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LeProT1, a Transporter for Proline, Glycine Betaine, and γ-Amino Butyric Acid in Tomato Pollen

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During maturation, pollen undergoes a period of dehydration accompanied by the accumulation of compatible solutes. Solute import across the pollen plasma membrane, which occurs via proteinaceous transporters, is required to support pollen development and also for subsequent germination and pollen tube growth. Analysis of the free amino acid composition of various tissues in tomato revealed that the proline content in flowers was 60 times higher than in any other organ analyzed. Within the floral organs, proline was confined predominantly to pollen, where it represented >70% of total free amino acids. Uptake experiments demonstrated that mature as well as germinated pollen rapidly take up proline. To identify proline transporters in tomato pollen, we isolated genes homologous to Arabidopsis proline transporters. *LeProT1* was specifically expressed both in mature and germinating pollen, as demonstrated by RNA in situ hybridization. Expression in a yeast mutant demonstrated that LeProT1 transports proline and γ-amino butyric acid with low affinity and glycine betaine with high affinity. Direct uptake and competition studies demonstrate that LeProT1 constitutes a general transporter for compatible solutes.

INTRODUCTION

The accumulation of nontoxic osmolytes, which may be part of a general adaptation to adverse environmental conditions, has been recognized in many organisms, including bacteria, algae, plants, and animals. The major osmolytes found in microorganisms and plants are nonreducing sugars (sucrose and trehalose), polyols (glycerol, sorbitol, and mannitol), amino acids (glutamate and proline) or quaternary ammonium compounds (QACs; e.g., glycine betaine), and tertiary sulfonium compounds (Delauney and Verma, 1993; Rhodes and Hanson, 1993; Serrano, 1996). The increase of compatible solutes is achieved either by altering metabolism (increasing biosynthesis and/or decreasing degradation) or by transport (increased uptake and/or decreased export).

Lack of water is an important limitation to agricultural production in most areas. In addition, the presence of salt in many soils further decreases water availability for plants. During their life span (which in natural environments can exceed thousands of years), plants frequently experience low water potentials. Due to their immobility, plants have had to develop efficient strategies to cope with water and salt stress. When exposed to osmotic stresses, plants accumulate compatible solutes, such as proline and QACs. For example, in potato, proline accumulates in both cytosol and plastids to concentrations of up to 100 mM in response to water stress (Büssis and Heineke, 1998).

Investigations into the accumulation of compatible solutes in plants have focused on their metabolism, and one approach to improve crop performance under water stress conditions has been to alter the metabolism of compatible solutes. Transgenic plants overexpressing different key enzymes of biosynthetic pathways showed higher concentrations, compared with wild-type plants, of compatible solutes, such as mannitol, proline, or glycine betaine, and increased tolerance, at least under certain stress conditions (Tarczynski et al., 1993; Kavi Kishor et al.,1995; Hayashi et al., 1997; Hare et al., 1998). In comparison, very little is known about the transport of compatible solutes within the plant. An indication that transport processes may play a role in adaptation to water stress derives from the finding that in alfalfa exposed to water deficiency, a massive increase of proline is found in the phloem sap (Girousse et al., 1996). Similarly, glycine betaine was shown to be phloem mobile and is translocated during moderate stress conditions (Ladyman et al., 1980; Mäkelä et al., 1996). This translocation may be required to supply stressed domains of the plant that otherwise would be unable to respond.

The accumulation of compatible solutes also occurs in plant tissues, such as pollen or seeds, that undergo

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dehydration during their maturation. Proline represents the most abundant free amino acid in the pollen from Petunia and from many grass species (Bathurst, 1954; Zhang et al., 1982). Glycine betaine appears to be utilized for the same purpose in other species. Spinach and wheat accumulate higher concentrations of glycine betaine in anthers and pollen, respectively, than in the rest of the plant (Pearce et al., 1976; Gorham et al., 1985). Studies with sorghum indicate a positive correlation between pollen viability and proline content, and high concentrations of proline may allow germinating pollen grains to survive at unfavorable temperatures (Zhang and Croes, 1983; Lansac et al., 1996).

The male gametophyte or pollen grain has no intercellular connections, that is, plasmodesmata, to the sporophytic tissue and is therefore dependent on an external supply of compatible solutes or of nutrients that can be converted to osmoregulatory compounds. In addition to the accumulation of compatible solutes, import of carbon and nitrogen is necessary for development of the pollen grain in the anthers. Pollen thus seems to be an ideal subject in which to study the role of transport in nutrition and development.

Recently, genes encoding transporters that can mediate the transport of proline have been identified. Within the amino acid transporter (ATF) superfamily, the amino acid permeases (AAPs) mediate proton-coupled uptake of structurally diverse amino acids, including proline, whereas the proline transporters (ProTs) preferentially transport proline but no other amino acids (Rentsch et al., 1996, 1998). The concept that the ProTs might be involved in the accumulation of proline in Arabidopsis derives from the findings that *AtProT2* is induced under water stress, whereas *AAP* genes are downregulated, and that *AtProT1* is expressed to higher levels in flowers. In contrast, betaine translocation has only been described at the physiological level, but no betaine transporter has been identified in plants thus far.

To study the accumulation of nutrients and compatible solutes in tomato pollen as a model system, we analyzed the distribution of proline in the plant and the accumulation and uptake of proline in pollen. Furthermore, we isolated putative transporters for proline. The uptake and accumulation of proline in developing and germinating pollen in tomato plants correlated with the induction of a pollen-specific member of the ProT family, *LeProT1*. Biochemical characterization revealed that besides proline, LeProT1 is an efficient transporter for glycine betaine and the stress-induced amino acid γ-amino butyric acid (GABA).

RESULTS

Content and Uptake of Compatible Solutes in Tomato Flowers and Pollen

The free amino acid composition was determined in different organs of fully developed tomato plants grown in soil. The data presented in Table 1 show that the content of proline in flowers was significantly higher than in any other organ analyzed and represents more than one-third of free amino acid in flowers but <5% of the total free amino acids in other tissues (leaf, root, and fruit) for which proline is one of the minor amino acids. Compared with source leaves, the

Table 1. Concentration of Free Amino Acids in Various Tomato Organs[a]

Amino Acid	Leaf (Source)	Root	Flower	Fruit
Ala	195 ± 47	30 ± 2	293 ± 101	168 ± 63
Gly	31 ± 8	<10	52 ± 14	56 ± 17
Val	33 ± 7	27 ± 3	468 ± 14	116 ± 26
Ile	22 ± 2	16 ± 1	38 ± 9	72 ± 18
Leu	31 ± 2	20 ± 2	55 ± 8	57 ± 21
Phe	37 ± 5	14 ± 4	35 ± 5	190 ± 22
Pro	58 ± 14	57 ± 19	3252 ± 188	20 ± 7
Thr	60 ± 6	24 ± 2	68 ± 6	84 ± 16
Ser	155 ± 21	29 ± 5	175 ± 68	99 ± 17
Asn	30 ± 7	131 ± 9	1201 ± 310	91 ± 8
Gln	333 ± 41	667 ± 137	1674 ± 579	4705 ± 643
Tyr	12 ± 3	<10	22 ± 1	58 ± 18
Asp	334 ± 69	49 ± 2	280 ± 102	110 ± 35
Glu	529 ± 13	114 ± 25	510 ± 154	280 ± 74
Lys	20 ± 4	12 ± 1	80 ± 24	53 ± 15
His	11 ± 2	11 ± 3	34 ± 7	43 ± 14
Arg	<10	<10	11 ± 1	76 ± 15

[a]Organ tissue was pooled from 10 different fully developed tomato plants grown in soil in the greenhouse. Results are means ±SE of three independent experiments. Data are expressed as nanomoles per gram fresh weight of tissue.

concentration of asparagine and valine was also significantly higher in flowers. Within the flower, the proline concentration was highest in pollen and represents >70% of total free amino acids (Table 2). Because other plant species, for example, spinach, accumulate glycine betaine instead of proline in pollen, the content of glycine betaine was determined (Gorham et al., 1985). The betaine content in different tomato organs, however, was below the detection limit (<0.1 nmol mg^{-1} fresh weight of tissue). This is consistent with the low amount of glycine betaine found in leaves of tomato (Weretilnyk et al., 1989). Controls were performed with *Alcea rosea* extracts to ensure that glycine betaine could be detected under the given experimental conditions (~4 nmol mg^{-1} fresh weight) (Gorham, 1996).

The high concentration of proline in pollen indicates that proline may serve either as osmotic protection during dehydration or as a nutrient for pollen germination. The high proline content present in mature pollen grains could be derived either from endogenous biosynthesis or from sporophytic supply of proline. Therefore, uptake of carbon-14 was measured. As shown in Figure 1, ^{14}C-labeled proline can be taken up by mature pollen and by pollen after germination, indicating that respective transport proteins are present.

Identification of Tomato Proline Transporters

To identify proline transport systems in tomato, cDNA libraries from tomato flowers and leaves were screened under nonstringent conditions by using the Arabidopsis proline transporters (Rentsch et al., 1995) as probes. Approximately 7.5×10^5 plaque-forming units from two libraries with similar complexity were screened. Homologous cDNAs were identified in both the flower and leaf cDNA libraries. *LeProT1* was isolated 105 times from the flower library and once from the leaf library. In contrast, *LeProT2* was obtained eight and seven times from the flower and leaf libraries, respectively, whereas only a single *LeProT3* clone was isolated from the leaf library. Different polyadenylation sites were found in LeProT1 (positions 156, 190, and 301 after the stop codon) and LeProT2 (positions 118 and 195 after the stop codon). Sequence comparisons show that the proteins encoded by *LeProT1*, *LeProT2*, and *LeProT3* are similar to each other and to the Arabidopsis proline transporters (63 to 70% identity) and belong to the ATF superfamily that also contains the more distantly related AAP family of ATFs (Figure 2A; Rentsch et al., 1998). The open reading frames with a length of 441, 439, and 442 amino acids are similar to Arabidopsis ProTs and represent polypeptides with a molecular mass of ~48.5 kD containing nine to 11 putative membrane-spanning regions. Comparison of ProTs shows that the Arabidopsis clones are more closely related to each other than to those from tomato, indicating that none of the transporters from tomato is an ortholog of *AtProT1* or *AtProT2* (Figure 2A). Investigations of the topology of the amino acid permease AtAAP1, also named neutral system II amino acid transporter (NAT2), revealed 11 putative transmembrane domains with the N terminus in the cytoplasm and the C terminus facing outside the cell (Chang and Bush, 1997). Hydrophobicity analysis of the entire ATF superfamily, assuming a similar orientation of the N and C termini as shown for AtAAP1/NAT2, predicted a topological model with nine transmembrane domains (Figure 2B).

Interestingly, in the hydrophilic loop between the predicted fourth and fifth transmembrane domains, both LeProT2 and LeProT3 contain an amino acid motif resembling the putative 14-3-3 protein recognition site R-(S)-$X_{1,2}$-pS-X-(P), where the second serine can be phosphorylated (pS, phosphoserine; Figure 2C; Aitken, 1996). Proteins bearing such a motif can be subject to post-translational regulation by 14-3-3 proteins. These interactions with 14-3-3 proteins lead to either inactivation or activation. In LeProT1, the serine residue required for phosphorylation is replaced by a histidine, whereas in AtProT1 and AtProT2, the sequence is not conserved.

LeProT1 Is Specifically Expressed in Pollen

RNA gel blot analyses showed that *LeProT1* is specifically expressed in tomato flowers (Figure 3A). *LeProT1* expression increased during flower development and was highest at anther dehiscence (Figure 4A), consistent with the accumulation of proline in developing flowers. In contrast, the Δ^1-pyrroline-5-carboxylate synthase gene (*tomPRO2*), encoding the key enzyme of proline biosynthesis, was not induced but rather was downregulated (Fujita et al., 1998). Dissection of flowers revealed that expression of *LeProT1* is restricted to anthers (Figure 3B), and the high transcript levels in this organ correlate with proline being the main amino acid in pollen. Anthers from which pollen was partially removed showed reduced levels of the *LeProT1* mRNA, indicating that *LeProT1* is expressed in the pollen grain. The expression in pollen was confirmed by in situ hybridization experiments showing high expression in the pollen grains with the antisense but not with the sense transcript (Figures 5A and 5B). Weak background staining could be observed in the sporophytic tissues in both sense and antisense hybridization.

The accumulation of *LeProT1* mRNA late in pollen development may indicate a role during germination. To test this, pollen was germinated in vitro and RNA was isolated after 1 and 5 hr of incubation when ~50 to 70% of the pollen had germinated and the pollen tubes had reached a length of ~1 to 2 and >10 times the diameter of the pollen grain, respectively (Figure 4B). Only slight changes in expression could be detected over the period investigated. Whole-mount in situ hybridization revealed that during pollen tube growth, *LeProT1* and actin as well as tubulin (data not shown) mRNAs are localized in the growing tube (Figures 6A to 6C). Asymmetric mRNA distribution between tube and body of the pollen grain had been observed previously in maize; however, the experiments shown here do not exclude the

Table 2. Concentration of Free Amino Acids in Different Organs of the Flower[a]

Amino Acid	Sepal	Petal	Carpel	Stamen	Pollen[b]
Ala	28	119	293	186	1,323
Val	326	319	719	834	901
Ile	50	53	55	69	338
Leu	76	64	96	73	291
Phe	111	107	116	57	367
Pro	61	239	151	8,559	69,010
Thr/Ser	215	436	316	1,108	1,065
Tyr	30	31	45	44	285
Asn	38	69	<10	1,399	4,206
Asp	323	136	192	284	278
Glx[c]	730	815	1,481	1,721	533
Lys	66	58	80	91	637
His	27	30	36	135	320
Arg	21	17	18	12	77

[a]Floral organs were dissected from fully developed flowers that had already released pollen. Results represent one typical experiment that has been repeated independently with comparable results. Data are expressed as nanomoles per gram fresh weight of tissue.
[b]Data for pollen might be overestimated because of the low water content.
[c]Gln and Glu.

possibility of differential accessibility of the pollen grain body and the pollen tube during staining (Torres et al., 1995).

For *LeProT2* and *LeProT3*, no expression could be detected in any of the organs analyzed or in fruits at different stages or at different time points during the day by using RNA gel blot hybridization. For *AtProT2* from Arabidopsis, it had been shown that expression was induced under water and salt stress conditions. Despite their exhibiting clear water deficiency symptoms after drought, salt, or polyethylene glycol treatment, no expression of *LeProT2* or *LeProT3* could be detected in tomato plants. To show that *LeProT2* and *LeProT3* are indeed tomato genes, we performed gel blot analyses of tomato genomic DNA to demonstrate that *LeProT1*, *LeProT2*, and *LeProT3* are present in the tomato genome (Figure 7).

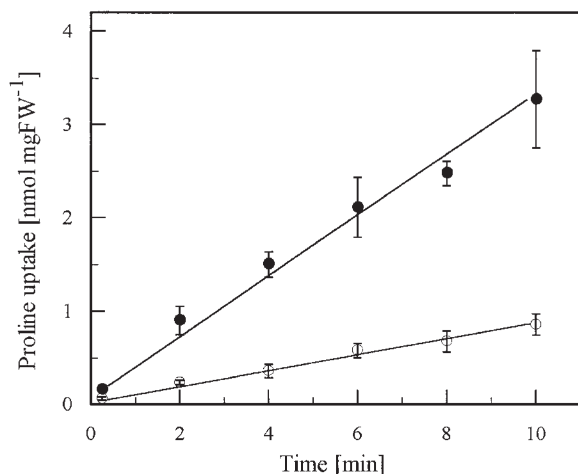

Figure 1. Time-Dependent Uptake of Radiolabeled Proline into Tomato Pollen.

Nongerminated (open circles) and germinated (filled circles) pollen was incubated in the presence of 500 μM L-^{14}C-proline. At the times indicated, pollen was removed from the incubation medium. Values are ±SD. FW, fresh weight.

Characterization of Transport Activity of LeProT1

The yeast strain 22574d, carrying mutations in the general amino acid (*gap1*), proline (*put4*), and GABA (*uga4*) permeases, is unable to grow on citrulline, proline, or GABA as the sole nitrogen source (Jauniaux et al., 1987). Transformation of 22574d with expression vectors (pDR195 or pFL61), *LeProT1*, *LeProT2*, and *LeProT3* cDNAs in pDR195, and positive controls, namely, *AtProT1* in pFL61 and the amino acid permease *AtAAP2* in pFL61, showed that only the two positive controls and *LeProT1* mediate growth on selective concentrations of proline (Figure 8; Kwart et al., 1993; Rentsch et al., 1996). *LeProT1* was also able to restore growth on selective GABA concentrations but not on citrulline or glycine betaine (data not shown). In contrast, two independent cDNA clones of *LeProT2* and the one for *LeProT3* did not restore growth under either condition. The presence of putative 14-3-3 recognition sites could lead to inactivation of LeProT2 and LeProT3 by yeast 14-3-3 proteins (Van Heusden et al., 1995). Site-directed mutagen-

esis was used to introduce a putative 14-3-3 recognition site into LeProT1$^{(H\text{-}212\rightarrow S\text{-}212)}$ and to destroy the motif in LeProT2$^{(S\text{-}210\rightarrow N\text{-}210)}$. Both changes had no effect on the growth phenotype, that is, LeProT1$^{(H\rightarrow S)}$ was still active, whereas LeProT2$^{(S\rightarrow N)}$ was inactive. Chimeric proteins between the N- or C-terminal half of LeProT1 and the corresponding part of LeProT2 were also not functional.

Uptake experiments with ^{14}C-labeled proline in yeast cells expressing *LeProT1* showed that uptake activity increased with decreasing pH and was higher at pH 4.5 than at pH 5.5 or 6.5 (15 and 2% of transport rate at pH 4.5, respectively). This pH dependence of transport as well as inhibition of proline uptake by the protonophore 2,4-dinitrophenol (100 μM 2,4-dinitrophenol inhibits proline uptake by >95%) indicate that LeProT1 mediates proline transport by cotransport of H^+ similar to the AAPs (Boorer and Fischer, 1997). Together with the fact that LeProT1 is targeted to the plasma membrane in yeast, this may indicate that LeProT1 is also localized at the plasma membrane in plants. The transport activity determined at pH 4.5 showed a Michaelis–Menten constant for proline of 1.9 ± 0.26 mM. Competition experiments of L-proline uptake in the presence of a fivefold excess of competitors showed that proteinogenic amino acids, for example, alanine, glutamate, and histidine, competed only weakly (Figure 9). As observed for the Arabidopsis proline transporters, hydroxyproline did not compete for proline uptake mediated by LeProT1. The toxic proline analog azetidine-2-carboxylic acid (A2C) competed weakly, whereas L-and D-proline were efficient competitors. Growth assays indicated that GABA can also be transported, and indeed, GABA and β-alanine, a structural analog of GABA, competed efficiently for proline uptake. GABA uptake by ProTs has been confirmed by a detailed characterization of the Arabidopsis transporter AtProT2 (K.E. Breitkreuz, B.E. Shelp, R. Schwacke, W.N. Fischer, and D. Rentsch, submitted manuscript).

In bacteria and animals, transporters have been identified that mediate the transport of either proline and betaine or GABA and betaine (Csonka, 1989; Yamauchi et al., 1992). The accumulation of betaines and other QACs during stress has also been shown in plants (Hanson et al., 1994). Competition of proline uptake by LeProT1 expressed in yeast showed that the QAC glycine betaine and its precursors, betaine aldehyde and choline, were strong competitors. Although *Saccharomyces cerevisiae* has an endogenous choline transport system, the strong inhibition by choline does not seem to result from a competition for the driving force of uptake, because proline uptake mediated by the broad specific amino acid permease AtAAP2 was not reduced in the presence of a 10-fold excess of choline (data not shown). Trigonelline, a betaine present in high concentrations in many legume seeds, has been found to induce nodulation (*nod*) gene expression in alfalfa seeds and is also present in tomato (Tramontano et al., 1986; Weretilnyk et al., 1989; Phillips et al., 1992). However, trigonelline only weakly competed for L-proline uptake.

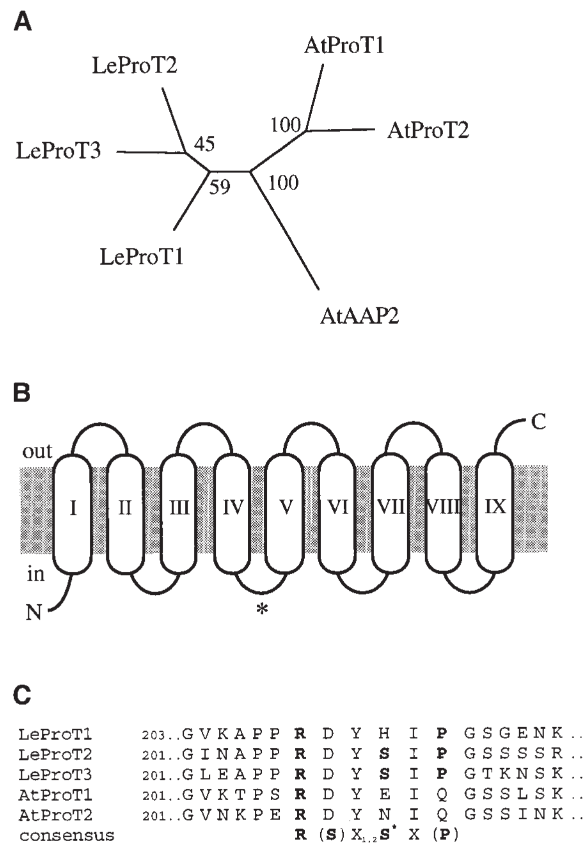

Figure 2. Comparison of the Deduced Amino Acid Sequences of LeProTs.

(A) Computer-aided analysis of the homologies between LeProTs and related proteins. The analysis was performed using the PHYLIP program package (Felsenstein, 1995) with the aligned sequences of LeProTs, AtProTs (Rentsch et al., 1996), and AtAAP2 (Kwart et al., 1993). The numbers indicate the occurrence of a given branch in 100 bootstrap replicates of the given data set. AtAAP2 was used as the outgroup.

(B) Structural model of LeProTs. The model is based on prediction of transmembrane domains for all members of the ATF family (Rentsch et al., 1998; DNASTAR, London; tmap, Milpetz and Argos, 1995), assuming a similar orientation of the N and C termini, as shown for AtAAP1/NAT2 (Chang and Bush, 1997). The position of the putative 14-3-3 binding motif in LeProT2 and LeProT3 is marked by an asterisk.

(C) Amino acid sequence at the putative 14-3-3 recognition site in LeProTs and AtProTs and the 14-3-3 consensus recognition site (Aitken, 1996). The phosphorylated serine residue is marked by an asterisk; residues conserved between the 14-3-3 consensus site and the ProTs are given in boldface.

To determine whether strongly competing compounds were transported by LeProT1, we measured uptake of ^{14}C-labeled glycine betaine and ^{14}C-labeled GABA in yeast cells expressing *LeProT1* (Table 3). Both GABA and glycine betaine were efficiently taken up by the cells, and transport

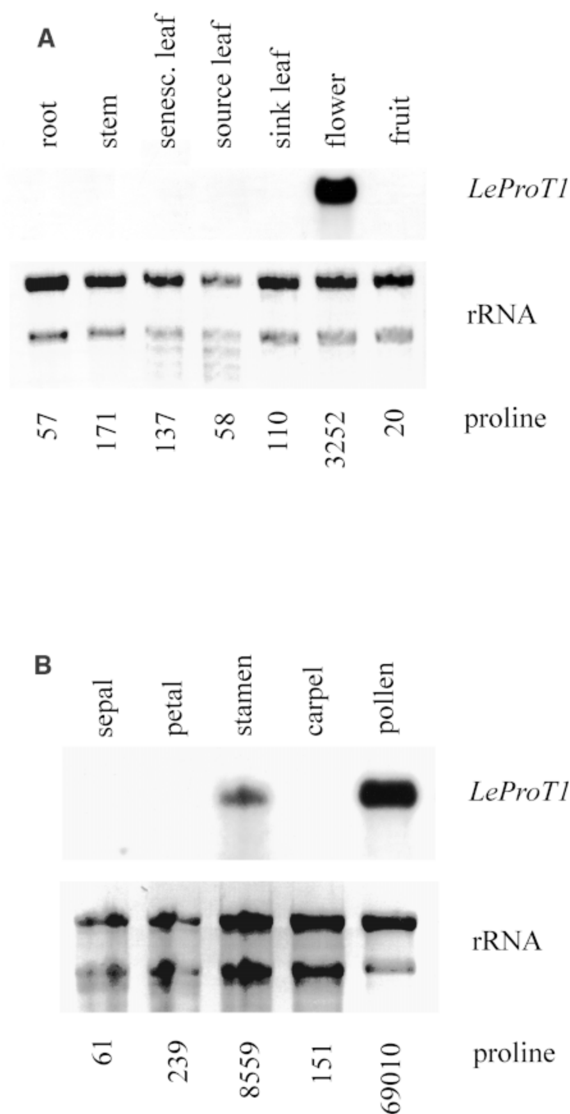

Figure 3. Expression of *LeProT1* in Tomato Organs.

Expression was analyzed by RNA gel blot hybridization by using ^{32}P-labeled full-length cDNAs from *LeProT1* as probe. The ethidium bromide staining of rRNA is shown at bottom. Proline concentrations were determined from the same plant material (from Tables 1 and 2) and are given as nanomoles per gram fresh weight of tissue.

(A) Determination of organ-specific *LeProT1* expression. Total RNA (20 μg) from developing leaves, mature leaves, senescing (senesc.) leaves, stems, flowers, fruits, and roots was analyzed.

(B) Expression of *LeProT1* in different organs of the flower. Total RNA (6 μg) from sepals, petals, anthers from which part of the pollen was removed, carpels, and pollen was analyzed.

activity was reduced by proline, GABA, and glycine betaine, respectively. The K_m determined for glycine betaine was 110 ± 34 μM. Thus, LeProT1 has a higher affinity for glycine betaine than for proline. Neither LeProT2 nor LeProT3 could mediate uptake of radiolabeled proline, GABA, or betaine. Whether LeProT2 and LeProT3 recognize a different substrate, are regulated, require additional subunits, or are not correctly targeted to the plasma membrane in yeast requires further analysis.

DISCUSSION

Bacteria adapt to high external osmolarity by increasing internal solute concentrations. These stress-induced compounds either are synthesized de novo (e.g., glutamate or trehalose) or are imported from the surrounding medium (e.g., K^+, proline, or glycine betaine). In *Escherichia coli*, three uptake systems for proline have been described. PutP is required for proline uptake when used as a carbon or nitrogen source, whereas ProP and ProU import proline and glycine betaine during osmotic stress (Csonka, 1989). Externally applied proline has long been known to alleviate growth inhibition caused by osmotic stress (Christian, 1955a, 1955b), indicating that uptake of compatible solutes plays an important role in adaptation to osmotic stress. Furthermore, proline-overproducing mutants of bacteria and *Nostoc muscorum* exhibit improved salinity tolerance (Csonka, 1989; Singh et al., 1996). The accumulation of proline and glycine betaine may allow osmotic adjustment without the accumulation of high salt concentrations, but compatible solutes might also function by stabilization of proteins and membranes (Csonka, 1989; Serrano, 1996). In addition, the protective role of compatible solutes for enzyme activities is supported by in vitro studies (Rhodes and Hanson, 1993).

Under stress conditions, higher plants also accumulate stress-related compounds and compatible solutes, such as proline, betaine, GABA, and a variety of carbohydrates (Hanson and Hitz, 1982; Serrano, 1996). Proline was shown to accumulate predominantly in plastids and the cytosol but not in the vacuole, which is consistent with its osmoprotective role (Delauney and Verma, 1993; Büssis and Heineke, 1998). Besides its presumptive role in osmotic adjustment and as a compatible solute that protects proteins, proline may function as a sink for energy and reducing equivalents, as a source of nitrogen upon relief of stress, as a means of reducing acidity, or as a radical scavenger (Blum and Ebercon, 1976; Ahmad and Hellebust, 1988; Smirnoff and Cumbes, 1989; Venekamp et al., 1989; Saradhi and Saradhi, 1991). In plants, proline could thus play different roles in different tissues or conditions. Similar functions have been suggested for other compatible solutes (Hare et al., 1998).

Similar to bacteria, multicellular organisms also have two principal strategies to accumulate solutes. Either the solute accumulation is cell autonomous via de novo synthesis from

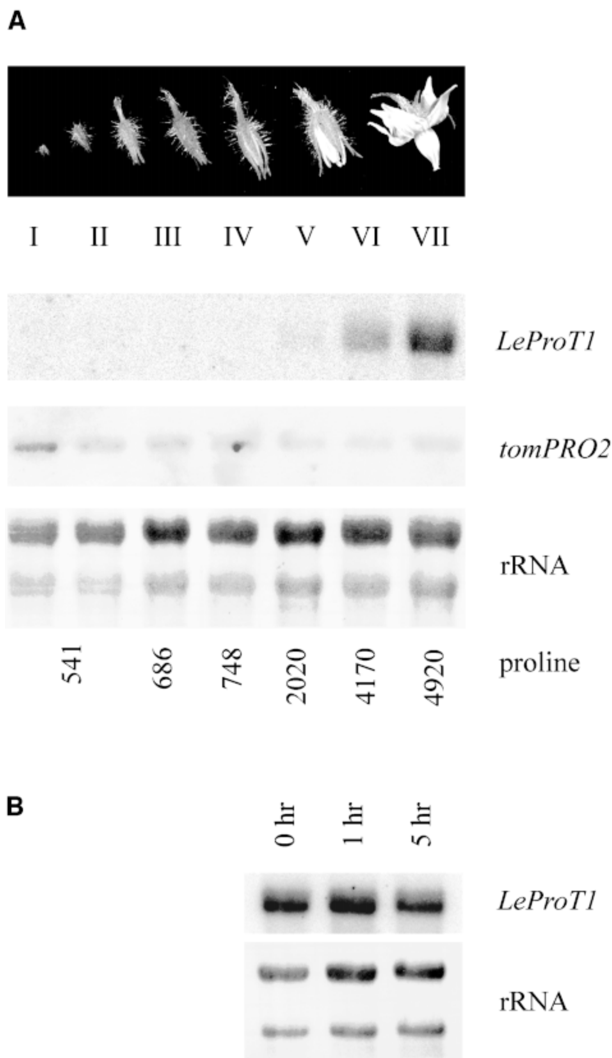

Figure 4. Developmental Control of *LeProT1* Expression.

Expression was analyzed by RNA gel blot hybridization using ^{32}P-labeled full-length cDNAs from *LeProT1* as probe. The ethidium bromide staining of rRNA is shown at bottom. Proline concentrations are given as nanomoles per gram fresh weight of tissue.

(A) Developmental control of *LeProT1* expression in floral organs. Total RNA (13 μg) from buds and flowers of different stages of development was analyzed by RNA gel blot hybridization. As a control, expression of *tomPRO2* is shown. Stages (I to VII) of flower development are shown at the top. Stages I and II were combined for determination of amino acid concentration.

(B) Expression of *LeProT1* during in vitro pollen germination. Total RNA (8 μg) of mature pollen (0 hr) and pollen germinated for 1 and 5 hr was analyzed.

existing precursors or compatible solutes are synthesized, for example, in nonstressed cell types, and subsequently transported intercellularly to water-stressed cells or other sinks for compatible solutes. Genes encoding proteins involved in proline biosynthesis are expressed in many cell types; thus, many cells seem to have the ability to synthesize proline, betaines, or GABA. Long-distance transport within the plant via the phloem has also been shown for proline and betaine (Ladyman et al., 1980; Girousse et al., 1996; Mäkelä et al., 1996).

Identification of a Transporter for Proline, Betaines, and GABA

The first plant ProTs were isolated by heterologous complementation of yeast mutants deficient in proline uptake. Two classes of clones were identified in Arabidopsis. The AAPs mediate relatively nonspecific uptake of a variety of amino acids, whereas the ProTs have been characterized as being highly specific for proline (Fischer et al., 1995, 1998; Rentsch et al., 1996). For a more detailed analysis of their function and physiological role, homologous genes were isolated from tomato by using the Arabidopsis *ProT* cDNAs as probes.

The simplest way to determine substrate specificity mediated by a plant transporter is by using growth assays of mutant yeast strains expressing the transporter cDNA. *LeProT1* expressed in yeast mediated growth on selective concentrations of proline and GABA but not on citrulline or glycine betaine. Competition studies indicated that other substrates besides proline might be transported. Indeed, direct transport assays clearly demonstrated that LeProT1 is a high-affinity transport system for glycine betaine that also mediates uptake of proline and GABA. The discrepancy between growth and uptake concerning betaines is probably due to lack of degradative enzymes in yeast. Due to the seemingly low similarity of the substrates, the finding that glycine betaine and GABA are transported in addition to proline might be surprising. However, GABA can be present in various conformations and adopts a structure that is very similar to proline (Christensen et al., 1994).

A similar study investigating the role of the Arabidopsis AtProT2 supports the finding that GABA is a substrate for ProTs (K.E. Breitkreuz, B.E. Shelp, R. Schwacke, W.N. Fischer, and D. Rentsch, submitted manuscript). In addition, the efficient competition of L-proline uptake by D-proline and the glycine betaine precursors choline and betaine aldehyde indicates that the substrate specificity is even broader. Although tomato does not seem to accumulate glycine betaine, transport of phosphorylcholine from roots to shoots in the xylem of young tomato plants has been demonstrated, and it would be interesting to test whether phosphorylcholine is recognized by the same transport system (Martin and Tolbert, 1983). The substrate specificity of LeProT1 is in agreement with the bacterial ProP and ProU system mediating transport of a variety of compatible solutes, that is,

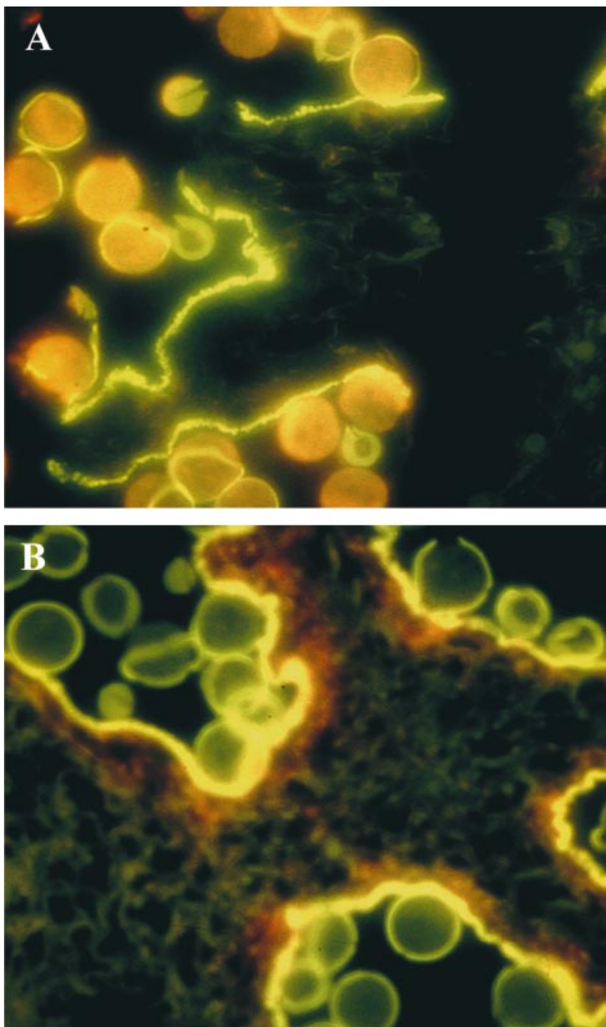

Figure 5. In Situ Localization of *LeProT1* Expression in Anthers.

In situ hybridization of the proline transporter mRNA (*LeProT1*) in anthers was visualized using the fluorescent dye 2-hydroxy-3-naphtoic acid-2′-phenylanilide phosphate and Fast Red TR giving orange fluorescence. The green color in the sense-transcript control is due to autofluorescence of the pollen grains. Pollen diameter is 15 μm.
(A) Cross-sections hybridized with antisense transcripts.
(B) Cross-sections hybridized with sense transcripts.

proline and betaines. In mammals and yeast, transporters have been described that mediate translocation of either GABA and proline or betaines and GABA, although these carriers do not share significant sequence similarities to the ProTs (Jauniaux et al., 1987; Jauniaux and Grenson, 1990; Yamauchi et al., 1992; Munck et al., 1994). However, the recently identified mammalian vesicular GABA transporter shares low but significant homology with the plant ATF superfamily that contains both ProTs and AAPs (McIntire et al., 1997; Rentsch et al., 1998).

Accumulation of Proline in Pollen

LeProT1 expression was exclusively detected in tomato pollen. *LeProT1* mRNA accumulated late in pollen development, suggesting a role in proline accumulation from anther tissue. Furthermore, expression was found in germinating pollen, indicating that it might also be involved in pollen nutrition. The expression data in mature and germinating pollen were verified by in situ hybridization. Dissection of open flowers revealed that proline is confined to pollen, where it represents >70% of total free amino acids. Proline accumulates not only in cells exposed to water stress but also in naturally desiccating tissues such as pollen, which loses up to 90% of its water content during maturation. Proline and, in some plant species, betaines are thought to serve as compatible solutes preventing destruction of cellular functions during desiccation in pollen. A positive correlation was observed for proline content and pollen survival (Lansac et al., 1996).

By using direct uptake experiments, we could verify that both mature and germinating pollen grains are able to take up proline from the external medium. Interestingly, the levels of precursors for proline biosynthesis (glutamate or ornithine) and also of other amino acids do not change during flower development (data not shown). Furthermore, expression of the genes encoding the key enzyme of proline biosynthesis from glutamate, *tomPRO1* or *tomPRO2*, was not increased in pollen; however, the presence of additional genes or biosynthesis from ornithine cannot be excluded (Fujita et al., 1998). Although it is generally assumed that the accumulation of compatible solutes is a cell-autonomous response, transport might play an essential role in leaves under water stress and in pollen, and specific accumulation of proline may require selective transport systems. Because no increased expression of *tomPRO* genes was found in flowers, either other genes encoding proteins involved in proline biosynthesis or an external supply by long-distance transport may be envisaged. The data presented here do not exclude the possibility that other transporters contribute to the uptake and accumulation of proline and other amino acids into pollen. The AAPs are nonselective and also mediate proline transport; however, none of the members of this family has been shown to be expressed in pollen. A gene that is related to the lysine/histidine transporter (LHT1) from Arabidopsis, namely, *NsAAP1* from tobacco, was found to be expressed in pollen (Chen and Bush, 1997; Lalanne et al., 1997). However, LHT1 does not seem to transport proline efficiently, and no function has been shown for NsAAP1.

The affinity of LeProT1 for glycine betaine (K_m = 110 μM) is higher than for proline (K_m = 1.9 mM). The K_m value for proline is approximately five times higher than for the stress-regulated ProT2 from Arabidopsis. Because the intracellular

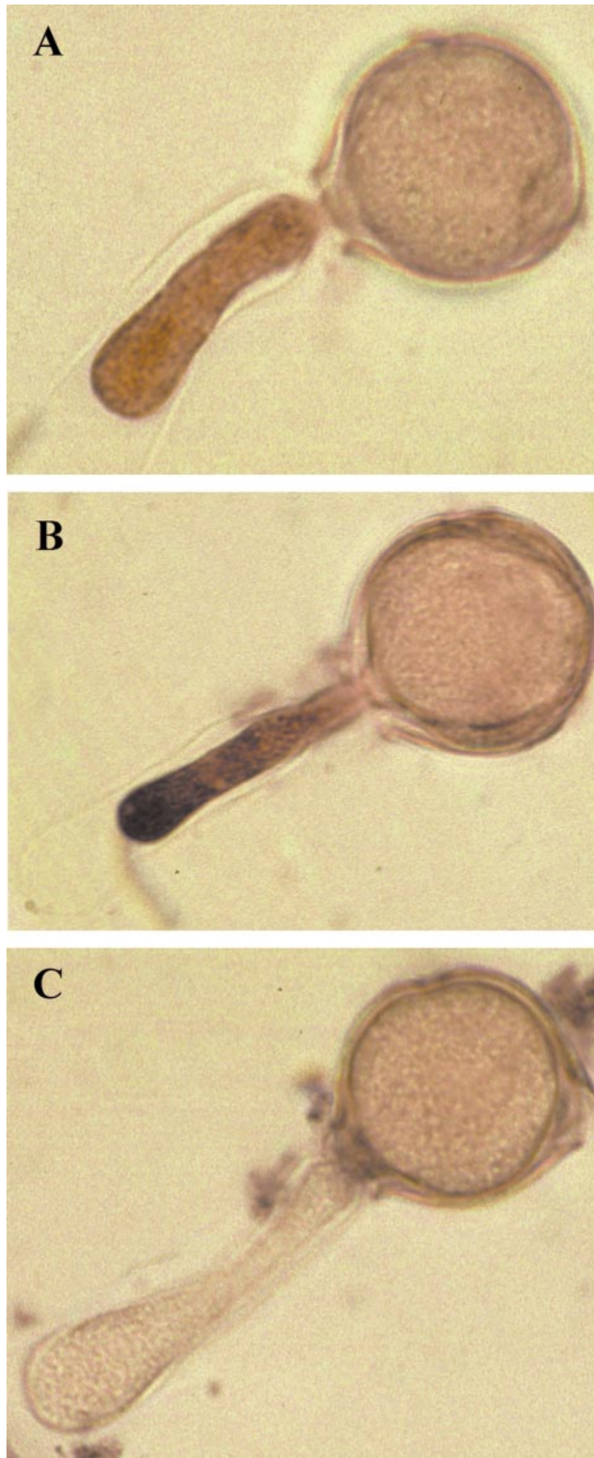

Figure 6. Whole-Mount in Situ Localization of the Proline Transporter (*LeProT1*) and Actin (*NtACT*) mRNAs in Germinating Tomato Pollen.

The accumulation of specific mRNAs was observed in situ using digoxigenin-labeled riboprobes and detected with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. *NtACT* is an expressed sequence tag of a tobacco pollen library (L. Bürkle, B. Stadelhofer, and W.B. Frommer, unpublished observations). Diameter of the body of the pollen grain is 15 μm.

(A) Localization of *LeProT1* mRNA in growing pollen tubes using the antisense probe.

(B) Localization of actin mRNA in the pollen tubes using the antisense probe.

(C) Localization of *LeProT1* mRNA in growing pollen tubes using the sense probe.

concentration of proline in pollen exceeds 50 mM, active proline uptake is required. Although not tested directly in these experiments, LeProT1 is assumed to be H^+ coupled and able to accumulate proline against a concentration gradient, as are the other members of the ProT and AAP family (Fischer et al., 1995; Rentsch et al., 1996). The extracellular concentration of proline in the tapetum and the style is unknown. The high K_m of LeProT1 for proline suggests that the concentration must be in the millimolar range. Because no glycine betaine was detected in pollen of tomato, the transport activity at least in the case of tomato pollen may not be physiologically relevant.

Potential Functions of Proline Transporters in Germinating Pollen

LeProT1 transcripts are also present during pollen germination, indicating that uptake of compatible solutes and/or proline is also required during pollen tube growth. In various plant species, mRNA synthesis is not required for the early events in germination, and even some proteins needed for germination are present already in the pollen grain at the time it is released from the anthers (Mascarenhas, 1993). In addition, at least for the more abundant mRNAs, there seems to be no difference between the mRNAs present in the ungerminated pollen grain and those synthesized during germination and pollen tube growth. Many of the transcripts accumulating during pollen maturation seem to be necessary to guarantee the rapid germination and elongation rates of the pollen tube (1 cm/hr, up to 50 cm; Mascarenhas, 1993). During this rapid growth, supply from the pistil is necessary (Labarca and Loewus, 1973). Uptake systems for other compounds, namely, sucrose in the germinating pollen tube, have been described in tobacco (R. Lemoine and W.B. Frommer, unpublished data). Proline could serve as a compatible solute during pollen dehydration and as the primary energy source during rapid tube elongation, that is, as a source for hydroxyproline-rich proteins for pollen tube elongation (Zhang et al., 1982).

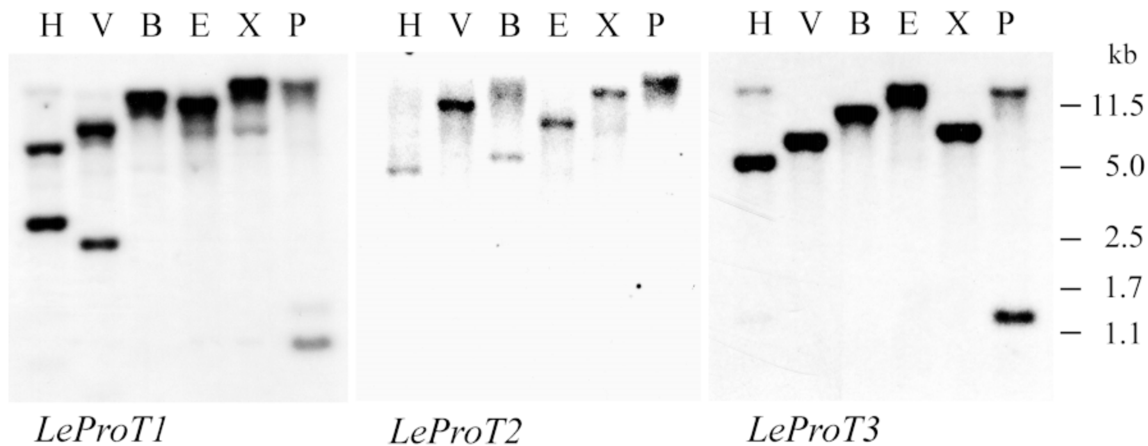

Figure 7. DNA Gel Blot Analysis of the Proline Transporter Genes.

Genomic DNA (10 μg) from tomato was digested with different restriction enzymes and analyzed by DNA gel blot hybridization using the indicated ^{32}P-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 lengths in kilobases.

Potential Regulation and Function of LeProT2 and LeProT3

The function of LeProT2 and LeProT3 remains unclear. Both cDNAs were unable to functionally complement deficiencies in proline or GABA transport when expressed in yeast and could not mediate transport of glycine betaine. However, it was not determined whether LeProT2 and LeProT3 transport substrates were not tested in these experiments or whether the transporters were not expressed in functional form in yeast (as discussed below).

The presence of a putative 14-3-3 target site in LeProT2 and LeProT3 favors the hypothesis that the transporters might be subject to post-translational regulation by 14-3-3 proteins. In plants, regulation of enzyme activity by 14-3-3 proteins has been demonstrated for the plasma membrane ATPase and nitrate reductase (Oecking et al., 1994, 1997; Bachmann et al., 1996; Moorhead et al., 1996). The RDYSIP motif in LeProT2 and LeProT3 fits the consensus target sequences proposed by Aitken (1996), namely, $R(S)X_{1,2}pSXP$, and could be seen as intermediate between the two different binding sequences, namely, RSXpSXP and RXY/FXpSXP, determined by Yaffe et al. (1997). It is not known whether 14-3-3 binding to the LeProT sites could be inhibitory or could activate transport activity. Because expressed plant 14-3-3 proteins can replace their yeast paralogs, the yeast endogenous 14-3-3 proteins might recognize and inactivate LeProT2 and LeProT3, or phosphorylation of the 14-3-3 binding site, as required for regulation of the plasma membrane ATPase, may not occur in yeast.

Explanations for the lack of transport activity include (1) inefficient targeting, as described for numerous membrane proteins expressed in yeast (e.g., Villalba et al., 1992); (2) the lack of additional required subunits; (3) a differential localization, for example, in the chloroplast or mitochondria; (4) a difference in the substrate specificity; or (5) a different transport mechanism, that is, uniport or antiport. Efflux transporters are required, for example, after stress release to remove the compatible solute, as found in *Corynebacterium glutamicum* (Ruffert et al., 1997). It is clear that chloroplasts and mitochondria require proline import, whereas the vacuole may have an export activity because proline concentration is low compared with the cytosol during stress (Büssis and Heineke, 1998). Because no obvious differences were found compared with LeProT1 or the Arabidopsis ProTs in the primary sequences, indicating the presence of specific targeting sequences, this may seem an improbable explanation. However, in the case of plasma membrane and tonoplast aquaporins, no obvious targeting signals have been identified either. Further experiments are required to determine the significance of the putative 14-3-3 binding domains and the seeming inactivity of the transporters.

Conclusions

A functional transporter was identified that is specifically expressed in tomato pollen and shares significant similarity to the high-affinity proline transporters from Arabidopsis. LeProT1 mediates low-affinity proline and GABA transport as well as high-affinity glycine betaine transport when expressed in yeast. During maturation, pollen was found to accumulate proline. The correlation of LeProT1 mRNA accumulation and the demonstration of proline uptake by pollen indicate a function for this transporter in supplying pollen with proline during both maturation and germination. The

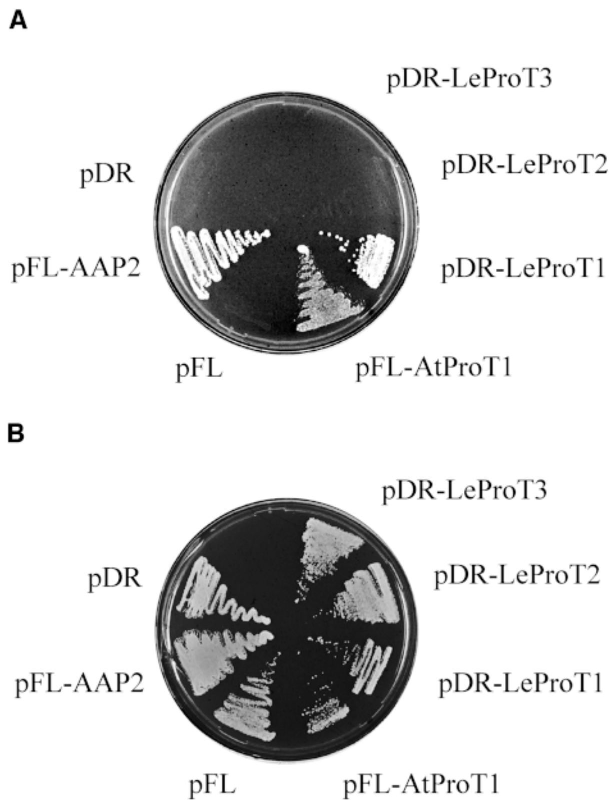

Figure 8. Complementation of Growth of a Citrulline, Proline, and GABA Uptake–Deficient Yeast Strain (22574d) by LeProTs, AtProT1, and the Amino Acid Permease AtAAP2.

Growth of 22574d expressing LeProT1, LeProT2, LeProT3, AtProT1, AtAAP2, and the strain transformed with the respective vectors pDR195 and pFL61 is shown.

(A) Minimal medium supplemented with proline as the sole nitrogen source.

(B) Minimal medium supplemented with ammonium sulfate as nitrogen source.

ProTs represent general stress amino acid and compatible solute transporters. It is thus conceivable that different members of the family serve different functions, such as in pollen nutrition, but also in water and salt stress responses, as shown for the Arabidopsis *AtProT2.*

METHODS

Plant Growth and Stress Treatments

Plants (*Lycopersicon esculentum* cv Moneymaker) were grown in the greenhouse for 4 to 5 weeks, and material was harvested from different organs and parts of the flower. All material was harvested at approximately noon to minimize variations due to diurnal fluctuations. For stress experiments, tomato plants were grown hydroponically in Hoagland's solution for 3 weeks before the stress treatment. Plants were frozen in liquid nitrogen at different time points.

DNA and RNA Manipulations and Sequence Analysis

Two tomato (*L. esculentum* var UC82b) cDNA libraries, one from above-ground portions of nonflowering tomato plants and one from tomato flowers (Schmid et al., 1992; Bischoff et al., 1996), were screened using *AtProT1* and *AtProT2* cDNAs as probes (Rentsch et al., 1996). Positive clones were plaque purified and excised in vivo. Both strands were sequenced with an automated sequencer (ABI Prism373; Applied Biosystems, Weiterstadt, Germany) by using synthetic oligonucleotides (ARK Scientific, Darmstadt, Germany). The nucleotide sequences have EMBL accession numbers AF014808 (*LeProT1*), AF014809 (*LeProT2*), and AF014810 (*LeProT3*).

Chimeric constructs between the N- and C-terminal parts of *LeProT1* and *LeProT2* were made at a conserved AvrII site. Site-directed mutagenesis with a mismatching primer was used to replace the Ser with an Asn at position 210 in *LeProT2.* Similarly, His was replaced by Ser at position 212 in *LeProT1.* Both polymerase chain reaction–amplified fragments were sequenced to confirm that no other modification occurred.

Genomic DNA was isolated according to Murray and Thompson (1980). RNA was isolated according to Prescott and Martin (1987), separated on formaldehyde gels, and transferred to nylon membranes. The complete *LeProT* cDNAs were used as radioactive

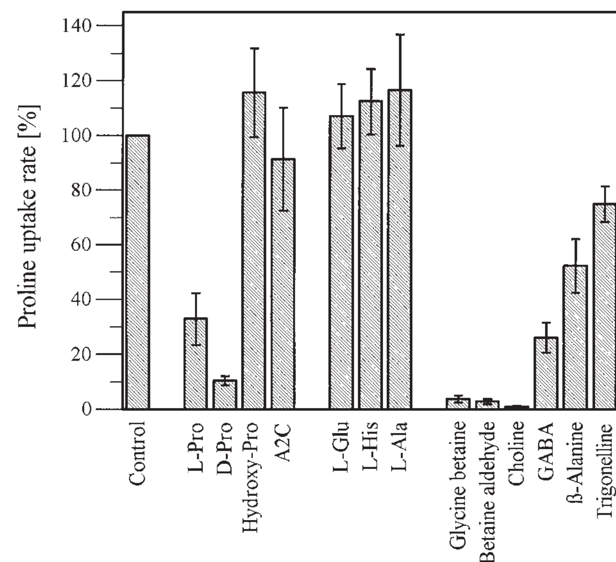

Figure 9. Biochemical Properties of LeProT1.

Competition of 1.5 mM L-^{14}C-proline uptake into yeast cells (22574d) expressing *LeProT1* in the presence of a fivefold excess of the respective substrate. The uncompeted uptake rate was taken as 100% corresponding to 1.76 pmol of L-proline min^{-1} mg^{-1} fresh weight. The means +SE of at least three independent competition experiments are shown. Hydroxy-Pro, 4-hydroxy-L-proline; A2C, L-azetidine-2-carboxylic acid.

Table 3. Competition among Proline, Glycine Betaine, and GABA Uptake in Yeast Cells Expressing *LeProT1*[a]

	Uptake Rate (Percentage of Control)[b]		
	^{14}C-Proline	^{14}C-Glycine Betaine	^{14}C-GABA
Control	100	100	100
Proline	24.6 ± 2.2	63.4 ± 8.8	28.4 ± 6
Glycine betaine	10.1 ± 1.5	21.6 ± 6.2	ND[c]
GABA	24.3 ± 2.0	ND	26.4 ± 3

[a]Uptake of 1.5 mM L-^{14}C-proline, L-^{14}C-glycine betaine, and ^{14}C-GABA, respectively, was measured in the presence of a fivefold excess of the respective substrates.
[b]Absolute uptake rates correspond to 4.3 ± 0.8 pmol of proline min^{-1} mg^{-1} fresh weight of tissue, 1.9 ± 0.5 pmol betaine min^{-1} mg^{-1} fresh weight of tissue, and 1.5 ± 0.6 pmol GABA min^{-1} mg^{-1} fresh weight of tissue and represent the mean of at least three independent measurements ±SE.
[c]ND, not determined.

probes and hybridized at stringent conditions, as described by Görlach et al. (1994). Washes were performed twice for 20 min in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 68°C. Experiments were repeated independently at least twice.

Yeast Growth, Transformation, and Selection

LeProT1, *LeProT2*, and *LeProT3* were isolated from pBluescript II SK− (Stratagene, La Jolla, CA) by cleavage with PstI and XhoI, made blunt ended with the Klenow fragment of DNA polymerase I, and inserted into the blunt-ended NotI site of pDR195 (Rentsch et al., 1995). *Saccharomyces cerevisiae* 22574d (*MAT*α, *ura3-1*, *gap1-1*, *put4-1*, and *uga4-1*; Jauniaux et al., 1987) was transformed with these constructs (Dohmen et al., 1991), and transformants were selected on nitrogen-free medium supplemented with 20 mg of glucose mL^{-1} and 0.5 mg of proline mL^{-1} medium. For nonselective conditions, the cells were grown in SD medium (Adams et al., 1998).

Transport Assays (Yeast)

For standard uptake studies, yeast cells were grown to logarithmic phase. Cells were harvested at an $OD_{600\ nm}$ of 0.5, washed twice in water, and resuspended in buffer A (0.6 M sorbitol and 50 mM potassium phosphate, pH 4.5) to a final $OD_{600\ nm}$ of 6. Before the uptake measurements, the cells were supplemented with 100 mM glucose and incubated for 5 min at 30°C. To start the reaction, 100 μL of this cell suspension was added to 100 μL of the same buffer containing 9.25 kBq ^{14}C-labeled L-proline, γ-amino butyric acid (GABA; Amersham, Braunschweig, Germany), or glycine betaine (Biotrend, Cologne, Germany) and appropriate amounts of the respective unlabeled amino acids. The transport activity of the yeast mutant 22574d transformed with the empty vector pDR195 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, GABA, and glycine betaine uptake was performed by adding a fivefold molar excess of the respective competitors to the standard assay. The uptake of carbon-14 was determined by liquid scintillation spectrometry. Transport measurements were repeated independently and represent the mean of at least three experiments.

Transport Assays (Pollen)

Pollen was collected in liquid nitrogen and stored at −70°C. For uptake studies, pollen was either germinated for 1 hr (see whole-mount in situ hybridization), centrifuged for 1 min at 1500 rpm, and resuspended in 300 μL of buffer A (0.6 M sorbitol and 50 mM potassium phosphate, pH 5.0), or resuspended in 300 μL of buffer A directly before the reaction was started. To start the reaction, 400 μL of buffer A containing 18.5 kBq ^{14}C-labeled L-proline and unlabeled proline leading to a final concentration of 500 μM was added. Samples were removed after 15 sec and 2, 4, 6, 8, and 10 min, transferred to 5 mL of ice-cold buffer A containing 10 mM unlabeled proline, filtered on glass fiber filters, and washed twice with 5 mL of buffer A containing 10 mM unlabeled proline. The uptake of carbon-14 was determined by liquid scintillation spectrometry. Transport measurements were repeated independently and represent the mean of at least three measurements.

Determination of Amino Acids

Different organs of tomato were frozen in liquid nitrogen, ground with a mortar and pestle, and subsequently extracted with 70% methanol heated to boiling and centrifuged. The pellet was washed twice with 70% methanol and 20% methanol, respectively. The combined supernatants were evaporated to dryness and redissolved in lithium diluent Li220 (Pickering Lab, Mountain View, CA). After ultrafiltration, the sample was separated by HPLC on a cation–exchange column (high-efficiency fluid column, 3 mm × 150 mm; Pickering Lab) using lithium as eluant. The amino acids were derivatized with ninhydrine before photometric detection at 440 and 570 nm for proline and all other amino acids, respectively.

Determination of Glycine Betaine

Plant material was ground with a mortar and pestle and extracted twice with methanol–chloroform-H_2O (12:5:1). The combined supernatants were evaporated and redissolved in H_2O. Further purification by ion exchange was done as described by Gorham (1984). Samples were evaporated twice to dryness and both times redissolved in deuterium oxide and subsequently filtered on celite. ^{1}H-NMR analysis was performed as described by Larsen et al. (1987) using 3-(trimethylsilyl)-1-propane sulfonate sodium salt as the internal standard.

In Situ Hybridization

Both sense and antisense transcripts were synthesized starting with 1 μg of template using the digoxigenin RNA labeling kit (Boehringer Mannheim) and hydrolyzed in carbonate buffer to an average length of 300 nucleotides. Both labeling reaction and hydrolyzed probes were checked on an agarose gel. The hydrolyzed probes were precipitated (0.4 M LiCl, 250 μg mL^{-1} tRNA, and 2.5 volumes ethanol) at

−20°C for 20 min. Pellets were sedimented, washed with 70% ethanol, and resuspended in water.

Whole Mount

Pollen grains were collected at approximately noon and stored in liquid nitrogen until use. Pollen was germinated for 1 hr in darkness at 28°C in medium described by Read et al. (1993). Germinated pollen grains were fixed in 4% paraformaldehyde in PBS (PBS is 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, and 270 μM KCl) for 2 hr at room temperature (S. Heuer, unpublished data). Samples were treated as described by Torres et al. (1995), except that the proteinase K concentration was 10 μg mL^{-1}, and there was a refixation step in 4% paraformaldehyde in PBS for 20 min and subsequent washing in PBS (S. Heuer, unpublished data). Prehybridization was performed for 3 hr at 50°C, and hybridization was performed overnight at 50°C with a probe concentration of 5 μg mL^{-1}. Hybridization buffer and wash conditions were as described by Torres et al. (1995), except that the RNase concentration was 40 μg mL^{-1}. The probe was detected by an alkaline phosphatase–coupled antidigoxigenin antibody and visualized using 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, following the manufacturer's instructions (Boehringer Mannheim).

Sections

Plant tissue fixation, embedding, and hybridization were performed as described by Yu et al. (1998). Sections were 3 μm thick. Staining was performed as described above but using the fluorescent dyes 2-hydroxy-3-naphtoic acid-2′-phenylanilide phosphate and Fast Red TR (4-chloro-2-methylbenzenediazonium hemi-zinc chloride salt) (Boehringer Mannheim). Fluorescence was viewed under blue light excitation.

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