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REVIEW ARTICLE

Proline metabolism and transport in plant development

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Abstract Proline fulfils diverse functions in plants. As amino acid it is a structural component of proteins, but it also plays a role as compatible solute under environmental stress conditions. Proline metabolism involves several subcellular compartments and contributes to the redox balance of the cell. Proline synthesis has been associated with tissues undergoing rapid cell divisions, such as shoot apical meristems, and appears to be involved in floral transition and embryo development. High levels of proline can be found in pollen and seeds, where it serves as compatible solute, protecting cellular structures during dehydration. The proline concentrations of cells, tissues and plant organs are regulated by the interplay of biosynthesis, degradation and intra- as well as intercellular transport processes. Among the proline transport proteins characterized so far, both general amino acid permeases and selective compatible solute transporters were identified, reflecting the versatile role of proline under stress and non-stress situations. The review summarizes our current knowledge on proline metabolism and transport in view of plant development, discussing regulatory aspects such as the influence of metabolites and hormones. Additional information from animals, fungi and bacteria is included, showing similarities and differences to proline metabolism and transport in plants.

Keywords Proline · Plant · Metabolism · Transport · Regulation · Development

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Introduction

The amino acid proline contains a secondary amino group and is thus unique among the proteinogenic amino acids. Proline plays a crucial role for cellular metabolism both as a component of proteins and as free amino acid. Due to its cyclic structure, proline has a restricted conformational flexibility, which determines the arrangement of the peptide chain around it, and as a consequence leads to stabilization or destabilization of secondary structures of protein conformation. In addition to its role in primary metabolism as a component of proteins, the free amino acid proline is one of the most widely distributed compatible solutes that accumulate in plants and bacteria during adverse environmental conditions such as drought, high salinity or low temperatures (Bohnert et al. 1995; Sleator and Hill 2002). The role of proline as compatible solute and the importance of proline metabolism under stress conditions in plants have been a field of intense research and the current state of knowledge including novel findings and open questions is covered by several recent and excellent reviews (Kavi Kishor et al. 2005; Verbruggen and Hermans 2008; Szabados and Savouré 2010). Although these stress-related issues cannot be ignored, the present review will focus primarily on the

role of proline during plant development as well as on regulation of proline metabolism and transport.

Proline biosynthesis

The anabolic branch of proline metabolism was first described in bacteria, where proline is synthesized from glutamate in three steps. Glutamate is phosphorylated by γ-glutamyl kinase using ATP and the resulting glutamyl-γ-phosphate is reduced to glutamic-γ-semialdehyde (GSA) by glutamic-γ-semialdehyde dehydrogenase. The reversible cyclization of GSA to Δ^1-pyrroline-5-carboxylate (P5C) is spontaneous and followed by the reduction of P5C to proline, catalysed by Δ^1-pyrroline-5-carboxylate reductase (P5CR) using NADPH (Csonka 1989). Proline biosynthesis in other kingdoms follows the same pathway, albeit differences exist. The γ-glutamyl kinase and γ-glutamyl phosphate reductase enzymes are conserved in bacteria (*ProB*, *ProA*) and yeast (*PRO1*, *PRO2*), whereas in plants and animals both activities are combined in a bifunctional enzyme called Δ^1-pyrroline-5-carboxylate synthetase (P5CS) that requires ATP as well as NADPH (Fig. 1; Tomenchok and Brandriss 1987; Csonka 1989; Hu et al. 1992; Aral et al. 1996). Activities of γ-glutamyl kinase in bacteria and yeast and of P5CS in plants represent the rate-limiting step of proline biosynthesis and are regulated through allosteric inhibition by the endproduct proline (Csonka et al. 1988; Hu et al. 1992; Sekine et al. 2007). One *P5CS* gene has been identified in man and mouse, which, due to alternative splicing, encodes two P5CS isoforms that differ in sensitivity to ornithine inhibition (Aral et al. 1996; Hu et al. 1999). Mutations in the human *P5CS* gene lead to metabolic abnormalities resulting in multiple disorders such as neurodegeneration, cataracts and connective tissue disorder, suggesting that P5CS is essential for proline and/or arginine supply (Baumgartner et al. 2005; Bicknell et al. 2008; Hu et al. 2008a). Plant genomes usually contain two homologous genes that encode P5CS proteins, e.g. Arabidopsis, *Medicago sativa*, *Medicago truncatula* and *Oryza sativa* (Strizhov et al. 1997; Ginzberg et al. 1998; Hien et al. 2003; Armengaud et al. 2004). The expression patterns indicate that *P5CS* paralogs have different functions during plant life and development, although the specific roles differ among plant species (see Kavi Kishor et al. 2005 for review). Analysis of Arabidopsis *p5cs1* and *p5cs2* knockout mutants confirmed the non-redundant function of the isoforms for plant development and stress tolerance (Székely et al. 2008; see below). Recent phylogenetic analysis suggested that *P5CS* genes underwent duplication several times independently after monocot and dicot divergence, allowing for the observed functional specialization (Turchetto-Zolet et al. 2009). The sequence identity of plant P5CS proteins ranges between 65 and 98% in different species (Szabados, unpublished).

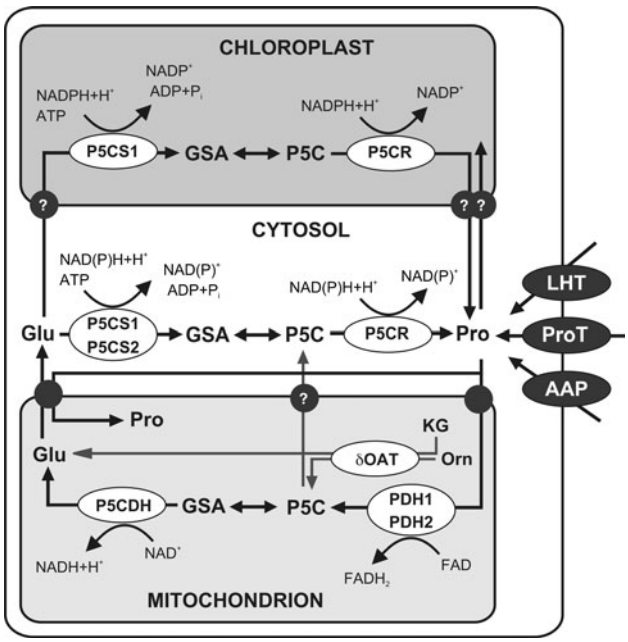

Fig. 1 Model of proline metabolism and transport in Arabidopsis cells. Substrates are shown in *black*: *Pro* proline, *Glu* glutamate, *Orn* ornithine, *P5C* pyrroline-5-carboxylate, *GSA* glutamic-γ-semialdehyde, *KG* α-ketoglutarate. Enzymes are *encircled*: *P5CS* P5C synthetase, *P5CR* P5C reductase, *PDH* proline dehydrogenase, *P5CDH* P5C dehydrogenase, *δOAT* ornithine-δaminotransferase. Transporters are shown as *dark grey circles*: *AAP* amino acid permease, *ProT* proline transporter, *LHT* lysine-histidine transporter, transporters that have only been described physiologically are shown as *circles* without label; proposed transporters are indicated by a *question mark*

For the second enzyme of proline biosynthesis, P5CR, two isoforms were biochemically identified in pea and spinach, although it is currently unclear, whether the two isoforms originate from one or two genes (Williamson and Slocum 1992; Murahama et al. 2001). In the Arabidopsis genome, P5CR is encoded by a single gene, though alternative splicing might generate two different proteins (Verbruggen et al. 1993; Hare and Cress 1996; http://fgcz-atproteome.unizh.ch; http://www.arabidopsis.org). The human genome contains two P5CR and one P5CR-like gene and although localization in the cytosol and/or loose association with the cytosolic side of the outer mitochondrial membrane was described, for one P5CR isoform co-localization with mitochondrial P5CS was recently shown (Dougherty et al. 1992; Hu et al. 2008b; Reversade et al. 2009). In plants, P5CR activity has been detected in the cytosol and in plastids of several plant species, and also the P5CS isoforms differ in their distribution between these two compartments, depending on environmental conditions (see Szabados and Savouré 2010 for review).

In an alternative pathway of proline synthesis, the activity of ornithine-δ-aminotransferase (δOAT) converts ornithine

and α-ketoglutarate to P5C and glutamate by transamination (see Stránská et al. 2008 for review). Arabidopsis mutants lacking δOAT activity were unable to mobilize nitrogen from arginine or ornithine but showed normal proline accumulation, supporting a role for δOAT mainly in arginine degradation (Funck et al. 2008). The localization of δOAT in mitochondria probably generates a barrier to direct utilization of δOAT-generated P5C by P5CR, which is localized in the cytosol or in plastids (Funck et al. 2008). In intact plant mitochondria fed with ornithine very little release of P5C was detected, supporting the concept that glutamate is an obligate intermediate in the conversion of ornithine to proline in plants (Elthon and Stewart 1982). The situation seems to be different in mammalian cells, where δOAT, P5CS and at least one P5CR isoform might co-localize in mitochondria (Aral and Kamoun 1997; Hu et al. 2008b; Reversade et al. 2009). In humans, δOAT deficiency is associated with high ornithine levels in blood and urine (Ramesh et al. 1991). Patients suffer from gyrate atrophy, a degenerative disease of the choroid and retina of the eye that leads to blindness (Mitsubuchi et al. 2008).

Degradation of proline

Proline degradation in eukaryotes takes place in mitochondria and thus in plants is spatially separated from the biosynthetic pathway. Proline catabolism starts with the oxidation of proline to P5C by proline dehydrogenase (PDH), using FAD as cofactor. P5C is subsequently converted to glutamate by pyrroline-5-carboxylate dehydrogenase (P5CDH) using NAD^+ (Fig. 1). Whereas in eukaryotes two enzymes catalyse these subsequent steps in proline degradation, in bacteria both mono- and bifunctional enzymes exist (Tanner 2008). Two homologous genes have been identified to encode proline dehydrogenases in Arabidopsis and tobacco (Mani et al. 2002; Ribarits et al. 2007; Verbruggen and Hermans 2008; Funck, unpublished), while data from other plant species are scarce. In contrast, the enzyme catalysing the second step of proline degradation (P5CDH) is encoded by a single copy gene in all monocot and dicot species analysed so far (Deuschle et al. 2001; Ayliffe et al. 2005; Mitchell et al. 2006). Biochemical analysis revealed the presence of two P5CDH activities with slightly different characteristics in *Nicotiana plumbaginifolia* and *Zea mays* (Elthon and Stewart 1982; Forlani et al. 1997). At present it is not clear if both activities arise from a single gene or if a second *P5CDH* gene is present in these species.

Proline toxicity

Despite the essential function of proline in primary metabolism and the accumulation of large amounts of proline in stressed plant tissues, exogenous supply of proline to non-stressed plants causes toxic effects (Bonner et al. 1996; Hellmann et al. 2000; Hare et al. 2002). Symptoms of proline intoxication included alteration in chloroplast and mitochondrial ultrastructure as well as several features of programmed cell death (Hare et al. 2002; Deuschle et al. 2004). In Arabidopsis mutants deficient in PDH1 or P5CDH, proline supply inhibited de-etiolation and caused an early arrest of seedling development (Nanjo et al. 2003; Deuschle et al. 2004; Miller et al. 2009; see Verbruggen and Hermans 2008 for review).

In human cell cultures, both PDH and P5CDH are direct targets of the central apoptotic integrator p53, whereby PDH has pro-apoptotic activity, while P5CDH counteracts induction of apoptosis (Donald et al. 2001; Yoon et al. 2004; Phang et al. 2008). Induction of apoptosis was attributed to ROS formation by misdirected electrons from PDH and rapid cycling of proline and P5C between PDH and P5CR in mitochondria. A similar cycle was recently proposed for plants, based on the observation that overexpression of *PDH* or deficiency in *P5CDH* caused mitochondrial ROS formation (Miller et al. 2009). It remains to be investigated whether the intact mitochondrial membrane constitutes an efficient barrier which prevents cycling of proline and P5C in plants, possibly controlled by a to date hypothetical P5C transporter. Further information on the role of proline metabolism as a redox shuttle between different compartments is covered in a recent review by Szabados and Savouré (2010).

Proline in plant development

The level of free proline varies considerably in different plant organs and is usually higher in reproductive organs than in vegetative tissues (Chiang and Dandekar 1995; Fujita et al. 1998; Armengaud et al. 2004). High concentrations of proline have been reported in plant organs during endogenously controlled dehydration, e.g. in seeds or pollen (see below). Furthermore, a high leaf-to-root ratio for proline has been found in several plant species including Arabidopsis, lentil and common bean (Hua et al. 1997; Sánchez et al. 2001; Misra and Saxena 2009). The amount of free proline in the leaf decreases during leaf maturation in *Vicia faba* and *Brassica juncea* (Venekamp and Koot 1984; Madan et al. 1994). Within the leaf blades of *Chrysanthemum indicum*, proline shows an uneven distribution pattern (Amberger-Ochsenbauer and Obendorfer 1988) as was also detected between lower epidermis and the rest of the leaf in Arabidopsis (Lehmann and Rentsch, unpublished). In barley, the preferential accumulation of proline in epidermis and vascular bundles was only observed under stress conditions (Zúñiga et al. 1989). The

biological significance of these differences has not been investigated so far.

Being a precursor for hydroxyproline-rich proteins, proline is an essential cell wall component. The antisense inhibition of Arabidopsis *AtP5CS1* led to malformation of leaf parenchyma, epidermal and vascular tissues accompanied by a decrease in proline and hydroxyproline content of the cell wall (Nanjo et al. 1999). This phenotype was not observed for *atp5cs1* knockout mutants, suggesting that it might partially be attributed to impaired expression of *AtP5CS2* due to high sequence similarity of the two *P5CS* genes (Székely et al. 2008). On the other hand, an increased proline synthesis was shown to affect leaf size and tissue formation as well, suggesting that proline homeostasis has to be tightly controlled (Mauro et al. 1996; Trovato et al. 2001; Mattioli et al. 2008). Interestingly, the expression of *35S:AtP5CS1* resulted in a decrease of *AtP5CS1* transcript level at later stages of plant development, leading to a reduction of leaf proline content and stunted growth similar to the phenotype of *AtP5CS1* antisense lines (Mattioli et al. 2008). High expression of *AtP5CS2* in leaf primordia and callus cells indicates that rapidly dividing and growing cells have a high demand for proline (Strizhov et al. 1997; Székely et al. 2008).

Proline during flower development

First indications for a role of proline in flower formation came from studies using transgenic plants with enhanced or repressed *P5CS* expression. Kavi Kishor et al. (1995) reported that tobacco plants constitutively expressing moth bean *VaP5CS* developed more flowers under high salinity conditions than the wildtype, although this difference might be attributed to the protective effects of proline as a compatible solute. Furthermore, Arabidopsis plants carrying an *AtP5CS1* antisense construct displayed an impaired elongation of the inflorescence internodes and reduced fertility in the absence of stress (Nanjo et al. 1999). Externally supplied L- but not D-proline could suppress the mutant phenotype (Nanjo et al. 1999). In axenic cultures of moth bean, the exogenous application of proline caused changes of flowering time, the length of the flowering period and the number of flowers (Saxena et al. 2008).

A stimulating effect on floral transition was also observed in tobacco plants, where expression of *RolD*, a bacterial enzyme catalyzing the direct conversion of ornithine to proline, resulted in early bolting, precocious flowering and the enhanced formation of axillary flower buds (Mauro et al. 1996; Trovato et al. 2001). The P5CS-dependent proline synthesis from glutamate was associated with flower development in several studies. In Arabidopsis, expression of AtP5CS-GFP fusion proteins under the control of their native promoters revealed that in flowers AtP5CS1-GFP was present exclusively in anthers, whereas AtP5CS2-GFP was found in inflorescence meristems, flower primordia and flower buds (Székely et al. 2008). However, in situ mRNA hybridization analysis detected indistinguishable expression patterns for both *P5CS* genes in all tissues tested, including vegetative and floral shoot apical meristems as well as axillary meristems (Mattioli et al. 2009b). Arabidopsis *AtP5CS2* is an early target gene of the transcription factor CONSTANS that promotes flowering in response to day length (Samach et al. 2000). Moreover, the overexpression of *AtP5CS1* caused an early flowering phenotype, further supporting the hypothesis that the proline level is implicated in floral transition (Mattioli et al. 2008). Since an *atp5cs1* T-DNA insertion mutant has a late flowering phenotype that was enhanced when *atp5cs1* plants were heterozygous for an *atp5cs2* insertion, both *P5CS* genes appear to be involved in flowering control (Mattioli et al. 2009a, b).

The analysis of numerous plant species revealed that proline is one of the most abundant amino acids in angiosperm pollen, while it is less abundant in gymnosperm pollen (Khoo and Stinson 1957; Krogaard and Andersen 1983; Lansac et al. 1996; Nepi et al. 2009). It was suggested that proline might act as a compatible solute in the dehydrated pollen grain or function as a source of energy and as metabolic precursor during pollen germination (Zhang and Croes 1983; Mutters et al. 1989; Schwacke et al. 1999). Interestingly, the high proline content of tomato pollen does not correlate with the relatively low abundance of *P5CS* transcripts (Fujita et al. 1998). In contrast, Arabidopsis P5CS1-GFP was detected in the pollen grains, indicating that there might be species-related differences (Székely et al. 2008). In tobacco, proline is the most abundant amino acid in mature anthers (Horner and Pratt 1979). Pollen of Arabidopsis and tobacco contains high amounts of proline despite the elevated expression level of *PDH*, indicating that *PDH* transcripts might accumulate without immediate translation into protein, as appears to be the case for numerous mRNA species present in pollen (Mascarenhas 1993; Nakashima et al. 1998; Ribarits et al. 2007; Lehmann and Rentsch, unpublished).

Pollen is symplasmically isolated and therefore dependent on membrane transport to sustain its development. Transcriptome analysis suggested that transporter genes become overrepresented as the pollen matures and identified substantial expression of the proline transporter *AtProT1* in mature pollen grains (Honys and Twell 2004; Bock et al. 2006). Since the amino acid level in mature pollen of *atprot1* knockout plants is unchanged, it is at present not clear to which extent import and endogenous biosynthesis contribute to proline accumulation in Arabidopsis pollen (Lehmann and Rentsch, unpublished).

Proline is also found in foliar, extrafloral and floral nectar where it is suggested to play a role in taste, pollinator attraction, egg laying in honeybees, insect feeding behaviour and serves as energy source for insects (Carter et al. 2006; Nepi et al. 2009).

Proline during seed development

Immature seeds of *Vicia faba* accumulated proline before ripening, leading to the assumption that proline might be important for the development of generative organs (Venekamp and Koot 1984). Several studies reported a high proline level in Arabidopsis seeds (Chiang and Dandekar 1995; Schmidt et al. 2007), although data from other plant species are scarce. However, genes encoding enzymes of proline metabolism are expressed during seed development of various plants, including Arabidopsis, tomato, *Medicago truncatula* and rice (Verbruggen et al. 1993; Fujita et al. 1998; Armengaud et al. 2004; Hur et al. 2004). The antisense repression of *AtP5CS1* caused a delay in radicle emergence during Arabidopsis seed germination, suggesting that proline synthesis might replenish the $NADP^+$ pool and therefore activate the oxidative pentose phosphate pathway (Hare et al. 2003). In Arabidopsis, concomitant expression of genes for proline synthesis (*P5CS*, *P5CR*) and proline degradation (*PDH*, *P5CDH*) has been repeatedly demonstrated during seed development, raising the question about a possible function of proline cycling (Verbruggen et al. 1993, 1996; Hua et al. 1997; Deuschle et al. 2004; Székely et al. 2008). While the distribution of *P5CS1* and *P5CS2* transcripts appears to overlap during embryogenesis, the expression of P5CS-GFP fusion proteins revealed a different subcellular localization of the two isoforms (Székely et al. 2008; Mattioli et al. 2009b). Both P5CS-GFP fusion proteins were detected throughout all stages of embryogenesis, but P5CS2-GFP was found in the cytoplasm while P5CS1-GFP formed a dotted pattern in the embryo that did not co-localize with chloroplasts, mitochondria or nuclei (Székely et al. 2008). P5CS2 appears to be more important for seed development, since an insertion in the *AtP5CS2* gene causes embryo abortion, while *atp5cs1* mutants are able to produce viable seeds (Székely et al. 2008; Mattioli et al. 2009b). Embryo lethality in heterozygous *atp5cs2* plants might be caused by premature desiccation of homozygous embryos that develop more slowly than wildtype or heterozygous embryos (Funck, unpublished). The presence of external proline allowed the rescue of homozygous *atp5cs2* mutant embryos in axenic culture, which developed to fertile plants that produced viable seeds under certain conditions (Székely et al. 2008; Funck, unpublished). Detailed analysis of *atp5cs2* embryos revealed an increased frequency of missing or disoriented cell planes as well as polyembryony, a phenotype that could be partially rescued by treatment with 10 μM proline (Mattioli et al. 2009b). The reason for the observed malformations is still largely unknown. Mattioli et al. (2009b) showed that exogenous treatment with proline promotes cell division and causes induction of the cell cycle gene *CYCB1.1*. P5CR appears to be required during embryogenesis in Arabidopsis as well, since two independent *atp5cr* T-DNA insertion mutants are embryo lethal (Meinke et al. 2008; http://www.seedgenes.org).

Regulation of proline metabolism

Light and metabolites

One of the first factors demonstrated to influence proline accumulation was the intensity and duration of light exposure (Hanson and Tully 1979; Joyce et al. 1984). The level of free proline in leaves was reported to increase in the light and decrease in the dark (Sanada et al. 1995; Hayashi et al. 2000). Light-dependent changes in the availability of reducing equivalents or the oscillation of the relative water content have been suggested to act as regulators of proline levels (Hanson and Tully 1979; Hayashi et al. 2000). Expression analysis revealed that *AtP5CS* and *AtPDH* genes are conversely regulated by light and dark conditions, but it is unclear whether this adaptation is attributed to direct light-signalling or light-dependent metabolic or hormonal signals (Hayashi et al. 2000; Ábrahám et al. 2003). In barley, the light-related fluctuations of proline were more pronounced in shoots than in roots, associating this regulatory pathway to photosynthetically active tissues (Sanada et al. 1995).

Photosynthesis increases the level of sugars, whose promoting influence on proline accumulation was demonstrated in several studies (Stewart et al. 1966; Pesci 1993). High external concentrations of sucrose and glucose caused a stronger accumulation of proline in *Brassica napus* leaf discs than did mannitol or sorbitol (Larher et al. 1993). This regulation is probably due to transcriptional control of proline metabolism enzymes, since the *AtP5CS* mRNA level increases, whereas *AtPDH1* and *AtPDH2* expression is repressed by sucrose (Kiyosue et al. 1996; Nakashima et al. 1998; Hellmann et al. 2000; Hanson et al. 2008). However, many studies reporting an enhanced proline accumulation as response to external sugars have been performed using high sugar concentrations, thus osmotic and sugar-derived signals cannot be easily separated (Stewart et al. 1966; Larher et al. 1993; Pesci 1993; Verslues and Bray 2006).

The role of sucrose in the negative regulation of *AtPDH1* and *AtPDH2* was demonstrated to involve bZIP

transcription factors (Weltmeier et al. 2006; Hanson et al. 2008). AtbZIP11, whose translation is inhibited by sucrose-specific signalling, was suggested to act upon sucrose partitioning between source and sink tissues (Rook et al. 1998). Later, AtbZIP11 was shown to regulate expression of *AtPDH2* (Hanson et al. 2008). AtbZIP53 was shown to control *AtPDH1* expression preferably as a heterodimer with group-C bZIP proteins (Weltmeier et al. 2006). One of the *cis*-elements recognized by AtbZIP11 and other group-S bZIP transcription factors was previously identified as proline- or hypoosmolarity-responsive element (PRE) in *AtPDH1* and other genes (Satoh et al. 2002, 2004). A promoting effect of proline on *PDH1* expression has been demonstrated in several studies (Kiyosue et al. 1996; Peng et al. 1996; Verbruggen et al. 1996; Nakashima et al. 1998). However, the accumulation of proline under stress conditions does not lead to increased *PDH1* expression, indicating a dominant mechanism that represses *PDH1* expression during abiotic stress (Kiyosue et al. 1996; Verbruggen et al. 1996).

The availability of nitrogen has also been demonstrated to affect proline accumulation in several plants species. The proline content in tobacco leaves increases if plants are grown on medium containing high concentrations of NH_4NO_3 (Kavi Kishor et al. 1995). In leaves and roots of common bean (*Phaseolus vulgaris*), δOAT activity was positively correlated with proline concentrations over a broad concentration range of NH_4NO_3, whereas P5CS activity was inversely regulated (Sánchez et al. 2001, 2002). It remains an open question if a direct conversion of ornithine to proline without participation of P5CS is possible under conditions of high nitrogen abundance.

Hormones

The effect of abscisic acid (ABA) on the expression of *P5CS* and *P5CR* during abiotic stress has been described in a number of studies and reviewed previously (Savouré et al. 1997; Hare et al. 1999; Verslues and Bray 2006). Chiang and Dandekar (1995) suggested that the endogenously determined desiccation during seed maturation might also involve an ABA-dependent regulation of proline biosynthesis. ABA is a key regulator of seed development, promoting the accumulation of storage and LEA proteins (Finkelstein et al. 2002). The expression of *AtP5CS1* and *AtP5CS2* genes is strongly and moderately ABA-responsive, respectively (Strizhov et al. 1997; Ábrahám et al. 2003). The salt-dependent induction of *AtP5CS* expression is reduced in *aba* and *abi1* mutants, suggesting that ABA and ABI1-dependent signalling are essential for high levels of gene activation (Strizhov et al. 1997). A link between ABA and proline might also be provided by sugars, as several ABA-deficient or ABA-insensitive mutants are impaired in their response to sugars as well (Gibson 2005). Verslues and Bray (2006) demonstrated an inhibitory effect of sucrose on ABA-induced proline accumulation and suggested the transcription factor ABI4 to connect ABA- and sugar-signalling in the regulation of proline accumulation. Furthermore, the ABA-dependent induction of *P5CS1* expression was prevented by pre-treatment with brassinolide, but the physiological role of this effect is still unknown (Ábrahám et al. 2003). During Arabidopsis seed germination, brassinolide and ABA were shown to behave as antagonistic stimuli (Steber and McCourt 2001).

Secondary messengers such as nitric oxide (NO) or reactive oxygen species such as hydrogen peroxide (H_2O_2) are known to mediate ABA signals and affect proline metabolism under stress conditions (Desikan et al. 2002; Neill et al. 2008; Wang and Song 2008; Yang et al. 2009). In the green alga *Chlamydomonas reinhardtii*, NO treatment enhanced the copper-induced proline accumulation and increased the expression of *P5CS* (Zhang et al. 2008). By contrast, the increase of proline levels in *Brassica rapa* plants under high salinity conditions was less pronounced when plants were simultaneously treated with NO, possibly due to less efficient reduction of PDH activity (López-Carrión et al. 2008). In Arabidopsis, NO donors stimulated *P5CS1* expression and repressed *PDH1* expression, while NO scavengers had inverse effects (Zhao et al. 2009). Mutant analyses suggested that nitrate reductase rather than NO synthase is responsible for NO-mediated regulation of proline accumulation and concomitant freezing tolerance (Zhao et al. 2009). A connection between the accumulation of proline and ROS was established studying pathogen interaction in Arabidopsis. Avirulent pathogens induce ROS generation in infected leaves, and local proline accumulation was observed in Arabidopsis challenged with avirulent *Pseudomonas syringae* strains, though a direct causal relationship has not been demonstrated (Fabro et al. 2004). In maize seedlings, exogenous hydrogen peroxide treatment induced proline accumulation by activation of the biosynthetic pathway, including P5CS, δOAT and arginase (Yang et al. 2009).

Another plant regulator that appears to be involved in the regulation of proline metabolism is salicylic acid (SA). Several recent studies reported a promoting effect of SA on proline accumulation even in the absence of stress (Kanade 2008; Yusuf et al. 2008; Misra and Saxena 2009). Treatment with 0.5 mM SA elevated the proline content in the shoots of lentil seedlings by a factor of 4–5, possibly by enhancing P5CR activity and decreasing the activity of PDH (Misra and Saxena 2009). The impact of SA on proline accumulation was further increased under high salinity conditions, leading to the assumption that the stress-protective effect of SA might partially be achieved via control of proline metabolism (Misra and Saxena 2009). Treatment with SA also increased peroxidase

activity, as did the exogenous supply of proline (Ötztürk and Demir 2002; Yusuf et al. 2008). Interestingly, the SA-associated regulation of proline metabolism enzymes was largely restricted to the shoots of lentil, whereas the roots showed weak changes for the parameters tested (Misra and Saxena 2009). Likewise, SA treatment increased proline accumulation in barley shoots but not in roots (El-Tayeb 2005). Since SA was also reported to increase photosynthetic rates in several crop species including maize, barley and soybean (Pancheva et al. 1996; Khan et al. 2003; Khodary 2004), these data suggest that SA-mediated signals might regulate proline metabolism in photosynthetically active tissues. Furthermore, SA was demonstrated to be a regulator of flowering time in Arabidopsis (Martínez et al. 2004) and is thus, similar to proline synthesis, associated with reproductive development as well as stress responses. SA signalling is also involved in the induction of the hypersensitive response and *P5CS2* expression after infection with avirulent *Pseudomonas syringae* strains in Arabidopsis (Fabro et al. 2004). Plants with low SA levels due to mutation of *EDS5* or overexpression of a bacterial salicylate hydroxylase (NahG) did not show enhanced *P5CS2* expression or proline accumulation in response to pathogen attack (Fabro et al. 2004).

Proline transport

Translocation of proline

Evidence connecting changes in proline content with transport processes comes from developmental as well as stress-related studies. The proline deposition in the elongation zone of maize roots at low water potential was not achieved by biosynthesis but by an increase in proline transport (Verslues and Sharp 1999). Another study using maize seedlings demonstrated that proline utilization exceeds biosynthesis and that proline, provided by degradation of proline-rich storage proteins of the endosperm, is imported into the seedling (Raymond and Smirnoff 2002). Similarly, the accumulation of proline in maturing grapevine berries was not associated with increased *P5CS* expression, suggesting a contribution of transport processes (Stines et al. 1999).

Proline has been detected in both phloem and xylem sap in several plant species (Weibull et al. 1990; Bialczyk et al. 2004). The amount of proline in the phloem sap of alfalfa was elevated under water stress, indicating that long-distance transport might be important for stress-related changes in metabolism and/or stress tolerance (Girousse et al. 1996). Girdling experiments revealed increased proline levels in leaves and reduced proline concentrations in sink organs, also suggesting proline translocation from stressed leaves to culms and roots (Tully et al. 1979). However, antisense repression of glutamine synthetase in phloem cells of tobacco led to a reduced proline content in different organs as well as in phloem and xylem cells, indicating that proline might also be synthesized within the phloem cells and directly depends on glutamate availability (Brugière et al. 1999).

Proline transporters

Plant transporters mediating proline uptake across the plasma membrane have been identified both in the amino acid transporter (ATF) or amino acid/auxin permease (AAAP) family and in the APC (amino acid–polyamine–choline) family (Rentsch et al. 2007; Table 1). Though some members of the cationic amino acid transporters (CATs), belonging to the APC family, transport neutral amino acids, none of them recognizes proline efficiently and therefore their role will not be discussed further (Frommer et al. 1995; Su et al. 2004; Hammes et al. 2006). In the ATF/AAAP family, transporters that recognize proline have been identified in different subfamilies, namely in the amino acid permease (AAP) family, the lysine-histidine transporter (LHT) family and the proline transporter (ProT) family. AAPs mediate proton-coupled uptake of glutamate (aspartate) and neutral amino acids including proline (Frommer et al. 1993; Fischer et al. 1995, 2002; Okumoto et al. 2002; Lee et al. 2007; Schmidt et al. 2007). The affinity of different AAPs for proline determined in heterologous expression systems varied between 60 and 500 μM and was similar to the affinities for other amino acids (Table 1). LHTs transport neutral amino acids (including proline) and acidic amino acids with high affinity (Chen and Bush 1997; Lee and Tegeder 2004; Hirner et al. 2006). Again the affinity of LHTs for proline ($K_m \approx 10$ μM; Table 1) was in the same range as the affinity for other amino acids (Hirner et al. 2006). In contrast to transporters of the AAP and LHT family, ProTs transport proline but no other proteinogenic amino acids (Rentsch et al. 1996). Further studies showed that ProTs from Arabidopsis, tomato and the mangrove *Avicennia marina* also transport glycine betaine, though only the latter is a glycine betaine accumulating species (Breitkreuz et al. 1999; Schwacke et al. 1999; Waditee et al. 2002; Grallath et al. 2005). Furthermore, the three Arabidopsis ProTs and ProT1 of tomato also transport the stress-induced compound γ-aminobutyric acid (GABA), while GABA was not a substrate for the mangrove proline transporters (AmTs) (Breitkreuz et al. 1999; Schwacke et al. 1999; Waditee et al. 2002; Grallath et al. 2005). Consistent with the latter result, the affinity of the AtProTs for GABA was much lower than for proline or glycine betaine (4.5 mM compared to 0.5 and 0.2 mM,

Table 1 Affinity of plant transporters for proline

Species	Name	Accession number	Affinity for proline (μM)	Expression pattern
ATF/AAAP (amino acid transporter family/amino acid/auxin permease) gene family				
ProT proline transporter [substrates: proline, glycine betaine (GABA)[a]]				
Arabidopsis	AtProT1	At2g39890	427 ± 17 [S.c.][b]	Phloem or phloem parenchyma in all organs[b], pollen[n]
	AtProT2	At3g55740	500 ± 5 [S.c.][b]	Root cortex and epidermis[b], leaf after wounding[b], seedling after salt and drought stress[o]
	AtProT3	At2g36590	999 ± 36 [S.c.][b]	Epidermis of leaf and sepal[b]
Tomato	LeProT1	AF014808	1,900 ± 260 [S.c.][c]	Pollen[c]
Barley	HvProT	AB073084	25.1 [S.c.][d]	Root (root cap, cortex, stele, phloem) after salt stress[d,p]
Rice	OsProT	AB022783	a.n.d. [X.l.][e]	In all organs[e]
Avicennia marina	AmT1	AB075902	430 [E.c.][f]	After salt stress in leaf and root[f]
	AmT2	AB075903	320 [E.c.][f]	After salt stress in leaf and root[f]
AAP *amino acid permease* (substrates: neutral amino acids and glutamate, aspartate[a])				
Arabidopsis	AtAAP1	At1g58360	60 [S.c.][g] 1,900 [X.l.][h]	Flower, endosperm, developing embryo (storage parenchyma and outer epidermal cells), root epidermis, root tip, root hairs[i,q,r,s,t]
	AtAAP2	At5g09220	140 ± 20 [S.c.][i]	Stem, vasculature in leaf and root, phloem of silique, flower[i,q,r]
	AtAAP3	At1g77380	250 ± 25 [S.c.][j]	Root (phloem, endodermis in root tip), tip of filament (anther), vasculature of cotyledons[q,u]
	AtAAP4	At5g63850	134 ± 25 [S.c.][j]	Stem and source leaf, sink leaf[q]
	AtAAP5	At1g44100	500 ± 25 [S.c.][j]	Source leaf, stem, flower, root, silique[q]
	AtAAP6	At5g49630	67 ± 21 [S.c.][k]	In all organs, xylem parenchyma in aerial parts, root (site of lateral root initiation)[o,v]
LHT 'lysine/histidine' transporter (substrates: neutral and acidic amino acids[a])				
Arabidopsis	AtLHT1	At5g40780	10 ± 0.5 [S.c.][l]	Leaf epidermis and mesophyll, root tip, stem, petals, sepals[l,w]
	AtLHT2	At1g24400	13 ± 3 [S.c.][k]	Flower (tapetum, pollen)[k,x]
APC (amino acid–polyamine–choline) family				
CAT *cationic amino acid transporters* [substrates: neutral and cationic amino acids (lysine)[a]]				
Arabidopsis	AtCAT1	At4g21120	3,000 [S.c.][m]	Flower, major veins of root and leaf, silique, stem[m]

Affinities were determined using different heterologous expression systems. Only amino acid transporters for which proline transport was determined are included

[S.c.] Saccharomyces cerevisiae, *[X.l.] Xenopus laevis*, *[E.c.] Escherichia coli*, *a.n.d.* affinity not determined

[a]Rentsch et al. 2007, [b]Grallath et al. 2005, [c]Schwacke et al. 1999, [d]Ueda et al. 2001, [e]Igarashi et al. 2000, [f]Waditee et al. 2002, [g]Frommer et al. 1993, [h]Boorer et al. 1996, [i]Kwart et al. 1993, [j]Fischer 1997, [k]Lee and Tegeder 2004, [l]Hirner et al. 2006, [m]Frommer et al. 1995, [n]Lehmann and Rentsch, unpublished, [o]Rentsch et al. 1996, [p]Ueda et al. 2007, [q]Fischer et al. 1995, [r]Hirner et al. 1998, [s]Lee et al. 2007, [t]Sanders et al. 2009, [u]Okumoto et al. 2004, [v]Okumoto et al. 2002, [w]Chen and Bush 1997, [x]Foster et al. 2008

respectively Grallath et al. 2005). While the selectivity of rice ProT has not been investigated in detail, barley HvProT recognized only L-proline efficiently, though with a higher affinity than any of the other ProTs (Igarashi et al. 2000; Ueda et al. 2001). These data on substrate selectivity of AAPs, LHTs and ProTs show that in plants both transporters with low and high selectivity for proline exist, indicating a role in general transfer of nitrogen and in proline-specific functions, respectively.

A separation in general amino acid transport and selective proline uptake is also found in the yeast *Saccharomyces cerevisiae*, where the general amino acid permease Gap1p and the proline transporter Put4p together mediate the major part of proline uptake (Lasko and Brandriss 1981). Whereas Gap1p transports all proteinogenic amino acids with low affinity, Put4p only transports GABA, alanine and glycine in addition to proline and recognizes proline with high affinity (Grenson et al. 1970;

Lasko and Brandriss 1981; Jauniaux et al. 1987; Regenberg et al. 1999). The amino acid permeases Agp1 and Gnp1p may contribute to residual low affinity proline uptake (Andréasson et al. 2004).

Also in bacteria, the accumulation of compatible solutes is controlled by synthesis, uptake and export, though uptake is preferred over biosynthesis provided that proline or glycine betaine is available (Kempf and Bremer 1998; Roeßler and Müller 2001). A range of secondary active transporters (e.g. the *E. coli* H^+/proline symporter ProP) and binding protein-dependent ABC transporters (e.g. *E. coli* ProU) mediate the uptake of proline and/or glycine betaine and related substrates (Csonka 1989; Wood et al. 2001). Under osmotic stress, these transporters may be regulated via both increased gene expression and higher activity (Wood et al. 2001), and some were additionally shown to function as osmosensors (Morbach and Krämer 2002; Wood 2006). In addition to osmolyte uptake systems, *E. coli* uses the PutP transporter for uptake of proline as a nitrogen and carbon source (Csonka 1989). *PutP* expression is repressed by the trifunctional PutA protein (combining PDH, P5CDH and regulatory functions in a single protein) in the absence of proline and becomes activated once the PutA protein is recruited to the membrane during proline degradation (Tanner 2008; Zhou et al. 2008).

The fact that in different kingdoms many transporters that belong to completely different gene families recognize both proline and glycine betaine argues for similarities in substrate recognition. Interestingly, BGT1, a member of the sodium- and chloride-dependent neurotransmitter transporter (SCL6) family in mammals transports GABA, glycine betaine and proline, though proline transport activity was lower (Matskevitch et al. 1999; Chen et al. 2004). Like many of the proline and glycine betaine transporters in bacteria and plants, *BGT1*-expression is regulated by changes in osmolarity and a role of BGT1 in osmoregulation in kidney was postulated (Chen et al. 2004).

Physiological role of plant proline transporters

So far, all functionally characterized proline transporters operate as cellular uptake systems, whereas information on carrier-mediated export is missing (Rentsch et al. 2007). Using knockout mutants, in planta transport studies confirmed the selectivity determined in heterologous systems (Hirner et al. 2006; Lee et al. 2007; Svennerstam et al. 2007; Näsholm et al. 2009). The complementary though overlapping expression patterns indicate specific physiological functions, but partial redundancy might also be expected. As members of the AAP and LHT families are not selective for proline, they rather play a role in general acquisition and allocation of nitrogen in the plant. Indeed, results from Arabidopsis *lht1*, *aap8* and *aap1* mutants as well as overexpression of *Vicia faba AAP1* in *Vicia narbonensis* and pea revealed complex and sometimes transient changes in amino acid levels (Rolletschek et al. 2005; Hirner et al. 2006; Schmidt et al. 2007; Weigelt et al. 2008; Sanders et al. 2009). Changes in proline levels in these mutants might also originate from stress-induced alteration of proline metabolism.

The selectivity of ProTs for proline and other compatible solutes indicates a specific role in proline homeostasis under stress and non-stress conditions. Expression analyses supported this function, as *ProT* expression was generally related to low water availability and/or high proline (or glycine betaine) levels. Under salt stress, proline accumulation is accompanied by an increased expression of Arabidopsis *ProT2*, mangrove *AmT1*, *2* and *3* as well as *HvProT* (Rentsch et al. 1996; Ueda et al. 2001; Waditee et al. 2002; Table 1). Likewise, the high transcript abundance of *AtProT1* and *LeProT1* in pollen and of *AtProT3* in the epidermis correlated with an elevated proline content (Schwacke et al. 1999; Lehmann and Rentsch, unpublished). *AtProT1* transcripts were found in different organs in addition to pollen, but expression was confined to the phloem, pointing to a role in long-distance transport (Grallath et al. 2005). Similarly, expression of rice *OsProT* was detected in all organs analysed (Igarashi et al. 2000; Table 1).

In spite of these correlations, there are only few reports showing a direct role of ProTs in proline transport in planta. The Arabidopsis knockouts mutants *atprot1, atprot2* or *atprot3* did not reveal phenotypic differences or altered proline content in the absence or presence of abiotic stress, indicating compensation by other transporters or altered proline metabolism (Lehmann and Rentsch, unpublished). However, the overexpression of *HvProT* in Arabidopsis resulted in reduced biomass and decreased proline levels in shoots, an effect that could be compensated by exogenous supply of low concentrations of proline (Ueda et al. 2008). Elevated PDH mRNA and activity in these plants suggested that altered transport may induce proline degradation, leading to a reduction of overall proline content (Ueda et al. 2008). On the other hand, root cap specific expression of *HvProT* in Arabidopsis resulted in higher proline levels in root tips and enhanced root elongation (Ueda et al. 2008), supporting a role of proline in organ development.

Intracellular transport

As proline metabolism is confined to distinct cellular compartments (Fig. 1), intracellular transport of proline is required. Proline biosynthesis takes place in the cytosol and probably in chloroplasts under stress conditions (Szabados

and Savouré 2010). Thus, at least in the absence of stress proline import into plastids is necessary. Furthermore, transfer into mitochondria is essential for proline catabolism. Whereas to our knowledge, information on proline transport into or out of plastids is lacking, proline uptake into mitochondria has been demonstrated to be mediated by two transport systems, i.e. a proline uniporter as well as a proline/glutamate antiport system (Elthon et al. 1984; Di Martino et al. 2006), though a reversible switch of the transport mode as shown for other mitochondrial carriers cannot yet be excluded (Krämer 1998). Likewise, in mammals, a glutamate/proline antiporter was characterized (Atlante et al. 1996). However, no genes encoding intracellular transporters have been identified in plants so far (Picault et al. 2004).

Much information is meanwhile available on proline metabolism and its regulation and compartmentation, but still the various physiological functions proposed for free proline lack mechanistic explanations that are unambiguously substantiated by experimental evidence. Likewise, the role of proline translocation and the identity of intracellular proline transport systems are still largely unknown. More detailed analyses might reveal novel and interesting links between metabolism and transport, which may contribute to a better understanding of the role and regulation of proline homeostasis.

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