

RESEARCH PAPER

# *In planta* function of compatible solute transporters of the AtProT family

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Received 1 June 2010; Revised 25 August 2010; Accepted 14 September 2010

## Abstract

The three proline transporters of *Arabidopsis thaliana* (AtProTs) transport the compatible solutes proline and glycine betaine and the stress-induced compound  $\gamma$ -aminobutyric acid when expressed in heterologous systems. The aim of the present study was to show transport and physiological relevance of these three AtProTs *in planta*. Using single, double, and triple knockout mutants and AtProT-overexpressing lines, proline content, growth on proline, transport of radiolabelled betaine, and expression of AtProT genes and enzymes of proline metabolism were analysed. AtProT2 was shown to facilitate uptake of L- and D-proline as well as [<sup>14</sup>C]glycine betaine *in planta*, indicating a role in the import of compatible solutes into the root. Toxic concentrations of L- and D-proline resulted in a drastic growth retardation of AtProT-overexpressing plants, demonstrating the need for a precise regulation of proline uptake and/or distribution. Furthermore evidence is provided that AtProT genes are highly expressed in tissues with elevated proline content—that is, pollen and leaf epidermis.

**Key words:** *Arabidopsis*, compatible solute, epidermis, pollen, proline, transport.

## Introduction

Organic solutes that accumulate at high concentrations in the cytoplasm in response to abiotic stress without interfering with primary metabolism are classified as ‘compatible’ solutes. The best known compatible solutes in plants are proline, glycine betaine, sugars, and polyols (Yancey, 2005; Verbruggen and Hermans, 2008). How exactly compatible solutes fulfil their protective role during stress is still a matter of debate; it appears that there exists a range of mechanisms such as supporting osmotic adjustment, protection of cellular structures, and regulation of cellular redox potential (Hare *et al.*, 1998). High levels of compatible solutes are found not only under stress conditions, but also in plant organs that undergo dehydration as part of their development, such as pollen and seeds (Krogaard and Andersen, 1983; Chiang and Dandekar, 1995; Mondal *et al.*, 1998; Schwacke *et al.*, 1999; Schmidt *et al.*, 2007). The key enzyme of proline biosynthesis,  $\Delta^1$ -pyrroline-5-carboxylate synthetase (P5CS), is encoded by two differen-

tially regulated genes in *Arabidopsis thaliana* (Strizhov *et al.*, 1997). AtP5CS1 is required for proline accumulation following osmotic stress, whereas AtP5CS2 is associated with embryo development (Székely *et al.*, 2008).

The presence of compatible solutes in the phloem sap indicates that long-distance transport might be important for metabolism and/or stress tolerance (Girousse *et al.*, 1996; Mäkelä *et al.*, 1996). Likewise, uptake of compatible solutes from the soil can improve the plant’s resistance to adverse environmental conditions (Räsänen *et al.*, 2004). Although transporters for polyols (Klepek *et al.*, 2005), glycine betaine, and proline (Rentsch *et al.*, 1996; Schwacke *et al.*, 1999) have been identified, their physiological role is poorly understood.

Amino acid transporters mediating the transport of proline have been identified in different gene families (Rentsch *et al.*, 2007; Lehmann *et al.*, 2010). The family of proline transporters (ProTs), with three members in

*Arabidopsis*, transports proline, but no other proteinogenic amino acids (Rentsch *et al.*, 1996). Although the ProTs were originally described as proline-selective transporters, later studies showed that in contrast to other amino acid permeases, ProTs from *Arabidopsis*, sugar beet, the mangrove *Avicennia marina*, and tomato, as well as barley ProT2 also transport glycine betaine (Rentsch *et al.*, 1996; Schwacke *et al.*, 1999; Waditee *et al.*, 2002; Grallath *et al.*, 2005; Yamada *et al.*, 2009; Fujiwara *et al.*, 2010). The tomato ProT1, barley ProT2, and the three *Arabidopsis* ProTs also recognize the stress-related compound  $\gamma$ -aminobutyric acid (GABA), though the affinity of the AtProTs and HvProT2 for GABA is lower than for proline or glycine betaine (Breitkreuz *et al.*, 1999; Grallath *et al.*, 2005; Fujiwara *et al.*, 2010). The plasma membrane localization of AtProTs (Grallath *et al.*, 2005), HvProT2 (Fujiwara *et al.*, 2010), and sugar beet ProT (BvBet/ProT1; Yamada *et al.*, 2009) suggests a function as a cellular uptake system in proline- and glycine betaine-accumulating species.

Although the intracellular localization, substrate selectivity, and affinity of the three *Arabidopsis* ProTs are similar, differences in expression indicate different roles *in planta* (Grallath *et al.*, 2005). *AtProT1:GUS* staining was detected in the phloem of all organs analysed, suggesting a role in long-distance transport of proline (Grallath *et al.*, 2005). *AtProT2:GUS* staining is found in the epidermis and cortex in roots and is detectable in leaves only after wounding. *AtProT3* expression is restricted to the epidermis in leaves (Grallath *et al.*, 2005). The expression of *ProT* family members is often associated with increased levels of proline. For example, tomato *ProT1* transcripts are found exclusively in pollen that has high concentrations of proline (Schwacke *et al.*, 1999). Transcript levels of *Arabidopsis ProT2* and of the three mangrove *ProT* homologues increase in response to salt stress, as do the concentrations of proline and glycine betaine, respectively (Chiang and Dandekar, 1995; Rentsch *et al.*, 1996; Hibino *et al.*, 2001; Waditee *et al.*, 2002). Salt stress also increases mRNA levels of *HvProT* in barley roots (Ueda *et al.*, 2001). Despite these correlative changes, a role for ProTs in the translocation of proline or glycine betaine has been demonstrated only recently. Transgenic *Arabidopsis* lines overexpressing *HvProT* showed reduced shoot biomass and decreased proline accumulation (Ueda *et al.*, 2008). Conversely, root cap-specific expression of *HvProT* resulted in increased accumulation of proline in the root tip and enhanced root elongation (Ueda *et al.*, 2008).

To reveal the contribution of individual *Arabidopsis* ProTs to the distribution of proline within the plant, the focus of the present study was on organs and growth conditions known to be associated with *AtProT* expression. Using *atprot* knockout plants and overexpressing lines, it is demonstrated that AtProT1 and AtProT2 mediate proline transport *in planta*. It is shown that proline is unevenly distributed between the lower leaf epidermis and the remainder of the leaf, and that proline is the major free amino acid in mature *Arabidopsis* pollen, correlating with high expression of *AtProT3* and *AtProT1*, respectively.

## Materials and methods

### Plant growth and transformation

*Arabidopsis thaliana* L. ecotype Columbia (Col-0) or Wassilewskija (Ws) and mutant lines were cultivated in a growth chamber under a photoperiod of 16 h of light ( $100\text{--}150 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and 8 h of darkness. Plants were grown in soil with a day/night temperature of 22 °C/18 °C at 65%/60% relative humidity.

The following *Arabidopsis* T-DNA insertion lines were used in this work: *atprot1-1* (Salk\_018050, ecotype Columbia), *atprot2-1* (4B14, Feldmann line, ecotype Wassilewskija), *atprot2-2* (CSJ1230, Wisconsin, ecotype Wassilewskija), *atprot2-3* (Salk\_067508, ecotype Columbia), and *atprot3-2* (Salk\_083340, ecotype Columbia), (Wisconsin lines: Arabidopsis Knockout Facility of the University of Wisconsin, Krysan *et al.*, 1999; Salk lines: Alonso *et al.*, 2003; Feldmann lines: PCR screening of mutants obtained from the ABRC stock center, Columbus, OH, USA, according to Krysan *et al.*, 1996). The insertion sites were verified by sequencing flanking regions which were amplified by PCR using T-DNA left border primers (for Salk lines, 5'-GCGTGGACCGCTTGCTGCAACT-3'; for Feldmann line, 5'-GATGCACCTCGAAATCAGCCAATTTAGAC-3'; for Wisconsin line, 5'-CATTTTATAATAACGCTGCGGACATCTAC-3') and gene-specific primers (for *atprot1-1*, 5'-GGCAACAGTGAGGCAACCAGT-3' and 5'-CATAGCTTTGTCATAGCATTC-3'; for *atprot2-1*, 5'-GCAGTTGAACAATTCGATCTCGAAGTCCC-3' and 5'-GAAGCAAACATTGAGCCAATGCCATAGC-3'; for *atprot2-2*, 5'-GTGTGAAAGCTTAAAGTGTGAAGAAGT-3' and 5'-ACCCTAGTTTTTCGCTATTAGGTCAAGACT-3'; for *atprot2-3*, 5'-TAAACAGTGCCTATGTGTG-3' and 5'-AGATCGATGACACTGACCTGT-3'; for *atprot3-2*, 5'-ACAATAACCATTGGAGAGG-3' and 5'-GAAAAGTGTAGCGGC-3'). Lines were back-crossed twice with the wild-type and selfed to isolate homozygous lines. Double and triple knockout lines were generated by crossing homozygous single *atprot* mutants (Col-0 background).

Plants were transformed by *Agrobacterium tumefaciens* (GV3101 pMP90) mediated gene transfer using the floral dip method (Clough and Bent, 1998).

For axenic culture, surface-sterilized *Arabidopsis* seeds were vernalized at 4 °C in the dark for 2 d. Plants were cultivated for 10 d in square plates (12 cm) with 70 ml of AM medium per plate [ $2.2 \text{ g l}^{-1}$  MS salts (Murashige and Skoog, 1962), 1% sucrose]. The medium contained 0.05% MES (pH 5.7) and was solidified with 0.7% agar.

### DNA and RNA work

For overexpression in *Arabidopsis*, the *AtProT1*-cDNA and *AtProT2*-cDNA including 5'- and 3'-untranslated region sequences were isolated from pFL61 (Minet *et al.*, 1992; Rentsch *et al.*, 1996) and introduced into the *Sma*I and *Xba*I sites of pBinAR (Bevan, 1984).

Extraction of total RNA and RNA gel blot analysis were performed according to a phenol-SDS extraction method and a formaldehyde-formamide protocol, respectively (Ausubel *et al.*, 1994). The fragments used for detection were labelled with [ $\alpha$ - $^{32}$ P]dCTP using the Megaprime Kit (Amersham). For detection of *AtProT1* expression, a 464 bp sequence was amplified from the *AtProT1* cDNA using the primers 5'-GAGAATTCAGGCTCTAATGGTAAGAC-3' and 5'-CATATGAGTACATGGACA-CA-3'. *AtProT2* and *AtP5CS* expression was detected using the entire cDNAs for the labelling procedure. The fragment amplified from the *AtP5CSI*-cDNA might detect *AtP5CSI* and *AtP5CS2* mRNA due to high homology of the transcripts. For detection of *AtPDH* (proline dehydrogenase) a 1600 bp fragment of the *AtPDH1* cDNA was amplified using a gene-specific primer 5'-CCCAACCTCTGATCTCC-3' and a vector primer, possibly also detecting *AtPDH2* transcripts.

For expression analysis of *AtProT3*, reverse transcription was performed using the RETROscript Kit (Ambion) with 2  $\mu\text{g}$  of total RNA. The primers 5'-ACAATAACCATTGGAGAGG-3'

and 5'-AAATCCAATAAGAATAAATACG-3' were used to amplify a full-length transcript of *AtProT3* by reverse transcription-PCR (RT-PCR).

#### Amino acid analysis

The content of free proline in the leaf was determined as described by Bates *et al.* (1973). For HPLC analysis, free amino acids from mature and germinated pollen were extracted as described by Bielecki and Turner (1966) and analysed by ARC (Analytical Research and Services, University of Bern) according to Bidlingmeyer *et al.* (1984).

#### Preparation of *Arabidopsis* leaf epidermis

*Arabidopsis* plants were grown under long-day conditions for 3–4 weeks. The salt treatment was performed 24 h prior to the experiment by adding NaCl solution to the plants until a concentration of ~200 mM NaCl was reached in the pot. After the leaf margin had been removed using a razor blade, the leaf was placed between two pieces of double-sided adhesive tape attached to a strip of plastic wrap. Tissues were separated by detaching the pieces of tape and immediately frozen in liquid nitrogen. A sample was composed of 10 leaves harvested from 10 individual plants [resulting in fresh weights of 10–20 mg (epidermis) and 60–100 mg (rest of the leaf)].

#### [<sup>14</sup>C]Glycine betaine uptake

Sixteen seedlings per genotype (64 seedlings in total) were grown for 10 d in square plates (12 cm) containing 70 ml of AM medium (0.6% agar). Per plate, 16 agar plugs (7 mm) were removed with a cut plastic pipette. To reach a final concentration of 500 μM glycine betaine, each of the holes was filled with 150 μl of labelling solution {14.6 mM glycine betaine, 1.3 μM [<sup>14</sup>C]glycine betaine (2 TBq mol<sup>-1</sup>) in liquid AM}. After 24 h the radioactivity was equally distributed in the plate (not shown). At each time point (6, 24, and 48 h) 10 seedlings per genotype were removed and washed thoroughly at 4 °C with AM containing 500 μM glycine betaine. For scintillation counting, seedlings were solubilized overnight using 500 μl of SOLUENE 350 (Perkin Elmer®). After addition of 4 ml of scintillation cocktail ULTIMA GOLD™ XR (Perkin Elmer®) the samples were counted using a Beckman LS6500 scintillation counter.

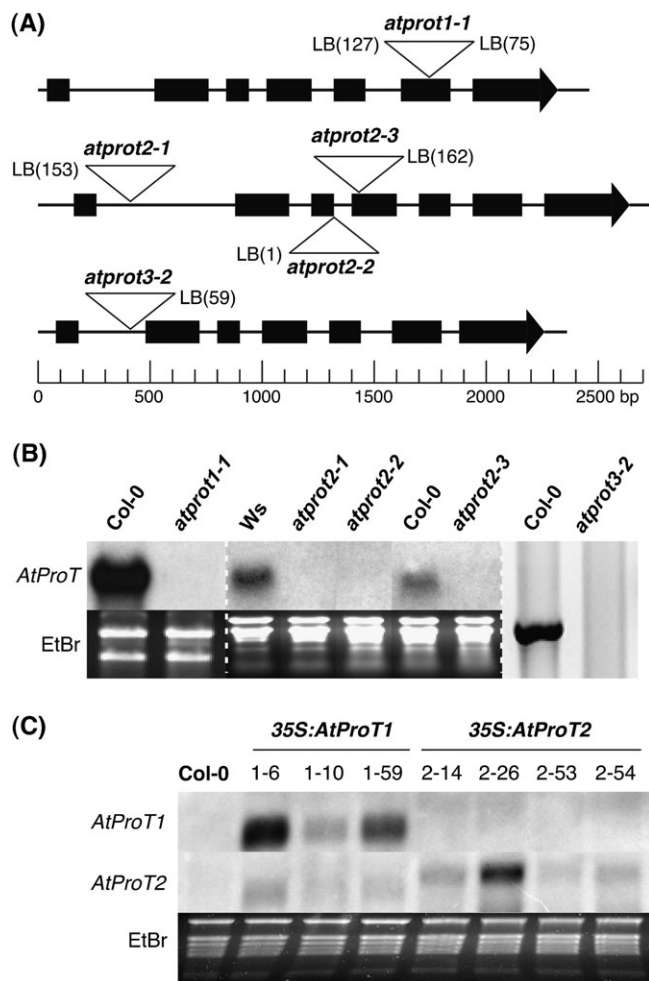
#### Pollen harvest and in vitro pollen germination

Mature pollen was harvested in bulk from several trays of flowering plants onto a nylon mesh (mesh size 15 μm), using a vacuum cleaner as described by Johnson-Brousseau and McCormick (2004). For germination, the pollen was transferred to an agar medium (pH 6.0) composed as described in Fan *et al.* (2001), but without myo-inositol. The pollen was transferred to the medium (5.5 cm plates) either by dipping the nylon mesh onto the agar surface or by placing the mesh on the medium (in the case of subsequent RNA extraction) and germinated in a humid chamber at 28 °C for 6 h. Due to this experimental set-up, the fresh weight of germinated pollen could not be determined. The extraction yielded similar amounts of amino acids per g pollen fresh weight from Col-0 and *atprot1-1* plants. The relative amount of an amino acid was determined as the mean of three samples, each extracted from several nylon meshes with pollen.

## Results

### Characterization of *atprot* T-DNA insertion and 35S:*AtProT*-overexpressing lines

An overview of the T-DNA insertion sites is given in Fig. 1A. *AtProT* transcript levels were determined in tissues



**Fig. 1.** Molecular characterization of *atprot* T-DNA insertion mutants and *AtProT*-overexpressing lines. (A) Schematic representation of the exon–intron structure of *AtProT1*, *AtProT2*, and *AtProT3* including the T-DNA integration sites. Genomic sequences were drawn to scale. Black boxes represent exons. The distance of the insertion site from the exon–intron border was verified by sequencing and is indicated as nucleotides in parentheses. (B) Expression of *AtProT* genes in several T-DNA insertion lines examined by northern blot (*AtProT1* and *AtProT2*) or RT-PCR (*AtProT3*). RNA was isolated from pollen (*AtProT1*), salt-stressed seedlings (*AtProT2*), or leaves (*AtProT3*). A dashed line separates individual gels. The lanes of the RNA gel blot analysis of *AtProT2* expression were regrouped for clarity. (C) RNA gel blot analysis of 35S:*AtProT1* and 35S:*AtProT2* lines (leaf tissue). Autoradiographic pictures in B and C have been adjusted in brightness levels.

where high expression of the respective gene had been shown (Rentsch *et al.*, 1996; Grallath *et al.*, 2005; see below). RNA gel blot analysis showed that in the *atprot1-1* mutant (Fig. 1B) and the three *atprot2* lines (*atprot2-1*, *atprot2-2*, and *atprot2-3*, Fig. 1B), the respective transcripts cannot be detected and thus the insertion lines can be considered as null mutants. In the *atprot3-2* mutant, no full-length transcript of *AtProT3* could be amplified by RT-PCR (Fig. 1B). A fragment of ~1000 bp downstream of the insertion site that contains an open reading frame was transcribed (data not shown), but expression of the

respective 3' end fragment did not complement the proline transport-deficient *Saccharomyces cerevisiae* mutant strain 22574d, suggesting that the truncated *AtProT3-2* transcript did not produce a functional protein in plants (data not shown; Jauniaux *et al.*, 1987). Double and triple insertion lines were generated using the lines *atprot1-1*, *atprot2-3*, and *atprot3-2*. Additionally, lines overexpressing *AtProT1* or *AtProT2* under control of the constitutive 35S promoter were generated. Two lines of each construct were selected for further analyses, one line showing moderately (*35S:AtProT1-59*, *35S:AtProT2-14*) and one line showing strongly (*35S:AtProT1-6*, *35S:AtProT2-26*) increased *AtProT* mRNA levels in the T<sub>3</sub> generation (Fig. 1C).

When grown in soil or axenically, none of these single, double, and triple knockout mutants and overexpression lines showed phenotypical differences (e.g. shoot size and development, root length, flowering time). Also, the proline content of shoots and roots of plants grown in axenic culture and of rosette leaves of plants grown in soil did not differ between wild-type, knockouts, and overexpressing lines (not shown).

#### *AtProT2* mediates uptake of glycine betaine from the rhizosphere

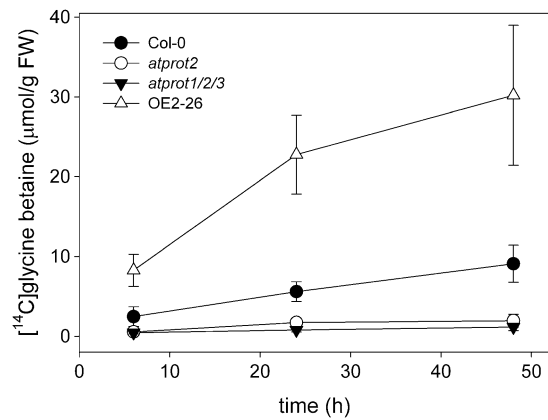
Heterologous expression of the *AtProTs* in *S. cerevisiae* demonstrated that the affinity for glycine betaine is higher than for L-proline (Grallath *et al.*, 2005). Glycine betaine does not seem to be metabolized in *Arabidopsis* and no transporter other than the *AtProTs* has been reported to mediate transport of glycine betaine. As *AtProT2* expression was primarily found in the cortex and epidermis of the roots (Grallath *et al.*, 2005), a role for *AtProT2* in the uptake of proline or glycine betaine into roots was suggested.

The uptake of [<sup>14</sup>C]glycine betaine in seedlings of wild-type plants, *atprot2-3* mutant, triple knockout (*atprot1-1 atprot2-3 atprot3-2*), and *35S:AtProT2-26* lines showed that uptake was time dependent and highest in the overexpressing line (Fig. 2). Wild-type seedlings accumulated more [<sup>14</sup>C]glycine betaine than the *atprot2* or the triple knockout mutant. No significant difference was observed between the latter. The overexpressing line accumulated three times as much [<sup>14</sup>C]glycine betaine as the wild type, which in turn imported 4.5 times the amount detected in *atprot2* knockout plants, indicating that *AtProT2* is the main uptake system for glycine betaine in roots.

#### *AtProT1* and *AtProT2* transport L-proline in planta

Some D-amino acids such as D-serine or D-alanine have been shown to be detrimental for plant growth and development (Erikson *et al.*, 2004). Transport studies using heterologous expression systems demonstrated that *AtProT2* transports D- and L-proline at similar rates (Breitkreuz *et al.*, 1999; CG and DR, unpublished).

It was found that D-proline inhibited *Arabidopsis* root growth in a dose-dependent manner and differences in D-proline transport between wild-type and mutant plants



**Fig. 2.** Uptake of [<sup>14</sup>C]glycine betaine. Time-dependent uptake of 500 µM [<sup>14</sup>C]glycine betaine in Col-0, *atprot2-3*, triple knockout (*atprot1-1 atprot2-3 atprot3-2*), and *35S:AtProT2-26* (OE) seedlings. Values represent the mean of 10 separately measured seedlings ±SD. Comparable results were obtained in several biologically independent experiments. The differences between the wild type and all three mutant lines are statistically significant (one-way ANOVA, Scheffé test).

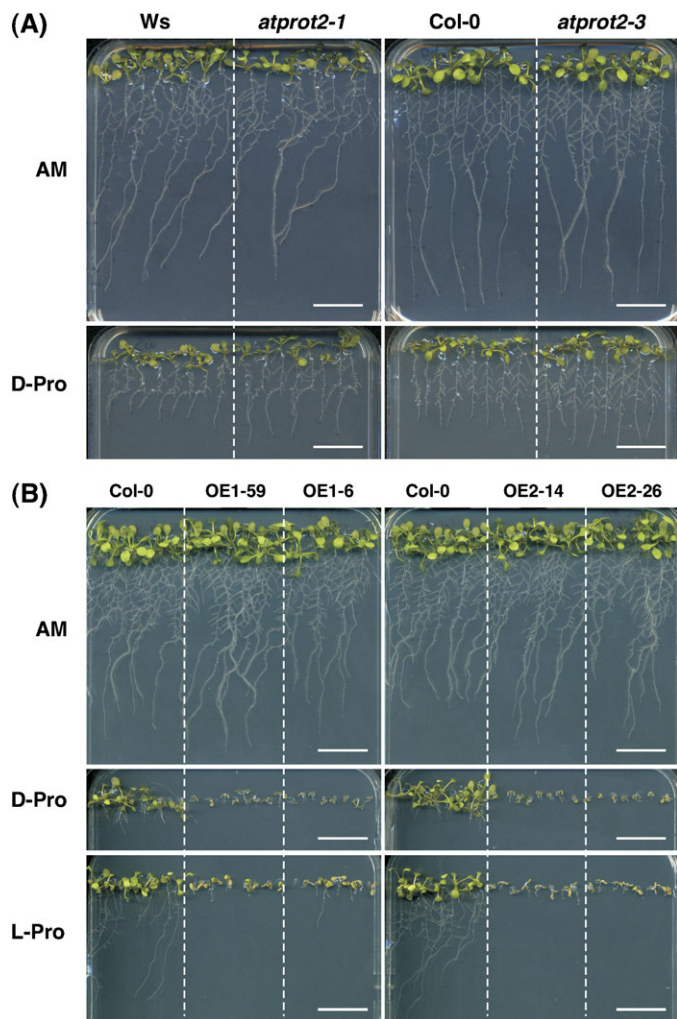
were assessed. Two independent *atprot2* mutants and the respective wild-type plants were germinated on AM medium and transferred to AM medium supplemented with 8 mM D-proline. D-Proline inhibited root growth of *atprot2* seedlings to a lesser extent than growth of wild-type roots (Fig. 3A), indicating that the loss of *AtProT2* activity decreased the net uptake of D-proline. Growth of the triple knockout line resembled that of the *atprot2* mutant (not shown).

Similarly, plants overexpressing *AtProT1* or *AtProT2* were grown on AM medium supplemented with 4 mM D-proline and compared with the wild type (Fig. 3B). All genotypes showed a strong phenotypic response to D-proline. Shoot growth and root elongation were reduced in wild-type plants, while development of all tested *35S:AtProT* lines was arrested shortly after unfolding of cotyledons (Fig. 3B).

Previous studies have demonstrated that high concentrations of exogenous L-proline also impair *Arabidopsis* growth (Hellmann *et al.*, 2000; Mani *et al.*, 2002). When plants were grown on AM medium containing 50 mM L-proline, shoot and root development was moderately inhibited in wild-type seedlings, whereas chlorosis and early cessation of growth was observed in *35S:AtProT* plants (Fig. 3B). Taken together these results demonstrate that *AtProT1* and *AtProT2* mediate proline transport *in planta*.

#### High proline level in *Arabidopsis* leaf epidermis

Zúñiga *et al.* (1989) demonstrated that in barley seedlings, proline distributes unevenly between leaf epidermis and mesophyll in water-stressed but not in control plants, whereas the concentration of glycine betaine is elevated in the epidermis of both stressed and unstressed barley leaves. As *AtProT3* is expressed in the lower epidermis but not in the mesophyll of leaves (Grallath *et al.*, 2005), proline

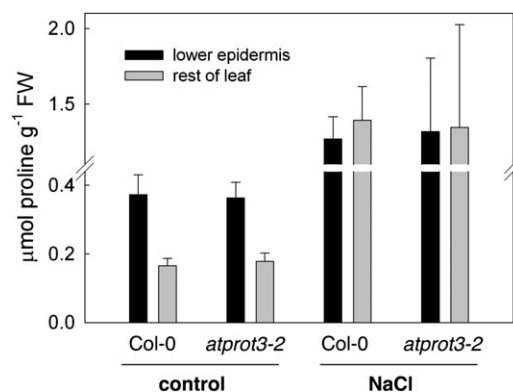


**Fig. 3.** Growth on D-proline and high concentrations of D-proline. (A) *atprot2-1* and *atprot2-3* seedlings and the respective wild-type plants were germinated on AM medium and transferred to AM or medium supplemented with 8 mM D-proline for 5 d. Scale bar 2 cm. (B) Growth of Col-0 and 35S:*AtProT* (OE) seedlings was compared 10 d after germination on AM medium, AM supplemented with 4 mM D-proline and AM containing 50 mM L-proline. Scale bar=2 cm. (This figure is available in colour at *JXB* online.)

distribution between the lower epidermis and the remaining leaf tissues was determined under control and under salt stress conditions, but found to be comparable in the wild type and the *atprot3-2* mutant (Fig. 4). Interestingly, the concentration of proline in the lower epidermis was 1.5–2 times higher than the average concentration in the remaining leaf tissues (Fig. 4). Upon salt treatment, proline concentrations increased in particular in the remaining leaf tissues, so that the difference in concentrations between the lower epidermis and remaining leaf tissues was no longer observed (Fig. 4).

#### Altered glutamate and arginine levels in germinating pollen of the *atprot1* mutant

Pollen has repeatedly been reported to contain a high amount of free proline (Krogaard and Andersen, 1983;

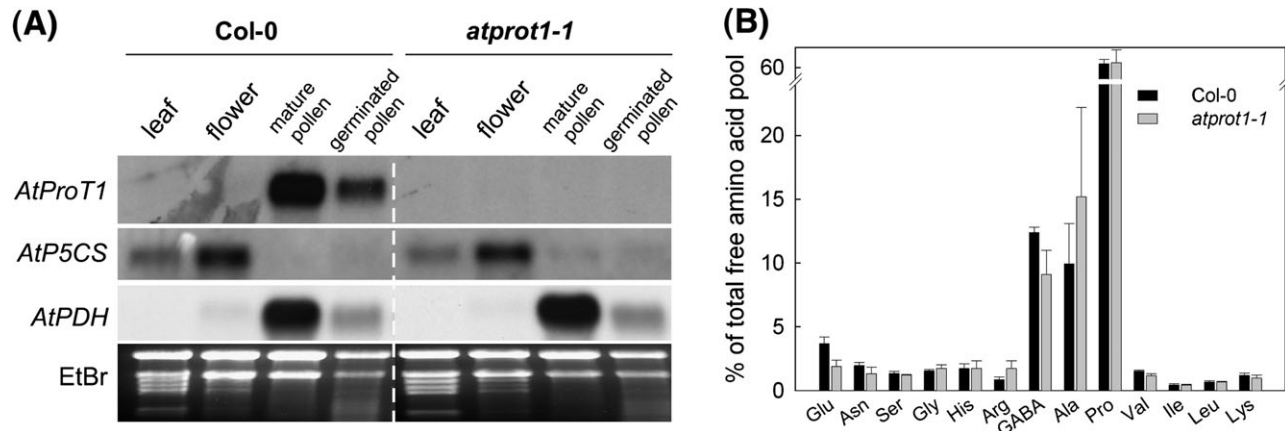


**Fig. 4.** Proline distribution within the leaf. The proline content in the lower epidermis and in the rest of the leaf was determined in wild-type and *atprot3-2* plants by ninhydrin assay. Plants were grown on soil for 3–4 weeks and watered normally or treated with 200 mM NaCl for 24 h prior to the analysis. Values represent the mean of six samples  $\pm$ SD.

Mondal *et al.*, 1998; Schwacke *et al.*, 1999). Consistently, proline was found to be the most abundant amino acid in mature and germinated *Arabidopsis* pollen, accounting for 60–65% of the free amino acid pool (Supplementary Fig. S1 available at *JXB* online; Fig. 5B). Previously, expression of *AtProT1* had been demonstrated in roots, leaves, and flowers (Rentsch *et al.*, 1996). The present analysis shows that the *AtProT1* transcript level is particularly high in pollen compared with leaf and flower tissue (Fig. 5A).

Of the mRNA species present in mature pollen, many appear to be translated into protein only upon the onset of pollen germination (Mascarenhas, 1993). Two independent pollen transcriptome analyses detected an increase in *AtProT1* transcript abundance during late stages of pollen maturation, suggesting that *AtProT1* functions in post-pollination processes (Honys and Twell, 2004; Bock *et al.*, 2006). Based on these observations, expression of *AtProT1* was compared between mature and *in vitro* germinated pollen and found to decrease during pollen germination (Fig. 5A). Mature pollen of Col-0 and *atprot1-1* plants does not differ in amino acid composition and content (Supplementary Fig. S1 at *JXB* online). In contrast, germinating pollen of *atprot1-1* plants displayed minor differences regarding the free amino acids—that is, a lower and higher percentage of glutamate and arginine, respectively (Fig. 5B). The high level of GABA in germinating pollen of both genotypes might result from stress during *in vitro* germination and subsequent harvest (Shelp *et al.*, 1999). Despite the altered concentrations of glutamate and arginine in *atprot1-1* mutant pollen, *in vitro* germination assays and an *in vivo* transmission analysis did not reveal differences in germination rate or a significantly biased distribution of the *AtProT1* alleles in the offspring (not shown).

Furthermore, transcript levels of *AtP5CS* ( $\Delta^1$ -pyrroline-5-carboxylate synthetase), the enzyme catalysing the first step in proline biosynthesis, and of *AtPDH* (proline dehydrogenase), the enzyme catalysing the oxidation of proline to pyrroline-5-carboxylate (P5C), were analysed by



**Fig. 5.** Molecular and biochemical analysis of the *atprot1-1* mutant. (A) Expression of *AtProT1*, *AtP5CS*, and *AtPDH* in different organs of Col-0 and *atprot1-1* plants examined by RNA gel blot analysis. The dashed line separates the half of the gel loaded with wild-type RNA from that loaded with RNA from *atprot1-1* plants, which was mirrored for clarity. The autoradiographic pictures have been adjusted in brightness levels. (B) Free amino acid composition in germinated pollen of Col-0 and *atprot1-1* plants. The relative amount of an amino acid is given as the mean of three composite samples  $\pm$ SD.

RNA gel blot in the *atprot1-1* knockout (Fig. 5A). *AtP5CS* transcripts were abundant in leaf and flower tissue but could hardly be detected in pollen, in both Col-0 and *atprot1-1* plants. In contrast, expression of *AtPDH* was high in pollen and decreased during pollen germination (Fig. 5A); however, lower *AtPDH* mRNA levels might also be caused by repression of *PDH* expression by sucrose present in the germination medium (Hanson *et al.*, 2008). Similar results were obtained from several independent batches of plants, suggesting that there are no major differences in expression of *AtP5CS* and *AtPDH* between wild-type and *atprot1-1* plants.

## Discussion

### *AtProTs* mediate proline and glycine betaine transport in planta

Evidence is provided that *in planta*, *AtProTs* transport D- and L-proline or glycine betaine, which were previously identified as substrates of *AtProTs* using heterologous expression systems (Breitkreuz *et al.*, 1999; Grallath *et al.*, 2005). First, the contribution of *AtProT1* and *AtProT2* to proline transport *in planta* is demonstrated by treatment with toxic concentrations of L-proline as well as D-proline, which are the cause of severe growth defects in *35S:AtProT* plants. Secondly, after 6, 24, and 48 h seedlings of *AtProT2*-overexpressing line accumulated more [ $^{14}$ C]glycine betaine than the wild type, which in turn contained more radiolabelled substrate than the *atprot2* and the triple knockout plants. As the *atprot2* mutant accumulates <25% of the [ $^{14}$ C]glycine betaine imported by the wild type, *AtProT2* seems to be the main glycine betaine uptake system in roots. *AtProT2* expression is found in the epidermis and cortex of roots (Grallath *et al.*, 2005), tissues which are involved in the uptake and radial transport of substances from the root medium. Because *AtProT2* expression is induced under salt

stress (Rentsch *et al.*, 1996), the acquisition of compatible solutes from the rhizosphere might improve growth of *Arabidopsis* under water stress, as observed for seedlings of acacia (Räsänen *et al.*, 2004), and is a common strategy found among bacteria (Sleator and Hill, 2002).

### The proline distribution within the leaf changes under salt stress

The differential distribution of solutes between leaf tissues such as epidermis, mesophyll, and vascular bundles has been demonstrated in several plant species (Fricke *et al.*, 1994; Karley *et al.*, 2000). Here, it was shown that in *Arabidopsis*, the proline level in the lower leaf epidermis is higher than in the rest of the leaf (Fig. 4). Compared with mesophyll cells, the water status of cells in the lower epidermis is constantly challenged by stomatal and cuticular transpiration. Furthermore, a high vacuolar osmotic pressure in epidermal cells requires a high solute content in the cytosol, thus the concentration of compatible solutes is expected to be elevated.

Under salt stress, this differential accumulation was no longer observed, suggesting that the stress-related increase of proline is stronger in the mesophyll or in the vasculature than in the epidermis. In potato mesophyll cells, the cellular increase in proline in response to osmotic stress is primarily due to accumulation of proline in the chloroplast stroma (Büssis and Heineke, 1998). An *AtP5CS1*-green fluorescent protein (GFP) fusion protein has been reported to re-localize from the cytosol into the chloroplasts when *Arabidopsis* mesophyll cells are subjected to hyperosmotic stress (Székely *et al.*, 2008). Therefore, less accumulation of proline in the epidermis under stress may also reflect the general scarcity of chloroplasts in this tissue. However, the highest increase in proline in water-stressed barley leaves was detected in the vasculature and the epidermis, whereas the proline level in mesophyll protoplasts remained rather constant (Zúñiga *et al.*, 1989), demonstrating species-

dependent differences in the distribution of compatible solutes under stress. To understand the physiological relevance of this phenomenon, it would be important to dissect the contribution of proline synthesis and transport, for instance by means of plants further impaired in the expression of *AtP5CS* or *AtLHT1*, an amino acid and proline transporter that is also expressed in the epidermis of *Arabidopsis* (Hirner *et al.*, 2006).

#### *Loss of AtProT1 activity changes amino acid composition in germinating but not in mature pollen*

The accumulation of proline in *Arabidopsis* pollen argues for a role in stabilizing cellular structures during dehydration, high and low temperatures, or as a metabolic precursor and source of energy, as was proposed for other plants (Stanley and Linskens, 1974; Zhang and Croes, 1983; Muters *et al.*, 1989; Lansac *et al.*, 1996; Schwacke *et al.*, 1999). Székely *et al.* (2008) detected AtP5CS–GFP in *Arabidopsis* pollen, suggesting that biosynthesis contributes to proline accumulation. However, the low abundance of *P5CS* transcripts in pollen of *Arabidopsis* and tomato observed in other studies supports the idea that proline accumulation is attributed to import processes, though post-transcriptional regulation cannot be excluded (Fig. 5A; Fujita *et al.*, 1998; Schwacke *et al.*, 1999). Accumulation of proline which is independent of changes of *P5CS* expression has also been reported for tissues other than pollen—that is, in developing grapevine berries—again pointing to the contribution of proline transport or regulation at the post-transcriptional level (Stines *et al.*, 1999).

The present data show that *AtP5CS* transcripts are present in flowers, but absent from pollen (Fig. 5A), suggesting that the proline generated in other parts of the flower may be imported into pollen. As pollen is symplasmically isolated, membrane transport is essential in sustaining its development during maturation and germination. It cannot be predicted whether transcripts that accumulate late in pollen development, such as *AtProT1* mRNA, are translated in mature pollen, germinating pollen, or both (Mascarenhas, 1990). Likewise, the concomitant accumulation of *AtPDH* mRNA and high amounts of proline in mature pollen (Fig. 5A, Supplementary Fig. S1 at *JXB* online) suggests that *AtPDH* transcript abundance does not reflect PDH activity. Proline degradation has been detected in germinating pollen of *Petunia* (Zhang and Croes, 1983); therefore, translation of PDH might only start with the onset of pollen germination.

Germinated wild-type pollen differs from germinating *atproT1* pollen particularly in those amino acids that are closely linked with proline metabolism (glutamate and arginine). Glutamate is the metabolic precursor of GABA and proline; it also represents the end-product of the degradation of proline and arginine. Thus it appears that during pollen germination the transport activity of AtProT1 affects amino acids other than proline, an effect that needs to be further investigated.

#### *How do AtProTs contribute to proline transport in the plant?*

None of the *atproT* T-DNA insertion lines (single, double, or triple knockouts) and overexpression lines analysed revealed differences in proline content, though the present results do not exclude changes at the subcellular or tissue level. Either transport of proline through AtProTs is not involved in the regulation of proline levels *in planta*, or plants can compensate for the lack or increase of AtProT activity—that is, other amino acid transporters and/or the regulation of proline metabolism might compensate for alterations in proline distribution. The findings of Ueda *et al.* (2008) suggest that metabolism responds to changes in proline transport. Overexpression of *HvProT* in *Arabidopsis* led to an increase in *AtPDH* expression and activity, parallel to a decrease in proline content of leaves (Ueda *et al.*, 2008). The authors conjectured that enhanced accumulation of proline in leaves of *35S:HvProT* plants might induce elevated *AtPDH* activity. Though altered expression of proline-metabolizing enzymes was not observed in the present mutant lines, altered proline transport may feed back directly on the metabolic pathway or alter flow through the pathway, keeping proline levels unchanged.

A functional overlap between AtProTs and transporters from other families is probably one of the main reasons why *atproT* mutants do not show a strong phenotype. The amino acid permease AtAAP2, expressed in major veins of leaves and stems (Hirner *et al.*, 1998), or AtAAP3 in the root phloem (Okumoto *et al.*, 2004) might counterbalance the altered AtProT1 activity in phloem tissue. In pollen, the lack of AtProT1 may be compensated by carriers with comparable expression patterns, for example by AtLHT5 or AtLHT7 (Foster *et al.*, 2008; Hruz *et al.*, 2008). Similarly, loss of AtProT2 function could be mitigated by the activity of AtLHT1 or AtAAP1, which mediate amino acid uptake into the root epidermis (Hirner *et al.*, 2006; Lee *et al.*, 2007; Svennerstam *et al.*, 2007). Interestingly, AtLHT1 expression was also described in the leaf epidermis (Hirner *et al.*, 2006), suggesting partial functional redundancy with AtProT3. To improve our understanding of their physiological significance, future analyses could benefit from studying plants impaired in the function of multiple transporters that show overlapping expression patterns and substrate selectivity.

## Supplementary data

Supplementary data are available at *JXB* online.

**Figure S1** Free amino acid composition of mature Col-0 and *atproT1-1* pollen. The relative amount of an amino acid is given as the mean of four composite samples  $\pm$ SD. Per gram pollen fresh weight, the extraction yielded between 0.75 mmol and 0.95 mmol amino acids.

## Acknowledgements

This work was supported by grants from the Swiss National Science Foundation SNF (3100-064918 and SNF 3100A0-

107507). We wish to thank Elisabeth Kuslys and Marianne Suter Grottemeyer for their technical assistance, Rebecca Alder and Christopher Ball for taking care of the plants, and Willi Tanner for designing the pollen harvesting system. We thank Mirco Hecht for testing the activity of the truncated AtProT3 protein. We are also grateful to Mechthild Tegeder (Washington State University, Pullman) for helpful comments on the manuscript.

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