Proline Metabolism and Its Functions in Development and Stress Tolerance

Maurizio Trovato, Giuseppe Forlani, Santiago Signorelli, and Dietmar Funck

1 Introduction

Most green plants are fully autotrophic organisms and can produce their entire biomass from inorganic molecules with the help of light energy captured by photosynthesis. Energy from photosynthesis is thereby not only needed to reduce CO_2 to carbohydrates but also to assimilate nitrogen, phosphorus, and sulfur from inorganic salts for the biosynthesis of proteins and nucleic acids (Buchanan et al. 2000; Taiz et al. 2018). In contrast, most non-photosynthetic organisms, including animals and humans, depend on the uptake of organic material both as energy source and as building material (Hill et al. 2016). This fundamental difference exists since the development of oxygenic photosynthesis by cyanobacteria, which were later converted to endosymbiotic chloroplasts in eukaryotic algae and plants (Nozaki 2005; Zimorski et al. 2014). It is therefore not surprising that despite the use of identical building blocks in all living organisms, i.e., nucleotides, amino acids, and carbohydrates, the pathways to acquire or synthesize these building blocks are not identical in distantly related groups of organisms. Knowledge about human metabolism can therefore only serve to guide investigations of regulatory and metabolic pathways in plant primary metabolism but not as a direct template.

M. Trovato (✉)
Department of Biology and Biotechnology, Sapienza University of Rome, Rome, Italy
e-mail: maurizio.trovato@uniroma1.it

G. Forlani
Department of Life Science and Biotechnology, University of Ferrara, Ferrara, Italy

S. Signorelli
Department of Plant Biology, Universidad de la República, Montevideo, Uruguay

D. Funck (✉)
Department of Biology, Division of Plant Physiology and Biochemistry, University of Konstanz, Konstanz, Germany
e-mail: dietmar.funck@uni-konstanz.de

M. A. Hossain et al. (eds.), *Osmoprotectant-Mediated Abiotic Stress Tolerance in Plants*, https://doi.org/10.1007/978-3-030-27423-8_2

An additional layer of complexity is added by the fact that many primary metabolites are used for additional specific purposes in plants. This and the following two chapters focus on the amino acid proline, which is an essential constituent of most proteins but serves additionally as a compatible solute with important functions in stress defense, as a signaling molecule, and as a precursor for secondary metabolites in some plant species. The functions and regulation of proline metabolism and accumulation in stress defense are summarized in detail in the following chapter, while other chapters of this book will specifically focus on the physiological function and agronomic potential of proline uptake from external sources (Chaps. 4 and 9) and on the role of proline as a signaling molecule in stress adaptation (Chap. 11). The present chapter summarizes the current knowledge about the biochemical pathways of proline metabolism and its functions in regulating plant development and physiology both in the absence or presence of stress.

2 Proline Biosynthesis and Degradation: Enzymes and Their Subcellular Localization

The concentration of free proline in a plant cell is determined largely by four metabolic processes, namely, biosynthesis and degradation of proline as well as consumption of proline for protein biosynthesis and release of proline during protein degradation (Hildebrandt 2018). Additionally, the distribution of proline among different sub-compartments of the cell is not uniform and proline is distributed within the plant by long-distance transport along vascular bundles and locally by transport across the plasma membrane or through plasmodesmata. This section will focus on the anabolic and catabolic enzymes and their subcellular localization, whereas the next section will integrate this information with the known transport routes to establish metabolic pathways.

2.1 Proline Biosynthetic Enzymes

In prokaryotes, three enzymes have been identified that can synthesize proline: ornithine cyclodeaminase (OCD), pyrroline-2-carboxylate reductase (P2CR), and pyrroline-5-carboxylate reductase (P5CR) (Fig. 1). OCD uses NAD^+ as a cofactor during the cyclization and subsequent deamination of ornithine. In the proposed reaction mechanism, NAD^+ is transiently reduced and later re-oxidized during enzyme regeneration and proline release (Goodman et al. 2004). OCD is encoded by *rolD* on the transfer DNA (T-DNA) of *Rhizobium rhizogenes* (better known under its traditional name *Agrobacterium rhizogenes*) and an integrated copy of such a T-DNA has been found in genomic DNA of *Catharanthus roseus* (GenBank accession DQ852612). Endogenous proteins with homology to OCD can be

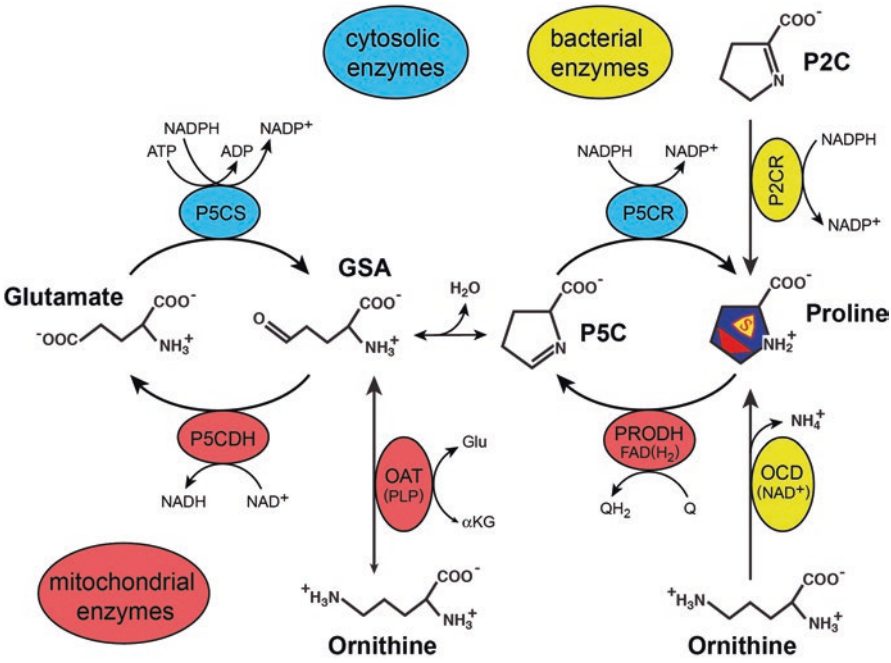

Fig. 1 Substrates, enzymes, and cofactors of proline metabolism. Blue and red circles indicate cytosolic and mitochondrial enzymes, respectively. The yellow color of OCD indicates the prokaryotic origin of this protein. Proline is depicted in its super proline dress to emphasize its many important functions in adaptation, defense, and development of plants. GSA glutamate-5-semialdehyde, OAT ornithine-δ-aminotransferase, OCD ornithine cyclodeaminase, P2C pyrroline-2-carboxylate, P2CR P2C reductase, P5C pyrroline-5-carboxylate, P5CDH P5C dehydrogenase, P5CR P5C reductase, P5CS P5C synthetase, PLP pyridoxal phosphate, ProDH proline dehydrogenase

identified in plant genomes, but an in-depth analysis of the Arabidopsis OCD homolog (At5g52810) did not yield any evidence of OCD activity or any other function in proline biosynthesis (Sharma et al. 2013). Pyrroline-5-carboxylate (P5C) and pyrroline-2-carboxylate (P2C) are reduced to proline by P5CR or P2CR, respectively, under consumption of NAD(P)H (Fichman et al. 2015). Bacterial P2CRs often have dual specificities for the conversion of P2C to proline or Δ^1-piperidine-2-carboxylate to pipecolate and function in trans-3-hydroxy-L-proline degradation or pipecolate biosynthesis (Watanabe et al. 2014). Early biochemical studies reported or postulated P2CR activity in few plant species (Meister et al. 1957; Mestichelli et al. 1979), and in several plant genomes, hypothetical P2CRs are annotated. However, the molecular identification of a plant P2CR has not been reported so far and the hypothetical P2CRs do not align unambiguously to characterized P2CRs. Therefore, P5C is at present the only confirmed precursor for proline biosynthesis in plants.

P5CR has been cloned or purified from a number of plant species and was found to form large homo-oligomers (Delauney and Verma 1990; Forlani et al. 2015; Funck et al. 2012; Ma et al. 2008; Murahama et al. 2001; Ruszkowski et al. 2015). The first crystal structure of P5CR from rice (*Oryza sativa*) revealed a decameric structure consisting of a ring of five dimers, which is in agreement with most molecular mass estimates for P5CR from other plant species (Forlani et al. 2015). P5CR can use both NADH and NADPH to reduce P5C to proline, and in the absence of $NAD(P)^+$, higher turnover rates were obtained with NADH. However, the affinity of P5CR for NADPH is much higher and even low concentrations of $NADP^+$ inhibited the reaction of Arabidopsis (*Arabidopsis thaliana*) P5CR with NADH (Giberti et al. 2014). At physiological pH values, the reaction is nearly unidirectional toward proline formation, while at high pH (>9) also the reverse reaction can be detected, because P5C is highly labile at elevated pH (Rena and Splittstoesser 1975) (unpublished data by G. Forlani). Unfortunately, proline-dependent formation of NAD(P)H by soluble plant extracts at high pH is often erroneously interpreted as ProDH activity (see below). Most plant species contain a single P5CR gene, and in Arabidopsis, a P5CR:GFP fusion protein was detected exclusively in the cytosol (Funck et al. 2012). After cell fractionation, the major part of the P5CR activity was detected in the soluble protein fraction in many plant species, whereas some authors also reported P5CR activity in chloroplast-enriched fractions (Murahama et al. 2001; Noguchi et al. 1966; Rayapati et al. 1989). It remains to be clarified if P5CR, which lacks a conserved chloroplast transit peptide in all analyzed genomes, can be imported into plastids by an unconventional mechanism or can be partially attached to plastids during isolation.

P5C is formed nonenzymatically by cyclization of glutamate-5-semialdehyde (GSA), which is an equilibrium reaction in aqueous solution. Two plant enzymes are known to produce GSA: ornithine-δ-aminotransferase (OAT) and P5C synthetase (P5CS). The first plant *OAT* gene was isolated by trans-complementation of an *Escherichia coli* strain unable to synthesize P5C with a cDNA clone from *Vigna aconitifolia* (Delauney et al. 1993). OAT is localized in the mitochondria and uses pyridoxal phosphate as cofactor in the transfer of the δ-amino group of ornithine to α-ketoglutarate, yielding GSA and glutamate (Funck et al. 2008; Roosens et al. 1998; Stránská et al. 2008). For mammalian OAT, it has been shown that it also catalyzes the reverse reaction in certain tissues, although the chemical equilibrium is far to the side of GSA and glutamate (Strecker 1965).

The second enzyme, P5CS, reduces glutamate to GSA in a two-step reaction consuming ATP and NADPH. All land plants and animals have bifunctional P5CS enzymes, whereas prokaryotes and some unicellular green algae and fungi have separate γ-glutamyl kinase and glutamyl-γ-phosphate reductase enzymes (Fichman et al. 2015; Hu et al. 1992; Zhang et al. 1995). A previous report describing the occurrence of prokaryote-like γ-glutamyl kinase and glutamyl-γ-phosphate reductase genes in tomato (*Solanum lycopersicum*) was most likely an artifact, because no such genes are present in genomic sequences of tomato (Fujita et al. 1998). In the initial reaction, the γ-glutamyl kinase domain of P5CS uses ATP to phosphorylate the γ-carboxy group of glutamate, and in the second reaction, glutamyl-γ-phosphate

is reduced to GSA under consumption of NADPH. The γ-glutamyl kinase activity is inhibited by millimolar concentrations of proline and mutations identified in bacterial enzymes were used to engineer feedback-insensitive variants of P5CS in plants (Hu et al. 1992; Zhang et al. 1995). The first plant *P5CS* gene was isolated by complementation of a proline synthesis-deficient *E. coli* strain (Hu et al. 1992). Most plant species have at least two P5CS isoforms, of which one can be regarded as a housekeeping gene, while others are induced by stress to enable proline accumulation (Kim and Nam 2013; Signorelli and Monza 2017; Székely et al. 2008; Turchetto-Zolet et al. 2009; Wang et al. 2014). Initial characterization of the Arabidopsis P5CS proteins by GFP fusion indicated that both isoforms are cytosolic in non-stressed plants but may be imported into plastids upon osmotic stress (Székely et al. 2008), whereas our own data indicate exclusive cytosolic localization (Funck et al. 2019). Similarly, GFP fusions of two out of three P5CS isoforms from *Medicago truncatula* co-localized with the small subunit of ribulose bisphosphate carboxylase/oxygenase in root hairs, but like in the Arabidopsis P5CS sequences, no typical chloroplast transit peptides are present in the protein sequences (Kim and Nam 2013). By cell fractionation and Western blot, corn (*Zea mays*) P5CS2 was detected exclusively in the cytosol and not in the organelle fraction (Wang et al. 2014). Because glutamate, ATP, and NADPH can be used as substrates by many different enzymes (e.g., glutamine synthetase or P5C dehydrogenase, see below), a specific assay of P5CS activity in crude plant or organelle extracts has not been reported so far, and thus the subcellular localization awaits biochemical confirmation.

2.2 Proline Degradation Enzymes

For the degradation of excess proline that is not used for protein synthesis or as compatible solute, also a single enzyme is known in plants. Proline dehydrogenase (ProDH), previously also referred to as proline oxidase, is an FAD-containing enzyme at the inner mitochondrial membrane, which oxidizes proline back to P5C while transferring the obtained electrons to the mitochondrial electron transport chain and thus fueling respiratory ATP production (Elthon and Stewart 1981; Huang and Cavalieri 1979; Schertl et al. 2014). Structural studies of Put1, the ProDH of baker's yeast (*Saccharomyces cerevisiae*), strongly indicate that the electrons are transferred via the tightly bound FAD cofactor to ubiquinone (Moxley et al. 2017; Wanduragala et al. 2010). A *ProDH* gene from Arabidopsis has been independently identified by homology searches and by screening for genes that respond rapidly to changes in the water status (Kiyosue et al. 1996; Peng et al. 1996; Verbruggen et al. 1996). Many plant genomes contain a single *ProDH* gene, while an early genome duplication in the *Brassicaceae* led to two isoforms in Arabidopsis with further multiplications occurring in *Brassica* species (Faes et al. 2015; Funck et al. 2010; Mani et al. 2002). ProDH activity has been exclusively detected in mitochondria unless the reverse reaction of P5CR at high pH was erroneously assigned to ProDH

(see above; (Huang and Cavalieri 1979; Schertl et al. 2014). C-terminal GFP fusion proteins of both Arabidopsis ProDH isoforms were targeted to the mitochondria in stably transformed plants, whereas a transient transformation assay provided evidence for chloroplast targeting of ProDH2 (Funck et al. 2010; Van Aken et al. 2009).

P5C produced by ProDH has three potential fates: It can be converted to proline by P5CR, or it can be linearized to GSA and be converted to ornithine by OAT or to glutamate by P5C dehydrogenase (P5CDH, recently suggested to be renamed as glutamate semialdehyde dehydrogenase, GSALDH, to better reflect the actual substrate and the evolutionary relationship to the aldehyde dehydrogenase family (Tanner 2019)). The latter is the last metabolic enzyme that is discussed here in detail. P5CDH activity was first characterized in corn mitochondria, and 20 years later the first *P5CDH* gene from Arabidopsis was identified by functional cloning (Deuschle et al. 2001; Elthon and Stewart 1981). In most annotated plant genomes, *P5CDH* is a single-copy gene except for polyploid species (Ayliffe et al. 2005; Deuschle et al. 2001; Korasick et al. 2019). Biochemical analyses of isolated corn mitochondria provided evidence for two P5CDH isoforms with distinct pH optima (Elthon and Stewart 1982), and for the single-copy *P5CDH* gene in the *Zea mays* genome, several predicted splicing variants are annotated (NCBI gene ID: 100193220). P5CDH is a soluble enzyme in the mitochondrial matrix and prefers NAD^+ over $NADP^+$ as electron acceptor during the oxidation of GSA to glutamate (Forlani et al. 1997). In many bacteria, ProDH and P5CDH activities are combined in a single enzyme that allows direct substrate channeling, and recently it was reported that also the two plant enzymes might be physically linked through interaction with the inhibitory protein DROUGHT AND FREEZING RESPONSIVE GENE 1 (DFR1; (Ren et al. 2018).

Especially in fragrant rice but also in some other plant species, proline and P5C were also identified as potential precursors for the production of 2-acetyl-1-pyrroline, the main constituent of the typical flavor (Wakte et al. 2017; Yoshihashi et al. 2002). However, a recent metabolomic and genomic study in rice proposed putrescine-derived 4-aminobutanal as immediate precursor for 2-acetyl-1-pyrroline and challenged the direct involvement of proline or P5C (Daygon et al. 2017).

3 Proline Transport and Metabolic Pathways

Proline is most likely synthesized exclusively in the cytosol but is needed for protein biosynthesis also in mitochondria and plastids. In leaves of osmotically stressed potato (*Solanum tuberosum*) plants, the highest concentrations of proline were reported for chloroplasts (Büssis and Heineke 1998). Additionally, a substantial part of protein degradation occurs in the vacuole, and proline degradation takes place in mitochondria. After release from stress, the high concentration of proline rapidly decreases, primarily by ProDH- and P5CDH-dependent degradation (Deuschle et al. 2004; Nanjo et al. 1999b). Therefore, efficient transport proteins for proline must exist in most intracellular membranes, but their molecular identity is only beginning to be revealed.

3.1 Intracellular Proline Transport

Isolated mitochondria from monocot seedlings can use proline and P5C/GSA as substrates for respiratory O_2 consumption, but in Arabidopsis mitochondria proline-dependent respiration was only detected when the expression of ProDH1 was stimulated by proline treatment prior to the isolation of mitochondria (Boggess et al. 1978; Cabassa-Hourton et al. 2016; Elthon and Stewart 1982). No carriers for proline or P5C/GSA in mitochondria have been molecularly identified so far. However, biochemical analyses provided evidence that the import of proline into mitochondria is dependent on a proton gradient and at least two transporters, a proline uniporter and a proline/glutamate antiporter, are present in the inner mitochondrial membrane (Di Martino et al. 2006; Elthon et al. 1984). Recently, several members of the mitochondrial carrier family (MCF) were shown to mediate glutamate transport, but no evidence for proline transport activity has been obtained yet (Monne et al. 2018; Porcelli et al. 2018). Even less data is available on amino acid transport across the chloroplast membranes, where so far only a malate/glutamate antiporter (DiT2 in the inner envelope) and a transporter for neutral amino acids in the outer envelope (OEP16) have been characterized (Pohlmeyer et al. 1997; Renné et al. 2003). In the vacuolar membrane of yeast, the family of AMINO ACID VACUOLAR TRANSPORTERS (AVT) has been characterized, and recently it was shown that Arabidopsis homologues AVT3A and AVT3C complement the defects of *avt3/avt4* double mutant yeast cells (Fujiki et al. 2017). These proteins are localized in the vacuolar membrane in Arabidopsis and functional studies suggested that they mediate the ATP-dependent export of several amino acids, including proline, from the vacuole. This suggestion is in agreement with evidence from potato showing that proline concentration in the cytosol can be 260-fold greater than in the vacuole, indicating the presence of an active transport system (Büssis and Heineke 1998).

3.2 Pathways for Proline Biosynthesis and Degradation

The lack of information about the activity and specificity of transport proteins in intracellular membranes makes it difficult to draw definite conclusions about the metabolic pathways of proline biosynthesis and degradation that occur in vivo. Biosynthesis of proline from glutamate by the sequential action of P5CS and P5CR appears to be the predominant pathway, especially for stress-induced proline accumulation. Accordingly, both *P5CS* and *P5CR* are essential genes in Arabidopsis, and double mutations in *P5CS1* and *P5CS2* are gametophytic lethal, as no fertile *p5cs1/p5cs2* mutant pollen is formed, while homozygous *p5cr* mutant embryos were observed but aborted at a very early developmental stage (Funck et al. 2010; Mattioli et al. 2012). These observations demonstrate that no other pathway can produce sufficient amounts of proline for successful sexual reproduction. An alternative pathway of proline biosynthesis from ornithine has been assumed based on radiotracer studies, co-expression analyses, and analogy to mammals (da Rocha

et al. 2012; Mestichelli et al. 1979; Roosens et al. 1998). To actually bypass P5CS activity, this pathway depends on export of GSA/P5C produced from ornithine by OAT in mitochondria, which was detectable in isolated corn mitochondria but is difficult to assess in vivo due to the high reactivity and inherent instability of GSA/P5C at neutral pH (Elthon and Stewart 1982; Mezl and Knox 1976). When mitochondria were incubated with proline, the production of glutamate was two orders of magnitude higher than GSA/P5C production, and also in Arabidopsis plants treated with external proline, GSA/P5C content stayed below the detection limit of 50 nmol/g fresh weight unless *p5cdh* mutants were used (Boggess et al. 1978; Deuschle et al. 2004). Much higher GSA/P5C contents were reported in a study using plants overexpressing *ProDH* and, together with unchanged GSA/P5C to proline ratios, were interpreted as evidence for a proline-GSA/P5C cycle between the cytosol and mitochondria (Miller et al. 2009). However, Miller et al. (2009) did not provide evidence that the employed color reaction is specific for GSA/P5C in crude plant extracts and the production of glutamate by both OAT and P5CDH makes it difficult to exclude that ornithine only stimulates the P5CS-dependent pathway of proline biosynthesis. Similarly, there is at present no evidence that proline degradation might yield ornithine instead of glutamate in plants, as it has been proposed for certain mammalian tissues (Ginguay et al. 2017).

3.3 Intercellular Proline Transport

In contrast to organellar proline transporters, which remain elusive so far, numerous proline transporters were identified that are localized in the plasma membrane. Several members of the amino acid/auxin permease (AAAP) family mediate amino acid-proton symport (Dinkeloo et al. 2018). Among these, members of the amino acid permease (AAP) and lysine histidine transporter (LHT) subfamilies transport proline along with a rather broad range of both neutral and charged amino acids (Fischer et al. 1995; Hirner et al. 2006). Members of the proline transporter (ProT) subfamily have rather narrow substrate specificity and transport proline, glycine betaine, or γ-aminobutyric acid (GABA) (Lehmann et al. 2011). A recent analysis of Arabidopsis *aap1* mutants suggested that AAP1 could contribute to the uptake of proline from the growth substrate (Perchlik et al. 2014; Wang et al. 2017). However, little is known about the concentrations of free proline in natural soils and its relevance for plant nutrition or communication, indicating that the major function of AAPs and ProTs is the redistribution of proline within the plant. Amino acids are transported in both the xylem and the phloem, and several members of the AAP family were shown to contribute to phloem loading in source leaves or retrieval of amino acids along the transport route (Tegeder and Hammes 2018). Apoplastic phloem loading requires that amino acids are released by source cells into the intercellular space and also loading of the dead xylem vessels in roots requires export of amino acids and other solutes by the surrounding living cells. In 2012, the SILIQUES ARE RED 1 (SIAR1/UMAMIT18) protein was identified in Arabidopsis as the first transporter that can mediate bidirectional amino acid transport, depending on the

electrochemical gradient across the membrane (Ladwig et al. 2012). SIAR1 is part of a protein family with 44 members in Arabidopsis, of which several members have been characterized in the meantime as broad specificity amino acid exporters and which were therefore named "usually multiple amino acids move in and out transporters" (UMAMITs) (Besnard et al. 2016, 2018; Müller et al. 2015). Also, plasmodesmata are a possible route for amino acid transport between connected cells, but to our knowledge, no specific transport mechanisms have been described so far.

4 Proline Biosynthesis and Degradation: Spatial and Temporal Regulation

The diverse functions of proline metabolism, which must sustain the variable requirements of protein synthesis, while playing multiple additional physiological functions and responding to environmental and biotic stimuli, are reflected in, and derived from, an elaborate network of gene and enzyme activity regulation (Fig. 2). Most of the known regulatory mechanisms for proline metabolism operate at the transcriptional level and appear to distinguish between stress and normal physiological conditions. Key to this distinction is the capability to detect and respond to different inputs via several signaling pathways that result in expression or activation of specific transcription factors (TFs). Accordingly, the promoters of all genes coding for proline metabolic enzymes that were characterized so far are especially rich in confirmed or predicted TF recognition elements (Fichman et al. 2015; Zarattini and Forlani 2017). Further regulatory mechanisms were found to act epigenetically or posttranscriptionally on gene expression, or allosterically on enzyme activities. Far less is known about posttranslational modifications or regulated degradation of proline metabolic enzymes and transporters. Most of the knowledge about regulatory mechanisms of proline metabolism has been obtained by analysis of Arabidopsis wild-type plants or mutants. As indicated above, duplications and functional diversification of proline metabolic genes occurred several times independently in different plant taxa. Therefore, it is at present unknown how much of the knowledge obtained in Arabidopsis can be directly transferred to different species (Mattioli et al. 2018; Signorelli and Monza 2017; Turchetto-Zolet et al. 2009). We will focus on the knowledge gained for Arabidopsis and indicate it specifically, when data from other plants are described as well.

4.1 Regulation of Genes Coding for Proline Biosynthesis Enzymes

As discussed above, the short pathway converting glutamate into GSA and P5C into proline is the most important and probably unique route of proline synthesis in higher plants. Compelling evidence indicates that P5CS, the first enzyme of glutamate-derived proline synthesis, is under most conditions the rate-limiting

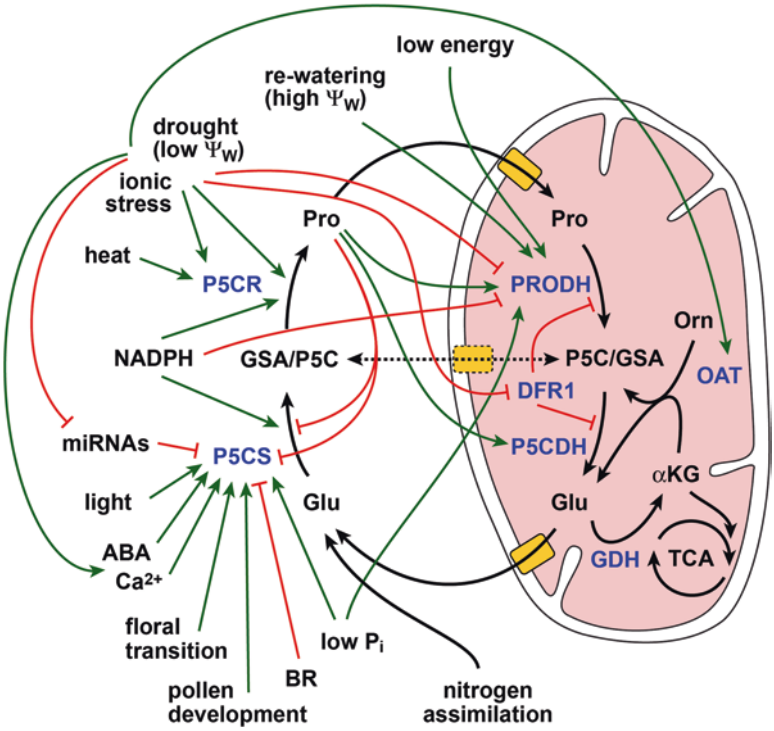

Fig. 2 The regulatory network controlling proline metabolism. Enzymes are given in blue letters and metabolic fluxes as solid black arrows. The uncertain transport of P5C/GSA across the mitochondrial membrane is indicated by a dashed arrow. Green and red lines indicate induction and repression, respectively. Lines ending at an enzyme name indicate regulation of gene expression, while lines ending at the metabolic flux indicate posttranslational regulation. For reasons of simplicity, low water potential and high ionic strength are depicted as a single regulatory unit, although they probably use partly independent signaling cascades. ABA abscisic acid, αKG α-ketoglutarate, BR brassinosteroids, DFR1 drought and freezing regulated gene 1, GDH glutamate dehydrogenase, Glu glutamate, GSA glutamate-5-semialdehyde, OAT ornithine-δ-aminotransferase, OCD ornithine-cyclodeaminase, Orn ornithine, P5C pyrroline-5-carboxylate, P5CDH, P5C dehydrogenase, P5CR P5C reductase, P5CS P5C synthetase, P_i Phosphate, Pro proline, ProDH proline dehydrogenase, TCA tricarboxylic acid cycle, Ψ_w water potential

enzyme of proline synthesis in higher plants. This evidence derives from the strict correlation between *P5CS* expression and proline accumulation (Hu et al. 1992; Peng et al. 1996; Savouré et al. 1995; Strizhov et al. 1997; Yoshiba et al. 1999) and from the effects of *P5CS* overexpression (Kavi Kishor et al. 1995; Per et al. 2017) and antisense inhibition (Nanjo et al. 1999b) or knockout mutations (Mattioli et al. 2008; Székely et al. 2008). Accordingly, the overall rate of proline biosynthesis is predominantly determined by the temporal and spatial regulation of *P5CS* gene expression.

The two Arabidopsis *P5CS* genes are located on chromosome 2 and 3 and share the same genomic structure with 20 exons sharing a nucleotide identity ranging

from 80% to 94%. A higher degree of difference is found in the promoter regions, the 5′ and 3′ untranslated sequences and introns, including variations in putative splicing sites, which might give rise to four different *P5CS1* and two different *P5CS2* transcripts included in the current annotation (ARAPORT11) of the Arabidopsis genome (https://www.Arabidopsis.org). The four different /P5CS1/ transcripts are derived from two splice variants and two alternative transcription initiation sites, while in *P5CS2* a single-splice variant skipping exon 3 is annotated. Skipping of exon 3 of *P5CS1* produces nonfunctional transcripts and has been experimentally confirmed as potential mechanism underlying differential drought tolerance of several natural accessions from different regions (Kesari et al. 2012).

The expression of *P5CS1* and *P5CS2* in Arabidopsis has been analyzed by northern blot, in situ hybridization, and analysis of transgenic plants carrying promoter-GUS and promoter-gene-GFP fusion constructs (Abrahám et al. 2003; Fabro et al. 2004; Mattioli et al. 2018; Mattioli et al. 2009; Strizhov et al. 1997; Yoshiba et al. 1999). There are some minor discrepancies between the results, most likely attributable to different cultivation conditions and analysis techniques. The prevailing picture is that both isoforms have partially overlapping expression patterns, whereby *P5CS1* is expressed more strongly in aboveground tissues and differentiated cells, whereas *P5CS2* expression levels are highest in regions of active cell division. In flowers, both *P5CS* isoforms are almost exclusively expressed in developing microspores and pollen (Mattioli et al. 2018). *P5CS1* and, to a lesser extent, *P5CS2* transcription is rapidly induced by drought and salt stress, with light and abscisic acid (ABA) as key inducing signals (Abrahám et al. 2003; Feng et al. 2016; Strizhov et al. 1997). An independent pathway seems to mediate induction of *P5CS1* expression in response to phosphate starvation (Aleksza et al. 2017). Proline-, brassinolide-, and phospholipase-dependent signaling were identified as negative regulators of *P5CS* expression and probably contribute to the rapid downregulation after relief from stress (Abrahám et al. 2003; Thiery et al. 2004). Bioinformatic analyses of the promoters of *P5CS1* and *P5CS2* showed that the promoter of *P5CS1* is enriched in putative binding sites for TFs related to abiotic stress, such as ABA response elements, AP2/EREBP, ERF2, DREB/CBF, and MYB binding sites (Fichman et al. 2015). The promoter of *P5CS2*, on the contrary, is enriched in putative regulatory elements for TFs related to biotic stresses such as HD-HOX, AP2/EREBP, MYB, WRKY, and bZIP (Fichman et al. 2015). Additionally, the promoter of *P5CS2* contains putative binding sites for TFs related to flowering time, such as *SQUAMOSA PROMOTER BINDING-LIKE* (*SPL*) and bHLH factors, and related to pollen development and function such as WRKY2 and WRKY34 (Mattioli et al. 2018).

Most studies on stress-induced or developmental accumulation of proline report good correlation between *P5CS* transcript levels and proline content, indicating that proline biosynthesis is predominantly regulated at the level of transcription. Early studies describing proline accumulation in tomato and grapevine in the absence of increased *P5CS* transcript levels can now be explained by the presence of second *P5CS* isoforms in these species that were unknown at the time (Fujita et al. 1998; Stines et al. 1999). More direct evidence for post-transcriptional regulation of *P5CS* expression was obtained by computational identification of matching micro-RNAs

in potato and chickpea (Shui et al. 2013; Yang et al. 2013). Expression levels of some micro-RNAs during stress were negatively correlated with *P5CS* transcript levels, but direct proof for their involvement in *P5CS* regulation is still missing. Epigenetic regulation caused by modulations of the methylation pattern of specific genes may also contribute to *P5CS* regulation. Changes in DNA methylation induced by environmental stresses or by developmental stimuli are known to modulate both plant stress tolerance and developmental processes, respectively (Bastow et al. 2004; Chinnusamy and Zhu 2009; Karan et al. 2012; Richards 2006). In rice, differential methylation of a *P5CS* gene has been proposed as a mechanism for trans-generational stress memory (Zhang et al. 2013). As mentioned above, another level of regulation is added by allosteric inhibition of the γ-glutamyl kinase activity of plant P5CS proteins by proline (Hu et al. 1992; Zhang et al. 1995). It is so far unknown if and how feedback inhibition of P5CS may be overcome in tissues or conditions where proline accumulation is desired. Immunoblot analyses of Arabidopsis P5CS1 protein levels in different protein phosphatase 2C mutants indicated the presence of posttranslational modifications or mechanisms to regulate protein stability (Bhaskara et al. 2015).

The concept of P5CS catalyzing the rate-limiting step in proline biosynthesis is contested by several studies reporting higher proline content, especially under stress conditions, upon overexpression of P5CR (De Ronde et al. 2004; Ma et al. 2008; Szoke et al. 1992). The most detailed analysis of *P5CR* expression has again been performed in Arabidopsis. In particular, a *P5CR promoter-GUS* fusion construct showed ubiquitous expression with the highest expression levels in areas of active cell division, in guard cells, and in reproductive tissues, especially pollen and developing seeds (Hua et al. 1997). Similarly, a *P5CR promoter-gene-GFP* fusion construct was expressed ubiquitously in leaves and roots, with highest expression in the root tip (Funck et al. 2010). The 5'-UTR of *P5CR* was found to mediate posttranscriptional regulation by stabilizing *P5CR* transcripts under heat and drought stress while at the same time inhibiting translation, resulting in unchanged protein levels despite strongly increased transcript levels, thus raising the question how P5CR keeps up with increased P5CS-mediated GSA/P5C production during stress (Hua et al. 2001). The biochemical properties of P5CR might solve this apparent conflict, as the activity of purified P5CR was stimulated by high ion concentrations when NADPH was available as electron donor (Forlani et al. 2015; Giberti et al. 2014). Phosphoproteomics studies have revealed two directly adjacent phosphorylation sites at T237 and S238 of Arabidopsis P5CR, but information about the possible function of P5CR phosphorylation is not available (Schulze et al. 2015).

As discussed in Sect. 3.2, it is at present unclear whether OAT-mediated production of GSA constitutes an alternative route for proline biosynthesis or whether it stimulates proline synthesis merely by increasing the level of glutamate. The spatial distribution of *OAT* expression has not been analyzed in Arabidopsis, while in pea the highest activity was detected in cotyledons, followed by true leaves, roots, and seeds (Taylor and Stewart 1981). In pine seedlings, *OAT* transcript levels were highest in the radicle and peaked transiently after germination (Canas et al. 2008). In young Arabidopsis and rice seedlings as well as in radish cotyledons and cashew

leaves, OAT activity or gene expression was induced in response to salt or drought stress (da Rocha et al. 2012; Liu et al. 2018; Roosens et al. 1998; You et al. 2012). Rice *OAT* expression was additionally induced by heat, ABA, brassinolide, and auxin treatment (You et al. 2012). Arabidopsis *oat* knockout mutants developed normally and had unchanged proline content but were unable to utilize arginine as nitrogen source for growth (Funck et al. 2008). In contrast, deletion of *OAT* in rice caused fertility defects and lower proline content together with general symptoms of nitrogen deficiency (Liu et al. 2018). In summary, the available data supports an essential role of OAT in recycling of nitrogen from arginine degradation, but does not demonstrate or exclude the existence of an alternative route for proline biosynthesis.

4.2 Regulation of Genes Coding for Proline Catabolic Enzymes

Since the transporters that mediate the uptake of proline into mitochondria have not been molecularly identified, we know virtually nothing about the regulation of this transport. Once cytosolic proline is imported into mitochondria, it can either be used for mitochondrial protein synthesis or it can be oxidized to glutamate by the sequential action of ProDH and P5CDH (see Sect. 2.2). Copy numbers of *ProDH* genes have not been thoroughly analyzed in available genomes except in Brassicaceae, where an early family-specific genome duplication produced two copies that were further multiplied in the genus *Brassica* (Faes et al. 2015). In Arabidopsis, the best-characterized species, it was shown that both genes, *ProDH1* and *ProDH2*, encode functional proteins with nonredundant but partially overlapping functions (Funck et al. 2010). P5CDH is encoded by a single-copy gene in Arabidopsis and in cereals, while no systematic searches in other plant genomes were reported (Ayliffe et al. 2005; Deuschle et al. 2001). As for proline biosynthesis, most studies on temporal and spatial regulation of proline catabolism were performed in Arabidopsis and we will therefore focus on this species, being aware that this knowledge might not be readily transferred to other plants with different gene copy numbers.

ProDH1, the more extensively characterized proline catabolic gene, is after a weak and transient induction repressed by dehydration but is rapidly and strongly induced by rehydration (Kiyosue et al. 1996; Peng et al. 1996; Verbruggen et al. 1996). In addition, *ProDH1* expression is induced by proline and hypoosmolarity and during HR-mediated pathogen defense but repressed by hyperosmolarity (Cecchini et al. 2011; Kiyosue et al. 1996; Monteoliva et al. 2014; Verbruggen et al. 1996; Yoshiba et al. 1999). More interesting in respect to plant development is the pattern of *ProDH1* expression under non-stressed conditions. Weak constitutive expression of *ProDH1* was observed in most organs of Arabidopsis, while in root tips and in flowers, particularly in pollen grains, stigmata, carpels, and developing seeds, the promoter activity was higher (Nakashima et al. 1998). Analysis of orthol-

ogous *ProDH1* genes in *Brassica* species revealed a very similar expression pattern (Faes et al. 2015). These findings are particularly interesting because they imply that the molecular mechanisms that reduce proline degradation and support accumulation under stress may be quite different from those active in proline accumulation during reproductive development.

Detailed analysis of the Arabidopsis *ProDH1* promoter revealed an ACTCAT motif responsible for proline and hypoosmolarity-mediated induction of *ProDH1* (Nakashima et al. 1998; Satoh et al. 2002). The ACTCAT motif is a typical binding site for basic leucine zipper (bZIP) TFs of the S1-group, and among these AtbZIP53 and AtbZIP1 were shown to physically interact with the promoter of *ProDH1* and to mediate induction of gene expression in response to proline, hypoosmolarity, and low sugar or energy levels (Dietrich et al. 2011; Satoh et al. 2002; Weltmeier et al. 2006). The activity of the *ProDH1* promoter was shown to be additionally controlled by the interaction between ARR18 and bZIP63, the former being a type-B response regulator that functions as a positive osmotic stress response regulator in Arabidopsis seeds, the latter a negative regulator of seed germination upon osmotic stress (Veerabagu et al. 2014). Furthermore, ROS- and redox-mediated signaling was reported to regulate *ProDH1* expression, but the precise mechanisms remain to be determined (Shinde et al. 2016). Immunoblot analyses of ProDH1 protein levels in leaf extracts or isolated mitochondria yielded multiple bands, indicating that ProDH1 may be subject to posttranslational modifications or alternative processing during mitochondrial import (Bhaskara et al. 2015; Cabassa-Hourton et al. 2016; Schertl et al. 2014).

The pattern and regulation of *ProDH2* expression appear largely different from *ProDH1*: *ProDH2* promoter activity was mainly detected in vascular tissue and in the abscission zone of sepals, petals, and stamina (Funck et al. 2010). In contrast to *ProDH1*, transcript levels of *ProDH2* were induced during senescence and by salt stress, whereas the repression by high sugar concentrations and the induction by proline and during pathogen defense were similar for both isoforms (Cecchini et al. 2011; Funck et al. 2010). Similar to *P5CS1*, expression of *ProDH2* was induced by phosphate starvation (Aleksza et al. 2017). Averaged over the entire seedlings or tissues, the expression level of *ProDH2* was much lower compared to *ProDH1*, and accordingly, deletion of *ProDH2* had no influence on the capacity of isolated mitochondria for proline-dependent respiration, whereas for mitochondria isolated from *prodh1* mutants, proline-dependent respiration was undetectable (Cabassa-Hourton et al. 2016; Funck et al. 2010). The strong and specific expression of *ProDH2* in the vascular system and its strong downregulation in the presence of sucrose are consistent with the report of Hanson et al. (2008) that identified *ProDH2*, along with *ASPARAGINE SYNTHETASE1* (*ASN1*) as two of the early targets of bZIP11, a transcription factor induced by SUCROSE NON-FERMENTING1 RELATED KINASE1 (SnRK1) in response to energy deprivation (O'Hara et al. 2013; Weiste et al. 2017). SnRK1 and bZIP11 also provide a direct link between proline metabolism and trehalose signaling and metabolism, which is discussed in more detail in Chap. 8 of this book.

Arabidopsis mutants for either *ProDH1* or *ProDH2* (Funck et al. 2010; Nanjo et al. 2003), as well as transgenic plants with antisense-mediated repression of *ProDH1* and *ProDH2* (Cecchini et al. 2011; Mani et al. 2002), have been generated. While no phenotypic or developmental aberrations were observed under normal conditions, these mutants exhibited enhanced proline accumulation under stress conditions, but stress tolerance and pathogen defense were weakened (Cecchini et al. 2011; Sharma et al. 2011). An unexpected, and as yet unexplained, observation is the hypersensitivity of *prodh1* mutants to exogenous proline under non-stressed conditions (Funck et al. 2010; Nanjo et al. 2003). Toxicity of proline supply was also observed in non-stressed wild-type plants but was proposed to be linked to ProDH activity, with either excess P5C production or excess electron load on the mitochondrial electron transport chain as harmful effects (Hellmann et al. 2000; Miller et al. 2009).

A crucial role in preventing proline toxicity was attributed to P5CDH, either by P5C/GSA detoxification or by withdrawing P5C/GSA from the proposed P5C-proline cycle (Deuschle et al. 2004; Deuschle et al. 2001; Miller et al. 2009). *P5CDH* expression was observed constitutively in Arabidopsis leaves, where it increased with leaf age, and to a lesser extent in roots (Deuschle et al. 2004). In reproductive organs, *P5CDH* was strongly expressed in pollen, developing embryos, and aborted seeds. External proline supply stimulated *P5CDH* expression, although with slower kinetics compared to *ProDH1*, whereas no prominent changes in transcript levels were observed in response to salt stress or pathogen infection (Cecchini et al. 2011; Deuschle et al. 2001; Monteoliva et al. 2014). In contrast to Arabidopsis *P5CDH*, the *P5CDH* gene from flax (*Linum usitatissimum*) was strongly induced by pathogen attack, but only when the plant encountered a virulent rust strain that did not elicit a hypersensitive response (Ayliffe et al. 2002; Mitchell et al. 2006). No upstream elements of *P5CDH* regulation have been identified so far, but transcript levels might be regulated posttranscriptionally by double-strand RNA formation with transcripts from the overlapping *SIMILAR TO RCD ONE 5* (*SRO5*) (Borsani et al. 2005). Recently, a posttranslational mechanism for the regulation of ProDH and P5CDH activity was identified: Binding of DFR1 to both ProDH and P5CDH was found to inhibit their enzymatic activity and could thus explain how proline accumulation and high levels of *ProDH* expression can occur simultaneously (Ren et al. 2018). Strong expression of *DFR1* was observed in inflorescences and in response to salt, drought, and cold stress.

In summary, the knowledge about the regulation of genes and enzymes involved in proline metabolism supports important contributions to stress tolerance and pathogen defense but also to sexual reproduction and other developmental processes both in the absence and presence of stress. Regulation of proline metabolism was found to occur at multiple levels, and therefore we need to be careful when inferring physiological functions from mere correlations between proline content, gene expression, and phenotypic observations.

5 Developmental Processes Influenced by Proline Metabolism

5.1 Proline and Plant Development

The idea that proline may be an active player in plant development, besides being one of the 21 amino acids used for protein synthesis, began to be accepted only at the end of the last century, when different groups detected, under non-stressed conditions, large amounts of proline in the reproductive organs of some plant species (Chiang and Dandekar 1995; Fujita et al. 1998; Mutters et al. 1989; Schwacke et al. 1999; Venekamp and Koot 1988; Walton et al. 1991). Similarly, upregulation of proline biosynthesis genes was reported in flowers, fruits, and seeds of plants not subjected to evident biotic or abiotic stress (Armengaud et al. 2004; Fujita et al. 1998; Schmidt et al. 2007; Schwacke et al. 1999; Vansuyt et al. 1979). Overall, these data indicated that proline levels could locally increase even in the absence of stress. In the vegetative Arabidopsis rosette before floral transition, for example, Chiang and Dandekar (1995) found a percentage of proline, relative to the total amino acidic pool, ranging from 1% to 3% in striking contrast to up to 26% in reproductive tissues after the floral transition (Chiang and Dandekar 1995). A similar result was reported by Schwacke et al. (1999) who measured a proline content in tomato flowers 60 times higher than in any other organ analyzed. The striking difference in proline concentrations between vegetative and floral tissues suggested that proline might play a special role in plant reproduction while raising the problem of the origin of the accumulated proline. As described in Sect. 3, the distribution of proline in plants is subjected to a complex regulation, involving long-distance transport between tissues through vascular vessels (Girousse et al. 1996), active transport from cell to cell and between different cell compartments (Lehmann et al. 2011; Rentsch et al. 1996; Schmidt et al. 2007; Schwacke et al. 1999), direct synthesis within target tissues (Chiang and Dandekar 1995; Mattioli et al. 2018), selective catabolism (Kiyosue et al. 1996; Nanjo et al. 1999b), and the rates of protein synthesis and degradation (Hildebrandt 2018). The complexity of these regulations, by itself, was suggestive of some special importance of proline in plant development, particularly in the reproductive phase. Indeed, although proline is relatively common in plant proteins, because of the frequent occurrence of long stretches of proline and/or hydroxyproline residues in a number of cell wall proteins, such as extensins, arabinogalactan-proteins, and hybrid proline-rich (Hyp/Pro-rich) proteins (Kavi Kishor et al. 2015), it seemed unlikely that, under non-stressed conditions, such large amounts of proline would be accumulated only for the requirements of protein synthesis.

However, differently from stress-induced proline accumulation, a phenomenon generally considered beneficial to plant cells, proline accumulation in the absence of stress drew little attention and was mostly attributed to some type of prior or undetected stress. Chiang and Dandekar (1995), for example, hypothesized that the high content of proline found in anthers and pollen grains of Arabidopsis could

function as a compatible osmolyte to protect pollen grains from the water stress caused by the natural process of dehydration during pollen maturation. A significant step toward the understanding of the role of proline in plant development came from the study of the hairy root syndrome induced by infection with the soil bacterium *Rhizobium rhizogenes*, formerly known as *Agrobacterium rhizogenes* (Trovato et al. 2018). The capability of *R. rhizogenes* to reprogram plant development and induce de novo root synthesis on differentiated tissues has been long studied as a paradigm of plant development control and relies on the integration of a transfer DNA (T-DNA) into the plant genome. It turned out that *rolD*, one of the four "root locus" (*rol*) genes in the T-DNA responsible for hairy root induction, codes for an ornithine cyclodeaminase (OCD), which converts ornithine into proline and ammonium (Trovato et al. 2001). This finding, along with the above-cited proline accumulation in floral organs of plants grown in optimal conditions, disclosed a novel role for proline in plant development, and we now know that proline is critically involved in a number of developmental processes, such as root elongation, floral transition, pollen fertility, and embryo development.

5.2 Germination

Seed germination, a developmental process of enormous physiological and economic relevance, has been sometimes reported to be positively correlated with proline accumulation, particularly under stress conditions, although the observations are rather scarce and a clear-cut demonstration of the involvement of proline in germination is still lacking. Because of the beneficial role that proline accumulation, or more probably proline metabolism, exerts on plant cells under stressful conditions, it may be difficult to distinguish a generic improvement of stress tolerance from a specific effect on seed germination.

Notwithstanding this, a limited number of authors have reported that the accumulation of proline and/or the upregulation of proline biosynthesis genes can improve seed germination rates. Roosens et al. (2002) reported that overexpression of Arabidopsis OAT increased proline biosynthesis and germination rates in transgenic tobacco (*Nicotiana tabacum*) plants under osmotic stress conditions. Similarly, transgenic tobacco plants overexpressing a feedback-insensitive variant of *Vigna aconitifolia* P5CS accumulated high levels of proline and exhibited higher germination rates under stress (Zonglie et al. 2000). A few reports also described positive effects of proline pretreatment on germination rates (Hua-long et al. 2014; Kubala et al. 2015; Posmyk and Janas 2007). However, this procedure, known as "osmopriming," can also function with different compatible solutes and might be dependent on the provision of a carbon and nitrogen source rather than a specific effect of proline.

The more convincing and exhaustive report claiming a positive role of proline metabolism on Arabidopsis germination comes from a study published by Hare et al. (2003) who observed that proline biosynthesis and the oxidative pentose phosphate pathway (OPPP) were induced in parallel during Arabidopsis seed germination.

Antisense inhibition of *P5CS1* (which most likely silences both *P5CS1* and *P5CS2* expression due to the high sequence similarity) delayed seed germination, whereas external proline supply inhibited germination and this inhibition was relieved by addition of artificial electron acceptors. Hare et al. (2003) proposed that proline biosynthesis served to lower the NADPH/$NADP^+$ ratio, which is known to stimulate the OPPP in many organisms and may be needed to provide sufficient ribose for nucleotide synthesis in the geminating seed (Shetty and Wahlqvist 2004).

5.3 Root Growth

In addition to being an essential component of protein biosynthesis in any growing tissue, proline also seems to play a role as a modulator of cell division, especially in the root elongation zone (Biancucci et al. 2015; Wang et al. 2014). This novel role ascribed to proline is not completely surprising as the elongation of the hairy roots induced by transformation by *R. rhizogenes* was originally ascribed to the action of *rolD*, later recognized as a proline producing OCD (Trovato et al. 2001; White et al. 1985). A specific requirement for proline metabolism was also reported in the elongation of Arabidopsis and corn primary roots at low water potential (Verslues and Skarp 1999; Sharma, 2011). Sharma et al. (2011) proposed that proline synthesized and accumulated in leaves was transferred to the root, where it was degraded to provide energy and building blocks for sustained root growth. In non-stressed Arabidopsis seedlings, exogenous proline supplementation, at micromolar concentration, was shown to induce root elongation and branching (Mattioli et al. 2009). Contrarily, exogenous supply of proline at millimolar concentrations inhibited root growth with symptoms resembling programmed cell death (Hellmann et al. 2000). Arabidopsis mutants with strongly reduced capacity to synthesize proline (*p5cs1/p5cs1;P5CS2/p5cs2)* displayed reduced root growth by reduction of the area of active cell division in the root meristem (Mattioli et al. 2009). In both Arabidopsis and corn *p5cs* mutants, reduced root growth was correlated with decreased expression levels of cyclins and other cell cycle-related genes, suggesting a link between proline or proline biosynthesis and cell cycle regulation (Mattioli et al. 2009; Wang et al. 2014).

5.4 Flowering

After the first demonstration of the importance of the *rol* genes in hairy root induction (White et al. 1985), *rolD/OCD* from *R. rhizogenes* has been overexpressed in tobacco, tomato, and Arabidopsis (Bettini et al. 2003; Falasca et al. 2010; Mauro et al. 1996). The ectopic expression of *rolD*, driven by its own promoter, was subjected to a complex developmental regulation and eventually led to early flowering and formation of increased numbers of flowers (Trovato et al. 1997). Transgenic tobacco plants

expressing *rolD* under the control of its own promoter reached anthesis 60 to 75 days before untransformed plants, produced abundant and long-lasting inflorescences, and exhibited an overall altered morphology with height reduction and bract-like leaves (Mauro et al. 1996). In addition, in vitro flower formation on tissue explants was stimulated in *rolD* transgenic plants, presumably by RolD-mediated conversion of ornithine to proline (Mauro et al. 1996; Trovato et al. 2001). Switchgrass (*Panicum virgatum*) plants overexpressing a heterospecific *P5CS* gene flowered earlier than control plants and produced more tillers after mowing (Guan et al. 2018). In transgenic Arabidopsis plants overexpressing an additional copy of *P5CS1* driven by the strong CaMV 35S promoter, the time until flowering induction was shortened and axillary coflorescences proliferated, especially in short-day conditions (Mattioli et al. 2008). The overexpression of the transgenic *P5CS1* copy was only transient though, and soon after the floral transition, a downregulation of both *P5CS1* and *P5CS2* took place, likely because of gene silencing (Mattioli et al. 2008). Unfortunately, most of the numerous studies on P5CS overexpression in other plant species focused on drought and salt stress tolerance and did not systematically analyze flowering. Consistent with a role of proline synthesis in flowering induction, upregulation of both proline biosynthesis (*P5CS, P5CR*) and transport (*ProT*) genes has been reported, under normo-osmotic conditions, in reproductive organs, such as flowers, inflorescences, and anthers (Savouré et al. 1997; Schwacke et al. 1999; Verbruggen et al. 1993). Intriguingly, also the expression of the proline catabolic genes (*ProDH, P5CDH*) was reported to increase in reproductive tissues in the absence of stress (Deuschle et al. 2001; Verbruggen et al. 1996), in contrast with the steep downregulation of these genes observed under stress conditions (Kiyosue et al. 1996; Peng et al. 1996). It is as yet unknown, whether upregulation of proline catabolic genes by high proline concentrations under non-stressed conditions causes rapid metabolic cycling or whether posttranscriptional mechanisms like the interaction with DFR1 limit the rate of proline degradation (Ren et al. 2018).

The antisense expression of *P5CS1* (likely affecting both *P5CS1* and *P5CS2*, see above) has been shown to inhibit Arabidopsis bolting (Nanjo et al. 1999a). Ambiguous observations have been reported for insertional mutants: in two labs, growth and flowering of *p5cs1* single mutants were not different from wild-type plants, whereas in a third lab, a delay in the onset of bolting was observed (Funck et al. 2012; Mattioli et al. 2008; Székely et al. 2008). For *p5cs2* single mutants and near-double mutants (*p5cs1/p5cs1*, *P5CS2/p5cs2*), a generally slower development was observed (Funck et al. 2012; Mattioli et al. 2012). Similarly, silencing of *P5CS2* expression in *Lotus japonicus* produced several lines with defects in flower and seed formation (S. Signorelli, unpublished observations). Altogether, these data indicate that both *P5CS1* and *P5CS2* can modulate flowering and suggest that proline plays a role in floral transition, bolting, and coflorescence emergence.

In Arabidopsis, and probably all flowering plants, multiple signaling pathways respond to a range of environmental (photoperiod, cold, heat) and endogenous (metabolites, gibberellin, age) stimuli and converge to induce the conversion of vegetative shoot meristems into floral meristems (Khan et al. 2014; Srikanth and Schmid 2011). CONSTANS, one of the master regulators of the photoperiodic

pathway of flowering induction, has been identified as an inducer of *P5CS2* in Arabidopsis (Samach et al. 2000). FLOWERING LOCUS C (FLC) was identified as inducer of *P5CS1* along with ELONGATED HYPOCOTYL 5 (HY5), which was found to be a crucial factor in the light-dependent induction of *P5CS1* by stress, indicating that proline may indeed contribute to the light-dependent regulation of flowering (Abrahám et al. 2003; Chen et al. 2018; Feng et al. 2016; Hayashi et al. 2000). Recently, a number of plant species, belonging to different taxonomic groups, have been reported to flower rapidly after exposure to a wide range of different stressors (Wada and Takeno 2010). Since the responses to many types of stress involve proline accumulation, it is tempting to speculate that stress-induced flowering and proline-induced flowering in non-stressed plants may rely on a common mechanism. The distribution of proline under normal physiological conditions, however, seems partly different from that found under stress conditions: In Arabidopsis, a locally and temporally confined increase of proline in the shoot apical meristem at floral transition has been reported, whereas, under stress conditions, proline is accumulated at high levels in all the tissues of the plant (Mattioli et al. 2008). Overall, the body of accumulated evidence points to proline as a modulator of floral transition, although its mechanism of action, the genes involved in this process, and the interaction with other regulatory pathways still need to be revealed in detail.

5.5 Pollen Fertility

Among floral organs, the highest proline contents have been observed in the pollen of many plant species including Arabidopsis, tomato, dandelion (*Taraxacum officinale*), willow (*Salix sp.*), and petunia (*Petunia hybrida*) (Auclair and Jamieson 1948; Chiang and Dandekar 1995; Hong-qi et al. 1982; Schwacke et al. 1999). In grass pollen, proline was the most abundant amino acid, accounting for up to 1.65% of pollen dry weight (Bathurst 1954). Proline was the most abundant amino acid in anthers of devil's trumpet (*Datura metel*) and the only one found to increase during pollen development (Sangwan 1978). In addition to this correlative evidence, recently proline has been shown to be essential for pollen development and fertility by two research groups, who independently reported that in Arabidopsis *p5cs1/p5cs2* double-mutant pollen was misshaped and infertile (Funck et al. 2012; Mattioli et al. 2012). The morphological abnormalities were accompanied by lack of storage compounds and nuclei and appeared late in pollen development, starting from stage 11 of anther development. The requirement for proline biosynthesis was specific for pollen, because only the pollen failed to transmit both *p5cs* mutant alleles simultaneously, whereas *p5cs1/p5cs2* double mutant egg cells showed almost no compromised fertility. Importantly, exogenous L-proline, supplemented in planta to developing anthers of p5cs1/p5cs1 P5CS2/p5cs2 near-double mutant plants, allowed the formation of fully developed and fertile *p5cs1/p5cs2* double mutant pollen (Mattioli et al. 2012). Quite surprisingly, Arabidopsis plants carrying mutations in *P5CR*, the gene coding for the second and final step of proline biosynthesis, are embryo lethal, but

not male sterile, presumably because P5CR is an exceptionally long-lived protein (Funck et al. 2012). High expression of the specific proline transporter ProT1 in the pollen of tomato and Arabidopsis raised the question, whether proline is imported during pollen development or is synthesized cell autonomously (Grallath et al. 2005; Schwacke et al. 1999). By targeting *P5CS* expression to different tissues in Arabidopsis anthers, Mattioli et al. (2018) demonstrated that only proline synthesized within developing pollen grains can fully restore fertility of *p5cs1/p5cs2* double mutant pollen. Consistently, both *P5CS1* and *P5CS2* genes exhibit a strong and specific expression in microspores and pollen grains but are essentially unexpressed in surrounding sporophytic tissues of the anther, as shown by β-glucuronidase (GUS) analysis, and inferred by bioinformatic analysis of *P5CS1* and *P5CS2* promoters (Mattioli et al. 2018).

5.6 Embryo Development

The analysis of *p5cs2* knockout mutants in Arabidopsis has disclosed an essential role of proline in plant embryogenesis. Three research groups have independently isolated and characterized two *p5cs2* T-DNA insertion mutants (Funck et al. 2012; Mattioli et al. 2009; Székely et al. 2008). Quite surprisingly, despite the high sequence similarity shared by the two paralogous genes, and although the same pattern of expression was detected for both P5CS1 and P5CS2 transcripts by in situ hybridisation of sections of shoot apical meristems and embryos (Mattioli et al. 2009; Székely et al. 2008), *p5cs2*, but not *p5cs1* mutants, are embryo lethal suggesting a specific role of P5CS2 or posttranscriptional repression of P5CS1 activity during embryogenesis. P5CS2-GFP fusion proteins were uniformly distributed in the cytosol of Arabidopsis embryos, whereas P5CS1-GFP formed cytoplasmic speckles, possibly indicating that P5CS1 is inactivated by aggregation (Székely et al. 2008). Spraying flowers with proline, induction of *P5CS1* expression by salt stress, or in vitro cultivation of immature seeds allowed rescuing homozygous *p5cs2* mutants, which were retarded in development but produced viable seeds under favorable conditions (Funck et al. 2012; Mattioli et al. 2009; Székely et al. 2008). The reason why homozygous *p5cs2* embryos die only in the siliques of heterozygous, but not of homozygous, mutants is not yet fully understood. Potentially, the slowly developing homozygous mutants are aborted when the faster-growing wild-type and heterozygous embryos in neighboring seeds reach maturity. In addition, microscopic analysis of the malformed *p5cs2* embryos revealed various aberrations typically associated with a defective cell cycle, such as anomalous orientations of cellular division planes, indicating that low proline levels may similarly inhibit cell cycle progression in embryos as in the root meristem (Mattioli et al. 2009). In corn *pro1* mutants, in which the independently evolved *P5CS2* of corn is inactivated, storage compounds in the seed endosperm of homozygous mutant seeds were strongly reduced, but formation of viable embryos still occurred (Wang et al. 2017). However, without exogenous proline supply, *pro1* homozygous mutants were seedling lethal and successful propagation has not been reported.

In addition to P5CS2, also P5CR, the enzyme involved in the second and final step of proline synthesis, is essential for embryogenesis as shown by Funck et al. (2012), who characterized two *p5cr* mutants, found in the Salk collection and annotated as embryo lethal in the SeedGenes database (SeedGenes Project. http://seedgenes.org). Intriguingly, any attempt to rescue homozygous *p5cr* embryos by proline supplementation was ineffective, differently from *p5cs2* mutants. Expression of a P5CR-GFP fusion protein under control of the endogenous *P5CR* promoter, which is active in developing embryos, reverted the embryo-lethal phenotype, while CaMV-35S-driven overexpression of P5CR-GFP in vegetative tissues was ineffective (Funck et al. 2012). These results indicate that, similarly to the situation in the pollen, long-distance transport of proline cannot fully substitute for local biosynthesis in tissues that critically depend on proline.

5.7 Role of Proline Metabolism in Development Under Stress

The accumulation of proline both during certain developmental processes and in response to stress is frequently regarded as two different phenomena which, accordingly, have been treated as separate chapters in this book. However, the large increase in proline content observed in reproductive tissues of most plant species is similar to that observed after many different types of stress, thus posing the question whether the function of proline may be similar in both cases. This seemingly simple question is particularly difficult to tackle because there are some hypotheses but no generally accepted idea of what the function of proline may be, neither under stress conditions nor during development. In addition, also the concept of stress is not as simple as it may seem. What is stress? According to Lichtenthaler (1998), any "unfavorable condition or substance that affects or blocks a plant's metabolism, growth, or development is regarded as stress." It may well be that many "normal" physiological conditions, such as seed and pollen maturation or high-intensity light, are more demanding for a plant than mild environmental stress, such as a transient period of drought or a moderate reduction of the average temperature. According to Chiang and Dandekar (1995), stronger proline accumulation was observed in tissues with low water content, such as embryos and pollen grains, which successfully entered a developmentally induced process of desiccation without loss of cellular and tissue viability. The most probable benefit of proline accumulation in these tissues is based on the kosmotropic properties of proline by which it helps to protect enzymes and membranes of plant cells with low water content (see Chap. 3). In contrast, the increase in proline concentration following osmotic stress may not be sufficient to protect cells because, as discussed in the following chapter, the amount of proline accumulated is typically not sufficient to counterbalance the decrease in the cellular osmotic potential. Another possibility is that proline in (some) reproductive tissues may be a precautionary measure in case of future adverse conditions in order to protect important plant organs and improve the fitness of the species. Consistent with this hypothesis is the observation that *p5cs1* mutants have no aberrant

phenotype under normal conditions but exhibit hypersensitivity to salt and hyperosmolarity stress (Sharma et al. 2011; Székely et al. 2008). On the other hand, as described in detail in the following chapter, regardless of proline accumulation being a response to stress or part of a developmental program, it remains still unclear if its beneficial effects are mediated by accumulation per se or by increased metabolic turnover. As indicated above and discussed in more detail in the next chapter, biosynthesis and degradation of proline have the capacity to change the redox state of the cytosol and the mitochondria, respectively, and may additionally modulate the levels of reactive oxygen species. Since far less details are known about the regulation of proline metabolism during normal development, it is at present difficult to predict downstream effects, although it is tempting to speculate that the accumulation of proline under stress and its accumulation during development are two sides of the same coin. Further studies on proline-dependent signal transduction and actual flux rates of proline metabolism and of the exchange of proline between different tissues or cell types will be needed to fully understand how proline exerts its stress protective and developmental functions.

References

Abrahám E, Rigó G, Székely G, Nagy R, Koncz C, Szabados L (2003) Light-dependent induction of proline biosynthesis by abscisic acid and salt stress is inhibited by brassinosteroid in Arabidopsis. Plant Mol Biol 51:363–372

Aleksza D, Horváth GV, Sándor G, Szabados L (2017) Proline accumulation is regulated by transcription factors associated with phosphate starvation. Plant Physiol 175:555–567. https://doi.org/10.1104/pp.17.00791

Armengaud P, Thiery L, Buhot N, Grenier-de March G, Savouré A (2004) Transcriptional regulation of proline biosynthesis in *Medicago truncatula* reveals developmental and environmental specific features. Physiol Plant 120:442–450. https://doi.org/10.1111/j.0031-9317.2004.00251.x

Auclair JL, Jamieson CA (1948) A qualitative analysis of amino acids in pollen collected by bees. Science 108:357–358. https://doi.org/10.1126/science.108.2805.357

Ayliffe MA, Mitchell HJ, Deuschle K, Pryor AJ (2005) Comparative analysis in cereals of a key proline catabolism gene. Mol Gen Genomics 274:494–505

Ayliffe MA, Roberts JK, Mitchell HJ, Zhang R, Lawrence GJ, Ellis JG, Pryor TJ (2002) A plant gene up-regulated at rust infection sites. Plant Physiol 129:169–180

Bastow R, Mylne JS, Lister C, Lippman Z, Martienssen RA, Dean C (2004) Vernalization requires epigenetic silencing of FLC by histone methylation. Nature 427:164. https://doi.org/10.1038/nature02269

Bathurst NO (1954) The amino-acids of grass pollen. J Exp Bot 5:253–256. https://doi.org/10.1093/jxb/5.2.253

Besnard J, Pratelli R, Zhao C, Sonawala U, Collakova E, Pilot G, Okumoto S (2016) UMAMIT14 is an amino acid exporter involved in phloem unloading in Arabidopsis roots. J Exp Bot 67:6385–6397. https://doi.org/10.1093/jxb/erw412

Besnard J, Zhao C, Avice JC, Vitha S, Hyodo A, Pilot G, Okumoto S (2018) Arabidopsis UMAMIT24 and 25 are amino acid exporters involved in seed loading. J Exp Bot 69:5221–5232. https://doi.org/10.1093/jxb/ery302

Bettini P, Michelotti S, Bindi D, Giannini R, Capuana M, Buaiatti M (2003) Pleiotropic effect of the insertion of *Agrobacterium rhizogenes rolD* gene in tomato (*Lycopersicon esculentum* Mill). Theor Appl Genet 107:831–836. https://doi.org/10.1007/s00122-003-1322-0

Bhaskara GB, Yang T-H, Verslues PE (2015) Dynamic proline metabolism: importance and regulation in water limited environments. Front Plant Sci 6:484. https://doi.org/10.3389/fpls.2015.00484

Biancucci M, Mattioli R, Moubayidin L, Sabatini S, Costantino P, Trovato M (2015) Proline affects the size of the root meristematic zone in Arabidopsis. BMC Plant Biol 15:263. https://doi.org/10.1186/s12870-015-0637-8

Boggess SF, Koeppe DE, Stewart CR (1978) Oxidation of proline by plant mitochondria. Plant Physiol 62:22–25

Borsani O, Zhu J, Verslues PE, Sunkar R, Zhu JK (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. Cell 123:1279–1291. https://doi.org/10.1016/j.cell.2005.11.035

Buchanan BB, Gruissem W, Jones RL (2000) Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville

Büssis D, Heineke D (1998) Acclimation of potato plants to polyethylene glycol-induced water deficit II. Contents and subcellular distribution of organic solutes. J Exp Bot 49:1361–1370

Cabassa-Hourton C et al (2016) Proteomic and functional analysis of proline dehydrogenase 1 link proline catabolism to mitochondrial electron transport in *Arabidopsis thaliana*. Biochem J 473:2623–2634. https://doi.org/10.1042/bcj20160314

Canas RA, Villalobos DP, Diaz-Moreno SM, Canovas FM, Canton FR (2008) Molecular and functional analyses support a role of Ornithine-{delta}-aminotransferase in the provision of glutamate for glutamine biosynthesis during pine germination. Plant Physiol 148:77–88

Cecchini NM, Monteoliva MI, Alvarez ME (2011) Proline dehydrogenase contributes to pathogen defense in Arabidopsis. Plant Physiol 155:1947–1959. https://doi.org/10.1104/pp.110.167163

Chen Q, Zheng Y, Luo L, Yang Y, Hu X, Kong X (2018) Functional FRIGIDA allele enhances drought tolerance by regulating the P5CS1 pathway in *Arabidopsis thaliana*. Biochem Biophys Res Commun 495:1102–1107. https://doi.org/10.1016/j.bbrc.2017.11.149

Chiang HH, Dandekar AM (1995) Regulation of proline accumulation in *Arabidopsis thaliana* (L) Heynh during development and in response to desiccation. Plant Cell Environ 18:1280–1290. https://doi.org/10.1111/J.1365-3040.1995.Tb00187.X

Chinnusamy V, Zhu J-K (2009) Epigenetic regulation of stress responses in plants. Curr Opin Plant Biol 12:133–139. https://doi.org/10.1016/j.pbi.2008.12.006

da Rocha IM, Vitorello VA, Silva JS, Ferreira-Silva SL, Viégas RA, Silva EN, Silveira JA (2012) Exogenous ornithine is an effective precursor and the delta-ornithine amino transferase pathway contributes to proline accumulation under high N recycling in salt-stressed cashew leaves. J Plant Physiol 169:41–49. https://doi.org/10.1016/j.jplph.2011.08.001

Daygon VD et al (2017) Metabolomics and genomics combine to unravel the pathway for the presence of fragrance in rice. Sci Rep 7:8767. https://doi.org/10.1038/s41598-017-07693-9

De Ronde JA, Cress WA, Kruger GH, Strasser RJ, Van Staden J (2004) Photosynthetic response of transgenic soybean plants, containing an Arabidopsis *P5CR* gene, during heat and drought stress. J Plant Physiol 161:1211–1224

Delauney AJ, Hu CA, Kishor PB, Verma DP (1993) Cloning of ornithine delta-aminotransferase cDNA from *Vigna aconitifolia* by trans-complementation in Escherichia coli and regulation of proline biosynthesis. J Biol Chem 268:18673–18678

Delauney AJ, Verma DP (1990) A soybean gene encoding delta 1-pyrroline-5-carboxylate reductase was isolated by functional complementation in *Escherichia coli* and is found to be osmoregulated. Mol Gen Genet 221:299–305

Deuschle K et al (2004) The role of δ^1-pyrroline-5-carboxylate dehydrogenase in proline degradation. Plant Cell 16:3413–3425

Deuschle K, Funck D, Hellmann H, Däschner K, Binder S, Frommer WB (2001) A nuclear gene encoding mitochondrial Δ^1-pyrroline-5-carboxylate dehydrogenase and its potential role in protection from proline toxicity. Plant J 27:345–356. https://doi.org/10.1046/j.1365-313X.2001.01101.x

Di Martino C, Pizzuto R, Pallotta ML, De Santis A, Passarella S (2006) Mitochondrial transport in proline catabolism in plants: the existence of two separate translocators in mitochondria isolated from durum wheat seedlings. Planta 223:1123–1133

Dietrich K, Weltmeier F, Ehlert A, Weiste C, Stahl M, Harter K, Dröge-Laser W (2011) Heterodimers of the Arabidopsis transcription factors bZIP1 and bZIP53 reprogram amino acid metabolism during low energy stress. Plant Cell 23:381–395. https://doi.org/10.1105/tpc.110.075390

Dinkeloo K, Boyd S, Pilot G (2018) Update on amino acid transporter functions and on possible amino acid sensing mechanisms in plants. Semin Cell Dev Biol 74:105–113. https://doi.org/10.1016/j.semcdb.2017.07.010

Elthon TE, Stewart CR (1981) Submitochondrial location and electron transport characteristics of enzymes involved in proline oxidation. Plant Physiol 67:780–784

Elthon TE, Stewart CR (1982) Proline oxidation in corn mitochondria : involvement of NAD, relationship to ornithine metabolism, and sidedness on the inner membrane. Plant Physiol 70:567–572

Elthon TE, Stewart CR, Bonner WD (1984) Energetics of proline transport in corn mitochondria. Plant Physiol 75:951–955

Fabro G, Kovacs I, Pavet V, Szabados L, Alvarez ME (2004) Proline accumulation and *AtP5CS2* gene activation are induced by plant-pathogen incompatible interactions in Arabidopsis. Mol Plant-Microbe Interact 17:343–350

Faes P et al (2015) Molecular evolution and transcriptional regulation of the oilseed rape proline dehydrogenase genes suggest distinct roles of proline catabolism during development. Planta 241:403–419. https://doi.org/10.1007/s00425-014-2189-9

Falasca G, Altamura MM, D'Angeli S, Zaghi D, Costantino P, Mauro ML (2010) The *rolD* oncogene promotes axillary bud and adventitious root meristems in Arabidopsis. Plant Physiol Biochem 48:797–804. https://doi.org/10.1016/j.plaphy.2010.06.002

Feng XJ, Li JR, Qi SL, Lin QF, Jin JB, Hua XJ (2016) Light affects salt stress-induced transcriptional memory of *P5CS1* in Arabidopsis. Proc Natl Acad Sci U S A 113:E8335–e8343. https://doi.org/10.1073/pnas.1610670114

Fichman Y, Gerdes SY, Kovács H, Szabados L, Zilberstein A, Csonka L (2015) Evolution of proline biosynthesis: enzymology, bioinformatics, genetics, and transcriptional regulation. Biol Rev Camb Philos Soc 90:1065–1099. https://doi.org/10.1111/brv.12146

Fischer WN, Kwart M, Hummel S, Frommer WB (1995) Substrate specificity and expression profile of amino acid transporters (AAPs) in Arabidopsis. J Biol Chem 270:16315–16320

Forlani G, Bertazzini M, Zarattini M, Funck D, Ruszkowski M, Nocek B (2015) Functional properties and structural characterization of rice delta(1)-pyrroline-5-carboxylate reductase. Front Plant Sci 6:565. https://doi.org/10.3389/fpls.2015.00565

Forlani G, Scainelli D, Nielsen E (1997) δ^1-Pyrroline-5-carboxylate dehydrogenase from cultured cells of potato (purification and properties). Plant Physiol 113:1413–1418

Fujiki Y, Teshima H, Kashiwao S, Kawano-Kawada M, Ohsumi Y, Kakinuma Y, Sekito T (2017) Functional identification of AtAVT3, a family of vacuolar amino acid transporters, in Arabidopsis. FEBS Lett 591:5–15. https://doi.org/10.1002/1873-3468.12507

Fujita T, Maggio A, Garcia-Rios M, Bressan RA, Csonka LN (1998) Comparative analysis of the regulation of expression and structures of two evolutionarily divergent genes for Delta1-pyrroline-5-carboxylate synthetase from tomato. Plant Physiol 118:661–674

Funck D, Eckard S, Müller G (2010) Non-redundant functions of two proline dehydrogenase isoforms in Arabidopsis. BMC Plant Biol 10:70. https://doi.org/10.1186/1471-2229-10-70

Funck D, Mattioli R, Biancucci M, Mosca L, Trovato M (2019) Proline biosynthesis: localisation matters. Paper presented at the 32nd Conference Molecular Biology of Plants, Dabringhausen, Germany,

Funck D, Stadelhofer B, Koch W (2008) Ornithine-δ-aminotransferase is essential for arginine catabolism but not for proline biosynthesis. BMC Plant Biol 8:40. https://doi.org/10.1186/1471-2229-8-40

Funck D, Winter G, Baumgarten L, Forlani G (2012) Requirement of proline synthesis during Arabidopsis reproductive development. BMC Plant Biol 12:191. https://doi.org/10.1186/1471-2229-12-191

Giberti S, Funck D, Forlani G (2014) Δ^1-Pyrroline-5-carboxylate reductase from *Arabidopsis thaliana*: stimulation or inhibition by chloride ions and feedback regulation by proline depend on whether NADPH or NADH acts as co-substrate. New Phytol 202:911–919. https://doi.org/10.1111/nph.12701

Ginguay A, Cynober L, Curis E, Nicolis I (2017) Ornithine aminotransferase, an important glutamate-metabolizing enzyme at the crossroads of multiple metabolic pathways. Biology 6. https://doi.org/10.3390/biology6010018

Girousse C, Bournoville R, Bonnemain J-L (1996) Water deficit-induced changes in concentrations in proline and some other amino acids in the phloem sap of alfalfa. Plant Physiol 111:109–113

Goodman JL, Wang S, Alam S, Ruzicka FJ, Frey PA, Wedekind JE (2004) Ornithine cyclodeaminase: structure, mechanism of action, and implications for the mu-crystallin family. Biochemistry 43:13883–13891. https://doi.org/10.1021/bi048207i

Grallath S, Weimar T, Meyer A, Gumy C, Suter-Grotemeyer M, Neuhaus JM, Rentsch D (2005) The AtProT family. Compatible solute transporters with similar substrate specificity but differential expression patterns. Plant Physiol 137:117–126

Guan C, Huang YH, Cui X, Liu SJ, Zhou YZ, Zhang YW (2018) Overexpression of gene encoding the key enzyme involved in proline-biosynthesis (*PuP5CS*) to improve salt tolerance in switchgrass (*Panicum virgatum* L.). Plant Cell Rep 37:1187–1199. https://doi.org/10.1007/s00299-018-2304-7

Hanson J, Hanssen M, Wiese A, Hendriks MMWB, Smeekens S (2008) The sucrose regulated transcription factor bZIP11 affects amino acid metabolism by regulating the expression of *ASPARAGINE SYNTHETASE1* and *PROLINE DEHYDROGENASE2*. Plant J 53:935–949. https://doi.org/10.1111/j.1365-313X.2007.03385.x

Hare P, Cress W, van Staden J (2003) A regulatory role for proline metabolism in stimulating *Arabidopsis thaliana* seed germination. Plant Growth Regul 39:41–50

Hayashi F, Ichino T, Osanai M, Wada K (2000) Oscillation and regulation of proline content by *P5CS* and *ProDH* gene expressions in the light/dark cycles in *Arabidopsis thaliana* L. Plant Cell Physiol 41:1096–1101. https://doi.org/10.1093/pcp/pcd036

Hellmann H, Funck D, Rentsch D, Frommer WB (2000) Hypersensitivity of an Arabidopsis sugar signaling mutant toward exogenous proline application. Plant Physiol 123:779–789

Hildebrandt TM (2018) Synthesis versus degradation: directions of amino acid metabolism during Arabidopsis abiotic stress response. Plant Mol Biol 98:121–135. https://doi.org/10.1007/s11103-018-0767-0

Hill RW, Wyse GA, Anderson M (2016) Animal physiology. Sinauer Associates, Sunderland

Hirner A et al (2006) Arabidopsis LHT1 is a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. Plant Cell 18:1931–1946. https://doi.org/10.1105/tpc.106.041012

Hong-qi Z, Croes AF, Linskens HF (1982) Protein synthesis in germinating pollen of *Petunia*: role of proline. Planta 154:199–203. https://doi.org/10.1007/BF00387864

Hu CA, Delauney AJ, Verma DP (1992) A bifunctional enzyme (delta 1-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. Proc Natl Acad Sci U S A 89:9354–9358

Hua XJ, van de Cotte B, Van Montagu M, Verbruggen N (1997) Developmental regulation of pyrroline-5-carboxylate reductase gene expression in Arabidopsis. Plant Physiol 114:1215–1224

Hua XJ, van de Cotte B, Van Montagu M, Verbruggen N (2001) The 5′ untranslated region of the *At-P5R* gene is involved in both transcriptional and post-transcriptional regulation. Plant J 26:157–169

Hua-long L, Han-jing S, Jing-guo W, Yang L, De-tang Z, Hong-wei Z (2014) Effect of seed soaking with exogenous proline on seed germination of rice under salt stress. J Northeast Aric Univ 21:1–6. https://doi.org/10.1016/S1006-8104(14)60062-3

Huang AHC, Cavalieri AJ (1979) Proline oxidase and water stress-induced proline accumulation in spinach leaves. Plant Physiol 63:531–535

Karan R, DeLeon T, Biradar H, Subudhi PK (2012) Salt stress induced variation in DNA methylation pattern and its influence on gene expression in contrasting rice genotypes. PLoS One 7:e40203. https://doi.org/10.1371/journal.pone.0040203

Kavi Kishor P, Hong Z, Miao GH, Hu C, Verma D (1995) Overexpression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol 108:1387–1394. https://doi.org/10.1104/pp.108.4.1387

Kavi Kishor PB, Hima Kumari P, Sunita MSL, Sreenivasulu N (2015) Role of proline in cell wall synthesis and plant development and its implications in plant ontogeny. Front Plant Sci 6:544. https://doi.org/10.3389/fpls.2015.00544

Kesari R et al (2012) Intron-mediated alternative splicing of Arabidopsis *P5CS1* and its association with natural variation in proline and climate adaptation. Proc Natl Acad Sci U S A 109:9197–9202. https://doi.org/10.1073/pnas.1203433109

Khan MR, Ai XY, Zhang JZ (2014) Genetic regulation of flowering time in annual and perennial plants. Wiley Interdiscip Rev RNA 5:347–359. https://doi.org/10.1002/wrna.1215

Kim G-B, Nam Y-W (2013) A novel Δ^1-pyrroline-5-carboxylate synthetase gene of *Medicago truncatula* plays a predominant role in stress-induced proline accumulation during symbiotic nitrogen fixation. J Plant Physiol 170:291–302. https://doi.org/10.1016/j.jplph.2012.10.004

Kiyosue T, Yoshiba Y, Yamaguchi-Shinozaki K, Shinozaki K (1996) A nuclear gene encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregulated by proline but downregulated by dehydration in Arabidopsis. Plant Cell 8:1323–1335. https://doi.org/10.1105/tpc.8.8.1323

Korasick DA et al (2019) Structural and biochemical characterization of Aldehyde Dehydrogenase 12, the last enzyme of proline catabolism in plants. J Mol Biol 431:576–592. https://doi.org/10.1016/j.jmb.2018.12.010

Kubala S, Wojtyla Ł, Quinet M, Lechowska K, Lutts S, Garnczarska M (2015) Enhanced expression of the proline synthesis gene *P5CSA* in relation to seed osmopriming improvement of *Brassica napus* germination under salinity stress. J Plant Physiol 183:1–12. https://doi.org/10.1016/j.jplph.2015.04.009

Ladwig F et al (2012) *Siliques Are Red1* from Arabidopsis acts as a bidirectional amino acid transporter that is crucial for the amino acid homeostasis of siliques. Plant Physiol 158:1643–1655. https://doi.org/10.1104/pp.111.192583

Lehmann S, Gumy C, Blatter E, Boeffel S, Fricke W, Rentsch D (2011) *In planta* function of compatible solute transporters of the AtProT family. J Exp Bot 62:787–796. https://doi.org/10.1093/jxb/erq320

Lichtenthaler HK (1998) The stress concept in plants: an introduction. Ann N Y Acad Sci 851:187–198. https://doi.org/10.1111/j.1749-6632.1998.tb08993.x

Liu C et al (2018) Ornithine δ-aminotransferase is critical for floret development and seed setting through mediating nitrogen reutilization in rice. Plant J 96:842–854. https://doi.org/10.1111/tpj.14072

Ma L, Zhou E, Gao L, Mao X, Zhou R, Jia J (2008) Isolation, expression analysis and chromosomal location of *P5CR* gene in common wheat (*Triticum aestivum* L.). S Afr J Bot 74:705

Mani S, Van De Cotte B, Van Montagu M, Verbruggen N (2002) Altered levels of proline dehydrogenase cause hypersensitivity to proline and its analogs in Arabidopsis. Plant Physiol 128:73–83

Mattioli R, Biancucci M, El Shall A, Mosca L, Costantino P, Funck D, Trovato M (2018) Proline synthesis in developing microspores is required for pollen development and fertility. BMC Plant Biol 18:356. https://doi.org/10.1186/s12870-018-1571-3

Mattioli R, Biancucci M, Lonoce C, Costantino P, Trovato M (2012) Proline is required for male gametophyte development in Arabidopsis. BMC Plant Biol 12:236. https://doi.org/10.1186/1471-2229-12-236

Mattioli R, Falasca G, Sabatini S, Altamura MM, Costantino P, Trovato M (2009) The proline biosynthetic genes *P5CS1* and *P5CS2* play overlapping roles in Arabidopsis flower transition but not in embryo development. Physiol Plant 137:72–85. https://doi.org/10.1111/j.1399-3054.2009.01261.x

Mattioli R, Marchese D, D'Angeli S, Altamura MM, Costantino P, Trovato M (2008) Modulation of intracellular proline levels affects flowering time and inflorescence architecture in Arabidopsis. Plant Mol Biol 66:277–288. https://doi.org/10.1007/s11103-007-9269-1

Mauro ML, Trovato M, De Paolis A, Gallelli A, Costantino P, Altamura MM (1996) The plant oncogene *rolD* stimulates flowering in transgenic tobacco plants. Dev Biol 180:693–700. https://doi.org/10.1006/dbio.1996.0338

Meister A, Radhakrishnan AN, Buckley SD (1957) Enzymatic synthesis of L-pipecolic acid and L-proline. J Biol Chem 229:789–800

Mestichelli LJ, Gupta RN, Spenser ID (1979) The biosynthetic route from ornithine to proline. J Biol Chem 254:640–647

Mezl VA, Knox WE (1976) Properties and analysis of a stable derivative of pyrroline-5-carboxylic acid for use in metabolic studies. Anal Biochem 74:430–440

Miller G, Honig A, Stein H, Suzuki N, Mittler R, Zilberstein A (2009) Unraveling δ^1-pyrroline-5-carboxylate-proline cycle in plants by uncoupled expression of proline oxidation enzymes. J Biol Chem 284:26482–26492. Epub 22009 Jul 26427

Mitchell HJ, Ayliffe MA, Rashid KY, Pryor AJ (2006) A rust-inducible gene from flax (*fis1*) is involved in proline catabolism. Planta 223:213–222

Monne M et al (2018) Uncoupling proteins 1 and 2 (UCP1 and UCP2) from *Arabidopsis thaliana* are mitochondrial transporters of aspartate, glutamate, and dicarboxylates. J Biol Chem 293:4213–4227. https://doi.org/10.1074/jbc.RA117.000771

Monteoliva MI, Rizzi YS, Cecchini NM, Hajirezaei MR, Alvarez ME (2014) Context of action of proline dehydrogenase (ProDH) in the hypersensitive response of Arabidopsis. BMC Plant Biol 14:21. https://doi.org/10.1186/1471-2229-14-21

Moxley MA, Zhang L, Christgen S, Tanner JJ, Becker DF (2017) Identification of a conserved histidine as being critical for the catalytic mechanism and functional switching of the multifunctional Proline utilization A protein. Biochemistry 56:3078–3088. https://doi.org/10.1021/acs.biochem.7b00046

Müller B et al (2015) Amino acid export in developing Arabidopsis seeds depends on UmamiT facilitators. Curr Biol 25:3126–3131. https://doi.org/10.1016/j.cub.2015.10.038

Murahama M, Yoshida T, Hayashi F, Ichino T, Sanada Y, Wada K (2001) Purification and characterization of Delta(1)-pyrroline-5-carboxylate reductase isoenzymes, indicating differential distribution in spinach (*Spinacia oleracea* L.) leaves. Plant Cell Physiol 42:742–750

Mutters RG, Ferreira LGR, Hall AE (1989) Proline content of the anthers and pollen of heat-tolerant and heat-sensitive cowpea subjected to different temperatures. Crop Sci 29:1497–1500

Nakashima K, Satoh R, Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K (1998) A gene encoding proline dehydrogenase is not only induced by proline and hypoosmolarity, but is also developmentally regulated in the reproductive organs of Arabidopsis. Plant Physiol 118:1233–1241. https://doi.org/10.1104/pp.118.4.1233

Nanjo T, Fujita M, Seki M, Kato T, Tabata S, Shinozaki K (2003) Toxicity of free proline revealed in an Arabidopsis T-DNA-tagged mutant deficient in proline dehydrogenase. Plant Cell Physiol 44:541–548

Nanjo T et al (1999a) Biological functions of proline in morphogenesis and osmotolerance revealed in antisense transgenic *Arabidopsis thaliana*. Plant J 18:185–193

Nanjo T, Kobayashi M, Yoshiba Y, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (1999b) Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. FEBS Lett 461:205–210. https://doi.org/10.1016/S0014-5793(99)01451-9

Noguchi M, Koiwai A, Tamaki E (1966) Studies on nitrogen metabolism in tobacco plants. Agric Biol Chem 30:452–456. https://doi.org/10.1080/00021369.1966.10858622

Nozaki H (2005) A new scenario of plastid evolution: plastid primary endosymbiosis before the divergence of the "Plantae," emended. J Plant Res 118:247–255. https://doi.org/10.1007/s10265-005-0219-1

O'Hara LE, Paul MJ, Wingler A (2013) How do sugars regulate plant growth and development? New insight into the role of trehalose-6-phosphate. Mol Plant 6:261–274. https://doi.org/10.1093/mp/sss120

Peng Z, Lu Q, Verma DPS (1996) Reciprocal regulation of Δ^1-pyrroline-5-carboxylate synthetase and proline dehydrogenase genes controls proline levels during and after osmotic stress in plants. Mol Gen Genet 253:334–341. https://doi.org/10.1007/pl00008600

Per TS, Khan NA, Reddy PS, Masood A, Hasanuzzaman M, Khan MIR, Anjum NA (2017) Approaches in modulating proline metabolism in plants for salt and drought stress tolerance: Phytohormones, mineral nutrients and transgenics. Plant Physiol Biochem 115:126–140. https://doi.org/10.1016/j.plaphy.2017.03.018

Perchlik M, Foster J, Tegeder M (2014) Different and overlapping functions of Arabidopsis LHT6 and AAP1 transporters in root amino acid uptake. J Exp Bot 65:5193–5204. https://doi.org/10.1093/jxb/eru278

Pohlmeyer K, Soll J, Steinkamp T, Hinnah S, Wagner R (1997) Isolation and characterization of an amino acid-selective channel protein present in the chloroplastic outer envelope membrane. Proc Natl Acad Sci U S A 94:9504–9509. https://doi.org/10.1073/pnas.94.17.9504

Porcelli V et al (2018) Molecular identification and functional characterization of a novel glutamate transporter in yeast and plant mitochondria. Biochim Biophys Acta 1859:1249–1258. https://doi.org/10.1016/j.bbabio.2018.08.001

Posmyk MM, Janas KM (2007) Effects of seed hydropriming in presence of exogenous proline on chilling injury limitation in *Vigna radiata* L. seedlings. Acta Physiol Plant 29:509–517. https://doi.org/10.1007/s11738-007-0061-2

Rayapati PJ, Stewart CR, Hack E (1989) Pyrroline-5-carboxylate reductase is in pea (*Pisum sativum* L.) leaf chloroplasts. Plant Physiol 91:581–586

Ren Y et al (2018) DFR1-mediated inhibition of proline degradation pathway regulates drought and freezing tolerance in Arabidopsis. Cell Rep 23:3960–3974. https://doi.org/10.1016/j.celrep.2018.04.011

Rena AB, Splittstoesser WE (1975) Proline dehydrogenase and pyrroline-5-carboxylate reductase from pumpkin cotyledons. Phytochemistry 14:657–661. https://doi.org/10.1016/0031-9422(75)83010-X

Renné P, Dreßen U, Hebbeker U, Hille D, Flügge U-I, Westhoff P, Weber APM (2003) The Arabidopsis mutant *dct* is deficient in the plastidic glutamate/malate translocator DiT2. Plant J 35:316–331. https://doi.org/10.1046/j.1365-313X.2003.01806.x

Rentsch D, Hirner B, Schmelzer E, Frommer WB (1996) Salt stress-induced proline transporters and salt stress-repressed broad specificity amino acid permeases identified by suppression of a yeast amino acid permease-targeting mutant. Plant Cell 8:1437–1446

Richards EJ (2006) Inherited epigenetic variation - revisiting soft inheritance. Nat Rev Genet 7:395. https://doi.org/10.1038/nrg1834

Roosens NH, Al Bitar F, Loenders K, Angenon G, Jacobs M (2002) Overexpression of ornithine-delta-aminotransferase increases proline biosynthesis and confers osmotolerance in transgenic plants. Mol Breed 9:73–80

Roosens NH, Thu TT, Iskandar HM, Jacobs M (1998) Isolation of the ornithine-delta-aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana*. Plant Physiol 117:263–271. https://doi.org/10.1104/pp.117.1.263

Ruszkowski M, Nocek B, Forlani G, Dauter Z (2015) The structure of *Medicago truncatula* delta(1)-pyrroline-5-carboxylate reductase provides new insights into regulation of proline biosynthesis in plants. Front Plant Sci 6:869. https://doi.org/10.3389/fpls.2015.00869

Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer ZS, Yanofsky MF, Coupland G (2000) Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science 288:1613–1616. https://doi.org/10.1126/science.288.5471.1613

Sangwan RS (1978) Change in the amino-acid content during male gametophyte formation of *Datura metel in situ*. Theor Appl Genet 52:221–225. https://doi.org/10.1007/BF00273893

Satoh R, Nakashima K, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2002) ACTCAT, a novel *cis*-acting element for proline- and hypoosmolarity-responsive expression of the *ProDH* gene encoding proline dehydrogenase in Arabidopsis. Plant Physiol 130:709–719. https://doi.org/10.1104/pp.009993

Savouré A, Jaoua S, Hua X-J, Ardiles W, Van Montagu M, Verbruggen N (1995) Isolation, characterization, and chromosomal location of a gene encoding the Δ^1-pyrroline-5-carboxylate synthetase in *Arabidopsis thaliana*. FEBS Lett 372:13–19. https://doi.org/10.1016/0014-5793(95)00935-3

Savouré A, Jaoua S, Hua XJ, Ardiles W, Van Montagu M, Verbruggen N (1997) Abscisic acid-independent and abscisic acid-dependent regulation of proline biosynthesis following cold and osmotic stresses in *Arabidopsis thaliana*. Mol Gen Genet 254:104–109. https://doi.org/10.1007/s004380050397

Schertl P, Cabassa C, Saadallah K, Bordenave M, Savouré A, Braun HP (2014) Biochemical characterization of proline dehydrogenase in Arabidopsis mitochondria. FEBS J 281:2794–2804. https://doi.org/10.1111/febs.12821

Schmidt R, Stransky H, Koch W (2007) The amino acid permease AAP8 is important for early seed development in *Arabidopsis thaliana*. Planta 226:805–813. https://doi.org/10.1007/s00425-007-0527-x

Schulze WX, Yao Q, Xu D (2015) Databases for plant phosphoproteomics. Methods Mol Biol 1306:207–216. https://doi.org/10.1007/978-1-4939-2648-0_16

Schwacke R, Grallath S, Breitkreuz KE, Stransky E, Stransky H, Frommer WB, Rentsch D (1999) LeProT1, a transporter for proline, glycine betaine, and gamma-amino butyric acid in tomato pollen. Plant Cell 11:377–392. https://doi.org/10.1105/tpc.11.3.377

Sharma S, Shinde S, Verslues PE (2013) Functional characterization of an ornithine cyclodeaminase-like protein of *Arabidopsis thaliana*. BMC Plant Biol 13:182. https://doi.org/10.1186/1471-2229-13-182

Sharma S, Villamor JG, Verslues PE (2011) Essential role of tissue-specific proline synthesis and catabolism in growth and redox balance at low water potential. Plant Physiol 157:292–304. https://doi.org/10.1104/pp.111.183210

Shetty K, Wahlqvist ML (2004) A model for the role of the proline-linked pentose-phosphate pathway in phenolic phytochemical bio-synthesis and mechanism of action for human health and environmental applications. Asia Pac J Clin Nutr 13:1–24

Shinde S, Villamor JG, Lin W, Sharma S, Verslues PE (2016) Proline coordination with fatty acid synthesis and redox metabolism of chloroplast and mitochondria. Plant Physiol 172:1074–1088. https://doi.org/10.1104/pp.16.01097

Shui XR, Chen ZW, Li JX (2013) MicroRNA prediction and its function in regulating drought-related genes in cowpea. Plant Sci 210:25–35. https://doi.org/10.1016/j.plantsci.2013.05.002

Signorelli S, Monza J (2017) Identification of Δ^1-pyrroline 5-carboxylate synthase (*P5CS*) genes involved in the synthesis of proline in *Lotus japonicus*. Plant Signal Behav 12:e1367464. https://doi.org/10.1080/15592324.2017.1367464

Srikanth A, Schmid M (2011) Regulation of flowering time: all roads lead to Rome. Cell Mol Life Sci 68:2013–2037. https://doi.org/10.1007/s00018-011-0673-y

Stines AP, Naylor DJ, Høj PB, van Heeswijck R (1999) Proline accumulation in developing grapevine fruit occurs independently of changes in the levels of *Δ¹-Pyrroline-5-Carboxylate Synthetase* mRNA or protein. Plant Physiol 120:923–923. https://doi.org/10.1104/pp.120.3.923

Stránská J, Kopecný D, Tylichová M, Snégaroff J, Sebela M (2008) Ornithine delta-aminotransferase: an enzyme implicated in salt tolerance in higher plants. Plant Signal Behav 3:929–935

Strecker HJ (1965) Purification and properties of rat liver ornithine δ-transaminase. J Biol Chem 240:1225–1230

Strizhov N et al (1997) Differential expression of two *P5CS* genes controlling proline accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in Arabidopsis. Plant J 12:557–569. https://doi.org/10.1111/j.0960-7412.1997.00557.x

Székely G et al (2008) Duplicated *P5CS* genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. Plant J 53:11–28. https://doi.org/10.1111/j.1365-313X.2007.03318.x

Szoke A, Miao GH, Hong Z, Verma DP (1992) Subcellular location of δ^1-pyrroline-5-carboxylate reductase in root/nodule and leaf of soybean. Plant Physiol 99:1642–1649

Taiz L, Zeiger E, Møller IM, Murphy A (2018) Fundamentals of plant physiology. Oxford University Press, New York/Oxford

Tanner JJ (2019) Structural biology of proline catabolic enzymes. Antioxid Redox Signal 30:650–673. https://doi.org/10.1089/ars.2017.7374

Taylor AA, Stewart GR (1981) Tissue and subcellular localization of enzymes of arginine metabolism in *Pisum sativum*. Biochem Biophys Res Commun 101:1281–1289

Tegeder M, Hammes UZ (2018) The way out and in: phloem loading and unloading of amino acids. Curr Opin Plant Biol 43:16–21. https://doi.org/10.1016/j.pbi.2017.12.002

Thiery L, Leprince AS, Lefebvre D, Ghars MA, Debarbieux E, Savouré A (2004) Phospholipase D is a negative regulator of proline biosynthesis in *Arabidopsis thaliana*. J Biol Chem 279:14812–14818. https://doi.org/10.1074/jbc.M308456200

Trovato M, Maras B, Linhares F, Costantino P (2001) The plant oncogene *rolD* encodes a functional ornithine cyclodeaminase. Proc Natl Acad Sci U S A 98:13449.13453. https://doi.org/10.1073/pnas.231320398

Trovato M, Mattioli R, Costantino P (2018) From *A. rhizogenes* RolD to plant P5CS: exploiting proline to control plant development. Plan Theory 7:108

Trovato M, Mauro ML, Costantino P, Altamura MM (1997) The *rolD* gene from *Agrobacterium rhizogenes* is developmentally regulated in transgenic tobacco. Protoplasma 197:111–120. https://doi.org/10.1007/BF01279889

Turchetto-Zolet AC, Margis-Pinheiro M, Margis R (2009) The evolution of pyrroline-5-carboxylate synthase in plants: a key enzyme in proline synthesis. Mol Gen Genomics 281:87–97

Van Aken O, Zhang B, Carrie C, Uggalla V, Paynter E, Giraud E, Whelan J (2009) Defining the mitochondrial stress response in *Arabidopsis thaliana*. Mol Plant 2:1310–1324. https://doi.org/10.1093/mp/ssp053

Vansuyt G, Vallee J-C, Prevost J (1979) La pyrroline-5-carboxylate réductase et la proline déhydrogénase chez *Nicotiana tabacum* var. Xanthi n.c. en fonction de son développement. Physiologie végétale 19:95–105

Veerabagu M et al (2014) The interaction of the Arabidopsis response regulator ARR18 with bZIP63 mediates the regulation of *PROLINE DEHYDROGENASE* expression. Mol Plant 7:1560–1577. https://doi.org/10.1093/mp/ssu074

Venekamp JH, Koot JTM (1988) The sources of free proline and asparagine in field bean plants, *Vicia faba* L., during and after a short period of water withholding. J Plant Physiol 32:102–109

Verbruggen N, Hua XJ, May M, Van Montagu M (1996) Environmental and developmental signals modulate proline homeostasis: evidence for a negative transcriptional regulator. Proc Natl Acad Sci U S A 93:8787–8791. https://doi.org/10.1073/pnas.93.16.8787

Verbruggen N, Villarroroel R, Van Montagu M (1993) Osmoregulation of a pyrroline-5-carboxylate reductase gene in *Arabidopsis thaliana*. Plant Physiol 103:771–781

Verslues PE, Skarp RE (1999) Proline accumulation in maize (*Zea mays* L.) primary roots at low water potentials. II. Metabolic source of increased proline deposition in the elongation zone. Plant Physiol 119:1349–1360

Wada KC, Takeno K (2010) Stress-induced flowering. Plant Signal Behav 5:944–947. https://doi.org/10.4161/psb.5.8.11826

Wakte K, Zanan R, Hinge V, Khandagale K, Nadaf A, Henry R (2017) Thirty-three years of 2-acetyl-1-pyrroline, a principal basmati aroma compound in scented rice (*Oryza sativa* L.): a status review. J Sci Food Agric 97:384–395. https://doi.org/10.1002/jsfa.7875

Walton EF, Clark CJ, Boldingh HL (1991) Effect of hydrogen cyanamide on amino acid profiles in kiwifruit buds during bud-break. Plant Physiol 97:1256–1259

Wanduragala S, Sanyal N, Liang X, Becker DF (2010) Purification and characterization of Put1p from *Saccharomyces cerevisiae*. Arch Biochem Biophys 498:136–142. https://doi.org/10.1016/j.abb.2010.04.020

Wang G et al (2014) Proline responding1 plays a critical role in regulating general protein synthesis and the cell cycle in Maize. Plant Cell 26:2582–2600. https://doi.org/10.1105/tpc.114.125559

Wang T, Chen Y, Zhang M, Chen J, Liu J, Han H, Hua X (2017) Arabidopsis AMINO ACID PERMEASE1 contributes to salt stress-induced proline uptake from exogenous sources. Front Plant Sci 8:2182. https://doi.org/10.3389/fpls.2017.02182

Watanabe S, Tanimoto Y, Yamauchi S, Tozawa Y, Sawayama S, Watanabe Y (2014) Identification and characterization of trans-3-hydroxy-l-proline dehydratase and Delta(1)-pyrroline-2-carboxylate reductase involved in trans-3-hydroxy-l-proline metabolism of bacteria. FEBS Open Bio 4:240–250. https://doi.org/10.1016/j.fob.2014.02.010

Weiste C et al (2017) The Arabidopsis bZIP11 transcription factor links low-energy signalling to auxin-mediated control of primary root growth. PLoS Genet 13:e1006607. https://doi.org/10.1371/journal.pgen.1006607

Weltmeier F et al (2006) Combinatorial control of Arabidopsis proline dehydrogenase transcription by specific heterodimerisation of bZIP transcription factors. EMBO J 25:3133–3143. https://doi.org/10.1038/sj.emboj.7601206

White FF, Taylor BH, Huffman GA, Nester EW (1985) Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. J Bacteriol 164:33–44

Yang J, Zhang N, Ma C, Qu Y, Si H, Wang D (2013) Prediction and verification of microRNAs related to proline accumulation under drought stress in potato. Comput Biol Chem 46:48–54. https://doi.org/10.1016/j.compbiolchem.2013.04.006

Yoshiba Y, Nanjo T, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Stress-responsive and developmental regulation of *Delta(1)-pyrroline-5-carboxylate synthetase 1* (*P5CS1*) gene expression in *Arabidopsis thaliana*. Biochem Biophys Res Commun 261:766–772

Yoshihashi T, Huong NT, Inatomi H (2002) Precursors of 2-acetyl-1-pyrroline, a potent flavor compound of an aromatic rice variety. J Agric Food Chem 50:2001–2004

You J, Hu H, Xiong L (2012) An ornithine δ-aminotransferase gene *OsOAT* confers drought and oxidative stress tolerance in rice. Plant Sci 197:59–69. https://doi.org/10.1016/j.plantsci.2012.09.002

Zarattini M, Forlani G (2017) Toward unveiling the mechanisms for transcriptional regulation of proline biosynthesis in the plant cell response to biotic and abiotic stress conditions. Front Plant Sci 8. https://doi.org/10.3389/fpls.2017.00927

Zhang CS, Lu Q, Verma DP (1995) Removal of feedback inhibition of delta 1-pyrroline-5-carboxylate synthetase, a bifunctional enzyme catalyzing the first two steps of proline biosynthesis in plants. J Biol Chem 270:20491–20496

Zhang CY, Wang NN, Zhang YH, Feng QZ, Yang CW, Liu B (2013) DNA methylation involved in proline accumulation in response to osmotic stress in rice (*Oryza sativa*). Genet Mol Res 12:1269–1277. https://doi.org/10.4238/2013.April.17.5

Zimorski V, Ku C, Martin WF, Gould SB (2014) Endosymbiotic theory for organelle origins. Curr Opin Microbiol 22:38–48. https://doi.org/10.1016/j.mib.2014.09.008

Zonglie H, Karuna L, Zhongming Z, Verma DPS (2000) Removal of feedback inhibition of Δ1-Pyrroline-5-Carboxylate Synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol 122:1129–1136. https://doi.org/10.1104/pp.122.4.1129