehydrated, and deproteinized for 30 min by using proteinase K (1 $\mu\text{g}/\text{mL}$) at 37°C. ^{35}S -labeled sense and antisense transcripts of *ProT1* were hydrolyzed to an average length of 0.2 kb and used for hybridization, as described previously (Schmelzer et al., 1989). Slides were coated with NTB2 nuclear track emulsion (Tecnomara, Fernwald, Germany), exposed for 15 days, and viewed by dark-field light microscopy.

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